Spinosyn, a potent insecticide, is a novel tetracyclic polyketide decorated with n-forosamine and tri-O-methyl-L-rhamnose. Spinosyn rhamnosyltransferase (SpnG) is a key biocatalyst with unique sequence identity and controls the biosynthetic maturation of spinosyn. The rhamnose is critical for the spinosyn insecticidal activity and cell wall biosynthesis of the spinosyn producer, Saccharopolyspora spinosa. In this study, we have functionally expressed and characterized SpnG and the three enzymes, Gdh, Epi, and Kre, responsible for dTDP-l-rhamnose biosynthesis in S. spinosa by purified enzymes from Escherichia coli. Most notably, the substrate specificity of SpnG was thoroughly characterized by kinetic and inhibition experiments using various NDP sugar analogs made by an in situ combination of NDP-sugar-modifying enzymes. SpnG was found to exhibit striking substrate promiscuity, yielding corresponding glycosylated variants. Moreover, the critical residues presumably involved in catalytic mechanism of Gdh and SpnG were functionally evaluated by site-directed mutagenesis. The information gained from this study has provided important insight into molecular recognition and mechanism of the enzymes, especially SpnG. The results have made possible the structure-activity characterization of SpnG, as well as the use of SpnG or its engineered form to serve as a combinatorial tool to make spinosyn analogs with altered biological activities and potency.

Functional Characterization and Substrate Specificity of Spinosyn Rhamnosyltransferase by in Vitro Reconstitution of Spinosyn Biosynthetic Enzymes

Spinosad, a mixture of spinosyns A and D (~85 and 15%, respectively) produced by the actinomycete Saccharopolyspora spinosa, has been proven highly effective against many chewing insect pests and designated as a reduced risk pesticide excellent in both environmental and mammalian toxicological considerations (1). Spinosyns have thus attracted increasing efforts on structural modifications to improve their insecticidal activity as well as to prevent possible resistance problems (2, 3). Structurally, spinosyns are novel glycosylated macrolides consisting of a 21-carbon tetracyclic lactone decorated with tri-O-methyl-l-rhamnose and n-forosamine (see Scheme 1). The tetracyclic aglycone scaffold is delicately constructed first by type I polyketide (PK-I)2 synthases, followed by proposed postmodification events, including FAD-oxidation at C-15, Michael addition between C-3 and C-14, γ-dehydration at C-11, and Diels-Alder cyclization (4, 5). The spinosyn biosynthesis genes recently cloned and sequenced are located in an ~80-kb cluster of the S. spinosa genome, except that the four genes involved in l-rhamnose (l-Rha) biosynthesis, gtt (NDP-glucose synthase), gdh (NDP-glucose 4,6-dehydratase), epi (NDP-4-keto-6-deoxyglucose epimerase), and kre (NDP-4-ketorhamnosyl reductase), are located in three different regions of the genome (6). Previous genetic experiments have suggested that the polyketide aglycone is biosynthesized prior to immediate attachment of l-Rha by the rhamnosyltransferase SpnG, followed by consecutive tri-O-methylation of the l-Rha on resulting spinosyn rhamnosyl pseudoaglycone (SRPG) and then by the addition of n-forosamine to give spinosins (Scheme 1) (4, 7). The four l-Rha biosynthetic genes were proposed to be required for both cell wall biosynthesis and spinosyn production. However, none of the above observations has ever been demonstrated directly in vitro or at the enzymatic level of bio-transformation. Moreover, it has been shown that l-Rha is absolutely required for the reported insecticidal activity, and its alteration can improve insecticidal activity (2). Nonetheless, spinosyn derivatives generated of this kind are still limited and are restricted to in vivo experiments only. Most notably, SpnG exhibits unique sequence identity among known secondary metabolite glycosyltransferases (Gtfs). The unique sequence feature may dictate a novel structure-activity relationship. In light of the above facts, it is therefore an interesting and important task to explore the substrate specificity of SpnG to uncover the molecular recognition and mechanism controlled by SpnG as well as its combinatorial potential for future generation of spinosyn variants with improved or altered biological activities.

The work here reports the heterologous expression, purification, and characterization of gdh, epi, and kre in Escherichia coli as well as those of spnG responsible for attachment of Rha to spinosyn aglycone. In addition, site-directed mutagenesis was carried out on Gdh and SpnG to verify or explore substrate specificity and functional roles of proposed active site residues.

This work was supported by National Science Council, Taiwan, Grants NSC90-2113-M-009-014 and NSC98-2113-M-009-036 (to H.-T. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The on-line version of this article (available at http://www.jbc.org) contains supplemental Scheme S1, Tables S1–S4, and Figs. S1–S11.

To whom correspondence should be addressed. Tel.: 886-3-5131595; Fax: 886-3-5719605; E-mail: chiu@mail.nctu.edu.tw.
Using the four purified enzymes, in vitro one-pot biosyntheses of dTDP-\(\gamma\)-rhamnose (dTDP-Rha) and SRPG were successfully demonstrated. In particular, the substrate specificity of SpnG was systematically probed by various NDP-sugar variants, some of which were made in situ from in vitro tandem reactions of NDP-sugar biosynthetic enzymes. The role of TDP in molecular recognition of TDP-sugars by SpnG was also characterized by inhibition kinetic experiments by dTDP. Upon exploration of SpnG substrate specificity, a number of spinosyn pseudoglycane variants were also generated, and the steady-state kinetic parameters were determined. The results have provided valuable molecular insight into the catalytic mechanism of SpnG and as PK-1 Gtfs in general and have set new, solid groundwork for combinatorial biosynthesis of spinosyns (8, 9).

**EXPERIMENTAL PROCEDURES**

**Materials**—*E. coli* and *Pfu* DNA polymerase were purchased from Stratagene. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs and Invitrogen. The pUC19 cloning vector and the pET expression vectors were purchased from NEB and Novagen, respectively. DNA primers were purchased from Operon Biotech Inc. Unless specified otherwise, all chemicals were purchased from Sigma.

**Bacterial Strains, Culture Conditions, and DNA Manipulation**—*S. spinosa* ATCC49460 was cultivated at 30 °C, 250 rpm in Bennett’s medium for 42 h in baffled flasks for genomic DNA isolation by the Qiagen Genomic-tip system (10). *E. coli* XL1-Blue was used for DNA manipulation, and *E. coli* BL21-Codon Plus (DE3)-RP was used as the host strain for protein expression. All *E. coli* strains were grown at 37 °C in Luria-Bertani (LB) medium with antibiotics (100 \(\mu g/ml\) ampicillin or 30 \(\mu g/ml\) kanamycin). Molecular cloning experiments were performed as described by Sambrook et al. (11).

**Construction of Plasmids**—The DNA fragments flanking each of *gdh*, *epi*, *kre*, and *spnG* were cloned individually from *S. spinosa* and maintained in pUC19 plasmids for easy manipulation. Designed based on 3′- and 5′-end DNA sequences, the oligonucleotide primers for PCR amplification (GeneAmp PCR system 2400; Applied Biosystems) are listed in Table S1. Each gene was subsequently amplified by PCR on the pUC19 plasmid as a PCR template, with the primers introduced with appropriate restriction sites as listed in Table S2. The PCR products (996 bp for *gdh*, 615 bp for *epi*, 927 bp for *kre*, and 1182 bp for *spnG*) were cloned into pUC19 by the Smal site. The resulting plasmids were digested with appropriate restriction endonucleases, and gene fragments were subsequently cloned into vectors pET21b (gdh), pET30b (epi), and pET19b (kre and spnG), generating pYL3, pYS11, pYS20, and pKC3, respectively.

**Sequence Analyses**—Nucleotide and amino acid sequences were analyzed by Vector NTI 9.0 and online analysis tools, including BLAST and Jpred (both available on the World Wide Web), using the default parameter settings.

