To the Graduate Council:

I am submitting herewith a dissertation written by Michael Gregory Abel entitled "Structure/function anaylsis of the *Saccharomyces cerevisiae* α-factor receptor (the *STE2* gene product) and its tridecapeptide ligand (the α-factor pheromone)." 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.

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Associate Vice Chancellor and
Dean of The Graduate School

# Structure/function analysis of the *Saccharomyces cerevisiae* α-factor receptor (the *STE2* gene product) and its tridecapeptide ligand (the α-factor pheromone)

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Michael Gregory Abel

December 1996

# Dedication

I would like to dedicate this dissertation to my parents, *Dr. Michael Abel* and *Dr. Peggy O'Neal Abel*, and to my two brothers, *Roger* and *Randy Abel*. I was only able to achieve this lofty goal by utilizing the strength and fortitude gained through the examples set by my parents. Thank you Mom and Dad, for the love and support that I always knew was there.....I love you. Thank you Roger and Randy, for the lives we shared together growing up and the new found relationships that we share now. I always hope for happiness and security for both of you.

I would also like to dedicate this work to my new family, *Anne* and *Wesley*, for the happiness they have brought into my life, I love you both. Thank you Anne for riding the roller coaster with me for the rest of your life and for your intense and unquestioning love. Thank you Wesley for your love and friendship and always making me proud. I will always try my best to make the two of you happy and I will always be your best friend and lobster.

## Acknowledgments

I cannot adequately express my feelings to the people that I have come in contact with during my time here at the University of Tennessee. The graduate experience in and of itself is an extremely difficult time of learning and enlightenment both on a professional as well as a personal level. Fate chose this point in my life to also test my mettle with a variety of personal traumas that made me, for the first time in my life, question whether I would be able to finish something that I started. I can say now, finally arriving at the end of my graduate career and looking ahead to the next period in my life, that I have accomplished this not solely by myself but with the help and support of the people I would like to acknowledge in print.

First, I would like to thank the members of my dissertation committee, *Dr. David Brian*, *Dr. Stuart Riggsby*, and *Dr. Bruce McKee*, for their critical reviews of my work and a special thank you to *Dr. Fred Naider* of the College of Staten Island for his support and interest in my research as well as my career. It has been a great pleasure to know Dr. Naider and to learn from such a driven, pure scientist; two heads <u>are</u> better than one.

I would like to thank the members of the Becker lab, both past and present, that have each in their own way helped shape me as a researcher. Thanks to *Dr. Munira Basrai*, *Dr. Kumar Alagramam*, *Dr. Steve Marcus*, *Dr. Mark Mason*, *David Miller*, *Dr. Melinda "the baby machine" Hauser*, *Keith "computer help desk" Henry*, *Jeff "Wilgus" Wiles*, *John "Wiles" Wilgus*, *Jingu "epitope" Gang*, and *Greg "Goober" Anderson*. I would also like to thank the ladies of the Microbiology department, *Paula Keaton*, *Carole Vosdingh*, and *Frances Cox* who were always quick with a smile and always

able to help me with any question or request (thanks for the paper and verbal sparing matches too, Francis!). I am grateful to *Angus "slacker/hemocytometer stockholder" Dawe*, *Mark "slacker/human megaphone" Lubkowitz*, and *David "slacker/looooooser" Barnes* for their unique personalities and insights. I have greatly enjoyed the many scientific and nonscientific conversations, beer outings and parties, as well as the total remorseless carnage heaped onto the carcass boy. I sincerely hope to be able to maintain both a personal and professional relationship with each of you.

A hearty THANK YOU goes to *Hui-Jen Lu* for all of her diligent help and insights that can be found in a majority of the work in this publication. Lu has been my right hand woman for quite a while and I could not have made it to this point without her; I owe her a large debt of gratitude.

I must acknowledge my good friend, collegue, and fellow α-factor compadre', *Dr. Angela McKinney*, for her perpetual good mood and infectious laugh. I know we will be good friends from now on (or I will have to go to Pennsylvania and start spreading rumors about her).

I want to acknowledge my friend *Dr. Peggy Maher* and wish her the best things that life has to offer. Even though we will not spend our lives with each other, I will always look back on our time together with great love and fondness and look forward to hearing about her great achievements.

I could not possibly express in words the thanks I have for *Dr. Guy Caldwell*. More than anyone could possibly know, his friendship has helped me through this entire experience and made me a better person; "I love you.....man!". I do not know of a finer scientist and truthfully hope to accomplish just a fraction of what he will accomplish in his career; I will be

extremely successful if I can. Thank you for sharing the distractions of pool, fantasy baseball, softball, and all the *in vivo* yeast metabolism byproduct studies which helped me maintain my sanity and cheery disposition (Go 'boys!). Thank you to *Dr. Kim Caldwell* for her friendship and support as well, I hope all of us will always find the time to share the ups and downs of our lives.....thank you two from the bottom of my heart.

Finally, I wish to offer my greatest thanks and appreciation to *Dr. Jeff Becker* for his patience and friendship during the highs and lows of my graduate career and personal life. He allowed me a loose rein to learn the foundations of good science but provided a constant critical critique of my work. His unwavering confidence in me and my ability gave me the fortitude to press on and grow and his door was always open when I needed. I sincerely want to thank him for training me as a complete scientist and allowing me to become so intimately involved in all aspects of research. He entrust me with the writing of papers and grants, the free development of my research project, and allowed me the experience to become a good editor, speaker, and educator. I enjoy and respect my profession because he always showed me that, even in the face of bad results and shredded submissions, science was a passion and adventure to be attacked with enthusiasm and conquered.

The examples set by Dr. Becker and Dr. Caldwell are the reasons I am the scientist I am today and I can only praise them the best way I know how, by trying to be the best scientist I can by emulating them. I thank them both and am honored that I can call them both my friends.

# Abstract

During the life cycle of the yeast *Saccharomyces cerevisiae*, haploid cells of opposite mating type can fuse during sexual conjugation to form a diploid cell. In preparation for conjugation, haploid cells secrete small diffusible peptide molecules [α-factor, a tridecapeptide pheromone and **a**-factor, a modified dodecapeptide pheromone] that bind specifically to cell surface receptors found on the opposite mating type cell.

The basic structure of the receptors (Ste2p for α-factor and Ste3p for **a**-factor) is evolutionarily conserved and places them among the 7-transmembrane, G-protein coupled super-family of hormone receptors. Part I of this dissertation is an overview of the life and times of the yeast *Saccharomyces cerevisiae* as it relates to this work.

Part 2 of this dissertation is a specific study of one residue of the α-factor pheromone. This section describes the possible function of the $His^2$ side chain of the α-factor and its contributions to binding to the α-factor receptor and propagation of the extracellular mating signal. While conclusions drawn from assays performed on the altered position 2 side chain analogs indicate that the $His^2$ residue is not essential for binding or biological activity alone, this residue does contribute to the overall efficient transduction of the mating signal.

Part 3 of this dissertation takes a broad look at the entire α-factor pheromone by alanine-scanning the entire peptide. Both the *L*- and *D*-isomers of alanine were synthesized in place of each natural amino acid to produce an Ala-scanned "library" of α-factor analogs. Biological and binding assays performed with these peptides on cells expressing the wild-

type α-factor receptor led us to propose functional domains of the peptide that play dominant roles in signal transduction, receptor interaction, and stabilization of the biologically relevant structure of the α-factor itself.

In Part 4, an analysis of the α-factor receptor is carried out using random and site-directed mutagenesis to try to pinpoint those residues critical for pheromone binding and signal transduction. Receptors with random mutations were generated and screened for biological response to antagonist peptides. Mutant receptors containing multiple mutations that respond to antagonist molecules were identified by sequencing as candidates with residues responsible for association with the ligand and/or propagation of the mating signal. These candidate residues were targeted for site-directed mutagenesis and tested for an antagonist responsive phenotype. Four mutations were independently introduced into the α-factor receptor. One mutant receptor (S259P) demonstrated ligand dependent biological response to all peptides tested. The S219P mutation demonstrated biological response to α-factor, some antagonist peptides and the synergist, but not to other antagonists. The F55V mutant receptor responded only to α-factor and the synergist peptide and not to any antagonist analogs. The last mutant receptor (I263V) only responded to α-factor.

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# PART 1

General Introduction

# CHAPTER I

## An overview of cellular communication in the yeast *Saccharomyces cerevisiae*

The yeast *Saccharomyces cerevisiae*, commonly referred to as "Baker's yeast", is a unicellular organism that exists as either a haploid or diploid cell (Fig. 1). This eukaryote has been used quite extensively as a research tool and model system to gain insights into different aspects of cellular function including protein trafficking (Pryer, et al., 1992; Marcus, et al., 1990), cell cycle regulation (Chang & Herskowitz, 1990), receptor-ligand interactions (Levin, et al., 1993; Raths, et al., 1988; Marsh, 1992; Houen, et al., 1996), signal transduction (Clark, et al., 1994; Stephan & Blumer, 1994), and signal adaptation (Chen & Konopka, 1996; Weiner, et al., 1993). This field of research involves many investigators examining different components of the cellular communication puzzle. However, the molecular determinants between the receptor and ligand, and critical residues involved in the transduction of the mating signal, have not yet been fully ascertained. This body of work has attempted to answer some of the questions concerning the interaction(s) and signal propagation mechanisms utilized by peptide pheromones and their receptors.

The use of *S. cerevisiae* as a model system has many advantages: (1) yeast genetics, physiology, and biochemistry are well documented in the literature and are well established fields with many tried and proven techniques useful for the manipulation and study of yeast; (2) yeast is easily

**Figure 1 :** The life cycle of the yeast *Saccharomyces cerevisiae*. Two haploid cells that unite to form a diploid cell differ in mating type (black and white circles represent the nuclei of opposite mating types called **a** and $\alpha$). Under favorable growth conditions, diploid cells reproduce asexually by budding. When such a cell is deprived of essential nutrients, however, it undergoes meiosis within a saclike structure called an ascus, and the yeast chromosomes assort independently in Mendelian fashion to yield four haploid ascospores. If the ascus is returned to favorable growth conditions, pairs of spores of opposite mating type within it may fuse to form diploid cells, which will proceed to grow and bud as before. On the other hand, each ascospore can remain haploid and can give rise to a haploid colony. Haploid cells of different mating types produce mating pheromones that bind to cell surface receptors on opposite mating type cells and initiate the mating response. Two cells of opposite mating type that have been induced for sexual conjugation by the mating pheromones can fuse and produce diploid prodigy which continue vegetative growth (adapted from Darnell, et al., 1990).

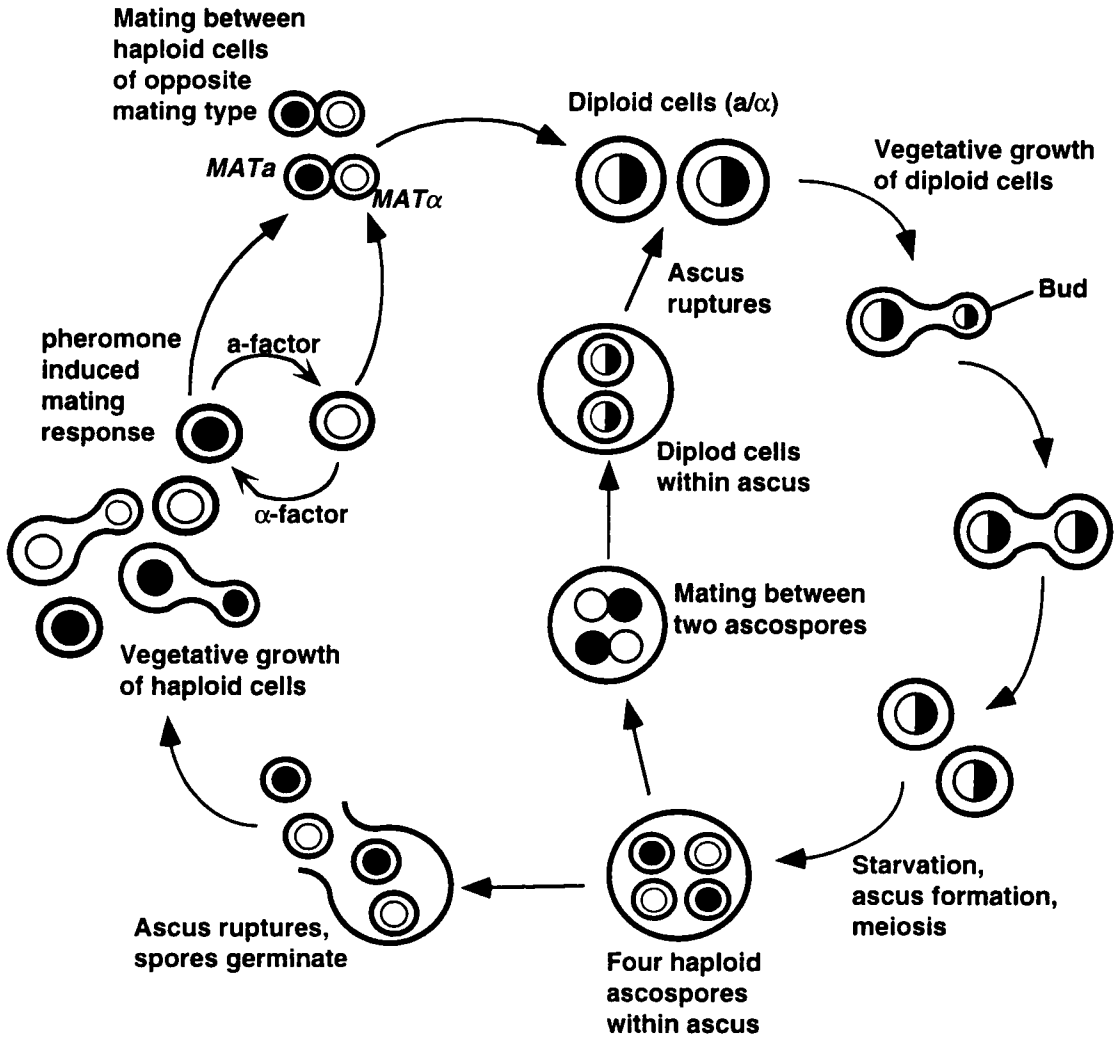



**Figure 1 :** The life cycle of the yeast *Saccharomyces cerevisiae*. Adapted from Darnell, et al., 1990.

handled and genetically manipulated; (3) powerful molecular cloning techniques such as gene disruptions are available for use; (4) yeast can exist in either a haploid or diploid state; (5) yeast are inexpensive to maintain and grows rapidly; (6) yeast are eukaryotes sharing fundamental properties of cell biology as well as a high degree of conservation of proteins with multicellular eukaryotes; (7) there is a high degree of genetic traceability in yeast (Botstein & Fink, 1988) and recently; (8) the entire genome has been sequenced (see the *Saccharomyces* Genome Database). There are a variety of citations and review articles that support this model system (for reviews see Sprague & Thorner, 1992; Marsh, 1991; Kurjan, 1992; Jeansonne, 1994).

The action of hormones (molecules secreted by organs of an animal which act on other tissues or organs in the same animal) and pheromones (molecules secreted by an organism which act on another organism) upon binding to their respective receptors is one of the initial events involved in cell to cell communication. Secreted peptides regulate many cellular functions through the action of receptors found on the surfaces of their target cells. In recent years, with the advances in molecular biology and the understanding of cellular function in terms of molecular properties, it has become apparent that receptors are an extremely important mediating component of cell behavior, such as growth, secretion, contraction, motility, adhesion, and mating.

One important group of signal transducing receptors is the structurally conserved 7-transmembrane, G-protein coupled receptors (7TGCR, Fig. 2, for review see Strader, et al., 1995). The ever growing family of G-protein coupled receptors exhibit conserved structural and

**Figure 2 :** The basic structure of 7-transmembrane G-protein coupled receptors. The receptor (far left) contains hydrophobic α-helical regions that pass back and forth through the plasma membrane 7 times resulting in an extracellular $NH_2$-terminus and a cytosolic COOH-terminus. The receptor is associated with a heterotrimeric (α, β, γ subunits), guanine-nucleotide (GDP) binding protein (G-protein). The α-subunit of the G-protein is *N*-myristoylated (****) while the γ-subunit is modified by a farnesyl group (++++) that is required for function.

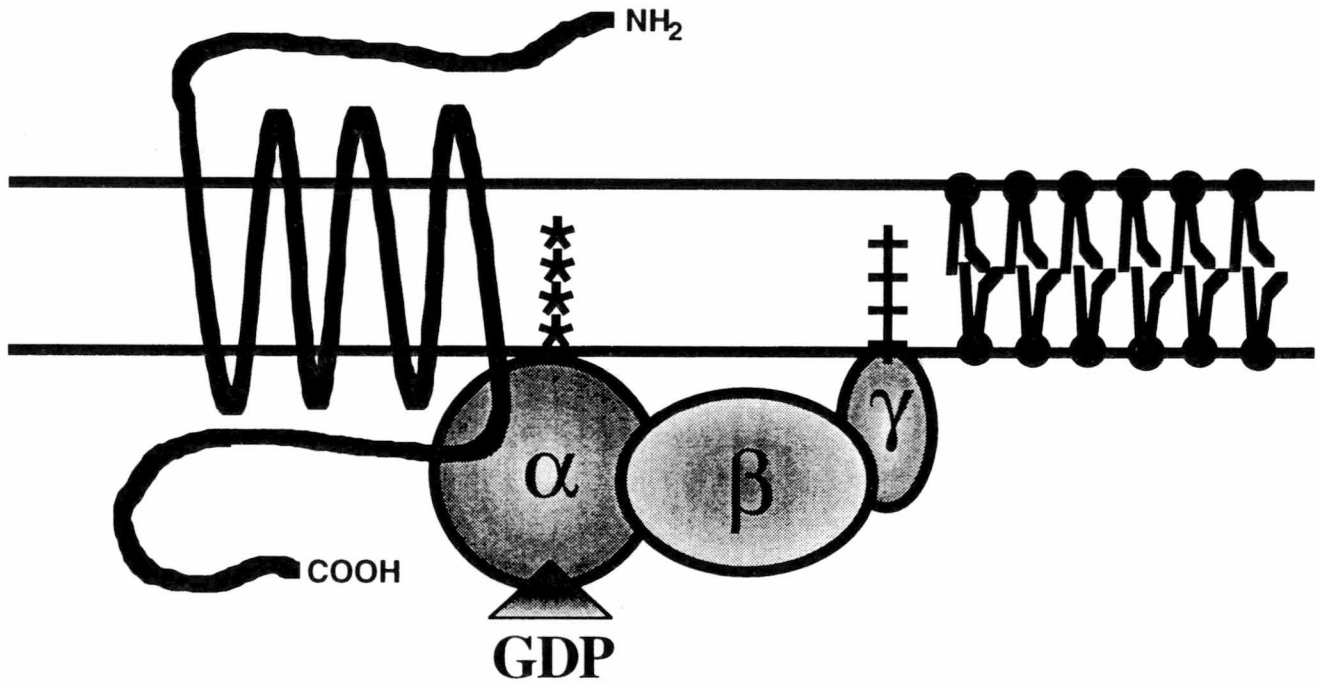



**Figure 2 :** Basic structure of 7-transmembrane G-protein coupled receptors

topological features, most notably, an extracellular amino terminus, intracellular carboxy terminus, and seven transmembrane spanning domains (Findlay & Pappin, 1986; Findlay & Eliopoulos, 1990). Due to the inherent difficulties in obtaining accurate diffraction patterns of membrane protein crystals and the subsequent lack of x-ray crystallographic data, this predicted structure of the 7TGCR family was initially based on electron diffraction (Henderson & Unwin, 1975) and limited proteolysis studies of bacteriorhodopsin (Ovchinnikov, et al., 1979). Since then, more and more evidence has confirmed this structural motif in a variety of cloned receptor proteins (Baldwin, 1993).

Some of the members of this 7TGCR super-family include the α- and β- adrenergic receptors, gonadotropin-releasing hormone receptor, rhodopsin, bacteriorhodopsin, serotonin receptor, dopamine receptor, substance P and K receptors, and muscarinic receptor, as well as the **a**- and α-factor receptors found in yeast. The deduced amino acid sequences of the genes encoding these receptors exhibit many structural similarities (O'Dowd, et al., 1992), indicative of a common structural theme. This structural homology likely parallels similarities in function and regulation and allows investigators to compare experimental data elucidated in one member of the family to other members.

Many receptors act through G-proteins in mammalian systems (Iyengar & Birnbaumer, 1990; Dohlman, et al., 1991) and G-protein coding genes have been identified in a variety of eukaryotes (Ma, et al., 1990; Fino-Silva & Plasterk, 1990; Yarfitz, et al., 1988; Kumagai, et al., 1989; Dietzel & Kurjan, 1987). Most of the research attention on this signaling system in yeast has focused on the receptor/G-protein coupling. The G-

proteins found to be associated with this class of receptors are heterotrimeric guanine nucleotide binding proteins located on the inner surface of the plasma membrane. The α-subunit ($G_\alpha$) specifically binds guanine nucleotides and has been shown to be *N*-myristoylated and membrane associated (Blumer & Thorner, 1990; Stone, et al., 1991). Myristoylation is required for activity of $G_\alpha$ but apparently not for its membrane association (Stone, et al., 1991). The β- and γ-subunits form a tightly linked dimer ($G_{\beta\gamma}$) and purify as a complex (Stryer & Bourne, 1986). In mammalian systems, the $G_\alpha$ subunit is the signaling component of the G-protein (Linder & Gilman, 1992) but in yeast, it has been shown that the Gβγ dimer carries out the propagation of the mating signal (Nakayama, et al., 1988a; Whiteway, et al., 1989). The Gγ subunit is modified by a farnesyl ($C_{15}$) group which is required for its function (Nakayama, et al., 1988b; Finegold, et al., 1990) and appears to facilitate interaction of the $G_{\beta\gamma}$ dimer with membranes (Muntz, et al., 1992). Upon ligand binding, a putative conformational change takes place causing receptor stimulation of the G-protein, catalyzing the release of GDP from the α-subunit and replacement with GTP (Blumer & Thorner, 1991). In yeast, the βγ subunit dimer is released from the α subunit and regulates downstream effectors propagating the mating cascade signal.

Cellular communication between the haploid mating types of *S. cerevisiae* is initiated by the reciprocal action of diffusible peptide pheromones **a**- and α-factor which specifically bind to mating receptors (Ste2p, Ste3p) expressed on the cell surfaces of *MATα* and *MAT**a*** cells, respectively (Fig. 3; for review see Sprague & Thorner, 1992). The α-factor receptor (Fig. 4) is encoded by the *STE2* gene and expressed on *Mat**a***

**Figure 3:** Cellular communication between mating types. Haploid cells of opposite mating type (*MAT***a**, *MAT*$\alpha$) of the yeast *Saccharomyces cerevisiae* secrete diffusible peptide pheromones (**a**-factor, $\alpha$-factor) which bind to 7-transmembrane G-protein coupled receptors (Ste3p, Ste2p) expressed on the cell surface. Binding of the peptides to their respective receptors initiates the mating response.

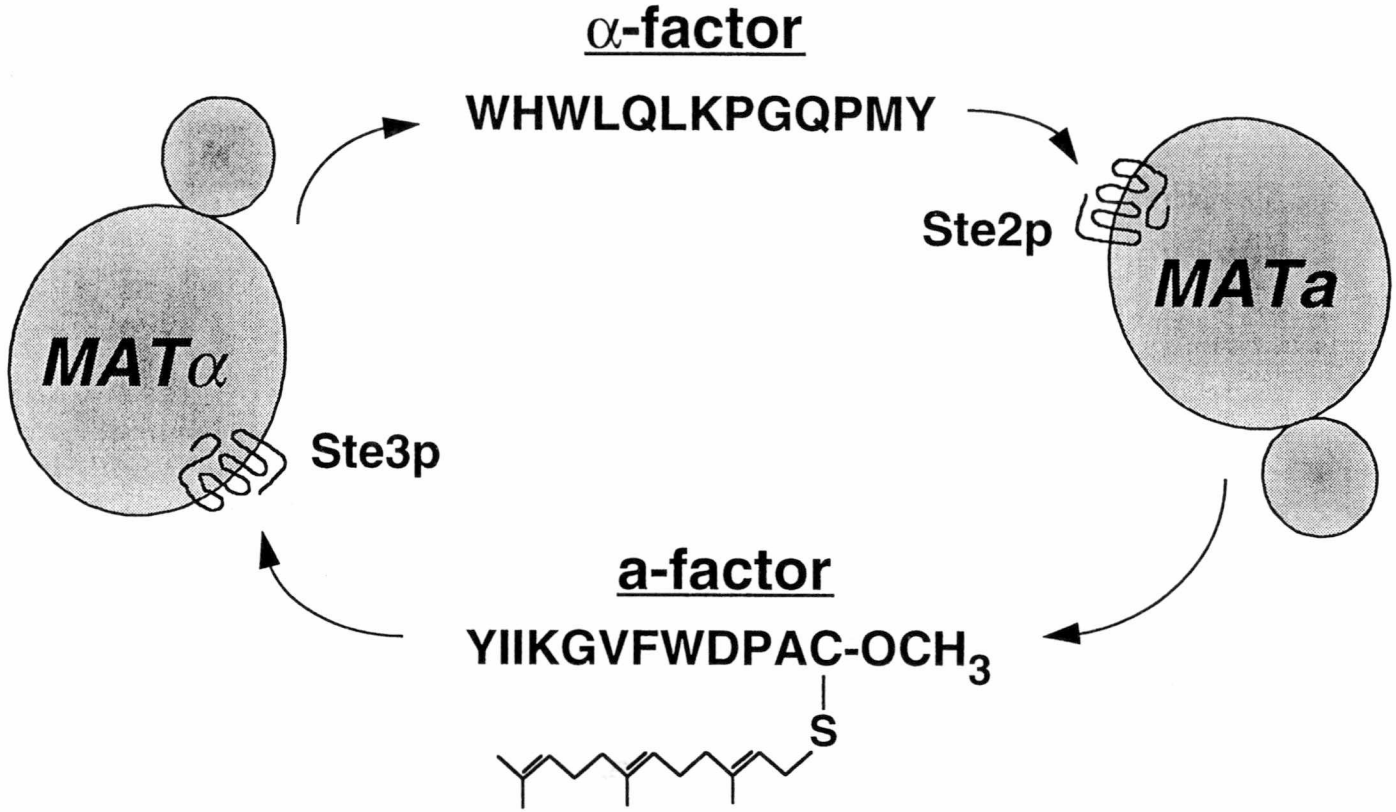



**Figure 3 :** Cellular communication between mating types

**Figure 4 :** Model of the Ste2p (α-factor receptor). Model showing the extracellular domains (**E1 - E4**), the transmembrane domains (**M1 - M7**), the internal domains (**I1 - I3**), and the cytoplasmic tail (**C**). The topological structure of the receptor was elucidated from studies carried out by Cartwright & Tipper (1991), from hydropathy plot analysis, and comparison to other similar receptors in the superfamily of G-protein linked transmembrane receptors (ie. rhodopsin, adrenergic receptors, neurotransmitter receptors, etc.). Balls represent amino acids which are represented by their single letter designations; Extra, extracellular; PM, plasma membrane; Cyto, cytosolic.

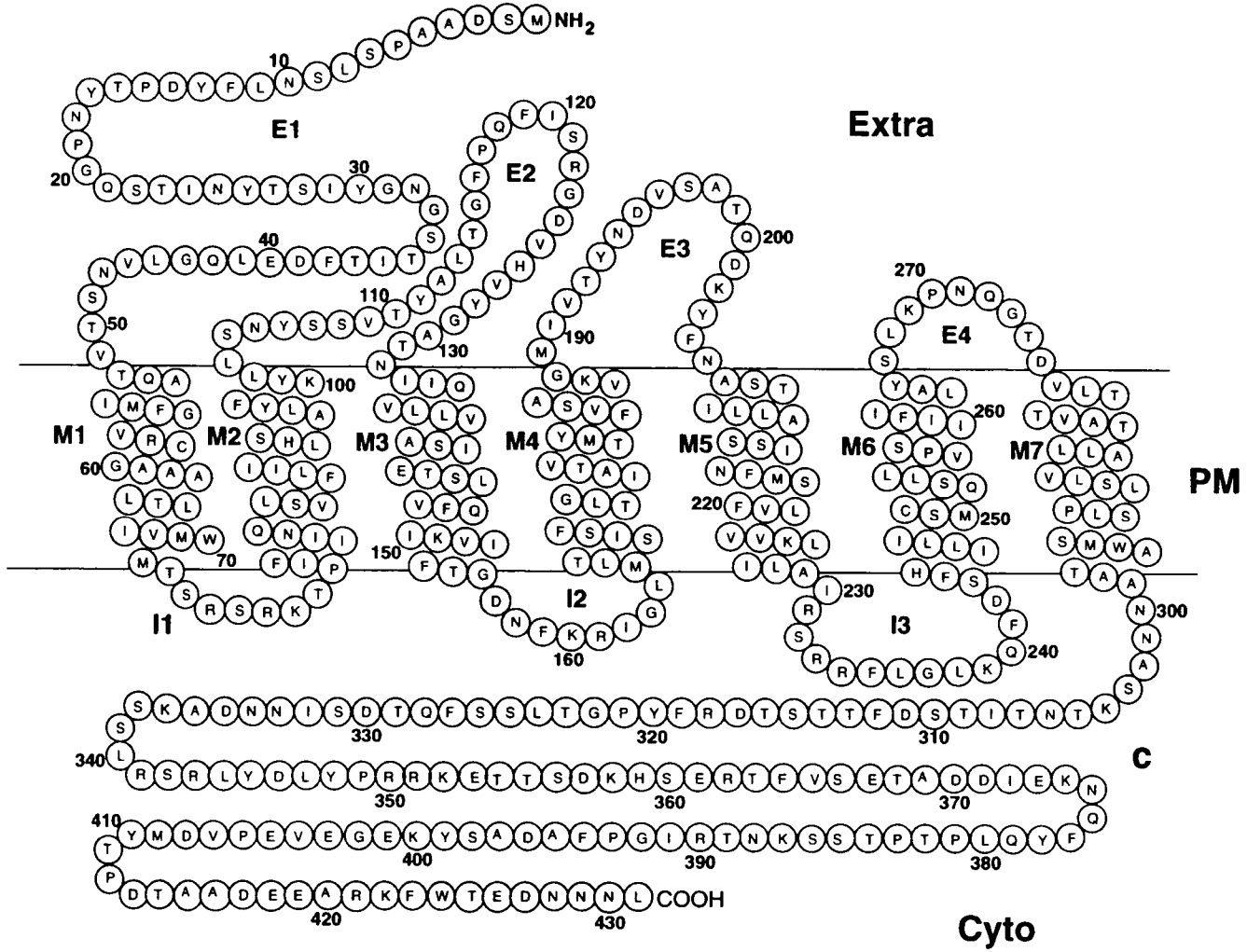



**Figure 4 :** Model of the Ste2p (α-factor receptor).

cells (Blumer, et al., 1988; Burkholder & Hartwell, 1985; Nakayama, et al., 1985). It is a 49 kDa protein encoded by a 1293 bp open reading frame translated into 431 amino acids (Burkholder & Hartwell, 1985). The N-terminal end of the protein is located on the extracellular side of the plasma membrane while the C-terminal end is located on the cytoplasmic side (Cartwright & Tipper, 1991; Clark, et al., 1988). It has been shown to be the specific binding protein of the α-factor pheromone (Fig. 5; Konopka & Fields, 1992; Bender & Sprague, 1989; Jenness, et al., 1986). Receptors have also shown the ability to bind another class of peptides termed antagonists. Antagonists are peptide pheromones that bind to receptors but do not elicit a phenotypic response. Antagonists usually compete with normal biogenic agonists for binding (either by binding in the same "pocket" or in a position that blocks agonist access to its binding "pocket"). Antagonists are a valuable tool for use in the study of receptor structure and function (Mukai, et al., 1992). Many previously described (Eriotou-Bargiota, et al., 1992; Raths, et al., 1988; Naider & Becker, 1986) as well as recently discovered (see Part 3 in this manuscript) antagonists are extensively used in this body of work.

*MATα* cells secrete α-factor (WHWLQLKPGQPMY) and *MAT**a*** cells export a dodecapeptide called **a**-factor (YIIKGVFWDPAC(farnesyl)-OMe) that is post-translationally modified by farnesylation and C-terminal carboxy-methylation. Studies of the biosynthesis of the α-factor pheromone (Fig. 6, Fuller, et al., 1988) have shown it is encoded by two genes, *MFα1* and *MFα2* (Kurjan & Herskowitz, 1982) which code for 165 and 120 amino acid pheromone precursors (prepro-α-factor), respectively, and is secreted by the cell through the classical vesicle-based secretory

**Figure 5 :** Amino acid structures of the *Saccharomyces cerevisiae* mating pheromones. The α-factor pheromone is an unmodified tridecapeptide while the **a**-factor pheromone is a posttranslationally modified dodecapeptide which is carboxymethylated and farnesylated on $Cys^{12}$.

