VOL. 53 NO. 11, NOV. 2000 THE JOURNAL OF ANTIBIOTICS pp. 1257 — 1264

Biosynthetic Intermediates of the Tetradehydro Cyclic Dipeptide Albonoursin Produced by *Streptomyces albulus* KO-23

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(Received for publication May 22, 2000)

The cell-free extract of an albonoursin-producing strain *Streptomyces albulus* KO-23 catalyzes the conversion of cyclo(L-Leu-L-Phe) (**1**) to albonoursin (**2**). At the early stage of this conversion, two compounds were newly formed prior to albonoursin synthesis in the reaction mixture. These compounds were isolated and identified as (*Z*)-3-benzylidene-6-isobutyl-2,5-piperazinedione (**4**) and (*Z*)-3-benzyl-6-isobutylidene-2,5-piperazinedione (**3**). The cell-free extract also catalyzed the conversion of compound **3** or **4** to albonoursin. From these results, albonoursin was found to be biosynthesized *via* these compounds from cyclo(L-Leu-L-Phe). These didehydro diketopiperazines exhibited no inhibitory activity toward the first cleavage of sea urchin embryo in contrast to the higher cytotoxicity for albonoursin, indicating that dehydrogenation at α,β-positions of both amino acid residues in diketopiperazines is required for cytotoxicity.

Streptomyces albulus KO-23[1] produces albonoursin (**2**)[2], a tetradehydro derivative of cyclo(L-Leu-L-Phe), diketopiperazine (DKP) **1**. Albonoursin exhibits pronounced inhibitory activity toward pronuclear fusion of sea urchin eggs[2]. We found that the strain had a novel enzyme activity converting the DKP **1** to albonoursin, which was effectively extracted into a cell-free extract[3].

During the conversion of the DKP **1** to albonoursin, dehydrogenation occurs at two α,β-positions in both the Phe- and the Leu-residues, and therefore, the conversion is presumed to proceed *via* the formation of intermediates. In this paper, we report the biosynthetic intermediates involved in converting the DKP **1** to albonoursin.

Materials and Methods

Materials

Cyclo(L-Leu-L-Phe) (**1**) was prepared from L-Leu-L-Phe (SIGMA) by the method of KOPPLE and GHAZAARIAN[4] as described in the previous paper[5].

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 PSM agar medium containing per liter 200 g of peeled potato, 20 g of sucrose and 12 g of malt extract (DIFCO), and 15 g of agar. 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 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 $CaCO_3$ (pH 7.3). Cultivation was carried out at 28°C on a rotary shaker (180 rpm).

Preparation of Cell-free Extract

After cultivating on a rotary shaker (180 rpm) at 28°C for 48 hours, the cells were harvested by centrifugation, washed with 0.85% NaCl, and suspended in 10 mM sodium phosphate buffer (pH 8.0) to make a thick slurry. The cell-free extract was prepared by disrupting the harvested cells with an ultrasonic oscillator (150W with cooling for 2 minutes, KUBOTA Insonator 201M).

Enzyme Assay[6]

The assay for the enzyme activity converting the DKP **1** to albonoursin was carried out at 37°C by measuring an

increase in absorbance at 317 nm, λ_{max} of albonoursin. The reaction mixture contained 0.29 μmol of the DKP **1** (in 50 μl of DMSO), 4 μmol of sodium phosphate buffer (pH 8.0), and an enzyme solution in a total volume of 0.5 ml.

One unit of the conversion activity is defined as the amount of enzyme that catalyzes the formation of one μmole of albonoursin per minute under the standard conditions.

Analytical Methods

^{1}H-NMR spectra were recorded in DMSO-d_6 with a Varian VXR-500 instrument, while UV and mass spectra were obtained with Shimadzu UV-3000 and JEOL SX-102A equipment, respectively. The reaction mixture and fractions containing biosynthetic intermediates were analyzed by TLC and HPLC. TLC analyses were performed on silica-gel TLC (E. Merck, Kieselgel 60 F_{254}, Art 5554) using benzene-ethyl acetate (6:4) as the developing solvent. UV-absorption spectral analysis of compounds on TLC plates was carried out using a Shimadzu CS-9000 flying-spot scanner. HPLC analyses were performed on an Inertsil ODS-3 column (i.d. 4.6×250 mm, GL Sciences) with UV detection at 256 nm, unless otherwise stated. The DKP **1**, albonoursin (**2**), and compounds **3** and **4** eluted in 9.2, 19.0, 8.7, and 15.3 minutes, respectively, with 60% methanol at a flow rate of 1.0 ml/minute.

