Identification of essential biosynthetic genes and a true biosynthetic product for thioviridamide

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Thioviridamide (1), a thioamide-containing cyclic peptide (Fig. 1), was isolated from the mycelial extract of Streptomyces olivoviridis NA05001 as an apoptosis inducer in cells transformed with adenovirus oncogenes (Hayakawa et al., 2006a, b). Thioamide-containing peptides are rarely found in natural sources and biosynthetic studies have been reported on only apomethanobactin and thioviridamide (Banala and Sussmuth, 2010; Izawa et al., 2013; Krentz et al., 2010). The biosynthetic gene cluster of 1 (Fig. 1) was identified as tvaA - tvaO by heterologous production of 1 in Streptomyces lividans expressing the 15 genes (Izawa et al., 2013). This unique antibiotic is assumed to be ribosomally synthesized from the precursor peptide gene tvaA and post-translationally modified. A putative regulatory gene (tvaB) and 10 sequential genes without noncoding sequences (tvaC - tvaL) in the cluster appear to be required for the biosynthesis of 1.

To identify the essential biosynthetic genes for 1, putative regulatory genes tvaM, tvaN and tvaO were removed from pWHM3-TV A, a plasmid containing tvaA - tvaO. A 2.6 kbp fragment from an XhoI site to tvaL was amplified from S. olivoviridis NA05001 genomic DNA by using KOD plus DNA polymerase (Toyobo, Osaka, Japan) and primers 5'-GGAGGAACTCGAGGAATCGGGCCGTA TCA-3' and 5'-AATAAGCCTTGAAGCGTGCCCCGG CGGTGCTTTCT-3' with an additional HindIII site (underlined). The fragment was swapped with a 7.4 kbp fragment from the XhoI site to the cloning site of pWHM3-TV Amini, which contained the thioviridamide biosynthesis gene cluster lacking tvaM, tvaN and tvaO. The expression plasmid was introduced into S. lividans TK23 and the transformant was cultivated in Erlenmeyer flasks containing a medium consisting of 2.5% glucose, 1.5% soybean meal, 0.2% dry yeast, 0.4% CaCO3 and 10 µg/mL thiostrepton (pH 6.2) on a rotary shaker at 27°C for 4 days. The mycelial acetone extract was analyzed by HPLC (YMC-Pack R-ODS-7, 80% MeOH - 0.2% H3PO4, A254), which revealed a peak corresponding to 1 (Fig. 2b). The transformants bearing pWHM3-TV A and pWHM3-TV Amini showed almost the same productivity (Figs. 2a and 2b). This result established the 12 genes (tvaA - tvaL) as being essential for thioviridamide biosynthesis.

One of the unique features of 1 is an N-terminal 2-hydroxy-2-methyl-4-oxopentanoyl (HMOP) group. This C6 moiety is considered to be originated from the serine (C3) residue of TvaA and an additional C3 unit. Acetone ad-
Fig. 2. HPLC analyses of thioviridamide (1) and prethioviridamide (2).

Mycelial acetone extract of *S. lividans* carrying pWHM3-TVA (a) or pWHM3-TVAmini (b). Acetone extract (c), methanol extract (d) and methanol extract treated with acetone (e) of the cultured mycelium of *S. lividans* carrying pWHM3-TVAmini. Purified 2 (f) and acetone-treated 2 (g). a, b: YMC-Pack R-ODS-7, 80% MeOH - 0.2% H₃PO₄, A₂₅₄. c–g: Senshu-Pak PEGASIL ODS, 82% MeOH - 5 mM disodium hydrogen citrate, A₂₅₄.
ducts are sometimes isolated as artifacts of the extraction procedure. We then investigated whether 1 was an acetone adduct or not. The cultured mycelium of S. lividans carrying pWHM3-TV-Amini was extracted with acetone or methanol, and the extracts were analyzed by HPLC (Senshu-Pak PEGASIL ODS, 82% MeOH - 5 mM disodium hydrogen citrate, A254). A peak corresponding to 1 was detected in the acetone extract but not in the methanol extract (Figs. 2c and 2d). The methanol extract was treated with an equal volume of acetone at room temperature for 24 hours and then subjected to HPLC, which revealed a peak due to 1 (Fig. 2e). These results indicated that 1 was an acetone adduct of an unknown true metabolite.

The HPLC chromatogram of the mycelial methanol extract showed a peak at a retention time of 8.0 min and the peak disappeared with the addition of acetone (Figs. 2d and 2e). We then attempted to isolate a metabolite corresponding to the HPLC peak. The fermented broth (2 L) of S. lividans carrying pWHM3-TV-Amini was centrifuged and the mycelium was extracted with methanol. After evaporation, the aqueous concentrate was extracted with ethyl acetate. The extract was dissolved in chloroform and precipitated by the addition of 10 volumes of hexane. The precipitate was applied to a silica gel column, which was washed with chloroform-methanol (10:1) and eluted with chloroform-methanol (2:1). The eluate was subjected to preparative HPLC (Senshu-Pak PEGASIL ODS, 20 × 250 mm) with 75% methanol containing 5 mM disodium hydrogen citrate. A peak fraction was concentrated and extracted with ethyl acetate. The extract was evaporated to dryness to give a colorless powder which we refer to as 2 (1.2 mg).

A reaction mixture for the acetone adduct formation consisted of 10 μl of a methanol solution of 2, 190 μl of 1 mM Tris-HCl (pH 8.0) and 800 μl of acetone. The mixture was allowed to stand at room temperature for 48 hours and then analyzed by HPLC. As estimated by HPLC peak areas in Figs. 2f and 2g, 2 was mostly converted to 1 under this condition. From this result, 2 was identified as a precursor and a true biosynthetic product for 1, and named prethioviridamide.

The molecular formula of 2 was determined to be C_{53}H_{87}N_{14}O_{9}S_{7}^{+} by high-resolution FAB-MS, \((m/z) 1287.4823 [M^{+}], \) calcd 1287.4825) and was consistent with a loss of C_{3}H_{6}O from 1. The \(^{1}H\) NMR spectrum of 2 in acetonitrile-d_{3} (Fig. 3) was similar to that of 1, and signals due to HMOP were not observed in 2. A singlet methyl at δ 2.37 was assigned to an acetyl group based on its \(^{1}H\)-\(^{13}C\) long-range coupling with a ketone carbonyl carbon (δ 196.4) in the HMBC spectrum (Bax and Summers, 1986). Although further NMR analysis was difficult because of its instability in a concentrated solution, the N-terminal structure of 2 was established as a pyruvyl group by the molecular formula and the conversion to 1 (Fig. 1). The pyruvyl moiety was considered to be formed by dehydration and hydrolysis of the N-terminal serine residue via enamine-imine tautomerization. Recently, an N-terminal lactyl derivative of 1, JBIR-140, has been reported (Izumikawa et al., 2015). This reduced metabolite might be produced from 2 by unknown reductase.

The biological activity of 2 was evaluated using normal and transformed 3Y1 rat fibroblasts (Zaitsu et al., 1988). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% glucose, and treated with various concentrations of the samples for 72 hours. The relative cell viability was measured as absorbance at 540 nm, after the cells were incubated with 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
(MTT) for 4 hours. The IC₅₀ values of 1 and 2 were 9.0 and 16 nM against Ad12-3Y1 cells, 27 and 21 nM against E1A-3Y1 cells, and 980 and 1400 nM against parent 3Y1 cells, respectively. The two compounds showed similar potency and selectivity, indicating that the N-terminal structure does not significantly affect the activity of these peptides. Further biosynthetic studies are in progress.

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