component of peak group IV in a biologically active state and to ascertain the structure of this extremely important disulfide moiety.

Registry No. Vitamin K_1 reductase, 9032-20-6; vitamin K_1 2,3-epoxide reductase, 55963-40-1.

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Biological Activity and Conformational Isomerism in Position 9 Analogues of the Des-1-tryptophan,3-β-cyclohexylalanine-α-factor from *Saccharomyces cerevisiae*†

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ABSTRACT: Analogues of the des-1-tryptophan,3-β-cyclohexylalanine-α-factor of *Saccharomyces cerevisiae*, where the glycyl residue of position 9 was replaced by D-Ala, L-Ala, D-Leu, and L-Leu, were synthesized and evaluated by morphogenesis assays and circular dichroism spectroscopy. Synthesis was accomplished in solution phase with mixed anhydrides and *p*-nitrophenyl active esters as the coupling agents. All crude dodecapeptides were purified to >98% homogeneity by preparative high-performance liquid chromatography on a reversed-phase column. The Gly^9, D-Ala^9, and D-Leu^9 analogues elicited morphogenic alterations in *MATa* strains of *S. cerevisiae* at concentrations of 1–2 μg/mL and exhibited similar CD patterns in both trifluoroethanol and tris(hydroxymethyl)aminomethane buffer, pH 7.4. In contrast, the L-Ala^9 and L-Leu^9 analogues were more than 200 times less active in the morphogenesis assay and had markedly different CD spectra. These results demonstrate that the position 9 residue plays an important role in determining the biological activity and solution conformation of α-factor. We suggest the presence of a type II β-turn in the Lys^7–Gln^{10} region when the α-factor assumes its biologically active conformation.

Sexual conjugation between haploid cells of *Saccharomyces cerevisiae* is induced by peptide pheromones, termed *a* factor and α-factor. The α-factor, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr, is secreted by *MAT* α-cells and upon interaction with *MATa* cells inhibits DNA replication and cell division (Throm & Duntze, 1970). The dodecapeptide α-factor (lacking N-terminal Trp) is also biologically active. Detailed studies on structure–activity relationships in tridecapeptide and dodecapeptide pheromones have been reported

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(Masui et al., 1979; Khan et al., 1981; Samokhin et al., 1979; Shenbagamurthi et al., 1983). A preliminary NMR study on the α-factor in water led to the hypothesis of three β-turns comprising residues His^2–Gln^5, Lys^7–Gln^{10}, and Gln^{10}–Tyr^{13} of this pheromone (Higashijima et al., 1980). More recently it has been concluded that the N-terminus of α-factor is α-helical and that β-turns are present in the central and C-terminal domains of the peptide (Higashijima et al., 1984).

The α-factor contains a Pro-Gly sequence at positions 8 and 9 in its primary sequence. Statistical analyses of over 400 β-turns in 29 globular proteins suggest that the Pro-Gly sequence has a high probability of forming a β-turn (Chou & Fasman, 1978). Indeed, several studies on low molecular weight model compounds showed that the Pro-Gly unit can be readily accommodated in such chain reversals (Bush et al., 1978; Gierasch et al., 1981; Urry et al., 1974). Venkatachalam (1968) conducted studies on steric interactions in systems containing three peptide bonds. These studies concluded that a type II β-turn $\phi_2\psi_2$ (−60°, 120°), $\phi_3\psi_3$ (80°, 0°) can be formed if the $i + 1$ residue is proline and the $i + 2$ residue is glycine. Moreover, these studies predicted that whereas an L-Ala residue in position $i + 2$ would destabilize such a turn, a D-Ala residue would be readily accommodated.

In previous studies on structure–activity relationships in the des-Trp^1,Cha^3-α-factor,[1] we found that replacement of Gly^9 by D-Ala^9 did not diminish the morphogenic activity of the pheromone (Shenbagamurthi et al., 1983). This finding would be expected if the Pro^8-Gly^9 residues were part of a type II β-turn. We decided to study further the Lys^7–Gln^{10} domain of α-factor in an attempt to correlate primary structure, biological activity, and conformation. Accordingly, we have synthesized analogues in which Gly^9 was replaced by D-Ala, L-Ala, D-Leu, or L-Leu. In this paper we report the synthesis, biological activity, and circular dichroism of the position 9 analogues. Our studies provide evidence that the Pro^8-Gly^9 residues of the α-factor exist in a type II β-turn when the peptide assumes its biologically active conformation.

Materials and Methods

Chemicals. All amino acids were of the L configuration unless otherwise specified. Most of the protected amino acid derivatives were purchased from Bachem. β-Cyclohexyl-L-alanine (Cha), Boc-Cha, and Boc-D-Leu were prepared in our laboratory by standard procedures and were characterized by thin-layer chromatography, melting points, and optical rotation. All the solvents used were analytical grade supplied by Fisher.

Analytical Procedures. Melting points were determined by capillary method and are uncorrected. High-performance liquid chromatography (HPLC, analytical) was carried out on a Waters system consisting of a M-6000 solvent pump and U6K injector, linked to a Waters 450 variable-wavelength UV monitor with an 8-μL flow cell. The μBondapak C_{18} column (10 μm, 30 cm × 0.39 cm i.d.) was also from Waters. All solvents were HPLC grade (Fisher); water was glass-distilled. Purity of samples was determined with two mobile phases (methanol–water–trifluoroacetic acid and acetonitrile–water–trifluoroacetic acid). Detection was usually at 220 nm. A waters Prep LC/System 500 was used for the purification of the dodecapeptides. Optical rotations of the peptide were determined in the solvents indicated in parentheses where the concentrations are in grams of peptide per 100 mL of solvent. The circular dichroism spectra were recorded on a Jasco Model J-500C spectropolarimeter. All spectra were taken at room temperature. Cells with path lengths of 0.01, 0.02, and 0.2 cm were employed.

