The Journal of Antibiotics (2019) 72:106–110
https://doi.org/10.1038/s41429-018-0127-y

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BRIEF COMMUNICATION

Geninthiocins C and D from *Streptomyces* as 35-membered macrocyclic thiopeptides with modified tail moiety

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Received: 24 July 2018 / Revised: 1 November 2018 / Accepted: 12 November 2018 / Published online: 27 November 2018

Abstract

Geninthiocin is a thiopeptide with 35-membered macrocyclic core moiety. It has potent anti-Gram-positive (G^+) bacteria activity. Herein, we reported two new congeners (**2**-**3**) of geninthiocin (geninthiocin A, **1**) from *Streptomyces* sp. CPCC 200267, and designated them as geninthiocins C and D, whose structures were determined by NMR. Geninthiocins A, C and D had the same 35-membered macrocyclic core moiety, but possessed a -Dha-Dha-NH_2, -Dha-Ala-NH_2, and -NH_2 tail, respectively. Besides, the Ala residue in geninthiocin C was determined as L- configuration by C_3 Marfey's method. In vitro assays indicated that geninthiocins C-D showed no antibacterial activity, in contrast to the potent anti-G^+ bacteria activity displayed by geninthiocin A. Therefore, the -Dha-Dha-NH_2 tail of geninthiocin A played an important role in its potent activity against G^+ bacteria.

Thiopeptides are a class of peptide antibiotics produced by some bacteria, especially *Streptomyces*. They have potent activity against various bacterial pathogens, and exhibit potential as lead compounds for antimicrobial drug against methicillin-resistant *Staphylococcus aureus* and penicillin-resistant *Streptococcus pneumoniae*, etc [1, 2]. Natural thiopeptides such as thiostrepton and nosiheptide are veterinary antibiotic and animal feed additive, and semi-synthetic thiopeptide LFF571 is under clinical trial against *Clostridium difficile* infection in humans [3].

Thiopeptides are structurally comprised of a macrocyclic core moiety and a tail moiety. They are divided into four families according to the size of macrocyclic core: 26-membered, such as thiocillin, thiostrepton, and nosiheptide; 29-membered, such as GE37468, amythiamicin, and GE2270A; 32-membered, such as lactazoles; and 35-membered, such as berninamycin, TP-1161, and geninthiocin [4–6]. Most thiopeptides have in their tails modified amino acid residues such as thiazole, oxazole, oxazoline, and dehydroalanine (Dha), and a terminal amine group.

Thiopeptides are a growing class of antibiotics with various biological activities [2, 7–12]. Among them, geninthiocin (hereafter designated as geninthiocin A, Fig. 1) is a 35-membered thiopeptide discovered from *Streptomyces* sp. DD84 with *tipA* promoter inducing activity and potent antibacterial activity, and val-geninthiocin (hereafter designated as geninthiocin B, Fig. 1) is a close analog of geninthiocin A identified from *Streptomyces* sp. RSF18 with antibacterial activity comparable to that of geninthiocin A [13].

In the course of exploring secondary metabolites from a soil isolate *Streptomyces* sp. CPCC 200267 with strong anti-G^+ bacteria activity, we identified two new congeners of geninthiocin A. Herein, structures of the two congeners and their antibacterial activities were described, which revealed a preliminary structure-activity relationship (SAR) concerning the tail moiety and antibacterial activity of 35-membered thiopeptides.

The agar culture of *Streptomyces* sp. CPCC 200267 was extracted with ethyl acetate (EtOAc). The EtOAc extract revealed three peaks with similar UV absorption profiles by reversed-phase HPLC (27.4, 27.0, and 25.9 min; Fig. S1),

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41429-018-0127-y) contains supplementary material, which is available to authorized users.

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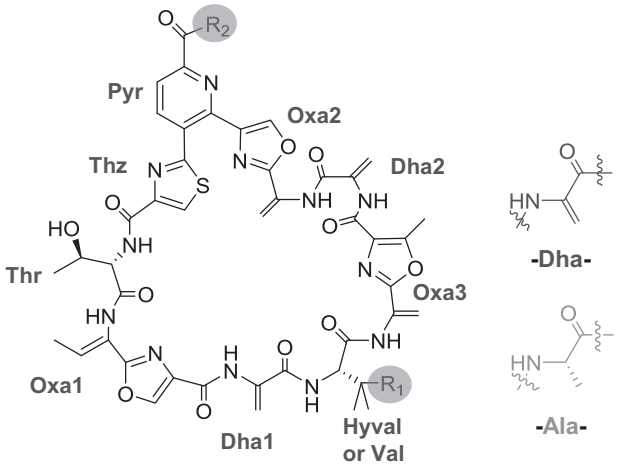
which corresponded to three structure-related compounds, **1** (major component), **2** and **3** (minor components), respectively. They were purified by a procedure of EtOAc extraction, silica gel and ODS column chromatography and semi-preparative HPLC (Supplementary material), and then identified by NMR as geninthiocin A (**1**) and its two new congeners (**2**-**3**).