**α-factor**

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

**a-factor**

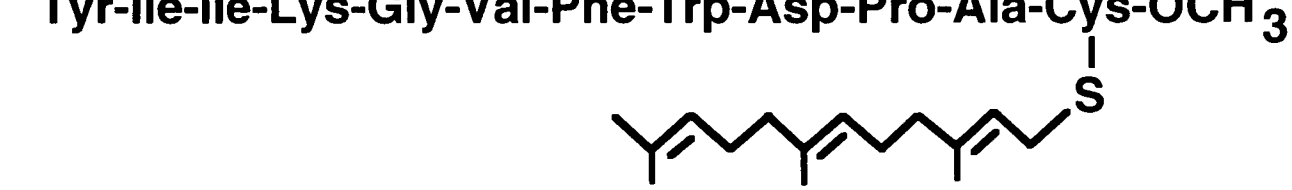
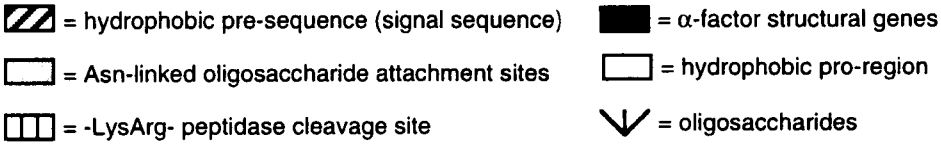



**Figure 5 :** Amino acid structures of the *Saccharomyces cerevisiae* mating pheromones.

**Figure 6 :** Processing of post-translational prepro-α-factor in *Saccharomyces cerevisiae*. Biosynthesis and maturation of the *MFα1* gene product has been described in detail (Fuller, et al., 1988).



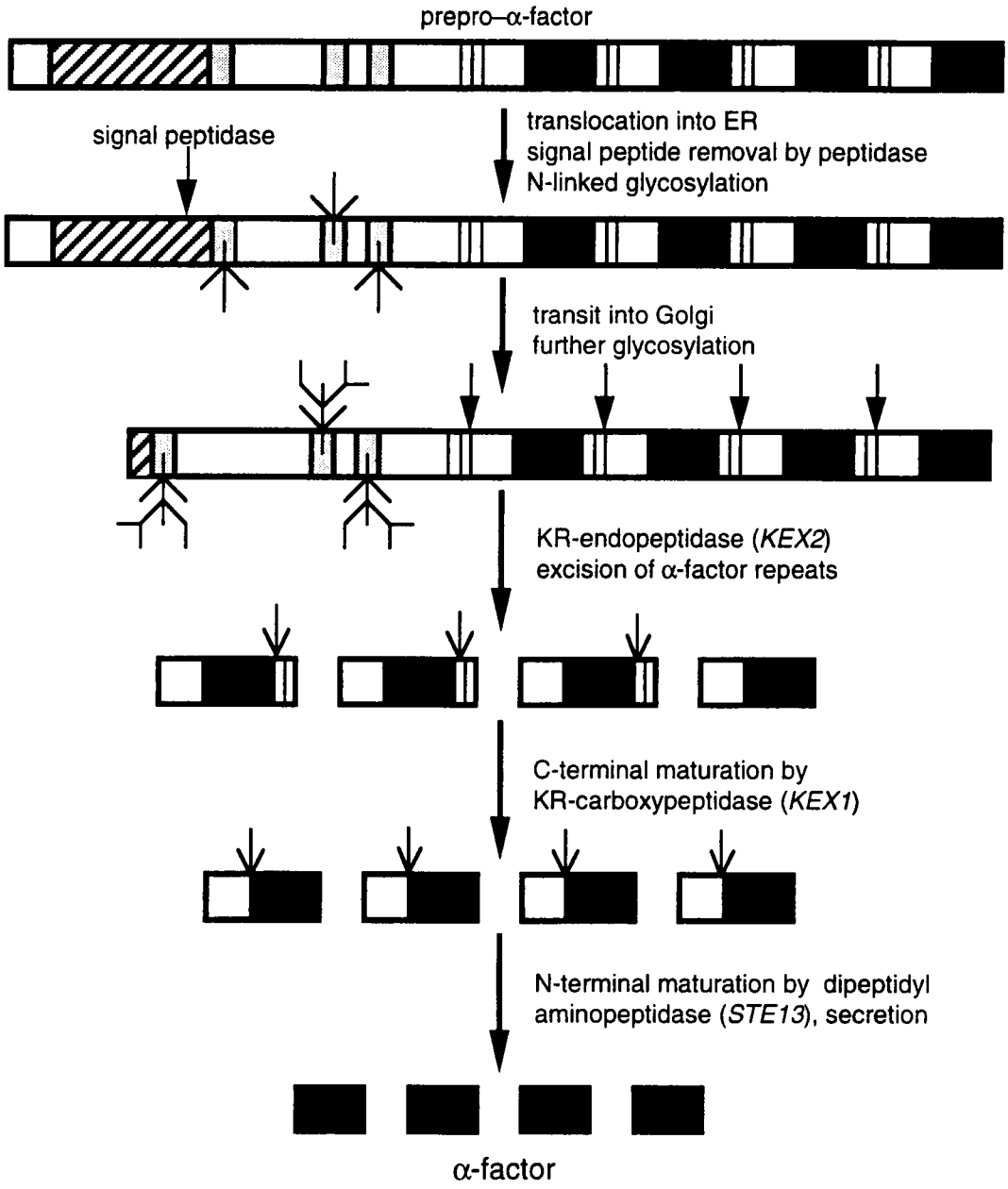



**Figure 6 :** Processing of post-translational prepro-α-factor in *Saccharomyces cerevisiae.*

pathway. Pheromone/receptor interaction transduces a signal across the plasma membrane which initiates a phosphorylation cascade that up regulates the transcription and translation of mating specific genes (Fig. 7). The pathway activated by the receptor-pheromone complex is common to both the **a**- and α-cells. The binding of the ligand to receptor transduces a signal via a putative conformational change in the receptor to a heterotrimeric G-protein. The βγ dimer of the G-protein dissociates from the α-subunit and regulates a proposed effector molecule (Nakayama, et al., 1988; Finegold, et al., 1990). The βγ dimer also has been shown to interact with the MAP kinase scaffold protein Ste5p (Hasson, 1992; Whiteway, et al., 1995) and therefore is physically linked to the MAP kinase cassette which may transmit the mating signal. Currently though, an effector molecule is still believed to transduce the signal through a number of protein kinases [Ste20p (Leberer, et al., 1992); Ste11p (Rhodes, et al., 1990); Ste7p (Clark, et al., 1988); Kss1p (Courchesne, et al., 1989); Fus3p (Elion, et al., 1990)] which phosphorylate and activate each successive kinase in a cascading series to a transcriptional activator (Ste12p; Errede & Ammerer, 1989). Specific interactions between the mitogen-activated protein (MAP) kinases Kss1p and Fus3p and the upstream MAP kinase kinase Ste7p have been recently identified and may provide evidence of one way the cell ensures the fidelity in signal transmission and signal pathway insulation. (Bardwell, et al., 1996). Activated Ste12p binds to pheromone response elements (PRE's; Kronstad, et al., 1987) and induces transcription of mating specific genes (for review see Sprague & Thorner, 1992).

**Figure 7** : The signal transduction pathway of *Saccharomyces cerevisiae*. Binding of pheromone (**a**-factor or α-factor) to its receptor (Ste2p, Ste3p, respectively) induces a putative conformational change that is transduced to an associated heterotrimeric G-protein (α, β, γ). Receptor mediated exchange of GTP for GDP on the $G_\alpha$-subunit induces the dissociation of $G_\alpha$ from the $G_{\beta\gamma}$ dimer which propagates the mating signal to an unknown effector (**?**). A phosphorylation cascade is initiated through a series of protein kinases (Ste20p, Ste5p, Ste11p, Ste7p, Fus3p, Kss1p) by the effector. An activated transcriptional activator presumably through phosphorylation (Ste12p*) binds to DNA at a specific sequence termed the pheromone response element (PRE) and initiates transcription of mating specific genes (*AGA2*, ect.). If sexual conjugation is not accomplished, the cell desensitizes to the external signal through a variety of mechanisms such as targeting ligand bound receptors for sequestration and degradation by phosphorylation (**-P**) and ubiquitination (**-U**). $G_{\beta\gamma}$ signaling is attenuated by re-association with the $G_\alpha$ subunit that has been "activated" for trimer formation by the GTPase activity of the Sst2p (◀, Dohlman, et al., 1996).

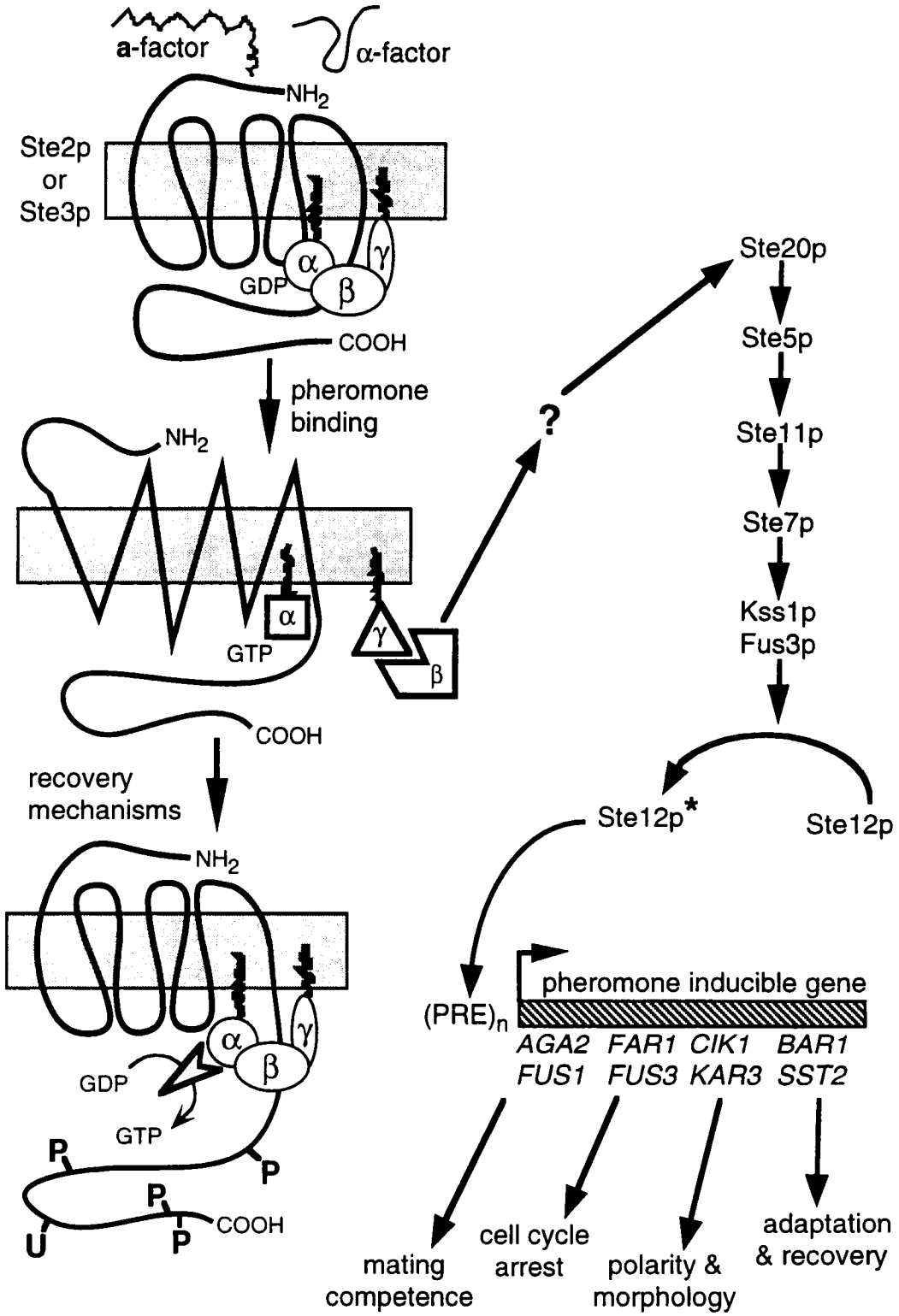



**Figure 7 :** The signal transduction pathway of *Saccharomyces cerevisiae*.

In response to pheromone binding, cell cycle progression is blocked late in the $G_1$ phase (Pringle & Hartwell, 1981). If a haploid cell that has been "activated" for sexual conjugation cannot find a mating partner, the cell must be able to recover from its $G_1$ growth arrest and continue mitotic growth (Moore, 1984; Moore, 1987). *Saccharomyces cerevisiae* has adapted many desensitization mechanisms for the recovery from pheromone induced growth arrest. The *BAR1* protease (Chan & Otte, 1982a,b; Sprague & Herskowitz, 1981) cleaves the α-factor peptide thereby inactivating its signaling capability. Ligand induced receptor down-regulation by endocytosis (Schandel & Jenness, 1994; Jenness & Spatrick, 1986) removes activated receptors from the cell surface along with bound ligand for eventual degradation in the vacuole (Jones, 1991). Receptor desensitization by C-terminal hyper-phosphorylation (Blumer, et al., 1988; Reneke, et al., 1988) in the presence of ligand has been described as another mechanism of adaptation to the mating signal (Chen & Konopka, 1996). The protein Sst2p appears to be important to the desensitization and recovery process of the cell (Chan & Otte, 1982a,b; Steden, et al., 1989). It has been shown to act through direct association with the $G_\alpha$ subunit of the G-protein (Gpa1p) and is the prototype for the newly recognized RGS (regulators of G-protein signaling) family (Dohlman, et al., 1996). It shows homology to many GTPase activating proteins (Bourne et al., 1991; Tanaka, et al., 1990) and colocalizes with Gpa1p in the plasma membrane, which is consistant with a role in binding to and affecting the activity of Gpa1p (Dohlman, et al., 1996). Finally, recent studies have suggested that pheromone induced ubiquitination (for review see Varshavsky, 1992) of the α-factor receptor cytoplasmic tail acts as a signal

for endocytosis leading to subsequent degradation of the receptor-ligand complex (Hicke & Riezman, 1996).

As the length of this introduction attests, there is a great deal of information known about the pheromones, receptors, and cellular communication in *Saccharomyces cerevisiae*. Parts 2, 3, and 4 attempt to add to this knowledge base by closely looking at contributions of specific residues of the α-factor in binding and signaling (Parts 2 & 3), as well as residues of the receptor involved in binding and signal transduction (Part 4).

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# PART 2

## The functional consequences of substitutions of the $His^2$ residue of the *Saccharomyces cerevisiae* tridecapeptide, α-factor.*

* Portions of Part 2 appear in modified form as **Levin, Y., Khare, R. K., Abel, G., Hill, D., Eriotou-Bargiota, E., Becker, J. M., and Naider, F.** 1993. Histidine$^2$ of the α-factor of *Saccharomyces cerevisiae* is not essential for binding to its receptor or for biological activity. *Biochemistry* **32** : 8199. Syntheses of α-factor analogs used in this study were carried out in the laboratory of Dr. Fred Naider, Department of Chemistry, C. U. N. Y., College of Staten Island, NY 10314.

# CHAPTER I

## Introduction

Previous structure-activity relationship studies of the *Saccharomyces cerevisiae* α-factor mating pheromone (WHWLQLKPGQPMY) have led to the conclusion that the His residue in position 2 is essential for biological activity. Deletion of this residue resulted in a decrease in activity by a factor of $10^8$ and replacement of His by Leu, Phe, and *D*-His resulted in virtually inactive pheromones (Masui, et al., 1977; Masui, et al., 1979; Naider & Becker, 1986). Recently, a detailed analysis of the coupling of the *STE2*-encoded α-factor receptor with G-proteins showed that the affinity of the pheromone for its receptor at pH 8.0 was almost one order of magnitude lower than that at pH 6.0 (Blumer & Thorner, 1990). Based on this result, the authors hypothesized that protonation of $His^2$ of α-factor may stabilize the conformation of the peptide in the most appropriate form for binding. We have shown that alterations at the amino terminus of the pheromone lead to α-factor antagonists. desTrp$^1$[Ala$^3$]α-factor and desTrp$^1$[Phe$^3$]α-factor were not biologically active but competed with the binding of α-factor to its receptor (Shenbagamurthi, et al., 1983; Raths, et al., 1988; Xue, et al., 1989). Similarly, a truncated pheromone (desTrp$^1$desHis$^2$[Nle$^{12}$]α-factor) was not active biologically but antagonized the activity of native α-factor and competed with the binding of the radiolabelled pheromone with the receptor (Eriotou-Bargiota, et al., 1992).

Histidine is often found in the active site of enzymes and is a functional residue in many biologically active peptides. The imidazole side chain of histidine may act as a general acid and/or a general base, may serve as a nucleophilic center, and provides a focal point for hydrogen bonding to water or ligands within the active site of enzymes. Normally, a catalytic function is not ascribed to histidinyl residues in peptide hormones because signal transmission from hormone/pheromone to the cell is believed to occur upon binding of peptide ligands to their cognate receptors. However, histidinyl residues in peptide hormones may enter into electrostatic or hydrogen bond interactions necessary to stabilize the biologically active conformation of the peptide or of the peptide-receptor complex. Thus, it is reasonable that histidine might play an important role in triggering signal transduction during hormone binding. Therefore, we decided to carry out a detailed structure-activity analysis to elucidate the importance of the His side chain in $\alpha$-factor activity and receptor binding. In Part 2, we present the synthesis, characterization and biological activities of seven $His^2$ analogs of the $\alpha$-factor mating pheromone.

# CHAPTER II

## Materials and Methods

*Chemicals and solvents*——All chemicals and solvents used in these studies were reagent grade and purchased from either Aldrich Chemical Co. or Fisher Scientific. Most of the Boc-protected amino acids were from Bachem Inc., Torrance, CA. Boc-β-(2-thienyl)-*L*-alanine was purchased from Bachem Inc., and the *DL* isomer of this compound was prepared starting from the amino acid using ditertiarybutyl dicarbonate in dioxane/0.5*M* NaOH (v/v). Boc-β-(3-pyridyl)-*L*-histidine and Boc-3-methyl-*L*-histidine were the gift of Bachem Inc. The position of the methyl group on the imidazole ring of these compounds were confirmed by 2D NMR spectroscopy.

*Peptide Synthesis*——All peptides prepared for use in this study contain norleucine (Nle) in place of methionine in position 12 of the α-factor. Nle is isosteric for Met and previous studies have shown that α-factor and [Nle$^{12}$]α-factor have identical activity (Raths et al., 1988). Replacement of Met by Nle results in an improved peptide synthesis and peptides which are more stable on storage. The [His$^{2}$]α-factor analogs were synthesized by starting with a Boc-Tyr(2-BrZ)phenylacetamidomethyl resin (PAM) and building up the peptide chain as described previously (Xue et al., 1989). The chemical structures of the position 2 side chains are shown in Figure 1.

**Figure 1 :** Chemical structures of the position 2 side chains. The side chain of the $His^2$ residue (R) in α-factor is replaced by the side chains represented (both *L*- and *D*-isomers of β-thienylalanine, 1-methylhistidine, 3-methylhistidine) to produce the position 2 α-factor analogs named.

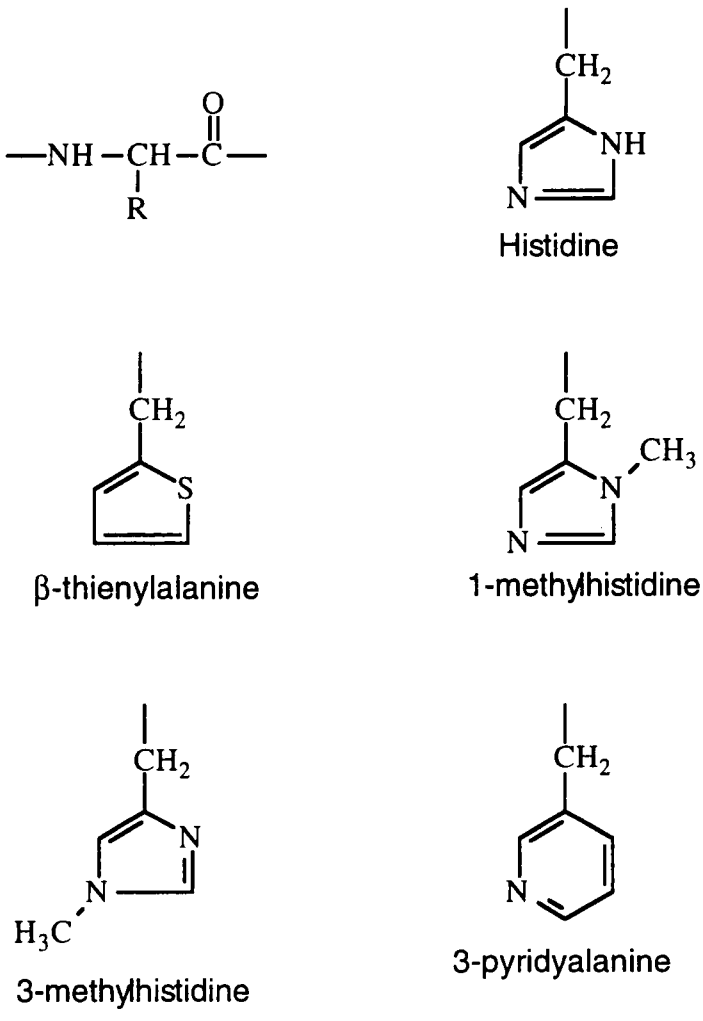


**Figure 1 :** Chemical structures of the position 2 side chains.

All residues were coupled using either diisopropylcarbodiimide or diisopropylcarbo-diimide/hydroxybenzotriazole activation. Coupling was monitored using the Kaiser test (Kaiser et al., 1970) and coupling reacts were repeated to ensure completeness. Boc protection was used for the Lys and Trp residues, respectively. The Boc group was usually deprotected using 45% TFA and 2% DMS in $CH_2Cl_2$. However, 4N HCl in dioxane was employed for deprotection of BocGln. In general, 0.167mmol of resin was used in the preparation of each analog. The final peptide products were cleaved from the PAM resin using HF/anisole (9:1) at 0°C for 1 hr. Peptides were extracted from the resin using 25-30% acetic acid and purified by preparative reversed phase high performance liquid chromatography (HPLC). The yield of purified peptide ranged from 13% to 48%.

*Characterization of Peptides*——All peptides were at least 98% homogeneous as judged by gradient reversed phase HPLC on a $C_{18}$ column, gave one ninhydrin positive spot on silica thin layers using both butanol:acetic acid:water (4:1:2) and butanol:acetic acid:water:pyridine (15:3:12:10) as mobile phase, and had the expected FAB-MS molecular ions. The UV spectra of all peptides in distilled water was measured using a Varian Instruments DMS-300 UV-Visible spectrophotometer. As judged by the presence of the 287.5 absorption shoulder and the calculated extinction coefficient, all analogs had two intact Trp residues. The molar extinction coefficient of the [Nle$^{12}$]α-factor was determined to be 10576 ($M^{-1} \cdot cm^{-1}$) at 287.5nm using absorption measurements and elemental

nitrogen analysis. This value was used to calculate the actual concentration of all pheromone solutions used for bioassays and for measurement of the specific rotation. Rotations were determined using a Jasco Dip-370 Digital polarimeter and are reported for solutions in distilled water. The amino acid analysis of the [1-MeHis$^2$,Nle$^{12}$]α-factor showed the expected ratios and that no free His was present in either this peptide or the starting 1-MeHis. A summary of the physical properties of the analogs used in this study appears in Table 1.

*Mass and NMR Spectroscopy, Capillary Zone Electrophoresis and Amino Acid Analysis*——The molecular weight of all peptides was determined using fast atom bombardment mass spectroscopy (FAB-MS) at the University of Tennessee as previously described (Levin, et al., 1993). Amino acid analysis and capillary zone electrophoresis were performed by the peptide group at Hoffman-LaRoche, Inc. as described (Levin, et al., 1993).

*Biological Assays of α-factor analogs*——The biological activity of the [His$^2$]α-factor analogs was assayed using a growth arrest (halo) assay as described previously (Raths, et al., 1988). Activity was examined using wild type (2180-1A), supersensitive (RC629[*sst1*]), and temperature sensitive (50B$^{ts}$) mutants of *S. cerevisiae*. The *sst1* mutation results in the loss of the *BAR1* protease, a secreted enzyme, which cleaves the α-factor (Chan & Otte, 1982). Strains 2180-1A, and 50B$^{ts}$ secrete *BAR1* protease, but strain 50B$^{ts}$ contains a mutant *STE2* gene that encodes an α-factor receptor inactivated by incubation of cells at 34$^o$C. At 23$^o$C, the *STE2* receptor of strain 50B$^{ts}$ is fully functional.

**Table 1:** Analytical and physical chemical characteristics of [His$^2$]α-factor analogs. (*a*) FAB-MS are monoisotopic values and refer to [M + $H^+$]. (*b*) Chromatography was carried out on a $C_{18}$ reversed-phase column using a water/$CN_3CN$/TFA gradient from 80:20:0.025 to 50:50:0.025 over 35 min. (*c*) $R_f$(A) = butanol/AcOH/$H_2O$ (4:1:2); $R_f$(B) = butanol/AcOH/$H_2O$/pyridine (15:3:12:10).

*Table 1 - Analytical and physical chemical characteristics of [2]-His factor analogs*

| number | peptide | FAB-MS [a] calc'd | FAB-MS [a] obs'd | specific rotation [α] | K' [b] | $R_f$(A) [c] | $R_f$(B) [c] |
|---|---|---|---|---|---|---|---|
| II. | β-*L*-ThienylAla[2] | 1681.9 | 1681.5 | -47.5 | 9.47 | 0.57 | 7.4 |
| III. | β-*D*-ThienylAla[2] | 1681.9 | 1681.6 | -59.6 | 11.00 | 0.58 | 6.5 |
| IV. | 1-*L*-MeHis[2] | 1679.9 | 1680.0 | -41.3 | 7.06 | 0.46 | 3.7 |
| V. | 1-*D*-MeHis[2] | 1679.9 | 1679.6 | -88.6 | 6.59 | 0.46 | 4.1 |
| VI. | 3-*L*-MeHis[2] | 1679.9 | 1679.7 | -51.5 | 7.28 | 0.43 | 3.5 |
| VII. | 3-*D*-MeHis[2] | 1679.9 | 1680.0 | -67.3 | 6.68 | 0.49 | 7.1 |
| VIII. | 3-*L*-PydAla[2] | 1676.9 | 1676.7 | -45.4 | 7.14 | 0.51 | 3.7 |

In separate experiments, the ability of nonactive analogs to compete with the activity of the parent ([Nle$^{12}$]α-factor) was analyzed by placing the analog on a disk which was about 15 mm from a second disk containing the parent compound. Antagonism was evidenced by the appearance of bean-shaped zones of inhibition for the α-factor (E.-Bargiota, et al., 1992). The effects of pheromone analogs on the cellular morphology of strain RC629 were measured as previously described (E.-Bargiota, et al., 1992).

*Binding Competition Assay*——Competition of bound [$^{3}$H, Nle$^{12}$]α-factor by unlabeled [Nle$^{12}$]α-factor analogs were carried out as previously described (Raths, et al., 1988). In general, *S. cerevisiae* strain Jena [*Mata cry1 bar1-1 ade2-1 his4-580 lys2 tyr1 SUP4-3*], (ATCC# 4202-15-3; Jenness, et al., 1983) was grown in YM-1 medium (0.5% yeast extract, 1% peptone, 1% succinic acid, 0.6% NaOH, 1% dextrose, 0.67% yeast nitrogen base without amino acids, 0.001% adenine sulfate, and 0.001% uracil) at 22$^{o}$C overnight in a rotary circulating air incubator. The cell culture was counted using a hemocytometer to determine when the culture was ready for harvesting. At 22$^{o}$C, the generation time for the Jena strain was 220 min. The cells were harvested at 1 x 10$^{7}$ cells/ml by centrifugation at 5,000 x *g* at 4$^{o}$C (JA-10 rotor, Beckman Model J-21C centrifuge). The pelleted cells were washed two times in ice cold YM-1i medium (YM-1 medium with 0.065% sodium azide, 0.094% potassium fluoride, and 0.38% tosyl(N-α-*p*)-*L*-arginine methyl ester (TAME) added) and resuspended to 1.25 x 10$^{9}$ cells/ml in YM-1i to be used as a cell stock suspension. Final assay concentrations of the unlabeled His$^{2}$ analogs were between 3 x 10$^{-5}$ *M* and 1 x 10$^{-9}$ *M*. The concentrations of the analogs were determined from

the $A_{280}$ for each peptide from extinction coefficients calibrated for [Nle$^{12}$]α-factor from quantitative elemental analysis.

The binding assay was started by the addition of 100 µl of a 1:1 mixture of [$^{3}$H, Nle$^{12}$]α -factor ($9.3 \times 10^{-7}$ *M*, 9.24 Ci/mmol) and the appropriate dilution of unlabeled analog to 400 µl of the cell suspension. Duplicate 20 µl aliquots were removed after vortexing and put into scintillation vials (tips were ejected directly into the vials) to determine total labeled ligand concentration. All cells, peptide dilutions, and media were kept on ice until the start of the assays. Cells were prewarmed for 10 min. at room temperature and then 20 min. incubations with peptides were carried out at room temperature. Assays were staggered by 2 or 3 minutes to allow for multiple sample handling and consistent incubation times. After the 20 min. incubation, duplicate samples of 200 µl were diluted in ice cold YM-1i medium and filtered over 1% bovine serum albumin presoaked GN-6 Metricel filters (Gelman Sciences, Inc., Ann Arbor, MI) under house vacuum. The dilution tube was then rinsed twice with ice cold YM-1i and filtered over the same filter. Filters were directly washed with cold YM-1i before being released from vacuum and placed in scintillation vials for counting. Binding of labeled α-factor to filters in the absence of cells was less than 20 cpm. Specific binding is defined as mean bound cpm/total mean cpm (x 100). Mean bound cpm is the average of multiple binding point determinations for each analog concentration tested (n=4) and total mean cpm is the average of the total counts incubated with each analog (n=4). Results for each analog were expressed as a percent of total binding in the absence of an analog competitor.

*HPLC purification of [$^{3}$H, Nle$^{12}$]α-factor*——Synthetic [Nle$^{12}$]α-factor was labeled by reduction of dihydroproline containing α-factor by the TR3 hydrogenation procedure of Amersham International as described previously (Raths, et al., 1988). Labeled peptide was purified by HPLC using a μBondapak $C_{18}$ 10mm column (3.9 mm x 300 mm) from Waters Chromatography Division on a Beckman System Gold HPLC. Unpurified labeled peptide (dissolved in ethanol/water 1:1) was dried *in vacuo* and redissolved in sterile water. The peptide solution was injected into the HPLC and eluted with a water/acetonitrile gradient of 0-40%. Fractions containing peptide eluting from the column similarly to unlabeled α-factor were collected and counted for radioactivity in a scintillation counter. All collected fractions were dried, resuspended in sterile water, combined, and the concentration and specific activity of the purified labeled peptide was determined.

*Reversed-Phase HPLC*——The final purification and characterization of the His$^{2}$ analogs was carried out on a Waters Instruments HPLC equipped with two Model 510 pumps, a Model 481 or Model 490 variable wavelength detector and a U6K injector. Preparative separations were carried out on 19 mm x 300 mm $C_{18}$ reversed-phase column; analytical runs were performed on a 3.9 mm x 300 mm $C_{18}$ reversed-phase column and a 3.9 mm x 300 mm CN reversed-phase column. Separations were accomplished using acetonitrile/water/trifluoroacetic acid gradients.

# CHAPTER III

## Results

*Synthesis and Characterization of His$^2$ Analogs*——The synthesis of the [His$^2$]analogs of the α-factor was accomplished efficiently using the solid phase approach on a PAM resin. In general, crude peptides were isolated in 80-95% yield after HF cleavage and contained one or two dominant products. In the case of the β-thienylalanine analog, the crude product prepared from the *DL* amino acid mixture exhibited nearly equal quantities of the diastereomeric isomers (Fig 2A). These were readily separated by preparative HPLC to yield both the *D*- and the *L*- isomers. The latter compound eluted first from the HPLC column. An independent synthesis starting with the *L* isomer of β-thienylalanine gave a product with an identical elution time to that of the first eluted product from the synthesis using the *DL* mixture. Analysis of the biological activities of these analogs confirmed the identity of the [β-*L*-thienylalanyl$^2$]α-factor (see below).

During the synthesis of the 1-methylhistidine and the 3-methylhistidine containing analogs, we observed significant heterogeneity in the crude products. Two major products, in unequal ratio, appeared in the chromatograms of the peptides cleaved from the PAM resins (Fig. 2B & 2C). We suspected that the observed heterogeneity was the result of racemization of the urethane protected methylhistidines during activation and coupling. Significant racemization has been reported for 1- and 3-alkyl histidines during carbodiimide activated addition to growing peptide

**Figure 2 :** HPLC plots of crude synthesis products. *Panel A* - HPLC of crude product from synthesis of [β-*DL*-thienylalanyl$^{2}$,Nle$^{12}$]α-factor. *Panel B* - HPLC of crude product from synthesis of [1-*L*-methylhistidinyl$^{2}$,Nle$^{12}$]α-factor. *Panel C* - HPLC of crude product from synthesis of [3-*L*-methylhistidinyl$^{2}$,Nle$^{12}$]α-factor. Chromatography was performed on a $C_{18}$ column using the following gradients: *A* - water/$CH_3CN$/TFA from 70:30:0.025 to 30:70:0.025 over 20 min. *B & C* - water/$CH_3CN$/TFA from 80:20:0.025 to 40:60:0.025 over 20 min.

