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Novel macrolactam compound produced by heterologous expression of a large cryptic biosynthetic gene cluster of *Streptomyces rochei* IFO12908

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Abstract.

In the course of our studies on the heterologous expression of giant biosynthetic genes, we discovered a novel cryptic biosynthetic gene cluster in *Streptomyces rochei* IFO12908. During our efforts to express biosynthetic genes using the host SUKA strain derived from *Streptomyces avermitilis*, a novel polyene macrolactam compound designated as JBIR-156 was produced. We report herein the cloning and heterologous expression of the JBIR-156 biosynthetic gene cluster, and the isolation, structure determination, and cytotoxic activity of this novel compound.

Screening for bioactive microbial secondary metabolites has been performed for more than 70 years. The search for bioactive natural metabolites has resulted in great benefits to humankind, although the rate of discovery of skeletally-novel compounds from bacteria has significantly decreased over time. (ref. 1, 2) New technologies to discover novel natural compounds are strongly desired. To overcome this problem, new techniques to express cryptic biosynthetic gene clusters for secondary metabolites are needed to access novel compounds. Furthermore, aside from the classical techniques including improvement of cultural conditions for wild-type strains, heterologous expression technology is considered to be one of the most promising strategies since the resultant production of microbial secondary metabolites can be performed under a common platform.

On the other hand, *Streptomyces* is known as a representative microorganism to produce potent natural products. (ref. 3) Recent advances in genome sequencing projects have revealed that a large number of biosynthetic gene clusters are encoded in the genome of *Streptomyces*. For the expression of biosynthetic gene clusters, genetically engineered *Streptomyces* strains such as *Streptomyces coelicolor* M1152, M1154, and *Streptomyces avermitilis* derived SUKA strains, have been developed. (ref. 4-6) In these engineered strains, endogenous metabolic pathways were removed to avoid competition of substrate usages between endogenous and exogenously introduced secondary metabolites. (ref. 7) In addition, this engineered host can afford a clean metabolic background, which makes it easier to isolate relevant compounds.

Despite the many heterologous expression experiments for the production of microbial secondary metabolites performed over the past two decades, (ref. 8) only a few examples of the heterologous expression of biosynthetic gene clusters over 100 kb in size have been reported (ref. 9) because it is extremely difficult to clone and introduce such giant biosynthetic gene clusters into the host strain. During the course of our focused efforts to establish a technique for the heterologous expression of large size biosynthetic gene clusters, we developed our technology for cloning larger biosynthetic gene clusters employing BAC vectors (up to 300 kb genome libraries can be prepared with BAC vectors). As a result of our studies, we succeeded in the heterologous production of mediomycin,

neomediomycin, (ref. 10) and quinolidomicin. (ref. 11) To express cryptic and/or unreported gene clusters with our system, we searched biosynthetic gene clusters larger than 100 kb from our in-house genome sequence data bank consisting of more than 100 actinomycete strains. Among such large biosynthetic gene clusters, we selected a gene cluster including type-I PKSs with glutamate mutase genes, which could produce a macrolactam compound in the genome of *Streptomyces rochei* IFO12908. As well known that macrolactam compounds can exhibit potent cytotoxic activities. On the other hand, macrolactam compounds are extremely difficult to obtain because of highly vulnerable character. It was expected that such unstable compounds showing strong bioactivities can be obtained by applying our heterologous expression techniques. Heterologous expression of the BAC clone containing a whole biosynthetic gene cluster of the type-I PKS resulted in the production of a novel polyene macrolactam compound designated as JBIR-156 (**1**). Here we report the cloning, heterologous expression of the gene cluster, isolation, structure determination, and brief biological activities of **1**.

We scanned the draft genome sequence of *S. rochei* IFO12908 strain and found a biosynthetic gene cluster over 100 kb which encodes 5 polyketide synthases (PKSs). Among the PKS modules of JBIR-156 (**1**), a total of 9 of the 10 modules include the dehydratase (DH) and ketoreductase (KR) domains, which compose the double bonds at β-keto groups of acyl-carrier protein (ACP)-bound intermediates. Therefore, this biosynthetic product is expected to consist of highly conjugated double bonds. The putative biosynthetic gene cluster including adjacent genes responsible for the production of the compound was estimated at 140 kb at most. For cloning of the gene cluster, a BAC DNA library of the *S. rochei* IFO12908 strain was prepared as previously described. (ref. 12, 13) A clone, pKU518F6K295_P1_P9-M23 (insert size: 234 kb), containing the biosynthetic gene cluster was picked up by PCR screening which amplified the upstream and downstream of the target gene cluster from the constructed DNA library. The pKU518F6K295_P1_P9-M23 was introduced into the SUKA32 strain to obtain a transformant, SUKA32::pKU518F6K295_P1_P9-M23. The obtained clone was cultivated in 50-mL test tubes, each containing 15 mL of a seed medium consisting

of 0.5% glucose, 1.5% Soya Flour and 0.5% yeast extract. The test tubes were shaken on a reciprocal shaker (320 r.p.m.) at 27 ˚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 of 2% glycerol, 1% molasses, 0.5% casein, 0.1% polypepton and 0.4% $CaCO_2$ (adjusted pH to 7.2 before sterilization), and cultured on a rotary shaker (180 r.p.m.) at 27 ˚C for 5 days.

