NEW ANTITUMOR ANTIBIOTIC, LL-D05139β

FERMENTATION, ISOLATION, STRUCTURE DETERMINATION AND BIOLOGICAL ACTIVITIES

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The LL-D05139 complex, containing LL-D05139β and azaserine, was recovered from the fermentation filtrate of *Glycomyces harbinensis* (NRRL 15337). A chemically defined medium was developed which favored the production of LL-D05139β. Antibiotic LL-D05139β was isolated from the fermentation filtrate by adsorption on granular carbon and further purified by chromatography on microcrystalline cellulose. Acid hydrolysis of LL-D05139β gave one molar equivalent each of alanine and serine. Both amino acids were found to have the L-configuration by GC analysis on a chiral column and alanine was assigned to be the *N*-terminal amino acid by Edman degradation. This information coupled with IR, UV, ^{1}H NMR, ^{13}C NMR and MS spectral data allowed us to assign the structure of LL-D05139β as alanylazaserine. LL-D05139β demonstrated greater antibacterial and biochemical induction assay activities than azaserine. The two drugs showed similar antitumor activities.

The LL-D05139 complex was produced by *Glycomyces harbinensis*, isolated from a soil sample collected in the vicinity of Harbin, China.[1] The activity in the fermentation broth was initially detected as a cell-wall biosynthesis inhibitor by the use of a β-lactam supersensitive mutant of *Escherichia coli*. The antibiotic complex inhibited the growth of *Proteus mirabilis* and *Staphylococcus aureus* but not their respective L-forms.[2] The complex was also found to be active in the biochemical induction assay (BIA),[3] a detection system for agents directly or indirectly initiating DNA damage, which has been used for antitumor drug screening.[4]

The fermentation, isolation, structure determination and biological activities of LL-D05139β (**1**) are described in this paper.[5~8] The other component of the complex, LL-D05139α (**2**), was identified as azaserine, a known antitumor antibiotic.[9,10]

Fermentation

Early analytical work showed that *Glycomyces harbinensis* (NRRL 15337) produced a mixture of two components with LL-D05139α (azaserine) predominating. Fermentation studies were initiated to selectively improve the yield of LL-D05139β. A variety of complex carbohydrate and nitrogen sources were tested and all resulted in substantial yields of the α component only. However, the use of a chemically defined medium led to fermentations with enhanced yields of LL-D05139β ($\sim$330 μg/ml) and reduced yields of LL-D05139α ($\sim$95 μg/ml). The composition of the seed medium and fermentation media for production of α (medium A) and β (medium B) are shown in Table 1. Seed medium (50 ml in 250-ml Erlenmeyer flasks) was inoculated with mycelial and spore scrapings from slant surfaces and was incubated on a rotary shaker (200 rpm) at 28°C for 3 days. A 5.0-ml

Table 1. Seed and production media for the LL-D05139 antibiotics.

Seed medium[a] (g/liter)		Production medium A[a] (g/liter)		Production medium B[a] (g/liter)	
Glucose	10	Glucose	10	Glucose[c]	20
Dextrin	20	Bacto-peptone[b]	5	$NaNO_3$	1
Yeast extract	5	Molasses	20	$FeSO_4 \cdot 7H_2O$	0.1
NZ-Amine A	5	$CaCO_3$	1	$MgSO_4 \cdot 7H_2O$	0.2
$CaCO_3$	1			$CaCO_3$	5

[a] The pH of all media was adjusted to 6.8±0.2; tap water was used for all media preparation.
[b] Difco Laboratories.
[c] Sterilized separately.

Table 2. Physico-chemical properties of antibiotic LL-D05139β.

