KAZUSAMYCIN B, A NOVEL ANTITUMOR ANTIBIOTIC

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A novel antibiotic, kazusamycin B ($C_{32}H_{46}O_7$, MW 542), was isolated from the fermentation broth of *Streptomyces* sp. No. 81-484 and the structure was established mainly on the basis of its physico-chemical properties. Unambiguous ^{13}C NMR spectral analysis of kazusamycin B has been also accomplished. Kazusamycin B possesses potent cytocidal activities against L1210 (IC_{50} 0.0018 μg/ml) and P388 (IC_{100} 0.0016 μg/ml) leukemia cells *in vitro*.

In the course of a continuing search for novel antitumor antibiotics of microbial origin, it was found that *Streptomyces* sp. No. 81-484 produces under certain conditions a new antitumor antibiotic, kazusamycin B (**1**), as one of the major products together with already known congeners.

In preceding papers[1~3], we reported the production, isolation, physico-chemical properties and biological activities of kazusamycin A (previously reported as kazusamycin (**2**)) and the taxonomy of the producing organism, *Streptomyces* sp. No. 81-484[1,2]. This paper describes the fermentation, isolation, physico-chemical and biological properties, and chemical structure of a novel antitumor antibiotic, kazusamycin B (**1**).

Stock cultures of the producing organism were inoculated into 500-ml Sakaguchi flasks containing 100 ml of the seed medium and the flasks were incubated at 28°C for 4 days on a reciprocal shaker. The resulting culture (2.5 liters) was then transferred to a 200-liter fermentor containing 120 liters of the production medium and the fermentation was carried out at 28°C for 124 hours.

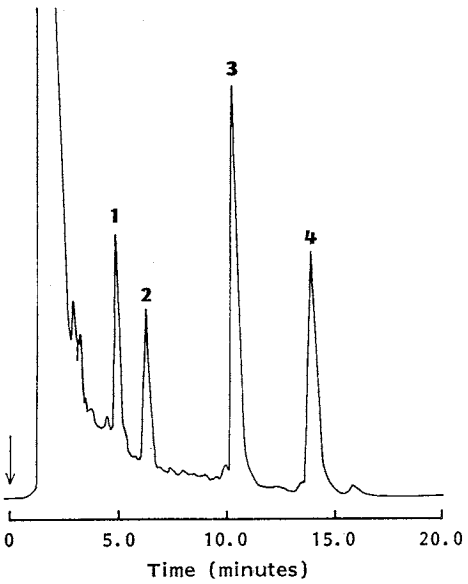
The fermentation broth was mixed with Hyflo Super-Cell and filtered. The broth filtrate was adsorbed on a column of Amberlite XAD-7 and the column washed firstly with water, then with 20% aq methanol; the active components were eluted with 60% aq methanol. The mycelial cake was extracted separately with methanol. After the aq methanol solutions containing active components were mixed and concentrated *in vacuo* to 6 liters, pH was adjusted to 6.8 with conc HCl and active substances were extracted with ethyl acetate. The ethyl acetate extract was concentrated *in vacuo* and subjected twice to silica gel column chromatography. The active fractions were combined and subjected to preparative HPLC (Fig. 1). Kazusamycin B (**1**) was isolated together with kazusamycin A (**2**)[1,2] and leptomycins A (**3**) and B (**4**)[4,5]. Identification of **2**~**4** was accomplished by comparison of UV, IR, MS, ^1H and ^{13}C NMR and $[\alpha]_D^{20}$ data with those reported in the literature[2,4,5] and further confirmed by direct comparison with authentic specimens (TLC and HPLC).

UV (Fig. 2) and IR (Fig. 3) spectra of kazusamycin B are almost superimposable on those of

kazusamycin A (**2**)[1,2] and leptomycins A (**3**) and B (**4**)[4,5]. It was concluded that kazusamycin B possesses the same skeleton as **2**~**4**. Field desorption mass spectrometry (FD-MS) of kazusamycin B gave peaks at *m/z* 543 ($M^+ + 1$) and 565 ($M^+ + Na$) (Table 1).

