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BUTHIOBATE: A POTENT INHIBITOR FOR YEAST CYTOCHROME P-450 CATALYZING 14α-DEMETHYLATION OF LANOSTEROL[1]

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SUMMARY: Buthiobate (S-*n*-butyl S'-*p*-*tert*-butylbenzyl N-3-pyridyldithiocarbonimidate), a fungicide, inhibited 14α-demethylation of lanosterol catalyzed by a reconstituted enzyme system consisting of cytochrome P-450 ($P\text{-}450_{14\text{-}DM}$) and NADPH-cytochrome P-450 reductase both purified from *Saccharomyces cerevisiae*. Concentration of buthiobate necessary for the 50% inhibition was 0.3 μM and this value was markedly lower than those of metyrapone and SKF-525A. Buthiobate bound stoichiometrically to $P\text{-}450_{14\text{-}DM}$ and induced Type II spectral change of the cytochrome. Buthiobate inhibited lanosterol-dependent enzymatic reduction of the cytochrome. These facts indicate that buthiobate binds to $P\text{-}450_{14\text{-}DM}$ with high affinity and acts as a potent inhibitor on the cytochrome.

In 1976, Kawase and Kato (1) reported that buthiobate (S-*n*-butyl S'-*p*-*tert*-butylbenzyl N-3-pyridyldithiocarbonimidate), a fungicide, inhibited the incorporation of radioactivity from [2-^{14}C]mevalonate into 14-demethylated sterols by *Saccharomyces cerevisiae* and caused large accumulation of the radioactivity in lanosterol. This finding strongly suggested that buthiobate inhibits lanosterol 14α-demethylation by *S. cerevisiae*. As described in the preceding papers (2-4), lanosterol 14α-demethylation in *S. cerevisiae* is catalyzed by a cytochrome P-450 (named $P\text{-}450_{14\text{-}DM}$ for convenience). In this communication, we describe effects of buthiobate on $P\text{-}450_{14\text{-}DM}$ and provide evidence indicating that this compound is a potent and specific inhibitor for this cytochrome P-450 species.

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EXPERIMENTAL PROCEDURES

$P\text{-}450_{14\text{-}DM}$ and NADPH-cytochrome P-450 reductase were purified from microsomes of semi-anaerobically grown cells of *S. cerevisiae* as described in (5) and (6), respectively.

Lanosterol 14α-demethylase activity of the reconstituted system consisting of $P\text{-}450_{14\text{-}DM}$ and the reductase was assayed as described in (3). Buthiobate was added to the reaction mixture as dimethylsulfoxide solution.

Enzymatic reduction of $P\text{-}450_{14\text{-}DM}$ was measured in a reaction mixture (2.0 ml) consisting of 0.32 nmol $P\text{-}450_{14\text{-}DM}$, 0.13 unit NADPH-cytochrome P-450 reductase, 35 nmol lanosterol dispersed in 0.125 ml water with 200 nmol dilauroyl phosphatidylcholine, 15 μmol glucose, 2 units glucose oxidase, 2600 catalase units catalase, various concentrations of buthiobate and 0.1 M potassium phosphate buffer, pH 7.5. The mixture was bubbled with CO and left to stand for 2 min at 30°C. Then, 0.3 μmol NADPH was added and the reduction of $P\text{-}450_{14\text{-}DM}$ was followed with a Hitachi 156 dual-wavelength spectrophotometer.

Binding of buthiobate to $P\text{-}450_{14\text{-}DM}$ was determined spectrophotometrically. $P\text{-}450_{14\text{-}DM}$ dissolved in 0.1 M potassium phosphate buffer, pH 7.0, containing 20% glycerol was titrated with 0.2 mM buthiobate/dimethylsulfoxide and resulting spectral change was recorded with a Shimadzu UV-300 recording spectrophotometer.

Buthiobate was kindly supplied by Dr. Toshiro Kato of Sumitomo Chemical Co., Takarazuka, Japan.

