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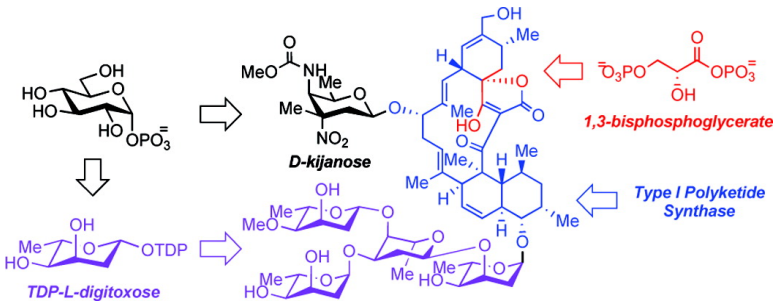

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Elucidation of the Kijanimicin Gene Cluster: Insights into the Biosynthesis of Spirotetronate Antibiotics and Nitrosugars

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Abstract: The antibiotic kijanimicin produced by the actinomycete *Actinomadura kijaniata* has a broad spectrum of bioactivities as well as a number of interesting biosynthetic features. To understand the molecular basis for its formation and to develop a combinatorial biosynthetic system for this class of compounds, a 107.6 kb segment of the *A. kijaniata* chromosome containing the kijanimicin biosynthetic locus was identified, cloned, and sequenced. The complete pathway for the formation of TDP-L-digitoxose, one of the two sugar donors used in construction of kijanimicin, was elucidated through biochemical analysis of four enzymes encoded in the gene cluster. Sequence analysis indicates that the aglycone kijanolide is formed by the combined action of a modular Type-I polyketide synthase, a conserved set of enzymes involved in formation, attachment, and intramolecular cyclization of a glycerate-derived three-carbon unit, which forms the core of the spirotetronate moiety. The genes involved in the biosynthesis of the unusual deoxysugar D-kijanose [2,3,4,6-tetradeoxy-4-(methylcarbamyl)-3-*C*-methyl-3-nitro-D-*xylo*-hexopyranose], including one encoding a flavoenzyme predicted to catalyze the formation of the nitro group, have also been identified. This work has implications for the biosynthesis of other spirotetronate antibiotics and nitrosugar-bearing natural products, as well as for future mechanistic and biosynthetic engineering efforts.

Kijanimicin (**1**, Chart 1) is a spirotetronate antibiotic isolated from *Actinomadura kijaniata*, a soil actinomycete. It has a broad spectrum of antimicrobial activity against Gram-positive bacteria, anaerobes, and the malaria parasite *Plasmodium falciparum*,[1] and it also shows antitumor activity.[2] The structure of kijanimicin (**1**) consists of a pentacyclic core, which is equipped with four L-digitoxose (**2**) units and a rare nitrosugar, 2,3,4,6-tetradeoxy-4-(methylcarbamyl)-3-*C*-methyl-3-nitro-D-*xylo*-hexopyranose, commonly known as D-kijanose (**3**). More than 60 kijanimicin-related spirotetronate-type compounds have been reported. Most are made by strains of high-GC Gram-positive bacteria (Actinomycetes), including *Streptomyces*,[3−8] *Micromonospora*,[9−12] *Actinomadura*,[1,13,14] *Saccharothrix*,[15] and *Verrucosispora*.[16] A species of *Bacillus* has also been identified as a producer of a member of this class of compounds.[17] Nearly all members of this class exhibit both antibacterial and antitumor activities, and many possess other biological activities. Well-known examples include chlorothricins (**4**, Chart 1), the anticholesterolemic agents;[18,19] tetronothiodin, a cholecystokinin B (CCK-B) inhibitor;[4] MM46115, an antiviral drug effective against parainfluenzae virus 1 and virus 2;[13] and tetrocarcins (see tetrocarcin A, **5**, Chart 1) and arisostatins, both of which have been shown to have therapeutic potential as inducers of apoptosis.[20−23] In a recent study, a collection of tetrocarcin

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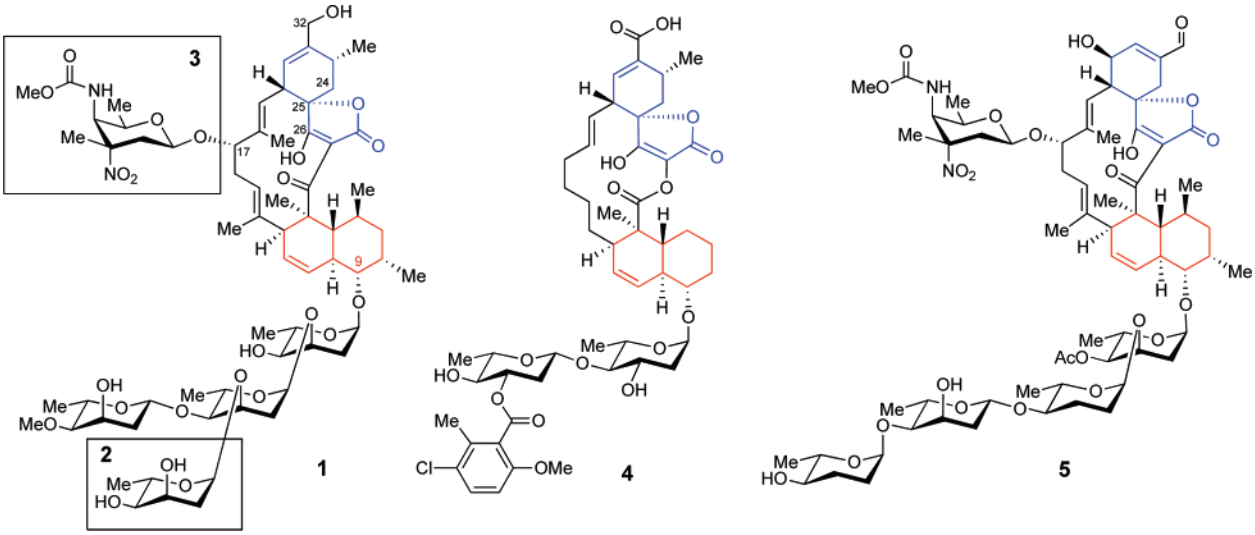
Chart 1



analogues was prepared synthetically, and some of them showed improved apoptosis-inducing activity.[24] Hence, compounds of this class have broad therapeutic potential worthy of further study and exploitation.

The complexity of the spirotetronate-containing aglycone cores of kijanimicin (**1**) and related compounds makes them attractive and challenging synthetic targets. Yoshii and co-workers reported the chemical synthesis of (+)-tetronolide, the aglycone of tetrocarcin A (**5**), in 1991.[25] Enantioselective synthesis of (-)-chlorothricolide, the aglycone of chlorothricin (**4**), was accomplished by Roush and Sciotti in 1994.[26] Both syntheses employed intramolecular Diels−Alder cyclizations to construct the spirotetronate and octahydronaphthalene portions of the aglycones. Kijanolide (**23**, Scheme 2) has not yet been chemically synthesized, but the preparation of an advanced *seco*-acid intermediate was recently reported.[27]

Biosynthetic studies of chlorothricin (**4**) have been performed by Floss and co-workers.[28] Through feeding of labeled acetate and propionate, it was found that the chlorothricolide aglycone (**6**) is primarily polyketide-derived, except for C22−C24, which are uniformly labeled after feeding of [$^{13}C_3$]-glycerol (Figure 1). Their studies also established that the precursor of the two sugars present in chlorothricin is glucose. Similar observations were made in the biosynthetic studies of tetrocarcin A (**5**), where feeding experiments confirmed a polyketide origin for the tetronolide aglycone (**7**, Figure 1).[29] These results suggest a common biosynthetic route for spirotetronate antibiotics involving assembly of the aglycone by the combined action of a modular type I polyketide synthase (PKS), the incorporation of a glycerol-derived three-carbon unit, and two intramolecular cyclization reactions to form the spirotetronate (highlighted in blue in **1**, **4**, and **5** in Chart 1) and the octahydronaphthalene (highlighted in red in **1**, **4**, and **5** in Chart 1) ring systems. The resulting aglycone cores are then modified by glycosylation with various deoxysugars and by other tailoring reactions to yield the final products.

There exist a number of challenging questions regarding the biosynthetic construction of this class of compounds. Particularly, we are interested in identifying the three-carbon aglycone precursor, understanding the mode of incorporation of this unit into the spirotetronate core, and elucidating the mechanisms of spirotetronate and octahydronaphthalene ring formation. Also, several spirotetronate antibiotics, such as kijanimicin (**1**), lobophorin B,[30] tetrocarcins (**5**),[11] and arisostatin A,[31] carry the unusual nitrosugar D-kijanose (**3**), whose biosynthesis likely involves new chemistry. To address these interesting questions, we undertook the identification and sequencing of the kijanimicin biosynthetic gene cluster in *A. kijaniata*.

Deoxysugar moieties of natural products are known to play critical roles in their bioactivity;[32−34] thus, glycodiversification of natural products holds promise for future drug development efforts.[35,36] A survey of known spirotetronate natural product structures shows diverse glycosylation patterns, particularly in the oligosaccharide chain attached to the C-9 position. The structure of the oligosaccharide chain at this position of tetrocarcin (**5**) was shown to be an important modulator of its Bcl-2 inhibition activity, suggesting that glycodiversification at C-9 may lead to spirotetronate derivatives with improved therapeutic value.[24] In the case of kijanimicin (**1**), this position is occupied by a branched tetrasaccharide chain composed of

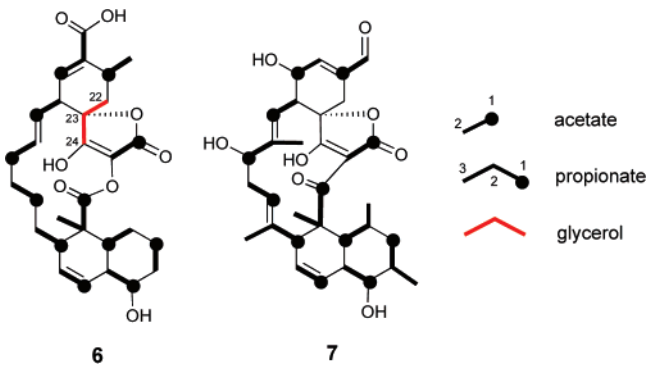


Figure 1. Structures of chlorothricolide (**6**) and tetronolide (**7**), summarizing labeling patterns observed by feeding ^{13}C-labeled precursors.

