The Journal of Antibiotics (2015) 68, 707–710
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www.nature.com/ja

NOTE

Avellanin C, an inhibitor of quorum-sensing signaling in *Staphylococcus aureus*, from *Hamigera ingelheimensis*

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The Journal of Antibiotics (2015) **68,** 707–710; doi:10.1038/ja.2015.50; published online 6 May 2015

Hamigera is one of the least studied genera of Eurotiales in terms of secondary metabolism compared with metabolically prolific genera such as *Penicillium*, *Aspergillus*, *Paecilomyces*, *Monascus* and *Talaromyces*.[1] Although thousands of metabolites are known from *Aspergillus* and *Penicillium*,[2] only 20–30 compounds have been reported from *Hamigera*.[3] In our previous assessment of chemodiversity in representative strains of *Hamigera*, cyclic peptides known as avellanins and PF1171 were found to be widely produced by a range of this species.[4] Meanwhile, one of the studied strains of *H. ingelheimensis* was observed to be producing an unknown congener, designated avellanin C (**1**) instead of the known cyclic peptides (Figure 1).[4] In this paper, we describe the isolation and structure determination of **1** along with its quorum-sensing inhibitory activity.

H. ingelheimensis NRRL 29060 was cultured in A-3M liquid medium for 6 days on a rotary shaker and the whole culture broth was extracted with 1-butanol. After evaporation, the crude extract was subsequently subjected to silica gel and octadecylsilyl (ODS) column chromatography, followed by HPLC purification to yield avellanin C (**1**).

Avellanin C (**1**) was obtained as a white amorphous solid. HR-ESITOFMS analysis gave a pseudomolecular ion $[M+Na]^+$ at *m/z* 618.2681 corresponding to the molecular formula of $C_{34}H_{37}N_5O_5$ (Δ −0.6 mmu, for $C_{34}H_{37}N_5O_5Na$), which was consistent with the NMR data. ^{13}C NMR and HSQC spectral analyses revealed the presence of 34 carbons assignable to five carbonyl carbons, four sp^2 quaternary carbons, 10 sp^2 methines, four sp^3 methines, five sp^3 methylenes and two methyls. The ^1H NMR spectrum displayed resonances for the aromatic protons, four methine protons ascribed to the α-protons of amino acids and three NH protons. These spectral features suggested the peptidic structure of **1** and the presence of aromatic groups within.

Further analysis of two-dimensional NMR data led to the identification of five amino-acid components (Figure 2, Table 1). Alanine (Ala) was confirmed by COSY correlations for NH (δ_H 7.23)/CH (δ_H 4.69)/CH_3 (δ_H 1.43) and an HMBC correlation from the methyl protons to a carbonyl carbon (δ_C 171.8). The other aliphatic amino acid was proline (Pro) that was deduced from sequential COSY correlations for CH (δ_H 4.68)/CH_2 (δ_H 1.97/1.43)/CH_2 (δ_H 2.09/1.86)/CH_2 (δ_H 3.61/3.56) along with long-range correlations from the α-methine proton to the δ-methylene (δ_C 51.3) and a carbonyl carbon (δ_C 173.7). In addition, a spin system comprising four contiguous aromatic protons (δ_H 7.32, 7.10, 7.46 and 8.43) was recognized in the COSY spectrum and was extended to a 1,2-disubstituted benzene ring by HMBC correlations from these protons to the quaternary carbons at δ_C 123.4 and 136.5. Correlations were also detected from the proton at δ_H 7.32 to a carbonyl carbon (δ_C 169.5) and from an NH proton (δ_H 9.86, s) to the aromatic carbons (Ant-2 and Ant-6), indicating an anthranilic acid unit (Ant). The remaining aromatic protons and carbons could be assigned to two phenyl groups by considering the molecular formula and NMR data. Each phenyl ring was linked to a methylene carbon (Phe-C3: δ_C 37.0; *N*MePhe-C3: δ_C 32.9) by long-range correlations from the methylene protons to the aromatic carbons and from the aromatic protons to the methylene carbons (Table 1). The methylenes were, respectively, COSY correlated to a methine (δ_C 54.5 and 57.4), which was further connected to a carbonyl carbon by HMBC correlations from the methylene and methine protons to the downfield carbons at δ_C 169.4 (Phe-C1) and 169.1 (*N*MePhe-C1). One of the methines was further COSY correlated to an NH proton (δ_H 6.49, d) to provide a phenylalanine residue (Phe), whereas another methine was long-range coupled to a nitrogen-bonded methyl protons (δ_H 2.97) establishing an *N*-methylphenylalanine residue (*N*MePhe). Connectivity among the five amino-acid components was determined by HMBC correlations from Phe-NH and Phe-H2 to Ala-C1, Ant-NH to Phe-C1, Pro-H5 to Ant-C1, *N*-Me to Pro-C1, and Ala-NH to *N*MePhe-C1, completing the cyclic peptide structure for **1** (Figure 2).

