Expression, Purification, and Characterization of Two *N*,*N*-Dimethyltransferases, TylM1 and DesVI, Involved in the Biosynthesis of Mycaminose and Desosamine†

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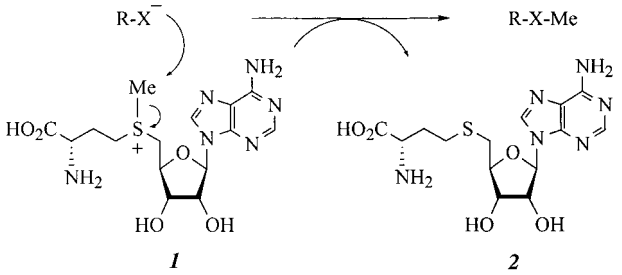
Received March 27, 2002

ABSTRACT: Methylation catalyzed by an *S*-adenosylmethionine- (AdoMet-) dependent methyltransferase is an effective means to alter the hydrophilicity and/or nucleophilicity of a molecule. While a large number of enzymes capable of catalyzing methylation at carbon, oxygen, sulfur, and nitrogen atoms are known, only a few are able to catalyze N,N-dimethylation. Mycaminose and desosamine are aminohexoses found in several macrolide antibiotics, such as tylosin and methymycin, respectively. Both sugars contain a C-3 *N*,*N*-dimethylamino group which has been shown to confer the biological activity of these unusual sugars. Recently, sequence analysis as well as genetic studies has led to the assignment of *tylM1* in the tylosin biosynthetic gene cluster and *desVI* in the methymycin biosynthetic gene cluster as genes encoding the corresponding *N*,*N*-dimethyltransferases. To verify the proposed roles of the *tylM1* and *desVI* genes, we have overexpressed and purified their encoded products, synthesized the predicted substrates, and characterized the catalytic function of these proteins. Our studies showed that TylM1 and DesVI are homodimeric proteins and have nearly identical biochemical properties. These enzymes do not have strong preference for binding either the unmethylated substrate or the monomethylated intermediate. It is the chemical reactivity of the nitrogen functional group that determines the relative rate of a particular methylation step. Thus, our results not only establish TylM1 and DesVI as new members of a small family of enzymes that are capable of catalyzing N,N-dimethylation of an amino group but also provide evidence indicating that the methylation catalyzed by AdoMet-dependent methyltransferases proceeds in a stepwise manner and is nucleophilic in nature.

Methylation catalyzed by *S*-adenosylmethionine- (**1**, AdoMet-)[1] dependent enzymes is one of the most common reactions in biological systems (*1*, *2*). A large number of methyltransferases that catalyze methylation at carbon, oxygen, sulfur, and nitrogen atoms are known. This class of enzymes owes its versatility to AdoMet, the electrophilic nature of whose methylsulfonium cation renders it an excellent alkylating agent for a variety of nucleophiles. The enzyme catalysis proceeds with nucleophilic attack at the sulfonium methyl carbon in AdoMet followed by cleavage of the methyl−sulfur bond, resulting in methylation at the nucleophilic site. Such an alkylation accompanied by neutralization of the positive charge on the sulfonium cation of AdoMet and formation of a neutral species, *S*-adenosylhomocysteine (**2**), is thermodynamically favorable. However,



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reactions catalyzed by methyltransferases are generally sluggish with k_{cat} values in the range of 1 s^{-1} (*3*). Interest-

† This work was supported in part by National Institutes of Health Grants GM35906 and GM54346. L.Z. was a recipient of a Graduate School Dissertation Fellowship of the University of Minnesota. H.-w.-L. thanks the National Institute of General Medical Sciences for a MERIT Award.

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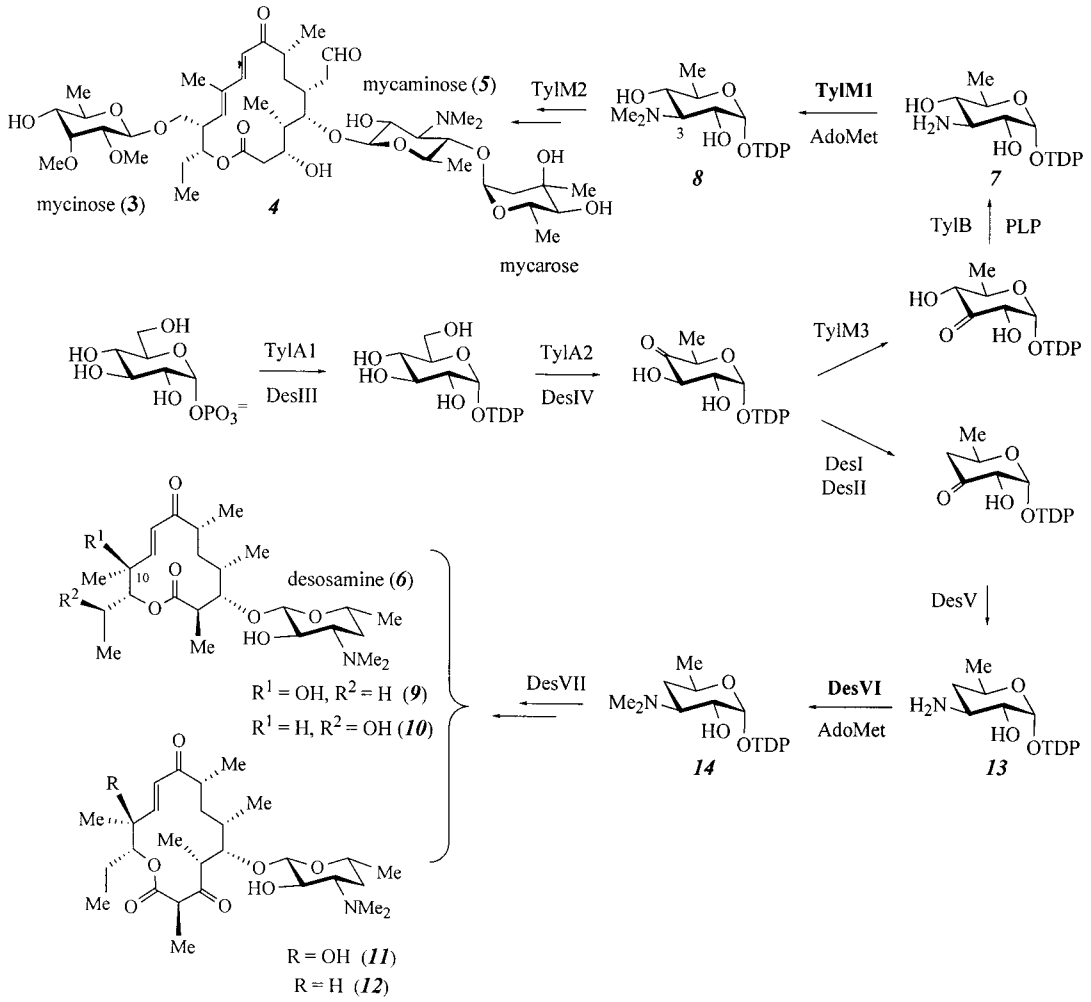
[1] Abbreviations: AdoMet, *S*-adenosylmethionine; BSA, bovine serum albumin; Cbz, carbobenzyloxy; COMT, catechol *O*-methyltransferase; DEAD, diethyl azodicarboxylate; DEAE, diethylaminoethyl; DIPEA, *N*,*N*-diisopropylethylamine; DMAP, (dimethylamino)pyridine; DPPA, diphenylphosphoryl azide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; IPTG, isopropyl thio-*β*-galactoside; LB medium, Luria−Bertani medium; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; *m*-CPBA, *m*-chloroperbenzoic acid; Ni-NTA, nickel nitrilotriacetic acid; ORF, open reading frame; PCC, pyridinium chlorochromate; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TDP, thymidine diphosphate; TSB, tryptic soy broth.

10.1021/bi020245j CCC: $22.00
Published on Web 06/27/2002

Scheme 1



ingly, methyl transfer from AdoMet to its acceptor is intrinsically a slow reaction in aqueous solution (*2*). Thus, the enzyme-catalyzed methyl transfer, despite its lethargic rate, is still significant compared to the uncatalyzed reaction.

This seemingly simple reaction catalyzed by methyltransferases on a wide variety of substrates involved in a myriad of biological processes serves many important functions in natural product biosynthesis. An excellent example is the methylation of the 6-hydroxy group of erythromycin A to give clarithromycin, which has much improved pharmacokinetic properties than erythromycin (*4*). The O-methylation of mycinose (**3**) in tylosin (**4**) catalyzed by macrocin methyltransferase (TylF) is another interesting case (*5, 6*). This reaction is the final and the rate-limiting step in the production of tylosin (**4**) in *Streptomyces fradiae*. Introduction of multiple copies of the *tylF* gene has been demonstrated to effectively increase the yield of tylosin in fermentations of *S. fradiae* (*7*). In addition, methylation of rRNA by the products of *erm*-type genes is known as a common mechanism of macrolide resistance (*8*−*10*). In particular, the *tlrD* gene product in *S. fradiae* has been shown to be a methyltransferase which is capable of monomethylating residue A-2058 in 23S rRNA, conferring resistance to lincosamide, macrolide, and streptogramin B type antibiotics (*11, 12*). Clearly, methyltransferases play significant roles in many biological processes. However, our knowledge of these enzymes, especially those involved in natural product biosynthesis, is limited, since most of them have never been isolated and/or fully characterized. Reported in this paper are our studies of two methyltransferases, TylM1 and DesVI, that catalyze the N,N-dimethylation steps in the biosynthesis of mycaminose (**5**) in *S. fradiae* and desosamine (**6**) in *Streptomyces venezuelae*, respectively (*13*−*16*).

D-Mycaminose (**5**) is an essential structural component of the macrolide antibiotic tylosin (**4**) produced by *S. fradiae*. Early genetic studies led to the isolation of the entire tylosin biosynthetic gene cluster from *S. fradiae* (*17*−*19*), among which 12 open reading frames (ORFs) in the *tylCK*, *tylLM*, and *tylIBA* regions were shown to be involved in the biosynthesis of mycaminose and mycarose (*19*−*22*). Most of the ORFs in these regions are assigned on the basis of sequence similarities to other sugar biosynthetic genes, especially those reported by Cundliffe and co-workers, who have also sequenced the *tylIBA* and *tylLM* segments of the *tyl* cluster (*23*−*25*). The tentative identification of these genes has allowed initial speculation of their roles, leading to a possible route for mycaminose biosynthesis shown in Scheme 1. Evidence gathered from our recent genetic as well as biochemical experiments revealed that TylM1 is the methyltransferase responsible for the last N,N-dimethylation step (**7** → **8**) in the biosynthetic pathway to mycaminose (*20*).

Investigation of the formation of D-desosamine (**6**), a 3,4,6-trideoxy-3-amino sugar, in *S. venezuelae* had allowed the identification of DesVI as another *N,N*-dimethyltranferase

involved in unusual sugar biosynthesis (*26*, *27*). *S. venezuelae* is a producer of methymycin (**9**), neomethymycin (**10**), pikromycin (**11**), and narbomycin (**12**), all of which contain D-desosamine as the sole sugar component. Seven open reading frames, *desI*−*desVII*, in the methymycin/pikromycin cluster from *S. venezuelae* have been assigned as genes involved in desosamine biosynthesis (Scheme 1) (*28*−*30*). Their assignments are based on literature precedents, especially on studies of the erythromycin cluster (*31*, *32*). The fact that the sugar component of the new metabolites isolated from the *desVI* deletion mutant strain is devoid of N-methylation provided convincing evidence sustaining the assignment of *desVI* as the *N*-methyltransferase gene (*26*). The assigned role of DesVI to catalyze the conversion of **13** to **14** was further confirmed by our recent biochemical study of the purified DesVI protein (*27*). Presented in this paper are the detailed accounts on the purification and biochemical characterization of TylM1 and DesVI and the comparison of the catalytic properties of these two *N*-methyltransferases involved in unusual sugar biosynthesis.

EXPERIMENTAL PROCEDURES

Materials. Cosmids pHJL309 and pSET552, used as PCR templates for *tylM1*, were kindly provided by Dr. Eugene Seno of Eli Lilly Research Laboratories. *Escherichia coli* strains DH5α and HB101 were purchased from Bethesda Research Laboratories (Gaithersburg, MD). Vectors pET17b-(+) and pET28b(+) and an overexpression host, *E. coli* BL21(DE3), were aquired from Novagen Inc. (Madison, WI). Culture media were products of Difco (Detroit, MI), and DEAE-Sepharose CL-6B and FPLC column MonoQ H/R 10/10 were products of Pharmacia (Uppsala, Sweden). DNA polymerase *pfu* was obtained from Stratagene (La Jolla, CA). GeneClean DNA purification kits were purchased from BIO101 Inc. (La Jolla, CA). DNA ladders (100 bp and 1 kb) and agarose were from Gibco BRL (Grand Island, NY). DNA minipreps were performed using the Wizard DNA purification kits from Promega (Madison, WI). Oligonucleotides used in the PCR amplification of desired inserts were prepared by Gibco BRL and used without further purification. Restriction endonucleases were from either Amersham (Arlington Heights, IL), Gibco BRL, or Promega. All chemicals were of analytical grade or the highest quality commercially available. All electrophoretic reagents, except for the agarose which is obtained from Gibco BRL, were purchased from Bio-Rad (Hercules, CA).

General. NMR spectra were recorded on Varian 300 or 500 MHz spectrometers. ^{13}C NMR spectra were recorded with proton broad-band decoupling, and the reported spin couplings are from phosphorus. ^{31}P NMR spectra were recorded with proton decoupling and externally referenced with 85% phosphoric acid. For NMR spectra recorded in D_2O, *t*-BuOH was added as an internal reference; the chemical shifts are 1.27 and 31.2 ppm for ^{1}H NMR and ^{13}C NMR, respectively. Fast atom bombardment (FAB) and chemical ionization (CI) mass spectra were recorded by the MS facility at the Department of Chemistry, University of Minnesota. Analytical thin-layer chromatography was carried out on Merck silica gel 60 G-254 plates, and the spots were visualized either under UV light or by heating plates previously stained with solutions of vanillin/methanol/H_2SO_4 (0.75:97.75:1.5) or phosphomolybdic acid (7% in EtOH). The concentration of protein was determined by Bradford's method (*33*) using bovine serum albumin as the standard. The relative molecular mass and purity of enzyme samples were determined using SDS−polyacrylamide gel electrophoresis as described by Laemmli (*34*). The native molecular masses of these protein samples were determined by a gel filtration method reported by Andrews (*35*). The N-terminal amino acid sequences were determined by the Microchemical Facility of the Institute of Human Genetics, University of Minnesota.

*Synthesis of TDP-3-amino-3,6-dideoxyglucopyranose (**7**).* Chemical synthesis of the TylM1 substrate (**7**) was carried out according to the reaction sequence delineated in Scheme 2.

