Acceptor Specificity of *Salmonella* GDP-Man:αLRha1→3αDGal-*PP*-Und *β*1→4-Mannosyltransferase: A Simplified Assay Based on Unnatural Acceptors

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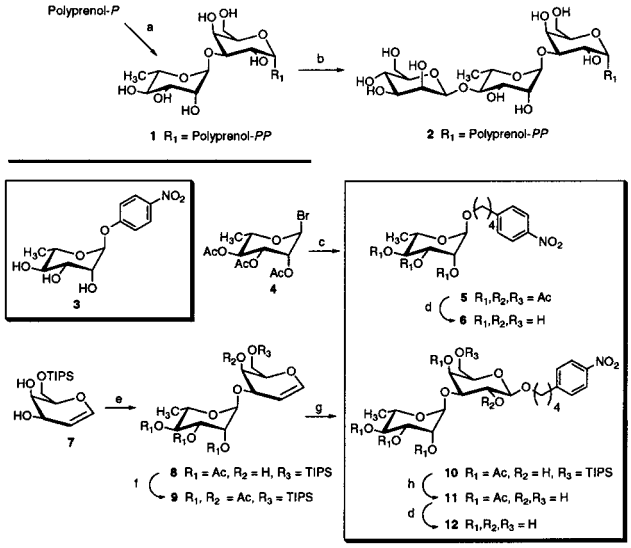
The first unique mannosyl transfer in the biosynthesis of the common core of most Asn-linked eukaryotic glycoproteins is catalyzed by GDP-α-D-Man:Glc*N*Ac*β*1→4Glc*N*Ac*β*1→4-*PP*-Dol *β*1→4-mannosyltransferase.[1,2] Unfortunately, due to low expression levels and enzyme lability, the chemical mechanism for this key reaction remains undetermined. Interestingly, dolichol-dependent biosynthesis shares striking similarities with bacterial undecaprenol-dependent glycosylation.[3] This relationship, in conjunction with the superior biochemical and genetic manipulatability of bacterial systems, renders prokaryotic mannosyltransferases attractive as accessible models for their eukaryotic counterparts. One such model mannosyltransferase, GDP-α-D-Man:αRha1→3αGal-*PP*-Und *β*1→4-mannosyltransferase (ManT$^{\beta 4}$) is involved in the biosynthesis of the *Salmonella* group E1 *O*-antigen repeat unit (**2**, Scheme 1).[4] The biosynthesis of the natural acceptor for this enzyme, αRha1→3αGal-*PP*-Und (**1**), is initiated by enzyme-catalyzed galactosyl-1-phosphate transfer from UDP-Gal to undecaprenol phosphate,[5] with subsequent loss of UMP, followed by enzyme-catalyzed rhamnosyl transfer from TDP-Rha and loss of TDP.[6] Of the reagents required for the biosynthesis of **1**, UDP-α-D-Gal:*P*-Und α-galactose-1-phosphoryl transferase (Gal-1-*P*T), TDP-*β*-L-Rha:Gal-*PP*-Und 1→3-rhamnosyltransferase (RhaT$^{\alpha 3}$) and TDP-*β*-L-Rha are not commercially available.[7] Thus, we report the synthesis of simplified analogues of **1** (Scheme 1) and compare their abilities to function as ManT$^{\beta 4}$ acceptors. In addition to providing a new method for the practical synthesis of Gram negative cell wall antigens,[8] this work reveals a new quantitative ManT$^{\beta 4}$ assay and begins to map acceptor requirements for this intriguing enzyme.

The first analogue, *p*-nitrophenyl-*β*-L-Rha (**3**), is commercially available, whereas Koenigs−Knorr[9] synthesis of **5** (64% α, α/*β* = 9:1), from bromide **4**[10] and 4-(4-nitrophenyl)-1-butanol, followed by Zémplen deacylation efficiently provided **6** (85%). Synthesis of the disaccharide analogue **12** was initiated with Koenigs−Knorr coupling of glycal **7**[11] and **4** to give **8** (69% α, α/*β* = 10:1).[12] The corresponding glycal **9** was epoxidized using 3,3-dimethyldioxirane[13] to provide the 1,2-anhydro derivative, which furnished crystalline **10** (43% α, α/*β* = 1:9) in the presence of zinc chloride and 4-(4-nitrophenyl)-1-butanol.[14] Deprotection provided the potential mannosyltransferase substrate **12** (84% α, α/*β* = 12:1). To test the potential acceptors, **3**, **6**, and **12** were individually incubated with GDP-α-D-Man or GDP-α-D-[*U*-^{14}C]-Man and extracts from a ManT$^{\beta 4}$ overexpressing strain,[15] and the reaction progress was monitored by HPLC.[16]

