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NOTE

Structure and biosynthetic implication of 5*R*-(*N*-acetyl-L-cysteinyl)-14*S*-hydroxy-dihydrokalafungin from a mutant of the *act*VA-ORF4 gene for actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2)

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Actinorhodin (ACT, **1**), an aromatic polyketide produced by *Streptomyces coelicolor* A3(2),[1] belongs to a class of benzoisochromanequinone (BIQ) antibiotics (Figure 1a).[2] Because **1** has a symmetrical structure in which two BIQ units are coupled via a C–C bond, its enzymatic formation in a regiospecific manner has drawn considerable attention. ACT biosynthesis is controlled by the *act* cluster, which comprises 22 genes, and the functions of several genes remain elusive. To characterize one such gene, the deletion mutant of *act*VA-ORF4 (Δ*act*VA-4) was constructed (Supplementary Figure S1). Analysis of ACT-related metabolites produced by this mutant led to the identification of two characteristic compounds: (1*R*,3*S*)-6,9-dihydroxy-1-methyl-5,10-dioxo-3,4,5,10-tetrahydro-1*H*-naphtho-[2,3-*c*]-pyran-3-ylacetic acid, known as 8-hydroxy-dihydrokalafungin (DHK-OH, **2**) and (1*R*,3*S*)-5,10-dihydroxy-1-methyl-6,9-dioxo3,4,6,7,8,9-hexahydro-1*H*-naphto-[2,3-*c*]-pyran-3-ylacetic acid, known as 8-hydroxy-3,4,7,8-tetrahydrokalafungin (THK-OH, **3**; Figure 1a).[3] The structures of **2** and **3** correspond to the monomeric unit of **1**, thus strongly suggesting *act*VA-4 to be an essential component for dimerization at C-10 of ACT biosynthesis. Further metabolite analysis of the Δ*act*VA--4 culture broth revealed the presence of a third unidentified ACT-related metabolite (compound Z; Figure 1b), which is absent in the wild-type broth. This turned out to be a new shunt product of ACT biosynthesis; the structure elucidation and biosynthetic implication of this new product are presented in this paper.

The Δ*act*VA-4 mutant was inoculated into tryptic soy broth medium for seed culture[4] and grown on a rotary shaker at 200 r.p.m. and 30 °C for 2 days. Aliquots of the seed culture were transferred to R5MS liquid medium and grown as described previously.[5] HPLC analysis of the culture broth showed that the UV-VIS spectrum of compound Z is similar to that of 5,14-epoxy-kalafungin (Supplementary Figure S2).[6] LC/HRESIMS provided a molecular formula of $C_{21}H_{23}NO_{10}S$ (*m/z* $[M\text{-}H]^-$ calcd for $C_{21}H_{22}NO_{10}S$, 480.0964. found, 480.0977), suggesting the attachment of a moiety containing nitrogen and sulfur atoms. Subsequently, the ethyl acetate (EtOAc) extract obtained from the large-scale culture (6.6 l) was subjected to silica-gel column chromatography eluting with EtOAc/hexane (6:4, 4:6 and 2:8, stepwise) and acetone/methanol (MeOH; 9:1). The fraction containing compound Z was subsequently subjected to preparative HPLC twice (TOSOH TSK-gel ODS-100 V, 7.8 × 300 mm, 50 °C, solvent A: water containing 0.01% TFA, solvent B: acetonitrile containing 0.01% TFA. First preparation: 25% B isocratic, 2.0 ml min^{-1}. Second preparation: 20% B isocratic, 2.5 ml min^{-1}) to yield a pure compound (8.2 mg).

Compound Z was isolated as a white amorphous solid: $[\alpha]_D^{23}$+116° (*c* 0.10, MeOH); UV λ_{max} nm (log ε) in MeOH: 208 (4.31), 232 (4.23), 260 (sh, 4.00), 354 (3.70); IR (ATR) ν_{max} cm^{-1} 3383 (hydroxyl), 1692 and 1651 (carbonyl). NMR data of compound Z in dimethylsulfoxide (DMSO)-d_6 indicated the existence of 21 carbons and at least 19 protons (Table 1). Three doublet–doublet signals at 7.29, 7.43 and 7.73 p.p.m. in ^{1}H-NMR and their HMBC correlations indicated a 2,3-dihydro-8-hydroxy-1,4-naphthoquinone moiety. Further comparison of the NMR data between compound Z and dihydrokalafungin (DHK, **4**, Figure 1a) strongly suggested the presence of a pyran ring moiety with a carboxyl group. Compound Z seemed to be a derivative of **4**; however, the signals assigned to C-5 (δ_C 58.6) and C-14 (δ_C 76.0) indicated attachment of hetero atoms to C-5 and C-14, respectively, and a single bond connection between C-5 and C-14. The COSY spectrum indicated the connectivity of the methylene peak (δ_C 31.7; δ_H 2.46, 2.57) with methine (δ_C 51.2; δ_H 4.14), which in turn correlated with the NH proton (δ_H 8.12;

