# EPITHIENAMYCINS. II

## ISOLATION AND STRUCTURE ASSIGNMENT

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At least six distinct β-lactam antibiotics of the epithienamycin family are produced by a strain of *Streptomyces flavogriseus* MB 4638. Each of the six can be isolated in substantially pure form by column chromatography using Dowex 1, Amberlite XAD-2 and Biogel packings. The structures were established by comparison of the ultraviolet, proton magnetic resonance and mass spectral characteristics with those of thienamycin and its derivatives. All six compounds contain the carbapenem ring system which is also found in thienamycin. They differ from each other and from thienamycin by chemical modifications and/or stereoisomerism. Enzymatically deacetylated epithienamycin A has the properties of an isomer of thienamycin.

In the accompanying paper[1], we have described the fermentation conditions for production of a new family of β-lactam antibiotics, the epithienamycins (ETMs) and have listed the antibacterial activities of isolated components. We here describe the techniques used for isolation of the six major antibacterial products of the producing organism, *Streptomyces flavogriseus*. The experimental basis for the structural assignments are reported. We also describe the procedure for enzymatically deacetylating saturated epithienamycins, and present evidence that deacetylated epithienamycin A is an isomer of thienamycin.

## Materials and Methods

### Chemicals

Dowex-1 resins were obtained from the Dow Chemical Corp., AG-1, hydroxylapatite, DEAE-cellulose and Biogel were from BioRad Laboratories. Amberlite XAD-2 is a product of Rohm and Haas. Tris was from Sigma, and $^3$H-acetic anhydride was purchased at 400 mCi/mmole from New England Nuclear Corp. and diluted with nonradioactive acetic anhydride immediately before use. Morpholinopropane sulfonic acid (MOPS) was from Calbiochem. All other chemicals were reagent grade.

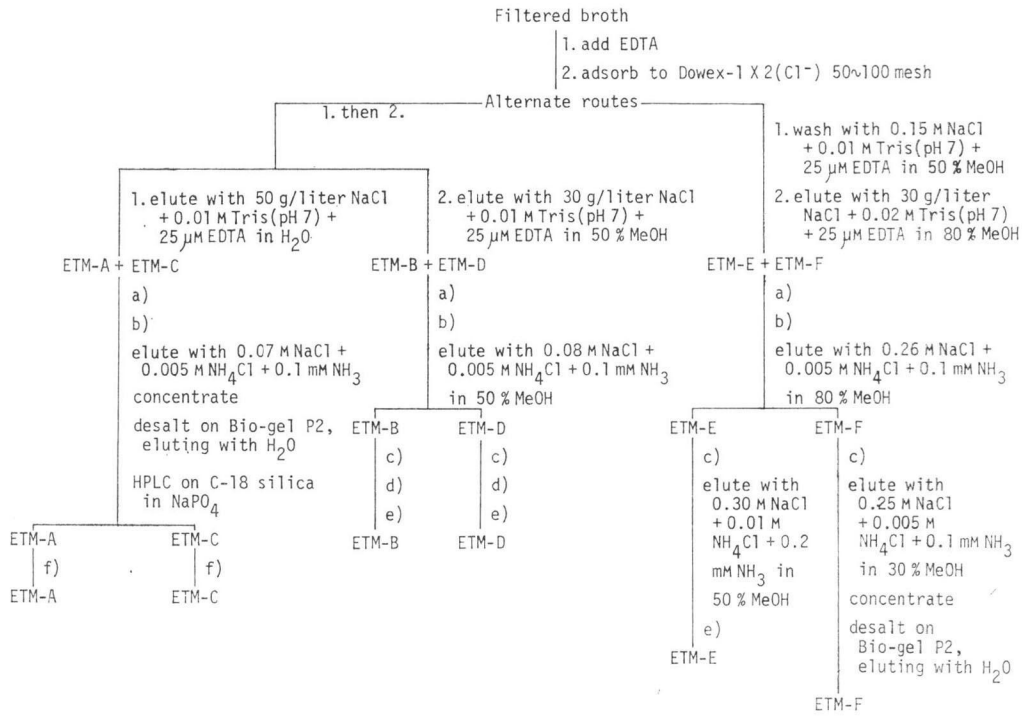
### Isolation

The general procedures for the isolation of the six major antibiotics of the MB 4638 broth in substantially pure form are depicted in Fig. 1. All operations, except the XAD-2 chromatography, were carried out in the cold. Deionized water was used for all elutions, and pH was adjusted to the range 6.5~7.5 as soon as practical after elution. Solutions in water were stored at 0°C over ice for short periods. Column fractions in 50% methanol were maintained at −20°C if they were to be stored for longer than a few hours. If fractions were to be stored for more than one week, methanol was added to bring the methanol concentration to >50% (if not already in methanol), and the pooled fractions were stored at −80°C.

The individual steps were varied somewhat for the isolation of particular components as below:

(1) Initial absorption to Dowex-1X2 ($Cl^-$): To approximately 9 volumes of clarified broth EDTA is added to 0.25 mM, and the broth is adsorbed to one volume of resin. Bed height is usually 20 cm

Fig. 1. Purification procedure for epithienamycins.[g]




a) concentrate, adjust pH to 6.5, apply to Amberlite XAD-2, elute with deionized water
b) adsorb to AG-1 X 4(Cl⁻) minus 400 mesh (40~50 cm length)
c) adsorb to AG-1 X 2(Cl⁻) minus 400 mesh (40~50 cm length)
d) elute with 0.07 M NaCl + 0.005 M $NH_4Cl$ + 0.1 mM $NH_3$ in 50 % 2-propanol
e) concentrate, add MeOH to 50 %, desalt on Bio-gel P2, elute with 50 % MeOH
f) HPLC on C-18 silica, elute with $H_2O$
g) All samples are lyophilized for storage after the final step.

for laboratory scale (1~15 liters) preparations. For isolation of ETMs A through D, elution is with 3 bed volumes of 5% NaCl, (to elute ETMs A and C) followed by 4 bed volumes of 3% NaCl in 50% (v/v) methanol (to elute B and D). The eluting solutions also contain 0.025 mM EDTA and 0.01 M tris, pH 7.0.

