Functional Characterization of the *jadI* Gene As a Cyclase Forming Angucyclinones

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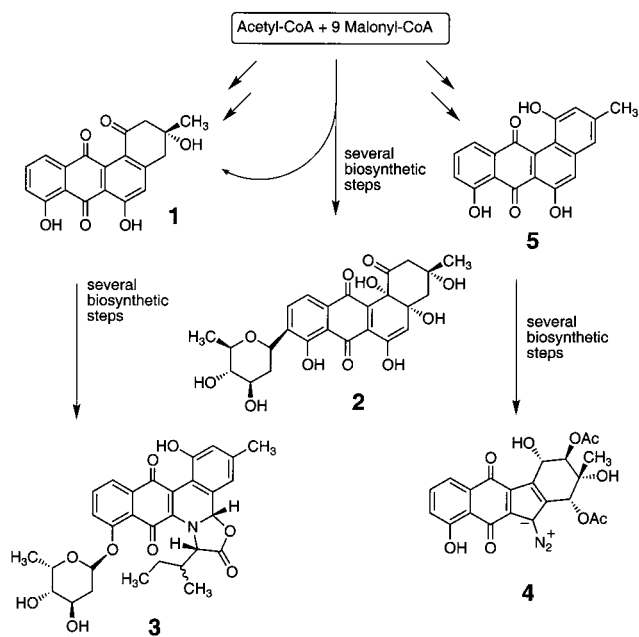
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Abstract: A gene designated *jadI* from the jadomycin B producer *Streptomyces venezuelae* has homology to the tetracenomycin pathway *tcmI* gene and encodes a putative cyclase for angucyclinone biosynthesis. Expression in *Streptomyces lividans* of a jadomycin (*jad*) gene cassette composed of the minimal polyketide synthase (PKS, *jadABC*), a cyclase (*jadD*), a ketoreductase (*jadE*), and *jadI* leads to production of several yellow compounds in liquid culture. Rabelomycin (**1**), a known angucyclinone, results from dehydration and oxidation of a new product, UWM6, isolated from the culture extracts. Characterization of UWM6 by UV, MS, and NMR analyses revealed a new angucyclinone structure, 4-hydroxy-12b*H*-12-deoxyrabelomycin. Production of these angucyclinones occurs only when *jadI* is present in the cassette, suggesting an essential cyclase phenotype for this gene. Engineered replacement of *jadI* in the *jad* cassette with the *tcmI* cyclase gene, and similar replacement of *tcmI* in a functional *tcm* PKS cassette with *jadI*, leads to the same set of aromatic decapolyketides produced by either cassette in the absence of *jadI*. From these results we conclude that both cyclases are nonfunctional out of their normal context.

Introduction

Tetracyclic aromatic polyketides known as angucyclines due to the angled orientation of the fourth ring were first isolated from bacterial cultures over thirty years ago.[1] This large class of secondary metabolites, many possessing antibiotic or antitumor activity, includes the least modified example, rabelomycin (**1**), as well as highly modified forms produced as in aquayamycin (**2**) via oxygenation and glycosylation, or as in jadomycin B (**3**) by additional ring formation via insertion of amino acids, or as in kinamycin D (**4**) by ring contraction (see Figure 1 for structures of **1**−**4**). Inclusion of a compound in the angucycline class requires a polyketide biosynthetic origin based on the benz[*a*]anthracene skeleton; most examples possess a benz[*a*]-anthraquinone skeleton derived from a decapolyketide.[1] Biosynthetic studies of angucyclines in their producing strains have shown that **1** is produced by a blocked mutant of *Streptomyces fradiae*, which normally produces **2**,[2] and is also a minor product in a strain producing the vineomycins.[3] Rabelomycin (**1**) has been implicated as an intermediate in the biosynthesis of **3** by using gene disruption to generate mutants of *Streptomyces venezuelae*.[4] The earliest identifiable intermediate for the production of **4** in *Streptomyces murayamaensis* is dehydro-rabelomycin (**5**) which interestingly does not derive from **1**.[5]

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(1) Rohr, J.; Thiericke, R. *Nat. Prod. Rep.* **1992**, *9*, 103−137.

(2) Rohr, J.; Schönewolf, M.; Udvarnoki, G.; Eckardt, K.; Schumann, G.; Wagner, C.; Beale, J. M.; Sorey, S. D. *J. Org. Chem.* **1993**, *58*, 2547−2551.

(3) Imamura, N.; Kakinuma, K.; Ikekawa, N.; Tanaka, H.; Omura, S. *J. Antibiot.* **1982**, *35*, 602−608.

(4) Yang, K.; Han, L.; Ayer, S. W.; Vining L. C. *Microbiology* **1996**, *141*, 123−132.

Figure 1. Angucyclines derived from aromatic polyketide synthases.

No intermediate prior to a complete angucyclinone has been identified in any of the latter biosynthetic pathways, suggesting the possibility that the carbon chain of the decapolyketide precursor may have to be folded and cyclized in a concerted fashion.

Iterative type II polyketide synthases (PKS) generate aromatic polyketides.[6] It is the variations in chain length, regiospecific

(5) (a) Seaton, P.; Gould, S. J. *J. Am. Chem. Soc.* **1987**, *109*, 5282−5284. (b) Gould, S. J.; Hong, S.-T.; Carney, J. R. *J. Antibiot.* **1998**, *51*, 50−57.

Scheme 1. Structures of Aromatic Polyketides Produced from *tcm* PKS Gene Cassettes Expressed in *S. lividans*[a]
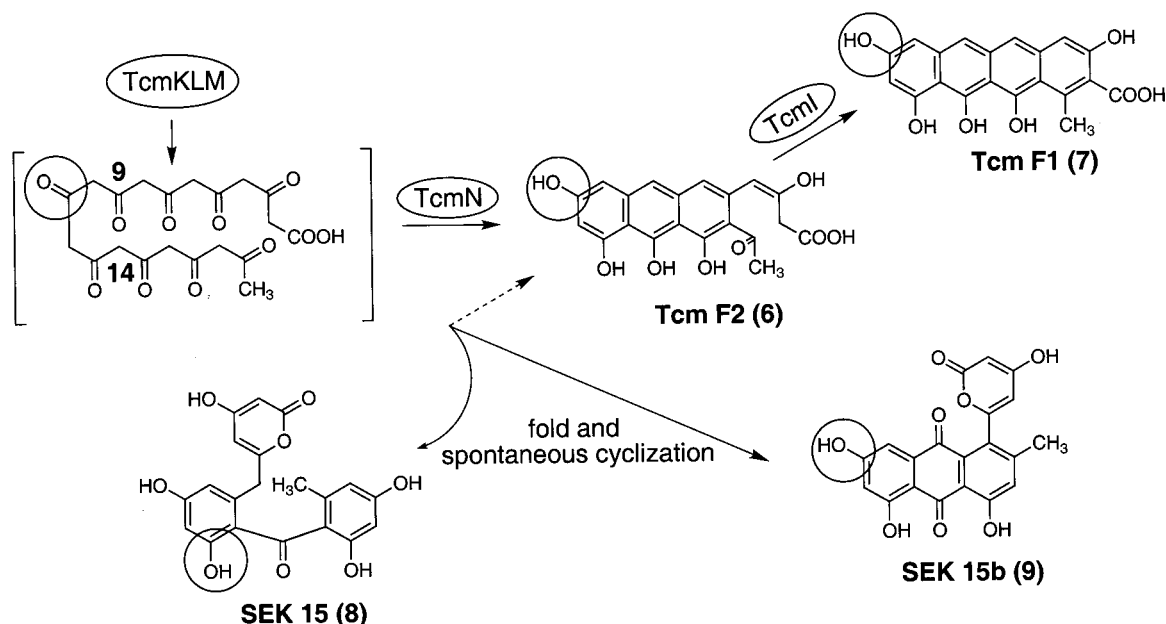
[a] The unreduced carbon is circled (C-11 of the decaketide chain after folding where the aldol condensation between carbons C-9 and C-14 is favored).

folding and cyclization, ketoreduction, and other modifications catalyzed by these enzymes that give rise to the large number and variety of secondary metabolites known as aromatic polyketides. The minimal PKS, made up of the β-ketoacyl synthase units, KS_α and KS_β, and an acyl carrier protein (ACP), is an enzyme complex that builds on a starter unit (e.g., acetyl-CoA) by repetitive addition of two-carbon units (via an enzyme-bound form of malonyl-CoA) until the polyketide backbone is fully formed to a chain length specified by the minimal PKS. This hypothesis is based on studies of artificially constructed minimal PKS gene clusters that, expressed in vivo, give rise to spontaneously folded and cyclized aromatic polyketides with a specific number of carbon atoms (see Schemes 1 and 2: structures **8**−**11**).[7] This concept has recently been exploited to prove that the aureolic acid class of antibiotics is derived from a single decaketide chain[8] and to identify functional PKS genes directly isolated from soils.[9]

A high degree of gene sequence conservation among polyketide-producing strains has facilitated the study of engineered recombinant genes. When expressed from artificial gene cassettes in proper context, the genes produce enzymes known to modify the polyketide chain processively, which may result in hybrid polyketides and thus may represent a potential for creating new chemical structures.[6,7,10] However, processing is subject to some limitations associated with polyketide chain length and also the regiospecific ketoreduction and cyclization reactions catalyzed by the PKSs. For instance, the *dpsE* ketoreductase is apparently unable to modify octaketides formed by the *act* minimal PKS, whereas the *jadE* ketoreductase is able to reduce an octaketide.[11] From structural analysis of a growing

library of hybrid, aromatic polyketides produced by strains harboring engineered gene cassettes, a set of design rules has been deduced.[10b] A recent addition to the rule of chain length determination is that the cyclase enzymes involved in first ring cyclization and aromatization can influence the final chain length,[12a,b] a property that was originally thought to reside with the minimal PKS enzymes. Also, there is evidence from a study of the *whiE* PKS genes[13] that the growing polyketide chain may dissociate from the enzyme complex in the absence of a cyclase and undergo several different cyclization modes spontaneously.

