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Overexpression of *hgc1* increases the production and diversity of hygrocins in *Streptomyces* sp. LZ35†

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Shanren Li,‡[a] Chunhua Lu,‡[a] Jinhuan Ou,[a] Jingjing Deng,[a] Yuemao Shen*[a]

Manipulation of pathway regulation is an efficient strategy to increase the secondary metabolite production. The production of hygrocins in *Streptomyces* sp. LZ35 was previously increased by overexpression of the *hgc1*, a LAL-family pathway-specific activator gene. In this study, we have further characterized the product of the *hgc1*-overexpressed mutant and three new hygrocins were isolated with the aid of chromophore-guided fractionation. The structure of hygrocins H - J (**1** − **3**) were determined by 1D and 2D NMR spectroscopic data and high-resolution mass spectrometry. Hygrocin H (**1**) was determined as 2,19-dehydrated-hygrocin C; Hygrocin I (**2**) and J (**3**) were shown to be 13,14-*seco*-hygrocin H and 13,14-*seco*-2,19-dehydrated hygrocin F, respectively. Hygrocin H showed toxicity to human tumor MDA-MB-231, PC3 and HeLa cell lines (IC_{50} = 2.4, 1.7, and 0.8 μM, respectively),while hygrocins I and J were inactive at 50 μM against all the tested cell lines.

Introduction

Ansamycins are an important family of natural products that exhibit a range of biological activities, including the Hsp90 inhibitor geldanamycin,[1] the RNA polymerase inhibitor rifamycin,[2] and the antiproliferative maytansinoids.[3] The hygrocins, structurally diverse naphthalenic ansamycins, were first isolated from *Streptomyces hygroscopicus* in 2005, have been shown to possess anti-bacterial and anti-cancer activity.[4, 5] Additionally, the hygrocins C−G were recently isolated from the *gdmAI*-disrupted *Streptomyces* sp. LZ35.[5] The intriguing structures and excellent bioactivity of hygrocins have encouraged us to search for more congeners.

In many species of *Streptomyces*, the secondary metabolites biosynthetic genes are clustered on the chromosome or plasmids.[6] The biosynthesis of each type of antibiotics is usually controlled by regulatory proteins, especially by transcriptional activators. Overexpression of pathway-specific activator genes have been reported to lead to increased production of the corresponding antibiotics.[7] Recently, the biosynthesis of hygrocins has been studied, and found that Hgc1 is a specific LAL-type activator in hygrocin biosynthesis.[8] To increase the production of hygrocins for facilitating isolation, a strain SR101OEhgc1 was constructed by constitutive overexpression (OE) of *hgc1*.[8] By metabolic profiling using HPLC/DAD, we found that the hygrocin congeners produced by SR101OEhgc1 are more abundant than expected. Further fractionation and detailed isolation by a combination of various column chromatographic methods with the aid of diode array detection of naphthoquinone or naphthohydroquinone chromophore led to the identification of three new hygrocin congeners, namely hygrocins H−J (**1**−**3**) (Fig.1). Herein, we report the isolation, structure elucidation and cytotoxicity evaluation of the three new hygrocin analogues which illuminates the flexibility and diversity of hygrocin biosynthesis.

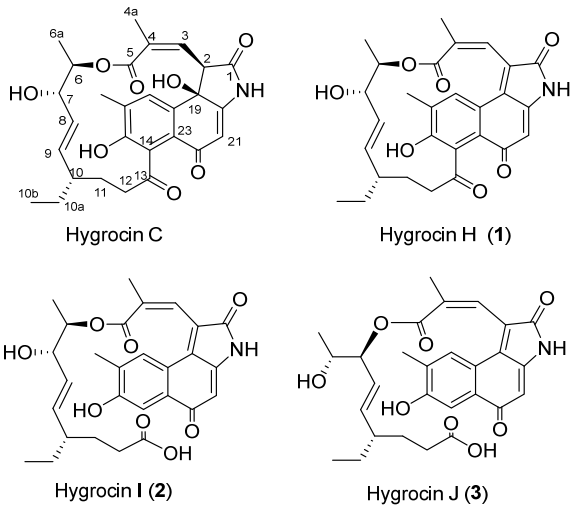


Figure 1. Structures of compounds **1** - **3** and hygrocin C.

