Running title: Tirandamycin inhibits menaquinone biosynthesis

Identification of Tirandamycins as Specific Inhibitors of the Futalosine Pathway

Yasushi Ogasawara, Kensuke Kondo, Ayumi Ikeda, Rikako Harada, and Tohru Dairi*

Graduate School of Engineering, Hokkaido University, Sapporo, Hokkaido 060-8628, Japan

*Corresponding author. E-mail: dairi@eng.hokudai.ac.jp

Keywords: futalosine pathway / inhibitor / menaquinone / tirandamycin

Menaquinone (MK) is an essential compound because it is an obligatory component of the electron transfer pathway in microorganisms. In *Escherichia coli*, MK was shown to derive from chorismate via a pathway involving eight enzymes, designated MenA–H (Figure 1a, canonical pathway).[1, 2] However, we revealed that an alternative pathway (Figure 1b, futalosine pathway)[3-7] was operating in some microorganisms including *Helicobacter pylori*, which causes gastric carcinoma. As humans and some useful intestinal bacteria, such as lactobacilli, possess the classical pathway, and MK biosynthesis is essential for the survival of microorganisms, the futalosine pathway is an attractive target for the development of specific anti-*H. pylori* drugs. In this study, we purified compounds from metabolites produced by actinomycetes and identified tetramic acid antibiotics, tirandamycins A and B, as specific inhibitors targeting the futalosine pathway.

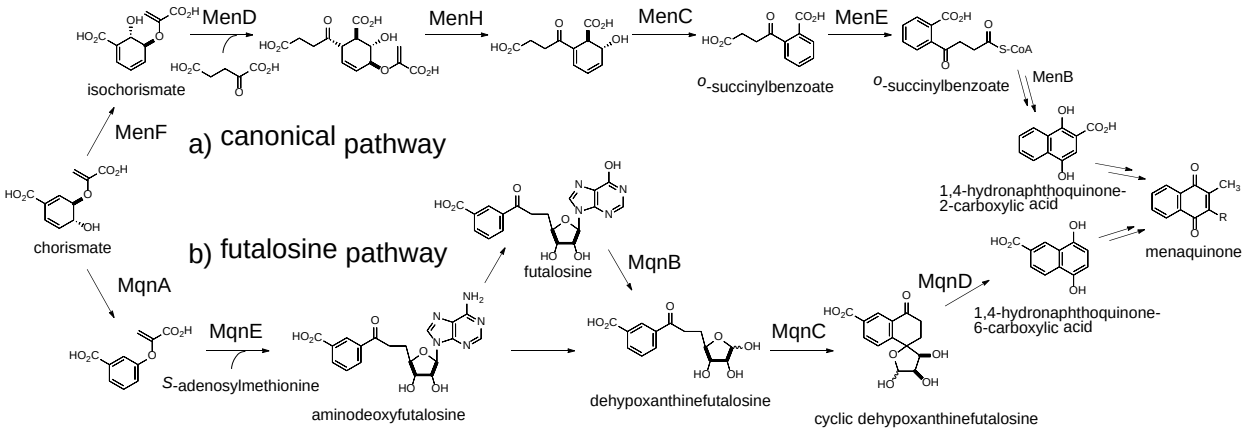

Figure 1 Schematic of the menaquinone biosynthetic pathways: (a) the canonical pathway and (b) the futalosine pathway

To identify compounds that specifically inhibit the futalosine pathway, we employed a previously developed screening method.[8] For the initial screening, we used the paper disk method and employed two closely related *Bacillus* strains, *Bacillus subtilis* strain 168 and

Bacillus halodurans C-125, as the test organisms. By genome sequencing, these two strains had been shown to possess a high degree of similarity.[9] However, *B. subtilis* strain 168 and *B. halodurans* C-125 use the classical pathway and the futalosine pathway, respectively, for the biosynthesis of MK. We therefore proposed that a compound inhibiting the biosynthesis of MK in the futalosine pathway may specifically repress the growth of *B. halodurans* C-125 only. To test this, we first screened candidate compounds for their ability to specifically inhibit *B. halodurans* C-125 using a paper disk assay. We tested approximately 3200 culture broths (2800 actinomycetes broths and 400 fungi broths). Of these, 18 culture broths were found to specifically inhibit the growth of *B. halodurans* C-125 (0.6%). In particular, the growth of *B. halodurans* C-125 was clearly inhibited in the presence of sample No. SF2910, but this inhibition was reversed by adding MK (0.1 mg ml^{-1}), even in the presence of sample No. SF2910. This result suggested that sample No. SF2910 contained a compound that specifically inhibited the futalosine pathway. Therefore, we next investigated active components in sample No. SF2910.

