BACTERIOSTATIC EFFECT OF 4,7-DICYANOBENZOFURAZAN DUE TO INACTIVATION OF 2,3-DIHYDROXYISOVALERATE DEHYDRATASE

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As part of our research on benzofurazans (BZs), we have reported the bacteriostases of BZs in *Escherichia coli*, which may be due to $O_2^{\cdot-}$ produced within *E. coli* in the presence of dioxygen (O_2). Incubation of *E. coli* with 4,7-dicyanobenzofurazan (**1**) lowered the 2,3-dihydroxyisovalerate dehydratase activity detectable in extracts from these cells. Addition of branched chain amino acids such as valine and leucine protected *E. coli* from growth inhibition by compound **1**, though it could not protect *E. coli* from the damage by paraquat (PQ). Addition of Fe(III)-tris[N-(2-pyridylmethyl)-2-aminoethyl]amine (Fe-TPAA), a novel superoxide dismutase mimic, protected the dehydratase in a dose-dependent manner, which confirms that inactivation of the dehydratase is largely due to production of $O_2^{\cdot-}$. The possibility was discussed that the bacteriostatic effect of compound **1** is due to the inactivation of 2,3-dihydroxyisovalerate dehydratase.

KEYWORDS benzofurazan; oxygen toxicity; 2,3-dihydroxyisovalerate dehydratase; superoxide; 4,7-dicyanobenzofurazan

We have already reported on the toxicity of benzofurazans (BZs) in *Escherichia coli*,[1-3] which is due to an increase in the intracellular flux of superoxide ($O_2^{\cdot-}$) by incubating aerobic *E. coli*. The $O_2^{\cdot-}$ production in *E. coli* through the action of 4,7-dicyanobenzofurazan (**1**) (Chart 1) was confirmed using the cytochrome *c* reduction method and ESR spectra.[2,3] Paraquat (PQ) (Chart 1) is also considered to increase the intracellular flux of $O_2^{\cdot-}$. However, compound **1** had a bacteriostatic, and not bacteriocidal, effect[3], while PQ had both effects. We now present the differences between BZs and PQ toxicities in *E. coli*.

In order to identify the bacteriostases of BZs, we compared BZs with PQ on $O_2^{\cdot-}$ production in *E. coli* and examined the protective effect of branched chain amino acids on bacteriostases by BZs. *E. coli* B B_{12}^-, ATCC 29682, was used throughout. *E. coli* was cultured at 37 °C in glucose minimal (GM) medium.[1] The following materials were added to the systems at different concentrations and in different combinations: compound **1**, PQ, valine and leucine. The growth was monitored in terms of changes in turbidity at 600 nm using a test tube (diameter = 15 mm) attached to the flask with a Coleman Junior II spectrophotometer. As shown in Fig. 1 A, compound **1** inhibited the growth of *E. coli* at 4 μM, but the effect was markedly relieved by adding valine and/or leucine. The growth inhibition caused by PQ could not be suppressed even by adding valine or leucine (Fig. 1 B). Remarkable protective effects against the toxicity of compound **1** could not be observed by adding other amino acids such as Gly, Lys, Asn, Gln, His, Arg, Ala, Thr, Ser, Pro, Glu, Tyr, Cys, Asp, Trp, Phe or Met. These results suggest that compound **1** causes serious damage to biosynthesis of branched chain amino acids such as valine and leucine. Fridovich and coworkers have reported that 2,3-dihydroxyisovalerate dehydratase, which catalyzes an essential step in the pathway for biosynthesis of branched chain amino acids, is susceptible to $O_2^{\cdot-}$ through the actions of PQ in *E. coli*.[4] Thus, we examined whether this growth inhibition is due to inactivation of 2,3-dihydroxyisovalerate dehydratase by $O_2^{\cdot-}$ produced through the action of compound **1**.

The preparation of *E. coli* cell lysate and the assay were carried out according to the method of Fridovich.[4] *E. coli* cell suspensions used were about 5.2 x 10^8 cells/ml (OD_{600} = 0.65). DL-Sodium-2,3-dihydroxyisovalerate was synthesized according to the method of Cioffi and Berg.[5] One unit of the dehydratase activity was defined as that causing the accumulation of 1 mM α-ketoisovalerate per 15 min under the conditions shown in ref. 4.