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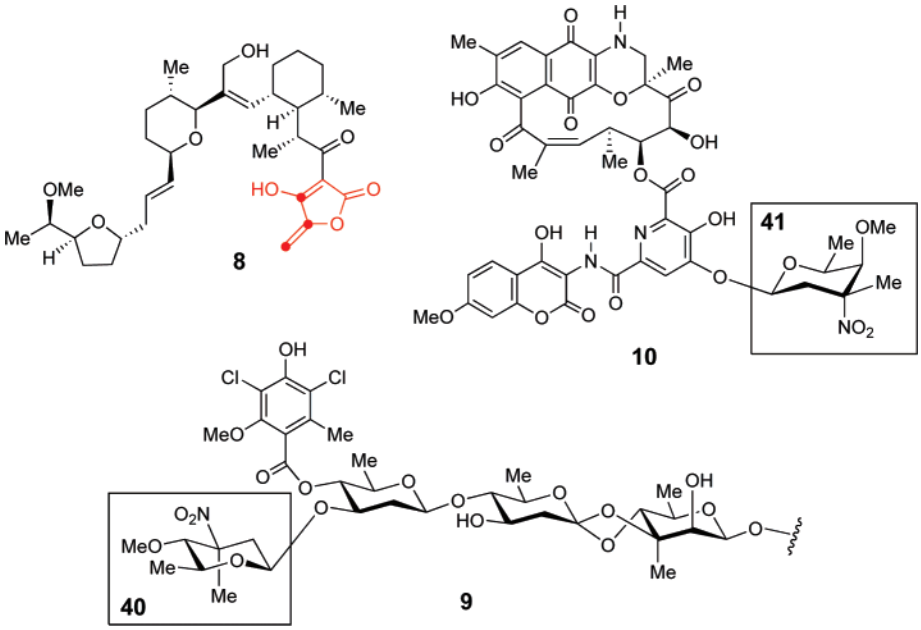
Chart 2



both α(1→3)- and β(1→4)-linked L-digitoxose (**2**) residues. Thus, another important goal of this study is to characterize the kijanimicin sugar biosynthesis and glycosylation pathways, particularly those involved in the biosynthesis and glycosyl transfer of the L-digitoxose residues, so as to lay a foundation for glycodiversification at this position of kijanimicin and other spirotetronate antibiotics.[33]

Here, we report the complete nucleotide sequence of the kijanimicin biosynthetic locus and functional prediction of encoded genes, resulting in a predicted biosynthetic scheme for kijanimicin formation. A complete *in vitro* functional characterization of the TDP-L-digitoxose biosynthetic pathway encoded therein is also reported. During our characterization work, gene cluster sequences of the biosynthetically related compounds chlorothricin (**4**)[37] and tetronomycin (**8**, Chart 2)[38] were reported, and a pathway for chlorothricin biosynthesis was proposed.[37] The availability of these sequences provided an opportunity to use sequence comparison to identify the core set of genes responsible for formation of the spirotetronate moiety. This analysis has revealed shared biosynthetic features between the glycerate component of spirotetronate natural products and the methoxymalonate unit found in several polyketide-derived natural products. Comparison of the kijanimicin cluster to those of other nitrosugar-containing natural products, evernimicin[39] and rubradirin (**9** and **10**, Chart 2),[40] enabled identification of the gene likely to be responsible for formation of the nitro moiety of these sugars. This work sets the stage for mechanistic studies of the reactions of kijanimicin biosynthesis and for the glycodiversification of spirotetronate antibiotics.

Experimental Section

Bacterial Strains, Plasmids, and Growth Conditions. *Actinomadura kijaniata* SCC1256 (ATCC 31588) was obtained from American Type Culture Collection and was maintained on ISP medium 2. For genomic DNA extraction, a seed culture of *A. kijaniata* was grown in liquid V15 medium[41] for 24−30 h, diluted 1:10 into V15-P medium,[41] and grown for 40−48 h. Preparation of *A. kijaniata* spore suspensions was performed as described by Kieser et al.[42] *Escherichia coli* DH5α, purchased from Bethesda Research Laboratories (Gaithersburg, MD), was used for routine cloning experiments. *E. coli* XL-1 Blue MRF′, purchased from Stratagene (La Jolla, CA), was used for cosmid manipulations. Plasmids SuperCos 1 and pBluescript II SK(+), used for cosmid library construction and cosmid fragment subcloning, respectively, were also purchased from Stratagene. Genes *kijB1* and *kijC2* were amplified from cosmid pHZ4-6, and *kijD10* and *kijD11* were amplified from cosmid pHZ14. Vector pET28b(+) and the overexpression hosts *E. coli* BL21 (DE3) pLysS and *E. coli* Rosetta II (DE3) were purchased from Novagen (Madison, WI).

Materials. Enzymes and molecular weight standards used for the cloning experiments were products of Invitrogen (Carlsbad, CA) or New England Biolabs (Ipswich, MA). Ni-NTA agarose and kits for DNA gel extraction and spin miniprep were obtained from Qiagen (Valencia, CA). *pfu* DNA polymerase was purchased from Stratagene (La Jolla, CA), and growth medium components were acquired from Becton Dickinson (Sparks, MD). The digoxigenin (DIG)-labeling and probing kit for colony hybridization assays was purchased from Boehringer Mannheim (Mannheim, Germany). Antibiotics and chemicals were products of Sigma-Aldrich Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). Bio-gel P2 resin and all reagents for sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) were purchased from Bio-Rad (Hercules, CA), with the exception of the prestained protein molecular weight marker, which

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was obtained from New England Biolabs. Amicon YM-10 filtration products were purchased from Millipore (Billerica, MA). The CarboPac PA1 high-performance liquid chromatography (HPLC) column was obtained from Dionex (Sunnyvale, CA), and the Mono-Q H/R 16/10 fast protein liquid chromatography (FPLC) column was obtained from Pharmacia (Uppsala, Sweden). Oligonucleotide primers for cloning of *kijB1*, *kijD10*, *kijD11*, and *kijC2* were prepared by Integrated DNA Technologies (Coralville, IA).

General. Protein concentrations were determined by the method of Bradford[43] using bovine serum albumin as the standard. The relative molecular mass and purity of enzyme samples were determined using SDS−PAGE as described by Laemmli.[44] NMR spectra were acquired on either a Varian Unity 300 or 500 MHz spectrometer, and chemical shifts (δ in ppm) are reported relative to that of the solvent peak (δ 4.65 for deuterated water in ^{1}H NMR). DNA sequencing was performed by the Core Facilities of the Institute of Cellular and Molecular Biology at the University of Texas. Mass spectra were obtained by the Mass Spectrometry Core Facilities in the College of Pharmacy and Department of Chemistry and Biochemistry at the University of Texas. Kinetic data were analyzed by nonlinear fit using Grafit5 (Erithacus Software Ltd.). Standard genetic manipulations of *E. coli* were performed as described by Sambrook et al.[45]

Identification and Cloning of the Kijanimicin Gene Cluster. Genomic DNA of *A. kijaniata* was isolated according to a procedure suggested by the manufacturer of the SuperCos 1 Cosmid Vector Kit (Stratagene), except that the cell pellet was first washed with 10 mM EDTA and then digested by lysozyme (2.5 mg/mL final concentration) at 30 °C for 3 h in solution A (10 mM NaCl, 20 mM Tris·HCl, pH 8.0, 1 mM EDTA) prior to the DNA isolation. For the construction of *A. kijaniata* SCC1256 genomic library, chromosomal DNA was partially digested with *Sau*3AI, isolated by ethanol precipitation, and ligated to SuperCos 1 that had been subjected to linearization by *Xba*I, CIAP treatment, and then digestion with *Bam*HI. Gigapack III Gold packaging extract (Stratagene) was used to perform the *in vitro* packaging based on the manufacturer's protocol. A conserved portion of the TDP-D-glucose 4,6-dehydratase gene, *tylA2*, from the tylosin gene cluster of *Streptomyces fradiae* labeled with DIG was used as the initial probe for colony hybridization to identify cosmids that contain portions of the *kij* cluster. Overlapping cosmids spanning the entire cluster were subsequently identified by colony hybridization and were mapped by restriction analysis.

Sequencing and Analysis. Cosmids pHZ8, pHZ14, pHZP1, pHZP5, and pHZ4-6, which span the entire *kij* cluster, were digested with either *Apa*I or *Bam*HI, and 1−5 kb fragments were subcloned into pBluescript II SK(+). Both strands of pBluescript II SK(+) subclones were sequenced using a combination of primer walking and sequencing using M13 universal primers. Sequencing data were obtained using a capillary-based AB 3700 DNA analyzer. DNA sequencing data were assembled and analyzed using Vector NTI Suite program (Version 6.0, InforMax, Inc., Bethesda, MD). Open reading frame assignments were made with the assistance of Vector NTI software and the NCBI ORF Finder interface. Protein sequence homology was analyzed using the NCBI BLAST server.[46]

Gene Amplification and Cloning of *kijB1, kijD10, kijD11,* and *kijC2*. Genes *kijB1*, *kijD10*, *kijD11* and *kijC2* were each PCR-amplified from appropriate cosmids using primers with engineered *Nde*I and *Hin*dIII (*Xho*I in the case of *kijB1*) restriction sites at the 5′ and 3′ termini, respectively. The primers used include kijB1-28-N-up (5′-GGAATTC**CATATG**AGCGCCATCCTCGGCC-3′) and kijB1-28-X-down (5′-CCG**CTCGAG**CCAGAGAGAACTCAGGCAG-3′) for *kijB1*; kijD10-28-N-up (5′-TAGGGGAGTT**CATATG**GAGAATCCGGCGAA-3′) and kijD10-28-H-down (5′-GGCTCCTGAA**AAGCTT**CTAAACCCGGGTCA-3′) for *kijD10*; kijD11-28-N-up (5′-AGGACATG**CATATG**AGCGGTATCGAGATCG-3′) and kijD11-28-H-down (5′-GGTGTCGCGG**AAGCTT**CCGCCCGGCTCAGA-3′) for *kijD11*; and kijC2-28-N-up 5′-GGAGGACC**AGCATATG**AGGCTCGCAGCGAC-3′ and kijC2-28-H-down 5′-GTCTGAAACA**AGCTT**CGTCAGGAATTCGCG-3′ for *kijC2*. Engineered restriction sites are shown in bold, start codons in bold underlined, and stop codons underlined. The PCR-amplified gene fragments were purified, digested with the appropriate restriction enzymes, and ligated into the pET28b-(+) vector digested with the same enzymes. The resulting plasmids were used to transform *E. coli* strains BL21(DE3)pLysS [in the cases of *kijB1*/pET28b(+), *kijD10*/pET28b(+), *kijD11*/pET28b(+)] or Rosetta II(DE3) [in the case of *kijC2*/pET28b(+)] for protein overexpression. Each enzyme was expressed as an *N*-terminal-His_6-tagged (*N*- and *C*-terminal His_6-tagged in the case of KijB1) protein.

Growth of *E. coli* KijB1, KijD10, KijD11, and KijC2 Recombinant Strains. An overnight culture of *E. coli* BL21(DE3)pLysS-*kijB1*/pET28b(+) strain grown in Luria−Bertani (LB) medium containing 50 μg/mL kanamycin and 35 μg/mL chloramphenicol at 37 °C was used (1 mL each) to inoculate 6 × 1 L of the same growth medium. These cultures were incubated at 37 °C with vigorous shaking until the OD_{600} reached 0.5. Protein expression was then induced by addition of isopropyl D-thiogalactoside (IPTG) to a final concentration of 0.1 mM, and the cells were allowed to grow at 37 °C for an additional 16 h. The cells were harvested by centrifugation at 4500*g* for 15 min and stored at −80 °C until lysis. The growth of *E. coli* BL21(DE3)pLysS-*kijD10*/pET28b(+) and BL21(DE3)pLysS-*kijD11*/pET28b(+) strains followed a similar procedure, except IPTG (1.0 mM) induction was carried out on cultures precooled to 14 °C after the OD_{600} reached ∼0.4. Subsequently, cultures were grown at 14 °C for 18 h. The growth of *E. coli* Rosetta II(DE3)-*kijC2*/pET28b(+) strain was carried out, after inoculation, at 30 °C for 24 h without IPTG induction.

