L-671,329, A NEW ANTIFUNGAL AGENT

II. STRUCTURE DETERMINATION

CAROL F. WICHMANN, JERROLD M. LIESCH and ROBERT E. SCHWARTZ

Department of Natural Products Research,
Merck Sharp & Dohme Research Laboratories,
P.O. Box 2000, Rahway, N.J. 07065-0900, U.S.A.

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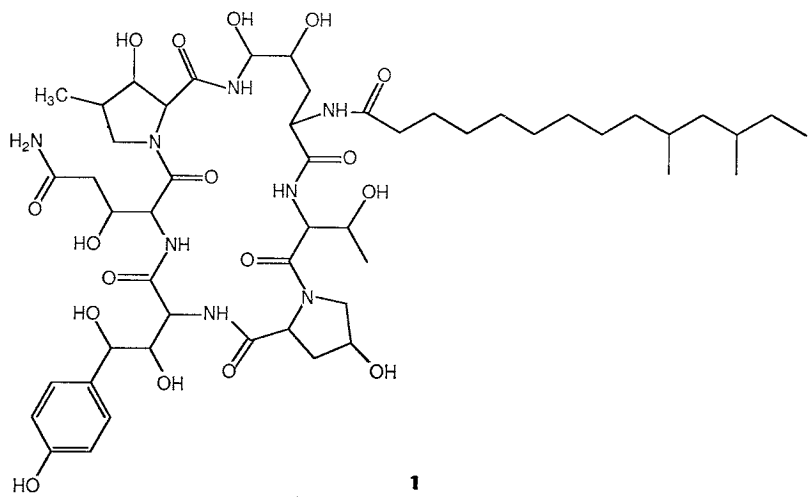
Based on spectroscopic data L-671,329, isolated from a filamentous fungus ATCC 20868, has been assigned the structure **1**. The compound is a lipopeptide antifungal agent and a structural analog of echinocandin B.

In the search for novel antifungal agents, L-671,329 was isolated from a culture of a filamentous fungus (deposited as ATCC 20868). The compound is a new member of the echinocandin structural family. We report here the structure determination for L-671,329. The fermentation, isolation and biological activities of this compound are discussed in the accompanying papers[1,2].

Materials and Methods

Mass spectral data were obtained on a Finnigan MAT 212 mass spectrometer at 90 eV. The high-resolution fast atom bombardment (HRFAB)-MS data was obtained on the same instrument using the peak matching method with a matrix of dithiothreitol-dithioerythritol-LiI spiked with polyethylene glycol (PEG)1000 as the internal reference. Samples were hydrolyzed in 6 N HCl in tightly capped vials at 110°C for 18 hours and then evaporated to dryness under a stream of nitrogen. The hydrolysate residue was derivatized with a 1 : 1 mixture of bis(trimethylsilyl)trifluoroacetamide(BSTFA) - pyridine (or BSTFA-d_9 - pyridine) at 50°C for 30 minutes. GC-MS analyses were carried out using a J&W DB-5 Durabond capillary column (15 m×0.3 mm, 25 μm film) with an initial temperature of 70°C (held for 2 minutes) programmed to 250°C at 5 degree/minute. Components were identified by interpretation of their mass spectra and by comparison to library spectra.



