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I am submitting herewith a dissertation written by Effimia Eriotou Bargiota entitled "Structure-Function Studies of the Tridecapeptide Mating Pheromone of *Saccharomyces cerevisiae*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Jeffrey M. Becker, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Provost
and Dean of the Graduate School

STRUCTURE-FUNCTION STUDIES OF THE TRIDECAPEPTIDE MATING PHEROMONE OF *SACCHAROMYCES CEREVISIAE*

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Effimia Eriotou Bargiota

August 1990

DEDICATION

With love I dedicate this dissertation to my husband and son. Through achievement of this goal, I can only hope that I have made them proud.

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ABSTRACT

An analog of α-factor, the *Saccharomyces cerevisiae* tridecapeptide mating pheromone (Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr), in which the side chains of Lys^{7} and Gln^{10} were covalently linked, was synthesized using solid phase methodologies. The structure of the peptide was verified by chemical analyses. Cyclo^{7,10}[Nle^{12}]α-factor caused growth arrest and morphological alterations in *S. cerevisiae MATa* cells qualitatively identical to those induced by linear pheromone and was one-fourth to one-twentieth as active as the linear α-factor depending upon the *S. cerevisiae* strain tested. Competion binding studies were consistent with the relative activities of the linear and cyclic peptides. Hydrolysis of the cyclic peptide by the target cells did not lead to opening of the ring and was less rapid than that of linear α-factor. The α-factor antagonist des-Trp^{1}-[Ala^{3},Nle^{12}]α-factor reversed the activity of the cyclic analog, and cyclo^{7,10}[Nle^{12}]α-factor was not active at the restrictive temperature in a temperature-sensitive receptor mutant. These results support the conclusion that the cyclic α-factor occupies the same binding site within the receptor as is occupied by the natural pheromone. The cyclic α-factor represents a rare example of an agonist among covalently constrained congeners of small linear peptide messengers.

Amino- and carboxy-terminus truncated peptides were derived from linear- and cyclic-α-factor using aminopeptidase M or carboxypeptidase A. Their structures were verified by chemical analyses. des-Trp^{1}- or des-Tyr^{13}- linear or cyclic α-factor peptides showed reduced agonist activity. des-Trp^{1},His^{2}-linear or cyclic peptides were antagonists of α-factor activity. des-Nle^{12},Tyr^{13}- linear or cyclic α-factor peptides exhibited synergistic activity with α-factor (increased the growth inhibition of *S. cerevisiae* caused by α-factor), although neither compound showed any agonist activity when tested alone. The exact mechanism of action of the synergistic peptides is still unkown.

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CHAPTER 1

LITERATURE REVIEW

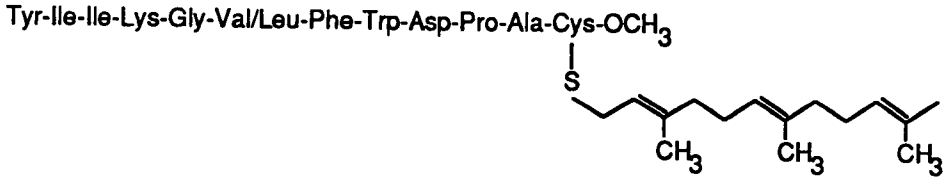
Saccharomyces cerevisiae, a nonfilamentous Ascomyces, has played a key role in the development of many of the important concepts of biochemistry. For example, fractionation of alcoholic fermentation extracts led to the discovery and characterization of enzymes and coenzymes. *S. cerevisiae* was also a principal contributor in the early research of vitamins and in the elucidation of the glycolytic pathway. In the field of genetics, *S. cerevisiae* contributed to the development several concepts, such as gene conversion, cytoplasmic inheritance, and the elucidation of the action of supersuppressors. The existence of both mitosis and meiosis as propagation procedures and the availability of both biochemical and genetic techniques make the microorganism an excellent model for the study of eukaryotic genetics.

Cells in a multicellular organism need to communicate in order to regulate their function. Communication between cells that are found at a distance occurs through chemical signals. In animals, one of these mechanisms involves the endocrine cells that secrete hormones which travel through the bloodstream to influence target cells distributed throughout the body. The first step of information transfer from a hormone to a target cell is the binding of the signaling molecule to its receptor. How the signal is transferred, what molecules mediate this transfer, and whether these molecules are modified are questions that, when answered, provide a molecular understanding of the system. The changes that occur may be covalent modifications of the hormone/receptor and/or changes in the conformation of the molecules. When a chemical substance functions as a hormone but it transfers a signal from one organism to another one, it is called a pheromone. Pheromones are produced by many insects and fungi.

Two mating types, **a** and α, can be dististinguished in *Saccharomyces cerevisiae.* Yeast cells of the **a** mating type secrete into the medium a pheromone of eleven amino acids called **a**-factor. (Wilkinson and Pringle, 1974; Betz *et al.*, 1977; Betz and Duntze, 1979). This peptide (Fig. 1) has a farnesyl moiety attached to the sulfur atom of the carboxyl-terminal cysteine residue and a terminal methyl ester group (Anderegg *et al.*, 1988). Both modifications are required for the peptide to exhibit full activity (Marcus, S. and Becker, J., personal communication). When this polypeptide is added to cultures of α mating type cells, the cells do not initiate DNA synthesis or bud formation but continue to grow and form pear shaped cells. Alpha-factor, a polypeptide of thirteen amino acids (Fig. 1), is produced by cells of α mating type and causes cells of the **a** mating type to undergo the same physiological changes as those seen in α-cells incubated with **a**-factor (Dunze *et al.*, 1970; Bucking-Throm *et al.*, 1973; Stotzler *et al.*, 1976). Neither **a**-factor nor α-factor has any effect on the cell type that produces them. In 1983, Jenness *et al.* provided genetic and biochemical evidence for an α-factor receptor using a binding assay of α-factor pheromone to yeast cells. The corresponding binding assay of **a**-factor to α-cells has not yet been accomplished.

According to Herskowitz and Oshima (1981), Sprague *et al.* (1983), and Bender and Sprague (1989) the characteristic mating type specific features of the **a** and α yeast cells are under the control of a region on chromosome III termed the mating type locus (*MAT*). *MATα* encodes for two regulators, α1 and α2 (Sprague *et al.*, 1983; Johnson and Herskowitz, 1985). *MATα1* is required to activate transcription of the α-factor and the **a**-factor receptor genes (the so called α-specific genes). *MATa* encodes also for two proteins, **a**1, **a**2. **a**1 is not required for mating but in conjuction with the α2 product, promotes sporulation in **a**/α cells. **a**2's role is unknown (Herskowitz, 1988). According to Nasmyth and Shore (1987), both *MATα1* and *MATα2* may act at some promoters via a common transcription factor that has been called Pheromone and Receptor Transcription

Natural a-factor (*MFa1* gene product contains Val at position six, whereas *MFa2* gene product contains Leu at this position):



α-factor:

Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr

Cyclic α-factor:

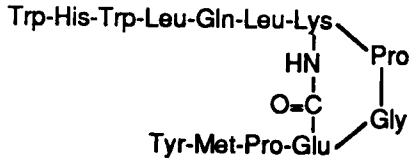


Figure 1. Structures of *S. cerevisiae* mating pheromones and cyclic α-factor.

Factor (PRTF). PRTF binding sites are required for upstream activation of both **a**- and α-specific genes. In the case of the structural gene for α-factor and the **a**-factor receptor gene, α1 (the protein product of *MATα1*) and PRTF act synergistically. In the case of α-factor receptor gene, α2 binds to sequences which overlap a PRTF binding site which is essential for promoter activity. Therefore, α2 may function at this promoter by excluding PRTF to negatively regulate *STE2* (α-factor receptor gene) expression at the level of mRNA production. *MATa/α* diploids are unable to mate but can be induced to undergo meiosis and sporulation. The generally accepted model is depicted in Fig. 2.

The ability to respond to pheromones is controlled by several genes *STE2, STE3, STE4, STE5, STE7, STE11, STE12, STE18, GPA1* (or *SCG1*), *CDC36,* and *CDC39 SST1* (*BAR*), and *SST2* (Table 1). The *STE2* gene (encoding for the α-factor receptor) and the *STE3* gene (encoding for the **a**-factor receptor) have been cloned and the primary structure of the polypeptide has been deduced from the gene sequence (Nakayama *et al.*, 1985; Burkholder and Hartwell, 1985; Hagen *et al.*, 1986). Hydropathy analysis of the predicted *STE2* and *STE3* proteins suggests that the proteins have seven transmembrane domains. This type of predicted structure is seen also in the mammalian β-adrenergic, the muscarinic acetylcholine, and the rhodopsin receptors (Julius *et al.*, 1988; Dixon *et al.*, 1986; Kobilka *et al.*, 1987; and Nathans and Hogness, 1984). The two proteins share no amino acid sequence similarities, which is expected as the two appear to have identical functions in the two mating types.

The *STE2* gene product has been shown biochemically to be the yeast protein that binds to α-factor (Blumer *et al.*, 1988). The bulk of the pheromone-binding activity was found in the pellet of a 100,000g centrifugation, consistent with the view that the receptor resides in the plasma membrane. The α-factor receptor is hyperphosphorylated in response to pheromone at its carboxy-terminal hydrophilic domain (Reneke *et al.*, 1988). This domain is not essential for pheromone binding since receptor mutants producing a carboxy-

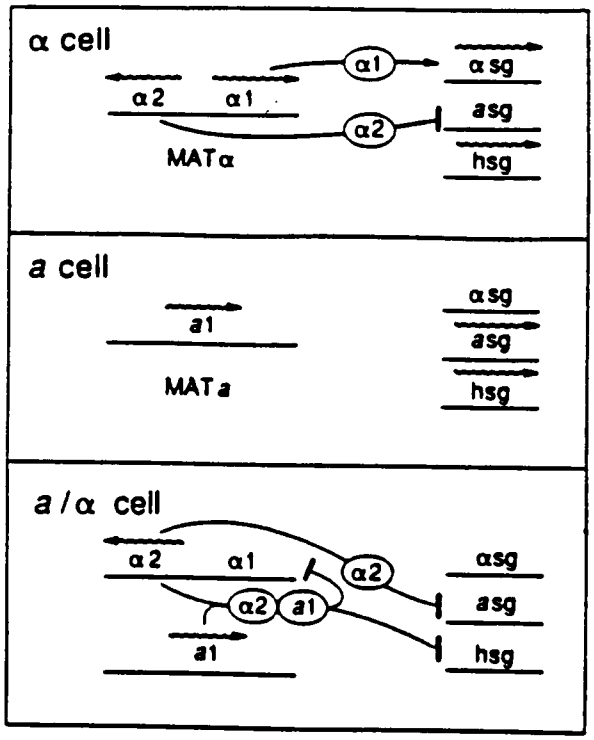


Figure 2. Control of expression of cell-type-specific gene sets by the mating type locus. The mating type locus is drawn to the left and the cell-type-specific gene sets (αsg, α-specific genes; asg, a-specific genes; hsg, haploid specific genes) are drawn to the right. The mating type loci, *MATα* and *MATa*, encode regulatory activities (α1 and α2, by *MATα* , and a1 and a2 by *MATa*) which govern transcription of mating-type-specific genes, as shown above. Wavy arrows indicate transcription. Pointed arrowheads indicate stimulation of gene expression; lines with blunt ends indicate inhibition of gene expression (Herskowitz, 1987).

Table 1. Genes Controlling the Ability to Respond to Pheromones.

Gene	Function	Expression
STE2	α-factor receptor	**a**-cells only
STE3	**a**-factor receptor	α-cells only
STE4	β-subunit of the G-binding protein	**a** and α-cells
STE5	Unknown	**a** and α-cells
STE7	Protein kinase	**a** and α-cells
STE11	Protein kinase	**a** and α-cells
STE12	Unknown	**a** and α-cells
STE18	γ-subunit of the G-binding protein	**a** and α-cells
GPA1 or *SCG1*	α-subunit of the G-binding protein	**a** and α-cells
CDC36	Unknown	**a** and α-cells
CDC39	Unknown	**a** and α-cells
SST1 (*BAR1*)	α-factor protease	**a**-cells only
SST2	Desensitization	**a** and α-cells

(Newlon, 1988; Herskowitz, 1988)

terminal truncated receptor can bind their ligand as shown with chemical cross-linking experiments and the biological response of truncated receptor-containing strains in the presence of α-factor. However, the carboxy-terminal hydrophilic domain is required for desensitization and internalization of the receptor by regulating receptor number and adaptation to pheromone (Reneke *et al.*, 1988; Konopka *et al.*, 1988; Jenness and Spatrick, 1986).

Both the STE2 and STE3 receptors share a common signal transduction pathway (Bender and Sprague, 1986; Nakayama *et al.*, 1987) (Fig. 3). Studies have identified genes encoding the α, β, and γ subunits of a G-binding protein (Miyajima *et al.*, 1987; Dietzel and Kurjan, 1987; Marsh and Herskowitz, 1988; Whiteway *et al.*, 1989). The evidence of their function is based mostly on genetic studies. The roles of the G-protein subunits in the signal transduction system of the yeast have been inferred from the behavior of mutants lacking these gene products. From such studies it has been deduced that the Gα (encoded by the *GPA1* or *SCG1* gene) subunit in wild-type strains inhibits cell response in the absence of the mating factor whereas the β and γ subunits (encoded by the *STE4* and *STE18* genes respectively) normally promote response to mating factors (Dietzel and Kurjan, 1987; Whiteway, 1988).

The work presented in this dissertation deals with the first step of pheromone-receptor interaction, namely the binding of the pheromone to its receptor. In this study we use α-factor, a tridecapeptide (NH-Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr-COOH) which is normally produced and secreted by *S. cerevisiae* α-mating type cells. The hypotheses we tested were: Does this peptide which is a linear peptide and which is flexible (when in solution) assume a specific configuration when it interacts with its receptor? Which part of the molecule interacts with the receptor and which part transfers the information? The answers to these questions have been of interest to our lab for some time. In fact, studies from our lab using α-factor analogs and proton NMR (Jelicks *et al.*,

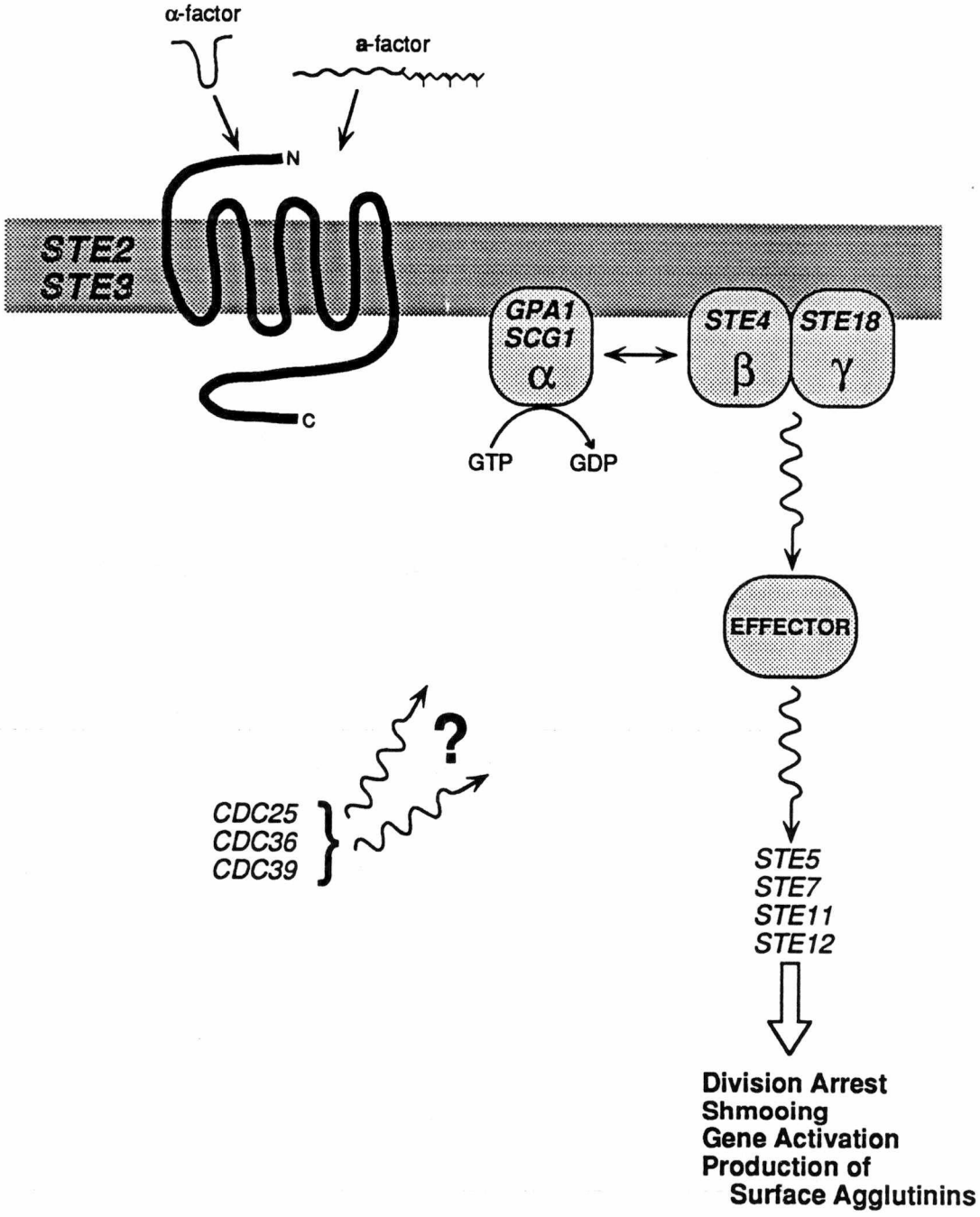


Figure 3. Schematic representation of the pheromone response pathway.