Compound **1** was obtained as white amorphous powder. Its molecular formula was determined as $C_{50}H_{49}N_{15}O_{15}S$ by HR-ESIMS (Fig. S4). The 1D and 2D NMR data of **1** (Fig. S7-S12) were highly consistent with those of geninthiocin A reported in the literature [6], which indicated that **1** was geninthiocin A. The NMR data of **1** were assigned completely as indicated in Table S1.



geninthiocin A (**1**): R_1= OH R_2= Dha3-Dha4-NH_2
geninthiocin B : R_1= H R_2= Dha3-Dha4-NH_2
geninthiocin C (**2**): R_1=OH R_2= Dha3-Ala-NH_2
geninthiocin D (**3**): R_1=OH R_2= NH_2

Fig. 1 Chemical structures of geninthiocins A-D. Pyr (pyridine); Thz (thiazole); Thr (threonine); Oxa (oxazole); Dha (dehydroalanine); Hyval (hydroxyvaline); Val (valine); Ala (alanine)

Compound **2** was obtained as white amorphous powder. Its molecular formula was determined by HR-ESIMS as $C_{50}H_{51}N_{15}O_{15}S$ (Fig. S15), two hydrogen atoms more than **1**. Compound **2** showed very similar NMR data to **1** except the appearance of new signals for a methyl group (δ_H 1.32 (d, $J = 7.2$ Hz), δ_C 18.0) and a sp^3-hybridized methine (δ_H 4.34 (q, $J = 7.2$ Hz), δ_C 49.3), and the loss of signals for a terminal methylidene (δ_H 6.04 (s) and 5.70 (s), δ_C 106.4; δ_C 135.4) in **1**. The COSY correlations of Ala-NH (δ_H 8.62)/Ala-Hα (δ_H 4.34)/Ala-H$_3\beta$ (δ_H 1.32), and HMBC correlations from the terminal -NH_2 (δ_H 7.39, 7.02) to Ala-C = O (δ_C 174.2) and Ala-Cα (δ_C 49.3) further revealed that an Ala residue had replaced the Dha4 residue in **1** (Fig. 2). All the other structural parts of **2** were the same as **1** deduced from their highly similar 1D and 2D NMR data (Fig. S18-S24). Therefore, **2** was determined as an analog of **1** with an Ala residue taking the place of Dha4 residue in **1**. The MS^2 spectra comparison between **1** and **2** further supported the amino acid replacement (Fig. S3, S14). The stereochemistry of **2** was established by C_3 Marfey's method and ROESY spectrum. The configuration of Ala, Thr, and Hyval in **2** were determined to be L- by C_3 Marfey's method (Fig. S36) [14]. The vinyl methyl group in Oxa1 was determined as Z-form based on ROESY correlation between the methyl proton (δ_H 1.76) and the amide proton (δ_H 9.63) in Oxa1 (Fig. 2). Compound **2** was designated by us as geninthiocin C. Its NMR data were assigned completely as indicated in Table 1.

Compound **3** was obtained as white amorphous powder. Its molecular formula was determined by HR-ESIMS as $C_{44}H_{43}N_{13}O_{13}S$ (Fig. S26), which was $C_6H_6N_2O_2$

