Heterologous Biosynthesis of Truncated Hexaketides Derived from the Actinorhodin Polyketide Synthase[†]

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Heterologous expression of the actinorhodin polyketide synthase in the recombinant host *Streptomyces lividans* K4-114 led to the characterization of three new minor polyketides, the novel hexaketides BSM1 and BSM3 and 9′-hydroxyaloesaponarin II, in addition to known anthraquinone and aromatic octaketides. The structures of BSM1 and BSM3 imply that these compounds are derived from a C-5-reduced hexaketide intermediate, suggesting that the timing of the ketoreduction reaction in the actinorhodin biosynthetic pathway may take place during the polyketide elongation process rather than after the completion of the octaketide chain as previously suggested.

Aromatic or type II polyketide synthases (PKSs) are enzyme complexes composed largely of monofunctional proteins that give rise to the aromatic polyketide family of natural products that are common among actinomycetes.[1−3] Products of this secondary metabolic pathway include benzoisochromanequinones (i.e., actinorhodin and granaticin), tetracyclines, anthracyclines (i.e., daunorubicin), angucyclines (i.e., jadomycin), tetracenomycins, and aureolic acids (i.e., mithramycin). Each PKS contains a "minimal" set of proteins [the two ketosynthase subunits KS_α and KS_β (also referred to as the chain length factor[4,5]), an acyl carrier protein (ACP), and malonyl-CoA:ACP acyltransferase] that are required for polyketide biosynthesis. Additional PKS subunits, including ketoreductases (KRs), cyclases (CYCs), aromatases (AROs), oxygenases, etc., are responsible for modification of the nascent poly-β-carbonyl intermediate to form a specific cyclized polyketide product.

Pioneering studies by Khosla, Hopwood, and co-workers in the 1990s on the development of an elegant *Streptomyces* host−vector expression system for the construction of novel polyketides provided new insight in aromatic polyketide assembly.[5−7] A large series of novel polyketides were generated by expressing incomplete or hybrid combinations of type II PKS gene sets,[1] leading not only to a better understanding of aromatic PKS specificity but to the emergence of the field of combinatorial biosynthesis.[8,9] The expression of the *act* minimal PKS genes (*actI*-ORFs1-3) together with the KR *actIII*, the ARO *actVII*, and the CYC *actIV* from the shuttle vector pRM5 in *Streptomyces coelicolor* CH999 was one of the first reported combinatorial biosynthetic experiments.[5] The recombinant strain *S. coelicolor* CH999, in which the actinorhodin biosynthetic gene cluster was chromosomally deleted, resulted in an ideal host for producing engineered polyketides because of its low-level background polyketide synthesis. The characterization of the resultant anthraquinones 3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid (DMAC) (**1**) and aloesaponarin II (**2**) was instrumental in the elucidation of the early stages in the biosynthesis of actinorhodin (Scheme 1). When pRM5 was alternatively expressed in the actinorhodin-deficient recombinant host *S. lividans* K4-

114,[10] although **1** and **2** were the major polyketides produced, the octaketides mutactin (**3**), EM18 (**4**), and dehydromutactin (**5**) were detected along with several uncharacterized polyketides.[11] Here, we describe the structures of the **2** derivative 9′-hydroxyaloesaponarin II (**6**) and the two novel truncated polyketides BSM1 (**7**) and BSM3 (**8**). The structures of the hexaketides **7** and **8**, which are reduced at C-5, imply that the timing of the ketoreduction reaction in the actinorhodin biosynthetic pathway may occur during the polyketide elongation process rather than after the completion of the octaketide chain as previously suggested.[12]