1988; Naider *et al.*, 1989; and Shenbagamurthi *et al.*,1985) have suggested that in its active conformation the peptide forms a β-bend. Computer model analysis shows that with Type II β-bend angles, the ε-amine of lysine (residue 7) and the γ-carbonyl of glutamine (residue 10) can come within 1.7 angstrom from each other. The C-N bond distance of an amide is 1.2 angstrom, a distance close to 1.7 angstrom.

Since there was a suggestion that the β-bend is the active conformation of the peptide and it was feasible to synthesize a peptide with a conformation close to that of a β-bend, a cyclic α-factor analog has been chemically synthesized in which the side chains of Lys and Gln were covalently linked through an amide bond. This modification causes a bend to be maintained involving the residues 7-10 believed to be involved in the β-bend. Chapter 2 of my dissertation is involved with the synthesis, purification, verification of the structure of cyclic α-factor (achieved by amino acid analysis, peptide sequencing, Fast Atom Bombardment mass specroscopy, and Proton NMR spectroscopy) and determination of its biological activity. We found that cyclic α-factor was highly active. When compared to the linear α-factor, it was found to be one-fourth to one-twentieth as active depending on the strain used.

Based on the bioactivity of the cyclic α-factor, we envisioned two ways (Fig. 4) through which a β-bend formation stabilized interaction between the receptor and α-factor. The first was that the β-bend as such was what interacts with the receptor; the second was that the amino and carboxyl ends of α-factor were what interacts with the receptor. Chapter 3 examines whether the β-bend or the ends of the pheromone are the signalling moieties (the parts of the molecule which bind to the receptor and carry the biological activity) of the α-factor. To investigate the proposed model we decided to create "truncated" α-factor and cyclic α-factor peptides by removing amino acids from the carboxy- and amino-terminus. If the β-bend was the signalling moiety, removal of amino

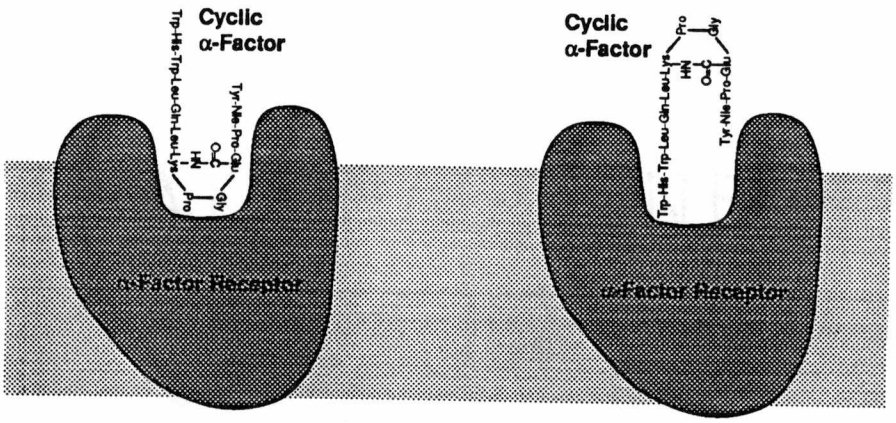


Figure 4. Model of possible α-factor receptor-cyclic α-factor interactions.

acids from the cyclic peptide would not affect its biological activity. If the amino and carboxy end segments were the signalling moiety, activity would decrease when amino acids were removed from the end tails of the peptide.

CHAPTER 2

A COVALENTLY CONSTRAINED CONGENER OF THE *SACCHAROMYCES CEREVISIAE* TRIDECAPEPTIDE MATING PHEROMONE IS AN AGONIST

Introduction

Recent studies have demonstrated quite elegantly that the mating process in *Saccharomyces cerevisiae* represents a powerful paradigm to understand the mode of action of peptide hormones (Herskowitz, 1988; Herskowitz and Marsh, 1987). Yeast cells secrete small peptides which play an essential role in the sexual conjugation of these eukaryotes (Sprague *et al.*; Cross *et al.*, 1988). The genes coding for the receptors for these peptide pheromones have been cloned and sequenced (Burkholder and Hartwell, 1985; Nakayama *et al.*, 1985). The deduced protein sequences of these receptors are compatible with an overall topology that closely resembles those of several mammalian hormone receptors (Lefkowitz and Caron, 1988). G-proteins are thought to be the transducers of the signal from pheromone- receptor to an, as yet unknown, effector system (Miyajima *et al.*, 1987; Dietzel and Kurjan, 1987). Given the wealth of biological, biochemical and genetic information available on *S. cerevisiae* and the ease with which this organism is both grown in the laboratory and subjected to molecular manipulation, great insights into receptors and peptide hormone-receptor interaction should be forthcoming from this system. The peptide pheromones which control mating in yeast are α-factor, a tridecapeptide with the sequence WHWLQLKPGQPMY (Stotzler *et al.*, 1976), and a-factor, a lipopeptide with the structure, YIIKGVFWDPAC(S-Farnesyl)$COCH_3$ (Anderegg *et al.*, 1988). The latter peptide has only recently been characterized and has not been subjected to structure-activity investigations. In contrast, numerous studies have been

conducted on the relationships between primary structure, the biological activity, and conformation of α-factor. In particular, proton NMR studies in solution (Jelicks *et al.*, 1988) and in the presence of lipid (Naider *et al.*, 1989), coupled with the biological activities of analogs that can and cannot form β-bends (Shenbagamurthi *et al.*, 1985), have led us to suggest that the α-factor is a flexible peptide which assumes a folded conformation when it assumes its biologically active structure. In order to test this hypothesis, we undertook the synthesis of α-factor in which covalent constraints were placed on the distribution of its allowable conformations. In chapter 2 we discuss strategies used to synthesize a cyclic analog of α-factor in which an amide bond has been formed between the side chains of residues 7 and 10. This covalent linkage of side chains causes a bend to be maintained in the center of the pheromone involving those residues (Lys^7-Pro^8-Gly^9-Gln^{10}) that we believe form a Type II β-turn. The biological activity of the cyclic α-factor provides new insights into the mode of action of this yeast mating pheromone.

Materials and Methods

Solid-Phase Synthesis.

The solid-phase syntheses of [Nle^{12}]α-factor, des-Trp^1-[Ala^3,Nle^{12}]α-factor, [Lys^7(Ac),Nle^{12}]α-factor and cyclo^{7,10}[Nle^{12}]α-factor were carried out manually starting with N- α-Boc-Tyr(2-BrZ)- OCH_2-PAM-resin (0.48 mmol/g of resin), which was prepared by the procedure of Tam et al. (1979). The Boc group was used for all N- α-protections. The side chain protecting groups were Trp(For), His(Tos), Lys(2-ClZ) for the [Nle^{12}]α-factor and des-Trp^1-[Ala^3,Nle^{12}]α-factor, Trp(For), His(Tos), Lys(Fmoc) for the [Lys^7(Ac),Nle^{12}]α-factor, and Trp(For), His(Tos), Lys(Fmoc), Glu(OFm) for the cyclic peptide. A typical cycle consisted of the following procedures (15 ml solvent/g of

resin): (1) CH_2Cl_2, 3 x 1 min; (2) 45% TFA/CH_2Cl_2/2% DMS, 1 x 1 min; (3) 45% TFA/CH_2Cl_2/2% DMS, 1 X 30 min; (4) CH_2Cl_2, 6 x 1 min; (5) 10% DIEA/CH_2Cl_2, 1 X 2 min; (6) 10% DIEA/CH_2Cl_2, 1 X 5 min; (7) CH_2Cl_2, 5 X 1 min; (8) Boc-amino acid (3 equiv) in CH_2Cl_2 added to the vessel followed by DIPC, diisopropylcarbodiimide (3 equiv), shaken for 2 h; (9) CH_2Cl_2, 4 x 1 min; (10) 5% DIEA/CH_2Cl_2, 1 x 2 min; (11) CH_2Cl_2, 3 x 1 min; (12) DMF, 1 x 1 min; (13) Boc-amino acid (1 equiv) in CH_2Cl_2/DMF (1:1) added to the vessel followed by DIPC (1 equiv), shaken for 1 h; (14) DMF, 3 x 1 min. The procedures of the cycle immediately following the incorporation of BocGln were different to the above protocol: (1) dioxan, 3 x 1 min; (2) 4 N HCl/dioxan, 1 x 1 min; (3) 4 N HCl/dioxan, 1 x 30 min; (4) dioxan, 3 x 1 min; (5) $CHCl_3$, 3 x 1 min; (6) 10% DIEA/$CHCl_3$, 1 x 2 min; (7) 10% DIEA/$CHCl_3$, 1 x 5 min; (8) $CHCl_3$, 3 x 1 min; (9) CH_2Cl_2, 2 x 1 min; (10) DIPC (3 equiv) in CH_2Cl_2 added to the vessel followed by Boc-amino acid (3 equiv) in CH_2Cl_2, shaken for 2 h; (11) CH_2Cl_2, 3 x 1 min; (12) $CHCl_3$, 1 x 1 min; (13) 5% DIEA/$CHCl_3$, 1 x 2 min; (14) $CHCl_3$, 3 x 1 min; (15) DMF, 1 x 1 min; (16) DIPC (1 equiv) in CH_2Cl_2/DMF (1:1) added to the vessel followed by Boc-amino acid (1 equiv) in CH_2Cl_2/DMF, (1:1), shaken for 1 h; (17) DMF, 3 x 1 min. From Leu^4 on, the first and second couplings were carried out in CH_2Cl_2/DMF (1:1) and DMF, respectively. For BocGln, the HOBt active ester was preformed in DMF at 0°C for 20 min using DIPC as activating agent and used in both couplings. In the cases where a third coupling was required, as revealed by Kaiser test (Kaiser *et al.*, 1970), 1 equiv Boc-amino acid, DIPC and HOBt in DMF was added and the reaction was allowed to proceed for 1 h at room temperature. One exception to this was the third coupling of $BocTrp^1$ (For) for which 1 equiv symmetrical anhydride in DMF was used. After BocGlu(OFm), or BocLys(Fmoc) were incorporated for the cyclic peptide or the [Lys^7(Ac),Nle^{12}]α-factor, respectively, the DIEA concentration was lowered to 5% DIEA in CH_2Cl_2 in both protocols.

Cyclization.

The BocTrp(For)-His(Tos)-Trp(For)-Leu-Gln-Leu-Lys(Fmoc)- Pro-Gly-Gln(OFm)-Pro-Nle-Tyr(2-BrZ)-PAM-resin (1 g) was washed with DMF (3 x 1 in), reacted with 20% piperidine/DMF (1 x 2 min and 1 x 30 min) to remove the Fmoc and OFm groups, and washed with DMF (2 x 1 min), MeOH (2 x 1 min), CH_2Cl_2 (2 x 1 min), and DMF (1 x 1 min). BOP (5 equiv) in DMF containing 1.5% DIEA was added and the vessel was shaken for 2 h. After washing with DMF (3 x 1 min), CH_2Cl_2 (2 x 1 min) and DMF (1 x 1 min), the resin was treated again with 20% piperdine/DMF (1 x 5 min), and washed with DMF (3 x 1 min) and CH_2Cl_2 (3 x 1 min). The Kaiser test showed that a trace of free amine was present. After a second coupling as above (3 equiv BOP) a negative Kaiser test was attained.

Acetylation.

BocTrp(For)-His(Tos)-Trp(For)-Leu-Gln-Leu-Lys(Fmoc)-Pro- Gly-Gln-Pro-Nle-Tyr(2-BrZ)-PAM-resin (1 g) was washed with DMF (3 x 1 min), treated with 20% piperdine/DMF (1 x 2 min and 1 x 30 min) to remove the Fmoc group, and washed with DMF (2 x 1 min), MeOH (2 x 1 min), CH_2Cl_2 (2 x 1 min) and DMF (1 x 1 min). Acetic anhydride (6 equiv) and DIEA (7 equiv) in DMF was added, and the vessel was shaken for 2 h. After washing with DMF (3 x 1 min), CH_2Cl_2 (2 x 1 min) and DMF (1 x 1 min), the resin was treated again with 20% piperidine/DMF (1 x 5 min), and washed with DMF (4 x 1 min). A second acetylation was performed with the same reagent as above, and complete acetylation was confirmed by the Kaiser test.

HF Cleavage.

Before the cleavage of the peptide from the resin, the N terminal Boc group was deprotected with 45% TFA/CH_2Cl_2/2% DMS for 30 min, and the resin was washed with

CH_2Cl_2, MeOH, and CH_2Cl_2 (three times each) and dried *in vacuo* overnight. One gram of the resin was mixed with 1.5 ml 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). Cleavage proceeded at 0-2°C for 1.5 h. After evaporation of HF, the residue was washed with precooled ether and then ethyl acetate (3 times each). The dodecapeptide was extracted with 6% aqueous acetic acid, the three tridecapeptides were extracted with 25% aqueous acetic acid, and the extracts were combined and lyophilized. The yield of the crude product was about 90% for the three linear peptides and 70% for the cyclic peptide based on the starting amine content on the resin.

Deformylation and Detosylation.

In order to remove the formyl group from the two tryptophan residues, the crude products from HF cleavage were dissolved in 20 ml 1M piperidine in H_2O/DMF (1:1) and the mixture was placed in a refrigerator overnight. After evaporation of the solvents at room temperature in vacuo, 1M aqueous HOAc (10 ml) was added, and the solution was lyophilized. Analytical HPLC analysis showed that the retention time was different after the piperidine treatment of the [Nle^{12}]α-factor; the deprotected peptide moved faster (0.3 min) than the protected peptide. No change in retention time was observed for the [Lys^7(Ac),Nle^{12}]α-factor and the cyclic peptide, suggesting that the formyl group had been completely removed during the treatment of the resin with piperidine in DMF prior to cyclization. The tosyl group on histidine has been long considered to be completely removed by HF (Tam and Merrifield, 1987). Therefore, no attempt was made to detosylate the crude [Nle^{12}]α-factor and des-Trp^1-[Ala^3,Nle^{12}]α-factor. However, during the purification of the [Nle^{12}]α-factor it was noticed that the side product at 13.9 min slowly transformed into the peptide at 9.1 min (Fig. 5A). This observation stimulated us to treat the crude cyclic peptide with HOBt in water and acetonitrile at room temperature for 2 h

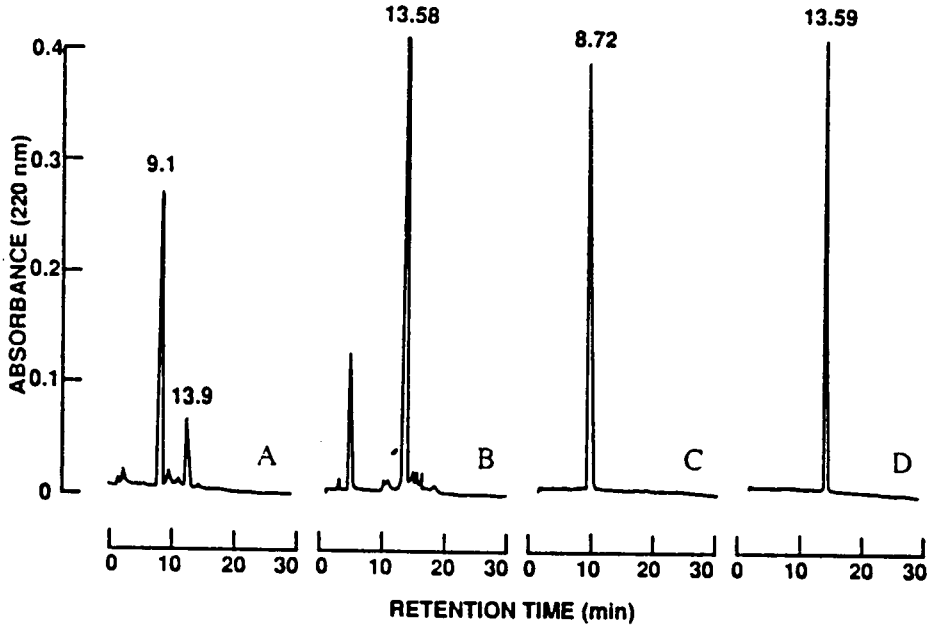


Figure 5. HPLC of [Nle^{12}]α-factor and des-Trp^{1}-[Ala^{3},Nle^{12}]α-factor crude and purified products. A, HPLC of the crude HF cleavage product from the solid phase synthesis of [Nle^{12}]α-factor. B, HPLC of the crude HF cleavage product from the solid phase synthesis of des-Trp^{1}-[Ala^{3},Nle^{12}]α-factor. C, purified product from A. D, purified product from B. Chromatographic separations were carried out on a C_{18} reversed phase column using a water/CH_3CN/trifluoroacetic acid gradient from 80:20:0.025 to 40:60:0.025 over 20 min for A and C and a water/ CH_3CN/trifluoroacetic acid gradient from 95:5:0.025 to 60:40:0.025 over 20 min for B and D. The flow rate was 1.5 ml/min, and detection was by absorbance at 220 nm.

prior to purification. The peak at 17.1 min in the crude peptide (Fig. 6A) completely disappeared after HOBt treatment (Fig. 6B). Based on these results, the protected [Lys^7(Ac),Nle^{12}]α-factor resin was treated with HOBt in DMF for 1 h prior to HF cleavage, and consequently, the crude peptide from HF cleavage was obtained without the significant tosylated side product (data not shown) as compared with the crude [Nle^{12}]α-factor (Fig. 5A) and the crude cyclic peptide (Fig. 6A).

