Enzymes in Oligosaccharide Synthesis: Active-Domain Overproduction, Specificity Study, and Synthetic Use of an α-1,2-Mannosyltransferase with Regeneration of GDP-Man[1]

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The catalytic domain of a membrane-bound α-1,2-mannosyltransferase (ManT) from yeast has been overexpressed in *E. coli*, at a level of approximately 0.7–0.8 units per L with a specific activity of about 1 U/mg (based on α-methyl mannoside) after purification. The *E. coli* strain has been deposited in ATCC (#77379) and will be available to the public. The isolated ManT is stable in 30% methanol or 20% acetone. It accepts mannose, mannobiose, and *O*-mannosylglycopeptides as acceptors. A multiple enzyme system with *in situ* regeneration of GDP-mannose suitable for large-scale synthesis of mannosides and mannosyl glycopeptides has been developed.

Oligosaccharide synthesis based on sugar nucleotide-dependent glycosyltransferases proceeds regio- and stereoselectively under mild reaction conditions without multiple protection and deprotection steps.[2] Glycosyltransferases, however, are difficult to obtain (β-1,4-galactosyltransferase is the only one commercially readily available), and the enzymatic synthesis requires sugar nucleotide regeneration for large-sacle processes.[3]

More than 50 glycosyltransferase genes have been cloned and sequenced from bacteria, yeast, and mammalian cells, and documented in the Genebank (IntelliGenetics, Inc.). The availability of these sequences provides us an opportunity to overexpress glycosyltransferases in large quantities and use them for oligosaccharide synthesis. Of the eight sugar nucleotides commonly used as donor substrates for mammalian glycosyltransferases, five of which (i.e., UDP-Glc, UDP-Gal, GDP-Fuc, CMP-NeuAc, and UDP-glucuronic acid) have the regeneration system available for large-scale processes.[5] The enzymes required for the regeneration of GDP-Man, UDP-GlcNAc, and UDP-GalNAc have been reported,[4,5] although regeneration of these sugar nucleotides has not been demonstrated. As part of our efforts to develop glycosyltransferase-based enzymatic procedures for the synthesis of complex oligosaccharides and glycopeptides, we report here the overproduction and specificity study of the soluble catalytic domain of an α-1,2-mannosyltransferase (ManT)[6] and application of this enzyme coupled with regeneration of guanosine 5′-diphosphomannose (GDP-Man) to the synthesis of mannose-containing oligosaccharides and glycopeptides.

The α-1,2-mannosyltransferase in the yeast *Saccharomyces cerevisiae* is a membrane-bound enzyme that transfers mannose from GDP-mannose to the terminal mannose residue of O-linked dimannosyl proteins (eq 1).[6]

$$\text{Man}\alpha1,2\text{Man}\alpha\text{Thr(Ser)-Protein} \xrightarrow[\alpha\text{-1,2-mannosyltransferase}]{\text{GDP-Man}} \text{Man}\alpha1,2\text{Man}\alpha1,2\text{Man}\alpha\text{Thr(Ser)-protein} \quad (1)$$

Similar to known glycosyltransferases and glycosidases of the mammalian Golgi apparatus,[7] this ManT contains a short N-terminal domain followed by a membrane-spanning region and a large catalytic domain. Since it has been shown that the catalytic domains of many glycosyltransferases are more stable and as active as the membrane-bound enzymes,[8] we have constructed a secretion vector harboring the gene encoding the catalytic domain of the mannosyltransferase for overexpression in *E. coli*.[9] It was, however, found in this study that some O-linked monomannosyl peptides are better substrates than mannose and mannobiose for the enzyme.

Results and Discussion

Overexpression and Purification of the Mannosyltransferase. The gene encoding the protein sequence 31–442 of ManT from *S. cerevisiae* was cloned by the PCR method with two designed primers (Table I). The PCR insert (1.4 kb) corresponding to ManT gene was digested with XbaI and SalI and was ligated into vector pFlag[10] to construct the plasmid pManflag20 (Figure 1). The plasmid was then transformed to *E. coli* XL1-Blue strain[11] for overproduction of the enzyme.

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Table I. Primers Used in the Amplication of α-1,2-Mannosyltransferase Gene

primer Manflag5:

5′ ATATTTCTAGAAGAACTCAGCAATATATT

Xba I structure gene of N-terminal

primer Manflag3:

5′ GCGCGTCGACTTATTACTCACGGAATTTTTTCCA

Sal I stop structure gene of C-terminal

We found that the activity of ManT expressed in *E. coli* was dependent on the culture condition. When the cells were cultured in the LB medium or M9 medium at 37 °C with the inducer (isopropyl β-D-thiogalactopyranoside, IPTG) concentration at 1 mM (standard conditions), no mannosyltransferase activity was detected in the culture. In contrast, when the cells were grown at 30 °C in M9-Ca medium[12] in the presence of a very low concentration of IPTG (<0.01 mM), significant amounts of ManT activities were obtained. This result indicated that at these conditions the recombinant protein was properly folded and secreted in the *E. coli* periplasmic space.[13,14] The optimal concentration of IPTG was found to be around 0.005 mM. At this concentration, 1 L of culture yields 0.7–0.8 units[15] of ManT.

Since most of the active enzyme is secreted in the periplasmic space, purification of the recombinant protein is very straightforward. After the periplasmic fraction is prepared from the cells as described in the Experimental Section, the fraction can be directly used for the synthetic purpose, although minor phosphatase activities were also found in this fraction. To prevent the decomposition of the sugar nucleotide during the enzymatic mannosylation reaction, an inhibition cocktail (~1% volume) containing 100 mM ATP,[16] 1 mM theophylline,[17] and 1 mM 2,3-dimercaptopropanol[18] was added.

After a Superose column chromatography, further purification of the ManT was carried out by FPLC on a Mono Q column to obtain the protein with a specific activity of about 1 unit/mg. The purity of the protein was assessed by SDS–polyacrylamide gel electrophoresis and was estimated to be greater than 85% with a molecular weight of 50 kDa.