Purification of His_6-Tagged KijB1, KijD10, KijD11, and KijC2. All purification steps were carried out at 4 °C according to the Ni-NTA manufacturer's protocol with minor modifications. Thawed cells were resuspended in lysis buffer (2 mL/g cells) containing 10% (v/v) glycerol. After incubation with lysozyme for 30 min, cells were disrupted by sonication using 12 × 30 s pulses with 1-min pauses between each pulse. The lysate was centrifuged at 10000*g* for 20 min, and the supernatant was subjected to Ni-NTA chromatography. In brief, Ni-NTA slurry (10 mL) was washed three times with lysis buffer before use. The soluble protein fraction was incubated with washed Ni-NTA beads on a rotator at 4 °C for 2 h. Lysate and beads were then loaded onto a column, which was allowed to drain and then washed with wash buffer containing 10% glycerol (70 mL). Bound protein was eluted using elution buffer containing 10% glycerol (12 mL) and collected in 8 × 1.5 mL portions, which were pooled and dialyzed against 3 × 1 L of buffer. All buffers used in KijC2 purification contained 1 mM dithiothreitol (DTT), and the elution buffer used in KijC2 purification contained 15% glycerol. All buffers used in KijB1 purification contained 20% glycerol. The dialysis buffers used in the purification of each protein were different and are as follows: for KijB1, 50 mM NaH_2PO_4, 300 mM NaCl, and 20% glycerol, pH 8.0; for KijD10, 50 mM Tris·HCl, 2 mM EDTA, 1 mM DTT, 10% glycerol, pH 7.5; for KijD11, 50 mM NaH_2PO_4, 300 mM NaCl, 15% glycerol, pH 8.0; and for KijC2, 50 mM NaH_2PO_4, 300 mM NaCl, 20% glycerol, 1 mM DTT, pH 8.0. After dialysis, protein was concentrated by filtration using a YM-10 filter. The collected protein was then flash-frozen in liquid nitrogen and stored at −80 °C until use. Yields (mg/L culture) and purities of proteins were as follows: KijB1, 8.4 mg/L, >80% purity; KijD10, 10.0 mg/L, >80% purity; KijD11, 16.7 mg/L, >80% purity; and KijC2, 14.3 mg/L, >60% purity.

Enzymatic Synthesis of TDP-4-keto-6-deoxy-D-glucose (13). The large-scale enzymatic preparation of the KijB1 substrate, TDP-4-keto-6-deoxy-D-glucose (**13**, see Scheme 1), was performed by a published

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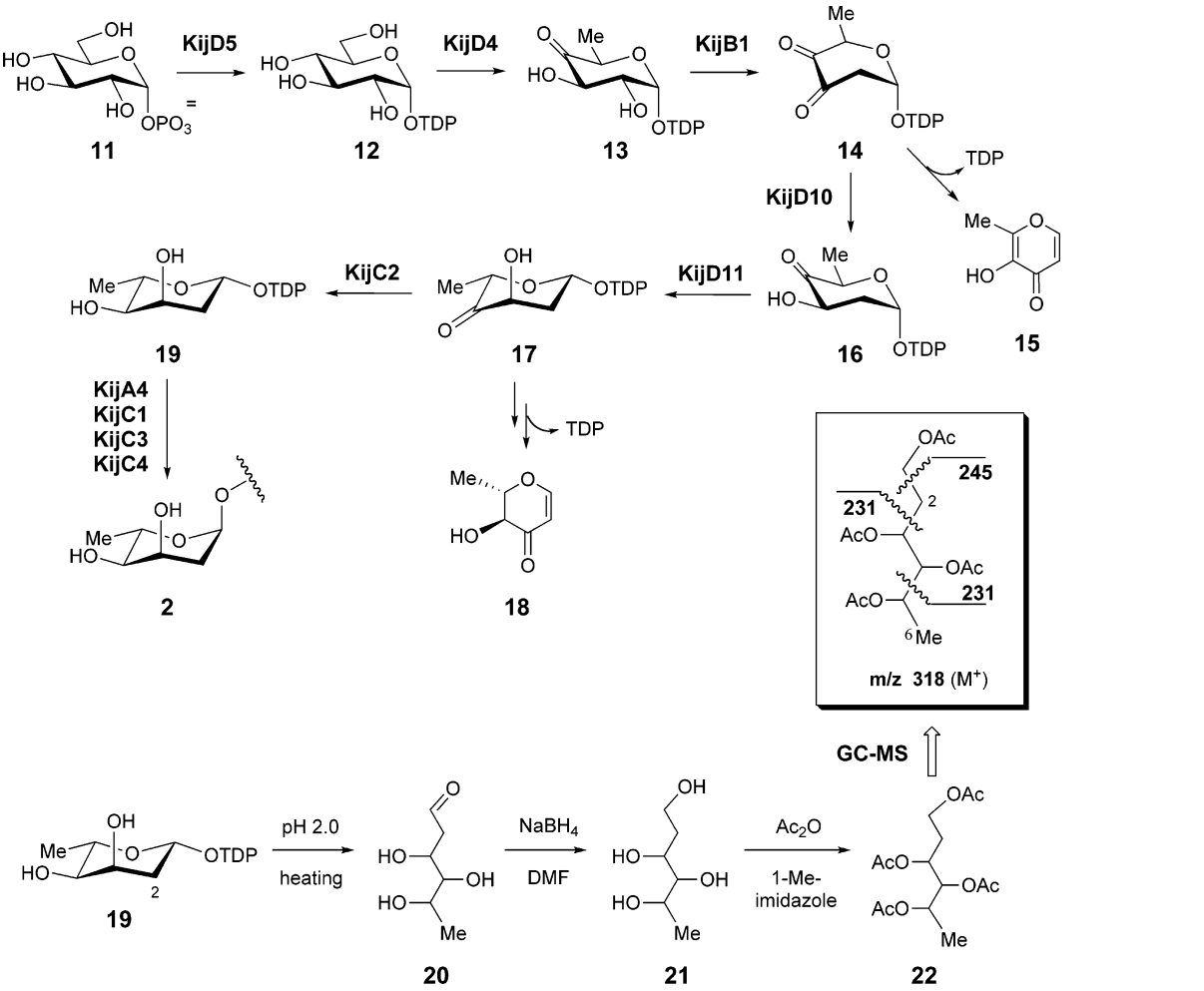
(44) Laemmli, U. K. *Nature* **1970**, *227*, 680−685.
(45) Sambrook, J.; Russell, D. W. *Molecular Cloning: A Laboratory Manual*, 3rd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 2001.
(46) www.ncbi.nlm.nih.gov/blast.

Scheme 1



"two-stage, one-pot" procedure.[47,48] High-resolution ESI-MS of **13** (C-4 hydrated form): calcd for $C_{16}H_{25}N_2O_{16}P_2$ $(M - H)^-$, 563.0674; found 563.0632.

Enzymatic Preparation of TDP-4-keto-2,6-dideoxy-D-glucose (16). Compound **16** (Scheme 1) was prepared from **13** using TDP-4-keto-6-deoxy-D-glucose 2,3-dehydratase (TylX3, the KijB1 homologue) from the tylosin gene cluster of *S. fradiae*[49] and the expressed TDP-3,4-diketo-2,6-dideoxy-D-glucose 3-ketoreductase (KijD10). A typical reaction mixture contained 23.8 mg (14 mM) of TDP-4-keto-6-deoxy-D-glucose (**13**), TylX3 (160 nM), KijD10 (360 nM), and NADPH (16.8 mM) in 3.1 mL of 50 mM Tris·HCl, pH 7.5. The reaction was incubated at 25 °C for 12 h, during which time reaction progress was monitored by HPLC with a Dionex Carbopac PA1 column. The HPLC program consisted of elutions with a gradient of water as solvent A and 500 mM ammonium acetate (adjusted to pH 7.0 with aqueous NH_3) as solvent B, where the gradient ran from 5 to 20% B over 15 min, from 20 to 60% B over 20 min, from 60 to 100% B over 2 min, wash at 100% B for 3 min, and from 100 to 5% B over 5 min, and concluded with re-equilibration at 5% B for 15 min. The flow rate was 1 mL/min, and the detector was set at 267 nm. When the reaction was greater than 80% complete, enzymes were removed by filtration through Microcon YM-10, and the filtrate was separated on a Bio-gel P2 gel filtration column (25 mm × 100 cm) prewashed with 25 mM NH_4HCO_3 and run at a flow rate of 12 mL/h with 25 mM NH_4HCO_3 as the eluant. Fractions (8 mL each) exhibiting UV absorbance at 267 nm were concentrated by partial lyophilization, and the identity and purity of the compounds in each fraction were analyzed by HPLC. Fractions containing TDP-4-keto-2,6-dideoxy-D-glucose (**16**) were pooled and concentrated by partial lyophilization. The concentration of **16** (>90% purity) was determined spectrophotometrically based on a molar extinction coefficient (ϵ) of 9600 M^{-1} cm^{-1} at 267 nm. The typical yield of **16** from 25 mg of starting material **13** ranges from 2 to 10 mg. ^1H NMR (300 MHz, D_2O) of **16**[50] (a mixture of hydrate and keto forms): δ 1.10 (3H, d, J = 6.4 Hz, 5-Me of the hydrate form), 1.18 (3H, d, J = 6.4 Hz, 5-Me of the keto form), 1.72−1.78 (1H, m, 2-H_{ax}), 1.82 (3H, s, 5″-Me), 2.05 (1H, m, 2-H_{eq}), 2.20−2.30 (2H, m, 2′-H), 3.91 (1H, dd, J = 12.9, 5.9 Hz, 3-H), 3.94 (1H, q, J = 6.4 Hz, 5-H), 4.06 (3H, m, 4′-H, 5′-H), 4.51 (1H, m, 3′-H), 5.50 (1H, dd J = 6.6, 4.0 Hz, 1-H), 6.24 (1-H, t, J = 6.9, 1′-H), 7.64 (1H, s, 6″-H). ^{31}P NMR (121 MHz, D_2O): δ −10.6 (d, J = 19.7 Hz), −12.6 (d, J = 19.7 Hz). High-resolution ESI-MS of **16**: calcd for $C_{16}H_{25}N_2O_{15}P_2$ $(M - H)^-$, 547.0725; found, 547.0691.

HPLC Activity Assays for KijB1, KijD10, KijD11, and KijC2. The general assay procedure for each protein involved incubation of the reaction mixture at 25 °C, from which aliquots (30 μL) were removed at various time points and quenched by flash-freezing in liquid nitrogen. Thawed at 4 °C, the mixture was filtered through a Microcon YM-10 membrane to remove enzyme and stored at −80 °C until HPLC analysis. HPLC analysis was performed with a Dionex Carbopac PA1

(47) Takahashi, H.; Liu, Y.-n.; Liu, H.-w. *J. Am. Chem. Soc.* **2006**, *128*, 1432−1433.
(48) Melançon, C. E., III; Hong, L.; White, J. A.; Liu, Y. N.; Liu, H.-w. *Biochemistry* **2007**, *46*, 577−590.
(49) Chen, H.; Agnihotri, G.; Guo, Z.; Que, N. L. S.; Chen, X.; Liu, H.-w. *J. Am. Chem. Soc.* **1999**, *121*, 8124−8125.
(50) Draeger, G.; Park, S.-H.; Floss, H. G. *J. Am. Chem. Soc.* **1999**, *121*, 2611−2612.

column using 20 μL of sample for each injection. The elution program was identical to that described above. The KijB1 activity assay mixture (100 μL) consisted of TDP-4-keto-6-deoxy-D-glucose (**13,** 1 mM) and KijB1 (20 μM in 50 mM KH_2PO_4, pH 7.5). The retention times were 33.6 min for **13** and 1.7 min for the degradation product maltol (**15**, Scheme 1). The KijD10 activity assay mixture (300 μL) contained **13** (1 mM), TylX3[49] (160 nM), KijD10 (360 nM), and NADPH (1.2 mM) in 50 mM Tris·HCl, pH 7.5, 20% glycerol. The retention time for TDP-4-keto-2,6-dideoxy-D-glucose (**16**) was 32.3 min. The KijD11 activity assay mixture (180 μL) contained **16** (1 mM) and KijD11 (100 nM) in 50 mM Tris·HCl, pH 7.5. The retention time of TDP-4-keto-2,6-dideoxy-L-allose (**17**) was 35.4 min, and that of (2*S*,3*S*)-2-methyl-3-hydroxy-4-keto-2,3-dihydropyran (**18**) was 2.4 min. The KijC2 activity assay mixture (180 μL) consisted of **16** (1 mM), KijD11 (100 nM), KijC2 (10 μM), NADH (1.4 mM), and $MgCl_2$ (10 mM) in 50 mM Tris·HCl, pH 7.5, 10% glycerol. The retention time of TDP-L-digitoxose (**19**) was 34.3−34.6 min, depending in its concentration in the sample. For confirmation of maltol (**15**) formation in the KijB1 reaction activity assay, the 1.7 min peak was collected from analytical HPLC and subjected to ESI-MS. High-resolution MALDI-MS of **15**: calculated for $C_6H_6O_3$ (M − H), 126.0317; found, 126.0312.

