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Synthesis and Biological Activity of Amino Terminus Extended Analogues of the α Mating Factor of *Saccharomyces cerevisiae*†

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ABSTRACT: The synthesis and biological activity are reported for extended analogues of the secreted tridecapeptide α-factor (Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr) from *Saccharomyces cerevisiae*. Peptides with Ala, Glu-Ala, Ala-Glu-Ala, or Glu-Ala-Glu-Ala attached to the amino terminus of α-factor were synthesized by the solid-phase method on a (phenylacetamido)methyl (PAM) resin, using a combination of dicyclohexylcarbodiimide- and 1-hydroxybenzotriazole-accelerated active ester coupling procedures. Free peptides were obtained by hydrogen fluoride (HF) cleavage in the presence of appropriate scavengers. Normal high HF cleavage and "low–high" HF cleavage were equally effective in liberating the desired product from the PAM resin. Yields of pure peptide ranged from 9% to 17%. All of the extended α-factors, which represent sequences of pro-α-factor coded for in the *MFα1* structural gene, caused morphological aberrations (shmoo assay) in strain X2180-1A (*MATa*) the same as those caused by the tridecapeptide. The 14-peptide was equally active compared to the native α-factor whereas the 17-peptide was 5–10-fold less active. The analogues also arrested to various degrees (halo assay) the growth of *S. cerevisiae* RC629 (*MATa sst1*) and *S. cerevisiae* RC631 (*MATa sst2*), two supersensitive mutants, and were converted to pheromones of equal activity by treatment with V8 protease. A temperature-sensitive receptor mutant responded to all the peptides at the permissive but not the restrictive temperature. An α-factor antagonist, des-Trp^1,Ala^3-α-factor, inhibited activity of all extended peptides. These results confirm that all the extended peptides interact with the same receptor and that this receptor can accommodate additional residues at the amino terminus of the α-factor.

Cells of the α mating type of *Saccharomyces cerevisiae* secrete a low molecular weight peptide, termed α-factor, which is required for sexual conjugation between α haploids (*MATα*) and *a* haploids (*MATa*) of this yeast (Thorner, 1981). The α-factor specifically inhibits DNA replication and initiates an aberrant elongation of *MATa* cells, called "shmooing", which forms the basis for a biological assay of the mating factor. The sequence of two peptides responsible for α-factor activity, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr and His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr, was determined by Stotzler et al. (1976). The tridecapeptide is the predominant species responsible for mating factor activity. Direct evidence has been obtained that the α-factor encoded by the *MFα1* gene is biosynthesized as a larger precursor polypeptide, prepro-α-factor (Emter et al., 1983; Julius et al., 1983, 1984a,b). This precursor contains four identical tandem repeats of the mature pheromone sequence, each separated from the other by spacer regions of six to eight residues of nearly identical sequence: Lys-Arg-Glu-Ala-Glu-Ala and Lys-Arg-Glu-Ala-Glu(or Asp)-Ala-Glu-Ala.

Previous genetic and biochemical studies of mutations affecting processing and secretion of prepro-α-factor (Achstetter & Wolf, 1985; Julius et al., 1983, 1984a), as well as analysis of the organization of the α-factor structural gene *MFα1* (Kurjan, 1985; Kurjan & Herskowitz, 1982; Singh et al., 1983), provide strong evidence that the first proteolytic processing event in α-factor maturation is cleavage at the carboxyl side of the Lys-Arg pair of the spacer regions. Such cleavage would result in four propheromone fragments containing four to six residues, Glu-Ala-Glu-Ala or Glu-Ala-Glu(or Asp)-Ala-Glu-Ala, attached to the amino terminus of the mature tridecapeptide α-factor. Three of these fragments would also have the Lys-Arg pair attached to their carboxyl ends. The final processing events to yield tridecapeptide would be the result of the combined action of carboxypeptidase ysc (which cleaves Lys-Arg) and dipeptidyl aminopeptidase yscIV (which cleaves X-Ala sequences) (Wolf, 1986).

Nonmating *ste13* mutants of *MATα* cells bearing defects in the dipeptidyl aminopeptidase do not produce normal α-factor (Julius et al., 1983). Rather, α-factor reported to contain Glu-Ala-Glu-Ala or Asp-Ala-Glu-Ala on the amino terminus of a tridecapeptide was isolated from cultures of *ste13*

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Table I: *S. cerevisiae* Strains Used

strain	genotype	relevant phenotype	source
X2180-1A	*MATa gal2*	wild type	YGSC[a]
RC618	*MAT rme ade2-1 ural his6 met1 can1 cyh2* GAL	parent of RC629 and RC631	R. Chan
RC629	*MATa rme sst1-2 ade2-1 ural his6 met1 can1 cyh2* GAL	supersensitive to α-factor	R. Chan
RC631	*MATa sst2-1 rme ade2-1 ural can1 cyh2* GAL *his6 met1*	supersensitive to α-factor	R. Chan
50B	*MATa SUP4-3 ste2(ts) cry1-1 his4-580 trp1 ade2-1 tyr1 lys2*	temperature sensitive α-factor receptor	YGSC[a]

[a] Yeast Genetic Stock Center.

mutants. It was proposed that the presence of extra negatively charged residues reduced the biological potency by at least 2 orders of magnitude. In contrast, α-factor molecules containing a single additional amino-terminal alanine (generated by V8 protease cleavage) had essentially the same biological activity as mature α-factor.

Studies by Schwyzer and co-workers (Erne et al., 1985; Sargent & Schwyzer, 1986) indicate that residues near the amino terminus of a peptide often play a critical role in the biological activity of a peptide hormone. In particular, such residues can influence integration of a peptide into the lipid membrane, thereby leading to an increased probability for binding to the receptor. Precursors of α-factor contain residues (Glu and/or Ala) with a high statistical probability of assuming an α helix (Chou & Fasman, 1978). Helicity near the amino terminus has been suggested as an important component in facilitating specific interactions between various neuropeptides and lipid membranes (Schwyzer, 1985).