Enzyme Stability. The enzyme retained more than 90% activity after 4 days in a buffer at 20 °C solution (100 mM Tris–HCl, 5 mM $MgCl_2$ buffer, pH 7.4). However, at a higher temperature (37 °C) the enzyme showed a half-life of about 4 h. The oligosaccharide synthesis was therefore conducted at 25–30 °C.

Organic Solvent Effects. Since a low concentration of organic solvent is needed to dissolve some of *O*-glycopeptides used as acceptors in this work, the effect of organic solvent on the mannosyltransfer reaction was investigated. The enzyme tolerates up to 30% of methanol and 20% acetone–buffer solution. The transferase remains about

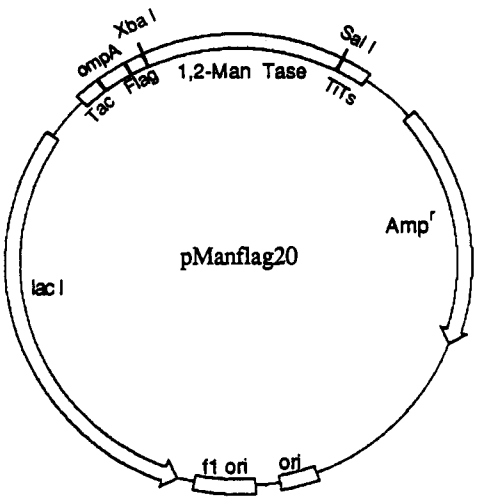

Figure 1. Genetic map of α-1,2-mannosyltransferase expression plasmid.

80% active in a 10% DMF-buffer solution and about 50% active in a 10% acetonitrile–buffer solution but loses 95% of activity when the concentrations of these organic solvents increase to 20%. Thus, when needed in the assay and synthesis, 10% acetone or up to 25% methanol–buffer solution is utilized.

Substrate Specificity. Previous studies on the native α-1,2-mannosyltransferase showed that the enzyme transferred mannose to α-methyl mannopyranoside and mannobiose.[19] The active domain of this enzyme prepared in this study also has a similar substrate specificity (Table II). It accepts α-methyl mannopyranoside (**1**), mannose (**2**), and Manα(1,2)ManαOMe (**3**) as substrates with K_m of 57, 193, and 28 mM, respectively. For comparison, the K_m value of mannose for the native enzyme was 100 mM.[19] The C-6 modified α-methyl mannopyranosides such as 6-deoxy-ManαOMe (**4**), 6-azido-6-deoxy-ManαOMe (**5**), and 6-amino-6-deoxy-ManαOMe (**6**) are poor substrates. *p*-Nitrophenyl α-mannopyranoside and other monosaccharides with an *S*-configuration at the 2 position, such as D-altrose, D-idose, D-talose, D-arabinose, and D-lyxose are not substrates. The mannosidase inhibitior, 1-deoxymannojirimycin,[20] was neither a substrate nor an inhibitor of ManT.

O-Mannosylpeptides were found to be good substrates for this enzyme. For example, Cbz-Thr(α-Man)-Val-OMe (**7**) is comparable with ManαOMe as an acceptor, but has a lower K_m value. The N-terminal deprotected compound **8** and longer peptide analog **9** are also good substrates. Interestingly, *O*-glycopeptide **10** with the peptide sequence Tyr-Thr-Val[21] has a K_m value of 0.7 mM for ManT. This value is 10 times smaller than that of compound **7** and 80 times smaller than that of ManαOMe (**1**). This result suggests that ManT prefers certain peptide sequences in *O*-mannosylpeptides.

Oligomannose is the backbone structure component of N-linked and O-linked glycoproteins in yeast and mam-

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(15) One unit transfers 1.0 μmol of mannose from GDP-mannose to methyl α-D-mannopyranoside per min at 30 °C.
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(20) Synthesized via fructose diphosphate aldolase catalyzed condensation followed by catalytic intramolecular reductive amination. Kajimoto, T.; Liu, K. K.-C.; Chen, L.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 6187.
(21) Tanner, W. *Eur. J. Biochem.* **1991**, *196*, 185. A good substrate for dolichylphosphate D-mannose:protein *O*-D-mannosyl transferase to put the first mannose to peptide in *S. cerevisiae*.

Table II. Relative Rates and K_m (Acceptor) of the Mannosyltransfer Reaction (GDP-Man + Acceptor) Catalyzed by the Recombinant α1,2-Mannosyltransferase

compd	$V_{relative}$[a]	K_m (mM)
Manα-OMe (1)	1.0	57
mannose (2)	0.46	193
Manα1,2Manα-OMe (3)	0.62	28
6-deoxy-Manα-OMe (4)	0.06	
6-azido-6-deoxy-Manα-OMe (5)	0.02	
6-amino-6-deoxy-Manα-OMe (6)	0.07	
Cbz-Thr-Val-OMe (7) |α Man	0.71	7.8
H-Thr-Val-OMe (8) |α Man	0.68	
Cbz-Thr-Val-Gly-Ala-NH_2 (9) |α Man	0.55	
Boc-Tyr-Thr-Val-OMe (10) |α Man	0.35	0.7
Cbz-Thr-Val-OMe (11) |α Man |α1,2 Man	0.17	26
6-*O*-tosyl-Manα-OMe (12)[b]	0.00	
p-nitrophenyl α-mannopyranoside (13)[b]	0.00	

[a] Calculated as the ratrio of V_{max}. [b] For other unacceptable substrates tested, see text.

malian cells. Such oligosaccharides are constructed by the highly ordered addition of monosaccharide units to the growing oligosaccharide chain. Recent studies on the biosynthesis of O-linked carbohydrate chains in *S. cerevisiae* suggested that the native α-1,2-mannosyltransferase was responsible for the transfer of the third mannose to the growing O-linked carbohydrate chains[22] (eq 1). However, comparing the kinetic data of compound **7** and **11**, the recombinant active domain of ManT seems to be more active on monomannosyl *O*-glycopeptides than on dimannosyl glycopeptides. The reason for this observation is not clear yet.

