http://dx.doi.org/10.5012/bkcs.2014.35.5.1365

Highly Active Analogs of α-Factor and Their Activities Against *Saccharomyces cerevisiae*

Hee Jun Ahn, Eun Young Hong,† Dong Hoon Jin,‡ and Nam Joo Hong*

*School of Biotechnology, Yeungnam University, Gyungsan City, Gyungbuk 712-749, Korea. *E-mail: njhong@ynu.ac.kr*
†School of Chemical and Biological Engineering, Seoul National University, Seoul 151-742, Korea
‡Asan Institute for Life Science, Department of Convergence Medicine, College of Medicine, University of Ulsan, Seoul 138-736, Korea
Received November 21, 2013, Accepted January 13, 2014

Thirteen analogs of tridecapeptide α-factor (WHWLQLKPGQPMY) of *Saccharomyces cerevisiae* with *C*- or *N*-terminal Trp extension and isosteric replacement by Aib at position 8 and 11, Trp at position 13, D-Ala at position 9, and Orn and Glu at position 6 were synthesized and assayed for their biological activity. Receptor binding assay was carried out using our newly developed spectrophotometric method with detector peptide **14**. *C*- or *N*-terminal extended analogs, α-factor-$[Trp]_n$ (n =1-5) **1**-**5** and $[N\text{-}Trp]_1$-α-factor **6**, were all less active than native α-factor and gradual decreases in both activity and receptor affinity were observed with greater Trp extension. Trp-substituted analog at position 13, [Trp^{13}]α-factor **7**, exhibited about 2-fold reductions in both activity and receptor affinity. Aib-substituted analogs, [Aib^8]α-factor **8** and [Aib^{11}]α-factor **9**, showed 5- to 10-fold reduction in activity as well as 3-fold reduction in receptor affinity compared to native α-factor. [Orn^6]α-factor **10** demonstrated strong potency with a 7.0-fold increase in halo activity as well as 1.8-fold increase in receptor affinity compared to native α-factor. For two double substituted analogs, [Glu^6,D-Ala^9]α-factor **12** showed the slightly decreased potency in halo activity compared to analog **10**, whereas [Orn^6,D-Ala^9]α-factor **11** exhibited 15-fold higher halo activity as well as nearly 3-fold higher receptor affinity compared to native α-factor.

Key Words : α-Factor, Analogs, *S. cerevisiae*, Activity

Introduction

Native α-factor, tridecapeptide (WHWLQLKPGQPMY), is secreted by *Saccharomyces cerevisiae MATa* haploid cells and recognized by a receptor that is coded by gene (*STE2*), which is expressed in *S. cerevisiae MATa* haploid cells.[1] The α-factor pheromone receptor, Ste2p, belongs to the large family of G protein-coupled receptors (GPCRs).[2-4] Even since α-factor was isolated and characterized in 1977,[5] many groups have reported studies on the structure-activity relationship (SAR) of α-factor.[4] Most studies have focused on tridecapeptide (α-factor) or dodecapeptide pheromone lacking an *N*-terminal Trp (des-Trp^1-α-factor), which is another pheromone produced by post-proteolytic processing during α-factor maturation. The both 13- and 12-peptides have been reported as showing similar activities in the nanogram per milliliter range. However, removal of Trp^1 residues from the 13-peptide generally reduces the activity of resulting 12-peptide.[6-9]

[Nle^{12}]α-factor, an isosteric replacement of Met,[12] is widely accepted as an alternative probe to native α-factor, and has several advantages over α-factor. Importantly, it is stable against autoradiolysis,[4,8,10] and it does not undergo oxidation leading to methionine sulfoxide. In addition, Nle^{12} substitution was reported to increase the stability of this pheromone against the endopeptidase-induced degradation in *MATa* cells, resulting in maintenance of activity for a longer period.[11,12] Surface-associated endopeptidase, which is involved in cleavage of the peptide bond between residues Leu^6 and Lys^7 of α-factor, has strong effects on the potency of α-factor, and thus increased stability against endopeptidase-mediated degradation is significant to maintain potency. However, despite such structural characteristics of [Nle^{12}]α-factor, its activity is not as strong as that of native α-factor.[6,8,27]

The presence of central residues Lys^7-Pro^8-Gly^9-Gln^{10} forming a type II β-turn has been verified in conformation studies as a major structural feature of biologically active pheromone.[4,13,14,43] To rigidify the central region of α-factor based on this β-turn structure, various cyclic analogs of α-factor possessing a covalent bridge between $Lys^7$and Gln^{10} (cyclo$^{7\text{-}10}$ α-factor) were synthesized as β-turn probe.[15,16] Subsequently, cyclic lactam with different ring size,[16,17] cyclic disulfide,[18,19] and cyclic γ-lactam[20] were designed to induce a more favorable turn structure for the pheromone. However, such cyclization approaches to conformational rigidification generally reduce potency by 5% to 25% regardless of the adopted cyclic structures.[16,17] Among the many synthetic β-turn peptidomimetics restricted by cyclization, (R)-γ-lactam conformational constraint incorporating [3-(R)-amino-2-oxo-1-pyrrolidineacetamido] in place of Pro-Gly at residues 8 and 9 was reported to have the best activity only equal to that of parent peptide, [Nle^{12}]α-factor, whereas others were not as active as [Nle^{12}]α-factor.[20]

Recently, to establish the identities of Ste2p receptor residues mediating interactions with ligands, an affinity label-

ing approach involving the photoactivatable groups attached to the α-factor backbone was developed.[28,30,44,51-53,55,56] *p*-Benzoyl-L-phenylalanine (Bpa) and 3,4-Dihydroxyphenylalanine (DOPA) was reported to have a desirable property for this purpose, although incorporation of these groups at various positions in α-factor have been shown to result in a less than 30-fold decrease in receptor affinity.[44,51,55] Biotin (Bio) tagging modification of DOPA analog (Bio[7]-[DOPA[13]]α-factor) and 7-Nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)[30,53,54] developed for the detection of ligand-receptor conjugates were shown to reduce activity to about 33% that of parent α-factor.[44,56] Although the conjugation strategy of photoactivatable groups is not focused on enhancing potency, all of these analogs display receptor affinities varying from slightly better than that of α-factor to about 6-fold lower.[53,54]

Most SAR studies on the α-factor have compared the activities of synthetic analogs with that of the parent Nle[12]-tridecapeptide, [Nle[12]]α-factor.[4,8,10-12,25,44,46] Early studies also made comparisons with the parent dodecapeptide, des-Trp[1]-α-factor.[4,42,47-50] However, not many reports have directly compared activity values with that of native tridecapeptide α-factor.[4,13,27,39,45] In our investigation, [D-Ala[9],Nle[12]]α-factor was shown to have one of the highest activities in halo assay (about 5-fold increase compared to that of [Nle[12]]α-factor), but no comparison data with native tridecapeptide α-factor have been obtained.[10]

Although hundreds of α-factor analogs have been reported over the last thirty years,[4,42,43,51,53] most of these synthetic analogs are known to markedly reduce activity, and only a few have activities comparable with that of the native α-factor. Surprisingly, none of these analogs exceed higher than 3- to 4-fold activity compared to that of native α-factor.[4,42] In this paper, to enhance the biological activity of native α-factor, we designed a series of α-factor analogs in which Trp was attached to the *C*-terminus in stepwise fashion without alteration of the main chain and unnatural amino acids such as 2-aminoisobutyric acid (Aib) and Ornithine (Orn) were substituted within the main chain. *C*-terminal elongated analogs have general structures such as α-factor-[Trp]$_n$, where subscript n refers to the number of Trp residues from 1 to 5. Such designs are expected to afford peptide analogs favorable structural characteristic for enhanced interactions with the receptor buried in the hydrophobic membrane due to the increased hydrophobic nature of the analogs.[21] In addition, we have designed and synthesized a new detector peptide, [Orn[6]]α-factor-[Cys]$_3$ **14**, that makes spectrophotometric measurement of receptor affinity possible. The relative affinities of achromic competing peptides can be rapidly determined by measuring their effect on binding of the detector peptide. We have used this method to determine the affinities of analogs for the cognate GPCR of *S. cerevisiae MATa*.

