16-DEETHYLINDANOMYCIN (A83094A), A NOVEL PYRROLE-ETHER ANTIBIOTIC PRODUCED BY A STRAIN OF *STREPTOMYCES SETONII*

TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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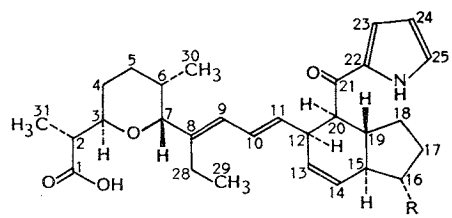
16-Deethylindanomycin (A83094A) is a novel pyrrole-ether antibiotic produced by a strain of *Streptomyces setonii*. The antibiotic, which is structurally similar to indanomycin (X-14547A), is active *in vitro* against Gram-positive bacteria as well as coccidia.

During the process of screening actinomycetes for novel antimicrobial substances, a new strain of *Streptomyces setonii*, isolated from a sample of soil collected in the state of Montana, U.S.A., was found to produce a new antibiotic. The active compound, which remained associated with the producing biomass, was isolated and found to be structurally similar to the pyrrole-ether X-14547A (indanomycin)[1,2]. This paper describes the taxonomy and fermentation studies on the producing organism as well as the isolation and characterization of A83094A (the structure shown in Fig. 1).

Fig. 1. The chemical structures of A83094A and X-14547A.



A83094A R = H

X-14547A R = CH_2CH_3 (26, 27)

Materials and Methods

Taxonomic Methods

The methods recommended by the International Streptomyces Project (ISP) for the characterization of *Streptomyces* species[3], and methods recommended by GORDON *et al.*[4] have been followed. ISCC-NSB Centroid Color Charts, standard sample No. 2106[5] and the Color Harmony Manual[6] were used to assign color names to the reverse side and to the aerial spore mass respectively. Morphology was studied using an optical light microscope. A scanning electron microscope (SEM) was used to study the spore surface ornamentation. Melanoid pigment production (chromogenicity) was determined with ISP No. 1 (Tryptone - yeast extract broth), ISP No. 6 (peptone - yeast extract iron agar), and ISP No. 7 (tyrosine agar). The isomer of diaminopimelic acid (DAP) and the carbohydrates in hydrolysates of whole cells were established by the chromatographic methods of BECKER *et al.*[7,8]. Phosphatase and urease were determined by methods described by BLAZEVIC and EDERER[9]. Gelatin liquefaction was used for the determination of proteinase activity. Resistance to antibiotics was measured by padding antibiotic sensitivity discs onto the surface of seeded ISP No. 2 agar plates. Starch hydrolysis was determined by testing for the presence of starch with iodine on ISP No. 4 (inorganic salts - starch agar) plates.

Fermentor Inoculum

The culture was propagated on a medium containing glucose 0.25%, soluble starch 0.5%, yeast extract 0.125%, NZ-Amine A (Sheffield Products) 0.125%, $CaCO_3$ 0.025% and agar 2% in deionized water (pH 7.2 prior to sterilization). Agar slope cultures were incubated 7 days at 30°C. Fermentor inoculum was prepared by inoculating wide-mouth 250-ml Erlenmeyer flasks either from agar slopes or submerged cultures stored in liquid nitrogen. These vessels contained 50 ml of a medium composed of glucose 1.0%, soluble starch 2.0%, yeast extract 0.5%, NZ-Amine A 0.5% and $CaCO_3$ 0.1% in deionized water, adjusted to pH 7.2 with aqueous NaOH prior to autoclaving. After incubation at 30°C for 48 hours on a rotary shaker, the resulting mycelial suspension was transferred to 2-liter flasks containing the same medium and incubated an additional 24 hours, then used to inoculate fermentors (2%).

Fermentors

Fermentations were conducted at 30°C in fully baffled vessels of conventional design with a total capacity of 165 liters. They contained 115 liters of a medium composed of glucose 4%, soluble starch 1%, fish meal 1% and $CaCO_3$ 0.5% in deionized water. Dissolved oxygen was maintained at 40% of air saturation with 0.34 atmospheres of internal head pressure.

