STUDIES ON THE MODE OF ANTIFUNGAL ACTION OF PRADIMICIN ANTIBIOTICS

I. LECTIN-MIMIC BINDING OF BMY-28864 TO YEAST MANNAN IN THE PRESENCE OF CALCIUM

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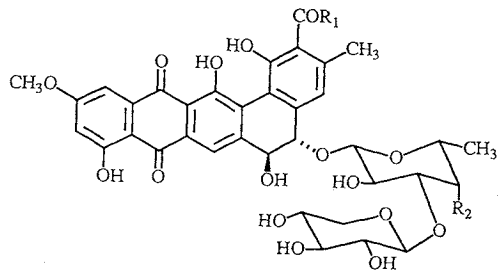
BMY-28864 (BMS-181184), a water-soluble pradimicin derivative, specifically bound on the yeast cell surface in the presence of calcium, which was considered to be the initial step that triggered chain reactions leading to the fungicidal action. Close cause-effect relationships of the cell wall binding of BMY-28864 with its antifungal activity and potassium leakage induction were observed by *Candida albicans* and *Saccharomyces cerevisiae* in the presence and absence of calcium. Using mannan and methyl α-D-mannopyranoside as specific sugars, the mode of binding of BMY-28864 to sugar was examined *in vitro* in the presence of calcium. Quantitative component analysis revealed that the precipitate of BMY-28864 with methyl α-D-mannopyranoside and calcium was a ternary complex possessing a molar component ratio of 2.1 : 4.3 : 1.0. These findings altogether proved that BMY-28864, although not protein, recognized specific sugars such as mannose in the same manner as lectin, and that the ternary complex formation of BMY-28864 with specific sugar and calcium was the first step for expression of the selective antifungal action of the pradimicin.

Pradimicins and benanomicins, which are antifungal and antiviral antibiotics produced by actinomycetes,[1~3] belong to a recently discovered family of benzo[*a*]naphthacenequinone compounds represented by chemical structures in Fig. 1.[4,5] They are selectively antifungal and antiviral without significant acute toxicity in mice as well as antibacterial activity on Gram-positive and Gram-negative bacteria.[4,6] Considered from their selective antifungal and antiviral activity profiles and no antifungal cross resistance with 5-fluorocytosine, ketoconazole and amphotericin B,[6,7] this family of antibiotics were assumed to have a novel mode of antifungal action.

In previous papers,[8~10] the anticandidal activities of pradimicin A and BMY-28864 were reported to depend on their abundant adsorption on the cell wall of *Candida albicans* in the presence of calcium ion. Consequently the detailed study on

Fig. 1.



	R_1	R_2
BMY-28864	$-NHCH(CH_2OH)COOH$ (*R*)	$-N(CH_3)_2$
Pradimicin A	$-NHCH(CH_3)COOH$ (*R*)	$-NHCH_3$
Benanomicin A	$-NHCH(CH_3)COOH$ (*R*)	$-OH$
Pradimicin C (Benanomicin B)	$-NHCH(CH_3)COOH$ (*R*)	$-NH_2$

the mechanism of antifungal action of pradimicins using a representative derivative was considered to be essential from the biochemical and clinical viewpoints, particularly because a new antifungal agent with a novel action mode has been eagerly sought for in clinical therapy of fungal infections in AIDS patients. For this purpose, BMY-28864, which is a hydrophilic derivative semi-synthesized from pradimicin FA-2,[11] was selected among a variety of pradimicin derivatives, as it is expected to be of clinical interest based on its potent antifungal activity, good water-solubility and least cytotoxicity.[12]

This paper describes that the antifungal action of BMY-28864 began with lectin-mimic specific binding of the pradimicin to cell wall mannan which depended on the formation of a ternary complex of BMY-28864 with mannan and calcium.

Materials and Methods

Yeasts, Fungi, Bacteria and Mannan Preparations

Candida albicans A9540 and *Saccharomyces cerevisiae* ATCC 9763 were cultivated at 28°C for 24 hours under shaking in 500 ml Erlenmeyer flasks containing 100 ml each of YPD medium (1% yeast extract, 2% polypeptone and 2% glucose). *Saccharomyces cerevisiae* X2180-1A (wild type) and X2180-1A5-mnn2((1→2)-mannosyl transferase II-defective strain) were gifts from Dr. C. E. BALLOU.[13,14]

Other microbes were cultured and assayed as described before.[8,9]

Linear and branched polymannosaccharide preparations employed in Table 5 were prepared from α-1,6-mannan[13] and wild type mannan by partial acetolysis as reported elsewhere.[13,15]

Antimicrobial Assays

MIC was determined by the agar dilution method on YNB (Difco) + glucose + PB agar medium, pH 7.0, in the presence and absence of 200 μM calcium chloride for fungi; and on nutrient agar medium in the presence of 1 mM calcium chloride for bacteria.

Adsorption to Freeze-dried Cells

Adsorption of BMY-28864 was measured by incubating freeze-dried cells (1.0 mg for fungi, and 5 mg for bacteria) with 60 μg/ml BMY-28864·HCl for 30 minutes at 30°C in the presence and absence of 200 μM calcium chloride in 1 ml of 50 mM sodium phosphate, pH 7.0. After incubation, the mixtures were centrifuged at 5,000 rpm for 5 minutes to provide the supernatants and the cells. The amount of BMY-28864 adsorbed to the cells was calculated by subtracting the absorbance reading of the test supernatant at 498.4 nm from that of the no-cell control.