Fig. 1. HPLC of kazusamycins B (**1**) and A (**2**) and leptomycins A (**3**) and B (**4**).

Column conditions: Column, YMC A-303, 4.6$\phi \times$150 mm; solvent, MeOH - 0.01 M H_3PO_4 (75:25); flow rate, 1 ml/minute; temp, 40°C; detector, UV_{220}.



Structure elucidation of kazusamycin B (**1**) was mainly done by comparison of the physicochemical data with those of kazusamycin A (**2**)[1,2] and leptomycins A (**3**) and B (**4**)[4,5]. In the ^{13}C NMR spectrum of kazusamycin B, 32 signals were observed which were classified into $CH_3 \times 7$, $CH_2 \times 3$, $CH \times 16$, $C{=}O \times 3$ and three quarternary

Fig. 2. UV spectrum of kazusamycin B (**1**) (MeOH).

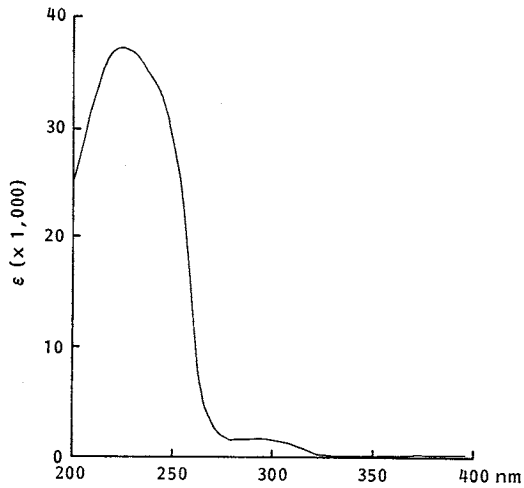


Fig. 3. IR spectrum of kazusamycin B (**1**) (KBr).

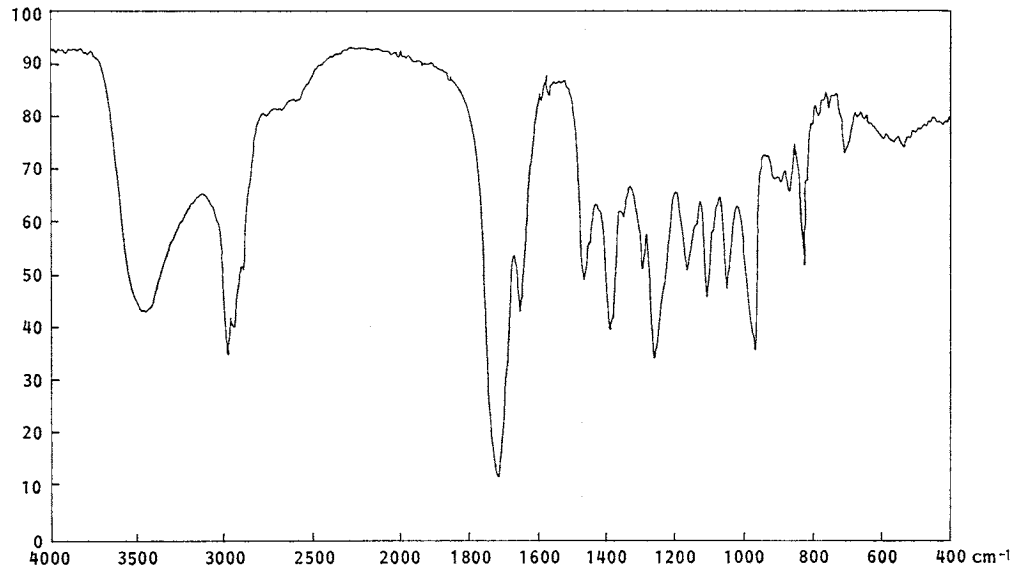


Table 1. Mass spectral data of kazusamycin B (1) and related compounds.