Isolation of A83094A

The fermentation broth (50 liters from a total volume of 115 liters) was filtered, using a filter aid (2% Hyflo Supercel, Johns-Manville Products Corp.). The filtered broth contained a broad spectrum antimicrobial activity unrelated to the compound of present interest, and was saved for separate investigation. The mycelial cake was extracted with 12 liters of MeOH, and the crude methanolic extract was recovered by filtration through a pad of Hyflo Supercel prepared in MeOH. Water (4 liters) was added to the filtrate and the resulting solution concentrated under reduced pressure to a volume of 2 liters. This aqueous solution was extracted four times with 2 liters of EtOAc. The combined organic layers were washed with water and evaporated under reduced pressure to yield 24.5 g crude residue.

The crude EtOAc extract residue was dissolved in 75 ml $CHCl_3$ and chromatographed on a column of 900 g silica gel (Merck Grade 62, 60~200 mesh) using as eluting solvents 12 liters of $CHCl_3$ - MeOH (99.5 : 0.5) then 7 liters of $CHCl_3$ - MeOH (9 : 1). The (99.5 : 0.5) $CHCl_3$ - MeOH fraction was found to contain no antimicrobial activity and was discarded. The (9 : 1) $CHCl_3$ - MeOH fraction containing activity against *Bacillus subtilis* was concentrated under reduced pressure to an oily yellow residue.

The oily residue (9.2 g) was further purified by chromatography on a column of 1,500 g Sephadex LH-20 (Pharmacia Fine Chemicals, 25~100 μm) using MeOH as the eluting solvent. The fractions were examined by bioassay against *B. subtilis* and by TLC on silica gel plates (Silica gel 5721, Merck-Darmstadt) developed with $CHCl_3$ - MeOH (94 : 6). Detection was made under UV light at 254 nm followed by bioautography against *B. subtilis*. Active fractions were combined and MeOH removed *in vacuo* to give a pale yellow oil.

The semi-purified oil (1.27 g) was dissolved in 10 ml dichloromethane and chromatographed on a Silica gel Prep Pak (Waters Prep/LC System 500A) using a linear solvent gradient elution system from dichloromethane to dichloromethane - MeOH (94 : 6) with 5 liters in each gradient reservoir. Forty 250-ml fractions were collected. Each fraction was evaluated by bioassay against *B. subtilis* and by TLC on silica gel plates developed with $CHCl_3$ - MeOH (94 : 6). Fractions containing A83094A were combined and solvent removed under reduced pressure. The resulting pale yellow oil was dissolved in 2 ml diethyl ether. When subjected to sudden vacuum, the oil converted to a foam which was dried *in vacuo*, yielding 1.0 g pure A83094A.

Physico-chemical Methods

Electron impact mass spectra (EI-MS) were determined on a Varian-MAT 731 mass spectrometer using an ionizing energy of 70 eV. Samples were introduced directly into the ion source.

NMR data were obtained on a Bruker WM270 spectrometer at 270 MHz for ^1H and 67.9 MHz

for ^{13}C. The 1D data were obtained in $CDCl_3$ using 32K of memory, 90 degree pulse width and no relaxation delays. ^{13}C and ^{1}H assignments were made by proton homonuclear decoupling, $^{1}H/^{13}C$ heteronuclear correlations, DEPT experiments and comparison to the shifts of X-14547A[10].

Results and Discussion

Taxonomy

Cultural Characteristics

Culture A83094 grew well on both complex and defined media. Aerial hyphae were produced on the common ISP media. The color of the spore mass was predominantly white. Yellow was produced occasionally. The reverse color was yellow to yellow-brown. No distinctive nor soluble pigments were produced. Table 1 presents the cultural characteristics of A83094 after an incubation period of 3 weeks at 30°C.

Morphological Characteristics

Culture A83094 had long chains of spores arranged in typical Rectus-flexibilis (RF) morphology, as shown in Fig. 2. The spore surface ornamentation is smooth (SM); spore shape is oblong and averages 1.0×0.6 μm in size, as shown in Fig. 3.

Physiological Characteristics

Culture A83094 produced acid from arabinose, cellobiose, fructose, galactose, glucose, glycerol, glycogen, inositol, lactose, maltose, mannitol, mannose, melibiose, rhamnose, ribose, salicin, trehalose and xylose. Acid was not produced from adonitol, cellulose, dextrin, dulcitol, ethanol, erythritol,

Table 1. Cultural characteristics of A83094.

