Structure–Activity Relationships in the Dodecapeptide α-Factor of *Saccharomyces cerevisiae*: Position 6 Analogues Are Poor Inducers of Agglutinability†

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Received June 26, 1984

ABSTRACT: Five des-Trp[1],Cha[3],X[6] analogues of α-factor, where X = Ala, Val, Ile, Nle, or D-Leu and X = Leu in the natural α-factor sequence, were prepared by solution-phase techniques utilizing isobutyl chloroformate or 1-hydroxybenzotriazole accelerated active esters as the coupling agents. Purification to 98% or greater homogeneity was accomplished by high-performance liquid chromatography on a reversed-phase μBondapak C_{18} column with methanol/water/trifluoroacetic acid as the mobile phase. Three of the synthesized analogues (X^6 = Val, Ile, Nle) induced morphogenesis and increased agglutinability in *a* cells. These substitutions demonstrate that a γ-branched side chain at position 6 is not essential for biological activity. All of the active analogues induced morphogenesis at lower concentrations than they induced enhanced agglutinability. These results and other structure–activity relationships [Baffi, R. A., Shenbagamurthi, P., Terrance, K., Becker, J. M., Naider, F., & Lipke, P. (1984) *J. Bacteriol. 158*, 1152–1156] indicate that the agglutination and morphological responses to α-factor can be varied independently. Replacement of Leu[6] with Ala or D-Leu resulted in inactive analogues that were not antagonistic for α-factor activity. Cell-mediated hydrolysis experiments indicated that the biological activities of the α-factor analogues are independent of their rates of degradation. All position 6 analogues were hydrolyzed more slowly than the parent compound, suggesting that the enzyme which degrades α-factor is highly specific for the native structure.

Saccharomyces cerevisiae has developed an elegant system that allows haploid *MATa* and *MATα* mating types to fuse, forming an *a*/α diploid cell. Both mating types secrete peptide pheromones that affect the opposite mating type in preparation for sexual conjugation (Thorner, 1981). The α-factor was purified from culture medium of α-haploid cells by Duntze and co-workers, who showed it to be a tridecapeptide with the following sequence: Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr (Duntze et al., 1973; Stotzler & Duntze, 1976; Stotzler et al., 1976, 1977). An active dodecapeptide mating pheromone lacking the N-terminal Trp was isolated also from culture medium (Stotzler et al., 1976).

α-Factor-treated *a* cells exhibit altered morphologies termed "shmoos" (Levi, 1956), enhanced agglutinability (Betz et al., 1978; Fehrenbacher et al., 1978; Terrance & Lipke, 1981), and inhibition of initiation of DNA synthesis (Throm & Duntze, 1970). Since α-factor does not inhibit RNA synthesis or cell wall assembly, *MATa* cells continue to grow without budding (Lipke et al., 1976; Tkacz & MacKay, 1979). The shmoo morphology is the basis for the biological assay for α-factor, which can be measured by using phase contrast microscopy. Enhanced agglutinability is associated with greater expression of cell surface glycoprotein agglutinins, and a cocentrifugation assay was devised to monitor this activity (Terrance & Lipke, 1981). Inhibition of DNA synthesis is associated with the arrest of the cell cycle in the G1 phase (Bucking-Throm et al., 1973; Samokhin et al., 1981).

The mating process in *S. cerevisiae* appears to share some features of peptide hormone action in higher eukaryotic systems (Cuatrecasas & Hollenberg, 1976). The α-factor peptide triggers several responses from *MATa* cells that are associated with sexual conjugation (Duntze et al., 1970). In addition, *MATa* cells have the ability to enzymatically degrade α-factor (Ciejek & Thorner, 1979; Finkelstein & Strausberg, 1979; Manness & Edelman, 1978). The primary scission occurs between Leu[6] and Lys[7], producing fragments that are not biologically active (Ciejek & Thorner, 1979).

α-Factor has been synthesized by several groups using either solution-phase or solid-phase methods and displays the entire spectrum of responses elicited by the biological pheromone (Ciejek et al., 1977; Khan et al., 1980; Masui et al., 1977; Samokhin et al., 1979; Shenbagamurthi et al., 1983; Stotzler et al., 1977). Structure–activity studies indicate that some residues are essential for activity while other residues can be modified or replaced (Masui et al., 1979; Samokhin et al., 1979; Shenbagamurthi et al., 1983; Stotzler et al., 1977). Little information is available concerning the influence of the residue in position 6. To date, only the des-Trp[1],D-Leu[6]-dodecapeptide was examined, and it was reported to have an activity 10^8-fold less than that of des-Trp[1],L-Leu[6]-α-factor (Masui et al., 1979). Furthermore, the D analogue was resistant to proteolytic degradation by *a* cells. Given the observation that the Leu[6]-Lys[7] peptide bond is specifically cleaved by *a* cells, we decided to explore the influence of residue 6 on biological activity, pheromone hydrolysis, and solution conformation in greater detail. In a previous report the des-Trp[1],Cha[3]-dodecapeptide was found to be more active than the des-Trp[1]-α-factor (Shenbagamurthi et al., 1983). The replacement of Trp[3] by β-cyclohexylalanine simplifies both the synthesis and conformational analysis of these materials.

