STUDIES ON A NEW POLYETHER ANTIBIOTIC, Ro 21-6150

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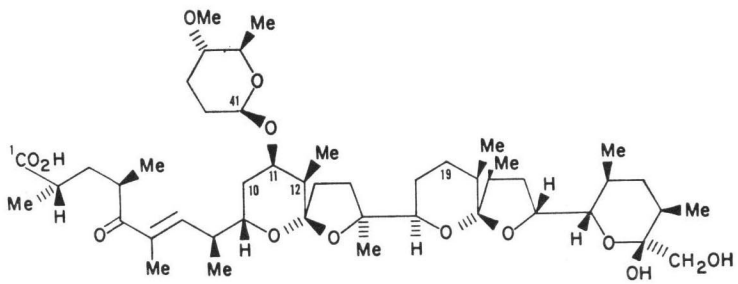
A new polyether antibiotic, Ro 21-6150, has been isolated from culture broths of *Streptomyces hygroscopicus*, strain X-14563. Ro 21-6150 has ionophore properties and is active *in vitro* against gram-positive bacteria.

In the course of searching for new antimicrobial agents, a new polyether antibiotic, Ro 21-6150, was discovered. Some of the polyether antibiotics reported earlier are lasalocid (X-537A),[1,2] nigericin (X-464),[3] X-206,[4] grisorixin,[5] monensin,[6] salinomycin,[7] septamycin[8] A204A,[9] and most recently lysocellin.[10] This group of antibiotics is active *in vitro* against gram-positive bacteria and mycobacteria, and several of the members have been reported as anticoccidal agents.[11] The structure of Ro 21-6150, recently determined by X-ray analysis of the silver salt,[12] is shown in Fig. 1. In this report we present results of a taxonomic study of the producing organism and describe the fermentation production, isolation and biological properties of the new antibiotic.

Fig. 1. Structure of Ro 21-6150



Taxonomy

Among isolates of streptomyces from a soil sample, X-14563 was selected on the basis of antibacterial activity exhibited against gram-positive bacteria. It was identified as a strain of *Streptomyces hygroscopicus* on the basis of the following observations:

(1) Microscopic characteristics:

X-14563 products a substrate mycelium which does not fragment into spores, and an aerial mycelium which forms non-segmented spiral chains of spores (Plates 1 and 2); the chains have a rugose surface.[13]

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Plates 1 and 2. Scanning electron micrographs of *Streptomyces hygroscopicus* strain X-14563. Cultures were grown on oatmeal agar (ISP-3) for 14 days.

Plate 1. Rugose non-segmented spores on a spiral chain, 6,046×.

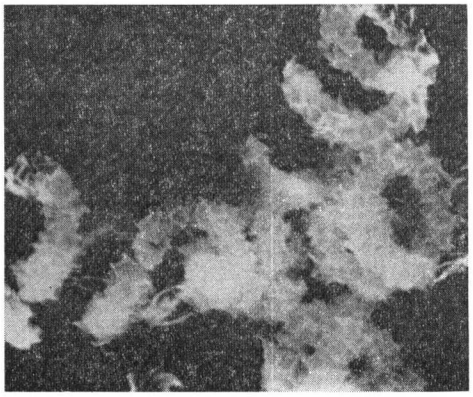
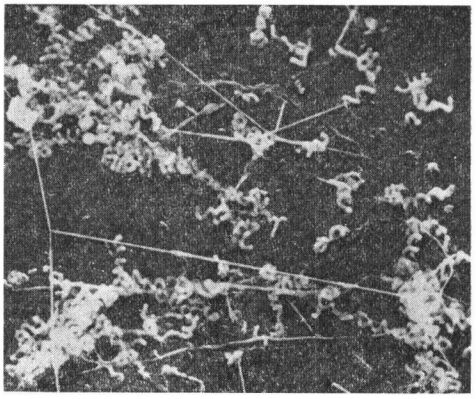


Plate 2. Spiral spore chain, 667×.



