Echinosporamicin, a New Antibiotic Produced by *Micromonospora echinospora* ssp. *echinospora*, *LL*-P175

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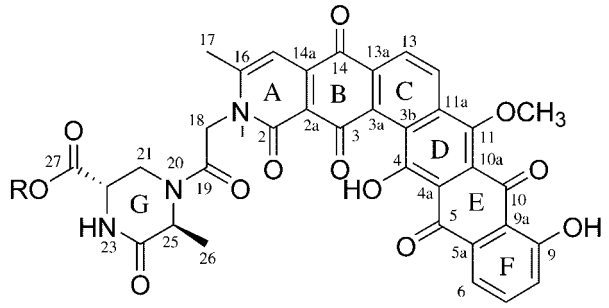
Echinosporamicin (**1**), a novel antibiotic containing an aromatic polycyclic system and a piperazinone moiety, was isolated from the fermentation broth of a new strain of *Micromonospora echinospora* subspecies *echinospora*, *LL*-P175. The structure of this compound was determined by spectroscopic analysis by using variable-temperature NMR techniques. Compound **1** exhibited potent activity against methicillin-resistant *Staphylococci* and vancomycin-resistant *Enterococci* strains. The methyl, ethyl, and benzyl esters showed improved antibacterial activity against *Streptococci*.

Introduction. – Bacterial resistance to antibiotics has emerged as a serious problem for public health [1]. Vancomycin and teicoplanin, two closely related glycopeptides, are commonly considered the last defense for infections caused by methicillin-resistant *Staphylococcus aureus* [2]. Unfortunately, the occurrence of vancomycin-resistant *Enterococci* has been increasing each year [3][4], and cases of vancomycin-resistant *S. aureus* have been reported recently [5][6]. Therefore, the search for new antibiotics that are effective against vancomycin-resistant isolates and with distinctly different chemical structures is particularly appealing. In the course of our continuing research to discover novel antibiotics from microbial sources [7][8], we isolated a new antibiotic, designated echinosporamicin (**1**), from the fermentation broth of a strain of *Micromonospora echinospora* ssp. *echinospora*, *LL*-P175. This compound, containing an aromatic polycyclic system and a piperazinone moiety, showed potent activity against methicillin-resistant *Staphylococci* and vancomycin-resistant *Enterococci* strains. In this paper, the production, structural determination, and antibacterial activity of **1** are reported.



1 R = H
2 R = Me
3 R = Et
4 R = ^{i}Pr
5 R = Bz

Results and Discussions. – Strain *LL*-P175 was isolated from a soil sample collected in a tidepool near Ventura, California, in 1998. A morphological study and 16S rDNA sequencing experiment classified this strain closely related to *Micromonospora echinospora* ssp. *echinospora*. The antibiotic, echinosporamicin (**1**), was purified as a red powder from the fermentation broth of *LL*-P175.

The molecular formula of **1** was determined by high-resolution *Fourier*-transform ion-cyclotron-resonance (FT-ICR) mass spectrometry to be $C_{35}H_{25}N_3O_{12}$. The absorption at λ_{max} 470 nm in the UV spectrum indicated an extended conjugation system. An analysis of ^{1}H- and ^{13}C-NMR spectral data, aided by DEPT and HSQC experiments, allowed the assignment of resonance signals for three Me, two CH_2, two aliphatic CH, and six aromatic CH groups, along with 22 quaternary C-atoms in the range between δ 100 and 187. The structure and relative configuration of **1** was established by spectroscopic means.

