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NOTE

NW-G03, a related cyclic hexapeptide compound of NW-G01, produced by *Streptomyces alboflavus* 313

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In the previous paper,[1] we described the novel cyclic hexapeptide antibiotic, NW-G01, produced by *Streptomyces alboflavus* 313. It is structurally related to himastatin[2,3] and chloptosin,[4] including a chlorinated pyrroloindoline derivative, but is significantly different in the amino-acid content. In the further study of secondary metabolites of *S. alboflavus* 313, another novel cyclic hexapeptide NW-G03, a related structural compound of NW-G01, was discovered as a trace constituent from fermentation broth of this strain. The difference of chemical structure between NW-G03 and NW-G01 is only an amino-acid residue. The bioassay results showed that NW-G03 also showed strong antibacterial activity against gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), but was ineffective against gram-negative bacteria. NW-G03 also inhibited the growth of three tumor cell lines *in vitro*.

In this paper, the isolation, structure elucidation, physicochemical properties and *in vitro* biological activity of compound NW-G03 are described.

NW-G03 was isolated from the fermentation broth of NW-G01-producing *S. alboflavus* 313, as a trace metabolite of interest. As described in our previous paper,[1] briefly, the supernatant (100 l) of the fermentation broth was adsorbed onto a macroporous resin (HPD400, Cangzhou Baoen, Hebei, China), followed by elution with methanol. The elution was evaporated to yield a residue (60 g). The residue was applied onto a silica gel column and eluted with EtOAc/MeOH (from 100:0 to 0:100). Then the antimicrobial fraction was purified by RP-HPLC (Hypersil C_{18}, 20 mm×250 mm, 10 μm) with methanol/water (75:25) as mobile phase to yield two new compounds NW-G01(168 mg) and NW-G03(11.2 mg). Unfortunately, satisfactory crystals of NW-G03 for X-ray *anal* could not be obtained.

The physicochemical properties of NW-G03 are summarized in Table 1. It was soluble in dimethyl sulfoxide, methanol and ethyl acetate, but insoluble in water and acetone. It displayed positive color reactions to iodine vapor and argenti nitras solution though it was negative against Molish and $FeCl_3$.The molecular formula of NW-G03 was determined to be $C_{35}H_{47}N_{10}O_7Cl$ (*m/z*, found 755.3384$[M+H]^+$, calcd 755.3396) on the basis of HR ESI-MS measurement, and the presence of chlorine was suggested by the isotope abundance peaks in the MS spectrum. UV (MeOH) absorptions at λ_{max} 208 nm and λ_{max} 346 nm indicated the presence of aromatic ring(s) in the molecule.

The ^{13}C and ^1H NMR data of NW-G03 are summarized in Table 2. At first, the molecular formula $C_{35}H_{47}N_{10}O_7Cl$ was determined by HR ESI-MS (Table 1). As the UV and ^{13}C and ^1H NMR spectra of NW-G03 were quite similar to those of NW-G01, the structure of which was determined by single crystal X-ray diffraction.[5,6] The structure of NW-G03 (Figure 1) was elucidated by comparison with

Table 1 Physico-chemical properties of compound NW-G03

Properties	*Compound NW-G03*
Appearance	White powder
m.p.	193–195 °C
Molecular formula	$C_{35}H_{47}N_{10}O_7Cl$
HR-ESI-MS[m/z]	
Found	755.3384 $[M+H]^+$
Calcd	755.3396 for $[M+H]^+$
UV λ_{max}^{MeOH} nm(ε)	208
$[\alpha]_D^{20}$ (c 0.1, MeOH)	−25°
Solubility	
Soluble	DMSO, Methanol, EtOAc
Insoluble	H_2O, Acetone
Test	
Positive	I_2, $AgNO_3$
Negative	$FeCl_3$, Molish

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Table 2 NMR data of compound NW-G03 and NW-G01 (^{1}H 500 MHz, ^{13}C 125 MHz $CDCl_3$)

