THE ISOLATION AND CHARACTERIZATION OF THE NEW ANTIBIOTIC U-60,394

Sir:

We have isolated a new antibiotic (U-60,394) which has the molecular formula $C_{20}H_{13}N_3O_6$. In this paper we report the production, isolation and physical properties of antibiotic U-60,394.

The producing organism was discovered in our soil screen in a soil obtained from the state of Washington, U.S.A. It has been characterized taxonomically and has been designated *Streptomyces woolensis* DIETZ and LI sp. nov. (UC® 5997, NRRL 12113).

A seed broth was prepared by inoculating the culture into a sterilized medium consisting of glucose (10 g/liter), Difco peptone (10 g/liter) and Difco yeast extract (2.5 g/liter) in deionized water. The broth (100 ml per 500-ml flask) was shaken on a rotary shaker at 28°C for 72 hours; the pH rose from 6.5 to 8.0±0.5. This broth was inoculated at the rate of 5 ml/100 ml into the production medium which consisted of 10 g/liter each of cerelose, buffalo starch, phytone (BBL), 5 g/liter $CaCO_3$ and 2 g/liter NaCl in tap water (pH 7.0±0.2 after sterilization). When this was incubated in flasks as described above the antibiotic was produced concurrently with a gradual rise in pH (to pH 8.5±0.3 after 4 or 5 days). The antibiotic was detected using a dipped disc assay on agar trays seeded with either *Sarcina lutea* UC® 130 or *Streptococcus pyogenes* UC® 6055.

The broth was filtered over a bed of Dicalite 4200 filter aid and the filter cake was washed with deionized water. The combined filtrate and wash pool was adjusted to pH 3±1 with sulfuric acid. Three successive extractions with 1/3 volumes of methylene chloride removed all the antibiotic from the aqueous pool. The combined organic phases were dried with magnesium sulfate, filtered and concentrated to dryness on a rotary evaporator. The yellow residue was dissolved in methanol and silica gel was added to the solution. The solvent was removed on a rotary evaporator so that the residue was adsorbed onto the silica gel.

The silica gel-sample mixture was used either as a pre-column connected in series to the chromatographic column or it was used to replace the first several centimeters of silica gel in the chromatographic column. In one case, 10 liters of beer yielded 540 mg of residue which was dissolved in 75 ml methanol and adsorbed onto 5 g of silica gel as described. This loaded silica gel-sample mixture was used to replace an equal amount of silica gel from the top of a 1.5×60 cm column of silica gel which was slurry-packed with 2% methanol in chloroform (v/v). The antibiotic was eluted by gradually adding methanol to the eluting solvent to a maximum of 10% methanol (v/v).

The active fractions were pooled and concentrated to give 110 mg of a yellow solid which gave a single spot (Rf 0.4) on Analtech glass silica gel plates using a mixture of ethyl acetate - acetone - water (8: 5: 1, v/v). The antibiotic was detected using its reaction with ferric chloride spray, its yellow color and bioautography on *S. pyogenes* UC® 6055. It also reacted with bromocresol green spray but was ninhydrin-negative.

The product was dissolved in hot methanol from which fine, greenish-yellow needles deposited on cooling, m. p. 265~266°C (dec.). This is antibiotic U-60,394.

The UV spectrum of this material is shown in Fig. 1. The solvent was methanol and the maxima shift with pH. The neutral spectrum was the same as that shown for added acid (solid line). Since antibiotic U-60,394 reacted with ferric chloride spray the presence of a phenolic moiety can be suspected.

The PMR spectrum (Fig. 2) showed three exchangeable protons (12, 11 and 10.3 ppm) along with ten aromatic/olefinic protons (7.2~8.4 ppm). The CMR spectrum (Fig. 3) had twenty lines between 114 and 168 ppm (Table 1). The IR spectrum as a mineral oil mull (Fig. 4) showed a carbonyl at 1765 cm^{-1} and bands at 1540 and 1300 cm^{-1} suggestive of a nitro group.