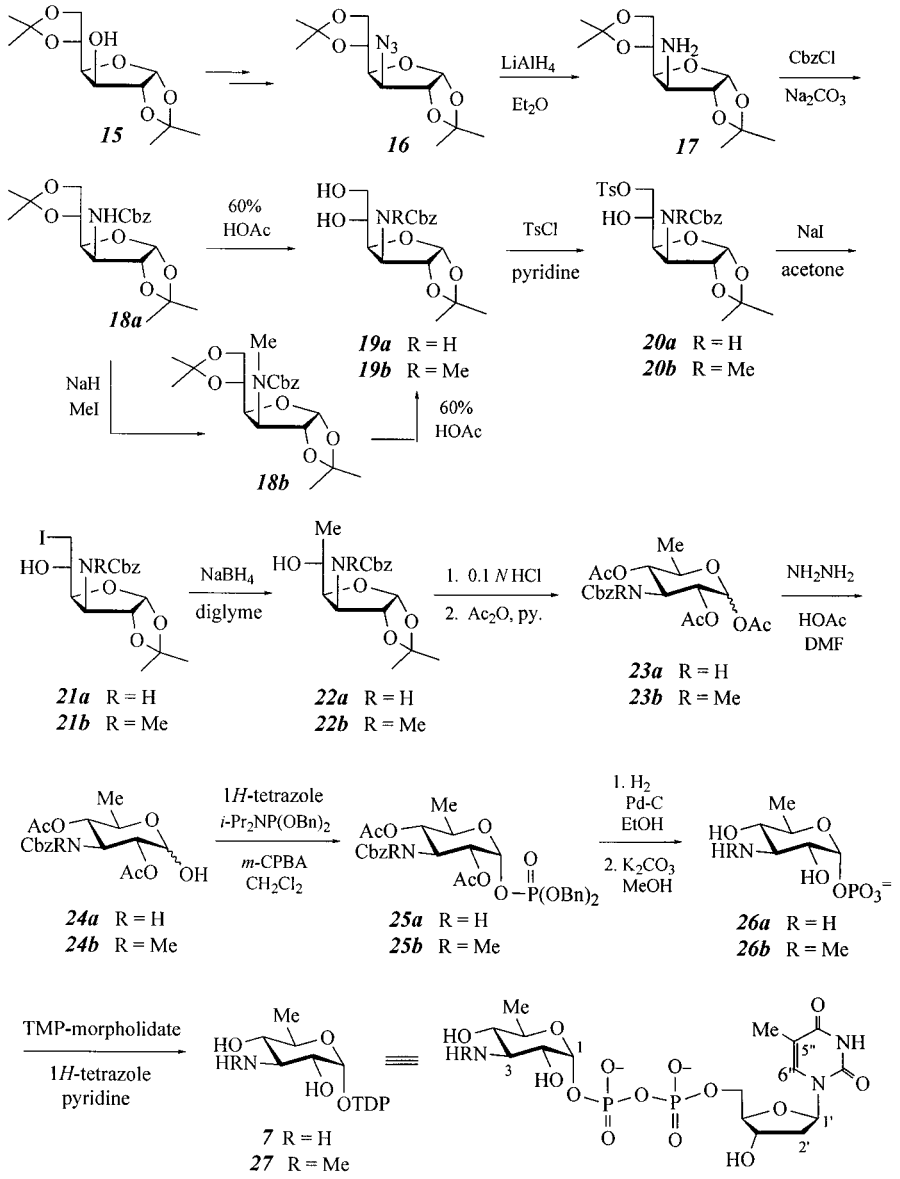
*3-Amino-3-deoxy-1,2:5,6-di-O-isopropylidene-*α*-D-glucofuranose (**17**)* (*36*). A solution of 3-azido-3-deoxy-1,2:5,6-di-*O*-isopropylidene-α-D-glucofuranose (**16**) (*37*) (6.75 g, 23.6 mmol) in dry ethyl ether (200 mL) containing suspended lithium aluminum hydride (3.4 g, 94.8 mmol) was heated under reflux for 2 h. The reaction was cooled on ice, and 3.4 mL of water, 3.4 mL of 15% aqueous NaOH, and 10.2 mL of water were added slowly in sequence to quench the extra reducing agent. After the mixture was stirred for 20 min, the solid residue was filtered off. The filtrate was washed with brine, dried, and evaporated. The residue was purified by column chromatography on silica gel (acetone/ethyl acetate = 3:1) to give **17** in 81.0% yield (4.96 g, 19.1 mmol): ^{1}H NMR ($CDCl_3$) δ 1.27 (3H, s), 1.34 (3H, s), 1.39 (3H, s), 1.48 (3H, s), 3.54 (1H, s), 3.94−4.04 (2H, m), 4.12−4.22 (2H, m), 4.83 (1H, d, J = 3.3 Hz), 5.88 (2H, d, J = 3.3 Hz); ^{13}C NMR ($CDCl_3$) δ 25.3, 26.1, 26.8, 26.9, 57.4, 68.2, 73.0, 81.4, 86.3, 105.1, 109.4, 111.6.

*3-N-Carbobenzyloxyamino-3-deoxy-1,2:5,6-di-O-isopropylidene-*α*-D-glucofuranose (**18a**).* Carbobenzyloxy chloride (4.2 mL, 30 mmol) was slowly added to an ice-cold mixture of **17** (4.5 g, 17.3 mmol) and sodium carbonate (2.5 g, 26.0 mmol) in 250 mL of water/dioxane (v/v = 1:1). After being stirred at 0 °C for 2 h, 250 mL of water was added, and the mixture was extracted with ethyl acetate (3 × 200 mL). The organic extracts were pooled, washed with brine, dried, and evaporated. The residue (**18a**) was directly subjected to acid hydrolysis in the next step without further purification.

*3-N-Carbobenzyloxyamino-3-deoxy-1,2-O-isopropylidene-*α*-D-glucofuranose (**19a**).* Aqueous acetic acid solution (60%, 150 mL) was mixed with **18a** obtained from the previous step, and the resulting solution was stirred at 60 °C for 4 h (*38*). After dilution with water (100 mL), the solution was extracted with ethyl ether (3 × 150 mL). The organic extracts were combined, washed with saturated sodium bicarbonate, dried, and evaporated. The residue was purified by column chromatography on silica gel, eluting with ethyl acetate in hexanes (1:1) to give **19a** as a white amorphous solid in 83% yield (5.07 g, 14.4 mmol): ^{1}H NMR ($CDCl_3$) δ 1.30 (3H, s), 1.50 (3H, s), 3.68 (1H, dd, J = 7.2, 11.4 Hz, 6-H), 3.77−3.81 (2H, m, 5-H, 6-H), 4.10 (1H, dd, J = 3.0, 7.6 Hz, 4-H), 4.22 (1H, dd, J = 3.0, 7.2 Hz, 3-H), 4.53 (1H, d, J = 3.6 Hz, 2-H), 5.07−5.18 (2H, m, PhCH_2), 5.50 (1H, d, J = 7.2 Hz, NH), 5.82 (1H, d, J = 3.6 Hz, 1-H), 7.31−7.40 (5H, m, PhHs).

*3-N-Carbobenzyloxyamino-3-deoxy-6-O-(p-toulenesulfonyl)-1,2-O-isopropylidene-*α*-D-glucofuranose (**20a**).* To the solution of **19a** (4.9 g, 13.8 mmol) and dry pyridine (25 mL)

Scheme 2



in dry methylene chloride (100 mL) at 0 °C was added *p*-toluenesulfonyl chloride (2.89 g, 15.2 mmol). After being stirred at 0 °C for 20 min, the reaction was allowed to warm to room temperature, and the reaction was continued for additional 18 h. Water (250 mL) was then added, and the resulting mixture was extracted with methylene chloride (3 × 150 mL). The combined organic extracts were washed with 1 N HCl, saturated sodium bicarbonate, and brine in sequence, dried, and evaporated. The residue was chromatographed on silica gel, eluting with a gradient of ethyl acetate in hexanes (from 20% to 50%) to give **20a** as a white solid in 90% yield (6.31 g, 12.4 mmol): ^{1}H NMR ($CDCl_3$) δ 1.28 (3H, s), 1.51 (3H, s), 2.43 (3H, s), 3.83−4.13 (3H, m), 4.20−4.34 (2H, m), 4.51 (1H, d, J = 3.6 Hz), 5.06−5.16 (2H, m), 5.23 (1H, d, J = 7.5 Hz), 5.78 (1H, d, J = 3.3 Hz), 7.26−7.37 (6H, m), 7.78 (2H, d, J = 8.1 Hz).

*3-N-Carbobenzyloxyamino-3,6-dideoxy-6-iodo-1,2-O-isopropylidene-*α*-*D*-glucofuranose (**21a**).* A solution of **20a** (6.10 g, 12.0 mmol) and sodium iodide (5.3 g, 90.0 mmol) in acetone (200 mL) was refluxed for 4 h. After being cooled, water was added to the reaction mixture, and the resulting mixture was extracted with chloroform (3 × 150 mL). The organic phase was combined, washed with brine, dried, and evaporated. The residue was chromatographed on silica gel using a gradient elution of $CHCl_3$ in hexanes (from 10% to 40%) to yield **21a** as a colorless amorphous solid in 75% yield (4.17 g, 9 mmol): ^{1}H NMR ($CDCl_3$) δ 1.30 (3H, s, Me), 1.50 (3H, s, Me), 3.32 (1H, dd, J = 7.0, 10.5 Hz, 6-H), 3.47 (1H, dd, J = 3.0, 10.5 Hz, 6-H), 3.55−3.60 (1H, m, 5-H), 4.10−4.14 (1H, m, 4-H), 4.28 (1H, dd, J = 3.0, 7.5 Hz, 3-H), 4.54 (1H, d, J = 3.5 Hz, 2-H), 5.12−5.20 (2H, m, PhCH_2), 5.26 (1H, d, J = 7.5 Hz, NH), 5.82 (1H, d, J = 3.5 Hz, 1-H), 7.34−7.40 (5H, m, PhHs); ^{13}C NMR ($CDCl_3$) δ 9.8, 26.3, 26.6, 57.2, 67.7, 68.4, 81.0, 84.2, 104.3, 112.5, 128.3, 128.6, 128.7, 136.1, 156.6.

*3-N-Carbobenzyloxylamino-3,6-dideoxy-1,2-O-isopropylidene-*α*-*D*-glucofuranose (**22a**).* To a solution of **21a** (4.0 g, 8.6 mmol) in anhydrous diglyme (100 mL) was added sodium borohydride (1.6 g, 43.5 mmol). The mixture was stirred at room temperature for 24 h under nitrogen atmosphere. The solvent was then removed under reduced pressure. After addition of water (200 mL), the solution was

extracted with ethyl ether (3 × 150 mL), and the organic extracts were combined, washed with brine, dried, and evaporated. The residue was purified by column chromatography on silica gel using a gradient elution of $CHCl_3$ in hexanes (from 5% to 50%) to give **22a** as an off-white solid in 92% yield (2.67 g, 7.9 mmol): ^{1}H NMR ($CDCl_3$) δ 1.26 (3H, d, J = 6.6 Hz, 5-Me), 1.30 (3H, s, Me), 1.50 (3H, s, Me), 3.02 (1H, br s, 5-OH), 4.02 (1H, dd, J = 3.6, 5.4 Hz, 3-H), 4.07−4.18 (2H, m, 3, 5-Hs), 4.63 (1H, d, J = 3.6 Hz, 2-H), 5.05−5.16 (2H, m, PhCH_2), 5.85 (1H, d, J = 3.6 Hz, 1-H), 6.23 (1H, d, J = 5.4 Hz, NH), 7.26−7.36 (5H, m, PhHs); ^{13}C NMR ($CDCl_3$) δ 14.2, 26.1, 26.6, 57.5, 60.4, 65.8, 67.1, 80.7, 84.3, 104.2, 111.9, 128.2, 128.3, 128.6, 131.5, 136.1, 156.5.

*1,2,4-Tri-O-acetyl-3-N-carbobenzyloxyamino-3,6-dideoxy-*D*-glucopyranose (**23a**).* To a solution of 0.1 N HCl (200 mL) were added **22a** (2.60 g, 7.70 mmol) and dioxane (40 mL). The resulting mixture was stirred at 80 °C for 5 h. After being cooled, Amberlite 401 (OH^-) resin was added, and the resulting suspension was stirred for a few minutes. The resin was filtered off, the solvent was removed in vacuo, and the residue was dried under vacuum overnight to obtain the crude product. To this crude product were added freshly distilled acetic anhydride (30 mL) and dry pyridine (5 mL). The reaction mixture was left stirring at room temperature overnight. The reaction was quenched by the addition of water (150 mL) and extracted with ethyl acetate (3 × 150 mL). The organic extracts were pooled, washed (sequentially with saturated sodium bicarbonate, brine, and water), dried, and evaporated. The solid residue was purified by flash chromatography on silica gel (ethyl acetate in hexanes, 1:3) to give **23a** in 97% yield (3.20 g, 7.5 mmol). This sample contained both anomers, of which 94% was the β isomer as judged by ^{1}H NMR. ^{1}H NMR ($CDCl_3$) of the β isomer: δ 1.22 (3H, d, J = 6.0 Hz, 5-Me), 1.93 (6H, s, 2×OAc), 2.10 (3H, s, OAc), 3.73 (1H, dq, J = 10.0, 6.0 Hz, 5-H), 4.03 (1H, q, J = 10.0 Hz, 3-H), 4.64 (1H, t, J = 10.0 Hz, 4-H), 4.84 (1H, d, J = 10.0 Hz, NH), 4.89 (1H, dd, J = 8.0, 10.0 Hz, 2-H), 5.03−5.09 (2H, m, PhCH_2), 5.70 (1H, d, J = 8.0 Hz, 1-H), 7.26−7.35 (5H, m, PhHs). ^{13}C NMR ($CDCl_3$) of the β isomer: δ 17.3, 20.4, 20.5, 20.9, 55.8, 66.8, 70.7, 72.0, 73.3, 92.0, 128.0, 128.1, 128.5.

*2,4-Di-O-acetyl-3-N-carbobenzyloxyamino-3,6-dideoxy-*D*-glucopyranose (**24a**).* A solution of **23a** (3.20 g, 7.5 mmol) and hydrazine acetate (2.04 g, 22.1 mmol) in 100 mL of DMF was stirred at 50 °C overnight (*39*). The reaction was quenched by adding 200 mL of water, followed by extracting with methylene chloride (3 × 150 mL). The organic extracts were pooled, washed with saturated sodium bicarbonate, brine, and water (in sequence), and dried. After the solvent was removed under reduced pressure, the desired product (**24a**) was isolated by flash chromatography (ethyl acetate in hexanes, 1:3) on silica gel as an off-white solid in 88% yield (2.53 g, 6.6 mmol).

*2,4-Di-O-acetyl-1-dibenzylphosphoryl-3-N-carbobenzyloxyamino-3,6-dideoxy-*α-D*-glucopyranose (**25a**).* Dibenzyl *N*,*N*-diisopropylphosphoramidite (3.9 g, 11.3 mmol) was added to a solution of **24a** (2.10 g, 5.7 mmol) and 1*H*-tetrazole (1.56 g, 22.6 mmol) in dry methylene chloride (150 mL) (*40*). After being stirred at room temperature for 2 h, the reaction was cooled to −78 °C with a dry ice−acetone bath, and *m*-CPBA (1.95 g, 11.3 mmol) in methylene chloride (20 mL) was added slowly. The reaction mixture was allowed to warm to room temperature over a period of 1 h, and the reaction was continued for an additional 1.5 h. Finally, the reaction was diluted with ethyl ether (100 mL), and the organic phase was successively washed with ice-cold saturated sodium thiosulfate, saturated sodium bicarbonate, and water. After being dried and concentrated, the remaining residue was chromatographed on silica gel with a gradient elution of ethyl acetate in hexanes (5−50%) to give the pure α isomer of **25a** in 62% yield (2.28 g, 3.5 mmol): ^{1}H NMR ($CDCl_3$) δ 1.06 (3H, d, J = 6.3 Hz, 5-Me), 1.79 (3H, s, OAc), 1.90 (3H, s, OAc), 4.00 (1H, dq, J = 10.0, 6.6 Hz, 5-H), 4.30 (1H, q, J = 10.0 Hz, 3-H), 4.64 (1H, t, J = 10.0 Hz, 4-H), 4.80 (1H, d, J = 10.0 Hz, NH), 4.98 (1H, m, 2-H), 5.01−5.15 (6H, m, PhCH_2), 5.80 (1H, dd, J = 3.3, 6.6 Hz, 1-H), 7.24−7.30 (15H, m, PhHs); ^{13}C NMR ($CDCl_3$) δ 17.2, 20.4, 20.5, 51.8, 66.7, 68.1, 69.4 (d, J = 5.5 Hz), 69.6 (d, J = 5.5 Hz), 70.1 (d, J = 7.9 Hz), 73.2, 94.1 (d, J = 6.2 Hz), 127.9−135.5 (PhCs), 156.12, 170.4; ^{31}P NMR (121 MHz, $CDCl_3$) δ −2.98.

*3-Amino-3,6-dideoxy-1-phosphoryl-*α*-*D*-glucopyranose (**26a**).* To the solution of **25a** (1.9 g, 2.92 mmol) in ethanol (40 mL) were added sodium bicarbonate (491 mg, 5.84 mmol) and 10% palladium on carbon (100 mg). Hydrogenation was carried out under atmospheric pressure of hydrogen gas for 40 min. The reaction mixture was filtered through a Celite column, and solvent was removed by rotary vaporization to give the crude product. Without purification, this crude product was dissolved in methanol (50 mL) and mixed with potassium carbonate (2 equiv), and the resulting solution was stirred at room temperature overnight. The potassium carbonate solid was filtered off, and the solvent was removed in vacuo to give **26a** as a potassium salt with an estimated 50% combined yield (414 mg, 1.5 mmol): ^{1}H NMR (D_2O) δ 1.09 (3H, d, J = 6.3 Hz, 5-Me), 3.16 (1H, t, J = 10.2 Hz, 4-H), 3.23 (1H, t, J = 10.2 Hz, 3-H), 3.60 (1H, m, 2-H), 3.82 (1H, m, 5-H), 5.27 (1H, dd, J = 3.6, 7.2 Hz, 1-H); ^{13}C NMR (D_2O) δ 16.3, 53.9, 68.0, 69.4 (d, J = 6.5 Hz), 71.3, 92.5 (d, J = 5.3 Hz).

