Characterization of Heronamide Biosynthesis Reveals a Tailoring Hydroxylase and Indicates Migrated Double Bonds

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Characterization of Heronamide Biosynthesis Reveals a Tailoring Hydroxylase and Indicates Migrated Double Bonds

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Heronamides belong to a growing family of β-amino acid polyketide macrolactams (βPMs) with an unsaturated side chain. The biosynthetic gene cluster for heronamide F was identified from the deep-sea-derived *Streptomyces* sp. SCSIO 03032. The involvement of the gene cluster in heronamide biosynthesis was confirmed by the functional characterization of the P450 enzyme HerO as an 8-hydroxylase for tailoring heronamide biosynthesis. The presence of migrated double bonds in the conjugated diene-containing side chain of heronamides was confirmed by feeding experiments with labeled small carboxylic acid molecules. This study is the first demonstration of migrated double bonds in βPMs with an unsaturated side chain.

Introduction

Heronamides contain a conjugated diene side chain that is proposed to be biosynthetically derived from a β-amino acid component, and thus belong to β-amino acid-containing polyketide macrolactams (βPMs). βPMs are a growing class of natural products with diverse biological activities.[1] Heronamides A–C (**4**–**6**, Scheme 1) were reported from a marine-derived actinomycete in 2010;[2] the stereochemistry of **4** was reassigned in 2013.[3] Recently, we reported heronamides D–F (**1**–**3**),[4] whose side chains are two carbons shorter than those of **4**–**6**, from the deep-sea-derived *Streptomyces* sp. SCSIO 03032. This species also produces bisindole alkaloids and α-pyridone antibiotics.[5] Although displaying no antibiotic or anticancer activity,[2,4] heronamides were initially found to elicit unexpected effects on cell morphology,[2] and were recently shown to have unique membrane-binding abilities.[6]

Recent characterization of a number of βPM biosynthetic gene clusters has revealed a common strategy of using a variety of β-amino acids as starter units in type I polyketide synthase (PKS) pathways.[1] Well-studied examples include vicenistatin (a β-aminoisobutyrate starter unit),[7] incednine (a β-aminobutyrate starter unit),[8] and hitachimycin (an *S*-β-phenylalanine starter unit).[9] In structural analogy to heronamides, a side chain of six or eight carbons is also present in the other βPMs (Scheme 1), such as BE-14106 (**7**), ML-449 (**8**), cremimycin (**9**), and aureoverticilactam (**10**). The biosynthetic gene clusters of BE-14106 (**7**) and ML-449 (**8**) have been deciphered by Zotchev and co-workers.[10] The side chains in **7** and **8** were proposed to be incorporated as aminoacyl starter units with glycine as the amino donor; however, the incorporation mechanisms remain unclear.[10] Recently, the formation of a β-aminoacyl starter unit (3-aminononanoate) in the biosynthesis of **9** was biochemically established by Eguchi and co-workers:[11] a unique amino transfer mechanism involving Michael addition of a glycine by the fatty acyl-CoA thioesterase CmiS1, and oxidative hydrolysis to remove a glyoxylate unit by the FAD-dependent glycine oxidase CmiS2.[11] Intriguingly, a protection/deprotection strategy was revealed for vicenistatin biosynthesis: addition and removal of an α-amino acid by a set of five enzymes (VinJKLMN).[7] The presence of VinJKLMN homologues in other βPMs pathways suggests that this protection/deprotection strategy is common in the biosynthesis of βPMs.[1,7]

A substantial understanding of the biosynthesis of βPMs has been achieved in recent years.[1] However, little is known about the mechanism of formation of their unsaturated side chains. Hydroxylation at C-8 (e.g., in **1**–**8**, Scheme 1) has been proposed as a tailoring step accomplished by a P450 enzyme. However, none of the enzymes has been functionally verified. In this study, the gene cluster for the biosynthesis of **3** was identified in *Streptomyces* sp. SCSIO 03032. The P450 enzyme HerO was biochemically characterized as the C-8 hydroxylase that tailors heronamide biosynthesis. Feeding experiments with labeled small carboxylic acid molecules indicated unusual migrated double bonds in the unsaturated side chain of heronamides.

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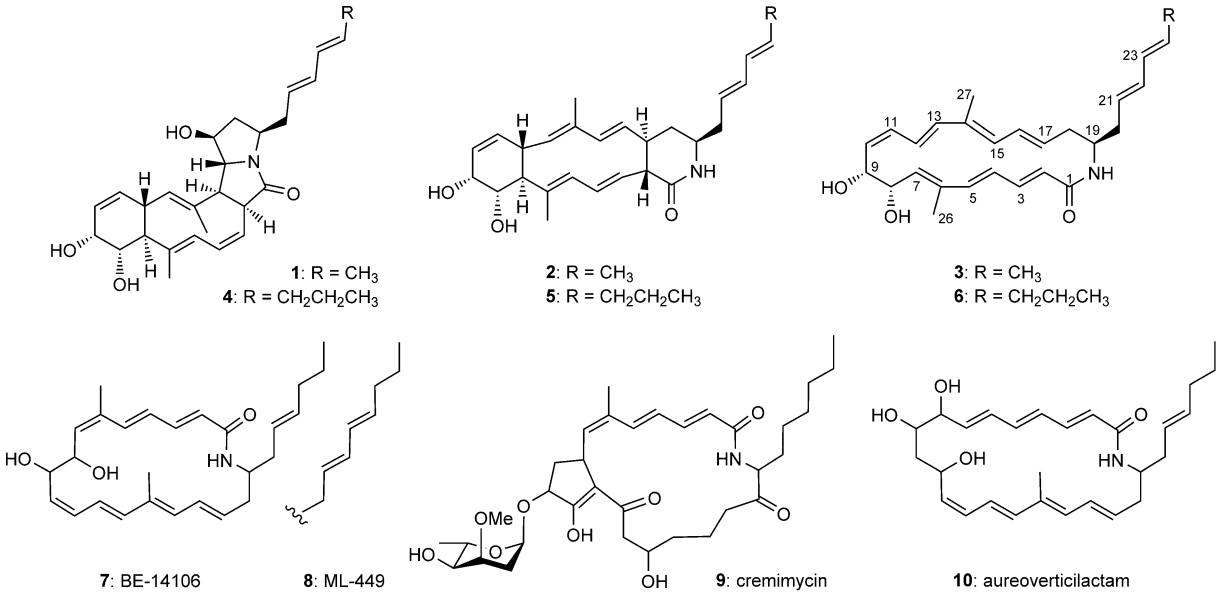


