# CISPENTACIN, A NEW ANTIFUNGAL ANTIBIOTIC

## II. *IN VITRO* AND *IN VIVO* ANTIFUNGAL ACTIVITIES

TOSHIKAZU OKI, MINORU HIRANO, KOZO TOMATSU†, KEI-ICHI NUMATA and HIDEO KAMEI

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

(Received for publication June 3, 1989)

Cispentacin ((−)-(1*R*,2*S*)-2-aminocyclopentane-1-carboxylic acid) is a new antifungal antibiotic possessing potent anti-*Candida* activity. The 50% inhibitory concentration ($IC_{50}$) and $IC_{100}$ values of cispentacin against clinical isolates of *Candida albicans* were in the ranges 6.3~12.5 and 6.3~50 μg/ml, respectively, by turbidimetric measurement in yeast nitrogen base glucose medium. No significant activity was seen against any yeasts and molds when tested by the agar dilution method using three different agar media; KNOPP's agar, yeast extract-glucose-peptone agar and Sabouraud dextrose agar. This antibiotic demonstrated good therapeutic efficacy against a systemic *Candida* infection in mice by both parenteral and po administrations. The 50% protection dose ($PD_{50}$) values after single iv and po administrations were 10 and 30 mg/kg, respectively. It was also effective in a systemic infection with *Cryptococcus neoformans* and in both lung and vaginal infections with *C. albicans* in mice. Cispentacin did not induce acute lethal toxicity at 1,000 mg/kg by iv injection and 1,500 mg/kg by ip and po administrations in mice.

In the course of antifungal screening, cispentacin, (−)-(1*R*,2*S*)-2-aminocyclopentane-1-carboxylic acid was isolated from the fermentation broth of *Bacillus cereus* strain No. L450-B2. Cispentacin is a water-soluble and amphoteric compound, and possesses excellent *in vivo* antifungal activity against experimental infection with *Candida albicans* despite its apparently low *in vitro* activity. In the present study, cispentacin was investigated for its *in vitro* activity against both bacteria and fungi, and for *in vivo* activity against various experimental fungal infections with *C. albicans* and other fungi in mice.

## Materials and Methods

### Antifungal Agents

Cispentacin was prepared at Bristol-Myers Research Institute, Ltd., Tokyo[1]. Amphotericin B (Fungizone, sodium deoxycholate complex) and 5-fluorocytosine were purchased from Squibb Japan Inc. and Sigma Chemical Company, respectively. The above three compounds were used by dissolving in saline for both *in vitro* and *in vivo* experiments except for a turbidimetric method where they were dissolved in 0.1 M phosphate buffer (pH 7.0). Ketoconazole was obtained from Janssen Pharmaceuticals and used as a suspension in saline containing one drop Tween 80.

### *In Vitro* Antifungal Activity

The MIC's of the antifungal agents were determined by an agar dilution method on KNOPP's agar (KA), yeast extract-glucose-peptone agar (YGPA) and Sabouraud dextrose agar (SDA) media. A 3-μl of fungal suspension containing $10^6$ cells/ml was applied to the surface of the antibiotic-containing agar plates with a multiinoculator. After incubation at 28°C for 44 hours, the lowest concentration of antibiotic

† Deceased.

causing virtually complete inhibition of fungal growth (MIC) was determined. Susceptibility of *Candida* species was determined by a turbidimetric method using micro-liquid culture as follows: *Candida* species were shake-cultured in YGP medium (glucose 1.5%, peptone 1.0%, yeast extract 0.4%, $K_2HPO_4$ 0.05% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 7.0) at 28°C for 18 hours. The culture (0.1 ml) was inoculated into a fresh YGP medium (5 ml) and incubated at 28°C for 4 hours. The fungal suspension containing $1 \times 10^7$ cells/ml was diluted 1,000 times with a fresh YNG medium (yeast nitrogen base 0.67%, glucose 1.0% and amikacin 50 U/ml, pH 7.0). A volume of 50 $\mu$l of the test compound solution in 0.1 M phosphate buffer (pH 7.0) and 200 $\mu$l of the fungal suspension were mixed in wells of a 96-well microtiter plate and incubated without agitation at 37°C for 40 hours. The cell growth was measured by an autoreader at 620 nm. The turbidity of the culture without the test compound was used as a control (0%) and fresh YNG medium as 100% inhibition control. Fifty % inhibitory concentration ($IC_{50}$) and $IC_{100}$ values were estimated from the values of 8 concentrations according to the method described by GALGIANI and STEVENS[2].