Conversion Reaction of the DKP 1 and Isolation of Compounds 3 and 4

A hundred and twenty-five mg of the DKP **1** was treated with the cell-free extract containing 3.1 units of the enzyme in 250 ml of reaction mixture at pH 8.0 and 37°C for 20 hours.

The precipitate in the reaction mixture, which contained a large portion of albonoursin produced, was fractionated with ethyl acetate-water and the resulting ethyl acetate soluble fraction was suspended in methanol to give a supernatant rich in compounds **3** and **4**. This fraction and an ethyl acetate extract of the supernatant of the reaction mixture were combined (41.1 mg) and subjected to preparative HPLC (ODS-3, i.d. 20×250 mm, GL Sciences), eluting with 60% methanol [10.0 ml/minute flow rate, 2-ml fractions]. The fraction rich in compounds **4** was further subjected the preparative HPLC to give compound **4** (1.69 mg). Compound **3**-rich fraction was also obtained by preparative ODS-HPLC, but was found to contain the DKP **1** by ODS-HPLC analysis with UV detection at 208 nm, not at 256 nm because the DKP **1** has little absorption at 256 nm and eluted immediately after compound **3** on ODS-HPLC with 60% methanol. The careful preparative HPLC of compound **3**-rich fraction give 1.39 mg of pure compound **3**.

Conversion Reaction of Compound 3 or 4

The reaction mixture contained 0.05 μmol of substrate in 10 μl of DMSO, 4 μmol of sodium phosphate buffer (pH 8.0), and 0.0025 units of the cell-free extract in a total volume of 0.1 ml. After incubation at 37°C for 0.5 and 24 hours, the products were determined by ODS-HPLC.

Bioassay for Cytotoxicity

The method for assaying cytotoxicity for sea urchin (*Hemicentrotus pulcherrimus*) embryos was as described previously[2]. The first cleavage of sea urchin embryos treated at doses higher than MID (minimum inhibitory dose) was blocked.

Results

Detection of Biosynthetic Intermediates of Albonoursin

We have previously reported that the cell-free extract of *Streptomyces albulus* KO-23 catalyzes the conversion of the DKP **1** to albonoursin[2]. Silicagel-TLC analysis of the reaction mixture revealed that two unknown compounds with higher polarity than albonoursin were formed at an early stage of the conversion and gradually disappeared. UV-absorption spectral analysis of these compounds using a flying-spot scanner strongly suggested that these compounds might be didehydro derivatives of the DKP **1**, in which either of the α,β-positions of the amino acid residues was dehydrogenated (Fig. 1)

Isolation of Biosynthetic Intermediates

The possible biosynthetic intermediates could not be analyzed with UV detection at 317 nm, a maximum absorption wavelength of albonoursin, at which we analyzed the reaction mixture by HPLC in the previous paper[2] describing albonoursin synthesis. Thus, in this paper, we determined the intermediates with UV detection at 256 nm. A typical chromatogram of the reaction mixture is shown in Fig. 2. HPLC analyses with several different UV wavelengths revealed that compounds **3** and **4** were cyclo(dehydroLeu-Phe), dehydroLeu-DKP, and cyclo(Leu-dehydroPhe), dehydroPhe-DKP, respectively. And therefore, the dehydroLeu-DKP **3** and the dehydroPhe-DKP **4** corresponded to the compounds having a Rf value 0.13 and 0.30, respectively, on silica-gel TLC (Fig. 1).

Fig. 1. TLC chromatogram of reaction mixture and absorption spectra of the compounds formed in the mixture.