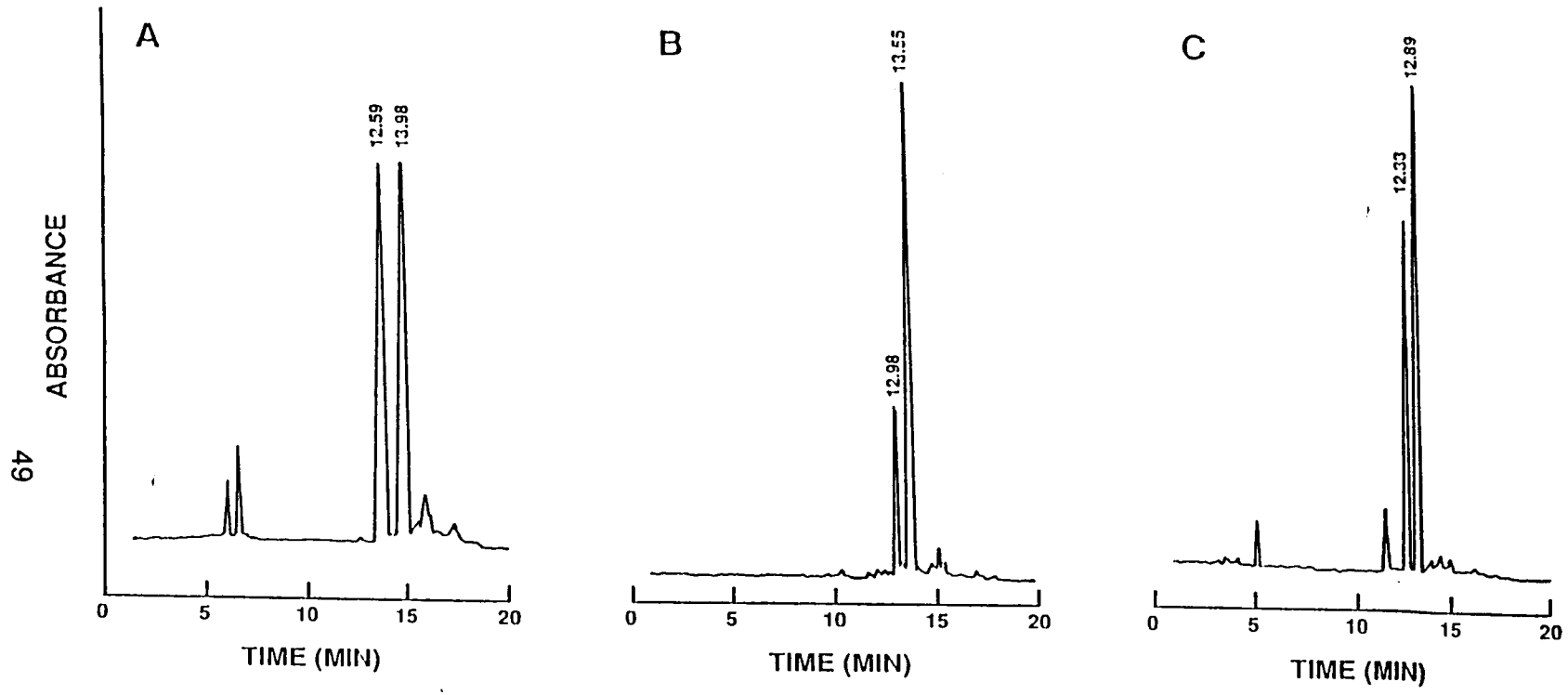


**Figure 2:** HPLC plots of crude synthesis products.

chains (Stewart & Young, 1984; Windridge & Jorgensen, 1971). On the basis of this assumption, it was reasonable that the smaller peaks in panels B (~21%) and C (~40%) were due to the incorporation of the *D*-isomer. To check this assumption, we purified both peaks to near homogeneity. NMR analysis of each peak from the 1-methylhistidine synthesis indicated that both peptides contained 1-methylhistidine and they exhibited identical molecular ions as judged by FAB-MS analysis. In addition, amino acid analysis showed that neither the starting Boc-1-methylhistidine nor the product peptide contained as much as 1% free histidine. It is interesting to note that in the case of the methyl histidine analogs, the peptide with a *D*-residue in position 2 elutes from the column first. This is opposite to the mobility observed for the β-thienylalanine analogs.

The position 2 analogs were characterized for homogeneity using gradient elution on reversed-phase $C_{18}$ columns (Figure 3) and reversed-phase CN column (data not shown). Similar results were obtained on both columns; however, much sharper peaks were obtained on the $C_{18}$ column. The peptides ranged from 96.4% homogeneous ([1-*L*-MeHis$^2$,Nle$^{12}$]α-factor; peptide IV) to 99.5% homogeneous ([3-*L*-PydAla$^2$,Nle$^{12}$]α-factor; peptide VIII). Most importantly, from the perspective of this study, by comparison of retention times and the coinjection of [Nle$^{12}$]α-factor (Figure 3, panels E and H), the HPLC analysis allowed the following conclusions: 1) The [β-*L*-thienylAla$^2$,Nle$^{12}$]α-factor contains less than 0.1% material with a chromatographic mobility equal to that of the [Nle$^{12}$]α-factor. 2) The [β-*D*-thienylAla$^2$,Nle$^{12}$]α-factor contains a maximum of 0.5% of the homologous *L*-isomer. 3) The [1-*D*-MeHis$^2$,Nle$^{12}$]α-factor does not contain a measurable amount of the homologous *L*-isomer.

**Figure 3 :** HPLC of purified peptides. **(A)** [β-*L*-thienylAla$^2$,Nle$^{12}$]α-factor (peptide II). **(B)** [β-*D*-thienylAla$^2$,Nle$^{12}$]α-factor (peptide III). **(C)** [1-*L*-MeHis$^2$,Nle$^{12}$]α-factor (peptide IV). **(D)** [1-*D*-MeHis$^2$,Nle$^{12}$]α-factor (peptide V). **(E)** [1-*D*-MeHis$^2$,Nle$^{12}$]α-factor and [Nle$^{12}$]α-factor (peptides V + I, arrow indicates [Nle$^{12}$]α-factor peak). **(F)** [3-*L*-MeHis$^2$,Nle$^{12}$]α-factor (peptide VI). **(G)** [3-*D*-MeHis$^2$,Nle$^{12}$]α-factor (peptide VII). **(H)** [3-*D*-MeHis$^2$,Nle$^{12}$]α-factor and [Nle$^{12}$]α-factor (peptide VII + I, arrow indicates [Nle$^{12}$]α-factor peak). **(I)** [3-*L*-PydAla$^2$,Nle$^{12}$]α-factor (peptide VIII). Chromatography was run on a $C_{18}$ column using a gradient of water/$CH_3CN$/TFA from 80:20:0.025 to 50:50:0.025 over 35 min.

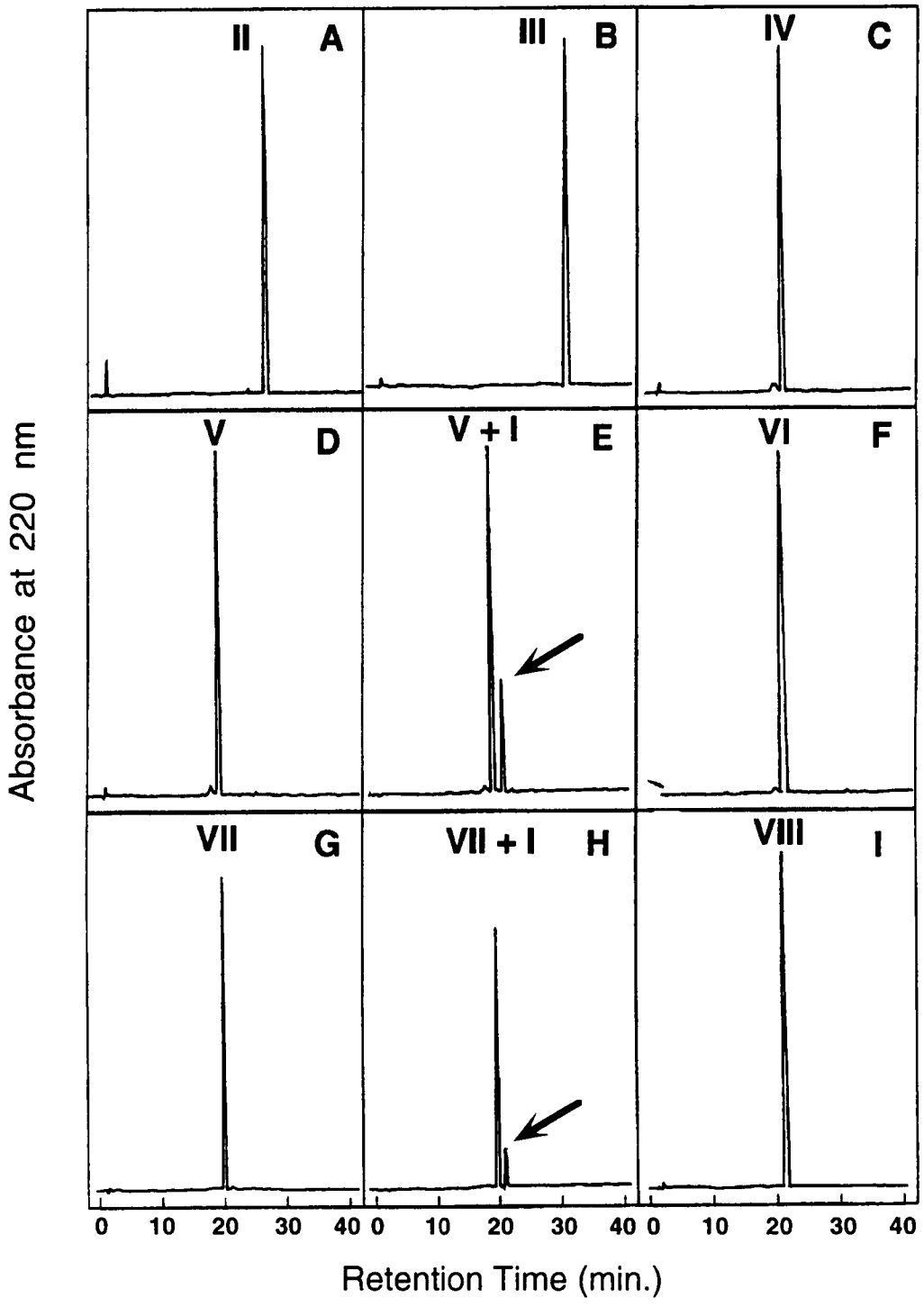


**Figure 3 :** HPLC of purified peptides.

4) The [3-*D*-MeHis$^2$,Nle$^{12}$]α-factor contains approximately 1% of an impurity with a mobility equal to that of the corresponding *L*-isomer. 5) The similar retention times of the [1-*L*-MeHis$^2$,Nle$^{12}$]-, [3-*L*-MeHis$^2$,Nle$^{12}$]-, and [3-*L*-PydAla$^2$,Nle$^{12}$]α-factor analogs and [Nle$^{12}$]α-factor (Table I) makes it impossible to prove using HPLC that these samples do not contain the parent pheromone. However, it is proven that the small impurities found in the other analogs are not the [Nle$^{12}$]α-factor.

To gain additional insights into the homogeneity of the [1-*L*-MeHis$^2$,Nle$^{12}$]-, [3-*L*-MeHis$^2$,Nle$^{12}$]-, and [3-*L*-PydAla$^2$,Nle$^{12}$]α-factors, these compounds were subjected to capillary zone electrophoresis. Under the conditions used, excellent separation of the above peptides and the [Nle$^{12}$]α-factor were obtained (data not shown). The results indicate that the [3-*L*-PydAla$^2$,Nle$^{12}$]α-factor contains less than 1% of the parent compound and that the [1-*L*-MeHis$^2$,Nle$^{12}$]α-factor and the [3-*L*-MeHis$^2$,Nle$^{12}$]α-factor have approximately 3% of an impurity with an electrophoretic mobility similar to that of [Nle$^{12}$]α-factor.

All peptides isolated from the solid phase syntheses were purified to >98% homogeneity and gave the expected FAB-MS molecular ions (Table 1). The results of these syntheses show that under our coupling conditions, Boc-3-methylhistidine is more prone to racemization than Boc-1-methylhistidine. Interestingly, in contrast to the results with the methylhistidines, the synthesis of the β-3-pyridylalanine analog did not result in appreciable racemization. The conclusions of the chromatographic and spectroscopic analyses are in harmony with the relative activities found for the methylhistidine containing peptides in the halo assay (see below).

*Biological Activity of [His$^2$]Analogs*——The biological activity of peptides I-VIII (Table 2) was determined using both wild type and supersensitive strains. None of the peptides tested were active against strain 50B$^{ts}$ at the restrictive temperature (34°C). This was true for both active and inactive analogs and indicates that the effects seen with all active analogs were due to interaction with the α-factor receptor. In general, we found that the relative activity of the [His$^2$]analogs was similar in different strains. Peptides III, V and VII (*D*-isomer forms of position 2 analogs) showed no activity in the halo assay against strains 2180-1A, RC629 and 50B$^{ts}$ at the highest concentrations tested. In addition, at high concentration, peptide VII (5 μg/ml) and peptide V (20 μg/ml) induced morphogenesis of strain RC629, whereas the β-*D*-thienylalanine containing peptide (peptide III) had no activity in the morphogenesis assay at concentrations up to 100 μg/ml (Figure 4). Replacement of the imidazole ring with a thiophene ring (peptide II versus peptide I) resulted in a 5 to 100-fold drop in activity, methylation of either imidazole nitrogen (peptides IV and VI) resulted in a 5 to 10-fold decrease in activity, whereas replacement of the imidazole ring with a pyridyl moiety (peptide VIII) resulted in only a 2 to 5-fold decrease in activity. Peptides III, V, and VII, which were inactive up to 50 μg/disk in the halo assay against all strains, antagonized the activity of the native pheromone using RC629 as the test organism (data not shown). Bean-shaped halos were observed for the α-factor when the competing inactive peptide was applied adjacent to the α-factor peptide at a 5 to 20-fold excess and is indicative of a competitive antagonist (Scannel & Pruess, 1974). The nature of the inhibition of activity is supported by direct binding competition studies using radioactive pheromone (see below).

**Table 2 :** Biological and biochemical properties of [His$^2$]α-factor analogs. (*a*) Growth arrest was determined by the formation of halos in lawns of three strains (2180**a**, RC629, and 50B$^{ts}$) represented. The value shown is the minimal amount of peptide (μg applied to disk) that caused a halo. (*b*) Cell morphologies were determined by the formation of shmoo-shaped cells in response to peptide. The value shown in the table is the amount of peptide causing formation of shmoo-shaped cells in 40-50% of the cell population. (*c*) Antagonism was determined by competition for halo formation by peptide analogs. (*d*) Binding competition was determined as described in E.-Bargiota, et al., (1992) *Biochemistry* **31** : 551. $K_d$ determined by the method of Cheng & Prusoff (1973).

***Table 2 - Biological and biochemical properties of [His$^2$]α-factor analogs***

| number | peptide | Halo [a] | | | shmoo [b] | Antagonist [c] | Binding [d] |
|---|---|---|---|---|---|---|---|
| | | 2180a | RC629 | 50B$^{ts}$ | (μg/ml) | | ($K_d$ x $10^{-7}$ *M*) |
| I. | [Nle$^{12}$]α-factor | 1.0 | 0.05 | 0.5 | 0.1 | ----- | 0.2 |
| II. | β-*L*-ThienylAla$^2$ | 5.0 | 5.0 | 5.0 | 0.5 | ----- | 7.4 |
| III. | β-*D*-ThienylAla$^2$ | >10 | >50 | >10 | >100 | yes | 6.5 |
| IV. | 1-*L*-MeHis$^2$ | 5.0 | 0.5 | 1.0 | 0.1 | ----- | 3.7 |
| V. | 1-*D*-MeHis$^2$ | >10 | >50 | >10 | 20.0 | yes | 4.1 |
| VI. | 3-*L*-MeHis$^2$ | 5.0 | 0.5 | 1.0 | 0.5 | ----- | 3.5 |
| VII. | 3-*D*-MeHis$^2$ | >10 | >50 | >10 | 5.0 | yes | 7.1 |
| VIII. | 3-*L*-PydAla$^2$ | 5.0 | 0.10 | 1.0 | 0.1 | ----- | 3.7 |

**Figure 4:** Effects of [Nle$^{12}$]α-factor and various position 2 substituted analogs on cellular morphology of *Saccharomyces cerevisiae* RC629. The symbols represent [Nle$^{12}$]α-factor (○, peptide I), [3-*L*-PydAla$^{2}$,Nle$^{12}$] α-factor (□, peptide VIII), [1-*D*-MeHis$^{2}$, Nle$^{12}$]α-factor (△, peptide V) and [β-*D*-thienylAla$^{2}$, Nle$^{12}$]α-factor (●, peptide III) at the indicated concentrations.

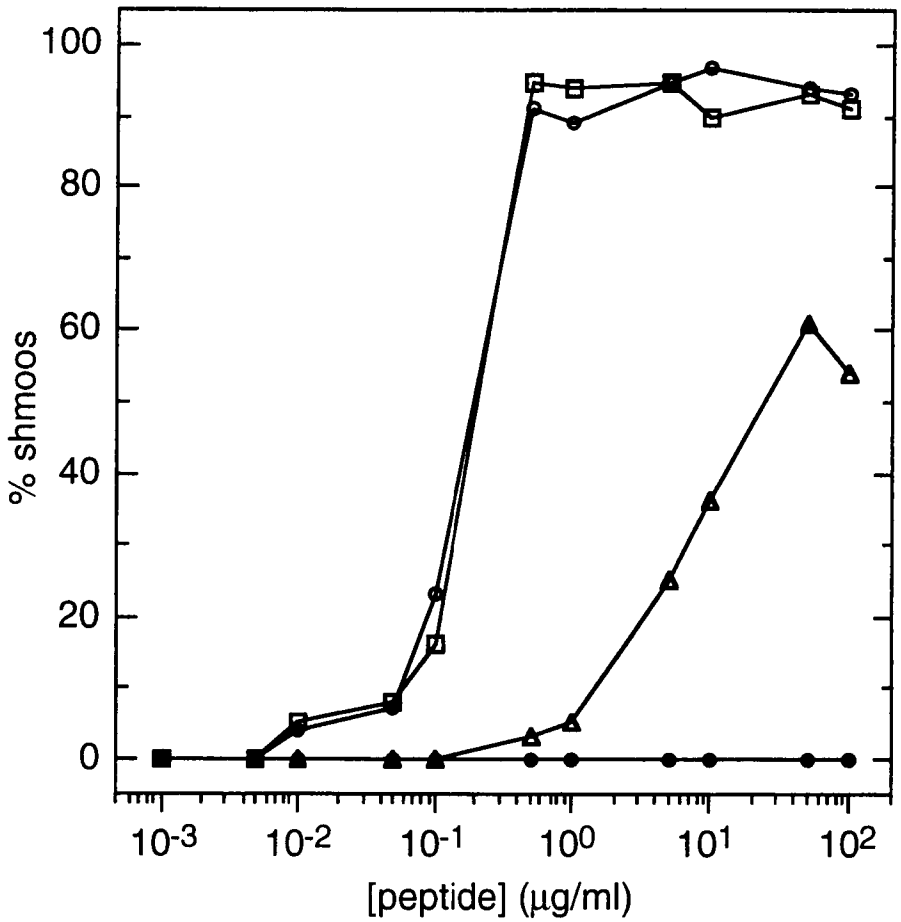


**Figure 4:** Effects of [Nle$^{12}$]α-factor and various position 2 substituted analogs on cellular morphology of *Saccharomyces cerevisiae* RC629.

*Competition With Binding to the Receptor*——The ability of the [$His^2$]analogs to bind to the α-factor receptor was determined directly using competition binding assays with radioactively labelled [$Nle^{12}$]α-factor. Binding competition curves for all analogs were parallel to those measured for the parent pheromone and complete displacement of the agonist was achieved at high concentration (Figure 5). The competition assays indicate that 50% of the α-factor is displaced from the receptor by the position 2 analogs at concentrations of 2-4 x $10^{-6}$ *M* (Table 2). This is approximately one order of magnitude lower than the $K_d$ observed for the parent pheromone (Figure 5). There was not a significant difference in the binding constants found for the biologically active and the inactive pheromones.

**Figure 5 :** Competition binding of [$^{3}$H,Nle$^{12}$]α-factor by unlabeled [Nle$^{12}$]α-factor and position 2 analogs. *Panel A* - The curves represent [$^{3}$H,Nle$^{12}$]α-factor plus [Nle$^{12}$]α-factor (○), [3-*D*-MeHis$^{2}$,Nle$^{12}$]α-factor (■), [1-*L*-MeHis$^{2}$,Nle$^{12}$]α-factor (●), and [3-*L*-MeHis$^{2}$,Nle$^{12}$]α-factor (▼). *Panel B* - The curves represent [$^{3}$H,Nle$^{12}$]α-factor plus [Nle$^{12}$]α-factor (○), [1-*D*-MeHis$^{2}$,Nle$^{12}$]α-factor (Δ), [β-*D*-thienylAla$^{2}$,Nle$^{12}$]α-factor (●), and [3-*L*-PydAla$^{2}$,Nle$^{12}$]α-factor (❑). The data shown are the means of triplicate determinations, with each curve representative of two separate experiments.

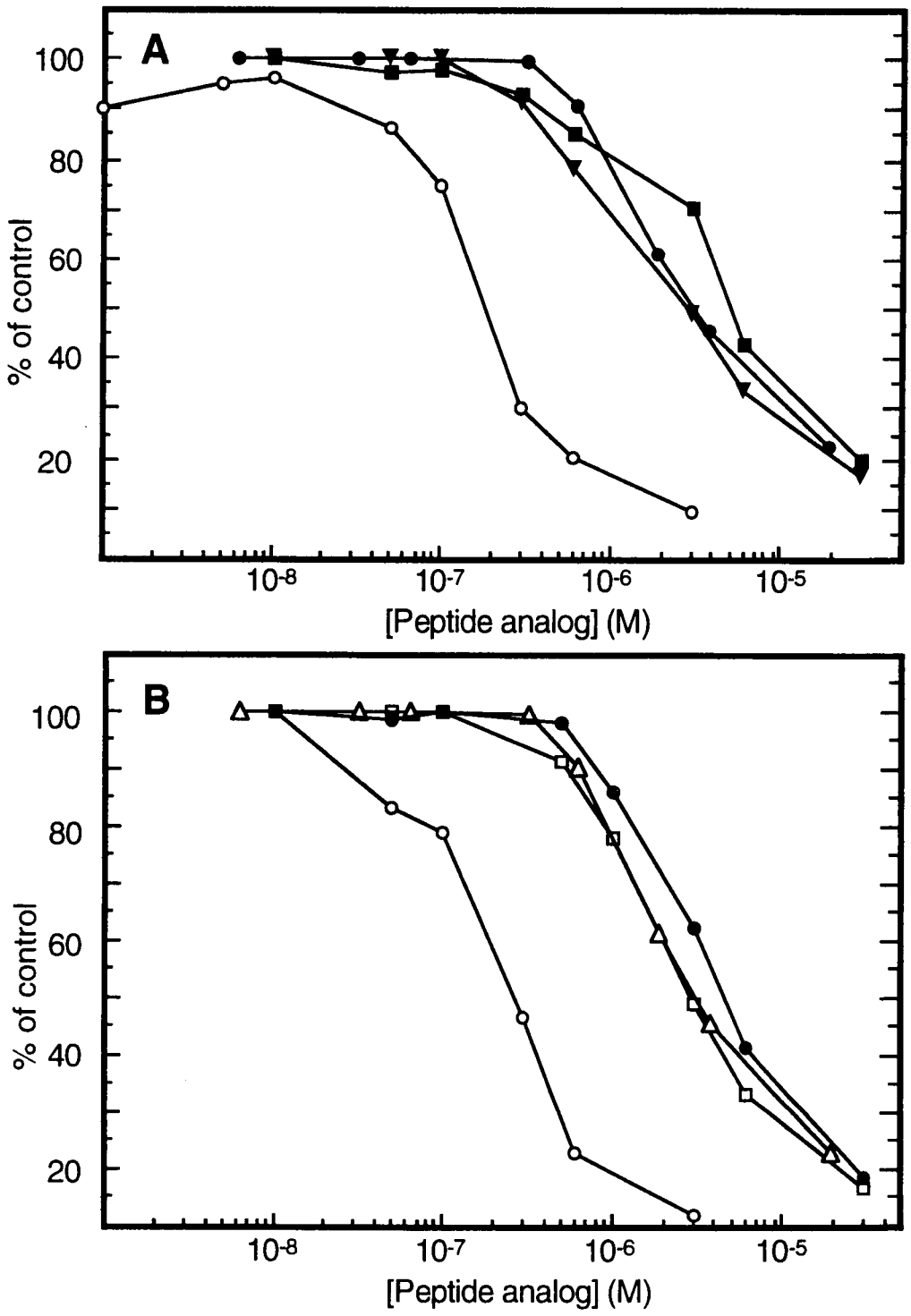


**Figure 5 :** Competition binding of [$^{3}$H,Nle$^{12}$]α-factor by unlabeled [Nle$^{12}$]α-factor and position 2 analogs.

# CHAPTER IV

## Discussion

Histidine residues in peptides and proteins have been associated with significant influences on biological activity. In addition to the well-documented cases of the involvement of the imidazole moiety of histidine in the active site of many enzymes (Fersht, 1985; Walsh, 1979), histidinyl residues of peptide ligands have also been shown to influence binding to protein receptors. In a series of thorough experiments, it was shown that the His residue of the S-peptide (residues 1-20) of ribonuclease A influenced both binding of S-peptide to S-protein (residues 21-124) and the activity of the resulting peptide-protein complex. In particular, replacement of $His^{12}$ in the S-peptide by 3-methylhistidine or 3-pyridylalanine resulted in the formation of a catalytically inactive complex while impairing the binding of the S-peptide to the S-protein by 10-fold and 0-fold, respectively. In contrast, replacement of $His^{2}$ by 1-methylhistidine resulted in a 100-fold decrease in binding to S-protein but the complex which formed retained about 20% of the activity of ribonuclease A (Van Batenburg, et al., 1977). Furthermore, replacement of the imidazole moiety of $His^{12}$ with pyrazole or 4-fluoroimidazole resulted in an inactive ribonuclease despite the fact that the modified S-peptides bound well to the S-protein (Dunn, et al., 1974).

A number of studies on histidine-containing hormones have measured effects of histidine deletion, replacement, or modification on the

resulting bioactivity and receptor binding of the analogs. Deletion of the N-terminal $His^1$ of vasoactive intestinal peptide (VIP) gave an analog with comparable diminution of biological and binding activity compared to the native 28 amino acid hormone (O'Donnell, et al., 1991). In contrast, replacement of histidine by alanine resulted in a 100 to 200-fold decrease in bioactivity, but binding was diminished only 3-fold (Bolin, et al., 1990). Interestingly, Na-acetylation of VIP resulted in increased hormone potency and led the authors to conclude that the acetyl function retained the features of the imidazole of histidine required for biological activity (Bolin, et al., 1990). Replacement of histidine by norvaline in the tripeptide hormone TRH (thyrotropin releasing hormone) gave an analog with reduced binding to the high affinity receptor and total loss of thyrotropin releasing capacity (Vonhof, et al., 1990), whereas replacement of $His^1$ of LHRH (luteinizing hormone releasing hormone) by *D*-Phe(4-Cl) resulted in an analog possessing high binding affinity but negligible bioactivity (Ljungqvist, et al., 1991). A number of analogs of this latter antagonist are being used as model compounds for studying cancer cells (Segel-Abramson, et al., 1992) and are used in human medicine as antitumor agents (Schally, et al., 1990). Investigations on angiotensin II showed that $His^6$ could be replaced by Gln, but not Asn, with retention of full bioactivity, leading the authors to conclude that the Gln side chain is an isostere for the imidazole of His (Pottorf, et al., 1990). Based on the literature, it is clear that histidine can have a marked influence on hormone activity and that in certain cases bioactivity is correlated with binding, whereas in others these two characteristics are dissociable.

The results reported herein clearly indicate that the imidazole side chain of the $His^2$ residue is not essential for biological activity of α-factor. Specifically, replacement of this moiety with a thiophene or 3-pyridyl ring led to analogs with 1/100 to 1/2 the biological activity of the parent pheromone. In addition, the NH of the imidazole ring could be changed to a N-methyl group with significant retention of activity (Table 2). These results suggest that pheromone induced responses do not require either protonation or the NH of the imidazole ring.

It is interesting that a change in the chirality of residue 2 from *L* to *D* results in a marked (at least 50 to 100 fold) loss in activity. For all of the *D*-diasteromers, the nearly complete loss of activity is supported by the observation that these inactive analogs antagonize the activity of the [$Nle^{12}$]α-factor. Indeed, all of the [$His^2$]analogs bind to the receptor and exhibit binding constants similar to those of active homologs. Therefore, we conclude that antagonism can be attributed to displacement of the agonist from the receptor. Moreover, the binding of the thienylalanine analogs provides additional evidence that protonation of $His^2$ is not required for binding.

The finding that both active and inactive analogs bind to the pheromone with similar affinities implies that the biological activity of the position 2 analogs cannot be correlated solely with receptor occupancy. Furthermore, our results show that pheromone binding and signal transduction can be uncoupled. Studies on glucagon (Unson, et al., 1991) and human chorionic gonadotropin (Willey & Leidenberger, 1989) also led to the conclusion that receptor binding and biological activity are dissociable. In all these cases, uncoupling was achieved by modification,

replacement or deletion of a histidine residue. In the case of α-factor, the side chain of histidine plays an extremely crucial role in the information transduction induced by the pheromone. The ability of this side chain to trigger the mating cascade reflects both the specific atoms present in the side chain and their stereochemical orientation. Changing the chirality of the side chain from *L*- to *D*- is sufficient to cause significant loss of activity without significant loss in the ability of the pheromone to bind to the receptor.

The individual atoms of the imidazole ring mediate the transduction of the pheromone signal as well. Thus, changing the two nitrogens of the His side chain to a sulfur and a carbon results in a 100-fold decrease in activity. The five membered ring of the His side chain is not absolutely essential since it can be replaced by the six membered ring in the β-pyridylalanine analog with only a two to five-fold decrease in activity. Furthermore, the steric bulk of the side chain can be increased by methylation without total loss in activity. It appears that the 3-nitrogen of the imidazole side chain may be especially important for high pheromone potency. Thus, deletion of this nitrogen, as in [Phe$^2$]α-factor (Masui, et al., 1979) or in the β-thienylalanine analogs, results in a marked drop in activity, whereas retention of this nitrogen, as in the 3-pyridylalanine analog, leads to an analog with high activity.

The emerging picture includes the influence of a variety of electronic and steric factors in triggering the response pathway via the side chain of residue 2. Additional analysis of this side chain might prove especially fruitful in clarifying the atomic basis for mating factor activity. However, there remains the possibility that some of the changes caused by

modification of the position 2 side chain are due to overall conformational effects on the pheromone or on the resulting pheromone-receptor complex. Therefore, we are currently carrying out conformational analyses of α-factor analogs using high resolution NMR spectroscopy in both organic and aqueous solvents.

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# PART 3

Structure/function analysis of the *Saccharomyces cerevisiae* tridecapeptide mating pheromone ($\alpha$-factor) utilizing alanine-scanning substitutions.

# CHAPTER I

## Introduction

*Saccharomyces cerevisiae* is a eukaryote that can exist in either a haploid or diploid state and can undergo asexual as well as sexual reproduction. In the haploid state, mating is mediated by the reciprocal action of diffusible peptide pheromones, **a**- and α-factor. Upon exposure to the appropriate pheromone, haploid cells exhibit a variety of characteristic responses, including cell cycle arrest in $G_1$, morphological transformation (termed "shmooing"), and the synthesis of cell surface agglutinins in the preparation for cell and nuclear fusion (for reviews see Lipke & Kurjan, 1992; Sprague & Thorner, 1992).

The α-factor pheromone is an unmodified tridecapeptide coded for by the *MFα1* and *MFα2* genes (Kurjan & Herskowitz, 1982; Singh, et al., 1983). The predominant species of pheromone secreted by *MATα* cells has the sequence $Trp^1$ $His^2$ $Trp^3$ $Leu^4$ $Gln^5$ $Leu^6$ $Lys^7$ $Pro^8$ $Gly^9$ $Gln^{10}$ $Pro^{11}$ $Met^{12}$ $Tyr^{13}$ (Stotzler, et al., 1976). The peptide pheromone binds to a corresponding G protein-coupled, 7-transmembrane-domain receptor that is encoded by the *STE2* gene and expressed on the cell surface of *MAT**a*** cells [for reviews see Blumer & Thorner, 1991; Marsh, 1991].

