TIRANDALYDIGIN, A NOVEL TETRAMIC ACID OF THE TIRANDAMYCIN-STREPTOLYDIGIN TYPE

I. TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION AND BIOLOGICAL ACTIVITY

JAMES P. KARWOWSKI, MARIANNA JACKSON, ROBERT J. THERIAULT, GRANT J. BARLOW, LYLE COEN, DENA M. HENSEY and PATRICK E. HUMPHREY

Pharmaceutical Products Research and Development, Abbott Laboratories, Abbott Park, Illinois 60064, U.S.A.

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Tirandalydigin is a new tetramic acid antibiotic which was discovered in a screen designed to find compounds with activity against pathogenic anaerobic bacteria. It was named tirandalydigin because it possesses structural features that are common to both tirandamycin and streptolydigin. The producing culture, strain AB 1006A-9, is a *Streptomyces* and was compared to the streptomycetes that synthesize tirandamycin and streptolydigin. It is closely related to the former culture and was named *Streptomyces tirandis* subsp. *umidus*. Tirandalydigin has MICs in the range of 0.5 to 32 μg/ml against many pathogenic anaerobes, streptococci, enterococci and legionellae.

Tirandalydigin is a new tetramic acid antibiotic isolated from the fermentation broth of *Streptomyces tirandis* subsp. *umidus* strain AB 1006A-9. This compound was discovered in a screen designed to find compounds with activity against pathogenic anaerobic bacteria. This paper describes the taxonomy of the producing microorganism and the fermentation and biological activity of the antibiotic. Portions of this work have been reported previously[1]. A paper was published earlier detailing the isolation and structural elucidation of tirandalydigin[2].

Materials and Methods

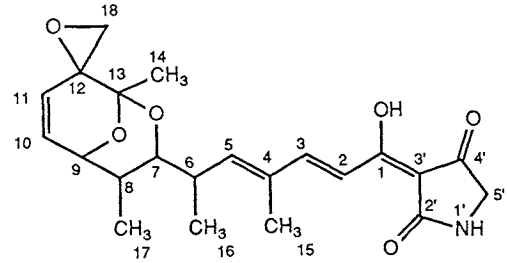
Microorganisms and Reference Antibiotics

The culture that produces tirandalydigin was isolated from soil collected in a salt marsh in North Carolina, U.S.A. *S. tirandis* var. *tirandis* NRRL 3689 and *S. lydicus* NRRL 2433 were obtained from the National Center for Agricultural Utilization Research, United States Department of Agriculture, Peoria, Illinois, U.S.A. The bacteria used to obtain the activity spectra of tirandalydigin, tirandamycin A and streptolydigin were from the stock culture collection in our laboratory and from the American Type Culture Collection (ATCC). Tirandamycin A and streptolydigin were produced and isolated in our laboratories.

Taxonomic Studies

The chemical structure of tirandalydigin formally represents a hybrid between those of tirandamycin and streptolydigin (Fig. 1). We compared strain AB 1006A-9 with the producers of these antibiotics, *S. tirandis* var. *tirandis* NRRL 3689[3] and *S. lydicus* NRRL 2433[4]. Methods and media described by the International Streptomyces Project (ISP)[5], WAKSMAN[6] and GORDON *et al.*[7] were used to determine most of the taxonomic characteristics. Incubation for cultural characteristics and carbon utilization was at 28°C for 21 days. Production of H_2S was determined by method 2 of SMIBERT and KRIEG[8], and a modification of KUTZNER's technique[9] was used to observe reduction of nitrate. Color names were assigned to the mycelial and diffusible pigments on the basis of the Inter-Society Color Council-National Bureau of

Fig. 1. Structures of tirandalydigin (**1**), streptolydigin (**2**) and tirandamycin A (**3**).



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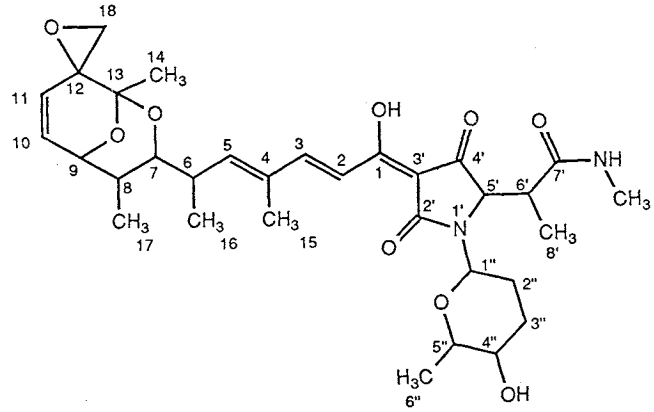

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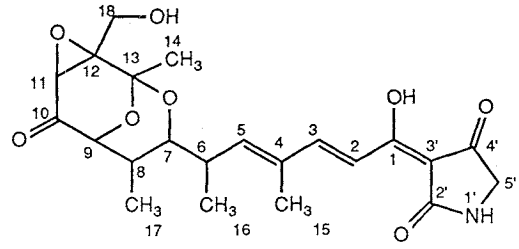

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Standards (ISCC-NBS) Centroid Color Charts[†]. Analysis of the whole-cell diaminopimelic acid isomer was done by the method of BECKER *et al.*[10].