RESULTS AND DISCUSSION

14α-Demethylation of lanosterol catalyzed by the reconstituted system (3) was inhibited by buthiobate (Fig. 1). Buthiobate did not inhibit NADPH-cytochrome *c* reductase activity catalyzed by the cytochrome P-450 reductase used in the reconstituted system (data not shown). Accordingly, it could be assumed that buthiobate interacted with $P\text{-}450_{14\text{-}DM}$ and inhibited the lanosterol demethylation. The concentration of buthiobate required for the 50% inhibition

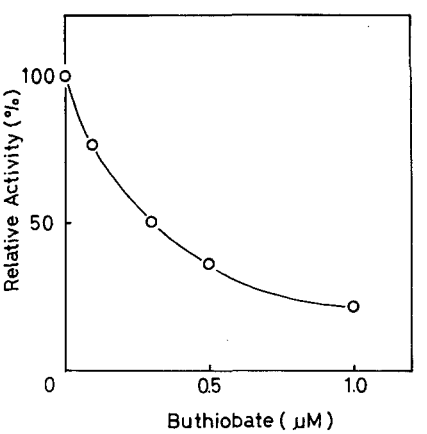

Fig. 1. Inhibition of lanosterol 14α-demethylase activity of the reconstituted system by buthiobate. Lanosterol 14α-demethylase activity of the reconstituted system consisting of 0.28 nmol $P\text{-}450_{14\text{-}DM}$ and 0.13 unit reductase was assayed in the presence of indicated concentrations of buthiobate. Buthiobate was dissolved in dimethylsulfoxide and dimethylsulfoxide concentration in the reaction mixture was fixed at 0.25% in all experiments.

was assumed to be 0.3 μM (Fig. 1). This concentration was extremely low to compare with those of metyrapone (0.1 mM) and SKF-525A (0.2 mM), well known cytochrome P-450 inhibitors, necessary for 50% inhibition of the demethylase activity.

Buthiobate caused a spectral change of P-$450_{14\text{-DM}}$ (Fig. 2A) providing a direct evidence for the binding of this compound to the cytochrome. The absorp-

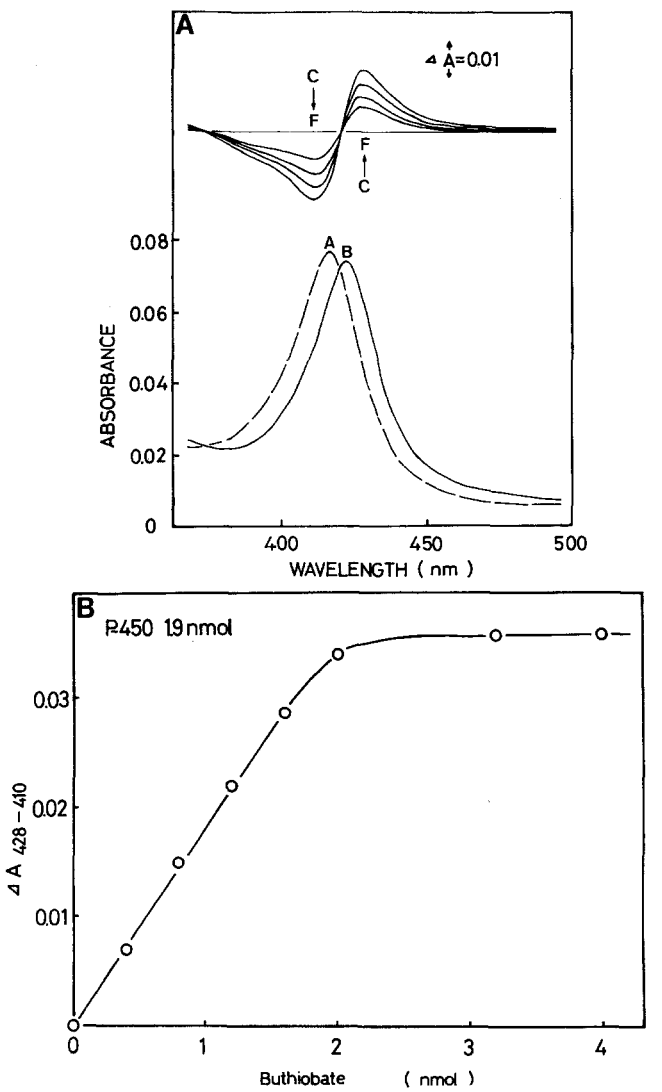

Fig. 2. Effect of buthiobate on the absorption spectrum of ferric P-$450_{14\text{-DM}}$. A: P-$450_{14\text{-DM}}$ (0.67 μM in 0.1 M potassium phosphate buffer, pH 7.0, containing 20% glycerol) was titrated with various concentrations of buthiobate. Spectrum A; before addition of buthiobate. Spectrum B; after the addition of 1.43 μM buthiobate. Spectra C-F; difference spectra observed in the presence of 0.29 (C), 0.43 (D), 0.57 (E) and 1.07 μM (F) buthiobate. B: P-450 (1.9 nmol) was titrated with indicated amount of buthiobate as A.

