Identification of pulvomycin as an inhibitor of the futalosine pathway

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Abstract

Menaquinone is an essential cofactor in the electron-transfer pathway for bacteria. Menaquinone is biosynthesized from chorismate using either the well-known canonical pathway established by pioneering studies in model microorganisms or the futalosine pathway, which we discovered in *Streptomyces*. Because *Helicobacter pylori*, which causes stomach cancer, uses the futalosine pathway and most beneficial intestinal bacteria including lactobacilli use the canonical pathway, the futalosine pathway will be a great target to develop antibiotics specific for *H. pylori*. Here, we searched for such compounds from metabolites produced by actinomycetes and identified pulvomycin from culture broth of *Streptomyces* sp. K18-0194 as a specific inhibitor of the futalosine pathway.

Menaquinone (MK) is an indispensable lipid-soluble electron carrier in the respiratory chain for most bacteria. Two completely different MK biosynthetic pathways have been discovered. One is the well-known canonical pathway identified in model microorganisms such as *Escherichia coli* and *Bacillus subtilis* (Figure 1a). This pathway involves MenH–MenI, which biosynthesize MK from chorismate via *o*-succinylbenzoate as an intermediate.(ref. 1, 2) The other is the futalosine pathway that we previously identified in *Streptomyces coelicolor* (Figure 1b).(ref. 3–7) Analysis of publicly available genome database revealed that *Helicobacter pylori*, which causes stomach cancer, uses the futalosine pathway, while most beneficial intestinal bacteria including lactobacilli employ the canonical pathway. Thus, the futalosine pathway is an attractive target for the development of specific anti-*H. pylori* drugs. In this study, we searched for specific inhibitors targeting the futalosine pathway from metabolites produced by actinomycetes.