Amino acid analyses were performed at the Weizmann Institute, Rehovot, Israel, or at Hoffmann-La Roche, Nutley, NJ. Peptides were hydrolyzed in sealed tubes in 6 N HCl at 100 °C for 24 h. For peptides containing methionine, anisole was added as a scavenger.

Synthesis of Des-Trp^1,Cha^3,X^9-α-factor Analogues. Synthesis of all peptides was achieved in solution with mixed anhydrides (Meienhofer, 1979) and 1-hydroxybenzotriazole-accelerated active ester coupling procedures (Bodansky, 1979). The des-Trp^1,Cha^3,X^9 analogues (X = L-Ala, L-Leu, or D-Leu) were prepared by coupling the heptapeptide Boc-His(Boc)-Cha-Leu-Gln-Leu-Lys(Z)-Pro to various pentapeptides, X-Gln-Pro-Met-Tyr-OBzl, as previously described for the synthesis of the des-Trp^1,Cha^3,D-Ala^9 analogue (Shenbagamurthi et al., 1983). The different pentapeptides were prepared in a stepwise fashion starting with Boc-Met and Tyr-OBzl. The protected dodecapeptides were subjected to transfer hydrogenation and acidolysis and then purified on a C_{18} reversed-phase column. Homogeneity of all peptides was confirmed by silica thin-layer chromatography employing the following solvent systems: (A) dichloromethane–methanol–acetic acid (20:3:1.0); (B) 1-butanol–acetic acid–water (4:1:5, upper phase); (C) 1-butanol–acetic acid–water–pyridine (15:3:12:10). R_f values were reported as R_f(A), R_f(B), and R_f(C). Details of the synthesis are given below.

Synthesis of Boc-X-Gln-Pro-Met-Tyr-OBzl. (*A*) *Boc-Met-Tyr-OBzl* (*I*). This dipeptide was synthesized as published earlier by Khan et al. (1981): yield 9.0 g (90%); mp 102 °C; $[\alpha]^{25}_D$ +1.35° (*c* 2.4, THF); R_f(A) 0.85; compound homogeneous on a C_{18} reversed-phase column, K' = 1.38 (CH_3OH–H_2O, 500:500).

(*B*) *Boc-Pro-Met-Tyr-OBzl* (*II*). The protected dipeptide (9.0 g, 17.9 mmol) was dissolved in a mixture of methylene chloride–trifluoroacetic acid (50 mL, 1:1 v/v) containing anisole (5 mL). The clear solution was left at room temperature for 0.5 h and then evaporated to dryness. The residue after precipitation twice with 2-propanol–ether was isolated and dried over P_2O_5 and NaOH. The trifluoroacetate salt of the dipeptide thus obtained was neutralized with *N*-methylmorpholine (2 mL, 17.9 mmol) in dimethylformamide (35 mL) and coupled with the mixed anhydride formed from Boc-Pro-OH (3.0 g, 17.9 mmol), in the presence of *N*-methylmorpholine (2.0 mL, 17.9 mmol) and isobutyl chloroformate (2.3 mL, 17.9 mmol) in tetrahydrofuran (15 mL) at −15 °C. The reaction mixture was stirred for 1 h at −10 °C and for 2 h at room temperature. At this time, the solvent was evaporated to dryness, and the residue was taken up in ethyl acetate and washed 3 times each with 5% citric acid, water, and 5% $NaHCO_3$ and water. The organic layer was dried over $MgSO_4$ and concentrated. The residue was crystallized from ethyl acetate–petroleum ether: yield 10.0 g (84%); mp 139–41 °C; $[\alpha]^{25}_D$ −46.1° (*c* 1.3, CH_3OH); product homogeneous on a C_{18} reversed-phase column, K' = 1.57 (CH_3OH–H_2O, 500:500); R_f(A) 0.67; NMR ($CDCl_3$) δ 7.34 (m, 5 H, Ar), 6.92 (d, 2 H, Ar-Tyr), 6.68 (d, 2 H, Ar-Tyr), 5.66 (s, 1 H, HO-Tyr), 5.09 (AB q, 2 H, CH_2-Ar), 4.84 (m, 1 H, α-CH), 4.52 (m, 1 H, α-CH), 4.2 (m, 1 H, α-CH), 2.01 (s, 3 H, SCH_3), and 1.40 [s, 9 H, $(CH_3)_3$].

[1] Abbreviations: Boc, *tert*-butoxycarbonyl; CD, circular dichroism; Cha, β-cyclohexylalanine; Me_2SO, dimethyl sulfoxide; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Bzl, benzyl ester; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

(*C*) *Boc-Gln-Pro-Met-Tyr-OBzl* (*III*). The Boc group from tripeptide II (10.0 g, 16.6 mmol) was removed as described for I. The trifluoroacetate salt was then neutralized with *N*-methylmorpholine (1.85 mL, 16.6 mmol) in dimethylformamide (40 mL) at 0 °C, folowed by addition of hydroxybenzotriazole (2.5 g, 16.6 mmol) and Boc-Gln-ONp (5.7 g, 16.6 mmol). The reaction mixture was worked up as described for II. The product was crystallized twice from ethyl acetate–ether: yield 9.0 g (75%); mp 165–167 °C; $[\alpha]^{25}_D$ −54.0° (*c* 1.0, CH_3OH); tetrapeptide homogeneous on a C_{18} reversed-phase column, K' = 0.93 (CH_3OH–H_2O, 500:500); R_f(A) 0.6; NMR ($CDCl_3$) δ 7.51 (d, 1 H, NH), 7.32 (m, 5 H, Ar), 7.01 (d, 1 H, NH), 6.91 (d, 2 H, Ar-Tyr), 6.68 (d, 2 H, Ar-Tyr), 5.66 (s, 1 H, HO-Tyr), 5.31 (d, 1 H, NH), 5.09 (AB q, 2 H, CH_2-Ar), 4.62 (m, 1 H, α-CH), 4.32 (c, 3 H, α-CH), 2.01 (s, 3 H, SCH_3), and 1.40 [s, 9 H, $(CH_3)_3$].

