BIOMODIFICATION OF ALBOCYCLINE BY *STREPTOMYCES VENEZUELAE*

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The macrolide antibiotic albocycline, when added to the fermentations of *Streptomyces venezuelae* UC-2560 (WC-3627) underwent a gradual loss of antimicrobial activity. The inactive product of this biotransformation was isolated from the fermentations and was shown to be 2,3-dihydroalbocycline.

Microbial modification of known antibiotics, as an approach to the search for new potentially useful antibiotics has been explored with a number of antibiotics with varying degree of success[1,2,3]. In most cases, however, substances with decreased or no antimicrobial activity were obtained. Nevertheless, these results are important since they offer a valuable insight into the structure-bioactivity relationship.

In this paper we wish to describe another case of antibiotic modification, namely the reduction of the macrolide antibiotic albocycline by *Streptomyces venezuelae* to antimicrobially inactive 2,3-dihydroalbocycline. Albocycline has been isolated from the fermentations of three strains of *Streptomyces* by NAGAHAMA, *et al.*[4,5] who also established the structure as a macrocyclic lactone without any carbohydrate moiety[6,7,8]. The same antibiotic has been isolated from the fermentations of *Streptomyces maizeus* in these laboratories where it was known for some time as ingramycin[9].

Experimental

Microorganism and Cultivation

Streptomyces venezuelae UC-2560 (WC-3627) was seeded from a soil stock into the seed medium of the following composition: glucose (25 g/liter) and Pharmamedia (25 g/liter) in tap water. Following 48 hours of growth on a rotary shaker (28°C, 250 rpm), the culture was used as the inoculum (5%) for the fermentation medium described by MAEZAWA, *et al.*[10]

Substrates for Biomodification

Albocycline was isolated from the fermentation of *Streptomyces maizeus* UC-5130 (NRRL-3508) according to published procedure[9].

Radioactive albocycline labeled with tritium in the $-OCH_3$ group (see structure in Fig. 3) was isolated from the fermentations of *S. maizeus* containing methyl labeled L-methionine (L. SLECHTA, J. CIALDELLA, unpublished).

Detection of Albocycline Modification

Five series of 250-ml Erlenmeyer flasks, each containing 25 ml of the fermentation medium, were inoculated with 1.25 ml of the seed culture of *S. venezuelae*. One series of flasks served as control. To the remaining four series, albocycline was added in increasing concentrations (50~200 μg/ml, final concentrations). The flasks were incubated on a rotary shaker (28°C, 250 rpm). One flask from each series was removed from the shaker at various times and the contents were harvested by centrifugation in a graduated centrifuge tube. The volume of the mycelial pellet served as an estimate of the *S. venezuelae* growth. The supernatant media were assayed by the paper disc-agar diffusion method against *Bacillus subtilis* UC-564 (measurement of picromycin production by *S. vene-*

zuelae) and against *Penicillium oxalicum* UC-1268 which determined specifically the concentrations of albocycline.

In the experiment with radioactive albocycline (7.95×10^5 cpm/mg), the antibiotic was added to 50 ml of inoculated medium in a 250-ml Erlenmeyer flask in the final concentration of 100 μg/ml. Aliquots of 10 ml were removed at zero time and after 24 and 48 hours of incubation. After removal of the mycelia by centrifugation, the supernatant media were used for total radioactivity measurements and thin-layer chromatography. Each sample of the medium was extracted with equal volume of Skellysolve B and the extracts were again used for radioactivity measurement and thin-layer chromatography. The samples were chromatographed on silica gel plates (Uniplate, Analtech, Inc.) with ethyl acetate - cyclohexane (1 : 1, v/v) as the solvent system. After development, 5-mm wide strips of the silica gel were scraped off the plates directly into the counting vials and the radioactivity was determined by conventional scintillation counting techniques.

Isolation of the Biomodification Product

One gram of albocycline dissolved in 100 ml of ethanol was added to 10 liters of the fermentation medium in a Virtis fermentor inoculated with *S. venezuelae*. After 72 hours incubation, when albocycline could no longer be detected by its *P. oxalicum* activity, the fermentation was harvested and extracted twice with 5 liters of Skellysolve B. The extracts were combined and evaporated in a rotary flash evaporator to yield 1.26 g of yellow oil. This material was chromatographed on a silica gel column (200 g of silica gel, 38×3.9 cm) with ethyl acetate - cyclohexane (1 : 1, v/v) as the solvent. Fractions of 100 ml were collected and analyzed by the above described thin-layer system using 40% sulphuric acid spray for detection. Fractions 6~9 were found to contain only one substance giving a dark pink color after spraying and gentle heating. The Rf of this compound was 0.57 which was in agreement with the Rf of the radioactive biomodification product detected when radioactive albocycline was used. These fractions were combined and evaporated to yield 200 mg of colorless oil. Attempts to crystallize this material were unsuccessful.

