STUDIES ON THE MODE OF ANTIFUNGAL ACTION OF PRADIMICIN ANTIBIOTICS

III. SPECTROPHOTOMETRIC SEQUENCE ANALYSIS OF THE TERNARY COMPLEX FORMATION OF BMY-28864 WITH D-MANNOPYRANOSIDE AND CALCIUM

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Sequence of reactions in the process of ternary complex formation of BMY-28864 with D-mannopyranoside and calcium was spectrophotometrically determined under more strict analytical conditions using metal-free preparations of sugars and the pradimicin derivative at a bandpass slit width of 1 nm. In the first phase of ternary complex formation, BMY-28864 stereospecifically recognized and bound to D-mannopyranoside in the absence of calcium, which was revealed by a visible absorption maximum shift of *ca.* 8 nm. Subsequently, the BMY-28864-D-mannopyranoside conjugate reacted with calcium to yield the ternary complex, which was detected by an additional visible absorption maximum shift of *ca.* 8 nm. When the three components were mixed at the same time, both phases simultaneously occurred to produce the ternary complex which was accompanied by a visible absorption maximum shift of 16 nm in total. Based on this two-phased reaction sequence, the mechanism of ternary complex formation of BMY-28864 with D-mannopyranoside and calcium was reexamined in details. Terminal D-mannopyranoside was confirmed to be essential as BMY-28864-specific sugar receptor by *in vitro* analysis and animal cell experiments. While calcium, strontium and cadmium behaved similar in the *in vitro* ternary complex formation, the yeast and animal cell experiments showed that only calcium played a dual role as a base in the ternary complex formation and as an effector in physiological disturbances leading to cell death.

In previous papers[1~6], the antifungal activities of pradimicin derivatives were positively correlated with their lectin-mimic ternary complex formation by validating the essential roles of the C-18 carboxyl group and the C-5 thomosamine moiety of pradimicin derivatives. In consequence, characterization of the sugar-recognizing ability of pradimicin derivatives in the light of lectin biochemistry using highly-purified metal-free sugar and pradimicin preparations was considered to be informative and indispensable, as calcium and metal impurities in reagents probably interact or compete with the calcium component of the ternary complex.

The findings described in the preceding paper[6] that BMS-184497 or BMY-28864 methyl ester still retains as ability to show a visible absorption maximum shift of *ca.* 8 nm in spite of the absence of the free C-18 carboxyl group; and that the breadth of the visible absorption peak shift depends on the status of the C-18 carboxyl group (*ca.* 8 nm for the methyl ester and *ca.* 16 nm for the free acid) allowed the hypothesis that the process of ternary complex formation might be divided into the sugar-recognization phase and the calcium salt formation phase. Therefore, the authors have attempted to elucidate the sequence of events in the visible absorption peak shift which seemed to be parallel with the process of ternary

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complex formation of BMY-28864 with D-mannopyranoside and calcium. In effect, by means of a higher level of spectrophotometric analysis using metal-free reagents and solvents at a bandpass slit width of 1 nm, they successfully separated the sugar recognition phase and the calcium salt formation phase in the ternary complex formation.

The current paper describes the precise spectrophotometric reanalysis of the ternary complex formation of metal-free BMY-28864 with metal-free mannan and calcium; and proposes a two-phased sequential mechanism of ternary complex formation which is demonstrable by the stepup shift of the visible absorption maximum of BMY-28864. Based on this reaction sequence, terminal D-mannopyranoside is shown to be necessary as BMY-28864 receptor. Unreplaceable dual role of calcium is also proved as both a base in the ternary complex formation and a physiological effector for final lethal actions.

Materials and Methods

Purification of Mannan and Sugar Preparations

For complete avoidance of possibly significant contamination with metal impurities from water, distilled water was replaced by pure water for HPLC (Product No. 042-16973, Wako Pure Chemical Industries, Ltd.).

Commercially available mannan (Product No. M7504, Sigma Chemical Co.) and methyl α-D-mannopyranoside (Product No. M6882, Sigma Chemical Co.) preparations were further purified by Chelate Cellulofine column chromatography (Product No. 810170, Seikagaku Kogyo Co., Ltd.) which is widely acknowledged to remove effectively metal impurities. The contents of calcium and other metal elements in these column-chromatographed preparations of mannan and methyl α-D-mannopyranoside were confirmed to be below a claimed detection level of 0.001 μg/mg (0.0001%) of the atomic absorption spectrometer employed, respectively[5].

Other sugars were purified with small columns containing *ca.* 0.5 ml Chelate Cellulofine, but used without confirmation by atomic absorption spectrometry because of material scarcity.

Purification of BMY-28864

BMY-28864 sodium salt in pure water was passed through a Chelate Cellulofine chromatographic column. Fractions containing BMY-28864 were combined and subjected to atomic absorption spectrometry for measurement of the contents of calcium and other metal ions. The freeze-dried metal-free preparation of BMY-28864 employed throughout this study was confirmed to be completely devoid of calcium and other metal ions.

UV-visible Spectrophotometric Analysis

BMY-28864 (1.15 mM; 100 μl), 200 μl of 25 mg/ml metal-free yeast mannan or 1 M sugar and 700 μl of pure water were mixed at pH 7.0 in the presence and absence of 1 mM calcium chloride or a metal salt and incubated at room temperature for a period of time. After centrifugation at 15,000 rpm for 5 minutes, the supernatant was subjected to UV-visible spectrophotometry using a bandpass slit of 1.0 nm in a Beckman DU-70 UV-visible spectrophotometer.

