Published in final edited form as:

Curr Chem Biol. 2015 ; 9(2): 123–141. doi:10.2174/2212796810666160216221610.

Novel Citronellyl-Based Photoprobes Designed to Identify ER Proteins Interacting with Dolichyl Phosphate in Yeast and Mammalian Cells

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Abstract

Background—Dolichyl phosphate-linked mono- and oligosaccharides (DLO) are essential intermediates in protein N-glycosylation, C- and O-mannosylation and GPI anchor biosynthesis. While many membrane proteins in the endoplasmic reticulum (ER) involved in the assembly of DLOs are known, essential proteins believed to be required for the transbilayer movement (flip-flopping) and proteins potentially involved in the regulation of DLO synthesis remain to be identified.

Methods—The synthesis of a series of Dol-P derivatives composed of citronellyl-based photoprobes with benzophenone groups equipped with alkyne moieties for Huisgen “click” chemistry is now described to utilize as tools for identifying ER proteins involved in regulating the biosynthesis and transbilayer movement of lipid intermediates. *In vitro* enzymatic assays were used to establish that the photoprobes contain the critical structural features recognized by pertinent enzymes in the dolichol pathway. ER proteins that photoreacted with the novel probes were identified by MS.

Results—The potential of the newly designed photoprobes, m-PAL-Cit-P and p-PAL-Cit-P, for identifying previously unidentified Dol-P-interacting proteins is supported by the observation that

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CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

Figure S1, S2, ¹H NMR spectra for compounds 5, 6, 10, 11, 12, 14a–b, 15a–b, 16a–b, 17a–b, 18a–b, ³¹P NMR spectra for compounds 5 and 6, low resolution mass spectra for compounds 5 and 6, and Tables S1–S6 containing concise summary data of all protein hits detected by proteomic analysis are available.

they are enzymatically mannosylated by Man-P-Dol synthase (MPDS) from Chinese Hamster Ovary (CHO) cells at an enzymatic rate similar to that for Dol-P. MS analyses reveal that DPM1, ALG14 and several other yeast ER proteins involved in DLO biosynthesis and lipid-mediated protein O-mannosylation photoreacted with the novel probes.

Conclusion—The newly-designed photoprobes described in this paper provide promising new tools for the identification of yet to be identified Dol-P interacting ER proteins in yeast and mammalian cells, including the Dol-P flippase required for the “re-cycling” of the glycosyl carrier lipid from the luminal monolayer of the ER to the cytoplasmic leaflet for new rounds of DLO synthesis.

Keywords

Click chemistry; dolichol; flippases; photo-affinity probes; proteomics

1. INTRODUCTION

The role of dolichol phosphate (Dol-P)-linked mono- and oligosaccharides (DLOs) as intermediates in protein N-glycosylation, O-mannosylation, C-mannosylation and glycosylphosphatidylinositol (GPI) anchor biosynthesis in yeast, plants and mammalian cells is now well-documented [1, 2]. In yeast and mammalian cells these lipid-mediated pathways begin with the biosynthesis of dolichol-linked precursors on the cytoplasmic leaflet of the endoplasmic reticulum (ER), and require translocation to the luminal leaflet to complete the assembly of the glycoproteins and GPIs (Fig. 1).

Because the unassisted transbilayer movement of glycosylphosphorylpolyisoprenols is extremely slow [3, 4], transverse diffusion is believed to require a specialized class of membrane proteins, termed flippases, to maintain physiological rates of glycoconjugate biosynthesis [1, 5–8]. Many of the ER-associated proteins involved in these assembly processes are known. However, the ER proteins mediating the transbilayer movement (flipping) of Man-P-Dol [1, 9, 10], Glc-P-Dol [1, 11], and Man₅GlcNAc₂-P-P-Dol (M5-DLO) [12–15] to the luminal leaflet, after they are formed on the cytoplasmic monolayer, as well as the ‘recycling’ of Dol-P [16] back to the cytoplasmic surface (Fig. 1) remain to be identified. It is also likely that there are other proteins, like Lec35 [17, 18] and RFT1 [12, 14, 15, 19], that play regulatory roles in the biosynthesis and utilization of Dol-P, Man-P-Dol, Glc-P-Dol and DLOs that have not been described. Attempts to identify the eukaryotic flippases of the protein glycosylation pathways by mutational screening or synthetic lethality approaches have failed for reasons that are not entirely clear. Furthermore, bioinformatic and phylogenetic approaches using various bacterial proteins (Wzx [20], GtrB [21], ArnE [22], MsbA [23], PglK [24]) as model flippases have proven unproductive since these proteins contain very limited conserved sequence homology. It is possible that the flippases are essential proteins, or as it has been suggested recently, that the flippase activity resides in another essential protein as a ‘moonlighting’ activity [25–27].

As a new approach to detect flippases and other previously unidentified Dol-P and DLO-interacting proteins in yeast and mammalian cells we have devised a method which combines the high selectivity afforded by photoactive analogues with the power of

proteomic analysis. This method employs two novel water-soluble Photo-Active Ligands (PAL), m-PAL-Cit-P (**5**) and p-PAL-Cit-P (**6**), illustrated in Fig. 2A, derived from a short-chain analogue of dolichol, citronellol, containing an ω -terminal photoactive benzophenone. Citronellol is a ten-carbon isoprenoid with a reduced α -isoprene. The photoprobes are equipped with an alkyne moiety to facilitate Huisgen “click” chemistry-mediated conjugation of reporter tags for rapid and sensitive detection by rhodamine fluorescence or biotin enabled affinity enrichment of photo-adducted proteins [28–30]. These novel “click” chemistry capable photoprobes provide new tools for identifying the Dol-P flippase involved in recycling of the Dol-P molecules released during the Man-P-Dol and Glc-P-Dol-mediated glycosylation reactions and Dol-P formed by cleavage of Dol-P-P by CWH8 [31, 32] when it is discharged from mature DLOs during protein N-glycosylation reactions on the luminal monolayer of the ER [1, 2] (Fig. 1).

2. MATERIALS AND METHODS

2.1. Materials

GDP-Man was obtained from Sigma-Aldrich; [2-³H]mannose (15 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). GDP-[³H]mannose was synthesized enzymatically from [2-³H]mannose as described [33]. All other solvents and reagents were purchased from VWR (EM Science-Omnisolv high purity) and Aldrich and used as received. During chemical and enzymatic syntheses, reaction temperature refers to the external bath. Synthetic products were purified by silica gel flash chromatography (EtOAc/hexane) unless otherwise noted. All RP-HPLC was performed on an Agilent 1200 HPLC system equipped with a microplate autosampler, diode array and fluorescence detector using a Varian Dynamax, 10 μ m, 300 Å, C-18 (10 mm x 250 mm) column and eluted with a gradient mobile phase and flow rate of 4.5 mL/min: 0–3 min, 90% A; 3–18 min, 0% A; 18–20 min, 0% A; 20–23 min, 90% A; and monitored at 254 nm & 210 nm [A: Water with 0.1% TFA, B: CH₃CN with 0.1% TFA]. ¹H NMR spectra of were obtained in CDCl₃ or CD₃OD and ¹H and ³¹P of phosphates in CD₃OH/D₂O/CDCl₃ with a Varian Inova spectrometer operating at 400 MHz (¹H), 100.6 MHz (¹³C), 161.8 MHz (³¹P).

Chemical shifts are reported in ppm from CDCl₃ internal peak at 7.24 ppm for ¹H or TSP in D₂O at 0 ppm for ¹H; H₃PO₄ as an external reference, 0 ppm for ³¹P. High resolution electrospray ionization (ESI) mass spectra of the photoprobes **5**, **6**, **7** and **8** were recorded on an ABSciex Triple-TOF 5600 mass spectrometer (ABSciex, Framingham, MA, USA). The mass resolution was greater than 40,000. Samples were directly infused to an ESI source using an Eksigent micro-LC system with a flow rate of 20 μ L/min.

2.2. Synthesis of Affinity Tagged Heterobifunctional Dol-P Photoaffinity Probes (refer to Scheme 1)

2.2.1. Synthesis of Compound 10—A mixture of *S*-citronellol (10 g, 64 mmol), dihydropyran (8.1 g, 96 mmol) *p*-toluene sulfonic acid (100 mg) in dichloroethane (50 mL) was stirred at room temperature (rt) overnight. The reaction mixture was diluted with 10 mL of water, extracted with CH₂Cl₂, washed with aq. sat. Na-HCO₃, brine, dried over MgSO₄,

concentrated *in vacuo* and purified by silica gel column chromatography to give 2-((S)-3,7-dimethylocta-6-en-1-yl)oxy)tetrahydro-2H-pyran as a colorless oil (14.4 g, 94%).

