Toxicities of Dicyanobenzofurazans with Formation of Superoxide in *Escherichia coli*

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The toxicities of some benzofurazans (BZs), benzofurazan (1), 4,7-dimethylbenzofurazan (2), 4,7-dibromobenzofurazan (3), 4-bromo-6-cyanobenzofurazan (4), 4,7-dicyanobenzofurazan (5) and 4,5-dicyanobenzofurazan (6), were examined on *Escherichia coli*. Compound 5 at 4 μM and compound 6 at 7 μM completely inhibited the growth of *E. coli* in a simple nutritionally restricted medium (GM medium). These compounds were more toxic in GM medium than in a nutritionally rich medium (YE medium), which contained yeast extract as an additive in GM medium. Compound 4 also inhibited the growth of *E. coli* at 300 μM in GM medium. The toxicities of BZs were in the order of $1 \approx 2 \approx 3 < 4 \ll 5 \approx 6$. Compounds 4, 5 and 6 induced manganese-superoxide dismutase (Mn-SOD) and catalase activities of *E. coli* in YE medium. The induced SOD and catalase provide a defense against the potential cytotoxicities of O_2^- and H_2O_2. The rate of dioxygen uptake in cyanide-resistant respiration of *E. coli* was dependent on the concentration of 5, and was correlated with the induction of SOD and catalase. The reduction potentials of BZs followed the order of $1 \approx 2 < 3 < 4 < 5 \approx 6$. Compounds 5 and 6, which had redox potentials higher than those of the other BZs, are thought to be more readily reduced in the living system. The present results suggest that the toxicities of compounds 5 and 6 to *E. coli* are due to their reduction within the *E. coli* cell and their reoxidization by molecular dioxygen to form superoxide (O_2^-) and hydrogen peroxide (H_2O_2). Compound 4 was also suggested to damage *E. coli* through the same mechanism, though it was less toxic.

Keywords superoxide; hydrogen peroxide; benzofurazan; *E. coli*; superoxide dismutase; catalase; oxygen toxicity; herbicide; toxicity; induction

There has been considerable interest in compounds which enhance the generation of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in biological systems.[1] Such compounds, including heteropentalenes,[2] paraquat,[3] bleomycin[4] and menadione,[5] often have various biological activities. Benzofurazan and its derivatives (BZs) (Chart 1) have the *o*-quinoid form which may increase the production of O_2^- and H_2O_2 *in vivo*. Generally, quinone compounds are readily reduced by reduced nicotinamide adenine dinucleotide (phosphate) (NAD(P)H) with or without diaphorase to form semi-quinone radicals, which react with dioxygen to generate univalent reduced species, superoxide (O_2^-) and/or divalent reduced species, hydrogen peroxide (H_2O_2).[5] Superoxide and hydrogen peroxide are very toxic to living cells.[6] Quinones, which have the potential to increase the production of O_2^- and/or H_2O_2 in living cells, can also cause serious damage but may be applicable as anticancer drugs if they show selective toxicity. 4,7-Dicyanobenzofurazan (**5**) and 4,5-dicyanobenzofurazan (**6**) have herbicidal activity,[7] though the biological mechanism involved is not known as yet. In order to elucidate the mechanism of toxicity, we examined whether various BZs, benzofurazan (**1**), 4,7-dimethylbenzofurazan (**2**), 4,7-dibromobenzofurazan (**3**), 4-bromo-6-cyanobenzofurazan (**4**), 4,7-dicyanobenzofurazan (**5**) and 4,5-dicyanobenzofurazan (**6**), can mediate the electron flow from the respiratory chain to dioxygen.

In this paper, we report that compounds **4**, **5** and **6** promote the formation of more O_2^- and H_2O_2 than other BZs in *Escherichia coli* and also that their toxicities are related to both the redox potentials and the increased contents of superoxide dismutase (SOD) and catalase, which are defense enzymes against the active oxygen species, O_2^- and H_2O_2, respectively.

Materials and Methods

Escherichia coli B B_{12}, ATCC 29682, which was used throughout, was provided kindly by Prof. Fridovich (Department of Biochemistry, Duke University, NC, U.S.A.). It was cultured at 37 °C in a water bath shaker at 100 rpm with a ratio of flask volume to culture volume of 5:1, and 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. Glucose minimal (GM) medium[2a] contained, per liter, $MgSO_4 \cdot 7H_2O$, 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. Yeast extract (YE) medium[2a] contained 0.5% yeast extract from DIFCO Laboratories in GM medium.

