STUDIES ON THE MODE OF ANTIFUNGAL ACTION OF PRADIMICIN ANTIBIOTICS

II. D-MANNOPYRANOSIDE-BINDING SITE AND CALCIUM-BINDING SITE

TOMOKAZU UEKI, KEI-ICHI NUMATA, YOSUKE SAWADA, MAKI NISHIO, HIROAKI OHKUMA, SOICHIRO TODA, HAJIME KAMACHI, YASUO FUKAGAWA and TOSHIKAZU OKI

Bristol-Myers Squibb Research Institute, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan

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Based on the structure-activity relationship data of BMY-28864 and related pradimicin derivatives, the calcium salt-forming ability and the D-mannopyranoside-specific visible absorption maximum shift of BMY-28864 were analysed in the ternary complex formation of BMY-28864 with D-mannopyranoside and calcium. The free C-18 carboxyl group of BMY-28864 was proved to be the sole site for binding to calcium, while no hydroxyl groups of the aglycone were involved in calcium salt formation. The stereospecific D-mannopyranoside-recognizing ability of BMY-28864 was completely abolished by removal of the C-5 disaccharide moiety, and, more particularly, of the C-5 thomosamine moiety. Close relationship of these findings with the antifungal action was also supported by the *in vitro* antifungal assay and the potassium leakage induction test.

In previous papers[1~4], the *in vitro* antifungal activities of pradimicin and benanomicin derivatives on yeasts were shown to be specifically expressed only in the presence of calcium. Using BMY-28864, a water-soluble pradimicin derivative, specific binding of the pradimicin to yeast cells was proved to depend on the ternary complex formation of BMY-28864 with mannan and calcium at a molar ratio of 2:4:1[5]. This highly stereospecific binding of BMY-28864 to the mannose unit (more generally, the specific sugar-recognizing ability of the pradimicin and benanomicin family of antibiotics) is biochemically worth studying, as it is currently unexplicable by the widely accepted concepts of receptor-ligand binding in the light of lectin and carbohydrate sciences. Lectins have been considered to recognize specific sugars based on the intrinsic properties of their peptide components, whereas the pradimicin and benanomicin family of compounds are not peptides. Under these circumstances, it is crucially important and essential to more precisely elucidate the mechanism of ternary complex formation of pradimicins with specific sugars and calcium in critical comparison with lectins. This type of knowledge is not only biochemically useful for receptology, but also clinically important from the viewpoint of selective toxicity of final pradimicin drugs in hosts, as sugars are essential cellular components of host animals to be treated with pradimicin, and assumed to exist ubiquitously at significant concentrations in a variety of forms throughout therapy.

In this paper, the structure-activity relationship of BMY-28864 and related pradimicin derivatives is analyzed for identification of the moieties of BMY-28864 responsible for binding to D-mannopyranoside and calcium and for induction of the visible absorption maximum shift. In brief, only the free C-18 carboxyl group of BMY-28864 serves to bind to calcium as salt, while the C-5 disaccharide moiety is essential for specific recognition of and binding to D-mannopyranoside.

Correspondence should be addressed to JUN OKUMURA, Bristol-Myers Squibb Research Institute, 2-9-3 Shimo-meguro, Meguro-ku, Tokyo 153, Japan.

Materials and Methods

Fermentation and Isolation of BMY-28567 and Pradimicin Analogs

BMY-28567 (pradimicin A) and other pradimicin analogs as starting materials for chemical synthesis of pradimicin derivatives were produced by fermentation and isolated as reported in a previous paper[6].

Influences of Incubation Conditions on the Visible Absorption Peak of BMY-28864

Incubation pH: Reaction mixtures which contained 100 μM BMY-28864 with or without 1 mM calcium chloride at the indicated pH's were incubated at 30°C for 30 minutes. After incubation, the reaction mixtures were centrifuged at 14,000 rpm for 5 minutes. The supernatants were employed for determination of the visible absorption maximum of BMY-28864 by scanning visible spectrophotometry (400~600 nm) with a Beckman DU-70 UV-visible spectrophotometer.

Incubation Temperature: BMY-28864 (1.15 mM, 100 μl), 200 μl of 25 mg/ml yeast mannan (Product No. M7504, Sigma Chemical Co.), 690 μl of distilled water (pH 7.0) and 10 μl of 100 mM $CaCl_2$ were mixed and incubated for 30 minutes at the indicated temperatures for 30 minutes. Control tests were carried out without yeast mannan in the presence of 1 mM calcium chloride. After incubation, the reaction mixtures were centrifuged at 14,000 rpm for 5 minutes. The supernatants were subjected to scanning visible spectrophotometry in a wavelength range of 400 to 600 nm.