Sugar specificity was tested at 200 mM in the presence of 115 μM BMY-28864 and 1 mM calcium chloride. After observation of the visible absorption peak shift, the reaction mixtures were incubated at 30°C for 60 minutes and centrifuged for 5 minutes at 14,000 rpm for precipitation check.

Detailed comparison of calcium, strontium and cadmium in the sugar-dependent visible absorption peak shift of BMY-28864 was done by using reaction mixtures which consisted of 115 μM BMY-28864, 200 mM sugar and 1 mM calcium, strontium or cadmium chloride at pH 7.0.

Comparison of Anthracycline Compounds with BMY-28864

Doxorubicin was purchased from Sigma Chemical Co. (Product No. D1515), while aklavin, carminomycin I and daunomycin were from the authentic sample stock of the authors' laboratory. Using

the following three types of mixtures, the effect of mannan on the visible absorption peak of a compound was examined: (1) 1 mg/ml anthracycline or BMY-28864 (pH 7.0) 100 μl and water (pH 7.0) 900 μl; (2) 1 mg/ml anthracycline or BMY-28864 (pH 7.0) 100 μl, 10 mg/ml mannan 200 μl and water (pH 7.0) 700 μl; (3) 1 mg/ml anthracycline or BMY-28864 (pH 7.0) 100 μl, 10 mg/ml mannan 200 μl, 100 mM calcium chloride 10 μl and water (pH 7.0) 690 μl. After incubation at 30°C for 10 minutes, the reaction mixtures were centrifuged at 14,000 rpm for 5 minutes. The supernatants were subjected to scanning spectrophotometry in the range of 400 to 600 nm.

Antifungal Assay and the Potassium Leakage Test

Antifungal assay method and the potassium leakage test were described previously[5].

Cultivation of Rat Leukemia Cells in the Presence and Absence of 1-Deoxymannojirimycin[7]

Rat basophilia leukemia-1 cells (RBL-1; ATCC CRL-1378) were cultivated at 37°C in 20 ml of 20% bovine serum-supplemented RPMI medium 1640 (No. 320-187PJ, GIBCO) in a CO_2 incubator. The test concentration of 1-deoxymannojirimycin (product No. D9160, Sigma Chemical Co.) was set a 50 μg/ml (final).

The control (untreated) and the 1-deoxymannojirimycin-treated RBL-1 cells were transferred twice a week in the fresh medium described above, at an initial density of 10^4 cells/ml in the absence and presence of 1-deoxymannojirimycin, respectively.

The RBL-1 cells were collected by centrifugation for 5 minutes at 1,500 rpm and 4°C, washed twice with physiological saline and resuspended in physiological saline at a density of 10^7 cells/ml.

Binding of BMY-28864 to the RBL-1 Cells

One half milliliter of 1.15 mM BMY-28864, 50 μl of 100 mM calcium chloride and 5.0 ml of the 1-deoxymannojirimycin-treated or untreated RBL-1 cell suspension were mixed and incubated at 30°C for 30 minutes. After incubation, the cells were recovered by centrifugation at 4°C and 1,500 rpm for 5 minutes, followed by rinsing in physiological saline.

The cell-bound BMY-28864 was released from the cell surface by suspending the cells in a mixture of 100 μl of DMSO and 100 μl of 0.2 N NaOH. After centrifugation, the content of BMY-28864 in the supernatant was measured by visible spectrophotometry at 498.4 nm.

Thymidine Incorporation Tests Using the RBL-1 Cells

After 4 transfers, the control and the 1-deoxymannojirimycin-treated RBL-1 cells were cultivated for 2 days and collected as described above for thymidine incorporation tests.

Twenty microliter (2×10^5 cells) of the 1-deoxymannojirimycin-treated or untreated RBL-1 cell suspension was distributed into each microplate well and then mixed with 10 μl of 20 mM calcium chloride, 20 μl of 1.15 mM or an appropriately diluted BMY-28864 solution and 150 μl of the RPMI medium described above. The incorporation of radiolabeled thymidine into the cells was started by adding 20 μl of [*methyl*-^{3}H]-thymidine (TRK 758, specific activity 0.88 Ci/mmol, 5 mCi/ml; Amersham Co.) to the cell suspension. Mitomycin C was employed as a positive reference and similarly treated. After incubation at 37°C for the indicated periods of time in a CO_2 incubator, the RBL-1 cells were harvested from each microplate well on a glass fiber beta-plate mat. The radioactivity was measured in a Pharmacia-Wallac 1205 Betaplate liquid scintillation counter system.

Results

UV-visible Spectrophotometric Profiles of Metal-free BMY-28864 in the Presence and Absence of Metal-free D-Mannopyranoside and Calcium

In previous studies[5,6], unnegligible contents of metal impurities in key reagents (for example, the calcium contents in the mannan, methyl α-D-mannopyranoside and BMY-28864 preparations employed in the previous paper[6] were found to be 0.05, 0.03 and 0.02 μg/mg, respectively) suggested possibilities that some of previously described results might be inaccurate; and/or that, if reanalysed with metal-free preparations, different or more precise results might be obtained. Actually this speculation was found to

Fig. 1. Spectrophotometric sequence analysis of the BMY-28864-mannan-Ca^{2+} ternary complex formation.

a: BMY-28864 (control), b: BMY-28864 + calcium, c: BMY-28864 + mannan, d: BMY-28864 + mannan + calcium.