[a] *Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, Jinan 250012, PR China. E-mail: yshen@sdu.edu.cn; Tel: 086-531-88382108*
‡These authors contributed equally to this work.
†Electronic Supplementary Information (ESI) available: See DOI: 10.1039/x0xx00000x

Table 1. ^{1}H and ^{13}C NMR data of compounds **1** - **3** in CD_3OD

Position	**1**		**2**		**3**	
	^{1}H *J* = Hz	^{13}C	^{1}H *J* = Hz	^{13}C	^{1}H *J* = Hz	^{13}C
1		172.4[a] (s)		171.4 (s)		171.5 (s)
2		Not observed		126.6 (s)		126.6 (s)
3	6.96 br s	128.4 (d)	7.54 s	129.8 (d)	7.53 d (1.2)	129.6 (d)
4		137.6 (d)		137.5 (s)		137.5 (s)
4a	2.25 d (1.3)	20.9 (q)	1.92 br s	16.3 (q)	1.92 d (1.4)	16.2 (q)
5		167.3 (s)		167.9 (s)		167.8 (s)
6	4.89 (overlapped in D_2O)	74.6 (d)	5.08 dq (6.1, 5.2)	75.3 (d)	3.98 dq (6.4, 6.4)	69.7 (d)
6a	0.94 d (6.4)	13.4 (q)	1.35 d (6.1)	16.7 (q)	1.26 d (6.4)	19.5 (q)
7	3.87 q (1.7)	71.2 (d)	4.20 t (5.2)	75.4 (d)	5.25 t (6.4)	81.2 (d)
8	4.61 dd (15.5, 9.1)	128.5 (d)	5.52 dd (15.5, 8.3)	131.3 (d)	5.57 m	127.6 (d)
9	5.38 dd (15.5, 1.5)	137.2 (d)	5.57 dd (15.5, 6.3)	138.2 (d)	5.58 m	141.2 (d)
10	1.59 m	43.7 (d)	1.95 m	45.5 (d)	1.98 m	45.7 (d)
10a	1.53 m,0.99 m	26.5 (t)	1.46 m, 1.31 m	29.1 (t)	1.56 m, 1.35 m	28.9 (t)
10b	0.74 t (7.3)	12.5 (q)	0.90 t (7.2)	12.2 (q)	0.89 t (7.3)	12.2 (q)
11	1.34 m, 1.20 m	31.8 (t)	1.75 m, 1.46 m	31.1 (t)	1.79 m, 1.56 m	31.2 (t)
12	2.82 m	39.5[a] (t)	2.28 m	33.1, (t)	2.33 m	33.5 (t)
13		211.9[a] (s)		177.9 (s)		178.5[a](s)
14		Not observed	7.44 s	113.7 (d)	7.41 s	113.8 (d)
15		156.1[a] (s)		159.7 (s)		159.7 (s)
16		132.8[a] (s)		131.8 (s)		131.7 (s)
16a	2.28 br s	17.0 (q)	2.24 s	16.3, (q)	2.20 s	16.6 (q)
17	7.67 s	131.2 (d)	7.43 s	131.5 (d)	7.38 s	131.4 (d)
18		131.2 (s)		122.6 (s)		122.6 (s)
19		134.1 (s)		137.3 (s)		137.2 (s)
20		155.8[a] (s)		153.9 (s)		153.8 (s)
21	5.93 s	106.0 (d)	5.89 s	106.1 (d)	5.87 s	106.2 (d)
22		186.8 (s)		186.3 (s)		186.3 (s)
23		129.4 (s)		131.7 (s)		131.7 (s)

[a]Those signals were estimated from HMBC correlations.

[b]Coupling constants are presented in Hertz. Unless otherwise indicated, all proton signals integrate to 1H.

