Isolation and structural determination of a new antibacterial compound demethyl-L-681,217 from Streptomyces cattleya

Note

Isolation and structural determination of a new antibacterial compound demethyl-L-681,217 from *Streptomyces cattleya*

Running headline: Structural determination of demethyl-L-681,217

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MAIN TEXT

Secondary metabolites including polyketides, nonribosomal peptides and hybrid polyketide-peptides have diverse chemical structures and a wide variety of bioactivities.[1] Polyketides and nonribosomal peptides are synthesized by large enzyme complexes such as polyketide synthases (PKS) and nonribosomal peptide synthetases (NRPS).[2] These biosynthetic proteins are typically encoded in neighboring loci and organized in gene clusters ranging from several to over 200 kb in length. NRPS and PKS employ a very similar strategy like an assembly line for the biosynthesis of two distinct classes of natural products.[3-5] In addition, NRPS/PKS hybrid biosynthetic systems give structurally more diverse compounds by the combination of NRPS and PKS like building blocks on one assembly line.[6]

The biosynthetic gene cluster of a polyketide kirromycin[7] (**3**, Fig. 1b) was identified from the genome sequence of *Streptomyces collinus*[8-10] and kirromycin was indicated to be biosynthesized by a large hybrid PKS/ NRPS gene cluster. Among the related compounds including efrotomycin[11], dihydromocimycin,[12] heneicomycin,[13] factumycin,[14] and kirromycin[15], only the biosynthesis of kirromycin has been reported so far.[8-10] Previously, a new kirromycin analogue L-681,217 (**2**, Fig. 1a) which lacked of pyridine ring was isolated from *Streptomyces cattleya* ATCC 39203.[16] Based on the results, we performed chemical investigation on a MeOH extract of *S. cattelya* NBRC

14057 (type strain) to search for new kirromycin analogues. As a result, a new kirromycin analogue demethyl-L-681,217 (**1**) was isolated as an antibacterial compound from *S. cattelya* along with a known compound L-681,217 (**2**). As the genome sequence of the type strain of *S. cattletya* was previously determined,[17] the biosynthetic gene cluster was searched for and found based on the similarity to that of kirromycin. Here we describe the isolation and structure determination of a new kirromycin analogue demethyl-L-681,217 (**1**) and discuss the possible biosynthetic gene cluster.

The cultivation of *S. cattleya* NBRC 14057 was performed with 5 L of ISP2 agar media. After 7 days of cultivation, cells of spore and aerial hyphae were harvested and extracted with MeOH. The MeOH extract was subjected to a CHP-20P column, washed with 60% MeOH, and eluted with 80% MeOH. The 80% MeOH fraction was repeatedly subjected to preparatory HPLC to yield a new compound **1**, along with a known compound **2** (Fig. S1).

The compound **1** was isolated as a white amorphous powder. High resolution ESI-TOF-MS analysis of **1** gave an $[M+H]^+$ ion at *m/z* 668.3372 (Fig. S2), consistent with a molecular formula of $C_{35}H_{51}NO_{10}$ (calculated $[M+H]^+$ ion at *m/z* 668.3410). The molecular formula of **2** was also confirmed to be $C_{36}H_{53}NO_{10}$ (Fig. S3) in the same manner. To obtain further information on the chemical structure, NMR experiments