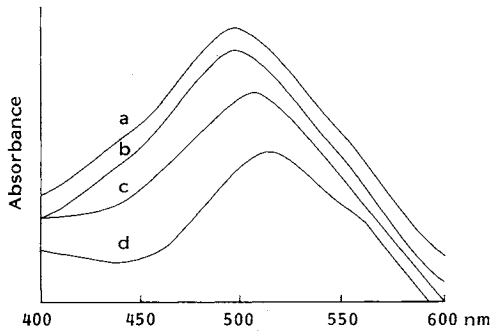
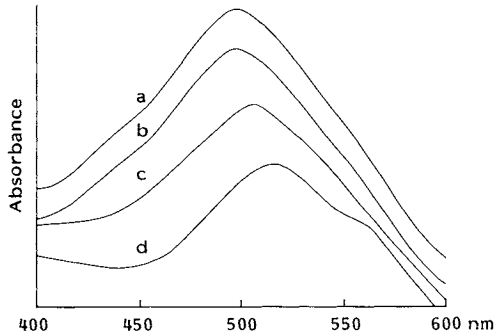


Fig. 2. Spectrophotometric sequence analysis of the BMY-28864-methyl-α-D-mannopyranoside (Me-Man)-Ca^{2+} ternary complex formation.

a: BMY-28864 (control), b: BMY-28864 + calcium, c: BMY-28864 + Me-Man, d: BMY-28864 + Me-Man + calcium.



be true by preliminary experiments using a variety of crude sugar and BMY-28864 preparations. Thus, with special care taken against possible interference by metal impurities, all reagents and solvents involved in the ternary complex formation were upgraded as much as possible or subjected to chelate cellulose column chromatography.

In addition, as the bandpass slit of a UV-visible spectrophotometer is empirically known to produce possibly critical differences in precise spectral analysis depending on the type of spectrum, all spectrophotometric recordings in this paper were carried out at a bandpass slit width of 1.0 nm, whereas the width employed in previous papers[5,6] was 2.0 or 4.0 nm.

Figs. 1 and 2 reproduce the UV-visible spectra of metal-free BMY-28864 recorded anew in the presence of metal-free mannan and methyl α-D-mannopyranoside, respectively[6].

More particularly, taken together with the observation in the preceding paper[6] that mannan shifted the visible absorption maximum of BMS-184497 or BMY-28864 methyl ester by 8 nm, the mannan-related changes in the visible absorption peak of BMY-28864 (Fig. 1) are explicable as follows: The visible absorption peak of BMY-28864 at 499 nm (Fig. 1, a) remains uninfluenced by addition of calcium (Fig. 1, b), while it steps up to 507 nm upon addition of mannan (Fig. 1, c). As reported previously[5], simultaneous addition of mannan and calcium leads to a peak shift of 16 nm (Fig. 1, d). As a result, it is reasonable to think that the peak shift of 16 nm is inducible only when BMY-28864 reacts first with mannan to produce the BMY-28864-mannan conjugate which in turn reacts with calcium, yielding the ternary complex. Validity of this reaction sequence was also confirmed by the experimental finding that mixing of BMY-28864 calcium salt with mannan produced neither the visible absorption peak shift nor precipitates in the form of the ternary complex.

The same conclusion is drawn from Fig. 2, where the effect of methyl α-D-mannopyranoside is shown.

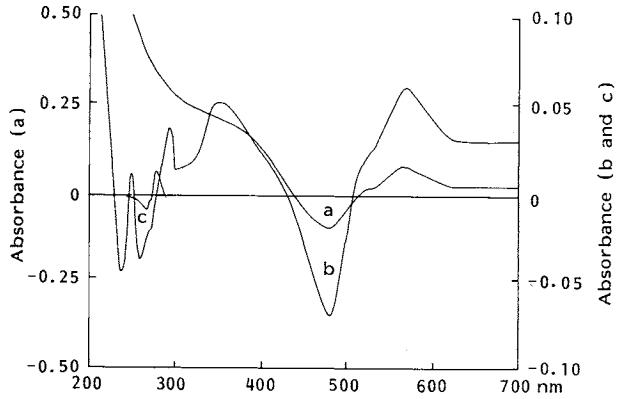
In a separate approach to the sequence analysis, BMY-28864 methyl ester which is lacking in the free C-18 carboxyl group was similarly analysed. As calcium has no chance to bind to the pradimicin molecule on account of the methyl-protected C-18 carboxyl group, addition of mannan always led to the visible absorption peak shift of 8 nm, regardless of the presence of calcium (charts not shown), which also substantiates the above reaction sequence.

Scheme 1. Relationship of the order of mannan and Ca^{2+} addition with the visible absorption peak shifts of BMY-28864 and BMS-184497.

	Start	First step		Second step	
BMY-28864	499 nm	+mannan	507 nm	$+Ca^{2+}$	515 nm
	499 nm	$+Ca^{2+}$	499 nm	+mannan	499 nm
BMS-184497 (BMY-28864 methyl ester)	499 nm	+mannan	507 nm	$+Ca^{2+}$	507 nm
	499 nm	$+Ca^{2+}$	499 nm	+mannan	507 nm

Fig. 3. Difference spectral analysis of the BMY-28864-mannan and the BMY-28864-methyl α-D-mannopyranoside (Me-Man) interaction in the absence of calcium.

a: BMY-28864+mannan, b: BMY-28864+Me-Man, c: concanavalin A+Me-Man.



* Excerpted from reference 13.