(2) Amberlite XAD Chromatography: The initial Dowex-1 eluate of each epithienamycin is concentrated under reduced pressure until a thick slurry of sodium chloride has precipitated. The liquid is filtered or pipetted away from the precipitate, and the pH adjusted to 6.5. The concentrate is then applied to an Amberlite XAD-2 column which had been previously washed with 5 volumes each of 60% (v/v) aqueous acetone, deionized water and 5% NaCl. The loading ratio used is 40 $A_{220}$ units of concentrate per ml of packed resin. Columns of 50 cm to 80 cm are employed, and the ETMs are eluted with deionized water. The volume of concentrate applied should be less than one-fourth of the volume of resin used. The column is eluted at room temperature taking fractions of 1/4 column volume each. The fractions are chilled in ice as they are eluted and are assayed by bioactivity and by hydroxylamine-extinguishability.

(3) AG-1×4 (minus 400 mesh) Chromatography: Efficient elution of ETMs B, D, E and F require some organic solvent in the eluent. For ETMs B and D, 50% aqueous methanol was normally employed. For E and F, both 80% and 50% methanol are used.

(4) Biogel P2 Desalting: For desalting of ETMs B, D and E on Biogel P2 (200~400 mesh), the columns are packed, washed and eluted with 0.02 mM ammonia in 50% (v/v) aqueous methanol. The

sample is applied in 50% methanolic solution. For desalting of ETMs A, C and F, packing and elution is in 0.02 mM ammonia in deionized water.

(5) Lyophilization: Desalted samples are concentrated and then shell-frozen and lyophilized for storage under vacuum in 14 ml screw-cap vials with split rubber stoppers (Virtis). Best results are obtained by lyophilization in a vacuum chamber containing temperature controlled shelves maintained below 0°C.

Other Chromatographic Methods

In addition to the general procedure outlined above, additional purification steps have been used for ETMs A, C and D.

ETMs A and C are not completely separated by the general procedure and may be resolved by high pressure liquid chromatography using Merck Lichrosorb RP 18 (10 micron) packing (ES Industries) by elution at room temperature with phosphate buffer in the pH range of 5~6.3. The phosphate may be removed by a subsequent pass over the same column, using water as eluent. Mixtures of up to 1.5 mg were resolved on a 9 mm×30 cm column.

For epithienamycin D, a final step of purification is accomplished by passage of a small volume of aqueous solution over a bed (>100 volumes) of AG-50×2 (200~400 mesh), sodium form. The epithienamycin is retarded slightly relative to NaCl and some residual UV-absorbing impurities, and a small additional purification may be thus accomplished.

Chemical Assay

As does thienamycin[2], epithienamycins react with hydroxylamine and produce a substance with greatly diminished absorbance at 308 nm. This provides the basis for a quantitative assay. The solution to be assayed is brought to 0.05 M in potassium phosphate, pH 7.4 by adding 0.05 volume of a solution containing 0.8 M $K_2HPO_4$ and 0.2 M $KH_2PO_4$. Then 0.01 volume of 1.0 M hydroxylamine hydrochloride is added. The absorbance at 308 nm (or 300 nm for saturated epithienamycin side chains) is measured over time. The final corrected absorbance decrease is taken as the hydroxylamine-extinguishable absorbance at 308 nm ($HAEA_{308}$). In crude preparations (HAEA <10% of total absorbance), corrections for background changes in UV absorbance are made by taking that portion of the UV absorbance decrease having an apparent first-order half life of 0.5~3.0 minutes.

E% values are calculated from the weights of samples after lyophilization of a known volume of known absorbance. Since most samples were of only 1~3 mg in total weight, these values are estimated to be accurate to only ±20% as judged by reproducibility.

Purity with respect to other organic materials was judged by the absence of extraneous peaks in the $^1$H-NMR spectrum, and with respect to inorganic contaminants by conductivity.

Proton Magnetic Resonance (PMR) Spectrometry

Sodium salts of the epithienamycins (0.5~2.5 mg) are dissolved in 0.4 ml of 99.9% $D_2O$ and spectra are taken on a Varian HA-100D or SC-300 spectrometer using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as internal standard. No buffers are added.

Mass Spectrometry (MS)

Ammonium salts of samples to be analysed (50~100 μg) are prepared by passing the sodium salts in $H_2O$ over an AG-50×2 ($NH_4^+$) column, followed by adjustment of pH to 7.5, concentration to 1 mg/ml and lyophilization. The samples are dissolved in 50% (v/v) bis-trimethylsilyl trifluoroacetamide (BSTFA) in dimethylformamide immediately before use.

Low resolution spectra are taken on an LKB-9000 mass spectrometer *via* the direct-probe inlet system at 70 e.v. ionization energy periodically, at increasing temperatures. High-resolution data for selected ions are obtained on a Varian MAT-731 instrument by the peak matching method.