Harvesting of *E. coli* Cells and Preparation of Cell-Free Extract Cultures were grown for 7 h and then cells were harvested by centrifugation at 13000 × *g* for 5 min at 4 °C. After resuspension in 50 mM potassium phosphate (pH 7.8) including 0.1 mM ethylenediaminetetraacetic acid (EDTA), the cells were harvested by centrifugation at 10000 × *g* for 10 min at 4 °C and washed three times with the phosphate buffer. Cells were suspended with 0.5—2.0 ml of the buffer and sonically disrupted in an ice bath with Branson sonifier (Cell Disruptor 200). Lysates were clarified at 13000 × *g* for 10 min at 4 °C. The resulting cell-free extract was stored in ice until use.

Specific Activities of SOD and Catalase The specific activities of SOD and catalase were assayed as previously described.[8] Protein was estimated by the Lowry procedure with bovine serum albumin (Sigma Chemical Co.) as a standard.[9] Electrophoresis was performed in 7% polyacrylamide gels[10] and SOD isozymes were visualized with the activity stain as previously described.[11]

The method of Gregory and Fridovich was modified for the analysis of catalase isozymes from *E. coli*.[12] Catalase isozymes were separated by electrophoresis on 7% polyacrylamide gel.[13] Cell-free extract was added to 20% sucrose solution in order to prevent convective mixing and electrophoresed in 0.188 M bicine-imidazole (pH 7.8) at 4 °C. Bromophenol blue was used as the tracking dye. Gels were removed at the end of the run and incubated individually for 45 min at 25 °C in 50 mM potassium phosphate buffer (pH 7.0) containing 0.05 mg/ml horseradish peroxidase (Sigma Chemical Co., Type I). After the incubation, gels were soaked for 10 min in 20 mM H_2O_2 in potassium phosphate buffer solution at 25 °C, rinsed with distilled water three times, and then individually kept for 20 min in the dark in the potassium phosphate buffer solution containing 0.5 mg/ml 3,3'-diaminobenzidine·4HCl (Dojin Laboratories) at 25 °C. The achromatic bands of catalase activities were visible against a brown-colored background.

Oxygen Uptake in Cyanide-Resistant Respiration Oxygen uptake by cell suspensions was measured at 35 °C using a Clark-type polarographic electrode (YSI. No. 5331, Yellow Springs Instrument Co., Ltd.) with and

without **5** according to the method of Hassan and Fridovich.[3c] The cyclic voltammograms of BZs at 20 mM in CH_3CN solution were measured using an RRDE-1 rotating ring-disk electrode (Nikko Keisoku). The CH_3CN solution contained 0.1 M tetraethylammonium perchlorate as the supporting electrode.

Preparation of Compounds 1, 2, 3, 4, 5 and 6 Benzofurazan (**1**) was prepared by reduction of benzofurazan *N*-oxide (benzofuroxan)[14] with hydroxylamine.[15] 4,7-Dimethylbenzofurazan (**2**)[16] and 4,7-dibromobenzofurazan (**3**)[17] were prepared by reduction of the corresponding benzofuroxan with triphenylphosphine, respectively. 4-Bromo-6-cyanobenzofurazan (**4**), a novel compound, was prepared according to the following method. 4,6-Dibromobenzofurazan (450 mg) and cuprous cyanide (300 mg) dissolved in dimethylformamide (2 ml) were stirred at 150 °C for 2 h. Dimethylformamide was removed under reduced pressure, and the residue was extracted with dichloromethane (100 ml). After evaporation of the dichloromethane, the crude mixture was subjected to silica gel preparative thin layer chromatography (TLC) (Merck Co., No. 5717) and compound **4** was eluted with dichloromethane : *n*-hexane = 9 : 1. The yield was 10%. mp 99—101 °C. ^{1}H-NMR ($CDCl_3$/TMS) δ: 7.97 (1H, d, J = 1.5 Hz), 8.38 (1H, d, J = 1.5 Hz). *Anal.* Calcd for $C_7H_2BrN_3O$: C, 37.53; H, 0.90; N, 18.76. Found: C, 37.59; H, 0.95; N, 18.81. MS *m/z*: 222.9379 (M^+). 4,5-Dicyanobenzofurazan (**5**) and 4,7-dicyanobenzofurazan (**6**) were prepared by reaction of the respective bromoheterocycle with cuprous cyanide in dimethylformamide.[18]

