Synthesis and Biological Activity of Fluorescent Yeast Pheromones†

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ABSTRACT: The mating pheromones of *Saccharomyces cerevisiae* and derivatives of these pheromones have been synthesized and tested for biological activity in a solution-phase assay. The effects of native α-factor and **a**-factor on the growth of target cells in these assays were identical. A derivative of α-factor in which the amino terminus was modified with the fluorescent probe, 6-amino-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)hexanoyl, was only slightly less active than the unmodified pheromone. Derivatives of **a**-factor that contain various alkyl groups in place of the farnesyl moiety of the native pheromone were also synthesized and tested for biological activity. A derivative in which the farnesyl moiety is substituted with an unbranched decyl group exhibited activity identical to that of the natural pheromone, whereas a derivative that contains an unbranched pentyl group exhibited significantly lower biological activity than native **a**-factor. The derivatives of **a**-factor have in addition been modified to incorporate 6-amino-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) at the terminus of the alkyl chains. A derivative with the probe attached to a decyl chain displayed activity similar to that of the native pheromone, whereas the same modification on a pentyl chain produced a derivative with very low activity. The fluorescence spectra of the modified α-factor and **a**-factors were measured in methanol, aqueous solution, and aqueous solution containing phospholipid vesicles. The fluorescence of the probes depends on the environment of the pheromones and can be used to monitor the association of the pheromones with the lipid bilayer.

The transduction of signals across the plasma membrane of eukaryotic cells is initiated by the specific binding of diffusible ligands to receptor proteins on the surface of a cell. Binding of a ligand to a receptor causes changes in the conformation of the receptor protein that ultimately result in the transmission of the signal into the cell. The signal itself can take the form of ion currents across the plasma membrane, phosphorylation of specific target proteins in the cytosol, release of second messenger molecules into the cytosol, or a combination of these.

In order to understand the signaling process, one must first understand how ligands are recognized by and are associated with their receptors. Although the binding of a ligand to a receptor is generally assumed to involve a simple two-state equilibrium between the free and bound forms of the ligand, it has also been suggested that nonspecific partitioning of some ligands into the lipid bilayer of the plasma membrane may precede the specific binding of the ligands to receptors (Sargent & Schwyzer, 1986). It can be argued that a division of the binding process into a separate partitioning of the ligand into the membrane bilayer and a subsequent recognition of the ligand by the receptor within the two-dimensional confines of the membrane would greatly accelerate the kinetics of receptor activation and could preferentially stabilize a conformation of the ligand that is recognized by the receptor.

In order to study the interactions of ligands with membranes and with receptors, it is useful to incorporate spectroscopic probes into the ligands. The binding steps can then be monitored by following changes in the signal of the probe. Extrinsic fluorescent probes are particularly well suited for this purpose because they can be detected at extremely low concentrations, and because their excitation and emission wavelengths can often be chosen to minimize interference from background signals in the biological samples. Care must be taken, however, to ensure that the modification does not significantly alter the biological activity of the fluorescent ligand.

The work presented here describes the synthesis and characterization of fluorescent derivatives of α-factor, **a**-factor, and a series of nonfluorescent **a**-factor derivatives. α-Factor and **a**-factor are peptide pheromones from the yeast, *Saccharomyces cerevisiae* (Naider & Becker, 1986). The pheromones are secreted by haploid yeast cells—α-factor by α-type cells and **a**-factor by **a**-type cells. They bind to G protein-coupled receptors on haploid cells of the opposite mating type and initiate the cellular responses involved in conjugation (Herskowitz & Marsh, 1987; Blumer & THorner, 1991). The binding of radiolabeled α-factor to the α-factor receptor has been reported previously (Jenness et al., 1983; Blumer et al., 1988; Raths et al., 1988), but the importance of nonspecific membrane interactions has not yet been examined directly. Such interactions have been suggested, however, from the results of studies using nuclear magnetic resonance to examine the conformation of α-factor (Wakamatsu et al., 1986, 1987; Jelicks et al., 1989). The fluorescent pheromones described here display normal biological activity and will provide quantitative information about early

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steps in the binding of the pheromones to the receptors. They may also prove useful in monitoring the subcellular location of the receptors by fluorescence techniques.

MATERIALS AND METHODS

Materials

The reagents used in the organic syntheses were purchased from Aldrich Chemical (Milwaukee, WI). The silica gel (Davisil, Grade 633, type 60A) for flash chromatography was obtained from Fisher Scientific (Pittsburgh, PA). The protected amino acids and the reagents used for the synthesis of the peptides were purchased from either Bachem California (Torrance, CA) or Novabiochem (La Jolla, CA). All of the solvents were HPLC grade and were used without further purification.

Methods

Chemical Syntheses. (A) 10-Phthalamidyl-1-decanol. 10-Bromo-1-decanol (20 mmol) was dissolved in DMF[1] (20 mL). Potassium phthalamide (24 mmol) was added to this solution, and the suspension was left to reflux. After 24 h, the solvent was removed by rotary evaporation. The residue was dissolved in 2% methanol/chloroform, and the phthalamidyldecanol was isolated by flash chromatography through silica gel. The isolated yield of product was typically greater than 90%. ^{1}H NMR ($CDCl_3$) (300 MHz) δ 7.81 (m, 2H, aromatic), 7.69 (m, 2H, aromatic), 3.64 (m, 4H, 2 methylenes), 1.65 (m, 2H, methylene), 1.55 (m, 2H, methylene), 1.27 (br s, 12 H, 6 methylenes).