Scheme 1. Naturally occurring βPMs with side chains.

Results and Discussion

Cloning, identification, and bioinformatics analysis of the heronamide biosynthetic gene cluster

We recently discovered the piericidin A1 biosynthetic gene cluster from *Streptomyces* sp. SCSIO 03032 by genome sequencing.[5c] Bioinformatics analysis by comparing the draft genome sequence with genes coding for the biosynthesis of **7** and **8**[10] led to the identification of putative heronamide biosynthetic genes ("*her*") distributed over seven contigs (50–53, 64, 156, and 216; see Figure S1 in the Supporting Information). Positive clones covering contigs 51, 53, 64, and 216 were screened from the previously constructed, SuperCos1-based genomic library of *Streptomyces* sp. SCSIO 03032,[5c] by PCR with primers ctg51F/R, ctg53F/R, ctg64F/R, ctg64F1/R1, and ctg216F/R (Table S1). This led to the identification of four overlapping cosmids spanning the *her* gene cluster (Figure S1). The gaps between neighboring contigs were bridged by PCR or subcloning. Finally, the heronamide biosynthetic gene cluster (GenBank accession KP742963) was unambiguously identified (Figure 1).

Despite a small difference in genetic organization, the enzymes encoded in the *her* gene cluster showed high amino acid similarity and identity to those in the biosynthesis of **7** (*bec*) and **8** (*mla*; Table 1, Figure S2), including eight PKSs (HerA1A2BCDEFG) for construction of the scaffold, two enzymes (HerIU) for the synthesis of a β-aminoacyl starter unit, five enzymes (HerJKLPS) conserved in βPMs for protection/deprotection, a P450 enzyme (HerO), and four enzymes (HerHMNR) putatively responsible for regulation and transportation. The high similarity between the three gene clusters (*her*, *bec*, and *mla*) indicates similar biosynthetic pathways, hence the only difference should lie in the biosynthesis of their different side chains.

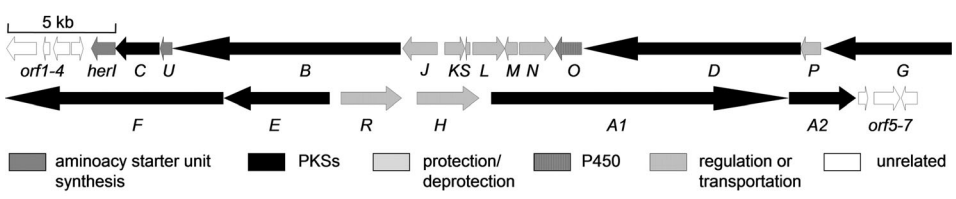


Figure 1. Heronamide gene cluster (*her*).

Characterization of the P450 enzyme HerO as the C-8 hydroxylase

In order to confirm the involvement of the *her* gene cluster in the biosynthesis of heronamides, *herO* (encoding P450) was inactivated by insertional mutation with an apramycin-resistance gene cassette by PCR-targeting (Figure 2A).[12] A double-crossover event in the resulting mutant (HER001) was verified by a PCR method (Figure 2B). HPLC analysis revealed that HER001 accumulated **11**, which is distinct from **3** (Figure 2C, traces i, ii). The molecular formula of **11** was established as $C_{27}H_{35}NO_2$ by HRESIMS (*m/z* 406.2729 $[M+H]^+$, calcd: 406.2741; Figure S3). The ^{1}H NMR data of **11** were highly similar to those of **3** (Figure S3, Table S2);[4] the only difference was that an oxymethine ($\delta_H = 5.33$ ppm) in **3** was replaced by a methylene ($\delta_H =$ 3.10, 1.68 ppm), thus indicating the absence of the 8-OH in **11**. In order to improve solubility and the NMR signals, the acetylated derivative **11a** was prepared (Figure 2D). It was identified as 9-acetyl-8-deoxy-heronamide F by HRESIMS, ^{1}H and

Table 1. Deduced genes of the *her* gene cluster and proposed functions.