In this study, we continue our efforts to understand the interaction of α-factor with its receptor, Ste2p. Previously, Masui et al. (1979) synthesized a variety of peptide analogs of the pheromone to determine the effect of amino acid substitution on morphological changes using wild type

*MATa* cells. Since wild type *S. cerevisiae* cleaves α-factor, the biological activities determined by Masui and coworkers also reflected the relative stability of their analogs to the *BAR1* protease encoded by the *SST1* gene (Chan & Otte, 1982). We have refined the study of the pheromone-receptor interaction by assaying both cellular responses and receptor binding activities using extended, substituted, and truncated α-factor analogs in strains lacking the *BAR1* protease (Tallon, et al., 1987; Raths, et al., 1988; Eriotou-Bargiota, et al., 1992; Levin, et al., 1993). Our earlier studies concentrated on dodecapeptide analogs lacking $Trp^1$ and included α-factor derivatives with side chain and termini modifications (Naider & Becker, 1986). In addition, structure-function analyses of synthetic analogs (Xue, et al.,1989; Xue, et al., 1996) and biophysical analysis by our group (Jelicks, et al., 1988; Jelicks, et al., 1989; Gounarides, et al., 1993; Naider, et al., 1992) and others (Higashijima, et al., 1984; Wakamatsu, et al., 1987) have allowed us to suggest that a Type II β-turn in the center of the peptide may be necessary for full biological activity. Finally, our investigations (Raths, et al., 1988; Eriotou-Bargiota, et al., 1992) have resulted in the discovery of α-factor antagonists which provide valuable insights into the function of the mating pheromone (Marsh, 1992; Stephan & Blumer, 1994).

Mapping of peptide-receptor interactions has relied heavily on the development of structure-activity relationships for substituted peptide analogs and truncated peptide fragments (for review see Strader, et al., 1995). Synthetic modification of peptide hormones involving the systematic replacement of each residue with alanine (alanine-scanning) is now accepted as a method to identify specific side chains that modulate the

interactions between polypeptide hormones and their receptors (Regoli, et al., 1984; Cunningham & Wells, 1989). This approach was also used to examine peptide-receptor interactions in small hormones such as α-melanocyte stimulating hormone (Sahm, et al., 1994), neuropeptide Y (B.-Sickinger, et al., 1994), endothelin-1 (Tam, et al., 1994), and the tachykinin "minimum fragment" (Boyle, et al., 1994). In Part 3, we report the synthesis, characterization, biological activity, and receptor affinity of 26 Ala-scanned analogs of α-factor. The results permit us to pinpoint residues that play dominant roles in signal transduction, receptor interaction, and stabilization of the biologically relevant structure of the pheromone.

# CHAPTER II

## Materials and Methods

*Synthesis of [Nle$^{12}$]α-factor and Ala-scanned α-factor analogs*—— *L*-norleucine, which is isosteric with *L*-methionine, was incorporated at position 12 to replace the original *L*-methionine in all of the analogs except [Ala$^{12}$] and [*D*-Ala$^{12}$]α-factor (Fig. 1). This replacement was shown previously to result in an analog with equal activity and receptor affinity to that of the native pheromone (Raths, et al., 1988). The insertion of Nle in place of Met improves the synthesis and the stability of the resulting peptide. The solid phase syntheses of all the Ala-scanned α-factors were carried out automatically on an Applied Biosystems 433 peptide synthesizer (Applied Biosystems, Foster City, California) starting with *N*-α-Fmoc-Tyr(OtBu)-Wang resin (0.65 mmol/gram resin, Advanced ChemTech, Louisville, Kentucky) except for the syntheses of [Nle$^{12}$,Ala$^{13}$]α-factor and [Nle$^{12}$,*D*-Ala$^{13}$]α -factor. In these cases, Fmoc-Ala (or Fmoc-*D*-Ala) was loaded onto a Wang-resin using *p*-(*N*,*N*-dimethylamino) pyridine-catalyzed esterification with dicyclohexylcarbodiimide in 1-methyl-2-pyrrolidinone followed by benzoic anhydride capping. 9-Fluorenylmethoxy-carbonyl (Fmoc) was employed for all *N*-α-protections while the side chain protecting groups were Trp(tBoc), His(Trt), Gln(Trt) and Lys(tBoc). The '0.1-mmol FastMoc' chemistry of Applied Biosystems was utilized for the peptide chain elongation with an HBTU/HOBt/DIEA catalyzed single-coupling using

**Figure 1:** Alanine substituted synthetic analogs of [Nle$^{12}$]α-factor. *D*- and *L*-isomers of alanine were synthesized in place of the native residue to study the structural and functional significance of each amino acid in the pheromone. Norleucine (Nle) was synthesized in place of the native methionine to stabilize the peptide (see Materials and methods).

| | |
|---|---|
| **W H W L Q L K P G Q P M Y** | wild type α-factor |
| **W H W L Q L K P G Q P Nle Y** | synthetic α-factor |

| (L)-Ala isomers of α-factor | (D)-Ala isomers of α-factor |
|---|---|
| **A**HWLQLKPGQPNleY | **A**HWLQLKPGQPNleY |
| W**A**WLQLKPGQPNleY | W**A**WLQLKPGQPNleY |
| WH**A**LQLKPGQPNleY | WH**A**LQLKPGQPNleY |
| WHW**A**QLKPGQPNleY | WHW**A**QLKPGQPNleY |
| WHWL**A**LKPGQPNleY | WHWL**A**LKPGQPNleY |
| WHWLQ**A**KPGQPNleY | WHWLQ**A**KPGQPNleY |
| WHWLQL**A**PGQPNleY | WHWLQL**A**PGQPNleY |
| WHWLQLK**A**GQPNleY | WHWLQLK**A**GQPNleY |
| WHWLQLKP**A**QPNleY | WHWLQLKP**A**QPNleY |
| WHWLQLKPG**A**PNleY | WHWLQLKPG**A**PNleY |
| WHWLQLKPGQ**A**NleY | WHWLQLKPGQ**A**NleY |
| WHWLQLKPGQP**A**Y | WHWLQLKPGQP**A**Y |
| WHWLQLKPGQPNle**A** | WHWLQLKPGQPNle**A** |

**Figure 1:** Alanine substituted synthetic analogs of [Nle$^{12}$]α-factor.

4 equivalents of protected amino acid and a 30 min. coupling time followed by an $Ac_2O$/HOBt/DIEA capping (10 min).

*Peptide Cleavage*——The *N*-α-deprotected peptide resin was washed thoroughly with 1-methyl-2-pyrrolidinone and dichloromethane and dried *in vacuo* for 2 hours. The cleavage was carried out in a mixture of trifluoroacetic acid (10 ml), crystalline phenol (0.75 g), ethane-1,2-dithiol (0.25 ml), thioanisole (0.5 ml) and water (0.5 ml) at room temperature for 1.5 hours. After evaporation of trifluoroacetic acid under reduced pressure, the residue was precipitated and thoroughly washed with ethyl ether and extracted into 20% aqueous acetonitrile.

*Purification and Characterization*——The crude peptide was purified by reversed phase HPLC (Hewlett-Packard Series 1050) on a semi-preparative Waters μBondapak $C_{18}$ (19x300 mm) column. Wavelength set for peptide detection was 220 nm. The cleavage product (45 mg) was dissolved in about 4 ml of aqueous acetonitrile (20%) containing 0.025% TFA, applied onto the column, and eluted with a linear gradient of water and acetonitrile both containing 0.025% TFA from 0 to 55% acetonitrile over 2 hours at a flow rate of 5 ml/min. The fractions were collected and analyzed at 220 nm by reversed phase HPLC (Hewlett-Packard Series 1050) on an analytical Waters μBondapak $C_{18}$ column (3.9 x 300 mm). The fractions of over 99% homogeneity were combined and lyophilized. The peptide purity was judged with analytical HPLC using two different solvent systems and with high performance capillary electrophoresis

(Hewlett-Packard 3D-CE Model G1600A) using a Hewlett-Packard CE capillary (L=56 cm, ID=50 mm) at 30 kV in acidic buffer (0.1*M* phosphate solution, pH=2.5). CE detection was at 220 nm. FAB-mass spectrometry was carried out at the University of Tennessee using a VG ZAB-EQ mass spectrometer equipped with a flow FAB ion source of an Ion Tech fast atom gun.

*Determination of the extinction coefficients for some typical Ala-scanned analogs*——The purified peptides with different Trp/His/Tyr combinations were weighed and dissolved volumetrically into deionized water. The resultant peptide solution was analyzed by amino acid analysis after complete hydrolysis at Brigham and Women's Hospital (Boston, MA) to determine the molar concentration. Absorption measurements were made on the same solutions using an ultraviolet spectrophotometer (Varian Model Cary1E). Molar extinction coefficients were obtained using the measured absorbance and the peptide concentration deduced from the amino acid analysis. Based on this analysis, the following $\varepsilon_{280}$ values were calculated: Peptides with $Trp^{3}$,$Tyr^{13}$ 7500 $M^{-1}\cdot cm^{-1}$; with $Trp^{1}$,$Trp^{3}$,$Tyr^{13}$ 13,500 $M^{-1}\cdot cm^{-1}$; with $Trp^{1}$,$Tyr^{13}$ 6800 $M^{-1}\cdot cm^{-1}$; with $Trp^{1}$,$Trp^{3}$ 13,500 $M^{-1}\cdot cm^{-1}$.

*Strains and Media*——The *Saccharomyces cerevisiae* strains used were *S. cerevisiae* RC629 [*MAT***a** *bar1-1* (supersensitive to α-factor due to mutation in the *BAR1* protease, allelic to *sst1*)] used in the "halo" and morphogenesis assays and *S. cerevisiae* Jena (ATCC# 4202-15-3) [*MAT***a**

*cry1 bar1-1 ade2-1 his4-580 lys2 trp1 tyr1 sup4-3*] used in the binding competition assay (Jenness, et al., 1986).

For the binding competition assay, cells were grown in YM-1 medium (0.5% yeast extract, 1% peptone, 1% succinic acid, 0.6% NaOH, 1% dextrose, 0.67% yeast nitrogen base without amino acids, 0.001% uracil, 0.001% adenine sulfate). All peptide dilutions, cell washes, and reaction incubations were carried out in YM-1 inhibitor (YM-1i) medium (YM-1 medium with 0.065% sodium azide, 0.1% KF, 0.4% TAME added). YM-1i medium is filtered through a 0.2 μm filter (Gelman Sciences VacuCap 90) and chilled on ice prior to use. For the growth arrest (halo) and morphogenesis assays, RC629 cells were grown in YEPD broth (1% yeast extract, 2% peptone, 2% dextrose).

*Growth arrest (halo) assay*——*S. cerevisiae* strain RC629 cells were grown overnight at 30°C in YEPD broth with shaking, diluted to 1 x $10^5$ cells/ml in fresh YEPD broth, and then grown to 1 x $10^6$ cells/ml. One ml of the cell suspension was mixed with 3 mls of noble agar (1%) and then overlaid onto YEPD plates (100 mm x 15 mm). Peptides were added in various amounts (between 0.078 μg and 10 μg) in 10 μl volumes to sterile filter discs (8 mm, Difco) and then placed on the solidified overlay of cells. Plates were incubated at 30°C for 24 hrs. and then observed for clear zones of growth inhibition (halos) around the disks. The data for biological potency were expressed as the diameter of the halo produced (including the diameter of the disk). The minimum value for growth arrest in this assay

is 9 mm, which represents the diameter of the filter disk and a 1 mm zone of inhibition.

*Antagonism and Synergism Assay*——Lawns of RC629 were overlaid onto YEPD plates as described in the growth arrest assay. Sterile disks were placed adjacent to each other so that the disk containing the test peptide would lie at the periphery of the halo formed by [Nle$^{12}$]α-factor. One disk was impregnated with 1μg of [Nle$^{12}$]α-factor in 10 μl $H_2O$, and the other disk was impregnated with various amounts of the test peptide in 10 μl $H_2O$. Plates were incubated as described in the growth arrest assay and the effects on halo formation noted.

*Morphogenesis (shmoo) assay*——Strain RC629 was grown at 30°C to 1x10$^6$ cells/ml in YEPD. Ninety μl of cells was aliquoted into wells of a 96-well microtiter plate (Corning, round bottom). To each well, 10μl of different concentrations of [Nle$^{12}$]α-factor alone, selected ala-scanned analogs alone, or [Nle$^{12}$]α-factor mixed with a 12X excess of selected ala-scanned analogs was added, and the mixture was incubated at 30°C with shaking (200 rpm). [Nle$^{12}$]α-factor concentrations tested were: (5.0 μg/ml, 1.0, 0.5, 0.1, 0.05, 0.025, 0.0125, 0.00625, 0.00313, 0.00156, and 0.00078). Fifteen μl of 37% formaldehyde (Sigma) was added to each well at the indicated times to fix the cell morphology. Total cells and total number of cells with shmoo morphology (an elongated, pear-shaped cell without a constricted neck at the bud site) were counted by visual inspection using a microscope

(Leitz) under 320X power. The percentage of shmoos was calculated by dividing total shmooing cells by total cells (x100).

*Binding competition assay*——Competition of bound [$^3$H]α-factor by unlabeled Ala-scanned analogs were carried out as previously described (Raths, et al., 1988). In general, Jena-cells were grown in YM-1 medium at 22°C overnight in a rotary circulating air incubator. The cell culture was counted using a hemocytometer to determine when the culture was ready for harvesting. At 22°C, the generation time for the Jena strain was 220 min. The cells were harvested at 1 x $10^7$ cells/ml by centrifugation at 5,000 x *g* at 4°C (JA-10 rotor, Beckman Model J-21C centrifuge). The pelleted cells were washed two times in ice cold YM-1i medium and resuspended to 1.25 x $10^9$ cells/ml in YM-1i to be used as a cell stock suspension. Final assay concentrations of the unlabeled Ala-scanned analogs were 1 x $10^{-5}$, 5 x $10^{-6}$, 1 x $10^{-6}$, 5 x $10^{-7}$, 1 x $10^{-7}$, and 5 x $10^{-8}$ *M*. The concentrations of the Ala-scanned analogs were determined from the $A_{280}$ for each peptide. Extinction coefficients used to calculate concentrations were based on the number and the position of Trp residues in each peptide (see above).

The binding assay was started by the addition of 100 µl of a 1:1 mixture of [$^3$H]α -factor (6 x $10^{-9}$ *M*, 8.26 Ci/mmol) and the appropriate dilution of unlabeled ala-scanned analog to 400 µl of the cell suspension. Duplicate 20 µl aliquots were removed after vortexing and put into scintillation vials (tips were ejected directly into the vials) to determine total labeled ligand concentration. All cells, peptide dilutions and media were kept on ice until the start of the assays. Cells were prewarmed for 10 min.

at room temperature and then 20 min. incubations with peptides were carried out at room temperature. Assays were staggered by 2 or 3 minutes to allow for multiple sample handling and consistent incubation times. After the 20 min. incubation, duplicate samples of 200 µl were diluted in YM-1i and filtered over 1% bovine serum albumin presoaked GN-6 Metricel filters (Gelman Sciences, Inc., Ann Arbor, MI) under house vacuum. The dilution tube was then rinsed twice with ice cold YM-1i and filtered over the same filter. Filters were directly washed with cold YM-1i before being released from vacuum and placed in scintillation vials for counting. Binding of labeled α-factor to filters in the absence of cells was less than 20 cpm. Specific binding is defined as (mean bound cpm/total mean cpm) x 100. Mean bound cpm is the average of multiple binding point determinations for each analog concentration tested (n=4) and total mean cpm is the average of the total counts incubated with each analog (n=4). Results for each analog were expressed as a percent of total binding in the absence of the analog.

*HPLC purification of [$^3$H]α-factor*——Synthetic [Nle$^{12}$]α-factor was labeled by reduction of dihydroproline containing α-factor by the TR3 hydrogenation procedure of Amersham International as described previously (Raths, et al., 1988). Labeled peptide was purified by HPLC using a µBondapak $C_{18}$ 10mm column (3.9 mm x 300 mm) from Waters Chromatography Division on a Beckman System Gold HPLC. Unpurified labeled peptide (dissolved in ethanol/water 1:1) was dried *in vacuo* and redissolved in sterile water. The peptide solution was injected into the

HPLC and eluted with a water/acetonitrile gradient of 0-40%. Fractions containing peptide eluting from the column similarly to unlabeled α-factor were collected and counted for radioactivity in a scintillation counter. All collected fractions were dried, resuspended in sterile water, combined, and the concentration and specific activity of the purified labeled peptide was determined.

# CHAPTER III

## Results

*Synthesis and characterization of alanine-substituted α-factor analogs*—The crude product obtained from the TFA-cleavage of the resin-bound α-factor analogs contained approximately 50~85% of the major component as indicated by analytical reversed-phase HPLC. Semi-preparative HPLC purification yielded peptides with a homogeneity greater than 99% as judged with analytical HPLC using both acetonitrile-water and methanol-water elution systems (Table 1) as well as high performance capillary electrophoresis (data not shown). The recovered yields of all peptides varied from 40% to 70%. FAB-MS gave the calculated monoisotopic mass values for the molecular ion to within one dalton (Table 1). Analogs containing $Ala^1$, $Ala^2$, $Ala^3$, $Ala^4$ or $Ala^{13}$ replacements were subjected to amino acid analysis during the calibration of the extinction coefficients (see Materials and Methods, Chapter II). In each case, the expected ratios were obtained. The extinction coefficient measurements also confirmed the presence of either one or two Trp residues in the alanine scan analogs.

*Biological activity of Ala-substituted [$Nle^{12}$]α-factor analogs in a growth arrest (halo) assay*——The biological activity of the Ala-scanned analogs was determined using *S. cerevisiae* strain RC629. Increasing amounts of pheromone caused an increase in growth arrest as measured by halo size

**Table 1 :** Characteristics of Ala-scanned analogs of α-factor.
(*a*) *L*- and *D*-alanine scanned isomers of α-factor with a general sequence of [Nle$^{12}$]α-factor. (*b*) $K' = (V_{retention} - V_{void})/V_{void}$, measured on $C_{18}$ reversed-phase analytical HPLC with a (I) water/acetonitrile (both containing 0.025% TFA) linear gradient from 85% water to 50% water in 40 minutes or with a (II) methanol/water (both containing 0.025% TFA) linear gradient from 80% water to 20% water in 30 min.
(*c*) Determined by FAB-MS.

Table I

*Characteristics of Ala-scanned analogs of α-factor*

| peptide[a] | residue replaced | K'[b] I | K'[b] II | calc. $[M+1]^+$ | found[c] $[M+1]^+$ |
|---|---|---|---|---|---|
| Ala[1] | Trp | 7.49 | 6.37 | 1550.8 | 1551.1 |
| Ala[2] | His | 11.0 | 8.81 | 1599.9 | 1600.3 |
| Ala[3] | Trp | 5.01 | 6.57 | 1550.8 | 1551.1 |
| Ala[4] | Leu | 8.04 | 6.84 | 1623.8 | 1624.5 |
| Ala[5] | Gln | 5.23 | 7.68 | 1608.9 | 1609.2 |
| Ala[6] | Leu | 2.42 | 6.73 | 1623.8 | 1624.5 |
| Ala[7] | Lys | 10.2 | 8.73 | 1608.8 | 1608.9 |
| Ala[8] | Pro | 8.83 | 7.51 | 1639.9 | 1640.1 |
| Ala[9] | Gly | 7.85 | 7.62 | 1679.9 | 1680.1 |
| Ala[10] | Gln | 3.31 | 7.88 | 1608.9 | 1609.5 |
| Ala[11] | Pro | 6.44 | 7.62 | 1639.9 | 1640.5 |
| Ala[12] | Nle | 7.28 | 7.13 | 1623.8 | 1624.5 |
| Ala[13] | Tyr | 7.43 | 7.49 | 1573.9 | 1573.9 |
| *D*-Ala[1] | Trp | 6.69 | 6.74 | 1550.8 | 1551.4 |
| *D*-Ala[2] | His | 3.86 | 8.94 | 1599.9 | 1599.9 |
| *D*-Ala[3] | Trp | 2.31 | 6.69 | 1550.8 | 1551.0 |
| *D*-Ala[4] | Leu | 3.29 | 6.93 | 1623.8 | 1623.9 |
| *D*-Ala[5] | Gln | 5.77 | 7.90 | 1608.9 | 1609.0 |
| *D*-Ala[6] | Leu | 6.38 | 6.20 | 1623.8 | 1623.9 |
| *D*-Ala[7] | Lys | 8.80 | 8.83 | 1608.8 | 1608.9 |
| *D*-Ala[8] | Pro | 3.74 | 7.12 | 1639.9 | 1639.9 |
| *D*-Ala[9] | Gly | 8.42 | 8.00 | 1679.9 | 1679.9 |
| *D*-Ala[10] | Gln | 8.73 | 8.04 | 1608.9 | 1609.0 |
| *D*-Ala[11] | Pro | 8.93 | 8.34 | 1639.9 | 1640.0 |
| *D*-Ala[12] | Nle | 7.37 | 6.83 | 1623.8 | 1623.9 |
| *D*-Ala[13] | Tyr | 8.26 | 7.84 | 1573.9 | 1574.0 |

(Fig. 2A). These data were plotted as halo size versus the amount of pheromone added to the cell lawn and linearized by regression analysis (Fig. 2B). To compare the relative activities of different peptides, the amount of peptide causing a halo size of 15mm was determined from the regression line. For most peptides at least two dose response values were above and below the intersection point and extrapolation of the data was not necessary (Fig. 2B and Table 2).

In the *L*-alanine scanned series, the amount of pheromone causing a 15 mm zone of inhibition varied from 0.22 μg ([Ala$^{1}$,Nle$^{12}$]α-factor) to 2.8 μg ([Ala$^{9}$,Nle$^{12}$]α-factor) (Table 2). These activities represent an approximately 3.5-fold increase and an approximately 3.7-fold decrease in activity, respectively, compared to [Nle$^{12}$]α-factor. Placement of an *L*-Ala residues in positions 4, 5, 6, 8, or 11 had at most about a 40% effect on activity, whereas *L*-Ala at positions 2, 3, 7 and 13 caused approximately a 3-fold decrease in potency. In general, substitution with an *L*-Ala residue had a relatively modest effect on the biological activity of the pheromone as judged by the growth arrest assay.

Several of the *D*-Ala containing analogs (positions 2, 3, 4 and 10) exhibited no activity in the growth arrest assay at amounts up to 10 μg/disk. This reflected greater than a 100-fold decrease in potency. Substitution of *D*-Ala at positions 5 or 8 had only a modest effect on potency whereas *D*-Ala at positions 1, 7, and 11 resulted in analogs which were 2-4 times less active than [Nle$^{12}$]α-factor. [*D*-Ala$^{6}$,Nle$^{12}$]α-factor and [*D*-Ala$^{13}$,Nle$^{12}$]α-factor were approximately 20-fold less active than the parent pheromone. Interestingly, [*D*-Ala$^{9}$,Nle$^{12}$]α-factor was the most active pheromone in

**Figure 2 :** The halo assay measuring the biological activity of the peptide analogs. ***A***- The growth arrest activity of [Nle$^{12}$]α-factor (pictured) or analogs (not pictured) was recorded as the diameter of the halo (in mm) produced by various amounts of the test peptide as shown. ***B***- The size of the halo produced was plotted vs. amount of peptide tested. The amount of peptide required to produce a 15mm halo was determined from the first-order regression line equation (for [Nle$^{12}$]α-factor this value was 0.764μg as shown) and used to compare the activities of the analogs relative to [Nle$^{12}$]α-factor (see Act. ratios, Table 2).

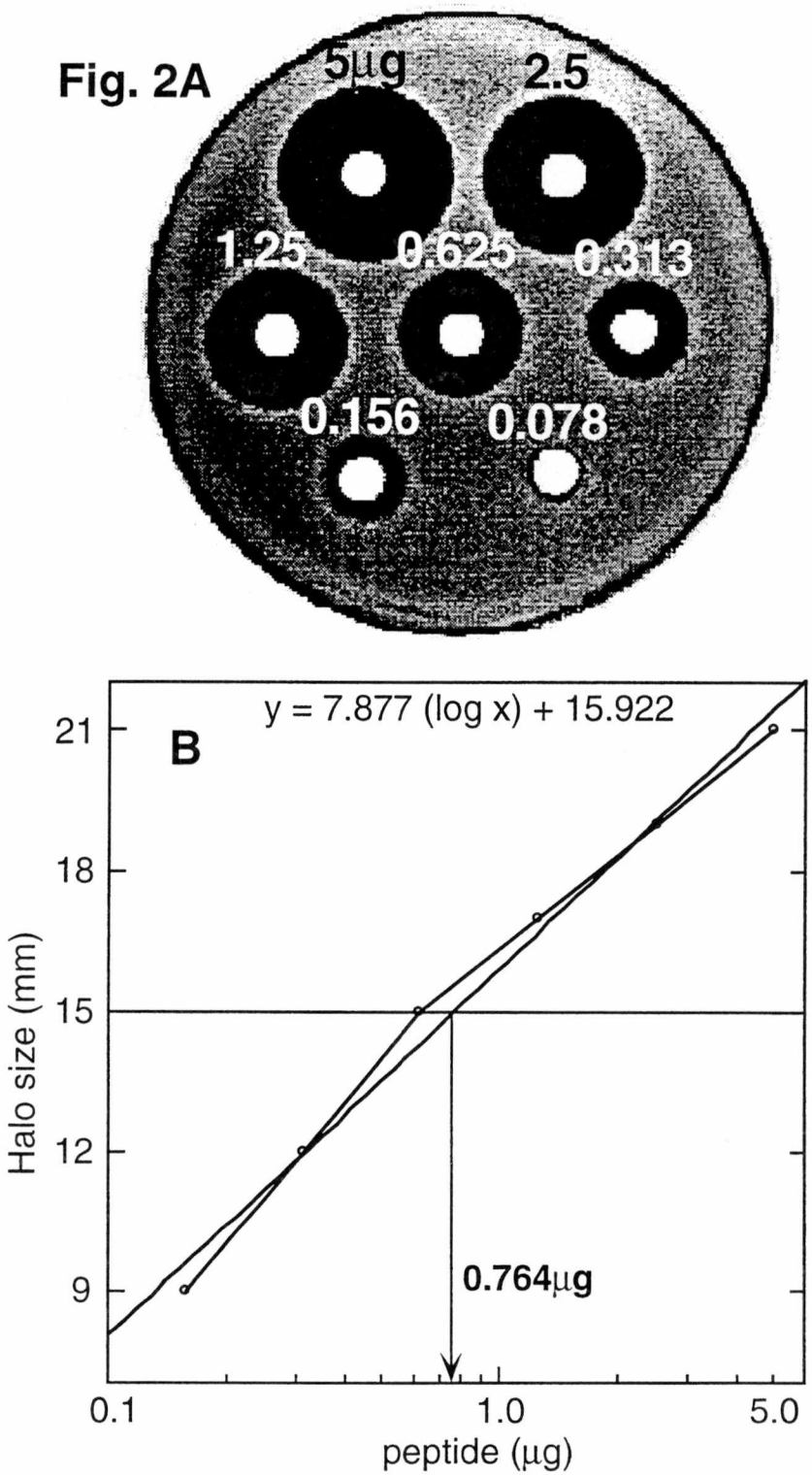


**Figure 2 :** The halo assay measuring the biological activity of the Ala-scanned analogs.

**Table 2 :** Biological activity and binding affinities of the Ala-scanned α-factor analogs. (*a*) Peptide analogs designated by position of alanine substitution and synthesized with norleucine$^{12}$ in place of native methionine$^{12}$ except for the *L*- and *D*-Ala$^{12}$ peptides. (*b*) Comparisons of biological activity among analogs is shown by the amount of peptide (μg) required to produce a growth arrest halo of 15mm interpolated from a 1$^{st}$ order regression line equation and rounded to the nearest 0.01 (see Figure 2B). (*c*) The ratio of biological activity represents the amount of [Nle$^{12}$]α-factor required to produce a 15mm halo divided by the amount of peptide analog required to produce a 15mm halo. (*d*) The amount of analog required to inhibit binding of [$^{3}$H,Nle$^{12}$]α-factor by 50% ($IC_{50}$) as measured by a binding competition assay (see Figure 4). The $IC_{50}$ of [Nle$^{12}$]α-factor is 1.4 x $10^{-8}$*M* (n=7). (*e*) The $IC_{50}$ ratio represents the $IC_{50}$ of [Nle$^{12}$]α-factor divided by the $IC_{50}$ of the analog (x1000). (*f*) na - no activity. (*g*) The analogs *D*-Ala$^{6}$ and *D*-Ala$^{13}$ did not produce a halo of 15mm or greater at any concentration tested; the biological activity reported for these two analogs was extrapolated. (*h*) The analogs *D*-Ala$^{12}$ and *D*-Ala$^{13}$ were not able to demonstrate 50% binding competition at the highest concentration tested.

## Table 2

*Biological activity and binding affinities of the Ala-scanned α-factor analogs*

| peptide [a] | Biol. act. [b] | Act. ratio [c] | $IC_{50}$ [d] ($\mu M$) | $IC_{50}$ ratio [e] |
|---|---|---|---|---|
| Nle$^{12}$α-factor | 0.76 | 1.00 | 0.014 | 1000 |
| Ala$^{1}$ | 0.22 | 3.46 | 1.00 | 14 |
| Ala$^{2}$ | 2.14 | 0.36 | 1.60 | 9 |
| Ala$^{3}$ | 2.14 | 0.36 | 0.60 | 23 |
| Ala$^{4}$ | 0.82 | 0.94 | 0.40 | 35 |
| Ala$^{5}$ | 0.53 | 1.44 | 0.08 | 175 |
| Ala$^{6}$ | 0.92 | 0.83 | 3.20 | 4 |
| Ala$^{7}$ | 2.23 | 0.34 | 0.29 | 48 |
| Ala$^{8}$ | 0.58 | 1.32 | 0.16 | 88 |
| Ala$^{9}$ | 2.80 | 0.27 | 5.00 | 3 |
| Ala$^{10}$ | 1.91 | 0.40 | 1.50 | 9 |
| Ala$^{11}$ | 1.13 | 0.68 | 0.90 | 16 |
| Ala$^{12}$ | 0.42 | 1.82 | 2.20 | 6 |
| Ala$^{13}$ | 2.30 | 0.33 | 7.80 | 2 |
| *D*-Ala$^{1}$ | 2.41 | 0.32 | 1.80 | 8 |
| *D*-Ala$^{2}$ | na [f] | --- | 2.30 | 6 |
| *D*-Ala$^{3}$ | na | --- | 0.27 | 52 |
| *D*-Ala$^{4}$ | na | --- | 0.43 | 33 |
| *D*-Ala$^{5}$ | 0.72 | 1.06 | 0.88 | 16 |
| *D*-Ala$^{6}$ | 13.60 [g] | 0.06 | 8.20 | 2 |
| *D*-Ala$^{7}$ | 3.30 | 0.23 | 19.50 | 1 |
| *D*-Ala$^{8}$ | 0.93 | 0.82 | 4.20 | 3 |
| *D*-Ala$^{9}$ | 0.13 | 5.79 | 0.01 | 1400 |
| *D*-Ala$^{10}$ | na | --- | 22.00 | 1 |
| *D*-Ala$^{11}$ | 2.02 | 0.38 | 24.00 | 1 |
| *D*-Ala$^{12}$ | 5.47 | 0.14 | >41.00 [h] | <0.3 |
| *D*-Ala$^{13}$ | 14.10 [g] | 0.05 | >46.00 [h] | <0.3 |

either the *L*-Ala or *D*-Ala scanned series exhibiting 5.8-fold higher activity than [Nle$^{12}$]α-factor.

*Antagonistic and Synergistic Ala-Scanned Analogs——The D*-Ala substitutions in positions 2, 3, 4, and 10 resulted in a total loss of biological activity as measured in the halo assay, yet were able to compete (albeit very weakly in some cases) for binding to the α-factor receptor (see *Competition for binding to the STE2 receptor*). Peptides that bind to receptors but do not elicit a signal are termed antagonists, while peptides that enhance the potency of the native α-factor without having any activity of their own are termed synergists. We tested these 4 analogs for antagonism/synergism (described in Materials and Methods), and found that when the peptides containing *D*-Ala$^3$ or *D*-Ala$^4$ were placed on a lawn of cells proximate to [Nle$^{12}$]α-factor, the growth arrest halos caused by this pheromone became noticeably flattened on the side adjacent to the analog (Fig 3A). The analog with a *D*-Ala$^2$ substitution demonstrated a very weak competition of the α-factor induced growth arrest (Fig. 3A). These antagonistic responses are similar to those previously described for desTrp$^1$desHis$^2$[Nle$^{12}$]α-factor (Fig 3A; E.-Bargiota et al.,1992). The antagonism caused by the *D*-Ala$^2$ analog is very weak (Fig. 3A). In contrast, the growth arrest halo caused by [Nle$^{12}$]α-factor was markedly elongated when [*D*-Ala$^{10}$,Nle$^{12}$]α-factor was placed adjacent to this pheromone (Fig 3B). This synergistic effect was previously noted with desMet$^{12}$desTyr$^{13}$α-factor (Fig. 3B; E.-Bargiota et al., 1992).

