Combined Role of Two Tryptophane Residues of α-Factor Pheromone

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Amide analogs of tridecapeptide α-factor (WHWLQLKPGQPMYCONH$_2$) of *Saccharomyces cerevisiae*, in which Trp at position 1 and 3 were replaced with other residues, were synthesized to ascertain whether cooperative interactions between two Trp residues occurred upon binding with its receptor. Analogs containing Ala or Aib at position 3 of the peptide [Ala^3]α-factor amide (**2**) and [Aib^3]α-factor amide (**5**) exhibited greater decreases in bioactivity than analogs with same residue at position one [Ala^1]α-factor amide (**1**) and [Aib^1]α-factor amide (**4**), reflecting that Trp^3 may plays more important role than Trp^1 for agonist activity. Analogs containing Ala or Aib in both position one and three **3**, **6** exhibited complete loss of bioactivity, emphasizing both the essential role and the combined role of two indole rings for triggering cell signaling. In contrast, double substituted analog with D-Trp in both positions **9** exhibited greater activity than single substituted analog with D-Trp **8** or deleted analog **7**, reflecting the combined contribution of two tryptophane residues of α-factor ligand to activation of Ste2p through interaction with residue Tyr^{266} and importance of the proper parallel orientation of two indole rings for efficient triggering of signal G protein coupled activation. Among ten amide analogs, [Ala^{1,3}]α-factor amide (**3**), [Aib^{1,3}]α-factor amide (**6**), [D-Trp^3]α-factor amide (**8**) and [des-Trp^1,Phe^3]α-factor amide (**10**) were found to have antagonistic activity. Analogs **3** and **6** showed greater antagonistic activity than analogs **8** and **10**.

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

Introduction

The 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) associated with signaling system present in diverse living organism from higher animals to microbes. GPCRs are involved in the control of many aspects of metabolism and divers processes such as pain perception, growth and blood pressure regulation, and viral pathogenesis.[2] The ubiquitous nature of GPCRs together with their highly specific ligand recognition makes them an important target for therapeutic agents. Ste2p has been studied as a model for peptide-response GPCRs, since it has several advantages over other systems. First, the cost of growing yeast in large fermentors is small compared with the costs of other cells maintenance and growth. Second, it is possible to construct, express, and purify mutant versions of receptors easily without the complications of recombinant virus selection.[3]

Previous studies on the structure-activity relationship (SAR) of α-factor provided the understanding on the interaction of this peptide with its GPCR, Ste2p and the biologically active conformation of the tridecapeptide.[4-9,42] The various studies such as SAR, point mutation and photoaffinity labeling focused on the influence of the C- and N-terminal residues of α-factor on pheromone activity, and the receptor binding affinity have provided the more detailed role of the specific site.[10,39,46-50] In summary, residues near the C-terminus contributed strongly with receptor binding to Ste2p and a secondary role in GPCR activation,[40,41] whereas those near the N-terminus of α-factor played an important role in triggering cell signaling through Ste2p or in stabilizing the activated state of this receptor.[4,10,30,40] Two tryptophane residues at position 1 and 3 in the N-terminal end are the key residues in promoting the interactions between α-factor and a region of the receptor, which connected directly in stimulating the pheromone responsive G protein pathway.[30,40,41]

Membrane phospholipids play an important role in the receptor recognition process by peptide pheromones. It was reported that the physiological activity of α-factor pheromone revealed close connection on the affinity with phospholipid membrane.[36-38] Since the target of α-factor is a membrane-associated protein, the Ste2p receptor, it can be conceivable that a certain type of hydrophobic residues in the ligand can aid in localization of the pheromone to the membrane of the target cell,[28,29] thereby leading to an increased probability for binding to the receptor. Study of the transferred nuclear overhauser effect revealed that the deletion of Trp or substitution of Trp with Ala or Gly appreciably weakened the interactions with the hydrophobic interior of phosphatidylcholine vesicle, reflecting that two tryptophane at position 1 and 3 in the N-terminal end were key residues in the preferential insertion of N-terminus into

phospholipid membrane upon the absorption of α-factor into *S. cerevisiae* membrane.[13,31]

The conformational analyses of Trp[1]-bearing active analogs of α-factor with NMR and CD revealed that N-terminal region, Trp-His-Trp-Leu-Gln-Leu, formed a stable α-helix together with β-turn conformation in the central region, Pro[8]-Gly[9].[14-20] According to the report, high agonist activity attributed to a stable α-helix. Thus, incorporation of D-residue counterpart at position 2-5 precluded the formation of stable α-helix, resulting in remarkable drop in activity.[32] Genetic screening approach to identify novel α-factor agonist and antagonist using libraries of a mutated α-factor encoding gene also revealed that two tryptophanes at position 1 and 3 were important for agonist activity together with Pro[8] and Gly.[9,39]

According to the recent model for α-factor bound to Ste2p,[30,44,52] residues in the region near the extracellular end of the sixth transmembrane segment (TMD6) and the third extracellular loop (EC3) of the receptor (receptor site 2)[30] implicates in receptor signaling. Tyr[266] is part of the region that recognizes the N-terminal portion of α-factor and plays a critical role in responding to α-factor leading to the transformation of Ste2p into an activated state.[52] The model has also suggested the possibility that Tyr[266] on TMD6 is oriented away from the surface of the helix bundle toward the surrounding lipids upon binding of ligand and possibility for interaction between aromatic residues in the α-factor ligand and Tyr[266] on TMD6. Such implication of Tyr[266] in interaction with ligand is indirectly supported by various model approaches.[30,45,53,54]