(B) 10-Amino(N-NBD)-1-decanol. 10-Phthalamidyl-1-decanol (5 mmol) was dissolved in absolute ethanol (22 mL). Hydrazine (95%, 6 mmol) was then added from a solution in absolute ethanol (1.5 mL), and the solution was stirred overnight at room temperature. The solution was then refluxed for 4 h, and, after cooling, the resulting solid was collected by filtration and washed with 95% ethanol. The filtrate was dried by rotary evaporation. The resulting solid was dissolved in 1 M NaOH (50 mL), combined with that previously collected, and extracted with several volumes of diethyl ether. The ether layers were then combined and dried by rotary evaporation. The resulting solid was dissolved in CH_3CN (15 mL), and 7-nitrobenz-2-oxa-1,3-diazol-4-yl chloride (NBD-Cl) (1.2 equiv) was added slowly to the stirred solution. The reaction was left for 2 h at room temperature before removing the solvent by rotary evaporation. The fluorescent 10-amino(*N*-NBD)-1-decanol was purified by flash chromatography through silica gel with 5% methanol/chloroform as the eluent. The isolated yields were 50−70%. ^{1}H NMR ($CDCl_3$) (300 MHz) δ 8.50 (d, 1H, aromatic), 6.20 (br s, 1H, NH), 6.17 (d, 1H, aromatic), 3.65 (t, 2H, methylene), 3.50 (q, 2H, methylene), 1.79 (m, 2H, methylene), 1.48 (m, 2H, methylene), 1.33 (br s, 12H, 6 methylenes).

(C) 5-Amino(N-NBD)-1-pentanol. 5-Amino-1-pentanol (10 mmol) was dissolved in CH_3CN (20 mL) and reacted with NBD-Cl (12 mmol) for 2 h at room temperature, and the fluorescent product was purified as above. The isolated yield was 80%. ^{1}H NMR ($CDCl_3$) (300 MHz) δ 8.51 (d, 1H, aromatic), 6.19 (d, 1H, aromatic), 3.53 (q, 2H, methylene), 3.45 (t, 2H, methylene), 1.96 (m, 2H, methylene), 1.86 (m, 2H, methylene), 1.66 (m, 2H, methylene).

(D) 1-Bromo-5-amino(N-NBD)pentane and 1-Bromo-10-amino(N-NBD) decane. 5-Amino(*N*-NBD)-1-pentanol or 10-amino(*N*-NBD)-1-decanol was dissolved in CH_3CN to 0.1 M. This solution was treated with CBr_4 (1.2 equiv) at room temperature with stirring. Triphenylphosphine (1.2 equiv) was then slowly added to the solution, allowing each addition of the solid to dissolve before another was added. The solution was stirred for 4−8 h at room temperature before removing the solvent by rotary evaporation. The resulting solid was kept under high vacuum overnight. The product was isolated by flash chromatography through silica gel using chloroform as the mobile phase. The isolated yield was 80%. ^{1}H NMR ($CDCl_3$) (300 MHz): 1-Bromo-5-amino-(*N*-NBD)pentane: δ 8.50 (d, 1H, aromatic), 6.26 (br s, 1H, NH), 6.19 (d, 1H, aromatic), 3.53 (q, 2H, methylene), 3.47 (t, 2H, methylene), 1.97 (m, 2H, methylene), 1.87 (m, 2H, methylene), 1.69 (m, 2H, methylene). 1-Bromo-10-amino-(*N*-NBD)decane: δ 8.51 (d, 1H, aromatic), 6.26 (br s, 1H, NH), 6.18 (d, 1H, aromatic), 3.49 (q, 2H, methylene), 3.41 (t, 2H, methylene), 1.85 (m, 4H, 2 methylenes), 1.3 (br s, 12 H, 6 methylenes).

(E) N,N-Bis(Boc)-L-cystine-(OMe)$_2$. *N,N*-Bis(Boc)-L-cystine (1 mmol) was dissolved in anhydrous DMF (10 mL) and treated with iodomethane (4 mmol) with stirring. *N,N*-Diisopropylethylamine (DIEA) (4 mmol) was then added, and the reaction was left at room temperature for 2 h before removing the solvent and excess iodomethane by rotary evaporation. The resulting solid was dissolved in 10% acetone/hexanes, and the product was isolated by flash chromatography through silica gel. The isolated yield was greater than 90%. ^{1}H NMR ($CDCl_3$) (300 MHz) δ 5.38 (br d, 2H, amides), 4.60 (q, 2H, 2 $C_\alpha H$), 3.77 (s, 6H, 2 methyl esters), 3.15 (d, 4H, 2 methylenes), 1.45 (s, 18H, 6 methyls).

(F) N-Boc-L-cysteine and N-Boc-L-cysteine-OMe. *N,N*-Bis(Boc)-L-cystine or *N,N*-bis(Boc)-L-cystine-(OMe)$_2$ (2 mmol) and tris(2-carboxyethyl)phosphine hydrochloride (2 mmol) were suspended in a mixture of DMF (2 mL) and H_2O (0.5 mL) under argon. For the reduction of the esterified cystine, the suspension was treated with pyridine (8 mmol), whereas the free acid form was treated with DIEA (8 mmol). After stirring under argon for 30 min at room temperature, the solution was acidified with a small volume of 1.2 M HCl, transferred to a larger volume of 1.2 M HCl (100 mL), and extracted with 2 volumes of diethyl ether. The ether layers were combined and dried by rotary evaporation. The resulting oil was dissolved in a small volume of tetrahydrofuran that had been sparged with argon. These solutions were used for the subsequent preparations of the *S*-alkylcysteine derivatives.

