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Characterization of JadH as an FAD- and NAD(P)H-Dependent Bifunctional Hydroxylase/Dehydrase in Jadomycin Biosynthesis

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As a major class of aromatic polyketide natural products, the angucyclines display significant diversity in structure and bioactivity. Like most other bacterial aromatic polyketides, angucyclines are derived from polyketide chains assembled by the minimal type II polyketide synthases, which consist of a ketosynthase α and β heterodimer complex and a cognate acyl carrier protein.[1] The polyketide chains are then modified by accessory enzymes (ketoreductase and cyclase/aromatase) to generate the key angucycline intermediates, UWM6 (**1**) or its analogues.[2,3] Subsequent modification steps catalyzed by tailoring enzymes afford the hundreds of structurally diverse angucyclines that have been identified.[4] A large number of different kinds of enzymes are involved in the tailoring steps of angucyclines. Investigation of these enzymes will help us to understand the mechanism of biosynthetic assembly of these molecules, and this knowledge is required for combinatorial engineering to produce novel angucyclines. Among all the tailoring steps, C12 monooxygenation is remarkable in that it takes place in all angucycline biosynthetic pathways,[4] and investigation of the enzyme catalyzing this reaction should shed significant light on the biosyntheses of the whole angucycline family.

The first characterized enzyme catalyzing angucycline C12 monooxygenation is JadH from jadomycin (JD) pathway. JD refers to a series of angucycline molecules with a unique pentacyclic benz[*b*]oxazolophenanthridine skeleton.[5–7] They exhibit good bioactivity against Gram-positive bacteria, including the methicillin-resistant *Staphylococcus aureus*.[8] Recently, JD B (**2**; Scheme 1) was shown to inhibit the activity of Aurora-B kinase.[9] The *jad* gene cluster was cloned from *Streptomyces venezuelae* ISP5230 in the early 1990s, and the JD biosynthetic pathway has been thoroughly analyzed.[10–12] JadH was proposed to catalyze the C12 monooxygenation and 4a,12b-dehydration from mainly in vivo investigations.[13,14] It was also shown to convert 2,3-dehydro-UWM6 (**3**) to dehydrorabelomycin (**4**) and **1** to rabelomycin (**5**) in vitro (Scheme 2).[13] Studies on the JadH homologues, (for example, PgaE (55.9% identity) from the gaudimycin A (**6**),[15,16] CabE (55.1% identity) from the gaudimycin B (**7**),[15,16] OvmOI (54.4% identity) from the oviedomycin (**8**),[17] and GilOI (40.1% identity) from the gilvocarcin V (**9**) gene clusters,[3,18] in other angucycline pathways also support the C12 monooxygenation function of JadH (Scheme 1).

However, two questions remain when comparing the function of JadH with its homologues. First, the biosynthesis of **9** should evoke a hydroquinone instead of a quinone intermediate,[3] which suggests that C12 hydroxylation is the real reaction catalyzed by JadH homologues in the **9** biosynthesis. It is also consistent with the similarities between JadH homologues and typical FAD-dependent aromatic hydroxylases in primary, secondary and tertiary structures.[13,19] Second, non-enzymatic 4a,12b-dehydration has been observed previously in the spontaneous conversion from **1** to **5** (Scheme 2),[2] and the 4a-hydroxyl groups are kept in some angucyclines (for example, **6**, **7** and simocyclinone D8 (**10**)[20]), hence raising the question whether the 4a,12b-dehydration is catalyzed by the JadH homologues or whether it is just a non-enzymatic conversion. These intriguing questions motivated us to further characterize the function of angucycline C12 monooxygenation enzymes, such as JadH.