¹H NMR (400 MHz, CDCl₃) δ 0.84 (d, *J* = 6.4 Hz, 3H), 1.10–1.18 (m, 1H), 1.28–1.42 (m, 2H), 1.49–1.83 (m, 13H), 1.75–1.83 (m, 1H), 1.88–2.01 (m, 2H), 3.33–3.42 (m, 1H), 3.45–3.50 (m, 1H), 3.70–3.78 (m, 1H), 3.81–3.87 (m, 1H), 4.54–4.55 (m, 1H), 5.04–5.09 (m, 1H).

To a suspension of SeO₂ (660 mg, 6.0 mmol), salicylic acid (805 mg, 5.8 mmol) in CH₂Cl₂ (75 mL) was added 70% *tert*-butyl hydroperoxide (22.5 mL, 0.163 mol). The resulting solution was allowed to stir for 20 min to homogeneity at room temperature before being placed at 0°C. 2-((S)-3,7-dimethylocta-6-en-1-yl)oxy)tetrahydro-2H-pyran (14 g, 58.3 mmol) in CH₂Cl₂ (10 mL) was added and stirred for 1 h at 0°C, warmed to rt and stirred for 24 h. The reaction mixture was diluted with 100 mL of toluene and concentrated under reduced pressure. The residue was then dissolved in toluene, washed sequentially with 5% NaHCO₃, saturated CuSO₄, aqueous Na₂S₂O₃, water, brine, dried over MgSO₄, filtered, concentrated under reduced pressure and purified by flash chromatography on silica gel to provide alcohol **10** (6.9 g, 59%) and the corresponding aldehyde (3.6 g, 30%). The yields are based on recovered 2-((S)-3,7-dimethylocta-6-en-1-yl)oxy)tetrahydro-2H-pyran.

(6*S*,*E*)-2,6-dimethyl-8-((tetrahydro-2H-pyran-2-yl)oxy)oct-2-en-1-ol **10** ¹H NMR (400 MHz, CDCl₃) δ 0.84 (d, *J* = 8.8 Hz, 3H), 1.11–1.18 (m, 1H), 1.28–1.40 (m, 2H), 1.42–1.67 (m, 10H), 1.71–1.78 (m, 1H), 1.93–2.01 (m, 2H), 3.29–3.38 (m, 1H), 3.40–3.45 (m, 1H), 3.66–3.73 (m, 1H), 3.75–3.82 (m, 1H), 3.90 (s, 2H), 4.48–4.51 (m, 1H), 5.32 (t, *J* = 7.2 Hz, 1H).

(6*S*,*E*)-2,6-dimethyl-8-((tetrahydro-2H-pyran-2-yl)oxy)oct-2-enal ¹H NMR (400 MHz, CDCl₃) δ 0.92 (d, *J* = 8.8 Hz, 3H), 1.40–1.80 (m, 14H), 2.30–2.43 (m, 2H), 3.33–3.42 (m, 1H), 3.45–3.50 (m, 1H), 3.70–3.78 (m, 1H), 3.81–3.90 (m, 1H), 4.55–4.60 (m, 1H), 6.50 (t, *J* = 6.4 Hz, 1H), 9.40 (s, 1H).

2.2.2. Synthesis of Ester 11—To a stirred solution of alcohol **10** (1.22 g, 4.76 mmol), ethyl-3-hydroxy-benzoate **9** (0.95 g, 5.71 mmol), Ph₃P (1.50 g, 5.71 mmol) in THF (40 mL) at 0°C, was added DEAD (40% in toluene, 2.5 mL, 5.71 mmol). The resultant reaction mixture was stirred for 1 h at 0°C and then warmed to rt and stirred overnight. The reaction mixture was diluted with sat. NaHCO₃ (5 mL), concentrated *in vacuo*, extracted with CH₂Cl₂, dried over MgSO₄, filtered, concentrated and purified by flash chromatography on silica gel to provide compound **11** (1.98 g, 83%).

Ethyl 3-(((6*S*,*E*)-2,6-dimethyl-8-((tetrahydro-2H-pyran-2-yl)oxy)oct-2-en-1-yl)oxy)benzoate, **11** ¹H NMR (400 MHz, CDCl₃) δ 0.88 (d, *J* = 8.8 Hz, 3H), 1.18–1.25 (m, 1H), 1.45–1.68 (m, 12H), 1.70 (s, 3H), 1.76–1.81 (m, 1H), 1.98–2.14 (m, 2H), 3.32–3.42 (m, 1H), 3.44–3.50 (m, 1H), 3.70–3.77 (m, 1H), 3.81–3.88 (m, 1H), 4.33 (q, *J* = 7.2, 14.4 Hz, 2H), 4.40 (s, 2H), 4.53–4.55 (m, 1H), 5.52 (t, *J* = 6.8 Hz, 1H), 7.00 (ddd, *J* = 0.8, 2.4, 8.0 Hz, 1H), 7.29 (t, *J* = 8.8 Hz, 1H), 7.53–7.60 (m, 2H).

2.2.3. Synthesis of Weinreb Amide, 12—To a stirred solution of ester **11** (2 g, 4.9 mmol) and Me(MeO)NH•HCl (748 mg, 7.6 mol) in THF (20 mL) at –20°C, was added a

solution of *i*-PrMgCl in THF (8 mL, 2 M) over 15 min maintaining the temperature below -5°C . The reaction mixture was stirred at -20°C for 35–45 min before being brought up to rt. The reaction mixture was quenched with sat. aq. NH_4Cl (5 mL), extracted with CH_2Cl_2 , washed with brine, dried over MgSO_4 , filtered, concentrated under reduced pressure and purified by silica gel column chromatography to afford compound **12** (1.8 g, 87%).

3-(((6S,E)-2,6-dimethyl-8-((tetrahydro-2H-pyran-2-yl)oxy)oct-2-en-1-yl)oxy)-N-methoxy-N-methylbenzamide, **12** ^1H NMR (400 MHz, CDCl_3) δ 0.87 (d, $J = 8.8$ Hz, 3H), 1.16–1.24 (m, 1H), 1.33–1.85 (m, 13H), 2.00–2.12 (m, 2H), 3.31 (s, 3H), 3.34–3.42 (m, 1H), 3.44–3.50 (m, 1H), 3.53 (s, 3H), 3.70–3.77 (m, 1H), 3.80–3.90 (m, 1H), 4.36 (s, 2H), 4.52–4.54 (m, 1H), 5.50 (t, $J = 6.8$ Hz, 1H), 6.97 (ddd, $J = 1.2, 2.8, 8.0$ Hz, 1H), 7.16–7.27 (m, 3H).

2.2.4. Synthesis of **13a** (3-bromophenoxy) (tert-butyl)dimethylsilane and **13b** (4-bromophenoxy)(tert-butyl)dimethylsilane—

To a stirred solution of either bromophenol (2 gm, 1.17 mmol) and imidazole (1.98 g, 2.9 mmol) in 1,2-dichloroethane (20 mL) at rt was added tert-butyldimethylsilylchloride (1.89 g, 1.26 mmol). The resulting solution was refluxed for 3 h and then brought to rt. The reaction mixture was poured into sat. aq. NH_4Cl (5 mL), extracted with CH_2Cl_2 , washed with brine, dried with MgSO_4 , filtered, concentrated under reduced pressure and purified by silica gel column chromatography to give either **13a** (3-bromophenoxy)-*t*-butyldimethylsilane (3.15 g, 95%) or **13b** (4-bromophenoxy)-*t*-butyldimethylsilane (3.25 g, 98%) as colorless oils.

(3-bromophenoxy)-t-butyldimethylsilane **13a** ^1H NMR (400 MHz, CDCl_3) δ 0.2 (s, 6H), 1.00 (s, 9H), 6.72–6.74 (m, 1H), 6.97–7.06 (m, 3H).

(4-bromophenoxy)-t-butyldimethylsilane **13b** ^1H NMR (400 MHz, CDCl_3) δ 0.10 (s, 6H), 0.85 (s, 9H), 6.70 (d, $J = 8.8$ Hz, 2H), 7.30 (d, $J = 8.8$ Hz, 2H).

2.2.5. Synthesis of Benzophenones 14a and 14b—To a stirred solution of either bromophenol **13a** or **13b** (1 g, 3.48 mmol) in THF (4 mL) at -78°C , was added dropwise *t*-butyl lithium (1 M in pentane, 4 mL, 6.96 mmol) and stirred for 1.5 to 2 h at -78°C . Weinreb amide **12** (1.45 gm, 3.4 mmol) in THF (5 mL) was then added dropwise and allowed to warm up to rt and stirred overnight. The reaction mixture was quenched with sat. aq. NH_4Cl (2 mL), extracted with CH_2Cl_2 , washed with brine, dried over MgSO_4 , filtered, evaporated under reduced pressure and purified by silica gel column chromatography to give **14a** (660 mg, 81%) or **14b** (947 mg, 87%). The yields are based on recovered starting amide **12**.