Fermentation Studies

Streptomyces AB 1006A-9 was grown on yeast extract - malt extract agar slants incubated at 28°C for 7 days and stored at 4°C. The seed medium consisted of glucose monohydrate 1.5%, soy flour 1.5%, yeast extract (Difco) 0.1%, NaCl 0.1% and $CaCO_3$ 0.1%. This medium was prepared with distilled water with no pH adjustment. Inoculum for antibiotic production was prepared in two stages. In the first step, 500 ml Erlenmeyer flasks containing 100 ml of seed medium were inoculated with spore suspensions prepared from slant cultures. These flasks were incubated for 96 hours, and then 5% inoculum was transferred to 2-liter Erlenmeyer flasks containing 600 ml of the same medium. After 72 hours, 5% inoculum was transferred to a production fermenter. Seed vessels were incubated at 28°C on a rotary shaker (5.6-cm stroke) at 250 rpm.

[†] ISCC-NBS Color-Name Charts Illustrated with Centroid Colors. U.S. Dept. of Comm. supp. to NBS Cir., 553, Washington, D.C., 1976.

The fermentation medium consisted of glucose monohydrate (added after sterilization) 2%, Lexein F-152 liquid peptone (Inolex) 1%, molasses (Del Monte Brer Rabbit green label) 0.5%, whole yeast (Universal Foods Red Star) 0.5%, $CaCO_3$ 0.2% and XFO-371 antifoam (Ivanhoe Chemical Co.) 0.01%. This medium was also prepared with distilled water without pH adjustment. Fermentation was carried out in a 150-liter vessel containing 80 liters of medium. The temperature was controlled at 28°C. The agitation rate was 200 rpm, the air flow rate was 0.7 vol/vol/minute and the head pressure was 0.35 kg/cm^2. The fermentation broth was harvested at 168 hours.

Cell growth was evaluated as packed cell volume by centrifuging the fermentation broth in a graduated tube at $600 \times g$ for 30 minutes. Carbohydrate utilization was determined by analyzing for reducing sugars in hydrolyzed fermentation broth. Two ml of 2 N H_3PO_4 were added to 5 ml of mycelium free fermentation broth, and this mixture was heated at 121°C, cooled and centrifuged. The supernatant was analyzed by HOFFMAN's method[11].

HPLC

Levels of tirandalydigin in fermentation broths were determined by HPLC. Broths were prepared for analysis by adjustment to pH 3 and extracting twice with one half volume of methylene chloride. The extracts were combined, solvent was removed under reduced pressure and the residue was reconstituted in MeOH to a concentration 50 times that of the original fermentation broth. Analyses were performed using a Waters model 6000A solvent delivery system and a U6K injector. Extracts were chromatographed on a 4.6×150 mm column packed with 7 μm C18 Adsorbosphere HS (Alltech) with a mobile phase consisting of acetonitrile - 0.1% H_3PO_4 (50 : 50) at pH 3. Tirandalydigin was detected at 350 nm using a Kratos SF 770 UV detector; it eluted as a sharp peak at 11 minutes at a flow rate of 1 ml/minute.

In Vitro Activity

MICs of tirandalydigin, tirandamycin and streptolydigin were determined by the agar dilution method†. All test cultures were incubated at 35°C. Anaerobes were grown on Wilkins-Chalgren agar and observed after 48 hours. The enterococci were grown on Mueller-Hinton agar, and the other aerobes were grown on brain heart infusion agar. These cultures were incubated for 16~20 hours. The legionellae were grown on buffered charcoal yeast extract agar in the presence of 5% CO_2 and observed at 24 hours.