For easy comprehension, the reaction sequence of ternary complex formation is illustrated in Scheme 1 using BMY-28864 and BMY-28864 methyl ester. If BMY-28864 first reacts with calcium to give BMY-28864 calcium salt, no further reaction occurs with D-mannopyranoside, perhaps because tightly packed BMY-28864 calcium salt makes it difficult for mannan to access to the sugar-binding site of the BMY-28864 molecule in the salt (lower row). The ternary complex formation starts with the specific-sugar recognition phase where one mole of BMY-28864 catches two moles of D-mannopyranoside (upper row). The first phase is spectrophotometrically demonstrable by the initial absorption peak shift of 8 nm. The calcium salt formation phase occurs in turn where two moles of the BMY-28864-D-mannopyranoside conjugate are bound *via* one mole of calcium, yielding the ternary complex consisting of 2 moles of BMY-28864, 4 moles of D-mannopyranoside and one mole of calcium. The second phase is also spectrophotometrically detectable by the additional peak shift of 8 nm.

Fig. 3 shows the UV-visible spectrophotometric difference spectra of BMY-28864 before and after

Table 1. Spectrophotometric comparison of BMY-28864 with anthracycline compounds in the presence of mannan and Ca^{2+}.

Compound	λ_{max} (nm)			Mannan precipitability (%)
	Control	+Mannan	+Ca^{2+} and mannan	
BMY-28864	498.5	508.8	515.0	100
Doxorubicin	481.5	481.5	481.5	0
Aklavin	435.0	435.0	435.0	0
Carminomycin I	493.5	493.5	493.5	0
Daunomycin	480.0	480.0	480.0	0

addition of mannan and methyl α-D-mannopyranoside.

For convenience of comparison, the difference spectrum of concanavalin A before and after methyl α-D-mannopyranoside addition is copied in Fig. 3 (spectrum c) from reference 13. It is astonishing to note that, in the UV absorption range from 240 to 290, BMY-28864 (Fig. 3, b) and concanavalin A (Fig. 3, c) share similar profiles bearing a dip (*ca.* 260 and 266 nm), a shoulder (*ca.* 280 and 275 nm) and a peak (293 and 282 nm, respectively) in the presence of methyl α-D-mannopyranoside. In the visible absorption range of BMY-28864 above 300 nm which is characteristic of pradimicin derivatives, mannan (Fig. 3, a) and methyl α-D-mannopyranoside (Fig. 3, b) give comparable curves with two peaks (341 and 565 nm), two shoulders (390 and 520 nm) and one dip (475 nm).

As a result, from the UV and visible spectrophotometric characteristics of pradimicin described above, the visible absorption maximum shift was again selected as a general measure for precise recharacterization of interactions among the three components of the ternary complex.

Comparison of BMY-28864 with Anthracyclines in Visible Absorption Maximum Shift Inducibility

Like the pradimicin and benanomicin family of compounds, the anthracycline family of antitumor antibiotics which are composed of the anthracyclinone aglycone and sugars are also colored in red or orange. As only the benzo[*a*]naphthacenequinone ring system and the disaccharide moiety of pradimicin are involved in the sugar recognition and color development, spectrophotometric comparison of pradimicin with anthracycline was assumed to reveal the spectrophotometric uniqueness of pradimicin, or, at least, to serve to contrast the novel characteristics of pradimicin as sugar-recognizing agent.

Table 1 summarizes the results of spectrophotometric comparison of BMY-28864 with doxorubicin, aklavin, carminomycin I and daunomycin in the visible absorption maximum shift together with mannan precipitability data in the presence of mannan and calcium. Although a wide variety of aromatic compounds have not yet been examined, BMY-28864 (and probably, the pradimicin and benanomicin family of compounds, too) seems to be very unique in both the visible absorption maximum shift inducibility and the mannan precipitability, currently at least in comparison with the red-colored anthracycline compounds.

Comparative Recharacterization of Calcium and Other Metal Ions in the Mannan-dependent Visible Absorption Maximum Shift Inducibility

(a) Comparison of Metal Ions

Comparative effects of metal ions on the cell adsorbability and potassium leakage of BMY-28864 were reported in a previous paper[5]. In the context of the two-phased sequential mechanism of visible

Table 2. Comparative effects of metal ions on the visible absorption maximum of BMY-28864 in the absence and presence of mannan.

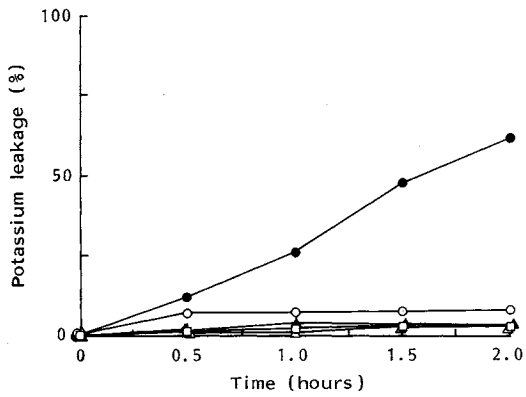
Metal ion	Ionic radius (Å)	Atomic radius (Å)	λ_{max} (nm)		
			Without mannan	With mannan	Difference
Control	—	—	499.8	506.0	6.2
Ca^{2+}	1.05	1.96	498.8	515.3	16.5
Sr^{2+}	1.18	2.13	500.3	516.3	16.5
Cd^{2+}	0.99	1.48	509.3	513.0	6.3
Ba^{2+}	1.38	2.17	499.8	506.8	7.0
Mg^{2+}	0.75	1.60	499.8	503.6	3.6
Zn^{2+}	0.80	1.32	499.8	498.5	−0.7
Co^{2+}	0.78	1.25	503.3	505.5	2.2
Ni^{2+}	0.74	1.32	513.0	513.0	0.0
Cu^{2+}	0.58	1.27	536.3	536.5	0.2
Mn^{2+}	0.83	1.12	509.8	506.5	−3.2
Fe^{2+}	0.80	1.24	503.5	505.3	1.8
Al^{3+}	0.55	1.43	505.3	505.0	−0.3
Fe^{3+}	0.67	1.24	466.3	440.0	−26.3

Table 3. Comparison of calcium, strontium and cadmium in the yeast cell adsorption and potassium leakage induction of BMY-28864.

