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Bioconversion capacity of *Streptomyces* sp GE44282, a producer of the antibiotics heneicomycin and aurodox

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***Streptomyces* sp GE44282 was isolated in the course of a screening program for novel antibiotics. It co-produces heneicomycin and aurodox, two kirromycin-type antibiotics, which differ by the presence of an hydroxyl group at the C30 position of aurodox. Heneicomycin is converted into aurodox both by growing and resting cells of *Streptomyces* sp GE44282 and by the producer of aurodox, *Streptomyces goldiniensis* ATCC 21386. This bioconversion of heneicomycin is substrate-specific and is not observed using the producer of heneicomycin, *Streptomyces filippiniensis* NRRL 11044. The three strains show very similar taxonomic characteristics. These results suggest that heneicomycin is a precursor of aurodox, the production of which depends on the bioconversion capability expressed by the strain.**

Keywords: antibiotic; fermentation; microbial transformation; hydroxylation; aurodox; heneicomycin; *Streptomyces goldiniensis*; *Streptomyces filippiniensis*

Introduction

Sixteen distinct antibiotics have been described in the literature [3] which are analogous to kirromycin (**1**) (Figure 1). These antibiotics inhibit bacterial protein synthesis by acting on the Elongation Factor Tu (EF-Tu) [15,16]. They inhibit anaerobes, neisseriae and streptococci [7,8] and are inactive against *Staphylococcus aureus* [10]. They have been developed as animal growth-promoting food additives and for the control of *Treponema hyodysenteriae* infections in pigs.

In the course of a screening program for novel antibiotics, we isolated *Streptomyces* sp GE44282 (Lepetit Culture Collection) which co-produced the kirromycin-type antibiotics aurodox (**2**) and heneicomycin (**3**) (Figure 1). These antibiotics were identified by FAB-MS, ^{1}H and ^{13}C-NMR spectroscopy and by comparison with the standard antibiotics obtained by fermentation of *Streptomyces goldiniensis* ATCC 21386 [4] and *Streptomyces filippiniensis* NRRL 11044 [22]. These streptomycetes are not reported in the literature to co-produce mixtures of aurodox and heneicomycin.

This paper describes the fermentation and the bioconversion capability of the *Streptomyces* sp GE44282 and compares the organism's taxonomy with the producers of aurodox and of heneicomycin.

Materials and methods

Microorganisms

Streptomyces sp GE44282 was isolated from a soil sample collected in Kenya. *Streptomyces goldiniensis* ATCC 21386, which produces aurodox; *Streptomyces filippiniensis* NRRL 11044, which produces heneicomycin; *Streptomyces lavendulae* ATCC 31312, which produces factumycin; and *Streptomyces cattleya* ATCC 39203, which produces L-681, 217, were all obtained from public collections [see 3 for references]. *Streptomyces* sp NRRL 15496, which produces SB22484 [17], and *Actinoplanes* sp A8924, which produces kirromycin [3], were from the Lepetit strain collection.

Taxonomy

Spore suspensions of *Streptomyces* sp GE44282, *S. goldiniensis* ATCC 21386 and *S. filippiniensis* NRRL 11044 were prepared for probabilistic identification, as described by Williams *et al* [20]. The organisms were tested for the 71 characters recorded in the major and minor probability matrices and the diagnostic table proposed by Langham *et al* [12], using the determination and test methods described by Williams *et al* [20]. Data were evaluated using the MATIDEN program [18].

Morphological observations on strain GE44282 were made on cells held on water agar (agar 15 g L^{-1} in tap water, pH 7.0) after 10–20 days incubation at 28° C. Cell wall analysis was made according to Staneck and Roberts [19].

Antibiotics

Aurodox, heneicomycin, factumycin [9], L-681, 217 [11], SB22484 [6] and kirromycin [2,21] were obtained by fermentation. They were extracted from the broth with solvents, were purified by chromatography on Sephadex LH-20 resin with methanol elution and were characterized by mass spectroscopy [5].

Fermentation

The strains were maintained on agar slants of ISP no 1 medium [1]. Precultures and fermentations were carried out at 28° C in 500-ml Erlenmeyer flasks containing 100 ml of medium stirred on a rotary shaker at 200 rpm.