***In Situ* NMR Assay for KijD11 and Characterization of the Products.** A reaction mixture (600 μL) containing 10 mM TDP-4-keto-2,6-dideoxy-D-glucose (**16**), 50 mM KH_2PO_4 buffer (pH 7.5), and 5% (v/v) D_2O was prepared in an NMR tube. After shimming and initial peak integration in a 500 MHz NMR spectrometer, the sample was removed from the tube, mixed thoroughly with glycerol-free His_6-tagged KijD11 (final concentration 10 μM), and transferred back to the same NMR tube. Data were acquired at 5 min intervals for 180 min. The identity of the degradation product (2*S*,3*S*)-2-methyl-3-hydroxy-4-keto-2,3-dihydropyran (**18**) was determined on the basis of the spectral data generated during the *in situ* assay. At the end of the reaction, the sample was also subjected to correlation spectroscopy (COSY) to confirm the assignments. ^{1}H NMR (500 MHz, D_2O) of **18**: δ 1.41 (3H, d, J = 6.9 Hz, 2-Me), 4.12 (1H, d, J = 12.4 Hz, 3-H), 4.26−4.34 (1H, m, 2-H), 5.37 (1H, d, J = 5.8 Hz, 5-H), 7.46 (1H, d, J = 5.8 Hz, 6-H).

Enzymatic Synthesis, Purification, and Characterization of TDP-L-digitoxose (19). TDP-4-keto-2,6-dideoxy-D-glucose (**16**, 5 mg) was incubated with KijD11 (100 nM), KijC2 (10 μM), NADH (1.4 mM), and $MgCl_2$ (10 mM) in a total volume of 9.4 mL of 50 mM Tris·HCl buffer, pH 7.5, 10% glycerol to give a final concentration of 1 mM for **16**. This reaction mixture was divided into 200 μL aliquots, which were incubated at 25 °C for 16 h with gentle shaking. After this time, only trace amounts of substrate remained, and a new peak with retention time 34.3 min, corresponding to TDP-L-digitoxose (**19**), was visible by HPLC. The new product was isolated by FPLC using a Mono-Q 16/10 column. The sample was eluted by a gradient of water as buffer A and 250 mM aqueous NH_4HCO_3 as buffer B, where the gradient ran from 0 to 40% B over 5 min, held at 40% B for 5 min, then increased to 100% B over 20 min. The column was then washed using 100% buffer B for 12 min and re-equilibrated with 100% buffer A for 18 min. The flow rate was 4 mL/min, and the detector was set at 280 nm. The product peak was eluted at 65% B. Fractions containing the product were pooled and stored at −80 °C prior to desalting on a Bio-gel P2 column (15 × 100 mm) which had been prewashed with 1 L of water. After sample loading, the column was run at a flow rate of 5 mL/min using water as the eluant, with 8 mL fractions collected. Fractions showing UV absorption at 267 nm were collected and lyophilized, and the identity and purity of the compounds in each fraction were analyzed by HPLC as described above. Fractions containing **19** were combined, concentrated to 100 μL by lyophilization, and further purified by HPLC using a C_{18} column and isocratic water elution. The flow rate was 1 mL/min, and the detector was set at 267 nm. The TDP-L-digitoxose-containing peak, which eluted at approximately 5 min, was collected and concentrated by lyophilization, yielding about 500 μg of **19**. Low-resolution MALDI-MS of **19**: calculated for $C_{16}H_{26}N_2O_{14}P_2$ (M + H), 532; found, 532. Attempts to obtained high-resolution MS data (FAB, CI, ESI) and a ^{1}H NMR spectrum were unsuccessful.

To gain further evidence for the identity of TDP-L-digitoxose (**19**), the purified **19** was chemically derivatized for GC/MS analysis according to a previously reported procedure.[51] Specifically, compound **19** was acidified to pH 2.0 with concentrated HCl, boiled for 10 min to remove the TDP moiety, and then neutralized with concentrated KOH. The mixture was lyophilized to dryness and redissolved in 0.6 mL of 0.5 M NH_4OH. The resulting compound, L-digitoxose (**20**), was then mixed with dry DMF (1.0 mL) containing $NaBH_4$ (21 mg, 0.55 mmol). The reaction was stirred at 40 °C for 90 min and then slowly quenched with 0.1 mL of glacial acetic acid. To peracetylate **21** were added 1-methylimidazole (0.2 mg, 2.5 μmol) and freshly distilled acetic anhydride (6.0 mL), and the resulting mixture was stirred at room temperature for 1 h. The reaction was quenched with 5.0 mL of methanol at 0 °C. The products were extracted with chloroform three times, and the combined organic extracts were washed twice with water, dried over anhydrous sodium sulfate, filtered, and concentrated. The resulting sample, containing digitoxosyl tetraacetate (**22**), was then subjected to GC/EI-MS analysis. Low-resolution GC/EI-MS of **22**: 318 (the parent peak), 231 (C1−C4 and C3−C6 fragments), 245 (C2−C6 fragment).

Results and Discussion

Cloning and Sequencing of Kijanimicin Biosynthetic Genes. The conserved region of the TDP-glucose 4,6-dehydratase gene (*tylA2*) from *S. fradiae* was chosen as the probe for initial cosmid library screening because the presence of deoxysugars in the kijanimicin structure suggested that the cluster would contain genes for their biosynthesis; furthermore, TDP-activated sugars are generally used in the deoxysugar biosynthetic pathways characterized to date in Actinomycetes. Accordingly, a cosmid library was constructed from *A. kijaniata* genomic DNA, packaged into phage, and used to transfect *E. coli* XL-1 Blue MRF′ cells. Partial sequencing of several cosmids that gave positive hybridization results, together with additional cosmid library screening using probes designed from sequenced portions of the cluster, led to identification of 11 contiguous 25−40 kb cosmid inserts that appeared to span the entire gene cluster. Five cosmids, pHZ4-6, pHZP1, pHZP5, pHZ14, and pHZ8, were chosen for sequencing, yielding the sequence of 107.6 kb of contiguous DNA. Forty-four open reading frames (ORFs) (Figure 2) were identified within this region, with 35 being assigned to the kijanimicin (*kij*) cluster. These genes and their proposed functions are listed in Table 1. Introduction of exogenous DNA into *A. kijaniata* by various methods and conditions, including protoplast transformation, conjugation, and protoplast electroporation, was attempted. Unfortunately, this strain was recalcitrant to these genetic manipulations, so kijanimicin biosynthesis could not be studied by genetic methods.

Polyketide Assembly. Sequence analysis revealed the presence of five modular polyketide synthase genes (*kijS1*−*kijS5*) covering approximately 61 kb in the *kij* cluster (Figure 2). Together these genes encode a PKS complex comprised of a loading module and 11 extender modules (Figure 3). The nomenclature adopted for these PKS genes reflects the presumed order of action of the five proteins. Interestingly, *kijS4* is separated by 16.3 kb from the downstream end of *kijS5*, away from the other four PKS genes. The structure of kijanolide (**23**)

(51) Weigel, T.; Liu, L.; Liu, H.-w. *Biochemistry* **1992**, *31*, 2129−2139.

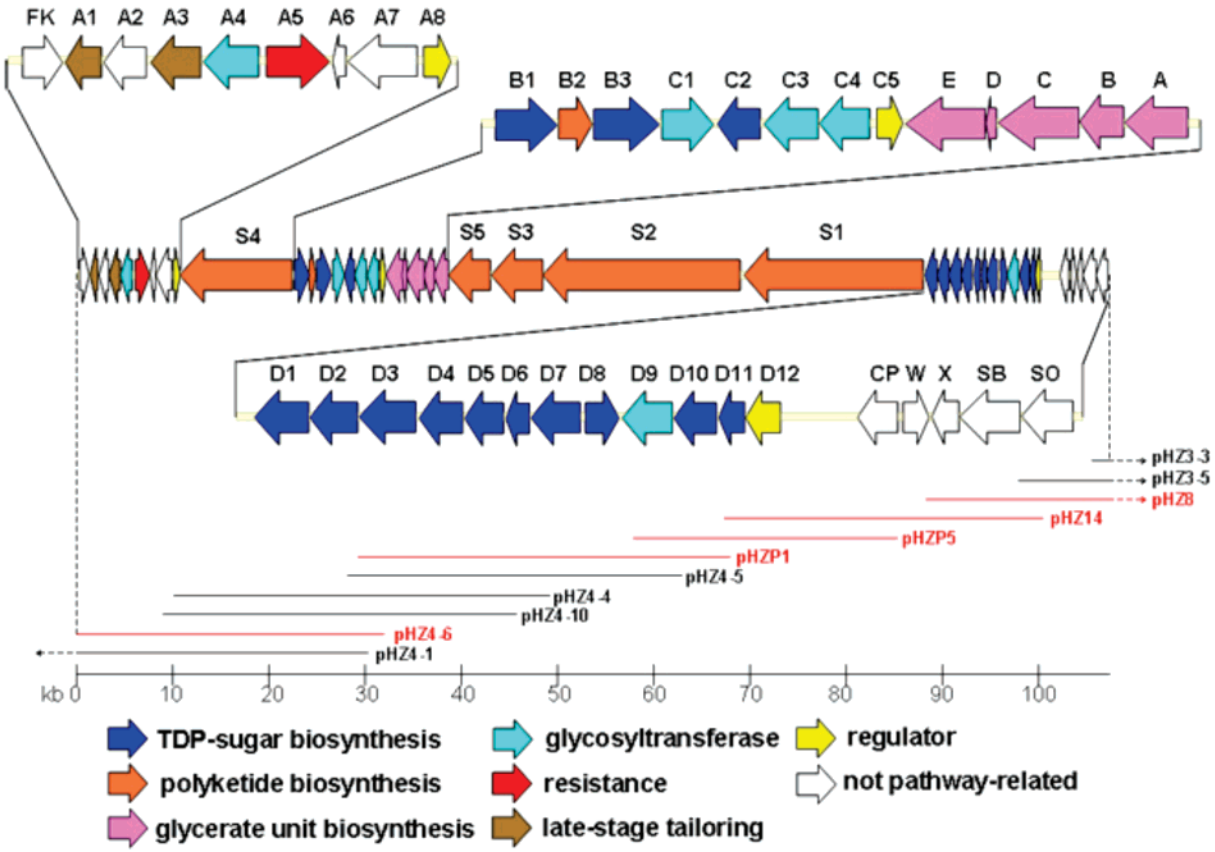


Figure 2. The 106.7 kb region of the *Actinomadura kijaniata* chromosome containing the kijanimicin gene cluster, showing open reading frames (ORFs) and cosmid boundaries. Cosmids shown in red were used to determine the nucleotide sequence of the cluster.

and the domain organization of *kijS1*−*kijS5* indicate that *kijS1* encodes the loading module along with three extender modules (1−3), and *kijS2* encodes the following four modules (4−7). The remaining three PKS genes are smaller, with *kijS3* encoding module 8, *kijS4* encoding modules 9 and 10, and *kijS5* encoding module 11. The domain architecture and the predicted structures of ACP-bound polyketide intermediates are shown in Figure 3.