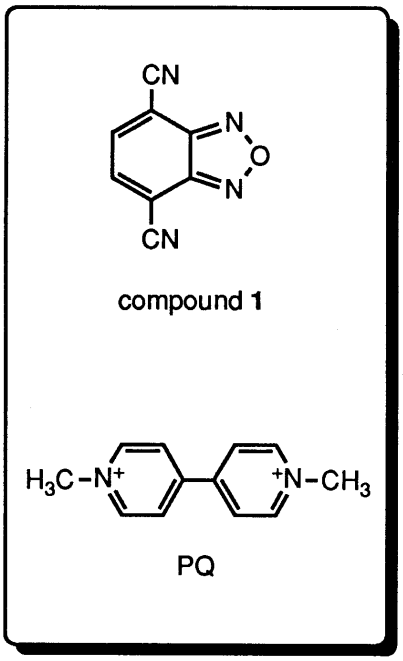

Chart 1

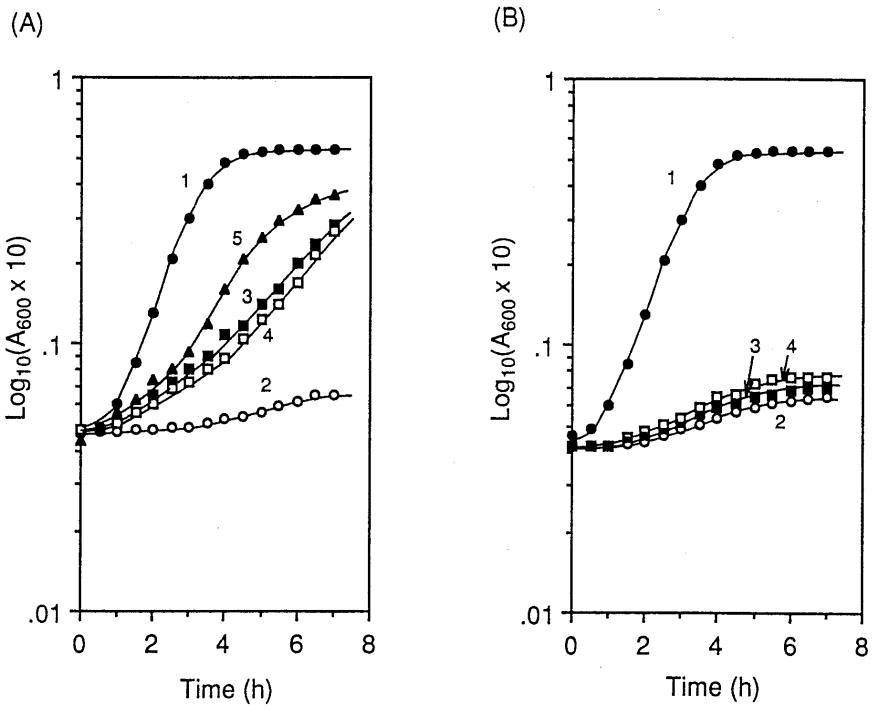

Fig. 1. Effect of Valine and/or Leucine on Toxicity of Compound 1 and PQ against *E. coli* Growth

(A): The GM medium contained the following compound(s): line 1, no additives; line 2, 4 μM of compound **1**; line 3, 4 μM of compound **1** and 1 mM of valine; line 4, 4 μM of compound **1** and 1 mM of leucine; line 5, 4 μM of compound **1**, 1 mM of valine and 1 mM of leucine.

(B): The GM medium contained the following compound(s): line 1, no additives; line 2, 7 μM of PQ; line 3, 7 μM of PQ and 1 mM of valine; line 4, 7 μM of PQ and 1 mM of leucine.