Stock cultures (5 ml) of washed mycelium, maintained

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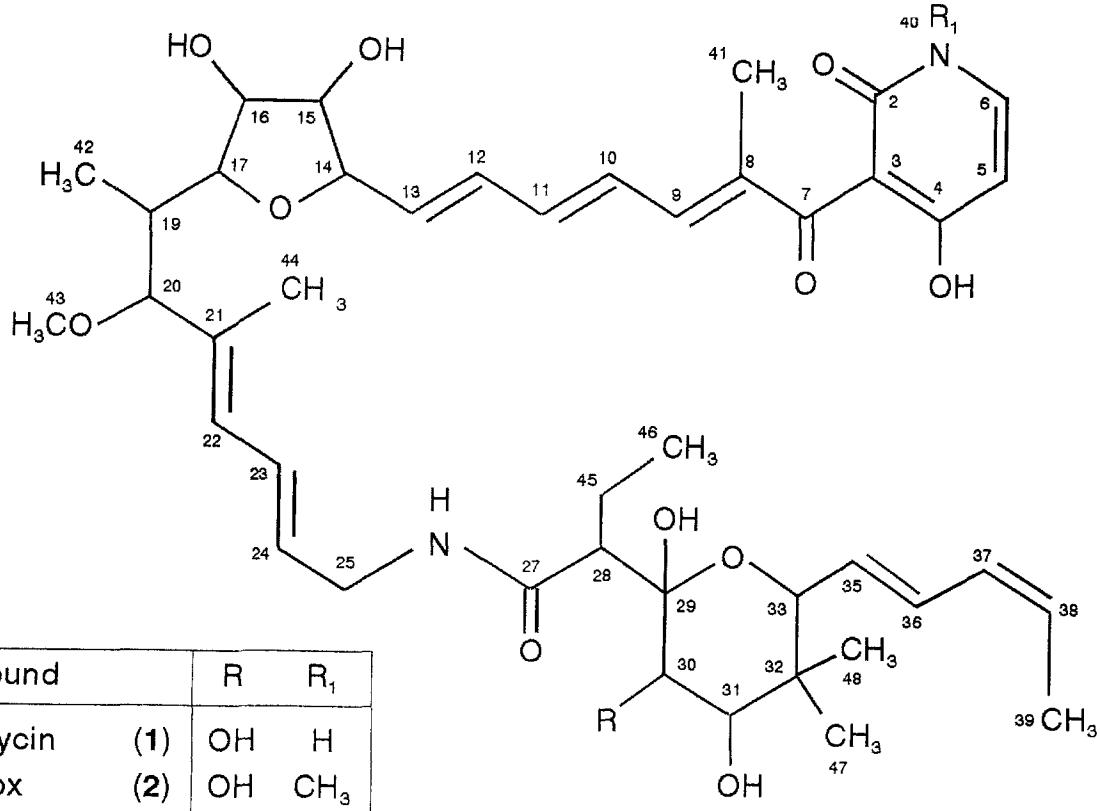

Compound		R	R_1
Kirromycin	(1)	OH	H
Aurodox	(2)	OH	CH_3
Heneicomycin	(3)	H	CH_3

Figure 1 Structures of kirromycin (1), aurodox (2) and heneicomycin (3)

frozen at −80° C, were inoculated into 100 ml of V6 medium consisting of (g L^{-1}): glucose (Carlo Erba, Milan, Italy) 10; Acas (Costantino & Co, Favria (TO), Italy) 5; yeast extract (Costantino & Co) 5; peptone (Costantino & Co) 5; casein hydrolysate (Costantino & Co) 3; NaCl 1.5; adjusted to pH 7.5 and then steam sterilized at 121° C for 20 min. After 72 h incubation, the mycelium was centrifuged at 3500 × *g* for 10 min and then was washed twice with an equal volume of sterile water. Five milliliters of the suspension were inoculated in 100 ml of the A-195 production media consisting of (g L^{-1}): glycerol (Carlo Erba) 50; corn steep (Roquette Italia, Cassano Spinola (AL), Italy) 10; soybean flour (Cerestar, Ravenna, Italy) 10; peptone (Costantino & Co) 5; NaCl 3; $CaCO_3$ 5; adjusted to pH 7.0 and steam sterilized at 121° C for 20 min. Fermentation was carried out for 168 h. Biomass was measured as the volume of the packed cells after centrifugation at 1200 × *g* for 10 min in a 50-ml graduated conical polypropylene tube (Falcon, Becton Dickinson, Oxford, UK). Concentrations of the antibiotics were determined by HPLC analysis.

