

ORIGINAL ARTICLE

Discovery and antibacterial activity of glabramycin A–C from *Neosartorya glabra* by an antisense strategy

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Treatment of drug-resistant bacteria is a significant unmet medical need. This challenge can be met only by the discovery and development of new antibiotics. Antisense technology is one of the newest discovery tools that provides enhanced sensitivity for detection of antibacterials, and has led to the discovery of a number of interesting new antibacterial natural products. Continued utilization of this technology led to the discovery of three new bicyclic lactones, glabramycins A–C, from a *Neosartorya glabra* strain. Glabramycin C showed strong antibiotic activity against *Streptococcus pneumoniae* (MIC 2 µg ml⁻¹) and modest antibiotic activity against *Staphylococcus aureus* (MIC 16 µg ml⁻¹). The isolation, structure, relative configuration and antibacterial activity, and plausible biogenesis of these compounds have been discussed.

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INTRODUCTION

The discovery and development of clinically useful antibiotic classes, such as, the aminoglycosides, macrolides and tetracyclines, have clearly shown that bacterial protein synthesis is a viable target for antibacterial drug discovery.^{1,2} The bacterial ribosome responsible for protein synthesis consists of a small 30S and a large 50S subunit. The ribosomal subunit (aka S4), referred to as RpsD, is a component of the 30S subunit.^{3–5} The RpsD protein is encoded by the *rpsD* gene, which is essential and resides in an operon containing only one other gene, *SAV1718*, which is a nonessential gene.^{6,7} Therefore, this gene was selected as a drug-target for the discovery of antibacterial agents.

For antibiotic discovery targeting the *rpsD* gene, we constructed a *Staphylococcus aureus* S1-782B strain expressing antisense RNA under xylose control, leading to hypersensitivity against RpsD inhibitors. To implement this approach for drug discovery, we designed a two-plate assay in which one plate was seeded with an *rpsD* antisense *S. aureus* strain and the other with an *S. aureus* EP 167 (control) strain. A similar general methodology led to the discovery of platensimycin and platencin.^{8–13} The screening of over 138 000 microbial extracts against *rpsD* two-plate antisense whole-cell assay led to the isolation of a series of interesting new compounds exemplified by lucensimycins,^{14–16} coniothyrione,¹⁷ pleosporone,¹⁸ phaeosphenone,¹⁹ and okilactomycin.²⁰ Continued screening and follow-up of one of the active extracts produced by *Neosartorya glabra* led to the isolation of three macro-lactones namely, glabramycin A–C (1–3, Figure 1). The isolation, structure elucidation, relative configuration and antibacterial activity of the glabramycins (1–3) are described.

RESULTS AND DISCUSSION

The producing organism was isolated from a soil sample collected from Candamia, Spain. The strain was identified as *Neosartorya glabra* by sequencing the large subunit of DNA of the D1D2 region and by tracing the phylogenetic relationships. The strain was grown in a submerged fermentation for 14 days and compounds were extracted with an equal volume of acetone. The extract was chromatographed on Amberchrome, a capture resin, followed by reversed-phase C₈ HPLC to give 1 (7 mg, 7 mg l⁻¹), 2 (1.9 mg, 1.9 mg l⁻¹) and 3 (1 mg, 1 mg l⁻¹), each as yellow gums.

Glabramycin A (1), the most abundant of the three compounds, showed a molecular formula C₂₂H₂₄O₆ as deduced from the HRESIFTMS data. The ¹³C NMR spectrum and distortionless enhancement by polarization transfer (DEPT) analysis of 1 showed signals for one methyl, four methylenes, ten methines and seven quaternary carbons (Table 1). The ¹H NMR spectrum showed signals for four olefinic methine doublet of doublets with *J*=15 and 11 Hz, and two methine doublets with *J*=15 Hz. The COSY correlations of these six olefinic methines indicated the presence of an *E*-triene in which each end terminated with a quaternary carbon. The methine (δ_H 5.90) at one end of the triene chain showed heteronuclear multiple bond correlations (HMBCs) to a carboxyl carbon at δ_C 170.3, and a carboxylic group was thus placed at one end of the triene. The terminal methine (δ_H 6.00) at the other end of the triene chain showed HMBC correlations to two quaternary olefinic carbons resonating at δ_C 167.4 and δ_C 108.6, allowing for further extension of the triene chain.

