NEW ANTIBIOTIC U-64846: FERMENTATION, ISOLATION AND CHARACTERIZATION

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Antibiotic U-64846 is a new entity with the molecular formula $C_{18}H_{35}ClN_4O_9$ (MW 486). It is a very water soluble, reddish solid which decomposes above 300°C and which is air-sensitive. The antibiotic is produced by *Streptomyces braegensis* and it inhibits a variety of Gram-positive bacteria. Acidic hydrolysis gave 3,7-diaminoheptanoic acid. The antibiotic gives ^1H NMR, ^{13}C NMR, IR and UV spectra which indicate it is not closely related to known antibiotic families.

Antibiotic U-64846 is a new entity which has the molecular formula $C_{18}H_{35}ClN_4O_9$. The data detailed below do not allow one to suggest a unique structure but the data preclude its inclusion in known antibiotic families. Isolation of antibiotics such as this one requires careful attention, particularly in devising methods to separate the active, organic compound from inorganic salts.

Microorganisms

Antibiotic U-64846 is produced by a new microorganism which has been characterized as *Streptomyces braegensis* Dietz sp. nov., UC 8284, NRRL 12567. The organism was found in our soil screening operation after manipulation of an acidic soil obtained from British Honduras.

Materials and Methods

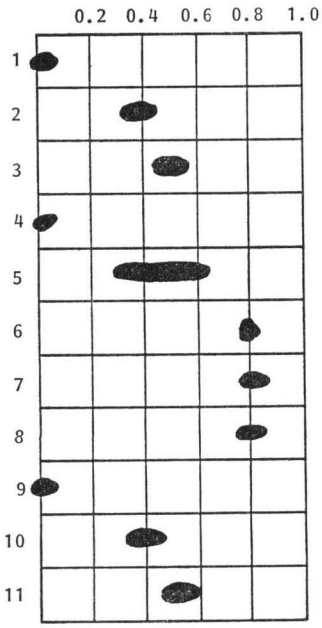
All solvent ratios are given as v/v. The production of antibiotic U-64846 was measured by the paper disc agar diffusion method using *Staphylococcus aureus* UC 80 or *Streptococcus pyogenes* UC 6055 as the test organism. Useful paper chromatographic systems are summarized in Fig. 1. Thin-layer chromatography with methanol - water, 1: 1 on Analtech silica gel plates gave an Rf of 0.3. On the same plates, but using the upper phase from chloroform - methanol - 17% NH_4OH, 2: 1: 1, the Rf was 0.6[1]. On the TLC systems the antibiotic was visualized on the plates using bioautography, phosphor quenching, iodine vapor, and with phosphomolybdic acid or permanganate-periodate sprays. High voltage paper electrophoresis was also used[2]. These experiments were routinely run at 60 V/cm using Whatman #1 paper and mobilities were referenced to streptothricin. With the pH 1.8 buffer[2], antibiotic U-64846 migrated toward the cathode 60% as far as did streptothricin. It migrated toward the anode 42% of the distance streptothricin did (toward the cathode) in a pH 8.7, volatile buffer consisting of 4-methylmorpholine - pyridine - glacial acetic acid - water, 23: 15: 0.4: 1964.

Fermentation

All fermentations were conducted under submerged culture conditions in 500-ml Erlenmeyer flasks containing 100 ml of medium. Seed cultures were prepared in a medium containing in g/liter: blackstrap molasses 4, dextrin 20, peptone (Difco) 10, yeast extract (Difco) 4, L-asparagine 0.2, $CoCl_2 \cdot 6H_2O$ 0.001. The medium was adjusted to pH 7.2 before sterilization. The seed flasks were inoculated with a frozen agar plug of *S. braegensis* UC 8284 and incubated on a rotary shaker (250 rpm, 6-cm stroke) at

Fig. 1. The paper chromatographic pattern of antibiotic U-64846 when bioautographed on *S. aureus* UC 80.

