L-681,217, A NEW AND NOVEL MEMBER OF THE EFROTOMYCIN FAMILY OF ANTIBIOTICS

AUGUST J. KEMPF*, KENNETH E. WILSON, OTTO D. HENSENS,
RICHARD L. MONAGHAN, SHELDON B. ZIMMERMAN
and EUGENE L. DULANEY

Merck Sharp and Dohme Research Laboratories,
Rahway, N.J., U.S.A.

(Received for publication June 16, 1986)

L-681,217 is a new broad spectrum antibiotic isolated from fermentation broth. The compound is a structurally unique member of the efrotomycin family of growth permittant antibiotics.

Efrotomycin[1] was discovered in the Merck laboratories several years ago. Aurodox[2], or antibiotic X5108[3] as it was called first, was found in another laboratory at about the same time. The two closely related compounds represented a new chemical class of antibiotics exhibiting growth permittant activity. Other antibiotics belonging to this class have been reported. Kirromycin[4] (=mocimycin[5]), dihydromocimycin[6], heneicomycin[7], factumycin[8], azdimycin[9] and kirrothricin[10] are all reported to have closely related chemical structures. In our search for new and interesting fermentation products, a culture was found which produces several components exhibiting various biological activities. One of these components, designated L-681,217, proved to be a structurally unique member of the efrotomycin-aurodox group of antibiotics. The fermentation, isolation, physico-chemical and structural characterization, as well as biological properties of L-681,217 are described in this paper.

Materials and Methods

Assay Methods

Biological Assays: Initial purification studies were monitored by disc-diffusion assays on nutrient agar plates seeded with *Vibrio percolans* ATCC 8461 (MB1272) as test organism.

HPLC Analysis: A method was developed for the quantitative analysis of L-681,217. The system employed a DuPont 10 μm Zorbax ODS reverse-phase column (25 cm $\times$ 0.46 cm), maintained at 28°C with a circulating water bath, and a solvent system of MeOH - 0.01 M potassium phosphate pH 6.5 (40 : 60) at a flow rate of 1.0 ml/minute. A Waters Associates Model 6000A pump and Model U6K loop sample injector were used. The column effluent was monitored at 288 nm using an LDC Spectromonitor II model 1202 UV detector. The UV monitor output was recorded on a Honeywell Model 195 strip chart recorder and quantitated using a Spectra-Physics System I digital computing integrator. The retention time for L-681,217 under the above conditions was 716 seconds.

Preparative HPLC: The system consisted of an Altex Model 110A Solvent Metering Pump, a Rheodyne Model 7120 injector equipped with an 8 ml sample loop, a DuPont Zorbax ODS prep column (25 cm $\times$ 2.12 cm) and a Gilson Escargot fraction collector. The mobile phase was MeOH - 0.05 M sodium citrate pH 3.3 (50 : 50). Column effluent was monitored using an LDC Spectromonitor II UV detector equipped with a flow cell of 1 mm path length and coupled to a Honeywell Model 195 strip chart recorder.

Physico-chemical Methods: UV absorption spectra were obtained with a Beckman UV 5260

spectrophotometer. The IR spectra were determined in KBr using a Nicolet Model 7199 FTIR spectrometer. ^{1}H NMR spectra were recorded in D_2O at 23°C on a Varian XL200 spectrometer using 2,2-dimethyl-2-silapentane-5-sulfonate sodium salt as internal standard. ^{13}C NMR spectra were recorded in CD_3OD - $CDCl_3$ (1 : 4) at 10°C on a Varian SC300 spectrometer with TMS as internal standard. Fast atom bombardment mass spectra (FAB-MS) and electron impact mass spectra (EI-MS) were collected on a MAT731 spectrometer.