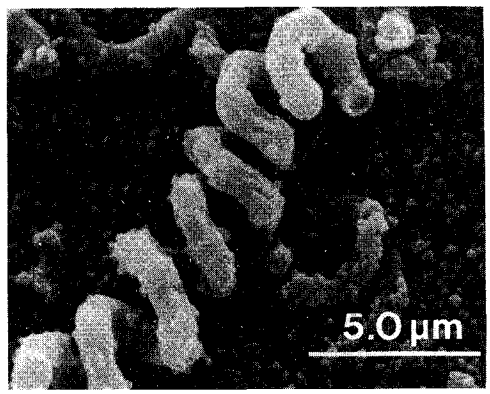
Results and Discussion

Taxonomy

Analysis of whole cell hydrolysates of AB 1006A-9 revealed the presence of LL-diaminopimelic acid (type I cell wall[12]). Scanning electron microscopy shows that this isolate forms spores in chains of open spirals (Fig. 2). The mature aerial spore mass is gray on the media tested. These properties indicate that strain AB 1006A-9 is a *Streptomyces* belonging to section *Spira*, in the gray series of PRIDHAM *et al.*[13].

The cultural characteristics of strain AB 1006A-9, *S. tirandis* var. *tirandis* NRRL 3689 and

Fig. 2. Scanning electron micrograph of a spore chain of strain AB 1006A-9 grown on ISP-4 medium.



† National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. Approved standard M7-A. Reference agar dilution procedure for antimicrobic susceptibility testing of anaerobic bacteria. Approved standard M11-A. National Committee for Clinical Laboratory Standards, Villanova, 1985.

Table 1. Cultural characteristics of strain AB 1006A-9, *S. tirandis* var. *tirandis* NRRL 3689 and *S. lydicus* NRRL 2433.

Medium		AB 1006A-9	*S. tirandis* var. *tirandis* NRRL 3689	*S. lydicus* NRRL 2433
Yeast extract-malt extract agar (ISP No. 2)	G:	Abundant	Abundant	Abundant
	AM:	Medium gray (265)[a] Coalesces to black (267)	Medium gray (265) with white bloom	Light brownish gray (63)
	R:	Deep yellowish brown (75)	Dark olive brown (96)	Strong yellowish brown (74)
	SP:	Absent	Absent	Absent
Oatmeal agar (ISP No. 3)	G:	Moderate	Abundant	Moderate
	AM:	Medium gray (265) Coalesces to black (267)	Medium gray (265) and light gray (264)	Medium gray (265) and white (263)
	R:	Grayish yellow (90)	Grayish yellow (90)	Grayish yellow (90)
	SP:	Absent	Absent	Absent
Inorganic salts-starch agar (ISP No. 4)	G:	Abundant	Abundant	Moderate
	AM:	Purplish gray (233) Coalesces to black (267)	Purplish gray (233) and light brownish gray (63)	Medium gray (265) and yellowish white (92)
	R:	Dark bluish gray (192) and moderate yellowish brown (77)	Dark bluish gray (192) and moderate yellowish brown (77)	Light yellowish brown (76)
	SP:	Absent	Absent	Absent
Glycerol-asparagine agar (ISP No. 5)	G:	Abundant, pocked	Abundant	Abundant
	AM:	Medium gray (265) and white (263)	Light gray (264) and light grayish yellowish brown (79)	White (263), not sporulated
	R:	Medium gray (265), light grayish yellowish brown (79) and moderate reddish brown (43)	Light yellowish brown (76)	Grayish yellow (90)
	SP:	Absent	Absent	Absent
Peptone-yeast extract-iron agar (ISP No. 6)	G:	Moderate	Moderate	Abundant
	AM:	Absent	Absent	Absent
	R:	Dark grayish yellowish brown (81)	Dark grayish yellowish brown (81)	Grayish yellow (90)
	SP:	Strong; brownish black (65)	Strong; brownish black (65)	Absent
Tyrosine agar (ISP No. 7)	G:	Abundant	Abundant	Abundant
	AM:	Light purplish gray (232)	Light brownish gray (63)	Medium gray (265) and white (263)
	R:	Dark grayish brown (62)	Brownish black (65)	Dark orange yellow (72)
	SP:	Present; dark grayish yellowish brown (81)	Present; dark grayish yellowish brown (81)	Absent
Nutrient agar	G:	Moderate	Moderate	Moderate
	AM:	Absent	Light gray (264) with white bloom	Sparse; white bloom
	R:	Grayish yellow (90)	Light yellowish brown (76)	Grayish yellow (90)
	SP:	Weak; light yellowish brown (76)	Light yellowish brown (76)	Absent
Calcium malate agar	G:	Abundant	Abundant	Poor
	AM:	Absent	Sparse; white (263)	Absent
	R:	Grayish yellow (90)	Pale yellow (89)	—
	SP:	Absent Calcium solubilized	Absent Calcium solubilized	Absent
CZAPEK's agar	G:	Abundant	Moderate	Moderate
	AM:	Sparse; yellowish gray (93)	Absent	White (263)
	R:	Deep yellowish brown (75)	Pale yellow (89)	Grayish yellow (90)
	SP:	Very weak	Absent	Absent

[a] Color and number in parenthesis follow ISCC-NBS Centroid Color Charts.
G: Growth, AM: aerial mycelium, R: reverse, SP: soluble pigment.

