NORTHIENAMYCIN AND 8-*EPI*-THIENAMYCIN, NEW CARBAPENEMS FROM *STREPTOMYCES CATTLEYA*

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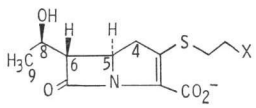
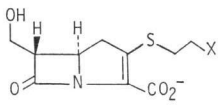
Two new carbapenem antibiotics, northienamycin and 8-*epi*-thienamycin have been isolated from culture broth of *Streptomyces cattleya* grown under conditions for thienamycin production. The isolation, structure elucidation and *in vitro* antibacterial spectra of the new carbapenems are reported. In addition, comparison of the *in vitro* potency of the corresponding formamidine derivatives to that of MK787 is presented.

The discovery of thienamycin (**1**) in fermentation broth of *Streptomyces cattleya* in 1976 introduced a new class of naturally occurring β-lactam antibiotics, the carbapenems.[1] Co-produced with thienamycin are *N*-acetylthienamycin[2] and *N*-acetyldehydrothienamycin.[3] Since 1976, the carbapenem family has grown to include approximately 34 structurally defined members.[4~21]

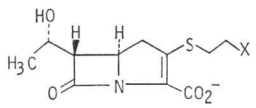
During subsequent development work on the isolation of thienamycin, two new carbapenems, 9-northienamycin (**2**, northienamycin) and 8-*epi*-thienamycin (**3**) were detected. This paper describes the isolation, structural elucidation, and antibacterial spectra of these two compounds. In addition, a comparison of the antibacterial potency of the formamidine derivatives of **2** and **3** relative to that of *N*-formimidoylthienamycin (MK787) is presented.



1 (Thienamycin) X: NH_3^+ (5*R*,6*S*,8*R*)
4 (MK-787) X: NH–CH=NH_2^+



2 (Northienamycin) X: NH_3^+
5 X: NH–CH=NH_2^+



3 (8-*epi*-Thienamycin) X: NH_3^+
6 X: NH–CH=NH_2^+

Materials and Methods

Chemicals

Thienamycin and *N*-formimidoylthienamycin (MK787) are products of Merck Sharp & Dohme Research Laboratories[22,23]. The latter was prepared from thienamycin by the reported procedure[23]. Chromatographic media were obtained from the following sources: Dowex AG 1X2, Dowex 50WX2 and Aminex A-5 from Bio-Rad Laboratories, Richmond, CA; Amberlite XAD-2 from Rohm and Haas Co., Philadelphia, and Sephadex G-75 from Pharmacia Fine Chemicals, Piscataway, NJ; Amberlite XAD-2, -400 mesh was prepared by milling commercial resin and sieving. Reference antibiotics were cephalothin (Keflin; Eli Lilly & Co.) and carbenicillin disodium (Pyopen, Beecham-Massengill). All other chemicals were of reagent grade.

Fermentation

Production of thienamycin by fermentation of *S. cattleya* was by reported procedures.[22]

Antibacterial Assays

Antibacterial activity of partially purified samples was determined by agar disc diffusion assay against *Staphylococcus aureus* ATCC 6538P using thienamycin or MK787 as standard. Disc diffusion assay results reported in Table 3 employed the KIRBY-BAUER method[24] modified only by the use of an agar thickness of 0.2 cm. For the determination of MICs, broth dilution studies were conducted by the microtiter technique. Volumes of 1.5 μl of culture containing 10^5 colony-forming units (CFU) were transferred by use of a Dynatech MIC-2000 inoculator to microtiter wells containing 0.1 ml of a twofold serial dilution of the antibiotics in Mueller-Hinton broth. The lowest concentration showing an absence of visible turbidity or sediment after 20 hours of incubation at 35°C was designated the MIC. Organisms listed in Tables 3 and 4 include both isolates of typical antibiotic sensitivity and isolates selected for their resistance to β-lactam antibiotics currently in use.