On the basis of the above considerations and our interest in the relationship between the primary structure of α-factor and its biological activity (Naider & Becker, 1986), we decided to synthesize extended analogues of α-factor and subject them to both biochemical and biophysical analysis. In this paper we report the synthesis and biological activities of tetra-, penta-, hexa-, and heptadecapeptides corresponding to the sequence of primary translation products of α-factor coded by the *MFα1* gene. We conclude that these amino terminus extended α-factors interact with the same receptor as does the secreted tridecapeptide.

Materials and Methods

Chemicals and Yeast Strains. Boc-Tyr[1] (2-bromo-Z) PAM resin (0.49 mmol of N/gm) was synthesized by the procedure of Mitchell et al. (1978). All Boc-amino acids were from Bachem Inc., Torrence, CA, except for Boc-Trp(CHO), which was from Peptides International, Louisville, KY. Dichloromethane (Fisher), dimethylformamide (J. T. Baker Chemical Co.), acetonitrile (Fisher), and methanol (Fisher) were either reagent or HPLC grade. TFA, DIEA, DCC, HOBt, *p*-cresol, *p*-thiocresol, and DMS were from Aldrich and were all reagent grade. Anhydrous HF was purchased from Matheson. Tridecapeptide α-factor was synthesized by solid-phase procedures as described below. Yeast nitrogen base without ammonium sulfate or amino acids and noble agar were from Difco, yeast extract and peptone were from BBL, and V8 protease was from Sigma. In Table I are listed the strains of *S. cerevisiae* used in this study.

HPLC Procedures. A Waters analytical HPLC instrument consisted of Waters 510 pumps, an on-line Waters λ-Max 481 variable-wavelength UV spectrophotometer, and a Waters 680 automated gradient controller attached to an M730 data module. A Waters μBondapak C_{18} (300 × 3.90 mm i.d.) column was used for analytical separations. HF reactions were carried out in the SAM II HF apparatus (Biosearch). Preparative HPLC was done on a C_{18} silica (10 μm, 5.0 × 30 cm Waters Associates) column equilibrated with 40% methanol and 0.025% TFA in water. Samples were loaded and then eluted with a methanol/H_2O/TFA step gradient using a 2% increase of methanol concentration. Deformylation of Trp was achieved by using 1 M piperidine in 60% aqueous DMF solution for 24 h at 0 °C.

Synthetic Protocols. Syntheses were performed manually with 4.0 g of starting resin (0.49 mmol/g) for each synthetic cycle. All residues were double coupled regardless of the results of the Kaiser test (Kaiser et al, 1970), which was performed prior to and after each coupling. A typical cycle is as follows: (1) TFA/DCM/DMS (40:58:2 v/v/v, 75 mL, 1 × 1 min); (2) TFA/DCM/DMS (40:58:2 v/v/v, 75 mL, 1 × 30 min); (3) DCM (75 mL, 3 × 1 min); (4) 10% DIEA/DCM (75 mL, 1 × 10 min); (5) DCM (75 mL, 3 × 1 min); (6) Boc-amino acid (3 equiv) in DCM (15 mL) added to resin vessel, and shaken at room temperature with DCC (3 equiv) for 2 h; (7) DCM (75 mL, 3 × 1 min); (8) 5% DIEA/DCM (75 mL, 1 × 10 min); (9) DCM (75 mL, 3 × 1 min); (10) Boc-amino acid (1 equiv) and DCC (1 equiv) in 25 mL of DCM were shaken at room temperature for 1 h; (11) DCM (75 mL, 3 × 1 min); (12) methanol (75 mL, 2 × 1 min); (13) DCM (75 mL, 3 × 1 min). The resin was then suspended in 100 mL of DCM and stored overnight. For Gln the HOBt/DCC-accelerated active ester coupling procedure in DMF replaced steps 6 and 10.

Cleavage and Purification. The N^{α}-Boc group was removed from the completed peptide–resins, and the peptides were cleaved from the resin by the low–high HF method of Tam et al. (1983). Low HF cleavage was carried out in liquid HF/DMS/*p*-cresol/*p*-thiocresol (25:65:7.5:2.5 v/v). High HF cleavage was run in liquid HF/*p*-cresol/*p*-thiocresol (90:7.5:2.5 v/v) at 0 °C for 1 h. The crude peptides were washed with EtOAc and anhydrous ether to remove scavengers. The peptides were then eluted from the resin with 6% HOAc solution. Extracts were evaporated to a low volume at room temperature in vacuo and lyophilized.

After isolation of crude peptides, analytical HPLC was performed to determine their purity. In all cases, except for the heptadecapeptide, the major peak represented the expected product. In the heptadecapeptide, it was found that incomplete removal of the formyl group caused recovery of a heterogeneous product. However, after treatment with piperidine, one major peak corresponding to product was obtained.

The crude peptide was purified by reversed-phase chromatography on C_{18} silica with a CH_3OH/H_2O/TFA mobile-phase step gradient. All final products were greater than

[1] Abbreviations: Boc, *tert*-butoxycarbonyl; DCC, dicyclohexylcarbodiimide; DCM, methylene chloride; DIEA, *N,N*-diisopropylethylamine; DMF, dimethylformamide; DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide; Et_2O, diethyl ether; EtOAc, ethyl acetate; FT-NMR, Fourier transform nuclear magnetic resonance; HF, hydrogen fluoride; HOAc, acetic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; PAM, (phenylacetamido)methyl; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TMS, tetramethylsilane; tosyl, *p*-toluenesulfonate; YEPD, yeast extract–peptone–dextrose medium; YNB, yeast nitrogen base medium; Z, benzyloxycarbonyl. Standard abbreviations for amino acid derivatives and peptides are according to IUPAC–IUB Commission on Biochemical Nomenclature (1975).

98% homogeneous as judged by analytical HPLC on a reversed-phase C_{18} column (CH_3OH/H_2O/TFA or CH_3CN/H_2O/TFA) and gave one ninhydrin-positive, UV-positive spot on silica thin layers. The purified peptides were characterized by amino acid analysis (Table II) and 400-MHz FT-NMR spectroscopy.