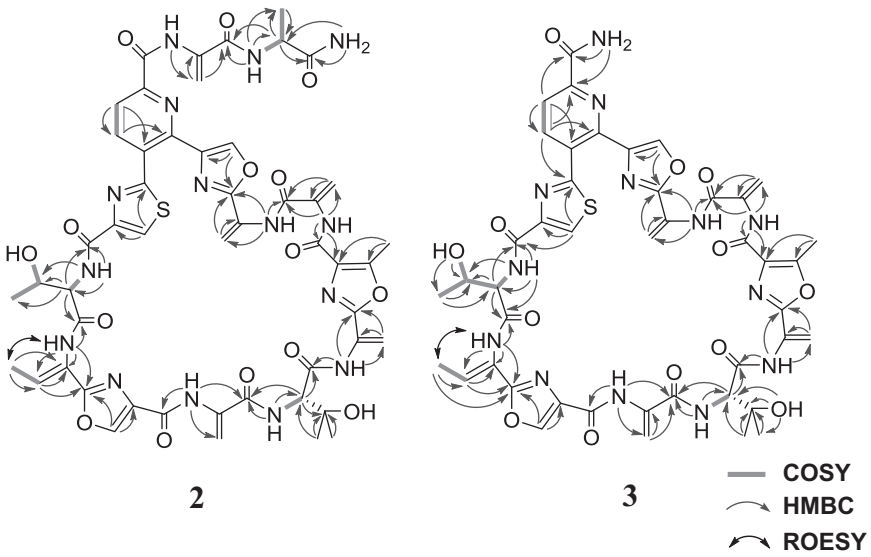


Fig. 2 Key COSY, HMBC, and ROESY correlations of **2** and **3**

Table 1 NMR data of geninthiocins C (**2**) and D (**3**)

Unit	Position	2		3	
		δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
Thz	C-2	163.4		164.1	
	C-4	149.7		149.5	
	CH-5	127.2	8.50	126.8	8.50
	CO	160.2		160.3	
Thr	NH		8.02 (d, 9.0)		7.96 (d, 9.0)
	αCH	58.1	4.61 (dd, 9.0, 3.0)	57.9	4.61 (dd, 9.0, 3.0)
	βCH	67.6	4.29 (m)	67.6	4.33 (m)
	γCH_3	20.8	1.15 (d, 6.0)	20.8	1.15 (d, 6.6)
	OH		5.01 (d, 4.2)		4.93 (d, 5.4)
	CO	169.1		169.1	
Oxa1	NH		9.63		9.59
	αC	123.4		123.3	
	βCH	129.9	6.56 (q, 7.2)	129.8	6.55 (q, 7.2)
	γCH_3	14.1	1.76 (d, 6.6)	14.1	1.75 (d, 7.2)
	C-2	159.7		159.6	
	C-4	136.3		136.3	
	CH-5	143.0	8.71	143.0	8.72
	CO	158.7		158.6	
Dha1	NH		9.40		9.45
	αC	133.7		133.7	
	βCH_2	104.0	6.46, 5.89	104.0	6.47, 5.91
	CO	164.0		163.9	
Hyval	NH		8.24 (d, 8.4)		8.26 (d, 8.4)
	αCH	62.1	4.64 (d, 8.4)	62.0	4.69 (d, 8.4)
	βC	71.3		71.4	
	γCH_3	27.6	1.23	27.7	1.23
	γCH_3	26.5	1.22	26.3	1.21
	OH		5.18		5.17
	CO	169.6		169.7	
Oxa3	NH		9.63		9.53
	αC	128.8		128.7	
	βCH_2	106.0	6.12, 5.67	105.5	6.20, 5.66
	C-2	155.5		155.3	
	C-4	129.5		129.4	
	C-5	154.8		155.0	
	CH_3-5	11.9	2.62	11.9	2.64
	CO	159.8		159.8	
Dha2	NH		9.37		9.35
	αC	134.1		134.4	
	βCH_2	106.1	6.38, 5.78	106.4	6.33, 5.70
	CO	163.0		163.0	
Oxa2	NH		9.82		9.52
	αC	129.6		129.4	
	βCH_2	111.7	5.72, 5.72	110.6	5.65, 5.74
	C-2	158.5		157.9	
	C-4	139.4		140.2	
	CH-5	140.8	8.60	141.9	9.05
Pyr	C-2	147.1		147.0	
	C-3	130.6		128.9	
	CH-4	141.2	8.53 (d, 7.8)	141.1	8.32 (d, 7.8)
	CH-5	121.7	8.25 (d, 8.4)	121.0	8.11 (d, 8.4)
	C-6	149.7		150.9	
	CO	161.5		165.3	
Dha3	NH		10.61		
	αC	134.1			
	βCH_2	104.1	6.50, 5.92		
	CO	163.2			
Ala	NH		8.62 (d, 7.2)		
	αCH	49.3	4.34 (q, 7.2)		
	βCH_3	18.0	1.32 (d, 7.2)		
	CO	174.2			
	NH_2		7.39, 7.02		8.41, 7.90

