Systematic Analysis of the *Saccharomyces cerevisiae* α-Factor Containing Lactam Constraints of Different Ring Size†

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ABSTRACT: Eight cyclic analogs and corresponding linear homologs of the α-factor mating pheromone (WHWLQLKPGQPMY) of *Saccharomyces cerevisiae* were synthesized using solid-phase procedures on a phenylacetamidomethyl support. On-resin lactamization of the side chains of residues 7 and 10 to form rings containing from 14 to 18 atoms was effected by the BOP reagent. All peptides were highly homogeneous and gave expected molecular ions by FAB mass spectrometry. The constrained analogs had biological activities varying from 10% to less than 0.1% of that of [Nle^{12}]-α-factor. In all cases, cyclic analogs with Glu in position 10 were more active than the homolog with Asp at this position. This trend was also found with the corresponding linear pheromones, suggesting that a γ-carbonyl in position 10 is an important determinant of pheromone potency. The cyclic peptides had from 50- to 20000-fold lower affinities for the α-factor receptor than for [Nle^{12}]-α-factor, as judged using a competition binding assay. Circular dichroism studies indicate that the cyclic lactam-containing region of cyclo^{7,10}[Orn^{7}, Glu^{10},Nle^{12}]-α-factor retains a β-turn-like structure similar to that found in the corresponding model tetrapeptide. The results show that covalently constrained analogs of the linear pheromone can maintain biological activity, despite binding poorly to the receptor, and indicate that a β-turn-like structure in the center of the pheromone allows signal transduction.

The search for the biologically active conformation of a peptide entails the application of a combination of physical, biochemical, and synthetic methodologies. Extensive studies using spectroscopic techniques such as circular dichroism and nuclear magnetic resonance spectroscopy have investigated conformational aspects of linear peptides in solution and in the presence of lipid. Although these procedures often result in conclusions regarding peptide conformation, the inherent flexibility of many linear peptides makes suspect the relationship of the solution structure to the biologically relevant conformation. Attempts to surmount these uncertainties have used a variety of solvent conditions and studies in the presence of lipids, cryoscopic solvents at low temperature, or solvents of high viscosity to mimic the binding environment (Motta *et al.*, 1988).

An alternative to these procedures is to constrain the flexibility of the peptide by covalent modification (Veber *et al.*, 1979; Rizo & Gierasch, 1992). In particular, backbone and side chain cyclization can result in rigidified peptides that may closely mimic the bound state (Veber *et al.*, 1981; Hruby *et al.*, 1982). A number of such studies have resulted in superactive agonists and antagonists that have excellent potential both in drug design and in the quest for understanding the bound conformation of biologically active peptides. Cyclic peptides have the obvious advantage that they are more amenable to conformational analysis because the ϕ,ψ distributions are significantly narrowed.

The α-factor mating pheromone of *Saccharomyces cerevisiae* is a linear tridecapeptide (WHWLQLKPGQPMY) involved in sexual conjugation between the opposite haploid cells of this yeast. The peptide has been studied extensively as a model for understanding the biochemistry of mammalian peptide hormones (Sprague & Thorner, 1992). ^{1}H NMR[1] studies on this peptide indicate that the Lys^7−Gln^{10} fragment assumes a type II β-turn and that this conformational feature is an important factor in the bioactive state of the molecule (Jelicks *et al.*, 1988; Gounarides *et al.*, 1993). Recently, we showed that cyclo^{7,10}[Nle^{12}]-α-factor, which contains a side chain lactam, retains significant, but not full, biological activity (Xue *et al.*, 1989). This finding led to the conclusion that the pheromone must be bent when it binds to its receptor.

In recent years, a number of laboratories have used side chain lactamization to constrain regions of linear peptide hormones (Al-Obeidi *et al.*, 1989; Struthers *et al.*, 1990; Roques, 1992). Important insights into the biologically active conformation of growth hormone-releasing hormone

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[1] Abbreviations: Ac, acetyl; Boc, *tert*-butoxycarbonyl; BOP, (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; 2-BrZ, 2-bromobenzyloxycarbonyl; Dab, L-2,4-diaminobutyric acid; DIEA, *N,N*-diisopropylethylamine; DIPC, diisopropylcarbodiimide; Dhp, 3,4-dehydro-L-proline; DMF, *N,N*-dimethylformamide; DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide; Dpr, L-2,3-diaminopropionic aicd; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethyloxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; Me, methyl; Nle, norleucine; NMR, nuclear magnetic resonance; NOESY, 2D nuclear Overhauser spectroscopy; OBzl, benzyl ester; OFm, 9-fluorenylmethyl; PAM, phenylacetamidomethyl; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Ph, phenyl.

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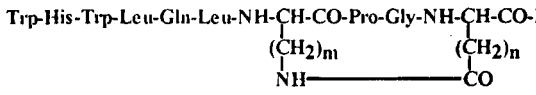

C(4)2: m = 4; n = 2 **C41**: m = 4; n = 1
C32: m = 3; n = 2 **C31**: m = 3; n = 1
C22: m = 2; n = 2 **C21**: m = 2; n = 1
C12: m = 1; n = 2 **C11**: m = 1; n = 1

FIGURE 1: Chemical structures of cyclic analogs of α-factor. **C(4)2** represents a D-Lys[7] residue. In other analogs, all residues are L.

have been achieved using $i - (i + 4)$ lactams to stabilize an α-helix (Fry *et al.*, 1992). Since a β-turn involves four residues, $i - (i + 3)$ lactamization might be expected to stabilize this conformation. Side chain cyclization between the ε-amine of Lys[7] and the γ-carboxyl of Glu[10] in cyclo[7,10]-[Nle[12]]-α-factor results in an 18-membered ring that spans the middle of the pheromone. It is possible that this ring causes significant distortions in the backbone ϕ,ψ angles, resulting in an analog with good but not equal potency to the native pheromone. In order to study the influence of side chain lactam formation on both the bioactivity of the α-factor and the conformation of the peptide in the cyclic ring, we have initiated a systematic investigation of the effect of ring size on the bioactivity of the pheromone. In this paper, we report on the synthesis, purification, and biological characterization of eight cyclic α-factor analogs in which the ring size is varied from 18 to 14 atoms.

