ISOLATION AND CHARACTERIZATION OF A NEW ANTIBIOTIC U-62162

L. SLECHTA, J. I. CIALDELLA, S. A. MIZSAK and H. HOEKSEMA

Research Laboratories, The Upjohn Company, Kalamazoo, Michigan 49001, U.S.A.

(Received for publication December 28, 1981)

A new antibiotic U-62162 has been isolated from the fermentations of *Streptomyces verdensis* Dietz, sp. n. (UC-8157). The compound has been characterized and its gross structure has been elucidated. The antibiotic inhibited the growth of Gram-positive bacteria (particularly *Staphylococcus aureus*) but was inactive in experimentally infected animals.

During the course of routine soil screening for antibiotic producing microorganisms, a new species of the genus *Streptomyces* was isolated. It was studied by DIETZ and designated as *Streptomyces verdensis* Dietz, sp. n. (UC-8157).

This species was found to produce a new antibiotic U-62162 and in this paper we report the fermentation conditions, the isolation procedure and the chemical and biological properties of this antibiotic.

Microorganisms

The antibiotic producing microorganism was a new soil isolate, classified as *Streptomyces verdensis* Dietz, sp. n. (UC-8157).

Analytical Methods

Samples of the fermentation media and of various fractions obtained during the isolation procedure were chromatographed on silica gel plates (Analtech, Inc.) developed in chloroform - methanol (9: 1, v/v). In this system, the antibiotic U-62162 had an Rf value of 0.5 and was detected by bioautography against *S. aureus* (UC-76).

The antibiotic production was determined by the paper disc agar diffusion method employing *S. aureus* (UC-76) as the assay organism.

The NMR spectra were determined in acetone-d_6, chloroform-d_1 and dimethylsulfoxide-d_6 on Varian XL-200 and Bruker WM 500 instruments. The ultraviolet light absorption spectra were obtained with a Cary 15 spectrophotometer. The infrared spectrum was determined in Nujol mull using a Digilab FTS 14D instrument.

Fermentation

Seed cultures of *S. verdensis* (UC-8157) were prepared in a medium composed of (in g/liter): glucose monohydrate 25.0 and Pharmamedia 25.0; in tap water and pH adjusted with 1 N KOH to 7.2 prior to sterilization. The seed flasks were inoculated with one agar plug of the culture and incubated at 28°C for 3 days on a rotary shaker (300 rpm, 6-cm stroke).

The fermentation medium contained (in g/liter): Brer Rabbit Molasses 30.0, glucose monohydrate 5.0, Wilson's liquid peptone 10.0, sodium glutamate 2.0 and calcium carbonate 5.0, adjusted to pH 7.2 with 1 N NaOH. The fermentations were performed in a Virtis Fermenter at 28°C and the aeration rate was 6 liters of air per minute. Ucon was used as antifoaming agent. The fermenter was equipped with an automatic pH control unit set between pH 6 and pH 7.5. The upper limit of the fermentation pH was found to be critical since the antibiotic U-62162 is very unstable at pH higher than 8. The

maximum antibiotic production occurred after 24 hours of fermentation in spite of the fact that the growth of the producing organism (measured as volume of packed mycelium in a centrifuge tube) was only about one fourth of the maximum growth observed 72 hours of fermentation.

Isolation

The fermentation broth was filtered with the aid of diatomaceous earth at harvest pH (7.4). The filtrate was acidified with 6 N H_2SO_4 to pH 4.5 and was extracted twice with half volume of methylene chloride. The combined extracts were washed with water and evaporated to dryness in a rotary flash evaporator, yielding 710 mg of solid residue/10 liters of the filtered medium. A 250 mg portion of the material was chromatographed over a column (25×2.5 cm) of silica gel buffered to pH 5.8 with KH_2PO_4, using chloroform - methanol (94: 6, v/v) mixture as solvent. The active fractions were combined and evaporated to dryness. The yield was 90 mg. Three grams of similarly prepared material were further purified by a 300 transfer counter current distribution (100 ml phase) in the system afforded by ethyl acetate - ethanol - cyclohexane - water (2: 3: 3: 2, v/v). The residue from tubes 120 to 150 was recrystallized from ethyl acetate+petroleum ether (60~70°C b.p.) affording 1 g of white, needle-like crystalline material; m.p. 96~98°C; $[\alpha]_D^{25}+74°$ (*c* 0.97, acetone).