Solvent systems: 1) 1-butanol - water (84: 16), 2) 1-butanol - water (84: 16) and 0.25% *p*-toluenesulfonic acid, 3) 1-butanol - acetic acid - water (2: 1: 1), 4) 2% piperidine (v/v) in 1-butanol - water (84: 16), 5) 1-butanol - water (4: 96), 6) 1-butanol - water (4: 96) +0.25% *p*-toluenesulfonic acid, 7) 0.5 M phosphate buffer pH 7.0, 8) 0.075 M NH_4OH saturated with methyl isobutyl ketone, lower phase, 9) toluene - methanol - water (1: 1: 2), 10) 1-butanol - water (84: 16) and 2% *p*-toluenesulfonic acid, 11) methanol - 15% aqueous sodium chloride (4: 1). The paper used is impregnated with 0.1 M Na_2SO_4.



28°C for 3 days. The fermentation medium contained in g/liter: black-strap molasses 4, corn starch 25, dextrin 5, brewers yeast 2, Kay soy (Archer Daniel Co., Midland, MI) 13, corn steep liquor 8, KH_2PO_4 3, mineral salt solution A 2 ml/liter, mineral solution B 1 ml/liter. Mineral solution A contained in g/100 ml: $MgSO_4 \cdot 7H_2O$ 5, $MnSO_4 \cdot H_2O$ 0.3, $FeSO_4 \cdot 7H_2O$ 1, $ZnSO_4 \cdot 7H_2O$ 0.3, $CoCl_2 \cdot 6H_2O$ 0.1 (adjusted to pH 2 with conc H_2SO_4). Mineral solution B contained in g/100 ml: KH_2PO_4 5, KCl 10. The fermentation medium was adjusted to pH 7.2 before sterilization. The fermentation flasks were inoculated with 5 ml of seed culture and incubated at 28°C on a rotary shaker. Peak antibiotic titers were usually obtained after 4~5 days. At that point inhibition zones of 26~27 mm were measured with undiluted broth in the disc agar plate diffusion assay using *S. pyogenes* UC 6055 as the indicator organism.

Isolation

The fermentation broth (9 liters, pH 7.6) was filtered over a pad of Dicalite and the filtrate was percolated over a bed of Amberlite XAD-2 resin (Rohm and Haas) which measured 6×70 cm. The resin was washed with 4 liters of water followed by 4 liters of water - acetone, 9: 1. The antibiotic was eluted with water - acetone, 4: 1. The acetone was removed on a rotary evaporator and the aqueous solution was lyophilized. The resulting solids were chromatographed on silica gel using a step gradient starting with methanol - water, 2: 1 and ending with water. The antibiotic eluted with the water-rich solvent and could be detected by UV absorbance at 290 nm. The appropriate fractions were pooled. The pool was adjusted to pH 7 and percolated over a bed of DEAE cellulose (OH^-) (Whatman, DE52) which had been slurry-packed in methanol - water, 1: 2. The column was washed with water and the antibiotic was eluted with 0.05 M NaCl solution. The fractions containing the antibiotic were pooled and the pool was desalted using another cycle over Amberlite XAD-2 resin as described above. The eluate pool was lyophilized to yield a white to brown powder (150 mg/liter of broth).

Brown powder obtained as described (2.8 g) was subjected to 700 transfers on a Craig Countercurrent Distribution machine (CCCD, 10 ml/phase/tube). The solvent consisted of the upper and lower phases obtained after equilibration of 1-butanol - methanol - glacial acetic acid - water in the ratio 12: 2: 1: 15. The peak tube was tube 221 (K 0.46). Tubes 190~260 were pooled on the basis of fit to