Adsorption to Intact Candida Cells

After 24 hours' cultivation in the YPD medium, *Candida albicans* A9540 cells were harvested at 4°C by centrifugation at 3,000 rpm for 10 minutes; and rinsed twice in physiological saline. A mixture containing 5×10^7 yeast cells, 100 μl of 1.10 mM BMY-28864·HCl, 10 μl of a 100 mM metal chloride solution and 890 μl of physiological saline was incubated for 2 hours at 30°C. After centrifugation at 5,000 rpm for 5 minutes at 4°C, the supernatant was employed for determination of the potassium content by atomic absorption spectrometry. The control tests with and without BMY-28864 contained no metal chloride.

For determination of BMY-28864 adsorbed to cells, the intact cells were collected by centrifugation and rinsed twice in physiological saline, followed by suspension in 1 ml of DMSO so that all the BMY-28864 adsorbed to the cells might be released into DMSO. The concentration of BMY-28864 in the DMSO solution was read at 498.4 nm by UV/visible spectrophotometry (Beckman DU-70), and the difference in absorbance of the test from the no-metal chloride control gave the amount of BMY-28864 adsorbed to the cells.

The total amount of potassium in the intact cells was recovered by boiling the cells at 100°C for 10 minutes. Centrifugation of the boiled cells yielded the supernatant which was subjected to atomic absorption spectrometry.

In the control, 22.4 ppm of potassium/5×10^7 cells/ml was observed and employed as 100% for calculation of the percent potassium leakage.

Adsorption to Cell Wall Components and Enzymes

Chitin was obtained from Yaizu Suisan Co., Shizuoka, Japan. Chitobiose (Sigma's Catalog No. D 1523), chitosan (C 0792), glucan (G 5011), yeast mannan (M 7504), carboxypeptidase Y (C 3888), invertase (I 4504), trypsin (T 8642), chymotrypsin (C 3142) and alkaline phosphatase (P 8639) were purchased from Sigma Chemical Co.

Cell wall component or an enzyme was dissolved in 1 ml (final volume) of 50 mM MOPS (3-(6-[*N*-morpholino]propanesulfonic acid), pH 7.4, containing 1 mM calcium chloride, and incubated at 30°C for 30 minutes with 138 μg/ml (152 μM) BMY-28864·HCl (A at 498.4 nm=2.43). After centrifugation at 14,000 rpm and 4°C for 5 minutes, the concentration of BMY-28864 in the supernatant was spectrophotometrically read at 498.4 nm in a UV/visible spectrophotometer. The amount of bound BMY-28864 was calculated by subtracting the absorbance of the test supernatant from that of the control.

Comparison of *Candida albicans* A9540 with *Saccharomyces cerevisiae* ATCC 9763 in BMY-28864 Adsorption at 1 mM Calcium Chloride

Intact cells were prepared as described above.

Immobilized cells were prepared by treating intact cells with 3% glutaraldehyde at room temperature for 60 minutes, followed by freeze-drying.

Yeast cells (1×10^8 cells) were suspended in 50 mM MOPS, pH 7.0, or physiological saline, containing 1 mM calcium chloride; and mixed with 60 μg (66 nmol), 120, 180, 240, 300, 360, 420 and 480 of BMY-28864·HCl in a total volume of 8 ml. After incubation at 30°C for 30 minutes, the cells were recovered by centrifugation at 4°C and 5,000 rpm for 10 minutes, and then rinsed twice in physiological saline. The BMY-28864 adsorbed to the cells was released by suspending in 1 ml of DMSO, and the concentration of BMY-28864 in the DMSO solution was read by UV/visible spectrophotometry at 498.4 nm.

Binding constant (Ka) and the number of binding sites (n) were calculated by the method of T. L. STECK and D. F. H. WALLACK.[16]

Antagonistic Effects of Mannan Preparations and Sugars on the Antifungal Activity of BMY-28864 against *Saccharomyces cerevisiae*

Antifungal activity of BMY-28864 against *Saccharomyces cerevisiae* was assayed by the agar well diffusion method on MA agar medium (agar 1.3%, sodium glutamate 0.15%, glucose 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%, NaCl 0.01% and biotin 0.00025% in 0.8 mM phosphate + 100 mM MOPS, pH 7.2, containing 1 mM $CaCl_2$). Sugar solution in 100 mM MOPS, pH 7.2 (80 μl; varied concentrations), and 105 μM BMY-28864 (20 μl) were mixed and subjected to the agar diffusion assay using *Saccharomyces cerevisiae* as tester. After incubation at 28°C for 20 hours, the diameters of the inhibition zones were measured, and the 50% antagonistic concentration of a sugar was read from the dose-halo calibration curve.