tion spectrum of the buthiobate-P-$450_{14\text{-DM}}$ complex (curve B of Fig. 2A) was essentially superimposable on those of pyridin complexes of the cytochrome and P-450_1 of rabbit liver microsomes (7,8). So, it can be assumed that the spectral change was due to the binding of the pyridyl moiety of buthiobate to the heme iron of the cytochrome. The spectral change was saturated when one mole of buthiobate was bound to one mole of the cytochrome (Fig. 2B) indicating that buthiobate formed one to one complex with the cytochrome. Addition of $Na_2S_2O_4$ to the buthiobate-P-$450_{14\text{-DM}}$ complex changed its absorption spectrum as shown by curves A through E in Fig. 3 indicating the formation of the buthiobate-ferrous P-$450_{14\text{-DM}}$ complex. A brief bubbling of CO converted the buthiobate complex to the reduced-CO compound showing Soret peak at 447 nm (curve F of Fig. 3). This fact indicated that buthiobate did not denature P-$450_{14\text{-DM}}$ to a P-420 form. It was also suggested that buthiobate on ferrous P-$450_{14\text{-DM}}$ was readily replaced by CO. As described in the preceding paper (3), lanosterol 14α-demethylase activity of P-$450_{14\text{-DM}}$ was inhibited by 80% under the gas mixture of

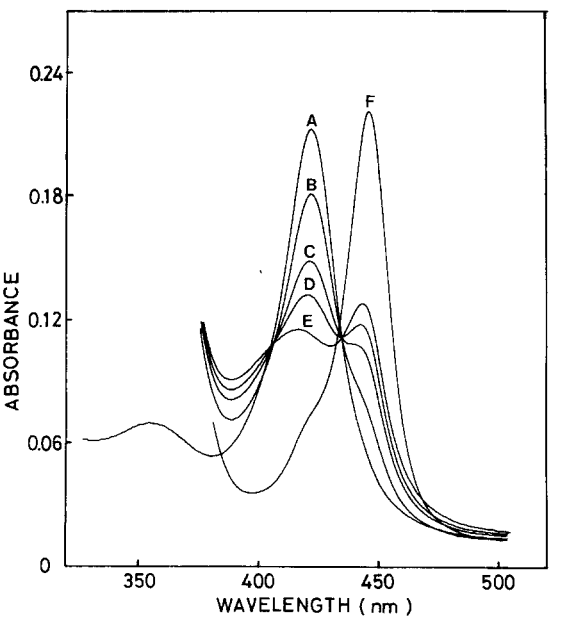

Fig. 3. Reduction of the buthiobate-P-$450_{14\text{-DM}}$ complex with $Na_2S_2O_4$. P-$450_{14\text{-DM}}$ (2.15 μM in 0.1 M potassium phosphate buffer, pH 7.0, containing 20% glycerol) was saturated with 8 μM buthiobate and reduced with $Na_2S_2O_4$. Spectrum A; before addition of $Na_2S_2O_4$. Spectra B-E; 2 (B), 4 (C), 6 (D) and 20 min (E) after the addition of $Na_2S_2O_4$. Spectrum F; after brief bubbling with CO through the sample of spectrum E.

$CO:O_2$ = 95:5. So, the above finding suggested that the inhibition by buthiobate of the demethylase activity was not due to the competition between the inhibitor and O_2.

