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Antifungal thiopeptide cyclothiazomycin B1 exhibits growth inhibition accompanying morphological changes via binding to fungal cell wall chitin

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ABSTRACT

Cyclothiazomycin B1 (CTB1) is an antifungal cyclic thiopeptide isolated from the culture broth of *Streptomyces* sp. HA 125-40. CTB1 inhibited the growth of several filamentous fungi including plant pathogens along with swelling of hyphae and spores. The antifungal activity of CTB1 was weakened by hyperosmotic conditions, and hyphae treated with CTB1 burst under hypoosmotic conditions, indicating increased cell wall fragility. CTB1-sensitive fungal species contain high levels of cell wall chitin and/or chitosan. Unlike nikkomycin Z, a competitive inhibitor of chitin synthase (CHS), CTB1 did not inhibit CHS activity. Although CTB1 inhibited CHS biosynthesis, the same result was also obtained with a non-specific proteins inhibitor, cycloheximide, which did not reduce cell wall rigidity. These results indicate that the primary target of CTB1 is not CHS, and we concluded that CTB1 antifungal activity was independent of this sole inhibition. We found that CTB1 bound to chitin but did not bind to β-glucan and chitosan. The results of the present study suggest that CTB1 induces cell wall fragility by binding to chitin, which forms the fungal cell wall. The antifungal activity of CTB1 could be explained by this chitin-binding ability.

1. Introduction

Several fungal genera are causative agents of infectious diseases in plants and humans. Plants show defense responses against fungal infection. However, heat and drought stress in addition to insect attack enhance the infectivity of pathogenic fungi including *Fusarium* genus, *Aspergillus*, and *Mucor* spp. against plants. Fungi-caused plant diseases result in vast economic losses in agriculture. Additionally, deep-seated mycoses are especially serious for immunocompromised patients including those administered immunosuppressive agents after organ transplantation or with AIDS.[1] Most mycoses are triggered by opportunistic infection with fungi such as *Aspergillus*, *Candida*, and *Fusarium* spp.[2] In addition, an outbreak of *Fusarium* keratitis was recently reported in contact lens users.[3] Corneal infection with *Fusarium* spp. results in vision loss.

Fungi, like plants and animals, are eukaryotes; therefore, preferentially attacking fungi without adverse effects against their host is difficult. As fungal infectious diseases in plants and humans increase, the development of antifungal antibiotics with new modes of action is greatly needed. In the present study, we have elucidated the mode of action of an antifungal thiopeptide, cyclothiazomycin B1 (CTB1) (Fig. 1). In our laboratory, CTB1 was isolated from the culture broth of a *Streptomyces* sp. strain as inducing growth inhibition against several filamentous fungi along with morphological changes in hyphae. CTB1 has been reported to inhibit DNA-dependent RNA synthesis by bacteriophage RNA polymerases and growth of phytopathogenic *Cochliobolus miyabeanus*.[4] However, its mode of action against fungi including phytopathogens has not been reported.

We found that CTB1 inhibited the growth of several filamentous fungal strains including *Mucor* and *Fusarium* spp. at low concentrations (0.020–0.33 μM) and showed no cytotoxicity against the mammalian cultured cell lines tested, suggesting that CTB1 may

Abbreviations: 5FU, 5-fluorouracil; ATCC, American Type Culture Collection; CHS, chitin synthase; CHX, cycloheximide; CTB1, cyclothiazomycin B1; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; FGSC, Fungal Genetics Stock Center; IFO, Institute for Fermentation Osaka; ME, malt extract; MFC, minimum fungicidal concentration; MIC, minimum inhibitory concentration; NBRC, NITE Biological Resource Center; NCTC, National Collections of Type Cultures; NZ, nikkomycin Z; TCA, Trichloroacetate.

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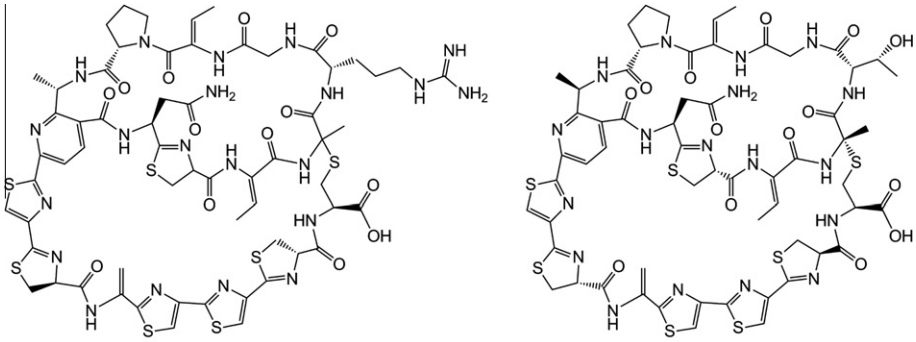

Figure 1. Structures of cyclothiazomycins.

be a potential candidate antifungal agent with fewer adverse effects. Therefore, we investigated its detailed mode of action using *Mucor mucedo* IFO 7684 as a test fungus and found that CTB1 appears to act by increasing the fragility of the cell wall via chitin binding.

2. Materials and methods

2.1. Isolation of CTB1

A strain of *Streptomyces* sp. HA 125-40 producing CTB1 was isolated from a soil sample collected on Osaka City University campus. CTB1 was purified from the culture broth, and its chemical structure was confirmed partially based on a previously described method.[4]

2.2. Microbial strains and culture

Microbial strains were obtained from the Institute for Fermentation Osaka (IFO; Osaka, Japan), the National Collections of Type Cultures (NCTC; London, UK), the American Type Culture Collection (ATCC; Manassas, VA), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM; Braunschweig, Germany), the NITE Biological Resource Center (NBRC; Chiba, Japan), and the Fungal Genetics Stock Center (FGSC; Kansas City, KS).

Precultivation was performed as follows. Bacterial strains were cultured in 3% nutrient broth (NB, Nissui) at 37 °C for 16 h. Yeast strains were cultured in 1% yeast extract, 2% peptone, and 2% D-glucose (YPD) medium at 30 °C for 16 h. Filamentous fungal strains were cultured in 2.5% malt extract (ME, Oriental Yeast) medium at 30 °C for 16 h.

2.3. Antimicrobial susceptibility test

Minimum growth inhibitory concentrations (MICs) for bacterial and fungal strains were determined by a slightly modified broth microdilution method outlined by the National Committee for Clinical Laboratory Standards.[5] Briefly, 50 μL of cell suspension (2×10^6 cells or spores/mL) was loaded into each well of 96-well flat-bottom plates. After 50 μL of drug-containing medium was loaded into each well, plates were incubated for 24 h at 37 °C or 30 °C for bacterial and yeast/fungal strains, respectively. MIC was defined as the lowest drug concentration to produce a prominent decrease in turbidity compared with control growth. Minimum fungicidal concentrations (MFCs) for fungal strains were determined as follows. After determining the MIC, 100 μL of each culture was added into 3 mL of a drug-free ME broth. After 24-h incubation, MFC was determined as the lowest concentration of test compounds in which no recovery of microorganism was observed. All assays were performed at least three times on separate occasions.

2.4. Cytotoxicity

Human promyelocytic leukemia cell line HL-60, human monocytic leukemia cell line THP-1, pig renal proximal tubule epithelial cell line LLC-PK1, and mouse embryonic fibroblast cell line NIH3T3 were obtained from the Health Science Research Resources Bank (Osaka, Japan). These cell lines were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Roche, Kaiseraugst, Switzerland), 100 μg/mL penicillin, and 100 μg/mL streptomycin. The cells were grown in a humidified incubator at 37 °C under 5% CO_2 and used for assays during the exponential phase of growth.

CTB1 cytotoxicity against the mammalian cell lines described above was examined using the Alamar blue assay in 96-well.[6] Cell suspensions (50 μL) were plated into 96-well plates at 2×10^6 cells/mL and grown for 2 h. Various concentrations of CTB1 (0.13–65.5 μM) were then added. Wells containing 100 μL of growth medium without cells were prepared to determine background. After 24-h incubation, 10 μL of Alamar blue dye reagent (TREK Diagnostic Systems, Cleveland, OH) was added to each well, and the plates were incubated for an additional 2 h. After incubation with Alamar blue dye, fluorescence was detected with a GENios fluorescence spectrophotometer (TECAN, Männedorf, Switzerland) at excitation wavelength 565 nm and emission wavelength 595 nm. Cell viability was calculated by the following equation: $100 \times [(\text{drug sample}) - (\text{background})]/[(\text{control}) - (\text{background})]$. IC_{50} was defined as the drug concentration resulting in 50% viability.