(3-((tert-butyldimethylsilyl)oxy)phenyl)(3-(((6S,E)-2,6-dimethyl-8-((tetrahydro-2H-pyran-2-yl)oxy)oct-2-en-1-yl)oxy)phenyl)methanone **14a** ^1H NMR (400 MHz, CDCl_3) δ 0.19 (s, 6H), 0.89 (d, $J = 8.8$ Hz, 3H), 0.95 (s, 9H), 1.18–1.80 (m, 18H), 2.00–2.10 (m, 2H), 3.30–3.42 (m, 1H), 3.43–3.50 (m, 1H), 3.70–3.80 (m, 1H), 3.81–3.90 (m, 1H), 4.40 (s, 2H), 4.52–4.58 (m, 1H), 5.50 (t, $J = 8.0$ Hz, 1H), 7.02–7.04 (m, 1H), 7.09–7.21 (m, 1H), 7.27–7.35 (m, 6H).

(4-((tert-butyldimethylsilyl)oxy)phenyl)(3-(((6*S*,*E*)-2,6-dimethyl-8-((tetrahydro-2*H*-pyran-2-yl)oxy)oct-2-en-1-yl)oxy)phenyl)methanone **14b** ¹H NMR (400 MHz, CDCl₃) δ 0.19 (s, 6H), 0.78 (d, *J* = 6.8 Hz, 3H), 0.85 (s, 9H), 1.00–1.10 (m, 1H), 1.20–1.75 (m, 10H), 1.90–2.00 (m, 2H), 3.20–3.40 (m, 2H), 3.60–3.80 (m, 2H), 4.25 (s, 2H), 4.40 (s, 2H), 5.40 (t, *J* = 8.0 Hz, 1H), 6.80 (d, *J* = 8.8 Hz, 2H), 6.95–7.00 (m, 1H), 7.15–7.30 (m, 3H), 7.65 (d, *J* = 8.8 Hz, 2H).

2.2.6. General Procedure for the synthesis of phenols 15a and 15b—To either silyl ether **14a** or **14b** (500 mg of **14a**, 600 mg of **14b**) dissolved in THF (6 mL) was added TBAF (1.1 eq.) drop wise at rt. and stirred overnight. The reaction mixture was concentrated under reduced pressure and purified by silica gel flash column chromatography directly to give compounds **15a** (375 mg, 94%) and **15b** (320 mg, 67%).

(3-(((6*S*,*E*)-2,6-dimethyl-8-((tetrahydro-2*H*-pyran-2-yl)oxy)oct-2-en-1-yl)oxy)phenyl)(3-hydroxyphenyl)methanone **15a** ¹H NMR (400 MHz, CDCl₃) δ ¹H NMR (400 MHz, CDCl₃) δ 0.80 (d, *J* = 8.8 Hz, 3H), 1.05–1.25 (m, 1H), 1.24–1.40 (m, 1H), 1.41–1.60 (m, 2H), 1.61–1.69 (m, 4H), 1.70–1.80 (m, 1H), 1.90–2.10 (m, 2H), 3.28–3.37 (m, 1H), 3.44–3.84 (m, 1H), 3.56 (s, 2H), 4.51–4.56 (m, 1H), 5.42 (t, *J* = 6.0 Hz, 1H), 7.00–7.08 (m, 3H), 7.19–7.28 (m, 7H).

(3-(((6*S*,*E*)-2,6-dimethyl-8-((tetrahydro-2*H*-pyran-2-yl)oxy)oct-2-en-1-yl)oxy)phenyl)(4-hydroxyphenyl)methanone **15b** ¹H NMR (400 MHz, CDCl₃) δ 0.88 (d, *J* = 8.8 Hz, 3H), 1.10–1.80 (m, 16H), 1.95–2.10 (m, 2H), 3.37–3.46 (m, 1H), 3.48–3.54 (m, 1H), 3.72–3.81 (m, 1H), 3.84–3.92 (m, 1H), 4.40 (s, 2H), 4.56–4.60 (m, 1H), 5.45 (t, *J* = 8.0 Hz, 1H), 6.85 (d, *J* = 8.8 Hz, 2H), 7.06–7.10 (m, 1H), 7.28–7.36 (m, 3H), 7.75 (d, *J* = 8.8 Hz, 2H).

2.2.7. General Procedure for the Synthesis of Propargyl Ethers 16a–b—To the solution of either **15a** or **15b** (**15a**; 300 mg, 0.66 mmol, **15b**; 250 mg, 0.55 mmol) in dry THF (6 mL) cooled to 0°C was added 60% sodium hydride in mineral oil (1.3 eq.) and stirred for 1 h. Freshly distilled propargyl bromide (1.1 eq. 0.71 mmol) in THF (1 mL) was added in dropwise. The resultant reaction mixture was stirred overnight, quenched with water (5 mL), concentrated and extracted with CH₂Cl₂, washed with brine, dried over MgSO₄, filtered, concentrated under reduced pressure and purified by silica gel flash column chromatography to give **16a** (254 mg, 94%) and **16b** (155 mg, 94%). The yields are based on recovered phenols **15a** or **15b**.

(3-(((6*S*,*E*)-2,6-dimethyl-8-((tetrahydro-2*H*-pyran-2-yl)oxy)oct-2-en-1-yl)oxy)phenyl)(3-(prop-2-yn-1-yloxy)phenyl)methanone **16a** ¹H NMR (400 MHz, CDCl₃) δ 0.80 (d, *J* = 8.8 Hz, 3H), 1.05–1.21 (m, 1H), 1.24–1.40 (m, 1H), 1.41–1.60 (m, 2H), 1.61–1.70 (m, 4H), 1.71–1.80 (m, 1H), 2.00–2.20 (m, 2H), 2.60 (t, *J* = 2.2 Hz, 1H), 3.28–3.37 (m, 1H), 3.44–3.50 (m, 1H), 3.64–3.73 (m, 1H), 3.80–3.85 (m, 1H), 4.40 (s, 2H), 4.60 (s, 1H), 4.75 (s, 2H), 5.55 (t, *J* = 6.0 Hz, 1H), 7.10–7.20 (m, 2H), 7.30–7.50 (m, 6H).

(3-(((6*S*,*E*)-2,6-dimethyl-8-((tetrahydro-2*H*-pyran-2-yl)oxy)oct-2-en-1-yl)oxy)phenyl)(4-(prop-2-yn-1-yloxy)phenyl)methanone **16b** ¹H NMR (400 MHz, CDCl₃) δ 0.90 (d, *J* = 8.8 Hz, 3H), 1.16–1.28 (m, 1H), 1.35–1.76 (m, 8H), 1.78–1.85 (m, 1H), 2.00–2.18 (m, 2H), 2.57

(t, $J = 2.2$ Hz, 1H), 3.37–3.47 (m, 1H), 3.48–3.55 (m, 1H), 3.72–3.81 (m, 1H), 3.84–3.92 (m, 1H), 4.40 (s, 2H), 4.56–4.60 (m, 1H), 4.77 (d, $J = 2.2$ Hz, 2H), 5.54 (t, $J = 8.0$ Hz, 1H), 7.04 (d, $J = 8.8$ Hz, 2H), 7.10–7.13 (m, 1H), 7.25–7.37 (m, 3H), 7.84 (d, $J = 8.8$ Hz, 2H).

2.2.8. General Procedure for Synthesis of Isoprenols 17a–b—The mixture of either **16a** or **16b** (**16a**; 150 mg, 0.306 mmol, **16b**; 140 mg, 0.285 mmol), *p*-toluene sulfonic acid (50 mg) in anhydrous methanol (5 mL) was stirred at rt overnight. The reaction mixture was concentrated, diluted with water (5 mL), extracted with CH₂Cl₂ washed with NaHCO₃, brine, dried over MgSO₄, filtered, concentrated under reduced pressure and purified by silica gel column chromatography to provide compounds **17a** and **17b** (100 mg of **17a**, 80%, 96 mg of **17b**, 83%).

