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BRIEF COMMUNICATION

Aplasmomycin and boromycin are specific inhibitors of the futalosine pathway

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Abstract

We searched for specific inhibitors of the futalosine pathway, non-canonical pathway of menaquinone biosynthesis operating in *Helicobacter pylori*, from metabolites produced by actinomycetes. Aplasmomycin, a boron-containing macrodiolide, was isolated from *Streptomyces* sp. K15-0223 as a specific inhibitor of the futalosine pathway. We also showed boromycin, an analog of aplasmomycin, had similar activity.

Menaquinone (MK) is a lipid-soluble electron carrier and a crucial component in the respiratory chain for many bacteria. Two distinct biosynthetic pathways of MK have been identified in bacteria. In *Escherichia coli*, MK is biosynthesized using the well-studied canonical pathway, which requires eight enzymes designated MenA–H (Fig. 1a). [1, 2] An alternative pathway, which we discovered in *Streptomyces*, utilizes futalosine as a biosynthetic intermediate (Fig. 1b, futalosine pathway). [3–7] Bioinformatic analysis showed that the latter pathway also operates in human pathogens such as *Helicobacter pylori*, which causes stomach cancer. Because MK biosynthesis is essential for the survival of microorganisms, and most useful intestinal bacteria, such as lactobacilli, employ the canonical pathway, the futalosine pathway is an attractive target for the development of specific anti-*H. pylori* drugs.

We previously developed a screening method to identify compounds that specifically inhibit the futalosine pathway. [8, 9] In brief, we employed the paper disk assay using two closely related *Bacillus* strains, *Bacillus subtilis* 168 and *Bacillus halodurans* C-125, as test organisms. These two strains had been shown to possess a high degree of similarity by genome analysis. For the biosynthesis of MK, however, *B. subtilis* strain 168 and *B. halodurans* C-125 use the canonical pathway and the futalosine pathway, respectively. Because compounds inhibiting the futalosine pathway would repress the growth of *B. halodurans* C-125 but not of *B. subtilis* 168, we selected samples that specifically inhibited *B. halodurans* C-125. We examined approximately 2000 actinomycete culture broths. Of these, seven culture broths (0.35%) were found to inhibit the growth of *B. halodurans* C-125 but not of *B. halodurans* C-125. We further selected samples that specifically inhibited the futalosine pathway by testing whether the growth of *B. halodurans* C-125 was recovered by adding MK (0.1 mg ml^{-1}) into the culture broth during liquid cultivation, and identified a cultured broth of *Streptomyces* sp. K15-0223.

To investigate the active compound in the culture broth, *Streptomyces* sp. K15-0223 was cultivated in a 100-ml test tube containing 10 ml TSB medium on a rotary shaker (200 rpm) at 27 °C for 3 days. A portion of the seed culture (0.5 ml) was transferred into a 500-ml Erlenmeyer flask containing 50 ml of modified YEME medium (sucrose 17%, glucose 1%, Difco yeast extract 0.3%, Difco malt extract 0.3%, Difco peptone 0.5%, pH 7.0) and cultivated on a rotary shaker (200 rpm) at 27 °C for 5 days. After removing the cells by centrifugation, the supernatant (3 l) was extracted with the same volume of chloroform, at neutral pH, three times. The organic layer was washed with brine, dried over anhydrous sodium sulfate, and concentrated in

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vacuo. The residue was subjected to silica gel flash column chromatography using hexane-ethyl acetate (3:1) as the eluant. The bioactive fractions were collected and evaporated in vacuo. The resulting residue was dissolved in a small volume of 50% aqueous acetonitrile and further purified by HPLC (column: Kanto Mightysil Aqua RP-18 column (250 × 4.6 mm), mobile phase: 46% aqueous acetonitrile isocratic, flow rate: 1 ml min^{-1}, detection: photo diode array detector 190–400 nm). After fractionation and paper disk assay, the active component (**1**) eluted at a retention time of about 4.7 min (Figure S1). By repetitive HPLC purification, we obtained 0.7 mg of compound **1** from 3 l culture broth. The high resolution ESI-MS and ^{13}C NMR spectra of **1** indicated that its molecular formula was $C_{40}H_{61}O_{14}B$ ($[M\text{-}H]^-$ calculated for $C_{40}H_{60}O_{14}{}^{10}B^-$: 774.4118; found: 774.4137 and $[M + Na]^+$ calculated for $C_{40}H_{61}O_{14}{}^{10}BNa^+$: 798.40829; found: 798.4105). The observed isotopic pattern of the MS spectrum was in good agreement with that of the simulation. Analysis of 1D (^1H and ^{13}C) and 2D (COSY, TOCSY, HSQC, HMBC, and NOESY) NMR resonances and correlations revealed a polycyclic polyketide structure. By comparing the spectral data with a previous reference, compound **1** was confirmed to be aplasmomycin (**1**, Fig. 2). [10–12]