2.5. Hyperosmotic or hypoosmotic treatment

M. mucedo IFO 7684 and *F. oxysporum* NBRC 5942 were incubated without shaking at a density of 10^6 spores/mL in ME broth with or without 0.026 μM CTB1 at 30 °C for 24 h unless otherwise stated. Hyphae for the following experiments were harvested by centrifugation at 1000*g* for 5 min unless otherwise stated.

A paper disc method was used for the hyperosmotic shock assay.[7] Paper discs containing 4.9 nmol CTB1 or a chitin synthase (CHS) inhibitor, nikkomycin Z (NZ), were placed on ME agar plate with or without 1.2 M sorbitol seeded with 10^5 spores/mL. The plates were incubated at 30 °C for 2 days. After incubation, the diameter of inhibitory zones was measured. Hypoosmotic shock assay was performed using hyphae treated with distilled water. Hyphae incubated with 0.026 μM CTB1 or 0.081 μM NZ were

harvested and suspended in distilled water for 10 min. After treatment, the hyphae were observed under a microscope.

2.6. Cell wall staining with calcofluor white

Cell wall polysaccharides including chitin and chitosan were stained with calcofluor white dye.[8] Hyphae of *M. mucedo* IFO 7684 incubated with 0.026 μM CTB1 or 0.081 μM NZ for 24 h were used for the staining. Hyphae were mixed with 0.1% calcofluor white in 10% glycerol on a slide glass and then covered with a coverslip. The stained hyphae were observed under a fluorescence microscope (excitation wavelength, 330 nm; emission wavelength, 385 nm).

2.7. Effect of CTB1 on chitin synthase activity in permeabilized germinated spores

CHS activity was assayed based on uptake of radioactive *N*-acetylglucosamine (GlcNAc) from UDP-GlcNAc into the permeabilized germinated spores.[9] *M. mucedo* IFO 7684 spores (2×10^6 spores/mL) incubated with shaking for 6.5 h were used for the preparation of permeabilized cells. The following operations were performed at 0 °C: the spores were washed twice with TGEM buffer (50 mM Tris–HCl, 1 mM EGTA, 1 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, pH 7.5), treated with TGEM buffer containing 33% glycerol (v/v) for 10 min, and washed twice with TGEM buffer. Finally, the spores were suspended in TGEM buffer with 2 vol. equiv of the initial spore suspensions and used as the enzyme solution for CHS assay.

The assay mixtures for CHS, containing 29 μL reaction buffer (100 mM MES, 20 mM $MgCl_2$, pH 6.5), 131.0 μM CTB1 or 403.7 μM NZ (both resulting in 4 μg/mL drug final concentration), and 20 μL enzyme solution, were incubated at room temperature for 10 min. Then, 50 μL of substrate solution (0.525 μCi/mL [U-^{14}C]-UDP-GlcNAc (PerkinElmer Life & Analytical Sciences, Wellesley, MA), 0.92 mM UDP-GlcNAc, 0.80 mM GlcNAc) was added.[10] The reaction mixtures were incubated at 30 °C for 5 or 60 min, and the reaction was stopped by suspending 40 μL reaction solution in 100 μL 10% trichloroacetate (TCA) on ice. The acid-insoluble fractions were harvested through glass filters (diameter 24 mm; Whatman, Middlesex, UK). The filters were washed with 10% TCA and then ethanol, dried, and immersed in 5 mL scintillation cocktail (toluene containing 0.5% DPO and 0.03% POPOP; Dojindo, Kumamoto, Japan). Radioactivity was quantified with a liquid scintillation counter (LS 6500, Beckman, Fullerton, CA).

2.8. Effect of CTB1 on nucleic acid and protein biosynthesis

An assay for biosynthetic activity of total nucleic acids (DNA and RNA) was performed using the following method.[11] *M. mucedo* IFO 7684 at 2×10^6 spores/mL was incubated with shaking in 3 mL ME medium. After 5.5 h, the hyphae were treated with 0.13 μM CTB1 or 0.77 mM 5-fluorouracil (5FU), which is an inhibitor of nucleic acid synthesis, for 15 min. After the addition of 0.1 μCi/mL [8-^{14}C]-adenine (Moravek Biochemicals, Brea, CA), the culture was further incubated for 5 or 30 min. The reaction was stopped by mixing 500 μL of hyphal suspension into an equal volume of 10% TCA on ice. The acid-insoluble fractions were harvested through a glass filter, and the filter was washed first with 10% TCA then ethanol, dried, and immersed in 5 mL scintillation cocktail. Radioactivity was quantified with a liquid scintillation counter.

For quantitation of DNA synthesis, the same protocol was used with minor modifications. After the 5- or 30-min incubation, 500 μL of hyphal suspension was mixed with an equal volume of 1 M NaOH and incubated at 60 °C for 30 min, then 1 mL 20% TCA was added on ice. Acid-insoluble fractions were harvested through glass filters for radioactivity quantitation. The quantity of RNA synthesis was estimated from the difference between DNA synthesis and total nucleic acid synthesis.

Total protein biosynthesis was assayed by the same method as nucleic acid biosynthesis with slight modifications. *M. mucedo* IFO 7684 at 2×10^6 spores/mL was incubated with shaking in 3 mL ME medium. After 5.5 h, the hyphae were treated with 0.13 μM CTB1 or 0.36 mM cycloheximide (CHX), which is an inhibitor of protein synthesis, for 15 min. After the addition of 0.1 μCi/mL [U-^{14}C]-leucine (American Radiolabeled Chemicals, St. Louis, MO), the culture was further incubated for 5 or 30 min. The reaction was stopped by mixing 500 μL of hyphal suspension with an equal volume of 10% TCA on ice. Acid-insoluble fractions were harvested for radioactivity quantitation. For leucine uptake into hyphae, the same method was used with slight modifications. The hyphae withdrawn from culture were harvested through glass filters without TCA treatment, washed with ME medium, dried, and then immersed in 5 mL scintillation cocktail. Radioactivity was then measured.

2.9. Assay for chitin synthase in CTB1-treated germinated spores

This assay was performed in the same manner as described above with slight modifications. Germinated spores treated with 2.61 μM CTB1 or 0.36 mM CHX for 1 h were used for preparation of an enzyme solution for CHS assay. The reaction mixtures for CHS, containing 30 μL of reaction buffer and 20 μL of enzyme solution, were added to 50 μL of substrate solution. After incubation at 30 °C for 5 or 60 min, the reaction was stopped by mixing 40 μL of the mixture with 100 μL of 10% TCA on ice. Acid-insoluble fractions were harvested through glass filters for radioactivity quantitation.

2.10. Effect of cycloheximide on cell wall rigidity

Cell wall rigidity was confirmed by the incubation of hyphae with distilled water as described above. Hyphae of *M. mucedo* IFO 7684 incubated with 0.36 mM CHX were suspended in distilled water for 10 min and then observed under a microscope.

2.11. Affinity of CTB1 for cell wall polysaccharides

To estimate the binding affinity of CTB1 for cell wall polysaccharides, chitin (Wako, Osaka, JPN), chitosan (Wako), and curdlan (Wako), which are main water-insoluble components of the fungal cell wall, were used. Each polymer ground at 10 mg/mL, sonicated, and centrifuged at 15,000*g* for 5 min. The supernatant was lyophilized to estimate the polymer dry weight. The supernatant (1 mL) contained 0.3, 0.5, and 0.5 mg of chitin, chitosan, and curdlan, respectively. Each lyophilized polymer was suspended in distilled water at a concentration of 0.5 mg/mL. The polymer suspension was mixed with 6.55 μM CTB1. The suspensions were incubated with gentle shaking at 30 °C for 30 min and then filtered with Ultrafree-MC 0.45-μm filters (Millipore, Bedford, MA) to remove the polysaccharides. Residual CTB1 contained in the filtrates was analyzed using HPLC with a L-column ODS (4.6 × 250 mm, Chemical Evaluation and Research Institute, Japan) at a flow rate of 1.0 mL/min and 75% methanol (v/v) as a mobile phase. Detection was performed at 270 nm.

2.12. Affinity of CTB1 for chemically deacetylated chitin

Chitin was chemically deacetylated using a previously described method with slight modifications.[12] A suspension of ground chitin (10.5 mg) in 47% aq NaOH (1.0 mL) was heated at

110 °C for 1 h under argon atmosphere. The resulting mixture was treated with 50% aq ethanol (10 mL). The precipitate was collected by filtration and washed with water until the washings were neutral, followed by further washings with ethanol. The crude product on the filter paper was dried in vacuo to afford partially deacetylated chitin (0.7 mg), which was ninhydrin-positive.[13]

The obtained deacetylated chitin (0.5 mg/mL) was suspended with distilled water containing 6.55 μM CTB1. The suspension was incubated with gentle shaking at 30 °C for 30 min and then filtered with Ultrafree-MC 0.45-μm filters to remove deacetylated chitin. CTB1 contained in the filtrates was quantified using HPLC as described in Section 2.11.