Thus far, artificial gene sets have been constructed from type II PKS genes that specify linearly-fused polycyclic aromatic polyketides. Our first attempt at including PKS genes from an angucycline-producing strain led to the conclusion that the presumed *jadI* cyclase did not function according to design rules established for linear PKSs.[11] Furthermore, a tricyclic, aromatic product folded correctly at C-9 to allow an intramolecular aldol reaction that would take place at C-7/C-12 was not identified in the constructs containing *jad* genes. A linear, tricyclic aromatic polyketide Tcm F2 (**6**) (Scheme 1) is an intermediate of the tetracenomycin (tcm) biosynthetic pathway, and **6** undergoes a final cyclization of the fourth ring catalyzed by the TcmI cyclase, producing Tcm F1 (**7**).[14] Similarly, aklanonic acid, a linear, tricyclic aromatic intermediate of daunorubicin biosynthesis has been isolated from a *Streptomyces lividans* host strain transformed with a cassette of the *dps* PKS genes.[15] Two possibilities for the failure to achieve the anticipated results

(6) Hopwood, D. A. *Chem. Rev.* **1997**, *97*, 2465−2497.

(7) Zawada, R. J.; Khosla, C. *Trends Biotechnol.* **1996**, *14*, 335−341.

(8) Kantola, J.; Blanco, G.; Hautala, A.; Kunnari, T.; Hakala, J.; Mendez, C.; Ylihonko, K.; Mantsala, P.; Salas, J. *Chem. Biol.* **1997**, *4*, 751−755.

(9) Seow, K.-h.; Meurer, G.; Gerlitz, M.; Wendt-Pienkowski, E.; Hutchinson, C. R.; Davies, J. *J. Bacteriol.* **1997**, *179*, 7360−7368.

(10) (a) McDaniel, R.; Ebert-Khosla, S.; Fu, H.; Hopwood, D. A.; Khosla, C. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11542−11546. (b) McDaniel, R.; Ebert-Khosla, S.; Hopwood, D. A.; Khosla, C. *Nature* **1995**, *375*, 549−554.

(11) Meurer, G.; Gerlitz, M.; Wendt-Pienkowski, E.; Vining, L. C.; Rohr, J.; Hutchinson, C. R. *Chem. Biol.* **1998**, *4*, 433−443.)

(12) (a) Kramer, P. J.; Zawada, R. J.; McDaniel, R.; Hutchinson, C. R.; Hopwood, D. A.; Khosla, C. *J. Am. Chem. Soc.* **1997**, *119*, 635−639. (b) Zawada R. J. X.; Khosla, C. *J. Biol. Chem.* **1997**, *272*, 16184−16188.

(13) Yu, T.-W.; Shen, Y.; McDaniel, R.; Floss, H. G.; Khosla, C.; Hopwood, D. A.; Moore, B. S. *J. Am. Chem. Soc.* **1998**, *120*, 7749−7759.

(14) (a) Shen, B.; Hutchinson, C. R. *Science* **1993**, *262*, 1535−1540. (b) Shen, B.; Nakayama, H.; Hutchinson, C. R. *J. Nat. Prod.* **1993**, *56*, 1288−1293. (c) Summers, R. G.; Wendt-Pienkowski, E.; Motamedi, H.; Hutchinson, C. R. *J. Bacteriol.* **1993**, *175*, 7571−7580.

(15) (a) Grimm, A.; Madduri, K.; Ali, A.; Hutchinson, C. R. *Gene* **1994**, *151*, 1−10. (b) Rajgarhia, V. B.; Strohl, W. R. *J. Bacteriol.* **1997**, *179*, 2690−2696.

Scheme 2. Structures of Aromatic Polyketides Produced from *jad* PKS Gene Cassettes Expressed in *S. lividans*[a]
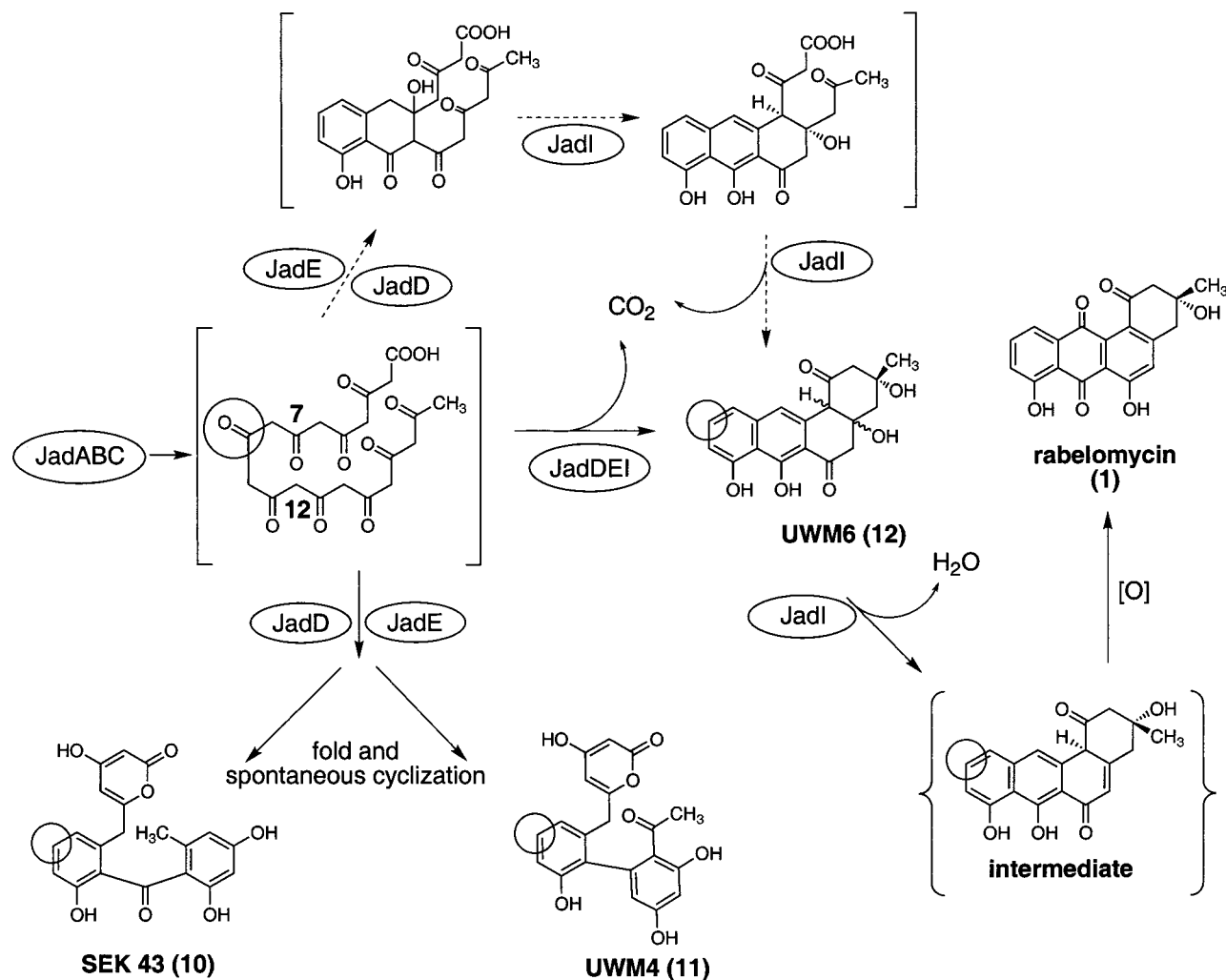
[a] The reduced carbonyl carbon C-9 where folding occurs is indicated by a circle. Structures within brackets indicate [hypothetical] or {partially characterized} intermediates.

with *jad* genes were considered: additional unknown enzyme activities may be required, or the PKS complex expressed from an artificial cassette may function aberrantly.

