AknK Is an L-2-Deoxyfucosyltransferase in the Biosynthesis of the Anthracycline Aclacinomycin A†

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ABSTRACT: The antitumor drug aclacinomycin A is a representative member of the anthracycline subgroup that contains a C_7-O-trisaccharide chain composed of L-2-deoxysugars. The sugar portion of the molecule, which greatly affects its biological activity, is assembled by dedicated glycosyltransferases; however, these enzymes have not been well-studied. Here we report the heterologous expression and purification of one of these enzymes, AknK, as well as the preparation of dTDP-L-2-deoxysugar donors, dTDP-L-2-deoxyfucose and dTDP-L-daunosamine, and the monoglycosyl aglycone, rhodosaminyl aklavinone. Our experiments show that AknK catalyzes the addition of the second sugar to the chain, using dTDP-L-2-deoxyfucose and rhodosaminyl aklavinone, to create the L-2-deoxyfucosyl-L-rhodosaminyl aklavinone. AknK also accepts an alternate dTDP-L-sugar, dTDP-L-daunosamine, and other monoglycosylated anthracyclines, including daunomycin, adriamycin, and idarubicin, to build alternate disaccharides on variant anthracycline backbones. Remarkably, AknK also catalyzes a tandem addition of a second L-2-deoxyfucosyl moiety, albeit with reduced activity, to the natural disaccharide chain to produce L-deoxyfucosyl-L-deoxyfucosyl-L-rhodosaminyl aklavinone, a variant of the natural aclacinomycin A. These results demonstrate that AknK may be a useful enzyme for the chemoenzymatic synthesis of anthracycline variants.

Anthracyclines make up a class of microbial natural products with potent antitumor activity. Among them, daunomycin (daunorubicin) **1** and adriamycin (doxorubicin) **2** (Figure 1), produced by *Streptomyces peucetius*, are clinically used in United States to treat cancer (*1*). Aclacinomycin A (aclarubicin) **3**, produced by *Streptomyces galilaeus*, is used in Japan and France for the treatment of acute leukemias and non-Hodgkin's lymphomas (*2*, *3*). These anthracyclines achieve their antitumor activities by intercalating DNA duplexes and stabilizing topoisomerase II-mediated double-stranded DNA breaks. The accumulation of double-stranded DNA fragments triggers the programmed cell death of cancer cells as well as normal proliferating cells (*4*).

Anthracycline compounds are composed of a conserved tetracyclic aglycone, 7,8,9,10-tetrahydro-5,12-naphthacenequinone, and diversified L-deoxysugar moieties. The tetracyclic aglycones are assembled by type II polyketide synthases (PKSs) through nine cycles of malonyl-CoA addition, cyclization, and oxidative transformations. The assembled aglycones are subsequently glycosylated at the C_7-OH position by dedicated anthracycline glycosyltransferases in the later stages of anthracycline biosynthesis (*1*, *2*). In the biosynthesis of daunomycin **1** and adriamycin **2**, the glycosyltransferase (Gtf), DnrS, transfers L-daunosamine, a 2,3,6-trideoxy-3-amino-L-hexose, to the aglycone scaffold (*5*). In contrast, aclacinomycin A **3** is representative of an anthracycline subfamily with a trisaccharide chain, consisting of L-rhodosamine, L-2-deoxyfucose, and L-cinerulose A. The terminal sugar initially added to the disaccharyl anthracycline is believed to be L-rhodinose, which is then converted to L-cinerulose, a 2,3,6-trideoxy-4-keto-L-hexose. Interestingly, only two Gtf genes, *AknS* and *AknK*, have been identified so far in the biosynthetic gene cluster of the aclacinomycin A-producing strain (*3*, *6*), raising the possibility that one of the Gtfs may catalyze the addition of two sugar moieties during aclacinomycin A maturation.

Kinetic analysis and crystallographic characterization of anthracycline−DNA complexes indicate that the sugar moiety appended at the C_7-OH position serves as a minor groove binder and is critical for the interaction between DNA and anthracyclines (*7*−*10*). Therefore, many efforts have been made to chemically synthesize new anthracycline-like antitumor compounds by changing the sugar moiety appended to the aglycone (*11*−*15*). Among them, the monosaccharide-containing idarubicin **4** and epirubicin **5** are in clinical use, and the disaccharide-containing MEN10755 **6** is in clinical trials (Figure 1) (*16*−*18*). However, the synthesis of these compounds, especially the disaccharide-containing derivatives, is not a trivial operation (*11*, *14*, *15*). Since the post-assembly line decorations of anthracycline aglycones are carried out by anthracycline Gtfs, it may become feasible to utilize these enzymes to prepare novel anthracycline derivatives containing various sugar modifications. Like many natural product Gtfs, the biochemical

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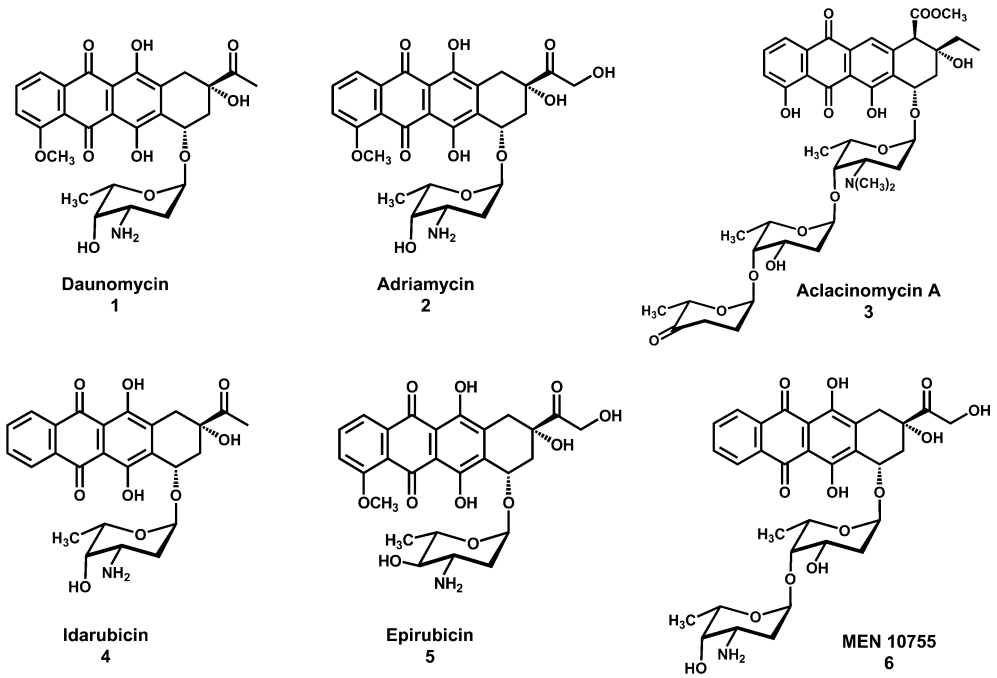


FIGURE 1: Anthracycline antitumor agents.

information about these anthracycline glycosyltransferases is scarce because of the tripartite difficulty in obtaining purified Gtfs, the acceptors, and the NDP-deoxysugar donors.

In this paper, we report the cloning, heterologous expression, and purification of AknK, a putative Gtf from the aclacinomycin A operon (*3*). We also report the synthesis of dTDP-L-daunosamine **10** and dTDP-L-2-deoxyfucose **14** and demonstrate that they are good substrates for AknK in the glycosylation of several monoglycosylanthracyclines. Furthermore, we find that AknK catalyzes a tandem glycosylation reaction, the double addition of L-2-deoxyfucose. These results lay the groundwork for the enzymatic generation of anthracycline analogues using anthracycline Gtfs.

MATERIALS AND METHODS

Materials

The pET-22b *Escherichia coli* expression vector was purchased from Novagen. *E. coli* Top10 and BL21(DE3) competent cells were purchased from Invitrogen. Primers were ordered from Intergrated DNA Technology. *Pfu* DNA polymerase was purchased from Stratagene. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. The Ni−NTA resin was purchased from Qiagen, and the Econo column was purchased from Bio-Rad. Daunomycin, doxorubicin, aclacinomycin A, and idarubicin were purchased from Sigma. Epirubicin was purchased from Calbiochem.

