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NOTE

Novel thioviridamide derivative—JBIR-140: heterologous expression of the gene cluster for thioviridamide biosynthesis

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Thioviridamide (**1**) is an *N*-acylated undecapeptide antibiotic that induces apoptosis selectively in E1A-transformed cells.[1] The most unique structural feature of thioviridamide is the presence of five thioamide bonds between the amino acids.[2] Recently, the thioviridamide biosynthesis gene cluster of *Streptomyces olivoviridis* NA05001 was identified, and the heterologous expression of thioviridamide in *Streptomyces lividans* TK23 was demonstrated.[3] Thioviridamide is synthesized by the posttranslational modification of a ribosomal precursor peptide containing a VMAAAASIALHC sequence.[3] Heterologous expression of a bacterial artificial chromosome (BAC) clone prepared from *S. olivoviridis* OM13 containing the entire gene cluster for thioviridamide biosynthesis in *S. avermitilis* SUKA17 strain,[4,5] which is the suitable host for heterologous expression, resulted in the expression of a novel thioviridamide derivative, JBIR-140 (**2**), together with thioviridamide. Here, we report the isolation, structure determination and biological activity of **2**.

The BAC genomic library of *S. olivoviridis* OM13 was prepared according to a previously reported method.[4] *S. olivoviridis* OM13 genome was partially digested with *Bam*HI in an agarose gel and then subjected to contour-clamped homogeneous electric field electrophoresis. Fragments (size, 100–130 kb) were then excised from the gel. The purified DNA fragments were ligated to the *Bam*HI fragment of the integrating BAC vector pKU503.[5] The ligated DNAs were transformed into *Escherichia coli* NEB 10-β cells (New England Biolabs, Ipswich, MA, USA) by electroporation.[4,5] Each BAC clone was stored in five 384-well plates containing Plusgrow II (100 μg ml^{-1} ampicillin and 20% glycerol) at − 80 °C. Clones carrying the entire gene cluster for thioviridamide biosynthesis were screened by PCR amplification using two pairs of primers corresponding to the upstream and downstream regions of the gene cluster (upstream primer pair, forward: 5′-CA GATGGACACGTACACGCAGAC-3′ (corresponding to the region from nucleotides 5539 to 5561 of the thioviridamide biosynthesis gene cluster[3]) and reverse: 5′-CACGTTCGTAGTAGCTGTCGGA GA-3′ (6187–6164[3]); downstream primer pair, forward: 5′-AGT GGAGCGGTGCTACGACATC-3′ (28 124–28 145[3])) and reverse: 5′-T CCGGTACAGCTTCTTGTACTCCA-3′ (27 567–27 544[3])). The amplification was performed under the following conditions: denaturation at 95 °C for 5 min, 35 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 60 s, and a final incubation at 72 °C for 5 min, and then soaked at 12 °C. One of the BAC clones, pKU503thvP3-F8 (inset size: 71 900 bp), contained the entire gene cluster for thioviridamide biosynthesis. Unmethylated pKU503thvP3-F8 was prepared by transforming the plasmid into *E. coli* GM2929 *hsdS*::Tn*10* cells.[5] The resultant DNA preparation was introduced into *S. avermitilis* SUKA17 by polyethylene glycol-assisted protoplast transformation.[4,5] Integration of the plasmids was confirmed by neomycin resistance and PCR analysis using the above-mentioned primer pairs.

SUKA17 carrying pKU503thvP3-F8 was cultivated in 50-ml test tubes, each containing 15 ml of a seed medium consisting of 0.5% glucose (Kanto Chemical, Tokyo, Japan), 1.5% soybean meal (Nisshin Oillio Group, Tokyo, Japan) and 0.5% yeast extract (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at pH 7.5 (adjusted before sterilization). The test tubes were shaken on a reciprocal shaker (320 r.p.m.) at 28 °C for 2 days. Aliquots (2.5 ml) of the culture were transferred into 500-ml baffled Erlenmeyer flasks filled with 100 ml of a production medium consisting 4.0% β-cyclodextrin (Kanto Chemical, Tokyo, Japan), 0.5% glycerin, 2.0% pharmamedia (Traders Protein, Lubbock, TX, USA), 5 mg l^{-1} $CuSO_4$, 5 mg l^{-1} $MnCl_2$ and 5 mg l^{-1} $ZnSO_4$ at pH 7.2 (adjusted before sterilization), and were cultured on a rotary shaker (180 r.p.m.) at 28 °C for 5 days.