*In Vitro* Antibacterial Activity

*In vitro* antibacterial activity of cispentacin was tested comparatively with kanamycin by an agar dilution method using nutrient agar (NA, meat extract 0.5%, peptone 0.5%, NaCl 0.25% and agar 1.5%, pH 7.0) and Davis synthetic agar (DA, $K_2HPO_4$ 0.7%, $KH_2PO_4$ 0.3%, sodium citrate 0.05%, $(NH_4)_2SO_4$ 0.1%, $MgSO_4$ 0.01%, glucose 0.2% and agar 1.5%, pH 7.0) media. A 3-$\mu$l of bacterial suspension containing $10^4$ cells/ml was applied to the surface of antibiotic-containing agar plate and MIC was determined after incubation at 37°C for 40 hours.

*In Vivo* Antifungal Activity

Systemic Fungal Infections: The *in vivo* therapeutic efficacy of cispentacin was examined comparatively with amphotericin B, 5-fluorocytosine and ketoconazole against systemic infections with *Candida albicans* A9540 ($10^6$ cells/mouse), 5-fluorocytosine-resistant *C. albicans* YA22851 ($5.5 \times 10^5$ cells/mouse), *Cryptococcus neoformans* IAM 4514 ($10^6$ cells/mouse) and *Aspergillus fumigatus* IAM 2034 ($10^7$ cells/mouse) in normal mice. *C. albicans* and *C. neoformans* were cultured at 28°C for 18 and 48 hours, respectively, in YGP medium and suspended in saline. *A. fumigatus* was cultured at 28°C for 7 days on YGP agar slant and the spores were suspended in saline. Male ICR mice weighing 20 to 24 g were infected intravenously with approx 10 times of the median lethal doses of each fungus as shown above. Groups of 5 mice at each dose level were given with the test compounds by various dosing routes and schedules. The 50% protection dose ($PD_{50}$) was calculated by the method of LITCHFIELD and WILCOXON[3] from the survival rate recorded 20 days after the fungal challenge. Untreated animals died 7 to 15 days post infection.

Systemic *Candida tropicalis* Infection in Immunocompromised Mice: Groups of 5 male ICR mice weighing 20 to 24 g were treated intraperitoneally with 200 mg/kg of cyclophosphamide 4 days before intravenous *C. tropicalis* CS-07 infection at $2.3 \times 10^6$ cells/mouse (10 times of $LD_{50}$). Test compounds were administered orally to the mice once immediately after the fungal challenge.

Lung *C. albicans* Infection: Groups of 5 male ICR mice weighing 20 to 25 g were treated intraperitoneally with 200 mg/kg of cyclophosphamide 4 days before the lung infection with *C. albicans* A9540. The mice were anesthetized with ether and were infected with a 70-$\mu$l of *C. albicans* cell suspension containing $10^6$ cells through an intranasal route on day 0. Test compounds were administered intravenously to the mice immediately after the inoculation followed by once a day dosing on days 2 and 4. The $PD_{50}$ was calculated from the survival rate recorded 20 days after the fungal challenge.