Results and discussion

The molecular formula $C_{28}H_{29}NO_7$ of **1** was established by HRESIMS (*m/z* 492.2030 $[M + H]^+$). The ^{1}H NMR spectrum (Table 1) displayed the presence of four methyl groups (δ_H 0.74, t; 0.95, d; 2.28, br s and 2.25, s), two oxymethine protons (δ_H 4.89, m and 3.87, q) and five aromatic or double-bond protons (δ_H 7.67, s; 6.96, s; 5.93, s; 5.38, dd and 4.61, dd). The ^{13}C NMR and HMQC data of **1** revealed the presence of four methyl carbons (δ_C 13.4, 12.5, 17.0 and 20.9), three methylene carbons (δ_C 26.5, 31.8 and 39.5), two oxymethine carbons (δ_C 71.2 and 74.6), twelve aromatic or olefinic carbons (δ_C 106.0, 126.3, 128.4, 128.5, 128.4, 131.2, 132.8, 134.1, 137.6, 137.2, 156.1 and 155.8), and two carboxyls (δ_C 167.3 and 172.4), one α,β-unsaturated keto carbon (δ_C 186.8) and one keto carbon at δ_C 211.9 (ESI Figure S5 – S7). The ^{1}H and ^{13}C NMR spectral data of **1** were almost identical to those of hygrocin C.[5] The major difference between hygrocin C and **1** are the changes of chemical shifts at C-19 (δ_C 72.6 d in hygrocin C, δ_C 131.4 s in **1**), and C-2 (δ_C 53.5 d, δ_H 4.67 in hygrocin C, and δ_C 126.3 s in **1**), which indicated that the carbon-carbon double bond was formed between C-19 and C-2 due to dehydration. Additionally, the structure of **1** was further confirmed by HMBC and ^{1}H-^{1}H COSY correlations (Fig. 2, ESI Figure S8 – S9). The relative configurations of **1** was established similar to those of hygrocin C by coupling constants (*J* = 15.5 Hz) between H-8 and H-9 and the relative downfield shift of the allylic methyl group C-4a (δ_C 20.9).

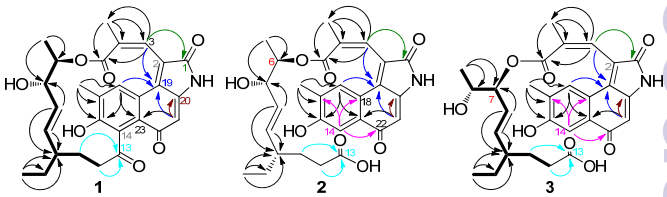


Figure 2. Selected ^{1}H-^{1}H COSY (▬) and HMBC (→) correlations for compounds **1** – **3**

Hygrocins I (**2**) and J (**3**) were obtained both as red powder with $[\alpha]_D^{20}$ -18 (*c* 0.40, CH_3OH) and $[\alpha]_D^{25}$ -30 (*c* 0.42, CH_3OH), and HRESIMS data indicated that **2** and **3** have the same molecular formula of $C_{28}H_{31}NO_8$ (*m/z* 510.2045 $[M + H]^+$). Detailed comparison of the NMR data (Table 1) of **2** and **1** revealed the apparent differences. The ^{1}H NMR spectra revealed the presence of an aromatic proton at δ_H 7.44 (H-14). The changes of chemical shift at C-14 (δ_C 128.4s in **1**, δ_C 113.7d in **2**) and C-13 (δ_C 211.9s in **1**; δ_C 177.9s in **2**) indicated the breakage of C-13/C-14 bond, which was further supported by the HMBC correlations from H-14 to C-16, C-18 and C-22

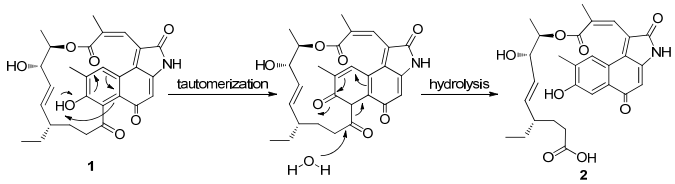
(Fig. 2). Thus, compound **2** was determined to be 13,14-*seco*-hygrocin H.

Hygrocin J (**3**), the 1D- and 2D-NMR data revealed that this metabolite represents a homologue of **2**, but differs in the ester linkage of side chain (Fig. 1 and 2). The downfield shift of H-7 (δ_H 5.25) and upfield shift of H-6 (δ_H 3.98) indicated the formation of a C-7/5 ester linkage instead of a C-6/5 in **3** (Table 1), which is similar to the difference between hygrocins E and F.[5] Therefore, compound 3 was determined to be 13,14-*seco*-2,19-dehydrated hygrocin F.

Hygrocins H-J were tested for their cytotoxicities against human tumor MDA-MB-231, PC3 and HeLa cell lines. Hygrocin H was found to be toxic to MDA-MB-231, PC3 and HeLa cell lines with IC_{50} of 2.4, 1.7, and 0.8 μM, respectively, while hygrocins I and J were inactive at the concentration of 50 μM, which suggested that the *ansa* ring was important for the biological activity.