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All NMR spectra were recorded on a Varian XL-400 NMR spectrometer. ^{1}H spectra were recorded in CD_3OD and DMSO-d_6 at 400 MHz; chemical shifts are given in ppm referenced to CD_3OD at 3.30 ppm as internal standard. ^{13}C spectra were recorded in CD_3OD at 100 MHz using Waltz 16 proton decoupling; chemical shifts are given in ppm referenced to CD_3OD at 49.0 ppm as internal standard. ^{1}H-^{1}H chemical shift correlation spectra (COSY): Spectra were recorded in both CD_3OD (3 mg sample in 0.5 ml) and DMSO-d_6 (2 mg sample in 0.5 ml) using the standard pulse sequence[3]. A 2K×2K data set was accumulated in 512 increments with 32 or 64 transients for each value of t1. The delay time between scans was 1.0 second. ^{1}H-^{13}C chemical shift correlation spectra (HETCOR): Spectra were recorded in CD_3OD (25 mg sample in 0.5 ml) using the standard pulse sequence[4]. A 512×4K data set was accumulated in 64 increments with 896 transients for each value of t1. The delay time between scans was 1.0 second and the experiment was optimized for $^{1}J_{CH}=140$ Hz. The related experiment was used to establish long-range connectivities, optimizing for a multiple bond ^{13}C-^{1}H coupling constant of 7 Hz. The 512×4K data set was accumulated as above with 1,408 transients for each value of t1. Pure-absorptive mode two dimensional (2D)-nuclear Overhauser effect (NOESY): Spectra were recorded in DMSO-d_6 (2 mg sample in 0.5 ml, degassed) using the standard pulse sequence with phase-sensitive detection[5]. 1K×1K data sets were accumulated in 64 increments with 192 transients for each value of t1. Mix times were 0.1, 0.2, 0.4 and 1.0 second and the delay times between scans were 2.0, 1.5, 1.5 and 1.0 second, respectively, for the four experiments.

The UV absorption spectrum was measured with a Beckman DU-70 instrument affording UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 225 (sh, 140) and 274 (14). The IR absorption spectrum was obtained with a Perkin-Elmer model 1750 instrument using a multiple internal reflectance cell (MIR, ZnSe) affording IR (neat) cm^{-1} 3350 (br), 2925 and 1650 (br).

Results and Discussion

The empirical formula $C_{51}H_{82}N_8O_{17}$ was determined by HRFAB-MS (calcd for $C_{51}H_{82}N_8O_{17}$+Li: 1085.5958, found: 1085.6146). ^{13}C NMR data, including fully-decoupled and 'gated' decoupled experiments in CD_3OD at 100 MHz, indicated a carbon count of 51 carbons and a hydrogen count of 66 carbon-bound hydrogens consistent with the MS empirical formula and inferring the presence

Fig. 1. Amino acid subunits for L-671,329.

(A) Dihydroxyhomotyrosine, (B) hydroxyglutamine, (C) methylhydroxyproline, (D) hydroxyproline, (E) threonine, (F) dihydroxyornithine.

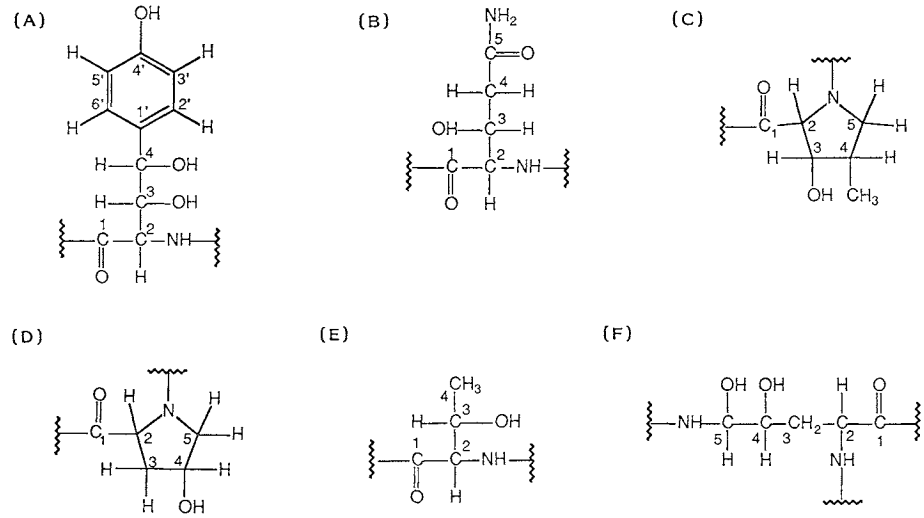


Fig. 2. ^{1}H-^{1}H chemical shift correlation (COSY) NMR spectrum of L-671,329 at 25°C in CD_3OD at 400 MHz.

