ChemPubSoc Europe

DOI: 10.1002/cbic.201600095

CHEMBIOCHEM Full Papers

Involvement of the Baeyer–Villiger Monooxygenase IfnQ in the Biosynthesis of Isofuranonaphthoquinone Scaffold of JBIR-76 and -77

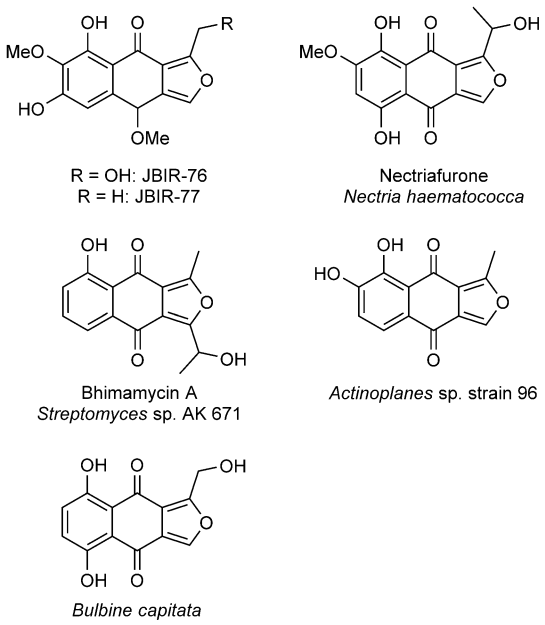
Yohei Katsuyama[+],[a] Kaoru Sone[+],[a] Ryutaro Satou,[a] Miho Izumikawa[†],[b] Motoki Takagi,[b] Manabu Fujie,[c] Noriyuki Satoh,[c] Kazuo Shin-ya,[d] and Yasuo Ohnishi*[a]

JBIR-76 and -77 are isofuranonaphthoquinones (IFNQs) isolated from *Streptomyces* sp. RI-77. Draft genome sequencing and gene disruption analysis of *Streptomyces* sp. RI-77 showed that a type II polyketide synthase (PKS) gene cluster (*ifn* cluster) was responsible for the biosynthesis of JBIR-76 and -77. It was envisaged that an octaketide intermediate (C_{16}) could be synthesized by the minimal PKS (IfnANO) and that formation of the IFNQ scaffold (C_{13}) would therefore require a C–C bond cleavage reaction. An *ifnQ* disruptant accumulated some shunt products (C_{15}), which were presumably produced by spontaneous cyclization of the decarboxylated octaketide intermediate. Recombinant IfnQ catalyzed the Baeyer–Villiger oxidation of 1-(2-naphthyl)acetone, an analogue of the bicyclic octaketide intermediate. Based on these results, we propose a pathway for the biosynthesis of JBIR-76 and -77, involving IfnQ-catalyzed C–C bond cleavage as a key step in the formation of the IFNQ scaffold.

Introduction

JBIR-76 and -77 are isofuranonaphthoquinone (IFNQ) derivatives that were isolated from *Streptomyces* sp. RI-77 (Scheme 1);[1] a broad range of IFNQs have been isolated from a wide variety of organisms, including plants and actinomycetes (Scheme 1).[2–7] Some of these IFNQ derivatives have been reported to have interesting biological properties, including antioxidant and antiplasmodial activities.[3] Notably, some plant-derived IFNQs have been reported to exhibit inhibitory effects against mosquito glutathione transferase.[8] Based on their structures, IFNQ derivatives appear to be synthesized by polyketide synthases (PKSs), but their biosynthetic pathways have not yet been elucidated.

Aromatic polyketides isolated from *Streptomyces* are generally synthesized by type II PKSs, which are complexes of monofunctional proteins.[9–11] Ketosynthase (KS), chain length factor (CLF), and acyl carrier protein (ACP) are essential components for the synthesis of polyketides in type II PKSs;[9–11] the single set of these proteins is defined as the "minimal PKS". KS and CLF form a heterodimer that catalyzes an iterative decarboxylative condensation reaction between malonyl-ACP and an acyl-ACP to synthesize a polyketide chain. The resulting polyketide chain is then further modified by tailoring enzymes, such as oxidoreductases and cyclases, to produce a final polyketide product.[9–11]



Scheme 1. Structures of JBIR-76 and -77 and related IFNQs isolated from microorganisms and a plant.

[a] *Dr. Y. Katsuyama,[+] K. Sone,[+] R. Satou, Dr. Y. Ohnishi*
Department of Biotechnology
Graduate School of Agricultural and Life Sciences, The University of Tokyo
1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657 (Japan)
E-mail: ayasuo@mail.ecc.u-tokyo.ac.jp

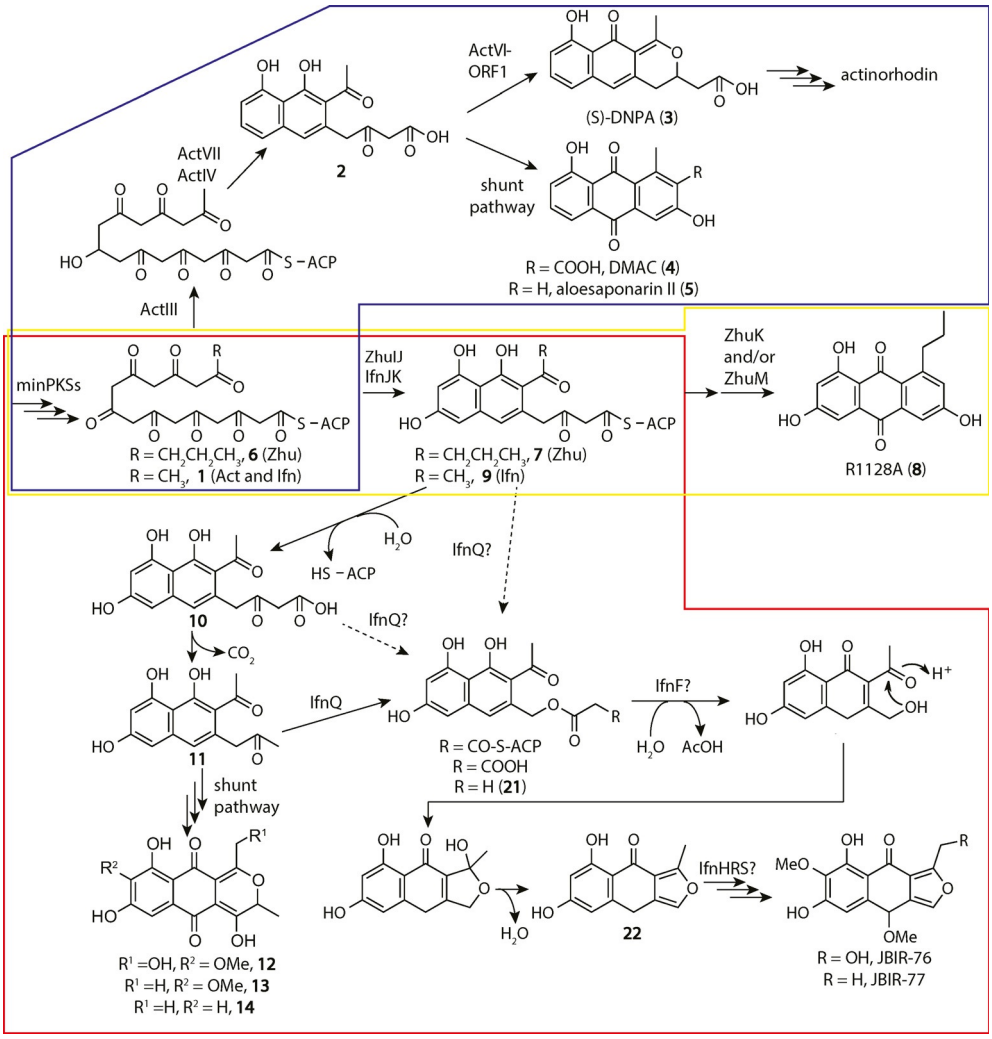
[b] *Dr. M. Izumikawa, Dr. M. Takagi*
Japan Biological Informatics Consortium (JBIC)
2-4-7 Aomi, Koto-ku, Tokyo 135-0064 (Japan)