Vaginal *C. albicans* Infection: Groups of 5 female ICR mice weighing 20 to 24 g were treated subcutaneously with 0.5 mg/kg of estradiol benzoate 3 days before and 2 days after the vaginal infection with *C. albicans* A9540. A 10-$\mu$l of cell suspension of *C. albicans* containing $10^8$ cells/ml was inoculated intravaginally on day 0. Test compounds were administered orally to the mice once a day on days 0 to 4. On the day 7, vaginal exudate was sampled with a thin glass rod and spread on an YGP agar plate containing 100 $\mu$g/ml of chloramphenicol. The plate was incubated at 28°C for 2 days. Viable cells count was made and graded by the following scores:

| No. of cells/plate | Score |
|---|---|
| >459 | 4 |
| 146~458 | 3 |
| 47~145 | 2 |
| 15~ 46 | 1 |
| 0~ 14 | 0 |

Acute Toxicity in Mice

Cispentacin was dissolved in saline and administered once to groups of 5 male ICR mice at the dosed volume of 0.1 ml per 10 g body weight by the iv, ip and po routes. The animals were observed daily for 10 days for physical and behavioral signs of toxicity.

## Results

*In Vitro* Antifungal and Antibacterial Activities

MICs for cispentacin and amphotericin B against 14 different fungi and yeasts are shown in Table 1. Only partial inhibition of the cell growth of *C. albicans* and *Trichophyton mentagrophytes* was observed at 50 and 200 μg/ml of cispentacin on YGPA medium. No growth inhibition was seen against the other fungi on this agar medium. However, by a turbidimetric method, cispentacin exhibited *in vitro* anti-*Candida* activity with the $IC_{50}$ values of 6.3~12.5 μg/ml against various strains of *C. albicans*, 100 μg/ml against *C. tropicalis* and 3.1~6.3 μg/ml against *Candida krusei* as shown in Table 2. The $IC_{100}$ values were 6.3~50 μg/ml against *C. albicans*, >100 μg/ml against *C. tropicalis* and 6.3~12.5 μg/ml against *C. krusei*. Cispentacin showed weak activity only against *Bacillus subtilis* PCI 219 at 25 μg/ml on Davis synthetic agar medium.

*In Vivo* Antifungal Activity against Systemic Fungal Infections in Mice

The therapeutic efficacy of cispentacin against systemic fungal infections in mice was comparatively

Table 1. Antifungal spectrum of cispentacin and amphotericin B by an agar dilution method.

| Test organism | MIC (μg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | Cispentacin | | | Amphotericin B | | |
| | KA | YGPA | SDA | KA | YGPA | SDA |
| *Candida albicans* IAM 4888 | >100 | >200 (50) | >400 | 12.5 | 1.6 (0.8) | 1.6 |
| *C. albicans* A9540 | >100 | >200 (50) | >400 | 25 | 3.1 (1.6) | 1.6 |
| *Cryptococcus neoformans* D 49 | >100 | >200 | >400 | 1.6 | 1.6 (0.8) | <0.2 |
| *C. neoformans* IAM 4514 | >100 | >200 | >400 | 1.6 | 1.6 (0.8) | <0.2 |
| *Aspergillus fumigatus* IAM 2530 | >100 | >200 | >400 | 50 | 3.1 (1.6) | 1.6 |
| *A. fumigatus* IAM 2034 | >100 | >200 | >400 | 50 | 3.1 (1.6) | 1.6 |
| *A. flavus* NRRL 484 | >100 | >200 | >400 | 100 | 3.1 (1.6) | 3.1 |
| *Fusarium moniliforme* A2284 | >100 | >200 | >400 | 50 | 25 (6.3) | 12.5 |
| *Piricularia oryzae* D91 | ND | >200 | >400 | ND | 1.6 (0.8) | 3.1 |
| *Trichophyton mentagrophytes* D155 | ND | >200 (50) | >400 | ND | 3.1 (1.6) | 6.3 |
| *T. mentagrophytes* No. 4329 | ND | >200 | >400 | ND | 3.1 (1.6) | 3.1 |
| *Sporothrix schenckii* IFO 8158 | >100 | >200 | >400 | >100 | 25 (3.1) | 50 (3.1) |
| *Petriellidium boydii* IFO 8078 | >100 | >200 | >400 | >100 | 100 (3.1) | >100 |
| *Mucor spinosus* IFO 5317 | >100 | >200 | >400 | >100 | 3.1 (1.6) | 0.8 |

( ): Concentration showing partial inhibition.
ND: Not determined.
KA: pH 4.9, YGPA: pH 6.6, SDA: pH 7.0.

