Biosynthesis of the antituberculous agent caprazamycin: Identification of caprazol-3″-phosphate, an unprecedented caprazamycin-related metabolite

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Taro Shiraishi, Noboru Hiro, Masayuki Igarashi, Makoto Nishiyama, and Tomohisa Kuzuyama

1 Biotechnology Research Center, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
2 Institute of Microbial Chemistry, 3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141-0021, Japan

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Caprazamycins, produced by Streptomyces sp. MK730-62F2, are liponucleoside antibiotics composed of uridine, 5-amino-5-deoxy-β-D-ribose, a characteristic diazepanone ring, tri-O-methyl-β-D-rhamnose moiety, and an acyl moiety (Fig. 1) (Igarashi et al., 2005). They form a structurally analogous mixture containing several different alkyl side-chains. These antibiotics display potent antituberculous activity in vitro against not only drug-susceptible but also multi drug-resistant Mycobacterium tuberculosis strains, whereas they exhibit no significant toxicity to mice (Igarashi et al., 2005). The activity is attributed to inhibition of the bacterial translocase I, which is essential for peptidoglycan biosynthesis. Because caprazamycins possess such excellent antibacterial properties, they are expected to be promising compounds for the development of antituberculous drugs. For example, several semisynthetic caprazamycin derivatives, such as caprazene-1″-alkylamides and caprazol-1″-alkylamides, have been prepared and their evaluation has been reported (Ishizaki et al., 2013; Miyake et al., 2014; Takahashi et al., 2013).

In addition to the chemical synthesis of such attractive antibiotics, biosynthetic studies have also been conducted; all of the cpz genes (DDBJ/EMBL/GenBank, accession numbers FJ490409 and HM051054) required for caprazamycin biosynthesis have been cloned, and the heterologous production of caprazamycin has been demonstrated using Streptomyces coelicolor M512 (Kaysser et al., 2009a, b). However, the biosynthetic pathway of caprazamycin is not fully understood: only six cpz genes (cpz14–19) have been functionally assigned and characterized in the biosynthetic pathway from uridine monophosphate (UMP) leading to 5′-O-(5″-amino-5″-deoxy-β-D-ribose)-5″-C-glycyluridine, an early-stage intermediate of caprazamycin biosynthesis (Fig. S1) (Barnard-Briston et al., 2012; Chi et al., 2011; Yang et al., 2011). None of the enzymes responsible for the biosynthetic pathway after the early-stage intermediate leading to caprazamycin have been characterized.

It has been proposed that the cpz23 gene is essential for caprazamycin biosynthesis and is involved in β-hydroxyacyl transfer during the biosynthesis of β-hydroxyacylated caprazol, because Cpz23 shows sequence similarity to certain hydrolases (Kaysser et al., 2009a). However, experimental evidence for the function of Cpz23 has not been documented in caprazamycin biosynthesis. In this study, we constructed the cpz23-deleted mutants (Δcpz23) derived from the caprazamycin-producing Streptomyces sp. MK730-62F2 and identified a novel metabolite that accumulated in the mutant. Here, we report the purification and structural elucidation of this metabolite related to caprazamycin biosynthesis.

To gain insight into the function of Cpz23, we deleted the cpz23 gene in the genome of Streptomyces sp. MK730-62F2 by double cross-over recombination (Fig. S2a). The deletion of cpz23 was unequivocally confirmed by PCR.
Production of caprazamycin and caprazol-3″-phosphate.

a, Extracted ion count (XIC) chromatograms at m/z 1146.593 ± 0.017 corresponding to [M+H]+ of caprazamycins A and B. i) wild-type, ii) Δcpz23/pSE101cpz23, and iii) Δcpz23. LC conditions: mobile phase A, water + 0.1% formate; mobile phase B, acetonitrile + 0.1% formate; 10–90% B over 5 min, 90% B for 2.5 min, and then 10% A for 2.5 min, at a flow rate of 0.4 ml/min. b, XIC chromatograms at m/z 656.182 ± 0.011 corresponding to [M+H]+ of the unknown caprazamycin-related metabolite. The metabolite was later identified as caprazol-3″-phosphate.

The resultant Δcpz23 mutant and the complemented mutant (Δcpz23/pSE101cpz23) as well as wild-type MK730-62F2 were cultured for 6 days at 27°C in the medium for caprazamycin production (Igarashi et al., 2003), and the metabolites were analyzed using a Triple TOF5600 LC-MS spectrometer (AB Sciex, Tokyo, Japan) equipped with a CAPCELL PAK C18 IF column (2.0 mm × 50 mm). The analysis revealed that MK730-62F2 produced caprazamycin B, whereas in the Δcpz23 mutant, caprazamycin B production was totally abolished (Fig. 2a). The production was partially restored by complementation with cpz23 expressed in the Δcpz23 strain (Fig. 2a). These results are consistent with the requirement of the cpz23 gene for caprazamycin biosynthesis. Because the mutant abolished caprazamycin production, we further analyzed the whole broth and cell extract of the mutant. We anticipated that the mutant would accumulate a novel biosynthetic intermediate. The metabolites were analyzed by the LC-MS system described above equipped with an ACQUITY UPLC BEH Amide column (2.1 mm × 50 mm; Waters, Tokyo). By LC-MS analysis, we found that an unidentified metabolite with a retention time of 4.3 min (Fig. 2b) accumulated in the cell extract of the Δcpz23 mutant. This metabolite was also detected in the cell extract of the wild-type and the Δcpz23/pSE101cpz23 complemented mutant, but the accumulation was much lower than that in the Δcpz23 mutant (Fig. 2b).