Plasmid Construction

The *AknK* gene was PCR[1] amplified from plasmid pSgc15.1, a gift from S. Torkkell and colleagues (*3*), using the following primer pair: 5′-AAAAAAGAATTCATGAAGGTCCTGTTCAACACG-3′ and 5′-AAAAAAAAGCTTCTGCTCCCCTCGGACGGTGCC-3′. The restriction sites are underlined. The PCR product was subcloned into the pET-22b vector using *Eco*RI and *Hin*dIII sites. The *AknK* gene sequence of the resulting plasmid, pET-22b-AknK-His_6, was confirmed before it was transformed into BL21(DE3) competent cells.

Heterologous Expression and Purification of AknK

The *E. coli* BL21(DE3) transformants were inoculated in 10 mL of LB medium containing 100 μg/mL ampicillin, and were grown at 37 °C overnight. The overnight culture was added to 1 L of LB medium supplemented with 100 μg/mL ampicillin. The cell growth was maintained at 37 °C until the OD_{600} reached 0.6, at which point it was induced with 0.3 mM IPTG at 15 °C for an additional 20 h. The cells were harvested by centrifugation, and the cell pellets were resuspended in buffer A [25 mM Tris (pH 8.0), 5 mM imidazole, and 500 mM NaCl]. The cells were lysed by being passed once through a French cell press at 15 000 psi. After centrifugation at 14000*g* for 20 min, the cell lysates were mixed with 1 mL of pre-equilibrated Ni−NTA resin at 4 °C for 1 h before they were loaded into an Econo column at a flow rate of 1 mL/min. The column was washed with buffer A followed by buffer A with 30 mM imidazole. The proteins were eluted off the column with buffer A supplemented with 200 mM imidazole. The desired fractions as determined by 12% SDS−PAGE were combined, transferred to a Slide-A-Lyzer dialysis cassette (10 kDa molecular weight cutoff), and dialyzed twice against 2 L of dialysis buffer [50 mM Tris (pH 8.0), 100 mM NaCl, 2 mM DTT, and 10% (v/v) glycerol]. The protein concentration was determined from its theoretical extinction coefficient at 280 nm (60 855 M^{-1}

[1] Abbreviations: PCR, polymerase chain reaction; LC−MS, liquid chromatography−mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; DMSO, dimethyl sulfoxide; Tris, tris(hydroxymethyl)aminomethane; dTDP, 2′-deoxythymidine 5′-diphosphate; IPTG, isopropyl L-thio-β-D-galactoside; DTT, dithiothreitol; BSA, bovine serum albumin; DMAP, (dimethylamino)pyridine; TMS-I, iodotrimethylsilane; DIPEA, diisopropylethylamine; Cbz, benzyloxycarbonyloxy; TOCSY, total correlation spectroscopy; ROESY, rotating frame Overhauser enhancement spectroscopy.

cm^{-1}). The protein samples were aliquoted as 100 μL fractions, flash-frozen in liquid nitrogen, and stored at −80 °C.

Reagents for Synthesis of dTDP-Deoxysugars

For the synthesis of dTDP-sugars, all chemicals were purchased from Aldrich or Sigma, unless otherwise noted, and used without further purification. Solvents were reagent-grade and were further dried when necessary. Analytical thin-layer chromatography was performed on glass plates pre-coated with silica gel (250 μm, Sorbent Technologies), with detection by UV and/or spraying with H_2SO_4 (50%). Flash chromatography was carried out on silica gel (60 Å, 32−63 μm), purchased from Sorbent Technologies. Analytical HPLC of reaction mixtures was performed on a Hewlett-Packard 1100 series instrument using a Phenomenx Luna 5 μm C18 column (250 mm × 4.6 mm). Compounds bearing a thymidine chromophore were monitored at an absorbance of 270 nm. Reactions were monitored by HPLC using gradient A (H_2O/0.1% NH_4HCO_3 to 100% MeOH/0.1% NH_4HCO_3 linear gradient over the course of 25 min). Preparative HPLC was performed on a Varian ProStar instrument using a Phenomenex Luna 10 μm C18 column (250 mm × 50 mm). NMR spectra were recorded on a Varian Inova 400 or 500 MHz spectrometer. Mass spectra (ESI) were recorded at the Mass Spectroscopy Facility at the Department of Chemistry, Princeton University.

Synthesis of dTDP-L-Daunosamine and dTDP-L-2-Deoxyfucose

*1,4-Di-O-acetyl-3-N-benzyloxycarbonyl-L-daunosamine **8***. To a solution of daunosamine hydrochloride (*19*, *20*) (**7**, 200 mg, 1.10 mmol) in MeOH (15 mL) were added Et_3N (0.23 mL, 1.65 mmol) and Cbz-succinimide (0.550 g, 2.20 mmol). The solution was stirred for 15 min and then evaporated to dryness. Purification of the residue by flash chromatography (1:1 petroleum ether/EtOAc) gave the *N*-Cbz derivative (297 mg, 96%); R_f = 0.86 (8:1 CH_2Cl_2/MeOH). This compound (297 mg, 1.06 mmol) was dissolved in pyridine (2 mL), and Ac_2O (0.35 mL) and DMAP (3 mg) were added to the solution. After the mixture had been stirred for 4 h, the reaction was quenched by adding MeOH and evaporated to dryness. The residue was dissolved in CH_2Cl_2 and washed with 1 N HCl (twice), H_2O, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried ($MgSO_4$), filtered, and evaporated, and the oily residue was purified by flash chromatography (4:1 petroleum ether/EtOAc) to give **8** as a mixture of anomers (347 mg, 90%, 2:1 α/β): ^{1}H NMR (400 MHz, $CDCl_3$) for **8α** δ 7.35 (m, aromatic), 6.23 (s, 1-H), 5.12 (m, CH_2Ph), 4.79 (d, $J_{3,4}$ = 8.4 Hz, 4-H), 4.33 (m, 3-H), 4.19 (q, $J_{5,6}$ = 6.4 Hz, 5-H), 2.16 (s, $2COCH_3$), 2.04−1.89 (m, 2-H_2), 1.12 (d, $J_{5,6}$ = 6.4 Hz, 6-H_3); ^{1}H NMR (400 MHz, $CDCl_3$) for **8*β*** δ 7.35 (m, aromatic), 5.77 (d, $J_{1,2}$ = 9.2 Hz, 1-H), 5.12 (m, CH_2Ph), 4.84 (d, $J_{3,4}$ = 7.7 Hz, 4-H), 4.08 (m, 3-H), 3.83 (q, $J_{5,6}$ = 6.7 Hz, 5-H), 2.07, 2.04 (2 s, $2COCH_3$) 2.02−1.77 (m, 2-H_2), 1.18 (d, $J_{5,6}$ = 6.7 Hz, 6-H_3); LRMS (ESI) for $C_{18}H_{23}NO_7$ (365.15) 365 ($[M]^+$).