Scheme 1. *Salmonella* Group E$_1$ *O* Antigen Biosynthesis (upper) and the Corresponding Unnatural ManT$^{\beta 4}$ Acceptors (lower)[a]



[a] Gal-1-*P*T/UDP-Gal, RhaT$^{\alpha 3}$/TDP-Rha. (b) ManT$^{\beta 4}$/GDP-Man. (c) AgOTf, 1,1,3,3-tetramethylurea, 4-(4-nitrophenyl)-1-butanol, CH_2Cl_2, 0 °C. (d) NaOMe, MeOH, 20 min. (e) AgOTf, 2,6-di-*tert*-butylpyridine, CH_2Cl_2, −40 °C. (f) Ac_2O, pyridine. (g) DMDO, 0 °C, CH_2Cl_2; 4-(4-nitrophenyl)-1-butanol, $ZnCl_2$, −78 °C → 20 °C, THF. (h) TBAF, THF.

Assays of ManT$^{\beta 4}$ extracts with **3** or **6** revealed no observed ManT$^{\beta 4}$-catalyzed glycosylation. However, assays containing **12** (retention time of 13.90 min.) unveiled the time-dependent formation of a new product with a retention time of 12.99 min (Figure 1a). Molecular weight determination of the new product was consistent with mannosylation of **12**,[17] and assays containing **12** in the presence of GDP-[*U*-^{14}C]Man demonstrated the time-dependent incorporation of [*U*-^{14}C]Man into the product peak

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(1) Abbreviations: Asn, asparagine; Dol, dolichol; Gal, D-galactose; GDP, guanosine diphosphate; Glc, D-glucose; Glc*N*Ac, 2-*N*-acetyl-D-glucose; Man, D-mannose; Rha, L-rhamnose (6-deoxy-L-mannose); TDP, thymidine diphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; Und, undecaprenol.

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(12) The use of 6-*O*-triisopropylsilyl greatly influenced regioselectivity, and the use of di-*tert*-butylpyridine gave slightly higher yields than either 2,4,6-collidine (50−55%) or 1,1,3,3-tetramethylurea (40%). Peracetylated rhamnal or pertriisopropylsilylated rhamnal failed to give a stable epoxide product with DMDO. Our rationale to synthesize **12**, as opposed to the 4-nitrophenyl disaccharide (not tested), was to provide both a hydrophobic handle reminiscent of **1** and stability to chromophore hydrolysis under alkaline assay conditions.

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(14) Galactal coupling in the absence of rhamnose reproducibly furnished [4-(nitrophenyl)-1-butyl]-*β*-D-galactopyranoside in >90% yield.

