THIENAMYCIN, A NEW β-LACTAM ANTIBIOTIC

I. DISCOVERY, TAXONOMY, ISOLATION AND PHYSICAL PROPERTIES*

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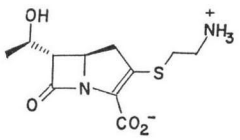
A new β-lactam antibiotic, named thienamycin, was discovered in culture broths of *Streptomyces* MA4297. The producing organism, subsequently determined to be a hitherto unrecognized species, is designated *Streptomyces cattleya* (NRRL 8057). The antibiotic was isolated by adsorption on Dowex 50, passage through Dowex 1, further chromatography on Dowex 50 and Bio-Gel P2, and final purification and desalting on XAD-2. Thienamycin is zwitterionic, has the elemental composition $C_{11}H_{16}N_2O_4S$ (M.W.=272.18) and possesses a distinctive UV absorption (λ max=297 nm, ε=7,900). Its β-lactam is unusually sensitive to hydrolysis above pH 8 and to reaction with nucleophiles such as hydroxylamine, cysteine and, to a lesser degree, the primary amine of the antibiotic itself. The latter reaction results in accelerated inactivation at high antibiotic concentrations.

Thienamycin**, a β-lactam antibiotic with the unique structure shown in Fig. 1[1,2] was discovered in the course of screening soil microorganisms for production of inhibitors of peptidoglycan synthesis in Gram-positive and Gram-negative bacteria. Taxonomic studies of the producing organism MA4297 resulted in its assignment to a new streptomycete species which has been named *Streptomyces cattleya*. Thienamycin was co-produced in broths as a component of a complex of β-lactam antibiotics, including penicillin N, cephamycin C and what was subsequently established to be the N-acetyl derivative of thienamycin itself[3]. Thienamycin could be distinguished from previously described natural products by its unusual and highly desirable antibacterial spectrum[4]; activity is relatively high against Gram-positive bacteria, and extends over the full range of Gram-negative bacteria, including *Pseudomonas aeruginosa*. Of equal note, its activity is undiminished when tested against organisms resistant by virtue of β-lactamases to penicillins and cephalosporins.

Fig. 1. Chemical structure of thienamycin



This paper describes taxonomic studies of the producing organism, the production of thienamycin by fermentation, the sequence of chromatographic procedures used to isolate it in an essentially pure form, and the physical and chemical properties of the purified antibiotic.

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** This antibiotic is the first representative of a family of des-thia-carbapenem nucleus antibiotics in which the enamine portion of the fused 5-member ring bears a thioethylamine moiety. From this structural feature, the name thienamycin (thī′ēn-ə-mī′sən) is derived.

Materials and Methods

Fermentation

Inocula for tank fermentations were prepared in a seed medium consisting of 10 g yeast autolysate (Ardamine®, Yeast Products Corporation), 10 g glucose, 182 mg KH_2PO_4, 190 mg Na_2HPO_4 and 50 mg $MgSO_4 \cdot 7H_2O$ per liter of distilled water adjusted to a final pH of 6.5. A 250-ml baffled flask containing 50 ml of this medium was inoculated with a lyophilized culture, slant or frozen vial of MA4297 and incubated at 28°C for 24 hours on a gyrorotatory shaker at 160 rpm. The resulting growth was scaled-up in the following two stages: It was used to inoculate at a ratio of 1: 50, 5 2-liter baffled flasks each containing 500 ml of seed medium. After 24 hours of incubation as described in the prior step, this growth was added at a ratio of 1: 500 to 467 liters of seed medium in a 756-liter stainless steel fermentor. This tank was operated for 24 hours at 28°C with an agitation rate of 130 rpm and an airflow of 0.3 m^3/min. The antifoam agent, Polyglycol 2000 (Dow Chemical Co.), was added as required up to a maximum volume ratio of 0.1%. After 24 hours 453 liters of this growth were transferred to a 5,670-liter stainless steel fermentor containing 4,082 liters of production medium. The latter consisted of 25 g cerelose, 15 g corn steep liquor, 10 g distiller's solubles, 5 g cottonseed media (Pharmamedia®, Trader's Protein) and 10 mg $CoCl_2 \cdot 6H_2O$ per liter of tap water. After adjustment of pH to 7.3, 3 g $CaCO_3$ and 2.5 ml Polyglycol 2000 were added per liter. The tank was operated at 24°C with an agitation rate of 70 rpm and an airflow of 1.54 m^3/min.