(G) S-Pentyl-, S-Decyl-, and S-(Amino(N-NBD)alkyl)-L-cysteine Derivatives. The appropriate alkyl bromide was dissolved in DMF, under argon, to 0.4 M. *N*-Boc-L-cysteine (1 equiv) was added from a 2 M stock solution in THF, and DIEA (6 equiv) was next added with stirring. For alkylations using bromopentane or 1-bromo-5-amino(*N*-NBD)pentane, the reactions were at 60 °C for 1 h. All other reactions were at 100 °C for 1 h. The primary products were *N*-Boc-

[1] Abbreviations: Ahx, L-2-aminohexanoic acid; Boc, *tert*-butoxycarbonyl; DCM, dichloromethane; DIEA, *N,N*-diisopropylethylamine; DMA, dimethylacetamide; DMF, *N,N*-dimethylformamide; Fmoc, (9-fluorenylmethoxy)carbonyl; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NMP, *N*-methylpyrrolidinone; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

cysteines that had been alkylated at both the thiol and the carboxylic acid. The undesirable esters were hydrolyzed by diluting the reaction mixtures (1:100) into a 50% acetone/1 M NaOH solution and stirring at room temperature for 20 min. The diluted solution was then acidified with HCl, concentrated by rotary evaporation, and extracted several times with diethyl ether. The ether layers were combined and dried by rotary evaporation, and the products were isolated by flash chromatography through silica gel with 7% methanol/chloroform. The isolated yields were greater than 90%. ^{1}H NMR ($CDCl_3$) (300 MHz): *N*-Boc-*S*-pentyl-L-cysteine: δ 5.45 (br d, 1H, amide), 4.46 (q, 1H, $C_\alpha H$), 2.98 (br d, 2H, methylene), 2.56 (t, 2H, methylene), 1.56 (m, 2H, methylene), 1.45 (s, 9H, 3 methyls), 1.33 (m, 4H, 2 methylenes), 0.89 (t, 3H, methyl). *N*-Boc-*S*-decyl-L-cysteine: δ 5.40 (br d, 1H, amide), 4.49 (q, 1H, $C_\alpha H$), 2.99 (d, 2H, methylene), 2.54 (t, 2H, methylene), 1.54 (m, 2H, methylene), 1.44 (s, 9H, 3 methyls), 1.25 (br s, 14H, 7 methylenes), 0.87 (t, 3H, methyl). *N*-Boc-*S*-(5-amino-(*N*-NBD)pentyl)-L-cysteine: δ 8.51 (d, 1H, aromatic), 6.77 (br s, 1H, NH), 6.20 (d, 1H, aromatic), 5.45 (br s, 1H, amide), 4.51 (q, 1H, $C_\alpha H$), 3.52 (q, 2H, methylene), 3.01 (d, 2H, methylene), 2.61 (t, 2H, methylene), 1.84 (m, 2H, methylene), 1.67 (m, 2H, methylene), 1.57 (m, 2H, methylene), 1.45 (s, 9H, 3 methyls). *N*-Boc-*S*-(10-amino(*N*-NBD)decyl)-L-cysteine: δ 8.50 (d, 1H, aromatic), 6.39 (br s, 1H, N-H), 6.19 (d, 1H, aromatic), 5.37 (d, 1H, amide), 4.50 (q, 1H, C_α-H), 3.50 (q, 2H, methylene), 2.99 (d, 2H, methylene), 2.56 (t, 2H, methylene), 1.83 (m, 2H, methylene), 1.59 (m, 2H, methylene), 1.45 (s, 9H, 3 methyls), 1.30 (br s, 12H, 6 methylenes).

(H) N-Boc-S-pentadecyl-L-cysteine-OMe. *N*-Boc-L-cysteine-OMe (1 mmol) was dissolved in DMF (3.5 mL). Pentadecyl bromide (1.2 mmol) was added to the stirred solution followed by DIEA (4 mmol). The reaction was carried out under argon for 45 min at 80 °C. The solvents were removed by rotary evaporation, and the product was isolated by flash chromatography through silica gel with 10% acetone/hexanes. The isolated yield was 90%. ^{1}H NMR ($CDCl_3$) (300 MHz) δ 5.35 (br d, 1H, amide), 4.53 (q, 1H, $C_\alpha H$), 3.76 (s, 3H, methyl), 2.96 (d, 2H, methylene), 2.51 (t, 2H, methylene), 1.55 (m, 2H, methylene), 1.45 (s, 9H, 3 methyls), 1.26 (br s, 24H, 12 methylenes), 0.89 (t, 3H, methyl).

(I) N-Boc-S-pentadecyl-L-cysteine. *N*-Boc-*S*-pentadecyl-L-cysteine-OMe (1 mmol) was dissolved in acetone (30 mL). To this solution was added 0.5 M KOH (30 mL), and the mixture was stirred for 20 min at room temperature before neutralizing with HCl. The solvents were removed by rotary evaporation, and the resulting solid was resuspended in 1 M HCl (50 mL) and extracted with 3 volumes of diethyl ether. The ether layers were combined, and the solvent was removed by rotary evaporation. The isolated yield was greater than 90%. ^{1}H NMR ($CDCl_3$) (300 MHz) δ 5.38 (br s, 1H, amide), 4.50 (q, 1H, $C_\alpha H$), 3.00 (d, 2H, methylene), 2.56 (t, 2H, methylene), 1.57 (m, 2H, methylene), 1.44 (s, 9H, 3 methyls), 1.24 (br s, 24H, 12 methylenes), 0.89 (t, 3H, methyl).