2.13. Affinity of CTB1 for cell wall obtained from filamentous fungi

Cell wall was obtained from *M. mucedo* IFO 7684 and *F. oxysporum* NBRC 5942, which demonstrated sensitivity to CTB1. Hyphae were incubated for 24 h, harvested, and washed twice with distilled water. One half-volume of glass beads was added to hyphal suspensions, and the mixture was vortexed for 5 min. The dissociated cell suspension was then centrifuged at 4500*g* for 5 min to obtain fractions containing cell wall.[14] These fractions were washed twice with distilled water and used as isolated cell wall. Mixtures of 6.55 μM CTB1 with cell wall fractions were incubated for 3 h at 30 °C and centrifuged at 8000*g* for 5 min. CTB1 contained in the supernatant was analyzed using HPLC as described in Section 2.11.

2.14. Effect of protein kinase A (PKA)-disturbing agents on CTB1 activity

A PKA inhibitor, H8, and a PKA activator, dibutyl cyclic AMP (dbcAMP), were used in this assay.[15] They were dissolved in water. Both agents showed no growth inhibitory activity against *M. mucedo* at 100 μM. The MIC of CTB1 against *M. mucedo* in medium containing 100 μM H8 or dbcAMP was determined by the method described in Section 2.3. The hyphae were also observed under a microscope.

2.15. Reconstruction of cell wall in spheroplasts

The spheroplasts of *M. mucedo* hyphae were prepared by lysing cell wall with lytic enzymes.[16] *M. mucedo* IFO 7684 incubated for 18 h was harvested and washed twice with phosphate buffer (pH 6.8) containing 0.6 M sorbitol (P buffer). Hyphae were incubated in P buffer containing 1% yatalase (Ozeki, Hyougo, Japan) and 20 U chitosanase (Wako) for 3 h at 30 °C with gentle shaking.[17] After confirmation of cell wall lysis, spheroplasts were washed twice with P buffer and then grown in ME medium containing 0.6 M sorbitol with 0.13 μM CTB1 or 0.40 μM NZ. Hyphae were observed under a microscope at 0, 6, 12, 18, and 24 h from initiation of incubation.

2.16. Chemicals

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Drugs including CTB1 were dissolved in *N*,*N*-dimethylformamide (DMF) unless otherwise stated.

3. Results

3.1. Isolation and chemical structure of CTB1

The chemical structure of the isolated antifungal antibiotic from a strain of *Streptomyces* sp. was re-elucidated as follows. FABMS measurements revealed that its molecular weight was 1527.3. The antibiotic was found to be ninhydrin negative, but acid-hydrolysate positive. Furthermore, an amino acid analysis revealed that the antibiotic contained aspartic acid, glycine, cysteine, arginine, and proline, indicating that the antibiotic is a cyclic peptide. According to degradation experiments and NMR analysis, the antibiotic was elucidated to be the same substance as CTB1 (Fig. 1), which was reported as an RNA polymerase inhibitor.[4] The purity of CTB1 (370 mg) isolated from 700 L culture was 98.5%.

3.2. Antimicrobial activity and cytotoxicity of CTB1

The MIC of CTB1 was examined by a broth microdilution method with slight modifications against 8 bacteria (*Staphylococcus aureus* NCTC 8530, *S. aureus* IFO 12732, *Micrococcus luteus* IFO 3333, *Proteus vulgaris* IFO 3851, *Pseudomonas aeruginosa* IFO 3080, *Bacillus subtilis* IFO 3007, *Bacillus licheniformis* IFO 12107, *Escherichia coli* IFO 3545), 5 yeast (*Saccharomyces cerevisiae* IFO 0203, *S. cerevisiae* ATCC 7754, *Schizosaccharomyces pombe* IFO 0342, *Candida albicans* IFO 1061, *Rhodotorula mucilaginosa* IFO 0001), and 17 filamentous fungi strains (Table 1). CTB1 did not show any antimicrobial activity against the bacterial and yeast strains tested, but exhibited antifungal activity against 12 strains of filamentous fungi including phytopathogens. CTB1-sensitive species included *Mucor*, *Penicillium*, *Fusarium*, and *Gibberella* spp. MFCs against fungal strains were also determined. Fungicidal activities of CTB1 were not observed at concentrations less than 6.55 μM. Thus, the action of CTB1 was regarded to be at least fungistatic around MIC. The hyphae of *M. mucedo* IFO 7684 and *F. oxysporum* NBRC 5942 treated with or without CTB1 at MICs were observed under a microscope. The hyphae of CTB1-treated *M. mucedo* IFO 7684 were thicker than untreated hyphae and demonstrated morphologically abnormal branching and swelling at the tips (Fig. 2A and B). CTB1 also induced morphological abnormality in *F. oxysporum* hyphae treated with CTB1 (Fig. 2C and D). Furthermore, *F. oxysporum* spores treated with CTB1 were very swollen (Fig. 2D). The cytotoxicity of CTB1 against 4 mammalian cell lines was assessed by the Alamar blue assay. CTB1 did not inhibit their proliferation or differentiation up to 65.5 μM (data not shown).

3.3. Effect of hyperosmotic or hypoosmotic treatment on CTB1-treated hyphae

Cell wall-disturbing antibiotics such as NZ and micafungin are known to induce morphological abnormalities in filamentous fungi.[18] Thus, we examined the effect of CTB1 on cell wall rigidity

Table 1
Antifungal activity of CTB1

Test organism	MIC[a]	
	μg/mL	μM
Mucor mucedo IFO 7684	0.039	0.026
M. javanicus IFO 4569	0.078	0.051
Fusarium oxysporum NBRC 5942	0.50	0.33
F. oxysporum IFO 7152	0.25	0.16
F. oxysporum NBRC 30700	0.25	0.16
F. solani NBRC 5899	0.25	0.16
F. solani NBRC 8505	0.25	0.16
F. sporotrichioides NBRC 33236	0.031	0.020
F. avenaceum NBRC 33237	0.25	0.16
Gibberella zeae NBRC 8850	0.063	0.041
G. fujikuroi NBRC 6349	0.25	0.16
Penicillium chrysogenum IFO 4626	0.63	0.41
Aspergillus niger ATCC 6275	>1000	
A. fumigatus IFO 5840	>1000	
A. nidulans FGSC A4	>1000	
Neurospora sitophila DSM 1130	>1000	
Rhizopus oryzae IFO 4766	>1000	

[a] MIC: Minimum growth inhibitory concentration.

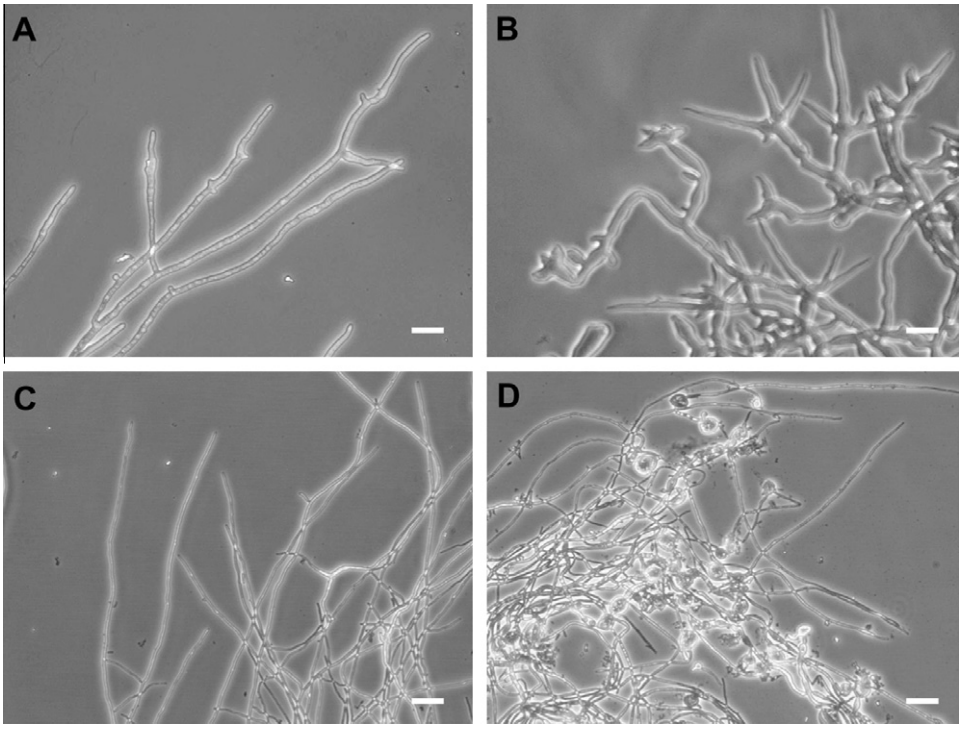

Figure 2. CTB1-induced morphological changes in *M. mucedo* and *F. oxysporum*. *M. mucedo* IFO 7684 (A and B) and *F. oxysporum* NBRC 5942 (C and D) were grown at 30 °C for 24 h in ME medium containing 0 (A and C) or 0.026 μM CTB1 (B and D). Bar = 20 μm.

