MM 42842, A NEW MEMBER OF THE MONOBACTAM FAMILY PRODUCED BY *PSEUDOMONAS COCOVENENANS*

II. PRODUCTION, ISOLATION AND PROPERTIES OF MM 42842

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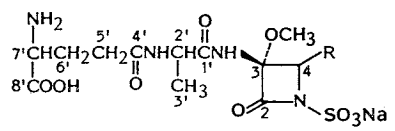
A new member of the monobactam family of β-lactam antibiotics, designated MM 42842, has been detected in a culture of *Pseudomonas cocovenenans*. The production, isolation and some properties of the antibiotic are described. Structural studies show MM 42842 to be closely related to the previously described antibiotic sulfazecin.

During a programme of screening soil micro-organisms for antibiotics, the production of a new β-lactam antibiotic, designated MM 42842, by a bacterial culture has been detected. The previous paper[1] described the identification of the producing organism as *Pseudomonas cocovenenans* 326-32B. MM 42842 (**2**) has been shown to be a new member of the monobactam series of β-lactam antibiotics[2] closely related to the previously described sulfazecin[3] (**1**) (Fig. 1). MM 42842 differs from other naturally occurring monobactam antibiotics in having a 4-methyl substituent. Interestingly both the MM 42842 and sulfazecin producing cultures also yield members of the bulgecin family of antibiotics[4] (S. J. Box and S. R. SPEAR; unpublished data).

Fig. 1. Structures of sulfazecin and MM 42842.



Sulfazecin (SQ 26,445) (**1**) R = H
MM 42842 (**2**) R = CH_3

This paper describes fermentation conditions for the production of MM 42842 and methods for its extraction. Details of the structural studies and some physico-chemical and biological properties of MM 42842 are also reported.

Materials and Methods

The IR spectra were recorded on a Perkin-Elmer 983 spectrophotometer. ^1H NMR spectra were obtained at 250 MHz on a Bruker WM250 instrument and ^{13}C NMR data on a Jeol GX 270, the latter using sodium trimethylsilyl propionate as an external standard. The mass spectrum was recorded on a VG ZAB 1F spectrometer.

Fermentation Conditions

P. cocovenenans 326-32B was grown on agar of the following composition (g/liter): Tryptone (Oxoid) 5.0, Neutralized soya peptone (Oxoid) 5.0, glucose 10.0, fructose 10.0, Purified agar (Oxoid) 18.0, pH 7.0.

Sterile deionized water (10 ml) was added to an agar slope of the organism in a McCartney bottle and a suspension of cells prepared. Portions of this suspension (0.3 ml) were used to inoculate the

fermentation stage medium (50 ml) in 250-ml Erlenmeyer flasks closed with foam plastic plugs. The fermentation medium had the same composition as the agar maintenance medium but without the addition of agar.

The flasks were incubated on a gyratory shaker at 28°C. All media were sterilized at 121°C for 15 minutes.

Detection Methods

Levels of MM 42842 in fermentation and extraction samples were monitored using antibacterial activity determined by the agar - diffusion method with either *Acinetobacter lwoffii* LSS7 or *Pseudomonas aeruginosa* ES48 as a test organism. These test organisms are β-lactam hypersensitive variants of the parent strains.

Purification

The following column media were used in the purification procedure for MM 42842 (Fig. 2): Amberlite IRA-458; acrylic based strongly basic anion exchange resin from Rohm and Haas Co., Philadelphia, U.S.A. Biogel P2; polyacrylamide gel filtration medium from BioRad Laboratories, Richmond, California, U.S.A. Cellulose CC31; microgranular cellulose from Whatman Ltd., Maidstone, Kent, UK.

The HPLC system comprised a Waters Model 6000A solvent delivery system, Rheodyne model 7125 injector and Waters RCM 100 radial compression unit containing a C_{18} Radi-pak cartridge (Waters Associates, Harrow, Middlesex, UK; Rheodyne Inc., Cotati, California, U.S.A.). Monitoring was with a Hewlett-Packard 1040 diode array detector at 220 nm (Hewlett-Packard Ltd., Bracknell, Berkshire, UK).