† This work was supported in part by Grants GM 22086 and GM 22087 from the National Institutes of Health, Grant PCM 8202414 from the National Science Foundation, and a PSC-CUNY Research Award.

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Accordingly, we have prepared des-Trp[1],Cha[3],X[6]-α-factor analogues, where X[6] = Ala, Val, Ile, D-Leu, and Nle. The effects of these analogues on cell morphology, cell agglutinability, and cell-mediated hydrolysis of the pheromone were compared to those of the parent pheromone (des-Trp[1],Cha[3]-dodecapeptide).

Materials and Methods

Chemicals. All amino acids were of the L configuration unless otherwise specified. The solvents were analytical grade. Yeast nitrogen base without amino acids was from Difco Labs. Further details on the sources of reagents and chemicals can be found in supplementary material, which is available upon request (see paragraph at end of paper regarding supplementary material).

Analytical Procedures. The following solvent systems were utilized for thin-layer chromatography (TLC)[1] on silica gel plates (Brinkman) for amino acid derivatives and peptides: (A) methylene chloride/methanol/acetic acid (9:1.5:0.5), (B) 1-butanol/acetic acid/water (4:1:5, upper phase), and (C) 1-butanol/acetic acid/water/pyridine (15:3:12:10); the R_f values are reported as R_f(A), R_f(B), and R_f(C). Optical rotations of the peptides were determined in the solvents indicated in parentheses, where the concentrations are in grams of peptide per 100 mL of solvent.

Melting points were determined by the capillary method and are uncorrected. High-performance liquid chromatography (HPLC, analytical) was carried out by using a Waters system consisting of a M-6000 solvent pump and U6K injector, linked to a Waters 450 variable wavelength UV monitor with an 8-μL flow cell. The μBondapak C_{18} column (10 μm, 30 cm × 0.39 cm i.d.) was also from Waters. All solvents were HPLC grade (Fisher); water was glass distilled. Purity of samples was determined in two solvent systems (methanol/water/trifluoroacetic acid and acetonitrile/water/trifluoroacetic acid). Detection was usually at 220 nm. A Waters Prep LC/System 500 was used for the purification of the dodecapeptides.

Amino acid analyses were performed at Rockefeller University, New York, or at Hoffmann-La Roche, Nutley, NJ. Peptides were hydrolyzed in sealed tubes in 6 N HCl at 100 °C for 24 h. For peptides containing methionine, anisole was added as a scavenger.

Yeast Strains. Haploid strains X2180-1A (*MATa*) and X2180-1B (*MATα*) were grown in minimal medium containing Difco yeast nitrogen base (2.2 g/L), $(NH_4)_2SO_4$ (4.5 g/L), and glucose (20 g/L). All cells were grown at 25 °C to mid-log phase for use as the inoculum in the morphogenesis and agglutination assays.

Morphogenic Activity. The morphogenesis assay was carried out in plastic microtiter plates obtained from Falcon as described previously (Shenbagamurthi et al., 1983). For the purpose of this paper the induction of altered morphologies by α-factor will be termed morphogenesis, and a pheromone causing altered morphologies is called a morphogen. Each well had 100 μL of medium containing 3×10^2 *a* cells and serial 2-fold dilutions of the appropriate synthetic analogue. The lowest concentration causing visible morphogenesis after a 4-h incubation at 25 °C was designated 1 unit of activity (Duntze et al., 1973).

Agglutination Activity. The agglutination potency was determined by the cocentrifugation assay of Terrance & Lipke (1981). *MATa* cells at a concentration of 2×10^7 cells/mL were incubated with 10-fold serial dilutions of the synthetic analogues. After a 20-min incubation, the *MATa* cells were mixed with *MATα* cells, and agglutination was determined in quadruplicate. The concentration inducing half the increase in agglutinability of that induced by 1 unit/mL biological α-factor was determined from interpolation of dose–response curves (Baffi et al., 1984).