When antibiotic U-60,394 was dissolved in methanol and shaken at 45 psi H_2 with a little palladium on charcoal for 16 hours, a bioinactive compound was formed. The new compound was ninhydrin-positive and remained at the origin in the tlc system described above. This is consistent with the reduction of a nitro group to an amine.

High resolution mass spectrometry of the underivatized antibiotic showed the molecular ion at m/z 391.0801 (theory for $C_{20}H_{13}N_3O_6$ is 391.0804). Derivatization with triSil Z (Pierce Chemical Company, Rockford, IL 61105) showed

Fig. 1. The UV spectrum of antibiotic U-60,394 in methanol.
$c=0.01182$ g/liter

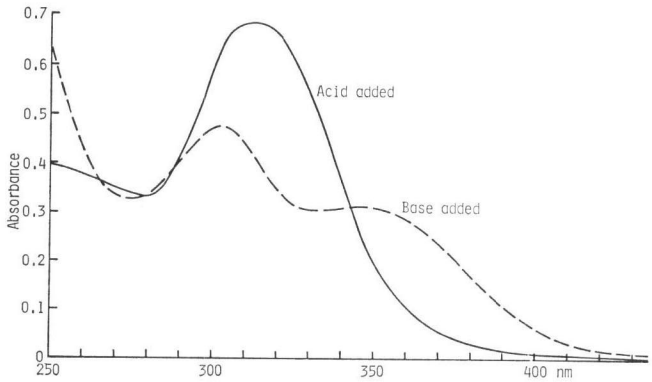


Fig. 2. PMR Spectrum of U-60,394 in d_6-DMSO with internal TMS at 100 MHz.

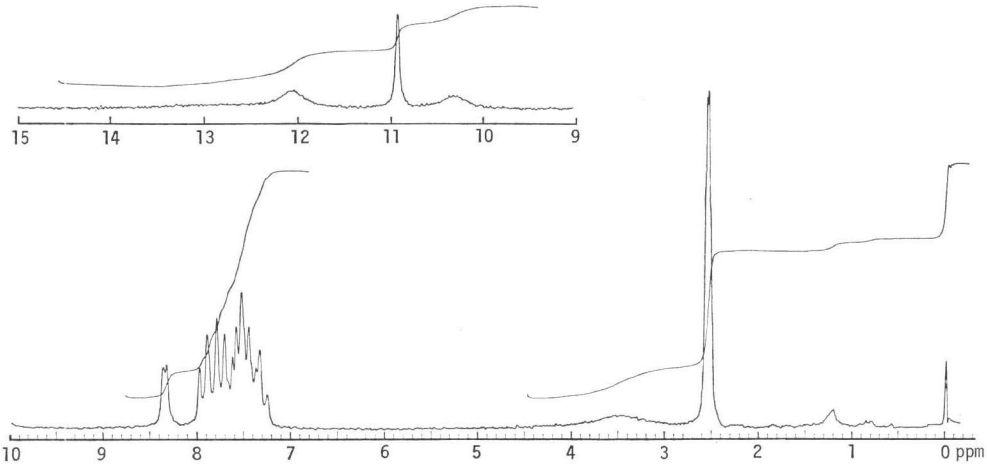


Fig. 3. CMR Spectrum of U-60,394 in d_6-DMSO with internal TMS.