Calcium Concentration: Reaction mixtures contained 100 μM BMY-28864, 5 mg/ml yeast mannan and calcium chloride in a concentration range of 0.1 to 10 mM. Control tests contained no yeast mannan. After incubation at 30°C for 30 minutes, the reaction mixtures were centrifuged at 14,000 rpm for 5 minutes. The supernatants were employed for determination of the visible absorption maximum of BMY-28864 by scanning spectrophotometry from 400 to 600 nm.

Analytical Methods

UV-visible Spectrophotometric Analysis: BMY-28864 (1.15 mM, pH 7.0) was dissolved in distilled water or 50 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), pH 7.4. For interaction with mannan (Product No. M6882, Sigma Chemical Co.), 100 μl of 1.15 mM BMY-28864 was mixed with 200 μl of 10 mg/ml yeast mannan and 700 μl of distilled water or 50 mM MOPS, pH 7.4, in the presence and absence of 1 mM calcium chloride. After incubation at 30°C for 2 hours, the mixture was centrifuged for 5 minutes at 15,000 rpm and the supernatant was subjected to scanning UV-visible spectrophotometry in a wavelength range from 200 to 700 nm.

For interaction with a variety of simple sugars, the final sugar concentrations were all set at 200 mM, and the mixtures were centrifuged without incubation to give the supernatants which were analyzed by scanning UV-visible spectrophotometry.

Calcium Salt Formation: A pradimicin derivative (1.15 mM, 100 μl) was mixed with 100 μl of 10 mM calcium chloride and 800 μl of distilled water at pH 7.0, and incubated at 30°C for 18 hours. If precipitation was observed, the reaction mixture was centrifuged for 5 minutes at 15,000 rpm. The precipitates were washed twice with distilled water 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 pradimicin contents by atomic absorption spectrometry and visible spectrophotometry at 498.4 nm, respectively[5]. The molar ratio of Ca^{2+}: Pradimicin was calculated from the contents of Ca^{2+} and pradimicin in the DMSO solution.

In Vitro Antifungal Assay: MIC on *Candida albicans* A9540 was determined by serial dilution in YNBG-PB liquid medium (Difco), pH 7.0, in the presence of 200 μM calcium chloride[5].

Potassium Leakage Induction Test: *Candida albicans* A9540 was cultivated and harvested as described in a previous paper[5]. One milliliter of the yeast cell suspension (5×10^7 cells) was mixed with 100 μl of a pradimicin solution, 10 μl of 100 mM calcium chloride and 890 μl of physiological saline. After incubation at 30°C for 2 hours, the cells and the supernatant were separated by centrifugation at 15,000 rpm and 4°C for 10 minutes. The control test was run without pradimicin derivative.

The supernatant solution was directly subjected to measurement of the potassium content by atomic absorption spectrometry. The control cells were boiled for 10 minutes at 100°C for release of the total amount of potassium into the supernatant which was subjected to atomic absorption spectrometry. In the control test, a reference potassium content of 23.2 ppm/5×10^7 cells was obtained and employed at 100% for calculation of the percent potassium leakage.

Chemical Synthesis of Pradimicin Derivatives

BMY-28864: This semi-synthetic pradimicin derivative as hydrochloride salt was prepared by the method of OKI *et al.*[7].

BMS-184497: Thionyl chloride (25 μl, 0.35 mmol) was added dropwise to a stirred solution of 100 mg BMY-28864 (0.117 mmol) in 50 ml of dry methanol. After stirring for one day at room temperature, 25 μl of thionyl chloride was added and the mixture was further stirred for one day. The solvent was removed by evaporation under reduced pressure and the residue was taken into 30 ml of ethyl ether. The insoluble matters were collected by filtration to give 102 mg (96% yield) BMS-184497 (BMY-28864 methyl ester) as hydrochloride. MP 190°C (dec.); IR ν (KBr) cm^{-1} 1725, 1610, 1290, 1050; ^1H NMR (400 MHz, DMSO-d_6) 1.38 (3H, d, J=6 Hz, 5-CH_3), 2.30 (3H, s, 3-CH_3), 2.99 (6H, s, 4'-$N(CH_3)_2$), 3.05~3.10 (3H, m, 4'-H, 2''-H and 5''-H), 3.67 (3H, s, $COOCH_3$), 3.73 (2H, dd, J=5 and 10 Hz, 17-CH_2), 3.76 (1H, dd, J=6 and 12 Hz, 5''-H), 3.91 (3H, s, OCH_3), 4.48~4.60 (4H, m, 5-H, 6-H, 1''-H and 17-H), 4.82 (1H, d, J=8 Hz, 1'-H), 6.95 (1H, d, J=3 Hz, 10-H), 7.30 (1H, s, 4-H), 7.31 (1H, d, J=3 Hz, 12-H), 8.07 (1H, s, J=7 Hz, 7-H), 8.14 (1H, d, J=7 Hz, CONH), 12.88 (1H, s, OH), 13.80 (1H, br, OH); FAB-MS m/z 885 $(M+H)^+$.