including ^{1}H, ^{13}C, DEPT-135, HSQC, HMBC, DQFCOSY, NOESY and TOCSY spectra of **1** and **2** were performed in acetone-d_6 (Fig. S4-10). Identification of the structure of a known compound **2** was performed by 2D NMR spectra (Fig. 1d). Briefly, five partial structures were indicated by five proton-proton spin systems (bold line, **2** in Fig. 1d), established by TOCSY and DQF-COSY spectra. The HMBC correlations from H36 to C14, C15, and C16 indicated the connection between the partial structure of C2 to C14 and that of C16 to C19. The HMBC correlation from H16 to C14 also supported this connection. The presence of a carboxyl residue at C1 was confirmed by HMBC correlation from H2 to C1. The methoxy residue was assigned as attached at C14 by the HMBC correlation from H35 to C14. The HMBC correlations from H19 and H37 to C21 indicated the connection between the partial structure of C16 to C19 and that of C22-C37-C38. The HMBC correlations from H22 and H24 to C23 indicated the connection between partial structure of C22-C37-C38 and that of C24-C25. The HMBC correlations from H25 and H27 to C26 indicated the connection between partial structure of C24-C25 and that of C27 to C33. The NOESY correlation between H25 and H27 also supported this connection. The methyl residue was indicated as attached to C26 by the HMBC correlations from H39 to C25, C26, and C27. The presence of a tetrahydropyran ring was confirmed by HMBC correlation

74 from H27 to C23. Regarding the assignment of tetrahydrofuran ring, a NOESY
75 correlation between H8 and H11 was not observed, although a NOESY correlation
76 between H9 and H11 was observed. In comparison with the reported NMR data of the
77 related compound,[18] the similarity of the chemical shifts indicated the presence of
78 tetrahydrofuran ring.
79 The structure determination of **1** was accomplished by the comparison with the NMR
80 data of **2** (Table S1). Judging from the molecular weight (Fig. S2 and S3), the
81 discrepancy between **1** and **2** was expected to be one methylene residue. As a result of
82 interpretation of NMR data, the chemical shifts of **1** and **2** were almost identical except
83 for a methyl and a methylene residue (Table S1). Methyl residue (δH 0.94, triplet) and
84 methylene residue (δH 1.63 and 1.72, multiplet) was lacking in the NMR spectrum of **1**
85 and new methyl residue (δH 1.20, doublet) was observed in the NMR spectrum of **1**.
86 The C37 methyl group (δH 1.20, doublet) was connected to C22 by correlation of
87 HMBC (Fig. 1c). In the same manner with **2**, five proton-proton spin systems (bold
88 line, **1** as shown in Fig 1c) were established by TOCSY and DQF-COSY spectra. The
89 connections of the smaller spin systems were established by HMBC correlations shown
90 in Fig. 1c (one way arrows). Regarding the assignment of the tetrahydropyran ring,
91 HMBC correlation from H27 to C23 was not observed. The proton and carbon

chemical shifts at position 8, 11, 23, 27 were almost identical with those of L-681,217 (Table S1). Thus, we concluded the structure to be **1** as shown in Fig. 1a.

The antimicrobial activities were measured by using a paper disk diffusion assay against bacterial strains (*Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens, Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Streptomyces antibioticus*), yeast strains (*Saccharomyces cerevisiae,Schizosaccharomyces pombe, Kloeckera apiculata*), and fungal strains (*Aspergillus niger, Aspergillus oryzae, Mucor hiemalis*). At the dosage of 10 μg/disk, **1** and **2** showed the inhibitory zones of 13 mm and 18mm in diameter, respectively, only against *S. antibioticus*. Interestingly, neither compound showed any antimicrobial activity against other tested microorganisms under these conditions.

As the complete genome sequence of *S. cattleya* NRRL 8057 (NBRC 14057) is available publicly,[17] we searched for the loci encoding the biosynthesis of the two compounds L-681,217 and demethyl-L-681,217 (L-681,217s) in the genome sequence, and consequently found a large hybrid PKS/NRPS gene cluster shown in Table S2. This cluster encoded five modular PKSs lacking internal AT domains (trans-AT type PKSs), two acyltransferases, one NRPS, two cytochrome P450s and one methyltransferase, which show high sequence homologies to enzymes for kirromycin synthesis [10]. Because

