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NOTE

Germicidins H–J from *Streptomyces* sp. CB00361

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α-Pyrones and natural products featuring an α-pyrone moiety are well known from bacteria,[1] fungi,[1] plants[2] and animals,[3] and they exhibit a wide range of biological activities.[1–3] The germicidins are a family of microbial α-pyrone natural products, featuring a 4-hydroxy-α-pyrone core with varying alkyl substitutions at C-3 and C-6 (Figure 1). Identified as the first autoregulators of spore germination in *Streptomyces*, germicidin A (**1**) and B were first isolated from *Streptomyces viridochromogenes* NRRL B-1551[4] and subsequently re-isolated from *S. coelicolor* A3(2), together with germicidin C (**2**) and D (**3**).[5] Other members of this family include: isogermicidin A and B, isolated in an effort to mine the *S. coelicolor* A3(2) genome for novel polyketides, together with **1**, **2** and germicidin B;[6] germicidin F and G, isolated from *Streptomyces* sp. HKI0576 during the biosynthetic study of the divergolides, along with **1** and germicidin B;[7] surugapyrone A (same as **3**),[8] isolated from *S. coelicoflavus* USF-6280 as a free radical scavenger; the violapyrones,[9] isolated from *S. violascens* as antibacterial antibiotics; the photopyrones,[10] isolated from *Photorhabdus luminescens* as a new family of bacterial signaling molecules; the myxopyronins[11] and corallopyronins,[12] isolated from *Myxococcus fulvus* Mx f50 and *Corallococcus coralloides* Cc c127, respectively, as novel inhibitors of bacterial RNA synthesis; the dactylfungins,[13] isolated from *Dactylaria parvispora* D500 as novel antifungal antibiotics; the phytotoxic α-pyrones,[14] isolated from *Pestalotiopsis guepinii* as the causal agents of hazelnut twig blight; the csypyrones,[15,16] isolated from recombinant *Aspergillus oryzae* strains in an effort to mine the *A. oryzae* genome for novel polyketides; and most recently, during the revision of the current manuscript, several α-pyrones with varying hydroxyl substitutions from three marine-derived *Nocardiopsis* strains[17] (Supplementary Figure S1).

Biosynthetic studies of selected members of the germicidin family of natural products have revealed distinct mechanisms for the formation of the C-3 and C-6 dialkylated 4-hydroxy-α-pyrone core. While stand-alone ketosynthases have been proposed to catalyze the condensation of two acyl carrier protein (ACP)-tethered β-ketoacyl intermediates to afford the characteristic myxopyronin[18] and photopyrone[10] scaffolds, the majority of this family of natural products is found to be biosynthesized by type III polyketide synthases (PKSs).[7,15,16,19–23] The hallmark feature of type III PKSs is to catalyze the iterative elongation of diverse acyl-CoA starter units with malonyl-CoA as an extender unit to form poly-β-ketoacyl-CoA intermediates that can undergo cyclization via Claisen and/or aldol reactions, followed by dehydration, to afford aromatic products.[19,20] Remarkably, germicidin synthase (Gcs) was found to prefer β-ketoacyl-ACP intermediates in fatty acid biosynthesis as starter units, exhibiting a broad substrate flexibility toward varying acyl-ACPs, and catalyze one cycle of elongation using malonyl-, methylmalonyl- or ethylmalonyl-CoA as an extender unit.[6,21–23] Gcs therefore represents an emerging subfamily of bacterial type III PKSs that cross talks with fatty acid biosynthesis, exploitation of which *in vitro* as biocatalysts has indeed resulted in the production of a focused library of polyketides with varying starter and extender units.[6,21–23] However, it is not known if the new germicidin analogs generated *in vitro* are true metabolites of Gcs or its homologs in *Streptomyces* species *in vivo*.

Here we report the discovery of six germicidins (**1–6**) and one keto acid (**7**) from *Streptomyces* sp. CB00361 (Figure 1a). Germicidin I (**5**) is a new compound, and germicidin H (**4**), J (**6**) and keto acid **7** are isolated for the first time as natural products. The seven compounds were tested for antibacterial activities, but no activity was detected under all conditions tested.