*TDP-3-amino-3,6-dideoxy-*α*-*D*-glucopyranoside (**7**).* The dipotassium salt of **26a** (63.9 mg, 225 μmol) was dissolved in 10 mL of water, applied to a cation-exchange column packed with Amberlite IR-120 resin (Et_3NH^+, 1.0 × 30 cm), and eluted with water (100 mL). The fractions containing **26a** were pooled, and the solvent was removed under reduced pressure. Coevaporation with methanol (2 × 5 mL) and drying in vacuo gave 3-amino-3,6-dideoxy-α-D-glucopyranonsyl phosphate as a triethylammonium salt (85.0 mg). The content of triethylamine in the sample was estimated to be 1.35 equiv as judged by ^{1}H NMR. This triethylammonium salt of **26a** and thymidine 5′-monophosphomorpholidate 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt (246 mg, 360 μmol) were coevaporated with dry pyridine (3 mL) three times. To this dry mixture were added 1*H*-tetrazole (47 mg, 675 μmol) and dry pyridine (5 mL), and the solution was stirred at room temperature for 3 days (*40*). The solvent was removed, and the solid residue was coevaporated with water (2 × 2 mL). The residue was purified by size exclusion chromatography using a Bio-Gel P-2 column (1 × 140 cm). The elution buffer was 20 mM ammonium bicarbonate, and the flow rate was 6.0 mL/h. Fractions containing the desired product were pooled and lyophilized to give **7** in 60% yield

(73.7 mg, 135 μmol): ^{1}H NMR (D_2O) δ 1.10 (3H, d, J = 6.3 Hz, 5-Me), 1.74 (3H, s, 5″-Me), 2.17−2.22 (2H, m, 2′-Hs), 3.18 (1H, t, J = 10.2 Hz, 4-H), 3.24 (1H, t, J = 10.2 Hz, 3-H), 3.61 (1H, ddd, J = 3.1, 3.3, 10.2 Hz, 2-H), 3.77−3.87 (1H, m, 5-H), 3.99−4.01 (3H, m, 4′-H, 5′-Hs), 4.41−4.44 (1H, m, 3′-H), 5.40 (1H, dd, J = 3.3, 7.2 Hz, 1-H), 6.17 (1H, dd, J = 6.9, 7.2 Hz, 1′-H), 7.55 (1H, s, 6″-H); ^{13}C NMR (D_2O) δ 12.8, 17.6, 39.7, 55.9, 66.6 (d, J = 6.0 Hz), 70.1, 70.6 (d, J = 8.4 Hz), 72.1, 73.3, 86.2, 86.4 (d, J = 9.2 Hz), 95.6 (d, J = 6.1 Hz), 112.8, 138.5, 153.0, 167.9; ^{31}P NMR (D_2O) δ −13.47 (d, J = 20.7 Hz), −11.75 (d, J = 20.7 Hz); high-resolution MALDI-MS calcd for $C_{16}H_{26}N_3O_{14}P_2$ [$(M + H)^+$] 546.0890, found 546.0894.

Synthesis of the Monomethylated Intermediate Found in the TylM1 Reaction (27). Chemical synthesis of the monomethylated intermediate (**27**) was performed following the reaction sequence delineated in Scheme 2.

*3-(N-Methyl-N-benzyloxycarbonyl)amino-3-deoxy-1,2:5,6-di-O-isopropylidene-α-D-glucofuranose (**18b**).* A crude product of **18a** (27.6 g, 74 mmol) in dry THF (1 L) was treated with 60% sodium hydride (5 g, 0.13 mol) for 30 min at 0 °C. To this mixture was added iodomethane (6 mL, 96 mmol). After being stirred for 2 h, the resulting mixture was poured into water (2 L) and extracted with ethyl acetate. The combined organic extracts were concentrated, and the residue was chromatographed on silica gel (5% methanol in chloroform) to yield the N-methylated product **18b** as a mixture of two diastereomers in 84% yield (24 g, 62 mmol): ^{1}H NMR ($CDCl_3$) δ 1.31 (9H, m), 1.50 (3H, s), 2.93 (3H, s), 2.99 (3H, s), 4.23−3.98 (5H, m), 4.72 (1H, s), 4.92 (1H, s), 5.13 (2H, m), 5.82 (1H, br s), 6.06 (1H, br s), 7.33 (5H, m); ^{13}C NMR ($CDCl_3$) δ 25.4, 26.0, 26.8, 26.9, 67.3, 67.7, 72.9, 73.3, 81.6, 84.8, 105.3, 106.1, 109.5, 110.9, 127.8, 128.1, 128.5, 136.6, 156.1; high-resolution CI-MS calcd for $C_{21}H_{30}NO_7$ [$(M + H)^+$] 408.2022, found 408.2018.

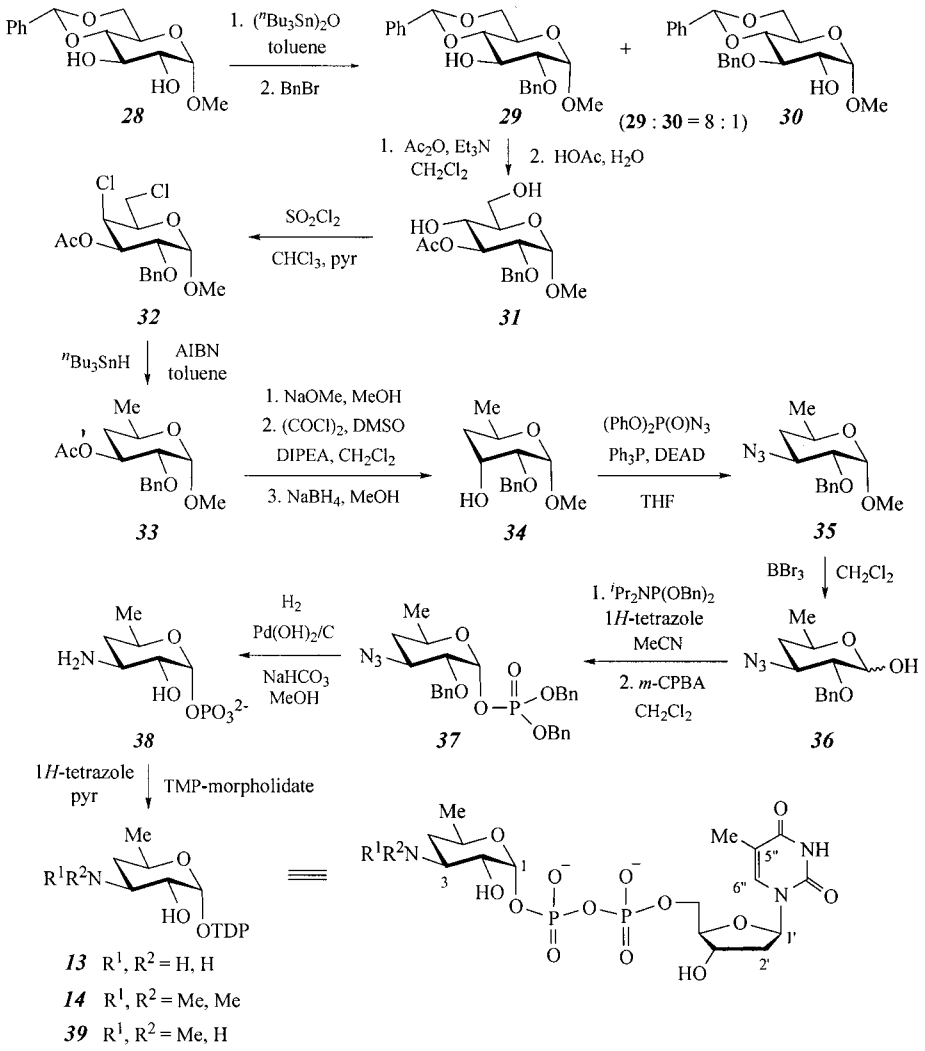
*3-(N-Methyl-N-benzyloxycarbonyl)amino-3-deoxy-1,2-O-isopropylidene-α-D-glucofuranose (**19b**), 3-(N-Methyl-N-benzyloxycarbonyl)amino-3-deoxy-6-O-(p-toluenesulfonyl)-1,2-O-isopropylidene-α-D-glucofuranose (**20b**), 3-(N-Methyl-N-benzyloxycarbonyl)amino-3,6-dideoxy-6-iodo-1,2-O-isopropylidene-α-D-glucofuranose (**21b**), 3-(N-Methyl-N-benzyloxycarbonyl)amino-3,6-dideoxy-1,2-O-isopropylidene-α-D-glucofuranose (**22b**), 1,2,4-Tri-O-acetyl-3-(N-methyl-N-benzyloxycarbonyl)amino-3,6-dideoxy-D-xylo-hexopyranose (**23b**), 2,4-Di-O-acetyl-3-(N-methyl-N-benzyloxycarbonyl)amino-3,6-dideoxy-D-glucopyranose (**24b**), and 2,4-Di-O-acetyl-1-dibenzylphosphoryl-3-(N-methyl-N-benzyloxycarbonyl)amino-3,6-dideoxy-α-D-glucopyranose (**25b**).* These compounds were synthesized following similar procedures as described for the preparation of their non-N-methylated counterparts and were fully characterized accordingly. The experimental details are not repeated here for the sake of saving space.

*TDP-3-N-methylamino-3,6-dideoxy-α-D-glucopyranose (**27**).* A suspension of starting material **25b** (0.77 g, 1.1 mmol) and 10% Pd/C catalyst (180 mg) in a solution of 1:1 MeOH/EtOAc (40 mL) was subjected to atmospheric hydrogenation at room temperature for 10 min. The mixture was filtered through Celite. The filtrate was neutralized with triethylamine and concentrated in vacuo to give a colorless oil. This oily residue was mixed with a solution of potassium carbonate (0.5 g) in a mixture of methanol (20 mL) and H_2O (6 mL) and stirred at room temperature for 14 h. The mixture was neutralized with Dowex-50W (H^+) resin. The resin was filtered off, and the filtrate was concentrated. The residue was mixed with 10% Pd/C (100 mg) in methanol (30 mL) and was stirred under hydrogen gas at room temperature for 12 h. The mixture was filtered through Celite, and the filtrate was neutralized with triethylamine. The solvent was evaporated; the residue was dissolved in water and lyophilized to give the coupling precursor **26b** in 48% yield (200 mg, 550 μmol). A mixture of **26b** (41 mg, 110 μmol) and thymidine 5′-monophosphomorpholidate 4-morpholine-*N,N*′-dicyclohexylcarboxamidine salt (200 mg, 290 μmol) was coevaporated with dry pyridine (2.5 mL) three times to form a white powder. To the above solid was added dry pyridine (2.0 mL) and 1*H*-tetrazole (30 mg, 450 μmol), and reaction was stirred for 60 h at room temperature. After concentration under reduced pressure, the residue was purified on a Bio-Rad P2 column (2 × 110 cm) eluted with 50 mM ammonium bicarbonate. The desired fractions were pooled and lyophilized to give **27** as a white powder in 85% yield (53 mg, 94 μmol): ^{1}H NMR (D_2O) δ 1.04 (3H, d, J = 6.3 Hz), 1.68 (3H, s), 2.13 (2H, m), 2.57 (3H, s), 3.25 (2H, m), 3.73 (2H, m), 3.94 (3H, m), 4.37 (1H, s), 5.35 (1H, dd, J = 7.2, 3.3 Hz), 6.10 (1H, t, J = 6.9 Hz), 7.49 (1H, s); ^{13}C NMR (D_2O) δ 11.6, 16.3, 29.7, 38.5, 60.9, 65.4 (d, J = 5.2 Hz), 66.7 (d, J = 8.9 Hz), 69.0, 70.9, 84.9, 85.1 (d, J = 9.5 Hz), 96.3 (d, J = 6.5 Hz), 111.6, 137.3, 151.7, 160.3, 166.5; ^{31}P NMR (D_2O) δ −10.6 (d, J = 20.7 Hz), −12.5 (d, J = 20.7 Hz); high-resolution FAB-MS calcd for $C_{17}H_{28}N_3O_{14}P_2$ [$(M - H)^-$] 560.1047, found 560.1046.

*Synthesis of TDP-3-amino-3,4,6-trideoxy-α-xylo-hexopyranose (**13**).* Chemical synthesis of the DesVI substrate (**13**) was performed following the reaction sequence delineated in Scheme 3.

*Methyl 3-O-Acetyl-2-O-benzyl-α-D-glucopyranoside (**31**) (41).* A solution of starting material **29** (30.4 g, 82 mmol) (*42*), acetic anhydride (165 mL), dried triethylamine (200 mL), dried methylene chloride (300 mL), and a catalytic amount of 4-dimethylaminopyridine was stirred at room temperature for 3 days. After the reaction was complete, the reaction mixture was poured into a solution of ice water and ether, and the resulting mixture was stirred for 30 min. The organic layer was washed with 1 N HCl, water, saturated sodium bicarbonate, and brine and then dried. After removal of solvent, the crude brownish oil was added with a solution of 80% acetic acid, 1% trifluoroacetic acid, and water (300 mL). After being stirred overnight, the solvent was removed under reduced pressure. Water was added to the residual oil and then evaporated under reduced pressure again. The crude product was subjected to gradient column chromatography (50% ethyl acetate in hexanes to 20% ethyl acetate in MeOH). The product (**31**) was obtained as a brownish oil in 59% yield (15.8 g, 48 mmol): ^{1}H NMR ($CDCl_3$) δ 2.09 (3H, s, OAc), 2.20 (1H, s, 6-OH), 3.10 (1H, d, J = 5.3 Hz, 4-OH), 3.39 (3H, s, OMe), 3.49 (1H, dd, J = 3.8, 9.8 Hz, 2-H), 3.58 (1H, ddd, J = 5.3, 9.5, 9.5 Hz, 4-H), 3.65 (1H, ddd, J = 3.8, 3.8, 9.5 Hz, 5-H), 3.78−3.86 (2H, m, 6-H), 4.63 (1H, d, J = 12.2 Hz, PhC*H*H), 4.66 (1H, d, J = 3.8 Hz, 1-H), 4.67 (1H, d, J = 12.2 Hz, PhC*H*H), 5.23 (1H, dd, J = 9.5, 9.8 Hz, 3-H), 7.3−7.4 (5H, m, PhHs); ^{13}C NMR ($CDCl_3$) δ 20.9, 55.0, 61.2, 69.1, 71.0, 72.8, 74.8, 76.7, 97.6, 127.7, 127.8, 128.3, 137.6, 172.5; high-resolution CI-MS calcd for $C_{16}H_{22}O_7$ ($M + H^+$) 327.1444, found 327.1433.

Scheme 3



*Methyl 3-O-Acetyl-2-O-benzyl-4,6-dichloro-4,6-dideoxy-*α-D-*galactopyranoside (**32**).* To a cooled solution (0 °C) of the starting material **31** (1.31 g, 4.0 mmol) in dried pyridine (5 mL) and dried chloroform (10 mL) was added sulfuryl chloride (4 mL) dropwise (*43*, *44*). The resulting yellowish solution was slowly warmed to room temperature and was stirred continuously overnight. At the end of the reaction, the reaction mixture was poured into a solution of ethyl acetate and ice water. The aqueous layer was extracted with ethyl acetate, and the combined organic layers were washed with 1 N HCl, water, saturated sodium bicarbonate, and brine and dried. The crude product was purified by a gradient column chromatography on silica gel (10−35% ethyl acetate in hexanes) to give **32** as a white solid in 52% yield (0.75 g, 2.07 mmol): ^{1}H NMR ($CDCl_3$) δ 2.12 (3H, s, OAc), 3.43 (3H, s, OMe), 3.59 (1H, dd, J = 6.7, 11.5 Hz, 6-H), 3.63 (1H, dd, J = 7.0, 11.5 Hz, 6-H), 3.95 (1H, dd, J = 3.9, 10.3 Hz, 2-H), 4.21 (1H, ddd, J = 1.2, 6.7, 7.0 Hz, 5-H), 4.62 (1H, d, J = 12.2 Hz, PhC*H*H), 4.67 (1H, dd, J = 1.2, 3.4 Hz, 4-H), 4.68 (1H, d, J = 3.9 Hz, 1-H), 4.76 (1H, d, J = 12.2 Hz, PhCH*H*), 5.31 (1H, dd, J = 3.4, 10.3 Hz, 3-H), 7.3−7.4 (5H, m, PhHs); ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.8, 42.8, 59.9, 68.7, 70.4, 73.2, 73.6, 98.6, 127.8, 128.0, 128.5, 137.8, 170.1; high-resolution CI-MS calcd for $C_{16}H_{21}Cl_2O_5$ ($M + H^+$) 363.0766, found 363.0770.

