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ANTIFUNGAL EVALUATION OF PSEUDOLARIC ACID B, A MAJOR CONSTITUENT OF *PSEUDOLARIX KAEMPFERI*

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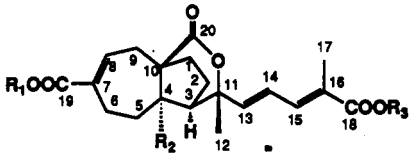
ABSTRACT.—Pseudolaric acid B [**1**] was isolated and identified as the main antifungal constituent of *Pseudolarix kaempferi* using bioassay-directed fractionation. Pseudolaric acid B was active against *Trichophyton mentagrophytes, Torulopsis petrophilum, Microsporum gypseum,* and *Candida* spp., while its methylated or hydrolyzed derivatives were not active against these same organisms. The minimum inhibitory concentrations and minimum fungicidal concentrations of pseudolaric acid B [**1**] against *Candida* and *Torulopsis* species were comparable with those of amphotericin B. The in vivo activity of pseudolaric acid B was evaluated in a murine model of disseminated candidiasis. Pseudolaric acid B [**1**] reduced the number of recovered colony-forming units significantly at different dosages. Infected mice treated intravenously with pseudolaric acid B [**1**] also had a longer survival time than those treated with vehicle alone.

With the increase in the number of immunocompromised patients in the last decade, especially from the AIDS epidemic, there has been a concomitant increase in the number of life-threatening mycoses (1,2). The antifungal agents currently available to counter these mycoses are limited either by ineffectiveness or by their toxicity (1,3). Therefore, there is a critical need for new antifungal agents that are more effective, fungicidal, and less toxic.

In the continuation of our efforts toward the identification of prototype antifungal agents from plants (4), an herb, *Pseudolarix kaempferi* Gordon (Pinaceae), has been investigated. The root and trunk barks of *P. kaempferi* have been used in the treatment of dermatological fungal infections in Chinese folkloric medicine. It has been reported that the EtOH extract of *P. kaempferi* was active against dermatomycoses (5). The chemical constituents of *P. kaempferi* have been investigated. Thus, pseudolaric acid B [**1**], pseudolaric acid A, glucosides of pseudolaric acid A and B and some other minor components have been identified from the bark (6–8) while several cytotoxic triterpenoids have been isolated from the seeds (9). It was reported that pseudolaric acids A and B had significant cytotoxicity for different tumor cell lines. In disease-oriented human tumor cell lines, pseudolaric acid B was active against CNS cancer TE671, melanoma SK-MEL-5, and ovarian cancer A2780 (10). Pseudolaric acids A and B also caused the death of rat embryos by decreasing blood flow in both the endo- and myometrium (11). However,



	R_1	R_2	R_3
1	CH_3	OAc	H
2	CH_3	OAc	CH_3
3	CH_3	OAc	β-D-glc
4	H	OAc	H
5	H	OH	H
6	CH_3	OH	H
7	CH_3	OAc	K
8	CH_3	OAc	Na

there has been no systematic investigation regarding the antifungal principle of *P. kaempferi*. Using bioassay-directed fractionation, pseudolaric acid B [**1**] was identified as the major antifungal constituent of *P. kaempferi*. Compound **1** was particularly active against *Candida* and *Torulopsis* species, common pathogens in immunocompromised patients, with a fungicidal activity against *C. albicans* comparable to that of amphotericin B. Pseudolaric acid B [**1**] was also active against *Trichophyton mentagrophytes* and *Microsporum gypseum*. No activity against bacteria and *Cryptococcus neoformans* was observed. The isolation and identification of pseudolaric acid B [**1**] and the evaluation of in vitro and in vivo antifungal activity of pseudolaric acid B and its derivatives are described in this report.

RESULTS AND DISCUSSION

The EtOH extract of the bark of *P. kaempferi* was evaluated for in vitro antibacterial and antifungal activity and showed strong activity against the opportunistic pathogen *C. albicans* B311. The extract was then subjected to separation and bioassay-directed fractionation. Compound **1** was identified as the major compound with strong anticandidal activity. The ir spectrum of **1** displayed the presence of ester groups (1740 cm^{-1}) and a free acid group (1720 cm^{-1}). A band at 255 nm (log ϵ 2.84) in the uv spectrum of **1** revealed a conjugated double bond system. The ^{1}H-nmr spectrum of **1** exhibited four olefinic protons, three of them coupled in an ABX system, four methyl groups, and one exchangeable proton. The ^{13}C-nmr spectrum of **1** showed four carbonyl carbons at δ_C 173.2, 172.8, 169.4, and 168.0 and six olefinic carbons. One carbonyl corresponded to a methyl ester and another to an acetate. APT and DEPTGL nmr experiments indicated that four of these six olefinic carbons had protons attached, which was consistent with the conclusion drawn from the ^{1}H-nmr data. Treatment of **1** with CH_2N_2 afforded compound **2**. The presence of a new methoxy signal at δ_H 3.76 (3H, singlet) in the ^{1}H-nmr spectrum indicated that compound **1** had one free carboxylic group. Comparing the spectral data (^{1}H- and ^{13}C-nmr, ir) of **1** with those reported by Z.-L. Li *et al.* led to the conclusion that **1** was pseudolaric acid B (6). The fully assigned ^{13}C-nmr data are summarized in Table 1.