UV Assay

Total carbapenem concentration in solution was determined by a difference spectrophotometric assay based on the reaction of the carbapenem chromophore (λ_{max} 297~300 nm) with hydroxylamine at pH 7 to form products exhibiting marginal absorbance at λ_{max}[22]. Since compounds **1**~**6** all possess the same carbapenem chromophore and have similar λ_{max} values, it was assumed that all compounds have the same molar extinction coefficient (ε) at λ_{max}. Crystalline MK787 monohydrate, λ_{max} 298 nm (ε 9,670, 99% NH_2OH extinction), was selected as standard. Based on this standard, pure carbapenems have the following $E^{1\%}_{1cm}$, λ_{max} values: **1** (355), **2** (374), **3** (355), **4** (323), **5** (338), **6** (323). The thienamycin sample assayed 85% pure.

HPLC Assay

HPLC assays were carried out using a Waters Assoc. Model 6000A pump and a Waters Assoc. Model U6K injector. Column effluent was monitored at 297~300 nm with an LDC SpectroMonitor II Model 1202 UV detector equipped with a flow cell of 10 mm pathlength and a Honeywell Model Electronik 195 recorder. A Spectra Physics Autolab System I Computing Integrator quantitated detector output. All carbapenem pairs were satisfactorily resolved at 30°C using a Waters 10 μm C_{18} μ-Bondapak column (4 mm ID×300 mm) in 0.01 M potassium phosphate pH 7.0. At a flow rate of 1.2 ml/minute, the retention times (seconds) of the antibiotics are as follows: **2** (283), **5** (350), **1** (435), **3** (465), **4** (562), **6** (615). Column performance deteriorated significantly with age. The retention times of all components decreased markedly although the relative order of elution did not change. Analysis of **1**/**3** mixtures was also performed using an Aminex A-5 column (4.6 mm ID×240 mm). The column was operated at ambient temperature in 0.01 M sodium phosphate pH 7.0 containing 25 μM EDTA. The flow rate was 0.2 ml/minute. Retention times of **1** and **3** were 2,180 seconds and 2,510 seconds respectively.

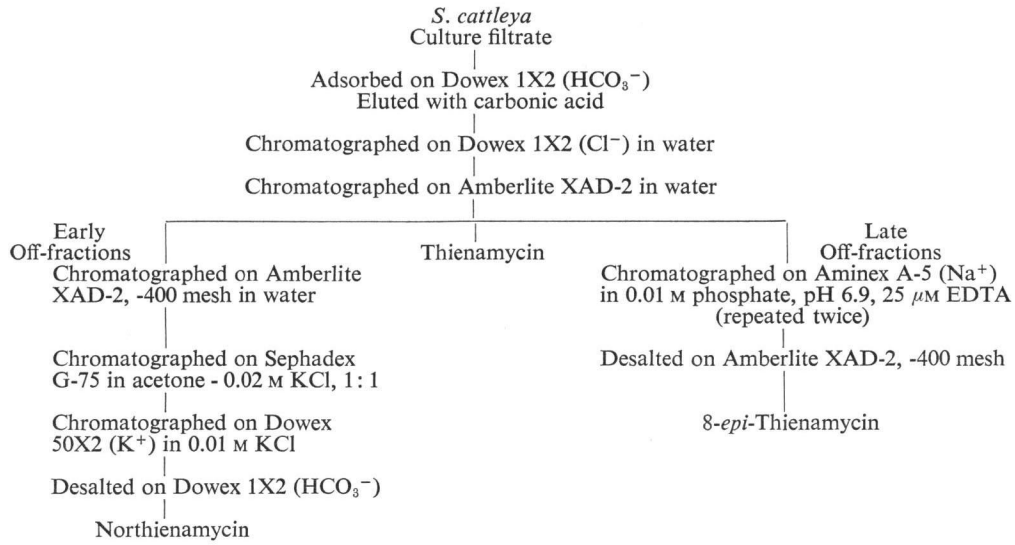
Analytical Instrumentation

NMR spectra were recorded on a Varian SC-300 spectrometer at 6°C in 0.02 M sodium phosphate pD 7.8. Low resolution MS data were collected at 70 eV on an LKB Model 9000 spectrometer. High resolution MS data were measured at 100 eV using a Varian MAT 731 spectrometer. IR, UV, and CD spectra were determined on the following respective instruments: Nicolet Model 7199 FTIR spectrometer, Beckman Model 5260 spectrophotometer, and Jasco Model J-41A spectropolarimeter.