Our goal in the present work was to establish which gene or combination of genes characterized from the jadomycin pathway[16a] would, when expressed in vivo, yield the earliest angucycline intermediates. Once requirements for cyclization to give a tri- or tetracyclic molecule had been established, we investigated how the *jadI* cyclase and *jadE* ketoreductase genes might function to give hybrid polyketides when combined with genes from the well-characterized *tcm* cluster. Some studies of ketoreductases for proper folding and subsequent cyclization of polyketides have been reported.[10,11] The corollary experiment was investigated by inserting *tcmI* into the *jad* cluster.

In addition to evaluating the design rules of PKS systems by including genes needed for angucycline formation, the approach described here can shed light on biosynthetic pathways of antibiotics such as **3**, by allowing the identification of early intermediates.

Results

Cloning and Characterization of *jadI*. A 2.4-kb *Eco*RI−*Bam*HI fragment containing the 5′ end (nt 1−21) of *jadA*[16a]

was subcloned from λ clone LH7 DNA.[16a] Sequencing and codon preference analysis[16b] of this fragment located two open reading frames (ORFs) upstream of *jadA* and with the same orientation: *jadI*, 329 bp encoding 109 amino acids in a 12 456 Da polypeptide and *jadJ*, 1754 bp encoding 584 amino acids in a 61 926 Da polypeptide (Figure 2A). The predicted stop codon for *jadI* overlaps the start codon for *jadA*; *jadJ* is positioned 112 nt upstream of *jadI*. A database search of deduced amino acid sequences using BlastP showed 80% identity to a putative polyketide cyclase gene in *S. fradiae*,[17] 54% identity to *whiE* ORFVII from *Streptomyces coelicolor* A(3)2,[18] 49% to the putative PKS ORF11 from *Actinomadura hibisca*,[19] 48% to *curG* a postulated cyclase from *Streptomyces cyaneus*,[20] and 40% to *tcmI*, the well-established cyclase gene from *Streptomyces glaucescens*.[14a] Alignment of the deduced amino acid sequences of these genes with the *jadI* product is shown in Figure 2B.

The *jadI* Cyclase Gene is Required for Biosynthesis of Angucycline Intermediates. In a previous study no tetracyclic angucycline metabolites were produced when the engineered plasmid pWHM1221 (Table 1), which contains a cassette of

(16) (a) Han, L.; Yang, K.; Ramalingam, E.; Mosher, R. H.; Vining, L. C. *Microbiology* **1994**, *140*, 3379−3389. (b) Deveraux, J.; Haeberli, P.; Smithies, O. *Nucleic Acids Res.* **1984**, *12*, 387−395.

(17) Decker, H.; Haag, S. 1995, NCBI database, S5811.
(18) Davis, N. K.; Chater, K. F. *Mol. Microbiol.* **1990**, *4*, 1679−1691.
(19) Dairi, T.; Hamano, Y.; Igarashi, Y.; Furumai, T.; Oki, T. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1445−1453.
(20) Bergh, S.; Uhlen, M. *Gene* **1992**, *117*, 131−136.

A

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      EcoRI BamHI        .          .        .           .          .         .         .         .
   1  GAATTCTGACGGATCCCTGAATCGGTGAGTCTTGAACGAAGTCCTGGACCGGAACAAGCTTGTCGTGAAGGTCCGGTGCACCGTCTTCGAGCCCTTTCGG
 101  GAAAAGCGGAACGGGTACGGAGAAGGCGAGTCGATCAGGAGTTGAAACCGTCGTGCGCAAGGTGCTCATCGCCAACCGTGGCGAAATCGCTGTCCGTGTG
                                             jadJ > fM  R  K  V  L  I  A  N  R  G  E  I  A  V  R  V
 201  GCCCGGGCGTGCCGGGACGCGGGTATCGGCAGCGTGGCCGTGTACGCCGAGCCGGACCGGGATGCTCTGCATGTCCGGGCGGCTGACGAGGCGTTCGCTC
       A  R  A  C  R  D  A  G  I  G  S  V  A  V  Y  A  E  P  D  R  D  A  L  H  V  R  A  A  D  E  A  F  A
 301  TGGGCGGTGACACTCCGGCGACCAGTTATCTGGACATGGCGAAGGTGTTGCAGGCCGCCGCGGACTCGGGTGCGGACGCGGTCCACCCCGGTTACGGGTT
       L  G  G  D  T  P  A  T  S  Y  L  D  M  A  K  V  L  Q  A  A  A  D  S  G  A  D  A  V  H  P  G  Y  G  F
 401  CCTTTCGGAGAACGCGGAGTTCGCGCAGGCGGTCCTCGACGCGGGTCTGACGTGGATCGGTCCGCCGCCGCAGGCGGATCCGGGATCTGGGTGACAAGGTG
       L  S  E  N  A  E  F  A  Q  A  V  L  D  A  G  L  T  W  I  G  P  P  P  Q  A  I  R  D  L  G  D  K  V
 501  GCGGCCCGTCACATCGCCCAGCGTGCCGGGGCCCCGCTGGTGGCCGGCACGCCCGACCCGGTCTCGGGTGCGGACGAGGTCGTGGCGTTCGCCGAGGAGC
       A  A  R  H  I  A  Q  R  A  G  A  P  L  V  A  G  T  P  D  P  V  S  G  A  D  E  V  V  A  F  A  E  E
 601  ACGGTCTGCCGATCGCGATCAAGGCGGCGTTCGGTGGCGGTGGCCGTGGTCTGAAGGTGGCGCGGACGCTGGAGGAGGTCCCGGAGCTGTACGACTCCGC
       H  G  L  P  I  A  I  K  A  A  F  G  G  G  G  R  G  L  K  V  A  R  T  L  E  E  V  P  E  L  Y  D  S  A
 701  GGTCCGTGAGGCGGTGGCGGCGTTCGGGCGGGGCGAGTGCTTCGTGGAGCGGTACCTGGACAAGCCGCGGCACGTGGAGACGCAGTGCCTGGCCGACCAG
       V  R  E  A  V  A  A  F  G  R  G  E  C  F  V  E  R  Y  L  D  K  P  R  H  V  E  T  Q  C  L  A  D  Q
 801  CACGGCAACGTGGTCGTCGTCTCGACGCGTGACTGCTCGCTGCAGCGCCGTCACCAGAAGCTGGTGGAGGAGGCTCCGGCGCCGTTCCTGTCGGAGGCTC
       H  G  N  V  V  V  V  S  T  R  D  C  S  L  Q  R  R  H  Q  K  L  V  E  E  A  P  A  P  F  L  S  E  A
 901  AGAACGCGGAGCTGTACGCGGCCGTCGAAGGCGATCCTGAAGGAGGCCGGTTACGTCGGTGCCGGCACGGTGGAGTTCCTCGTCGGCACGGACGGCACGAT
       Q  N  A  E  L  Y  A  A  S  K  A  I  L  K  E  A  G  Y  V  G  A  G  T  V  E  F  L  V  G  T  D  G  T  I
1001  CTCGTTCCTCGAGGTCAACACCCGTCTGCAGGTGGAGCACCCGGTGACGGAGGAGGTCACGGGGATCGACCTGGTGCGGGAGATGTTCCGGATCGCGGAC
       S  F  L  E  V  N  T  R  L  Q  V  E  H  P  V  T  E  E  V  T  G  I  D  L  V  R  E  M  F  R  I  A  D
1101  GGTGAGGAGCTCGGCTACGGCGACCCGGAGATCCGGGGTCACTCCTTCGAGTTCCGGATCAACGGTGAGGACCCGGGCCGTGGTTTCCTCCCGGCGCCCG
       G  G  E  E  L  G  Y  G  D  P  E  I  R  G  H  S  F  E  F  R  I  N  G  E  D  P  G  R  G  F  L  P  A
1201  GCACCGTCACCGAGTTCACGCCGCCGACCGGTCCGGGTGTCCGCCTCGACGCGGGTGTGGAGTCGGGTTCCGTGATCGGCCCCGCGTGGGACTCGCTGCT
       P  T  V  T  E  F  T  P  P  T  G  P  G  V  R  L  D  A  G  V  E  S  G  S  V  I  G  P  A  W  D  S  L  L
1301  TGCGAACGTGATCGTGACCGGTGCGAGCCGTGAGCAGGCGCTGCAGCGTGCGGCGCGTGCCGTGGCGGAGTTCCGCGTCGAGGGCATGGCGACCGCGATC
       A  N  V  I  V  T  G  A  S  R  E  Q  A  L  Q  R  A  A  R  A  V  A  E  F  R  V  E  G  M  A  T  A  I
1401  CCCTTCCACCAGGCGGTGGTGACCGACCCGGACTTCACCGCGGACCCGTTCCGGGTGCACACGCGGTGGATCGAGACGGAGTTCGTCAACGAGATCAAGC
       P  F  H  Q  A  V  V  T  D  P  D  F  T  A  D  P  F  R  V  H  T  R  W  I  E  T  E  F  V  N  E  I  K
1501  CGTTCGCGCCGGCGGGGGAGGCGGACGAGGACGAGGCCGGCCGCGAGACGATCGTGGTCGAGGTCGGCGGCAAGCGTCTTGAGGTCTCCCTGCCGTCCTC
       P  F  A  P  A  G  E  A  D  E  D  E  A  G  R  E  T  I  V  V  E  V  G  G  K  R  L  E  V  S  L  P  S  S
1601  GCTCGGGATGACGCTGGCCCGGACGGTCTTGCGGCGGGTGCCGAAGCCGAAGCGGCGGGCGTCGAAGAAGGTCCGGGCTTCGGCCGCCTCGGGTGACACC
       L  G  M  T  L  A  R  T  V  L  R  R  V  P  K  P  K  R  R  A  S  K  K  V  R  A  S  A  A  S  G  D  T
1701  CTCGCCTCCCCGATGCAGGGCACGATCGTGAAGGTCGCGGTCGAGGAGGGCCAGGAGGTCAAGGAAGGCGATCTGATCGTCGTCCTGGAGGCCATGAAGA
       L  A  S  P  M  Q  G  T  I  V  K  V  A  V  E  E  G  Q  E  V  K  E  G  D  L  I  V  V  L  E  A  M  K
1801  TGGAACAGCCCCTCAACGCCCACCGCTCCGGCACCGTGAAGGGCCTGACCGCCGAAGTCGGCGCGTCCGTCACCTCCGGCGCCACCATCTGCGACATCAA
       M  E  Q  P  L  N  A  H  R  S  G  T  V  K  G  L  T  A  E  V  G  A  S  V  T  S  G  A  T  I  C  D  I  K
      EcoRI
1901  GGACTGAATTCACCCGCCGCCCCCGCGCGCCCTTTCGTACCACCCCGCTATTTCCCTTCGCTTTCCCTGCCTTCTTCAGCACGTCCTTTTCCAGGACTTC
       D  *                                                                                      EcoRI
2001  TTCCGGTGAGGAGAAACACATGCACAGCACTCTGATCGTGGCCCGAATGGAACCCGGATCGAGCACCGACGTGGCGAAGCTGTTCGCCGAATTCGACGCC
            jadI > fM  H  S  T  L  I  V  A  R  M  E  P  G  S  S  T  D  V  A  K  L  F  A  E  F  D  A
                   PvuII