	NW-G03		*NW-G01*	
Position	δ_C *(p.p.m.)*	δ_H *(p.p.m., J=Hz)*	δ_C *(p.p.m.)*	δ_H *(p.p.m., J=Hz)*
Valine				
CO	170.1		175.4	
C_α	54.8	5.44 dd, *J*=10,7.5	54.1	5.45 dd, *J*=10, 7.5
C_β	29.7	2.13 m	30.2	2.01 m
C_γ	17.4	0.97 d, *J*=6.5 (3H)	18.4	0.99 d, *J*=6.5 (3H)
$C_{\gamma'}$	19.7	0.98 d, *J*=6.5 (3H)	19.6	0.98 d, *J*=6.5 (3H)
NH		7.62 d, *J*=10		7.68 d, *J*=10
Trp deriv (6-chloro-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-caboxylic acid)				
CO	172.2		172.2	
C_2	61.7	5.05 d, *J*=8.5; 2.69 d, *J*=14.0;	61.6	5.16 d, *J*=8.5; 2.72 d, *J*=14.0;
C_3	39.4	2.13 dd, *J*=14.0, 8.5 (2H)	39.2	2.03 dd, *J*=14.0, 8.5
C_{3a}	89.6		89.9	
3a-OH				6.08 s
C_{3b}	129.8		129.9	
C_4	124.3	7.21 d, *J*=8.0	123.8	7.22 d, *J*=8.0
C_5	120.2	6.79 dd, *J*=8.0, 2.0	120.2	6.81 dd, *J*=8.0, 2.0
C_6	135.1		135.0	
C_7	111.6	6.65 d, *J*=2.0	111.7	6.69 d, *J*=2.0
C_{7a}	148.6		148.3	
C_{8a}	85.5	5.14 d, *J*=5.5	86.3	5.17 m
8-NH		5.93 d, *J*=5.0		5.96 d, *J*=5.0
Piperazic Acid (PA-1)				
CO	170.7		170.2	
C_α	52.1	5.44 m	50.6	5.30 dd, *J*=6.5, 2.0
C_β	25.4	2.35 m, 1.72 m, (2H)	24.9	2.17 d, *J*=14; 1.89 m (2H)
C_γ	19.5	2.40 m, 1.50 m, (2H)	21.8	1.60 m; 1.48 m, (2H)
				3.04 dd, *J*=13.5, 3.5
C_δ	48.4	3.06 dd, *J*=13.5, 3.5;	48.3	2.80 m, (2H)
		2.77 m, (2H)		
C_δ–NH				4.45 d, *J*=10
N-methylalanine				
CO	173.4		174.6	
C_α	49.7	5.64 q, *J*=6.5	49.9	5.62 q, *J*=7.0
C_β	14.5	1.25 d, *J*=6.5 (3H)	14.5	1.23 d, *J*=6.5 (3H)
N–CH_3	30.9	2.89 s, (3H)	30.9	2.87 s, (3H)
Piperazic Acid (PA-2)				
CO	170.2		170.0	
C_α	51.0	5.60 d, *J*=4.0	52.0	5.39 d, *J*=4.0
C_β	24.3	2.35 m; 1.72 m, (2H)	25.3	2.30 d, *J*=14;
				1.73 m (2H)
C_γ	18.8	2.20 m; 1.96 m, (2H)	19.9	2.40 m; 1.48 m (2H)
C_δ	47.9	3.17 m; 2.77 m, (2H)	47.9	3.14 m; 2.70 m (2H)
C_δ–NH		4.84 d, *J*=12.5		4.82 d, *J*=12.5
6-ene-*piperazic Acid*				
CO	173.9		172.6	
C_α	44.7	5.30 m	43.9	5.33 br s, *J*=5.5
C_β	20.0	2.35 m; 1.52 m, (2H)	24.3	2.80 m; 1.80 m (2H)
C_γ	20.2	2.15 m, (2H)	19.3	2.40 m;1.48 m (2H)
C_δ	142.3	6.98 m	46.6	3.14 m; 2.70 m (2H)
				(δ-NH) 4.73 d, *J*=12.5

Abbreviations: br, broad; d, doublet; dd, doublet of doublets; m, multiplet; q, quartet; s, singlet.