Most of SAR studies on α-factor focused on [Nle[12]]α-factor, especially on an isosteric replacement of Met,[12] since it revealed several better advantages over natural α-factor. Importantly, it did not undergo oxidation leading to methionine sulfoxide, which occurred in natural α-factor, and it was stable against autoradiolysis.[4,8,10] In addition, Nle[12] substitution was reported to have increased stability of pheromone against the endopeptidase degradation by *MATa* cell, retaining its activity for long period.[11,12] Despite of such tactical advantages, potency of [Nle[12]]α-factor was not as active as natural α-factor, either equivalent to that of natural α-factor[8] or less active than α-factor in both the shmoo assay and halo assay for assessing growth arrest.[6,27] Anyhow, considering the importance of the direct comparison of bioactivity with natural pheromone, ten amide analogs reported here were designed on the base of natural α-factor sequence, instead of [Nle[12]]α-factor. Then, since most of α-factor analogs reported to date was carboxylic acid at C-terminus, amide modification was adopted at C-terminus, which was expected to provide the information of anionic effect at C-terminal and amidation effect on antagonism upon binding to receptor. Analogs, which were substituted at position 1 and 3 simultaneously, were not reported to date, therefore, modifications solely focused on the replacement of two tryptophanes to elucidate the combined role of two tryptophane indole rings in triggering signal transduction. Moreover, since our recent study demonstrated that the C-terminal

extension of α-factor with tryptophane was designed to impose more favorable structural characteristic through the enhanced hydrophobicity on a pheromone ligand, and it led to remarkable increase in activity in the shmoo assay.[57] The present study was conducted to investigate whether the effect of tryptophane tale could cause the increased affinity to lipid membrane or/and enhanced stimulation to receptor. In this paper, the synthesis, characterization, and biological activities of ten Trp[1,3] substituted amide analogs of the α-factor mating pheromone were presented.

Experimental Procedure

Synthesis of α-Factor Amide Analogs Using Rapidamide Resin[21-24]. All α-factor amide analogs were prepared using a solid phase strategy. All reagents and solvents in the solid phase peptide synthesis were analytical grade and were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA), VWR Scientific (Piscataway, NJ), and Aldrich. High performance liquid chromatography grade dichloromethane, acetonitrile, methanol, and water were purchased from VWR Scientific and Fisher Scientific (Springfield, NJ). Synthesis of target peptides were carried out manually by RAMP cartridge (Response Biomedical Co. Canada) using preloaded Rapidamide Resin (33 μmol, 160 mg of resin, Dupont, Bo.). The elongation of the peptide chain was carried out with 2.5 equivalents of *N*-9-fluorenylmethoxycarbonyl (Fmoc) amino acids activated with pentafluorophenyl (PFP) using an *N*-hydroxybenzotriazole (HOBT: 3.0 eq.)/1-methyl-2-pyrrolidone (NMP: 1 mL). The side chain protecting groups were His(Trt)-OH, Lys(*t*-Boc)-OH, Gln(Trt)-OH, Tyr(*t*-Bu) and Trp(t-Boc)-OH. Every coupling was accomplished for 3 h at room temperature. Fmoc was deprotected using piperidine/DMF (50/50 v/v) for 10 mins. The reaction completeness was checked by Kaiser test. The first 10-amino acid fragment was synthesized by single coupling and then double coupling was 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 NMP and CH_2Cl_2 and dried *in vacuo* for 4-5 h. The peptide was 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) for 16 h. 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 crude peptide so obtained was purified by reversed phase high performance liquid chromatography (Shimazu Prominence HPLC) on a semipreparative Vydac 218TP54 C_{18} reverse phase polymer column with detection at 220 nm. Because of the strong aggregating tendency of the peptide 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

0.05% trifluoroacetic acid and 0-100% CH_3CN over 90 min with a flow rate of 2 mL/min. Fractions were analyzed by reversed phase high performance liquid chromatography (Shimazu Prominence HPLC) on an analytical Vydac Everest C_{18} reversed phase polymer column with detection at 220 nm. Fractions of over 97% homogeneity were pooled and subjected to lyophilization. For the amino acid analysis, the hydrolyzed peptides by 6 N HCl at 150 °C were subjected to HPLC and eluted with a linear gradient of 6% acetonitrile/ sodium acetate trihydrate/water and 0-100% acetonitrile/ water over 15 min with detection at 254 nm.

Synthesis of Natural α-Factor Using Trityl Resin[21-24]. Synthesis of natural α-factor was carried out manually by RAMP cartridge (Response Biomedical Co. Canada) using preloaded NH_2-Trp-2-chloro-Trityl Resin (0.53 mmol/g resin, Novabiochem, La Jolla, CA) on 0.1-mmol scale. The elongation of the peptide chain was carried out with an 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU: 8 eq.)/*N*-hydroxybenzotriazole (HOBT: 8 eq.)/4-methylmorpholine (16 eq.) catalyzed coupling step using 8 equivalents of *N*-protected amino acids with 9-fluorenylmethoxycarbonyl (Fmoc). Fmoc was used for all *N*-α-protections whereas the side chain protecting groups were His(Trt)-OH, Lys(*t*-Boc)-OH, Gln(Trt)-OH, Tyr(*t*-Bu) and Trp(*t*-Boc)-OH. The first 10-amino acid fragment was synthesized by single coupling and then double coupling was 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 peptide was cleaved and purified by the same procedure of above amide synthesis. Analytical HPLC of final product showed 99% homogeneity.

Organisms. Two strains of *S. cerevisiae* 2180-1A [*MATa SUC2 mal0 gal2 CUP1*] and 7925 [*MATa his3-532 trp1 gal2*] were used for the growth arrest and halo assay of various α-factor analogs. Both strains were purchased from Korean Collection for Type Cultures. The strains were maintained and grown on YEPD medium (yeast extract: 1%, peptone: 2%, dextrose: 2%) at 30 °C.

