GLIDOBACTINS A, B AND C†, NEW ANTITUMOR ANTIBIOTICS

I. PRODUCTION, ISOLATION, CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITY

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New antitumor antibiotic glidobactins A, B and C were isolated from the cultured broth of a gliding bacterium, *Polyangium brachysporum* sp. nov. No. K481-B101. They are novel molecules carrying the common cyclic tripeptide nucleus substituted with different α,β,γ-δ-unsaturated fatty acids. Glidobactins exhibit broad inhibitory activity against fungi and yeasts, and prolong the life span of mice inoculated with P388 leukemia cells.

In the course of our systematic search for microbial metabolites effective against experimental tumors, an unusual Gram-negative bacterial strain No. K481-B101 (ATCC 53080), which was designated as *Polyangium brachysporum* sp. nov., was found to produce a complex of novel antitumor antibiotics[1,2]. They were recovered from the fermentation broth by extraction with organic solvent and purified by a series of chromatographies to yield three components, glidobactins A, B and C. As described in the following paper, structural studies demonstrated that glidobactins A, B and C are acylated cyclic tripeptides hitherto unobserved in nature[3]. This paper presents the production, isolation, chemical properties and biological activity of glidobactins A, B and C.

Antibiotic Production

The stock culture of *P. brachysporum* K481-B101 was propagated at 28°C for 3 days on agar slant medium composed of soluble starch 0.5%, glucose 0.5%, meat extract 0.1%, yeast extract 0.1%, NZ-case 0.2%, NaCl 0.2%, $CaCO_3$ 0.1% and agar 1.6% (pH 7.0). A well grown agar slant was used to inoculate the vegetative medium consisting of corn starch 2%, soybean meal 3%, $MgSO_4 \cdot 7H_2O$ 0.3% and $CaCO_3$ 1% (pH 7.0, before sterilization). After incubation at 28°C for 3 days on a rotary shaker (250 rpm), 5 ml of the growth was transferred into a 500-ml Erlenmeyer flask containing 100 ml of the production medium having the same composition as the vegetative medium.

The antibiotic production was monitored by the paper-disk agar diffusion method using *Candida albicans* A9540 as the test organism. The fermentation was continued for 4 days at 28°C on a rotary shaker until the antibiotic production reached a maximum of 100 μg/ml.

The fermentation was also carried out in stir-jar fermentors. A 500-ml portion of the seed culture obtained by flask fermentation was used to inoculate 10 liters of the production medium in 20-liter vessels. The fermentation was carried out at 28°C with agitation at 250 rpm and aeration at 10 liters per minute. The antibiotic production reached a maximum of 150 μg/ml after 40 hours'

† Glidobactins A, B and C are originally celled as BU-2867T A, B and C or BMY-28119, -28262 and -28120, respectively.

fermentation.

Isolation and Purification

The whole fermentation broth (48 liters) was centrifuged with the aid of a Sharples centrifuge. The mycerial cake was homogenized with 7 liters of methanol and the mixture stirred for 1 hour. After removal of the insolubles by filtration, the methanol extract was evaporated to an aqueous solution which was combined with the broth filtrate and extracted with butanol (24 liters). The extract was concentrated to 0.5 liter which was poured into *n*-hexane (3.5 liters) under vigorous stirring to precipitate the crude antibiotic (41 g). This solid was chromatographed on a column of silica gel (Wakogel C-200, 760 ml) eluting with ethyl acetate and increasing amounts of methanol (0~50%). The bioactivity eluted was detected by the paper-disc assay using *C. albicans* A9540 as the test organism. The active fractions were combined and evaporated to yield pale yellow powder of glidobactin complex (13 g). A 200-mg portion of this solid was chromatographed on a reverse-phase silica gel column (Merck Kieselgel 60 silanized, 100 ml) using ethanol - water (3 : 7 to 5 : 5) as eluants. The eluate was monitored by antifungal bioassay and TLC (silanized, ethanol - water (55 : 45)). The first active fractions were combined and evaporated under reduced pressure to afford pure white solid of glidobactin A (61 mg). This solid was crystallized from aqueous methanol to deposit colorless needles (34 mg). Evaporation of the second and third active fractions yielded glidobactins B (1 mg) and C (11 mg), respectively. Glidobactin C was crystallized from methanol as fine needles. Repetition of the above reverse-phase silica gel chromatography afforded a total of 3.9 g of glidobactin A, 44 mg of glidobactin B and 342 mg of glidobactin C.