BMY-28754: Thionyl chloride (3 ml) was dropwise added to a stirred solution of 290 mg pradimicin A in 50 ml of ethanol, and was stirred for one hour at 0°C and then for a further two hours at room temperature. After concentration to dryness *in vacuo*, the residue was subjected to reversed-phase silica gel column chromatography (ODS-A60, Yamamura Chemical Lab., 2.0 × 32 cm). Products were eluted by increasing stepwise the concentration of acetonitrile in 0.15% KH_2PO_4, pH 3.5, starting with the ratio of 2:8 and ending with the ratio of 1:1. Eluate fractions containing BMY-28754 were combined and concentrated under reduced pressure for removal of the organic solvent. The aqueous concentrate was applied on a Diaion HP-20 column for desalting. After rinsing with distilled water, the product was eluted with 80% aqueous acetone (pH 3.0 with HCl). Concentration of the red eluate to dryness yielded 263 mg BMY-28754 hydrochloride. MP 218~221°C (dec.).

BMY-28946 (Desxylosyl BMY-28864) and BMY-28962 (BMY-28864 Aglycone): A solution of 50 mg BMY-28864 in a mixture of 5 ml of dioxane and 1 ml of 1 N HCl was heated for 8 hours on a steam bath and then neutralized with 1 N NaOH. After the dioxane was removed by evaporation *in vacuo*, the remaining aqueous solution was charged on a Diaion HP-20 chromatographic column (1.8 × 25 cm). The column was rinsed with water and eluted with 80% aqueous acetone, pH 3.0. Evaporation of the solvent *in vacuo* gave a deep red solid which was subjected to successive purification by reversed-phase silica gel column chromatography (ODS-A60, Yamamura Chemical Lab., 2.1 × 25 cm) with 35% CH_3CN in 0.15% phosphate buffer, pH 3.5, and Diaion HP-20 column chromatography (1.8 × 25 cm) with 80% aqueous acetone to yield 7.8 mg (17%) desxylosyl BMY-28864 (BMY-28946) and 11.5 mg (33%) of the aglycone (BMY-28962).

BMY-28946: MP >180°C (dec.); IR ν (KBr) cm^{-1} 3400, 1730, 1610, 1380, 1260, 1070; UV (0.01 N NaOH) λ_{max} nm (ε) 211 (38,100), 318 (14,200), 496 (12,500); ^1H NMR (DMSO-d_6+D_2O) 1.23 (3H, d, J=7 Hz, 6'-CH_3), 2.29 (3H, s, 3-CH_3), 2.75 (6H, s, 4'-$N(CH_3)_2$), 3.16 (1H, m, 4'-H), 3.30 (1H, m, 2'-H), 3.74 (4H, m, 17-CH_2, 3'-H and 5'-H), 3.91 (3H, s, 11-OCH_3), 4.38~4.45 (3H, m, 5-H, 6-H and 17-H), 4.60 (1H, d, J=8 Hz, 1'-H), 6.73 (1H, d, J=3 Hz, 10-H), 6.89 (1H, s, 4-H), 7.12 (1H, d, J=3 Hz, 12-H), 7.77 (1H, s, 7-H). SI-MS m/z 740 $(M+2H)^+$.

BMY-28962: MP >200°C (dec.); IR ν (KBr) cm^{-1} 3240, 1720, 1605, 1340, 1305, 1165; UV (0.01 N NaOH) λ_{max} nm (ε) 212 (34,500), 319 (15,200), 498 (14,000); ^1H NMR (DMSO-d_6+D_2O) 2.34 (3H, s, 3-CH_3), 3.73 (2H, m, 17-CH_2), 3.91 (3H, s, 11-OCH_3), 4.22 (1H, d, J=11.1 Hz, 5-H), 4.27 (1H, d, J=11.1 Hz, 6-H), 4.45 (1H, t, J=4.9 Hz, 17-H), 6.92 (1H, d, J=2 Hz, 10-H), 7.06 (1H, s, 4-H), 7.28 (1H, d, J=2 Hz, 12-H), 8.08 (1H, s, 7-H); SI-MS m/z 567 $(M+2H)^+$.