**HPLC Methods**—All HPLC analyses were performed by Agilent 1100 series. The analytical SAX HPLC analyses, monitored at 267 nm, were carried out on a SAX column (Agilent Zorbax SAX; 5 \(\mu m\), 4.6 × 250 mm) with a linear gradient of potassium phosphate (60–90 mm in 30 min, pH 3.5). A semipreparative SAX column (Agilent Zorbax SAX; 5 \(\mu m\), 9.4 × 250 mm) was used to prepare dTDP-sugars with a linear gradient of ammonium formate (0.5–80 mm in 40 min, pH 3.5). Gtf reactions were analyzed on a C18 column (Agilent Zorbax ODS; 5 \(\mu m\), 4.6 × 250 mm) with a linear gradient of 22.5–70% acetonitrile, 22.5–5% methanol, and 55–25% ammonium acetate (65 mM, pH 7.0) in 5 min, and the elution was monitored at 246 nm. Purification of spinosyn A aglycone and pseudoaglycone products was carried out on a semipreparative reverse-phase HPLC (RP-HPLC) column (Agilent Zorbax ODS; 5 \(\mu m\), 9.4 × 250 mm) with a linear gradient of acetonitrile (25–12.5%), methanol (22.5–30%), and ammonium acetate (52.5–57.5%, 65 mM, pH 7.0) in 65 min.

**Protein Overexpression**—To overexpress *gdh*, *epi*, and *kre*, *E. coli* BL21 (DE3) cells, transformed with pYL3, pYS11, and pYS20, respectively, were grown at 37 °C in LB medium with appropriate antibiotics until \(A_{600}\) reached 0.5. After induction with isopropyl \(\beta-d\)-thiogalactopyranoside (IPTG), the culture grew at the designated temperature for 10 h. For Gdh, Epi, and Kre, the culture was induced with 50 \(\mu M\) IPTG at 15 °C, 250 \(\mu M\) IPTG at 25 °C, and 250 \(\mu M\) IPTG at 30 °C, respectively. To express SpnG, the cells co-transformed with pKC3 and pG-KJE7 (12) were induced with 0.2% (v/v) arabinose and 50 \(\mu M\) IPTG at 15 °C for 20 h. The cells were then harvested by centrifugation (3,200 \(\times g\), 15 min).

**Protein Purification**—For Gdh, cells resuspended in 30 ml of buffer A (20 mM Tris-HCl, pH 7.8) were broken by two passages through a French press cell (Spectronic Instruments) at 16,000 p.s.i. Cell debris was removed by centrifugation (20,000 \(\times g\), 1.5 h, 4 °C). The supernatant was loaded onto Ni2+-nitrilotriacetic acid superflow resin (4 ml; Qiagen) and washed with buffer A (120 ml). Gdh was eluted with a stepwise imidazole gradient (2–500 mM). The desired fractions, as confirmed by SDS-PAGE, were concentrated by Centriprep YM-10 (Millipore) with 20 mM Tris-HCl (pH 7.8 in 15% glycerol) and then stored at −20 °C. For Epi and Kre, cell pellets harvested from 2 liters of culture were resuspended in buffer B (20 mM Tris-HCl, 1 mM NaCl, 9 mM MgCl2, pH 7.3), followed by the similar procedure described above. The desired fractions were exchanged with Tris-HCl buffer (20 mM Tris-HCl, pH 7.3, 9 mM MgCl2, 1 mM dithiothreitol, 15% glycerol). For SpnG, the harvested cells were resuspended in buffer C (20 mM Tris-HCl, pH 9.0, 150 mM NaCl). SpnG was finally eluted with buffer C containing 150 mM imidazole. The eluted SpnG fractions were exchanged with 20 mM Tris-HCl (pH 8.0 in 15% glycerol) by Centriprep YM-30 and then stored at −20 °C. The concentration of each purified protein was determined by Lowry assay (13). Unless specified otherwise, the above conditions were used for enzyme preparation in this study.

**Construction of Gdh and SpnG Mutants**—To generate Gdh and SpnG mutants, the plasmids pYL3 and pKC3, respectively, were used as DNA templates for QuikChange site-directed mutagenesis (Stratagene) with primers listed in Tables S3 and S4. The mutants were expressed and purified as described for the wild type.

**Kinetic Characterization of Gdh and Mutants**—Reaction mixtures (18 \(\mu l\)) containing TDP-Glc (125 \(\mu M\) to 6 mM), NAD\(^+\) (2 mM), and Gdh (18 pmol) were incubated at 30 °C for 10 min. The activities of Gdh and mutants were measured in duplicate.
Substrate Specificity of Spinosyn Rhamnosyltransferase

and spectrophotometrically at 318 nm and also confirmed by SAX HPLC analysis for dTDP-6-deoxy-\(\alpha\)-xylo-4-hexulose (KDG) (3) (see Fig. 5) formation. The extinction coefficient of \(3\) under this condition was 6,500 \(\text{m}^{-1} \text{cm}^{-1}\), as reported by Gross et al. (14).

Steady-state Kinetic Measurements of SpnG and Mutants—To determine the steady-state kinetic parameters for aglycone (5) and NDP-sugars, the experiments were conducted by varying the concentration of one substrate while the other was saturated. \(K_m\) and \(k_{cat}\) for NDP-sugars were determined by varying the NDP-sugar concentration with aglycone (5) held at 4.5 \(\mu\)M. A typical assay containing NDP-sugar, spinosyn aglycone (in DMSO), and SpnG in 35 \(\mu\)l of reaction (100 mM Tris-HCl, pH 9.0, 2.5 mM MgCl\(_2\)) was carried out at 30 °C, quenched by adding cold methanol (70 \(\mu\)l), flash-frozen by liquid nitrogen, and then stored at -80 °C overnight. After removal of the precipitated protein by centrifugation, the supernatant was analyzed by RP-HPLC using the conditions described under “HPLC Methods.” The product formation was monitored at \(A_{246}\) and verified by mass spectrometry and NMR. For TDP-Glc (2), SpnG was held at 25 \(\mu\)M, and the TDP-Glc concentration was varied (0.25–30 mM). For KDG (3), SpnG was held at 100 \(\mu\)M with KDG varied (0.5–20 mM). For TDP-Rha (4), SpnG was held at 500 \(\mu\)M with TDP-Rha varied (0.05–15 mM). For dTDP-6-deoxy-\(\delta\)-glucose (TDP-6dGlc) (10), SpnG was held at 25 \(\mu\)M with TDP-6dGlc varied (0.1–4 mM). For dTDP-\(\delta\)-olivose (TDP-Oli) (8), SpnG was held at 5 \(\mu\)M with TDP-Oli varied (0.18–5.71 mM). The SpnG reactions for 2, 4, 8, and 10 were quenched at 6 min, whereas for 3 it was quenched at 1.5 h.

The determination of \(K_m\) and \(k_{cat}\) for aglycone (5) was conducted with various concentrations (0.05–3.5 mM) of aglycone, whereas NDP-sugar (TDP-Rha or TDP-Glc) was fixed at 10 \(\mu\)M. Each experiment was conducted in duplicate. The kinetic parameters of SpnG mutants were obtained by similar procedures. Quenched samples were manipulated and analyzed by RP-HPLC, as described. The kinetic parameters were deduced by fitting the HPLC integration data with Michaelis-Menten equation.

