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A Novel Potent Cell Cycle Inhibitor Dehydrophenylahistin

—Enzymatic Synthesis and Inhibitory Activity toward Sea Urchin Embryo—

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A novel dehydrogenated cyclic dipeptide named as dehydrophenylahistin (ΔPLH) was effectively prepared from a fungal metabolite (−)-phenylahistin by the enzymatic conversion catalyzed by the cell-free extract of *Streptomyces albulus* KO-23, an albonoursin-producing actinomycete. ΔPLH exhibited more than 1,000 times as high potent inhibitory activity toward the first cleavage of sea urchin embryos as (−)-phenylahisitn which has been reported to be a cell cycle inhibitor and more than 10,000 as high as albonoursin, indicating that ΔPLH is a promising leading compound for anticancer drugs.

Cyclic dipeptides (CDP), which are formed by cyclization of two α-amino acids, and their analogs are widely distributed in secondary metabolites of organisms[1]. Although some dehydrogenated CDPs are known to exhibit cell cycle inhibition, the systematic synthesis of dehydrogenated CDPs has not been tried because of the low yield by organic synthetic methods.

We previously reported that *Streptomyces albulus* KO-23, which produced a bioactive dehydrogenated CDP, albonoursin, had a biosynthetic pathway from cyclo (L-Leu-L-Phe), CFL, to albonoursin[2,3], and that the enzymes for albonoursin biosynthesis effectively catalyzed the bioconversion of CDPs other than CFL to the corresponding dehydrogenated CDPs[4].

Furthermore, among the dehydro derivatives of cyclic dipeptides that we prepared, tetradehydro derivatives were found to exhibit cytotoxicity, whereas didehydro derivatives or cyclic dipeptides did not[4]. These results strongly suggest the presence of double bonds at the α,β-positions in both amino acid residues, and hence a planar structure of a diketopiperazine ring with two exo double bonds is required for higher inhibitory activity.

Recently, one of the authors in this paper reported that a novel didehydro cyclic dipeptide, (−)-phenylahistin [cyclo (ΔisoprenylHis-L-Phe), (−)-PLH], isolated from a fungal culture represents a new class of colchicine-like microtubule binding agents and exhibits cytotoxicity against a variety of tumor cell lines[5~8].

Based on our assay data for dehydro CDPs and the structure of (−)-PLH, we speculated that the tetradehydro derivative of (−)-PLH might exhibit the highest cytotoxicity among the previously reported dehydrogenated CDPs, and could be a candidate for cancer chemotherapy. In this paper, we reported the effective enzymatic conversion of (−)-PLH to dehydrophenylahistin (ΔPLH) and its biological activity.

Materials and Methods

Materials

CFL was obtained from Bachem AG (Switzerland). (±)-PLH was purified from the culture of *Aspergillus ustus* NSC-F038, and (+)- and (−)-PLHs were prepared from (±)-PLH by a chiral HPLC as described in the previous paper[5].

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Preparation of the Cell-free Extract for Enzymatic Conversion

Cultivation of an albonoursin-producing strain, *Streptomyces albulus* KO-23, and preparation of its cell-free extract was carried out by the methods described in the previous paper[4].

Enzyme Assay

In the previous paper[9], phenazinemethosulfate (PMS) was found to act as a good cofactor for the conversion of CFL to albonoursin. Thus, CFL dehydrogenation activity was measured by the cofactor-coupling assay. The assay was carried out at 37°C by measuring an decrease in absorbance at 600 nm, λ_{max} of dichlorophenol indophenol (DCIP). The reaction mixture contained 0.29 μmol of CFL (in 50 μl of DMSO), 4 μmol of sodium phosphate buffer (pH 8.0), 0.06 μmol of PMS and 0.04 μl of DCIP, 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, which equals to the reduction of two μmole of DCIP, under the standard conditions in the cofactor-coupling assay.