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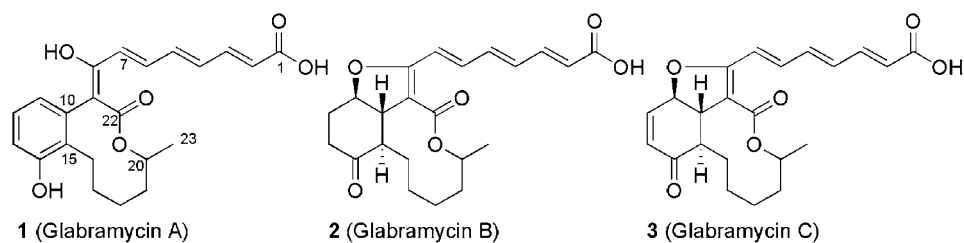


Figure 1 Structures and relative configurations of glabramycins A–C (1–3).

Table 1 ^1H (600 MHz) and ^{13}C (125 MHz)-NMR assignments of glabramycin A (1), B (2) and C (3)

	1 <i>CD₃OD</i>		2 <i>CD₃OD</i>		3 <i>CDCl₃</i>	
Position	δ_{C}	δ_{H} , mult, <i>J</i> in Hz	δ_{C}	δ_{H} , mult, <i>J</i> in Hz	δ_{C}	δ_{H} , mult, <i>J</i> in Hz
1	170.3		170.1		170.8	
2	124.4	5.90, d, 15	124.5	6.00, d, 15	122.2	5.90, d, 15
3	145.6	7.30, dd, 15, 11	145.2	7.30, dd, 15, 11	145.5	7.40, dd, 15, 11
4	136.0	6.60, dd, 15, 11	136.2	6.60, dd, 15, 11	134.5	6.57, dd, 15, 11
5	140.4	6.54, dd, 15, 11	140.3	6.80, dd, 15, 11	140.0	6.75, dd, 15, 11
6	136.9	7.10, dd, 15, 11	137.3	7.00, dd, 15, 11	135.9	6.97, dd, 15, 11
7	129.1	6.0, d, 15	123.3	7.20, d, 15	122.9	7.32, d, 15
8	167.4		164.5		163.1	
9	108.6		110.9		112.0	
10	125.3		49.4	3.46, t, 9.6	44.0	3.62, dd, 10, 4.5
11	124.9	6.49, dd, 7.5, 1.0	82.0	5.00, m	77.5	5.09, dd, 8.5, 4.5
12	126.9	6.95, t, 7.5	35.9	2.20, m, 2.40, m	137.6	6.87, dd, 10.5, 4.5
13	115.9	6.73, dd, 10, 7.5	26.0	2.20, m, 2.40, m	132.5	6.23, d, 10.5
14	157.4		213.3		198.7	
15	131.8		50.6	2.60, m	48.3	2.46, m
16	27.3	2.07, m 2.70, m	23.7	1.46, m 1.62, m	22.9	2.05, m 1.40, m
17	29.5	1.94, m 1.87, m	25.2	1.80, m 1.30, m	23.6	1.53, m 1.89, m
18	22.6	1.34, m 1.53, m	24.9	1.78, m 1.88, m	32.4	1.65, m 1.76, m
19	33.9	1.49, m 1.83, m	32.9	1.56, m 1.83, m	24.8	1.38, m 1.77, m
20	73.5	5.30, m	74.1	4.94, m	73.2	5.00, m
22	164.7		170.1		164.9	
23	18.5	1.10, d, 6.5	20.3	1.28, d, 6.5	20.6	1.30, d, 6.5

Three of the remaining olefinic methines coupled with each other, with $J=7.5$ Hz indicating the presence of a 1, 2, 3-tri-substituted phenyl group. The only non-olefinic methine proton resonating at δ_{H} 5.30 showed heteronuclear multiple quantum coherence (HMQC) correlation to an oxygenated carbon resonating at δ_{C} 73.5. The methyl group resonating at δ_{H} 1.10 showed HMBC correlations to the methine carbon C-20 (δ_{C} 73.5) and to the methylene at C-19 (δ_{C} 33.9). This observation allowed for the placement of the methyl group on the oxygenated carbon C-20. The COSY correlations from the methyl protons, H₃-23 at δ_{H} 1.10 to the methylene H₂-16 at δ_{H} 2.07 and δ_{H} 2.7, established the C16–C20–C23 spin system. The HMBC correlations of the terminal methylene protons of the alkyl chain at δ_{H} 2.70 and δ_{H} 2.07 to carbons δ_{C} 131.8 (C-15), 125.3 (C-10) and 157.4