Purification and Characterization.

The crude peptide from HF cleavage was purified by reversed-phase, semi-preparative HPLC (Waters Associates) on a Waters μ-Bondapak C_{18} column (19 x 150 mm). The cleavage product (250-350 mg) was dissolved in about 18 ml H_2O/CH_3CN containing 1% TFA, and one third of this solution (about 6 ml) was applied onto the column and eluted with a linear gradient of H_2O (0.025% TFA)/CH_3CN, from 0 to 60% CH_3CN over 60 min at a flow rate of 6 ml/min. One min fractions were collected, and the desired peptide generally eluted sharply within 3 fractions. The [Nle^{12}]α-factor and the dodecapeptide were pure (>99%) after one purification, but the [Lys^7(Ac),Nle^{12}]α-factor and the cyclic peptide required a second chromatography on the same column. The total yields of the four purified peptides, on the basis of the starting loading of the amine on the resin, were 51%, 67%, 45%, and 30% for the [Nle^{12}]α-factor, des-Trp^1-[Ala^3,Nle^{12}]α-factor, [Lys^7(Ac),Nle^{12}]α-factor and cyclo^{7,10}[Nle^{12}]α-factor, respectively. The purified peptides were analyzed with analytical HPLC on a Waters μBondapak C_{18} column (3.9 x 300 mm), and all were found to be over 99% homogenous (e.g. Figs. 5B, 5D, 5C). The purity of the peptides was also checked by TLC on silica gel and a single spot in two systems for each peptide was observed with both UV and ninhydrin detections. Amino acid analysis gave the results very close to the calculated values (Table 2). The presence of Trp in the synthetic peptides was confirmed by 400 MHz ^{1}H-NMR and ultraviolet

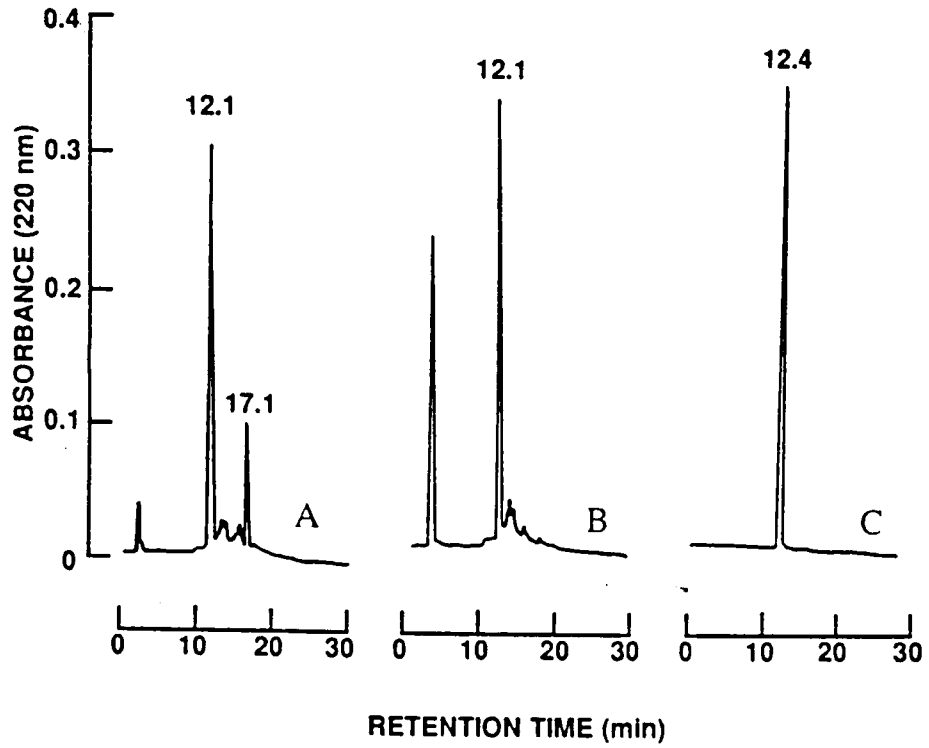


Figure 6. HPLC of cyclic [Nle^{12}]α-factor crude and purified products. A, HPLC of the crude HF cleavage product from the synthesis of cyclic [Nle^{12}]α-factor. B, HPLC of the product in A after treatment with HOBt in water and CH_3CN for 2 h. C, HPLC of purified cyclic α-factor. Chromatography conditions were as in Fig. 5.

Table 2. Chemical and Physical Properties of α-factor analogs.

Analysis	Peptide[a]			
	[Nle^{12}]α-factor	Dodecapeptide	[Lys^{7}(Ac),Nle^{12}]α-factor	Cyclic peptide
Amino Acid				
Ala		0.97		
Glu	2.10	2.00	1.92	2.08
Gly	1.14	1.07	1.06	1.05
His	0.79	0.97	0.86	0.93
Leu	1.98	2.01	2.04	1.93
Lys	0.88	1.00	1.05	1.06
Nle	0.99	1.00	1.05	0.97
Pro	2.01	1.93	1.88	1.80
Tyr	1.06	1.05	1.14	1.16
Yield(%)	51	67	45	30
R_F, solvent A[b]	0.41	0.27	0.46	0.49
R_F, solvent B[b]	0.51	0.44	0.59	0.65
K' value[c]	2.8	4.9	3.8	4.3
FAB mass spectrometry				
Found				1649.5
Calculated				1648.9

[a]The peptides were: [Nle^{12}]α-factor, WHWLQLKPGQP(Nle)Y; dodecapeptide, HALQLKPGQP(Nle)Y; [Lys^{7}(Ac),Nle^{12}]α-factor, WHWLQLK(Ac)PGQP(Nle)Y; and cyclic peptide, cyclo^{7,10}[Nle^{12}]α-factor.

[b]Solvent system A: butanol/acetic acid/water (4:1:2); solvent system B: butanol/acetic acid/water/pyridine (15:3:12:10). R_F values were determined using TLC on silica.

[c]K' values for [Nle^{12}]α-factor, [Lys^{7}(Ac),Nle^{12}]α-factor, and cyclic peptide were determined for a μBondapak C_{18} column using a 20-60% acetonitrile gradient (0.025% trifluoroacetic acid) over 20 min, and the K' value for the dodecapeptide was for the same column using a 5-40% acetonitrile gradient (0.025% trifluoroacetic acid) over 20 min.

spectroscopy. Peptide sequencing was carried out by Hoffman LaRoche Inc., Nutley, N.J. The peptide (150 μg) was dissolved in 100 μl of 70% CH_3CN and 400 μl of 0.1% CF_3COOH. The analysis was performed using an Applied Biosystem gas phase sequencer model 470A. Phenylthiohydantoin (PTH) amino acid derivatives were identified "on-line" with an ABI model 120A PTH analyzer. FAB mass spectrum was obtained with a VG ZAB EQ mass spectrometer equipped with a flow FAB ion source of an Ion tech fast atom gum. Approximately 2 μl of a peptide solution (1.0 nmol/μl) dissolved in acetonitrile:water (2:1), 0.1% trifluoroacetic acid was dispersed on the stainless steel target in a matrix of monothioglycerol. The accelerating voltage of the mass spectrometer was maintained in 8 kV, while 8 keV xenon atoms at a discharge current of 1 mA were used to bombard the sample. Spectra were recorded in the "multi-channel acquisition" mode from m/z 800 to 1800 at 20 sec/decade by the VG 11-250 J data system. Instrument resolution (m/Δm) was set to 2000. Peptide sequencing for the cyclic peptide confirmed residues 1-6, 8-9, and 11-13. The cyclo^{7,10}[Nle^{12}]α-factor had the calculated molecular ion as determined by FAB mass spectrometry.

Saccharomyces cerevisiae Strains Used.

The sources and relevant genotype of the strains used in this chapter are: *S. cerevisiae* 2180-1A(*MATa*) from the Yeast Genetics Stock Center, Berkeley, CA; *S. cerevisiae* RC629(*MATa sst1-2*, supersensitive to α-factor by virtue of a mutation in the *BAR1* protease [allelic to *SST1*] which hydrolyzes α-factor) from R. Chan; *S. cerevisiae*-RC631(*MATa sst2-1*, supersensitive to α-factor because of a mutation in a desensitization response) from R. Chan (Chan and Otte, 1982); *S. cerevisiae* 4202-15-3(*MATa bar1-1*, a strain used in binding studies) from D. Jenness (Raths *et al.*, 1988 and Jenness *et al.*,1983), and *S. cerevisiae* 50B(*MATa ste2^{ts}*, temperature sensitive for the α-factor receptor *STE2*) from Yeast Genetics Stock Center.

Growth Arrest (Halo Assay).

YEPD plates (yeast extract, 2%; peptone, 1%; dextrose, 2%; and agar, 2%) were overlayed with 4 ml of *S. cerevisiae MATa* cells (strains 2180-1A, RC629, 4202-15-3, RC631 and 50B; 2.5 x 10^5 cells/ml) in noble agar (0.825%). Filter disks (sterile blanks from DIFCO), 8 mm in diameter, were 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 24 h, except for strain 50B (*ste2^{ts} MATa*) for which duplicate plates were made for the same concentration of peptides and one set of plates was incubated at 23°C and the other at 34°C. Following incubation, the plates were observed for clear zones (halos) around the disks. The data were expressed arbitrarily as the diameter of the halo including the diameter of the disk. Therefore, a minimum value for growth arrest is 9 mm which represents the disk diameter (8 mm) and a small zone of inhibition. Neither the presence of the disk nor agar in the medium intefered with the results. The effect of α-factor analogs on yeast cells was the same in the presence or absence of the disk; the halo was the same diameter when a disk was used or when the peptide was applied directly to the lawn of cells. Agar does not interfere with the results because an independent assay performed in liquid medium (shmoo assay) gave the same order of relative activities for all the analogs. Therefore, we believe that peptides do not differ in diffusing through the agar or in binding to agar molecules.

Antagonist Reversal Assay.

YEPD plates were overlayed with 4 ml of RC629 cells (2.5 x 10^5 cells/ml) in noble agar. Filter disks were placed on the overlay and 10 µl of either α-factor (0.05 mg/ml) or cyclic α-factor (0.4 mg/ml) were pipetted onto the disks. An additional disk was placed with its center at the periphery of the expected halo and des-Trp^1-[Ala^3,Nle^{12}]α-factor was added at 20X, 10X, and 2X and 10X, 5X, and 1X the concentration of α-factor or cyclic

α-factor, respectively. The plates were incubated at 30°C for 24 h and then observed for antagonism of α-factor or cyclic α-factor activity shown as reversal of growth arrest by the antagonist.

HPLC Analysis of Cell Mediated Peptide Hydrolysis.

A culture of 2180-1A was grown at 30°C with shaking to log phase (1×10^7 cells/ml) in YEPD. A portion of these cells and α-factor or analog was added to fresh YEPD so that the final concentrations were 1.2×10^5 cells/ml and 5 μg/ml of peptide. The suspensions were incubated at 30°C and at 2 h intervals 2 ml of the cell suspension were filtered (0.45 μm filters) and the culture medium analyzed by HPLC on a Waters μBondapak C_{18} column with a gradient mobile phase of 25-40% acetonitrile with 0.025% TFA.

Competition of Bound [^{3}H]α-Factor by Unlabeled α-Factor Analogs.

Competition of bound [^{3}H]α-factor by unlabeled cyclic α-factor was measured as described previously (Raths *et al.*, 1988) with the following changes. The final assay concentrations of the peptides were: 6×10^{-10}, 3.6×10^{-9}, 6×10^{-9}, 3.6×10^{-8}, 6×10^{-8}, 3.6×10^{-7}, 6×10^{-7} and 3.6×10^{-6} M for [Nle^{12}]α-factor; and 7.4×10^{-9}, 4.4×10^{-8}, 7.4×10^{-8}, 4.4×10^{-7}, 7.4×10^{-7}, 4.4×10^{-6}, 7.4×10^{-6} and 1.48×10^{-5} M for cyclic α-factor. Typically, the binding competition was started by the addition of 50 μl of [^{3}H]α-factor (9.3×10^{-7} M, 7.57 Ci/mMol) to 400 μl of the cell suspension (1.25×10^9 cells/ml) and 50 μl of the appropriate unlabeled analog.

Results

Synthesis of α-Factor Analogs

The efficient synthesis of a cyclic analog of α-factor required that all precursors be prepared in optimum yield. Prior to the preparation of cyclo^{7,10}[Nle^{12}]α-factor we attempted to improve our synthesis of the tridecapeptide. In previous studies, α-factor and several analogs were prepared using both solution phase (Khan *et al.*, 1981; Shenbagamurthi *et al.*, 1983) and solid phase (Tallon *et al.*, 1987) strategies. It was found that the isosteric substitution of Nle for Met at position twelve decreased side reactions, improved yield, and resulted in a pheromone with activity equal to that of the natural tridecapeptide (Raths *et al.*,1988). In the present study, we made one change in our coupling and deprotection cycle that proved to be extremely important. Rather than removing the Boc protecting group on Gln^5 and Gln^{10} using trifluoroacetic acid, we used HCl in dioxane to deprotect these residues. Marked improvement in the homogeneity of the crude peptide cleaved from the resin was noted. As seen in Fig. 5, the crude product from the synthesis of [Nle^{12}]α-factor contained primarily one major product (Fig. 5A). The peak at 13.9 min in Fig. 5A was subsequently shown to be due to incomplete removal of the tosyl group from histidine (see below). Interestingly, this same type of side product was found in all tridecapeptides but was not observed in the dodecapeptide (Fig. 5B). In this latter compound, His is the amine terminal residue. Apparently, when His is at an interior position or when it is flanked by large hydrophobic residues, removal of the Tos protecting group is more difficult.

After purification of these analogs using HPLC, peptides of greater than 99% purity (Figs. 5C, 5D) were isolated in yields of 51% and 67%, for the tridecapeptide and dodecapeptide, respectively. The best yield that was obtained previously during solid phase synthesis of [Nle^{12}]α-factor was 25% (Raths *et al.*, 1988) and the yield first

reported for the solid phase synthesis of α-factor was 2% (Ciejek *et al.*, 1977). It is clear that the utilization of HCl in an organic solvent in place of trifluoroacetic acid should be considered for problematic residues and that this inorganic acid might lead to dramatic improvement in the synthesis of longer peptides if it were more extensively employed. In addition to the use of HCl in dioxane, our improved synthesis also employed reversed addition of DIPC and the protected amino acid. This procedure has been found to lead to improvement during other solid phase syntheses (Barany and Merrifield, 1979). As judged using a five minute Kaiser test, the last four residues (Trp^1-Leu^4) all required a third coupling, and special care was taken to assure that these couplings went to completion. We also carried out the HF cleavage for 1.5 h at 0-2°C in the present study as compared to 1 h at 0°C in previous syntheses. The longer cleavage seems to lead to an increased yield of crude peptide without any deleterious effect on the quality of the crude peptide. The synthesis of cyclo^{7,10}[Nle^{12}]α-factor (see Fig. 7) was accomplished by using an OFm ester for protection of γ-carboxyl of Glu^{10} and Fmoc protection for the ε-amine of Lys^7 following a strategy recently developed by Felix and coworkers (Felix *et al.*, 1988). After incorporation of the OFm group, the neutralization step in the synthetic cycle was accomplished using a lower concentration of DIEA. Although Fmoc and OFm groups are reported to be stable to tertiary amines such as DIEA in CH_2Cl_2 (Atherton and Shepherd, 1987), we and others (Felix *et al.*, 1988) have observed slow cleavage of these groups on extended incubation with 5-10% DIEA in CH_2Cl_2. It seemed prudent, therefore, to lower the DIEA concentration to 5%. At DIEA concentrations below 5%, neutralization was incomplete and required repetitive treatment with the base. After assembly of the complete peptide, the side chain protecting groups were removed with piperidine and cyclization was carried out using the BOP reagent. We observed that the crude cyclic product isolated after HF cleavage from the resin contained a significant impurity at 17.1 min (Fig. 6A). Treatment of the crude product with HOBt resulted in the disappearance of the impurity

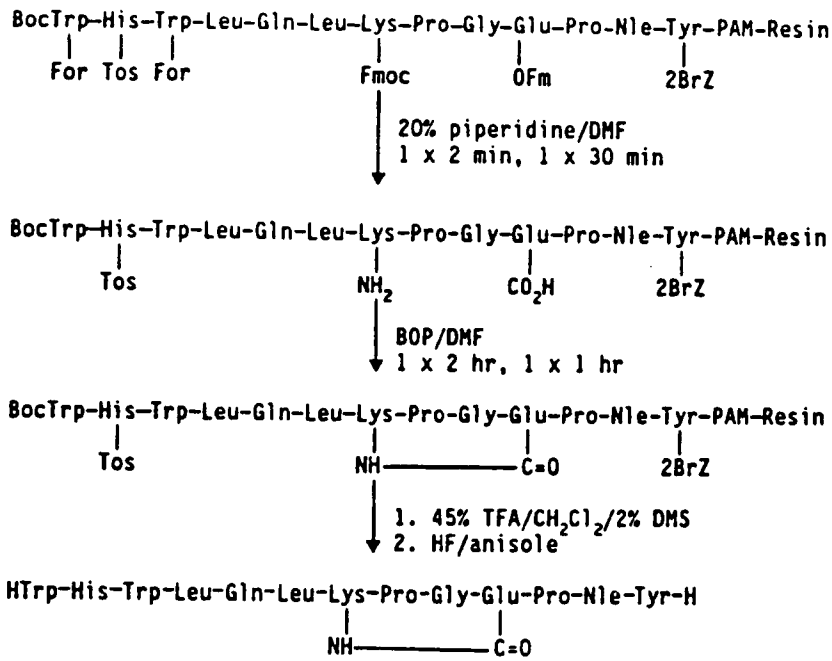


Figure 7. Synthetic scheme for cyclo^{7,10}[Nle^{12}]α-factor. TFA, trifluoroacetic acid.

from the chromatogram suggesting that it represented tosylated cyclic peptide. Subsequent to this finding, we have routinely treated the crude products from HF cleavage with HOBt if this side product occurs. It should be noted that our synthetic strategy requires treatment of the peptide with piperidine prior to cyclization. This may result in partial deprotection of Trp(For) and some destruction of Trp during HF cleavage. Nevertheless, the overall yield for the purified cyclic peptide (Fig. 6C) was 30%.