The two *seco*-derivatives hygrocins I and J (**2** and **3**, respectively) may derive from a spontaneous reversed-Claisen reaction, in which deprotonation of phenolic oxygen and protonation of the alpha carbon leads to a highly conjugated, resonance-stabilized tautomer, which is then vulnerable to hydrolysis and ring opening (Scheme 1). Basic or acidic conditions might promote this reaction. We speculate that the high degree of conjugation in the tricyclic system of the hygrocins allows the formation of the tautomer and the otherwise difficult cleavage of the C-C bond between the phenolic moiety and macrocycle.

The intermediates of ansamycin have been observed in mutated pathways of rifamycin.[9] And the recently reported the *seco*-variants of ansamycins divergolides M and N are shunt products of the biosynthetic pathway and *seco*-divergolide L maybe formed by decarboxylation after spontaneous hydrolysis of the macrolide.[10] Whereas, *seco*-hygrocin congeners (**2** and **3**) are different in that the C-C bond at the aromatic ring is cleaved. Therefore, the *seco*-variants **2** and **3**, isolated from the strain SR101OEhgc1 in this study, represented the novel examples of natural *seco*-ansamycins.



Scheme 1. Models for the formation of seco-hygrocin H (**2**)

Experimental section

General experimental procedures

HRESIMS were carried out on an LTQ-Orbitrap XL. NMR spectra were measured on Bruker DRX-600 MHz NMR spectrometer (Bruker Daltonics Inc., Billerica, Massachusetts) with tetramethylsilane (TMS) as an internal standard. Reversed-phase (RP) C18 silica gel for column chromatography (CC) was obtained from Merck (Darmstadt, Germany) and Sephadex LH-20 from GE Amersham Biosciences (Piscataway, New Jersey). Silica gel (200-300 mesh) for CC and silica gel GF_{254} for TLC were purchased from Qingdao Marine Chemical Ltd. (Qingdao, China). High-performance liquid chromatography (HPLC) was performed using ZORBAX XDB-C18 (5 μm). Semi preparative column (9.4×250 mm). All solvents used were of analytical grade. Compounds were visualized under UV light and by spraying with H_2SO_4/EtOH (1:9, v/v) followed by heating.

Strain and fermentation

Strain SR101OEhgc1 was constructed by our previously work.[8] The strain was cultured and fermented for 11 d in petri dishes laid with *ca*. 20 mL ISP3 medium (1.5% agar, 2% oatmeal, 0.1% trace element solution, pH 7.2) with a total volume of 10 litres at 28°C.

Extraction and isolation

To extract the metabolites, the culture of SR101OEhgc1 was diced and extracted three times overnight with EtOAc/MeOH/AcOH (80:15:5, *v/v/v*) at room temperature and partitioned between EtOAc and doubly-distilled water until the EtOAc layer was colorless. The EtOAc soluble fraction was dried with sodium sulfate (anhydrous) and the solvent was removed under vacuum to afford the EtOAc extract. The EtOAc extract was sequentially solvent-partitioned into petroleum ether and methanol soluble extracts. The methanol extract (2.0 g) was subjected to CC over Sephadex LH-20 (140 g) eluted with acetone to obtain 8 fractions, i.e. Fr. 1-8. HPLC analysis indicated that Fr. 6 and Fr.7 contained compounds with differential absorption (ESI Figure S1). Fr. 6 (396 mg) was further subjected to MPLC over RP-18 silica gel 40 g, 12 subfraction were obtained from the elution of 30%-100 mL, 40%-100 mL, 50%-200 mL, 70%-100 mL and 100%-100 mL MeOH in water respectively, 16 mL for each gradient. 1-6 obtained from 30%, 7-12 from 40%, 13-24 from 50%, 25-31 from 70% and 32-35 from 100% MeOH. According to TLC results, 1-12, 13-15, 16-20, 21-31 and 32-35 were combined and marked as Fr. 6a, Fr. 6b, Fr. 6c (20 mg), Fr. 6d and Fr. 6e, respectively. HPLC analysis of the constituents of Fr. 6a-e indicated that compounds with differential absorption existed inFr. 6c. Fr. 6c (20 mg) was finally purified by semi-preparative reverse-phase HPLC (Agilent 1260 instrument; ZORBAX Eclipse XDB-C18 5 μm, column ID: 9.4 × 250 mm, flow rate: 4 mL/min, elution: CH_3CN/H_2O (35-65, *v/v*), UV detections at 274 nm) to afford 1 (t_R 7.7 min, 6 mg (ESI Figure S2). Fr. 7 (478 mg) was further subjected to MPLC over RP-18 silica gel 40 g, 12 subfraction were obtained and marked as Fr. 7a-7M. Fr. 7d (50 mg) was subjected to Sephadex LH-20 (80 g) eluted with acetone to obtain Fr. 7d1 (ESI Figure S3). Fr. 7d1 (12 mg) was finally purified by semi preparative reverse-phase HPLC (Agilent 1260 instrument; ZORBAX Eclipse XDB-C18 5 μm, column ID: 9.4 × 250 mm, flow rate: 4 mL/min, elution: CH_3CN/H_2O (40-60, v/v), UV detections at 274 nm) to afford 3 (t_R 8.5 min, 3 mg) and 2 (t_R 9.2 min, 3 mg) (ESI Figure S4).