*Methyl 3-O-Acetyl-2-O-benzyl-4,6-dideoxy-*α-D-*xylo-hexopyranoside (**33**).* A solution of dichloride **32** (0.75 g, 2.0 mmol), tributyltin hydride (2.53 g, 8.6 mmol), and AIBN (50 mg, 0.3 mmol) in toluene (30 mL) was heated to reflux for 24 h. The solvent was removed under reduced pressure, and the residue was purified by silica gel chromatography, eluted first with a linear gradient of ether in hexanes (0−30%) and followed by a solution of 40% ethyl acetate in hexanes. The desired product **33** was isolated as a colorless oil in 96% yield (0.6 g, 1.9 mmol): ^{1}H NMR ($CDCl_3$) δ 1.16 (3H, d, J = 5.9 Hz, 5-Me), 1.31 (1H, ddd, J = 11.5, 12.0, 12.7 Hz, 4-H_{ax}), 2.01 (3H, s, OAc), 2.12 (1H, ddd, J = 2.2, 5.2, 12.7 Hz, 4-H_{eq}), 3.37 (3H, s, OMe), 3.47 (1H, dd, J = 3.5, 10.0 Hz, 2-H), 3.94 (1H, qdd, J = 5.9, 2.2, 12.0 Hz, 5-H), 4.64 (1H, d, J = 12.5 Hz, PhC*H*H), 4.67 (1H, d, J = 3.5 Hz, 1-H), 4.68 (1H, d, J = 12.5 Hz, PhCH*H*), 5.22 (1H, ddd, J = 5.2, 10.0, 11.5 Hz, 3-H), 7.1−7.2 (5H, m, PhHs); ^{13}C NMR ($CDCl_3$) δ 20.6, 21.2, 38.4, 55.0, 62.9, 70.2, 72.8, 77.8, 98.6, 127.4, 127.8, 128.3, 138.2, 170.3; high-resolution CI-MS calcd for $C_{16}H_{26}O_5N$ ($M + NH_4^+$) 312.1811, found 312.1802.

*Methyl 2-O-Benzyl-4,6-dideoxy-*α-D-*allo-hexopyranoside (**34**).* To a solution of **33** (6.7 g, 23 mmol) in 30 mL of methanol was added a methanol solution containing a catalytic amount of sodium methoxide. The reaction was

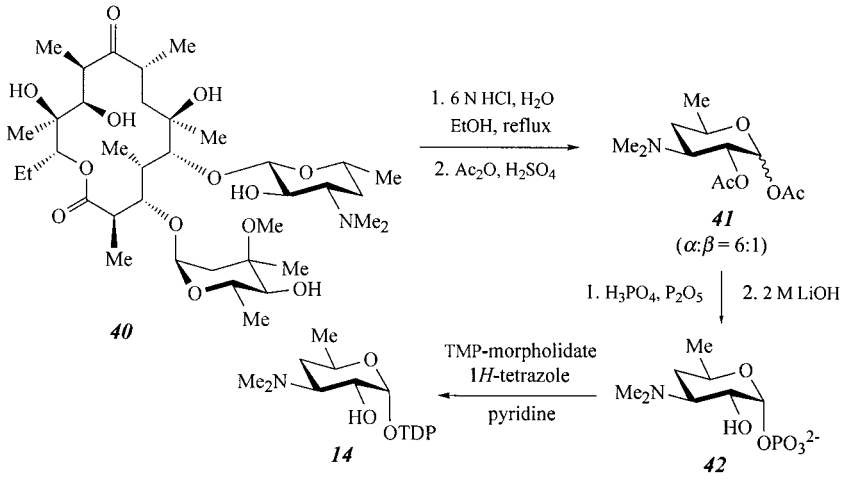
stirred at room temperature for 3 h to hydrolyze the acetyl group. After completion, the reaction mixture was mixed with Amberlite IR-120 (H^+) resin and filtered through glass wool. The filtrate was concentrated to afford the deacylated product as a clear oil. This crude product was subjected to oxidation which was initiated by preparing a solution of oxalyl chloride (4.0 mL, 46 mmol) in dry methylene chloride (100 mL) at −78 °C. This solution was mixed with dried dimethyl sulfoxide (6.4 mL, 91 mmol) and stirred for 30 min, during which time the temperature was allowed to rise to −65 °C. To this reaction mixture was added a solution of the crude deacylated product in methylene chloride (100 mL), and the resulting mixture was stirred for 1 h from −65 to −45 °C. The reaction was then treated with dry diisopropylethylamine (31.7 mL, 182 mmol) for 1 h while the temperature was raised to 0 °C. The reaction was quenched with 1 N HCl and diluted with methylene chloride. The organic layer was washed with water, dried, and concentrated. This crude 3-keto sugar was dissolved in methanol (50 mL) and cooled to 0 °C. To this solution was added solid sodium borohydride (6.0 g, 0.15 mol) in small portions. After being stirred overnight, the reaction was quenched with 1 N HCl and filtered through Celite, and the filtrate was concentrated under reduced pressure. The desired product (**34**), after purification by gradient column chromatography on silica gel (10−60% ether in hexanes), was obtained as a clear oil in 69% overall yield (4.12 g, 16 mmol): ^{1}H NMR ($CDCl_3$) δ 1.20 (3H, d, J = 6.2 Hz, 5-Me), 1.48 (1H, ddd, J = 2.6, 11.5, 14.3 Hz, 4-H_{ax}), 1.97 (1H, ddd, J = 3.0, 3.0, 14.3 Hz, 4-H_{eq}), 3.45 (3H, s, OMe), 3.46 (1H, dd, J = 4.4, 7.0 Hz, 2-H), 4.16 (1H, qdd, J = 6.2, 3.0, 11.5 Hz, 5-H), 4.18 (1H, ddd, J = 2.6, 3.0, 7.0 Hz, 3-H), 4.59 (1H, d, J = 12.1 Hz, PhC*H*H), 4.76 (1H, d, J = 4.4 Hz, 1-H), 4.78 (1H, d, J = 12.1 Hz, PhCH*H*), 7.3−7.4 (5H, m, PhHs); ^{13}C NMR ($CDCl_3$) δ 20.5, 39.70, 55.5, 59.4, 65.4, 70.1, 74.0, 99.7, 127.90, 127.97, 128.5, 137.6; high-resolution CI-MS calcd for $C_{14}H_{24}O_4N$ ($M + NH_4^+$) 270.1705, found 270.1700.

*Methyl 3-Azido-2-O-benzyl-3,4,6-tideoxy-*α*-*D*-xylo-hexopyranoside (**35**).* To a mixture of **34** (3.85 g, 15.3 mmol), triphenylphosphine (6.14 g, 23.4 mmol), diphenylphosphoryl azide (5.04 mL, 23.4 mmol) in dried THF (200 mL) was slowly added diethyl azodicarboxylate (3.68 mL, 23.4 mmol) at −78 °C under nitrogen (*45*, *46*). The reaction was stirred overnight at room temperature. After removal of solvent, the residual orange oil was subjected to gradient column chromatography on silica gel (10−60% ether in hexanes) to afford the desired product (**35**) as a light yellowish oil in 81% yield (3.65 g, 13.1 mmol): ^{1}H NMR ($CDCl_3$) δ 1.16 (3H, d, J = 6.3 Hz, 5-Me), 1.28 (1H, ddd, J = 9.8, 11.8, 13.1 Hz, 4-H_{ax}), 1.94 (1H, ddd, J = 2.2, 4.9, 13.1 Hz, 4-H_{eq}), 3.34 (1H, dd, J = 3.4, 9.8 Hz, 2-H), 3.36 (3H, s, OMe), 3.85 (1H, ddd, J = 4.9, 9.8, 9.8 Hz, 3-H), 3.90 (1H, qdd, J = 6.3, 2.2, 11.8 Hz, 5-H), 4.59 (1H, d, J = 3.4 Hz, 1-H), 4.65 (1H, d, J = 12.1 Hz, PhC*H*H), 4.77 (1H, d, J = 12.1 Hz, PhCH*H*), 7.2−7.4 (5H, m, PhHs); ^{13}C NMR (125 MHz, $CDCl_3$) δ 20.6, 38.3, 55.1, 58.0, 63.1, 72.7, 79.5, 97.8, 128.0, 128.1, 128.5, 137.7; high-resolution CI-MS calcd for $C_{14}H_{23}N_4O_3$ ($M + NH_4^+$) 295.1770, found 295.1773.

*3-Azido-2-O-benzyl-3,4,6-trideoxy-*D*-xylo-hexopyranose (**36**).* To a cooled solution of **35** (6.86 g, 25.0 mmol) in dry methylene chloride (20 mL) was added boron tribromide (12.0 mmol, 12.0 mL of a 1 M solution in methylene chloride) dropwise at −78 °C (*47*). The reaction was stirred at 0 °C and was closely monitored by TLC until the starting material was all consumed. The remaining boron tribromide was quenched by solid sodium bicarbonate, and the reaction mixture was filtered through Celite. The solution was concentrated, and the crude product was purified by gradient column chromatography on silica gel (10−60% ether in hexanes) to give **36** as a yellowish oil, which solidified upon drying under vacuum. The product was a mixture of α and β isomers, and the combined yield was 55% (3.64 g, 13.8 mmol): ^{1}H NMR ($CDCl_3$) δ 1.17 (3H, d, J = 6.3 Hz, 5-Me of α anomer), 1.26 (3H, d, J = 6.3 Hz, 5-Me of β anomer), 1.33 (1H, ddd, J = 12.1, 12.2, 13.1 Hz, 4-H_{ax} of α anomer), 1.37 (1H, ddd, J = 12.2, 12.2, 13.1 Hz, 4-H_{ax} of β anomer), 1.93 (1H, ddd, J = 2.0, 5.5, 13.1 Hz, 4-H_{eq} of β anomer), 1.95 (1H, ddd, J = 2.0, 4.8, 13.1 Hz, 4-H_{eq} of α anomer), 3.14 (1H, dd, J = 7.4, 9.5 Hz, 2-H of β anomer), 3.53 (1H, ddd, J = 5.5, 9.5, 12.2 Hz, 3-H of β anomer), 3.62 (1H, qdd, J = 6.3, 2.0, 12.2 Hz, 5-H of β anomer), 3.66 (1H, dd, J = 3.4, 9.8 Hz, 2-H of α anomer), 3.88 (1H, ddd, J = 4.8, 9.8, 12.1 Hz, 3-H of α anomer), 4.16 (1H, qdd, J = 6.3, 2.0, 12.2 Hz, 5-H of α anomer), 4.66 (1H, d, J = 7.4 Hz, 1-H of β anomer), 4.68 (1H, d, J = 11.7 Hz, PhCH*H* of α anomer), 4.76 (1H, d, J = 11.7 Hz, PhC*H*H of α anomer), 4.78 (1H, d, J = 10.7 Hz, PhC*H*H of β anomer), 4.95 (1H d, J = 10.7 Hz, PhCH*H* of β anomer), 5.21 (1H, d, J = 3.4 Hz, 1-H of α anomer), 7.2−7.4 (10H, m, PhHs of α and β anomers); ^{13}C NMR ($CDCl_3$) δ 20.68 (α), 20.73 (β), 38.00 (α), 38.05 (β), 57.9 (α), 61.4 (β), 63.6 (α), 68.6 (β), 72.8 (α), 74.6 (β), 79.5 (α), 82.3 (β), 91.0 (α), 97.6 (β), 127.9, 128.14, 128.18, 128.36, 128.42, 128.6, 137.3 (α), 137.8 (β); high-resolution CI-MS calcd for $C_{13}H_{21}N_4O_3$ ($M + NH_4^+$) 281.1613, found 281.1606.

*Dibenzylphosphoryl 3-Azido-2-O-benzyl-3,4,6-trideoxy-*α*-*D*-xylo-hexopyranoside (**37**).* To a solution of **36** (3.64 g, 13.8 mmol) and 1*H*-tetrazole (1.92 g, 27.4 mmol) in dried acetonitrile (20 mL) was added slowly diisopropylphosphoramidite (7.90 mL, 1.91 mmol) at room temperature under nitrogen (*40*). After being stirred for 5 h, the reaction mixture was cooled to 0 °C, and then a suspension of *m*-chloroperbenzoic acid (8.6 g, 27.4 mmol, 55% pure) in methylene chloride (20 mL) was added. After being stirred for an additional 30 min, the reaction mixture was diluted with methylene chloride, washed with saturated sodium thiosulfate and saturated sodium bicarbonate, dried, and concentrated. After purification by gradient column chromatography on silica gel (10−60% ether in hexanes), the desired product (**37**) was obtained as a clear oil in 71% yield (5.16 g, 9.86 mmol): ^{1}H NMR ($CDCl_3$) δ 1.23 (3H, d, J = 6.7 Hz, 5-Me), 1.35 (1H, ddd, J = 11.7, 12.2, 13.3 Hz, 4-H_{ax}), 1.93 (1H, ddd, J = 2.2, 4.8, 13.3 Hz, 4-H_{eq}), 3.39 (1H, ddd, J = 3.1, 3.1, 9.9 Hz, H-2), 3.76 (1H, ddd, J = 4.8, 9.9, 12.2 Hz, H-3), 4.05 (1H, qdd, J = 6.7, 2.2, 11.7 Hz, H-5), 4.65 (1H, d, J = 11.3 Hz, PhC*H*H), 4.76 (1H, d, J = 11.3 Hz, PhCH*H*), 5.01 (2H, d, J = 7.6 Hz, (PhC*H*HO)$_2$PO$_2$), 5.07 (2H, d, J = 8.3 Hz, (PhCH*H*O)$_2$PO$_2$), 5.92 (1H, dd, J = 3.1, 6.7 Hz, H-1), 7.2−7.4 (15H, m, Ph); ^{13}C NMR ($CDCl_3$) δ 20.4 (C-6), 37.5 (C-4), 57.3 (C-3), 65.6 (Ph*C*H$_2$), 69.0 (d, J = 6.0 Hz, (Ph*C*H$_2$O)$_2$PO$_2$), 69.2 (d, J = 5.0 Hz, (Ph*C*H$_2$O)$_2$PO$_2$), 72.3 (C-5), 78.5 (d, J = 8.1 Hz, C-2), 95.3 (d, J = 6.0 Hz, C-1), 126.8, 127.4, 127.6, 127.73, 127.76, 127.85, 127.93, 128.1, 128.4, 128.45, 128.46, 135.6

Scheme 4



(d, J = 7.0 Hz), 135.7 (d, J = 7.0 Hz), 137.0, 138.3; ^{31}P NMR ($CDCl_3$) δ −3.6 (s); high-resolution FAB-MS calcd for $C_{27}H_{30}N_3O_6PNa$ ($M + Na^+$) 546.1770, found 546.1770.

*3-Amino-3,4,6-tideoxy-*α*-*D*-xylo-hexopyranoside 1-Phosphate (**38**).* A solution of **37** was stirred with 10% $Pd(OH)_2$/C and sodium bicarbonate in degassed methanol under atmospheric hydrogen for 2 days (*48*). The catalyst was removed by filtration through Celite, and the filtrate was concentrated. The product (**38**) was isolated as a white solid. The yield was up to 78% depending on the purity of the starting material: ^{1}H NMR (D_2O) δ 1.19 (3H, d, J = 6.2 Hz, 5-Me), 1.52 (1H, ddd, J = 11.3, 11.3, 12.6 Hz, 4-H_{ax}), 2.16 (1H, ddd, J = 2.0, 4.0, 12.6 Hz, 4-H_{eq}), 3.62 (1H, ddd, J = 4.0, 11.3, 10.6 Hz, 3-H), 3.66 (1H, ddd, J = 2.6, 2.9 10.6 Hz, 2-H), 4.24 (1H, qdd, J = 6.2, 2.0, 11.3 Hz, 5-H), 5.46 (1H, dd, J = 2.9, 7.0 Hz, 1-H); ^{31}P NMR ($CDCl_3$) δ 3.08 (s); FAB-MS *m*/*e* 587.1 ($M + H^+$), 609.1 ($M + Na^+$).