2101  TCCGAGATGCCGCATCTCATGGGGACGCGGACGCCGTCAGCTGTTCTCGTACCGCGGCCTCTACTTCCACCTCCAGGACTTCGACGCCGACAACGGCGGCG
       S  E  M  P  H  L  M  G  T  R  R  R  Q  L  F  S  Y  R  G  L  Y  F  H  L  Q  D  F  D  A  D  N  G  G
2201  AGCTCATCGAGCGGGCCAAGACCGACCCCCGCTTCGTGGGCATCAGCGAGGACCTGAAGCCGTTCATCGAGGCCTACGACCCGGCCACCTGGCGCTCGCC
       E  L  I  E  R  A  K  T  D  P  R  F  V  G  I  S  E  D  L  K  P  F  I  E  A  Y  D  P  A  T  W  R  S  P
2301  CGCCGACGCCATGGCCACCCGCTTCTACAACTGGGAGGCGAACGCGTGACCGCCGACGCGTCGTCATCACCGGCATCGAGGTCCTCGCCCCCGGTGGCA
       A  D  A  M  A  T  R  F  Y  N  W  E  A  N  A  *         jadA >  fM  T  A  R  R
      BamHI
2401  CCGGATCC
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B

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      -  M  H  .  T  L  I  V  A  R  M  .  P  .  S  A  .  D  V  A  .  L  F  A  E  S  D  -  G  T  E  L  P  H  L  .  G  V  R
Jadl  -  M  H  S  T  L  I  V  A  R  M  E  P  G  S  S  T  D  V  A  K  L  F  A  E  F  D  -  A  S  E  M  P  H  L  M  G  T  R   37
Urd   -  M  H  S  T  L  I  V  A  R  M  E  P  R  S  A  E  D  V  A  R  L  F  S  E  F  D  -  G  T  D  M  P  H  R  M  G  T  R   37
Prm11 -  M  D  R  F  L  I  V  A  R  M  S  P  S  S  E  K  E  V  A  L  F  A  E  S  D  E  G  T  E  L  P  E  V  A  G  T  V   38
CurG  -  M  H  H  A  L  I  V  A  R  I  A  R  S  P  A  Q  D  I  A  G  V  F  A  A  S  D  P  G  -  E  L  R  H  L  V  G  V  T   37
WhiE  -  M  H  H  T  L  I  V  A  R  M  A  P  G  A  A  P  D  I  A  K  V  F  A  E  S  D  S  G  -  E  L  P  H  L  V  G  V  N   37
Tcml  M  A  Y  R  A  L  M  V  L  R  M  D  P  A  D  A  E  H  V  A  A  F  A  E  H  D  -  T  T  E  L  P  L  E  I  G  V  R   38

      R  R  S  L  F  S  F  -  .  G  L  Y  F  H  L  I  E  .  D  -  E  D  .  G  E  R  I  .  .  A  .  .  H  P  E  F  V  .  I
Jadl  R  R  Q  L  F  S  Y  -  R  G  L  Y  F  H  L  Q  D  F  D  A  D  N  G  G  E  L  I  E  R  A  K  T  D  P  R  F  V  G  I   75
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Prm11 S  R  S  L  L  S  F  -  H  G  L  Y  F  H  L  T  E  V  E  -  E  S  T  D  R  T  L  N  G  I  H  E  H  P  E  F  V  R  L   75
CurG  Q  R  S  L  F  Q  F  G  D  -  V  Y  M  H  F  I  E  A  G  -  A  G  P  G  P  R  I  A  K  V  T  G  H  P  E  F  V  D  L   74
WhiE  R  R  S  L  F  E  F  G  D  G  V  Y  M  H  L  I  E  S  D  -  E  D  P  A  P  T  I  G  H  P  E  F  F  R  Q  V   75
Tcml  R  R  V  L  F  R  F  -  H  D  L  Y  M  H  L  I  E  A  D  -  D  D  I  M  E  R  L  Y  Q  A  R  S  H  P  L  F  Q  E  V   75

      S  E  .  L  .  P  Y  V  .  A  Y  D  P  .  T  W  R  S  P  A  D  A  M  A  R  .  F  Y  .  W  E  A  -  -  -  -  -  -
Jadl  S  E  D  L  K  P  F  I  E  A  Y  D  P  A  T  W  R  S  P  A  D  A  M  A  T  R  F  Y  N  W  E  A  N  A                  109
Urd   S  E  D  L  K  P  F  I  A  A  Y  D  P  D  T  W  R  S  P  A  D  A  M  A  Q  R  F  Y  H  W  T  A  L                     108
Prm11 S  R  Q  L  S  G  H  V  Q  A  Y  D  P  K  T  W  R  S  P  A  D  A  M  A  R  E  F  Y  R  W  E  A  G  T  G  V  V  R  R   114
CurG  S  R  K  L  E  A  Y  V  S  P  Y  D  P  A  T  W  R  S  P  R  D  A  M  G  R  C  F  Y  H  W  E  R  D                     107
WhiE  S  E  R  L  E  P  Y  V  S  A  Y  D  P  A  T  W  R  G  P  K  D  A  M  A  R  C  F  Y  R  W  E  R  T  P  A  G            111
Tcml  N  E  R  V  G  Q  Y  L  T  P  Y  -  A  Q  D  W  E  E  L  K  D  S  K  A  E  V  F  Y  S  W  T  A  P  D  S               109
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Figure 2. (A) DNA and deduced amino acid sequences of the region containing *jadI* and *jadJ* cloned from *S. venezuelae*. Only restriction sites of interest are shown. Putative translational start and stop sites are shown in bold and ribosome binding sites are underlined. The DNA sequence is available from GenBank/EMBL under accession number AF126429. (B) Amino acid sequence comparisons of putative aromatic polyketide cyclases. Shaded regions show 3 or more identical amino acids.