A: Thin layer chromatogram on silica-gel of cyclo(L-Leu-L-Phe) (**1**), albonoursin (**2**), and the ethyl acetate extract of the reaction mixture.

B: UV-absorption spectra of conversion products from cyclo(L-Leu-L-Phe).

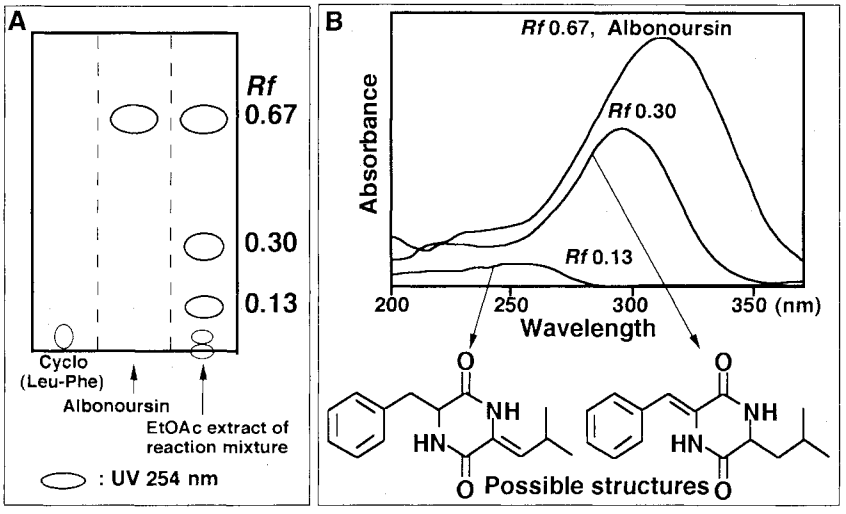
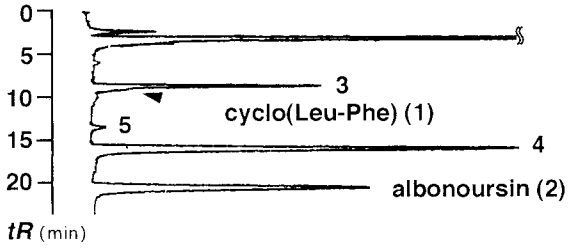


Fig. 2. Typical chromatogram of the reaction mixture on ODS-HPLC.



To isolate the biosynthetic intermediates effectively, we examined their optimal production. The optimum conditions for albonoursin synthesis by the cell-free extract[3] were expected to be inadequate for the accumulation of the biosynthetic intermediates. Thus, for the optimum production of the intermediates, we tested four different reaction conditions by varying the reaction temperature and the amounts of enzyme. As shown in Fig. 3, under the condition D, which is the best for the albonoursin production, the biosynthetic intermediates were not accumulated in the reaction mixture as expected above. We selected condition B as the best for producing compound **4**. When a heat-treated cell-free extract was used as an enzyme preparation, neither compound **3** nor **4** was detected in the reaction mixture, suggesting that the compounds shown in Fig. 3 were derived from the DKP **1** by the enzyme-catalyzed reactions.

As albonoursin has low solubility in methanol and any other solvents, it is difficult to isolate compound **3** or **4** in an albonoursin-rich fraction by chromatography. Thus, it is essential to remove albonoursin from the reaction mixture as much as possible prior to chromatography. Methanol extractions were the most effective for separation of products into albonoursin-rich and dehydro DKPs-rich fractions.

