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NOTES

Glomecidin, a Novel Antifungal Cyclic Tetrapeptide Produced by *Streptomyces lavendulae* H698 SY2

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In the course of our screening program for antifungal substances, we have found a novel cyclic tetrapeptide antibiotic named glomecidin (**1**, Fig. 1) in the fermentation broth of *Streptomyces* sp. H698 SY2. The strain was isolated from a soil sample collected in Kochi, Japan and was later classified to belong to *Streptomyces lavendulae* on the basis of taxonomic characterizations.[1,2] In this paper, we report the production, isolation, structure elucidation and biological activities of glomecidin (**1**).

A slant culture of the producing organism was inoculated into 500-ml Sakaguchi flasks each containing 100 ml of a seed medium consisting of soluble starch 0.5%, glucose 0.25%, maltose 0.25% and yeast extract 0.2% (pH 7.0 before sterilization). After incubating on a reciprocal shaker at 27°C for 2 days, 2 ml of this seed culture was transferred into each of 500-ml Sakaguchi flasks containing 100-ml of a production medium composed of soluble starch 1%, glucose 1%, soybean meal 2%, yeast extract 0.5%, NaCl 0.25% and $CaCO_3$ 0.3% (pH 7.0 before sterilization). The fermentation was carried out at 27°C for 4 days on a reciprocal shaker. The progress of the fermentation was monitored by bioactivity testing against a plant pathogenic fungus *Glomerella cingulata*. The same bioactivity test directed the following isolation and purification procedure.

The broth filtrate (4 liters) obtained from the culture broth by centrifugation and filtration was adsorbed on Amberlite XAD-2 resin, which was washed with water and eluted with 80% MeOH. The eluate was concentrated and the resultant aqueous solution was adjusted at pH 8.0 and extracted three times with *n*-BuOH. The *n*-BuOH extract was concentrated *in vacuo* and lyophilized. The residue was dissolved in 0.05 M AcOH-AcONa buffer (pH 4.0) and loaded on a SP-Sephadex C-25 (Na^+) cation exchange column which was pre-equilibrated in 0.05 M AcOH-AcONa buffer (pH 4.0). The column was washed with 0.01 M NaCl aqueous solution and eluted with 0.1 M NaCl aqueous solution. Fractions active against *Glomerella cingulata* were combined and desalted by adsorption on an Amberlite XAD-2 column. After washing the column with water, the active fraction was eluted with 80% MeOH. The eluate was concentrated and further purified by reversed phase HPLC (YMC-Pack CN, 6.0×150 mm) with 1 mM phosphate buffer (pH 7.0) and MeOH (7 : 3). Desalting with BOND ELUTE C-18, concentration and lyophilization yielded white powder of glomecidin (10 mg).

The physico-chemical properties of glomecidin (**1**) are summarized in Table 1. It showed positive ninhydrin reaction and negative fluorescamine[3] or Sakaguchi reaction, indicating the presence of a secondary amino group, but no guanidino group. The molecular formula of **1** was determined to be $C_{27}H_{37}N_7O_7$ from HRFAB-MS data (found $(M+H)^+$, *m/z* 572.2837; calcd for $C_{27}H_{38}N_7O_7$, *m/z* 572.2833).

The structure of **1** was mainly deduced on the basis of 1D and 2D NMR experiments in CD_3OD. The ^{13}C NMR and DEPT spectra of **1** revealed the presence of 2 methyls, one methoxyl, 4 methylenes, 13 methines and 7 quaternary (including 4 CO) carbons. The ^1H and ^{13}C NMR spectra of **1** suggested a peptide structure, and 2D NMR (^1H-^1H COSY, HSQC, NOESY, HMBC) experiments revealed the presence of four amino acid residues in the molecule. They are isoleucin (Ile), histidine (His), *O*-methyltyrosine (*O*-MeTyr) and one unusual amino acid residue (**2**) (Fig. 1). The molecular formula and the unsaturation index of **1** indicated that the remaining formula for the amino acid residue (**2**) should be $C_5H_8N_2O_3$ ($C_5H_{10}N_2O_4$ as an amino acid) with a cyclic structure. The ^1H-^1H COSY, HMBC and NOESY correlations observed in the molecule are shown in Fig. 2. In the case of **2**, it was revealed by ^1H-^1H COSY and HSQC that two oxygenated methine carbons (δ_H 4.95, δ_C 78.3 and δ_H 4.20, δ_C 73.9) should be adjacent to each other, and the former methine carbon linking to the α-carbon (C-13). The latter oxygenated methine was also revealed by

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Table 1. Physico-chemical properties of glomecidin (**1**).

