HHS Public Access

Author manuscript

Curr Top Pept Protein Res. Author manuscript; available in PMC 2018 April 24.

Published in final edited form as:

Curr Top Pept Protein Res. 2017 ; 18: 133–151.

a-Factor: a chemical biology tool for the study of protein prenylation

Veronica Diaz-Rodriguez[1] and **Mark D. Distefano**[1]

[1]Department of Chemistry, University of Minnesota, 207 Pleasant St. SE, Minneapolis, Minnesota 55455, United States

Abstract

The mating pheromone **a**-factor is a lipidated dodecapeptide found in the budding yeast *S. cerevisiae*. The biosynthesis of this peptide encompasses the same three-step processing pathway (farnesylation of C-terminal cysteine, C-terminal proteolysis and C-terminal methyl esterification) as Ras proteins, mutated forms of which have been found in ~30% of human cancers. For the mating of two haploid yeast cells into a diploid cell to happen, the **a**-factor pheromone travels to the cell surface of the opposite mating cell, where it binds and activates a G-protein coupled receptor. This lipidated-peptide/protein interaction has caught the attention of researchers studying protein prenylation, and studies have shown that this peptide can be used as a model system to understand the role of prenyl groups in protein-protein interactions. Here, we review the different methods used for the synthesis of **a**-factor and **a**-factor analogs containing C-terminal cysteine esters and the assays developed for detecting pheromone bioactivity and quantitation of pheromone efficiency. Also, we review crucial peptide modifications that have been used to investigate relationships between the structure and activity of this lipopeptide with its receptor Ste3p. Finally, we aim to discuss recent and future applications of **a**-factor as a chemical biology tool to study protein prenylation. These include the use of photo crosslinking reactions to map peptide/receptor interactions, the addition of fluorophore tags to visualize the peptide binding, and the use of bio-orthogonal reactions for protein labeling and protein purification.

Keywords

a-factor; farnesylation; farnesyltransferase; isoprenoid; Ste24; protein prenylation; Rce1

INTRODUCTION

Protein prenylation is a post-translational modification that occurs in eukaryotic cells and consists of the attachment of a prenyl group (C_{15}-farnesyl or C_{20}-geranylgeranyl) to the C-terminal cysteine of specific proteins containing a C-terminal CaaX box sequence. In this four amino acid sequence, C is a cysteine, “a” is an aliphatic residue and X is a residue that defines the type of prenyl addition (farnesylation or geranylgeranylation). Ras is a farnesylated protein important in cell signaling that regulates cell growth, cell differentiation

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

and cell survival. Mutant forms of Ras are found in approximately 30% of human cancers making prenylation a research target for the development of cancer treatment [1, 2].

The prenylation of proteins consists of a three-step processing pathway that involves three different enzymes. For protein farnesylation, the soluble protein farnesyltransferase (PFTase) attaches a farnesyl group to the thiol functionality of a cysteine near the C-terminus, followed by proteolytic cleavage of the “” tripeptide by a membrane-attached protease. Subsequent methyl esterification of the C-terminal cysteine by a carboxymethyltransferase membrane-bound enzyme produces a hydrophobic protein that is targeted to the membrane for proper functioning (Figure 1) [3]. These modifications have also been associated with protein-protein interactions, intracellular trafficking and protein stabilization [4, 5].

The field of protein prenylation started in the early 1980’s after the analysis of mating pheromones, secreted by several fungal species, contained an farnesyl group [6]. Subsequent analysis of pheromones from other fungal species provided evidence for the methyl esterified C-terminus and the importance of these modifications for the bioactivity of the pheromones [6-11]. One of the most studied lipidated pheromones is the 12-mer peptide **a**-factor from *S. cerevisiae*. Studies on **a**-factor have provided extensive knowledge in the prenylation field including the identification of the CaaX-processing enzymes [12]. The highly hydrophobic **a**-factor peptide has caught the attention of researchers working in the area of protein prenylation due to the similarity of this peptide with the C-terminal portion of farnesylated proteins. Similar to these proteins, the farnesyl moiety and the methyl ester group incorporated at the C-terminal cysteine of the **a**-factor peptide have been shown to be critical for its bioactivity [13, 14]. Marcus and coworkers demonstrated that analogs of this dodecapeptide lacking both modifications were totally inactive [15]. Also, it is believed that the purpose of the farnesyl moiety is to increase the hydrophobicity of the peptide since studies have shown that replacement of the farnesyl moiety with less hydrophobic substituents significantly decreased its activity [15]. Because it has been shown that the farnesyl group is necessary for **a**-factor activity, this demonstrates the important function of the farnesyl group in protein-protein interactions in addition to its role in membrane targeting [16].

Due to the lack of availability of **a**-factor and simple methods for its synthesis, the use of this peptide as a chemical tool for studying protein prenylation has been underappreciated. Recently, our group took the initiative to develop new methods for the facile synthesis of **a**-factor and other analogs to capitalize on the **a**-factor/receptor system as a simple model to understand the function of prenyl modifications of many proteins in cells [17-19]. Due to the similarity of **a**-factor with important farnesylated proteins, this peptide could serve as a useful system to study the processing of prenylated proteins and whether proteins are able to recognize and interact with the farnesyl group.

Mating of yeast: an overview

The budding yeast *Saccharomyces cerevisiae* is a single-celled eukaryote that can exist either as a diploid (cell containing two sets of chromosomes) or haploid form (cell containing only one set of chromosomes) [16]. In its haploid state *S. cerevisiae* can exist as either of two types of cells, the α-type or the **a**-type. Each one of these haploid cells secretes

a polypeptide mating pheromone that stimulates the cell surface receptors on the opposite cell type, which then results in a cellular mating response between two different cell types [20-25]. A representation of the yeast mating is shown in Figure 2.

Mating pheromones

α-Type cells secrete the mating pheromone α-factor, which is a tridecapeptide with the sequence WHWLQLKPGQPMY. **a**-Type cells secrete the mating pheromone **a**-factor, which is a farnesylated dodecapeptide with the sequence YIIKGVFWSPAC(farnesyl)-OMe [20]. The α-factor targets the receptor Ste2p that is located on **a**-type cells,[21] while the **a**-factor targets the receptor Ste3p that is located on α-type cells [23]. Ste2p and Ste3p are G protein-coupled receptors (GPCRs) that initiate the mating response.

GPCRs

GPCRs are one of the largest classes of cell-surface receptor proteins. More than 1,000 proteins of this class have been identified to date [22]. These proteins are important due to their function in signal transduction pathways [26]. There are three essential components that characterize the signaling process through GPCRs including (1) the membrane bound receptor with seven transmembrane helical segments that binds the signaling molecule, (2) a guanosine nucleotide-binding protein (G protein) that activates the effector enzyme and (3) an effector enzyme that generates an intracellular second messenger. The overall role of GPCR is to indirectly regulate the activity of the effector enzyme which is typically a different plasma-membrane-bound protein [26]. The binding of pheromones (α-factor or **a**-factor) to their corresponding receptors activates a signaling cascade. This cascade starts with the exchange of bound GDP (guanosine diphosphate) for GTP (guanosine triphosphate) followed by dissociation of the coupled G protein into its α and βγ subunits on the cytoplasmic side of the plasma membrane. A variety of events, including G_1 growth arrest, cellular elongation, gene induction and agglutinin biosynthesis [22], are the result of the signaling pathway and culminate in cell fusion of the haploid cells (α-type and **a**-type) to produce a diploid progeny [20].

a-Factor: biogenesis, export, and receptor interaction

Even though both **a**-factor and a-factor are functional analogs in yeast cells, which interact with membrane-bound GPCRs and stimulate them to cause the signaling events that result in mating, their biogenesis and secretion follow different mechanisms. The α-factor pheromone is biosynthesized and secreted via a “classical” secretory pathway [27-29]. In this pathway, proteins are synthesized by ribosomes and enter the endoplasmic reticulum. A series of steps that involve different transformations of the Golgi apparatus help in the progression of protein post-translational modifications and secretion to the outer cell membrane. The α-factor precursors are a 165 and 120 amino acid chains from the redundant gene products MFα1 and MFα2 [30, 31]. The MFα1 precursor contains an N-terminal signal sequence, a “pro” region, and four copies of the α-factor 13-mer sequences linked by spacers that have proteolytic cleavage sites for multiple proteases [28]. During the different steps of the secretory pathway, the precursor is modified and cleaved in the last transformation step in the Golgi apparatus where it generates four copies of the final a-

factor. Finally, secretory transport vesicles move the final product to the membrane for secretion.

Production of a-factor precursors

Interestingly, the **a**-factor follows a very different biosynthesis pathway than that of the α-factor (Figure 4). The **a**-factor precursor peptides come from 36 and 38 amino acid sequences, genes products of MFa1 and MFa2. These genes are homologous and functionally redundant with few different amino acid residues [32]. The **a**-factor biosynthesis starts in the endoplasmic reticulum where the **a**-factor precursor undergoes a series of steps that modify its structure at the C-terminus (3 steps) and the N-terminus (2 steps) to form the mature peptide in that same location. After maturation, the peptide interacts with Ste6p, a membrane-bound protein located at the plasma membrane that exports **a**-factor outside of the cell (1 step) [23, 33-36].