The N-His-tagged JadH (JadH-*N*-His) was expressed and purified as described previously.[13] To overexpress native JadH in *Escherichia coli*, plasmid pET23b-jadH was first introduced into *E. coli* BL21. However, no JadH expression was detected although different culture temperatures and IPTG concentrations were tested. We noticed that several codons (CGG and CCC) seldom used in *E. coli* appeared frequently in the *jadH* gene. The plasmid was then transformed into the *E. coli* host Rosetta gami™ B LysS, which harbors plasmids for *E. coli*-rare codons. Although it was not very efficient, native JadH was expressed as soluble protein in the Rosetta host. About 0.4 mg of colorless native JadH was purified from the cell extracts of 1 L cultured cells. Purified native JadH and JadH-*N*-His were examined on 10% SDS-PAGE. Both of them migrated as a single band with a molecular mass consistent with the predicted sizes (57 kDa for native JadH (Figure S1 in the Supporting Information) and 61 kDa for JadH-*N*-His (Figure 1)).

JadH was proposed to be an FAD-dependent enzyme based on it containing a typical Rossmann fold dinucleotide-binding motif (GXGXXG/A), which is responsible for binding the adenosine moiety of FAD,[21] and a GD motif interacting with the flavin moiety of FAD (Figure S2).[22] Purified JadH-*N*-His is yellow and its UV/visible spectrum was consistent with the presence of a flavin cofactor with absorbance maxima at 366 and 450 nm (Figure S3). After JadH-*N*-His was denatured by acidification (5 μL of 1 M HCl was added to 50 μL of 10 μM

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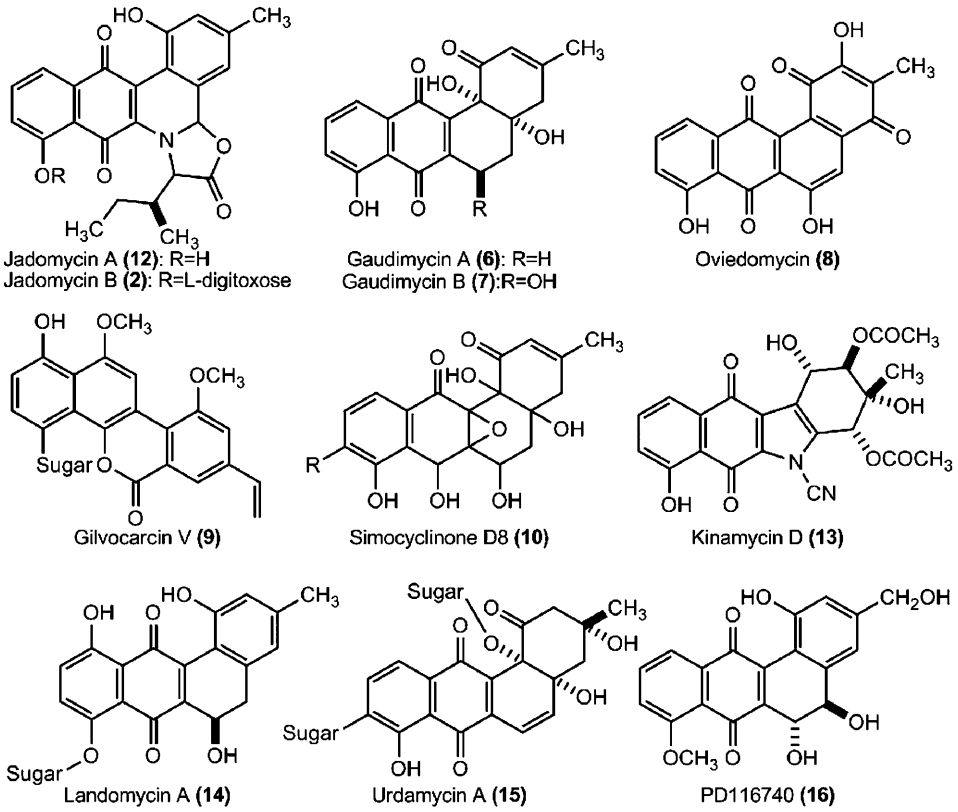
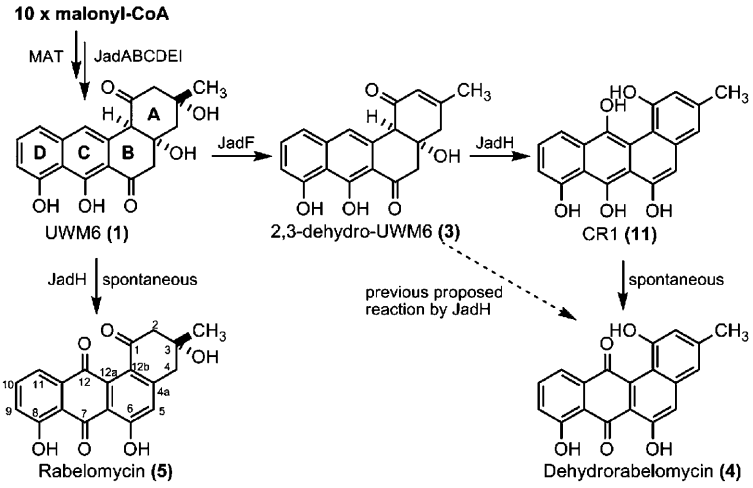


