Binding of Benanomicin A to Fungal Cells in Reference to its Fungicidal Action

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An antifungal antibiotic, benanomicin A, binds in the presence of Ca^{2+} to susceptible fungi and some bacteria, but not to antibiotic-resistant bacteria and mammalian cells. With the susceptible yeast *Saccharomyces cerevisiae*, benanomicin A binds similarly to whole cells and to protoplasts. Studies using benanomicin A and three structurally related derivatives suggested that a carboxylic acid in the D-alanine moiety and a sugar moiety in the benanomicin A molecule are essential for both binding and antifungal activities against growing *S. cerevisiae*. An amino substituent on the sugar moiety can be replaced with a hydroxyl group without the loss of activities. Benanomicin A binds to various yeast mannans which differ in glycosidic linkages. These results indicate that binding of benanomicin A to the mannan portion of fungal cells is essential for exertion of the antifungal activity.

Benanomicin A produced by *Actinomadura sporax*[1,2] is a red-colored antibiotic consisting of benzo[*a*]naphthacenequinone, D-alanine and a disaccharide (Fig. 1).[3] Previous studies demonstrated that benanomicin A inhibits growth of a wide range of pathogenic fungi including *Candida* spp., *Aspergillus* spp. and *Cryptococcus neoformans*, that parenteral doses of the antibiotic protected mice against a lethal infection with these fungi, and that it was well tolerated by mice and other experimental animals.[4,5]

At an early stage of our investigation, it was observed that a red-colored ring was formed in the marginal region of inhibitory zones on agar medium implanted with a test fungus when an agar diffusion assay with the antibiotic was performed. In addition, fungal cells aggregated and showed a red color when they were treated with the antibiotic in liquid medium. These findings strongly suggested that substantial amounts of benanomicin A are bound to fungal cells when the antibiotic and cells are incubated together.

The present study was undertaken to get information on the binding property of benanomicin A to yeast cells in order to further elucidate the mode of its antifungal action. For this purpose the benanomicin A-related compounds benanomicin B, its methyl ester and benanomicinone (Fig. 1), as well as several different mannan preparations, were used to define the molecular basis of the binding activity. Preliminary results were reported elsewhere.[6,7]

Materials and Methods

Benanomicins, Related Compounds, Mannan Preparations and Other Chemicals

Benanomicins A and B, benanomicin B methyl ester and benanomicinone were prepared as previously reported.[3,8] Chemically modified mannans containing various glycosidic linkages were prepared from the mannans of the following fungal species by $NaBH_4$ reduction under alkaline conditions according to the literature.[9~11] Modified mannan 1A, containing α-1,2- and α-1,3-linkages, was obtained from *Saccharomyces cerevisiae* X2180-1A; modified mannan 1A-5, containing α-1,6-linkage, from *S. cerevisiae* X2180-1A-5; modified

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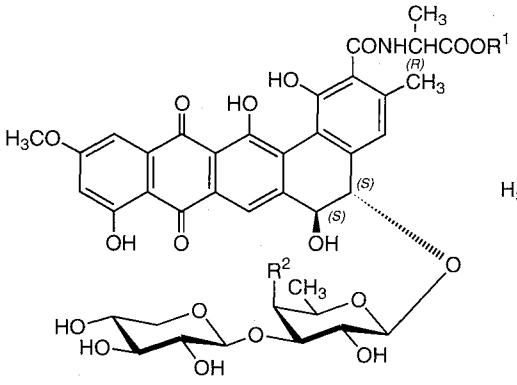
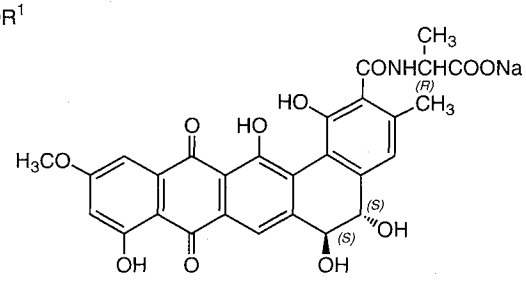
Fig. 1. Structures of benanomicins A and the related compounds.