A detailed analysis of 2D-NMR spectra (*Table 1*), including COSY and HMBC, led to the identification of two moieties of **1**, an aromatic polycyclic bis-quinone **I**, including C(2), C(2a) to C(16), and C(17)[1]), and a substituted piperazinone **II** (see *Fig. 1*). For the bis-quinone moiety, the presence of the anthraquinone substructure (rings D, E, and F) was indicated by the chemical-shift data of C(5) at δ 187.2 and C(10) at 186.5 and by a number of HC correlations in the HMBC spectrum. In particular, strong two- and three-bond HMBC correlations were observed from OH–C(4) to C(3b), C(4), and C(4a), from H–C(6) to C(5), C(8), and C(9a), from H–C(7), to C(5a), and C(9), from H–C(8) to C(6) and C(9a), from OH–C(9) to C(8), C(9), and C(9a), and from MeO_3 to C(11). The supporting evidence for this substructure was found in several four-bond correlations including those from H–C(7) to C(5) and from H–C(8) to C(10) in an HMBC spectrum acquired with a more sensitive 5 mm *Bruker-TXI CryoProbe*™ (500 MHz). This anthraquinone substructure was fused to a second quinone structure (rings A, B, and C), indicated by strong three-bond HMBC correlations from H–C(12) to C(3b), C(11), and C(13a), from H–C(13) to C(3a), C(12), and C(14), from H–C(15) to C(2a), C(14), C(14a), C(16), and C(17), and from Me(17) to C(15) and C(16). The assignment of the second quinone substructure was also supported by four-bond correlations from H–C(15) and H–C(13) to C(3), observed in the HMBC spectrum acquired with the *CryoProbe*. In ring A, C(2a), C(14a), C(15), C(16), C(17), H–C(15), and Me(17) showed dual signals.

The piperazinone moiety was elucidated by analysis of COSY and HMBC spectral data. All H- and C-atoms associated with this moiety displayed dual signals, indicative of the presence of two interchangeable isomers. For both isomers, COSY data identified two homonuclear spin systems, CH_2CHNH (C(21), C(22), and

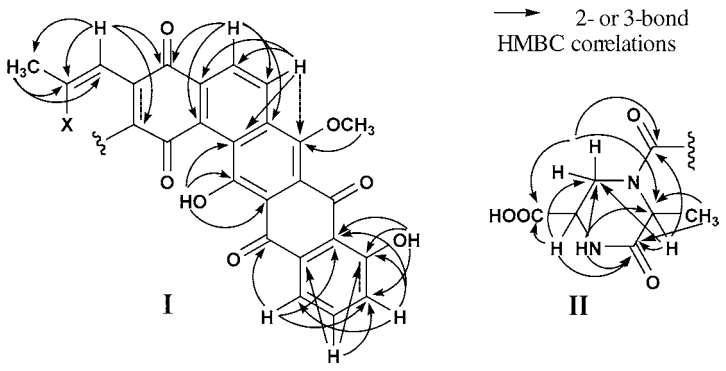


Fig. 1. *Moieties* **I** *and* **II** *of echinosporamicin* (**1**)

[1]) Arbitrary numbering; for the systematic name, see *Exper. Part*.

Table 1. *^{1}H- and ^{13}C-NMR Spectral Data ((D$_6$)DMSO) of Echinosporamicin* (**1**) at 25°[a]). δ in ppm, J in Hz. Arbitrary numbering[1]).