The C-6 modified α-methyl mannopyranosides used in the specificity study were synthesized from **1** (Scheme I). Tosylation of the 6-OH of **1** in pyridine at 0 °C, followed by substitution and reduction, generated compounds **12**, **5**, and **6**, respectively. Bromination of the 6-hydroxyl group of **1** and subsequent acetylation afforded compound **14**. Reduction of **14** with Bu_3SnH followed by deprotection gave 6-deoxy derivative **4**.

O-Mannosylpeptides in Table II were prepared chemically, and the peptide chain was then extended either chemically or enzymatically as shown in Scheme II. The *O*-glycopeptide **16** was synthesized by glycosylation of peptide Cbz-Thr-Val-OMe with tetraacetyl-*O*-D-mannopyranosyl bromide in the presence of silver trifluoromethanesulfonate.[23] Compound **10** was synthesized from **17** by standard peptide coupling with Boc-Tyr(Bzl)-OH. Deacylation of **16** in NaOMe/MeOH at 0 °C afforded compound **7**. Compound **9** was synthesized via coupling of **7** with H-Gly-Ala-NH_2 catalyzed by a subtilisin mutant 8397[24] in 80% DMF (v/v) solution. This protease was developed for use in DMF and was proven to be an effective catalyst for the synthesis of *O*- and *N*-glycopeptides.[25]

Synthesis of Mannose-Containing Oligosaccharides and Glycopeptides with Regeneration of GDP-Man. As illustrated in Scheme III, the multienzyme system started with mannose 1-phosphate which was synthesized from mannose in three steps in this laboratory.[26] Mannose 1-phosphate reacted with GTP catalyzed by GDP-mannose pyrophosphorylase (EC 2.7.7.22) from yeast cells[27] to form GDP-Man.[28] GDP-Man was consumed by ManT to give the mannosyl oligosaccharide or glycopeptide, and the released GDP was again converted to GTP by pyruvate kinase (PK, EC 2.7.7.9) and phospho(enol)pyruvate (PEP). The inorganic pyrophosphate resulted from the reaction was hydrolyzed to inorganic phosphate by inorganic pyrophosphatase (EC 3.6.1.1) to shift the equilibrium and to avoid its inhibition.

In a representative synthesis of Cbz-Thr(α-Man1,2α-Man)-Val-OMe (**11**), a reaction mixture (2 mL, 100 mM Tris, pH 7.5, 5% acetone, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mM EDTA, 5 mM NaN_3, 1 mM ATP, 0.01 mM theophylline, 0.03 mM 2,3-dimercaptopropanol, and 0.05 mM phenylmethylsulfonyl fluoride) containing Cbz-Thr(α-Man)-Val-OMe (**7**) (100 mg, 95 mM), mannose 1-phosphate (60 mg, 100 mM), GDP (9 mg, 10 mM), PEP (47 mg, 100 mM), pyruvate kinase PK (50 U), dried yeast cells (50 mg), ManT (0.4 U), and inorganic pyrophosphatase (1 U) was slightly stirred at room temperature for 60 h and then centrifuged. The supernatant was lyophilized and extracted with methanol. After removal of the methanol, the product was purified by silica gel column chromatography ($CHCl_3$:CH_3OH:H_2O = 6:3:0.5, v/v/v) to afford Cbz-Thr(α-Man1,2α-Man)-Val-OMe (**11**) (31 mg) in 41% overall yield based on consumed Cbz-Thr(α-Man)-Val-OMe. The disaccharide **3** was prepared in 38% yield in a similar manner.

Conclusion

In summary, we have developed an efficient expression system for overproduction of the catalytic domain of a membrane-bound α-1,2-mannosyltransferase. In M9-CA medium and at very low inducer concentration, the active domain of ManT was properly folded and secreted into the *E. coli* periplasm. The recombinant enzyme accepts mannose, mannobiose, and *O*-mannosylglycopeptides as substrates. A multiple enzyme system with *in situ* regeneration of GDP-mannose suitable for the large-scale synthesis of mannosyloligosaccharides and *O*-mannosylpeptides has been developed based on this enzyme. Work is in progress to apply this methodology to the preparation of other glycosyltranferases.

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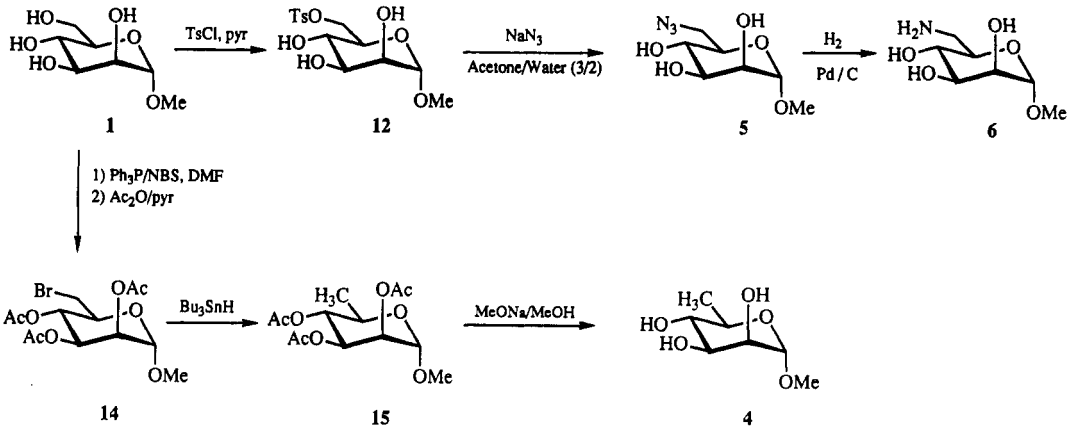
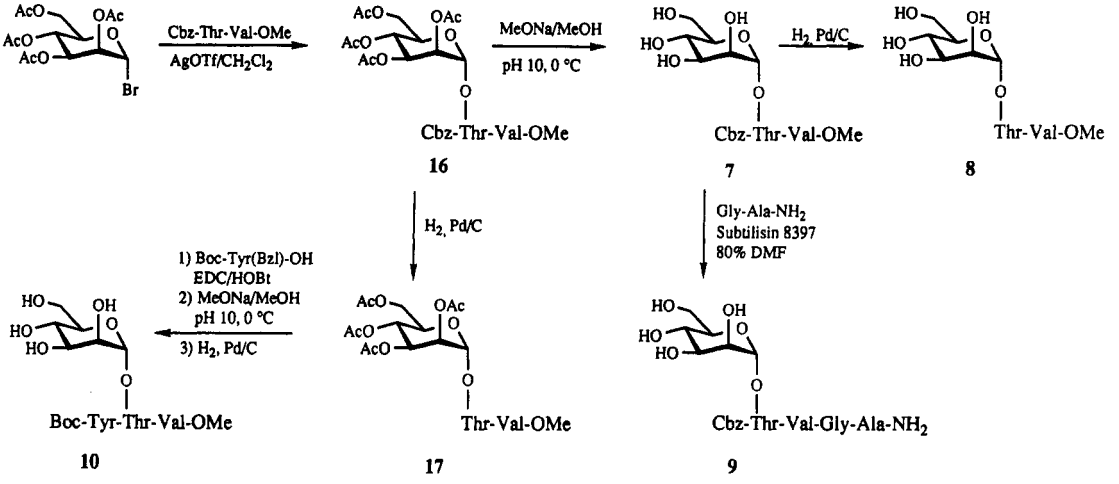
Scheme I. Synthesis of C-6 Modified α-Methyl Mannopyranoside