Metal chloride (1 mM)	Adsorbed BMY-28864 ($\mu g/5 \times 10^7$ cells)	Potassium leakage	
		+BMY-28864 (%)	−BMY-28864 (%)
$CaCl_2$	40.7	62	4
$SrCl_2$	17.6	8	4
$CdCl_2$	9.8	3.5	2.5

Fig. 4. Time courses of BMY-28864-dependent potassium leakage in the presence of calcium and strontium.

● BMY-28864+Ca^{2+}, ○ BMY-28864+Sr^{2+}, ▲ control, △ Sr^{2+} only, □ Ca^{2+} only.



absorption peak shift, the influences of divalent and trivalent metal ions on the visible absorption peak of BMY-28864 were compared in the absence and presence of mannan. The results are presented in Table 2.

It is worth noting that, in the absence of mannan, metal ions such as Ca^{2+}, Sr^{2+}, Ba^{2+}, Mg^{2+} and Zn^{2+} have no direct influence on the visible absorption maximum of BMY-28864 (499 nm), whereas the other ions positively or negatively shift the peak. It seems reasonable to say that metal ions should be doubly checked for both the ternary complex formability and the direct spectrophotometric influence on the benzo[*a*]naphthacenequinone ring. Taken with the effects of metal chlorides on yeast cell adsorbability and potassium leakage inducibility reported in a previous paper[5], Table 2 seems to suggest that, regardless of the direct influences on the visible absorption maximum, strontium, calcium and cadmium, which have ionic radii from 1.18 to 0.99 Å, are necessary for BMY-28864 to form the ternary complex with D-mannopyranoside.

(b) Detailed Comparison of Calcium, Strontium and Cadmium

Using BMY-28864 and *Candida albicans* A9540, the effects of calcium, strontium and cadmium on cell adsorbability and potassium leakage inducibility were compared. Table 3 shows that calcium has the

most potent effect, followed by strontium. Time courses of BMY-28864-dependent potassium leakage are presented in Fig. 4 for calcium and strontium.

In detailed comparison with calcium, strontium shifts the visible absorption maximum of BMY-28864 to the same extent (16 nm) (Table 2), fixes about half the calcium-dependent BMY-28864 adsorption on the yeast cells (Table 3), but induces negligible potassium leakage (Fig. 4), which means that calcium is highly selective and indispensable in a later stage for lethal action on intact candida cells.

Spectrophotometric Recharacterization of Sugar Compounds

(a) Specificity of Sugars in the Calcium-dependent Visible Absorption Maximum Shift of BMY-28864

As previously reported[6,9], simultaneous presence of BMY-28864 (and other pradimicin and benanomicin derivatives) with mannan and calcium causes the visible absorption maximum of BMY-28864 to rise by 16 nm by virtue of the spectrophotometric characteristics of the benzo[*a*]naphthacenequinone ring structure. Sugar specificity in the calcium-dependent visible absorption maximum shift of BMY-28864 was recharacterized with a variety of hexose, pentose and related sugar derivatives (Table 4).

Table 4 apparently discloses the following sugar-recognizing characteristics of BMY-28864:

1. BMY-28864 recognizes and binds to D-mannose, but not L-mannose. More particularly, BMY-28864 has an ability to recognize the absolute configuration of D-mannopyranoside.
2. In addition to D-mannopyranoside, BMY-28864 recognizes D-arabinose, D-lyxose and D-fructose, and produces visible absorption maximum shifts of 13, 21 and 17 nm, respectively. The stereochemical reasons why the three monosaccharides listed above are specific to BMY-28864 are illustrated in Fig. 5, based on the information of lectin biochemistry[10]. In a previous paper[5], the absolute configurations

Table 4. Specificity of sugars in the calcium-dependent visible absorption maximum shift of BMY-28864.

Compound	λ_{max} (nm)	Shift	Precipitability (%)	Compound	λ_{max} (nm)	Shift	Precipitability (%)
Control	500.3			*p*-Nitrophenyl β-D-mannopyranoside	516.0	15.7	100
Pentoses				*p*-Aminophenyl α-D-mannopyranoside	516.0	15.7	100
D-Arabinose	513.5	13.2	100	D-Fructose	517.3	17.0	100
L-Arabinose	500.2			L-Fucose	499.7		
Methyl β-D-arabinopyranoside	516.0	15.7	100	L-Rhamnose	499.6		
D-Ribose	500.2			D-Sorbose	500.0		
D-Lyxose	521.3	21.0	100	Disaccharides			
D-Xylose	499.1			Lactose	500.0		
Hexoses				Maltose	499.8		
D-Allose	500.0			Sucrose	501.0		
D-Altrose	499.8			Turanose	500.2		
D-Galactose	499.8			Amino sugars			
L-Galactose	499.6			2-Acetylamino-D-glucose	499.0		
D-Glucose	500.0			2-Acetylamino-D-mannose	499.5		
Methyl α-D-glucopyranoside	500.0			2-Acetylneuramic acid	500.5		
D-Gulose	499.6			2-Amino-D-glucose	500.0		
D-Idose	500.2			2-Amino-D-mannose	499.8		
D-Talose	500.0			Sugar alcohols			
D-Mannose	517.3	17.0	100	Galactitol	499.0		
L-Mannose	500.0			Mannitol	499.0		
D-Mannose 6-phosphate	499.9			Sorbitol	509.2	8.9	100
Methyl α-D-mannopyranoside	516.2	15.9	100				
Methyl β-D-mannopyranoside	516.0	15.7	100				
p-Nitrophenyl α-D-mannopyranoside	516.0	15.7	100				

of the C-2 and C-4 hydroxyl groups were claimed to determine the sugar specificity to BMY-28864. Fig. 5 additionally reveals importance of the absolute configuration of the C-3 hydroxyl group.