Experimental Procedure

Synthesis of α-Factor Analogs.[22-24] All α-factor analogs were prepared using a solid phase strategy. All reagents and solvents in solid phase peptide synthesis were of analytical grade and purchased from Calbiochem-Novabiochem Corporation (San Diego, CA), VWR Scientific (Piscataway, NJ), and Sigma Aldrich. High performance liquid chromatography grade dichloromethane, acetonitrile, methanol, and water were purchased from VWR Scientific and Fisher Scientific (Springfield, NJ). Automated synthesis of target peptides was carried out on an ASP 48S peptide synthesizer (Peptron Inc.) using preloaded NH_2-Trp-2-chloro-Trityl Resin (0.53 mmol/g resin, Novabiochem, La Jolla, CA) on a 0.1-mmol scale. Elongation of the peptide chain was carried out using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU: 8 eq.)/*N*-hydroxybenzotriazole (HOBT: 8 eq.)/4-methylmorpholine (16 eq.) along with eight equivalents of a 9-fluorenylmethoxycarbonyl (Fmoc) amino acids. Fmoc was used for all *N*-α-protections, whereas the side chain protecting groups were His(Trt)-OH, Lys(*t*-Boc)-OH, Orn(*t*-Boc)-OH, Gln(Trt)-OH, and Trp(*t*-Boc)-OH. For the first 10-amino acid fragment, single coupling, followed by double coupling were used to avoid formation of deletion peptides. Acetic anhydride capping was used after each coupling to improve the purity of the peptide.

After complete chain assembly, the *N*-α-deprotected peptidyl resin was washed thoroughly with 1-methyl-2-pyrrolidinone and CH_2Cl_2 and then dried *in vacuo* for 4–5 h. The peptide was then cleaved from the resin support with simultaneous side chain deprotection using trifluoroacetic acid (90 mL), 1,2-ethanedithiol (2.5 mL), thioanisol (2.5 mL), triisopropylsilane (2.5 mL) and water (2.5 mL). Filtrates from the cleavage mixture were collected, combined with trifluoroacetic acid washes of the resin, concentrated under reduced pressure, and treated with cold ether to precipitate the crude product.

The obtained crude peptide was purified by reversed-phase high performance liquid chromatography (Shimazu Prominence HPLC) on a preparative Vydac Everest C18 reverse phase polymer column (250 mm × 22 mm) with detection at 220 nm. Due to the strong tendency of the peptide to aggregate in water, the crude product (5 mg) was dissolved in 1 mL of 50% trifluoroacetic acid/water, applied to the column, and eluted with a linear gradient of CH_3CN/water containing 0.1% trifluoroacetic acid and 10 to 75% CH_3CN over 90 min with a flow rate of 1.5 mL/min. Fractions were analyzed by reversed-phase high performance liquid chromatography (Shimazu Prominence HPLC) on an analytical Vydac Everest C18 reversed phase polymer column with detection at 220 nm. Fractions with over 98% homogeneity were pooled and subjected to lyophilization. Molecular weights of the final peptides were assessed by LC/MS (Agilent HP1100 series).

Organisms. Strain *S. cerevisiae* 7925 [*MATa his3-532 trp1 gal2*] was used for growth arrest and halo assay of various α-factor analogs. The strain was purchased from the Korean Collection for Type Cultures and was maintained and grown on YEPD medium (yeast extract: 1%, peptone: 2%, dextrose: 2%) at 30 °C.

Morphogenesis (5% Shmoo) Assay.[10,25] Strain *S. cerevisiae* 7925 (*MATa*) was grown at 30 °C with shaking (200 rpm/sec) to early log phase in YEPD broth (yeast extract: 1%, peptone: 2%, dextrose: 2%) supplemented with 2% glucose. Cells were then harvested by centrifugation at 6,000 rpm/sec (IBRD IB4, Hanil Science Centrifuge), washed twice with sterile distilled water, resuspended in YEPD broth to 4.0×10^6 cells/mL, and placed on ice. Dilution series (0.5-10 μL/100 μL; 5-100 μg/100 μL) of peptide analogs was prepared in plastic test tubes using peptide stock solution (6×10^{-3} M, 1000 μg/100 μL) with YEPD as the diluents. Ten microliter of the 1.0×10^6 cells/mL was then added to 90 μL of each of the peptide solutions, after which suspensions were incubated at 30 °C for 4 h with 200 rpm/sec of shaking. The cells were placed on ice, after which 10 μL portions were placed in a hemocytometer and observed by visual inspection using a microscope (Olympus CHD) under 400× magnification in order to quantify the total cells as well as the number of cells with shmoo morphology (elongated, pear-shaped cells without a constricted neck at the bud site). Shmoo cells were counted repeatedly in three separate areas containing 400 total cells. The percentage of shmoo cells was calculated by dividing the number of shmoo cells by the total cell numbers. To compare relative activities of different analogs, the amount of analog causing 5% shmoo morphology (20 shmoo cells/400 total cells) was determined from the regression line.

Growth Arrest (halo) Assay (Fig. 2(a) and (b)).[10,25] Two microliter (2.4×10^3 cells/mL) of *S. cerevisiae* 7925 cells suspension (1.2×10^6 cells/mL) was diluted in 6 mL of YEPD [yeast extract (1%, w/v), peptone (2%, w/v) and dextrose (2%, w/v)] containing 2% agar and layered on a plate. Ten microliters of each peptide solutions at various concentrations (6×10^{-3} M, 0.01 μL-10 μL/10 μL) was pipette onto filter disks (8 mm in diameter) from Advantec, Toyo Roshi. Plates were incubated at 30 °C for 24 and 48 h and then observed for clear zones (halos) around the disks. Data were expressed as the diameter of the halo including the diameter of the disk. The minimum value for growth arrest was 9 mm, which represents the disk diameter (8 mm) with a small zone of inhibition. All assays were carried out at least three times, and variations in halo size were no larger than 2 mm at any amount of applied peptide. Reported averages are averages of tests. Differences in diffusion of the various analogs in agar medium had no effect on biological activities in the halo assay. Similar trends were obtained for these analogs when activities were ranked within a single assay, as measured by halo assay and morphogenesis. Each halo assay was carried out at least three times with virtually identical regression line obtained.

Synergism Assays (Fig. 2(c)). Synergism assay was used to determine whether or not analogs with no growth arrest activity are capable of antagonizing native pheromone activity. Lawns of *S. cerevisiae* strain 7925 suspended in YEPD were layered onto plates as described in the growth arrest assay. Specifically, sterile disks were placed adjacent to each other so that the disk containing the test peptide would lie at the periphery of the halo formed by the standard analog, [Orn^6]α-factor **10**. One disk was impregnated with 100 μg (6.0×10^{-2} μmol) of [Orn^6]α-factor **10** in 10 μL of H_2O, and the other disk was impregnated with 150 μg (9.0×10^{-2} μmol) of the test peptide **1-5** in 15 μL of H_2O. Plates were incubated as described in the halo assay, and the effects on halo formation were noted.

Steady-state Saturation Binding with Detector, [Orn^6]α-Factor-[Cys]$_3$ (Fig. 4). To obtain steady-state saturation binding for Scatchard analysis, cells were grown and harvested as described above with the following changes. Cell stock suspension at a concentration of 3.0×10^{11} cells/mL was diluted to final assay concentration of 2.5×10^{11} cells/mL. The cell suspension (1 mL) were then dispensed into seven round bottom plastic test tubes with a volume of 5 mL, after which various amounts of [Orn^6]α-factor-[Cys]$_3$ **14**, [0.12 μL (8.8×10^{-10} mol, 7.1×10^{-7} M), 0.6 μL (4.4×10^{-9} mol, 3.5×10^{-6} M), 1.2 μL (8.8×10^{-9} mol, 7.0×10^{-6} M), 1.8 μL (1.3×10^{-8} mol, 1.0×10^{-5} M), 2.4 μL (1.8×10^{-8} mol, 1.4×10^{-5} M), 3.6 μL (2.7×10^{-8} mol, 2.1×10^{-5} M), 6 μL (4.4×10^{-8} mol, 3.5×10^{-5} M), 12 μL (8.9×10^{-8} mol, 7.1×10^{-5} M), 18 μL (1.3×10^{-7} mol, 1.1×10^{-4} M)] at 4.73×10^{-3} M (1 mg detector/100 μL saline water) were added to each test tube. Saline water was added to obtain a final volume of 2 mL. Initiation of binding was processed with continuous vortexing (2,000 rpm) on a Finemixer Mx 2 (Finepcr, Korea) at 22 °C. After 35 min, all test tubes were centrifuged for 15 min at 9,000 rpm, after which supernatant (about 1.25 mL) was removed and filtered through 0.2 μm Acrodisc LC syringe filters. Portions (950 μL) of the filtrates were dispensed into a cuvette (1 cm path length) containing 50 μL of Ellman reagent (10 mM), after which the absorbance was measured at 412 nm to determine the concentration of bound detector for each experimental portion. The total bound concentration of bound detector was calculated based on the decrease in absorbance after binding process for 35 min. To determine the level of nonspecific binding, α-cells were tested at the same concentrations using the same method. Total counts per minute bound to α-cells were average 10% of total count per minute bound to α-cells.