(*D*) *Boc-Ala-Gln-Pro-Met-Tyr-OBzl* (*IV*). The Boc group from tetrapeptide III (1.0 g, 1.3 mmol) was removed as described for I. The trifluoroacetate salt was neutralized with *N*-methylmorpholine (0.15 mL, 1.3 mmol) and coupled to the mixed anhydride of Boc-Ala-OH (0.26 g, 1.4 mmol) as described for II. The product after the usual workup was crystallized from ethyl acetate–ether: yield 0.98 g (90%); mp 88 °C; $[\alpha]^{25}_D$ −83.2° (*c* 1, CH_3OH); product homogeneous on a C_{18} reversed-phase column, K' = 0.96 (CH_3OH–H_2O, 500:500); R_f(A) 0.56; NMR (Me_2SO-d_6) δ 9.24 (s, 1 H, HO-Tyr), 8.28 (d, 1 H, NH), 8.06 (d, 1 H, NH), 7.91 (d, 1 H, NH), 7.30 (c, 5 H, Ar), 6.97 (d, 2 H, Tyr), 6.89 (d, 1 H, NH), 6.81 (s, 1 H, H_2N-Gln), 6.63 (d, 2 H, Tyr), 5.04 (s, 2 H, CH_2-Ar), 4.3–4.4 (c, 4 H, α-CH), 3.97 (m, 1 H, α-CH), 2.01 (s, 3 H, SCH_3), 1.36 [s, 9 H, $(CH_3)_3$], and 1.13 (d, 3 H, CH_3).

(*E*) *Boc-Leu-Gln-Pro-Met-Tyr-OBzl* (*V*). This pentapeptide was synthesized from Boc-Leu-OH and Boc-Gln-Pro-Met-Tyr-OBzl as described for IV: yield 0.4 g (88%); mp 96 °C; $[\alpha]^{25}_D$ −68.6° (*c* 1, CH_3OH); product homogeneous on a C_{18} reversed-phase column, K' = 1.94 (CH_3OH–H_2O, 500:500); R_f(A) 0.51; NMR (Me_2SO-d_6) δ 9.26 (s, 1 H, HO-Tyr), 8.30 (d, 1 H, NH), 8.07 (d, 1 H, NH), 7.92 (d, 1 H, NH), 7.24–7.36 (c, 5 H, Ar), 6.97 (d, 2 H, Ar-Tyr), 6.87 (d, 1 H, NH), 6.85 (s, 1 H, H_2N-Gln), 6.63 (d, 2 H, Ar-Tyr), 5.04 (s, 2 H, CH_2-Ar), 4.3–4.4 (c, 4 H, α-CH), 3.96 (m, 1 H, α-CH), 2.00 (s, 3 H, SCH_3), 1.36 [s, 9 H, $(CH_3)_3$], 0.86 (d, 3 H, CH_3-Leu), and 0.83 (d, 3 H, CH_3-Leu).

(*F*) *Boc-D-Leu-Gln-Pro-Met-Tyr-OBzl* (*VI*). This was synthesized from Boc-D-Leu-OH and Boc-Gln-Pro-Met-Tyr-OBzl in a manner similar to IV: yield 0.41 g (90%); mp 98 °C; $[\alpha]^{25}_D$ −48.6° (*c* 1.1, CH_3OH); product homogeneous on a C_{18} reversed-phase column, K' = 1.93 (CH_3OH–H_2O, 500:500); R_f(A) 0.50; NMR (Me_2SO-d_6) δ 9.27 (s, 1 H, HO-Tyr), 8.30 (d, 1 H, NH), 8.08 (d, 2 H, NH), 7.24–7.36 (c, 5 H, Ar), 6.98 (d, 2 H, Ar-Tyr), 6.85 (d, 1 H, NH), 6.83 (s, 1 H, H_2N-Gln), 6.63 (d, 2 H, Ar-Tyr), 5.04 (s, 2 H, CH_2-Ar), 4.3–4.4 (c, 4 H, α-CH), 3.95 (m, 1 H, α-CH) 2.00 (s, 3 H, SCH_3), 1.36 [s, 9 H, $(CH_3)_3$], 0.86 (d, 3 H, CH_3-Leu), and 0.83 (d, 3 H, CH_3-Leu).

(*G*) *Boc-His(Boc)-Cha-Leu-Gln-Leu-Lys(Z)-Pro-OH* (*VII*). The heptapeptide was synthesized by the procedure described earlier by Khan et al. (1981): yield 0.96 g (72%); mp 204–205 °C; $[\alpha]^{25}_D$ −36.5° (*c* 1.2, DMF); R_f(A) 0.65.

(*H*) *His-Cha-Leu-Gln-Leu-Lys-Pro-Ala-Gln-Pro-Met-Tyr·3TFA* (*VIII*). The boc group was removed by acidolysis at room temperature for 30 min from IV (0.152 g, 0.19 mmol) in trifluoroacetic acid–methylene chloride (6 mL, 1:1 v/v) containing anisole (0.15 mL). After the solvent was removed in vacuo, the residue was triturated with ether, and the product was filtered, washed with ether, and dried over P_2O_5 and NaOH in a vacuum desiccator. The trifluoroacetate salt of the pentapeptide thus obtained was neutralized with *N*-methylmorpholine (0.021 mL, 0.19 mmol) in dimethylformamide (2 mL) at −10 °C. It was coupled with the mixed anhydride formed from VII (0.21 g, 0.172 mmol) in the presence of *N*-methylmorpholine (0.019 mL, 0.172 mmol) and isobutyl chloroformate (0.022 mL, 0.172 mmol) in a mixture of tetrahydrofuran and dimethylformamide (2 mL, 1:1 v/v) at −10 °C. The reaction mixture was stirred at −10 °C for 1 h and then left for 3 h stirring at room temperature. The solvent was evaporated in vacuo, and the residue was triturated with 5% citric acid. The product was filtered, washed thoroughly with water, and dried in a vacuum desiccator over H_2SO_4 and NaOH. It was precipitated from tetrahydrofuran–ether, yield 0.25 g (77%). The crude product was used without further purification to prepare the corresponding free peptide.