[c] *M. Fujie, Dr. N. Satoh*
Okinawa Institute of Science and Technology Graduate University
1919-1 Tancha, Onna-son, Kunigami-gun, Okinawa 904-0495 (Japan)

[d] *Dr. K. Shin-ya*
National Institute of Advanced Industrial Science and Technology (AIST)
2-4-7 Aomi, Koto-ku, Tokyo 135-0064 (Japan)

[+] *These authors contributed equally to this work.*

[†] *Deceased on 23 December 2015.*



Scheme 2. Putative pathway for the biosynthesis of JBIR-76 and -77. This biosynthetic pathway is compared with those of actinorhodin and R1128A. Red, blue, and yellow boxes indicate the putative biosynthetic pathways for JBIR-76/77, actinorhodin, and R1128A, respectively.

Actinorhodin and R1128 derivatives are representative of the polyketides synthesized by type II PKSs, and the pathways involved in their biosynthesis have been studied extensively (Scheme 2). For the biosynthesis of actinorhodin, the minimal PKS (Act-1, 2, 3) synthesizes the octaketide intermediate **1** from eight molecules of malonyl-CoA.[12–14] The dihydroxybicyclic intermediate **2** is then synthesized by the ActIII-catalyzed reduction of the keto group at position 9[15] followed by two cyclization reactions (catalyzed by cyclases ActVII and ActIV), which proceed by aldol condensation.[16,17] Subsequent ActVI-ORF1-catalyzed reduction of this material affords 4-dihydro-9-hydroxy-1-methyl-10-oxo-3-*H*-naphtho-[2,3-c]-pyran-3-(*S*)-acetic acid (DNPA, **3**), which is a key precursor in the biosynthesis of actinorhodin.[18–20] In the absence of ActVI-ORF1, the dihydroxybicyclic intermediate **2** is converted to dihydroxymethylanthraquinone-2-carboxylic acid (DMAC, **4**) and aloesaponarin II (**5**) by a nonenzymatic process.[17,21]

In contrast, the biosynthesis of R1128 starts with the preparation of a short-chain acyl-ACP intermediate by an initiation module.[22] The resulting acyl-ACP is then used as a substrate by the minimal PKS and converted into the different octaketide intermediate **6**.[10,23] The biosynthetic pathway to R1128, unlike actinorhodin, lacks a specific octaketide reductase such as ActIII.[23] The cyclases (i.e., ZhuI and ZhuJ) therefore cyclize the unmodified octaketide **1** to give the trihydroxybicyclic intermediate **7**, which is presumably cyclized by a nonenzymatic process in a manner similar to that observed for the formation of DMAC in the biosynthesis of actinorhodin.[23,24] Subsequent oxidation reactions catalyzed by ZhuK and/or ZhuM result in the formation of R1128A (**8**).[23]

Baeyer–Villiger monooxygenases are flavin-dependent monooxygenases that catalyze the cleavage of C–C bonds adjacent to keto groups to give the corresponding esters.[25] Although some Baeyer–Villiger monooxygenases are involved in ketone catabolism in bacteria, many are involved in the biosynthesis of secondary metabolites. For example, several Baeyer–Villiger monooxygenases are involved in the modification of polyketide scaffolds synthesized by type II PKSs.[26–28] These enzymes catalyze C–C bond cleavage reactions that induce rearrangement of scaffolds of aromatic polyketides, and are there-

fore important for the structural diversity of aromatic polyketides. MtmOIV, GilOII, and JadG are examples of this type of Baeyer–Villiger monooxygenase; these enzymes are involved in the biosynthesis of mithramycin,[27] gilvocarcin[28] and jadomycin,[28] respectively.

Here, we analyzed the pathway involved in the biosynthesis of JBIR-76 and -77 (Scheme 2). Genome sequencing and gene inactivation experiments revealed that a type II PKS gene cluster is responsible. This cluster was named *ifn* (isofuranonaphthoquinone). We focused our attention on *ifnQ*, which encodes a putative Baeyer–Villiger monooxygenase. Inactivation of *ifnQ* and an in vitro enzymatic assay of IfnQ indicated that this Baeyer–Villiger monooxygenase catalyzed the cleavage of a C–C bond in a bicyclic intermediate, which could be the key step in the biosynthesis of the IFNQ scaffold of JBIR-76 and -77. Based on these results, we propose a pathway for the biosynthesis of JBIR-76 and -77, and provide an important insight into the biosynthesis of compounds containing an IFNQ scaffold.