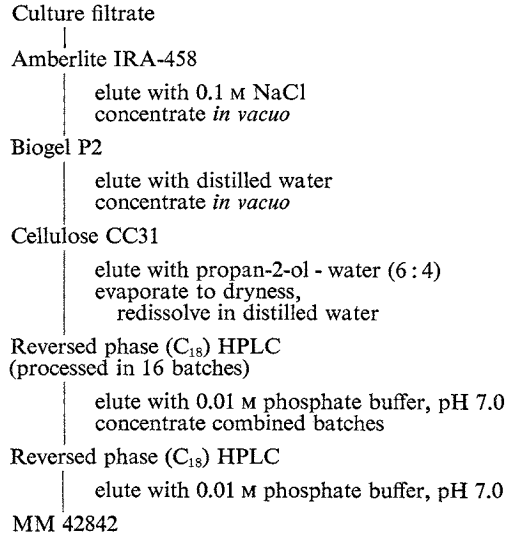
Antibacterial Evaluation

MICs were determined by a 2-fold dilution microtitre method. Todd-Hewitt broth (Oxoid) was used for *Streptococcus* sp. and Nutrient broth (Oxoid No. 2) was used for all other organisms. Final inoculum level was approximately 10^5 cfu per ml.

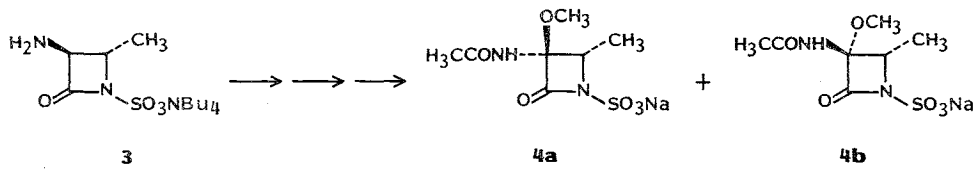
Acid Hydrolysis of MM 42842

MM 42842 (2 mg) was hydrolyzed in 6 M HCl at 110°C for 24 hours under nitrogen in a sealed tube. After derivatisation with dansyl chloride the hydrolysate was examined by HPLC on a chiral β-cyclodextrin column.

Fig. 2. Purification procedure for MM 42842.



Scheme 1.



Preparation of (4*S*)-3-Acetylamino-3-methoxy-4-methyl-2-oxoazetidine-1-sulfonic Acid Sodium Salt (4)

The starting material **3** (Scheme 1) was prepared from L-threonine using essentially the method of FLOYD *et al.*[5] A methylthio moiety was introduced by the action of methyl methanethiosulfonate and 1,8-diazabicyclo[5.4.0]undec-7-ene (M. J. PEARSON and S. C. FINCH; personal communication) on the SCHIFF's base of **3**. After acylation, the sulfide was oxidised to the sulfoxide with *m*-chloroperbenzoic acid and subsequent treatment with MeOH in the manner of KAURA and PEARSON[6] afforded the inseparable mixture of diastereoisomers **4a** and **4b** in a 3:2 ratio: Isomer **4a** ^{1}H NMR (D_2O) δ 1.37 (3H, d, J=6.3 Hz, CH_3), 2.11 (3H, s, $COCH_3$), 3.48 (3H, s, OCH_3), 4.25 (1H, q, J=6.3 Hz, 4-H); isomer **4b** ^{1}H NMR (D_2O) δ 1.45 (3H, d, J=6.3 Hz, CH_3), 2.07 (3H, s, $COCH_3$), 3.49 (3H, s, OCH_3), 4.32 (1H, q, J=6.3 Hz, 4-H).

Results

Fermentation and Purification Studies

The fermentation profile for *P. cocovenenans* showed that peak titres were reached at approximately 48 hours. Production of antibiotic activity was closely related to the growth of the organism.