In Situ Kinetic Characterization of SpnG for dTDP-\(\delta\)-rhamnose—In situ generation of TDP-Rha (0.05–14 \(\mu\)M) as the sugar donor for SpnG was achieved by tandem reaction of Gdh, Epi, and Kre. In 12.5 \(\mu\)l of reaction, TDP-Glc was incubated with 0.65 nmol of Gdh at 30 °C for 20 min. After the complete conversion to 3, the solution was added Kre (0.11 nmol), Epi (1.63 nmol), and NADPH (14.4 \(\mu\)M) to give a total volume of 20.5 \(\mu\)l, followed by an additional incubation of 1 h at 30 °C. The quantitative conversion to 4 was confirmed by SAX HPLC. Without further purification, the reaction mixture was adjusted to the optimal condition (pH 9.0, 2.5 \(\text{mM} \text{MgCl}_2\)) described for SpnG reactions with the addition of aglycone (5) (157.5 nmol) and SpnG (17.5 pmol) in a final reaction of 35 \(\mu\)l. After incubation at 30 °C for 6 min, each reaction was quenched and analyzed by RP-HPLC. Each reaction was conducted in duplicate.

Inhibition by dTDP—An inhibition study was conducted by measuring the SRPG (6) formation catalyzed by SpnG in the presence of dTDP. With aglycone (5) saturated, the TDP-Rha concentration was varied (0.25–1.74 \(\mu\)M) in the presence of various concentrations (0, 0.5, 1.5, and 3.0 mM) of dTDP. The reaction was carried out in duplicate under the same condition described for SpnG kinetics. The data obtained with various inhibitor concentrations were fitted to the equation describing competitive inhibition (Equation 1).

\[
v = V_{max}/(1 + (K_m/5)(1 + I/K)) \tag{Eq. 1}
\]

Production and Characterization of dTDP-6-deoxy-\(\alpha\)-xylo-4-hexulose (3) and dTDP-\(\delta\)-rhamnose (4)—The Gdh product (KDG) (3) was prepared by incubation of TDP-Glc with Gdh. TDP-Rha (4) was generated in large scale by starting with incubation of TDP-Glc with Gdh to complete the KDG formation, followed by the addition of Epi, Kre, and NADPH. All experimental and spectrometric details are presented in the supplemental material.

Preparation of Spinosyn Aglycone Substrate (5)—Spinosyn A aglycone was prepared by the method described previously (15) with modifications in the isolation procedure (supplemental material).

Production and Structural Characterization of Various Pseudoaglycone Products—SRPG (6) was prepared by a tandem reaction where TDP-Rha (4) was enzymatically prepared with Gdh, Epi, and Kre, followed by glycosylation with SpnG. Spinosyn glucosyl pseudoaglycone (SGPG) (7) was generated by reaction of TDP-Glc with SpnG. Enzymatic syntheses of spinosyn oligosyl pseudoaglycone (14) and 6-deoxy-glucosyl pseudoaglycone (15) were achieved by reactions of TDP-Oli and TDP-6dGlc with SpnG, respectively. Experimental and spectrometric details are described in the supplemental material.

RESULTS

Heterologous Expression and Purification of the dTDP-\(\delta\)-Rhamnose Biosynthetic Enzymes and Spinosyn Rhamnosyltransferase in E. coli—In this study, gdh, epi, kre, and spnG were cloned into pET, expressed, and purified to near homogeneity in E. coli for in vitro functional characterization, as described under “Experimental Procedures.” As a result, the expression of Gdh and Epi, both with a C-terminal His\(_{14}\) tag, afforded soluble proteins to near homogeneity in decent quantities (17.5 and 17.0 mg/liter of culture, respectively). However, the recombinant C-terminal His\(_{14}\)-tagged forms of Kre and SpnG gave little soluble protein. To overcome the solubility problems, the DNA fragment harboring kre was subsequently subcloned into pET19b for expression as an N-terminal His\(_{14}\)-tagged protein. An optimal expression condition of N-His\(_{14}\)-tagged Kre was achieved by induction with 250 \(\mu\)M IPTG at 30 °C and gave ~1.4 mg/liter of culture protein to near homogeneity. A similar strategy, however, failed to improve SpnG expression significantly, although an expression at lower temperature (e.g. 8 and 15 °C) was also attempted. The situation was resolved by co-expression of SpnG with a combination of chaperones (GroEL, GroES, Dnak, DnaJ, and GrpE) encoded by pG-KJE7 (12), successfully affording ~2.4 mg of protein/liter of culture to near homogeneity. As revealed by SDS-PAGE (Fig. 1), molecular masses of purified Gdh, Epi, Kre, and SpnG were found to be about 37, 23, 36, and 44 kDa, respectively, consistent with the predicted values.
Functional Characterization of dTDP-4-Rhamnose Biosynthetic Enzymes—To functionally characterize Gdh, both TDP-Glc and UDP-4-glucose (UDP-Glc) were tested as possible substrates for enzymatic activity. Consequently, Gdh displayed a robust dehydratase activity for TDP-Glc. As shown in Fig. 2, the SAX HPLC analysis of Gdh with TDP-Glc showed a complete formation of the product 3 within 1 min. NMR and MALDI-TOF characterization of the product matched favorably with the literature data (16) of KDG. Gdh, however, showed no detectable activity toward UDP-Glc. In addition, Gdh exhibited maximal activity at pH 7.8 and displayed a NAD(P)⁺-dependent activity. Gdh gave a 3.5-fold increase of activity in the presence of 2 mM NAD⁺. Unlike NAD⁺, NADP⁺ gave no improvement in Gdh activity but, instead, inhibited Gdh when it exceeded 0.5 mM. The kinetic parameters $K_m$ and $k_{cat}$ were determined to be $0.28 \pm 0.03$ mM and $51.1 \pm 1.4$ min⁻¹, respectively, for Gdh with TDP-Glc.

Subsequently, Epi and Kre were examined for their enzymatic activities in an attempt to complete TDP-Rha biosynthesis (Scheme 1). Whereas KDG was incubated with Epi in the absence of Kre, dTDP and maltol (decomposed products) accumulated along with consumption of KDG in a time-dependent manner, as verified by SAX HPLC with commercial standards. Since KDG was incubated simultaneously with Epi, Kre, and the required co-substrates, TDP-Rha (4) was successfully afforded (Fig. 2), as confirmed by subsequent MALDI-TOF and NMR spectroscopy. Hence, the in vitro characterization of the three enzymes firmly established that Gdh, Epi, and Kre are responsible for the TDP-Rha biosynthesis in S. spinosa. In addition, Kre displayed no reductase activity toward KDG within 6 h, indicating that Kre possesses substrate specificity, and no side product will be generated during the in vitro TDP-Rha biosynthetic route under the described condition. To prepare TDP-Rha in situ for later glycosylation with SpnG, an in vitro tandem enzymatic reaction of TDP-Glc with Gdh, Epi, and Kre altogether was accomplished as described under “Experimental Procedures.” Consequently, the reaction succeeded in ready quantitative conversion of TDP-Glc to TDP-Rha.

Characterization and Site-directed Mutagenesis of Gdh—In NDP-deoxysugar biosynthesis, dTDP-4-glucose 4,6-dehydratase occupies the key branch point from which a series of structural modifications occur to give diverse NDP-deoxysugars (17). Gdh showed high sequence similarity (75.4%) and identity (65.9%) with DesIV from Streptomyces venezuelae (Fig. S1). By sequence alignments and homology modeling of Gdh on DesIV as a template (18) (available on the World Wide Web), several potential catalytic residues (e.g. Tyr-151, Glu-129, and Asp-128) were identified in Gdh for functional elucidation in the catalytic mechanism (Scheme 2).

Gdh contains the Y-X3-Y fingerprint of short chain dehydrogenase/reductase family, suggesting that Tyr-151 may be the active site amino acid involved in the NAD(P)H binding motif (Wierenga motif) (19). Tyr-151 may also serve as a base responsible for the deprotonation of the hydroxyl group at C-4 and subsequently as an acid to reprotonate the ketene intermediate at C-5. Disruption of Tyr-151, in Gdh Y151F, completely abolished the Gdh activity, supporting its roles in catalysis (Table 1). As shown in Scheme 2, the dehydration at C-6 would require a general acid/base counterparts. Indeed, the Y151F mutation suffered a 183-fold decrease in $k_{cat}$ and the D128E mutation gave an 18-fold reduction in $k_{cat}$ and a relat...
Notably, the phosphoryl oxygen binding activity of Asn-180 failed to be rescued by the replacement with an aspartate in Gdh N180D, implicating the direct electrostatic interaction between the phosphoryl oxygen and the Asn-180 side chain.