	R^1	R^2
Benanomicin A sodium salt	Na	OH
Benanomicin B sodium salt	Na	NH_2
Benanomicin B methyl ester	CH_3	$NH_3^+Cl^-$



Benanomicinone sodium salt

mannan P, containing β-1,2-linkage, from *Pichia pastoris* IFO0948; modified mannan A from *Candida albicans* NIH A-207 (serotype A). The modified mannans contained 96.0~99.5% carbohydrate content (determined by the phenol-sulfuric acid method), 0.1~0.2% protein (determined by the Folin method), 0.08~0.97% phosphate (determined by the Ams-Dubin method) and 0.7 ppm calcium. The *Saccharomyces* mannan used in the experiments of gel filtration was that derived from *S. cerevisiae* (Sigma M 7504, Sigma Chemical Company, St. Louis). EGTA (Ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*',*N*'-tetraacetic acid), $CaCl_2$ and other chemicals were commercial products from Wako Chemicals (Tokyo). Concanavalin A and FITC-labeled concanavalin A were from Sigma.

Spectrophotometric Analyses

UV and visible spectra were determined in aqueous solution with a Hitachi UV spectrometer. Optical rotations were determined with a JAS DIP-360 digital polarimeter using a 1-cm cell. Aqueous solutions of 0.05% benanomicin A and 0.20~0.60% glycan or sugar were used. Flow cytometry was conducted using a FAC Star, Benton Dickinson, California, U.S.A.

Fungal, Bacterial and Tumor Cell Strains

S. cerevisiae X2180-1A was kindly supplied by Dr. Y. OSUMI, University of Tokyo. All other fungal strains including *Candida albicans* Y-2, *Cryptococcus neoformans* IMC F-10 and *Aspergillus fumigatus* TIMM1775 and two bacterial strains, *Corynebacterium xerosis* NCTC9775 and *Escherichia coli* NIHJ were from culture collections at the Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd. or the Research Center for Medical Mycology, Teikyo University. P388 leukemia cells and NRK (normal rat kidney) cells were cell lines stocked in the Pharmaceutical Research Center, Meiji Seika Kaisha, Ltd.

Preparation of *Saccharomyces* Protoplasts

The washed cells of *S. cerevisiae* X2180-1A were suspended in 1/15 M sodium phosphate buffer containing 20 mM EDTA, 1 mM mercaptoethanol and 1.2 M sorbitol as an osmotic stabilizer to give a cell suspension at a concentration of 1×10^8 cells/ml. The cell suspension was mixed with Zymolyase 100T (Seikagaku Kogyo Co., Tokyo) suspended in the same buffer at a final concentration of 100 μg/ml. The mixture was incubated at 27°C for 30 minutes with gentle shaking. Completion of protoplast formation was confirmed under a microscope by the observation that almost all cells burst after dilution with water. Protoplasts thus prepared were collected by centrifugation (1,500 rpm, 5 minutes), washed three times with 1/15 M sodium phosphate buffer containing 1.2 M sorbitol, and then resuspended in an appropriate medium for use.

Determination of Mannose Content in Whole Cells and Protoplasts of *S. cerevisiae*

The washed cells (10^8 cells) of *S. cerevisiae* X2180-1A or its protoplasts were mixed with 1 ml of 1 N sulfuric acid, heated at 100°C for 5 hours, and neutralized with barium carbonate. The mixture was centrifuged, and the supernatant and washings were combined, concentrated, and subjected to HPLC analysis under the following conditions: column, Shim-pack ISA-07/S2504 (4.0 × 250 mm); mobile phase, gradient of 0.1 M borate buffer (pH 8.0) and 0.4 M borate buffer (pH 9.0); flow rate, 0.7 ml/minutes; temperature, 65°C; detection, post-column fluorescence using 1% arginine-boric acid.[12]

Determination of Benanomicin A Binding to Fungal Cells

The fungal cultures were grown at 30°C for 48 hours (24 hours in *Aspergillus* sp.) in YPG medium (yeast extract 0.5%, Polypeptone 1.0% and glucose 2.0%). The bacterial cultures were grown at 37°C for 24 hours in Nutrient broth (Difco). The fungal or bacterial cells were harvested by centrifugation at 3,000 rpm for 5 minutes, and washed three times with sterilized water or saline.