Thienamycin Assay Procedure

Thienamycin content of broths and partially purified samples were determined by a disc-diffusion assay using *Staphylococcus aureus* ATCC 6538P incorporated into nutrient agar supplemented with 0.2% yeast extract. Typically in this assay, 0.01 μg of thienamycin (applied as a 0.1 μg/ml solution to a 14-mm paper disc) produced a 25 mm zone of seeded agar layer 0.2 cm in depth.

A differential spectrophotometric assay was employed during later stages of purification and for analysis of the purified antibiotic's stability. Samples were buffered with 10 mM potassium phosphate, pH 7, and reacted for 30 minutes at 23°C with 10 mM NH_2OH (freshly prepared from $NH_2OH \cdot HCl$ by exact titration with alkali). From the difference in absorbance measured at 297 nm between an unreacted and reacted sample, the concentration of thienamycin was established using a $\Delta\varepsilon^{1cm}_{297nm} = 7{,}465$. For example, solutions showing a $\Delta A = 1.0$ contained 36.5 μg/ml of the antibiotic. This assay was found closely proportional to the bioassay even with samples having a purity, by weight, as low as 1%.

Analytical Procedures

Amino acid analyses were conducted on a model KLA-5 Hitachi-Perkin-Elmer analyzer. Microanalysis of sulfur content was performed by the energy-dispersive analysis of electron-induced X-ray emission using an EDAX system mounted on an AMR-600 scanning electron microscope. Molecular weight determinations were made on a Model E analytical ultracentrifuge using the short-column sedimentation technique and UV absorption optics to monitor the sedimentation boundary of materials absorbing at 300 nm. A partial specific volume of 0.60 cc/g was assumed in calculating the apparent molecular weight. Circular dichroism and optical rotatory dispersion spectra were recorded on a Carey Model 60 spectropolarimeter. Field desorption mass spectra were obtained using a Varian MAT CH5DF spectrometer equipped with the Spectra-System 100 Data Analyzer.

Chemicals

Chromatographic media were obtained from the following sources: Dowex 50 and Dowex 1 resins from Dow Chemicals, Midland, MI; Bio-Gel P-2 from Bio-Rad, Richmond, CA; XAD-2 from Rohm and Haas Co., Philadelphia, PA. 2-(N-Morpholino)ethanesulfonic acid (MES) and 3-(N-morpholino)propanesulfonic acid (MOPS) were obtained as the free acid and sodium salts from Calbiochem, La Jolla, CA.

Results

Taxonomic Studies

The thienamycin-producing microorganism, MA4297, was isolated from a soil sample originating in New Jersey, U.S.A. The morphological and cultural characteristics of this isolate as determined by methods described in SHIRLING and GOTTLIEB[5] are listed in Tables 1, 2 and 3. The most striking macroscopic characteristic of the culture when grown on various solid media is the orchid pigmentation of the sporulated aerial mycelium. The micromorphology, as shown in Fig. 2, comprises sporophores in compact spirals, occurring as side and terminal branches on the aerial mycelium. Spores examined by electron microscopy, Fig. 3, are ellipsoidal to cylindrical in shape, $0.9\,\mu \times 1.2\,\mu$ in length, show a smooth surface and occur in chains of more than 10.

A search of the classification keys for the genus *Streptomyces* in the standard references showed no species to have the combination of orchid pigmentation, micromorphology and the absence of melanoid pigment which are characteristic of MA4297. This justified its assignment to a new *Streptomyces* species for which was chosen the name *cattleya* (derived from the botanical orchid genus of that name and the use of the noun as a color name)[6] to denote the notable pigmentation of the sporulated aerial mycelium. This culture has been deposited with accession number NRRL 8057 with the Northern Regional Research Laboratories, Peoria, Illinois, U.S.A.

Fermentation

The time course of thienamycin production as monitored by the disc-diffusion assay is shown in Fig. 4. As is the case with many antibiotic fermentations involving streptomycetes, the maximum rate of antibiotic synthesis occurred only after substantial depletion of the major carbohydrate source, dextrose. The peak thienamycin levels shown in Fig. 4 were $1 \sim 4\,\mu$g/ml*, a range typical of both laboratory shake-flask and tank fermentations in this medium with the wild-type isolate of MA4297.

Fig. 2. Spiral morphology of *Streptomyces cattleya* grown on tomato paste-oatmeal agar; electron micrograph (×7,600).

