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Absolute Structure of Prunustatin A, a Novel GRP78 Molecular Chaperone Down-Regulator

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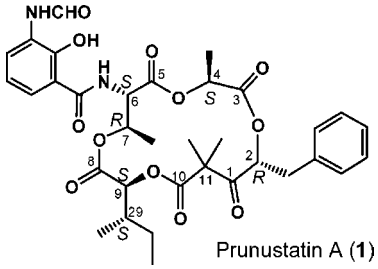
ABSTRACT



In the course of our screening program for regulators of a molecular chaperone GRP78 expression, we isolated a novel inhibitor of GRP78 expression, designated as prunustatin A, from *Streptomyces violaceoniger* 4521-SVS3. The planar structure of prunustatin A was determined to be an oxidized type of the neoantimycin family. Its absolute stereochemistry was established to be 2*R*, 4*S*, 6*S*, 7*R*, 9*S*, and 29*S* by analyzing chemically degraded components obtained from the derivative of prunustatin A.

GRP78 acts as a molecular chaperone in the endoplasmic reticulum (ER) to promote protein folding. The enhancement of ER stress response has been proven to play a role in mechanisms of resistance to chemotherapy and hypoglycemic stress in solid tumors.[1] The ER stress response causes an increase in gene expression of a number of ER chaperones such as GRP78/Bip and GRP94.[2] Thus, substances that directly down-regulate *grp78* transcription are expected to be promising drugs for the treatment of cancer. In the course of our screening program for regulators of a molecular chaperone GRP78 expression, we isolated a novel inhibitor of GRP78 expression, designated as prunustatin A (**1**), from *Streptomyces violaceoniger* 4521-SVS3 (Figure 1). The planar structure of prunustatin A has been elucidated by a variety of NMR spectral analyses.[3] **1** is a member of neoantimycin family,[4,5] but the absolute stereochemistry of these analogues was not fully investigated. To the best of

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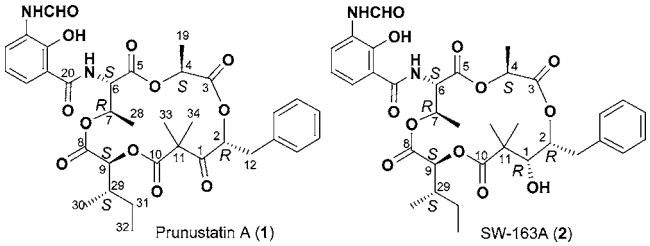

Figure 1. Structure of prunustatin A (**1**) and SW-163A (**2**).

our knowledge, few compounds such as **1**, verrucosidins,[6,7] and versipelostatin[8−10] were reported to inhibit the expression of GRP78 elicited by glucose deprivation. Since verucoccidin and the formylaminosalicylate moiety of antimycins were reported as the respiratory inhibitor, the mode of action mechanisms of **1** is attributed to respiratory inhibition. Recently, we succeeded in isolating the derivative of **1**, of which the formylaminosalicylate moiety was replaced by benzoate moiety.[11] Contrary to our speculation, the activity to suppress the expression of GRP78 still remained in this derivative, which suggested the exsistance of other target molecules. In addition, the exact target molecules of versipelostatin have not been proven yet. Since the stereochemistry of active substances frequently contributes to their mode of action mechanisms, the elucidation of the absolute configuration of **1** is strongly desired, which will provide the three-dimensional structural information between **1** and versipelostatin.[10] In this paper, we report herein the absolute stereochemistry of **1**.

Preparation of Hydrolyzed Components. Despite recent progress in the NMR-based configuration analysis of natural products,[10,12,13] chemical degradation in combination with partial synthesis is still considered the sole reliable method for determining the stereochemistry. As **1** mainly consists of cyclized amino and α-hydroxy acid moieties, the determination of the stereochemistry of **1** was performed by analyzing the absolute configuration of each component obtained by alkaline and acidic hydrolysis. However, the racemization could occur at the C-2 position in **1** during the reaction of alkaline hydrolysis, so that we employed SW-163A (**2**)[14] instead of **1**, which was also produced by the prunustatin-producing strain and is considered to be a reduced type of **1** at the C-1 position.