In addition to the five known octaketides **1−5**, which all share a common structural motif consistent with a C7/C12 first cyclization event (Scheme 1), HPLC analysis of the K4-114/pRM5 polyketides suggested the presence of several new shunt products (Figure 1). UV analysis of compounds with chromatographic properties similar to those of the anthraquinones **1** and **2** hinted at at least four additional anthraquinone derivatives. The **2** derivative 9′-hydroxyaloesaponarin II (**6**) was purified by reversed-phase chromatography and characterized by (^{13}C,^{1}H) gradient-enhanced HSQC and HMBC spectroscopy. HRESIMS supported a formula of $C_{15}H_{10}O_5$ (m/z = 269.0463 [M − H]$^-$, Δ −1.3 mmu), which contains one oxygen atom more than that of aloesaponarin II (**2**). Comparison of the ^{1}H and ^{13}C NMR data of **2** and the more hydrophilic **6** indicated that the only difference in **6** was the absence of the 9′-methyl group. Rather it was replaced with a hydroxymethyl group consisting of a methylene at δ 5.50 that coupled to an exchangeable proton at δ 5.03. HMBC correlations from the H-9′ methylene triplet to the phenolic carbons C-7, C-8, C-11, C-12 and its ROESY correlation to H-8 supported the attachment of the hydroxymethyl group at C-9. The oxidized **6** is probably derived from a post-PKS hydroxylation of **2** by a *S. lividans*-encoded monooxygenase.

Several uncharacterized polyketides of slightly greater polarity than the known **3−5** were also evident by HPLC (Figure 1). To our surprise BSM1 (**7**) had the molecular formula $C_{12}H_{10}O_4$ (m/z = 219.0665 [M + H]$^+$, Δ +0.8 mmu) on the basis of HRFABMS, suggestive of a truncated hexaketide rather than a full length octaketide that is characteristic of all *act* shunt products described to date. Inspection of the NMR spectral data indicated that **7** had the same structure from carbons 2 to 12 as the octaketide SEK34b (**9**),[13] but differed at the carboxy terminus (Table