The actinomycete that was used for the preparation of sample No. SF2910 was cultivated in a 100-ml Erlenmeyer flask containing 15 ml seed medium (starch 2.5%, glucose 2.0%, polypeptone 0.7%, wheat germ 0.6%, yeast extract 0.45%, soybean meal 0.3%, Lab-Lemco-Powder (Merck) 0.3%, $CaCO_3$ 0.2%, pH 7.2) on a rotary shaker (220 rpm) at 28°C for 3 days. A portion of the medium was transferred into a 500-mL Erlenmeyer flask containing 80 mL of production medium (malt syrup 4.0%, soybean meal 2.0%, cotton seed meal 1.0%, sungrain F2 0.5%, soybean oil 0.3%, $CaCO_3$ 0.3%, $FeSO_4$ 0.001%, $CoCl_2$ 0.0001%, $NiCl_2$ 0.0001%, pH 7.2) and cultivated on a rotary shaker (220 rpm) at 28°C for 5 days. After centrifugation to remove cells, the supernatant was extracted with the same volume of ethyl acetate, at neutral

pH, three times. The organic layer was washed with brine, and concentrated *in vacuo*. The residue was dissolved in a small volume of acetonitrile and analyzed by HPLC (column: Kanto Mightysil Aqua RP-18 column (250 × 4.6 mm); mobile phase: 37% aqueous acetonitrile supplied with 0.1% formic acid; flow rate: 1 ml min^{-1}; detection: photo diode array detector 190–400 nm). By fractionation of each peak detected at 210 nm, we identified two active components (**1** and **2**, Figure S1). The UV-visible spectra of the two compounds were almost identical, suggesting that they are congeners. Compound **1** was purified by preparative HPLC, yielding 3.9 mg as a pale yellow solid. The high resolution ESI-MS of **1** revealed a molecular formula of $C_{22}H_{27}NO_8$ ($[M-H]^-$ calculated for $C_{22}H_{26}NO_8$ 432.1664; found 432.1671). Analysis of the 1D NMR (^1H and ^{13}C) and 2D NMR (COSY, HSQC and HMBC) spectra of compound **1** (Table S1 and Figure S2–7) revealed the partial structure from C-1 through C-18. Although 2D correlations were not observed on the remaining NMR signals, the signals for two carbonyl carbons (178.5 and 193.3 ppm), a quaternary carbon (101.5 ppm), and a methylene group (δ_C 52.3 ppm and δ_H 3.73 ppm, 2H) together with characteristic UV-vis spectrum established the tetramic acid structure in **1** (Figure 2). Comparing spectral data with a previous reference, compound **1** was confirmed as tirandamycin B.[10-12] Purified **2** was not obtained in sufficient quantity to allow NMR structural analysis, but was likely to be a tirandamycin on the basis of HR-ESI-MS (*m*/*z*: [M-H] calculated for $C_{22}H_{26}NO_7$ 416.1715; found 416.1724).

The minimum inhibitory concentration (MIC) value of **1** against *B. halodurans* C-125 was calculated to be 1 μg mL^{-1} by measuring the OD_{600} of liquid cultures containing various concentrations of **1**, and no growth inhibition for *Bacillus subtilis* strain 168 was observed up to 100 μg mL^{-1} of **1**. Tirandamycins A and B, originally isolated from *Streptomyces tirandis* in 1971,

are antibiotics that exhibit antimicrobial activity against a number of bacteria with an MIC range of 1–10 μg mL^{-1}.[12, 13] *In vitro* experiments have revealed that tirandamycins inhibit the chain initiation and elongation steps of RNA polymerase transcription.[14] Among tirandamycin-sensitive bacteria, some use a canonical MK biosynthetic pathway (*Bacillus megaterium*, *Clostridium pasteurianum*, *Streptococcus pyogenes,* and *Bacteroides fragilis*) and others use a futalosine pathway (*Streptomyces prasinus*). Therefore, tirandamycins are suggested to target both transcription and the futalosine pathway.