The loading module (L) contains a KS-Q domain, which is responsible for decarboxylation of the ACP-bound malonate starter unit to form acetyl-*S*-ACP-L at the beginning of polyketide formation. Sequence comparison of the AT domains in the *kij* cluster (in particular residues at positions 93, 197, 198, and 200, whose identities are known to correlate with substrate specificity)[52,53] with known PKS systems predicts that modules L, 1−3, 5, and 8−10 are specific for methylmalonyl-CoA, whereas modules 4, 6, 7, and 11 are specific for malonyl-CoA. For the most part, this is consistent with the structure of kijanolide (**23**). Exceptions occur in modules L and 2, which appear to be malonyl-CoA specific, based on the structure of the final product. Analysis of the dendrogram of the *kij* AT domains shows that AT-L and AT-2 are closely related and constitute a subclass of predicted *kij* methylmalonyl-CoA specific AT domains. It is therefore conceivable that these two domains are exceptions to the predictive model and do indeed incorporate malonyl-CoA.

The presence of a conserved Asp residue, which correlates with the observed D-configuration of KR domain products[54] in each of the *kij* KR domains, predicts that all domains should exhibit specificity for the D-product. This is consistent with the chirality of the C-9 and C-17 hydroxyl groups in the structure of kijanolide (**23**), which both have the D-configuration. The remnants of a highly diverged and obviously inactive ketoreductase domain, designated KR11, are found in KijS5. The DH domains in modules 4 and 8 are predicted to be inactive on the basis of the structure of kijanolide (**23**), which has hydroxyl groups at C-9 and C-17. In accordance with this observation, the histidine thought to be the catalytic base[55] has been replaced by tyrosine in DH-4 and DH-8. Last, KijB2 is homologous to thioesterases thought to be involved in polyketide "editing", that is, the removal of aberrant polyketide intermediates stalled on the PKS assembly line. Thus, KijB2 likely serves this function.

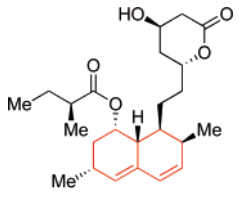
Kijanolide Maturation. In view of the structure of the linear polyketide intermediates predicted from analysis of the *kij* PKS, it is clear that the octahydronaphthalene ring structure of kijanolide (**23**, highlighted in red in Scheme 2) is formed by an intramolecular cyclization between diene (C10−C13) and dienophile (C4, C5) which resembles a "Diels−Alder-type"

(52) Haydock, S. F.; Aparicio, J. F.; Molnar, I.; Schwecke, T.; Khaw, L. E.; Koenig, A.; Marsden, A. F. A.; Galloway, I. S.; Staunton, J.; Leadlay, P. F. *FEBS Lett.* **1995**, *374*, 246−248.
(53) Yadav, G.; Gokhale, R. S.; Mohanty, D. *J. Mol. Biol.* **2003**, *328*, 335−363.
(54) (a) Reid, R.; Piagentini, M.; Rodriguez, E.; Ashley, G.; Viswanathan, N.; Carney, J.; Santi, D. V.; Hutchinson, C. R.; McDaniel, R. *Biochemistry* **2003**, *42*, 72−79. (b) Caffrey, P. *ChemBioChem* **2003**, *4*, 654−657.
(55) Wu, J.; Zaleski, T. J.; Valenzano, C.; Khosla, C.; Cane, D. E. *J. Am. Chem. Soc.* **2005**, *127*, 17393−17404.

Table 1. Kijanimicin Biosynthetic Gene Cluster ORFs and Their Predicted Functions

gene	BLAST functional assignment	predicted function in kijanimicin biosynthesis
FK	fructokinase	not pathway related
kijA1	sugar *O*-methyltransferase	4-*O*-methylation of terminal digitoxose and/or kijanose
kijA2	oxidoreductase/aldo/ketoreductase	not pathway related
kijA3	P450 monooxygenase	hydroxylation of aglycone C-32 methyl group
kijA4	glycosyltransferase	digitoxosyltransferase
kijA5	efflux permease	kijanimicin export
kijA6	heat shock protein GroES	not pathway related
kijA7	heat shock protein GroEL	not pathway related
kijA8	tet^R-type regulatory protein	pathway regulation
kijS4	polyketide synthase	PKS modules 9, 10
kijB1	sugar 2,3-dehydratase	digitoxose, kijanose biosynthesis
kijB2	thioesterase	PKS editing
kijB3	FAD-dependent oxidoreductase	formation of kijanose methylcarbamate moiety
kijC1	glycosyltransferase	digitoxosyltransferase
kijC2	sugar 4-ketoreductase	digitoxose biosynthesis
kijC3	glycosyltransferase	digitoxosyltransferase
kijC4	glycosyltransferase	digitoxosyltransferase
kijC5	tet^R-type regulatory protein	pathway regulation
kijE	*C*-terminal domain, α/β-hydrolase superfamily; *N*-terminal domain, dihydrolipoamide acyltransferase	*C*-terminal domain, thioesterase, formation of five-membered lactone; *N*-terminal domain, transfer of glycerate from KijD to CoA
kijD	ACP	glycerate carrier protein
kijC	FkbH homologue	formation of glyceryl-*S*-KijD from 1,3-BPG (**29**)
kijB	ketoacyl acyl carrier protein synthase III	condensation of glycerate and C-2 of polyketide chain
kijA	FAD-dependent oxidoreductase	aglycone cyclization to form spirotetronate
kijS5	polyketide synthase	PKS module 11
kijS3	polyketide synthase	PKS module 8
kijS2	polyketide synthase	PKS modules 4−7
kijS1	polyketide synthase	PKS loading, modules 1−3
kijD1	sugar 3-*C*-methyltransferase	kijanose biosynthesis
kijD2	sugar 3-aminotransferase	kijanose biosynthesis
kijD3	FAD-dependent oxidoreductase	amine oxidation of kijanose
kijD4	E_{od} (NDP-sugar 4,6-dehydratase)	digitoxose, kijanose biosynthesis
kijD5	E_p (sugar nucleotidyltransferase)	digitoxose, kijanose biosynthesis
kijD6	NADPH-dependent flavin reductase	flavoenzyme reductase
kijD7	sugar 4-aminotransferase	kijanose biosynthesis
kijD8	SAM-dependent methyltransferase	*N*-methylation of C-4 amine of kijanose
kijD9	glycosyltransferase	kijanosyltransferase
kijD10	sugar 3-ketoreductase	digitoxose biosynthesis
kijD11	sugar 5-epimerase	digitoxose biosynthesis
kijD12	tet^R-type regulatory protein	pathway regulation
CP	D-Ala, D-Ala carboxypeptidase	not pathway related
orfW	conserved hypothetical protein	not pathway related
orfX	permease	not pathway related
SB	selenium binding protein	not pathway related
SO	sulfite oxidase/nitrate reductase	not pathway related

coupling reaction (Scheme 2, **24** → **25**). However, a comparison of genes in the chlorothricin (*chl*)[37] and kijanimicin clusters failed to reveal any obvious candidates to encode an enzyme with this activity. In the case of the polyketide-derived natural product lovastatin (**26**), which also contains an octahydronaphthalene ring system (highlighted in red), *in vitro* work with the purified PKS showed that it is capable of converting a linear *N*-acetylcysteamine mimic of the appropriate enzyme-bound polyketide intermediate to the correct isomer of the octahydronaphthalene moiety.[56] By analogy to lovastatin, it is possible that the *kij* PKS module 10 catalyzes this reaction when the polyketide intermediate is tethered to the ACP of this module (Scheme 2). In this scenario, polyketide intermediate **27** would undergo DH-10-catalyzed dehydration to give dienophile **24**, which would then undergo cyclization to form the octahydronaphthalene-containing polyketide **25**. This reaction might also be catalyzed within module 11 or perhaps by an enzyme further downstream in the kijanimicin biosynthetic pathway.



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Labeled precursor feeding experiments performed on chlorothricin (**4**) using [$^{13}C_3$]-pyruvate and [$^{13}C_3$]-lactate showed no incorporation of radiolabels, indicating that neither pyruvate nor lactate is a direct precursor of the three-carbon unit (C24−C26).[28] It was suggested that this fragment might be produced via a primary metabolic route in which glycerol is an intermediate. Examination of the kijanimicin gene cluster reveals a group of five ORFs (*kijABCDE*) immediately downstream from and transcriptionally coupled to *kijS5* (Figure 2), implicating a functional link between these gene products and the final steps in PKS assembly. Interestingly, ORFs with high sequence similarity to *kijA*−*E* were found adjacent to each other within several polyketide natural product gene clusters, including those

(56) Auclair, K.; Sutherland, A.; Kennedy, J.; Witter, D. J.; Van, den Heever, J. P.; Hutchinson, C. R.; Vederas, J. C. *J. Am. Chem. Soc.* **2000**, *122*, 11519−11520.