Competition for Detector Binding by Competitor α-Factor Analogs (Fig. 5 and 6). Competition for bound detector, [Orn^6]α-factor-[Cys]$_3$ **14**, by competitive peptides was measured by the following protocol. In general, *S. cerevisiae* strain 7925 was grown to a concentration of 1×10^7 cells/mL and harvested by centrifugation at 3,000 g for 15 min at 4 °C. Pelleted cells were washed three times in ice-cold saline water and resuspended in saline water to a concentration of 2.5×10^{11} cell/mL. The cell suspension (1 mL) was dispensed into eight test tubes with a volume of 5 mL. Various amounts of competitors [6.0×10^{-3} M, 0.5 μL (2.95×10^{-9} mol/1.31 mL, 2.25×10^{-6} M)-40 μL (2.36×10^{-7} mol/1.31 mL, 1.8×10^{-4} M)] and 2.2 μL (6.0×10^{-3} M) of detectors (1.17×10^{-8} mol/1.31 mL, 8.96×10^{-6} M) were added to each tube, after which total volumes were adjusted to a final volume of 2 mL using saline water. The binding reaction was processed by continuous vortexing (2,000 rpm) on a Finemixer Mx 2 (Finepcr, Korea) at 22 °C. After 35

min, all test tubes were centrifuged for 15 min at 9,000 rpm, after which supernatant (about 1.25 mL) was removed and filtered through 0.2 μm Acrodisc LC syringe filters. Portions (950 μL) of the filtrates were dispensed into a cuvette (1 cm path length) containing 50 μL of Ellman reagent (10 mM), after which absorbance was measured at 412 nm to determine the concentration of bound detector for each experimental portion. Results for each analog were expressed as a percentage of total binding in the absence of competitor. Each binding assay was carried out at least three times, and virtually identical curves were obtained. IC_{50} values were determined based on the concentration of competitor that inhibits detector binding by 50%. Values were calculated as the 50% point on the regression line incorporating the appropriate competition curve from Figures 5 and 6. The K_i values were calculated by dividing the experimentally determined concentration providing 50% binding displacement by $[1+ H_T/K_D]$, where H_T = concentration of detector and K_D = dissociation constant of detector.[58]

Results

Synthesis and Characterization of Peptide Analogs. Native α-factor is a tridecapeptide with the following primary structure: NH_2-Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr-COOH. Analogs of this peptide are designated according to IUPAPC convention.[26] Thus, if Orn is substituted for Leu at position 6, the analogs become designated [Orn^6]α-factor. α-Factor-[Trp]$_n$ represents *C*-terminal extended analogs. The subscript n denotes the number of Trp residues. For example, α-factor-[Trp]$_1$ refers to a *C*-terminal extended analog with one Trp while α-factor-[Trp]$_2$ represents two Trp residues at the *C*-terminus. In the same manner, [*N*-Trp]$_1$-α-factor refers to an *N*-terminal extended analog with one Trp.

All of target analogs investigated in this paper were efficiently synthesized on trityl resin using a solid phase approach. In general, the crude products of TFA cleavage obtained from the resin contained approximately 85 to 95% of the major components, as indicated by analytical reversed-phase HPLC. Preparative HPLC purification yielded peptides with a homogeneity greater than 98%, as determined by analytical HPLC using an acetonitrile/water/TFA gradient system. Molecular masses of detector peptide, [Orn^6]α-factor-[Cys]$_3$ **14**, assessed by LC/MS were consistent with expected values within 1 Da (Fig. 1). Retention times and molecular weights of all final analogs are presented in Table 1.

Morphogenesis Activity of Peptide Analogs (5% Shmoo Assay). The most widely reported activity of α-factor is its effects on the shape and budding of *S. cerevisiae* 7925 *MATa* cells. Incubation of all α-factor analogs with *MATa* cells resulted in formation of aberrant cell morphologies (shmoo shape) as well as cessation of budding. Concentrations of synthetic peptides that induced 5% aberrant morphogenesis ranged from 0.5 μL/100 μL to 15 μL/100 μL (5 μg-150 μg/100 μL). All *C*-terminal extended α-factor analogs with Trp **1-5** showed lower activities than native α-factor. α-Factor-[Trp]$_5$ **5** was the least potent analog by a factor of five in terms of induction of 5% aberrant morphogenesis as com-

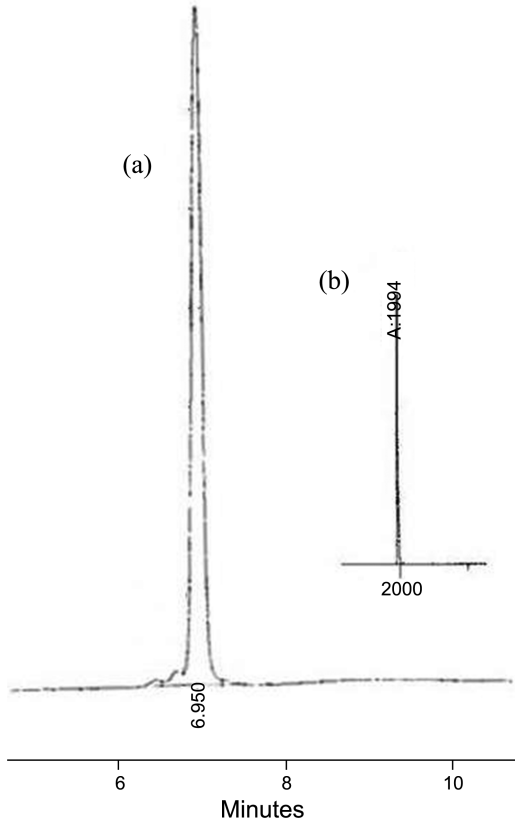

Figure 1. HPLC and mass spectroscopy analyses of synthetic native α-factor **13**. (a) HPLC peak of purified α-factor on a reversed-phase C_{18} column eluted with $CH_3CN/H_2O/CF_3COOH$ (20 to 40% CH_3CN, 0.025% CF_3COOH). Peak area was 99%. (b) The observed mass of the peptide as determined by LC/MS (Agilent HP1100 series) was good agreement with mass expected by the mass calculation; expected α-factor, 1682.84 Da.

Table 1. Analytical properties of synthetic α-factor analogs

No	Peptide	Retention times[a] (min)	Molecular weights	
			Expected	Found
1	α-factor-[Trp]$_1$	6.98	1868.92	1870
2	α-factor-[Trp]$_2$	7.51	2055.00	2056
3	α-factor-[Trp]$_3$	7.98	2241.08	2242
4	α-factor-[Trp]$_4$	8.32	2427.16	2428
5	α-factor-[Trp]$_5$	8.77	2613.24	2614
6	[*N*-Trp]$_1$-α-factor	7.04	1868.93	1870
7	[Trp^{13}]α-factor	6.96	1705.85	1706
8	[Aib^8]α-factor	6.43	1670.84	1671
9	[Aib^{11}]α-factor	6.42	1670.82	1671
10	[Orn^6]α-factor	6.15	1683.89	1684
11	[Orn^6,D-Ala^9]α-factor	6.99	1697.91	1699
12	[Glu^6,D-Ala^9]α-factor	6.89	1712.81	1714
13	native α-factor	6.20	1682.81	1684
14	[Orn^6]α-factor-[Cys]$_3$	6.95	1992.92	1994

[a]The product was dissolved in 50% trifluoroacetic acid and eluted with a linear gradient of CH_3CN/water containing 0.1% trifluoroacetic acid and 10 to 75% CH_3CN over 90 min with a flow rate of 1.5 mL/min.

pared to native α-factor. Gradual reduction of activity occurred as Trp residues were extended at the *C*-terminus; 2.0-fold decrease for α-factor-[Trp]$_1$ **1**, 3.0-fold decrease for α-factor-[Trp]$_2$ **2**, 4.0-fold decrease for α-factor-[Trp]$_3$ **3**, 5.0-fold decrease for α-factor-[Trp]$_4$ **4**, and 5.2-fold decrease for α-factor-[Trp]$_5$ **5** in the amount of analog required to cause 5% aberrant morphogenesis as compared to native α-factor. *N*-Terminal extended analog with Trp, [*N*-Trp]$_1$-α-factor **6**, showed a 2-fold decrease in activity as compared to native α-factor. Similar trends were found for *C*- and *N*- terminal extended analogs using two strains, *S. cerevisiae* 7925 and 7951. Strain 7951 showed slightly higher sensitivity than strain 7925 in the assay.[60] [Trp^{13}]α-factor **7** showed 57% activity compared to native α-factor. On the other hand, analogs substituted with unnatural amino acids in internal regions, [Aib^8]α-factor **8** and [Aib^{11}]α-factor **9**, showed 60 to 66% activity compared to native α-factor. Analog with Leu replaced by Orn at position 6, [Orn^6]α-factor **10**, resulted in a 2-fold increase in activity. For two double-substituted analogs, [Glu^6,D-Ala^9]α-factor **12** exhibited a 2.2-fold increase in activity while [Orn^6,D-Ala^9]α-factor **11** showed a 3.3-fold increase in activity compared to native α-factor based on the amount of pheromone causing 5% aberrant morphogenesis (20 shmoos/400 cells).