Protected dodecapeptide (250 mg) was dissolved in methanol (9 mL), and freshly prepared Pd black (~300 mg) was added. The reaction mixture was treated with 90% formic acid (0.6 mL) and stirred at room temperature for 8 h. The catalyst was removed by filtration, and the filtrate was concentrated in vacuo and dried in a vacuum desiccator over P_2O_5 and NaOH. The residue was then treated with methylene chloride–trifluoroacetic acid (3 mL, 1:1 v/v) containing anisole (0.1 mL) at room temperature for 30 min. The product was then precipitated with excess ether, filtered, washed with ether, and dried, yield 0.24 g (95%). This crude dodecapeptide was dissolved in 3 mL of methanol–water–trifluoroacetic acid (400:600:0.25 v/v) and injected onto a reversed-phase C_{18} column of the Prep LC/System 500, which had been equilibrated with the same solvent system. The column was eluted, the fractions were analyzed on an analytical HPLC, and those corresponding to the main peak were pooled and lyophilized: yield 54 mg (21%); $[\alpha]^{25}_D$ −58.8° (*c* 0.17, CH_3COOH); product >98% homogeneous on a C_{18} reversed-phase column, K' = 1.21 (CH_3OH–H_2O–CF_3COOH, 550:450:0.25), K' = 1.19 (CH_3CN–H_2O–CF_3COOH, 300:700:0.25); R_f(B) 0.11, R_f(C) 0.59. Amino acid analysis was as follows: Ala, 1.00; Glu, 1.99; His, 0.94; Leu, 1.98; Lys, 1.00; Met, 0.95; Pro, 1.95; Tyr, 0.98; NH_3, 2.17.

(*I*) *His-Cha-Leu-Gln-Leu-Lys-Pro-Leu-Gln-Pro-Met-Tyr·3TFA* (*IX*). The protected heptapeptide VII was coupled with H_2N-Leu-Gln-Pro-Met-Tyr-OBzl·TFA by the mixed-anhydride procedure as described for VIII, yield 80%. The crude protected dodecapeptide (260 mg) was deblocked as described for Ala-containing dodecapeptide. The deblocked peptide was purified as in case of VIII: yield 117 mg (45%); $[\alpha]^{25}_D$ −65.8° (*c* 0.19, CH_3COOH); R_f(B) 0.17, R_f(C) 0.63; product >99% homogeneous on a C_{18} reversed-phase column, K' = 0.63 (CH_3OH–H_2O–CF_3COOH, 600:400:0.25), K' = 1.0 (CH_3CN–H_2O–CF_3COOH, 330:670:0.25). Amino acid analysis was as follows: Glu, 2.00; His, 0.97; Leu, 2.87; Lys, 1.00; Met, 0.94; Pro, 2.01; Tyr, 0.99; NH_3, 2.02.

(*J*) *His-Cha-Leu-Gln-Leu-Lys-Pro-D-Leu-Gln-Pro-Met-Tyr·3TFA* (*X*). This was prepared in a fashion similar to the procedure described for IX: yield 89 mg (23%); $[\alpha]^{25}_D$ −40.6° (*c* 0.17, CH_3COOH); R_f(B) 0.21, R_f(C) 0.65; product >99% homogeneous on a C_{18} reversed-phase column, K' = 1.11 (CH_3OH–H_2O–CF_3COOH, 600:400:0.25), K' = 1.25 (CH_3CN–H_2O–CF_3COOH, 330:670:0.25). Amino acid analysis was as follows: Glu, 2.02; His, 0.97; Leu, 2.83; Lys, 1.01; Met, 0.93; Pro, 2.05; Tyr, 0.98; NH_3, 1.90.