We executed comparative analyses of the transformant and null strains of SUKA32. According to the structural prediction of the biosynthetic gene cluster of JBIR-156, **1** could show characteristic polyene UV absorption. A specific peak showing polyene UV absorption was appeared in the extract of the mycelial cake of transformant. The fermentation broth (1 L) was centrifuged to collect a mycelial cake, which was extracted with 200 mL of acetone three times. Acetone was evaporated *in vacuo*, and the residual aqueous layer was extracted with ethyl acetate twice. The resultant ethyl acetate layer was concentrated to dryness (321 mg). The sample was subjected to ODS RP-MPLC (Purif-Pack ODS-60 Shoko Scientific Co., Yokohama, Japan) by using a H_2O–MeOH stepwise solvent system (0%, 40%, 60%, 80%, and 100% MeOH). The 80% MeOH fraction was collected and lyophilized (30.7 mg). The fraction was further purified by RP HPLC employing a Develosil RPAQUEOUS-AR5 C_{30} column (20 i.d. × 150 mm, Nomura Chemical, Seto, Japan), in isocratic mode with 55% CH_3CN (flow rate 10 ml min^{-1}) to afford a pure sample of **1** (3.6 mg).

Compound **1** was obtained as a brownish powder. $[\alpha]^{24}_{D}$ –25 (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 264 nm (4.22), 303 nm (4.53), 313 nm (4.54), 348 nm (4.11); IR (ATR) ν_{max} 3400 and 1646 cm^{-1}. The UV spectrum of **1** suggested that **1** consisted of a polyene structure. Its molecular formula was established as $C_{24}H_{29}NO_2$ by HR-ESIMS ($[M+H]^+$, *m/z* 364.2270, calcd for $C_{24}H_{30}NO_2$, 364.2271). The structure determination of **1** was performed based on ^1H, ^{13}C NMR and a series of 2D NMR analyses, double quantum filtered COSY (DQF-COSY), gradient-enhanced heteronuclear single quantum coherence with adiabatic pulses (HSQCAD), and gradient-selected heteronuclear multiple bond correlation using adiabatic pulses (HMBCAD). The ^{13}C and ^1H NMR data for **1** are listed in Table 1. The well-resolved DQF-COSY spectrum of **1** indicated two partial structures.

The sequence from a doublet olefinic methine proton 2-H (δ_H 5.64) to an olefinic methine proton 18-H (δ_H 6.15) through olefinic methine protons 3-H (δ_H 6.33), 4-H (δ_H 7.35), 5-H (δ_H 6.87), 6-H (δ_H 6.33), 7-H (δ_H 6.55), 8-H (δ_H 6.62), 9-H (δ_H 5.69), aliphatic methylene protons 10-H_2 (δ_H 2.27, 2.95), an oxymethine proton 11-H (δ_H 4.47, δ_C 68.7), olefinic methine protons 12-H (δ_H 5.60), 13-H (δ_H 5.90), 14-H (δ_H 6.13), 15-H (δ_H 6.38), 16-H (δ_H 5.97), and 17-H (δ_H 6.36) was observed. Another ^{1}H sequence was established by the ^{1}H spin system from an olefinic methine proton 19-H (δ_H 6.17) to an amide proton NH (δ_H 7.98) *via* olefinic methine protons 20-H (δ_H 6.50), 21-H (δ_H 5.19), a methine proton 22-H (δ_H 3.40) which in turn coupled to methyl protons 24-H_3 (δ_H 0.92) and nitrogen atom connected methylene protons 23-H_2 (δ_H 2.35, 3.39, δ_C 45.9). Although, the $^3J_{H,H}$ value between 18-H and 19-H (11.3 Hz) was read from a coupled HSQC spectrum, the linkage between C-18 and C-19 was confirmed by the HMBC correlation as described *vide infra*.