The absolute configuration of **1** was determined by applying Marfey's method.[5] The acid hydrolysate of **1** was derivatized with L-FDLA (1-fluoro-2,4-dinitrophenyl-5-L-leucinamide) and the HPLC retention times were compared with L-FDLA derivatives of standard

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Received 13 February 2015; revised 28 March 2015; accepted 2 April 2015; published online 6 May 2015

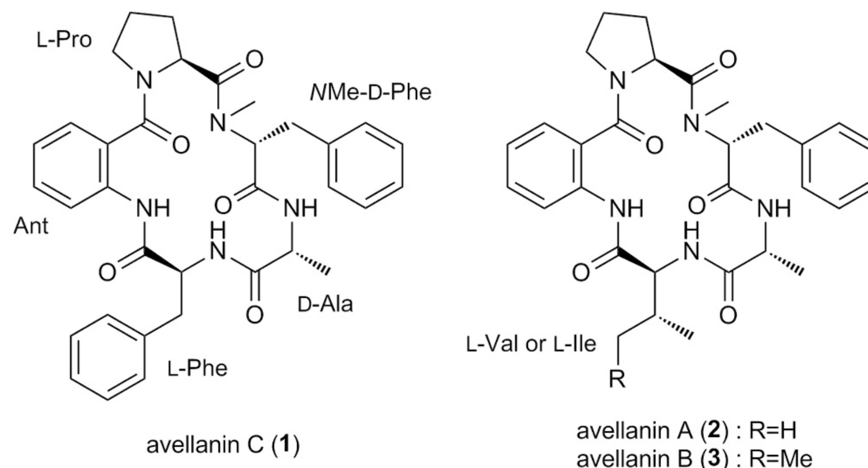

Figure 1 Structures of avellanins.

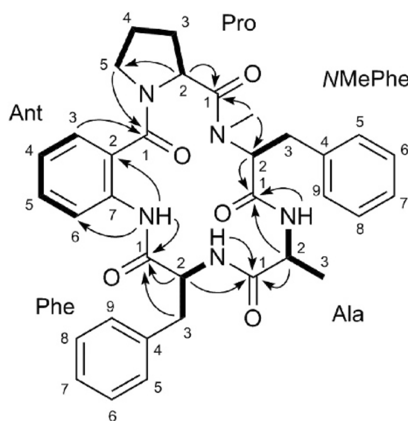

Figure 2 ^{1}H-^{1}H COSY and key HMBC correlations for **1**.

amino acids (Supplementary Figure S6). The acid hydrolysate of **1** contained D-Ala, L-Pro, L-Phe and *N*-Me-D-Phe. Therefore, the absolute configuration of **1** was established as depicted in Figure 1.