Scheme 1. Structures of angucyclines, the gene clusters of which have been cloned.



Scheme 2. Proposed biosynthetic pathway from UWM6 (**1**) to dehydrorabelomycin (**4**).

enzyme solution), the prosthetic group was separated with JadH-*N*-His by centrifugation (12000 *g*, 30 min), and confirmed to be FAD by HPLC with an authentic FAD standard. The ratio between JadH-*N*-His and FAD was determined to be 1:0.93; this indicates that one FAD molecule binds to one JadH protein.

Purified native JadH was colorless, and no FAD-specific absorption was observed in its UV/visible spectrum; this indicates that FAD was removed during the purification process. When testing the native JadH under standard conditions (0.2 μM purified enzyme, 4 μM FAD, 125 μM NADPH, and 100 μM **3** incubating 60 s at pH 8.0, 32 °C), it could convert **3** to **4** efficiently. However, if FAD were eliminated from this system, no enzyme activity could be observed; this confirms that FAD is indispensable for JadH bioactivity.

The specific activity of native JadH was determined to be 4.1 ± 0.1 μM min^{-1} mg^{-1}, and it was 4.0 ± 0.1 μM min^{-1} mg^{-1} for JadH-*N*-His. This suggests that the influence of the N-terminal His-tag on JadH activity is insignificant. In the following studies, JadH-*N*-His was used to characterize this enzyme.

The quaternary structure of JadH was examined by blue native-PAGE. A single band observed at about 60 kDa showed that JadH-*N*-His might be a monomer (Figure S4). When the standard assay was carried out in anaerobic conditions with JadH-*N*-His, neither decrease of the substrate **3** nor production of any new compound was observed, proving that O_2 was necessary for the JadH-catalyzed reaction. It also indicates that the 4a,12b-dehydration step is after or coupled to the C12 oxygenation reaction.

Detection of the utilization of NAD(P)H by JadH revealed that no conversion was observed without NADPH in JadH enzymatic assay, showing that NADPH was necessary for JadH activity. When substituting NADPH with NADH in the standard assay conditions, the specific activity was determined to be 3.2 ± 0.1 μM min^{-1} mg^{-1}; this shows that NADH was also an efficient cofactor for JadH.

The optimal pH of JadH-*N*-His was determined to be 8.5 by assays ranging in pH from 6.5 to 9.5 at 30 °C. The optimal reaction temperature was then determined to be 32 °C by varying from 24 °C to 40 °C with an interval of 2 °C at pH 8.5. The steady-state kinetic properties of the enzyme were investigated at 32 °C in 50 mM Tris–HCl buffer pH 8.5 (Figure 2). The apparent K_m for **3**, determined at 250 μM NADPH, was 153.8 ± 47.1 μM, The value for k_{cat} was determined to be 10.7 ± 1.5 s^{-1}.