Circular Dichroism

Circular dichroism spectra were taken on a Cary Model 60 spectropolarimeter. Solutions in water were adjusted to give an absorbance of 3 at the point of the ~300 nm absorbance maximum. Cells of 1 cm path length were used, and the specific ellipticity was calculated using concentrations estimated from the ultraviolet absorption.

### $^{3}$H-*N*-Acetylthienamycin

Lyophilized thienamycin powder, 117 mg, is added to 2 ml of dimethylformamide which has been dried over a molecular sieve. To this suspension 250 mCi of $^{3}$H-acetic anhydride (300 mg) are added and the mixture is stirred at 0°C for 3 minutes, at which time all solids have dissolved. The reaction is terminated by addition of 40 ml $H_2O$ and 1 ml of 1 M tris base, and this solution brought to pH 7.0 by addition of 1 M NaOH. After further dilution to 500 ml with water, the sample is applied to a column (2.15×42 cm) of AG-1-X4 ($Cl^-$, minus 400 mesh) and is eluted and desalted on Biogel P2 as described for epithienamycin A. Portions of the lyophilized product are dissolved in 0.05 M MOPS buffer, pH 7.6 and used within one week.

### Deacetylation Assay

Typically, assays for deacetylating activity are conducted in 6×25 mm tubes in which 25~75 $\mu$l of enzyme solution are added to 25 $\mu$l of a solution of 1 or 2 mg/ml $^{3}$H-*N*-acetylthienamycin in 0.05 M MOPS buffer, pH 7.6. After 2~18 hours at 28°C the mixture is diluted into 1.8 ml of 0.01 M Na-acetate, and 0.2 ml of a 50% (packed volume) slurry of activated charcoal are added. The suspensions are stirred several times during 5 minutes at 0°C, and then centrifuged. The radioactivity in 1 ml of supernatant is measured by liquid scintillation counting. Background values are equivalent to 0.2% of the acetylated thienamycin, and >98% of the released acetate remain in the supernatant. Conversion of 0.2% of substrate is unambiguously detectable. The rate of release of acetate is linear with substrate concentration up to 2 mg/ml. Linearity with respect to added enzyme is often poor at crude stages, but improves with purification. Sodium or potassium chloride, or divalent metal ions such as $Co^{++}$ may be slightly stimulatory (less than 2×), but are not employed in assays or preparative reactions. Activity increases with pH up to 8.5.

### Purification of Epithienamycin Deacetylase from Hog Kidneys

Hog kidney tissue apparently contains several enzymes capable of deacetylating epithienamycins. The purification of one such enzyme is outlined here.

Frozen hog kidneys are thawed at 5°C overnight, and the fat and membranous portions are cut away. To 200 g are added 200 ml of cold 0.05 M MOPS buffer, pH 7.6 and the tissue is homogenized in a blendor. Temperature is kept below 20°C. Additional MOPS buffer (400 ml) is added and the homogenate is centrifuged. The supernatant is fractionated by addition of solid ammonium sulfate, and that portion precipitating between 45% and 95% saturation (at 5°C) is resuspended in a minimum volume of MOPS buffer and dialysed overnight into MOPS. At this stage, 2 mg/ml of protein will deacetylate 0.4% of substrate in 2 hours at 28°C.

The sample is applied to a column (8×100 cm) of Sephadex G-100, and eluted with MOPS buffer at 3~5°C. Several peaks of activity are usually observed, and the lower molecular weight fractions with specific activity equal to or greater than the input value are pooled for further processing. The pooled fractions have a specific activity 1.5 to 2× that of the applied sample. The enzyme is precipitated by addition of ammonium sulfate to 90% saturation and the redissolved precipitate dialysed into 0.01 M tris-HCl buffer, pH 7.4. The sample is applied to a column (4.9×16 cm) of DEAE-cellulose, and the column is rinsed with 100 ml of 0.01 M tris pH 7.0, and eluted at 5°C with 2 liters of a linear gradient of 0~0.5 M NaCl in 0.01 M tris pH 7.0. A single peak of activity is observed between 1.2 and 1.4 liters of eluted volume, and fractions with specific activity equal to or greater than the input value are pooled. This pool is concentrated to a final volume of 1.3 ml in an Amicon pressure cell with a UM-10 membrane. The final concentration is 55 mg/ml. When incubated with an equal volume of 2 mg/ml $^{3}$H-*N*-acetylthienamycin in 0.05 M MOPS buffer, pH 7.6 for 4 hours, this fraction deacetylated 39% of the substrate at 28°C and 47% at 37°C. This fraction was used for the deacetylation of epithienamycin A described below.

An additional increase in specific activity may be obtained by passing the DEAE fraction enzyme in 0.01 M sodium phosphate, pH 6.5, through a column of hydroxylapatite. The deacetylating activity passes through while most of the protein is adsorbed.

### Desacetyl Epithienamycin A

In a typical preparation, 0.45 ml of concentrated deacetylase (55 mg/ml, above) were added to

2.5 mg of lyophilized epithienamycin A, followed by 10 $\mu$l of 1 M MOPS buffer, pH 7.7, and the reaction was kept at 28°C. Degradation of the lactam ring was monitored by diluting 5 $\mu$l aliquots to 0.5 ml periodically, and measuring the $HAEA_{300}$ value. After 3 hours, 52% of the original $HAEA_{300}$ had been lost, and the reaction was terminated by addition of 0.5 ml $H_2O$ and chilling to 0°C. This sample was applied directly to a column (0.66 cm × 7.5 cm) of AG-1 × 2 ($Cl^-$, 200 ~ 400 mesh), and eluted with deionized water at room temperature. The deacetylated epithienamycin was retarded slightly behind the protein. Fractions rich in $HAEA_{300}$ were pooled and titrated to pH 6.7 with 1.3 $\mu$l of 1 M NaOH. A total of 16 $HAEA_{300}$ units were recovered, equal to 60% of the total $HAEA_{300}$ applied to the column, and to 28% of the starting material. This sample was further purified by concentration to 1.5 ml and re-chromatography on an AG-1 column with bed dimensions 1.5 × 17 cm, as above. Fractions with little contamination by protein were pooled, concentrated, and lyophilized.