Avellanin C (**1**) was inactive in antimicrobial or cytotoxic assay but displayed inhibitory effects on the quorum-sensing signaling in *Staphylococcus aureus*. The expression of virulence factors in *S. aureus* is controlled by the accessory gene regulator (*agr*).[6] The *agr* expression is in turn triggered by high concentrations of autoinducing peptide pheromone that is self-produced by *S. aureus* to recognize its own cell density in a specific microenvironment.[7] Disturbance of quorum sensing by small molecules is currently attracting attention as a new strategy for the treatment of staphylococci infections.[8,9] Inhibition of *agr* signaling pathway by **1** was assessed by measuring the luminescence intensity from the *S. aureus* transformant that carried a plasmid-encoding luciferase gene under *agr*P3 promoter.[10] Luminescence emission from the reporter strain was decreased by the treatment with **1** dose dependently at 0.5–200 μM with an IC_{50} value of 4.4 μM (Figure 3). The growth of this strain was unaffected by **1** within the same concentration range (data not shown).

EXPERIMENTAL PROCEDURE

General procedure

Optical rotation was measured using a JASCO DIP-3000 polarimeter (JASCO Corporation, Tokyo, Japan). UV spectrum was recorded on a Hitachi U-3210 spectrophotometer (Hitachi, Ltd., Tokyo, Japan). IR spectrum was measured on a PerkinElmer Spectrum 100 (Perkin-Elmer Inc., Fremont, CA, USA). NMR spectra were obtained on a Bruker AVANCE 500 spectrometer (Bruker BioSpin K. K., Kanagawa, Japan) in $CDCl_3$ using the signals of the residual solvent protons (δ_H 7.27) and carbons (δ_C 77.0) as an internal standard. HRESITOFMS was recorded on a Bruker microTOF focus. LC-MS data were analyzed on an Agilent HP1200 system (Agilent Technologies Japan Ltd., Tokyo, Japan) and the Bruker microTOF focus.

Fermentation

Hamigera ingelheimensis NRRL29060 was obtained from Agricultural Research Service, United States Department of Agriculture, Peoria, Illinois, USA. Strain NRRL29060 was grown on a potato dextrose (PDA) agar plate and, subsequently, inoculated into 500-ml K-1 flasks each containing 100 ml of the V-22 seed medium consisting of soluble starch 1%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, tryptone (Difco Laboratories, Detroit, MI, USA) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3% (pH 7.0). The flasks were placed on a rotary shaker (200 r.p.m.) at 30 °C for 2 days. The seed culture (3 ml) was transferred into 500-ml K-1 flasks each containing 100 ml of the A-3M production medium consisting of glucose 0.5%, glycerol 2%, soluble starch 2%, Pharmamedia (Traders Protein, Memphis, TN, USA) 1.5%, yeast extract 0.3% and Diaion HP-20 (Mitsubishi Chemical Co., Kanagawa, Japan) 1%. The pH of the medium was adjusted to 7.0 before sterilization. The inoculated flasks were placed on a rotary shaker (200 r.p.m.) at 30 °C for 6 days.

Extraction and isolation

At the end of the fermentation period, 50 ml of 1-butanol was added to each flask, and the mixture was shaken for 1 h. The mixture was centrifuged at 6000 r.p.m. for 10 min and the organic layer was separated from the aqueous layer containing the mycelium. Evaporation of the solvent gave 2.44 g of crude extract from a 2 l culture. The extract was subjected to silica gel column chromatography with a step gradient of $CHCl_3$-MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1 and 0:1 v/v). Fraction 3 (10:1) was concentrated to provide 0.84 g of brown solid, which was then fractionated by reversed phase ODS column chromatography with a gradient of MeCN/0.1% HCO_2H (2:8, 3:7, 4:6, 5:5, 6:4, 7:3 and 8:2 v/v). Fractions 6 (7:3) was concentrated and extracted with EtOAc. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to give a pale yellow solid (160 mg). A portion (40 mg) of this material was purified by preparative HPLC (Cosmosil 5PE-MS, 10 × 250 mm, 4 ml min^{-1}, UV detection at 254 nm) with MeCN/0.1% HCO_2H (53:47), followed by evaporation and extraction with EtOAc, to give avellanin C (**1**, 19 mg, t_R 16.5 min).