C-terminal processing

The first steps of **a**-factor maturation encompass the modification of the C-terminal sequence of the peptide via the protein farnesylation pathway. First, the enzyme farnesyltransferase (FTase) recognizes the C-terminal CaaX motif (CVIA in **a**-factor) and mediates farnesylation of the cysteine thiol. This FTase contains two subunits Ram1 (β-subunit) and Ram2 (α–subunit). The farnesylation of **a**-factor has been shown to be critical for subsequent biosynthesis steps and biological activity of the pheromone, since the prenyl group facilitates membrane targeting of the peptide where other enzymes reside [2, 37, 38].

Second, the farnesylated peptide is recognized by one of two multi-spanning membrane proteases localized in the ER membrane including Rce1 (Ras-converting enzyme) and Ste24 [36, 39-42]. These enzymes have redundant activity towards the proteolytic cleavage of the –aaX tripeptide (-VIA) of **a**-factor precursor leaving behind a product with a C-terminal cysteine. While both proteins have the same type of activity towards aaX cleavage, they do not have a homologous sequence and the only feature they share is being multi-spanning membrane proteins [39, 42]. It is hypothesized that their mechanisms of actions are also different but there is no strong evidence of that since the purification of Rce1 in its active form has not yet been accomplished.

The third step in the process is the methyl esterification of the C-terminal cysteine. This step is catalyzed by the isoprenyl cysteine carboxymethyl transferase (ICMT), Ste14p, also a membrane-bound protein. This enzyme transfers a methyl group from S-adenosylmethionine (SAM) to the free carboxylate of the farnesylated cysteine. The product of this enzymatic reaction is the fully C-terminally-modified **a**-factor precursor [43-45].

N-terminal processing

The second set of reactions in **a**-factor maturation involves N-terminal modifications leading to the final mature pheromone. Two different proteins catalyze proteolysis of the N-terminal sequence. The enzyme Ste24p mediates the cleavage between residues T7 and A8 followed by the enzyme Axl1 mediating cleavage between residues N^{21} and Y^{22} [46]. There have been multiple studies that demonstrate the dual activity of Ste24p as a C-terminal and N-

terminal protease of **a**-factor precursor peptides [40, 42, 47, 48]. Michaelis et al. suggested that although there is a single catalytic site, there could be two substrate recognition regions within Ste24p [12]. The Axl1 is a zinc metalloprotease that only cleaves the targeted sequence after Ste24p premoves the first residues of the **a**-factor N-terminal sequence. While the N-terminal proteolysis reaction mediated by Ste24p is known to occur on the cytosolic face of the ER membrane, the precise location of the Axl1 protein is not yet known [49] although it is predicted that Axl1 is not a multi-spanning membrane protein and that it could be a soluble protein [12].

Export of a-factor

Once the mature dodecapeptide is synthesized from its precursor, the protein Ste6p mediates the export of **a**-factor via a nonstandard mechanism. This protein acts as a pump, which transports **a**-factor out of the cell, in an unconventional way that does not involve a "classical" protein secretory pathway [33, 50, 51]. There is evidence that supports that the Ste6p protein recognition site does not depends highly on sequence-specific amino acid code. This was supported by the ability of Ste6p to recognize *Schizosaccharomyces pompe* mating pheromone M-factor (a prenylated, carboxymethylated peptide with amino acid sequence dissimilar with **a**-factor) [52].

Receptor interaction

After **a**-factor exits the cell by its nonclassical export mechanism, it travels to the surface of *MAT*a cells (opposite mating cell type) where it interacts with its G-protein coupled receptor Ste3p [53]. A sequence of events is triggered upon interaction of **a**-factor with its receptor due to stimulation of an intracellular signal transduction pathway. The main events are cell-cell fusion, followed by nuclear fusion and diploid formation [54]. Since the presence of this receptor is essential for mating, haploid *MAT*a cells lacking Ste3p are sterile. The fundamentals of the interaction between **a**-factor and Ste3p are not as clear as the counterpart system α-factor/Ste2p receptor [21, 55-60]. The structural difference between the two mating pheromones is the main reason for this information gap (Figure 3). α-Factor is a hydrophilic peptide that shows specific binding with its receptor. In contrast, **a**-factor is quite hydropobic due to the presence of both the farnesyl and carboxymethyl ester groups. These highly hydrophobic structural properties complicate pheromone/receptor binding studies due to non-specific binding of the peptide with membranes.

A number of studies have shown that both the **a**-factor sequence and its C-terminal modifications are necessary for optimal activation of Ste3p and mating of cells [15, 61]. Other efforts to understand **a**-factor structural components that directly affect bioactivity-include sequence mutations and truncations [62, 63], synthesis of isoprenoid-modified analogs, [13-15, 17, 64] and C-terminal ester [15, 18, 19, 61, 64] analogs. The goal of this article is to discuss in detail how these studies have contributed to the understanding of **a**-factor structure and its influence in **a**-factor activity.

Bioassays for a-factor activity

When the pheromones bind the receptors on their target cells, a number of cellular responses and events occur that are necessary for mating. These include, growth arrest of the cell,

competence for nuclear fusion, production of cell surface agglutinins, induction of gene transcription, and a morphological change of the haploid cells termed shmooing. There are different types of bioactivity assays that have been developed to measure the activity of the **a**-factor peptide. These methodologies have been developed based on the signaling events that occur upon pheromone release and/or binding with its receptor. These include the Shmoo formation assay, the FUS1-lacZ induction assay, the mating restoration assay, and the halo (growth arrest) assay [12].

Shmoo assay

One of the first methods used to detect **a**-factor activity was through shmooing formation of *MAT*a observed microscopically. Shmooing is the name given to the morphological alteration of the haploid yeast cells formed in response to a pheromone from the opposite mating type. The cellular bulge produced is called the “shmoo” by its resemblance to the 1950’s cartoon character. A typical experiment consists of preparing a series of dilutions of **a**-factor and **a**-factor analogs and adding them to a cell suspension containing *MAT***a** and *MAT*a cells. After incubation, cells are placed in a hemocytometer and observed in a microscope to quantitate the total number of shmoo cells vs. unbudded cells (percent shmoos). The end point of activity is defined by the concentration of **a**-factor that causes 50% of shmoos [12, 15, 65, 66].

FUS1-lacZ induction assay

The FUS1-lacZ induction assay is used to measure pheromone response by evaluating its ability to induce transcription of β-galactosidase from the pheromone responsive promoter element FUS1. The end point of activity of this assay is defined by the concentration of **a**-factor required to induce a response in β-galactosidase [13, 15]. Due to its complexity and lack of sensitivity, this assay is not the first option when it comes to the detection of pheromone activity.

Mating restoration assay

The mating restoration assay quantifies the minimum amount of pheromone that is necessary to produce conjugation of haploid cells to form diploid cells. A typical experiment consists of the preparation of a mixed lawn of *MAT*a and *MAT***a** cells defective in **a**-factor production (*mfa1 mfa2*). Both *MAT* cells contains auxotrophies that inhibit the growth of unmated haploid cells but allows for the newly formed diploid cells to grow on a synthetic depleted (SD) medium. **a**-Factor and **a**-factor analogs are spotted onto the mixed lawn of cells. Incubation for two days produces colonies of diploid cells where the pheromone was spotted. This method has also been used to quantitatively measure the efficiency of mating of the pheromones. To do this, **a**-factor and **a**-factor analogs dilutions are prepared and plated selectively for diploids. This assay has been shown to be accurate over at least six orders of magnitude and have been used to test mutations of proteins involved in the biogenesis of **a**-factor, mutations of the **a**-factor itself, and to test synthetic peptide analogs with modifications within the C-terminal ester or farnesyl moiety [15, 23, 67].

Halo assay

The halo assay (also, growth arrest assay) is a test that detects the G1 cell growth inhibition of haploid cells that occur as a consequence of pheromone/receptor binding. For this assay, a plate containing a lawn of *MATa* containing the hypersensitizing mutation sst2 is spotted with different dilutions of the pheromone. Cells detecting the presence of **a**-factor will develop an observable "halo" representing a zone of inhibition. The amount of active **a**-factor is reflected by the width of the "halo". The end-point of activity is defined as the minimum amount of pheromone added that causes an observable halo. It is important to prove that the growth arrest was caused by presence of **a**-factor and not because of toxicity of the compounds. A control experiment that determines if the compounds are toxic consists on the treatment of a different yeast strain (LM102), which is not susceptible to **a**-factor, with the synthesized peptides. Using this strain, no growth arrest is expected in the presence of the peptides. Using this assay, researchers have observed and quantified the biological activity of **a**-factor and different **a**-factor analogs. Peptides with 1% to 5% wild type activity tend to show a significant halo in this assay making it the most sensitive and commonly used assay for the detection and quantitation of pheromone efficacy [15, 23, 68].

In 1991, Marcus et al. performed an analysis in which they compared the activity of **a**-factor and several synthetic analogs using these four different assays [15]. An important observation was that the difference in activity of the **a**-factor analogs compared to the natural **a**-factor varied based on the assay used. This was due to the biochemical challenges presented when working with the **a**-factor structure. In that study, all the analogs tested had the same amino acid sequence, but since changes were incorporated into the prenyl group and the C-terminal ester, the overall physical characteristics of the peptides such as hydrophobicity (which changes solubility and degradation susceptibility), varied significantly. Unfortunately, this makes the comparison of analogs challenging since the conditions of each assay and therefore the specific physiological response measurements will differ. The mating restoration assay and the halo assay are the most commonly used assays due to their sensitivity and ability to measure **a**-factor activity qualitatively and quantitatively [67].

