THE DISCOVERY, FERMENTATION, ISOLATION, AND STRUCTURE OF ANTIBIOTIC A33853 AND ITS TETRAACETYL DERIVATIVE

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The structure of antibiotic A33853, isolated from the culture broth of *Streptomyces* sp., NRRL 12068, is reported. The structure was deduced from an X-ray crystallographic study of its tetraacetyl derivative. Tetraacetyl A33853 is unique because it contains an anhydride moiety, an unexpected product from the reaction of A33853 with acetic anhydride and pyridine.

In the course of our screening for new antibiotics, *in vitro* antibacterial and antiviral activities were detected in the crude broths and various extracts of *Streptomyces* sp., NRRL 12068. The producing organism was isolated from an Alaskan soil sample. This biologically active compound designated A33853 was found to be a novel antibiotic, the structure of which was elucidated through X-ray diffraction studies of its tetraacetyl derivative.

Fermentation

Stock cultures of *Streptomyces* sp., NRRL 12068, were propagated on a medium containing potato dextrin 0.8%, enzyme-hydrolyzed casein 0.2%, beef extract 0.1%, yeast extract 0.1%, KCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001% and washed agar 2.0% in deionized water. The preautoclaving pH was adjusted to 7.0 with aqueous sodium hydroxide. This medium was inoculated with spores and incubated at 34°C for 7~10 days. Mature cultures were suspended in calf serum and lyophilized.

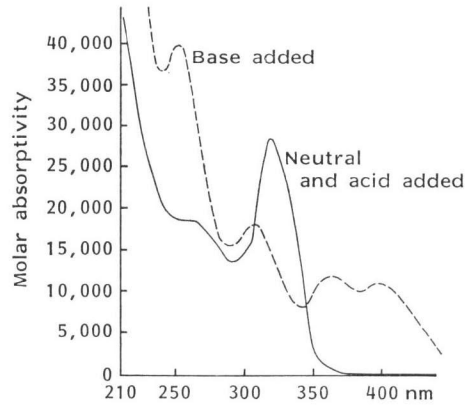
Fermentor inoculum was grown in wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of a medium composed of glucose 1.5%, soybean meal 1.5%, corn steep liquor 1.0%, NaCl 0.5% and $CaCO_3$ 0.02% in tap water. Flasks were inoculated either from sporulated agar slant cultures or lyophilized pellets. They were incubated 48 hours at 30°C on a shaker rotating at 250 rpm in a 5-cm diameter circle. The resulting culture was either used directly to provide a 1% (v/v) level of inoculum to flask fermentors or was transferred serially in the same medium to produce sufficient inoculum for larger, stirred vessels.

Fermentations were conducted either in wide-mouth 250-ml Erlenmeyer flasks containing 50 ml of media or in fully baffled, stirred vessels of conventional design with a total capacity of 165 liters and a 1:1 height-diameter ratio for the 100 liters of medium. The medium contained tapioca dextrin 7%, corn meal 0.5%, O.M. Peptone (Amber Laboratories) 1%, $MgSO_4 \cdot 7H_2O$ 0.4% and $CaCO_3$ 0.2%. The level of dissolved oxygen was maintained above 60% of air saturation. Production of the A33853 antibiotics was monitored quantitatively by a disc-plate agar diffusion technique employing *Bacillus subtilis* as the test organism. A typical time-course of the fermentation in a 165-liter fermentor is shown in Table 1.

Table 1. Typical time-course of the A33853 fermentation in a 165-liter fermentor.

Fermentation age (hours)	pH	Solids (vol %)	Antibiotic titer (μg/ml)
0	7.3	2	0
16	7.3	3	0
42	7.9	6	430
64	8.0	8	400
88	8.1	9	365
112	8.1	9	320

Fig. 1. The UV spectrum of the tetraacetyl A33853 in dioxane.



Isolation

The filtrate from 188 liters of whole fermentation broth was adjusted to pH 6.5 and extracted with an equal volume of chloroform to give 125 liters of extract. The chloroform phase was dried with sodium sulfate, filtered, and concentrated under reduced pressure to a volume of 500 ml, whereupon a yellowish crystalline material separated. The crystals were filtered, washed with cold chloroform, and dried *in vacuo* to give 9.16 g of crude A33853.