### Enzymatic Transfer of Rhamnose onto Spinosyn Aglycone by SpnG

- The capability of SpnG to transfer L-Rha from the natural substrate (TDP-Rha) onto spinosyn aglycone (SAG) was examined by the incubation assays with authentic TDP-Rha, SAG, and SpnG at appropriate reaction time intervals. Consequently, the RP-HPLC analysis revealed the formation of SRPG (retention time 12.01 min) (Fig. 3, profile A), and the reaction reached completion in 6 min. Upon extensive NMR analyses of 6, designation of each proton was established by COSY spectroscopy. The small coupling constant ($J_{1,2} = 0.5$ Hz) from the rhamnosyl moiety designated the $\alpha$-glycosidic linkage. These results readily revealed the attachment of L-Rha moiety to SAG. Subsequently, SpnG was kinetically characterized. A maximal activity for SpnG was observed at pH 9.0, accompanied by a gradual increase from pH 6.0 to pH 8.5 and a dramatic drop while pH increased from 9.0 to 10.5. Moreover, exogenous divalent metal ion was found not to be necessary for SpnG activity. However, the presence of 2.5 mM MgCl$_2$ allowed SpnG activity to increase and reach the optimum by ~2.2-fold. In contrast, SpnG gained only, at the most, a 1.3-fold increase of activity with 2.5 mM MnCl$_2$.

Steady-state kinetic experiments by varying TDP-Rha resulted in a $K_m$ of 0.60 ± 0.01 mM and a $k_{cat}$ of 12.1 ± 0.28 min$^{-1}$ for TDP-Rha (Fig. 4A). Likewise, the $K_m$ and $k_{cat}$ for SAG was determined to be 0.14 ± 0.01 mM and 8.78 ± 0.16 min$^{-1}$, respectively (Fig. 4B). SpnG exhibited classical Michaelis-Menten kinetic behavior (data summarized in Table 2), where no...
substrate inhibition was observed with either dTDP-sugar or SAG substrate. Hence, SpnG displayed a robust Gtf activity with a moderate $K_m$ for dTDP-sugar and relatively small $K_m$ for the aglycone substrate, giving a catalytic efficiency of 62.71 mM/1002 1 min/1002 1. Importantly, the above results provide the first in vitro demonstration of sugar transfer activity of SpnG in E. coli and in enzymatic level.

**Discovering Substrate Promiscuity of SpnG—** Spinosyn analogues altered in the rhamnose moiety were found to display improved activity against larvae of *Heliothis virescens* (1, 2). Unfortunately, so far they were generated only by chemical or genetic means and severely limited in number and diversity. Nevertheless, most naturally occurring spinosyns retain the common rhamnosyl moiety, implying no apparent substrate promiscuity for SpnG. To explore the substrate specificity of this key player for future combinatorial biosynthesis, TDP-Glc was first examined as an alternative substrate. Interestingly, a new product was generated, as revealed in RP-HPLC analyses of SpnG reactions with TDP-Glc and SAG (Fig. 3, profile B). The product was subjected to fast atom bombardment mass spectrometry and NMR analyses and subsequently identified to be the expected product, SGPG (7). The one-dimensional NMR spectrum gave the coupling constant $J_{1,2} = 7.5$ Hz, indicating a $\beta$-linkage between the sugar moiety and aglycone scaffold. The NMR spectroscopic results of SRPG and SGPG were in good agreement with the inverting catalytic mechanism classified in the CAZy system (available on the World Wide Web) (21). Furthermore, SpnG was kinetically characterized with TDP-Glc for comparison in substrate specificity. As shown in Table 2, $K_m$ and $k_{cat}$ for TDP-Glc were found to be 1.21 ± 0.12 mM and 0.68 ± 0.02 min$^{-1}$, respectively (Fig. 4, inset in A), leading to a 2-fold increase in $K_m$, and an 18-fold drop in $k_{cat}$ as compared with those for TDP-Rha. Parallel experiments were conducted to determine the kinetic parameters for SAG by replacing the sugar donor with TDP-Glc. Consequently, the $K_m$ for SAG was found to be 0.11 ± 0.01 mM, and the $k_{cat}$ was found to be 0.65 ± 0.01 min$^{-1}$ (Fig. 4, inset in B). As compared with those obtained with the natural sugar donor (TDP-Rha), the $K_m$ remained unchanged, and the $k_{cat}$ decreased ~13-fold for SAG when TDP-Glc was used. These

**FIGURE 3.** HPLC analytical profiles of glycosylation of aglycone 5 by SpnG. The assay mixture consisting of NDP-sugar (1 mM), aglycone 5 (0.5 mM), MgCl$_2$ (2.5 mM), SpnG (25 μM), and Tris-HCl (100 mM, pH 9.0) was incubated at 30 °C for different periods of time, as indicated. Trace profile A, TDP-Rha (4) was used as the sugar donor; trace profile B, TDP-Glc (2) was used as the NDP-sugar substrate. The reaction was quenched at each time point and subjected to analytical C$_{18}$ RP-HPLC analysis (retention time in min), as described under “Experimental Procedures.” The resulting glycosylated products 6 and 7 are as indicated in A and B, respectively.

Substrate Specificity of Spinosyn Rhamnosyltransferase
Substrate Specificity of Spinosyn Rhamnosyltransferase

FIGURE 4. A, Michaelis-Menten plots of SpnG kinetics for NDP-sugar kinetic parameters. Each velocity was determined at various concentrations of TDP-Rha (A) by saturating SAG (S), as described under “Experimental Procedures.” In the inset of A, TDP-Glc (2) was utilized as the sugar donor for SpnG kinetics with saturating SAG. B, Michaelis-Menten plots for steady-state kinetic parameters for SAG. Each velocity was determined at various concentrations of SAG (S) by saturating TDP-Rha (A), as described under “Experimental Procedures.” In the inset of B, TDP-Glc (2) was used, instead, as the sugar substrate for SpnG kinetics with various concentrations of SAG (S).

interesting results concluded that SpnG was able to accept a different NDP-sugar as an alternative substrate and exhibited relaxed substrate specificity toward TDP-Glc, albeit with an ~36-fold drop in catalytic efficiency.

In Vitro Reconstitution of Spinosyn Rhamnosyl Pseudoaglycone Biosynthetic Pathway—To expand the potential applications of SpnG in glycosylation with various NDP-sugars generated by a series of NDP-sugar biosynthetic enzymes, an in vitro mixing of SpnG with a group of dedicated NDP-sugar enzymes needs to be examined for glycosylation efficiency in the presence of various required co-factors. Therefore, an attempt to reconstitute an in vitro the spinosyn pseudoaglycone biosynthesis by a multienzyme reaction system was made with purified Gdh, Epi, Kre, and SpnG together with the required co-factors. In our initial attempt, TDP-Glc was treated with simultaneous incubation of the four enzymes and required co-substrates. Consequently, SRPG and SGPG were both obtained as analyzed by RP-HPLC, reasonably, due to the substrate promiscuity of SpnG. To circumvent the SGPG formation, in another attempt, TDP-Glc was first incubated with Gdh, followed by the addition of Epi, Kre, and co-factors to complete TDP-Rha formation and then by the addition of SpnG and co-substrates/cofactor. Under such conditions, Gdh, Epi, and Kre indeed quantitatively converted TDP-Glc to TDP-Rha in the presence of NAD(P)H, as verified by SAX HPLC analysis. Consequently, SpnG was successfully generated as the only glycosylated product by this in vitro tandem multienzyme reaction, further enabling us to characterize the Gtf activity by in situ generation of TDP-Rha. In the in situ condition, SpnG exhibited normal Michaelis-Menten kinetics characterized by a Km of 1.57 ± 0.15 mM and a kcat of 12.30 ± 0.28 min⁻¹. In comparison with those obtained directly with authentic TDP-Rha, in the in situ tandem system, SpnG displayed a 2.6-fold increase in Km and only a modest variation in kcat. Together, these results are rather encouraging and indicate that the in vitro biosynthetic reconstitution of glycosylated spinosyn analogues (e.g. SRPG and SGPG) is feasible under the tandem reaction condition, and the components (cosubstrates or cofactors) to support in vitro NDP-sugar biosyntheses and glycosylation had little effect on the catalytic behavior of SpnG. This feature can be very helpful for future applications in in vitro combinatorial biosynthesis of NDP-sugars and spinosyn glycosides.