Generally, FAD in FAD-dependent monooxygenases can be reduced to $FADH^-$ by NAD(P)H, and its absorption at 450 nm would decrease sharply.[23] The reduction can take place with or without substrate in many cases, like the well-characterized *para*-hydroxybenzoate hydroxylase.[24] However, no absorption decrease at 450 nm was observed when 6 μM JadH-*N*-His was mixed with 6, 12, or 18 μM NADPH in 50 mM Tris–HCl at differ-

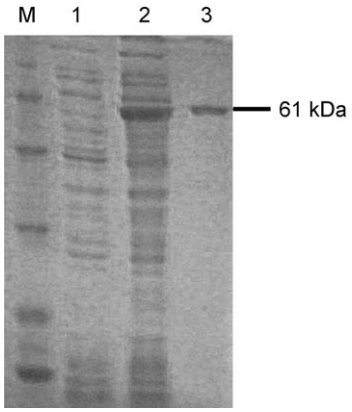


Figure 1. SDS-PAGE of JadH-*N*-His expression and purification. Lane M, protein marker; lane 1, *E. coli* BL21 harboring pET30a as control; lane 2, pET30a-JadH-*N*-His in *E. coli* BL21; lane 3, purified JadH-*N*-His.

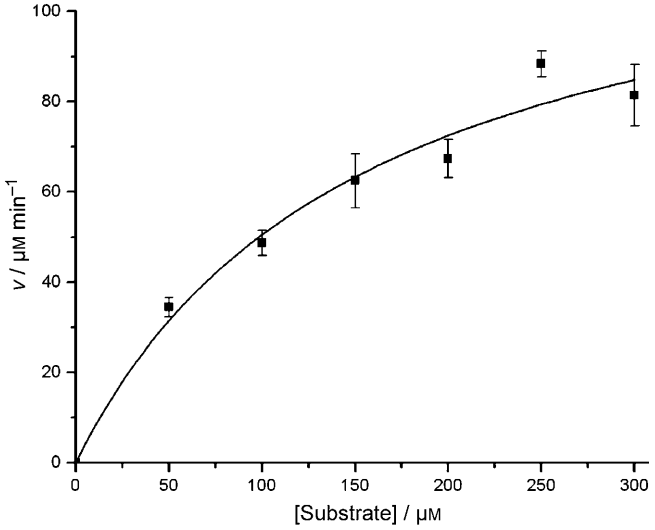


Figure 2. Plot of initial velocity versus substrate concentration of 2,3-dehydro-UWM6 (**3**) for the JadH-*N*-His-catalyzed reaction.

ent pHs (6.5 to 9.5 with 0.5 intervals). Notably, the absorption at 450 nm dramatically dropped within 20 s by adding 10 μM **3** to the JadH-*N*-His (6 μM) and NADPH (6 μM) mixture, indicating a conformational change of JadH triggered by substrate binding made FAD accessible to NAD(P)H. Similar phenomena have also been observed in some other FAD-dependent hydroxylases.[25] After this initial rapid formation of $FADH^-$ and a subsequent lag phase (from 20 to 30 s), the absorbance began to increase due to the formation of product **4**, which absorbs strongly at 450 nm (Figure S5).

Strikingly, a new compound with an HPLC retention time at 11.7 min was observed at lower temperature ($<20\,^{\circ}C$) when we assayed JadH carefully at different conditions (Figure 3). This new compound, designated as CR1 (**11**), was very unstable and spontaneously converted to **4** in air under basic conditions such as the pH 8.5 Tris–HCl buffer used for the enzymatic assay. The rate of this oxidization decreased dramatically as pH decreased from 8.5 to 7.4, as shown in Figure S6 (Supporting Information). Quantitative HPLC analysis of this oxidization at