Table 2. Utilization of carbon sources by strain AB 1006A-9, *S. tirandis* var. *tirandis* and *S. lydicus*.

Carbon source	AB 1006A-9	*S. tirandis* var. *tirandis* NRRL 3689	*S. lydicus* NRRL 2433	Carbon source	AB 1006A-9	*S. tirandis* var. *tirandis* NRRL 3689	*S. lydicus* NRRL 2433
Adonitol	−	−	++	D-Melezitose	++	−	++
L-Arabinose	++	++	++	D-Melibiose	++	++	++
Cellulose	−	−	−	D-Raffinose	+	++	++
Dulcitol	−	−	−	L-Rhamnose	++	++	−
D-Fructose	++	++	++	Salicin	+	+	+
D-Galactose	++	++	++	D-Sorbitol	−	++	++
D-Glucose	++	++	++	Starch	−	−	−
m-Inositol	++	++	++	Sucrose	++	++	++
Lactose	++	++	++	D-Trehalose	++	++	++
D-Mannitol	++	++	++	D-Xylose	++	++	++
D-Mannose	++	++	++				

++: Good utilization, +: poor utilization, −: did not utilize.

Table 3. Comparison of physiological characteristics of strain AB 1006A-9 with *S. tirandis* var. *tirandis* and *S. lydicus*.

Test	*Streptomyces* AB 1006A-9	*S. tirandis* var. *tirandis* NRRL 3689	*S. lydicus* NRRL 2433
Starch hydrolysis	+	+	+
H_2S production	+	+	−
Nitrate reduction	+(weak)	+(strong)	+(very weak)
Melanin formation on:			
Peptone-yeast extract-iron agar	+	+	−
Tyrosine agar	+	+	−
Temperature range for:			
Growth	21 to 37°C No growth at 42°C	21 to 42°C No growth at 54°C	21 to 42°C No growth at 54°C
Sporulation	21 to 37°C	21 to 32°C, less at 37°C	21 to 32°C
NaCl tolerance	4% but not 7%	4% but not 7%	10% but not 13%
Decomposition of:			
Adenine	+	+	+
Casein	+	+	+
Xanthine	−	−	+
Hypoxanthine	+	+	+

S. lydicus NRRL 2433 are compared in Table 1. Their carbon source utilization patterns and physiological characteristics are shown in Tables 2 and 3, respectively. The data in these tables show that strain AB 1006A-9 closely resembles *S. tirandis* var. *tirandis* but is quite different from *S. lydicus*. We searched the descriptions of streptomycetes published by the International Streptomyces Project[14~17] and BERGEY'S Manual[18,19] and found no other species that fits strain AB 1006A-9 as well as the NRRL 3689 culture. The producer of tirandalydigin is distinguishable from *S. tirandis* var. *tirandis*, however, by its inability to use sorbitol as a sole source of carbon, by a slightly narrower temperature range for growth and by its tendency to form moist droplets, coalescing the aerial spore chains, on several agar media. This latter characteristic is frequently seen in streptomycetes, but we never observed it with *S. tirandis* var. *tirandis* NRRL 3689. To recognize the similarity to *S. tirandis* var. *tirandis* and the unique moisture droplet feature, we have named the producer of tirandalydigin *S. tirandis* subsp. *umidus*.

In the course of screening for antianaerobe antibiotics we isolated producers of tirandamycin and streptolydigin several times. These cultures were found in diverse soils collected in nine locations in the United States in 1981 and 1982. The soils ranged from alkaline desert soils in the southwestern United States to acid soils from an Ohio forest and New Jersey farmland. The producers of streptolydigin were identical to *S. lydicus*. The producers of tirandamycin were variable and only one resembled *S. tirandis* var. *tirandis*. HAGENMAIER *et al.*[20] have reported tirandamycin B production by a culture different from *S. tirandis* var. *tirandis*, which they identified as *S. flaveolus*. Streptomycetes which produce tetramic acid antibiotics seem to be widely dispersed. It would be interesting to learn the differences in the molecular genetics and biochemical pathways which lead to the biosynthesis of tirandamycin, streptolydigin and tirandalydigin.