Attachment of Cysteine Derivatives to Merrifield Resin. Each *N*-Boc-L-cysteine derivative was attached to chloromethyl resin using potassium fluoride (Stewart & Young, 1984). The amount of amino acid incorporated was determined by weight.

Synthesis of **a***-Factor Analogues.* In a typical synthesis, the substituted resin was treated with trifluoroacetic acid to remove the Boc protecting group (Stewart & Young, 1984). *N*-Fmoc-amino acids were used to complete the synthesis of the peptide. Each amino acid was attached using a mixture of the protected amino acid (4 equiv), (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP) (4 equiv), and DIEA (6 equiv) in a final volume of 12 mL of NMP (g of resin)$^{-1}$. The completed peptide was cleaved from the resin by methanolysis and then deprotected using TFA and the appropriate scavengers (Stewart & Young, 1984). The peptides were purified by reverse-phase HPLC on a Vydac C_{18} column in 0.1% TFA with a linear gradient of CH_3CN. The molecular mass [$(M + H)^+$] of $S^{\beta 12}$-pentyl-**a**-factor was 1495.7 amu (calcd 1496.1). The molecular mass [$(M + H)^+$] of $S^{\beta 12}$-pentyl-**a**-factor was 1674.7 amu (calcd 1674.2). The molecular mass [$(M + H)^+$] of $S^{\beta 12}$-decyl-**a**-factor was 1565.8 amu (calcd 1566.2). The molecular mass [$(M + H)^+$] of $S^{\beta 12}$-(10-amino(*N*-NBD)decyl)-**a**-factor was 1744.8 amu (calcd 1744.3). The molecular mass [$(M + H)^+$] of $S^{\beta 12}$-pentadecyl-**a**-factor was 1635.7 amu (calcd 1636.4).

Synthesis of [Ahx^{12}]-α-factor. The peptide [Ahx^{12}]-α-factor differs from the wild-type pheromone by the substitution of L-2-aminohexanoic acid (Ahx) for methionine at position 12.[2] To attach the first amino acid, Wang resin (2.5 g, 0.69 mmol g^{-1}) was mixed with *N*-Fmoc-*O-tert*-butyl-L-tyrosine (2 equiv) in DMA/DCM (1:5) (12.5 mL) and stirred in an ice bath. *N,N'*-Diisopropylcarbodiimide (2.2 equiv) in DMA (2.5 mL) was then added to the solution. After 5 min, (dimethylamino)pyridine (0.1 equiv) dissolved in DMA/DCM (1:5) (2.5 mL) was added. After 20 min, *N*-methylmorpholine (1 equiv) was added, and the suspension was stirred for 4 h at room temperature. The degree of amino acid substitution was determined by measuring the absorbance of Fmoc-piperidine produced by a small amount of the resin (1-2 mg) upon treatment with 50% piperidine/DMF for 5 min (ϵ_{301} = 7800 M^{-1} cm^{-1}) (Grandas et al., 1989). The substitution was 0.33 mmol g^{-1}. The remaining hydroxyl groups were blocked by reaction with benzoyl chloride. The synthesis of the peptide was completed using the methods described for the analogues of **a**-factor. The completed peptide was deprotected and cleaved from the resin with TFA and purified by HPLC. The molecular mass [$(M + H)^+$] of this peptide was 1665.8 amu (calcd 1666.1).

Synthesis of **a***-Factor.* Wild-type **a**-factor was produced by reacting farnesyl bromide with an **a**-factor peptide that contained an unmodified cysteine. The peptide was synthesized by the standard method, but with an *S*-tritylcysteine. Deprotection with TFA generated a reactive thiol on the cysteine. The **a**-factor peptide (30 nmol) was dissolved in DMF/H_2O/0.5 M NH_4HCO_3 (4:1:1) (140 μL). Farnesyl bromide (280 nmol) in DMF (40 μL) was added, and the mixture was stirred for 15 min at 40 °C. The solution was then acidified with glacial acetic acid (180 μL) and dried. The **a**-factor was purified by HPLC as described above. The molecular mass [$(M + H)^+$] of the **a**-factor was 1629.8 amu (calcd 1630.3).

Synthesis of 6-Amino(N-NBD)hexanoyl-[Ahx^{12}]-α-factor. The [Ahx^{12}]-α-factor (3.1 μmol) was dissolved in 40 mM

[2] L-2-Aminohexanoic acid is the systematic name for L-norleucine.

Name	Structure
[Ahx^{12}]-α-factor (α-factor)	Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Ahx-Tyr
6-Amino-*N*-NBD-hexanoyl-[Ahx^{12}]-α-factor (NBD α-factor)	NH-'Trp'-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Ahx-Tyr
a-factor	Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-OCH$_3$ (Farnesyl)
S$^{\beta 12}$-Pentyl-**a**-factor (Pentyl **a**-factor)	Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-OCH$_3$ (CH$_2$)$_4$CH$_3$
S$^{\beta 12}$-(5-Amino-*N*-NBD-pentyl)-**a**-factor (Pentyl-NBD **a**-factor)	Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-OCH$_3$ (CH$_2$)$_5$NH-NBD
S$^{\beta 12}$-Decyl-**a**-factor (Decyl **a**-factor)	Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-OCH$_3$ (CH$_2$)$_9$CH$_3$
S$^{\beta 12}$-(10-Amino-*N*-NBD-decyl)-**a**-factor (Decyl-NBD **a**-factor)	Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-OCH$_3$ (CH$_2$)$_{10}$NH-NBD
S$^{\beta 12}$-Pentadecyl-**a**-factor (Pentadecyl **a**-factor)	Tyr-Ile-Ile-Lys-Gly-Val-Phe-Trp-Asp-Pro-Ala-Cys-OCH$_3$ (CH$_2$)$_{14}$CH$_3$

FIGURE 1: Synthetic yeast pheromones and their derivatives. Common names used in the text are indicated within parentheses.

