NEW ANTITUMOR ANTIBIOTIC, LL-C10037α

FERMENTATION, ISOLATION AND STRUCTURE DETERMINATION

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A new antitumor antibiotic, LL-C10037α was isolated from the fermentation filtrate of a *Streptomyces* by adsorption, partition and reverse phase column chromatography. Its chemical structure was determined by ^{1}H NMR, ^{13}C NMR, UV, IR and mass spectral data. LL-C10037α is a 7-aminoepoxysemiquinone and is related to the epoxyquinone class of antibiotics.

Antitumor antibiotic LL-C10037α[1], produced by a member of the genus *Streptomyces*, was discovered during our screening for new broad spectrum antibiotics. This paper describes the fermentation conditions, the isolation procedures, and the chemical structure of this new antibiotic.

Fermentation

The producing culture, LL-C10037, was maintained on agar slants of yeast extract - malt extract medium consisting of glucose 4.0 g, yeast extract 4.0 g, malt extract 10.0 g and agar 20.0 g per liter of water. Incubation was at 28°C.

The seed inoculum for the fermentation was prepared by transferring spore and mycelial scrapings from a slant into a 250-ml Erlenmeyer flask containing 50 ml of a medium composed of glucose 10.0 g, soluble starch 20.0 g, yeast extract 5.0 g, N-Z Amine A 5.0 g and $CaCO_3$ 1.0 g per liter of water. The pH was adjusted to 7.2 before sterilization. The inoculated flask was incubated on a rotary shaker at 28°C and 200 rpm for 48 to 72 hours.

A 5.0 ml volume of seed inoculum was then transferred to a 500-ml Erlenmeyer flask containing 100 ml of a fermentation medium composed of glucose 10.0 g, Bacto Peptone 5.0 g, molasses (cane) 20.0 g and $CaCO_3$ 1.0 g per liter of water. The fermentation flasks were incubated under the same conditions as the seed inoculum, and sampled and assayed over a period of 3 to 6 days.

Peak activity was obtained at day 5, as determined by agar-well diffusion assays against strains of *Klebsiella pneumoniae*, *Escherichia coli* and *Proteus mirabilis*.

Isolation

The procedure for the isolation of antibiotic LL-C10037α is depicted in Fig. 1. Most of the antibacterial activity was found in the fermentation filtrate. Thus, 9 liters of the fermentation broth were filtered, and the filtrate was passed through a column (2.5 cm$\times$60 cm) of Ambersorb XE-348 at 16 ml/minute. The effluent and the water wash of the column was discarded and the antibiotic was eluted from the resin at 2 ml/minute with a linear gradient of 0$\sim$80% aqueous acetone over a period of 8 hours. The fractions active against *K. pneumoniae* were pooled, concentrated to remove acetone, and the residual aqueous solution was lyophilized to yield 3.65 g of crude antibiotic LL-C10037α. A 3.4 g portion of this crude preparation was further purified by chromatography on a Sephadex LH-20 column

Fig. 1. Process for the isolation of antibiotic LL-C10037α.

Fermentation filtrate (9 liters)
↓
Ambersorb XE-348 column chromatography
washed with H_2O
eluted with 0~80% aq Me_2CO gradient
↓
Crude LL-C10037α (3.65 g)
↓
Sephadex LH-20 column chromatography
eluted with MeOH - H_2O, 10: 90
↓
Partially purified LL-C10037α (1.65 g)
↓
C_{18} Porasil B column chromatography
eluted with H_2O
↓
Pure LL-C10037α (805 mg)

(4.4 cm×85 cm) equilibrated with water - methanol, 90: 10, and eluted with the same solvent mixture at 1.2 ml/minute. Concentration and lyophilization of the active fractions yielded 1.65 g of partially purified antibiotic LL-C10037α. The final purification was carried out on a reverse phase column chromatographic system. A 210 mg sample of the above partially purified antibiotic was dissolved in 1.5 ml of water and loaded on a C_{18} Porasil B column (1.5 cm×100 cm) equilibrated with water. The column was eluted with water at 2 ml/minute and the fractions were analyzed by TLC (Rf 0.62, Analtech RPS plate, H_2O - MeOH, 95: 5, visualized by UV_{254} quenching) as well as bioassay. The desired fractions were pooled and lyophilized to yield 115 mg pure antibiotic LL-C10037α.

