Novel Modifications to the Farnesyl Moiety of the **a**-Factor Lipopeptide Pheromone from *Saccharomyces cerevisiae*: A Role for Isoprene Modifications in Ligand Presentation†

Angus L. Dawe,‡,§ Jeffrey M. Becker,‡ Ying Jiang,|| Fred Naider,*,|| Jeffrey T. Eummer,⊥ Yong Qi Mu,⊥ and Richard A. Gibbs⊥

Department of Microbiology and Program in Cellular, Molecular and Developmental Biology, University of Tennessee, Knoxville, Tennessee 37996, Department of Chemistry, College of Staten Island, Staten Island, New York 10314, and Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, Wayne State University, Detroit, Michigan 48202

Received April 25, 1997; Revised Manuscript Received July 31, 1997⊗

ABSTRACT: The **a**-factor of *Saccharomyces cerevisiae* is a dodecapeptide pheromone [YIIKGVFWDPAC-(farnesyl)-OCH_3] in which posttranslational modification with a farnesyl isoprenoid and carboxymethyl group is required for full biological activity. Utilizing novel synthetic techniques and a well-characterized array of biological assays, we prepared original modifications to the farnesyl moiety of the pheromone in order to assess the importance of this part of the lipopeptide for biological activity. Specifically, the 3-methyl group was replaced to create analogs containing the ethyl, vinyl, *tert*-butyl, and phenyl moieties at the 3-position of the farnesyl chain. Subsequent biological analyses demonstrated that all of these modifications render an active pheromone, with the vinyl and ethyl analogs exhibiting higher activity than the native **a**-factor. However, the level of activity varied with the modification; the bulkier and more hydrophobic groups (*tert*-butyl and phenyl) exhibited lower biological activity than the smaller moieties (ethyl and vinyl). Furthermore, two analogs with phenyl substitutions that differ only in the presumed isomerization of the allylic double bond show up to an 8-fold difference in bioactivity. It has previously been surmised that the role of isoprenoid additions is solely to target the attached polypeptides to membranes by increasing their hydrophobicity. However, these studies demonstrate that even modest structural changes to the isoprenoid can significantly affect biological activity. These results are clearly inconsistent with a simple hydrophobic role for the isoprenoid and instead illustrate that it plays an active role in mediating optimal **a**-factor/receptor interaction.

Mating between the two haploid mating types of the budding yeast *Saccharomyces cerevisiae* is mediated by two diffusible peptide pheromones, termed **a**-factor and α-factor (for review see refs *1* and *2*). Although functionally equivalent in their roles as ligands for specific receptors that activate a signal transduction cascade and result in a number of specific cellular responses, these pheromones are structurally distinct. An unmodified 13 amino acid peptide, α-factor[1] is secreted by α-cells and is responsible for eliciting the mating response from the target **a**-cell via the Ste2p receptor (*3*, *4*). In contrast to α-factor, **a**-factor is a 12 amino acid lipopeptide (Figure 1), posttranslationally modified by the addition of a farnesyl group and a carboxymethyl ester to its C-terminal cysteine residue (*5*). The target of **a**-factor is the *STE3* gene product, a specific receptor located on the surface of the α-cell (*4*, *6*, *7*). In the case of both pheromones, binding to the receptor results in the transmission of an intracellular G-protein-mediated signal and subsequent physiological changes such as growth arrest at G1 of the cell cycle and induction of specific genes. These changes ultimately result in nuclear fusion and formation of diploid progeny.

Using a synthetic approach, Marcus et al. (*8*) demonstrated that both the farnesyl and carboxymethyl modifications are required for full biological activity of the *S. cerevisiae* **a**-factor. Nonmethylated or nonfarnesylated versions had activities of 1.5% and 0.1% that of **a**-factor, respectively, while removal of both moieties resulted in a pheromone that was only active at concentrations 40 000-fold greater than **a**-factor when tested by a growth arrest assay. Substitution of the farnesyl group with other additions (methyl, hexade-

† This work was supported in part by National Institutes of Health Grants GM22086 and GM22087 to J.M.B. and F.N. and CA67292 to R.A.G. R.A.G. is also the recipient of an American Cancer Society Junior Faculty Research Award (JFRA-609). J.T.E. was supported in part by a predoctoral assistanship from the Wayne State University MBRS program (GM08167), and Y.Q.M. was supported in part by a Rumble fellowship from Wayne State University.

* To whom correspondence should be addressed: Phone (718)-982-3896; fax (718)-982-3910; e-mail Naider@postbox.csi.cuny.edu.

‡ University of Tennessee.

§ Present address: Department of Pharmacology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854.

|| College of Staten Island.

⊥ Wayne State University.

⊗ Abstract published in *Advance ACS Abstracts*, September 15, 1997.

[1] Abbreviations: **a**-factor, YIIKGVFWDPAC(farnesyl)-OCH_3; α-factor, WHWLQLKPGQPMY; HPLC, high-performance liquid chromatography; FAB-MS, fast-atom bombardment mass spectrometry; HRE-IMS, high-resolution electron impact mass spectrometry; NMR, nuclear magnetic resonance spectrometry; HOBt, 1-hydroxybenzotriazole; DIEA, diisopropylethylamine; DIPC, diisopropylcarbodiimide; TFA, trifluoroacetic acid; DMS, dimethylsulfide; MeCN, acetonitrile; DMF, *N*,*N*-dimethylformamide; MeOH, methanol; DMSO, dimethyl sulfoxide; DIBAL-H, diisobutylaluminum hydride; BOP, benzotriazol-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate.