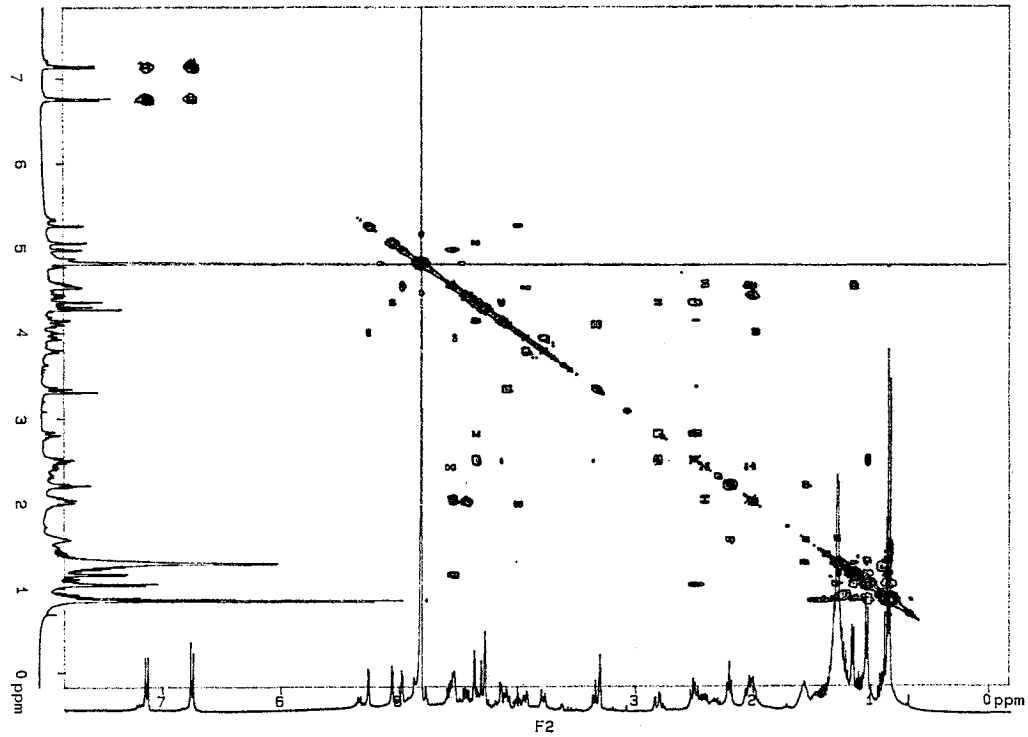
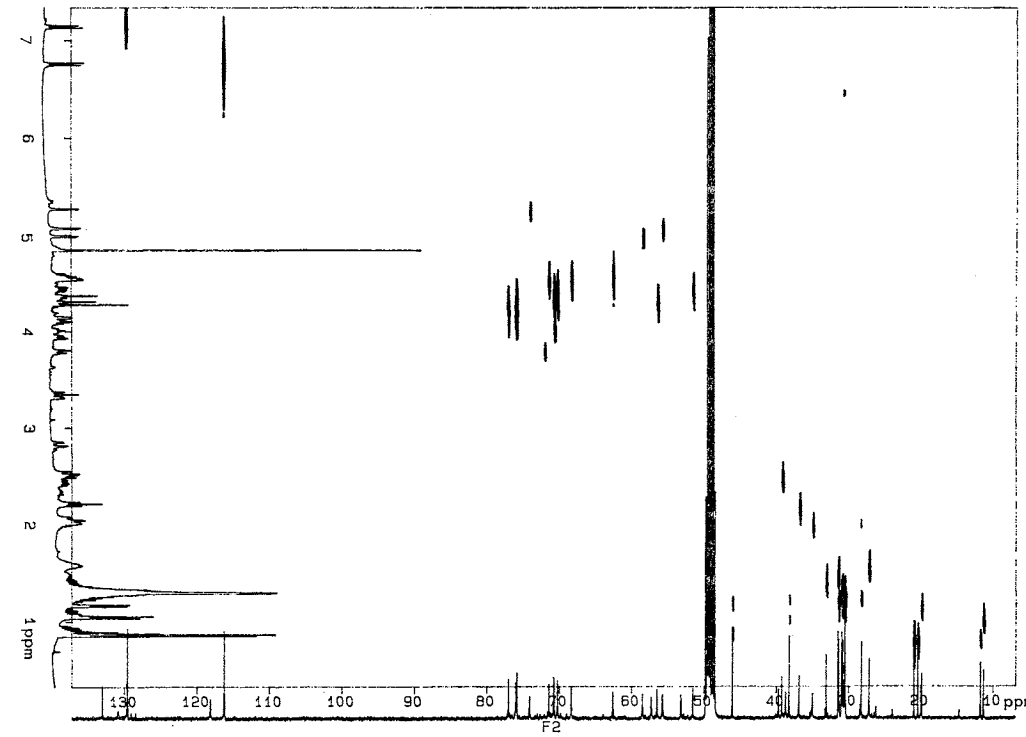