The fermentation broth (2 l) was centrifuged, and the pelleted mycelial cake was extracted with acetone (400 ml), two times. The mycelium was removed by filtration, and the acetone extract was evaporated under reduced pressure. The residual aqueous concentrate

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was partitioned between EtOAc and water (100 ml) three times. The combined EtOAc extract was evaporated to dryness and the dried residue (867 mg) was applied on a normal-phase medium-pressure liquid column (Purif-Pack SI-30; Shoko Scientific, Yokohama, Japan), and successively eluted using a hexane-EtOAc solvent system (0, 5, 10, 20 and 25% EtOAc), followed by a $CHCl_3$-MeOH solvent system (0, 2, 5, 10, 20 and 50% MeOH). The eluate (50% MeOH, 37 mg) was further purified by preparative reversed-phase HPLC using a CAPCELL PAK C_{18} MGII column (5.0 μm, 20 i.d. × 150 mm; Shiseido, Tokyo, Japan) fitted with a 2996 photodiode array detector (Waters, Taunton, MA, USA) and a 3100 mass detector (Waters), and was developed with 75% aqueous MeOH containing 50 mM ammonium formate for 20 min (flow rate, 10 ml min^{-1}), followed by 90% aqueous MeOH containing 50 mM ammonium formate for 20 min (flow rate, 10 ml min^{-1}) to yield **1** (2.5 mg, retention time = 20.7 min) and **2** (15.7 mg, retention time = 31.0 min).

Compound **2** was obtained as a colorless amorphous powder ($[\alpha]_D^{24} - 162$ (*c* 0.17, in MeOH)]; UV λ_{max} (ε) in MeOH: 247 (28 900) and 272 (31 400) nm). The molecular formula was determined by high-resolution ESI-MS to be $C_{53}H_{88}N_{14}O_9S_7^+$ (found: 1288.4912 [M^+], calculated for $C_{53}H_{88}N_{14}O_9S_7^+$: 1288.4898), indicating that it was smaller than **1**, which had additional C_3H_4O atoms. The presence of hydroxy and amide moieties in **2** was established from its IR spectrum (ν_{max} (attenuated total reflection): 3450, 1670 and 1520 cm^{-1}).

Direct connectivity between the protons and carbons was established from the heteronuclear single-quantum coherence spectrum. The ^{13}C and ^1H NMR spectroscopic data for **2** are listed in Table 1. The ^1H and ^{13}C NMR spectra of **2** resembled those of **1**, which indicated that **2** was produced by the heterologous expression system. Therefore, **2** was confirmed as biosynthesized by the RiPS (ribosomal peptide synthesis) pathway. Taking into consideration the NMR spectra and the biosynthetic gene cluster, the amino-acid residues and the sequence of **2** except for *N*-terminal cap moieties were concluded to be identical to that of **1**.

Analyses of the double quantum-filtered-COSY and constant time-HMBC[6] spectra reconfirmed the presence of a Val, a Met, an Ile, a Leu, a Cys-like, a β-hydroxy-N^1, N^3-dimethylhistidinium (hdmHis) and five Ala amino-acid residues, in addition to an ethenamine moiety, as shown in Figure 1b. A sulfide linkage between the two partial structures was elucidated by ^1H–^{13}C long-range coupling from an olefinic proton (δ_H 5.47), which was in turn ^1H–^1H coupled to an

Table 1 ^{13}C and ^1H NMR spectroscopic data for JBIR-140 (2) in CD_3OD

	δ_C	δ_H, multiplicity (J, in Hz)		δ_C	δ_H, multiplicity (J, in Hz)
LA			avCys		
1	173.4		1	173.7	
2	65.3	4.48, q (7.3)	2	53.5	3.92, dd (10.3, 3.5)
3	25.9	1.46, d (7.3)	3	36.6	3.18, dd (11.5, 10.3)
Val′					2.99, dd (11.5, 3.5)
1	202.9		5	97.9	5.47, d (7.6)
2	64.4	5.47, d (5.5)	6	135.0	7.26, d (7.6)
3	34.6	2.26, dq (6.9, 5.5)	Ile		
4	20.2	0.96, d (6.9)	1	174.6	
5	18.7	0.94, d (6.9)	2	63.0	4.00, d (11.1)
Met′			3	37.1	1.96, m
1	202.8		4	27.3	1.68, m
2	65.2	5.48, dd (7.5, 6.1)			1.30, m
3	36.5	2.32, dd (14.2, 7.5)	5	11.2	0.99, t (7.2)
		2.19, dd (14.2, 6.1)	6	15.9	1.02, d (6.9)
4	31.0	2.56, m	Ala 5		
6	15.4	2.08, s	1	176.0	
Ala′ 1			2	53.6	4.02, q (6.8)
1	204.3		3	17.4	1.43, d (6.8)
2	61.5	5.31, q (6.2)	Leu		
3	20.4	1.60, d (6.2)	1	174.5	
Ala′ 2			2	53.0	3.99, m
1	205.3		3	40.4	1.52, m
2	62.1	5.24, q (6.1)	4	25.7	1.68, m
3	20.5	1.64, d (6.1)	5	23.8	0.86, d (6.4)
Ala′ 3			6	20.9	0.81, d (6.4)
1	207.6		hdmHis		
2	61.4	5.30, q (6.9)	CO	167.6	
3	20.3	1.67, d (6.9)	α	58.8	4.26, d (8.7)
Ala 4			β	62.6	5.97, d (8.7)
1	174.3		2	138.7	8.79, s
2	57.6	4.61, q (7.1)	4	135.0	
3	16.9	1.55, d (7.1)	5	124.1	7.64, s
			N^1-Me	36.4	3.95, s
			N^3-Me	35.0	4.01, s