L-681,217s are structurally related to kirromycin and the genome of *S. cattleya* NRRL 8057 harbors no other such gene cluster, we assumed this to be the biosynthetic gene cluster for L-681,217s and then bioinformatically analyzed. The module and domain organization of the hybrid PKS/NRPS gene cluster is shown in Figure 2. This cluser harbors 15 modules, which are very similar to 15 of 17 modules present in the kirromycin-synthetic gene cluster.[10] According to the collinearity rule of the modular PKS/NRPS assembly line[6] and the similarity to kirromycin biosynthetic pathway, we predicted the chemical structure of the backbone synthesized by these enzymes. The domain organization of these PKS and NRPS proteins well agreed with the backbone structures of L-681,217s except for the difference at C25. Since module 4 has a DH-KR pair as the optional domain, the C-C bond between C25 and C26 would be double in L-681,217s, but it is actually single and C25 has a hydroxyl group. Hence, we assumed the DH domain in module 4 is inactive. The analysis of the PKS and NRPS module organization indicates that the biosynthesis starts with loading of an acetyl-CoA to SCAT_3576. The polyketide chain is extended by the PKS modules (modules 1 to 5) of SCAT_3576 and SCAT_3577, then condensed with glycine by the hybrid NRPS/PKS SCAT_3578, and further extended by SCAT_3579 to SCAT_3581. During or after release of the linear precursor of L-681,217s, this intermediate must undergo cyclization

128 to yield two intramolecular rings and modifications. *O*-methylation of the keto group at
129 C14 is presumably catalyzed by the methyltransferase of SCAT_3585. Cytochrome
130 P450 of SCAT_3583 or SCAT_3588 is predicted to add an oxygen molecule at C26,
131 yielding a hydroxyl group. Demethyl-L-681,217 is produced if module 5 loads
132 methylmalonyl-CoA, whereby L-681,217 is produced if module 5 loads ethylmalonyl-
133 CoA. However the situation appears to be more complex than that, as two discrete
134 acyltransferases are encoded as SCAT_3584 and SCAT_3586, respectively. SCAT_3584
135 contains two acyltransferase (AT) domains whose substrates are predicted to be
136 malonyl-CoA. In contrast, SCAT_3586 contains only a single AT domain, which is
137 predicted to recognize methylmalonyl-CoA as the substrate by the analysis using
138 antiSMASH[19]. Like KirCI and KirCII for kirromycin, one of the acyltransferases may
139 be responsible for loading the ACPs needed for extension of the polyketide chain with
140 malonyl-CoA, whereas the other may catalyze the loading the ACPs of modules 5, 9
141 and 14 with alkylmalonyl-CoA such as methylmalonyl-CoA or ethylmalonyl-CoA.
142 Although each of C15 and C24 also has a methyl side chain, these methyl groups are
143 not derived from incorporation of methylmalonyl-CoA, but modules 3 and 8 incorporate
144 malonyl-CoA molecules in the elongating polyketide chain and then the
145 methyltransferase (MT) domains in these modules methylate the positions. Above all,

146 we concluded that this gene cluster is responsible for the synthesis of L-681,217s and
147 proposed the biosynthetic pathway, although genetic experiments such as gene
148 distruption are needed to completely prove it.

149 **Materials and Methods**

150 **Bacterial strains**

151 The producing strain *Streptomyces cattleya* NBRC 14057 and test microorganisms
152 (bacterial strains including, *Escherichia coli* NBRC 102203, *Pseudomonas aeruginosa*
153 NBRC 12689, *Serratia marcescens* NBRC 102204, *Bacillus subtilis* NBRC 13719,
154 *Staphylococcus aureus* NBRC 100910, *Micrococcus luteus* NBRC 3333; *Streptomyces*
155 *antibioticus* NBRC 3117, yeast strains including *Saccharomyces cerevisiae* NBRC
156 2376, *Schizosaccharomyces pombe* NBRC 0340, *Kloeckera apiculata* NBRC 0154;
157 fungal strains including *Aspergillus niger* NBRC 33023, *Aspergillus oryzae* NBRC
158 4290, *Mucor hiemalis* NBRC 9405) for antimicrobial assays were obtained from the
159 NBRC culture collection (NITE Biological Resource Center, Tokyo, Japan).