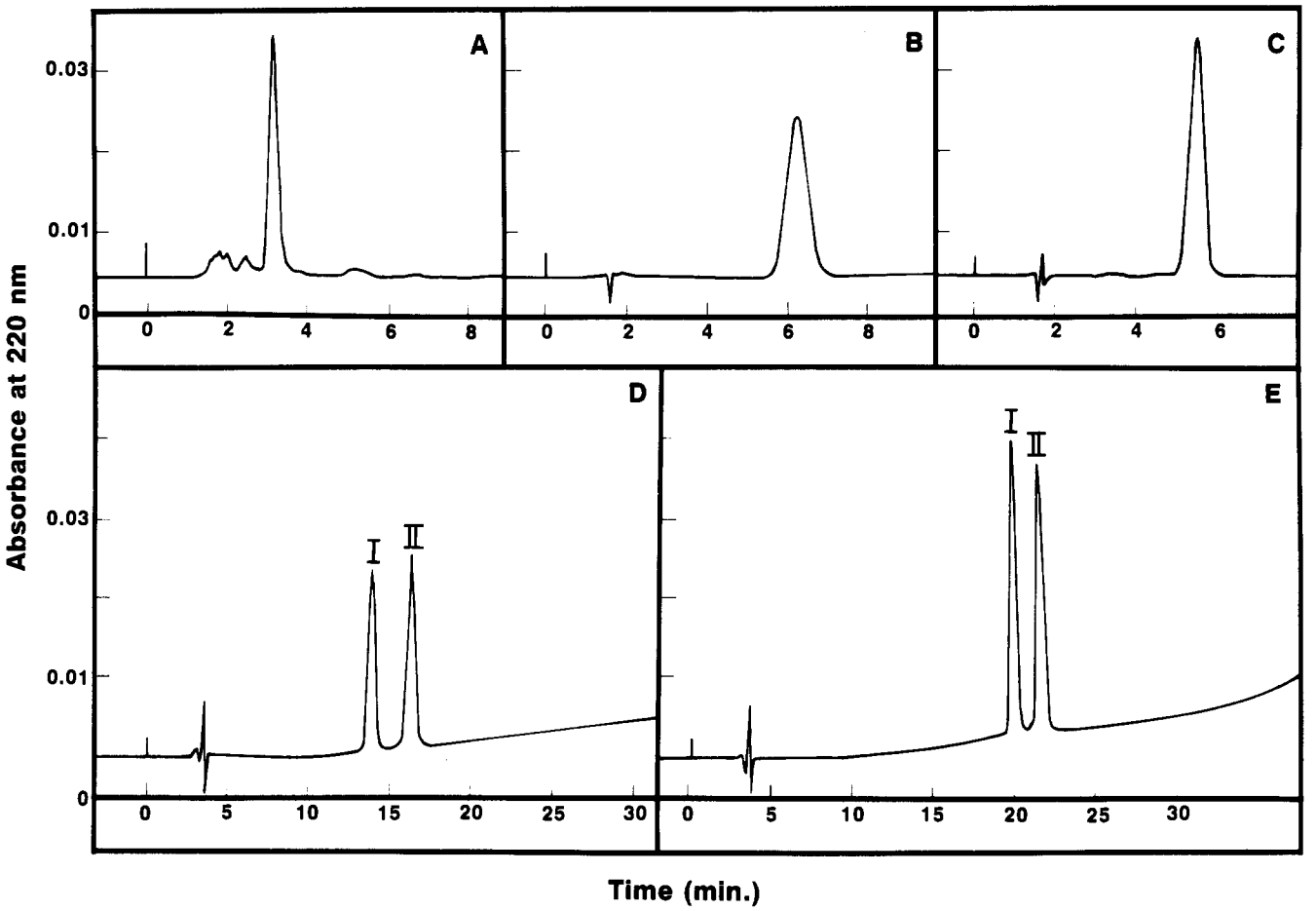

FIGURE 1: HPLC of des-Trp[1],Cha[3],X[9]-α-factors on a reversed-phase C_{18} column. (Panel A) Crude des-Trp[1],Cha[3],D-Leu[9]-α-factor before purification. Mobile phase CH_3OH–H_2O–CF_3COOH (600:400:0.25). (Panels B and C) Des-Trp[1],Cha[3],-D-Leu[9]-α-factor after purification; (panel B) mobile phase CH_3OH–H_2O–CF_3COOH (500:500:0.25); (panel C) mobile phase CH_3CN–H_2O–CF_3COOH (280:720:0.25). (Panel D) Gradient elution of a mixture of (I) des-Trp[1],Cha[3],L-Ala[9] and (II) des-Trp[1],Cha[3],D-Ala[9] dodecapeptides. Gradient from 21 to 36% CH_3CN. (Panel E) Gradient elution of a mixture of (I) des-Trp[1],Cha[3],L-Leu[9] and (II) des-Trp[1],Cha[3],D-Leu[9] dodecapeptides. Gradient from 21 to 36% CH_3CN.

Biological Methods. (*A*) *Yeast Strains.* Haploid strains X2180-1A (*MATa*) and X2180-1B (*MAT*) were grown in the minimal medium of Difco yeast nitrogen base (2.2 g/L), $(NH_4)_2SO_4$ (4.5 g/L), and glucose (20 g/L). All cells were grown at 25 °C to mid-log phase for use as the inoculum in the morphogenesis assays.

(*B*) *Morphogenic Activity.* The morphogenesis assay was carried out in plastic tubes (Falcon) as described previously (Khan et al., 1981). For the purpose of this paper, the induction of altered morphologies by α-factor will be termed morphogenesis, and a pheromone causing altered morphologies is called a morphogen. Each tube had 1 mL of medium containing 2×10^6 *a* cells and serial 2-fold dilutions of the appropriate synthetic analogue. The pheromone activity was determined by counting the number of unbudded cells and the number of cells with altered morphologies after a 4-h incubation of *S. cerevisiae* X2180-1A with the α-factor analogue. These values were then compared with control cultures.

(*C*) *Cell-Mediated Hydrolysis of Peptides. MATa* cells at 2×10^6 cells/mL from a mid-log phase culture were incubated with the various analogues at concentrations that induced shmoo formation. The supernatent was separated from the cells at various time intervals by centrifugation (Beckman microfuge) and the supernatant frozen and chromatographed within 24 h or chromatographed directly on a μBondapak C_{18} column (Waters Associates) with CH_3CN–H_2O–CF_3COOH as the mobile phase. Breakdown of the pheromones was determined by plotting the area under the peak corresponding to either the remaining dodecapeptide or the N-terminal pentapeptide cleavage product vs. time.

Results and Discussion

Synthesis of Position 9 Analogues. The synthesis of the des-Trp[1],Cha[3],X[9] analogues was carried out in a manner similar to that previously described for a variety of α-factor analogues (Khan et al., 1981; Shenbagamurthi et al., 1983). This procedure, which coupled Pro to the X residue, was found to give reasonable yields when X = Gly, L-Ala, L-Leu, D-Ala, or D-Leu. No diminution in coupling yield was observed as the steric bulk of the side chain was increased. Preparative high-pressure liquid chromatography on a C_{18} reversed-phase column using isocratic elution with a CH_3OH–H_2O–CF_3CO-OH mobile phase resulted in peptides that were greater than 98% pure in two HPLC systems (Figure 1A–C). We observed that the L and D diastereomers had different K' values and were readily separable on reversed-phase HPLC (Figure 1D,E). Other diastereomeric peptides have been found to separate under similar conditions (Rivier et al., 1977; 1979; Spatola et al., 1981), and we found that diastereomeric position 6 analogues of the des-Trp[1],Cha[3]-α-factor also separated on reversed-phase HPLC (Baffi et al., 1985). The final dodecapeptides were homogeneous on silica thin layers and gave the expected amino acid ratios. The 200-MHz NMR spectroscopy confirmed that coupling took place as evidenced by peaks characteristic of the leucyl, alanyl, methionyl, tyrosyl, histidyl, and lysyl residues.