Table 1. PKS Gene Constructs, Phenotypes, and Resulting Polyketide Metabolites

plasmid	CYC	KS$_\alpha$	KS$_\beta$	ACP	KR	CYC	metabolites[a]
pWHM725[b]		*tcmK*	*tcmL*	*tcmM*		*tcmN*	**6, 7**, (**8**), (**9**)
pWHM1230	*jadI*	*tcmK*	*tcmL*	*tcmM*		*tcmN*	**6**
pWHM1231	*jadI*	*tcmK*	*tcmL*	*tcmM*			
pWHM1220		*jadA*	*jadB*	*tcmM*		*tcmN*	**6**, (**7**)
pWHM1232	*jadI*	*jadA*	*jadB*	*tcmM*		*tcmN*	**6**, (**7**)
pWHM1233		*jadA*	*jadB*	*jadC*		*jadD*	(**6**), **8, 9**
pWHM1234	*jadI*	*jadA*	*jadB*	*jadC*		*jadD*	(**6**), **8, 9**
pWHM1235	*tcmI*	*jadA*	*jadB*	*jadC*		*jadD*	(**6**), **8, 9**
pWHM1221		*jadA*	*jadB*	*jadC*	*jadE*	*jadD*	**10**, (**11**)
pWHM1236	*tcmI*	*jadA*	*jadB*	*jadC*	*jadE*	*jadD*	**10**, (**11**)
pWHM1237	*jadI*	*jadA*	*jadB*	*jadC*	*jadE*	*jadD*	**1, 12**
pWHM1238	*jadJ*	*jadI*	*jadA*	*jadB*	*jadC*	*jadE*	*jadD*	**1, 12**

[a] Major metabolites identified are shown with minor metabolites in parentheses. *[b]* This construct was expressed in *S. glaucescens* 1077 to verify *tcmI* activity.[14c] All others were expressed in *S. lividans* 1326.
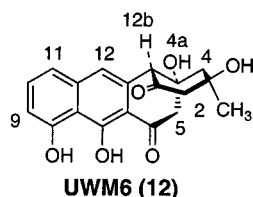
Figure 3. Three-dimensional structural representation and numbering of a possible isomer of UWM6 (**12**).

jadomycin B biosynthetic cluster genes encoding the minimal PKS, a ketoreductase, a cyclase (JadD), and a region upstream of *jadA* believed to include *jadI*, was expressed in a heterologous host.[11] When the DNA segment containing *jadI* and *jadJ* was incorporated into pWHM1221 to give pWHM1238, a *S. lividans* strain transformed with pWHM1238 grew as bright yellow colonies. We speculated that the addition of *jadJ*, a gene of unknown function immediately upstream of *jadI*, was needed for angucycline production. However, pWHM1237, obtained from pWHM1238 by removal of *jadJ*, also gave yellow pigmented colonies when the plasmid was introduced into *S. lividans*. A comparison of the deduced amino acid sequence of the *jadI* region in pWHM1221 with the *jadI* sequence revealed that pWHM1221 lacked a 24 amino acid segment at the N terminus (contained in the small *Eco*RI segment, Figure 2A). We therefore investigated the production of angucyclines in *S. lividans* transformed with pWHM1237 and pWHM1238.

From liquid shaken-flask cultures the known benz[*a*]anthraquinone, **1**, was isolated as well as an unprecedented angucyclinone **12** (Figure 3), characterized by UV, MS, and NMR analyses. Time-course analyses by HPLC revealed that **12** is a precursor of **1** via sequential dehydration and oxidation steps; these appear to occur spontaneously in liquid culture or when **12** was spotted on silica gel TLC plates. A third angucyclinone component identified in culture extracts by HPLC was a chromatographically unresolvable mixture (1:1 by ^{1}H NMR) of two isomers that apparently formed by nonenzymatic dehydration of **12** at C-4a. Although this component accumulated in liquid cultures in variable amounts, it also readily oxidized to **1** (see below).

When grown on R2YENG agar plates, *S. lividans* harboring the *jad* gene cassette pWHM1237 gave bright yellow colonies that contrasted with tan-colored transformants of pWHM1221 or pWHM3 (Table 1). Acidification, extraction, and TLC analysis of liquid R2YENG cultures inoculated with the yellow colonies showed that **1** and at least one other yellow metabolite were produced within 24 h; production of the yellow metabolites rapidly diminished after 24 h. HPLC analysis of organic extracts with photodiode array detection showed three peaks, one of

which coeluted with authentic **1** and gave an identical UV spectrum. The production ratios for these three peaks varied considerably among culture flasks and production occurred only after inoculation with colonies from freshly transformed plates. The other two unknown metabolite peaks gave similar UV spectra with a λ_{max} at lower wavelength (408 nm) than given by the quinone moiety of **1** (422 nm). This initial result suggested that at least one unknown was an immediate precursor of the benz[*a*]anthraquinone **1**. Although our *jad* cassette did not contain the *jadG* gene that has strong sequence similarity to known anthrone oxidases[4] (e.g., *tcmH*[21]), introduction of oxygen at C-12 to give a benz[*a*]anthraquinone may occur nonenzymically in shaken-flask liquid cultures or may be an enzymic biotransformation mediated by *S. lividans*. Unlike cultures of *S. lividans* transformed with pWHM1237 grown in liquid medium for 24 or 48 h, cultures grown on R2YENG agar plates for 10 days yielded only **1**.

Subsequent structural data supported the hypothesis that these intermediates are precursors of **1**. A sample mixture of purified metabolites was analyzed by low resolution LC−MS operating in the CI mode to give $(M + H)^+$ ions with *m/z* values of 339 (**1**) and 343 (**12**). Although **1** and **12** were stable in the dry state, the third intermediate had converted to **1** prior to LC−MS analysis. A 1-D ^{1}H NMR spectrum recorded within a day of isolating yellow material corresponding to the unknown intermediate showed that the material had converted to a mixture of **1** (50%, estimated by ^{1}H NMR) and two closely related intermediates (1:1 by ^{1}H NMR), possibly dehydration products of **12**. When the nonenzymic conversion of pure **12** to **1** on a TLC plate was monitored by HPLC, a peak corresponding to the intermediate was detected but remained at low levels, presumably because it converted at a high rate to **1**, which accumulated. On the basis of HREIMS (*m/z* 324.0990, $C_{19}H_{16}O_5$) and 1-D ^{1}H NMR spectra the intermediate is undoubtedly a dehydration product of **12**, and it either contains a double bond across carbons C-4 and C-4a or is the α,β-unsaturated ketone that has resulted from dehydration at C-4a and C-5. Evidence for a dehydration product includes a vinylic proton signal (1H, δ 6.10, either H-4 or H-5), a singlet for H-12b at δ 3.82, and loss of geminal coupling for the H-4 or H-5 methylene protons in the ^{1}H NMR spectrum of this intermediate. The multiplicity and chemical shifts of the other signals were otherwise very similar to those recorded for **12**, indicating a similar carbon skeleton.

All of the analytical data (UV, CIMS, and ^{1}H NMR) for **1** isolated from *S. lividans* cultures transformed with *jad* genes (pWHM1237) was identical to an authentic sample of **1**, proving that *jadI* functions as a cyclase for angucycline production.

(21) Shen, B.; Hutchinson, C. R. *Biochemistry* **1993**, *32*, 6656−6663.

Table 2. Nuclear Magnetic Resonance Data for **12**

position number[a]	[1]H, δ, m, ($J_{H,H}$ Hz)[b]	[13]C, $\delta^{c,d}$	HMBC correlation[b]	NOESY correlation[b]
1		207		
2	2.50 d (14)	51.3	3, 4, 12b	5, 13
	2.74 d (14)		3, 13	5, 13
3		76.1		
4	1.98 d (15)	42.8	2, 3, 4a, 12b	5, 13
	2.02 d (15)			
4a		76.2		
5	2.98 d (17)	50.8	4, 4a, 6, 12b	2, 4
	3.02 d (17)			
6		202.5		
6a		108.4		
7	−OH, 15.9 s	167.1		
7a		113.4		
8	−OH, 9.67 s	158.4	8, 9	
9	6.92 d (8.8)	112.2	7a, 8, 11	10
10	7.53 t (8.8)	133.7	8, 11a	9, 11
11	7.13 d (8.8)	119.1	9, 11a, 12	10
11a		139.5		
12	6.76 s	117.8	6a, 11, 11a, 12a, 12b	
12a		132.7		
12b	3.99 s	62.2	1, 4, 4a, 12, 12a	
13	1.30 s	30.9	2, 3, 4	2, 4

[a] Numbering for benz[a]thracene skeleton as in Figure 3. [b] Bruker DMX-500, TXI-5-mm probe; CDCl₃/TMS. [c] Bruker AM-300; CD₂Cl₂. [d] Assignments were confirmed with an HMBC pulse sequence modified to remove the 1-bond coupling purge pulse which obviated the need for a separate HSQC experiment.