MATERIALS AND METHODS

Except for N^{α}-Boc-Orn(N^{δ}-Fmoc), N^{α}-Boc-Dab(N^{γ}-Fmoc), and N^{α}-Boc-Dpr(N^{β}-Fmoc), all protected amino acids were purchased from BaChem Inc. (Torrance, CA). N^{α}-Boc-Orn-(N^{δ}-Fmoc) and N^{α}-Boc-Dab(N^{γ}-Fmoc) were prepared in 90% and 63% overall yields, respectively, from Boc-Gln and Boc-Asn (Waki *et al.*, 1981; Stanfield *et al.*, 1990). N^{α}-Boc-Dpr(N^{β}-Fmoc) was prepared by the procedure of Stanfield *et al.* (1990). N^{α}-Boc-Glu(γ-OFm) and N^{α}-Boc-Asp(β-OFm) were prepared from N^{α}-Boc-Glu(γ-OBzl) and N^{α}-Boc-Asp-(β-OBzl) as previously described (Bolin *et al.*, 1989). All synthetic derivatives were purified to over 99% homogeneity, as judged by reversed-phase HPLC, and characterized by 200 MHz ^{1}H NMR spectroscopy before being used in solid-phase peptide synthesis. All peptides were synthesized with Nle in place of Met[12] of the native α-factor. Previous studies have shown Nle to be an isosteric substitution with full retention of the biological activity of [Nle[12]]-α-factor in comparison to α-factor (Raths *et al.*, 1988). All cyclic analogs synthesized are given a **C** prefix, with the corresponding linear homologs being given an **L** prefix. The numbers following the prefix indicate the number of CH_2 groups on the side chains of residues 7 and 10, respectively. For example, cyclo[7,10][Orn[7],Glu[10],Nle[12]]-α-factor is designated **C32** (Figure 1), whereas the corresponding linear peptide [Orn[7],Glu[10],Nle[12]]-α-factor is designated **L32**.

Peptide Synthesis

N^{α}-Boc-Tyr(2-BrZ) was reacted with 4-(bromomethyl)-phenylacetic acid phenacyl ester to produce N^{α}-Boc-Tyr(2-BrZ)-$OCH_2PhCH_2COOCH_2COPh$. This product was reduced using Zn/HOAc to N^{α}-Boc-Tyr(2-BrZ)-OCH_2PhCH_2-COOH (yield, 77%) (Tam *et al.*, 1979). The free acid was coupled with aminomethyl polystyrene resin (0.759 mmol/g of resin) using BOP in DMF to give to N^{α}-Boc-Tyr(2-BrZ)-OCH_2-PAM-resin.

Solid-Phase Synthesis. The solid-phase syntheses of the α-factor analogs were carried out manually starting with N^{α}-Boc-Tyr(2-BrZ)-OCH_2-PAM-resin (0.54 mmol/g of resin), following the procedures described by Xue *et al.* (1989). The Boc group was used for all N^{α} protections. The side chain protecting groups were Trp(For), His(Tos), D-Lys(Fmoc), Glu(OFm), Orn(Fmoc), Dab(Fmoc), Dpr(Fmoc), Lys(Fmoc), and Asp(OFm). A typical cycle consisted of the following procedures (15 mL of solvent/g of resin): (1) CH_2Cl_2, 3 × 1 min; (2) 50% TFA in CH_2Cl_2 or 48% TFA in CH_2Cl_2 (2% DMS) (from Trp[3] on), 1 × 1 min; (3) 50% TFA in CH_2Cl_2 or 48% TFA in CH_2Cl_2 (2% DMS) (from Trp[3] on), 1 × 30 min; (4) CH_2Cl_2, 3 × 1 min; (5) Kaiser test; (6) 10% DIEA in CH_2Cl_2, 1 × 2 min; (7) 10% DIEA in CH_2Cl_2, 1 × 5 min; (8) CH_2Cl_2, 3 × 1 min; (9) N^{α}-Boc-amino acid (3 equiv) in CH_2Cl_2 added to the vessel followed by DIPC (3 equiv), 3 h; (10) CH_2Cl_2, 6 × 1 min; (11) Kaiser test; (12) 5% DIEA in CH_2Cl_2, 1 × 2 min; (13) CH_2Cl_2, 3 × 1 min; (14) N^{α}-Boc-amino acid (1 equiv) in CH_2Cl_2 added to the vessel followed by DIPC (1 equiv), 1 h; (15) CH_2Cl_2, 6 × 1 min; (16) Kaiser test.

In cases where the N^{α}-Boc-amino acids did not dissolve well in CH_2Cl_2, such as for Boc-Glu(OFm), Boc-Leu, Boc-His(Tos), and Boc-Trp(For), the couplings were carried out in CH_2Cl_2/DMF (1:1, v/v) and the resin was washed with DMF prior to and after the coupling step, 2 × 1 min. The procedures of the cycle immediately following the incorporation of Boc-Gln were different from the preceding protocol: (1) dioxane, 3 × 1 min; (2) 4 N HCl in dioxane, 1 × 1 min; (3) 4 N HCl in dioxane, 1 × 30 min; (4) dioxane, 3 × 1 min; (5) $CHCl_3$, 3 × 1 min; (6) Kaiser test; (7) 10% DIEA in $CHCl_3$, 1 × 2 min; (8) 10% DIEA in $CHCl_3$, 1 × 5 min; (9) $CHCl_3$, 3 × 1 min; (10) CH_2Cl_2, 3 × 1 min; (11) DIPC (3 equiv) in CH_2Cl_2 added to the vessel followed by Boc-amino acid (3 equiv) in CH_2Cl_2, 3 h; (12) CH_2Cl_2, 3 × 1 min; (13) Kaiser test; (14) $CHCl_3$, 3 × 1 min; (15) 5% DIEA in $CHCl_3$, 1 × 2 min; (16) $CHCl_3$, 3 × 1 min; (17) CH_2Cl_2, 3 × 1 min; (18) DIPC (1 equiv) in CH_2Cl_2 added to the vessel followed by Boc-amino acid (1 equiv) in CH_2Cl_2, 1 h; (19) CH_2Cl_2, 6 × 1 min; (20) Kaiser test. In the cases in which a third coupling was required, as revealed by the Kaiser test, 1 equiv each of Boc-amino acid, DIPC, and HOBt in DMF were added, and the reaction was allowed to proceed for 1−3 h. This approach always resulted in a negative Kaiser test.

Cyclization. Cyclization was carried out on the resin-bound peptide prior to HF cleavage to reduce any intermolecular cross-links using BOP to effect amide bond formation. For example, the Boc-Trp(For)-His(Tos)-Trp(For)-Leu-Gln-Leu-D-Lys(Fmoc)-Pro-Gly-Glu(OFm)-Pro-Nle-Tyr(2-BrZ)-PAM-resin (1.53 g) was washed with DMF (3 × 1 min), reacted with 20% piperidine in DMF (1 × 2 min and 1 × 30 min) to remove the Fmoc and OFm groups, and washed with DMF (2 × 1 min), MeOH (2 × 1 min), and CH_2Cl_2 (2 × 1 min). The free amino groups were indicated by a positive Kaiser test. The resin was washed with DMF (2 × 1 min) and shaken with BOP (5 equiv) in DMF (containing 1.5% DIEA) for 3 h to effect the amide bond formation. The resin was subjected to the Kaiser test after being washed with DMF (2 × 1 min), MeOH (2 × 1 min), and CH_2Cl_2 (2 × 1 min). A positive Kaiser test indicated incomplete cyclization, and additional BOP (3 equiv) in DMF (contain-

