Cystothiazoles A and B, New Bithiazole-type Antibiotics from the Myxobacterium *Cystobacter fuscus*

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New bithiazole-type antibiotics, cystothiazoles A (C$_{20}$H$_{26}$N$_2$O$_4$S$_2$) and B (C$_{20}$H$_{26}$N$_2$O$_5$S$_2$), have been isolated from a culture broth of the myxobacterium, *Cystobacter fuscus*. The gross structures of these compounds were elucidated by spectroscopic analysis, and their absolute stereochemistry was determined by chemical degradation of cystothiazole A. Cystothiazole A inhibits fungi and human tumor cells, whereas it is inactive against bacteria. The antifungal activity appears to result from the inhibition of submitochondrial NADH oxidation. Although these compounds are structurally related to the known antibiotic myxothiazol, cystothiazole A was more active against fungi and less cytotoxic than myxothiazol.

In recent years a number of novel secondary metabolites have been discovered from myxobacteria, which have now been recognized as a rich source of new antibiotics. One of the most significant examples are the epothilons1), which are attracting attention of many researchers2,3). In the course of our search for bioactive compounds from myxobacteria by using an inhibition assay against the phytopathogenic fungus, *Phytophthora capsici*4), we found two new antifungal substances from a myxobacterium strain AJ-13278, which was identified as *Cystobacter fuscus*. These antibiotics termed cystothiazoles A and B (Fig. 1) are structurally related to myxothiazol, an antibiotic from the myxobacterium, *Myxococcus fulvus*5), and show a broad antifungal spectrum. This paper describes the production, isolation, physico-chemical properties, structural elucidation, absolute stereochemistry, and biological activity of cystothiazoles.

### Microorganisms

Bacterial strain AJ-13278 was isolated from a soil sample collected at Kamakura, Kanagawa, Japan. According to the *Bergey's Manual of Determinative Bacteriology*, the strain was identified as *Cystobacter fuscus*, a member of fruiting gliding bacteria, myxobacteria. This strain was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Tsukuba, Japan, as FERM P-15997.

### Fermentation

A slant culture of *C. fuscus* grown on vy/2 medium (baker's yeast 0.5%, CaCl$_2$·H$_2$O 0.1%, cyanocobalamin 0.5 µg/ml, Bacto agar 1.5%, pH 7.2) was used throughout this work. A loopful of the slant culture was inoculated into a 300-ml Erlenmeyer flask containing 70 ml of a seed medium [Casitone (Difco) 1%, baker's yeast 0.5%, Malt extract (Difco) 0.2%, Yeast extract (Difco) 0.2%, Yeast extract (Difco) 0.1%, MgSO$_4$·H$_2$O 0.1%, Mg$_3$(PO$_4$)$_2$·8H$_2$O 0.3%, HEPES 1%, Bacto agar 0.05%]. The flask was shaken on a rotary

![Figure 1. Structures of cystothiazoles A and B.](image-url)
shaker (180 rpm) for 3 days at 28°C. Five milliliters of the seed culture thus obtained were inoculated into a 500-ml Erlenmeyer flask containing 100ml of a production medium. The production medium was the same as the seed medium except that 2% (w/v) adsorber resin SP207 (Mitsubishi Chemical Co.) was added to promote the productivity of the active substances. The fermentation was done on a rotary shaker (180 rpm) at 28°C for 4 days.

Isolation

The bacterial cells and the adsorber resin were collected from 2.5-liter culture broth and extracted with a mixture of acetone and methanol. The extract was partitioned between 60% aqueous methanol and dichloromethane, and the dichloromethane fraction was chromatographed on silica gel. The ethyl acetate fraction, which was active against P. capsici at 1 µg/disc, was subjected to silica gel medium-pressure liquid chromatography to give several active fractions. The most active fraction was purified by recrystallization to yield 31.6mg of cystothiazole A as colorless needles. A more-polar active fraction was separated by silica gel column chromatography followed by TLC to give an active fraction, which was further purified by normal-phase HPLC to give 1.7mg of cystothiazole B as a colorless powder.

Physico-chemical Properties

Table 1 summarizes the physico-chemical properties of cystothiazoles A and B. Both compounds were soluble in methanol, ethanol, aceton, ethyl acetate, chloroform, and benzene, slightly in hexane, and scarcely in water. Positive FAB-MS showed the pseudomolecular ion (M + H)+ at 423 for cystothiazole A and 439 for cystothiazole B, and high-resolution FAB-MS revealed the molecular formulae C20H26N2O4S2 and C20H26N2O5S2, respectively. The molecular formula of cystothiazole A was also supported by elemental analysis. The UV spectra of cystothiazoles A (Fig. 2) and B are quite similar, suggesting that these compounds are congeners possess-
ing a common chromophore. The absorption bands at 1705, 1625, and 1150 cm\(^{-1}\) in the IR spectrum of cystothiazole A (Fig. 3) are indicative of the presence of an \(\alpha,\beta\)-unsaturated ester.