(2) Cell wall analyses:

An isomer of diaminopimelic acid different from the *meso* form was found as a component of the cell wall of X-14563 by the method of BECKR *et al.*[14] This fact taken together with the observed microscopic characteristics places X-14563 in the genus *Streptomyces*.[16]

(3) Macroscopic characteristics on selected media:

Results obtained from culturing strain X-14563 on various ISP[16] and other media are summarized in Table 1. Observations were made after incubation of the agar plates at 28°C for 14 days. The Color Harmony Manual (Container Corp. of America, 4th Edition, 1958) was used as a reference for the colors described. Based on these results, and the hygroscopic patches observed on certain media, strain X-14563 is clearly a member of the *Streptomyces hygroscopicus* group as described by WAKSMAN and HENRICI, 1948.[17] A comparison of X-14563 with *S. hygroscopicus* NRRL 2387 and some of the subspecies described in the literature is given in Table 2. Strain X-14563 is clearly different from the neotype strain of *S. hygroscopicus* NRRL 2387 in melanin production and carbon utilization patterns. The most closely related cultures are the subspecies *ossamyceticus* and *aabomyceticus*, but they do not correspond to X-14563 in all of the characteristics given. Although X-14563 may represent a new subspecies of *S. hygroscopicus*, such a consideration is being postponed until the internal subdivision of the species is better understood through careful comparative studies.

Fermentation

Vegetative inoculum was prepared in a medium containing the following (g/liter): tomato pomace 5.0, distillers soluble 5.0, O.M. peptone (Oscar Mayer & Co., Madison, Wis.) 5.0, debittered dried yeast 5.0, corn starch 20.0, $CaCO_3$ 1.0, and K_2HPO_4 1.0. The pH of this medium was adjusted to 7.0 before autoclaving. Spores of *S. hygroscopicus* X-14563 were inoculated into 4 liters of this medium and the culture incubated on a rotary shaker (250 rpm) for 72 hours at 28°C. The resulting growth was used to inoculate a 100-gallon fermentor containing 60 gallons of the previously described tomato pomace medium supplemented with 0.01% SAG 4130, a silicone defoamer (Union Carbide Corp.). The tank was stirred at a rate of 280 rpm

Table 1. Cultural characteristics of strain X-14563

Medium	Amount of growth Degree of sporulation	Spore mass color	Reverse color of substrate mycelium
Yeast-malt extract agar (ISP-2)	moderate growth, well sporulated, liquid droplets	2*ih* (dark covert gray), 2*ch* (ivory tint) where isolated colonies and 1*ca* (canary yellow)	2*ic* (honey gold) at edge and 3*lq* (adobe brown) center
Oatmeal agar (ISP-3)	moderate growth, well sporulated	3*iq* (beige brown), *b* (oyster white) at edge	2*fc* (covert gray) center to 2*cb* (ivory tint) at edge
Inorganic salts-starch agar (ISP-4)	moderate growth, well sporulated	2*fe* (covert gray), 2*ch* (ivory tint) in isolated colonies, a few patches of 1½ *ea* (light yellow)	1½ *ga* (butter yellow)
Glycerol-asparagine agar base plus 1 % glycerol (ISP-5)	poor growth, poor sporulation	*b* (oyster white) in isolated patches where sporulated	2*ba* (pearl)
Peptone-yeast extract-iron agar (ISP-6)	moderate to abundant growth, moderate sporulation	2*cb* (ivory tint)	
Tyrosine agar (ISP-7)	abundant growth, well sporulated	1*ga* (light lemon yellow) in center to *b* (oyster white) at edge 2*fe* (covert gray)	
Sporulation agar, ATCC medium No. 5[a)]	abundant growth, well sporulated, liquid droplets	3*ig* (beige brown) 1*ea* (canary yellow) in scattered patches	2*nl* (covert brown) in center 1½ *ga* (butter yellow) at edge
CZAPEK-DOX with 1.5 % agar	moderate to abundant growth, sparse sporulation	*b* (oyster white)	2*ec* (biscuit)
Thermoactinomyces fermentation medium (Bacto) with 1.5 % agar	moderate to abundant growth, well sporulated, liquid droplets	1*ea* (canary yellow) mostly, to 2*ba* (pearl) at edges	2*le* (mustard), 1½ *ea* (light yellow) at edge
Amidex agar[b)]	moderate growth, well sporulated, liquid droplets	3*ih* (beige gray) where dense, 2*ba* (pearl) where less dense	2*ac* (bamboo), 2*ca* (light ivory) at edge
SABOURAUD dextrose agar	moderate to abundant growth, moderate sporulation	2*ba* (pearl) with patches of 1½ *ea* (light yellow)	
Tomato agar[c)]	moderate growth, abundant sporulation	3*ih* (beige gray) to 3*fe* (silver gray) with spots of 3*dc* (natural) and 1½ *ea* (light yellow)	
Starch agar[d)]	sparse growth, sparse sporulation	2*dc* (natural)	