Table 1: Chemical and Physical Properties of Cyclic Analogs

	amino acid analysis										R_f[b]				
peptide	Asx	Glx	Gly	His	Leu	Lys	Nle	Pro	Tyr	Trp[a]	solvent A	solvent B	K'[c]	FAB-MS[d]	calculated mass (amu)
C(4)2		1.86	0.94	0.88	2.21	1.02	0.96	2.05	1.07	ND[e]	0.46	0.68	6.92	1649.0	1648.8
C32		2.03	1.20	0.91	1.92		0.94	2.01	1.07	1.92	0.43	0.65	6.90	1635.0	1634.8
C22		1.75	0.97	1.10	2.18		0.93	1.93	1.02	2.13	0.44	0.64	6.85	1620.1	1620.8
C12		1.82	0.99	1.02	1.86		ND	2.11	1.21	ND	0.44	0.68	6.84	1607.4	1606.8
C41	0.92	1.04	1.12	0.89	2.03	0.91	0.98	2.04	1.07	ND	0.51	0.69	7.14	1635.0	1634.8
C31	0.93	0.91	1.01	1.04	1.87		ND	2.10	1.15	ND	0.54	0.67	6.99	1621.5	1620.8
C21	1.08	0.88	0.97	1.01	1.80		ND	2.09	1.16	ND	0.51	0.66	6.89	1607.3	1606.8
C11	1.16	0.92	0.96	1.01	1.81		ND	2.02	1.14	ND	0.51	0.66	6.87	1593.3	1592.8

[a] In two cases (**C32** and **C22**), special care was taken during hydrolysis to obtain the Trp content. In the other peptides, the presence of Trp residues is verified by the FAB-MS values. [b] Solvent system A, butanol/acetic acid/water (4:1:2, v/v/v); solvent system B, butanol/acetic acid/water/pyridine (4:1:2:1, v/v/v/v). R_f values were determined using TLC on silica. [c] K' values were determined for a μBondapak C_{18} column using a 20−60% acetonitrile gradient (0.025% TFA) over 20 min. [d] The values represent monoisotopic masses. [e] ND, not done.

Table 2: Chemical and Physical Properties of Linear Analogs

	amino acid analysis										R_f[b]				
peptide	Asx	Glx	Gly	His	Leu	Lys	Nle	Pro	Tyr	Trp[a]	solvent A	solvent B	K'[c]	FAB-MS[d]	calculated mass (amu)
L(4)2		1.93	1.01	0.91	2.22	1.12	0.86	1.97	0.99	ND[e]	0.43	0.59	5.99	1666.9	1666.8
L42		2.24	1.03	0.84	1.95	0.98	1.04	1.96	0.96	ND	0.34	0.53	5.33	1666.9	1666.8
L32		1.93	1.01	0.89	1.96		0.95	2.18	1.09	1.91	0.44	0.59	6.06	1653.0	1652.8
L22		1.97	1.04	0.98	2.15		0.86	2.07	1.10	1.88	0.45	0.61	6.14	1638.1	1638.8
L12		1.77	1.00	1.05	1.87		ND	2.13	1.19	ND	0.47	0.63	6.19	1625.4	1624.8
L41	1.07	1.02	1.13	0.88	1.83	1.01	0.84	2.20	1.03	ND	0.48	0.59	6.11	1653.0	1652.8
L31	1.09	0.87	0.97	1.00	1.77		ND	2.18	1.13	ND	0.49	0.58	6.11	1639.4	1638.8
L21	1.21	0.98	1.09	1.06	2.02		ND	2.35	1.26	ND	0.44	0.60	6.13	1625.3	1624.8
L11	1.08	0.87	0.97	1.01	1.85		ND	2.07	1.15	ND	0.47	0.61	6.20	1611.2	1610.8

[a] In two cases (**L32** and **L22**), special care was taken during hydrolysis to obtain the Trp content. In the other peptides, the presence of Trp residues is verified by the FAB-MS values. [b] Solvent system A, butanol/acetic acid/water (4:1:2, v/v/v); solvent system B, butanol/acetic acid/water/pyridine (4:1:2:1, v/v/v/v). R_f values were determined using TLC on silica. *c* K' values were determined for a μBondapak C_{18} column using a 20−60% acetonitrile gradient (0.025% TFA) over 20 min. [d] The values represent monoisotropic masses. [e] ND, not done.

ing 1.5% DIEA) was added and the mixture was shaken overnight (total coupling time, 12 h). A negative Kaiser test was obtained, which indicated the absence of free amino groups. The on-resin cyclization of other cyclic peptides followed the preceding protocol. Increased difficulty with amide bond formation was encountered as the size of the lactam ring decreased. For example, cyclization to form **C11** required a total of 28 h of coupling time.

HF Cleavage. It was found to be beneficial to deprotect other side chain protecting groups before HF cleavage (Xue *et al.*, 1989). In particular, cleaner crude products were obtained when the tosyl group on His was removed using HOBt. The tosyl group on His^2 was deprotected with 5% HOBt in DMF (1 × 2 min and 1 × 30 min), and the amino-terminal Boc group was deprotected with 50% TFA in CH_2Cl_2 (1 × 2 min and 1 × 30 min). The formyl groups on Trp^1 and Trp^3 were deprotected during the treatment with 20% piperidine in DMF used to remove the Fmoc and OFm groups prior to cyclization (see above). The resin was washed with CH_2Cl_2 (2 × 1 min), MeOH (2 × 1 min), CH_2Cl_2 (2 × 1 min), and MeOH (2 × 1 min) and dried in vacuo overnight. One gram of the resin was mixed with 1 mL of anisole in a Kel-F HF reaction vessel, and HF was condensed at −78 °C under reduced pressure to a total volume of 15 mL (HF + anisole + resin). The HF cleavage was allowed to proceed at −5 to 0 °C for 1 h. After evaporation of HF, the residue was washed with ethyl ether to remove scavenger, extracted with 20−50% acetic acid, and lyophilized. The yields of the crude peptides, based on the starting amine content of the aminomethyl resin, were in the ranges 32−58% for the cyclic analogs and 35−83% for the linear analogs.

Tritiated [Nle^{12}]-α-factor was prepared as previously described (Raths *et al.*, 1988). Briefly, α-factor containing dehydroproline (Dhp) in place of proline in positions 8 and 11 and norleucine at position 12 in place of methionine was synthesized by standard methods of solid-phase peptide synthesis. [Dhp^8,Dhp^{11},Nle^{12}]-α-factor was tritiated by Amersham Corp. by the T-3 method, involving reduction by tritium gas. The crude tritiated peptide was purified to homogeneity by HPLC as previously described (Raths *et al.*, 1988). Purity and specific activity were monitored by TLC, HPLC, amino acid analysis, and biological activity. Control reductions with hydrogen gas indicated that [Nle^{12}]-α-factor was fully regenerated (Raths *et al.*, 1988).