## Results

### Isolation

Each of the six major epithienamycins (ETMs) has been isolated free of the other components and from essentially all detectable organic impurities. The purity of the final products is estimated to be greater than 90% for components A, C and F. For components B, D and E the desalting on Biogel is incomplete, and 10 ~ 50% of the weight of the final product apparently consists of residual NaCl, as the products had only trace amounts of detectable organic impurities by NMR analysis.

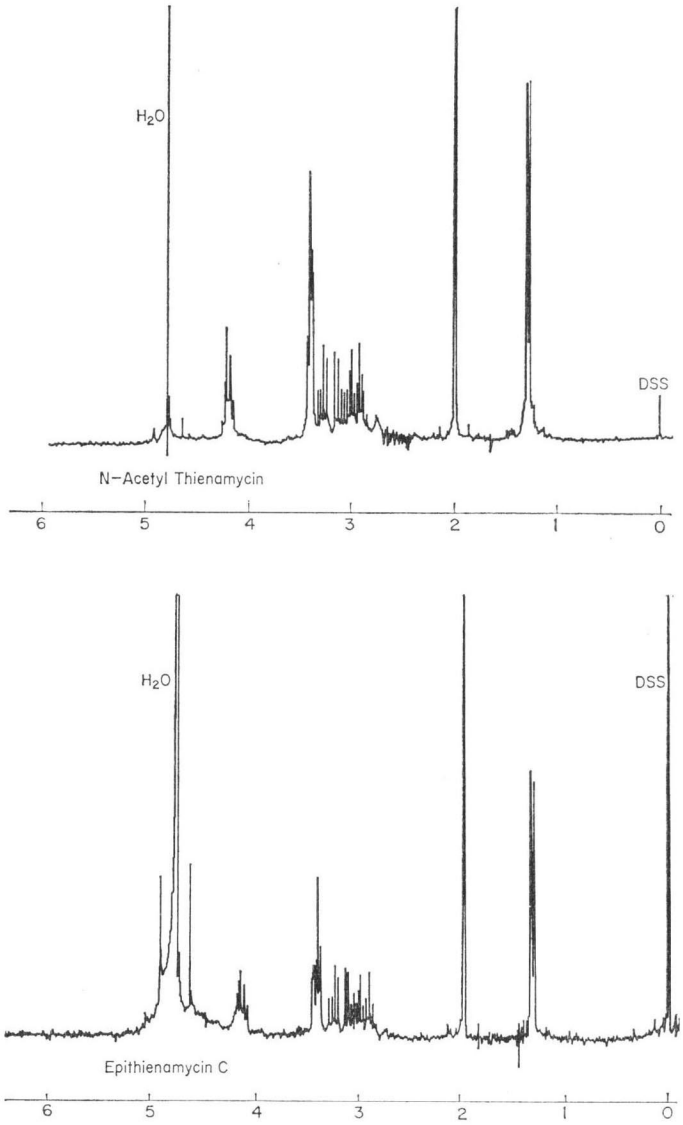
The estimated yield of the final products from crude broths varies with the product, being approximately 11% for component A and 3% for component B with the yields of other components being between these values. Exact yields could not be determined, as no accurate method was developed for measuring the quantities of each component in crude broth.

All six of the ETMs can be absorbed to and eluted from Dowex-1 ($Cl^-$) resin; however, the conditions for elution vary widely. Structural studies show that ETMs E and F have two negative charges at neutral pH, whereas A through D have one. This difference is reflected in their mobilities on Dowex-1, where E and F require both organic solvent (≥30% methanol) and high salt (≥0.25 M) for reasonably efficient elution. In addition, the unsaturated side chain in ETMs B, D and E cause a substantial retardation on Dowex resins relative to the saturated analogues, and all three require some organic solvent in the eluent. Thus components A and C are easily separated from the others by elution with salt in the absence of organic solvent; components B and D may then be eluted by salt in 50% methanol. The doubly charged components E and F require even higher proportions of organic solvent, or a longer elution time.

In the subsequent chromatography on AG-1 (minus 400 mesh), components B and D are separated from each other, and likewise components E and F are readily separated. Components A and C, however, are not separated on this ion exchange resin but can be completely separated by reverse phase HPLC (See Materials and Methods).

A variable partial separation of ETMs A and C is achieved during retardation chromatography on Amberlite XAD-2. Component C elutes somewhat later than A on XAD-2. The degree of separation is monitored by measuring the ratio of bioactivity to hydroxylamine-extinguishable absorption at 300 nm (or 308 nm for B and D). As this ratio is about ten-fold lower for C than for A, the two are easily distinguished. The ratio is likewise much lower for D than for B. In runs where separation at the XAD-2 stage provides less than 20% contamination of one component with the other, the two components are pooled separately and further purified as separate batches.