Growth Arrest (Halo) Assay. α-Factor is known to arrest growth of *MATa* cells. Therefore, halo assay provides an easy and direct measure of pheromone activity. α-Factor and its analogs arrested growth of cells, and bioactive analogs presented clear zones around the disk (Fig. 2(a) and (b)). Increasing amounts of pheromone promoted growth arrest, as measured by halo size. These data were plotted in a graph as halo size versus amount of pheromone added to cell lawn, and then linearized by regression analysis (Fig. 3). To compare relative activities of the different analogs, the amount of peptide causing a halo size of 15 mm was determined from the regression line. Relative halo activities of the analogs against strain 7925 are presented in Table 2.

All *C*- and *N*-terminal extended α-factor analogs with Trp showed lower activities compared to native α-factor. Further, *C*-terminal extended analogs showed similar activities as observed in the halo assay. None of the analogs except for α-factor-[Trp]$_1$ **1** showed clear zones up to a volume of 10 μL, whereas α-factor-[Trp]$_1$ **1** showed weak activity (12 mm zone/10 μL). Interestingly, α-factor-[Trp]$_2$ **2** presented a blurry zone after 24 h (11 mm/10 μL) that soon disappeared after 48 h. *N*-Terminal extended analog with one Trp, [*N*-Trp]$_1$-α-factor **6**, presented 3-fold higher halo activity than its *C*-terminal elongated counterpart, α-factor-[Trp]$_1$ **1**. [Trp^{13}]α-factor **7** showed a 0.5-fold decrease in activity as compared to native α-factor. For the series of analogs substituted with unnatural amino acids in the internal region ([Aib^8]α-factor **8**, [Aib^{11}]α-factor **9**, and [Orn^6]α-factor **10**), Aib alterations resulted in 5 to 10-fold decrease in activity compared to native α-factor, and alteration at position 8 caused an additional drop in activity. Substitution of Leu, a hydrophobic amino acid, by positively charged Orn at position 6 resulted in analog **10**, which showed 7.0-fold

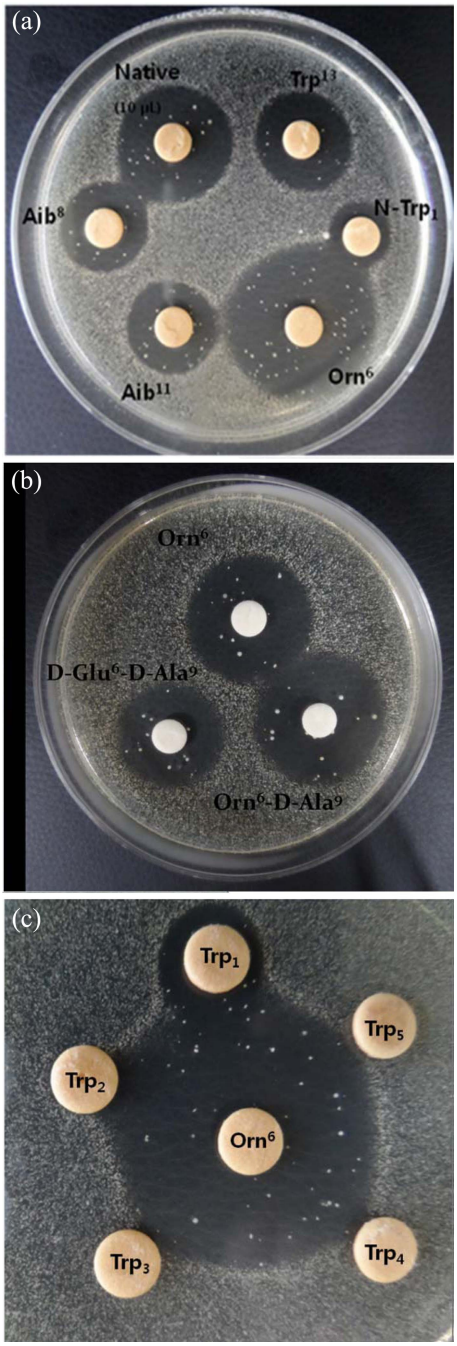

Figure 2. Halo assays. (a) Results of halo assay for α-factor analogs. Synthetic analogs were placed on a disk, which was placed at the center of a lawn of strain *S. cerevisiae* 7925. Growth arrest was indicated by a halo. Analogs (native = native α-factor **13**, Trp^{13} = [Trp^{13}]α-factor **7**, N-Trp$_1$ = [*N*-Trp]$_1$-α-factor **6**, Orn^6 = [Orn^6]α-factor **10**, Aib^{11} = [Aib^{11}]α-factor **9**, Aib^8 = [Aib^8]α-factor **8**, peptide amount = 10 mL (100 mg/disk) were placed on disks at the center of growth arrest. (b) Analogs (Orn^6 = [Orn^6]α-factor **10**, Orn^6,D-Ala^9 = [Orn^6,D-Ala^9]α-factor **11**, and Glu^6,D-Ala^9 = [Glu^6,D-Ala^9]α-factor **12**, peptide amount = 1 mL/10 mL (10 mg/10 mL of saline water). (c) Halo assays demonstrating synergistic activity of biologically inactive Trp extended analogs (panel **1**-**5**). Lawn of strain 7925 was challenged with 10 mL (100 mg/10 mL) of [Orn^6]α-factor **10** and 15 mL (150 mg/15 mL) of weak active or inactive analogs, α-factor-[Trp]$_1$ **1**-α-factor-[Trp]$_5$ **5**. Analogs demonstrated synergism as represented by a broaden neck at the proximal surfaces of the halos.

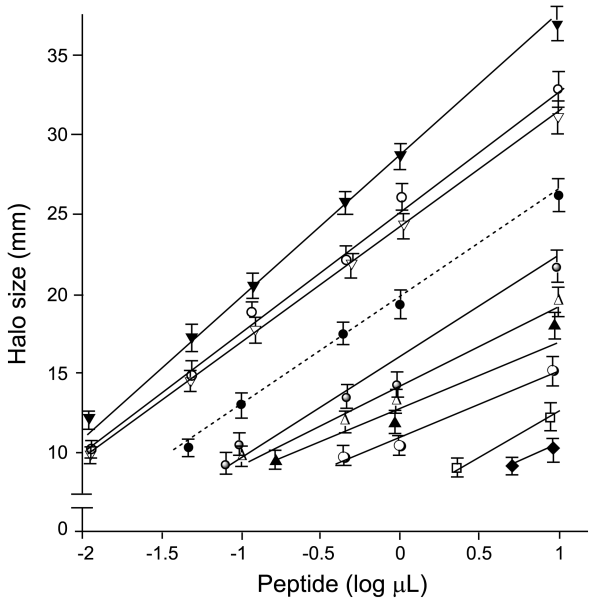

Figure 3. Bioactivity of α-factor analogs by halo assay in *S. cerevisiae* 7925. [Orn^6,D-Ala^9] α-factor (▼), [Orn^6]α-factor (○), [Glu^6,D-Ala^9]α-factor (▽), native α-factor (●), [Trp^{13}]α-factor (◒), [Aib^{11}]α-factor (△), [Aib^8]α-factor (▲), [*N*-Trp]1α-factor (◔), α-factor-[Trp]$_1$ (□), α-factor-[Trp]$_2$ (◆). No activity; α-factor-[Trp]$_3$, α-factor-[Trp]$_4$, α-factor-[Trp]$_5$.

higher activity in comparison with native α-factor in the halo assay. For two double substituted analogs, [Orn^6,D-Ala^9]α-factor **11** showed a 15-fold increase in activity while [Glu^6,D-Ala^9]α-factor **12** exhibited a 6-fold increase in activity compared to native α-factor based on a halo size of 15 mm.