Structural Analysis of Cyclo^{7,10}[Nle^{12}] α-Factor

The structure of the cyclic analog was characterized by a number of complementary procedures. Amino acid analysis (Table 2) supported the presence of all residues except for Trp. The presence of this latter residue was confirmed using 400 MHz 1H-NMR which exhibited resonances at 10.92 ppm and 10.82 ppm characteristics of Trp indole NH protons. The integration of the indole NH protons relative to the Tyr aromatic protons indicated that two Trp residues were present in cyclo^{7,10}[Nle]α-factor. Using COSY spectroscopy, nearly all resonances in the tridecapeptide have been assigned. Most significantly the broad resonance found for the N-ε of Lys in the linear molecule (7.72 ppm; Jelicks *et al.*, 1988) changed to a sharp amide-like triplet at 7.73 ppm in the cyclic pheromone (data not shown). To further support the structure of the molecule a sequence analysis was performed (see Methods). The sequence obtained, with the cycle number, indicated in parentheses was: Trp(1), His(2), Trp(3), Leu(4), Gln(5), Leu(6), Blank (7), Pro(8), Gly (9), unknown (10), Pro(11), Nle(12), Tyr(13). This data is consistent with a peptide containing a linkage between residue 7 (Lys) and residue (10). Finally, fast atom bombardment mass spectroscopy, indicated a molecular ion at 1650 + 1 daltons (Fig. 8). This compares favorably with the molecular weight of 1648.9 calculated for the cyclic pheromone. No molecular ion was observed at 1667 which would correspond to the linear

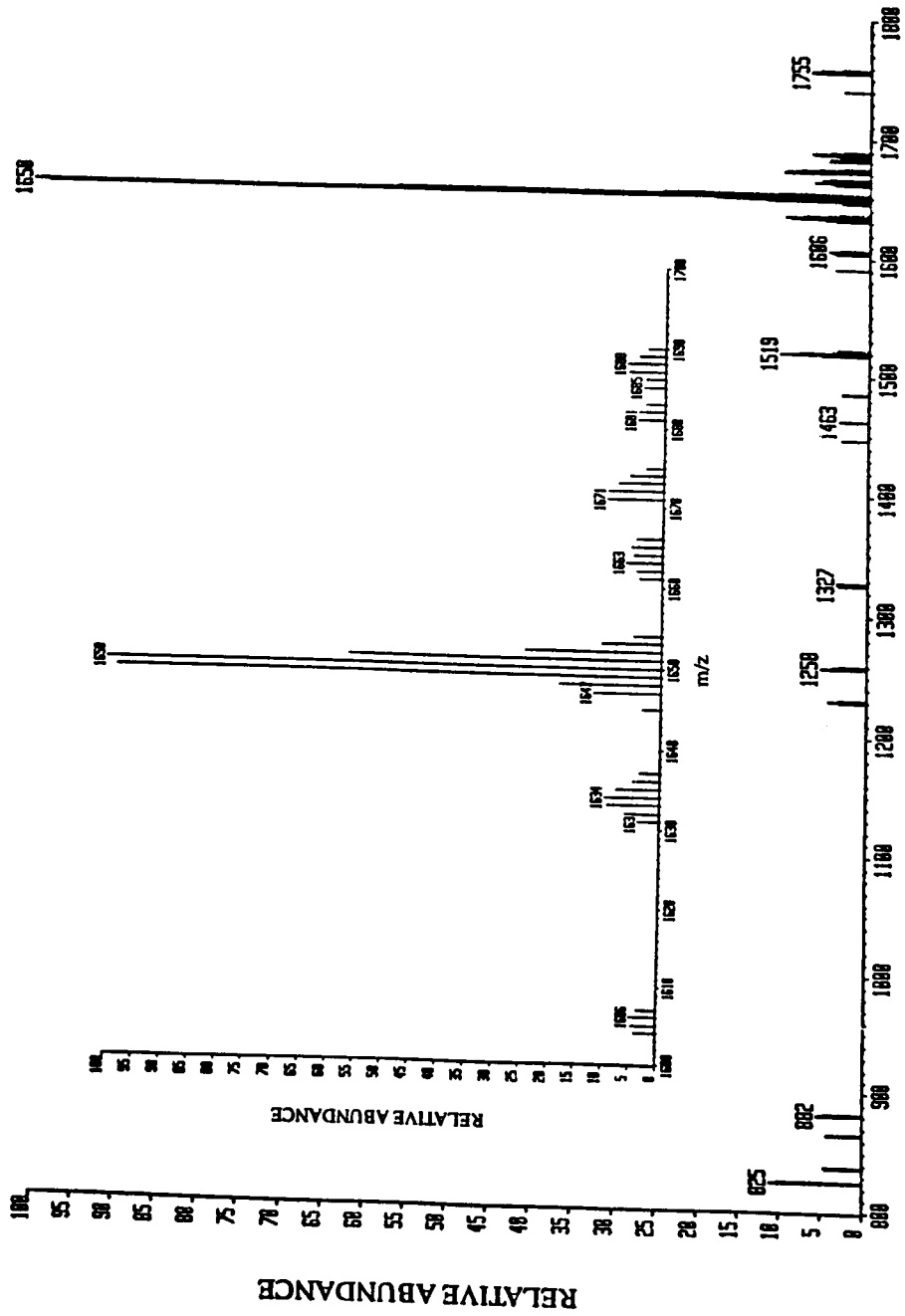


Figure 8. Fast atom bombardment-mass spectroscopy of cyclo^{7,10}[Nle^{12}]α-factor.

pheromone. We believe that these results strongly support our conclusion that we have prepared cyclo^{7,10}[Nle^{12}]α-factor.

Biological Activity of the Cyclic α-Factor

The activity of the cyclic analog in the growth arrest assay was investigated (Table 3). This constrained molecule proved to be highly active causing growth arrest at 2 μg/disk with the wild type tester *S. cerevisiae* 2180-1A. In comparison with the linear [Nle^{12}]α-factor in the growth arrest assay, the cyclic compound was one-fourth as active using a wild type tester strain, one-fifth as active with *S. cerevisiae* 631 (a *sst2*-supersensitive strain) and one-twentieth as active against *S. cerevisiae* 629 and *S. cerevisiae* 4202-15-3 (*sst1*-supersensitive mutants). The increased sensitivity of three supersensitive strains to both cyclic α-factor and the linear tridecapeptide indicated that the tester strains were responding similarly to both peptides. Since cyclo^{7,10}[Nle^{12}]α-factor contains an acylated lysine side chain, we also compared its activity with a linear tridecapeptide which had an acetyl group on the Lys^{7} amine. This latter pheromone had biological activities intermediate between those for the underivatized tridecapeptide and the cyclic peptide.

Stability of the Cyclic α-factor

The recovery of yeast cells from sex pheromones may be mediated in part by the extracellular hydrolysis of these peptides (MacKay *et al.*, 1988; Ciejek and Thorner, 1979) and by a poorly understood mechanism which has been termed desensitization (Moore, 1984). We examined the recovery of strain 2180-1A by determining the proportions of shmoos (growth-arrested cells) in a culture exposed to α-factor and cyclic α-factor. At 5 μg/ml of peptide, the start of recovery was about 12 h and 14 h for cyclic α-factor and α-factor, respectively. The stability of cyclo^{7,10}[Nle^{12}]α-factor in the presence of *S. cerevisiae MATa* cells was evaluated by HPLC analysis of the culture medium taken at

Table 3. Growth Arrest of Four Strains of *S. cerevisiae* by α-Factor Analogs. Results are represented as average of at least three determinations of the diameter of the zone of inhibition in mm. In all cases, the three values averaged were within 2 mm of each other. Peptides are as follows: α-factor, WHWLQLKPGQP(Nle)Y; cyclic α-factor, cyclic^{7,10}[Nle^{12}]α-factor; Lys^{7}(Ac)α-factor, [Lys^{7}(Ac),Nle^{12}]α-factor[a].

Peptide	2180-1A			RC631 (*sst2*)			RC629(*sst1*) or 4202-15-3		
	α-Factor	Cyclic α-Factor	Lys^{7}(Ac)-α-Factor	α-Factor	Cyclic α-Factor	Lys^{7}(Ac)-α-Factor	α-Factor	Cyclic α-Factor	Lys^{7}(Ac)-α-Factor
μg/disc					mm				
10	20	14	16	36	35	35	32	23	27
4	16	11	14	35	33	32	30	19	24
2	12	9	11	30	29	30	27	17	22
1	11	0	10	28	28	25	24	12	20
0.5	9	0	0	26	21	19	22	8	16
0.1	0	0	0	20	0	10	14	0	0
0.05	ND[b]	ND	ND	15	ND	ND	11	ND	ND
0.01	0	0	0	0	0	0	0	0	0

[a]All strains grow equally well in the medium used (YEPD).
[b]ND, not determined.

various time points during recovery. The peak area of the three pheromones all decreased exponentially with time and the same degradation product appeared for all pheromones (Fig. 9). This degradation peak at 18.1 min correlates with the expected hexapeptide N-terminal portion of α-factor which is known to be a product of degradation of α-factor by the *BAR* protease (Ciejek and Thorner 1979; Naider and Becker, 1986). The half- lives calculated from Fig. 9 are 2.2 h, 4.2 h, and 10 h for [Lys^{7}(Ac),Nle^{12}] tridecapeptide, [Nle^{12}]tridecapeptide, and cyclo^{7,10}[Nle^{12}]α-factor, respectively. As seen in Fig. 9, during the first 4 hours there is no evidence of breakdown of cyclo^{7,10}[Nle^{12}]α-factor, and even after a 12 hour incubation, no [Nle^{12}]α-factor is apparent in the culture medium. Additional results with cyclo^{7,10}[Nle^{12}]α-factor and 1.2 x 10^{6} cells/ml for 20 h showed the same profile of degradation products as seen in Fig. 9. Thus, we expect that the growth arrest experiments (Table 3) and the HPLC analyses contain the same hydrolysis products. Furthermore, we have synthesized Glu^{10}[Nle^{12}]α-factor. This molecule would result if the amide bond between the Lys ε-amine and Glu- γ-carbonyl were hydrolyzed. When this peptide was added to a 19 h degradation mixture of the cyclic α-factor, a peak appeared (at 15.8 min) which would be between the amine terminal hexapeptide (18.1 min) and the unknown peak near 12 minutes as seen in Fig. 9. Thus there is no indication that the cyclic peptide is degraded to a linear pheromone during the bioassay. Our results, therefore provide convincing evidence that the activity of the cyclic pheromone is due to the intact molecule and not due to the hydrolysis of the amide bond between the side chains of Lys^{7} and Glu^{10}.

Reversal of Activity by an Antagonist

des-Trp^{1}-[Ala^{3},Nle^{12}]α-factor is an antagonist of α-factor activity (Naider and Becker, 1986). This analog binds to the pheromone receptor but does not trigger a biological response (Raths *et al.*, 1988). We tested whether des-Trp^{1}-[Ala^{3},Nle^{12}]α-factor

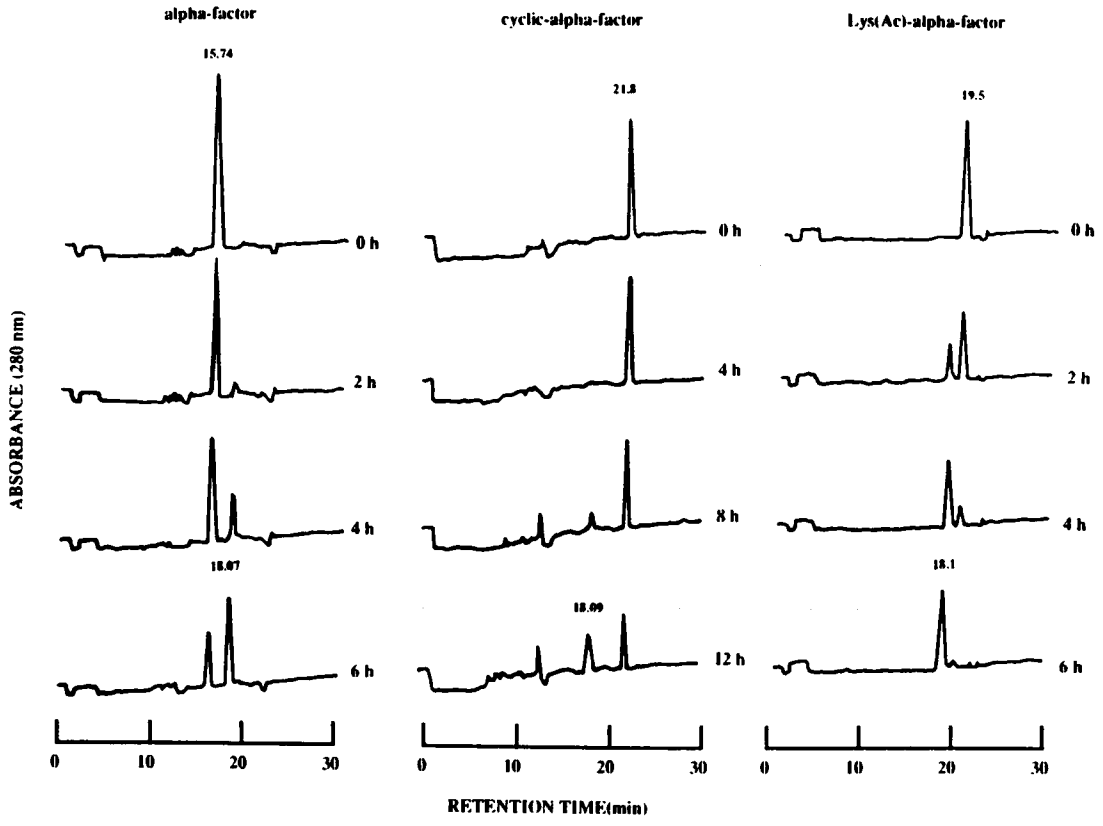


Figure 9. Effect of cell cultures on the extracellular concentrations of α-factor, cyclic α-factor, and [Lys^{7}(Ac),Nle^{12}]α-factor. *S. cerevisiae* 2180-1A cells (1.2 x 10^5 cells/ml) were incubated with 5 μg/ml pheromone. At 2-h intervals, portions of the supernatant were removed, centrifuged to separate cells and medium, and the medium was chromatographed on a C_{18}-reversed phase column. For the cyclic α-factor, HPLC results from 4-h intervals are shown.

inhibited the biological activity of cyclo^{7,10}[Nle^{12}]α-factor. To a lawn of *S. cerevisiae* RC629(*MATa*), [Nle^{12}]α-factor and cyclo^{7,10}[Nle^{12}]α-factor were spotted. A disk containing des-Trp^{1}-[Ala^{3},Nle^{12}]α-factor was placed within the growth inhibition zone (halo) expected to be formed from linear and cyclic peptide. Amounts of α-factor and cyclic α-factor (as indicated in Fig. 10) used in this experiment were based on amounts yielding 22-24 mm halos. The ratios of antagonist to α-factor and cyclic α-factor were 2X, 10X, 20X and 1X, 5X,and 10X, respectively, and were based on the relative affinities of these pheromones for the receptor. After growth for 24 h it was apparent (Fig. 10) that the antagonist reversed the growth inhibition of both [Nle^{12}]α-factor and cyclo^{7,10}[Nle^{12}]α-factor. These findings support studies with temperature sensitive mutants that suggest that the cyclic pheromone binds to the same receptor as the native α-factor (see below).