**Figure 3 :** Halo assays demonstrating antagonistic and synergistic activity of biologically inactive Ala-scanned analogs. A lawn of strain RC629 was challenged with 1μg of [Nle$^{12}$]α-factor (α, disk in center of growth arrest halo) and 15μg of peptide analogs (*D*-Ala$^{2}$-, *D*-Ala$^{3}$-, *D*-Ala$^{4}$-, *D*-Ala$^{10}$-, desTrp$^{1}$desHis$^{2}$[Nle$^{12}$]α-factor a known antagonist [desW$^{1}$H$^{2}$], and desMet$^{12}$desTyr$^{13}$-α-factor a known synergist [desM$^{12}$Y$^{13}$]). ***A***- The analogs *D*-Ala$^{2}$-, *D*-Ala$^{3}$-, and *D*-Ala$^{4}$- demonstrated varying degrees of antagonism as represented by the flattened halos. The same amount of a known antagonist (desW$^{1}$H$^{2}$) is shown for comparison. ***B***- The *D*-Ala$^{10}$- analog demonstrated a synergistic effect as seen by the elongation of the [Nle$^{12}$]α-factor halo towards the disk containing [*D*-Ala$^{10}$,Nle$^{12}$]α-factor. For comparison, the same amount of a known synergist (desM$^{12}$Y$^{13}$) and antagonist (desW$^{1}$H$^{2}$) as well as a $H_2O$ control are shown.

Fig. 3A

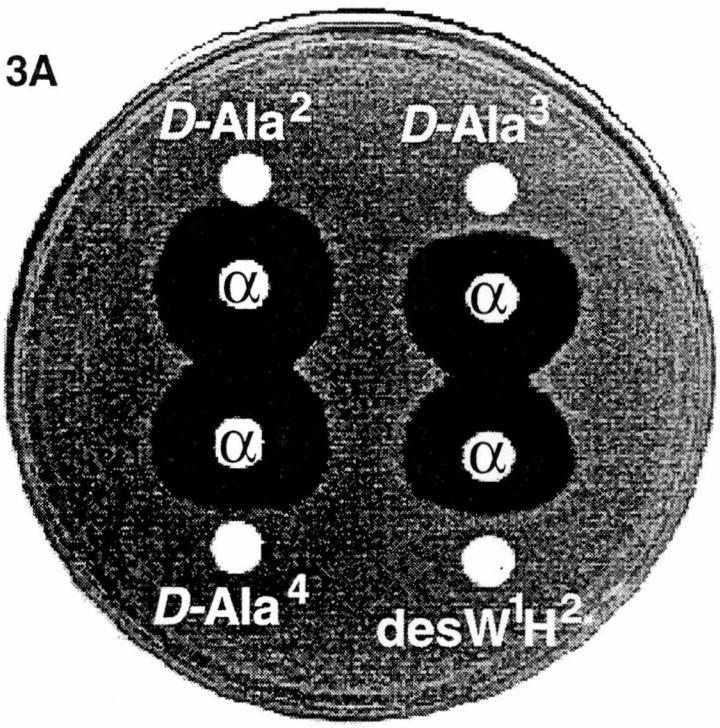


Fig. 3B

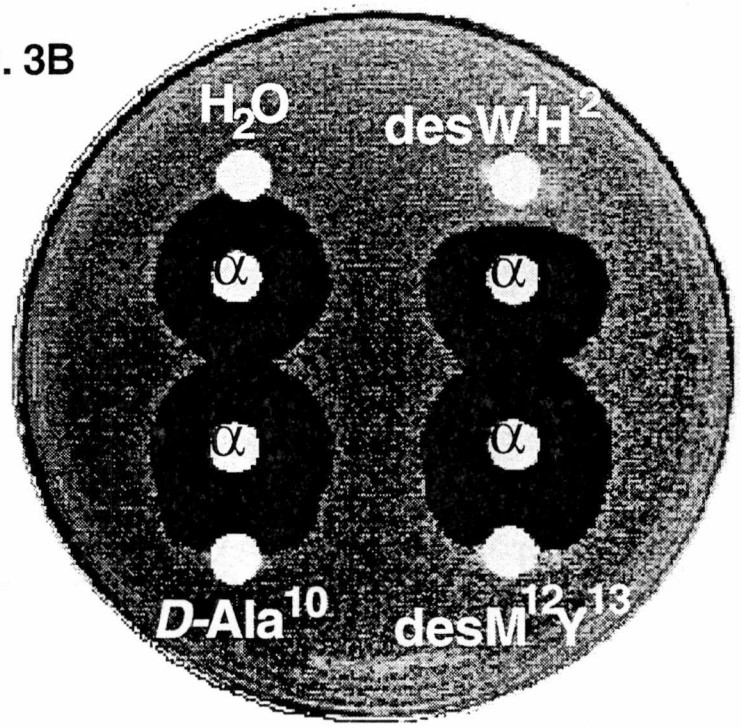


**Figure 3 :** Halo assays demonstrating antagonistic and synergistic activity of biologically inactive Ala-scanned analogs.

*Morphogenesis (shmoo) and recovery assays*——To further evaluate the biological activity of the Ala-scanned analogs of [Nle$^{12}$]α-factor we examined their activity in a cell morphogenesis assay (shmoo assay). In this experiment, cells challenged with pheromone cease vegetative growth and become elongated and pear shaped. We found that the trends of the alanine-scanned series in the morphogenesis assay were similar to those found in the growth arrest assay in that the *D*-Ala containing peptides were often significantly less active than their *L*-Ala containing homologs (data not shown). Excluding the antagonists and synergist mentioned above, the relative activities varied by 12-fold in the *L*-Ala series and 120-fold in the *D*-Ala series as judged by the growth arrest assay. In contrast, only a 4-fold variation was observed using the shmoo assay on the same set of peptides. Furthermore, the recovery of the shmoos induced by challenge with various analogs differed appreciably (see below).

Neither [*D*-Ala$^3$,Nle$^{12}$]α-factor, [*D*-Ala$^4$,Nle$^{12}$]α-factor or [*D*-Ala$^{10}$, Nle$^{12}$]α-factor caused a change in cell shape at concentrations of up to 5 μg/ml which was more than 50 times the concentration of [Nle$^{12}$]α-factor required to cause 50% shmooing (Fig 4A). This lack of biological response correlates with the lack of response seen in the growth arrest assay. In contrast, [*D*-Ala$^2$, Nle$^{12}$]α-factor, which was not active and showed a small competition with the parent pheromone in the growth arrest assay (Fig. 3A), exhibited a partial activity in the shmoo assay (Fig 4A). When RC629 cells were challenged with [Nle$^{12}$]α-factor mixed with a 12-fold excess of either *D*-Ala$^4$ or *D*-Ala$^{10}$ analogs, 0.7 μg/ml or 0.009 μg/ml of [Nle$^{12}$]α-factor, respectively, was required to induce 50% shmooing (Fig 4B).

**Figure 4A:** Morphogenesis assays of [Nle$^{12}$]α-factor and selected alanine-scanned analogs. Morphogenesis induction of the analogs inactive in the growth arrest (halo) assay. Symbols represent [Nle$^{12}$]α-factor (○), *D*-Ala$^{2}$- (▼), *D*-Ala$^{3}$- (□), *D*-Ala$^{4}$- (■), and *D*-Ala$^{10}$- (◆) analogs.

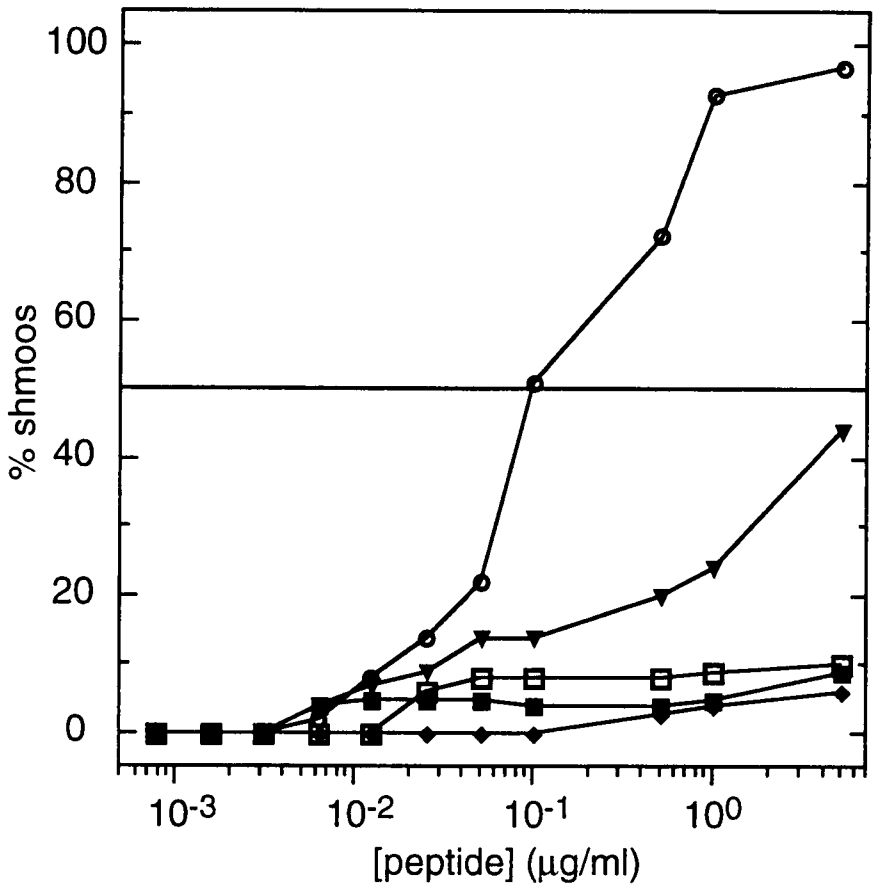


**Figure 4A :** Morphogenesis assays of [Nle$^{12}$]α-factor and selected alanine-scanned analogs.

**Figure 4B:** Morphogenesis induction by selected analogs. The ability to induce morphogenesis ("shmooing") by [Nle$^{12}$]α-factor alone (○), [Nle$^{12}$]α-factor mixed with a 12-fold excess of [*D*-Ala$^{4}$,Nle$^{12}$]α-factor (●), or [Nle$^{12}$]α-factor mixed with a 12-fold excess of [*D*-Ala$^{10}$,Nle$^{12}$]α-factor (▲) is shown as a function of [Nle$^{12}$]α-factor concentration scored at 4 hrs. post pheromone challenge.

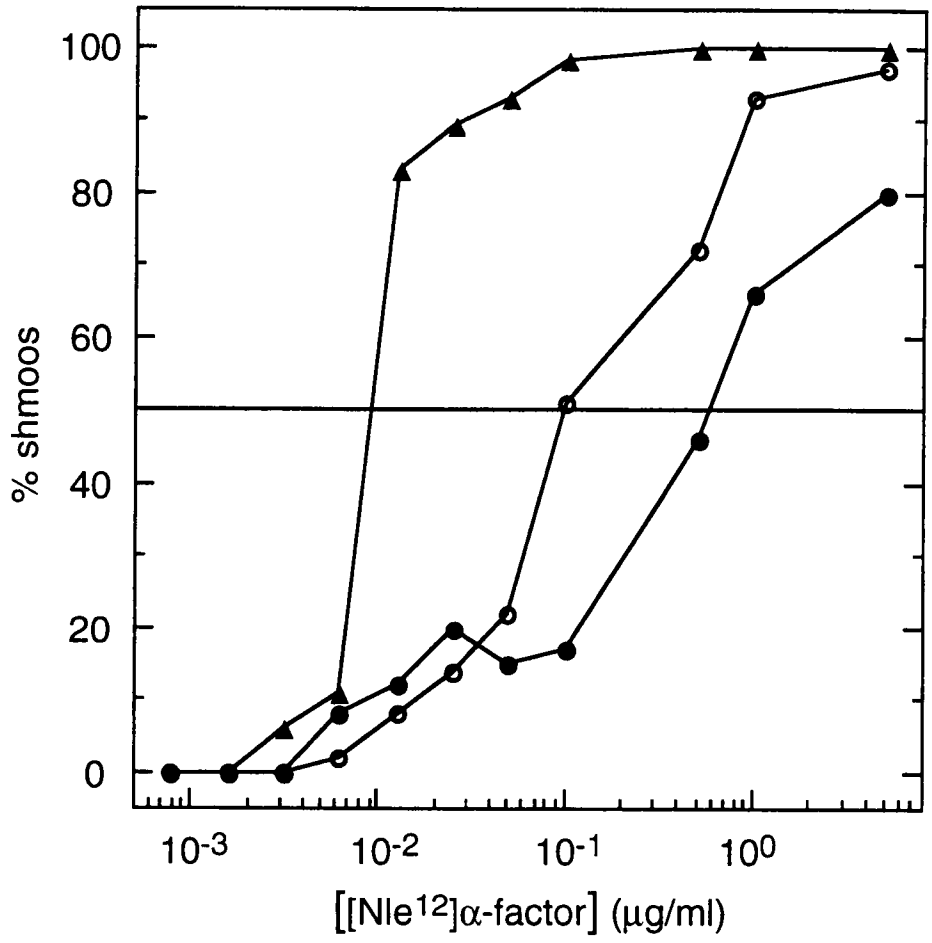


**Figure 4B :** Morphogenesis induction by selected analogs.

[Nle$^{12}$]α-factor alone caused 50% shmooing at a concentration of 0.1 μg/ml. *D*-Ala$^3$ affected [Nle$^{12}$]α-factor shmooing similarly to *D*-Ala$^4$ (data not shown). These findings indicate that the both the *D*-Ala$^3$ and the *D*-Ala$^4$ containing pheromones are antagonists, whereas the *D*-Ala$^{10}$ containing peptide is a synergist as judged by a cell morphogenesis assay.

The partial agonist activity observed for [*D*-Ala$^2$,Nle$^{12}$]α-factor in the "shmoo" assay was unexpected given the findings of the growth arrest assay. The morphogenesis response of cells challenged with *D*-Ala$^2$ and [Nle$^{12}$]α-factor was therefore measured over time. The test strain RC629 when challenged with *D*-Ala$^2$ exhibited a rapid recovery as compared to the recovery of cells induced by [Nle$^{12}$]α-factor (Fig. 4C). [Nle$^{12}$]α-factor arrested cells were fully recovered at 8 hrs. after initial pheromone challenge while *D*-Ala$^2$ challenged cells were "recovered" by 5 hrs. and demonstrated unusual cell morphologies (see Fig. 6 in Discussion section).

*Competition for binding to the STE2 receptor*—Binding affinity of the Ala-substituted analogs for the α-factor receptor was determined by measuring the competition between the analogs and [$^3$H]α-factor. Binding curves of representative analogs are shown in Fig. 5 and the concentrations of peptide required to displace 50% of [$^3$H]α-factor from the receptor are given in Table 2. Replacement of a naturally occurring residue with either *L*- or *D*-Ala almost always resulted in a decrease in affinity for the receptor. In the *L*-Ala scanned series the drop in affinity varied from 6-fold ( [Ala$^5$,Nle$^{12}$]α-factor) to 500-fold ([Ala$^{13}$,Nle$^{12}$]α-factor). The decrease in affinity is lowest for replacements at positions 5, 7, 8. Except for

**Figure 4C:** The duration and extent of morphogenesis. The duration and extent of morphogenesis induction was measured for [Nle$^{12}$]α-factor (○) at 1.0 μg/ml and for the *D*-Ala$^{2}$-analog at 5.0 μg/ml (●) and 1.0 μg/ml (▼).

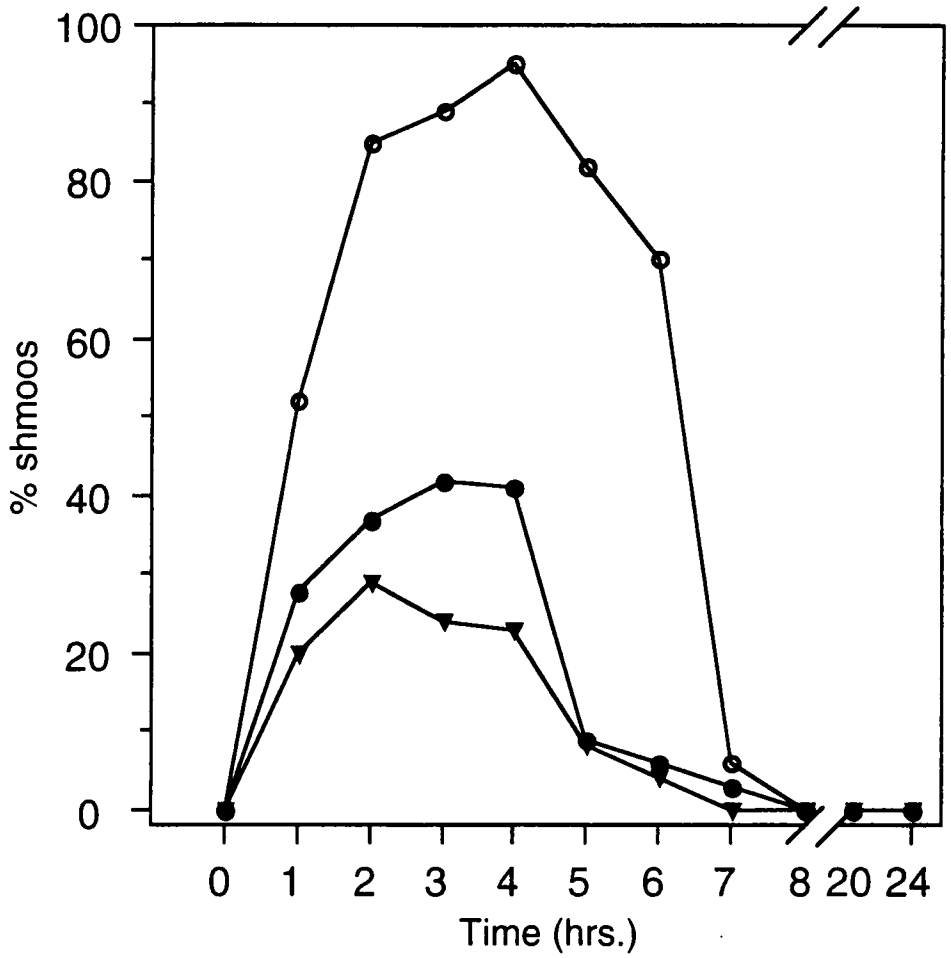


**Figure 4C :** The duration and extent of morphogenesis.

**Figure 5:** Competition binding assay of [$^{3}$H,Nle$^{12}$]α-factor. Binding of the Ala-scanned analogs to 4202-15-3 (*Mata*) cells was performed in competition with [$^{3}$H]α-factor as described under "Materials and Methods". The concentration required to displace 50% of labeled [$^{3}$H]α-factor ($IC_{50}$) was determined from the graphs and compared to the $IC_{50}$ of unlabeled [Nle$^{12}$]α-factor. The binding curves of some representative compounds are shown: [Nle$^{12}$]α-factor (○), [Ala$^{7}$,Nle$^{12}$]α-factor (●), [Ala$^{11}$,Nle$^{12}$]α-factor (▲), [*D*-Ala$^{11}$,Nle$^{12}$]α-factor (■), [*D*-Ala$^{13}$,Nle$^{12}$]α-factor (◆).

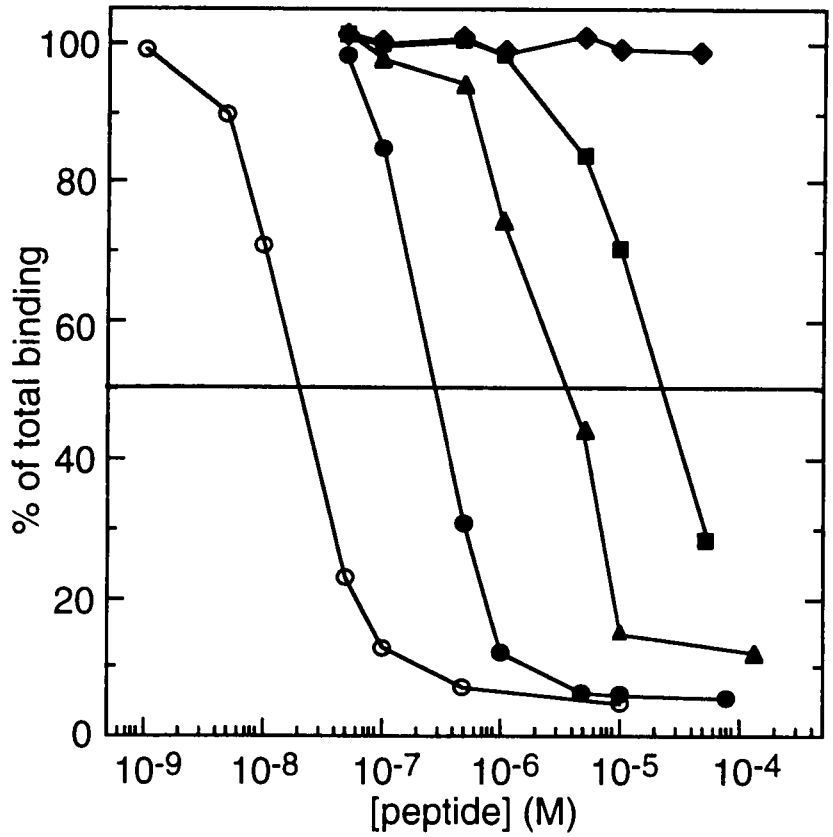


**Figure 5 :** Competition binding assay of [$^{3}$H,Nle$^{12}$]αfactor.

replacement at position 11, which leads to a 60-fold reduction in affinity, placement of Ala at any of the 5 carboxyl terminal residues resulted in a drop in affinity of greater than 100-fold. In contrast, except for position 2, where replacement with *L*-Ala leads to a 110-fold drop in affinity, placement of *L*-Ala at any of the 5 amine terminal residues resulted in a drop of affinity of 70-fold or lower. The trend found for *D*-Ala substitution paralleled the findings in the *L*- Ala series with the exception that the magnitude of the drop was greatly increased for residues near the center (residues 5, 7, 8) and carboxyl terminus (residues 10-13) of the pheromone. For example, the ratio of the $IC_{50}$ values for the *L*- and *D*- analogs is between one and two for substitutions in residues 1-4 whereas it is >10 for replacement at positions 5, 7, 8, 10, 11, 12 and perhaps 13. Noteably, the only peptide with an increased affinity compared with [$Nle^{12}$]α-factor is [*D*-$Ala^{9}$,$Nle^{12}$]α-factor which binds 50% more avidly than the parent peptide and more than 400 times better than [$Ala^{9}$,$Nle^{12}$]α-factor.

# CHAPTER IV

## Discussion

In this study, all 13 amino acids comprising the α-factor pheromone were systematically replaced with *L*-alanine or *D*-alanine to identify those residues involved in the mating response. In Table 2, we present the biological and binding assay data as ratios of activity or affinity compared to [Nle$^{12}$]α-factor for easier comparison of the signaling and binding abilities of the different analogs. From an analysis of these data and from previous studies, a picture has emerged in which α-factor may be divided into parts that may be dominant in various functional aspects of the pheromone. Although the domains are not absolute in their delineation, we discuss below the data organized in structure-function domains.

*N-terminal signaling domain*—The *L*-Ala$^{1}$ analog of [Nle$^{12}$]α-factor showed a >3-fold increase in biological activity in the halo assay even though the binding of this analog was 71-fold less than that of [Nle$^{12}$]α-factor (Fig. 5, Table 2). This finding suggests that although Trp$^{1}$ contributes to the free energy of binding ($\Delta G^{o}$ for [Nle$^{12}$]α-factor and [Ala$^{1}$,Nle$^{12}$]α-factor were -10.75 kcal/mole and -8.25 kcal/mole, respectively), the side chain of this bulky residue may somehow attenuate signal transduction. For example, the binding of tryptophan to the receptor may negatively affect the isomerization of the receptor to its activated state. This possibility would be consistent with the extended ternary complex model postulated by Samama, et al. (1993). In this model, 7-transmembrane, G protein-coupled

receptors can exist in two conformations: inactive and active. Binding of agonists to receptor induces isomerization to the activated state of the receptor or stabilizes this activated state. Only the activated state of the receptor forms a ternary complex with ligand and G-protein that initiates signal transduction. If $Trp^1$ in α-factor is somehow preventing or slowing down receptor isomerization, then an alanine substitution in position 1 might release the attenuating effect of the residue (increase activity) and also show a decrease in binding affinity of the peptide due to loss of a favorable $Trp^1$ interaction with Ste2p.

Masui, et al. (1979) reported that des$Trp^1$[$Nle^{12}$]α-factor was as active as wild type α-factor and that replacement of $Trp^1$ with *D*-$Trp^1$, $Tyr^1$, or $Lys^1$ caused a severe loss of bioactivity. We previously showed that des$Trp^1$[$Nle^{12}$]α-factor was 16-fold less active than the parent pheromone and had a 10-fold lower binding affinity (Raths, et al., 1988; Shenbagamurthi, et al., 1983). Recent studies of the role of $Trp^1$ in binding and signaling showed that $Gly^1$ and $Leu^1$ analogs of [$Nle^{12}$]α-factor had 20- to 60-fold lower receptor affinity, respectively, whereas these peptides were slightly more active than the parent pheromone in a growth arrest assay (Y. L. Zhang and H.-F. Lu, *personnal communication).* These results parallel the findings with [$Ala^1$,$Nle^{12}$]α-factor and provide a clear example of a separation of signaling and binding at this position of the peptide.

The similar decrease in binding affinity observed for both the *L*-$Ala^1$ and *D*-$Ala^1$ analogs suggests that the position 1 methyl side chain of the substituted alanine residue does not strongly interact with the receptor. The approximately 10-fold decrease in biological activity of the *D*-$Ala^1$

analog compared to the *L*-Ala$^1$ homolog may reflect a negative influence of the *D*-side chain on receptor isomerization. This would be consistent with the extremely low activity of the *D*-Trp$^1$ analog reported by Masui, et al. (1979).

In Part 2, we concluded that while the imidazole of His$^2$ was not essential for pheromone activity or binding to the receptor, the chemical composition of the side chain of residue 2 did play a crucial role in the biological activity induced by α-factor (Levin, et al., 1993). Replacement of this ring with a thiophene moiety (β-*L*-thienylalanine) resulted in a large decrease in bioactivity and a more modest decrease in receptor affinity. The corresponding β-*D*-thienylalanine-containing pheromone lost activity almost completely but still bound strongly to the receptor. The Ala-scanned analogs studied herein provide additional insights into the importance of the position two side chain. The Ala$^2$-analog was one of the weakest agonists in the *L*-alanine scan series. It also exhibited a 114-fold drop in binding affinity. Interestingly, the corresponding *D*-Ala$^2$-homolog was completely inactive in the growth arrest assay (scored at 24 hours), had low receptor affinity, and was a weak antagonist (Table 2, Figure 3A). However, [*D*-Ala$^2$,Nle$^{12}$]α-factor did elicit a partial morphogenesis response when tested in the shmoo assay which was scored at 4 hrs. after pheromone challenge (Fig. 4B). This raised the possibility that these seemingly contradictory responses from the growth arrest and morphogenesis assays were due to the nature of the recovery of the yeast strain to [*D*-Ala$^2$,Nle$^{12}$]α-factor. A recovery assay of [Nle$^{12}$]α-factor and the *D*-Ala$^2$- containing analog showed that strain RC629 responded weakly to

the *D*-Ala$^2$- analog and recovered faster to continue vegetative growth (budding of daughter cells) as compared to [Nle$^{12}$]α-factor (Fig. 4C). The unusual morphologies and quick recovery of RC629 cells in response to [*D*-Ala$^2$,Nle$^{12}$]α-factor indicated, perhaps, a reversion to vegetative growth as opposed to a true recovery from $G_1$ arrest (Fig. 6). Thus, cells may be stimulated just enough to initiate a morphological response but not enough to sustain the response or initiate the normal recovery pathway(s). The results with the Ala-scanned analogs are consistent with our conclusion that whereas the His side chain of the native α-factor is not essential, it is important for signaling and also contributes to the free energy of binding.

The position 3 and 4 Ala-scan analogs had very similar influences on pheromone binding. In both cases, a modest reduction in receptor affinity was observed for both the *L*- and *D*- diastereomers. In contrast, the Ala$^4$ analog had high potency, whereas the Ala$^3$ analog was almost three times less active than [Nle$^{12}$]α-factor, and both the *D*-Ala$^3$ and the *D*-Ala$^4$ peptides had no activity in the growth arrest or morphogenesis assays (Fig. 4B and Table 2). The low potency of the Ala$^3$ analog indicates that the Trp$^3$ indole moiety may positively affect activation of the receptor. The lack of activity of both analogs with *D*-residues in positions 3 and 4 and their relatively high receptor affinity together suggests that these residues at the amine terminus play a very important role in signal transduction but are not the major determinants of pheromone binding. On this basis, we suggest that the first four residues at the N-terminus of α-factor are important residues in the formation of a "signaling domain" for this peptide (Fig. 7).

**Figure 6 :** Induction and recovery of RC629 cells from [Nle$^{12}$]α-factor or [*D*-Ala$^{2}$,Nle$^{12}$]α-factor challenge. RC629 cells (panel A) challenged with [Nle$^{12}$]α-factor or [*D*-Ala$^{2}$,Nle$^{12}$]α-factor demonstrate morphogenesis within 1 to 2 hrs. after addition of peptide. Cells challenged with [Nle$^{12}$]α-factor formed normal shmoos at 2 hrs. (panel B), recovered and started budding (vegetative growth) by 8 hrs. post pheromone challenge (panel C). *D*-Ala$^{2}$ challenged cells demonstrated unusual "elongated" morphologies (panel D, 2 hrs. exposure) and buds formed rapidly (3 hrs. post challenge) at the shmoo tip (panel E).

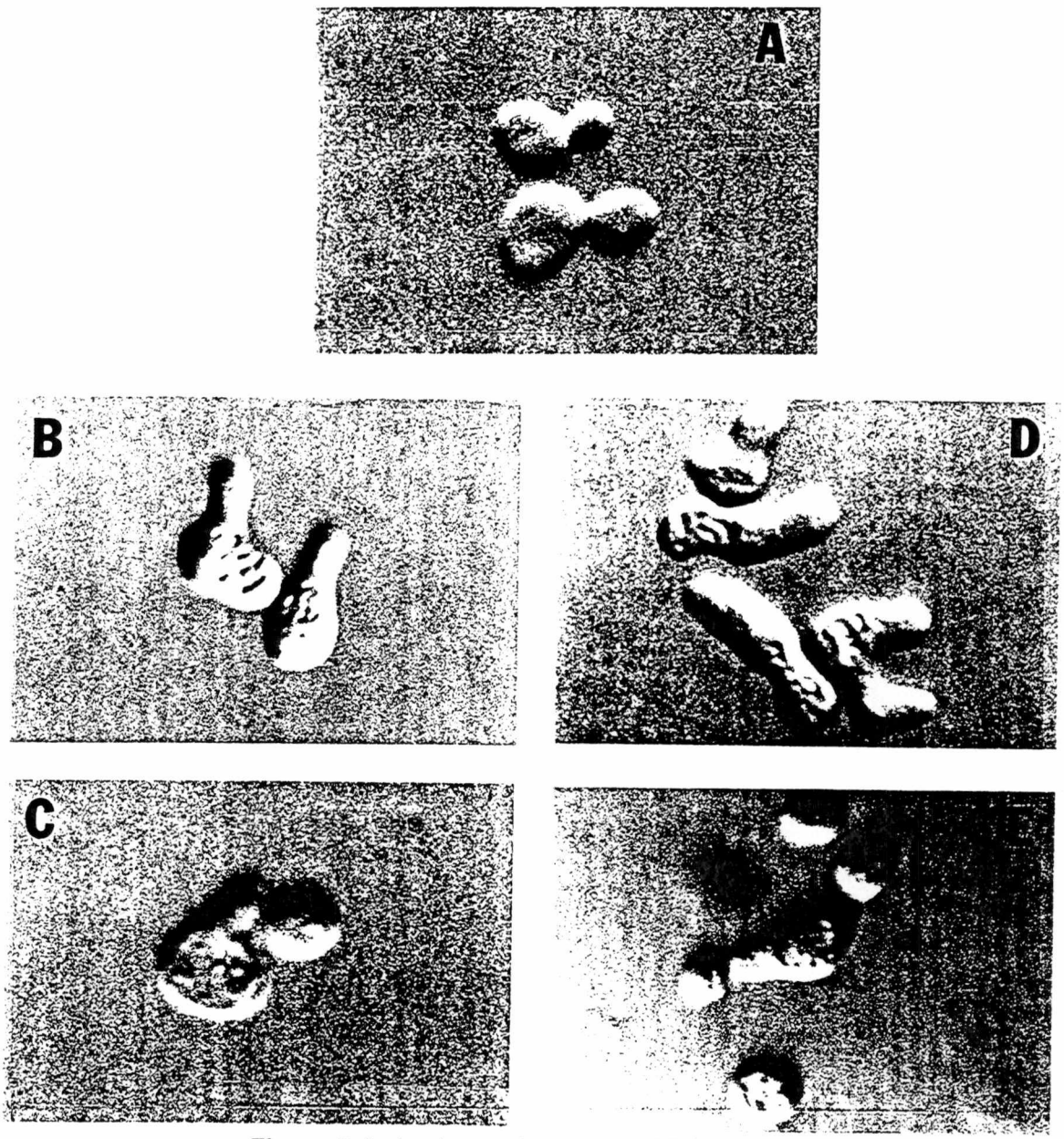


**Figure 6:** Induction and recovery of RC629 cells from [Nle$^{12}$]α-factor or [*D*-Ala$^{2}$,Nle$^{12}$]α-factor challenge.