Synergistic Effect of *C*-terminal Extended Analogs with Trp. All five *C*-terminal extended Trp analogs (**1-5**) were tested for antagonism or synergism (Fig. 2(c)). Three of the analogs, α-factor-[Trp]$_3$ **3**, α-factor-[Trp]$_4$ **4**, and α-factor-[Trp]$_5$ **5**, were virtually devoid of activity while the remaining two analogs, α-factor-[Trp]$_1$ **1** and α-factor-[Trp]$_2$ **2**, were weakly active in the halo assay against strain 7925. Therefore, we tested their synergistic effects to determine whether or not the three inactive analogs (**3-5**) have intrinsic halo activities. All five analogs (**1-5**) were placed on a lawn of strain 7925 in close proximity to [Orn^6]α-factor **10**, and all changes in the shape of the standard halo was noted. An antagonistic response, as represented by a flattened halo, was not detected. Instead, elongated halos of [Orn^6]α-factor **10** toward the test analog-containing discs were observed for all *C*-terminal extended analogs, as evidenced by a broadened neck at the proximal surface. This indicates that test analogs (**3-5**) induced a synergistic effect or very weak intrinsic activity. The size of broadened neck indicated the relative potency of the competing analogs. Based on the shape of the halo, activities gradually decreased as the number of Trp residues increased.

Steady-state Saturation Binding with Detector, [Orn^6]α-factor-[Cys]$_3$ 14. For steady-state saturation binding, the same procedure was performed as previously described,[8] except that the *S. cerevisiae* strain used in the assay was 7925. Since a given number of cells are expected to possess a finite number of receptors, saturation was assessed by examining binding as a function of increasing detector concentration using 2.5×10^{11} cells. A Scatchard plot was derived from the saturation binding data (Fig. 4).[8,59] The straight line of the Scatchard plot indicates that the interaction of detector

Table 2. Biological activity of α-factor analogs in *S. cerevisiae* 7925

No	Peptide	Shmoo[a] (μL/100 μL)	Relative activity	Halo[b] (μL/15 mm)	Relative activity
1	α-factor-[Trp]$_1$	4.0 ± 0.2	50	32 ± 2	1
2	α-factor-[Trp]$_2$	6.0 ± 0.2	33	90 ± 5	0.4
3	α-factor-[Trp]$_3$	7.5 ± 0.2	27	NA	NAc
4	α-factor-[Trp]$_4$	9.5 ± 0.2	21	NA	NA
5	α-factor-[Trp]$_5$	10.5 ± 0.2	19	NA	NA
6	[*N*-Trp]$_1$-α-factor	4.0 ± 0.2	50	9.5 ± 1.0	3
7	[Trp^{13}]α-factor	3.0 ± 0.2	57	0.70 ± 0.05	50
8	[Aib^8]α-factor	3.5 ± 0.2	60	3.5 ± 0.3	10
9	[Aib^{11}]α-factor	3.0 ± 0.2	66	1.6 ± 0.1	22
10	[Orn^6]α-factor	1.0 ± 0.1	200	0.052 ± 0.005	700
11	[Orn^6,D-Ala^9]α-factor	0.6 ± 0.1	330	0.024 ± 0.002	1,500
12	[Glu^6,D-Ala^9]α-factor	0.9 ± 0.1	220	0.060 ± 0.005	600
13	native α-factor	2.0 ± 0.2	100	0.36 ± 0.03	100

[a]Cell morphogenesis was determined by the formation of shmoo-shaped cells in response to peptide. Shmoo cells were counted separately at three differnet areas containing 400 total cells. The percentage of shmoos was calculated by dividing total shmooing cells by total cells. To compare the relative activities of different analogs, the amount of analog causing 5% shmoo (20 shmoos/400 total cells) was determind from the regression line. [b]Halo assay as measured by the growth arrest. For each peptide (6×10^{-3} M), 0.01 μL (0.1 μg)-10 μL (100 μg) were spotted on discs which were placed upon YEPD plates containing *S. cerevisiae* 7925 (1.2×10^6 cells). At 24 h, the zone of growth inhibition was determined by the halo diameter in centimeters. The size of the halo produced was plotted vs the amount of peptide tested (Fig. 3). The amount of the peptide required to produce a 15 mm halo was determined from the first order regression line equation and used to compare the activities of the analogs relative to the native α-factor (0.36 mL/15 mm). [c]NA = no activity

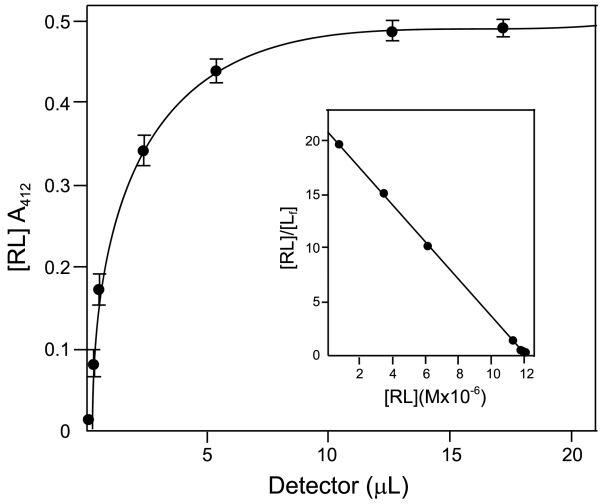

Figure 4. Saturation binding of detector, [Orn^6]α-factor-[Cys]$_3$, to *S. cerevisiae* 7925. The inset represents a Scatchard plot of the binding data; K_D = 4.96 × 10^{-7} M. For experimental details, see "Experimental procedure". Detector (4.96 × 10^{-3} M) was incubated for 35 min with strain 7925 (●) at the cell concentration of 2.5 × 10^{11} cells/mL. [RL] = bound detector, [L_f] = unbound free detector.

14 with its receptor can be characterized by one equilibrium dissociation constant (K_D). The slope of the line yielded a K_D of 4.96 × 10^{-7} M. Furthermore, the x intercept revealed that a-cells contained approximately 29,500 binding sites per cell.

Competition for Detector Binding by Achromic Competitors. Binding affinity of the achromic synthetic analogs to its cognate receptor in *S. cerevisiae MATa* cells was determined by measuring competition between increasing concentrations of synthetic competitors and a constant concentration of detector, [Orn^6]α-factor-[Cys]$_3$ **14**, using a spectrophotometer. The affinities of these competitors for their receptor are represented by IC_{50} values, which indicate 50% of the specific ligand binding. Binding curves of representative analogs are shown in Figures 5 and 6, whereas their IC_{50} values were given in Table 3. The extension with Trp at *C*- or *N*-terminal resulted in a decrease in affinity for the receptor. Similarly, *C*-terminal extended analogs showed reduced receptor affinity as that observed in the halo assay. Both growth arrest activity and receptor affinity decreased gradually as the number of Trp residues increased. *C*- or *N*-terminal extended analogs with only one Trp residue (**1**,**6**) showed similar receptor affinity (approximately 30% reduction), which was quite different from that observed in the 5% halo assay. Analogs with Aib substituted at positions 8 or 11, [Aib^8]α-factor **8**, [Aib^{11}]α-factor **9**, showed a 5- to 10-fold decrease in receptor affinity compared to native α-factor, although alteration at position 8 caused an additional drop in receptor affinity as that observed in the halo assay. Replacement of Tyr with Trp at position 13, [Trp^{13}]α-factor **7**, led to 2-fold reduction in receptor affinity. Replacement with Orn, [Orn^6]α-factor **10**, showed significantly increased receptor affinity, as expected from the halo assay result, and this analog showed 80% higher binding activity than native

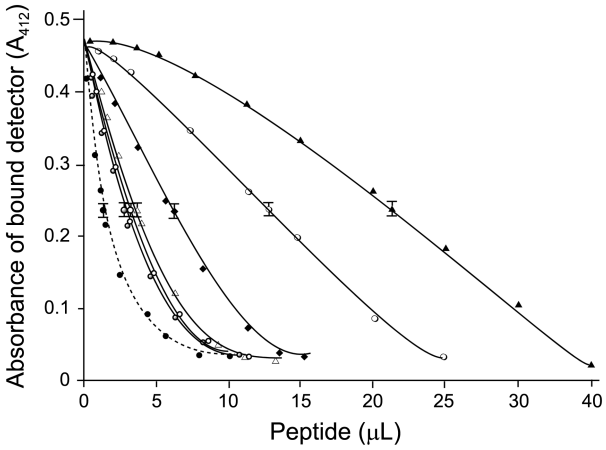

Figure 5. Competition for binding of detector, [Orn^6]α-factor-[Cys]$_3$, by synthetic α-factor analogs. α-Factor and synthetic analogs binding to *S. cerevisiae* 7925 cells were performed in competition with detector as described under "Experimental procedure". The symbols represent competition of [Orn^6]α-factor-[Cys]$_3$ binding by native α-factor (●), [*N*-Trp]$_1$α-factor (◒), α-factor-[Trp]$_1$ (○), α-factor-[Trp]$_2$ (△), α-factor-[Trp]$_3$ (◆), α-factor-[Trp]$_4$ (❍), α-factor-[Trp]$_5$ (▲).