Chemical synthesis of a-factor

a-factor is a dodecapeptide containing amino acids in all classifications: aliphatic (Ala, Ile, Val), aromatic (Phe, Trp, Tyr), polar neutral (Cys), acidic (Asp), basic (Lys), and with unique properties (Pro, Gly). The peptide contains a C-terminal cysteine modified with a methyl ester in the C-terminus and a farnesyl lipid moiety linked to the side-chain thiol forming a thioether. The sequence of **a**-factor consists of 12 amino acid residues, of which 59% are hydrophobic, 8% are basic, 8% are acidic, and the other 25% are neutral. These high percent of neutral and hydrophobic amino acids combined with the hydrophobicity of the farnesyl group and methylated C-terminus makes this peptide insoluble in aqueous solutions and only soluble in a few organic compounds. This increases the challenges for its synthesis and purification.

When incorporating prenyl groups into peptides using solid phase chemistry, the major challenge is the release of the peptide from the solid support. Currently, due to the use of

basic conditions for the coupling and deprotection cycles of peptide synthesis, acidic conditions are used for the release of protecting groups from the side chains of amino acids and to cleave the peptide from the resin. When a prenyl group, such as farnesyl, is treated under the acidic conditions used in SPPS, a series of isomers of identical molecular weight are obtained due to a reaction of the acid with the double bonds of the prenyl groups. The mixture obtained is complex, making the compound difficult to purify and decreasing the yield of the desired product [69].

However, such challenges have changed due to developments in solid phase peptide synthesis, including mild acidic conditions for cleavage, new specialized protecting groups, and customized resins, bases and coupling reagents for the different sequence possibilities. Even though conventional methods can be used to synthesize peptides incorporating the 20 natural amino acids and a large variety of unnatural amino acids or synthetic molecules, they are not universal for any modification. For this reason, many studies in the field of peptide chemistry focus on the development of new methods that overcome specific challenges in peptide synthesis.

Here, different approaches used for the synthesis of **a**-factor and recent advances in increasing the yield and reducing side reactions while constructing the peptide on resin are described. These methods are applicable for the facile construction of **a**-factor and a variety of **a**-factor analogs with modifications in the sequence, ester moiety and prenyl group.

Solution/solid based methods

Two of the first strategies used for the synthesis of **a**-factor consisted of incorporating the farnesyl group later in the synthesis to avoid exposing the prenyl groups to acidic conditions [15, 64, 70-72]. For the conventional solution phase peptide synthesis, a synthetic Cys(farnesyl)-OCH_3 residue was coupled in solution to Fmoc-Ala to give Fmoc-Ala-Cys(farnesyl)-OCH_3. The synthesis of the N-terminal decapeptide was also performed in solution and required 3+2 and 5+5 fragment couplings. Construction of the protected dodecapeptide was accomplished by a 10+2 fragment condensation of the decapeptide and the Ala-Cys(farnesyl)-OCH_3 in solution. The second strategy used also involved the 10+2 fragment condensation between the farnesylated C-terminal dipeptide and a decapeptide. However, in this latter case, the decapeptide was assembled via solid phase peptide synthesis. Apart from providing a way to make **a**-factor itself, these methods are extendable to the construction of **a**-factor analogs with different modifications at the C-terminus and the cysteine alkyl side chain. Even though these protocols were time consuming and produced peptides in low yield (28% and 25%, respectively), the successful total synthesis of the **a**-factor pheromone facilitated structure/activity analysis of this peptide. These results also motivated peptide chemists to create more convenient routes synthesis of lipidated peptides including **a**-factor for the study of protein prenylation.

Esterification of complete sequence

In an effort to simplify the labor-intensive methods described above, a different route using only solid phase peptide synthesis was proposed [14, 24]. In this method the **a**-factor sequence was assembled on resin using Fmoc/*t*Bu conditions. The protected peptide was

removed from the resin and esterified in a single step. *tert*-Butyl protection was used for the aspartic acid to avoid its esterification in the cleavage step. However, a competing oxidiation reaction of the cysteine thiol was observed that presumably caused low yields of the desired final peptide. Yields on the methanolysis reaction were not reported but several repetitions were necessary to drive the reaction to completion.

Hydrazine linker strategy

More recently, our group used a hydrazine-containing resin for the synthesis of **a**-factor and benzophenone-containing analogs modified at the cysteine side chain [17]. This method was originally developed for the synthesis of peptides containing a C-terminal methyl ester and was successful for the synthesis of fully protected lipidated peptides [73]. The method consists of attaching the first residue (Cys) to a hydrazinobenzoyl AM NovaGel™ resin via a hydrazide linkage. The peptide sequence is then assembled using conventional Fmoc/*t*Bu protection conditions. After peptide assembly, the peptide-containing resin is exposed to Cu(I) oxidation of the hydrazide linkage yielding a diazo-ester intermediate. Spontaneous alcoholysis of the intermediate in the presence of methanol produces the methyl ester-functionalized peptide. Two different strategies were used to synthesize **a**-factor in that work. First, farnesylation of the unprotected peptide on resin was followed by the copper oxidation. This resulted in traces of the desired product, presumably due to the interference of unprotected side-chains with the cleavage reaction or oxidation of reactive functional groups. A less ideal but more successful attempt was to release the fully-protected peptide from the resin using Cu(I) oxidation followed by side-chain deprotection and farnesylation in solution to obtain **a**-factor. Even though this method produced the desired peptide, yield and purity after peptide cleavage were not improved compared to the previous strategies. Nevertheless, it was the first time after ~20 years that **a**-factor was prepared without requiring the use of HF for side-chain deprotection since the peptides were assembled using Fmoc protection in lieu of Boc protection for masking of the α-amino group during SPPS.

Trimethylsilyldiazomethane method

In order to facilitate the synthesis of lipidated peptide, a different method was established aiming for the synthesis of **a**-factor in biology laboratories lacking specialist equipment and chemicals, such as the HF, used in previous synthesis. This methodology consisted of using trimethylsilyldiazomethane to methylate a fully protected peptide acid in solution [66]. For the synthesis of **a**-factor, the peptide sequence was synthesized using Fmoc/*t*Bu SPPS conditions. A commercially available 2-chlorotrityl chloride resin pre-loaded with Fmoc-Cys(Trt) residue was used for the peptide synthesis. After peptide assembly, resin cleavage was performed using low concentrations of acid (1% TFA) in order to avoid deprotection of the side chains. The fully protected peptide was methylated using trimethylsilyldiazomethane, followed by side-chain deprotection and farnesylation in solution. The use of trimethylsilyldiazomethane as a methylating agent provided a more straight forward methodology avoiding the need to use anhydrous solvents and oxygen gas required for the Cu(I) oxidation of the hydrazide linkage reported by Mullen et al. [17].

Trityl side-chain anchoring

A versatile methodology for the synthesis of peptides with C-terminal cysteine methyl esters using trityl side-chain anchoring was recently developed in our group [18, 19]. We envisioned a strategy that will allow the preparation of peptides with C-terminal cysteine esters of varying amino acid sequences (natural and unnatural) and varying alkyl groups at the ester position. Hence, we used the commercial Trt-containing resin to couple Fmoc-Cys-OR via its thiol functionality. The peptide was then synthesized by SPPS using Fmoc/*t*Bu protection followed by side-chain deprotection and cleavage under mild acidic conditions. We were able to use this method for the preparation of **a**-factor in high yields and purity via thiol alkylation with farnesyl bromide in solution. The methodology has also been used to make **a**-factor analogs containing fluorophores appended onto the lysine residue, different alkyl esters at the C-terminus, as well as peptides incorporating modified isoprenoids. Since the farnesylation reaction is performed in solution, this method is not limited to lipidated peptides.

Structure-activity studies of a-factor

The similarity of the C-terminal portion of **a**-factor with farnesylated proteins, that otherwise do not share functional similarities, is an interesting element that highlights the importance of CaaX processing in proteins. Also, the remarkable differences in biosynthetic pathways and structures of **a**-factor and a-factor, while causing related signaling after receptor activation, raise the question of why are they different and why does **a**-factor needs to be farnesylated and further processed. One of the first synthetic **a**-factor analogs synthesized, to study whether the farnesyl and carboxymethyl ester group were essential for activity, lacked both modifications. This changed the peptide structure to one that was less hydrophobic with a free C-terminal cysteine acid. The activity of this peptide significantly decreased to 0.0025% compared to 100% activity for the wild type **a**-factor [15]. This result showed that the sequence alone is not responsible for activity and confirmed the importance of these modifications. This raised the question of whether both the farnesyl and the methyl ester are essential, if only one of them has a major role in activity or if the sequence has a major role but needs the hydrophobic modification for membrane targeting. Due to the challenges presented in the purification of membrane proteins, no crystal structure of the receptor bound to the **a**-factor has been solved. In this section, we summarize the structure-activity studies that have been performed in the past 25 years using synthetic analogs of **a**-factor and cell mutations that change the structure of the peptide. Specifically, we aim to give an account of studies highlighting the individual importance of the peptide sequence, the farnesyl group, and the ester group. We also review how changes in spatial orientation and the combination of structure modifications affect the mating response.