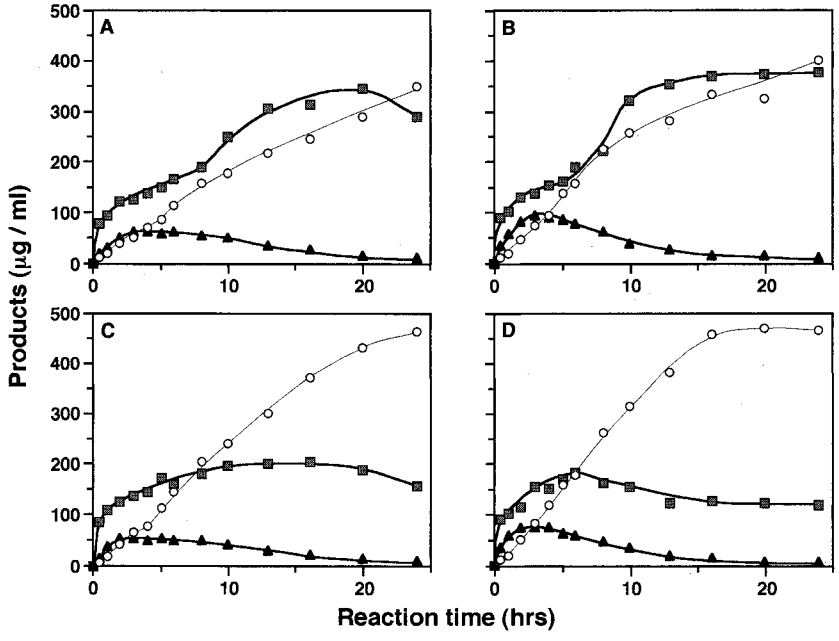
Under condition B, 125 mg of the DKP **1** was treated with cell-free extract for 20 hours. After the conversion, 1.39 mg of compound **3** and 1.69 mg of compound **4** were obtained by a combination of methanol extraction and preparative ODS-HPLC.

Identification of Intermediates

EI-mass analysis of compound **4** revealed that the compound has a molecular weight of 258, two mass-units lower than the DKP **1** and two mass-units higher than albonoursin, indicating that the compound could be a didehydro derivative of the DKP **1**. The presence of an

Fig. 3. Time-course of conversion of the DKP **1** to albonoursin.

The conversion of the DKP **1** to albonoursin was carried out under four different reaction conditions; 0.0125 units/ml at 37°C (A), 0.0125 units/ml at 50°C (B), 0.025 units/ml at 37°C (C), and 0.025 units/ml at 50°C (D). The amount of compounds **3** (▲) and **4** (■), and albonoursin **2** (○) formed in the reaction mixtures were determined by ODS-HPLC.



olefinic proton at δ 6.68 in the ^{1}H-NMR spectrum of the compound indicated that the compound has an additional double bond compared with the DKP **1**. A comparison of a UV-spectrum of compound **4** with that of the DKP **1** or albonoursin strongly suggested that the compound has a double bond at the Phe residue, not at the Leu residue. The geometry of the α,β-bond in the dehydrophenylalanine residue was assigned to be *Z* by an NOESY analysis, which indicated the presence of NOE between the NH proton at δ 9.90 and the phenyl proton at δ 7.49. From these results, compound **4** was identified as (*Z*)-3-benzylidene-6-isobutyl-2,5-piperazinedione (Fig. 4).

EI-mass, ^{1}H-NMR and UV-absorption spectral analyses suggested that the compound **3** was the dehydroLeu-DKP. The geometry of the α,β-bond in the dehydroleucine residue was assigned to be *Z* by an NOESY analysis, which indicated the presence of NOE between the NH proton at δ 9.70 and the methine proton at δ 2.61. From these results, compound **3** was identified as (*Z*)-3-benzyl-6-isobutylidene-2,5-piperazinedione (Fig. 4).

Conversion of the DidehydroDKPs **3** and **4** to Albonoursin

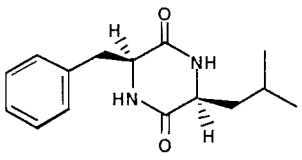
We tested the ability of the cell-free extract to catalyze the conversion of the didehydroDKPs **3** and **4** as well as the DKP **1** to albonoursin. From both of the didehydroDKPs **3** and **4**, albonoursin was formed (Fig. 5). The dehydroLeu-DKP **3** was much effectively converted to albonoursin than the dehydroPhe-DKP **4**.