Fermentation

Seed Medium: Seed culture was prepared in three stages from a lyophile of a strain of *Streptomyces cattleya* (ATCC 39203). The first two stages were grown in baffled 250-ml Erlenmeyer flasks each containing 44 ml of seed medium. The final seed stage was grown in baffled 2-liter Erlenmeyer flasks each containing 500 ml of seed medium. Seed medium consisted of (g/liter) glucose (1.0), soluble starch (10.0), beef extract (3.0), Ardamine pH (5.0), NZ-Amine Type E (5.0) and $MgSO_4 \cdot 7H_2O$ (0.05) dissolved in distilled water. A 2-ml per liter aliquot of phosphate buffer, consisting of KH_2PO_4 (9.1% by weight) and Na_2HPO_4 (9.5% by weight), was added. The pH of the medium was adjusted to 7.0~7.2. After pH adjustment $CaCO_3$ was added to a final medium concentration of 0.5 g/liter. Flasks were incubated at 28°C for 24 hours on a rotary shaker (5 cm throw, 220 rpm).

Production Medium: A 5% transfer of the seed culture was used to inoculate four 14-liter Brunswick Model MF14 fermentors, each containing 9.5 liters of production medium. Production medium consisted of (g/liter) glucose (10.0), asparagine (1.0), K_2HPO_4 (0.1), $MgSO_4 \cdot 7H_2O$ (0.5), yeast extract (0.5), $CaCO_3$ (3.0) and (mg/liter) $MnSO_4 \cdot H_2O$ (10.0), $FeSO_4 \cdot 7H_2O$ (10.0), $CaCl_2$ (1.0), H_3BO_3 (0.56), $CoCl_2 \cdot 2H_2O$ (0.25) and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.19) suspended in distilled water and adjusted to pH 7.0~7.2. Polyglycol P-2000 was added during fermentation to control foam. Fermentation was continued for 90 hours at 28°C at an agitation rate of 400 rpm and an air flow of 3 liters/minute.