Aplasmomycin (**1**) is a boron-containing macrodiolide antibiotic that was originally isolated from the culture broth

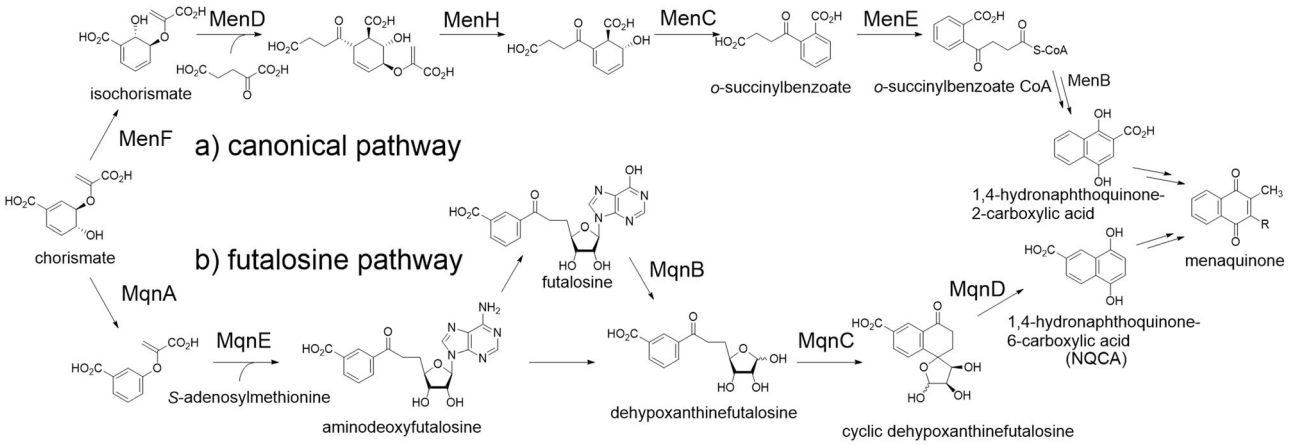

Fig. 1 Two distinct biosynthetic pathways of menaquinone. (**a**) the canonical pathway and (**b**) the futalosine pathway