A second compound [**3**] with weak antimicrobial activity was also isolated. It was observed that **3** was a glycosylated derivative of **1** by comparison of their spectral data. The ^{1}H-nmr spectrum exhibited a doublet (J=8.0 Hz) at δ_H 5.84. The signals at δ_C 96.3 (CH), 79.4 (CH), 78.4 (CH), 74.2 (CH), 70.9 (CH), and 62.1 (CH_2) in the ^{13}C-nmr spectrum of **3**, revealed that the sugar was glucose. The upfield shift of the free carboxylic group from δ_C 173 to δ_C 167 in the ^{13}C-nmr spectrum indicated that the glucose was linked to C-18. Hydrolysis of **3** under basic conditions in MeOH resulted in the isolation of two products. The less polar compound was identified as the methyl ester of pseudolaric acid B [**2**] and the polar product was identified as pseudolaric acid B [**1**]. Thus, compound **3** was identified as pseudolaric acid B-β-D-glucoside. The data reported here are in agreement with those of pseudolaric acid B-β-D-glucoside as reported by Z.-L. Li *et al.* (7).

In order to evaluate the features necessary for antifungal activity, several derivatives of pseudolaric acid B [**1**] were prepared using previously reported methods (8). Hydrolysis of **1** with 2% KOH at room temperature for 15 min resulted in the preparation of compound **4**. Fabms showed a peak at *m/z* 425 $[M+Li]^+$. High-resolution fabms gave a pseudomolecular ion at *m/z* 425.1768, corresponding to an elemental formula of $C_{22}H_{26}O_8Li$ (calcd for 425.1788). The ^{1}H- and ^{13}C-nmr spectra of **4** were very similar to those of pseudolaric acid B, except that the methoxyl signal at C-19 was absent. Thus, compound **4** was identified as pseudolaric acid C_2 (9).

TABLE 1. ^{13}C-Nmr Data of Pseudolaric Acid B [**1**] and Its Derivatives **3**–**6**.[a]

Carbon	Compound				
	1	**3**	**4**	**5**	**6**
C-1	33.2 (2)	33.5 (2)	33.3 (2)	35.9 (2)	34.6 (2)
C-2	24.2 (2)	24.6 (2)	24.3 (2)	25.2 (2)	24.1 (2)
C-3	49.2 (1)	49.6 (1)	49.3 (1)	54.7 (1)	53.5 (1)
C-4	90.0 (0)	89.9 (0)	90.0 (0)	79.6 (0)	79.1 (0)
C-5	30.6 (2)	31.0 (2)	30.6 (2)	34.3 (2)	33.3 (2)
C-6	20.0 (2)	20.4 (2)	19.7 (2)	20.5 (2)	19.4 (2)
C-7	134.4 (0)	134.8 (0)	133.8 (0)	136.2 (0)	134.0 (0)
C-8	141.6 (1)	142.2 (1)	144.4 (1)	142.5 (1)	142.9 (1)
C-9	27.6 (2)	28.1 (2)	27.9 (2)	28.1 (2)	26.8 (2)
C-10	55.2 (0)	55.6 (0)	55.3 (0)	55.6 (0)	54.6 (0)
C-11	83.6 (0)	83.8 (0)	83.7 (0)	83.6 (0)	83.8 (0)
C-12	28.4 (3)	28.2 (3)	28.5 (3)	28.4 (3)	27.9 (3)
C-13	144.4 (1)	145.3 (1)	144.5 (1)	145.2 (1)	144.1 (1)
C-14	121.6 (1)	121.3 (1)	121.7 (1)	121.3 (1)	120.9 (1)
C-15	138.6 (1)	138.4 (1)	138.8 (1)	136.9 (1)	137.1 (1)
C-16	127.8 (0)	127.9 (0)	127.8 (0)	129.5 (0)	127.8 (0)
C-17	12.5 (3)	12.6 (3)	12.6 (3)	13.0 (3)	12.1 (3)
C-18	173.2 (0)	167.0 (0)	173.5 (0)	170.4 (0)*	175.3 (0)*
C-19	168.0 (0)	167.9 (0)	172.6 (0)	170.6 (0)*	168.6 (0)*
C-20	172.8 (0)	173.0 (0)	172.7 (0)	174.9 (0)*	170.3 (0)*
CH_3O	52.0 (3)	51.8 (3)	—	—	51.5 (3)
CH_3CO	21.7 (3)	21.4 (3)	21.8 (3)	—	—
CH_3CO	169.4 (0)	169.6 (0)	169.4 (0)	—	—
Glucose					
C-1′		96.3 (1)			
C-2′		74.2 (1)			
C-3′		79.4 (1)			
C-4′		70.9 (1)			
C-5′		78.4 (1)			
C-6′		62.1 (2)			

[a]The number in parentheses indicates the number of attached hydrogens as determined by the ATP and DEPTGL experiments. $CDCl_3$ was used for **1**, **4**, and **6**, while **3** and **5** were recorded in pyridine-d_5. Data bearing an asterisk may be reversed in the same column.

Treatment of pseudolaric acid B with 2% KOH at room temperature overnight yielded compound **5**, which had a molecular formula of $C_{20}H_{24}O_7$ (high-resolution fabms *m/z* [M+1] 377.1599, calcd for $C_{20}H_{25}O_7$, 377.1600). The ^{1}H- and ^{13}C-nmr spectral data indicated that compound **5** was demethoxydeacetoxypseudolaric acid B (9).

Refluxing of **1** in MeOH with KOH afforded compound **6**. Purification of **6** by recrystallization from MeOH gave colorless crystals with a molecular formula of $C_{21}H_{26}O_7$ (high-resolution fabms *m/z* $[M+Li]^+$ 397.1844, calcd for $C_{21}H_{26}O_7Li$, 397.1838). The ^{1}H- and ^{13}C-nmr spectra indicated that the acetoxy group at C-4 was removed. Thus, compound **6** was determined to be pseudolaric acid C (9).