A 1.0 g sample of crude A33853 was dissolved in 15 ml of a 1:1 mixture of acetonitrile and aqueous 1 N sodium hydroxide. This solution was filtered, and the pH of the filtrate was adjusted to 7.0 with 1 N aqueous hydrochloric acid. Four successive extractions with equal volumes of chloroform removed most of the antibiotic from the aqueous pool. The chloroform extracts were combined, concentrated to a volume of about 10 ml, and added to 100 ml of methanol. This solution was concentrated under reduced pressure until a precipitate separated. The precipitate was collected by filtration, washed with a small volume of cold chloroform, and dried under vacuum to yield 423 mg of light yellow-green crystals. This preparation appeared as a single spot on silica gel thin-layer chromatography plates that had been developed in acetonitrile - water (9: 1) and detected by bioautography using a strain of *Staphylococcus aureus*.

Physical-chemical Characterization of A33853

A33853 is an acidic compound, belonging to the small group of oxazole antibiotics. Electron impact mass spectrometry showed the molecular ion at *m/z* 391 which, with the elemental analysis gives an empirical formula $C_{20}H_{13}N_3O_6$. IR, ^{1}H NMR and ^{13}C NMR spectral data agree well with that given for U-60,394, a new antibiotic reported recently by DOLAK and JOHNSON[1]. The structure of U-60,394, however, was not reported and a reference sample was not obtainable. A33853, when crystallized from chloroform - methanol, gave a mp of 310~315°C (dec). The reported mp for U-60,394 is 265~266°C (dec). A33853 is optically inactive at 365 and 589 nm in pyridine. It showed titratable groups with *pKa* values of 7.25, 10.0 and 12.21 when titrated in 66% dimethylformamide. No *pKa* data were reported for U-60,394[1]. The UV spectrum for A33853 in dioxane is practically identical with the UV spectrum of the tetraacetyl A33853 (Fig. 1), and both spectra differ only slightly from the UV spectrum of U-60,394. The reported base added spectrum of U-60,394 in methanol shows only one maximum at about 350~355 nm, while the corresponding spectra of A33853 and its tetraacetyl derivative in dioxane show two maxima each at 360 nm and 400 nm. For comparison the IR spectrum of A33853 in KBr is shown in

Fig. 2. IR spectrum of A33853 (KBr).

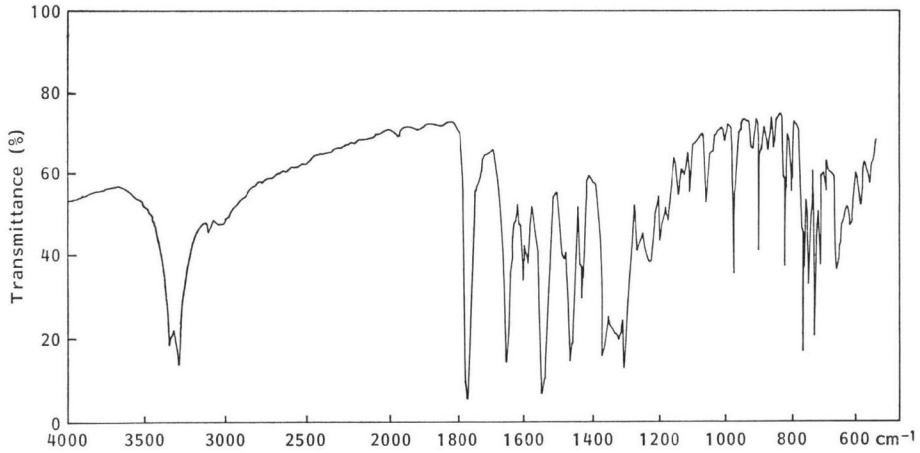


Fig. 3. IR spectrum of tetraacetyl A33853 (Nujol mull).