**Figure 7:** Model of the possible functionally dominant domains of α-factor. A model of proposed domains for the α-factor pheromone and their major functional contributions to the mating response is shown. Amino acids are represented by their single letter designations; Nle is norleucine (see text).

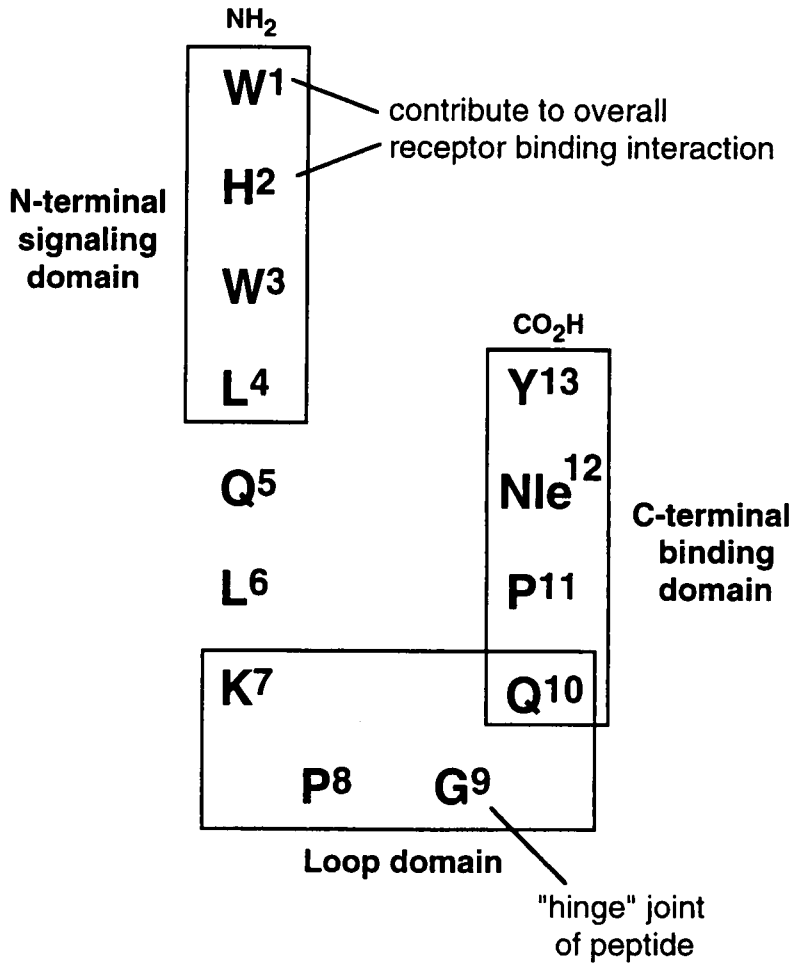


**Figure 7 :** Model of the possible functionally dominant domains of α-factor.

The strong antagonism exhibited by both [*D*-Ala$^{3}$,Nle$^{12}$]α-factor and [*D*-Ala$^{4}$,Nle$^{12}$]α-factor is consistent with the above conclusion.

The separation of binding and signal transduction activities has been documented for other peptide hormones. Deletion of the first two amino acids of human parathyroid hormone (hPTH) did not affect the cAMP-receptor binding capacity but the stimulatory potential of the hormone was lost (Marx, et al., 1995). Uncoupling of receptor binding and biological activity was also demonstrated with the glucagon hormone utilizing amino acid substitutions and deletions in the N-terminal portion of the peptide (Unson, et al., 1991).

*The C-terminal binding domain of α-factor*—In general, the most striking effect of alanine replacement at the carboxyl terminus is a marked drop in binding affinity. This outcome is clearest for position 13 where both *L*- and *D*-alanine replacements resulted in the lowest receptor affinities in their respective series. In the *D*-alanine scan series, replacement at positions 10, 11 and 12 also resulted in a greater than 1000-fold drop in binding affinity. Given this observation, the approximately 2.6-fold drop in growth arrest activity for [*D*-Ala$^{11}$,Nle$^{12}$]α-factor is surprisingly modest. Indeed, all of the alanine scan analogs with replacements at the carboxyl terminus (residues 10-13) still induce a rather efficient signal transmission. If the isomerization associated with the extended ternary complex model is applicable to this pheromone-receptor paradigm, it appears that insertion of residues with methyl side chains into positions 10-13 facilitate receptor isomerization (activation) in comparison to the native hormone. Based on the above, we suggest that the carboxyl end of the native pheromone is an

important determinant of receptor affinity (Fig. 7) and that the tyrosine side chain makes a significant contribution to the free energy of binding ($\Delta G^o$ for *L*-Ala$^{13}$ and *D*-Ala$^{13}$ analogs were -7.04 and -6.00 kcal/mole, respectively, in comparison to -10.75 for [Nle$^{12}$]α-factor).

The *D*-Ala$^{10}$ analog did not elicit growth arrest. However, it enhanced the activity of [Nle$^{12}$]α-factor in both bioassays (Fig. 3B and Fig. 4). Furthermore, this peptide exhibited more than a 1000-fold decrease in binding affinity as compared to [Nle$^{12}$]α-factor (Table 2). Synergy had been previously demonstrated for a truncated analog of α-factor lacking residues 12 and 13 (E.-Bargiota, et al., 1992). However, the mechanism of action of the previously described synergist has not been elucidated. The ability of the synergists to enhance the efficacy of the native pheromone without eliciting a mating response suggests that these peptides may lower the activation energy required for receptor isomerization. The synergist may occupy a binding site on the receptor leading to a partial conformational change which would "prime" the receptor for activation. Subsequent binding of native α-factor to this primed receptor would complete receptor isomerization and lead to signal transduction. Because the receptors were previously primed for transduction, less actual pheromone would be required to induce a response. The [*D*-Ala$^{10}$,Nle$^{12}$]α-factor represents the first example of a full length α-factor analog that acts as a synergist. It provides additional evidence that changes near the carboxyl terminus of the peptide play a role in this phenomenon.

*The loop region of α-factor*—Residues 7-10 were previously suggested to assume a turn that was a critical feature of the biologically active conformation of α-factor (Jelicks, et al., 1988; Gounarides, et al., 1993; Xue, et al., 1996). The results with the Ala-scan peptides provide additional information on the role of each residue in this region of the pheromone. The fact that both the *L*- and *D*- Ala$^{8}$ analogs have high biological activity suggests that the proline side chain is not essential for a productive interaction with the receptor. Indeed, the relatively high binding affinities of [*L*-Ala$^{7}$,Nle$^{12}$]α-factor and [*L*-Ala$^{8}$,Nle$^{12}$]α-factor indicate that the side chains of residues 7 and 8 are not major contributors to the binding energy (Table 2). However, the large dropoff in binding affinity of the analogs with *D*- residues in positions 7, 8 and 10 indicates that this region may be essential for the proper orientation of the carboxyl terminus of the pheromone to give an optimal interaction with the receptor or are necessary for overall structural stability of the peptide. The position 10 side chain is an important contributer to both activity and affinity and change in the chirality of this residue results in a synergist (see above). The observation that an *L*- or *D*- replacement at position 9 leads to low activity/weak binding and high activity/strong binding, respectively, is consistent with a turn around Gly$^{9}$ which is stabilized by *D*-Ala$^{9}$ and destabilized by *L*-Ala$^{9}$ (Gounarides, et al., 1993) and implicates this position as a "hinge" joint critical for the active conformation of α-factor. Chiral requirements in the β-turn region of the peptide hormone glucagon have been shown to affect binding and signaling and suggests a probable "bioactive" conformation of the hormone (Azizeh, et al., 1996). A

comprehensive study of conformational memories (Guarnieri, et al., 1995) of the gonadotropin-releasing hormone (GnRH) suggested that a distictive β-turn region was necessary for high affinity association with the GnRH receptor. Mutations in the turn region lead to disruptions in the structural conformations of the analogs that lead to low affinity binding (Guarnieri & Weinstein, 1996). On the basis of the above observations, we suggest that the center of the pheromone constitutes a loop region that orients the signaling and binding domains of α-factor for optimal interactions with the receptor and provides structural stability for the functionally active ends of the peptide (Fig. 7).

*Conclusion*—The alanine-scanning analysis of the α-factor provides new insights into the way in which this peptide folds in space, binds to the receptor, and triggers a response. Information has been gleaned concerning the contribution of each amino acid side chain of the peptide to both biological activity and receptor affinity. As suggested by Strader, et. al. (1995), this analysis constitutes one part of a two dimensional analysis to reveal which ligand components are interacting with specific residues on the receptor protein. Given the model in Figure 7, we can speculate that "partners" for the phenolic side chain of $Tyr^{13}$, the indole side chain of $Trp^{1}$, and the imidazole side chain of $His^{2}$ will be important constituents of the binding and/or signaling interactions. Thus, our model may provide important guidance for future investigations on the molecular basis of pheromone-receptor interactions in yeast.

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# PART 4

Structure/function analysis of the *Saccharomyces cerevisiae* α-factor receptor (*STE2* gene product) using random and site-directed mutagenesis.

# CHAPTER I

## Introduction

The haploid mating types of the yeast *Saccharomyces cerevisiae* prepare for conjugation through a signaling pathway initiated by the reciprocal action of diffusible peptide pheromones **a**- and α-factor which specifically bind to mating receptors expressed on the cell surfaces of Matα and Mat**a** cells, respectively (for review see Sprague & Thorner, 1992). Mat**a** cells express the α-factor receptor which is encoded by the *STE2* gene. Ste2p is a member of the structurally similar superfamily of seven-transmembrane G-protein coupled receptors (7TGCR; Blumer, et al., 1988; Burkholder & Hartwell, 1985; Nakayama, et al., 1985; for review see Strader, et al., 1995). This super-family includes the receptors for a wide range of hormone and neurotransmitters such as the α- and β-adrenergic, olfactory, neurokinin, and gonadotropin-releasing hormone receptors (for review see Birnbaumer & Birnbaumer, 1996).

Ligand binding to these receptors is thought to cause a conformational change which is propagated to an associated heterotrimeric guanine-nucleotide regulatory protein (G-protein). The concept of receptor isomerization has been demonstrated in some systems (Hwa, et al., 1996; Lefkowitz, et al., 1993; Gilman, 1987) and described by a two-state model of receptor activation (Leff, 1995). Recently, evidence has been presented for a conformational change in the α-factor receptor upon ligand binding (Bukusoglu & Jenness, 1996). Receptor stimulation of the G-protein

causes the $G_\alpha$-subunit to exchange GDP for GTP (Hepler, et al., 1992). GTP exchange causes $G_\alpha$ to release the $G_{\beta\gamma}$ dimer which in yeast cells activates a MAP kinase signaling cascade. Activation of a transcriptional activator (Ste12p) by components of the cascade (Song, et al., 1991) induces transcription of mating specific genes which in turn cause morphological changes (Barnes, et al., 1990) and cell cycle arrest in $G_1$ of the yeast cell (Pringle & Hartwell, 1981) in preparation for sexual conjugation.

Functional analyses of this receptor family have been carried out using receptor chimeras and site-directed mutagenesis. Binding domains and determinants of selective efficacy, potency, and signal transduction have been identified in 7TGCRs using chimeric proteins in such systems as the $\beta_2$-adrenergic receptor (Samama, et al., 1993), opioid receptor (Xue, et al., 1994), and the dopamine receptor (Kozell, et al., 1994). Xue (1994) constructed receptor chimeras between the $\mu$ and $\kappa$ opioid receptors and identified binding regions of the receptor that differentiated between peptide and non-peptide ligands. D1/D2 dopamine chimeras were used to identify transmembrane region VII as having an important role in D1 and D2 receptor ligand selectivity (Kozell, et al., 1994). Receptor chimeras between $\beta_2$- and $\alpha_{1B}$-adrenergic receptors provided evidence of the processes involved in receptor activation through isomerization and extends the ternary complex model (DeLean, et al., 1980) of agonist promoted formation of an active agonist/receptor/G-protein complex (Samama, et al., 1993).

While chimeric receptors can identify regions of function, site-directed mutagenesis is used to pinpoint the residue(s) of these receptors

that play active roles in the binding of ligands and propagation of external signals. For example, mutation of a glutamate residue in the human C5a receptor decreased receptor affinity for hC5a by up to 10-fold (Monk, et al., 1995) and defined a second binding site on the receptor. Site-directed mutagenesis of a single tyrosine residue on the 7$^{th}$ transmembrane domain of the $AT_{1A}$ angiotensin II receptor had no effect on ligand binding but severely impaired association of the receptor with phospholipase C (Marie, et al., 1994) identifying a linkage between receptor activation and signal propagation.

Also, double reciprocal mutations in transmembrane domians II & VII of the gonadotropin-releasing hormone receptor (Zhou, et al., 1993) and receptor chimeras of the m2/m5 muscarinic acetylcholine receptors (Liu, et al., 1995) provide evidence for interactions between helices which provide an empirical basis to define the spatial orientation of the transmembrane (TM) helix bundle of this type of receptor protein.

Identification of receptor binding sites in many neurotransmitter receptors has been accomplished using site-directed mutagenesis such as alanine-substitutions in the angiotensin II receptor of eleven conserved residues of which 5 appear to provide angiotensin II binding sites (Yamano, et al., 1995). A serine to alanine substitution in the 3$^{rd}$ transmembrane domain of the angiotensin II receptor completely abolished peptide agonist and antagonist binding (Groblewski, et al., 1995). Alanine substitutions in transmembrane domains of the A2a adenosine receptor (Kim, et al., 1995) and the neurotensin receptor (Huang, et al., 1994) have identified residues important for ligand binding. Finally, binding determinants of the thyrotropin-releasing hormone receptor was developed through evidence

of direct binding interactions by way of site-directed mutagenesis in residues of the 3rd transmembrane domain (Perlman, et al., 1994).

Because the α-factor receptor is thought to be organized in similar functional domains as other 7TGCRs (Dohlman, et al., 1991), information gleaned from the study of *Saccharomyces cerevisiae* cellular communication may be able to contribute to the understanding of function of the whole family. Many labs have used the *Saccharomyces cerevisiae* mating response pathway to gain insights into the structure and function of 7TGCRs. Mutational analysis of the 3rd cytoplasmic loop has implicated this domain as a multifunctional regulatory domain that controls pathway activation and/or desensitization. Six ste2$^{sst}$ alleles of the receptor were identified by random mutagenesis of the 3rd cytoplasmic loop that demonstrated increased α-factor sensitivity (Stefan & Blumer, 1994). Clark, et al. (1994) performed a systematic mutational analysis of the 3rd loop region using ala-scanning substitutions to provide clues to the function and physical interaction(s) of the receptor with the G-protein.

A constitutively active mutant of the α-factor receptor was described by Konopka, et al. (1996) in which a Pro258Leu substitution caused constitutive activation of the signaling pathway equivalent to about 45% of wild-type levels observed for α-factor stimulation. This mutation is predicted to alter the structure of transmembrane 6 which may effect a change in adjacent sequences in the third intracellular loop that are thought to function in G-protein coupling.

Lastly, domain swapping and substitutions in the hydrophobic core (transmembrane domains) of the α-factor have identified regions and residues involved in ligand specificity, signal transduction, and mating

response recovery (Sen & Marsh, 1994; Marsh, 1992). Receptor domains from *Saccharomyces kluyveri* α-factor receptor were substituted for the positionally homologous domains of the *Saccharomyces cerevisiae* α-factor receptor to study binding domains of these receptors for their respective peptide hormones (Sen & Marsh, 1994). Non-contiguous domains of the receptor that include TM1 through TM3 and part of TM6 through the end of TM7 including the connective loops between the TM domains was concluded to house the ligand specificity determinants.

Random mutations of the α-factor receptor by the previously mentioned lab (Marsh, 1992) identified 6 single or double mutations that confered unique phenotype mating responses. One mutation (M54I) only affected ligand specificity of the receptor while other mating responses remained the same as wild-type. A mutant receptor containing the double mutation (L255S-S288P) was able to induce *FUS1*-lacZ transcription but the cell did not arrest in $G_1$ of the cell cycle. Another double mutant (S145L-S219L) demonstrated normal response to pheromone but recovered more rapidly from growth arrest than normal. A stop codon in the C-terminal tail region caused a lack of internalization of the receptor and therefore a deficiency in desensitization to the mating signal but did not effect agonist binding or signal transduction. Lastly, two single mutations (S145L, A229L) caused an increase in sensitivity to α-factor possibly by "relaxing" the receptor to a more readily induced isomerization state.

Therefore, in following with the previously described investigations and to add to the understanding of this family of proteins, Part 4 is a study, using random and site-directed mutagenesis, of those residue(s) of the α-factor receptor that are critical for the binding of the α-factor ligand as well

as residues involved in transduction of the mating signal. We used PCR based random and site-directed mutagenesis as opposed to chemical or *E.coli* mutD mutagenesis to try to produce all types of mutations possible in the *STE2* gene. The chemicals used for mutagenesis only produce certain mutations in DNA and may not provide a comprehensive number of mutations to allow for a thorough study of receptor function. Theoretically, PCR based mutagenesis will introduce random mutations at any position of the DNA fragment being amplified. In the end, the mutated DNA produced after PCR amplification will contain all possible base pair substitutions at all positions within the fragment amplified.

Mutant α-factor receptors have been identified by their biological responses to molecules that the receptors do not normally respond. Marsh (1992) identified receptor mutants that responded to a pheromone from *Saccharoyces kluyveri*, a close yeast relative of *Saccharomyces cerevisiae*. Our approach of mutant receptor screening involved the use of synthetic peptides of the natural α-factor pheromone to identify those mutant receptors that would respond to them. Our lab along with our collaborative lab in New York, has designed, synthesized, and biologically characterized many new antagonist and synergistic peptide analogs that are valuable for he study of receptor function and mechanism of action.

# CHAPTER II

## Materials and Methods

*Media and Sequencing*——Sequencing was carried out manually using a dideoxy DNA sequencing kit from United States Biochemical as per kit instructions (Sequenase Version 2.0) and automatically by the Molecular Biology Resourses Facility (MBRF) located on the campus of the University of Tennessee using a Perkin Elmer GeneAmp 2400 PCR machine and ABI 373 sequencer. For automated sequencing, dye termination flourescent sequencing was performed using the Flourescent Sequencing kit from Perkin Elmer which utilizes AmpliTaq DNA polymerase FS. Synthetic dextrose (SD) minimal medium (2% dextrose, 2% agar for plates, 0.67% yeast nitrogen base without amino acids, Difco) was supplemented with amino acids (Sigma) when appropriate by adding histidine [H] (20 μg/ml), lysine [K] (30 μg/ml), methionine [M] (20 μg/ml), and/or tryptophan [W] (20 μg/ml). Complex YEPD medium (1% yeast extract, 2% peptone, 2% dextrose, 2% agar for plates, Difco) was used to grow yeast under nonselective conditions.

*Random mutagenesis of STE2 using a modified PCR* ——Random mutations were introduced into the *STE2* gene by use of a modified version of a PCR based random mutagenesis protocol (Muhlrad, et al., 1992) as described below. The *STE2* gene was amplified with pAB539 as the template using the primers XHOIA (CATATCCAAGACTCGAGAATGTCTGA

TGCG) and XHOIB (CTACCACTACTCTCGAGATGTTTATTATG) using the following conditions: 50ng of pAB539 (Fig. 1; Burkholder & Hartwell, 1985) as template; 50nM each of XHOIA and XHOIB primers; 200μM $MnCl_2$; 200μM each dCTP, dGTP, dTTP, and 40μM dATP; 1X PCR buffer (50mM Tris, 500μg/ml BSA, 0.5% Ficoll, 1mM Tartrazine, 2mM $MgCl_2$); 2.5 Units of *Taq* DNA polymerase (Promega) in 25μl total volume. Hot start PCR was used for amplification by heating the reaction mix (without *Taq* polymerase) to 94°C for 5 min., transfering to ice for 5 min., and then adding 2.5 Units of *Taq* polymerase and cycling 25 times at 94°C for 30 sec., 58°C for 2 min., and 72°C for 1.5 min. Point mutations in primer sequences (underlined) introduced flanking XhoI restriction sites that allowed for subcloning of mutagenic PCR products into the unique Sal I site of the vector pAD54 (Fig. 2; Colicelli, et al., 1989) to produce a series of yeast expression vectors (pSTEAD; Fig. 3) containing a "library" of mutant receptor genes.

*FUS1-lacZ screen for antagonist responsive mutant receptors*——The pSTEAD "library" of plasmids was transformed (Gietz, et al., 1992) into yeast strain LM23-16az (Mat**a** *ste2-i4 bar1 leu2 ura3 his4 lys5 met1* [*FUS1*-lacZ::*URA3*]) (Marsh, 1992), plated on synthetic dextrose minimal medium supplemented with lysine and methionine (SD+KM) and grown at 30°C for 72 hrs. LacZ induction was assayed as previously described (Marsh, 1992). Transformant colonies were replica plated onto SD+KM plates overlayed with filters (Whatman #50) and with or without 10 μg/ml desTrp$^1$desHis$^2$[Nle$^{12}$]α-factor antagonist (E.-Bargiota, et al., 1992) and incubated for 6 hrs. at 30°C. Filters were quick frozen in liquid nitrogen for 20 sec. and transfered to a second filter (Whatman #3) that had been

**Figure 1:** The yeast/bacterial shuttle vector pAB539. The yeast/bacterial shuttle vector YEp13 with a 4.3Kb chromosomal insert which includes the ORF of the *STE2* gene cloned into the unique BamHI site constructed by Anne Burkholder (Burkholder & Hartwell, 1985). Extra DNA, chromosomal DNA flanking the *STE2* ORF; 2μ, yeast 2 micron origin of replication; ori, bacterial origin of replication; bla, β-lactamase gene (ampicillin resistance); *LEU2*, leucine biosynthesis gene 2; *STE2*, α-factor receptor gene; START, ATG of *STE2*; STOP, termination codon of *STE2*. Some restriction site positions are approximate.

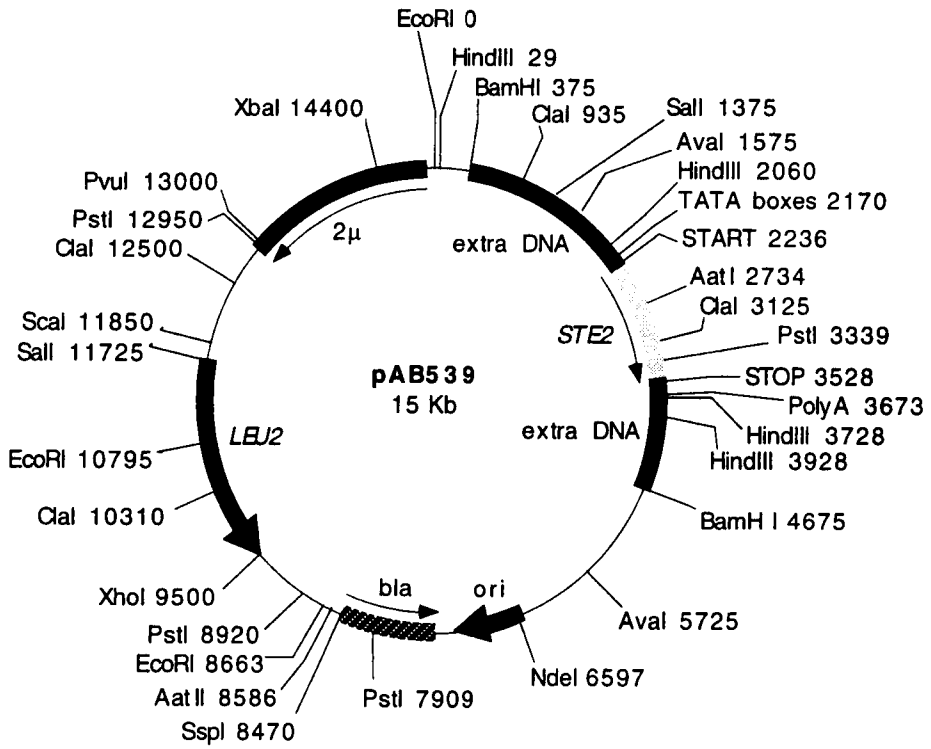


**Figure 1:** The yeast/bacterial shuttle vector pAB539.

**Figure 2:** The yeast/bacterial shuttle vector pAD54. pAD54 was used to subclone PCR generated mutant *STE2* gene fragments at a unique Sal I site. The ligation of the fragments is carried out so that the gene is inframe with read-through of the alcohol dehydrogenase 1 gene constitutive promotor ($ADH1_p$). Transcription starts at the ATG before the Lerner epitope sequence which adds 18 amino acids to the N-terminal end of the α-factor receptor upon translation. $ADH1_t$, alcohol dehydrogenase 1 gene terminator sequence; ori, bacterial origin of replication; bla, β-lactamase gene (ampicillin resistance); *LEU2*, leucine biosythesis gene 2; 2μ, episomal origin of replication; YPYDVPDYA, protein epitope sequence that is recognized by the antibody 12CA5.

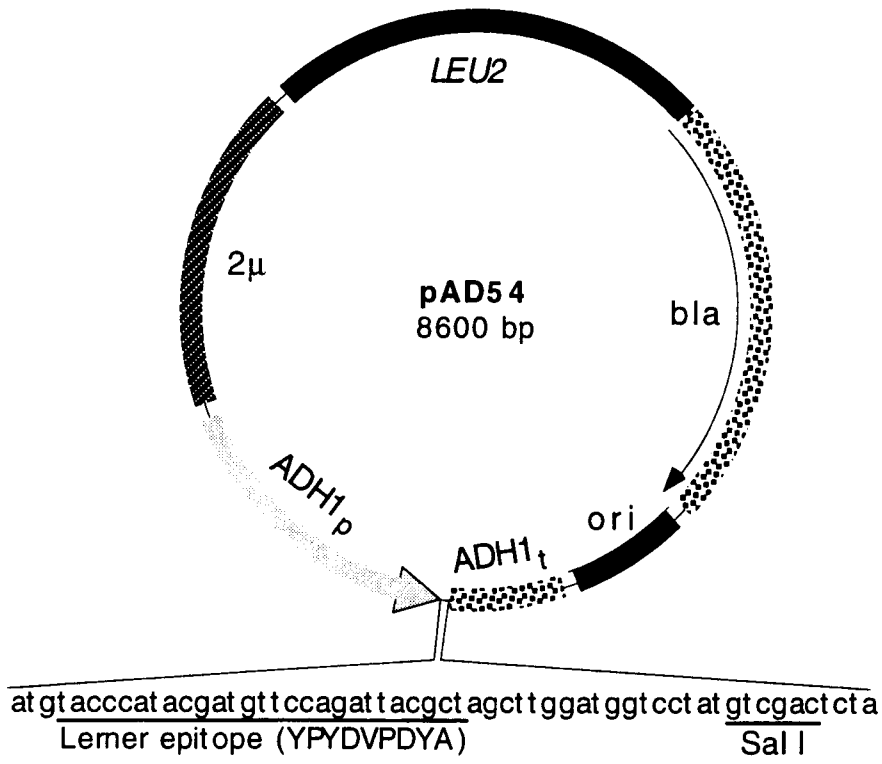


**Figure 2:** The yeast/bacterial shuttle vector pAD54.

**Figure 3:** The yeast/bacterial shuttle vector pSTEAD. Vector was constructed by ligating PCR generated mutant *STE2* gene products in-frame into the unique Sal I site downstream of the alcohol dehydrogenase 1 constitutive promoter ($ADH1_p$) in vector pAD54 (see Fig. 4). Transcription is initiated at an ATG 5' to the Lerner epitope and adds 54 bps (18 amino acids) to the N-terminal end of the α-factor receptor protein. $ADH1_t$, alcohol dehydrogenase 1 gene terminator sequence; ori, bacterial origin of replication; bla, β-lactamase gene (ampicillin resistance); *LEU2*, leucine biosythesis gene 2; 2μ, episomal origin of replication; *STE2*, α-factor structural gene; YPYDVPDYA, protein epitope sequence that is recognized by the monoclonal antibody 12CA5 available from Berkeley Antibody Co.

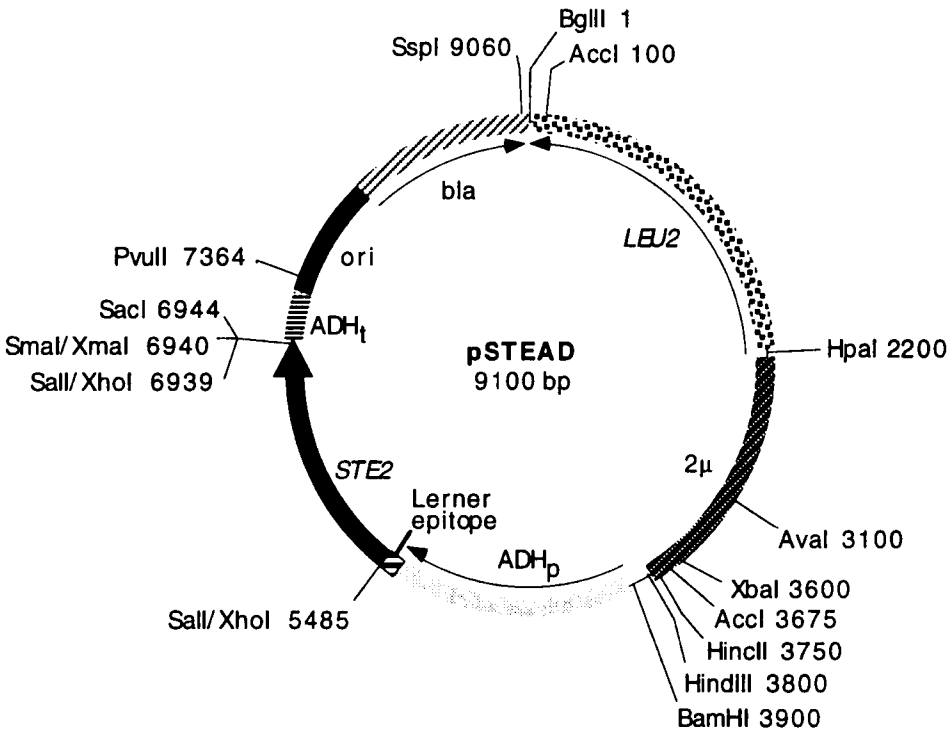


**Figure 3:** The yeast/bacterial shuttle vector pSTEAD.

soaked with 2.5 mls Z-buffer (60mM $Na_2HPO_4 \cdot 7H_2O$, 40mM $NaH_2PO_4 \cdot H_2O$, 10mM KCl, 1mM $MgSO_4 \cdot 7H_2O$, 50mM β-mercaptoethanol, pH 7.0) containing 0.03% X-gal. The filters were incubated at 30°C for 1-3 hrs. to allow for any blue color development from β-galactosidase production. Filters were aligned with the original transformant plates and corresponding colonies to replicated colonies that turned blue were isolated for further testing.

*Growth arrest (halo) assay and sequencing of antagonist responsive mutant α-factor receptors*——Colonies responding to antagonist as measured in the *FUS1*-lacZ assay were isolated and tested for further biological response in the growth inhibition (halo) assay. Response to 1μg [$Nle^{12}$]α-factor or 10μg desTrp$^1$desHis$^2$[$Nle^{12}$]α-factor was measured as previously described (Eriotou-Bargiota, et al., 1992). SD+KM plates were overlayed with 4mls of a yeast colony demonstrating a positive response to antagonist in the *FUS1*-lacZ assay in Noble agar at a cell density of 2.5 x $10^5$ cells/ml. Filter disks (8 mm sterile blanks, Difco) were placed on the overlay and 1 μg of [$Nle^{12}$]α-factor in 10 μl and 10 μg of antagonist in 10 μl were spotted onto the disks. Biological response to the peptides was measured as either a positive response (any zone of inhibition) or negative response (no zone) after 36 hrs. incubation at 30°C. Plasmids from the transformed yeast colonies that demonstrated unusual phenotype(s) to [$Nle^{12}$]α-factor and/or antagonist were isolated (Ward, 1990), amplified in *E. coli* and purified using a plasmid maxiprep kit (Qiagen, Inc.) as per manufacturers instructions. Plasmids were manually sequenced using Sequenase Version 2.0 DNA sequencing kit (United States Biochemical).

*Construction of expression vector for site-directed mutagenesis*——A yeast/bacterial shuttle vector was constructed by starting with the vector pRS314 (Fig. 4, Sikorski & Hieter, 1989) linearized with SpeI enzyme (New England Biolabs, NEB) and purified with the Wizard DNA clean-up kit according to manufacturers protocol (Promega). A 1.8 Kb PCR fragment containing the *STE2* ORF plus 316 bps upstream promoter region from the start ATG and 163 bps downstream region from the stop codon of *STE2* was amplified from the vector template pAB539 using the primers SPEIA (GCTTTGCAATGAAACACTAGTATCCGCTAAG) and SPEIB (GTGGTTTC TACCACTAGTTACGAGATGTTTATTATG). The PCR conditions used were as follows: 40ng pAB539, 100nM each primers SPEIA and SPEIB, mix of 200μM each dNTP, 1X DeepVent polymerase buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl (pH 8.8), 2mM $MgSO_4$, 0.1% Triton X-100), 1 Unit DeepVent DNA polymerase (NEB), for 25 cycles of 94°C (1 min.), 46°C (2 min.), 72°C (3 min.) with a final extension of 72°C for 10 min. Point mutations (underlined in primer sequence) introduce SpeI enzyme restriction sites into the ends of the PCR fragment. PCR fragments were subsequently digested with SpeI and purified with the Wizard PCR preps DNA purification system (Promega). PCR fragment and linear pRS314 vector were ligated together using the standard protocol accompanying the Ligator rapid DNA ligation and screening kit (Epicenter Technologies) producing the vector pGA314.WT (Fig. 5). The entire *STE2* gene and flanking regions was sequenced by the MBRF and confirmed that the subcloned region was wild type sequence with no spurious mutations.