(equivalent to Dha-Dha residues) less than geninthiocin A. Compound **3** also showed very similar NMR data to **1** except the loss of signals for two Dha residues. It had an identical 35-membered macrocylic core moiety to **1** because of its highly similar 1D and 2D NMR data to **1** (Fig. S29-S35). The HMBC correlations from the terminal -NH_2 (δ_H 7.90, 8.41) to the carbonyl carbon (δ_C 165.3) and C-6 (δ_C 150.9) of pyridine-2-carboxylate proved that **3** lost Dha-Dha residues in its tail (Fig. 2). Therefore, **3** was determined as another analog of **1** without Dha-Dha residues in its tail. The ROESY correlation between the methyl proton (δ_H 1.75) and the amide proton (δ_H 9.59) in Oxa1 of **3** was observed, indicating that the vinyl methyl group in Oxa1 was in Z-form (Fig. 2). Both Thr and Hyval residues in **3** must take the L-configuration (the same as **2**) because **3** shared the same prepeptide with **2** in biosynthesis (Fig. 3). Compound **3** was designated by us as geninthiocin D (Fig. 1). Its NMR data were assigned completely as indicated in Table 1.

We proposed a possible production relationship of geninthiocins A, C, and D (**1**–**3**) based on the biosynthetic mechanism of geninthiocin A and the structural differences of them (Fig. 3). Besides, we eliminated the very small chance that geninthiocin C (**2**) may come from a specific mutant carrying a base change in the structural gene encoding the core peptide for geninthiocin biosynthesis (leading to the translation of Ala instead of Ser in geninthiocin biosynthesis), by choosing randomly well separated single colonies of *Streptomyces* sp. CPCC 200267 for

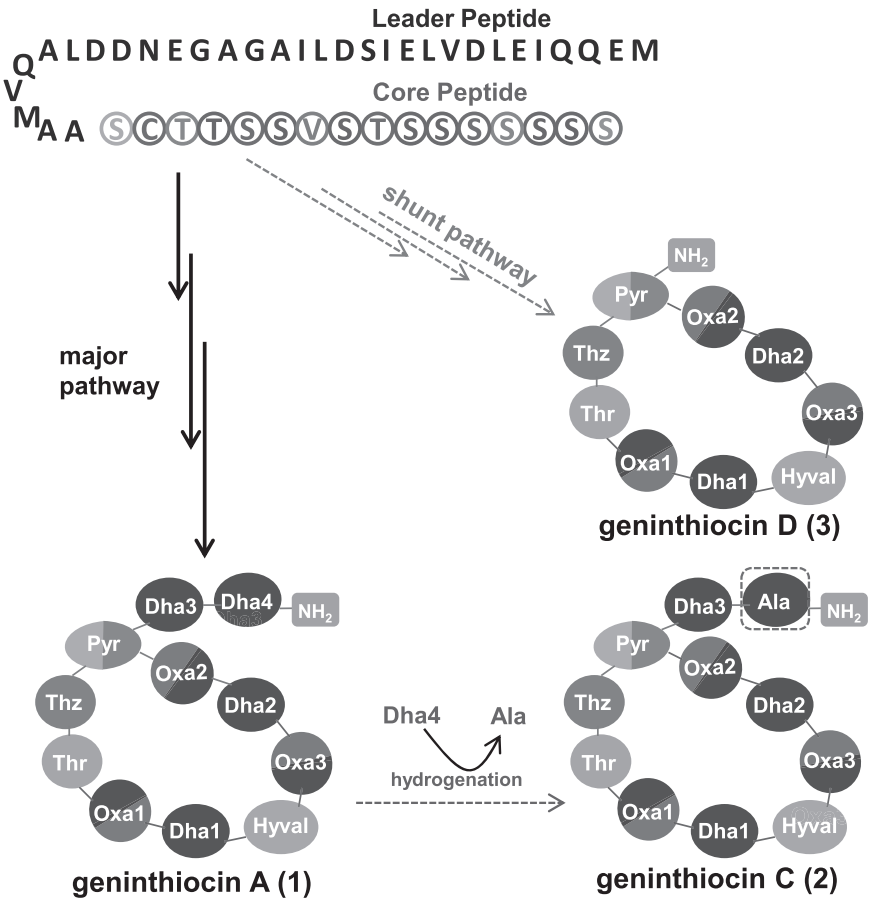


Fig. 3 The proposed biogenesis of geninthiocins A, C, and D (**1**-**3**)

culture and then analysis of geninthiocin(s) they produced. All the ten single colonies produced the same profile of geninthiocins as the original *Streptomyces* sp. CPCC 200267 did (Fig. S37), i.e. geninthiocins A (**1**) as major component and C-D (**2**-**3**) as minor components. Thus, we demonstrated that geninthiocin C (**2**) was not the metabolite from a specific mutant of *Streptomyces* sp. CPCC 200267. It was a congener in geninthiocin A (**1**) production, and should be the product of an unidentified enzyme-catalyzed hydrogenation of geninthiocin A (**1**) at Dha4 residue, which generated the only L-Ala residue in geninthiocin C (**2**, Fig. 3). We believe that geninthiocin D (**3**) is a shunt product in geninthiocin A (**1**) biosynthesis. It may come from the incorrect removing of two more Ser residues from *C*-terminal of the core peptide in geninthiocin A (**1**) biosynthsis (Fig. 3).