Appearance	Pale yellow amorphous solid
Stability	Decomposes rapidly below pH 5.0, fairly stable in weakly basic solutions
$[\alpha]_D^{26}$	+57±5° (*c* 0.19, H_2O)
MW (FAB-MS) *m/z*	244
UV $\lambda_{max}^{H_2O}$ nm (ε)	250 (646) (Fig. 1)
IR (KBr) cm^{-1}	3600~3150, 3150~2500, 2120, 1680, 1620, 1525, 1390, 1345 (Fig. 2)
TLC[a] (Rf)	0.27 (1-propanol - H_2O, 80:20), 0.35 for azaserine; 0.62 (MeOH - H_2O, 95:5), 0.3 for azaserine
HPLC (R_{vol})	6.0 ml[b], 3.2 ml for azaserine

[a] Merck Silica gel 60. Detected by bioautography against *Klebsiella pneumoniae* AD, $UV_{254\,nm}$ quenching and ninhydrin spray.
[b] Column: Nucleosil 10, $N(CH_3)_2$, 4.0 mm×25 cm; solvent: MeOH - 2-propanol - H_2O (75:5:20); detector: UV, 254 and 280 nm, both at 0.05 aufs; flow: 1.5 ml/minute.
FAB-MS: Fast atom bombardment mass spectra. R_{vol}: Retention volume.

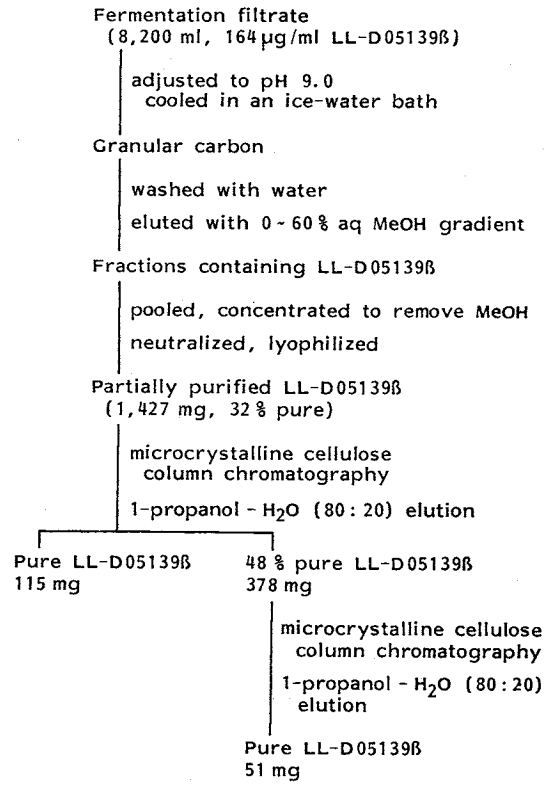
portion of the seed was inoculated into the production medium (100 ml in 500-ml Erlenmeyer flasks), which was then incubated under the same conditions and harvested after 5~6 days.

The ratio of the components produced and the antibiotic titer in fermentation broths were monitored by bioautography against *Klebsiella pneumoniae* AD using the TLC solvent system, methanol - water (95:5), and by HPLC (Table 2).

Isolation

The procedure for the isolation of LL-D05139β from fermentations conducted in medium B is depicted in Scheme 1. Due to its poor stability in acid, solutions containing LL-D05139β were kept between pH 7 and 9.5 throughout the isolation process. A 9-liter portion of the fermentation broth was filtered and the filtrate (8,200 ml, containing 164 μg/ml of LL-D05139β, as determined by HPLC analysis), was adjusted to pH 9.0 by adding dilute NaOH. The filtrate was then cooled in an ice-water bath and was passed through a column (3.2×85 cm) of granular

Scheme 1. Process for the isolation of antibiotic LL-D05139β.



carbon at 16 ml/minute. The column was first washed with one bed volume of water and was then eluted at 2.6 ml/minute with a linear gradient of water to water - methanol (40 : 60) over a period of 6 hours. Fractions were collected every 5 minutes and analyzed by TLC (Table 2) and an agar-diffusion assay using *E. coli* as the test organism. Concentration, neutralization (with dilute aqueous HCl) and lyophilization of the active fractions yielded 1,427 mg of partially purified (32% pure) LL-D05139β.