(C-14) established its connection to the phenyl group. The HMBC correlation of the methine proton (δ_{H} 5.30) to an ester carbonyl at δ_{C} 164.7 (C-22) allowed for the linkage of this carbonyl and the methine in the form of a lactone ring. Finally, the ester carbonyl C-22 was connected to the olefinic carbon C-9 (δ_{C} 108.6) to form the macrocyclic lactone, to satisfy the degrees of unsaturation and the molecular formula. This assignment was supported by the ^{13}C chemical shifts of C-8, C-9 and C-22. The substitution around the phenyl ring was confirmed by HMBC correlations of the aromatic protons and supported by HMBC correlations of the benzylic methylene protons (Figure 2).

The structures for glabramycins, B (2) and C (3), were determined by comparison of ^{13}C and ^1H NMR spectral data with 1 (Table 1).

Glabramycin B (**2**) exhibited a molecular formula, C₂₂H₂₆O₆. The ¹H NMR spectrum of **2** showed only six olefinic protons of trienoic acid moiety, indicating the presence of a saturated six-membered ring system. The COSY spectrum showed an extended spin system comprising C20(C23)-C15-C10-C14. The HMBC correlations of H-10 (δ_H 3.46), H₂-12 (δ_H 2.20, 2.40) and H₂-16 (δ_H 1.46, 1.62) to the downfield carbonyl C-14 (δ_C 213.3) allowed the placement of a carbonyl group at C-14 in the cyclohexanone ring. The ether bridge between C8 and C11 made the dihydrofuran ring that satisfied the molecular formula. The relative configurations at C-10, C-11 and C-15 were established from the magnitude of the scalar couplings. H-10 resonated as a triplet with *J*=9.6 Hz because of axial-axial couplings both from H-11 and H-15, thus establishing an anti relationship between these three protons and a *trans*-ring fusion.

Glabramycin C (**3**) showed a molecular formula that was isomeric to that of **1**. Comparison of the ¹H and ¹³C NMR spectra of **3** with those of compound **2** indicated the presence of a pair of olefinic protons, δ_H 6.23 (d, *J*=10.5 Hz) and 6.87 (dd, *J*=10.5, 4.5 Hz), which were assigned to H-13 and H-12 by COSY correlations and confirmed by their HMBC correlations to an upfield shifted C-14 carbonyl (δ_C 198.7), thus assigning structure **3** for glabramycin C (Table 1).

On the basis of these data, structures **1**, **2** and **3**, with relative configurations, were assigned for glabramycins A, B and C, respectively. Biogenetically, these compounds are likely to originate from a

polyketide pathway by condensation of 11 acetate units to a undeca-ketide, which likely undergoes cyclization, reduction and dehydration to produce compound **1**, which then produces compounds **3** and **2**.

Biological activities

All three glabramycins were tested in the *S. aureus* antisense *rpsD*-sensitized two-plate differential sensitivity assay. Glabramycin C showed the most potent activity in this assay and showed a minimum detection concentration (MDC) of 62 μg ml⁻¹. At this concentration, a more than 5 mm zone differential was observed with a 10.78 mm zone of inhibition on the antisense plate vs a 5.63 mm zone of inhibition on the control plate. The size of the zone of clearance was dose dependent, and at 500 μg ml⁻¹, it produced a zone of clearance of 16.89 and 6.8 mm on the antisense and the control plate, respectively. The other two compounds were less active. Glabramycin A was approximately four-fold less active and showed intermediate activity with MDC of 250 μg ml⁻¹, producing zone sizes of 11.24 and 6.25 mm on the antisense and the control plate, respectively. The MDC of glabramycin B was greater than 500 μg ml⁻¹. Glabramycin C showed better activity against a panel of bacteria used in this assay. It inhibited *S. aureus* growth with MIC values of 16 μg ml⁻¹ (Table 2). Glabramycin C exhibited a similar activity against *Bacillus subtilis*, but was less active against *Enterococcus faecalis* (MIC > 32 μg ml⁻¹). The best activity was against *Streptococcus pneumoniae* regardless of the medium used, and inhibited the growth with an MIC value of 2 μg ml⁻¹. Glabramycins A and B were significantly less active, see Table 2. None of these compounds inhibited growth of Gram-negative bacteria or fungi *Candida albicans*. Mechanistically, glabramycin A, the most abundant of the three, showed 2–3-fold preferential inhibition of RNA synthesis (IC₅₀ 10 μg ml⁻¹) compared with DNA and protein synthesis in a macromolecular synthesis assay (Figure 3). Inhibition of RNA synthesis, IC₅₀ (10 μg ml⁻¹), of *S. aureus* is 10 times more potent than the MIC value (100 μg ml⁻¹) against the same *S. aureus* strain. Why the compounds discovered in this assay preferentially inhibit RNA synthesis rather than the expected protein synthesis is not clear and requires further investigation.