Gene	Size[a]	Putative function	Closest homologue[b]	Other βPM homologues clusters
orf1	461	FAD dependent oxidoreductase	SIRAN2303 (CDR05344, 77/84)	
orf2	100	hypothetical protein	M271_11240 (AGP53848, 74/85)	
orf3	256	type 11 methyltransferase	WP_014056794 (64/72)	
orf4	180	HxlR family transcriptional regulator	WP_026400921 (72/79)	
herI	370	glycine oxidase	BecI (ACO94457, 77/86)	MlaI,CmiS2
herC	694	type I polyketide synthase	MlaC (ACO94486, 75/84)	BecC, CmiP2
herU	187	fatty acyl-CoA thioesterase	MlaU (ACO94487, 82/88)	BecU, CmiS1
herB	3555	type I polyketide synthase	BecB (ACO94460, 79/85)	MlaB, CmiP7
herJ	532	AMP-dependent acyl-CoA synthetase/ligase	MlaJ (ACO94489, 79/87)	BecJ, CmiS6, VinN, IdnL1, HitB, MmlL
herK	313	acyltransferase	MlaK (ACO94490, 77/84)	BecK, CmiS5, VinK, IdnL2, HitC, MmlJ
herS	78	peptidyl carrier protein	MlaS (ACO94491, 83/91)	BecS, CmiS4, VinL, IdnL6, HitD, MmlM
herL	504	NRPS adenylation domain protein	MlaL (ACO94492, 82/89)	BecL, CmiS3, VinM, IdnL7, HitE, MmlK
herM	198	TetR family transcriptional regulator	MlaM (ACO94493, 80/85)	BecM
herN	520	MFS transporter	MlaN (ACO94494, 78/86)	BecN
herO	415	P450 monooxygenase	MlaO (ACO94495, 77/87)	BecO, CmiM4
herD	3376	type I polyketide synthase	MlaD (ACO94496, 77/83)	BecD
herP	311	putative proline iminopeptidase	MlaP (ACO94497, 84/91)	BecP, CmiM6, VinJ, IdnL5, HitF, MmlP
herG	1997	type I polyketide synthase	BecG (ACO94470, 74/82)	MlaG, CmiP6
herF	3389	type I polyketide synthase	MlaF (ACO94499, 77/85)	BecF, CmiP8
herE	1648	type I polyketide synthase	MlaE (ACO94500, 72/81)	BecE, CmiP7
herR	946	LuxR-type transcriptional regulator	LuxR (AHH94860, 44/59)	
herH	966	LuxR-type transcriptional regulator	MlaH (ACO94482, 64/75)	BecH
herA1	4620	type I polyketide synthase	BecA (ACO94456, 65/75)	MlaA1
herA2	1048	type I polyketide synthase	MlaA2 (ACO94484, 76/83)	
orf5	146	hypothetical protein	WP_030106204 (58/69)	
orf6	397	transcriptional regulator	RtcB (WP_031045282, 83/90)	
orf7	248	methyltransferase	WP_037777959 (45/64)	

[a] Amino acids. [b] Accession numbers; values indicate % identity/similarity.

^{13}C NMR (Figure S4, Table S3), so **11** from the *ΔherO* mutant was 8-deoxy-heronamide F (Figure 2D), thus indicating that HerO is an 8-hydroxylase to tailor **3** biosynthesis.

Cytochrome P450 enzymes generally require redox partners to activate molecular oxygen for their catalytic activities.[13] Sherman and co-workers engineered the FMN/Fe_2S_2-containing reductase domain (RhFRED) in $P450_{RhF}$ from *Rhodococcus* sp. NCIMB 9784[14] into a generally effective redox partner for in vitro characterizing bacterial P450s.[15] To further validate the function of HerO, the *herO* gene was fused to RhFRED to afford a self-sufficient recombinant cytochrome P450 in *Escherichia coli* (Figure S5). The purified recombinant HerO protein was capable of converting **11** into **3** in the presence of NADPH or NADH (Figure 2C, traces iii, iv); the yield of **3** increased with incubation time (Figure S6). In control assays lacking NAD(P)H or HerO, **11** remained unchanged (Figure 2C, traces v, vi). These data unambiguously confirmed HerO as an 8-hydroxylase and demonstrated the involvement of the *her* gene cluster in heronamide biosynthesis. The in vivo function of HerO homologues in BE-14106 (**7**) and cremimycin (**9**) have been implicated by gene knockout.[10a,11] However, this work provides the first biochemical confirmation of the 8-hydroxylating function of HerO in the biosynthesis of βPMs.

Feeding with isotope-labeled small carboxylic acids

In the conjugated diene-containing side chains of **1**–**3**, both double bonds ($\Delta^{21,22}$ and $\Delta^{23,24}$) are at atypical β,γ positions, in contrast to the general α,β position generated by the canonical PKS assembly line.[16] To gain more insights, we fed sodium [1-^{13}C]acetate, [1-^{13}C]propionate, or [2-^{13}C,D_3]acetate to cultures of *Streptomyces* sp. SCSIO 03032. The NMR spectra of **3** isolated from these were compared (Figure 3, Figure S7): high levels of enrichments were observed at nine positions (C-1, C-3, C-7, C-9, C-11, C-15, C-17, C-19, and C-21) when sodium [1-^{13}C]acetate was fed; with sodium [1-^{13}C]propionate, enrichment was found at three positions (C-5, C-13 and C-23); and with sodium [2-^{13}C,D_3]acetate, enrichment was at nine positions (C-2, C-4, C-8, C-10, C-12, C-16, C-18, C-20, and C-22). From the ^{13}C DEPT NMR spectrum (Figure S8), no incorporation of deuterium was detected after feeding with sodium [2-^{13}C,D_3]acetate. A similar absence of incorporation of deuterium from [2-^{13}C,D_3]acetate was reported in polyketide biosynthesis, attributed to significant washout of deuterium in the conversion of perdeuterated acetyl-CoA to malonyl-CoA and in the subsequent incorporation of malonyl-CoA as the PKS extender unit.[17] It should be noted that minor enrichment at C-5, C-13, and C-23 was also observed when feeding with sodium [1-^{13}C]acetate (Figure 3A). Similar phenomena were observed when investigating the biosynthetic origin of cremimycin by feeding with [1-^{13}C]acetate, and were explained by the conversion of [1-^{13}C]acetyl-CoA into [1-^{13}C]succinyl-CoA in the tricarboxylic acid (TCA) cycle, and further into [1-^{13}C]methylmalonyl-CoA to serve as polyketide starter or as extender units.[18]