Geninthiocins A, C, and D (**1**–**3**) differed only at tail moiety of their structures, which provided us the opportunity to explore tail variations on their antibacterial activity. In vitro MIC assay revealed that geninthiocins C and D (**2**–**3**) lost activities against G^+ bacteria (Table S2) [15, 16]. Thus, the -Dha-Dha-NH_2 tail of geninthiocin A (**1**) was essential for its potent anti-G^+ bacteria activity.

The structure-activity relationship (SAR) of thiopeptides is very complex. While the macrocyclic core moiety is essential for the anti-G^+ bacteria activity, the tail moiety is also indispensible for some thiopeptides (Table S3 for a brief summary concerning the SAR of Dha-containing tail moiety and anti-G^+ bacteria activity of some thiopeptides). For example, tail modification of 26-membered thiopeptides such as siomycin, nocathiacin, and thiostrepton resulted in various effects on anti-G^+ bacteria activity (nearly no changes, decreases or increases) [17–20], and tail modification of 29-membered thiopeptides such as thiomuracin A and baringolin exerted little influences on anti-G^+ bacteria activity [8, 9]. Our study of geninthiocins provided the first example for 35-membered thiopeptides concerning the SAR of tail moiety and anti-G^+ bacteria activity. It may also provide some references for predicting the SAR of berninamycins [4], another 35-membered thiopeptide with very similar structure to geninthiocins.

Geninthiocin A (**1**): white amorphous powder; UV (MeOH) λ_{max} (log ε) 240 (4.78) and 324 (3.79) nm; IR (KBr) ν_{max} 3344.5, 2916.1, 1763.2, 1665.4, 1503.3, 1201.7, 1106.3, 995.6, and 890.1 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz) data, see Table S1. ESIMS *m/z* 1132.46 $[M + H]^+$, 1154.57 [M +

Na]$^+$; (+) HRESIMS *m/z* 1132.3354 $[M + H]^+$ (calcd for $C_{50}H_{50}N_{15}O_{15}S$, 1132.3326, ppm = 2.5).

Geninthiocin C (**2**): white amorphous powder; UV (MeOH) λ_{max} (log ε) 238 (4.83) and 324 (3.90) nm; IR (KBr) ν_{max} 3342.8, 2921.0, 1765.9, 1660.8, 1503.6, 1202.4, 1105.7, 1025.6, and 889.1 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz) data, see Table 1. ESIMS *m/z* 1134.51 $[M + H]^+$, 1156.58 $[M + Na]^+$; (+) HRESIMS *m/z* 1134.3490 $[M + H]^+$ (calcd for $C_{50}H_{52}N_{15}O_{15}S$, 1134.3483, ppm = 0.7).

Geninthiocin D (**3**): white amorphous powder; UV (MeOH) λ_{max} (log ε) 239 (4.89) nm; IR (KBr) ν_{max} 3338.7, 2975.8, 1765.2, 1668.9, 1503.9, 1387.8, 1202.3, 1100.9, and 887.8 cm^{-1}; ^1H NMR (DMSO-d_6, 600 MHz) and ^{13}C NMR (DMSO-d_6, 150 MHz) data, see Table 1. ESIMS *m/z* 994.31 $[M + H]^+$, 1016.42 $[M + Na]^+$; (+) HRESIMS *m/z* 994.2906 $[M + H]^+$ (calcd for $C_{44}H_{44}N_{13}O_{13}S$, 994.2897, ppm = 1.0).

Acknowledgements We gratefully acknowledge Nuclear Magnetic Resonance Center (Institute of Materia Medica, CAMS & PUMC) and Analytical Center of Drugs (Institute of Medicinal Biotechnology, CAMS & PUMC) for the NMR and MS analyses. This work was supported by CAMS Initiative for Innovative Medicine (CAMS-I2M-3-012) and National Infrastructure of Microbial Resources (No. NIMR-2018-3).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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