Buthiobate inhibited the lanosterol-dependent reduction of P-$450_{14\text{-DM}}$ in the reconstituted system (Fig. 4). On the other hand, buthiobate showed no effect on the chemical reduction of the cytochrome with $Na_2S_2O_4$. In addition, the inhibition by buthiobate of the cytochrome reduction was incomplete and the rate of reduction observed in the presence of excess buthiobate (curve B of Fig. 4) was comparable to that observed in the absence of lanosterol. Based on these facts, inhibition of the enzymatic reduction of P-$450_{14\text{-DM}}$ by buthiobate was likely to be due to the disappearance of the enhancement by lanosterol of the enzymatic reduction rate of the cytochrome caused by the binding of the inhibitor to the cytochrome. Buthiobate concentration necessary for 50% inhibition of

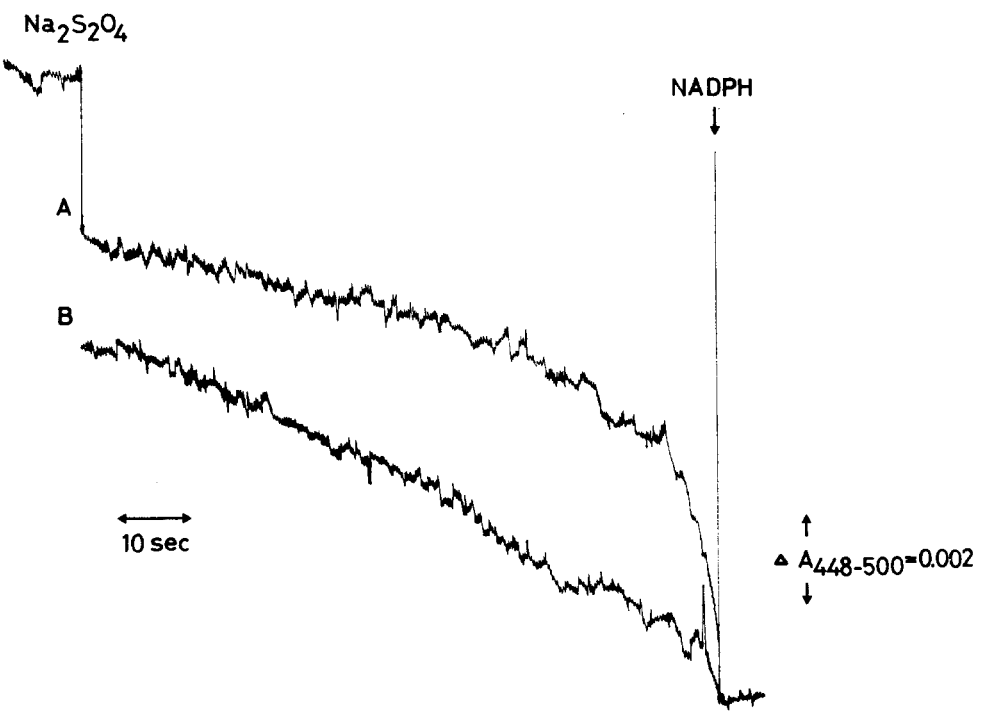

Fig. 4. Inhibition of the lanosterol-dependent enzymatic reduction of P-$450_{14\text{-DM}}$ by buthiobate. P-$450_{14\text{-DM}}$ reduction was assayed as described in EXPERIMENTAL PROCEDURES in the presence (B) or absence (A) of buthiobate. Concentrations of P-$450_{14\text{-DM}}$ and buthiobate were 0.16 μM and 1.0 μM, respectively. Buthiobate was dissolved in dimethylsulfoxide and dimethylsulfoxide concentration in the reaction mixture was fixed at 0.25% in all experiments.

$P\text{-}450_{14\text{-}DM}$ reduction was assumed to be 0.15 μM. This concentration was close to that caused 50% inhibition of the lanosterol demethylation (Fig. 2). This fact indicated close relationship between inhibitory effect of buthiobate on the lanosterol demethylation and the lanosterol-dependent enzymatic reduction of $P\text{-}450_{14\text{-}DM}$.

Taken all observations together, it can be concluded that buthiobate binds to $P\text{-}450_{14\text{-}DM}$ with high affinity and inhibits the lanosterol-dependent reduction and the catalytic activity of the cytochrome. In addition, it was reported by Hata *et al.* (9) that Δ^{22}-desaturation of ergosta-5,7-dien-3β-ol to ergosterol in yeast microsomes, which has been considered to be a cytochrome P-450-dependent reaction (9,10), was not inhibited by buthiobate. This finding suggested the possibility that buthiobate specifically inhibits $P\text{-}450_{14\text{-}DM}$.

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