The washed microbial cells were suspended in DULBECCO's phosphate buffered saline (pH 7.5, DPBS, Sigma) supplemented with 1 mM $CaCl_2$ and adjusted to 2×10^7 cells per ml by counting with a hemocytometer. To 1 ml of the cell suspension was added an equal volume of aqueous solution of benanomicin A (200 μg/ml). After stirring for 5 minutes, the mixture was incubated statically at 27°C for fungi or 37°C for bacteria for 30 minutes, and centrifuged at 3,000 rpm for 3 minutes. The sedimented cells were washed by centrifugation three times with DPBS, and the antibiotic bound to the cells was extracted with 2 ml of dimethyl sulfoxide (DMSO) by stirring for 5 minutes, then recovered from the supernatant after centrifugation at 3,000 rpm for 3 minutes. The concentration of the antibiotic in the supernatant was determined by measuring the optical density at 490 nm with a UV/visible spectrophotometer, and the amount of the antibiotic bound to 10^7 cells was calculated. Viable yeast cells were counted using a conventional colony count technique on YPG agar plate. *Aspergillus fumigatus* conidia were collected on a GF/c filter, and binding amount was expressed on a weight basis owing to the difficulty of cell counting.

Determination of Benanomicin A Binding to Tumor Cells, NRK cells and Erythrocytes

P388 leukemia cells were grown at 37°C in RPMI-1640 medium (Nissui) supplemented with 10% fetal calf serum. The cells were collected by centrifugation at 1,000 rpm for 5 minutes, washed with the same warmed medium, and resuspended in DPBS containing 1 mM $CaCl_2$. NRK cells were grown at 37°C in DULBECCO's modified Eagle medium (DMEM, Nissui) containing 50% fetal calf serum. Experimental cell suspension of P388 leukemia cells and NRK cells at a concentration of 1.6×10^6 and 2×10^6 cells/ml, respectively, was inoculated at a volume of 1 ml into each plastic dish (Falcon 3001). The cells adhering to dishes were washed with DMEM, and suspended in DPBS containing 1 mM $CaCl_2$. Murine erythrocytes were obtained from blood taken from descending cava of male Crj:CDl (ICR) mice under anesthesia, and washed with DPBS by centrifugation at 3,000 rpm for 5 minutes. The sedimented erythrocytes (0.4 ml) were finally suspended in DPBS (0.6 ml) containing 1 mM $CaCl_2$ at a cell concentration of 1×10^6 cells/ml.

Benanomicin A was added to an experimental cell suspension at a final drug concentration of 100 μg/ml, and the mixture was incubated at 37°C for 30 minutes. After centrifugation at 3,000 rpm for 5 minutes, the supernatant was removed, and extracted with 200 μl of DMSO. The determination of bound benanomicin A in the extract was made using the same method as used for experiments with microbial cells as above mentioned.

Determination of Antimicrobial and Cytotoxic Activities

Antimicrobial activity of benanomicin A was determined by the agar dilution method on Kimmig agar for fungi, and nutrient agar (Difco) for *Escherichia coli*, or by the broth dilution method in a liquid medium consisting of Bactopeptone 1.0%, beef extract 0.3% and NaCl 3.0% for *Corynebacterium* sp. The activity was expressed as the minimum growth-inhibitory concentration (MIC).

Cytotoxicity of benanomicin A against P388 leukemia cells and NRK cells was determined by the staining technique. P388 leukemia cells were suspended in RPMI-1640 containing 10% fetal calf serum and NRK cells in DMEM containing 5% fetal calf serum at a concentration of 1×10^6 and 2×10^6 cells/ml, respectively. The cell suspension in a volume of 4 ml was incubated in the presence of benanomicin A at 37°C for 3 days in 5% CO_2 atmosphere. Number of surviving cells was measured by staining with 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyltetrazolium bromide. Those drug concentrations killing 50% of the tested cells were defined as IC_{50} values.