160 **Isolation of demethyl-L-681,217**

161 *Streptomyces cattleya* NBRC 14057 was cultured using 5 L of ISP2 agar medium for 7
162 days at 30 °C. The aerial hyphae and spore cells on the agar surface were harvested
163 with a steel spatula. Double volume of MeOH was added to the harvested cells,

164 followed by filtration with paper filter (Whatman No. 1, GE Healthcare Life Sciences,
165 Little Chalfont, UK). The MeOH extract was concentrated to an aqueous residue
166 using a rotary evaporator. The aqueous residue was subjected to open column
167 chromatography using hydrophobic resin CHP-20P (Mitsubishi Chemical, Tokyo,
168 Japan), eluted with 10% MeOH, 60% MeOH, 80% MeOH, and 100% MeOH. The 80%
169 MeOH fraction was concentrated, and subjected to HPLC separation using an ODS
170 column (4.6 × 250 mm, Wakopak Handy-ODS, Wako Pure Chemical Industries, Ltd.,
171 Osaka, Japan), with elution (linear gradient of 25 to 55% MeCN containing 0.05%
172 trifluoroacetic acid at the flow rate of 1 ml/min for 20 min, monitoring 220 nm
173 absorbance) to yield 1.3 mg of demethyl-L-681,217 and 2.0 mg of L-681,217 at the
174 retention times of 17.2 and 18.8 min, respectively (Fig. S1).

175 **NMR experiments**

176 Two NMR samples were prepared by dissolving **1** or **2** in 500 μl of acetone-d_6,
177 respectively. 1D ^{1}H, ^{13}C, DEPT-135, and all 2D NMR spectra were obtained on a
178 Bruker Avance800 spectrometer with quadrature detection (Bruker BioSpin,MA, USA).
179 The 1D ^{1}H, ^{13}C, DEPT-135 spectra were recorded at 25 °C with 11 ppm for proton and
180 240 ppm for carbon. The following 2D ^{1}H-NMR spectra were recorded at 25 °C with 10
181 ppm spectra widths in *t1* and *t2* dimensions in the phase sensitive mode by States-TPPI[17]

182 method: 2D double quantum filtered correlated spectroscopy (DQF-COSY), recorded
183 with 512 and 1024 complex points in *t1* and *t2* dimensions; 2D homonuclear total
184 correlated spectroscopy (TOCSY) with MLEV-17 mixing sequence, recorded with
185 mixing time of 120 ms, 512 and 1024 complex points in *t1* and *t2* dimensions; 2D nuclear
186 Overhauser effect spectroscopy (NOESY), recorded with mixing time of 300 ms, 256 and
187 1024 complex points in *t1* and *t2* dimensions. 2D ^{1}H–^{13}C heteronuclear single quantum
188 correlation (HSQC) and heteronuclear multiple bond connectivity (HMBC) spectra were
189 acquired at 25 °C in the echo-antiecho mode[18] or in the absolute mode, respectively. The
190 ^{1}H–^{13}C HSQC and HMBC spectra were recorded with 1024 and 512 complex points for
191 12 ppm in the ^{1}H dimension and 160 ppm in the ^{13}C dimension or for 10 ppm in the ^{1}H
192 dimension and 210 ppm in the ^{13}C dimension, respectively, at a natural isotope abundance.
193 All NMR spectra were processed using XWINNMR (Bruker). Before Fourier
194 transformation, the shifted sinebell window function was applied to *t1* and *t2* dimensions
195 except for the HMBC spectrum. All ^{1}H and ^{13}C dimensions were referenced to acetone-
196 d_6 at 25°C.

197 **ESI-MS experiments**

198 ESI-TOF-MS spectra were recorded using a JEOL JMS-T100LP mass spectrometer.
199 For high resolution ESI-TOF-MS analysis, reserpine was used as an internal standard for

caliblation.