Scheme II. Synthesis of *O*-Mannosylpeptides



Experimental Section

General. All chemicals were purchased from commercial sources as reagent grade. Fast protein liquid chromatography (FPLC) was performed on a Pharmacia system composed of two P-500 pumps, a GP-250 gradient programmer, and a single-path UV-1 monitor. The UV–visible spectrum was recorded on a Beckman DU-70 spectrometer. SDS–PAGE was carried out on a Pharmacia Phast System. ^1H and ^{13}C NMR spectra were recorded on a Bruker AMX-500 spectrometer. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE mass spectrometer under fast atom bombardment (FAB) conditions. Thin-layer chromatography was conducted on Baker Si250F silica gel TLC plates with fluorescent indicator. Column chromatography was conducted with silica gel, grade 62, 60-200 mesh, and 150 Å. Enzymes such as pyruvate kinase and inorganic pyrophosphatase were purchased from Sigma Co. The vector pFlag-1 was purchased from International Biotech. Inc. (New Haven, CT).

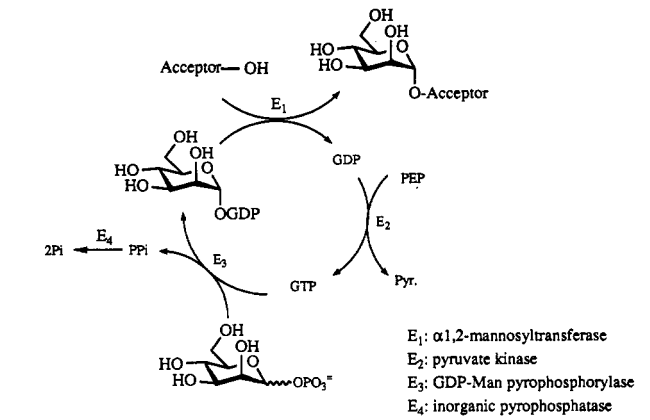
Amplification of the α-1,2-Mannosyltransferase Gene from Yeast DNA. A PCR amplification was performed in a 100-μL reaction mixture containing 1 μL (0.5 μg) of yeast (*Saccharomyces cerevisiae*) DNA,[11] 400 nmol of the primers Manflag5 and Manflag3, 200 mM of different dNTPs, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% Triton X-100, and 2 units of *Thermus aquaticus* DNA polymerase. The reaction was overlayed with 100 μL of mineral oil and subjected to 30 cycles of amplifications.

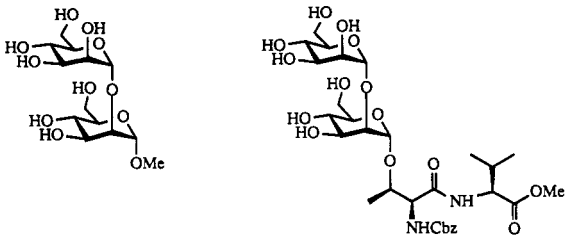
Construction of the α-1,2-Mannosyltransferase Expression Vector. The DNA obtained from the PCR amplification was extracted with phenol/chloroform and precipitated with ethanol at −70 °C for 30 min. The precipitated DNA was centrifuged and washed with 70% ethanol. The DNA pellet (about 500 μg) was then dissolved in a restriction enzyme buffer and digested with XbaI and SalI (40 U each) at 37 °C for 2 h. The digested DNA was then recovered by phenol/chloroform extraction and ethanol precipitation. The DNA was redissolved in 50 μL of TE buffer (pH 7.7) and purified on 0.7% agarose gel. The DNA band corresponding to 1.4 kb was separated from the agarose gel and purified with Gene Clean kit (Bio-101 Co., San Diego) and was used as an insert. The vector prepared from digestion of pFlag-1 DNA (1 mg) with 40 units of XbaI and SalI was recovered with ethanol precipitation after extraction with phenol/chloroform and further purified by agarose gel as described in the insert preparation. The insert was then ligated to the vector and transformed to *E. coli* XL1-Blue strain and plated on LB agar plates which contained 100 μg/mL of ampicillin.