Medium	Cultural characteristics	Medium	Cultural characteristics
ISP No. 2	G: Abundant	CZAPEK'S	G: Good
	R: 72.d.OY		R: 89.p.Y
	Am: Good: White		Am: Fair: White
	Sp: None		Sp: None
ISP No. 3	G: Good	Glucose yeast - extract agar	G: Good
	R: 90.gy.Y		R: 79.1.gy.yBr
	Am: Poor: White		Am: None
	Sp: None		Sp: None
ISP No. 4	G: Abundant	Glucose asparagine	G: Good
	R: 77.m.yBr		R: 94.1.01 Br
	Am: Abundant: White		Am: Fair: White
	Sp: None		Sp: None
ISP No. 5	G: Abundant	Nutrient agar	G: Good
	R: 68.s.OY		R: 93.y Gray
	Am: Abundant: Pale yellow		Am: None
	Sp: Light-brown		Sp: None
ISP No. 7	G: Abundant	Tomato paste oatmeal agar	G: Abundant
	R: 68.s.OY		R: 77.m.yBr
	Am: Abundant: White		Am: Good: White
	Sp: None		Sp: None
Calcium malate	G: Good: (hydrolysis)	Tap water agar	G: Poor
	R: 68.s.OY		R: 93.y Gray
	Am: Good: White		Am: Trace: White
	Sp: None		Sp: None

G: Growth, R: reverse, Am: aerial mycelium, Sp: soluble pigment.

Fig. 2. Aerial mycelia of culture A83094 on glycerol-asparagine agar (ISP No. 5) at 3 weeks incubation at 30°C, 160× magnification.

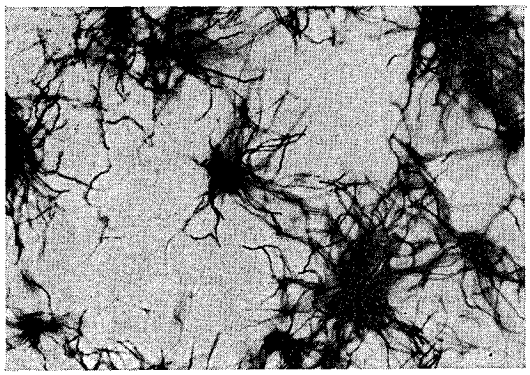
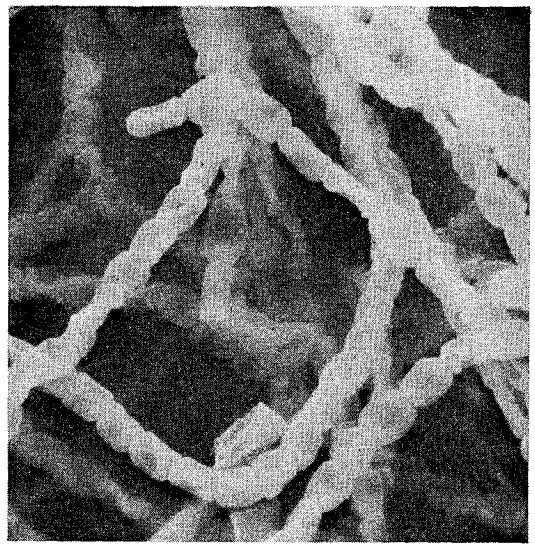


Fig. 3. Electron micrograph of sporophores of A83094 on glycerol-asparagine agar (ISP No. 5) at 3 weeks incubation at 30°C.

Bar represents 1.0 μm.



inulin, melezitose, α-methyl-D-glucoside, raffinose, sorbitol, sorbose, sucrose and xylitol. It utilized acetate, butyrate, citrate, formate, lactate, malate, oxalate, propionate, pyruvate, succinate and tartrate, but no benzoate.

Culture A83094 decomposed adenine, allantoin, casein, hypoxanthine, calcium malate, tyrosine, urea, xanthine, esculin and testosterone, but not elastin, guanine, hippurate or starch. It liquefied gelatin, reduced nitrate, produced hydrogen sulfide, catalase and phosphatase. Culture A83094 did not produce melanoid pigments, nor was it resistant to lysozyme. It was resistant to 30 μg cephalothin, 10 U of benzylpenicillin, 30 μg nalidixic acid, 300 U polymyxin B, and 5 μg trimethoprim per disc. Culture A83094 was able to tolerate NaCl up to 11%, and grew at temperatures between 5~37°C.

Cell-wall Analysis

Hydrolyzed whole cells contain the LL isomer of DAP. Sugars present were galactose, glucose and ribose. The cell-wall type (8) is Type I, the sugar pattern[11] is Type NC.