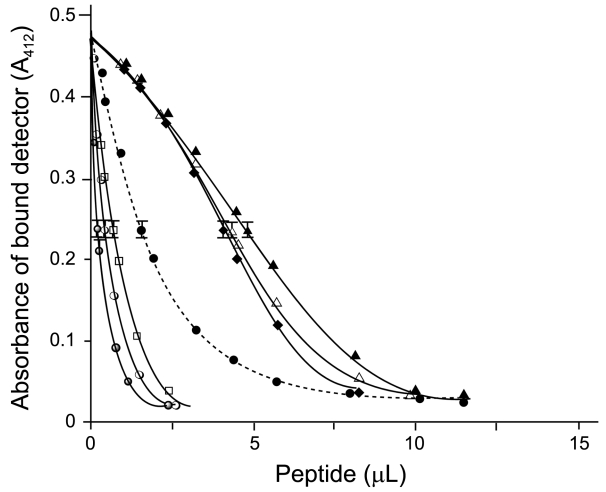

Figure 6. Competition for binding of detector, [Orn^6]α-factor-[Cys]$_3$, by synthetic α-factor analogs. α-Factor and synthetic analogs binding to *S. cerevisiae* 7925 cells were performed in competition with detector as described under "Experimental procedure". The symbols represent competition of [Orn^6]α-factor-[Cys]$_3$ binding by [Orn^6,D-Ala^9] α-factor (◒), [Glu^6,D-Ala^9] α-factor (❍), [Orn^6]α-factor (□), native α-factor (●), [Trp^{13}]α-factor (◆), [Aib^{11}]α-factor (△), [Aib^8]α-factor (▲).

α-factor. For two double-substituted analogs with oppositely charged residue at position 6, [Orn^6,D-Ala^9]α-factor **11** showed a nearly 3-fold increase in receptor affinity, whereas [Glu^6,D-Ala^9]α-factor **12** showed only a 2.2-fold increase in receptor affinity compared to native α-factor based on *Ki* value.

Discussion

Membrane phospholipids play an important role in the

Table 3. Concentration for 50% inhibition of binding (IC_{50}) and binding dissociation constants (K_i) for α-factor and its analogs in *S. cerevisiae* 7925

No	Peptide	IC_{50}[a] (M)	K_i[b] (M)	Relative affinity
1	α-factor-$[Trp]_1$	$(9.53 \pm 0.95) \times 10^{-6}$	$(4.74 \pm 0.47) \times 10^{-7}$	68
2	α-factor-$[Trp]_2$	$(1.03 \pm 0.10) \times 10^{-5}$	$(5.12 \pm 0.51) \times 10^{-7}$	63
3	α-factor-$[Trp]_3$	$(1.96 \pm 0.19) \times 10^{-5}$	$(9.75 \pm 0.97) \times 10^{-7}$	33
4	α-factor-$[Trp]_4$	$(3.51 \pm 0.35) \times 10^{-5}$	$(1.75 \pm 0.17) \times 10^{-6}$	19
5	α-factor-$[Trp]_5$	$(5.25 \pm 0.52) \times 10^{-5}$	$(2.61 \pm 0.26) \times 10^{-6}$	12
6	$[N\text{-}Trp]_1$-α-factor	$(9.15 \pm 0.91) \times 10^{-6}$	$(4.55 \pm 0.45) \times 10^{-7}$	70
7	$[Trp^{13}]$α-factor	$(1.59 \pm 0.16) \times 10^{-5}$	$(7.91 \pm 0.79) \times 10^{-7}$	40
8	$[Aib^{8}]$α-factor	$(2.59 \pm 0.26) \times 10^{-5}$	$(1.29 \pm 0.13) \times 10^{-6}$	25
9	$[Aib^{11}]$α-factor	$(1.83 \pm 0.18) \times 10^{-5}$	$(0.91 \pm 0.09) \times 10^{-6}$	36
10	$[Orn^{6}]$α-factor	$(3.57 \pm 0.35) \times 10^{-6}$	$(1.78 \pm 0.17) \times 10^{-7}$	180
11	$[Orn^{6}$,D-$Ala^{9}]$α-factor	$(2.19 \pm 0.22) \times 10^{-6}$	$(1.09 \pm 0.11) \times 10^{-7}$	290
12	$[Glu^{6}$,D-$Ala^{9}]$α-factor	$(2.94 \pm 0.30) \times 10^{-6}$	$(1.46 \pm 0.14) \times 10^{-7}$	220
13	native α-factor[c]	$(6.47 \pm 0.64) \times 10^{-6}$	$(3.22 \pm 0.32) \times 10^{-7}$	100
	native α-factor[d]	–	3×10^{-7}	–
	native α-factor[e]	$(1.4 \pm 0.14) \times 10^{-8}$	1.3×10^{-8}	–
14	$[Orn^{6}]$α-factor-$[Cys]_3$	–	$(4.96 \pm 0.50) \times 10^{-7}$	65

[a]IC_{50} = the concentration of competitor which inhibits binding of by 50%. Values were calculated as the 50% point on a regression line incorporating the points of the appropriate competition curve from Figure 4-5. [b]$K_D = K_i$ calculated according to Linden.[58] [c]This is our value determined with *S. cerevisiae* 7925. [d]This is the value obtained by Scatchard analysis with *S. cerevisiae* 4202-15-3.[59] [e]This is the value obtained by competition study with *S. cerevisiae* 4202-15-3.[8] [f]K_D as determined form Scatchard analysis of the $[Orn^{6}]$α-factor-$[Cys]_3$ binding isotherm.

receptor recognition process by peptide pheromones. It has been reported that the physiological activity of α-factor pheromone is closely correlated with affinity for the phospholipid membrane.[36-38] Since the target of α-factor is a membrane-associated protein, Ste2p receptor, a certain type of hydrophobic moiety attached to α-factor could aid pheromone localization to the target cell membrane, as do many other hormone such as Ras protein,[29,36-38] thereby leading to increased probability of binding. However, integration of a pheromone into the cellular lipid bilayer depends on the type of modification applied.[21,36-38] Alanine scanning study previously examined the roles of *C*- and *N*-terminal residues of α-factor in pheromone activity and receptor binding affinity.[10,39] According to the study, *C*-terminal residues contribute strongly to binding to Ste2p receptor as well as G protein-coupled receptor (GPCR) activation.[40,41] On the other hand, those near the *N*-terminus of α-factor are responsible for triggering cell signaling through Ste2p or stabilizing the activated state of this receptor.[4,10,30,40,57] The two *N*-terminal Trp residues at position 1 and 3 are the key residues in the interaction between α-factor and the region of the receptor that promotes stimulation of the pheromone-responsive G protein pathway.[30,40,41] In addition, two *N*-terminal Trp residues preferentially insert into the cell membranes following interaction of α-factor with the phospholipid membrane.[13,31] Based on the biological activity of lipopeptide conjugates and the combined role of the two Trp residues,[57] *C*-terminal extended analogs of α-factor with various number of Trp residues can be used to study highly potent α-factor analogs.