Competition Studies. In competition assays, the biologically active mating factor was present at a constant concentration (10 units/mL) in all wells of the microtiter plates, and the competitor was 10-fold serially diluted. Competition was judged by the ability of the competitor to eliminate shmoo formation by the biologically active mating factor. In competition assays for agglutination, the biologically active mating factor was present at a constant concentration (10 units/mL), and the competitor was 10-fold serially diluted. Competition was judged by the ability of the competitor to eliminate increased agglutination caused by the biologically active mating factor.

Cell-Mediated Hydrolysis of Peptides. MATa cells at 2×10^6 cells/mL from a mid-log phase culture were incubated with the various analogues at concentrations that induced shmoo formation. The supernatant was separated from the cells at various time intervals by centrifugation (Beckman microfuge) and the supernatant frozen and chromatographed within 24 h or chromatographed directly on a μBondapak C_{18} column (Waters Associates) with $CH_3CN/H_2O/CF_3COOH$ as the mobile phase.

Synthesis of Peptides. The des-Trp[1],Cha[3],X[6]-dodecapeptides were prepared by coupling a protected heptapeptide Boc-His(Boc)-Cha-Leu-Gln-X-Lys(Z)-Pro-OH to Gly-Gln-Pro-Met-Tyr-OBzl by use of the mixed anhydride coupling procedure (Meienhofer, 1979). The assembly of the penta- and heptapeptide utilized both isobutyl chloroformate activation and *p*-nitrophenyl active esters. Deprotection was accomplished by using either trifluoroacetic acid in methylene chloride, methanesulfonic acid in formic acid/methylene chloride, or transfer hydrogenolysis. For the most part the strategies employed followed procedures described previously for the synthesis of other analogues of the α-factor (Khan et al., 1981; Shenbagamurthi et al., 1983). Specific details for the synthesis of the des-Trp[1],Cha[3],Ile[6]-dodecapeptide are given in supplementary material, which is available on request. The physical properties of the position 6 analogues are summarized in Table I.

Results

Preparation of α-Factor Analogues. Synthesis of the des-Trp[1],Cha[3],X[6]-α-factor analogues was accomplished by using a combination of mixed anhydride (Meienhofer, 1979) and accelerated active ester coupling reactions (Bodansky, 1979) in solution phase. As discussed in previous communications, the deprotected dodecapeptides were found to contain a number of impurities on HPLC analysis. These impurities result from either incomplete coupling of the hepta- and pentapeptide segments used to assemble the dodecapeptide or side reactions during the deprotection step. A number of attempts were made to improve the 7 plus 5 coupling step, including the use of HOBt, DCC–HOBt, and a variety of solvents. In no case, however, did these attempts significantly

[1] Abbreviations: Ac, acetyl; AcOH, acetic acid; Boc, *tert*-butoxycarbonyl; *n*-BuOH, 1-butanol; CD, circular dichroism; Cha, β-cyclohexylalanine; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; HOBt, 1-hydroxybenzotriazole; EtOAc, ethyl acetate; Et_2O, diethyl ether; EtOH, ethanol; HPLC, high-performance liquid chromatography; IBC, isobutyl chloroformate; MeOH, methanol; MSA, methanesulfonic acid; Nle, norleucine; NMM, *N*-methylmorpholine; OBzl, benzyl ester; OMe, methyl ester; ONp, *p*-nitrophenyl ester; PE, petroleum ether; THF, tetrahydrofuran; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; Z, benzyloxycarbonyl.

Table I: Summary of Chemical and Physical Properties of Position 6 Analogues of Des-Trp[1],Cha[3]-α-factor[a]

position 6 residue	Ala	Cha	Gln	Gly	His	Ile	Leu	Lys	Met	Nle	Pro	Tyr	Val	R_f(B)[b]	R_f(C)[b]	K'[c]	$[\alpha]^{24}_D$[d] (deg)
Ala	1.1	1.1	2.0	1.1	0.9		0.9	1.0	0.9		1.8	0.9		0.18	0.48	2.0	−25.0
Ile		1.1	2.1	1.0	1.0	0.9	1.0	0.9	1.0		2.0	0.9		0.23	0.52	6.5	−34.6
D-Leu		1.1	2.1	0.9	1.1		1.8	1.0	1.0		2.1	0.9		0.26	0.55	7.5	−32.0
Nle		1.1	2.0	1.0	1.1		1.0	0.9	1.0	1.0	2.1	1.0		0.24	0.52	8.1	−44.0
Val		1.1	2.1	0.9	1.0		1.0	1.0	1.0		1.9	1.0	1.0	0.23	0.48	4.9	−26.3