*TDP-3-amino-3,4,6-trideoxy-*α*-*D*-xylo-hexopyranose (**13**).* As described for the synthesis of **7**, a mixture of **38** (120 mg, 280 μmol) and TMP-morpholidate (0.58 g, 840 μmol) was dried azotropically by addition of anhydrous pyridine and removal of solvent under vacuum. After being repeated twice, a solution of 1*H*-tetrazole in anhydrous pyridine was added, and the reaction mixture was stirred under nitrogen at room temperature for 3 days (*40*). The solvent was evaporated under reduced pressure, and the white solid residue was redissolved in water. The crude product was purified by two consecutive P2 column chromatographies (1.5 × 75 cm and 2.5 × 120 cm, eluted with 25 mM NH_4HCO_3), followed by gradient FPLC on a MonoQ column (eluted with 0−0.5 M NH_4HCO_3). Compound **13** was isolated as a white solid in 28% yield (42 mg, 78 μmol): ^{1}H NMR (D_2O) δ 1.24 (3H, d, J = 6.3 Hz, 5-Me), 1.50 (1H, ddd, J = 11.7, 12.3, 12.7 Hz, 4-H_{ax}), 1.93 (3H, s, 5″-Me), 2.27 (1H, ddd, J = 3.0, 4.0, 12.7 Hz, 4-H_{eq}), 2.40 (2H, m, 2′-Hs), 3.59 (1H, ddd, J = 4.0, 10.7, 12.2 Hz, 3-H), 3.74 (1H, ddd, J = 3.4, 2.9, 10.7 Hz, 2-H), 4.20 (3H, m, 4′- and 5′-Hs), 4.29 (1H, ddq, J = 3.0, 11.7, 6.3 Hz, 5-H), 4.62 (1H, dt, J = 3.0, 5.8 Hz, 3′-H), 5.63 (1H, dd, J = 3.4, 7.3 Hz, 1-H), 6.35 (1H, t, J = 6.8 Hz, 1′-H), 7.71 (1H, s, 6″-H); ^{13}C NMR (D_2O) δ 11.6, 19.5, 35.3, 38.5, 48.9, 65.4, 69.5 (d, J = 6.6 Hz), 70.9, 84.9, 85.0, 85.3 (d, J = 10.1 Hz), 95.0 (d, J = 8.0 Hz), 111.7, 137.3, 151.7, 166.5; ^{31}P NMR (D_2O) δ −12.4 (d, J = 20.9 Hz), −10.5 (d, J = 20.9 Hz); high-resolution FAB-MS calcd for $C_{16}H_{28}N_3O_{13}P_2$ ($M + H^+$) 532.1097, found 532.1098.

*Preparation of TDP-desosamine (**14**).* To facilitate confirmation of the identity of the turnover product, a convenient protocol to make the expected product, TDP-desosamine (**14**), was developed as shown in Scheme 4.

*Acetyl 2-O-Acetyl-3-dimethylamino-3,4,6-trideoxy-*D*-xylo-hexopyranoside (**41**).* A solution of erythromycin (**40**, 14 g, 19 mmol), 6 N HCl (250 mL), and ethanol (100 mL) was refluxed for 4 h and then cooled to room temperature (*49*). The dark solution was transferred to a separatory funnel and washed with chloroform. The color of the dark aqueous solution became light brownish after several washes. The aqueous layer was collected and concentrated under reduced pressure. The crude product was redissolved in acetic anhydride (100 mL) and cooled to 0 °C. To this solution was slowly added concentrated H_2SO_4 (4 mL), and the resulting mixture was stirred overnight. The reaction mixture was then poured into ice water and stirred for another 1 h. After neutralization with concentrated ammonium hydroxide, the aqueous solution was extracted with methylene chloride. The combined organic layers were dried and concentrated in vacuo. The desired product (**41**) was obtained as a brownish oil in 89% yield (4.4 g, 17 mmol): high-resolution CI-MS calcd for $C_{12}H_{22}NO_5$ ($M + H^+$) 260.1498, found 260.1501.

*α-*D*-Desosamine 1-Phosphate (**42**).* A mixture consisting of **41** (0.15 g, 0.58 mmol), crystal phosphoric acid (0.3 g), and a trace amount of P_2O_5 was heated at 80−90 °C under vacuum for 90 min (*50*). After being cooled for several minutes, a solution of 2 M lithium hydroxide was added to the above mixture, and the resulting cloudy solution was heated at 80−90 °C for 60 min to hydrolyze pyrophosphate. The resulting cloudy brownish solution was treated with charcoal and then filtered through Celite. The filtrate was concentrated to about 3 mL, and the resulting yellowish solution was loaded onto a Dowex 50W (cyclohexylammonium·H^+) column and eluted with water. The fractions were analyzed by TLC (developed with *i*-PrOH/1 M NH_4OAc = 2:1 and stained by I_2 vapor), and those containing product were collected and lyophilized. The yellowish solid residue was redissolved in 95% ethanol and filtered. The filtrate was concentrated to dryness, redissolved in small amount of water, loaded onto a P2 column (1.5 × 120 cm), and eluted

with water. The fractions containing the desired product (**42**) were pooled and lyophilized. The product was converted to a cyclohexylammonium salt by passing the sample through a Dowex 50W (cyclohexylammonium·H^+) column: ^{1}H NMR (D_2O) δ 1.11 (3H, d, J = 6.1 Hz, 5-Me), 1.46 (1H, ddd, J = 11.8, 11.9, 12.3 Hz, 4-H_{ax}), 2.00 (1H, ddd, J = 3.0, 3.4, 12.3 Hz, 4-H_{eq}), 2.72 (6H, s, NMe_2), 3.63 (1H, ddd, J = 3.4, 10.9, 11.8 Hz, 3-H), 3.73 (1H, dd, J = 3.0, 10.9 Hz, 2-H), 4.15 (1H, qdd, J = 6.1, 3.0, 11.9 Hz, 5-H), 5.39 (1H, dd, J = 3.0, 6.8 Hz, 1-H); ^{13}C NMR (D_2O) δ 19.7 (C-6), 29.1 (C-4), 38.5 (br, NMe_2), 61.9 (C-3), 64.8 (C-5), 67.1 (d, J = 6.6 Hz, C-2), 93.5 (d, J = 5.0 Hz, C-1); ^{31}P NMR (D_2O) δ 2.79 (s).

*TDP-desosamine (**14**).* A mixture of TMP morpholidate (0.68 g, 990 μmol) and desoamine 1-phosphate (**42**, 155 mg, 330 μmol) was dried three times by coevaporation with anhydrous pyridine. The resulting solid was dissolved in pyridine, mixed with 1*H*-tetrazole (5−7 equiv), and stirred under nitrogen at room temperature for 3−5 days (*40*). The workup followed a similar procedure as described before for **13**. The crude product was purified first by P2 column chromatography (2.5 × 120 cm) and then by gradient FPLC on a MonoQ column (0−0.5 M NH_4HCO_3). The desired product (**14**) was isolated in 78% yield (144 mg, 257 μmol): ^{1}H NMR (D_2O) δ 1.24 (3H, d, J = 6.4 Hz, 5-Me), 1.62 (1H, ddd, J = 11.7, 11.7, 12.7 Hz, 4-H_{ax}), 1.90 (3H, s, 5″-Me), 2.12 (1H, ddd, J = 3.4, 3.4, 12.7 Hz, 4-H_{eq}), 2.3−2.4 (2H, m, 2′-Hs), 2.83 (6H, s, NMe_2), 3.76 (1H, ddd, J = 3.4, 11.2, 11.7 Hz, 3-H), 3.88 (1H, ddd, J = 2.9, 3.1, 11.2 Hz, 2-H), 4.1−4.2 (3H, m, 4′−H, 5′-Hs), 4.28 (1H, qdd, J = 6.4, 3.4, 11.7 Hz, 5-H), 4.59 (1H, dt, J = 3.4, 5.4 Hz, 3′-H), 5.64 (1H, dd, J = 3.1, 7.1 Hz, 1-H), 6.33 (1H, t, J = 6.8 Hz, 1′-H), 7.70 (1H, s, 6″-H); ^{13}C NMR (D_2O) δ 11.6 (5″-Me), 19.7 (C-6), 29.1 (C-4), 38.5 (C-2), 38.6 (br, NMe_2), 62.0 (C-3′), 65.5 (d, J = 5.1 Hz, C-5′), 65.8 (C-5), 66.7 (d, J = 8.1 Hz, C-2), 70.9 (C-3′), 84.9 (C-1′), 85.2 (d, J = 10.1 Hz, C-4′), 95.2 (d, J = 6.0 Hz, C-1), 111.7 (C-5″), 137.3 (C-6″), 151.7 (C-2″), 166.5 (C-4″); ^{31}P NMR (D_2O) δ −12.4 (d, J = 20.8 Hz), −10.6 (d, J = 20.8 Hz); high-resolution CI-MS calcd for $C_{18}H_{32}N_3O_{13}P_2$ ($M + H^+$) 560.1411, found 560.1410.

Cloning of the tylM1 and desVI Genes. Two oligonucleotide primers complementary to the sequence at each end of the *tylM1* gene were prepared to amplify this gene from the cosmid pHJL309, provided by Dr. Eugene Seno of Eli Lilly Research Laboratories, for cloning into the pET17b(+) expression vector (*20*). The start primer, 5′-GGC**CATATG**-GCCCATTCATCCGCC-3′, contains a *Nde*I restriction site (in bold). The halt primer, 5′-CGC**GAATTC**TCACCGGGT-TTCTCCCTT-3′, contains an *Eco*RI restriction site (in bold) immediately downstream of the stop codon. Similarly, oligonucleotide primers were designed to amplify the *desVI* gene for cloning into the pET28b(+) expression vector to produce the N-terminal His_6-tagged fusion protein. The start primer, 5′-GGCC**CATATG**TACGAAGTCGACCACG-3′, contains a *Nde*I restriction site (in bold). The halt primer, 5′-GCGC**GAATTC**TCAGGCGGGGACGCCGAC-3′, has an *Eco*RI (in bold) restriction site. The template used for *desVI* amplification was a previously constructed cosmid, pLZ4 (*26*, *27*). The PCR-amplified *tylM1* and *desVI* DNA fragments were purified, digested with *Nde*I and *Eco*RI, and ligated into the *Nde*I/*Eco*RI sites of pET17b(+) and pET28b-(+), respectively. Positive clones were identified by digestion of the plasmid DNA with appropriate restriction enzymes and visualization of the excised insert by staining the agarose gel with ethidium bromide after electrophoresis. The plasmid DNAs of the positive clones were then used to transform *E. coli* BL21(DE3). The general methods and protocols for recombinant DNA manipulations were as described by Sambrook et al. (*51*).

Purification of TylM1 Protein. All purification operations were carried out at 4 °C, except for the FPLC step, which was run at room temperature. Unless otherwise specified, all buffers contained 0.5 mM DTT and 0.1 mM AdoMet, the latter of which was freshly added each time due to its low hydrolytic stability.

Step 1: Growth of E. coli Cells. An overnight culture of *E. coli* BL21(DE3)-pET17b(+)/*tylM1*, grown in LB medium supplemented with ampicillin (100 μg/mL) at 37 °C, was diluted 250-fold with 4 L of the same medium and incubated at 30 °C until the OD_{600} reached 0.6. The culture was then chilled with cold tap water for 15 min, induced with IPTG to a final concentration of 1.0 mM, and allowed to grow for an additional 15 h at 24 °C. The cells were harvested by centrifugation at 6500*g* for 5 min at 4 °C and stored at −20 °C. The typical yield was 6 g of wet cells/L of culture.

Step 2: Crude Extract Preparation. Thawed cells were suspended in 100 mL of 50 mM potassium phosphate buffer (pH 7.5) and disrupted with four 60 s sonication bursts, with 1 min cooling intervals between bursts. Cell debris was removed by centrifugation at 16000*g* for 25 min.

Step 3: Ammonium Sulfate Precipitation. To the supernatant from the previous step were added ammonium sulfate crystals in three portions over 25 min to 50% saturation. After being stirred for 2 h, the precipitated proteins were collected by centrifugation at 15000*g* for 20 min and resuspended in a minimal amount (80 mL) of 50 mM potassium phosphate buffer, pH 7.5. The resulting protein solution was desalted by dialysis against 1 L of the same butter for 6 h. The buffer was changed three times.

Step 4: DEAE-Sepharose CL-6B Chromatography. The dialysate from step 3 was applied to a DEAE-Sepharose CL-6B column (2.5 × 24 cm) which was preequilibrated with 50 mM potassium phosphate buffer, pH 7.5. The column was washed with 100 mL of the same buffer, followed by a linear gradient between 400 mL of buffer A (50 mM of potassium phosphate, pH 7.5) and 400 mL of buffer B (buffer A plus 0.5 M NaCl). The flow rate was set at 1.5 mL/min, and fractions of 12 mL were collected throughout the entire gradient elution. The fractions containing TylM1 protein, as determined by SDS−PAGE, were pooled, concentrated to 10.3 mL by ultrafiltration, and desalted using 50 mM potassium phosphate buffer, pH 7.5.

Step 5: FPLC MonoQ Chromatography. The material from step 4 was further purified by FPLC on a MonoQ HR (10/10) column using buffer A (50 mM potassium phosphate, pH 7.5) and buffer B (buffer A plus 0.5 M NaCl). The elution profile started with a linear gradient of 0−35% B in 20 min, and this was followed by a wash with 100% buffer B for 5 min. The flow rate was 3 mL/min, and the detector was set at 280 nm. The desired protein, which was eluted as a sharp peak with a retention time of 8.0 min, was collected, concentrated by ultrafiltration, desalted with 50 mM potassium phosphate (pH 7.5), and stored at −80 °C.

Purification of DesVI Protein. All purification operations were carried out at 4 °C, and 15% glycerol, 0.1 mM DTT, and 0.1 mM AdoMet were included in the lysis, washing, elution, and dialysis buffers.

Step 1: Growth of E. coli Cells. An overnight culture of *E. coli* BL21(DE3)-pET28b(+)/DesVI, grown in LB medium supplemented with kanamycin (40 μg/mL) at 37 °C, was used to inoculate 6 L of LB supplemented with kanamycin. Generally, the amount of inoculation was 2 mL of the overnight culture/L. The culture was incubated at 37 °C until its OD_{600} reached 0.6. It was then chilled with tap water briefly, followed by the addition of IPTG to a final concentration of 0.5 mM. After an additional 18 h of growth at 25 °C, the cells were harvested by centrifugation at 5000*g* for 8 min at 4 °C and stored at −20 °C.

Step 2: Crude Extract Preparation. Cells from 6 L of culture were suspended in 100 mL of lysis buffer (50 mM sodium phosphate, 150 mM NaCl, 10 mM imidazole, pH 8.0) and disrupted with five 1 min sonication bursts, with 1 min cooling intervals between bursts. Cellular debris was removed by centrifugation at 15000*g* for 25 min. The supernatant was transferred to an Erlenmyer flask for Ni-NTA resin treatment.

Step 3: Ni-NTA Chromatography. To the above crude extract was added 2 mL of Ni-NTA slurry (1.0 mL of resin), and the resulting mixture was incubated for 1 h with gentle shaking at 4 °C. The resin slurry was transferred into an empty column (1 × 25 cm), and the column was washed with 100 mL of washing buffer containing 20 mM imidazole. DesVI protein was then eluted from the column using 10 mL of the elution buffer (50 mM sodium phosphate, 150 mM NaCl, 250 mM imidazole, pH 8.0). The eluted protein was desalted by dialyzing against 1 L of 50 mM potassium phosphate buffer (pH 7.5) with four changes of buffer.

Step 4: FPLC MonoQ Chromatography. The protein obtained from step 3 was further purified by FPLC on a MonoQ HR (10/10) column using buffer A (50 mM potassium phosphate, 10% glycerol, pH 7.5) and buffer B (buffer A plus 0.5 M NaCl). The elution profile started with a linear gradient of 0−50% buffer B in 20 min, followed by a wash with 100% buffer B for 5 min. The flow rate was 3 mL/min, and the detector was set at 280 nm. DesVI protein was eluted at 52% buffer B. It was concentrated by ultracentrifugation, desalted with 50 mM potassium phosphate buffer (pH 7.5), and stored at −80 °C.

Molecular Weight Determination. The molecular weight of the native protein was determined by size exclusion chromatography using a FPLC equipped with a Superdex 200 HR 10/30 column. The column was eluted with 50 mM sodium phosphate buffer containing 0.15 M NaCl (pH 7.0), and the flow rate was 0.8 mL/min. Calibration of the column (V_i) was achieved using the protein standards from Sigma. The void volume (V_0) of the column was measured using blue dextran. A linear fit to a plot of the molecular weight versus V_i/V_0 was used to estimate the native molecular mass (M_r) (*35*). The subunit molecular mass was estimated by SDS−PAGE as described by Laemmli (*34*).