2 also consists of an amino acid and amino acid derived α-hydroxy acid residues classified into a 3,4-dihydroxy-2,2-dimethyl-5-phenylpentanoic acid, a lactic acid, a 2-hydroxy-3-methylpentanoic acid, a threonine, and a 3-formylamino-2-hydroxybenzoic acid moieties. **2** (10 mg) was hydrolyzed with 3 N NaOH for 10 min at 95 °C. Then, 6 N HCl was added to this reaction mixture to adjusted the pH to 2−3 followed by the extraction with ethyl acetate. The aqueous layer was further subjected to acid hydrolysis for 12 h to obtain threonine (**3**) and lactic acid (**4**) residues. The organic layer from alkaline hydrolysis was concentrated to dryness, and the dried residue was dissolved in saturated $NaHCO_3$ aqueous solution followed by the extraction with diethyl ether to provide 5-benzyl-4-hydroxy-3,3-dimethyldihydrofuran-2-one (**5**, 3.2 mg, 99% purity), which was spontaneously converted from 3,4-dihydroxy-2,2-dimethyl-5-phenylpentanoic acid in acidic condition.[15] The residual aqueous layer was adjusted to pH 2−3 with 6 N HCl and re-extracted with EtOAc to afford 2-hydroxy-3-methylpentanoic acid (**6**, 1.3 mg). This protocol seemed to afford **6** with no major hydrolysis byproducts (more than 90% purity) according to the ^{1}H NMR spectrum of **6**.

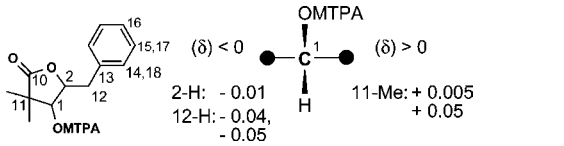

Figure 2. MTPA ester of 5-benzyl-4-hydroxy-3,3-dimethyldihydrofuran-2-one (**5**) and application of modified Mosher's method.

Absolute Stereochemistry of the Threonine (3) and Lactic Acid (4) Residues. The absolute configuration at C-6 and C-7 in **3** was determined by Marfey's method,[16] which is applicable for the determination of the stereochemistry of amino acids. After acid hydrolysis, the reaction solution was adjusted to neutral and evaporated in vacuo. The residue was dissolved in 0.1 M $NaHCO_3$ and successively 10 mM N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide (FDAA) in acetone was added. The mixture was kept at 70 °C for 10 min with frequent shaking. After workup with the addition of 0.2 N HCl, the filtered reaction mixture was subjected to HPLC (Cosmosil ODS 5C18-MS, 4.6 i.d. × 250 mm, 15% aq CH_3CN with 0.05% TFA). The standard D- and L-threonine and D- and L-*allo*-threonine were reacted with FDAA in the same manner as described above. The threonine residue (**3**) obtained from **2** was determined to be L-threonine (retention time: FDAA conjugated **3**, 42.5 min; L-Thr, 42.5 min; D-Thr, 83.0 min; L-*allo*-Thr, 46.2 min; D-*allo*-Thr, 57.5 min).

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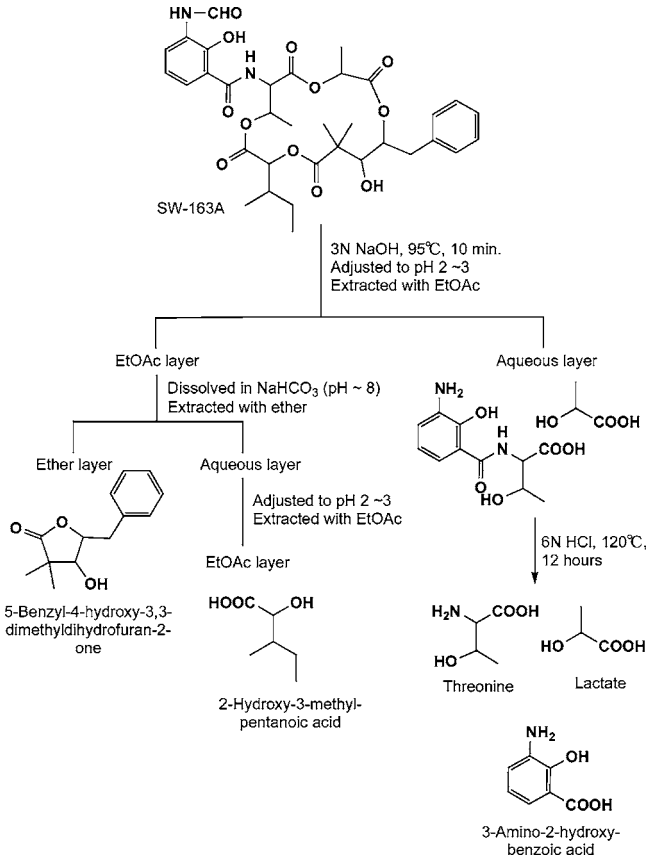
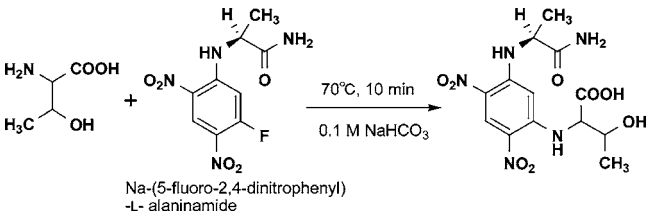
Scheme 1. Degradation of **2**