S0006-2960(97)00975-6 CCC: $14.00

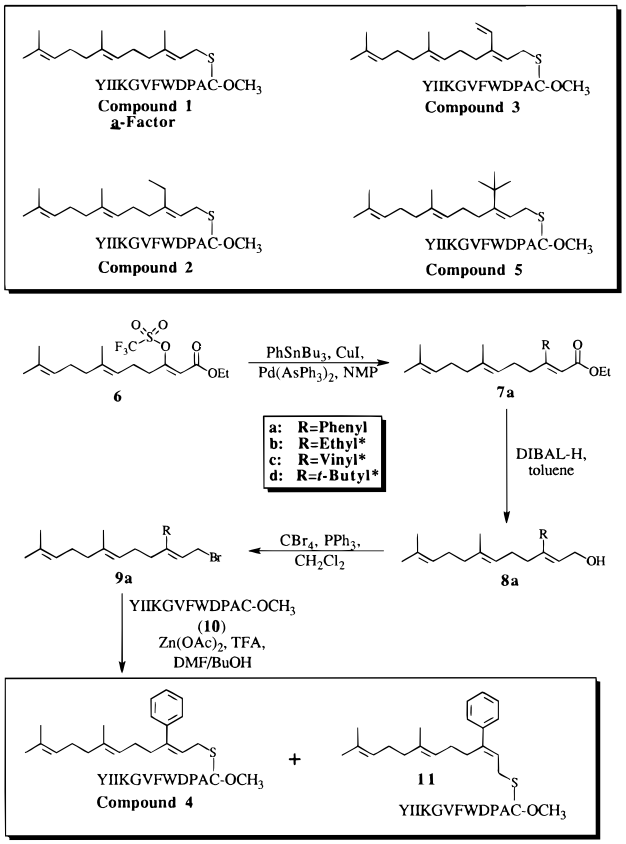

FIGURE 1: Structure of **a**-factor (**1**) and lipopeptide analogs (**2**−**5**) used in this study and synthetic scheme for compounds **4** and **11**. *Compounds **7b** and **7c** were prepared in the same manner as shown for **7a** using the appropriate organotin reagent (*28*), but preparation of **7d** required the use of a different alkylation procedure (*21*).

canyl, prenyl, geranyl and benzyl) failed to restore activity to wild-type levels under the same assay conditions but did abrogate the loss to varying degrees. In particular, the benzyl and geranyl variations restored activity up to 3% and 12%, respectively, and geranylgeranylated **a**-factor had an activity of 25% that seen with the farnesylated pheromone in growth arrest assays (*8*, *9*).

The exact function of the farnesyl group of **a**-factor has yet to be determined. However, isoprenyl modifications are not restricted to the mating pheromones of fungi. It has been established over the past several years that in all eucaryotic cells studied a significant number of proteins (including the monomeric and heterotrimeric G proteins) are isoprenylated at a carboxyl-terminal cysteine in the same manner as the **a**-factor (*10*). In particular, the Ras protein product of the *ras* oncogene is farnesylated, and this modification is essential for its membrane localization and role in cellular transformation (*11*). Mutant forms of the Ras protein are found in ~30% of human cancers, and this has led to intense interest in inhibitors of protein farnesylation as potential anticancer agents (*12*, *13*) and in the fundamental biochemistry of protein isoprenylation (*14*). The exact function of the farnesyl group of the Ras protein has also yet to be determined. Initial studies indicated a role for the farnesyl moiety in membrane attachment of the Ras protein (*15*, *16*). However, recent studies have demonstrated that even without membrane binding the farnesyl group of Ras is required for its proper interaction with other proteins in the signal transduction pathway (*17*−*19*). This provides evidence that prenyl−protein interactions (*20*) are also important for Ras protein activity. Although certain modified farnesyl groups have been attached to the Ras protein, such studies on Ras are limited by the need to use the enzyme protein−farnesyl transferase to modify the protein (*18*, *19*). For example, note that 3-*tert*-butylfarnesyl diphosphate is an exceptionally poor substrate for yeast protein−farnesyl transferase (*21*). In contrast, **a**-factor provides an ideal model system to study farnesyl function, as a variety of farnesyl-modified analogs can be prepared entirely via chemical synthesis. Furthermore, the resulting analogs can be fully characterized for purity and identity by HPLC, FAB-MS, and NMR, and a variety of well-characterized biological assays are available to quantitate their bioactivity (22).

Since the target of **a**-factor is a membrane-associated protein, the Ste3p receptor (*4*, *6*, *7*), it has been suggested that a function of the farnesyl group is to aid in localization of the pheromone to the membrane of the target cell (*9*, *23*−*25*). Previously, Epand et al. (*26*) investigated the ability of various additions to effect the association of **a**-factor with membranes, suggesting that the level of insertion of the pheromone into an artificial lipid bilayer depends on the type of modification applied. These findings were borne out by complementing studies of Shahinian and Silvius (*27*), who noted the stronger association of fluorescently labeled doubly-modified lipopeptides with vesicles as compared to singly-modified versions.

In this work, we attempt to examine more precisely the role of the farnesyl group in the interaction of **a**-factor with yeast cells by examining the biological activities of novel lipid substitutions containing various hydrocarbon substituents in place of the 3-methyl group of the farnesyl moiety. We found that replacement of the 3-methyl group of farnesyl with ethyl, vinyl, phenyl, or *tert*-butyl moieties alters the biological activity of the pheromone up to 8-fold and that isomerization of one double bond from *trans* to *cis* has a similar effect. These results denote the first time the effect of such a modest modification, changing one methyl group on the farnesyl side chain, on the biological activity of a lipopeptide or lipoprotein has been determined. The current study represents the beginning of a true structure/activity study of the farnesyl moiety, which has been enabled by the development of a novel and flexible synthetic route to a variety of farnesyl analogs (*21*, *28*).

These findings are clearly incompatible with a simple hydrophobic model for isoprenoid function. We speculate that such modifications to the farnesyl moiety of **a**-factor alter its specific mode of associaton with the membrane bilayer which, in turn, affects the subsequent interaction of the peptide portion with the membrane-localized receptor. This leads to altered signaling and, hence, altered bioactivity of the pheromone analog. Thus, the implication from this work is that isoprenyl moieties are not simply modes of membrane localization but also play an active role in presentation of an attached ligand to its target.

EXPERIMENTAL PROCEDURES

Materials. Protected amino acids, all of the L configuration, were purchased from Bachem Inc. (Torrance, CA) and Advanced ChemTech (Louisville, KY). 1-Hydroxybenzotriazole (HOBt) was purchased from Advanced ChemTech. Diisopropylethylamine (DIEA), diisopropylcarbodiimide (DIPC), trifluoroacetic acid (TFA), anisole, dimethylsulfide

(DMS), and all reagents used for the synthesis of the 3-substituted farnesyl bromides were purchased from Aldrich Chemical Co. (Milwaukee, WI). Solvents used for synthesis were purchased from VWR Scientific and Fisher Scientific. HPLC-grade acetonitrile (MeCN), *N*,*N*-dimethylformamide (DMF), dichloromethane (CH_2Cl_2), methanol (MeOH), and water were all purchased from VWR Scientific.