Activity Assay for TylM1 and DesVI. A HPLC-based assay was developed for TylM1 and DesVI by monitoring the product formation at 267 nm with an Adsorbosphere SAX column (5 μm, 4.6 × 250 mm). A typical assay mixture for TylM1 contained 1.0 mM substrate (**7**), 2.2 mM AdoMet, and an appropriate amount of TylM1 in 58 μL of 50 mM potassium phosphate buffer (pH 7.5). A typical assay mixture for DesVI contained 1.2 mM substrate (**13**), 2 mM DTT, 10 mM of AdoMet, and an appropriate amount of DesVI in 50 μL of 50 mM potassium phosphate buffer (pH 7.5). The incubations were carried out at room temperature (ca. 24 °C) and terminated by heat denaturing the protein at 90 °C for 4 min. A suitable amount of reaction mixture was withdrawn at time intervals and subjected to HPLC analysis. In the TylM1 assay, a linear gradient from 0% to 20% buffer B between buffer A (50 mM potassium phosphate, pH 3.5) and buffer B (500 mM potassium phosphate, pH 3.5) over 12 min gave good separation among the substrate (**7**, 8.5 min), the intermediate (**27**, 9.1 min), and the product (**8**, 10.1 min). In the DesVI assay, a linear gradient from 0% to 20% buffer B between buffer A and buffer B over 20 min gave sufficient separation between the substrate (**13**, 6.5 min), the intermediate (**39**, 9.3 min), and the product (**14**, 17.0 min). The thermostability of each set of substrate, intermediate, and product was examined by HPLC analysis. No degradation was discernible in the heat-treated samples (90 °C for 10 min). Precautions were also taken to limit the overall conversion to be within 10% and thus ensure initial rate measurement.

Production and Characterization of the TylM1 Product. A preparative scale incubation was carried out in which 7.0 μmol of substrate (**7**), 40 μmol of AdoMet, and 0.05 μmol of TylM1 were incubated in 1.5 mL of 50 mM potassium phosphate buffer (pH 7.5) at 24 °C for 4 h. After the conversion was completed as determined by HPLC analysis, the TylM1 enzyme was removed using a Centricon-10, and the product (**8**) was isolated by a FPLC MonoQ HR (10/10) column, eluted with a linear gradient of 0−0.4 M ammonium bicarbonate buffer over 25 min. The flow rate was 3 mL/min, and the detector was set at 267 nm. Under these conditions, **8** was eluted with a retention time of 12.2 min. This peak was collected, lyophilized, and subjected to spectroscopic analysis: ^{1}H NMR (D_2O) δ 1.11 (3H, d, J = 6.3 Hz, 5-Me), 1.75 (3H, d, J = 1.2 Hz, 5″-Me), 2.18−2.22 (2H, m, 2′-Hs), 2.85 (6H, s, NMe_2), 3.36−3.46 (2H, m, 3-H, 4-H), 3.82−3.87 (2H, m, 2-H, 5-H), 4.01−4.02 (3H, m, 4′−H, 5′-Hs), 4.42−4.46 (1H, m, 3′-H), 5.42 (1H, dd, J = 7.2, 3.3 Hz, 1-H), 6.18 (1H, dd, J = 6.9, 6.7 Hz, 1′-H), 7.56 (1H, d, J = 1.2 Hz, 6″-H); high-resolution MALDI-MS calcd for $C_{18}H_{29}N_3O_{14}P_2$ [$(M + H)^+$] 574.1203, found 574.1196.

Characterization of the Monomethylated Intermediate in the TylM1 Reaction. When the aforementioned incubation was quenched at an earlier time, two products could be detected by HPLC (see Activity Assay for TylM1). The one with a retention time of 10.1 min was determined to be the dimethylated product (**8**). The other peak with a retention time of 9.1 min was established to be the monomethylated product (**27**) by a radioactive tracer experiment using [C^3H_3]-AdoMet (10 μCi, diluted with cold AdoMet) as the methyl donor in the assay mixture. After incubation at 24 °C for 5 min, the reaction was quenched, and the reaction mixture was subjected to HPLC analysis. Both peaks (with retention times of 10.1 and 9.1 min) were collected and their radioactivities measured. Since this monomethylated product did not accumulate significantly (<15%) under the normal incubation conditions, a single turnover experiment was carried out to generate more of the desired material for

structural determination. Accordingly, TylM1 protein (50 mg, 0.91 μmol of enzyme dimer) was incubated with an excess amount of AdoMet in the absence of substrate in 100 mM potassium phosphate buffer (pH 8.0). After 1 h of incubation, the free AdoMet was removed by a Centricon-10 microconcentrator with three rounds of dilution/concentration. A stoichiometric amount of substrate (**7**, 1.6 μmol) was then added, and the resulting mixture was incubated at 24 °C for 10 min, followed by quenching at 90 °C for 4 min. As expected, under the single turnover conditions, compound **27** was produced as the major product. The exact mass of this monomethylated product was determined by high-resolution MALDI-MS: calcd for $C_{17}H_{27}N_3O_{14}P_2$ [$(M + H)^+$] 560.1047, found 560.1023. The identity of this compound was eventually confirmed by comparison with the synthetic standard.

Preparation and Characterization of the DesVI Product. A preparative scale incubation to make **14** was carried out in which 5.7 μmol of substrate (**13**), 30 μmol of AdoMet, 2 μmol of DTT, and 1.8 mg of DesVI were incubated in 1 mL of 50 mM potassium phosphate/15% glycerol (pH 7.5) at 25 °C for 3 h. After the conversion was complete as judged by HPLC analysis as described above, the DesVI enzyme was removed using a Centricon-10 microconcentrator, and the product (**14**) was isolated by size exclusion chromatography on a P2 column (2 × 100 cm) eluted with 0.5 M ammonium bicarbonate. The fractions containing the product were combined, concentrated, and further purified by a FPLC MonoQ HR (10/10) column. A linear gradient of 0−0.15 M ammonium bicarbonate buffer over 15 min was used to elute the desired product. The flow rate was 3 mL/min, and the monitor was set at 267 nm. The corresponding peak with a retention time of 8.0 min was collected, lyophilized, and subjected to spectroscopic analysis: ^{1}H NMR (D_2O) δ 1.24 (3H, d, J = 6.4 Hz, 5-Me), 1.62 (1H, dt, J = 12.7, 11.7 Hz, 4-H_{ax}), 1.90 (3H, s, 5″-Me), 2.12 (1H, dt, J = 12.7, 3.4 Hz, 4-H_{eq}), 2.3−2.4 (2H, m, 2′-Hs), 2.83 (6H, s, NMe_2), 3.76 (1H, dt, J = 11.7, 3.4 Hz, 3-H), 3.88 (1H, dt, J = 11.7, 3.1 Hz, 2-H), 4.1−4.2 (3H, m, 4′-H, 5′-Hs), 4.28 (1H, ddq, J = 11.7, 3.4, 6.4 Hz, 5-H), 4.59 (1H, dt, J = 5.4, 3.4 Hz, 3′-H), 5.64 (1H, dd, J = 7.1, 3.1 Hz, 1-H), 6.33 (1H, t, J = 6.8 Hz, 1′-H), 7.70 (1H, s, 6″-H); ^{13}C NMR (D_2O) δ 11.6, 19.7, 29.1, 38.5, 38.6 (br, NMe_2), 62.0, 65.5 (d, J = 5.1 Hz), 65.8, 66.7 (d, J = 8.1 Hz), 70.9, 84.9, 85.2 (d, J = 10.1 Hz), 95.2 (d, J = 6.0 Hz), 111.7, 137.3, 151.7, 166.5; ^{31}P NMR (D_2O) δ −12.4 (d, J = 20.8 Hz), −10.6 (d, J = 20.8 Hz); high-resolution FAB-MS calcd for $C_{18}H_{30}N_3O_{13}P_2$ ($M + H^+$) 560.1410, found 560.1400.

Assay of AdoMet Binding to TylM1 Protein. A mixture containing 3 mg of TylM1 and 3.0 μCi of [C^3H_3]AdoMet in 250 μL of 100 mM potassium phosphate buffer (pH 8.0) was incubated at 24 °C for 1 h. The mixture was then applied to a prepacked 10-DG column (1.0 × 10 cm) and eluted with 100 mM potassium phosphate buffer (pH 8.0). Fractions of 250 μL were collected, and the radioactivity of each fraction was measured by a liquid scintillation counter.

Effects of Mg^{2+} Ions in TylM1 Catalysis. Two independent experiments were carried out to investigate whether Mg^{2+} plays a role in TylM1 catalysis. First, various amounts of Mg^{2+} (over a range of 0−5 mM) were included in the assay mixture to examine if Mg^{2+} could accelerate the reaction rate. In a separate set of experiments, different amounts of EDTA (0−10 mM) were added to the assay solution to probe its effect on the activity of TylM1. Assays were conducted as described above.

Time Courses of Methylation. The time courses of mono- and dimethylation catalyzed by TylM1 were determined by HPLC following the consumption of substrate and the formation of monomethylated and dimethylated products. A typical assay mixture for TylM1 consisted of 10 mM AdoMet, 0.5 mM **7**, 10.9 μM TylM1, and 200 μL of 50 mM potassium phosphate (pH 7.5). Aliquots of 5 μL were withdrawn at appropriate time intervals, boiled, and subjected to the aforementioned HPLC analysis using the SAX anion-exchange column. The conversion of substrate to monomethylated and dimethylated products was estimated on the basis of the integration of the corresponding peaks. The data were fitted into the rate laws for an irreversible unimolecular consecutive reaction [substrate (A) → monomethylated intermediate (B) → dimethylated product (C)]. The rate laws for an irreversible unimolecular consecutive reaction of (A → B → C) are $v_A = -dx/dt = k_1x$, $v_B = k_1x - k_2y$, and $v_C = k_2y$, where x, y, and z are the concentrations of A, B, and C at time t, respectively. Assuming the initial concentration (at time 0) of A is a and those of B and C are nil, data fitting to any two of the following three equations could solve k_1 and k_2: $x = a\exp(-k_1t)$, $y = ak_1[\exp(-k_1t) - \exp(-k_2t)]/(k_2 - k_1)$, and $z = a[1 - k_2\exp(-k_1t)/(k_2 - k_1) + k_1\exp(-k_2t)/(k_2 - k_1)]$.

Determination of the Kinetic Parameters for TylM1 Catalysis. Assays were carried out by varying the concentrations of the substrate to determine the K_m and k_{cat} of the first methylation step under saturating conditions of AdoMet. A typical assay mixture contained 2.2 mM AdoMet, 2.2 μM TylM1, and various amounts of substrate **7** (ranging from 0.044 to 0.994 mM) in 56 μL of 50 mM potassium phosphate buffer (pH 7.5). After incubation at 24 °C for 3 min, the reactions were terminated by denaturing the enzyme at 90 °C for 4 min. Aliquots of 5 μL were withdrawn and subjected to HPLC analysis. The kinetic constants of AdoMet of this step were determined under the saturating conditions of substrate. The incubation mixture contained 4.5 μM TylM1, 1.28 mM **7**, and various amounts of AdoMet ranging from 0.045 to 1.33 mM in 56 μL of 50 mM potassium phosphate buffer (pH 7.5). Likewise, determination of the kinetic parameters of the second methylation step was performed by holding the concentrations of TylM1 (0.43 μM) and AdoMet (2.14 mM) constant, while varying that of the monomethylated intermediate (**27**) over a range of 0.043−0.667 mM in a total volume of 56 μL of 50 mM potassium phosphate buffer (pH 7.5). Similarly, the kinetic constants for AdoMet of this step were determined under the conditions in which the concentrations of TylM1 (0.56 μM) and the monomethylated **27** (0.89 mM) were held constant while that of AdoMet was varied from 0.050 to 1.0 mM. In all cases, the incubation was continued for 3 min at 24 °C and terminated by heat denaturation. The reaction mixture was then subjected to HPLC analysis. The values of the kinetic parameters were estimated by fitting the experimental data to the Michealis−Menten equation.

Determination of the Kinetic Parameters for DesVI Catalysis. Assays were carried out as described above by varying the concentration of substrate (**13**) to determine the K_m and k_{cat} of the first methylation step under saturating conditions of AdoMet. A typical assay mixture of 50 μL

contained 3.0 mM AdoMet, 0.5 mM DTT, 0.27 μM DesVI, and various amounts of **13** (ranging from 0.105 to 1.045 mM) in 50 mM potassium phosphate/15% glycerol (pH 7.5). After incubation at 24 °C for 3 min, the reactions were terminated by denaturing the enzyme in boiling water for 5 min. Aliquots of 20 μL were withdrawn and subjected to HPLC analysis. Experimental data were fit into the Michealis−Menten equation to determine the kinetic parameters.

Determination of the Kinetic Constants of the DesVI Substrate in the TylM1 Reaction. The efficiency of TylM1 to process the DesVI substrate (**13**) was examined by determining the K_m and k_{cat} of the conversion under saturating conditions of AdoMet. A typical assay mixture contained 3.0 mM AdoMet, 0.5 mM DTT, 3.19 μM TylM1, and various amounts of **13** (ranging from 0.105 to 1.045 mM) in 50 μL of 50 mM potassium phosphate/15% glycerol (pH 7.5). After incubation at 24 °C for 3 min, the reaction was quenched by denaturing the enzyme in boiling water for 5 min. Aliquots of 20 μL were withdrawn and subjected to HPLC analysis. The values of the kinetic parameters were estimated by fitting the experimental data to the Michealis−Menten equation.

Determination of the Kinetic Constants of the TylM1 Substrate in the DesVI Reaction. Assays were also carried out to determine the K_m and k_{cat} of the TylM1 substrate (**7**) when processed by DesVI. A typical assay mixture contained 3.0 mM AdoMet, 0.5 mM DTT, 2.67 μM DesVI, and various amounts of **7** (ranging from 0.073 to 1.024 mM) in 50 μL of 50 mM potassium phosphate buffer/15% glycerol (pH 7.5). After incubation at 24 °C for 3 min, the reactions were terminated by denaturing the enzyme in boiling water for 5 min. Aliquots of 20 μL were withdrawn and subjected to HPLC analysis. Experimental data were fit into the Michealis−Menten equation to determine the kinetic parameters.

Replacement of the desVI Gene by tylM1 in a desVI Deletion Mutant of S. venezuelae. The *tylM1* gene was amplified by PCR from cosmid pSET552 using the following primers: 5′-CCGG**GGATCC**CACTTCACCGGGTTTCTCC-3′ and 5′-GCGC**TCTAGA**ATGGCCCATTCATCCGCC-3′. This fragment was digested with *Xba*I/*Bam*HI. Meanwhile, plasmid pDLZ3001, which was constructed in an earlier work to make a *desVI* deletion mutant (*26*), was also digested with *Xba*I/*Bam*HI, and the larger fragment was isolated as the vector for cloning of *tylM1*. Subsequent ligation of the amplified *tylM1* gene and the digested pDLZ3001 plasmid resulted in the generation of pDLZM1. The 3.1 kb *Eco*RI/*Hin*dIII insert of pDLZM1 was then recloned into a conjugation vector pKC1139 (apramycin resistant) to give pM1. The plasmid pM1, which was initially propagated in *E. coli* DH5α, was transformed to *E. coli* S17-1 (*52*) and then introduced by conjugal transfer into the previously prepared KdesVI mutant of *S. venezuelae* in which the *desVI* gene had already been replaced by a thiostrepton gene (*tsr*) (*53*). The resulting conjugants were allowed to grow on sporulation agar plates for several generations without added antibiotics. They were subsequently inoculated into 5 mL of TSB (tryptic soy broth) for growth at 29 °C without added antibiotics. Overnight cultures were diluted 10^{-5}−10^{-7}-fold with TSB and plated out on sporulating agar plates to screen for thiostrepton-sensitive ($Thio^S$) and apramycin-sensitive (Apr^S) clones. Two such colonies in each case, KdesVI/tylM1-1 and KdesVI/tylM1-3 for the *tylM1*-containing mutants and KdesV/tylB-2 and KdesV/tylB-4 for the *tylB*-containing mutant, were chosen for further investigation.