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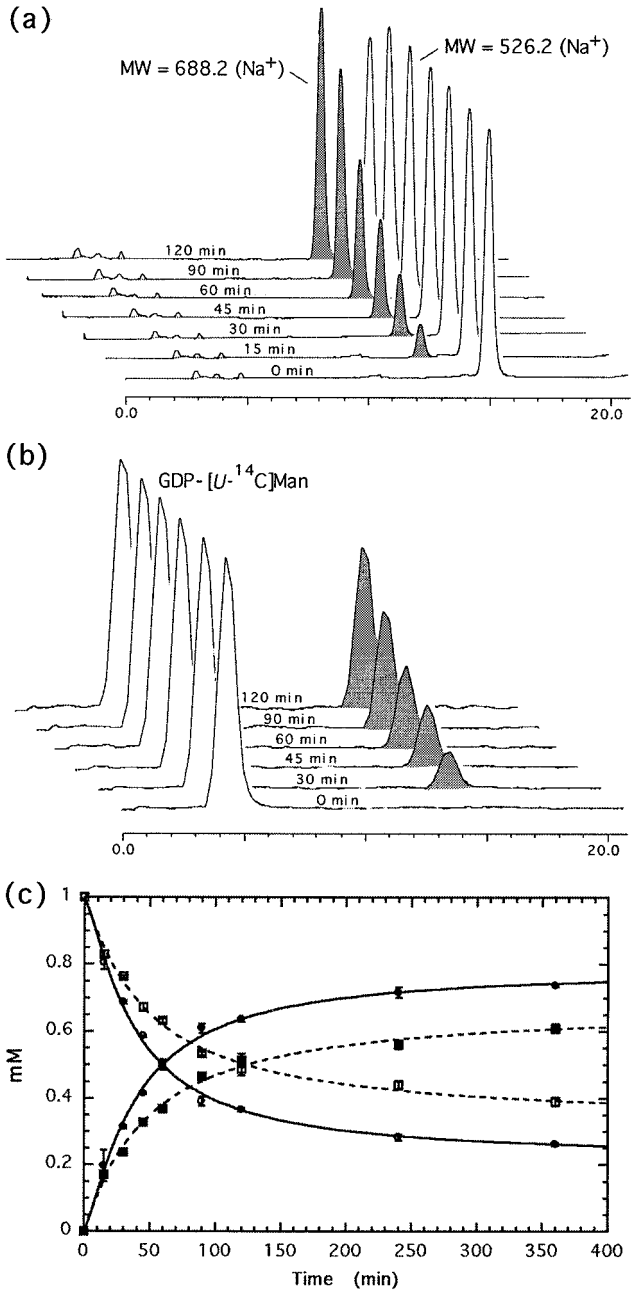

Figure 1. (a) Time dependence of an assay containing **12**, GDP-Man, and ManT$^{\beta 4}$ extracts monitored at 325 nm. (b) Time dependence of an assay containing **12**, GDP-[*U*-^{14}C]Man, and ManT$^{\beta 4}$ extracts monitored by flow scintillation counting. (c) Plots of the disappearance of **12** (○, from Figure 1a) and GDP-Man (□, from Figure 1b) and the corresponding formation of the trisaccharide product (●, from Figure 1a; ■, from Figure 1b).

(Figure 1b). A plot of the peak area versus time from Figure 1a or b revealed the time-dependent formation of the new product ($A_{325} = 18.0\ \mu$M min^{-1}; [^{14}C] = 15.0 μM min^{-1}) and the decrease of **12** ($A_{325} = 23.0\ \mu$M min^{-1}; [^{14}C] = 13.0 μM min^{-1}) are linear for the first 30 min (Figure 1c).[18] In addition, control reactions containing **12**, GDP-Man, and pET11a-BL21 extracts (identical *Escherichia coli* extracts but lacking ManT$^{\beta 4}$ expression) or **12** and ManT$^{\beta 4}$ extracts (but lacking GDP-Man) showed no change over 120 min (data not shown), indicating that the mannosylation of **12** is clearly dependent upon the expression of both the exogenous ManT$^{\beta 4}$ and GDP-Man. Finally, the newly formed trisaccharide could only be cleaved by commercially available β-mannosidases, confirming the Man-Rha linkage as beta.[19]

The advantage of the strategy which we present over existing routes to 3-*O*-α-L-rhamnopyranosyl-D-galactopyranosides[20] is in providing a 3-*O*-α-L-rhamnopyranosyl-D-galactal which can be readily derivatized from the reducing end. We have exploited this strategy to provide a simplified chromophoric substrate which allows for the first ManT$^{\beta 4}$ quantitative assay system. Cumulatively, the presented results are consistent with the ManT$^{\beta 4}$-catalyzed formation of trisaccharide **2** and therefore provide further confirmation of the original *wba*O gene assignment put forth by Liu et al.[4a] The observed lack of mannosylation of **3** and **6** indicate that ManT$^{\beta 4}$ requires at least part, if not all, of the galactosyl moiety; however, acceptance of **12** indicates that ManT$^{\beta 4}$ does not require the Und-*PP* portion of the native acceptor **1**.