3. For recognition by BMY-28864, the C-2 hydroxyl group of mannose should be free, as 2-amino-D-mannose and 2-acetylamino-D-mannose induce no peak shift and are regarded to be unrecognizable and noninteractive.
4. Phosphorylation at the C-6 hydroxyl group makes D-mannopyranoside unrecognizable by BMY-28864. In lectin, the nonreducing C-6 hydroxyl group of D-mannopyranoside is known to be important for recognition.
5. BMY-28864 accepts both the α and the β anomer of D-mannopyranoside (with or without aliphatic or aromatic substituent at C-1 OH).
6. Fucose and xylose, although structurally related or identical with the sugar components of the disaccharide moiety of BMY-28864, have no influence on BMY-28864.
7. The sugar-binding site of a lectin subunit accommodates one mole of specific sugar, whereas BMY-28864 catches two moles of specific sugar to fill the sugar-binding pocket[5].
8. There is no plausible explanation available about the reason why sorbitol induces visible absorption peak shift and precipitates with BMY-28864, but it is worth pointing out that sorbitol, although linear, has the same configurations as D-mannopyranose at the C-2, C-3 and C-4 hydroxyl groups.

In conclusion, as is known for lectin-specific sugars[11], the pradimicin-specific hexoses and pentoses in Table 4 are explained as is illustrated in Fig. 5. In brief, for stereospecific recognition by BMY-28864, the C-2, C-3 and C-4 hydroxyl groups of mannose should be free and have the absolute configurations required for D-mannopyranoside. Moreover, the substituent at C-5 (CH_2OH for mannopyranoside and H for lyxose; see Fig. 5) is importantly involved in sugar specificity to BMY-28864. Considered from the consistent observation that mannan is highly specific and efficient in recognition of and binding to BMY-28864, it is safe to think for now that only the terminal D-mannopyranoside of mannan can satisfactorily in reality meet such structural requirements and environment conditions for BMY-28864 recognition and binding.

Fig. 5. Structural requirements for BMY-28864-specific monosaccharides.

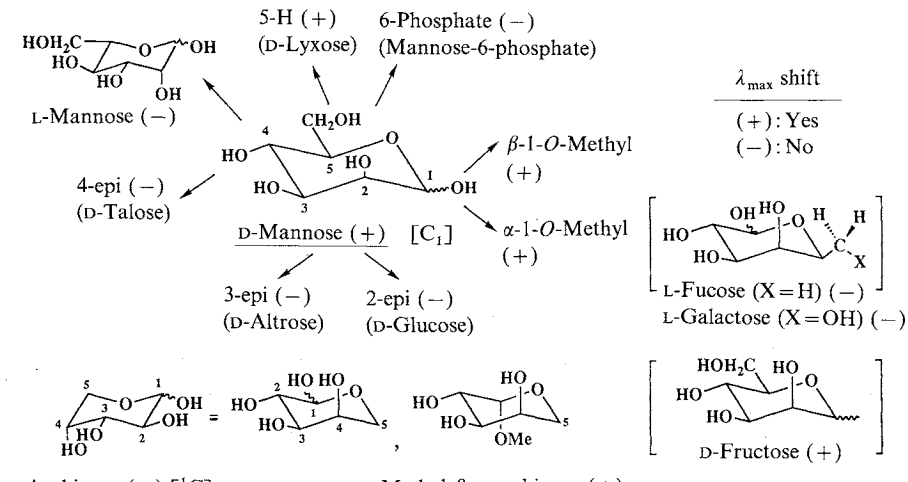


Fig. 6. Effect of the mannan concentration on the visible absorption maximum of BMY-28864 in the absence of calcium.

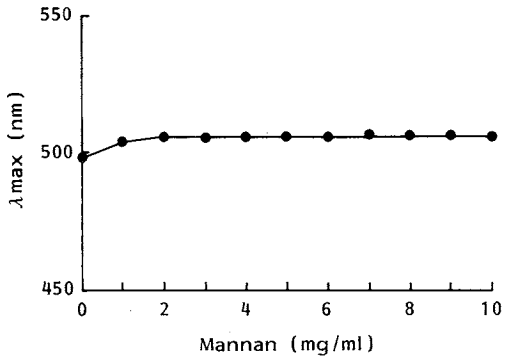


Table 5. Comparison of calcium, strontium and cadmium in the sugar-dependent visible absorption maximum shift of BMY-28864.