a) American Type Culture Collection, Catalog of Strains, 11th Edition, 1974.
b) Amidex (Corn Products Co., Decatur, I11) 1 %, N-Z-Amine A 0.2 %, beef extract 0.1 %, yeast extract 0.1 %, $CaCl_2 \cdot 2H_2O$ 0.0014 %, agar 2 %, pH adj. to 7.3.
c) Tomato paste 2 %, dextrose 1 %, K_2HPO_4 0.1 %, WILSON'S medopeptone 0.1 %, $CaCO_3$ 0.2 %, agar 1.5 %, pH 6.8~7.3.
d) Soluble starch 0.25 %, agar 2 % in Actinomyces broth (Difco).

and an air flow of 3 cfm was maintained. Additional defoamer was added as needed. The fermentation broth was assayed for antibiotic activity by an agar diffusion method using *Bacillus megaterium* ATCC 8011. This test organism is insensitive to venturicidin A, an antibiotic coproduced along with Ro 21-6150. The fermentation was terminated after 7 days.

Table 2. Comparison of strain X-14563 with *S. hygroscopicus* and various subspecies

Test[a]	*Streptomyces hygroscopicus*						
	Strain X-14563[c]	NRRL 2387	Subspecies				
			ossamy-ceticus	*angustmy-ceticus*	*decoyicus*	*glebosus*	*aabomy-ceticus*
Color of spore mass	gray	gray	gray, white	gray, white	gray, white	gray, white	gray
Spore chain	spiral, non-segmented	spiral, non-segmented	spiral, segmented	spiral, segmented	spiral, segmented	spiral, not typical	spiral, non-segmented
Spore chain or wall surface appearance	rugose	rough	smooth	smooth	smooth	ridged, or smooth	rugose
H_2S production (ISP-6 medium)	−		+				−
Melanin production (ISP-7 medium)	weakly +	−	+	−	−	−	±
D-Glucose utilization[b]	+	+	+	+	+	+	+
D-Xylose 〃	+	+	+	−	+	+, ±	+
L-Arabinose 〃	+	+	+	−	−	−	±
L-Rhamnose 〃	+	+	+	−	−	−	+
D-Fructose 〃	+	+	+		+	+	+
D-Galactose 〃	+		+	±	+	+	+
Raffinose 〃	+	−?	+	±	−	+	+
D-Mannitol 〃	+	+	+	+	+	+	+
i-Inositol 〃	+	−	+	−	+	+	+
Salicin 〃	± to +	+	−	−	−	−	−
Sucrose 〃	+	−?	+	+		+	+
Cellulose 〃	−		±				−
Gelatin hydrolysis	+	+slow	−	+ weak	+ weak	−	+
Starch hydrolysis	+	+	+	+	+	+	+
Reverse side pigmentation	−		−	−			−
Soluble pigment produced	−	yellow	yellow to greenish brown	−		−	pale yellow, pink
Streptomycin sensitivity (10μg disc)	+	+			+	+	
NaCl (%) tolerance	≤5 %	≥7, but <10			≥10, but <13	≥10, but <13	
Growth temperature, C°	10–37		28–35		18, but <55		>5 but <50 mesophilic
Antibiotic production	Ro 21-6150 Venturi-cidin A		Ossamy-cin	Angust-mycin	Psicofura-nine	Glebomy-cin	Aabomycin A