Structural Elucidation and Absolute Stereochemistry

The \(^1\)H and \(^{13}\)C NMR data for cystothiazole A were summarized in Table 2. The assignment of the direct connectivity between protons and carbons was estab-
lished by HETCOR experiments. The presence of two partial structures, an isopropyl group and -CH(CH₃)-CH(OR)CH=CH-, were easily determined from spin-spin coupling patterns in the ¹H NMR of cystothiazole A. The signals in the aromatic region of the ¹H and ¹³C NMR spectra were presumed to correspond to a bithiazole substructure as in myxothiazol⁵) by comparison of NMR data of both compounds. The bithiazole substructure was confirmed by ¹H-¹³C long-range couplings determined by the heteronuclear multiple-bond correlation (HMBC) spectrum. The HMBC data are shown in Table 2. The above partial structures and the rest of the molecule [three methoxyl groups, an olefinic methine (C-2), and quaternary carbons (C-1 and C-3)] were also connected by the HMBC experiment to give the gross structure of cystothiazole A. The E geometry of the trisubstituted double bond at C-2 was evidenced by difference NOE data (H-2/3-OMe).

The gross structure of the minor product cystothiazole B was elucidated by comparison with the spectral data for cystothiazole A. The ¹H NMR data (Table 2) were similar to those for cystothiazole A except for the data for the isopropyl group (C-14 to C-16): the signals due to the methine proton H-14 disappeared and the methyl protons H-15 and H-16 were observed at a lower field as a singlet. These findings and the fact that the molecular formula of cystothiazole B is more than that of cystothiazole A by one oxygen suggest that cystothiazole B is 14-hydroxycystothiazole A.

The stereochemistry of cystothiazole A was determined by degradation experiments. Ozonolysis of cystothiazole A followed by an oxidative treatment with hydrogen peroxide and methylation with diazomethane gave dimethyl 2-methoxy-3-methylsuccinate. The ¹H NMR data for this diester was superimposable on the known threo isomer and its absolute configuration was determined to be 2R,3R by the specific rotation values ([α]D +32°; lit.⁶ [α]D +35°), establishing the 4R,5S configuration of cystothiazole A. Both cystothiazoles A and B show similar ¹H NMR data (H-4 and H-5) and specific rotations, indicating that the absolute stereochemistry of cystothiazole B is identical with that of cystothiazole A.

### Biological Properties

The minimum inhibitory amounts of cystothiazoles A and B against Phytophthora capsici were determined to be 0.05 and 1 μg/disc, respectively. For cystothiazole A, which was obtained in sufficient amount for the evaluation, the antimicrobial properties are investigated. The antimicrobial spectrum of cystothiazole A was summarized in Table 3 in comparison with myxothiazol⁷). Cystothiazole A was active against all fungi tested, but had no effect on bacteria. MICs were measured by serial dilution media [Potato dextrose broth (Difco)] ranging from 0.1 to 6.3 μg/ml. Although such a tendency of activity was same as that of myxothiazol, the potency of cystothiazole A was mostly higher.

Cystothiazole A was also tested for the in vitro cytotoxicity by using human colon carcinoma HCT-116 and human leukaemia K562 cells (Table 4). The IC₅₀ values of cystothiazole A were 110–130 ng/ml, which were significantly higher than those of myxothiazol.

Since the mode of action of myxothiazol is known to be the inhibition of NADH oxidation of submito-
Table 4. Cytotoxic activity of cystothiazole A and myxothiazol.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC_{50} (ng/ml)</th>
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<tr>
<td></td>
<td>Cystothiazole A</td>
</tr>
<tr>
<td>HCT-116</td>
<td>130</td>
</tr>
<tr>
<td>K562</td>
<td>110</td>
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chondrial membrane fraction^{8,9}, the effect of cystothiazole A was tested. As shown in Fig. 4 both antibiotics showed comparable activity. Thus, the doses required for 50% inhibition of cystothiazole A and myxothiazol were 1.8 and 2.1 μM, respectively.