Bioconversion in growing and resting cell systems

A solution of heneicomycin (5 mg ml^{-1}) was prepared in DMSO: 22 mM phosphate buffer (pH 5.8) (1:1 v/v) and was added to the culture grown for 24 h in medium A-195 to give a final concentration of 50 μg ml^{-1}. The control fermentation was in the presence of DMSO and phosphate buffer and no antibiotic.

Resting cells were prepared by washing the mycelium grown for 24 h in A-195 medium, and resuspending it in the original volume of the broth with physiological solution (NaCl 9 g L^{-1}). Heneicomycin was added to the resting cells at 25, 50, 75 μg ml^{-1} final concentrations. The kirromycin-type antibiotics SB 22484, L681, 217 and factumycin were added at 50 μg ml^{-1} concentration.

The bioconversions were carried out in 500-ml Erlenmeyer flasks as described for the fermentations.

HPLC determination of aurodox and heneicomycin in fermentation broths

The mycelium was removed by centrifugation at 3500 × *g* for 10 min. The supernatant fluid was mixed with an equal volume of acetonitrile and was centrifuged at 15000 × *g* for 2 min. The supernatant fluid was analyzed on a Beckmann ODS 4.6 × 250-mm column eluted at 1 ml min^{-1} with acetonitrile: 32 mM ammonium formate (40:60 v/v). The UV detection was at 320 nm. Kirromycin was used as the external standard. The specific absorbances of kirromycin, heneicomycin and aurodox were considered identical for the quantitative analyses. This assumption does not cause a significant error as the three antibiotics have ident-

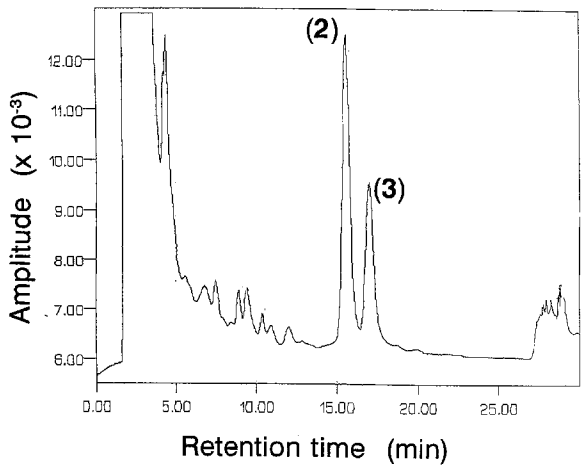

Figure 2 HPLC profile of aurodox (**2**) and heneicomycin (**3**) in the culture broth at 72 h of *Streptomyces* sp GE44282 fermentation

ical chromophores and differ structurally only in small molecular weight substituents.

A typical HPLC profile of heneicomycin and aurodox after 72 h of fermentation is shown in Figure 2.

Results

Identification of the strains

Isolate GE44282 was identified as a *Streptomyces* sp. It contains LL-diaminopimelic acid in its cell wall and, grown on water agar, it shows spore chains of the spiralis type.

The probabilistic identification of *Streptomyces* sp GE44282, *S. goldiniensis* ATCC 21386 and of *S. filippiniensis* NRRL 11044 reveals respectable Wilcox probabilities for all three strains using both of the probability matrices. The identification scores of the three strains are reported in Table 1. In an assessment of all of the data produced by the MATIDEN program, however, there were fewer characters against the identification of the three strains as *Streptomyces prasinosporus* compared to that of *Streptomyces cyaneus* for all three strains. The next best identification gave different taxa for each of the three strains in both probability matrices with a Wilcox probability of $<1 \times 10^{-4}$

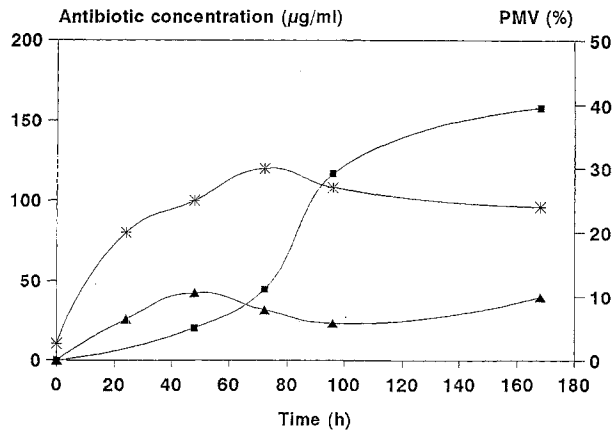

Figure 3 Growth (*) and time course of heneicomycin (▲) and aurodox (■) production in fermentation using *Streptomyces* sp GE44282