	Molecular formula	MW	SI-MS	FD-MS
Kazusamycin B (1)	$C_{32}H_{46}O_7$	542	543	565
			387	543
Kazusamycin A (2)	$C_{33}H_{48}O_7$	556	557	579
			401	557
Leptomycin A (3)	$C_{32}H_{46}O_6$	526	527	
			371	
Leptomycin B (4)	$C_{33}H_{48}O_6$	540	541	
			385	

SI-MS: Secondary ion mass spectra.

Table 2. ^1H and ^{13}C NMR spectral data of kazusamycins B (1) and A (2) (in $CDCl_3$).

No.	δ_H		δ_C	
	Kazusamycin B (1)	Kazusamycin A (2)	Kazusamycin B (1)	Kazusamycin A (2)
1	—	—	164.4 s	164.3 s
2	6.02 1H, dd	6.01 1H, dd	119.9 d	120.0 d
3	6.97 1H, dd	6.97 1H, dd	151.7 d	151.7 d
4	2.54 1H, ddq	2.54 1H, ddq	33.5 d	33.5 d
5	5.01 1H, ddd	4.99 1H, ddd	81.3 d	81.5 d
6	5.69 1H, dd	5.72 1H, dd	123.2 d	122.6 d
7	6.74 1H, d	6.64 1H, d	131.1 d	130.2 d
8	—	—	129.6 s	135.6 s
9	5.25 1H, d	5.23 1H, d	138.6 d	136.8 d
10	2.69 1H, m	2.68 1H, m	32.2 d	32.1 d
11	2.09 2H, ddd	2.10 2H, ddd	40.7 t	40.8 t
12	5.63 1H, ddd	5.64 1H, ddd	128.8 d	129.0 d
13	6.02 1H, d	6.01 1H, d	134.9 d	134.8 d
14	—	—	139.3 s	139.2 s
15	5.04 1H, d	5.04 1H, d	122.1 d	122.0 s
16	3.89 1H, ddd	3.89 1H, ddd	53.8 d	53.8 d
17	—	—	215.0 s	215.1 s
18	2.80 1H, dq	2.80 1H, dq	48.0 d	47.8 d
19	3.64 1H, dd	3.64 1H, dd	73.9 d	73.9 d
20	1.76 1H, m	1.76 1H, m	33.4 d	33.5 d
21	1.94 1H, dd	1.94 1H, dd	45.6 t	45.6 t
	2.23 1H, dd	2.22 1H, dd		
22	—	—	160.7 s	160.8 s
23	5.69 1H, br s	5.69 1H, br	116.9 d	116.8 d
24	—	—	170.6 s	170.6 s
25	1.07 3H, d	1.07 3H, d	12.3 q	12.3 q
26	1.83 3H, s	2.21 2H, q	20.4 q	26.5 t
27	—	1.06 3H, t		13.6 q
28	0.97 3H, d	0.98 3H, d	20.8 q	20.9 q
29	1.87 3H, s	1.86 3H, s	13.3 q	13.3 q
30	3.89 1H, dd,	3.89 1H, dd,	62.5 t	62.5 t
	3.58 1H, dd	3.59 1H, dd		
31	1.19 3H, d	1.19 3H, d	12.3 q	12.3 q
32	0.78 3H, d	0.78 3H, d	13.4 q	13.5 q
33	2.12 3H, s	2.13 3H, s	18.5 q	18.6 q

Fig. 4. Two-dimensional ^{1}H-^{13}C chemical shift correlation map of kazusamycin B (**1**) in $CDCl_3$.