Protective Effects of Mannan and Methyl α-D-Mannopyranoside (Me-Man) on *Candida albicans* Protoplasts in the Presence of BMY-28864

Intact *Candida albicans* cells were transformed to protoplasts by the method of I. TAKANO and K. ARIMA.[17,18] More particularly, *Candida albicans* A9540 was cultivated at 28°C for 24 hours in YPD medium and then harvested at 4°C by centrifugation at 1,500 rpm for 10 minutes. The cell pellet was washed twice with physiological saline and preincubated at 30°C for 30 minutes in 60 mM EDTA, pH 7.0, containing 0.2% mercaptoethanol. The treated yeast cells were recovered by centrifugation and suspended at a density of 10^7 cells/ml in 0.6 M KCl containing 0.2% mercaptoethanol. Zymolase 100T (Catalog No. 120493; Seikagaku Kogyo Co., Ltd) was added to the cell suspension at a final concentration of 20 μg/ml and incubated at 30°C for 60 minutes. The protoplasts were recovered by mild centrifugation and washed twice with 0.6 M KCl. The protective effects of 0.1 mg/ml mannan and 100 mM Me-Man were observed by incubating the protoplasts (10^7 cells/ml) and 50 μg/ml (55 μM) BMY-28864·HCl at 30°C for the indicated periods of time in NS medium (SDM medium[19] in which the sorbitol was replaced with 0.6 M KCl). By

centrifugation at 1,500 rpm and 4°C for 5 minutes, the protoplasts were recovered and resuspended in 1 ml of 0.6 M KCl containing 100 mM ethylene glycol-*bis*-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), pH 7.0. After incubation at 30°C for 15 minutes, the numbers of protoplasts were counted with a hemocytometer before and after dilution with water so that the count of the protoplasts was obtained as balance between the two observed numbers of cells.

Interaction of BMY-28864 with Hexoses

(1) Precipitation of BMY-28864 with hexoses at 1 mM calcium chloride.

Hexoses were dissolved in 0.6 M KCl, 50 mM MOPS, pH 7.4, and distilled water (adjusted to pH 7.0), and then mixed with 136 μg/ml (final concentration, 150 μM) BMY-28864·HCl. The final sugar concentrations were all set at 200 mM. After incubation at 30°C for 30 minutes, the reaction mixtures were centrifuged at 5,000 rpm for 5 minutes and the concentrations of BMY-28864 remaining in the supernatants were spectrophotometrically read at 498.4 nm. If a hexose precipitated with BMY-28864, the amount of BMY-28864 adsorbed to that hexose was calculated by subtracting the absorbance reading at 498.4 nm of the supernatant from that of the no-sugar control.

(2) Inhibition by a specific hexose of BMY-28864 adsorption to immobilized *Saccharomyces cerevisiae* cells at 1 mM calcium chloride.

BMY-28864 (1.10 mM, 100 μl), 500 μl of a specific hexose solution in a final concentration range of 200 to 12.5 mM, 10 μl of 100 mM calcium chloride, 290 μl of 50 mM MOPS buffer, pH 7.0, and 3.4×10^7 cells (1 mg) of freeze-dried cells were incubated at 30°C for 30 minutes. The control test contained no specific sugar. After centrifugation at 14,000 rpm for 5 minutes, the precipitates were collected and washed twice with 1 ml each of 50 mM MOPS buffer, pH 7.0. The BMY-28864-calcium-sugar precipitates were dissolved in 1 ml of DMSO and the optical density of the DMSO solution was read at 498.4 nm for determination of the BMY-28864 content.

Kinetics of Inhibition by Me-Man of BMY-28864 Adsorption to Immobilized *Saccharomyces cerevisiae* Cells in the Presence of 1 mM Calcium Chloride

Inhibitory effect of 50 mM Me-Man on BMY-28864 adsorption was kinetically examined by mixing 6.8×10^7 cells (2 mg) of immobilized *Saccharomyces cerevisiae* cells with BMY-28864 (concentrations indicated in Fig. 4) at 1 mM of calcium chloride in a total volume of 8 ml, followed by incubation at 30°C for 30 minutes. After centrifugation and rinsing, the cells were suspended in 1 ml DMSO. The supernatant was collected by centrifugation and the content of BMY-28864 was measured by UV/visible spectrophotometry at 498.4 nm.

Quantitative Component Analysis of the BMY-28864-sugar-calcium Complex

(1) Molar ratio of BMY-28864 to calcium in complexes containing a variety of specific sugars.

A variety of sugars (500 μl; final concentrations 200 mM), 100 μl of 1.10 mM BMY-28864, 100 μl of 10 mM $CaCl_2$ and 300 μl of distilled water were mixed and incubated at 30°C for 30 minutes. The control contained no calcium chloride. The mixtures were centrifuged at 14,000 rpm for 5 minutes, and the precipitates, if formed, were recovered and washed three times with the corresponding sugar solutions at 200 mM. The washed precipitates were dissolved in 1.0 ml DMSO and divided into halves which were employed for determination of the concentration of BMY-28864 by UV/visible spectrophotometry and of the calcium content by atomic absorption spectrometry, respectively.

(2) Molar ratio of Me-Man to BMY-28864 in the complex yielded in the presence of 1 mM calcium chloride at varied BMY-28864 concentrations.

Me-Man (250 μl; final concentration 50 mM), 75, 100, 150 or 200 μl of 1.10 mM BMY-28864, 10 μl of 100 mM $CaCl_2$ and 665, 640, 590 or 540 μl of distilled water were mixed to make 1.0 ml mixtures; and allowed to react at 30°C for 30 minutes. After centrifugation at 14,000 rpm for 5 minutes, the supernatants (970 μl) were removed from the reaction vessels, and the precipitates were dissolved in 970 μl of distilled water containing no calcium. A half-milliliter aliquots were subjected to quantitative analyses of Me-Man and BMY-28864 by the phenol sulfuric acid method[20] and UV/visible spectrophotometry, respectively.