Antimicrobial assays

By using a paper disk diffusion assay (6mm i.d., thick type) in the same manner of our previous report,[20] the antimicrobial activity of **1** and **2** was mesured against all the test microorganism. Compunds **1** and **2** was dissolved in MeOH at the concentration of 1 mg/mL. After all the test microroganisms were inoculated onto ISP2 agar medium, paper disks (10 μg each/disk) were placed onto the surface of the agar medium, and paper disk with MeOH (10 μL) was used as a negative control. After incubation for 2 days at 30°C, the diameter of the inhibitory zone was measured for evaluation of antimicrobiol activity.

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Conflict of interest

The authors had no conflict of interest in undertaking this project.

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278 **Figure legends**

279

280 Figure 1. **a**, Chemical structures of demethyl-L-681,217 (**1**) and L-681,217 (**2**); **b**,

281 Chemical structure of kirromycin (**3**); **c**, Key 2D NMR correlations for structure

282 determination of **1**; **d**, Key 2D NMR correlations for structure determination of **2**

283

284 Figure 2. Proposed biosynthetic pathway of demethyl-L-681,217 (**1**) and L-681,217 (**2**).

285 A, adenylation; ACP acyl carrier protein; C, condensation; DH, dehydratase; DH*,

286 inactive DH; KS, ketosynthase; KR, ketoreductase; MT, methyltransferase; PCP,

287 peptidyl carrier protein.

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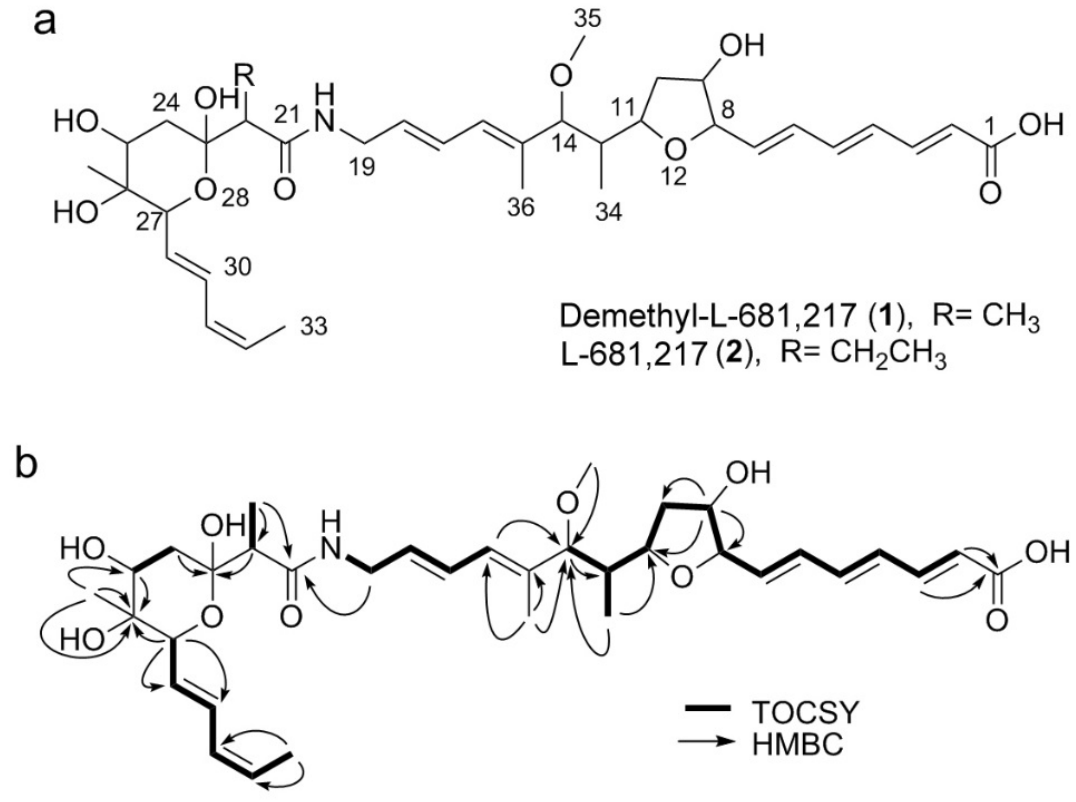