Abbreviations: avCys, *S*-(2-aminovinyl)cysteine moiety; hdmHis, β-hydroxy-N^1, N^3-dimethylhistidinium; LA, N-terminal lactate moiety.
NMR spectra were recorded on a Varian NMR System 500NB LM (Agilent Technologies, Santa Clara, CA, USA) in CD_3OD with the residual solvent peak as the internal standard (δ_C 49.0, δ_H 3.31 p.p.m.).

olefinic proton (δ_H 7.26, $J=7.6$ Hz), to the β-methylene carbon (δ_C 36.6) in the cysteine-like moiety, no coupling between the olefin proton and the β-methylene protons (δ_H 3.18, 2.99) and the molecular formula of **2**, to generate an *S*-(2-aminovinyl)cysteine moiety (avCys). An *N*-terminal lactate moiety (LA) was also established instead of 2-hydroxy-2-methyl-4-oxopentanoic acid in **1** as follows: the ^1H–^1H spin correlation between a methyl proton (δ_H 1.46) and an oxymethine proton (δ_H 4.48), as well as a ^1H–^{13}C long-range coupling between the oxymethine proton and an amide carbonyl carbon (δ_C 173.4), established the presence of LA.

Three-bond correlations from α-methine protons to thioamide carbons established five thioamide linkages between Ala 4 and Ala′ 3, Ala′ 3 and Ala′ 2, Ala′ 2 and Al ′1, Ala′ 1 and Met′, and Met′ and Val′. To finalize the structure of **2**, five amide bonds between Val′ and LA, Ile and avCys, Ala5 and Ile, Leu and Ala5 and hdmHis and Leu were identified by three-bond couplings from α-methine protons to amide carbonyl carbons. A ^1H–^{13}C long-range coupling from an olefin proton (δ_H 7.26) to a carbonyl carbon (δ_C 167.6) indicated the presence of an additional amide linkage between the carbonyl group of hdmHis and the ε-amino group of avCys. The two partial structures should be directly joined from the molecular formula of **2**. Finally, an amide linkage between avCys and Ala4 established the planar structure of **2**, an *N*-terminal lactate derivative of thioviridamide (**1**), as shown in Figure 1a. The *N*-terminal 5-dimethyl-2-oxohexanoate of polytheonamides A and B and the *N*-terminal lactate of epilancin 15X have been reported to be derived from threonine and serine, respectively.[7,8] In the case of thioviridamide, although heterologous expression of the thioviridamide biosynthesis gene cluster resulted in the production of both **1** and **2**, *S. olivoviridis* OM13 expressed only **1**. Posttranslational modification genes related to *N*-terminal modification have not been identified in the thioviridamide biosynthesis gene cluster. Further biosynthetic studies of **1** and **2** are in progress.

The absolute configurations of the standard amino-acid residues in **2** were determined using the Marfey's method.[9] Compound **2** was subjected to complete acid hydrolysis (6 N HCl, 110 °C for 16 h), followed by reaction with FDAA (N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-alaninamide)