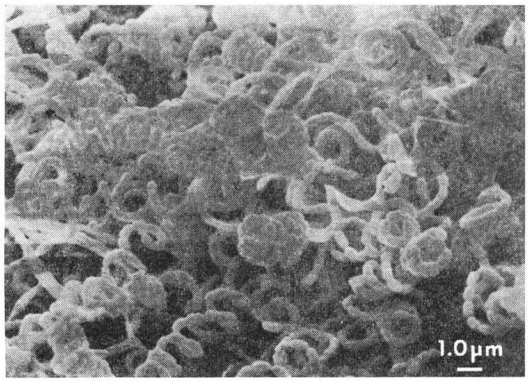
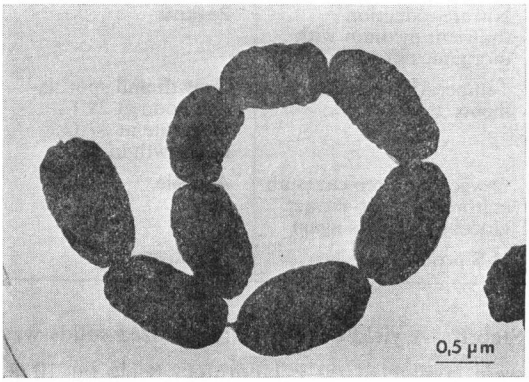


Fig. 3. Spores of *Streptomyces cattleya* grown on tomato paste-oatmeal agar; electron micrograph (×38,000)



* The activity of broths as measured by *Staphylococcus aureus* ATCC 6538P is almost entirely attributable to thienamycin *per se*; intrinsic potencies against this strain of the co-produced antibiotics, penicillin N, cephamycin C and N-acetyl thienamycin are, respectively, 1/1,500, 1/1,000 and 1/8 that of thienamycin and concentrations of the first two were 50 μg/ml or less, and of the third, less than 2 μg/ml (J. S. KAHAN & T. W. MILLER, unpublished results).

Table 1. Cultural characteristics of strain MA4297

Agar medium	Aerial mycelium	Vegetative mycelium	Soluble pigment
Tomato paste-oatmeal	Orchid (10 gc)* mixed with white	Reverse: tan, flat, spreading	None
CZAPEK DOX	Sparse, pinkish white	Colorless, flat, spreading	None
Egg albumin	Orchid (10 gc) mixed with lighter shades of orchid and some white	Tan with grayed-orchid cast, flat, spreading	None
Glycerol asparagine	Orchid (10 gc) mixed with some white	Reverse: tan with gray-pink cast, flat, spreading	None
Yeast extract-malt extract	Orchid (10 gc) mixed with pinkish white	Tan	None
Peptone-iron-yeast extract	none	Tan	Slight browning of medium
Tyrosine	Mixture of orchid (10 gc) and white	Tan	None
Potato plug	Sparse, grayish-pinkish white	Moderate growth, tan	None

* Color number designations taken from Color Harmony Manual, 4th edition, Container Corporation of America, Chicago, Illinois, 1958.

Table 2. Physiological properties of strain MA4297

Property observed	Characteristics
Action on milk	Broth – partial peptonization, becoming alkaline Agar – positive hydrolysis of casein
Gelatin liquefaction	Moderate
Hydrolysis of starch	Moderate
Melanin production (Peptone-iron-yeast extract agar, tyrosine agar)	None
Nitrate reduction (nutrient medium with inorganic nitrogen source)	Positive
Temperature requirements	Growth and sporulation good at 28°C, moderate at 37°C; no growth at 50°C.
Oxygen requirement (stab culture in yeast-extract, glucose and salts agar)	Aerobic
H_2S production	Negative

Table 3. Utilization of carbohydrates by strain MA4297

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

Isolation of Thienamycin

The isolation sequence and operating conditions are shown in Table 4. The material balance cited therein was obtained from a typical pilot plant batch starting with 4,000 liters of the broth shown in Fig. 4, having a titer of 2.4 μg/ml of thienamycin. The broth was filtered with 4% w/v of filter aid on a filter press prior to Step I. A yield of 99 g of lyophilized solids was obtained at the end of Step II. Subsequent steps were conducted on a laboratory scale on 10 g portions of the Step II solids, and in that case for which material balance figures are cited, yielded 18 mg of substantially pure, desalted thienamycin after Step Vb.

