EPITHIENAMYCINS—NOVEL β-LACTAMS RELATED TO THIENAMYCIN

I. PRODUCTION AND ANTIBACTERIAL ACTIVITY

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The epithienamycins are cell wall active antibiotics structurally related to *N*-acetylthienamycin. We have found forty-three isolates of *Streptomyces flavogriseus* which are capable of producing members of the epithienamycin family. Six major epithienamycin components, and xanthomycin, have been isolated from fermentation broth. Fermentation conditions can be varied to enrich for certain members of the epithienamycin family. All six components show activity *in vitro versus* a broad spectrum of bacterial species. The weight potencies vary 27 fold from the most active to least active.

During the last several years there has been an increase in the discovery rate of new, naturally occurring β-lactams. The independent discovery of the cephamycins by Merck and Eli Lilly Research Laboratories[1,2] ended a long fallow period in which no new β-lactam antibiotics were found in nature. The subsequent isolation of thienamycin[3], clavulanic acid[4], the nocardicins[5], the olivanic acids[6,7], MC696-SY2A and B[9], PS-5[8], and PS-6[10], confirmed that the possibilities for discovery of new β-lactams from soil microorganisms had not yet been exhausted[30]. We report here the discovery, production and *in vitro* antibacterial activity of another group of naturally occurring β-lactam antibiotics, the epithienamycins, which are produced as metabolites of several newly isolated strains of *Streptomyces flavogriseus*.

A companion paper[11] will describe the isolation procedure and the basis for the structure assignments. A preliminary account of this work has been presented elsewhere[12,13].

Materials and Methods

Chemicals

Thienamycin is a product of the Merck Sharp and Dohme Reasearch Laboratories[3]. Desacetylepithienamycin A was prepared by deacetylation of epithienamycin A as described in the accompanying paper[11]. *Bacillus cereus* penicillinase was obtained as a solution from Difco (Bacto-Penase). All other chemicals are reagent grade.

Taxonomic Studies

The cultural caracteristics of the microorganisms which produce the epithienamycins were determined by use of the media and methods described in SHIRLING and GOTTLIEB[14]. The taxonomic descriptions in BERGEY's Manual (8th Edition)[15], in SHIRLING and GOTTLIEB[16~19] and in WAKSMAN[20], were used to compare the epithienamycin producers with recognized genera and species of actinomycetes.

Two strains of *Streptomyces flavogriseus* designated MA 4434 and MA 4600 have been placed on permanent deposit with the culture collection of the Northern Regional Research Laboratories of the U.S. Department of Agriculture, Peoria, I11., U.S.A., under accession numbers NRRL 8139 and NRRL

8140. These cultures have also been deposited in Japan at the Fermentation Research Institute, Chiba-Ken, Japan, under accession numbers 3792 and 3793. A subisolate of MA 4434 designated MA 4638 has also been deposited under accession numbers NRRL 11020 and Research Institute 4284.

Fermentation Studies

Laboratory fermentations were run in 250 ml Erlenmeyer flasks. Flasks were incubated on a rotary shaker with a 5 cm throw at 220 revolutions per minute. Unbaffled Erlenmeyer flasks containing 40 ml of medium and 2 ml of mature seed broth were used for production. Production flasks were incubated at 24°C or 28°C for 3~5 days. Baffled Erlenmeyer flasks, incubated at 28°C for 1 or 2 days, were used for seed development. Seed flasks contained 50 ml of medium composed of 10.0 g per liter dextrose, 10.0 g per liter yeast autolysate (ardamine pH), 0.05 g per liter $MgSO_4 \cdot 7H_2O$ and 2.0 ml per liter phosphate buffer. The final pH was 6.5. Phosphate buffer contained 91.0 g per liter KH_2PO_4 and 95.0 g per liter Na_2HPO_4.