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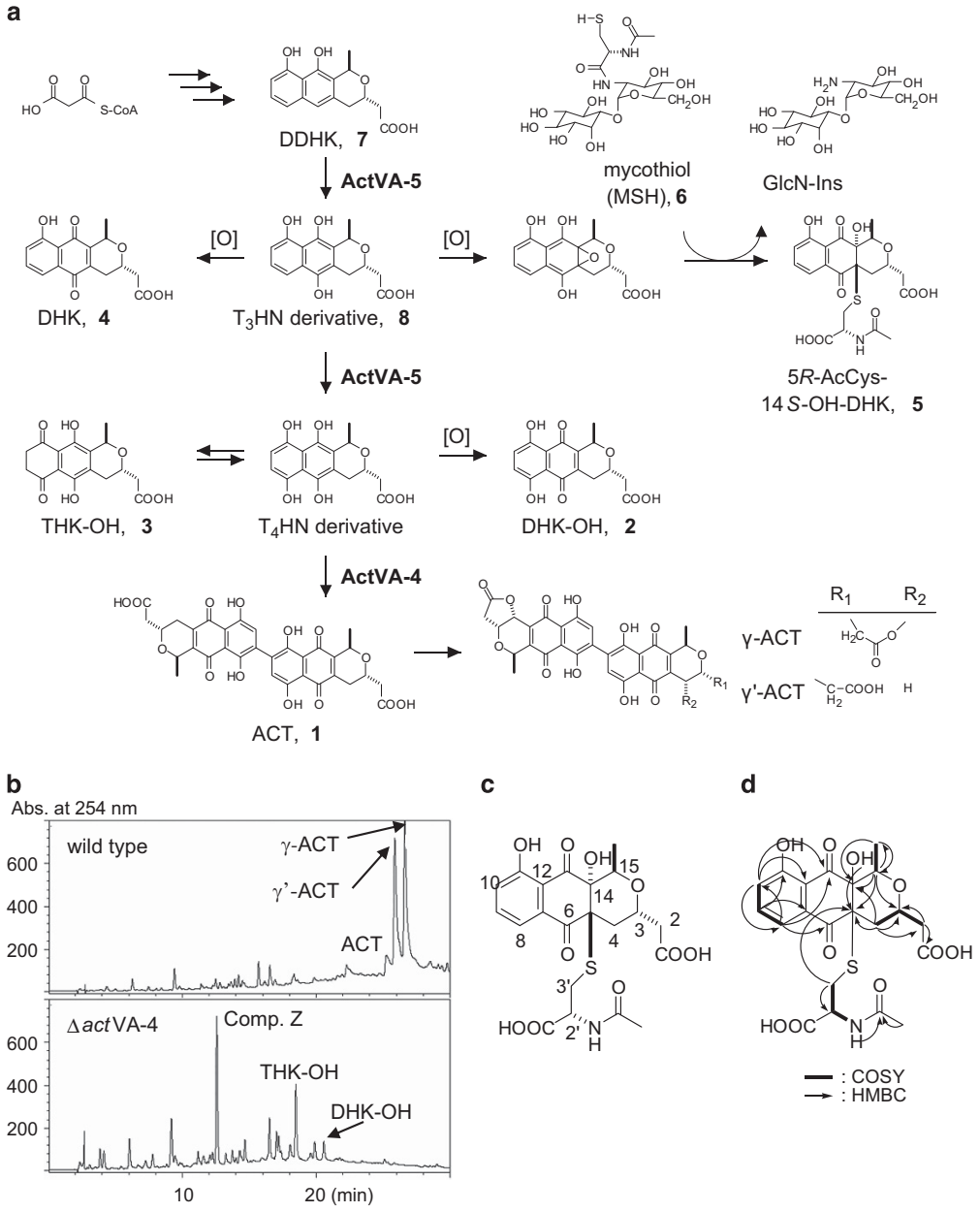

Figure 1 The proposed biosynthetic pathway of ACT and structural elucidation of **5**. (**a**) The biosynthetic relationship of the metabolites mentioned in this study. (**b**) HPLC profiles of ethyl acetate extracts from liquid culture of wild type (top) and Δ*act*VA-4 (bottom) under the following conditions: TOSOH TSK-gel ODS-100 V, 4.6 mm i. d. ×150 mm, 40 °C, 10–95% aqueous acetonitrile containing 0.5% acetic acid, 1.0 ml min^{-1}. Chromatograms were monitored by absorbance at 254 nm. (**c**) Structure of **5**. Numbering of positions is based on the biosynthetic order. (**d**) Correlations of COSY (bold line) and HMBC (arrow).

Figure 1d). These data, as well as the additional signals derived from the acetyl (δ_C 22.3, 169.3; δ_H 1.75) and carboxyl (δ_C 171.4) groups, indicated the existence of an *N*-acetyl cysteine (AcCys) moiety. The key HMBC correlation could be successfully detected between the methylene proton (δ_H 2.46, 2.57) and C-5 (δ_C 58.6), thus clearly indicating that the AcCys moiety attaches to C-5 via a sulfur atom.