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 conversion was monitored by ODS-HPLC: column; Inertsil ODS-3 (4.6×250 mm, GL Sciences), solvent; 70 % methanol, flow rate; 1.0 ml/minute, detection; diode array detector (HITACHI L-7455, UV 200~400 nm).

Bioconversion of PLH

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

Isolation of PLH Dehydrogenation Products

Fifty mg of (±)-PLH (containing about 30% (−)-PLH) was treated with the cell-free extract containing 3.1 units of the enzyme in 100 ml of reaction mixture at pH 8.0 and 50°C for 24 hours with shaking. During the reaction, yellow crystals were precipitated in the reaction mixture. Crystals were collected and dissolved in small amounts of MeOH for removing the inpurities from enzyme preparation. HPLC analysis indicated that the MeOH soluble fraction contained high purity compound **2**. Crystallization with MeOH/water gave 5.6 mg of pure compound **2**. The remaining solution was subjected to preparative HPLC (ODS-3, i.d. 20×250 mm, GL Sciences), eluting with 70% methanol [10.0 ml/minute flow rate, 5-ml fractions]. This preparative HPLC gave 7.9 mg of pure compound **2** and 0.9 mg of compound **1**.

Bioassay for Cytotoxicity

The method for assaying cytotoxicity for sea embryos was as described previously[10]. Three species of sea urchins, *Hemicentrotus pulcherrimus*, *Scaphechinus mirabilis*, and *Temnopleurus toreumaticus*, were used for the bioassay. The first cleavage of sea urchin embryos treated at concentrations higher than MIC (minimum inhibitory concentration) was blocked.

Results

Enzymatic Conversion of Phenylahisitn (PLH)

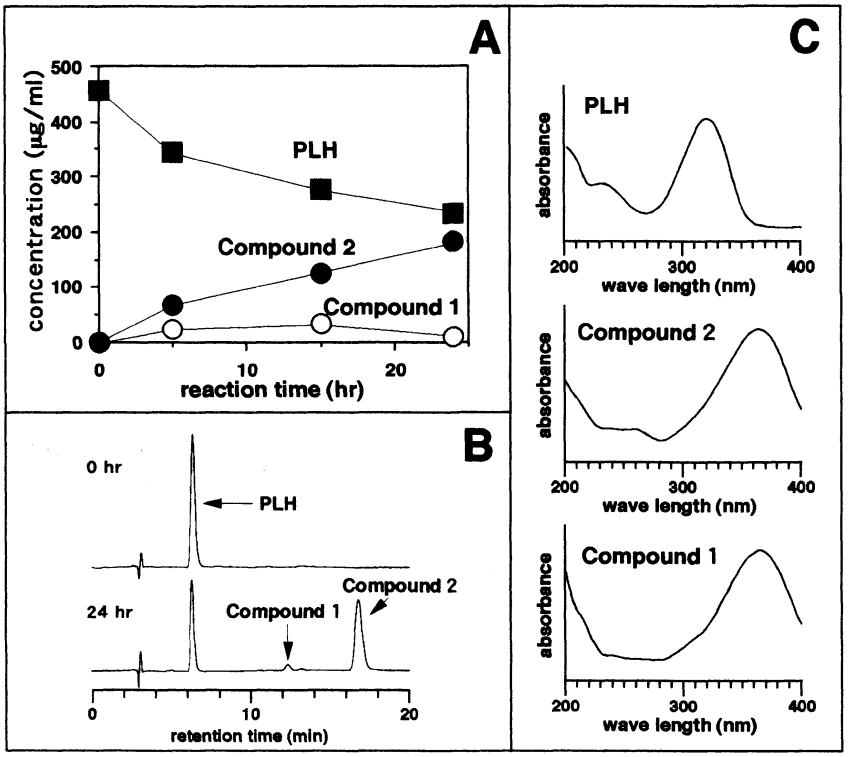
Enzymatic conversion of PLHs, (±)-PLH, (+)-PLH, and (−)-PLH, by the cell-free extract of *S. albulus* KO-23 was carried out by the method described in Materials and Methods. DAD-HPLC analysis of the reaction mixture revealed that (−)-PLH was completely consumed, while (+)-PLH was not at all (data not shown) and that the products from (−)-PLH were possibly dehydro PLH judged by their UV-spectra. (±)-PLH was also effectively converted to the products, but the substrate, possibly (+)-PLH, remains in the reaction mixture after the conversion.