(S,E)-(3-((8-hydroxy-2,6-dimethyloct-2-en-1-yl) oxy)phenyl)(3-(prop-2-yn-1-yloxy)phenyl)methanone **17a** ¹H NMR (400 MHz, CDCl₃) δ 0.89 (d, $J = 8.8$ Hz, 3H), 1.20–1.30 (m, 1H), 1.32–1.50 (m, 2H), 1.55–1.70 (m, 1H), 1.75 (s, 3H), 2.00–2.20 (m, 1H), 2.58 (t, $J = 2.2$ Hz, 1H), 3.60–3.80 (m, 2H), 4.42 (s, 2H), 4.75 (t, $J = 2.2$ Hz, 2H), 5.55 (t, $J = 6.0$ Hz, 1H), 7.10–7.20 (m, 2H), 7.30–7.50 (m, 6H).

(S,E)-(3-((8-hydroxy-2,6-dimethyloct-2-en-1-yl) oxy)phenyl)(4-(prop-2-yn-1-yloxy)phenyl)methanone **17b** ¹H NMR (400 MHz, CDCl₃) δ 0.87 (d, $J = 8.8$ Hz, 3H), 1.04–1.24 (m, 1H), 1.32–1.41 (m, 2H), 1.52–1.62 (m, 2H), 1.70 (s, 3H), 1.98–2.10 (m, 2H), 2.54 (t, $J = 2.2$ Hz, 1H), 3.59–3.70 (m, 2H), 4.40 (s, 2H), 4.75 (d, $J = 2.2$ Hz, 2H), 5.50 (t, $J = 8.0$ Hz, 1H), 7.03 (d, $J = 8.8$ Hz, 2H), 7.08–7.12 (m, 1H), 7.22–7.34 (m, 3H), 7.82 (d, $J = 8.8$ Hz, 2H).

2.2.9. General Procedure for the Synthesis of Bromides 18a and 18b—To a stirred solution of Ph₃P (118.7 mg, 0.45 mmol) and CBr₄ (150 mg, 0.45 mmol) in CH₂Cl₂ (5 mL) at 0°C was added either compound **17a** or **17b** (**17a**; 100 mg, 0.27 mmol, **17b**; 92 mg, 0.23 mmol) in CH₂Cl₂ (1 mL) and stirred for 45 min. The reaction mixture was concentrated under in vacuum and the residue purified by silica gel column chromatography to give bromides **18a** and **18b** (108 mg of **18a**, 93%, 98 mg of **18b**, 92%).

(S,E)-(3-((8-bromo-2,6-dimethyloct-2-en-1-yl) oxy)phenyl)(3-(prop-2-yn-1-yloxy)phenyl)methanone **18a** ¹H NMR (400 MHz, CDCl₃) δ 0.82 (d, $J = 8.8$ Hz, 3H), 1.12–1.20 (m, 2H), 1.21–1.31 (m, 1H), 1.50–1.68 (m, 2H), 1.70 (s, 3H), 1.80–1.90 (m, 1H), 2.00–2.10 (m, 2H), 2.50 (t, $J = 2.2$ Hz, 1H), 3.30–3.45 (m, 2H), 4.38 (s, 2H), 4.70 (t, $J = 2.2$ Hz, 1H), 5.48 (t, $J = 6.0$ Hz, 1H), 7.00–7.20 (m, 2H), 7.21–7.40 (m, 6H).

(S,E)-(3-((8-bromo-2,6-dimethyloct-2-en-1-yl) oxy)phenyl)(4-(prop-2-yn-1-yloxy)phenyl)methanone **18b** ¹H NMR (400 MHz, CDCl₃) δ 0.90 (d, $J = 8.8$ Hz, 3H), 1.20–1.25 (m, 2H), 1.31–1.45 (m, 1H), 1.78 (s, 3H), 1.60–1.80 (m, 4H), 1.81–1.95 (m, 1H), 2.00–2.18 (m, 2H), 2.68 (t, $J = 2.2$ Hz, 1H), 3.38–3.50 (m, 2H), 4.42 (s, 2H), 4.75 (t, $J = 2.4$ Hz, 2H), 5.58 (t, $J = 8.0$ Hz, 1H), 7.00 (d, $J = 8.8$ Hz, 2H), 7.10–7.15 (m, 1H), 7.20–7.40 (m, 3H), 7.80 (d, $J = 8.8$ Hz, 2H) LR Mass (M⁺) 469.1.

2.2.10. General Procedure for Preparation of Monophosphates 5 and 6—

Bis(tetra-*n*-butylammonium) hydrogen phosphate was prepared by titrating 85% phosphoric acid with 40% tetra-*N*-butylammonium hydroxide to pH 9.8 and lyophilizing the resultant solution. To a solution of either bromide **18a** or **18b** (50 mg, 0.10 mmol) in CH₃CN (3 mL) was added (n-Bu)₄N₂HPO₄ (3 eq. 185 mg) in dry CH₃CN (3 mL) at rt and stirred overnight. The reaction mixture was concentrated and extracted with Et₂O. The organic extracts were discarded and the residue was suspended in 4 mL ion exchange buffer (25 mM NH₄HCO₃ in 2% (v/v) *i*-PrOH/water). The resultant white solution was loaded onto a pre equilibrated 6×20 cm column of Dowex AG 50W-X8 (100–200 mesh) cation-exchange resin (NH₄⁺ form). The flask was washed with buffer (2 x 2 mL) and loaded onto the column before eluting with 50 mL of ion exchange buffer. The eluent was lyophilized to a white solid. This solid was dissolved in 25 mM solution of NH₄HCO₃ buffer (2 mL), purified by RP-HPLC (retention time about 8.7–9.2 min) and the collected fractions lyophilized to give the compounds **5** and **6** (13.8 mg of **5**, 25%, 10.2 mg of **6**, 18.5%) as a white powder.

*(S,E)-3,7-dimethyl-8-(3-(3-(prop-2-yn-1-yloxy) benzoyl)phenoxy)oct-6-en-1-yl phosphate **5**, ¹H NMR (400 MHz, D₂O) δ 0.58 (d, *J* = 8.8 Hz, 3H), 0.70–0.80 (m, 1H), 0.90–1.03 (m, 1H), 1.05–1.50 (m, 5H), 1.51–1.75 (m, 2H), 2.50 (t, *J* = 2.2 Hz, 1H), 3.60–3.70 (m, 2H), 3.80–4.0 (m, 2H), 4.30 (t, *J* = 2.2 Hz, 2H), 5.10 (t, *J* = 8.0 Hz, 1H), 6.70–7.00 (m, 8H) ³¹P NMR (161.8 MHz, D₂O) δ 0.5 (s, 1P) LR Mass (M-1) 486.18 HR Mass (M-1) Found 485.1721 Calc 485.1729.*

*(S,E)-3,7-dimethyl-8-(3-(4-(prop-2-yn-1-yloxy) benzoyl)phenoxy)oct-6-en-1-yl phosphate **6**, ¹H NMR (400 MHz, D₂O) δ 0.60 (d, *J* = 8.8 Hz, 3H), 0.70–0.85 (m, 1H), 0.90–1.03 (m, 1H), 1.05–1.50 (m, 6H), 1.60–1.80 (m, 2H), 2.60 (t, *J* = 2.2 Hz, 1H), 3.60–3.70 (m, 2H), 3.90–4.0 (m, 2H), 4.40 (t, *J* = 2.2 Hz, 2H), 5.20 (t, *J* = 8.0 Hz, 1H), 6.60–7.00 (m, 6H), 7.36 (d, *J* = 8.8 Hz, 2H) ³¹P NMR (161.8 MHz, D₂O) δ 0.5 (s, 1P) LR Mass (M-1) 486.18 HR Mass (M-1) Found 485.1726 Calc 485.1729.*

2.3. Preparation of ER-enriched Membrane Fractions from Rat Liver, CHO Cells and Yeast

ER-enriched membrane fractions were prepared from fasted Sprague-Dawley adult rats as described previously [9].

CHO Lec15, lacking MPDS activity [34], and CHO K1 cells were cultured in Ham's F12, 10 % fetal calf serum in 5 % CO₂ at 37°C in tissue culture treated plastic petri dishes. Cells were released from the dishes by brief incubation in PBS, 10 mM EDTA at rt, recovered by centrifugation at 1000 x g, 10 min, washed with ice-cold 10 mM HEPES-OH pH 7.4, 0.25 M sucrose two times and lysed by sonication (3 × 15 second pulses) with a Kontes cell disruptor at 40 % full power at 4°C. Unbroken cells and debris were removed by centrifugation at 1,000 x g, 10 min and the membrane fraction recovered by centrifugation at 100,000 x g, 20 min.

ER-enriched membrane fractions were prepared from logarithmically growing *S. cerevisiae* (strain S288c) as described elsewhere [15].