Morphogenesis (shmoo) Assay[10,25]. Two strains of *S. cerevisiae* 2180-1A and 7925 were grown at 30 °C with shaking (200 rpm/sec) to early log phase in YEPD broth supplement with 2% glucose. The cells were then harvested by centrifugation at 8,000 rpm/sec (IBRD IB4, Hanil Science Centrifuge), washed twice with sterile distilled water, resuspended to 1.2×10^6 cells/mL in YEPD broth, and placed on ice. Dilution series (0.001 μg-1000 μg/mL) of peptide analogs were prepared in plastic test tubes with YEPD as the diluents using peptide stock solution (1000 μg/100 μL). Each of the peptide solutions (100 μL) were then added to the mixture of 100 μL of DMF and 800 μL of the 1.0×10^6 cells and the suspensions were incubated at 30 °C for 5 h with 200 rpm/sec of shaking. At the completion of the incubation period, the cells were placed on ice, and then 10 μL portions were placed in a hemocytometer and observed microscopically to quantitate the total cells and total number of cells with shmoo morphology (an elongated, pear-shaped

cell without a constricted neck at the bud site) by visual inspection using a microscope (Olympus CHD) under × 400 power. The percentage of shmoos was calculated by dividing total shmooing cells by total cells.

Growth Arrest (halo) Assay[10,25]. YEPD plated [yeast extract (1%, w/v), peptone (1%, w/v), dextrose (2%, w/v) and agar (2%, w/v)] were overlaid with 4 mL of *S. cerevisiae* 2180-1A and 7925 (2.7×10^6 cells/mL) cell suspension in 0.825% Noble agar respectively. Filter disks (Advantec, Toyo Roshi), 8 mm in diameter, were placed on the overlay and impregnated with 10-μL portions of peptide solutions at various concentrations (0.1 μg-10 μg/1 μL). The plates were incubated at 30 °C for 24 h and then observed for clear zones (halos) around the disks. The data were expressed as the diameter of the halo including the diameter of the disk. Therefore, a minimum value for growth arrest is 9 mm, which represents the disk diameter (8 mm) and a small zone of inhibition. All assays were carried out at least three times with no more than a 2-mm variation in halo size at a particular amount applied for each peptide. The values reported represent averages of the tests. Differences in diffusion of the various analogs in the agar medium did not contribute to the differences in the biological activities in the halo assay. Similar trends were obtained for these analogs when activities were ranked within an assay as measured by the halo and morphogenesis.

Antagonism Assays. The assay was used to measure whether analogs with no growth arrest activity were capable of antagonizing activity of the natural pheromone. In the assay, lawns of strain 7925 were overlaid onto YEPD plates, as described in the growth arrest assay: Sterile disks were placed adjacent to each other, so that the disk containing the test peptide would lie at the periphery of the halo formed by the standard analog, [Ala³]α-factor amide (**2**). One disk was impregnated with 150 μg of [Ala³]α-factor amide (**2**) in 10 μL of H_2O, and the other disk was impregnated with equal amounts of the test peptide in 10 μL of H_2O. Plates were incubated as described in the halo assay and the effects on halo formation noted.

Results and Discussion

Synthesis and Characterization of Peptide Analogs. α-Factor amide is a tridecapeptide amide of the following primary structure: Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr-$CONH_2$. Amide analogs of this peptide are designated according to IUPAPC convention.[26] Thus, if 2-aminoisobutyric acid (Aib) is substituted for tryptophane at position 2, the analogs is designated [Aib²]α-factor amide. The removal of tryptophane at residue 1 gives [des-Trp¹]α-factor amide. Synthesis of all of target amide analogs investigated in this paper was accomplished efficiently using solid phase approach on a Rapidamide resin. In general, the obtained crude products of the TFA cleavage of the bound analogs from resin contained approximately 43-58% of the major peak as indicated by analytical reversed phase HPLC. Based on the HPLC peak patterns of the crude products, resultants
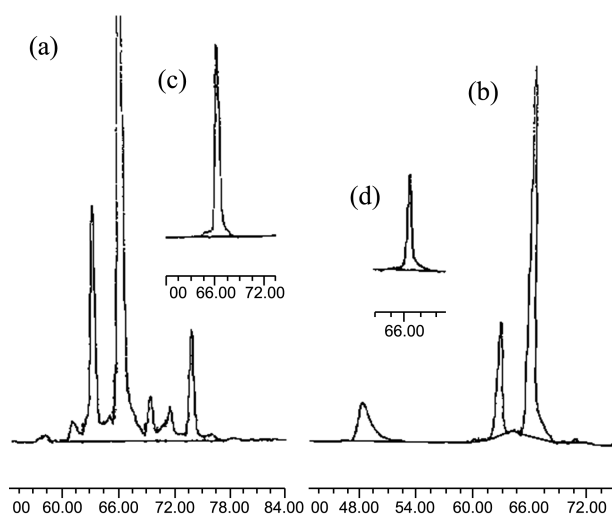
Figure 1. (a) HPLC of crude product from [Ala[1]]α-factor amide **1**. Major peak was detected at 67.5 min. Similar elution patterns of HPLC were obtained with [Ala[3]]α-factor amide **2**, [Aib[1]]α-factor amide **4** and [Aib[3]]α-factor amide **5**, [des-Trp[1]]α-factor amide **7**, [D-Trp[3]]α-factor amide **8**, [D-Trp[1,3]]α-factor amide **9**. (c) HPLC of purified product from [Ala[1]]α-factor amide **1**. (b) HPLC of crude product from [des-Trp[1],Phe[3]]α-factor amide **10**. Major peak was detected at 66.4 min. Similar elution patterns of HPLC were obtained with [Ala[1,3]]α-factor amide **3** and [Aib[1,3]]α-factor amide **6**. (d) HPLC of purified product from [des-Trp[1],Phe[3]]α-factor amide **10**. Chromatography was performed on a C_{18} column using the following gradients: CH_3CN/water containing 0.05% trifluoroacetic acid and 0-100% CH_3CN over 90 min with a flow rate of 2 mL/min.