Screening for Positive Clones. Since the host strain *E. coli* does not contain yeast α-1,2-mannosyltransferase gene, only the positive clones which contain this gene will show the PCR amplification product of 1.4 kb when primers Manflag5 and Manflag3 are used. Therefore, the PCR method was used for the screening. Thirty-three colonies were randomly selected from plates and lysed with 50 μL of a cell lysing buffer (20 mM Tris–HCl containing 1% Triton X-100 and 2 mM EDTA, pH 8.5). Heated with boiling water for 5 min, the solution was used as a DNA template source for PCR amplification. The procedure for the PCR amplification was the same as that described in the amplification of α-1,2-mannosyltransferase gene except 3 μL of the cell lysing solution was used to replace yeast DNA. Three positive clones were identified. The resulted α-1,2-mannosyltransferase expression vector was shown in Figure 1.

Growing Transformed *E. coli* Strain. The transformed *E. coli* strain was grown on M9-CA medium ($Na_2HPO_4 \cdot 7H_2O$, 12.8 g; KH_2PO_4, 3.0 g; NaCl, 0.5 g; NH_4Cl, 1.0 g; technical grad casamino acids, 20.0 g; water, 1 L) containing 1 mM $CaCl_2$ and 100 μg/mL of ampicillin to mid-logarithmic phase (OD_{600} 0.5–0.6) at 37 °C and then induced with 0.005 mM IPTG for 12 h at 30 °C with shaking.

Scheme III. Synthesis of Mannosyloligosaccharides with Regeneration of GDP-Man





3 (38%) 11 (41%)

Preparation of ManT from *E. coli* Periplasmic Space.[19] All steps were carried out at 4 °C. The culture from 5 L of grown *E. coli* cells was centrifuged at 10000*g* for 10 min. The cell pellet was resuspended in 200 mL of 20% sucrose, 10 mM Tris–HCl (pH 7.6). To the suspension was added 4 mL of EDTA (0.5 M), and the sample was incubated on ice for 30 min and then centrifuged for 5 min at 4 °C. After removal of the supernatant the pellet was resuspended in 20 mL of cold distilled water. The suspension was incubated for 30 min on ice and then centrifuged for 5 min. The supernatant (the periplasmic fraction) was carefully removed, and then 1 mL of 2 M Tris–HCl, 100 mM $CaCl_2$ solution was slowly added to the supernatant. After incubation on ice for 10 min, the suspension was centrifuged for 5 min and the precipitates were discharged. The supernatant was then dialyzed against 100 mM Tris–HCl (pH 7.6) for 8 h at 0 °C. Activity assay showed the resulting periplasmic fraction (20 mL) has approximately 3.7 units of ManT activity.

α-1,2-Mannosyltransferase Activity Assay. GDP-Man (^{14}C) (10 mM, 10 mL), α-methyl mannopyranoside (500 mM, 10 μL), and the enzyme solution (10 μL) were mixed in a buffer containing 50 mM Hepes (pH 7.2), 0.1% Triton, and 10 mM $MnCl_2$. The mixture was incubated at 30.0 °C for 1 h. The unreacted GDP-mannose was removed by adding QAE-Sephadex (400 μL) to the solution. After the resin was removed by centrifugation, the radioactivity of the solution was counted in 10 mL of scintillation fluid. Controls were carried out by omitting the acceptor or the enzyme.

Enzyme Stability Study. The enzyme was incubated at room temperature in 100 mM Tris-HCl buffer, 5 mM $MgCl_2$, pH 7.4, containing 0.5 mM dithiothreitol. At different time intervals, 10-μL aliquots were taken and assayed for mannosyltransferase activity as described above.

Organic Solvent Effect Study. GDP-Man (^{14}C) (10 mM, 10 μL) and methyl mannopyranoside (500 mM, 10 μL) were mixed with certain volume of an organic solvent and H_2O to achieve the desired organic solvent concentration. Then the enzyme (10 μL) was added to the mixture. The solution was incubated at 30.0 °C for 1 h. The enzyme activity was measured as described in the enzyme assay section.

Substrate Specificity and Enzyme Kinetics. The substrate specificity was determined in a 30-μL assay solution containing 150 mM substrate, 3.3 mM GDP-Man, overproduced ManT, 50 mM Hepes (pH 7.2), 0.1% Triton, and 10 mM $MnCl_2$. The mixture was incubated at 30 °C for 1 h, and the formation of product was determined by the radioactivity assay. Initial velocities were measured as described in the enzyme assay at various concentration of acceptors for kinetic studies. From the Lineweaver–Burk plots of the data were determined K_m and V_{max}.

GDP-mannose Pyrophosphorylase. Yeast (*S. cerevisiae*) was grown in YM broth (yeast extract, 3 g, malt extract, 3 g; Bacto peptone, 5 g; Bacto dextrose, 10 g/L of distilled water, pH 6.2) with supplement of 1% sucrose. The culture was grown at 30 °C with shaking (250 rpm) for 36 h and then centrifuged at 8000*g* (4 °C) for 20 min to recover the cells. The cells were washed once with Tris–HCl buffer (100 mM, pH 7.5 with 5 mM $MgCl_2$ and 1 mM mercaptoethanol) and then lyophilized and used as the GDP-mannose pyrophosphorylase source.

Methyl 2,3,4-Tri-*O*-acetyl-6-bromo-6-deoxy-α-D-mannopyranoside (14). Triphenylphosphine (5.4 g, 20.6 mmol) was added portionwise to a cooled solution of methyl α-D-mannopyranoside (2.0 g, 10.3 mmol) and NBS (3.66 g, 20.6 mmol) in DMF (100 mL) at 0–5 °C, and the mixture was heated at 50 °C for 2 h. After cooling, MeOH (5 mL) was added dropwise and the mixture was concentrated. The residue was acetylated with Ac_2O (20 mL) and pyridine (30 mL). The product was purified by silica gel column chromatography with toluene–EtOAc (10:1) to give 14 (1.74 g, 44%): ^1H NMR (500 MHz, $CDCl_3$) δ 2.00, 2.07, 2.15 (s, 3H, 3 × OAc), 3.41–3.49 (m, 2H, H-6), 3.46 (s, 3H, OMe), 3.98 (ddd, 1H, J = 3.0, 8.0, 9.5 Hz, H-5), 4.74 (d, 1H, J = 1.65 Hz, H-1), 5.19 (t, 1H, J = 9.92 Hz, H-4), 5.23 (dd, 1H, J = 1.70, 3.42 Hz, H-2), 5.33 (dd, 1H, J = 3.42, 9.97 Hz, H-3); ^{13}C {^1H} NMR ($CDCl_3$) δ 20.60, 20.69, 20.79, 31.32, 55.31, 68.64, 68.77, 69.41, 69.89, 98.35, 169.77, 169.96; HRMS calcd for $C_{13}H_{19}O_8BrCs^+$ (M + Na^+) 514.9318, found 514.9318.