The choice of isolation methods and conditions suitable for concentrating thienamycin from large volumes of broth were constrained by the polar, zwitterionic character of the antibiotic over the narrow range of pH at which it is stable. Initial desalting and concentration on Dowex 50 Na^+ was

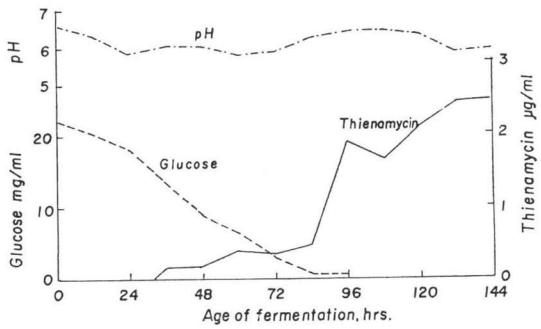
Table 4. Chromatographic steps in the isolation of thienamycin from broth filtrate

Chromatographic medium	Adsorption	Elution*	Material balance		
			Yield (%)		Purity μg/mg
			per step	overall	
I. Dowex 50×4, Na^+ 20/50 equilibrated with H_2O	10 CV broth filtrate adjusted to pH 4.5	Eluted with 2% pyridine, collected 0.6 through 1.6 CV, effluent concentrated 10-fold by evaporation *in vacuo*	16	16	—
II. Dowex 1×2, Cl^- 50/100 equilibrated with H_2O	0.67 CV of Step I concentrate adjusted to pH 7.3	Eluted with H_2O, collected 1.25 through 2.2 CV effluent lyophilized	67	10	10
III. Dowex 50×8, lutidinium, 200/400 equilibrated with eluant	0.02 CV of eluant containing 80 mg/ml of Step II solids	Eluted with 0.1 M 2,6, lutidinium acetate, pH 6.3 collected 0.7 through 0.9 CV effluent lyophilized	63	6.3	42
IV. Bio-Gel P-2 equilibrated with eluant	0.01 CV of eluant containing 55mg/ml of Step III solids	Eluted with 0.1 M 2,6, lutidinium acetate, pH 7 collected 0.65 through 0.7 CV effluent lyophilized	40	2.5	240
Va. XAD-2 equilibrated with eluant	0.05 CV of eluant containing 23mg/ml of Step IV solids	Eluted with 10mM K-phosphate, pH 7, collected 1.1 through 3.3 CV effluent concentrated 30-fold by evaporation *in vacuo*	80	2.0	—
b. XAD-2 equilibrated with H_2O	0.07 CV of Step Va concentrate	Eluted with H_2O collected 1.1 through 3.3 CV effluent lyophilized	82	1.64	940

* Unit of volume, CV, is the volume of the resin bed in each chromatographic step.

conducted at 6°C and a short contact time, but resulted nevertheless in low yields. Subsequent steps employing ion-exchange resins relied largely on adsorption, rather than ion exchange, as judged by the early appearance of antibiotic in the effluent.

Fig. 4. Time-course of fermentation of *Streptomyces cattleya* illustrating pH, glucose consumption and thienamycin production.



Evaluation of chromatographic Steps I through IV relied on gravimetric criteria of purity, and their performance could be monitored by differential refractometry. However, as a result of the low initial titre of broths, low overall yields and the necessity to employ a nominally volatile supporting buffer, these criteria became less reliable in guiding the final steps needed to achieve purity. It was at this point that the observation was made of the coordinate loss of bioactivity and absorbance in the 300 nm region of the UV spectrum, when thienamycin is exposed to unusually low concentrations of hydroxylamine and cysteine. Difference-spectra between unreacted and reacted crude preparations revealed the likely spectrum of the underlying pure antibiotic, and the succeeding XAD-2 Step was pursued in a non-UV-absorbing supporting buffer with the objective of spectrophotometric purity.

Relatively weak adsorption effects account for the manner in which Step V is conducted. Input material was applied as a narrow zone to the resin bed and advantage was taken of the retardation of

antibiotic relative to impurities in the first cycle, and relative to the phosphate buffer in the second cycle, thereby achieving final desalting.