	δ(H) (500 MHz)		δ(C) (100 MHz)		HMBC (500 MHz, $J = 8$ Hz)
	isomer **A**	isomer **B**	isomer **A**	isomer **B**	
C(2)			157.71[b])	157.66[b])	
C(2a)			119.79	119.90	
C(3)			181.95	181.95	
C(3a)			137.44	137.44	
C(3b)			124.65	124.65	
C(4)			161.01	161.01	
OH–C(4)	15.37	15.37			C(3a)[c]), C(3b), C(4), C(4a), C(5)[c])
C(4a)			110.08	110.08	
C(5)			187.19	187.19	
C(5a)			132.74	132.74	
H–C(6)	7.77 (*m*)	7.77 (*m*)	118.54	118.54	C(5), C(8), C(9a)
H–C(7)	7.75 (*m*)	7.75 (*m*)	136.87	136.87	C(5)[c]), C(5a), C(8), C(9)
H–C(8)	7.35 (br. *d*, $J = 7.0$)	7.35 (br. *d*, $J = 7.0$)	124.43	124.43	C(6), C(9), C(9a), C(10)[c])
C(9)			161.76	161.76	
OH–C(9)	12.90	12.90			C(8), C(9), C(9a)
C(9a)			117.32	117.32	
C(10)			186.48	186.48	
C(10a)			120.10	120.10	
C(11)			151.79	151.79	
C(11a)			138.54	138.54	
H–C(12)	8.52 (*d*, $J = 8.7$)	8.52 (*d*, $J = 8.7$)	128.40	128.40	C(3b), C(11), C(13), C(13a)
H–(13)	8.279 (*d*, $J = 8.7$)[b])	8.282 (*d*, $J = 8.7$)[b])	126.79	126.79	C(3)[c]), C(3a), C(11a), C(12), C(14)
C(13a)			133.85	133.85	
C(14)			180.81	180.81	
C(14a)			141.56[b])	141.49[b])	
H–C(15)	6.75 (br. *s*)[b])	6.74 (br. *s*)[b])	100.52	100.52	C(2a), C(3)[c]), C(14), C(16), C(17)
C(16)			155.40	155.62	
Me(17)	2.42	2.39	21.04	21.20	C(15), C(16)
CH$_2$(18)	*ca.* 5.10	*ca.* 5.10	45.70	45.58	C(2)[d]), C(16)[d]), C(19)[d])
	ca. 5.20	*ca.* 5.20			
C(19)			164.43	163.87	
H$_{ax}$–C(21)	3.39 (*dd*, $J = 14.4, 3.6$)	4.69 (br. *d*, $J = 14.4$)	37.29	40.41	C(19), C(25), C(27)
H$_{eq}$–C(21)	4.69 (br. *d*, $J = 14.4$)	4.34 (br. *d*, $J = 14.4$)			C(19), C(25), C(27)
H–C(22)	4.02 (*m*)	4.18 (*m*)	52.55	53.10	C(21), C(24), C(27)
H–N(23)	8.20 (*d*, $J = 2$)	8.26 (*d*, $J = 2$)			C(21), C(24), C(25)
C(24)			168.53	168.76	
H–C(25)	4.60 (*q*, $J = 7.2$)	4.55 (*q*, $J = 7.2$)	52.70	50.95	C(19), C(21), C(24), C(26)
Me(26)	1.57 (*d*, $J = 7.2$)	1.31 (*d*, $J = 7.2$)	17.46	15.96	C(24), C(25)
C(27)			172.30	172.30	
MeO	4.00 (*s*)	4.00 (*s*)	62.74	62.74	C(11)

[a]) Two sets of signals were observed for many H- and C-atoms with a ratio of *ca.* 45 : 55, owing to the presence of two interchangeable isomers **A** and **B**. [b]) Assignments for isomers **A** and **B** may be reversed. [c]) Weak correlations. [d]) Observed at 55°.

N(23)) and CHMe (C(25) and C(26)). In the HMBC spectrum, the two- and three-bond H,C correlations clearly defined a 6-carboxyl-substituted 3-methylpiperazin-2-one moiety with a carbonyl attached to N(4)[1]).

The elements remaining after accounting for the bis-quinone and piperazinone moieties **I** and **II** were C_2H_2NO, based on the molecular formula. The NMR data indicated the presence of a quaternary C-atom with dual signals at δ 157.71 and 157.66, and a CH_2 group with δ 45.70 and 45.58, which were correlated in the HSQC spectrum to a broad proton signal centered at δ 5.15 (2 H). This CH_2 signal, although broad at 25°, was significantly sharpened at 55° to give two sets of *AB* systems. The HMBC spectrum (55°) displayed 3-bond C,H correlations between these two protons (CH_2(18)) and signals of C(2), C(16), and C(19). These correlations allowed the assembly of these elements into an *N*-alkyl-pyridinone part that connects the two moieties to give the planar structure of echinosporamicin (**1**).

An NOE study confirmed the structural assignment and determined the rotameric nature of **1** (rotamers **A** and **B**; *cf. Fig. 2*). In a ROESY plot (55°), cross-peaks were observed between Me(17) and CH_2(18) for both isomers. Moreover, a ROESY cross-peak was observed between CH_2(18) and H–C(25) for isomer **A**, and between CH_2(18) and H_{eq}–C(21) for isomer **B**. These NOE data implied that the presence of the dual ^{1}H- and ^{13}C-NMR signals associated with the piperazinone moiety was caused by two distinctive rotamers, **A** and **B**, along the C(19)–N(20) amide bond (*Fig. 2*). The broadening of the CH_2(18) signal could be attributed to partially restricted rotation along the N(1)–C(18) and C(18)–C(19) bonds, owing to steric hindrance. The relative configuration at C(22) and C(25) was determined by NOE observations and ^{1}H,^{1}H coupling constants. For both rotamers, a cross-peak between H_{ax}–C(21) and Me(26) was observed in the ROESY plot, which required an axial Me(26). The small ^3J(21ax,22) and ^3J(21eq,22) were indicative of an equatorial H–C(22) and thereby an axial COOH group (*Fig. 2*).