Purification and Characterization

The crude peptides were analyzed by reversed-phase analytical HPLC on a Waters μBondapak C_{18} column (3.9 × 300 mm). Crude peptides usually were dissolved in 20−40% CH_3OH/H_2O/0.025% TFA and purified by reversed-phase, semipreparative HPLC on a Waters μBondapak C_{18} column (19 × 150 mm or 19 × 300 mm). The purification was carried out with a linear gradient of H_2O (0.025% TFA) and CH_3CN (0.025% TFA), from about 20% CH_3CN to about 60% CH_3CN, over 60 min at a flow rate of 6 mL/min. Up to 200 mg of crude material could be purified in a single run. The linear analogs were usually over 99% pure after a single purification. Cyclic analogs needed additional chromatographic separations. All analogs exhibited one spot on silica thin layers in two solvent systems using UV and ninhydrin detection (Tables 1 and 2). Amino acids analyses were carried out at the Wistar Institute, and FABMS was measured at the University of Tennessee Mass Spectrometry

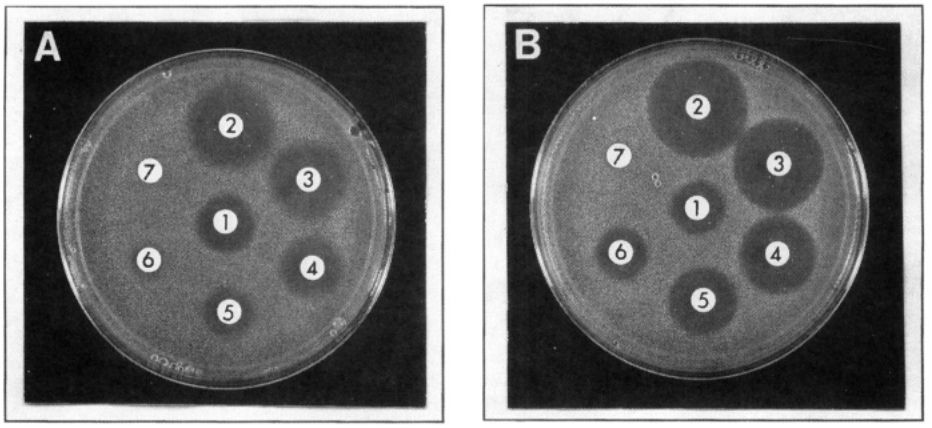

FIGURE 2: Growth arrest of *S. cerevisiae* RC629(**MAT***a sst1-2*) by α-factor analogs. Peptides were added to sterile disks placed on a lawn of RC629 cells and incubated until well-developed halos of growth inhibition could be observed. (A) [Nle[12]]-α-factor at 0.2 μg (disk 1) and **C12** at 10, 5, 2, 1, 0.5, and 0.2 μg (disks 2−7, respectively). (B) [Nle[12]]-α-factor at 0.2 μg (disk 1) and **L12** at 10, 5, 2, 1, 0.5, and 0.2 μg (disks 2−7, respectively).

Center. ^{1}H NMR spectra were acquired on a 400 MHz JEOL GX-400 spectrometer or a Bruker 200 MHz spectrometer. Data processing was performed on a SUN Sparc IPC workstation utilizing the NMRI 1.4.1 software package.

CD Measurements

All CD measurements were performed on a Jasco J-500 spectropolarimeter using circular quartz cells, with a path length of 0.01 cm at ambient temperature (~25 °C). The instrument was calibrated with *d*-(+)-10-camphorsulfonic acid. Spectra were obtained in the range 190−260 nm, at a scan speed of 20 nm/min and using a time constant of 16 s. Peptide solutions were prepared in HPLC grade water and methanol. All spectra were corrected by subtracting the solvent absorption recorded under identical conditions. The CD spectra are reported in terms of molar ellipticity, $[\theta]_M$ (deg cm^2 dmol^{-1}), wherein the actual molecular weight of the peptide and not the mean residue molecular weight was used to calculate the ellipticity. The CD additivity experiment was carried out following a method described by Fasman and co-workers (Perczel *et al.*, 1993). Specifically, Ac-cyclo[Orn-Pro-Gly-Glu]-NH_2 (0.44 mg, 1 μmol) and **L32** (2.00 mg, 1 μmol) were dissolved in a mixture of 1 mL of water and 1 mL of MeOH. In the additivity experiment, for molar ellipticity calculations, the average concentration, 0.61 mg/mL, was used with a modified molecular weight of 1217 (average molecular weight of the two peptides).

Growth Arrest Assay

The biological activity of the α-factor analogs was assayed using a growth arrest assay (halo) as described previously (Raths *et al.*, 1988). Briefly, peptides to be tested are added to sterile filter disks (diameter = 6 mm) placed on a lawn of *S. cerevisiae* RC629. The amount of peptide is systematically lowered until no growth arrest (as indicated by a halo around the disk) is observed. Activity was measured as the diameter (mm) of the growth arrest halo on a lawn of *S.* cerevisiae cells. At least three determinations were made and averaged. The values of each experiment were within 2 mm of each other. Results for a typical experiment are given in Figure 2, where the end points for **C12** and **L12** are 0.5 μg/disk for this determination, although the zone diameters clearly are different. All activities were examined using a supersensitive mutant (RC629 (*sst1*)) of *S. cerevisiae*. The *sst1* mutation results in the loss of *BAR1* protease, a secreted enzyme that cleaves the α-factor between residues 6 and 7 (Chan & Otte, 1982). Thus, the biological activities and the results of receptor binding assays (see the following) are not influenced by peptide degradation.

Binding Competition Assay

Competition of bound, tritiated [Nle[12]]-α-factor by unlabeled α-factor analogs was measured by the following protocol. *S. cerevisiae* strain 4202-15-3 (*MAT***a** *cryl bar1-1 ade2-1 his 4-580 lys2 tyrl SUP4-3)* was grown in YM-1 medium [5 g/L yeast extract, 10 g/L peptone, 6.7 g/L yeast nitrogen base without amino acids, 0.01 g/L adenine, 0.01 g/L uracil, 10 g/mL succinic acid, 6 g/L sodium hydroxide, and 10 g/mL glucose at a final pH of 5.8] overnight at 30 °C with shaking at 120 rpm to 1 × 10^7 cells/mL. Cells were harvested by centrifugation (6000*g* for 10 min at 4 °C), and the resultant pellet was washed twice with ice-cold YM-1+i medium (YM-1 containing 10 mM NaN_3, 10 mM KF, and 10 mM *p*-tosyl-L-arginine methyl ester) and resuspended to 1.25 × 10^9 cells/mL in YM-1+i. Final assay concentrations of the unlabeled competitors were between 3 × 10^{-5} and 1 × 10^{-9} M, as determined from the A_{287} for each peptide and the extinction coefficients calibrated for [Nle[12]]-α-factor from quantitative elemental analysis. The reaction was started by the addition of 100 μL of an analog/[^{3}H] α-factor mix (120 μL of appropriate unlabeled α-factor analog mixed with 120 μL of labeled α-factor 9.3 × 10^{-7} M, 9.24 Ci/mmol) to 400 μL of cell suspension. Identical competition curves were obtained using a 10-fold lower concentration of radioactive ligand.