Characterization of UWM6 (12). The maximum at 408 nm in the UV spectrum of UWM6 indicated a highly conjugated system, however without the quinone moiety present in **1**. Low-resolution chemical ionization mass spectrometry (CIMS) gave an M + H ion at $m/z = 343$ for the formula $C_{19}H_{18}O_6$ with an unsaturation number indicating four rings and seven double bonds. High-resolution electron impact EIMS confirmed the formula for **12** (calcd 342.1103, found 342.1096) and showed sequential loss of two molecules of H_2O and a CH_3 group to give fragment ions of $m/z = 324$, 306, and 291, respectively. The base peak ion of $m/z = 282$ indicated loss of 60 amu, which would correspond to loss of fragments for H_2O and C_2H_2O. Several key features of the 1-D ^{1}H NMR spectrum led to the angucyclinone structure proposed in Figure 3. Sharp singlets at δ 15.9 and 9.7, each integrating for 1H, were assigned to the phenolic protons at C-7 and C-8, respectively. Both protons exchanged with D_2O and have very similar chemical shifts to phenolic protons in the anthracycline ring system, deduced for the aglycone portion of mithramycin, a member of the aureolic acid group of antitumor antibiotics.[22] These chemical shifts contrast with the phenolic proton chemical shifts of δ 11.6 and 12.2 for the benz[a]anthraquinone skeleton of **1**. Another unusual feature of the 1-D ^{1}H NMR spectrum was a sharp singlet at δ 4.0 that did not exchange with D_2O and integrated for 1H. This singlet was assigned to the bridge methine at C-12b (δ 62.2) on the basis of the results of an HMBC NMR experiment (Table 2) where 2-bond correlations to quaternary carbons for ketone C-1, hydroxylated bridge carbon C-4a, aromatic carbon C-12a, and 3-bond correlations to methylene carbon C-4 and aromatic carbon C-12 were evident. The remaining carbon assignments and corresponding proton assignments were readily deduced from the HMBC spectrum (summarized in Table 2).

Assignment of stereochemistry for the three chiral centers was less straightforward. Both the circular dichroism (CD) spectrum (MeOH, nm ($[\Theta]^{25}$) 400 (−850), 328 (+1400), 298 (−4400), 275 (−2000), 255 (+1100), 228 (−700)) and NMR

(22) Thiem, J.; Meyer, B. *Tetrahedron* **1981**, *37*, 551−558.

analyses support one predominant diastereomer of UWM6. All three methylene carbons (C-2 and C-4 of ring A, and C-5 of ring B) have nonequivalent protons, each evident as a pair of doublets (Table 2). An attempt to define the relative stereochemistry at carbons C-4a and C-12b was based on possible conformations from Dreiding models and results of a 2-D NOESY NMR experiment. The configuration at C-3 for **1** had been assigned as *R* from biosynthetic evidence based on the isolation of **1** from blocked mutants of the urdamycin producer *S. fradiae*.[2] The *R* configuration at C-3 was also deduced from an X-ray crystal structure of the angucycline, sakyomicin A.[23] From the NOESY spectrum it was clear that the methylene protons attached to C-2 and C-5 are in a close proximity because they gave strong cross-peaks. This result ruled out the two isomers with trans configuration between rings A and B because either configuration would place C-2 and C-5 distant from one another. For the cis configuration, flexibility of ring A results in several possible conformations (restricted to boat forms) for the two possible isomers. The NOESY correlation from H-2 and H-5 supports a conformation of ring A folded down as depicted in Figure 3 for one isomer ((4a*R*, 12b*R*)-4-hydroxy-12b*H*-12-deoxyrabelomycin). However, definitive data were not obtained because such a conformation for ring A of either isomer would not be expected to give a NOESY correlation between H-12b and either of the C-2 protons or the C-3 methyl group to confirm this choice or exclude alternatives. Although the 4a*S*,12b*S* configuration would be expected to give a NOESY cross-peak between H-12b and the C-3 methyl, the absence of such a cross-peak precludes establishment of the relative stereochemistry of **12**. This question should be revisited once the role of **12** is confirmed by studies of the *jad* PKS enzymes in vitro.

Cyclase Function is Abolished When *tcmI* and *jadI* Are Interchanged in Their Respective Gene Cassettes. We constructed a series of artificial gene cassettes made up of *tcm* genes and *jad* genes (Table 1) to assess how the *tcmI* and *jadI* cyclases might function in cooperation with other PKS enzymes. Because the absence of a full length *jadI* gene had resulted in the formation of shunt products SEK43 (**10**) and UWM4 (**11**),[11] the structures of **1** and **12** produced from in vivo expression of *jad* genes in engineered PKS cassettes confirm the function of the *jadI* protein as a cyclase required for angucyclinone production. Thus, it is unlikely that a specific enzyme of the *S. lividans* host strain is involved in the formation of these angucyclines.

We examined whether *jadI* provides a means to produce hybrid polyketides by genetically engineering artificial gene cassettes using other PKS genes. We first tested the ability of *jadI* to function with the well-studied *tcm* PKS genes by replacing *tcmI* in pWHM725[14c] with *jadI* (pWHM1230). Both pWHM1230 and pWHM1232 (*tcmKL*[14c] replaced with *jadAB*) produced TcmF2 (**6**) with trace amounts of Tcm F1 (**7**) (Scheme 1), which must arise by spontaneous cyclization of **6** because the same metabolite profile was obtained with pWHM1220 (pWHM1232 without *jadI*).[9] As previously reported, expression of *tcmN* efficiently directs nonreduced decaketides to **6**,[10,24] and the presence of *jadI* in pWHM1230 did not influence this outcome. An unexpected result was the apparent disruption of the *tcm* minimal PKS by the addition of *jadI* (pWHM1231); this construct was expected to produce **8** and **9** with trace amounts of **6**, but did not produce any metabolites. The previous

(23) Irie, H.; Mizuno, Y.; Kouno, I.; Nagasawa, T.; Tani, Y.; Yamada, H.; Taga, T.; Osaki, K. *J. Chem. Soc., Chem. Commun.* **1983**, 174−175.
(24) Shen, B.; Summers, R. G.; Wendt-Pienkowski, E.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1995**, *117*, 6811−6821.

conclusion that *jadD* cannot function with unreduced decaketides to form angucyclic compounds[11] was reinforced when *jadD* was unable to operate with the *jad* minimal PKS in the construct pWHM1233, which produced the same set of metabolites (**6**, **8**, and **9**) as the *tcm* minimal PKS.[24] Addition of *tcmI* or *jadI* to pWHM1233 to give respectively, pWHM1234 and pWHM1235, did not influence metabolite production (Table 1).

In showing by TLC and HPLC analyses that similar aromatic polyketides were produced from these constructs whether *jadI* or *tcmI* were included, we have established that neither the *tcmI* nor the *jadI* cyclase gene is able to function out of context. Furthermore, when expressed together in vivo, *jadD* and *jadI* give enzymes that appear to act cooperatively only on C-9 reduced decaketides to produce angucycline intermediates (pWHM1237). This contrasts with the *tcmN* and *tcmI* cyclases, which function independently to first produce TcmF2 (**6**) by the action of the TcmN protein cooperating with the *tcm* minimal PKS and then to convert **6** to Tcm F1 (**7**) by the action of the TcmI protein.[14]

Discussion

Current understanding of iterative type II PKS systems, both in regard to enzyme function and the potential for novel product formation, is based solely on the class of aromatic polyketides with linearly-fused rings.[7,10,24,25] Our goal was to identify genes needed to produce angucyclinones such as **1** and then to evaluate these genes in different contexts using a combinatorial approach. In the present work this involved the reassembly of *tcm* cyclase genes with *jad* PKS genes or that of *jad* cyclase genes with *tcm* PKS genes. The TcmI protein was previously shown to be a linear decapolyketide cyclase for fourth-ring cyclization of **6** to **7** (Scheme 1). Although the results described here with the JadI cyclase in a Jad PKS cluster suggest involvement of a fourth-ring cyclization, we never observed or isolated any tricyclic substrate for the JadI cyclase (see Scheme 2).

Comparison of the JadI protein with other aromatic polyketide cyclases showed high degrees of amino acid sequence similarity. It showed very strong similarity to a putative polyketide cyclase associated with urdamycin biosynthesis.[17] Urdamycins are decapolyketide angucyclines produced by strains of *S. fradiae*. At lower sequence similarity, JadI resembled WhiE-OrfVII, a putative cyclase from *Streptomyces coelicolor*.[18] Comparable sequence similarities were found between JadI and ORF11, a putative PKS gene from the pradimycin (a C_{24} angucycline)-producing strain *Actinomadura hibisca*,[19] as well as between JadI and CurG, a putative cyclase from another spore pigment-producing strain, *Streptomyces curacoi*.[20] Of this related group of cyclases in the databases TcmI had the lowest sequence similarity to JadI. Among the six proteins compared, only TcmI and now JadI have well-established functions.