Importance of C-terminal methyl ester on a-factor activity

The presence of a C-terminal methyl ester found in a number of pheromones has been probed to study the consequences of hydrophobic modifications on lipopeptide bioactivity. In order to define the role that the presence of the ester alone has on bioactivty, Marcus et al. synthesized an **a**-factor analog retaining the farnesyl group but lacking the C-terminal methyl ester [15]. This peptide manifests a 100-fold decrease in activity instead of the

100,000-fold decrease observed for the peptide lacking both modifications. This demonstrated that both modifications have important roles, even when the farnesyl group appears to have a major function. The increase of activity based on the presence of a more hydrophobic group in place of a carboxylic acid was tested on the pheromone tremerogen A-10 [74, 75]. This peptide also contains a farnesylated cysteine at the C-terminus but unlike **a**-factor it does not have or need the methyl ester moiety to be active. A peptide analog of tremerogen A-10 containing a C-terminal amide showed an increase in activity to a level above that of the wild-type non-methylated peptide. Also, a peptide analog of **a**-factor modified at the C-terminus with an amide showed higher bioactivity that an analog containing a free carboxylate. This may be related to the charge neutralization that occurs when the anionic carboxylate is neutralized by methylation and its effect on membrane binding. An example of this phenomenon is the incorporation of amides or non-charged moieties to mask the charge of free carboxylate groups on drug carrier molecules in order to facilitate their entrance into the cell [76-79].

Marcus et al. had previously showed that while there is a requirement for hydrophobicity at the C-terminal site, excessive hydrophobicity provided by larger groups caused a decrease in activity similar to the one observed with a free carboxylate group [15]. To better map the hydrophobicity requirements and hydrophobicity range of the methyl ester position, our group synthesized new analogs containing ethyl, isopropyl, and benzyl ester groups at the C-terminus of **a**-factor [19]. The observed activity for the ethyl ester analog and the isopropyl ester analog, were 200% and 50%, respectively, that of the wild-type **a**-factor indicating that these substitutions in place of the naturally occurring methyl ester have minimal effects on biological activity. More striking results were observed with the benzyl ester, which showed 1.2% activity indicating that replacement of the methyl ester moiety present in wild type **a**-factor with a benzyl ester group results in an approximately 100-fold reduction in activity. Overall, these results confirmed that small changes in the size of the C-terminal alkoxy group have minimal effects on the bioactivity of **a**-factor. Taken together, these results and the existing literature suggest that there might be a specific ester group size range required for optimum activity of **a**-factor when the rest of the peptide structure remains intact. By calculating the molecular volumes of ester groups (incorporated in **a**-factor analogs) [15, 19, 64] and analyzing activity percent as a function of ester group size (Table 1) we found that when the ester group exceeds 70 $Å^3$, there is a precipitous drop in activity. This suggests that there could be a defined binding pocket within the receptor that interacts with the C-terminal region of **a**-factor. In the following table, we present all the **a**-factor analogs found in literature that contain modifications in the C-terminal ester motif while the rest of the **a**-factor peptide remains unmodified.

Importance of the farnesyl group on a-factor activity

Since its discovery, the prenylation of proteins and peptides has been recognized as a biologically important post-translational modification required for proper localization and function of mammalian and yeast Ras and maximal biological activity of several mating pheromones including **a**-factor [15]. To obtain a perspective on the efforts made to elucidate the specific role of prenylation in the peptide structure of **a**-factor we review in this section the **a**-factor analogs that have been synthesized containing modifications at its farnesyl site.

In 1991, Marcus et al. synthesized an **a**-factor analog containing a C-terminal methyl ester but lacking the farnesyl group on the C-terminal cysteine. The result of the halo assay and the mating assay showed a decrease in bioactivity of 1,000-fold [15]. This confirmed a major role of the farnesyl moiety for receptor binding and mating. The research that followed that initial report was aimed to answer questions about specific structural properties of the farnesyl moiety that made this hydrophobic group critical for activity or receptor binding, compared to any other hydrophobic chain or lipids found in nature. Different analogs have been synthesized containing changes spanning different modifications of the farnesyl group including entities with carbon chains of different lengths, branched carbon chains, alkene functionalities, and aromatic groups (Table 2). Using the bioactivity data measured for such analogs, our group and others, have developed **a**-factor variants containing fluorophores, photocleavable groups and bioorthogonal groups integrated within the farnesyl group for different applications discussed later in this review.

The first set of **a**-factor analogs prepared by Marcus et al., to study modifications of the farnesyl group included aliphatic, benzylic, prenyl, and geranyl substituents [15]. These peptides were analyzed using four different assays that showed varied results. The data obtained using the halo assay, the most commonly used assay for testing **a**-factor bioactivity and the one used in this review for literature comparison, showed that only the prenyl-containing **a**-factor analog (12% active) has significant bioactivity while the other analogs showed ~100-fold decrease in their activity when compared to the wild-type **a**-factor. When testing the peptides using a mating restoration assay, analogs containing the prenyl (25% active), the geranyl (200% active), and the benzyl (25% active) showed bioactivity results that were close to **a**-factor. With these results in hand, they concluded that the presence of the farnesyl group is not specifically required for high biological activity. To complement that work, Caldwell et al. analyzed the biosynthesis of **a**-factor adding a mutation in the **a**-factor gene to produce a geranylgeranylated product instead of the usual farnesylated one [63]. After analysis using different assays they found that the mutated peptide as well as a synthetic geranylgeranylated peptide retained bioactivity. If there is a binding pocket in the Ste3p receptor specific for the hydrophobic portion of **a**-factor, these results suggest that this pocket is flexible and can accommodate larger groups (such geranylgeranyl) and bulkier groups (including benzyl).

Another set of farnesyl-modified **a**-factor analogs were constructed by Sherill et al. which were based on unbranched aliphatic chains with the purpose of evaluating the importance of branched isoprenoid groups for optimal bioactivity [14]. An **a**-factor analog containing a pentyl group (on cysteine) showed a lower activity (~100-fold) when compared to the farnesyl, while a decyl-containing **a**-factor demonstrated wild type bioactivity. These results suggest that a branched lipid is not essential but that a certain level of hydrophobicity is required for receptor binding. A decrease in activity was also observed, in a study by Marcus et al. [15], for an analog containing a methyl group in place of the farnesyl. However, hydrophobicity does not seem to be the only role of the isoprenoid group since addition of more hydrophobic groups (hexadecanyl) onto the cysteine thiol resulted in a significant decrease of pheromone activity.

A few years later Dawe et al. reported several additional **a**-factor farnesyl analogs [13]. These novel modifications consisted of replacing the 3-methyl group of the farnesyl to create analogs with ethyl, vinyl, *tert*-butyl and phenyl moieties at the 3-position. These farnesyl modifications caused significant increases in the bioactivity up to 8-fold higher than the natural pheromone. Once again this demonstrated that isoprenoid-modified **a**-factor analogs are capable of initiating a biological response, but to varying degrees depending on the nature of the modification. The effect of such modifications on the biological activity of **a**-factor suggested that they altered the specific mode of association with the membrane and that consequently affected the peptide/receptor interaction.

More recently, Mullen et al. synthesized new **a**-factor analogs with photocrosslinkable isoprenoid modifications in efforts to elucidate recognition of these synthetic groups by the Ste3p receptor and with future plans of using those systems to map direct interactions of the receptor with the isoprenoid motif of **a**-factor [17]. The synthetic peptides include benzophenone-containing isoprenoid analogs with C_5 and C_{10} chains. These peptides showed bioactivity results comparable to the natural **a**-factor, opening the door to the design of different **a**-factor analogs containing these modifications at different sites. Our group has also synthesized other **a**-factor analogs containing bioorthogonal azide and alkyne functionalities integrated within the isoprenoid group and shown that those analogs retain near-wild type bioactivity [80].

Importance of the amino acid sequence on a-factor activity

After Marcus et al. demonstrated that the farnesyl and methyl ester groups are both important bioactivity determinants in the **a**-factor pheromone structure [15], considerable focus went into making analogs of **a**-factor to better understand the contributions of CaaX modifications on this pheromone and other prenylated systems (Table 3). In 1994, Caldwell et al. altered residues via mutation within the **a**-factor sequence and create analogs with several amino acid truncations to define other molecular determinants of bioactivity [62]. With these experiments they elucidated **a**-factor residues that may have an essential role in the peptide/receptor interaction. This experiment showed that sequential loss of residues from the N-terminus negatively affects the activity of the pheromone. Minimal bioactivity loss was observed when NH-terminal tyrosine was not present. Further truncation of Ile^2 resulted in an additional activity loss of an order-of-magnitude, while removing Ile^3 and Lys^4 did not cause any additional effect. Removal of the N-terminal sequence Tyr-Ile-Ile-Lys-Gly resulted in an additional order-of-magnitude reduction in activity that was more significant with the further removal of Val and Phe. After truncation of the N-terminal eight residues a completely inactive tetrapeptide with the sequence DPAC(Far)OCH_3 was obtained. This experiment demonstrated that N-terminal residues in **a**-factor sequence do not have a major effect on bioactivity until more than half of the residues are removed.