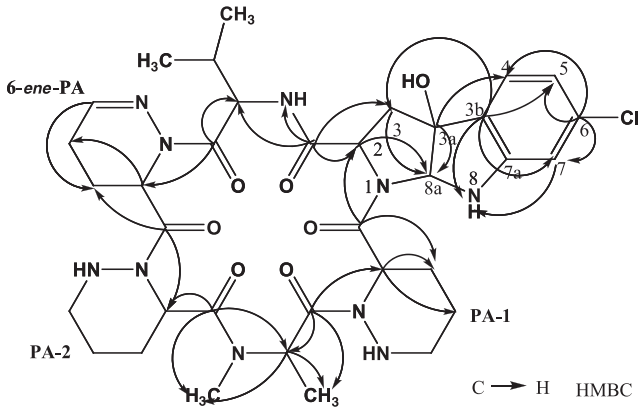

Figure 1 Key HMBC of compound NW-G03.

the ^{1}H and ^{13}C NMR data with those of NW-G01 (Table 2), which was finally established by application of a series of 2D NMR techniques. From the ^{1}H and ^{13}C NMR spectra of NW-G03, typical signals for a cyclic peptide were clearly discovered. There were six amide carbonyl carbons (δ 173.9, 173.4, 172.2, 170.7, 170.2 and 170.1) in the ^{13}C NMR spectrum.

The ^{13}C NMR (DEPT) spectrum showed 35 carbon signals, which were attributed to four methyl carbons, nine methylene carbons, eight methine carbons, three aromatic methine carbon, one sp^2 methine carbon, one oxygenated quaternary carbon, two quaternary carbons, one chloridated quaternary carbon and six carbonyl carbons by *anal* of the DEPT and HSQC spectra.

In the ^{1}H NMR spectrum, large coupling (J=7.5 Hz) were observed between the two γ methyl groups at δ 0.97, 0.98 and the β methine proton at δ 2.13, diagnostic of an isopropyl group. A smaller coupling

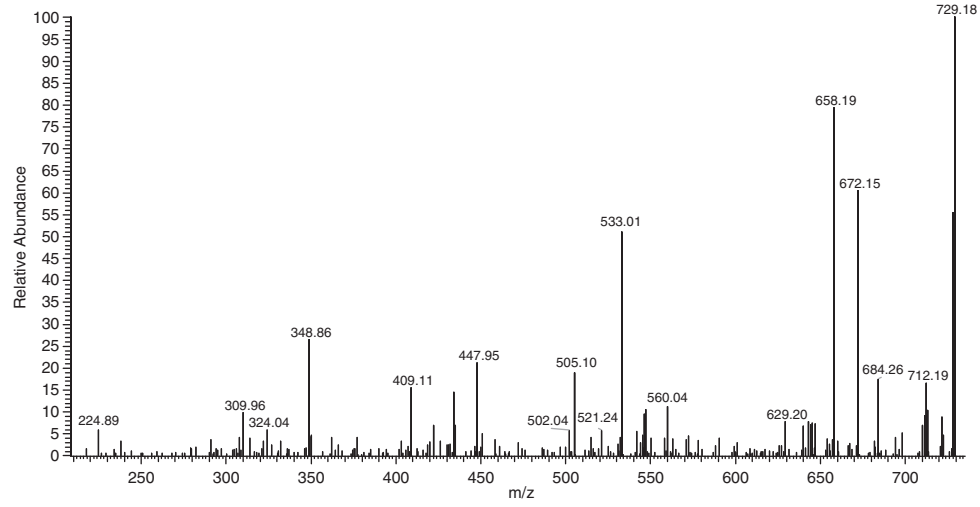

Figure 2 The MS/MS picture of NW-G01 (A mass peak of 739 is missing).