Binding of cyclo^{7,10}[Nle^{12}]α-factor to the α-factor Receptor

To measure directly whether cyclo^{7,10}[Nle^{12}]α-factor interacted with the α-factor receptor, binding competition studies and response of a temperature-sensitive receptor mutant were carried out. Radioactive α-factor binding to *S. cerevisiae MATa* cells was measured in the presence of the cyclic peptide. Starting with the same concentration of radioactive peptide (10^{-7}M), 20 to 40-fold more cyclo^{7,10}[Nle^{12}]α-factor than [Nle^{12}]α-factor was needed to reduce binding by 50% (Fig. 11). This lower affinity was consistent with the 20-fold less activity for growth arrest found for cyclo^{7,10}[Nle^{12}]α-factor in 4202-15-3, the strain used for binding studies. *S. cerevisiae* strain 50B(*ste2^{ts}*) was used to measure growth arrest at the permissive and restrictive temperatures. At 24°C α-factor and cyclo^{7,10}[Nle^{12}]α-factor inhibited growth, but at the restrictive temperature of 34°C neither peptide arrested cells (data not shown).

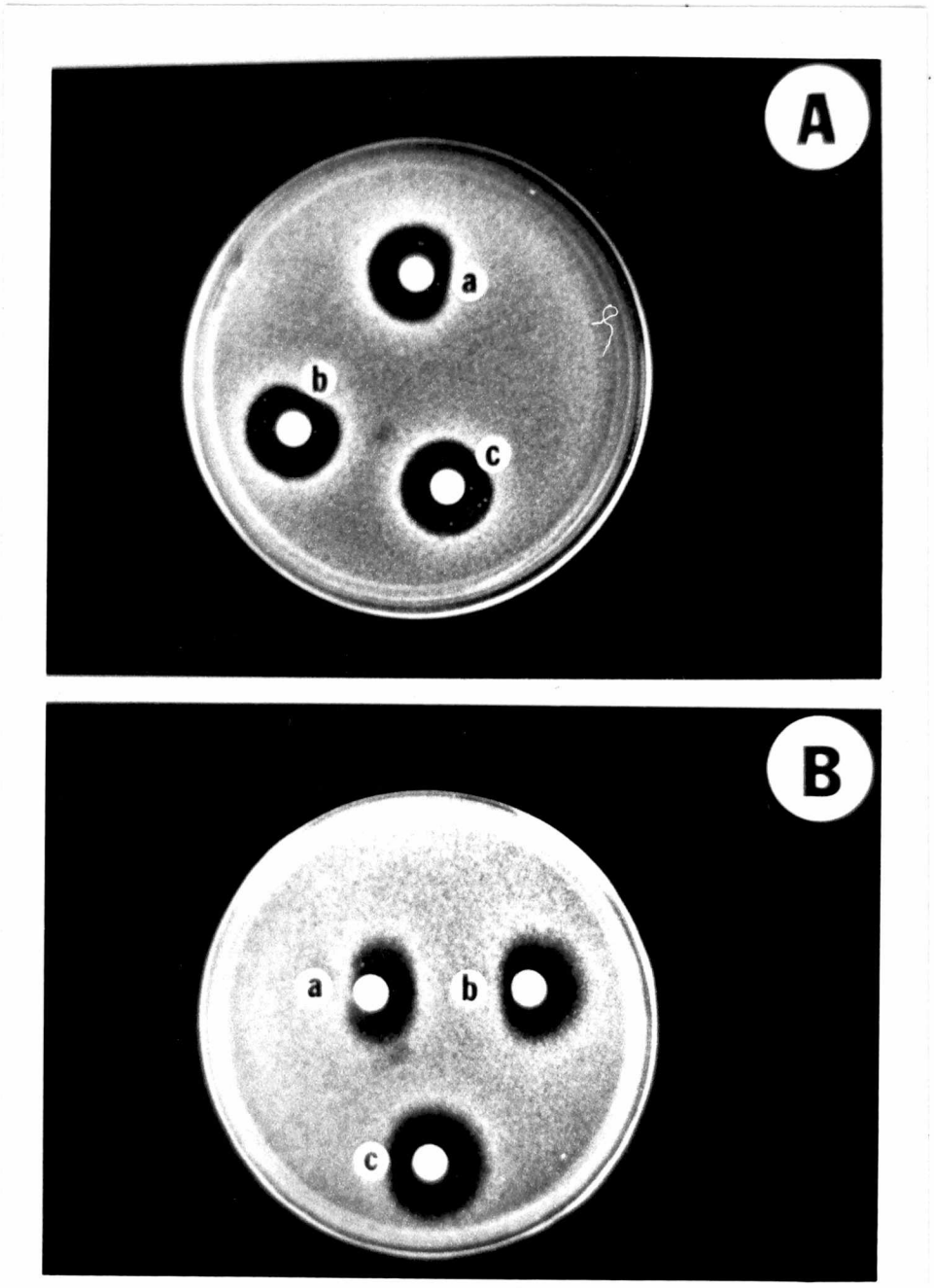


Figure 10. Reversal of α-factor growth inhibition of *S. cerevisiae* RC629 by des-Trp^{1}-[Ala^{3},Nle^{12}]α-factor. A, [Nle^{12}]α-factor (0.5 μg/disk) in the center and des-Trp^{1}-[Ala^{3},Nle^{12}]α-factor at a, 10 μg/disk (20-fold); b, 5 μg/disk (10-fold); or c, 1 μg/disk (2-fold). B, cyclic α-factor (4 μg/disk) in the center and des-Trp^{1}-[Ala^{3},Nle^{12}]α-factor at a, 40 μg/disk (10-fold); b, 20 μg/disk (5-fold); or c, 4 μg/disk (1-fold).

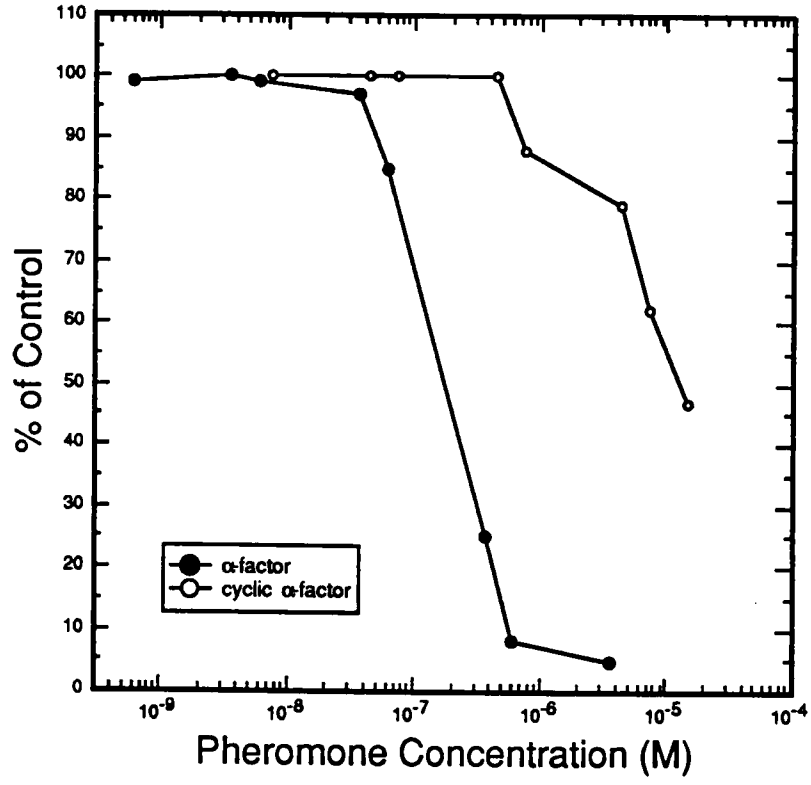


Figure 11. Competition for binding of α-[^{3}H]factor by unlabeled α-factor and cyclo^{7,10}-[Nle^{12}]α-factor. α-Factor and analog binding to 4202-15-3 (*MATa sst1*) cells was performed in competition with α-[^{3}H]factor as described under "Materials and Methods." Data shown are the means of triplicate determinations, with each curve representative of two separate experiments. The *symbols* represent competition of α-[^{3}H]factor by [Nle^{12}]tridecapeptide, and cyclo^{7,10}-[Nle^{12}] α- factor as indicated in the figure.

Discussion

The data presented in this chapter show that a cyclic analog of the α-factor retains significant biological activity. This finding represents the first example of a conformationally restricted α-factor and provides important new information concerning the structural requirements of the pheromone which are necessary for activity. The cyclo^{7,10}[Nle^{12}]α-factor was designed on the basis of previous studies which indicated that the Type II β-turn involving the Lys-Pro-Gly-Gln sequence might be important for recognition by the receptor (Jelicks *et al.*, 1988; Naider *et al.*, 1989; Shenbagamurthi *et al.*, 1985). From this perspective, it is somewhat disappointing that the cyclic pheromone exhibited lower activity than its linear homologue. If a Type II β-turn were necessary for activity one might have expected that cyclic analog containing this turn would be super active. Nevertheless, detailed physical chemical studies are needed to determine the exact structure assumed by the cyclic pheromone. Although a computer model binding analysis shows that a type II β-turn with only slight distortions of idealized dihedral angles could occur when the ε- amine of lysine is bonded to the γ-carbonyl of glutamic acid, such distortions might lower the free energy of binding and lead to reduced receptor affinity. Despite the above limitations it is clear that the α- factor receptor can recognize a pheromone which contains a bend at the center of the molecule.

The covalent linkage involving Lys^7 and Glu^{10} greatly restricts the number of degrees of freedom available to these residues. However, the remaining residues at the amine and carboxyl terminus can still assume a myriad of structures. Linkage of Lys^7 and Glu^{10} would still permit the two ends of the pheromone to interact with each other and the receptor. If such interactions are important it is not difficult to understand the drop in activity observed with the cyclic analog since the conformational restriction at the center of the molecule might prevent optimal binding to the receptor. It is interesting to note that

although the natural pheromones from *S. cerevisiae* (WHWLQLKPGQPMY) and *S. kluyveri* (WHWLSFSKGEPMY) differ in structure they cross react with their respective receptors (Marsh and Herskowitz, 1988; McCullough and Herskowitz,1979). The common feature of these pheromones are the four residues at the amino terminus and the three residues at the carboxyl end. This observation may indicate that interactions between the peptide termini and the receptor are critical for pheromone activity. In the next chapter we investigate the biological importance of the interaction of amine and carboxyl segments of the α-factor with the *STE2* receptor.

Analysis of the relative activity of different mating factors must consider the possible extracellular hydrolysis of the pheromones. *S. cerevisiae* wild type contains a peptidase coded by the *BAR1* gene (allelic to *SST1*) which rapidly degrades α-factor (Chan and Otte, 1982; Sprague and Herskowitz, 1981). In *S. cerevisiae* RC629 and 4202-15-3, which are mutants for the *BAR1* protease, there is a 20-fold difference between sensitivity to α-factor versus cyclic α-factor. This value correlates well to the 20 to 40-fold difference in binding of α-factor versus cyclic α-factor to the receptor in strain 4202-15-3. Strain 2180-1A produces the protease. In this case, one has to account for the different stabilities of α-factor and cyclic α-factor towards the protease in addition to their difference in binding. Because cyclic α-factor is more stable than α-factor, the increased relative activity in the growth arrest assay (4-fold less active) as compared to the RC629 (20-fold difference), is expected. Previous structure activity studies had indicated which amino acids in the primary sequence had a major influence on activity. For example, these studies showed that His^2 and Tyr^{13} were essential for activity (Masui *et al.*, 1979). We note that the [Lys^7(Ac),Nle^{12}]α-factor is almost as active as the parent peptide, although it is cleaved more rapidly (Table 2, Fig. 9). This finding definitively proves that a chargeable ε-amine at position 7 of the tridecapeptide pheromone is not essential for activity. Similar results have been reported for dodecapeptide α-factor analogs (Shenbagamurthi *et al.*, 1983). In light

of the more rapid hydrolysis of [Lys^7(Ac),Nle^{12}]α-factor, it is surprising to note that this analog showed a greater difference in activity versus α-factor against the *sst1* mutant strains as compared to 2180-1A. This apparent discrepancy can not be explained.

During recent years, several laboratories have prepared constrained peptides in order to learn about the biologically active conformation of a given peptide hormone (Hill *et al.*, 1988; Baniak *et al.*, 1987). To our knowledge, in every case except one (Sawyer *et al.*, 1983), the constrained peptide had little or no activity as an agonist. A cyclic analog of the tridecapeptide α-melanocyte-stimulating hormone (α-MSH) in which two Cys residues replaced Met and Gly at positions 4 and 10, respectively, yielded a hormone that was 10 to 100-fold more potent in a skin in vitro bioassay and 6-fold more active in vivo in restoring grooming activity (Sawyer *et al.*, 1983). Although the conformational restriction of the [Cys^4,Cys^{10}]-cyclic melanotrophin may lead to the increased activity, the possibility of greater stability or compartmentalization was not ruled out, and direct receptor binding was not assessed. In all other examples of cyclic peptides, the constrained analogs were antagonists. Although antagonists may have profound biological significance, they are not satisfactory probes for the conformation of the active hormone at the receptor. Clearly a full understanding of biological activity requires not only binding to the receptor, but also triggering of the biological response. Although an antagonist binds, it does not trigger. Therefore, antagonists provide insights into the requirements of the binding site but do not give critical information on the orientation of side chain and main chain atoms that are responsible for the biological activity. From this perspective the cyclic α-factor appears to be an extremely exciting molecule. It is an agonist that is biologically active in the same concentration range found for the natural pheromone. A detailed knowledge of its three-dimensional configuration might provide heretofore unavailable information on the "biological active" conformation of a peptide hormone. Finally, the yeast α-factor system has great advantages for conducting physical-chemical studies. One can obtain high

receptor densities and mutations in specific amino acid residues of the α-factor receptor in this model system. The ability to study α-factor bound to its receptor is an exciting potential extension of this work.

CHAPTER 3

TRUNCATED α-FACTOR ANALOGS ANTAGONIZE OR SYNERGIZE BIOLOGICAL ACTIVITY OF THE PHEROMONE: EVIDENCE FOR ALLOSTERIC INTERACTIONS IN THE RECEPTOR

Introduction

Communication between cells is mediated by an elegant information system which involves the specific recognition of effector molecules by membrane-bound receptors and subsequent signal transduction to the intracellular machinery (Lefkowitz and Caron, 1988). The molecular interactions between messenger molecules and their receptors and the pathway of signal transduction remains one of the most intensively studied areas of cellular biology. Such cell to cell communication and intricate signal transduction mechanisms occur throughout nature and have been studied in lower eukaryotes such as *Saccharomyces cerevisiae* as well. Sexual conjugation in *S. cerevisiae* is influenced by diffusible peptides, the **a**-factor and the α-factor (Herskowitz, 1988; Cross *et al.*, 1988) which are recognized by membrane bound receptors (Burkholder and Hartwell, 1985; Nakayama *et al.*, 1985). Based on the sequence of the genes encoding receptors, significant structural similarity is predicted among the yeast α- and **a**-factor receptors and receptors as diverse as rhodopsin and those for β-adrenergic and cholinergic agents (Lefkowitz and Caron, 1988). The yeast signalling system manifests remarkable similarities to hormonal systems of higher eukaryotes not only in the receptor structural motif, but also in the signal transduction through GTP-binding proteins (Dietzel and Kurjan, 1987; Miyajima *et al.*, 1987; Marsh and Herskowitz, 1988; Whiteway *et al.*, 1989; Blumer and Thorner, 1990).

NMR studies in solution (Jelicks *et al.*, 1988) and in lipid (Jelicks *et al.*, 1989), and analysis of the activity of conformationally restricted (Shenbagamurthi *et al.*, 1985) and conformationally constrained analogs (Xue *et al.*, 1989) led to the conclusion that the α-factor may be bent when bound to its receptor. If this is correct, it is possible that this bend directly interacts with the receptor, or that the bend allows residues which are far away in the primary structure to fold into the biologically active conformation (Figure 4, page 10). The latter possibility suggests that residues near the end of the molecule might be critical for activity. In order to probe this possibility, we decided to prepare and evaluate both linear and cyclic analogs of the α-factor that are truncated at either the amine or carboxyl terminus. Our studies led to the discovery of two new α-factor antagonists and of analogs of this pheromone which synergize its activity. These latter compounds represent the first known example where a truncated analog of a biologically active peptide stimulates the activity of the native peptide. The activity of the synergists can be explained in terms of a model whereby an allosteric interaction potentiates pheromonal activity.

Materials and Methods

Organisms, Media and Growth Conditions.

The *Saccharomyces cerevisiae* strains used in this chapter are *S. cerevisiae* 2180-1A *MATa* from the Yeast Genetics Stock Center, Berkeley, CA; *S. cerevisiae* RC629 *MATa sst1-2* (supersensitive to α-factor by virtue of a mutation in the BAR1 protease [allelic to *sst1*] which hydrolyzes α-factor) from R. Chan; and *S. cerevisiae* RC631 *MATa sst2-1* (supersensitive to α-factor because of a mutation in the desensitization response) from R. Chan.

Amino Acid Analysis

The amino acid analysis was performed in the Analytical Services Facility of the University of Tennessee. It involved precolumn derivatization with PITC (phenolisothiocyanate) to make PTC (phenolthiocarbonyl) amino acid derivatives. Hydrolysis was achieved using boiling HCl containing 1% (v/v) phenol. After hydrolysis, the sample was injected on a C_{18} Novapak column (Waters) and analyzed with a 721 data system (Waters).