Table 2. *In vitro* anti-*Candida* activity of cispentacin, amphotericin B and 5-fluorocytosine by a turbidimetric method.

| | Inhibition concentration (μg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | Cispentacin | | Amphotericin B | | 5-Fluorocytosine | |
| | $IC_{50}$ | $IC_{100}$ | $IC_{50}$ | $IC_{100}$ | $IC_{50}$ | $IC_{100}$ |
| *Candida albicans* IAM 4888 | 6.3 | 12.5 | 0.8 | 0.8 | 0.1 | 0.4 |
| *C. albicans* A9540 | 12.5 | 25 | 0.8 | 1.6 | 0.2 | 1.6 |
| *C. albicans* YA15049 | 6.3 | 12.5 | 0.8 | 0.8 | 0.2 | 0.8 |
| *C. albicans* YA15051 | 6.3 | 12.5 | 0.8 | 0.8 | 0.2 | 0.8 |
| *C. albicans* YA22802 | 6.3 | 12.5 | 0.8 | 0.8 | 0.2 | 0.4 |
| *C. albicans* YA22804 | 6.3 | 6.3 | 0.8 | 0.8 | 0.1 | 0.2 |
| *C. albicans* YA22851 | 6.3 | 6.3 | 0.8 | 0.8 | 0.8 | >100 |
| *C. albicans* YA22853 | 6.3 | 12.5 | 0.8 | 0.8 | 0.8 | >100 |
| *C, albicans* YA22578 | 12.5 | 50 | 0.8 | 0.8 | 0.8 | >100 |
| *C. albicans*YA25810 | 12.5 | 50 | 0.8 | 0.8 | 0.1 | 0.4 |
| *C. tropicalis* CS-07 | 100 | >100 | 0.8 | 0.8 | 0.1 | 0.2 |
| *C. krusei* IAM 4489 | 6.3 | 12.5 | 0.8 | 0.8 | 0.1 | 0.2 |
| *C. krusei* YA15052 | 3.1 | 6.3 | 0.8 | 0.8 | 0.1 | 0.2 |

Table 3. *In vivo* antifungal activity of cispentacin and other antifungal agents against systemic *Candida* infections in mice ($n=5$).

| Compound | $PD_{50}$ (mg/kg/dose)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | *C. albicans* A9540 | | | | | *C. albicans*[b] YA22851 | *C. tropicalis* CS-07 |
| | Dosing route and schedule[c] | | | | | Dosing route and schedule[c] | Dosing route and schedule[c] |
| | Single iv | Qd × 6 iv | Bid × 2 im | Single po | Bid × 2 po | Bid × 2 po | Single po |
| Cispentacin | 10 | 1.9 | 8.8 | 30 | 9.0 | 4.9 | 81 |
| Amphotericin B | 0.42 | 0.14 | 0.15 | >4.0 | 0.60 | 0.65 | 0.78 |
| 5-Fluorocytosine | >100 | 60 | >100 | >100 | >200 | >200 | 75 |
| Ketoconazole | 44 | NT | 70 | >100 | 56 | NT | NT |

[a] $PD_{50}$ value was calculated on day 20 after the fungal challenge.
[b] 5-Fluorocytosine-resistant strain.
[c] The first dosing was done immediately after the fungal challenge:
Qd × 6, once daily for 6 days; Bid × 2, twice a day for 2 days.
NT: Not tested.
Inoculum size: $10LD_{50}$.