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.0 (2.5 mL). Succinimidyl 6-amino(*N*-NBD)hexanoate (3.1 μmol, Molecular Probes) was dissolved in CH_3CN (2.5 mL). The solutions were combined and stirred overnight at 37 °C. The reaction mixture was dried under reduced pressure and separated by reverse-phase HPLC. Three NBD-labeled products were observed, all of which were eluted from the C_{18} column at higher concentrations of CH_3CN than the unmodified peptide. The purified products were identified by digesting them with thermolysin, isolating the thermolytic fragments by HPLC, and identifying the isolated fragments by amino acid analysis. One product was modified at the amino terminus, one was modified at Lys^7, and one was modified at both sites. The peptide that contained a single modification at the amino terminus was used for all subsequent experiments. The molecular mass [$(M + H)^+$] of this peptide was 1942.7 amu (calcd 1943.1).

Activity Assays. The biological activities of the synthetic peptides were monitored initially using halo assays (Manney, 1983). For quantitative analysis of the activity of the pheromones, however, a modified version of a growth-arrest assay developed by Moore (1983) was used. In each case, stock cultures of haploid *S. cerevisiae* cells, AH216α (*MAT*α, *leu2-3*, *leu2-112*, *his3*, *pho5*, *pho3*) and AH216**a** (*MATa*, leu2-*3*, *leu2-112*, *his3*, *pho5*, *pho3*) (Meyhack et al., 1982), were grown to near saturation in YPD meduim (2% yeast extract, 1% peptone, 1% dextrose). These cultures could be kept at 4 °C for approximately 1 month before being replaced. For the quantitative assays, cells were diluted from the stock cultures to roughly 30 000 cells mL^{-1} in YPD. The diluted solutions were divided into culture tubes and vigorously shaken in an orbital shaker at 30 °C to initiate growth of the cells. After 1 h, the cultures were treated with various concentrations of pheromones from stock solutions in 50% ethanol. To control for the effects of the added ethanol on cell growth, all cultures received identical volumes of 50% ethanol. The cultures were shaken in the dark for an additional 5 h at 30 °C, which allowed the cells to grow approximately two generations. Aliquots were then either plated on YPD-agar or analyzed by particle counting on a coulter counter to determine the final cell densities. Cells that had been plated were grown on the solid medium at 30 °C for 2.5 days before counting the colonies. The results obtained by the two different methods agreed exactly.

Peptide Concentrations. The concentration of peptide in the stock solutions was determined by quantitative amino acid analysis. Aliquots were subjected to acid hydrolysis and analyzed on an ion-exchange HPLC system (Spectra Physics) equipped with a post-column reactor (Pickering Laboratories).

Fluorescence Spectra. All fluorescence spectra were obtained on an SLM-Aminco SPF-500C spectrofluorometer using 5 nm bandwidths. The spectra shown are uncorrected.

RESULTS

Synthesis of the Pheromones. The synthetic pheromones are summarized in Figure 1. The trivial names of the peptides given there will be used for all subsequent discussion. α-Factor was synthesized with an Ahx at position 12

in place of the methionine that is present in the natural pheromone (Stötzler & Duntze, 1976). This substitution increases the stability of the peptide and has no measurable effect on biological activity (Raths et al., 1988). The method used to synthesize α-factor has not, to our knowledge, been described elsewhere. The use of the coupling agent, PyBOP, reduced the coupling times to approximately 5 min, and no double couplings were needed. Upon cleavage and deprotection, the peptide was found to be extremely pure by HPLC, and reasonable yields were obtained. The structure of the product was confirmed by amino acid analysis, mass spectroscopy, and biological activity assays. To produce the fluorescent derivative, purified α-factor was reacted with an activated NBD derivative under conditions that favored labeling of the amino terminus (Naider et al., 1990). Proteolysis of the purified products by thermolysin and subsequent amino acid analysis of the repurified fragments allowed the position of the modification to be assigned (not shown). Mass spectroscopy confirmed that the α-factor contained a single modification.

The synthesis of **a**-factor has been reported previously (Xue et al., 1989). The published method involved a combination of solution- and solid-phase techniques and used protected amino acids that are not commercially available. The synthesis reported here was done entirely by solid-phase techniques using standard Fmoc-amino acids. After synthesis, the peptide backbone was cleaved from the resin and esterified in a single step. To avoid esterification of the aspartic acid side chain, it was important to use the *tert*-butyl protecting group rather than the benzyl (Stewart & Young, 1984). All of the protecting groups were then removed using TFA. At this stage, the cysteine was susceptible to oxidation, however, and during the subsequent farnesylation, some of the peptide was oxidized to the disulfide. The extent of the oxidation could be minimized by removal of oxygen from the solutions, but some of the peptide was always consumed by this competing reaction. The structure of native **a**-factor was confirmed by amino acid analysis, mass spectroscopy, and biological activity assays.