The stereochemistry of the C-4 position which was included in the lactate moiety was determined by utilizing a chiral column according to the method described previously as follows.[17] The acid hydrolysate, which was independently prepared, was neutralized with 5 N NaOH and evaporated to dryness, and the residue was taken up in DMF and treated with *p*-bromophenacyl bromide in the presence of KF at 50 °C for 12 h. The reaction mixture was concentrated to dryness and separated by preparative silica gel-TLC (*n*-hexane:EtOAc = 3:1) to give *p*-bromophenacyl lactate. According to the retention time in HPLC on a chiral stationary phase (Chiralcel OJ, 4.6 i.d. × 250 mm; *n*-hexane: 2-propanol = 9:1), the lactate derived from **2** was elucidated to be L-lactate (observed, 46.4 min; standard L-lactate derivative, 46.4 min; standard D-lactate derivative, 49.6 min).

Scheme 2. Synthesis of the FDAA Derivative of **3**



Conclusively, the stereochemistry at the C-4 position in **2** was established as *S*.

Determination of Stereochemistry of the Lactone Moiety (5). The absolute configuration at the C-1 position in **2** was established by modified Mosher's method[18] as follows. The lactone compound (**5**), which was obtained from the ether layer of alkaline hydrolysis of **2**, was reacted with (+)-MTPA or (−)-MTPA chloride to give (*R*)- and (*S*)-MTPA ester, respectively (Table 1). The proton chemical

Table 1. ^{1}H (500 MHz) NMR Data for the (*S*)- and (*R*)-MTPA Esters of **5**

no.	*S*-MTPA ester δ_H (multiplicity, J = Hz)	*R*-MTPA ester δ_H (multiplicity, J = Hz)
2-Me	1.33	1.32
	1.11	1.06
4	4.77	4.78
5	2.785 (dd, J = 14.5, 9.5)	2.825 (dd, J = 14.5, 9.5)
	2.66 (dd, J = 14.5, 4.0)	2.71 (dd, J = 14.5, 4.0)
6	7.115 (dd, J = 7.5, 1.5)	7.158 (dd, J = 7.5, 1.5)
7	7.425 (m)	7.425 (m)
8	7.54 (m)	7.53 (m)

shifts at the C-2 and C-12 positions in the (*R*)-MTPA ester derivative of **5** appeared at a lower field than those of the (*S*)-MTPA ester derivative. On the other hand, the proton chemical shifts of both singlet methyl moieties located at the C-11 position in the (*R*)-MTPA ester derivative were observed at a higher field than those of the (*S*)-MTPA ester derivative. Thus, the absolute stereochemistry at the C-1 position in **5** was deduced to be the *R* configuration.

The stereochemistry at the C-2 position in **2** was confirmed by the determination of the relative configuration between C-1 and C-2 employing the *J*-based method.[19] The small coupling constant values for $^3J_{6H-7H}$ revealed that these protons are in the *gauche* orientation. Moreover, the small coupling constants ($^2J_{1C-2H}$ and $^2J_{2C-1H}$) indicate that H-1 and an oxygen atom at C-2, and H-2 and an oxygen atom at C-1 are both in the *anti* location as shown in Figure 3.

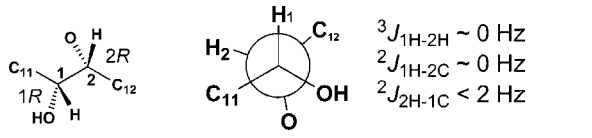


Figure 3. The absolute stereochemistry of C-1 and C-2 in **2**.

Considering these relative stereochemistries, the C-2 position was assigned as *R* by comparing with the absolute configuration of C-1 (*R*).

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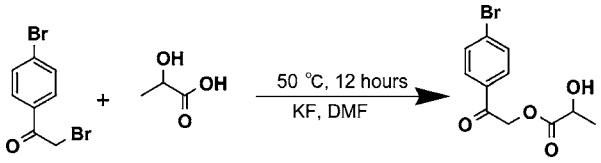
Scheme 3. Preparation of the FDAA Derivative of **3**