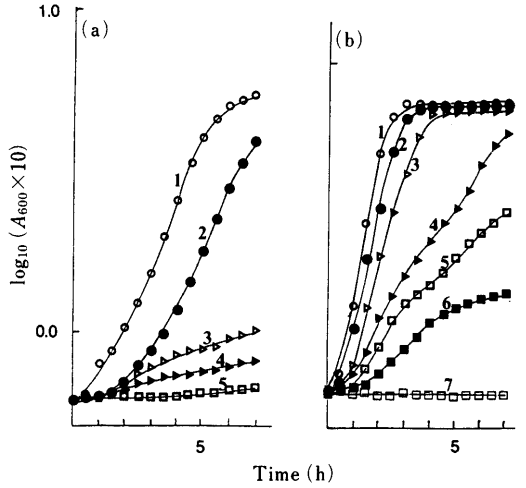


Fig. 1. Effect of Compound **5** on Growth of *E. coli* in GM (a) and YE Media (b)

(a) 1, no addition; 2, 1 μM; 3, 2 μM; 4, 3 μM; 5, 4 μM. (b) 1, no addition; 2, 5 μM; 3, 10 μM; 4, 50 μM; 5, 100 μM; 6, 200 μM; 7, 400 μM.

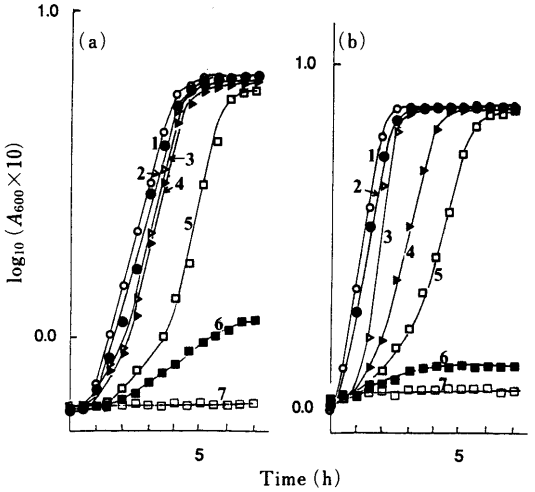


Fig. 2. Effect of Compound **6** on Growth of *E. coli* in GM (a) and YE Media (b)

(a) 1, no addition; 2, 1 μM; 3, 2 μM; 4, 3 μM; 5, 4 μM; 6, 5 μM; 7, 7 μM. (b) 1, no addition; 2, 5 μM; 3, 10 μM; 4, 50 μM; 5, 100 μM; 6, 200 μM; 7, 400 μM.

Results

Effects of Benzofurazans 1—6 on Growth of *E. coli* *E. coli* was aerobically exposed to a wide range of concentrations of **4**, **5** and **6** in GM and YE media. As shown in Figs. 1 and 2, **5** and **6** completely inhibited the growth of *E. coli* at 4 and 7 μM, respectively, in GM medium, and at 400 and 400 μM, respectively, in YE medium. The cells were more sensitive to **5** and **6** in GM medium than in YE medium and the toxic effect was dose-dependent. On the other hand, **4** was effective at 300 μM in GM medium and 3.0 mM in YE medium (Fig. 3), while compounds **1**, **2** and **3** did not inhibit the growth at all. BZ toxicities were in the order of **1** ≈ **2** ≈ **3** < **4** ≪ **5** ≈ **6** (Chart 1).

Effects of Compounds 4, 5 and 6 on Induction of Specific Activities of SOD and Catalase We have reported that heteropentalenes can increase the intracellular production of $O_2^{\cdot-}$, strikingly increasing the rate of biosynthesis of SOD.[2a] The toxicities of **4**, **5** and **6** to *E. coli* also appear to be due to the formation of $O_2^{\cdot-}$ within *E. coli* cells. Thus, we measured the specific activities of SOD and catalase in *E. coli* exposed to **4**, **5** and **6**. Table I shows the levels of SOD and catalase present in *E. coli* grown in GM and YE media over a range of concentrations of **4**, **5** and **6**. These compounds induced SOD production in the cells in a dose-dependent manner in the presence of yeast extract, but not in its absence. Catalase production was also induced dramatically by **5** and **6**. Compound **4** was less effective than compounds **5** and **6**, that is, a higher concentration of compound **4** was needed for induction of specific activities of SOD and catalase, as shown in Table I. As the pro-