The H_2O-soluble potassium and sodium salts of **1** were also prepared. A 400-mg sample of **1** was dissolved in 20 ml MeOH, the solution was cooled in an ice bath, and 1 equivalent of ice-cooled KOH in MeOH was added. After the addition of KOH the solvent was evaporated *in vacuo* at room temperature and a H_2O-soluble (3 mg/ml) solid [**7**] was obtained. The ir spectrum exhibited the presence of lactone and methyl ester groups (1740, 1720, and 1703 cm^{-1}). When a solution of the powder was acidified with dilute HCl and then extracted with $CHCl_3$, a pure compound was recovered which had

TABLE 2. Antimicrobial Activities of Pseudolaric Acid B [1] and Its Derivatives.[a]

Organisms	Compound							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
C. albicans	+++	±	+	±	±	+	+++	+++
A. flavus	−	−	−	−	−	+	−	−
A. fumigatus	++	−	−	−	−	+	++	++
C. neoformans	−	−	−	−	−	+	−	−
T. mentagrophytes	+++	−	−	−	++	−	+++	+++
M. gypseum	+++	−	−	−	−	−	+++	+++
S. cerevisiae	−	−	−	−	−	−	−	−
E. coli	−	−	−	−	−	−	−	−
S. aureus	−	−	−	−	−	−	−	−
B. subtilis	−	−	−	−	−	−	−	−
P. aeruginosa	−	−	−	−	−	−	−	−
M. intracellulare	+	+	−	−	+	+	−	−

[a]Test compounds were dissolved in DMSO. Inhibitory zones were recorded in mm from the edge of the agar well to the edge of the zone. The codes were assigned as follows: −, no inhibitory zone; ±, <1 mm; +, 1–2 mm; ++, 3–6 mm; +++, 7–12 mm; ++++, >12 mm. Amphotericin B and streptomycin were used as positive antifungal and antibacterial controls, respectively.

superimposable ir and ^{1}H-nmr spectra with those of **1**. Thus, compound **7** was identified as potassium pseudolarate B. Sodium pseudolarate B [**8**] was prepared using the same procedure (NaOH/MeOH).

In vitro antimicrobial evaluation of **1** showed strong activities against *Candida* species, *Torulopsis petrophilum, Trichophyton mentagrophytes,* and *Microsporum gypseum* (Tables 2 and 3). Neither the methylated derivative **2** nor any of the hydrolyzed compounds **4**–**6** were active against the tested organisms. Compound **3** exhibited only marginal anticandidal activity and no activity against *T. mentagrophytes* or *M. gypseum.*

The fact that **2** and **4**–**6** lost antifungal activity indicates that the presence of the acetoxyl group and the C-18 free acid are necessary for antifungal activity. These results are parallel to those observed from a study of the antitumor activity (10). The weak anticandidal activity of **3** could be attributed to the hydrolysis of **3** into **1** during

TABLE 3. Qualitative Activity of Pseudolaric Acid B [**1**], Potassium Pseudolarate B [**7**], and Amphotericin B (AmB) Against *Candida* and *Torulopsis* Species.[a]

Yeasts	Compound		
	1	**7**	AmB
C. albicans B311	+++	+++	+++
C. albicans ATCC 10231	+++	+++	+++
C. magnoliae ATCC 12573	±	±	+++
C. parapsilosis ATCC 20224	+++	+++	++
C. stellatoidaes SA-I	++++	+++	+++
C. tropicalis ATCC 20326	+++	+++	+++
C. tropicalis ATCC 20021	+++	+++	+++
C. tropicalis LM 64	++++	++++	+++
T. petrophilum ATCC 20225	+++	+++	+++
T. cremoris NRRL 7495	++	++	+++

[a]Test compounds were dissolved in DMSO. Inhibitory zones were recorded in mm from the edge of the agar well to the edge of the zone. The codes were assigned as follows: −, no inhibitory zone, ±, <1 mm; +, 1–2 mm, ++, 3–6 mm; +++, 7–12 mm; ++++, >12 mm. Amphotericin B was used as positive antifungal control.

TABLE 4. Quantitative Activity of Pseudolaric Acid B [**1**], Potassium Pseudolarate B [**7**], and Amphotericin B (AmB) Against *Candida* and *Torulopsis* Species.[a]

Fungi	**1**	**7**	AmB
	MIC/MFC	MIC/MFC	MIC
C. albicans B311	1.56/3.12	3.12/6.25	1.56
C. albicans ATCC 10231	1.56/3.12	1.56/6.25	3.12
C. parapsilosis ATCC 20224	3.12/12.5	3.12/25.0	1.56
C. stellatoidaes SA-I	1.56/6.25	3.12/6.25	3.12
C. tropicalis ATCC 20326	1.56/3.12	1.56/6.25	1.56
C. tropicalis ATCC 20021	1.56/3.12	3.12/6.25	3.12
C. tropicalis LM 64	0.78/6.25	0.78/6.25	1.56
T. petrophilum ATCC 20225	0.78/1.56	0.78/12.5	0.20
T. cremoris NRRL 7495	12.5/12.5	12.5/25.0	3.12

[a]Minimum inhibitory concentration (MIC) values were determined using the twofold serial broth dilution method and are expressed in μg/ml.

incubation, since **1** could be detected when **3** was incubated in Sabouraud dextrose broth with *C. albicans* B311 at 37°. No **1** was detected when **2** was incubated under the same conditions.

It is worth noting that **1** is not active against *Cryptococcus neoformans*, which indicates that it might have an antifungal mechanism different from that of amphotericin B since the latter compound is active against both *C. albicans* and *C. neoformans*. Significant differences in the composition of the cell wall between *C. albicans* and *C. neoformans* have been observed (12).

Table 4 shows the minimum inhibitory concentrations (MIC) of **1** and **7** determined using the macrobroth dilution assay. MIC values of **1** and **7** were within two dilutions of that of amphotericin B for most of the *Candida* species and *T. petrophilum*. Initial evaluation of the minimum fungicidal concentrations (MFC) was accomplished by subculturing from MIC tubes showing no growth onto drug-free agar plates. Using this technique, the MFC values of **1** and **7** for most of the *Candida* and *Torulopsis* species tested were within two dilutions (twofold) of the MICs (Table 4).