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Supporting Information Available: Experimental details and characterization data for **4**-**6**, **8**-**12** (3 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(15) PCR amplified *wba*O (encoding ManT$^{\beta 4}$) from pPR1330[4b] was cloned into a T7-driven pET11a-BL21 system, grown to an $OD_{600} = 0.55$ at 37 °C with shaking (250 rpm), the growth temperature reduced to 20 °C, and protein expression induced with 1.0 mM IPTG. Rifampicin (75 μg mL^{-1}) was added 2 h after induction, and the cultures were allowed to grow for an additional 12−14 h. Cells were harvested (2000*g*, 15 min), resuspended in 50 mM Tris-HCl, 1 mM EDTA, pH 8.5, and lysed by sonication. The cellular debris was removed by centrifugation (2000*g*, 15 min), and the membrane fractions were isolated from the supernatant by centrifugation (30000*g*, 1 h), resuspended in 50 mM Tris-HCl, 1 mM EDTA, pH 8.5, and stored at −80 °C until used. SDS-PAGE revealed the desired ManT$^{\beta 4}$ as approximately 70−80% of the total membrane-associated protein.

(16) A typical assay consisted of 10 mM acceptor, 10 mM GDP-α-D-Man, 10 mM $MgCl_2$, 25 μL crude extracts in a total volume of 50 μL. After an appropriate incubation period at 37 °C, an equal volume of MeOH was added, denatured proteins were removed by centrifugation, and 20 μL was analyzed by reverse-phase chromatography (Microsorb-MV C-18, 4.6 × 250 mm, 75% 10 mM NaH_2PO_4, 25% CH_3CN, pH 7.5, 0.75 mL min^{-1}, $\lambda = 325$ nm). The ϵ_{325} for **3** (7820 M^{-1} cm^{-1}), **6** (2340 M^{-1} cm^{-1}), and **12** (2310 M^{-1} cm^{-1}) used for these assays were determined experimentally (75% 10 mM NaH_2PO_4, 25% CH_3CN, pH 7.5). Radiolabeled assays in addition contained 3.3 μM GDP-α-D-[*U*-^{14}C]Man and were monitored with a Packard 150TR flow scintillation analyzer. Although the lipid moiety of **12** is inverted from that of **1**, **12** is the major synthetic product, and assays with the minor α-analogue revealed a similar rate of ManT$^{\beta 4}$-catalyzed mannosylation.

(17) A mixture of 1.0 mM **12**, 1.2 mM GDP-Man, 10 mM $MgCl_2$, 1.2 mg ManT$^{\beta}$.[4] 50 mM Tris-Cl, 1 mM EDTA, pH 8.5 in a total volume of 750 μL was incubated at 37 °C for 4 h, the reaction was stopped by adding an equal volume of methanol, and denatured protein was removed by centrifugation (16000*g*, 15 min). The supernatant (0.5 mL) was chromatographed on a Licrosorb RP-18 (7 μm) semipreparative column with a mobile phase of 25% CH_3CN, 75% H_2O, flow rate of 1.5 mL min^{-1}. The trisaccharide fraction (20−22 min) and disaccharide fraction (24−26 min) were collected, concentrated, and submitted for mass spectral analysis.

(18) The difference of this rate versus ManT$^{\beta 4}$-catalyzed mannosylation of **1** (275 nM min^{-1}) is partially attributed to technical difficulties associated with the selective extraction of **2**.[7]

(19) A mixture of 1.0 mM **12**, 1.2 mM GDP-Man, 3.32 μM GDP-α-D-[*U*-^{14}C]Man, 10 mM $MgCl_2$, 960 μg ManT$^{\beta}$.[4] 50 mM Tris-Cl, 1 mM EDTA, pH 8.5 in a total volume of 500 μL was incubated at 37 °C for 4 h the reaction stopped by adding an equal volume of methanol and denatured protein removed by centrifugation (16000*g*, 15 min). The recovered supernatant was evaporated and the corresponding dried reaction mixture dissolved in 180 μL of 200 mM NaH_2PO_4, 1 mM EDTA pH 5.5 buffer and separated into three equal aliquots. To the first was added 0.5 U of α-mannosidase (Sigma), to the second was added 0.04 U of β-mannosidase (Sigma), and to the third buffer was added as a control. After incubation at 37 °C for 4 h, the reactions were analyzed by HPLC as previously described.[16] Although this result confirms the postulated ManT$^{\beta 4}$ stereoselectivity,[4] the confirmation of the postulated regioselectivity is currently in progress.

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