[a] Amino acid ratios are rounded to the nearest tenth. The expected amino acids with the number of residues in parentheses for des-Trp[1],Cha[3],X[6]-dodecapeptide, where X is not designated, are Cha (1), Gln (2), Gly (1), His (1), Leu (1), Lys (1), Met (1), Pro (2), and Tyr (1). [b] Chromatography conducted on thin layers of silica. System B, 1-butanol/acetic acid/water (4:1:5, upper phase); system C, 1-butanol/acetic acid/water/pyridine (15:3:12:10). [c] K' is defined as $(V_p - V_f)/V_f$, where V_p = the elution volume for the peptide and V_f = the breakthrough volume. The value reported is for a C_{18} reversed-phase column using methanol/water/trifluoroacetic acid (400:600:0.25) as the mobile phase. [d] c 0.11, CH_3COOH.

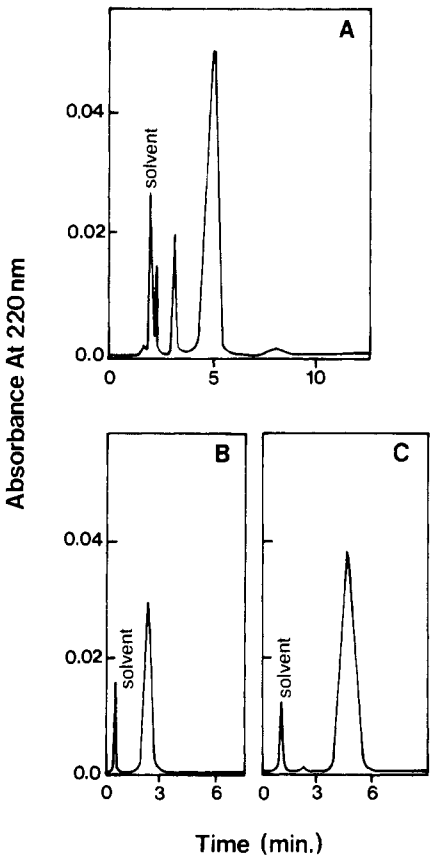

FIGURE 1: HPLC of des-Trp[1],Cha[3],Val[6]-α-factor on a C_{18} reversed-phase column. (Panel A) Crude peptide before purification; mobile phase, $CH_3OH/H_2O/CF_3COOH$ (400:600:0.25). (Panel B) Peptide after purification; mobile phase, $CH_3CN/H_2O/CF_3COOH$ (200:800:0.25). (Panel C) Peptide after purification; mobile phase, $CH_3OH/H_2O/CF_3COOH$ (400:600:0.25).

improve the homogeneity of the crude deprotected dodecapeptide or the yield of pure peptide. Despite this difficulty we were able to obtain essentially homogeneous dodecapeptides after a one-step isocratic purification on reversed-phase silica using a $CH_3OH/H_2O/CF_3COOH$ mobile phase (Figure 1). All des-Trp[1],Cha[3],X[6]-dodecapeptides (X = Ala, Ile, D-Leu, Val, Nle) were homogeneous on silica thin layers, were >98% pure on HPLC using two mobile phases, and gave the expected amino acid ratios (Table I). The retention time on reversed-phase columns correlated with the hydrophobicity of the position 6 side chain with the Nle[6] analogue eluting last off the column and the Ala[6]-dodecapeptide eluting first. The diastereomeric L-Leu[6] and D-Leu[6] analogues exhibited different retention times on reversed-phase chromatography with the D-Leu[6] analogue (K' = 7.5) being retained more strongly than the L-Leu[6]-dodecapeptide (K' = 7.0). The separation of diastereomeric peptides on HPLC has been noted previously (Rivier & Burgus, 1979).

Table II: Biological Activity of Des-Trp[1],Cha[3],X[6]-α-factors

α-factor analogue	morphogenesis act. (ng/mL)	agglutination act. (ng/mL)	ratio
des-Trp[1] [a]	720 (12)[c]	8 (2)[c]	90
des-Trp[1],Cha[3],L-Leu[6] [b]	270 (12)[c]	35 (2)[c]	8.0
des-Trp[1],Cha[3],Ala[6]	NA[d]	NA[e]	
des-Trp[1],Cha[3],Val[6]	2650 (3)[c]	12000 (3)[c]	0.2
des-Trp[1],Cha[3],Ile[6]	1500 (3)[c]	5200 (3)[c]	0.3
des-Trp[1],Cha[3],D-Leu[6]	NA[d]	NA[e]	
des-Trp[1],Cha[3],Nle[6]	270 (6)[c]	1200 (3)[c]	0.2

[a] Natural sequence dodecapeptide. [b] Parent compound for position 6 analogues. [c] Number of independent trials. [d] Not active at 500 μg/mL. [e] Not active at 50 μg/mL.