Purification and Characterization. Peptides were purified and analyzed by reversed-phase high-performance liquid chromatography (Hewlett-Packard HPLC Series 1050 or 1090) on Waters semipreparative μBondapak C_{18} column (19 × 300 or 3.9 × 300 mm) using water/acetonitrile/TFA and water/methanol/TFA elution systems with detection at 220 nm. Homogeneity was also assessed on silica gel thin-layer chromatography (silica gel 60 precoated aluminum sheet from E. Merck, Germany) developed in *n*-butanol/acetic acid/water (4:1:5 upper layer, v/v/v) and *n*-butanol/acetic acid/water/ethyl acetate (1:1:1:1, v/v/v/v) using UV light (254 nm) and ninhydrin visualization. Amino acid analysis, carried out at Brigham and Women's Hospital (Boston, MA), and FAB-MS, done at University of Tennessee, were employed for product characterization. Proton NMR spectra of the peptides were run in 100% DMSO-d_6 on a Varian Instruments Unity 600 MHz spectrometer. NMR spectra of the farnesyl intermediates were run in $CDCl_3$ on a GE QE 300 MHz spectrometer.

Synthesis of 3-Substituted Farnesyl Analogs (Figure 1)

3-Phenyl-7,11-dimethyl-2Z,6E,10-dodecatrien-1-o1 ***(8a)***. In a dry 50 mL flask, phenyl ester **7a** [128 mg, 0.390 mmol; prepared from triflate **6** as previously described (*28*)] was dissolved in toluene (2.0 mL of HPLC grade, dried over 4 Å sieves) under argon. The solution was cooled to −78 °C and 1.18 mL of diisobutylaluminum hydride (DIBAL-H; 1.0 M in toluene) was slowly added. After 2 h at −78 °C, the reaction mixture was quenched with saturated aqueous potassium sodium tartrate. The organic layer was separated and the aqueous layer was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with water (1 × 20 mL) and brine (1 × 20 mL) and dried over anhydrous magnesium sulfate. The solvent was then concentrated *in vacuo* and the crude product was purified by flash chromatography (9:1 hexane/ethyl acetate) to give pure **8a**. Yield 61 mg, 55%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.51 (s, 3 H), 1.60 (s, 3 H), 1.68 (s, 3 H), 1.98 (m, 6 H), 2.41 (t, 2 H), 4.03 (d, 2 H), 5.09 (br s, 2 H), 5.68 (t, 1 H), 7.13 (d, 1 H), and 7.30 (m, 4 H). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 16.03, 17.73, 25.73, 26.49, 26.69, 38.98, 39.69, 60.23, 123.51, 123.95, 124.30, 125.66, 127.05, 128.09, 128.18, 131.35, 139.92, and 144.45. HREIMS *m*/*z* calcd for $C_{20}H_{28}O$, 284.2140 (M^+); found, 284.2134.

3-Ethyl-7,11-dimethyl-2E,6E,10-dodecatrien-1-o1 ***(8b)***. The 3-ethyl ester **7b** [54.7 mg, 0.195 mmol; prepared from triflate **6** as previously described (*28*)], was dissolved in toluene (1.0 mL) and reacted with DIBAL-H (0.59 mL, 0.585 mmol), as described above for **8a**, to give **8b**. Yield 24 mg, 53%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.99 (t, 3 H), 1.60 (s, 6 H), 1.68 (s, 3 H), 2.00 (m, 10 H), 4.17 (d, 2 H), 5.10 (br d, 2 H), and 5.38 (t, 1 H). ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 13.70, 23.51, 25.72, 26.49, 26.70, 36.38, 39.68, 59.06, 122.78, 123.85, 124.27, 131.35, and 135.30. HREIMS *m*/*z* calcd for $C_{16}H_{28}O$, 236.2140 (M^+); found, 236.2141.

1-Bromo-3-phenyl-7,11-dimethyl-2Z,6E,10-dodecatriene ***(9a)***. In a 25 mL flask, 3-phenyl alcohol **8a** (22 mg, 0.078 mmol), carbon tetrabromide (34 mg, 0.101 mmol), and triphenylphosphine (22 mg, 0.082 mmol) were dissolved in CH_2Cl_2 (3.0 mL, distilled from CaH_2). After 2.5 h at room temperature, the solvent was removed. The residue was treated with hexane (∼5 mL). The hexane solution was then centrifuged, removed from the precipitate with a pipet, transferred to a second flask, and concentrated. This procedure was repeated 5−6 times. This bromide could either be used directly in the next step or first purified by reversed-phase HPLC (to remove residual alcohol **8a**) using a linear gradient of 30% A/70% B to 100% B over 30 min (A, water; B, MeCN; column, Waters Novapak C_{18} 10 mm × 100 mm Radial-Pak cartridge; flow rate, 2 mL/min; UV monitoring at 214 and 254 nm). The retention time of **9b** was 18 min, and 9.0 mg (33%) of the purified bromide was obtained. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.49 (s, 3 H), 1.51 (s, 3 H), 1.59 (s, 3 H), 1.9−2.1 (m, 6 H), 2.42 (t, 2 H), 3.88 (d, 2 H), 5.08 (narrow m, 2 H), 5.78 (t, 1 H), and 7.2−7.5 (m, 5 H).

1-Bromo-3-ethyl-7,11-dimethyl-2E,6E,10-dodecatriene ***(9b).*** 3-Ethyl alcohol **8b** (24.4 mg, 0.104 mmol) was treated with CBr_4 (44.8 mg, 0.135 mmol), Ph_3P (28.6 mg, 0.109 mmol), and 2 mL of CH_2Cl_2, as described above for 3-phenyl bromide **9a**, to give **9b** (22.5 mg; 72%), which was used directly in the next step. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.04 (t, 3 H), 1.60 (2 s, 6 H), 1.683 (s, 3 H), 1.95−2.2 (m, 10 H), 4.03 (d, 2 H), 5.09 (narrow m, 2 H), and 5.49 (t, 1 H).

1-Bromo-3-vinyl-7,11-dimethyl-2Z,6E,10-dodecatriene ***(9c)***. 3-Vinyl alcohol **8c** [25 mg, 0.106 mmol; prepared as previously described (*28*)] was treated with CBr_4 (46 mg, 0.138 mmol), Ph_3P (29 mg, 0.11 mmol) and 0.70 mL of CH_2Cl_2, as described above for 3-phenyl bromide **9a**, to give **9c** (16 mg; 51%), which was used directly in the next step. ^{1}H NMR (300 MHz, $CDCl_3$), δ 1.59 (s, 3 H), 1.61 (s, 3 H), 1.68 (s, 3 H), 1.95−2.3 (m, 8 H), 4.14 (d, 2 H), 5.1 (narrow m, 2 H), 5.3 (d, 1 H), 5.45 (d, 1 H), 5.67 (t, 1 H), and 6.7 (dd, 1 H).

