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INHIBITION OF *E. coli* GROWTH BY FULLERENE DERIVATIVES AND INHIBITION MECHANISM

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Abstract: Cationic ammonium fullerene derivatives (C_{60}-*bis*(*N,N*-dimethylpyrrolidinium iodide) and C_{60}-*bis*(*N*-methylpiperazinium iodide)) suppressed *E. coli* growth, whereas an anionic derivative (C_{60}-dimalonic acid) did not. Both cationic derivatives inhibited *E. coli* dioxygen consumption. Inhibition of energy metabolism is concluded to be a mechanism of the growth inhibition effect of fullerene derivatives.

Fullerene is a new type of carbon allotrope that was discovered in 1985.[1] It has a unique cage-like shape, and a great deal of attention has been focused on its properties. Several years' wide study of fullerene and its analogs have revealed many aspects of their physical properties and chemical reactivities.

Investigation of the biological effects of fullerene and its derivatives with regard to the development of new and efficient pharmaceuticals is also interesting.[2] In contrast to studies in the physical and chemical fields, biological activities have not been fully investigated, mainly because the high hydrophobicity and poor solubility of the compounds in aqueous media make the study of fullerene and its derivatives difficult.[2] However, introducing hydrophilic groups in a C_{60} core by chemical modification has partly solved this problem.[3-5] Recently some interesting results in this field have been reported, including active oxygen quenching activity,[4-8] neuroprotective activity,[8] anti-tumor activity (photodynamic therapy),[9] and HIV protease inhibition activity (anti-HIV activity).[10] We have been investigating the model metabolic reactions of fullerene.[11]

We previously examined the effect of water-soluble fullerene derivatives (**1**, **2**, and **3** in Fig. 1) on the active oxygen generating system and found their O_2^- quenching activity.[4, 7] We also showed that C_{60}-dimalonic acid, **1**, actually decreased the toxicity of active oxygen generators in *E. coli*.[12] Later, the effect of other fullerene derivatives on the toxicity of active oxygen generators in *E. coli* was studied. During this study, we found the bacteriostatic effect of C_{60}-*bis*(*N,N*-dimethylpyrrolidinium iodide), **2**. Da Ros *et al.* reported

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the antimicrobial activity of **4** (Fig. 1).[13] These results encouraged us to investigate the inhibition of *E. coli* growth by fullerene derivatives and the inhibition mechanism.

Here we show that cationic C_{60} derivatives exhibited a bacteriostatic effect and inhibited glucose induced dioxygen uptake by *E. coli*. The inhibition of energy metabolism is thought to be a mechanism of the bacteriostatic effect of fullerene derivatives.

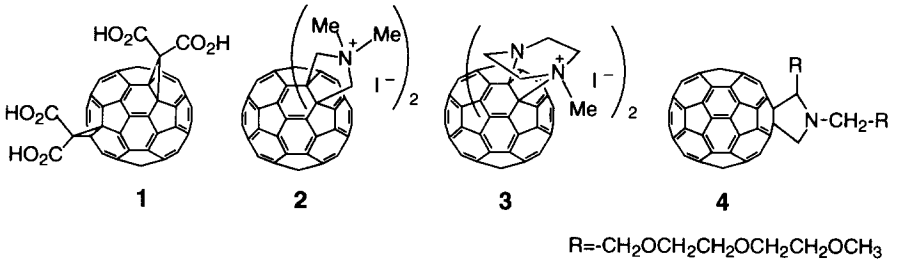

Fig. 1 Fullerene derivatives

Fullerene derivatives used in this study are listed in Fig. 1. **1**, an equatorial isomer of dimalonic acid derivative, was prepared as described by Lamparth and Hirsch,[3] and we have already reported the synthetic methods of C_{60}-*bis*(*N*,*N*-dimethylpyrrolidinium iodide), **2** and C_{60}-*bis*(*N*-methylpiperazinium iodide), **3**.[4] **2** and **3** are mixtures of regioisomers.

E. coli B B_{12}^-, ATCC 29682, was used throughout. *E. coli* was cultured at 37 °C in a water bath shaker at 100 rpm with a flask volume to culture volume ratio of 5:1. Growth was monitored in terms of changes in turbidity at 630 nm using a test tube (diameter = 15 mm) attached to the flask with a photoelectric colorimeter. The culture medium contained $MgSO_4 \cdot 7H_2O$ 0.2 g, citric acid·H_2O 2.0 g, K_2HPO_4 10.0 g, $NaNH_4HPO_4 \cdot 4H_2O$ 3.5 g, vitamin B_{12} 1.0 mg, and glucose 5.0 g in one liter. Fullerene derivatives **1**, **2**, and **3** were dissolved in DMSO and then added to the medium. The final DMSO concentration was less than 2.0 %, and 2.0 % DMSO did not affect *E. coli* growth.