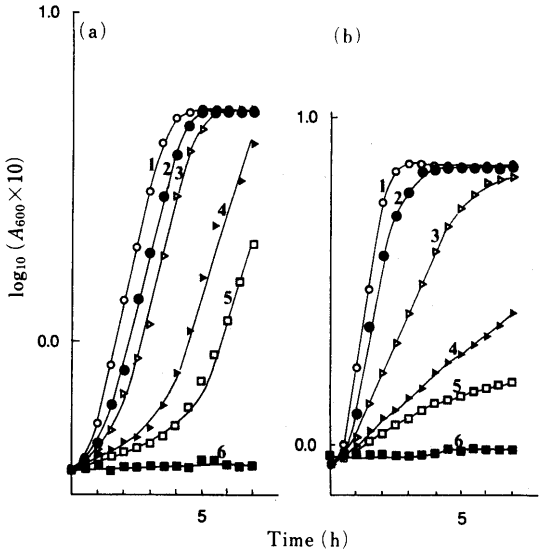
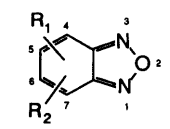


Fig. 3. Effect of Compound **4** on Growth of *E. coli* in GM (a) and YE Media (b)

(a) 1, no addition; 2, 30 μM; 3, 50 μM; 4, 100 μM; 5, 150 μM; 6, 300 μM. (b) 1, no addition; 2, 0.5 mM; 3, 1.0 mM; 4, 1.5 mM; 5, 2.0 mM; 6, 3.0 mM.



benzofurazans (BZs)

	R_1	R_2
1	H	H
2	4-CH_3	7-CH_3
3	4-Br	7-Br
4	4-Br	6-CN
5	4-CN	7-CN
6	4-CN	5-CN

Chart 1

TABLE I. SOD and Catalase Synthesis in *E. coli* Grown in Different Media in the Presence of Compound **4**, **5** or **6**[a]

Medium	Benzofurazan (μM)	SOD (units/mg protein)	Catalase (units/mg protein)
	Compound **4**		
GM	0	8	12
	30	7	12
	50	7	12
	100	7	17
YE	0	26	13
	500	39	27
	1000	44	64
	1500	48	72
	Compound **5**		
GM	1	9	14
	2	15	25
YE	5	25	7
	10	39	10
	50	44	18
	100	47	80
	200	54	158
	Compound **6**		
GM	1	8	10
	2	9	11
	3	8	10
	4	8	13
	5	9	18
YE	5	28	18
	10	29	21
	50	44	40
	100	49	90

a) Cells were grown for 7 h at 37 °C at each concentration of **4**, **5** or **6** prior to sonication and assay for SOD. Inocula were taken from 15 h cultures grown in GM and YE media.

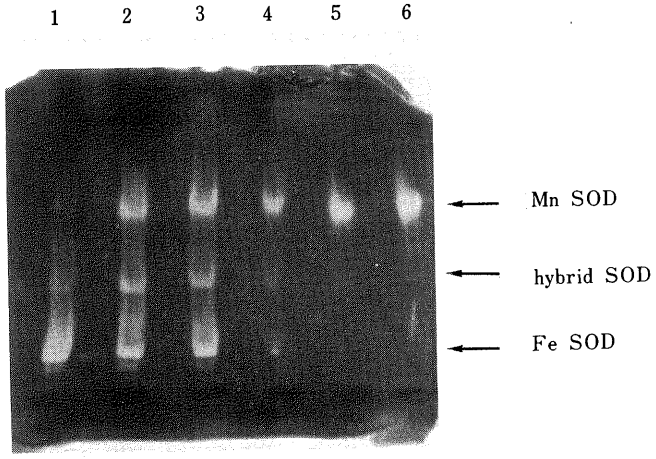


Fig. 4. Effect of Compound **5** on Synthesis of SOD Isozymes

E. coli was grown aerobically for 7 h in YE medium with the following levels of **5**. 1, no addition; 2, 5 μM; 3, 10 μM; 4, 50 μM; 5, 100 μM; 6, 200 μM. Samples containing 2.5 U/mg protein specific activity of SOD were applied to 7% polyacrylamide gels. After electrophoresis, the gels were stained for activity.

duction of neither SOD nor catalase was induced in the absence of yeast extract, *E. coli* cells were damaged at low concentrations of **5** and **6** in GM medium. The cells in GM medium were unable to defend themselves against the cytotoxicity of the active oxygen species whose production was enhanced by **5** and **6**.