Formation of Geometric Isomer of the DehydroPhe-DKP **4**

During the purification of compounds **3** and **4**, we isolated compound **5**, which eluted at 12.9 minutes on an Inertsil ODS-3 column with 60% methanol at a flow rate of 1.0 ml/minute. EI-mass and UV-absorption spectra of the compound **5** were the same as those of compound **4**. The ^{1}H-NMR spectrum of compound **5** was very similar to that of compound **4**, except that the signal of the olefinic proton in the dehydroPhe residue of compound **5** (δ 6.48) were slightly shifted upfield compared with that of compound **4** (δ 6.68), suggesting that compound **5** would be a

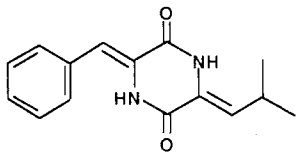
Fig. 4. Spectral data and structures of the DKP **1** and its conversion products.

Cyclo(Leu-Phe) (1)



FABMS *m/z* 261 (M+ H)$^+$
UV (MeOH) λ_{max}, nm (ε)
247 (110), 252 (280)
256 (340), 263 (260)
$[\alpha]_D^{24}$= + 38.0° (*c* 0.100, AcOH)
^{1}H-NMR, δ (500MHz, DMSO-*d* 6)
0.12 (1H, m)
0.58 (3H, d, 6.4)
0.63 (3H, d, 6.7)
0.75 (1H, m)
1.42 (1H, m)
2.83 (1H, dd, 4.9, 13.4)
3.13 (1H, dd, 3.7, 13.4)
3.47 (1H, m)
4.16 (1H, ddd, 1.5, 3.7, 4.9)
7.13 (2H, d, 7.9)
7.22 (1H, t, 7.6)
7.27 (2H, dd, 7.6, 7.9)
8.07 (1H, br.s)
8.09 (1H, br.s)

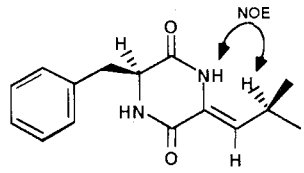
Albonoursin (2)



CIMS *m/z* 257 (M+ H)$^+$
UV (MeOH) λ_{max}, nm (ε)
235 (9000), 317 (25,400)

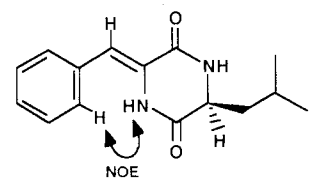
^{1}H-NMR, δ (500MHz, DMSO-*d* 6)
0.98 (6H, d, 6.7)
2.97 (1H, dsep, 6.7, 10.4)
5.70 (1H, d, 10.4)
6.74 (1H, s)
7.31 (1H, t, 7.6)
7.40 (2H, d, 7.6)
7.50 (2H, d, 7.6)
9.96 (1H, s)
10.34 (1H, s)

compound 3 (*Z*)-3-benzyl-6-isobutylidene DKP



EIMS m/z
258 (M$^+$, 100), 167 (65), 91 (70)
UV (MeOH) λ_{max}, nm (ε)
210 (11,000), 240 (9,500)
$[\alpha]_D^{19}$= -57.8° (*c* 0.116, DMSO)
^{1}H-NMR, δ (500MHz, DMSO-*d* 6)
0.70 (3H, d, 6.4)
0.83 (3H, d, 6.4)
2.61 (1H, dqq, 6.4, 6.4, 10.4)
2.89 (1H, dd, 4.9, 13.7)
3.11 (1H, dd, 4.0, 13.7)
4.29 (1H, dd, 4.0, 4.9)
5.25 (1H, d, 10.4)
7.11 (2H, d, 8.2)
7.20 (2H, dd, 7.0, 8.2)
7.22 (1H, t, 7.0)
8.20 (1H, s)
9.70 (1H, s)

compound 4 (*Z*)-3-benzylidene-6-isobutyl DKP



EIMS *m/z*
258 (M$^+$, 64), 202 (100), 173 (90)
UV (MeOH) λ_{max}, nm (ε)
224 (9,300), 307 (13,100)
$[\alpha]_D^{20}$= -54.6° (*c* 0.273, DMSO)
^{1}H-NMR, δ (500MHz, DMSO-*d* 6)
0.89 (3H, d, 6.4)
0.90 (3H, d, 6.7)
1.57 (1H, dd, 6.5, 13.8)
1.62 (1H, dd, 5.5, 13.8)
1.82 (1H, qq, 6.4, 6.7)
3.94 (1H, ddd, 2.4, 5.5, 6.5)
6.68 (1H, s)
7.30 (1H, t, 7.3)
7.39 (2H, dd, 7.3, 7.3)
7.49 (2H, d, 7.3)
8.53 (1H, d, 2.4)
9.90 (1H, s)

Fig. 5. Enzymatic conversion of the DKP **1**, the dehydroLeu-DKP **3** and the dehydroPhe-DKP **4** to albonoursin.