**Figure 4:** The yeast/bacterial shuttle vector pRS314. The 4.8 Kb plasmid used as the parent vector for subcloning mutant *STE2* gene fragments produced by PCR mutagenesis. *CEN6/ARSH4*, centromeric and autonomously replicating sequence H4; *TRP1*, tryptophan biosynthesis gene 1; f1(+), origin of single strand replication; lacZ, β-galactosidase gene for blue/white selection (disrupted by DNA insertion in polylinker); ori, bacterial origin of replication; bla, β-lactamase gene (ampicillin resistance). Arrows indicate direction of transcription.

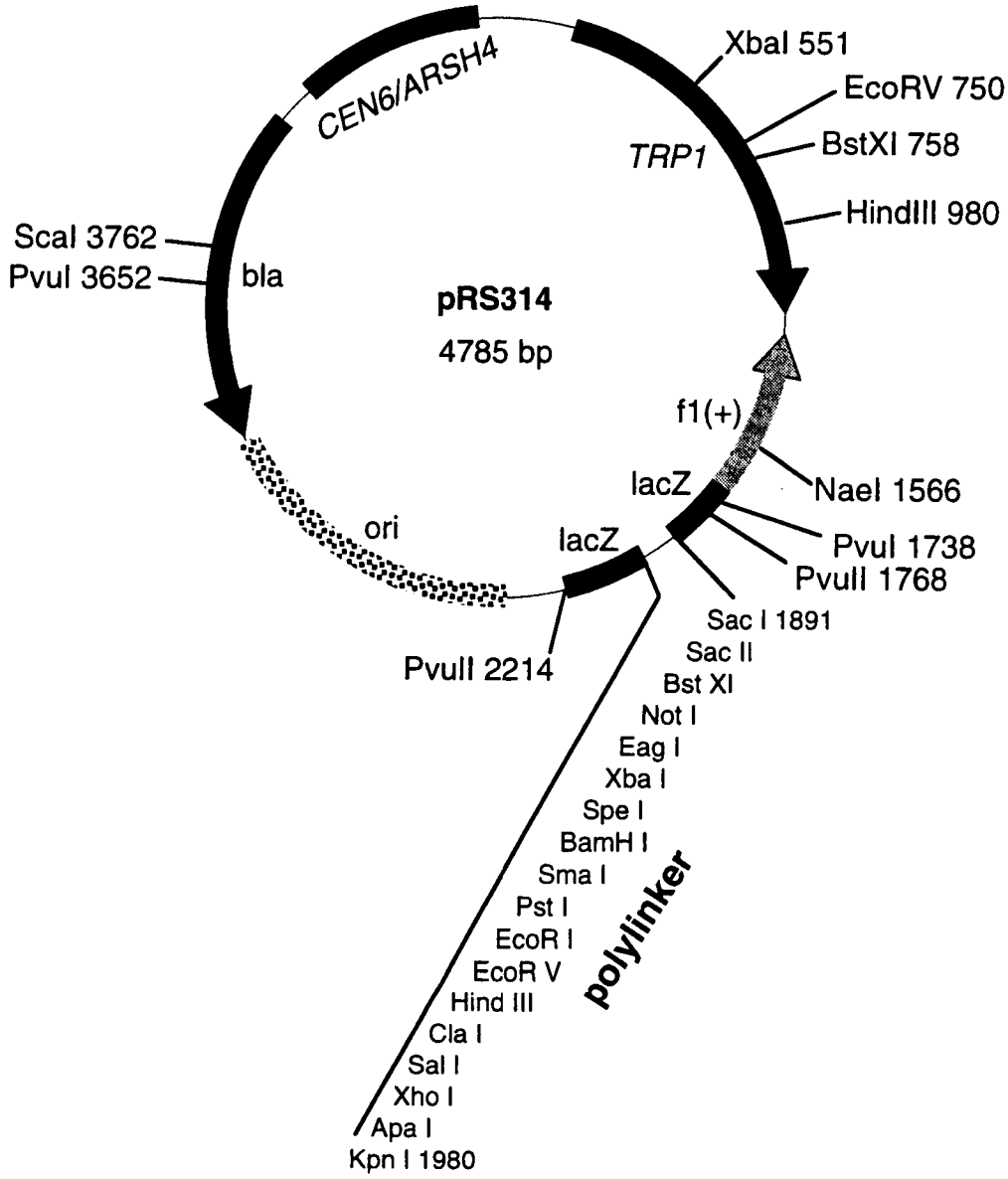


**Figure 4:** The yeast/bacterial shuttle vector pRS314.

**Figure 5:** The yeast/bacterial shuttle vector pGA314.WT. This vector contains a 1.8Kb PCR fragment that includes the *STE2* ORF, 316 bps of upstream promoter region from the START ATG, and 165 bps of downstream region after the termination codon cloned into the SpeI restriction site of pRS314 in the (+) orientation (5' to 3' coding strand reads in the clockwise direction). *TRP1*, tryptophan biosythesis gene 1; f1(+), viral f1 origin of single stranded replication; lacZ, β-galactosidase gene; *STE2*, α-factor receptor structural gene; ori, bacterial origin of replication; bla, β-lactamase gene (ampicillin resistance); *CEN6/ARSH4*, centromeric/autonomously replicating sequence H4 for yeast. Restriction sites with stars (*) are unique.

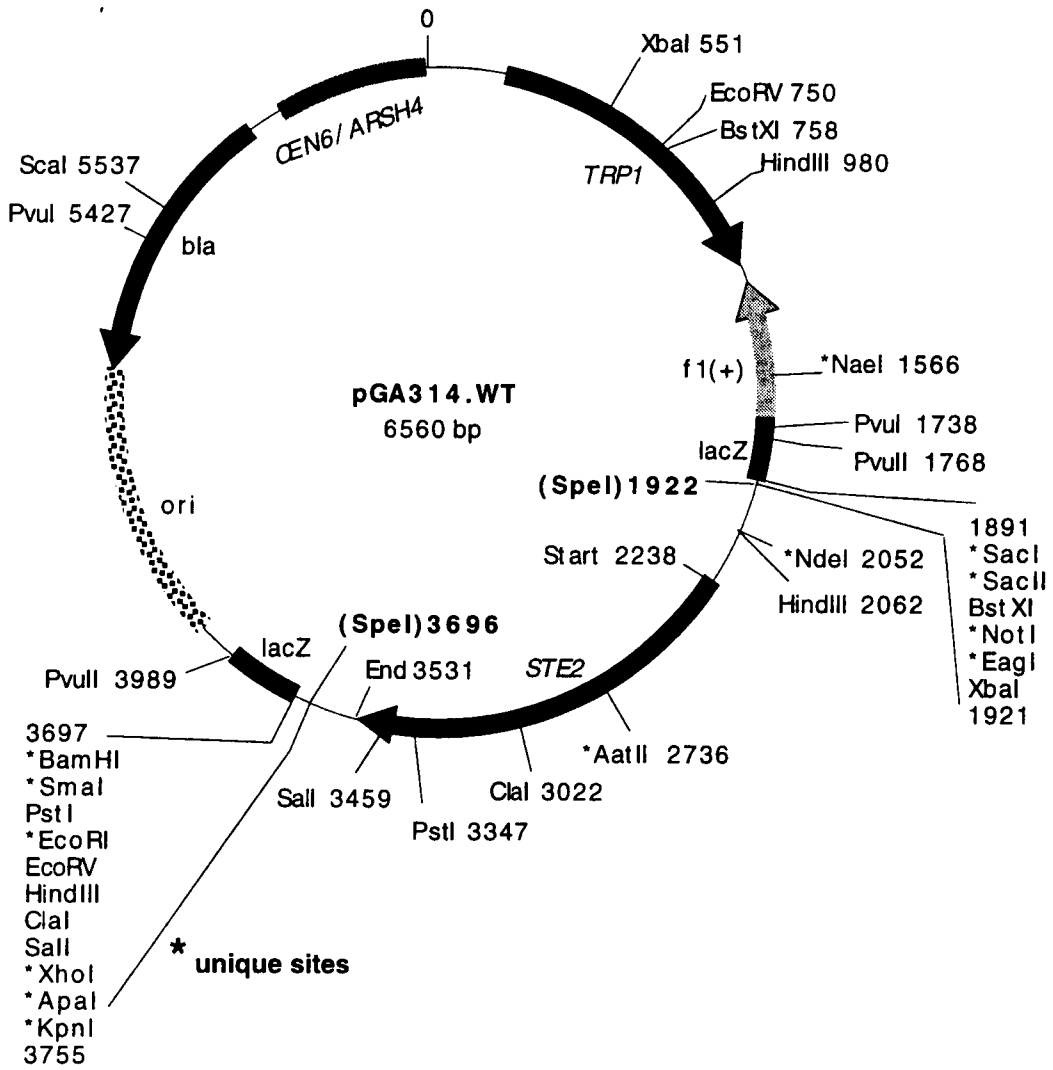


**Figure 5:** The yeast/bacterial shuttle vector pGA314.WT.

*Site-directed mutagenesis of STE2*——Site-directed mutagenesis of the *STE2* gene was carried out using a modified version of overlap extension PCR (Ho, et al., 1989) as described below (see Fig. 6). The first round of amplification required 200 ng pGA314.WT DNA, 5 μM dNTPs, 1.0 μM primer A or B (for primer sequences, see below), 1.0 μM of each mutagenic primer, 1X *Taq* DNA polymerase buffer (50mM KCl, 10mM Tris-HCl, 0.1% Triton X-100, 2mM $MgCl_2$, NEB), and 2.5 Units *Taq* DNA polymerase (NEB) in a total volume of 100μl for 25 cycles of 94°C (1 min.), 50°C (2 min.), and 72°C (3 min.). PCR fragments generated during the first round of PCR were gel purified using the Geneclean I kit (Bio101, Inc.) and used as template DNA for a second round of PCR-overlap extension mutagenesis. The second round of amplification was performed using 200ng of the 5'-half mutagenic fragment, 200ng of the 3'-half mutagenic fragment, 1X *Taq* DNA polymerase buffer, 100μM dNTPs, and 1 Unit *Taq* DNA polymerase in a total volume of 10μl for 10 cycles of 94°C (1 min.), 55°C (2 min.), and 72°C (3 min.). A third round of amplification was performed to produce the full length *STE2* gene with point mutations (underlined in primer sequences below) that introduced single amino acid substitutions in the translated protein product. Conditions for the third round of amplification were 1 μl of round 2 reaction mix, 1.0 μM primer A, 1.0 μM primer B, 1X *Taq* DNA polymerase buffer, 5 μM dNTPs, and 2.5 Units *Taq* DNA polymerase in a total volume of 100 μl for 30 cycles of 94°C (1 min.), 50°C (2 min.), and 72°C (3 min.).

Full length third round PCR reaction products are subcloned into the vector pCR2.1 (Invitrogen TA cloning kit) and amplified in *E. coli* strain DH5α. Bacterial transformants were randomly chosen and plasmid DNA

**Figure 6:** Site-directed overlap extension PCR mutagenesis. Using the vector pGA314.WT as template in round 1 amplification with primers A & B and complementary mutation (*) containing oligos, two mutation containing fragments of *STE2* are generated (5'- and 3'-half fragments). The two fragments are purified and used as template for round two PCR extension. The complementary overlapping region of each fragment primes the extension of the full length product (flanking primers A & B are not used in round 2). An aliquot of the round 2 PCR mix is used to spike the third round of PCR amplification which includes the flanking primers A & B. Any full length products produced during round 2 provide template for the flanking primers to amplify. The final amplified product contains the desired mutation in both strands of DNA and can be subcloned for further study.

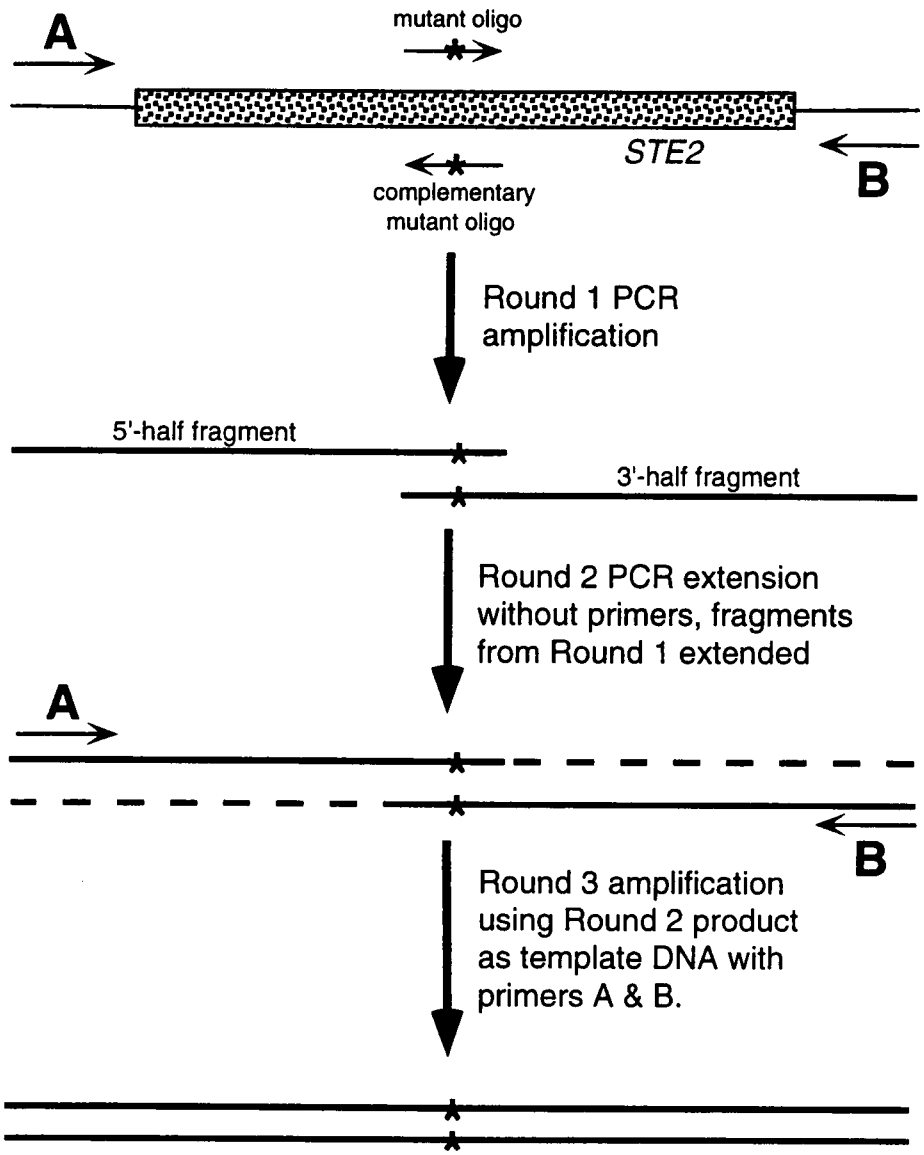


**Figure 6:** Site-directed overlap extension PCR mutagenesis.

was isolated from each (Sambrook, et al., 1989). Insert containing plasmids were digested with EcoRI and the 1.8Kb *STE2* gene fragment was isolated using the Geneclean I DNA purification kit and subcloned into the unique EcoRI site of pRS314 to produce the plasmids pGA314.F55V, pGA314.S219P, pGA314.S259P, and pGA314.I263V (pGA314.WT vector that now contains a *STE2* gene with the designated mutation, see Fig. 5 for vector design). Two other vectors were produced designated pGA314.Q118stop and pGA314.K239R,S259P,S325Y that contained extra mutations not originally designed. During the amplification of DNA by PCR, some thermostable polymerases will make errors in base pair extension and introduce mutations into the DNA fragment being amplified (Leung, et al., 1990; Cadwell & Joyce, 1992). Vector pGA314.Q118stop contains a mutation that introduced a stop codon in the second extracellular loop region of Ste2p. Vector pGA314.K239R,S259P,S325Y contains two extra mutations that introduce amino acid substitutions in the 3$^{rd}$ cytoplasmic loop (K239R) and C-terminal tail (S325Y) along with the planned S259P mutation in the 5$^{th}$ transmembrane domain.

Primer pairs used for the initial round of amplification reactions to introduce single point mutations were primer A (GGATAACAATTTCACA CAGG) with primer F55VF (GGCCATTATGGTTGGTGTCAG), primer A with primer S259PF (GTTGGTTCCACCGATAATATT), primer A with primer I263VF (GATAATATTCGTCCTCGCATAC), primer A with primer S219PF (GCATCCTCAATAAACTTTATGCCATTTGTCCTGGTAGTTAAATTGATTTTA GC), primer B (TCGCTATTACGCCAGCTGG) with primer F55VR (CTGACA CCAACCATAATGGCC), primer B with primer S259PR (AATATTATCGGTG GAACCAAC), primer B with primer S219PR (GCTAAAATCAATTTAACTAC

CAGGACAAATGGCATAAAGTTTATTGAGGATGC), and primer B with primer I263VR (GTATGCGAGGACGAATATTATC). All primers were synthesized by BioServe BioTechnologies (Laurel, MD), shipped lyopholized, and resuspended in sterile water.

# CHAPTER III

## Results

*Expression vector construction*——Two expression vectors were constructed for subsequent use in random and site-directed mutagenesis, yeast expression, and bacterial amplification. Initially, PCR fragments containing mutant *STE2* ORFs were cloned into the vector pAD54 (Fig. 2) to produce the plasmid series pSTEAD (Fig. 3). Subsequently, the pSTEAD plasmid series was transformed into a yeast strain deficient in the expression of the native Ste2p. The mutant receptor proteins expressed from this series of plasmids were screened for their response to antagonist peptides. The plasmid DNA from those clones that demonstrated a positive response to α-factor analogs that normally do not elicit a response was isolated and the corresponding *STE2* gene was sequenced.

For site-directed mutagenesis, the *STE2* promoter and ORF were subcloned into the yeast shuttle vector pRS314 to produce the plasmid pGA314.WT (see Materials and Methods). The plasmid was sequenced by the MBRF and confirmed the wild-type sequence of the *STE2* gene. Confirmation of gene sequence is very important using any type of PCR based mutagenesis technique due to the relatively high incidence of spurious mutations introduced by many of the thermostable polymerases used for PCR amplification. Two out of the six *STE2* genes sequenced after site-directed mutagenesis contained extra mutations not originally designed into them. Activity of pGA314.WT was checked by growth arrest

response to α-factor in the strain LM102. The zone of growth inhibition of LM102 expressing the α-factor receptor from either pGA314.WT or pAB539 (Burkholder & Hartwell, 1985) to 1 μg of [Nle$^{12}$]α-factor was equivalent (data not shown).

*Random mutagenesis of STE2 and sequence analysis*——The plasmid pAB539 was used as the template DNA for PCR based random mutagenesis of the *STE2* gene. *Saccharomyces cerevisiae* strain LM23-16az (a Mata yeast strain that expresses a non-functional α-factor receptor) was transformed with the "library" of mutant *STE2* gene containing plasmids (pSTEAD) and screened for ligand dependent biological response to an antagonist peptide by *FUS1*-lacZ induction (see Materials and Methods). Approximately 40,000 colonies were screened and a total of 144 *FUS1*-lacZ positive colonies were isolated and tested further for biological response (Figure 7). These 144 colonies were retested for *FUS1*-lacZ induction and 83 false positive clones did not respond to antagonist in the second screen and were discarded. The high number of false positive colonies isolated during the first screening round likely is due to the process of selecting positively responding colonies. The transformed colonies were replica plated onto filters that were quick frozen and then exposed to buffer containing X-gal. Blue color production on the filter represented a positive response. The filters were then lined up with the original plates containing the transformed yeast colonies. In some cases, the close proximity of colonies on the original plates did not allow for the specific identification of positive clones. Therefore, 3 to 5 colonies in the immediate area corresponding to the blue color production on the filter

**Figure 7:** Phenotype screen of randomly generated mutant receptors. (*a*) The yeast strain LM23-16az was transformed with the pSTEAD series of vectors which contain *STE2* genes with random mutations. (*b*) *FUS1*-lacZ induction measured by assay as described in Materials and Methods from L. Marsh (1992). (*c*) Growth arrest in the halo assay was measured as a positive response for any zone of inhibition and as a negative response if there was not a zone of growth inhibition. One μg of [Nle$^{12}$]α-factor was spotted for response to α-factor and 10 μg of desTrp$^{1}$[Ala$^{3}$,Nle$^{12}$]α-factor or desTrp$^{1}$desHis$^{2}$[Nle$^{12}$]α-factor for response to antagonist. (*d*) The plasmid DNA from six representative clones was sequenced to identify the residues mutated.

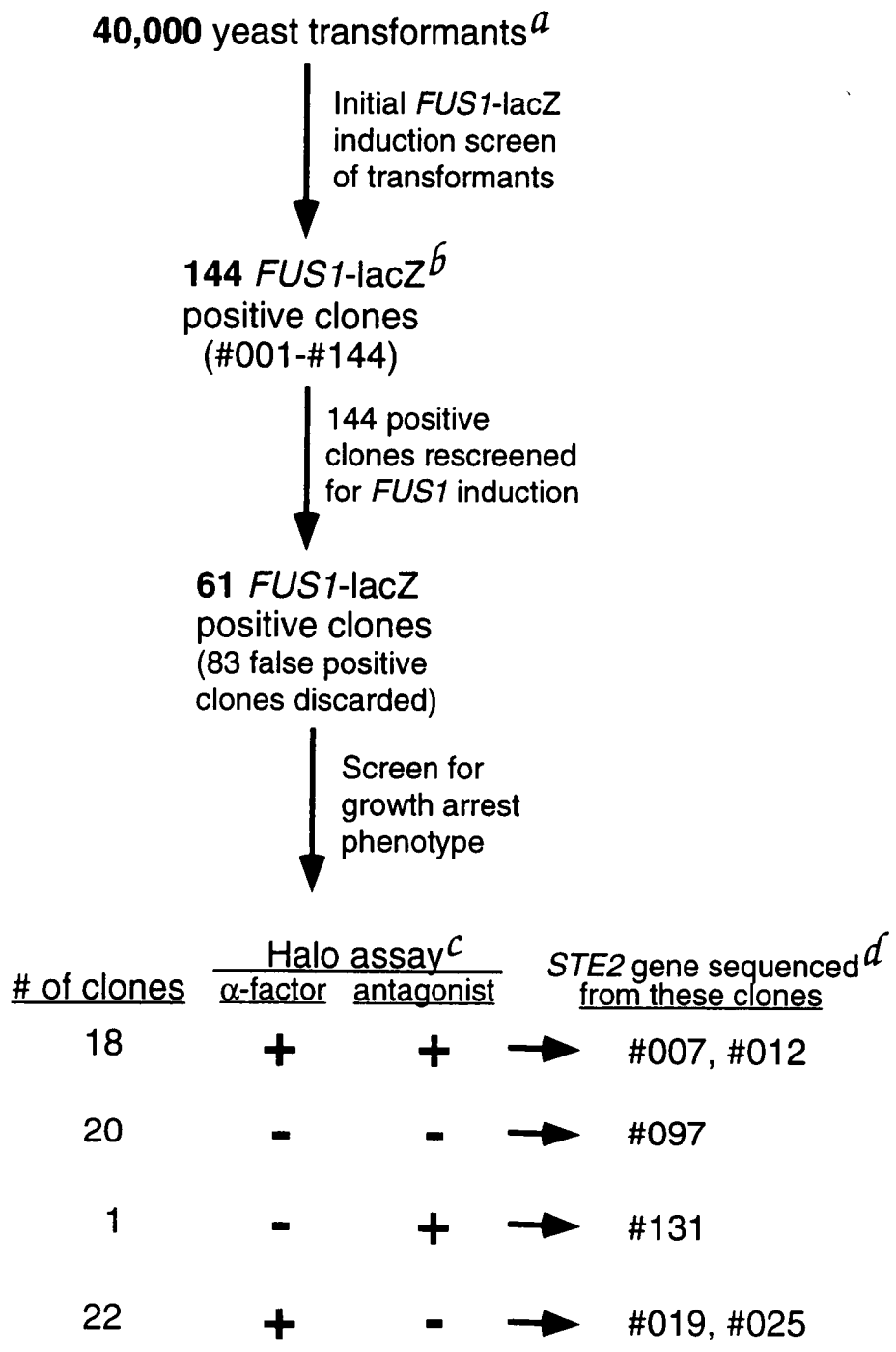


**Figure 7:** Phenotype screen of randomly generated mutant receptors.

were isolated and tested a second time for *FUS1*-lacZ induction. Consequently, many non-responsive clones were isolated in the original screen.

The remaining 61 β-galactosidase positive colonies were individually plated as lawns on selective medium plates and tested for growth arrest response to 3 μg of [Nle$^{12}$]α-factor and 10 μg of desTrp$^{1}$desHis$^{2}$[Nle$^{12}$]α-factor or 10 μg of desTrp$^{1}$[Ala$^{3}$,Nle$^{12}$]α-factor. Eighteen colonies demonstrated varying degrees of growth arrest (zones of growth inhibition from 9mm to 21mm) to both antagonist and [Nle$^{12}$]α-factor and 20 colonies demonstrated no growth arrest response to either antagonist or [Nle$^{12}$]α-factor. One isolate (#131) initially demonstrated a halo response to desTrp$^{1}$[Ala$^{3}$,Nle$^{12}$]α-factor and no response to [Nle$^{12}$]α-factor. The remaining 22 isolates responded only to [Nle$^{12}$]α-factor to varying degrees (9mm to 25mm diameter zones of growth inhibition) and not at all to either antagonist.

Plasmid DNA from one or two *FUS1*-lacZ positive clones representative of each phenotype class (MR007, MR012, MR019, MR025, MR097, MR131; Table 1) was purified and the *STE2* gene was sequenced to identify residues mutated during PCR mutagenesis. The mutated *STE2* genes contained between 10-19 bp changes (Table 2) that translated into 5-10 residue mutations per receptor protein (see Table 1).

*Site-directed mutagenesis of STE2*——Specific residues identified during the random mutagenesis of the *STE2* gene were targeted for site-directed mutagenesis based on three criteria: 1) Residue was changed in isolated receptors from random PCR mutagenesis and demonstrated an antagonist

**Table 1:** Mutant α-factor receptors demonstrating unique phenotypes to [Nle$^{12}$]α-factor and antagonist pheromones. The plasmid DNA from vectors containing *STE2* genes which code for receptors that demonstrate unique phenotypes to [Nle$^{12}$]α-factor and antagonist pheromones was sequenced and translated to identify residues mutated during PCR mutagenesis. (*a*) Mutations were infered from sequence data (n=2 for each receptor) and are represented by single letter amino acid designation. (*b*) Any zone of growth inhibition is represented as a positive (+) response (n=3) however, distinctions between degree of response were not done for positive clones. (*c*) For mutant receptors #MR007, #MR012, #MR019, and #MR025, the antagonist used for growth arrest response was desTrp$^{1}$desHis$^{2}$[Nle$^{12}$]α-factor (3.0 μg) while for mutant receptors #MR097 and #MR131, the antagonist used was desTrp$^{1}$[Ala$^{3}$,Nle$^{12}$]α-factor (3.0 μg). (*d*) Synthetic [Nle$^{12}$]α-factor (3.0 μg) was used for assay of response to native pheromone.

## Table 1

*Mutant α-factor receptors demonstrating unique phenotypes to [Nle$^{12}$]α-factor and antagonist pheromones.*

| | Mutation[a] | | | | |
|---|---|---|---|---|---|
| Mutant | N-term. domain (residues 1-50) | Transmembrane domain (51-299) | C-term. domain (300-431) | Halo response[b] to: Antagonist[c] | Halo response[b] to: α-factor[d] |
| MR007 | none | N84D<br>M165T<br>F182L<br>N216S<br>I230T<br>D242G | I308V<br>F327S<br>E403G | + | + |
| MR012 | none | S259P<br>I263V | A369G<br>M409T<br>D417G | + | + |
| MR019 | E40G | L93F<br>Q149R | N375S<br>D417G<br>K422E<br>F423S | − | + |
| MR025 | F38L | R76G<br>G156S<br>M165I<br>L255M<br>S267G<br>T278S | N301S<br>T354R<br>K374N<br>G402A | − | + |
| MR097 | S28F<br>N46D | F116L<br>L284P | K304I<br>K374E<br>L380S<br>T382A | − | − |
| MR131 | none | F55V<br>N132S<br>I142N<br>S219P | T313S<br>R318G<br>L324P<br>N334D<br>R343S<br>D357G | + | − |

**Table 2:** Nucleotide mutations of the *STE2* gene. The nucleotides found to be mutated from the wild-type sequence of the *STE2* gene in the 6 clones sequenced are listed. (*a*) Mutations of the *STE2* gene are shown represented by the nucleotide of the wild-type sequence, followed by the position in the ORF of the nucleotide from the start ATG of *STE2* (the A being #1) and then followed by the mutant nucleotide. (*b*) Nucleotide mutations that did not result in a substitution in the translated Ste2p were designated as conserved). (*c*) The amino acid substitution that resulted from the nucleotide mutation in the *STE2* gene is shown by the three letter designation for the wild-type residue, followed by the position in the α-factor receptor protein relative to the $NH_2$-terminus methionine, and then followed by the resulting substituted residue.

**Table 2:** *Nucleotide mutations of the STE2 gene.*

| MR007 | MR019 | MR012 | MR025 |
|---|---|---|---|
| T30C[a] (conserved[b]) | A119G (Glu40Gly[c]) | T711C (conserved) | T112C (Phe38Leu) |
| T144C (conserved) | G279C (Leu93Phe) | T775C (Ser259Pro) | A227G (Arg76Gly) |
| A250G (Asn84Asp) | A446G (Gln149Arg) | A787G (Ile263Val) | A289G (conserved) |
| T375C (conserved) | T711C (conserved) | C1106G (Ala369Gly) | G467A (Gly156Ser) |
| T494C (Met165Thr) | A915G (conserved) | T1226C (Met409Yhr) | G495T (Met165Ile) |
| T544C (Phe182Leu) | A1124G (Asn375Ser) | A1250G (Asp417Gly) | G501T (conserved) |
| T591C (conserved) | A1209G (conserved) | T1281C (conserved) | T591C (conserved) |
| A647G (Asn216Ser) | A1250G (Asp417Gly) | | T711C (conserved) |
| T689C (Ile230Thr) | A1264G (Lys422Glu) | **MR131** | T756C (conserved) |
| T711C (conserved) | T1268C (Phe423Ser) | T42C (conserved) | T763A (Leu255Met) |
| A725G (Asp242Gly) | | T163G (Phe55Val) | A799G (Ser267Gly) |
| T763C (conserved) | **MR097** | T396C (Asn132Ser) | A810G (conserved) |
| A879G (conserved) | C83T (Ser28Phe) | T425A (Ile142Asn) | A832T (Thr278Ser) |
| A922G (Ile308Val) | A136G (Asn46Asp) | A642G (conserved) | A902T (Asn301Ser) |
| T980C (Phe327Ser) | T346C (Phe116Leu) | T655C (Ser219Pro) | C1061G (Thr354Arg) |
| A1208G (Glu403Gly) | T711C (conserved) | T711C (conserved) | A1122T (Lys374Asn) |
| | T851C (Leu284Pro) | A867G (conserved) | G1205C (Gly402Ala) |
| | A911T (Lys304Ile) | A937T (Thr313Ser) | |
| | A963T (conserved) | A952G (Arg318Gly) | |
| | A1120G (Lys374Glu) | A971G (Leu324Pro) | |
| | T1139C (Leu380Ser) | A1000G (Asn334Asp) | |
| | A1144G (Thr382Ala) | A1029T (Arg343Ser) | |
| | A1257G (conserved) | A1070G (Asp357Gly) | |

responsive growth arrest phenotype, 2) Residue was located in a transmembrane domain, and 3) Residues were in close proximity to previously described receptor mutations demonstrating unique phenotypes (Marsh, 1992; Konopka, et al., 1996). PCR based site-directed mutagenesis (see Materials and Methods) of the wild-type *STE2* gene was carried out to produce 4 mutant receptors each expressing one of the following mutations: F55V, S219P, S259P, or I263V (Figure 8). Two other mutant receptors were generated during site-directed mutagenesis that were not designed. One mutant receptor contained a triple mutation (K239R, S259P, and S325Y) while another mutant receptor contained a stop codon in the 2$^{nd}$ extracellular loop (Q118stop) that would produce a receptor protein truncated after the 2$^{nd}$ transmembrane domian. This truncated receptor was tested for response to [Nle$^{12}$]α-factor and antagonist and did not respond to either (data not shown). All six of the above described mutant receptors were sequenced (n=2) by the MBRF and sequences compared to the published wild-type sequence of *STE2* (Burkholder & Hartwell, 1985; Nakayama, et al., 1985).

