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All the eight stereoisomers of (+)-preussin (1b), an antifungal agent inhibiting the growth of fission yeast and human cancer cells, were synthesized in two steps by non-stereoselective reactions and chromatographic separation, starting from L- and D-*N*-protected-phenylalaninal (2). Their bioassay revealed all of the stereoisomers to be almost equally bioactive.

Key words: antifungal; antibacterial; cell growth inhibitor; apoptosis; preussin

Preussin (L-657,398) (**1b**) was first isolated from the fermentation broth of *Aspergillus ochraceus* ATCC22947 and *Preussia* sp.[1)] and exhibits antifungal and antibacterial activities.[2)] More recently, its selective inhibitory activity against cell growth of the fission yeast *ts* mutants defective in *cdc2*-regulatory genes[3)] and apoptosis-induction activity by inhibiting cdk2-cyclin E kinase in human cancer cells[4)] have been reported. Although a number of total syntheses of **1b** and its stereoisomers have been reported[5)] including our stereoselective synthesis,[5u)] details of the stereochemistry-bioactivity relationships have remained unclear. We report here a two-step synthesis of each of the eight stereoisomers of preussin (**1b**) and their bioactivities.

Materials and Methods

Infrared spectra were recorded by a Jasco FT/IR-620 spectrometer, and ^{1}H-NMR spectra were recorded by a Jeol JNM-A500 spectrometer (500 MHz). Chemical shift values are reported in δ ppm and referenced to the residual proton signal of $CDCl_3$ (7.24 ppm). Specific rotation values were measured with a Jasco DIP-1000 polarimeter, and mass spectra were obtained with a Jeol JMS-SX102 instrument. Open-column chromatography was performed with Fuji Silysia Chromatorex® NH (100–200 mesh), and analytical thin-layer chromatography was performed on Merck silica gel 60 F_{254}.

(2S,3RS)-2-Benzyloxycarbonylamino-3-hydroxy-1-phenyltetradecan-5-one (**3a** + **3b**). To a solution of LDA prepared from diisopropylamine (160 μl, 1.14 mmol) and *n*-butyllithium (1.61 M in hexane, 660 μl, 1.06 mmol) in THF (7.5 ml) was added a solution of 2-undecanone (200 μl, 0.969 mmol) in THF (0.5 ml) at -78°C, and the mixed solution was stirred at -78°C for 30 min. To this mixture was added a solution of (*S*)-2-benzyloxycarbonylamino-3-phenylpropanal[6)] [(*S*)-**2**, 300 mg, 1.02 mmol] in THF (1 ml) at -78°C. After the solution had been stirred at -78°C for 30 min, a solution of acetic acid (100 μl) in Et_2O (0.5 ml) was added, and the reaction mixture was diluted with EtOAc. The organic layer was successively washed with a saturated $NaHCO_3$ solution and brine, dried over $MgSO_4$ and concentrated. The residue was purified by chromatography (hexane:EtOAc = 4:1) to give a colorless waxy solid of a diastereomeric mixture of **3a** and **3b** (370 mg, 82%, **3a**:**3b** = 1:2.2). An aliquot of this mixture was chromatographed again to obtain pure **3a** and **3b** for a ^{1}H-NMR analysis. **3a**: $[\alpha]_D^{22}$ -44.0° (*c* 0.92, $CHCl_3$). FABMS *m*/*z* (MH^+): 454. HR-FABMS *m*/*z* (MH^+): calcd. for $C_{28}H_{40}NO_4$, 454.2957; found, 454.2972. IR ν_{max} (film) cm^{-1}: 3500, 3333, 2925, 2853, 1694, 1531, 1235, 1225, 1050, 750, 699. ^{1}H-NMR δ_H ($CDCl_3$, at 55°C): 0.87 (3H, t, J = 6.7 Hz), 1.24–1.30 (12 H, m), 1.50 (2H, m), 2.32 (2H, t, J = 7.3 Hz), 2.49 (1H, br. d, J = 17.7 Hz), 2.58 (1H, dd, J = 17.7, 9.2Hz), 2.90–2.92 (2H, m), 3.40 (1H, br. m), 3.75 (1H, br. m), 4.01 (1H, br. m), 5.04–5.12

