Cyclo(His–Phe) was effectively converted to its dehydro derivatives by the enzyme of *Streptomyces albulus* KO-23, an albonoursin-producing actinomycete. Two types of dehydro derivatives were isolated from the reaction mixture and identified as cyclo(ΔHis–ΔPhe) and cyclo(His–ΔPhe). This is the first report on cyclo(His–ΔPhe) and the enzymatic preparation of both compounds. Cyclo(ΔHis–ΔPhe), a tetra(dehydro cyclic dipeptide, exhibited a minimum inhibitory concentration of 0.78 μmol/ml inhibitory activity toward the first cleavage of sea urchin embryos, in contrast to cyclo(His–ΔPhe) that had no activity. The finding that the isoprenylated derivative of cyclo(ΔHis–ΔPhe), dehydrophenylahistin, had 2,000 times higher activity than cyclo(ΔHis–ΔPhe) indicates that an isoprenyl group attached to an imidazole ring of the compound was essential for the inhibitory activity.

**Key words:** dehydro cyclic dipeptide; enzymatic conversion; inhibitor for cell division; diketopiperazine; albonoursin

Cyclic dipeptides (CDPs), diketopiperazine ring-containing compounds, are widely distributed in nature, and their dehydro derivatives are known to exhibit unique bioactivities.\(^1\)

We have previously reported that *Streptomyces albulus* KO-23 had a biosynthetic enzyme converting cyclo(Leu–Phe), CFL, to its dehydro derivative albonoursin,\(^2,3\) and that the enzyme effectively catalyzed the bioconversion of CAF, CFF, CFG and CFV to their corresponding dehydro CDPs.\(^4\) We have recently prepared a novel dehydro CDP dehydrophenylahistin (ΔPLH; 2) from fungal metabolite (−)-phenylahistin (−)-PLH; 1) by the use of this enzyme.\(^5\)

ΔPLH (2) exhibited potent cytotoxic activity and higher than that of the known anticancer drugs, taxol, vinblastine and vincristine, indicating that this novel compound could be a candidate for an anticancer drug.\(^5\) Although there is not much difference in structure between ΔPLH [cyclo(ΔisoprenylHis–ΔPhe)] (2) and (−)-PLH [cyclo(ΔisoprenylHis–L-Phe)] (1) (Fig. 1), ΔPLH exhibited more than 1000 times the inhibitory activity toward the first cleavage of sea urchin embryos.
as (–)-PLH (1). Thus, structure-activity relationship studies are needed to develop ΔPLH and its analogs as anticancer drugs.

This paper describes enzymatic preparation of the dehydro derivatives of cyclo(His–Phe), CFH (3), which has no isoprenyl group, in contrast to PLH (1) and ΔPLH (2), which has such a group, and a comparison of their activities to those of PLH (1) and ΔPLH (2).

**Materials and Methods**

**Materials.** CFH (3) was obtained from Bachem AG (Switzerland). All other reagents were commercially available.

**Enzyme preparation and assay.** Cultivation of the albonoursin-producing strain, *S. albulus* KO-23, and preparation of its cell-free extract were carried out by the methods described in our previous paper.4) The CFL preparation of its cell-free extract were carried out by *albonoursin*-producing strain, *Hemi-centrotus pulcherrimus*, *Scaphechinus mirabilis*, and *Temnopleurus toreumaticus*, were used for the bioassay. The first cleavage of sea urchin embryos was blocked when treated at doses higher than MID (minimum inhibitory dose).

**Results**

**Enzymatic conversion of CFH**

The enzyme converting CFL to albonoursin exhibited a wide substrate specificity for cyclic dipeptides, especially those containing a Phe residue, including PLH [cyclo(ΔisoprenylHis–Phe)], a CFH analog, indicating that CFH was possibly converted to its dehydro derivatives.