As a part of the Natural Products Library Initiative at The Scripps Research Institute, we aim at discovering natural products from Actinomycetales that are isolated from unexplored and underexplored ecological niches and unavailable in public strain collections.[24] Strain CB00361 was isolated from a bamboo grove in Changning county, Sichuan province, China, and was classified as a *Streptomyces* species on the basis of phylogenetic analysis (Supplementary Figure S2). A 6 l fermentation of *S.* sp. CB00361 was carried out and seven compounds (**1–7**) were isolated (Supplementary Information). Their structures were elucidated based on NMR and high-resolution (HR)-ESI-MS analysis.

Compounds **1–3** were confirmed as germicidin A,[4–7] germicidin C[5,6] and germicidin D,[5,8] respectively, upon comparisons of their ^{1}H and ^{13}C NMR data with those published in the literature

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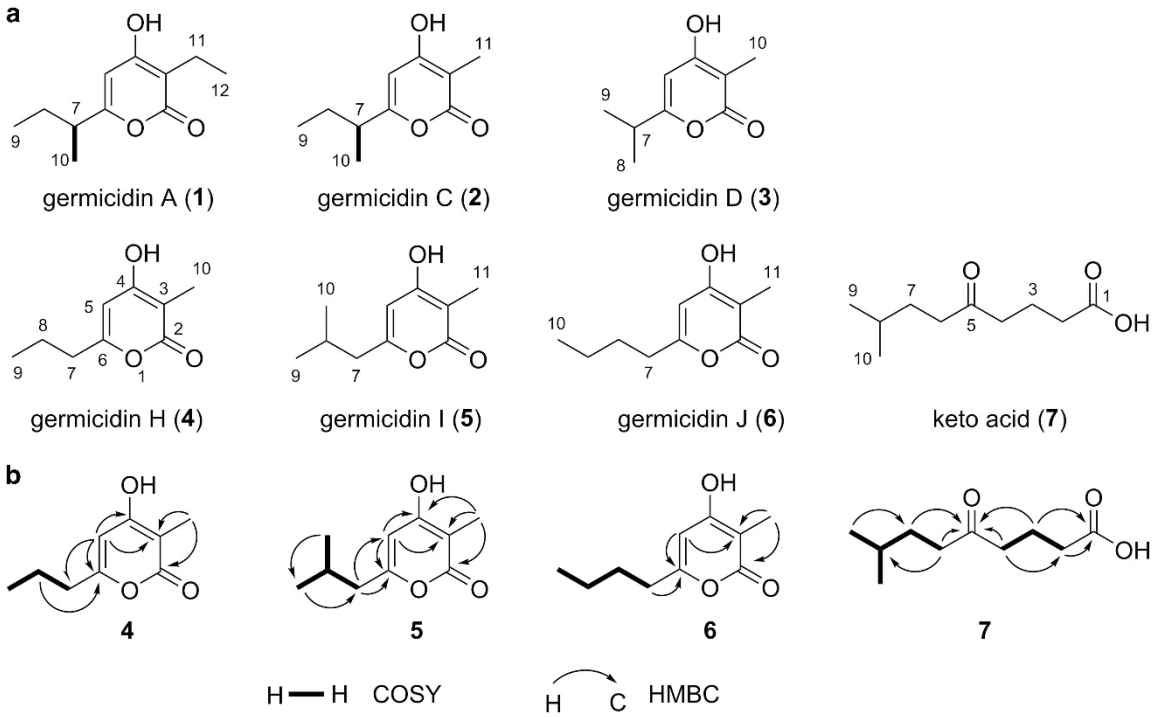

Figure 1 Six germicidins (**1**–**6**) and one keto acid (**7**) isolated from *S.* sp. CB00361. (**a**) The structures of germicidin A (**1**), C (**2**), D (**3**), H (**4**), I (**5**), J (**6**) and keto acid (**7**). (**b**) The key COSY and HMBC correlations supporting the structures of germicidin H (**4**), I (**5**), J (**6**) and keto acid (**7**).