The outcome of these labeling studies was entirely consistent with the organization of *her* PKSs to assemble nine mole-

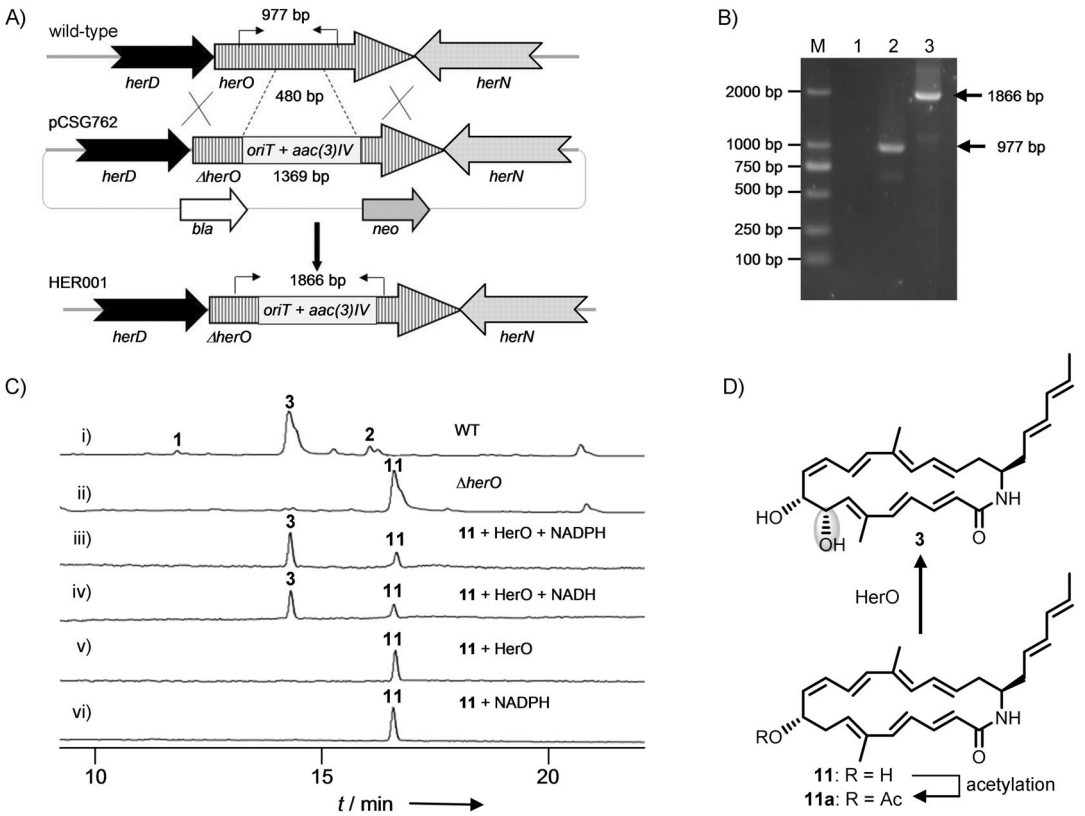


Figure 2. Characterization of HerO. A) *herO* inactivation. The mutant HER001 was constructed by replacing a 480 bp internal *herO* fragment with a 1369 bp DNA fragment containing *oriT* and *acc3(IV)* in the delivery plasmid pCSG762 (Table S4), thereby resulting in a double cross-over recombination event. The locations of the PCR primers are indicated with small arrows. B) Gel electrophoresis of PCR products: lane 1: negative control (water); lane 2: *Streptomyces* sp. SCSIO 03032; lane 3: HER001. C) HPLC analysis of metabolite profiles: i) wild-type *Streptomyces* sp. SCSIO 03032, ii) *ΔherO* mutant HER001, iii) HerO in vitro assay containing **11** (100 μM), HerO (2 μM), and NADPH (2 mM), iv) assay with NADPH in place of NADH, v) assay without NADPH, vi) assay without HerO. D) Scheme for HerO-catalyzed reaction.

cules of malonyl-CoA and three of methylmalonyl-CoA, and as well as with the predicted substrate specificity of the AT domains (methylmalonyl-CoA for three ATs in the loading module of HerA1, modules 5 and 9, and malonyl-CoA for the other nine ATs, Figure S9) (Scheme 2).[19] Given that the loading module of HerA1 contains a KS_Q domain (Figure S9),[20] a methylmalonyl-CoA should be loaded as a starter unit, rather than a propionyl-CoA. Taken together, the labeling results demonstrate that C-21/C-22 were indeed derived from a malonyl-CoA unit, and that C-23/C-24/C-25 were derived from a methylmalonyl-CoA unit, thus indicating that the formation of the two double bonds $\Delta^{21,22}$ and $\Delta^{23,24}$ should involve migrations.