After the reaction was started, two 20 μL samples were removed and counted to determine the total amount of radioactivity present. Duplicate reactions were started 3 min after initial reaction for each analog tested. At 30 min, two 200 μL portions of cell suspension were added separately to 2 mL of YM-1+i and filtered over GN-6 Metricel filters (Gelman Sciences, Inc., Ann Arbor, MI) presoaked in 1% bovine serum albumin. The reaction tubes were then rinsed twice with 2 mL of ice-cold YM-1+i medium, with each rinse filtered over the same filter. Finally, each filter was rinsed twice with 2 mL of YM-1+i medium. The nonspecific binding of labeled α-factor to the filters was less than 100 cpm. This represented less than 5% of the total counts. The filters were counted to 5 mL of Budget-solve counting cocktail (Research Products International Corp., Mt. Prospect,

IL). All assays and manipulations were done in siliconized borosilicate tubes or vials. Equilibrium binding was reached after a 30 min incubation of cells and pheromone, with or without competitor, as indicated by identical binding and competition curves in control experiments using a 90 min incubation time (data not shown). The binding of the radioactively labeled [Nle^{12}]-α-factor was highly specific as the radioactive counts were reduced to background levels by the addition of high levels of unlabeled pheromone. The binding curve for α-factor has been previously obtained in the infinite dilution experiment by us and others (Raths *et al.*, 1988; Blumer *et al.*, 1988; Jenness *et al.*, 1986) to show that there is no evident cooperativity in the binding between α-factor and its receptor.

RESULTS

Synthesis and Characterization of Cyclic α-Factor Analogs. The lactam-containing α-factor analogs were all synthesized on PAM resins using the strategy previously described for the cyclo^{7,10}[Nle^{12}]-α-factor (Xue *et al.*, 1989). Cyclization was carried out on the resin using BOP as a coupling reagent. All crude peptides (both cyclic and linear) contained one major product and similar side products. In a parallel investigation of the synthesis of corresponding model tetrapeptides (e.g., Ac-cyclo[Lys-Pro-Gly-Glu]-NH_2), we observed a major impurity in the crude product (Rao *et al.*, 1995). This impurity has been isolated and shown to be the cyclic dimer that results from interpeptide amide bond formation. Similar interchain reactions have been reported during the syntheses of cyclic analogs of enkephalin (Schiller *et al.*, 1985). In the present case, it appears that inclusion of an Xxx-Pro-Gly-Zzz sequence in the center of a large peptide (such as the [Nle^{12}]-α-factor) impedes interchain reactions. This may be a consequence of a general steric effect that prevents interchain contacts or of the fact that cyclization is favored when the Xxx-Pro-Gly-Zzz sequence is present in the center of the tridecapeptide. The cyclic lactam-containing tridecapeptides were purified to near homogeneity using preparative reversed-phase chromatography. Yields of the final products varied over 5−20%. The yields of the analogous linear peptides were 8−35%.

Chemical and Spectroscopic Characterization of α-Factor Analogs. All peptides subjected to bioassay were >99% pure as judged by acetonitrile/water/trifluoroacetic acid gradients on reversed-phase HPLC and greater than 97% pure using methanol/water/trifluoroacetic acid gradients (Figure 3). The HPLC analysis permits us to conclude that the cyclic analogs contained less than 0.1% of the corresponding linear homolog. We also coinjected [Nle^{12}]-α-factor with the purified analogs and ascertained that the parent pheromone was not present in any of these peptides (data not shown). Therefore, it is highly unlikely that the activity of the cyclic lactams is due to contamination by either [Nle^{12}]-α-factor or the corresponding linear homolog. This conclusion is supported by the more than 100-fold variation in the activity of the cyclic analogs and by the fact that several cyclic analogs (e.g., **C22** and **C12**) have as much as 10% of the activity of [Nle^{12}]-α-factor (see Table 3). Neither of these observations can be explained by spurious impurities that might be picked up during HPLC purification.

The peptides had the expected amino acid ratios and monoisotopic masses within 0.7 amu of the calculated values (Tables 1 and 2). The linear and cyclic analogs differ by 18 amu, indicating formation of the amide bond between the side chains of residues 7 and 10. Several of the peptides were characterized by 1D and 2D NMR spectroscopy and gave the expected resonances. The $K^7{}_\alpha-P^8{}_\delta$ connectivity that appeared in the NOESY spectra of **C32** and **C42** indicates that the amide bond involving Pro^8 is in a *trans* configuration in these 17- and 18-membered lactam-containing tridecapeptides (data not shown). A detailed conformational analysis of all these cyclic tridecapeptides, using ^{1}H NMR and quantitative NOE approaches, is currently in progress.

Circular Dichroism Studies on Cyclic and Linear Tridecapeptides. In order to gain insights into the conformational preferences of the lactam-containing α-factor analogs, we measured the circular dichroism spectra of **C32** and ap-

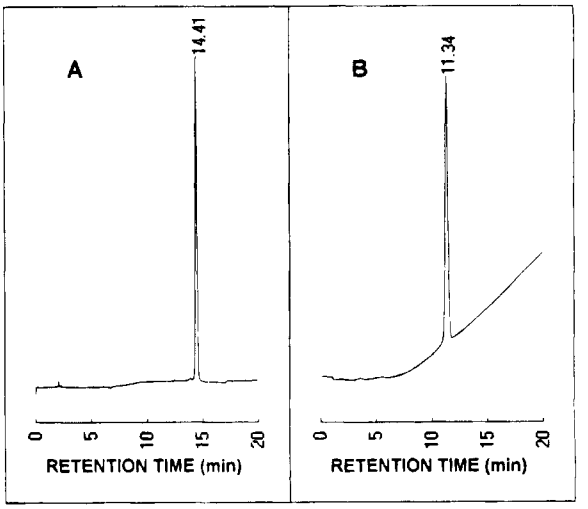

FIGURE 3: (A) HPLC of **C22** in a H_2O/CH_3CN system. Chromatography was performed on a C_{18} column using a 20−60% CH_3CN gradient (0.025% TFA) over 20 min. (B) HPLC of **C22** in a H_2O/MeOH system. Chromatography was performed on a C_{18} column using a 50−90% MeOH gradient (0.025% TFA) over 20 min.

Table 3: Bioactivity and Receptor Affinity of Cyclic and Linear α-Factor Analogs

peptide	ring size	bioactivity[a] (μg/disk)	IC_{50}[c] (M × 10^7)	IC_{50} analog/IC_{50} ([Nle^{12}]-α-factor
C(4)2	18	>10 (>200)[b]	100	50
C42	18	0.5 (10)	150	75
C32	17	10 (200)	600	300
C22	16	0.5 (10)	2200	1100
C12	15	0.5 (10)	2000	1000
C41	17	>10 (>200)	>10000	>5000
C31	16	10 (200)	45000	22500
C21	15	5 (100)	10000	5000
C11	14	>50 (>1000)	>10000	>5000
L(4)2		5 (100)	700	350
L42		0.1 (2)	10	5
L32		0.1 (2)	30	15
L22		0.05 (1)	600	300
L12		0.1 (2)	260	130
L41		10 (200)	1400	700
L31		5 (100)	10000	5000
L21		5 (100)	2700	1350
L11		5 (100)	10000	5000

[a] The minimum amount of pheromone causing growth arrest of *S. cerevisiae* RC629. The quantities (μg/disk) represent the average of at least three determinations. [b] The numbers in parentheses are the ratio of the end point amounts for the analog to that of [Nle^{12}]-α-factor that caused growth arrest at a minimum of 0.05 μg/disk. [c] Peptide concentration needed to reduce binding by 50%. IC_{50} of [Nle^{12}]-α-factor = 2.0×10^{-7} M.