The partially purified LL-D05139β was chromatographed in two batches over a microcrystalline cellulose column (2.5×110 cm) equilibrated with 1-propanol - water (80 : 20) and eluted with the same solvent mixture at 2 ml/minute. Fractions containing pure LL-D05139β were pooled and the 1-propanol was azeotroped off *in vacuo* with an additional 1/2 volume of water. The aqueous solution was then neutralized and lyophilized to yield 115 mg of pure LL-D05139β. Side fractions were rechromatographed under the same conditions to yield an additional 51 mg of pure LL-D05139β.

When a complex medium (such as medium A) was used for the fermentation, LL-D05139β could not be adsorbed from the fermentation filtrate by granular carbon, presumably due to the lower titer of LL-D05139β and the presence of large amounts of azaserine and other interfering impurities. In this case, the fermentation filtrate was passed through a column of granular carbon to remove azaserine and some other impurities. The column effluent which contained LL-D05139β was then passed through a weak anion-exchange resin such as Amberlite IR45 (OH^-) or an anion-exchange Sephadex such as DEAE- or QAE-Sephadex. LL-D05139β was eluted from the anion exchanger with dilute aqueous base and desalted *via* a granular carbon column and further purified as described above. In general, the overall recovery of LL-D05139β from a complex medium fermentation was poor due to the additional processing steps required for this inherently unstable compound.

Physico-chemical Properties

Antibiotic LL-D05139β was obtained as a pale yellow amorphous solid. It is soluble in water and lower alcohols and is fairly stable in weakly basic solutions but decomposes rapidly below pH 5.0. Analytical standards (0.1 mg/ml in methanol) could be kept at −20°C for 2 months with no appreciable decomposition. Selected physico-chemical properties of antibiotic LL-D05139β are listed in Table 2.

Structure Elucidation

The UV spectrum (Fig. 1) of LL-D05139β is identical to that of azaserine and indicative of a diazo-ester functionality. The decomposition of both antibiotics in dilute acid was followed by monitoring their absorbance at 250 nm. Both decomposed completely after they were exposed to 0.02 M HCl in methanol for 1 hour. The IR spectrum (Fig. 2) of LL-D05139β showed

Fig. 1. UV spectrum of LL-D05139β, 20 μg/ml solutions.

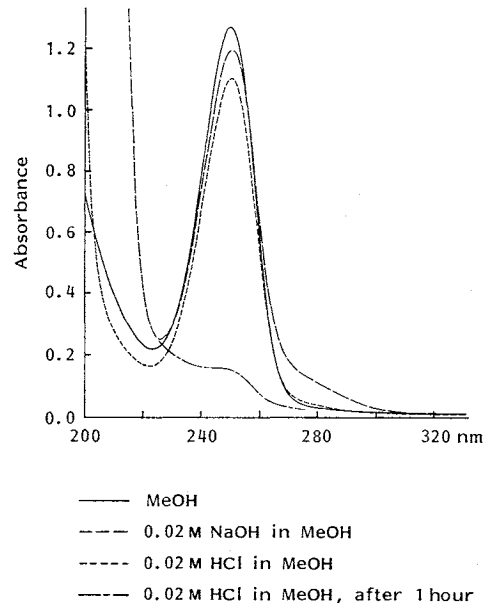


Fig. 2. IR spectrum of LL-D05139β (KBr disc).