The enzymatic conversion of CFH by a cell-free extract of *S. albulus* KO23 was carried out under the optimum pH (8.0) and temperature (50 °C) for albonoursin synthesis. After a 24-hr reaction, three newly produced compounds were detected at tR 4.0 (compound 4), 9.2 (compound 5) and 11.7 min (compound 6) in the HPLC chromatogram. No conversion was apparent when using a heat-denatured cell-free extract, indicating that compounds 4–6 had been formed by an enzymatically catalyzed reaction. The UV spectrum of compound 6 obtained by repetitive HPLC analyses with seven different single-wavelength detection (256, 300, 320, 340, 360, 380 and 400 nm) was very similar to that of ΔPLH, suggesting this compound to be CΔFH, a tetradehydro derivative, and that compound 5, which exhibited the same UV spectrum as that of compound 6, might be a geometrical isomer of compound 6. The UV absorption maximum at near 300 nm of compound 4 is very similar to that at 307 nm of CΔFL,7) indicating that 4 might be CΔFH, a didehydro derivative of CFH.

**Bioconversion of CFH.** The reaction mixture contained 0.05 μmol of a substrate (10 μl of DMSO), 0.9 μmol of a sodium phosphate buffer (pH 8.0) and 0.0145 units of the enzyme in a final volume of 100 μl. The reaction was carried out at 50 °C for 24 hrs and stopped by adding 900 μl of MeOH. The conversion process was monitored by HPLC as just described above.

**Isolation of the CFH dehydrogenation products.** Fifty mg of CFH (3) was treated with a cell-free extract containing 4.35 units of the enzyme in 100 ml of the reaction mixture at pH 8.0 and 50 °C for 24 hrs with shaking.

After centrifuging the reaction mixture, the supernatant was extracted with EtOAc. The EtOAc extract (11.5 mg) was subjected to preparative HPLC (ODS-3 column, φ20 × 250 mm, GL Sciences), eluting with 60% methanol [10.0 ml/min flow rate, 5-ml fractions]. This operation gave 1.15 and 2.27 mg of pure compounds 5 and 6, respectively. The remaining water-soluble fraction after EtOAc extraction was concentrated to dryness, and the resulting residue (35.9 mg) was extracted with 70% MeOH. The 70% MeOH extract was subjected to preparative HPLC under the same conditions as those just described above, giving 2.33 mg of pure compound 4.

**Bioassay for cytotoxicity.** The method for assaying the cytotoxicity toward sea urchin embryos was as previously described.6) Three species of sea urchins, *Hemi-centrotus pulcherrimus*, *Scapechinus mirabilis*, and *Temnopleurus toreumaticus*, were used for the bioassay. The first cleavage of sea urchin embryos was blocked when treated at doses higher than MID (minimum inhibitory dose).
similar under these four conditions. Thus, the optimum reaction conditions for preparing the CFH dehydro products were determined to be 0.435 units/ml of the enzyme and 50 °C. Compound 5 could hardly be detected in any sample just after the reaction, but gradually increased with the decrease of compound 6. This phenomenon strongly supports compound 5 being a geometrical isomer of compound 6.

Fifty mg of CFH was treated under the optimum reaction conditions. After EtOAc fractionation of the reaction mixture, compounds 5 and 6 were recovered in the EtOAc extract, and compound 4 was retained in the H2O layer. Finally, 2.33, 1.15 and 2.27 mg of pure compounds 4, 5 and 6 were obtained by the purification method described in the Materials and Methods section.

**Structural elucidation of compounds 4, 5 and 6**

An El-mass analysis of compound 6 revealed that it had a molecular weight of 280, four mass units lower than that of CFH, indicative of a tetrahydro derivative of CFH. This was confirmed by the molecular formula of C15H22O2N4 obtained by HREIMS measurements (Table 1). The UV spectrum of this compound (λmax, nm (ε): 205 (14,800), 351 (27,100)) was similar to that of ΔPLH (λmax, nm (ε): 205 (16,600), 363 (35,300)). In its 1H-NMR spectrum, the presence of two olefinic protons at δ 6.77 and δ 7.02 and the loss of both α-protons in the Phe and His residues of CFH strongly suggested that compound 6 was CΔFΔH. The NOESY analysis gave observed NOE between the NH proton in the ΔPhe residue (δ 8.09) and the phenyl proton (δ 7.37), and not between the NH proton and the olefinic proton (δ 7.02), indicating the geometry of the double bond in the ΔPhe residue to be Z. The amide NH proton in the ΔHis residue was observed at a relatively low magnetic field, δ 11.91, indicating the presence of a hydrogen bond between this proton and the imidazole nitrogen. NOE was not observed between the NH proton (δ 11.91) and the olefinic proton (δ 6.77) in the ΔHis residue. These data indicate the geometry of the double bond in the ΔHis residue to be Z. Compound 6 was identified from these data as 3Z-benzylidene-6Z-(1H-imidazol-4-yl-methylene)-2,5-piperazinedione (Z,Z-CΔFΔH) (Fig. 2).