a) Inoculum for these tests was prepared in tryptone yeast extract broth (ISP-1 medium); the cells were washed before use.

b) These tests were done in Carbon-Utilization agar (Bacto ISP-9 medium) containing 1.0 % of the indicated carbon source.

c) These data were obtained experimentally; data for all other strains were taken from the literature.[13,17~24]

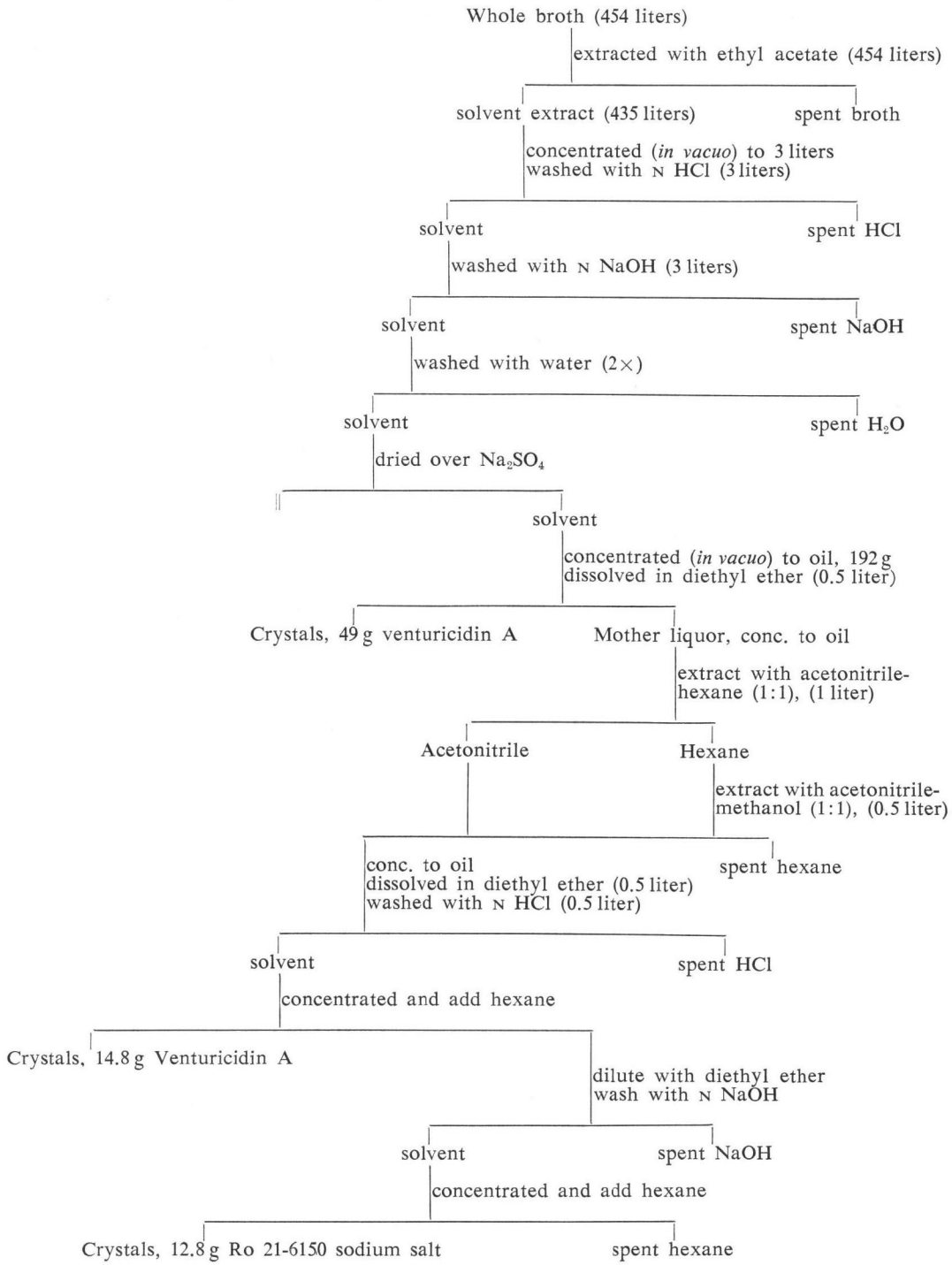
Isolation and Characterization

A flow diagram for the isolation of Ro 21-6150 and venturicidin A is given in Fig. 2. The separation of the two antibiotics is accomplished by crystallization of venturicidin A under

conditions where the free acid of Ro 21-6150 is soluble.

Venturicidin A was identified by comparison with an authentic sample. Ro 21-6150 sodium salt was obtained as colorless needles, m.p. 235°C dec., $[\alpha]_D^{25}$ +95° (*c* 1, $CHCl_3$). Anal. calcd.

Fig. 2. Isolation procedure for Ro 21-6150 sodium salt



for $C_{47}H_{77}O_{13}Na$ (873.13): C 64.65, H 8.89, Na 2.63. Found: C 64.72, H 8.87, Na 2.45. Ro 21-6150 has a U.V. max at 235 nm (ε 14,000) in ethanol. The IR and NMR spectra are shown in Figs. 3, 4, respectively. The presence in the NMR ($CDCl_3$) of a methyl singlet at δ 1.88 (s, 3, $C\underline{H}_3$-C=C) and peaks at δ 2.58 (m, 1, C=CH-C$\underline{H}$) and δ 6.65 (d, 1, C=C$\underline{H}$-CH, J=10 Hz) are indicative of the chromophore of Ro 21-6150 which is the same as that previously reported for dianemycin.[25]

Fig. 3. IR Spectra of Ro 21-6150 (KBr)