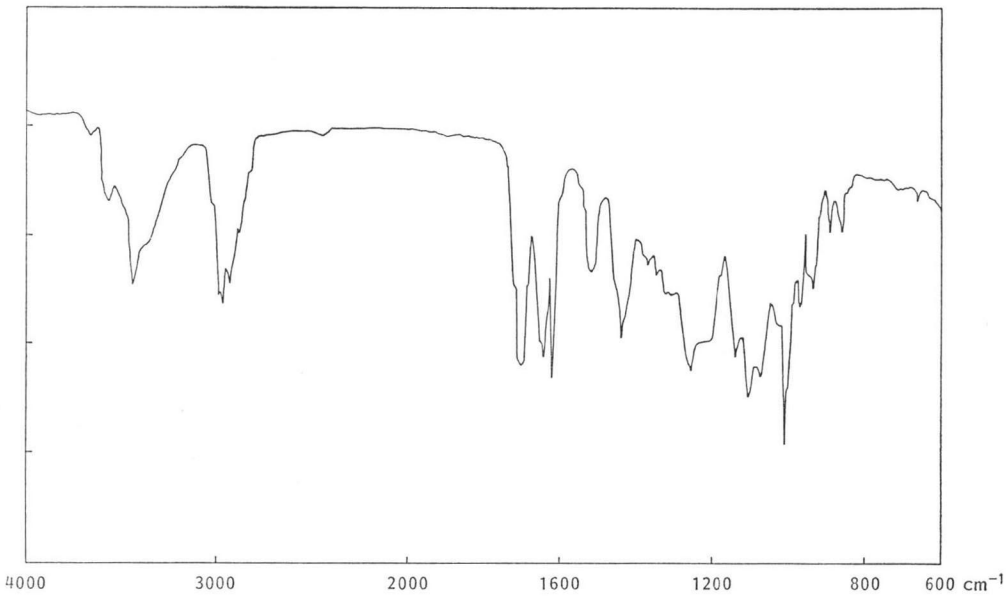
Results

Isolation

L-681,217 was found in culture filtrate. Thus, 34 liters of filtered broth, containing 5.0 g of the antibiotic by HPLC assay, was adsorbed at pH 7.0 on 3.7 liters of Amberlite XAD-2 resin. The column was washed with 6 liters of deionized water and eluted with 8 liters of acetone - water (20 : 80) followed by 10 liters of acetone - water (60 : 40). Eluate fractions were collected and analyzed by HPLC, as well as bioassay. Fractions 1 and 2 were 4 liters each, fractions 3 through 7 were 2 liters each. Fractions 2 through 4 were combined and evaporated to 1 liter. Fifteen ml of 1 M citric acid was added to adjust the pH to 3.4. This solution was then extracted twice with 1 liter portions of ethyl acetate. The combined ethyl acetate extract was dried with anhydrous sodium sulfate and concentrated to 150 ml. The antibiotic was back-extracted into 150 ml of water maintained at pH 7.5 by the addition of 1 M sodium hydroxide. The aqueous phase was then lyophilized to give 5.2 g of L-681,217 sodium salt as a powder (84% pure by weight).

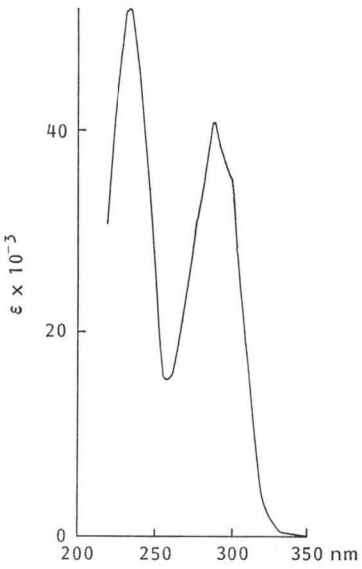
Five hundred mg of lyophilized sodium salt were dissolved in 3.7 ml of methanol - 0.05 M sodium citrate pH 3.3 (50 : 50) and chromatographed on a DuPont Zorbax ODS prep column (25 cm × 2.12 cm) in the same solvent. The flow rate was 10 ml/minute. After a 50-ml forecut, 15-ml fractions were collected and assayed by HPLC and bioassay. Fractions 24 through 45, containing essentially pure antibiotic, were combined and concentrated to remove the methanol. Desalting was accomplished as follows: the aqueous concentrate was adjusted to pH 5.0 and extracted twice with an equal volume of ethyl acetate. The combined extract was dried with anhydrous sodium sulfate. After the addition of 150 ml of water to the extract, the mixture was gently stirred, and the pH of the aqueous layer

Fig. 1. IR spectrum of L-681,217 methyl ester in $CHCl_3$.



was adjusted to 6.5 with 1 M sodium hydroxide. The ethyl acetate - water mixture was then concentrated to remove the organic phase. A small amount of methanol was added to clarify the resultant aqueous suspension of the antibiotic. The methanol - water solution was then lyophilized to give 175 mg of pure L-681,217 as the sodium salt.

Fig. 2. UV absorption spectrum of L-681,217 sodium salt in MeOH.



Preparation of the Methyl Ester of L-681,217

Eleven mg of pure L-681,217 sodium salt was dissolved in 5 ml of water and acidified by adding 3 ml of 0.1 M sodium citrate, pH 2.0. The mixture was extracted three times with 5 ml of chloroform. The combined extract, containing L-681,217 free acid, was dried over anhydrous sodium sulfate and treated at 0°C with a slight excess of ethereal diazomethane solution. After 2 minutes, excess diazomethane was removed under a stream of nitrogen. The solution was dried over sodium sulfate and evaporated to 9.6 mg of oily residue. The product was chromatographed on 0.5 ml of E. Merck Silica Gel 60 (40~63 micron), being eluted with ethyl acetate - methylene chloride (75:25) and with ethyl acetate. Appropriate fractions were combined and concentrated to afford 7.3 mg of L-681,217 methyl ester.

Table 1. UV absorption of L-681,217.

Solvent	λ_{max} nm (ε)
MeOH	298 (sh, 35,500), 288 (41,100), 278 (sh, 31,600), 234 (52,100)
H_2O at pH 6	300 (sh, 32,000), 280 (38,900), 278 (sh, 30,500), 234 (52,000)
H_2O - 0.1 N HCl	310 (sh, 28,800), 299 (33,000), 285 (sh, 27,000), 232 (51,100)
H_2O - 0.1 N NaOH	300 (sh, 19,600), 288 (35,600), 278 (sh, 31,200), 230 (51,400)

Fig. 3. 200 MHz ^1H NMR spectrum of L-681,217 sodium salt in D_2O.