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(3H, m), 7.17–7.35 (10H, m). **3b**: $[\alpha]_D^{22}$ +9.9° (*c* 1.19, $CHCl_3$). FABMS *m/z* (MH^+): 454. HR-FABMS *m/z* (MH^+): calcd. for $C_{28}H_{40}NO_4$, 454.2957; found, 454.2953. IR ν_{max} (film) cm^{-1}: 3321, 2923, 2851, 1694, 1538, 1455, 1260, 1015, 750, 699. ^{1}H-NMR δ_H ($CDCl_3$, at 55°C): 0.87 (3H, t, *J*=6.7 Hz), 1.26–1.50 (12H, m), 1.54 (2H, m), 2.35 (2H, t, *J*=7.3 Hz), 2.55 (1H, dd, *J*=17.7, 8.5 Hz), 2.62 (1H, dd, *J*=17.7, 3.1 Hz), 2.84 (1H, dd, *J*=14.0, 7.9 Hz), 2.99 (1H, dd, *J*=14.0, 4.9 Hz), 3.43 (1H, m), 3.87 (1H, br. m), 3.98 (1H, br. m), 4.72 (1H, br. m), 5.01 (2H, s), 7.17–7.33 (10H, m).

(2S,3R,5R)-2-Benzyl-3-hydroxy-1-methyl-5-nonylazolidine [(+)-**1a**], *(2S,3S,5R)-2-benzyl-3-hydroxy-1-methyl-5-nonylazolidine* [(+)-**1b**, preussin], *(2S,3R,5S)-2-benzyl-3-hydroxy-1-methyl-5-nonylazolidine* [(+)-**1c**] and *(2S,3S,5S)-2-benzyl-3-hydroxy-1-methyl-5-nonylazolidine* [(+)-**1d**]. A suspension of **3a**+**3b** (140 mg, 0.301 mmol) and 20% $Pd(OH)_2$/C (7 mg) in MeOH (9 ml) was stirred vigorously under H_2 (1 atm) at room temperature. During the first 1 h, the starting material disappeared by silica gel TLC, and the newly-appearing intermediates were slowly reduced to very polar secondary amines. After 30 h, 35% aq. HCHO (100 μl) was added to the reaction mixture, and hydrogenation was continued at room temperature for another 1 h. The reaction mixture was filtered through Celite, and the filtrate was concentrated. The residue was purified by chromatography (hexane:EtOAc=3:1, 1:1; CH_2Cl_2:MeOH=19:1, 9:1) to give a colorless oil of (+)-**1a** (60 mg, 6%), (+)-**1b** (12.5 mg, 13%), (+)-**1c** (25.7 mg, 27%) and (+)-**1d** (5.8 mg, 6%). (+)-**1a**: R_f 0.53 (50% EtOAc/hexane); $[\alpha]_D^{25}$ +0.93° (*c* 0.39, $CHCl_3$). IR ν_{max} (film) cm^{-1}: 3365, 2925, 2854, 1496, 1456, 1360, 1125, 1034, 699. ^{1}H-NMR δ_H ($CDCl_3$): δ 0.88 (3H, t, *J*=6.3 Hz), 0.96 (1H, d, *J*=3.0 Hz), 1.21–1.26 (15H, m), 1.66–1.77 (3H, m), 2.36 (3H, s), 2.44 (2H, m), 2.56 (1H, dd, *J*=13.0, 9.6 Hz), 3.07 (1H, dd, *J*=13.0, 4.4 Hz), 4.03 (1H, m), 7.22–7.31 (5H, m). (+)-**1b**: R_f 0.39 (50% EtOAc/hexane); $[\alpha]_D^{25}$ +28.7° (*c* 1.14, $CHCl_3$). The spectral data for (+)-**1b** were identical with those of our previously synthesized preussin.[5u] (+)-**1c**: R_f 0.21 (50% EtOAc/hexane); $[\alpha]_D^{25}$ +45.1° (*c* 0.315, $CHCl_3$). IR ν_{max} (film) cm^{-1}: 3335, 2925, 2854, 1496, 1456, 1030, 733, 699. ^{1}H-NMR δ_H ($CDCl_3$): 0.88 (3H, t, *J*=6.2 Hz), 1.21–1.34 (15H, m), 1.53 (1H, dd, *J*=14.2, 5.1 Hz), 1.68 (1H, m), 2.22 (1H, dd, *J*=13.4, 10.8 Hz), 2.39 (1H, ddd, *J*=14.2, 8.6, 6.4 Hz), 2.46 (3H, s), 2.69 (1H, m), 2.98 (1H, dd, *J*=13.4, 4.2 Hz), 3.18 (1H, dd, *J*=10.6, 4.2 Hz), 3.91 (1H, d, *J*=6.4 Hz), 7.14–7.31 (5H, m). (+)-**1d**: R_f 0.11 (50% EtOAc/hexane); $[\alpha]_D^{25}$ +57.1° (*c* 1.86, $CHCl_3$). IR ν_{max} (film) cm^{-1}: 3335, 2925, 2853, 1496, 1455, 1376, 1169, 1127, 1051, 742, 699. ^{1}H-NMR δ_H ($CDCl_3$): 0.88 (3H, t, *J*=6.9 Hz), 1.12–1.26 (15H, m), 1.66–1.74 (2H, m), 1.93–2.12 (1H, m), 2.45 (3H, s), 2.84–2.97 (3H, m), 3.22 (1H, m), 3.97 (1H, m), 7.19–7.30 (5H, m).