V8 Protease Digestion of Extended α-Factors. The following solutions were pipetted into borosilicate glass test tubes: 45 μL of α-factor or extended α-factor (222 μg/mL), 5 μL of 1 M HOAc, and 740 μL of 50 mM NH_4HCO_3, pH 7.8. After vortexing, 10 μL of V8 protease (1 mg/mL in 50 mM NH_4HCO_3) was added to each tube (controls without V8 protease were prepared concurrently), and the resulting reaction mixtures were incubated for 10 h in a 37 °C rotary water bath shaker. After incubation, the reaction mixtures were quenched with 343 μL of glacial HOAc, lyophilized, and then resuspended with 100 μL of sterile distilled water. The resulting solutions were then tested in the halo assay.

Biological Assay for Shmoo Formation. A culture of X2180-1A(*MATa*) was grown at 30 °C with shaking to early log phase (85 Klett units, blue filter with Klett colorimeter) in YNB supplemented with 0.5% ammonium sulfate and 2% glucose. The cells were then harvested by centrifugation at 1000*g*, washed twice with sterile distilled water, resuspended to 4.0×10^6 cells/mL in YNB, and placed on ice. Dilution series (1:2, 80–1.25 μg/mL) of tridecapeptide and each of the extended α-factors were prepared in borosilicate glass tubes with YNB as the diluent. Five hundred microliters of the 4.0×10^6 cell suspension was then added to 500 μL of each of the peptide solutions, and the suspensions were incubated at 30 °C for 3.5 h in a rotary water bath shaker. At the completion of the incubation period, the cells were placed on ice, and then 10-μL portions were placed in a hemocytometer and observed microscopically to quantitate the total number of cells, shmoos, and unbudded cells.

Halo Assay. YEPD plates were overlayed with 4 mL of *MATa* cells (2.5×10^5 cells/mL) in noble agar (0.825%). Filter disks (Whatman No. 1 or Whatman 3MM) were than placed on the overlay, and 10-μL portions of peptide solutions at various concentrations were pipetted onto the disks. The plates were incubated at 30 °C for 48 h and then observed for clear zones (halos) around the disks, an indication of the arrest of cell growth. The diameter of the clear zone was determined by subtracting the diameter of the disk from the diameter of the zone of inhibition.

Competition Assay. Ten microliters of 1 mg/mL des-Trp[1],Ala[3]-α-factor, a dodecapeptide with the sequence His-Ala-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr, was added to 10-μL portions of 0.1 mg/mL solutions of α-factor or each of the extended α-factors. Ten-microliter portions of these mixtures were then tested in the halo assay. Controls without des-Trp[1],Ala[3]-dodecapeptide were tested concurrently.

RESULTS

Synthesis of Extended α-Factors. In previous studies on the α-factor, solution-phase techniques were utilized to synthesize both the naturally occurring tridecapeptide and a variety of analogues and derivatives (Naider & Becker, 1986). In contrast, tridecapeptide α-factor and all of the extended analogues investigated in this paper were prepared by using solid-phase peptide synthesis. As indicated under Materials and Methods, our protocol employed dicyclohexylcarbodiimide activation for most amino acids. In almost all cases qualitative ninhydrin analysis (Kaiser test) indicated virtually complete reaction after the first coupling step. Nevertheless, we routinely included a second coupling step to minimize deletion sequences. Protecting groups were chosen to avoid small amounts of side-chain deprotection during removal of the Boc group and to minimize alkylation during final cleavage from the resin. Our synthesis utilized both the normal Merrifield resin [(chloromethyl)polystyrene] and the PAM resin. Judging by weight gain, we observed that approximately 2.5% of the peptide chains were lost per coupling cycle with the former resin whereas less than 1% of the chains were lost per coupling cycle using the PAM resin. The yield of purified peptide also increased markedly with the PAM resin. For example, the final yield of tetra- and pentadecapeptide was 3% and 5% respectively, starting with Merrifield resin, and 11% and 10%, respectively, starting with the PAM resin (Table II).

Table II: Chemical and Physical Properties of Extended α-Factors

	α-factor[a]	14-peptide	15-peptide	16-peptide	17-peptide
amino acid analysis					
Ala	0.00	1.00	0.96	2.13	1.91
Glu	1.98	2.12	2.87	3.06	3.96
Gly	1.08	1.12	1.08	1.11	1.16
His	0.96	0.88	1.00	0.96	0.87
Leu	2.01	1.95	2.01	2.00	1.99
Lys	0.99	0.91	0.98	0.93	1.01
Pro	2.01	2.19	2.15	2.13	2.03
Met	0.95	0.97	0.93	0.99	1.01
Tyr	1.03	1.02	1.04	1.06	1.07
Trp[b]					
yield (%)	17[c]	11 (3)	10 (5)	9	11
$[\alpha]_D$	−23.2[d]	−23.6	−25.8	−30.4	−30.1
$R_{f,A}$[e]	0.06	0.03	0.03	0.03	0.02
$R_{f,B}$	0.63	0.63	0.58	0.58	0.55
K'	7.51[f]	7.67	7.82	7.91	8.17

[a] Factor sequence: Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr. [b] The Trp content of these peptides was determined by using ultraviolet absorption spectroscopy. All peptides exhibited the characteristic Trp absorption pattern and were judged to have two Trp residues on the basis of A_{289} values at pH 9 with an extinction coefficient of 4500 (Khan et al., 1981). [c] The yields are those obtained by using a PAM resin. Numbers in parenthesis reflect yields with a normal Merrifield resin. [d] The various peptides have different amounts of bound water. The concentration of α-factor was 1.3 mg/mL in acetic acid. The other peptides were adjusted to the same concentration on the basis of the A_{289} values of solutions of the pheromones in acetic acid. [e] Solvent system A = butanol/acetic acid/water (4:1:5 upper phase). Solvent system B = butanol/acetic acid/water/pyridine (15:3:12:10). [f] K' values are reported for a 20–40% acetonitrile gradient (0.025% TFA) over 30 min.