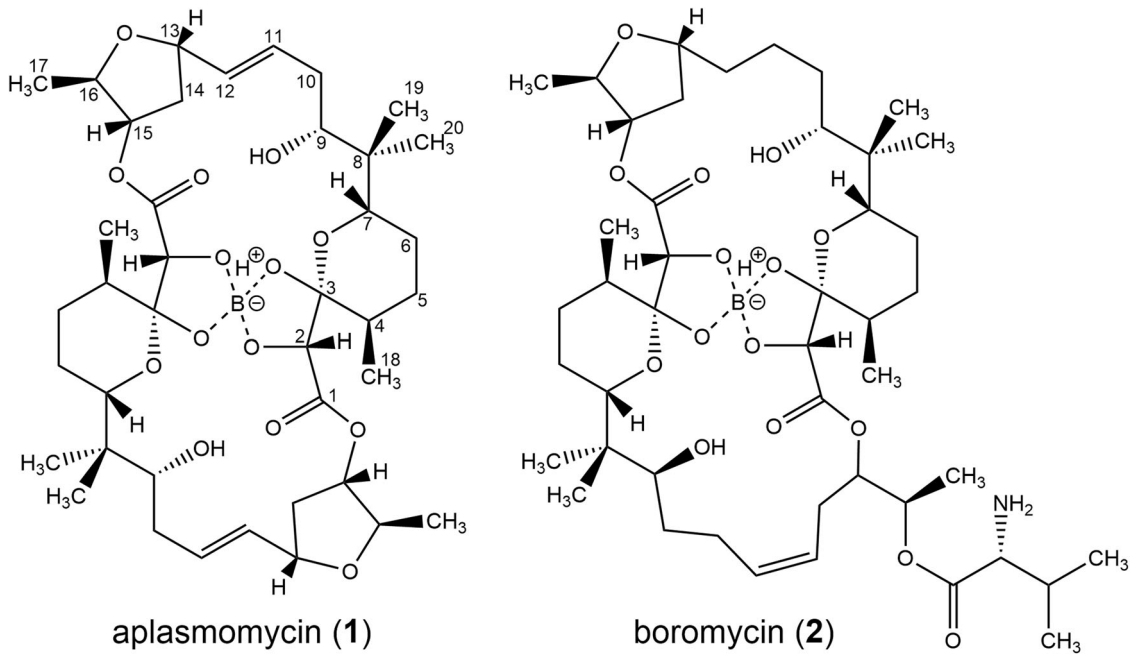

Fig. 2 Structures of aplasmomycin (**1**) and boromycin (**2**)

of *Streptomyces griseus* SS-20. [13] Aplasmomycin shows antimicrobial activity against Gram-positive bacteria with a minimum inhibitory concentration (MIC) range of 0.78–6.25 μg ml^{-1}, and exhibits inhibitory activity against plasmodium infections in vivo. To confirm whether **1** inhibited an enzyme in the futalosine pathway, the above-mentioned growth recovery assay was employed. As shown in Figure S11, the growth inhibition of *B. halodurans* C-125 by compound **1** (100 ng ml^{-1}) was clearly recovered by adding 0.1 mg ml^{-1} MK (Figure S11A). The MIC value of **1** against *B. halodurans* C-125 was calculated to be 40 ng ml^{-1} by measuring the OD_{600} of liquid cultures containing various concentrations of **1**, while no growth inhibition was observed for *Bacillus subtilis* 168 with up to 1 μg ml^{-1} of **1**. In the previous report, the antimicrobial activity of **1** was evaluated only against bacteria utilizing the canonical MK biosynthetic pathway (*Staphylococcus aureus*, *Bacillus anthracis*, *Corynebacterium bovis*, and *Mycobacterium smegmatis*). [13] The low MIC value of **1** against *B. halodurans* C-125 indicated that **1** is a potent inhibitor of the futalosine pathway. Because boromycin (**2**) is structurally similar to **1** [14] we also tested whether **2** had the same activity. Compound **2** inhibited the growth of *B. halodurans* C-125 (MIC: 10 ng mL^{-1}) but not the growth of *B. subtilis* 168 with up to 500 ng ml^{-1}, and the growth of *B. halodurans* C-125 was recovered by supplementation with MK, indicating that **2** also inhibited the futalosine pathway (Figure S11B).

We next investigated the target step of **1** in the futalosine pathway. The only compound available for the experiment was 1,4-hydronaphthoquinone-6-carboxylic acid (NQCA). We previously showed that NQCA was able to recover growth of mutants disrupted at the SCO4506 (*mqnA*) and SCO4550 (*mqnC*) genes, both of which participate in earlier biosynthetic steps than NQCA biosynthesis.[4] Using similar methods, we examined whether the growth of *B. halodurans* C-125 was recovered when NQCA (0.04 mg ml^{-1}) was added to cultures containing purified **1** (100 ng ml^{-1}). As shown in Figure S11A, *B. halodurans* C-125 was unable to grow in the presence of both **1** and NQCA, suggesting that compound **1** inhibited a step after the formation of NQCA.

To date, several compounds including branched fatty acids [8], tirandamycin [9], polyunsaturated fatty acids [15], the lasso peptide siamycin I [15], and a transition state analog of nucleosidases (BuT-DADMe-ImmA) [16], have been identified as specific inhibitors targeting the futalosine pathway. In the present study, we showed that the boron-containing macrodiolides aplasmomycin and boromycin are potent inhibitors of the futalosine pathway for the first time. Our findings could be useful in the design of more potent futalosine pathway inhibitors.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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