Recovery of Benanomicin A from Preloaded Fungal Cells

C. albicans Y-2 cells (4.4×10^7 cells) were mixed with 100 μg of benanomicin A in a volume of 1 ml, and incubated for 30 minutes at room temperature. After centrifugation at 3,000 rpm for 5 minutes, the yeast cells sedimented were resuspended in 1 ml of the indicated solvent and incubated at 25°C for 5 minutes to extract the benanomicin A bound to the cells. The extract was subjected to HPLC analysis to determine cell-bound benanomicin A under the following conditions: Precolumn, Cosmosil $10C_{18}$ 4.6 × 50 mm; column, Cosmosil $5C_{18}$ 4.6 × 150 mm; mobile phase, 0.5% KH_2PO_4–CH_3OH (1:1); flow rate, 1 ml/minute; temperature, 40°C and UV detection at 280 nm.

Measurement of Lytic Activity against Fungal Protoplasts

Protoplasts of *S. cerevisiae* X2180-1A were suspended in YNB-S-PB (YNB supplemented with 1.2 M sorbitol and 1/15 M sodium phosphate buffer, pH 7.0) supplemented with 1% glucose, and adjusted to a concentration of 1×10^6 cells/ml. The protoplast suspension was dispersed in a volume of 180 μl into each well of flat bottom microtiter plates (96 wells) which received 20 μl of a solution of benanomicin A or another test drug. Tween 80 was added at a concentration of 1% in the experiments when necessary. The plates were incubated

at 37°C and the optical density of each well was read at 620 nm using an automated reader (Titertek Multiskan MC, Labsystem and Flow Laboratory). When 10^6 protoplasts were applied, a decrease of 0.1 optical density was given by lysis of approximately 10^3 protoplasts.

Determination of Hemolysis of Murine Erythrocytes

The experimental procedure was similar to that described by TANIGUCHI *et al.*[13] To a 1% suspension (5 ml) of murine erythrocytes in HANK's solution (pH 7.5) in test tubes was added an aqueous solution of benanomicin A (5 μl). After standing at room temperature for 30 minutes, the test tubes were shaken at a rate of 200 strokes per minutes for 5, 10, 20, 30 and 60 minutes. After centrifugation at 3,000 rpm, hemoglobin in the supernatant was measured at 540 nm with a UV/visible spectrophotometer. The rate of hemolysis was calculated using a control value without Folin as 100%.

Gel Filtration Analysis of Interaction of Benanomicin A with *Saccharomyces* Mannan

Ten mg of *Saccharomyces* mannan (Sigma) was dissolved in 200 μl of distilled water. One hundred μl of aqueous solution of benanomicin A (8 mg/ml) was mixed with 200 μl of aqueous solution of *Saccharomyces* mannan. After incubation at room temperature for 5 minutes, the reaction mixture was passed through a column of Sephadex G-50 (15 ml), and developed with water. Effluent was collected in 1-ml fractions. With each fraction, optical density at 490 nm and antifungal activity against *C. albicans* cells were determined.

To test the effect of Ca^{2+} on benanomicin A-*Saccharomyces* mannan interaction, the reaction mixture containing 400 μg of benanomicin A and 5 mg of *Saccharomyces* mannan was supplemented with 20 μg of $CaCl_2$, adjusted to a final volume of 350 μl, and passed through a Sephadex column.

Results

Requirement of Ca^{2+} for Binding of Benanomicin A to Fungal Cells

Table 1 shows effects of $CaCl_2$ and EGTA on the binding of benanomicin A to the susceptible yeast, *C. albicans*. Addition of Ca^{2+} significantly stimulated the bindings of antibiotic to cells. The concurrent addition of EGTA with Ca^{2+} reduced the binding of benanomicin A, to near baseline.

These results suggested that Ca^{2+} is required for antibiotic binding. Accordingly, all of the following experiments were carried out using a medium containing Ca^{2+} unless otherwise stated.