A comparison was also made of the efficiency of the low–high HF method and the normal HF method in cleaving the peptide from the resin. HPLC analysis revealed that these protocols released crude peptide of similar homogeneity from the resin. We note that the low–high method efficiently removed the *N*-formyl group from tryptophan in the tridecapeptide (data not shown), but removal was less complete in the elongated peptides. Indeed, comparison of the crude heptadecapeptide generated by normal HF and low–high HF conditions shows that they are virtually identical (Figure 1A–C). In cases where a careful comparison was conducted, the yield of pure peptide was the same from either cleavage method. We conclude that for the α-factor-like peptides there is no advantage in using the low–high cleavage procedure. Rather, the extra time required by this type of cleavage is clearly not warranted.

All crude peptides were purified to >98% homogeneity as judged by gradient HPLC (e.g., Figure 1D) and thin-layer chromatography (Table II). Gradient HPLC on a reversed-phase column with an acetonitrile/water/trifluoroacetic acid mobile phase showed that the extended α-factors migrated in order of their molecular weights with the tridecapeptide eluting

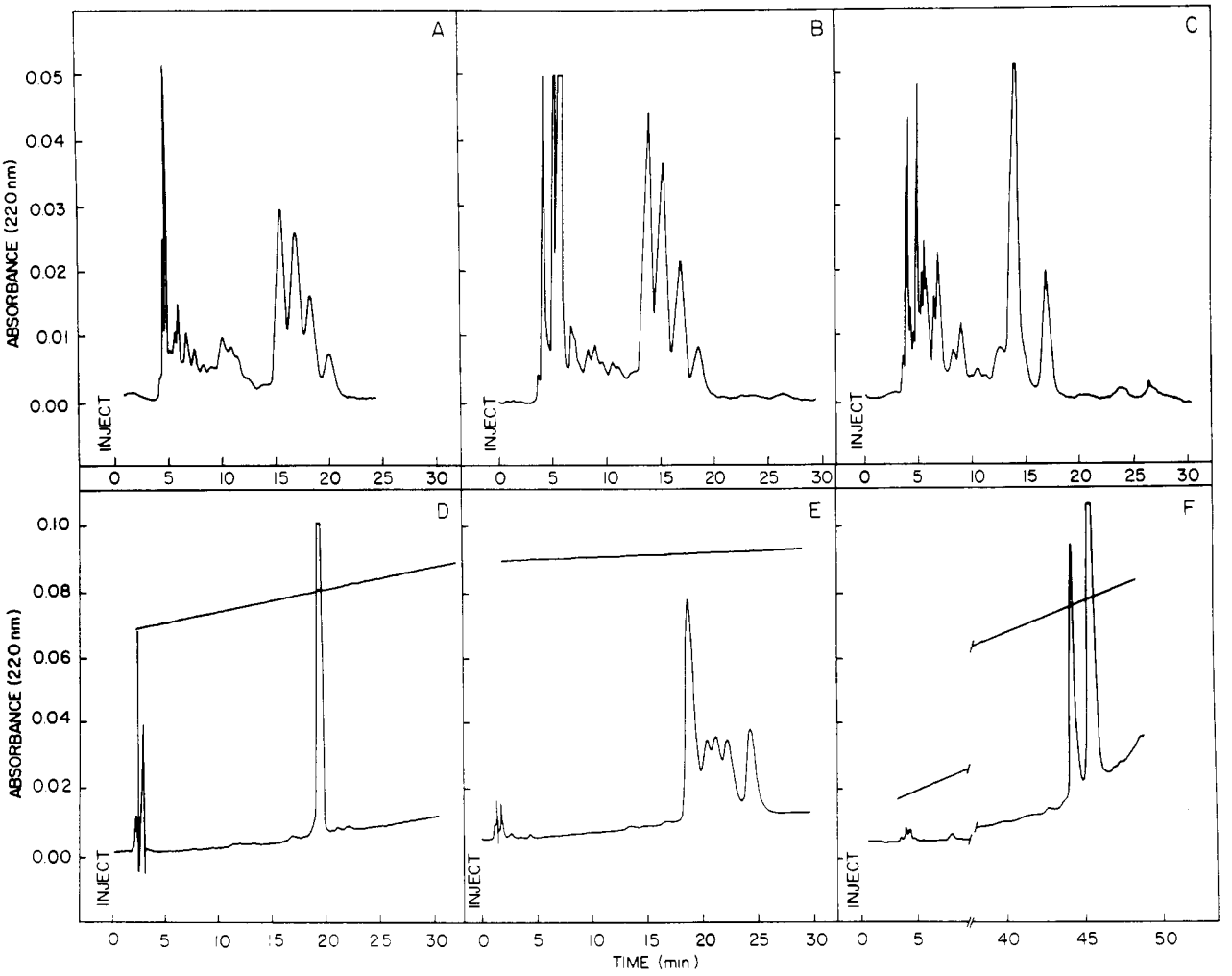

FIGURE 1: High-performance liquid chromatography of elongated α-factors on a reversed-phase C_{18} column. (Panel A) Crude heptadecapeptide from cleavage of PAM resin using high HF conditions. (Panel B) Crude heptadecapeptide from cleavage of PAM resin using low–high HF conditions. (Panel C) Crude heptadecapeptide from cleavage by either high HF or low–high HF conditions followed by treatment with piperidine. For separations shown in panels A–C the mobile phase was $CH_3CN/H_2O/CF_3COOH$ (29:71:0.025). (Panel D) Gradient elution of purified tetradecapeptide with $CH_3CN/H_2O/CF_3COOH$ as the mobile phase (20% to 40% CH_3CN, 0.025% CF_3COOH). (Panel E) Gradient elution of a mixture of the tridecapeptide through heptadecapeptide. In this experiment an excess of tridecapeptide was injected. Gradient from 20% to 26% CH_3CN (0.025% CF_3COOH). (Panel F) Gradient elution of a mixture of the tetradecapeptide and the heptadecapeptide. In this experiment an excess of heptadecapeptide was injected. The mobile phase was an acetonitrile/phosphoric acid/water gradient from 0.1% H_3PO_4 containing 0.5% CH_3CN to 0.045% H_3PO_4 containing 55% CH_3CN (Julius et al., 1983).

first (Figure 1E and Table II). The assignment of the individual peaks in this chromatogram was carried out by comparing mobilities of the mixture with those of the isolated pheromones and by coinjecting individual pheromones added into the mixture of the five peptides. Similar results were observed for the tridecapeptide and heptadecapeptide by using an acetonitrile/water/phosphoric acid mobile phase (Figure 1F). These findings are in contrast to those of Julius et al. (1983), who reported that α-factor* had a lower K' than authentic α-factor.