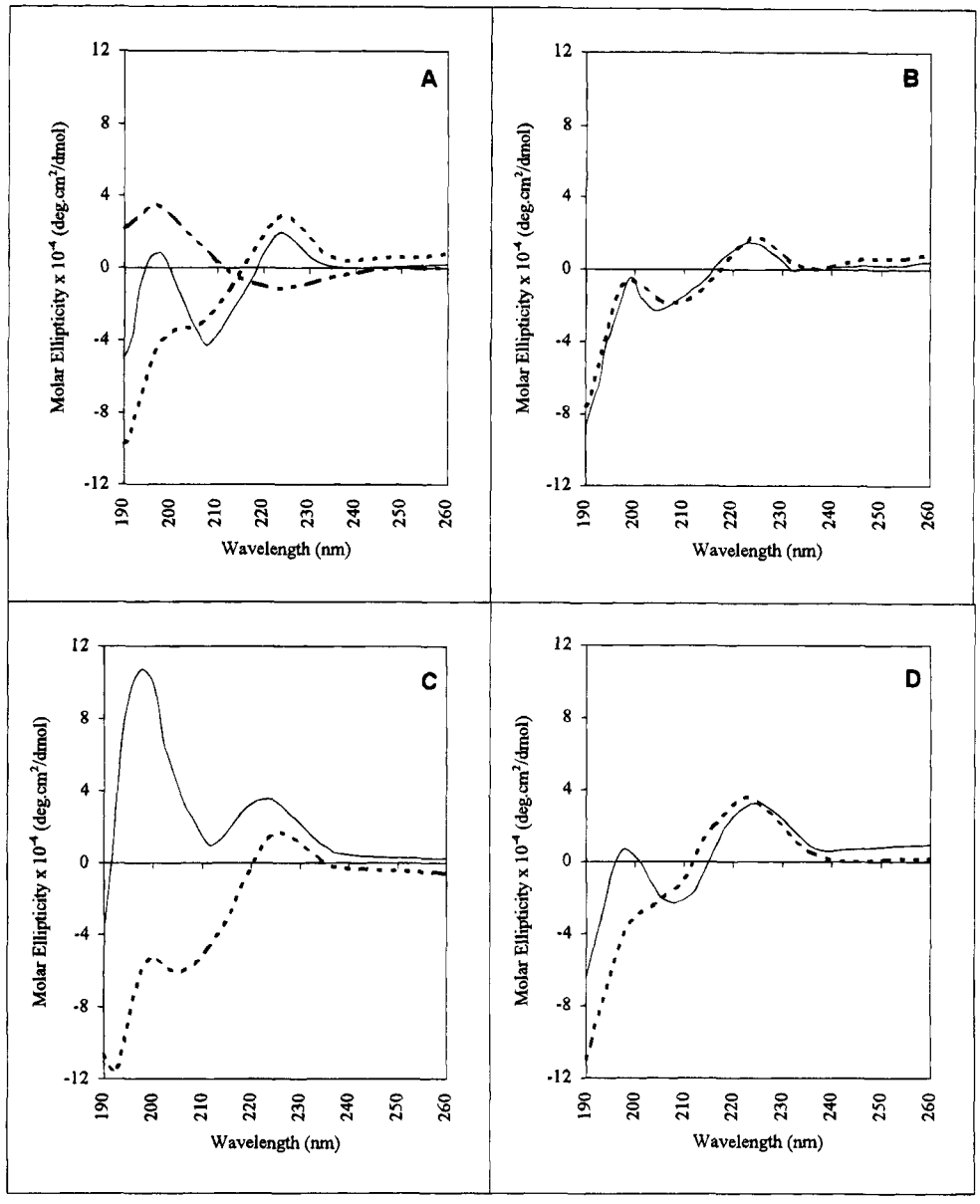

FIGURE 4: Circular dichroism of [Nle[12]]-α-factor analogs in methanol/water: (A) 1.0 mg/mL **C32** (—), 1.0 mg/mL **L32**, (- - -), 1.0 mg/mL Ac-cyclo[Orn-Pro-Gly-Glu]-NH_2 (— - -); (B) experimental (—) and computed (- - -) CD spectra of equimolar mixtures of **L32** and Ac-cyclo[Orn-Pro-Gly-Glu]-NH_2; (C) 1.0 mg/mL **C12** (—), 1.0 mg/mL **L12** (- - -); (D) 1.0 mg/mL **C21** (—), 1.0 mg/mL **L21** (- - -).

propriate controls. Previous CD studies on α-factor concluded that this linear peptide is predominantly unstructured in aqueous buffer and becomes more ordered in organic solvents or the presence of lipid (Higashijima et al., 1983; Shenbagamurthi et al., 1985). The CD pattern of **L32** in water/methanol (1:1, v/v) shows a broad minimum below 195 nm, a shoulder at 207 nm, and positive ellipticity between 217 and 235 nm (Figure 4A). Such a curve would be associated with a predominantly disordered peptide. In contrast, the CD pattern for a model tetrapeptide (Ac-cyclo-[Orn-Pro-Gly-Glu]-NH_2), which represents the cyclized central region of **C32**, exhibits a negative minimum at 223 nm and a maximum at 198 nm in methanol/water (Figure 4A). The general shape of this curve is typical of those found for peptides in β-turn structures (Woody, 1974). The CD curve for **C32** (Figure 4A) differs appreciably from those for **L32** and the cyclic tetrapeptide. However, CD spectra obtained on an equimolar mixture of **L32** and Ac-cyclo-[OrnProGlyGlu]-NH_2 or by computer summation of the patterns for **L32** and *Ac*-cyclo[OrnProGlyGlu]-NH_2 in Figure 4A are qualitatively similar to that found for **C32** (compare Figure 4A,B). On the basis of these results, it is reasonable to conclude that the central region of **C32** retains the turn structure found in the cyclic tetrapeptide. We also measured the CD spectra of the other cyclic analogs and their linear homologs in methanol/water (1:1, v/v). All of the linear analogs exhibit CD patterns that are similar to that of **L32** (for example, compare Figure 4A,C,D). In contrast, as was found for **C32**, the CD patterns for the cyclic lactam-containing tridecapaptides all differ markedly from that of the linear homolog (Figure 4C,D). A more detailed understanding of the conformational preferences of the center of the cyclic pheromones will be forthcoming upon completion of our NMR analysis.

Biological Activity of Lactam-Containing α-Factor Analogs. The biological activity of the lactam analogs was determined using *S. cerevisiae* RC629. The most potent cyclic analogs had end points in the growth arrest assay of 0.5 μg/disk (**C42**, **C22**, **C12**; Figure 2 and Table 3). The activities of the cyclic analogs varied from 10% (**C12**) to less than 0.1% (**C11**) of the activity of the parent [Nle[12]]-α-factor (Table 3). In all cases, the series containing Glu

in position 10 (compounds where $n = 2$; see Figure 1) had higher potency than the corresponding analog with Asp. For example, the **C42** tridecapeptide was at least 20-fold more potent than the homologous **C41** pheromone. Except for the ornithine-containing peptides (**C32** and **C31**), the difference was usually at least 1 order of magnitude. The lactam containing D-Lys in position 10 (**C(4)2**) was inactive at all concentrations tested.