Physico-chemical Properties

Glidobactins A and C were obtained as colorless needles while glidobactin B was isolated as white crystalline powder. The three components displayed very similar physico-chemical and spectroscopic properties but they were distinguished from each other by the TLC and the HPLC systems as described in Table 1. Glidobactins are readily soluble in methanol, ethanol, butanol and dimethyl sulfoxide, slightly soluble in chloroform, acetonitrile and ethyl acetate and practically insoluble in

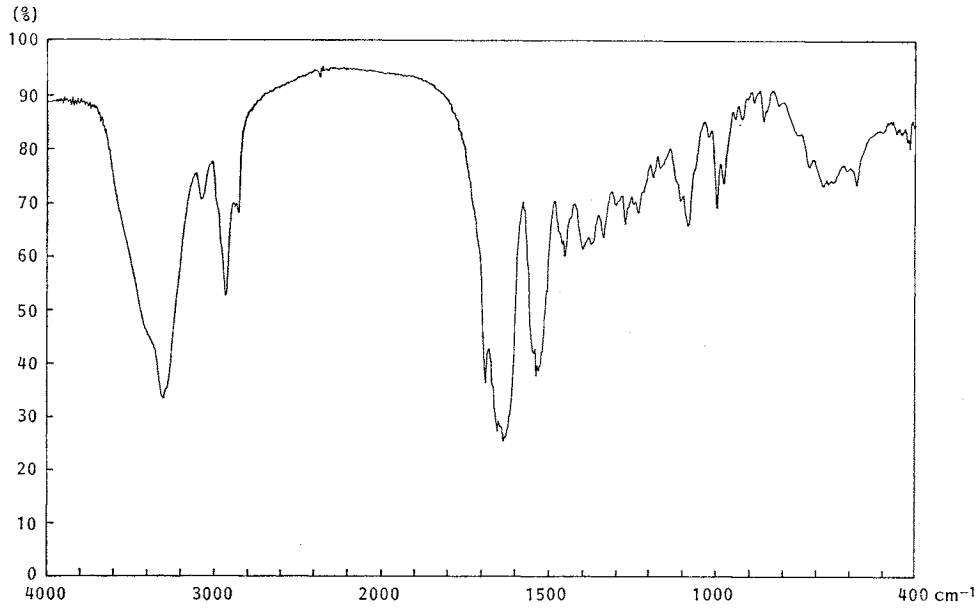
Table 1. Physico-chemical properties of glidobactins A, B and C.

	A	B	C
Nature	Colorless needles	White powder	Colorless needles
MP (°C)	259~261	232~234	273~275
$[\alpha]_D^{24}$ (*c* 0.5, MeOH)	−111°	−92°	−104°
Microanalysis:			
Calcd for	$C_{27}H_{44}N_4O_6 \cdot \frac{1}{2}H_2O$:	$C_{29}H_{46}N_4O_6 \cdot 1\frac{1}{2}H_2O$:	$C_{29}H_{48}N_4O_6$:
	C 61.22,	C 60.71,	C 63.48,
	H 8.56,	H 8.61,	H 8.82,
	N 10.58	N 9.77	N 10.21
Found:	C 60.90,	C 60.89,	C 63.48,
	H 8.65,	H 8.31,	H 8.91,
	N 10.47	N 9.23	N 10.16
EI-MS (*m/z*)	520 (M^+)	546 (M^+)	548 (M^+)
UV λ_{max}^{MeOH} nm (ε)	261 (35,000)	261 (28,000)	261 (36,000)
TLC* (Rf)	0.45	0.41	0.34
HPLC** (Rt)	6.43	7.93	11.33

EI-MS: Electron impact mass spectrum, Rt: retention time.

* Silanized plate; EtOH - H_2O (55 : 45). ** Lichrosorb RP-18; EtOH - H_2O (65 : 35).