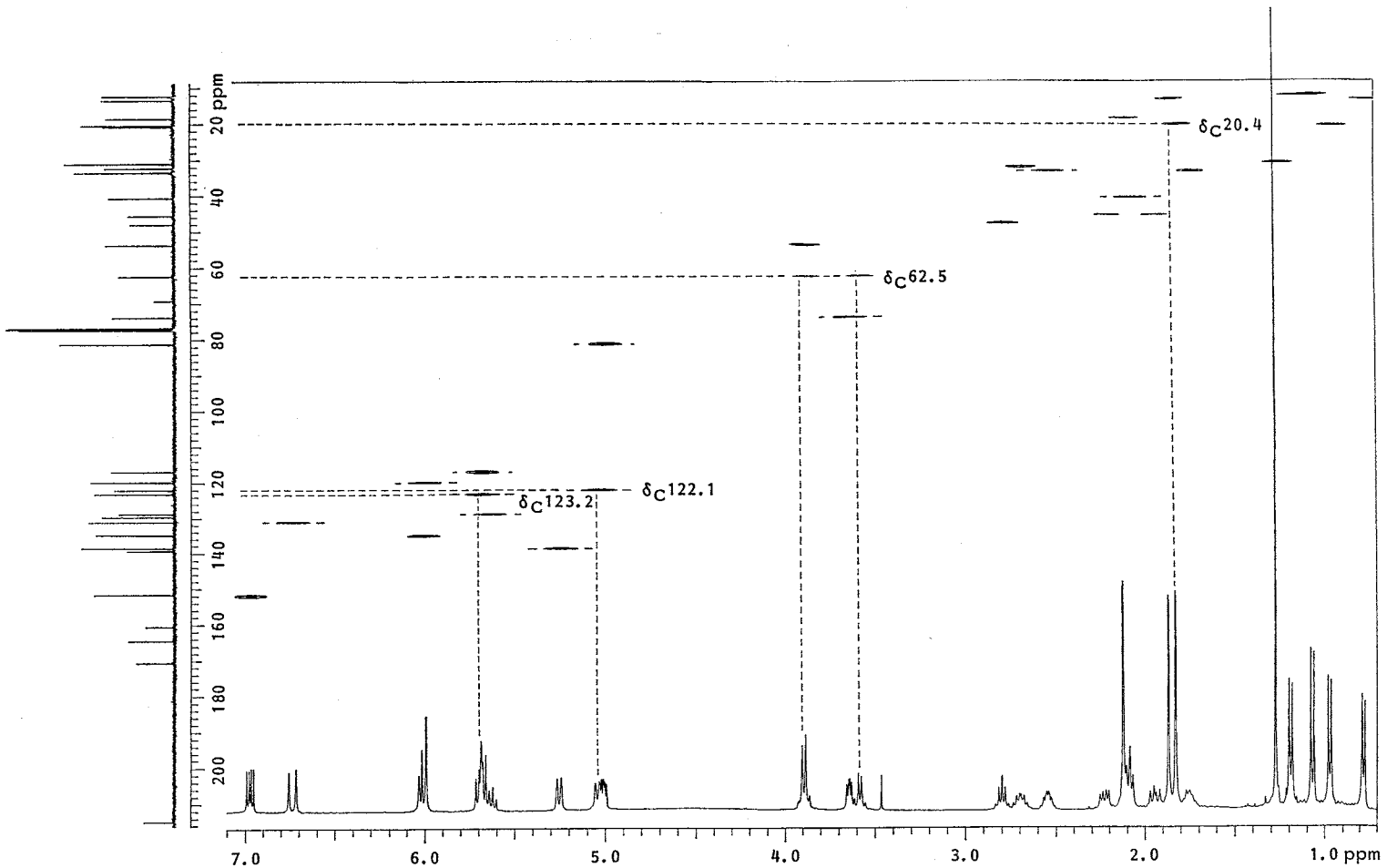
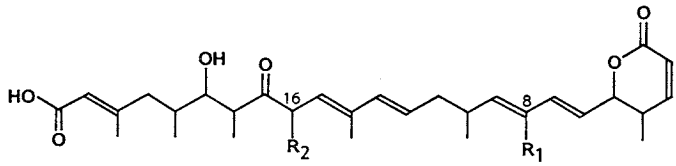


Fig. 5. Structures of kazusamycin B (**1**) and related compounds.