Results and Discussion

Identification of the gene cluster involved in the biosynthesis of JBIR-76 and -77

The draft genome sequence of the JBIR-76 and -77 producer *Streptomyces* sp. RI-77 was determined, and two type II PKS gene clusters were found. One is similar to that involved in the biosynthesis of UT-X26,[29] and we therefore postulated that the other cluster (*ifn*) is responsible for the biosynthesis of JBIR-76 and -77 (Figure 1). Thus, we constructed a mutant of

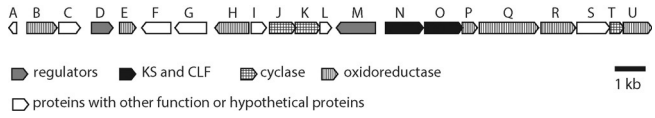


Figure 1. Gene cluster for the biosynthesis of JBIR-76 and -77 showing lengths and directions of the open reading frames. Letters above indicate gene names ("*ifn*" is omitted for clarity).

the KS-encoding gene *ifnN*: the core region of *ifnN* was substituted with *aphII* to confer neomycin resistance. The *Streptomyces* sp. RI-77 *ifnN*::*aphII* mutant did not produce JBIR-76 or -77 (Figure 2 A and B), thus clearly demonstrating that *ifn* is responsible for the biosynthesis of JBIR-76 and -77.

Prediction of the JBIR-76 and -77 biosynthesis pathway

The genes encoded by the *ifn* gene cluster were subjected to in silico analysis (mainly BLAST search; Table 1 and Figure 1). IfnN and IfnO show high sequence homology to the enzymes KS (80% identity) and CLF (71%), respectively, involved in the biosynthesis of actinorhodin.[10,12–14] In addition, *ifn* does not contain any genes related to the formation of unusual starter units,[11,30–32] for example, genes for the initiation module in R1128 biosynthesis.[22] This suggests that IfnN and IfnO catalyze

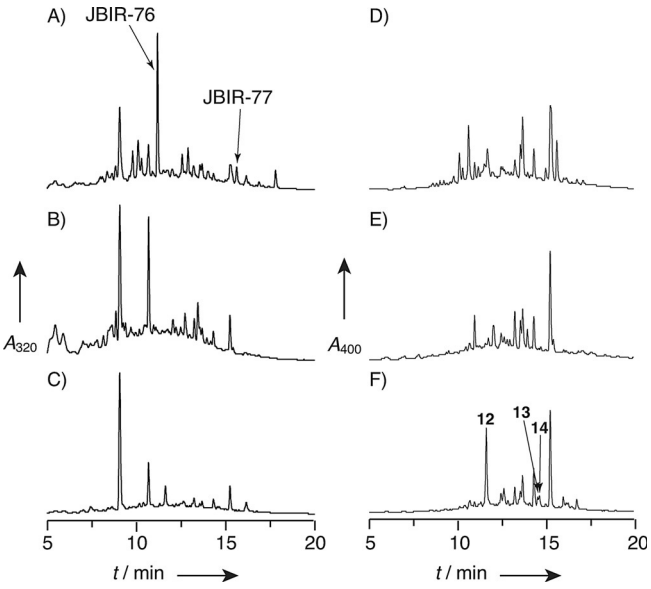


Figure 2. LC-MS analysis (320 nm) of the metabolites produced by *Streptomyces* sp. RI-77 and its mutants: A) wild-type *Streptomyces* sp. RI-77 produces JBIR-76 and -77; B) *Streptomyces* sp. RI-77 *ifnN*::*aphII* does not produce JBIR-76 or -77; C) *Streptomyces* sp. RI-77 Δ*ifnQ* does not produce JBIR-76 or -77, but accumulates **12**, **13**, and **14**. D)–F) Corresponding absorbances at 400 nm.

a similar reaction to that catalyzed by the minimal PKS in the biosynthesis of actinorhodin and that they synthesize the same octaketide intermediate (**1**) from eight molecules of malonyl-CoA (Scheme 1). However, this octaketide intermediate consists of 16 carbons, and is inconsistent with the core structures of JBIR-76 and -77, both of which have 13 carbons (excluding two carbons of the methoxy groups). Therefore, it seems likely that the biosynthesis of JBIR-76 and -77 involves a C–C bond cleavage reaction.

The *ifn* gene cluster contains three genes (*ifnJ*, *ifnK*, and *ifnT*) that encode putative polyketide cyclases. IfnJ is a cyclase fused to a domain of unknown function at its N terminus; the cyclase domain is homologous to ZhuI (60.1% identity).[23,24] IfnK shows high sequence homology to ZhuJ (78.3%).[10,23] IfnT belongs to the SnoaL family of cyclases, but does not show significant similarity to any characterized enzyme. ZhuI and ZhuJ catalyze the formation of the A and B rings, respectively, of the octaketide intermediate in the biosynthesis of R1128 derivatives (Scheme 2).[10,23,24] Therefore, IfnJ and IfnK likely catalyze similar reactions in the biosynthesis of JBIR-76 and -77. Taken together, the trihydroxybicyclic intermediate **9** is a probable precursor of JBIR-76 and -77 (Scheme 2). This is supported by the fact that an ActIII homologue, which could reduce the keto group of octaketide,[15] is not encoded in the *ifn* gene cluster.

As mentioned above, the biosynthesis of JBIR-76 and -77 would require a C–C bond cleavage reaction, and it was assumed that trihydroxybicyclic intermediate **9** was the substrate for this reaction. The *ifn* gene cluster was therefore searched for a gene encoding an enzyme that could catalyze such a reaction: a flavin-dependent monooxygenase (IfnQ) that possesses the Baeyer–Villiger monooxygenase motif, FXGXXXHXXXW(P/

Table 1. Predicted functions of genes in the *ifn* gene cluster.

