U-56,407, A NEW ANTIBIOTIC RELATED TO ASUKAMYCIN: ISOLATION AND CHARACTERIZATION

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U-56,407, a bright yellow, crystalline antibiotic was isolated from fermentations of *Streptomyces hagronensis* (strain 360). This antibiotic was extracted from fermentation broths with halogenated hydrocarbons and purified by silica gel chromatography and crystallization. U-56,407 is active *in vitro* against Gram-positive bacteria but not Gram-negative organisms. It failed to demonstrate *in vivo* activity in experimentally infected mice. Physical-chemical characterization of U-56,407 supports a molecular formula of $C_{29}H_{32}N_2O_7$ and a structure related to the antibiotic asukamycin.

During the course of soil screening for antibiotics, a new species of streptomycete designated *Streptomyces hagronensis* strain 360 (UC 5875) was found to produce two solvent extractable antibiotics. One component, U-56,407, is active only against Gram-positive organisms while the other inhibits principally the Gram-negative bacteria. The antibiotics were readily separated on silica gel TLC plates and columns using differential bioautography on *Staphylococcus aureus* and *Klebsiella pneumoniae*. This paper describes the taxonomy of the producing organism, its fermentation and the extraction and purification of U-56,407 by silica gel chromatography and crystallization. The application of spectroscopic methods (UV, IR, NMR & MS) as well as physical-chemical procedures resulted in the structural characterization of this new antibiotic which belongs to a small group of compounds probably derived from dehydroquinic acid in the shikimic acid pathway.

Taxonomy

The organism producing U-56,407 was isolated from soil obtained from the State of Washington. Although it resembled *Streptomyces lemensis* (NRRL 8170) microscopically and by reverse color pattern using Ektachrome photography, it was differentiated from this organism by other taxonomic criteria. Since the new organism produced globomycin[1], in addition to U-56,407, it was compared with the 5 cultures reported to produce this cyclic peptide, *S. hagronensis*, *S. halstedii*, *S. hygroscopicus*, *S. neohygroscopicus*, and *Streptoverticillium cinnamomeum*. It was differentiated from all but *S. hagronensis* by microscopic and Ektachrome characteristics. Although the culture producing U-56,407 differed from *S. hagronensis* in its growth characteristics on D-xylose and L-rhamnose, its similarities to this organism led to its designation as *S. hagronensis* strain 360 (UC 5875).

Fermentation

The seed medium for the *S. hagronensis* culture contained 10 g/liter of glycerol, 10 g/liter of Difco peptone and 0.5 ml/liter of Ucon defoaming agent.

The medium was prepared in tap water adjusted to pH 7. The seed fermentation was carried out at 28°C in a 20-liter vessel with an aeration rate of 10 liters/minute (back pressure 700 g/cm^2) and an agitation speed of 400 rpm. The seed was harvested at 2 days.

The fermentation medium contained 20 g/liter of Con Pro corn gluten meal, 10 g/liter of Con

Pro soluble starch, 20 g/liter glycerol, g/liter of Cal Carb $CaCO_3$ and 0.2 ml/liter of Ucon in tap water adjusted to pH 7.5. The fermentation, using a 5% inoculum of seed, was carried out at 28°C in a 250-liter tank at an agitation rate of 280 rpm and an aeration rate of 200 liters/minute (back pressure 700 g/cm^2). The tank was harvested at 3 days and *Staphylococcus aureus* was used to ascertain the level of U-56,407 present.

Isolation

Unfiltered fermentation broth was stirred with an equal volume of CH_2Cl_2. Dicalite, 20% w/v, was added and, after thorough mixing, the broth was filtered. The filter cake was washed with 1/2 broth volume of CH_2Cl_2 which was then combined with the filtrate. After separation, the solvent extract was dried overnight with anhydrous Na_2SO_4 and evaporated in a rotary evaporator under water aspirator vacuum at a temperature which did not exceed 40°C. The oily residue which resulted was dissolved in 3 volumes of CH_2Cl_2 - CH_3OH (92.5: 7.5). This solution was mixed with 3 volumes of hexane. The precipitate which formed with filtered off, washed several times with hexane and air dried. The potency of this crude solid was determined using a disc-plate bioautographic assay on *S. aureus*. The composition of the solid was determined by TLC analysis on silica gel plates (K6F, Whatman Inc., Clifton, NJ) using a mobile phase of CH_2Cl_2 - CH_3OH (92.5: 7.5) or THF - C_6H_{12} (4: 1).

After drying, the plates were viewed under 254 nm light or were bioautographed on *S. aureus* seeded agar trays[2]. U-56,407 has an Rf of 0.45 in the CH_2Cl_2/CH_3OH mobile phase and 0.55 in the THF/C_6H_{12} mobile phase.