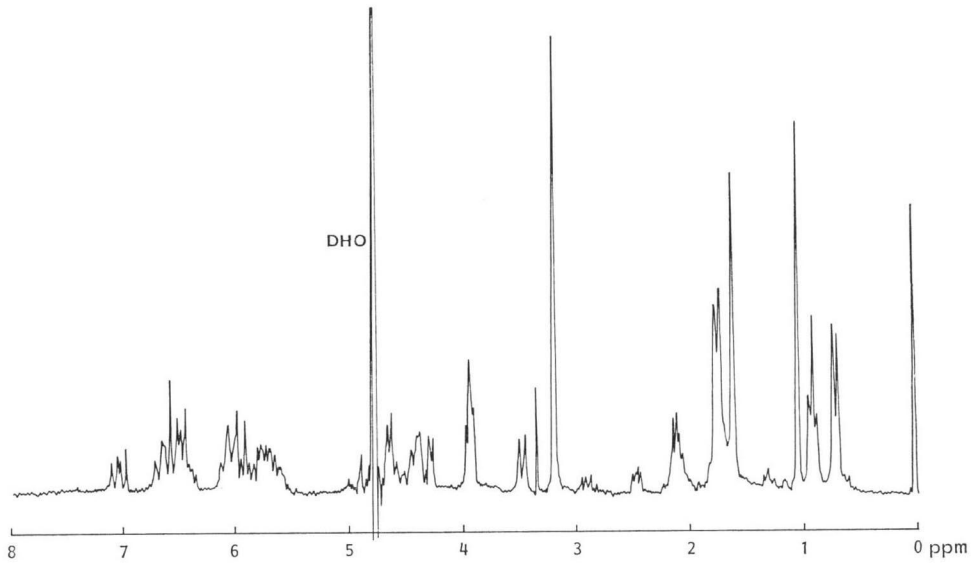
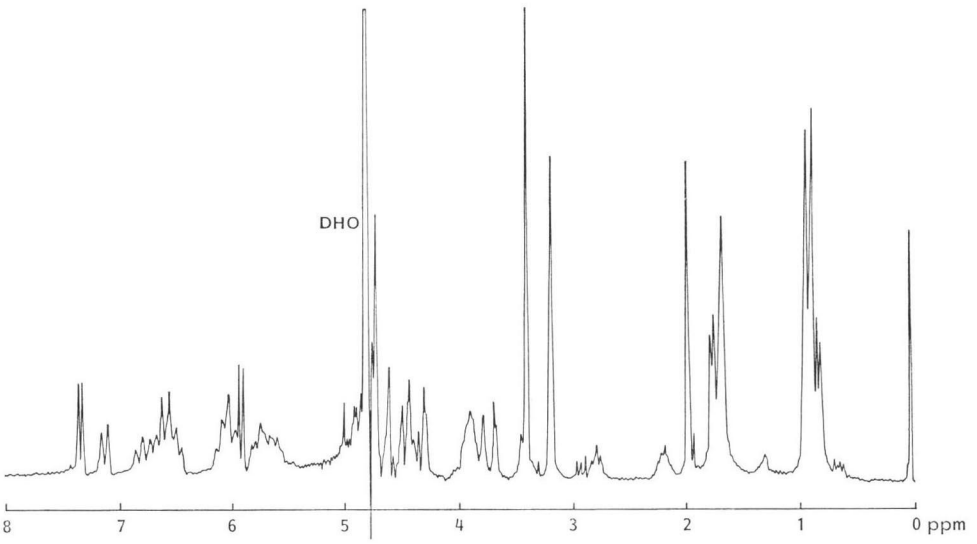


Fig. 4. 200 MHz ^1H NMR spectrum of aurodox sodium salt in D_2O.



Characterization

L-681,217 sodium salt is an off-white amorphous powder. It is soluble in water, methanol and acetone but only slightly soluble in methylene chloride. The antibiotic does not afford useful MS

Table 2. ^{13}C NMR data (20% CD_3OD - $CDCl_3$).