The isolation procedure for MM 42842 is shown in Fig. 2. Primary extraction of MM 42842 was achieved, using the acidic nature of the antibiotic, by chromatography on the strongly basic ion exchange resin Amberlite IRA-458. Some difficulty was experienced in obtaining pure preparations of the metabolite by classical chromatographic techniques. However, reversed phase HPLC proved a valuable approach in this respect. The final step was elution in a dilute buffer, and the MM 42842 was eluted in a small volume. Good spectral and biological data were obtained without further desalting of the product.

Structural Studies

The IR spectrum of MM 42842 (**2**) showed a characteristic monocyclic β-lactam absorption band at 1765 cm^{-1} and also sulfonic acid absorptions at 1250 and 640 cm^{-1}, typical of monobactams[7] (see Table 1).

An examination of the ^{1}H NMR data obtained on MM 42842 (**2**) indicated a close similarity with data obtained by Takeda workers on sulfazecin (**1**).[8] However, notable differences in the spectrum of MM 42842 were evident, *i.e.* the appearance of an extra methyl doublet at 1.34 ppm (J=6.3 Hz), an extra methine at 4.24

Table 1. Physico-chemical properties of MM 42842.

Appearance	Colourless powder
$[\alpha]_D^{20}$	+34° (*c* 0.3, H_2O)
UV	End absorption
Molecular formula	$C_{13}H_{21}N_4O_9SNa$
FAB-MS	
Positive ion	455 (M+Na), 433 (M+H), 361, 347, 325, 303 (M+H−Glu)
Negative ion	431 (M−H), 409 (M−Na), 323, 321, 301 (M−H−Glu)
IR ν_{max} (KBr) cm^{-1}	1765, 1660, 1540, 1250, 640

FAB-MS: Fast atom bombardment mass spectrum.

Table 2. A comparison of ^{13}C NMR spectra of MM 42842 and sulfazecin.

MM 42842		Reported for sulfazecin[a]	Position	$\Delta\delta$[b]
δ ppm (D_2O)	135° DEPT[c]			
16.0	CH_3		4-CH_3	—
19.3	CH_3	19.2 (q)	3′	+0.1
29.1	CH_2	29.1 (t)	6′	0.0
34.0	CH_2	34.1 (t)	5′	−0.1
52.9	CH or CH_3	52.9 (d)	2′ or 7′	0.0
55.7	CH or CH_3	55.8 (q)	OCH_3	−0.1
57.0	CH or CH_3	57.0 (d)	7′ or 2′	0.0
67.4	CH or CH_3	57.2 (t)	4	+10.2
96.2	Quaternary	93.7 (s)	3	+2.5
164.7	Quaternary	164.7 (s)	C=O	0.0
176.8	Quaternary	176.4 (s)	C=O	+0.4
177.5	Quaternary	177.2 (s)	C=O	+0.3
178.8	Quaternary	178.8 (s)	C=O	0.0

[a] ref 3. We have added an increment of +2.0 ppm to the literature chemical shifts in order to account for differences in referencing procedure, thereby giving closest agreement between the two sets of figures.
[b] $\Delta\delta=\delta$(MM 42842)$-\delta$(sulfazecin).
[c] CH and CH_3 signals up, CH_2 signals down. Quaternary signals are absent in this experiment but are observable in the spin-echo spectrum. DEPT: Distortionless enhancement by polarization transfer.

ppm (J=6.3 Hz) and the absence of the C-4 methylene normally found at 3.6 ppm.

Similar differences were noted in the ^{13}C NMR data (Table 2) obtained on MM 42842, in particular the presence of an extra methyl signal at 16.0 ppm in MM 42842 and the appearance of a low-field methine carbon at 67.4 ppm in place of the methylene carbon at 57.2 ppm reported for sulfazecin. Furthermore, the only significant chemical shift changes ($\Delta\delta$) occurring, on going from MM 42842 to sulfazecin are associated with the C-3 and C-4 carbons and these shifts are entirely consistent with the presence of a methyl substituent at C-4 in MM 42842. On the basis of these results the structure **2** is proposed for MM 42842.