Kazusamycin B (**1**)	$R_1=CH_3$	$R_2=CH_2OH$
Kazusamycin A (**2**)	$R_1=CH_2CH_3$	$R_2=CH_2OH$
Leptomycin A (**3**)	$R_1=CH_3$	$R_2=CH_3$
Leptomycin B (**4**)	$R_1=CH_2CH_3$	$R_2=CH_3$

carbons (Table 2). Because a signal corresponding to a carbinol methylene moiety [δ_C 62.5 (C-30)], which was observed in the ^{13}C NMR of **2**, was also observed in the ^{13}C NMR of kazusamycin B (Fig. 4), it was concluded that **1** possessed three active hydrogens in the molecule and the number of hydrogens was calculated to be 46. From the observations described above, the molecular formula of **1** was established to be $C_{32}H_{46}O_7$ (MW 542), corresponding to one CH_2 less than kazusamycin A (**2**), one oxygen more than leptomycin A (**3**) and one CH_2 less and one oxygen more than leptomycin B (**4**).

In the ^1H NMR spectrum of kazusamycin B (**1**), most of the ^1H NMR signals of **1** corresponded to those of **2**, except for methyl signals (Table 2). Namely, seven methyl signals of **1** were divided into four doublets (δ_H 0.78, 0.97, 1.07 and 1.19) and three singlets (δ_H 1.83, 1.87 and 2.12), whereas the seven methyl signals of **2** were divided into four doublets (δ_H 0.78, 0.98, 1.07 and 1.19), two singlets (δ_H 1.86 and 2.13) and a triplet (δ_H 1.06). It was estimated that in the chemical structure of **1**, an ethyl group of **2** was replaced with a methyl group. Because an ethyl signal, which had been observed at δ_H 1.06 (3H, t) and 2.21 (2H, q) in the ^1H NMR of **2** disappeared in the ^1H NMR of **1**, whereas other six methyl signals of **1** corresponded to those of **2**, it was concluded that a methyl signal at δ_H 1.83 or 1.87 was the newly appeared signal in **1**. As it will be mentioned later, a methyl signal at δ_H 1.87 corresponding to δ_C 13.3 in the ^1H-^{13}C HETCOR experiments (Fig. 4) was assigned to the C-14-methyl (C-29) group of **2**. Thus, it was concluded that a methyl signal at δ_H 1.83 (δ_C 20.4) was the newly appeared signal of **1** and was assigned to C-8-methyl (C-26). Signals assigned to C-6~C-9 were shifted according to the change described above (Table 2). From all of the accumulated data described above, the structure of kazusamycin B was established (Fig. 5).

On the other hand, because the ^1H NMR assignments had been made through decoupling study, an unambiguous ^{13}C NMR assignment of **1** has been accomplished through the analysis of ^1H-^{13}C chemical shift correlation map (Fig. 4) and LSPD experiments. Especially, assignments of methyl signals of **1** have been completed straightforwardly through the analysis of two-dimensional (2D) NMR spectral data. Signals corresponding to newly appeared methyl moiety was observed at δ_H 1.83 (3H, s) and δ_C 20.4, whereas the ethyl signal at the C-8 position of kazusamycin A (**2**) [δ_H 1.06 (3H, t) and 2.21 (2H, q) and δ_C 13.6 and 26.5] disappeared. The following correlation has also been established: δ_C 122.1 (C-15)–δ_H 5.04 (C-15-H) and δ_C 123.2 (C-6)–δ_H 5.69 (C-6-H).

Assignments of three carbonyl carbons (δ_C 164.4, 170.6 and 215.0) and three quarternary carbons (δ_C 129.6, 139.3 and 160.7) have been accomplished through the combination of ^1H-^{13}C HETCOR (2D) NMR spectrum (Fig. 4) and LSPD experiments. When the signal at δ_H 6.97 (C-3-H) was ir-

Table 3. Antimicrobial spectrum of kazusamycin B (1) and related compounds.

