LL-AF283 ANTIBIOTICS, CYCLIC BIPHENYL PEPTIDES

CONWAY C. CHANG, GEORGE O. MORTON, JOHN C. JAMES, MARSHALL M. SIEGEL, NYDIA A. KUCK, RAYMOND T. TESTA and DONALD B. BORDERS*

Medical Research Division, Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y. 10965, U.S.A.

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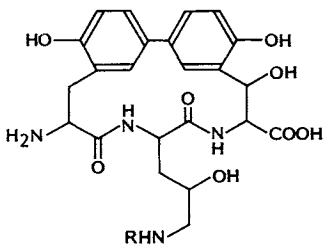
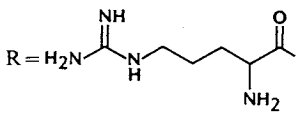
The isolation of water soluble, basic antibiotics LL-AF283α and β from the fermentation of *Streptomyces filipinensis* was previously reported[1,2]. The antibiotic complex contained four components, and the structures of LL-AF283α and β are now reported[3]. In the course of this study, it was found that LL-AF283α is identical to the peptide antibiotic biphenomycin A (WS-43708A)[4~7], reported by Fujisawa Pharmaceutical Co. The stereochemistry of biphenomycin A was recently reported by KANNAN and WILLIAMS[8].

The LL-AF283 antibiotic complex was formed by the fermentation of *S. filipinensis*, which was isolated from a soil sample collected in the Dominican Republic. The components of antibiotic complex in the conventional *in vitro* tests were active at low concentration only against *Corynebacterium xerosis*. However, the antibiotics protected mice against lethal infections caused by *Staphylococcus*, *Streptococcus pneumoniae* and *Escherichia coli* with very low toxicity[1,2]. The ED_{50} of LL-AF283α by single subcutaneous administration was about 1 mg/kg against both the Smith and Rose strains of *Staphylococcus aureus*[3].



LL-AF283α (1) R=H
(Biphenomycin A)



LL-AF283β (2) R=H_2N

The antibiotic complex was previously isolated from the fermentation broth by a batch process on activated carbon[1]. Ion-exchange chromatography with Amberlite IRC-50 (NH_4^+ form) and CM-Sephadex (NH_4^+ form) eluted with $HCOONH_4$ was used to obtain LL-AF283α and β as the formate salts. The partially purified LL-AF283β has now been further purified by chromatography on a Sephadex LH-20 column eluted with 3% butanol in water to obtain the major component β.

Although LL-AF283α and β obtained from the ion exchange chromatography step appeared to be relatively pure by ^1H and ^{13}C NMR, HRFAB mass spectral and FAB-MS/MS analyses revealed the presence of minor components in both purified preparations. The physical properties of the LL-AF283 antibiotics are shown in Table 1. The major components were designated as LL-AF283α ($C_{23}H_{28}N_4O_8$, HRFAB-MS (M+H) 489.1985) and LL-AF283β ($C_{29}H_{40}N_8O_9$, HRFAB-MS (M+H) 645.2978), and the minor components as LL-AF283α_1 ($C_{23}H_{28}N_4O_7$, HRFAB-MS (M+H) 473.2036) and LL-AF283β_1 ($C_{29}H_{40}N_8O_8$, HRFAB-MS (M+H) 629.3015). The similarities in the fragmentation patterns in the FAB-MS/MS demonstrated the structural similarities between the pairs of major and minor components. The 16 daltons

Table 1. Physical properties of LL-AF283 antibiotics.

	α	α_1	β	β_1
MW (HRFAB-MS)	489.1985	473.2036	645.2978	629.3015
Molecular formula	$C_{23}H_{29}N_4O_8$	$C_{23}H_{29}N_4O_7$	$C_{29}H_{41}N_8O_9$	$C_{29}H_{41}N_8O_8$
UV nm	264 (0.1 N HCl), 290 (0.1 N NaOH)		264 (0.1 N HCl), 290 (0.1 N NaOH)	
Elemental analysis	C 51.08 H 6.77 N 11.83 O 27.88		C 50.28 H 6.77 N 15.32 O 26.09	
Optical rotation	$[\alpha]_D^{25}$ −6.1±2.3 (*c* 1.310 in 0.1 N HCl)		$[\alpha]_D^{25}$ −28.6±3 (*c* 0.874 in 0.01 N HCl)	