Ability of Des-Trp[1],Cha[3]-X[6]-dodecapeptides To Induce Altered Yeast Morphologies. Incubation of *a* haploids with the natural α-factor results in cessation of growth and the formation of nonbudded elongated cells termed shmoos (Levi, 1956). This change in morphology of yeast cells has been extensively employed as a measure of the activity of the mating pheromone and various analogues (Masui et al., 1979; Shenbagamurthi et al., 1983; Thorner, 1981). Certain position 6 analogues were observed to induce the formation of morphologically aberrant shapes upon incubation with *S. cerevisiae* X2180-1A. The morphological changes caused by the position 6 analogues were similar to those induced by other dodecapeptides but different from those induced by the tridecapeptide pheromone (Shenbagamurthi et al., 1983). Since the analogues examined are all related to the des-Trp[1],Cha[3] pheromone, we compare the effects of position 6 substitution to this parent compound (Table II). We found that the Nle[6] analogue caused morphogenesis at a concentration of 270 ng/mL. This was virtually identical with the activity of the pheromone containing the naturally occurring L-leucine at position 6. In contrast, replacement of L-leucine by L-isoleucine or L-valine resulted in a 6- or 10-fold reduction, respectively, in biological activity as judged by the morphogenesis assay. Neither the L-Ala[6]-dodecapeptide nor the D-Leu[6]-dodecapeptide induced aberrant cell morphologies at concentrations up to 500 μg/mL. Thus these analogues were at least 2000 times less active than their parent compound.

Position 6 Analogues Induce Increased Agglutinability of a Cells. The position 6 analogues that affected the morphology of *MATa* cells also increased the agglutinability of these yeasts (Table II). Dose–response curves for the des-Trp[1],Cha[3]-dodecapeptide and the Ile[6] and Val[6] analogue are represented in Figure 2. It is clear that for various analogues the concentration of pheromone that causes half-maximal agglutination varies over several orders of magnitude. In contrast to the results of the morphogenesis assay, replacement of Leu[6] by Nle[6] in the des-Trp[1]-α-factor causes a 34-fold reduction

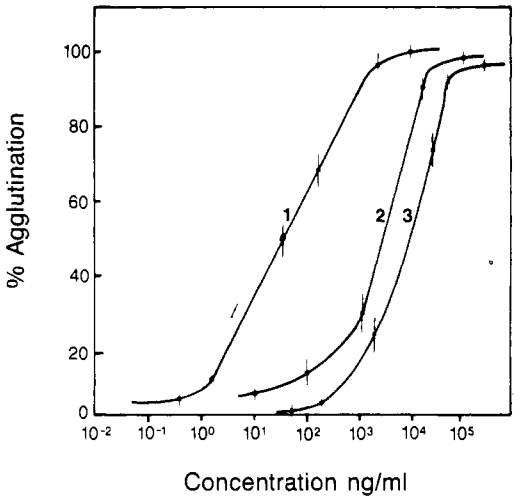

FIGURE 2: Concentration dependence of pheromone-induced agglutination. *MATa* cells were incubated with various concentrations of pheromone as described under Materials and Methods. Percent agglutination is relative to biologically induced and uninduced controls: (1) des-Trp[1],Cha[3]-α-factor, (2) des-Trp[1],Cha[3],Ile[6]-α-factor, and (3) des-Trp[1],Cha[3],Val[6]-α-factor.

in the ability of the pheromone to induce increased agglutination (Table II). Similarly, replacement by Ile[6] and Val[6] results in 150- and 340-fold decreases in agglutination activity, respectively. Neither the Ala[6] analogue nor D-Leu[6] analogue was active in the agglutination assay at concentrations up to 50 μg/mL. These analogues are at least 1500 times less active in this assay, therefore, than the parent compound. In addition to the failure of the Ala[6] and D-Leu[6] analogues to elicit changes in the shape or agglutinability of *MATa* cells, these pheromones did not compete with the biological activity of the parent compound (des-Trp[1],Cha[3]-dodecapeptide) at ratios of 500:1.