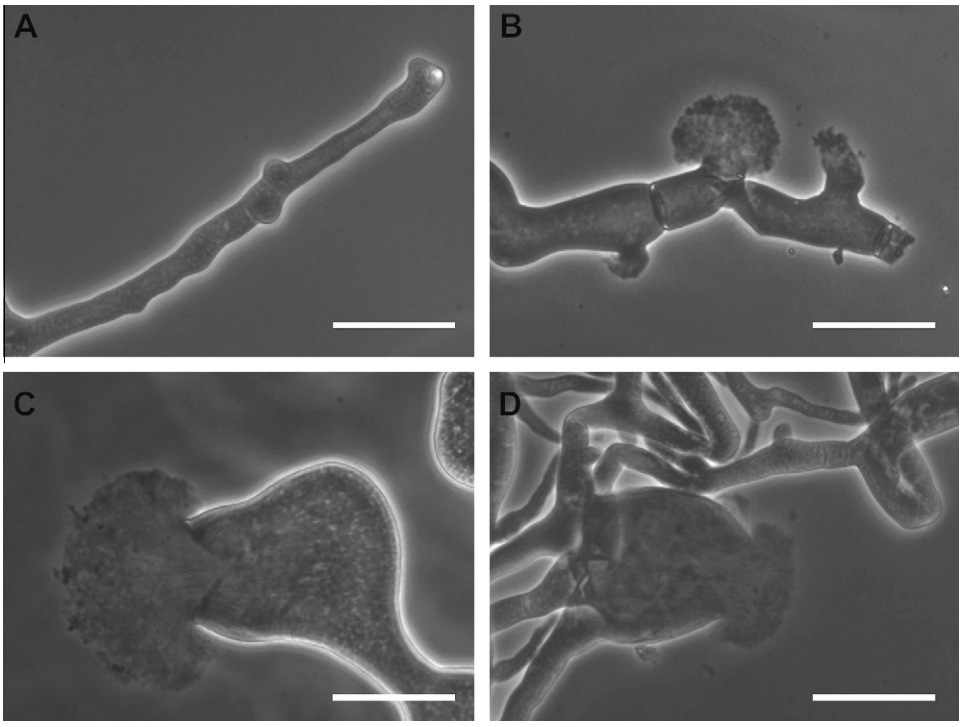

Figure 3. CTB1-treated hyphae under hypoosmotic conditions. The hyphae of *M. mucedo* IFO 7684 were incubated at 30 °C for 24 h in ME medium containing no drugs (A), 0.026 μM CTB1 (B and C), or 0.081 μM NZ (D). The hyphae were treated with distilled water for 10 min prior to microscopic observation. Bar = 20 μm.

using an osmotic shock assay. Hyperosmotic assays were performed for *M. mucedo* IFO 7684 and *F. oxysporum* NBRC 5942 using a paper disc method in ME medium with or without 1.2 M sorbitol. NZ, an inhibitor of CHS, was used as a positive control. The potency of CTB1 and NZ antifungal activities was indicated as the diameter of growth inhibitory zones. Hyperosmotic treatment reduced the antifungal activity of NZ against both *M. mucedo* IFO 7684 and *F. oxysporum* NBRC 5942, from 34 to 23 mm and from 22 to 0 mm, respectively. The inhibitory zones produced by CTB1 disappeared under hyperosmotic conditions.

Hypoosmotic assay was performed using *M. mucedo* IFO 7684 by treatment with distilled water. Control hyphae were not

affected by hypoosmotic treatment (Fig. 3A); whereas the hyphae treated with CTB1 and NZ burst and leaked cytosolic constituents (Fig. 3B–D). Bursts were frequently observed at the tips of hyphae (Fig. 3B) and areas of swelling (Fig. 3C and D). These results revealed that CTB1, like NZ, may increase the fragility of *M. mucedo* IFO 7684 and *F. oxysporum* NBRC 5942 cell wall.

3.4. Staining of chitin and chitosan with calcofluor white

Species of CTB1-sensitive filamentous fungi tend to contain relatively high chitin and/or chitosan content in their cell wall.[19] Chitin and chitosan contained in the cell wall of *M. mucedo* IFO 7684 were stained with calcofluor white, and stained hyphae were observed under a fluorescence microscope (Fig. 4). The surface of control hyphae was stained uniformly around the cell wall and septum, emitting intense fluorescence. In contrast, swelling hyphae treated with CTB1 or NZ demonstrated non-specific fluorescence compared to control hyphae.

3.5. Effect of CTB1 on chitin synthase

The effect of CTB1 on CHS activity was determined by measurement of radioactive-GlcNAc uptake from [U-^{14}C]-UDP-GlcNAc into acid-insoluble fractions in the permeabilized germinated spores of *M. mucedo* IFO 7684 using NZ as a positive control. The results shown in Figure 5A indicated the uptake of GlcNAc into acid-insoluble fractions for 55 min. NZ decreased CHS activity to 69% compared to control. In contrast, CTB1 did not inhibit CHS activity.

3.6. Effect of CTB1 on nucleic acid and protein biosynthesis

The biosynthesis of nucleic acids and proteins was determined by measurement of [8-^{14}C]-adenine or [U-^{14}C]-leucine uptake into acid-insoluble fractions of *M. mucedo* IFO 7684 hyphae. As positive controls, 5FU and CHX were used as biosynthetic inhibitors of nucleic acids and proteins, respectively. RNA and DNA synthesis shown in Figure 5D were indicated as the uptake of [8-^{14}C]-adenine into acid-insoluble fractions for 25 min. RNA and DNA synthesis were both decreased by 5FU to 75% and 61% compared to control. CTB1 slightly decreased only DNA synthesis, to 84%, but did not affect RNA synthesis. Total protein synthesis, shown as black bars in Figure 5C, was indicated as the uptake of [U-^{14}C]-leucine into acid-insoluble fractions for 25 min. CHX greatly decreased total protein synthesis to 0.7% compared to control. CTB1 demonstrated weaker inhibition, decreasing protein synthesis to 37%. This inhibition could not be explained solely by the inhibition of leucine uptake into whole hyphae. The leucine uptake into hyphae was greater than that into acid-insoluble fractions for both CTB1 and CHX (Fig. 5C, gray bars), indicating that CTB1 appears to inhibit protein synthesis.