Determination of the Stereochemistry of the 2-Hydroxy-3-methylpentanoic Acid Moiety (6). The absolute configuration of the stereogenic center at C-29 and C-9 can be determined by using NOE analysis combined with *J* coupling analysis. The relative configuration between C-29 and C-9 was determined as follows. A large $^3J_{H-H}$ coupling constant (10 Hz) between 9-H and 29-H suggested that these protons were in the *anti* orientation. In the same way, small $^3J_{C-H}$ coupling constants (~2 Hz) between 9-H and C-30 ($^3J_{30C-9H} \approx 2$ Hz) and 9-H and C-31 ($^3J_{31C-9H} \approx 2$ Hz) suggested that C-30 and C-31 were both in the *gauche* orientation and two possible relative structures were constructed as shown in Figure 4 A,B. An NOE between the

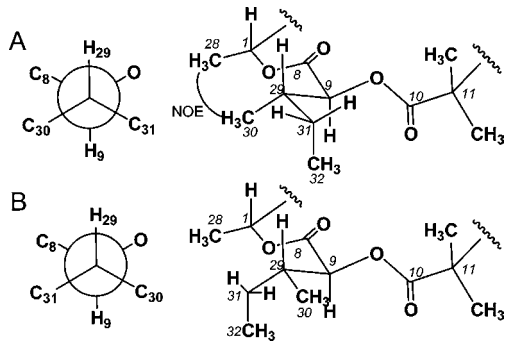


Figure 4. Relative stereochemistry of C-9 and C-29 in **2**.

doublet methyl signal of 28-H (δ_H 1.31) and the doublet methyl signal of 30-H (δ_H 0.87) in the high-resolution NOESY spectrum reasonably explains the relative configuration at C-9 and C-29 to be *S** and *S**, respectively.

An optical rotation (−33.8, *c* 0.18, in $CHCl_3$) of 2-hydroxy-3-methylpentanoic acid (**6**) obtained from the organic layer of the alkaline hydrolysis of **2** established the absolute structure unambiguously as 9*S* and 29*S*, respectively (reference data for (2*S*,3*S*)-2-hydroxy-3-methylpentanoic acid: $[\alpha]^D$ −21.6 (*c* 1.0, in $CHCl_3$)).[20,21] The corresponding ^{1}H NMR spectrum and HPLC retention time between the methyl ester derivative of **6** and the authentic sample of (2*S*,3*S*)-methyl 2-hydroxy-3-methylpentanoate, which was derived from L-isoleucine, also supported the stereochemistry of **6**.

Absolute Stereochemistry of 1. To determine the final stereochemical structure of **1**, we applied the Dess−Martin Periodinane (DMP)[22] to obtain the selective oxidized type of **2** at the C-1 position. The optical rotation of the oxidized type of **2** [+22.8 (*c* 0.24, $CHCl_3$)] was almost the same as that of **1** [+21.2 (*c* 0.01, $CHCl_3$)]. The ^{1}H NMR data and MS spectrum (*m/z*) and retention time of analyzed HPLC supported that the oxidative type of **2** possesses the same stereochemistry as **1**. The reduction of the ketone moiety in **1** with $NaBH_4$ unexpectedly afforded only one product. The ^{1}H NMR spectrum of this reduced product from **1** completely corresponded with that of **2**. Conclusively, the absolute stereochemistry of prunustatin A was determined as 2*R*, 4*S*, 6*S*, 7*R*, 9*S*, and 29*S* as shown in Figure 1. Although the planar structures of **1** and **2** are closely similar to that of neoantimycin, it is interesting that the absolute stereochemistry at C-1 and C-2 of **1** and **2** is opposite that reported for neoantimycin (1*R* and 2*R*).[23]

1 showed more potent inhibitory activity than **2** against GRP78 expression induced by 2-deoxyglucose stimulation in HT1080 cells which were transformed by the luciferase reporter gene inserted under GRP78 promoter. Thus, the redox state at the C-1 position is considered to be essential for exhibiting stronger biological activity. **1** belongs to the neoantimycin family, which is well-known as a respiratory inhibitor. In our preliminary biological studies, mitochondrial respiratory inhibitors specifically suppressed the induction of GRP78 expression elicited by glucose starvation, but not by chemically inducing ER stresses such as tunicamycin treatment. It showed that mitochondrial function plays an significant role in exerting ER stress responces, and it also gave us an new idea for targeting a solid tumor. Studies on detailed biological activities of **1** are now underway.

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Supporting Information Available: Selected spectra used for configuration assignments of **1** and **2**; ^{1}H NMR of **1**, **2**, **5**, **6**, *R*- and *S*-MTPA esters of **5**, standard (2*S*,3*S*)-2-hydroxy-3-methyl pentanoic acid, the oxidized derivative of **2**, and reduced derivative of **1**, NOESY, CT-HMBC, *J*-resolved HMBC for measurement of $^{2,3}J_{C-H}$ of **2**, MS spectrum of **5**, **6**, and the MTPA ester of **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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