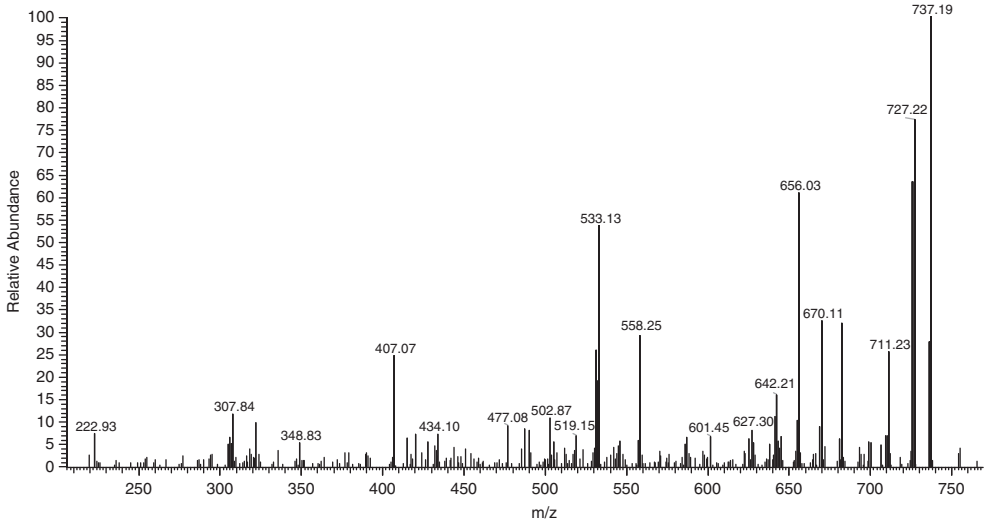

Figure 3 The MS/MS picture of NW-G03.

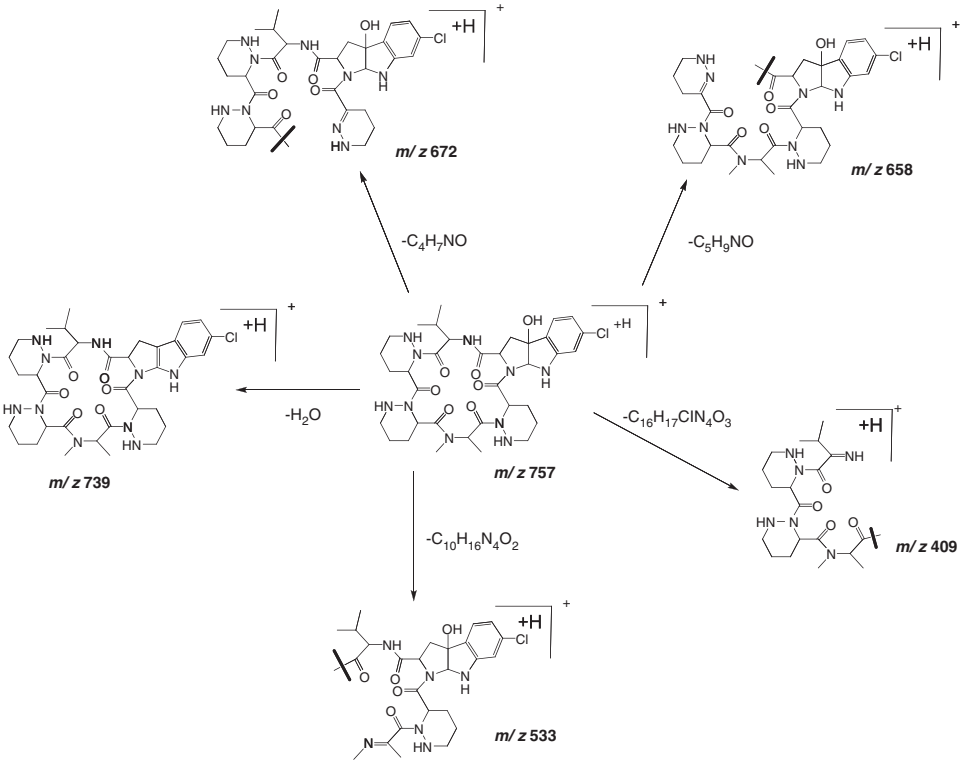

Figure 4 The diagram of the key fragmentations of NW-G01.