Anal. Calcd. for $C_{23}H_{33}NO_6$: C 65.83, H 7.93, N 3.34.
Found: C 65.33, H 7.81, N 2.60.

High resolution MS, *m/z* 419. Theory for $C_{23}H_{33}NO_6$: 419.2308. Found: 419.2303.

Physical Characteristics and Structural Considerations

The antibiotic U-62162 exhibited absorption at 235 nm (ε 14000) with an inflexion at 276 nm (ε 8150) in its ultraviolet spectrum, suggestive of an α,β unsaturated ketone or aldehyde. The infrared spectrum supported this with characteristic bands at 1685 cm^{-1} and 1638 cm^{-1} (Fig. 1). The additional absorptions observed in the carbonyl region were eventually ascribed to a carboxylic acid (1702 cm^{-1}), and a secondary amide (1650 cm^{-1} and 1518 cm^{-1}). Analytical and mass spectral considerations indicated a molecular formula of $C_{23}H_{33}NO_6$, and the extraction characteristics suggested that the antibiotic U-62162 was an acid.

When this information was combined with the ^{13}C NMR and ^1H NMR data (Table 1), the structure shown in Fig. 2 was assigned to the antibiotic U-62162. This places the antibiotic U-62162 in the family

Fig. 1. IR spectrum of antibiotic U-62162.

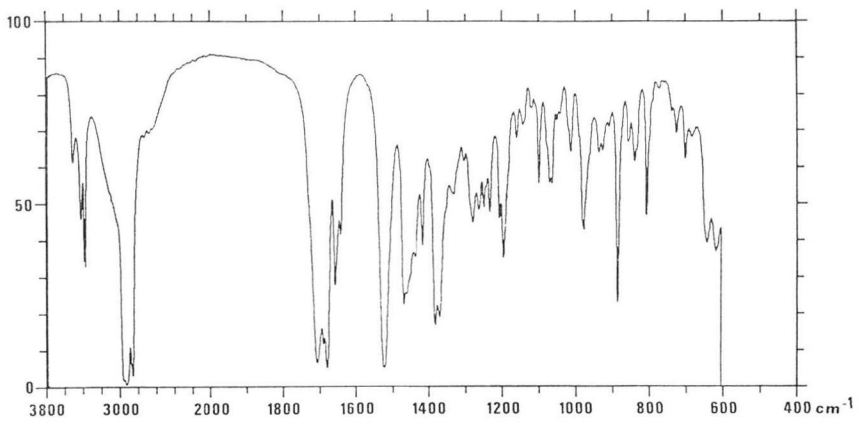
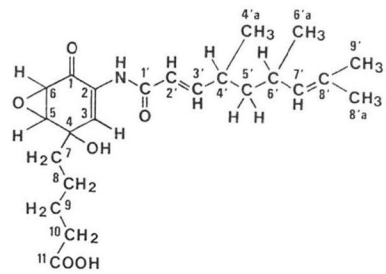


Table 1. Antibiotic U-62162: Summary of NMR data obtained in acetone-d_6.