Bioassay

The antibiotic potency of broth is determined by an agar disc-diffusion assay performed with *Vibrio percolans* ATCC 8461 (MB 1272). The culture is incubated in nutrient broth plus 0.2% yeast extract medium overnight on a rotary shaker at 28°C and then diluted to an optical density of 0.220 at 660 nm. A 33.2 ml portion of this diluted culture is added to 1.0 liter of nutrient broth-yeast extract agar medium at 45°C. The inoculated agar-containing medium is poured into 100×15 mm plastic petri dishes (5.0 ml per plate).

A one-half inch diameter filter paper disc is wetted with 0.1 ml of filtered or centrifuged broth, and the disc applied to the agar surface. The plates are incubated overnight at 25°C. One unit per ml is that concentration of antibiotic which produces a zone equivalent to that of a cephaloridine solution of 12.5 μg/ml. For bioassays other than on crude broths, purified epithienamycin A is also used as a standard. The purity of the standard is checked by observing the absorbance decrease at 300 nm upon reaction with hydroxylamine[11]. Epithienamycin A free of other antibiotics was assigned a specific bioactivity of 250 units per hydroxylamine-extinguishable absorption unit at 300 nm, approximately 6.2 bioassay units per microgram.

Antibacterial Spectrum Measurements

The antibacterial spectra were measured against a variety of laboratory strains of bacteria, which are not necessarily representative of clinical strains. The general procedure for preparing agar plates seeded with bacteria for zone of inhibition measurements involved: 1) diluting the overnight cultures to $A_{660}=0.22$; 2) adding one volume of diluted inoculum to 30 volumes of 2% agar-containing medium at 45°C; and 3) pouring the agar to a depth of 1.7 mm in 100×100 mm square petri dishes. Exceptions: for organisms 1 and 6, a spore suspension is used instead of an overnight culture; for organisms 8, 22 and 23 the inocula are diluted into 9 volumes of agar; and for organisms 9, 10, 11 and 24 dilutions are into 14 volumes of agar.

The samples described in Table 2 are prepared as (A) peak fractions from Biogel desalting columns (Epithienamycins A and C, *N*-acetylthienamycin), or from a Dowex-1 desalting column (desacetylepithienamycin A), or (B) solutions of the lyophilized antibiotics in distilled water. Concentrations are estimated by ultraviolet absorbance at the 300~308 nm maximum, and adjusted to the concentrations listed by dilution with distilled water. See reference 11 for details of preparation and A_{300} values.

Samples are applied directly to the plate surface as droplets of *ca.* 12~15 μl, and allowed to dry by exposure to air. Plates are incubated overnight at 28°C for assays 1~7, 11~21 and 24~26, and at 37°C for assays 8~10, 22 and 23.

Penicillinase Susceptibility

Assay plates of *Vibrio percolans* MB 1272 containing penicillinase are prepared as described for antibacterial spectrum measurements except that to 100 ml of inoculated agar, 2 ml of *Bacillus cereus* penicillinase (Difco, Bacto-Penase) containing 10^6 units of penicillinase, are added immediately before the plates are poured.

Identical antibiotic samples are placed on *V. percolans* plates with and without penicillinase, and the zone size difference (no penicillinase)−(+penicillinase) in millimeters is taken as a measure of penicil-

linase susceptibility.

Urinary Recovery

Five female CD2 mice (Charles River), weight approximately 20 g, were each injected subcutaneously with 0.5 ml of a solution of thienamycin, desacetylepithienamycin A, or epithienamycin A, at *ca.* 30 μg/ml. An additional 0.5 ml of water was injected to increase total urine output. The five mice per compound were kept in a single metabolism cage. Urine samples were rinsed into collecting tubes at 0.5, 1, 2, and 4 hours after injection. Recovery was measured by bioassay. No activity was detected for any sample in the 2~4 hours interval.