The □, ▨, ▨ and ▩ indicate the amounts of the DKP **1**, the dehydroPhe-DKP **4**, the dehydroLeu-DKP **3**, and albonoursin.

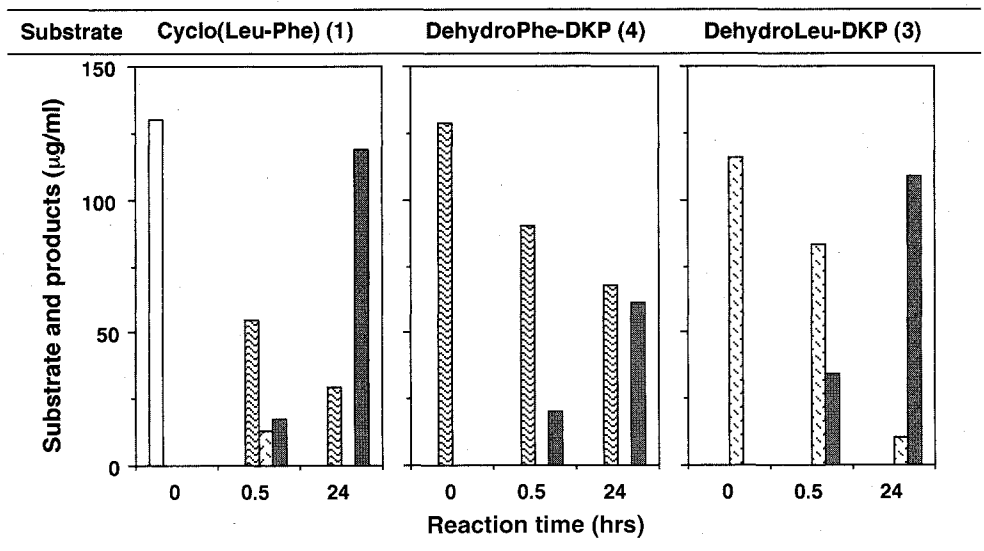
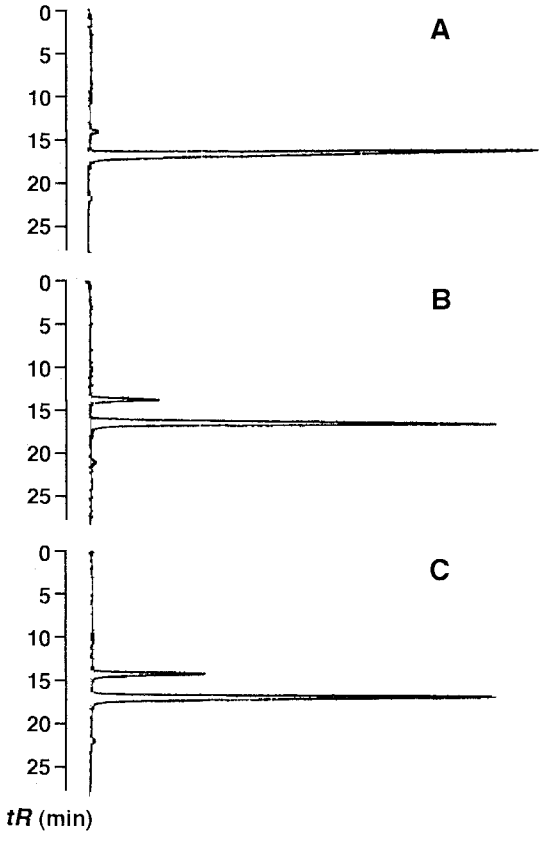


Fig. 6. Geometrical isomerization of the dehydroPhe-DKP **4**.