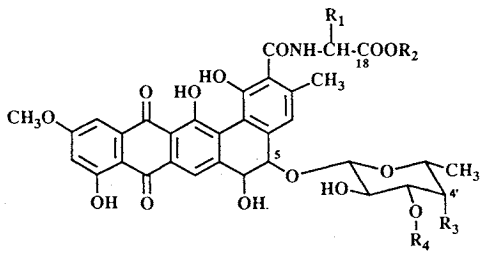
BMY-28634 (pradimicin AG-11 or B), BMY-28749 (pradimicin AG-1) and BMY-28750 (pradimicin AG-2) were prepared as described in a previous paper[8].

Results

Calcium-binding Site of BMY-28864

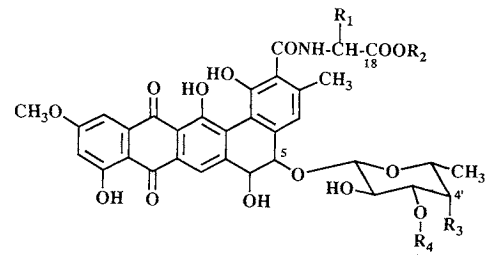
Previous results of quantitative analysis revealed that the calcium-BMY-28864-methyl α-D-

Fig. 1. Structures of BMY-28864 and related pradimicin derivatives.



Drivative	R_1	R_2	R_3	R_4
BMY-28864	CH_2OH	H	$N(CH_3)_2$	β-D-Xylose
BMS-184497	CH_2OH	CH_3	$N(CH_3)_2$	β-D-Xylose
BMY-28567	CH_3	H	$NHCH_3$	β-D-Xylose
BMY-28754	CH_3	C_2H_5	$NHCH_3$	β-D-Xylose
BMY-28946	CH_2OH	H	$N(CH_3)_2$	H
BMY-28634	CH_3	H	$NHCH_3$	H
BMY-28749	CH_3	CH_3	$NHCH_3$	H
BMY-28962	CH_2OH	H	(No sugar moiety at all)	
BMY-28750	CH_3	H	(No sugar moiety at all)	

Table 1. Analysis of the calcium-binding site.



Derivative	R_2	Ca^{2+} salt formability	Ca^{2+}/ Pradimicin
BMY-28864	H	Yes	0.50
BMS-184497	CH_3	No	0.0
BMY-28567	H	Yes	0.44
BMY-28754	C_2H_5	No	0.0
BMY-28946	H	Yes	0.48
BMY-28634	H	Yes	0.45
BMY-28749	CH_3	No	0.0
BMY-28962	H	Yes	0.57
BMY-28750	H	Yes	0.48

mannopyranoside ternary complex possesses a molar component ratio of 1 : 2 : 4[5]. For further elucidation of the mechanism of ternary complex formation, it was prerequisite to identify which moieties of the BMY-28864 molecule are involved in binding to the other two components. Without clear identification of the responsible moieties, the studies on the mode of antifungal action of pradimicin derivatives and the comparative characterization of their specific-sugar-recognizing ability would be less conclusive and meaningful particularly for future biochemical and clinical use of this interesting family of natural antibiotics and synthetic derivatives.

Using BMY-28864 and structurally-related pradimicin derivatives (see Fig. 1), the calcium-binding site of BMY-28864 was analysed first. As the terminal C-18 carboxyl group and the 4 hydroxyl groups of the aglycone of BMY-28864 were taken into account as possible calcium-binding sites, the calcium salt-forming abilities of the pradimicin derivatives were examined in the absence of mannan as the sugar component.

It is obvious from Table 1 that only the free C-18 carboxyl group of BMY-28864 is responsible for binding to calcium, as the pradimicin alkyl esters (BMS-184497, BMY-28754 and BMY-28749) form no calcium salts, whereas the other pradimicin derivatives, which all possess the free C-18 carboxyl group, yield the calcium salts at a molar Ca^{2+}/pradimicin ratio of 1 : 2. This molar ratio is identical with that observed in the ternary complex of BMY-28864 with methyl α-D-mannopyranoside and calcium[5], showing that the carboxyl group of the pradimicin derivatives stoichiometrically forms the salt with calcium. Consequently, at least as far as BMY-28864 is concerned, it is unambiguously ruled out that any one of the hydroxyl groups of the aglycone is responsible for binding to calcium. It also seems unlikely that pradimicin and benanomicin derivatives other than BMY-28864 have different calcium-binding sites other than the free C-18 carboxyl group.

D-Mannopyranoside-recognizing Site of BMY-28864

(1) UV-visible Absorption Profiles of BMY-28864 before and after Mannan-dependent Ternary Complex Formation in the Presence of Calcium.