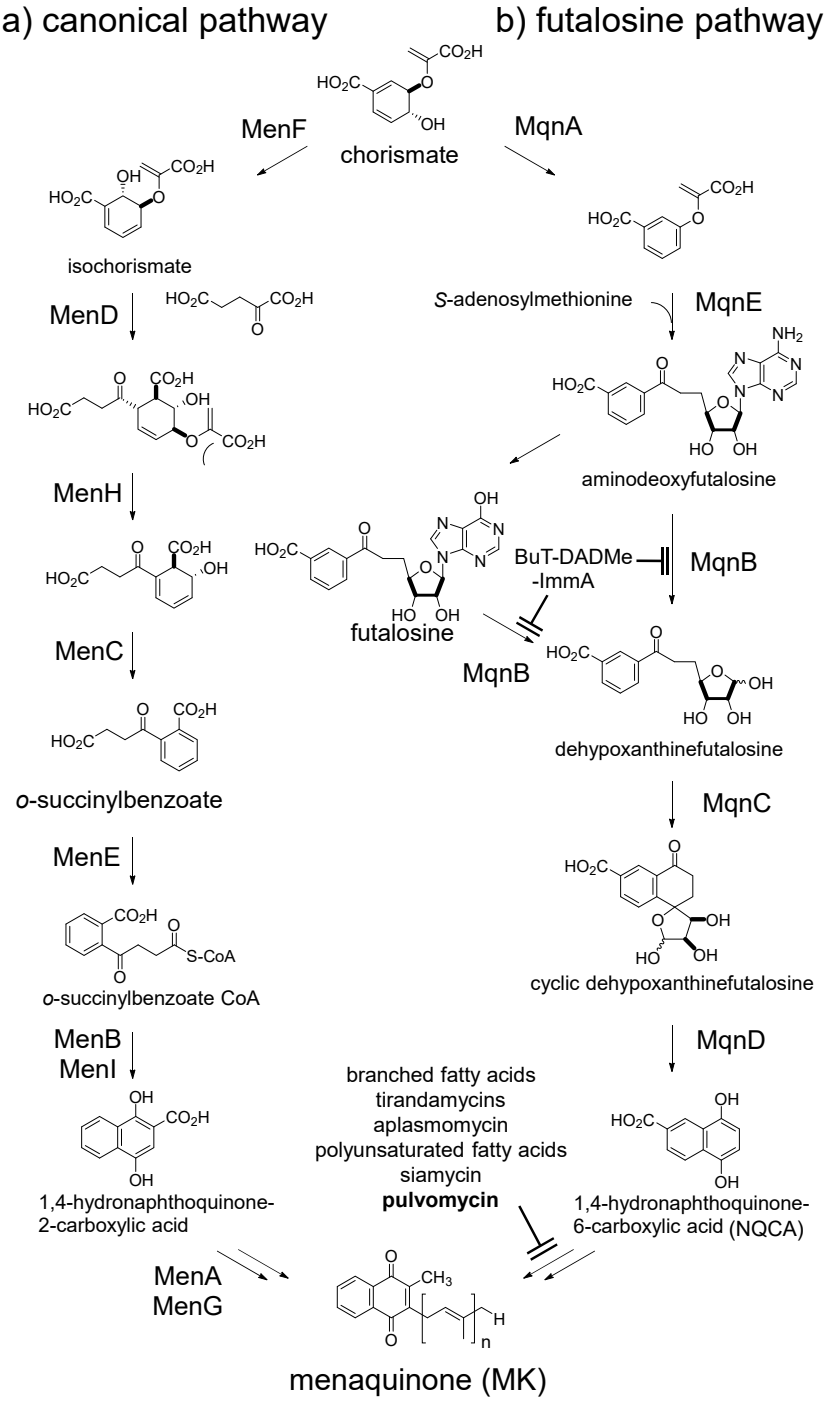

Figure 1. The two known menaquinone biosynthetic pathways. a) The canonical pathway and b) the futalosine pathway. In b), The targets of the known futalosine pathway inhibitors are shown.

To identify samples containing futalosine pathway-specific inhibitors, the culture broths of approximately 1,000 actinomycetes were examined using a previously developed screening method.(ref. 8–10) In this method, two closely related *Bacillus* strains, *Bacillus halodurans* C-125 and *Bacillus subtilis* 168, were used as test organisms.(ref. 11) Although these strains exhibit a high degree of genome similarity, *B. halodurans* C-125 uses the futalosine pathway while *B. subtilis* 168 uses the canonical pathway. Considering that samples containing specific inhibitors would suppress the growth of *B. halodurans* C-125 but not of *B. subtilis* 168, we selected such samples by paper disk assays and obtained 14 culture broths (1.4%) as candidates. The candidates were further examined by a growth recovery assay with commercially available menaquinone-4 (MK-4) supplementation. Consequently, we found that the growth inhibition of *B. halodurans* C-125 by one sample (K18-0194) was recovered by supplementation of MK-4 (0.1 mg ml^{-1}). We thus selected the culture broth of K18-0194 for further investigation. The strain K18-0194 was isolated from soil in Chiba Prefecture, Japan, and identified as *Streptomyces* based on the morphology and 16S rRNA gene sequence.

To isolate the active compound in the culture broth, we grew *Streptomyces* sp. K18-0194 in a 100-ml test tube containing 10 ml TSB medium on a rotary shaker (200 rpm) at 28°C. After 48 h, a 0.5 mL portion of the seed culture was transferred into a 200-ml baffled Erlenmeyer flask containing 50 ml of the production medium (oatmeal 1%, pharmamedia 1%, glucose 0.5%, corn steep powder 0.5%, K_2HPO_4 0.5%, $MgSO_4 \cdot 7H_2O$ 0.5%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $ZnSO_4 \cdot 7H_2O$ 0.0001%, $CoCl_2 \cdot 6H_2O$ 0.0001%, $MnCl_2 \cdot 4H_2O$ 0.0001%, $CuSO_4 \cdot 5H_2O$ 0.0001%, pH 7.0) and cultivated on a rotary shaker (200 rpm) at 30°C for 48 h. Subsequently, the culture broth (200 ml) was mixed with an equal volume of ethanol and the supernatant obtained by centrifugation was