(2R,3R,5R)-2-Benzyl-1-methyl-5-nonyltetrahydro-1H-3-pyrrol [(−)-**1a**], *(2R,3R,5S)-2-benzyl-1-methyl-5-nonyltetrahydro-1H-3-pyrrol* [(−)-**1b**], *(2R,3S,5S)-2-benzyl-1-methyl-5-nonyltetrahydro-1H-3-pyrrol* [(−)-**1c**] and *(2R,3S,5R)-2-benzyl-1-methyl-5-nonyltetrahydro-1H-3-pyrrol* [(−)-**1d**]. In the same manner as that described for the synthesis of (+)-**1a-d**, (−)-**1a-d** were obtained in two steps starting from (*R*)-**2**. (−)-**1a**: $[\alpha]_D^{25}$ −1.6° (*c* 0.580, $CHCl_3$). (−)-**1b**: $[\alpha]_D^{25}$ −28.7° (*c* 1.01, $CHCl_3$). (−)-**1c**: $[\alpha]_D^{25}$ −62.9° (*c* 2.64, $CHCl_3$) (−)-**1d**: $[\alpha]_D^{25}$ −47.9° (*c* 0.365, $CHCl_3$). The IR and ^{1}H-NMR spectral data for (−)-**1a-d** were respectively identical with those for (+)-**1a-d**.

Fission yeast growth inhibition assay. The bioactivity of the synthesized stereoisomers of preussin was evaluated for their growth-inhibitory activity against the fission yeast, *Schizosaccharomyces pombe*, by a paper disk assay. The fission yeast *cdc25*^{ts} mutant, which is ultra-sensitive to natural preussin, was cultured in a YES liquid medium containing 3% glucose, 0.5% yeast extract, and 75 μg/ml each of adenine, leucine and uracil to the mid-log phase at 26.5°C, and then mixed into YES agar at the concentration of 1×10^5 cells/ml. The gradually diluted drugs were spotted on to ϕ 8 mm paper disks. These paper disks were air-dried and then placed on an agar plate containing the fission yeast cells. After cultivating at 26.5°C for 2 days, the diameter of the inhibitory zone on each paper disk was measured.

Results and Discussion

Our synthetic strategy includes an aldol reaction and reductive pyrrolidine formation of the resulting γ-amino ketone derivative (Fig. 1) which is basically the same as our previous stereoselective synthesis,[5u] except for the stereoselectivity of each reaction. The aldol reaction between the known L-*N*-Cbz-phenylalaninal [(*S*)-**2**][6] and the lithium enolate of 2-undecanone in THF gave a 1:2.2 mixture of **3a** and **3b** in an 82% yield. The stereochemistry of each product was assigned by the analogy of its ^{1}H-NMR data with those of the corresponding *N*-methoxycarbony derivative which had previously been obtained by the stereoselective aldol reaction with the zinc enolate of 2-undecanone in CH_2Cl_2.[5u] The mixture of **3a** and **3b** was then subjected to hydrogenation [H_2, $Pd(OH)_2$/C, and then aq. HCHO] which caused Cbz deprotection and intra- and intermolecular reductive amination to form *N*-methylpyrrolidine in one pot. This reaction was also non-stereoselective, and a mixture of the four diastereomers was obtained. For-

