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ORIGINAL ARTICLE

NW-G01, a novel cyclic hexapeptide antibiotic, produced by *Streptomyces alboflavus* 313: II. Structural elucidation

Zhengyan Guo[1], Zhiqin Ji[1], Jiwen Zhang[1], Jing Deng[1], Ling Shen[2], Wei Liu[1,3] and Wenjun Wu[1]

NW-G01 is a novel cyclic hexapeptide antibiotic produced by *Streptomyces alboflavus* 313. Its relative structure was established by HR-ESI-MS, IR, 1D and 2D NMR techniques, the absolute structure was determined using a combination of single-crystal X-ray diffraction and Marfey's method finally. The antibiotic consists of L-valine, (3*S*)- and (3*R*)-piperazic acids, *N*-methyl-D-alanine and (*2S,3aR,8aS*)-6-chloro-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid.
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Keywords: cyclic hexapeptide antibiotic; NW-G01; *Streptomyces alboflavus* 313; structural elucidation

INTRODUCTION

In the course of a screening program for new antibiotics, NW-G01, a novel cyclic hexapeptide antibiotic, was isolated from the fermentation broth of *Streptomyces alboflavus* 313. The chemical structure of NW-G01 is composed of a molecule of valine, *N*-methylalanine, a chlorinated pyrroloindoline derivative, and three molecules of piperazic acids (Figure 1). NW-G01 is structurally related to himastatin[1] and chloptosin,[2] two cyclic hexapeptide antitumor antibiotics, but is significantly different in the amino-acid content. Another important difference from them is that the two reported antibiotics are the dimers of hexapeptide. The bioassay results showed that NW-G01 had strong antibacterial activity against gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus*, but was ineffective against gram-negative bacteria.

The taxonomy of the producing strain, fermentation, isolation, physicochemical properties and antibacterial activities of the antibiotic have been previously reported.[3] In this paper, the structure elucidation of NW-G01 will be described.

RESULTS

Physicochemical properties

The molecular formula of NW-G01 was determined to be $C_{35}H_{49}N_{10}O_7Cl$ (*m/z*, found 757.3640[M+H]$^+$, calcd 757.3552) on the basis of HR-ESI-MS measurement, and the presence of chlorine was suggested by the isotope abundance peaks in the MS spectrum. The IR spectrum of NW-G01 exhibited absorption bands at 3425, 1638, 1114 and 1073 cm^{-1}, indicating peptidic moieties in the molecule, and UV (MeOH) absorptions at λ_{max} 218 nm and λ_{max} 346 nm indicated the presence of aromatic ring(s).

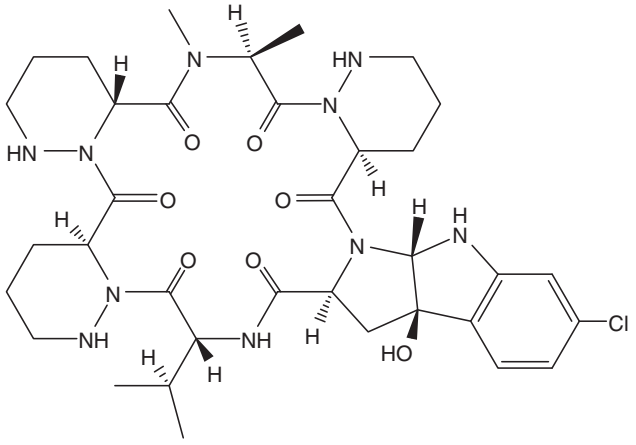

Figure 1 The structure of NW-G01.

Structure elucidation

The ^{13}C and ^{1}H NMR data of NW-G01 are summarized in Table 1. The ^{1}H and ^{13}C NMR spectra of NW-G01 showed typical signals for a cyclopeptide. There were six amide carbonyl carbons (δ 175.4, 174.6, 172.6, 172.2, 170.2 and 170.0) in the ^{13}C NMR spectrum. The ^{13}C NMR (DEPT) spectrum showed 35 carbon signals, which were attributed to four methyl carbons, ten methylene carbons, eight methine carbons, three aromatic methine carbon, one oxygenated quaternary carbon, two quaternary carbons, one chloridated quaternary carbon, six carbonyl carbons by analysis of DEPT and heteronuclear single quantum coherence spectra.