Physical and Chemical Properties of Thienamycin

Thienamycin, isolated following Step Vb of the above process, is a white hygroscopic solid, freely soluble in water and sparingly soluble in methanol. The physical properties of the antibiotic are listed in Table 5; IR and UV absorption spectra are shown in Figs. 5 and 6. It should be noted that the specific rotation and UV extinction coefficients cited in Table 5 were computed on the basis of the following estimate for thienamycin content in the reference sample: On analysis, a 5.67% weight loss occurred on drying at room temperature for 4 hours under vacuum, and the elemental composition found was C 47.68%, H 6.22% and N 11.48%. From an energy dispersive analysis of X-rays emitted by

Table 5. Physical properties of thienamycin

Molecular weight:	Predicted for $C_{11}H_{16}N_2O_4S$	272.18
	by field desorption M.S.	272
	by sedimentation	282
Rotation:	$[\alpha]_D^{27}$ (c 0.1, H_2O*)	+82.7
ORD	Single positive COTTON effect	peak 311 nm trough 242 nm
CD	Positive maximum Negative minimum	287.5 nm 216 nm
IR (nujol mull)	sharp peak at broad peaks at	1765 cm^{-1} 1650~1550 cm^{-1} 2800~2500 cm^{-1} 3500~3100 cm^{-1}
UV	(H_2O*, pH 4~8)	maximum 296.5nm $E_{1cm}^{1\%}$ 290 ε 7900 min 242 nm $E_{1cm}^{1\%}$ 88
	(H_2O, pH 2†)	max 309 nm
	(H_2O, pH 12†)	max 300.5nm

* Rotation and UV spectra measured in 10 to 30 mM K-phosphate buffer at pH 7 or at the pH values specified.

† Spectra recorded immediately after pH adjustment.

Table 6. Chromatographic mobility of thienamycin

Solvent system	Chromatographic medium	Rf
Butanol – acetic acid – water (40: 10: 50)	Avicel TLC	0.43
Ethanol – water (70: 30)	Silica gel G	0.49
	Cellulose TLC	0.45~0.50
	Avicel TLC	0.48
	Silica gel HF (Analtech) TLC prewashed with EtOH	0.44
	Whatman No. 1 paper (descending)	0.63
N-Propanol – water (70: 30)	Silica gel G	0.31
	Avicel TLC	0.36

Fig. 6. Ultraviolet absorption spectrum of thienamycin in 10 mM MOPS, pH 7 before and after reaction with neutral 10 mM NH_2OH for 25 minutes.

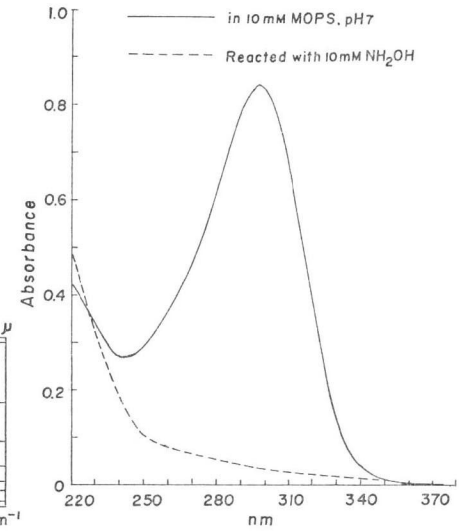
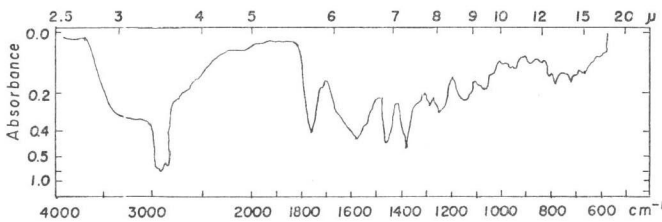


Fig. 5. Infrared absorption spectrum of thienamycin (nujol mull)



the antibiotic under the beam of a scanning electron microscope, sulfur was established to be the sole element of atomic weight greater than 19 present in significant proportions. Further application of the technique to a sample that had been combined with a known proportion of phosphate ion (serving as an internal reference), yielded sulfur content of $11 \pm 0.75\%$. These results taken together were consistent with the empirical formula $C_{11}H_{16}N_2O_4S \cdot (NH_3)_{0.28}$ having the predicted composition: C 47.68%, H 6.13%, N 11.52%, S 11.57% and O 23.1%. It was thus inferred that antibiotic purified by these methods had a thienamycin content of 92.7%, the remainder representing a partial ammonium salt of the carboxylic acid and tightly absorbed water. The organic moiety represented in the empirical formula is consistent with the results of structural studies, performed for the most part on the N-acetyl-methyl ester of thienamycin[1,2], and predicts a molecular weight equal to that found by field desorption mass spectrophotometry and analytical centrifugation (Table 5).