Appearance	White powder
Molecular weight	571.6
Molecular formula	$C_{27}H_{37}N_7O_7$
FAB-MS (m/z)	572 $(M+H)^+$, 594 $(M+Na)^+$
HRFAB-MS (m/z)	
Found	572.2837 $(M+H)^+$
Calcd.	572.2833 for $C_{27}H_{38}N_7O_7$
UV λ_{max}(MeOH)	282 nm
Color reaction	
Positive	Ninhydrin
Negative	Fluorescamine, Sakaguchi
Solubility	
Soluble:	MeOH, DMSO, H_2O
Insoluble:	$CHCl_3$, EtOAc
Rf on TLC	0.25[a], 0.48[b]
HPLC retention time[c]	12.5 minutes

[a]Silica gel TLC (Merck No. 5715): *n*-PrOH-EtOAc-H_2O(7:1:1)
[b]Silica gel TLC (Merck No. 5715): CH_2Cl_2-MeOH-H_2O(10:5:1)
[c]Conditions: See the text.

^{1}H-^{1}H COSY to be bonded to a methylene. Judging from the chemical shifts of the methylene (δ_C 52.8, δ_H 2.87 and δ_H 3.12), this methylene was inferred to be adjacent to a nitrogen atom. The HMBC correlations were also detected from this methylene to each of the above-mentioned two oxygenated methine carbons (δ_C 78.3 and δ_C 73.9). On the basis of these findings, the amino acid (**2**) has been deduced to be 4,5-dihydroxypiperidazine-3-carboxylic acid (DHPC). The full NMR assignments of all protons and carbons of the four amino acid residues in **1** were thus established as shown in Table 2.

The sequence of the four amino acids in **1** was determined as shown in Fig. 2 by the following NOESY and HMBC experiments. The NOESY correlations were observed between the following pairs of protons: the αH of Ile (δ_H 4.39) and the αH of His (δ_H 4.57), the αH of His and the αH of DHPC (δ_H 3.98), the αH of DHPC and the αH of *O*-MeTyr (δ_H 3.94), and the αH of *O*-MeTyr and the αH of Ile. In addition, a benzene ring proton (22-H or 24-H) of *O*-MeTyr correlated with 14-H (δ_H 4.95) of DHPC in the NOESY experiment, supporting their adjacency. Furthermore, an HMBC correlation was observed between the α proton of DHPC at δ_H 3.98 and the carbonyl carbon of His at δ_C 172.5.

In order to determine the absolute configuration of the constituent amino acids, complete acid hydrolysis of **1** was carried out in a sealed tube with 6 N HCl at 120°C for 24 hours. The resultant hydrolysate was developed by two-dimensional silica gel TLC in *n*-BuOH - AcOH - H_2O (3 : 1 : 1) for the first dimension and in phenol - MeOH - H_2O (3 : 1 : 2) for the second dimension. On this 2D TLC, the four spots of the amino acids were detected by fluorescamine or ninhydrin, among which the three spots corresponding to Ile, His, *O*-MeTyr were identified by comparing with each authentic sample (Fig. 3). By spraying highly diluted fluorescamine in acetone and detecting

Fig. 1. Structure of glomecidin (**1**).

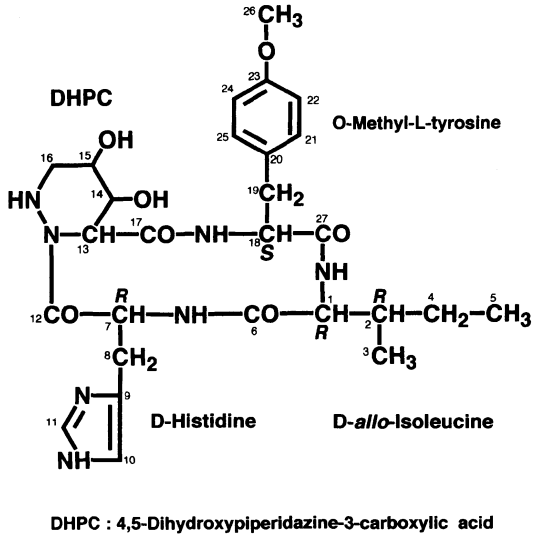
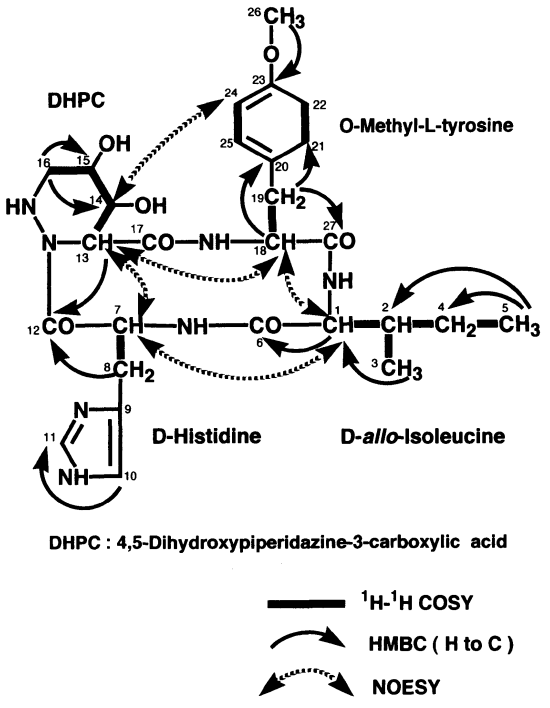