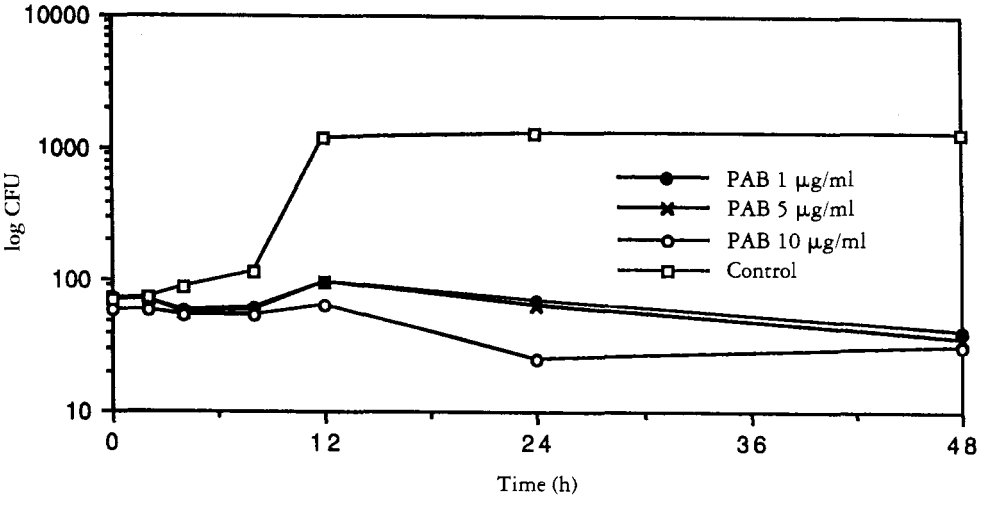


FIGURE 1. Time-to-kill curve of pseudolaric acid B [**1**] against *Candida albicans* B311 in Sabouraud dextrose broth.

TABLE 5. Body Weights of Mice Following Administration of **1** and **7** for Fourteen Consecutive Days.[a]

Compound and Dose	Body Weight (Mean±SD)		
	Day 1	Day 14	Day 21
1 40 mg/kg/day, ip	22.5±0.8	24.1±1.2	24.7±1.7
Vehicle 10% (DMSO/H_2O)	23.2±1.5	24.6±1.0	25.9±0.7
7 40 mg/kg/day, ip	24.2±0.8	25.4±1.3	25.6±1.3
Vehicle (0.9% Saline)	22.8±1.0	24.3±1.3	25.5±1.9

[a]Six CD-1 female mice were used in each group. Mice were weighed each week and were observed for four weeks.

However, when the fungicidal activity of pseudolaric acid B [**1**] was further tested by determination of time-to-kill curves against *C. albicans* B311 (Figure 1), there was less convincing evidence of strong fungicidal activity. Although a rapid decrease in the number of viable cells was observed at concentrations of 1, 5, and 10 μg/ml, and the viable cell counts remained at relatively low levels compared with the control, the counts were not reduced to zero even at 10 μg/ml.

Pseudolaric acid B [**1**] is relatively nontoxic. The reported iv and ip LD_{50} values of **1** in mice were 423 and 316 mg/kg, respectively (13). Pseudolaric acid B was administered via the intraperitoneal route at a dose of 40 mg/kg for 14 consecutive days. Mice were examined daily for physical and behavioral signs of toxicity for 4 weeks, and weighed weekly. No unusual behavioral changes were observed and the body weight changes of drug-treated mice were consistent with those of vehicle-treated mice (Table 5).

Kidneys are the primary target of candidal infections and the ability of **1** to reduce *C. albicans* in the infected kidneys was evaluated using a mouse candidiasis model (14). The mice treated with pseudolaric acid B [**1**] were found to consistently exhibit a significant ($p<0.05$) reduction in the number of recovered colony-forming units (CFU) of *C. albicans* as compared with vehicle-treated control groups (Table 6). When **1** was administered intravenously (via the lateral tail veins) at doses of 10 and 20 mg/kg 18 and 48 h after infection, the number of CFU recovered was reduced significantly as compared

TABLE 6. Recovery of *C. albicans* from Kidneys After Treatment with **1** or **3**.[a]

Compound	Route	Dosage	CFU/mg	% Reduction	*p*
1	iv	Control	8.80×10^3	—	—
		20 mg/kg	3.60×10^3	59.60	0.03
		10 mg/kg	4.10×10^2	95.34	0.00
		AmB, ip	1.19×10^1	99.86	0.00
1	sc	Control	2.94×10^3	—	—
		20 mg/kg	6.85×10^2	76.70	0.02
		10 mg/kg	7.35×10^2	75.00	0.01
		3 mg/kg	8.74×10^2	70.27	0.05
		AmB, ip	5.36×10^0	99.82	0.00
3	ip	Control	2.64×10^4	—	—
		20 mg/kg	2.65×10^4	N/A	N/A
		7 mg/kg	1.41×10^4	46.59	0.24
		3 mg/kg	2.40×10^4	9.91	0.19
		AmB, ip	1.10×10^1	99.96	0.00

[a]Vehicle control for iv and ip administration was 10% DMSO/H_2O, while sesame oil was used for sc injection. Amphotericin B (AmB) was dissolved in 10% DMSO/H_2O and was delivered ip at 1 mg/kg.