General assignments (ppm)		
5	CH_3	(9.9, 10.7, 12.0, 12.9, 13.5) q
4	CH_2	(21.0, 39.0, 39.2, 41.5) t
2	CH	(40.0, 56.6) d
1	CH_3O	(56.2) q
6	CH-O	(72.0, 74.6, 74.8, 77.8, 84.3, 89.7) d
1	C-O	(74.4) s
1	$C<^{O}_{O}$	(98.7) s
13	CH=	(125.9, 126.2, 127.0, 128.2, 128.6, 129.3, 129.5, 129.7, 131.5, 133.0, 133.6, 138.8, 142.9) d
1	C=	(136.5) s
2	COX	(173.3, 176.0) s

data underivatized. Silylation produces a penta-trimethylsilyl derivative which exhibits a weak molecular ion at m/z 1,019 (M^+) by EI-MS. The molecular formula $C_{51}H_{93}NO_{10}Si_5$ was assigned to the derivative based on high resolution MS measurements on the more prominent fragment ion $M^+-TMSOH-CH_3$ (m/z 914.4905 measured, 914.4910 calcd for $C_{47}H_{90}NO_9Si_4$; m/z 947 for the penta-($^2H_9$-trimethylsilyl) derivative). Antibiotic L-681,217 has accordingly the molecular formula $C_{36}H_{53}NO_{10}$ (MW 659). In agreement with this result, L-681,217 methyl ester exhibits a strong molecular ion at m/z 673 by FAB-MS and fragment ion M^+-H_2O (m/z 655.3720 measured, 655.3720 calcd for $C_{37}H_{53}NO_9$) by high resolution EI-MS.

Table 3. Antibacterial spectrum of L-681,217 (255 μg/ml).

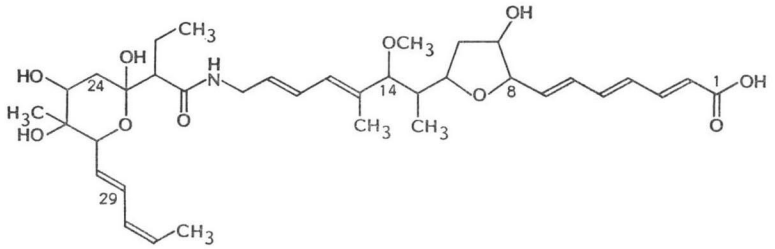
Organism, MB No. (ATCC No.)	Inhibition zone diameter (mm)
Bacillus subtilis 964 (6633)	12
Bacillus sp. 633	12
Corynebacterium pseudodiphtheriticum 261	12
Micrococcus luteus 1101 (9341)	25
Staphylococcus aureus 108 (6538P)	10
Streptococcus agalactiae 2875	10
Vibrio percolans 1272 (8461)	18
Proteus vulgaris 838 (21100)	16
P. vulgaris 2112 (episome)	18
P. vulgaris 1012	13
P. mirabilis 3126	10H*
Erwinia atroseptica 1159 (4446)	0
Escherichia coli 60 (9637)	17
E. coli 1418	16
Pseudomonas aeruginosa 2824	12H*
P. stutzeri 1231 (11607)	0
Brucella bronchiseptica 965 (4617)	13
Enterobacter aerogenes 835	15
Klebsiella pneumoniae 1264	18
Xanthomonas vesicatoria 815	15

* H: Hazy.