Biological Activity of Linear α-Factor Analogs. Linear model peptides identical to the lactam analogs except for a hydrolyzed lactam bond were evaluated using the same bioassays (Figure 2, Table 3). These peptides differ from the constrained peptides in that the amine and carboxyl side chains of residues 7 and 10 are ionizable. It is unclear which linear peptide most closely models the lactam, since the nitrogen in the lactam ring is acylated whereas the carbonyl is N-alkylated. The activities of [Nle^{12}]-α-factor and [Glu^{10},-Nle^{12}]-α-factor (**L42**) are very similar. We decided, therefore, to use linear analogs with a free amine at the position 7 side chains and a free COOH at the position 10 side chain for comparative purposes in this investigation. We again found that, in all cases, the series with Glu at residue 10 was significantly more potent than corresponding peptides with Asp at this position. For example, **L32** was 50 times more potent than **L31** against RC629. All linear peptides within each series (Glu^{10} or Asp^{10}) had similar activities.

Competition for Binding to the STE2 Receptor. The affinity for the α-factor receptor was determined by measuring the competition between the analogs and tritiated [Nle^{12}]-α-factor. In all cases, the concentration of analog required to displace 50% of the tritiated pheromone from the receptor was at least 15-fold higher than that of [Nle^{12}]-α-factor and a minimum of 3-fold higher than that of the **L42** analog (Table 3). In general, the lactams with Glu at position 10 exhibited higher receptor affinity than those with Asp at this position. This was also true for the corresponding linear homologs. The relative receptor affinities varied from nearly 300-fold (compare **L31** with **L32**) to as low as 5 (compare **C22** with **C21**). As the size of the lactam ring decreased from 18 members to 15 members in the Glu series, the affinity for the receptor decreased by approximately 15-fold. Interestingly, the cyclic compound containing D-Lys had a higher affinity for the receptor than the corresponding linear homolog. This is the only case where a cyclic lactam analog was a stronger binder than the linear homolog.

DISCUSSION

Previous studies on a constrained congener of α-factor concluded that the pheromone was likely in a bent structure when bound to its receptor (Xue *et al.*, 1989). Complementary biophysical analysis on linear and disulfide-constrained analogs suggested that the middle of the α-factor assumes a β-turn (Jelicks *et al.*, 1988; Naider *et al.*, 1992; Gounarides *et al.*, 1994). In this paper, we report a biochemical analysis of eight synthetic cyclic analogs (and their linear counterparts) of the mating pheromone containing a systematic variation in the size of the constrained region. The growth arrest and binding assays used in this investigation were carried out on strains lacking the BAR protease, the enzyme that cleaves α-factor. Thus, pheromone degradation does not influence our findings, and the results reflect the true potency of the analogs and their relative receptor affinities.

Earlier investigations on the α-factor have provided important insights into residues that influence the activity of the pheromone (Masui *et al.*, 1979; Naider & Becker, 1986). Our goal in this investigation was to relate the activity of the pheromone to spatial constraints conferred by side chain cyclization in the center of the peptide. Before addressing this issue, we note that the results of the growth arrest analysis provide new knowledge concerning molecular groupings that influence the activity of the pheromone. Specifically, the γ-carbonyl of residue 10 was found to be a critical determinant of the potency of the pheromone. Thus, all compounds with Glu at this position are more active than those with Asp. This is true regardless of whether the pheromones are cyclic or linear. The growth arrest assay also gives evidence that the length of the side chain at position 7 is not decisive for optimal activity; in the linear series containing either Asp^{10} or Glu^{10}, the activity of the pheromones varies very little as the side chain in position 7 is changed from $(CH_2)_4NH_3^+$ to $(CH_2)NH_3^+$ (Table 3). The fact that the ε-amine in position 7 of the native α-factor is not essential for activity has been noted previously (Samokhin *et al.*, 1979; Shenbagamurthi *et al.*, 1983). Comparison of corresponding linear and cyclic analogs shows that the linear peptides were always the most potent. However, the ratio of activities varied from a high of 100 for **L32** versus **C32** to lows of 2 and 5 for the **L31/C31** and **L12/C12** homologs, respectively. The D-Lys-containing linear peptide had $^1/_{100}$ the activity of the parent compound.

The bioactivity of the cyclic analogs does not vary in a simple manner with the size of the lactam ring. Among the most potent α-factor analogs are those containing 18-, 16-, and 15-membered rings (**C42**, **C22**, and **C12**). These peptides, which all contain Glu^{10}, show virtually no change in activity as the ring size decreases. In contrast, the 17-membered ring analog in this series (**C32**) has relatively low activity. The 17-membered ring analog in the Asp series (**C41**) also has very low activity. However, in the Asp series, the potency of all analogs is very low, with the 14-membered lactam analog (**C11**) having the lowest activity. In comparing the two series it is clear that the specific composition and conformation of the ring are more important than ring size in affecting biological activity.

The most striking outcome of the binding competition studies is that any change in the side chains of the residues in positions 7 or 10 leads to a decrease in affinity for the receptor. A decrease in the length of the position 7 side chain from four methylenes to three methylenes results in 3-fold and 7-fold drops in affinity in the linear Glu^{10} and the linear Asp^{10} series, respectively. A change in the side chain from Glu to Asp in the Lys- and Orn-containing linear pheromones results in 140- and 330-fold decreases in affinity, respectively. As was true for biological potency, the γ-carbonyl appears to have a greater influence on receptor affinity than the ε-NH moiety. In Glu-containing analogs, covalent linkage of the side chains of residues 7 and 10 further reduces the affinity for the receptor by 3−20-fold. Such a modification in the Asp series, where receptor affinities are extremely low in both the linear and cyclic peptides, has at most a 4-fold effect.

The concept of a ligand binding to a receptor and not elucidating a response has long been noted in the literature. The ultimate example is a pure competitive antagonist, which binds strongly to the agonist binding site but does not transduce signal. Less well understood would be a ligand that binds weakly, yet results in strong signal transduction. In the present study, we observed a clear dissociation

between binding and biological potency, as defined by the absolute amount of a pheromone that results in a biological response. Specifically, **L42** and **L22** have nearly identical bioactivities against strain RC629, but the latter compound binds 60 times less effectively. Similarly, **C12** and **C22** have the same potency as **C42** in the halo assay, yet the latter compound binds more than an order of magnitude more avidly. For structurally similar compounds such as **L42** and **L22** to exhibit equal potency and a nearly 2 orders of magnitude variation in affinity would require the poorly bound pheromone (**L22**) to be an extremely efficient transducer of signal. This latter comparison attempts to normalize the potency of the pheromones at identical receptor occupancies. Although this approach is appealing, there are several specific problems associated with its application to our analysis: it requires the accurate determination of K_i values and a direct correlation of binding to bioactivity. The poor binding of several of our analogs (Table 3), even at millimolar concentrations, precludes the determination of their binding constants. Given the current lack of understanding of the atomic interactions between bound pheromone and receptor, we cannot offer an unequivocal explanation for the apparent highly efficient signalling by certain poorly bound analogs.