The extended α-factor peptides were characterized by ^1H NMR. Complete assignments of the ^1H NMR spectrum have been made and are consistent with the expected amino acid composition (Linda Hughes and Michelle Broido, unpublished results). A representative ^1H NMR spectrum for the 15-peptide in HOD appears in Figure 2. Amino acid ratios were consistent with expected values (Table II). Although the yields of the solid-phase synthesis are not outstanding (9–17%), they reflect a major improvement over previous reports on the α-factor (Naider & Becker, 1986). Previous experience suggests that the low yields can be attributed to the high percentage of residues such as Trp, Met, His, and Tyr which are known to undergo a variety of side reactions during solid-phase peptide synthesis. Higher yields could probably be obtained by a procedure that avoids HF treatment of the assembled peptide–resin conjugate.

Morphogenesis Activity of Extended α-Factors. The most widely reported activity of α-factor is its effect on the shape and budding of *MATa* cells of *S. cerevisiae*. All extended α-factors (14-peptide to 17-peptide) when incubated with *S. cerevisiae* X2180-1A (*MATa*) caused formation of aberrant morphologies (the shmoo shape) and cessation of budding (Figure 3). Under the conditions of our assay the natural α-factor causes 50% aberrant morphologies at 1 μg/mL; 100% of the cells are affected at 2 μg/mL. The tetradecapeptide is only slightly less active, causing 50% aberrant morphology at 2 μg/mL and 100% affected cells at 5 μg/mL. Gradual loss in activity is observed with additional chain length. The heptadecapeptide is 50% active at about 5 μg/mL and affects 100% of the cells only at 40 μg/mL. It is interesting to note that the drop-off of activity with chain length is approximately linear. Previous studies have shown that α-factor can be degraded at high cell densities (Moore, 1983). We, therefore, carried out morphogenesis assays at cell concentrations of 2 $\times 10^3$ cells/mL. Under these conditions no degradation of the tridecapeptide α-factor is expected to occur during the 3-h

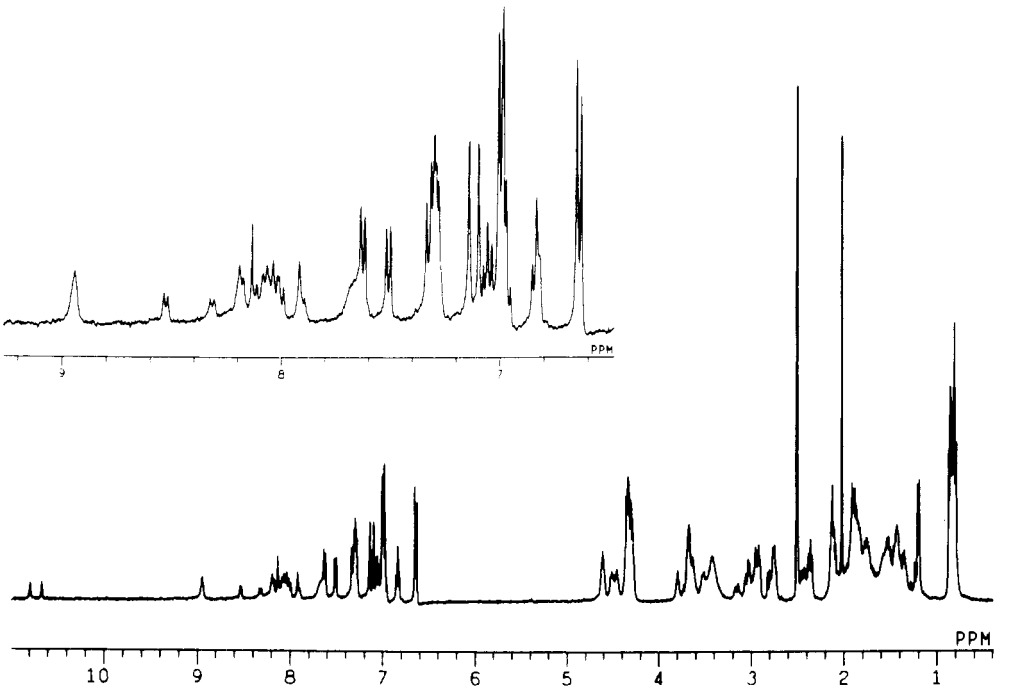

FIGURE 2: 400-MHz ^1H NMR spectrum of the pentadecapeptide Glu-Ala-Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr in DMSO-d_6. The spectrum is referenced to TMS, but this peak is not shown because of its size. The insert represents the NH and aromatic side chain regions of the spectrum. Representative assignments include Trp indole NH, 10.66 and 10.80 ppm; His C_2H, 8.95 ppm Tyr ring protons, 6.64 and 7.04 ppm; α-CH region, 4.30–4.68 ppm; Ala CH_3, 1.18 ppm; Leu CH_3, 0.82 and 0.78 ppm.