Orf	Amino acids	Proposed function	Protein homology	Amino acids identity
ifnA	96	acyl carrier protein	acyl carrier protein [*Saccharopolyspora hirsuta*] AAA26490.1	52/80 (65%)
ifnB	330	dehydrogenase	putative acyl-CoA dehydrogenase [uncultured soil bacterium V167] ACX83623.1	51/122 (42%)
ifnC	247	phosphopantetheinyl transferase	putative phosphopantetheinyl transferase [*Streptomyces gancidicus*] WP_016435292.1	101/247 (41%)
ifnD	250	regulator	SARP family transcriptional regulator [*Streptomyces aureofaciens*] ACK77758.1	125/244 (51%)
ifnE	194	FMN reductase	multimeric flavodoxin WrbA [*Streptomyces venezuelae* ATCC 10712] YP_006880813.1	116/192 (60%)
ifnF	319	esterase	lipase/esterase [*Amycolatopsis mediterranei* U32] YP_003766633.1	168/316 (53%)
ifnG	361	hypothetical protein	Aln6 [*Streptomyces* sp. CM020]	143/320 (45%)
ifnH	377	$FMNH_2$-dependent monooxygenase	oxygenase LndZ5 [*Streptomyces globisporus*] AAR16420.1	186/374 (50%)
ifnI	161	hypothetical protein	–	–
ifnJ	287	polyketide cyclase	granaticin polyketide synthase bifunctional cyclase/dehydratase [*Streptomyces himastatinicus*] WP_009714484.1	145/279 (52%)
ifnK	265	polyketide cyclase	ZhuJ cyclase [*Streptomyces* sp. R1128] AAG30196.1	198/249 (80%)
ifnL	132	lipocalin-like domain	EsmH2 [*Streptomyces antibioticus*] AFB35638.1	67/134 (50%)
ifnM	434	regulator	LuxR family transcriptional regulator [*Stackebrandtia nassauensis* DSM 44728] YP_003512161.1	209/426 (49%)
ifnN	422	KS alpha	polyketide synthase [*Saccharopolyspora hirsuta*] AAA26488.1	344/419 (82%)
ifnO	415	chain length factor	polyketide synthase chain length factor [*Streptomyces vietnamensis*] ADO32787.1	290/403 (72%)
ifnP	244	two-component oxidase	actinorhodin polyketide dimerase [*Streptomyces coelicolor* A3(2)] NP_629242.1	86/152 (57%)
ifnQ	638	Baeyer–Villiger monooxygenase	4-hydroxyacetophenone monooxygenase [*Streptomyces chartreusis*] WP_010034394.1	364/638 (57%)
ifnR	383	$FMNH_2$-dependent monooxygenase	AlnT hydroxylase [*Streptomyces* sp. CM020] ACI88867.1	187/385 (49%)
ifnS	358	methyltransferase	methyltransferase [*Verrucomicrobium spinosum*] WP_009963450.1	210/336 (63%)
ifnT	141	polyketide cyclase	hypothetical protein AMED 0961 [*Amycolatopsis mediterranei* U32] YP_003763182.1	41/114 (36%)
ifnU	315	enoyl reductase	alcohol dehydrogenase GroES domain-containing protein [*Catenulispora acidiphila* DSM 44928] YP_003115275.1	193/308 (63%)

D),[33] showed sequence similarity to the Baeyer–Villiger monooxygenase 4-hydroxyacetophenone monooxygenase from *Pseudomonas fluorescens* (46.8% identity).[34] This enzyme catalyzes the oxidation of 4-hydroxyacetophenone to 4-hydroxyphenyl acetate, and it was therefore hypothesized that IfnQ catalyzes the Baeyer–Villiger monooxygenation of **9** to afford cleavage of an appropriate C–C bond. Three substrates were considered as candidates for the IfnQ-catalyzed Baeyer–Villiger reaction: octaketide intermediate **9** synthesized by IfnJ and IfnK, β-keto acid **10** synthesized by the hydrolysis of **9**, the ketone **11** formed by decarboxylation of **10** (Scheme 1).

Analysis of the *ifnQ* deletion mutant

An *ifnQ* deletion mutant was constructed to examine the function of IfnQ. The *ifnQ* gene was deleted in-frame, thereby resulting in *Streptomyces* sp. RI-77 Δ*ifnQ*, which was unable to produce JBIR-76 and -77 (Figure 2C). Three compounds accumulated in the culture of *Streptomyces* sp. RI-77 Δ*ifnQ* (Figure 2F); these were isolated and analyzed by spectroscopic methods to elucidate their structures. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) revealed the molecular formulae of **12**, **13**, and **14** (Table 2), and NMR analysis revealed their structures (Figure 3; Table 3). All three have a benzo[*g*]isochromene scaffold, and the core structure is composed of 15 carbons, thus suggesting that they are shunt products synthesized by the spontaneous cyclization and oxi-

Table 2. HR-ESIMS analysis of **12**, **13**, and **14**.

Compound	Molecular formula	Observed *m/z*	Calculated *m/z*
12	$C_{16}H_{14}O_8$	333.0612 $[M-H]^-$	$C_{16}H_{13}O_8 = 333.0610$
13	$C_{16}H_{14}O_7$	317.0656 $[M-H]^-$	$C_{16}H_{13}O_7 = 317.0661$
14	$C_{15}H_{12}O_6$	287.0559 $[M-H]^-$	$C_{15}H_{11}O_7 = 287.0556$

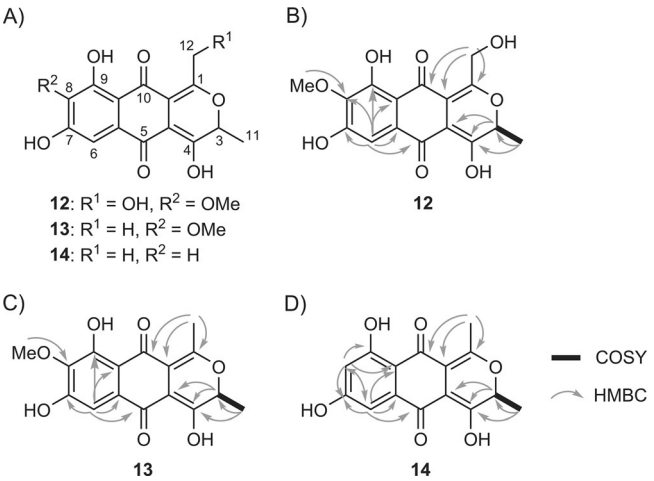


Figure 3. NMR analysis of **12**, **13**, and **14**. A) Compounds **12**, **13**, and **14**. HMBC and COSY signals for B) **12**, C) **13**, and D) **14**.

Table 3. ^{13}C and ^{1}H NMR data for **12**, **13**, and **14**.

	12[a]		**13**[b]		**14**[b]	
Position	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		158.5		158.9		158.8
3	5.39 (q, J=6.5 Hz, 1H)	60.7	5.4 (q, J=6.5 Hz, 1H)	63.2	5.44 (q, J=6.5 Hz, 1H)	63.3
4		162.6		162.6		162.6
4a		115.2		117.5		117.9
5		178.5		180.7		181.4
5a		131.5		133.0		139.2
6	7.18 (s, 1H)	109.0	7.26 (s, 1H)	109.6	7.13 (d, J=2 Hz, 1H)	110.2
7		157.9		157.6		168.3
8		139.3		140.7	6.48 (d, J=2 Hz, 1H)	109.1
8-OMe	3.82 (s, 3H)	59.9	3.96 (s, 3H)	61.0		–
9		157.5		158.5		167.3
9a		111.5		113.6		112.1
10		184.7		186.6		185.9
10a		116.7		118.0		118.1
11	1.43 (d, J=6.5 Hz, 3H)	21.3	1.55 (d, J=6.5 Hz, 3H)	21.4	1.54 (d, J=6.5 Hz, 3H)	21.3
12	4.82 (s, 2H)	54.4	2.71 (s, 3H)	13.8	2.71 (s, 3H)	13.6

[a] ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC spectra recorded in [D_6]DMSO on a JEOL JNM-A500 NMR system. The solvent peak was used as an internal standard (δ_C=39.7, δ_H=2.49 ppm). [b] ^{1}H (600 MHz) and ^{13}C (125 MHz) NMR spectra recorded in [D_6]DMSO on a Varian 600 NB CL NMR system. The solvent peak was used as an internal standard (δ_C=39.7, δ_H=2.49 ppm).

dation of a decarboxylated octaketide intermediate prior to the Baeyer–Villiger monooxygenation (**9**, **10**, or **11**). Compounds with similar structures have been isolated from another IFNQ producer, *Nectria haematococca*.[5] These compounds might be synthesized by a similar shunt pathway.