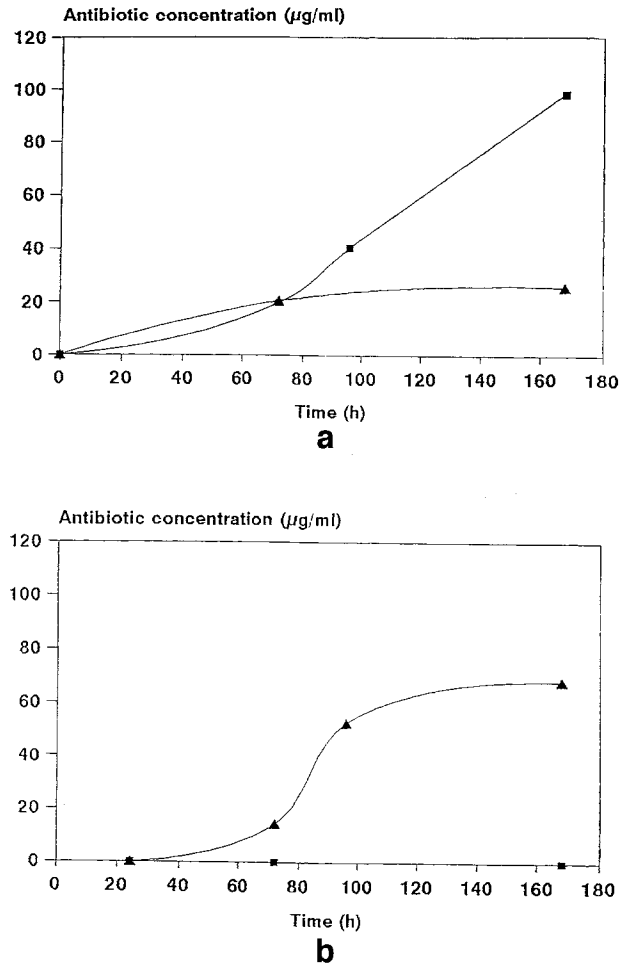

Figure 4 Time course of heneicomycin (▲) and aurodox (■) production in fermentations of *S. goldiniensis* ATCC 21386 (**a**) and *S. filippiniensis* NRRL 11044 (**b**)

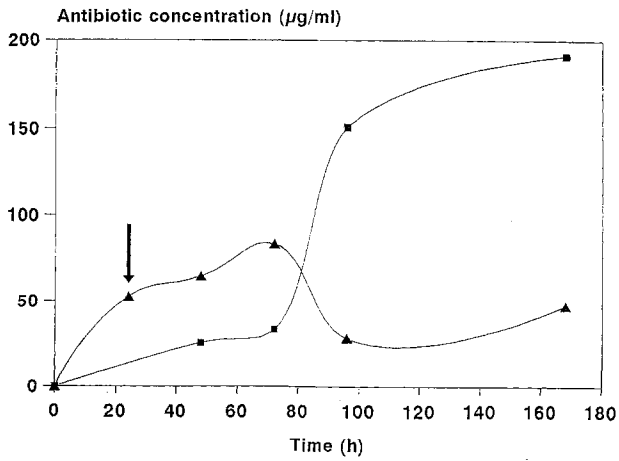

Figure 5 Heneicomycin (▲) and aurodox (■) levels in fermentation of *Streptomyces* sp GE44282 after the addition (indicated by the arrow) of 50 μg ml^{-1} exogenous heneicomycin

for the minor clusters and between 1×10^{-2} and 4.7×10^{-4} for the major clusters. Taxonomic distance and standard error values were within acceptable ranges. These data indicate that the strains are very similar.