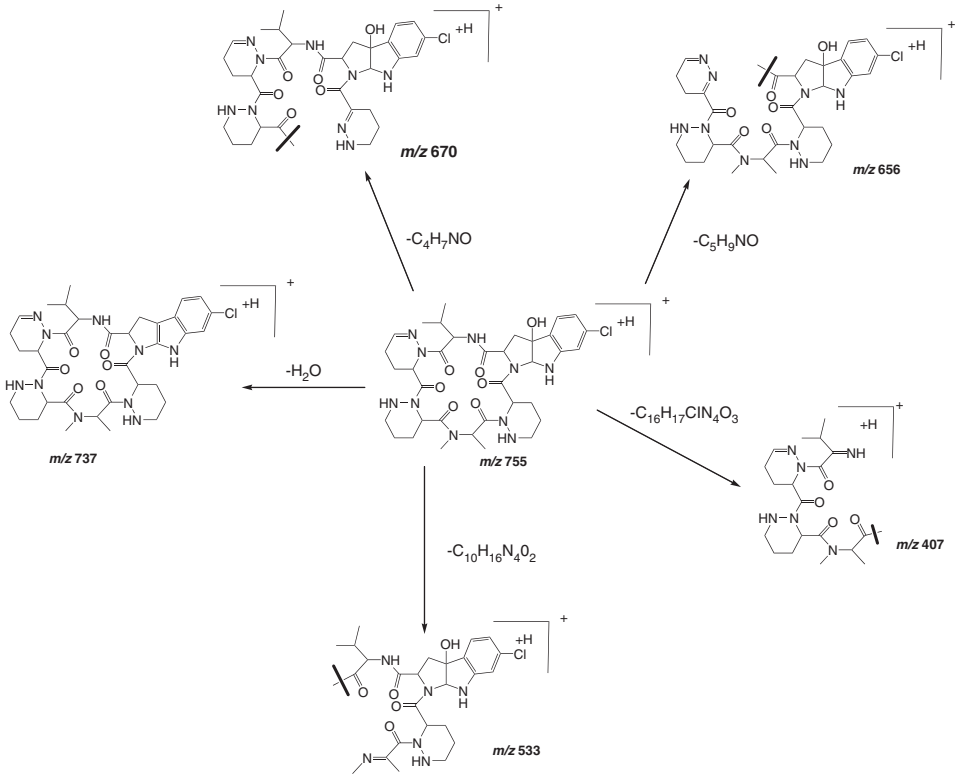

Figure 5 The diagram of the key fragmentations of NW-G03.

(J=5.5 Hz) was observed between the β C–H (δ 2.13) and α C–H (δ 5.44). A large coupling (J=10 Hz) between the α C–H (δ 5.44) and the N–H (δ 7.62) completed the spin system. The carbonyl carbon at δ 170.1 showed long-range heteronuclear coupling with α C–H (δ 5.44). The above information indicated the presence of a valine moiety.

According to the same manner described as above, the distinct coupling (J=6.5 Hz) were displayed between the methyl protons (δ 1.25) and the methine protons (δ 5.64) from ^{1}H NMR spectrum, and the long-range couplings (HMBC) from the methyl single peak protons (δ 2.89) to the methine carbon (δ 49.7), and from the methyl protons (δ 1.25) to a carbonyl carbon (δ 173.4) diagnosed the presence of a *N*-methylalanine moiety.

Two 3-proton spin systems, including an aromatic ABX system (δ 7.21 (1H, d, J=8.0 Hz,); 6.79(1H, dd, J=8.0, 2.0 Hz,); 6.65 (1H, d, J=2.0 Hz)), and an aliphatic ABX system (δ 5.05 (1H, d, J=8.5 Hz); 2.69 (1H, d, J=14.0 Hz,); 2.13 (1H, dd, J=14.0, 8.5 Hz)) were evident from the ^{1}H-NMR spectrum. Additionally, other elements of the fragment included an amide proton (δ 5.93), a quaternary carbon (δ 89.6), a carbonyl carbon (δ 172.2) and a chlorine atom. In comparison with spectral data of compound NW-G01,[5,6] a chlorinated pyrroloindoline derivative(6-chlorl-3a-hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-b]indole-2-carboxylic acid) was deduced in molecular structure.

In comparison with NW-G01, the remaining residues of NW-G03 included two molecules of piperazic acid (PA) and a molecule of dehydrogenated PA. As only one remaining methenyl carbon (δ 142.3) was present, a *δ-dehydro*-piperazic acid was diagnosed easily.

To further verify the structural relationship between NW-G01 and NW-G03, The MS/MS experiments of NW-G01 and NW-G03 were carried out and the MS/MS pictures were showed in the Figures 2 and 3, respectively. From the two MS/MS, some main fragment ion peaks were belonged and the fragmentations were determined in the Figures 4 and 5. In the Figures 4 and 5, the NW-G01 fragment ions (*m/z* 739, 672, 658 and 409) including PA were two more mass units than NW-G03 fragment ions (*m/z* 737, 670, 656 and 407) including PA, furthermore, the fragmentations of NW-G01 and NW-G03 contained the same fragment ion (*m/z* 533) without PA part. So, the above results also confirmed that the other structural parts of two compounds were completely uniform except the PA part (C=N) of NW-G03.