*Thymidine 5′-(4-O-Acetyl-3-N-benzyloxycarbonyl-α,β-L-daunosaminyl Diphosphate) **9***. Lactol (**8**, 100 mg, 0.27 mmol) was azeotroped with toluene (twice), dissolved in benzene (2.5 mL), and placed under argon. TMS-I (42 μL, 0.297 mmol) was added dropwise to the solution. After being stirred for 10 min, the reaction mixture was lyophilized. TDP tetrabutylammonium salt (*21*) (0.240 g, 0.27 mmol) was dissolved in a mixture of benzene (1.5 mL) and CH_2Cl_2 (2 mL). DIPEA (47 μL, 0.27 mmol) was added, and this solution was added to the iodide at 10 °C. The solution was allowed to stir for 3 h while warming to room temperature. The progress of the reaction was monitored by analytical HPLC [gradient A, t_R(TDP) = 2.4 min, t_R(**9α**) = 12.70 min, and t_R(**9*β***) = 12.75 min]. The reaction was quenched with 0.1 mL of DIPEA and 0.1 mL of MeOH, and the mixture was concentrated and purified by preparative HPLC. Fractions containing predominantly the β-isomer were pooled and evaporated to give the ammonium salt of **9** (40 mg, 21%, 1:2 α/β) as a white powder: ^{1}H NMR (400 MHz, D_2O) δ 7.71 (s, β-6-H), 7.68 (s, α-6-H), 7.33−7.27 (m, aromatic), 6.26−6.20 (m, β-1′-H, α-1′-H), 5.68 (d, $J_{1,2}$ = 7.1 Hz, α-1″-H), 5.26 (t, J = 8.2 Hz, β-1″-H), 5.07−4.94 (m, α,β-4″-H, CH_2Ph), 4.54−4.48 (m, α,β-3′-H), 4.35 (q, $J_{5,6}$ = 6.0 Hz, α-5″-H), 4.13−4.04 (m, α,β-4′-H, α,β-5′-H_2, α-3″-H), 3.90−3.84 (m, β-3″-H, β-5″-H), 2.27−2.19 (m, α,β-2′-H_2, α-2″-H_a), 2.10 (s, thymidine CH_3), 2.05−1.98 (m, α-2″-H_b, β-2″-H_a), 1.86 (s, α,β-$COCH_3$), 1.67 (m, β-2″-H_b), 1.08 (d, $J_{5,6}$ = 6.4 Hz, β-6″-H_3), 1.04 (d, $J_{5,6}$ = 6.4 Hz, α-6″-H_3); ^{31}P NMR (162 MHz, D_2O) δ −10.83, −12.82; LRMS (ESI) for $C_{26}H_{35}N_3O_{16}P_2$ (707.15) 706 ($[M - H]^-$).

*Thymidine 5′-(α,β-L-Daunosaminyl Diphosphate) **10***. The protected TDP-sugar (**9**, 10 mg, 14 μmol) was dissolved in an MeOH/H_2O/Et_3N mixture (2:2:1, 5 mL) and stirred for 16 h. The reaction mixture was concentrated and dissolved in MeOH (5 mL) and H_2O (5 mL). To this solution was added Pd/C (10 mg), and the mixture was hydrogenated (1 atm) for 1 h. The progress of the reaction was monitored by analytical HPLC [gradient A, t_R(**10*β***) = 6.20 min and t_R(**10α**) = 6.25 min]. The reaction mixture was filtered through a pad of Celite, concentrated, and purified by preparative HPLC. Fractions with retention times of 6.20 min were pooled to give the ammonium salt of **10*β*** (3 mg, 38%) as a white solid: ^{1}H NMR (400 MHz, D_2O) δ 7.69 (s, 1H, 6-H), 6.36 (t, J = 7.1 Hz, 1H, 1′-H) 5.20 (t, J = 7.8 Hz, 1H, 1″-H), 4.64 (m, 1H, 3′-H), 4.16 (m, 3H, 4′-H, 5′-H_2), 3.72 (q, $J_{5,6}$ = 6.4 Hz, 1H, 5″-H), 3.46 (d, $J_{4,3}$ = 2.4 Hz, 1H, 4″-H), 3.02 (m, 1H, 3″-H), 2.37−2.31 (m, 2H, 2′-H_2), 2.00 (m, 1H, 2″-H_a) 1.90 (s, 3H, thymidine CH_3), 1.58−1.50 (m, 1H, 2″-H_b), 1.21 (d, $J_{5,6}$ = 6.41 Hz, 3H, 6″-H_3); ^{31}P NMR (162 MHz, D_2O) δ −10.62, −12.95; LRMS (ESI) for $C_{16}H_{27}N_3O_{13}P_2$ (531.10) 530 ($[M - H]^-$).

*3,4-Di-O-acetyl-2-deoxy-β-L-fucospyranosyl Phosphate **12***. 3,4-Di-*O*-acetyl-L-fucal (**11**, 0.100 g, 0.47 mmol) (*22*, *23*) was azeotroped with benzene (twice) and dissolved in benzene (2 mL), and HCl(g) was bubbled through the solution for 15 min. After removal of the solvent, the residue was azeotroped with benzene to remove excess HCl (*24*). The oily residue was dissolved in CH_2Cl_2 (1 mL), and the pH was brought to 9 by dropwise addition of DIPEA. This solution was cooled to 0 °C, and a solution of $Bu_4NH_2PO_4$ (0.319 g, 0.94 mmol) and DIPEA (0.164 mL, 0.94 mmol) in CH_2Cl_2 (2 mL) was added dropwise. The reaction mixture was allowed to stir for 2 h while warming to room temperature, at which point it was concentrated and purified by reversed phase HPLC (H_2O/0.1% NH_4HCO_3 mixture for 5 min and then a linear gradient to a 100% MeOH/0.1%

NH_4HCO_3 mixture over the course of 50 min, at a flow rate of 45 mL/min). Evaporation of the product-containing fractions (R_f = 0.90, 1:1 H_2O/MeOH, C_{18} TLC) gave **12** as a white powder (30 mg, 22%): ^{1}H NMR (400 MHz, CD_3OD) δ 5.21 (m, $J_{1,P}$ = 8.1 Hz, 1H, 1-H), 5.06−5.02 (m, 2H, 3-H, 4-H), 3.82 (q, $J_{5,6}$ = 6.4 Hz, 1H, 5-H), 2.12 (s, 3H, $COCH_3$), 2.09−2.07 (m, 1H, 2-H_a), 1.95 (s, 3H, $COCH_3$), 1.90−1.82 (m, 1H, 2-H_b), 1.16 (d, $J_{5,6}$ = 6.2 Hz, 3H, 6-H_3); ^{31}P NMR (162 MHz, CD_3OD) δ −0.25 ($J_{1,P}$ = 8.2 Hz): LRMS (ESI) for $C_{10}H_{17}O_9P_2$ (312.16) 311 ([M − H]$^-$).

*Thymidine 5′-(3,4-Di-O-acetyl-2-deoxy-β-L-fucopyranosyl diphosphate) **13***. Phosphate (**12**, 40 mg, 0.09 mmol) and TMP morpholidate (96 mg, 0.14 mmol) were azeotroped with pyridine (thrice) and then dissolved in pyridine (0.5 mL). Tetrazole (22 mg, 0.28 mmol) was added to this solution, and the reaction mixture was stirred under argon for 2 days. The progress of the reaction was monitored by analytical HPLC [gradient A, t_R(TMP morpholidate) = 8.8 min and t_R(**13**) = 8.1 min]. After removal of the solvent, the residue was dissolved in 0.1% aqueous NH_4HCO_3 (7 mL) and extracted with diethyl ether (7 mL). The water layer was purified by preparative HPLC to give the ammonium salt of **13** (44 mg, 79%) as a white powder: ^{1}H NMR (400 MHz, D_2O) δ 7.75 (s, 1H, 6-H), 6.36 (m, 1H, 1′-H), 5.20 (t, $J_{1,2}$ = $J_{1,P}$ = 8.7 Hz, 1H, 1″-H), 5.08−5.04 (m, 2H, 3″-H, 4″-H), 4.58 (m, 1H, 3′-H), 4.15 (m, 2H, 4′-H, 5′-H_2), 3.72 (q, $J_{5,6}$ = 6.0 Hz, 1H, 5″-H), 2.37−2.31 (m, 1H, 2′-H_2), 2.17 (s, 3H, $COCH_3$), 2.03 (m, 1H, 2″-H_a) 1.97 (s, 3H, thymidine CH_3), 1.91 (s, 3H, $COCH_3$), 1.84 (m, 1H, 2″-H_b), 1.16 (d, $J_{5,6}$ = 6.4 Hz, 3H, 6″-H_3); ^{31}P NMR (162 MHz, D_2O) δ −10.8, −13.0; LRMS (ESI) for $C_{20}H_{30}N_2O_{16}P_2$ (616.33) 615 ([M − H]$^-$).