Physico-chemical Characteristics and Structural Assignment

Antibiotic LL-C10037α was obtained as a non-crystalline, fluffy, white powder, soluble in water and lower alcohols. It is fairly stable in acid and unstable in base. Listed in Table 1 are selected physico-chemical properties of antibiotic LL-C10037α.

The ^{13}C NMR spectrum of LL-C10037α showed a carbonyl peak at 189.3 ppm characteristic of an α,β-unsaturated ketone or aldehyde. This was supported by the carbonyl absorption at 1665 cm^{-1} in its IR spectrum (Fig. 2) and the absorptions at 212 and 275 nm in its UV spectrum (Fig. 3). Since there was no absorption due to an aldehyde proton in the ^1H NMR spectrum of LL-C10037α, the presence of an α,β-unsaturated ketone was established. The presence of an amide linkage in LL-C10037α was indicated by the carbonyl peak at 169.4 ppm in its ^{13}C NMR spectrum and confirmed by the two characteristic bands at 1690 and 1545 cm^{-1} in its IR spectrum.

High resolution mass spectral data and elemental analysis assigned the molecular formula of LL-C10037α to be $C_8H_9NO_4$ requiring five degrees of unsaturation. Combining this with the information above and the rest of the ^{13}C NMR and ^1H NMR data, structure **1** was assigned to antibiotic LL-C10037α. The NMR data of the epoxide carbons and protons are consistent with those reported

Table 1. Physico-chemical properties of antibiotic LL-C10037α.

Appearance	White amorphous powder
MP	153°C (dec)
$[\alpha]_D^{26}$	$-155\pm10°$ (c 0.1, H_2O)
Anal	Calcd for $C_8H_9NO_4$: C 52.5, H 4.9, N 7.6.
	Found: C 51.9, H 4.8, N 7.5.
MW (exact mass)	Calcd for $C_8H_9NO_4$: 183.0531.
	Found: 183.0534.
UV $\lambda_{max}^{H_2O}$ nm (ε)	212 (10,970), 275 (3,510) (Fig. 3)
IR (KBr) cm^{-1}	3260, 3070, 1690, 1665, 1545 (Fig. 2)
Rf (TLC)	0.62 (absorbant: Analtech RPS plate, solvent: H_2O - MeOH, 95: 5, detection: bioautography, UV_{254} quenching)
R_{vol} (HPLC)	12.2 ml (column: μBondapak, 3.9 mm×30 cm, solvent: 0.1 M NH_4OAc, pH 5.0, detector: UV 254 and 280 nm, both at 0.05 AUFS, flow: 1.6 ml/minute)

Fig. 2. IR absorption spectrum of LL-C10037α (KBr disc).

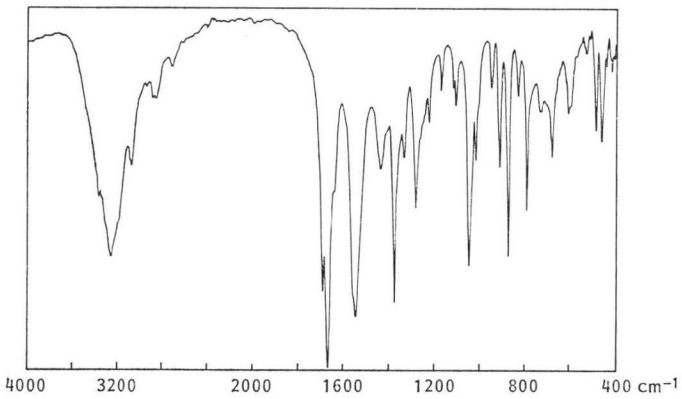
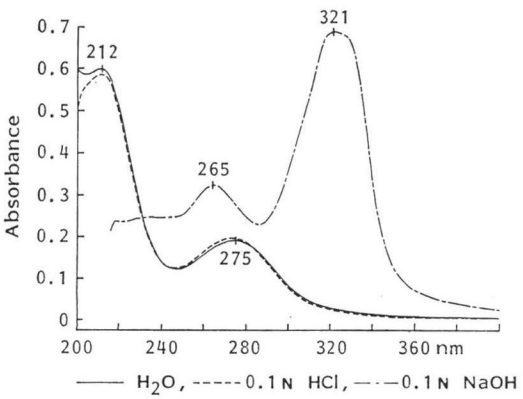
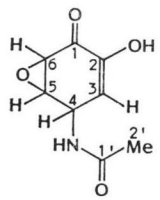