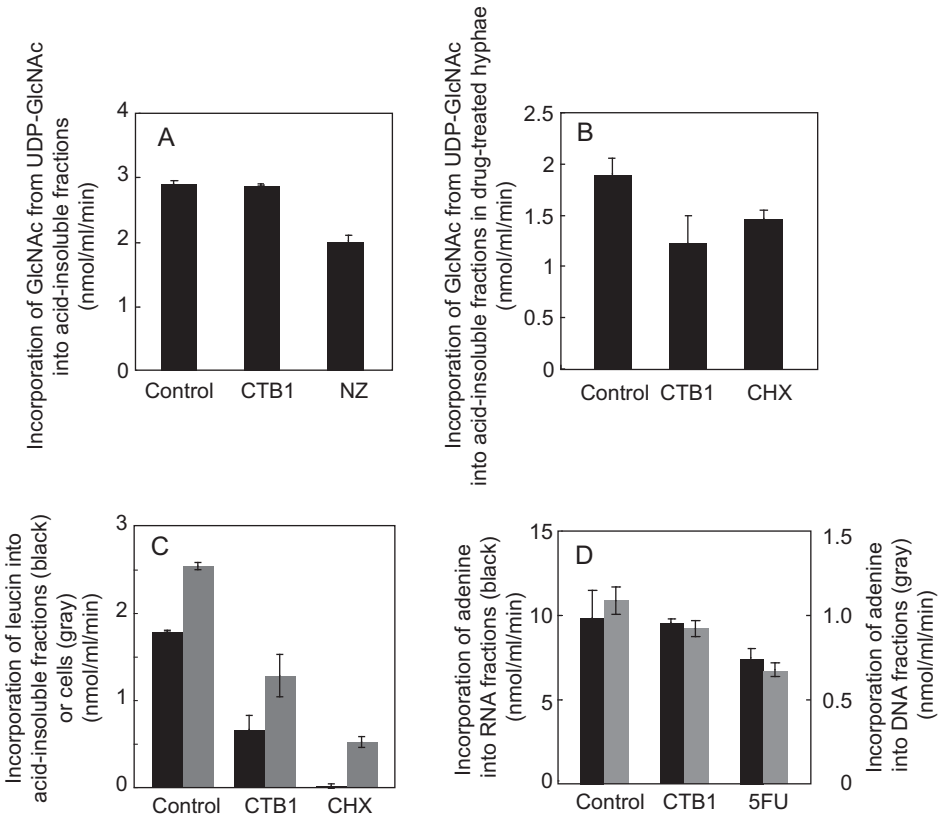

Figure 4. Effect of CTB1 on CHS activity (A), de novo synthesis of chitin synthase protein (B), and biosynthesis of total proteins (C) and nucleic acids (D). (A) CHS activity was indicated by the incorporation of GlcNAc from [^{14}C]-UDP-GlcNAc into acid-insoluble fractions of *M. mucedo* IFO 7684 permeabilized germinated spores at 30 °C for 55 min. The concentrations of CTB1 and NZ were 2.61 mM and 8.07 mM (both 4 mg/mL). (B) The de novo synthesis of CHS protein was indicated by CHS activity in permeabilized germinated spores treated with CTB1 or CHX. *M. mucedo* IFO 7684 germinated spores were treated with 2.61 μM CTB1 or 0.36 mM CHX for 1 h. After drug treatment, the germinated spores were permeabilized by treatment with 33% glycerol (v/v). CHS activity was assayed as described above. (C) The biosynthesis of total proteins was indicated by the incorporation of [^{14}C]-leucine into acid-insoluble fractions (black) at 30 °C for 55 min. The incorporation of leucine into hyphae was also measured (gray). The concentrations of CTB1 and CHX were 0.13 μM and 0.36 mM, respectively. (D) The biosynthesis of DNA and RNA was indicated by the incorporation of [^{14}C]-adenine into acid-insoluble fractions showing NaOH-unstable (black, RNA biosynthesis) or -stable (gray, DNA biosynthesis) material at 30 °C for 25 min. The concentrations of CTB1 and 5FU were 0.13 μM and 0.77 mM, respectively. All values are means ± standard deviations (n = 3).

3.7. Chitin synthase activity in CTB1-treated germinated spores

This assay was performed using an enzyme solution prepared from germinated spores treated with CTB1 or CHX. CHS activity was determined by measurement of radioactive-GlcNAc uptake from [U-^{14}C]-UDP-GlcNAc into the permeabilized germinated spores. The activity in permeabilized germinated spores treated with CTB1 decreased to 64% compared to control (Fig. 5B). The activity of CHX-treated spores also decreased, similar to CTB1, but CTB1 demonstrated slightly stronger inhibition than CHX. These results demonstrate that both CTB1 and CHX reduce the CHS activity of germinated spores by restricting protein biosynthesis. Furthermore, hypoosmotic conditions did not affect the morphology of CHX-treated hyphae (data not shown).

3.8. CTB1 affinity to cell wall related-polysaccharides or cell wall obtained from filamentous fungi

The affinity of CTB1 to polysaccharides was estimated by quantitation of CTB1 in filtrates of their mixture. The polysaccharides tested included chitin, chitosan, and a type of β-glucan, curdlan, which are main components of fungal cell wall, and chemically deacetylated chitin. This assay revealed that chitosan and β-glucan demonstrated no affinity for CTB1, but chitin had massive affinity for CTB1 (Fig. 6). The affinity of CTB1 to deacetylated chitin was decreased, similar to chitosan (Fig. 6). Both cell walls obtained from *M. mucedo* IFO 7684 and *F. oxysporum* NBRC 5942 had no affinity to CTB1 (data not shown).

3.9. Effect of PKA-disturbing agents on CTB1 activity

H8 and dbcAMP were used as inhibitor and activator of PKA, respectively.[15] They did not demonstrate antifungal activity against *M. mucedo* IFO 7684 at 100 μM. In the presence of 100 μM H8 or dbcAMP, the MIC of CTB1 against *M. mucedo* IFO 7684 decreased to 0.013 μM. Thus, PKA-disturbing agents were found to enhance the antifungal activity of CTB1. Alone, they did not induce morphological changes in hyphae (Fig. 7A, C, and E), but they intensified morphological abnormalities in the presence of CTB1 (Fig. 7B, D, and F).

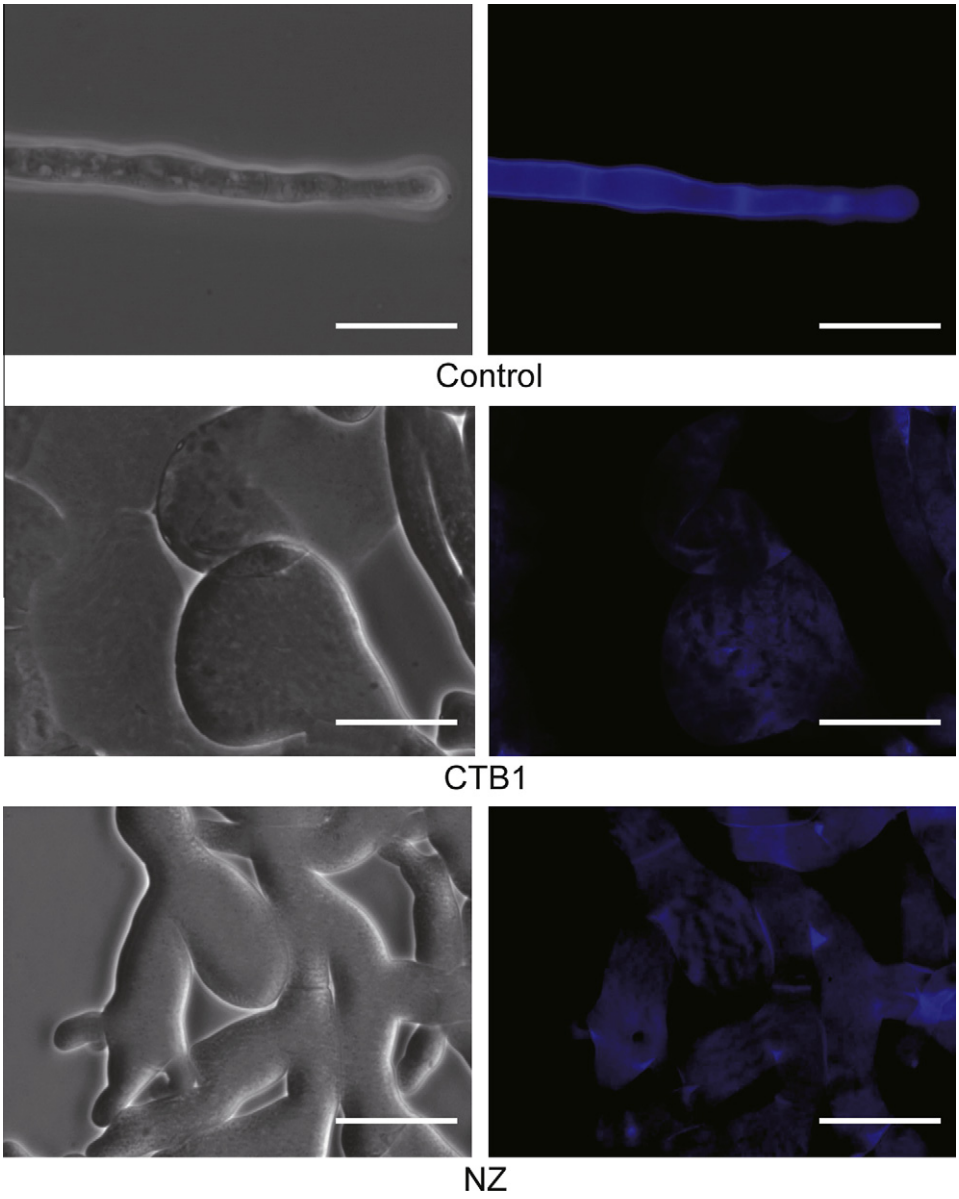

Figure 5. Cell wall staining with calcofluor white. The hyphae of *M. mucedo* IFO 7684 were incubated at 30 °C for 24 h in ME medium containing of 0.026 μM CTB1 or 0.081 μM NZ. The hyphae were stained with calcofluor white and observed under a phase-contrast (left) or fluorescence (right) microscope. Bar = 20 μm.