As noted in Table 5, a 12-nm red-shift in the UV absorption spectrum is observed immediately following adjustment of pH to 2. From spectra taken at a series of intermediate pH values (in 30 mM K-phosphate with appropriate amounts of additional HCl), transitions in initial absorbance* at wavelengths between 290 and 320 nm were found to have their midpoint at pH 3.08. This is inferred to be the pKa_1 of thienamycin. A reliable estimate of pKa_2 could not be obtained spectrophotometrically, owing to the small magnitude of the red-shift (4 nm) and the rapidity of hydrolysis at alkaline pH's. Since thienamycin shows insignificant electrophoretic mobility at pH 7 in K-phosphate, it is likely that $pKa_2 \geq 8$.

The mobility of thienamycin when subjected to thin-layer or paper chromatography in several solvent systems is listed in Table 6. In most cases, localization was achieved by bioautography and by the quenching of fluorescence excited by incident UV light. In addition, positive reactions were observed with ninhydrin (a peach-colored product evolving upon heating) and with the iodoplatinate reagent[7] (a slow bleaching at room temperature).

Amino acid analysis of acid hydrolysates of the purified antibiotic (6 N HCl, 112°C for 18 hours) revealed a single major, as yet unidentified ninhydrin-reactive component, having a retention time midway between that of alanine and glycine and producing a ninhydrin product whose absorbance measured at 440 nm is three-fold greater than that measured at 540 nm. When thienamycin is oxidized with cold performic acid followed by hydrogen bromide prior to acid hydrolysis[8], one major ninhydrin-reactive product was found and identified by TLC and amino acid analysis as taurine. It was formed in a yield of 0.8 μmoles for each mg of antibiotic entering the reaction sequence.

A chemical property of particular utility in the identification and analysis of thienamycin is its unusual susceptibility to inactivation by dilute solutions of hydroxylamine and cysteine. For example, antibiotic activity and UV absorbance (measured at 297 nm) were extinguished in proportion and with a half-life of 5.7 minutes in reaction mixtures containing 50 μM thienamycin and 4 mM NH_2OH at pH 7, 20°C. A half-life of 2.5 minutes was observed when the reagent was cysteine. These reactions are significantly more rapid than their classical counterparts for penicillin. Expressed as bimolecular rate constants for purposes of comparison with the studies of NAKKEN *et al.*[9], the hydroxylamine and cysteine inactivation of thienamycin proceeded at 31 and 69 liters/mol.min. compared with 18 and 2 liters/mol.min., respectively, in the case of benzylpenicillin. As in the case of penicillin, the reaction

* To take into account hydrolysis, spectra were taken at several time intervals at a given pH and back-extrapolated to the moment of acidification.

of thienamycin with SH-bearing reagents lacking a vicinal amine (*e.g.*, glutathione, β-mercaptoethanol and thioglycollic acid) occurred at less than 1% of the rate found at comparable concentrations of cysteine.

The hydroxylamine induced quenching of absorbance has been used, as mentioned earlier, to estimate from difference-spectra the presence and amount of thienamycin in crude preparations having a preponderance of UV absorbance unrelated to the antibiotic. It is also routinely used in the spectrophotometric assay of pure preparations, particularly those stored in aqueous solutions, since degradates resulting from thienamycin hydrolysis have been observed to develop a hydroxylamine insensitive absorbance with a maximum at *ca.* 280 nm. As shown in Fig. 6, 94.5% of the absorbance at 297 nm of a freshly prepared solution of purified thienamycin is extinguished following hydroxylamine treatment according to the conditions specified.

Stability of Thienamycin in Aqueous Solution

The stability of dilute solutions of thienamycin at differing pH's, and the supporting buffer's influence on stability are shown in Table 7. Stability is optimal between pH 6 and 7, declining with unusual rapidity above that range. By way of comparison, the stability of thienamycin between pH 2 and pH 5 is comparable to that reported for benzylpenicillin[10], but at pH 7 it is 5-fold and at pH 8.2, 100-fold less stable than penicillin in phosphate and borate buffers[11]. It is likely that both of these buffers catalyze β-lactam hydrolysis for, as shown in Table 7, stability is diminished when PO_4 buffer concentrations are increased. Furthermore, superior stability is obtained at pH 8.2 when triethylamine is employed in place of phosphate or borate. Buffers with primary amines, such as glycylglycine,