examined with amphotericin B, 5-fluorocytosine and ketoconazole. Anti-*Candida* activity was determined by various dosing routes and schedules as summarized in Table 3. Against the systemic *C. albicans* A9540 infection, cispentacin was highly active by iv, intramuscular and po administrations. The $PD_{50}$ values of orally administered cispentacin was 9.0 mg/kg by twice a day dosing for 2 days and it was approximately 6 times more potent than ketoconazole. By single po administration, cispentacin demonstrated significant anti-*Candida* activity showing a $PD_{50}$ value of 30 mg/kg, while amphotericin B, 5-fluorocytosine and ketoconazole was inactive at 4.0, 100 and 100 mg/kg, respectively. Against 5-fluorocytosine-resistant *C. albicans* YA22851, cispentacin was remarkably effective by twice a day dosing for 2 days. Its $PD_{50}$ value was 4.9 mg/kg, approximately two times more potent than that against *C. albicans* A9540 by the

Table 4. *In vivo* antifungal activity of cispentacin and other antifungal agents against systemic *Cryptococcus* or *Aspergillus* infection in mice ($n=5$).

| Compound | $PD_{50}$ (mg/kg/dose)[a] | | | | | |
|---|---|---|---|---|---|---|
| | *C. neoformans* IAM 4514 | | | | | *A. fumigatus* IAM 2034 |
| | Dosing route and schedule[b] | | | | | Dosing route and schedule[b] |
| | Single iv | Qd × 6 iv | Bid × 2 im | Single po | Bid × 5 po | Qd × 6 iv |
| Cispentacin | >50 | 42 | >50 | >200 | 36 | >100 |
| Amphotericin B | 0.36 | 0.35 | 0.33 | 3.6 | 1.4 | 0.28 |
| 5-Fluorocytosine | >100 | >100 | >100 | >200 | >200 | >100 |
| Ketoconazole | >50 | NT | >100 | >100 | >100 | NT |

[a] $PD_{50}$ value was calculated on day 20 after the fungal challenge.
[b] The first dosing was done immediately after the fungal challenge:
Qd × 6, once daily for 6 days; Bid × 2 or × 5, twice a day for 2 days or 5 days.
NT: Not tested.
Inoculum size: $10LD_{50}$.

same dosing schedule. This antibiotic also exhibited significant therapeutic efficacy against *C. tropicalis* infection in immunocompromised mice by a single po administration. As summarized in Table 4, cispentacin was inactive against systemic *A. fumigatus* infection but was active against systemic *C. neoformans* infection by once daily administration for 6 days and twice a day for 5 days with the $PD_{50}$ values of 42 and 36 mg/kg, respectively.

*In Vivo* Antifungal Activity against Lung and Vaginal Infections with *C. albicans* in Mice

As shown in Fig. 1, intravenously administered cispentacin or amphotericin B significantly increased the survival rate of mice with lung candidiasis, their $PD_{50}$ values were 28 and 0.7 mg/kg, respectively. As shown in Table 5, orally administered cispentacin demonstrated significant therapeutic efficacy with the $PD_{50}$ value of 76 mg/kg against *Candida* vaginal infection.

Table 5. Antifungal activity of cispentacin and other antifungal agents against vaginal *Candida albicans* A9540 infection in mice[a] ($n=5$).