The naturally occurring dodeca- and tridecapeptide α-factors induce morphogenesis at concentrations 100 times those necessary for induction of agglutination (Baffi et al., 1984). In previous studies we have observed that the potency in the morphogenesis and agglutination assays varied somewhat for synthetic analogues; however, in every case except one the pheromone induced agglutinability at concentrations lower than those inducing morphogenesis. As seen in Table II the Nle[6]-, Ile[6]-, and Val[6]-dodecapeptides are 3–5-fold better inducers of morphogenesis than they are inducers of increased agglutinability. This finding should be contrasted with that for the parent compound des-Trp[1],Cha[3],Leu[6]-dodecapeptide, which is an 8-fold better morphogen than it is an inducer of agglutinability.

Hydrolysis of Position 6 Analogues by S. cerevisiae X2180-1A (MATa). When *a* haploids of *S. cerevisiae* are treated with the naturally occurring α pheromone, they eventually recover from G1 arrest and resume normal asexual reproduction (Thorner, 1981). A major aspect of recovery is degradation of the pheromone by proteolytic cleavage (Ciejek & Thorner, 1979; Finkelstein & Strausberg, 1979; Manness & Edelman, 1978). The ability of whole cells to cleave the position 6 analogues was examined by using an HPLC assay that determines disappearance of the intact pheromone and appearance of the degradation product. As seen in Figure 3, incubation of the des-Trp[1],Cha[3]-α-factor with 2 × 10^6 cells/mL results in a reduction of the peak that elutes at 7.0 ± 0.1 min and the appearance of a new peak at 10.5 ± 0.1 min. The peak at ~7.0 min is associated with the intact pheromone whereas the peak at 10.5 min has a mobility identical with (the synthetic standard) His-Cha-Leu-Gln-Leu. Material eluting at retention times less than 6 min is associated

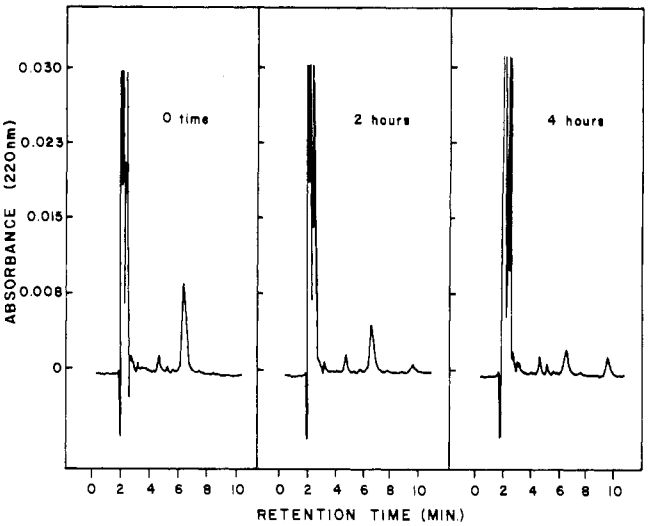

FIGURE 3: Cell-mediated hydrolysis of des-Trp[1],Cha[3]-α-factor. The pheromone (10 μg/mL) was incubated with *S. cerevisiae* X2180-1A (*MATa*) (2 × 10^6 cells/mL) at 30 °C. At various times portions were removed, separated from cells by centrifugation, and analyzed by reversed-phase HPLC. The mobile phase was $CH_3CN/H_2O/CF_3COOH$ (280:720:0.35). Panels represent 0-, 2-, and 4-h time points as shown.

Table III: Degradation Rates of Synthetic α-Factors

peptide	$t_{1/2}$ (min)[a]
natural sequence tridecapeptide	70
des-Trp[1],Cha[3]	105
des-Trp[1],Cha[3],Ala[6]	>360[b]
des-Trp[1],Cha[3],Ile[6]	>360[b]
des-Trp[1],Cha[3],Nle[6]	240
des-Trp[1],Cha[3],Val[6]	>360[b]
des-Trp[1],Cha[3],D-Leu[6]	>360[b]

[a] Whole cells (2 × 10^6 cells/mL) were incubated with the peptide (10 μg/mL) in yeast nitrogen base at 30 °C. At various times portions were removed and examined by HPLC (see legend to Figure 3). The $t_{1/2}$ was determined by plotting the area under the peak corresponding to the pheromone vs. time on a semilog graph. [b] Not degraded after a 6-h incubation.

with the yeast nitrogen base, the medium used for the experiment. These results suggest that the des-Trp[1],Cha[3] analogue is hydrolyzed by cleavage at the Leu[6]–Lys[7] peptide bond, a result expected on the basis of the known pathway of degradation of the natural sequence α-factor. The natural sequence dodecapeptide has a $t_{1/2}$ of 70 min when incubated at a peptide concentration of 10 μg/mL with 2 × 10^6 cells/mL. Under identical conditions, the des-Trp[1],Cha[3] analogue has a $t_{1/2}$ of 105 min and the Nle[6] analogue has a $t_{1/2}$ of 240 min. None of the other position 6 analogues were degraded during a 6-h incubation with whole cells (Table III). In addition, des-Trp[1],Cha[3],Ala[6]-dodecapeptide does not inhibit the degradation of the natural sequence tridecapeptide when both are present at concentrations of 10 μg/mL.