Table III: Growth Arrest of *S. cerevisiae* by Extended α-Factors

	halo diameter (mm) for									
	RC629 (*MATa sst1-2*), amount spotted (μg/plate)[b]					RC631 (*MATa sst2-1*), amount spotted (μg/plate)				
peptide	10	1	0.1	0.05	0.01	10	1	0.1	0.05	0.01
tridecapeptide[a]	30	20	10	4	0	35	25	14	9	0
tetradecapeptide	30	20	11	7	0	33	25	14	9	0
pentadecapeptide	24	15	3	0	0	31	22	12	8	0
hexadecapeptide	23	13	2	0	0	30	21	12	7	0
heptadecapeptide	8	5	0	0	0	30	21	11	6	0

[a] Represents mature α-factor. The longer peptides have the sequences Ala, Glu-Ala, Ala-Glu-Ala, and Glu-Ala-Glu-Ala, respectively, attached to the amino terminus of α-factor. [b] Concentrations adjusted to that of α-factor by using absorbance at 289 nm.

assay (Baffi et al., 1985). At these low cell densities the minimum concentration of pheromone that caused aberrant cell formation was 5–10-fold greater for the heptadecapeptide than it was for the tridecapeptide α-factor. Intermediate end points were found for the 14–16-peptides. Thus, pheromone destruction does not appear to be a significant factor in affecting the morphogenesis activity of the extended α-factors. Similar trends were found with supersensitive strains and a halo assay (see below) and in studies on the relative ability of the elongated peptides to induce agglutination (data not shown).

Effects of α-Factors on Cell Growth. α-Factor is known to arrest growth of *MATa* cells. Halo assays, therefore, provide an easy and direct measure of pheromone activity. The α-factor and the four elongated analogues all arrested the growth of *S. cerevisiae* RC629 (*MATa sst1*). As little as 0.05 μg of pheromone caused a well-defined halo in a lawn of the supersensitive mutant (Figure 4 and Table III). Careful analysis revealed that the 14-peptide was as active as natural α-factor, the 15-peptide and 16-peptide were 2–5-fold less active, and the 17-peptide was 20–100-fold less active. The differences in activity between the different peptides at 1 μg/plate were eliminated when the peptides were incubated for 10 h at 37 °C with V8 protease (Figure 4). This result suggests that each peptide is metabolized to the same end

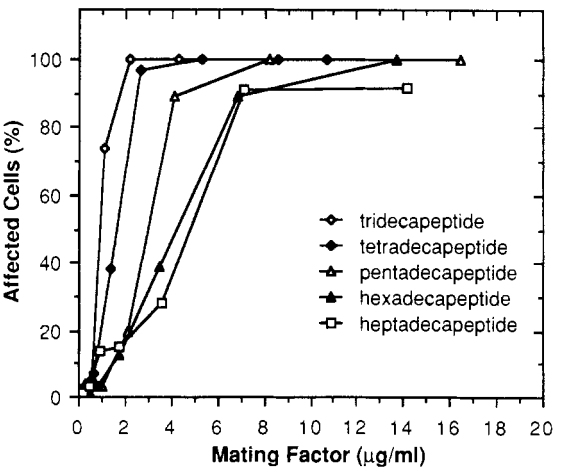

FIGURE 3: Effect of various peptides on the cell shape of *S. cerevisiae* X2180-1A (shmoo assay). Cells were incubated with the indicated concentration of pheromone under the conditions described under Materials and Methods, and the percent of aberrant cells were determined. Peptide concentration was adjusted to that of α-factor by using the absorbance value at 289 nm.

product. For the 17-peptide this end product was shown to have identical mobility on a C_{18} reversed-phase column as the authentic tetradecapeptide (data not shown). Similar results

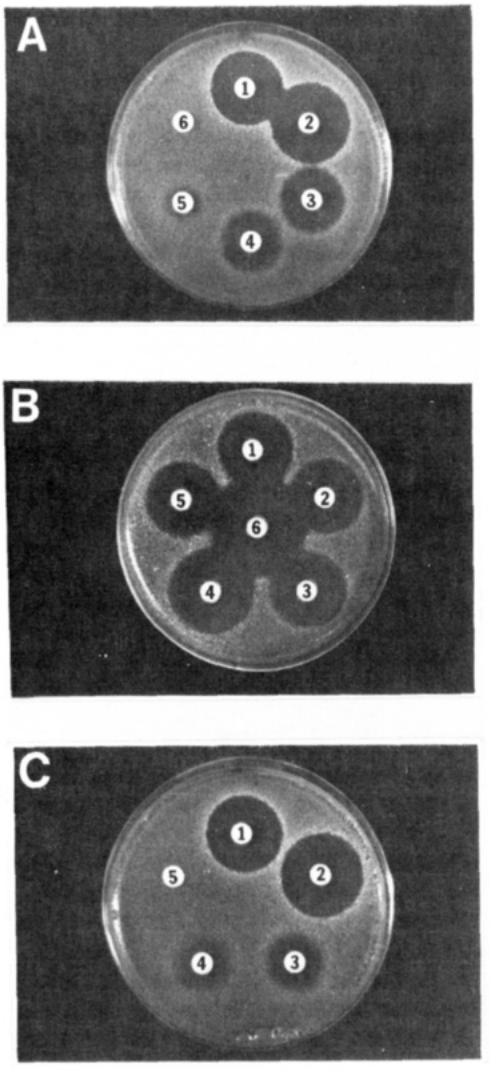

FIGURE 4: Growth arrest by various peptides of *S. cerevisiae* RC629 (*MATa sst1-2*). (Panel A) The indicated peptide (1 μg) was applied to each disk: tridecapeptide (1), tetradecapeptide (2), pentadecapeptide (3), hexadecapeptide (4), heptadecapeptide (5), and des-Trp[1],Ala[3]-dodecapeptide (6). (Panel B) Disks 1–5 represent 1 μg of each peptide incubated with V8 protease: tridecapeptide (1), tetradecapeptide (2), pentadecapeptide (3), hexadecapeptide (4), and heptadecapeptide (5). Disk 6 is the untreated tridecapeptide. (Panel C) The indicated peptide (1 μg) was added in the presence of 10 μg of des-Trp[1],Ala[3]-dodecapeptide: tridecapeptide (1), tetradecapeptide (2), pentadecapeptide (3), hexadecapeptide (4), and heptadecapeptide (5).

were observed when *S. cerevisiae* RC631 (*MATa sst2*) was used as the tester strain. However, with the *sst2* mutant the activity of the 17-peptide was nearly equal to that of the tridecapeptide α-factor (Table III). For comparison with the above results, we studied the parental strain, RC618 (*MATa*), from which the supersensitive mutants were generated. This wild type did not respond to α-factor or the elongated analogues at concentrations below 1 μg/mL. In addition, des-Trp[1],Ala[3]-α-factor, an antagonist of α-factor activity (Shenbagamurthi et al., 1983), reversed growth arrest in strain RC629 caused by the elongated α-factors (Figure 4). Under the conditions used for the experiment summarized in Figure 4 only slight competition was found with the trideca- and tetradecapeptides. Complete reversal of the activity of these peptides could be achieved, however, when the concentration of the antagonist was increased.