[1]Institute of Pesticide Science, Northwest Agricultural & Forestry University, Shaanxi, China; [2]School of Life Sciences, Anhui Agricultural University, Anhui, China and [3]Institute for Control of Agrochemicals, Shaanxi, China
Correspondence: Professor W Wu, Institute of Pesticide Science, Northwest Agricultural & Forestry University, Xinong 61# Yangling, Shaanxi 712100, China.
E-mail: wuwenjun@nwsuaf.edu.cn
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Table 1 NMR data of compound NW-G01 (^{1}H 500 MHz, ^{13}C 125 MHz CDCl3)

Position	δ_C *(125 MHz, p.p.m.)*	δ_H *(500 M Hz, p.p.m., J=Hz)*	^1H-^1H *COSY* (δ_H *p.p.m.)*	*HMBC* (δ_H *p.p.m.)*
Valine				
CO	175.4			5.45, 2.01, 7.68, 5.33
C_α	54.1	5.45 dd, *J*=10, 7.5	7.68, 2.01	2.01, 7.68, 0.98, 0.99
C_β	30.2	2.01 m	0.99, 0.98, 5.45	0.99, 0.98, 5.45, 7.68
C_γ	18.4	0.99 d, *J*=6.5 (3H)	2.01	0.98, 2.01, 5.45
$C_{\gamma'}$	19.6	0.98 d, *J*=6.5 (3H)	2.01	0.99, 2.01, 5.45
NH		7.68 d, *J*=10	5.45	
Trp derivative (6-chloro-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-caboxylic acid)				
CO	172.2			5.45, 7.68, 5.16, 2.03
C_2	61.6	5.16 d, *J*=8.5	2.03, 2.72	7.68, 2.03, 2.72
C_3	39.2	2.72 d, *J*=14.0; 2.03 dd, *J*=14.0, 8.5	5.16	5.16, 5.17
C_{3a}	89.9			5.16, 5.17, 5.96
3a-OH		6.08 s		
C_{3b}	129.9			6.81, 6.69, 7.22
C_4	123.8	7.22 d, *J*=8	6.81	
C_5	120.2	6.81 dd, *J*=8, 2	7.22	6.69, 7.22
C_6	135			7.22, 6.81, 6.69
C_7	111.7	6.69 d, *J*=2		6.81
C_{7a}	148.3			6.69, 7.22
C_{8a}	86.3	5.17 m,		5.16, 2.03, 5.96
8-NH		5.96 d, *J*=5		
Piperazic acid (PA-1)				
CO	170.2			5.16, 5.17, 5.30, 1.89
C_α	50.6	5.30 dd, *J*=6.5, 2.0	2.17, 1.89	1.89, 1.48, 2.17
C_β	24.9	2.17 d, *J*=14; 1.89 m (2H)	5.30, 1.48, 1.60; 5.30, 1.60, 1.48	5.30, 1.60, 1.48, 3.04, 2.80
C_γ	21.8	1.60 m; 1.48 m, (2H)	2.17, 1.89, 3.04; 2.17, 1.89, 3.04, 2.80	5.30, 3.04, 2.80, 1.89, 2.17
C_δ	48.3	3.04 dd, *J*=13.5, 3.5;		
		2.80 m, (2H)	1.60, 1.48; 1.48	1.60, 1.48, 2.17, 1.89
C_δ-NH		4.45 d, *J*=10		
N-*methylalanine (MeAla)*				
CO	174.6			5.30, 5.62, 1.23
C_α	49.9	5.62 q, *J*=7	1.23	1.23, 2.87
C_β	14.5	1.23 d, *J*=6.5 (3H)	5.62	5.62
N-CH_3	30.9	2.87 s, (3H)		5.62
Piperazic acid (PA-2)				
CO	170			5.62, 2.87, 5.39, 2.30, 1.73
C_α	52	5.39 d, *J*=4	2.30, 1, 73	2.30, 1.73, 2.40, 1.48
C_β	25.3	2.30 d, *J*=14; 1.73 m (2H)	2.40, 5.39, 1.48; 2.40, 5.39, 1.48	5.39, 2.40, 1.48, 3.14, 2.70
C_γ	19.9	2.40 m; 1.48 m (2H)	2.30, 1.73, 3.14, 2.70	5.39, 2.30, 1.73, 3.14, 2.70, 4.82
C_δ	47.9	3.14 m; 2.70 m (2H)	2.40, 1.48, 4.82	2.40, 1.48, 2.30, 1.73, 4.82
C_δ-NH		4.82 d, *J*=12.5	2.40, 1.48	
Piperazic acid (PA-3)				
CO	172.6			5.39, 4.82, 5.33, 2.80, 1.80
C_α	43.9	5.33 brs, *J*=5.5	1.80	2.80, 1.80, 2.40, 1.48
C_β	24.3	2.80 m; 1.80 m (2H)	2.40, 1.48; 2.40, 1.48, 5.33	5.39, 2.40, 1.48, 3.14, 2.70
C_γ	19.3	2.40 m; 1.48 m (2H)	2.80, 1.48, 2.70, 3, 14	5.39, 2.80, 1.80, 3.14, 2.70
C_δ	46.6	3.14 m; 2.70 m (2H)	2.40, 1.48	2.40, 1.48, 2.80, 1.80
δ-NH		4.73 d, *J*=12.5		