As described in a previous paper[5], only D-mannopyranoside and D-fructose precipitated with BMY-28864 and calcium as a result of the ternary complex formation, whereas other hexoses such as D-galactose and D-glucose, and hexosamines such as 2-amino-D-mannose yielded no precipitation. It is interesting to note that the presence and configurations of the C-2 and C-4 hydroxyl groups of hexose determine precipitability of the pradimicin derivative in the form of ternary complex. As previously reported[5,9], coexistence of BMY-28864 or a pradimicin derivative with mannan and calcium induces the upward shift of the visible absorption maximum around 500 nm which is related to the characteristic red color of the benzo[*a*]naphthacenequinone ring structure.

Fig. 2 shows the UV-visible absorption spectra of BMY-28864 with calcium in the presence and absence of mannan. Spectrophotometric effect of mannan is apparent from shift of the visible absorption maximum of BMY-28864 from 499 nm to 516 nm.

Detailed effects of mannan addition on the UV-visible absorption profile of BMY-28864 are summarized as follows:

(a) Visible absorption peak of BMY-28864 shifts from 499 to 516 nm;

(b) a shoulder is generated around 560 nm;

(c) visible absorption intensity increases in a range of 300 to 440 nm;

(d) visible absorption intensity decreases in a range of 440 to 510 nm;

(e) UV absorption intensity decreases below 300 nm.

Methyl α-D-mannopyranoside produced a spectrum nearly identical to that of mannan (spectrum not shown), but differed in markedly low absorption below 300 nm.

Among these characteristics, the visible absorption maximum shift around 500 nm was selected as a general index to characterize the sugar-recognizing property of pradimicin derivatives in this paper.

(2) Reliability of the Visible Absorption Maximum Shift of BMY-28864.

Reliability of the visible absorption maximum shift of BMY-28864 as a dependable measure for

Fig. 2. Significance of mannan in the spectrophotometric profile change of BMY-28864.

a: BMY-28864+Ca^{2+} (control), b: BMY-28864+Ca^{2+}+mannan.

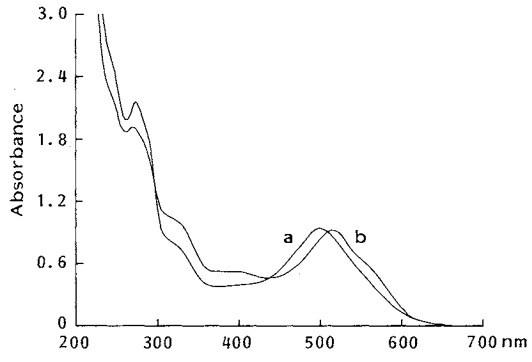


Fig. 3. Effect of the incubation pH on the visible absorption maximum of BMY-28864 in the presence and absence of calcium.

△ BMY-28864+Ca^{2+}, ○ BMY-28864 only.

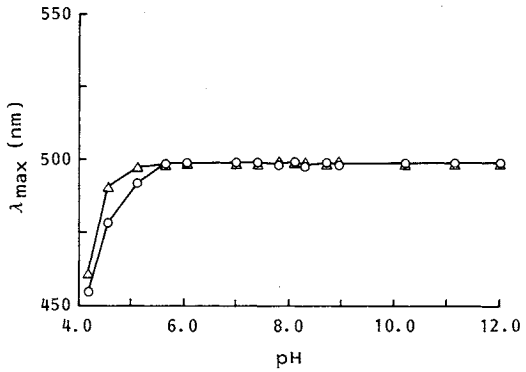


Fig. 4. Effect of the incubation temperature on the visible absorption maximum of BMY-28864 in the presence and absence of mannan.

● BMY-28864 + Ca^{2+} + mannan, ○ BMY-28864 + Ca^{2+}.

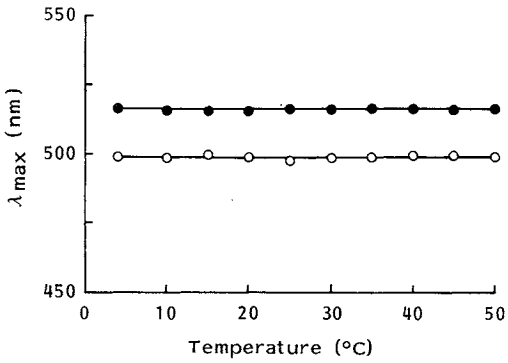
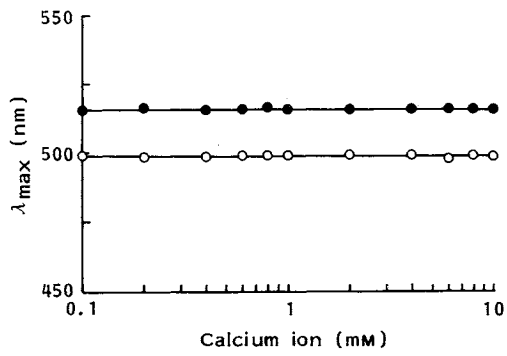


Fig. 5. Effect of the calcium concentration on the visible absorption maximum of BMY-28864 in the presence and absence of mannan.