A methanol solution of purified (*Z*)-3-benzylidene-6-isobutyl-2,5-piperazinedione was left at room temperature for several days. After 0 (A), 3 (B), and 7 days (C), the solution was analyzed by ODS-HPLC.



geometrical isomer of compound **4**. From these data and the NOESY spectra, the compound **5** was identified as (*E*)-3-benzylidene-6-isobutyl-2,5-piperazinedione.

During the enzymatic conversion of the DKP **1**, the amount of compound **5** formed in the reaction mixture did not increase. On the other hand, purified compound **4** in methanol was spontaneously converted into compound **5** at room temperature (Fig. 6). This phenomenon was not observed at −30°C.

Cytotoxic Activity

These didehydroDKPs **3** and **4**, exhibited no inhibitory activity toward the first cleavage of sea urchin embryo in contrast to the higher cytotoxicity for albonoursin (Table 1).

Table 1. Cytotoxicity for sea urchin embryos.

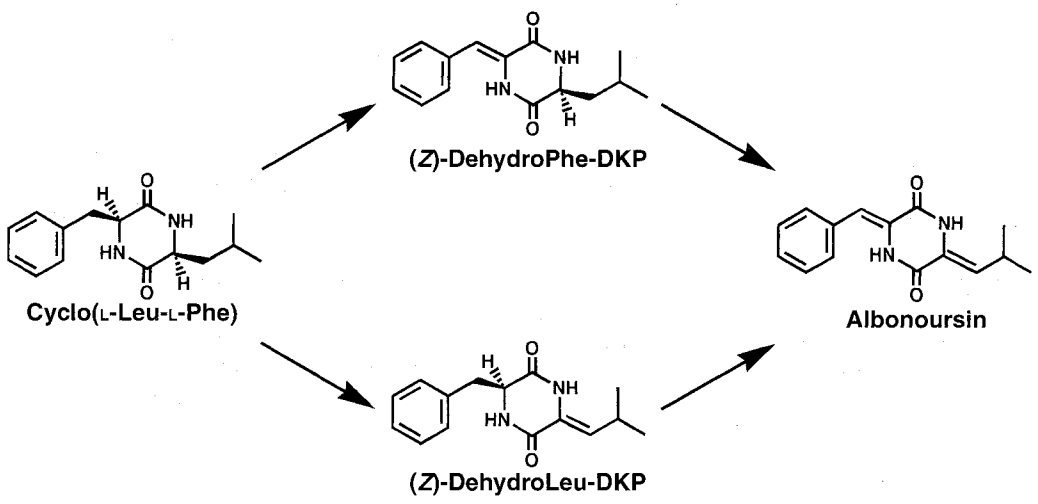
Compounds	Cytotoxicity (MID, μg/ml)
Compound **3**, (Z)-dehyroLeu-DKP	> 25.0
Compound **4**, (Z)-dehyroPhe-DKP	> 25.0
Cyclo(L-Leu-L-Phe) (**1**)	> 25.0
Albonoursin (**2**)	6.3

Discussion

We report the isolation and identification of the dehydroLeu-DKP **3** and the dehydroPhe-DKP **4** in the reaction mixture of the enzymatic conversion of the DKP **1** to albonoursin. This is the first report of the presence of these didehydro-cyclo(Leu-Phe)s in nature, although they have already been prepared by organic synthesis[7~9]. Both the didehydro-DKPs **3** and **4** were formed from the DKP **1** prior to albonoursin production and decreased during long-time incubation (Fig. 3). Furthermore, both the didehydroDKPs **3** and **4** were converted to albonoursin by the cell-free extract (Fig. 5). Therefore, albonoursin was found to be biosynthesized *via* the dehydroLeu-DKP **3** and/or the dehydroPhe-DKP **4** (Fig. 7).