Migrated double bonds

We propose that migration events occur during the PKS assembly, putatively from two unusual β,γ-dehydrations catalyzed by DH domains in modules 1 and 2 of HerA1 (Scheme 2), as discussed below. These β,γ-dehydration events would lead to intermediate **13**, which undergoes another round of chain extension by module 3 to provide intermediate **14**. It should be noted that module 3 is located on two proteins, HerA2 and HerC, with a truncated DH domain, the N and C termini of which are on HerA2 and HerC, respectively (Scheme 2). Similar truncated PKS modules were found for BE-14106 (BecA/BecC), ML-449 (MlaA2/MlaC), and cremimycin (CmiP2/CmiP3). In analogy to the biosynthesis of the β-aminoacyl starter unit by the fatty acyl-CoA thioesterase CmiS1 and the FAD-dependent glycine oxidase CmiS2 in cremimycin,[11] the resulting intermediate **14** would undergo a Michael addition of a glycine by HerU (CmiS1 homologue) to provide **15**, from which a glyoxylate unit would be removed by HerI (CmiS2 homologue) by oxidative hydrolysis to yield **16**. HerU/HerI homologues are also found in BE-14106 (BecU/BecI) and ML-449 (MlaU/MlaI, Table 1),[10] thus suggesting a similar mechanism for the formation of the corresponding β-aminoacyl starter units in BE-14106 and ML-449. Taking into account the catalytic mechanisms of HerU and HerI (Figure S10), as proposed for CmiS1 and CmiS2,[11] the migrated double bonds are unlikely to be generated during nitrogen incorporation, accomplished by HerU and HerI (Figure S10).

Migrated double bonds are rare in polyketide natural products; examples include ansamitocin,[21] rhizoxin,[22] bacillanene,[23] corallopyronin A,[24] and microsclerodermins.[25] Two mechanisms have been proposed for generating such migrated double bonds: direct β,γ-dehydration, and isomerization of the double bond after α,β-dehydration.[22–24] Direct evidence has been provided for DH-domain-catalyzed β,γ-dehydration