The results in Table I show that compound **1** caused inactivation of the dehydratase and this inactivation was $O_2^{-\cdot}$-dependent. The exposure was done in the presence of chloramphenicol to prevent synthesis of proteins. Assays of aliquots of these cultures demonstrated that chloramphenicol was adequate to completely suppress cell growth and protein synthesis (data not shown). Continuous $O_2^{-\cdot}$ production in *E. coli* should keep lowering the extent of the dehydratase activity in the cells. Fe(III)-tris[N-(2-pyridylmethyl)-2-aminoethyl]amine (Fe-TPAA) acts as a potent superoxide dismutase (SOD) mimic within *E. coli*.[6,7] As shown in Table II, adding Fe-TPAA allowed the recovery of the dehydratase activity, dependent on its concentration. Fe-TPAA could protect the dehydratase from $O_2^{-\cdot}$, which confirms that inactivation of the dehydratase is largely due to intracellular production of $O_2^{-\cdot}$.

As part of our research concerning reactive oxygen species, we have already reported on the toxicity of BZs in *E. coli*, which is due to increasing the intracellular flux of $O_2^{-\cdot}$ by incubating aerobic *E. coli*. Adding branched chain amino acids such as valine and leucine relieves the growth-inhibiting activity of compound **1**. Continuous exposure of *E. coli* to compound **1** causes inactivation of 2,3-dihydroxyisovalerate dehydratase. The activity of the dehydratase recovers more quickly with omission of O_2, and the inactivation of the dehydratase is suppressed by Fe-TPAA, a SOD mimic. Brown and Yein have

TABLE I. Inactivation of the Dehydratase by Compound 1

	Relative activity of dehydratase (%)	
	Aerobic	Anaerobic
Control	100 (1.85 units/mg)	100 (1.71 units/mg)
Compound 1 15μM	55	91

For anaerobic experiments, cultures were grown in the air with constant shaking until log phase (OD_{600} = 0.45) and were then exposed to compound 1 at 37 °C anaerobically for 40 min, after adding chloramphenicol. The culture was made anaerobic by bubbling with Ar.

TABLE II. Protective Effect of Fe-TPAA on Inactivation of the Dehydratase

	Relative activity of dehydratase (%)
Control	100 (2.30 units/mg)
Compound 1 15 μM	58
Compound 1 15 μM + Fe-TPAA 1 μM	78
Compound 1 15 μM + Fe-TPAA 10 μM	91
Compound 1 15 μM + Fe-TPAA 30 μM	97

After grown aerobically until OD_{600} = 0.2, cultures were exposed to various concentrations of Fe-TPAA and cells were grown until OD_{600} = 0.45 and then exposed to compound 1 for 40 min at 37 °C after adding chloramphenicol.

reported the dehydratase as the site of hyperbaric oxygen poisoning in branched chain amino acid biosynthesis.[8] These results suggest that inactivation of the dehydratase is the cause of the bacteriostatic effect of compound 1. Though compound 1 has the ability to produce greater amounts of $O_2^{\cdot-}$ than PQ, it is less toxic.[3] This suggests the importance of production site(s) of $O_2^{\cdot-}$, rather than production amounts, within the *E. coli* cell. Kitzler and Fridovich have proposed that the bacteriostatic and bacteriocidal effects of PQ involve attack upon distinct targets within the cell and that the bacteriocidal effect differs in kind, not merely in degree, from its bacteriostatic effect.[9] It is probable that PQ damages not only the dehydratase but also DNA, membranes, or other essential macromolecules, while compound 1 mainly inactivates the dehydratase, and so adding valine and leucine can reduce the toxicity of the compound 1. We reported that the *E. coli* strain used in this communication retains PQ but does not retain compound 1 after washing.[3] Compound 1 has high permeability through the *E. coli* cell membrane. The difference between compound 1 and PQ in the inactivation of 2,3-dihydroxyisovalerate dehydratase may be interpreted by the different ability of the two compounds to penetrate the *E. coli* cell membrane. Thus, compound 1 should be less toxic than PQ, because the one-electron reduced form of compound 1 is ready to exit from the cell.

Further studies on target(s) for the lethal effect of PQ are in progress.

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