The sequence linkage of the amino-acid residues in NW-G03 was established by the *anal* of HMBC data, which showed correlation from the α-methine protons of amino-acid residue to carbonyl carbon of the neighboring residues (Figure 1).

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)[3,5,7–9] and subsequent HPLC *anal* of the acid hydrolysate of NW-G03. The results of Marfey's *anal* revealed the presence of D-valine in the acid hydrolysate of NW-G03.

Antibacterial activity was measured by the micro-broth dilution method in 96-well culture plates by employing Mueller-Hinton broth (Hangzhou Microbial Reagent Co. Ltd), according to the Standard of National Committee for Clinical Laboratory (NCCLS).[10] The compound NW-G03 exhibited significant activity against several gram-positive bacteria (Table 3) with MIC values of less than 25 μg ml^{-1}. No obvious inhibitory effects were observed against gram-negative bacteria at a concentration of 200 μg ml^{-1}. The tested gram-positive bacteria were *Bacillus cereus* 1.1846, *Bacillus subtilis* 1.88, *S. aureus* 1.89, MRSA 212 and the gram-negative bacteria were *Escherichia coli* 1.1636 and *Pseudomonas aeruginosa* 1.2031.

Table 3 MICs of NW-G03 against the bacteria

Name of the tested bacteria	*MIC of NW-G03(μg ml^{-1})*	*MIC of Ampicillin (μg ml^{-1})*
Bacillus cereus (1.1846)	12.25	50
Bacillus subtilis (1.88)	25	50
Staphylococcus aureus (1.89)	3.07	6.13
MRSA (No. 212)	6.13	>100
Escherichia coli (1.1636)	>200	>100
Pseudomonas aeruginosa (1.2031)	>200	>100

Table 4 Inhibition of NW-G03 against three tumor cells (30 μg ml^{-1})

	Inhibition rate (%)	
Name of cell lines	*NW-G03*	*Taxol*
Bel-7402	45.1	55.5
HCT116	69.3	69.5
H460	42.0	51.1

In addition, antitumor activities of NW-G03 against three human tumor cell lines were tentatively evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) method.[11–15] As summarized in Table 4, the *in vitro* compound inhibited the growth of cell lines BEL-7402, HCT-116 and H460 in the concentration of 30 μg ml^{-1}.

In the isolation process of compound NW-G01, NW-G03, a trace constitutent showed antimicrobial activity was discovered. HR ESI-MS measurement exhibited that the molecular formula of NW-G03 only had two less hydrogen atoms than that of NW-G01. Furthermore, ^{1}HNMR and ^{13}CNMR spectral data revealed that these two compounds were highly similar skeleton structures except only one difference that NW-G03 contained a dehydrogenated PA moiety, whereas NW-G01 included a saturated PA moiety. Additionally, the valine residue of NW-G03 was also the D configuration by using Marfery's method, which agreed with the valine absolute configuration of NW-G01.[5,6] The other residues configurations were confirmed in accordance with compound NW-G01, because the standard substances of these residues were not directly purchased. Therefore, compound NW-G03 is also structurally characterized as an octadeca-membered cyclic hexapeptide composed of D-valine moiety, *N*-methyl-D-alanine, PAs and a chlorined pyrroloindoline moiety and NW-G03 and NW-G01 were considered as a class of cyclic hexapeptide antibiotics.

In our current study, it was found that NW-G03 had higher antibacterial activities than ampicillin against *B. cereus* 1.1846, *B. subtilis* 1.88, *S. aureus* 1.89 and MRSA 212, especially to MRSA. It was homophilic with antibacterial spectrum of NW-G01,[1] himastatin[7] and chloptosin.[4] Surprisingly, NW-G03 was found to show strong activity against human hepatoma cell line BEL-7402, human colon cancer cell line HCT-116 and human large-cell lung carcinoma cell line H460 cultured *in vitro*. Further pharmacological studies and an investigation of the mechanism of action are now underway.

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