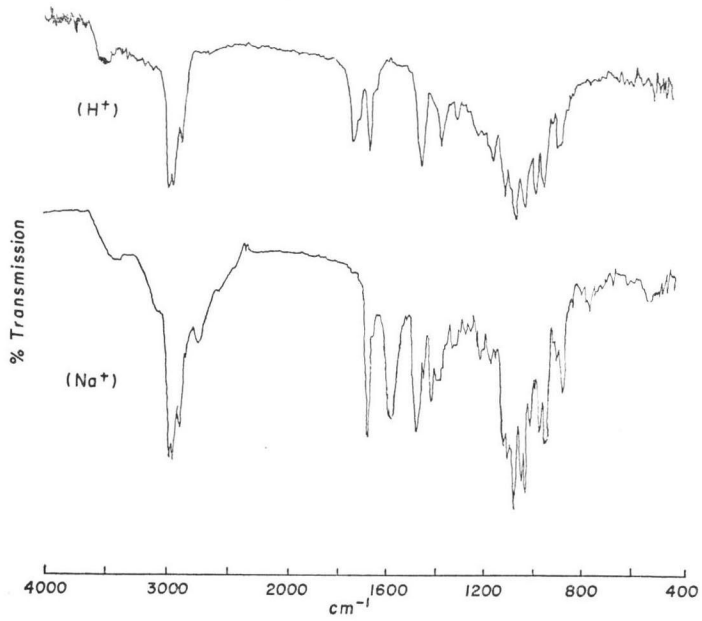
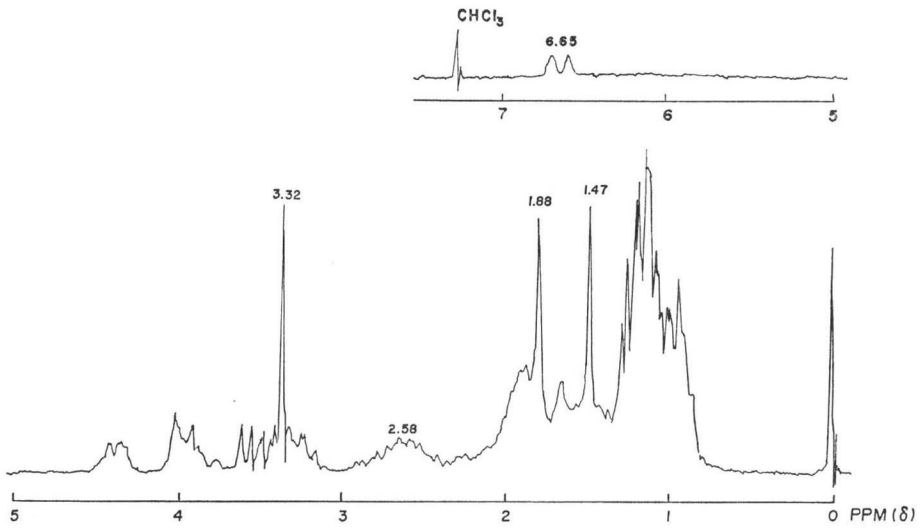


Fig. 4. NMR Spectrum of Ro 21-6150 (100 MHz, $CDCl_3$)



Biological Properties

The ionophore characteristics of Ro 21-6150 were studied using the U-tube method described

by ASHTON and STEINRAUF.[26] Ro 21-6150 was found to facilitate the transport of $^{86}Rb^{+}$ across a chloroform barrier and was similar to dianemycin and nigericin with respect to this property.

The antibacterial spectrum of Ro 21-6150 was determined by an agar diffusion method (Table 3). It is mostly active against gram-positive bacteria including *Mycobacterium phlei*, but also shows some activity against fungi and yeast; no activity was observed against gram-negative bacteria. Ro 21-6150 is an effective coccidiostat as determined in chickens infected with *Eimeria tenella*. The toxicity of the antibiotic in mice is 55 mg/kg p.o.

Table 3. Antimicrobial spectrum of Ro 21-6150

Organism	M.I.C.* (mcg/ml)
Staphylococcus aureus ATCC 6538P	0.2
Sarcina lutea ATCC 9341	0.2
Bacillus megaterium ATCC 8011	0.1
Bacillus sp. E ATCC 27859	0.02
Bacillus subtilis NRRL 558	0.4
Bacillus sp. TA ATCC 27860	0.4
Mycobacterium phlei ATCC 355	0.2
Streptomyces cellulosae ATCC 3313	1.6
Paecilomyces varioti ATCC 26820	0.8
Penicillium digitatum ATCC 26821	1.6
Candida albicans NRRL 477	25
Saccharomyces cerevisiae ATCC 4266	25
Pseudomonas aeruginosa ATCC 8709	>100
Proteus vulgaris ATCC 6380	>100
Escherichia coli ATCC 27856	>100
Klebsiella pneumoniae ATCC 27858	>100
Serratia marcescens ATCC 27857	>100
Serratia sp. 101	>100
Acinetobacter calcoaceticus ATCC 10153	>100

* Lowest two-fold dilution giving a zone of inhibition in an agar-well diffusion assay.

Following completion of these studies, we obtained a sample of antibiotic A-130-A (Shionogi & Co., Ltd.) and found it identical to Ro 21-6150 by IR and NMR criteria.

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