*Thymidine 5′-(2-Deoxy-β-L-fucopyranosyl Diphosphate) **14***. dTDP-sugar (**13**, 30 mg, 49 μmol) was dissolved in an MeOH/H_2O/Et_3N mixture (2:2:1, 5 mL). The solution was stirred for 16 h, evaporated to dryness, and purified by preparative HPLC, affording **14** (15 mg, 58%) as its ammonium salt (gradient A, t_R = 6.15 min): ^{1}H NMR (400 MHz, D_2O) δ 7.70 (s, 1H, 6-H), 6.36 (t, $J_{1,2a}$ = $J_{1,2b}$ = 6.8 Hz, 1H, 1′-H), 5.19 (t, $J_{1,2}$ = $J_{1,P}$ = 8.9 Hz, 1H, 1″-H), 4.61 (m, 1H, 3′-H), 4.16 (s, 3H, 4′-H, 5′-H_2), 3.87 (dt, $J_{3,4}$ = 4.2 Hz, $J_{3,2}$ = 12.6 Hz, 1H, 3″-H), 3.69 (q, $J_{5,6}$ = 6.4 Hz, 1H, 5″-H), 3.57 (bs, 1H, 4″-H), 2.40−2.33 (m, 1H, 2′-H_2), 2.10 (m, 1H, 2″-H_a), 1.91 (s, 3H, thymidine CH_3), 1.73−1.65 (m, 1H, 2″-H_b), 1.27 (d, $J_{5,6}$ = 6.41 Hz, 3H, 6″-H_3); ^{31}P NMR (162 MHz, D_2O) δ −10.78, −13.01; LRMS (ESI) for $C_{16}H_{26}N_2O_{14}P_2$ (531.03) 530 ([M − H]$^-$).

*Preparation of Rhodosaminyl Aklavinone **15***

Rhodosaminyl aklavinone **15** was prepared by limited acid-catalyzed hydrolysis of commercially available aclacinomycin A **3** with 0.1 M HCl in dry methanol at 25 °C for 30 min. Compound **15** was purified using a Beckman Gold Nouveau HPLC system with a Vydac semi-prep C_{18} column using a gradient from 0 to 100% acetonitrile in 0.1% trifluoroacetic acid (TFA) and H_2O over the course of 20 min at a flow rate of 3 mL/min. The product-containing fractions were combined and lyophilized. The mass of purified **15** ([M + H]$^+$ = 570.6) was confirmed by LC−MS, and the purity was greater than 95%.

Characterization of the First Glycosylation Reaction Catalyzed by AknK

dTDP-L-deoxysugars and the anthracycline monoglycosides were incubated with 500 nM to 5 μM AknK in 50 μL of reaction buffer [75 mM Tris (pH 7.5), 12 mM $MgCl_2$, 1 mg/mL BSA, and 20% (v/v) DMSO] at 25 °C for 1−5 min. An aliquot (25 μL) of the reaction mixture was quenched with 125 μL of methanol. The samples were centrifuged for 2 min at 13 000 rpm. The supernatants were subjected to HPLC analysis with a Vydac small pore C_{18} column, and the products were monitored at 435 nm. The molecular weights of the desired products were confirmed by LC−MS or MALDI-TOF. The peaks for the diglycosylated products and remaining monoglycosylated substrates were integrated, and the product concentration was deduced from its percentage of the total peak area.

*Preparation of 2-Deoxyfucosyl Rhodosaminyl Aklavinone **16** and Characterization of the Second Glycosylation Catalyzed by AknK*

Compound **16** was enzymatically synthesized with 10 μM AknK under the following conditions: 75 mM Tris (pH 7.5), 1 mM dTDP-L-2-deoxyfucose **14**, 2 mM rhodosaminyl aklavinone **15**, 12 mM $MgCl_2$, 1 mg/mL BSA, and 20% (v/v) DMSO. The reaction mixture was kept at 25 °C overnight. The desired product **16** was purified with a Vydac small pore C_{18} column {65:35 MeOH/H_2O [50 mM NH_4OAc (pH 7.0)], 1 mL/min}. The molecular weight of the purified product was confirmed by MALDI-TOF and NMR analysis: ^{1}H NMR ($CDCl_3$, 500 MHz) δ 12.70 (s, 1H, 4-OH), 12.04 (s, 1H, 6-OH), 7.85 (dd, $J_{1,3}$ = 1.1 Hz, $J_{1,2}$ = 7.4 Hz, 1H, 1-H), 7.71 (m, 2H, 2-H, 11-H), 7.32 (dd, $J_{1,3}$ = 1.2 Hz, $J_{2,3}$ = 8.4 Hz, 1H, 2-H), 5.5 (d, $J_{1′,2′}$ = 3.4 Hz, 1H, 1′-H), 5.3 (m, 1H, 7-H), 5.1 (d, $J_{1′,2′}$ = 3.1 Hz, 1H, 1″-H), 4.5 (q, $J_{5′,6′}$ = 6.4 Hz, Hz, 1H, 5″-H), 4.1 (m, 1H, 3″-H), 4.12 (s, 1H, 10-H), 4.02 (q, $J_{5′,6′}$ = 6.5 Hz, 1H, 5′-H), 3.77 (s, 1H, 4′-H), 3.70 (s, 3H, 15-H_3), 3.65 (s, 1H, 4″-H), 2.52 (dd, $J_{7,8}$ = 4.3 Hz, $J_{8a,\ 8b}$ = 15.0 Hz, 1H, 8-H_a), 2.36 (d, $J_{8a,8b}$ = 15.0 Hz, 1H, 8-H_b), 2.21 [s, 6H, $N(CH_3)_2$], 2.13 (m, 1H, 3′-H), 2.08 (m, 1H, 2″-H_a), 1.83 (m, 1H, 2′-H_a), 1.84 (dd, $J_{1′,2′}$ = 3.8 Hz, $J_{2a′,2b′}$ = 12.5 Hz, 1H, 2′-H_b), 1.83 (m, 1H, 2″-H_b), 1.75 (dt, $J_{13,14}$ = 7.4 Hz, $J_{13a,13b}$ = 14.0 Hz, 1H, 13-H_a), 1.53 (dt, $J_{13,14}$ = 7.3 Hz, $J_{13a,13b}$ = 14.0 Hz, 1H, 13-H_b), 1.29 (d, $J_{5′,6′}$ = 6.6 Hz, 3H, 6′-H_3), 1.22 (d, $J_{5′,6′}$ = 6.5 Hz, 3H, 6″-H_3), 1.09 (t, $J_{13,14}$ = 7.3 Hz, 3H, 14-H_3).

To determine the AknK activity for the second glycosylation reaction, 250−1000 μM dTDP-L-2-deoxyfucose **14** was mixed with 100 μM 2-deoxyfucosyl rhodosaminyl aklavinone **16** in 25 μL of reaction buffer [75 mM Tris (pH 7.5), 12 mM $MgCl_2$, 1 mg/mL BSA, and 20% (v/v) DMSO]. The reaction was initiated by adding 10 μM AknK, and the mixture was kept at 25 °C for 10−40 min before the reaction was quenched with 125 μL of methanol. The supernatant from the reaction was analyzed by reversed phase HPLC {66:34 MeOH/H_2O [50 mM NH_4OAc (pH 7.0)], 1 mL/min}. The molecular weight of the product was confirmed by MALDI-TOF. The peaks for the product **19** (t_R = 23.4 min) and the remaining **16** (t_R = 21 min) were integrated, and the product concentration was deduced from its percentage of the total peak area.