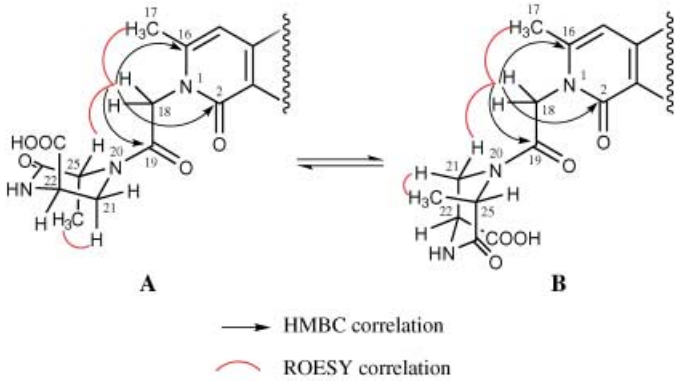


Fig. 2. *2D-NMR Correlations of* **1**, *observed at 55°*

The MS fragmentation was consistent with structure **1**, where the fragment ion due to loss of the piperazinone moiety by breaking the C(19) and N(20) amide bond was found to be an abundant signal at *m*/*z* 522 (40%) in a positive-ion ESI-MS source-fragmentation spectrum (collision energy 30 V).

Esterification of echinosporamicin (**1**) with alkyl halides in the presence of Na_2CO_3 led to the synthesis of its methyl, ethyl, isopropyl, and benzyl esters **2**–**5**. Compound **1** and its esters exhibited potent activity against *Gram*-positive bacteria, including methicillin-resistant *Staphylococci* and vancomycin-resistant *Enterococci*. *MIC* data obtained by the broth-dilution method [9] are listed in *Table 2*. The antibacterial activity against *Streptococci* for methyl, ethyl, and benzyl esters **2**, **3**, and **5** exceeded echinosporamicin (**1**). In an *in vivo* test, echinosporamicin (**1**) and its methyl ester **2** showed efficacy against *Staphylococcus aureus* infection at low concentrations in a mouse model (i.p.), with respective ED_{50}'s of 0.35 and 0.13 mg/kg.

Table 2. *Antimicrobial Activity of Echinosporamicin* (**1**) *and Its Ester Derivatives* **2**–**5**

	MIC [μg/ml]						
	1	**2**	**3**	**4**	**5**	Piperacillin	Vancomycin
Staphylococcus aureus GC 1131[a])	< 0.12	< 0.12	0.12	1	2	> 64	1
Staphylococcus aureus GC 4543	< 0.12	0.12	0.12	1	0.12	1	1
Staphylococcus aureus GC 2216	< 0.12	< 0.12	< 0.12	4	2	4	0.5
Enterococcus faecalis GC 4555	< 0.12	< 0.12	0.5	4	8	2	2
Enterococcus faecalis GC 2242[b])	< 0.12	< 0.12	0.25	4	2	2	> 64
Streptococcus pneumoniae GC 1894* (5% LHB)	16	1	0.5	2	0.5	2	< 0.12
Streptococcus pneumoniae GC 1894 + (THY)	8	2	1	4	0.25	1	< 0.12
Escherichia coli GC4559	> 64	> 64	> 64	> 64	> 64	1	> 64
Candida albicans GC3066	> 64	> 64	> 64	> 64	> 64	> 64	> 64

[a]) Methicillin-resistant strain. [b]) Vancomycin-resistant strain.

In conclusion, echinosporamicin (**1**), a novel antibiotic, was isolated from the fermentation broth of a new strain of *Micromonospora echinospora* ssp. *echinospora*, *LL*-P175. This compound exhibited potent activity against *Gram*-positive bacteria, including methicillin-resistant *Staphylococci* and vancomycin-resistant *Enterococci*. The methyl and ethyl esters showed improved activity against *Streptococci*.