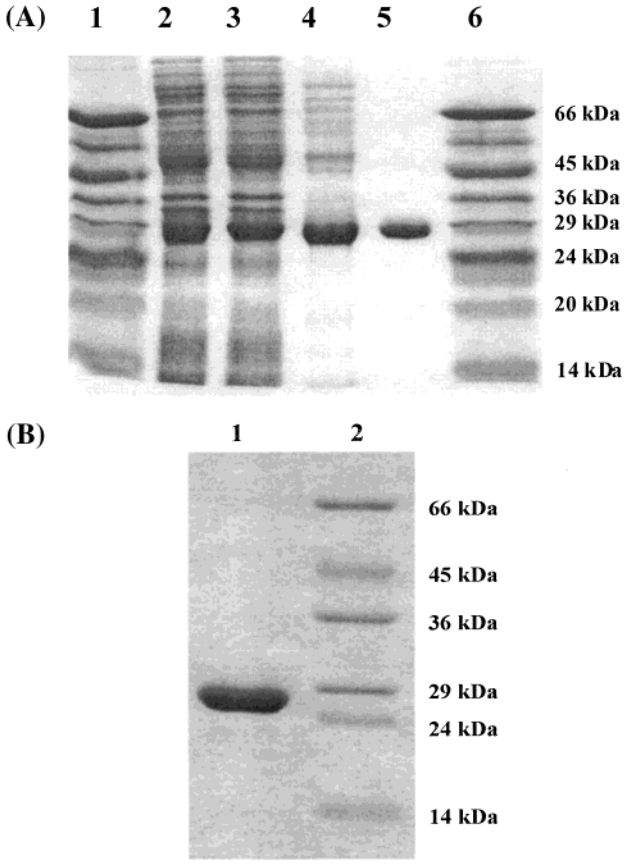


FIGURE 1: (A) SDS−polyacrylamide gel electrophoresis analysis of protein samples collected from different steps during TylM1 purification. Lanes: 1 and 6, molecular mass markers; 2, crude extract; 3, 50% $(NH_4)_2SO_4$ fractionation; 4, DEAE-Sepharose chromatography; 5, FPLC MonoQ 10/10 purification. (B) SDS−polyacrylamide gel electrophoresis analysis of purified DesVI protein. Lanes: 1, purified DesVI; 2, molecular mass markers.

RESULTS AND DISCUSSION

Purification and Characterization of TylM1 Protein. To study the function of TylM1, the *tylM1* gene was amplified by the polymerase chain reaction (PCR) and cloned into the *Nde*I/*Eco*RI sites of the expression vector pET17b(+). The resulting plasmid was used to transform *E. coli* BL21(DE3) cells, and the recombinant strain was highly effective in producing soluble TylM1, which accounts for more than 20% of the total proteins in the crude extract. As shown in Figure 1A, more than 200 mg of nearly homogeneous protein was obtained from 4 L of cell culture after two chromatographic steps. It should be mentioned that the inclusion of 0.1 mM AdoMet in buffers is crucial to prevent TylM1 from precipitating during the purification. The identity of TylM1 was confirmed by N-terminal peptide sequencing. The first 10 residues (A-H-S-S-A-T-A-G-P-Q) of this protein are identical to the translated *tylM1* sequence except for the deletion of the first methionine residue. The subunit molecular mass of 27 kDa, as revealed by SDS−PAGE, correlates well with the predicted value of 27427 Da based on the translated peptide sequence. Judging from a M_r of 55.2 kDa estimated by gel filtration, TylM1 must exist as a homodimer.

The electronic absorption spectrum of the purified protein shows no absorbance above 300 nm. The concentrated TylM1 protein is stable and can endure repeated freeze/thaw cycles without losing noticeable activity. It maintains its activity at −80 °C for at least 2 years. However, TylM1 is unstable at low concentrations, requiring the addition of BSA (1 mg/mL) to the assay buffer during kinetic studies of the enzyme.

Binding of AdoMet to TylM1 Protein. An essential characteristic for a methyltransferase is its capability to recognize and bind AdoMet. Initial evidence indicating that TylM1 is an AdoMet-dependent methyltransferase was derived from sequence alignment studies, which revealed the presence of a conserved AdoMet binding motif (^{53}L-L-D-V-A-C-G-T-G^{61}) in TylM1 (*32*, *54*−*57*). To test whether TylM1 can actually bind AdoMet, a binding study was conducted where [C^3H_3]AdoMet was incubated with the enzyme. A significant amount of radioactivity was found to coelute with the protein fraction when the incubation mixture was separated on a size exclusion column. This result is consistent with TylM1 being an AdoMet-dependent enzyme. While TylM1 can bind AdoMet in the absence of the amino sugar substrate, whether its catalysis proceeds via an ordered mechanism must await further kinetic experiments.

Metal Dependence in TylM1 Catalysis. A few AdoMet-dependent methyltransferases are known to require a divalent metal ion, mostly Mg^{2+}, for activity. For example, two *O*-methyltransferases (TylE and TylF) catalyzing the penultimate and terminal reactions of tylosin biosynthesis have been shown to be Mg^{2+} dependent (*5*, *6*). While the exact role played by Mg^{2+} in these enzymes remains obscure, the absolute requirement of Mg^{2+} for the activity of catechol *O*-methyltransferase (COMT) has been shown to be only in a structural context (*58*). Crystallographic study of COMT with bound AdoMet and in the presence of an inhibitor, 3,5-dinitrocatechol, revealed that Mg^{2+} is not directly involved in catalysis but assists in the organization of the active site by bringing together AdoMet and catechol. To investigate whether the TylM1-catalyzed reaction is Mg^{2+} dependent, the effects of exogenous Mg^{2+} and ion chelator on TylM1 activity were tested. Our results showed that the inclusion of different amounts of Mg^{2+} in the incubation had no effect on the catalysis, and the reaction rate remained unchanged when EDTA was added to the assay mixture. Evidently, TylM1 is not a Mg^{2+}-dependent methyltransferase.

Purification and Characterization of DesVI Protein. To study the function of DesVI, the *desVI* gene was PCR amplified and cloned into the *Nde*I/*Eco*RI sites of the expression vector pET28b(+). The resulting plasmid was used to transform *E. coli* BL21(DE3) cells, and the recombinant strain was grown at 30 °C in LB medium. Induction of this recombinant strain with IPTG led to the overexpression of the desired *desVI* gene product. This N-terminal His_6-tagged DesVI protein was purified to near homogeneity by a Ni-NTA column (Qiagen) followed by FPLC on a MonoQ column (Figure 1B) with a yield of 50 mg of DesVI/6 L of cell culture. The subunit mass of DesVI was estimated to be 28 kDa by SDS−PAGE, which agrees well with the calculated molecular mass of 28110 Da (including the His_6 tag). Further analysis by size exclusion chromatography revealed that DesVI exists as a homodimer in solution with a M_r of 47.4 kDa. The UV−vis spectrum of the purified enzyme is transparent above 300 nm. The inclusion of 0.1 mM AdoMet, 0.1 mM dithiothreitol (DTT), and 10% glycerol in buffers is essential to stabilize the DesVI protein during purification.

Synthesis of TDP-3-amino-3,6-dideoxy-α-D-glucopyranoside (7). Synthesis of the TylM1 substrate (**7**) was accomplished according to the reaction sequence delineated in Scheme 2. Starting from 1,2:5,6-di-*O*-isopropylidene-α-D-glucose (**15**), the key intermediate **17** bearing a 3-amino group was prepared in five steps (*59*−*61*), involving stereochemical inversion of the 3-hydroxyl group and introduction of the nitrogen functionality at C-3 by a nucleophilic displacement with sodium azide with an overall yield of 30%. After carbobenzyloxy chloride treatment to protect the 3-amino moiety, the 5,6-isopropylidene group was selectively cleaved to give **19a** (83% yield for two steps) (*38*). Subsequent C-6 iodination followed by $NaBH_4$ reduction gave the C-6 deoxygenated compound **22a**, which was converted to **24a** via acid treatment, peracetylation, and selective removal of the 1-*O*-acetyl group with hydrazine acetate in DMF (*39*) (45% overall yield from **22a**). Phosphate **26a** was obtained from **24a** in pure α form by *m*-CPBA oxidation of the corresponding phosphite (*40*), followed by hydrogenation and treatment with K_2CO_3 in methanol (50% yield from **24a**). After being passed through an Amberlite IR-120 cation-exchange column (Et_3NH^+), the triethylammonium salt of **26a** was reacted with thymidine 5′-monophosphomorpholidate in the presence of 1*H*-tetrazole in pyridine to give **7** (*40*), which was purified by a Bio-Gel P-2 column (60% yield). It should be noted that there was an intramolecular *O*- → *N*-acetyl migration (30%) when the K_2CO_3-mediated deprotection (**25a** → **26a**) was performed. This mixture was carried on, and the N-acetylated side product could be separated at the final purification step using Bio-Gel P-2 chromatography.

Isolation and Characterization of TylM1 Reaction Products. To test whether TylM1 is the desired methyltransferase, a mixture of TylM1, AdoMet, and **7** was incubated at 23 °C for 4 h. The product was isolated on a FPLC MonoQ column. Spectral characterization of the purified product confirmed that it is indeed the dimethylated hexose **8**. The *N*,*N*-dimethyl group appears at δ 2.85 as a sharp singlet in the ^{1}H NMR spectrum. The other noticeable change is the downfield shift of the C-2 proton signal which now overlaps with the signal of the C-5 proton. The enzymatic conversion is quantitative. Clearly, TylM1 is an AdoMet-dependent *N*,*N*-dimethyltransferase.

Interestingly, when the reaction was quenched at an earlier time, two products could be detected by HPLC using an Adsorbosphere SAX column. The product with a retention time of 10.1 min is the dimethylated product **8**. The new product with a retention time of 9.1 min was identified to be the monomethylated intermediate **27** by several lines of evidence. When [C^3H_3]AdoMet was used as the methyl donor, both products became radiolabeled, and the specific radioactivity of the intermediate (**27**) was 50% of that of dimethylated product (**8**). This result suggested that the new product is likely a monomethylated species. Under normal turnover conditions, this intermediate did not accumulate to a large extent. As expected, when the supply of AdoMet was limited in the single turnover experiment, compound **27** was produced as the predominant product. The identity

of this compound as the monomethylated intermediate was verified by high-resolution MALDI-MS and, ultimately, by comparison with the synthetic standard.

Synthesis of the Monomethylated Intermediate Found in the TylM1 Reaction. As illustrated in Scheme 2, synthesis of the monomethylated standard **27** followed essentially the same synthetic sequence as for its free amino counterpart, **7**, except for the N-methylation step (**18a** → **18b**), which was achieved by treatment with sodium hydride and iodomethane. It is worth mentioning that efforts to remove all protecting groups by overnight hydrogenolysis of **25b** under basic conditions resulted in acetyl migration from C-2 to form the corresponding acetamide at C-3. Attempts to deprotect the acetyl group first by basic hydrolysis were also complicated by the unwanted scission of the anomeric dibenzyl phosphate. A stepwise sequence was later found to be most effective (*62*). The protocol involved partial hydrogenolysis to remove the *O*-benzyl groups first, followed by base hydrolysis to deprotect the acetyl groups, and prolonged hydrogenolysis to unmask the 3-amino group. Subsequent coupling of **26b** with TMP-morpholidate gave the desired product **27**. This chemically prepared monomethylated compound is a substrate for TylM1 and could be swiftly converted by TylM1 to the dimethylated product **8**. These results provided unambiguous evidence that TylM1 catalyzes an N,N-dimethylation reaction by way of a monomethylated intermediate.

*Synthesis of TDP-3-amino-3,4,6-trideoxy-*α*-*D*-xylo-hexopyranose (**13**).* The predicted substrate of DesVI, **13**, was synthesized from methyl 4,6-*O*-benzylideneglucoside (**28**) as delineated in Scheme 3. Preparation of the first key intermediate **33** was initiated by a selective monobenzylation mediated by bis(tributyltin) oxide (**29**:**30** = 8:1) (*42*), followed by acetylation, deprotection, dichlorination, and reduction (*43*, *44*) to give **33** with an overall yield of 14%. After hydrolysis, the configuration of 3-OH was inverted via Swern oxidation and $NaBH_4$ reduction to give **34** (77% yield from **33**). The amino group, masked as an azide moiety, was introduced at C-3 of **34** under Mitsunobu conditions (*45*, *46*) to give **35** in 81% yield. Subsequent demethylation at C-1 was effected by BBr_3 at low temperature (*47*), and the product was benzylphosphorylated to give **37** (34% yield). The final steps involved hydrogenation of **37** with $Pd(OH)_2$, Degussa type (*48*), and coupling of the resulting product with TMP-morpholidate (*40*).

*Preparation of TDP-*α*-*D*-desosamine (**14**).* To facilitate structural confirmation of the product obtained from the enzymatic reaction, the expected product, TDP-desosamine (**14**), was chemically prepared. As shown in Scheme 4, the preparation began with the acid hydrolysis of the commercially available erythromycin B (**40**) (*49*). The released desosamine was directly acetylated to give **41** in 89% yield. The crude product was relatively labile at room temperature; however, it was rendered more stable after neutralization by dissolving it in methylene chloride and washing with ammonium hydroxide and water. Direct phosphorylation of **41** using crystalline phosphoric acid followed by alkaline hydrolysis with lithium hydroxide (*50*) afforded desosamine 1-phosphate (**42**) as a mixture of α and *β* anomers. The desired α anomer of **42** was obtained in 12% yield after purification by Dowex 50W and Bio-Gel P-2 column chromatography. The subsequent coupling with TMP-morpholidate and the purification of the desired product

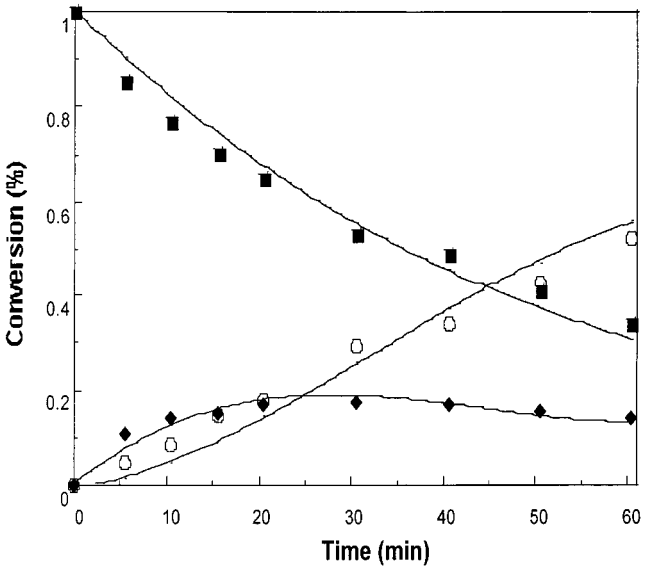


FIGURE 2: Time courses of TylM1-catalyzed *N*-methyl-transfer reactions [■, amino sugar substrate (**7**); ◆, monomethylated intermediate (**27**); ○, dimethylated product (**8**)]. See Experimental Procedures for details.

14 followed a procedure similar to that for the synthesis of **13**.

Isolation and Characterization of the DesVI Reaction Product. To test whether DesVI is the desired methyltransferase, the purified DesVI was incubated with its predicted substrate **13** and AdoMet at 27 °C for 3 h. At the end of this incubation, greater than 80% of **13**, which has a retention time of 6.5 min, was consumed, and a new peak appeared at 17.0 min. A subsequent large-scale incubation allowed the isolation and purification of this new compound. Spectral characterization confirmed that the purified product is indeed the desired TDP-desosamine (**14**). Interestingly, when the reaction was quenched at an earlier time, the monomethylated species (**39**) with a retention time of 9.3 min could also be detected. These findings are similar to those observed for the TylM1 reaction and unequivocally establish that DesVI is the required methyltransferase in the biosynthesis of desosamine. Clearly, DesVI alone catalyzes the N,N-dimethylation of the 3-amino group of its substrate in a stepwise manner.

Reaction Time Course of TylM1-Catalyzed Methylation. The time courses of TylM1-catalyzed mono- and dimethylations of **7** were studied to determine the rate constants of each step. The progress of the reaction was monitored over a period of 60 min by taking aliquots from the incubation at appropriate time intervals and subjected to HPLC analysis. The percent conversions of substrate (**7**) to monomethylated intermediate (**27**) and dimethylated product (**8**) were estimated on the basis of the integration of the corresponding HPLC peaks. The incubation was conducted under saturating concentrations of AdoMet. As illustrated in Figure 2, monotonic consumption of the substrate and the concomitant formation of the dimethylation product were observed. It was also noted that the monomethylated intermediate reached a steady state shortly after the reaction was initiated and did not accumulate to a significant amount throughout the entire process. When the data were fitted to the rate law of irreversible unimolecular consecutive reactions, rate constants of 0.020 and 0.062 min^{-1} for the respective mono- and

Table 1: Kinetic Parameters for the Methylation Catalyzed by TylM1 and DesVI on Various Substrates

enzyme	substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($min^{-1}\,mM^{-1}$)
TylM1	**7**	9.9	59.4	166.7
TylM1	**27**	32.5	46.8	694.4
TylM1	**13**	7.2	118.4	60.8
DesVI	**13**	92.0	307.4	299.3
DesVI	**7**	4.2	276.6	15.2

dimethylation steps of the TylM1 reaction were deduced. The data here clearly indicate that the monomethylated intermediate is not an aberrant shunt product prematurely leaking from the enzyme active site. It is kinetically competent and should be the true intermediate during catalysis.