We initially tried to synthesize the **a**-factor derivatives by the same method that had been used for native **a**-factor. Unfortunately, the various alkyl groups had such different reactivities—the larger groups reacting slowly, and the smaller ones reacting at sites other than the cysteine—that we were forced instead to incorporate the alkyl chains into the cysteine prior to assembly of the rest of the peptide backbone. In the first step, *N*,*N*-bis(Boc)cystine was reduced to *N*-Boc-cysteine using a phosphine reagent (Burns et al., 1991). To minimize oxidation, this and the subsequent step were performed under argon, using solvents that had been degassed and stored under argon. The Boc protecting group was necessary because of its stability to the subsequent basic conditions. The cysteine was then reacted with the appropriate alkyl bromide to produce a cysteine that contained both the desired thioether and an undesired ester. For all but the pentadecyl product, the ester was easily cleaved by saponification to yield the monosubstituted cysteine. The pentadecyl product could not be dissolved in an aqueous, alkaline solution, however, and *S*-pentadecylcysteine was produced instead by alkylation of a methylesterified *N*-Boc-cysteine. The subsequent saponification of the methyl ester was facile. The modified cysteine was next attached to the solid support. Because the cysteine was protected with Boc, Merrified resin was used instead of Wang resin. The rest of the peptide was synthesized by the same method described for the other peptides. The methanolysis was very efficient, and a single step removed most of the peptide. For quantitative removal of the peptide, however, the methanolysis was repeated several times.

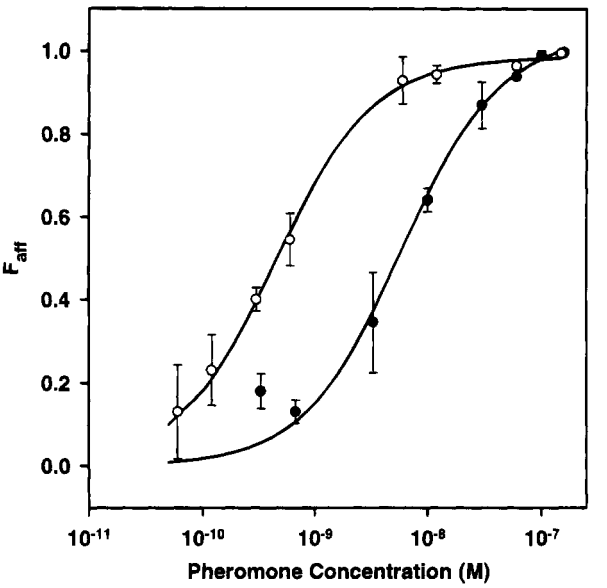

FIGURE 2: Growth-arrest assays. The activity of α-factor (○) and NBD α-factor (●) was measured as a function of concentration of pheromone. The activity is presented as the fraction of cells affected by the pheromone, F_{aff}, where $F_{aff} = (N_0 - N_t)/N_0$. In this equation, N_0 is the number of cells in an untreated culture and N_t is the number of cells in a culture treated with pheromone. The data were fit to the equation $F_{aff} = (F_{max}[P])(P_{50} + [P])^{-1}$, where [P] is the concentration of the pheromone, F_{max} is a constant that represents the maximum fraction affected (usually 0.5−0.7), and P_{50} is the concentration of pheromone that produces half the F_{max}. To compare the two curves, F_{max} was normalized to 1.0 for the two experiments. The error bars represent the standard deviation between cultures treated in duplicate. Values of P_{50} for the other pheromones are compiled in Table 1.

Biological Activities of the Pheromones. The yeast pheromones are responsible for initiating a series of events in haploid yeast cells that culminates in the fusion of two cells of opposite mating type (Blumer & Thorner, 1991). One of the earliest effects, and the one most sensitive to pheromone, is an arrest in the G_1 phase of the cell cycle (Moore, 1983). Consequently, a culture of haploid yeast cells treated with an active pheromone will grow to a lower cell density than a similar culture that was not treated with pheromone. In order to measure this activity accurately, a modified version of the growth-arrest assay developed by Moore (1983) was used. Solutions of the pheromones were diluted into liquid cultures of the yeast cells of the appropriate mating type, and the cells were allowed to grow for approximately two generation times. The number of cells present in each culture at the end of the incubation was then counted. These values were adjusted according to the number of cells present in a culture not treated with the pheromone to obtain the fraction of cells affected. Examples of the results of two of these assays are shown in Figure 2. The data from assays with α-factor and NBD α-factor are compared on one plot. This comparison demonstrates that small differences in pheromone activity are easily measured by this method. The activities of the pheromones are summarized in Table 1.

Table 1: Summary of Activities of the Pheromones

pheromone	P_{50}[a] (nM)	pheromone	P_{50}[a] (nM)
α-factor	0.5 ± 0.3	pentyl **a**-factor	5.8 ± 0.7
NBD α-factor	4 ± 1	decyl-NBD **a**-factor	2.0 ± 0.5
a-factor	0.3 ± 0.1	pentyl-NBD **a**-factor	23 ± 9
decyl **a**-factor	0.2 ● 0.1		

[a] Average of three or more separate assays.

The biological activity of the yeast pheromones can be decreased by degradation of the pheromone in the culture, and the extent of this degradation is dependent on the density of cells in the culture (Moore, 1983). In order to minimize this problem, we used very low densities of cells in our activity assays. To confirm that the pheromone added to the assay cultures was not being degraded, we performed control assays on cultures of different densities. Identical activities were obtained from assays with cultures that differed 10-fold in concentration of cells (not shown).