No compounds have earlier been reported from *N. glabra* strain, but a large number of biologically active compounds have been reported from genera *Neosartorya*, for example, the angiogenesis inhibitor, azaspirene.²¹ It seems that the most chemically studied

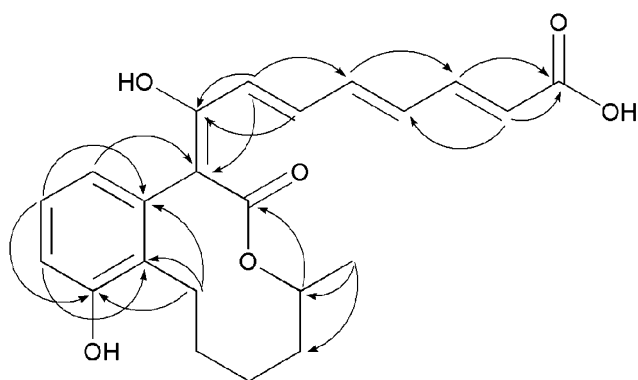


Figure 2 Heteronuclear multiple bond correlations (HMBC) of glabramycin A (**1**).

Table 2 Antibacterial activities (MIC, μg ml⁻¹)^a of glabramycins A–C (**1**–**3**)

Strains ^b	Phenotype	Strain #	1 ^c	2 ^c	3 ^c
<i>S. aureus</i>	meth ^S	ATCC 29213	>64	>64	16
<i>S. aureus</i>	meth ^S	MB2865	>64 (64)	>64	16 (8)
<i>S. aureus</i> (+50% human serum)	meth ^S	MB2865	>64	>64	>32
<i>S. pneumoniae</i> ^d	pen ^S , quin ^S , mac ^S	CL2883	>64	4	2
<i>S. pneumoniae</i> ^e	pen ^S , quin ^S , mac ^S	CL2883	32	64	2
<i>E. faecalis</i>	van ^S , mac ^R	CL8516	>64	>64	>32 (32)
<i>B. subtilis</i>	Wt	MB964	>64	>64	16
<i>H. influenzae</i>	Amp ^S , quin ^S , mac ^S	MB4572	>64	>64	>64
<i>E. coli</i>	Wt	MB2884	>64	>64	>32
<i>E. coli envA/toIC</i>	Wt	MB2884	64	>64	>32
<i>C. albicans</i>	Wt	MY1055	>64	>64	>32

Abbreviations: CAMHB, cation adjusted Mueller–Hinton broth; MIC, minimum inhibitory concentration; MIC80, minimum inhibitory concentration that inhibits 80% growth.

^aMIC determined using NLSI protocols.

^bAll strains were tested in CAMHB medium, unless mentioned otherwise, under National Committee for Clinical Laboratory Standards (NCCLS) guidelines.

^cThe data in parentheses are MIC80 values.

^dCAMHB +2.5% lysed horse blood medium.

^eIso-sensitized medium.