Response of Temperature-Sensitive Mutants of S. cerevisiae. A temperature-sensitive mutant of *S. cerevisiae* 50B (*ste2*[ts] *MATa*) has been characterized as having a functional receptor for α-factor at the permissive temperature (23 °C) but not the restrictive temperature (34 °C) (Jenness et al., 1983). When grown at 23 °C, this mutant was arrested by 10 μg of the 14–17-peptides (Table IV). In contrast, at the restrictive temperature neither the α-factor nor any of the elongated peptides inhibited growth of the mutant. These results provide strong evidence that all the extended analogues and the α-factor interact with the same receptor.

Table IV: Biological Activity of Various Pheromones against *S. cerevisiae* 50B (*ste2 MATa*)

pheromone	peptide[a] (μg)	halo diameter (mm) at 23 °C	halo diameter (mm) at 34 °C
tridecapeptide	1	20	0
tetradecapeptide	1	18	0
pentadecapeptide	1	15	0
hexadecapeptide	1	16	0
heptadecapeptide	10	10	0

[a] Concentrations adjusted to that of α-factor by using absorbance at 289 nm.

Discussion

The mode of sexual reproduction in *S. cerevisiae* provides an ideal model system in which to examine the molecular details of peptide hormone secretion and of peptide–cell interactions. Previous investigations have revealed that many mammalian hormones are biosynthesized as large polypeptide precursors which are enzymatically processed to bioactive molecules (Douglass et al., 1984; Docherty & Steiner, 1982). α-Factor has been shown to follow a very similar biosynthetic route (Julius et al., 1984a). In the *MFα1* gene the α-factor sequence is coded for as part of a 165-residue precursor containing four copies of the mature tridecapeptide pheromone (Kurjan & Herskowitz, 1982). Interestingly, studies on *ste13* mutants have led to the conclusion that loss of a membrane-bound dipeptidyl aminopeptidase can result in incorrect processing leading to the secretion of α-factor species elongated at the amino terminus of the pheromone (Julius et al., 1983). Indeed a mixture of these species has been isolated and subjected to amino acid analysis, enzymatic degradation, and sequence analysis (Julius et al., 1983). On the basis of these studies, it was concluded that the improperly processed pheromone, termed α-factor*, was primarily a heptadecapeptide with the sequence NH_2-Glu(or Asp)-Ala-Glu-Ala-Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr-COOH.

Inspection of chromatograms of α-factor* reveals that this material was quite heterogeneous (Julius et al., 1983). We decided, therefore, to synthesize a family of amino terminus elongated peptides corresponding to the translation products coded for by the *MFα1* structural gene. The synthetic peptides contained 13–17 residues and were highly homogeneous as judged by HPLC and thin-layer chromatography. The 17-peptide was retained on a C_{18} reversed-phase column more strongly than α-factor in both an acetonitrile/water/trifluoroacetic acid mobile phase and an acetonitrile/water/phosphoric acid mobile phase. In contrast, Julius et al. (1983) reported that α-factor* was removed from a C_{18} column at lower acetonitrile concentrations than the tridecapeptide. If this latter finding is correct, we conclude that the α-factor* does not correspond exactly to the heptadecapeptide. We should note that the amino acid analysis of α-factor* reveals nonnegligible amounts of Thr, Ser, and Phe (0.3 residue per molecule of α-factor*). None of the residues are found in the translation sequence of the extended pheromones.

The extended synthetic α-factors were all biologically active when tested against wild-type and supersensitive *MATa* haploids. In all cases, the tridecapeptide and tetradecapeptide had nearly identical activities. Similar findings on the activity of tetradecapeptide α-factor analogues have been reported previously (Julius et al., 1983; Samokhin et al., 1980). In contrast, the penta-, hexa-, and heptadecapeptides showed significantly lower activities. The relative potency of the elongated pheromones was markedly dependent on the assay employed and the *MATa* target cell. With strain X2180-1A (*MATa*) there was <1 order of magnitude difference in activity between the mature α-factor and the heptadecapeptide as judged by the shmoo assay (Figure 3). When growth inhibition is used to judge pheromone activity, the 17-peptide was >1 order of magnitude less active than the 13-peptide using RC629 (*MATa*), an *sst1* mutant, but almost equally active to the 13-peptide when tested against RC631 (*MATa*), an *sst2* mutant (Table III). These results are in contrast to findings on α-factor*, which was reported to be at least 2 orders of magnitude less active than mature α-factor as judged by growth arrest with RC687 *MATa* (*sst2-4*) (Julius et al., 1983). They also suggest that the failure of RC634 (*sst1*) to give a positive halo assay when tested against secreted peptides of the *ste13* mutant is not a general phenotype for *sst1* strains. The differences found by us and Julius et al. (1983) can be attributed to inherent differences in the *sst1* and *sst2* tester strains examined in the studies, to chemical differences between the synthetic peptides employed in this study and α-factor* (which may be a mixture of 17-mers, 19-mers, 21-mers, or other processing intermediates that would probably have lower biological activity than the shorter peptides we examined) isolated from the *ste13* mutant, or to the actual amounts of peptide tested in growth arrest assays. Julius et al. (1983) determined relative activities by spotting equivalent amounts of immuno-cross-reacting material, whereas we used molar equivalents of pure peptide based on 289-nm absorbance values.