Binding of Benanomicin A to Different Microbial Cells

Table 2 shows the binding of benanomicin A to four different species of fungi and two species of bacteria. Benanomicin A bound in a range of 7.5~25 μg/10^7 cells to all the antibiotic susceptible species of fungi (MIC, 1.56~12.5 μg/ml). Benanomicin A also bound to one antibiotic susceptible bacterium, *Corynebacterium xerosis* (MIC, 6.25 μg/ml), but did not bind to benanomicin resistant *E. coli* strain (MIC, 100 μg/ml). No substantial binding of benanomicin A was observed with any of mammalian cells tested, *viz.*, P388 leukemia cells, NRK cells or murine erythrocytes. Furthermore no cytotoxicity or hemolysis was observed with these cells and erythrocytes at 100 μg/ml.

In order to investigate the strength of benanomicin A binding to *C. albicans*, various solvents were used to

Table 1. Effect of $CaCl_2$ and EGTA on the binding of benanomicin A to *C. albicans*[a].

Addition of:		Amount of benanomicin A bound to 10^7 cells (μg)
$CaCl_2$ (mM)	EGTA (mM)	
0	0	0.2
0	2	0.2
1	0	13
1	2	1.9

[a] Initial concentration of benanomicin A, 100μg/ml. Initial inoculum of *C. albicans* Y-2 suspended in DPBS, 4.4×10^7 CFU/ml.

Table 2. Ability of benanomicin A to bind to and inhibit several fungi and bacteria.

Organism	Amount of benanomicin A bound to 10^7 cells (μg)	Minimum growth inhibitory concentration of benanomicin A (μg/ml)
Candida albicans Y-2	13	6.25
Saccharomyces cerevisiae X2180-1A	25	3.13
Cryptococcus neoformans IMC F-10	7.5	1.56
Aspergillus fumigatus TIMM1775	21*	12.5
Corynebacterium xerosis NCTC9775	4.5	6.25
Escherichia coli NIHJ	0.1	100

* μg/mg (dry weight).

Table 3. Binding and bioactivity of benanomicin A and related compounds to *C. albicans* and *S. cerevisiae*.

Compound	Whole cells of *C. albicans* Y-2		Whole cells of *S. cerevisiae*		Protoplasts of *S. cerevisiae*	
	Amount of benanomicin A bound ($\mu g/10^7$ cells)	Susceptibility to benanomicin A ($\mu g/ml$)*	Amount of benanomicin A bound ($\mu g/10^7$ cells)	Susceptibility to benanomicin A ($\mu g/ml$)*	Amount of benanomicin A bound ($\mu g/10^7$ cells)	Susceptibility to benanomicin A (%)**
Benanomicin A	13	6.25	25	1.56	8.0	99.9
Benanomicin B	18	12.5	30	1.56	28	99.9
Benanomicin B methyl ester	1	100	1	100	0.4	0
Benanomicinone	1	100	1	100	0.4	0

* Minimum growth inhibitory concentration.
** Decrease in number of intact protoplasts 120 minutes after addition of drug (50 $\mu g/ml$) in YNBD-S-PB medium.

extract prebound benanomicin A. The recovery of the antibiotic was very low when extracted with acetone (1.0%), moderate with water (14.9%), *N,N*-dimethylformamide (37.3%) or 5 mM EGTA (49.5%), but almost complete with DMSO (99.0%). These results suggested that the binding was not irreversible covalent bonding but reversible hydrogen bonding.

Correlation of the Structure of Benanomicin A with Its Binding Ability and Antifungal Activity