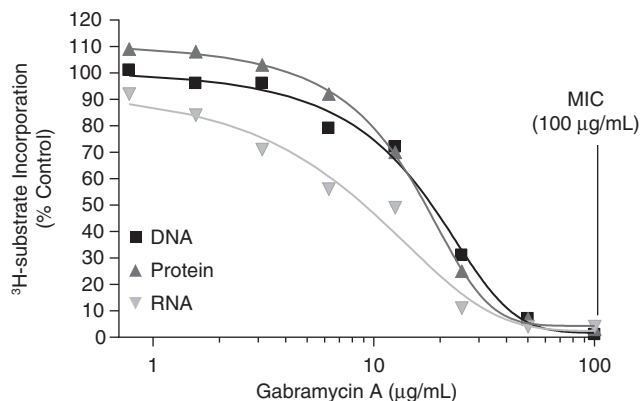


Figure 3 Macromolecular synthesis inhibition by glabramycin A (1) in *S. aureus*. No significant preference to inhibition of DNA, RNA and protein synthesis was observed.

species is *Neosartorya fischeri*, which is known to produce substance P inhibitors namely the fiscalins,²² the toxin fischerin²³ and the tremorgenic mycotoxins, fumitremorgins,²³ neosartorin,²⁴ and terreins.²⁵

In conclusion, it is evident that the antisense screening approach provides higher sensitivity and allows the discovery of antibiotics with weaker activity, exemplified by the discovery of the three new compounds, glabramycins A–C. One of the three compounds showed moderate antibacterial activity without being a general toxin and can be exploited further. Screening using an antisense sensitized *rpsD* strains of *S. aureus* led to the isolation of a number of antibacterial agents that do not preferentially inhibit protein synthesis but often RNA synthesis. Understanding of this phenomenon requires further study.^{14,15,17–19} Although lack of selectivity for protein synthesis inhibition by compounds discovered by *rpsD*-sensitized antisense screening is perplexing, it cannot be merely explained as a generalized technology artifact, because a similar screening approach using a *fabF*-sensitized strain led to the discoveries of platensimycin and platencin, highly selective fatty-acid synthesis inhibitors.^{10,11}

EXPERIMENTAL SECTION

For general experimental procedure, see, for example, Zhang *et al.*²⁶

Producing organism

The *N. glabra* (MF7030, F-155,700) strains were isolated from hot water-pasteurized soil collected from Candamia, near Valdefresno province of León, Spain. The ascospores and conidial states were observed on malt yeast extract agar and were readily recognized as a species of *Neosartorya* (Ascomycota, Eurotiales).

The DNA of strain F-155,700 was extracted and used as a template for polymerase chain reaction (PCR) reactions. The D1D2 region of the large subunit of ribosomal DNA (LSU rDNA) was amplified and sequenced to aid in identification and to infer phylogenetic relationships of the strains to other fungi. The sequences were used to query GenBank for similar ribosomal sequences. The best matches with the large subunit (LSU) region and the percentage similarities were: *N. glabra* (U28456) 99%.

Inoculum was prepared by inoculating agar plugs into a 250 ml Erlenmeyer flask containing 60 ml seed medium of the following composition: g l⁻¹ in distilled water (corn steep powder, 2.5; tomato paste, 40.0; oat flour, 10.0; glucose, 10.0; FeSO₄·7H₂O, 0.01; MnSO₄·4H₂O, 0.01; CuCl₂·2H₂O, 0.0025; CaCl₂·2H₂O, 0.001; H₃BO₃, 0.00056; (NH₄)₆MoO₂₄·4H₂O, 0.00019; ZnSO₄·7H₂O, 0.01). The pH was adjusted to 6.8 before autoclaving. The seed culture was incubated for 5 days at 22 °C on a gyratory shaker (220 rev min⁻¹)

before the inoculation of the production medium. The production medium, designated as WS80, consisted of g/l in distilled water (whole wheat flour (Pillsbury), 50; xylose, 40; and fructose, 40). The 100 ml medium aliquots was dispensed in 500 ml Erlenmeyer flasks, inoculated with 1% volume of the seed culture, and were agitated for 14 days at 22 °C.

Extraction and isolation of glabramycins

One l fermentation broth (10 flasks) was extracted with one l acetone by shaking for 1 h after the harvest. The acetone extract (2 l) was evaporated under reduced pressure to less than one liter and further diluted with 500 ml water and loaded onto a 50 cc Amberchrome CG161 m (Rohm & Haas, Reading, PA, USA) column. The column was eluted with a 100 min 10–100% aqueous methanol-gradient. The activity was detected in a late eluting band. The active fraction (600 mg) was further purified by repeated reversed-phase HPLC (50% aqueous CH₃CN + 0.1% TFA on a Zorbax SB C₈, 24×250 mm, Agilent Technologies, Santa Clara, CA, USA). The identical fractions were pooled from 10 runs and lyophilized to afford glabramycin A (7 mg, 7 mg/l), glabramycin B (1.9 mg, 1.9 mg/l) and glabramycin C (1 mg, 1 mg/l) as yellow gums.