As shown in the time-course experiments for albonoursin production (Fig. 3), the dehydroPhe-DKP **4** was produced more than the dehydroLeu-DKP **3** in any conditions, while the dehydroLeu-DKP **3** formed disappeared from the reaction mixture was faster than the dehydroPhe-DKP **4**. These results indicated that the conversion of the DKP **1** to the dehydroPhe-DKP **4** was much faster than that of the DKP **1** to the dehydroLeu-DKP **3**, and similarly that of the dehydroLeu-DKP **3** to albonoursin was faster than that of the dehydroPhe-DKP **4** to albonoursin (Fig. 3). The results in Fig. 5 surely indicated that the dehydroLeu-DKP **3** was a better substrate for albonoursin synthesis than the dehydroPhe-DKP **4**. From these facts, the four different reactions in the conversion of the DKP **1** to albonoursin (Fig. 7) were possibly catalyzed by at least the four distinct enzymes. This is the first report of the presence of this type of dehydrogenation enzyme, and therefore, the characterization of these enzymes will be a subject of considerable interest. Some other dehydro cyclic dipeptides and their analogs in nature might be biosynthesized by the similar enzymes.

Fig. 7. Biosynthetic pathway of cyclo(L-Leu-L-Phe) to albonoursin.



The control of these enzyme activities in the cell-free extract or cells allows us to regulate the production ratio of albonoursin, the dehydroLeu-DKP **3** and the dehydroPhe DKP **4**. Our preliminary experiments indicate that the enzymes catalyzed the conversion of several other cyclic dipeptides to their dehydro derivatives. Thus, this effective enzymatic conversion is promising for production of a variety of didehydro cyclic dipeptides as well as tetradehydro cyclic dipeptides.

We isolated (*E*)-dehydroPhe-DKP (**5**) from the reaction mixture in addition to (*Z*)-dehydroPhe-DKP (**4**). However, the content of (*E*)-dehydroPhe-DKP (**5**) did not increase during the conversion and was considerably lower than that of (*Z*)-dehydroPhe-DKP (**4**). On the other hand, (*E*)-dehydroPhe-DKP (**5**) was spontaneously formed from (*Z*)-dehydroPhe-DKP (**4**) when its solution was kept at room temperature. From these results, (*Z*)-dehydroPhe-DKP (**4**), not (*E*)-dehydroPhe-DKP (**5**), is proved to be the real intermediate in albonoursin biosynthesis. We previously reported[3] that (3*E*, 6*Z*)-3-benzylidene-6-isobutylidene-2,5-piperazinedione, a geometric isomer of albonoursin, (3*Z*, 6*Z*)-3-benzylidene-6-isobutylidene-2,5-piperazinedione, was detected in the fermentation broth of *Streptomyces albulus* KO23 in addition to albonoursin. On the other hand, neither (3*Z*, 6*E*)-3-benzylidene-6-isobutylidene-2,5-piperazinedione nor (*E*)-dehydroLeu-DKP was formed in either the fermentation broth or the enzymatic reaction mixture. The interconversion between albonoursin and (3*E*, 6*Z*)-3-benzylidene-6-isobutylidene-2,5-piperazinedione occurred in methanol solution. These results strongly suggested that geometric isomerization in the dehydrophenylalanine residue proceeded easily while that in the dehydroleucine residue was difficult.

That the dehydroLeu-DKP **3** and the dehydroPhe-DKP **4** have no cytotoxicity strongly suggests that the presence of double bonds at α,β-positions in both amino acid residues in cyclic dipeptides is required for higher cytotoxicity. This suggestion was supported by the result that 3,6-dibenzylidene-2,5-piperazinedione (didehydroPhe-DKP) exhibits pronounced cytotoxicity (data not shown). The introduction of double bonds at exo-position to the diketopiperazine ring fixes the residual side chains, and the resulting wide planar structure is possibly important for bioactivity.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture to HK (09660095). We are grateful to the SC-NMR laboratory of Okayama University and the MS laboratory of the Faculty of Agriculture, Okayama University for assistance in the ^{1}H-NMR and MS experiments, respectively. We are indebted to Mr. K. GODO of Ushimado Marine Laboratory, Faculty of Science, Okayama University, for helpful suggestions and a supply of sea urchins.

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