E. coli contains three forms of SOD — Mn-SOD,[19] Fe-SOD[20] and hybrid-SOD.[21] Hassan and Fridovich have

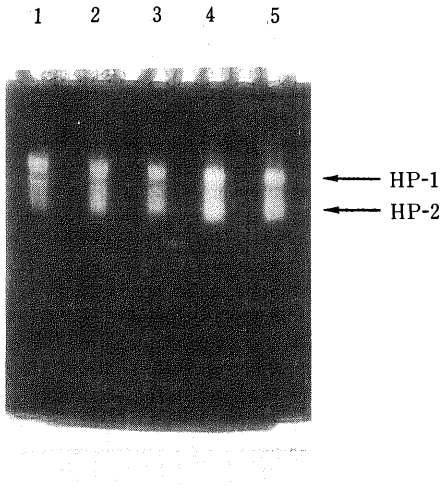


Fig. 5. Effect of Compound **5** on Synthesis of Catalase Isozymes

E. coli was aerobically grown for 7 h in YE medium with the following levels of **5**. 1, no addition; 2, 5 μM; 3, 10 μM; 4, 50 μM; 5, 100 μM. Samples containing 0.25 U of catalase were applied to 7% polyacrylamide gels. After electrophoresis, the gels were stained for activity.

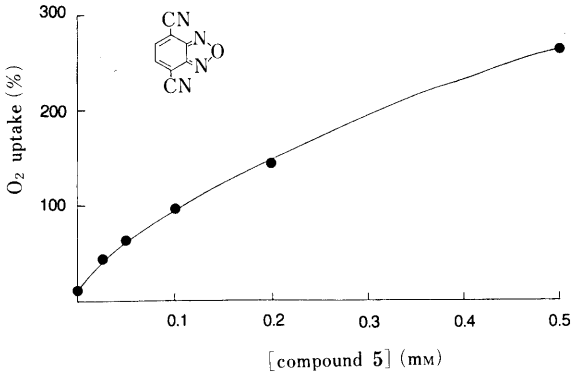


Fig. 6. Effect of Compound **5** on the Rates of Cyanide-Resistant Respiration in *E. coli*

Cells were grown for 7 h at 37 °C in GM medium, collected by centrifugation, and suspended in GM medium. The reaction mixture contained 40 μl of cells, 0.5% glucose and 1 mM KCN, and also GM medium to a final volume of 2 ml. Compound **5** was added after inhibition by CN^- had been established. Oxygen uptake is plotted on the ordinate, which presents the O_2 concentration consumed in the absence of KCN and **5** as 100%.

reported that Mn-SOD production is induced by paraquat in *E. coli* cells.[3c] We also examined whether **5** could induce Mn-SOD but not Fe-SOD when *E. coli* was grown in the presence of **5**. Previous studies[3] have shown that Fe-SOD is constitutive, being present even in anaerobically grown cells, whereas Mn-SOD is under repression control and is produced in response to intracellular O_2^- production. As shown in Fig. 4, aerobic growth in the presence of **5** led to a marked increase in Mn-SOD.

Both hydroperoxidases of *E. coli*,[13] HP-1 and HP-2, are effective catalases.[22] As shown in Fig. 5, samples containing 0.25 U of catalase were applied to 7% polyacrylamide gels. HP-1 and HP-2 had almost the same ratio of the intensity of the corresponding achromatic bands on each of runs 1—5 in Fig. 5, different from the results obtained in the electrophoresis of SODs. The results show that the production of both HP-1 and HP-2 increased during aerobic growth in the presence of **5**. Compound **5** was thus qualitatively similar to paraquat and heteropentalenes[2a] in its effects on SOD and catalase in *E. coli*.