A different study, on the substitution of amino acid residues within **a**-factor and determination of secretion of these peptides from the **a**-cells, provided data from two peptides that were secreted but were not active as the natural pheromones. Two peptides were chosen from a pool of peptides with residue variations at Gly^5, Val^6, Trp^8, Asp^9, and Ala^{11} positions. Both peptides (YIIKG**I**F**L**DPAC(Far)-OMe and YIIKG**I**F**K**DPAC(Far)-

OMe), with mutations at Val^6 and Trp^8, were chemically synthesized and tested for pheromone activity. These substitutions resulted in 16-25-fold decrease in biological activity based on results obtained in a growth arrest assay. This drop indicates a lack of optimal ligand-receptor interaction in the system when using these mutant pheromones.

Huyer et al. carried out an analysis where different mutations were made at most positions of the mature **a**-factor sequence [23]. That mutagenesis study revealed numerous mutations (at residues I^2, F^7, W^8, D^9, and P^{10}) that render them substrates for biogenesis and export even though they are inactive in subsequent signaling. This indicates that the amino acid sequence of **a**-factor is also critical for recognition or access to its Ste3p receptor.

Importance of stereochemistry and conformation on a-factor activity

Stereochemical and conformational aspects of a-factor activity have also been probed using synthetic analogs. In order to evaluate the influence of the topology of **a**-factor on its activity, Caldwell et al. synthesized **a**-factor analogs varying the chirality of cysteine (**59**, Table 4) and interchanging the farnesyl group and the methyl ester (**60-61**, Table 4) [62]. By inverting the chirality of cysteine (*D*-Cys) they obtained a pheromone with only 5% less activity than **a**-factor. Interestingly, when the farnesyl and methyl group positions were switched, the peptide analog retained significant activity (25% of the natural pheromone). These results indicated that the specific spatial orientation of the farnesyl group is not a key determinant on **a**-factor potency.

Based on an NMR study on **a**-factor conformation that indicated the possibility of a weak interaction between Val^6 and Trp^8, causing a possible bend in the middle of the peptide, Caldwell et al. designed two **a**-factor analogs (**57-58**, Table 4) where Gly^5 was replaced with either *D*- or *L*-alanine to evaluate the consequences in activity [81]. The study produced the first hyperactive pheromone with the *D*-Ala^5 containing analog that showed an activity 4-6-fold greater than the natural pheromone. The *L*-Ala^5-containing analog showed a 4-16-fold lower activity than **a**-factor. The hyperactivity exhibited by this peptide might reflect a preference for the bioactive conformation of the natural pheromone. To further study the nature of these results, Caldwell et al. published data on amino acid truncation and mutations (discussed above), in order to understand the importance of the **a**-factor sequence in the bioactivity of the peptide. From that study, they confirmed the importance of Val^6 and Trp^8 for activity and the flexibility of substituting Gly^5 with other amino acids. Using this information and with the purpose to clarify the structural implications of these findings, Zhang et al. prepared two **a**-factor analogs where they substituted the Lys^4Gly^5 or Gly^5Val^6 dipeptide units for a γ-lactam constraint (**62-63**, Table 4) [82]. They used (*R*)-3-amino-2-oxo-1-pyrrolidineacetic acid and (*S*)-3-amino-2-oxo-1-pyrrolidineacetic acid respectively, since they have both been successfully used to act as conformational constrains in reverse turns. After testing these peptides in a growth arrest assay, they found that one of them acted as a hyperactive agonist exhibiting 32-fold higher activity suggesting that the **a**-factor adopts a reverse turn as its bioactive conformation. These studies strongly suggest that a type II β-turn at Lys^4Gly^5 dipeptide position is a favorable conformation for higher activity.

Xie et al. constructed another set of peptides with similar activity as the natural pheromone (**64-68**, Table 4) [20]. The purpose of studying these peptides was to evaluate a possible correlation between partitioning of **a**-factor into the yeast membranes and optimal interaction of the peptide with its Ste3 receptor [13, 15, 20, 62, 63]. The peptides involved internal amino acid modifications at positions 4 and 5 to also evaluate the importance of the β-turn structure proposed previously. Their results indicated that a type II β-turn was promoted to compensate for the loss of the favorable Lys^4 side chain after incorporation of the new residues and that there was no correlation between the propensity of the pheromone to partition into the lipid and its biological activity.

These were the last two reports involving synthetic **a**-factor analogs in a period of eleven years. During that time many efforts focused on understanding the specific interactions between a-factor and its receptor Ste2p, probably with the goal of finding clues or possible similarities between the two different systems, that could assist in the development of approaches to understand **a**-factor/Ste3p interactions. In the next section, recent efforts made by our group to use **a**-factor as a chemical biology tool are described.

a-factor as a chemical biology tool to study protein prenylation

Photocrosslinkable a-factor analogs—As mentioned above, Ste3p is the receptor that binds **a**-factor. Becker et al. have worked extensively on the study of the other pheromone, the α-factor, and the mechanism of how it binds to its specific GPCR, Ste2p [21, 22, 55]. An important question about these systems (**a**-factor/Ste3p and α-factor/Ste2p) is how are these peptides interacting with their specific GPCR. Crosslinking experiments are a powerful tool for mapping peptide-receptor interactions [22, 83]. In this approach, a photoactivatable group is incorporated to the pheromone. After photolysis, this group is converted into an extremely reactive species causing covalent bonding between the pheromone and the receptor. With the addition of a molecular tag on the pheromone, it is possible to isolate the protein of interest and study the specific interactions between the farnesylated peptide and the receptor protein. Previous work elucidating the α-factor mechanism has shown that a biotin tag in the lysine residue of the peptide and substitution of some of its amino acid residues with the photoactivatable group *p*-benzoylphenylalanine (Bpa) does not significantly affect the activity of the peptide when compared to the wild type α-factor [22]. Cross-linking experiments have been used to demonstrate that the α-factor analogs bind to the Ste2p receptor in the **a**-type mating cell type. Also, these experiments have allowed the definition of the sites of interactions between the α-factor and its receptor [22]. It will be of great interest to use this approach to study the Ste2p/**a**-factor system since little is known about the specific interactions between **a**-factor and its receptor. Mullen et al. prepared analogs of the **a**-factor that incorporate benzophenone as the photoactivatable group as part of the farnesyl moiety [17]. These analogs showed a similar activity when compared to **a**-factor in a growth arrest assay. Considering this, the use of photoactivatable **a**-factor analogs in photocrosslinking experiments could allow a better understanding of the interactions involved in the **a**-factor/receptor system as well as the function of the farnesyl group. As a prelude to these experiments, related **a**-factor analogs incorporating photoactivatable isoprenoids have been used to study their interaction with Ste14p, the methyltransferase responsible for the last step in prenylated protein maturation [84].

Fluorescently labeled a-factor analogs—The addition of spectroscopic probes, such as fluorophores, into ligands has been advantageous for the analysis of interactions of ligands with membranes and receptors. For a successful study is important to ensure that the modification does not disturb the interactions of the ligand and the receptor. Our lab and others have incorporated different fluorophores into **a**-factor at different positions. Based on previous studies, that show essential residues in the sequence, modification of lysine does not significantly affect the activity of the pheromone. Therefore, this residue could serve as a position for moficiation. Recently, we reported the synthesis of an **a**-factor analog containing a 5-FAM fluorophore into the side-chain of the Lys^4 residue [18]. The 5-FAM fluorophore was selected due to its high fluorescence, low cost and ease of attachment to lysine side-chains during SPPS [22, 85]. We also synthesized an **a**-factor analog with an NBD group on the Lys 4 (data not published). This NBD group was chosen as a probe because its fluorescence intensity increases with an increase of hydrophobic environment. This means that it produce a strong fluorescence signal when the peptide is bound to a hydrophobic binding site such as a GPCR [21]. The fluorescent pheromones showed lower but significant bioactivity suggesting that they could be used as probes for binding studies. Future experiments using these peptides should allow the binding of **a**-factor to its receptor to be studied by following changes in the signal of the probe. Related **a**-factor analogs incorporating photoactivatable isoprenoids (for crosslinking) and fluorescent groups (for nonradioactive detection) have been used to study their interaction with Ste14p, the methyltransferase responsible for the last step in prenylated protein maturation [86].

a-factor analogs containing alkyne- and azide-modified isoprenoids

Previously, other groups have synthesized analogs of **a**-factor with isoprenoid variations in order to study the role of the prenyl group in the interaction of **a**-factor with the membrane bilayer and protein receptor [13-17, 64, 87]. Some isoprenoid modifications, particularly when the change is modest, have shown to have little effect on the bioactivity of **a**-factor. Azide- and alkyne-functionalized isoprenoids are particularly useful in metabolic labeling experiments where they can be used to monitor the “prenylome”, the entire complement of prenylated proteins within cells and to measure how their levels vary in different physiological and disease states [88-91]. Accordingly, we have used the **a**-factor system to evaluate the bioactivity of these modified isoprenoids. Thus, analogs of **a**-factor and the **a**-factor precursor peptides containing the alkyne- and azide-modified isoprenoids have been prepared and studied [80]. We incorporated the modified isoprenoids on the cysteine thiol functionality of peptides containing a C-terminal -VIA sequence, C-terminal Cys acid and C-terminal Cys methyl ester. The purpose was to evaluate the ability of these peptides to act as substrates of the post-prenylation processing enzymes Ste24p/Rec1p and Ste14p, respectively, using an *in vitro* assay. The bioactivity of the **a**-factor analogs containing the alkyne- and azide-modified isoprenoids was also analyzed (see Table 4). From these experiments we found that these prenylated peptides have similar and sometimes higher activity when compared with the farnesylated peptides. The peptides were processed by the post-prenylation membrane enzymes and efficiently caused growth arrest of yeast cells. These results, together with previous studies that show the substrate capabilities of these isoprenoid modifications with FTase and GGTase, we were able to demonstrate that these bioorthogonal chemical reporters are processed through the entire prenylation pathway and

are able to activate the signal transduction signal for mating with activity comparable to the natural pheromone. These results suggest the potential use of these modified isoprenoids for metabolic labeling of prenylated proteins/peptides in living cells without concern that they may interfere with normal cell physiology.