Table 7. Stability of dilute thienamycin in aqueous solution

	Buffer	pH	Temp. (°C)	Initial antibiotic concentration	Inactivation rate half-life (hr)*
A. Dependence on pH	K-Phosphate, 0.03 M	2	25°	0.1 mM	0.11
	〃 〃	3	〃	〃	0.50
	〃 〃	4	〃	〃	5.8
	〃 〃	7	〃	〃	94.0
	$Na_2B_4O_7$, 0.02 M	8.2	30°	〃	0.17
	〃 〃	8.8	〃	〃	0.095
	NaOH, 0.01 M	12.0	〃	〃	0.035
B. Dependence on buffer	Glycylglycine, 0.5 M	6.4	37°	0.1 mM	1.75
	2-(N-morpholino)ethane sulfonate (MES), 0.5 M	6.4	〃	〃	17.0
	$(NH_4)_2SO_4$, 0.1 M, MES, 0.02 M	6.8	〃	〃	3.0
	$(NH_4)_2SO_4$, 1.6 M, MES, 0.02 M	〃	〃	〃	0.41
	K-Phosphate, 1 M	7.0	23°	4 mM	2.4
	MES, 1 M	7.0	〃	0.05 mM	210.0
	TRIS, 0.2 M	7.5	37°	0.1 mM	0.55
	3-(N-morpholino)propane sulfonate (MOPS), 0.01 M	7.5	25°	0.1 mM	144.0
	MOPS, 0.01 M	8.0	25°	0.1 mM	96.0
	K-Phosphate, 0.03 M	8.2	30°	0.1 mM	0.3
	Triethylamine, 0.25 M	8.2	37°	0.1 mM	3.0

* Inactivation was measured by the differential spectrophotometric assay.

Table 8. Self-inactivation of thienamycin in concentrated solutions

Buffer	Initial concentration of antibiotic	Assay	Rate of degradation*	
			t_{30} (hr)	K″ ($\times 10^3$) [liter/(mol. min)]
2-(N-Morpholino) ethane sulfonate, 1 M, pH 7.0, 25°C	60 mM	UV	13.3	8.9
	140 mM	〃	7.3	7.1
	210 mM	〃	4.3	7.3
	620 mM	〃	3.1	3.8
3-(N-Morpholino) propane sulfonate, 1 M, pH 7.0, 25°C	210 mM	UV	6.78	4.9
		Bioactivity	3.38	9.5

* t_{30}: The time at which 30% of initial UV absorbance or bioactivity had disappeared.
K″: The apparent bimolecular rate constant for inactivation observed up to the t_{30} point.

Tris and ammonium ion are also found to inactivate thienamycin. Of the buffers examined for use in biological studies with thienamycin between pH 6 and 8, zwitterionic substituted morpholines, such as 2-(N-morpholino)ethane sulfonate and 3-(N-morpholino)propane sulfonate provide maximum stability, even when they are used at concentrations up to 1 M.

Of considerable practical importance, for example, during the latter stages of purification of thienamycin, was the observation that inactivation accelerates as concentration of the antibiotic is increased above approximately 10 mg/ml, regardless of the buffer employed. Solutions allowed to degrade above this concentration rapidly acquired a yellow and then brown color. Formation of the colored degradates, but not the primary inactivation reaction, required the presence of oxygen. Inactivation rates measured in solutions with initial concentrations from 16 to 170 mg/ml are listed in Table 8. Since the time course of degradation in concentrated solution, as monitored by loss of specific (hydroxylamine-extinguishable) UV absorbance at 297 nm, was intermediate between that of a first- and second-order reaction, the initial rates of degradation are expressed in 2 ways: (1) the time at which 30% of the initial UV absorbance had disappeared (t_{30}), (2) the apparent bimolecular rate constant (K″) based on a best-fit of UV absorbance measurements taken between 0 and 30% loss. The t_{30}'s are inversely proportional to concentration, while the apparent bimolecular rate constants remained approximately the same over a concentration range from 60 to 210 mM. Significantly, parallel measurements of antibiotic activity (Table 8, reaction 5) showed it to decline at twice the rate of UV absorbance at these concentrations.

The above kinetic findings can be explained by postulating aminolysis of the β-lactam by the primary amine of a second thienamycin molecule. A completely analogous reaction has been shown to occur with 6-aminopenicillanic acid and ampicillin[12]. The dimer formed initially should retain the UV chromophore of the amino donor, but is predicted to have a low order of biological activity. Supporting this hypothesis was the finding upon chromatography of concentrated solutions of thienamycin on gel-permeation columns (Bio-Gel P-2, with ethanol – H_2O, 20: 80 as eluant) of a species with hydroxylamine-extinguishable UV absorbance (λ max = 300 nm) migrating in advance of the peak of undegraded antibiotic. The new component possessed a ratio of antibacterial activity to UV absorbance only 2% that of the parent antibiotic, and migrated with a net negative charge on electrophoresis at neutral pH (J. S. KAHAN, R. T. GOEGELMAN and D. COLE, unpublished results).