Position	^{13}C NMR			^{1}H NMR		
	Chemical shifts (ppm)	Multiplicity	Couplings (H_2)	Chemical shifts (ppm)	Multiplicity	Couplings (H_2)
1	189.57	s	J_3=9.1, J_6=4.0			
2	130.02	s				
NH				8.25	Br. s	
3	129.77	d		7.43	d	J_5=2.71
4	70.56	s				
5	57.94	d	J_5=188, J_3 & J_7 Broadening	3.69	dd	J_3=2.71, J_6=4.0
6	53.03	d	J_6=189	3.56	d	J_5=4.0
7	40.77	t		1.82	m	with 8
8	22.77	t		1.43	m	with 7 & 9
9	25.24	t		1.63	m	with 8 & 10
10	33.33	t		2.28	m	with 9
11	173.90	s		12.1	(OH)	
1′	164.82	s				
2′	122.23	d		6.27	dd	J_3=15.31, J_4=1.01
3′	151.59	d		6.80	dd	J_2=15.31, J_4=7.58
4′	34.17	d		2.30	ddtq	$J_{4'a}$=6.7, $J_{3'}$=7.5 $J_{5'\alpha}$=7.8, $J_{5'\beta}$=6.5 $J_{2'}$=1.25
4′a	18.96	q		1.02	d	$J_{4'}$=6.70
5′	44.12	t		5′α, 1.33	ddd	$J_{5'\beta}$=13.5, $J_{4'}$=6.5 $J_{6'}$=8.5
				5′β, 1.26	ddd	$J_{5'\alpha}$=13.5, $J_{4'}$=7.8 J_6=6.1
6′	30.19	d		2.43	dddq	$J_{5'\alpha}$=8.5, $J_{5'\beta}$=6.1 $J_{7'}$=9.5, $J_{6'}$=$_\alpha$6.63
6′a	20.93	q		0.91	d	$J_{6'}$=6.63
7′	131.11	d		4.86	d of sept.	$J_{6'}$=9.5, $J_{9'}$=1.4
8′	128.72	s				$J_{8'a}$=1.4
8′a	17.42	q		1.61	dd	$J_{7'}$=1.4, $J_{9'}$=0.5
9′	25.24	q		1.65	Br. d	$J_{7'}$=1.4

of antibiotics such as asukamycin (**1**) and manumycin (**2**). The ^{13}C NMR indicated the presence of 23 carbons and 30 hydrogens, by off resonance decoupling. The ^{1}H NMR spectra displayed signals for all the protons required by the observed carbon multiplicities. By specific decoupling the hydrocarbon sidechain consisting of carbons 2′ through 9′ was established. The *trans* configuration at C-2′, C-3′ was apparent from the large coupling constant, $J_{2',3'}$=15.3 Hz. The upfield signal of C-2′ (122.23 ppm) and the downfield signal for C-3′ (151.59 ppm) as well as the downfield signals at C-2′ (6.27 ppm) and at the β position C-3′ (6.80 ppm) were all indicative of the carbonyl

Fig. 2. Structure of antibiotic U-62162.



character of C-1′. With additional decoupling studies the remaining 4 methylene groups were found to be in a single chain, linked on both ends to quaternary carbons. One of these (C-11) was a carbonyl by virtue of the downfield position of the C-10 triplet (2.28 ppm) in the ^{1}H NMR spectrum. When proton spectra were run in Me_2SO-d_6, the signals for 3 additional protons were seen, all exchangeable with D_2O. These included a broad peak at 12.1 ppm for COOH, a broad singlet at 9.04 ppm (NH), and a signal at 5.74 ppm which was assigned with the remaining heteroatom for OH. The carboxylic acid group could be immediately assigned to C-11.