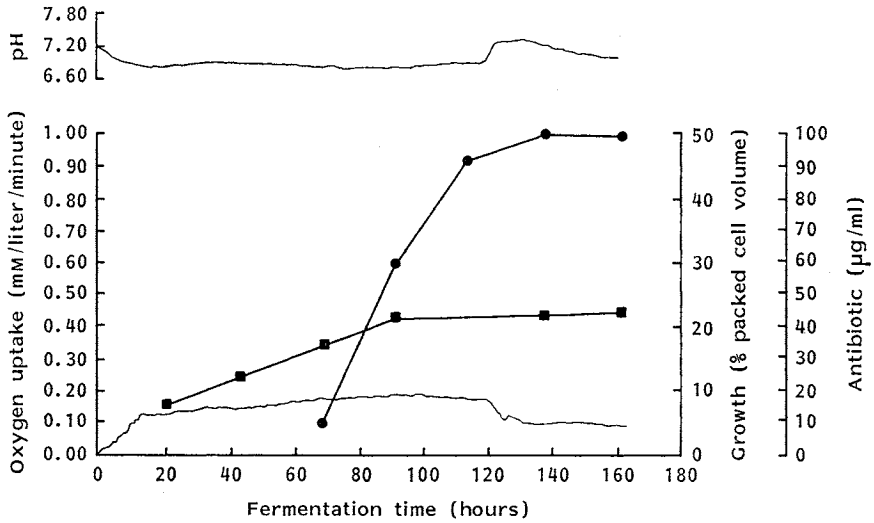
Identity of Strain A83094

The chemotaxonomic and general cultural characteristics are consistent with assignment of strain A83094 to the genus *Streptomyces* WAKSMAN and HENRICI 1943. Using the keys of NONOMURA[12], SZABO *et al.*[13], and WAKSMAN and HENRICI[14], and by comparison to published descriptions[15], culture A83094 is classified as a strain of *Streptomyces setonii* (Millard and Burr 1926) Waksman 1953[16].

Fermentation

A83094A was retained by the producing biomass and was not elaborated into the aqueous fermentation broth. Antibiotic production was therefore monitored by quantitating methanolic extracts of the fermentation solids through a disc-plate agar diffusion technique employing *B. subtilis* as the test organism. Qualitative determinations of A83094A were made with a TLC system employing Merck-Darmstadt Silica gel 5721 plates developed in chloroform - methanol (9 : 1). Chromatograms were bioautographed vs. *B. subtilis* grown in minimal nutrient agar. The Rf value in this system was 0.5. The biosynthesis of A83094A did not begin until about 60 hours after the fermentation was

Fig. 4. Typical time course of the A83094 fermentation.
● Antibiotic, ■ growth, — oxygen uptake.



initiated, when the trophophase was essentially completed. Antibiotic production occurred during the idiophase of the fermentation. A time course profile of the fermentation is shown in Fig. 4.

Physico-chemical and Biological Properties

A83094A is a pale yellow powder. MS *m/z* 465 ($C_{29}H_{39}NO_4$). It is soluble in acetone, acetonitrile, benzene, diethyl ether, ethyl acetate and lower alcohols. It is insoluble in *n*-hexane, *n*-pentane and water.

The *in vitro* antibacterial spectrum of A83094A is shown in Table 2. The acute toxicity determined as the LD_{50} in mice is 196.4 mg/kg ip, 630 mg/kg po. A83094A exhibits *in vitro* anticoccidial activity like that of certain polyether antibiotics. At a concentration of 0.31 μg/ml, it completely inhibits the development of *Emeria tenella*. However, the compound showed no efficacy *in vivo* when chicks infected with *E. tenella* or *Emeria acervulina* were fed a diet containing 200 μg/g A83094A.

Table 2. *In vitro* antibacterial spectrum of A83094A.