Scheme 1. Structures of SEK34 (**11**) and SEK34b (**9**) and Proposed Biosynthesis of Actinorhodin and the Shunt Products DMAC (**1**), Aloesaponarin II (**2**), Mutactin (**3**), EM18 (**4**), Dehydromutactin (**5**), 9′-Hydroxyaloesaponarin II (**6**), BSM1 (**7**), BSM3 (**8**), and BSM2 (**10**)[a]
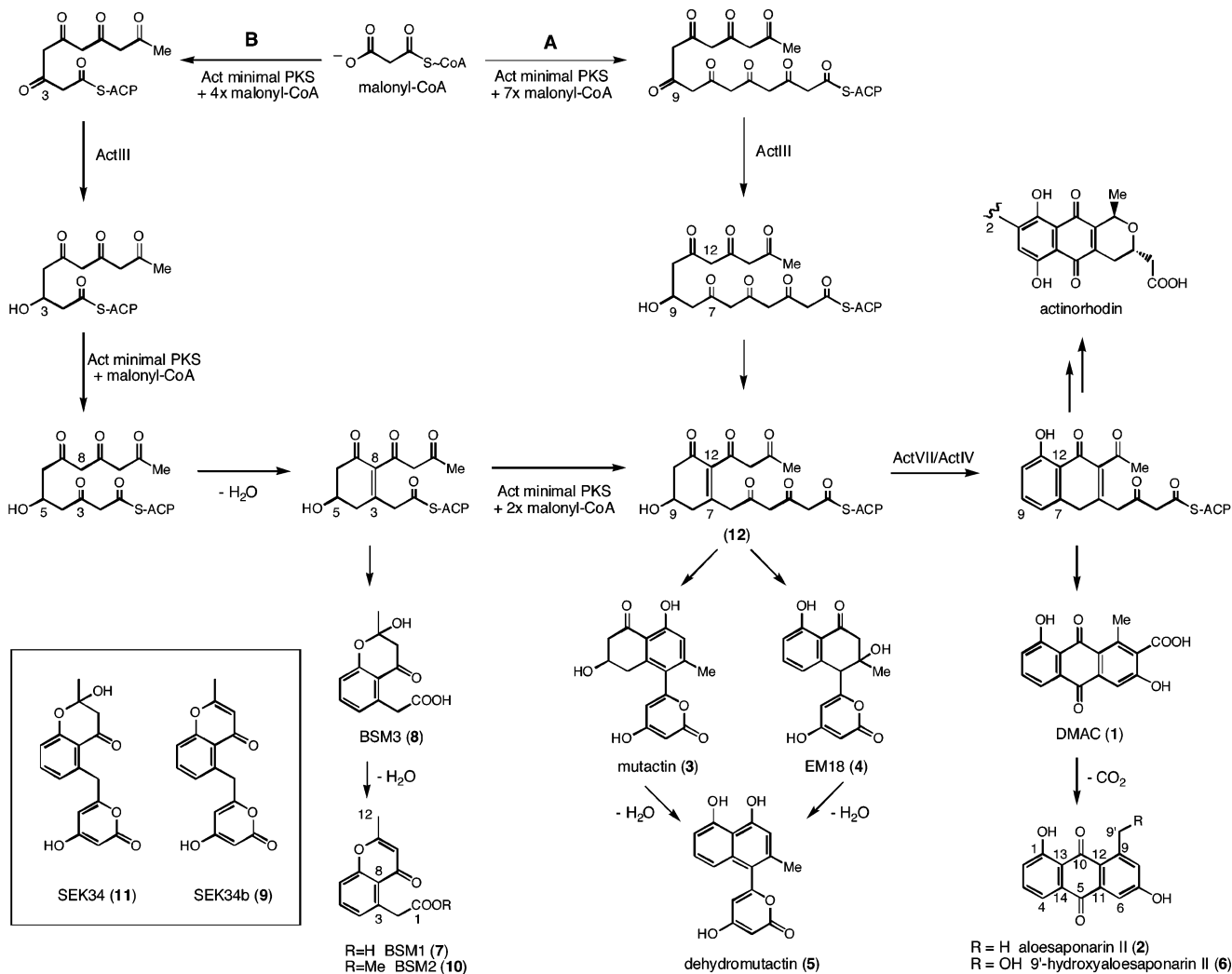
[a] The carbon atoms for most intermediates and products are labeled according to their position in the polyketide backbone, except for **2**, which is labeled according to the literature.[12]
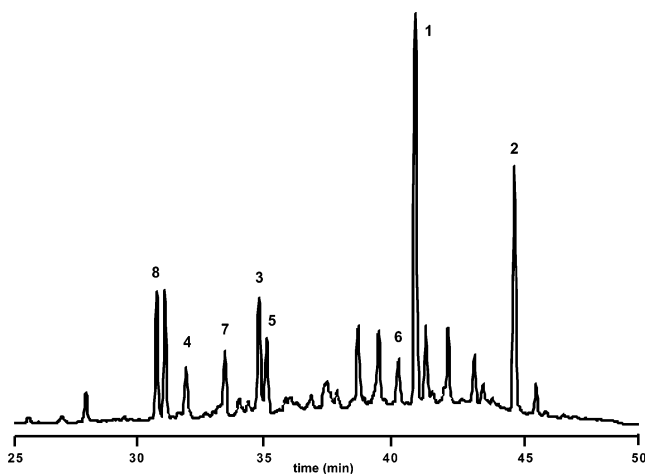

Figure 1. HPLC chromatogram of the *S. lividans* K4-114/pRM5 polyketides.

1). NMR established the presence of a carboxyl group (δ_H 12.0, δ_C 172.1) attached to the C-2 methylene rather than a 4-hydroxy-2-pyrone unit such as in the structurally related SEK34b and in **3**−**5**. Key HMBC correlations on the unnatural **7**-methyl ester BSM2 (**10**) (HRFABMS: *m/z*

= 233.0804 [M + H]+, Δ −1.0 mmu, $C_{13}H_{12}O_4$), a purified artifact resulting from acidic methanol workup, verified the assignment of the chromone unit and the methyl ester group (Table 1). Base hydrolysis of the methanolysis product **10** to the carboxylic acid **7** confirmed their structural relationship. A second naturally occurring hexaketide, BSM3 (**8**), was also characterized. Positive ion LR-APCIMS (*m/z* = 237.1 [M + H]+; 219.0 [M + H − H_2O]+) supported a molecular formula of $C_{12}H_{12}O_5$, which was suggestive of a hydrated form of **7**. Comparison of the ^{1}H NMR data with those for **7** as well as the analogous octaketide SEK34 (**11**)[13] supported the proposed structure of **8** (Table 1).