1-Bromo-3-tert-butyl-7,11-dimethyl-2Z,6E,10-dodecatriene ***(9d)***. 3-(*tert*-Butyl) farnesol **8d** [21 mg, 0.080 mmol; prepared from triflate **6** via ethyl 3-(*tert*-butyl)farnesoate **7d** as previously described (*21*)] was treated with CBr_4 (34 mg, 0.10 mmol), Ph_3P (22 mg, 0.080 mmol), and 0.50 mL of CH_2Cl_2, as described above for 3-phenyl bromide **9a**, to give **9d** (23 mg, 88%), which was used directly in the next step. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.21 (s, 9 H), 1.61 (s, 6 H), 1.68 (s, 3 H), 1.95−2.2 (m, 8 H), 4.23 (d, 2 H), 5.10 (narrow m, 2 H), and 5.42 (t, 1 H).

Synthesis of ***a****-Factor Analogs (Figure 1)*

The strategy employed to synthesize analogs of the **a**-factor with various substituents in place of the 3-methyl moiety involved the preparation of unfarnesylated **a**-factor and the thioalkylation of the SH group of this dodecapeptide using the appropriate alkyl bromide under acidic conditions. Unfarnesylated **a**-factor was synthesized by coupling Fmoc-Tyr-Ile-Ile-Lys(Fmoc)-Gly-Val-Phe-Trp-Asp(OFmoc)-Pro with (Ala-Cys-OMe)$_2$ using the BOP reagent as the coupling agent as previously described (*29*). The protecting groups were removed using piperidine and the resulting 24-mer was

judged to be >90% pure using HPLC. This compound was used without further purification to prepare unfarnesylated dodecapeptide methyl ester by reduction of the disulfide bond with Zn in 90% acetic acid/water. This approach always generated the sulfhydryl form of the peptide immediately prior to the thioalkylation reaction. Thioalkylation was accomplished under acidic conditions with zinc acetate as the catalyst following the procedure of Xue et al. (*30*) with the following modification. Given the fact that the alkyl bromides were available in very limited amounts and that they were not stable under the thioalkylation conditions, excess peptide was used and the reaction was monitored using analytical HPLC on a C_{18} reversed-phase column until all of the alkyl bromide had reacted. The thioalkylated peptide was then recovered from the reaction mixture by semipreparative HPLC. The amount of the final product varied from 1 to 7 mg. A typical procedure used for the synthesis of the 3-ethyl-substituted **a**-factor analog (compound **2**) follows.

*Synthesis of (3-Ethylfarnesyl)-**a**-factor (**2**).* Freshly generated Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-OMe (**10**, 12 μmol) was reacted with 3-ethylfarnesyl bromide (**9b**, 6 μmol) in a DMF/butanol/water (0.25% TFA) 2:1:1 solvent mixture in the presence of zinc acetate dihydrate (40 μmol) at 0 °C. The reaction mixture was stirred and monitored by analytical HPLC on a reversed-phase (C_{18}) column. When all of **9b** had disappeared, the mixture was diluted with water (1% TFA) abd extracted with ethyl ether to remove organic side products, and the product was recovered by semipreparative HPLC on a C_{18} reversed-phase column. A total of 7 mg (62% yield based on alkyl bromide) of product (greater than 98% homogeneity) was recovered after lyophilization of the HPLC fractions.

All products subjected to biological assay were greater than 98% homogeneous on analytical HPLC. All final peptides gave the expected amino acid ratios for Ala, Asp, Gly, Ile, Lys, Phe, Pro, Tyr, and Val. The presence of Trp was confirmed by absorbance measurements at 289 nm. The peptides gave the following monoisotopic mass values ($M + H)^+$ rounded to the nearest 0.1 Da: compound **2**, 1644.0 (calcd 1644.0); compound **3**, 1642.0 (calcd 1641.9); compound **4**, 1692.1 (calcd 1692.0); compound **5**, 1671.7 (calcd 1672.0); compound **11**, 1691.7 (calcd 1692.0).

Biological Assays

Yeast Strains and Genotypes. Saccharomyces cerevisiae strain RC757 [*MAT*α *sst2-1 rme1 his6 met1 can1 cyh2*] was a gift from Russell Chan (University of Cincinnati). *S. cerevisiae* SM1229 [*MAT**a*** Δ*mf**a**1*::*LEU2* Δ*mf**a**2*::*URA3 trp1 his4 can1*] was a gift from Susan Michaelis (Johns Hopkins University), and *S. cerevisiae* LM23-116AZ [*MAT*α *FUS1*::*lacZ(URA3) leu2 lys5 met1 ura3-52 ste2*::*TRP1*] was a gift from Lorraine Marsh (Albert Einstein University). *S. cerevisiae* X2180-1B [*MAT*α *SUC2 mal mel gal2 CUP1*] was obtained from the Yeast Genetic Stock Center.

FUS1−*lacZ Induction Assay.* This assay was performed as previously described (*8*) using strain LM23-116AZ, which carries a fusion of the pheromone-inducible *FUS1* promoter and the *lacZ* reporter gene. This facilitates a defined measurement of the level of pheromone-specific induction by assaying for β-galactosidase activity. Late log-phase cells were harvested and exposed to various concentrations of **a**-factor or the analogs for 2 h at 30 °C prior to testing for β-galactosidase. Results of three experiments at each concentration were plotted as units of activity (*8*) against concentration of **a**-factor or analog.

Growth Arrest Assay. Biological activity of these pheromone analogs was measured by examining their ability to induce a halo of growth arrest when spotted onto a lawn of RC757 cells, a strain supersensitive to **a**-factor. The end point of activity is defined as the minimum quantity of peptide resulting in the formation of an observed halo, as described previously (*8*). No variations in the activities of analogs from those values reported were seen across three experiments. All peptide stocks were verified to be approximately equal in concentration by absorbance at 279 nm ($\epsilon = 5600$ cm^2 mol^{-1}).

Mating Restoration and Induction Assays. Mating restoration was assessed as the ability of exogenously added synthetic peptide to induce the formation of constellations of diploid colonies from a mixture of two haploid strains, RC757 and SM1229. In the latter, the structural genes for **a**-factor, *MF**a**1* and *MF**a**2*, have been deleted so diploid formation is dependent on the addition of exogenous pheromone. The end point is defined as the minimum quantity of pheromone that is required to form a cluster of diploids on medium lacking supplements required for growth of either haploid strain, as previously described (*31*). No variations in the activities of analogs from those values reported were seen across three experiments. Mating induction in an aqueous environment was analyzed by modifying the method of Dorer et al. (*32*). The above strains were incubated for 5 h at 30 °C in a range of concentrations from 2 to 50 nM of each of the analogs. After recovery of cells by centrifugation, the number of diploid colonies formed in each case was determined by plating on diploid-selective medium [0.67 g/L yeast nitrogen base without amino acids (Difco), 2% glucose, and 1.5% agar]. The standard error of variation in the activities of analogs as seen across three experiments is represented as error bars. All peptide stocks were verified to be approximately equal in concentration by absorbance at 279 nm ($\epsilon = 5600$ cm^2 mol^{-1}).