The molecular weight of compound 4 was found to be 2 mass units lower than that of CFH, indicative of a didehydro derivative of CFH. This indication was confirmed by the molecular formula C13H14O2N4.
only one olefinic proton was observed in its 1H-NMR spectrum. The UV spectrum of this compound (λmax, nm (ε): 210 (13,500), 298 (12,600)) was similar to that of CΔFL (λmax, nm (ε): 224 (9,300), 307 (13,100)), suggesting that it was a ΔPhe-containing dehydro cyclic dipeptide. Only one olefinic proton was observed in its 1H-NMR spectrum, in contrast with two protons in that of compound 6. All signals in the spectrum were assigned to protons of CΔFH. NOE was observed between the NH proton in the Phe residue (δ 9.76) and the benzene proton (δ 7.40), but not between the NH proton and olefinic proton (δ 6.49), indicating that the geometry of the double bond in the ΔPhe residue was Z. Compound 4 was identified from these data as 3Z-benzylidene-6Z-(1H-imidazol-4-ylmethyl)-2,5-piperazinedione (Z-CΔFH) (Fig. 2).

**Inhibitory activity toward the first cleavage of sea urchin embryos**

As shown in Table 2, CΔFΔH exhibited more than 8 times higher inhibitory activity toward the first cleavage of sea urchin (Hemicentrotus pulcherrimus, Scaphechirus mirabilis or Temnopleurus toreumaticus) embryos than albonoursin, while CFH and CΔFH had no activity. Among these three sea urchins tested, H. pulcherrimus was found to be more sensitive to dehydro CDPs than other two sea urchins.

**Discussion**

We have reported here the preparation and identification of the dehydro derivatives of cyclo(His–Phe). This is the first report on the preparation of CΔFH. An enzymatic preparation of CΔFΔH has also never been reported before, although its organic synthesis has been achieved by Xenova Ltd. in their patent application.5) Xenova Ltd. has reported that the dehydro CDPs exhibited inhibitory activity against a plasminogen activator inhibitor, so the dehydro CDPs prepared by our enzymatic system could possibly exhibit the same activity.

Among the cyclo(His–Phe) analogs, tetradehydro derivative CΔFΔH exhibited inhibitory activity against cell division, while cyclic dipeptide CFH and didehydro derivative CΔFH did not (Table 1). This result confirmed our speculation that the presence of double bonds at the α,β-positions in both amino acid residues of CDPs was required for high inhibitory activity.4) We have previously reported that ΔPLH exhibited potent inhibitory activity toward cell division.5) Although CΔFΔH prepared in this study was structurally similar to ΔPLH, which has an isoprenyl group attached to an imidazol ring while CΔFΔH has not, its activity was 2,000 times less than that of ΔPLH. Kanoh et al. have reported that the activities of PLH analogs with a methyl group instead of an isoprenyl group were lower than that of PLH.9) These results indicate the presence of the isoprenyl group to be attributable to the expression of the higher activity of ΔPLH. In summary, the high inhibitory activity of ΔPLH is attributable to the following three structural units: (1) a uniplanar pseudo three-ring structure formed by the hydrogen bonding of diketopiperazine and imidazole rings, (2) the further widespread planar structure of ΔPhe, and (3) the isoprenyl group attached to the imidazole ring. Preliminary experiments to evaluate the bioactivity of ΔPLH indicate this compound to inhibit tubulin polymerization (data not shown). The unique structure of ΔPLH is thought to be necessary for expression of the high inhibitory activity. Novel dehydro CDPs with potent inhibitory activity for cell division will be designed and developed based on these findings.

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**References**


