Effective Production of Dehydro Cyclic Dipeptide Albonoursin
Exhibiting Pronuclear Fusion Inhibitory Activity

II. Biosynthetic and Bioconversion Studies

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Albonoursin production was greatly enhanced when cyclo (L-Leu-L-Phe) (CFL), a
tetrahydro derivative of albonoursin, was added to the 2-day culture of an albonoursin-
producing actinomycete, Streptomyces albulus KO-23. The increase in albonoursin production
paralleled the amount of CFL added. Furthermore, the resting cells of the strain catalyzed the
bioconversion of CFL to albonoursin. The optimum pH and temperature for the conversion
were found to be pH 10.0 and 50°C. The feeding experiments and the resting-cell reactions
revealed that albonoursin is biosynthesized by dehydrogenation of CFL in the actinomycete.
This is the first report for a dehydrogenation of amino acid residues at the α,β-positions in
cyclic dipeptides.

Materials and Methods

Materials
Cyclo (L-Leu-L-Phe), CFL, was prepared from L-Leu-L-
Phe (SIGMA) by the method of KOPPLE and GHAZAARIAN4).
1H-NMR spectra were recorded with a Varian VXR-500
instrument. UV and MS spectra were obtained with
Shimadzu UV-3000 and JEOL SX-102A instruments,
respectively. The product is determined to be sterically pure
by comparing its optical rotation obtained with a Jasco
DIP-360 polarimeter with that of CFL reported by NITECKI et al.5)
Cyclo (L-Leu-L-Phe), CFL. [α]D20 +38.0° (c 0.1,
CH3COOH). EIMS m/z (rel. int.): 260 (M+, 26.0), 204
(39.0), 169 (24.7), 141 (34.2), 113 (25.3), 91 (100.0). IR
υmax (KBr) cm⁻¹: 3300, 3191, 1657, 1494. UV λmax
(Methanol) nm (ε): 247 (110), 252 (280), 256 (338), 263
(260). NMR δH (DMSO-d6): 0.12 (1H, m), 0.58 (3H, d, 7=
6.4Hz), 0.63 (3H, d, J=6.7Hz), 0.75 (1H, m), 1.42 (1H,
m), 2.83 (1H, dd, J=4.9, 13.4Hz), 3.13 (1H, dd, J=3.7,
13.4Hz), 3.47 (1H, m), 4.16 (1H, ddd, J=1.5, 3.7, 4.9Hz,
7.13 (2H, d, J=7.9Hz), 7.22 (1H, t, J=7.6Hz), 7.27 (2H,
dd, J=7.6, 7.9 Hz), 8.07 (1H, br.s), 8.09 (1H, br.s).

Cultivation of an Albonoursin-producing Strain
Strain KO-23 was cultivated at 28°C for 14 days on agar
slants (10 ml in 18-mm dia. tubes) of Bennett's medium
containing per liter 1 g of yeast extract (Nacalai Tesque),
1 g of beef extract (DIFCO), 2 g of NZ Amine type A
(Humko Sheffield Chemical), 10 g of glucose, and 20 g of
agar at pH 7.3. A spore and aerial mycelium suspension was prepared by adding 10 ml of sterilized water containing two drops of Triton X-100. Forty µl of the spore suspension were used to inoculate a 200-ml Erlenmeyer flask containing 40 ml of medium KP containing per liter of 15.0 g of glucose, 10.0 g of glycerol, 10.0 g of Polypepton, 10.0 g of beef extract, and 4.0 g of CaCO₃ (pH 7.3). Cultivation was carried out at 28°C on a rotary shaker (180 rpm).

Fed-batch Cultivation
After 2-day cultivation described above, CFL solution in DMSO was fed to the culture of strain KO-23, keeping the DMSO final concentration below 10%. DMSO did not affect the growth of strain KO-23 at a concentration of 10%.

Resting Cell Reaction
Resting cells were prepared by centrifugation of a culture produced on a rotary shaker (180 rpm) in 40 ml of the medium KP at 28°C for 48 hours, followed by washing the cells twice with 0.85% NaCl and resuspending them in 8 ml of the physiological saline. The final concentration of resting cells prepared by this procedure was about 250 mg of wet cells per ml. The reaction mixture consisted of 4.5 ml of the suspension of the resting cells and 0.5 ml of CFL solution in DMSO. After appropriate times of incubation at 160 strokes/minute, the reaction was terminated by adding 1-ml aliquots of the reaction mixture to the 5 ml of methanol. After removing the cells by centrifugation, the albonoursin content was determined by HPLC.

Determination of Albonoursin
Culture broth obtained by fed-batch culture was separated into the supernatant and the cells by centrifugation. The supernatant was extracted with ethyl acetate and the cells with methanol-acetone (1:1). The ethyl acetate extract of the supernatant and the methanol-acetone extract of the cells were then subjected to TLC or HPLC analysis.

TLC analysis: One µl of the methanol solution of albonoursin-containing sample was spotted on a silica gel plate (E. Merck, Kieselgel 60 F₂₅₄, Art. 5554) and developed with benzene-ethyl acetate (6:4). Albonoursin (Rf=0.67) was determined under UV light (317 nm) by a Shimadzu flying-spot scanner CS-9000 in a range of 11.4–114 ng.

HPLC analysis: The albonoursin content was determined by HPLC on an Inertsil ODS-3 column (i.d. 4.6×250 mm, GL Sciences) with UV detection at 317 nm. Albonoursin eluted in 19.0 minutes with 60% methanol at a flow rate of 1.0 ml/minute.