Methyl 6-Deoxy-α-D-mannopyranoside (4). A solution of Bu_3SnH (1.97 g, 6.77 mmol; 1.82 mL) in toluene (40 mL) was added dropwise to a gently refluxing solution of 14 (1.70 g, 4.42 mmol) in toluene (40 mL) over 20 min, and the mixture was refluxed for 10 h. After cooling, the mixture was concentrated, and the residue was chromatographed on silica gel with toluene–EtOAc (30:1) to give methyl 2,3,4-tri-*O*-acetyl-6-deoxy-α-D-mannopyranoside. A solution of this compound (700 mg, 2.30 mmol) and methanolic MeONa (1 mL, 0.3 M solution) in MeOH (30 mL) was stirred for 2 h at room temperature. The mixture was neutralized by addition of Dowex 50W-X8, and then the resin was filtered off and the filtrate was concentrated to give compound 4 (327 mg, 80% yield). ^1H NMR (500 MHz, CD_3OD) δ 1.26 (d, 3H, J = 6.5 Hz, H-6), 3.33 (s, 3H, OMe), 3.37 (m, 1H), 3.51–3.55 (m, 1H), 3.59 (dd, 1H, J = 3.5, 9.5 Hz), 3.76 (dd, 1H, J = 2.0, 3.5 Hz), 4.54 (d, 1H, J = 1.5 Hz, H-1); ^{13}C {^1H} NMR (CD_3OD) δ 18.01, 55.12, 69.67, 72.25, 72.44, 73.90, 102.83; HRMS calcd for $C_7H_{14}O_5Na^+$ (M + Na^+) 201.0739, found 201.0745.

6-Tosyl-ManαOMe (12). α-Methyl mannopyranoside (1.5 g, 7.8 mmol) was dissolved in 20 mL of dry pyridine and cooled to 0 °C. Then tosyl chloride (2.0 g, 10.3 mmol) was slowly added. The reaction was kept stirring at 0 °C for 8 h. The solvent was removed *in vacuo*, and the residue was chromatographed with silica gel (eluting with hexane/EtOAc = 1/4) to afford the desired product (2.02 g, 74% yield): ^1H NMR (500 MHz, $CDCl_3$) δ 2.42 (s, 3H), 2.38 (s, 3H), 3.70–3.75 (m, 3H), 4.25–4.34 (m, 3H), 4.65 (s, 1H), 7.26–7.33 (m, 2H), 7.78–7.80 (m, 2H); ^{13}C {^1H} NMR ($CDCl_3$) δ 21.00, 55.01, 66.95, 69.58, 69.97, 70.45, 71.58, 128.01, 129.83, 129.98, 132.60, 145.00.

6-Azido-6-deoxy-ManαOMe (5). 6-Tosyl-ManαOMe (12) (100 mg, 0.28 mmol) was dissolved in 10 mL of solvents (acetone/H_2O = 3/2). Sodium azide (56 mg, 0.86 mmol) was added. The reaction was refluxed under N_2 for 12 h. Then the solvents were removed *in vacuo* and the residue was extracted with 50 mL of methanol and chromatographed with silica gel (eluting with $CHCl_3$/MeOH/H_2O = 6/3/0.5) to give the desired product (55 mg, 86% yield): ^1H NMR (500 MHz, CD_3OD) δ 3.30 (s, 3H), 3.32 (m, 2H), 3.46–3.54 (m, 3H), 3.70 (dd, 1H, J = 2.0, 3.5 Hz), 4.54 (d, 1H, J = 2.0 Hz); ^{13}C {^1H} NMR (CD_3OD) δ 52.92, 55.30, 69.45, 71.90, 72.33, 73.78, 102.80; HRMS calcd for $C_7H_{13}N_3O_5Na^+$ (M + Na^+) 242.0753, found 242.0761.

6-Amino-6-deoxy-ManαOMe (6). Hydrogenolysis of 5 (24 mg) in 2 mL of methanol in the presence of Pd/C produced the desired product (18 mg, 85% yield): ^1H NMR (500 MHz, D_2O)

δ 2.7 (dd, 1H, J = 7.0, 13.5 Hz), 2.90 (dd, 1H, J = 3.0, 13.5 Hz), 3.28 (s, 3H), 3.32–3.33 (m, 1H), 3.35 (t, 1H, J = 2.5 Hz), 3.55 (dd, 1H, J = 3.5, 9.0 Hz), 3.68 (dd, 1H, J = 2.0, 3.5 Hz), 4.53 (d, 1H, J = 1.5 Hz); ^{13}C {^1H} NMR (D_2O) δ 43.61, 55.30, 69.98, 72.01, 72.40, 102.81; HRMS calcd for $C_7H_5N_1O_5Na^+$ (M + Na^+) 216.0848, found 216.0853.