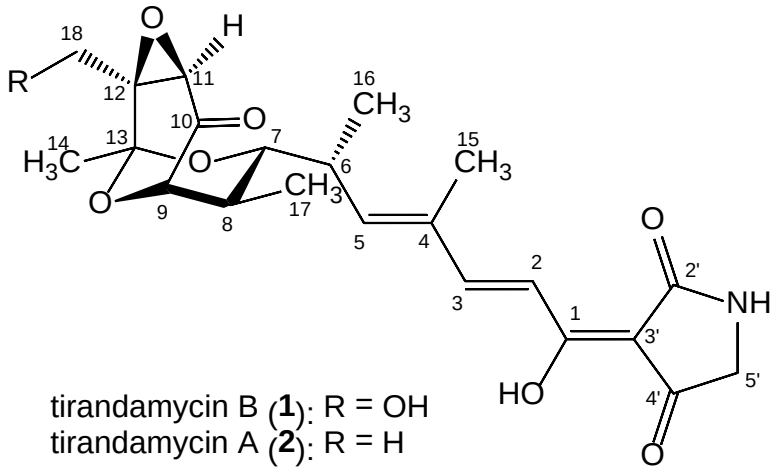

Figure 2 Structures of the tirandamycins

To confirm that the isolated compounds inhibit an enzyme in the futalosine pathway, we examined if the growth inhibition by **1** was recovered by co-administration of MK. Previously, the optimized concentration of MK for recovery assays was determined to be 0.1 mg ml^{-1} for the mutants disrupted at SCO4506 (*mqnA*) and SCO4550 (*mqnC*) of *Streptomyces coelicolor*.[4] In this study, we added 12.5, 25, 50, and 100 mg ml^{-1} of MK taking account of probable difference of uptake and utilization efficiency between the two strains. As shown in Figure S8, the growth inhibition of *B. halodurans* C-125 by compound **1** was clearly recovered

by adding MK in a concentration-dependent manner, and minimal effective concentration of MK was 0.05 mg ml^{-1}. These results suggested that tirandamycins **1** and **2** were specific inhibitors of the futalosine pathway in *B. halodurans* C-125. We next tried to examine which biosynthetic step in the futalosine pathway was inhibited by tirandamycins. However, we did not have sufficient quantities of the intermediate compounds in the futalosine pathway. The only compound available for the experiment was 1,4-hydronaphthoquinone-6-carboxylic acid (NQCA). We previously showed that NQCA is able to recover growth of the mutants disrupted at SCO4506 (*mqnA*) and SCO4550 (*mqnC*) genes, both of which participate in the earlier biosynthetic steps than NQCA biosynthesis.[4] Using similar methods, we examined whether the growth of *B. halodurans* C-125 was recovered when NQCA (0.05 mg ml^{-1}) was added to the medium containing purified compound **1**. The results indicated that *B. halodurans* C-125 was not able to grow in the presence of both **1** and NQCA (Figure S9). These results suggested that compound **1** inhibited a step after the formation of NQCA. These steps are thought to require three enzymes, a prenyltransferase, a methyltransferase, and a decarboxylase, although experimental evidence for this is lacking, but similar reactions are involved in the canonical pathway. The positional isomers, 1,4-hydronaphthoquinone-6-carboxylic acid and 1,4-hydronaphthoquinone-2-carboxylic acid, are involved in the futalosine pathway and the canonical pathway, respectively (Figure 1). Although it remains unclear how tirandamycins specifically inhibit an enzyme in the futalosine pathway, the molecular recognition of the enzymes, possibly due to subtle differences in the substrates, may be important to distinguish the two pathways of MK biosynthesis.