Biological Activity of Des-Trp[1],Cha[3],X[9]-α-factors. The effect of the des-Trp[1],Cha[3],X[9]-α-factors on the morphology

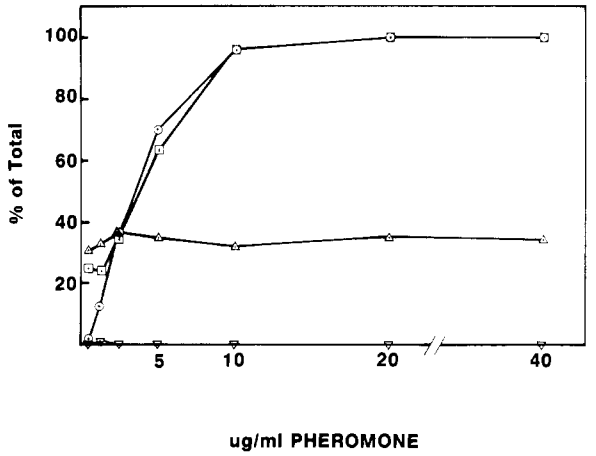

FIGURE 2: Morphogenic response of *S. cerevisiae* X2180-1A (*MATa*) cells to the des-Trp[1],Cha[3],X[9] dodecapeptides. Yeast cells were incubated with the specificed pheromone as described under Materials and Methods, and the number of unbudded or abberant cells were counted. The activity is reported as the percent of the total cells that have altered morphology or that are unbudded. Des-Trp[1],Cha[3],D-Leu[9]-α-factor: (□) unbudded; (○) altered morphology. Des-Trp[1],Cha[3],L-Leu[9]-α-factor: (△) unbudded; (▽) altered morphology.

of *S. cerevisiae* is summarized in Table I and Figure 2. The des-Trp[1],Cha[3],D-Ala[9] analogue causes 80% aberrant morphologies at a concentration of 1.25 μg/mL. At this concentration, 83% of the cells are also unbudded. This activity is nearly identical with that found for the dodecapeptide with Gly in position 9. In contrast, even at 200 μg/mL of the des-Trp[1],Cha[3],L-Ala[9] analogue only 23% of the cells are morphologically distorted and 27% are unbudded, which is equal to the number of unbudded cells found in control cultures. Thus, the replacement of Gly with L-Ala leads to at least a 200-fold decrease in activity. Although the D-Leu[9] analogue exhibited a slightly lower activity than the D-Ala[9]-α-factor, it was still more than 100 times as active as the des-Trp[1],Cha[3],L-Leu[9]-dodecapeptide. Similar results are found for cell number where a definite growth arrest is observed for the D analogues at concentrations near 1 μg/mL whereas the L analogues do not affect cell number until the pheromone concentration is greater than 100 μg/mL. Thus, for both leucine and alanine replacements, the D enantiomer results in much higher biological activity than its L homologue.

Degradation of α-Factor Analogues by S. cerevisiae. The effects of α-factor on *S. cerevisiae* are transient, and *a* cells recover after an 8–15-h incubation with this pheromone (Chan, 1977). Recovery is at least in part due to proteolysis of α-factor by an enzyme that is present on the yeast cell surface (Ciejek & Thorner, 1979; Finkelstein & Strausburg, 1979; Manney, 1983; Moore, 1983, 1984), and that is believed to be coded by the *Bar1* gene (Hicks & Herskowitz, 1976). Although the primary site of cleavage of α-factor is at the Leu[6]–Lys[7] peptide bond (Ciejek & Thorner, 1979), there is little information available on the effect of amino acid substitution on hydrolysis rates. Recently, we have developed a rapid and sensitive HPLC method for following pheromone degradation by whole cells (Baffi et al., 1985). Using this approach, we have determined that replacement of the Gly residue at position 9 with either L- or D-alanine has a marked influence on the hydrolysis rates of the pheromone. As shown previously, when the des-Trp[1],Cha[3]-α-factor is incubated with whole cells, major changes in the HPLC pattern of the culture medium occur with time (Baffi et al., 1985). Specifically, a peak with an elution time of 6.95 ± 0.2 min decreases with incubation time, and a peak appears with an elution time of

Table I: Effect of Position 9 Analogues on Cell Number and Cell Morphology

peptide	concn (μg/mL)	no. of cells	% aberrant	% unbudded
des-Trp[1],Cha[3],-D-Ala[9]	0.63	621	6	30
	1.25	516	80	83
	2.50	410	89	91
	5.00	391	95	96
	0.00	700	0.3	38
des-Trp[1],Cha[3],L-Ala[9]	0.63	523	0	25
	10.00	519	0.6	23
	50.00	517	4	25
	100.0	474	10	22
	200.00	416	23	27
	0.00	535	0.6	28
des-Trp[1],Cha[3],-D-Leu[9]	0.63	600	2	25
	1.25	497	13	24
	2.5	529	37	35
	5.0	485	70	63
	10.0	465	96	96
	0.00	765	0	32
des-Trp[1],Cha[3],-L-Leu[9]	0.63	700	0	31
	10.00	648	0.5	32
	50.00	668	4	32
	100.00	710	12	35
	200.00	740	27	32
	0.00	720	0.5	24
des-Trp[1],Cha[3],Gly[9]	0.63	510	14	37
	1.25	360	89	82
	2.5	384	97	91
	5.0	414	98	97
	0.00	610	0	36

10.5 ± 0.2 min that increases with incubation time. We ascribe this peak to His-Cha-Leu-Gln-Leu, the amino terminal pentapeptide of the dodecapeptide α-factor that results from proteolysis at the Leu[6]–Lys[7] peptide bond. To confirm this assignment, we synthesized His-Cha-Le-Gln-Leu and found it to have the identical mobility in our HPLC system.