Hygrocin H (**1**): yellow powder; $[\alpha]_D^{20}$ = [illegible] = +16.7 (*c* 0.35, CH_3OH). UV(MeOH) λ_{max}, 260, 295, 335, 380 nm. [illegible]

and ^{13}C NMR data, see Table 1. HRESIMS *m/z* 492.2030 $[M + H]^+$ (calcd. for $C_{28}H_{29}NO_7^+$, 492.2017).

Hygrocin I (**2**): red powder; $[\alpha]_D^{20}$ = -18 (*c* 0.40, MeOH); UV(MeOH) λ_{max}, 220, 260, 285, 335, 380 nm. ^1H and ^{13}C NMR data, see Table 1. HRESIMS *m/z* 510.2045 $[M + H]^+$ (calcd. for $C_{28}H_{29}NO_8^+$, 510.2122).

Hygrocin J (**3**): red powder; $[\alpha]_D^{20}$ = -30 (*c* 0.42, MeOH); UV(MeOH) λ_{max}, 220, 260, 285, 335, 380 nm. ^1H and ^{13}C NMR data, see Table 1. HRESIMS *m/z* 510.2045 $[M + H]^+$ (calcd. for $C_{28}H_{29}NO_8^+$, 510.2122).

Cytotoxicity Test

The *in vitro* antiproliferative activities were assessed with a sulforhodamine B (SRB) assay.[11] The test compounds and hygrocin C were dissolved in DMSO at 50 mM as a stock solution. Cells were seeded in triplicate in 96-well plates at a density of 5000 cells per well and incubated for 24 h in 0.1 mL of culture medium, leaving three wells without cell seeded as blank control. Then the medium in each well was exchanged with 0.1 mL of medium containing graded concentrations of compounds or same volume of DMSO for 24, 36, 48 or 72 h. Medium were discarded and 10% trichloroacetic acid (TCA) was added to cell monolayers and stained for 1 h at 4°C. TCA was removed by washed with distilled water for five times, after which 100 μL 4 mg/mL SRB (Sigma–Aldrich) was added and stained for 15 min at room temperature. After excess dye being removed by washing five times with 1% acetic acid, 200 μL 10 mM Tris base solution was used to dissolve protein-bound dye, then measured the OD at 570 nm wavelength by a microplate reader (M-3350, Bio-Rad). Growth inhibition rates were calculated by the following equation and Prism 5 (GraphPad Software, Inc.) was used to determine IC_{50}, which is defined as the concentration of compound that results in 50% growth inhibition at 72 h. Independent experiments were taken at least in duplicate to confirm the results. Growth inhibitory rate = $OD_{control\ well}$ - $OD_{sample\ well}$/$OD_{control\ well}$ - $OD_{blank\ well}$.

Conclusions

In summary, we have isolated and characterized three new hygrocin analogues from the LAL-family regulator gene *hgc1*-overexpressed strain SR101OEhgc1. In these compounds, hygrocin H showed toxicity to human cancer cell lines, but the *seco*-hygrocins I and J lost the activities with the inherent reactivity in ansamycin biosynthesis. This work not only demonstrates the overexpression of regulatory genes could be a useful strategy to increase antibiotic production, but also exhibits the unusual flexibility and diversity in ansamycin biosynthesis.

Acknowledgements

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