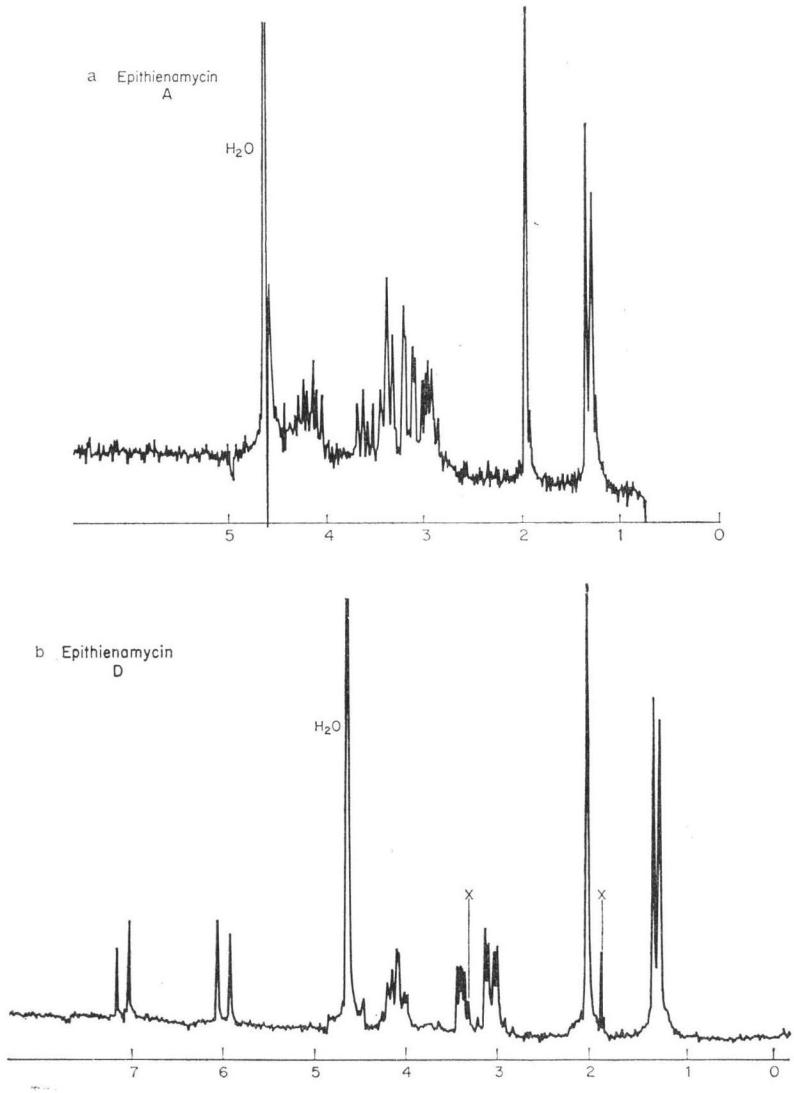
Fig. 2. 300 MHz $^1$H-NMR Spectra of *N*-acetylthienamycin and epithienamycin C. Scale in ppm.




Desalting on Biogel P2 is accomplished for ETMs A, C and F, by elution with water. A trace quantity (=0.02 mM) of ammonia is usually added to avoid an excessive drop in pH. However, in the small quantities employed in these experiments components B, D and E, tend to adsorb to the Biogel matrix, and columns for desalting of these compounds are packed in and eluted with 0.02 mM ammonia in 50% aqueous methanol.

One additional component has been observed in small quantities but not isolated in high purity and is designated epithienamycin G. This component is doubly charged by criterion of electrophoretic mobility and moves between ETMs E and F on AG-1 ($Cl^-$) columns. The difference spectrum after hydroxylamine extinction shows a peak at 300 nm, which distinguishes this material from the unsaturated side chain component E, ($\lambda$ max=308) and also from the sulfoxide MM 4550 observed by HOOD, *et al.*[3]

Fig. 3. 100 MHz $^1$H-NMR Spectra of epithienamycin A and epithienamycin D.
Scale in ppm. X=Impurity peak




to have a double maximum at 287 nm and 240 nm. By analogy with the existence of both *cis* and *trans* isomers of the non-sulfated components in broths of this organism, epithienamycin G is believed to be the 6-epimer (5, 6 *trans*) of epithienamycin F.

## Structure Identification

The assignment of structures to the ETMs was considerably simplified by their close resemblance to thienamycin (TM), whose structure and stereochemistry have been firmly established[2,4]. Comparisons of the ultraviolet (UV), circular dichroism (CD), proton magnetic resonance and mass spectra of the ETMs and TM derivatives have formed the basis for the following structural assignments.

### Epithienamycins A and C

$^1$H-NMR spectra of ETM-A and ETM-C closely resemble that of *N*-acetylthienamycin (*N*-Ac-TM),