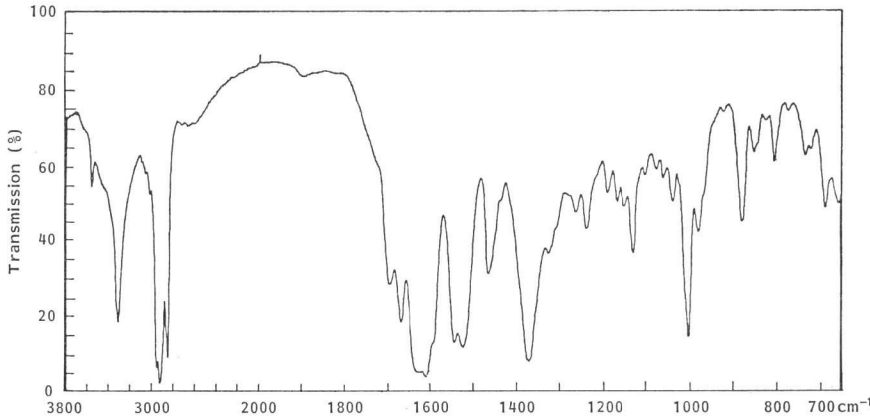
Purification

Crude U-56,407 was purified by silica gel chromatography and subsequent crystallization from solutions containing halogenated hydrocarbons and low molecular weight alcohols. The silica gel column was slurry packed with 100 g silica (Geduran-TM S160, EM Labs, Elmsford, NY) per g of crude U-56,407 to be processed using the CH_2Cl_2/CH_3OH mobile phase described above.

The column charge was prepared by mixing the crude solid extract with twice its weight of silica gel and slurrying this mixture in mobile phase. After evaporating the solvent, the resulting dry powder was charged onto the column and was eluted with the CH_2Cl_2/MeOH mobile phase at a linear velocity of 0.1 to 0.2 cm/minute. Fractions (20 ml) were collected and bioautographed on *S. aureus* and *Klebsiella pneumoniae* seeded trays. Those fractions giving zones of inhibition against *S. aureus* but not *K. pneumoniae* were pooled and the solvent was evaporated under vacuum resulting in a yellow powder. *K. pneumoniae* was used in testing the column fractions since the culture produced a Gram-negative activity ultimately identified as globomycin by IR, UV, ^{13}C NMR and mass spectrometry.

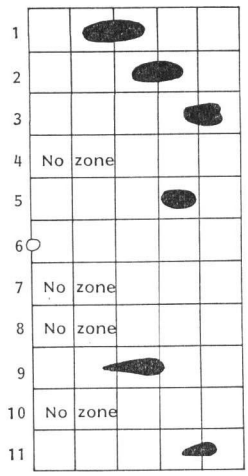
The yellow solid was washed several times with hexane and dried *in vacuo*. In many cases, this procedure was sufficient to produce highly purified U-56,407 but occasionally a second column was necessary to purify the antibiotic further. The second column contained silica gel as before but the mobile phase consisted of $CHCl_3$ - C_6H_{14} - CH_3OH (82: 11: 7). The solid isolated from the first column was charged onto the second column using the technique described above and the column was eluted at a mobile phase velocity of 0.1 cm/minute. Again, the antibiotic was isolated on the basis of those fractions which were active against *S. aureus* only. At this point, U-56,407 did not contain any other antibacterially active component as determined by bioautographic TLC using *S. aureus*. Visu-

Fig. 1. IR Spectrum of U-56,407 (Nujol).



alization of the TLC plates under 254 nm light prior to bioautography revealed the presence of only trace impurities. After pooling the active fractions and removing the solvent, the solid residue was taken up in CH_3OH (100 ml/g) containing sufficient CH_2Cl_2 to effect complete dissolution of the sample. Crystallization occurred at room temperature within 24 hours and after filtration and drying of the solid, TLC analysis using bioautography or fluorescence quenching (254 nm) revealed a single zone at Rf 0.5.

Fig. 2. The paper chromatographic pattern of U-56,407 bioautographed on *S. aureus*.



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) and 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) benzene - 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.

Chemical Characterization

Antibiotic U-56,407 is a yellow, crystalline (long, broad, flat plates), water insoluble compound with a molecular ion at m/z 521 (M+1) by field desorption mass spectroscopy and an equivalent weight of 519 by titration with base. Based on these data and elemental analyses [calcd. C 66.91, H 6.20, N 5.38, O 21.51; found C 66.28, H 6.23, N 5.49, O 22.0 (by difference)], a molecular formula of $C_{29}H_{32}N_2O_7$ is proposed. U-56,407 decomposes at 150°C and has a $[\alpha]_D^{25}$ +263° ($CHCl_3$). The UV maxima and log ε values are given in Table 1 while the infra-red spectrum and paper chromatographic pattern are

Table 1. UV Spectrum of U-56,407.