Figure1. Sugai *et al.*

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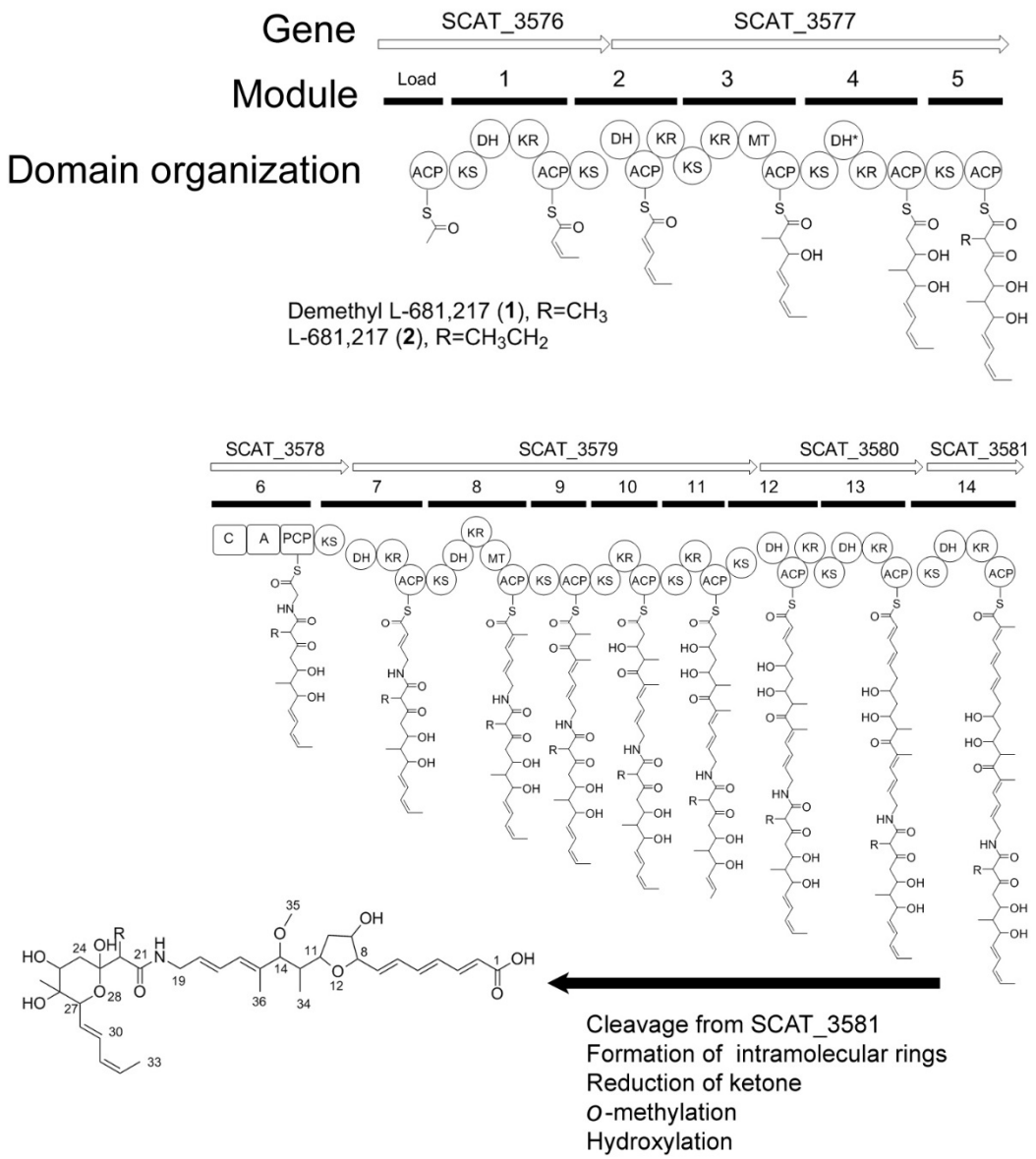

Figure 2. Sugai et al.