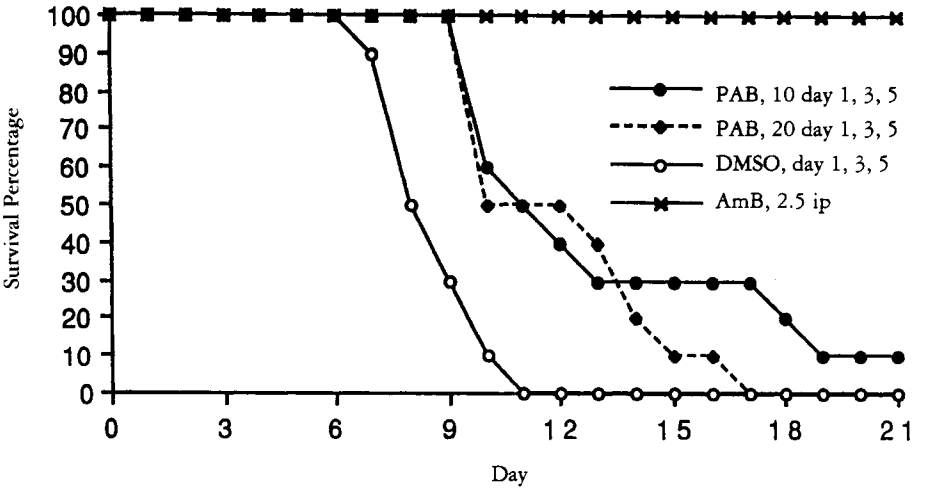


FIGURE 2. Survival of CD-1 mice after challenge with 10^6 *Candida albicans* B311 and treatment (iv) with pseudolaric acid B [**2**] at days 1, 3, and 5 post-infection.

to vehicle-treated mice (Table 6). When sesame oil was used as vehicle and the drug was delivered subcutaneously, the number of recovered CFU was reduced at all three dosages (3, 10, and 20 mg/kg). The reduction effect was moderate (70–76%) but significant ($p<0.05$).

In pseudolaric acid B glucoside **3**, the ester linkage is susceptible to hydrolysis under mild conditions. Thus, it was speculated that **3** may act as a masked form of **1**. However, when **3** was evaluated for efficacy in the mouse tissue-burden model, only marginal activity was observed at intraperitoneal doses of 3.5 and 7 mg/kg (10–47% reduction, $p>0.05$), while no effect was observed at a dose of 20 mg/kg (Table 6).

Pseudolaric acid B [**1**] was also evaluated for its ability to prolong the survival time of infected mice. Mice infected with 10^6 CFU of *C. albicans* were treated with **1** intravenously at days 1, 3, and 5 postinfection. At a dose of 10 mg/kg, **1** prolonged the survival time significantly (Figure 2). However, when **1** was delivered once daily via the intraperitoneal route (highest dose of 40 mg/kg), no activity was observed (data not shown). Although the reason for this is not clear, hydrolysis of **1** to an inactive derivative (e.g., **4**, **5**, or **6**) would be a reasonable explanation.

Based on these results, it can be concluded that pseudolaric acid B [**1**] is the major antifungal component in *P. kaempferi* and is active against most of the *Candida* and *Torulopsis* species tested. In murine disseminated candidiasis, **1** is therapeutically effective. Further studies on such properties as pharmacokinetic parameters, bioavailability, excretion, and metabolism would benefit future evaluation of the in vivo anticandidal activity of **1**.

Pseudolaric acid B [**1**] is also active against *Trichophyton mentagrophytes* and *Microsporum gypseum*, pathogens commonly found in dermatological infections. This may well explain why, in the People's Republic of China, the EtOH extract of the bark of *P. kaempferi* is still used for dematomycoses.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps were determined on a Fisher digital melting point analyzer Model 355 or Thomas-Hoover capillary melting point apparatus. Ir spectra were recorded in KBr using a Perkin-Elmer 281 B infrared spectrophotometer. Uv spectra were recorded in MeOH on a Perkin-Elmer Lamda 3B spectrophotometer, and the optical rotation data were collected using a Jasco DIP 370

digital polarimeter. ^{1}H- and ^{13}C-nmr spectra were obtained on a Varian VXR 300 spectrometer with standard pulse sequences operating at 300 MHz and 75 MHz, respectively. Nmr spectra were recorded in $CDCl_3$, unless otherwise stated. The chemical shift values are reported in ppm units, and the coupling constants are in Hz. Carbon multiplicities were determined by DEPTGL experiments. All ^{13}C-nmr assignments were based on DEPTGL, HETCOR, long-range HETCOR, and/or comparisons within the compound series. Chromatography was performed using Si gel (70–270 mesh), and tlc with Si gel 60 F_{254}. Tlc plates were developed in $CHCl_3$/MeOH and were visualized under uv light or by spraying with 10% H_2SO_4.

TEST ORGANISMS.—Organisms included in the study were selected because of their resistance or susceptibility to known antimicrobial agents, virulence, source, and clinical importance. Long-term stock cultures were either lyophilized or stored under sterile mineral oil and subcultures were maintained on agar slants and stored at 4°. Cultures were obtained from either the American Type Culture Collection (ATCC, Rockville, MD) or Northern Regional Research Laboratories (NRRL, Peoria, IL). *C. albicans* B311 is a clinical isolate (15) that was kindly provided by Dr. E. Balish, University of Wisconsin at Madison.

ANIMALS.—Female CD-1 mice (Charles River Breeding Laboratories, Inc., Portage, MI) weighing 22–24 g were used in models of disseminated candidiasis as described by Clark *et al.* (14). Animals were maintained in air-conditioned rooms at 72° on a 12-h light, 12-h dark cycle. Water and food were supplied *ad libitum*. Animals were quarantined and acclimated one week prior to initiation of experiments.

QUALITATIVE ANTIMICROBIAL ACTIVITY EVALUATION.—The agar-well diffusion assay (16) was used for evaluation of the antibacterial and antifungal activities of the extract, fractions, and pure compounds. Test organisms included *Aspergillus* species, *Candida* species, *Cryptococcus neoformans*, *Torulopsis* species, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Saccharomyces cerevisiae*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Mycobacterium intracellulare*.