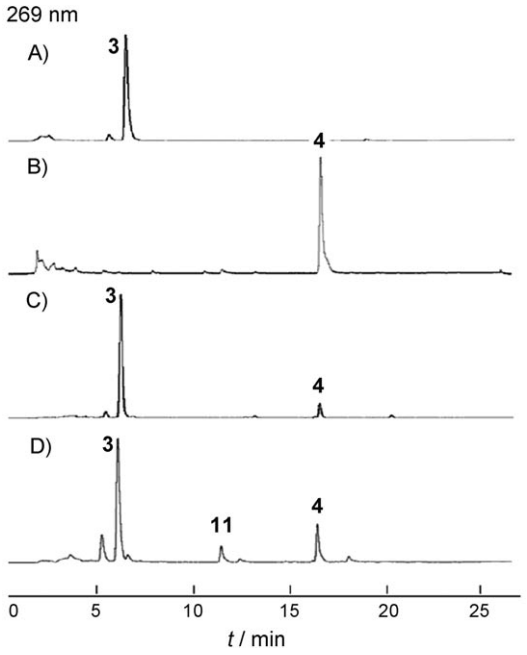


Figure 3. HPLC traces to show the reaction catalyzed by JadH. A) purified 2,3-dehydro-UWM6 (**3**), B) dehydrorabelomycin (**4**), C) JadH-*N*-His assay at 32 °C, and D) JadH-*N*-His assay at 10 °C.

32 °C, pH 8.5, the same condition for the kinetic study of JadH-*N*-His, showed a single-exponent decrement of **11**, and a pseudo-first-order reaction rate constant was deduced as $0.300 \pm 0.015\ min^{-1}$. Compound **4** was the only product of **11** oxidization, and its formation was coupled to the consumption of **11** as shown by HPLC (Figure S7; Supporting Information). Addition of JadH-*N*-His, NADPH and $NADP^+$ in a variety of combinations had no influence on the conversion rate; this shows that oxidation from **11** to dehydrorabelomycin was not enzyme catalyzed.

We scaled up the assay at 10 °C and purified **11** as a yellow solid by HPLC. The UV/visible spectrum of **11** showed absorbance peaks at 239, 299, and 372 nm, implying a highly conjugated system. Its molecular weight was determined to be 322 Da by HPLC-ESI-MS (*m/z* at 321.2 for negative-ion mode and 323.2 for positive-ion mode) and its formula was determined to be $C_{19}H_{14}O_5$ by high-resolution ESI-MS (*m/z* calcd: 345.073; found: 345.0741 $[M+Na]^+$). The structure of **11** was elucidated based on its NMR spectroscopy data (^1H and COSY; Table 1) and by the fact that it can be oxidized to **4** nonenzymatically.

Elucidation of the structure of **11** and studies on the conversion from **11** to **4** revealed that JadH actually catalyzed the 4a,12b-dehydration and C12 hydroxylation to form the hydroquinone compound **11**, and the quinone form **4** arose from subsequent spontaneous oxidation of **11** (Scheme 2). The 4a,12b-dehydration step was proposed to be catalyzed by JadH based on the following evidences: 1) spontaneous dehydration of **3** has never been observed during handling and purification of this compound; 2) both **11** and **4** are 4a,12b-dehydrated; 3) no other product was produced when the JadH assay was performed under different conditions (pH 6.5–9.5,

Table 1. ^{1}H NMR and gCOSY of CR1 (**11**).

Pos.	CR1 (**11**) in [D_6]acetone δ_H (J [Hz])	Pos.	CR1 (**11**) in [D_6]acetone δ_H (J [Hz])	gCOSY(H→H)
1		9	6.99 (d, 8.4, 1H)	10
2	6.80 (s, 1H)	10	7.68 (t, 7.8, 1H)	9, 11
3		11	7.34 (d, 7.2, 1H)	10
3-CH_3	2.39 (s, 3H)	11a		
4	7.11 (s, 1H)	12		
4a		12a		
5	7.18 (s, 1H)	12b		
6		OH-1	[a]	
6a		OH-6	11.91 (s, 1H)[a]	
7		OH-7	[a]	
7a		OH-8	[a]	
8		OH-12	11.92 (s, 1H)[a]	

[a] Interchangeable.