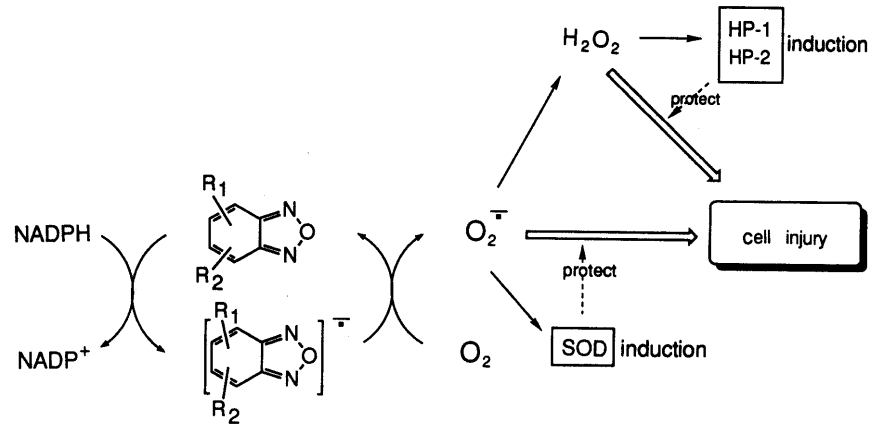


Chart 2. Proposed Mechanism of Benzofurazan Toxicity in *E. coli*

TABLE II. Half-Wave Potentials of Benzofurazan and Its Derivatives

Compound	$E_{1/2}$ (mV) *vs.* SCE (in CH_3CN)
1	−1440
2	−1570
3	−1043
4	−788
5	−455
6	−445

The $E_{1/2}$ potential *versus* a saturated calomel electrode (SCE) was determined by cyclic voltammetry in absolute CH_3CN. Tetraethylammonium perchlorate was used as the supporting electode.

Effects of Compound 5 on Cell Respiration *E. coli*, grown for 8 h in aerobic GM medium, was collected by centrifugation, suspended in 50 mM potassium phosphate at pH 7.0, and then placed under a Clark oxygen electrode. Such cells respired vigorously when glucose in GM medium was added, and the respiration was largely inhibited by 1 mM cyanide. Compound **5** relieved the inhibition even in the presence of cyanide. Figure 6 shows the rate of respiration as a function of **5** in the presence of the cyanide. The ordinate in Fig. 6 presents the O_2 concentration consumed in the absence of KCN and **5** as 100%. The cyanide-resistant respiration increased from 5% in the absence of **5** to approximately 262% in the presence of 0.5 mM **5**. The increase was dose-dependent. These results suggest that **5** can be reduced in *E. coli* and mediate electron transfer to dioxygen.

Redox Potentials of BZs We measured the redox potentials of BZs by cyclic voltammetry in CH_3CN. All of the BZs were reversibly reducible under anaerobic conditions. As shown in Table II, the reduction potentials of **5** and **6** were higher than those of the other BZs tested. The results show that **5** and **6** were more readily reduced. The potentials of BZs followed the order of $\mathbf{1} \approx \mathbf{2} < \mathbf{3} < \mathbf{4} < \mathbf{5} \approx \mathbf{6}$. It is noteworthy that higher redox potentials lead to an enhanced rate of dioxygen uptake, and thus **5** and **6** are the most efficient for reducing dioxygen.

Discussion

As a part of our work on benzofuroxans chemistry, the biological activity of benzofurazans was examined using *E. coli*. We have already reported the photoreaction of benzofuroxans to form the corresponding azepine diones and the reaction of benzofuroxans to the corresponding quinoxaline di-*N*-oxides catalyzed by molecular sieves[23] or silica gel.[24] Also, benzofurazan and its derivatives can be readily prepared by deoxygenation reaction of the corresponding benzofuroxans by triphenylphosphine.

This paper shows that the benzofurazan derivatives **5** and **6** increase the cyanide-resistant respiration of *E. coli* and cause increases in the biosynthesis of SOD and catalase. Also, **5** and **6** divert the electron flow within this organism from the normal cytochrome pathway to an $O_2^{\cdot-}$- and H_2O_2-producing pathway. *E. coli* minimizes their toxicities by inducing SOD and catalase production to scavenge $O_2^{\cdot-}$ and H_2O_2. The higher the redox potential of the BZ, the more the production of these enzymes was induced and the higher were the rates of BZ-dependent oxygen uptake by *E. coli*. We conclude that compounds **5** and **6** are reduced to the corresponding anion radicals in *E. coli* and are reoxidized by dioxygen with the production of $O_2^{\cdot-}$ and H_2O_2 and regeneration of the parent compounds (Chart 2). The rate-limiting step is thought to be the reduction of BZs, from the results of the redox potential.

Further studies on the general properties of other BZs having the potential to increase the production of $O_2^{\cdot-}$ and H_2O_2 in living cells are in progress.

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