Fig. 3. ^{13}C-^{1}H chemical shift correlation (HETCOR) NMR spectrum of L-671,329 at 25°C in CD_3OD at 100 MHz.



of 16 exchangeable hydrogens and 15 degrees of unsaturation/rings. Carbon types included 4× CH_3CH, 1× CH_3CH_2, 15× CH_2, 3× CH, 14× CHX, 4× CH=, 1× C=, 1× XC= and 8× XC=O, accounting for 11 degrees of unsaturation and suggesting 4 rings.

The structures of the amino acid subunits (see Fig. 1) were established by MS and NMR analysis. GC-MS of the trimethylsilyl (TMS) derivative of the total acid hydrolysate indicated the presence of one equivalent each of threonine, hydroxyproline, methylhydroxyproline, and hydroxyglutamic acid. ^1H and ^{13}C NMR data (CD_3OD, 400 and 100 MHz) including ^1H double resonance studies, ^1H-^1H COSY and ^{13}C-^1H HETCOR experiments (Figs. 2 and 3, respectively) confirmed the MS assignments and established the presence of the additional amino acid residues 3,4-dihydroxyhomotyrosine and dihydroxyornithine. Complete carbon and proton assignments for the amino acid subunits are presented in Table 1. These subunits account for 3 rings, 6 amide and one carboxyl groups, and $C_{35}H_{51}N_8O_{16}$. This leaves a $C_{16}H_{31}O$ structural fragment, which must contain the remaining carboxyl

Table 1. ^1H and ^{13}C NMR data for L-671,329[a].