C-terminal extended synthetic α-factor analogs **1-5** were all biologically active when tested against *S. cerevisiae* 7925. The relative potencies of *C*-terminal extended analogs **1-5** were all less active than that of native α-factor in both the shmoo and halo assays employing *S. cerevisiae* 7925. Their relative receptor affinities were also lower than that of native α-factor. Further, their relative potencies and affinities were clearly dependent on the number of Trp residues. The relative affinity and potency of the *C*-terminal extended analogs gradually decreased as number of Trp residues increased. Analog with one or two Trp residues (**1**,**2**,**6**) showed a similarly modest reduction in affinity (33% reduction) with weak bioactivity. In contrast, analogs with three to five Trp residues (**3-5**) showed negligible affinity values with no bioactivity. A similar result was reported with single *N*-terminal extension of Tyr.[27] The activity profiles of the elongated analogs suggest that α-factor receptor probably has a restricted binding site that can accommodate up to two additional residues at the *C*-terminus of tridecapeptide α-factor. In addition, the observation that *N*-terminal extended analog **6** showed almost three times higher activity compared to *C*-terminal extended analog **1** indicates that the *N*-terminal region plays a more important role in signal transduction, thus supporting the previous finding that the *N*-terminal region of α-factor is the major determinant of signal transduction.[4,10,42]

Based on our results, we cannot conclude whether the effects of Trp residue extension are due to increased lipid membrane affinity and/or enhanced receptor stimulation. A previous report found that dodecapeptide α-factor extended at the *C*-terminus with non-amino acids such as polyethylene glycol (PEG) is neither active in morphogenesis assay nor binding assay.[4,49] Therefore, the fairly high affinity of *C*-terminal extended analogs **1-6** ranging from 10% to 70%

compared to that of native pheromone indicated that Trp extension has certain additional effects upon receptor binding. These effects include penetration of the ligand toward receptor site 1, which is the hydrophobic *C*-terminal binding pocket surrounded by transmembrane domain helix bundles. However, the observed negligible halo activity of less than 1% clearly indicates that the potentially favorable membrane interaction could be dominated by potentially poorer receptor binding caused by additional Trp residues.

Our observation that activity gradually decreased in proportion to the increase in Trp residues does not correlate with our expectation of either more facile intercalation of Trp into the lipid bilayer or an active role for Trp in pheromone Ste2p receptor binding. The *C*-terminal residues of α-factor are responsible for the transition from an inactive to active state of the receptor form.[30] Therefore, the ratio of biological activity to receptor affinity (the dramatic decrease in halo activity versus modest reduction in receptor affinity, Tables 1 and 2 suggests that Trp extension may play negative regulatory role in receptor isomerization. This results in the relatively high population of the inactive state leading to a remarkable change in halo activity.

A previous conformational study on ligand in the receptor-bound state strongly suggested that a type II β-turn involving residues Lys^7-Gln^{10} in the center of α-factor is essential for receptor binding and agonist efficacy.[13,18,19,30,32] A study concerning cyclic α-factor analog mimicking β-turn structure in the center of α-factor provided additional proof that the constrained structure of the center of α-factor is deeply correlated with the potency of active analog.[18-20,30,32,43] These results show that the central β-turn near the receptor in the lipid membrane promotes interaction of the ligand with two sites in the receptor implicated in ligand binding (*C*-terminal region) and triggering of G protein signal transduction (N-terminal region).[4,30] Aib is sterically hindered by dialkylated side chains and thus its substitution generates a collapsed turn structure effectively.[34] Substituting Pro with Aib at position 8 and 11 causes 90% and 80% decreases in halo activity, respectively, as well as a 40% decrease in 5% shmoo assay compared to the native α-factor. Therefore, the dramatic reduction in activity strongly emphasizes critical role of the β-turn structures for pheromone activity.

Des-Tyr^{13}-α-factor, the deleted analog of Tyr at position 13, is 6-fold less active than native α-factor in shmoo assay (data not shown).[60] This supports an important role for Tyr^{13} in interaction with its receptor[4,30] and the lipid membrane.[13,31] According to previous studies,[13,31] during ligand-receptor recognition, Tyr directly binds to lipids and facilitates the active conformation of the ligand (bent structure). This plays a critical role in the high potency of α-factor. Only a 50% reduction in halo activity was observed upon Trp replacement at position 13, [Trp^{13}]α-factor **7**, whereas 60% reduction in affinity occurred. This was surprisingly modest, as more than 90% reduction in halo activity was observed for other analogs (**1**,**2**,**6**), along with 30% reductions in affinity. This outcome clearly emphasizes more dominant role for the aromatic ring of Tyr versus the hydroxyl group in triggering isomerization of the receptor to an active state, resulting in high bioactivity. However, these results indicate that the hydroxyl group of Tyr at position 13 is necessary for efficient receptor binding up to 60% affinity, supporting the notion that the phenolic hydroxyl group of Tyr^{13} of α-factor forms a hydrogen bond with the thiol group of Cys^{59} of TM1 near the extracellular face of the receptor pocket. This is necessary for the subsequent stabilization of cation-π interaction between the Arg^{58} guanidinium moiety in TM1 and the phenyl ring of Tyr^{13} of ligand.[45]

There are two cationic residues at positions 2 and 7, as well as six hydrophobic residues at position 1, 3, 4, 6, 12, and 13. Therefore, we examined the effects of ionic charge on biological activity. As seen in Tables 1 and 2, alteration of hydrophobic residue Leu^6 to the charged residues Orn and Glu (analogs **10**-**12**) increased in halo activity with high affinity compared to native α-factor **13**. To investigate whether the improved activities of the analogs (**10**-**12**) are attributed to an increased proteolytic stability, we carried out the time course for saturation binding study with detector, [Orn^6]α-factor-$[Cys]_3$ **14**. The saturation of receptor was completed by 30 min, after which the straight line of absorbance was remained for 2 h. This indicates no hydrolysis of pheromone analogs by membrane associated peptidase. We investigated whether the dramatic changes in activity and affinity in response to charged residue substitution are due to the resulting pheromone-receptor complex or membrane-interaction. [Glu^6,D-Ala^9]α-factor **12** showed the significant reductions in halo assay compared to [Orn^6,D-Ala^9]α-factor **11**. This supports the specific pheromone interaction with certain receptor site rather than a facilitated access to the receptor assisted by more favorable membrane interaction due to the additional charge.

We thoroughly examined synthetic analogs reported over the last 30 years since native α-factor was first isolated.[5] We found that most synthetic analogs reported to date are far less active than native α-factor, and only a few are about as active as α-factor.[4,42,43] As an explanation for this, previous review on α-factor pheromone described that α-factor and its receptor have evolved to have optimal complementarity.[4] To our knowledge, [D-Ala^9,Nle^{12}]α-factor is the most active analog with about 5-fold higher activity in halo assay,[10,13] compared with its parent analog, [Nle^{12}]α-factor. However, D-Ala incorporated analog of native α-factor at the same position, [D-Ala^9]α-factor, was found to have activity comparable to that of native α-factor.[13] This suggests that the SAR and activity of [Nle^{12}]α-factor are not similar to those of native α-factor.[6,27] Therefore, we reported activity values of all analogs as determined by comparison of their activities with that of native α-factor.

[Orn^6]α-factor **10** exhibited a 7.0-fold increase in halo activity and a 1.8-fold increase in receptor affinity as compared with those of native tridecapeptide α-factor. Accordingly, we synthesized two additional double substituted analogs, [Orn^6,D-Ala^9]α-factor **11** and [Glu^6,D-Ala^9]α-factor **12**, and measured their bioactivity as well as receptor affinity. Negatively charged analog **12** exhibited slightly

decreased potencies in activity assay compared to analog **10**, whereas positively charged analog **11** showed a nearly 3-fold higher affinity as well as 15-fold increased activity in halo assay compared to those of native α-factor **13**. Based on these results, further studies on analogs **11**, **12** are currently underway. We expect that these analogs may serve as important model compounds for future investigations into the molecular basis of pheromone-GPCR interactions.