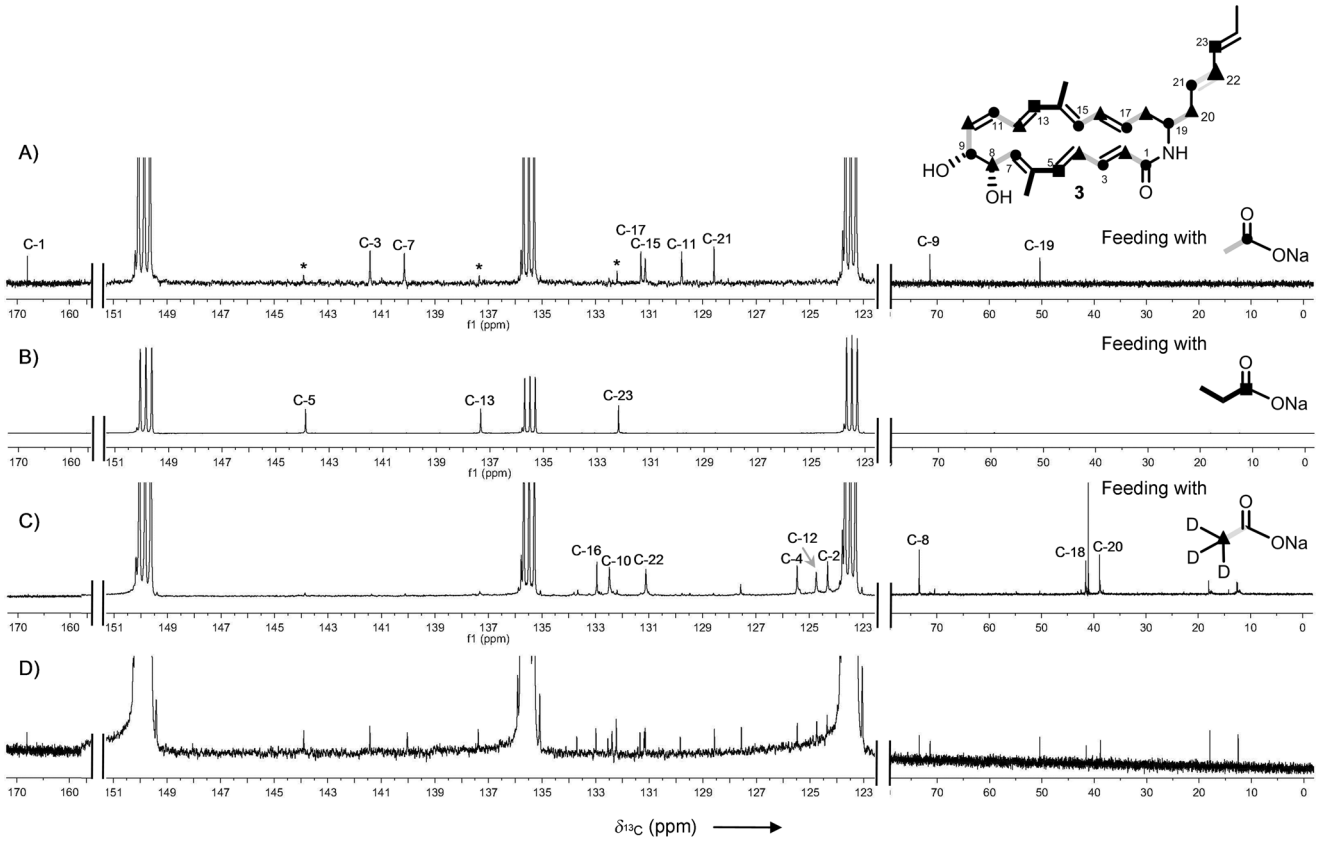


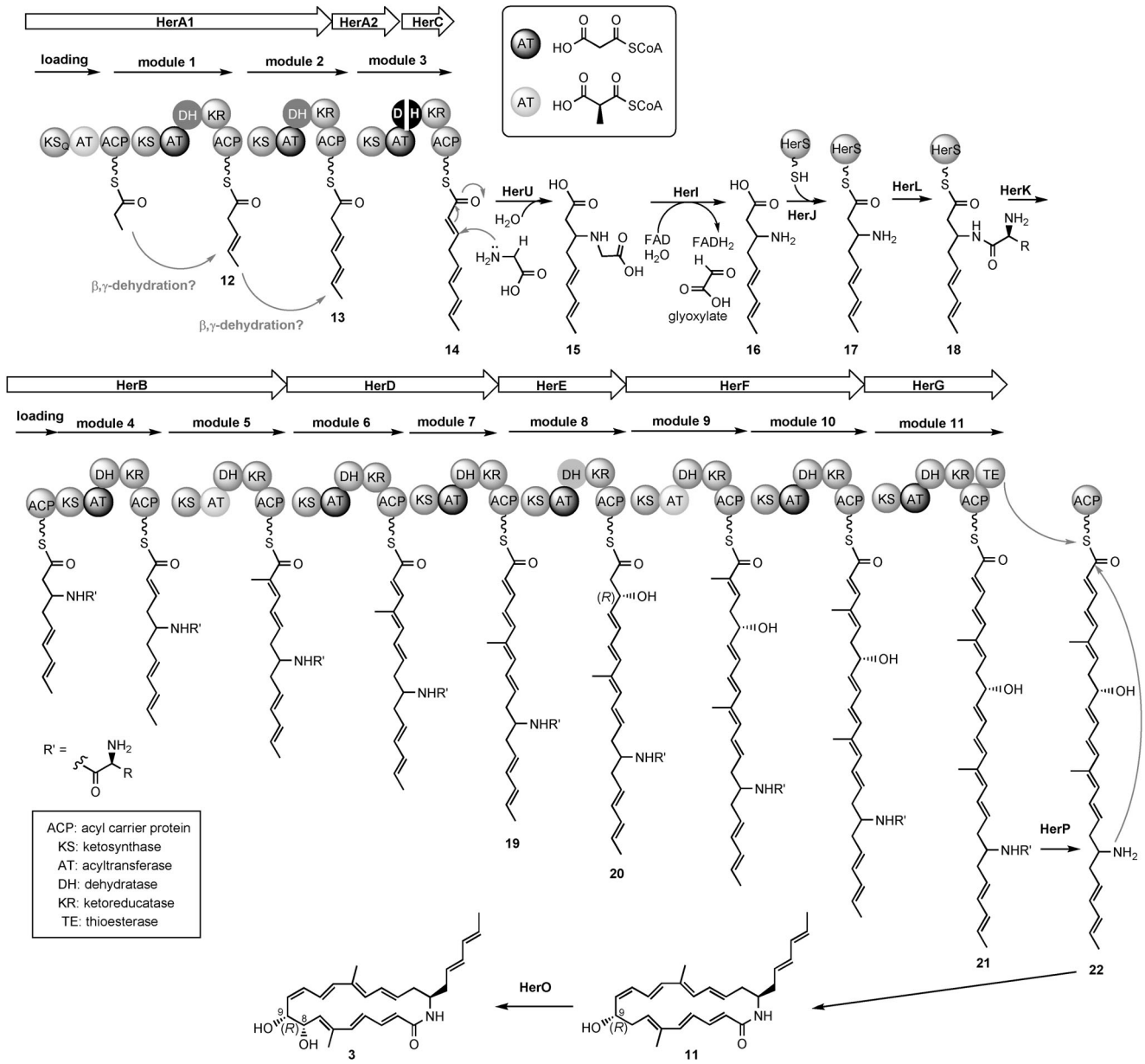
Figure 3. ^{13}C NMR spectra of labeled heronamide F (**3**) isolated from cultures of *Streptomyces* sp. SCSIO 03032 fed with A) sodium [1-^{13}C]acetate, B) sodium [1-^{13}C]propionate, or C) sodium [2-^{13}C,D_3]acetate. D) Control (**3** without labeling). The intact ^{13}C spectra are shown in Figure S7. The three asterisks in (A) indicated the minor enrichment at C-5, C-13, and C-23.

for migrated double bonds in rhizoxin[22] and bacillanene.[23] Interestingly, double-bond migration resulting from the isomerization of a normal α,β-double bond was reported to be mediated by a downstream "shift module" for rhizoxin[22] and corallopyronin A.[24] As no such shift module exists in the *her* PKS, the migrated double bonds in heronamides are most likely generated by DH-domain-catalyzed β,γ-dehydrations (Scheme 2). However, bioinformatics analysis of a number of DH domains predicted no conserved phylogenetic clades for those putatively involved in β,γ-dehydration (Figure S11). In our feeding of sodium [2-^{13}C,D_3]acetate, ideally two ^{2}Hs should be incorporated into C-20 (Figure S11) in the case of migrated double bonds by β,γ-dehydrations, whereas only one ^{2}H should be at C-20 if the migrated double bonds are caused by isomerizations after normal α,β-dehydrations (Figure S12). However, the complete absence of deuterium incorporation in **3** failed to provide direct evidence to support either mechanism for migrated double bonds in the biosynthesis of the unsaturated side chain of **3**.