CONCLUSION

Given that many prenylated proteins are involved in signal transduction pathways and diseases, peptide analogs of the proteins that are farnesylated are useful tools to study the interactions involved in these processes. A good example of a natural farnesylated peptide is the mating pheromone **a**-factor secreted by *S. cerevisiae*. This dodecapeptide contains a farnesylated Cys and a C-terminal methyl ester shown to be crucial for its activity. This system, where a small farnesylated peptide interacts with its cognate receptor, is an ideal simple system to study how other proteins recognize farnesylated molecules in cells. With facile methods for chemical synthesis in place, synthetic **a**-factor analogs have helped illuminate key aspects of the function of this prototypical molecule including the structure of the isoprenoid, the sequence of the peptide and the nature of the C-terminal ester group. However, the precise details of how the receptor Ste3p interacts with a-factor remain elusive. It is likely that chemical synthesis of a-factor analogs will play a role in addressing this question in future work.

Acknowledgments

This research was supported by the National Institutes of Health (GM084152 and GM106082). Additional support came from the National Science Foundation grant CHE-1308655 V.D.R. was supported by National Institute of Health Training Grants T32GM008700 and T32GM008347.

References

1. Ochocki JD, Distefano MD. MedChemComm. 2013; 4:476. [PubMed: 25530833]
2. Schafer WR, Kim R, Sterne R, Thorner J, Kim SH, Rine J. Science. 1989; 245:379. [PubMed: 2569235]
3. Zhang FL, Casey PJ. Annu Rev Biochem. 1996; 65:241. [PubMed: 8811180]
4. Ghomashchi F, Zhang X, Liu L, Gelb MH. Biochemistry. 1995; 34:11910. [PubMed: 7547927]
5. Seabra MC. Cell Signalling. 1998; 10:167. [PubMed: 9607139]
6. Kamiya Y, Sakurai A, Tamura S, Takahashi N, Abe K, Tsuchiya E, Fukui S, Kitada C, Fujino M. Biochem Biophys Res Commun. 1978; 83:1077. [PubMed: 708426]
7. Ishibashi Y, Sakagami Y, Isogai A, Suzuki A. Biochemistry. 1984; 23:1399.
8. Kamiya Y, Sakurai A, Tamura S, Takahashi N. Agric Biol Chem. 1979; 43:1049.
9. Kamiya Y, Sakurai A, Tamura S, Takahashi N, Tsuchiya E, Abe K, Fukui S. Agric Biol Chem. 1979; 43:363.
10. Sakagami Y, Isogai A, Suzuki A, Kitada C, Fujino M. Pept Chem. 1980; 17:1.
11. Sakagami Y, Yoshida M, Isogai A, Suzuki A. Science. 1981; 212:1525. [PubMed: 17790543]
12. Michaelis S, Barrowman J. Microbiol Mol Biol Rev. 2012; 76:626. [PubMed: 22933563]
13. Dawe AL, Becker JM, Jiang Y, Naider F, Eummer JT, Mu YQ, Gibbs RA. Biochemistry. 1997; 36:12036. [PubMed: 9315841]
14. Sherrill C, Khouri O, Zeman S, Roise D. Biochemistry. 1995; 34:3553. [PubMed: 7893651]
15. Marcus S, Caldwell GA, Miller D, Xue CB, Naider F, Becker JM. Mol Cell Biol. 1991; 11:3603. [PubMed: 2046670]

16. Xie HB, Shao Y, Becker JM, Naider F, Gibbs RA. J Org Chem. 2000; 65:8552. [PubMed: 11112575]
17. Mullen DG, Kyro K, Hauser M, Gustavsson M, Veglia G, Becker JM, Naider F, Distefano MD. Bioorg Med Chem. 2011; 19:490. [PubMed: 21134758]
18. Diaz-Rodriguez V, Mullen DG, Ganusova E, Becker JM, Distefano MD. Org Lett. 2012; 14:5648. [PubMed: 23121562]
19. Diaz-Rodriguez V, Ganusova E, Becker JM, Distefano MD. Biopolymers. 2013; 100:309.
20. Xie H, Becker JM, Gibbs RA, Naider F. J Pept Res. 2000; 55:372. [PubMed: 10863934]
21. Ding FX, Lee BK, Hauser M, Davenport L, Becker JM, Naider F. Biochemistry. 2001; 40:1102. [PubMed: 11170434]
22. Son CD, Sargsyan H, Naider F, Becker JM. Biochemistry. 2004; 43:13193. [PubMed: 15476413]
23. Huyer G, Kistler A, Nouvet FJ, George CM, Boyle ML, Michaelis S. Eukaryot Cell. 2006; 5:1560. [PubMed: 16963638]
24. Khouri O, Sherrill C, Roise D. Biochemistry. 1996; 35:14553. [PubMed: 8931552]
25. Anderegg RJ, Betz R, Carr SA, Crabb JW, Duntze W. J Biol Chem. 1988; 263:18236. [PubMed: 3056940]
26. Calebiro D, Nikolaev VO, Lohse MJ. Journal of Molecular Endocrinology. 2010; 45:1. [PubMed: 20378719]
27. Fuller RS, Sterne RE, Thorner J. Annu Rev Physiol. 1988; 50:345. [PubMed: 3288097]
28. Caplan S, Green R, Rocco J, Kurjan J. J Bacteriol. 1991; 173:627. [PubMed: 1987155]
29. Baker D, Hicke L, Rexach M, Schleyer M, Schekman R. Cell. 1988; 54:335. [PubMed: 3293799]
30. Kurjan J, Herskowitz I. Cell. 1982; 30:933. [PubMed: 6754095]
31. Kurjan J. Mol Cell Biol. 1985; 5:787. [PubMed: 3887136]
32. Michaelis S, Herskowitz I. Mol Cell Biol. 1988; 8:1309. [PubMed: 3285180]
33. Michaelis S. Semin Cell Biol. 1993; 4:17. [PubMed: 8095825]
34. Michaelis S, Chen P, Berkower C, Sapperstein S, Kistler A. Antonie van Leeuwenhoek. 1992; 61:115. [PubMed: 1580613]
35. Paumi CM, Chuk M, Snider J, Stagljar I, Michaelis S. Microbiol Mol Biol Rev. 2009; 73:577. [PubMed: 19946134]
36. Young SG, Fong LG, Michaelis S. J Lipid Res. 2005; 46:2531. [PubMed: 16207929]
37. He B, Chen P, Chen SY, Vancura KL, Michaelis S, Powers S. Proc Natl Acad Sci U S A. 1991; 88:11373. [PubMed: 1763050]
38. Powers S, Michaelis S, Broek D, Santa Anna-A S, Field J, Herskowitz I, Wigler M. Cell. 1986; 47:413. [PubMed: 3533274]
39. Boyartchuk VL, Ashby MN, Rine J. Science. 1997; 275:1796. [PubMed: 9065405]
40. Boyartchuk VL, Rine J. Genetics. 1998; 150:95. [PubMed: 9725832]
41. Lal M, Caplan M. Physiology. 2011; 26:34. [PubMed: 21357901]
42. Tam A, Schmidt WK, Michaelis S. J Biol Chem. 2001; 276:46798. [PubMed: 11581258]
43. Hrycyna CA, Clarke S. Mol Cell Biol. 1990; 10:5071. [PubMed: 2204804]
44. Hrycyna CA, Sapperstein SK, Clarke S, Michaelis S. EMBO J. 1991; 10:1699. [PubMed: 2050108]
45. Hrycyna CA, Wait SJ, Backlund PS Jr, Michaelis S. Methods Enzymol. 1995; 250:251. [PubMed: 7651156]
46. FujimuraKamada K, Nouvet FJ, Michaelis S. J Cell Biol. 1997; 136:271. [PubMed: 9015299]
47. Schmidt WK, Tam A, Michaelis S. J Biol Chem. 2000; 275:6227. [PubMed: 10692417]
48. Tam A, Nouvet FJ, Fujimura-Kamada K, Slunt H, Sisodia SS, Michaelis S. J Cell Biol. 1998; 142:635. [PubMed: 9700155]
49. Lord M, Inose F, Hiroko T, Hata T, Fujita A, Chant J. Curr Biol. 2002; 12:1347. [PubMed: 12176366]
50. Kuchler K, Sterne RE, Thorner J. EMBO J. 1989; 8:3973. [PubMed: 2686977]
51. McGrath JP, Varshavsky A. Nature. 1989; 340:400. [PubMed: 2569166]