Fast Atom Bombardment-Mass Spectrometry

FAB-mass spectrum was obtained with a VG ZAB EQ mass spectrometer equipped with a flow FAB ion source of an Ion Tech fast atom gun. Approximately 2 µl of a peptide solution (1.0 nmol/µl) dissolved in acetonitrile/water (2:1), 0.1% trifluoroacetic acid was dispersed on the stainless steel target in a matrix of monothioglycerol. The accelerating voltage of the mass spectrometer was maintained in 8 kV, whereas 8-keV xenon atoms at a discharge current of 1 mA were used to bombard the sample. Spectra were recorded in the "multichannel acquisition" mode from m/z 800 to 1800 at 20 s/decade by the VG 11-250 J data system. Instrument resolution (m/Δm) was set at 2,000. All the truncated peptides exhibited the calculated molecular ion as determined by FAB-mass spectrometry.

Carboxypeptidase A and Amino Peptidase M Digestion

20 units of carboxypeptidase A (Sigma) were incubated with 250 µl of cyclic α-factor (0.4 mg/ml in distilled water) for 3 h or with 100 µl of α-factor (1 mg/ml in distilled water) for 2.5 h at 25°C with shaking. In the case of aminopeptidase M (Pierce), 0.01 units of the enzyme (cross-linked to 4% beaded agarose) were incubated with 500 µl of cyclic-α-factor (0.4 mg/ml in 0.05 M phosphate buffer, pH 7.5) for 6 h or with 200 µl of α-factor (1 mg/ml in 0.05 M phosphate buffer, pH 7.5) for 3 h at 35°C. After incubation

the mixtures were centrifuged through a Centricon 10 filter column (Amicon, with 10,000 molecular weight cut-off) to remove the carboxypeptidase A (molecular weight of 36,000) or the aminopeptidase M (subunit molecular weight of 28,000) and left at -20°C overnight to destroy any remaining enzyme activity. Following removal of enzyme activity, the mixture was fractionated and purified using HPLC. For this purpose, a Waters μBondapak C_{18} column was employed using a water:CH_3CN:TFA gradient from 90:10:0.025 to 40:60:0.025 over 50 minutes. The flow rate was 1.4 ml/min and detection was at 200 nm.

Growth Arrest (Halo Assay)

YEPD plates were overlayed with 4 ml of *S. cerevisiae MAT***a** cells (strains 2180-1A, RC629, and RC631; 2.5 x 10^5 cell per ml) in Noble agar and the assay was performed as described in Chapter 2.

End-Point Shmoo Assay

A culture of *S. cerevisiae* 2180-1A and RC629 was grown at 30°C with shaking to log phase (1 x 10^7 cells/ml) in YEPD. A portion of these cells and α-factor or analog were added to fresh YEPD so that the final concentrations were 1 x 10^6 cells/ml and 0, 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 15, or 20 μg/ml of peptide. The suspensions were incubated at 30°C and after 4 h incubation a fraction was used to microscopically count the number of morphologically aberrant cells (shmoos) and the number of non-shmooing cells. From these results the proportion of shmoos was calculated for each concentration of the peptides tested.

Qualitatively, both the shmoo and the halo assays yielded the same relative order of activities for all α-factor analogs tested. However, the shmoo assay was a more sensitive

assay than the halo assay in that concentration of peptide yielding an effect was always lower in the shmoo assay.

Halo Competition

YEPD plates were overlayed with 4 ml of RC629 cells (2.5 x 10^5 cells/ml) in Noble agar. Filter discs were placed on the overlay, and 10 µl of α-factor (0.1 µg/ml) was pipetted onto the disks. An additional disk was placed so that its center rested at the periphery of the expected halo, and des-Nleu^{12}, Tyr^{13}-linear-α-factor, des-Nleu^{12},Tyr^{13}-cyclic-α-factor or des-Trp^1,His^2-linear-α-factor were added at 2, 10, 20, or 30 X the amount of α-factor. The plates were incubated at 30°C for 24 h and then observed for effects on α-factor activity. Reversal of growth arrest was shown by antagonists; whereas stimulation of growth arrest was shown by synergists.

Competition of Bound [^{3}H]α-Factor by Unlabeled α-Factor Analogs

Competition of bound [^{3}H]α-factor by unlabeled cyclic α-factor was measured as described previously (Raths *et al.*, 1988) with the following changes. The *S. cerevisiae* strain used in the assay was *S. cerevisiae* RC629 (*sst1*). The final assay concentration of the peptides were 5 x 10^{-6}, 2 x 10^{-6}, 1 x 10^{-6}, 5 x 10^{-7}, 2 x 10^{-7}, 1 x 10^{-7}, 5 x 10^{-8}, 2 x 10^{-8}, 1 x 10^{-8}, 5 x 10^{-9}, 2 x 10^{-9}, 1 x 10^{-9}, 5 x 10^{-10}, 2 x 10^{-10}, 1 x 10^{-10}, and 5 x 10^{-11} M for [Nleu^{12}]α-factor, des-Trp^1,His^2-α-factor, or des-Nle^{12},Tyr^{13}-α-factor.

Steady-State Saturation Binding

The procedure followed to obtain steady state saturation binding, was the same as described previously (Raths *et al.*, 1988) with the following changes. The *S. cerevisiae* strain used in the assay was *S. cerevisiae* RC629 (*sst1*). Dilutions of [^{3}H]α-factor (7.5 Ci/mmol) were made in order to achieve assay concentrations of [^{3}H]α-factor: 1 x 10^{-7}, 5 x

10^{-8}, 3 x 10^{-8}, 2 x 10^{-8}, 5 x 10^{-9}, 2 x 10^{-9} and 1 x 10^{-9} M. The synergistic peptide was used at the concentrations of 7 x 10^{-6}, or 1.4 x 10^{-6} M or no synergist.

Recovery from the Shmoo Assay and Detection of α-Factor Degradation Products Produced by the BAR1 Protease

A culture of 2180-1A cells was grown at 30°C with shaking to log phase (1 X 10^7 cells/ml) in YEPD. A portion of these cells and α-factor with or without des-Nle^{12},Tyr^{13}-α-factor were added to fresh YEPD so that the final concentrations were 1 X 10^6 cells/ml, 0.1 or 1 μg/ml of α-factor and 10 μg/ml of des-Nle^{12},Tyr^{13}-α-factor. The suspensions were incubated at 30°C. At 2-h intervals, a fraction was removed and examined microscopically to determine the proportion of shmoos (growth arrested cells). When the products of hydrolysis of α-factor were to be evaluated, the culture was filtered through a 0.45 μm filter to remove the cells, and the filtrate was analyzed by HPLC to determine the stability of α-factor when exposed to *MATa* cells. HPLC analysis was performed using a Waters μBondapak C_{18} column with a gradient mobile phase of 10-60% acetonitrile with 0.025% trifluoroacetic acid.

Results

Chemical Structure Characterization of the Truncated Peptides

The truncated analogs of the linear pheromone: des-Trp^1-α-factor [HWLQLKPGQP(Nle)Y], des-Trp^1,His^2-α-factor [WLQLKPGQP(Nle)Y], des-Tyr^{13}-α-factor [WHWLQLKPGQP(Nle)], and des-Nle^{12},Tyr^{13}-α-factor [WHWLQLKPGQP] as well as the analogous truncations of the cyclo^{7,10}[Nle^{12}]-α-factor were generated by treating the appropriate synthetic peptide (Xue et al., 1989) with either aminopeptidase M or carboxypeptidase A. The shortened fragments could be separated, isolated and purified

using reversed-phase high performance liquid chromatography (HPLC). Fragments of the pheromones were > 99% homogeneous as judged by HPLC and were characterized by both amino acid analysis and fast atom bombardment mass spectrometry (Table 4). Note that the carboxyl terminal truncations showed no tyrosine (WHWLQLKPGQP[Nle]), or, no tyrosine and norleucine (WHWLQLKPGQP), and that the desTrp^{1}His^2-α-factor and desTrp^{1}His^2-cyclo^{7,10}-α-factor showed total absence of histidine. The absence of these residues eliminates the possibility of contamination by the native pheromone.

Biological Activity of Truncated Peptides

The activity of the truncated analogs was tested with a growth arrest assay (as described in chapter 2 of this dissertation and in Xue et al., 1989) against three *MATa* haploid strains which represent both wild type and supersensitive phenotypes. The truncated peptides HWLQLKPGQP(Nle)Y and WHWLQLKPGQP(Nle), each lacking one amino acid, were approximately 10-fold and >40-fold less active, respectively, than the native α-factor against 2180-1A (Table 5). Similar losses of activity were observed for comparable truncated analogs of the cyclic peptide (Table 6). Removal of two amino acids from either the amino or carboxyl terminus of the linear or cyclic pheromone resulted in peptides which were virtually devoid of activity against strain 2180-1A or RC629 ($<10^4$ residual activity) (data not shown). Identical conclusions could be drawn using assay of cell shape as the criterion for biological activity (Fig. 12). In contrast to the results with the wild-type (2180-1A) or the *sst1* phenotype (RC629), all of the cyclic and linear truncations exhibited activity within 2 orders of magnitude of the parent peptide using an *sst2* tester strain (Table 7 and Fig. 12).

To further characterize the truncated analogs we investigated the influence of these peptides on the activity of the linear and cyclic tridecapeptide. As followed using a growth arrest assay, desTrp^1, His^2[Nle^{12}]-α-factor inhibited the activity of the parent compound

Table 4. Chemical Properties of Truncated α-Factor and Cyclic α-Factor Analogs[a].

Analysis	[Nle^{12}]a-factor	Linear α-Factor Derived Peptides				Cyclic α-Factor Derived Peptides			
		des-Tyr^{13}	des-Nle^{12}, Tyr^{13}	des-Trp^{1}	des-Trp^{1}, His^{2}	des-Tyr^{13}	des-Nle^{12}, Tyr^{13}	des-Trp^{1}	des-Trp^{1}, His^{2}
amino acid analysis									
Glu	2.10	1.91	2.00	1.97	1.94	1.88	1.94	1.89	1.92
Gly	1.14	1.16	1.02	1.11	1.00	1.14	1.00	1.06	1.02
His	0.79	0.97	0.89	0.96	0.00	1.01	0.92	0.91	0.00
Leu	1.98	2.08	2.04	1.89	1.87	2.05	2.06	1.95	2.00
Lys	0.88	1.05	0.93	0.88	0.92	1.03	0.97	0.96	0.96
Nle	0.99	0.92	0.00	1.16	1.12	0.91	0.00	1.18	1.18
Pro	2.01	2.01	2.12	2.14	2.03	1.98	2.11	2.12	1.94
Tyr	1.06	0.00	0.00	0.91	0.91	0.00	0.00	0.92	0.98
FAB-MS									
Found		1503	1390	1480	1343	1486	1372	1463	1326
Calculated		1503	1389	1480	1343	1485	1371	1463	1326

[a]The peptides were [Nle^{12}]α-factor, WHWLQLKPGQP[Nle^{12}]Y; des-Tyr^{13}, WHWLQLKPGQP[Nle^{12}]; des-Tyr^{13},Nle^{12}, WHWLQLKPGQP; des-Trp^{1}, HWLQLKPGQP[Nle^{12}]Y; des-Trp^{1}, His^{2}, WLQLKPGQP[Nle^{12}]Y.

Table 5. Halo Assay on des-Trp^1-α-Factor and des-Tyr^{13}-α-Factor

Peptide	2180-1A			RC631 (*sst2*)			RC629(*sst1*)		
(μg/disc)	α-Factor	des-Trp^1	des-Tyr^{13}	α-Factor	des-Trp^1	des-Tyr^{13}	α-Factor	des-Trp^1	des-Tyr^{13}
20	-	-	0	-	-	26	-	-	12
15	20	-	0	-	-	24	-	-	10
10	19	12	0	36	35	21	32	21	0
5	17	9	0	35	33	17	31	18	0
1	10	0	0	29	28	11	23	12	0
0.5	9	0	0	27	24	-	21	10	-
0.1	0	0	0	21	17	0	14	0	0
0.05	0	-	-	14	10	-	11	0	-
0.01	0	-	-	0	0	-	0	0	-

Table 6. Halo Assay on des-Trp^{1}-Cyclic-α-Factor and des-Tyr^{13}-Cyclic-α-Factor

Peptide	2180-1A			RC631 (*sst2*)			RC629(*sst1*)		
(μg/disc)	Cyclic	des-Trp^{1}	des-Tyr^{13}	Cyclic	des-Trp^{1}	des-Tyr^{13}	Cyclic	des-Trp^{1}	des-Tyr^{13}
20	-	0	0	-	22	27	-	0	0
15	14	0	0	-	20	24	-	0	0
10	12	0	0	36	18	22	23	0	0
5	0	0	0	34	14	19	20	0	0
1	0	0	0	28	10	12	12	0	0
0.5	0	0	0	21	-	0	8	0	0
0.1	0	0	0	0	0	0	0	0	0

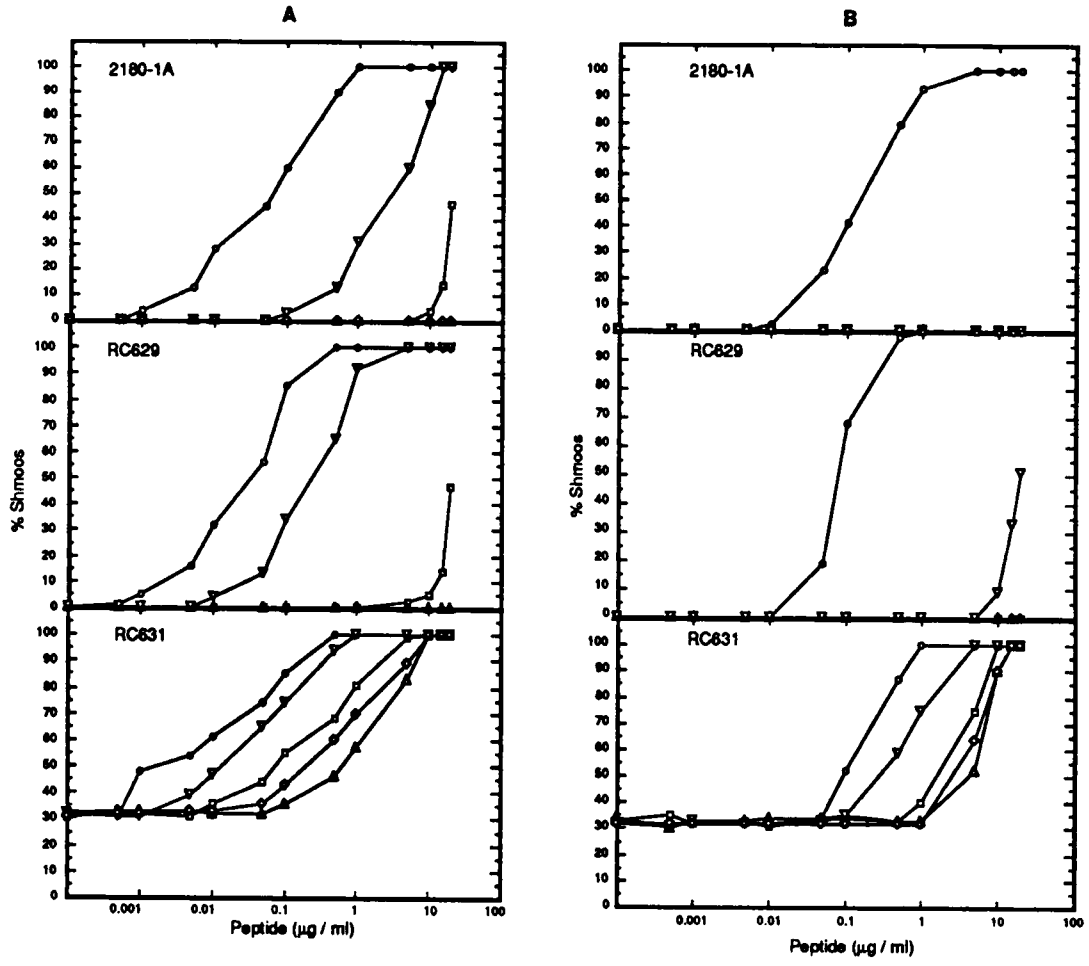


Figure 12. End point shmoo assay. A, Linear peptides; B, Cyclic peptides. The *symbols* represent: [Nle^{12}]α-factor, o, des-Trp^{1}[Nle^{12}]α-factor, ∇, des-Trp^{1},His^{2}-[Nle^{12}]α-factor, ◇, desTyr^{13}[Nle^{12}]α-factor, □, and desNle^{12},Tyr^{13}[Nle^{12}] α-factor, Δ.

Table 7. Halo Assay on des-Trp^1,His^2- and des-Nle^{12},Tyr^{13}-Linear and Cyclic Peptides Using the RC631 (*sst2*) strain.