In vitro analysis of IfnQ

To determine which compound (**9**, **10**, or **11**) is the natural substrate of IfnQ, we analyzed the activity of IfnQ in vitro. Recombinant IfnQ was produced with the pET system and *Escherichia coli* BL21(DE3) and purified by Ni^{2+} affinity chromatography (Figure 4A). LC-MS analysis revealed that purified IfnQ contained flavin adenine dinucleotide (FAD; data not shown), thus suggesting that this enzyme uses FAD as a cofactor. Given that the putative substrate of IfnQ was predicted to be unstable, we synthesized five substrate analogues (Figure 4B). Compounds **15** and **16** mimic heptaketide bicyclic intermediates in the free-acid and ACP-bound forms, respectively; *N*-acetylcysteamine (NAC) is known to mimic ACP.[35] Compounds **17**, **18**, and **19** mimic **9**, **10**, and **11**, respectively. When IfnQ was incubated with **15**, **16**, **17**, **18**, or **19** in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH), only **19** gave a new product, which was subsequently identified as **20** (Figure 4C and D). Given the predicted function of IfnQ, it was assumed that **20** would be 2-naphthalenemethyl

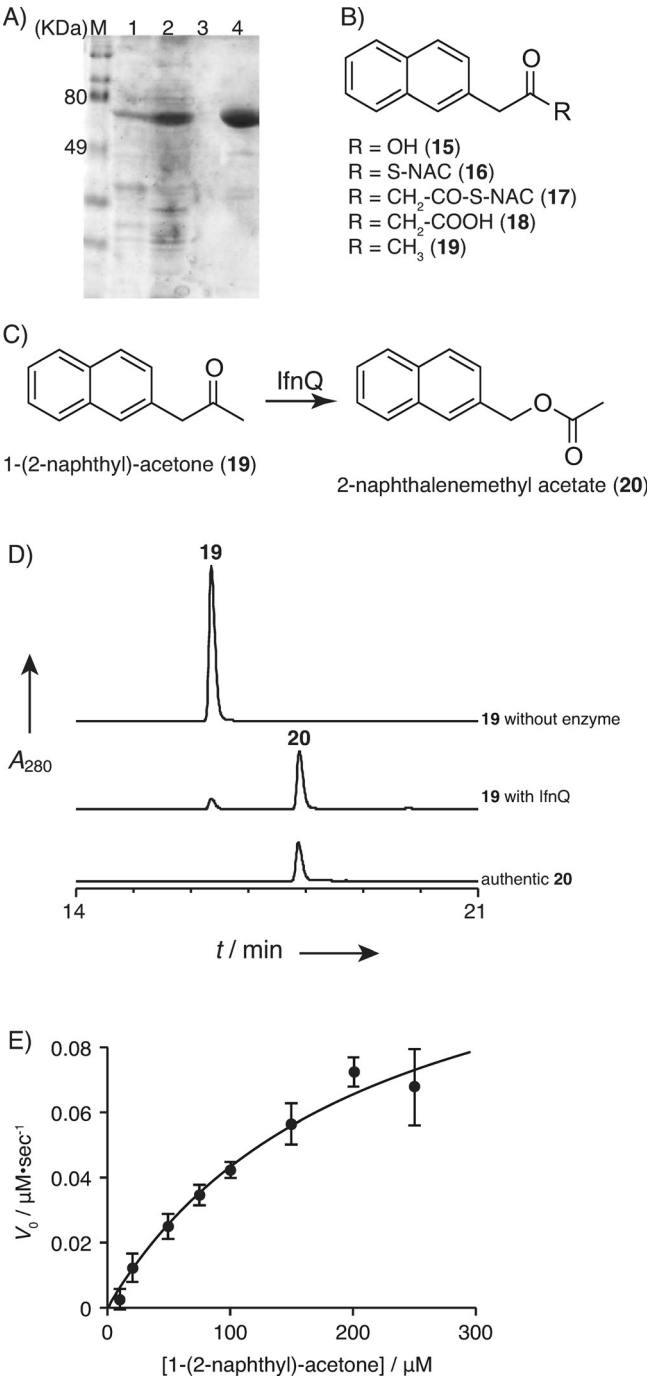


Figure 4. In vitro analysis of IfnQ. A) SDS-PAGE of recombinant IfnQ. Lane M: molecular weight marker; lane 1: insoluble fraction; lane 2: soluble fraction; lane 3: washings; lane 4: purified recombinant IfnQ. B) Substrates **15**, **16**, **17**, **18**, and **19** used for the in vitro analysis of IfnQ. C) IfnQ-catalyzed conversion of **19** to **20**, and D) LC-MS analysis. E) Michaelis–Menten plot of IfnQ-catalyzed formation of **19**; mean ± SEM (n=3).

acetate, which was prepared by chemical synthesis and used as an authentic standard. LC-MS analysis of the authentic 2-naphthalenemethyl acetate and the material produced by the incubation of **19** with IfnQ revealed that these two materials had identical retention times (Figure 4D) and UV spectra (data not shown). Because IfnQ was active without a flavin reduc-

tase, we concluded that this enzyme is a single-component FAD-dependent monooxygenase. The kinetic parameters for the synthesis of 2-naphthalenemethyl acetate by IfnQ were then measured (Figure 4E): k_{cat} and K_m were determined to be 0.045 s^{-1} and 210 μM, respectively. Based on these results, it was concluded that in vivo IfnQ catalyzes the Baeyer–Villiger oxidation of **11** (or its derivatives) to **21** (Scheme 2).