Glabramycin A (1): [α]_D²³+7.7 (c 0.44, CH₃OH), UV (CH₃OH) λ_{max} 278 (log ε 3.73), 333 (3.70), IR (ZnSe) ν_{max} 3369, 2936, 1684, 1627, 1580, 1498, 1457, 1377, 1284, 1252, 1210, 1142, 1052, 888 cm⁻¹, High Resolution Electro-spray Ionization Fourier Transformation mass spectrometry (HRESIFTMS) (*m/z*) 385.1648 (calcd for C₂₂H₂₄O₆+H, 385.1651), for ¹H and ¹³C NMR see Table 1.

Glabramycin B (2): [α]_D²³-0.8 (c 0.6, CH₃OH), UV (CH₃OH) λ_{max} 269 (log ε 3.39), IR (ZnSe) ν_{max} 3445, 2966, 2933, 1722, 1693, 1661, 1465, 1375, 1239, 1190, 1128, 1013 cm⁻¹, HRESIFTMS (*m/z*) 387.1802 (calcd for C₂₂H₂₆O₆+H, 387.1808), for ¹H and ¹³C NMR see Table 1.

Glabramycin C (3): [α]_D²³-1.3 (c 0.6, CH₃OH), UV (CH₃OH) λ_{max} 277 (log ε 3.49), IR (ZnSe) ν_{max} 3445, 2965, 2933, 1735, 1657 1466, 1386, 1349, 1307, 1284, 1259, 1184, 1151, 1130, 1094, 1011 cm⁻¹, HRESIFTMS (*m/z*) 385.1647 (calcd for C₂₂H₂₂O₆+H, 385.1651), for ¹H and ¹³C NMR see Table 1.

Two-plate differential sensitivity *rpsD* assay

Staphylococcus aureus cells (RN450) carrying plasmid S1-782B bearing antisense to *rpsD* (AS-RNA strain) or a vector (control strain) were inoculated from a frozen vial source into a tube containing 3 ml of Miller's LB Broth (Invitrogen, Carlsbad, CA, USA) plus 34 µg ml⁻¹ of chloramphenicol. Tubes were incubated at 37 °C at 220 r.p.m. for 18–20 h and kept at room temperature (23 °C) until use. Miller's LB broth was supplemented with 1.2% Select agar (Invitrogen), 0.2% glucose, 15 µg ml⁻¹ chloramphenicol and 12 mM of xylose (only for the antisense strain). The OD₆₀₀ of the culture was measured and diluted to 1/1000, and an OD 3.0 culture was inoculated. Next, 100 ml of the culture media was poured into each NUNC plate, the well-caster templates were placed into the agar and the agar was allowed to solidify. Thereafter, 20 µl of the test samples were added to the wells and the plates were incubated at 37 °C for 18 h and zones of inhibition were measured. MDC (minimum detection concentration) values were determined by two-fold serial dilution.

Antibiotic assay (MIC)

The MIC (minimum inhibitory concentration) against each of the strains was determined as described earlier and under the guidelines of the National Laboratory Standards Institute (NLSI). The Cells were inoculated at 10⁵cfu ml⁻¹, followed by incubation at 37 °C with a 2-fold serial dilution of compounds in the growth medium for 20 h. MIC is defined as the lowest concentration of an antibiotic inhibiting visible growth.

Macromolecular synthesis inhibition

The assay was carried out as described earlier. Briefly, mid-log (*A*₆₀₀=0.5–0.6) *S. aureus* growths were incubated with an increasing concentration of each inhibitor at 37 °C for 20 min with 1 µCi ml⁻¹ 6-[³H]thymidine, 1 µCi ml⁻¹ 5,6-[³H]uracil or 5 µCi ml⁻¹ 4,5-[³H]leucine, to measure DNA, RNA and protein synthesis, respectively. The reaction was stopped by the addition of 10% trichloroacetic acid and the cells were harvested using a glass fiber filter

(Perkin-Elmer Life Sciences, Waltham, MA, USA, 1205-401). The filter was dried and counted with a scintillation fluid.

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