Substrate Specificity of SpnG and Novel in Vitro Glyco-diversification—The important findings above prompted us to further explore, at the molecular level, the substrate promiscuity and combinatorial potential of SpnG with more NDP-sugar analogs. As listed in Fig. 5, these analogs include dTDP-6-deoxy-1-talose (TDH-Oli) (8), TDP-6-deoxy-1-talose (9), KDG (3), TDP-6dGl (10), dTDP-o-mannose (11), dTDP-6-galactose (12), and dTDP-xylose (13). Consequently, SpnG did exhibit an excellent degree of substrate promiscuity toward the sugar donor, including TDP-Oli, dTDP-xylose, KDG, and TDP-6dGl, thus generating the corresponding glycosylated products. dTDP-6-deoxy-1-talose, dTDP-o-mannose, and dTDP-6-galactose, however, did not serve as substrates for SpnG under

TABLE 2

| Substrates | Km (mM) | kcat (min⁻¹) | kcat/Km (min⁻¹/mM) |
|------------|---------|--------------|-------------------|
| Spinous aglycone | 0.14 ± 0.01 | 8.78 ± 0.16 | 62.71 |
| Spinous aglycone | 0.11 ± 0.01 | 6.65 ± 0.01 | 5.91 |
| TDP-Rha | 0.60 ± 0.01 | 12.1 ± 0.28 | 20.17 |
| TDP-Rha | 0.39 | 7.83 | 0.39 |
| TDP-Oli | 0.41 ± 0.04 | 2.31 ± 0.07 | 5.63 |
| dTDP-6-deoxy-1-talose | NA | ND |
| TDP-Glc | 1.23 ± 0.12 | 0.68 ± 0.02 | 0.56 |
| TDP-6dGl | 0.44 ± 0.06 | 1.95 ± 0.06 | 4.43 |
| KDG | 1.31 ± 0.18 | 0.02 ± 0.01 | 0.02 |
| dTDP-o-mannose | NA | ND |
| dTDP-o-galactose | NA | ND |
| UDP-o-glucose | NA | 0.00001 |
| dTDP (as an inhibitor) | 0.97 ± 0.16 |

a TDP-Rha as the sugar donor.
b TDP-Glc as the sugar donor.
c Kinetic parameters were obtained using TDP-Rha synthesized in situ by Gdh, Epi, and Kre.
d NA, kinetic parameters could not be determined by Michaelis-Menten equation.
e ND, not detectable (kcat < 0.00001 min⁻¹). See supplemental material for HPLC analysis.
g The SpnG reaction was conducted with TDP-Rha and spinosyn aglycone in the presence of dTDP as an inhibitor.
the described conditions. To better describe the content of substrate specificity, SpnG was further kinetically characterized on TDP-Oli, KDG, and TDP-6dGlc for comparison with TDP-Rha and TDP-Glc. In Table 2 are summarized the steady-state kinetic parameters for the substrate analogs examined. It should be noted that, for all of the NDP-sugar analogs serving as substrates, an alternation in the sugar structure essentially did not have a noticeable effect on $K_m$. However, such alternation did cause dramatic variation in $k_{cat}$, depending on the pattern of alternation. In comparison with the natural substrate TDP-Rha, the most extreme case came from KDG, where its $K_m$ remains essentially the same, but its $k_{cat}$ strikingly dropped more than 600-fold. On the other hand, the configuration (d- or l-form) of the sugar moiety seemed to contribute only a moderate effect on $K_m$ among the accepted substrates. Taking the kinetic behaviors of all of the NDP-sugar analogs into consideration, it may thus suggest that the catalytic rate of SpnG is greatly susceptible to the variation in sugar structure, but the active site of SpnG is remarkably flexible enough to accommodate the structural variants. It is possible that the variation could just disturb the correct positioning of C-1 of the sugar moiety for precise nucleophilic attack from the aglycone OH. The fact that dTDP-6-deoxy-L-talose, dTDP-D-mannose, and dTDP-D-galactose did not react with SpnG could simply be due to the severe shifting of the C-1 electrophile in position.

The correct positioning of the C-1 of the sugar moiety can be closely associated with the presence and stereochemistry of the hexose hydroxyl groups. For example, the absence of the C-2 OH in TDP-Oli paid the price of an ~5-fold decrease in $k_{cat}$ as compared with TDP-Rha, whereas an opposite stereocchemistry of the C-2 OH in dTDP-D-mannose (versus TDP-Glc) led to a total loss in catalytic activity. More examples confirmed that the change of the C-4 OH to a keto group in KDG suffered a severe drop in $k_{cat}$, an opposite stereocchemistry of the C-4 OH in TDP-6d-Tal (versus TDP-Rha) totally abolished SpnG activity, and an inverse configuration of the C-4 OH in dTDP-D-galactose (versus TDP-Glc) resulted in no measurable turnover.

Furthermore, UDP-Glc was tested as a sugar donor for SpnG to examine the substrate tolerance toward not only the sugar but also the nucleotide moiety. In our initial efforts, little Gtf activity was detected by RP-HPLC. However, the activity was later observed when SpnG of over 100 μM was used under a prolonged incubation time of over 12 h, thereby generating SGPG, as identified by co-elution with authentic SGPG. The enzymatic activity with UDP-Glc was not high enough to conduct the kinetic characterization for comparison with TDP-Glc. Nevertheless, this result suggested that SpnG displayed relatively strict specificity on the nucleotide portion of the NDP-sugar substrate, thereby resulting in a residual turnover number of $\sim 10^{-3} \text{ min}^{-1}$ for UDP-Glc.

**Inhibition of SpnG by dTDP**—To dissect the contribution of the NDP-sugar structure to molecular recognition adapted by SpnG, dTDP was examined as a potential inhibitor against TDP-Rha under the condition with saturating aglycone. As shown in the Lineweaver-Burk plot (Fig. 6), dTDP was characterized as a competitive inhibitor for TDP-Rha with an apparent $K_i$ of $0.97 \pm 0.16$ μM. In comparison with the $K_m$ of TDP-Rha (0.60 ± 0.01 μM) and that of TDP-Glc (1.21 ± 0.12 μM), dTDP showed no significant difference in binding affinity to SpnG. Importantly, the result may suggest that, in the dTDP-sugar structure, dTDP alone has a major contribution to molecular recognition by SpnG, and the sugar moiety may not contribute essentially to substrate binding, which is in excellent agreement with the substrate promiscuity displayed by SpnG earlier in this study.