Cbz-Thr(α-tetraacetyl-Man)-Val-OMe (16). To a solution of 2,3,4,6-triacetyl-D-mannopyranosyl bromide (2.40 g, 5.84 mmol) in dry dichloromethane (30 mL) was added at −20 °C Cbz-Thr-Val-OMe (2.00 g, 5.67 mmol) and silver triflate (2.92 g, 11.4 mmol). The suspension was stirred at −20 °C for 4 h and then filtered through a bed of Celite. The filtrate was washed twice with water and twice with saturated sodium bicarbonate. The organic layer was dried over anhydrous $MgSO_4$ and concentrated *in vacuo*, and the residue was chromatographed with silica gel (eluted with EtOAc/hexanes = 2/1) to give the desired product as a white solid (2.8 g, 72% yield): ^1H NMR (500 MHz, $CDCl_3$) δ 0.86 (dd, 6H, J = 4.5, 7.0 Hz), 1.20 (d, 3H, J = 1.0 Hz), 1.91 (s, 3H), 1.98 (s, 3H), 2.01 (s, 3H), 2.02 (s, 3H), 2.12 (m, 1H), 3.67 (s, 3H), 4.05–4.07 (m, 2H), 4.18–4.20 (m, 1H), 4.33 (t, 2H, J = 3.5 Hz), 4.44–4.45 (m, 1H), 5.00 (s, 1H), 5.08 (s, 2H), 5.16–5.24 (m, 3H), 5.81 (d, 1H, NH, J = 7.5 Hz), 6.82 (d, 1H, NH, J = 8.5 Hz), 7.25–7.32 (m, 5H); ^{13}C {^1H} NMR ($CDCl_3$) δ 16.53, 17.74, 18.71, 20.40, 20.53, 20.58, 30.65, 51.82, 57.31, 58.15, 62.35, 66.69, 67.10, 68.78, 68.83, 69.05, 76.18, 98.85, 127.40, 128.40, 135.81, 156.12, 168.71, 169.40, 170.43, 171.75; HRMS calcd for $C_{32}H_{44}N_2O_{15}Cs^+$ (M + Cs^+) 829.1796, found 829.1796.

Cbz-Thr(α-Man)-Val-OMe (7). To a solution of Cbz-Thr(α-tetraacetyl-Man)-Val-OMe (16) (200 mg, 0.28 mmol) in 20 mL of dry methanol at 0 °C was added 1% MeONa in dry methanol (about 500 μL) until pH 10.0. The solution was stirred at 0 °C for 1.5 h. Then Dowex H^+ was added to the reaction to pH 4.0. After filtration, the solvent was removed *in vacuo* to give the desired product as a colorless liquid (132 mg, 87% yield): ^1H NMR (500 MHz, D_2O) δ 0.85 (dd, 6H, J = 4.0, 7.0 Hz), 1.21 (d, 3H, J = 6.5 Hz), 2.04–2.07 (m, 1H), 3.26–3.54 (m, 2H), 3.62 (s, 3H), 3.64–3.68 (m, 2H), 3.69–3.74 (m, 2H), 4.10 (m, 1H), 4.24–4.29 (m, 2H), 5.02 (s, 2H), 7.19 (d, 1H, NH, J = 3.0 Hz), 7.20–7.28 (m, 5H), 8.10 (d, 1H, NH, J = 8.5 Hz); ^{13}C {^1H} NMR (D_2O) δ 18.52, 18.91, 19.36, 31.70, 48.66, 48.82, 49.00, 49.17, 49.33, 52.61, 59.19, 60.45, 62.80, 67.83, 68.50, 71.94, 72.26, 74.89, 77.13, 103.01, 128.82, 129.98, 129.41, 137.92, 158.50, 172.58, 173.26; HRMS calcd for $C_{24}H_{36}N_2O_{11}Cs^+$ (M + Cs^+) 661.1373, found 661.1392.

Thr(α-Man)-Val-OMe (8). Hydrogenolysis of Cbz-Thr(α-Man)-Val-OMe (7) (156 mg) in 10 mL of methanol in the presence of trifluoroacetic acid (30 μL) and Pd/C produced the desired product (130 mg, 87% yield): ^1H NMR (500 MHz, D_2O) δ 0.72 (dd, 6H, J = 2.5, 7.0 Hz), 1.22 (d, 3H, J = 6.5 Hz), 2.00 (m, 1H), 3.40 (t, 1H, J = 4.5 Hz), 3.48–3.52 (m, 2H), 3.55 (s, 3H), 3.64 (m, 2H), 3.91 (d, 1H, J = 4.0 Hz), 4.68 (d, 1H, J = 1.5 Hz); ^{13}C {^1H} NMR (D_2O) δ 17.42, 17.97, 18.27, 30.00, 52.95, 57.13, 58.86, 61.09, 66.83, 70.04, 70.43, 73.49, 75.25, 101.75, 167.96, 173.56; HRMS calcd for $C_{16}H_{30}N_2O_9Cs^+$ (M + Cs^+) 527.1006, found 527.1016.

Cbz-Thr(α-Man)-Val-Gly-Ala-NH_2 (9). Compound 7 (20 mg, 0.038 mmol), Gly-Ala-NH_2 (60 mg, 0.24 mmol), and 3.0 mg of subtilisin 8397 in 2.0 mL of 8:2 mixture of dimethylformamide and water (pH 8.5–9.0, adjusted with triethylamine) was incubated at room temperature for 2.5 h. After the solvents were removed, the glycopeptide (12 mg, 43%) was isolated by HPLC: ^1H NMR (500 MHz, CD_3OD) δ 0.87 (d, 3H, J = 3.0 Hz), 1.18 (d, 3H, J = 6.0 Hz), 1.28 (d, 3H, J = 7.0 Hz), 1.97 (m, 1H), 3.21 (q, 1H, J = 1.5 Hz), 3.51 (m, 2H), 3.61–3.64 (m, 3H), 3.74 (m, 1H), 3.83 (d, 2H, J = 3.5 Hz), 4.02 (d, 1H, J = 7.0 Hz), 4.20 (s, 2H), 5.02 (s, 3H), 7.20–7.31 (m, 5H); ^{13}C NMR (D_2O) δ 16.81, 17.84, 17.90, 30.51, 35.91, 49.40, 54.20, 59.30, 59.84, 61.10, 67.00, 67.62, 70.41, 70.51, 73.30, 76.10, 101.40, 115.60, 127.80, 128.30, 128.60, 129.01, 130.7, 136.50, 154.60, 172.20, 172.41, 174.48, 174.84; HRMS calcd for $C_{28}H_{43}N_5O_{12}Cs^+$ (M + Cs^+) 774.1963, found 774.1958.