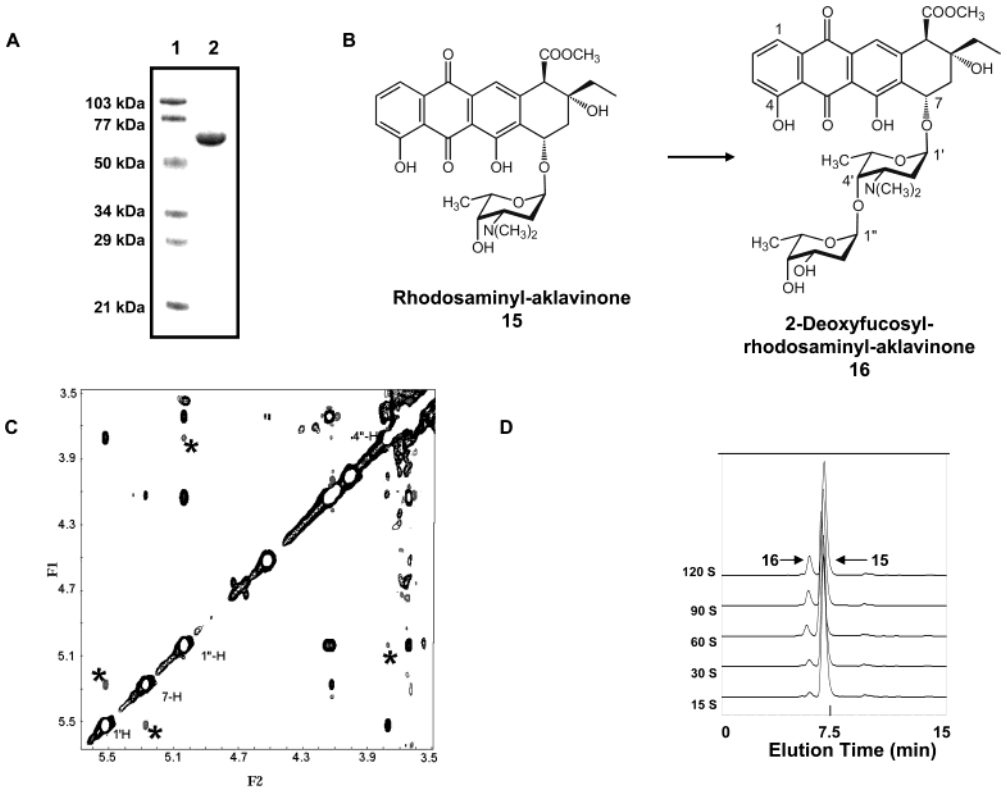


FIGURE 2: AknK transfers L-2-deoxyfucose to rhodosaminyl aklavinone **15** to yield 2-deoxyfucosyl rhodosaminyl aklavinone **16**. (A) SDS−PAGE (12%) analysis of purified C-terminally tagged His_6-tagged AknK (lane 2) along with molecular weight markers (lane 1). (B) Schematic representation of the conversion of **15** to **16**. (C) Detail of the overlaid TOCSY/ROESY spectrum of **16**. ROESY cross-peaks marked with an asterisk establish the connectivities between 1′-H of rhodosamine and 7-H of the aklavinone aglycone and 1″-H of 2-deoxyfucose and 4′-H of rhodosamine. (D) Formation of **16** over 3 min monitored by RP-HPLC.

RESULTS

Expression and Purification of AknK. Analysis of the gene cluster of the aclacinomycin A-producing strain *S. galilaeus* ATCC 31615 reveals two Gtf genes (*3*, *6*), *AknS* and *AknK*, that are strongly homologous to other Gtfs, such as *DnrS* (*5*). However, the functions of either of these Gtfs have not been defined. In this paper, we focus on the elucidation of AknK function. The *AknK* coding sequence was subcloned into a pET-22b expression vector and expressed recombinantly in *E. coli* as a C-terminal His_6-tagged protein. The protein was overproduced well and was soluble. The AknK protein behaved well in nickel affinity chromatography, leading to a purity of greater than 95% based on SDS−PAGE (Figure 2A), and the overall yield was 30 mg/L of culture. The protein appeared to be a 65 kDa protein based on its mobility on SDS−PAGE; however, the mass of AknK measured by MALDI ($[M + H]^+ = 50\,699$) agreed well with the calculated mass ($[M + H]^+ = 50\,685$), and the identity of the protein was confirmed by N-terminal sequencing (data not shown).

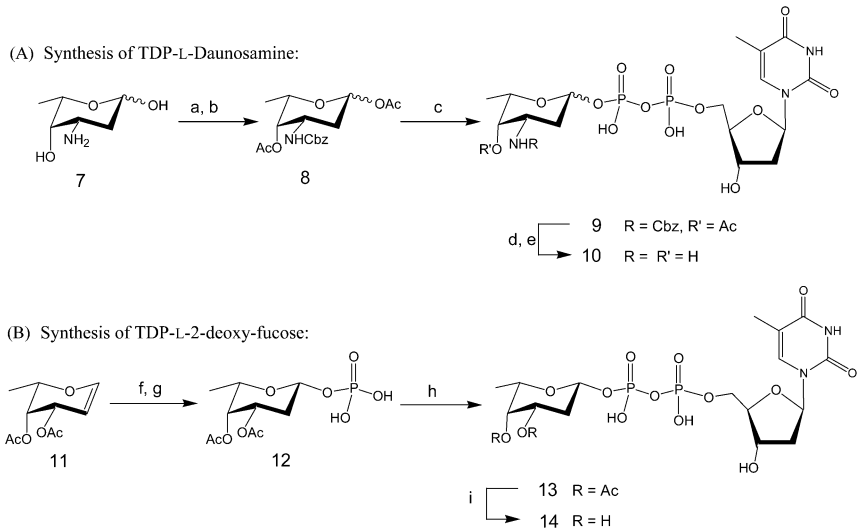
*Preparation of dTDP-L-Daunosamine **10** and dTDP-L-2-Deoxyfucose **14**.* To assay AknK for its Gtf activity requires the availability of the proposed nucleoside diphosphohexose donors. In monosaccharylanthracyclines such as daunomycin, the sugar is the 2,3,6-trideoxy-3-amino-L-hexose, daunosamine (*1*). N,N-Dimethylation of daunosamine yields L-rhodosamine, the proximal hexose in aclacinomycin A. The middle sugar in the aclacinomycin A trisaccharyl chain is the 2,6-dideoxy-L-hexose, 2-deoxyfucose (*6*). Most *Streptomyces* species use dTDP-sugars in antibiotic biosynthetic pathways, so the syntheses of dTDP-L-daunosamine **10** and dTDP-L-2-deoxyfucose **14** were undertaken.

dTDP-L-daunosamine **10** was synthesized according to a method first described by Hindsgaul (*21*) and adapted for the synthesis of UDP-L-*epi*-vancosamine (*25*). As shown in Scheme 1, L-daunosamine hydrochloride **7** (*19*, *20*) was converted to its acetylated *N*-Cbz derivative **8** and coupled to dTDP tetrabutylammonium salt via the anomeric iodide to give to the protected dTDP-L-daunosamine **9** as a 1:1 mixture of α- and *β*-anomers. Careful separation by reversed phase HPLC led to a mixture enriched in the *β*-anomer of **9** (1:2 α/*β*). Removal of the protecting groups and careful separation by HPLC then led to a pure sample of the desired *β*-isomer of **10**.

For the synthesis of dTDP-L-2-deoxyfucose **14**, a different approach was used. Diacetyl-L-fucal **11** (*22*, *23*) was converted to the 2-deoxyfucosyl chloride and coupled with tetrabutylammonium dihydrogen phosphate to give the corresponding phosphate **12** with high *β*-selectivity (1:9 α/*β*). Purification by reversed phase HPLC led to the pure *β*-anomer of **12**, which was coupled with TMP morpholidate to give TDP derivative **13**. Deacetylation then afforded the required dTDP-*β*-L-2-deoxyfucose **14**. The details of our studies directed toward the stereoselective synthesis of *β*-2-deoxyglycosyl phosphates, including application of this methodology to various 2-deoxy substrates, will be reported in due course (*25*).

AknK Transfers L-2-Deoxyfucose to Rhodosaminyl Aklavinone. To test the activity of AknK, the purified recombinant enzyme was incubated with potential substrates. This

Scheme 1: Synthesis of dTDP Sugars[a]



[a] Reagents and conditions. (A) dTDP-L-daunosamine **10**: (a) Cbz-succinimide, Et_3N, MeOH; (b) Ac_2O, DMAP, pyridine; (c) (i) TMS-I, CH_2Cl_2, −78 °C, (ii) TDP Bu_4N salt, DIPEA, CH_2Cl_2, −45 °C → rt; (d) Et_3N, MeOH, H_2O; (e) H_2/Pd-C, MeOH. (B) dTDP-L-2-deoxyfucose **14**: (f) HCl(g), benzene; (g) $Bu_4NH_2PO_4$, DIPEA, CH_2Cl_2; (h) TMP morpholidate, tetrazole, pyridine; (i) Et_3N, MeOH, H_2O.