Conclusion

In this study, we identified the JBIR-76 and -77 biosynthesis gene cluster and elucidated the critical role of the Baeyer–Villiger monooxygenase IfnQ in the construction of the IFNQ scaffold. Furthermore, we propose the following pathway for the biosynthesis of JBIR-76 and -77 on the basis of bioinformatics analysis and our experimental results (Scheme 2). First, IfnO and IfnN synthesize the octaketide intermediate **1** from eight molecules of malonyl-CoA. IfnJ and IfnK convert this intermediate into the trihydroxybicyclic intermediate **9**. Subsequent hydrolysis and decarboxylation reactions (enzymes unknown) convert **9** into **11**, which is the probable substrate of IfnQ. The IfnQ-catalyzed Baeyer–Villiger oxidation of **11** then gives ester **21**. This IfnQ-catalyzed C–C bond cleavage reaction is the key step in the formation of the IFNQ scaffold, because the hydroxy group synthesized during the hydrolysis of **21** would be sufficiently nucleophilic to attack the adjacent ketone and form a five-membered ring. Subsequent nonenzymatic dehydration completes the synthesis of the IFNQ scaffold (**22**). Further tailoring reactions would be required to synthesize JBIR-76 and -77.

This work has provided a deeper insight into the way in which type II PKS systems expand the structural diversity of their products. The results also provide a new example of the role of Baeyer–Villiger monooxygenases in the tailoring of polyketides.

Experimental Section

Materials: ISP-2 starch medium was prepared by dissolving Bacto yeast extract (0.4%), Bacto malt extract (1%) and soluble starch (0.4%) in water. The pH was adjusted to 7.2 prior to autoclaving.

Draft genome sequencing of *Streptomyces* sp. RI-77: Genome sequencing was performed on a 454 GS FLX sequencer (Roche). Shotgun and paired-end reads were assembled in Newbler (de novo sequence assembly software; Roche). The *ifn* gene cluster was deposited at the DNA Data Bank of Japan (DDBJ) under accession number LC125462.

Construction of *ifnN* and *ifnQ* mutants: The *ifnN* mutant (*Streptomyces* sp. RI-77 *ifnN*::*aphII*) was constructed by exchanging the region encoding Pro97 to Ala362 of IfnN with *aphII*, which confers neomycin resistance. The *ifnQ* mutant (*Streptomyces* sp. RI-77 Δ*ifnQ*) was constructed by deleting the region encoding Asp122 to Glu544 of IfnQ. Both mutants were obtained by conjugal transformation with *E. coli* ET12567/pUZ8002 (John Innes Centre, Norwich, UK) and screening of the double-crossover recombinant mutants. Correct gene disruption was confirmed by PCR.

Analysis of metabolites produced by *Streptomyces* sp. RI-77 and its mutants: *Streptomyces* sp. RI-77[1] and its mutants were inoculated into ISP-2 starch medium (100 mL) and cultivated at 30 °C for 7 days. The resulting culture was acidified with HCl (1 M) to pH < 2, and extracted with ethyl acetate. The organic extract was collected and evaporated to dryness to give a residue, which was dissolved in methanol for LC-ESI-MS analysis on an 1100 series HPLC system (Agilent Technologies) with a high-capacity trap plus (Bruker Daltonics) equipped with a Monobis C_{18} column (2×100 mm; Kyoto Monotech, Kyoto, Japan). The compounds were eluted with linear gradient of water and acetonitrile containing formic acid (0.1%).

Isolation and characterization of 12, 13, and 14: *Streptomyces* sp. RI-77 was inoculated into ISP-2 starch medium (1.5 L) and cultivated at 30 °C for 7 days. Amberlite XAD FPX66 (20 g, Organo, Tokyo, Japan) was then added to the culture, and the mixture was stirred at room temperature for 2 h. The mixture of cells and XAD was harvested by centrifugation and extracted with methanol. The solvent was then evaporated to dryness to give a residue, which was purified by medium pressure liquid chromatography (MPLC; Purif-Compact A, Shoko Scientific, Kanagawa, Japan) equipped with an ODS column (Purif-Pak ODS SIZE 60, Shoko Scientific). The compounds were eluted in a linear gradient from solvent A (methanol (10%) containing formic acid (0.1%)) to solvent B (methanol containing formic acid (0.1%)) over 20 min. Fractions containing compounds were evaporated to dryness and further purified on a Sephadex LH20 column (GE Healthcare). Compounds were eluted in methanol as a mobile phase; fractions containing compounds were evaporated to dryness, and the compounds were further purified on a Waters 600 HPLC system equipped with a C_{18}-AR-II column (10×250 mm, COSMOSIL, Nacalai Tesque, Kyoto, Japan). Compounds were eluted in linear gradient of methanol (50–100%) containing formic acid (0.1%). ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC spectra of **12** (2.8 mg) were recorded in [D_6]DMSO on a JNM-A500 NMR System (JEOL, Tokyo, Japan). ^{1}H NMR, ^{13}C NMR, COSY, HMQC, and HMBC spectra of **13** (0.6 mg) and **14** (0.5 mg) were recorded in [D_6]DMSO on a Varian 600 NB CL NMR system (Varian, Palo Alto, CA).

Synthesis of 2-naphthaleneacetyl-*N*-acetylcysteamine (16): 2-Naphthaleneacetyl-*N*-acetylcysteamine was synthesized as previously reported.[35] 2-Naphthaleneacetate (4 mmol, TCI, Tokyo, Japan), *N*-acetylcysteamine (NAC, 4 mmol, Sigma-Aldrich), triethylamine (4 mmol, Wako, Osaka, Japan), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSC, 4 mmol, Dojindo Molecular Technologies, Kumamoto, Japan) and 4-dimethylaminopyridine (DMAP, 6 mmol, Wako) were dissolved in dichloromethane (10 mL), and the mixture was stirred at room temperature for 8 h. The mixture was diluted with chloroform and washed with deionized water before being dried over anhydrous Na_2SO_4. The solvents were then evaporated under reduced pressure to give a residue, which was purified by MPLC on a silica gel column (Purif-Pak SI, SIZE 60, Shoko Scientific). The structure of 2-naphthaleneacetyl-*N*-acetylcysteamine was confirmed by ^{1}H NMR analysis.

Synthesis of 4-(2-naphthalene)-3-oxobutyryl-*N*-acetylcysteamine (17): 3-Oxo-4-naphthylbutyryl-*N*-acetylcysteamine thioester was synthesized according to a previously reported procedure.[18] 2-Naphthaleneacetate (4 mmol, TCI), Meldrum's acid (4 mmol, Wako), triethylamine (4 mmol), WSC (4 mmol) and DMAP (6 mmol) were dissolved in dichloromethane (10 mL), and the resulting mixture was stirred at room temperature for 8 h. The mixture was then diluted with chloroform and washed with deionized water before being dried over anhydrous Na_2SO_4. The solvents were evaporated under reduced pressure to give a residue, which was purified by

MPLC on a reversed-phase column (Purif-Pak ODS SIZE 60). The structure of 2-naphthaleneacetylmeldrum acid was confirmed by ^{1}H NMR.