To verify that the pheromones were completely dissolved in the culture medium and that they remained so during the assays, a control experiment that tested the amount of pheromone present in the solution at the end of an assay was performed. Low solubility was a concern, particularly for **a**-factor and the derivatives of **a**-factor, because of their obvious hydrophobic character. In this experiment, a standard growth-arrest assay with **a**-factor was performed. At the end of the assay, an aliquot was removed from each culture to determine the cell density, and the cultures were cleared of cells by centrifugation. The supernatants were then removed, inoculated with fresh cells, and assayed for effects of the pheromone on growth. The activity measured in each assay was identical. This result confirmed that the pheromone neither precipitated nor was degraded during the assay. This result also indicates that at the low cell densities used in these assays, only a small fraction of the total **a**-factor is adsorbed to the surface of the cells. Since **a**-factor is one of the most hydrophobic of the pheromones, its lack of precipitation, degradation, or adsorption to the cells suggests that the activities obtained with the other pheromones indeed represent the true concentrations of the pheromones in solution. In contrast, results obtained from assays of pentadecyl **a**-factor indicate that this pheromone is not well dissolved in the liquid culture. Although this derivative has biological activity, an accurate concentration necessary for half-maximal arrest of growth (P_{50}) cannot be reported at this time.

Fluorescence Measurements. To test the ability of the NBD to report a change in the solvent environment of the pheromone, we measured the fluorescence of the NBD-modified pheromones in various solvents. The results of these measurements are shown in Figure 3. When dissolved in methanol, all of the NBD-linked pheromones fluoresce much more intensely than when they are dissolved in aqueous solution. The fluorescence of the pheromones is also greatly enhanced when the pheromones are added to an aqueous solution that contains phospholipid vesicles. The fluorescence of decyl-NBD **a**-factor in the presence of a low concentration of lipid vesicles approaches that measured in methanol. In contrast, the fluorescence of pentyl-NBD **a**-factor was much less, even at higher concentrations of phospholipid vesicles. This result suggests that pentyl-NBD **a**-factor has a lower affinity for lipid bilayers than the decyl-

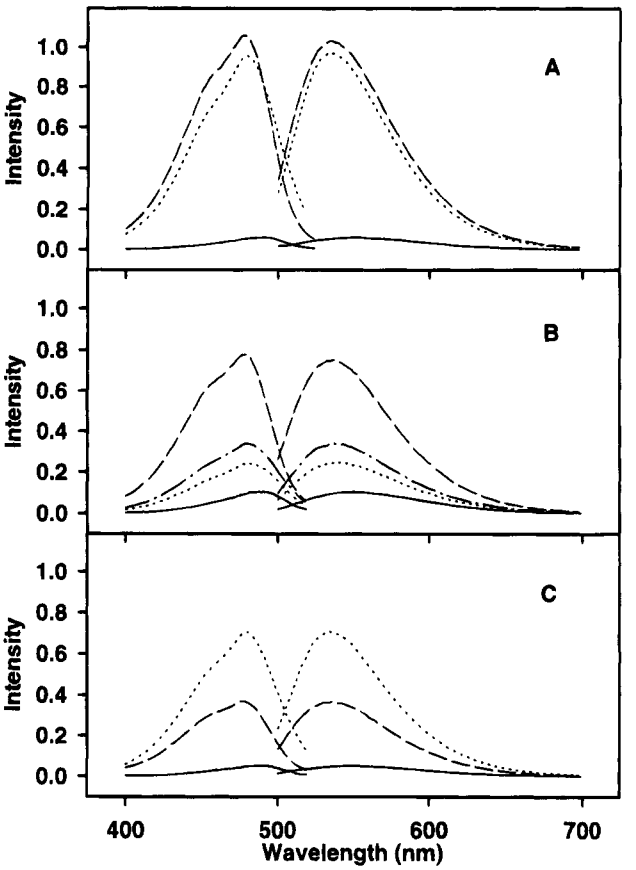

FIGURE 3: Fluorescence spectra of the NBD-labeled peptides. (A) Decyl-NBD **a**-factor, (B) pentyl-NBD **a**-factor, and (C) NBD α-factor. The concentration of peptide was 0.5 μM in each case. Spectra were measured in MeOH (– –), in an aqueous solution of 10 mM 4-morpholineethanesulfonic acid, pH 6.0/50 mM KCl (—), and in the aqueous solution plus either 0.2 mM (▪) or 0.4 mM (– • –) 1-palmitoyl-2-oleoylphosphatidylcholine/1-palmitoyl-2-oleoylphosphatidylglycerol (4:1) vesicles. Preparation of vesicles was as described (Maduke & Roise, 1993). Any background signal from the vesicles themselves was subtracted. The fluorescence intensities are given in arbitrary units.

NBD derivative. For NBD α-factor, the level of enhancement under these conditions was reversed; the fluorescence in the presence of the lipid vesicles was greater than that measured in methanol. In all cases, the effect of the vesicles on the fluorescence of the pheromones indicates that the pheromones are able to interact with lipid bilayers.