The main products of the most complete set of *jad* genes, as in pWHM1237, were all angucyclinones (**1**, **12**, and the unknown intermediate; Scheme 2). From the results of expressing pWHM1221 and pWHM1237 in *S. lividans* (Table 1), it is evident that *jadI* requires not only the *jadE* ketoreductase gene but also a second cyclase (*jadD*) to function. Activities of minimal PKSs customarily can be observed in the absence of the cognate or heterologous cyclases.[10a,12,13,24] The functions of pWHM1233 and pWHM1234 (Table 1) reflect the properties of the *jad* minimal PKS (pWHM1233) which, as expected, primarily makes SEK15 (**8**) and SEK15b (**9**)[10a,11] (Scheme 1), and the lack of an effect on this activity by addition of *jadI*

UWM5 (13)   **12-deoxyaklanonic acid (14)**
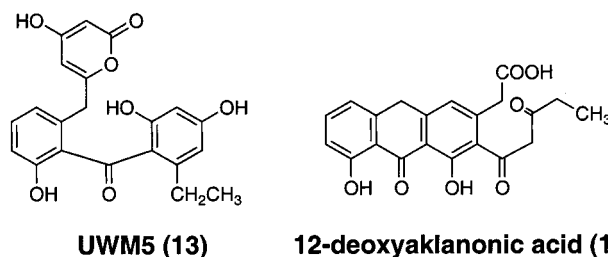
Figure 4. Structures of aromatic polyketide products **13** and **14** isolated from strains expressing artificial *dps* gene cassettes.[26]

(pWHM1234). Thus, it was surprising that pWHM1231 containing *jadI* plus the *tcm* minimal PKS genes did not produce the expected products **8** or **9**. Formation of **6** when *tcmN* was included in the latter construct (as pWHM1230 and pWHM1232) apparently excludes the trivial explanation that the *tcm* PKS genes were nonfunctional in pWHM1231. For some reason *jadI* prevented the normal function of the *tcm* minimal PKS.

The set of cassettes containing the minimal *jad* PKS genes and the *jadD* cyclase gene, but lacking the *jadE* ketoreductase gene, were constructed to test if *jadD* when coexpressed with *jadI* (pWHM1234) could result in a correctly folded, unreduced decaketide and cooperate with the other genes to give a C-10 unreduced angucycline. Expression of pWHM1235 tested if the TcmI and JadD cyclases could interact correctly to fold and cyclize an unreduced decaketide to produce **6** and possibly **7** where the JadD cyclase could not normally function on its own. Expression of these constructs (pWHM1233, pWHM1234, and pWHM1235) all produced the same polyketides **8** and **9** plus small amounts of **6** (Scheme 1). These results indicate that all of the heterologous PKSs tested functioned much like the *tcm* minimal PKS and that neither TcmI nor JadI could assist the JadD cyclase in forming a nonreduced, tri-, or tetracyclic linear polyketide. When the *tcmI* cyclase gene was placed in the context of the *jad* PKS genes containing a ketoreductase (*jadE*), it was nonfunctional as well, producing the same products **10** and **11** as pWHM1221.

We did not observe a bi- or tricyclic intermediate in the products obtained from expression of the *jad* PKS genes or their heterologous constructs in vivo. Consequently, we are tempted to conclude that JadI catalyzes all three cyclizations required for the biosynthesis of **1**, beyond formation of the first ring by JadD. Cooperation between JadI and a host cyclase is unlikely, since the inclusion of *jadI* alone redirected the *jadABCED* genes to produce true angucyclines (**1** and **12**) instead of monocyclic shunt products such as **10** and **11** (Scheme 2), but it cannot be excluded a priori.

In this regard, it is instructive to compare the *jad* PKS with the daunorubicin/doxorubicin (dps) PKS, which forms a linearly-fused tricyclic decapolyketide, 12-deoxyaklanonic acid (Figure 4, **14**), a precursor of daunorubicin and doxorubicin in *Streptomyces peucetius*[15a] and *Streptomyces sp.* strain C5.[15b] The biosynthesis of **14** also involves intermediates with one reduced aromatic ring, created by the action of the *dpsABCDEF* genes. The *dps* PKS does not employ *jadI* (or *tcmI*) homologues; instead, one other gene, *dpsY*[26] (and perhaps *dpsH* also[27]) acts along with the *dpsE* ketoreductase and *dpsF* first-ring cyclase/aromatase to make 12-deoxyaklanonic acid. When *dpsY* is absent, UWM5 is produced in *S. peucetius* (Figure 4, **13**)

(25) (a) Hutchinson, C. R.; Fujii, I. *Annu. Rev. Microbiol.* **1995**, *49*, 201–238. (b) Hutchinson, C. R. *Curr. Opin. Microbiol.* **1998**, *1*, 319–329.

(26) Lomovskaya, N.; Doi-Katayama, Y.; Filippini, S.; Natro, C.; Fonstein, L.; Gallo, M.; Colombo, A. L.; Hutchinson, C. R. *J. Bacteriol.* **1998**, *180*, 2379–2386.

(27) Gerlitz, M.; Meurer, G.; Wendt-Pienkowski, E.; Madduri, K.; Hutchinson, C. R. *J. Am. Chem. Soc.* **1997**, *119*, 7392–7393.

whereas the absence of *dpsH* has no obvious effect.[28] One could conclude from these facts that the *dps* PKS, like the *jad* PKS, employs at least two cyclases, perhaps in a stepwise manner. However, the issue is clouded by the fact that neither *dpsH* nor *dpsY* is required for 12-aklanonic acid biosynthesis by the *dps* PKS genes when they are expressed in *S. lividans*.[15,27] Therefore, the precise role of JadI (as well as JadJ) in the biosynthesis of 12-deoxyrabelomycin, including the question of bi- and tricyclic intermediates, will have to await studies with *S. venezuelae* mutants and purified Jad PKS enzymes.

In conclusion, we have characterized the function of *jadI* as an enzyme that facilitates cyclization of decapolyketides to give angucyclinones **1** and **12**. It appears that the enzymes for the minimal *jad* PKS and minimal *tcm* PKS function similarly and are exchangeable but that the cyclase genes from the *tcm* and *jad* biosynthetic pathways do not function when combined with the minimal PKS genes of the other's cluster. Although our current results imply that nonfirst ring cyclases cannot be switched between systems involving reduced and nonreduced first rings, future testing of *jadD* and *jadI* together in other angucycline systems, such as those represented by the dodeca-ketide *whiE* PKS[13] and pradimycin PKS,[20] may lead to novel aromatic polyketide structures.

Experimental Section

General Methods. UV (Shimadzu, model 1605), and CD (On-Line Instrument Systems) analyses were recorded in MeOH (Omnisolv, EM Science). LC−CIMS was performed on PE Sciex API100LC MS-based detector using a heated nebulizer as the ion source generator. The compounds were separated on a C-18 column (4.6 mm × 15 cm). HREIMS was carried out on a Kratos MS-80RFA spectrometer. The 1-D ^{13}C NMR spectrum of **12** was recorded on a Bruker AM3000 spectrometer in CD$_2$Cl$_2$ (Merck Sharp & Dohme, 99 atom % D), and 1-D ^{1}H NMR and 2-D NMR spectra (NOESY, HMBC) were recorded on a Bruker Avance DMX 500 with a 5-mm TXI inverse probe. Samples were dissolved in CDCl$_3$ (Aldrich, 100 atom % D) and referenced to internal TMS. The NOESY spectrum was obtained with a standard Bruker pulse program and a mixing time of 500 ms. The HMBC pulse program was modified to remove the 1-bond purge pulse so that 1-bond correlations were observed as off-set cross-peaks in the proton dimension.

Bacterial Strains, Plasmids. The *S. glaucescens* GLA.0 and 1077 strains,[29] *S. lividans* 1326,[30] are described elsewhere. The *ermE*p* promoter from pIJ4070 (obtained from Mervyn Bibb, John Innes Centre, England) inserted into the *Streptomyces* shuttle vector pWHM3[31] has been described as plasmid pWHM1250.[32] The plasmids pGEM-3Zf(−), and −7Zf(+) were purchased from Promega (Madison, WI), The *E. coli* strain used was DH5α.