2-Naphthaleneacetylmeldrum acid (4 mmol) and NAC (4 mmol) were added to anhydrous benzene (10 mL), and the mixture was stirred under reflux at 80 °C for 2 h. The mixture was then cooled to ambient temperature and diluted with chloroform before being washed with deionized water and dried over anhydrous Na_2SO_4. The solvents were evaporated under reduced pressure to give a residue, which was purified by MPLC on a silica gel column (Purif-Pak SI, SIZE 60). The structure of 4-(2-naphthalene)-3-oxobutyryl-*N*-acetylcysteamine was confirmed by ^{1}H NMR.

Synthesis of 4-(2-naphthalene)-3-oxobutyric acid (18): 4-(2-Naphthalene)-3-oxobutyric acid is unstable and was therefore synthesized immediately prior to the reaction. Methanol (5 μL) and NaOH (3 μL, 2 M) were added to **17** (100 mM) in dimethyl sulfoxide (DMSO), and the mixture was incubated at 60 °C for 2 h. The mixture was then cooled to ambient temperature and treated with methanol (7 μL) and HCl (3 μL, 2 M). The resulting mixture was added directly to the reaction mixture.

Synthesis of 1-(2-naphthyl)acetone (19): 1-(2-Naphthyl)acetone was synthesized as previously reported.[36] A mixture of 2-naphthylacetic acid (1 g, TCI), acetic anhydride (4 mL, Wako) and pyridine (4 mL, Wako) was heated at reflux under an atmosphere of nitrogen for 4 h. The mixture was then cooled to ambient and the solvents were evaporated under reduced pressure to give a residue, which was purified by MPLC on a silica gel column (Purif-Pak SI, SIZE 60). The structure of 1-(2-naphthyl)-acetone was confirmed by ^{1}H NMR.

Synthesis of 2-naphthalenemethyl acetate (20): 2-Naphthalenemethanol (1 mmol, TCI), acetic anhydride (2 mmol, Wako), and DMAP (0.05 mmol) were dissolved in dichloromethane (5 mL), and the mixture was stirred at room temperature for 8 h. The mixture was then diluted with chloroform and washed with deionized water before being dried over anhydrous Na_2SO_4. The solvents were evaporated under reduced pressure to give a residue, which purified by MPLC on a silica gel column (Purif-Pak SI, SIZE60). The structure of 2-naphthalenemethyl acetate was confirmed by ^{1}H NMR.

Purification of IfnQ: A 1.6 kb DNA fragment containing the IfnQ-encoding region was amplified from genomic *Streptomyces* sp. RI-77 by PCR with primers 5′-AA**CAT *ATG***GC GTTCA GCAGG CCGCT GCG-3′ (NdeI site in bold, start codon in italics) and 5′-TT**CTC GAG***TC AGCTG* ACATG GAGGT CGT-3′ (XhoI site in bold, stop codon in italics). The amplified fragment was inserted to pJET1.2 to obtain pJET1.2-*ifnQ*. The NdeI–XhoI fragment containing *ifnQ* was excised from pJET1.2 and inserted between the NdeI and XhoI sites of pET16b, thereby resulting in pET16b-*ifnQ*.

For the production of N-terminally His-tagged IfnQ, *E. coli* BL21(DE3) harboring pET16b-*ifnQ* was grown at 37 °C in ZYM-5052 medium[37] containing ampicillin (100 mg L^{-1}) to OD_{600} = 0.8. The culture was then cooled to 15 °C and incubated at this temperature for 32 h. The cells were harvested by centrifugation and resuspended in Tris·HCl (10 mM, pH 8.0) containing NaCl (200 mM) and glycerol (10%). After sonication, cellular debris was removed by centrifugation and filtration, and the clear lysate was subjected to purification by Ni^{2+} affinity chromatography using His-Accept (Nacalai Tesque). Imidazole was removed from the elution buffer by using a PD-10 column (GE Healthcare), and the purified protein was dissolved in Tris·HCl (10 mM, pH 8.0) with NaCl (200 mM) and glycerol (10%). The cofactor of IfnQ was identified by the following procedure. The IfnQ solution was mixed with an equal volume of methanol, and the resulting mixture was centrifuged to remove the precipitated protein. The supernatant was analyzed by LC-MS to compare the retention time of the extracted cofactor and that of FAD.

In vitro analysis of IfnQ reaction: The reaction mixture (100 μL) containing Tris·HCl (100 mM, pH 8.0), substrate analogue (200 μM), NADPH (250 μM), and IfnQ (1 μM) was incubated at 30 °C for 1 h, and the product was extracted with ethyl acetate. The organic layer was collected and evaporated to dryness to give a residue, which was dissolved in methanol (15 μL) for LC-MS analysis.

Kinetic analysis of IfnQ: For the analysis of the kinetic parameters of the IfnQ reaction, Tris·HCl (50 mM, pH 7.5, 100 μL) containing IfnQ (3 μM), 1-(2-naphthyl)-acetone (10–250 μM), NADPH (2 mM) was incubated at 30 °C. The initial velocity of the reaction was estimated by monitoring the decrease in absorbance at 340 nm as a result of the consumption of NADPH. The kinetic parameters were calculated by fitting the substrate concentration/initial velocity plot to Equation (1)

$$v = k_{cat} \times [E] \times [S]/(K_m + [S]) \quad (1)$$

Acknowledgements

This research was supported in part by a funding program for next-generation world-leading researchers from the Bureau of Science, Technology, and Innovation Policy, Cabinet Office, Government of Japan (to Y.O.), a Grant-in-Aid for Scientific Research on Innovative Areas from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT) (to Y. K.), and a grant from the New Energy and Industrial Technology Development Organization (NEDO) of Japan (to K.S. and N.S.).