10–40 °C); 4) compound **3** could not be modified by JadH in anaerobic conditions indicating that the 4a,12b-dehydration step was coupled to or occurred after C12 hydroxylation. JadH was therefore concluded to be a bifunctional hydroxylase/dehydrase converting **3** to **11**. Bifunctional hydroxylases/dehydrases have been reported previously, for example, CYP71E1 is a cytochrome P450-dependent hydroxylase/dehydrase involved in the biosynthesis of the cyanogenic glucoside dhurrin in *Sorghum bicolor*.[26] The characterization of JadH here presents an interesting case of FAD-dependent hydroxylase/dehydrase involved in polyketide biosynthesis.

Finally, the intermediacy of **4** and **11** was assessed by bioconversion assay by using *S. venezuelae* CH56, a *jadA* (encoding KSα) in-frame deletion mutant.[13,14] CH56 without feeding served as a negative control; whereas CH56 that was fed with purified **3** (a known intermediate of JD biosynthesis) was used as a positive control.[13,14] When **4** was fed to CH56, compounds **2** and **12** were both observed after 2 h incubation (Figure S8) like the positive control, these results clearly showed that **4** is an intermediate of JD biosynthesis. Because **11** could spontaneously convert to **4**, it is also expected to be an intermediate of JD biosynthesis. In fact, when **11** was fed to a CH56 culture under identical bioconversion conditions, it was completely oxidized to **4** in no more than 10 min, which was further converted to JDs (data not shown). The identification of both **4** and **11** as intermediates in JD biosynthesis filled a gap in the biosynthetic pathway between compounds **3** and **12**. These in vitro results also reflect the real function of JadH in *S. venezuelae*.

To our knowledge, JadH homologues have been found in all cloned angucycline gene clusters. This is consistent with the fact that all of these molecules possess a hydroxyl or keto group at their C12 position. These homologues include the aforementioned OvmOI,[17] PgaE, CabE,[14,16] GilOI,[18] and several other proteins such as KinO2 (69.2% identity) from the kinamycin (**13**),[27] LanE (54.5% identity) from the landomycin (**14**),[28] UrdE (54.9% identity) from the urdamycin (**15**),[29] Pd2O (55.5% identity) from the PD116740 (**16**),[30] and SimA7 (54.8% identity) from the simocyclinone D8 (**10**)[20] gene clusters (Scheme 1). Multiple sequence alignments reveal that the conserved Rossmann fold and GD motif for FAD binding[21,22] and DG motif for both FAD and NAD(P)H binding[31] are present in all ten proteins (Figure S2); so they are also expected to catalyze the C12 hydroxylation reaction in their biosynthetic pathways.

Besides JD, five other compounds (**8**, **9**, **13**, **14**, and **16**) also undergo 4a,12b-dehydration, implying that the JadH homologues involved in their biosynthesis are also bifunctional hydroxylases/dehydrases. This has already been implied in the case of **8** and **9** by in vivo data. Heterologous expression of the polyketide synthetic genes for **8** biosynthesis with various accessory oxygenase genes in *Streptomyces albus* had shown that the JadH homologue, OvmOI was required for the C12 oxygenation and 4a,12b-dehydration, because all compounds formed in the presence of OvmOI contained C12 keto and 4a,12b-double bond.[17] The C12 hydroxylated and 4a,12b-dehydrated compounds were also observed in *Streptomyces lividans* strain expressing **9** polyketide synthetic genes with GilOI.[3] In addition, inactivation of *gilOI* could be complemented by *jadH* in the heterologous *S. lividans* mutant strain; this strongly supports GilOI catalyzing the same reaction as JadH.[18]