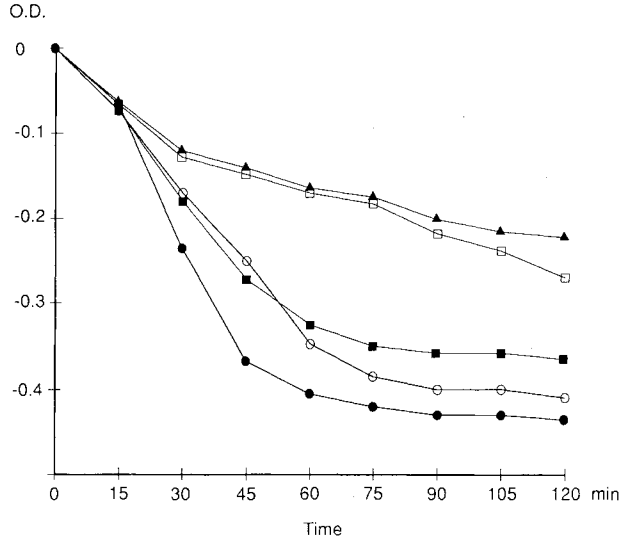
Table 3 shows the extent of binding of benanomicin A and related compounds to *Candida* and *Saccharomyces* cells and their protoplasts. Benanomicins A and B bound to both whole fungal cells and their protoplasts, in parallel to the growth-inhibitory activity (against the whole cells) and lytic activity (against the protoplasts). Benanomicin A bound to the whole cells of *S. cerevisiae* to a greater extent than to their protoplasts, but benanomicin B, a basic analog of benanomicin A, showed comparable binding to both whole cells and protoplasts. Analysis of the mannan content in the whole cells and protoplasts of *S. cerevisiae* X2180-1A, using the acid hydrolysates of samples, revealed that mannans in the whole cells and protoplasts were 20 and 2.5 $\mu g/10^7$ cells, respectively. A derivative of benanomicin B lacking either an acidic function (benanomicin B methyl ester) or the sugar moiety (benanomicinone) did not bind to nor inhibit *C. albicans* or *S. cerevisiae*.

Protoplast Lytic Activity of Benanomicin A

Figure 2 shows the time-course of lysis of *S. cerevisiae* protoplasts when treated with 20 $\mu g/ml$ of benanomicin A in YNBD-S-PB, as measured by a decrease in optical density. Concanavalin A which is known to bind to the mannan moiety of yeast cells was used as a reference. Benanomicin A was capable of inducing protoplast lysis, and the extent of this was further increased by the addition of 1% Tween 80. Concanavalin A, in contrast, at 1,000 $\mu g/ml$ induced only slight protoplast lysis which was accelerated in the presence of Tween 80. One percent Tween 80 alone showed no effect on the protoplast lysis. This indicated that benanomicin A, which shows the cytolytic action alone, has a different mechanism of action than concanavalin A that requires the Tween 80 for lysis.

Fig. 2. Time course of antifungal action of benanomicin A (20 $\mu g/ml$) and concanavalin A (1,000 $\mu g/ml$) on protoplast of *S. cerevisiae* X2810-1A with or without Tween 80 (1%).

○: Benanomicin A, ●: benanomicin A plus Tween 80, □: concanavalin A, ■: concanavalin A plus Tween 80.



As measured by flow cytometry, the relative fluorescence intensity of *S. cerevisiae* protoplasts in YNB-S-PB treated with FITC-concanavalin A (1,000 $\mu g/ml$) for 30 minutes was 311, and this was decreased to 192 after pretreatment with benanomicin A (25 $\mu g/ml$) for 5 minutes. The intensity of the protoplasts treated with

benanomicin A alone was 7.6. The decrease in fluorescence of the concanavalin A treated protoplasts following benanomicin A pretreatment suggested competitive binding of two compounds.

Interaction of Benanomicin A with *Saccharomyces* Mannan

We examined benanomicin A interaction with *Saccharomyces* mannan using a gel filtration technique with a column of Sephadex G-50. The elution pattern of an aqueous solution of benanomicin A showed a single red-colored peak (peak I) in the range of fraction 10 accompanied by a tailing (Fig. 3A). When a mixture of benanomicin A and yeast mannan at a weight ratio of 1:12 was passed through the Sephadex column, an additional red-colored peak (peak II) appeared in the range of fraction 6 (Fig. 3B). Mannan was exclusively recovered from peak II. Fraction 10 (peak I) was active in inhibiting fungal growth, whereas fraction 6 (peak II) showed no activity at all. When the mixture of benanomicin A and yeast mannan was charged to the column in the presence of 2 mM Ca^{2+}, there occurred an increase in the peak II area with a concomitant decrease in the peak I area (Fig. 3C). This indicated that benanomicin A bound to yeast mannan, and that in so doing it lost its antifungal activity.