	λ_{max} (nm)	log ε
λ_{max}^{MeOH}	265 (sh)	4.55
	315	4.78
$\lambda_{max}^{0.1N\ HCl\text{-}MeOH}$	265 (sh)	4.55
	315	4.78
$\lambda_{max}^{0.1N\ NaOH\text{-}MeOH}$	265 (sh)	4.64
	307	4.78

shown in Figs. 1 and 2.

The results of several chemical tests are given in Table 2.

The UV peak at 315 nm with a shoulder at 265 nm is suggestive of an extended conjugated chromophore probably containing a keto-enol system. The multiple bands in the IR between 1725 and 1590 cm^{-1} can be assigned to various C=O, C=C, C=N groups with the 1525 and 1542 cm^{-1} bands being consistent with amide II vibrations. The molecular formula gives rise to a ring/double bond number of 15 which also suggests a high degree of unsaturation.

The unsaturated character of U-56,407 is further supported by the presence of a complex group of peaks in the 5.8 to 7.2 ppm region of the 200 MHz ^1H NMR spectrum (Varian XL 200), Fig. 3. In addition, peaks between 0.7 and 2 ppm in this spectrum are indicative of an isobutyl substituent.

The 0.9 ppm doublet is assigned to the "a" methyl protons while the broad complex at 1.2 ppm is

Table 2.

$FeCl_3$	Negative	No phenols
Ninhydrin	Negative	No primary amines
Na_2SO_3 - H_2SO_4	Negative	No aldehydes
NH_2OH - HCl	Positive	Ketone present

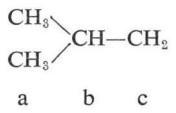


Fig. 3. 200 MHz ^1H NMR spectrum of U-56,407 (in DMSO).

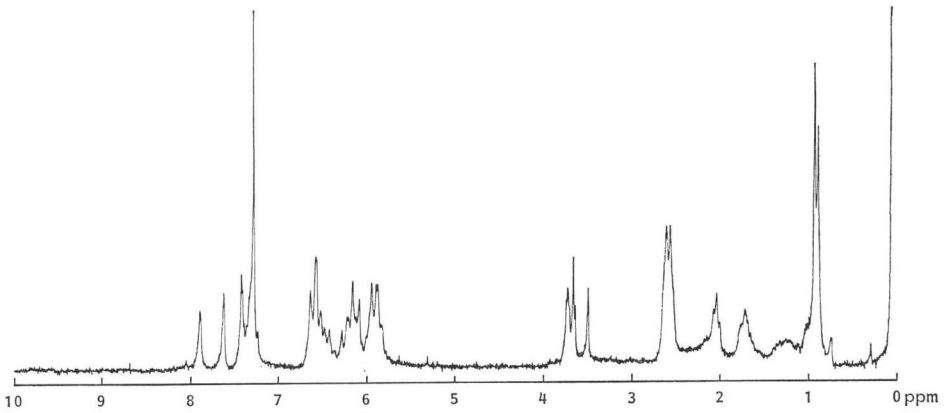
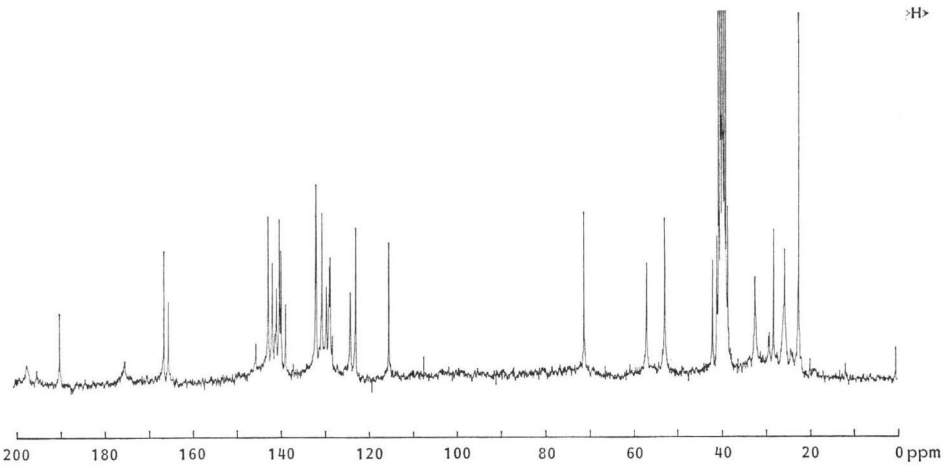
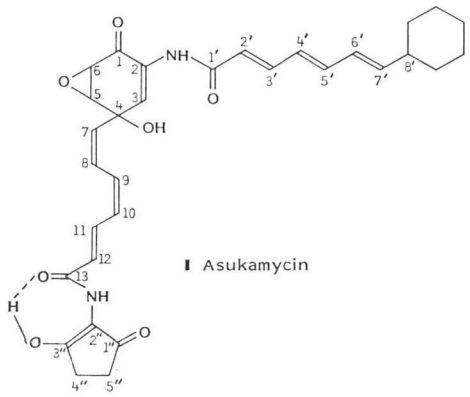
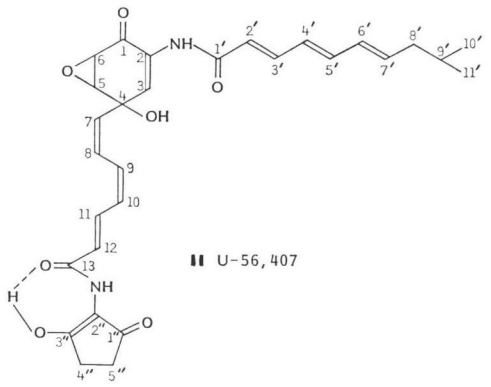