Fig. 2. ^{1}H-^{1}H COSY, HMBC and NOESY correlations for glomecidin (**1**).



fluorescence under 360 nm UV light, the above three amino acids were located on the TLC plate, which were then scratched off and were extracted from the silica gel with water. Absolute configuration of each amino acid was determined by HPLC using a chiral column by comparing its HPLC chromatogram with that of the authentic chiral amino acid under the same HPLC condition. The chiral HPLC analysis was carried out under the following conditions: column, SUMICHIRAL OA-5000, 4.6×150 mm; mobile phase, 2.0 mM $CuSO_4$, flow rate, 1.0 ml/minute, for Ile; 2.0 mM $CuSO_4$-MeOH (85 : 15), flow rate, 0.5 ml/minute, for His and *O*-MeTyr; detection, UV 254 nm. As a result, D-His, *O*-Me-L-Tyr and D-*allo*-Ile were elucidated as shown in Fig. 1. Due to unavailability of a sample of DHPA, the absolute configurations of the three carbons of DHPA still remain to be established.

Glomecidin exhibited growth inhibitory activity against several species of plant pathogenic fungi such as *Glomerella cingulata* (0.78 μg/ml), *Colletotrichum gloeosporioides* Penzig (0.78 μg/ml) and *C. lagenarium* (1.56 μg/ml). However, it did not show any significant activity against a series of other fungi, yeasts or bacteria.

Discussion

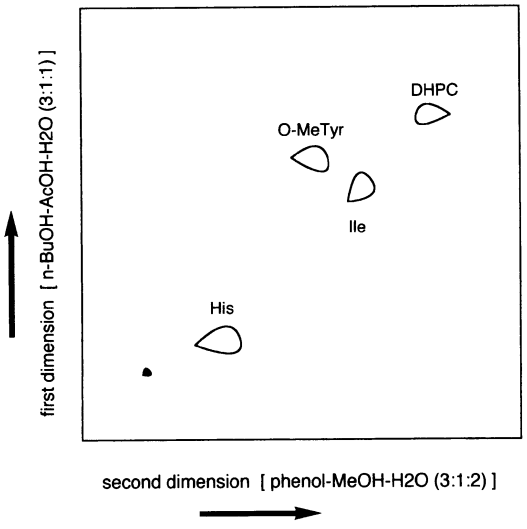
From the same strain, *Streptomyces lavendulae* H698 SY2, NAKAMURA *et al.* isolated an antifungal substance named ileumycin[1] in 1978, reporting its isolation, physico-chemical properties and biological activities. Neither NMR nor MS data was presented, hence no structure was revealed. When the physico-chemical properties of ileumycin and glomecidin are compared, their solubility and UV spectra are quite similar, but their Rf values on Silica gel TLC are slightly different. Glomecidin seems to be less polar and more lipophilic than ileumycin. The significant difference was that ileumycin gave on acid hydrolysis only one ninhydrin-positive spot, which was identified as isoleucin (the antibiotic was named after this fact), and in contrast, glomecidin yielded four amino acids including isoleucin on acid hydrolysis. The biological activities of both antibiotics, however, were quite similar and showed narrow and potent antifungal activities against several species of plant pathogenic fungi. However, glomecidin exhibited slightly weaker activity than the reported activity of ileumycin.[1] Direct comparison of the two substances is now impossible due to inaccessibility of an actual sample of ileumycin.

Table 2. ^{1}H and ^{13}C NMR data of glomecidin (**1**) in CD_3OD (400 MHz and 100 MHz, respectively).

Moiety	position	δ_C	δ_H (J in Hz)
D-*allo*-Ile	1	60.4	4.39 d(6.8)
	2	37.8	2.04 m
	3	16.2	1.07 d(6.8)
	4	26.0	1.30 m, 1.69 m
	5	11.6	0.94 t(7.4)
	6	173.1	
D-His	7	52.8	4.57 br
	8	32.3	2.82 m
	9	121.2	
	10	117.8	7.02 br s
	11	137.3	7.65 s
	12	172.5	
DHPC	13	63.6	3.98 m
	14	78.3	4.95 d(3.2)
	15	73.9	4.20 br d(4.4)
	16	52.8	2.87 m, 3.12 m
	17	177.0	
O-Me-L-Tyr	18	63.6	3.94 m
	19	30.8	2.76 m, 2.94 m
	20	129.6	
	21,25	130.9	7.08 d(8.8)
	22,24	115.2	6.80 d(8.8)
	23	160.2	
	26	55.7	3.76 s
	27	174.8	

DHPC: 4,5-Dihydroxypiperidazine-3-carboxylic acid.

Fig. 3. Amino acid separation of the hydrolysate of glomecidin on 2D Silica gel TLC.



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