Enzymatic Preparation of Compounds **1** and **2**

For preparation of the products from PLH, optimum reaction conditions were examined. (±)-PLH was used as a substrate for a large scale production because a large amounts of (−)-PLH were hard to prepare by a chiral HPLC. The substrate concentration was fixed at 0.5 mg/ml, the same as a CFL concentraton for the optimum albonoursin production. Two different enzyme concentrations, 0.145 units/ml and 0.290 units/ml, were tested. In these two conditions, the reaction rate and the amouts of products produced were almost similar. The enzyme concentration of the optimum prepartion of PLH bioconversion was determined to be 0.145 units/ml.

Figure 1 shows the conversion of (±)-PLH under the optimum reaction conditions. In the course of the reaction, compound **2** was generated as a main product with the

Fig. 1. Enzymatic conversion of (±)-phenylahistin.



A: Time course of conversion, B: ODS-HPLC chromatogram (detection: A_{340}) of EtOAc extract of reaction mixture, C: UV-spectra of phenylahistin and products.

decrease of the substrate. The UV spectrum strongly suggested that compound **2** is the dehydro product of PLH. A minor product **1**, which has the same UV spectrum as the compound **2**, is probably the geometrical isomer of **2**. As *Aspergillus ustus* produces PLH as a scalemic mixture (about 30% of (−)-PLH) not a racemate, (−)-PLH containing in the scalemic mixture of PLH was almost completely converted to compounds **1** or **2** after 24 hours incubation.

Fifty mg of (±)-PLH was treated under the optimum reaction conditions, and 13.4 mg of compound **2** and 0.93 mg of compound **1** were obtained by the purification method described in Materials and Methods.

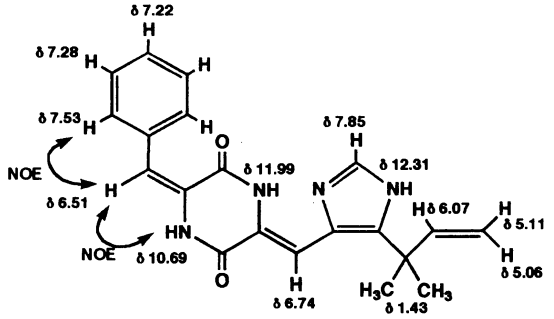
Structural Elucidation of Compounds **1** and **2**

EI-Mass analysis of compound **2** revealed that it had a molecular weight of 262, two mass units lower than that of PLH, indicative of a dehydro derivative of PLH. Comparison of UV spectra of PLH and compound **2**, and UV spectral data of other dehydro CDPs suggested that compound **2** had a double bond at a Phe residue. This is confirmed by its ^{1}H-NMR spectrum: the presence of an olefinic proton at δ 6.96 and the disappearance of methylene proton signals (δ 2.95 and δ 3.49) in PLH. The geometry of dehydrophenylalanine (ΔPhe) residue in compound **2** was assigned as *Z* because the NOE was observed between the NH proton (δ 8.04) and the benzene proton (δ 7.37), not between the NH proton and the olefinic proton (δ 6.96). From these results, compound **2** was identified as 3*Z*-benzylidene-6*Z*-[[5-(1,1-dimethyl-2-propenyl)-1*H*-imidazol-4-yl]methylene]-2,5-piperazinedione (Fig. 2).