**Discussion**

The cystothiazoles are bithiazole-type antibiotics, which are structurally related to myxothiazol. While cystothiazoles were isolated from *C. fuscus*, myxothiazol was from a different species of myxobacteria. Furthermore other similar antibiotics termed melithiazoles A and B were also isolated from another myxobacterium^{10}. Although these suggest the presence of a similar biosynthetic pathway in different species, myxothiazol and melithiazoles themselves were not detected in the extract of *C. fuscus*. In our assay system with the filamentous fungus *P. capsici*, cystothiazole A showed a potent inhibition, and the activity was observed up to an amount of 0.05 μg/disc. In a microscope observation, the filamentous branches decreased in both number and length. It is noteworthy that cystothiazole B is about 20-fold less active than cystothiazole A in spite of their high structural similarity. The extra hydroxyl group on the isopropyl group would serve as a detoxication factor by increasing hydrophilicity, or the isopropyl moiety may be responsible for the biological activity. The antimicrobial spectrum (Table 3) shows that the biological activity of cystothiazoles and myxothiazol is essentially same and confined within eukaryotes. The mechanism of action on fungi is supposed to be inhibition of respiration, since cystothiazole A interferes with NADH oxidation in a concentration similar to that of myxothiazol (Fig. 4). Although cystothiazole A showed potent *in vitro* cytotoxicity at IC_{50} of 110~130 μg/ml against tumor cells tested, the IC_{50} values were significantly (10-fold) higher than that of myxothiazol. Furthermore, antimicrobial activity of cystothiazole A is a little higher than that of myxothiazol. It seems thus likely that cystothiazole A could be more promising than myxothiazol in pharmaceutical applications.

**Experimental**

**General**

Organic extracts were dried over anhydrous Na_{2}SO_{4}. Evaporation of solvents was carried out with a rotary evaporator under reduced pressure (ca. 3 kPa). Fuji Silysia silica gel BW-300 was employed for column chromatography. Precoated silica gel 60 F_{254} and RP-18 WF_{254} plates (E. Merck) were used for thin-layer chromatography (TLC). HPLC was performed on a JASCO high-pressure gradient system with PU-980 pumps. Melting points were uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. IR spectra were recorded on a JASCO FT/IR-7000S. UV spectra were recorded on a JASCO Ubest-50 UV/VIS spectrophotometer. NMR spectra were recorded on a Bruker-ARX400 (400 MHz) or a JEOL EX-270 (270 MHz). NMR chemical shifts were referenced to the solvent peak of δ_{H} 7.26 (residual CHCl_{3}) or δ_{C} 77.0 ppm for CDCl_{3}. Mass spectra were recorded on a JEOL Mstation JMS-700 mass spectrometer in the FAB mode using m-nitrobenzyl alcohol as a matrix.

**Assay Method**

Antifungal activity of fermentation broths and chromatographic fractions was evaluated by a paper disc assay using *P. capsici*. In a typical assay, an agar piece
A stream of 4% ozone in oxygen was passed through a solution of cystothiazole A (22.1 mg, 0.052 mmol) in dichloromethane (2 ml) at $-78^\circ$C for 5 minutes. The solution was flushed with nitrogen and concentrated. The residue was dissolved in 90% formic acid (0.3 ml) and 30% hydrogen peroxide (0.3 ml) was added. After gentle heating the mixture was heated under reflux for 70 minutes. The mixture was concentrated, and the residue was dissolved in methanol (0.5 ml) and treated with ethereal diazomethane. The mixture was concentrated and the residue was chromatographed on silica gel (4 g) with hexane-ethyl acetate (9:1) and then 3:1. The fraction showing an Rf value of 0.41 on a silica gel TLC [hexane-ethyl acetate (3:1)] was further purified by normal-phase HPLC [Nomura Chemical Developis 60-5 (8 x 250 mm), hexane-ethyl acetate (3:1), flow rate 2 ml/minute, UV 210 nm] to give dimethyl (2R, 3R)-2-methoxy-3-methylsuccinate (Rt=22.5 minutes) as an oil: [z]$_D^22 +32^\circ$ (c 0.03, ether), $^1$H NMR (400 MHz, CDCl$_3$) $\delta$ 4.18 (1H, d, J=4.8 Hz), 3.78 (3H, s), 3.72 (3H, s), 3.44 (3H, s), 2.95 (1H, dq, J=7.2, 4.8 Hz), 1.20 (3H, d, J=7.2 Hz).

Acknowledgments

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References

5) GERTH, K.; H. IRSCHIK, H. REICHENBACH & W. TROWITZSCH: Myxothiazol, an antibiotic from My xo-


7) Myxothiazol was obtained by chromatographic separations from a myxobacterium isolated in our laboratory (Mycococcus fulvus M-219).