Peptide (μg/ml)	α-factor	des-Trp^1,His^2 α-factor	des-Trp^1,His^2 cyclic- α-factor	des-Nle^{12},Tyr^{13} α-factor	des-Nle^{12},Tyr^{13} cyclic- α-factor
20	-	-	24	19	18
15	-	-	22	17	15
10	36	28	20	15	11
5	-	25	13	12	10
1	28	19	0	0	0
0.5	26	16	0	0	0
0.1	20	10	0	0	0
0.05	15	0	0	0	0
0.01	0	0	0	0	0

(Fig. 13A). The reversal of the growth arrest, shown as a bean shaped halo in the presence of WLQLKPGQP(Nle)Y in contrast to a completely round one, along with the lack of activity of the undecapeptide demonstrate that the latter peptide is an antagonist. This conclusion is supported by the results of a competition binding assay (Fig. 14) where it is apparent that the des-Trp^1,His^2[Nle^{12}]α-factor competed effectively with α-factor for the receptor binding site. The analogous des$Trp^1$$His^2$-$cyclo^{7,10}$[$Nle^{12}$]-α-factor was also an antagonist although it is not as effective as the linear analog (Fig. 13B).

In the presence of either linear or cyclic WHWLQLKPGQP, the halo due to α-factor is elongated towards the undecapeptide containing disc (Fig. 15A, 15B). This elongation indicates that the analogs lacking the two carboxyl terminal residues increase mating factor activity. The potentiation of pheromonal activity is also seen in the shmoo assay (Fig. 16). At a concentration of WHWLQLKPQP (10 μg/ml), which has no effect on cellular morphology, the dose response of strain RC629 to α-factor shifts by nearly one order of magnitude (Fig. 16). Furthermore, whereas antagonists compete with α-factor for binding to its receptor the synergistic undecapeptide has no effect up to almost 10^{-4} M (Fig. 14). The synergistic activity of the WHWLQLKPQP peptide in strain RC629 is not due to its competition with α-factor degradation by the BAR1 protease (Table 8). The results in this Table show that α-factor is completely degraded after 4 h incubation by cells that produce the protease, whereas no proteolysis is seen at the same time period when the RC629 strain was used. Therefore, we do not expect the synergist to cause its effect by interfering with degradation of α-factor.

The competition for binding assay in Fig. 14 showed that the WHWLQLKPQP undecapeptide does not compete with α-factor for the binding site of the α-factor receptor. The synergist was added up to a 1,000 times the concentration of α-factor under conditions that synergy would have been readily apparent in the shmoo assay. If competition had occured, the curve of binding in the presence of synergist would have shown a downward

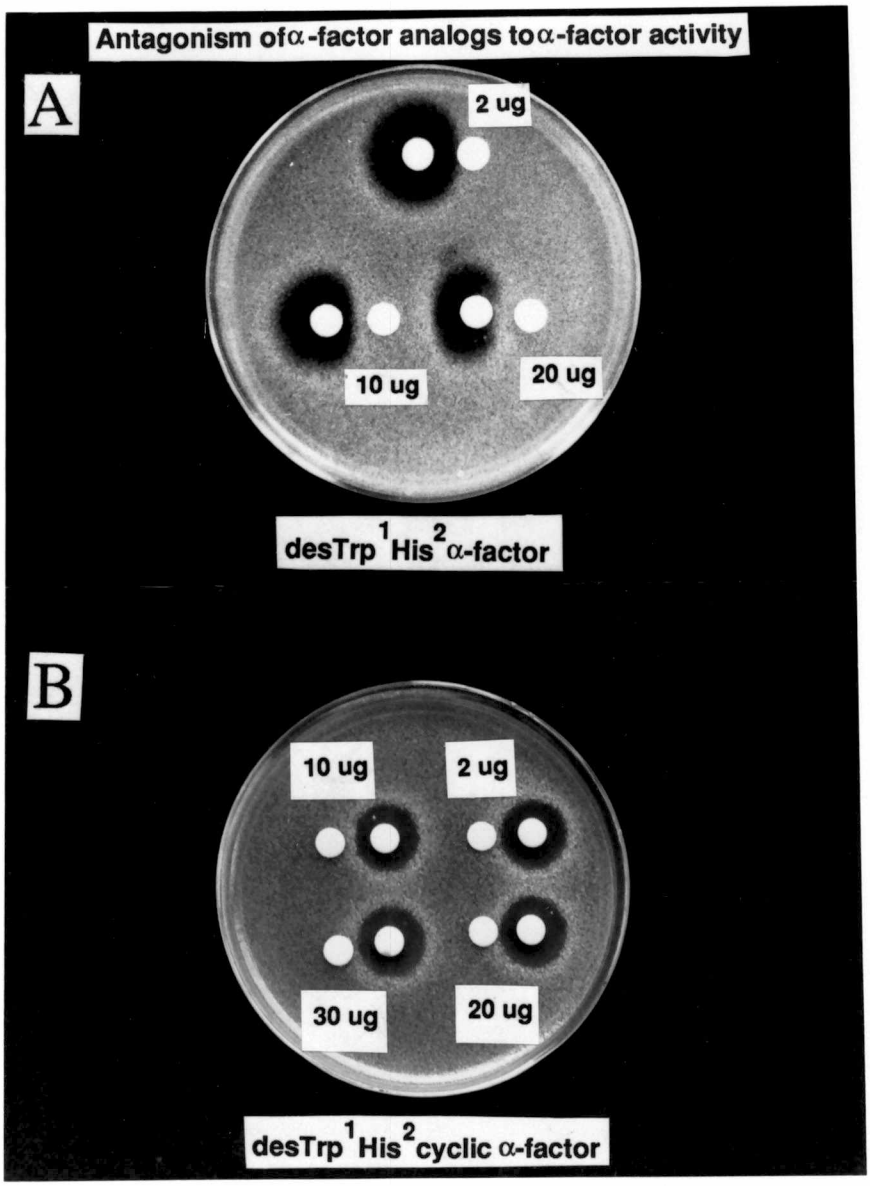


Figure 13. Effect of des-Trp[1],His[2]-α-factor analogs on α-factor activity. A, [Nle[12]]α-factor (1 μg/disk) on the disks that are surrounded by halos and des-Trp[1],His[2]-α-factor at a, 2 μg/disk (2-fold); b, 10 μg/disk (10-fold); or c, 20 μg/disk (20-fold). B, [Nle[12]]α-factor (1 μg/disk) on the disks that are surrounded by halos and des-Trp[1],His[2]-cyclo[7,10]-α-factor at a, 2 μg/disk (2-fold); b, 10 μg/disk (10-fold); c, 20 μg/disk (20-fold); or 30 μg/disk (30-fold).

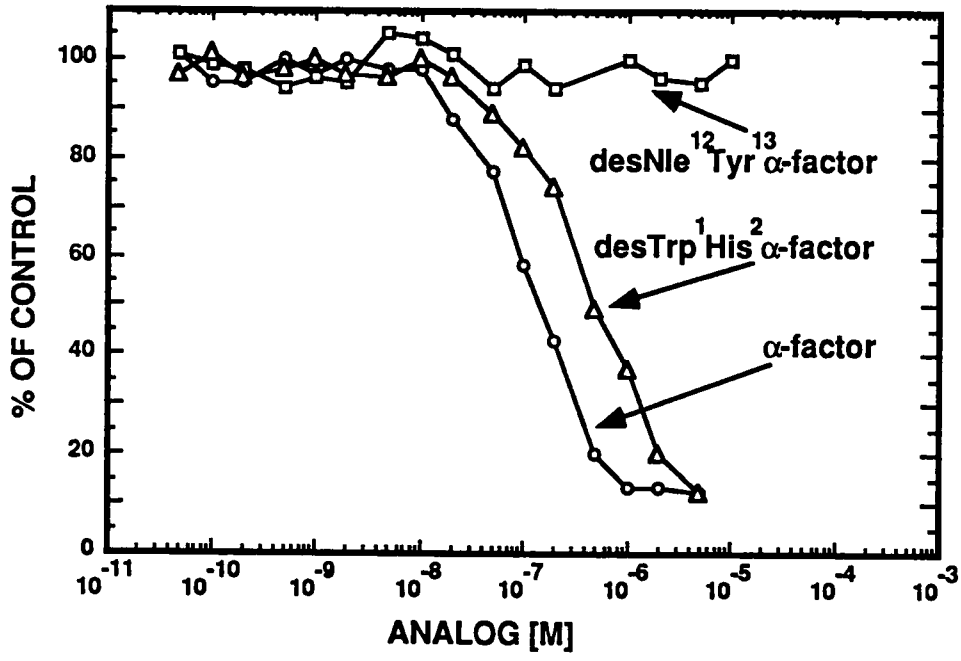


Figure 14. Competition of binding of [^{3}H]α-factor by unlabeled des-Trp^1,His^2-α-factor and des-Nle^{12},Tyr^{13}-α-factor. α-Factor binding to RC629 (*MATa sst1*) cells was performed in competition with [^{3}H]α-factor as described under "Materials and Methods". Data shown are the means of triplicate determinations, with each curve representative of two separate experiments. The *symbols* represent competition of [^{3}H]α-factor by [Nle^{12}]-tridecapeptide, des-Trp^1,His^2-α-factor, and des-Nle^{12},Tyr^{13}-α-factor as indicated in the figure.

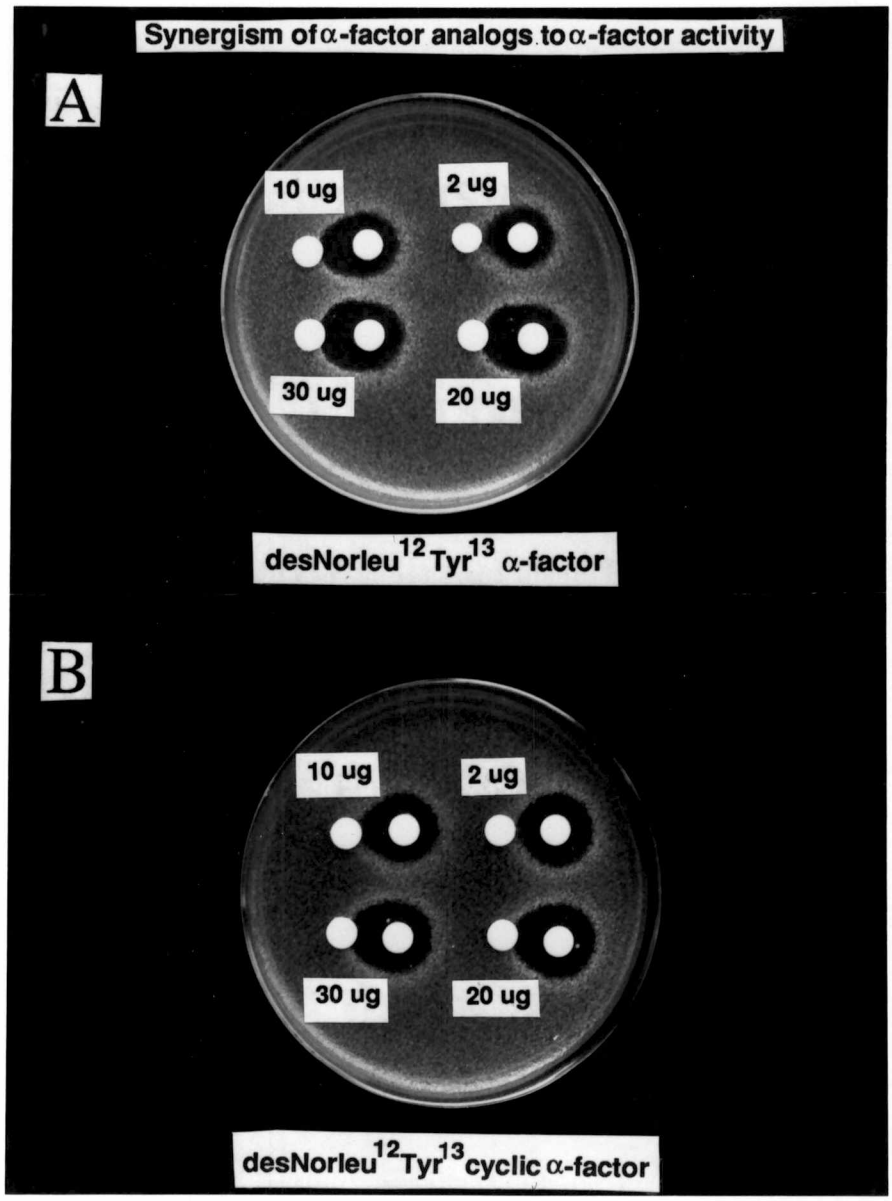


Figure 15. Effect of des-Nle^{12},Tyr^{13}-α-factor analogs on α-factor activity. A, [Nle^{12}]α-factor (1 μg/disk) on the disks that are surrounded by halos and des-Nle^{12},Tyr^{13}-α- factor at a, 2 μg/disk (2-fold); b, 10 μg/disk (10-fold); c, 20 μg/disk (20-fold); or 30 μg/disk (30-fold). B, [Nle^{12}]α-factor (1 μg/disk) on the disks that are surrounded by halos and des-Nle^{12},Tyr^{13}-cyclo^{7,10}-α-factor at a, 2 μg/disk (2-fold); b, 10 μg/disk (10-fold); c, 20 μg/disk (20-fold); or 30 μg/disk (30-fold).

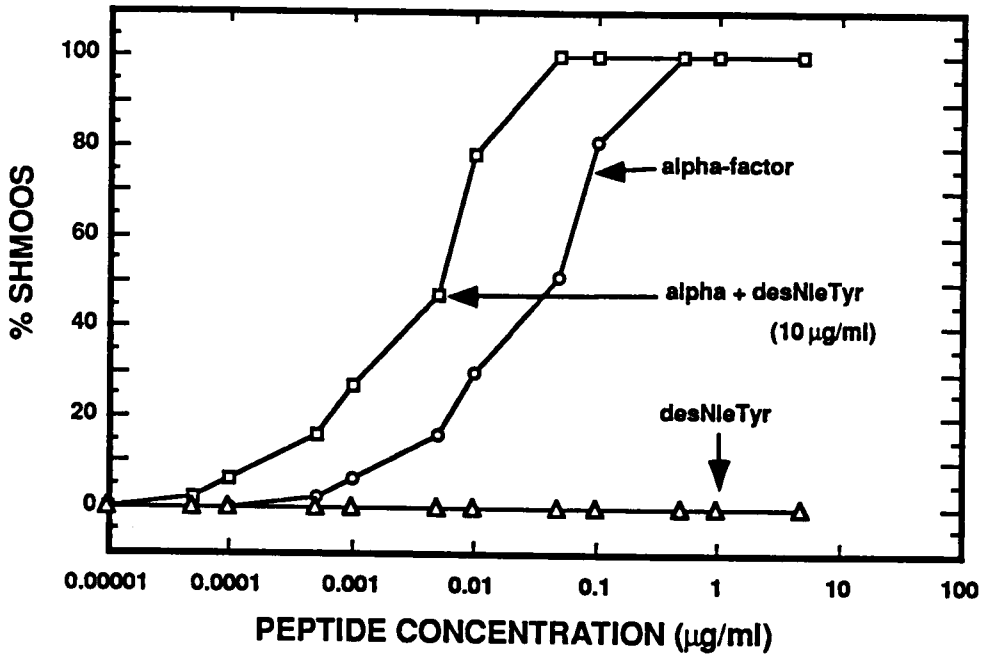


Figure 16. Effect on cellular morphology of RC629 (*MATa sst1*) cells of [Nle^{12}]α-factor in the presence or absence of des-Nle^{12},Tyr^{13}-α-factor.

Table 8. α-Factor Hydrolysis by Wild Type (2180-1A) and Mutant (RC629) Strains of *S. cerevisiae.*

	% of Peak Area (2180-1A)		% of Peak Area (RC629(*sst1*))	
Time (hours)	α-Factor	Degradation Product	α-Factor	Degradation Product
0	100	0	100	0
2	53	47	100	0
4	0	100	100	0
8	0	100	100	0

inflection like the curve of the antagonist (WLQLKPGQP[Nle]Y). However, from this experiment, we could not derive any differences in the affinity of binding of α-factor to its receptor in the presence or absence of the synergistic undecapeptide. To determine whether such a difference exists, we performed a steady state saturation binding assay (Fig. 17). In this experiment the binding of [^{3}H]α-factor was assayed either when used alone or in the presence of two different (but constant for each case) concentrations of the WHWLQLKPQP undecapeptide. As can be seen in Fig. 17, the three curves showing the specific binding of [^{3}H]α-factor to its receptor are superimposable. These results indicate that the affinity of the receptor for α-factor did not change, at least to a level that would be detectable by the experimental conditions used in the assay. In addition the maximal binding of α-factor (molecules bound/cell) would have been diminished in the presence of the synergist if this peptide had bound to α-factor binding sites.

Discussion

The data shown in Chapter 3 demonstrate the importance of residues near the end of the α-factor molecule for its biological activity. Removal of either the amino terminal or the carboxy terminal amino acid reduced the biological activity of α-factor. This is especially true in the case of Tyr^{13}. Results from two independent biological assays (growth arrest and shmoo formation) showed that the biological activity was markedly reduced when Tyr^{13} was removed from the molecule. This reduction in the activity of α-factor was expected as there is a previous report that synthetic des- Tyr^{13}- α-factor was found to have very low biological activity (Masui *et al.*, 1977). It is also known that modifications on the Tyr^{13} amino acid, such as iodination, results in a biologically inactive α-factor (Becker, J. and Naider, F., personal communication).