Table 1 Probabilistic identification data using the MATIDEN program

Strain designation	Identification	Wilcox probability	Taxonomic distance	Standard error
		Major cluster probability matrix		
GE 44282	*S. cyaneus*	0.9992161	0.4584648	1.747711
S. filippiniensis	*S. cyaneus*	0.9783565	0.4896836	2.544248
S. goldiniensis	*S. cyaneus*	0.9379182	0.5057569	2.954352
		Minor cluster probability matrix		
GE 44282	*S. prasinosporus*	0.9994552	0.4541744	3.3132818
S. filippiniensis	*S. prasinosporus*	0.9997157	0.4642254	3.39639
S. goldiniensis	*S. prasinosporus*	0.9477693	0.4993509	4.317278

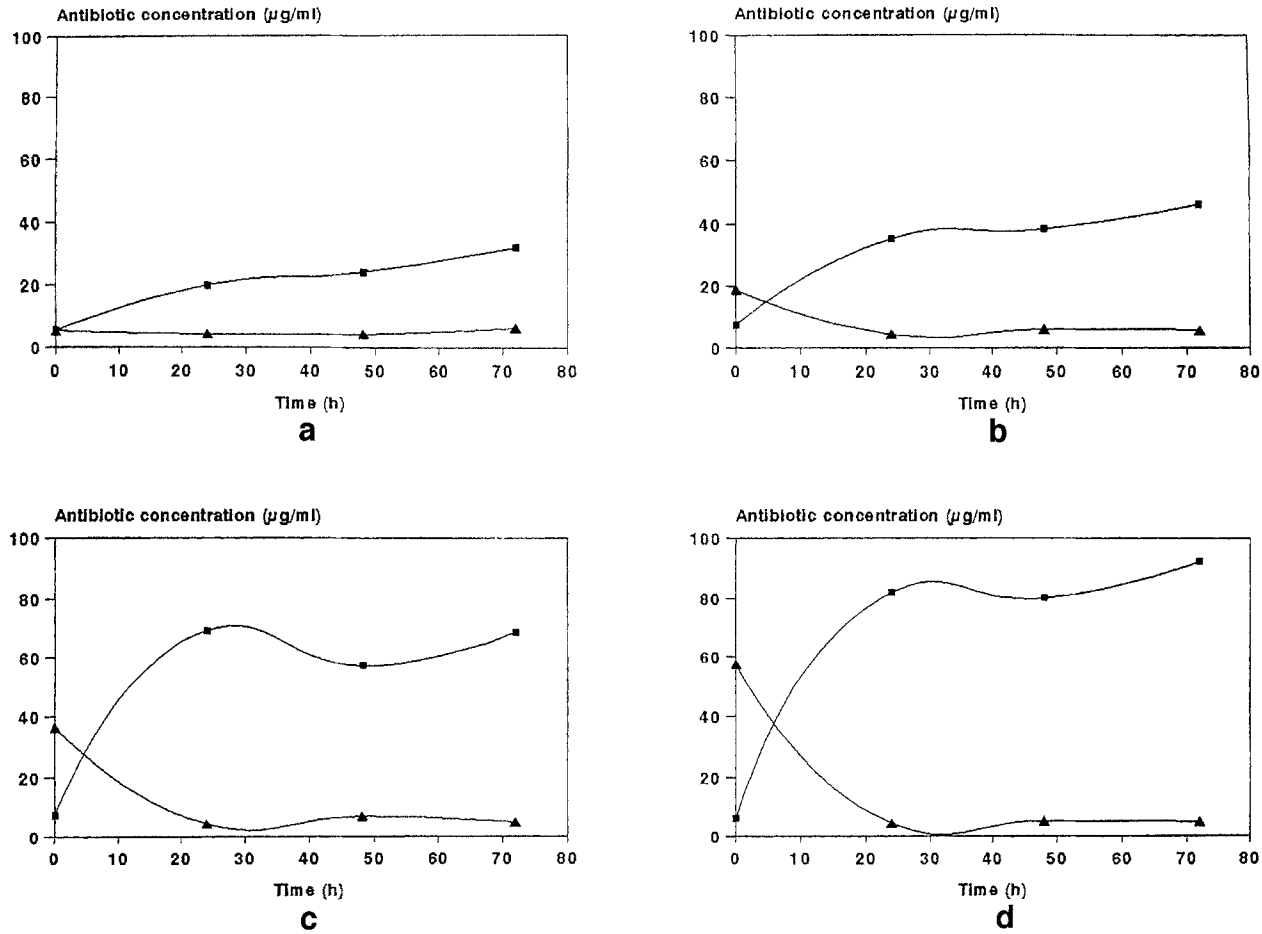

Figure 6 Heneicomycin (▲) and aurodox (■) levels after addition of 0 (**a**), 25 (**b**), 50 (**c**) and 75 (**d**) μg ml^{-1} exogenous heneicomycin to resting cells of *Streptomyces* sp GE44282