52. Christensen PU, Davey J, Nielsen O. Mol Gen Genet. 1997; 255:226. [PubMed: 9236781]
53. Hagen DC, McCaffrey G, Sprague G Jr. Proc Natl Acad Sci U S A. 1986; 83:1418. [PubMed: 3006051]
54. Leberer E, Thomas DY, Whiteway M. Curr Opin Genet Dev. 1997; 7:59. [PubMed: 9024634]
55. Ding FX, Lee BK, Hauser M, Patri R, Arshava B, Becker JM, Naider F. J Pept Res. 2002; 60:65. [PubMed: 12081627]
56. Naider F, Becker JM. Peptides. 2004; 25:1441. [PubMed: 15374647]
57. Bajaj A, Celic A, Ding FX, Naider F, Becker JM, Dumont ME. Biochemistry. 2004; 43:13564. [PubMed: 15491163]
58. Tantry S, Ding FX, Dumont M, Becker JM, Naider F. Biochemistry. 2010; 49:5007. [PubMed: 20420459]
59. Umanah GKE, Huang LY, Maccarone JM, Naider F, Becker JM. Biochemistry. 2011; 50:6841. [PubMed: 21728340]
60. Kim KM, Lee YH, Akal-Strader A, Uddin MS, Hauser M, Naider F, Becker JM. Pharmacol Res. 2012; 65:31. [PubMed: 22100461]
61. Ewenson A, Marcus S, Becker JM, Naider F. Int J Pept Prot Res. 1990; 35
62. Caldwell GA, Wang SH, Xue CB, Jiang Y, Lu HF, Naider F, Becker JM. J Biol Chem. 1994; 269:19817. [PubMed: 8051063]
63. Caldwell GA, Wang SH, Naider F, Becker JM. Proc Natl Acad Sci U S A. 1994; 91:1275. [PubMed: 8108401]
64. Xue CB, Becker JM, Naider F. Int J Pept Protein Res. 1991; 37:476. [PubMed: 1917304]
65. Duntze W, MacKay V, Manney TR. Science. 1970; 168:1472. [PubMed: 5445939]
66. O'Reilly N, Charbin A, Lopez-Serra L, Uhlmann F. Yeast. 2012; 29:233. [PubMed: 22641466]
67. Nijbroek GL, Michaelis S. Methods Enzymol. 1998; 292:193. [PubMed: 9711555]
68. Chan RK, Otte CA. Molecular and Cellular Biology. 1982; 2:11. [PubMed: 7050665]
69. Naider FR, Becker JM. Biopolymers. 1997; 43:3. [PubMed: 9174408]
70. Xue CB, Caldwell GA, Becker JM, Naider F. Biochem Bioph Res Co. 1989; 162:253.
71. Xue CB, Ewenson A, Becker JM, Naider F. Int J Pept Protein Res. 1990; 36:362. [PubMed: 2079391]
72. Xue CB, Becker JM, Naider F. Tet Lett. 1992; 33:1435.
73. Peters C, Waldmann H. J Org Chem. 2003; 68:6053. [PubMed: 12868949]
74. Fujino M, Kitada C, Sakagami Y, Isogai A, Tamura S, Suzuki A. Naturwissenschaften. 1980; 67:406.
75. Tsuchiya E, Fukui S, Kamiya Y, Sakagami Y, Fujino M. Biochem Biophys Res Commun. 1978; 85:459. [PubMed: 743289]
76. Turner RA, Weber RJ, Lokey RS. Org Lett. 2010; 12:1852. [PubMed: 20329782]
77. Beaumont K, Webster R, Gardner I, Dack K. Curr Drug Metab. 2003; 4:461. [PubMed: 14683475]
78. Zablocki JA, Tjoeng FS, Bovy PR, Miyano M, Garland RB, Williams K, Schretzman L, Zupec ME, Rico JG, Lindmark RJ, Toth MV, Mcmackins DE, Adams SP, Panzerknodle SG, Nicholson NS, Taite BB, Salyers AK, King LW, Campion JG, Feigen LP. Bioorg Med Chem. 1995; 3:539. [PubMed: 7648203]
79. Doh HJ, Cho WJ, Yong CS, Choi HG, Kim JS, Lee CH, Kim DD. J Pharm Sci. 2003; 92:1008. [PubMed: 12712420]
80. Diaz-Rodriguez V, Hsu ET, Ganusova E, Werst ER, Becker JM, Hrycyna CA, Distefano MD. Bioconj Chem. 2017; doi: 10.1021/acs.bioconjchem.7b00648
81. Caldwell GA, Wang SH, Dawe AL, Naider F, Becker JM. Biochem Biophys Res Comm. 1993; 197:1173. [PubMed: 8280131]
82. Zhang YL, Dawe AL, Jiang Y, Becker JM, Naider F. Biochem Biophys Res Comm. 1996; 224:327. [PubMed: 8702390]
83. Ruoho AE, Kiefer H, Roeder PE, Singer SJ. P Natl Acad Sci USA. 1973; 70:2567.
84. Hahne K, Vervacke JS, Shrestha L, Donelson JL, Gibbs RA, Distefano MD, Hrycyna CA. Biochem Biophys Res Comm. 2012; 423:98. [PubMed: 22634004]

85. Wollack JW, Zeliadt NA, Mullen DG, Amundson G, Geier S, Falkum S, Wattenberg EV, Barany G, Distefano MD. J Am Chem Soc. 2009; 131:7293. [PubMed: 19425596]
86. Vervacke JS, Funk AL, Wang YC, Strom M, Hrycyna CA, Distefano MD. Journal of Organic Chemistry. 2014; 79:1971. [PubMed: 24502619]
87. Epand RF, Xue CB, Wang SH, Naider F, Becker JM, Epand RM. Biochemistry. 1993; 32:8368. [PubMed: 8347633]
88. DeGraw AJ, Palsuledesai C, Ochocki JD, Dozier JK, Lenevich S, Rashidian M, Distefano MD. Chem Biol Drug Des. 2010; 76:460. [PubMed: 21040496]
89. Palsuledesai CC, Ochocki JD, Markowski TW, Distefano MD. Mol Biosys. 2014; 10:1094.
90. Palsuledesai CC, Distefano MD. ACS Chem Biol. 2015; 10:51. [PubMed: 25402849]
91. Suazo KF, Schaber C, Palsuledesai CC, Odom John AR, Distefano MD. Sci Rep. 2016; 6:38615. [PubMed: 27924931]

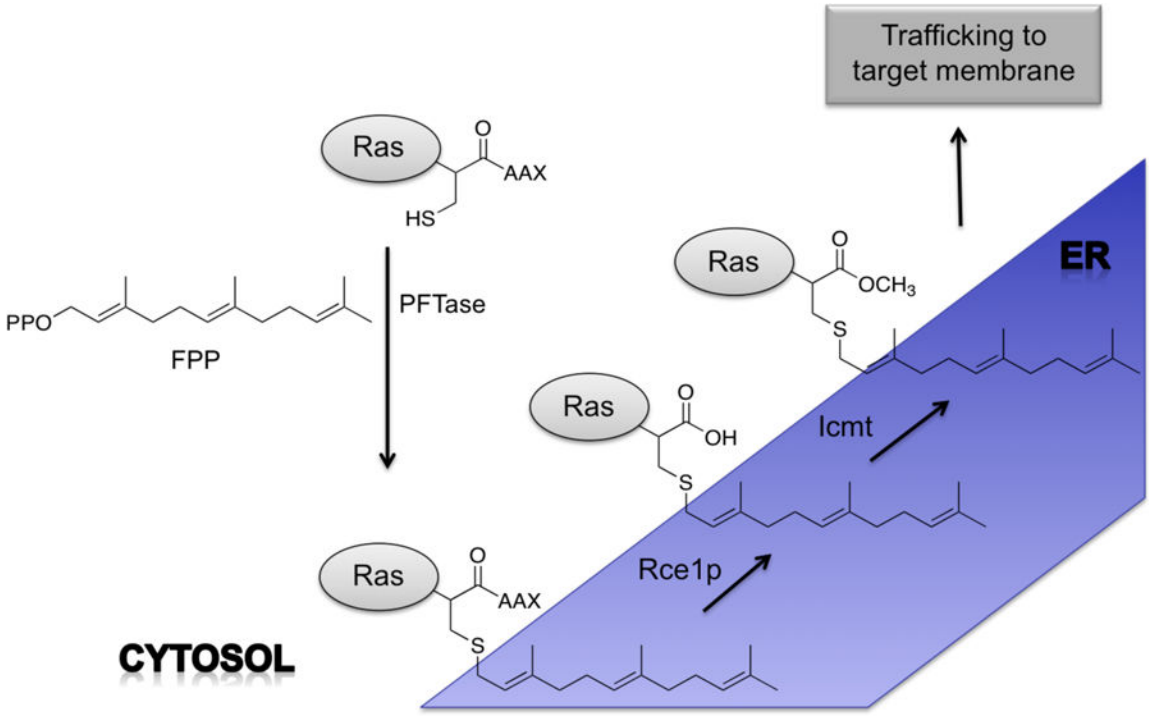

Figure 1.
General representation of the three-step prenylation pathway of Ras proteins.