were divided into two categories (Fig. 1): Firstly, three or four small peaks of side products appeared around one dominant product peak (Fig. 1(a)), and secondly, a couple of side products eluted prior to major peak (Fig. 1(b)). [Ala[1]]α-factor amide **1**, [Ala[3]]α-factor amide (**2**), [Aib[1]]α-factor amide (**4**), [Aib[3]]α-factor amide (**5**), [des-Trp[1]]α-factor amide (**7**), [D-Trp[3]]α-factor amide (**8**), [D-Trp[1,3]]α-factor amide (**9**) were categorized into the former pattern, and the latter cases were [Ala[1,3]]α-factor amide (**3**), [Aib[1,3]]α-factor

amide (**6**) and [des-Trp[1],Phe[3]]α-factor amide (**10**). Preparative HPLC purification yielded peptides with a homogeneity greater than 97% as judged with analytical HPLC using an acetonitrile/water/TFA gradient system (Fig. 1(c) and 1(d)). In order to verify the major peaks as authentic products, the final products were assessed by PTC amino acid analysis using RP-HPLC. Table 1 revealed the results of amino acid ratios, consistent with expected values. The relative mobility of the amide analogs on a reversed-phase C_{18} column using water/acetonitrile based linear gradients correlated with the apparent hydrophobicity of the analogs as [Ala[1,3]]α-factor amide (**3**) > [Aib[1,3]]α-factor amide (**6**) > [des-Trp[1],Phe[3]]α-factor amide (**10**) > [Ala[3]]α-factor amide (**2**) > [Aib[3]]α-factor amide (**5**) > [Ala[1]]α-factor amide (**1**) > [des-Trp[1]]α-factor amide (**7**) > [Aib[1]]α-factor amide (**4**) > [D-Trp[1,3]]α-factor amide (**9**) > [D-Trp[3]]α-factor amide (**8**).

Morphogenesis (Shmoo) Activity of Peptide Analogs. The most widely reported activity of α-factor is its effect on the shape and budding of *MATa* cells of *S. cerevisiae*. All α-factor analogs upon incubation with *S. cerevisiae* 2180-1A and 7925 (*MATa*) caused the formation of aberrant morphologies (shmoo shape) and cessation of budding. Minimum concentrations of the synthetic peptides induced morphogenesis ranged from 0.1 μg/mL to 40 μg/mL (0.1 μg/mL for natural α-factor). A goal of this study is to understand the role of two tryptophanes in their biological activities. Therefore, peptides of two tryptophanes substitution with other amino acid residues were designed and synthesized for the determination of their activity variations systematically. Three classes of modifications involving Ala, Aib, and D-Trp were utilized for this purpose for replacing two tryptophanes. As shown in Table 2 (d: ratio), all amide analogs showed lower activities than that of their respective natural α-factor analogs in shmoo assay. Among the ten amide analogs, [Aib[1]]α-factor amide (**1**) was the most potent analog, but less active by a factor of 4 for minimum concentration for induced aberrant morphogenesis in comparison with natural α-factor. Aib substituted analogs exhibited higher potency than other two classes of Ala and D-Trp analogs. Analogs with a sub-

Table 1. Analytical and physical properties of synthetic α-factor amide analogs

No	Analogs	Yields		Amino acid analysis										
		Crude	Final	Gln	Gly	His	Ala	Pro	Tyr	Met	Leu	Phe	Lys	Aib
1	[Ala[1]]α-factor amide	43.7	99.9	2.0(2)	1.0(1)	0.8(1)	1.1(1)	2.2(2)	1.0(1)	0.9(1)	2.2(2)	0.0(0)	1.2(1)	0.0(0)
2	[Ala[3]]α-factor amide	52.8	98.1	1.8(2)	1.0(1)	0.9(1)	0.9(2)	1.9(2)	1.0(1)	0.9(1)	2.1(2)	0.0(0)	1.1(1)	0.0(0)
3	[Ala[1,3]]α-factor amide	55.7	99.7	2.3(2)	1.3(1)	1.2(1)	1.9(2)	2.3(2)	1.0(1)	1.0(1)	2.3(2)	0.0(0)	0.9(1)	0.0(0)
4	[Aib[1]]α-factor amide	51.3	98.9	2.0(2)	1.1(1)	0.9(1)	0.0(0)	2.1(2)	1.0(1)	0.9(1)	2.1(2)	0.0(0)	1.0(1)	1.2(1)
5	[Aib[3]]α-factor amide	49.6	99.2	1.9(2)	1.0(1)	1.0(1)	0.0(0)	2.2(2)	1.0(1)	1.0(1)	2.1(2)	0.0(0)	1.1(1)	1.1(1)
6	[Aib[1,3]]α-factor amide	53.2	98.2	2.0(2)	1.1(1)	1.1(1)	0.0(0)	2.1(2)	1.0(1)	1.0(1)	2.0(2)	0.0(0)	1.1(1)	2.1(2)
7	[des-Trp[1]]α-factor amide	47.4	99.1	1.8(2)	0.9(1)	0.9(1)	0.0(0)	1.8(2)	1.0(1)	0.9(1)	1.9(2)	0.0(0)	0.9(1)	0.0(0)
8	[D-Trp[3]]α-factor amide	47.8	96.8	1.7(2)	0.9(1)	0.9(1)	0.0(0)	1.8(2)	1.0(1)	0.9(1)	1.9(2)	0.0(0)	1.0(1)	0.0(0)
9	[D-Trp[1,3]]α-factor amide	50.0	96.8	1.8(2)	1.1(1)	0.8(1)	0.0(0)	2.2(2)	1.0(1)	0.9(1)	2.2(2)	0.0(0)	1.1(1)	0.0(0)
10	[des-Trp[1],Phe[3]]α-factor amide	58.6	99.4	2.0(2)	1.1(1)	1.0(1)	0.0(0)	2.2(2)	1.0(1)	0.9(1)	2.2(2)	1.2(1)	1.2(1)	0.0(0)
11	α-factor	59.3	99.3	2.1(2)	1.0(1)	0.9(1)	0.0(0)	2.1(2)	1.0(1)	0.9(1)	2.1(1)	0.0(0)	1.1(1)	0.0(0)