References

1. Dohlamn, H. G. *Annu. Rev. Physiol.* **2002**, *64*, 129.
2. Schoneberg, T.; Schulz, A.; Biebermann, H.; Hermsdorf, T.; Rompler, H.; Sangkuhl, K. *Pharmaco. Ther.* **2004**, *104*, 173.
3. Klabunde, T.; Hessler, G. *Chembiochem*. **2002**, *3*, 928.
4. Naider, F.; Becker, J. M. *Peptides* **2004**, *25*, 1441.
5. Stotzler, D.; Duntze, W. *Eur. J. Biochem*. **1976**, *65*, 257.
6. Masui,Y.; Chino, N.; Sakakibara, S.; Tanaka, T.; Murakami, T.; Kita, H. *Biochem. Biophys. Res. Commun.* **1977**, *78*, 534.
7. Hong, N. J.; Park, Y. A. *Peptide Science Present and Future, Proceedings of the First International Peptide Symposium*; Shimonishi, Y., Ed.; Kluwer Academic Publishers: Tokyo, Japan, 1999; p 467.
8. Raths, S. K.; Naider, F.; Becker, J. M. *J. Biol. Chem*. **1988**, *263*, 17333.
9. Ciejek, E.; Thorner, J.; Geler, M. *Biochem. Biophys. Res. Commun.* **1977**, *78*, 952.
10. Abel, M. G.; Zhang, Y, L.; Lu, H. F.; Naider, F.; Becker, J. M. *J. Pept. Res*. **1998**, *52*, 95.
11. Ciejek, E.; Thorner, J. *Cell* **1979**, *18*, 623.
12. Chan, R. K.; Otte, C. A. *Mol. Cell. Biol*. **1982**, *2*, 11.
13. Gounarides, J. S.; Broido, M. S.; Becker, J. M.; Naider, F. *Biochemistry* **1993**, *32*, 908.
14. Jelicks, L. A.; Naider, F.; Shenbagamurthi, P.; Becker, J. M.; Broido, M. S. *Biopolymer* **1988**, *27*, 431.
15. Xue, C. B.; Eriotou-Bargiota, E.; Miller, D.; Becker, J. M.; Naider, F. *J. Biol. Chem*. **1989**, *264*, 19161.
16. Yang, W.; Mckinney, A.; Becker, J. M.; Naider, F. *Biochemistry* **1995**, *34*, 1308.
17. Antohi, O.; Marepalli, H. R.; Yang, W.; Becker, J. M.; Naider, F. *Biopolymers* **1998**, *45*, 21.
18. Albercio, F.; Hammer, R. P.; Garcia-Echeverria, C.; Molins, M. A.; Chang, J. L.; Munson, M. C. *Int. J. Pept. Prot. Res*. **1991**, *37*, 402.
19. Xue, C. B.; Mckinney, A.; Lu, H. F.; Jiang, Y.; Becker, J. M.; Naider, F. *Int. J. Pept. Prot. Res*. **1996**, *47*, 131.
20. Zhang, Y. L.; Marepalli, H. R.; Lu, H. F.; Becker, J. M.; Naider, F. *Biochemistry* **1998**, *37*, 12465.
21. Hong, N. J.; Jin, D. H.; Hong, E. Y. *Bull. Korean Chem. Soc*. **2009**, *30*, 599.
22. Bernhart, A.; Drewello, M.; Schutkowski, M. *J. Peptide Res*. **1997**, *50*(2), 143.
23. Hong, N. J. *Bull. Korean Chem. Soc*. **2010**, *31*, 874.
24. Kim, D. H.; Hong, N. J. *Bull. Korean Chem. Soc*. **2012**, *33*, 261.
25. Liu, S.; Henry, K.; Lee, B. K.;Wang, S. H.; Arshava, B.; Becker, J. M.; Naider, F. *J. Pept. Res.* **2000**, *56*, 24.
26. IUPAC-IUB *Joint Commission on Biochemical Nomenclature J. Biol. Chem*. **1985**, *260*, 14.
27. Siegel, E. G.; Gunther, R.; Schafer, H.; Folsch, U. R.; Schmidt, W. E. *Anal. Biochem.* **1999**, *275*, 109.
28. Kim, K. M.; Lee, Y. H.; Naider, F.; Uddin, M. S.; Akal-Strader, A.; Hauser, M.; Becker, J. M. *Pharm. Res*. **2012**, *65*, 31.
29. Goodsell, D. S. *Stem Cells* **2004**, *22*, 119.
30. Mathew, E.; Bajaj, A.; Connelly, S. M.; Sargsyan, H.; Ding, F. X.; Hajduczok, A. J.; Naider, F.; Dumont, M. E. *J. Mol. Biol*. **2011**, *409*, 513.
31. Wakamatsu, K.; Okada, A.; Miyazawa, T.; Masui, Y.; Sakakibara, S.; Higashijima, T. *Eur. J. Biochem*. **1987**, *163*, 331.
32. Higashijima, T.; Masui, Y.; Chino, N.; Sakakibara, S.; Kita, H.; Miyazawa, T. *Eur. J. Biochem*. **1984**, *140*, 163.
33. Dube, P.; DeCostanzo, A.; Konopka, J. B. *J. Biol. Chem*. **2000**, *275*, 26492.
34. Banerjee, R.; Chattopadhyay, S.; Basu, G. *Proteins* **2009**, *76*, 184.
35. Masui, Y.; Tanaka, T.; Chino, N.; Kita, H.; Sakakibara, S. *Biochem. Biophy. Res. Com.* **1979**, *86*, 982.
36. Hruby, V. J. *J. Pept. Res*. **2005**, *66*, 309.
37. Veronese, F. M.; Schiavon, O.; Pasut, G.; Mendichi, R.; Andersson, L.; Tsirk, A.; Ford, J.; Wu, G.; Kneller, S.; Davies, J.; Duncan, R. *Bioconjugate Chem.* **2005**, *16*, 775.
38. Banerjee, S.; Aher, N.; Patil, R.; Khandare, J. *J. Drug Deliv.* **2012**, *2012*, 1.
39. Manfredi, J. P.; Klein, C.; Herrero, J. J.; Byrd, D. R.; Trueheart, J.; Wiesler, W. T.; Fowlkes, D. M.; Broach, J. R. *Mol. Cell. Biol.* **1996**, *16*, 4700.
40. Umanah, G. K.; Huang, L.Y.; Maccarone, J. M.; Nider, F.; Becker, J. M. *Biochemistry* **2011**, *50*, 6841.
41. Kim, K. M.; Lee, Y. H.; Naider, F.; Uddin, M. S.; Akal-Strader, A.; Hauser, M.; Becker, J. M. *Pharm. Res*. **2012**, *65*, 31.
42. Naider, F.; Becker, J. M. *CRC Crit. Rev. Biochem.* **1986**, *21*(3), 225.
43. Garbow, J. R.; Breslav, M.; Antohi, O.; Naider, F. *Biochem.* **1994**, *33*, 10094.
44. Umanah, G. K.; Son, C.; Ding, F. X.; Naider, F.; Becker, J. M. *Biochem*. **2009**, *48*, 2033.
45. Tantry, S.; Ding, F. X.; Dumont, M.; Becker, J. M.; Naider, F. *Biochem.* **2010**, *49*, 5007.
46. Zhang, Y. L.; Lu, H. F.; Becker, J. M.; Naider, F. *J. Pept. Res*. **1997**, *50*, 319.
47. Baffi, R. A.; Becker, J. M.; Lipke, P. N.; Naider, F. *Biochem.* **1985**, *24*, 3332.
48. Khan, S. A.; Merkel, G. J.; Becker, J. M.; Naider, F. *Int. J. Pept. Res*. **1981**, *17*, 219.
49. Shenbagamurthi, P.; Baffi, R. A.; Khan, S. A.; Lipke, P.; Pousman, C.; Becker, J. M.; Naider, F. *Biochem.* **1983**, *22*, 1298.
50. Baffi, R. A.; Shenbagamurthi, P.; terrance, K.; Becker, J. M.; Naider, F.; Lipke, P. N. *J. Bact*. **1984**, *158*, 1152.
51. Henry, L. K.; Khare, S.; Son, C.; Babu, V. V.; Naider, F.; Becker, J. M. *Biochem*. **2002**, *41*, 6128.
52. Dube, P.; DeCostanzo, A.; Konopka, J. B. *J. Biol. Chem*. **2000**, *275*, 26492.
53. Ding, F. X.; Lee, B. K.; Hauser, M.; Davenport, L.; Becker, J. M.; Naider, F. *Biochem.* **2001**, *40*, 1102.
54. Ding, F. X.; Lee, B. K.; Hauser, M.; Patri, R.; Arshava, B.; Becker, J. M. *J. Pept. Res.* **2002**, *60*, 65.
55. Son, C. D.; Sargsyan, H.; Naider, F.; Becker, J. M. *Boichem*. **2004**, *43*, 13193.
56. Son, C. D.; Sargsyan, H.; Hurst, G. B.; Naider, F.; Becker, J. M. *J. Pept.* **2005**, *65*, 418.
57. Hong, E. Y.; Hong, N. J. *Bull. Korean Chem. Soc*. **2013**, *34*, 600.
58. Linden, J. *J. Cyclic Nucleotide Res*. **1982**, *8*, 163.
59. Jenness, D. D.; Burkholder, A. C.; Hartwell, L. H. *Cell* **1983**, *35*, 521.
60. Kim, D. H.; Hong, N. J. *Ann. Meet. Kor. Soc. Biochem. Mol. Biol.* 2012; p 149.