**Bioinformatic Analysis of SpnG**—To resolve the relationship between SpnG and other Gtfs in the NCBI data base (available on the World Wide Web), sequence alignments and evolutionary comparisons were conducted in this study, and the result is shown in Fig. 7. Interestingly, the Gtfs tended to group together according to the type of the aglycone substrate to be glycosylated, as illustrated with the six groups shown in Fig. 7. All members of Group 4 utilize the same sugar substrate (L-noviose), whereas Group 6 harbors vancomycin group Gtfs (Scheme S1) (23–25). Strikingly, SpnG displayed unique overall sequence identity and only grouped together with angucycline.
Substrate Specificity of Spinosyn Rhamnosyltransferase

FIGURE 7. Phylogenetic analysis of secondary metabolite glycosyltransferases. The phylogenetic analysis illustrates the evolutionary relationship among the Gtfs mostly involved in the biosyntheses of various secondary metabolites. Gtfs shown in the analysis are as follows (accession number in parentheses): CalG1 (AA70336), AknK (AA707120), DnrH (AAD4714), AknS (AAF73455), SnogE (AAF01809), DnrS (AAD15267), EryBV (AAD15267), NbmD (AA88353), TyMII (CAAS7472), EryCII (CAAS7410), MegDI (CACC3807), OligS (CAAO05641), NanS (AAP42861), Vinc (BAD08357), SpnP (AA23277), C1027-ORF19 (AAO06670), UrdGT1a (AA00214), LanG1 (AA013562), AviGT1 (AAK83182), MtmGII (AA55583), SnogZ (CAB59003), CmmGII (CAE17533), CmmGIV (CAE17547), MtmGII (AACEG298), AmpdDI (AAK35111), NysDI (AAF71773), SpsA (P39261), KyUGFT1 (BAB41017), UGT73K1 (AAAS5901), GtA (BAC56174), AveBI (BAA45092), CalG2 (AA70348), TyICV (AACE4824), Bgtfc (CAAS7565), GtD (AKS13352), GtB (AABB2923), GtE (AABB3133), GtA (AA489929), MurG (P17443), GtG (AA69578), LanG1 (AABB3135), CalG3 (AAM70351), ElmgT (CACC1613), SnogD (AAB0111), JadS (AABB4152), ColM (AABB65229), Vinc (AAB29785), NovM (AACE7506), CmmGII (CAE17548), MtmGII (AACE4927), UrdGT1b (AA00215), LanG3 (AAB13559), UrdGTc1 (AA00217), and SpnG (AACEG298). Most of the 54 Gtfs belong to the GT-B structural fold and inverting catalytic mechanism, with the exception of SpsA (GT-2, KyUGFT1 (GT-1), UGT73K1 (GT-1), GtA (GT-1), and MurG (GT-26) (marked with asterisks). In brackets are indicated the group numbers of closely related members in evolution. The type of aglycone for each group is as follows: type-II PK/anthracycline (1), PK-I macrolide (2), aureolic acid-type PK (3), aminocoumarin (4), angucycline (except SpnG) (5), and glycopeptide (6).

Gtfs (Group 5) instead of general PK-I macrolide Gtfs of Group 2. SpnG was found to be relatively far from most Gtfs studied so far, such as glycopeptide Gtfs GtFA (29.3% sequence similarity, 18.1% sequence identity), GtFB (29.8% similarity, 19.1% identity), and GtFD (28.6% similarity, 18.9% identity) (Fig. 7). This finding urged us to conduct a bioinformatic comparison of SpnG with NCBI Gtfs to see if they retain a common sequence domain. Hence, representative Gtfs from Fig. 7 were further categorized by sequence alignment according to the metabolite classification and the type of the sugar acceptor to be transferred (Fig. 8). However, the analysis revealed that SpnG retained the αβα motif previously proposed to participate in the Gtf catalysis and NDP-sugar binding (23–26), as illustrated by our homology modeling in Fig. 9 and secondary structure prediction in Fig. S2.

Of many DNA sequences proposed to code for antibiotic Gtfs in NCBI, spnG thus represents one with a unique sequence feature, possibly dictating a special structure-activity relationship in molecular enzymology and catalysis of antibiotic Gtfs. Thus far, only relatively few Gtfs have been characterized in vitro and at the mechanistic level, considering the vast number of Gtf sequences revealed in the data base (9, 23–25, 27–34). Especially, SpnG is involved in the biosynthetic construction of unique multicyclic or highly modified PK-I glycoside (4, 5). Unlike general PK-I macrolides (eg. erythromycin), spinosyn shows much more structural rigidity. Hence, discrepancy of aglycone binding residues in the enzyme active site would be expected between SpnG and general PK-I Gtfs.

Site-directed Mutagenesis and Kinetic Characterization of SpnG.—By sequence alignment of SpnG with GT-B Gtfs (Fig. 8 and Fig. S2), some candidates, including Thr-297, Thr-300, and Asp-319, could be speculated to participate in dTDP-sugar binding in SpnG (25, 26). The Thr-300 equivalent in MurG, Glu-269, was previously reported to contact both 2’- and 3’-ribose hydroxyls of UDP to discriminate against dTDP (26). Hence, SpnG was engineered by replacing Thr-300 with Asp and Val to examine its functional role and, if possible, to accept UDP-sugar. Consequently, the T300V mutant SpnG displayed not only no detectable activity for UDP-Glc but also no remaining activity for the natural sugar donor, TDP-Rha. On the other hand, no activity was observed by RP-HPLC for SpnG T300D when either TDP-Rha or UDP-Glc (even at 20 mM) was used. Notably, structural disruption was observed by CD spectrometry in SpnG T300D, which may result from the hydrophilic or anionic feature of the aspartate. In addition, Thr-297 positioned in the α-helix of the αβα motif was postulated to interact with the α-phosphate via the side chain hydroxyl. To probe its role, the T297A mutant was also constructed. As a consequence, T297A mutation completely abolished the Gtf activity of SpnG, suggesting that Thr-297 might play an essential role for substrate binding.

In the Gtf catalysis of inverting mechanism (21, 22), a general base has been proposed to abstract the hydroxyl proton of the sugar acceptor to initiate nucleophilic attack upon the C-1 of the pyranose ring. As shown in Fig. S2, two amino acids (eg. Asp-13 and Asp-322 in GtfB) have been previously proposed as the candidate to act as the base (23, 25, 34). In GtFD and MurG, the Asp-13 equivalent lied in the G(T/S)RGD motif and was suggested to be the potential catalytic base (25). Our replacement of the Asp-13 equivalent with Ala, in SpnG H13A, did lead to a complete loss of SpnG activity. However, this prop-
Substrate Specificity of Spinosyn Rhamnosyltransferase

In S. spinosa, the 4,6-dehydratase activity of Gdh was shown here to be much lower (137- and 270-fold) than those of RmlB (Salmonella enterica) and RffG (E. coli), respectively, in catalytic efficiency (35, 36). This is an interesting observation presumably associated with the fact that, unlike Gdh, RmlB and RffG mainly mediate the primary metabolic pathway for principle survival of the microorganisms. On the other hand, the Y-X_3-K signature motif characteristic of the short chain dehydrogenase/reductase family was proposed to take part in the active site, not only in the dehydratase (19) but also in other known short chain dehydrogenase/reductase enzymes (37–39).