Fig. 1. IR spectrum of glidobactin A (KBr disk).



n-hexane and water. They gave a positive response to Rydon-Smith reagent and colored upon spray of iodine or sulfuric acid on TLC plate. They were negative to ninhydrin, Sakaguchi, anthrone and Dragendorff reaction. Glidobactins A, B and C were analyzed for $C_{27}H_{44}N_4O_6$, $C_{29}H_{46}N_4O_6$ and $C_{29}H_{48}N_4O_6$, respectively, by the mass spectrometry and microanalyses. The UV spectra of the three components exhibited a single maximum at 261 nm in methanol which did not shift in acidic nor alkaline solution. The IR spectrum of glidobactin A in KBr (Fig. 1) showed strong amide bands at around 1630 and 1540 cm^{-1} and OH and/or NH absorption at 3300 cm^{-1}. Its ^1H NMR spectrum (Fig. 2) revealed the presence of six olefinic protons (δ 6.10, 6.12, 6.19, 6.23, 6.38 and 7.01) and four amide protons (δ 7.19, 7.56, 7.69 and 8.43). Three methyl signals (δ 0.87, t, 1.03, d and 1.24, d) were also observed in the spectrum. The ^{13}C NMR spectrum of glidobactin A (Fig. 3) showed more than 22 carbon signals including four carbonyl carbons, six olefinic carbons and three methyl carbons. Glidobactins B and C showed the ^{13}C NMR spectra very similar to that of glidobactin A except the presence of two more olefinic carbons in glidobactin B and two more methylene carbons in glidobactin C than in glidobactin A.

Antimicrobial Activity

The MICs of glidobactins A, B and C were determined for various microorganisms by the serial agar dilution method. Nutrient agar (Eiken) was used for bacteria and Sabouraud dextrose agar (Difco) for fungi. The inoculum size was adjusted to 10^4 cfu/ml for bacteria and $10^5 \sim 10^7$ cfu/ml for fungi.

Glidobactins A, B and C did not inhibit the growth of Gram-positive and Gram-negative bacteria at 50 μg/ml. The antifungal activities of glidobactin components are shown in Table 2. Component C showed potent antifungal activity against clinically important pathogenic fungi such as *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Trichophyton mentagrophytes* and *Mucor spinosus*. Components A and B were somewhat less active than component C, but their antifungal

Fig. 2. 400 MHz ^{1}H NMR spectrum of glidobactin A (70°C in DMSO-d_6).

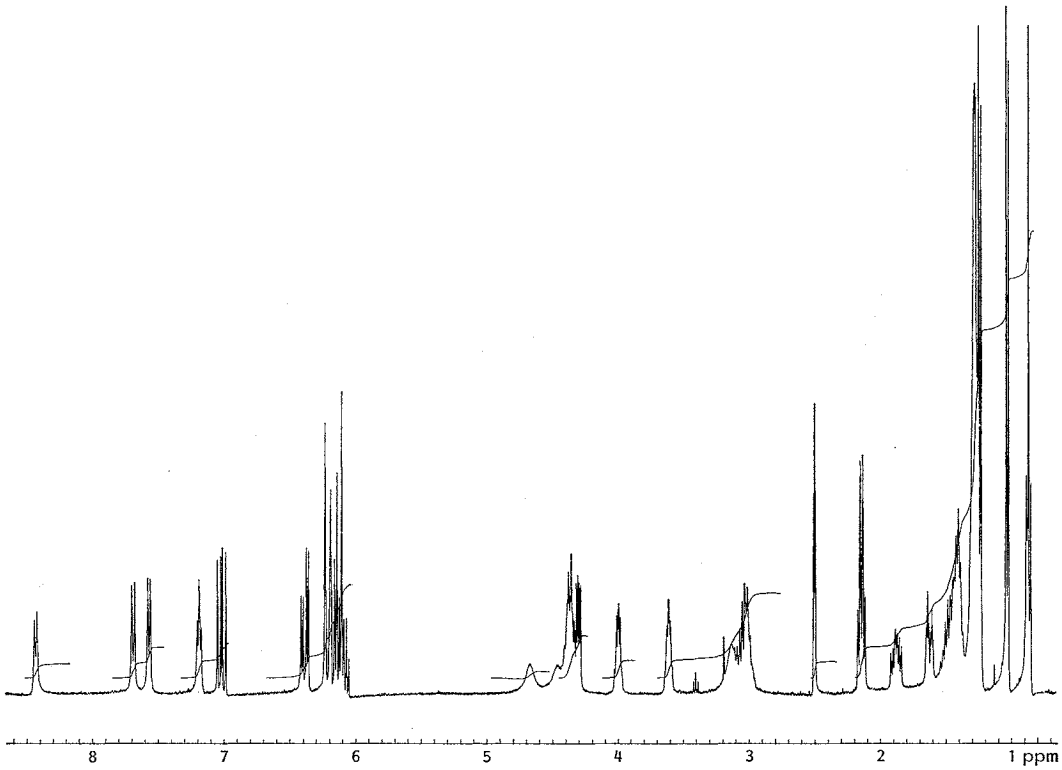


Fig. 3. 100 MHz ^{13}C NMR spectrum of glidobactin A (70°C in DMSO-d_6).