To date, several compounds including branched fatty acids[8], polyunsaturated fatty acids[15], a 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. It had been proposed that branched fatty acids and polyunsaturated fatty acids inhibited an enzyme that catalyzes the transfer of a prenyl side chain to the naphthoquinone moiety. Furthermore, BuT-DADMe-ImmA was shown to inhibit 6-amino-6-deoxyfutalosine *N*-ribosylhydrolase in the futalosine pathway. The present study revealed that tetramic acid antibiotics, tirandamycins A and B, also inhibited the futalosine pathway. Our findings could be useful in the design of more potent futalosine pathway inhibitors.

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research on Innovative Areas from MEXT, Japan (JSPS KAKENHI Grant Number 16H06452) and by the Urakami Foundation for Food and Food Culture Promotion to T. Dairi. We thank Meiji Seika Pharma Co., Ltd. for providing microbial cultures for screening. We also thank Dr. Eri Fukushi of the GCMS and NMR Laboratory, Graduate School of Agriculture, Hokkaido University, for analyzing the NMR spectra.

Supplementary Information accompanies this paper on *The Journal of Antibiotics* website.

1. Bentley R, Meganathan R. Biosynthesis of vitamin K (menaquinone) in bacteria. *Microbiol*. *Rev.* **46**, 241–280 (1982).

2. Meganathan R. Biosynthesis of menaquinone (vitamin K2) and ubiquinone (coenzyme Q): a perspective on enzymatic mechanisms. *Vitam*. *Horm.* **61**, 173–218 (2001).

3. Seto H, et al. Studies on a new biosynthetic pathway for menaquinone. *J*. *Am*. *Chem*. *Soc.* **130**, 5614–5615 (2008).

4. Hiratsuka T, et al. An alternative menaquinone biosynthetic pathway operating in microorganisms. *Science* **321**, 1670–1673 (2008).

5. Hiratsuka T, Itoh N, Seto H, Dairi T. Enzymatic properties of futalosine hydrolase, an enzyme essential to a newly identified menaquinone biosynthetic pathway. *Biosci*. *Biotechnol*. *Biochem.* **73**, 1137–1141 (2009).

6. Arakawa C, et al. Diversity of the early step of the futalosine pathway. *Antimicrob*. *Agents Chemother.* **55**, 913–916 (2011).

7. Mahanta N, Fedoseyenko D, Dairi T, Begley TP. Menaquinone biosynthesis: formation of aminofutalosine requires a unique radical SAM enzyme. *J*. *Am*. *Chem*. *Soc.* **135**, 15318–15321 (2013).

8. Tanaka R, et al. Branched fatty acids inhibit the biosynthesis of menaquinone in *Helicobacter pylori*. *J*. *Antibiot*. **64**, 151–153 (2011).

9. Takami H, et al. Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. *Nucleic Acids Res.* **28**, 4317–4331 (2000).

10. Meyer CE. Tirandamycin, a new antibiotic isolation and characterization. *J*. *Antibiot*. **24**, 558–560 (1971).

11. MacKellar FA, Grostic MF, Olson EC, Wnuk RJ. Tirandamycin. I. Structure assignment. *J*. *Am*. *Chem*. *Soc.* **93**, 4943–4945 (1971).

12. Hagenmaier H, Jaschke KH, Santo L, Scheer M, Zahner H. Metabiolic products of microorganisms. Tirandamycin B(author's transl). *Arch*. *Microbiol.* **109**, 65–74 (1976).

13. Karwowski JP, et al. Tirandalydigin, a novel tetramic acid of the tirandamycin-streptolydigin type. I. Taxonomy of the producing organism, fermentation and biological activity. *J*. *Antibiot.* **45**, 1125–1132 (1992).

14. Reusser F. Tirandamycin, an inhibitor of bacterial ribonucleic acid polymerase. *Antimicrob*. *Agents Chemother.* **10**, 618–622 (1976).

15. Yamamoto T, et al. Narrow-spectrum inhibitors targeting an alternative menaquinone biosynthetic pathway of Helicobacter pylori. *J*. *Infect*. *Chemother.* **22**, 587–592 (2016).

16. Wang S, et al. A picomolar transition state analogue inhibitor of MTAN as a specific antibiotic for *Helicobacter pylori*. *Biochemistry* **51**, 6892–6894 (2012).