Table 1 ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data for germicidins H–J (4–6) in acetone-d_6 and keto acid (7) in $CDCl_3$[a]

	Germicidin H (4)		*Germicidin I (5)*		*Germicidin J (6)*		*7*	
Positions	δ_C, *type*	δ_H (*J in Hz*)	δ_C, *type*	δ_H (*J in Hz*)	δ_C, *type*	δ_H (*J in Hz*)	δ_C, *type*	δ_H (*J in Hz*)
1							179.0, C	
2	166.2, C		165.0, C		165.0, C		33.1, CH_2	2.38, t (6.9)
3	98.1, C		97.6, C		97.5, C		18.6, CH_2	1.90, quint (7.2)
4	166.1, C		164.0, C		164.1, C		41.3, CH_2	2.51, t (7.2)
5	100.5, CH	6.03, s	100.0, CH	6.01, s	99.0, CH	6.01, s	210.8, C	
6	163.4, C		162.0, C		163.0, C		40.9, CH_2	2.40, t (7.7)
7	35.8, CH_2	2.41, t (7.6)	42.1, CH_2	2.31, d (7.2)	32.8, CH_2	2.44, t (7.1)	32.6, CH_2	1.46, m
8	20.9, CH_2	1.64, m	26.6, CH	2.01, m	28.9, CH_2	1.60, quint (7.5)	27.7, CH	1.51, m
9	13.6, CH_3	0.95, t (7.4)	21.5, CH_3	0.95, d (6.6)	21.8, CH_2	1.38, sextet (7.3)	22.3, CH_3	0.88, d (6.4)
10	8.6, CH_3	1.85, s	21.5, CH_3	0.95, d (6.6)	13.1, CH_3	0.93, t (7.4)	22.3, CH_3	0.88, d (6.4)
11			7.7, CH_3	1.86, s	7.7, CH_3	1.85, s		

[a]Assignments were based on COSY, HMBC and HSQC experiments.

(Supplementary Table S1). The absolute configuration at C-7 in **1** and **2** was established as '*S*' based on their specific rotation values, $[\alpha]^{25}_D$ +31 ($c = 0.08$, dimethyl sulfoxide) and $[\alpha]^{25}_D$ +18.5 ($c = 0.46$, dimethyl sulfoxide), respectively, which were in agreement with the published specific rotation values of germicidin A ($[\alpha]_D$ +22 ($c = 0.10$, CH_3OH))[5] and germicidin C ($[\alpha]_D$ +26 ($c = 0.30$, CH_3OH)).[5]

Compound **4** was obtained as a white powder. HR-ESI-MS analysis afforded an $[M+H]^+$ ion at *m/z* 169.0861, establishing the molecular formula of **4** as $C_9H_{12}O_3$ (calculated for $[M+H]^+$ ion at *m/z* 169.0864). The ^{1}H NMR spectrum of **4** showed resonances attributed to an aromatic methine group at δ_H 6.03 (s, H-5), two methyl groups at δ_H 1.85 (s, H_3-10) and δ_H 0.95 (t, $J = 7.4$ Hz, H_3-9), and two methylene groups at δ_H 2.41 (t, $J = 7.6$ Hz, H_2-7) and δ_H 1.64 (m, $J = 7.5$ Hz, H_2-8) (Table 1). The correlations of H_2-7/H_2-8 and H_2-8/H_3-9 in the COSY spectrum of **4** established that the methyl group at δ_H 0.95 and the two methylene groups form a propyl group (Figure 1b). The ^{13}C NMR spectrum of **4** showed four aliphatic carbon resonances, corresponding to the methyl group at δ_H 1.85 and the propyl group, and five other aromatic carbon resonances, representing a typical pattern of the 4-hydroxy-2-pyrone moiety in germicidins A–D.[4–8] In the HMBC spectrum of **4**, the correlations of H_2-8/C-6, H-5/C-3, H-5/C-4, H-5/C-6 and H-5/C-7 established the attachment of the propyl group at C-6 (Figure 1b), and the correlations of H_3-10/C-2 and H_3-10/C-3 established the attachment

of the methyl group at C-3. Taken together, **4** was identified as 4-hydroxy-6-propyl-3-methyl-2-pyrone. While **4** has been prepared recently from acyl-S-*N*-acetylcysteamines by employing a recombinant type I PKS as a biocatalyst,[24] this is the first time for **4** to be isolated as a natural product, and hence named germicidin H.