In Gdh, Tyr-151, Lys-154, and Thr-127 were implicated to be the catalytic triad activating the carbonyl or hydroxyl of the substrates by protonation or deprotonation, respectively. In this study, Y151F mutation caused an essentially lethal cutback (>170,000-fold) in catalytic activity of Gdh. Previous replacement of the Tyr equivalent with Phe, however, led to only 190-fold reduction in k_cat for RffG (36) and an ~2,600-fold decrease in k_cat for GMD (40). Our mutagenesis result may suggest that Tyr-151 in Gdh plays a more aggressive role in acting as a general acid/base during chemical catalysis, possibly due to unique characteristics of Gdh protein structure. Interestingly, in Gdh, Asp-128 and Glu-129 were also speculated to work together as a team of general acid and base, respectively, to facilitate the dehydration mechanism. Our mutagenesis finding that Gdh D128E displayed only a modest reduction in k_cat, as expected, could very well be attributed to the residual ability held by glutamate to protonate the hydroxyl at C-6. On the other hand, the E129A mutant was found to be completely inactive, reasonably resulting from an abolishment of its ability to abstract the proton at C-5 of the glucose moiety. Moreover, the catalytic roles of the Asp and Glu were similarly observed in RffG, whose mutants suffered from a >200-fold drop in k_cat and a slight increase in K_m (41). Based on the above results, the mutagenesis study has allowed us to characterize the intrinsic difference of Gdh, particularly involved in both pri-

![Image](73x313 to 385x461)

**FIGURE 8.** Sequence alignment of the αβα motif in secondary metabolite glycosyltransferases. The following glycosyltransferases (accession number in parentheses) were subjected to sequence alignment: SpnG (AAH23258), GtfB (AAB49293), GtfD (AAM31352), EryCIII (AAM31352), and SpnG (AAH23257). The conserved αβα motif is proposed to be involved in the binding of NDP-sugar and exhibits similar folding in these five glycosyltransferases. In the motif, the α helix and β sheet are shown in red and green, respectively. In MurG, UDP-GlcNAc is shown in blue, and side chains interacting with UDP-GlcNAc are shown in red. In SpnG, the potential residues of SpnG proposed for interaction with TDP-Rha are shown in blue.

![Image](97x102 to 517x214)

**FIGURE 9.** Ribbon representation of αβα motif in MurG, GtfA, GtfB, GtfD, and SpnG. The αβα motif structures of MurG, GtfA, GtfB, and GtfD are shown as reported (23–26), whereas that of SpnG was generated in this study by SWISS-MODEL with GtfB as a template (43) (available on the World Wide Web). The conserved αβα motif is proposed to be involved in the binding of NDP-sugar and exhibits similar folding in these five glycosyltransferases. In the motif, the α helix and β sheet are shown in red and green, respectively. In MurG, UDP-GlcNAc is shown in blue, and side chains interacting with UDP-GlcNAc are shown in red. In SpnG, the potential residues of SpnG proposed for interaction with TDP-Rha are shown in blue.
Substrate Specificity of Spinosyn Rhamnosyltransferase

mary and secondary metabolism, from other regular dehydratases.

Enzymatic characterization of Gtfs and their applications in combinatorial biosynthesis can be greatly benefited by an in situ, direct supply of NDP-sugar substrates to avoid tedious or impossible isolation of enzymatic products involved in in vitro NDP-sugar biosynthesis. By in situ coupling of Epi with Kre, KDG (3) in the reaction mixture of Gdh was completely converted to TDP-Rha in this study. Such a coupled system enhanced the efficiency of the TDP-Rha biosynthesis by direct reduction of the extremely unstable product (dTDP-6-deoxy-1-xilo-4-hexulose) of Epi with Kre. Interestingly, our investigation revealed that, in the coupled system, various Epi/Kre ratios in enzyme concentration resulted in no decomposition of dTDP-6-deoxy-1-xilo-4-hexulose, even at the highest ratio of 18:1. This observation was in good agreement with the previous postulation made by Messner et al. (37) that the epimerase and reductase might form an active complex and dTDP-6-deoxy-1-xilo-4-hexulose could remain enzyme-bound before its immediate conversion by the reductase.

To date, relatively few antibiotic Gtfs have been in vitro characterized to display substrate promiscuity for NDP-sugars or aglycone substrates, mostly due to the obstacles to gaining the soluble and active form of Gtf enzymes by heterologous expression and the serious limitation in availability of their natural or unnatural substrates (42). Notably, this work was made possible because of efficient heterologous expression and purification of the four enzymes from E. coli as well as availability of the aglycone substrate. Most importantly, successful in vitro reconstitution of TDP-Rha and SRPG, as well as other NDP-sugars and pseudoaglycones, by tandem reactions of various biosynthetic enzymes allowed us to characterize the catalytic efficiency and permissiveness of SpnG toward various NDP-sugar substrates, as summarized in Table 2.

Among 91 Gtf families reported thus far, SpnG was classified into the GT-1 family in CAZY according to stereochemistry and amino acid sequence similarity. The attachment of l-Rha and d-Glc onto aglycone reveals the β- and α-linkages, respectively, correlating with the inversion of the anomeric configuration by a single displacement mechanism of GT-1 Gtfs. For kinetic characterization, SpnG displayed a robust activity, with the presumed substrates, TDP-Rha and SAG, yielding SRPG as expected. This is the first in vitro evidence for the rhamnosyltransferase activity demonstrated in antibiotic Gtfs as well as in spinosyn (multicyclic macroide) biosynthesis. The optimal transferase activity of SpnG resided at the high pH, characteristic of Gtfs, as also found in vancomycin and pikromycin biosyntheses (24, 31).

It should be noted that SpnG here represented one of very few examples that the substrate specificity of the Gtf was thoroughly characterized by in vitro and in situ multienzyme systems. As demonstrated in this study by the dTDP inhibition experiment, TDP alone held the major contribution to the binding capability of SpnG to TDP-sugars, and the active site cavity binding the sugar moiety was found to be permissive enough to accommodate the sugar variants. However, our results (Table 2) from the probing of SpnG with various TDP-sugars suggested that the sugar moiety of TDP-sugar still controls the proper three-dimensional fitting of the entire molecule to the active site contour of SpnG, thereby affecting $k_{cat}$ by monitoring the correct positioning of the C-1 for the efficient nucleophilic attack from the aglycone or $K_m$ by tuning the actual interaction of TDP-sugars with the active site amino acids. For example, SpnG exhibited substrate promiscuity to utilize TDP-Glc as an alternative sugar donor and generate SGPG, where the substitution of the hydroxyl at C-6 and the overall configuration change from l - to d-sugar paid the price on the $k_{cat}$ reduced by ~18-fold, and the $K_m$ increased by 2-fold. TDP-Glc differs from TDP-Rha (the natural substrate) not only in the substituent and stereochemistry at C-3, C-4, and C-5 but also in the overall configuration and conformation of the sugar moiety. In addition to TDP-Glc, SpnG was also capable of transferring TDP-oli, KDG, dTDP-xylene, and TDP-6dGlc onto SAG. The striking relaxed specificity exhibited by SpnG to tolerate such dramatic changes, however, was accompanied by observed variations in kinetic parameters, further confirming the proposed role of each structural counterpart of the NDP-sugar in molecular recognition of SpnG.

Interestingly, SpnG was also found to catalyze d-glucosyl transfer from UDP-Glc, albeit with >60,000-fold reduction in $k_{cat}$ as compared with that of TDP-Glc. This result is, again, in good agreement with the concluded role of NDP on SpnG. In contrast, previous studies reported that MurG accepted only an UDP-sugar, and vancomycin group Gtfs utilized both UDP- and TDP-sugars (26, 40). For the discrimination between UDP-sugar and TDP-sugar, a Glu residue was observed in MurG to hydrogen-bond to the ribose 2'- and 3'-hydroxyls, whereas in vancomycin group Gtfs a replacement with Val or Leu was found (25), forming no hydrogen bond. SpnG, on the other hand, possessed a Thr at the corresponding position. The attempt to improve the binding capability of SpnG toward UDP, by replacing Thr-300 with an acidic Asp or a hydrophobic Val, consequently led to a complete loss of Gtf activity for both TDP-Rha and TDP-Glc. Presumably, the additional methyl group at the C-5" of the nucleotide moiety in TDP might also be a determinant factor for the substrate recognition of TDP-sugar by SpnG.