*Degradation of **a**-Factor and Analogs.* Lipopeptides to be analyzed for degradation were labeled with ^{125}I using the Iodogen iodinating reagent (Pierce Chemical Co.) as previously described (*31*, *33*). Cells of strain X2180-1B, an α-mating type haploid strain, were grown to late log phase in YEPD [yeast extract, 1% (w/v), peptone, 2% (w/v); and dextrose, 2% (w/v)] and harvested. Following resuspension at 1×10^8 cells/mL, 30 000−60 000 cpm of labeled peptide was added to 1 mL of cells. At regular time points, 100 μL aliquots of the reaction were removed and the cells were separated by centrifugation. The amount of degraded peptide in this supernatant was then assessed by mixing it with 100 mg/mL aluminum silicate suspension. Only intact peptide was retained by the silicate, which was removed by brief centrifugation prior to counting of both the resulting pellet and supernatant in a γ counter (LKB-Wallac Clinigamma 1272). The amount of degradation was determined by the number of counts in the supernatant as compared to the number retained by the silicate.

RESULTS

*Synthesis of **a**-Factor Analogs.* The synthetic route to the farnesyl-modified **a**-factors **2**−**5** utilized a convergent ap-

proach. The 3-phenyl, 3-ethyl, and 3-vinyl modified farnesyl groups were prepared starting from the triflate **6** and the appropriate organotin reagent, using our previously described palladium/CuI-catalyzed coupling route (*28*). A representative example, the synthesis of the 3-phenylfarnesyl **a**-factor **4**, is shown in Figure 1. The syntheses of compounds **6, 7a,** and **7b** have been previously reported (*28*). Reduction of the esters with DIBAL-H led to the production of alcohols **8a**−**c**. The conversion of the alcohols to the required bromides was best achieved using carbon tetrabromide and triphenylphosphine (*34*). Other bromination protocols were tried (*n*-bromosuccinimide/dimethylsulfide, phosphorous tribromide), but they were less efficient and gave impure bromide products. Frequently, the products from the CBr_4/PPh_3 procedure were sufficiently pure for direct use in the peptide farnesylation reaction.

The *tert*-butylfarnesyl group required for the preparation of analog **5** cannot be synthesized via the palladium/CuI-catalyzed coupling route. Instead, the triflate **6** was coupled with a lower order *tert*-butyl cyanocuprate reagent (tBuCuCNLi) to give ethyl 3-*tert*-butylfarnesoate **7d**. This ester was then reduced to the corresponding alcohol **8d** and converted to the bromide **9d** using the same procedures described above. The detailed procedures for the synthesis of compounds **7d** and **8d** have been previously described (*21*).

The carboxylmethylated, nonfarnesylated dodecapeptide **10** was prepared by a combination of solution and solid-phase peptide synthesis and then regiospecifically coupled with the appropriate farnesyl bromide using zinc/trifluoroacetic acid catalysis as described previously (*30*). As the allylic chloride analog of bromide **9c** had already been prepared (*28*), its coupling with peptide **10** was initially investigated. However, none of the desired product **3** was seen except under forcing conditions (large excess of the farnesyl chloride, extended reaction times). In a control reaction we compared the synthesis of **a**-factor using either farnesyl chloride or farnesyl bromide as the alkylating agent. With a 4-fold excess of these reagents and zinc acetate as a catalyst in DMF/butanol/0.25% aqueous TFA (2:1:1), thioalkylation was complete in 30 min with farnesyl bromide but only 10−20% complete with farnesyl chloride after 5 days. Therefore, the farnesyl bromides were prepared and utilized for the coupling reactions.

The coupling of the bromides with peptide **10** proceeded smoothly, although we detected some instability of these compounds under the thioalkylation conditions. In the case of the phenyl bromide **9a**, two farnesylated peptides were obtained. These compounds were separated by reversed-phase HPLC and both exhibited appropriate molecular weights by FAB-MS. These isomers were assigned structures **4** and **11**, on the assumption that the allylic double bond of compound **9a** isomerized during the acid-catalyzed coupling reaction. This may be due to the additional stabilization of the allylic carbocation derived from **9a** that is provided by the phenyl moiety. In order to provide further information on the structures of **4** and **11**, their 600 MHz proton NMR spectra were obtained. As expected, the spectra were similar to each other and the majority of the peaks were identical to those of the **a**-factor itself (*35*). However, significant differences were seen in the vinylic region (5.0−5.5 ppm) where the signals for the farnesyl H2, H6, and H10 proteins appear. The differences observed between the spectra of **4** and **11** were essentially consistent with their assigned structures.[2]

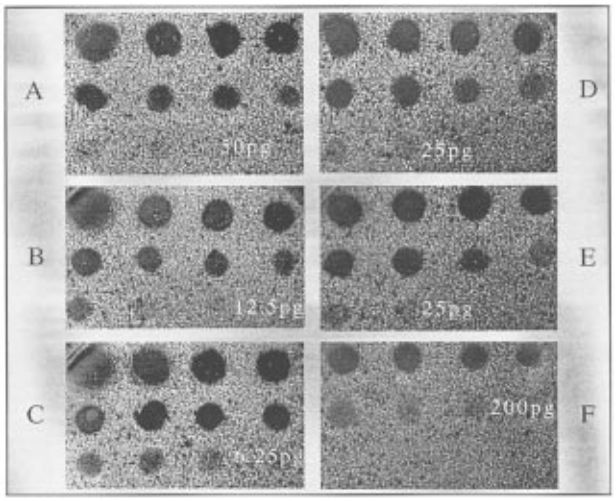

FIGURE 2: Growth arrest (halo) assay. Each analog was diluted in the same manner and spotted onto the surface of the lawn of RC757 cells. The first spot, as denoted by the halo of growth arrest in the upper left corner of each picture, corresponds to 6.25 ng, and subsequent spots are 1:2 serial dilutions from left to right and top to bottom. Activity of each analog was verified by performing at least three experiments. (A) Compound **1**; (B) compound **2**; (C) compound **3**; (D) compound **4**; (E) compound **5**; (F) compound **11**. The end points determined for each compound are indicated in each panel.