**Table 2.** Biological and biochemical properties of α-factor amide analogs

No	Analogs	Halo 7925		2180-1A[a]		Amount[c]	Shmoo[b] (mg/mL)	Ratio[d]
		10	30	10	30			
1	[Ala[1]]α-factor amide	11	14	11	15	30	0.6	6
2	[Ala[3]]α-factor amide	NA	12	10	14	40	4	40
3	[Ala[1,3]]α-factor amide	NA	NA	NA	NA	-	20	200
4	[Aib[1]]α-factor amide	11	15	12	16	25	0.4	4
5	[Aib[3]]α-factor amide	11	14	12	16	25	2	20
6	[Aib[1,3]]α-factor amide	NA	NA	NA	NA	-	15	150
7	[des-Trp[1]]α-factor amide	NA	10	10	11	80	6	50
8	[D-Trp[3]]α-factor amide	NA	NA	NA	NA	200	40	400
9	[D-Trp[1,3]]α-factor amide	NA	10	10	11	100	6	60
10	[des-Trp[1],Phe[3]]α-factor amide	NA	NA	NA	NA	200	30	300
11	α-factor	14	22	18	25	7	0.1	1

NA = not active. [a]Halo assay as measured by the growth arrest. For each peptide, 10 and 100 μg were spotted on discs which were placed upon YEPD plates with Noble agar containing strain *S. cerevisiae* 7947 (2.1 × 10^{6} cells). At 24 h, the zone of growth inhibition was determined by the halo diameter in centimeters. The halo sizes for 10 and 100 μg of each peptide are shown. [b]Values represented activity and points for shmoo formations in the microtiter assay as described under "Experimental procedure". Serial dilutions of each peptide were tested for shmoo-inducing effect. The end points shown indicate the last well in which morphological effect of the peptide was seen microscopically.

stituted residue at position one (**1,4**) showed about 5 times higher activities than analogs with a substitution at position three (**2,5**). The double substituted analogs with Ala (**3**) and Aib (**6**) showed significantly lower activity than single substituted analogs at position one (**1,2**) or three (**4,5**). In contrast to the above analogs, [D-Trp[1,3]]α-factor amide (**9**) showed similar activity or 16 times higher activity in comparison to single modified analogs, [des-Trp[1]]α-factor amide (**7**) and [D-Trp[3]]α-factor amide (**8**), respectively. In addition to the above results, three class of homologous series, [des-Trp[1], Phe[3]]α-factor amide (**10**), of which acid counterpart, [des-Trp[1], Phe[3]]α-factor with antagonist activity from previous reports,[4,43] revealed weak agonist activity against strain 2180-1A at concentration as high as 60 mg/10 mL, but no agonist activity against strain 7925 up to 100 mg/10 mL.

Growth Arrest (halo) Assay. The growth arrest assay carried out using *S. cerevisiae* wild type strain 2180-1A and 7925. The bioactive analogs presented clear zone around the disk (Fig. 2(a)). Increasing amounts of pheromone caused increases in growth arrest, as measured by halo size. These data were plotted, as halo size versus the amount of pheromone added to the cell lawn, and linearized by regression analysis. To compare the relative activities of different analogs, the amount of peptide causing a halo size of 15 mm was determined from the regression line. In general, the relative activity of the amide analogs was similar in two different strains. The relative halo activities of amide analogs against strain 2180-1A were presented in Figure 3. Strain 2180-1A showed one order of magnitude higher sensitivity than the strain 7925 in the assays (Table 2).

In the single Ala contained analogs (**1,2**), the amounts of pheromone causing a 15 mm zone of inhibition were 30 μg for [Ala[1]]α-factor amide (**1**) and 40 μg for [Ala[3]]α-factor amide (**2**), respectively (Table 2, c: ratio). These activities represented decreases in activity of approximately 4.5- and 6-folds in comparison with the natural α-factor (**11**, 7 μg/15
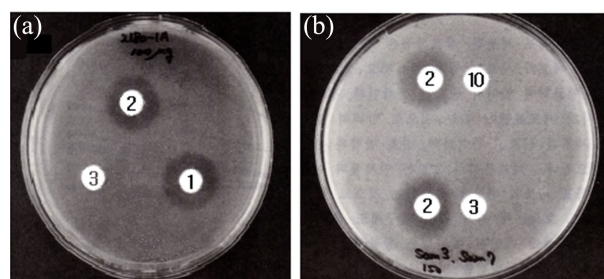
Figure 2. Halo assays. (a) Results of halo assay for α-factor amide analogs. Synthetic analogs were placed on the disk which appears at the center of growth arrest shown by a halo of no growth in the lawn of strain 2180-1A. Analogs (panel **2** = [Ala[3]]α-factor amide, panel **1** = [Ala[1]]α-factor amide and panel **3** = [Ala[1,3]]α-factor amide, peptide amount = 100 μg/disk) were placed on disks at the halo periphery as shown in the figure. (b) Halo assays demonstrating antagonistic activity of [des-Trp[1], Phe[3]]α-factor amide (panel **10**) and [Ala[1,3]]α-factor amide (panel **3**). A lawn of strain 7925 was challenged with 150 μg of [Ala[3]]α-factor amide (panel **2**) and inactive analogs, [des-Trp[1], Phe[3]]α-factor amide (panel **10** = 150 μg) and [Ala[1,3]]α-factor amide (panel **3** = 150 μg). The analogs demonstrated weak antagonism as represented by the flattened halos.