EI-Mass, UV and ^{1}H-NMR spectra of compound **1** indicated that this compound was a geometrical isomer of compound **2**. The geometry of dehydrophenylalanine residue in compound **2** was assigned as *E* because the NOE was observed between the NH proton (δ 10.69) and the olefinic proton (δ 6.51), not between the NH proton and the benzene proton (δ 7.53).

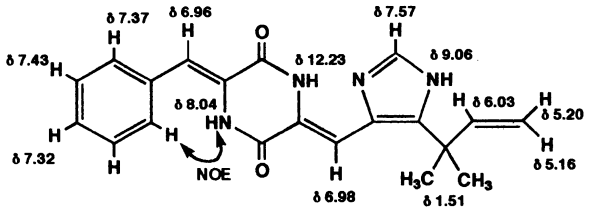
Fig. 2. Spectral data and sturctures of compounds **1** and **2** enzymatically prepared from PLH.

EIMS *m/z* : 348 (M^+, 100), 133 (29), 160 (20), 117 (15), 260 (14)
UV (MeOH) λmax, nm (ε) : 205 (14,300), 363 (24,400)
Proton-NMR (500 MHz, DMSO-*d6*) :
δ 1.43, 6H, s
δ 5.06, 1H, d (J=17.4)
δ 5.11, 1H, d (J=10.7)
δ 6.07, 1H, dd (J=10.7, 17.4)
δ 6.51, 1H, s
δ 6.74, 1H, s
δ 7.22, 1H, t (J=7.0)
δ 7.28, 2H, dd (J=7.0, 7.6)
δ 7.53, 2H, d (J=7.6)
δ 7.85, 1H, s
δ 10.69, 1H, s
δ 11.99, 1H, s
δ 12.31, 1H, br s



***(E)*-Dehydrophenylahistin (compound 1)**

EIMS *m/z* : 348 (M^+, 100), 133 (25), 160 (17), 260 (16)
UV (MeOH) λmax, nm (ε) : 205 (16,600), 363 (35,300)
Proton-NMR (500 MHz, CDCl3) :
δ 1.51, 6H, s
δ 5.16, 1H, d (J=17.4)
δ 5.20, 1H, d (J=10.7)
δ 6.03, 1H, dd (J=10.7, 17.4)
δ 6.96, 1H, s
δ 6.98, 1H, s
δ 7.32, 1H, d (J=7.0)
δ 7.37, 2H, d (J=7.3)
δ 7.43, 2H, dd (J=7.0, 7.3)
δ 7.57, 1H, s
δ 8.04, 1H, s
δ 9.06, 1H, br s
δ 12.23, 1H, s



***(Z)*-Dehydrophenylahistin (compound 2)**

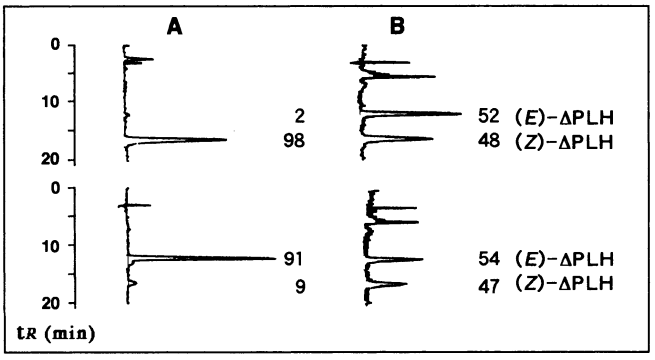
Compounds **1** and **2** are named as *Z*-dehydrophenylahistin (*Z*-ΔPLH) and *E*-dehydrophenylahistin (*E*-ΔPLH), respectively.

Interconversion between Geometrical Isomers **1** and **2**

We reported that one of albonoursin biosynthetic intermediates, *Z*-CΔFL, was spontaneously transformed to its geometircal isomer, *E*-CΔFL[11]. Thus, compound **2** was also presumed to be transformed from compound **1** without any enzymes. As shown in Fig. 3, compound **1** was generated from compound **2** in MeOH after two weeks at room temperature or *vice versa*.