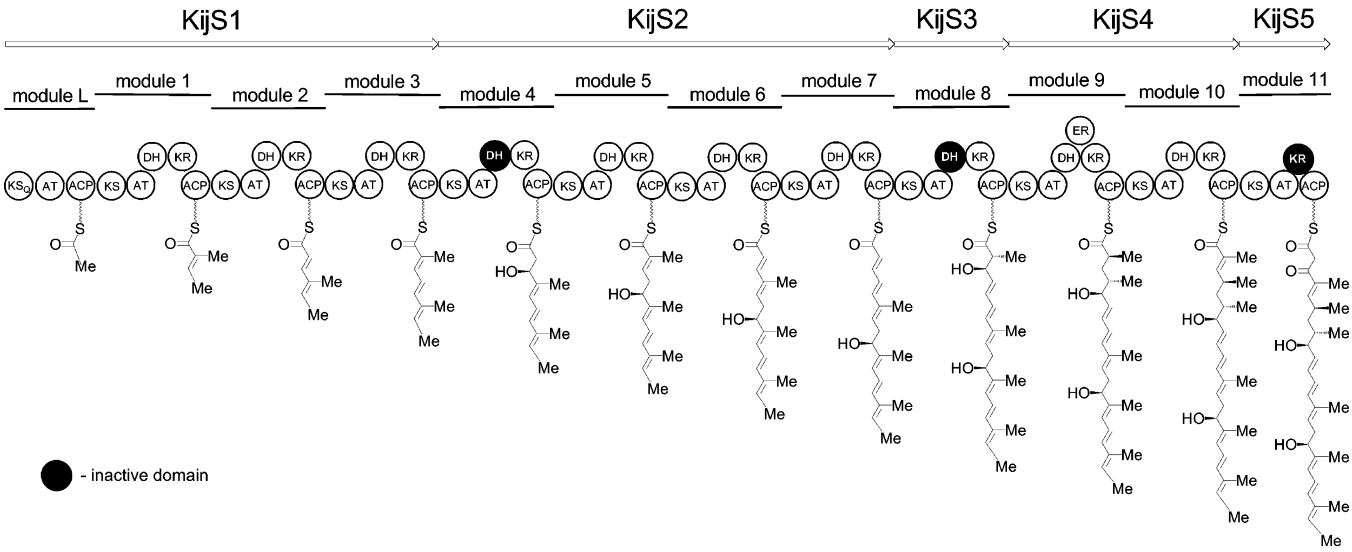
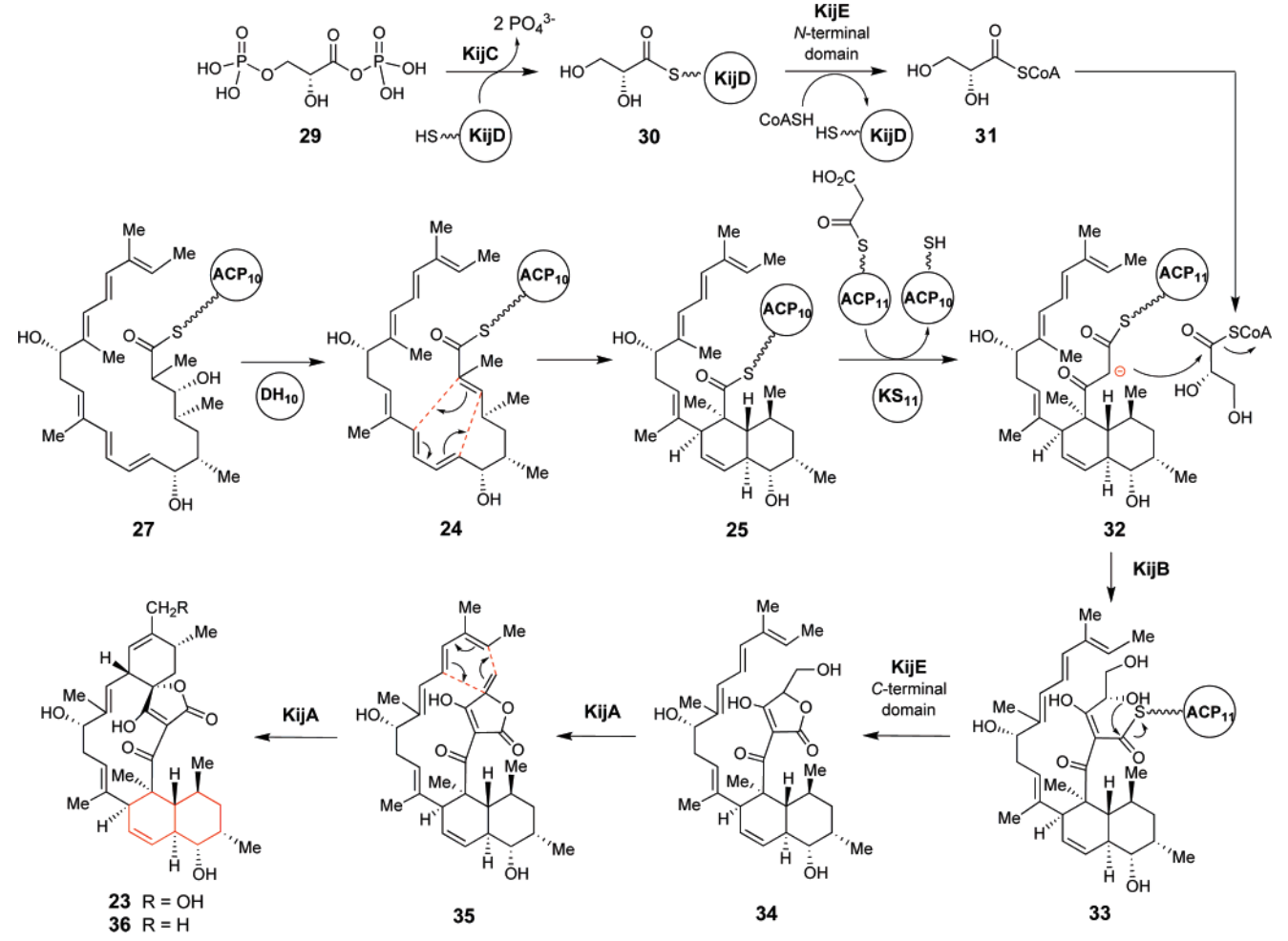


Figure 3. Kijaminicin modular polyketide synthase (PKS) encoded by *kijS1*−*S5*, showing modules L and 1−11 and predicted ACP-bound polyketide intermediates.

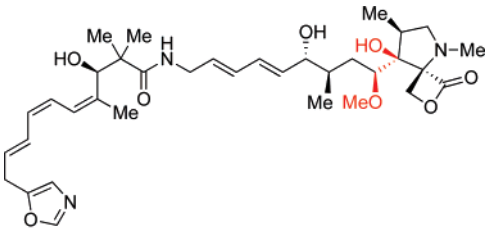
Scheme 2



of chlorothricin (**4**) and tetronomycin (**8**), suggesting that these enzymes work together. Cluster sequencing and genetic studies of chlorothricin biosynthesis led to the proposal that ChlD1, ChlD2, ChlD3, and ChlD4, which are homologues of KijC, KijD, the *N*-terminal domain of KijE, and the *C*-terminal domain of KijE, respectively, are involved in the biosynthesis of the three-carbon unit.[37] Hence, we propose that the five proteins encoded by the *kijABCDE* operon are involved in activation of the glycerol-derived three-carbon unit, its attachment to the polyketide chain, and subsequent cyclization steps required to generate the mature spirotetronate ring system (Scheme 2). Although this proposed scheme shares some similarity with that

proposed for chlorothricin, there are several important differences, which are highlighted below.

Analysis of the sequence of KijC reveals modest similarity to phosphatases (26−60% identity) in the haloacid dehalogenase (HAD) superfamily, which have been proposed to load a three-carbon glycolytic intermediate onto an ACP domain in methoxymalonyl-ACP biosynthesis in several natural product pathways.[57−62] Recent biochemical studies with OzmB, a KijC homologue from the biosynthetic pathway of oxazolomycin (**28**, methoxymalonyl unit shown in red), show that OzmB acts as a



28

bifunctional phosphatase/glyceryltransferase using D-1,3-bisphosphoglycerate (**29**) as a substrate to load a D-glycerate (**30**) unit onto a stand-alone ACP, OzmE, encoded in the same cluster.[61] Labeled precursor feeding experiments in ansamitocin and soraphen producers also implicate **29** as the source of their methoxymalonyl units.[62] These findings strongly suggest that KijC catalyzes the identical reaction as OzmB, loading **30** onto the stand-alone ACP, KijD (Scheme 2). The KijC homologue in the *chl* cluster, ChlD1, was also proposed to load a three-carbon glycolytic intermediate onto the stand-alone ACP domain ChlD2.[37]

KijE appears to be a bifunctional protein, with the *N*-terminal domain showing modest sequence homology (20−25% identity) to the dihydrolipoyl acyltransferase (E_2) subunit of several bacterial pyruvate dehydrogenase complexes, and the *C*-terminal domain showing homology to proteins of unknown function belonging to the α/*β*-hydrolase superfamily (32−56% identity). Since all homologues of KijE *N*- and *C*-terminal domains are encoded as discrete polypeptides, the bifunctionality of KijE is likely the result of a gene fusion event rather than a functional requirement. It had been demonstrated that E_2 in the pyruvate dehydrogenase complex uses a conserved histidine to transfer an acetyl group from the terminal sulfhydryl group of its lipoamide cofactor to coenzyme A, forming acetyl-CoA.[63] The KijE *N*-terminal domain possesses this conserved histidine residue (His227). Other KijE *N*-terminal domain homologues, such as chloramphenicol acetyltransferase, catalyze mechanistically similar acyl-CoA-dependent acyl-transfer reactions without the use of lipoamide. We therefore propose that the KijE *N*-terminal domain catalyzes acyl transfer of the glyceryl group from glyceryl-*S*-KijD to coenzyme A, forming glyceryl-CoA (**31**, Scheme 2). While gene disruption experiments demonstrated that ChlD3, the homologue of the KijE *N*-terminal domain, is essential for chlorothricin (**4**) formation, this enzyme was instead proposed to catalyze dehydration of **30** to form pyruvyl-*S*-ACP.[37]

KijB is homologous to *β*-ketoacyl-ACP-synthase III proteins such as *E. coli* FabH, which catalyzes decarboxylative condensation of malonyl-ACP and an acyl-CoA to form a chain-extended ACP-bound polyketide intermediate.[64] It is thus reasonable to propose that KijB performs a similar role, catalyzing the condensation between a carbanion generated at C-2 of kijanolide polyketide-*S*-ACP-11(**32**) and glyceryl-CoA (**31**) to form the branched chain intermediate **33** tethered to ACP-11 (Scheme 2). ChlM, the KijB homologue in the *chl* cluster, was shown by gene disruption to be non-essential for formation of **4** in the producing strain *Streptomyces antibioticus*.[37] This is surprising given the close association of *chlM*, *kijB*, and their homologues with the other genes thought to be involved in three-carbon unit utilization. It is possible that an enzyme capable of substituting for ChlM may be present in *S. antibioticus*.

The α/*β*-hydrolase superfamily, of which the KijE *C*-terminal domain is predicted to be a member, is a functionally diverse group of enzymes, many of which catalyze the hydrolysis of an ester, a thioester, or an amide group using a catalytic triad (Ser, Asp, His). Several members of this superfamily, such as polyhydroxyalkanoic acid (PHA) synthase, also catalyze ester bond formation using acyl-CoA donors. Analysis of the KijE *C*-terminal domain sequence showed the presence of the conserved catalytic triad (S457, D544, H571). Hence, the KijE *C*-terminal domain is likely a thioesterase that catalyzes the formation of the five-membered ring lactone intermediate **34** and the release of the polyketide from ACP-11. ChlD4, the homologue of the KijE *C*-terminal domain in the *chl* cluster, was shown to be essential for formation of **4** and was proposed to transfer the enolpyruvoyl moiety from the ACP (ChlD2) to C-1 of the tethered polyketide intermediate, releasing it from the PKS.[37] This is similar to our proposal, except that C−O bond formation would occur in an *inter*- rather than *intra*molecular manner.

KijA is homologous to several putative flavin-dependent monooxygenase/oxidoreductases found in Type-II PKS-derived antibiotic biosynthetic pathways, such as those of mithramycin, landomycin, and jadomycin. Recently, a KijA homologue, JadH (38% identity, 48% similarity), was shown *in vitro* to catalyze a dehydration reaction of a polyketide intermediate in jadomycin biosynthesis.[65] Also, a homologue of KijA, Tmn9, is present in the gene cluster of tetronomycin (**8**), which contains a tetronic acid moiety (highlighted in red in Chart 2, with red dots indicating carbons derived from glycerol). Taken together, these findings suggest that KijA catalyzes the dehydration of **34** to form the tetronic acid intermediate **35**, and that KijA also likely promotes intramolecular cyclization between the diene and dienophile of **35** via proximity and orientation effects, resulting in formation of the mature aglycone, 32-deoxy kijanolide (**36**),

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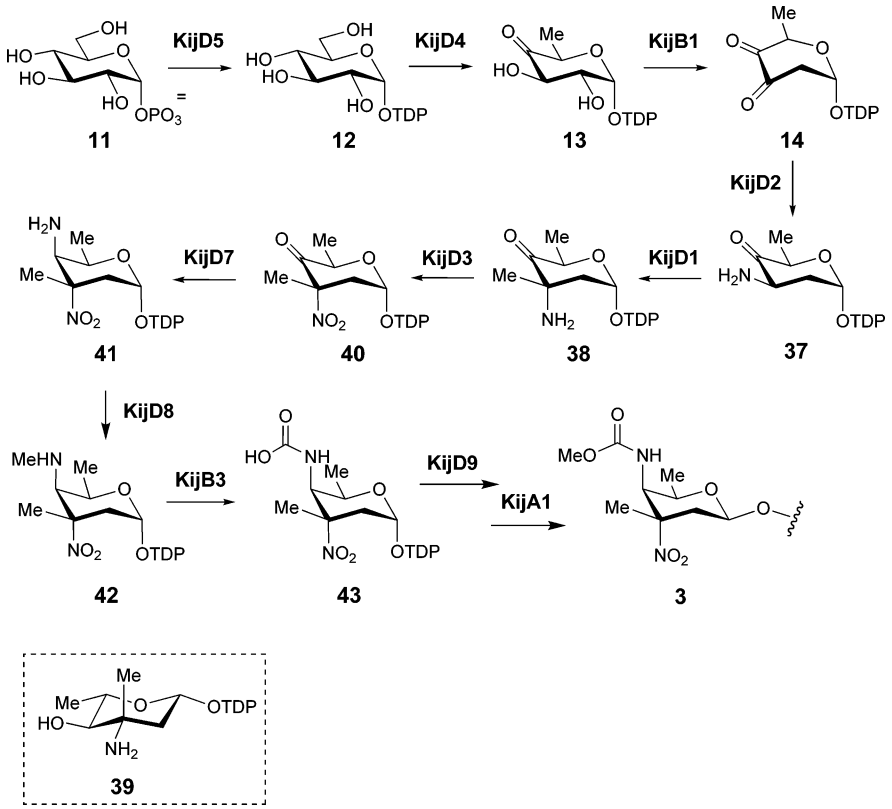
Scheme 3



in the KijA active site. The cyclization reaction may proceed via a concerted (i.e., Diels−Alder-like) or a stepwise reaction mechanism. Overall, the *kijABCDE* operon appears to constitute a functional unit encoding enzymes responsible for tetronic acid/spirotetronate formation in polyketide-derived natural products.