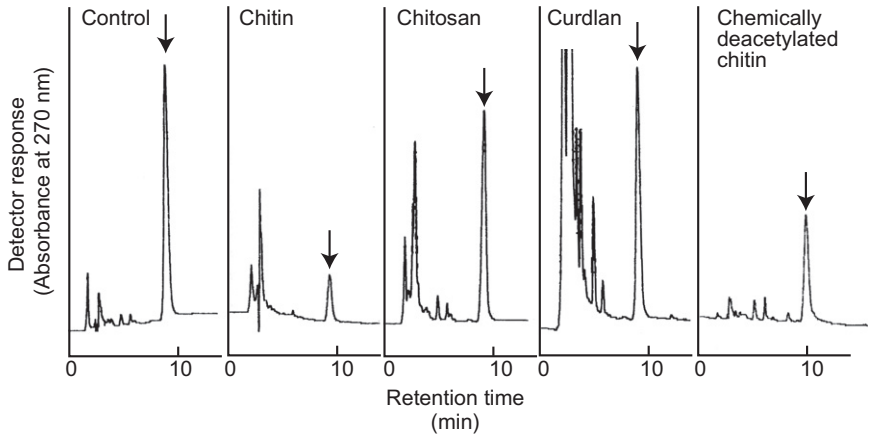

Figure 6. Affinity of CTB1 to cell wall-related polysaccharides. Mixtures of 6.55 μM CTB1 and 0.5 mg/mL of each polysaccharide were incubated at 30 °C for 30 min with shaking. After the removal of polysaccharides from the mixtures by filtration, the residual amount of CTB1 in filtrate was quantified using HPLC with a L-column ODS (4.6 × 250 mm, Chemical Evaluation and Research Institute, Japan) and 75% methanol (v/v) as a mobile phase. Arrows indicate the peak of CTB1. The relative peak area of residual CTB1 was 100, 16, 83, 98, and 54 for control, chitin, chitosan, curdlan, and chemically deacetylated chitin, respectively.

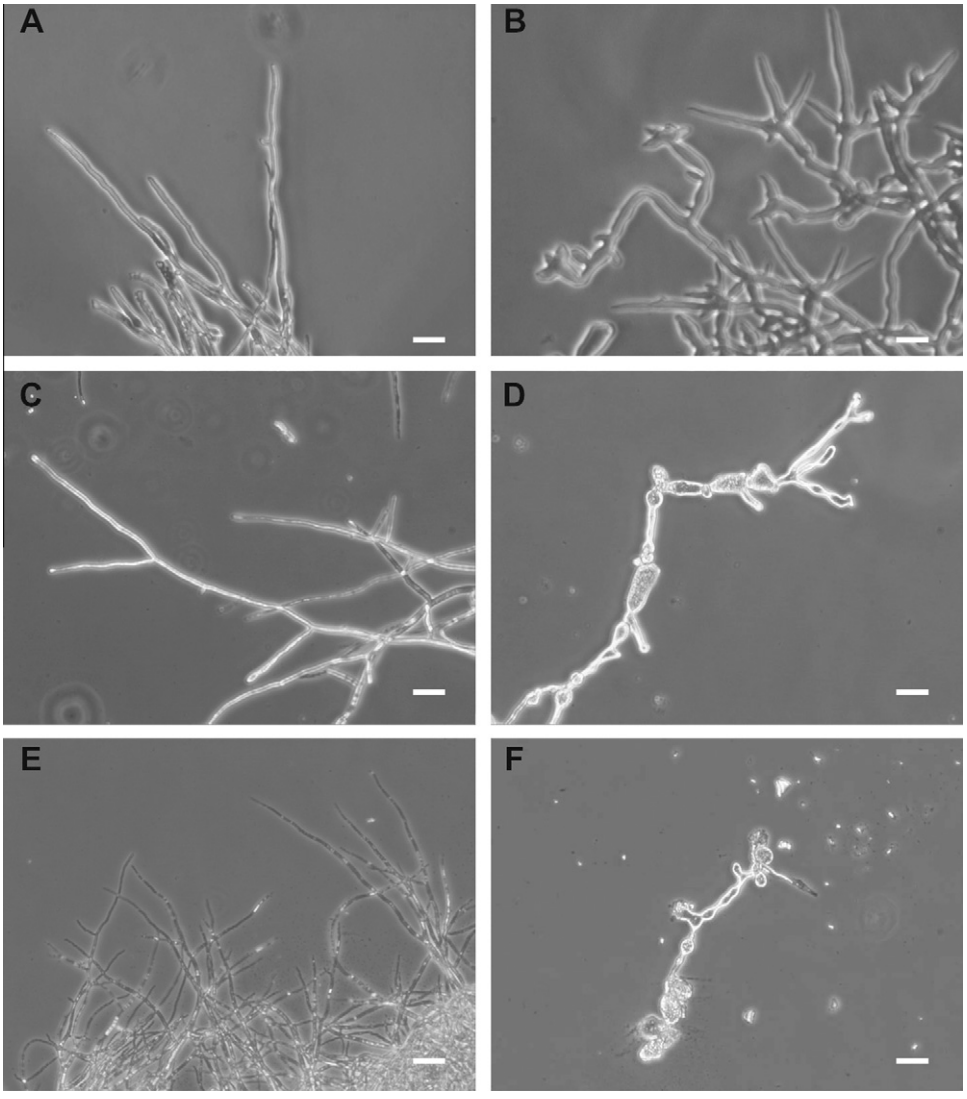

Figure 7. Effect of PKA-disturbing agents on morphological changes induced by CTB1. *M. mucedo* IFO 7684 was incubated in ME medium with no drugs (A), CTB1 (B), dbcAMP (C), dbcAMP and CTB1 (D), H8 (E), or H8 and CTB1 (F) at 30 °C for 24 h. The concentrations of CTB1 and PKA-disturbing agents were 0.026 μM and 100 μM, respectively. Bar = 20 μm.

3.10. Effect of CTB1 on cell wall regeneration in spheroplasts

Spheroplasts of *M. mucedo* IFO 7684 were prepared by treatment with chitosanase and yatalase for 3 h. The spheroplasts were then incubated with CTB1 or NZ and observed under a microscope at 0, 6, 12, 18, and 24 h (Fig. 8). The surface of spheroplasts, from which cell wall was removed, became transparent, so the cytoplasm could be clearly observed upon initial incubation. For a control, reconstruction of cell wall was observed from 6-h incubation, and the spheroplasts recovered to normal hyphae at 24 h. In CTB1-treated spheroplasts, cell wall regeneration was observed up to 12 h, but hyphae burst in spite of hyperosmotic conditions from 18 h. Although hyphae also burst in NZ-treated spheroplasts, they did not demonstrate cell wall reproduction.

4. Discussion

Cyclothiazomycin was first isolated as a rennin inhibitor, which is expected to be the most effective agent in hypertension therapy.[20] An analogue of cyclothiazomycin, CTB1, was isolated by screening for hyphal swelling formation in the plant pathogen *Cochliobolus miyabeanus*.[4] In addition, CTB1 demonstrated antifungal activity against other plant pathogens *Botrytis cinerea* Persoon and *Septoria nodorum*.[4] CTB1 inhibits DNA-dependent RNA synthesis using bacteriophage RNA polymerase.[4] However, its antifungal mechanism has remained unknown.

The results of antimicrobial susceptibility testing clearly indicated that CTB1 did not inhibit the growth of any bacteria or yeasts tested but did inhibit that of 12 filamentous fungi strains including phytopathogenic strains at low concentrations (0.020–0.33 μM), as described in Table 1. The CTB1-sensitive strains include *Mucor*, *Penicillium*, *Fusarium*, and *Gibberella* spp. The antifungal action of CTB1 was not fungicidal but fungistatic around the MIC. CTB1 at concentrations less than 65.5 μM did not inhibit the proliferation or differentiation of any mammalian cell lines tested. These results indicate that CTB1 selectively acts on filamentous fungi. Thus, CTB1 may be expected to contribute substantially to the development of new antifungal antibiotics with fewer adverse effects.