concentrated *in vacuo*. The residue was dissolved in water (7 ml) and was subjected to reverse phase column chromatography using a Sep-Pak Vac 35cc C18 column (Waters) with step gradient elution (20%, 40%, 80%, and 100% aq. acetonitrile). After concentration of the bioactive fractions eluted with 80% aq. acetonitrile, the resulting residue was further purified by HPLC (column: Kanto Mightysil Aqua RP-18 column (250 × 4.6 mm), column temperature: 40°C, mobile phase A: water, mobile phase B: acetonitrile, gradient: 0% solvent B for 0–5 min and a linear gradient to 100% solvent B for an additional 30 min, flow rate: 1 ml min^{-1}, detection: photo diode array detector 190–400 nm). By fractionation and bioassay, we identified the active compound eluted at a retention time of 28.6 min (Figure 2A). We collected the active compound by repetitive HPLC fractionation and obtained 0.7 mg of compound (**1**) as a pale yellow solid. The high resolution ESI-MS and ^{13}C NMR spectra of **1** indicated that its molecular formula was $C_{47}H_{66}O_{13}$ ($[M+Na]^+$ calculated for $C_{47}H_{66}O_{13}Na^+$: 861.4396; found: 861.4391). Analysis of the 1D- and 2D-NMR (^1H and ^{13}C, COSY, HSQC, HMBC and ROESY) spectra of compound **1** (Figures S1–9 and Table S1) established the planar structure from C-1 through C-47, although 2D correlation between C-1 and C-21 was not observed. By comparing the spectral data with a previous report together with its characteristic UV-vis spectrum, compound **1** was determined to be pulvomycin (**1**, Figure 2B).(ref. 12)

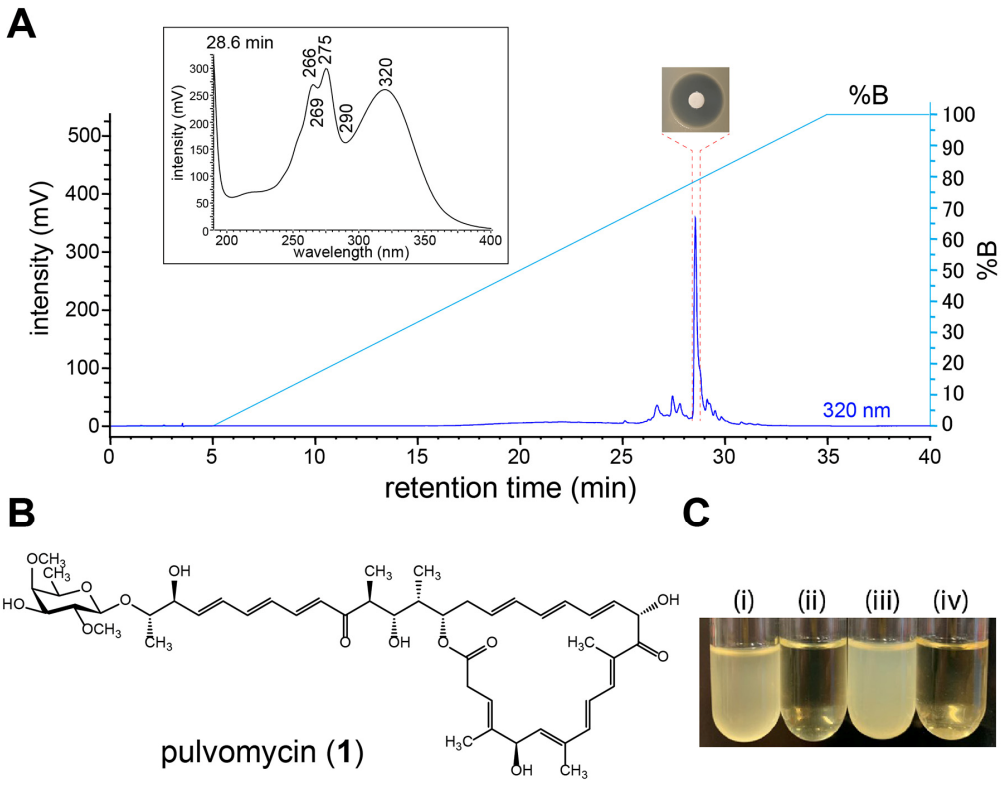

Figure 2. Isolation and characterization of the active compound. (**A**) Purification of **1** using HPLC by monitoring UV absorption at 320 nm (left scale) and the bioassay result against *B. halodurans* of the peak at retention time of 28.6 min. Gradient time program (%B) was shown with line (right scale). The inlet shows UV spectrum of the peak. (**B**) Structure of pulvomycin (**1**). (**C**) Growth recovery assay of *B. halodurans* C-125. (i) *B. halodurans* only, (ii) with **1** (0.2 μg ml^{-1}), (iii) with **1** (0.2 μg ml^{-1}) and MK-4 (100 μg ml^{-1}), and (iv) with **1** (0.2 μg ml^{-1}) and NQCA (20 μg ml^{-1}).