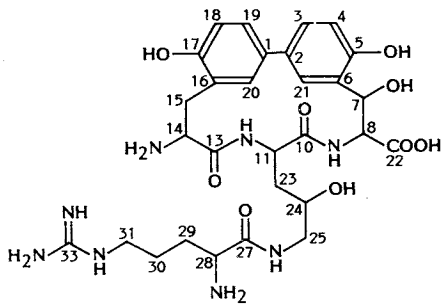


Table 2. ^{13}C NMR assignments for LL-AF283β in acidic and basic condition, 75 MHz.

	Chemical shift (ppm)		Δ (ppm)
	D_2O+DCl	$D_2O+NaOD$	
C-1	132.8	128.5	−4.3
C-2	132.8	130.4	−2.4
C-3	125.8	125.6	−0.2
C-4	116.6	118.6	+2.0
C-5	153.1	160.0	+6.9
C-6	128.3	130.2	+1.9
C-7[a]	65.5	69.8	+4.3
C-8[a]	65.3	66.9	+1.6
C-11	51.0	51.3	+0.3
C-14	58.6	58.6	0
C-15[a]	45.2	47.0	+1.8
C-16	120.6	125.9	+5.3
C-17	154.4	162.3	+7.9
C-18	117.2	120.2	+3.0
C-19	127.2	125.8	−1.4
C-20	130.5	128.8	−1.7
C-21	127.7	127.6	−0.1
C-23	29.5	29.7	+0.2
C-24	55.9	55.3	−0.6
C-25	38.2	38.0	−0.2
C-28	55.4	55.8	+0.4
C-29[a]	30.8	34.4	+3.6
C-30	24.9	25.3	+0.4
C-31	41.6	41.6	0
C-33	157.7	157.5	−0.2
Carbonyls	168.1	171.8	
(C-10,13,22,27)	168.7	172.5	
	172.4	173.9	
	173.3	176.5	
	178.1	179.0 (formate)	

[a] Non-aromatic signals which underwent considerable shift from acidic to basic conditions.

difference between α and α_1 and between β and β_1 suggested the presence of an extra hydroxyl group in the major components.

The UV spectra of both LL-AF283α and LL-AF283β components showed absorptions at 264 nm (ε 19,000 in water) with 26 nm bathochromic shifts to 290 nm upon treatment with base. The extinction coefficient of this chromophore suggested the presence of two isolated nonconjugated phenolic moieties. This assumption was substantiated by ^1H NMR spectra which contained signals for two trisubstituted aromatic rings with substitutions at 1, 3, and 4 positions for both components.

Table 3. ^1H NMR assignments for LL-AF283β (D_2O/NaOD), 300 MHz.

	Chemical shift (ppm)	Multiplicity	Coupling constant (J=Hz)
3-H	7.26	dd	8.2, 2.4
4-H	6.65	d	8.2
7-H	5.74	s	
8-H	4.90	s	
11-H	5.05	dd	8.5, 7.0
14-H	3.67	dd	2.6, 8.2
15-H	3.14	dd	8.2, 14.6
	2.92	dd	2.5, 14.6
18-H	6.71	d	8.2
19-H	7.18	dd	8.2, 2.4
20-H	6.85	d	2.4
21-H	7.25	d	2.4
23-H	2.0	m	
24-H	3.61	m	
25-H	2.68	dd	4.0, 13.4
	2.58	dd	7.5, 13.4
28-H	4.27	dd	5.0, 8.0
29-H	1.8	m	
30-H	1.6	m	
31-H	3.21	t	6.8

The nature of the carbon atoms was revealed from ^{13}C NMR studies, which showed that LL-AF283β has six aromatic =CH–, six aromatic quarternary carbons, five carbonyls, two CHOH, six methylenes and four methines. The LL-AF283α has six aromatic =CH–, six aromatic quarternary carbons, four carbonyls, two CHOH, three methylenes, and three methines. The ^{13}C NMR data of LL-AF283β in acidic and basic conditions were shown in Table 2.