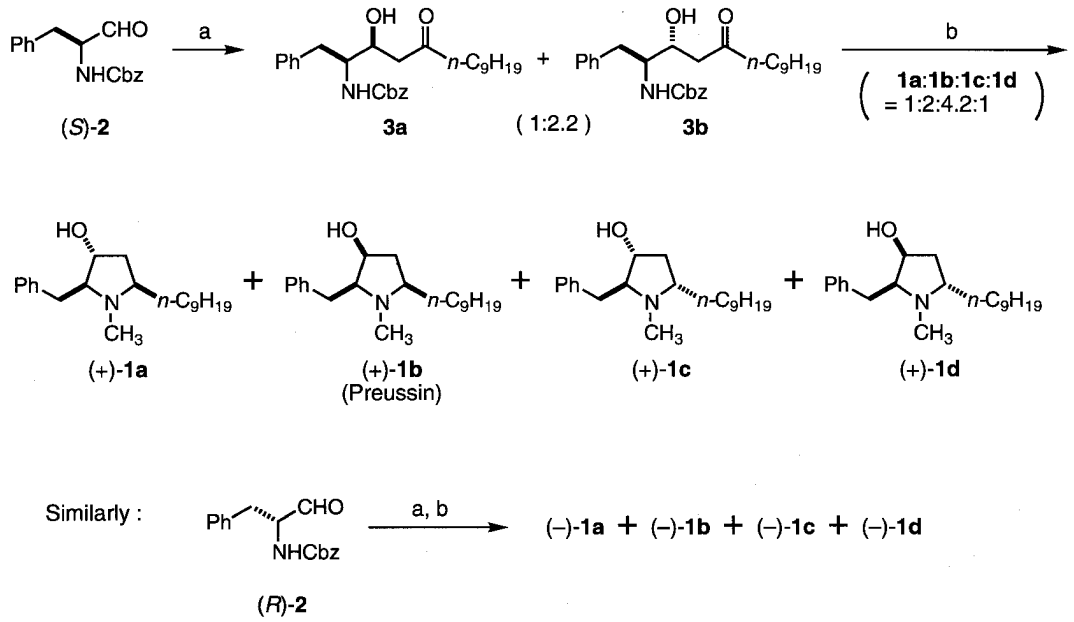

Fig. 1. Synthesis of All Stereoisomers of Preussin.
a) 2-Undecanone, LDA, THF, −78°C (82%). b) H_2, 20% $Pd(OH)_2/C$, MeOH then aq. HCHO [(+)-**1a** (6%), (+)-**1b** (13%), (+)-**1c** (27%), (+)-**1d** (6%)].

tunately, each stereoisomer was separable by chromatography with Chromatorex® NH, and pure (+)-**1a**, (+)-**1b**, (+)-**1c** and (+)-**1d** were obtained in 6%, 13%, 27% and 6% yields, respectively. The relative stereochemistry of each product was determined by comparing the ^{1}H-NMR data with those reported by Shimazaki *et al.*[5c] Similarly, (−)-**1a-d** were synthesized by starting from the (*R*)-isomer of **2**.

The inhibitory activities of the eight stereoisomers of preussin against cell growth of the fission yeast *cdc25*^{ts} mutant were tested, and the results are shown in Fig. 2. Surprisingly, the biological activity of each of these compounds was not particularly influenced by stereochemical factors, although (+)- and (−)-**1d** each showed slightly weaker activity. However, some of these compounds might exhibit inhibitory activities by enzymatic conversion from the inactive stereoisomer(s) to active isomer(s) in the yeast cell. To exclude this possibility, in the second assay, we tested the inhibitory activity against Cdk2-Cyclin E kinase *in vitro* by using the immunopurified kinase complex. However, contrary to the results previously reported,[4] preussin [(+)-**1b**] itself, as well as the other isomers, showed no inhibitory activity (data not shown). To investigate the effect of the stereochemical factors of these compounds in detail, the identification of other molecular targets of preussin is expected.

In conclusion, all the eight stereoisomers of preussin were concisely synthesized by starting from both enantiomers of **2** *via* non-stereoselective reactions and chromatographic separation. All of our synthetic diastereomers exhibited almost the same level of inhibitory activity against cell growth of the fission yeast.

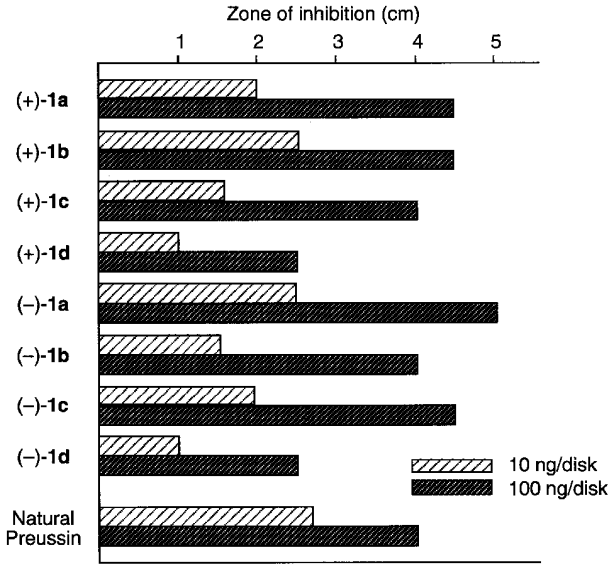

Fig. 2. Growth-inhibitory Activity by Stereoisomers of Preussin against the Fission Yeast *Cdc25*^{ts} Mutant.
The diameter of the inhibitory zone on a paper disk containing 100 ng (filled bar) and 10 ng (shaded bar) of a drug was measured.

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