2.4. Photolysis of Membrane Fractions from Rat Liver and Yeast

Membrane fractions from yeast and rat liver (5 mg/mL membrane protein) were photolyzed with gentle stirring at 0°C in the presence of 0.2 mM photoprobe (compounds **5** or **6**) in 0.1–0.2 mL Dulbecco's PBS for 60 min with a Model UVL-56 Blak-Ray Lamp (UVP Inc., San Gabriel, CA) long wave mineral light (366 nm) positioned 4 cm directly above the surface of the reaction mixture. Following photolysis, 5–10 µl of the reaction was removed for coupling to rhodamine-azide and the remainder was ligated to biotin-azide, as described in detail below.

2.5. Reaction of Photolabeled Proteins with Rhodamine-azide and Biotin-azide

Photolabeled proteins containing alkyne 'click chemistry' 'handles' were coupled to rhodamine-azide by copper (I)-catalyzed azide-alkyne cycloaddition essentially as described by Speers and Cravatt [28]. Reaction mixtures for cycloaddition catalyzed tagging with rhodamine-azide contained 0.2 % SDS, 1 mM Tris 2-carboxyethyl phosphine (TCEP), 0.1 mM Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methylamine], added from a 1.67 mM stock in t-butanol/DMSO (4:1), 0.02 mM tetramethylrhodamine 5-carboxamido-(6-azidohexanyl) (TAMRA), 1 mM CuSO₄ and 50 µg photolyzed protein (as described above) in a total volume of 0.05 mL Dulbecco's PBS. Following incubation for 60 min at room temperature in the dark, the reactions were diluted with 1x Laemmli buffer and resolved by SDS-PAGE. Fluorescent proteins were visualized using a Typhoon 9400 Variable Mode Imager (GE healthcare) running ImageQuant TL software. Reaction mixtures for copper(I)-catalyzed cycloaddition of biotin azide to photolabeled proteins were identical to the mixtures described above for rhodamine tagging, except that TAMRA is replaced with 0.2 mM biotin azide and the reaction was scaled to a final volume of 1 mL. Following incubation at room temperature for 1 h, reactions were processed for proteomic analysis as described below.

2.6. Analysis of Photolabeled Proteins by Proteomics

Photolabeled protein samples were processed by a modification of a method described by Martin and Cravatt [35]. After the click reaction described above, samples were transferred to 15 mL tubes, and diluted with 9 vol ice-cold methanol. The precipitated proteins were recovered by low-speed centrifugation and washed three times with 10 mL ice-cold methanol. After the final wash, protein pellets were resuspended in 1 mL of 1.2% SDS in PBS, heated to 100°C and sonicated briefly to dissolve completely. Solubilized samples were diluted with 5 mL PBS to 0.2% SDS final concentration. Biotinylated proteins were captured and recovered by incubation with streptavidin beads (Thermo Scientific, 0.1 mL, 50% slurry) in PBS/0.2 % SDS for 2 hours with rocking. Following stringent stepwise washing to remove un-biotinylated proteins with 0.2% SDS/PBS, PBS (3 times), 2 M urea in PBS (2 times), and 20 mM Tris pH 6.8 (3 times), the beads were transferred to 1.5 mL Protein Lo-Bind eppendorf tubes in 500 µl 6 M urea then reduced with TCEP (10 mM) and alkylated with iodoacetamide (20 mM) while rocking covered in foil. After washing with PBS, the beads were resuspended in 200 µl 2M urea/PBS supplemented with CaCl₂ (1 mM) for on-bead digestion with trypsin (Promega, 2 µg per 0.2 mL reaction) at 37°C overnight. Peptide products were recovered by solid-phase extraction using Phenomenex Strata-X 33u polymeric reversed phase cartridges. The solid-phase cartridges were prepared by washing

sequentially with 2 mL methanol, 2 mL 70% acetonitrile/0.1% TFA and 3 mL 5% acetonitrile/0.1% TFA. Tryptic peptides were loaded onto the cartridges in 5% acetonitrile/0.1% TFA, washed with 25 mL 5% acetonitrile/0.1% TFA, eluted with 0.6 mL 70% acetonitrile/0.1% TFA and 0.4 mL 90% acetonitrile/0.1% TFA, dried under N₂ and stored at -80°C until analysis by mass spectrometry as described below.

2.7. Liquid Chromatography-electrospray Ionization-tandem Mass Spectrometry (LC-ESI-MS/MS) Analysis

LC-MS/MS analysis was performed using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) coupled with an Eksigent Nanoflex cHiPLC™ system (Eksigent, Dublin, CA) and a nano-electrospray ionization source. The peptide samples were separated with a reversed phase cHiPLC column (75 µm × 150 mm) at a flow rate of 300 nL/min. Mobile phase A was water with 0.1% (v/v) formic acid while B was acetonitrile with 0.1% (v/v) formic acid. A 50 min gradient condition was applied: initial 3% mobile phase B was increased linearly to 50% in 24 min and further to 85% and 95% for 5 min each before it was decreased to 3% and re-equilibrated. The mass analysis method consisted of one segment with eight scan events. The 1st scan event was an Orbitrap MS scan (100–1600 m/z) with 60,000 resolution for parent ions followed by data dependent MS/MS for fragmentation of the 7 most intense ions with collision induced dissociation (CID) method.

The LC-MS/MS data were submitted to a local mascot server for MS/MS protein identification via Proteome Discoverer (version 1.3, Thermo Fisher Scientific, Waltham, MA) against a custom database containing the *S. cerevisiae* strain s288c proteome. Typical parameters used in the MASCOT MS/MS ion search were: trypsin digest with maximum of two miscleavages, cysteine carbamidomethylation, methionine oxidation, a maximum of 10 ppm MS error tolerance, and a maximum of 0.8 Da MS/MS error tolerance. A decoy database was built and searched. Peptide matches that pass the false discovery rate (FDR) filter (FDR target setting = 0.01) are assigned as high confidence matches. For MS/MS ion search, proteins with two or more high confidence peptides were considered unambiguous identifications without manual inspection. Proteins identified with one high confidence peptide were manually inspected and confirmed.

2.8. Assay for Man-P-Dol Synthesis

Reaction mixtures for the assay of Man-P-Dol synthase activity contained 50 mM HEPES pH 8, 10 mM MgCl₂, 5 mM 5'-AMP, 0.25 % CHAPS, 0–200 µM Dol-P (added exogenously as a dispersion in 1 % CHAPS), 1–50 µg membrane protein (partially purified *M. luteus* MPUS, yeast microsomes or rat liver microsomes) and 10 µM GDP-[³H]mannose (100–1500 cpm/pmol) in a total volume of 0.1 mL. Following incubation for 3 min at either 21°C (*M. luteus* partially purified enzyme), 30°C (yeast microsomes) or 37°C (rat liver microsomes), the enzymatic reaction was terminated by the addition of 40 vol CHCl₃/CH₃OH (2:1) and the synthesis of [³H]Man-P-Dol was assayed as described in detail elsewhere [36].

2.9. Assay for synthesis of water-soluble analogs of Man-P-Dol

Reaction mixtures (0.01 mL) for assay of water-soluble analogs of Dol-P as substrates for Man-P-Dol synthase were identical to those described above for Man-P-Dol synthesis. Following incubation the enzymatic reactions are terminated by the addition of 4 vol of CH₃OH and placed on ice for 5 min. Insoluble material was removed by centrifugation and the supernatant was transferred to a plastic tube and dried under a stream of nitrogen gas. The dried residue was redissolved in 5 μ L 50 % CH₃OH and 1 μ L was spotted onto a 10 cm plastic-backed cellulose thin layer sheet (Baker-Flex) and developed in butanol/ethyl acetate/acetic acid/water (4:3:2.5:4). The enzymatic product was located by scanning with a Bioscan AR2000 Radio-chromatoscanner (Bioscan, Washington, DC). The radioactive product zone was removed from the thin layer sheet by scraping with a razor blade and transferred to a 5 mL scintillation vial; the remainder of the lane was transferred to a separate vial. The cellulose was soaked briefly in 0.5 mL 1 % SDS and radioactivity was measured by scintillation spectrometry using a Packard TR2100 liquid scintillation spectrometer after the addition of 4 mL Econo Safe Complete Counting Cocktail (Research Products International Corp.). Enzymatic rates are calculated from the ratio of radioactivity incorporated into product to total radioactivity detected.