In the chemical catalysis of GT-1 antibiotic and GT-28 family Gtfs, two candidates were proposed as the general base to activate the nucleophilic substitution, one in the N-terminal domain and the other in the C-terminal domain (Fig. S2). Among vancomycin group Gtfs, the G(T/S)RGD motif in the N-terminal domain harbors the possible base, Asp (25), however, the catalytic role of the Asp as the base was found questionable in GtfB, because the D13A mutation in GtfB resulted in 2-fold reduction, suggesting its catalytic involvement (26). However, such Asp or His is not conserved and replaced with Thr in the case of UrdGT2 for urdamycin C-glycosylation, where the base was proposed elsewhere (33). On the contrary, the other potential catalytic base lies in the conserved DQ region of the αβα motif in the C terminus region. Unlike the Asp/His residue close to the N terminus, this DQ Asp is very well conserved among the antibiotic and distantly related Gtfs of the GT-1
family (Fig. 8) (23). The Asp can sometimes be substituted with Glu in a few cases, such as N-Gtfs in indolocarbazole glycosylation. Most importantly, a sufficient reduction of the Gtf activity was evidenced in GtfB D332A mutant, suggesting the DQ Asp to be the catalytic base (23). However, in SpnG both His-13 and Asp-316 were found to be critical in activity, since both of their mutations turned out to be lethal. As compared with other Gtfs, this observation is peculiar and may reflect the unique feature of SpnG in sequence and structural fold.

CONCLUSION

SpnG catalyzes the key O-glycosylation controlling the biosynthetic maturation of spinosyn. In light of its special sequence feature and aglycone substrate, SpnG represents a precious enzyme to uncover the mysterious relationship between protein sequence and aglycone substrate. SpnG illustrates the possibility of SpnG or its engineered form serving as a useful tool for combinatorial biosynthesis. Structural characterization of SpnG is currently under way by x-ray protein crystallography.

Acknowledgment—We thank HSP Research Institute, Japan, for providing the PG-KJE7 plasmid as a gift.

REFERENCES

1. Lopez, O., Fernandez-Bolanos, J. G., and Gil, M. V. (2005) Green Chem. 7, 431–442
2. Kirst, H. A., Creemer, L. C., Naylor, S. A., Pugh, P. T., Snyder, D. E., Winkle, J. R., Lowe, L. B., Rothwell, J. T., Sparks, T. C., and Worden, T. V. (2002) Curr. Top. Med. Chem. 2, 675–699
3. Alex, T., Brian, D. G., Mervyn, B., and Dylan, M. (2005) in World (PCT) patent WO 2005/049979 A2
4. Waldron, C., Matsuhashi, P., Rostock, P. R., Broughton, M. C., Turner, J., Madduri, K., Crawford, K. P., Merlo, D. J., and Balf, R. H. (2001) Chem. Biol. 8, 487–499
5. Martin, C. J., Timoney, M. C., Sheridan, R. M., Kendrew, S. G., Wilkinson, B., Staunton, J., and Leadlay, P. F. (2003) Org Biomol. Chem. 1, 1444–1447
6. Madduri, K., Waldron, C., and Merlo, D. J. (2001) J. Bacteriol. 183, 5632–5638
7. Madduri, K., Waldron, C., Matsuhashi, P., Broughton, M. C., Crawford, K., Merlo, D. J., and Balf, R. H. (2001) J. Ind. Microbiol. Biotechnol. 27, 399–407
8. Balf, R. H. (2006) Nat. Biotechnol. 24, 1533–1540
9. Bode, H. B., and Muller, R. (2007) Angew. Chem. Int. Ed. Engl. 46, 2147–2150
10. Kiser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) in Practical Streptomyces Genetics, pp. 117–118, John Innes Foundation, Norwich, UK
11. Sambrook, J., and Russell, D. W. (2001) in Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Nishihara, K., Kanemori, M., Kitagawa, M., Yanagi, H., and Yura, T. (1998) Appl. Environ. Microbiol. 64, 1694–1699
13. Lovley, D. R., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
14. Gross, J. W., Hegeman, A. D., Vestling, M. M., and Frey, P. A. (2000) Biochemistry 39, 13633–13640
15. Creemer, L. C., Kirst, H. A., and Paschal, J. W. (1998) J. Antibiot. 51, 795–800
16. Naundorf, A., and Klaflke, W. (1996) Carbohydr. Res. 285, 141–150
17. Thibodeaux, C. J., Melancon, C. E., and Liu, H. W. (2007) Nature 446, 1008–1016
18. Allard, S. T. M., Cleeland, W. C., and Holdem, H. M. (2004) J. Biol. Chem. 279, 2211–2220
19. Jornvall, H., Persson, B., Krook, M., Attian, S., Gonzalezduarte, R., Jeffery, J., and Ghosh, D. (1995) Biochemistry 34, 6003–6013
20. Allard, S. T. M., Giraud, M. F., Whitfield, C., Granger, M., Messner, P., and Naismith, J. H. (2001) J. Mol. Biol. 307, 283–295
21. Coutinho, P. M., Deleury, E., Davies, G. J., and Henriassit, B. (2003) J. Mol. Biol. 328, 307–317
22. Lairson, L. L., Henriassit, B., and Withers, S. G. (2008) Annu. Rev. Biochem. 77, 521–555
23. Mulichak, A. M., Losey, H. C., Walsh, C. T., and Garavito, R. M. (2001) Structure 9, 547–557
24. Mulichak, A. M., Losey, H. C., Lu, W., Wawrzak, Z., Walsh, C. T., and Garavito, R. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9238–9243
25. Mulichak, A. M., Lu, W., Losey, H. C., Walsh, C. T., and Garavito, R. M. (2004) Biochemistry 43, 5170–5180
26. Wu, B. Y., Zhang, Y. X., and Wang, P. G. (2001) J. Mol. Biol. 313, 553–563
27. Minami, A., and Eguchi, T. (2007) J. Am. Chem. Soc. 129, 5102–5107
28. Zhang, C. S., Griffith, B. R., Fu, Q., Albermann, C., Fu, X., Lee, I. K., Li, L. J., and Thrors, J. S. (2006) Science 313, 1291–1294
29. Yuan, Y. Q., Chung, H. S., Leimkuhler, C., Walsh, C. T., and Worden, T. V. (2005) J. Am. Chem. Soc. 127, 14128–14129
30. Borisova, S. A., Zhao, L. S., Melancon, C. E., Kao, C. L., and Liu, H. W. (2004) J. Am. Chem. Soc. 126, 6534–6535
31. Meyers, C. L. F., Oberton, M., Heide, L., Kaneh, D., and Walsh, C. T. (2004) Biochemistry 43, 15022–15036
32. Mittler, M., Bechthold, A., and Schulz, G. E. (2007) J. Mol. Biol. 372, 67–76
33. Bolam, D. N., Roberts, S., Proctor, M. R., Turkenburg, J. P., Dodson, E. J., Martinez-Fleites, C., Yang, M., Davis, B. G., Davies, G. J., and Gilbert, H. J. (2007) Proc. Natl. Acad. Sci. U. S. A. 104, 845–849
34. Minami, A., and Eguchi, T. (2007) J. Am. Chem. Soc. 129, 5102–5107
35. Zhang, C. S., Griffith, B. R., Fu, Q., Albermann, C., Fu, X., Lee, I. K., Li, L. J., and Thorsen, J. S. (2006) Science 313, 1291–1294
36. Wright, J. S., Melancon, C. E., Walsh, C. T., and Worden, T. V. (2005) J. Mol. Biol. 127, 527–534
37. Yuan, Y. Q., Chung, H. S., Leimkuhler, C., Walsh, C. T., and Worden, T. V. (2005) J. Am. Chem. Soc. 127, 14128–14129
38. Sambrook, J., and Russell, D. W. (2001) in Molecular Cloning: A Laboratory Manual, Norwich, UK
39. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
40. Walsh, C. T. (2006) Nature 443, 285–286
41. Kopp, J., and Schwede, T. (2004) Nucleic Acids Res. 32, D230–D234