Table 1a. $^1H$ NMR absorptions of epithienamycins.**

| | *N*-Acetyl-thienamycin | Epithienamycin A (890 $A_1$) | Epithienamycin B (890 $A_2$) | Epithienamycin C (890 $A_3$) | Epithienamycin D (890 $A_5$) | Epithienamycin E (890 $A_9$) | Epithienamycin F (890 $A_{10}$) |
|---|---|---|---|---|---|---|---|
| Configuration* at C5-C6-C8 | *R-S-R* | *R-R-S* | *R-R-S* | *R-S-S* | *R-S-S* | *R-R-S* | *R-R-S* |
| $CH_3$(9) d, $J$=6.5 Hz | 1.27 | 1.35 | 1.33 | 1.29 | 1.29 | 1.50 | 1.55 |
| $CH_3CO$ (s) | 1.98 | 1.98 | 2.06 | 1.98 | 2.05 | 2.04 | 2.02 |
| C(6)H d, d | 3.37 ($J$=3, 6) | 3.63 ($J$=5.2, 9.8) | 3.61 ($J$=5.0, 9.6) | 3.42 ($J$=2, 5) | 3.41 ($J$=3, 5) | 3.84 ($J$=5, ~10) | 3.89 ($J$=5.4, 9.2) |
| C(5)H, C(8)H | 3.96 (m) | 4.14 (m) | 4.19 (m) | 4.13 (m) | 4.16 (m) | 4.29 C(5)H ~4.85 C(8)H | 4.34 C(5)H ~4.83 C(8)H |
| $C(1)H_2$ | 3.17 (m) | 3.18 (m) | 3.08 (d, d; $J$=18.0, 9.8) | 3.13 (m) | 3.04 (d, d; $J$=17, 9) | 3.06 (d, d; $J$=18, 9) | 3.27 (m) |
| | | | 3.15 (d, d; $J$=18.0, 9.8) | | 3.12 (d, d; $J$=17, 9) | 3.32 (d, d; $J$=18, 9) | |
| $-CH_n-N$ | 3.39 (m) | 3.41 (m) | 7.13 (d, $J$=13.8) | 3.39 (m) | 7.11 ($J$=13.5) | 7.16 (d, $J$=13.8) | 3.43 (m) |
| $-CH_n-S$ | 2.93 (m) | 2.97 (m) | 6.03 (d, $J$=13.8) | 2.92 (m) | 6.00 ($J$=13.5) | 6.07 (d, $J$=13.8) | 3.03 (m) |

* See text.

** Spectra were recorded at 100 or 300 MHz in $D_2O$. Chemical shifts are given in ppm relative to internal DSS, coupling constants are given in Hertz.

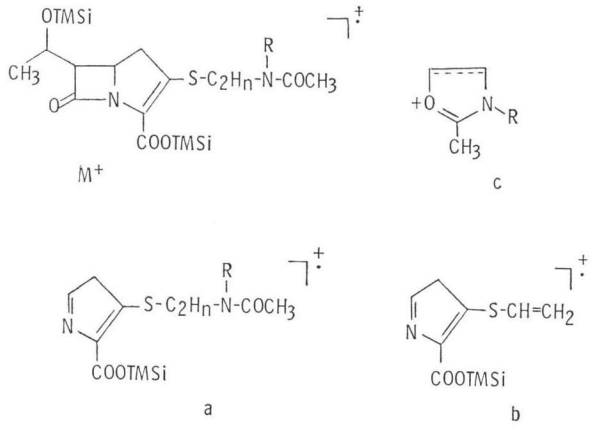
Table 1b. Trimethylsilyl-epithienamycins. Mass spectral data.

| | | ETM-A | ETM-B | ETM-C | ETM-D | ETM-E | ETM-F |
|---|---|---|---|---|---|---|---|
| n | | 4 | 2 | 4 | 2 | 2 | 4 |
| $M^+$ | R=TMSi | 530 | 528 | 530 | 528 | | |
| | R=H | 458.1742 | 456 | 458 | 456 | | |
| $(M-CH_3)^+$ | R=TMSi | 515.1931 | 513 | 515 | 513 | | |
| | R=H | 443.1495 | 441 | 443 | 441 | | |
| $M-TMSiHSO_4$ | | | | | | 366 | 368 |
| a | R=TMSi | 373.1422 | 371 | 373 | 371 | | |
| | R=H | 301.1034 | 299 | 301 | 299 | 299 | 301 |
| b | | 241.0590 | — | 241 | — | | 241 |
| c | R=TMSi | 158.1000 | 156 | 158 | 156 | 156 | 158 |
| | R=H | 86.0610 | 84 | 86 | 84 | 84 | 86 |
| $(TMSi)_2SO_4-CH_3$ | | | | | | 227.0224 | 227 |

with which they are isomeric as determined by MS analysis of their trimethylsilyl derivatives (Table 1). Indeed, $^1H$-NMR spectra of ETM-C and *N*-Ac-TM (Fig. 2) would be virtually indistinguishable if peak splitting were not observed in the spectrum of a mixture of the two compounds. Further evidence for close structural similarities among ETM-A, ETM-C and *N*-Ac-TM is provided by their UV and CD spectra. The similarities of their antibacterial profiles have been previously noted[1].

The spectra of ETM-A and ETM-C are interpreted in terms of stereoisomers of *N*-Ac-TM. Fig. 4 and Table 1b show the interpretation of all major mass spectral fragments which account for all por-

Fig. 4. Characteristic mass spectral fragments of trimethylsilyl-epithienamycins.*




* Compare Table 1b.

tions of the molecule. $^1$N-NMR spectra of ETM-C and *N*-Ac-TM show small vicinal coupling constants $J_{5,6} \leq 3$ Hz which, as has been shown previously for thienamycin, require *trans* orientation of the $\beta$-lactam hydrogens in both compounds. Since the two compounds are different, they must be stereoisomers at C8, with *N*-Ac-TM having the previously reported *R*-configuration and ETM-C the *S*-configuration. By comparison ETM-A, showing the larger $J_{5,6}=5$ Hz but retaining the positive CD maximum near 295 nm, has been assigned the *R*-configuration at both C5 and C6. The configuration at C8 could not be determined by $^1$H-NMR spectra alone, but was inferred to be *S* due to non-identity of the $^1$H-NMR spectra of desacetylepithienamycin A and the 5-(*R*), 6-(*R*), 8-(*R*) isomer of thienamycin prepared by total synthesis[11].