The stoichiometry required that the remaining unassigned atoms were associated with a single moiety, $C_6H_3O_2$(NH)(OH). Three carbons were required by the α,β unsaturated system, as suggested by the ultraviolet light spectrum. This was supported by the fact that the only unsaturated carbons left were a carbonyl (189.5 ppm), a vinyl singlet at 130.1 ppm and a vinyl doublet at 151.6 ppm with a corresponding proton signal at 7.43 ppm (d, J=2.71, H-3). The ^{13}C NMR also showed signals at 53.03 ppm ($^1J_{C\text{-}H}$=189 Hz) and at 57.94 ppm ($^1J_{C\text{-}H}$=188 Hz), which were assigned to a *cis* epoxide with corresponding protons observed at 3.56 ppm (d, J=4.0 Hz, H-6) and at 3.69 ppm (dd, J=4.0 and 2.71 Hz, H-5). The W-type long range coupling observed between H-3 and H-5 (J=2.71) indicated the ring being nearly planar and the epoxide protons equatorial. The last carbon of the six-membered ring was seen as a tertiary carbinol at 70.56 ppm, which specified this carbon as the attachment site of the hydroxyl group and of the aliphatic acid chain. The relative placement of these fragments in the ring was confirmed by the examination of long range couplings in the ^{13}C NMR spectra. Specific low power proton decoupling in the ^{13}C NMR spectrum revealed 3-bond coupling ($J_{C\text{-}H}$=9.1 Hz) between ketonic C-1 and the vinyl proton, which located the latter at C-3 and thus leaving C-2 (130.2 ppm) for the nitrogen. Since this nitrogen is non-basic, it became the acceptable site for the 9-carbon acyl chain, forming the amide consistent with the carbonyl shift at 164.82 ppm. The proton which was assigned unequivocably to C-6 by specific decoupling was also coupled long range to C-1 ($J_{C\text{-}H}$=4.0 Hz). In addition to the large 1-bond couplings already described for the epoxide carbons, C-5 showed additional long range couplings (3-bond) in the gated spectrum to both the vinyl proton at C-3 and the methylenic protons on C-7, confirming its linkage to the quaternary carbon (70.56 ppm). The gated spectrum of C-6 showed a clean doublet with no 2 or 3-bond coupling.

Biological Properties

Crystalline antibiotic U-62162 inhibited the growth of Gram-positive bacteria when tested by the standard agar-dilution method (Table 2).

Table 2. *In vitro* antibacterial activity of antibiotic U-62162.

Microorganism		Minimal inhibitory concentration (μg/ml)
S. aureus	UC 76	1.0
〃	UC 6685	1.0
〃	UC 6690	1.0
S. pyogenes	UC 159	125
S. faecalis	UC 694	7.8
S. marcescens	UC 131	1000
S. schottmuelleri	UC 126	>1000
S. flexneri	UC 143	>1000
P. stuartii	UC 6570	>1000
K. pneumoniae	UC 58	>1000
E. coli	UC 6783	>1000
Ps. aeruginosa	UC 95	>1000
A. naeslundii	UC 5920	250
P. acnes	UC 6564	15.6
P. maguns	UC 6258	3.9
P. aerogenes	UC 6319	15.6
E. leutum	UC 6327	31.2
C. bifermentans	UC 6507	31.2
C. novyi B	UC 6329	31.2
C. perfringens	UC 6509	31.2
C. difficile	UC 6834	31.2

Since this antibiotic was found to be particularly active in inhibiting the growth of *S. aureus*, it was evaluated *in vivo* in mice experimentally infected with *S. aureus*. The maximum tolerated dose in healthy animals was 12.5 mg/kg/day when administered intraperitoneally. The highest dose tested in infected animals in the standard CD_{50} test was 8 mg/kg/day and at this level no protection of the animals was detected.

Acknowledgments

The authors wish to thank Dr. C. W. FORD, Mr. K. F. STERN and Mr. G. E. ZURENKO for the *in vitro* and *in vivo* evaluation of antibiotic U-62162; thanks are also due to members of the Physical and Analytical Chemistry Department of The Upjohn Company for analytical and spectral data and to Mr. J. A. SHILEY for technical assistance.

References

1) KAKINUMA, K.; N. IKEKAWA, S. NAKAGAWA & S. ŌMURA: The structure of asukamycin, a possible shunt metabolite from 3-dehydroquinic acid in the shikimate pathway. J. Am. Chem. Soc. 101: 3402~3404, 1979
2) SCHROEDER, K. & A. ZEECK: Manumycin. Tetrahedron Lett. 1973: 4995~4998, 1973