In the controls, the BMY-28864 solution was replaced by distilled water. After incubation followed by centrifugation, 970 μl of the supernatant was removed; and the precipitate was mixed with 75~200 μl

of the BMY-28864 solution plus 895~770 μl distilled water in a total volume of 970 μl.

The net amount of Me-Man was calculated by subtracting the control value (corresponding to the color intensity of BMY-28864) from the test value (corresponding to the color intensity of Me-Man plus BMY-28864).

(3) Analysis of the three components in one and the same BMY-28864-sugar-calcium complex.

Two-milliliter reaction mixture containing 500 μl of 200 mM Me-Man (final concentration 50 mM), 400 μl of 1.10 mM BMY-28864 (final concentration 200 μg/ml; 220 μM), 20 μl of 100 mM calcium chloride (final concentration 2 mM) and 1,080 μl of distilled water was incubated at 30°C for 30 minutes, and then divided into two 1-ml aliquots.

One aliquot was employed for quantitation of the BMY-28864 and calcium contents. After centrifugation at 14,000 rpm for 5 minutes, the precipitate was collected by decantation, rinsed twice with 1 ml each of 200 mM Me-Man, and then dissolved in 1.0 ml of DMSO. The DMSO solution was divided into two halves which were used for determination of the calcium and BMY-28864 contents by atomic absorption spectrometry and UV/visible spectrophotometry at 498.4 nm, respectively.

The other aliquot was centrifuged at 14,000 rpm for 5 minutes and the supernatant (970 μl) was replaced by 970 μl of distilled water. The aqueous solution was divided into two halves. One half was subjected to the phenol sulfuric acid analysis for quantitation of Me-Man, whereas the other half, after diluted with 0.5 ml of 0.1 N NaOH, was used for spectrophotometric quantitation of BMY-28864.

The no-calcium control contained 20 μl of distilled water instead of the calcium chloride solution, and was treated in the same fashion as above to give the 100% control for adsorbed BMY-28864.

The no-BMY-28864 control was used to compensate the Me-Man content by subtracting the optical density of the disaccharide moiety of BMY-28864, as described above. More particularly, the no-BMY-28864 control contained 400 μl of distilled water instead of the BMY-28864 solution. After incubation, the control mixture was centrifuged and the supernatant (970 μl) was discarded. The remaining content was mixed with 770 μl of distilled water and 200 μl of the BMY-28864 solution, and then subjected to the phenol sulfuric acid analysis, yielding the optical density of Me-Man plus BMY-28864.

Results

Relationship of the Antifungal Activity with the Cell Adsorption of BMY-28864

Macroscopically it is easy to predict which fungi are sensitive to pradimicins, as sensitive strains are stained red with pradimicins on mixing. For further confirmation, the antimicrobial activity and adsorption profiles of BMY-28864 were examined for yeasts, fungi and bacteria. Results in Table 1 show that BMY-28864 has antifungal activities against yeasts and *Trichophyton mentagrophytes*, only in the presence

Table 1. Comparative MIC and cell adsorption data of BMY-28864 to microorganisms in the presence and absence of calcium chloride.

Microorganism	MIC (μg/ml)		Adsorbed BMY-28864[a]	
	$+CaCl_2$	$-CaCl_2$	$+CaCl_2$	$-CaCl_2$
Candida albicans A9540	6.3	>100	41	3
Cryptococcus neoformans IAM 4514	1.6	>100	12	4
Saccharomyces cerevisiae ATCC 9763	3.1	>100	52	6
Trichophyton mentagrophytes #4329	12.5	>100	14	6
Mucor spinosus IFO 5317	>100	>100	7	6
Bacillus subtilis ATCC 6633	>100	NT	0	0
Escherichia coli NIHJ JC-2	>100	NT	0	0
Micrococcus luteus	12.5	NT	3	2
Salmonella typhimurium IID 971	>100	NT	0	0

[a] In μg BMY-28864/mg freeze-dried cells.
NT: Not tested.

of calcium chloride; and that its antifungal activities are positively correlated with the amounts of BMY-28864 adsorbed to freeze-dried cells. Similar results of BMY-28864 adsorption were also obtained with intact microorganisms (data not shown).

Essential Role of Calcium in the Cell Adsorption and Potassium Leakage Induction of BMY-28864

It is important to recall that pradimicin derivatives have empirically been shown to express their antifungal activities solely in the presence of calcium by *in vitro* assays. As the first step for detailed elucidation of the antifungal action of BMY-28864, specificity of calcium ion was comparatively examined with a variety of metal chlorides in the cell adsorption and potassium leakage induction of BMY-28864. Table 2 apparently demonstrates that only calcium chloride among the metal chlorides tested is highly specific in both the yeast cell adsorption and the potassium leakage induction of BMY-28864. Cadmium may also have a similar but weaker effect.

Binding Kinetics of BMY-28864 to *Candida albicans* and *Saccharomyces cerevisiae* Cells

If BMY-28864 were adsorbed to fungi, the progresses of BMY-28864 binding to a variety of yeast cells would be traceable and analysable by binding kinetics.

The kinetic parameters of BMY-28864 adsorption to yeast cells are listed in Table 3, which reveals that *Candida albicans* A9540 and *Saccharomyces cerevisiae* ATCC 9763 have virtually the same kinetic properties; and that the immobilized yeast cells probably behave in the same manner as the intact cells, in binding of BMY-28864 in the presence of calcium chloride. Accordingly, adsorption data of BMY-28864 to dead yeast cells are considered to be reasonably applicable to intact yeast cells.