Agar plates were streaked with a solution of the 24-h-old test organism cultured in Sabouraud dextrose broth (SDB) (for fungi) or Eugon® broth (for bacteria). Cylindrical plugs were removed from the plates by means of a sterile cork borer to produce wells with a diameter of approximately 10 mm. To each well was added 100 μl of solution of an extract, fraction, or pure compound. Pure compounds were tested at a concentration of 1 mg/ml, while the extracts and fractions were tested at 20 mg/ml. The antifungal activities were recorded as the width (in mm) of the inhibition zone measured from the edge of the well to the outside edge of the inhibition zone following incubation of the plates at 37° for 24 or 48 h. The antimicrobial agents amphotericin B and streptomycin were included as standards in each assay.

DETERMINATION OF THE MIC AND MFC VALUES.—The twofold serial broth dilution assay (14, 16, 17) in yeast nitrogen broth (Difco) supplemented with 1% of glucose and 0.15% L-asparagine was used for determination of the MIC values. All compounds were initially tested using a concentration of 100 μg/ml in the first tube and serially diluted twofold to 0.20 μg/ml. The test organisms were grown in Sabouraud dextrose broth for 24 h at 37° at which time the cells were harvested by centrifugation (2,000 rpm). After centrifugation, the cells were washed twice and suspended in physiological saline solution to give a final concentration of 10^6 viable yeast cells/ml. Using a micropipette, an inoculum of 10 μl of the cell suspension of a fungus was added to each tube. The MIC value is defined as the lowest drug concentration to inhibit visible growth of the test organisms after 24 or 48 h of incubation at 37°. The antifungal agent amphotericin B was included as standard in each assay. Drug-free solution was also used as blank control.

After MIC data were recorded, the tubes with no growth were vortexed, and 10 μl of the broth were plated in triplicate on a SDA plate. After incubation at 37°, the results were recorded at 24 or 48 h. The MFC value is defined as the lowest concentration of drug showing no growth.

TIME-TO-KILL CURVE.—The time course of the candidacidal activity was determined by enumerating viable cells in 4.5 ml SDB of fungal suspension exposed to the drug. The initial inoculum was adjusted to about 10^6 CFU/ml (the viable cells were 2×10^4/ml in the broth). The test compound in 0.5 ml DMSO was then added to the culture at final concentrations of 1, 5, and 10 μg/ml. Samples of 10 μl were removed at fixed times, diluted appropriately if necessary, and were plated onto drug-free SDA plates. Colonies were counted after incubation at 37° for 24 h.

EXTRACTION AND BIOASSAY-DIRECTED ISOLATION.—The root bark and trunk bark of *P. kaempferi* were collected in the fall of 1987, in Jiangsu Province, People's Republic of China. The voucher specimens have been deposited in the Department of Phytochemistry, Nanjing College of Traditional Chinese Medicine, Nanjing, People's Republic of China. The powdered bark (1 kg) was extracted with 95% EtOH. After evaporation of the solvent, the residue (50 g) was redissolved in $CHCl_3$/MeOH, then adsorbed onto Celite® 545 and dried under reduced pressure. The dried powder was placed in a Soxhlet apparatus and extracted with hexane, $CHCl_3$, Me_2CO, and finally MeOH. The $CHCl_3$ and Me_2CO fractions were pooled (20 g), since

they had similar tlc patterns, and both exhibited strong activities against *Trichophyton* and *Candida* species. A portion of the combined fraction was chromatographed on a Si gel column and elution with $CHCl_3$-MeOH (19:1) afforded compounds **1** (3.5 g) and **2** (330 mg).

Pseudolaric acid B [**1**].—Colorless powder, mp 139–141°; $[\alpha]^{25}D$ −25.9° (*c*=0.9, MeOH) [lit. (6) mp 145–146°, $[\alpha]^{25}D$ −30.1° (EtOH)]; uv λ max (MeOH) 255 nm (log ε 2.84); ir ν max 3500–2700, 1740, 1720, 1682, 1635, 1605 cm^{-1}; ^1H nmr δ 7.24 (1H, br d, H-15), 7.2 (1H, m, H-8), 6.54 (1H, dd, *J*=15.1 and 11.4 Hz, H-14), 5.90 (1H, d, *J*=15.1 Hz, H-13), 3.70 (3H, s, OCH_3), 3.3 (1H, m, H-3), 3.06 (1H, dd, *J*=13.5 and 6.3 Hz, H-5), 2.86 (1H, dd, *J*=15.5 and 6.3 Hz, H-6), 2.73 (1H, dd, *J*=15.0 and 8.8 Hz, H-9), 2.58 (1H, ddd, *J*=15.0, 4.0, and 1.8 Hz, H-9), 2.1 (1H, m, H-6), 2.11 (3H, s, OAc), 1.94 (3H, d, *J*=1.2 Hz, CH_3-17), 1.8 (2H, m, H-1), 1.7 (2H, m, H-2), 1.7 (1H, m, H-5), 1.58 (3H, s, CH_3-12); ^{13}C-nmr data, see Table 1; fabms *m/z* 439 $[M+Li]^+$; hrfabms *m/z* $[M+Li]^+$ 439.1933 (calcd for $C_{23}H_{28}O_8Li$, 439.1944). The ir spectrum of **1** was superimposable upon that obtained from an authentic sample of pseudolaric acid B.