From these results, it was found that the enzyme in *Streptomyces albulus* KO23 converted a Phe residue in CDP to *Z*-ΔPhe residue, not *E*-ΔPhe, and then, *Z*-ΔPhe residue formed was slowly converted to *E*-form.

Fig. 3. Geometrical isomerization of compounds **1** and **2**.



A methanol solution of compounds **1** or **2** was left at room temperature for 2 weeks. After 0 (A) and 2 weeks (B), the solution was analyzed by ODS-HPLC. The ratios of ΔPLH isomers in a mixture were listed on their peak top in HPLC chromatograms (detection: A_{340}).

Table 1. Inhibitory activity toward the first cleavage of sea urchin embryo.

Compounds	Minimum inhibitory concentration (μg/ml)		
	Hemicentrotus pulcherrimus	*Scaphechinus mirabilis*	*Temnopleurus toreumaticus*
albonoursin	6.3	>13	>13
(-)-PLH	0.39	1.6	0.20
(+)-PLH	13	>13	6.3
(*Z*)-ΔPLH	0.00038	0.0061	0.0061

Inhibitory Activity toward the First Cleavage of Sea Urchin Embryos

As shown in Table 1, ΔPLH exhibited the most potent inihbitory activity, and its activity was more than 1,000 times higher than (−)-PLH and more than 10,000 times higher than albonoursin. Furthermore pleliminary results of the cytotoxicity assay against human cancer cell lines indicated that ΔPLH exhibited the potent inhibitory activity with the IC_{50} of nM order.

Discussion

The enzymes in the cell-free extract prepared from an albonoursin-producing actinomycete, *Streptomyces albulus* KO-23, effectively catalyzed the conversion of (−)-phenylahistin, a didehydro cyclic dipeptide, to dehydrophenylahistin, a tetradehydro cyclic dipeptide. This is the first report that cyclic dipeptide dehydrogenase also catalyzes the conversion of an isoprenyl cyclic dipeptide, one of modified cyclic dipeptides, to its dehydro derivative. We reported that this cell-free extract catalyzed the conversion of several cyclic dipeptides, cyclo (Leu-Phe), cyclo (Gly-Phe), cyclo (Ala-Phe) and cyclo (Phe-Phe) or didehydro cyclic dipeptide, cyclo (ΔLeu-Phe) and cyclo (Leu-ΔPhe) to their dehydro derivatives[2~4,11]. From these results, the enzyme from *S. albulus* KO23 is found to has a wide substrate specifity for CDPs, and therefore much promising catalysis for dehydro CDP production. Utilizing this character of the enzymes, many types of dehydro CDPs can be prepared even if substrate has some functional groups, which need the protection during reactions by organic synthesis.

(+)-PLH can not be converted to its dehydro derivative by this cell-free extract, indicating that cyclic dipeptide (CDP) dehydrogenase in *S. albulus* KO-23 is highly specific to L-amino acid-containing CDPs.

We previously reported that the presence of double bonds at the α,β-positions in both amino acid residues in CDPs is required for higher inhibitory activity[11]. This speculation is confirmed by the result that ΔPLH, a tetradehydro CDP, has higher activity than PLH, a didehydro CDP. From these results for biological activity of ΔPLH, the further widespread planar structure by ΔPhe, in addition to the uniplanar pseudo-three ring structure formed by the hydrogen-bonding of diketopiperazine and imidazole rings, is found to be needed for potent inhibitory activity for cell division.

ΔPLH prepared from PLH is very potent cytotoxic activity, which is the same level of or lower level than known anticancer drugs, indicating that this novel compound will be a candidate for an anticancer drug or a lead compound for better drugs. Effective production, other bioactivities and the mode of action studies for ΔPLH are now in progress.

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