Epithienamycins B and D

The $^1$H-NMR spectra of ETM-B and ETM-D (Table 1a) show no absorption for the methyl ne groups of an *N*-acetylcysteamine substituent. Instead, two one-proton doublets of a 1,2-*trans* di ib-stituted ethylene group are observed. These data and the mass spectral fragments shown in Fig. 4 (note the characteristic absence of fragments corresponding to b of $m/z=239$) suggest that B and D are the unsaturated analogues of A and C. Analysis of the C6-$^1$H-NMR absorption reveals $J_{5,6}=3$ Hz for ETM-D and $J_{5,6}=5.0$ Hz for ETM-B, demonstrating *trans* and *cis* $\beta$-lactam configurations, respectively. Positive circular dichroism peaks at $\lambda \sim 300$ nm indicate the absolute (*R*) configuration at C5. No direct information about configuration at C8 is available. However, it is assumed that B and D possess the same 8-(*S*) configuration as do A and C, since all are produced in the same fermentation. This presumption is supported by recent data from another laboratory[7], indicating that the saturated compounds are biosynthetic precursors of the unsaturated analogues.

Epithienamycins E and F

The ultraviolet-spectra, and the mass spectral and $^1$H-NMR data given in Table 1 clearly classify these components as epithienamycins with *cis*-$\beta$-lactam configuration. ETM-F contains the saturated and ETM-E the unsaturated *N*-acetylcysteamine substituent. Electrophoretic and column chromatographic mobilities (see Materials and Methods) indicate two negative charges in both components. $^1$H-NMR and mass data lead to the conclusion that these compounds are epithienamycins with sulfate esters

at C8. Compared to ETM-A, the $^1$H-NMR spectrum of ETM-F shows several signals shifted to lower field. The largest change, of $\delta \sim 0.7$, is observed for the C8 proton. Smaller chemical shift changes of 0.2 are observed for the methyl and C6 protons. Mass spectra of trimethylsilyl derivatives show abundant M-minus-methyl ions for $(TMSi)_2SO_4$ (*m/z* found: 227.0224; calc. 227.0230), which must be attributed to thermal elimination of trimethylsilyl-sulfate from the molecules.* In samples of increasing purity, a weaker phosphate signal (due to carry over from broth) diminishes to insignificance. With further heating the mass spectrum of the TMSi-derivative of an anhydro-epithienamycin is obtained which shows all significant fragments comprising the pyrolidine ring and the carboxy- and *N*-acetylcysteamine substituents. Pseudo-molecular ions of *m/z* 368 therefore indicate that sulfate elimination has occurred from the substituent at C6. Analogous data have been obtained and are tabulated for ETM-E, containing the unsaturated *N*-acetylcysteamine substituent. No data on absolute or relative stereochemistry were obtained for ETM-E and ETM-F, other than the coupling constants, $J_{5,6}$, for the $\beta$-lactam protons. Because ETMs E and F are believed to be derived from the non sulfated ETMs[7], it is assumed that they share the 5-(*R*) and 8-(*S*) configurations of ETMs A and B.

Desacetylepithienamycin A

To confirm that the product of the enzymatic deacetylation of epithienamycin A was indeed the expected isomer of thienamycin, ultraviolet and $^1$H-NMR spectra were measured. Desacetylepithienamycin A has a $\lambda$ max at 296 nm, and a minimum at 240 nm, with a $A_{296}/A_{240}$ ratio of 3.7 at pH 6.5. These values are similar to those of thienamycin. The $^1$H-NMR spectrum of the deacetylatedepithienamycin A (Table 2) is closely similar, but not identical, to the 6-(*R*), 8-(*R*) isomer of thienamycin prepared by total synthesis[11]. The only structural change caused by the deacetylation reaction therefore appears to be the loss of the *N*-acetyl group.

Table 2. Chemical shifts and coupling constants for deacetylated epithienamycin A.*

| $\delta$ (ppm) | $J$ (Hz) | Assignment |
|---|---|---|
| 1.34 (3H, d) | 6.4 | $CH_3CH$ |
| 3.1~3.2 (4H, m) | | $-CH_2CH_2-$ |
| 3.12 (1H, d, d) | 17.3, 9.2 | $CH_2CH$ |
| 3.27 (1H, d, d) | 17.3, 9.2 | |
| 3.67 (1H, d, d) | 9.5, 5.3 | $H_6$ |
| 4.18 (1H, d, q) | 9.5, 6.4 | $H_3$ |
| 4.33 (1H, d, t) | 9.5, 9.5, 5.5 | $H_5$ |

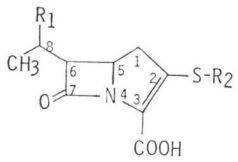
* 300 MHz spectrum, DSS as internal standard.

## Discussion

Three structural variations account for the six identified epithienamycins: a saturated or unsaturated *N*-acetylcysteamine substituent; the presence or absence of a sulfate esterified to the C8 hydroxyl; and a *cis* or *trans* $\beta$-lactam configuration. Table 3 summarizes the stereochemical assignments established or (in parentheses) inferred. Antibacterial potency and $\beta$-lactamase resistance are taken from reference 1. From Table 3 it can be seen that the 6-(*S*) epithienamycins (*trans* $\beta$-lactams) have overall potencies which are substantially lower than those of the 6-(*R*) epithienamycins (*cis* $\beta$-lactams). Nevertheless *N*-acetylthienamycin, which has the 6-(*S*) but 8-(*R*) configurations is as potent as the most potent epithienamycin. Thus the configurations at both C6 and C8 have a marked influence on antibacterial potency. Likewise, both steric centers are important in determining the susceptibility of the antibiotics to penicillinase (from *B. cereus*). Of the four unsulfated epithienamycins, the two with *trans* lactam rings have substantially lower susceptibility than those with a *cis*-lactam; but the susceptibility of the 8-(*R*) *N*-acetylthienamycin is even lower than that of the *trans* epithienamycins. In contrast to these differences, the epithienamy-

* Thermal elimination of a TMSi-sulfate or a di-TMSi-phosphate in the presence of residual silylating reagent will in our experience result in the formation of di-TMSi-sulfate or tri-TMSi-phosphate.