Production of heneicomycin and aurodox

The time course of fermentation in medium A-195 is shown in Figure 3. Biomass increased in the first 72 h and then remained almost constant. Heneicomycin was produced in the first 50 h up to a maximum level of 50 μg ml^{-1}, after which it decreased and remained almost constant at 30 μg ml^{-1}. Aurodox production was low during the first 60–70 h and then rapidly increased above 150 μg ml^{-1}. Medium A-195 sustained maximum production among 26 different media tested. Some media showed significant levels of production of aurodox but only traces of heneicomycin (data not shown).

In medium A-195, *S. goldiniensis* ATCC 21386 produced a low amount of heneicomycin initially and then aurodox up to 100 μg ml^{-1} (Figure 4a). The production was lower and delayed in comparison to *Streptomyces* sp GE44282. *S. filippiniensis* NRRL 11044 produced only heneicomycin with a peak concentration of 70 μg ml^{-1} after 168 h fermentation (Figure 4b).

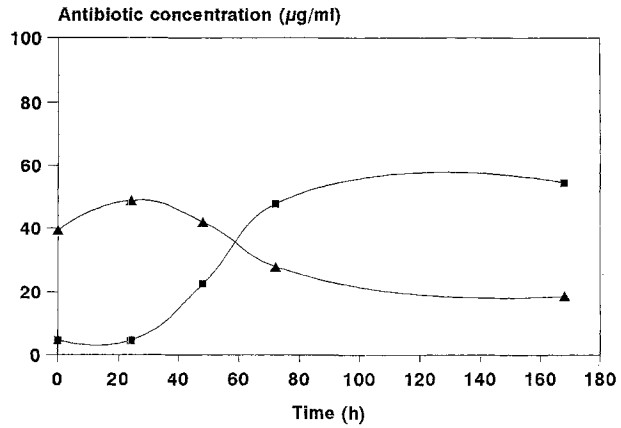

Figure 7 Heneicomycin (▲) and aurodox (■) levels afer addition of 50 μg ml^{-1} exogenous heneicomycin to resting cells of *S. goldiniensis* ATCC 21386

Bioconversion of heneicomycin

To investigate whether heneicomycin was produced and then progressively converted into aurodox, 50 μg ml^{-1} of exogenous heneicomycin was fed to *Streptomyces* sp GE44282 grown for 24 h. The viability and morphology of the strain was not altered and the total peak production increased from 160 to 190 μg ml^{-1} (Figure 5). In experiments using resting cells, *Streptomyces* sp GE44282 showed residual capacity to produce 30 μg ml^{-1} of aurodox (Figure 6a). However, when increasing amounts of exogenous heneicomycin were added (Figure 6b,c,d), the final concentrations of aurodox increased proportionally and the bioconversion was almost complete after 24 h incubation.

Bioconversion was also observed with the producer of aurodox but at a slower rate (Figure 7) whereas the producer of heneicomycin did not show any bioconversion capacity (data not shown).

The kirromycin-type antibiotics factumycin (**4**), L-681, 217 (**5**) and SB22484 (**6**) (Figure 8) were tested in the resting cell system of *Streptomyces* sp GE44282 and remained unmodified afer 168 h of incubation (data not shown).

Discussion

Streptomyces sp GE44282 co-produces the kirromycin-type antibiotics aurodox and heneicomycin, which differ structurally in the presence of an hydroxyl group on position C30 of the former. The time course of the fermentation in medium A-195 shows that peak production of heneicomycin occurs early followed by accumulation of aurodox. Moreover, the *Streptomyces* sp GE44282 transforms exogenous heneicomycin to aurodox with almost quantitative yields. These data indicate that heneicomycin is a precursor of aurodox. It appears also that the capability to bioconvert this precursor depends on the growth phase as the precursor is progressively synthesized and then converted into the final product.