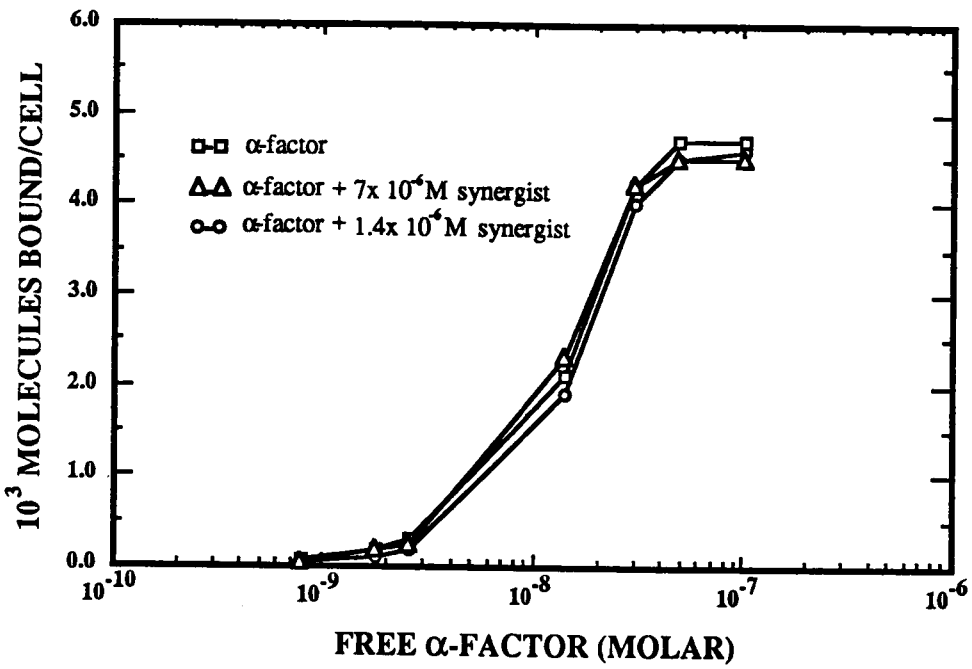


Figure 17. Steady-state saturation binding of [^{3}H]α-factor to a cells in the presence or absence of des-Nle^{12},Tyr^{13}-α-factor. For experimental details, see "Materials and Methods."

Unexpected and interesting results, however, were observed upon analysis of molecules in which two amino acids were removed from either end. The amino terminal truncated peptides were antagonists of α-factor activity. Antagonists can be extremely useful tools when studying the specificity of the ligand-receptor binding and the signalling pathway. For example, the documentation of the existence of spare receptors came from studies of receptor antagonism (Limbird, 1986) and the subdivision of β-adrenergic receptors into β_1 and β_2 and α-adrenergic receptors into α_1 and α_2 subtypes was based on the selectivity of different antagonists in blocking catecholamine effects in a variety of tissues (Berthelson and Pettinger, 1977). Through the use of an α-factor antagonist generated in the laboratories of J. M. Becker and F. Naider, Blumer and Thorner (1990) showed recently that the α-factor receptor was coupled biochemically to GTP-binding proteins. Analysis of the results in this chapter of one of the antagonists (WLQLKPGQP[Nle]Y) demonstrated that it bound with very high affinity to the receptor yet had no biological activity.

The carboxy terminal truncated peptides, on the other hand, potentiated α-factor activity. The synergists did not appear to bind to the α-factor binding site of the α-factor receptor. To our knowledge there is no example in the scientific literature of peptide hormone analogs acting as synergists. The potentiation we observed could result from a variety of molecular interactions.

1. In one explanation, the synergist could compete with degradation of α-factor leading to an apparent increase in α-factor concentration. α-Factor is cleaved by a secreted protease which is the product of the *BAR1* gene expressed in *MATa* strains (Chan & Otte, 1982; Sprague & Herskowitz, 1981). Indeed the synergist increased the time required for strain 2180-1A to recover from α-factor (Fig. 18). However, strain RC629 which exhibits potentiation is devoid of this peptidase (Chan & Otte, 1982). Furthermore, direct peptidase assays showed that in a four hour time period using 10^6 cells/ml no α-factor was cleaved

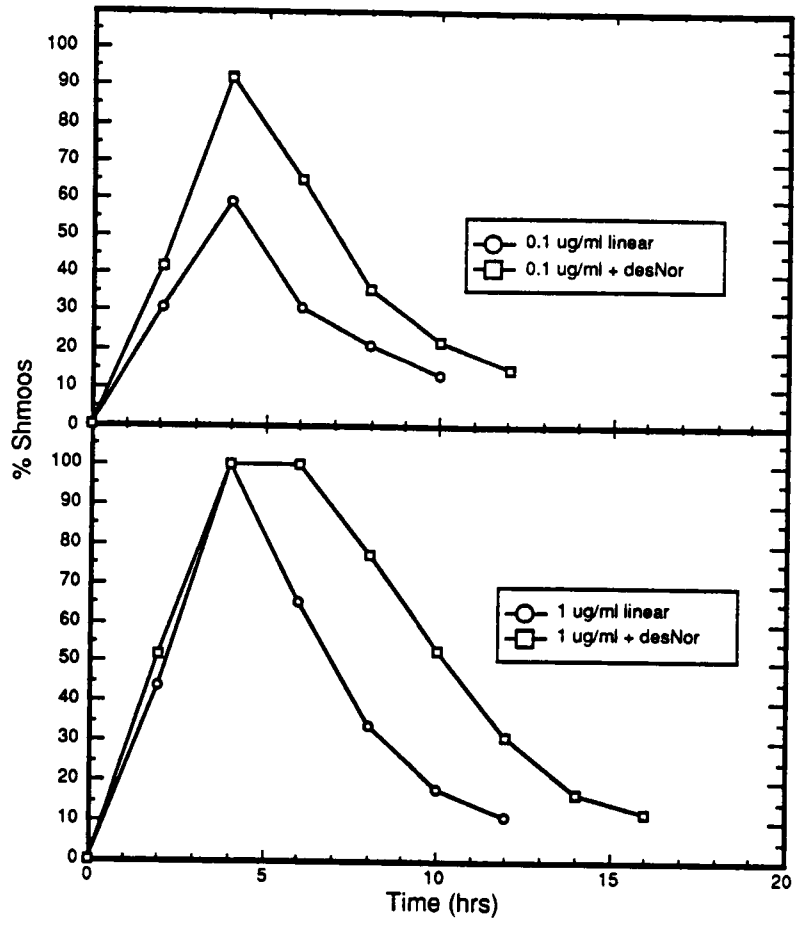


Figure 18. Recovery of cells from α-factor treatment. A culture of *S. cerevisiae* 2180-1A cells was grown at 30°C with shaking to log phase (1 X 10^7 cells/ml) in YEPD. A portion of these cells and α-factor with or without des-Nle^{12},Tyr^{13}-α-factor were added to fresh YEPD so that the final cell concentration was 1 X 10^6 cells/ml. The suspensions were incubated at 30°C and at 2 h intervals, fractions was removed and examined microscopically to determine the proportion of shmoos. A, 0.1 μg/ml of α-factor with or without 10 μg/ml des-Nle^{12},Tyr^{13}- α-factor. B, 1 μg/ml of α-factor with or without 10 μg/ml des-Nle^{12},Tyr^{13}- α-factor.

by RC629 whereas 100% of the pheromone was cleaved by an identical preparation from 2180-1A. The peptidase assay conditions reflect those found in the shmoo assay and we conclude that potentiation by the synergistic peptide is not due to competition with secreted protease.

2. It is possible that the synergist interacts with either the α-factor binding site on the α-factor receptor or another site on either the receptor or another protein. Such interaction could result in an allosteric potentiation of pheromonal signalling through either conformational alteration of the receptor or receptor oligomerization. There are several examples in the literature where it is believed that one of these phenomena is occuring. Limbird and Lefkowitz (1978) have speculated that in the presence of agonist the conformation of the β-adrenergic receptor changes. In 1979, Weiland *et al.* added that this conformationally altered form of the receptor is what interacts directly or indirectly with an intracellular messenger to cause activation. Schlessinger (1988) postulated that the EGF receptor forms oligomeric structures in the presence of agonist with a resulting higher affinity for ligand of oligomerized receptor than that of the monomeric receptor, whereas Mayo *et al.* (1989) postulated that EGF binds to both the receptor and another protein. The resultant hypothetical dimerization caused positive cooperativity to "draw in more receptor/ligand binding". Based on the Schlessinger and Mayo models, we can predict that the synergist binds to the same site as α-factor (with very low affinity) or to some other site of the receptor or another protein, causing the formation of oligomers . Oligomer formation alone does not trigger any response and so the synergist does not exhibit biological response by itself. However, oligomer formation changes the state of the receptor, so that when α-factor and synergist are present, the response is greatly enhanced. Indeed, in a study where α-factor was crosslinked into the receptor there was some evidence for multimeric receptor on acrylamide gels (Blumer *et al.*, 1988).

The binding competition studies suggest that the synergist does not bind to the same site as the native pheromone. However, we can not completely rule out the possibility that the synergist binds to the same receptor site with very low affinity, such that even at very high concentrations of the synergist we can not detect competition . To determine whether the synergist exerts its effect through a change of the affinity of the receptor, we measured the affinity of α-factor receptor for its ligand in the presence and absence of synergist (Fig. 17). Under the experimental conditions used we could not detect any change in the affinity of the receptor for its natural ligand in the presence of the synergist. The results from both binding experiments suggest that the effect of the synergist does not occur through binding to the α-factor binding site nor by increasing the affinity of the receptor for its natural ligand. However, it is difficult to imagine that the receptor or the yeast cell contains binding sites, specifically, for truncated peptides because contact with these molecules would not be expected under natural *in vivo* circumstances. Thus, we are led to conclude that the truncated peptides may interact with an additional site for α-factor either on the receptor or some other regulatory protein which has very low affinity for α-factor. The failure to see interaction in binding studies reported to date may be due to the difficulty found in whole cell and membrane based assays. A more conclusive experiment is required to test the possibility that the synergist binds with very low affinity to α-factor binding sites (such as it is undetected with the current assays). One way to test this hypothesis is to examine whether labeled synergist could be competed out by unlabeled α-factor.

All post-receptor events in both the pheromone response and recovery pathways appear to be common in both **a** and α-cells of *S. cerevisiae* (Bender and Sprague, 1986; Nakayama *et al.*, 1987; Blinder and Jenness, 1989). There is genetic and biochemical evidence that the pheromone signal transduction mechanism in *S. cerevisiae* is mediated by a guanine nucleotide binding protein complex (G-binding protein) (Dietzel and Kurjan,

1987; Miyajima *et al.*, 1987; Marsh and Herskowitz, 1988; Whiteway *et al.*, 1989; Blumer and Thorner, 1990). These proteins have been shown to serve as intermediates in the transmembrane signaling of several receptors (Casey and Gilman, 1988; Dohlman, *et al.*, 1987). They are composed of three different subunits (α, β and γ) (Fig. 3, page 8) which in the case of *S. cerevisiae* are encoded by the *SCG1* (or *GPA1*), *STE4* and *STE18*, genes, respectively. The GTP/GDP binding domain is found on the α-subunit. When a ligand binds to its receptor GDP is exchanged for GTP and the signal pathway is activated. It is generally accepted that this activation involves the disssociation of the α-subunit from the β-γ complex (Bourne, 1989). The signal is terminated when the GTP is hydrolyzed to GDP.

The RC631 strain used in this study is an *sst2* mutant. The *SST2* gene product has been only shown to be involved in recovery (Chan and Otte, 1982; Dietzel and Kurjan, 1987; and Blinder and Jenness, 1989) and it is thought to act through a mechanism independent of the recovery pathway controlled by the *SCG1* gene product (Blinder and Jenness, 1989). Mutants of the *SST2* gene are supersensitive to pheromone and the cells do not recover for prolonged periods of time even after removal of the pheromone. Furthermore, it appears that the *STE4* gene product requires the *SST2* gene product for function indicating the possibility that *SST2* may be involved at the G-binding protein level. Our results show that the *sst2* strain (RC631) does not follow the pattern of response that is shown by the wild type or the *sst1* mutant. Strain RC631 responds to all antagonists and synergists we tested as if they are agonists, although the response is not as great as that seen for α-factor. Based on our results and the model proposed by Blinder and Jenness (1989) we are tempted to give an additional function to the *SST2* gene product. In this scheme (Fig. 19), the *SST2* gene product acts independently of the *SCG1* (*GPA1*) to transfer the signal to and control the *STE4* gene product function. If an agonist binds to the receptor the signal is normally transferred through both *SST2* and

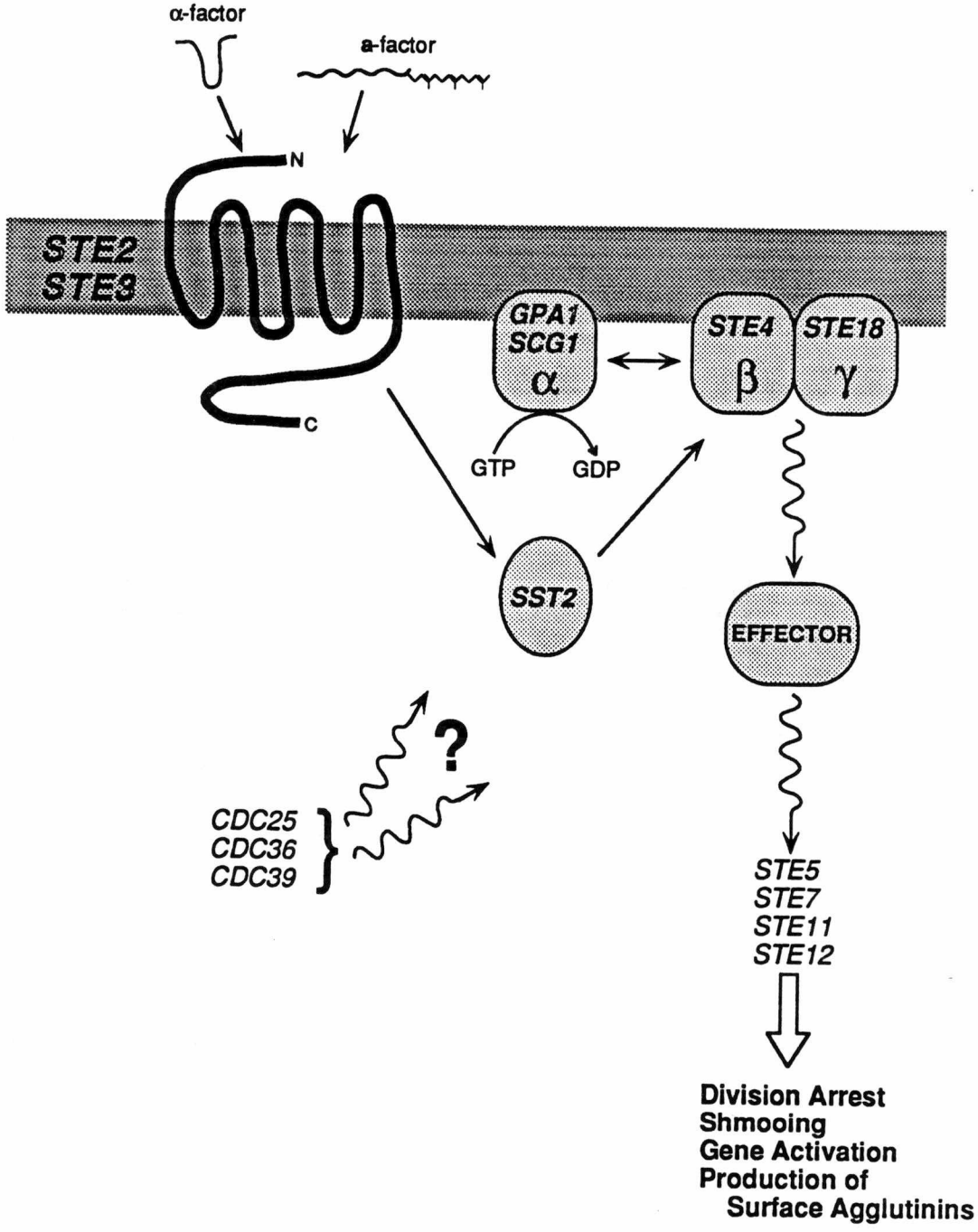


Figure 19. Model of the possible function of the *SST2* gene product in the pheromone response pathway.

SCG1 gene products to the *STE4* gene product. The wild type *SST2* gene product transfers the information to *STE4* gene product only if the substance is an agonist whereas the information from SCG1 is transfered to *STE4* gene product by agonists, antagonists and synergists we have generated. Dissociation of the G-binding protein subunits, however does not occur unless both *SST2* and *SCG1* gene products have interacted with the *STE4* gene product. In the case of *sst2* mutant there is no functional *SST2* gene product to "proof-read" the signal. Thus, even pheromone analogs that in wild type cells do not have any biological activity exhibit biological response in *sst2* cells.

In summary, we have shown that the ends of the α-factor molecule are important for its biological response. Truncations of two amino acids at either end of the molecule generate peptides which elicit no biological response in the wild type or the *sst1* mutant, but which exhibit either antagonistic or synergistic activity when tested in combination with α-factor. We believe that the synergistic activity seen is either due to an allosteric interaction mediated through the α-factor binding site or a receptor binding site other than that for α-factor. The fact that we were not able to observe effects of synergists in the binding assays we performed is attributed to the very low affinity exhibited by the synergist. Further research is necessary to dissect the exact mechanism of action of the synergism and the role of the *SST2* gene product in the signal transduction pathway.

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