Table 3. Properties of the epithienamycins.



| Antibiotic | $R_1$ | $R_2$ | Absolute configuration $C_5$ | $C_6$ | $C_8$ | β-Lactam configuration | Relative potency | Penicillinase resistance | $\lambda_{max}$ | E % |
|---|---|---|---|---|---|---|---|---|---|---|
| Thienamycin | -OH | $-CH_2CH_2NH_2$ | *R* | *S* | *R* | *trans* | 100 | High | 297 | 272 |
| N-Acetylthienamycin | -OH | $-CH_2CH_2NHCOCH_3$ | *R* | *S* | *R* | *trans* | 53 | High | 301 | 270 |
| Epithienamycin A | -OH | $-CH_2CH_2NHCOCH_3$ | *R* | *R* | *S* | *cis* | 29 | Low | 299+ | 230 |
| Epithienamycin B | -OH | $-CH{=}CHNHCOCH_3$ | *R* | *R* | (*S*) | *cis* | 63 | Low | 308‡ | 500 |
| Epithienamycin C | -OH | $-CH_2CH_2NHCOCH_3$ | *R* | *S* | *S* | *trans* | 3.4 | Moderate | 300+ | — |
| Epithienamycin D | -OH | $-CH{=}CHNHCOCH_3$ | *R* | *S* | (*S*) | *trans* | 2.3 | Moderate | 308‡ | 490 |
| Epithienamycin E | $-OSO_3H$ | $-CH{=}CHNHCOCH_3$ | (*R*) | *R* | (*S*) | *cis* | 34 | Moderate* | 308 | 370 |
| Epithienamycin F | $-OSO_3H$ | $-CH_2CH_2NHCOCH_3$ | (*R*) | *R* | (*S*) | *cis* | 29 | Moderate* | 300 | 210 |

* The tight binding of sulfated epithienamycins to *B. cereus* penicillinase may be responsible for the apparent sensitivity of these compounds.

\+ Minimum at 242 nm. The purest samples have $A_{300}/A_{242}$ ratios of 4.2~4.3.

‡ Second maximum at 228 nm, minimum at 262 nm. The purest samples have ratios $A_{308}/A_{262} \cong 2.1$ and $A_{308}/A_{228} \cong 1.14$.

cins which are unsaturated in the *N*-acetylcysteamine side chain have biochemical properties which are little changed from those of their saturated analogues.

The most marked difference between sulfated and unsulfated epithienamycins appears in the profoundly stronger penicillinase inhibitory capacity of the sulfated compounds. The sulfated compounds inhibit *B. cereus* β-lactamase at 0.001 μg/ml, one thousand times lower than the concentration at which the unsulfated compounds inhibit (G. DARLAND, unpublished results). This property of the sulfated epithienamycins has apparently led to their independent discovery, under the name of olivanic acids[3,5]. A 300-MHz $^1$H-NMR spectrum of a mixture of ETM-E and a sample of MM13902 (obtained from Beecham) was indistinguishable from the spectra of either of the two individual compounds, providing strong evidence that they are identical. A mixed spectrum has not been performed for ETM-F and MM17880, but comparison of the properties measured independently, as well as their occurrence together with ETM-E, suggests that these two compounds are also identical.

## Conclusion

In the several years since it was first reported that an antibiotic with a carbapenem ring system (thienamycin) is made by a *Streptomyces* species[10], other carbapenems have been discovered and their properties described. Although our first recognition of epithienamycins as novel antibiotics predated the discovery of thienamycin, the final elucidation of epithienamycin structures had to await the extensive studies on thienamycin which firmly established the structure of that related compound[4]. The subsequent comparison of the physical properties of epithienamycins with those of thienamycins allowed the assignment of structures for all six of the epithienamycins which have been isolated in substantial purity.

Since the preliminary report of this work appeared[8,9], other workers have reported compounds similar to ETMs A, B, C and D[7]. Based upon their reported structures and their occurrence together in the same fermentation broth with MM 13902, which we have shown to be identical to ETM-E, we believe that MM 22380, MM 22382, MM 22381 and MM 22383 are identical to ETMs A, B, C and D respectively. The relative antibacterial potencies of the olivanic acids[12] are also consistant with

this identification. Our conclusions regarding the correspondence of the Merck and Beecham products are set out in Table 4[4].

The epithienamycin family of antibiotics present an interesting series of naturally occurring structures related to thienamycin. The major differences observed in intrinsic potency, penicillinase resistance and penicillinase inhibitory activity, all associated with structural variations in the substituent at the 6-position, suggest a fertile area for further exploration by chemical modification or synthesis.

Table 4. Probable correspondence of epithienamycins and olivanic acids.

| Merck product | Beecham product |
|---|---|
| Epithienamycin A (890 $A_1$) | MM 22380 |
| Epithienamycin B (890 $A_2$) | MM 22382 |
| Epithienamycin C (890 $A_3$) | MM 22381 |
| Epithienamycin D (890 $A_5$) | MM 22383 |
| Epithienamycin E (890 $A_9$)* | MM 13902 |
| Epithienamycin F (890 $A_{10}$) | MM 17880 |

* Established by mixed PMR spectrum.

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