In the current model for the biosynthesis of actinorhodin and other C-9-reduced type II PKS products, the timing of ketoreduction at C-9 by the ActIII KR has been proposed to occur after the assembly of the complete poly-β-ketide chain (Scheme 1, path A).[1−3] All ketoreduced aromatic PKS products, including actinorhodin, oxytetracycline, aclacinomycin A, and enterocin, are similarly reduced at the ninth carbon from the carboxy terminus of the assembled polyketide irrespective of its chain length or the nature of the starter unit. Ketoreductases from these different systems have been successfully interchanged and shown to function with hybrid PKSs,[11,12,14−16] thereby supporting the conclusion that "the polyketide chain is completely

Table 1. NMR Data for BSM1 (**7**), BSM2 (**10**), and BSM3 (**8**)[a]

no.[b]	BSM2 (**10**)			BSM1 (**7**)		BSM3 (**8**)
	δ_C	δ_H[c]	HMBC	δ_C	δ_H[c]	δ_H[c]
1	171.1			172.1	12.0 (brs)	
2	39.6[d]	4.14 (s, 2H)	C-1, C-3, C-4, C-8	39.6[d]	4.09 (s, 2H)	3.77 (d, 16.5)
						3.95 (d, 16.5)
3	135.3			136.2		
4	128.6	7.24 (d, 7.8)	C-2, C-6, C-8	128.6	7.21 (d, 7.8)	6.83 (d, 8.0)
5	133.0	7.66 (t, 7.8)	C-3, C-6, C-7	132.9	7.64 (t, 7.8)	7.42 (t, 8.0)
6	117.6	7.51 (d, 7.8)	C-4, C-7, C-8, C-9	117.3	7.49 (d, 7.8)	6.90 (d, 8.0)
7	157.1			157.1		
8	121.2			121.4		
9	178.5			178.6		
10	110.8	6.13 (s)	C-8, C-9, C-11, C-12	110.9	6.12 (s)	2.60[e]
						2.98 (brd, 16.0)
11	165.4			165.3		
12	19.5	2.34 (s, 3H)	C-10, C-11	19.5	2.34 (s, 3H)	1.58 (s, 3H)
1-OMe	51.2	3.55 (s, 3H)	C-1			

[a] Spectra were obtained at 600 MHz for proton and 75 and 150 MHz for carbon and were recorded at 30 °C in DMSO-d_6. Chemical shifts are reported in δ (ppm). [b] Carbons are labeled according to their number in the polyketide backbone (Scheme 1). [c] Coupling constants are presented in hertz. Unless otherwise indicated, all proton signals integrate to 1H. [d] Carbon signal obscured by DMSO-d_6 solvent peak and thus determined by HSQC/HMBC. [e] Proton signal obscured by DMSO-d_6 solvent peak and determined by HSQC.

assembled before reduction of the keto group takes place".[12] Recent studies on the biosynthesis of enterocin and the wailupemycins in the marine actinomycete "*Streptomyces maritimus*" demonstrated a functional requirement of the enterocin PKS for its ActIII analogous KR EncD, implying that the enterocin-precursor polyketide chain is reduced during the elongation process rather than after its completion.[17] All other type II PKSs studied to date, including *act*,[12,14,18] however, do not require their endogenous KR for minimal PKS activity, thereby suggesting the possibility of two ketoreduction mechanisms in type II PKS pathways. The structures of the truncated hexaketides BSM1 (**7**) and BSM3 (**8**), which are reduced at C-5 and not C-9, imply that these compounds may be derived from a C-5-reduced hexaketide linear intermediate (Scheme 1, path B). If **7** and **8** are indeed biosynthetic shunt products and not catabolic products, then their production calls into question the timing of the ActIII reaction. Like that proposed for the biosynthesis of enterocin,[17] ActIII may reduce the target carbonyl group when it is in the β-position relative to the thioester carbonyl (Scheme 1, path B). Ketoreduction at an earlier stage in the polyketide elongation process may facilitate proper aldol cyclization of the first ring by limiting nonspecific cyclizations due to the deactivation of the reduced carbonyl carbon and the adjacent methylene groups. All reduced type II PKS-derived polyketides engineered to date share the same regiochemistry about the first cyclized ring regardless of chain length,[1] thus supporting this alternative model. Conversely, the truncated hexaketides **7** and **8** may be degradation products resulting from modifying enzymes present in the host strain *S. lividans* K4-114 not present in *S. coelicolor* involving retro-Aldol-type chemistry of the reactive β-diketo side chain of the C-9-reduced octaketide **12** to yield the hexaketides and acetoacetate.