^1H-^1H and ^1H-^{13}C correlation spectra in conjunction with investigations of coupling constants revealed information on fragments of the structure. The assignments of the ^1H NMR were shown in Table 3. The spectroscopic data (MS, UV, ^1H and ^{13}C NMR) and the unsaturation number indicated that LL-AF283α and LL-AF283β are macrocyclic peptides and revealed that LL-AF283α (**1**) was identical to WS-43708A. The optical rotation of LL-AF283α (Table 1) was less than that reported for biphenomycin A ($[\alpha]_D^{20}$ (c 0.1, 1 N HCl) which may indicate a difference in purity. The difference

between the α-component and the β-component was the extra arginine unit in the β-component as indicated from the HRFAB-MS, FAB-MS/MS data and the NMR spectra and was confirmed by the detection of arginine in the acid hydrolysate (6 N HCl, 120°C, 18 hours.)

A pH induced shift in the ^{13}C NMR was applied to determine the *N*-terminal and the *C*-terminal of the peptide sequences in the assembling of the fragments and led to the final structure of LL-AF283β (**2**). The sequence of the peptides could be determined by the induced shift from acidic to basic conditions; the β-carbon of the *N*-terminal underwent a downfield shift (3.1+0.8 ppm) and the α-carbon bearing the *C*-terminal underwent a downfield shift (2.5 ppm for α-carbon, 1.2 ppm for β-carbon). Not including aromatic carbon-13 signals, there were four signals (C-7, +4.3 ppm; C-8, +1.6 ppm; C-15, +1.8 ppm and C-29, +3.6 ppm) which underwent considerable shift from acidic to basic conditions as shown in the Table 2[9]. The deprotonation of the ammonium group caused considerable β-shift for C-15 and C-29 and indicated that arginine was linked to the peptides at the NH_2 of the ornithine. The shifts at the C-terminal (C-7, C-8) were due to deprotonation of the carboxylic acid which electrostatically affected the carbon atom bearing the hydroxyl group. This explanation was further substantiated by the very low field signals (5.7 ppm) for the hydrogen atom (7-H).

The ^{13}C NMR assignment of the biphenol system as shown in the Table 2 was derived from the ^{1}H-^{13}C correlation spectra and the pH induced shift upon the conversion of phenol to phenolate.

The antibacterial activity of LL-AF283α was tested by standard agar and broth dilution methods. As in the case of biphenomycin, the *in vitro* activity was influenced by the test medium. In agar dilution tests with inocula of approximately 10^4 cfu per spot, low concentration of LL-AF283α inhibited Gram-positive bacteria on semisynthetic medium or saline agar fortified with 10% rabbit blood. The antibiotic was not inhibitory in complex media such as nutrient, Trypticase-soy or Mueller-Hinton agars. Small addition of Trypticase-soy to synthetic media nullified activity. It was not effective against Gram-negative bacteria on any type of medium. In microbroth dilution tests with synthetic AOAC broth (Difco), the MIC of LL-AF283α and β against *S. aureus* Smith were $<0.12\,\mu g/ml$ and the MBC's were $>128\,\mu g/ml$. In broth tests "skips" were frequently observed. Also in agar diffusion tests with *S. aureus* Smith, zones of inhibition contained resistant colonies. Susceptibility of resistant colonies picked from a plate containing $16\,\mu g/ml$ of LL-AF283α, and colonies from the parent culture were determined by the broth dilution method. The MICs of LL-AF283α and β vs. the resistant cultures were $>128\,\mu g/ml$ and <0.12 vs. the parent culture. Both cultures were equally susceptible to vancomycin, tetracycline, cephalothin, cefotaxime, ampicillin, erythromycin, and tobramycin. Thus it appears that highly resistant mutants are selected on first exposure to LL-AF283α.

The *in vivo* activity of LL-AF283α was reported previously. The antibiotics were highly active in mice against infections with Gram-positive bacteria. In recent studies with *S. aureus* Smith infection in mice, LL-AF283α was five times more effective than the β-component and vancomycin on the basis of median effective doses. Against an infection with the LL-AF283α-resistant *S. aureus* strain, both the α and β components were ineffective while vancomycin was equally effective against infections with either the parent or resistant strains.

Thus our tests show that the antibacterial activities of LL-AF283α are similar to biphenomycin A and *in vitro* activities are influenced by media. Both the α and β components are highly effective against Gram-positive infection in mice.

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