Interaction of Benanomicin A with Chemically Modified Yeast Mannans

Table 4 shows the interaction of benanomicin A with modified yeast mannans that contain different glycosidic linkages: α-1,2 and α-1,3 (1A); α-1, 6 (1A-5); and β-1,2 (P). Utilizing the changes of absorption spectrum and optical rotation, all of these chemically modified mannans when added to an aqueous solution of benanomicin A produced the same spectral change. The absorption

Fig. 3. Gel filtration patterns of benanomicin A - *Saccharomyces* mannan complexes over Sephadex G-50.

A: Benanomicin A alone, B: benanomicin A plus *Saccharomyces* mannan (1:12 w/w), C: benanomicin A, *Saccharomyces* mannan plus $CaCl_2$ (2 mM).

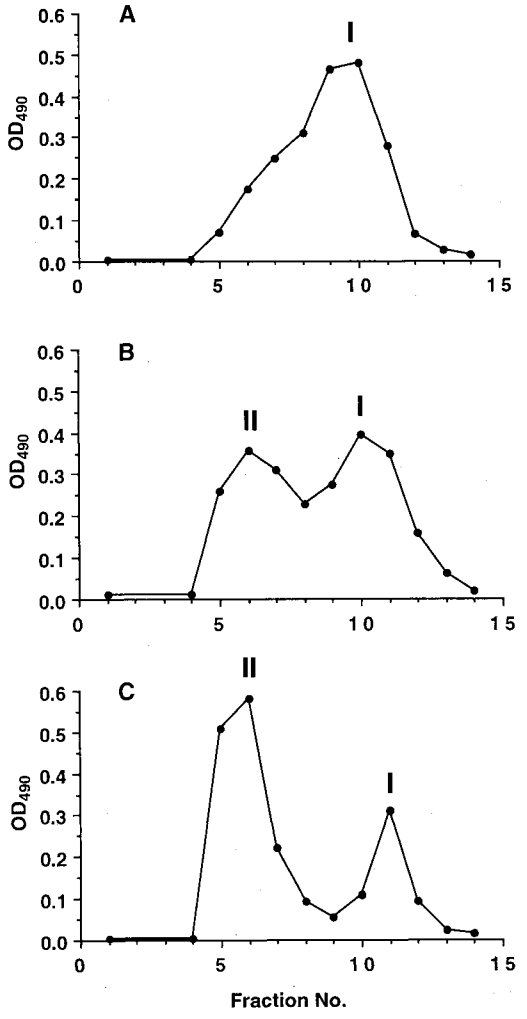


Table 4. Visible and rotational changes due to interaction between benanomicin A and modified yeast mannans containing various glycosidic linkages and D-mannose and dextran T-40*.

Addition (glycan)	Weight ratio (antibiotic: glycan)	Visible spectrum (H_2O)	Specific rotation** ($[\alpha]_D$, H_2O) Found	Calcd.
None (control)***		500 nm	+360°	
α-1,2-1,3-mannan (1A)	1:12	515, 565 (sh) nm	+372°	+102°
α-1,6-mannan (1A-5)	1:5	515, 565 (sh) nm	+784°	+152°
β-1,2-mannan (P)	1:4	515, 565 (sh) nm	+965°	+110°
Mannan (A)	1:5	515, 565 (sh) nm	+672°	+124°
D-Mannose	1:5	500 nm	+108°	+92°
Dextran T-40	1:25	500 nm	+240°	+252°

* An aqueous solution of glycan or sugar (0.20~0.60%, w/v) and benanomicin A (0.05%, w/v) in weight ratios of 1:4~1:12 was allowed to stand at room temperature for 5 minutes, and was subjected to measurement.

** Sum total of specific rotations of glycan or sugar and benanomicin A, provided that both values were additive.

*** An aqueous solution of benanomicin A alone.

peak at 500 nm for benanomicin A was shifted to a longer wavelength with formation of a new shoulder at 565 nm. The degree of red-shifting was dependent on the dose of yeast mannan. A maximum shift was observed at 515 nm at ratios of benanomicin A-mannan of 1:5~1:12. Concurrent with these spectral changes, the optical rotations of a mixture of benanomicin A and yeast mannan, especially with the modified mannan P containing β-1,2-linkage, were increased when compared with additive rotation values of the antibiotic and the yeast mannan.