Keywords: Baeyer–Villiger monooxygenase · C–C bond cleavage · isofuranonaphthoquinone · natural products · polyketides · *Streptomyces*

[1] K. Motohashi, M. Izumikawa, N. Kagaya, M. Takagi, K. Shin-ya, *J. Antibiot.* **2016**; DOI: 10.1038/ja.2015.135.
[2] P. Jetter, C. Steinert, M. Knauer, G. Zhang, T. Bruhn, J. Wiese, J. F. Imhoff, H-P Fiedler, G. Bringmann, *J. Antibiot.* **2013**, *66*, 719–726.
[3] Q. Zhang, A. J. Peoples, M. T. Rothfeder, W. P. Millett, B. C. Pescatore, L. L. Ling, C. M. Moore, *J. Nat. Prod.* **2009**, *72*, 1213–1215.
[4] Y. Yamamoto, Y. Kinoshita, G. Ran Thor, M. Hasumi, K. Kinoshita, K. Koyama, K. Takahashi, I. Yoshimura, *Phytochemistry* **2002**, *60*, 741–745.
[5] D. Parisot, M. Devys, J.-P. Férézou, M. Barbier, *Phytochemistry* **1983**, *22*, 1301–1303.
[6] M. Bezabih, S. Motlhagodi, B. M. Abegaz, *Phytochemistry* **1997**, *46*, 1063–1067.
[7] S. R. Jammula, S. B. Pepalla, H. Telikepalli, K. V. Jagannadha Rao, R. H. Thomson, *Phytochemistry* **1991**, *30*, 2427–2429.
[8] V. Muleya, R. Hayeshi, H. Ranson, B. Abegaz, M.-T. Bezabih, M. Robert, B. T. Ngadjui, F. Ngandeu, S. Mukanganyama, *J. Enzyme Inhib. Med. Chem.* **2008**, *23*, 391–399.
[9] E. S. Sattely, M. A. Fischbach, C. T. Walsh, *Nat. Prod. Rep.* **2008**, *25*, 757–793.
[10] A. Das, C. Khosla, *Acc. Chem. Res.* **2009**, *42*, 631–639.
[11] C. Hertweck, A. Luzhetskyy, Y. Rebets, A. Bechthold, *Nat. Prod. Rep.* **2007**, *24*, 162–190.

[12] M. A. Fernández-Moreno, E. Martínez, L. Boto, D. A. Hopwood, F. Malpartida, *J. Biol. Chem.* **1992**, *267*, 19278 – 19290.
[13] C. W. Carreras, C. Khosla, *Biochemistry* **1998**, *37*, 2084 – 2088.
[14] J. Dreier, A. N. Shah, C. Khosla, *J. Biol. Chem.* **1999**, *274*, 25108 – 25112.
[15] P. Javidpour, T. P. Korman, G. Shakya, S.-C. Tsai, *Biochemistry* **2011**, *50*, 4638 – 4649.
[16] W. R. Strohl, N. C. Connors, *Mol. Microbiol.* **1992**, *6*, 147 – 152.
[17] P. L. Bartel, C.-B. Zhu, J. S. Lampel, D. C. Dosch, N. C. Connors, W. R. Strohl, J. M. Beale Jr., H. G. Floss, *J. Bacteriol.* **1990**, *172*, 4816 – 4826.
[18] T. Itoh, T. Taguchi, M. R. Kimberley, K. I. Booker-Milburn, G. R. Stephenson, Y. Ebizuka, K. Ichinose, *Biochemistry* **2007**, *46*, 8181 – 8188.
[19] S. P. Cole, B. A. Rudd, D. A. Hopwood, C.-J. Chang, H. G. Floss, *J. Antibiot.* **1987**, *40*, 340 – 347.
[20] K. Ichinose, C. Surti, T. Taguchi, F. Malpartida, K. I. Booker-Milburn, G. R. Stephenson, Y. Ebizuka, D. A. Hopwood, *Bioorg. Med. Chem. Lett.* **1999**, *9*, 395 – 400.
[21] R. McDaniel, S. Ebert-Khosla, D. A. Hopwood, C. Khosla, *Science* **1993**, *262*, 1546 – 1550.
[22] E. S. Meadows, C. Khosla, *Biochemistry* **2001**, *40*, 14855 – 14861.
[23] T. Marti, Z. Hu, N. L. Pohl, A. N. Shah, C. Khosla, *J. Biol. Chem.* **2000**, *275*, 33443 – 33448.
[24] B. D. Ames, M.-Y. Lee, C. Moody, W. Zhang, Y. Tang, S.-C. Tsai, *Biochemistry* **2011**, *50*, 8392 – 8406.
[25] G. de Gonzalo, M. D. Mihovilovic, M. W. Fraaije, *ChemBioChem* **2010**, *11*, 2208 – 2231.
[26] Z. Xu, K. Jakobi, K. Welzel, C. Hertweck, *Chem. Biol.* **2005**, *12*, 579 – 588.
[27] M. P. Beam, M. A. Bosserman, N. Noinaj, M. Wehenkel, J. Rohr, *Biochemistry* **2009**, *48*, 4476 – 4487.
[28] N. Tibrewal, P. Pahari, G. Wang, M. K. Kharel, C. Morris, T. Downey, Y. Hou, T. S. Bugni, J. Rohr, *J. Am. Chem. Soc.* **2012**, *134*, 18181 – 18184.
[29] Z. Feng, D. Kallifidas, S. F. Brady, *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 12629 – 12634.
[30] A. Das, C. Khosla, *Chem. Biol.* **2009**, *16*, 1197 – 1207.
[31] J. A. Kalaitzis, Q. Cheng, D. Meluzzi, L. Xiang, M. Izumikawa, P. C. Dorrestein, B. S. Moore, *Bioorg. Med. Chem.* **2011**, *19*, 6633 – 6638.
[32] W. Zhang, B. D. Ames, S.-C. Tsai, Y. Tang, *Appl. Environ. Microbiol.* **2006**, *72*, 2573 – 2580.
[33] M. W. Fraaije, N. M. Kamerbeek, W. J. H. van Berkel, D. B. Janssen, *FEBS Lett.* **2002**, *518*, 43 – 47.
[34] N. M. Kamerbeek, M. J. H. Moonen, J. G. M. van der Ven, W. J. H. van Berkel, M. W. Fraaije, D. B. Janssen, *Eur. J. Biochem.* **2001**, *268*, 2547 – 2557.
[35] Y. Katsuyama, Y. Ohnishi, *Methods Enzymol.* **2012**, *515*, 359 – 377.
[36] K. Jozwiak, A. Y.-H. Woo, M. J. Tanga, L. Toll, L. Jimenez, J. A. Kozocas, A. Plazinska, R.-P. Xiao, I. W. Wainer, *Bioorg. Med. Chem.* **2010**, *18*, 728 – 736.
[37] F. W. Studier, *Prot. Exp. Pur.* **2005**, *41*, 207 – 234.

Manuscript received: February 16, 2016
Final article published: May 9, 2016