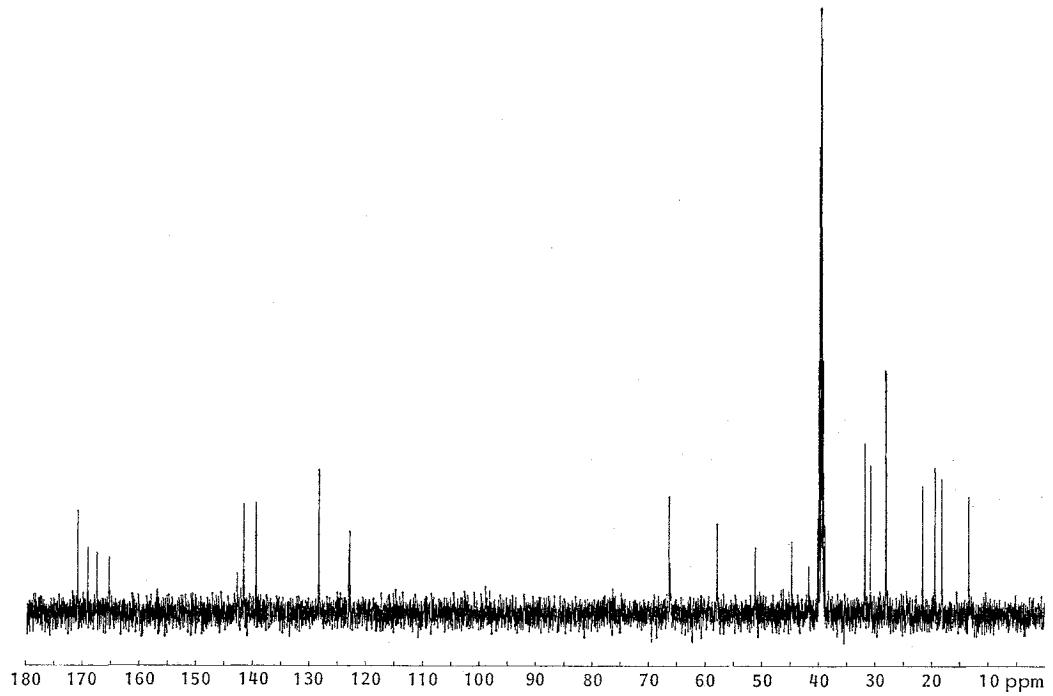


Table 2. Antifungal activity of glidobactins.

Test organisms	MIC (μg/ml)		
	Glidobactin A	Glidobactin B	Glidobactin C
Candida albicans IAM 4888	3.1	3.1	1.6
C. albicans A9540	1.6	1.6	0.8
Cryptococcus neoformans D 49	25	25	3.1
C. neoformans IAM 4514	25	25	3.1
Aspergillus fumigatus IAM 2530	1.6	3.1	0.8
A. fumigatus IAM 2034	1.6	3.1	0.8
A. flavus FA 21436	25	25	50
Fusarium moniliforme A 2284	>50	>50	>50
Piricularia oryzae D 91	>50	>50	>50
Trichophyton mentagrophytes D 155	25	12.5	1.6
T. mentagrophytes No. 4329	25	12.5	6.3
Blastomyces dermatitidis IFO 8144	50	50	>50
Sporothrix schenckii IFO 8158	>50	>50	>50
Petriellidium boydii IFO 8073	>50	>50	50
Mucor spinosus IFO 5317	1.6	0.8	0.2

Medium: Sabouraud dextrose agar.

Table 3. Effects on the cell growth and macromolecular biosynthesis.

	IC_{50} (μg/ml)				
	Cytotoxicity[a]		Inhibition of biosynthesis[b] vs. L1210 cells		
	B16	HCT-116	DNA	RNA	Protein
Glidobactin A	0.13	0.122	>100	>100	0.03
Glidobactin B	0.12	0.013	>100	>100	0.15
Glidobactin C	0.23	0.018	>100	>100	0.06
Doxorubicin	0.06	0.19	4.1	3.9	>100

[a] Tumor cells + drug $\xrightarrow[37°C]{72\text{ hours}}$ cell count (dye-uptake method).