2.10. Preparation of Mannosylated Photoprobes 7 and 8 for Mass Spectrometric Analysis

Reaction mixtures for the preparation of compounds **7** and **8** contained 50 μ M of the pertinent PAL-Cit-P acceptor substrate, 50 mM HEPES-NaOH, pH 7.4, 10 mM MgCl₂, 2.5 mM 5'-AMP, 1 mM sodium orthovanadate, 0.25 % CHAPS, 200 μ M GDP-Man and 0.6 mg partially purified Man-P-Und synthase (prepared as described elsewhere [37]) in a total volume of 10 mL. Following overnight incubation at rt, reactions were diluted with 5 mL water-saturated butanol and mixed vigorously. The two-phase mixtures were briefly centrifuged and the aqueous layer was removed and discarded. The butanol phase was washed two times by partitioning with 5 mL water saturated with butanol, diluted with 3 vol of methanol and applied to a 25 mL column of DEAE-cellulose, equilibrated with methanol. The DEAE column was eluted with 50 mL methanol, equilibrated into water using a 120 mL gradient from 100 % methanol to 100 % water, washed with an additional 50 mL water and eluted with a 200 mL gradient 0 to 1 M NH₄HCO₃. Ammonium bicarbonate gradient fractions of 4 mL were collected and monitored for product by A253. The synthetic product elutes in a broad peak at 0.5 M NH₄HCO₃. The fractions containing the synthetic product were pooled, supplemented with 1/10th vol 10x PBS and passed over a Waters Sep-Pak Vac RC C18 reverse-phase column (500 mg). The reverse phase column was washed with 10 mL PBS, 5 mL water and then eluted with 50 % EtOH. Fractions containing PAL-Cit-P-Man were combined, dried and stored at -20C° until analysis. The purified synthetic PAL-Cit-P-Man migrated as a single compound in thin layer chromatography on silica Gel G plates developed in CHCl₃/CH₃OH/H₂O (65:35:6). CHCl₃/CH₃OH/H₂O/NH₄OH (65:35:6:2) and CHCl₃/CH₃OH/H₂O/HAc (65:35:6:2) visualized by either UV quenching, anisaldehyde spray [38], Dittmer-Lester phospholipid spray [39] or iodine vapors and co-chromatographed with radioactive compound prepared using GDP-[2-³H]mannose.

β -Mannosyl-(S,E)-3,7-dimethyl-8-(3-(3-(prop-2-yn-1-yloxy)benzoyl)phenoxy)oct-6-en-1-yl phosphate (m-PAL-Cit-P-Man, **7**), LR Mass (M-1) 647.5 HR Mass (M-1) Found 647.2259

Calc 647.2257 β -Mannosyl-(S,E)-3,7-dimethyl-8-(3-(4-(prop-2-yn-1-yloxy)benzoyl)phenoxy)oct-6-en-1-yl phosphate (p-PAL-Cit-P-Man, **8**), LR Mass (M-1) 647.5 HR Mass (M-1) Found 647.2257 Calc 647.2257.

3. RESULTS AND DISCUSSION

3.1. Design and Utility of Citronellyl based Photoprobes

To devise a method to specifically tag and identify proteins that interact with Dol-P and DLOs we have designed water-soluble photoactive probes based on a short chain analogue of dolichol, citronellol. The design of these new photoprobes is an extension of an earlier strategy in which citronellyl-based water-soluble analogues of Man-P-Dol [9] and Glc-P-Dol [11] were used in transport assays to detect flippase activity of the pertinent ER proteins mediating their transverse diffusion from the cytoplasmic leaflet of the ER to the luminal monolayer. Citronellol is a 10 carbon isoprenyl alcohol containing an ω -terminal isoprene and the critical reduced α -isoprene unit characteristic of dolichols. The structural relationship between the native molecule, Dol-P, the 10 carbon water-soluble analogue, Cit-P, and the two Photo-Active Ligands (PAL), m-PAL-Cit-P (**5**) and p-PAL-Cit-P (**6**), is illustrated in Fig. 2A.

Studies from other laboratories [40] report that short-chain isoprenols modified with an ω -terminal benzophenone efficiently photo-insert into proteins when exposed to UV light (365 nm). Pilot studies (data not shown) revealed that a Cit-P derived photoprobe containing a *m*-benzophenone moiety was a superior substrate analogue for *Micrococcus luteus* Man-P-undecaprenol synthase (MPUS), and was therefore selected for further development. To facilitate purification and recovery of specifically tagged proteins following photoinsertion, the prenyl-benzophenone adduct was further modified with a propargyl ether substituent to incorporate a 'click' chemistry handle to form the final photo-active analogues (Fig. 2A, compounds **5** and **6**). The PAL-Cit-P analogues are water-soluble to concentrations of at least 10 mM and exhibit similar kinetic properties as naturally-occurring Dol-P when tested as acceptor substrates for Man-P-Dol synthase from several different organisms (see Table 2, below).

The addition of the propargyl 'click' chemistry handle provides an efficient means of identifying and recovering the photolabeled proteins, as illustrated in Fig. 2B. Following photoinsertion, the proteins can be ligated by copper-catalyzed cy-claddition, (Huisgen 'click' chemistry), to either rhodamine-azide, for fluorescence detection after SDS-PAGE separation, or to biotin-azide for recovery by streptavidin capture. Following a stringent wash, to remove non-specifically bound proteins, tryptic peptides from the specifically bound proteins can be released by on-column trypsinization, recovered by solid-phase extraction, and identified by MS/MS analysis.

The photo-crosslinking groups are incorporated into the probes near the substrate binding site, but with an orientation and molecular flexibility that minimally perturbs substrate-enzyme interactions. The utility of benzophenone photoprobes for specifically tagging target proteins has recently been reviewed [41, 42].

The photoprobe isoprenoid moieties have saturated α -isoprene units with (*S*)-stereochemistry, as found in dolichols [43, 44], linked to the proximal benzophenone ring via a *meta*-ether linkage. Sequential assembly of the substituted central benzophenone moiety allows complete regiochemical control of the relative orientation of the isoprenoid and the acetylene click handle (Scheme 1). Recently, logically similar experimental approaches have been used to probe the *Sacharomyces cerevisiae* isoprenoid interactome [45], phospholipid binding proteins of *S. cerevisiae* inner mitochondrial membranes [46], leukocyte-specific protein tyrosine kinase [47], proteins that recognize post-translationally modified histones [48] and to identify the molecular target of serum response factor-mediated signaling in PC-3 cells [49].

3.2. Synthesis and Structural Confirmation by NMR of Citronellyl-based Photoprobes

Unsymmetric disubstituted benzophenones have been previously prepared by coupling substituted aryl Weinreb amides with suitably functionalized aryl Grignard reagents [50]. As shown in synthetic Scheme 1, the key intermediate **12** was prepared by Mitsunobu coupling of THP protected 8-hydroxy-(+)-citronellol **10** with ethyl *m*-hydroxy benzoate **9** followed by conversion to the Weinreb amide **12** [51]. Benzophenones **15a–b** were prepared by reacting the amide **12** with either *meta*- or *para*-silyl protected phenyllithium **13a–b** followed by desilylation with TBAF [52]. The alkyne for subsequent bio-orthogonal chemistry was then introduced as a propargyl ether at the terminus of the probes [53]. Bromides **18a–b** were prepared by cleavage of THP ethers **16a–b** with PPTS/MeOH followed by reaction with $\text{CBr}_4/\text{Ph}_3\text{P}$. The bromides were converted to the phosphate photoprobes **5** and **6** by reaction with $(\text{nBu}_4)_2\text{HPO}_4$ in CH_3CN in moderate yield [54–56]. It is anticipated that the ketyl radicals formed upon irradiation with long wavelength UV light (320–380 nm) would insert into C-H bonds of Dol-P, Man-P-Dol, Glc-P-Dol and DLO binding proteins with high yield.

3.3. Recognition and Enzymatic Mannosylation of Dol-P Analogues by Membrane Fractions from CHO Cells, Yeast, Rat Liver and *M. luteus*

To confirm that the newly-synthesized photoprobes had the critical structural features recognized by pertinent enzymes in the dolichol pathway, they were tested as substrates for Man-P-Dol synthase (MPDS) in microsomal fractions from CHO cells. As seen in Table 1, photoprobes **5** and **6** were enzymatically mannosylated by MPDS associated with CHO microsomes at virtually the same initial rate as the natural substrate, Dol-P.

Supporting recognition of the photoprobe analogues by MPDS, very little detectable activity was observed when compounds **5** or **6** were incubated with microsomes from the CHO mutant Lec15 lacking MPDS activity due to a defective DPM2 subunit, which enhances the binding of Dol-P to the multi-subunit enzyme subunit [17, 57].