The last finding is consistent with the presence in the postulated dimer of 2 carboxylic acids, one primary amine and a weakly basic imine.

Discussion

The last 3 years have witnessed the description of several new classes of β-lactam antibiotics that diverge radically from the structural pattern established over prior decades by the natural product penicillins and cephalosporins. The first of these to be announced was the monocyclic β-lactam nocardicin[13], followed by the oxa-penam structure, clavulanic acid[14]. The nocardicins retain the acylamido substituent on the azetidinone ring, as in penicillins and cephalosporins, whereas clavulanic acid has the additional novelty of being unsubstituted at that position.

Thienamycin is the first structurally-elucidated member of a related series of carbapenem-nucleus antibiotics, including the olivanic acids[15] and the epithienamycins[16]*. The azetidinone in all three is directly alkylated by a hydroxy-ethyl group, in place of the classical acylamide. In the case of the olivanic acids, the hydroxyl is sulfonated as well. Variations on the thio-ethylamine side chain as found in thienamycin consist of N-acetylation in all other members of this group, oxidation in several to the N-acetyl-thio-vinylamine, and, in one of the olivanic acids, further oxidation of the sulfur to the sulfoxide.** Important stereochemical differences have been established among various members of the thienamycin and epithienamycin families; significantly, the substituents about the azetidinone ring of thienamycin have the *trans*-configuration, as opposed to the *cis*-configuration found among all natural penicillins and cephalosporins, and in several of the epithienamycins as well.

It is interesting to speculate on the factors that could account for the discovery at this late date in antibiotic research of such a constellation of new structures. The introduction of new screening procedures has been cited most frequently; as examples, a test strain hypersensitive to β-lactam antibiotics detected nocardicin, and an enzymatic screen to detect inhibitors of β-lactamase enabled the discovery both of clavulanic acid and the olivanic acids. The additional role of novel microbial cultures would appear to be particularly relevant in the case of thienamycin, for it is produced by the hitherto undescribed species *Streptomyces cattleya*. However, the basic biosynthetic steps involved in its formation cannot be unique to this organism, since the related families of olivanic acids and epithienamycins are produced by numbers of independent isolates of established species.

Emphasis on the detection *per se* of antibiotics should be balanced by the common experience that many more 'activities' are detected than are either regarded worthy of or found amenable to purification and characterization. In our experience, chemical instability was decisive in prolonging the purification and recognition of structure of thienamycin. The limited pH range tolerated by the antibiotic and its insignificant solubility in organic solvents obliged the use of inefficient purification procedures. Final steps of purification, made possible once it was recognized that the UV chromophore was truly associated with the antibiotic, yielded minute quantities of product. Subsequent structural studies depended in turn on the availability of newer techniques of high-resolution NMR and mass spectrometry. Experience gained in the isolation and structure analysis of thienamycin greatly facilitated the elucidation of the epithienamycins in these laboratories.

The potential lability of the β-lactam of thienamycin has received particular attention in this paper to provide guidance in the use of the antibiotic for *in vitro* studies. Neutral pH must be maintained by non-reactive buffers, and reaction with nucleophiles should be anticipated and where possible avoided; (for example, cysteine should not be used as a reductant in the antimicrobial susceptibility testing of anaerobes). Where stability may nevertheless be of concern, the measurement of specific (hydroxylamine-extinguishable) UV absorbance provides a convenient determination of intact β-lactam.

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* Prior to the initial disclosure of the structure of thienamycin[17], 2 laboratories independently reported the isolation of potent β-lactamase inhibitors from *Streptomyces* broths[18,19]. No structures were proposed at that time. These agents were subsequently established to be members of the olivanic acid series[15].

** N-Acetyl thienamycin[3], as well as the vinylogue, N-acetyl-dehydrothienamycin (J. S. KAHAN, unpublished results) have also been isolated from the *S. cattleya* fermentation.

The accelerated inactivation of thienamycin encountered in concentrated solutions of the antibiotic has so far not been controllable by the choice of buffering conditions. It must be conceded that this phenomenon is a significant determent to the use of thienamycin *per se* as a practical therapeutic. The goal of suppressing the inactivation reaction by chemical modification of thienamycin is under active pursuit in these laboratories.

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