A class of polycyclic pyridinone antibiotics, including albofungin [10], simaomicins [11], and cervinomycins [12] were previously reported to have antimicrobial, antifungal, and antitumor activity. The C-skeleton of echinosporamicin (**1**) differs from this class by having the γ-pyrone (=4*H*-pyran-4-one) moiety replaced by an 1,4-benzoquinone moiety (ring E). In addition, none of the compounds belonging to that class contain the piperazinone moiety present in **1**. Biologically, echinosporamicin (**1**) exhibited potent and selective activity against *Gram*-positive bacteria without significant activity for fungus, *Candida albicans*, and colon tumor cell line, HCT-116. The mode of action for echinosporamicin will be the subject of a separate report.

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Experimental Part

1. *Microorganism*. Strain *LL*-P175 was isolated from a soil sample obtained in a tidepool near Ventura, California, in 1998. This culture was preserved as frozen whole cells at $-150°$ with 20% glycerol.

The 16S rDNA sequence was determined for strain *LL*-P175 following isolation and direct sequencing of the amplified gene. The 16S rDNA sequence was compared to databases by the use of BLAST (basic local alignment search tool) to determine the phylogenetic position, and phylogenetic trees were generated by using two neighbor-joining tree algorithms. The DNA analysis led to the classification of this strain as closely related to *Micromonospora echinospora* ssp. *echinospora*.

Physiological and morphological studies of culture *LL*-P175 with standard methods confirmed that strain *LL*-P175 was closest to a type strain *M. echinospora* subspecies *echinospora* ATCC 15837^T [13]. However, these studies also revealed several differences between the two strains. Strain *LL*-P175 had moderate growth on galactose and mannitol, but no growth on raffinose, in comparison to *M. echinospora* subspecies *echinospora*

ATCC 15837^T, which had abundant growth on raffinose and galactose, but no growth on mannitol. *M. echinospora* subspecies *echinospora* ATCC 15837^T also exhibited good growth on tyrosine and peptone-iron agar, but culture *LL*-P175 had little or no growth on these substrates. Other differences include that culture *LL*-P175 could not liquefy gelatin and demonstrated good nitrate reduction; *M. echinospora* subspecies *echinospora* ATCC 15837^T could liquefy gelatine, and nitrate reduction was variable. These differences supported the creation of a new strain of *M. echinospora* subspecies *echinospora*, *LL*-P175.

2. *Seed Preparation*. The seed medium contained dextrose (1.0%), soluble starch (2.0%), yeast extract (0.5%), N-Z amine (type A, 0.5%), and $CaCO_3$ (0.1%) at pH 7.3. Four 25 mm × 150 mm glass culture tubes containing 8 ml each of the seed medium were inoculated with cells of *LL*-P175, cultured on ATCC agar medium #172 [14]. Sufficient inoculum from the agar culture was used to provide turbid primary-seed tubes after incubation at 28° for 72 h, at 200 rpm by using a gyro-rotary shaker with a 2-inch throw. The primary-seed pool (*ca.* 16% inoculum) was then used to inoculate four 250-ml *Erlenmeyer* flasks containing 50 ml of medium #172. These four secondary-seed flasks were incubated at 28° for 72 h at 200 rpm by using a gyro-rotary shaker.

3. *Fermentation*. The production medium contained glycerol (1.0%), soy peptone (0.5%), K_2HPO_4 (0.25%), NaCl (0.5%), $MgSO_4 \cdot 7\,H_2O$ (0.05%), KCl (0.05%), and agar (0.04%) at pH 7.0. A 7.5-l glass fermentor was prepared with 5.0 l of the production medium and inoculated with 200 ml of the secondary-seed fermentation and incubated at 28° for 6 days at 300 rpm under 5.0 l/min airflow. The fermentation was scaled up to 300 l to provide a gram quantity of **1** for structure characterization and derivatization as well as biological evaluation.