In order to ascertain whether α-factor destruction was a contributing factor in pheromone potency, we conducted the following experiment. The natural sequence tridecapeptide, which is the most labile pheromone that we have examined (Table III), was incubated with *a* cells in the morphogenesis assay. After a 4-h incubation, the cells were sedimented, and the supernatant was incubated with fresh *a* cells. Morphogenesis was determined on the fresh cells after an additional 4-h incubation. The lowest active concentration in both incubations, 61 ng/mL, was slightly lower than the mean threshold value reported previously (Baffi et al., 1984). These results show that significant destruction of the tridecapeptide

did not occur during the initial incubation.

Discussion

The results presented herein confirm that the ability of α-factor pheromones to induce agglutination can be modified independently of their ability to induce morphological changes in yeast cells. In reaching this conclusion, we have compared the concentration of α-factor necessary to induce a 50% increase in agglutination with the lowest concentration of pheromone that caused morphogenesis in *a* cells of *S. cerevisiae*. We have used such comparisons previously (Baffi et al., 1984), and our studies have shown that dose–response curves for both morphogenesis and induction of agglutination are usually a series of parallel curves (Figure 2 and unpublished data). We believe, therefore, that such a comparison is valid and provides insights into the variation of agglutination and morphogenic potencies with amino acid replacement in the α-factor.

Compared to the des-Trp^1,Cha^3-dodecapeptide (Leu^6 parent compound), the Nle^6 analogue is an equally good morphogen but a 34-fold poorer inducer of agglutination. Although the des-Trp^1,Cha^3,Ile^6-dodecapeptide shows a 6-fold decrease in morphogenic potency compared to the Leu^6 parent compound, it is 150 times less active in the agglutination assay. Similar results were observed for the des-Trp^1,Cha^3,Val^6 analogue, which is a 10-fold poorer morphogen and 340-fold poorer inducer of agglutinability. These findings suggest that the position 6 side chain is more important in determining the agglutination potency of α-factor than in determining its morphogenic potency. In previous studies most α-factor analogues caused increased agglutinability at concentrations that were significantly lower than those required to induce morphogenesis. The natural sequence dodecapeptide and tridecapeptide induce morphogenesis at concentrations that are approximately 100 times greater than the concentration needed to induce agglutination (Baffi et al., 1984). For the des-Trp^1,Cha^3,Nle^6 and des-Trp^1,Cha^3,Val^6 analogues the concentration determined in the morphogenesis assay was 5 times less than that determined in the agglutination assay. Thus the ratio of active concentrations for morphogenesis and agglutinability varies 500-fold among active α-factors.

The structure–activity studies show that a γ-branch (leucyl residue) at the position 6 side chain is not essential for biological activity, as linear (norleucyl) or β-branched (isoleucyl and valyl) analogues retain both morphogenic and agglutinogenic potencies. However, it is clear that β-branching decreases the activity of the dodecapeptides in both assays since both the Val^6 and Ile^6 analogues are significantly inferior pheromones compared to their Leu^6 or Nle^6 homologues. It also appears that biological activity of the α-factor pheromones requires a minimum hydrophobic bulk at the 6-position side chain as the Ala^6 analogue was inactive up to 500 000 ng/mL and was not able to compete with the activity of an active pheromone. The inactivity of the des-Trp^1,Cha^3,D-Leu^6 analogue may be a consequence of the chirality at this position, which changes the topological relationship of the side chain and the receptor. However, the CD spectrum of the D-Leu^6 analogue differs from those of the other peptides (unpublished data), and it is possible that this difference reflects conformational perturbations. Thus the inactivity of the D-Leu^6 analogue may result from the fact that the peptide does not assume the biologically active conformation.

In a recent study, Moore (1983) has presented kinetic data which suggest that agglutination and cell cycle arrest are induced at 100-fold lower concentrations of natural α-factor than induction of projection (shmoo) formation. She proposed that the differences in pheromone concentration required for the various biological activities might reflect two classes of α-factor receptors or different levels of receptor occupancy. The results of the present paper combined with previous findings (Baffi et al., 1984) support the multiple receptor hypothesis and are not compatible with models requiring variable receptor occupancy. This conclusion is based on our observation that certain analogues of α-factor can induce morphogenesis with a minimal increase in agglutinability while others can induce agglutination without morphogenesis.