Kinetic Parameters for Both Methylation Steps in the TylM1 Reaction. The kinetic parameters for the monomethylation step catalyzed by TylM1 were determined by following the rate of substrate (**7**) consumption. The values of K_m and k_{cat} were deduced by fitting the initial reaction rates to the Michaelis−Menten equation based on nonlinear regression. In these experiments, the concentration of one substrate (AdoMet or **7**) was kept fixed while that of the other was varied. When the concentration of **7** was maintained at 1.28 mM, the K_m for AdoMet was determined to be 98.9 ± 1.2 μM, and the k_{cat} was estimated to be 5.5 ± 0.2 min^{-1} (Figure 3A). Similarly, when the concentration of AdoMet was maintained at 2.2 mM, a K_m of 59.4 ± 6.1 μM was determined for the amino sugar substrate (**7**), and a k_{cat} of 9.9 ± 0.2 min^{-1} was obtained from curve fitting (Figure 3B). Following an analogous approach, the kinetic parameters were also determined for the second half of methylation by following the rate of formation of the dimethylated product (**8**) using chemically prepared **27** as the substrate. It was found that the K_m for AdoMet is 117.0 ± 1.6 μM with a k_{cat} of 51.4 ± 1.7 min^{-1} under conditions when **27** was kept at 0.89 mM (Figure 4A). The corresponding values of K_m and k_{cat} for the monomethylated intermediate **27** were determined to be 46.8 ± 3.4 μM and 32.5 ± 0.5 min^{-1}, respectively, when the concentration of AdoMet was maintained at 2.14 mM (Figure 4B). The apparent secondary rate constant (k_{cat}/K_m) of the sugar substrate for the second step is about four times that of the first step (see Table 1). This correlates well with the rate constants estimated by data fitting in the reaction time course study, in which the second step rate constant is about three times that of the first step. Since the second step has a considerably greater reaction rate, the intermediate **27** is not expected to accumulate during reaction, which again agrees well with the experimental observations. It is important to note that the K_m values for the substrate (**7**) and the monomethylated intermediate (**27**) are comparable, indicating that the binding interactions within the Michaelis complex of these two species should be similar. Thus, the higher rate of the second methylation step may be attributed to differences in the inherent reactivity of these two substrates, rather than to differences in sugar binding to TylM1. Since the monomethylated amino group in **27** is intrinsically a better nucleophile than the unsubstituted amino group in **7**, the reaction rate of this S_N2-type methyl transfer reaction is expected to be higher for the second half reaction than for the first methylation reaction.

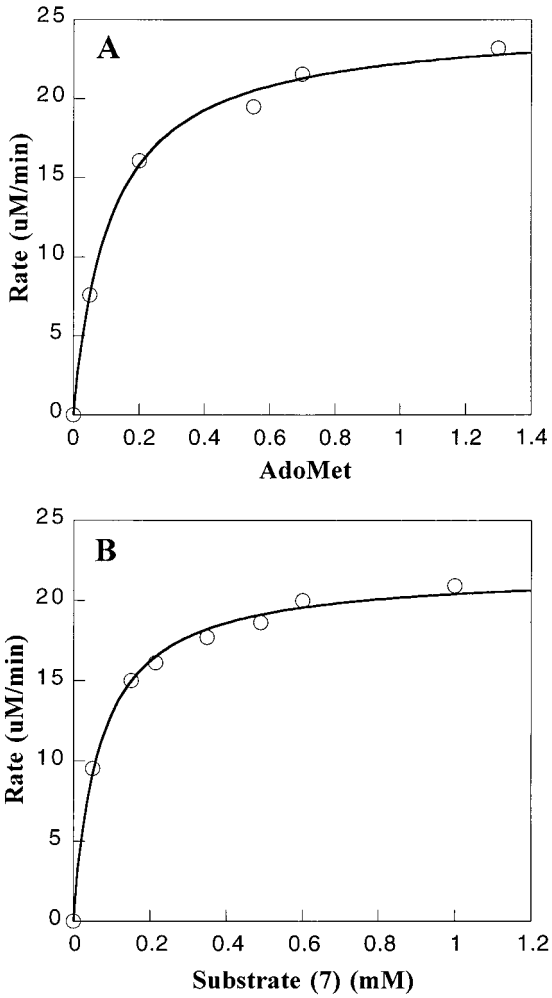


FIGURE 3: (A) Determination of steady-state kinetic parameters for the first methylation of amino sugar **7** catalyzed by TylM1. Rates of individual reactions were obtained by incubating varying amounts of AdoMet (0.045−1.33 mM) with 4.5 μM TylM1 and 1.28 mM amino sugar **7** as described in Experimental Procedures. The initial rates were plotted against the AdoMet concentrations (mM) to obtain the data shown above. (B) As above, rates of individual reactions were obtained by incubating varying amounts of amino sugar **7** (0.044−0.994 mM) with 2.2 μM TylM1 and 2.2 mM AdoMet as described in Experimental Procedures. The initial rates were plotted against the sugar substrate concentrations to obtain the data shown above.

Kinetic Parameters for the DesVI-Calalyzed Reaction. The kinetic parameters of this reaction were determined by following the rate of substrate (**13**) consumption, and by fitting the initial reaction rates to the Michaelis−Menten equation based on nonlinear regression. As shown in Table 1, the K_m and k_{cat} values for the amino sugar **13** were determined to be 307.4 μM and 92.0 min^{-1}, respectively, when the concentration of Ado-Met was maintained at 3.0 mM. Due to the limitation of resources and manpower, no attempt to synthesize the monomethylated intermediate (**39**) was pursued. Thus, no kinetic analysis of the second half of the reaction was performed.

Heterologous Expression of the tylM1 Gene in the desVI Deletion Mutant of S. venezuelae. Since both TylM1 and DesVI are AdoMet-dependent *N,N*-dimethyltransferases and their substrates are aminodeoxy sugars, it is likely that their in vivo catalytic roles are interchangeable. To explore this possibility, an experiment was designed to test whether

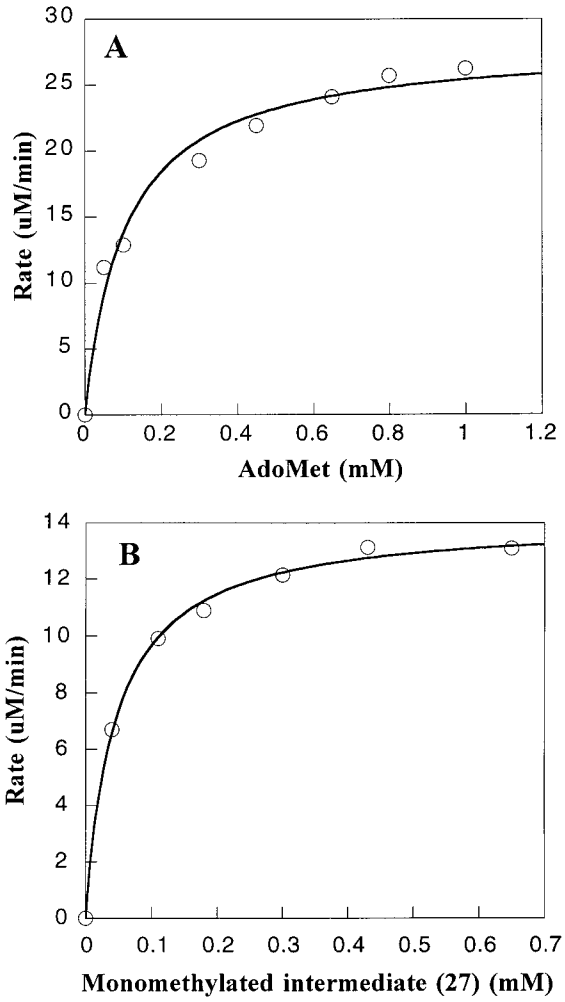


FIGURE 4: (A) Determination of steady-state kinetic parameters for the second methylation of amino sugar **27** catalyzed by TylM1. Rates of individual reactions were obtained by incubating varying amounts of AdoMet (0.050−1.0 mM) with 0.56 μM TylM1 and 0.89 mM monomethylated amino sugar **27** as described in Experimental Procedures. The initial rates were plotted against the AdoMet concentrations to obtain the data shown above. (B) As above, rates of individual reactions were obtained by incubating varying amounts of amino sugar **27** (0.043−0.667 mM) with 0.43 μM TylM1 and 2.14 mM AdoMet as described in Experimental Procedures. The initial rates were plotted against the sugar substrate concentrations to obtain the data shown above.

incorporation of the *tylM1* gene in the *desVI* deletion *S. venezuelae* mutant strain could restore the phenotype of the mutant strain to that of the wild type. In this experiment, the *tylM1* gene in the plasmid pM1 was introduced via conjugal transfer into KdesVI-21 (*26*), a previously constructed *S. venezuelae* mutant whose *desVI* gene was replaced by a thiostrepton-resistant gene (*53*). Screening of double crossover mutants resulted in six colonies, all of which had a thiostrepton-sensitive phenotype. Two of these colonies, KdesVI/tylM1-1 and KdesVI/tylM1-3, were selected and fermented in 50 mL of the methymycin production medium (*63*). The fermentation broth was centrifuged to remove the cellular debris and mycelia, and the supernatant was adjusted to pH 9.5 with concentrated KOH, followed by extraction with chloroform. Methymycin (**9**) and neomethymycin (**10**) were found to be the major products, and no 10-deoxymethynolide (the 10-deoxy aglycon of **9**) (*64*), which is the major metabolite produced by the *desVI* deletion mutant (*26*), was discernible. In comparison with the results obtained from a 50 mL culture of the wild-type strain prepared in parallel, it was estimated that 90% of methymycin/neomethymycin production was restored in the KdesVI/tylM1 strain. These results are consistent with TylM1 as a competent substitute for DesVI and also indicate that TylM1 has relaxed specificity toward its sugar substrate.

Determination of Kinetic Parameters for TylM1 and DesVI Using Alternate Substrates. To gain further information on the efficiency with which TylM1 and DesVI process alternative substrates, kinetic analyses of the TylM1 and DesVI catalysis using **13** and **7** as alternate substrates were performed. When the DesVI substrate (**13**) was used as an alternative substrate for the TylM1 reaction, a K_m of 118.4 μM for **13** and a k_{cat} of 7.2 min^{-1} were obtained. Similarly, using the TylM1 substrate (**7**) for the DesVI reaction, the values of K_m and k_{cat} were determined to be 276.6 μM and 4.2 min^{-1}, respectively. As summarized in Table 1, when TylM1 is used as the methyltransferase, the values of k_{cat} observed with **7** and **13** are similar. However, the value of K_m for **13** is twice that for **7**. On the contrary, when DesVI is used as the methyltransferase, there is a large difference between the values of k_{cat} observed for **7** and **13**, whereas the values of K_m for the two substrates are similar. Judging from the apparent secondary rate constants (k_{cat}/K_m), the difference between TylM1 and DesVI in catalyzing the methylation of **13** is 5-fold, whereas the difference increases to more than 10-fold when **7** is used as the substrate.

CONCLUSION

Methylation catalyzed by an AdoMet-dependent methyltransferase is an effective means to alter the hydrophilicity and/or nucleophilicity of a molecule. While a large number of enzymes capable of catalyzing methylation at carbon, oxygen, sulfur, and nitrogen atoms are known, only a few are able to catalyze N,N-dimethylation (*65*−*67*). A few representatives of this class include PEM-2, a phospholipid methyltransferase that catalyzes the methylation of phosphatidylethanolamine, preferentially the second and third methyl-transfer steps, to form phosphatidylcholine (*65*); RMT1, a protein−arginine methyltransferase that catalyzes both the N^G-monomethylation and N^G,N^G-asymmetric dimethylation of protein arginine residues, an important process in modulating mRNA splicing as well as growth factor localization and function (*67*, *68*); and TlrA, the product of a resistance gene isolated from the tylosin producer *S. fradiae*, catalyzes the dimethylation of a single base to N^6,N^6-dimethyladenine within 23S rRNA rendering the bacterial strain resistant to macrolide, lincosamide, and streptogramin B type (MLS) antibiotics (*66*). Interestingly, studies of the catalyses of TylM1 and DesVI have established their roles as the required methyltransferase involved in the biosynthesis of mycaminose and desosamine, respectively. Each enzyme is capable of catalyzing the N,N-dimethylation of an amino sugar, via a monomethylated intermediate, in a stepwise manner. Thus, our results introduce TylM1 and DesVI as new members of a small family of enzymes that are capable of catalyzing N,N-dimethylation.

TylM1 and DesVI are homodimeric proteins and have nearly identical biochemical properties. Similar to most AdoMet-dependent methyltransferases, the reactions cata-

lyzed by TylM1 and DesVI are likely initiated with the binding of AdoMet, followed by complex formation with the sugar substrate. After the first N-methylation of the C-3 amino group of **7** or **13**, the corresponding monomethylated intermediate (**27** or **39**) and the *S*-adenosylhomocysteine byproduct are expected to release from the enzyme active site. The recruitment of another molecule of AdoMet renders the enzymes ready for the second round of methylation, eventually leading to the desired dimethylated product (**8** or **14**). The proposed reaction sequence is consistent with the findings that these methyltransferases can bind AdoMet in the absence of amino sugar substrate, and the corresponding monomethylated compound, such as **27** or **39**, is a kinetically competent intermediate for the respective enzyme. As predicted, little monomethylated product accumulates in the fermentation broth, since the second methylation step is more facile than the first methylation reaction in the catalytic mechanism. Interestingly, the values of K_m for the unmethylated (**7**) and the monomethylated substrate (**27**) in the TylM1 reaction are comparable (59.4 $\pm$ 6.1 versus 46.8 $\pm$ 3.4 μmol). These two substrates should thus have similar binding affinity toward TylM1. The higher catalytic efficiency for the dimethylation step in TylM1 reaction may simply reflect the greater reactivity of the monoalkylated amino group in **27** than that of a naked amino group in **7**. Clearly, studies of these two enzymes have led to significant insight substantiating that methylation catalyzed by AdoMet-dependent methyltransferases is nucleophilic in nature.

It should be pointed out that the deduced sequence of *tylM1* reveals significant similarity to those of *desVI* (60% identity) (*29*), *eryCVI* from the erythromycin cluster of *Saccharopolyspora erythraea* (60% identity) (*31*, *32*), *snoX* from the nogalamycin cluster *of Streptomyces nogalater* (54% identity) (*69*), *rdmD* from the rhodomycin cluster of *Streptomyces purpurascens* (50% identity) (*70*), and *srmX* from the spiramycin cluster of *Streptomyces ambofaciens* (47% identity) (*71*). All of them contain a short consensus sequence near the N-terminus, L-L-D-V(I)-A-C-G-T-G, which is conserved for many AdoMet binding proteins (*54*−*57*). Although such a sequence analysis has allowed speculation of their catalytic roles as methyltransfereases, their actual functions have never been verified biochemically. Now that TylM1 and DesVI have been fully established as AdoMet-dependent *N*,*N*-dimethyltransferases, a similar role assigned to these proteins in their respective biosynthetic pathways can be considered to be more certain.

The present studies on TylM1 and DesVI not only expand our knowledge of AdoMet-dependent enzymes but also add to the tools available for the genetic manipulation of unusual sugar biosynthetic pathways. Since the sugar components of many secondary metabolites are known to be essential for the biological activity of the parent compounds, the ability to genetically engineer microorganisms to produce sugars that contain various structural alterations provides an alternative approach to the discovery of clinically useful compounds (*13*−*16*). TylM1 and DesVI, acting specifically on the amino group of a sugar substrate, are distinct members of this AdoMet-dependent *N*,*N*-dimethyltransferase family. Their catalyzed reactions could be used as convenient means to make subtle alterations to the chemical properties of the parent compounds due to the changes in size, hydrogen-bonding capacity, and propensity for protonation of the amino groups. With the addition of TylM1 and DesVI to the arsenal of well-characterized biosynthetic genes, our ability to prepare designed glycoconjugate analogues with novel tailored biological activities is significantly enhanced.

ACKNOWLEDGMENT

We express our great appreciation to Dr. Eugene Seno and Eli Lilly Research Laboratories for their generous gifts of the plasmids pSET552, pHJL309, pHJL311, and POJ190.

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BI020245J