In addition, the water-soluble analogues were tested as substrates for MPDS associated with membrane fractions from yeast and rat liver and for Man-P-undecaprenol synthase from *M. luteus*. As seen in Table 2, probes **5** and **6** exhibited K_m values and catalytic efficiencies similar to Dol-P for the mannosyltransferases from yeast, rat liver and *M. luteus*. Importantly, the addition of the propargyl ether to the benzophenone-Cit-P intermediate had no detectable impact on the affinities of the photo-active probes (data not shown). Adducing

evidence for the recognition of compound **5** by MPDS from *S. cerevisiae* was obtained by showing that MPDS activity was lost in a UV- and concentration-dependent reaction (Fig. 3).

Definitive proof of the formation of mannosylated probes **7** and **8** catalyzed by MPUS was confirmed by high resolution mass spectrometry (HRMS) and by analysis of the low resolution mass spectrum fragmentation pattern. The synthetic monophosphates, **5** and **6** were enzymatically mannosylated to prepare probes m-PAL-Cit-P-Man **7** and p-PAL-Cit-P-Man **8** using GDP-Man and partially purified MPUS from *M. luteus* [37]. Importantly, enzymatic reactions with either photoprobe yielded essentially a single product with a parent molecular ion of 647.5 daltons as well as a fragmentation pattern consistent with mannosylation of **5** (Fig. 4, **6**, Fig. S1).

Analysis of the mass spectra for mannosylated compounds **7** and **8** are similar and will only be described for compound **7**. Loss of water from the parent ion m-PAL-Cit-P-Man **7** gives the ion at m/z 629. Loss of mannose by cleavage of the anomeric oxygen bond gives isoprenoid phosphate **5** (m/z 485), which gives the minor ion at m/z 447 upon loss of the propargyl group. Alternative cleavage of the isoprenoid phosphate ester bond followed by loss of water gives the mannosyl phosphate ester observed at m/z 241. Cross-ring fragmentation of the mannose [58] gives the glycoaldehyde phosphodiester of **5** (m/z 527) after neutral loss of a tetrose. Neutral loss of the benzophenone from the m/z 527 ion gives the rose oxide-glycoaldehyde phosphodiester (m/z 275). Neutral loss of the isoprenoid from both the m/z 527 and m/z 275 ions gives glycoaldehyde phosphate (m/z 139). A major fragmentation pathway involves cleavage of the ether linkage between the proximal benzophenone ring and the beta isoprene giving either the propargyl substituted benzophenone (m/z 251) or (S)-3,7-dimethyl-5,7-octadiene-1-ol phosphate (m/z 233, rose oxide phosphate) after neutral loss of mannose.

These results establish that the newly-designed isoprenoid photoprobes **5** and **6** contain the critical structural features recognized by enzymes in the dolichol pathway. Moreover, the results demonstrate that the CHO and yeast MPDS are capable

3.4. Photoreaction of m/pPAL-Cit-P with *S. cerevisiae* Microsomes Reveals Several Pertinent Enzymes Involved in Lipid-mediated Glycosylation Reactions

Based on the encouraging results obtained with CHO and yeast microsomes, the utility of the photoactive, water-soluble analogs of Dol-P for the identification of novel Dol-P binding proteins was tested by incubating ER-enriched membrane fractions from *S. cerevisiae* with probes **5** or **6** in the presence and absence of UV light (366 nm). Following incubation at 4°C for 1 h, the reactions were solubilized with 0.1 % SDS and photolabeled proteins were either ligated to rhodamine azide and resolved by SDS-PAGE or ligated to biotin azide, purified by streptavidin capture, trypsinized on-column and analyzed by conventional proteomics. Fluorescence imaging, as described in *Materials and methods*, and western blotting with streptavidin-HRP (Thermo Scientific) revealed the presence of a variety of discreetly labeled photoprobe- and UV-light dependent protein bands (Fig. S2, panel A). Furthermore, photolabelling by compound **6** was concentration-dependent (Fig. S2, panel B)

and inhibited by competition with 5 mM Cit-P (Fig. S2, panel A, lane 7). Proteomic analysis of the specific biotin-tagged *S. cerevisiae* proteins identified a number of proteins that were bound by streptavidin agarose. Since it is impractical to quantitatively rank protein hits based on mass spectral data alone in 'shotgun' proteomic experiments [59], the identified proteins were sorted by topological analysis (TMHMM server available through the Center for Biological Sequence Analysis at <http://www.cbs.dtu.dk/services/TMHMM-2.0/>) and annotation of subcellular location available through the UniProt Consortium (www.uniprot.org). The results of these experiments are summarized in Table 3. Reactions without added photoprobe and either incubated in the dark or exposed to UV light (366 nm) yielded 63 and 18 identified proteins, respectively.

Reactions containing compounds **5** and **6**, but without UV light irradiation, identified 112 and 124 labeled proteins. However, 295 and 281 proteins were identified in reactions containing **5** and **6** following irradiation with UV light, respectively. The dramatic increase in the number of photolabelled proteins suggests active, UV-light catalyzed crosslinking of the probes to the additional proteins. Eliminating proteins that lack transmembrane domains (as determined by topological analysis using the TMHMM server available through the Center for Biological Sequence Analysis, Technical University of Denmark) and that do not localize to the ER, reduced the number of identified proteins in the reactions without photoprobe, but with and without UV light, to 2 and 3 respectively. Similarly, the reactions with **5** and **6**, but without exposure to UV light gave 12 and 10 ER membrane proteins, respectively. Importantly, the reactions containing **5** and **6** that were irradiated with UV light gave 54 and 51 ER-associated membrane proteins, respectively. The entire collection of proteins detected by LC MS/MS following trypsinization of the streptavidin-purified yeast proteins, along with peptide spectral data for each identified protein is available in Tables S1–S6 in supplemental materials. The photolabeled membrane proteins that are annotated as ER-associated are presented in Table 4. Among this collection of ER-associated membrane proteins are a number of proteins that utilize Dol-P as an acceptor substrate (DPM1 and Alg5) and Man-P-Dol as a mannosyl donor and produce Dol-P as an end product (PMT1, PMT2 and Alg12), as well as several that use acceptor and glycosyl donor substrates containing Dol-P-P (Alg1, Alg14, STT3 and OST2) (Table 4). Furthermore, these samples also contained a number of polytopic membrane proteins of uncertain cellular location or poorly characterized function, such as RSN1 (11 TMDs) YGL114W (12 TMDs) and PDR5 (14 TMDs), found in Table 5, that are currently under investigation (see Tables S4 and S6 in supplementary data for a complete list). It is interesting that some of the proteins photoreacted preferentially with one or the other of the regio-isomers (Tables 4 and 5), implying a degree of specificity in the interactions with the individual compounds. The specificity of the photoreaction is further supported by the observation that photolabeling was reduced by the addition of 5 mM Cit-P, but not by the addition of 5 mM naphthyl-P (data not included).

Despite continued interest in the membrane proteins that mediate the transmembrane movement of the dolichol-linked intermediates of the protein glycosylation pathways, they remain unidentified. Genetic, bioinformatic and phylogenetic approaches have failed to produce viable candidates [60, 61] and the technical difficulties inherent in performing

flippase assays have hindered conventional biochemical methods of purification and characterization of flippase active proteins [8, 62]. The development of these novel tools provides an effective approach with the potential to identify the Dol-P flippase required for “recycling” the many Dol-P molecules formed by lipid-mediated reactions on the luminal surface of the ER membrane (illustrated in Fig. 1), as well as other regulatory proteins like Lec35 [17, 18] that play a role in the utilization of Man-P-Dol and Glc-P-Dol for DLO synthesis. In addition, it is possible that the probes could detect critical ER proteins that are previously unknown binding partners in multi-subunit complexes similar to protein oligosaccharyltransferase [63–65], PMTs [66], *cis*-isoprenyltransferase/NogoB receptor [67, 68], Dol-P-Man Synthase [69] and the ALG13/ALG14 complex [70–72].

Finally, as noted above, MPDS in CHO and yeast cells, as well as a partially purified preparation of MPUS from *M. luteus* are capable of converting the PAL-Cit-P probes to the mannosylated analogues (compounds **7** and **8**). Studies with the mannosylated derivatives aimed at identifying the long sought after Man-P-Dol flippase are in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors express their appreciation to Manjula Sunkarna and Dr. Andrew J. Morris for assistance in obtaining mass spectrometric data and to Dr. Mark Lehrman (Department of Pharmacology, University of Texas, Southwestern) for generously providing CHO Lec15 cells. We acknowledge the University of Kentucky Proteomics Core that is partially supported by grants from the National Institute of General Medical Sciences (P20GM103486) and funds from the Office of the Vice President for Research of the University of Kentucky. This work was supported by NIH grant R01 GM66152 (HPS) and R01 GM102129 (CJW).