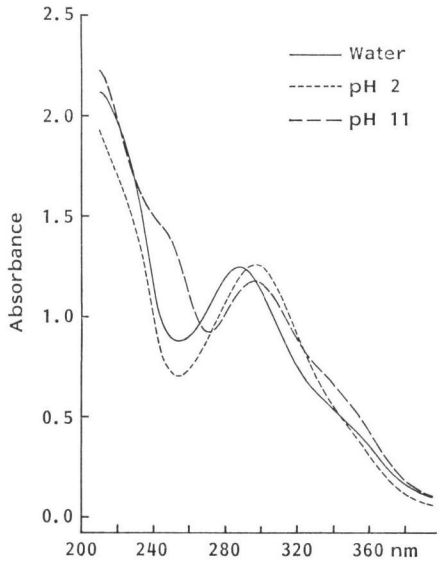
the theoretical curve (UV assay). The butanol and methanol were removed on a rotary evaporator. The aqueous solution was diluted with water and lyophilized. This procedure resulted in an oil reeking of acetic acid. The oil was dissolved in water and the new solution was lyophilized. The product was now a gray to reddish brown solid which was pure antibiotic U-64846. The yield was 1.3 g or about 50% of the CCCD feed.

Care had to be exercised throughout the work to avoid procedures which allowed solutions of the antibiotic to stand exposed to air unnecessarily. Colorless aqueous solutions of the antibiotic turn red overnight due to formation of a small amount of an unknown compound.

Characterization

The product obtained from CCCD above darkened but did not melt at even 350°C. High resolution FAB-MS experiments established that the molecular formula was $C_{18}H_{35}ClN_4O_9$. FAB-MS in both the positive and negative ion modes confirmed that the molecular ion appeared at *m/z* 486. Also, a pentatrimethylsilyl derivative (M^+ *m/z* 846) was prepared using bistrimethylsilylacetamide in pyridine (60°C, 30 minutes, sealed vial). EI and CI MS techniques gave no recognizable molecular ion or large fragments. Elemental analyses varied with the salt content of the various preparations. A typical analysis gave C 46.55, H 5.95, N 10.22, Cl 6.60, ash 5.61. Theory requires C 44.4, H 7.19, N 11.51, Cl 7.30. The equivalent weight found was 346 and $[\alpha]_D$ +69° (*c* 6.5, H_2O).

Fig. 2. The UV spectra of antibiotic U-64846 at 0.1 mg/ml.



The UV spectra (Fig. 2) show a maximum near 295 nm (ε 5,800) with minor shifts with pH. The IR spectrum (Fig. 3) shows a

Fig. 3. The IR spectrum of antibiotic U-64846 (reflectance plate method).

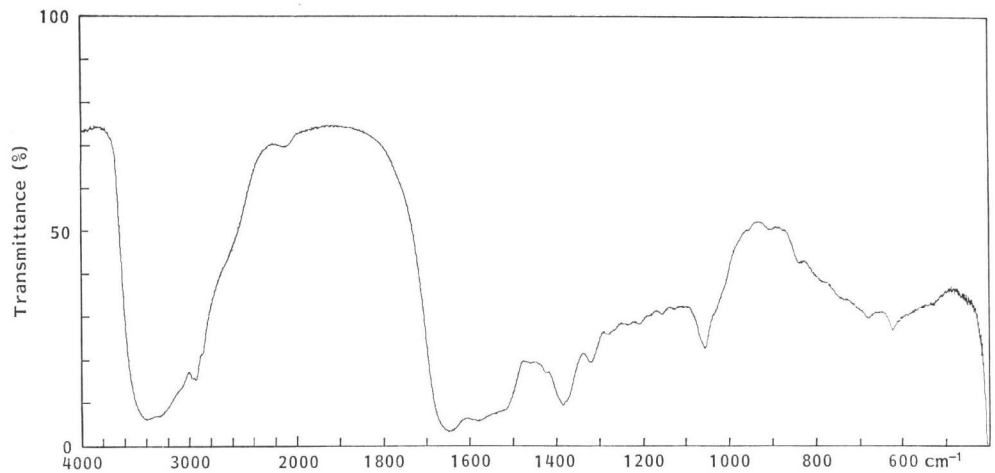


Table 1. The ^{13}C NMR spectra of antibiotic U-64846.

