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Novel GRP78 Molecular Chaperone Expression Down-regulators JBIR-04 and -05 Isolated from *Streptomyces violaceoniger*

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Abstract In the course of our screening program for regulators of the expression of GRP78 molecular chaperone, JBIR-04 (**1**) and -05 (**2**) were isolated from *Streptomyces violaceoniger* 4541-SVS3 as congeners of prunustatin A (**3**). The structures of **1** and **2** were determined by the analyses of the spectroscopic data. These compounds mainly consist of an amino acid and amino acid derived α-hydroxy acid residues. **1** and **2** inhibited the expression of GRP78 induced by 2-deoxyglucose in human fibrosarcoma HT1080 cells, but their activities were highly reduced compared with those of **3** and SW-163A.

Keywords JBIR-04 and -05, *Streptomyces violaceoniger*, neoantimycin, prunustatin A, SW-163A, molecular chaperone, GRP78

GRP78 acts as a molecular chaperone in the endoplasmic reticulum (ER) to promote protein folding. The enhancement of the ER stress response has been proven to play a role in mechanisms of resistance to chemotherapy and hypoglycemic stress in solid tumors [1], while the reduction of the ER stress response is involved in the pathology of central nervous diseases such as Alzheimer's and Parkinson's diseases [2]. The ER stress response causes an increase in gene expression of a number of ER chaperones such as GRP78/Bip and GRP94 [3]. In the course of our screening program for regulators of GRP78 molecular chaperone expression, we have isolated versipelostatin [4], a new member of neoantimycin [5] family, prunustatin A (**3**) [6], and SW-163A (**4**) [7]. Further searching for congeners from the prunustatin A-producing strain resulted in the isolation of JBIR-04 (**1**) and -05 (**2**). In this paper, we report herein the isolation, structure elucidation and brief structure-activity correlations of **1** and **2**.

The prunustatin-producing strain *S. violaceoniger* 4541-SVS3 was cultured at 27°C for 5 days in 500-ml Erlenmeyer flasks containing a medium consisting of 2.5% starch, 1.5% soy bean meal, 0.2% dry yeast and 0.4% $CaCO_3$ (pH 6.2 before sterilization). The collected mycelial cake from fermented whole broth (2 liters) was extracted with Me_2CO (400 ml). The extract was evaporated *in vacuo*, and the residual aqueous concentrate was extracted with EtOAc (150 ml×3). The organic layer residue (0.36 g) was separated on a silica gel flash column (Purif-Pack SI-60, Moritex) with a *n*-hexane - EtOAc linear gradient system (0～100% EtOAc). The 50～100% EtOAc eluate was further purified by preparative HPLC using PEGASIL-ODS (Senshu Pak, 20 i.d.×150 mm) developed with 85% aqueous acetonitrile to give **1** (1.1 mg, Rt 9.0 minutes) and **2** (2.63 mg, Rt 8.5 minutes).

The physico-chemical properties of **1** and **2** are summarized in Table 1. **1** and **2** were isolated as colorless amorphous and pale yellow amorphous solids, respectively. The molecular formulas of **1** and **2** were established as $C_{33}H_{39}NO_{10}$ and $C_{33}H_{42}N_2O_{11}$, respectively, on the basis of HR-ESI-MS analysis.