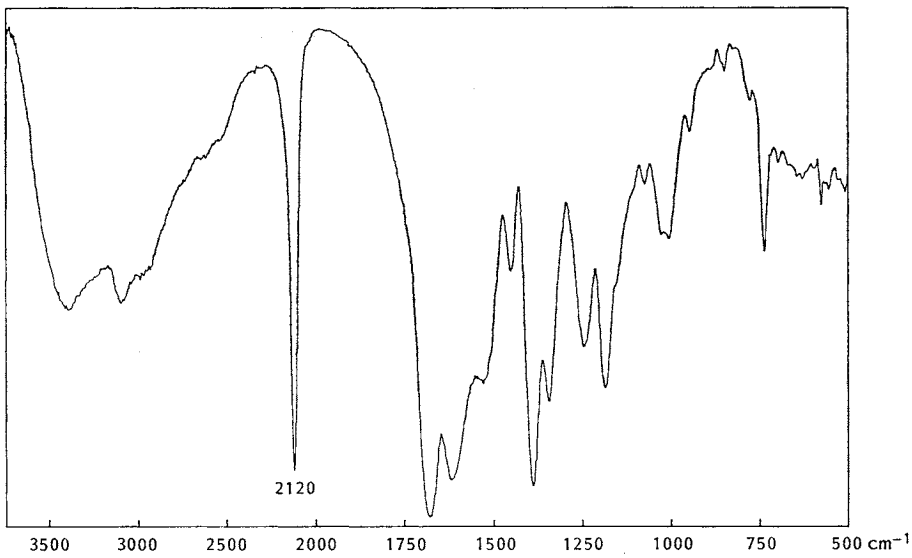
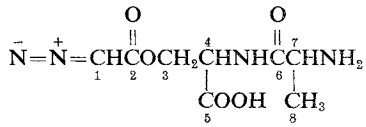


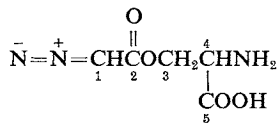
Table 3. ^{1}H and ^{13}C NMR chemical shifts of LL-D05139β and azaserine.

Position	^{1}H NMR (300 MHz, D_2O, δ)		^{13}C NMR (20 MHz, DMSO-d_6, δ)	
	LL-D05139β	Azaserine	LL-D05139β	Azaserine
1	5.21 (1H, s)	5.52 (1H, s)	59.1	60.9
2			171.1	171.7
3	4.33 (2H, m)	4.35 (2H, m)	65.5	64.0
4	4.25 (1H, m)	3.47 (1H, dd)	55.2	54.4
5			182.5	174.3
6			175.2	
7	3.79 (1H, q, J=6.9 Hz)		49.9	
8	1.34 (3H, d, J=7.1 Hz)		17.2	

a strong absorption at 2120 cm^{-1} consistent with the presence of a diazo-ester. The IR (KBr) spectrum of azaserine showed a similarly strong absorption at 2115 cm^{-1}. In addition, the ^{1}H and ^{13}C NMR spectra of LL-D05139β had peaks comparable to the signals attributed to the diazo-ester function of azaserine (Table 3).



LL-D05139β (**1**)



Azaserine (**2**, LL-D05139α)

Degradation studies (Scheme 2) were carried out on LL-D05139β in order to determine its amino acid composition. Acid hydrolysis (sealed tube, 6 M HCl, 104°C, 16 hours) of LL-D05139β released one molar equivalent each of alanine and serine as determined by standard amino acid analysis; no other amino acids were found. The amino acids contained in the acid hydrolysate were also converted to their *N*-trifluoroacetyl-*O*-methyl esters and were analyzed by the well-accepted gas chromatographic procedure with an asymmetric stationary

Scheme 2. Degradation studies of LL-D05139β.