mm). Whereas, for two Ala contained analog, [Ala[1,3]]α-factor amide (**3**) revealed no clear zone up to 100 μg. In the Aib contained series **4-6**, activity was same as series with Ala. Substitution with Aib residue had the slightly better effect on the biological activity of the pheromone than Ala substituents, as judged by the both halo and shmoo assays. [Aib[1]]α-factor amide (**4**) and [Aib[3]]α-factor amide (**5**) were 25 μg/15 mm and 25 μg/15 mm, respectively, reflecting one order of magnitude higher activity than the counterparts of Ala substituents **1**, **2**.

Deletion of Trp[1], [des-Trp[1]]α-factor amide (**7**, 80 μg/15 mm), resulted in an 11-fold drop in activity in comparison to natural α-factor (**11**, 7 μg/15 mm), and the replacement of Trp[3] by Phe in the deleted analog **7** caused additional 20-fold

drop in activity. Single substituted analog with D-isomer at position three, [D-Trp^3]α-factor amide (**8**, 200 μg/15 mm), exhibited the decreased potency, 30 times less active than natural α-factor (**11**), whereas double substituent with D-isomer at both positions one and three, [D-Trp^{1,3}]α-factor amide (**9**, 100 μg/15 mm), showed better result in activity, two times higher activity than single substituent **11**. This finding revealed adverse trend to the observed results with two series of Ala and Aib substituents.

Antagonistic Activity. The four analogs, **3**, **6**, **8** and **10**, which were virtually devoid of activity in the halo assay against strains 7925, were tested for antagonism (Fig. 2(b)). The four inactive analogs were placed on a lawn of strain 7925 in proximity to [Ala^3]α-factor amide (**2**), and the bean-shaped zone of inhibition was observed. The growth arrest halo of [Ala^3]α-factor amide (**2**) caused by the test analogs **3**, **6**, **8**, **10** became flattened on the side adjacent to the test analogs **3**, **6**, **8**, **10** (Fig. 2(b)) at identical concentration. Analogs **3**, **6** with similar antagonistic activity were found to be slightly more active at a given concentration (150 mg/disk) than analog **8**, **10**, as judged by the shape of the flattened halo. The antagonistic response of analog **8** was similar to analog **10**.

Discussion

The structure activity relationship study of synthetic analogs and genetic screening study using a mutated α-factor encoding gene have examined the influence of the residues of α-factor on pheromone activity and receptor binding affinity.[10,39] The results have detailed the role of each residue in addition to C-, N-terminal role associated with Ste2p and GPCR activation. Trp^1, Trp^3, Pro^8 and Gly^9 are important for agonist activity that connected directly to stimulation of the pheromone responsive G protein pathway and His^2, Leu^4, Leu^6, Pro^{11} and Tyr^{13} contribute to both agonist and antagonist activity.[30,40,41] Most of analogs obtained from these selection procedure sorely relies on the single replacement of each residue with an alternative. Moreover, such studies concerning the SAR of two tryptophanes have revealed different results in α-factor activities.[4,10] Meanwhile, investigation on the interaction between α-factor and lipid membrane showed that two tryptophane aromatic residues at N-terminus play a key role in inserting into membranes, when the association α-factor with phospholipid membranes occurs.[13,31] In this connection, uncertain mechanistic role of two tryptophanes together with the significance of the intrinsic hydrophobicity of pheromone on biological activity prompt us to design ten analogs substituted with various residues at position 1 and 3, collecting the data about the combined role of two tryptophanes on pheromone activity of α-factor.

Ala scanning analysis is a widely accepted method to determine which residues of peptide contribute to either the binding to the receptor or its activation. This approach provides general insights into the influence of pheromone on bioactivity and identification of specific side chains that

modulate the interactions between the peptide and receptor.[10] In the first step, two tryptophanes at position 1 and 3 were replaced with alanine to compare difference between the earlier studies focused mostly on single substitution and our study focused on double substitution. [Ala^1]α-factor amide (**1**) showed a 6-fold decrease in biological activity in the shmoo assay, whereas [Ala^3]α-factor amide (**2**) showed a 40-fold decrease in shmoo assay. [Ala^{1,3}]α-factor amide (**3**) exhibited 200-fold decrease in shmoo assay and no activity in halo assy. The lower potency of [Ala^3]α-factor amide (**2**) compared to [Ala^1]α-factor amide (**1**) indicates that the Trp^3 indole moiety affects more strongly activation of the receptor compared to that of Trp^1. The complete loss of activity of double substituent with Ala, [Ala^{1,3}]α-factor amide (**3**) strongly implies that two tryptophane residues at the amine terminus contribute together to the signal transduction. Antagonism effect exhibited by [Ala^{1,3}]α-factor amide (**3**) against [Ala^3]α-factor amide (**2**) supports above notion, although antagonistic response was observed against [Ala^3]α-factor amide (**2**). In addition, such antagonism effect together with the lack of activity of [Ala^{1,3}]α-factor amide (**3**) in halo assay supports the general acceptance that a major determinant of receptor binding is not the amine terminus of the native pheromone, but carboxyl terminus. It seems clear that the cooperative act of two tryptophane residues near amine terminus is essential in presentation to the site of pheromone Ste2p receptor which is specific for the receptor's activated conformation.