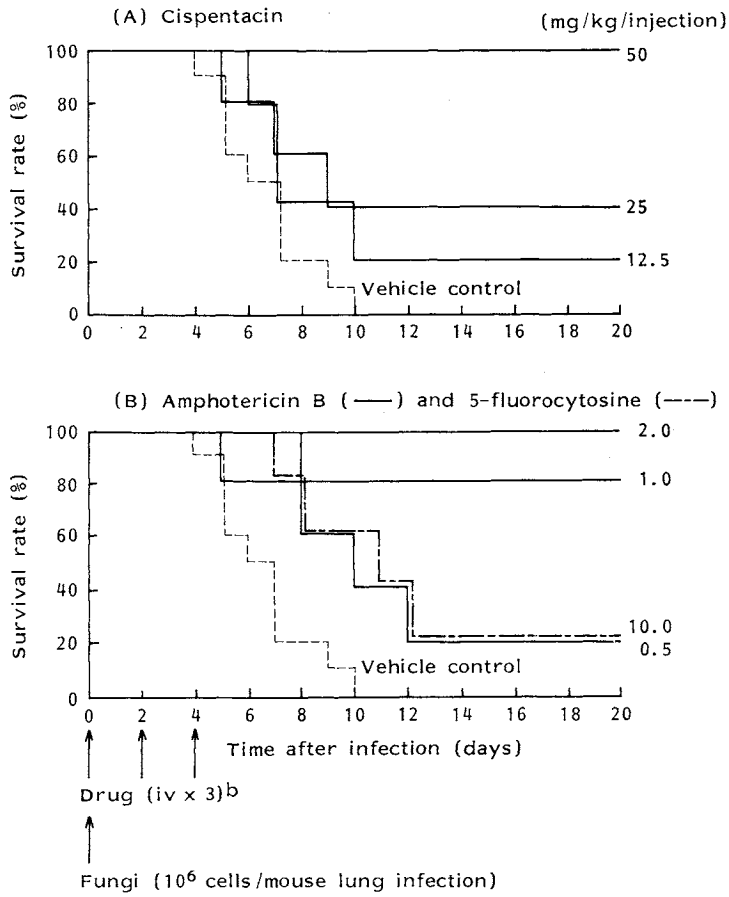
| Compound | Dose[b] (mg/kg/dose) | Lesion score (mean ± SE) | $PD_{50}$ (mg/kg/dose) |
|---|---|---|---|
| Vehicle control | — | 2.8±0.20 | — |
| Cispentacin | 100 | 0.6±0.27[c] | 76 |
| | 50 | 2.6±0.20 | |
| | 25 | 2.8±0.32 | |
| Amphotericin B | 0.25 | 0.8±0.37[c] | 0.19 |
| | 0.13 | 2.2±0.22 | |
| 5-Fluorocytosine | 200 | 2.2±0.49 | >200 |
| | 100 | 3.0±0.66 | |

[a] Female ICR mice (20~24 g) were treated subcutaneously with 0.5 mg/kg of estradiol benzoate 3 days before and 2 days after the fungal challenge.
[b] Test compounds were given to mice orally once daily for 5 days.
[c] Significantly different from control, $P<0.01$.
Inoculum size: $10^6$ cells/mouse.

## Acute Toxicity in Mice

No lethal toxicity was observed in mice given cispentacin at 1,000 mg/kg intravenously or 1,500 mg/kg intraperitoneally and orally. When cispentacin was administered at 1,000 mg/kg orally or 1,500 mg/kg intraperitoneally, muscle relaxation was observed in all mice 30 minutes after administration, and this continued for 5 days. By iv administration, the above effect was not observed even at 1,000 mg/kg.

Fig. 1. Antifungal activity of cispentacin and other antifungal agents against lung *Candida albicans* A9540 infection in immunocompromised mice[a].




[a] Male ICR mice (20~24 g) were injected intraperitoneally with 200 mg/kg cyclophosphamide 4 days prior to fungal infection.

[b] Drugs were intravenously injected immediately after the fungal challenge followed by once a day dosing on days 2 and 4.