Adsorption of BMY-28864 to Cell Wall Components and Enzymes in the Presence of Calcium Chloride

It is widely acknowledged that the yeast cell wall is composed of mannan, mannoprotein, glucan, chitin, *etc.*[21]; and that some enzymes such as invertase also contain small amounts of mannan.[21,22] For better characterization of BMY-28864 adsorption to yeast cells, commercially available cell wall

Table 2. Effects of metal chlorides on *Candida albicans* cell adsorption and potassium leakage induction of BMY-28864.

Metal chloride (1 mM)	Adsorbed BMY-28864 (in μg/test)	Potassium leakage (%)	
		+BMY-28864	−BMY-28864
$BaCl_2$	4.5	0	0
$CaCl_2$	36.1	58.5	0.1
$CdCl_2$	6.7	3.5	0
$CoCl_2$	0	0.1	0.1
$FeCl_2$	0	3.4	3.6
$MgCl_2$	7.5	0	0
$MnCl_2$	0	0.1	0
$NiCl_2$	0	0	0
$ZnCl_2$	0	0	0
$AlCl_3$	0	2.1	2.0
$FeCl_3$	0	3.5	4.2

Table 3. Scatchard analysis of BMY-28864 adsorption to *Candida albicans* and *Saccharomyces cerevisiae* cells at 1 mM calcium chloride.

Yeast	*Ka* (μM)[a]	n/cell[b]	r[c]
Candida albicans A9540	12	0.8×10^9	0.998
[immobilized cells]	11	1.0×10^9	0.999
Saccharomyces cerevisiae ATCC 9763	17	1.7×10^9	0.994
[immobilized cells]	16	1.8×10^9	0.986
Saccharomyces cerevisiae X2180-1A	11	0.8×10^9	0.984
X2180-1A5-mnn2	8	1.0×10^9	0.990

[a] Binding constant.
[b] Number of binding sites/cell.
[c] Correlation coefficient.

Table 4. Adsorption of BMY-28864 to cell wall components and enzymes in the presence of 1 mM calcium chloride.

Agent	Concentration	Adsorbed BMY-28864
Chitin	5.0 mg/ml	0 μg/test
Chitobiose	5.0	0
Chitosan	5.0	0
Glucan	5.0	0
Mannan	5.0	132 (100%)
Carboxypeptidase Y	1.25	103 (78%)
Invertase	5.0	132 (100%)
Trypsin	5.0	0
Chymotrypsin	5.0	0
Alkaline phosphatase	5.0	0

components, mannan-containing enzymes and mannan-free enzymes were tested for adsorption of BMY-28864 in the presence of 1 mM calcium chloride (Table 4). Among the cell wall components tested, only mannan completely adsorbs BMY-28864, suggesting the high specificity of mannan in BMY-28864 adsorption. It is interesting to note that BMY-28864 also binds to invertase, and less abundantly, to carboxypeptidase Y, probably through mannan. Taken all together, BMY-28864 seemed to be specifically adsorbed not only to yeast cell wall mannan but also to a variety of mannan-containing materials such as invertase.

Table 5. Antagonistic effects of hexoses and mannan preparations on the antifungal activity of BMY-28864 against *Saccharomyces cerevisiae*.

Sugar	50% antagonistic concentration (μg/ml)
Mannans	
Sigma M-7504	10
S. cerevisiae X2180-1A	15
S. cerevisiae X2180-1A5-mm2 (α-1,6-mannan)	15
Linear α-1,6-manno-oligosaccharides	
$M_{25\sim30}$ (25~30 mannose units)	2,000
$M_{20\sim25}$ (20~25 mannose units)	2,000
$M_{15\sim20}$ (15~20 mannose units)	2,000
$M_{9\sim10}$ (9~10 mannose units)	2,000
M_8 (8 mannose units)	2,500
M_5 (5 mannose units)	2,500
M_4 (4 mannose units)	3,500
M_3 (3 mannose units)	4,000
M_2 (2 mannose units)	3,000
Linear α-1,2-disaccharide	
M_2 (2 mannose units)	>4,000
Branched manno-oligosaccharide	
M_7 (7 mannose units[a])	4,000
Methyl glycosides	
Me-Man	4,000
Methyl α-D-glucopyranoside	>8,000
Monosaccharides	
D-Galactose	>8,000
D-Glucose	>8,000
D-Mannose	8,000

[a] Structure
$^1M_2-^1M_2-^1M_3-^1M$
$\quad|$
$^6M_2-^1M_2-^1M$

Antagonistic Effects of Hexoses and Mannan Preparations on the Antifungal Activity of BMY-28864

As mannan, a polymannosaccharide, abundantly exists on the yeast cell surface, exogenous addition of saccharides in the presence of BMY-28864 and calcium was expected to antagonize the *in vitro* antifungal activity of BMY-28864 by competitive binding.

Specificities of hexoses and mannan preparations in inhibition of BMY-28864 adsorption to yeast cells in the presence of calcium were indirectly compared by antifungal antagonism using *Saccharomyces cerevisiae* instead of *Candida albicans* as assay organism, because of technical difficulties in bioassay by the latter. The effect of a sugar is expressed in 50% antagonistic concentration.