From the HPLC analysis we were able to show that the Gly[9] and L-Ala[9] analogues had half-lives of 105 and 300 min, respectively, after incubation of 10 μg/mL pheromone with 2 × 10[6] cells/mL. The D-Ala[9] analogue was not degraded during a 6-h incubation. These results suggest that the biological activities of the position 9 analogues do not correlate with their degradation rates. The Gly[9] analogue, which is most rapidly degraded, and the D-Ala[9] analogue, which is not cleaved at all, have almost identical activity whereas the L-Ala[9] analogue with an intermediate rate of degradation is more than 200 times less active. Furthermore, the ability of the D-Ala[9] and Gly[9] analogues to induce morphogenesis at cell densities of 3 × 10[3] cells/mL is identical. Using an equation suggested by Moore (1983) that assumes first-order degradation kinetics and half-lives derived in our laboratory, we calculate that starting with 3 × 10[3] cells/mL 99.5% of the des-Trp[1],Cha[3],Gly[9]-α-factor remains in the culture medium after the morphogenesis is complete. Thus, at low cell densities both the Gly[9] and D-Ala[9] analogues remain intact for the duration of the morphogenesis assay (4 h). Studies on the degradation of the L-Leu[9] and D-Leu[9] analogues gave results that were similar to those with the alanine analogues. We conclude that the biological activities found for the various position 9 analogues reflect their affinity for the receptor and are not a function of the different rates of cellular hydrolysis of these pheromones.

CD Studies on Position 9 Analogues. In addition to effects on the rate of degradation, it is possible that D residues could have a pronounced influence on the conformation of the α-factor. A change in the solution conformation of the pheromone might manifest itself as a change in morphogenic ac-

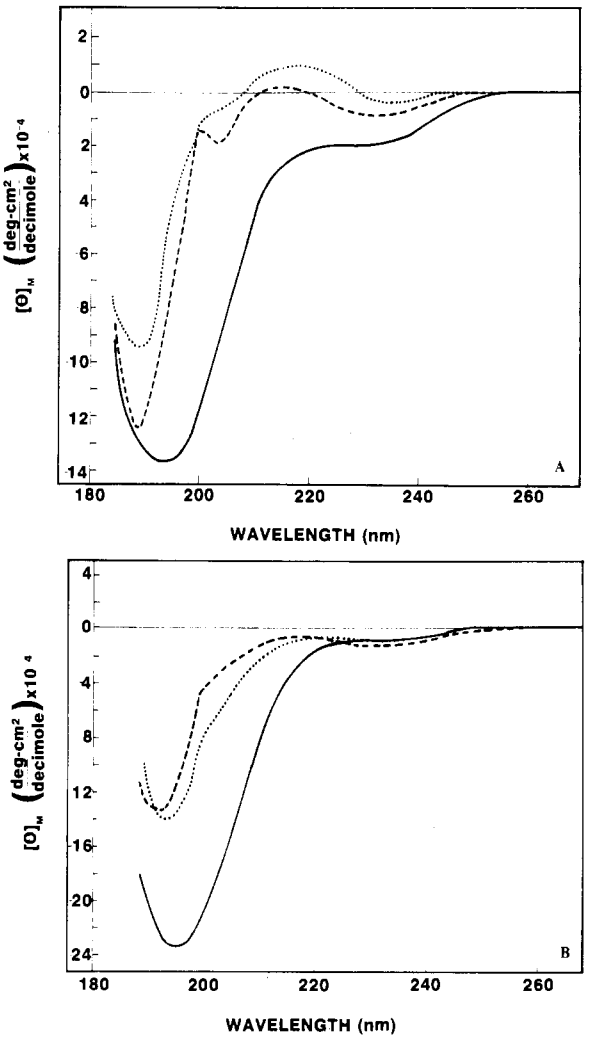

FIGURE 3: Circular dichroism of des-Trp[1],Cha[3],X[9]-α-factor in aqueous and organic solvents. (Panel A) Des-Trp[1],Cha[3]-α-factor (--); des-Trp[1],Cha[3]-D-Leu[9]-α-factor (···); des-Trp[1],Cha[3],L-Leu[9]-α-factor (—) in trifluoroethanol. Concentration 1 mg/mL. (Panel B) Des-Trp[1],Cha[3],-α-factor (···); des-Trp[1],Cha[3],D-Ala[9]-α-factor (--); des-Trp[1],Cha[3]-L-Ala[9]-α-factor (—) in Tris buffer (pH 7.4, 50 mM).

tivity. In order to investigate the conformation of the position 9 analogues in solution, CD studies were carried out.