α-Factor is known to be hydrolyzed by *MATa* cells. Thus, care was taken to monitor degradation of pheromone during the course of the bioassay, because those pheromones that are rapidly degraded might apppear to have low activities while those that are slowly degraded might give high activities. Our results suggest that degradation of the pheromones is not affecting our overall interpretation of the biological activities. (1) The morphogenesis assays are done at low cell densities (3×10^3 cells/mL). At its threshold concentration (61 ng/mL) the natural tridecapeptide was not significantly degraded during the morphogenesis assay since the supernatant medium retained full activity. In addition, Moore (1983) determined a second-order rate constant for α-factor inactivation by strain 381G and found, at low cell densities (10^2 cells/mL), that *S. cerevisiae* 381G and *S. cerevisiae* X2180-1A were arrested at nearly identical α-factor concentrations. Thus it may be assumed that the two strains degrade tridecapeptide at very similar rates. Using Moore's rate constant, we calculate that >99% of the natural α-factor should be present at the end of the shmoo assay with strain X2180-1A. Since all analogues of α-factor are degraded more slowly than the tridecapeptide, no significant degradation of these pheromones should occur during the course of the morphogenesis assay. (2) One might argue that due to the high cell densities used in the agglutination assays (2×10^7 cells/mL) the breakdown of pheromones is significant. Indeed, using our $t_{1/2}$ for degradation (Table III) and assuming first-order kinetics, we calculate that only 25% of the des-Trp^1,Cha^3-α-factor and 60% of the des-Trp^1,Cha^3,Nle^6-α-factor would remain after the agglutination assay (20-min incubation). We previously showed that for the tridecapeptide α-factor induction of increased agglutinability was complete after a 1-min incubation (Baffi et al., 1984). The des-Trp^1,Cha^3- and des-Trp^1,Cha^3,Nle^6-dodecapeptides also induce agglutination, rapidly causing greater than 60–70% of the final agglutinability increase within 2 min of exposure (unpublished data). Using our half-lives, we calculate that more than 90% of these pheromones remain intact after a 2-min incubation with the cell densities employed in the agglutination assay. (3) Finally, the most potent α-factor peptides in the agglutination assay are those that are degraded most rapidly (tridecapeptide, des-Trp^1-dodecapeptide, des-Trp^1,Cha^3-dodecapeptide), and the three position 6 analogues with the lowest agglutination activities (Ile^6, Val^6, Ala^6) are not degraded. Thus, if degradation is influencing the ratio of the potencies in the morphogenesis and agglutination assays, then the actual differences are greater than those that we report.

A great amount of information has accumulated on the genetics of the mating process in *S. cerevisiae* (Sprague et al., 1983). The genes involved in the *MAT* locus are being characterized, and the gene coding for α-factor precursor has been cloned (Kurjan & Herskowitz, 1982). Recently, a binding assay for the receptor has been developed by using ^{35}S-labeled α-factor, and mention was made of the cloning of the α-factor receptor gene (Jenness et al., 1983). Although

the pheromone employed in the binding assay was only 15% pure, it is now apparent that biochemical investigations can be conducted on the interaction of α-factor and its receptor. Important aspects of such investigations will be an analysis of the role that the various residues of α-factor play in binding to the receptor and the ability to study binding independently of degradation. Well-characterized analogues of α-factor will play an important role in these studies. It will be of great interest to determine the correlation of binding studies with the various biological responses that the mating pheromone triggers.

ACKNOWLEDGMENTS

We thank Charlotte Boney for excellent technical assistance.

SUPPLEMENTARY MATERIAL AVAILABLE

Description of reagents and chemicals used and details of the synthesis of the des-Trp[1],Cha[3],Ile[6]-dodecapeptide (5 pages). Ordering information is given on any current masthead page.

Registry No. α-Factor, 59401-28-4; des-Trp[1]-α-factor, 59401-29-5; des-Trp[1],Cha[3],L-Leu-α-factor, 78395-56-9; des-Trp[1],Cha[3],Ala[6]-α-factor, 96212-39-4; des-Trp[1],Cha[3],Val[6]-α-factor, 96212-40-7; des-Trp[1],Cha[3],Ile[6]-α-factor, 96212-41-8; des-Trp[1],Cha[3],D-Leu-α-factor, 96290-36-7; des-Trp[1],Cha[3],Nle-α-factor, 96212-42-9.

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