Results

Strain Description

The capacity for synthesis of the epithienamycins is widely distributed in nature. We have isolated from soils found worldwide 43 strains of *Streptomyces flavogriseus* which produce the epithienamycins. The epithienamycin producing cultures had branching sporophores consisting of straight to flexuous chains of spores forming tufts (Fig. 1). Spore chains are more than 10 spores in length. Spores are spherical to oval (0.9 μ×1.2 μ) in shape with a smooth surface. None of the cultures produced melanoid pigments. All of the cultures produced gray spores in mass. The gray varied from medium to dark on most media, becoming yellow-gray on glycerol asparagine and/or egg albumin agar. All produced the same pattern of carbohydrate utilization in which inositol, raffinose and sucrose as carbohydrate sources gave little or no growth. All cultures gave the same results on the physiological tests run. The cultural characteristics of two of the best producers, NRRL 8139 and NRRL 8140, are summarized in Tables 1, 2, and 3.

Fig. 1. Electron micrograph of *Streptomyces flavogriseus* MA 4434 (×1,200).

Obtained by the Ultrastructure and Histochemistry Unit of the Merck Sharp and Dohme Research Laboratories.

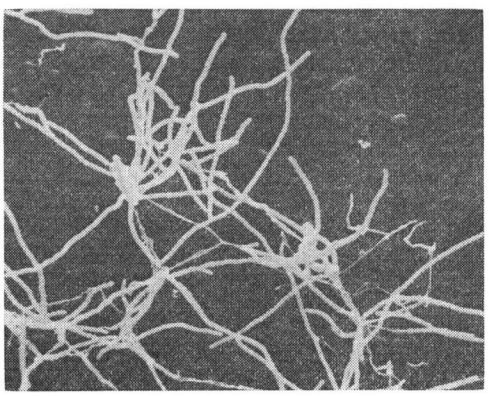

Table 1. Carbohydrate utilization of *S. flavogriseus*.

Carbohydrate	NRRL 8139	NRRL 8140
Glucose	+	+
Arabinose	+	+
Cellulose	−	−
Fructose	+	+
Inositol	−	±
Lactose	+	+
Xylose	+	+
Maltose	+	+
Mannitol	+	+
Mannose	+	+
Raffinose	−	±
Rhamnose	+	+
Sucrose	±	±

+, good growth; ±, poor growth; −, no growth.

Table 2. Physiological characteristics of *S. flavogriseus* strains.

Characteristic	NRRL 8139	NRRL 8140
Melanoid pigment production	Negative	Negative
Hydrolysis of casein	Positive	Positive
Decomposition of tyrosine	Negative	Negative
Hydrolysis of starch	Positive	Positive
Liquefaction of gelatin	Positive	Positive
Peptonization of milk	Positive	Positive

Table 3. Cultural characteristics of *S. flavogriseus* NRRL 8139 and NRRL 8140.

Medium	Color of aerial spore mass*	Color of reverse vegetative growth
Oatmeal agar	Light medium gray	Yellow-tan
Egg albumin agar	Medium gray mixed with yellowish-gray (2dc), old grayed yellow (2db)	Yellow-tan
Glycerol-asparagine agar	Light gray with yellowish tone (2dc)	Brown
Inorganic salts-starch agar	Medium gray	Yellow-tan
Yeast extract-malt extract agar	Dark gray	Dark brown

* Color Harmony Manual, 4th Edition (1958), Container Copy of America, Chicago.

The differences between the strains were in the degree of strength of yellow-tan vegetative growth and in degree of, or absence of, a strong yellow tone to the gray of sporulating aerial mycelium on at least one solid medium. While there were definite variations among the cultures studied, these were variations of degree rather than of characteristics, indicating that these cultures are distinct strains of the same species rather than different species. It was therefore concluded, after comparison with the description of recognized species, that the cultures producing epithienamycins were all strains of the same organism. That organism is *Streptomyces flavogriseus*.

The strains described in this paper are distinguishable from the olivanic acid producer *Streptomyces olivaceus* in that the sporophore structure formed straight to flexous chains in tufts rather than the open loose spirals often seen in *S. olivaceus*, that inositol and raffinose were not utilized and that a definite yellow tone to the vegetation growth and to the gray spore mass appeared in varying degree on certain standard media.