The CD spectrum of the des-Trp[1],Cha[3],Gly[9]-α-factor in trifluoroethanol is characterized by a weak trough at 234 nm ($[\theta]_M$ = −9000 deg cm^2 dmol^{-1}), a small positive ellipticity between 220 and 210 nm, a shoulder near 203 nm, and a strong transition centered at 190 nm ($[\theta]_M$ = −94000 deg cm^2 dmol^{-1}) (Figure 3A). Given the fact that the α-factor pheromone contains tyrosine and histidine, it is somewhat difficult to analyze its CD pattern. It is clear, however, that on the basis of the position of the π–π^* transition at 190 nm and the general shape of the CD curve that the majority of the peptide chromophores are not present in α-helical, β-sheet, or β-turn structures. When Gly[9] is replaced by D-Leu[9], a similar CD pattern is observed although there are slight differences in both the intensities and positions of all Cotton effects. In contrast, when Gly[9] is replaced by L-Leu[9], the CD pattern is quite different. The L-Leu[9] analogue exhibits a CD curve with a weak Cotton effect at 230 nm and a broad negative trough centered at 195 nm. The CD curve of the L-Leu[9] pheromone never shows positive ellipticity and does not exhibit the fine structure found for the Gly[9]- and D-Leu[9]-α-factors. The CD curve for the des-Trp[1],Cha[3],D-Ala[9]-α-factor in trifluoroethanol is qualitatively similar to those of the Gly[9] and D-Leu[9] peptides, whereas the L-Ala[9] pheromone has a CD spectrum similar to that of the L-Leu[9] analogue (data not shown). Thus, the CD curves for the position 9 analogues in trifluoroethanol may be separated into one class of curves for the Gly[9],D-Ala[9], and D-Leu[9] peptides and a second class of curves for the L-Ala[9] and L-Leu[9] analogues.

When the CD spectra for the position 9 analogues were measured in Tris buffer (50 mM, pH 7.4), similar results were observed. The CD curves for the Gly[9] and D-Ala[9] pheromones are still characterized by a weak trough at 235 nm, a slight shoulder near 205 nm, and a negative band at 192–193 nm (Figure 3B). In comparison to the spectrum for the analogous compound in TFE, there is approximately a 50% increase in the intensity of the π–π^* minimum and there is a slight red shift in the λ_{max}. The tendency to exhibit positive ellipticity between 220 and 210 nm is lessened in aqueous medium. In contrast to the glycine and D-X[9] analogues, the L-Ala[9] homologue in Tris buffer exhibits a CD pattern characterized by a weak minimum near 235 nm and a broad symmetrical trough centered at 195 nm (Figure 3B). Again, the CD curves may be grouped into two classes: one for Gly[9], D-Ala[9], and D-Leu[9] (data not shown) and the second for L-Ala[9] and L-Leu[9] (data not shown).

Recently, Higashijima et al. (1983) reported CD spectra for the tridecapeptide α-factor and various analogues in buffer and in the presence of lipid. The presence of tryptophan in these peptides results in a positive band between 235 and 220 nm. In other respects, the spectra reported by Higashijima et al. for tridecapeptide α-factors are similar to those found for our dodecapeptides.

A number of studies have appeared on the CD of LHRH in both aqueous and organic media (Mabrey & Klotz, 1976; Cann et al., 1979). The α-factor from *S. cerevisiae* activates mammalian gonadotrophs (Loumaye et al., 1982), and several investigators have suggested structural similarities between LHRH and the yeast mating pheromone (Geiger, 1978; Kitada et al., 1979; Stewart et al., 1979). In view of this homology, it is pertinent that the CD of LHRH in water is characterized by a weak positive maximum at 220 nm and a strong minimum at around 200–205 nm (Cann et al., 1979). A systematic investigation of LHRH concluded that this peptide exists as an ensemble of structures in both trifluoroethanol and water and that the far-ultraviolet CD spectra can be simulated by the spectra of its aliphatic- and aromatic-containing halves. Although differences exist between the CD of our α-factor analogues and LHRH, we believe that the spectra for the yeast sex pheromone are indicative of a predominantly disordered peptide in both trifluoroethanol and Tris buffer. The insertion of a D residue into a linear peptide can effect changes in the CD pattern of the resulting analogues. These changes can result from configurational and/or conformational factors. In the present study, the fact that the achiral Gly[9] analogues and the chiral D-Ala[9] and D-Leu[9] analogues manifest one class of CD patterns leads us to conclude that conformational factors dominate the chromophoric interaction manifested in the spectra. Our results suggest that the above peptides have similar distributions of ϕ, ψ angles in both water and trifluoroethanol and that the insertion of an L-Ala or L-Leu residue in position 9 appears to disturb this distribution, resulting in the observed change in spectral patterns.

It is interesting that the change in CD pattern for the L and D analogues parallels to a certain extent the changes in the biological activity of these peptides. Specifically, those peptides with a CD pattern similar to that of the Gly[9] dodecapeptide have high biological activity and those with perturbed CD

spectra have almost no activity. Since the Gly to D-X^9 change would retain the β-type II conformation whereas a Gly to L-X^9 change would disrupt this structure, our results suggest that a type II β-turn may contribute to the biologically active structure. This structure does not predominate in trifluorethanol or water but may be induced in the yeast membrane or on contact with the receptor. This conclusion is consistent with the observation that the tridecapeptide α-factor undergoes a conformational change in the presence of phospholipid vesicles (Higashijima et al., 1983)) and with the widely accepted premise that the conformation of a peptide in solution does not always reflect its conformation at the receptor. We are currently attempting to synthesize analogues that will lock in the β-turn between Lys^7 and Gln^{10}. Such analogues are expected to display an increased biological activity and spectral patterns more indicative of an ordered 2° conformation.

Registry No. I, 78395-24-1; II, 98990-18-2; III, 98990-19-3; IV, 98990-20-6; V, 98990-21-7; VI, 98990-22-8; VII, 98990-23-9; VIII, 99094-55-0; IX, 98990-25-1; X, 99094-57-2; des-Trp^1,Cha^3,D-Ala^9, 84280-08-0; des-Trp^1,Cha^3,Gly^9, 78395-56-9; Boc-Pro-OH, 15761-39-4; Boc-Gln-ONp, 15387-45-8; Boc-Ala-ONp, 15761-38-3; Boc-Leu-OH, 13139-15-6; Boc-D-Leu-OH, 16937-99-8.

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