The tabulated ^{13}C- and ^{1}H-NMR spectral data for **1** and **2**

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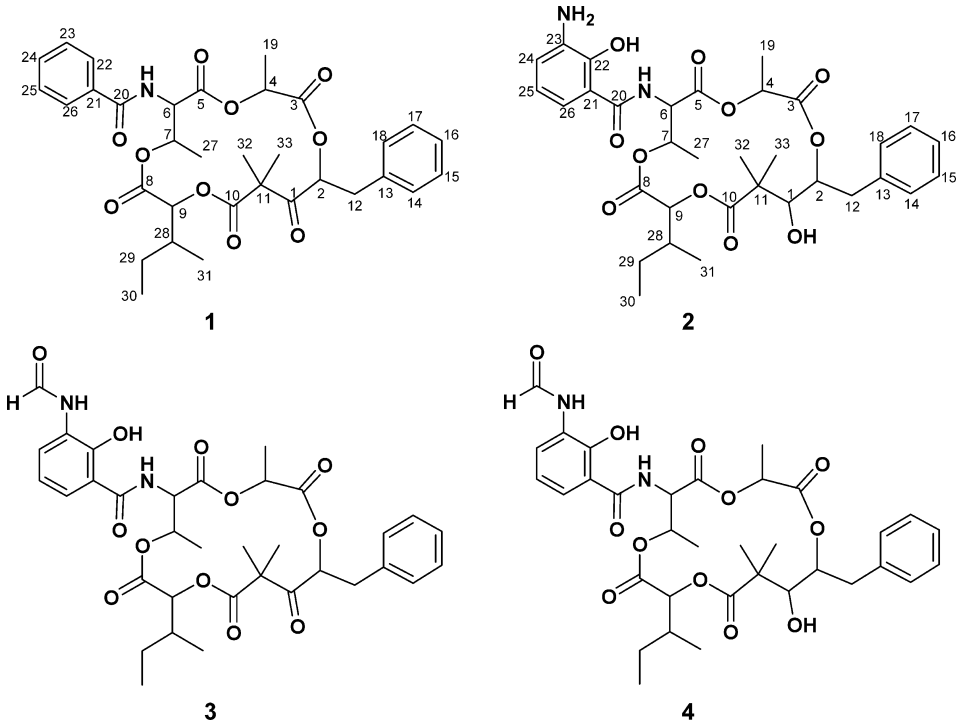

Fig. 1 Structures of JBIR-04 (**1**), JBIR-05 (**2**), prunustatin A (**3**) and SW-163A (**4**).

Table 1 Physico-chemical properties of JBIR-04 (**1**) and -05 (**2**)

	1	**2**
Appearance	Colorless amorphous solid	Pale yellow amorphous solid
Melting point	60∼62°C	104∼107°C
$[\alpha]_D^{22}$ (MeOH)	−28.6° (*c* 0.01)	−17.3° (*c* 0.03)
Molecular formula	$C_{33}H_{39}NO_{10}$	$C_{33}H_{42}N_2O_{11}$
HR-ESI-MS (*m*/*z*)		
found:	610.2664 $[M+H]^+$	643.2887 $[M+H]^+$
calcd:	610.2652	643.2867
UV λ_{max} (MeOH) nm (ε)	219 (10,000)	228 (37,300), 337 (5,800)
IR ν_{max} ($CHCl_3$) cm^{-1}	1750, 1720, 1670	3550, 3440, 1750, 1715, 1645

are shown in Table 2. The IR spectrum of **1** showed absorption of ester (ν_{max} 1750 and 1720 cm^{-1}) and amide (1670 cm^{-1}) groups. The direct connectivity of protons and carbons were established by the HSQC spectrum. The ^{1}H-^{1}H COSY and HMBC spectra established five partial structures (Fig. 2). The proton–proton correlations observed in the ^{1}H-^{1}H COSY include 14,18-H (δ_H 7.32), 15,17-H (δ_H 7.32) and 16-H (δ_H 7.25), revealing the presence of a benzene ring moiety. The ^{1}H-^{1}H couplings between an oxymethine proton 2-H (δ_H 5.784, δ_C 79.1) and methylene protons 12-H (δ_H 3.40, 3.15), together with ^{1}H-^{13}C long-range couplings in HMBC spectrum from 2-H, 12-H and 15,17-H to C-13 (δ_C 136.6) and 14,18-H to C-12 (δ_C 37.7) established a phenethyl residue. The protons of two singlet methyl groups 32-H (δ_H 1.42) and 33-H (δ_H 1.25) were each long-range coupled to an ester carbonyl carbon C-10 (δ_C 171.6), a quaternary carbon C-11 (δ_C 54.0) and a ketone carbonyl carbon C-1 (δ_C 203.7), which in turn were coupled to 2-H. These HMBC correlations indicated the successive connectivity of C-2, C-1 and C-11 as shown in Fig. 2. Thus, a 4-hydroxy-2,2-dimethyl-3-oxo-5-phenylpentanoic acid moiety was elucidated as a partial structure of **1** as shown in Fig. 2. A 2-hydroxypropionic acid moiety was established by ^{1}H-^{1}H correlation between