Test organisms	MIC (μg/ml)			
	1	2	3	4
Bacillus subtilis ATCC 6633	>100	>100	>100	>100
Staphylococcus aureus FDA 209P	>100	>100	>100	>100
Micrococcus luteus ATCC 9341	>100	>100	>100	>100
Escherichia coli NIHJ	>100	>100	>100	>100
Saccharomyces cerevisiae IFO 0283	>100	>100	>100	>100
Candida albicans IAM 4888	>100	>100	>100	>100
Aspergillus fumigatus IAM 2530	>100	>100	>100	>100
Rhodotorula rubra IFO 0001	>100	>100	>100	>100
Trichophyton mentagrophytes TIMM 1189	>100	>100	>100	>100
Schizosaccharomyces pombe IAM 4863	0.05	0.03	NT	0.03
Rhizopus javanicus IAM 6241	3.13	0.78	0.39	0.78

NT: Not tested.

radiated, the signal at δ_C 164.4 was simplified so that the signal was assigned to C-1. Because δ_C 215.0 has been assigned to the C-17 position, δ_C 170.6 was assigned to C-24. When δ_H 2.12 [C-22-methyl (C-33)] was irradiated, the signal at δ_C 160.7 was simplified, so that the signal was assigned to C-22. On the other hand, when the signal at δ_H 6.02, which was assigned to C-13-H, was irradiated the signal at δ_C 139.3 was simplified, so that δ_C 139.3 was assigned to C-14 and the remaining quarternary carbon signal (δ_C 129.6) was assigned to C-8.

From all of the observations described above, the unambiguous ^{13}C NMR assignments of kazusamycin B (1) have been accomplished as shown in Table 2.

The antimicrobial spectrum of kazusamycin B (1) is similar to that of kazusamycin A (2). The antibiotic was active against *Schizosaccharomyces pombe* IAM 4863 and *Rhizopus javanicus* IAM 6241, but inactive against Gram-positive and Gram-negative bacteria (Table 3). Kazusamycin B (1) showed strong cytotoxic activity against L1210 leukemia (IC_{50} 0.0018 μg/ml) and P388 leukemia (IC_{100} 0.0016 μg/ml) cells *in vitro*. We are presently investigating the antitumor activity against various kinds of murine tumors *in vivo* and the results will be reported elsewhere.

Discussion

It was found that *Streptomyces* sp. No. 81-484 produced a novel antibiotic, kazusamycin B (1) ($C_{32}H_{46}O_7$, MW 542), under certain conditions, together with formerly isolated congeners kazusamycin A[1~3] (PD 114721[6,7]) (2) and leptomycins A (3) and B[4,5] (PD 114720[6,7]) (4). It is interesting that the yield of 1, which has not been observed before[1] is now higher than that of 2.

The chemical structure of kazusamycin B (1) was established mainly on the basis of its physicochemical properties. An unambiguous ^{13}C NMR spectral assignment of 1 has also been made.

Kazusamycin B (1) possesses potent cytocidal activities against L1210 leukemia (IC_{50} 0.0018 μg/ml) and P388 leukemia (IC_{100} 0.0016 μg/ml) cells *in vitro*.

Experimental

General Experimental Procedures

Melting points were determined using a Yanagimoto MP-3 hot stage microscope and are uncorrected. UV spectra were recorded on a Shimadzu model UV 200S spectrophotometer and IR spectra on a Jasco model A-102 interferometer. Mass spectra were obtained with a Jasco model DX-300

mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Bruker WM-360 and Varian XL-400 instruments and run in $CDCl_3$ solution. Wacogel C-200 (Wako Pure Chemical Industries, Ltd.) was used for column chromatography and DC-Fertigplatten Kieselgel 60 (Merck) was used for TLC analysis.

Taxonomic Studies

Taxonomic studies of *Streptomyces* sp. No. 81-484 were reported in the previous paper[1].