Assignment	^1H (ppm)	$^1J_{HH}$ (Hz)	^{13}C (ppm)
Dihydroxyhomotyrosine			
C-1	—		168.80 s
C-2	4.31 d		56.31 d
C-3	4.27[c]m		76.86 d
C-4	4.28[c]m		75.72 d
C-1′	—		132.86 s
C-2′,C-6′	7.13 d	(8.6)	129.43 d
C-3′,C-5′	6.75 d	(8.7)	116.06 d
C-4′	—		158.22 s
Hydroxyglutamine			
C-1	—		172.16 s
C-2	5.06 d	(4.0)	55.59 d
C-3	4.35 ddd	(3.8, 3.8, 9.6)	70.61 d
C-4	2.50 dd	(9.7, 15.7)	39.53 t
	2.82 dd	(3.6, 15.5)	
C-5	—		176.88 s
Methylhydroxyproline			
C-1	—		172.40[b]s
C-2	4.36 d	(2.2)	70.08 d
C-3	4.14 dd	(2.2, 4.5)	75.84 d
C-4	2.48[c]m		39.07 d
C-5	3.34 dd	(9.6, 9.6)	53.01 t
	4.09 dd	(7.5, 9.4)	
C-6	1.04 d	(6.8)	11.20 q
Hydroxyproline			
C-1	—		173.12 s
C-2	4.58 dd	(7.4, 11.3)	62.43 d
C-3	2.06[c]m		38.54 t
	2.42 ddd	(1.4, 6.8, 13.0)	
C-4	4.54[c]m		71.26 d
C-5	3.78 br d	(10.9)	57.11 t
	3.93 dd	(3.3, 11.1)	
Threonine			
C-1	—		174.24 s
C-2	4.97 d	(3.6)	58.35 d
C-3	4.54[c]m		68.18 d
C-4	1.16 d	(6.3)	19.75 q
Dihydroxyornithine			
C-1	—		172.35 s
C-2	4.43 dd	(6.1, 11.1)	51.39 d
C-3	2.00[c]m		34.83 t
C-4	4.00 ddd	(2.6, 6.8, 8.9)	70.55 d
C-5	5.26 d	(2.7)	73.94 d
10,12-Dimethylmyristate			
C-1	—		175.47[b]s
C-2	2.21 t	(7.3)	36.69 t
C-3	1.57 m		28.07 t
C-4	1.28 m		30.61 t
C-5	1.29 m		30.33 t
C-6	1.26 m		31.19 t
C-7	1.28 m		30.76 t
C-8	1.28 m		27.00 t
C-9	1.06 m		38.10 t
	1.27 m		
C-10	1.48 m		31.29 d
C-11	0.92 ddd	(6.8, 6.8, 13.7)	45.93 t
	1.23 ddd	(6.7, 6.8, 13.5)	
C-12	1.39 m		32.94 d
C-13	1.10 dd	(7.3, 10.2)	30.37 t
C-14	0.87 t	(7.2)	11.64 q
C-15	0.85 d	(6.5)	20.78 q
C-16	0.85 d	(6.5)	20.25 q

[a] Spectra recorded at 400 and 100 MHz for ^1H and ^{13}C, respectively, in CD_3OD at 25°C. Chemical shifts in ppm referenced to CD_3OD as internal standard at 3.30 ppm for ^1H and 49.0 ppm for ^{13}C.

[b] Interchangeable assignments.

[c] Overlapping or obscured resonances.

function. The carboxyl group suggests the presence of an acyl chain and accounts for the only unsaturation in this fragment, inferring a cyclic peptidic structure reminiscent of the echinocandins[6,7].

Because of significant overlap in the proton spectrum, assignment of the acyl chain structure was based on consideration of the carbon data. Assignment of the cyclic peptide left a $C_{16}H_{31}O$ structural fragment containing $2\times CH_3CH$, $1\times CH_3CH_2$, $10\times CH_2$, $2\times CH$ and $1\times XC{=}O$. Carbon chemical shifts were calculated[8,9] for several possible structural alternatives and compared to the experimental values. The terminal methyl shift was particularly diagnostic, as the observed resonance at 11.20 ppm was characteristic of the terminal methyl shift in the structural fragment $CH(CH_3)(CH_2)_nCH_3$ where n=1. For n=2, 3 and 4, the calculated values for the terminal methyl resonance were downfield at approximately 14 ppm. Similarly, calculated shifts were compared with experimental values to place the second methyl branch. Tentative assignment of all carbon resonances in the C_{16} acyl chain was based on comparison with the calculated values (see Fig. 4). Slight differences in chemical shift be-

Fig. 4. Acyl chain fragment of L-671,329.