Table 5 demonstrates that the mannan preparations are good antagonists and over 100 times more antagonistic than the linear and branched polymannosaccharides and Me-Man, whereas monosaccharides such as D-glucose are not antagonistic even at 8 mg/ml. As the linear polymannosaccharides tested are derived from *Saccharomyces cerevisiae* X2180-1A5-mnn2, the two- or three-dimensional network or multiple branching structure of mannan seems important to efficiently adsorb BMY-28864 in the presence of calcium.

Protective Effects of Mannan and Me-Man on *Candida albicans* Protoplasts in the Presence of BMY-28864

Antagonistic effect of mannan on the *in vitro* antifungal activity of BMY-28864 against *Saccharomyces cerevisiae* (Table 5) indicates the probable competitive binding of exogenously added mannan and cell wall mannan to BMY-28864. As the cytoplasmic membrane of yeast is also known to contain mannan, the competition of cytoplasmic membrane mannan with exogenously added mannan in binding to BMY-28864 was examined by the time course of change in stability of *Candida albicans* protoplasts. Fig. 2 shows that exogenously added mannan and Me-Man significantly protect the protoplasts from BMY-28864-dependent lysis by competitive binding to BMY-28864.

Interactions of BMY-28864 with Sugars

(1) Specificity of sugars in precipitation of BMY-28864 in the presence of 1 mM calcium chloride.

Yeast mannans commonly contain multiple units of mannose, but greatly differ in components, compositions and linkages.[21,24] Although linear polymannosaccharides are far less effective than mannans in antagonism (Table 5), the mannose unit was assumed to play a central role in BMY-28864 adsorption. Therefore, in the presence of 1 mM calcium chloride, interactions of BMY-28864 with monosaccharides were compared at a sugar concentration of 200 mM in 0.6 M KCl, 50 mM MOPS, pH 7.4, and distilled water.

Table 6 demonstrates that only D-mannose, Me-Man and D-fructose among the tested hexoses produce precipitates with BMY-28864 in the three media. Considered from the high specificity of mannan in BMY-28864 adsorption, it is important to recall that the 3 hexoses listed above share the common configurations at the C-2 and C-4 hydroxyl groups (see Fig. 3), whereas 2-amino-mannose, 2-acetylamino-mannose and mannose 6-phosphate form no precipitates with BMY-28864.

(2) Inhibition by the specific hexoses of BMY-28864 adsorption to immobilized *Saccharomyces cerevisiae* cells at 1 mM calcium chloride

Clear difference of mannose from glucose and galactose in precipitation with BMY-28864 suggests a possibility that the precipitates of BMY-28864 with the seemingly specific hexoses such as mannose and Me-Man might not be simple mixtures but complexes containing BMY-28864 and components at fixed ratios. If this were the case, the adsorption of BMY-28864 to yeast cells in the presence of calcium would be selectively inhibited by the specific hexoses, depending on the degree of binding specificity, as is reported with lectin.[25,26]

Based on this assumption, the comparative inhibitory effects of fructose, mannose and Me-Man on adsorption of BMY-28864 to immobilized *Saccharomyces cerevisiae* cells were examined at 200, 100, 50, 25 and 12.5 mM.

Results in Table 7 apparently show that 50 mM Me-Man, D-mannose and D-fructose significantly inhibit the BMY-28864 adsorption, again support-

Fig. 2. Protective effects of mannan and Me-Man on *Candida albicans* protoplasts in the presence of BMY-28864.

■ Control, ● BMY-28864 (55 μmol/ml) + mannan (0.1 mg/ml), □ BMY-28864 (55 μmol/ml) + 100 mM Me-Man, ○ BMY-28864 (55 μmol/ml).

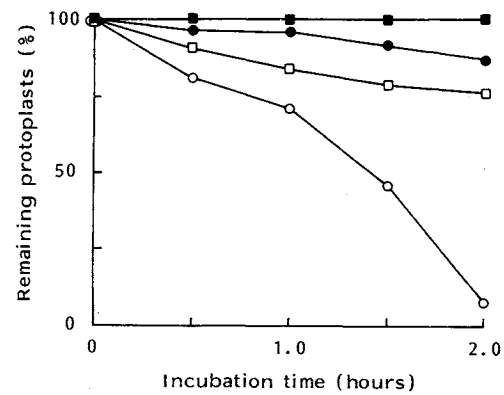
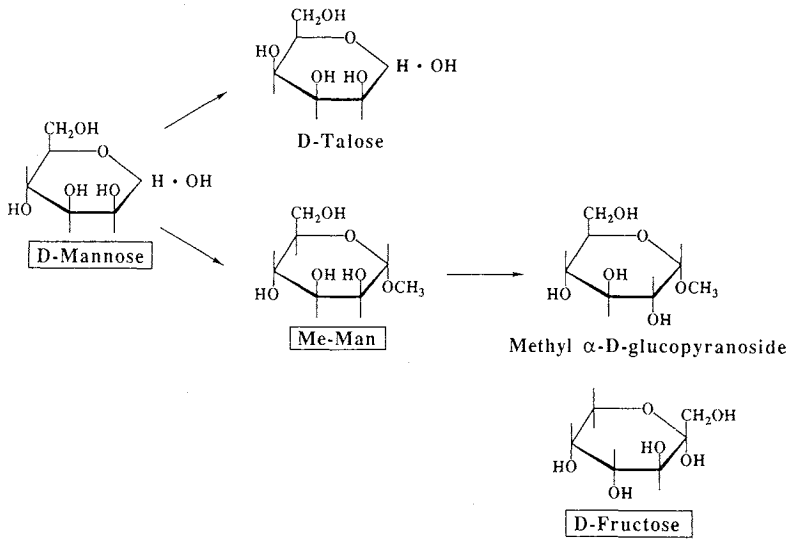


Table 6. Precipitation of BMY-28864 with 200 mM hexoses at 1 mM calcium chloride.