Fig. 3. UV spectrum of LL-C10037α, 0.01 mg/ml solutions.





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for epoxydon[2,3], panepoxydon[4], antibiotic U-62162[5], the enaminomycin[6] and antibiotic MT 35214[7].

Proton homonuclear *J*-correlation studies confirmed the structural fragment from C-3 to C-6. It also demonstrated clearly that the broad doublet of the amide proton at δ 5.762 is coupled to 4-H at δ 4.788. The carbon-13 to proton chemical shift correlation map of LL-C10037α unambiguously correlated its carbon-13 resonances to the corresponding proton resonances as shown in Table 2. Structure **1** also accounts for the extreme lack of stability of antibiotic LL-C10037α in basic solutions.

Table 2. ^{13}C NMR and ^{1}H NMR assignments of LL-C10037α.

Position	^{13}C NMR (DMSO-d_6, 20 MHz)		^{1}H NMR (DMSO-d_6, 300 MHz)		
	δ_C	Multiplicity	δ_H	Multiplicity	*J* (Hz)
1	189.3	s			
2	128.1	s			
2-OH			9.008	br s	
3	128.0	d	7.058	dd with 4, 5-H	
4	63.2	d	4.788	ddd with 3, 5-H & 4-NH	2.7, 3.1, 6.4
4-NH			5.762	br d with 4-H	6.4
5	53.6	d	3.766	ddd with 3, 4, 6-H	2.5, 3.1, 4.2
6	52.0	d	3.539	d with 5-H	4.2
1′	169.4	s			
2′	23.4	q	2.003	s	

Table 3. Antibacterial activity of LL-C10037α.

Organism	MIC (μg/ml)*	Organism	MIC (μg/ml)*
Escherichia coli CMC 84-11	64	*Citrobacter diversus* K 82-24	64
E. coli ATCC 25922	128	*Pseudomonas aeruginosa* 12-4-4	>128
Klebsiella pneumoniae AD	64	*P. aeruginosa* ATCC 27853	>128
Enterobacter cloacae CMC 84-4	128	*Staphylococcus aureus* SSC 82-31	128
Serratia marcescens CMC 83-27	128	*S. epidermidis* CMC 83-133	128
Proteus rettgeri ID 83-21	64	*Streptococcus faecalis* ATCC 29212	128
Morganella morganii ID 83-18	64	*Micrococcus luteus* PCI 1001	128
Providencia stuartii CMC 83-82	64	*Bacillus subtilis* ATCC 6633	128

* MICs were determined by the standard agar-dilution method in Mueller-Hinton agar.

Close examination of the Dreiding model of structure **1** showed that the configurations at C-4 and C-5 cannot be predicted by the observed coupling constants between 4-H and 5-H ($\sim$2.7 Hz).

A large number of epoxyquinone-class antibiotics as well as non-antibacterial metabolites have been reported[2~10]. To our knowledge, however, this is the first example of a γ-aminoepoxysemiquinone antibiotic.

Antibiotic LL-C10037α has poor activity against a limited number of Gram-positive and Gram-negative bacteria (Table 3). It is active against murine leukemia P388 with 29% increase in life-span of the treated mice relative to saline-treated controls at its optimum dose level (25 mg/kg by ip injection on days 1, 5 and 9 following tumor inoculation).

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