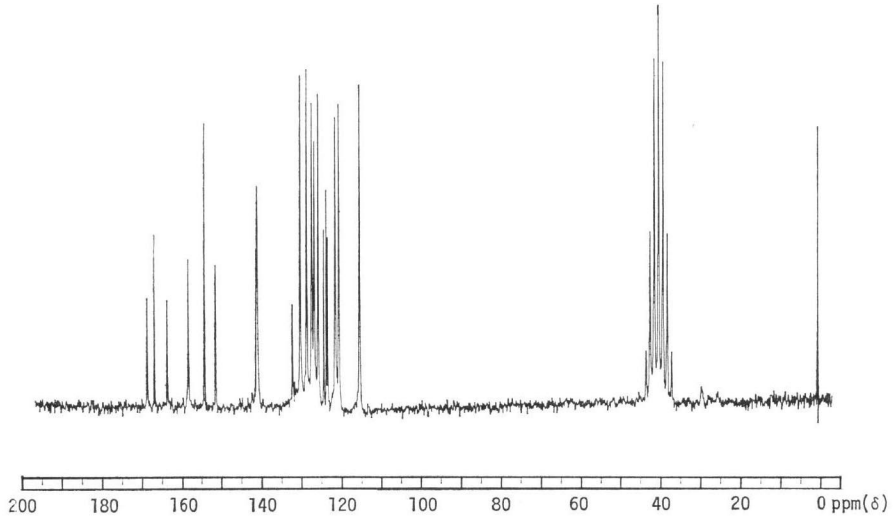


Table 1. CMR of antibiotic U-60,394.*

Shift (ppm)	Multiplicity	Shift (ppm)	Multiplicity	Shift (ppm)	Multiplicity	Shift (ppm)	Multiplicity
114.5	D	123.6	S	129.3	D	153.2	S
119.8	D	124.9	D	131.3	S	157.3	S
120.7	D	125.9	D	140.1	D	162.6	S
122.6	S	126.5	D	140.3	S	165.8	S
123.0	S	127.8	D	150.5	S	167.6	S

* The spectrum was taken on a Varian CFT-80 spectrometer. The sample was dissolved in d_6-DMSO with internal trimethylsilane as the reference.
D=doublet and S=singlet.

Fig. 4. Infrared spectrum of U-60,394 (Nujol).

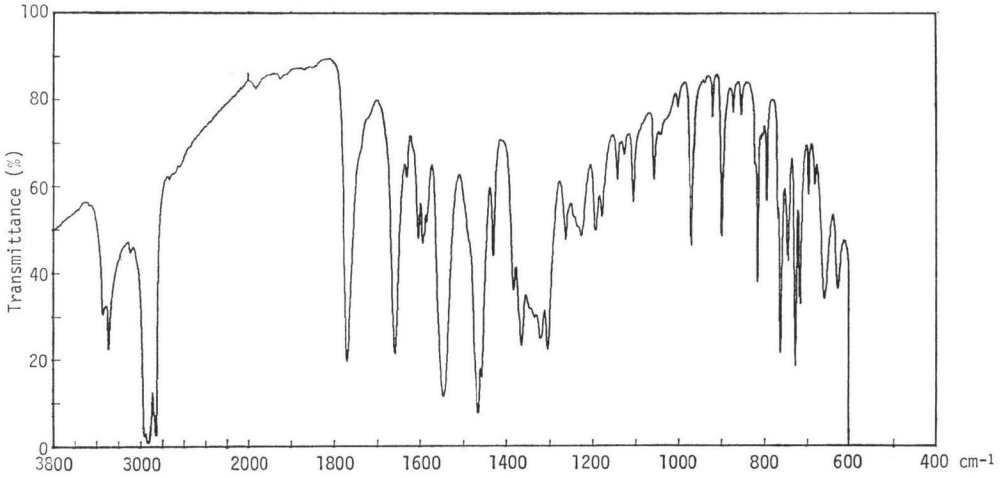
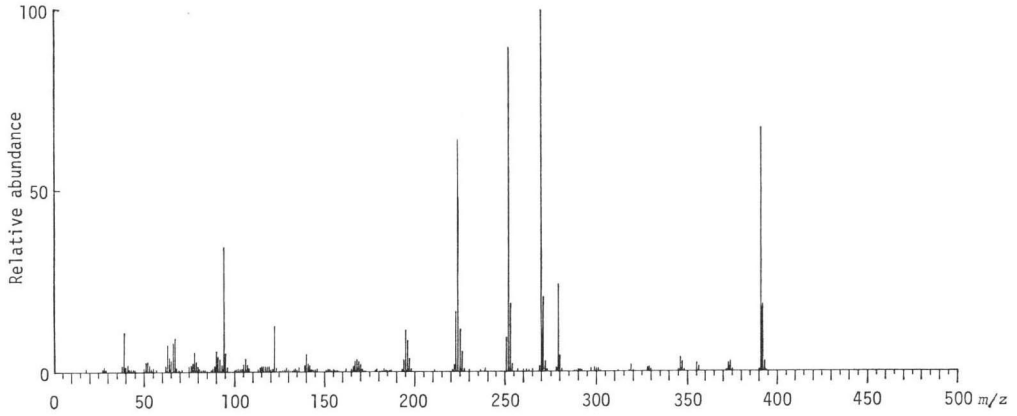