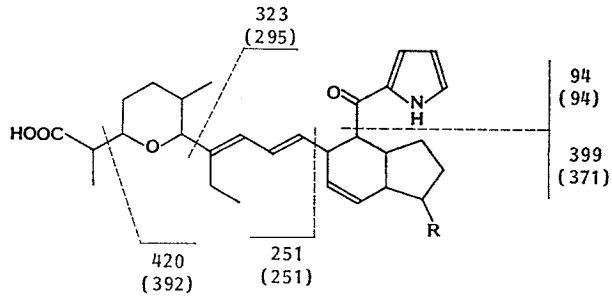
Test organism	MIC (μg/ml)
Staphylococcus aureus X1.1	4
S. aureus V41	4
S. aureus X400	4
S. aureus S13E	4
S. epidermidis 222	4
Streptococcus A C203	4
Streptococcus D X66	4
Streptococcus D 2041	4
S. pneumoniae Park I	2
Haemophilus influenzae C.L. (penicillin-sensitive)	>128
H. influenzae 76 (penicillin-resistant)	>128
Escherichia coli EC14	>128
Klebsiella X26	>128
Enterobacter aerogenes EB17	>128
E. cloacae EB5	>128
Salmonella X514	>128
Pseudomonas X528	>128
Serratia marcescens X99	>128
Shigella sonnei N9	>128
Morganella morganii PR15	>128
Proteus inconstans PR33	>128
Providencia rettgeri C24	>128
Citrobacter CF17	>128
Acinetobacter AC12	>128

Standard agar dilution method using nutrient agar. Tubes incubated for 24 hours at 37°C.

Structure Elucidation

The EI-MS of A83094A closely resembles that of X-14547A[2]. A number of peaks occurred at

Fig. 5. EI fragmentation of X-14547A and A83094A.



A83094A R=H
X-14547A R=Et
Figures in parentheses are for A83094A.

Table 3. The NMR data in $CDCl_3$ for A83094A and X-14547A[10].

Position	^{13}C (ppm)		^{1}H (ppm)	
	A83094A	X-14547A	A83094A	X-14547A
1	179.49	179.4	—	—
2	41.27	41.3	2.99	2.04
3	74.94	75.1	3.94	3.90
4	22.13	22.0	1.94/1.61	1.94/1.45
5	26.29	26.4	1.76/1.48	1.82/1.61
6	30.26	30.4	1.86	1.95
7	74.28	74.4	4.21	4.20
8	141.08	140.0	—	—
9	124.00	124.5	5.92	5.93
10	126.90	127.2	5.77	5.79
11	132.59	132.4	5.42	5.42
12	45.42	45.7	3.30	3.32
13	29.10	29.4	5.47	5.50
14	130.16	129.5	5.93	5.95
15	44.51	49.9	1.89	1.60
16	28.90	43.8	1.81/1.20	1.48
17	21.54	29.7	1.83/1.31	1.91/1.30
18	27.92	27.2	1.97/0.97	2.00/1.06
19	40.70	40.8	1.76	1.93
20	52.49	52.7	3.41	3.41
21	191.69	191.6	—	—
22	132.59	132.6	—	—
23	116.10	116.1	6.92	6.92
24	110.07	110.2	6.25	6.27
25	125.32	125.4	6.99	6.27
26	—	27.4	—	1.74/1.18
27	—	12.6	—	0.96
28	21.93	21.6	1.66	2.00/1.75
29	13.27	13.4	0.72	0.79
30	13.22	13.5	0.80	0.84
31	14.09	14.3	1.13	1.15

the same mass, and others differed in mass by 28 daltons. In particular, the molecular ion was peak matched to give a molecular formula of $C_{29}H_{39}NO_4$. Prominent peaks at *m/z* 251 and 94 occurred in the spectra of both compounds. The fragments at *m/z* 420, 399 and 323 in the spectrum of X-14547A

have their analogs at *m/z* 392, 371 and 295 respectively in the spectrum of A83094A. All of the MS data were consistent with the structure shown in Fig. 5 for A83094A.

The ^{13}C and ^{1}H NMR spectra were compared to the published data of X-14547A[10] (see Table 3). From the ^{13}C spectrum, it was clear that these compounds are related. The sp^2 region of the spectra agreed very well, indicating that this region had not been affected. The carbons representing C-2 to C-7 and C-28 to C-31 also agreed very well. The carbons most affected were C-15 to C-19 and C-26 and C-27. Those representing C-26 and C-27 were absent from A83094A. The loss of this ethyl group was also seen in the ^{1}H NMR spectrum. Carbons 15~17 experienced upfield shifts which is consistent with the loss of the ethyl at C-16. Carbon 18 experienced a small downfield shift; again, this is consistent with the loss of the 16-ethyl. The NMR data, therefore, support the structure proposed from the mass spectra data.

Because of its structural similarity to indanomycin, we propose the trivial name 16-deethylindanomycin for A83094A.

Acknowledgments

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