Acid form*	Base form**	Acid form*	Base form**
172.06, s	171.12, s	61.24, d	58.58, d
170.96, s	168.23, s	48.70, d	45.60, d
152.40, s	157.20, s	39.01, t	37.87, t
136.45, d	129.81, s	35.83, t	36.59, t
132.11, s	129.26, d		31.42, t
132.02, s	125.97, s	30.99, t	24.09, q
123.63, s	110.44, s	26.29, t	23.29, q
109.35, d	104.81, d	25.52, q***	22.51, t
71.57, s	69.25, s	21.64, t	19.29, t

* At pH 5.

** Conc NH_4OH was added to the pH 5 solution.

*** This line results from the coincidence of two methyl groups. The spectrum was run in D_2O at 53 MHz on a Varian XL-200 spectrophotometer. The abbreviations s, d, t and q, respectively represent singlet, doublet, triplet and quartet.

series of broad bands.

The ^{13}C NMR spectrum showed 18 lines. The shifts varied with the pH of the solution used. The spectra of the acid and salt forms of antibiotic U-64846 are summarized in Table 1. The salt form spectrum was obtained by adding concentrating ammonia to the solution of the acid form. This yielded a bright blue solution after 16 hours. The doublet at δ 48.7, the five triplets and one of the carbonyl singlets have been assigned to a 3,7-diaminoheptanoic acid moiety, the presence of which we deduce below on the basis of GC-MS work on the hydrolyzed antibiotic. We frequently observed a singlet at 167.4 ppm in the acid spectrum which we attribute to a decomposition product of the antibiotic. The spectra require that 20 covalently-bonded protons be present in antibiotic U-64846.

The 500 MHz ^1H NMR spectrum of antibiotic U-64846 is shown in Fig. 4 and summarized in Table 2. The peaks between 1 and 4 ppm appeared as broad multiplets at 200 MHz. Gated decoupling experiments and integration allowed us to identify the protons present in the 3,7-diaminoheptanoic acid moiety (*vide infra*). The peaks labelled F are assigned to a methylene α to a carbonyl. It is coupled to a methine (labelled D) which is α to an amino group. The methine is further coupled to the methylene group labelled I. Group I is also coupled to methylene group G which in turn is coupled to methylene group H. Group H is coupled to methylene group E. Methylene group E is α to an amino

Fig. 4. The 500 MHz ^1H NMR spectrum of antibiotic U-64846 in D_2O.
See Table 2.

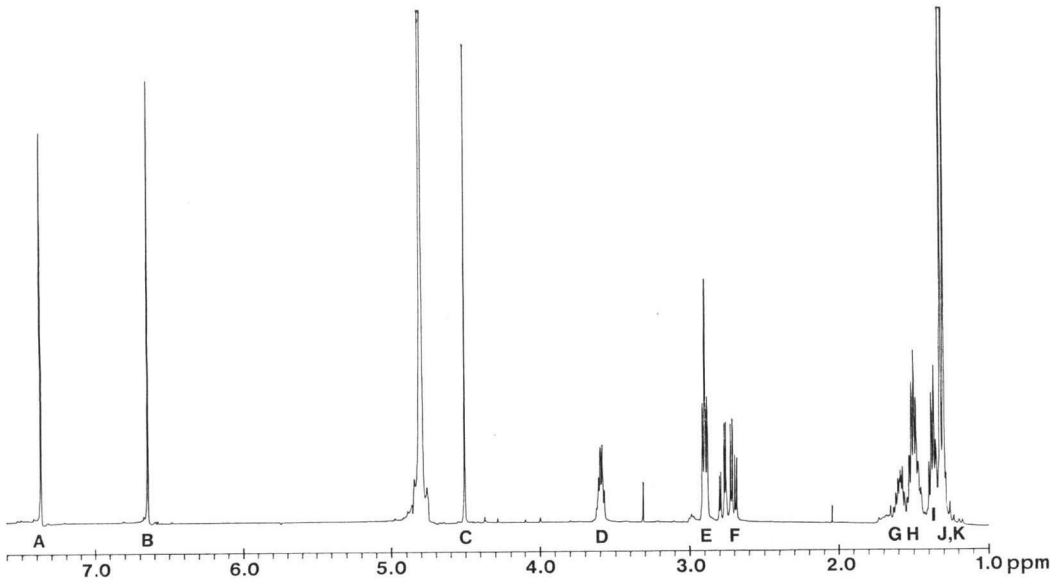


Table 2. The ^1H NMR spectrum of antibiotic U-64846.