Fig. 5. Mass spectrum of U-60,394.



that two trimethylsilyl groups were added easily (M^+, m/z 535) and that a third could be introduced more slowly (M^+, m/z 607). The low resolution mass spectrum of antibiotic U-60,394 is given in Fig. 5.

From the above data we infer that antibiotic U-60,394 is a $C_{20}H_{13}N_3O_6$ compound, is completely aromatic, has at least one phenolic hydroxyl, one nitro group and possibly one carboxyl group. Preliminary trials with the Computer

Table 2. Minimum inhibitory concentrations of U-60,394.*

Organism	MIC (mcg/ml)
Staphylococcus aureus UC 76	7.8
Staphylococcus aureus UC 570	2.0
Staphylococcus aureus UC 746	7.8
Streptococcus hemolyticus UC 152	≤0.5
Streptococcus faecalis UC 694	500
Escherichia coli UC 45	>1,000
Proteus vulgaris UC 93	>1,000
Klebsiella pneumoniae UC 58	>1,000
Salmonella schottmuelleri UC 126	>1,000
Pseudomonas aeruginosa UC 95	>1,000
Diplococcus pneumoniae UC 41	1.0

* The MIC's were determined using the microplate method with Brain Heart Infusion Agar.

Assisted Structure Elucidation program[1,2] indicated that numerous families of structures are possible which are consistent with all of these data. Structure work is continuing.

Antibiotic U-60,394 is active mainly against Gram-positive bacteria (Table 2). It was inactive in mice infected with *Streptococcus hemolyticus* UC® 152 when given subcutaneously at 132 mg/kg, the highest dose tested. It was not toxic to the infected mice at this dose nor to rats dosed at 100 mg/kg subcutaneously. Only 9% of the drug given to the rats appeared in the urine.

It is interesting to note that matchamycin has a molecular formula similar to that of antibiotic U-60,394 (but with one atom of copper). The possibility that antibiotic U-60,394 is related to matchamycin[3] cannot be entirely ruled out. However, we grew *Streptomyces amagasakensis* (the matchamycin producer) in the medium we describe for antibiotic U-60,394 and failed to detect production of antibiotic U-60,394. We also cultured *S. woolensis* DIETZ and LI sp. nov. in the medium described by KIMURA[3] and failed to detect production of matchamycin. The mass spectrum of authentic matchamycin did not resemble that of antibiotic U-60,394 when run under comparable conditions and the two antibiotics were easily separated by tlc. The NMR spectra of matchamycin have not been reported. The differences we noted between these two antibiotics can all be rationalized as being due to the presence of copper in matchamycin. A final decision on this point cannot be made with the available data although a close relationship seems unlikely.

Acknowledgements

The authors thank the following Upjohn workers: Dr. L. BACZYNSKYJ for the mass spectral data, G. E. ZURENKO for the *in vitro* results and K. F. STERN and C. LEWIS for the *in vivo* work. We are grateful to Dr. J. SHOJI of the Shionogi Research Laboratory, Osaka 553, Japan, for the generous sample of matchamycin.

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(Received August 26, 1980)

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