Swelling of hyphae induced by CTB1 in *M. mucedo* and *F. oxysporum* (Fig. 2) indicates its serious effect on cell wall rigidity. Cell wall-disturbing antibiotics such as NZ, micafungin, and anethole

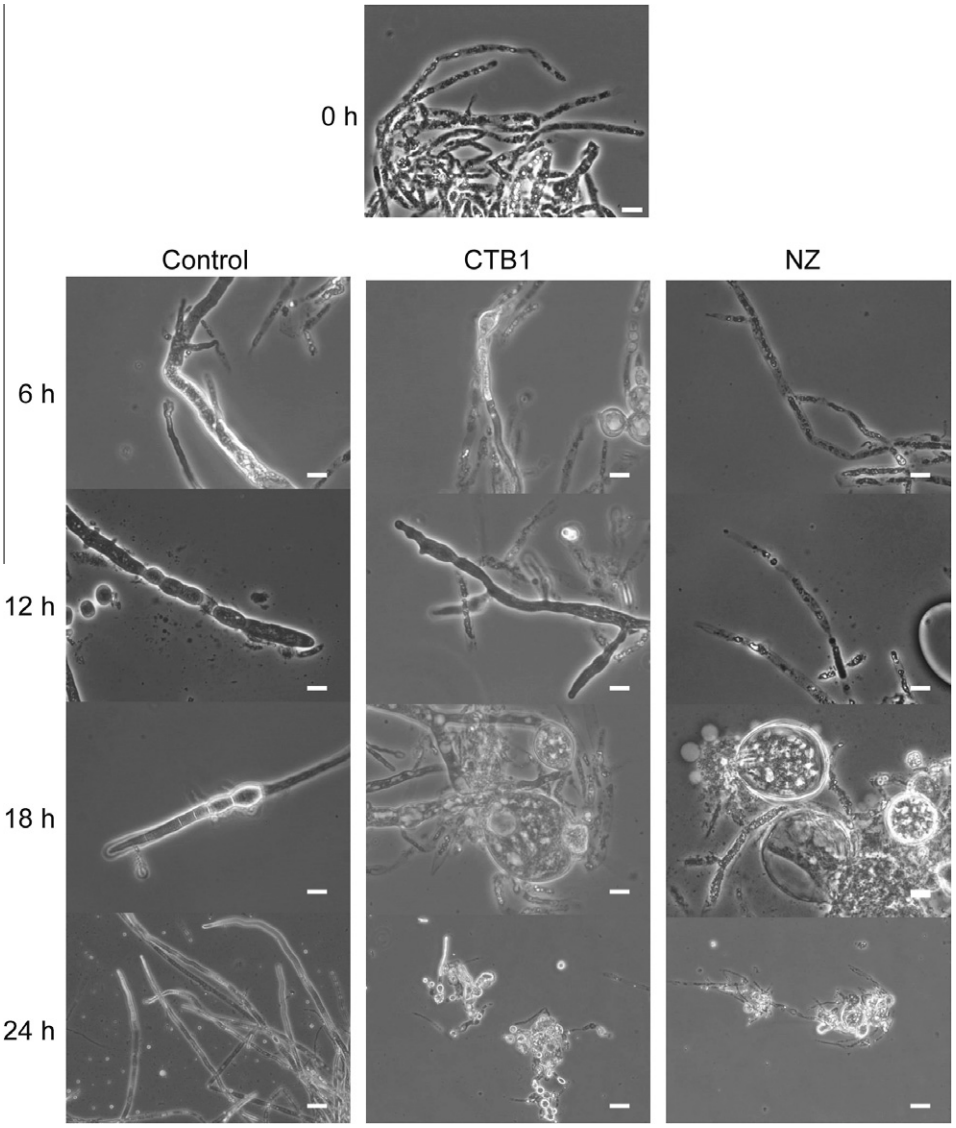

Figure 8. Cell wall regeneration in spheroplasts. *M. mucedo* IFO 7684 spheroplasts prepared with chitosanase and yatalase were incubated in ME medium containing 0.026 μM CTB1 or 0.081 μM NZ (both 40 ng/mL) at 30 °C. The hyphae were observed at 0, 6, 12 18, and 24 h. Bar = 20 μm.

are known to induce morphological abnormalities and inhibit the growth of filamentous fungi.[18] The cell wall contributes to protecting fungal cells from changes in osmotic pressure,[21] and hyperosmotic conditions are well known to weaken the antifungal activity of cell wall-disturbing agents.[22] Hyperosmotic treatment also greatly reduced CTB1 activity against both *M. mucedo* and *F. oxysporum*. In contrast, CTB1-treated *M. mucedo* hyphae burst at tips or branching sites under hypoosmotic treatment, similarly to NZ (Fig. 3).

These results suggest that CTB1 inhibits the growth of filamentous fungi by reducing cell wall rigidity. Thus, we focused on the metabolism of cell wall components, chitin or chitosan, as possible targets for CTB1 because CTB1-sensitive filamentous fungi tend to contain high chitin or chitosan levels in their cell walls.[19] The swelling hyphae of CTB1-treated *M. mucedo* demonstrated non-specific calcofluor white fluorescence compared to control hyphae. The distribution of the fluorescence was similar to that of NZ (Fig. 4). These results also indicate that CTB1, like NZ, induces cell wall abnormalities. Competitive inhibitors of CHS such as nikkomycins and polyoxin, disturb the biosynthesis of chitin in cell walls.[19c,23] While NZ inhibited CHS activity CTB1 did not, (Fig. 5A), indicating that CTB1 does not directly affect CHS activity.

CTB1 inhibits bacteriophage RNA polymerase, thereby restricting RNA synthesis.[4] Therefore, the effect of CTB1 on nucleic acid biosynthesis in *M. mucedo* IFO 7684 was determined. Both RNA and DNA biosynthesis were inhibited by 5FU. CTB1 slightly inhibited DNA biosynthesis but did not inhibit RNA biosynthesis. However, similar to CHX, CTB1 inhibited total protein biosynthesis in *M. mucedo* IFO 7684 (Fig. 5C, black bars). Total inhibition was partly reflected by the sole inhibition of leucine uptake into whole hyphae. As the leucine uptake into hyphae was larger than that into the acid-insoluble fractions for both CTB1 and CHX (Fig. 5C, gray bars), the inhibition of leucine uptake was estimated to be weak. The inhibition of total protein biosynthesis may also affect CHS biosynthesis in hyphae.

Although CTB1 inhibited CHS biosynthesis, its inhibition was weak. Thus, we concluded that the antifungal activity of CTB1 did not depend on the restriction of chitin biosynthesis occurred as a result from the inhibition of protein synthesis, because the same result was also obtained in the case of CHX, which does not reduce cell wall rigidity (data not shown). These results indicate that CHS inhibition is not the most likely site of CTB1 action. The effect of the slight inhibition of DNA biosynthesis on CTB1 activity is still not understood.

Chitin-binding substances such as calcofluor white and wheat germ agglutinin are also known as antifungal agents,[24] and the antifungal activity of calcofluor white against *Geotrichum lactis* decreases under hyperosmotic conditions such as 0.8 M sorbitol.[17b,24] β-Glucan and chitosan did not have affinity for CTB1, but chitin demonstrated a massive affinity (Fig. 6). Furthermore, chemically deacetylated chitin showed lower affinity for CTB1 (Fig. 6). In addition, CTB1 did not demonstrate affinity for cell wall obtained from *M. mucedo* or *F. oxysporum* (data not shown). This result may have reflected high chitosan and low chitin content in the cell wall. Chitosan is commonly found in the cell wall of *Fusarium* sp.[25] and zygomycetes including *Mucor* sp.[26] In particular, the vegetative cell walls of *M. rouxii* contain both chitin and chitosan in an approximate ratio of 1:3.[27]

The protein kinase A (PKA) pathway was reported to regulate hyphal growth polarity against *Neurospora crassa*.[28] Both an inhibitor of PKA, H8, and an activator of PKA, dbcAMP, enhanced the activity of CTB1 (data not shown) and significantly intensified the morphological abnormality of hyphae induced by CTB1 (Fig. 7B, D, and F). These results suggest that PKA-disturbing agents enhance CTB1 action, but the mechanism underlying this process remains to be understood.