Aib has a structure similar to Ala except for additional methyl group at the chiral center. Unlike Ala, two methyl groups at a α-carbon created significant steric effect which imposes dramatic constraint on the peptide backbone, forcing to assume a 3$_{10}$helix.[34] In this respect, to reduce a structural flexibility of linear α-factor and examine a possibility as an alternative tool for Ala scanning, we have incorporated Aib in place of Ala. Two single substituted analogs with Aib, [Aib^1]α-factor amide (**4**) and [Aib^3]α-factor amide (**5**), exhibited similar activity drops compared to natural α-factor in halo assay. In both cases, 4-fold reduction was observed. For the comparison with shmoo activities of Aib analogs **4**, **5**, with Ala counterparts **1**, **2**, the analog **4** was 1.5 times more potent than Ala counterpart **1**, whereas the analog **5** was almost 2 times more potent than Ala counterpart **2**. It seems that such a higher increase observed in [Aib^3]α-factor amide (**5**) ascribe to a more stabilized α-helical conformation through the influence of Aib incorporation at position 3. Such explanation is supported by both the previous report that amine terminal region of pheromone assumes α-helical conformation preferentially which thereby is responsible for pheromone activity[32] and the previous report that helicity is an important factor in facilitating specific interaction between various peptides and lipid membranes.[4,32] Additional methyl group of Aib seems to cause an additional activity increase owing to the facile approach to Ste2p through the increased penetration into lipid membrane without destabilizing α-helix. Neither the [Ala^{1,3}]α-factor amide (**3**) nor the [Aib^{1,3}]α-factor amide (**6**) showed any activity against both strains 2180-1A and 7925 in the halo assay, emphasizing

again the importance of combined role of two tryptophanes for agonist activity and indicating a relatively large hydrophobic binding region of Ste2p associated with N-terminus of ligand.

Some studies reported that the tridecapeptide α-factor and the dodecapeptide lacking the tryptophane at position 1 had equal activity in the shmoo assay.[5,6,42] In contrast, other studies reported the different result that removal of Trp[1] from pheromone resulted in a one order of magnitude drop in biological activity.[8,33] Given both such conflicting results and study for the cooperative role of two tryptophanes, we have examined the effect of Trp[1] on biological activity with amide analog of α-factor. Deletion of Trp[1], [des-Trp[1]]α-factor amide (**7**), resulted in a 11-fold drop for halo assay and 50-fold drop for shmoo assay in comparison to natural α-factor (**11**), respectively. Such a huge drop in activity together with results of Ala and Aib substituents is the result consistent with the latter report, although the comparison of the amide analog. The fact that conversion of the carboxyl terminus to a carboxamide caused the decrease in activity identified the cationic residue such as Arg[58] at the receptor binding site of C-terminus of α-factor which was known to involve in cation-π binding interaction with Tyr[13] of α-factor.[56] The further modification of [des-Trp[1]]α-factor amide (**7**) replaced Trp[3] by Phe **10** led to additional 20-fold drop for halo assay and about 250-fold drop for shmoo assay and incorporation of D-Trp[3] **8** in α-factor **11** led to 400-fold drop compared to α-factor (**11**) against shmoo assay, reflecting that position 3 requires L-isomer of Trp and plays more important role than Trp[1] for triggering cell signaling as observed with both Ala and Aib series.

In the comparison of amide analogs of D-Trp, single substituted analog, [D-Trp[3]]α-factor amide (**8**), exhibited the decreased potency, 30-fold less active in halo assay and 400-fold less active in shmoo assay than those of natural α-factor (**11**) respectively, whereas double substitutent, [D-Trp[1,3]]α-factor amide (**9**), was 14-fold less active in halo assay and 60-fold less active in shmoo assay respectively, exhibiting approximately 2 and 7 times higher activity of double substituent **9** than single substituent **8**. This is adverse trend to the results observed with above two series of Ala and Aib substituents. Both the [Ala[1,3]]α-factor amide (**3**) and [Aib[1,3]]α-factor amide (**6**) were devoid of activity, whereas [D-Trp[1,3]]α-factor amide (**9**) was about as active as that of amide analog **7**, indicating cooperative interaction between two Trp[1,3] upon binding to its receptor GPCR. The truncated analog study revealed that removal of two residues from N-terminus, [des-Trp[1],des-His[2]]α-factor, led to completely inactive analog which had antagonistic activity.[4,35] This results prompt us to test the antagonistic activity with four inactive analogs **3**, **6**, **8**, **10** for halo assays using strain 7925. Indeed, analogs **3**, **4** shows similar antagonistic activity against analog **2** in the similar range of amounts, whereas analogs **8**, **10** shows slightly weak antagonistic response compared to above two analogs **3**, **6** for halo assays using strain 7925. For the comparison of antagonist activity with the known standard antagonist, [des-Trp[1],Phe[3],Nle[12]]α-factor,[4,43] amide analog
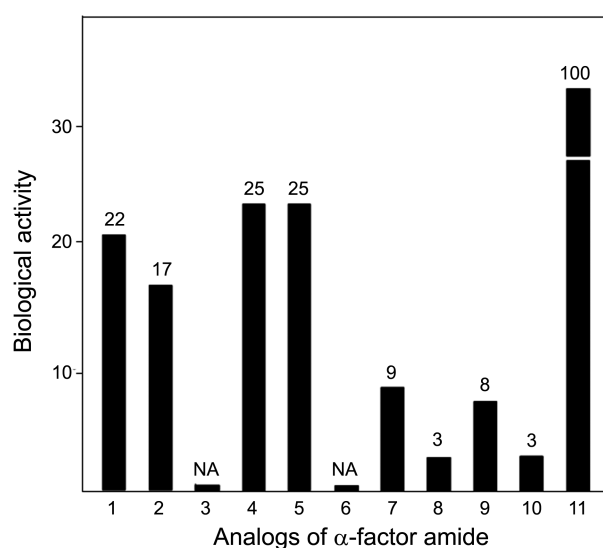
Figure 3. Bar graph of relative biological activities of α-factor and amide analogs. Values represent relative activity for halo assay with strain 2180-1A. Ratio was determined by comparison of concentration causing halo size 15 mm. Analogs number represent as following: **1**; [Ala[1]]α-factor amide, **2**; [Ala[3]]α-factor amide, **3**; [Ala[1,3]]α-factor amide, **4**; [Aib[1]]α-factor amide, **5**; [Aib[3]]α-factor amide, **6**; [Aib[1,3]]α-factor amide, **7**; [des-Trp[1]]α-factor amide, **8**; [D-Trp[3]]α-factor amide, **9**; [D-Trp[1,3]]α-factor amide, **10**; [des-Trp[1], Phe[3]]α-factor amide, **11**; natural α-factor. NA = not active.