● BMY-28864 + Ca^{2+} + mannan, ○ BMY-28864 + Ca^{2+}.



analysis of the specific-sugar-recognizing ability was examined under varied incubation conditions such as pH, temperature and calcium concentration.

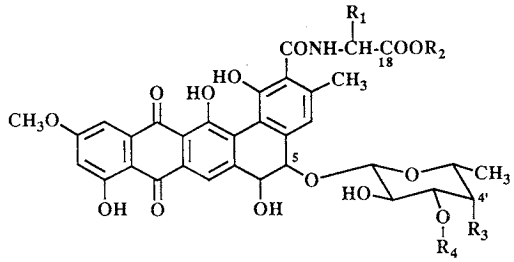
Figs. 3, 4 and 5 show that the visible absorption maximum shift of BMY-28864 is a qualitatively stable and reliable measure in wide ranges of incubation conditions such as pH (5.5~12), temperature (4~50°C) and calcium (>0.1 mM) concentration. The effect of the mannan concentration will be presented in the subsequent paper[10].

(3) Spectrophotometric Comparison of the Pradimicin Derivatives in the Mannan-dependent Visible Absorption Maximum Shift in the Presence of Calcium.

As the water-solubility greatly differs among the pradimicin derivatives employed, the sugar-dependent pradimicin precipitability is an unreliable measure for comparative analysis of the sugar-recognizing and/or sugar-binding abilities of pradimicin and benanomicin derivatives. Spectrophotometric maximum shift data, in contrast, were found to be dependable with good reproducibility, as they are not limited by water-solubility difference among the pradimicin derivatives. Results in Table 2 apparently demonstrate that the disaccharide moiety at C-5 is essential for the visible absorption maximum shift (and probably for precipitation resulting from ternary complex formation) (compare BMY-28962 and BMY-28750 with the other derivatives); and, more particularly, that the thomosamine or 4-*N*,*N*-dimethylamino-D-fucose moiety at C-5 plays a decisive role in interaction with mannan (compare BMY-28946, BMY-28634 and BMY-28749 with BMY-28864, BMY-28567 and BMY-28754, respectively). As benanomicin compounds in which the thomosamine moiety of the pradimicin analogs is replaced by D-fucose were also reported to show a visible absorption maximum shift[9], the type of the C-4′ substitute (the unsubstituted or substituted amino group versus the hydroxyl group) seems least important in the sugar-recognizing ability of the pradimicin derivatives.

It is worth mentioning that BMS-184497 (BMY-28864 methyl ester), BMY-28749 and BMY-28754 give about half (8~11 nm) the visible absorption maximum shift breadth (16 nm) as observed with BMY-28864. This clear difference in the breadth of visible absorption maximum shift served as an impetus to the subsequent study which will prove the important role of calcium in the visible absorption peak shift inducibility[10].

Table 2. Spectrophotometric analysis of the mannan-binding site.



Derivative	R_2	R_3	R_4	λ_{max} (nm) in the presence of Ca^{2+}	
				Without mannan	With mannan
BMY-28864	H	$N(CH_3)_2$	β-D-Xylose	499.3	515.6
BMS-184497	CH_3	$N(CH_3)_2$	β-D-Xylose	499.0	507.5
BMY-28567	H	$NHCH_3$	β-D-Xylose	496.0	515.4
BMY-28754	C_2H_5	$NHCH_3$	β-D-Xylose	509.4	520.5
BMY-28946	H	$N(CH_3)_2$	H	498.5	514.0
BMY-28634	H	$NHCH_3$	H	501.7	515.7
BMY-28749	CH_3	$NHCH_3$	H	499.5	507.0
BMY-28962	H	(No sugar moiety at all)		494.5	494.5
BMY-28750	H	(No sugar moiety at all)		495.7	495.7

The sugar-recognizing and/or the sugar-binding abilities of BMY-28864 are currently summarized as follows:

(a) The C-5 disaccharide (3-*O*-β-D-xylosyl-4-*N*,*N*-dimethylamino-D-fucose or xylosylthomosamine) moiety of BMY-28864 is essential for the visible absorption maximum shift, precipitability as a result of the ternary complex formation, potassium leakage inducibility and antifungal activity expression.