METHYLATION OF PSEUDOLARIC ACID B [**1**] TO [**2**].—Compound **1** (20 mg) dissolved in 10 ml Et_2O was reacted with CH_2N_2 solution in Et_2O at 0°. A colorless powder [**2**] was obtained after removal of the solvent. Compound **2** was an amorphous powder, mp 70°, uv λ max (MeOH) 256 nm (log ε 2.74); ir ν max 1730, 1700, 1633 cm^{-1}; ^1H nmr δ 7.2 (1H, m, H-8), 7.16 (1H, br d, H-15), 6.53 (1H, dd, *J*=15.0 and 11.4 Hz, H-14), 5.86 (1H, d, *J*=15.0 Hz, H-13), 3.76 (3H, s, OCH_3), 3.72 (3H, s, OCH_3), 3.30 (1H, br d, H-3), 3.07 (1H, dd, *J*=13.5 and 6.3 Hz, H-5), 2.89 (1H, br dd, *J*=15.5 and 6.3 Hz, H-6), 2.75 (1H, dd, *J*=15.0 and 8.8 Hz, H-9), 2.6 (1H, m, H-9), 2.12 (3H, s, OAc), 2.1 (1H, m, H-6), 1.95 (3H, d, *J*=1.2 Hz, CH_3-17), 1.8 (2H, m, H-1), 1.7 (2H, m, H-2), 1.7 (1H, m, H-5), 1.59 (3H, s, CH_3-12); fabms *m/z* 453 $[M+Li]^+$; hrfabms *m/z* $[M+Li]^+$ 453.2111 (calcd for $C_{24}H_{20}O_8Li$, 453.2101).

PSEUDOLARIC ACID B-β-D-GLUCOSIDE [**3**].—Amorphous powder, $[\alpha]^{25}D$ −30.4° (*c*=0.47, MeOH); uv λ max (MeOH) 258 nm (log ε 2.65); ir ν max 3400 (br), 1737, 1712, 1702, 1635 cm^{-1}; ^1H nmr (pyridine-d_5) δ 7.43 (1H, br d, H-15), 7.4 (1H, m, H-8), 6.63 (1H, dd, *J*=15.1 and 11.4 Hz, H-14), 6.41 (1H, d, *J*=15.1 Hz, H-13), 5.84 (1H, d, *J*=8.0 Hz, anomeric H), 3.61 (3H, s, OCH_3), 3.4 (1H, m, H-3), 3.19 (1H, dd, *J*=13.5 and 6.3 Hz, H-5), 2.99 (1H, dd, *J*=15.5 and 6.3 Hz, H-6), 2.81 (1H, dd, *J*=15.0 and 8.8 Hz, H-9), 2.63 (1H, ddd, *J*=15.0, 4.0, and 1.8 Hz, H-9), 2.2 (1H, m, H-6), 2.14 (3H, s, OAc), 1.82 (CH_3-17), 1.51 (3H, s, CH_3-12), 1.8 (5-H), 3.5–4.6 (sugar); hrfabms *m/z* $[M+Li]^+$ 601.2493 (calcd for $C_{29}H_{38}O_{13}Li$, 601.2472). These data are consistent with those reported (7). ^{13}C-nmr data, see Table 1.

HYDROLYSIS OF PSEUDOLARIC ACID β-D-GLUCOSIDE [**3**] TO **1** AND **2**.—Compound **3** (10 mg) dissolved in 2 ml 0.5 M HCl/MeOH was refluxed for 30 min. After removal of the solvent, the residue was extracted with Et_2O. Tlc analysis showed the presence of two compounds in the Et_2O extract. Purification of the Et_2O extract on a small Si gel column using $CHCl_3$-MeOH (19:1) as eluent afforded two compounds. The ir and ^1H-nmr spectra of the less polar compound were superimposable with those of **2**, and the polar product was identified as pseudolaric acid B [**1**].

HYDROLYSIS OF PSEUDOLARIC ACID B [**1**] TO **4**.—Pseudolaric acid B [**1**] (120 mg) was dissolved in 5 ml 2% KOH at room temperature. After 15 min, the solution was acidified (to pH 5) with 5% HCl and the precipitate was collected by filtration. After purification on a small Si gel column, **4** was isolated as the major component. Compound **4** exhibited: mp 160–162° [lit. (8) mp 178°]; $[\alpha]^{25}D$ −24.6° (*c*=0.47, MeOH); uv λ max (MeOH) 255 nm (log ε 2.81); ir ν max 3600–2700 (br), 1737, 1712, 1680, 1630 cm^{-1}; ^1H nmr δ 7.4 (1H, m, H-8), 7.26 (1H, br d, *J*=11.4 Hz, H-15), 6.56 (1H, dd, *J*=15.1 and 11.4 Hz, H-14), 5.92 (1H, d, *J*=15.1 Hz, H-13), 3.3 (1H, m, H-3), 3.07 (1H, br dd, *J*=13.5 and 6.3 Hz, H-5), 2.9 (1H, m, H-6), 2.8 (1H, m, H-9), 2.6 (1H, m, H-9), 2.14 (3H, s, OAc), 2.1 (1H, m, H-6), 1.8 (5H, m), 1.95 (3H, s, CH_3-17), 1.60 (3H, s, CH_3-12); ^{13}C-nmr data, see Table 1; fabms *m/z* 425 $[M+Li]^+$; hrfabms *m/z* $[M+Li]^+$ 425.1768 (calcd for $C_{22}H_{26}O_8Li$, 425.1788).

HYDROLYSIS OF PSEUDOLARIC ACID B [**1**] TO **5**.—100 mg of **1** were dissolved in 2 ml 2% KOH solution at room temperature and allowed to stand overnight. Acidification with HCl produced **5** which was collected by filtration. Colorless flakes of **5** were obtained after crystallization from MeOH. Compound **5** exhibited: mp 260° (dec); $[\alpha]^{25}D$ −93.1° (*c*=0.58, MeOH); uv λ max (MeOH) 255 nm (log ε 2.90); ir ν max 3470, 3200–2500 (br), 1720, 1702, 1675, 1590 cm^{-1}; ^1H nmr (pyridine-d_5) δ 7.9 (1H, m, H-8), 7.66 (1H, d, *J*=11.4 Hz, H-15), 6.84 (1H, dd, *J*=15.1 and 11.4 Hz, H-14), 6.03 (1H, d, *J*=15.1 Hz, H-13), 3.4 (1H, m, H-3), 3.2 (1H, m, H-5), 3.06 (1H, dd, *J*=15.5 and 6.3 Hz, H-6), 2.87 (1H, dd, *J*=15.0 and 8.8 Hz, H-9), 2.35 (1H, ddd, *J*=15.0, 4.0, and 1.8 Hz, H-9), 2.2 (1H, m, H-6), 2.03 (3H, s, CH_3-17), 2.0–1.7 (5H, m), 1.52 (3H, s, CH_3-12); ^{13}C-nmr data, see Table 1; fabms *m/z* 377 [M+1]; hrfabms *m/z* [M+1] 377.1599 (calcd for $C_{20}H_{25}O_7$, 377.1600).