Digitoxose Biosynthesis. Kijanimicin (**1**) contains five deoxysugars, of which four are L-digitoxose (**2**) and one is D-kijanose (**3**). On the basis of protein sequence alignments to known deoxysugar biosynthetic enzymes, the genes involved in the formation of these sugars can be identified and the overall biosynthetic pathways can be proposed. Since sequences of the sugar biosynthesis enzymes in the *kij* cluster closely resemble those of known TDP-sugar enzymes, all sugar biosynthesis enzymes in the kijanimicin pathway are expected to use TDP-activated substrates. The proposed biosynthetic pathways for L-digitoxose (**2**) and D-kijanose (**3**) are shown respectively in Schemes 1 and 3. The *kijD5* and *kijD4* genes encode D-glucose-1-phosphate thymidylyltransferase (E_p) and TDP-D-glucose 4,6-dehydratase (E_{od}), which catalyze the conversion of glucose-1-phosphate (**11**) to TDP-D-glucose (**12**), and **12** to TDP-4-keto-6-deoxy-D-glucose (**13**), respectively. Subsequent conversion of **13** to TDP-2,6-dideoxy-3,4-diketo-D-glucose (**14**) is catalyzed by KijB1, which is a sugar 2,3-dehydratase. In the TDP-L-digitoxose (**19**) pathway, the C-3 reduction of **14** is expected to be the next step and is catalyzed by the 3-ketoreductase KijD10. Since KijD10 shares high sequence identity with other TDP-sugar 3-ketoreductases, such as SpnN (47%),[66] Gra26 (52%), and OleW (55%),[50] which generate products with an equatorial C-3 hydroxyl group, the product of KijD10 is predicted to be TDP-4-keto-2,6-dideoxy-D-glucose (**16**). The *kjD11* gene encodes an epimerase, which likely catalyzes the conversion of **16** to TDP-4-keto-6-deoxy-L-allose (**17**). The final reduction of **17**, catalyzed by the 4-reductase KijC2, completes the biosynthesis of TDP-L-digitoxose (**19**).

To verify the proposed TDP-L-digitoxose biosynthetic scheme, each of the four proposed biosynthetic enzymes, KijB1, KijD10, KijD11, and KijC2, was individually overexpressed in *E. coli* as a His_6-tagged protein and purified by Ni-NTA affinity chromatography. To test its proposed 2,3-dehydratase activity, KijB1 was incubated with TDP-4-keto-6-deoxy-D-glucose (**13**, Figure 4, trace A), which was prepared enzymatically using a reported two-stage, one-pot methodology.[47] As was previously observed for other TDP-sugar 2,3- dehydratases (such as TylX3, Gra27, and OleV),[49,50] **13** was rapidly depleted, but the anticipated product TDP-3,4-diketo-2,6-dideoxy-D-glucose (**14**) could not be directly observed. Rather, a degradation product, maltol (**15**), formed by non-enzymatic elimination of TDP from **14**, was detected by high-resolution ESI-MS. This observation provided evidence for the role of KijB1 as a 2,3-dehydratase. In subsequent assays, the 2,3-dehydratase TylX3 from the mycarose pathway of *S. fradiae*[49] was used in lieu of KijB1 because TylX3 is more easily produced. Since reduction of **14** yields a more stable product, incubation of **13** with TylX3, KijD10, and NADPH resulted in its nearly complete conversion to a new compound (retention time of 32.3 min, Figure 4, trace B). Interestingly, no new peak was observed when NADH was substituted for NADPH. To definitively identify the new product, the same reaction was carried out on a preparative scale, and the product was purified and characterized by ^1H and ^{31}P NMR spectroscopies and high-resolution ESI-MS. The results clearly showed the product is TDP-4-keto-2,6-dideoxy-D-glucose (**16**), indicating that KijD10 is a 3-ketoreductase.

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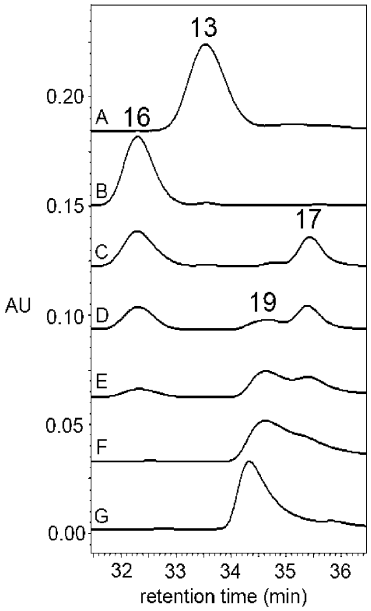

Figure 4. Enzymatic synthesis of TDP-L-digitoxose (**19**). (A) Purified TDP-4-keto-6-deoxy-D-glucose (**13**). (B) Incubation mixture containing **13** (1 mM), TylX3 (160 nM), KijD10 (360 nM), and NADPH (1.2 mM) in 50 mM Tris·HCl, pH 7.5, 20% glycerol showing formation of TDP-4-keto-2,6-dideoxy-D-glucose (**16**). (C) Incubation mixture containing **16** (1 mM) and KijD11 (100 nM) in 50 mM Tris·HCl, pH 7.5, showing formation of TDP-4-keto-2,6-dideoxy-L-allose (**17**). (D−G) TDP-4-keto-2,6-dideoxy-D-glucose (**16**, 1 mM), KijD11 (100 nM), KijC2 (10 μM), NADH (1.4 mM), and $MgCl_2$ (10 mM) in 50 mM Tris·HCl, pH 7.5, 10% glycerol after 1 h, 2, 4, and 8 h, respectively, showing time-dependent formation of **19**.

The activity of the proposed 5-epimerase KijD11 was examined next by incubation of purified **16** with the enzyme. HPLC analysis showed that the substrate was converted to a new product (retention time of 35.4 min, Figure 4, trace C), which rapidly degraded to TDP and a second compound (retention time of 2.4 min). Apparently, the KijD11 product is unstable and undergoes rearrangement and elimination of TDP to form a more stable pyran derivative with retention time of 2.4 min. A similar non-enzymatic degradation has been observed for the unstable TDP-sugar product of the Tyl1a reaction.[48] The KijD11 reaction and its products were further studied by an *in situ* ^{1}H NMR assay. Spectra were taken at 5 min intervals for 180 min (shown in Figure 5). Time-dependent disappearance of **16** and concomitant appearance of a new set of TDP-sugar signals are evident. Those signals corresponding to the KijD11 product, TDP-4-keto-2,6-dideoxy-L-allose (**17**), reached maximum intensity at ~20 min and then diminished. A third set of signals corresponding to the degradation product appeared after a short lag. The ^{1}H NMR and COSY spectra taken at the end of reaction allowed the verification of (2*S*,3*S*)-2-methyl-3-hydroxy-4-keto-2,3-dihydropyran (**18**) as the degradation product. Figure 6 shows the change in peak integration of the 5-methyl of **16**, 5-methyl of **17**, and 2-methyl of **18** over time, revealing the time course of the conversion of **16** to **17** by KijD11 and the subsequent degradation of **17** to **18**.

To test whether the KijD11 product (**17**) is the substrate for the proposed 4-ketoreductase KijC2, **16** was incubated with both KijD11 and KijC2 in the presence of NADH or NADPH. No new peak was observed in either incubation mixture. After repeating the assay under several conditions, formation of a new product (retention time of 34.3−34.6 min) was eventually detected in a reaction mixture containing **16**, KijD11, KijC2, NADH, and Mg^{2+} in 50 mM Tris·HCl buffer containing 10% glycerol (Figure 4, traces D−G). A significant decrease of the degradation product **18** of the KijD11 reaction was also noted in this assay mixture. These results are consistent with the formation of TDP-L-digitoxose (**19**) by the tandem action of KijD11 and KijC2. Formation of **19** was indicated by MALDI-MS analysis of the purified KijC2 product and later confirmed by GC/MS analysis of its peracetylated derivative **22**. Thus, the entire TDP-L-digitoxose biosynthesis pathway has been elucidated by *in vitro* biochemical characterization. This work provides compelling evidence indicating that the genes in the *kij* cluster are relevant to kijanimicin biosynthesis. These results also provide the foundation for future functional characterization of the glycosyltransferases involved in kijanimicin biosynthesis and for the *in vitro* glycodiversification of kijanolide.

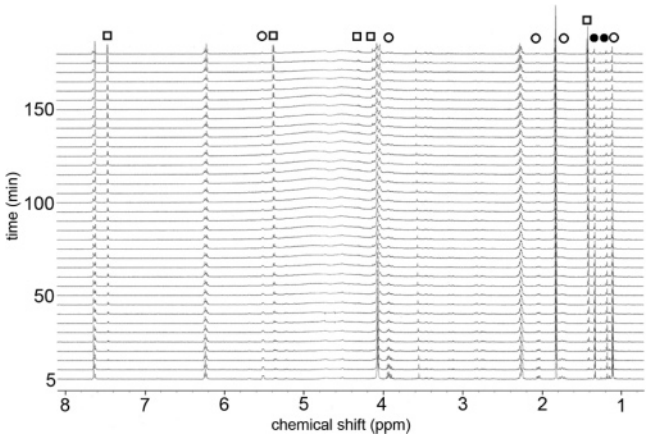

Figure 5. ^{1}H NMR stack plot of the KijD11 reaction (10 mM **16** and 10 μM Tyl1a in 50 mM KH_2PO_4 buffer, pH 7.5) monitored over 180 min. The signals corresponding to each compound are labeled: (○) from **16**, (●) from **17**, and (□) from **18** (see Experimental Section for details).

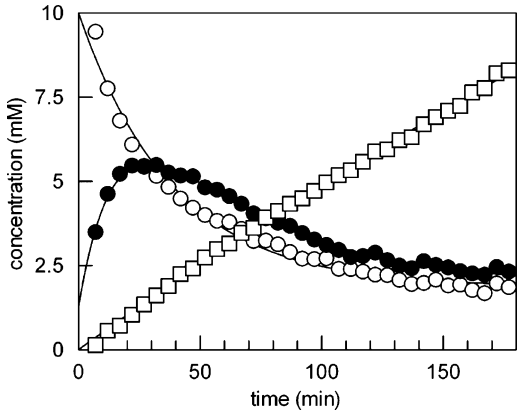

Figure 6. Plot of the integration of the ^{1}H NMR signals of the 5-methyl group of **16** (○) and **17** (●) and that of the 2-methyl group of **18** (□) during the *in situ* ^{1}H NMR assay (10 mM **16** and 10 μM KijD11 in 50 mM KH_2PO_4 buffer, pH 7.5, and 5% (v/v) D_2O) (see Experimental Section for details).