[b] L1210 cells + drug $\xrightarrow[37°C]{15\text{ minutes}}$ labeled precursor $\xrightarrow[37°C]{4\text{ hours}}$ radioactivity in acid-insoluble fraction.

spectra were similar to that of the latter.

Antitumor Activity

Effects on the cell growth and macromolecular biosynthesis were determined according to the published method[4,5]. As shown in Table 3, glidobactins A, B and C exhibited strong cytotoxicity against B16 and HCT-116 cells and inhibited protein synthesis.

The *in vivo* antitumor activity of glidobactin was determined in female CDF_1 and male BDF_1 mice. Lymphocytic leukemia P388 (CDF_1 and BDF_1 mice) and lymphoid leukemia L1210 (BDF_1 mice) were inoculated by intraperitoneal injection of 0.4 ml of diluted ascitic fluid containing 10^6 and 10^5 cells per mouse, respectively. Melanotic melanoma B16 (BDF_1 mice) was implanted in 0.5 ml of a 10%-tumor brei intraperitoneally. Test materials were dissolved in 0.9% saline containing 10% dimethyl sulfoxide and graded doses of them were administered to the mice intraperitoneally 24 hours after tumor implantation. Either olivomycin or mitomycin C was comparatively tested as a reference compound in the experiments. Glidobactins A, B and C showed antitumor activity against P388

Table 4. Antitumor activity of glidobactins against P388 leukemia.

	T/C (%) of MST[a]			
	Dose (mg/kg/day, ip[b])			
	3	1	0.3	0.1
Glidobactin A		140[c]	130	120
Glidobactin B	165	140	120	
Glidobactin C	155		130	
Olivomycin	140	135	100	

[a] Ratio of median survival time of test and control mice (CDF_1, $n=4$).
[b] Schedule 1: Treatments given once a day on days 1, 4 and 7.
[c] Values $\geqq 125\%$ indicate significant antitumor effect.

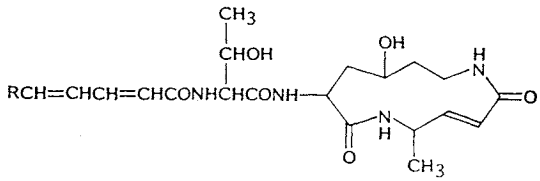
Table 5. Antitumor activity of glidobactins A and C.

	T/C (%) of MST[a]					
	Dose (mg/kg/day, ip[b])					
	2	1	0.5	0.25	0.13	0.06
P388 Leukemia						
Glidobactin A	67	228[c]	186	156	147	136
Glidobactin C	234	189	175	159	153	123
Mitomycin C		290	240	170	150	130
L1210 Leukemia						
Glidobactin A			129	118	118	106
Mitomycin C		140	141	129	129	106
B16 Melanoma						
Glidobactin A	Toxic	125	116	113	100	
Mitomycin C	63	181	163	141	131	

[a] Ratio of median survival time of test and control mice (BDF_1, $n=12$).
[b] Schedule 2: Treatments given once daily for 9 days.
[c] Values $\geqq 125\%$ indicate significant antitumor effect.

leukemia by the treatment schedule 1 (once a day on days 1, 4 and 7) by the following order in terms of minimum effective dose: A=C>B (Table 4). As shown in Table 5, glidobactins A and C were highly active against P388 leukemia by the treatment schedule 2 (once daily on days 1 to 9). However, glidobactin A showed moderate activity against L1210 leukemia and B16 melanoma. When the acute toxicity of glidobactins A and C determined in male *dd*Y mice by single intraperitoneal administration, the LD_{50} values were 8.1 and 25 mg/kg, respectively.

Fig. 4. Structures of glidobactins A, B and C.



A R=$CH_3(CH_2)_6$–
B R=$CH_3(CH_2)_4$–CH=CH$(CH_2)_2$–
C R=$CH_3(CH_2)_8$–

Discussion

Glidobactins A, B and C are novel antitumor antibiotics produced by a strain of *P. brachysporum* sp. They are colorless crystalline compounds having single UV absorption maximum at 261 nm. A combination of chemical and microbial degradations and spectral studies established that glidobactins A, B and C are unique acylated cyclic tripeptides and they differ from each other only in their acyl

side chain moieties (Fig. 4). Glidobactins A, B and C exhibited broad antifungal activity *in vitro* but their *in vivo* antifungal activity was marginal. They showed strong cytotoxicity against various tumor cells and prolonged the life span of mice implanted with P388 leukemia.

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