PREPARATION OF COMPOUND **6**.—157 mg of **1** were dissolved in 30 ml 0.5% KOH/MeOH. The mixture was refluxed for 30 min, then acidified with HCl. After removal of solvent, the residue (104 mg) was dissolved in Et_2O and washed with H_2O. Colorless needles [**6**] were obtained from the Et_2O extract by crystallization from MeOH. Compound **6** exhibited: mp 198–200° [lit. (8) 225° (Me_2CO)]; $[\alpha]^{25}D$ −78.6° (c=0.2, MeOH); uv λ max (MeOH) 255 nm; ir ν max 3460, 3000 (br), 1730, 1720, 1710, 1675 cm^{-1}; ^1H nmr δ 7.2 (2H, m, H-8, H-15), 6.52 (1H, dd, J=15.1 and 11.4 Hz, H-14), 5.93 (1H, d, J=15.1 Hz, H-13), 3.73 (3H, s, OCH_3), 1.95 (3H, s, CH_3-17), 1.56 (3H, s, CH_3-12); ^{13}C-nmr data, see Table 1; fabms *m/z* 397 $[M+Li]^+$; hrfabms *m/z* $[M+Li]^+$ 397.1844 (calcd for $C_{21}H_{26}O_7Li$, 397.1838).

PREPARATION OF COMPOUND **7**.—Compound **1** (400 mg) was dissolved in 20 ml MeOH, the solution was cooled in an ice bath, and 1 equivalent of KOH in MeOH was added. After removal of MeOH *in vacuo* at room temperature, a solid [**7**] was obtained: mp 156° (dec); ir ν max 3400 (br), 1740, 1720, 1705, 1640 cm^{-1}. When an aqueous solution of the powder was acidified with dilute HCl and extracted with $CHCl_3$, a compound was recovered which had superimposable ir and ^1H-nmr spectra with those of **1**. No hydrolyzed products such as **4**, **5**, or **6** were detected by tlc from the acidified MeOH solution of **7**.

PREPARATION OF COMPOUND **8**.—Compound **8** was prepared by treatment of **1** with NaOH/MeOH and was identified by the same procedures as mentioned above, mp 153° (dec); ir ν max 3400 (br), 1740, 1725, 1710, 1635 cm^{-1}.

EVALUATION OF TOXICITY.—Compounds **1** and **7** were each administered by intraperitoneal injection to groups of CD-1 female mice (n=6) at a dose of 40 mg/kg/day for 14 consecutive days. Vehicle controls (10% DMSO/H_2O for **1**, 0.9% saline solution for **7**) were administered with the same schedule. Animals were examined daily for physical and behavioral signs of toxicity and body weights were measured weekly. The body weight changes between the drug-treated group and the control were analyzed using the un-paired Student-t test.

IN VIVO EFFICACY EVALUATION.—*Infection of mice.*—The mouse model of disseminated candidiasis as described by Clark *et al.* (14) was utilized. Acute disseminated infections in mice were produced by intravenous injection of *C. albicans*. Briefly, cells harvested from an overnight growth of *C. albicans* NIH B311 in SDB were washed twice with saline solution and suspended in saline. The concentration of the cells was determined by counting in a hemacytometer and was adjusted to 2×10^6 cells/ml. Counts of CFU were confirmed by serial tenfold dilution and plating triplicate aliquots of the inoculum onto SDA. Mice were injected intravenously via the lateral tail veins with 0.5 ml of cell suspension (1×10^6 cells/mouse). The dose of 1×10^6 cells/mouse was verified to be 90% lethal within 7–10 days and capable of induction of systemic candidiasis in mice. The infected mice were randomly grouped, 10 in each group.

Recovery of C. albicans *from kidney*.—Experiments to determine the tissue burden of *C. albicans* in the kidneys of treated and untreated animals were conducted as previously described by Clark *et al.* (14). For ip or sc administration, test compounds were administered to infected mice at 7 and 24 h after inoculation; for iv injection, test compounds were delivered 18 and 42 h following inoculation. Twenty-four h after the final drug injection, mice were sacrificed with CO_2, and the kidneys were aseptically removed, weighed, and homogenized in 5.0 ml sterile saline solution. Following tenfold serial dilutions of the kidney homogenate, 100-μl aliquots were cultured in triplicate on SDA plates. Yeast colonies were enumerated for determination of CFU after about 16 h of incubation at 37°. The final result was recorded as CFU/mg tissue.

Survival study.—For evaluation of efficacy by the ip route, test compounds were given at varying doses beginning 7 h after inoculation and continuing once daily for 7 or 14 consecutive days; for evaluation by the iv route, compounds were administered on days 1, 3, and 5 post-inoculation. Infected mice in the control group were administered the appropriate solvent vehicle by following the same schedule for the test compounds. Amphotericin B at 2.5 mg/kg was used as positive control.

Statistical analysis.—The Wilcoxon nonparametric rank sum test was used in determining the p value for CFU recovery study. The unpaired Student-t test and ANOVA performed with StatView® software (Calabasas, CA), were used for the analysis of data obtained from survival experiments. Values of $p\leq0.05$ were considered significant.

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