Avellanin C (**1**): colorless amorphous; $[\alpha]^{25}_D$ +78 (*c* 0.40, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.57), 253 (4.12), 296 (3.45); IR (ATR) ν_{max} 3327, 2930, 1669, 1639, 1586 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESITOFMS $[M+Na]^+$ 618.2681 (calcd for $C_{34}H_{37}N_5O_5Na$, 618.2687).

Table 1 ^{1}H and ^{13}C NMR data for avellanin C (1) in $CDCl_3$.

Residue	*Position*	δ_C[a]	δ_H *mult (J in Hz)*[b]	*HMBC*[b,c]
Ala	1	171.8, qC		
	2	48.3, CH	4.69[d], m	Ala-1, 3, *N*MePhe-1
	3	18.0, CH_3	1.43, d (7.0)	Ala-1, 2
	NH		7.23[d]	*N*MePhe-1
Phe	1	169.4, qC		
	2	54.5, CH	4.95, ddd (9.0, 9.0, 4.5)	Phe-1, Phe-3, Phe-4, Ala-1
	3	37.0, CH_2	3.39, dd (14.5, 4.5)	Phe-1, 2, 4, 5
			3.07, dd (14.5, 9.0)	Phe-1, Phe-2, Phe-4, Phe-5
	4	136.4, qC		
	5, 9	129.1, CH	7.20[d]	Phe-3, 7
	6, 8	128.6[e], CH	7.30–7.31[d]	Phe-4, 8
	7	126.8[e], CH	7.24[d]	
	NH		6.49, d (9.0)	Phe-2, 3, Ala-1
Ant	1	169.5, qC		
	2	123.4, qC		
	3	127.3, CH	7.32[d]	Ant-1, 5, 7
	4	123.1, CH	7.10, ddd (8.0, 8.0, 1.0)	Ant-2, 6
	5	131.8, CH	7.46, ddd (8.0, 8.0, 1.0)	Ant-3, 7
	6	121.7, CH	8.43, d (8.0)	Ant-1, 2, 4
	7	136.5, qC		
	NH		9.86, s	Ant-2, 6, Phe-1
Pro	1	173.7, qC		
	2	56.3, CH	4.68[d], m	Pro-1, 3, 4, 5
	3	28.5, CH_2	1.97, m	Pro-1, 2, 4
			1.43[d], m	Pro-1, 4
	4	24.9, CH_2	2.09, m	Pro-3, 5
			1.86, m	Pro-3
	5	51.3, CH_2	3.61, ddd (10.5, 10.5, 6.0)	Pro-3, 4
			3.56, m	Pro-2, 3, 4, Ant-1
*N*MePhe	1	169.1, qC		
	2	57.4, CH	5.62, dd (12.5, 4.7)	*N*MePhe-1, 3, 4, NMe, Pro-1
	3	32.9, CH_2	3.80, dd (15.7, 4.7)	*N*MePhe-1, 2, 4, 5
			2.94, dd (15.7, 12.5)	*N*MePhe-1, 2, 4, 5
	4	137.2, qC		
	5, 9	128.2, CH	7.21[d]	*N*MePhe-3, 7
	6, 8	128.8[e], qC	7.30-7.31[d]	*N*MePhe-4, 8
	7	127.1[e], CH	7.24[d]	
	NMe	31.0, CH_3	2.97, s	*N*MePhe-1, Pro-1

[a]Recorded at 125 MHz, referenced to the residual solvent signal.
[b]Recorded at 500 MHz, referenced to the residual solvent signal.
[c]HMBC correlations are from proton(s) stated to the indicated carbon.
[d]Overlapping signals.
[e]Assignment interchangeable.

Acid hydrolysis of 1 and FDLA derivatization

A portion of **1** (0.5 mg) was hydrolyzed at 110 °C in 6 M HCl (200 µl) for 16 h and the reaction mixture was evaporated to dryness. A 0.1 M $NaHCO_3$ solution (100 µl) was added to the dried hydrolysate of **1**, as well as to standards of L-alanine (Ala), L-proline (Pro), L-phenylalanine (Phe) and *N*-Me-L-Phe (*N*MePhe).[11] A solution of L-FDLA and D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucinamide) in acetone (0.05 mg in 50 µl) was added to each reaction vial. Each vial was sealed and incubated at 55 °C for 2 h. To quench the reactions, 2 M HCl (50 µl) was added and then diluted with MeCN/0.2% HCO_2H (100 µl, 50:50).