*Biological response of mutant α-factor receptors to [Nle$^{12}$]α-factor and various analogs*——The four mutant receptors constructed by site-directed mutagenesis (for simplicity, the mutant receptors will be designated by their mutations only; F55V, S219P, S259P, and I263V), the truncated receptor (Q118stop) and wild-type Ste2p (WT) were tested for their growth arrest response to 3 μg of [Nle$^{12}$]α-factor and 10 μg of 5 or 6 α-factor analogs (Table 3). Previously published antagonists desTrp$^{1}$desHis$^{2}$[Nle$^{12}$]α-factor and desTrp$^{1}$[Ala$^{3}$,Nle$^{12}$]α-factor (Eriotou-Bargiota, et al., 1992;

**Figure 8:** Site-directed mutations introduced into the α-factor receptor. Mutations were introduced individually using a modified PCR protocol (see Materials and Methods and Figure 6). Extra, extracellular; PM, plasma membrane; Cyto, cytosolic. Amino acids are represented by single letter designations; first letter, native residue; number, residue position in protein; second letter, mutant residue. Circled mutations were specifically engineered into the α-factor receptor and boxed mutations were introduced during site-directed mutagenesis due to the intrinsic error rate of the thermostable polymerase during amplification.

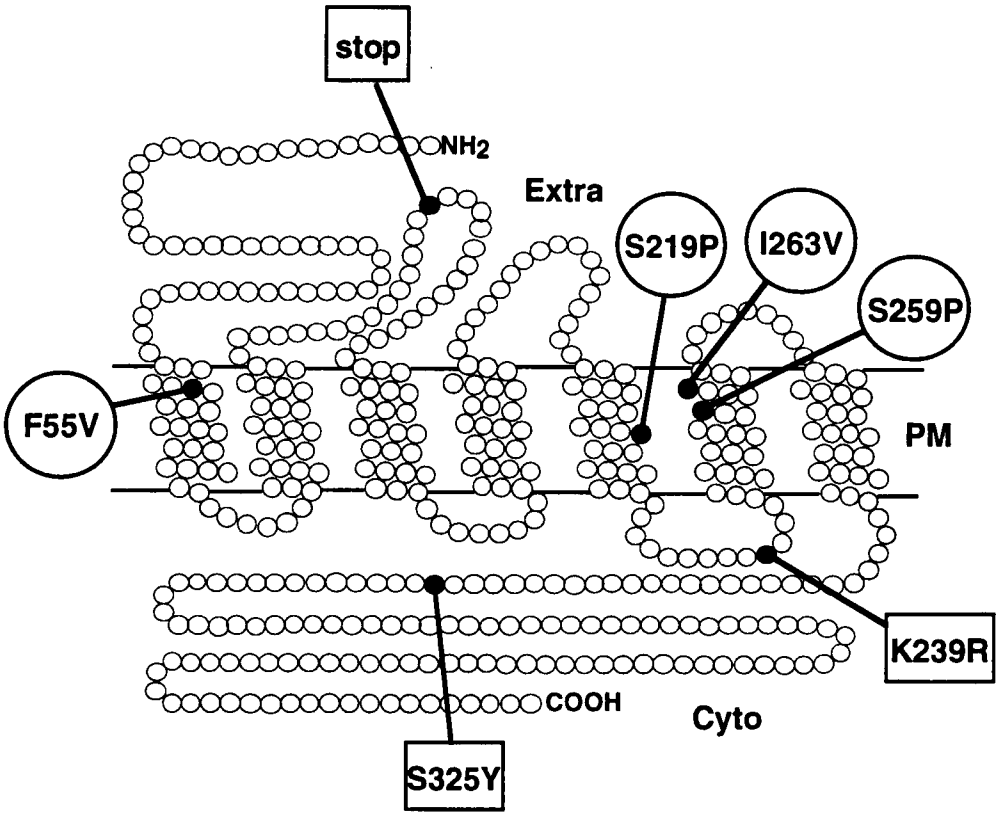


**Figure 8 :** Site-directed mutations introduced into the α-factor receptor.

**Table 3:** Growth arrest response of mutant α-factor receptors to various analogs. (*a*) The amounts of peptides used were 3μg of [Nle$^{12}$]α-factor and 10μg of all other analogs. The analogs tested are all antagonists to the α-factor receptor except [*D*-Ala$^{10}$,Nle$^{12}$]α-factor which is a synergist. All responses were ligand dependent. (*b*) The yeast strain LM102 (*MATa ste2-Δ1 FUS1*-lacZ::*URA3 bar1 ura3 leu2 his4 trp1 met1*) was a gift from Dr. Lorraine Marsh. (*c*) The parent plasmid used for the construction of the mutant vector expression plasmids was pRS314 (bla ori *CEN6 TRP1* f1(+) lacZ). (*d*) Receptor genes containing only single mutations were confirmed by MBRF sequencing except for S259P from which plasmid DNA has not yet been recovered. (*e*) The abbreviation nd stands for not done. (*f*) Halos were distinct after 24hrs. of lawn growth but filled in rapidly by 36hrs. (*g*) A partial response was observed where the halo was discernable but fuzzy. (*h*) The S259P single mutation has not been sequenced confirmed because the plasmid DNA has not yet been recovered from the cotransformed yeast colony demonstrating the antagonist responsive phenotype.

## Table 3

*Growth arrest response of mutant α-factor receptors to various analogs.*

| Strain[b] | Plasmid[c] | mutation[d] | Halo assay (mm)[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | α-factor | desW$^1$desH$^2$ | desW$^1$A$^3$ | *D*-A$^2$ | *D*-A$^3$ | *D*-A$^4$ | *D*-A$^{10}$ |
| LM102 | pGA314.WT | none | 33 | - | - | nd[e] | - | - | - |
| LM102 | pGA314.S219P | S219P | 34 | - | 27[f] | 18[g] | 17[f] | - | 22[f] |
| LM102 | pGA314.I263V | I263V | 31 | - | - | nd | - | - | - |
| LM102 | pGA314.Q118stop | truncated protein after TM2 | - | - | - | nd | - | - | - |
| LM102 | pGA314.F55V | F55V | 30 | - | - | - | - | - | 26 |
| LM102 | pGA314.S259P[h] | S259P | 25 | 22 | 24 | 20 | 23 | 24 | 24 |

Raths, et al., 1988) as well as newly identified antagonistic ([*D*-Ala$^2$,Nle$^{12}$]-, [*D*-Ala$^3$,Nle$^{12}$]-, [*D*-Ala$^4$,Nle$^{12}$]α-factor) and synergistic ([*D*-Ala$^{10}$,Nle$^{12}$]α-factor) peptide analogs of α-factor (Abel, et al., 1996, *in preparation*) were used to challenge mutant receptors. None of these analogs of α-factor ellicit a growth arrest response from a cell expressing a wild-type receptor. All responses discussed in this manuscript were ligand dependant (constitutively responding mutant receptors were never isolated due to the specific design of the screen).

Wild-type, F55V, S219P, S259P, and I263V receptors demonstrated a growth arrest response to synthetic [Nle$^{12}$]α-factor of between 25mm to 34mm diameter zones of inhibition. The truncated receptor (Q118stop) did not respond to any of the peptides tested against it. The mutant receptor S259P demonstrated a response to all peptides tested. Mutant receptor F55V also arrested growth in response to the [*D*-Ala$^{10}$,Nle$^{12}$]α-factor analog. I263V receptor did not show an unusual growth inhibition response to any analog tested. Finally, the S219P receptor demonstrated a positive growth arrest phenotype to the antagonist analogs desTrp$^1$[Ala$^3$,Nle$^{12}$]α-factor, [*D*-Ala$^2$,Nle$^{12}$]α-factor, and [*D*-Ala$^3$,Nle$^{12}$]α-factor and to the synergistic analog [*D*-Ala$^{10}$,Nle$^{12}$]α-factor but not to the antagonist desTrp$^1$desHis$^2$[Nle$^{12}$]α-factor. The halos produced by the S219P receptor from desTrp$^1$[Ala$^3$,Nle$^{12}$]-, [*D*-Ala$^3$,Nle$^{12}$]-, and [*D*-Ala$^{10}$,Nle$^{12}$]α-factor were distinct at 24hrs. post challenge but filled in rapidly by 36hrs. The halo produced by [*D*-Ala$^2$,Nle$^{12}$]α-factor in S219P was "fuzzy" and never showed any distinct zone of inhibition.

# CHAPTER IV

## Discussion

We have generated antagonist responsive mutant α-factor receptors using PCR based random mutagenesis and site-directed mutagenesis. Initially, the α-factor receptor was mutated utilizing the intrinsic error rate of the *Taq* DNA polymerase (Brail, et al., 1993; Ling, et al., 1991; Eckert & Kunkel, 1991) in a modified PCR protocol (Muhlrad, et al., 1992) to try to introduce single mutations randomly throughout the gene. The protocol was successful in that we were able to introduce seemingly random mutations in the *STE2* gene translating into residue substitutions in the receptor protein, but we were not able to titrate the number of mutations introduced into the gene. Therefore, the mutant *STE2* genes that were recovered from colonies responding to an antagonist α-factor analog contained between 10-19 bp mutations per *STE2* ORF corresponding to 5 to 10 residue substitutions per receptor. While information concerning the roles of specific residues of the receptor could not be elucidated from mutant receptors containing so many mutations, they did provide us with targets for site-directed mutagenesis.

The *FUS1*-lacZ induction by antagonist peptides was the initial screen used to identify receptors with functionally critical residue mutations. One hundred and forty-four lacZ positive colonies were isolated and screened further (see Fig. 7 for selection scheme). Eighty-three out of the 144 clones turned out to be false positives for *FUS1*-lacZ induction when

rescreened for blue color production on X-gal containing selective plates and were discarded. Twenty-two clones showed growth arrest in the presence of [Nle$^{12}$]α-factor but did not respond in the halo assay to challenge by antagonist. The fact that these colonies were *FUS1*-lacZ positive but growth arrest negative to the same antagonist peptide may indicate that components of the mating response pathway are differentially up-regulated by pheromone binding and *FUS1* induction is more easily accomplished by the cell than cell-cycle arrest. Also, the signal propagated by these mutated receptors may be just "loud" enough to induce the *FUS1* gene but not the machinery necessary for growth arrest. Two of these receptor clones were randomly chosen to be sequenced to identify mutated residues.

Twenty colonies isolated from the initial screen demonstrated no growth arrest phenotype to either antagonist analogs or [Nle$^{12}$]α-factor. The induction of the *FUS1* promoter by antagonist and the lack of cell cycle arrest by either antagonist or agonist makes this set of mutant receptors interesting for further study. Clone MR097 was selected at random as a representative of this phenotype set and plasmid DNA was isolated and sequenced to try to identify residue(s) responsible for this observed phenotype (see Table 1). This clone contained 11 bp mutations that translated into 8 amino acid substitutions in the receptor protein. Two residue substitutions were located in the N-terminal domain, 2 residues were mutated in the transmembrane domains, and 4 mutations were located in the C-terminal tail of Ste2p. Due to the redundancy of the genetic code, about 30% of the base mutations found for all the mutant

receptors sequenced were conserved and did not code for a new amino acid in the translated protein.

Eighteen clones demonstrated a relaxation of ligand specificity by responding to both antagonist and agonist peptides. Two representative clones (MR007, MR012) were randomly chosen for sequencing to elucidate those amino acids substituted in the receptor. MR007 contained 16 bp mutations that translated into 9 amino acid substitutions in the receptor protein. Six residue substitutions were located in the transmembrane domains or connecting loop regions. Three mutations were found in the cytoplasmic tail of the receptor. The mutant receptor MR012 responded to an antagonist as well as to [Nle$^{12}$]α-factor. This clone contained 7 bp mutations that translated into only 5 amino acid substitutions, the fewest of any responsive receptor isolated and sequenced. There were 3 residue mutations in the C-terminal tail region and only 2 mutated residues found in the transmembrane (TM) domain region. The two transmembrane substitutions were situated in close proximity to each other and located in the 6$^{th}$ TM domain region. This region is connected to the 3$^{rd}$ cytoplasmic loop that is believed to transduce the mating signal to a G-protein via a ligand dependant conformational change in the receptor. We chose these two residues for targets of site-directed mutagenesis that is described below.

Ligand specific conformational changes of the α-factor receptor have been recently investigated using limited trypsin digestion of the receptor in the presence of agonist and antagonist peptides (Bukusoglu & Jenness, 1996). In the native receptor, the α-factor peptide seems to promote a distinct conformational state that favors interaction of the third cytoplasmic

loop with the G-protein (Stefan & Blumer, 1994; Clark, et al., 1994) while an antagonist peptide promotes a different conformation other than the signal inducing isomerization state. The 18 clones of this phenotype class of mutant receptors may contain mutations that affect the isomerization state allowing for a receptor conformation energetically shifted towards the "on" or active conformation and a receptor in which a less energetically favorable binding event such as one involving an antagonist peptide is capable of inducing the propagation of the mating signal.

One clone (MR131) was isolated that demonstrated upon initial testing a reversal of ligand specificity by responding to an antagonist peptide but not to the agonist [Nle$^{12}$]α-factor. Upon testing this mutant receptor for biological response 2 years later, the antagonist response and agonist non-response was not reproducible. The initial screen for this mutant receptor was carried out in the strain LM23-16az. The second screen of this mutant receptor was done in the strain LM102 and it was believed that the inability to reproduce the initial phenotype might be due to strain differences. However, when mutant receptor #131 (MR131) was retested in the strain LM23-16az, it did not respond to antagonist as was origianally reported. The site-directed mutations engineered into Ste2p based on the MR131 sequence data however, do show unique responses to antagonist peptides. That indicates that some of the mutations in MR131 when expressed separately effect receptor function yet those same mutations together with the other nine found for MR131 do not produce an antagonist response. This observation may indicate that one or more of the other mutations found in MR131 could be reversing the antagonist phenotype seen for single residues mutations described in this section.

Mutant receptor MR131 contained 14 bp mutations that translated into 10 residue substitutions in the receptor protein. Six of the 10 changed residues were located in the C-terminal tail region while 4 substitutions were found in the 1st (F55V), 3rd (N132S, I142N), and 5th (S219P) transmembrane domains. The F55V mutation was interesting due to the close proximity of a mutation in the adjacent methionine (M54) residue described by Marsh (1992) playing a role in ligand specificity of the receptor. The S219P mutation in the 5th TM domain was interesting because of the nature of the mutation (a serine to a "kinked" proline residue) and the location in the protein (TM domain connecting the 3rd cytoplasmic loop). These two residues were therefore selected as targets for site-directed mutagenesis. The nature of this specificity reversal could possibly be explained by a mutation in a receptor residue that normally interacts directly with the native ligand and its loss eliminates normal receptor/ligand binding and discrimination. Antagonist peptides may now be able to occupy the receptor in a fashion that promotes functional isomerization.

As described above, we used the sequence data obtained from the 6 clones of the 4 phenotype classes of mutant receptors to identify specific residue targets of the receptor for site-directed mutagenesis to try to pinpoint which amino acids were responsible for the observed phenotypes. Criteria used to select residues for individual mutagenesis were, 1) The residue was mutated in the isolated receptors from the random PCR mutagenesis screen and demonstrated an antagonist responsive phenotype, 2) The mutant residue(s) were located in a transmembrane domain, and 3) Candidate residues were in close proximity to previously

described receptor mutations demonstrating unique phenotypes (Marsh, 1992; Konopka, et al., 1996) or within domains identified as confering ligand specificity for the α-factor receptor (Sen & Marsh, 1994).

Four specific mutations were introduced into the α-factor receptor to study receptor/ligand specificity and receptor isomerization by signal transduction (Fig. 9). The residue substitution I263V is located in the 6$^{th}$ transmembrane domain which is connected to the G-protein associated 3$^{rd}$ cytoplasmic loop. This amino acid mutation was not a dramatic change in side chain for the receptor but it was 1 of only 2 transmembrane domain mutations isolated from MR012 (Table 1) that demonstrated an antagonist responsive phenotype. However, this mutation did not alter the function of the receptor when compared to the wild type receptor (Table 3).

The mutant receptor containing the F55V substitution was selected as a potential critical residue in receptor function because it was found in the sequence of the random mutant receptor MR131 that demonstrated a switch in ligand specificity. This residue (phenylalanine 55) is immediately adjacent to a methionine 54 residue which had been previously described by Marsh (1992) to confer ligand specificity for the α-factor receptor. The F55V mutant receptor did respond to the synergistic analog [*D*-Ala$^{10}$,Nle$^{12}$]α-factor and also to [Nle$^{12}$]α-factor. The *D*-Ala$^{10}$ analog of does not have any activity in the growth arrest assay on its own, but does enhance the efficacy of [Nle$^{12}$]α-factor when incubated together (Part 3 of this dissertation). This observation suggests that the nature of the F55V mutation does not affect ligand specificity but more likely affects the receptor structure and the energy required for receptor isomerization.

**Figure 9:** View of the relative positions of residue substitutions introduced into the α-factor receptor. Single residue substitutions in the α-factor receptor generated for this study shown as circles. Mutations of the Ste2p described by other investigators as important for ligand descrimination (Marsh, 1992; M54I), receptor recovery (Marsh, 1992; S141L-S219L double mutant), mating specific gene induction (Marsh, 1992; L255S-S288P double mutant), and signaling (Konopka, 1996; P258L) are represented by triangles. Amino acids are represented by their single letter designations. M1 through M7 denote the transmembrane domains of the α-factor receptor. E1 through E4 are designations for the extracellular loop regions connecting the transmembrane domains and I1, I2, and I3 denote the intracellular domains of the receptor.

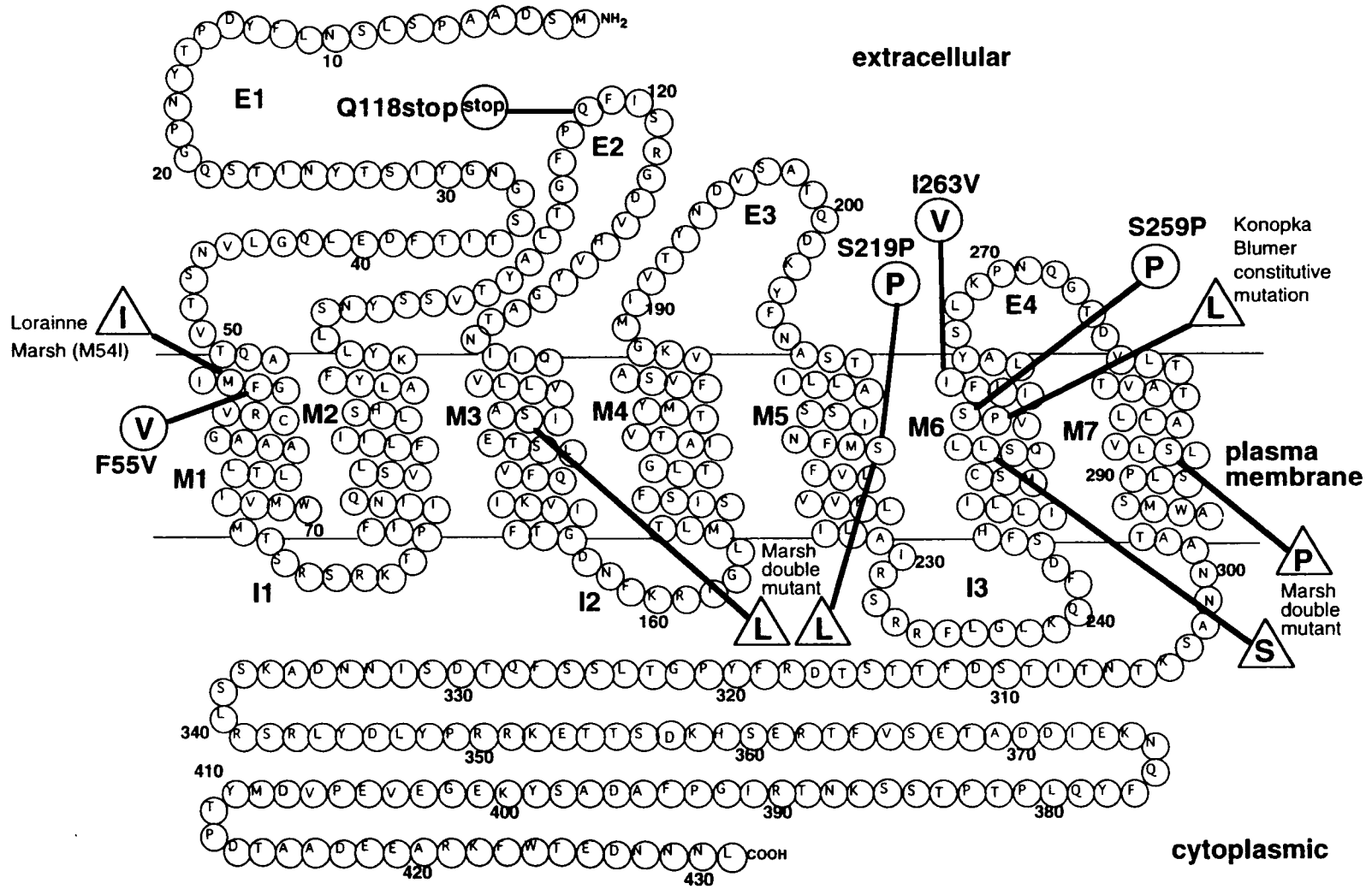


**Figure 9:** View of the relative positions of residue substitutions introduced into the α-factor receptor.

A truncated receptor was produced during an attempt at site-directed mutagenesis that contains a stop codon in the 2nd extracellular loop of the protein. This truncated receptor, if expressed and folded properly in the plasma mambrane of the cell, could possibly be used for binding studies when expressed in a receptorless strain to determine if binding components of the recepter are located in the 1st or 2nd transmembrane domains. The Q118stop truncated receptor could also be coexpressed with wild-type receptor for use in studies of the dimerization state of the receptor. Receptor dimerization has been described in many receptor signaling systems as necessary for activity (Herbert, et al., 1996; P.-Vinay & Finidori, 1995; Mynarcik & Whittaker, 1995; Declercq, et al., 1995; S.-Kroizman, et al., 1994). Receptor dimerization may be a possible explaination of the mode of action of the *D*-Ala$^{10}$ synergist.

Two serine to proline mutations in the α-factor receptor resulted in receptors that demonstrate dramatic changes in ligand specificity. The S219P mutation is located in the 5th transmembrane domain and the S259P mutation is found in the 6th transmembrane domain. These two domains are linked by the 3rd cytoplasmic loop that has been shown to be the link between the receptor and the G-protein (Whiteway, et al., 1989). Both mutations convert a serine residue to a proline in an α-helical region of the receptor protein. Proline has been established as a strong α-helix breaker when found in soluble proteins. However, prolines found in integral membrane proteins do not break the helical structure but possibly function to introduce "kinks" into the helices and thereby confer a degree of conformational flexibility to the protein transmembrane domain (Li, et al., 1996; Saier, Jr., 1994). While the signal transducing properties of the α-

factor seven-transmembrane receptor are assumed to be carried out by conformational changes in the receptor, the presence of prolines in the connective helices of the 3rd cytoplasmic loop might provide the flexibility necessary for that mode of signal transduction.

The S259P mutation produces a receptor that responds to all the antagonist and the synergist peptides tested. Residue 259 is adjacent to another proline 258 and the introduction of two prolines in the 6th transmembrane domain may confer too much flexibility to the receptor and therefore, any peptide ligand that can occupy the binding site of the receptor will trigger a response. A constitutively active receptor mutant was recently reported that contains a mutation in the just mentioned proline (P258L) which removes the flexible proline and replaces it with a "rigid" leucine residue. The introduction of the leucine residue may have locked the receptor in the "on" conformation and thus cause the constitutive phenotype observed.

The S219P mutation also may provide more flexibility to the 5th transmembrane domain and thereby provides a less descriminating receptor species. This mutant receptor does show a degree of specificity between antagonist peptides and could represent a mutation that affects actual ligand interaction with the receptor as well as the isomerization state of the receptor. Further study of these and other mutations are definitely warranted.

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# PART 5

Summary & Prospectus

# Summary and Prospectus

*Summary*——This dissertation has attempted to contribute to knowledge of cellular communication in eukaryotic organisms. Using *Saccharomyces cerevisiae*, we have studied the structural properties of the α-factor ligand required for the transduction of the extracellular mating signal. The ligand binds to the integral membrane α-factor receptor found on the cell surface which, through a putative conformational change, propagates the external signal to the internal cellular machinery. The α-factor receptor is a member of the super-family of 7-transmembrane G-protein coupled receptors that share a conserved structure and mechanism of action. These receptors are found throughout biology and are involved in many cellular functions as diverse as neurotransmission, pain and hunger perception, growth regulation, and odorant recognition. An understanding of sexual conjugation in *S. cerevisiae* could add to the understanding of how these other systems function and are regulated, and help in the design of drugs for regulation and control of such systems when they malfunction leading to disease states.

We have generated mutant α-factor receptors that provide information about the way the protein carries out its biological function. A comprehensive study of the α-factor pheromone was also carried out which led to the development of a model describing the possible functional domains of the peptide ligand.

The strains and techniques are in place for a more thorough examination of the binding and signaling residues critical for the interaction

with ligand and propagation of the mating signal by the α-factor receptor. Site-directed mutagenesis of the receptor can be achieved at any residue using the modified PCR protocol described in Part 3. Well established biological and binding assays as well as a large variety of α-factor analogs available for use provide the tools necessary to identify specific functions of receptor residues.

Information on the structure and functioning of 7TGCRs is still incomplete. This manuscript has added to the understanding of this system of cellular communication and has set in place the tools and techniques for continued study. Obviously, more randomly mutated receptors could be generated using the techniques reported in Part 4 to isolate and target other potentially critical residues of the α-factor receptor. Also, further site-directed mutations based on the results from the random mutants already generated could be introduced into the receptor using the PCR based technique described in Part 4.

From part 4 and the relevant literature, it appears that proline substitutions have a dramatic affect on receptor function. Inspection of the receptor tallies 15 prolines throughout Ste2p with 5 found in the extracellular loops, 7 in the cytoplasmic tail, and only three (P79 found in TM2; P259 in TM6; P290 in TM7) in the transmembrane domains (TM). Mutations in P79 and P290, the two transmembrane prolines not mutated in this study, should be pursued.

Binding assays using the mutant α-factor receptors generated in Part 4 should be done using synthetic [Nle$^{12}$]α-factor along with the many analogs available to Dr. Becker's laboratory. The Ala-scanned analogs synthesized in Part 3 for the study of the pheromone should be tested

against the mutant receptors. Their biological and binding profiles should be compared to the profile already established for this series of analogs with the wild-type receptor.

Double mutants could be introduced into the receptor to further study effects of prolines in the transmembrane domains. A S219P-S259P double mutation might provide information concerning conformational requirements of the receptor and the mechanism of signal transduction. Another double mutation, P258L-S259P, could show whether our serine to proline mutation would compensate for the constitutively active mutation found in Konopka's lab (Konopka, 1996).

Receptor binding studies could be carried out using the truncated receptor found in Part 4 (Q118stop). If the truncated receptor protein is translated and properly inserts into the plasma membrane, binding assays using [Nle$^{12}$]α-factor would identify TM1 and/or TM2 as domains containing binding contacts. The truncated receptor could be co-expressed with wild-type full length receptor for possible studies of receptor dimerization using synergistic peptides discovered from the work of Part 3 ([*D*-Ala$^{10}$,Nle$^{12}$]α-factor) and previously reported from this lab (desMet$^{12}$desTyr$^{13}$α-factor; E.-Bargiota, 1992). Furthermore, the mutant receptor F55V showing a biological response to [Nle$^{12}$]α-factor and also to the synergist [*D*-Ala$^{10}$,Nle$^{12}$]α-factor could also be used to study the possible phenomenon of receptor dimerization.

Investigations into the role of Tyr$^{13}$ could be carried out using synthetic analogs with different side chain configurations to determine if this residue contributes to the binding interaction of the receptor and ligand.

Dr. Larry Zhang from Dr. Naider's laboratory at CUNY has suggested that the charged residues in the TM domains might play a role in ligand binding and receptor structural integrity. Using PCR based mutagenesis, these charged residues could be easily substituted for study.

The mutant receptors already generated should be tested using other assays to determine function when challenged with agonist and antagonist peptides. Marsh (1992) produced a double mutant receptor (L255S-S288P) that was able to induce *FUS1*-lacZ transcription but was unable to mate or arrest in $G_1$ of the cell cycle. Our mutant receptors should be tested for their ability to carry out these other biological responses using the mating, shmoo, and *FUS1*-lacZ induction assays as well as the binding assay to determine $K_d$ values for the mutant receptors.

Although great progress has been achieved in the area of signal transduction and receptor organization in the recent years, more study is needed. The system and techniques established in this dissertation are intended to advance that study.

# Appendix

## List of Abbreviations

| | |
|---|---|
| [$^3$H]α-factor | [$^3$H-Pro$^8$,Nle$^{12}$]α-factor |
| µl | microliter |
| Ac | acetyl |
| Ala or A | alanine |
| Asn or N | asparagine |
| bp | base pair |
| BSA | bovine serum albumin |
| CE | capillary electrophoresis |
| cpms | counts per minute |
| C-terminal | carboxy terminal end of protein |
| desTrp$^1$ | tryptophan is missing from native peptide |
| desHis$^2$ | histidine is missing from native peptide |
| DIEA | diisopropylethylamine |
| dATP | 2'-deoxyadenosine 5'-triphosphate |
| dCTP | 2'-deoxycytidine 5'-triphosphate |
| dGTP | 2'-deoxyguanosine 5'-triphosphate |
| DMSO | dimethyl sulfoxide |
| dNTP | deoxynucleotide triphosphate |
| dTTP | 2'-deoxythymidine 5'-triphosphate |
| Ɛ | extinction coefficient |
| FAB-MS | fast-atom bombardment-mass spectroscopy |
| Fmoc | 9-fluorenylmethoxycarbonyl |
| GDP | guanine diphosphate |
| Gln or Q | glutamine |
| Gly or G | glycine |
| G-protein | guanine-nucleotide binding protein |
| GTP | guanine triphosphate |
| HBTU | 2-(*1H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate |
| HCl | hydrochloric acid |

## List of Abbreviations (cont'd)

| | |
|---|---|
| HF | hydrogen fluoride |
| His or H | histidine |
| HOBt | N-hydroxybenzotriazole |
| HPLC | high performance liquid chromatography |
| Kb | kilobase |
| kDa | kilodalton |
| KF | potassium floride |
| Leu or L | leucine |
| LHRH | luteininzing hormone releasing hormone |
| Lys or K | lysine |
| *M* | Molar |
| MBRF | Molecular biology resources facility |
| Me | methyl |
| Met or M | methionine |
| mmol | millimole |
| Nle | norleucine |
| NMR | nuclear magnetic resonance |
| N-terminal | amino terminal end of protein |
| ORF | open reading frame |
| PAM | Boc-Tyr(2-BrZ)phenylacetamidomethyl resin |
| PCR | polymerase chain reaction |
| Phe or F | phenylalanine |
| PRE | pheromone response element |
| Pro or P | proline |
| TFA | trifuoroacetic acid |
| TRH | thyrotropin releasing hormone |
| Trp or W | tryptophan |
| 7TGCR | 7-transmembrane G-protein coupled receptor |
| SD | synthetic dextrose medium |
| TAME | tosyl(N-α-*p*)-*L*-arginine methyl ester |
| tBoc | *tert*-butoxycarbonyl |

## List of Abbreviations (cont'd)

| | |
|---|---|
| tBu | *tert*-butyl |
| TFA | trifluoroacetic acid |
| Trt | trityl |
| Tyr or Y | tyrosine |
| UV | ultraviolet |
| VIP | vasoactive intestinal peptide |
| Wang resin | (4-hydroxymethyl)phenoxymethyl on 1% cross-linked polystyrene resin |

# Vita

Michael Gregory Abel was born in College Station, Texas on January 8, 1964. He attended schools in the public systems of the Bexar, Travis, and Hayes County Public School Systems, where he graduated from Jack C. Hayes High School in May, 1982. He entered Southwest Texas State University, San Marcos, Texas during August of 1982 and transferred to Texas A&M University, College Station, Texas in August of 1983 where he received a Bachelor of Sciences in Microbiology in December, 1989. He entered the Ph. D. program in Microbiology at the University of Tennessee, Knoxville in January of 1990 in the laboratory of Dr. Jeffrey M. Becker. During the course of his time in this program, he served as a Graduate Teaching Assistant (1990-1991) and as the President of the graduate student organization in the Department of Microbiology (1995-1996). He also represented the College of Liberal Arts as a graduate representative to the Student Government Association (1995-1996). He officially received a Doctor of Philosophy degree in Microbiology in December, 1996.

He is presently working as a postdoctoral fellow in the laboratory of Dr. Kathryn B. Horwitz at the University of Colorado Health Sciences Center in Denver, Colorado. He is studying novel molecular mechanisms of progesterone and estrogen receptor signaling as well as unique properties of isoforms of the receptors in relation to breast and uterine cancers.