Results

Northienamycin and 8-*epi*-thienamycin were isolated from *S. cattleya* fermented to produce thienamycin. Fermentation was carried out in complex medium as described previously.[22] The isolation process is summarized in Fig. 1. The first three steps are common with thienamycin isolation and have been reported in detail elsewhere.[25] Broth was first adsorbed on Dowex 1X2 (bicarbonate), 50~100 mesh. Crude thienamycin complex was eluted from the resin with carbonic acid and chromatographed in water on Dowex 1X2 (Cl^-), 50~100 mesh. To this point compounds **2** and **3** co-chromato-

Fig. 1. Isolation of northienamycin and 8-*epi*-thienamycin.



graphed with thienamycin. HPLC analyses later showed that the relative amounts of **1**, **2** and **3** are 90: 5: 5. Subsequent chromatography on Amberlite XAD-2, 20~50 mesh afforded essentially pure thienamycin. In addition, an early thienamycin off-fraction was found by agar disc diffusion assay to have more antibacterial activity than could be accounted for by the HPLC assay of thienamycin content. The new antibiotic component has been isolated and shown to be northienamycin. In a similar way, a late thienamycin off-fraction from the Amberlite XAD-2 step was analyzed by HPLC using an Aminex A-5 column. An effluent fraction was collected that exhibited less antibacterial activity than expected from the UV assay of the fraction for thienamycin. This result led ultimately to the isolation of 8-*epi*-thienamycin whose antibacterial potency is considerably less than that of thienamycin. Because of the pronounced instability of **2** and **3** in solution, successive isolation steps were executed with minimum delay.

The formamidine derivatives of northienamycin and 8-*epi*-thienamycin were most conveniently prepared by *N*-formimidoylation of thienamycin and isolation of **5** and **6** as minor constituents of the reaction product.

Isolation of Northienamycin

A sample of early thienamycin off-fractions from the Amberlite XAD-2 chromatography (Fig. 1) contained 48 mg of northienamycin and 520 mg of thienamycin. The sample, adjusted to pH 7, was chromatographed in two equal portions on 230 ml of Amberlite XAD-2, -400 mesh in water to remove thienamycin. The northienamycin rich cut was lyophilized to 340 mg of 10% pure antibiotic. The solid was reconstituted in 30 ml of acetone - 0.02 M aqueous KCl (pH 7) 1: 1, and chromatographed at 5°C in the same solvent on 400 ml of Sephadex G-75. Northienamycin concentration in column effluent peaked at 2.6 column volumes. About half of the eluted northienamycin was excluded from the rich cut because of impurities indicated by HPLC assay at 300 nm and 220 nm. The rich cut, containing 11 mg of **2**, was further purified by chromatography at 5°C on 200 ml of Dowex 50WX2 (K^+), 200~400 mesh in 0.01 M KCl. Appropriate fractions were pooled and adsorbed directly onto 40 ml of Dowex 1X2 (bicarbonate), 200~400 mesh at 5°C. After washing the column with water, the antibiotic was

eluted with cold carbonic acid. Lyophilization of active fractions afforded 3.8 mg of **2** as a white solid, judged to be 60% pure by UV assay.

Isolation of 8-*epi*-Thienamycin

A 180 mg sample of lyophilized late thienamycin off-fractions from the Amberlite XAD-2 chromatography (Fig. 1) contained 2.3 mg of 8-*epi*-thienamycin and 48 mg of thienamycin. The sample was chromatographed in twelve 15-mg portions on 8 ml of Aminex A-5 (Na^+). The eluting solvent was 0.01 M sodium phosphate pH 6.9 containing 25 μM EDTA and the flow rate was 0.4 ml/minute. The elution peak of **1** occurs at 1.8 column volumes and that of **3** at 2.1 column volumes. The combined rich cut from the twelve runs was rechromatographed in four portions on the same column and under the same conditions. A final third Aminex A-5 chromatography gave 0.82 mg of **3** containing less than 1% thienamycin. The product was concentrated to 1 ml and desalted by chromatography in water on 20 ml of Amberlite XAD-2, -400 mesh. Lyophilization gave 1.43 mg of **3** as a white solid, judged to be 44% pure by UV assay.