Biological Activity of ***a****-Factor Analogs*. In order to assess the effects of altered substitutions of the 3-methyl group of **a**-factor, a number of biological assays were employed. These utilized different characteristics of the pheromone response pathway, including growth arrest of α-cells in response to **a**-factor, induction of specific genes in response to **a**-factor, and mating between two strains that will not mate in the absence of exogenous pheromone. It has been previously established (*8*, *31*, *36*, *37*) that these assays are able to reflect the biological activity of a given analog.

In the growth arrest (halo) assay (Figure 2), lawns of cells of the supersensitive strain RC757 were subjected to a dilution series containing identical amounts of each analog. End points were defined as the least amount of pheromone that was capable of inducing growth arrest resulting in a

[2] For the native **a**-factor (*35*) and the closely related 3-ethyl **a**-factor **2**, the signals for the H2, H6, and H10 protons appear as overlapping triplets in the 5.0−5.15 ppm region. With compound **4**, the H2 proton signal shifts downfield to 5.45 ppm. The downfield shift in the H2 proton is consistent with the downfield shift seen with the H2 signals in **7a** and **8a**, when compared to the H2 protons in the 3-ethyl ester **7b** and alcohol **8b**. Previously, we have observed that the H6 and H10 proton signals in **7a** and **7c** overlap, while the signals for the H6 and H10 protons in the *2E* isomers of both these compounds appear as separate triplets at 5.0 and 5.1 ppm (*28*). In the NMR spectrum of **11**, the H2 signal again appears as a triplet at 5.45 ppm as with **4**, but the H6 and H10 signals appear as separate signals at 5.05 and 5.15 ppm, consistent with the pattern seen in the *2E* isomer model system. However, a more complex signal is seen at 5.05 ppm rather than the simple triplet expected. Thus the NMR spectrum of **4** is consistent with its assigned structure, while the NMR of **11** is mainly consistent with the assigned structure but more complex than expected. However, taking the NMR, HPLC, FAB-MS, and biological data together with the method of synthesis, we are reasonably confident of the structure assigned for **11**. In particular, the NMR confirms that a more significant isomerization of the farnesyl structure, such as the attachment of the cysteine sulfur to the C3 position via an S_N2' or S_N1' process, did not occur during the formation of **11**.

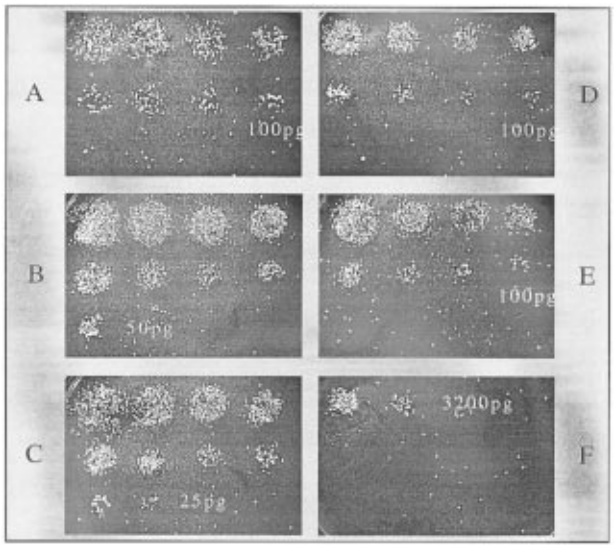

FIGURE 3: Mating restoration assay. Each analog was diluted in the same manner and spotted onto the mixed lawn of RC757 and SM1229 cells. The first spot, as denoted by the cluster of diploid colonies in the upper left corner of each picture, corresponds to 12.5 ng, and subsequent spots are 1:2 serial dilutions from left to right and top to bottom. The end point for each analog was verified by performing at least three experiments. (A) compound **1**; (B) compound **2**; (C) compound **3**; (D) compound **4**; (E) compound **5**; (F) compound **11**. The end points determined for each compound are indicated in each panel.

region of significantly reduced lawn density. Farnesylated synthetic **a**-factor (compound **1**) was used for reference as described in the Experimental Procedures and shows an end point of 50 pg. Each of the modified farnesyl additions, except compound **11** (discussed below), shows an end point that indicates a biological potency at least 2-fold greater than that of **a**-factor. End points for each pheromone analog were determined from at least three experiments and are as follows: **2**, 25 pg; **3**, 6.25 pg; **4**, 25 pg; **5**, 25 pg; **11**, 200 pg.

The end points in the growth arrest assay suggested that substituting the 3-methyl of the farnesyl group with other moieties altered the biological activity of the various **a**-factor analogs. To further assess this effect, mating restoration assays were performed. Mixed lawns of strains SM1229 and RC757 were plated onto medium on which diploid colonies are only able to form in the presence of exogenous **a**-factor or biologically active analog spotted using the regimen described in Experimental Procedures. In this case, the end points were defined as the least amount of pheromone that was capable of producing a distinct cluster of diploid colonies at the precise location where the compound was applied. Photographs of the mating restoration plates (Figure 3) demonstrate that the same overall comparative activity among compounds observed with the halo assay was reproduced, with end points as follows: **1** (**a**-factor), 100 pg; **2**, 50 pg; **3**, 25 pg; **4**, 100 pg; **5**, 100 pg; **11**, 3200 pg. It is interesting to note, however, that in all cases the mating assays showed a reduced enhancement of activity when compared to the growth arrest assay. We attribute this to the fact that the latter requires only the successful interaction of ligand and receptor followed by specific induction of genes responsible of cell cycle arrest, whereas the mating response requires many more steps including morphological changes, cell−cell contact, and successful cell and nuclear fusion, all while an auxotrophic selection pressure is maintained.

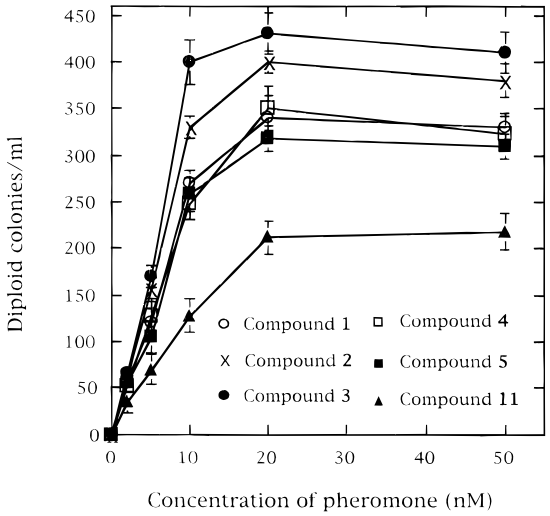

FIGURE 4: Mating induction assay. Each analog was tested at various concentrations for its ability to induce successful mating between strains SM1229 and RC757. The number of diploid colonies recovered from each experiment was determined after 2 days at 30 °C. Each point represents the average of three experiments.