Table 2 ^{13}C (150 MHz)- and ^{1}H (600 MHz)-NMR data for **1** and **2** in $CDCl_3$

	1		**2**	
	δ_C	δ_H	δ_C	δ_H
1	203.7		79.2	3.20 (d, 11.7)
2	79.1	5.784 (dd, 10.9, 2.9)	72.2	5.48 (dd, 9.7, 5.6)
3	169.1		170.0	
4	69.9	5.33 (q, 6.8)	69.1	5.47 (q, 7.0)
5	168.6		168.2	
6	56.1	5.21 (dd, 9.1, 2.9)	55.4	5.10 (dd, 8.8, 2.1)
7	72.1	5.777 (dq, 2.9, 6.8)	72.4	5.72 (dq, 2.1, 6.5)
8	168.7		168.3	
9	75.9	5.05 (d, 9.4)	75.4	4.67 (d, 8.5)
10	171.6		177.2	
11	54.0		45.5	
12	37.7	3.40 (dd, 14.5, 2.9)	40.0	3.17 (dd, 14.1, 5.6)
		3.15 (dd, 14.5, 10.9)		2.93 (dd, 14.1, 9.7)
13	136.6		136.97	
14, 18	129.7	7.32 (m)	129.4	7.20 (d, 7.9)
15, 17	128.7	7.32 (m)	128.8	7.27 (t, 7.9)
16	127.2	7.25 (m)	127.0	7.20 (m)
19	16.7	1.26 (d, 6.8)	17.4	1.00 (d, 7.0)
20	168.2		171.0	
21	133.6		113.0	
22	127.5	7.91 (d, 7.3)	149.9	
23	129.0	7.51 (t, 7.6)	137.02	
24	132.5	7.58 (t, 7.6)	118.7	6.86 (d, 7.6)
25	129.0	7.51 (t, 7.6)	119.1	6.75 (t, 7.9)
26	127.5	7.91 (d, 7.3)	114.8	6.96 (d, 7.9)
27	16.6	1.41 (d, 6.8)	16.6	1.32 (d, 6.5)
28	36.4	2.03 (m)	36.2	1.96 (m)
29	24.6	1.51 (m)	24.9	1.52 (m)
		1.17 (m)		1.20 (m)
30	10.5	0.89 (t, 7.8)	10.7	0.88 (t, 7.2)
31	14.2	0.90 (d, 7.6)	14.5	0.89 (d, 6.8)
32	22.7	1.42 (s)	27.2	1.42 (s)
33	20.8	1.25 (s)	22.0	1.32 (s)
1-OH				3.63 (d, 11.7)
6-NH		6.95 (d, 9.1)		7.03 (d, 8.8)
22-OH				12.03 (br.s)

an oxymethine proton 4-H (δ_H 5.33, δ_C 69.9) and a methyl proton 19-H (δ_H 1.26), and ^{1}H-^{13}C long-range correlations between 19-H and an ester carbonyl carbon C-3 (δ_C 169.1). The coupling between 9-H (δ_H 5.05) and a carbonyl carbon C-8 (δ_C 168.7), along with the sequence from 9-H to 30-H (δ_H 0.89) through 28-H (δ_H 2.03), which was additionally coupled to a methyl proton 31-H (δ_H 0.90), and 29-H (δ_H 1.51, 1.17) observed in the ^{1}H-^{1}H COSY spectrum of **1** established a 2-hydroxy-3-methylpentanoic acid moiety as shown in Fig. 2. The sequence from an amide proton 6-NH (δ_H 6.95) to a methyl proton 27-H (δ_H 1.41) through an α-methine proton 6-H (δ_H 5.21, δ_C 56.1) and an oxymethine proton 7-H (δ_H 5.777) was revealed. In addition to these correlations, a long-range coupling from the methine proton 6-H to an ester carbonyl carbon C-5 (δ_C 168.6) was observed. These results indicated the presence of a threonine residue. The proton–proton correlations observed in the COSY among 22,26-H (δ_H 7.91), 23,25-H (δ_H 7.51)