Isolation of *N*-Formimidoylnorthienamycin (**5**) and *N*-Formimidoyl-8-*epi*-thienamycin (**6**)

Thienamycin isolated by the process in Fig. 1 typically contains 2~5% each of northienamycin (**2**) and 8-*epi*-thienamycin (**3**). Conversion of 32 g of thienamycin to MK787 followed by Dowex 50WX2 chromatography[23] provided an MK787 tail fraction enriched in the corresponding formamidine derivatives of **2** and **3**. The fraction assayed 57 mg of **5**, 66 mg of **6**, and 500 mg of MK787. When chromatographed in water on 250 ml of Amberlite XAD-2, -400 mesh, the northienamycin derivative eluted at 1.4 column volumes (Rich Cut 1) while MK787 and **6** co-eluted at 2 column volumes (Rich Cut 2).

N-Formimidoylnorthienamycin (**5**)

An aliquot of Rich Cut 1 containing 27 mg of **5** was further purified by chromatography on 80 ml of Dowex 50WX2 (Na^+), 200~400 mesh. The eluant was 0.005 M sodium phosphate pH 7.6. After subsequent desalting on 8 ml of the same resin in water, the antibiotic was chromatographed in water on 20 ml of Amberlite XAD-2, -400 mesh. Freeze-drying afforded 17 mg of **5** as a white solid, estimated to be 64% pure by UV assay.

N-Formimidoyl-8-*epi*-thienamycin (**6**)

Rich Cut 2 was chromatographed three successive times on 300 ml of Dowex 50WX2 (K^+), 200~400 mesh. Each time the sample was charged in 0.1 M potassium phosphate pH 8.2 and eluted with 0.005 M KCl containing 25 μM EDTA, pH 7.2. Desalting of the final rich cut on Amberlite XAD-2, -400 mesh and subsequent lyophilization gave 11 mg of **6** as a white solid, 66% pure. The final sample was contaminated with 7% MK787. For antibacterial testing, the MK787 content was reduced to less than 1% by purification on Aminex A-5 in 0.01 M sodium phosphate pH 7.4.

Structure of Northienamycin and 8-*epi*-Thienamycin

It was suspected early in the isolation that **2** and **3** were closely related to thienamycin. The UV spectra of all three compounds exhibit single absorbance maxima at 297 nm, which are destroyed upon treatment with hydroxylamine at pH 7. Both **2** and **3** are unstable in solution and are not retained at pH 7 on Dowex 1X2 (Cl^-) resin, properties shared by thienamycin.[22] The antibacterial profiles are also similar to thienamycin, particularly with respect to anti-Pseudomonas activity (*vida infra*).

Purified **2** shows strong IR absorption at 1762 cm^{-1} and 1610 cm^{-1}, consistent with a β-lactam carbonyl and a carboxylate anion (Table 1). The molecular formula of **2** was established as $C_{10}H_{14}N_2$-

Table 1. Physicochemical properties of thienamycin (**1**), northienamycin (**2**), and 8-*epi*-thienamycin (**3**).

	1	**2**	**3**
Molecular formula	$C_{11}H_{16}N_2O_4S$ MW 272	$C_{10}H_{14}N_2O_4S$ MW 258	$C_{11}H_{16}N_2O_4S$ MW 272
UV (0.1 M phosphate pH 7) λ_{max} nm	297	297	297
CD (0.1 M phosphate pH 7) λ_{max} nm $[\theta]^{23}$	288.5 +23,900	290 +22,400	289 +23,200
IR (KBr) cm^{-1}	1764, 1580, 1389	1762, 1610, 1400	1758, 1590, 1396

Fig. 2. 300 MHz ^1H NMR spectrum of northienamycin.