(b) In the two sugar constituents of the C-5 disaccharide moiety, the thomosamine is indispensable, while the D-xylose seems to be unimportantly involved in sugar recognition.

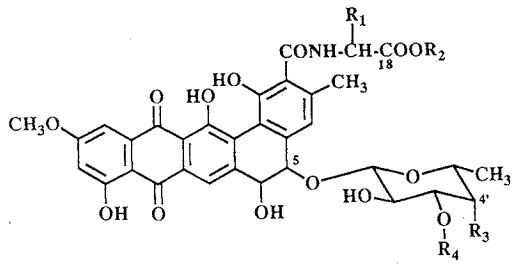
(c) As benanomicin derivatives were reported to generate the visible absorption maximum shift[9], the 4′-amino group (substituted or unsubstituted) of the thomosamine moiety is considered to play an insignificant role in sugar recognition. In other words, as far as the axial configuration is retained at C-4′, a hydroxyl, amino or substituted amino group can similarly play the essential function for recognition of specific sugars.

Consistent Relationship of the Antifungal Activity and Potassium Leakage Induction Data with the Structural Requirement Data for Calcium Salt Formation and Sugar Recognition

In previous papers[2,3,5], the close relationship among pradimicin derivatives of antifungal activity and potassium leakage data with cell adsorption data was repeatedly pointed out. For example, the time course of BMY-28864-dependent potassium leakage from *Candida albicans* A9540 in Fig. 6 shows the dose dependency of potassium leakage on the BMY-28864 concentration in the presence of calcium.

Table 3 collectively compares the antifungal activity, calcium salt formability, absorption peak shift inducibility and potassium leakage inducibility of BMY-28864 and the structurally related derivatives.

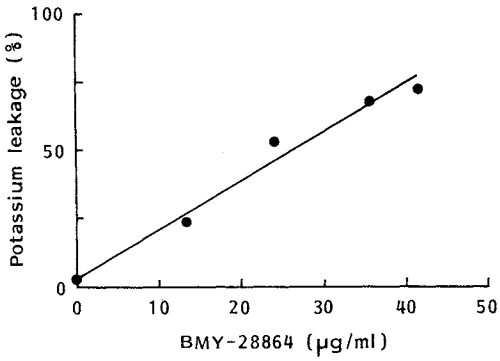
Table 3. Structure-antifungal activity relationship of pradimicin derivatives.



Derivative	R_2	R_3	R_4	MIC[a] (μg/ml)	Ca^{2+} salt formability	λ_{max} (nm) shift (with mannan and Ca^{2+})	K^{2+} leakage (%)
BMY-28864	H	$N(CH_3)_2$	β-D-Xylose	6.3	Yes	Yes (499.3→515.6)	58.5
BMS-184497	CH_3	$N(CH_3)_2$	β-D-Xylose	>100	No	Yes (499.0→507.5)	2.5
BMY-28567	H	$NHCH_3$	β-D-Xylose	3.1	Yes	Yes (496.0→515.4)	69.7
BMY-28754	C_2H_5	$NHCH_3$	β-D-Xylose	>100	No	Yes (509.4→520.4)	1.6
BMY-28946	H	$N(CH_3)_2$	H	6.3	Yes	Yes (498.5→514.0)	54.3
BMY-28634	H	$NHCH_3$	H	3.1	Yes	Yes (501.7→515.7)	29.4
BMY-28749	CH_3	$NHCH_3$	H	>100	No	Yes (499.5→507.0)	3.0
BMY-28962	H	(No sugar moiety at all)		>100	Yes	No (494.5→494.5)	0.9
BMY-28750	H	(No sugar moiety at all)		>100	Yes	No (495.7→495.7)	0.0

[a] Activity against *Candida albicans* A9540.

Fig. 6. Dependency of the potassium leakage on the BMY-28864 concentration.



In summary, the following conclusions are drawn from Table 3:

(a) Pradimicin derivatives which induce no visible absorption maximum shift in the presence of mannan (BMY-28962 and BMY-28750), even though the calcium salt-forming ability is retained, possess neither potassium leakage inducibility nor antifungal activity, probably because no binding to mannan results in nearly nil adsorptive condensation of the antibiotics on the yeast cell wall, which triggers no antifungal action on candida.

(b) Pradimicin derivatives which have no free C-18 carboxyl group (or no site for binding to calcium) (BMS-184497, BMY-28754 and BMY-28749), even though they can recognize and bind to D-mannopyranoside, are also devoid of potassium leakage inducibility and antifungal activity, presumably because, without calcium salt formability, the pradimicin derivatives cannot stay fixed on the yeast cell wall; or high concentrations of pradimicin and calcium are not generated locally on the yeast cytoplasmic membrane for expression of antifungal activity.