10 mimicking structurally the standard antagonist showed weak agonist activity for strain 2180-1A, reflecting weakest antagonism among four tested analogs **3**, **6**, **8**, **10** (Fig. 3). Summary of previous structure activity studies on antagonists of α-factor reported to date is as follow: For the dodecapeptides, [des-Trp[1]]α-factor, only two analogs [des-Trp[1],Ala[3]]α-factor and [des-Trp[1],Phe[3]]α-factor are only the known antagonists toward normal α-factor receptor.[4,42] Then, for the tridecapeptides, analogs with D-residues in position 2,3 or 4 such as [D-Ala[2]]-, [D-Ala[3]]-, [D-Ala[4]]-, [D-His[2]]-, and [D-Tyr[3]]-α-factor are also known to behave as antagonists.[4,10] In addition to these antagonists reported to date, we have found that [Ala[1,3]]-, [Aib[1,3]]- and [D-Trp[3]]-α-factor amide analogs **3**, **6**, **8** function as antagonist. Moreover, the finding from the comparison study of analogs with standard antagonist [des-Trp[1],Phe[3]]α-factor clearly indicates double substituted amide analogs **3**, **6** are more active than above single substituted antagonists. Antagonist has been useful tool to characterize various isomeric state of GPCR in the activation pathway.[4,30] Thus, our new strong antagonists are expected to play a part in characterization of receptor, specifically identification of interaction of pheromone with region of receptor associated with two tryptophanes of α-factor.

Importantly, consistent with the recent proposed model for α-factor bound to Ste2p,[30,44,52] our finding with double substitution approach concentrated on two tryptophanes supports a notion strongly that two aromatic rings of Trp[1,3] are implicated in interaction with Tyr[266] of receptor pocket near the extracellular end of TMD6. It is highly likely that aromatic ring of Tyr[266] inserts between two indole rings of Trp[1,3] leading to form a stable staggered parallel stacking

orientation of three aromatic rings, in the same manner as the base stacking in double helical structure of nucleic acid. Such stable orientation is not thought to be fully favorable with Phe[1,3], or Tyr[1,3] in place of Trp[1,3], what with relatively small size of phenyl ring and also side chain contact between three residues away. In addition, substitution of one of two tryptophanes with D-isomer is thought to cause a corruption of proper parallel orientation, affecting negatively on binding of N-terminus toward receptor site 2. This assumption is strongly supported by the deteriorated activity of our analogs such as [des-Trp[1],Phe[3]]α-factor amide (**10**) and [L-Trp[1],D-Trp[3]]α-factor amide (**8**) and other new antagonist reported lately, [D-Tyr[3], Lys[7](NBD)]α-factor.[30] Furthermore, the fact that substitution of both tryptophanes with two D-isomers, [D-Trp[1,3]]α-factor amide (**9**), causes greater than two or five times recovery in activity compared to above two analogs **8**, **10** emphasizes the importance of combined role *via* proper parallel orientation of two indole rings for efficient triggering of signal G protein coupled activation.

Our recent reports using analogs extended with varying number of tryptophane residues provided additional evidence for close correlation between the biological activity of α-factor pheromone and the tendency of integration of the pheromone into a lipid bilayer.[51] In addition to overall hydrophobicity of α-factor interacting with membrane, given the molecular model of the receptor site 2 and orientation of Tyr[266] surrounding by lipids layer near membrane surface, facile intercalation of the N-terminal residue of pheromone into the lipid layer is clearly thought to be required in the receptor recognition process. The possibility that two Trp[1,3] of the N-terminus contribute greatly to the insertion of pheromone into lipid membranes is certified by NMR study and various model compound studies on peptide.[31,52,55] However, the fact that analogs with D-isomer of Trp **8**, **9**, **11** differ in activity profiles with α-factor having only L-isomers emphasizes again that spatial orientation of two indole rings of Trp[1,3] plays highly essential role in promoting the interaction between α-factor and receptor site 2 apart from the role of Trp associated with membrane association,

Conclusively, the contribution of two tryptophane residues of α-factor ligand to activation of Ste2p, through interaction with residue Tyr[266], seems to be dominant, although single substitutions of tryptophane have effect on activation. Moreover, proper parallel orientation of two indole rings seems to be closely correlated with binding to receptor site 2.

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