Compound	λ_{max} (nm)		
	Ca^{2+}	Sr^{2+}	Cd^{2+}
Control	499.0	499.0	506.8
Pentoses			
D-Arabinose	513.3	513.5	506.3
D-Lyxose	521.5	510.0	508.5
D-Xylose	499.1	499.0	507.6
Methyl β-D-arabinopyranoside	516.0	519.5	516.5
Hexoses			
D-Fructose	517.3	520.0	517.3
D-Glucose	500.0	499.0	506.7
D-Mannose	517.3	520.5	515.8
Methyl α-D-mannopyranoside	516.2	516.8	515.3
Methyl β-D-mannopyranoside	516.0	522.5	516.8

(b) Reliability of the Sugar-dependent Visible Absorption Peak Shift as a General Measure

In the preceding paper[6], influences of incubation conditions such as pH, temperature and calcium concentration on the absorption peak shift were studied with BMY-28864. Using the metal-free mannan preparation, the effect of the mannan concentration was examined on the metal-free BMY-28864-dependent absorption peak shift in the absence of calcium (Fig. 6).

The mannan-dependent absorption peak shift of BMY-28864 (507 nm) in the absence of calcium is highly reliable above 1 mg/ml. This high reliability of the sugar-dependent absorption peak shift in the absence of calcium also substantiates separability of the sugar recognition phase and the calcium salt formation phase. There seems to be dose-dependency between the mannan concentration and the breadth of the peak shift below 1 mg/ml.

(c) Comparison of Strontium and Cadmium with Calcium in the Sugar-dependent Visible Absorption Maximum Shift of BMY-28864

Sugar specificity profiles in Table 5 demonstrate that strontium and calcium recognize the same specific sugars, while cadmium obviously differs from the former two metals in recognition of the free pentoses. This difference of cadmium in the sugar recognition profile seems to be related to the observation that, unlike calcium, cadmium induced no significant BMY-28864-dependent potassium leakage from intact candida cells[5].

Effects of BMY-28864 on Cultured Animal Cells Treated with 1-Deoxymannojirimycin

(a) Identification of Terminal D-Mannopyranoside as the BMY-28864-binding Receptor Site

Mannan has a three-dimensional mannose matrix with rate replacement by other sugars, and accordingly possesses many D-mannopyranoside terminals for binding to BMY-28864. Results of the sugar specificity test described above indicate that, in fact, only the terminal D-mannopyranoside of mannan can satisfy the structural requirements for BMY-28864 binding including the absolute configurations of the free C-2, C-3 and C-4 hydroxyl groups, although human immunodeficiency virus and cytomegalovirus possibly behave like yeasts[12].

As reported elsewhere[2], normal animal cells show no binding to BMY-28864 at all, probably because

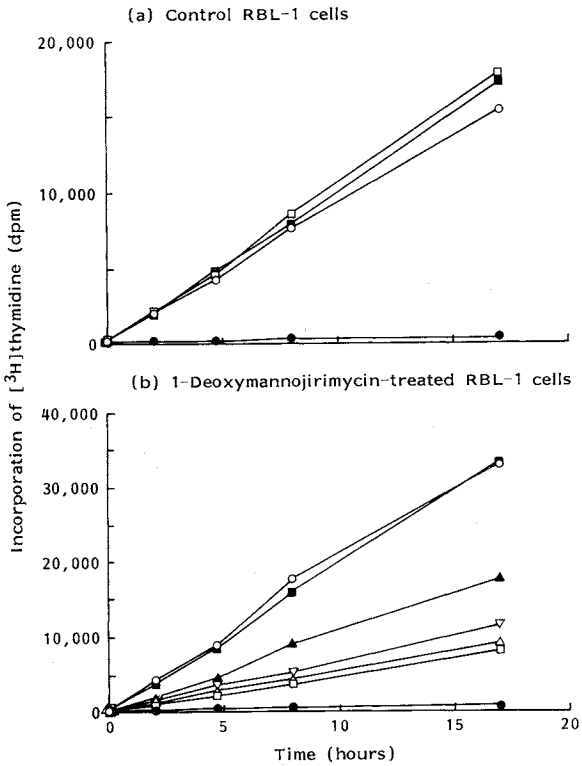
D-mannopyranoside terminals which are necessary for BMY-28864 binding are very scarce on the cell surface. As this working hypothesis seemed experimentally confirmable based on the finding of FUHRMANN *et al.*[7] that 1-deoxymannojirimycin, a mannosidase inhibitor, blocked the conversion of high-mannose to complex oligosaccharides, yielding many D-mannopyranoside terminals, rat basophilia leukemia cells (RBL-1) were cultivated in the presence and absence of 1-deoxymannojirimycin. It is obvious from Table 6 that, as expected, the normal or untreated RBL-1 cells exhibit no BMY-28864 adsorbability, as the cytoplasmic membrane consists of glycoproteins carrying the complex type of *N*-linked glycans with terminal sugars such as *N*-acetylglucosamine, galactose, sialic acid and fucose. The 1-deoxymannojirimycin-treated cells, on the other hand, adsorb a significant amount of BMY-28864 exactly like candida cells, as the membrane glycoproteins belong to the high mannose type with terminal mannose. This sharp contrast in BMY-28864 adsorbability before and after 1-deoxymannojirimycin treatment again proves the necessity of terminal D-mannopyranoside as BMY-28864 receptor.

(b) Inhibition of DNA Synthesis by BMY-28864

The control (untreated) and 1-deoxymannojirimycin-treated RBL-1 cells were compared in thymidine-incorporating ability, using mitomycin C as positive reference. Fig. 7 shows that the thymidine incorporation of the 1-deoxymannojirimycin-treated RBL-1 cells in the presence of calcium is inhibited by BMY-28864 in a dose-dependent manner (Fig. 7b), while the control (untreated) cells are virtually

Fig. 7. Effects of BMY-28864 on the thymidine incorporations by control and 1-deoxymannojirimycin-treated rat basophilia leukemia-1 (RBL-1) cells in the presence of calcium.