(c) Pradimicin derivatives which have both of the sugar-recognizing and binding ability and the calcium salt-forming ability (BMY-28864, BMY-28567, BMY-28946 and BMY-28634) express antifungal activity through some lethal actions such as potassium leakage induction as a result of ternary complex formation.

Discussion

As described in a previous paper[1], most pradimicin fermentation products are so water-insoluble that chemical modifications were inevitably attempted with success to yield virtually water-soluble pradimicin derivatives such as BMY-28864. The lipophilicity of pradimicin is largely ascribed to the pradimicin aglycone, whereas the disaccharide and amino acid moieties are intrinsically hydrophilic. Accordingly the overall lipophilicity or hydrophilicity of a pradimicin derivative results from the delicate total balance in solubility among the aglycone, the disaccharide moiety and the amino acid substituent. In practice, the pradimicin derivatives of this study greatly differ in water-solubility, which made it unacceptable to employ precipitability of a pradimicin derivative as a comparative measure for ternary complex formation. Under such circumstances, the UV-visible spectrophotometric characteristics of BMY-28864 were examined in search for a general measure to evaluate the ternary complex-forming ability. As detailed in the text, although the generation of a shoulder and the intensity change in spectrophotometric absorption are also observed before and after mannan addition, the visible absorption peak shift of *ca.* 16 nm was considered to be an appropriate replacement measure for precipitability to demonstrate comparatively the ternary complex-forming ability.

For detailed study on the mode of antifungal action of pradimicin derivatives and for further chemical derivation work, it was very interesting and urgent to identify clearly the calcium- and sugar-binding sites of BMY-28864 and other pradimicin derivatives, as the ternary complex formation is now assumed to be of absolute necessity for antifungal activity. From chemical viewpoints, some of the hydroxyl groups of the aglycone seemed to be acidic in nature and so to have a potential calcium salt-forming ability. Contrary to the authors' speculation, at least as far as the BMY-28864-related compounds are concerned, only the C-18 carboxyl group is responsible for calcium salt formation, whereas the hydroxyl groups of the aglycone probably have no effect as the alkyl esters produced no calcium salt.

As reported in a previous paper[5], it is believed that BMY-28864 and pradimicin and benanomicin derivatives, although not proteinaceous, behave like lectin, which means their sugar-binding pocket meets spacial or environmental requirements necessary for specific-sugar recognition and binding. To date, all pradimicin derivatives seem to be specific for mannose, but it cannot be explicitly ruled out that, as the antifungal activity has very often been focused on candida whose mannan is best suited for binding to pradimicin compounds, other types of pradimicin derivatives that recognize and bind to sugars other than mannose might have been left unnoticed as antifungally inactive derivatives. For now it seems reasonable to think that BMY-28864 and the antifungally active derivatives of this paper share the mannose-specific sugar binding characteristics. More particularly, the pradimicin aglycone and the thomosamine moiety of BMY-28864 serve to construct a specific mannose-binding pocket which stereospecifically recognizes and accommodates two moles of D-mannopyranoside. Lectins, on the other hand, are known to vary widely in specific-sugar recognition. Among well-characterized lectins, however, mannose-binding lectins such as concanavalin A and *Pisum sativum* lectin are assumed to be worth comparing with pradimicin derivatives, because the special requirements for the D-mannopyranoside-recognizing site would be at least partially equivalent. It is regrettable to say that no crystallographic data have yet been obtained for BMY-28864 and other pradimicin derivatives.

Structure-antifungal activity relationship study in this paper clearly demonstrates that, although there remains much to be studied, antifungally active pradimicin derivatives should have both the xylosyl-thomosamine moiety (at least the thomosamine moiety) at C-5 for recognition of and binding to mannan; and the free carboxyl group at C-18 for calcium salt formation. Once the spacial requirements for ternary complex formation of BMY-28864 are elucidated, more detailed and fruitful discussions will become possible about the substituents of the pradimicin aglycone for design of more clinically useful pradimicin derivatives.

In the meantime, BMY-28864 methyl ester or BMS-184497 gives a narrower breadth of absorption peak shift (*ca.* 8 nm) than BMY-28864 in the presence of mannan and calcium (Table 3). As the C-18 carboxyl group is protected by methyl, BMY-28864 methyl ester has no chance to form calcium salt, but its aglycone and disaccharide moiety are exactly the same as BMY-28864. Thus it is theoretically assumable that BMY-28864 methyl ester still retains the mannan-recognizing ability regardless of no calcium salt-forming ability.

This hypothesis is the starting point of the subsequent paper which will describe the reaction sequence analysis of ternary complex formation under more critical analytical conditions.

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