Compound	Precipitated BMY-28864 (μg) in		
	0.6 M KCl	50 mM MOPS	Water
Hexoses			
D-Allose	0	0	0
D-Altrose	0	0	0
D-Galactose	0	0	0
L-Galactose	0	0	0
D-Glucose	0	0	0
D-Gulose	0	0	0
D-Idose	0	0	0
D-Mannose	122 (93%)	131 (100%)	131 (100%)
D-Mannose 6-phosphate	0	0	0
Methyl α-D-glucopyranoside	0	0	0
Me-Man	131 (100%)	131 (100%)	131 (100%)
D-Talose	0	0	0
Ketohexoses			
D-Fructose	126 (96%)	131 (100%)	131 (100%)
L-Sorbose	0	0	0
Amino sugars			
N-Acetyl D-glucosamine	0	0	0
N-Acetyl D-mannosamine	0	0	0
N-Acetyl neuramic acid	0	0	0
D-Glucosamine	0	0	0
D-Mannosamine	0	0	0

Fig. 3. Structural requirements of sugar for binding to BMY-28864.

▭, BMY-28864 binding sugars.



ing the above-described assumption that BMY-28864 might behave like lectin by formation of complexes in the presence of calcium. Among the three hexoses, Me-Man seems to be more effective in precipitation with BMY-28864, presumably because of the methyl substituent at C-1.

Kinetics of Inhibition by Me-Man of BMY-28864 Adsorption to Immobilized *Saccharomyces cerevisiae* Cells in the Presence of 1 mM Calcium Chloride

For further confirmation of the working hypothesis that, on binding to the yeast cell wall, BMY-28864 might form a complex with mannan in the presence of calcium, the inhibitory effect of Me-Man on BMY-28864 adsorption to the immobilized yeast cells was analysed by the Scatchard plot according to the method of STECK and WALLACK.[20] Binding profiles of BMY-28864 to the yeast cells in the presence and absence of Me-Man are presented in Fig. 4.

Ka values in the presence and absence of Me-Man were calculated to be 22 and 18 μM, respectively, leading to an inhibition constant *Ki* of about 100 mM in the competitive inhibition mode.

Additional evidence for the complex formation of BMY-28864 with mannan and Me-Man in the presence of calcium was provided by UV peak shift from 498 nm to 515 nm before and after mixing. This characteristic UV shift was already reported for benanomicins by S. KONDO *et al.*[27] (For details, see a subsequent paper).

These findings together with above-described observations explicitly indicate the probability that BMY-28864 forms a complex with yeast cell wall mannan in the presence of calcium.

Quantitative Component Analysis of the Complex of BMY-28864 with a Specific Sugar in the Presence of Calcium

(1) Molar ratio of BMY-28864 to calcium in complexes containing the specific sugars.

If mixing of BMY-28864 with the specific sugars in the presence of calcium resulted in complex formation, on one hand, the molar ratio of BMY-28864 to calcium in the precipitates should remain constant irrespective of the type of the specific sugar. If the precipitate were a simple mixture and not a complex, on the other hand, the molar ratio of BMY-28864 to calcium would freely change depending on the mixing conditions. These two possibilities were examined using precipitates which were yielded from BMY-28864 with mannan, Me-Man, mannose and fructose in the presence of calcium chloride.

Table 8 clearly reveals that, although the molar content of sugar was not determined, the molar ratio of BMY-28864 to calcium is constant at 2 : 1 within

Table 7. Inhibitory effects of the specific hexoses on BMY-28864 adsorption to immobilized *Saccharomyces cerevisiae* cells.

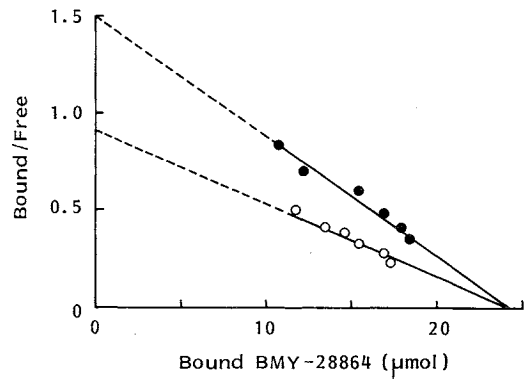
Hexose	Percent inhibition of BMY-28864 adsorption at (mM)				
	200	100	50	25	12.5
D-Fructose	10.7	11.0	6.2	0	0
D-Mannose	15.0	9.7	4.5	0	0
Me-Man	31.0	25.9	21.0	14.3	0

Table 8. Molar ratio of BMY-28864 to calcium in the precipitates with the specific sugars.