Nevertheless, on the basis of heronamide F, we propose that migrated double bonds should also be present in the side chains of βPMs such as BE-14106,[10a] ML-449,[10b] aureoverticillactam,[26] and heronamides A–C.[2]

Enzymes putatively involved in protection/deprotection

A set of five enzymes, HerJKLPS, was proposed to be involved in protection/deprotection in βPMs, as elucidated in the vicenistatin pathway.[7] These five enzymes have conserved analogues in most identified βPM biosynthetic gene clusters (Table 1), including those for BE-14106,[10a] ML-449,[10b] vicenistatin,[7] incednine,[8] hitachimycin,[9] cremimycin,[11] micromonolactam,[27] and salinilactam.[28] Briefly, the aminoacyl unit **16** would be installed with ACP HerS by the acyl-CoA synthase HerJ to produce **17** (Scheme 2). Compound **17** would then be equipped with an L-α-amino acid (e.g., L-alanine in vicenistatin)[7] to afford **18** by the adenylation enzyme HerL prior to transfer to the loading module of HerB by the acyltransferase HerK, thus preventing undesired lactam formation during polyketide elongation by HerBDEFG.[7] Bioinformatics analysis of all DH domains in *her* PKSs and comparison with those in BecE and MlaE revealed that the DH domain of module 8 in HerE should be inactive, as it lacks a conserved C-terminal motif, such as YGPxFxGLxxxWR or HPAxxD (Figure S9).[29] Similarly, the DH domains in BecE and MlaE were proposed to be inactive.[10] This would be consistent with the retaining of the 9-OH functionality in heronamides BE-14106 and ML-449 (Scheme 1). Furthermore, the presence of a B-type KR domain[30] with a con-



Scheme 2. Proposed biosynthetic pathway for heronamide **3**. The migrated double bonds are shown as generated by DH-domain-catalyzed β,γ-dehydrations, but isomerization-generated migrated double bonds cannot be ruled out.

served L(V)DD motif in HerE, BecE, and MlaE (Figure S9) suggests that the HerE-catalyzed conversion of **19** into **20** should generate an (*R*)-OH (Scheme 2), thereby eventually leading to the 9*R* configuration, as experimentally determined by Mosher's esterification for heronamides A (**4**),[3] D (**1**), and E (**2**).[4] Prior to lactam formation, HerP would cleave the protecting L-α-amino acid in **21** to yield **22** (Scheme 2). Such amide bond hydrolysis mechanisms have been demonstrated in the biosynthesis of zwittermicin,[31] xenocoumacin,[32] caerulomycin A,[33] and colibactin.[34] Upon release from the PKS assembly by the TE domain in HerG (Scheme 2), macrolactamization of **19** would give **11**, and hydroxylation of **11** by HerO would complete the biosynthesis of **3**.

Nonenzymatic conversion of 3 into both 1 and 2

Heronamide A (**4**) was proposed to be formed from **6** by $\Delta^{16,17}$ epoxidation, subsequent S_N2-mediated pyrrolidine ring formation, and $4\pi+6\pi$ tandem electrocyclization.[2] However, a recent study suggests that the cyclization of **6** to afford **4** could occur spontaneously.[6] Similarly, spontaneous conversion of **3** into both **1** and **2** was observed by stirring in DMSO solution under air for eleven days (Figure S13). Consistent with this nonenzymatic process, no candidate enzymes driving such cyclizations were found in the *her* gene cluster. HerR is unique to this cluster: homologues are not present in the *bec* or *mla* clusters. However, HerR is predicted to be a member of the family of LuxR-type transcriptional regulators, a group of path-

way-specific transcriptional activators that have been shown to positively regulate the production of secondary metabolites.[35] Thus, HerR is unlikely to act as a cyclase to transform **3** into **1** and **2**.

Conclusion

We have identified and characterized the heronamide biosynthetic gene cluster. The cytochrome P450 HerO was elucidated as an 8-hydroxylase tailoring heronamide F biosynthesis. Unusual migrated double bonds in the side chain of heronamides were confirmed by labeling studies and are proposed to be generated by DH-domain-catalyzed β,γ-dehydrations, thus adding another example of a polyketide natural product.

Experimental Section

Construction of the *ΔherO* mutant: PCR targeting for *herO* gene disruption in *Streptomyces* sp. SCSIO 03032 was performed according to previous methods.[5c] Briefly, the gene cassette comprising *aac(3)IV* and *oriT* was amplified from plasmid pIJ773 with primers herO-tarF and herO-tarR (Table S1). The purified PCR product was introduced into *E. coli* BW25113/pIJ790 containing the cosmid pCSG5–8F by electroporation to afford the apramycin-resistant positive plasmid. The detailed conjugation procedures for gene inactivation in *Streptomyces* sp. SCSIO 03032 were as described previously.[5c]

Isolation of compound 11 from the *ΔherO* mutant HER001: Cells of strain HER001 were inoculated into seed medium modified ISP3 (50 mL; oat meal (1.5%), $FeSO_4$ (0.0001%), $MnCl_2$ (0.0001)%, $ZnSO_4$ (0.0001)%, sea salt (3%), pH 7.2–7.4) in a 250 mL Erlenmeyer flask and cultivated on a rotary shaker (200 rpm) at 28 °C for 7 days. Production fermentation (total 12 L) was performed by inoculating seed culture (40 mL) into to 2 L Erlenmeyer flasks containing the same medium (400 mL), and culturing on a rotary shaker (200 rpm) at 28 °C for four days. Sterilized polystyrene resin (20 mL, 5 vol%; Amberlite XAD-16) was added to the medium (200 mL L^{-1}), and fermentation was continued for another day. The mycelia and polystyrene resin were separated by filtration through a metal sieve (40 mesh). The mycelia were extracted three times with acetone (12 L), and the acetone was removed under vacuum. The resin was washed twice with water and transferred to a glass column (Synthware 10×40 cm). The glass column was eluted with acetone (2 L), and acetone fractions were concentrated under vacuum to afford an aqueous residue, which was extracted four times with EtOAc (1.5 L). The extracts were combined and concentrated under vacuum to yield a dry crude extract (3.0 g). The crude extract was subjected to column chromatography in silica gel (300–400 mesh, 150 g), with elution in a gradient of $CHCl_3$/MeOH (100:0→0:100) to give four fractions (Fr1–4). Fr4 (2.4 g) was purified by reversed-phase MPLC (C18; 40×2.5 cm ID), by eluting with a linear gradient of H_2O/MeOH (0–100%, 20 mL min^{-1}, 200 min) to give four fractions (Fr4–1, –2, –3, –4). Compound **8**-deoxy-heronamide F (**11**, 15.0 mg) was obtained from Fr.4–4 by RP-HPLC.