The activity profile exhibited by the elongated pheromones suggests that the α-factor receptor has a binding site that can readily accommodate additional residues at the amino terminus of the tridecapeptide α-factor. The drop-off in activity with increasing peptide size can reflect either a steric interaction or the addition of two residues with ionizable side chains to the amino terminus. Since the receptor is most likely ensconced in a nonpolar lipid milieu, it is not clear whether the influence of the charged residues would be due to a primary effect on the peptide–receptor interaction or an indirect effect on the integration of the pheromone into the membrane. Additional studies with other analogues and isolated receptor should distinguish between these possibilities.

Previously, studies on analogues of α-factor gave evidence for the existence of more than one receptor (Baffi et al., 1984), although there is no genetic or equilibrium binding data supporting this hypothesis. The fact that the analogues were all active against a temperature-sensitive receptor mutant at the permissive temperature, but not at the restrictive temperature, suggests that they act on the same receptor. This conclusion is supported by the fact that des-Trp^1,Ala^3-α-factor antagonized the activity of all pheromones against *S. cerevisiae* RC629 (*MATa sst1*). This dodecapeptide also antagonized the activity of α-factor and several active analogues against wild-type *MATa* haploids (Shenbagamurthi et al., 1983). Surprisingly, the antagonist did not complete with the elongated peptides against the *sst2* (RC631) tester strain (data not shown). Control studies showed that the dodecapeptide is actually an agonist for this latter yeast strain. The *sst2* mutation which confers supersensitivity on *S. cerevisiae* has not been previously explained. The fact that these mutants respond to analogues that are totally inactive to other *MATa* haploids (more than 1000-fold less active) and that they respond to aberrant forms of α-factor produced by *Saccharomyces kluyveri* (McCullough & Herskowitz, 1979; Julius et al., 1983) indicates that the *sst2* mutation suppresses a receptor mutation, allowing the yeast to respond to pheromones differing in primary sequence. This would lend further support to the supposition that *sst1* and *sst2* loci confer supersensitivity through totally different biochemical pathways.

In summary, we have synthesized analogues of the α-factor corresponding to amino terminus extended peptides of the primary translation products of the *MFα1* gene. For the most part, biological activities were inversely proportional to the length of the peptide. That an antagonist interfered with the activity of all the peptides and none of the peptides were active against a receptorless mutant indicates strongly that all of these analogues interact with the same site on the target cell. These results, and the surprising finding that an antagonist for wild-type cells may be an antagonist for only one of the two supersensitive complementation groups, demonstrate again the value of structure–function studies in aiding our understanding of peptide–cell interactions.

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Registry No. *N*-Ala-α-factor, 110795-73-8; *N*-Glu-Ala-α-factor, 110795-74-9; *N*-Ala-Glu-Ala-α-factor, 110795-75-0; *N*-Glu-Ala-Glu-Ala-α-factor, 110795-76-1.

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Helical Formation in Isolated Fragments of Bovine Growth Hormone

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ABSTRACT: The peptide 109–133 was isolated from bovine growth hormone (bGH) and studied for helix formation in aqueous solutions. This fragment was shown to contain helical structure by far-ultraviolet circular dichroism in aqueous solutions. The amount of helix was dependent on pH and peptide concentration. The peptide has maximum helicity between pH 4 and 5 and at high peptide concentration. Under these conditions for maximal helix population, this fragment is approximately 100% helical. Secondary structure predictions suggest that residues 110–127 have a strong propensity to form an amphipathic helix. We have also studied a related peptide, 96–133, and show by gel filtration that it undergoes an increase in molecular weight that directly correlates with a coil to helix transition. A comparison of the helical content of 96–133 to 109–133 and circular dichroism studies of peptide 96–112 suggest that the helix of 96–133 is limited to the 109–133 region. Current models for α-helix formation predict that peptides the size of 109–133 should not contain measurable helicity in aqueous solutions. Our studies show that the unusual stability of helix 109–133 is due to electrostatic interactions and probable intermolecular packing between hydrophobic faces of the amphipathic surfaces of the helices. The implications of helix formation in these fragments to a framework model of protein folding for bGH are discussed.

The framework model of protein folding states that formation of secondary structure precedes tertiary structure (Kim & Baldwin, 1982). Previous folding studies of bovine growth hormone (bGH) are consistent with a framework-type model of folding (Brems et al., 1987). bGH is a member of the class of large pituitary polypeptide hormones and has 191 amino acids. Circular dichroism studies indicate that it is approximately 50% helical (Holladay et al., 1974). Equilibrium denaturation of bGH is not a two-state process, and at least four species have been identified: unfolded, native, intermediate (that contains helical structure but lacks tertiary structure), and an associated intermediate (that contains helical structure but lacks tertiary structure). Kinetic folding studies of bGH showed that formation of helical structure precedes the tertiary structure (Brems et al., 1987).

It is commonly accepted that the secondary structure of globular proteins is unstable in the absence of long-range tertiary interactions. This notion came from studies of protein fragments that corresponded to helical regions of the intact protein (Epand & Scheraga, 1968; Taniuchi & Anfinsen, 1969) and the two-state equilibrium denaturation of most proteins (Creighton, 1984). The general lack of stability of isolated secondary structure is sometimes considered as evidence against the feasibility of the framework model. Because of the unusual stability of bGH helical structure, we reasoned that it may be possible to find appropriate fragments of bGH that are helical in aqueous solution. If so, these highly stable helices would likely represent the helical regions that make up the framework of an early folding intermediate. Previous studies of fragments derived from bGH have revealed that 96–133 is biologically active and contains measurable helical structure in aqueous solutions (Chen & Sonenberg, 1977). We have reinvestigated the helical formation of this fragment and have obtained a truncated form, 109–133, that retains all of the helix that is observed in 96–133. The sequence of peptide 96–133 is as follows: VFTNS^{100}LVFGTSDRVY^{110}EKLKDLEEGI^{120}LALMRELEDG^{130}TPR.

Experimental Procedures

Materials

Fragment 96–133 was derived from bGH that was obtained by expression of bGH in *Escherichia coli* using recombinant

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