The connectivity between these two moieties was established as follows. In the HMBC spectrum, ^{1}H-^{13}C long-range couplings from the olefinic methine proton 17-H (δ_H 6.36) to an olefinic methine carbon C-19 (δ_C 125.9), and from an olefinic proton 20-H (δ_H 6.50) to an olefinic methine carbon C-18 (δ_C 123.3), established the connection between C-18 and C-19. A macrolactam structure was established by the ^{1}H-^{13}C long-range couplings from the olefinic protons 2-H, 3-H, 23-H_2 and the amide proton 23-NH to an amide carbonyl carbon C-1 (δ_C 166.9). Based on the index of hydrogen deficiency of **1** together with the proton and carbon chemical shifts, it was determined that a hydroxy group was deduced to be substituted at the position of C-11. The geometrical configurations at C-2, C-4, C-6, C-8, C-12, C-14, C-16, C-18 and C-20 were established as *Z*, *E*, *Z*, *Z*, *E*, *E*, *Z*, *Z* and *Z*, respectively, according to the $^3J_{H,H}$ values, ($J_{2,3} = J_{6,7} = J_{8,9} = J_{16,17} = J_{18,19} = J_{20,21}$ = 10 - 12 Hz and $J_{4,5} = J_{12,13} = J_{14,15}$ = 14 - 16 Hz). Thus, the gross structure including the geometry of **1** was established as shown in Figure 1.

We annotated the functions of the genes in the biosynthetic gene cluster as shown in Table S1 and a plausible biosynthetic pathway is illustrated in Figure S2. The biosynthetic gene cluster for JBIR-156 resembles those of polyene macrolactams such as vicenistatin, (ref. 14) ML-449 (ref. 15)

and sceliphrolactam. (ref. 16) The biosynthesis of the starter unit in the PKS of **1** occurs through the same pathway as that of vicenistatin. Biosynthesis is initiated from the conversion of L-glutamic acid to (2*S*,3*S*)-3-MeAsp by ORF H and I. Following this reaction, ORF K, J and E form an intermediate on a carrier protein ORF L, which is then loaded onto PKS by ORF F (Figure S2). (ref. 17) Consequently, the absolute configuration at the position of C-22 of **1** is predicted to be *S* as shown in Figure S2, because the methyl residue at C-22 is derived from (2*S*,3*S*)-3-MeAsp. The biosynthetic intermediates on the PKS for the formation of the macrolactam, which is predicted from the information of its domain organization, are shown in Figure S2. The intermediates on PKS deduced from the domain architecture coincide well with the structure of JBIR-156. The formation of absolute configurations of chiral carbons substituted by hydroxy residues in type-I PKS can be regulated by the sequence of KR domains. The KR domain on Module 6 that introduces the hydroxy residue at C-11 is of the A-type, forming an L-configuration of the ACP-bound intermediates. The other KR domains that reside with DH domains in the other modules are all B-type. Therefore, the putative absolute configurations at C-11 and C-22, which are substituted by the hydroxy and the methyl groups, respectively, are shown in Figure S2.

Since potent cytotoxic activities are often induced by polyene macrolactam compounds, we examined the cytotoxic activities of **1** against human ovarian adenocarcinoma SKOV-3, malignant pleural mesothelioma Meso-1 and T lymphoma Jurkat cells. As the result of cytotoxic test, **1** exhibited cytotoxicities against SKOV-3 and Jurkat cells with the IC_{50} values of 9.45 and 3.51 μM, respectively. **1** also showed weak cytotoxicity against Meso-1 (IC_{50} value: > 10 μM). The cytotoxic activities of **1** were almost the same magnitudes as those of etoposide (IC_{50} values of etoposide against SKOV-3, Meso-1 and Jurkat cells were 2.4 μM, 39.3 μM and 0.5 μM, respectively), while camptothecin showed more potent activities against SKOV-3, Meso-1 and Jurkat cells with IC_{50} values of 33 nM, 7,100 nM and 4.3 nM, respectively.

Compound **1** structurally resembles cyclamenol A (ref. 18), vicenistatin and NOVO4 (ref. 19) aglycons. Compound **1** was first isolated as the result of heterologous expression of the cryptic

biosynthetic gene cluster in SUKA host strain, from which endogenous biosynthetic gene clusters were knocked out. Since **1** consists of polyene moieties well known to be vulnerable to oxygen, **1** is degraded through the isolation process in which compounds are exposed to oxygen. The cultural metabolite of wild-type producing strain *S. rochei* IFO12908 contains many impurities, therefore it requires more isolation steps to obtain pure sample of **1**. Moreover, the production yield of **1** by wild type strain is about 4-fold less than that of the SUKA22 transformant. Comparatively the clean metabolic background and the higher production yield of heterologous expression in SUKA32 strain make it easier the isolation process. Thus, our heterologous expression system could be a promising technology for the production of even unstable compounds. Further studies on the heterologous expression of various cryptic biosynthetic genes are now underway.

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Notes

The authors declare no competing financial interest.

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