Preparation of 8-deoxy-heronamide F acetate (11a): A solution of **11** (8.0 mg) in pyridine (1 mL) and acetic anhydride (1 mL) was stirred overnight. The reaction was stopped, and the solution was reduced to dryness in vacuo. The residues were purified by PTLC (preparative thin-layer chromatography) to yield the acetate derivative (**11a**, 5.0 mg) as a yellow powder.

Feeding experiments: To isolate **3** in labeling studies with sodium [1-^{13}C]acetate, sodium [1-^{13}C]propionate and [2-^{13}C,D_3]acetate, spores of *Streptomyces* sp. SCSIO 03032 were inoculated into a 250 mL Erlenmeyer flask containing modified ISP3 medium (50 mL) and incubated at 28 °C on a rotary shaker (200 rpm) for three days. Full fermentation (total 1 L) was performed by inoculating the seed culture (1 mL) into 250 mL Erlenmeyer flasks containing modified ISP3 medium (50 mL) supplemented with sodium [1-^{13}C]acetate (10 mM), [1-^{13}C]propionate (10 mM) *or* [2-^{13}C,D_3]acetate, and cultured on a rotary shaker (200 rpm) at 28 °C for three days. [1-^{13}C]acetate-labeled **3** (4.0 mg), [1-^{13}C]propionate-labeled **3** (3.2 mg), and [2-^{13}C,D_3]acetate-labeled **3** (5.0 mg) were isolated as described for isolating **3** from the wild-type strain.[4]

Overexpression and purification of HerO: The *herO* gene was amplified from genomic DNA of *Streptomyces* sp. SCSIO 03032 with PCR primers herO-EF and herO-ER (Table S1). The product was digested with NdeI/EcoRI and inserted into pET28b-pikC_{D50N}-RhFRED,[15b] linearized with NdeI/EcoRI, to yield the *herO* expression plasmid pCSG614 after sequence confirmation. The plasmid was introduced to *E. coli* BL21(DE3) to produce the N-terminal His_6-tagged HerO-RhFRED fusion protein. Overnight *E. coli* cultures (5 mL) were inoculated into LB medium (250 mL) in eight 1 L flasks, and grown at 37 °C to $OD_{600} \approx 0.6$. After adding IPTG (0.1 mM), the cultures were grown at 16 °C for an additional 12 h. The cells were then collected by centrifugation, and washed twice with Tris·Cl (50 mM, pH 8.0). The cell pellets were resuspended in binding buffer (Tris·Cl (20 mM, pH 7.9), NaCl (500 mM), and imidazole (5 mM)) and sonicated. After centrifugation, the supernatant was filtered through a 0.45 μm membrane, loaded onto an Ni-NTA affinity column (Novagen), washed twice with wash buffer (Tris·Cl (20 mM, pH 7.9), NaCl (500 mM), imidazole (40 mM)), and then eluted with elution buffer (Tris·Cl (20 mM, pH 7.9), NaCl (500 mM), imidazole (200 mM)). The fractions containing HerO were desalted in a PD-10 column (GE Healthcare), and finally stored in storage buffer (Tris·Cl (50 mM, pH 8.0), glycerol (10%), dithiothreitol (1 mM)) at −80 °C, or dissolved in Tris·Cl (50 mM) for the large-scale enzymatic reaction.

HerO enzyme assays: The concentration of purified protein was determined by a Bradford assay.[36] For in vitro assay of HerO, a typical reaction was conducted in Tris·Cl (100 μL, 50 mM, pH 8.0) containing **11** (100 μM), HerO (2 μM), and NADPH or NADH (2 mM). The reaction mixtures were incubated at 28 °C and were stopped by adding ice-cold MeOH (100 μL). The assays were monitored by HPLC with a reversed-phase Luna C18 column (5 μm, 150× 4.6 mm; Phenomenex, Torrance, CA) as follows: UV detection at 254 nm; solvent A: acetonitrile (10%) in water with formic acid (0.1%); solvent B: acetonitrile (90%) in water; 5–100% B (0–20 min), 100% B (20–24 min), 100–5% B (24–25 min), 5% B (25–30 min); flow rate, 1 mL min^{-1}.

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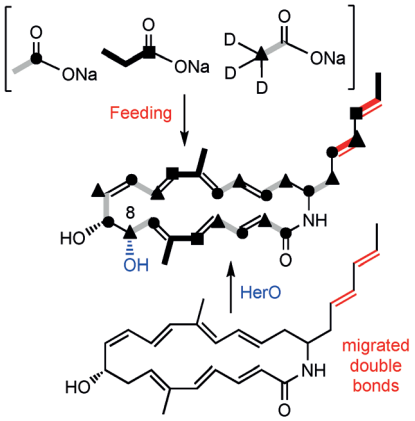
Migrated double bonds: A P450 enzyme HerO was functionally characterized as the C-8 hydroxylase tailoring heronamide biosynthesis. Migrated double bonds in the biosynthesis of the unsaturated side chain were indicated by feeding with labeled small carboxylic acid molecules.



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Characterization of Heronamide Biosynthesis Reveals a Tailoring Hydroxylase and Indicates Migrated Double Bonds

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