Fermentation

Stock cultures of the producing organism were inoculated into 500-ml Sakaguchi flasks each containing 100 ml of the seed medium consisting of glucose 2.0%, peptone 0.5%, dry yeast 0.3%, meat extract 0.5%, NaCl 0.5% and $CaCO_3$ 0.3% (pH 7.0). The flasks were incubated at 28°C for 4 days on a reciprocal shaker. The resulting culture (2.5 liters) was then transferred to a 200-liter fermentor containing 120 liters of a medium consisting of glucose 3.0%, meat extract 0.75%, dry yeast 0.3% and $MgSO_4 \cdot 7H_2O$ 0.2% (pH 7.0). Fermentation was carried out at 28°C for 124 hours.

Isolation of Kazusamycin B (1) and Related Compounds

The fermentation broth (110 liters) was mixed with 3% Hyflo Super-Cell (Johns-Manville Sales Co., U.S.A.), filtered and washed with 20 liters of water. The broth filtrate was absorbed on a column (5 liters) of Amberlite XAD-7 and the column washed firstly with water, then with 20% aq methanol, and eluted with 60% aq methanol. The active fractions were collected and concd *in vacuo* to 3 liters. The mycelial cake was extracted with 45 liters of methanol and the extract concd *in vacuo* to 3 liters. After both aq solutions (6 liters) were combined, pH was adjusted to 6.8 with conc HCl and active substances were extracted with ethyl acetate (2×6 liters). The organic extract was dried over Na_2SO_4 (anhydrous), concd *in vacuo* and applied to a column packed with silica gel (Wakogel C-200, 1,000 ml), and eluted with *n*-hexane - acetone (gradient). The active fractions were combined, dissolved in a small volume of benzene, chromatographed over a silica gel column and eluted with ethyl acetate. The resulting mixture was subjected to preparative HPLC (YMC A-343, $20\phi \times 250$ mm, Yamamura Chemical Lab. Co., Ltd.; UV 220 nm) with a solvent system of methanol and 0.05 M phosphoric acid (pH 2.3) (3 : 1). Each fraction was adjusted to pH 6.0 with 0.1 N NaOH, evaporated under reduced pressure and extracted with ethyl acetate. The organic solvent extract was evaporated to dryness *in vacuo*. Kazusamycin B (**1**, 347 mg) was isolated together with kazusamycin A (**2**, 253 mg)[1,2], leptomycins A (**3**, 611 mg) and B (**4**, 434 mg)[3,4].

Physico-chemical Properties of Kazusamycin B (1)

Kazusamycin B (**1**) was isolated as a pale yellow powder: MP 53~55°C; $[\alpha]_D^{20}$ −152.0° (*c* 0.77, $CHCl_3$); UV spectrum is shown in Fig. 2; IR spectrum is shown in Fig. 3; MS data are given in Table 1; ^{1}H and ^{13}C NMR spectral data are shown in Table 2; 2D ^{1}H-^{13}C chemical shift correlation map is shown in Fig. 4.

Antimicrobial Activity of Kazusamycin B (1) and Related Compounds

The antimicrobial spectra of kazusamycin B (**1**) and related compounds were determined by an agar dilution method (inoculum size: 10^6 cells/ml) using Mueller-Hinton agar medium for bacteria and Sabouraud agar for fungi. The minimum inhibitory concentration (MIC) was observed after 18-hour incubation at 37°C for bacteria or 48-hour incubation at 28°C for fungi. The results are shown in Table 3.

Effect of Kazusamycin B (1) on L1210 Leukemia and P388 Leukemia Cells

L1210 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum; the P388 mouse leukemic cells were maintained in the peritoneal cavity of CDF_1 mice. To determine the cytotoxicity of kazusamycin B (**1**), L1210 or P388 cells (1×10^4) in 1 ml of medium (RPMI-1640+ 10% FBS) containing various concentrations of the antibiotic were placed in a tissue culture plate (Falcon, 24-cell) and incubated for 72 hours at 37°C in a 5% CO_2-95% air atmosphere. At the end of the incubation period, the cells were counted by a hemacytometer.

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