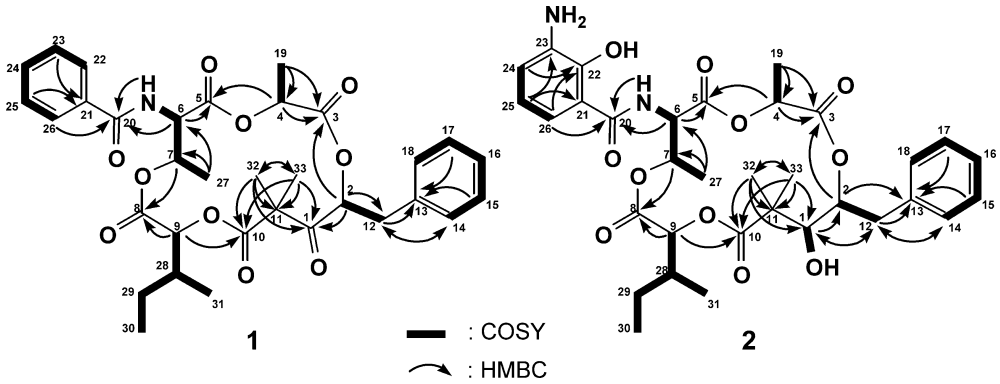

Fig. 2 Key correlations in 2D NMR of **1** and **2**.

and 24-H (δ_H 7.58) revealed the presence of a benzene ring moiety. The ^{1}H-^{13}C long-range couplings between 23,25-H and an aromatic quaternary carbon C-21 (δ_C 133.6), 22,26-H and an amide carbonyl carbon C-20 (δ_C 168.2) revealed a benzoic acid moiety. The connectivity of these partial structures was elucidated by the ^{1}H-^{13}C long-range couplings between 2-H and C-3, 4-H and C-5, 6-H and C-20, 7-H and C-8, and 9-H and C-10. In this manner, the structure of **1** was determined as shown in Fig. 1.