Pulvomycin (**1**) is a polyketide antibiotic that was originally isolated from an unidentified *Streptomyces* strain in 1957 and rediscovered in 1963 as labilomycin from *Streptomyces albosporeus* var. *labilomyceticus*.(ref. 13, 14, 15) As the name labilomycin implies, pulvomycin is

labile to light and heat and its structure was not determined until 1984.(ref. 12) Pulvomycin acts as a potent inhibitor of elongation factor Tu (EF-Tu) in protein synthesis by preventing the formation of the ternary complex between aminoacyl-tRNA and EF-Tu·GTP.(ref. 16–19) Pulvomycin shows antimicrobial activity against Gram-positive bacteria and its minimum inhibitory concentrations (MICs) for *Micrococcus luteus* (1 μg ml^{-1}), *Staphylococcus aureus* (2–4 μg ml^{-1}), *Staphylococcus saprophyticus* (2 μg ml^{-1}), *Staphylococcus epidermidis* (4 μg ml^{-1}), *Enterococcus faecium* (4 μg ml^{-1}), and *Enterococcus faecalis* (4–32 μg ml^{-1}), all of which use the canonical pathway for MK biosynthesis, have been reported.(ref. 20) By measuring the OD_{600} of liquid cultures containing various concentrations of **1**, we determined the MIC value of **1** against *B. halodurans* C-125 was 200 ng ml^{-1}, while its MIC value against *Bacillus subtilis* 168 was 10 μg ml^{-1}. We also confirmed that the growth inhibition of *B. halodurans* C-125 by compound **1** (200 ng ml^{-1}) was recovered by adding 0.1 mg ml^{-1} MK-4 (Figure 2C). These results indicated that **1** inhibited both the futalosine pathway and protein synthesis with higher affinity toward enzymes in the futalosine pathway.

We next investigated the inhibition target step of **1** in the futalosine pathway by growth recovery experiments. Because the intermediate 1,4-hydronaphthoquinone-6-carboxylic acid (NQCA) was readily available and we previously showed that NQCA is membrane-permeable by growth recovery experiments with *Streptomyces coelicolor* mutants disrupted at SCO4506 (*mqnA*) and SCO4550 (*mqnC*), the genes responsible for the biosynthetic steps before NQCA formation, we examined whether the growth of *B. halodurans* C-125 was recovered with NQCA supplementation.(ref. 4) As shown in Figure 2C, inhibition of *B. halodurans* C-125 by **1** (200 ng

ml^{-1}) was not recovered with supplementation of NQCA (0.02 mg ml^{-1}). Thus, compound **1** is suggested to inhibit a biosynthetic step between NQCA and MK. Although the detailed biosynthetic process from NQCA to MK is not clear at this point, it requires three enzymes, a prenyltransferase, a methyltransferase, and a decarboxylase. Because **1** contains a methyl-branched polyene moiety, **1** might inhibit the enzymes after the prenylation step by mimicking the isoprenyl side chain of a biosynthetic intermediate.

To date, branched fatty acids, tirandamycin, aplasmomycin, boromycin, polyunsaturated fatty acids, siamycin, and BuT-DADMe-ImmA have been identified as specific inhibitors of the futalosine pathway.(ref. 8–10, 21, 22) Among these, BuT-DADMe-ImmA was shown to inhibit 6-amino-6-deoxyfutalosine *N*-ribosylhydrolase (MqnB) while the others inhibit the conversion step from NQCA to MK. Although structure–activity relationship studies on **1** will be necessary to develop more specific inhibitors, the present study adds the polyene polyketide pulvomycin as a lead compound among futalosine pathway-specific inhibitors.

The authors declare no conflicts of interest.

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