PMR spectra of the isolated product of albocycline biomodification were obtained with Varian T60 and HA100 instruments in deuterochloroform using $SiMe_4$ as internal standard. Mass spectra were determined using underivatized compounds in a Hewlett Packard 5990A GC/MS instrument.

Results

The gradual, concentration dependent inactivation of albocycline added to the fermentations of *S. venezuelae* is shown in Table 1. In the low concentrations (50 and 100 μg/ml), albocycline had no effect on the *S. venezuelae* growth and on the picromycin production. At the concentration of 150 μg/ml, the added albocycline began to exert a toxic effect on *S. venezuelae* growth and at 200 μg/ml, the growth was almost completely inhibited. This effect on growth paralleled an inhibitory effect on picromycin production.

In order to follow the biomodification of albocycline, suggested by the demonstrated loss of

Table 1. Inactivation of albocycline added to *S. venezuelae* fermentations

Concentrations of added albocycline (μg/ml)	Concentrations of albocycline during the fermentation (μg/ml)					
	Fermentation time in hours					
	0	6	12	24	36	48
0	0	0	0	0	0	0
50	50	50	29	0	0	0
100	100	100	75	24	0	0
150	150	150	120	89	68	52
200	200	200	200	139	118	104

Table 2. Analyses of the *S. venezuelae* fermentations containing radioactive albocycline (100 μg/ml, 7.95×10^5 cpm/mg).

Fermentation period (days)	Radioactivity in medium (cpm/ml)	Radioactivity in Skellysolve B Extract (cpm/ml)	Radioactivity in extracted medium (cpm/ml)
0	79,500	78,000	trace
1	74,500	31,150	42,400
2	76,000	18,200	57,000

P. oxalicum activity, radioactive albocycline was added to the *S. venezuelae* fermentation and the aliquots thereof were analyzed as described above.

The data in Table 2 show that during the incubation, gradually larger amounts of the originally extractable radioactivity of albocycline remained in the water phase. Thin-layer chromatographic analyses of the three samples of the fermentation medium are shown in Fig. 1. As may be seen, the analysis of the medium at zero time revealed the presence of radioactive albocycline (Rf=0.70, confirmed by bioautography against *P. oxalicum*). After 24 hours of incubation, large amounts of radioactivity were found on the origin of the chromatogram. In addition to radioactive albocycline, a new radioactive substance with Rf=0.57 was detected. Analysis of the medium after 48 hours of incubation, when albocycline no longer could be detected microbiologically, revealed only the presence of the new radioactive compound (Rf=0.57) in addition to radioactivity on the origin of the chromatogram.

Fig. 1. Thin-layer chromatography of *S. venezuelae* fermentation containing radioactive albocycline (100 μg/ml, 7.95×10^5 cpm/mg). The bars represent the radioactivity in 5 mm wide strips of silica gel.
A: Medium at 0 time (50 μl).
B: Medium after 24 hours of incubation (50 μl).
C: Medium after 48 hours of incubation (50 μl).

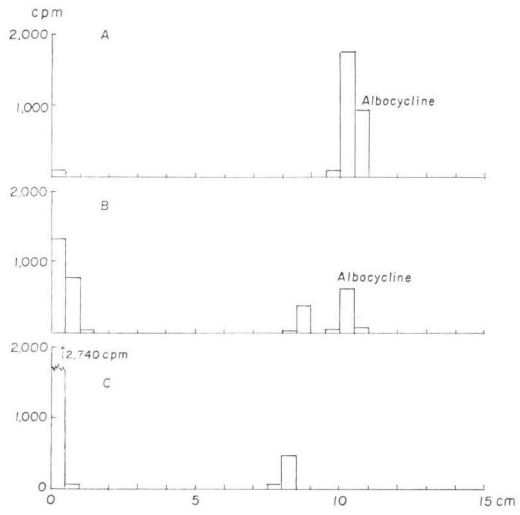
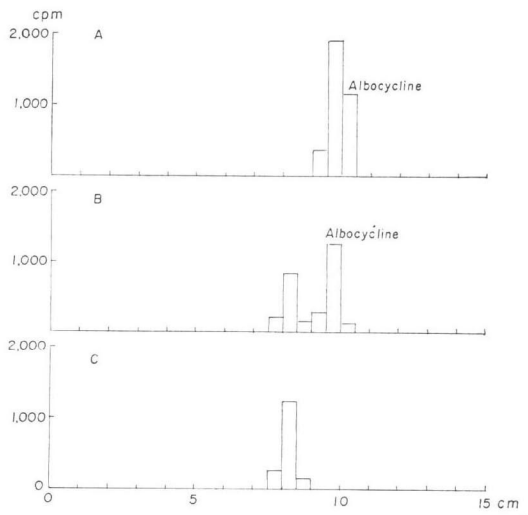


Fig. 2. Thin-layer chromatography of Skellysolve B extracts of *S. venezuelae* fermentation supplied with radioactive albocycline (100 μg/ml, 7.95×10^5 cpm/mg). The bars represent the radioactivity in 5 mm wide strips of the silica gel.
A: Extract of medium at zero time (50 μl).
B: Extract of the medium after 24 hours of incubation (100 μl).
C: Extract of the medium after 48 hours of incubation (100 μl).