The IR spectrum of **2** showed presence of the hydroxyl (3550 cm^{-1}) and amine (3440 cm^{-1}) functions in addition to the ester (ν_{max} 1750 and 1715 cm^{-1}) and amide (1645 cm^{-1}) groups which were also observed in **1**. UV absorption at 337 nm suggested the presence of a salicylate moiety as a chromophore in **2** [5∼8]. As observed in **1**, **2** also consists of an amino acid and amino acid derived α-hydroxy acid residues, in which the lactic acid, the 2-hydroxy-3-methylpentanoic acid, and the threonine moieties are common between **1** and **2** as shown in Fig. 2. The 4-hydroxy-2,2-dimethyl-3-oxo-5-phenylpentanoic acid and benzoic acid moieties in **1** were replaced by 3,4-dihydroxy-2,2-dimethyl-5-phenylpentanoic acid and 3-amino-2-hydroxybenzoic acid moieties in **2**, demonstrated as follows. In the ^{1}H-^{1}H COSY spectrum of **2**, the spin couplings between a hydroxyl proton 1-OH (δ_H 3.63) and an oxymethine proton 1-H (δ_H 3.20, δ_C 79.2), and between another oxymethine proton 2-H (δ_H 5.48, δ_C 72.2) and methylene protons 12-H (δ_H 3.17, 2.93) were observed. In the same manner as observed in **1**, the presence of a benzene ring moiety was also revealed in **2** as shown in Fig. 2. In the HMBC spectrum, ^{1}H-^{13}C long-range couplings from 1-H to C-2 and C-12 (δ_C 40.0), and from 12-H to an aromatic carbon C-13 (δ_C 136.97), which in turn long-range coupled to aromatic protons 15,17-H (δ_H 7.27), established a 3-phenyl-propane-1,2-diol moiety. In addition, ^{1}H-^{13}C long-range couplings were observed from two singlet methyl groups 32-H (δ_H 1.42) and 33-H (δ_H 1.32) to an ester carbonyl carbon C-10 (δ_C 177.2), a quaternary carbon C-11 (δ_C 45.5) and oximethine carbon C-1. Thus, the 3,4-dihydroxy-2,2-dimethyl-5-phenylpentanoic acid moiety was elucidated as a partial structure of **2** as shown in Fig. 2. The connectivity of these partial structures was elucidated by the ^{1}H-^{13}C long-range couplings between 2-H and C-3, 4-H and C-5, 7-H and C-8, and 9-H and C-10, which gave a macrocyclic structure of **2** (Fig. 2). The proton spin couplings among aromatic protons 24-H (δ_H 6.86), 25-H (δ_H 6.75) and 26-H (δ_H 6.96) indicated a 1,2,3-trisubstituted benzene ring moiety. The aromatic proton 26-H was long-range coupled to a carbonyl carbon C-20 (δ_C 171.0) at the *peri* position, which indicated that this carbonyl carbon was substituted at C-21. ^{1}H-^{13}C long-range couplings were observed from 25-H to quaternary aromatic carbons that possessed chemical shifts at δ_C 113.0 and δ_C 137.02. From the substituent at C-21, these carbon signals should be assigned to C-21 (δ_C 113.0) and C-23 (δ_C 137.02), respectively. The aromatic protons 24-H and 26-H were long-range coupled to C-22 (δ_C 149.9). By taking into consideration the ^{13}C chemical shifts of these aromatic carbons and molecular formula of **2**, a hydroxyl and an amine residue should be substituted at positions C-22 and C-23, respectively. Thus, the remaining unit of **2** was revealed to be the 3-amino-2-hydroxybenzoic acid moiety. The connectivity between the macrocyclic substructure and the 3-amino-2-hydroxybenzoic acid moiety was determined by the ^{1}H-^{13}C long-range couplings between 6-NH (δ_H 7.03), 6-H (δ_H 5.10) and C-20.

The structure of **1** resembled that of **3**, but contained of the non-substituted benzoate residue instead of the 3-(formylamino)-salicylate moiety. The structure of **2** resembled that of **4**, lacking only a formyl residue. The 3-(formylamino)-salicylate substructure is the representative functional moiety in the antimycin family, which exhibits

antirespiratory and antioxidative activities [8]. Thus, these compounds are extremely useful tools for identifying antirespiratory activity.

Human fibrosarcoma cell line HT1080 cells that were transformed with the luciferase reporter gene under the regulation of the GRP78 promoter produced luciferase in four fold greater quantity than did the control when treated with 10 mM of 2-deoxyglucose. In this evaluation system, **1** and **2** reduced the expression of the reporter gene with IC_{50} values of 2407 and 282 nM, respectively. In the same manner, the structurally related compounds **3** and **4** exhibited inhibitory activity in this assay with IC_{50} values of 12 and 23 nM, respectively. Since the activity of **2** was 12 times less than that of **4** and **1** was 200 fold less than **3**, the hydroxyl and formylamino residues on the benzene ring play an important role in enhancing the inhibitory effect against GRP78 expression induced by 2-deoxyglucose. These results suggested that an antirespiratory effect is not the mode of action of these compounds. Although the mode of action of versipelostatin was investigated in detail [9], its target is still unknown. Clarifying the target of these compounds is expected to result in elucidating novel and undiscovered pathways of GRP78 expression.

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