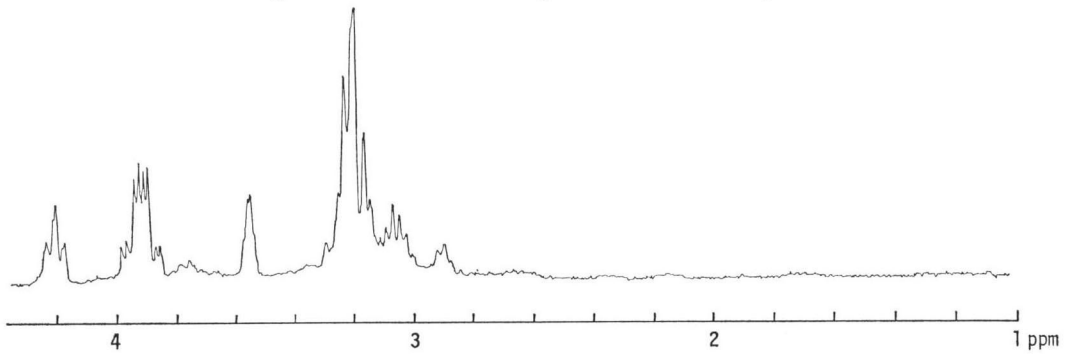
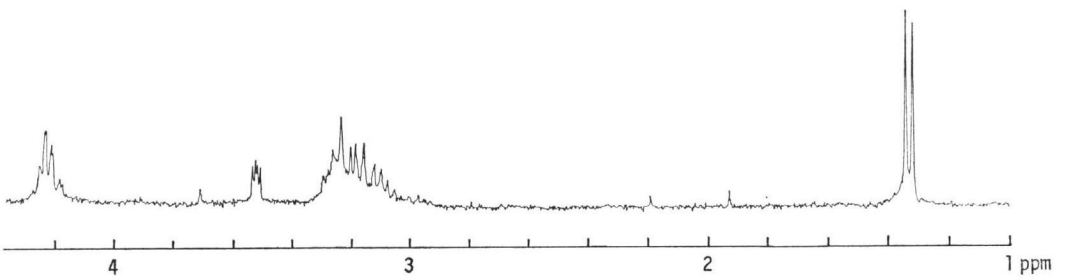


Fig. 3. 300 MHz ^1H NMR spectrum of 8-*epi*-thienamycin.



O_4S (MW 258) from high resolution MS studies of the tris-(trimethylsilyl) derivative (M^+ 474.1845, calcd 474.1860 for $C_{19}H_{38}N_2O_4SSi_3$). Thus, **2** has one carbon and two hydrogens less than thienamycin. The ^1H NMR spectrum of **2** (Fig. 2, Table 2) shows resonances corresponding to those of CH(5), CH(6), CH_2(4), and $-SCH_2CH_2N-$ of thienamycin. However, the spectrum of **2** lacks the CH_3(9) doublet of thienamycin at δ 1.27 and has an additional CH_2-X signal, appearing as an ABX pattern (J=12.0, 5.0, 4.5 Hz), centered at δ 3.92 Double irradiation at the center of the δ 3.56 methine multiplet causes collapse of the δ 3.92 methylene signal to an AB quartet (J=12.0 Hz) and of the δ 4.22 methine (dt, J= ~2.5, 8.5 Hz) to a triplet (J=8.5 Hz). This requires the sequence CH_2(X)–CH–CH(Y) of carbon atoms bearing protons in **2** where X and Y are of comparable deshielding effect. The data clearly establish **2** as a 9-nor analogue of thienamycin. Furthermore, the small coupling constant (J=2.5 Hz) between CH(5) and CH(6) requires a *trans* β-lactam stereochemistry.[26,27]

Antibiotic **3** has strong IR absorption bands at 1758 cm^{-1} (β-lactam carbonyl) and 1590 cm^{-1} (carboxylate anion). Compound **3** forms a tris-(trimethylsilyl) derivative having molecular formula C_{20}-