## Discussion

Cispentacin is a new antibiotic produced by *Bacillus cereus* strain No. L450-B2 exhibiting potent *in vivo* anti-*Candida* activity. *In vitro* antifungal activity was seen against a number of *C. albicans* isolates by turbidimetric analysis in YNG medium, but only partial inhibition of the cell growth of *C. albicans* at 50 $\mu$g/ml and no antibacterial activity at 100 $\mu$g/ml was seen by agar dilution methods. In a murine systemic infection with *C. albicans*, cispentacin demonstrated potent therapeutic efficacy after iv, intramuscular and oral administrations. In particular, cispentacin was effective by single iv and oral dosing with the $PD_{50}$ values of 10 and 30 mg/kg, respectively, suggesting that this antibiotic is well absorbed through GI tracts and has a long biological half-life. The preliminary pharmacokinetic experiments in mice demonstrated that cispentacin has high area under the curve (AUC) and is retained in the various tissues at a fairly high level along with a high blood level. Furthermore, cispentacin showed significant efficacy in experimental murine lung and vaginal candidiases and in a systemic cryptococcosis, but not in a systemic aspergillosis. Taking into consideration these paradoxical *in vitro* and *in vivo* results, microbiological and biochemical studies are being performed to determine the mechanism of action of this antibiotic.

In the acute toxicity experiment in mice, cispentacin did not show any lethality at 1,000 mg/kg by the

iv route and at 1,500 mg/kg by ip and oral route of administration. Reversible muscle relaxation was observed at higher ip and po doses.

Cispentacin is a simple amino acid-type of antibiotic having the chemical structure of (−)-(1*R*,2*S*)-2-aminocyclopentane-1-carboxylic acid. 2-Aminocyclopentane-1-carboxylic acid was synthesized by PLIENINGER and SCHNEIDER in 1959[4] and a series of aminocyclopentanecarboxylic acids and aminocyclohexanecarboxylic acids have been synthesized to determine some aspects of the spatial dimensions of GABA receptors in the frog spiral cord[5] and in the rat hippocampus[6], but no other biological activities have been reported. YAMAGUCHI *et al.*[7] recently isolated rather simple amino acid antifungal antibiotic, (*S*)-2-amino-5-hydroxy-4-oxopentanoic acid (RI-331), from a strain of *Streptomyces* species, which was active against yeasts but inactive against aspergilli *in vitro*. Orally administered RI-331 exhibited *in vivo* activity against murine systemic candidiasis with low acute toxicity.

Acknowledgment

The authors wish to thank Dr. H. KAWAGUCHI of this Institute for valuable discussion throughout the present study and K. SHIINA for the supply of cispentacin. The excellent technical assistance of M. YAMANAKA, Y. OBI, M. MORI, H. FUJIMURA and A. OHTA is greatfully acknowledged.

## References

1) KONISHI, M.; M. NISHIO, K. SAITOH, T. MIYAKI, T. OKI & H. KAWAGUCHI: Cispentacin, a new antifungal antibiotic. I. Production, isolation, physico-chemical properties and structure. J. Antibiotics 42: 1749～1755, 1989
2) GALGIANI, J. N. & D. A. STEVENS: Antimicrobial susceptibility testing of yeasts: a turbidimetric technique independent of inoculum size. Antimicrob. Agents Chemother. 10: 721～726, 1976
3) LITCHFIELD, J. T. & F. WILCOXON: A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96: 99～113, 1949
4) PLIENINGER, H. & K. SCHNEIDER: Die Anlagerung von Ammoniak an $\Delta^1$-Tetrahydrobenoesäure and Cyclopenten-(1)-carbonsäure-(1) und die eindeutige sterische Zuordnung der entstehenden $\beta$-Aminosäuren. Chem. Ber. 92: 1594～1599, 1959
5) NICOLL, R. A.: The effect of conformationally restricted amino acid analogues on the frog spinal cord *in vitro*. Br. J. Pharmacol. 59: 303～309, 1977
6) SEGAL, M.; K. SIMS & E. SMISSMAN: Characterization of an inhibitory receptor in rat hippocampus: a microiontophoretic study using conformationally restricted amino acid analogues. Br. J. Pharmacol. 54: 181～188, 1975
7) YAMAGUCHI, H.; K. UCHIDA, T. HIRATANI, T. NAGASE, N. WATANABE & S. OMURA: RI-331, a new antifungal antibiotic. Ann. N. Y. Acad. Sci. 544: 188～189, 1989