Table 1: Kinetic Parameters of AknK Using Authentic and Alternate Substrates

	K_m (μM)		
	monoglycosyl-aglycone	TDP-sugar	k_{cat} (min^{-1})
rhodosaminyl aklavinone **15** + TDP-L-2-deoxyfucose **14**	109.1 ± 5.2	148.9 ± 10.1	65.4 ± 1.2
rhodosaminyl aklavinone **15** + TDP-L-daunosamine **10**	104.4 ± 9.8	940 ± 2.7	2.1 ± 0.1
idarubicin **4** + TDP-L-2-deoxyfucose **14**	138.0 ± 3.7	531.0 ± 44.2	5.4 ± 0.1

included the aglycone aklavinone and dTDP-L-daunosamine **10**, which yielded no detectable product. To evaluate whether AknK catalyzes the addition of the second saccharyl moiety, L-2-deoxyfucose, AknK was incubated with dTDP-L-2-deoxyfucose **14** and rhodosaminyl aklavinone **15** (Figure 2B). The HPLC analysis of the reaction mixtures indicated the appearance of a new peak (Figure 2D), and its mass detected by MALDI ($[M + H]^+ = 701.1$) was in agreement with the calculated mass of the 2-deoxyfucosyl rhodosaminyl aklavinone product **16** ($[M + H]^+ = 700.7$). Extensive NMR analysis (TOCSY and ROESY) of **16** revealed the attachment of 2-deoxyfucose to the 4-hydroxyl of the rhodosaminyl moiety. The overlaid TOCSY/ROESY spectrum of **16** (see the detail in Figure 2C) shows ROESY cross-peaks, marked with an asterisk, between 1′-H of rhodosamine and 7-H of the aklavinone aglycone and, most importantly, 1″-H of 2-deoxyfucose and 4′-H of rhodosamine. These data and the characteristic small coupling constant ($J_{1',2'} = 3.1$ Hz) obtained for the anomeric 1″-H proton of 2-deoxyfucose unambiguously established the α-(1→4)-linkage of the disaccharide unit. Steady-state analysis of AknK resulted in a k_{cat} of 66.1 min^{-1}, indicating that AknK is a reasonably robust glycosyltransferase. The K_m values for dTDP-L-2-deoxyfucose **14** and aglycone **15** were 149 and 109 μM, respectively (Table 1 and Figure S1 of the Supporting Information). These results validate the fact that AknK is the Gtf that adds the second sugar in the aclacinomycin A biosynthesis.

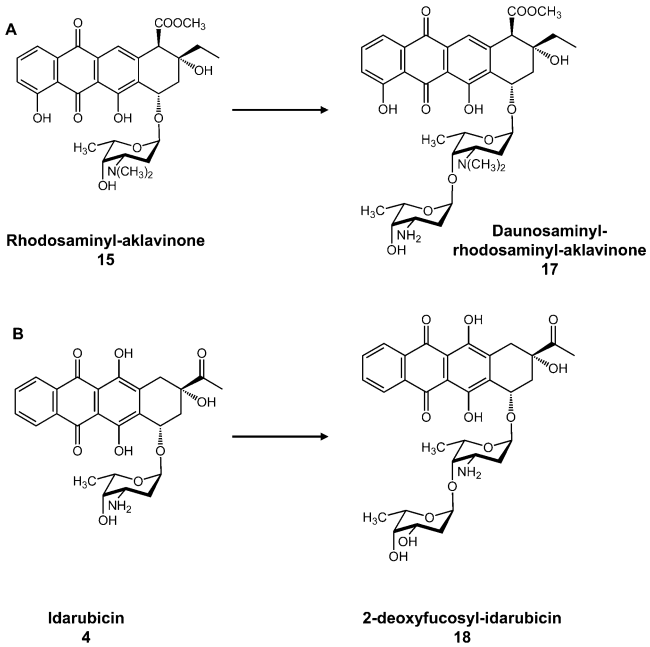


FIGURE 3: AknK uses various dTDP-2-deoxysugars and anthracycline monoglycoside to yield disaccharide-containing anthracycline variants. (A) Schematic representation of the conversion of rhodosaminyl aklavinone **15** to daunosaminyl rhodosaminyl aklavinone **17**. (B) Schematic representation of the conversion of idarubicin **4** to 2-deoxyfucosylidarubicin **18**.

AknK Transfers Daunosamine to Rhodosaminyl Aklavinone. AknK was then tested for its ability to accept an alternative activated sugar donor, dTDP-L-daunosamine **10**, a 3′-amino analogue of dTDP-L-2-deoxyfucose **14** (Figure 3A). Incubation of dTDP-L-daunosamine with authentic rhodosaminylanthracycline acceptor **15** in the presence of AknK for 30 min led to the appearance of a new peak detected by HPLC (Figure S2 of the Supporting Information). The mass of the peak characterized by MALDI-TOF ($[M + H]^+ = 700.3$) was in agreement with the calculated mass of expected product **17** ($[M + H]^+ = 699.8$). Steady-state analysis of AknK indicated that the enzyme catalyzes the addition of daunosamine at the maximal velocity of 2.1 min^{-1}

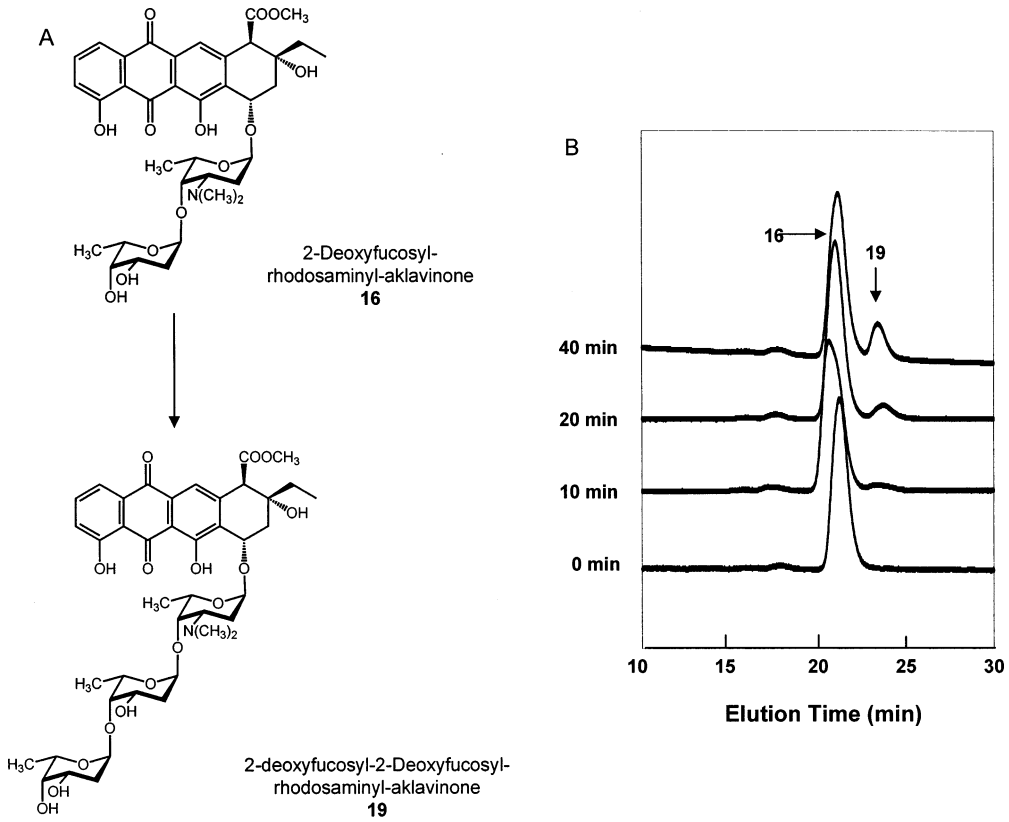


FIGURE 4: AknK transfers L-2-deoxyfucose to 2-deoxyfucosyl rhodosaminyl aklavinone **16**. (A) Schematic representation of the conversion of **16** to the trisaccharyl anthracycline analogue, 2-deoxyfucosyl-2-deoxyfucosyl rhodosaminyl aklavinone **19**. (B) Formation of **19** from 0 to 40 min monitored by RP-HPLC.