Construction of Plasmids. Table 1 lists the plasmids constructed in this work. Plasmid pWHM1237 was constructed by the ligation of a 0.8 kb *Bam*HI/*Pvu*II fragment containing the beginning of *jadI* from pJV58[16] and a 4.8-kb *Pvu*II/*Nsi*I segment from pWHM1221[11] into the shuttle vector pWHM1250 using the *Bam*HI/*Pst*I sites. Plasmid pWHM1236 was constructed by first subcloning *tcmI* as a 0.8 kb *Pst*I/*Bgl*II fragment from pWHM725[14c] into pGEM3Zf(−). A 0.4 kb *Hin*dIII/*Nae*I fragment was then removed and combined with a 4.7 kb *Xmn*I/*Nsi*I fragment from pWHM1221 containing the jad PKS genes and a

0.3 kb *Eco*RI/*Hin*dIII fragment containing *ermE*p* from pIJ4070 in a four-way ligation into the *Eco*RI and *Pst*I sites of pWHM3. The plasmid pWHM1235 was constructed by recovering a 2.2 kb *Xba*I/*Bgl*II fragment from pWHM1236, a 1.2 kb *Bgl*II/*Nsp*I fragment from pWHM1221, and a 1.4 kb *Sph*I/*Nsi*I fragment from pWHM1221 and ligating them jointly into the *Xba*I and *Pst*I sites of pWHM1250. The plasmid pWHM1234 was constructed in two steps by first recovering a 3.0 kb *Kpn*I/*Bgl*II fragment from pWHM1237, plus a 2.5 kb *Bgl*II/*Hin*dIII segment from pWHM1235, and cloning them jointly into pGEM7Zf(+) at the *Kpn*I/ *Hin*dIII sites. The resulting plasmid was then digested with *Xba*I and *Hin*dIII, and the 5.5 kb fragment was inserted into pWHM3 at the *Xba*I and *Hin*dIII sites to give pWHM1234. The plasmid pWHM1233 was constructed from pWHM1235 and pWHM1221 by cutting each with *Bgl*II/*Hin*dIII and ligating the vector-containing portion of pWHM1221 to the 2.5 kb fragment from pWHM1235. The plasmid pWHM1232 was derived from pWHM1220 by removing a 0.6 kb *Eco*RI/*Pst*I fragment and inserting three other fragments into the *Eco*RI/*Pst*I sites: a 0.3 kb *Eco*RI/*Bam*HI fragment from pIJ4070 plus the 0.8 and 1.8 Kb *Bam*HI/*Pvu*II fragments from pWHM1237. To construct pWHM1231 and pWHM1230 the *jadI* gene was added onto the *tcm* PKS genes by first removing *jadI* from pJV58 as a 0.4 kb *Eco*RI/*Bam*HI fragment and ligating this fragment plus the *ermE*p* promoter (0.3 kb *Eco*RI/ *Hin*dIII fragment from pIJ4070) into pGEM3Zf(−) at the *Eco*RI/*Bam*HI sites. Two fragments (0.4 kb *Eco*RI/ *Sst*I and 0.3 kb *Eco*RI/*Pst*I) were recovered from this intermediate construct and ligated into pWHM3 at the *Sst*I/*Bam*HI sites along with a 3.4 kb *Pst*I/*Bam*HI fragment from pWHM1200[11] for pWHM1231 or a 4.7 kb *Pst*I/*Bam*HI fragment from pWHM862[24] for pWHM1230.

Culture Conditions, Chemicals, and Other Biological Materials. *S. glaucescens* and *S. lividans* were grown in liquid R2YENG medium[33] (pH 6) for preparation of protoplasts, on R2YENG agar plates for protoplast regeneration, and in R2YENG liquid or agar for production of secondary metabolites. *S. glaucescens* spores were isolated after growth on HT medium[29] and *S. lividans* spores after growth on R2YE agar.[35] All transformed *Streptomyces* species were selected with thiostrepton (10 μg/mL in liquid and 50 μg/mL on solid media). Recombinant *E. coli* DH5α strains were grown in Luria−Bertani medium[36] containing ampicillin (150 μg/mL). Restriction enzymes, DNA ligase, and other molecular biology materials were purchased from standard commercial sources.

Metabolite Production and Analysis. DNA fragments containing the hybrid PKS expression cassettes under control of the *ermE*p* promoter were cloned into pWHM3 to give the plasmids described in Table 1 and were introduced by transformation into protoplasts of *S. lividans* and *S. glaucescens* strains by the method of Hopwood et al.[34] Transformants were selected by overlaying the plates with thiostrepton (final concentration of 10 μg/mL). Transformants were initially grown in culture tubes containing 5 mL of R2YENG medium plus thiostrepton for 1−2 days. The contents of the tube was transferred to 250-mL baffled flasks containing 15 mL of R2YENG with thiostrepton. At 1, 3, and 5 days, samples of culture (1 mL) were acidified with 100 μL of 1 M KH$_2$PO$_4$, pH 4.8, extracted with 0.25 mL of ethyl acetate, and concentrated under a stream of nitrogen. The resulting crude residues were resuspended in 20 mL of MeOH and subjected to chromatography on thin-layer chromatography (TLC) plates (silica gel 60 F$_{254}$, E. Merck, Darmstadt, Germany) developed in chloroform:methanol:acetic acid (40: 10:1.25). Secondary metabolites were detected with UV light (254 or 310 nm). Analyses of cultures with a Waters HPLC system [two LC pumps (models 6000A and 510), pump control module, photodiode array detector (model 996), Novapak C$_{18}$ column (3.9 × 150 mm) with guard column, and Millenium software] were performed as above,

(28) Lomovskaya, N.; Otten, S. L.; Fohnstein, L.; Doi-Katayama, Y.; Liu, X.-C.; Takatsu, T.; Inventi-Solari, A.; Breme, U.; Colombo, A. L.; Hutchinson, C. R. *J. Bacteriol.* **1999**, *181*, 305−318.

(29) Motamedi, H.; Wendt-Pienkowski, E.; Hutchinson, C. R. *J. Bacteriol.* **1986**, *167*, 575−580.

(30) Hopwood, D. A.; Kieser, T.; Wright, H. M.; Bibb, M. J. *J. Gen. Microbiol.* **1983**, *129*, 2257−2269.

(31) Vara, J.; Lewandowska-Skarbek, M.; Wang, Y.-G.; Donadio, S.; Hutchinson, C. R. *J. Bacteriol.* **1989**, *171*, 5872−5881.

(32) Madduri, K.; Kennedy, J.; Rivola, G.; Inventi-Solari, A.; Filippini, S.; Zanuso, G.; Colombo, A. L.; Gewain, K. M.; Occi, J. L.; Macneil, D. J.; Hutchinson, C. R. *Nat. Biotechnol.* **1998**, *12*, 69−74.

(33) Motamedi, H.; Hutchinson, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 4445−4449.

(34) Bormann, C.; Aberle, K.; Fiedler, H.-P.; Schrempf, H. *Appl. Microbiol. Biotechnol.* **1990**, *32*, 424−430.

(35) Hopwood, D. A.; Bibb, M. J.; Chater, K. F.; Kieser, T.; Bruton, C. J.; Kieser, H. M.; Lydiate, D. J.; Smith, C. P.; Ward, J. M.; Schrempf, H. *Genetic Manipulation of Streptomyces: A Laboratory Manual*; The John Innes Foundation: Norwich, U.K., 1985.

(36) Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular cloning: a laboratory manual,* 2nd ed.; Cold Spring Harbor Laboratory, Cold Spring Harbor: NY, 1989.

except that the extracted crude residues were resuspended in 50 μL of MeOH and 5 μL samples were injected (Rheodyne) onto the column. The solvent gradient used was: Solvent A = 0.1% formic acid in H_2O; solvent B = acetonitrile (Omnisolv, EM science); flow rate, 2.0 mL/ min, 0–10 min 100% A to 70% A:30% B (linear gradient, curve 6), 10–15 min, 70% A:30% B to 100% B (concave gradient, curve 9), 15–20 min, 100% B to 100% A (curve 9), then held at 100% A for 5 min.

The final analyses of *S. lividans* cultures freshly transformed with constructs listed in Table 1 were performed in duplicate and analyzed by TLC and HPLC at 48 h.

Isolation and Characterization of Angucyclinones. Preparative incubations of *S. lividans* harboring plasmid pWHM1237 were grown in 250-mL baffled Erlenmeyer flasks each containing R2YENG (50 mL) with thiostrepton (10 μg/mL). Three separate 500-mL incubations were acidified with formic acid to pH 4 and extracted by shaking with 2 × 500 mL EtOAc portions. After centrifugation to separate emulsions, the organic layers were combined and evaporated to dryness in vacuo. The combined crude extract residues (300 mg) were redissolved in 3 mL of MeOH and then fractionated by HPLC (as described above using a Prep Nova-Pak HR C$_{18}$, 7.8 × 300 mm column, gradient: 6.0 mL/ min, 0–30 min 100% A to 70% A:30% B (linear gradient, curve 6), 30–45 min, 70% A:30% B to 100% B (concave gradient, curve 9), 45–60 min, 100% B to 100% A, curve 9, and then held for 5 min). Elution profiles were monitored at 265/410 nm, and two peaks were collected at elution volumes 204–216, and 227–232 mL. The first broad peak was a mixture of **1** and unknown, and the second peak was pure **12** (3 mg), as determined by HPLC analysis. Fractions were pooled, concentrated under a stream of nitrogen, and extracted with dichloromethane, and the organic layers were evaporated to dryness under a stream of nitrogen. Further HPLC of the solid residues from the early collected fractions gave enriched samples of **1** and the unknown (estimated 90% pure by HPLC). However, the unknown spontaneously converted to **1**. Later, it was found that only **1** was produced by *S. lividans* transformed with pWHM1237 grown on solid media (200 mL of R2YENG agar, 30 °C, 10 d). The solid media was chopped, acidified, and extracted three times with 200-mL portions of CHCl$_3$, and the organic layer was evaporated to dryness in vacuo. This crude extract residue was subjected to TLC (EtOAc:toluene, 1:1) and recovered to afford 4 mg of **1**.