Fermentation

An HPLC scan of a typical fermentation broth extract is shown in Fig. 3. The time course of the production of tirandalydigin by strain AB 1006A-9 is shown in Fig. 4. The maximum yield of 64 mg/liter of tirandalydigin is reached on the 6th day.

Antimicrobial Activity

Tirandalydigin has an antimicrobial spectrum

Fig. 3. HPLC of a fermentation broth extract containing tirandalydigin.

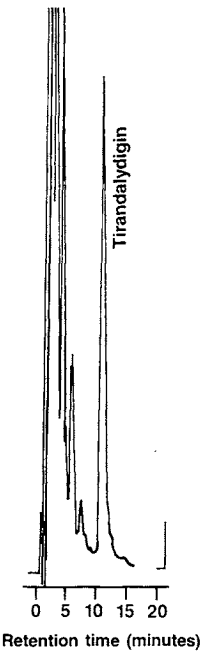


Fig. 4. Time course of the tirandalydigin fermentation.

● pH, ▲ tirandalydigin, △ growth, ○ reducing sugar.

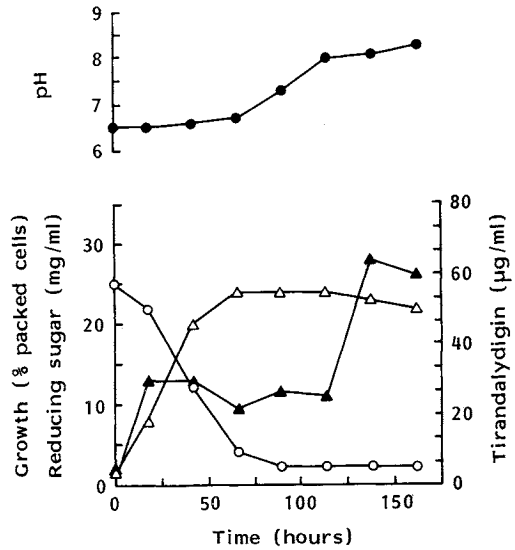


Table 4. Comparative potency of tirandalydigin, streptolydigin and tirandamycin A against anaerobic bacteria.

Test microorganism	MIC (μg/ml)		
	A	B	C
Bacteroides fragilis ATCC 25285	0.5	1	1
B. fragilis UC-2	0.5	1	1
B. fragilis SFM2906A	0.5	1	1
B. thetaiotaomicron ATCC 29742	0.5	2	1
B. melaninogenicus ATCC 25845	32	4	32
B. vulgatus 792	0.5	0.25	0.5
Clostridium difficile ATCC 9689	32	8	16
C. perfringens SFBC 2026	0.25	1	2
Peptostreptococcus magnus ATCC 29328	16	4	64
P. anaerobius ATCC 27337	0.06	0.12	0.12

A: Tirandalydigin, B: streptolydigin, C: tirandamycin A.

Table 5. Comparative potency of tirandalydigin, streptolydigin and tirandamycin A against aerobic bacteria.

Test microorganism	MIC (μg/ml)		
	A	B	C
Staphylococcus aureus ATCC 6538P	>100	50	>100
S. aureus CMX 686B	>100	100	>100
S. epidermidis 3519	>100	50	>100
Streptococcus pyogenes EES 61	25	3.1	12.5
S. pyogenes 930 CONST	3.1	3.1	12.5
S. bovis A5169	12.5	12.5	50
Escherichia coli Juhl	>100	>100	>100
Enterobacter aerogenes ATCC 13048	>100	>100	>100
Klebsiella pneumoniae ATCC 8045	>100	>100	>100
Providencia stuartii CMX 640	>100	>100	>100
Pseudomonas aeruginosa BMH 10	>100	>100	>100

A: Tirandalydigin, B: streptolydigin, C: tirandamycin A.

Table 6. Potency of tirandalydigin against enterococci and legionellae.

Test microorganism	MIC (μg/ml)
Enterococcus faecalis CMX 736F	16
E. faecalis CMX 729G	16
E. faecalis A-5168	16
E. faecalis CMX 663F	8
Legionella pneumophila ATCC 33152	8
L. pneumophila 2551	16
L. pneumophila 2552	8
L. bozemanii ATCC 33217	16
L. longbeachae ATCC 33462	32
L. micdadei ATCC 33204	1

that is similar to both tirandamycin A and streptolydigin as indicated in Tables 4 and 5. Table 4 shows that tirandalydigin has potent activity against a number of anaerobes. Table 5 indicates that, of the aerobes examined, only the streptococci are sensitive to tirandalydigin. Tirandalydigin also has moderate activity against enterococci and legionellae (Table 6).

Acknowledgments

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