Fermentation

The various strains of *S. flavogriseus* produce 8 antibacterially active compounds. These include six major epithienamycin components A~F, one minor antibiotic thought to be an epithienamycin component, and xanthomycin. Two of our most productive *S. flavogriseus* strains are NRRL 8139 and NRRL 8140.

NRRL 8139 produced predominately epithienamycins A~D at an average titer of 68 biological units per ml in medium 1 after 3 days incubation at 28°C. NRRL 8140 on the other hand produces predominately epithienamycins A and B plus smaller variable amounts of epithienamycins C and D along with xanthomycin, at an average titer of 95 biological units per ml in medium 1 after 3 days incubation at 28°C. No xanthomycin and an average titer of 88 biological units per ml of epithienamycins are produced in medium 2 by NRRL 8140 after 3 days incubation at 28°C.

Epithienamycins E and F, at 20~40 biological units per ml, are the predominant antibacterial compounds found in medium 3 when NRRL 8140 is grown in it for 3 days at 28°C. Xanthomycin alone was produced by NRRL 8140 when it was grown 3 days at 28°C in medium 4. Media compositions are in Table 4.

As has been found for thienamycin[8], fosfomycin[21] and gentamicin[22], cobalt is required for optimum epithienamycin production. Fermentation times and temperatures for optimum production vary depending on the *S. flavogriseus* strains used to produce the epithienamycins.

In general, media that are acidic and contain cobalt result in the best epithienamycin titers. Acidic media may enhance epithienamycin production by minimizing the degradation of synthesized β-lactams

Table 4. Media compositions.

	Carbon sources (g/liter)	Nitrogen sources (g/liter)	Salts and defoamers (g/liter)	Presterile pH
Medium 1	Dextrin (Amidex) 20	Tomato paste 20 Primary yeast 10	$CoCl_2 \cdot 6H_2O$ 0.005	7.2~7.4 (NaOH)
Medium 2	Dextrin (CPC) 30 Na citrate 1.5 Gluconic acid 1.5	Soybean meal 15 Autolysed yeast 1.5	$CoCl_2 \cdot 6H_2O$ 0.005	6.2 (NaOH)
Medium 3	Dextrin (CPC) 40	Distillers solubles 7 Autolysed yeast 5	$CoCl_2 \cdot 6H_2O$ 0.05	7.3 (NaOH)
Medium 4	Corn meal 20	Soybean meal 15 Distillers solubles 10	$CaCO_3$ 4.0 $CaCl_2$ 0.5 $MgSO_4 \cdot 7H_2O$ 0.1 $CoCl_2 \cdot 6H_2O$ 0.01 $FeSO_4 \cdot 7H_2O$ 0.01 Polyglycol P2000 2.5	6.5 (NaOH)

which occurs in alkaline conditions in the presence of nucleophiles[23]. The role of cobalt in thienamycin or epithienamycin synthesis is unknown. However, cobalt is known to alter the structural characteristics of glutamine synthetase[24]. Perhaps since glutamic acid is a synthetic precursor of thienamycin[25] cobalt may have a similar effect on *Streptomyces* glutamine synthetase.

Antibacterial Activity

All of the epithienamycins show cell wall inhibitory activity as judged by their ability to induce spheroplast formation in hypertonic media[28]. They are active against both Gram-positive and Gram-negative organisms (Table 5). The spectrum of activity of each of the epithienamycins is generally similar to that of *N*-acetylthienamycin, after overall absolute potency differences are discounted, with small but significant variations on some strains. In addition, the β-lactamase-sensitive epithienamycin A and epithienamycin B have diminished activity in certain β-lactamase producing organisms (*e.g.* #24).

Epithienamycins may be deacetylated by enzymes isolated from *Protaminobacter ruber*[31], *Alcaligenes faecalis*, or hog kidneys[11]. The deacetylated products (*e.g.* desacetylepithienamycin A, Table 5) show a substantially increased activity against *Pseudomonas* species relative to parent compounds. A similar difference is observed between the anti-pseudomonal potencies of acetylated and unacetylated thienamycin. Thus reduction of *Pseudomonas* activity by *N*-acetylation appears to be a general property of thienamycin-like antibiotics.