LIST OF ABBREVIATIONS

Dol-P	Dolichyl monophosphate
DLO	Dolichol-linked oligosaccharide
Man-P-Dol	Mannosylphosphoryldolichol
Glc-P-Dol	Glucosylphosphoryldolichol
Cit-P	Citronellyl phosphate
MPDS	Mannosylphosphoryldolichol synthase
MPUS	Mannosylphosphorylundecaprenol synthase
rt	Room temperature

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Fig. (1).

Topological model for the “recycling” of Dol-P formed during lipid-mediated glycosylation reactions on the lumenal leaflet of the ER and the cleavage of Dol-P-P released by OST during protein N-glycosylation. Dol-P = dolichyl monophosphate; PMT = protein mannosyltransferase. The numbers in red indicate the number of Dol-P producing reactions on the lumenal monolayer.

Fig. (2). Structures of newly-designed citronellyl-based Dol-P derivative photoprobes (panel A) and scheme for identifying photo-tagged proteins (panel B).

Fig. (3). Man-P-Dol synthase from *S. cerevisiae* is photoinactivated by exposure to UV light in the presence of photoprobe 5

Photolysis reactions contained 5 mM sodium phosphate, pH 7.4, 0.15 M NaCl, the indicated concentration of compound 5 and *S. cerevisiae* ER-enriched microsomal fraction (1 mg/mL membrane protein). Following exposure to UV light for 60 min, reactions were supplemented with compound 5 to a final concentration of 0.4 mM and an aliquot assayed for MPDS activity as described in *Materials and methods*.

Fig. (4). Negative ionization mode LCMS-ESI product ion spectrum of m-PAL-Cit-P-Man **7** (m/z 647.5) formed by MPUS catalyzed mannosylation of **5** (see text for interpretation). of converting them to the mannosylated derivatives **7** and **8** which will be valuable tools for detecting new Man-P-Dol interacting proteins such as Man-P-Dol flippase (Fig. 1).

Scheme (1).

(a) DEAD, Ph₃P, THF, (b) 1) Me(MeO)NH•HCl, THF, 2) *i*-PrMgCl (c) *t*-BuLi, THF, (d) TBAF, THF, (e) propargyl bromide, NaH, THF, (f) PPTS, MeOH, (g) Ph₃PCl₂, CH₃CN, (h) (n-Bu₄)₂HPO₄, CH₃CN, (i) GDP-Mannose, Man-P-Und synthase.

Table 1

Mannosylation of various water-soluble analogues of Dol-P by Man-P-Dol synthase in microsomal fractions from CHO K1 and Lec15 cells.^a

Substrate	CHO K1 (pmol/mg/min)	Lec15 (pmol/mg/min)
Dol-P	248 +/- 10.5	n. d.
Cit-P	52.5 +/- 8	1.7
5	235.5 +/- 1	n. d.
6	202.6 +/- 7.7	n. d.

^aMicrosomal fractions were prepared from CHO K1 or CHO Lec15 cells and assayed for Man-P-Dol synthase activity in the presence of 100 μ M exogenously added acceptor, as described in *Materials and Methods*. The data are average initial rates from three separate experiments. n. d. indicates 'none detected'.

Table 2

Comparison of kinetic constants of mannosyltransferase activities from membrane fractions from *S. cerevisiae*, rat liver and *M. luteus* for Dol-P and three water-soluble analogues^a.

Enzyme source	Substrate	Vmax (nmol/mg/min)	Km (mM)	Vmax/Km
Yeast	Dol-P	3.34	49.4	0.068
	Cit-P	10.81	166.5	0.065
	5	7.84	48.4	0.162
	6	4.36	20.3	0.215
Rat liver	Dol-P	0.68	23.7	0.029
	Cit-P	n.d.	400	n.d.
	5	1.00	29.0	0.034
	6	1.24	77.0	0.016
<i>M. luteus</i>	Dol-P	0.53	7.1	0.075
	Cit-P	0.32	66.5	0.005
	5	0.28	6.8	0.041
	6	0.25	10.7	0.023

^aKinetic constants were calculated from regression lines of double reciprocal plots of average initial rates of mannosyltransferase activity from three separate experiments as described in "Methods". Regression lines, determined by linear regression analysis using the Sigma Plot Linear Regression Tool, exhibited coefficients of determination between 0.95–1.00. Although Cit-P was clearly a substrate for rat liver Man-P-Dol synthase, the substrate concentration range employed did not permit an accurate determination of kinetic constants. n.d. = not determined.

Table 3Summary of proteins identified by photoreaction with 5^a.

Addition	UV	Total proteins	Membrane proteins	ER proteins
		<i>(number of proteins detected)</i>		
none	–	63	8	3
none	+	18	6	2
5	–	112	32	12
5	+	295	114	54
6	–	124	31	10
6	+	281	115	51

^aMembrane proteins were identified by the presence of at least one transmembrane α -helix detected using the TMHMM server; ER proteins were identified using the annotation available through the UniProt Consortium web site (www.uniprot.org).

Table 4

S. cerevisiae ER-associated proteins identified by photoreaction with PAL-Cit-P^a.

Acc	TMD	Description	5	6
P14020	1	Dolichol-phosphate mannosyltransferase DPM1	+	+
P16661	1	Chitobiosyldiphosphodolichol beta-mannosyltransferase ALG1	+	+
P33775	9	Dolichyl-phosphate-mannose--protein mannosyltransferase 1 PMT1	+	+
P31382	10	Dolichyl-phosphate-mannose--protein mannosyltransferase 2 PMT2	+	+
P38242	1	UDP-N-acetylglucosamine transferase subunit ALG14	+	+
P39007	13	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit STT3	+	+
P46964	3	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit OST2	+	+
P40350	1	Dolichyl-phosphate beta-glucosyltransferase ALG5	+	
P53730	9	Dol-P-Man:Man(7)GlcNAc(2)-PP-Dol alpha-1,6-mannosyltransferase ALG12	+	
P40318	14	ERAD-associated E3 ubiquitin-protein ligase DOA10 SSM4	+	+
P47190	10	Dolichyl-phosphate-mannose--protein mannosyltransferase 3 PMT3	+	+
Q12078	10	Iron transporter SMF3	+	
P39986	10	Manganese-transporting ATPase 1 SPF1	+	+
P38353	9	Sec sixty-one protein homolog SSH1	+	+
Q12144	5	Pore and endoplasmic reticulum protein of 33 kDa PER33	+	+
Q12164	5	Pore membrane protein of 33 kDa POM33	+	+
P47154	4	CAAX prenyl protease 1 STE24	+	+
P35723	3	Endoplasmic reticulum transmembrane protein 1 YET1	+	+
Q07451	3	Endoplasmic reticulum transmembrane protein 3 YET3	+	+
P36039	3	ER membrane protein complex subunit 3 EMC3	+	
P53173	3	ER-derived vesicles protein ERV14 ERV14	+	

^aMembrane fractions from *S. cerevisiae* were photolyzed in the presence of the indicated regio-isomer of PAL-Cit-P (para- or meta-). Photolabeled proteins were coupled to Biotin-azide, purified by streptavidin capture and identified by proteomics as described in "Materials and methods." These results are representative of at least three separate experiments. For a complete list of ER associated membrane proteins identified by interaction with p-PAL-Cit-P and m-PAL-Cit-P see supplementary Table S4 and Table S6, respectively.

Table 5

S. cerevisiae annotated as “cell membrane”-associated proteins identified by photoreaction with PAL-Cit-P^a.

Acc	TMD	Description	5	6
P33302	14	Pleiotropic ABC efflux transporter PDR5	+	
P53134	12	Putative oligopeptide transporter YGL114W	+	+
Q02785	11	ATP-dependent permease PDR12	+	+
P54862	11	Hexose transporter HXT11	+	+
P40885	11	Hexose transporter HXT9	+	+
P39003	11	High-affinity hexose transporter HXT6	+	+
P39004	11	High-affinity hexose transporter HXT6	+	+
P32466	11	Low-affinity glucose transporter HXT3	+	+
P32467	11	Low-affinity glucose transporter HXT4	+	+
Q03516	11	Uncharacterized protein RSN1		+
P53154	10	Glycerol uptake protein 1 GUP1		+
P40441	10	Putative hexose transporter 12 HXT12	+	+
P32804	8	Zinc-regulated transporter 1 ZRT1	+	+
P25619	7	30 kDa heat shock protein HSP30	+	+
P38079	7	Protein YRO2	+	+
Q12207	4	Non-classical export protein 2 NCE102	+	+
P53217	4	Uncharacterized membrane protein YGR026W	+	+

^aSee the legend to Table 4 for details.