The MS data indicated the presence of an oxygen atom in addition to the aforementioned structure, and the attachment of hydroxyl group at C-14 was reasonably confirmed on the basis of the HMBC correlation from the proton signal at 7.10 p.p.m. to C-5 (δ_C 58.6). Considering the assumption of the biosynthetic relationship with ACT, the structure of compound Z was elucidated as 5-(*N*-acetyl-L-cysteinyl)-14-hydroxy-dihydrokalafungin (5-AcCys-14-OH-DHK, Figures 1c and d). In 2D rotating frame NOESY (ROESY) analysis (Supplementary Figure S3), ROEs between 14-OH and H-4α, and between 14-OH and H-15 suggested the C-14 stereochemistry as (*S*)-configuration. Similarly, ROEs between H-16 and H-3, between H-3 and H-4β, and between H-4β and H-3' indicated the C-5 stereochemistry as (*R*)-configuration. The absolute structure of the compound was thus elucidated as 5*R*-(*N*-acetyl-L-cysteinyl)-14*S*-hydroxy-dihydrokalafungin (5*R*-AcCys-14*S*-OH-DHK, **5**; Figure 1c), which is (1*R*,3*R*,4a*R*,10a*S*)-4,4a,5,10,10a-hexahydro-9,10a-dihydroxy-5,10-dioxo-1-propyl-4a-[[(2*R*)-2-(acetylamino)-2-carboxyethyl]thio]-1*H*-Naphtho[2,3-*c*]pyran-3-acetic acid.

Certain Gram-positive bacteria, including streptomycetes, use mycothiol (MSH, **6**) to protect the cells against toxic and/or reactive

Table 1 NMR data of 5*R*-AcCys-14*S*-OH-DHK, 5, in DMSO-d_6

			5R-AcCys-14S-OH-DHK in DMSO-d_6			
Position	*δ_C (100 MHz)*	*δ_H (400 MHz)*		*COSY*	*HMBC*	*ROESY*
1	172.1					
2	40.3	2.42	dd, 15.6, 9.2	2b, 3	1, 3, 4	2b
		2.58	dd, 15.6, 3.2	2a, 3	1, 3, 4	2a
3	62.9	4.53	m	2a, 2b, 4a, 4b		2, 4b, 16
4	29.4	1.90	dd, 14.2, 1.2	3, 4α	5, 14	4α, 3, 3′
		2.15	dd, 14.2, 11.6	3, 4β	2, 3, 5	4β, 14-OH
5	58.6					
6	189.2					
7	133.0					
8	118.5	7.43	dd, 7.6, 1.2	9, 10	6, 9, 10, 11, 12, 13	
9	137.1	7.73	dd, 8.4, 7.6	8, 10	7, 8, 11, 12	
10	123.7	7.29	dd, 8.4, 1.2	8, 9	8, 11, 12, 13	
11	160.5	11.18	s (OH)		10, 11, 12	
12	114.6					
13	198.7					
14	76.0	7.10	broad s (OH)		5	4α, 15
15	72.8	4.15	q, 7.2	16	3, 5, 14, 16	16, 14-OH
16	15.6	1.62	d, 7.2	15	14, 15	3, 15
1′	171.4					
2′	51.2	4.14	ddd, 8.4, 8.0, 5.6	3′a, 3′b, 2′NH	1′, 3′, *N*-Ac-CO	3′a, 3′b, 2′NH
2′ NH		8.12	d, 8.4	2′	1′, 2′, *N*-Ac-CO	2′, *N*-Ac-Me
3′	31.7	2.46	dd, 12.4, 5.6	2′, 3′b	5, 2′	3′b
		2.57	dd, 12.4, 8.0	2′, 3′a	5, 2′	3′a
N-Ac-Me	22.3	1.75	s		2′, *N*-Ac-CO	
N-Ac-CO	169.3					

electrophiles. MSH has analogous functions to glutathione and can react with various toxic compounds to form MSH *S*-conjugates, which are then cleaved by an MSH *S*-conjugate amidase to release GlcN-Ins and a toxin-AcCys *S*-conjugate.[7,8] The metabolic genes for MSH are present in *S. coelicolor* A3(2),[9] and two AcCys adducts of **4** were identified from a recombinant *S. coelicolor* strain.[10] ActVA-ORF5 is a bifunctional flavin-dependent monooxygenase that catalyzes the oxygenation reactions at both the central (C-6) and lateral (C-8) rings of a tricyclic substrate, 6-deoxy-dihydrokalufungin (DDHK, **7**).[9] Formation of **5** apparently occurred via the ring-opening reaction of a 5,14-epoxy intermediate, which is most plausibly the 5,14-epoxide of a trihydroxynaphthalene (T_3HN) derivative (**8**; Figure 1a). A hydroquinone moiety of **8** was unstable and could be easily oxidized to an isolable quinone form. Although there is no report on epoxide derivatives of **1** from *S. coelicolor* strains, diepoxyactinorhodin has been isolated from a different *Stretpomyces* strain.[11] Another recent example related to **5** is the isolation of frenolicin C with the same substitution pattern of hydroxyl and AcCys groups but with a different carbon chain length of polyketide origin.[12]

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