In ^{1}H-^{1}H COSY spectrum, vicinal coupling signals were observed between the two methyl groups (δ 0.98, 0.99) and the methine proton (δ 2.01), the proton (δ 2.01) and another methine proton (δ 5.45), and the proton (δ 5.45) and the amide proton (δ 7.68). The long-range couplings in the HMBC spectrum from two methyl groups' protons (δ 0.98, 0.99) to the methine carbon

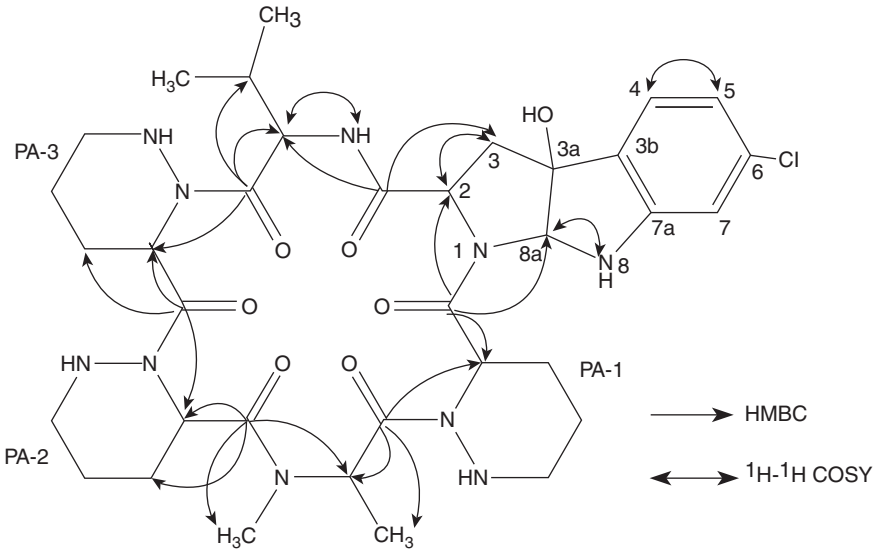

Figure 2 Key HMBC and 1H-1H COSY for NW-G01 in $CDCl_3$.

(δ 54.1) and the methine proton (δ 2.01) and the amide proton (δ 7.68) to a carbonyl carbon (δ 175.4) revealed the presence of the valine moiety.