Group label	Shift (δ)	Integral	Couplings
A	7.4	1H	Singlet
B	6.65	1H	Singlet
	4.8	water	
C	4.5	1H	Singlet
D	3.6	1H	$J_{DF1}=6.5$, $J_{DF2}=7.0$, $J_{DI}=7.0$
E	2.9	2H	$J_{EH}=7.0$
F	2.75	2H	$J_{F1F2}=16$, $J_{F1D}=6.5$, $J_{F2D}=7.0$
G	1.6	2H	*
H	1.5	2H	*
I	1.4	2H	$J_{IG}=7.0$, $J_{ID}=7.0$
J	1.32	3H	Singlet
K	1.30	3H	Singlet

* The overlap between groups G and H makes determination of the coupling constants tenuous at best.

group. See Table 2 for the coupling constants. Groups A, B and C are assigned to isolated protons and the two methyl groups are labelled J and K. The spectrum requires that there be 20 covalently-bonded protons in antibiotic U-64846.

3,7-Diaminoheptanoic Acid

A solution of antibiotic U-64846 (500 mg, 1.03 mmol) in 50 ml of 3 N HCl was heated at 50°C for 16 hours. The solution was cooled to 25°C and adjusted to pH 7 with 50% NaOH solution. This was passed over a 20 ml bed of Dowex 50 WX8 (H^+). The resin was washed with deionized water and the basic products were eluted with 1 N NH_4OH solution. The eluate was lyophilized to give a dark solid. A ^{13}C NMR at pH 4 of this solid in D_2O showed six lines at ppm 50.49 (d), 40.78 (t), 39.26 (t), 33.0 (t), 27.86 (t) and 23.03 (t). An 80 MHz ^1H NMR spectrum showed broad bands corresponding to groups labelled D to I in the spectrum of the intact antibiotic (Fig. 4).

A sample of the eluted product was converted to an *N*-trifluoroacetyl butyl ester (TAB derivative) by standard means[8]. The TAB derivative was injected onto an 80 cm×4 mm glass column packed with 3% OV-17 on high efficiency Chromosorb W. The instrument used was a Hewlett-Packard 5992A desk-top GC-MS operating with a temperature gradient of 5°C/minute starting from 150°C. Total

Fig. 5. The mass spectrum of the TAB amino acid obtained after acid hydrolysis of antibiotic U-64846. See Table 3.

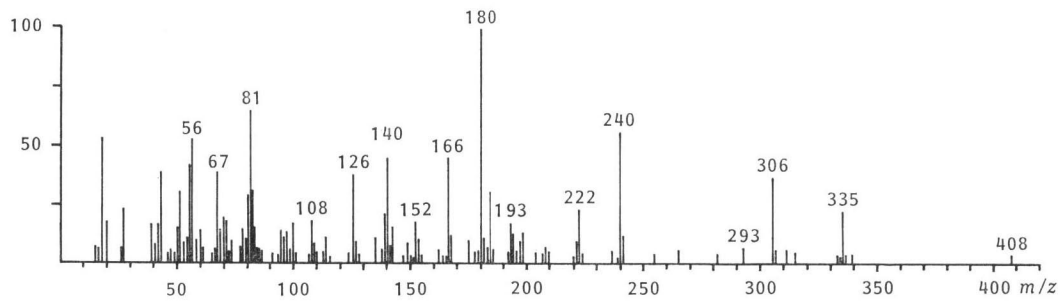
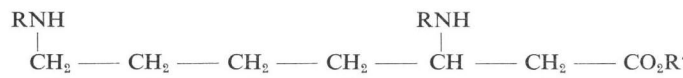


Fig. 6. A summary of the spectroscopic data assignable to the 3,7-diaminoheptanoic acid moiety present in antibiotic U-64846.