Biological Spectrum

Relative potencies of the epithienamycins were estimated by measuring the zone diameters produced on 35 strains of bacteria (including the 26 in Table 5), averaging the zone sizes obtained (excluding those with no zone) and correcting for the concentration differences using a slope of 3 to estimate potency differences. In as much as the diameters are approximately linearly dependent on the logarithm of concentration, this procedure yields a comparison of the geometric means of the potencies. The relative potencies of the epithienamycins compared to thienamycin is in Table 6. Thienamycin was arbitrarily assigned the value of 100 as the most potent antibiotic tested. It should be noted that epithienamycin B was the most potent *N*-acetylated epithienamycin in these tests. It also is evident that removing the

Table 5. Zone of inhibition diameters (mm).*

Antibiotic concentration (μg/ml)	Thienamycin	Desacetyl-epithienamycin A	N-Acetyl-thienamycin	Epithienamycins					
				A	B	C	D	E	F
	33	32	38	90	30	104	187	20	11
1. *Bacillus* sp. 633	34	38	28	35	31	17	17	35	27
2. *Proteus vulgaris* 1012	27	12	22	15	10	16	14	23	16
3. *Pseudomonas aeruginosa* 979	13	14	0	0	0	0	0	0	0
4. *Serratia marcescens* 252	20	29	29	23	10	17	18	18	12
5. *Staphylococcus aureus* 108	39	36	32	32	26	25	24	27	17
6. *Bacillus subtilis* 964	44	38	32	—	31	23	23	35	27
7. *Sarcina lutea* 1101	41	38	32	36	40	22	30	27	30
8. *Streptococcus faecalis* 753	15	22	5	17	10	0	0	0	0
9. *Brucella bronchiseptica* 965	19	18	21	23	21	17	18	13	0
10. *Salmonella gallinarum* 1287	28	27	28	33	25	17	20	31	25
11. *Vibrio percolans* 1272	33	30	28	31	31	33	37	29	30
12. *Xanthomonas vesicatoria* 815	20	22	22	22	21	12	16	25	17
13. *Proteus vulgaris* 838	22	27	28	32	29	15	18	32	27
14. *Escherichia coli* 1418	21	26	24	35	30	14	20	27	22
15. *Pseudomonas stutzeri* 1231	23	31	6	19	13	0	7	7	0
16. *Klebsiella pneumoniae* 1264	27	31	21	27	20	12	17	21	16
17. *Aerobacter aerogenes* 835	26	25	20	28	20	14	17	22	19
18. *Erwinia atroseptica* 1159	21	27	23	27	29	17	21	27	7
19. *Pseudomonas aeruginosa* 2824	30	30	4	0	0	0	0	0	0
20. *Corynebacterium pseudodiphtheriticum* 261	33	34	24	35	33	23	23	16	9
21. *Escherichia coli* 60	25	23	22	27	23	12	17	23	14
22. *Streptococcus faecium* 2820	13	22	—	22	—	—	—	7	0
23. *Streptococcus agalactiae* 2875	35	32	30	26	25	23	23	26	26
24. *Vibrio percolans* 2566 (res. ceph. C)	32	15	21	13	0	27	27	18	19
25. *Proteus vulgaris* 2112 (episome)	29	33	29	37	27	17	19	31	27
26. *Proteus mirabilis* 3126	22	22	23	25	23	7	9	24	26

* 15 μl drop applied to seeded agar 2 mm thickness.

N-acetyl group from epithienamycin A markedly increases relative potency while on the other hand the addition of the *N*-acetyl group to thienamycin markedly decreases relative potency. The potency change due to *N*-acetylation is particularly pronounced in *Staphylococcus aureus* as well as *Pseudomonas* species.

Table 7 summarizes the MIC values of epithienamycin B against some of our more sensitive strains. Values for thienamycin and *N*-acetylthienamycin against the same strains are included for comparison.