The above assay data indicates different levels of activity imparted by the different substitutions of the 3-methyl substituent of the **a**-factor farnesyl group that are of a similar magnitude to those reported by Marcus et al. (*8*) when the farnesyl group was replaced by hexadecanyl, prenyl, geranyl, and benzyl moieties. It is important to remember that both the halo assay and mating restoration assay rely on subjective observation of a 2-fold dilution series on a solid surface in which the exact concentrations of analogs are not known. In order to test the trends observed above in a more precise system, the response of target cells to pheromone was tested in an aqueous environment at defined concentrations. The mating induction assay relies on the inability of RC757 and SM1229 to mate without the addition of exogenous pheromone but differs from the restoration assay in that the actual number of diploid colonies formed in the presence of a given amount of **a**-factor, or analog, is determined. These assays were performed at a range of concentrations, which generated the response curves shown in Figure 4. Once again, it is clear that the ethyl and vinyl modifications (compounds **2** and **3**) show a larger increase in activity over normal **a**-factor than the other modified analogs, although there is little difference between analogs **4** and **5** and **a**-factor. No diploid colonies resulted from mating mixtures in which neither **a**-factor nor an analog was present.

In addition, strain LM23-116AZ was used to assess pheromone-dependent induction of the β-galactosidase reporter gene. A graph of β-galactosidase induction versus concentration of the various analogs tested (Figure 5) shows that compounds **2** and **3** are able to induce the pheromone response pathway to higher levels than **a**-factor, while the other modifications have an effect only similar to or a little less than **a**-factor. These results reflect the same overall trend previously observed in the other assays.

As described above, only one abnormal synthesis product was obtained: the presence of two farnesylated peptides from the coupling of peptide **10** with bromide **9a** (Figure 1). This resulted in the isolation and the characterization of the biological activities of two analogs, **4** and **11**, that are identical except for the presumed isomerization of the allylic

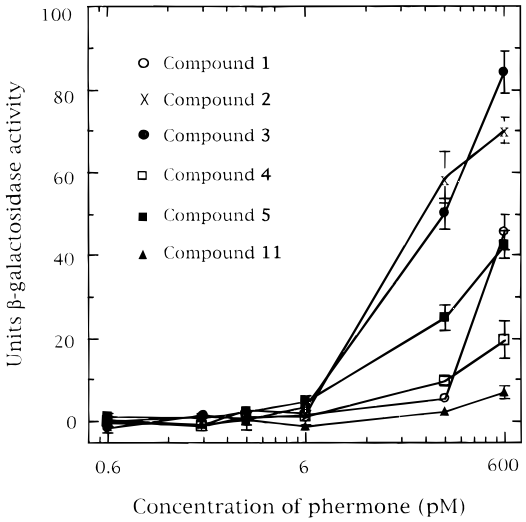

FIGURE 5: β-Galactosidase induction assay. Each analog was tested for its ability to induce transcription of β-galactosidase from the pheromone-responsive promoter element *FUS1* at a range of concentrations from 10 to 1000 pg/mL. Each point represents the average of at least three experiments. Error bars denoting the standard error for activity at each concentration of the various analogs are included.

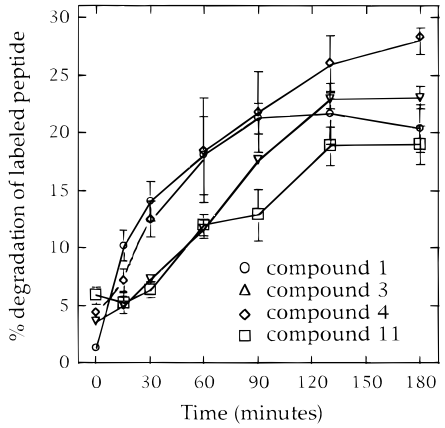

FIGURE 6: Degradation of compounds **1**, **3**, **4**, and **11** by α-cells. Amount of degradation was detemined by analyzing the amount of undegraded versus degraded ^{125}I-labeled peptide in the presence of α-cells over time, performed in triplicate. Error bars represent the standard error of each data point.

double bond. Such a structural difference appears to have a great effect on the biological activity of these pheromones, as can be seen in each of the assays described above. The activity of compound **11** is much less than that of **a**-factor in all of the assays (Figures 2−5), with up to a 32-fold reduction, as noted in the mating restoration assay. In contrast, compound **4** shows an activity at least as strong as that of **a**-factor in the equivalent experiment and up to 2-fold higher in the halo assay (Figure 2). Given that the only difference is the orientation of the peptide portion of the pheromone imparted by the differential isomerization, it is evident that this spatial change is very important for bioactivity.

An α-cell-specific **a**-factor-degrading peptidase activity was described by Marcus et al. (*33*). Conceivably, a peptide analog of **a**-factor with different levels of activity from the native pheromone could simply be more or less resistant to this peptidase. In order to determine whether this was the case, compounds **3**, **4**, and **11** were radioactively labeled with ^{125}I and subjected to a degradation assay. The results of this are shown in Figure 6 and demonstrate that all of the analogs labeled, and **a**-factor, are degraded by α-cells. No degradation was seen with **a**-cells (data not shown). Although there are slight differences in the rates of degradation, particularly during the first 60 min, they do not correspond to the observed differences in biological activity. Both of the phenyl variations appear to be degraded at a slower rate initially, yet this would only explain a small increase in activity and not the decrease observed with **11**. Also, compound **3**, the most active of all the analogs, is degraded at a rate comparable to **a**-factor-eliminating the possibility of a reduced rate being the reason for this peptide's enhanced bioactivity.