○ None, ■ Ca^{2+}, ▲ BMY-28864 (10.6 μg/ml), ▽ BMY-28864 (21.3 μg/ml), △ BMY-28864 (42.5 μg/ml), □ BMY-28864 (85 μg/ml), ● mitomycin C (10 μg/ml).



insensitive to BMY-28864 (Fig. 7a).

Results in Table 6 and Fig. 7 altogether apparently indicate that, once the animal cells which are normally BMY-28864-insensitive because of no binding-affinity are transformed to become BMY-28864-sensitive through newly generated binding-affinity, the biosyntheses of macromolecules such as DNA are severely damaged by BMY-28864, ultimately leading to cell death. Although there remains much to be studied, this important finding on the animal cells, together with previous observations on candida cells, suggests that the real action site of BMY-28864 probably is the cytoplasmic membrane where calcium and BMY-28864 exert lethal effects such as potassium leakage and DNA synthesis inhibition by an unknown mechanism.

Table 6. Adsorption of BMY-28864 on the untreated and 1-deoxymannojirimycin-treated rat basophilia leukemia-1 cells.

No. of transfers	Adsorbed BMY-28864 ($\mu g/5 \times 10^7$ cells)	
	Untreated	Treated
2	0.0	13.6
4	0.0	15.8
6	0.0	20.7

Discussion

As detailed previously[5], the antifungal activity of BMY-28864 begins with the ternary complex formation. In reality, BMY-28864 first reacts with mannan to produce the mannan-BMY-28864 conjugate which is subsequently treated with calcium to yield the ternary complex on the candida cell wall. The reversed sequence of reactions results in no ternary complex formation, which means no antifungal effect is produced. It is important to stress here that both the first sugar recognition and binding phase and the second calcium salt formation phase are necessary for antifungal action of BMY-28864, and any one of the two phases is insufficient, because coexistence of pradimicin and calcium at significant concentrations on the cytoplasmic membrane is probably essential so that the physiological functions of candida cells may be markedly disturbed, leading to the cell death. Validity of this working hypothesis on the mode of action of BMY-28864 was also supported by the 1-deoxymannojirimycin-treated RBL-1 cells in which newly-generated D-mannopyranoside terminals were positively correlated with the significant adsorption of BMY-28864 on the cytoplasmic membrane accompanied by physiological disturbances.

Scientific utility of the pradimicin and benanomicin family of compounds as biochemical tools in lectin studies is readily understandable from the fact that BMY-28864 functionally matches well with the recent version of definitions of lectins except for the chemical nature as protein[8]. Based on the spectrophotometric reanalyses in this paper, it is timely to summarize the common and different properties of BMY-28864 and lectins for future studies.

1. According to the MAKELA's classification of lectins[10], BMY-28864 belongs to group III; mannose- and glucose-specific type, as it recognizes D-mannopyranoside as specific sugar. Thus concanavalin A and *Pisum sativum*, *Lens culinalis* and *Vicia faba* lectins among a variety of lectins, for example, will be suitable plant lectin candidates to compare with BMY-28864 about recognition of specific sugars, although marked differences in molecular size and chemical nature might possibly make it meaningless to comparatively characterize them.
2. As most pradimicin derivatives have molecular weights below 1,000, their 3-dimensional specifications and structural requirements of the D-mannopyranoside-specific recognition pocket are assumed to be relatively facile to analyze, if they were obtained in crystal form. To date, crystallization has been attempted in vain.
3. It is hardly comprehensible why one mole of BMY-28864 accepts two moles of D-mannopyranoside, whereas one mole of a lectin subunit binds to one mole of specific sugar, even if the molecule size differs by a factor of 10 or higher. On account of the higher binding valence and lipophilicity, BMY-28864 precipitates with methyl α-D-mannopyranoside and calcium.
4. As described in the text, BMY-28864 to express the antifungal activity, calcium is necessary not only as a component of the ternary complex but also as an effector to trigger lethal actions. Concanavalin

A contains calcium and manganese, and the order of addition of the two metals to concanavalin A is important for full activity[11]. It is possible that concanavalin A needs manganese and calcium not only for lectin construction but also for physiological activity.

5. For lectin to efficiently bind to polysaccharide to yield precipitates, the type of the terminal sugar as well as the type and composition of the subsequent sugar chain is important. To date, BMY-28864 is known to recognize D-mannopyranoside; and to bind far less efficiently to free D-mannopyranoside and linear polymannopyranosides than to mannan. There is no information available about the reason why mannan is more efficient in binding to BMY-28864 than linear polymannopyranosides; and which type and composition of sugar chains are highly specific to BMY-28864. One plausible speculation is that the type, density and arrangement of the terminal D-mannopyranoside of mannan are all best suited for the pradimicin compound to form the ternary complex.
6. Mannose-specific plant lectins have recently been reported to be potent and selective inhibitors of human immunodeficiency virus and cytomegalovirus replication[12]. The pradimicin and benanomicin family of compounds which were reported to be antiviral[2,9] have potent inhibitory activity against human immunodeficiency virus. Although highly speculative, the D-mannopyranoside-specific binding property of pradimicin and benanomicin derivatives might play a decisive role in expression of the anti-HIV activity, too.
7. UV absorption difference spectra of concanavalin A and BMY-28864 seem to be worth precise analysis for recognition of and binding to specific sugar, as some common tendencies are observed in the profiles.

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