Table 6. Relative potencies of thienamycins and epithienamycins (Average of 35 strains).

	Relative potencies
Thienamycin	100
Desacetylepithienamycin A	96
Epithienamycin B	63
N-Acetylthienamycin	54
Epithienamycin F	42
Epithienamycin E	34
Epithienamycin A	29
Epithienamycin C	3.4
Epithienamycin D	2.3

Table 7. Minimum inhibitory concentrations.

Organism	Thienamycin	*N*-Acetylthienamycin	Epithienamycin B
Staphylococcus aureus 108	<0.01	0.082	0.12
Bacillus subtilis 964	<0.01	0.027	0.014
Strept. agalactiae 2875	0.020	0.027	0.041
Escherichia coli 60	0.31	0.74	0.12
Proteus vulgaris 838	0.62	0.25	0.12
Vibrio percolans 1272	0.04	0.027	0.014
Salmonella gallinarum 1287	0.31	0.027	0.041

Inocula of 10^3 organisms from an overnight culture were incubated in 2 ml MUELLER-HINTON broth containing antibiotic, for 18 hours at 37°C. The lowest concentration producing a clear tube is the MIC.

Table 8. Sensitivity of epithienamycins to penicillinase from *Bacillus cereus*.

Compound	Average zone size difference (mm*)
Thienamycin	0
Desacetylepithienamycin A	28
N-Acetylthienamycin	0
Epithienamycin A	24
Epithienamycin B	23
Epithienamycin C	8
Epithienamycin D	7
Epithienamycin E	10
Epithienamycin F	11

* *versus Vibrio percolans* 1272 with and without penicillinase.

Penicillinase Susceptibility

None of the epithienamycins appear susceptible to the *Enterobacter cloacae* P99 lactamase, but they vary widely in resistance to the *B. cereus* penicillinase. Data presented in Table 8 show that epithienamycins A and B are relatively sensitive to *B. cereus* penicillinase while on the other hand epithienamycins C and D are relatively resistant to this β-lactamase preparation. Thienamycin and *N*-acetylated thienamycin are completely resistant to this β-lactamase preparation. Desacetylepithienamycin A retained the β-lactamase sensitivity of the *N*-acetylated compound, epithienamycin A. A related pattern of sensitivities to the RTEM β-lactamase has been observed by others for olivanic acid components[34], which we believe are identical to the epithienamycins[11].

Urinary Recovery

Total urinary recoveries of epithienamycins injected subcutaneously into mice are quite low, approximately 1%. This is now known to be due to hydrolysis of the β-lactam ring by the kidney enzyme dehydropeptidase I, which shows no comparable degradative activity against penicillins or cephalosporins[32,33]. The enzyme is more active against thienamycin-type compounds with an *N*-acetyl group on the cysteamine side chain than against the unacetylated forms. Thus under conditions in which only 1% of administered epithienamycin A is found in the urine (materials and methods), 25% of thienamycin and 10% of desacetylepithienamycin A are recovered.

Discussion

We have found that *Streptomyces flavogriseus* produces at least six closely related cell wall active antibiotics with a wide range of potencies, some variations in their pattern of antibacterial activities, and major differences in their resistance to inactivation by penicillinase. These antibiotics have been named epithienamycins because they contain the same bicyclic ring system as does thienamycin[29], but are all epimeric to thienamycin at least one asymmetric center[11]. In spite of the close structural similarity of thienamycin and the epithienamycins, there is a striking difference in the frequency of isolation of organisms producing these two structural types. Over forty independently isolated actinomycete cul-

tures have been found to produce epithienamycins, whereas only one producer of thienamycin has been found in the same group of soil isolates.

The bicyclic carbapenem nucleus contained in the epithienamycins and thienamycins has also been observed in PS-5, PS-6, PS-7, MC696-SY2A, the olivanic acids and the carpetimycins[35]. We believe that six of the olivanic acids described[6,7,26] are identical to the six epithienamycins reported here. The bases for the structural conclusions are described in the accompanying paper[11].

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