These results are in accord with the polyketide biosyn-

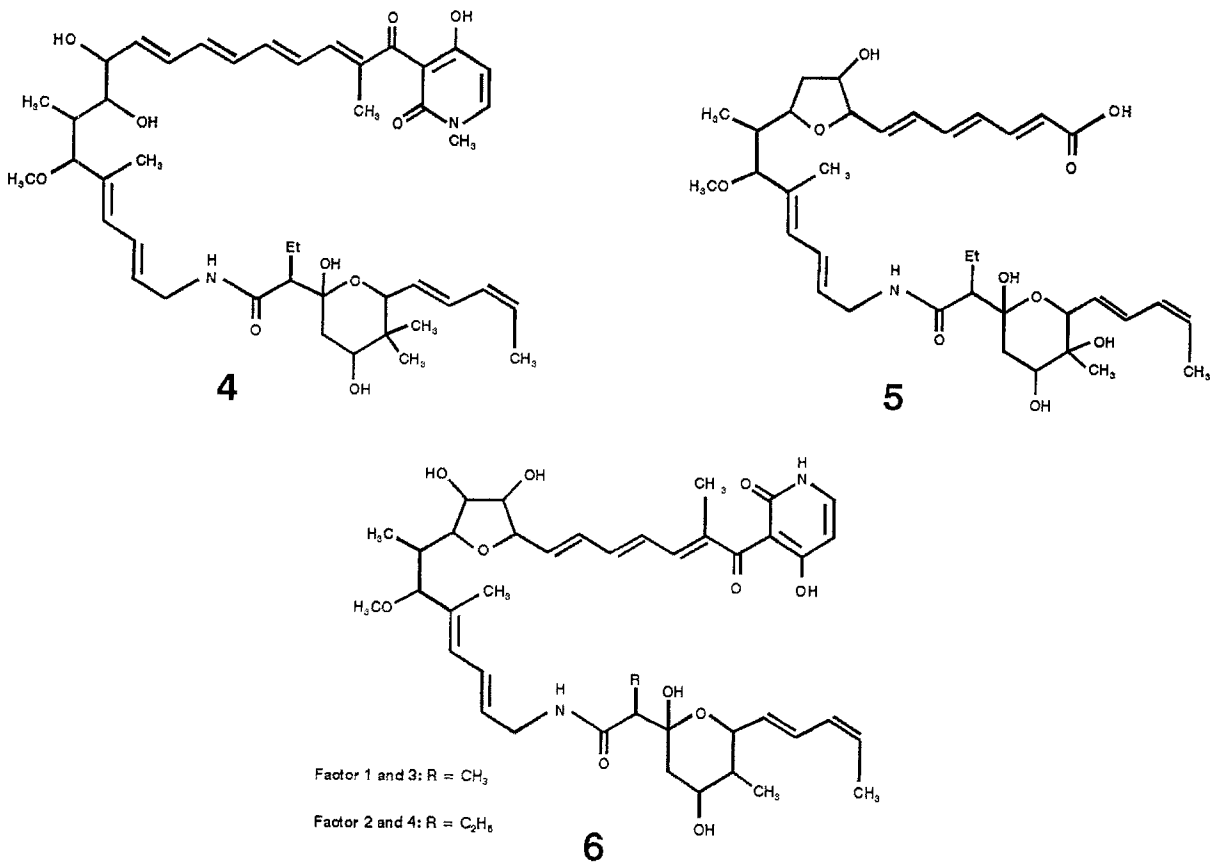

Figure 8 Structures of factumycin (**4**), L681, 217 (**5**) and SB22484 (**6**)

thesis of the kirromycin-type antibiotics [13,14]. These antibiotics are synthesised by polymerization of units of acetate-malonate. In the resulting polyketide backbone, the C30 position is derived from the methyl carbon of acetate. Consequently the C30 position is a methylene, as in the structure of heneicomycin, and the latter is the likely substrate for an additional transformation to aurodox.

In the experimental conditions described, this hydroxylation was substrate-specific. The antibiotics factumycin (**4**), L-681, 217 (**5**) and SB22484 (**6**) (Figure 8) lack the hydroxyl group at the position C30 as in the structure of heneicomycin. However, when tested, they were not bioconverted by the *Streptomyces* sp GE44282.

The producer of aurodox, *S. goldiniensis* ATCC 21386, also transforms heneicomycin into aurodox, although in a less efficient manner when compared to *Streptomyces* sp GE44282. As expected, the producer of heneicomycin does not show any bioconversion capability. This indicates that the production of heneicomycin or aurodox depends on the expression of bioconversion capability. All the strains, *Streptomyces* sp GE44282, *S. goldiniensis* ATCC 21386 and *S. filippiniensis* NRRL 11044, belong to the same taxon. They are probably natural mutants which differentiate in the productivity and capability to bioconvert heneicomycin.

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