Compound **5** was obtained as a white powder. HR-ESI-MS analysis afforded an $[M+H]^+$ ion at *m/z* 183.1016, establishing the molecular formula of **5** as $C_{10}H_{14}O_3$ (calculated for $[M+H]^+$ ion at *m/z* 183.1020). The ^{1}H NMR spectrum of **5** resembled that of **4** except that the propyl group in **4** was replaced by an isobutyl group in **5** at δ_H 2.31 (d, $J=7.2$ Hz, H_2-7), δ_H 2.01 (m, H-8) and δ_H 0.95 (d, $J=6.6$ Hz, H_3-9 and H_3-10) (Table 1). This difference between **4** and **5** was confirmed by key correlations in the COSY and HMBC spectra of **5** as summarized in Figure 1b. Therefore, **5** was identified as 4-hydroxy-6-isobutyl-3-methyl-2-pyrone, which was a new compound and named as germicidin I.

Compound **6** was obtained as a white powder. HR-ESI-MS analysis afforded an $[M+H]^+$ ion at *m/z* 183.1017, establishing the molecular formula of **6** as $C_{10}H_{14}O_3$ (calculated for $[M+H]^+$ ion at *m/z* 183.1020). The ^{1}H NMR spectrum of **6** resembled that of **5** except that the isobutyl group in **5** was replaced by a butyl group in **6** at δ_H 2.44 (t, $J=7.1$ Hz, H_2-7), δ_H 1.60 (quintet, $J=7.5$ Hz, H_2-8), δ_H 1.38 (sextet, $J=7.3$ Hz, H_2-9) and δ_H 0.93 (t, $J=7.4$ Hz, H_3-10) (Table 1). This difference was further confirmed upon analysis of key correlations in the COSY and HMBC spectra of **6** as summarized in Figure 1b. Thus, **6** was identified as 4-hydroxy-6-(1-butyl)-3-methyl-2-pyrone. Although **6** was also prepared from acyl-S-*N*-acetylcysteamines upon employing a recombinant type I PKS as a biocatalyst,[25] this is the first time that **6** has been isolated as a natural product, and hence named germicidin J.

Compound **7** was obtained as a colorless oil. HR-ESI-MS analysis afforded an $[M-H]^-$ ion at *m/z* 185.1179, establishing the molecular formula of **7** as $C_{10}H_{18}O_3$ (calculated for $[M-H]^-$ ion at *m/z* 185.1177). The ^{1}H NMR spectrum of **7** showed two coupling systems: (i) one consisted of resonances attributed to three methylene groups at δ_H 2.38 (t, $J=6.9$ Hz, H_2-2), δ_H 1.90 (quintet, $J=7.2$ Hz, H_2-3) and δ_H 2.51 (t, $J=7.2$ Hz, H_2-4) and (ii) the other consisted of resonances attributed to two methylene groups at δ_H 2.40 (t, $J=7.7$ Hz, H_2-6), δ_H 1.46 (m, H_2-7), one methine group at δ_H 1.51 (m, H-8) and two methyl groups at δ_H 0.88 (d, $J=6.4$ Hz, H_3-9 and H_3-10) (Table 1). Key correlations in the COSY and HMBC spectra of **7** established the two coupling systems contributed to one $-CH_2CH_2CH_2-$ moiety and one $(CH_3)_2CHCH_2CH_2-$ moiety (Figure 1b). The ^{13}C NMR spectrum of **7** showed eight aliphatic carbon resonances and two carbonyl carbon resonances (Table 1). The correlations of H_2-2/C-1, H_2-3/C-1, H_2-3/C-5, H_2-4/C-5, H_2-6/C-5 and H_2-7/C-5 in the HMBC spectrum of **7**, in combination with the coupling constants and chemical shifts of resonances in the ^{1}H NMR and ^{13}C NMR spectra, unambiguously established **7** as 8-methyl-5-oxo-nonanoic acid. While **7** has been prepared synthetically,[26] this is the first time that **7** is isolated as a natural product.