Fig. 4. 50 MHz ^{13}C NMR spectrum of U-56,407 (in DMSO).





I Asukamycin



II U-56,407

Table 3.

	U-56,407	Asukamycin	Manumycin
Color	Yellow	Yellow	Pale yellow
Solubility	Alcohols, halogenated hydrocarbons	Alcohols, halogenated hydrocarbons	Alcohols
mp °C (dec.)	150	188	140
Rotation	+263°	+181°	−185°
UV	315 nm (sh. 265)	315 nm (sh. 265)	325, 281 nm
IR	All major peaks correlate		Some peaks correlate
Mol. formula	$C_{29}H_{32}N_2O_7$ (MW 520)	$C_{31}H_{34}N_2O_7$ (MW 546)	$C_{31}H_{38}N_2O_7$ (MW 550)

assigned to the "b" proton and the 2.0 ppm peaks to the "c" protons. Examination of the 50 MHz ^{13}C NMR spectrum (Varian XL 200), Fig. 4, supports these assignments with the presence of chemical shifts at 22.3 (q), 28.0 (d) and 41.8 (t) ppm.

During interpretation and searching of the IR spectrum of U-56,407 using an IR spectral library compiled with a Perkin Elmer digitizer and data station, the spectra of asukamycin[3] and a related antibiotic manumycin[4] were found to be similar to the spectrum of U-56,407. A comparison of other physical-chemical properties of asukamycin (**I**) and manumycin with U-56,407, Table 3, supports the structural similarity.

The difference between the molecular formulas for U-56,407 and asukamycin is C_2H_2 (26 a.m.u.). In view of the spectral data which support the presence of an isobutyl group in the structure of U-56,407, it is proposed that the cyclohexyl group in asukamycin has been replaced by an isobutyl group leading to the structure (**II**) for U-56,407.

The correlation between the ^{13}C NMR spectra of asukamycin and U-56,407, Table 4, was examined to test the structural assignment. Some chemical shifts for U-62,162[5], a recently isolated antibiotic containing the 6 carbon-epoxide fused ring system, are included as additional evidence for certain assignments. The similarity in the chemical shifts and multiplicities of the spectral lines for the various compounds supports the proposed structure of U-56,407. Only weak broad bands were observed for the 1″ and 3″ carbon atoms at 198.0 and 175.8 ppm respectively in the ^{13}C NMR spectrum of U-56,407 run in DMSO. ŌMURA[3] does not report signals for these carbons in the asukamycin DMSO spectrum but did find singlets at 197.9 and 174.6 ppm in $CDCl_3$. A 50 MHz ^{13}C NMR spectrum of U-56,407 in

Table 4. ^{13}C NMR chemical shifts[a] and multiplicities for U-56,407 and asukamycin.