Sugar	BMY-28864 (nmol)	Calcium (nmol)	Ratio
Mannan (1 mg/ml)	123	60	2.1
Me-Man (200 mM)	123	65	1.9
D-Mannose (200 mM)	90	41	2.2
D-Fructose (200 mM)	74	30	2.5

Fig. 4. Kinetics of inhibition by Me-Man of BMY-28864 adsorption to immobilized *Saccharomyces cerevisiae* cells in the presence of 1 mM calcium chloride.

● Without Me-Man, ○ with 50 mM Me-Man.



allowable measurement error limits for the four sugars, indicating the complex formation of BMY-28864 with sugar and calcium.

(2) Molar ratio of Me-Man to BMY-28864 in the complex formed in the presence of 1 mM calcium chloride at varied BMY-28864 concentrations.

If the precipitate of BMY-28864 with sugar and calcium chloride were a true complex, changes in the BMY-28864 concentration would also be assumed not to lead to variation in the molar component ratio of BMY-28864 to the specific sugar in the precipitate.

Table 9. Molar ratio of Me-Man to BMY-28864 in the complexes formed with varied concentrations of BMY-28864 at 1 mM calcium chloride.

BMY-28864 concentration (μM)	Ratio of BMY-28864: Me-Man	
	In reaction mixture	In complex
83	1:602	1.0:2.1, 1.0:2.1
111	1:451	1.0:2.0, 1.0:2.2
166	1:301	1.0:2.0
220	1:225	1.0:2.1, 1.0:2.2

Using 50 mM Me-Man as specific sugar, the molar ratio of Me-Man to BMY-28864 was determined in the precipitates which were produced in the presence of 1 mM calcium chloride at varied BMY-28864 concentrations from 83 μM to 220 μM. The results of quantitative analysis are summarized in Table 9, which also clearly indicates the complex formation of BMY-28864 with Me-Man at a molar ratio of 1:2.

(3) Analysis of the three components in one and the same ternary complex of BMY-28864 with Me-Man and calcium.

The preceding findings revealed that the ternary complex of BMY-28864 with sugar and calcium probably had a molar ratio of BMY-28864 - sugar - calcium of 1:2:0.5. As this ratio was indirectly calculated based on the separate analytical results from independent experiments, it was essential to directly determine the molar ratio of the three components in one and the same preparation of the ternary complex.

For determination of the molar ratio of the three components in one and the same complex, 0.22 mM BMY-28864 was mixed with 50 mM Me-Man (molar mixing ratio = 1:227) and 2 mM calcium chloride, and the resulting complex was subjected to the component analysis. The molar component ratio of Me-Man : BMY-28864 : Ca in this complex was found to be 4.3 (301 nmol) : 2.1 (144 nmol) : 1.0 (70 nmol), which allows to conclude that one mole of calcium binds to 2 mol of BMY-28864 and 4 mol of Me-Man to form the ternary complex.

Discussion

As reported in previous papers,[1~6] pradimicin derivatives including BMY-28864 which are selectively antifungal and/or antiviral *in vitro* and *in vivo*, show no binding affinity to cultured animal cells at all, suggesting the potential application of BMY-28864 (and probably other pradimicin derivatives) to therapy of fungal infections especially in AIDS patients. As immuno-deficiency is very significant in AIDS, the antifungal activity of BMY-28864 without affinity to animal cells seems to be of prime value as antifungal agent. In addition, the pradimicin and benanomicin family of antibiotics clearly differ from conventional antifungal agents in calcium dependency and no cross resistance with azole compounds. These features of pradimicin analogs as antifungal agent motivated the authors to study the detailed mode of antifungal action of pradimicins from the biochemical and therapeutic viewpoints.

Recently the definition of lectin, a carbohydrate-binding protein, has been revised jointly by I. R. GOLDSTEIN, R. C. HUGHES, M. MONSIGNY, T. OSAWA and N. SHARON.[28] Interestingly, the results described in this paper clearly show that, although not protein, BMY-28864, which is a semi-synthetic derivative from naturally-occurring pradimicin analogs, is adsorbed to yeast mannan at two sugar-binding sites in the presence of calcium; that the binding of BMY-28864 to yeast mannan is inhibited by specific monosaccharides such as Me-Man, mannose and fructose; and that BMY-28864 binds to sugar-specific

enzymes such as invertase and carboxypeptidase Y, which all perfectly satisfies the essential functional requirements of lectin. Only one, but very critical, contradiction to the above-cited definition of lectin is that the pradimicin is not protein but benzo[*a*]naphthacenequinone.

Referring to the binding properties of BMY-28864 to candida as lectin-mimic agent, it is important to indicate that BMY-28864 recognizes D-mannose moieties in mannan by the two free hydroxyl groups at C-2 and C-4 of mannose. Consequently, taken all together, it will be appropriate hereafter to collectively characterize the pradimicin and benanomicin family of compounds as "carbohydrate-recognizing antibiotics".

In *Candida albicans*, mannan which composes the fibrillar, outer and inner layers of the cell wall, represents about 40% of the total cell wall polysaccharide and plays an important role not only as ligand but also as receptor on the cell surface. As it seems probable that BMY-28864 expresses a potent *in vitro* and *in vivo* antifungal activity on yeasts in lectin-mimic mode of binding, a group of pradimicin derivatives will also be valuable as biochemical tools for study of the adherence and receptor activities of *Candida albicans* and other yeasts.[21]

In a subsequent paper, the antifungal and mannan-binding properties of BMY-28864 and related analogs will be comparatively analyzed in the light of the ternary complex formation in a lectin-mimic manner.

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