(Table 1), a 30-fold drop compared to the addition of natural sugar donor **14**. The K_m for rhodosaminyl aklavinone **15** was 104 μM, and the K_m for dTDP-L-daunosamine **10** was 940 μM, a 9-fold increase compared to that of the authentic sugar donor. These results indicate that AknK accepts L-daunosamine as an alternative sugar substrate, albeit with a 270-fold decrease in catalytic efficiency (k_{cat}/K_m).

AknK Transfers L-2-Deoxyfucose and L-Daunosamine to Other Anthracycline Monoglycosides. AknK was then tested for it ability to accept alternative monoglycosylaglycone acceptors. The first monoglycosylanthracycline acceptor that was tested was idarubicin **4**. Idarubicin differs from aglycone **15** in both the side chains of the aglycone and the sugar moiety (Figure 3B). Incubation of dTDP-L-2-deoxyfucose **14** and idarubicin **4** in the presence of AknK resulted in the formation of a new peak as determined by HPLC analysis. The molecular weight of the peak measured by MALDI-TOF ($[M + H]^+ = 628.1$) was 130 mass units larger than that of idarubicin ($[M + H]^+ = 498.2$), validating the addition of L-2-deoxyfucosyl moiety to the idarubicin acceptor (Figure S3 of the Supporting Information) to give a disaccharylanthracycline **18**. The fact that idarubicin **4** bears a structural resemblance to natural aglycone substrate **15** and the observation that AknK indeed catalyzes the attachment of L-2-deoxyfucose to the 4-hydroxyl substituent of **15** suggest that a similar transformation would take place on idarubicin. Steady-state analysis showed that AknK catalyzes the addition of 2-deoxyfucose to idarubicin at the maximal rate of 5.6 min^{-1} (Table 1). The K_m values for idarubicin **4** and dTDP-L-2-deoxyfucose **10** are 137 and 562 μM, respectively. It is unclear why the alteration of the monoglycosylated aglycone acceptor results in a 4-fold increase in the sugar donor K_m. Further elucidation of the full kinetic mechanism is required to address this question. In addition to idarubicin, AknK catalyzes the transfer of L-2-deoxyfucose to two other widely used monoglycosylated anthracyclines, daunomycin **1** and adriamycin **2** (data not shown). Moreover, the enzyme is capable of transferring daunosamine to idarubicin **4** (data not shown). The steady-state kinetic studies on AknK for these alternative acceptors and L-deoxyhexoses remain to be carried out.

AknK Catalyzes the Tandem Transfer of L-2-Deoxyfucose to Rhodosaminyl Aklavinone. Aclacinomycin A **3** contains a trisaccharide chain attached to the C_7-OH position of the tetracyclic structure (Figure 1). However, only two Gtf genes, *AknS* and *AknK*, have been identified in the biosynthetic cluster from *S. galilaeus* (*3*, *6*), raising the possibility that one of these Gtfs is capable of a tandem transfer. Since AknK transfers the second sugar to the monoglycosyl intermediate **15**, we sought to evaluate whether AknK could transfer an additional deoxysugar to the disaccharide-containing anthracycline **16** and complete the trisaccharide chain (Figure 4A). Attempts to prepare compound **16** by limited acid-catalyzed hydrolysis of commercially available aclacinomycin A **3** yielded only minute quantities. Instead, **16** was prepared enzymatically by large-scale incubation of rhodosaminyl aklavinone **15** and dTDP-L-2-deoxyfucose **14** in the presence of AknK and was purified using reversed phase HPLC. The purified product was used to test a possible tandem glycosylation reaction with AknK.

Incubation of the disaccharide-containing anthracycline **16** and dTDP-L-2-deoxyfucose **14** in the presence of AknK gave

a new peak as determined by HPLC analysis (Figure 4B). The molecular weight of the new compound ($[M + H]^+ = 830.2$) agreed with the mass of expected trisaccharide-containing compound **19** ($[M + H]^+ = 830.9$). Compound **19** has been isolated from *S. galilaeus* mutant strain H075 containing a nonsense mutation in the *AknP* gene encoding dTDP-hexose 3-dehydratase (*26*, *27*). We analyzed the compound isolated from H075, a gift from S. Torkkell and colleagues, by HPLC and found that it has a retention time identical to that of the compound generated from **14** and **16** by AknK (data not shown), confirming that AknK was capable of appending L-2-deoxyfucose to disaccharide-containing compound **16** to generate trisacchride anthracycline **19**. Because of the limited quantities of **16**, the steady-state kinetic analysis of the second transfer reaction by AknK has not yet been accomplished. However, using 100 μM disaccharide-containing compound **16** and 1000 μM dTDP-L-2-deoxyfucose **14**, AknK catalyzes the transfer of the second sugar at a rate of 0.04 min^{-1}; the activity is not saturated in the concentration range of dTDP-L-2-deoxyfucose **14** that was used. The second transfer reaction catalyzed by AknK was thus ~3 orders of magnitude slower than the first sugar transfer reaction catalyzed by AknK. Evaluating the comparative rates of the first and second transfers with optimal dTDP-L-deoxyhexose substrates requires preparation of dTDP-L-rhodinose and dTDP-L-cinerulose.

DISCUSSION

Sugar modifications of many antibiotics play essential roles in maintaining the biological activities of these compounds (*28*−*31*). In addition to the anthracyclines described in this study, glycopeptide antibiotics (vancomycin, chloroeremomycin, and teicoplanin) (*32*), aminocoumarin-containing antibiotics (novobiocin, chlorobiocin, and coumermycin A1) (*33*), and macrolide antibiotics (erythromycin, pikromycin, and tylosin) (*34*, *35*) require sugar moieties for their full biological activities. The crystal structures of the large ribosomal subunit complexed with macrolide antibiotics show that the saccharide moieties extend toward the peptidyl transferase center and block the egress of nascent polypeptide chain (*34*, *35*). The cocrystal structures of novobiocin and chlorobiocin with the ATPase domain of DNA gyrase B revealed that noviose is indispensable in the formation of high-affinity interactions between antibiotics and their targets (*33*). The glycosyl tailoring by dedicated glycosyltransferases occurs after the aglycones have been assembled by polyketide synthases or nonribosomal peptide synthetases.

Although glycosylation is greatly important in maintaining the bioactivity of numerous natural products, only a few have been studied biochemically, including glycopeptide Gtfs (GtfA−E) (*36*−*39*), the novobiocin glycosyltransferase NovM (*40*), and an oleandomycin Gtf (OleD) (*41*). The difficulty in obtaining highly purified active Gtfs, aglycones, and NDP-deoxysugars has prevented biochemical studies on the natural product Gtfs. As a first limitation, only a small number of antibiotic Gtfs have been successfully expressed from heterologous systems in a soluble and active form for specificity and mechanistic studies. The aglycone substrates can be isolated from blocked mutant strains or obtained by partial degradation of mature glycosides as reported here. Although some Gtfs utilize UDP- or dTDP-glucose as a cosubstrate, most Gtfs transfer deoxysugars to the corresponding aglycones. Since the chemical synthesis of NDP-deoxysugars with the correct stereochemistry at C-1 (β) has been demanding, the difficulty in obtaining particular NDP-deoxy-D- or -L-hexoses has been the major obstacle in the study of glycosyltransferases *in vitro*.

In the biosynthesis of monosaccharide-containing anthracyclines, such as daunomycin **1** and adriamycin **2**, the glycosyltransferase DnrS transfers a single deoxysugar moiety, L-daunosamine, to the aglycone scaffold (*5*). In contrast, aclacinomycin A **3** has a trisacchride chain attached to the aglycone, and only two Gtf genes, *AknS* and *AknK*, have been revealed from its producing strain (*3*, *6*). Prior to this study, the functions of the two genes were yet to be defined. Other trisaccharide-containing anthracyclines display variations in the trisaccharide identity. Ciclamycin 0 uses L-2-deoxyfucose as the proximal sugar, and ciclamycin 4 uses L-2-deoxyfucose and L-rhodinose as the first two sugars (*42*). On the other hand, trisaccharyl anthracyclines with altered glycosylation patterns accumulate in several aclacinomycin A nonproducing mutant strains (*26*). These observations suggest that anthracycline Gtfs may have broad substrate specificity.