It is notable that four of these compounds (**6**, **7**, **10**, and **15**) still contain 4a-hydroxyl groups in the final products. JadH homologue, PgaE (involved in **6** biosynthesis) was proposed as not having 4a,12b-dehydrase activity by the fact that 4a,12b-dehydrated product was not observed in the in vitro bienzyme assays of PgaE and PgaM.[16] Sequence comparison of JadH and its homologues did not show clear differences between those deduced to catalyze 4a,12b-dehydration and those that probably not. Recently, the three-dimensional structures of PgaE and CabE (involved in the biosynthesis of **7**) were determined by X-ray crystallography,[19] which were similar to those structures of previously characterized flavin-dependent aromatic hydroxylases. The monomers of both enzymes consisted of three domains, the FAD-binding domain (residues 1–172 and 256–375), the middle domain (residues 173–255) and the C-terminal domain (residues 376–491) for PgaE. The FAD-binding and middle domains are essential for hydroxylation catalysis; whereas the precise function of the C-terminal domain is unknown, which resembled thioredoxin in fold, but lacked two conserved cysteine residues in thioredoxin.[16] Unfortunately, because no protein structure of FAD-dependent hydroxylase/dehydrase is solved yet, investigating the dehydration activity of JadH homologues by structure comparison is still difficult. The mutational analyses of JadH function are underway in our lab; and they should shed some light on the dehydration activities of this group of enzymes.

Experimental Section

General materials and methods: *E. coli* strains DH5α, BL21, and Rosetta gami^T B LysS were grown in Luria–Bertani broth.[32] Plasmid pJV69A, harboring the *jadFGHK* genes, has been described previously.[11] DNA restriction and ligation enzymes were purchased from TaKaRa (Shiga, Japan). Chemical agents that were used in this paper (NADPH, NADH, and FAD) were purchased from Sigma–Aldrich.

DNA manipulations, competent cells preparation and transformation were performed as described previously.[32] Protein concentration was determined by the Bradford method.[33] SDS-PAGE and Blue native-PAGE were performed as described.[34] Compound **3** was purified as described previously.[13] Homologous sequence database searching and multiple alignments were executed with BLASTP and ClustalX, respectively. Origin 6.1 was used to determine the enzyme kinetic parameters.

Expression and purification of N-terminal His-tagged JadH: The fragment containing *jadH* was cloned by PCR from pJV69A with *Pfu* DNA polymerase. The forward primer was 5′-GTGGGTACCGTGACCACCACCCCG-3′ (KpnI); and the reverse primer was 5′-GGAATTCACCGGGCCGCGCCGC-3′ (EcoRI). The cloned 1.6-kb fragment was digested with KpnI/EcoRI and inserted into pET30a to obtain plasmid pET30a-JadH-*N*-His. The resultant plasmid was then transformed into *E. coli* BL21. N-terminal His_6-tagged JadH (JadH-*N*-His) was produced and purified as previously described.[13]

Expression and purification of native JadH: Gene *jadH* was cloned by PCR by using 5′-GAGTGAGCCATATGACCACCACCCCGG-3′ (NdeI) and 5′-GGAATTCACCGGGCCGCGCCGC-3′ (EcoRI). After digestion with NdeI/EcoRI, the fragment was inserted into pET23b to construct pET23b-jadH. Then, pET23b-jadH was transformed into *E. coli* Rosetta gami^T B LysS. The culture procedure for JadH expression was similar to that of JadH-*N*-His production,[13] except the IPTG final concentration was 0.4 mM instead of 0.6 mM.