DISCUSSION

Using a well-defined assay, the biological activities of the native yeast pheromones and a variety of derivatives have been measured (Table 1). The concentration of α-factor required for half-maximal effect on growth, 0.5 nM, is in good agreement with the value of 0.25 nM reported previously (Moore, 1983). In the case of **a**-factor, a similar comparison is not possible; although a variety of synthetic **a**-factor derivatives have been described previously (Marcus et al., 1991), the activities of these pheromones were measured using assays in which an accurate effective concentration of pheromone cannot be determined. With our assays, however, we were able to determine that yeast strains of opposite mating type, which are otherwise isogenic, display identical sensitivity to α-factor and **a**-factor (Table 1). Although the binding of **a**-factor to the **a**-factor receptor has not yet been reported, this result suggests that the

pheromone receptors may have similar affinities for their ligands.

Modification of α-factor by the fluorescent probe decreased the activity of the pheromone somewhat but did not abolish it. This result is consistent with experiments that indicated that α-factor can tolerate modification of the amino terminus by either small molecules (Naider et al., 1990) or extra amino acids (Tallon et al., 1987). Consistent with previous reports (Raths et al., 1988; Naider et al., 1990), we also found that α-factor modified at Lys^7 displayed biological activity at relatively low concentrations (not shown).

The experiments with the **a**-factor derivatives show that the farnesyl moiety can be effectively replaced by a simple decyl group. The similar behavior of **a**-factor and decyl **a**-factor is consistent with lipid vesicle partitioning studies that showed that an unbranched C_{11} alkane confers the same lipophilicity to model peptides as a farnesyl group (Silvius & l'Heureux, 1994). Replacement of farnesyl with less hydrophobic groups decreases the activity of **a**-factor somewhat (Table 1), although the effects of these changes on the activity of the pheromone appear less significant than has been reported (Marcus et al., 1991).

We initially tried to synthesize fluorescent derivatives of **a**-factor by incorporating fluorescent probes into the native structure. Modification of the amino terminus or the ϵ-amine of Lys^4 by succinimidyl NBD hexanoate or NBD-Cl, however, resulted in the loss of activity of the pheromone. We therefore decided to incorporate a fluorescent probe at the terminus of the alkyl chain on the cysteine. This position appears to be much less important for interactions with the receptor than the peptide backbone. The activity of a fluorescent derivative of decyl **a**-factor, decyl-NBD **a**-factor, is somewhat lower than that of native **a**-factor. Others have shown, however, that the NBD group generally does not insert deeply into lipid bilayers (Chattopadhyay & London, 1987, 1988). Because of this, the modification of the decyl group by NBD probably decreases the effective hydrophobicity of the alkyl chain and consequently decreases the biological activity of the fluorescent pheromone. Comparison of the activities of pentyl-NBD and decyl-NBD **a**-factors suggests that an NBD **a**-factor that exactly matches the activity of native **a**-factor may be obtained by extending the alkyl group to C_{11} or C_{12}.

The activity assays show clearly that the pheromone receptors can recognize the fluorescent ligands. We have also demonstrated that the NBD label on the pheromones can report the solvent environment of the probe. When the fluorescent pheromones are dissolved in methanol or in an aqueous buffered solution containing lipid vesicles, the fluorescence of the pheromones is enhanced (Figure 3). This result demonstrates that the pheromones associate to some extent with lipid bilayers. This property was previously described by others with model membranes and high concentrations of unmodified pheromones (Wakamatsu et al., 1986, 1987; Jelicks et al., 1989; Epand et al., 1993). The NBD-labeled pheromones, however, can be used to quantify the binding properties of the pheromones with lipid bilayers in natural membranes at physiological concentrations.

We are currently using the labeled pheromones to examine the association of the pheromones with natural and artificial membranes. Support for the role of a membrane-partitioning step in the activation of certain receptors comes from studies with model peptide hormones (Kaiser & Kézdy, 1983, 1984). These hormones contain a short sequence of amino acids that is thought to be responsible for specific interactions with receptors and that cannot be altered without affecting the biological activity of the hormone. The hormones also contain a segment that is surface-active and that can be substituted by unrelated amino acid sequences as long as the biophysical properties of the altered sequences remain unchanged (Taylor et al., 1983; Moe & Kaiser, 1985; Minakata et al., 1989). Results from the published studies suggest that, in many cases, only a relatively small segment of a peptide hormone may actually be necessary for specific interactions with a receptor and that the rest of the molecule is simply responsible for associating this segment with the surface of the membrane in an appropriate orientation. In the case of the yeast pheromones, it is not yet clear which regions of the peptides are responsible for activation of the receptors, though a large number of modified pheromones have been tested for biological activity (Naider & Becker, 1986; Marcus et al., 1991). Our results with **a**-factor suggest that the role of the farnesyl moiety is to provide nonspecific interactions with the membrane bilayer. The hydrophobic amino acid side chains of α-factor presumably provide a similar function.

The availability of NBD-labeled pheromones will greatly facilitate the analysis of receptor activation in the yeast system and the involvement of a membrane-partitioning step in this process. Others have used fluorescent ligands to monitor interactions between ligands and receptors (Tota & Strader, 1990; Hwang et al., 1992). Although these assays require that the various forms of the ligand display distinct spectroscopic signals, they are highly sensitive, specific, and rapid, and they eliminate the need to separate the forms by physical methods. The interactions, therefore, can be monitored under true equilbrium conditions. Similar approaches have recently been used to measure the partitioning of surface-active molecules into the hydrophobic core of membrane bilayers (Rizzo et al., 1987; Rapaport & Shai, 1991, 1994; Swanson & Roise, 1992; Silvius & l'Heureux, 1994). A combination of these approaches with the fluorescent yeast pheromones should provide valuable information about the molecular mechanism of transmembrane signaling in the yeast pheromone response.

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