When D-mannose or dextran T-40 was added to an aqueous solution of benanomicin A, no shifting of the absorption maximum and no increase of optical rotation were observed under the conditions examined. Likewise, no change occurred when D-mannose, α- or β-cyclodextrin, dextran T-70, T-150, T-250, or amylose A was added to an aqueous solution of benanomicin A (data not shown). Benanomicinone which showed no binding to fungal cells did not cause significant spectral or rotational changes when mixed with yeast mannans (data not shown). These data indicate that benanomicin A binds specifically to yeast mannans irrespective of difference in the types of glycosidic linkages, but not to a monosaccharide D-mannose or D-glucose polymers.

Discussion

The present study demonstrate that benanomicin A binds to fungi and bacteria which are susceptible to the antibiotic when incubated in the presence of calcium ion. The antibiotic does not bind to bacterial and mammalian cells which are not inhibited by benanomicin A. Furthermore, derivatives of benanomicins A and B (methyl ester and benanomicinone) without antifungal activity did not bind to any fungi or bacteria tested. Thus, it appears that the cell-binding ability of benanomicin A is closely associated with its biological activity leading to its selective toxicity against fungal cells. Both a carboxylic acid function of the D-alanine moiety and the sugar moiety with or without an amino group are essential for the binding ability of benanomicin A. The antibiotic appears to bind to the fungal cells tightly through hydrogen bonding, since only part of it was extracted with water while almost all the benanomicin A was recovered when DMSO, a strong cleaving agent of hydrogen bond, was used as the extraction solvent. The kinetics of benanomicin A binding is discussed in a separate paper.[14]

Fungi susceptible to benanomicin A contain mannans or heteropolymeric mannans like galactomannan (*Aspergillus*), and susceptible bacteria also contain mannans (*Micrococcus lysodeikticus*[15]) and/or arabinomannans (*Mycobacterium* sp.[16]). Therefore, the selective binding of benanomicin A to fungal cells leads us to the assumption that mannan or mannan-derived components of fungal cell walls might act as the receptor molecule for benanomicin A binding. This postulation is supported by the finding that benanomicin A was capable of binding to yeast mannans with several different glycosidic linkages, but not to any glucose polymers so far examined. It was also demonstrated that Ca^{2+} is again essential for benanomicin A binding to mannan, as was previously reported for pradimicins[17~21] which are closely related to benanomicin A in chemical structure and biological activity[22].

Benanomicin A was demonstrated to be capable of binding not only to intact yeast cells but also to yeast protoplasts. It is known that mannan is also localized on yeast cell membranes as mannoproteins, although at a lower content than that found in yeast cell walls. On the basis of cell numbers, the amount of benanomicin A or B bound to the protoplasts was almost equal to that of the intact cells, while the mannan content of yeast protoplasts was approximately 1/8 that of intact yeast cells. This data suggest that benanomicin A binds to membrane-associated mannan or mannoproteins at a rate equal to or higher than that for cell wall mannans.

The ability of benanomicin A to bind selectively to yeast mannans appears similar to that of lectins. Concanavalin A is demonstrated to cause a change of fluidity of the cell membrane after binding to yeast cells.[23] In the context of interaction with *S. cerevisiae*, benanomicin A and concanavalin A are similar in that they require Ca^{2+} for binding to yeast cells, and that they bind yeast cells to coagulate and to lyse the yeast protoplasts in the presence of Tween 80. However, unlike benanomicin A, concanavalin A binds to D-mannose, and is not lethal to whole yeast cells.

In conclusion, benanomicin A binds selectively to mannan or mannan-derived polysaccharide moieties localized on the cell envelope of fungi and other susceptible microorganisms. The carboxylic acid group, the sugar moiety and the chromophore of benanomicin A molecule are involved in the binding for which Ca^{2+} is essential. Although the binding to fungal cells is necessary for benanomicin A to exert an antifungal action, it remains unanswered how the antibiotic-binding becomes lethal. Current experiments are under way to determine the mechanism of this antifungal action.

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