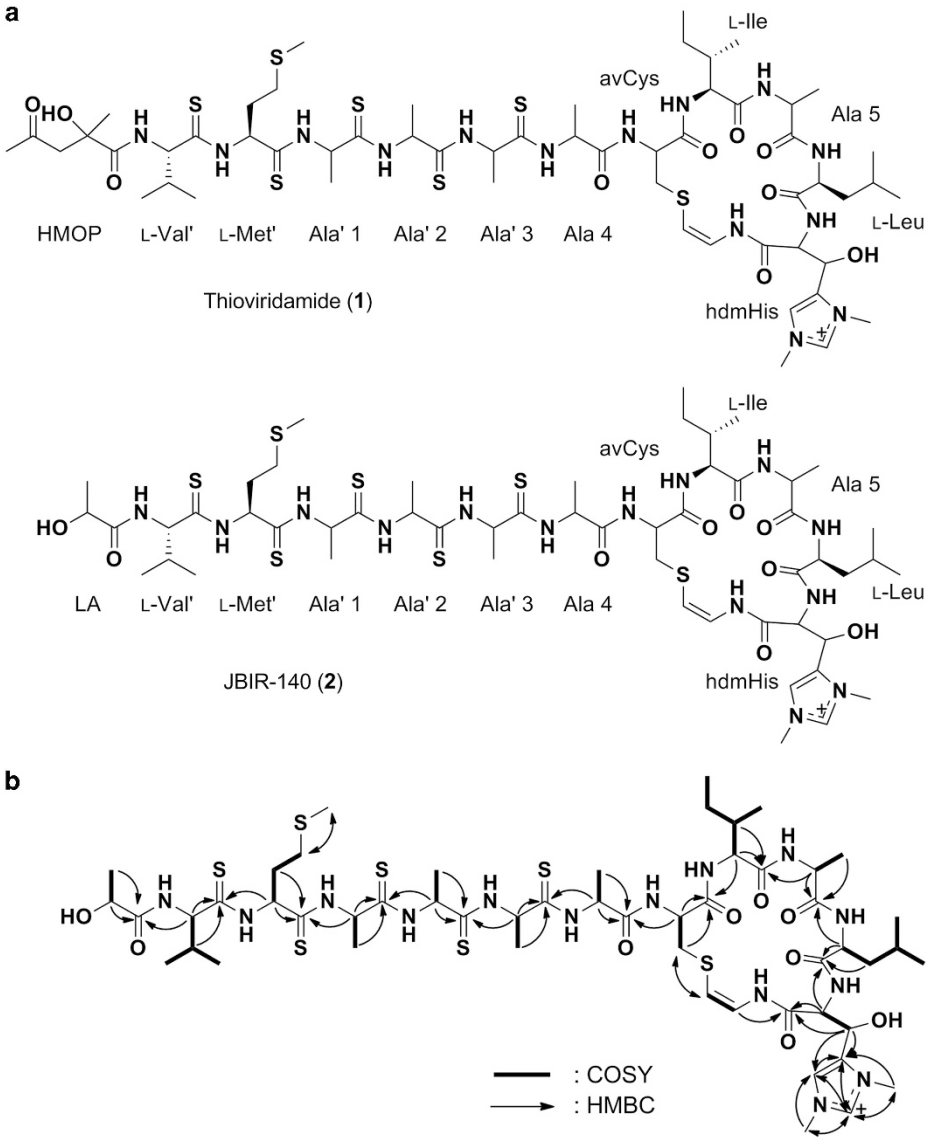

Figure 1 (**a**) Structures of thioviridamide (**1**) and JBIR-140 (**2**). (**b**) Correlations in the double quantum-filtered-COSY (DQF-COSY; bold lines) and constant time-HMBC spectroscopy (CT-HMBC; arrows) spectra of **2**.

to obtain the respective FDAA derivatives. The retention times of these FDAA amino-acid derivatives were established by monitoring the UV absorption at 340 nm using ultra-performance liquid chromatography and negative-ion mode ESI-MS. All derivatives were identified based on a comparison of their retention times, molecular formulae and UV spectra with those of standard amino acids derived from FDAA-conjugated compounds. The absolute configurations of the standard amino acids in **2** were identified as L-Val, L-Met, L-Ile and L-Leu. As two L-Ala and three D-Ala units were obtained, their locations could not be established conclusively.

The cytotoxic activities of **1** and **2** against human ovarian adenocarcinoma SKOV-3, malignant pleural mesothelioma Meso-1 and Jurkat cells were examined. All cell lines were seeded in 384-well plates at a density of 1000 cells per 20 μl per well and incubated at 37 °C in a humidified incubator with 5% CO_2. After 4 h, cells were treated with various concentrations of compounds for 72 h. The vehicle solvent (dimethylsulfoxide) was used as a control. Cell viabilities were measured using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt) by a colorimetric assay (Cell Counting Kit-8; Dojindo; Kumamoto, Japan). Compound **1** exhibited cytotoxic activities against SKOV-3, Meso-1 and Jurkat cells with the IC_{50} values of 38.0, 27.8 and 12.5 μM, respectively. Compound **2** exhibited two to three times higher activities against SKOV-3, Meso-1 and Jurkat cells with IC_{50} values of 10.8, 14.3 and 5.4 μM, respectively. Further studies on the absolute configuration and biological activities of **1** and **2** are under way.

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