4. *4-[(1,2,5,9,14,16-Hexahydro-10,15-dihydroxy-8-methoxy-3-methyl-1,5,9,14,16-pentaoxonaphthaceno[2,1-g]isquinolin-2-yl)acetyl]-5-methyl-6-oxopiperazine-2-carboxylic Acid* (**1**): *Islation and Purification*. A portion of the whole broth (1 l) was centrifuged at 3800 rpm, and the supernatant was extracted with BuOH (1 l). The org. layer was evaporated and the obtained brownish gum dissolved in DMSO (10 ml). The soln. was poured into MeOH/H_2O 1:1 (100 ml) at 0°. The precipitate, obtained by centrifugation, was chromatographed by reversed-phase HPLC (*C18* column (*YMC ODS-A*, particle size, 10 μm; 70 × 500 mm), 30–100% MeCN/H_2O + 0.01% CF_3COOH: **1** (45 mg). Red amorphous powder. UV (MeCN/H_2O 1:1): 248, 276 (sh), 325, 470, 490 (sh). ^{1}H- and ^{13}C-NMR: *Table 1*. HR-FT-ICR-MS: pos. mode: 680.15251 (MH^+, $C_{35}H_{26}N_3O_{12}^+$; calc. 680.15109); neg. mode: 678.13647 ($[M-H]^-$, $C_{35}H_{24}N_3O_{12}^-$; calc. 678.13654).

5. *Methyl Ester* **2**. To a soln. of **1** (100 mg) in anh. DMSO (2.5 ml) was added Na_2CO_3 (200 mg) and then MeI (138 μl). The mixture was stirred at r.t. for 2 h. Upon filtration, the soln. was acidified with CF_3COOH and then chromatographed by reversed-phase HPLC: **2** (32.2 mg). ESI-MS (pos.): 694 ($[M+H]^+$).

6. *Ethyl Ester* **3** *and Benzyl Ester* **5**. As described in *Exper. 5*, with **1** (50.0 mg) and EtI (89 μl) or $PhCH_2Br$ (132 μl), resp.: **3** (12.4 mg) and **5** (12.3 mg), resp. ESI-MS (pos.): **3**: 708 ($[M+H]^+$): **5**: 770 ($[M+H]^+$).

7. *Isopropyl Ester* **4**. To a soln. of **1** (50 mg) in anh. DMSO (2.0 ml) was added Na_2CO_3 (100 mg) and then ^{i}PrBr (104.2 μl). The mixture was heated at 60° for 1 h and the resulting mixture worked up as in *Exper. 5:* **4** (3.6 mg). ESI-MS (pos.): 722 ($[M+H]^+$).

REFERENCES

[1] H. Breithaupt, *Nat. Biotechnol.* **1999**, *17*, 1165 and ref. cit. therein.
[2] T. M. Perl, *Am. J. Med.* **1999**, *106* (*5A*), 26S and ref. cit. therein.
[3] E. Ginzburg, N. Namias, M. Brown, S. Ball, S. M. Hameed, S. M. Cohn, *Int. J. Antimicrob. Agents* **2000**, *16* (Suppl.), S39.
[4] I. J. Chopra, B. M. Hodgson, G. Poste, *Antimicrob. Agents Chemother.* **1997**, *41*, 497.
[5] K. Hiramatsu, H. Hanaki, *Curr. Opin. Infect. Dis.* **1998**, *116*, 653.
[6] A. Marchese, G. C. Schito, E. A. Debbia, *J. Chemother.* **2000**, July 12, Suppl. 2, 12.
[7] H. He, B. Shen, P. J. Petersen, W. J. Weiss, H. Y. Yang, T.-Z. Wang, R. G. Dushin, F. E. Koehn, G. T. Carter, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 279.
[8] F. Kong, N. Zhao, M. M. Siegel, K. Janota, J. S. Ashcroft, F. E. Koehn, D. B. Borders, G. T. Carter, *J. Am. Chem. Soc*. **1998**, *120*, 13301.
[9] National Committee for Clinical Laboratory Standards (NCCLS), Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standards: M7–A5′, Vol. 19, NCCLS, Villanova, PA, 2000.
[10] A. I. Gurevich, T. N. Deshko, G. A. Kogan, M. N. Kolosov, V. V. Kudryashova, V. V. Onoprienko, *Tetrahedron Lett.* **1974**, *33*, 2801.

[11] T. M. Lee, G. T. Carter, D. B. Borders, *J. Chem. Soc., Chem. Commun.* **1989**, *22*, 1771.
[12] S. Omura, A. Nakagawa, K. Kushida, G. Lukacs, *J. Am. Chem. Soc.* **1986**, *108*, 6088.
[13] G. M. Luedemann, B. Brodsk, *Antimicrob. Agents Chemother.* **1964**, *1963*, 116.
[14] 'ATCC Catalogue of Bacteria and Bacteriophages', 18 edn., American Type Culture Collection, Rockville, 1992, p. 422.

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