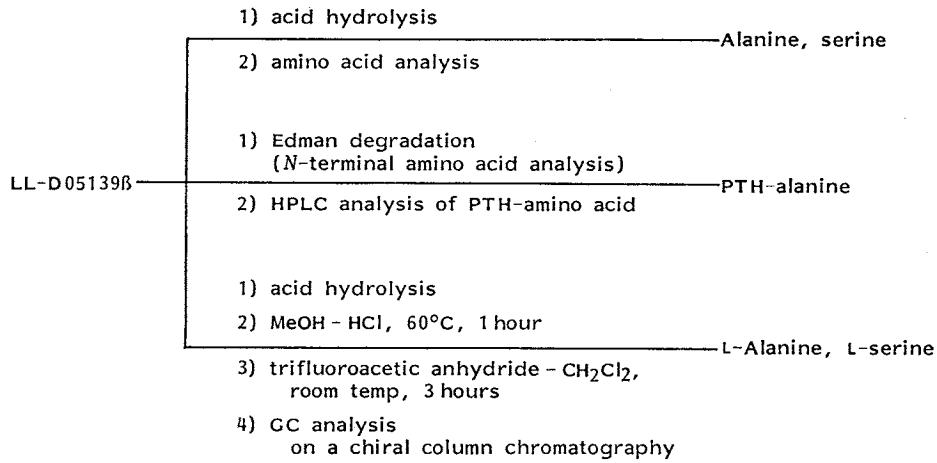


Table 4. Antimicrobial spectrum of LL-D05139β and azaserine.

Organism (strains tested)	MIC (μg/ml) range[a]	
	LL-D05139β	Azaserine
Staphylococcus aureus (3)	16	>256
Enterococcus sp. (2)	64	64~256
Bacillus subtilis (1)	16	32
Micrococcus luteus (1)	4	64
Escherichia coli (3)	4~8	32~128
Klebsiella pneumoniae (3)	4~128	64~>256
Enterobacter sp. (2)	128	>256
Serratia sp. (2)	32~64	256~>256
Proteus (indole +) (2)	16~64	256~>256
Pseudomonas aeruginosa (3)	128~>256	32~256
Acinetobacter calcoaceticus (2)	128~>256	4~16

[a] MICs were determined by the standard agar-dilution method in Mueller-Hinton medium.

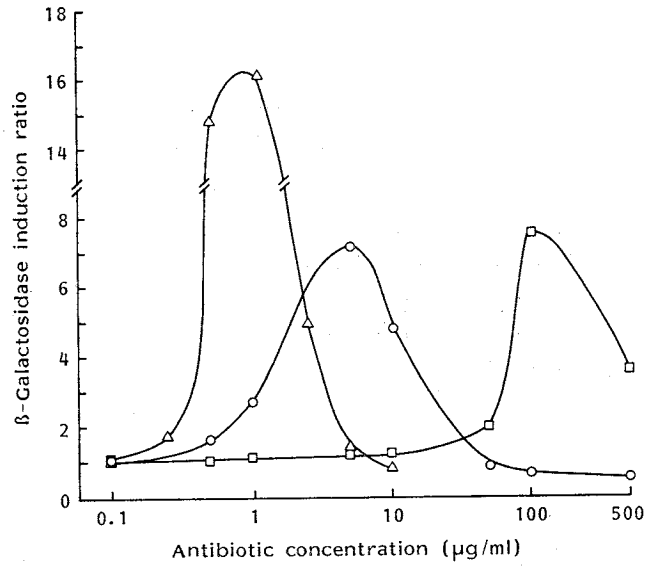
phase, *N*-lauroyl-L-valine-*tert*-butylamide.[11] Both alanine and serine derived from LL-D05139β were found to have the L-configuration. *N*-Terminal amino acid analysis of LL-D05139β using the Edman degradation procedure followed by HPLC analysis of the PTH-amino acid identified alanine as its *N*-terminal amino acid.[12,13] Thus, the chemical structure of LL-D05139β was assigned as alanylazaserine (**1**). The ^1H and ^{13}C NMR chemical shifts of LL-D05139β were assigned and correlated with those of azaserin (**2**) as sehown in Table 3.

Biological Activities

Antibiotic LL-D05139β is more active than azaserine against a spectrum of Gram-positive and Gram-negative bacteria (Table 4). Similarly, LL-D05139β is more active in the BIA with peak enzyme induction at ~5 μg/ml compared to ~100 μg/ml for azaserine (Fig. 3). LL-D05139β is less active than bleomycin in the BIA. LL-D05139β and azaserine showed camparable activity in the human lung and colon tumor clonogenic (stem cell)[14] assay and in the murine P388 leukemia *in vivo* test.

Fig. 3. The activity of LL-D05139β in the BIA.

LL-D05139β (○) was compared with azaserine (□) and bleomycin (△) for the induction of β-galactosidase synthesis in a modified version of the BIA.[15]



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