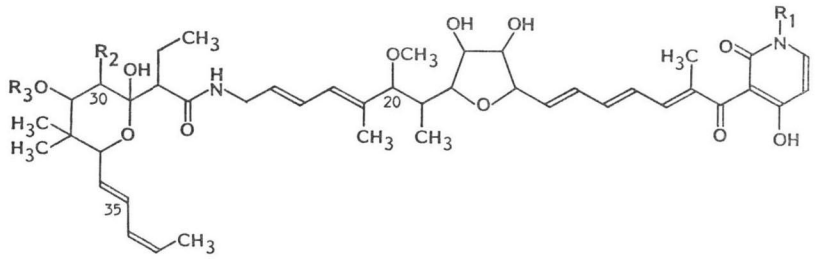
IR data indicate that a carboxylic acid group is responsible for the acidic nature of the antibiotic. This is based on the observation that the free acid form of L-681,217 exhibits an absorption band in KBr at 1689 cm^{-1} that shifts to 1543 cm^{-1} for L-681,217 sodium salt. The carboxyl group appears to be present as a conjugated trienoic acid moiety based on the IR absorption band at 1700 cm^{-1} for L-681,217 methyl ester (Fig. 1) and the UV absorption maximum in methanol at 288 nm (ε 41,100; Fig. 2 and Table 1). The remaining UV absorption maximum at 234 nm (ε 52,100) is consistent with two isolated diene chromophores. In addition the IR spectrum of L-681,217 methyl ester supports MS evidence for hydroxyl groups (3580 cm^{-1}, 3400 cm^{-1} broad) and identifies the single nitrogen of the antibiotic as a secondary amide (3450 cm^{-1}, 1646 cm^{-1}, 1520 cm^{-1}).

The ^1H NMR spectrum of L-681,217 sodium salt strongly resembles that of aurodox, a member of the efrotomycin class of growth permittant antibiotics (Figs. 3 and 4). Similarities are particularly

Fig. 5. Structures of L-681,217 and related antibiotics.



L-681,217 (**1**)



2 (**a** ~ **d**)

Efrotomycin (**2a**)	$R_1=CH_3$,	$R_2=OH$,	R_3=Disaccharide ($C_{15}H_{27}O_5$)
Aurodox (**2b**)	$R_1=CH_3$,	$R_2=OH$,	$R_3=H$
Kirromycin (**2c**)	$R_1=H$,	$R_2=OH$,	$R_3=H$
Heneicomycin (**2d**)	$R_1=CH_3$,	$R_2=H$,	$R_3=H$

striking for the aliphatic signals centered at δ 1.7 and in the olefinic region δ 5.5~6.9. The spectrum of L-681,217 however, lacks resonances at δ 1.97, 3.40, 5.95 and 7.36 corresponding to aurodox protons C(8)-CH_3, NCH_3 and CH(5) and CH(6). This, along with the fact that L-681,217 has likely a trienoic acid function and has one less nitrogen than aurodox, suggested that L-681,217 lacks the pyridone ring. ^{13}C NMR data (Table 2) combined with extensive NMR decoupling experiments and HR-MS studies led to the complete structure elucidation of antibiotic L-681,217 (**1**, Fig. 5). The details of the NMR analysis and stereochemistry will be reported in a subsequent paper.

Biological Properties

The antibacterial spectrum of L-681,217 at 255 μg/ml is shown in Table 3. The spectrum was obtained by dissolving the sodium salt in methanol - distilled water (10 : 90) and placing a sample droplet of 0.015 ml on the surface of seeded agar plates containing nutrient media. The methanol - water solution alone demonstrated no inhibition with any of the test organisms. L-681,217 is active *in vitro* against both Gram-positive and Gram-negative organisms.

L-681,217 has also been shown to exhibit growth permittant activity in chicks comparable to aurodox and mocimycin.

Conclusion

L-681,217 is a new member of the efrotomycin-aurodox family of antibiotics. The most note-

worthy novel structural feature in comparison with the efrotomycin-aurodox type is the absence of the pyridone group and termination of the chain instead in a carboxylic acid function. The compound is active *in vitro* against Gram-positive and Gram-negative organisms and *in vivo* shows growth permittant activity.

Acknowledgments

We thank Ms. CHERYL SCHULMAN and Ms. RUTH SYKES for fermentation support and Dr. JERROLD LIESCH and Mr. JACK SMITH for mass spectral studies. We also thank Drs. BRINTON MILLER and SCOTT FEIGHNER for growth permittance studies and Mr. MICHAEL SALVATORE and Ms. JANET SIGMUND for biological assays. We also acknowledge the support and cooperation of the other members of MSDRL who contributed to this work.

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