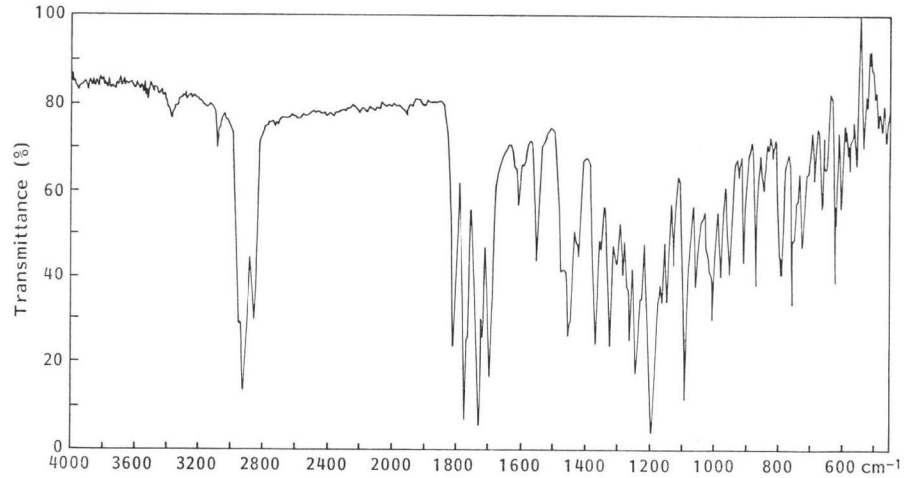


Fig. 2. At room temperature A33853 is soluble in dimethyl sulfoxide, pyridine and 0.1 N NaOH at concentrations of 5 mg/ml; it is less soluble in acetone, methanol, ethyl acetate, chloroform, tetrahydrofuran and water.

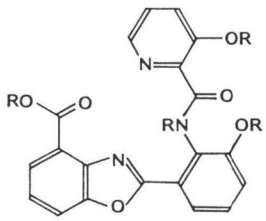
Comparing the data reported it is suggested that both A33853 and U-60,394 are either similar or identical compounds. A final analysis will be possible when additional data or a reference sample are available.

Preparation, Characterization, and Structure of Tetraacetyl A33853

A sample of A33853 was dissolved in hot pyridine and acetic acid anhydride. Crystals of the tetraacetyl compound precipitated during the following days at room temperature. The derivative was recrystallized from chloroform - ethanol to give the pure compound, mp 184~189°C. The IR absorption spectrum in Nujol mull is shown in Fig. 3, and the following distinguishable maxima are observed: 1810, 1775, 1766, 1731, 1719 and 1695 cm^{-1}. It shows the presence of six carbonyl groups and no hydroxyl absorptions. The electron impact mass spectrum showed the molecular ion m/z 559. Elemental analysis confirmed the empirical formula $C_{28}H_{21}N_3O_{10}$. Electrometric titration in 66% dimethyl-

Table 2. The ^1H NMR data of the tetraacetyl A33853, determined at 220 MHz (TMS standard).

Chemical shifts ($CDCl_3$, ppm)	J (Hz)
8.26	1.5, 4.4
8.24	— —
8.08	1.0, 7.8
7.86	1.1, 8.1
7.58	7.8, 7.8
7.50	1.3, 8.0
7.50	1.3, 8.0
7.46	8.0, 8.0
7.33	4.5, 8.2



A33853 (I) R=H
Tetraacetyl A33853 (II) R=CH_3CO

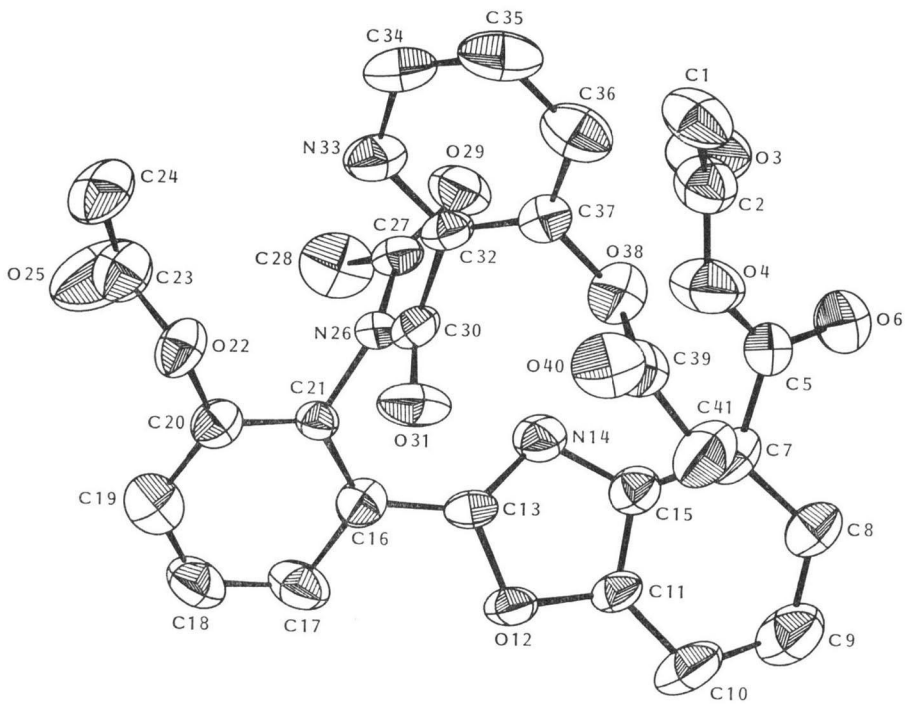
formamide showed no titratable groups, and the compound showed no specific rotation. The ^1H NMR data recorded in $CDCl_3$ are given in Table 2, chemical shifts are in ppm (tetramethylsilane standard) and coupling constants in Hz.