Experimental Section

Culture Conditions and Isolation of Polyketides from *S. lividans* K4-114/pRM5. *S. lividans* K4-114[10] was used as a host for transformation with pRM5[5] as previously described.[11] *S. lividans* K4-114 was cultured on R2YE agar for protoplast transformation and polyketide production, and transformants were selected with thiostrepton (20 μg/mL). *S. lividans* K4-114/pRM5 was grown on fresh solid R2YE (900 mL) for 5−7 days at 30 °C, during which time the plates became deeply pigmented. The culture was exhaustively extracted with 5% MeOH/EtOAc, dried over anhydrous MgSO$_4$, and evaporated in vacuo. The crude extract was then subjected to stepwise normal-phase silica gel (Aldrich, 28−200 mesh) flash column chromatography with 25% EtOAc/hexane, 50% EtOAc/hexane, EtOAc, and MeOH. Fractions were analyzed by reversed-phase C$_{18}$ analytical HPLC using a Phenomenex Synergi Hydro-RP column (150 × 4.6 mm, 4 μm) employing gradient elution from 100% H$_2$O (0.15% TFA) to 100% MeOH over 30 min at a flow rate of 0.5 mL/min with UV detection at 254 nm. Fractions eluting with 50% EtOAc/hexane and EtOAc were combined and subjected to HPLC purification. Compounds were purified using a YMC pack ODS-A HPLC column (250 × 20 mm, 10 μm), employing a gradient from 100% H$_2$O (0.15% TFA) to 100% MeOH over 50 min at a flow rate of 9.5 mL/min with UV detection at 254 nm. BSM3 (**8**, 0.7 mg), BSM1 (**7**, 0.7 mg), and 9′-hydroxyaloesaponarin II (**6**, 0.7 mg) eluted at 23, 27.5, and 37.5 min, respectively.

9′-Hydroxyaloesaponarin II (6): ^{1}H NMR (600 MHz, DMSO-d_6) δ (integration, multiplicity, coupling constants in hertz, assignment, *HMBCs*, **ROESYs**): 5.03 (2H, d, $J = 4.4$, H-9′, *C-7, C-8, C-11, C-12*, **H-8, 9′-OH**), 5.50 (1H, t, 9′-OH, $J = 4.4$, *C-7, C-9′*, **H-8, H-9′**), 7.33 (1H, d, $J = 7.8$, H-2, *C-4, C-13*, **H-3, H-4**), 7.46 (1H, brs, H-6, *C-5, C-8, C-12*), 7.64 (1H, d, $J = 7.8$, H-4, *C-2, C-5, C-13*, **H-2**), 7.65 (1H, brs, H-8, *C-6, C-12, C-9′*, **H-9, 9′-OH**), 7.73 (1H, t, $J = 7.8$, H-3, *C-1, C-14*, **H-2**), 11.3 (1H, s, 7-OH), 12.9 (1H, s, C1-hydroxyl, *C-1, C-2, C-13*); ^{13}C NMR (150 MHz, DMSO-d_6) δ 62.0 (C-9′), 111.8 (C-6), 116.4 (C-13), 118.4 (C-4 or C-8), 118.5 (C-4 or C-8), 120.8 (C-12), 124.3 (C-2), 132.5 (C-14), 133.8 (C-9), 136.2 (C-3), 136.9 (C-11), 151.4 (C-7), 161.4 (C-1), 182.4 (C-5), 189.2 (C-10).

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