Results
Fed-batch Culture
Since cyclo (t-Leu-t-Phe), a tetrahydro derivative of albonoursin, was a possible biosynthetic precursor of albonoursin, we carried out experiments feeding CFL to cultures of strain KO-23. In the preceding paper, we reported that albonoursin production increased after 2-day cultivation, indicating that a biosynthetic enzyme system for albonoursin was also highly expressed after two days. When CFL in DMSO was added to the culture after 2-day cultivation, albonoursin accumulation was enhanced with the enhancement proportional to the dose of CFL, as shown in Fig. 1. This result indicated that albonoursin is biosynthesized from CFL by the strain (Fig. 2).

Resting Cell Reaction
The ability of the resting cells to catalyze the bioconversion of CFL to albonoursin was tested. The resting cells suspended in 0.85% NaCl were found to catalyze the bioconversion. In the presence of 150 mg CFL per liter in the reaction mixture, albonoursin production increased with incubation time at 37°C and went up to over 130 mg/L after
Fig. 2. Bioconversion of CFL to albonoursin catalyzed by *Streptomyces albus* KO-23.

![Cyclo (L-Leu-L-Phe) to Albonoursin](image)

Fig. 3. Optimum pH and temperature of bioconversion of CFL to albonoursin catalyzed by resting cells of *Streptomyces albus* KO-23.

**Optimum pH**

We selected a Britton-Robinson buffer system for determination of the optimum pH for this bioconversion because a wide range of pH is available in this buffer. As shown in Fig. 3A, albonoursin production from CFL by the resting KO-23 cells was found to be high at alkaline pH with a maximum at pH 10.0.

**Optimum Temperature**

We determined the optimum temperature for this bioconversion at the optimum pH of the reaction (pH 10.0). The highest production was attained at 50°C for both 1 and 2 hours incubations (Fig. 3B).

**Effect of Substrate Concentration**

Production of albonoursin increased with increasing substrate concentration at 50°C and pH 10.0 (Fig. 4). At CFL concentrations of 150 and 500 mg per liter, the substrate was completely converted to albonoursin. In the presence of 1000 mg per liter of CFL, more than 600 mg of albonoursin per liter was produced after 12-hours incubation, and the conversion was still in progress at that time. The product from CFL converted by the resting cells was
Fig. 4. Albonourin production by resting cells.

The reaction was carried out at CFL concentrations of 150 (○), 500 (●), and 1000 (□) mg per liter at 50°C and pH 10.0.

isolated and identified as 3-benzylidene-6-isobutylidene-2,5-piperazinedione by direct comparison of its spectral data with those of the compound isolated from the culture broth of *Streptomyces alkalus* KO23 or prepared from CFL converted by the cell-free extract. Furthermore, its geometry was found to be (3Z, 6Z) by NOESY analysis, indicating that the compound had the same stereochemistry as albonoursin (Fig. 2).

Characterization of Resting Cells

Strain KO-23 attained its stationary phase after 2-day cultivation under the conditions described in Materials and Methods. The specific activity for the conversion, which was represented by the amounts of albonoursin produced per mg of wet cells, remained high from 1- through 5-day cultivation. These results indicated that the resting cells obtained from 2-day cultivation were the best as a catalyst for this bioconversion.

The stability of the enzyme system in the resting cells was tested at 5 and −80°C. The cells were gradually inactivated under both conditions, but over 80% of the activity remained in the cells stored at both temperatures for 5 days.

Discussion

In this paper, we described using an albonoursin-producing actinomycete for the bioconversion of CFL to albonoursin, which is an example of the biosynthesis of dehydroamino acid-containing peptides. We have already revealed that the cell-free extract of this actinomycete catalyzed the bioconversion and that the conversion required phenazine methosulfate as a cofactor and proceeded in the absence of O₂ (data not shown). These results strongly suggested that the reaction is a dehydrogenation, not a dehydration or an O₂-dependent oxidation.

Few studies have dealt with the biosynthetic pathway for dehydroamino acid-containing peptides, although microorganisms or plants have been known to produce a variety of these peptides. However, the biosynthetic pathway has been studied in lantibiotics, bacteriocins produced by lactic acid bacteria. Lanthionine residues in lantibiotics are known to be biosynthesized by condensation of dehydroamino acid and cysteine residues, constituting a post-translational modification of peptide chains. Dehydroamino acids involved in the posttranslational modification of lantibiotics are dehydroalanine from serine and dehydrobutyrylserine from threonine by a dehydration. Therefore, the reactions involved in lantibiotic biosynthesis are found to be different from the bioconversion reaction described in this paper. A second example is tryptophan side chain oxidase reported by Takai and Hayaishi. This enzyme catalyzes the formation of dehydrotryptophan, but requires O₂ for the reaction. Thus, this enzyme is also thought to be distinct from the albonoursin-forming enzyme. These facts suggest that the enzyme system catalyzing the bioconversion of CFL to albonoursin is the first example for the dehydrogenation of amino acid residues in peptides.

High productivity of albonoursin was attained by this bioconversion system using strain KO-23 (Fig. 2). Furthermore, we found in our preliminary experiments that this system was capable of synthesizing other dehydro cyclic dipeptides from the corresponding cyclic dipeptides. Therefore, this system would be promising for the production of novel bioactive dehydro cyclic dipeptides.

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References