Table 2. ^{1}H NMR data for thienamycin (**1**), northienamycin (**2**) and 8-*epi*-thienamycin (**3**).*

Assignment	**1**	**2**	**3**
CH_3(9)	1.27 (d, J=6.8 Hz)	—	1.33 (d, J=6.8 Hz)
CH_2(4)	~3.20 (m)	~3.2 (m)	~3.2 (m)
CH_2–S	~3.10 (m)	~3.2 (m)	~3.2 (m)
CH_2–N	~3.25 (m)	~3.2 (m)	~3.2 (m)
CH(6)	3.44 (dd, J=2.6, 6.0 Hz)	3.56 (m)	3.52 (dd, J=2.9, 4.3 Hz)
CH(5)	4.22 (dt, J=2.7, 9.2 Hz)	4.22 (dt, J=~2.5, 8.5 Hz)	4.20 (m)
CH_A(8)	4.23 (qn, J=6.5 Hz)	3.89 (dd, J=4.5, 12.0 Hz)	4.22 (m)
CH_B(8)	—	3.94 (dd, J=5.0, 12.0 Hz)	—

* Spectra were recorded at 300 MHz in 0.02 M phosphate pD 7.8 at 6°C.
Chemical shifts are reported in ppm relative to internal DDS.
Multiplicity: d, doublet; t, triplet; qn, quintet; m, multiplet.

$H_{40}N_2O_4SSi_3$ (HRMS M^+ 488.2019, calcd 488.2017). Therefore, the molecular formula of **3** is $C_{11}H_{16}N_2O_4S$ (MW 272), identical to that of thienamycin. From the close similarity of the ^{1}H NMR spectra of **3** and thienamycin, **3** must be a diastereomer of thienamycin (Fig. 3, Table 2). The CH(6) methine appears as a doublet of doublets (J=2.9, 4.3 Hz) at δ 3.52. Double irradiation of CH_3(9) at δ 1.33 collapsed the δ 4.22 CH(8) methine multiplet to a doublet (J=4.3 Hz). Therefore, CH(6) must be coupled to CH(5) with the 2.9 Hz coupling constant, thereby requiring β-lactam stereochemistry to be *trans*.[26,27]

Circular dichroism data on thienamycin, **2** and **3** are presented in Table 1. Molarity of solutions was determined by UV assay. The fact that all compounds exhibit positive molar ellipticities of similar magnitude suggests that **2** and **3** have the same absolute stereochemistry at C-5 as thienamycin, namely *R*.[28] Because β-lactam stereochemistry is *trans* and **3** is a diastereomer of thienamycin, the structures of **2** and **3** are 5(*R*),6(*S*)-9-northienamycin and 5(*R*),6(*S*)-8-*epi*-thienamycin respectively.

Table 3. Antibacterial spectrum of northienamycin and 8-*epi*-thienamycin.

Organism	Zone of inhibition diameter (mm), 6 mm disc				
	1 2 μg/disc	**2*** 6 μg/disc	**3*** 26 μg/disc	Carbenicillin 50 μg/disc	Cephalothin 30 μg/disc
Staphylococcus aureus 2985	34	32	36	36	33
S. aureus 2314**	31	27	32	18	30
Enterococcus sp. 2862	20	10***	21	24	18
Escherichia coli 2482	22	23	22	24	22
E. coli 2964**	22	22	23	0	15
Enterobacter cloacae 2647	22	22	22	21	20
E. cloacae 2646**	20	20	22	13	0
Klebsiella pneumoniae 2921	22	22	20	13	22
K. pneumoniae 2922**	21	21	19	0	18
Serratia sp. 2855	21	18	—	0	0
Proteus mirabilis 2830	15	16***	17	0	14
P. morganii 2833**	14	14***	17	0	0
Providencia sp. 2851	16	16***	—	22	14
Pseudomonas aeruginosa 2835	17	16	22	0	0
P. aeruginosa 2824	29	20	30	19	0

* Antibiotic contains <1% other carbapenems.
** Strain resistant to β-lactam antibiotics.
*** Resistant colonies within zone of inhibition.