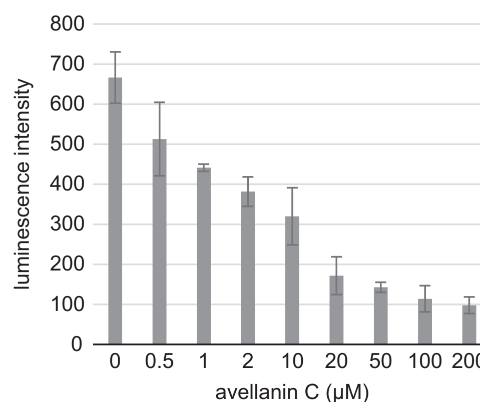

Figure 3 Inhibition of *agr* system in *Staphylococcus aureus* by **1**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

LC-MS analysis of Marfey's derivatives

FDLA derivatives of the hydrolysate and standards were analyzed by LC-MS using a Cosmosil AR-II C_{18} column (Nacalai Tesque Inc., Kyoto, Japan, 2.0 × 150 mm) eluted with MeCN-0.1% HCO_2H at a flow rate of 0.2 ml min^{-1}, monitoring by extracted ions under the negative ion mode with a gradient program (MeCN %: 25% for 0–4 min, 25–55% for 4–19 min, 55–100% for 19–25%, 100% for 25–33 min, 100–25% for 33–35 min). Retention times for the FDLA-derivatived amino-acid standards were 20.2 min for L-Ala and 22.7 min for D-Ala, 20.2 min for L-Pro and 20.2 min for D-Pro, 24.6 for L-Phe and 27.0 for D-Phe, and 25.3 for *N*-Me-L-Phe and 25.8 for *N*-Me-D-Phe. Acid hydrolysate of **1** contained D-Ala (22.6 min), L-Pro (20.1 min), L-Phe (24.5 min) and *N*-Me-D-Phe (25.9 min).

Biological assay

The *agr* inhibition assay was performed as described previously.[12] The strain used was *S. aureus agr* reporter strain, 8325-4, which carries plasmid pSB2035 encoding the *Photorhabdus luminescens* luciferase gene and *gfp* under control of the *agr*P3 promoter. An overnight culture of *S. aureus* 8325-4 (pSB2035) in Luria-Hewitt broth (LB) (10 g of tryptone, 5 g of yeast extract and 10 g of NaCl per liter) supplemented with 7 µg ml^{-1} of chloramphenicol at 30 °C with shaking was diluted in fresh LB broth (1:100) and 200 µl was dispensed into each well of a 96-well microplate (96 Well Round Bottom; Sigma-Aldrich, St Louis, MO, USA). Samples to be tested for quorum-sensing (QS) inhibition were dispensed into wells of microplate and the solvent in the sample was evaporated in a SpeedVac concentrator (Thermo Fisher Scientific Inc., Waltham, MA, USA) before adding the culture. *S. aureus* 8325-4 (pSB2035) and *S. aureus* 12600 were cultured in the same way but with solvent only as a control. The microplate was incubated at 37 °C with shaking, whereas the cell growth was monitored at OD_{620} every hour in the plate reader (Infinite F200 pro, Tecan Japan, Co. Ltd., Kanagawa, Japan). After 5 h, the OD_{620} was measured. For the measurement of luminescence, the culture was transferred into each well of new microplate (96F Black MicroWell, Thermo Fisher Scientific) and luminescence was measured by the plate reader.

ACKNOWLEDGEMENTS

This research was supported in part by Grants-in-Aid for Scientific Research (B) No. 24380050 from the Japan Society for the Promotion of Science to JN.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)