Kijanose Biosynthesis. D-Kijanose (**3**) is one of the most highly functionalized sugars found in nature. The biosynthesis of kijanose is expected to include several interesting and as yet uncharacterized enzymatic transformations likely involving unusual chemistry. As shown in Scheme 3, a pathway for the formation of **3** can be proposed on the basis of a combination of sequence analysis of enzymes encoded in the *kij* cluster and chemical intuition of functional group transformations in biological systems. The genes believed to participate in the

formation of kijanose are assigned on the basis of sequence similarities to other sugar biosynthetic genes. The steps leading up to formation of **14** are identical to those in the TDP-L-digitoxose (**19**) biosynthetic pathway.

The aminotransferase KijD2 is predicted to convert **14** to **37**, which is then converted to **38** by the methyltransferase KijD1. These two enzymes are homologous to EvaB and EvaC, which have been shown to catalyze identical reactions in the TDP-L-eremosamine (**39**) pathway of *Amycolatopsis orientalis*.[67] Oxidation of the 3-amino group of **38** to form **40** is likely catalyzed by the flavoprotein KijD3. KijD3 shares high sequence identity with EvdC from the evernimicin (**9**) biosynthetic pathway of *Micromonospora carbonacea* (61% identity, 72% similarity)[39] and with RubN8 from the rubradirin (**10**) biosynthetic pathway of *Streptomyces achromogenes* (57% identity, 68% similarity).[40] Evernimicin and rubradirin have in their structures the 2,3,6-trideoxy-3-*C*-methyl-3-nitrosugars L-evernitrose (**40**, see structure **9**) and D-rubranitrose (**41**, see structure **10**), respectively, which are similar in structure to **3**. Due to the high sequence identity among these three gene products and the lack of other oxidase proteins common to all three clusters, we propose that KijD3, EvdC, and RubN8 are oxidases that convert the C-3 amino groups of TDP-sugars to nitro groups. Since **40**, **41**, and **3** all possess identical 3-*C*-methyl-3-nitro functionalities and the evernimicin and rubradirin gene clusters share well-conserved three-gene biosynthetic operons encoding KijD1, KijD2, and KijD3 homologues, we propose that these three enzymes (3-aminotransferase, 3-*C*-methyltransferase, and the flavin oxidase) catalyze identical reactions in all three pathways, converting **14** → **37** → **38** → **40**.

Preliminary biochemical work on RubN4 and RubN6, the KijD2 homologue and 4-ketoreductase in D-rubranitrose biosynthesis, respectively, suggests that 4-ketoreduction of **14** occurs prior to 3-amino transfer in that pathway, and also demonstrates that RubN4 can convert **14** to **37**.[68] The putative NAD(P)H-dependent flavin reductase KijD6, which is located near KijD3 in the cluster, may assist KijD3 by regenerating reduced flavin. However, homologues of KijD6 are absent in the evernimicin and rubradirin clusters, so a generic reductase may be used in the amino oxidation in evernimicin and rubradirin biosyntheses. The spirotetronate antibiotics lobophorins[30] and arisostatins[31] have structural skeletons similar to that of kijanimicin and also possess D-kijanose moieties at C-17 of the aglycone. Interestingly, congeners of these compounds have been isolated carrying a 3-amino-D-kijanose rather than **3** at C-17. While this might suggest either that amine oxidation occurs after glycosyl transfer or that these kijanosyltransferases are promiscuous, the high degree of sequence identity among KijD3 homologues in spite of the marked structural differences among the aglycones of **1**, **9**, and **10** makes the latter possibility more likely. Next, the C-4 aminotransferase KijD7 is predicted to convert **40** to **41**. KijD7 is highly similar to SpnR (57% identity, 69% similarity), the sugar C-4 aminotransferase involved in TDP-D-forosamine biosynthesis in the spinosyn pathway of *Saccharopolyspora spinosa*.[66]

Since there are no reports on the biosynthesis of methylcarbamate moieties, it is not apparent what types of enzymatic activities might be required to construct this functional group present at C-4 of **3**. Whether its formation occurs on a TDP-sugar intermediate, such as **41**, or via a series of tailoring steps after the sugar precursor has been attached to the aglycone is also uncertain. One might envision either direct carboxylation of the C-4 amine of **41** or formylation followed by oxidation to the carboxylate as possible steps in this pathway. However, no genes with homology to known formyltransferases or carboxylating enzymes are found in the *kij* cluster. In view of the remaining unassigned genes in the *kij* cluster and basic biosynthetic logic, we speculate that formation of the methylcarbamate group might be a collaborative effort of three enzymes. The methyltransferase, KijD8, could be responsible for *N*-methyl transfer to the C-4 amine of **41**, resulting in **42**. The flavin-dependent oxidoreductase, KijB3, may then oxidize the methyl group to a carboxylate group, forming **43**. No homologues of KijB3 are encoded in the chlorothricin (**4**) or tetronomycin (**8**) clusters, suggesting that this enzyme catalyzes a step unique to kijanimicin biosynthesis. Finally, the sugar *O*-methyltransferase homologue KijA1 may catalyze the last step to generate D-kijanose (**3**). Since most sugar *O*-methyltransfer steps occur after glycosyl transfer in natural product pathways, it is possible that methylation of the carbamate moiety occurs in this manner.

Glycosyl Transfer Reactions and Tailoring Steps. In accordance with the presence of five deoxysugars in the structure of kijanimicin, there exist five glycosyltransferases (GTs) encoded in the *kij* gene cluster, KijA4, KijC1, KijC3, KijC4, and KijD9. Sequence comparison of these GTs reveals that KijD9 is significantly different from the other four GTs, suggesting that it catalyzes attachment of **3** or its precursor to the C-17 hydroxyl group of kijanolide (**23**) or 32-deoxykijanolide (**36**). The remaining GTs are highly similar to each other (51−60% pairwise identity) as well as to the chlorothricin GTs, ChlC6 and ChlC7, strongly suggesting they are digitoxosyltransferases responsible for construction the C-9 oligosaccharide of kijanimicin. It is not possible to predict the order in which these four GTs act. However, it is interesting to note that one of the terminal digitoxose residues is attached via a $\beta(1\rightarrow4)$ glycosidic linkage, indicating that the corresponding GT-catalyzed attachment of this sugar proceeds with retention of configuration. This is in contrast to the other digitoxosyltransferase-catalyzed reactions, which form $\alpha(1\rightarrow3)$ linkages and therefore should proceed with inversion of configuration. The discovery of GTs sharing such high sequence identity but employing different mechanisms is intriguing.

In addition to glycosyl transfer, there are two tailoring steps for which candidate genes can be identified in the *kij* cluster. The $\beta(1\rightarrow4)$-linked digitoxose residue is 4-*O*-methylated, which is likely to be catalyzed by KijA1, an enzyme homologous to known sugar *O*-methyltransferases. The C-32 methyl group of kijanolide is hydroxylated. KijA3 is homologous to P450 monooxygenases known to be involved in polyketide tailoring in macrolide antibiotic biosynthesis, and is thus the likely candidate for this reaction.

Regulation and Antibiotic Export. Three genes in the cluster, *kijA8*, *kijC5*, and *kijD12*, show homology to putative transcriptional regulator genes from various Gram-positive bacteria. KijA8 and KijC5 are of the tet^R class of transcriptional regulators, while KijD12 is homologous to various bacterial two-

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domain response regulator proteins which act as transcriptional activators. These proteins are likely involved in *kij* cluster-specific regulation of gene expression in response to environmental or cellular cues. The protein KijA5 is homologous to numerous antibiotic efflux transporters and is therefore expected to serve as the efflux pump for kijanimicin as part of a self-resistance mechanism.

Genes Likely Not Involved in Kijanimicin Biosynthesis. A total of 107.6 kb of the *A. kijaniata* chromosome were sequenced in this work. As discussed in previous sections, the 99.9 kb region between KijA1 and KjD12 appears to harbor all the necessary genes for the production of kijanimicin. The remaining nine genes in the sequenced region may not be involved in kijanimicin biosynthesis. These include the gene upstream of KijA1, exhibiting homology to fructokinases from primary metabolism, and the five genes downstream of KijD12, showing homology to a D-Ala-D-Ala carboxypeptidase, a conserved hypothetical protein, a permease of the major facilitator superfamily, a selenium binding protein, and a sulfite oxidase/nitrate reductase, respectively. These genes do not appear to be required for kijanimicin biosynthesis. The other three genes located within the proposed boundaries of the *kij* cluster are *kijA2*, *kijA6*, and *kijA7*. KijA6 and KijA7 are homologous to the chaperone proteins, GroES and GroEL, respectively. It is unusual to find chaperone-encoding genes in a secondary metabolite gene cluster, and homologues of these genes are absent in the related chlorothricin and tetronomycin gene clusters, suggesting that KijA6 and KijA7 may act in general cellular processes in *A. kijaniata*. KijA2 is homologous to NAD(P)H-dependent TDP-sugar reductases, such as TylC1, which catalyze 3-ketoreduction of TDP-3,4-diketo-2,6-dideoxy-D-glucose (**14**) to form TDP-4-keto-2,6-dideoxy-D-allose, the C-3 epimer of the KijD10 product **16**. However, neither digitoxose nor kijanose formation requires this activity, so it is unlikely that KijA2 plays a role in kijanimicin biosynthesis. It is possible that KijA2 diverts TDP-sugar intermediates from the kijanimicin pathway to that of another unknown natural product pathway present in *A. kijaniata*.

Conclusions

We have sequenced the kijanimicin biosynthetic gene cluster and carried out biochemical characterization of the pathway for the synthesis of TDP-L-digitoxose, the sugar donor used in the construction of the kijanimicin tetrasaccharide chain. Characterization of the digitoxose pathway provides a solid foundation from which both *in vitro* and *in vivo* functional elucidation of *kij* digitoxosyltransferases and glycodiversification of kijanolide can be experimentally addressed. Comparative genomic analysis of the enzymes encoded in the *kij* cluster with those of the chlorothricin (**4**) and tetronomycin (**8**) clusters enabled prediction of functions for enzymes of the kijanimicin biosynthetic pathway. Assembly of the spirotetronate aglycone 32-deoxykijanolide (**36**) is carried out by the concerted action of a type-I polyketide synthase likely having a Diels−Alder-like cyclization activity and a conserved set of enzymes which use a glyceryl unit for the construction of the spirotetronate ring system via a second likely Diels−Alder-type reaction. The conserved "glycerate utilization operon" *kijABCDE* is involved in the activation of glycerate, its condensation with the kijanolide linear polyketide precursor via C−C bond formation to give a branched chain intermediate, and subsequent lactonization, dehydration, and cyclization to form the mature spirotetronate group. By analogy to putative Diels-Alderases lovastatin nonaketide synthase (LNKS),[56] solanopyrone synthase,[69,70] and macrophomate synthase,[71−74] the putative Diels−Alder-type cyclases in kijanimicin biosynthesis form or activate a dienophile prior to cyclization. The steps involved in the formation of the highly modified deoxysugar D-kijanose have also been proposed, and a likely candidate for 3-amine oxidation of a D-kijanose pathway intermediate, the flavoprotein KijD3, has been identified. KijD3 shares no detectable sequence similarity or common cofactor requirement with AurF[75] or PrnD,[76] two enzymes proposed to carry out the oxidation of an amine to a nitro group in aureothin and pyrrolnitrin biosyntheses, respectively. Hence, KijD3 may represent a unique solution to the problem of amine oxidation in biological systems. Thus, the groundwork has been laid for functional and mechanistic studies of kijanimicin pathway enzymes and the exploitation of the deoxysugar pathways and glycosyltransferases of kijanimicin biosynthesis for the glycodiversification of spirotetronate natural products.

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