Although CTB1 ultimately caused the bursting of hyphae in *M. mucedo* spheroplasts recovering under hyperosmotic conditions, regeneration of the cell wall was also observed prior to bursting (Fig. 8). In contrast, NZ induced hyphal bursting similar to CTB1, but without any regeneration of the cell wall. The reason why CTB1-treated spheroplasts burst under hyperosmotic conditions such as 1.2 M sorbitol is unclear. Based on our hypothesis that the burst was caused by cell wall fragility, we suggest that CTB1 disturbs the fungal cell wall after regeneration of cell wall materials, unlike NZ, which completely inhibited cell wall regeneration. We predict that CTB1 binds to chitin to decrease cell wall rigidity. On the surface of hyphal growth points such as tips and branching sites, chitin and chitosan chains are crystallized into fibrils by chain–chain hydrogen bonds. The rigidity of the fungal cell wall is sustained by a meshwork assembled with these fibrils.[27,29] Calcofluor white has been reported to inhibit the normal crystallization of chitin chains and disturb this meshwork assembly.[30] From these findings, we hypothesize that CTB1 binds to chitin and subsequently disturbs the crystallization of chitin chains into fibrils and the meshwork assembly.

5. Conclusions

CTB1 inhibited the growth of several filamentous fungi including plant pathogens those with swelling of hyphae and spores. CTB1-sensitive fungal species contain high levels of chitin and/or chitosan in their cell walls. Although CTB1 did not inhibit CHS activity, CTB1 bound to chitin, not β-glucan or chitosan. We conclude that the growth inhibition caused by CTB1 could be explained by cell wall fragility induced via CTB1 binding to chitin. Furthermore, CTB1 did not inhibit the proliferation of mammalian cell lines tested. Taken together, these findings indicate that CTB1 may be a promising antifungal agent with fewer adverse effects.

References and notes

1. (a) Mathew, B. P.; Nath, M. *ChemMedChem* **2009**, *4*, 310; (b) Alvarez-Lerma, F.; Nolla, J.; Palomar, M.; León, M. A. *Enferm. Infecc. Microbiol. Clin.* **2003**, *21*, 83.
2. (a) Kauffman, C. A. *Proc. Am. Thorac. Soc.* **2006**, *3*, 35; (b) Rakhmanova, A. G.; Giaurgieva, O. K. h.; Gorshkov, G. I. *Klin. Med.* **1995**, *73*, 44.
3. Thomas, P. A.; Geraldine, P. *Curr. Opin. Infect. Dis.* **2007**, *20*, 129.
4. Hashimoto, M.; Murakami, T.; Funahashi, K.; Tokunaga, T.; Nihei, K.; Okuno, T.; Kimura, T.; Naoki, H.; Himeno, H. *Bioorg. Med. Chem.* **2006**, *14*, 8259.
5. 'Reference Method for Broth Dilution Antifungal Susceptibility Testing of Yeast', National Committee for Clinical Laboratory Standards; Tentative Standard M27-T, NCCLS, Wayne PA, 1995.
6. Nakayama, G. R.; Caton, M. C.; Nova, M. P.; Parandoosh, Z. *J. Immunol. Methods* **1997**, *204*, 205.
7. (a) Jault, J.-M.; Comte, J.; Gautheron, D. C.; Pietro, A. D. *J. Bioenerg. Biomembr.* **1994**, *26*, 447; (b) Kiso, T.; Fujita, K.; Ping, X.; Tanaka, T.; Taniguchi, M. *Antimicrob. Agents Chemother.* **2004**, *48*, 1739.
8. Hageage, G. J.; Harrington, B. *J. Lab. Med.* **1984**, *15*, 109.
9. Šesták, S.; Farkaš, V. *Anal. Biochem.* **2001**, *292*, 34.
10. Crotti, L. B.; Drgon, T.; Cabib, E. *Anal. Biochem.* **2001**, *292*, 8.
11. Hatano, K.; Morishita, Y.; Nakai, T.; Ikeda, F. *J. Antibiot.* **2002**, *55*, 219.
12. Kim, S.-H.; Nah, J.-W. *Korea Polym. J.* **1999**, *7*, 24.
13. Leane, M. M.; Nankervis, R.; Smith, A.; Illum, L. *Int. J. Pharm.* **2004**, *271*, 241.
14. Reusch, V. M.; Foster, J. L.; Haberkorn, D. S. *J. Bacteriol.* **1983**, *155*, 896.
15. (a) Schwartz, Z.; Lohmann, C. H.; Sisk, M.; Cochran, D. L.; Sylvia, V. L.; Simpson, J.; Dean, D. D.; Boyan, B. D. *Biomaterials* **2001**, *22*, 731; (b) Sorol, M. R.; Pereyra, E.; Mizyrycki, C.; Rossi, S.; Moreno, S. *Exp. Cell Res.* **2001**, *271*, 337.
16. (a) Lasker, B. A.; Borgia, P. T. *J. Bacteriol.* **1980**, *141*, 565; (b) Genthner, F. J.; Borgia, P. T. *J. Bacteriol.* **1978**, *134*, 349.
17. (a) Hamada, M.; Ohata, I.; Fujita, K.; Usuki, Y.; Ogita, A.; Ishiguro, J.; Tanaka, T. *J. Biochem.* **2006**, *140*, 851; (b) Roncero, C.; Durán, A. *J. Bacteriol.* **1985**, *163*, 1180.
18. (a) Tariq, V. N.; Devlin, P. L. *Fungal Genet. Biol* **1996**, *20*, 4; (b) Dennis, C. G.; Greno, W. R.; Brun, Y.; Youn, R.; Slocum, H. K.; Bernacki, R. J.; Lewis, R.; Wiederhold, N.; Holland, S. M.; Petraitiene, R.; Walsh, T. J.; Segal, B. H. *Antimicrob. Agents Chemother.* **2006**, *50*, 422; (c) Yutani, M.; Hashimoto, Y.; Ogita, A.; Kubo, I.; Tanaka, T.; Fujita, K. *Phytother. Res.* in press. doi:10.1002/ptr.3579.
19. (a) Datema, R.; Van Den Ende, H.; Wessels, G. H. J. *Eur. J. Biochem.* **1977**, *80*, 611; (b) Rizza, V.; Kornfeld, J. M. *J. Gen. Microbiol.* **1969**, *58*, 307; (c) Ruiz-Herrera, J. *Arch. Biochem. Biophys.* **1967**, *122*, 118; (d) Barran, L. R.; Schneider, E. F.; Wood, P. J.; Madhosingh, C.; Miller, R. W. *Biochim. Biophys. Acta* **1975**, *392*, 148.

20. (a) Aoki, M.; Ohtsuka, T.; Yamada, M.; Ohba, Y.; Yoshizaki, H.; Yasuno, H.; Sano, T.; Watanabe, J.; Yokose, K.; Seto, H. *J. Antibiot.* **1991**, *44*, 582; (b) Aoki, M.; Ohtsuka, T.; Itezono, Y.; Yokose, K.; Furihata, K.; Seto, H. *Tetrahedron Lett.* **1991**, *32*, 217; (c) Aoki, M.; Ohtsuka, T.; Itezono, Y.; Yokose, K.; Furihata, K.; Seto, H. *Tetrahedron Lett.* **1991**, *32*, 221; (d) Wang, J.; Yu, Y.; Tang, K.; Liu, W.; He, X.; Huang, X.; Deng, Z. *Appl. Environ. Microbiol.* **2010**, *76*, 2335.
21. Bowman, S. M.; Free, S. J. *Bioessays* **2006**, *28*, 799.
22. Kim, M.-K.; Park, H.-S.; Kim, C.-H.; Park, H.-M.; Choir, W. *Yeast* **2002**, *19*, 341.
23. (a) Decker, H.; Walz, F.; Bormann, C.; Zähner, H.; Fiedler, H. P.; Heitsch, H.; König, W. A. *J. Antibiot.* **1990**, *43*, 43; (b) López-Romero, E.; Ruiz-Herrera *J. Antonie Van Leeuwenhoek* **1976**, *42*, 261.
24. Ciopraga, J.; Gozia, O.; Tudor, R.; Brezuica, L.; Doyle, R. J. *Biochim. Biophys. Acta* **1999**, *1428*, 424.
25. Xu, G.-F.; Song, B.-A.; Bhadury, P. S.; Yang, S.; Zhang, P.-Q.; Jin, L.-H.; Xue, W.; Hu, D.-Y.; Lu, P. *Bioorg. Med. Chem.* **2007**, *15*, 3768.
26. Rinaudo, M. *Prog. Polym. Sci.* **2006**, *31*, 603.
27. Davis, L. L.; Bartnicki-Garcia, S. *J. Gen. Microbiol.* **1984**, *130*, 2095.
28. Bruno, K. S.; Aramayo, R.; Minke, P. F.; Metzenberg, R. L.; Plamann, M. *EMBO J.* **1996**, *15*, 5772.
29. Lesage, G.; Bussey, H. *Microbiol. Mol. Biol. Rev.* **2006**, *70*, 317.
30. Elorza, M. V.; Rico, H.; Sentandreu, R. *J. Gen. Microbiol.* **1983**, *129*, 1577.