DISCUSSION

The objective of these studies was to define further the role of the farnesyl moiety of the *Saccharomyces cerevisiae* **a**-factor. To this end, we have synthesized novel variations of the farnesyl moiety modified at the 3-position and examined the biological activities of the corresponding **a**-factor analogs in a number of well-characterized assay systems. The purpose of isoprenoid modifications is a basic question in the field of protein prenylation. Whether these additions serve to locate an attached peptide to membranes via lipid−lipid inteactions or bind to proteins through lipid−protein interactions has been a contentious issue (*14*, *20*, *38*, *39*), and evidence to support several models can be found in the literature. Investigation of the mammalian Ras protein in soluble assay systems (*18*, *19*, *40*) and the *S. cerevisiae* Ras protein *in vivo* (*17*) have demonstrated that the farnesyl group is required for biological activity in the absence of membrane binding, implying the involvment of prenyl−protein binding sites. In addition, the fact that prenyl cysteine analogs can block certain signal transduction pathways has been taken as evidence for prenyl binding sites on intracellular proteins (*41*−*43*). Moreover, the recently determined structure of the rhoGDI protein clearly indicates an isoprene binding site on this protein that interacts with the prenyl group of the Rho protein (*44*, *45*). However, studies in biological systems (*24*, *25*) and model membrane/prenylated peptide systems (*27*, *46*) suggest a purely hydrophobic, lipid−lipid based interaction between membranes and farnesylated peptides. Furthermore, Parish and Rando have summarized the evidence concerning the γ subunit of heterotrimeric G proteins and concluded that the isoprenoid moiety in this case also facilitates membrane attachment strictly through lipid−lipid interactions (*39*, *47*).

Previous studies from our laboratories, however, have indicated that hydrophobic factors alone do not predict the behavior of lipid-modified **a**-factor analogs. Replacement of the farnesyl group with the more hydrophobic hexadecanyl moiety leads to a drastic reduction in biological activity (*8*), and the more hydrophobic *S*-geranylgeranyl **a**-factor is also significantly less active than the natural compound (*9*). In the present study, analysis of the activities of the 3-substituted analogs demonstrated that all were capable of initiating a biological response, but to varying degrees depending on the nature of the modification. In Figure 7, the relative activity of each analog was determined by normalizing the values obtained for each compound in the various assays to that of **a**-factor. Clearly, the more hydrophobic 3-phenyl and 3-*tert*-butyl derivatives are less active than the 3-vinyl and 3-ethyl

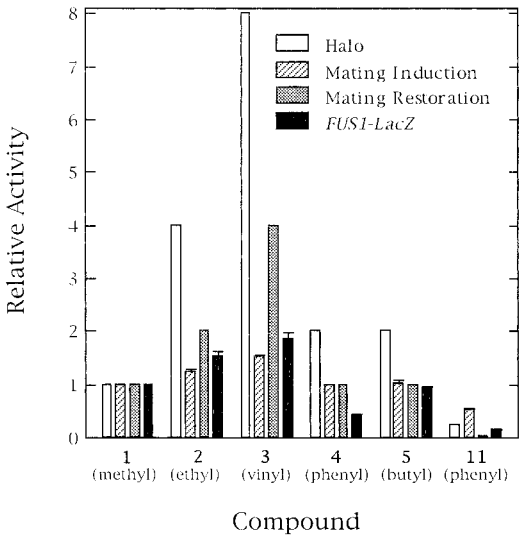

FIGURE 7: Relative consequence for bioactivity of novel 3-methyl substitutions to the farnesyl moiety of **a**-factor. End-point values for the activity of the analogs in each assay were compared to **a**-factor, except for the *FUS1−LacZ* data, in which the relative levels of induction at 1000 pg/ml were compared. Graphical evaluation represents the mean relative activity in each experiment normalized to that of **a**-factor. Error bars denoting the standard error are included for the liquid-based assays but not for the halo or mating restoration assays, which generate data in 2-fold increments. Compound designations are those indicated in Figure 1.

compounds in all assays. Large differences in biological activity between the two 3-phenyl isomers and the significantly higher biological activity of the 3-vinyl isomer than the most active of the 3-phenyl isomers (compound **4**) are also in direct conflict with the simple hydrophobic model.

The dramatic variation in activities associated with compounds **4** and **11** (Figure 7), the different isomers of the 3-phenyl-modified farnesyl group, is a point of particular interest. Previous biophysical studies by Epand and coworkers on lipid-modified **a**-factor analogs demonstrated that the farnesyl group results in the attached peptide being anchored more deeply in the membrane than a simple *n*-alkyl chain (*26*), and this correlated with the lower biological activity of the *n*-alkyl **a**-factor analog. The different spatial relationship of the peptide relative to the farnesyl moiety that is imparted by the isomerization of the 2*Z* double bond (**4**) to the 2E conformation (**11**) could influence the interaction of the lipopeptide with the Ste3p receptor. In general, it would appear that the significant yet relatively small differences in biological activity of **a**-factor analogs observed in the present and previous studies (*37*) are not consistent with a traditional tight ligand−receptor interaction. Moreover, in the only other case where modified farnesyl groups were attached to a peptide or protein, the changes in biological activity observed were also modest. Gelb and coworkers attached a series of farnesyl analogs (different from those described in the current study) to the Ras protein and then examined their ability to activate downstream kinases in a soluble system (*18*). As membranes were completely absent, the modest differences in biological activity observed (ca. 2-fold) were apparently due to relatively nonspecific prenyl−protein binding.

In conclusion, these studies indicate a role for the farnesyl group of the **a**-factor pheromone of *S. cerevisiae* that is clearly inconsistent with a simple hydrophobic model. Our data suggest either a specific lipid−lipid interaction between the farnesyl group and the yeast cell membrane or relatively nonspecific direct binding of the farnesyl group to a site on the Ste3p protein. Although we are not able to distinguish whether the farnesyl group acts via lipid−lipid interactions or prenyl−protein interactions, it is clear that subtle changes in the structure of the farnesyl group can result in a *significant decrease or increase* in the biological activity of the pheromone. Moreover, to the extent that these results can be extended to other lipopeptides and lipoproteins, they suggest that prenylation with 3-substituted farnesyl analogs may lead to alteration of their biological activity. Indeed, preliminary evidence indicates that prenylation of certain mammalian proteins with the 3-vinylfarnesyl moiety has profound biological consequences.[3]

[3] B. S. Gibbs, T. J. Zahn, Y. Q. Mu, J. S. Sebolt-Leopold, and R. A. Gibbs, manuscript in preparation.

ACKNOWLEDGMENT

We thank John Wilgus for assistance with the mating assays, Dr. H. R. Marepalli for running the 600 MHz NMR spectra, and Frances Cox and Paula Keaton for assistance with the manuscript. We thank Dr. Julia Dolence and Professor C. Dale Poulter (University of Utah) for helpful advice on the bromination of **8a**−**d**. The NMR facility at the College of Staten Island is operated by the CUNY Center for Applied Biomedicine and Technology and was supported by funds from NSF (BIR-9214560), the City University of New York, and the College of Staten Island.

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BI9709755