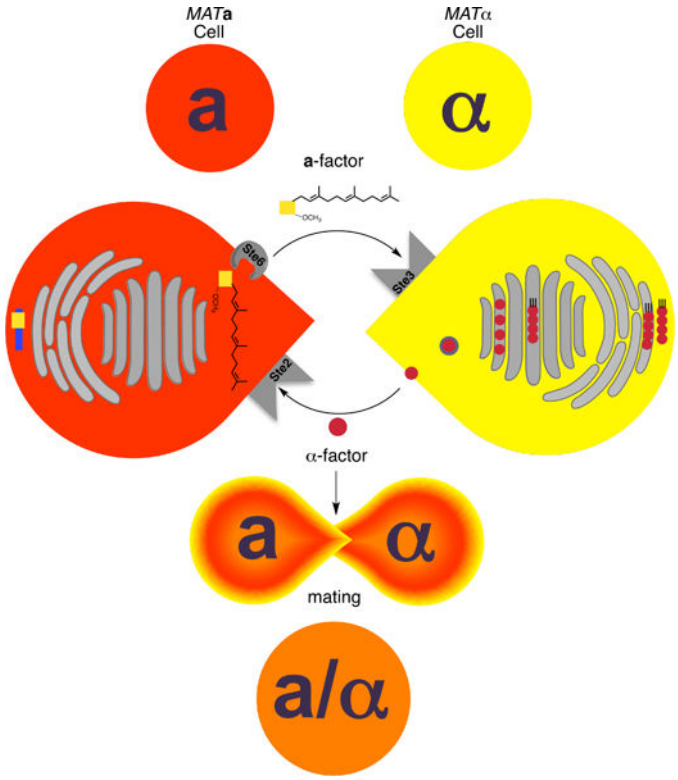

Figure 2.
Overview of the synthesis, secretion and biological activity of the mating pheromones (a-factor and a-factor) in yeast.

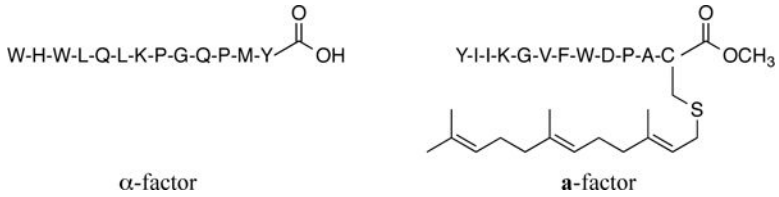

Figure 3.
Chemical structures of a-factor and a-factor pheromones.

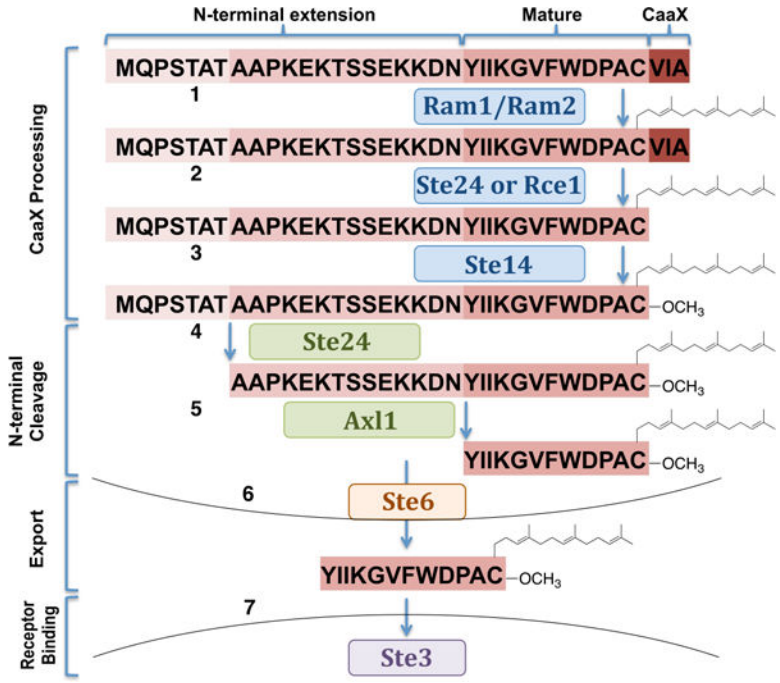

Figure 4.
Model for the biosynthesis of a-factor in *MAT*a cells.

Table 1

C-terminal ester modifications on the a-factor peptide structure reported in the literature.

Entry	Alkyl ester group (R) YIIKGVFWDPAC(Far)-OR	Wild-type activity (%)	R-OH group volume (Å^3)
1	-CH_3 (wild-type)	100	37.2
2	-H	1.5	19.3
3	-NH_2	6	21.9
4	-$CH_2CH_2CH(CH_3)_2$	0.8	104.2
5	-$CH_2CH{=}C(CH_3)_2$	1.5	97.99
6	-CH_2CH_3	200	54.0
7	-$CH(CH_3)_2$	48	70.6
8	-$CHCH_2C_6H_6$	1.2	109.0

Table 2

List of isoprenoid modifications on a-factor reported in the literature

Entry	Isoprenoid group (R^I) YIIKGVFWDPAC(R^I)-OCH_3	Wild-type activity (%)
9	H	0.1
10	Methyl	0.2
11	Hexadecanyl	1.5
12	Benzyl	1.5
13	Prenyl	12.0
14	Geranyl	3.0
15	Geranylgeranyl	25.0
16	$(CH_2)_4$-CH_3	5.2
17	$(CH_2)_5$-NBD	1.3
18	$(CH_2)_9$-CH_3	150.0
19	$(CH_2)_{10}$-NBD	15.0
20	3-ethylfarnesyl	200
21	3-vinylfarnesyl	800
22	3-*t*-butylfarnesyl	200
23	3-phenylfarnesyl	200
24	C5-benzophenone	48.0
25	C10-benzophenone	24.0
26	C10-alkyne	800
27	C15-alkyne	200
28	C15-azide	100
29	C15-dihydro-azide	100

Table 3

a-factor amino acid substitutions and sequence truncations.

Entry	Amino acid sequence	Wild-type activity (%)
30	YIIKGVFWDPAC(Far)-OCH_3	100.0
31	IIKGVFWDPAC(Far)-OCH_3	2.5
32	IKGVFWDPAC(Far)-OCH_3	0.25
33	KGVFWDPAC(Far)-OCH_3	0.25
34	GVFWDPAC(Far)-OCH_3	0.25
35	VFWDPAC(Far)-OCH_3	0.025
36	FWDPAC(Far)-OCH_3	0.01
37	WDPAC(Far)-OCH_3	0.0025
38	DPAC(Far)-OCH_3	>0.0025
39	**N**IIKGVFWDPAC(Far)-OCH_3	100.0
40	**D**IIKGVFWDPAC(Far)-OCH_3	0.1
41	Y**M**IKGVFWDPAC(Far)-OCH_3	38.0
42	Y**N**IKGVFWDPAC(Far)-OCH_3	0.1
43	YII**A**GVFWDPAC(Far)-OCH_3	100.0
44	YII**E**GVFWDPAC(Far)-OCH_3	100.0
45	YIIK**V**VFWDPAC(Far)-OCH_3	0.01
46	YIIK**C**VFWDPAC(Far)-OCH_3	25.0
47	YIIKGV**A**WDPAC(Far)-OCH_3	23.0
48	YIIKGV**Y**WDPAC(Far)-OCH_3	89.0
49	YIIKGV**N**WDPAC(Far)-OCH_3	0.001
50	YIIKGVF**C**DPAC(Far)-OCH_3	0.008
51	YIIKGVF**R**DPAC(Far)-OCH_3	0.008
52	YIIKGVFW**E**PAC(Far)-OCH_3	0.01
53	YIIKGVFWD**A**AC(Far)-OCH_3	1.0
54	YIIKGVFWD**Q**AC(Far)-OCH_3	0.1
55	YIIKGVFWDP**K**C(Far)-OCH_3	0.01
56	YIIKGVFWDP**S**C(Far)-OCH_3	92.0

Table 4

a-factor analogs with different spatial conformation.

Entry	a-factor conformational analog	Wild-type activity (%)
57	[*D*-Ala^{5}] **a**-factor	600.0
58	[*L*-Ala^{5}] **a**-factor	25.0
59	[*D*-Cys^{12}] **a**-factor	5.0
60	[*L*-Cys^{12}(CH_3)OFar] **a**-factor	25.0
61	[*D*-Cys^{12}(CH_3)OFar] **a**-factor	25.0
62	[(*R*)-g-lactam^{4,5}] **a**-factor	3,205.0
63	[(*S*)-g-lactam^{5,6}] **a**-factor	3.2
64	[*L*-Pro^{4}] **a**-factor	100.0
65	[*D*-Pro^{4}] **a**-factor	2.0
66	[*L*-Pro^{4}, *D*-Ala^{5}] **a**-factor	100.0
67	[*D*-Pro^{4}, *L*-Ala^{5}] **a**-factor	2.0
68	[*L*-Nle^{4}] **a**-factor	0.5
69	Z,E **a**-factor	100.0
70	E,Z **a**-factor	50.0
71	Z,Z **a**-factor	100.0