Boc-Tyr-Thr(α-Man)-Val-OMe (10). To a solution of 104 mg of Boc-Tyr(Bzl)-OH, 167 mg of H-Thr(α-tetraacetyl-Man)-Val-OMe (synthesized from hydrogenolysis of compound 14), 50 μL of triethylamine, and 44 mg of 1-hydroxybenzotriazole in 2 mL of dry methylene chloride at 0 °C was added 63 mg of 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride. The reaction was stirred at 0 °C for 12 h. The mixture was then evaporated to dryness under reduced pressure. Purification by a silica gel column with hexane/EtOAc (1/2, v/v) afforded 138 mg (65% yield) of Boc-Tyr(Bzl)-Thr(α-tetraacetyl-Man)-Val-OMe. This compound was then subjected to deacylation and hydrogenolysis as described for the preparation of compounds 7 and 8 to give the desired compound in 82% overall yield: ^1H NMR (500 MHz, CD_3OD) δ 0.85 (dd, 6H, J = 1.5, 7.0 Hz, Val $2CH_3$), 1.15 (d, 3H, J = 6.5 Hz, Thr CH_3), 1.28 (s, 9H, Boc), 2.05 (m, 1H, Val CH), 2.64 (dd, 1H, J = 9.5, 13.5 Hz, Tyr CH_2Ph), 3.20 (m, 1H), 3.51 (m, 2H), 3.59 (dd, 2H, J = 5.0, 9.0 Hz), 3.63 (s, 3H, Val OCH_3), 3.68 (m 1H), 3.71 (d, 1H, J = 2.5 Hz), 3.73 (d, 1H, J = 1.5 Hz), 4.11 (dd, 1H, J = 4.0, 6.5 Hz), 4.20 (t, 1H, J = 4.5 Hz), 4.23 (d, 1H, J = 6.5 Hz), 4.45 (d, 1H, J = 4.0 Hz, Man C_1-H), 6.58 (d, 2H, J = 8.5 Hz, Tyr Ph), 6.95 (d, 2H, J = 8.0 Hz, Tyr Ph); ^{13}C {^1H} NMR (CD_3OD) δ 18.56, 18.71, 19.41, 28.69, 31.79, 38.07, 52.65, 57.66, 58.13, 59.24, 62.92, 68.67, 72.01, 72.33, 75.01, 76.76, 103.02, 116.23, 129.23, 131.36, 157.28, 171.78, 173.36, 174.79; HRMS calcd for $C_{30}H_{47}N_3O_{13}Cs^+$ (M + Cs^+) 790.2163, found 790.1253.

Manα1,2Manα-OMe (3). A reaction mixture (100 mL, 100 mM Tris, pH 7.5, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mM EDTA, 5 mM NaN_3, 1 mM ATP, 0.01 mM theophylline, and 0.03 mM 2,3-dimercaptopropanol) containing α-methyl D-mannopyranoside (0.4 g, 20 mM), PEP (1.0 g, 427 mM), GDP (30 mg, 1 mM), dried yeast cells (0.4 g), pyruvate kinase PK (300 U), ManT (1.2 U), and inorganic pyrophosphate (2 U) was slightly stirred at room temperature for 72 h and then centrifuged. The supernatant was lyophilized and extracted with methanol. After removal of the methanol, the product was purified by silica gel column chromatography ($CHCl_3$:CH_3OH:H_2O = 6:3:0.5, v/v/v) to afford Manα1,2Manα-OMe (96 mg) in 38% overall yield based on consumed α-methyl D-mannopyranoside: ^1H NMR (D_2O) δ 3.41 (s, 3H), 3.60–3.80 (m, 6H), 3.86 (dd, 1H, J = 3.5, 10 Hz), 3.88 (dd, 1H, J = 3.5, 10 Hz), 3.90 (d, 1H, d, J = 1.5 Hz), 3.92 (d, 1H, J = 1 Hz), 3.97 (dd, 1H, J = 2, 3.5 Hz), 4.08 (dd, 1H, J = 2, 3.5 Hz), 5.02 (d, 1H, d, J = 2 Hz), 5.04 (d, 1H, J = 2.0 Hz); ^{13}C {^1H} NMR (D_2O) δ 54.90, 61.01, 61.24, 66.98, 67.03, 70.02, 70.29, 70.37, 72.63, 73.40, 78.62, 99.40, 102.43; HRMS calcd for $C_{13}H_{24}O_{11}Na^+$ (M + Na^+) 379.1216, found 379.1220.

Cbz-Thr(αManα1,2Man)-Val-OMe (7). The synthetic procedure was described in the text: ^1H NMR (500 MHz, D_2O) δ 0.83 (m, 6H), 1.21 (d, 3H, J = 4.5 Hz), 2.04 (m 1H), 3.55–3.62 (m, 15 H), 4.05 (m, 1H), 4.16 (m, 1H), 4.24 (m, 1H), 5.00 (s, 2H), 7.16–7.27 (m, 5H); ^{13}C {^1H} NMR (D_2O) δ 17.63, 17.68, 18.41, 30.10, 52.88, 58.68, 61.14, 61.21, 66.91, 67.19, 67.52, 70.03, 70.13, 70.47, 73.30, 76.47, 79.31, 99.84, 102.40, 127.87, 127.92, 128.60, 129.00, 172.82, 173.80; HRMS calcd for $C_{30}H_{46}N_2O_{16}Cs^+$ (M + Cs^+) 823.1902, found 823.1928.

Supplementary Material Available: Graphic illustrations of growth of cells in M9 medium with different inducer IPTG concentrations (Figure 2), stability study of α-1,2-mannosyltransferase at 20 °C (Figure 3), organic solvent effect on α-1,2-mannosyltransferase (Figure 4), and ^1H NMR and ^{13}C NMR spectra of compounds 3–12 (23 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.