For a dioxygen uptake experiment, *E. coli* was grown in a 60-ml medium for 18 hr at 37℃ and then collected by centrifugation. The pellet cells were washed twice with 50 mM potassium phosphate pH 7.8 containing 0.1 mM EDTA and again collected by centrifugation. The consumption of dioxygen was monitored polarographically with a Clark type electrode. The reaction solution contained 0.1 mM EDTA and 0.025 % glucose in 50 mM potassium phosphate pH 7.8. The reaction was started by the addition of 40 μl *E. coli* solution at 37℃. Fullerene derivatives were also dissolved in DMSO and then added to the reaction buffer. The final DMSO concentration was less than 1.0 %, and 1.0 % DMSO did not affect dioxygen consumption.

Figure 2 shows the effects of fullerene derivatives on *E. coli* growth. As we have previously reported, **1** did not affect growth up to 50 μM.[12] 100 μM of **1** suppressed growth slightly (Fig. 2A). Only 5 μM of **2** inhibited growth completely (Fig. 2B). **3** also showed growth inhibition activity, but the effect was lower

than that of **2** (Fig. 2C). **2** was most effective in growth inhibition.

To elucidate a mechanism of growth inhibition, dioxygen uptake by *E. coli* was examined in the presence of glucose. These fullerene derivatives seemed to inhibit energy metabolism in *E. coli*. Glucose was metabolized through a glycolytic pathway, TCA cycle, and then the respiratory chain, and finally dioxygen was consumed to produce H_2O.

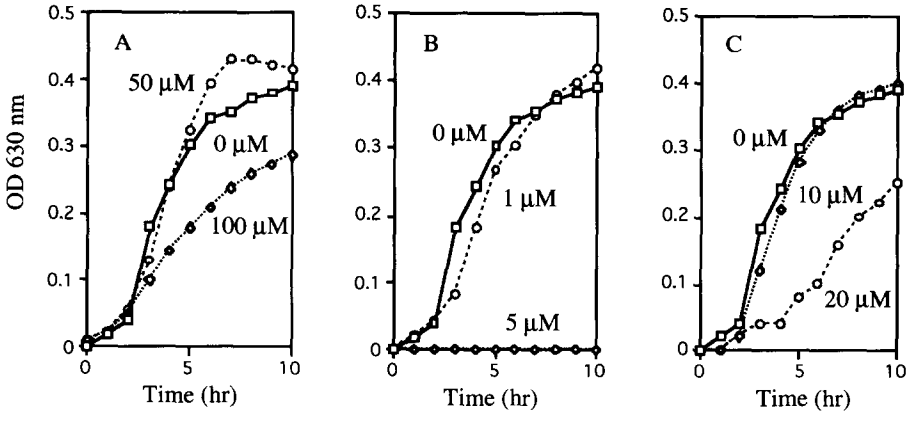

Fig. 2 Effect of fullerene derivatives on *E. coli* growth
Incubation conditions are described in the text. A: **1**, B: **2**, C: **3**.

As shown in Fig. 3A, the addition of an *E. coli* solution initiated dioxygen consumption in the presence of glucose. Dioxygen uptake rates were measured and are listed in Table 1 and shown as a dashed line in Fig. 3. In the presence of 50 μM of **2**, the initial dioxygen uptake rate was almost same as that of the rate without **2**, but after a short lag time (less than 30 seconds), consumption was inhibited (Fig. 3B). **3** also suppressed the consumption, but the effect was lower than that of **2** (Table 1). In this case, no remarkable initial lag time was observed. The addition of 50 μM of **1** did not affect consumption (Table 1).

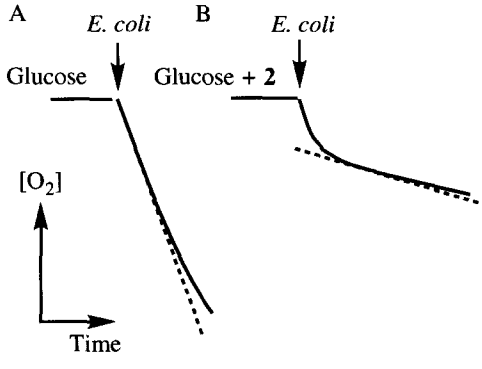

Fig. 3 Dioxygen consumption by *E. coli*

Table 1 Effect of fullerene derivatives on the dioxgen uptake

Fullerene (50 μM)	Dioxgen uptake rate (μM/min)
none	46.6
1	42.2
2	4.1
3	13.7

The result of dioxygen consumption inhibition was consistent with that of bacteriostatic effect. That is, **2** was most effective in both the bacteriostatic effect and the dioxygen uptake inhibition (inhibition of energy metabolism). **3** also showed both activities but was less effective than **2**. Finally, **1** had almost no activity. These results strongly indicate that the mechanism of the bacteriostatic effect was energy metabolism inhibition in *E. coli*. In the case of **2**, the observation of a short lag time may indicate that the fullerene derivative takes time to penetrate the cell.

Further studies for the energy metabolism inhibition mechanism of the fullerenes have been undertaken. We are now interested in activities of these cationic ammonium derivatives against a wide variety of microorganisms and cancer cells to develop antibacterial and anti-cancer drugs.

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