According to the same manner described as above, the coupling signals between the methyl protons (δ 1.23) and the methine protons (δ 5.62) were displayed from ^{1}H-^{1}H COSY spectrum, and the long-range couplings (HMBC) from the methyl single peak protons (δ 2.87) to the methine carbon (δ 49.9), and from the methyl protons (δ 1.23) to a carbonyl carbon (δ 174.6) indicated the presence of a *N*-methylalanine moiety.

Two three-proton spin systems, including an aromatic ABX system (δ 7.22 (1H, d, *J*=8.0Hz,); 6.81(1H, dd, *J*=8.0 Hz, 2.0 Hz,); 6.69 (1H, d, *J*=2.0 Hz)) and an aliphatic ABX system (δ 5.16 (1H, d, *J*=8.5 Hz); 2.72 (1H, d, *J*=14.0 Hz,); 2.03 (1H, dd, *J*=14.0, 8.5 Hz)), were evident from the ^{1}H-^{1}H COSY spectrum. In addition, other elements of the fragment included a hydroxy group (δ 6.08), an amide proton (δ 5.96), a quaternary carbon (δ 89.9), a carbonyl carbon (δ 172.2) and a chlorine atom. Combining the relative information with 2D NMR data (such as HMBC, heteronuclear single quantum coherence, NOESY and TOCSY) and the spectra data of himastatin[1] and chloptosin,[2] the molecular structure included the presence of a 6-chloro-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid moiety, a chlorinated pyrroloindoline derivative considered to be derived from tryptophan.

In ^{1}H-^{1}H COSY spectrum, there was strong coupling signals between the methylene protons (δ 3.04, 2.80) and the methylene protons (δ 1.60, 1.48), the methylene protons (δ 1.60, 1.48) and the methylene protons (δ 2.17, 1.89), and the long-range coupling (HMBC) from the methylene protons (δ 2.17, 1.89) to a carbonyl carbon (δ 170.2). In addition, this element included an amide proton (δ 4.45). Combining the key information with 2D NMR data and the relative references,[2,4,5] a piperazic acid moiety was considered. In the same manner, it was confirmed that another two molecules of piperazic acid moiety were in the hexapeptide antibiotic.

The sequence of the amino-acid residues in NW-G01 was established by the analysis of HMBC data, which showed correlation from the α-methine protons of amino-acid residue to carbonyl carbon of the neighboring residues (Figure 2).

Stereochemistry of NW-G01

Finally, the crystals (Figure 3) suitable for X-ray crystallographic analysis were obtained fortunately. The results obtained from this analysis defined the relative chemistry at all centers. The relative configurations of the PA-1, PA-3, MeAla and Trp derivatives were *S** and those of the valine and PA-2 derivatives were *R**. As these standard amino acids were not directly obtained except for D- and L-valine, the absolute configurations of the amino-acid residues were deduced from the direction of this known absolute configuration of valine.

The absolute configuration of the valine residues was determined by application of the Marfey's method using 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA)[6–8] and subsequent HPLC analysis of the acid hydrolysate of NW-G01. Marfey's analysis revealed the presence of L-valine in the acid hydrolysate of NW-G01, and the absolute configuration of MeAla was deduced as *D*-configuration based on X-ray crystal diffraction analysis. Thus, the absolute stereochemistry of amino acid groups composed of NW-G01 was determined and confirmed as L-valine, (*2S,3aR,8aS*)-6-chloro-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid, (*3S*)-piperazic acid (PA-1), *N*-methyl-D-alanine, (*3R*)-piperazic acid (PA-2) and (*3S*)-piperazic acid (PA-3).