To identify the unknown metabolite, we cultured the Δcpz23 mutant on a large scale (100 ml × 30) and purified the metabolite by using activated carbon (20–150 mesh; Nakalai Tesque, Kyoto) column chromatography, anion exchange (AG® 1-X8, Formate form: Bio-Rad, Tokyo) column chromatography, and preparative HPLC with a Develosil RPAQUEOUS-AR-5 column (200 × 250 mm; Nomura Chemical, Seto, Japan). Eventually, the unknown metabolite (12.1 mg) was purified as a white powder from the mycelium of the Δcpz23 mutant.

The structure of the purified metabolite was deduced by spectroscopic methods. The molecular formula was calculated to be C_{22}H_{34}N_{5}O_{16}P by positive high-resolution mass spectrometry (m/z 656.1816 [M+H]+; calculated mass for C_{22}H_{35}N_{5}O_{16}P+, 656.1811). The 1H- and 13C-NMR spectral features of the purified metabolite (Figs. S3–S5, Table S2) showed close similarities to caprazol (Fig. 3a; Miyake et al., 2014). However, the molecular formula differed from caprazol (C_{22}H_{33}N_{5}O_{16}P) by a value corresponding to a phosphate group. These spectral data suggested that the metabolite was a phosphorylated derivative of caprazol. Further, a noticeable difference in 1H-NMR spectra between caprazol and the purified metabolite was found in the downfield shift of the H-3″ signal (4.45 ppm in the purified metabolite compared to 4.03 ppm in caprazol). Together, these spectral data unequivocally established the structure of the unknown metabolite as caprazol-3″-phosphate, which has a core skeleton of caprazamycin with a phosphate group at the 3″ position but lacks the acyl moiety attached to the 3″-hydroxy group (Fig. 3b). Spin coupling constants of C-2″/P, C-3″/P, and C-4″/P also support this structure (Fig. S5). Caprazol-3″-phosphate is the first example of a phosphorylated derivative isolated from liponucleoside antibiotic-producing organisms.

It is intriguing that the Δcpz23 mutant accumulated the metabolite with a phosphate group that is not appended in caprazamycins. A non-negligible amount of this metabolite was detected even in the wild-type. A previous study has demonstrated that a hydroxyl group at the 3″-position of caprazamycin is essential for inhibiting translocase I activity (Dini et al., 2001). Phosphorylation of the 3″-hydroxyl group of liponucleoside antibiotic A-503083 by CapP kinase extinguishes its antibiotic activity (Yang et al., 2010). The cpz gene cluster contains two putative kinase homologues Cpz12 and Cpz27, which show no similarity to CapP but do show similarity to TmrB, which has been identified in the tunicamycin-resistant Bacillus subtilis (Noda et al., 1992). It has been speculated that TmrB binds tunicamycin and ATP and catalyzes the phosphorylation of the antibiotic tunicamycin, thereby extinguishing its antibacterial activity (Noda et al., 1995). By analogy with the case of TmrB, we suspect that Cpz12

Fig. 2. Production of caprazamycin and caprazol-3″-phosphate.

Fig. 3. Structures of (a) caprazol and (b) caprazol-3″-phosphate.
and/or Cpz27 catalyze(s) the phosphorylation of the 3'-hydroxyl group of caprazol or its derivative, thus reducing its antibacterial activity during caprazamycin biosynthesis. The phosphorylation may be a self-resistance mechanism against biosynthetic intermediates of caprazamycin in *Streptomyces* sp. MK730-62F2.

The identification of caprazol-3'-phosphate provides insight into the function of Cpz23. Cpz23 shows 33% sequence identity and 52% sequence similarity to SsfX3 acyltransferase, which catalyzes the C-4 acylation of salicylic acid in the tetracycline skeleton by using salicylic-CoA as a cofactor (Pickens et al., 2011) (Fig. S6). This high sequence similarity suggests that Cpz23 may catalyze the acylation of the 3'-hydroxy group in caprazol-3'-phosphate. Given that a structurally analogous mixture of caprazamycins containing several different acyl groups has been isolated, Cpz23 presumably displays promiscuous substrate specificity toward acyl groups. Because the acyl group is critically important for the antibacterial properties of caprazamycin (Hirano et al., 2008), the characterization of Cpz23 may lead to the production of promising caprazamycin derivatives.

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Supplementary Materials

Supplementary figures and tables are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

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