^1H NMR label:	E	H	G	I	D	F	(Table 2)
^{13}C NMR (ppm):	40.8	33.0	23.0	27.9	50.5	39.3	R=R'=H
^{13}C NMR (ppm):	39.0	35.8	21.6	26.3	48.7	39.0	(Table 1)

GC-MS: R=CF_3CO, R'=*n*-C_4H_9

M^+ *m/z* 408; loss of R'O yields *m/z* 335; cleavage at C_2–C_3 yields *m/z* 293 ion (diagnostic for β-amino acids).

Table 3. Major fragments from TAB derivative of 3,7-diaminoheptanoic acid.

m/z	Assignment
408	M^+
335	$M^+-C_4H_9O$
293	$M^+-C_4H_9OCOCH_2$
240	$M^+-C\text{-}1\sim C\text{-}7$
222	$335-CF_3CONH_2$
193	$306-CF_3CONH_2$
180	$293-CF_3CONH_2$
166	$193-CH_2=CH_2$
152	$180-CH_2=CH_2$
140	$180-CH_2=CH\text{-}CH$
126	$[CH_2=NHCOCF_3]^+$

Table 4. MICs (μg/ml) of antibiotic U-64846.

Organism	UC #	MIC (μg/ml)
Staphylococcus aureus	76	1,000
S. aureus	6685	500
Streptococcus pyogenes	152	31.2
S. pneumoniae	41	31.2
S. feacalis	694	>1,000
Escherichia coli	51	>1,000
Klebsiella pneumoniae	57	>1,000
Salmonella schottmuelleri	126	>1,000
Pseudomonas aeruginosa	95	>1,000

ion abundance detection showed a single peak with a retention time of 9.9 minutes. The fragmentation pattern of this peak is shown in Fig. 5.

The GC-MS analysis of TAB amino acids has long been studied[4~6] and they are known to fragment by predicable pathways. We have compiled a library of retention times and fragmentations of the TAB derivatives of 16 protein and 65 non-protein amino acids (The Upjohn Company Research Labs., unpublished results). TAB amino acid obtained from antibiotic U-64846 differed from any we had seen and in fact was the TAB derivative of 3,7-diaminoheptanoic acid. This amino acid has not previously been reported as a constituent of an antibiotic but it[7] and its di-*N*-carbobenzyloxy derivative[8] have been synthesized. The fragmentation pathways that support this identification are summarized in Table 3. Fig. 6 summarizes all the data supporting the contention that a 3,7-diaminoheptanoic acid is a constituent of antibiotic U-64846. The question of the nature of the linkage and the optical configuration of this seven carbon fragment remains unsettled.

Structural Consideration

Antibiotic U-64846 appears to represent a new antibiotic type. If the elements of 3,7-diaminoheptanoic acid are subtracted from the molecular formula ($C_{18}H_{35}ClN_4O_9$ less $C_7H_{15}N_2O_2$), one is left with a hypothetical $C_{11}H_{21}ClN_2O_7$ molecule. The hypothetical molecule must have two methyl groups and account for the UV chromophore observed in the intact antibiotic. Note that there are ten heteroatoms and only nine carbon atoms on which to place them in this structure.

The reddish color, high melting point and ease of oxidation of antibiotic U-64846 are vaguely reminiscent of the dnacins, which are produced by a *Nocardia* species[9].

Biological Data

Antibiotic U-64846 show activity vs. a variety of Gram-positive and anaerobic bacteria. The minimum inhibitory concentrations assayed in agar are listed in Table 4. The new antibiotic showed weak activity against influenza A (PR8) and equine rhinovirus in cell culture. It was inactive against herpes simplex types 1 and 2 in the same assay and against *Eimeria tenella* at 1 μg/ml. It did not protect mice infected with *S. pyogenes* UC 152 when it was administered subcutaneously at doses as high as 80 mg/kg.

Acknowledgments

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