The objective of this study has been to understand the role of AknK and to test its tolerance toward other substrates. The biochemical characterization of AknK required the purified enzyme, the monoglycosylaglycone acceptors, and NDP-L-deoxysugars. The combination of a low concentration of IPTG induction (300 μM) and low-temperature induction (15 °C) allowed us to express a C-terminally His_6-tagged AknK in *E. coli* as a soluble protein. The AknK protein was readily purified in good yield to homogeneity using nickel chelating chromatography. The authentic monoglycosylaglycone acceptor was acquired by limited acid-catalyzed hydrolysis of commercially available aclacinomycin A, whereas other monoglycosylated acceptors used in this study were from commercial sources. Although it is possible to produce dTDP-L-deoxysugars in minute quantities by enzymatic reconstitution of their biosynthesis pathway (*43*), we chose to prepare dTDP-L-2-deoxyfucose **14** and dTDP-L-daunosamine **10** by chemical synthesis.

AknK displays robust activity with the presumed authentic substrates, L-rhodosaminyl aklavinone **15** and dTDP-L-2-deoxyfucose **14**, yielding the expected product, L-2-deoxyfucosyl rhodosaminyl aklavinone **16**. This is the first piece of biochemical evidence validating the fact that AknK is the second Gtf in the pathway (and therefore suggesting that AknS will transfer the first sugar to the aklavinone). The robust activity of AknK also allowed us to study the tolerance of AknK toward both dTDP-L-deoxysugar donors and monoglycosylaglycone acceptors. In the deoxysugar donor category, AknK can utilize dTDP-L-daunosamine **10** as an alternative sugar donor to generate the disaccharylanthracycline. The substitution of the 3′-OH group in the 2-deoxyfucosyl moiety with the 3′-NH_2 group resulted in a 30-fold decrease in the transfer k_{cat}. In the future, we will investigate what other variations at C_3 and C_6 of the L-sugar ring can be tolerated by the enzyme. On the other hand, we found AknK was capable of transferring L-2-deoxyfucose and/or L-daunosamine to several commercially available monoglycosylanthracycline acceptors, including daunomycin **1**, adriamycin **2**, and idarubicin **4**, with reduced catalytic efficiencies. Perhaps not surprisingly, AknK failed to catalyze the addition

of L-2-deoxyfucose to epirubicin, a 4′-*epi*-hydroxy analogue of adriamycin **2**, suggesting the enzyme requires acceptor substrates with an axially oriented hydroxyl at the site of nucleophilic attack. Utilization of either the alternative acceptor or donor did not affect the K_m for the monoglycosyl acceptors but greatly increased the K_m for dTDP-sugar donors. Subsequent studies will be directed toward the kinetic and chemical mechanism of AknK. This Gtf belongs to the GT-B enzyme superfamily (*44*−*46*), only two members of which, MurG and OleD, have been studied mechanistically (*41*, *47*). Both enzymes follow a compulsory ordered bi-bi mechanism, where the nucleotide-sugar donor binds first to MurG while the macrolide acceptor binds first to OleD.

The most unexpected finding from this study is the ability of AknK to catalyze tandem additions of L-2-deoxyfucose to the rhodosaminyl aklavinone **15**, even though the second transfer is much slower than the first. This observation may explain why only two Gtfs, AknS and AknK, are found in the biosynthetic cluster while there are three steps required to build the trisaccharide chain to the anthracycline aglycone. The slow rate of the second deoxyfucosyl transfer could reflect the specificity for rhodinose or cinerulose in that step and must await the preparation of those dTDP-L-deoxyhexoses. We note that cinerulose with a 4-keto group is a chain-terminating residue so it is unclear if AknK could accomplish additional transfers beyond the second or, for that matter, how the monosaccharyl and the disaccharyl aklavinones can be positioned in the AknK active site for elongation.

Because of the clinical utility of anthracycline drugs for several tumor types, substantial efforts have been made to search for new anthracycline compounds. Given that the L-deoxyhexose moiety is required for the antitumor efficacy of anthracyclines (*18*, *48*), and mono- to trisaccharide chains are found, modifications of the carbohydrate portion are of interest. These compounds have been generated by feeding experiments or by multistep organic synthesis (*14*, *15*, *49*, *50*), and each approach has its constraints. In feeding experiments, the kind of sugar to be appended is limited by the identity and pool of dTDP-deoxysugars produced by the biosynthetic enzymes inside microbial cells, even with gene swapping protocols (*49*). In contrast, it is relatively easy to append one sugar to aglycones using organic synthesis, but much more difficult to build oligosaccharide chains with L-2-deoxysugars (*15*).

With recent progress in the biochemical characterization of antibiotic Gtfs, chemoenzymatic approaches become attractive for the rapid generation of antibiotic derivatives. We have previously reported the chemoenzymatic generation of several novel glycopeptide antibiotics by vancomycin Gtfs, GtfD and GtfE (*36*, *37*). The characterization of AknK in this study also indicates this enzyme can be useful for the chemoenzymatic generation of anthracycline analogues with various sugar substitutions. We found that AknK added L-2-deoxyfucose and L-daunosamine to several monoglycosylated anthracyclines, tolerating changes made on the aglycone side chains as well as the first sugar attached to the tetracyclic ring. In the AknK transfer of L-daunosamine to idarubicin **4**, all three elements, the aglycone and the identity of both L-sugars, are variant. Genetic and feeding experiments suggest that anthracycline Gtfs may have broad substrate specificity. For example, aclacinomycin-negative mutant strain KE303 has been used to make a series of glycosylated anthracyclines, and some of the them contain rhodinose at the second position of the sugar moiety (*50*). The prospect of tandem glycosylations by AknK adds another degree of diversity for the chemoenzymatic synthesis of anthracycline derivatives. With the formation of **19**, we have reproduced *in vitro* the deoxyfucosyl deoxyfucosyl rhodosaminyl aklavinone produced by *S. galilaeus* mutant H075 (*26*, *27*).

There are still several problems to solve to allow chemoenzymatic synthesis of glycosylated anthracycline derivatives. The first is to obtain at least one anthracycline Gtf that adds the first sugar to the aglycone. DnrS (*5*) and AknS (*6*) seem to be good candidates. We purified AknS in *E. coli* as a His_6-tagged protein in a soluble form; however, it was not active when incubated with aklavinone and dTDP-L-daunosamine **10**, and further studies will depend on the availability of dTDP-L-rhodosamine. All our efforts with DnrS expression in *E. coli* have to date yielded insoluble proteins and may require other heterologous hosts. On the other hand, the efficiency of AknK using an alternative substrate still needs to be improved. With progress in these two aspects, as well as the availability of more dTDP-L-deoxysugars, anthracycline Gtfs may become powerful catalysts for the generation of anthracycline analogues and may speed the discovery of new antitumor therapeutics.

CONCLUSIONS

We report here the purification of anthracycline glycosyltransferase AknK as well as the preparation of its substrates, monoglycosylated aglycone **15** and two dTDP-L-deoxyhexoses (dTDP-L-2-deoxyfucose **14** and dTDP-L-daunosamine **10**). Our studies of AknK not only reveal that AknK can catalyze the generation of a trisaccharide chain by tandem addition of L-2-deoxyfucose but also demonstrate that AknK accepts other monoglycosyl anthracyclines and dTDP-L-deoxysugar donors. These studies indicate that anthracycline Gtfs can be a useful tool for generating novel anthracycline compounds.

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SUPPORTING INFORMATION AVAILABLE

Additional experimental methods, determination of kinetic parameters of AknK using its natural substrates (Figure S1), transfer of L-daunosamine to rhodosaminyl alkavinone **15** by AknK (Figure S2), and transfer of L-2-deoxyfucose to an alternative acceptor (Figure S3), idarubicin **4**, by AknK. This material is available free of charge via the Internet at http://pubs.acs.org.

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