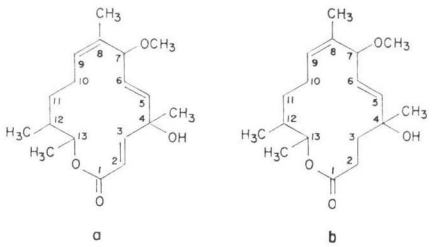
The chromatographic analyses of the Skellysolve B extracts of the three samples of the fermentation medium are shown in Fig. 2. The extract of the starting fermentation contained only the radioactive albocycline. As may be expected, the analysis of the extract of the 24-hour fermentation medium did not reveal any radioactivity at the origin of the chromatogram and only the new compound in addition to albocycline was found. The extract of the 48-hour fermentation medium, in terms of radioactivity, was found to contain only the new radioactive compound (Rf=0.57).

These results provided us with the guidelines for the isolation procedure of the product of the albomycin biomodification by *S. venezuelae* on a larger scale so that the structure could be determined and the biological properties evaluated.

Structure of the Biomodification Product

The molecular weight of the new substance determined by mass spectroscopy was 310, an increase of two mass units over that of albocycline. This suggested that hydrogenation of one of the three double bonds in the molecule of albocycline had occurred. Addition of the two hydrogens at any of these three sites could be readily recognized by the changes in the PMR spectrum. In albocycline, substituents at Δ^8 are recognized by the methyl-singlet (3H, δ 1.64) and the C-9 triplet (1H, δ 5.3); the two protons at Δ^5 are seen as a doublet (δ 5.73, $J_{5,6}$ 17 Hz) and a doublet of doublets (δ 5.70, $J_{5,6}$ 17 Hz, $J_{6,7}$ 5 Hz) whilst two doublets $J_{2,3}$ 13 Hz are ascribed to the protons at Δ^2. The latter are found at δ 6.86 and δ 5.84. The PMR spectra of the biotransformed material closely resemble those of albocycline, save for the disappearance of the δ 6.86 and δ 5.84 signals attributed to the Δ^2-protons. New absorptions between δ 1.4 and δ 2.4 equivalent to 4 protons are seen. These observations clearly show that the new substance is 2,3-dihydroalbocycline (Fig. 3).

Fig. 3. Structure of albocycline (*a*) and of 2,3-dihydroalbocycline (*b*).



Biological Evaluation of 2,3-Dihydroalbocycline

Albocycline and 2,3-dihydroalbocycline have been compared by the disc-agar diffusion test (400 μg per disc) against a variety of gram-positive and gram-negative bacteria and fungi. Albocycline was found to inhibit the growth of *P. oxalicum* and *S. aureus* as described earlier[9]. The biotransformation product, 2,3-dihydroalbocycline was completely inactive against all the microorganisms tested.

Discussion

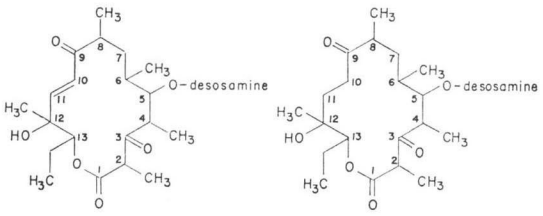
The antibiotic albocycline when added to the fermentation of *S. venezuelae*, underwent a gradual inactivation (Table 1) and the use of radioactive albocycline allowed the investigation of this process in more depth. It was found that after 48 hours of incubation, more than 70% of the label originally present in radioactive albocycline (and therefore extractable by Skellysolve B) was transformed into nonextractable materials, presumably the products of extensive degradation (Table 2).

About 23% of the albocycline label remained solvent extractable (Table 2) and the thin-layer chromatography revealed all the radio-activity to be present in a compound with a slightly different Rf from albocycline (Fig. 2). This substance was isolated and the structure was established as 2,3-dihydroalbocycline. This change in the molecule of albocycline completely abolished the antimicrobial

activity of the antibiotic.

The main antibiotic produced by the strain of *S. venezuelae* used in this study is picromycin[11,12]. Recently[13] a minor antibiotic component was isolated from *S. venezuelae* fermentations and the structure was established as 10,11-dihydropicromycin (Fig. 4). Evidence was also presented that picromycin is a precursor of 10,11-dihydropicromycin[13]. We suggest here that 2,3-dihydroalbocycline is generated by the same enzymatic mechanism since in both antibiotics the part of the molecule containing the double bond to be reduced is the same. On one carbon atom adjacent to the double bond, there is a carbonyl function and on the other adjacent carbon atom the substituents are a methyl and a hydroxyl group.

Fig. 4. Structure of picromycin (*a*) and 10,11-dihydropicromycin (*b*).



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