CD analysis of **C32** leads to the conclusion that the cyclized region of this tridecapeptide retains certain conformational features found in the corresponding model cyclic tetrapeptide (Figure 4A,B). Similar studies on the remaining analogs of [Nle^{12}]-α-factor showed that all of the cyclic peptides have significantly different CD patterns than their linear homologs (Figure 4C,D). We believe that these results suggest that the cyclization perturbs the conformation of the center of the pheromone and that the conformation of the cyclic lactam in the tridecapeptide is probably quite similar to that assumed by the corresponding model tetrapeptide. Detailed CD studies on the complete series of model tetrapeptides containing the constrained regions of the cyclic pheromones reported in this paper indicate that models of **C42** and **C22** assume structures that are nearly perfect type II β-turns (Rao *et al.*, 1995). The fact that the α-factor analogs corresponding to the tetrapeptide models that form the best type II β-turns are the most potent cyclic analogs reinforces our previous suggestion that a type II β-turn in the middle of the peptide may be an important determinant of the biologically active state (Shenbagamurthi *et al.*, 1983, 1985). Nevertheless, NMR studies in different solvents show that various amounts of conformational heterogeneity exist in these model tetrapeptides. Therefore, conclusions on the correlation between bioactivity and structure and on the exact conformations assumed by the central regions of the different cyclic tridecapeptides must await the outcome of detailed NMR, CD, and energy minimization studies that are currently in progress.

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REFERENCES

Al-Obeidi, F., de L. Castrucci, A. M., Hadley, M. E., & Hruby, V. J. (1989) *J. Med. Chem. 32*, 2555−2561.

Blumer, K. J., Reneke, J. E., & Thorner, J. (1988) *J. Biol. Chem. 263*, 10836−10842.

Bolin, D. R., Wang, C.-T., & Felix, A. M. (1989) *Org. Prep. Proced. Int. 21*, 67−74.

Chan, R. K., & Otte, C. A. (1982) *Mol. Cell. Biol. 2*, 11−20.

Fry, D. C., Madison, V. S., Greeley, D. N., Felix, A. M., Heimer, E. P., Frohman, L., Campbell, R. M., Mowels, T. F., Toome, V., & Wegrzynski, B. B. (1992) *Biopolymers 32*, 649−666.

Goundarides, J. S., Broido, M. S., Becker, J. M., & Naider, F. R. (1993) *Biochemistry 32*, 908−917.

Goundarides, J. S., Xue, C.-B., Becker, J. M., & Naider, F. R. (1994) *Biopolymers 34*, 709−720.

Higashijima, T., Fujimura, K., Masui, Y., Sakakibara, S., & Miyazawa, T. (1983) *FEBS Lett. 159*, 229−232.

Hruby, V. J. (1982) *Life Sci. 31*, 189−201.

Jelicks, L. A., Naider, F. R., Shenbagamurthi, P., Becker, J. M., & Broido, M. S. (1988) *Biopolymers 27*, 431−449.

Jenness, D. D., Burkholder, A. C., & Hartwell, L. H. (1986) *Mol. Cell. Biol. 6*, 318−320.

Kaiser, E., Colescott, R. K., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34*, 595−598.

Masui, Y., Tanaka, T., Chino, N., Kita, H., & Sakakibara S. (1979) *Biochem. Biophys. Res. Commun. 86*, 982−987.

Motta, A., Picone, D., Tancredi, T., & Temussi, P. A. (1988) *Tetrahedron 44*, 975−990.

Naider, F., & Becker, J. M. (1986) *CRC Crit. Rev. Biochem. 21*, 225−248.

Naider, F., Gounarides, J., Xue, C.-B., Bargiota, E., & Becker, J. M. (1992) *Biopolymers 32*, 335−339.

Perczel, A., Kollat, E., Hollosi, M., & Fasman, G. D. (1993) *Biopolymers 33*, 665−685.

Rao, M. H., Yang, W., Joshua, H., Becker, J. M., & Naider, F. (1995) *Int. J. Pept. Protein Res.* (in press).

Raths, S. K., Naider, F., & Becker, J. M. (1988) *J. Biol. Chem. 263*, 17333−17341.

Rizo, J., & Gierasch, L. M. (1992) *Annu. Rev. Biochem. 61*, 387−418.

Roques, B. P. (1992) *Biopolymers 32*, 407−410.

Samokhin, G. P., Lizlova, L. V., Bespalova, J. D., Titov, M. I., & Smirnov, V. N. (1979) *FEMS Microbiol. Lett. 5*, 435−438.

Schiller, P. W., Nguyen, T. M. D., Lemieux, C., & Maziak, A. (1985) *J. Med. Chem. 28*, 1766−1771.

Shenbagamurthi, P., Baffi, R., Khan, S. A., Lipke, P., Becker, J. M., & Naider, F. (1983) *Biochemistry 22*, 1298−1304.

Shenbagamurthi, P., Kundu, B., Raths, S., Becker, J. M., & Naider, F. (1985) *Biochemistry 24*, 7070−7076.

Sprague, G. F., Jr. & Thorner, J. W. (1992) in *Molecular and Cellular Biology of the Yeast Saccharomyces (Gene Expression)* (Jones, E. W., Pringle, J. R., & Broach, J. R., Eds.) Vol. 2, pp 657−744, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Stanfield, C. F., Felix, A. M., & Danho, W. (1990) *Org. Prep. Proced. Int. 22* (5), 597−603.

Struthers, R. S., Tanaka, G., Koerber, S. C., Solmajer, T., Baniak, E. L., Gierasch, L. M., Vale, W., Rivier, J., & Hagler, A. T. (1990) *Proteins: Struct. Funct., Genet. 8*, 295−304.

Tam, J. P., Kent, S. B. H., Wong, T. W., & Merrifield, R. B. (1979) *Synthesis 12*, 955−957.

Veber, D. F., Holly, F. W., Nutt, R. F., Bergstrand, S., Brady, S. F., Hirschmann, R., Glitzer, M. S., & Saperstein, R. (1979) *Nature 280*, 512−514.

Veber, D. F., Freidinger, R. M., Perlow, D. S., Paleveda, W. J., Jr., Holly, F. W., Strachan, R. G., Nutt, R. F., Arison, B. H., Homnick, C., Randall, W. C., Glitzer, M. S., Saperstein, R., & Hirschmann, R. (1981) *Nature (London) 292*, 55−58.

Waki, M., Kitajima, Y., & Izumuya, N. (1981) *Synthesis 4*, 266−267.

Woody, R. W. (1974) in *Peptides, Polypeptides and Proteins* (Blout, E. R., Bovey, F. A., Goodman, M., & Lotan, N., Eds.) pp 338−350, Wiley Interscience, New York.

Xue, C.-B., Eriotou-Bargiota, E., Miller, D., Becker, J. M., & Naider, F. (1989) *J. Biol. Chem. 264*, 19161−19168.

BI9416162