The antibacterial activities of compounds **1–7** were evaluated against the Gram-positive strains *S. aureus* ATCC 25923, *Bacillus subtilis* NCTC 2116 and *Mycobacterium smegmatis* ATCC 607, and the Gram-negative strain *Escherichia coli* ATCC 25922, using standard disk diffusion and broth dilution methods.[27] None of the compounds showed any inhibitory activities (up to 64 $\mu g\,l^{-1}$) against the four strains under the conditions tested. These results are consistent with the α-pyrones from the marine-derived *Nocardiopsis* strains[17] but differ from those of the violapyrones, which showed moderate inhibitory activities (4–32 $\mu g\,ml^{-1}$) against *S. aureus* ATCC 25923 and *B. subtilis* ATCC 6633 but no activity against *E. coli*.[8] The violapyrones have longer alkyl chains at C-6 than those of **1–6**, suggesting that a shorter alkyl chain at C-6 may diminish the antibacterial activity (Figure 1 and Supplementary Figure S1). These results revealed important structure–activity relationships for these C-3 and C-6 dialkylated 4-hydroxy-α-pyrone natural products.

Isolation of **1–7** from *S.* sp. CB00361 supports the proposal that Gcs and homologs are an emerging subfamily of type III PKSs in *Streptomyces* that cross talks with fatty acid biosynthesis, prefers β-ketoacyl-ACP intermediates from fatty acid biosynthesis as starter units, and utilizes malonyl-, methylmalonyl-, and ethylmalonyl-CoA as extender units, further expanding the biosynthetic repertoire of polyketide natural products. Given the close taxonomic relationship between *S.* sp. CB00361 and *S. coelicolor* A3(2) (Supplementary Figure S2), it is tempting to speculate that a Gcs homolog in *S.* sp. CB00361 would be responsible for the biosynthesis of **1–6**. Thus, in a mechanistic analogy to Gcs for germicidin biosynthesis in *S. coelicolor* A3(2),[6,21] we propose that the Gcs homolog in *S.* sp. CB00361 catalyzes one cycle of elongation, using varying β-ketoacyl-ACP intermediates from the fatty acid biosynthetic pathway as starter units and methylmalonyl- or ethylmalonyl-CoA as an extender unit, to

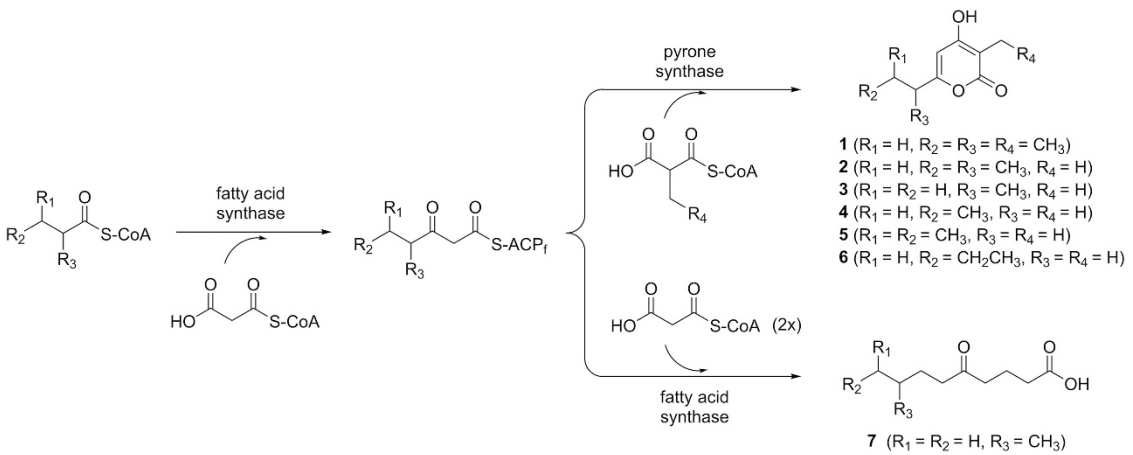

Figure 2 Proposed pathway for germicidin biosynthesis in *S.* sp. CB00361 featuring a pyrone synthase that utilizes acyl-ACP intermediates from fatty acid biosynthesis as starter units and methylmalonyl- and ethylmalonyl-CoA as extender units. ACP, acyl carrier protein.

produce **1–6**, and co-isolation of **7**, a shunt metabolite of fatty acid biosynthesis, featuring the starter unit of **3**, serves as additional evidence supporting the proposed cross talk between germicidin and fatty acid biosynthesis (Figure 2).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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