(A) Ethyl ester of 10,12-dimethylmyristate with calculated carbon chemical shifts given in ppm and (B) L-671,329 acyl chain with carbon assignments shown in ppm.

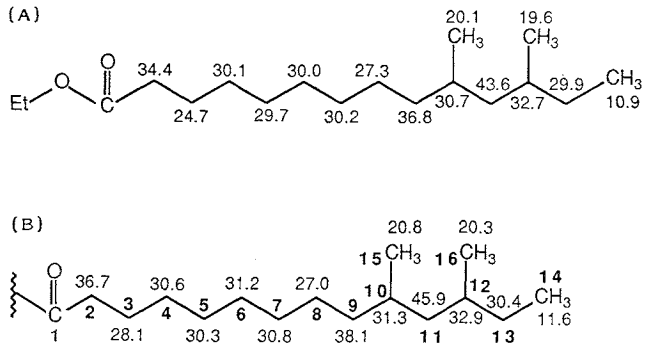
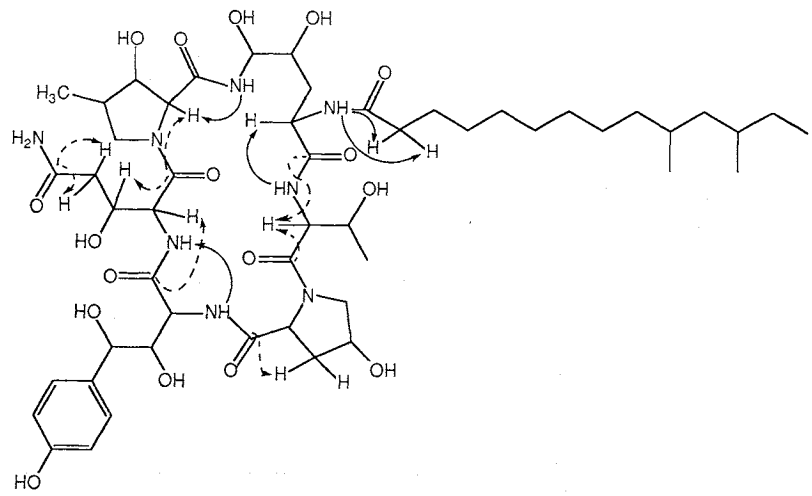


Fig. 5. The sequence of amino acid residues for L-671,329.



The sequence was established from nuclear Overhauser enhancement (NOE) data in DMSO-d_6 and multiple bond carbon hydrogen chemical shift correlation data in CD_3OD. The figure depicts ^1H-^1H NOEs as solid-line arrows and ^1H-^{13}C long-range chemical shift correlations as dotted-line arrows.

tween the calculated and experimental values are most likely due to solvent effects. Proton assignments followed from the ^{1}H-^{13}C HETCOR (see Fig. 4 and Table 1). Verification of the carbon and proton assignments was provided by analysis of COSY and double resonance proton experiments, which showed the predicted proton coupling patterns.

The sequence of the amino acid residues as in **1** was established by observation of nuclear Overhauser enhancements (NOEs) in DMSO-d_6†. Assignment of all resonances in the DMSO-d_6 proton spectrum was accomplished utilizing COSY and double resonance experiments. Of particular interest were the amide and α proton resonances. Pure absorptive mode NOESY[5] allowed observation of distance dependent NOEs resulting in the sequence assignment (see Fig. 5). The DMSO-d_6 data also confirmed that the hydroxyglutamic acid moiety observed by MS after hydrolysis was instead hydroxyglutamine in the intact peptide. Corroboration of structure **1** was obtained by a multiple-bond ^{1}H-^{13}C correlation experiment (selected for $J_{CH}=7$ Hz) which allowed assignment of the carbonyl carbons and confirmed the peptide sequence established by the NOE data.

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† During this work, these assignments were independently established for the related mulundocandin structure by MUKHOPADHYAY *et al.*[10].