Carbon No.	U-56,407	Asukamycin	U-62,162
1	189.6 (s)	189.2 (s)	189.6 (s)
2	128.2 (s)	127.9 (s)	130.0 (s)
3	130.1 (d)	129.8 (d)	129.8 (d)
4	70.8 (s)	70.5 (s)	70.6 (s)
5	56.6 (d)	56.4 (d)	57.9 (d)
6	52.6 (d)	52.4 (d)	53.0 (d)
7~12	Unassigned	Unassigned	
13	166.1 (s)	165.7 (s)	
1′	165.0 (s)	164.7 (s)	164.8 (s)
2′~6′	Unassigned	Unassigned	
7′	138.3 (d)	144.7 (d)	
8′	41.8 (t)	40.2 (d)	
9′	28.0 (d)	28.6 (broad t) cyclohexyl ring carbons	
10′, 11′	22.3 (q)		
1″	198.0 (s)	197.9 (s)	
2″	115.0 (s)	114.7 (s)	
3″	175.8 (s)	174.6 (s)	
4″	25.5 (t)	25.5 (t)	
5″	32.2 (t)	31.9 (t)	

Unassigned doublets:

U-56,407 142.3, 141.4, 140.4, 139.8, 139.4, 131.4, 131.4, 129.1, 128.4, 123.7, 122.5.

Asukamycin 142.0, 141.0, 140.4, 139.4, 139.0, 131.2, 128.8, 128.2, 127.4, 123.3, 122.2.

[a] ppm relative to TMS in DMSO (Varian XL 200).

Table 5. Antibacterial spectrum of U-56,407.

Organism	Zone of inhibition (mm)[a]
Bacillus subtilis UC 564	24
B. subtilis UC 6033 (Amicetin Resistant)	27
Staphylococcus aureus UC 80	24
S. aureus UC 3665 (Macrolide resistant)	21
S. aureus UC 6029 (Streptomycin resistant)	19
Micrococcus luteus UC 130	17
M. luteus UC 3883 (Erythromycin resistant)	22
M. luteus sens. UC 130 (pH 8.3)	22
Klebsiella pneumoniae UC 57	0
Escherichia coli UC 51	0
Salmonella schottmuelleri UC 126	0
Proteus vulgaris UC 93	0
Mycobacterium avium UC 159	16
Penicillium oxalicum UC 1268	18 (hazy)
Saccharobacillus pastorianus UC 1342	0
Pseudomonas aeruginosa UC 95	0
Rhodopseudomonas sphaeroides UC 3238	15
Streptococcus pyogenes UC 152	22
Clostridium perfringens UC 6509	23
Bacteroides fragilis UC 6513	24

[a] 80 μl of a 1 mg/ml methanolic solution applied to 12.5 mm Schleicher and Schuell paper discs.

Table 6. Minimum inhibitory concentrations of U-56,407.

Organism	MIC (μg/ml)
Staphylococcus aureus UC 76	320
S. aureus UC 6675	160
S. aureus UC 3665	320
S. aureus UC 6685	320
S. aureus UC 6690	320
Streptococcus pneumoniae UC 41	160
S. pyogenes UC 152	160
S. faecalis UC 694	>320
Escherichia coli UC 45	>320
E. coli UC 3833	>320
Klebsiella pneumoniae UC 58	320
Citrobacter freundii UC 3507	>320
Providencia stuartii UC 6570	>320
Serratia marcescens UC 131	>320
S. marcescens UC 6888	>320
Salmonella typhi UC 215	>320
S. schottmuelleri UC 126	>320
Shigella flexneri UC 143	>320
Enterobacter cloacae UC 3054	>320
Pseudomonas aeruginosa UC 95	>160
P. aeruginosa UC 9191	>160

Test method; agar dilution, pH 7.4

$CDCl_3$ contained medium intensity singlets at 198.0 and 175.3 ppm respectively. The 7′ carbon in asukamycin was assigned the chemical shift 144.7 (d) based on reported line positions of sp^3 carbon atoms bonded to sp^2 carbons (both carbons bearing only protons). The shift contributions of the α, β, and γ carbon atoms in the isopropyl group to the proposed 7′ carbon in U-56,407, suggested a chemical shift of 7 to 10 ppm upfield from the 144.7 ppm line in asukamycin. The doublet at 138.3 ppm in U-56,407 was assigned to the 7′ carbon based on these considerations.

Biological Properties

U-56,407 is active *in vitro* against Gram-positive organisms particularly *S. aureus* and *Bacillus subtilis*, Table 5. Minimum inhibitory concentrations against a variety of pathogenic bacteria are given in Table 6. U-56,407 is inactive *in vivo* at a maximum level of 320 mg/kg in mice experimentally infected with *S. aureus*. The antibiotic was not toxic to the animals at this level of administration. In view of the water insolubility of U-56,407, an apparent NH_4^+ salt was prepared on the supposition that it would display superior absorption characteristics. It was inactive *in vivo* and only marginally active *in vitro*.

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