^{1}H NMR data of the formamidine derivatives of **2** and **3** are as follows: *N*-formimidoylnorthienamycin (**5**) δ 3.18 (1 H, dd, J=17, ~9 Hz), 3.26 (1 H, dd, J=17, ~9 Hz), 3.0~3.3 (2 H, m), 3.60 (3 H, m), 3.90 (1 H, dd, J=12.0, 4.5 Hz), 3.95 (1 H, dd, J=12.0, 5.5 Hz), 4.20 (1 H, dt, J=≤3, 8.0 Hz), 7.86/7.88 (1 H, s, RNHCH=NH_2^+ conformers); *N*-formimidoyl-8-*epi*-thienamycin (**6**) δ 1.32 (3 H, d, J=6.5 Hz), ~3.2 (4 H, m), 3.51 (1 H, dd, J=5, 2.5 Hz), 3.62 (2 H, m), 4.20 (2 H, m), 7.84/7.86 (1 H, s, RNHCH=NH_2^+ conformers).

Table 4. Antibacterial potency of *N*-formimidoylnorthienamycin (**5**) and *N*-formimidoyl-8-*epi*-thienamycin (**6**).

Organism	MIC (μg/ml)		
	MK787	**5**	**6**
S. aureus 2822 (ATCC #25933)	≤0.016	≤0.016	≤0.098
S. aureus 2314*	≤0.016	0.12	0.39
E. coli 2821 (ATCC #25922)	0.12	0.50	6.2
E. coli 2895	0.25	4.0	6.2
E. cloacae 2647	0.12	0.5	6.2
E. cloacae 2646*	0.25	16	6.2
K. pneumoniae 2921	0.12	0.5	3.1
Klebsiella sp. 2888	0.50	4.0	6.2
P. aeruginosa 4369 (ATCC #27853)	2.0	8.0	6.2

* β-Lactamase producing strain.

Antibacterial Properties

Antibacterial activity of northienamycin (**2**) and 8-*epi*-thienamycin (**3**) is presented in Table 3. Data for thienamycin, cephalothin and carbenicillin are included for comparison. Both **2** and **3** exhibit good broad spectrum activity that includes *Pseudomonas aeruginosa*. Discounting differences in antibiotic potency, the three carbapenems have similar spectral profiles. Relative potencies of the antibiotics, determined from the geometric means of potencies of data in Table 3,[28] are the following: thienamycin 100, northienamycin 22, 8-*epi*-thienamycin 11. Resistant colonies were seen within the zone of inhibition of northienamycin against several organisms.

MIC results for formamidine derivatives **4**~**6** are presented in Table 4. *N*-Formimidoylnorthienamycin is more susceptible to P-99 β-lactamase of *Enterobacter cloacae* 2646 than either MK787 or *N*-formimidoyl-8-*epi*-thienamycin.

Discussion

Two new carbapenem antibiotics, northienamycin and 8-*epi*-thienamycin, have been isolated from *S. cattleya* fermentation. Broth titers are approximately 5% of that of co-produced thienamycin. Both compounds have *trans* β-lactam stereochemistry (5*R*, 6*S*) and the simple unsubstituted cysteamine side chain of thienamycin and NS-5.[12] Northienamycin is the first example of a carbapenem antibiotic with a one carbon side chain at C-6.

Although northienamycin and 8-*epi*-thienamycin are less potent antibacterial agents than thienamycin, both exhibit good activity against a range of organisms. Their greater effectiveness against *P. aeruginosa* strains compared to most other carbapenems can be attributed to the amino group in the cysteaminyl side chain. For instance epithienamycin C (MM 22381), the *N*-acetyl analogue of 8-*epi*-thienamycin, is reported ineffective against Pseudomonas.[28] Furthermore, epithienamycin C appears to have approximately one-third the overall antibacterial potency of 8-*epi*-thienamycin. Analogous results have been reported for thienamycin/*N*-acetylthienamycin and for epithienamycin A/deacetylepithienamycin A.[28]

The formamidine derivatives of northienamycin and 8-*epi*-thienamycin are less active *in vitro* than MK787.

Acknowledgments

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