DISCUSSION

The complete structure of NW-G01 was determined using spectral analysis and single-crystal X-ray diffraction. It is structurally characterized as an 18-membered cyclic hexapeptide composed of two amino-acid residues including an L-amino acid and nitrogen-bearing heterocyclic rings, including a chlorine atom on the pyrroloindoline moiety. The structure of NW-G01 is closely related to chloptosin, produced by *Streptomyces* MK498-98F14 stain, such as, the presence of the uniform chlorine atom on the pyrroloindoline moieties, 6-chloro-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole-2-carboxylic acid.[2] However, it is significantly different in the amino-acid content, especially, the former was a monomer and contained L-valine and *N*-methyl-D-alanine residues, but the latter was a dimer and included *O*-methyl-D-serine, D-valine and D-threonine groups.

From our previous study, it was found that NW-G01 had higher antibacterial activities than ampicillin against four tested gram-positive

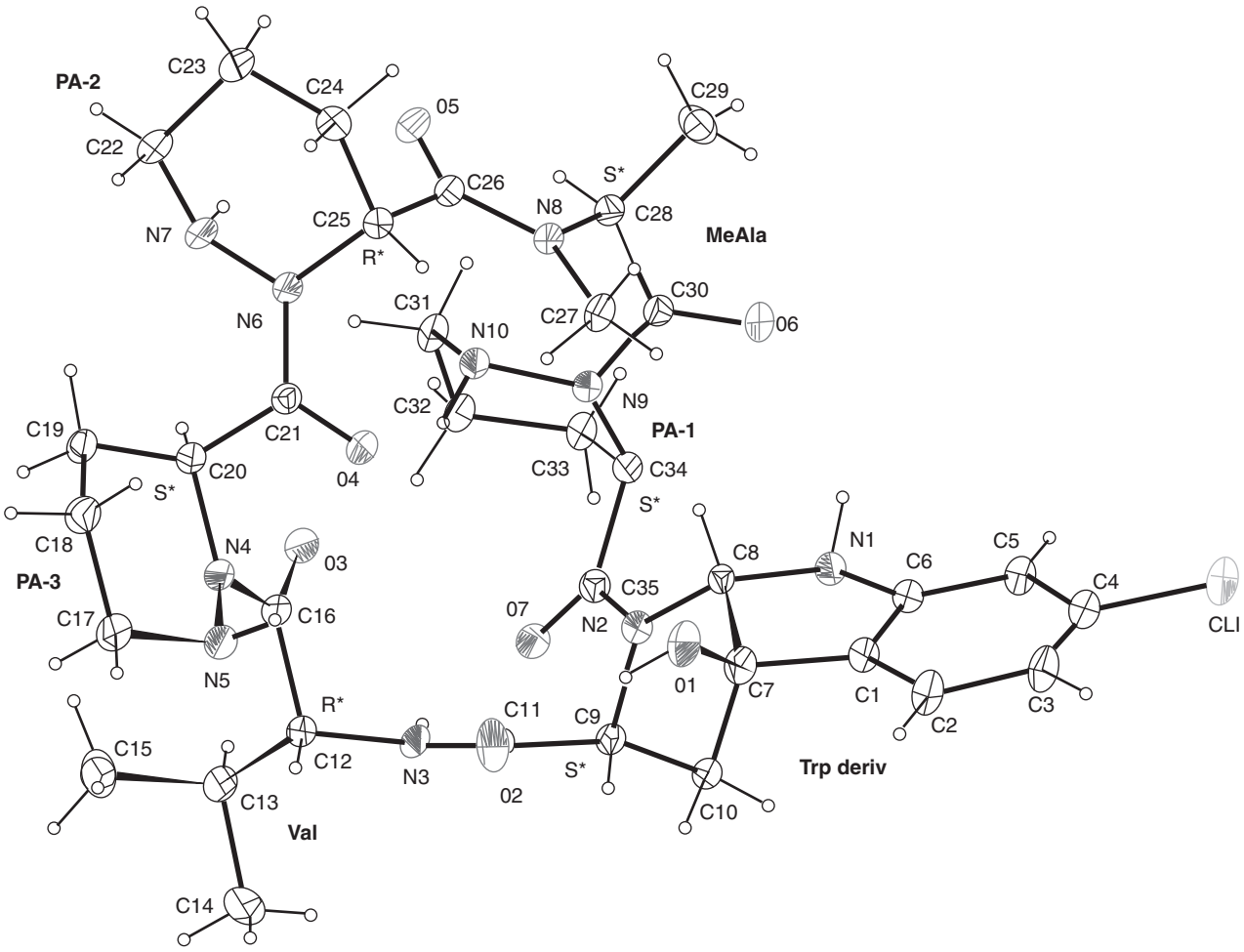

Figure 3 Molecular single-crystal X-ray diffraction photo of compound NW-G01.

bacteria, especially to methicillin-resistant *Staphylococcus aureus* (MIC 7.81 μg ml^{-1}),[3] and had strong activities against human hepatoma cell line BEL-7402 and human colon cancer cell line HCT-116 cultured *in vitro*. Further pharmacological studies and an investigation of the mechanism of action are now underway.

EXPERIMENTAL SECTION

General

HR-ESI-MS was measured on a Bruker APX500 analytical spectrometer (Bruker, Shanghai, China). ^{1}H- and ^{13}C-NMR spectra and 2D NMR were obtained in $CDCl_3$ on a Bruker AV-500 spectrometer, using $CDCl_3$ as solvent and TMS as an internal standard. IR spectra were recorded on a Bruker VECOR22 FT-IR spectrometer using KBr pellets. All solvents and chemicals were of analytical grade.

Marfey's analysis