Side Chain Stereochemistry

The following amino acids were detected on acid hydrolysis of MM 42842; D-glutamic acid (0.29 mg), L-glutamic acid (0.11 mg), D-alanine (0.13 mg) and L-alanine (0.07 mg). These results indicate that the side chain stereochemistry is mainly DD as in sulfazecin.[3] However, quantities of the L-enantiomers were detected suggesting that other side chain isomers may be present and indeed one such minor isomer is detectable in the ^1H NMR spectrum of MM 42842. No epimerisation of authentic D-glutamic acid or D-alanine takes place under the hydrolysis conditions.

C-4 Stereochemistry of MM 42842

The synthetic diastereomeric monobactams **4a** and **4b** were prepared as model compounds in order to compare the ^1H NMR chemical shifts of the 4-H and 4-CH_3 with the values obtained on MM 42842. The assignment of C-3 stereochemistry in the case of **4a** and **4b** was made on the basis of nuclear Overhauser experiments. Thus, irradiation of the methoxyl signal at 3.5 ppm in the diastereomeric mixture gave an enhancement of the signals at 1.45 ppm (the 4-CH_3 of isomer **4b**) and 4.25 ppm (the 4-H of isomer **4a**) and it follows therefore that these groups are *cis* to the methoxyl protons in the respective isomers. Thus the major isomer **4a** formed by addition of methanol to the acylimine intermediate[6] derived from methylthio compound must have a *trans* relationship between the C(4)-methyl and the

Table 3. A comparison of ^1H NMR data on MM 42842, **4a** and **4b**.

	δ ppm (D_2O) 4-H (J=6.3 Hz)	δ ppm (D_2O) 4-CH_3 (J=6.3 Hz)
MM 42842	4.24	1.34
4a	4.25	1.37
4b	4.32	1.45

methoxyl group. This is to be expected from steric considerations, the methanol adding from the least hindered face.

A comparison of the chemical shift data obtained on MM 42842, **4a** and **4b** has been made in Table 3 and a consideration of this information suggests that the relative stereochemistry about the C(3)-C(4) bond in MM 42842 is as found in **4a**.

Table 4. Antibacterial activity of MM 42842 by the microtitre method (see Materials and Methods).

Organism	MIC (μg/ml)
Escherichia coli 10418	64
Pseudomonas aeruginosa 1771P	>512
Proteus mirabilis C977	>512
Enterobacter cloacae N1	>512
Klebsiella aerogenes A	512
Serratia marcescens US32	512
Bacillus subtilis	256
Micrococcus luteus NCTC 8340	8
Staphylococcus aureus Oxford	32
S. aureus V573	>512
S. saprophyticus FL1	32
S. epidermidis 60137	256
Streptococcus pyogenes CN10	16
S. agalactiae Hester	8
S. faecalis I	64

Biological Properties

The antibacterial activity of MM 42842 was weak (Table 4) and was directed mainly against Gram-positive bacteria. This spectrum of activity contrasts with those of sulfazecin[8] and isosulfazecin[9] which are generally more active against Gram-negative bacteria.

Discussion

The production of β-lactam antibiotics by bacteria is well known. MM 42842 is of particular interest since it is the first naturally occurring monobactam antibiotic with a 4-methyl substituent. The results of the chiral amino acid analysis, showed that MM 42842 is the sulfazecin analogue with the D-glutamic - D-alanine side chain. However, the presence of both amino acids with the L-configuration albeit in small quantities, suggests the presence of further monobactam derivatives. It was not possible to confirm the presence of such compounds in the present study. Similar variations are also found in the case of sulfazecin and isosulfazecin.[2] Of interest in the case of *P. cocovenenans* 326-32B is its ability to produce members of the bulgecin family of antibiotics as well as the monobactam MM 42842. This property is in common with the previously described sulfazecin producing organism *Pseudomonas acidophilia* ATCC 31363.[2]

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