All the purification steps were carried at 4 °C. After the collected cells were lysed by sonication, the cell debris was removed by centrifugation (14000 *g*, 15 min); the supernatant was fractionated by ammonium sulfate precipitation. Proteins precipitated by 40–55% ammonium sulfate were collected by centrifugation (8000 rpm, 10 min). The remaining soluble proteins were then desalted and concentrated by centrifugation in 10 kDa molecular-weight cut-off ultrafiltration tubes twice at 4000 rpm. Subsequent chromatographic steps for obtaining purified JadH were performed on an ÄKTA FPLC system (GE Healthcare). During the purification process, SDS-PAGE and a JadH assay against **3** were used to monitor JadH. The desalted protein was first loaded onto a Resource Q anion exchange column (GE Healthcare) by using a gradient from 0 to 1 M NaCl in 20 mM Tris–HCl buffer (pH 8.0). The proteins eluting between 0.3 and 0.4 M NaCl were collected, concentrated, and subjected to gel filtration on a Superdex-200 column (GE Healthcare), which was eluted with 20 mM Tris–HCl, pH 7.0. Finally, the eluted fractions containing JadH were loaded on a Mono-Q anion exchange column (GE Healthcare) and washed with 20 mM Tris–HCl, pH 7.5 by using a 0 to 1 M NaCl gradient.

Enzyme assay of JadH: The standard enzyme assay was started by adding purified enzyme (0.2 μM; JadH or JadH-*N*-His) to the reaction mixture (100 μL; 50 mM Tris–HCl buffer, pH 8.0, 4 μM FAD, 125 μM NADPH and 100 μM substrate **3**). After incubating for 60 s at 32 °C, the reaction was terminated by HCl (1 μL, 1 M). A reaction with all components except the enzyme was treated in the same way and used as a control. The kinetic data was calculated by using the nonlinear curve fit (hyperbola equation) of Origin 6.1 program.

For enzyme assay at anaerobic conditions, the reaction mixture without adding JadH was degassed under vacuum and bubbled with nitrogen for 8 to 10 rounds in a sealed tube. JadH that had been treated with nitrogen was then injected to initiate reaction. When the reaction was finished, HCl (1 μL, 1 M) was injected to denature the enzyme.

Isolation of 11: After the JadH-catalyzed reaction at low temperature (10 °C) was terminated, the reaction mixture was injected to HPLC directly for separation by using a Waters SymmetryShield RP18 column (5 μm, 4.6×150 mm, Waters, Milford, MA). The elution solvents were H_2O with 0.1% TFA (solvent A) and MeCN with 0.1% TFA (solvent B). A 20-minute linear gradient from 25% to 100% solvent B was used. Compound **11** was collected and lyophilized for further MS and NMR spectroscopic analysis.

In vivo bioconversion assay: *S. venezuelae* CH56 was cultured following the jadomycin production procedure as described previously.[13] After being stimulated with EtOH (6%, *v/v*) and cultured at 28 °C, 250 rpm for further 48 h, the cells (30 mL cultures) were collected and washed twice with PBS buffer (5 mL, 50 mM, pH 7.2). They were resuspended in PBS buffer with isoleucine (5 mL, 50 mM pH 7.2, 30 mM isoleucine). Compounds **3**, **4** and **11** (20 μL solution in ethanol) were added to cell suspension (0.5 mL), and they were cultured at 28 °C, 250 rpm for 2 h. The cultures were then acidified by adding HCl (20 μL, 2 M) and extracted with EtOAc (0.4 mL). Finally, EtOAc extracts (0.3 mL) were concentrated and analyzed by HPLC.

Analytical and spectroscopic procedures: Related compounds were monitored by HPLC with a 5 μm Inertsil ODS-3 column (4.6× 250 mm, GL sciences, Torrance, CA). The elution solvents were the same as those for isolation of **11**. The percentage of solvent B (flow rate: 1.0 mL min^{-1}) changed linearly from 50 to 100% between 0 and 20 min, then stayed at 100% for 3 min. For LC–MS analysis, an Agilent 1100 HPLC system with Finnigan LCQ DecaXP ion trap mass spectrometer (Thermo Finnigan) was used. High-resolution ESI-MS was performed on a Bruker Apex IV FT-MS spectrometer (Fremont, CA). NMR spectroscopy of CR1 was collected on a Varian Unity VNS 600 MHz NMR spectrometer.

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Keywords: angucycline · biosynthesis · enzymes · hydroxylation/dehydration · jadomycin

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