The ^{13}C NMR spectrum was not recorded because of the compound's low solubility in common solvents. The UV spectrum in dioxane is shown in Fig. 1, the neutral and acidic spectra were identical (pH 4.0); the broken line shows the shift in basic solution (pH 10.0). This compound is soluble in pyridine at a concentration of 1 mg/ml, but is less soluble in acetone, methanol, ethyl acetate, tetrahydrofuran, water, 0.1 N NaOH and 0.1 N HCl.

The X-Ray Structure of Tetraacetyl A33853

The structure of A33853 (**I**) was deduced from an X-ray crystallographic study of its tetraacetyl derivative (**II**) on the assumption that no structural changes other than acetylation occurred during the preparation.

Fig. 4.



Tetraacetyl A33853 ($C_{28}H_{21}N_3O_{10}$, MW 559.5) crystallized from chloroform - ethanol as colorless prisms in the centric, monoclinic space group, $P2_1/a$. There are four molecules in a unit cell having the dimensions: $a=10.548\pm0.001$ Å; $b=15.303\pm0.003$ Å; $c=16.570\pm0.003$ Å and $\beta=101.13\pm0.01°$.

The calculated crystal density is 1.42 g/cm^3; the density determined by the flotation method is 1.40 g/cm^2. The intensities of 3,752 independent reflections were measured on a four-circle computer-automated diffractometer using monochromatic copper K_α radiation. Of these data, 2,634 were greater than two standard deviations above background and were taken as observed reflections. The structure was solved using the direct methods program, MULTAN, and refined by the leastsquares method to an R value of 0.072 with anisotropic temperature factors for all atoms except hydrogen, which were included at assumed positions. The conformation of the molecule is shown in Fig. 4. Tables of the atomic parameters, as well as bond distances and angles have been deposited with the Cambridge Crystallographic Data Center.

Table 3. Activity of A33853.

Organism	MIC (μg/ml)
Staphylococcus aureus 3055*	2.0
S. aureus 3074**	2.0
Streptococcus agalactiae 19F	6.25
Mycoplasma gallisepticum 29C	1.56
M. hyopneumoniae 55B	<0.78
M. hyorhinis 29E	1.56
M. synoviae 40A	1.56
Aeromonas liquefaciens 44B	3.12
Bordetella bronchiseptica 17D	>50
Escherichia coli 19B	>50
Pasteurella multocida (bovine) 17E	1.56
P. multocida (turkey) 60A	6.25
Pseudomonas sp. 61B	>50
Salmonella dublin 30F	>50
S. typhosa SA12***	2.0

* Benzylpenicillin-susceptible, clinical isolate.
** Benzylpenicillin-resistant, clinical isolate.
*** Clinical isolate.

The structure of the tetraacetyl derivative is unique because it contains an anhydride moiety, an unexpected product from the reaction of A33853 with acetic anhydride in pyridine under mild conditions.

Biological Properties of A33853

A33853 has shown *in vitro* antibacterial activity against benzylpenicillin susceptible and resistant *S. aureus*. The antibiotic was also active against *Salmonella typhosa* and other pathogenic bacteria (Table 3). It was inactive in mice infected with *S. aureus* when given subcutaneously at 70 mg/kg×2, the highest dose tested. A33853 was ineffective against *Mycoplasma gallisepticum* infections in chicks at 60 mg/kg subcutaneously. The LD_{50} in mice, ip, was >300 mg/kg.

In addition, the antibiotic was active against vaccinia, polio type III, herpes type I, Ann Arbor influenza type A, Echo 10, Maryland B influenza, and rhino viruses but no *in vivo* activity was detected. It has also shown *in vitro* activity against the protozoan organism *Eimeria tenella*, a major causative organism of coccidiosis, and the MIC's against *Trichomonas vaginalis* were <0.975 μg/ml. However, A33853 was inactive against both these organisms in animal models. The tetraacetyl derivative was less active than the parent compound in most test systems.

Acknowledgments

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Reference

1) DOLAK, L. A. & L. E. JOHNSON: The isolation and characterization of the new antibiotic U-60,394. J. Antibiotics 33: 1391~1394, 1980