Approximately 1.0 mg of NW-G01 was hydrolyzed with 100 μl of 6 N HCl at 110 °C for 24 h. The acid hydrolysate was evaporated to dryness and dissolved in 100 μl of 0.1 N HCl. To 50 μl of the acidic solution, 80 μl of 1 N $NaHCO_3$ and 400 μg FDAA (Marfey's reagent, J&K Chemical, Beijing, China) with 40 μl acetone was added, and the mixture was heated at 50 °C for 1 h. The reaction mixture was cooled to room temperature, neutralized with 40 μl 1 N HCl, and evaporated to dryness. The residue was dissolved in 40 μl of acetonitrile and the FDAA derivative solution was analyzed by reverse-phase HPLC. The analysis of the FDAA derivatives was performed on a SinoChrom ODS-BP (250×4.6 mm, 5 μm, Dalian Elite Analytical Instruments, Dalian, China) column maintained at 30 °C with UV detector at 340 nm. Acetonitrile and 50 mM triethylamine phosphate buffer (pH 5.0) were used as mobile phases (acetonitrile/triethylamine phosphate (40/60, v/v)) at a flow rate of 1.0 ml min−1.[9,10] The FDAA derivatives of the acid hydrolysate were identified by comparing the retention times with FDAA derivatized standard amino acids. The retention time of the FDAA derivatives of the acid hydrolysate was 32.3 min (L-Val; D-Val, 37.1 min).

X-ray crystallographic data of NW-G01

A needle-shaped crystal was obtained by slow evaporation of a methanol solution of the compound. Single-crystal X-ray diffraction measurements were made on a Bruker SMART 1000 CCD diffractometer with graphite-monochromated Mo $K\alpha$ radiation (λ=0.71073 Å) at 295(2) K. Crystal data: Mr=756.28, $C_{35}H_{48}N_{10}O_7Cl$, monoclinic, space group, *C2*, unit cell dimensions: a=32.4107(11) Å, b=11.1186(4) Å, c=14.4296(6) Å, β=114.8050(10)°, V=4720.1(3) Å^3, Z=4, D_{calcd}=1.064 mg m^{-3}, μ=0.130 mm^{-1}, F(000)=1604. Data collection and reduction: crystal size, 0.20×0.10×0.10 mm^3, θ range=2.31–25.00°, 12 659 reflections collected, 7383 independent reflections (R_{int}=0.0428), final R indices [$I>2\sigma(I)$]: R_1=0.0731, wR_2=0.1806, GOF=0.990.

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