Structure–Activity Relationships in the Dodecapeptide α Factor of *Saccharomyces cerevisiae*†

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ABSTRACT: Ten analogues of His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr, the dodecapeptide α factor of *Saccharomyces cerevisiae*, were synthesized by conventional solution phase techniques and purified by using high-performance liquid chromatography. The dodecapeptide was also synthesized attached at its carboxyl terminus to poly(ethylene oxide), a macromolecular protecting group. Analogues in which Lys[6] or His[1] was modified exhibited high biological activity as evidenced by their ability to elicit aberrant morphologies in *a* cells of *S. cerevisiae*. These results suggest that neither a free α-amine nor a protonatable side chain at position 6 is necessary for biological activity of the dodecapeptide α factor. Although Ala[2]- and Phe[2]-dodecapeptides were not biologically active, they competed with the natural α factor and several active analogues. Thus binding of the α factor is not sufficient to elicit a biological response; it appears that the side chain in position 2 is critical for triggering morphological alterations in *a* cells.

Cell to cell interaction in *Saccharomyces cerevisiae* mating types, termed *a* and α, is initiated by peptide pheromones (Thorner, 1980; Manney & Meade, 1977). The α cell produces a pheromone, designated α factor, which upon interaction with the target *a*-mating type cell affects DNA synthesis, in vitro activity of plasma membrane bound adenylate cyclase, cell–cell adhesion, and the morphology of the *a*-mating type (Thorner, 1980; Liao & Thorner, 1981). Most effects of the α factor appear to be specific for *a*-mating type *S. cerevisiae*. Indeed the biological activity of α factor is commonly monitored by following a transient morphological change in *a* cells, an elongation of the cell in lieu of budding, and is called the shmoo assay (Hartwell, 1970).

From the culture supernatant of the α-mating type cell, a dodecapeptide and a tridecapeptide have been isolated and are reported to have identical biological activity; the dodecapeptide lacks an amino-terminal tryptophanyl residue and may be an artifact of purification procedures. The sequence of the tridecapeptide pheromone has been determined (Stotzler et al., 1976), and it has been synthesized by using both solid phase and solution phase procedures (Masui et al., 1977; Ciejek et al., 1977).

Our initial studies on analogues of the dodecapeptide (Khan et al., 1981) showed that certain structure–activity relationships differed from those reported for analogues of the tridecapeptide α-factor sequence, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr (Masui et al., 1979). Specifically whereas the Ala[2] analogue of the tridecapeptide had a relatively high activity, the Ala[2] analogue of the dodecapeptide did not cause morphological aberrations in *a* cells. In this paper we report additional findings on a number of dodecapeptide analogues. Our results provide the first conclusive evidence that a protonatable α- or ε-amine is not necessary for biological activity of the pheromone and suggest that certain analogues can bind to the receptor without triggering morphological changes in *a* cells of *S. cerevisiae*.

Materials and Methods

Chemicals. Protected amino acids and amino acid active esters were purchased from Bachem Inc., Torrence, CA. Biotin *p*-nitrophenyl ester and *p*-nitrophenyl acetate were purchased from Sigma Chemical Co., St. Louis, MO, and α-Dns-L-histidine[1] hemihydrate was from Fluka/Tridom Chemical Inc., Hauppauge, NY. This latter amino acid derivative was converted to N^{α}-Dns-N^{im}-Boc-histidine by using the procedure of Khan & Sivanandaiah (1977). All other reagents and solvents are the purest commercially available.

Synthesis and Purification of α-Factor Analogues. All analogues of the dodecapeptide α factor were prepared in solution by using mixed anhydride (Meienhofer, 1979) and 1-hydroxybenzotriazole accelerated active ester coupling procedures (Bodansky, 1979). The tyrosine was protected as the benzyl ester and the ε-amine of lysine as the benzyloxycarbonyl derivative, and di-*tert*-butoxycarbonylhistidine was employed for amino-terminal protection. The protected dodecapeptide was assembled by coupling Pro[7] to Gly[8] by using the mixed anhydride technique. Details for specific steps in the synthesis have been previously reported (Khan et al., 1981). For the preparation of the Cha[2]-ε-Ac-Lys[6], Cha[2]-ε-biotinyl-Lys[6], and Trp[2]-ε-(Dns-Gly)-Lys[6] analogues the corresponding protected dodecapeptide was hydrogenated to remove the N^{ϵ}-benzyloxycarbonyl protecting group from lysine. The crude material was then treated with the appropriate *p*-nitrophenyl active ester to derivatize the N^{ϵ} terminus of lysine. An example of a typical procedure is given below. The final deprotected peptide was purified by using high-performance liquid chromatography (HPLC). The synthesis of the natural tridecapeptide was carried out by a modification of the previously described strategy.

Synthesis of His-Cha-Leu-Gln-Leu-Lys(Ac)-Pro-Gly-Gln-Pro-Met-Tyr. Boc-His(Boc)-Cha-Leu-Gln-Leu-Lys(Z)-Pro-Gly-Gln-Pro-Met-Tyr-OBzl (Khan et al., 1981) (302 mg, 0.16

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[1] Abbreviations: Ac, acetyl; AcOH, acetic acid; Boc, *tert*-butoxycarbonyl; *n*-BuOH, 1-butanol; Cha, β-cyclohexylalanine, DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; Dns, dansyl; EDTH, 1,2-ethanedithiol; HOBt, 1-hydroxybenzotriazole; H-OPEGM, monomethylpoly(ethylene oxide); MeOH, methanol; Nle, norleucine; NMM, *N*-methylmorpholine; OBzl, benzyl ester; ONp, *p*-nitrophenyl ester; ONSu, *N*-hydroxysuccinimide ester; OTcp, trichlorophenyl ester; Py, pyridine; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl.

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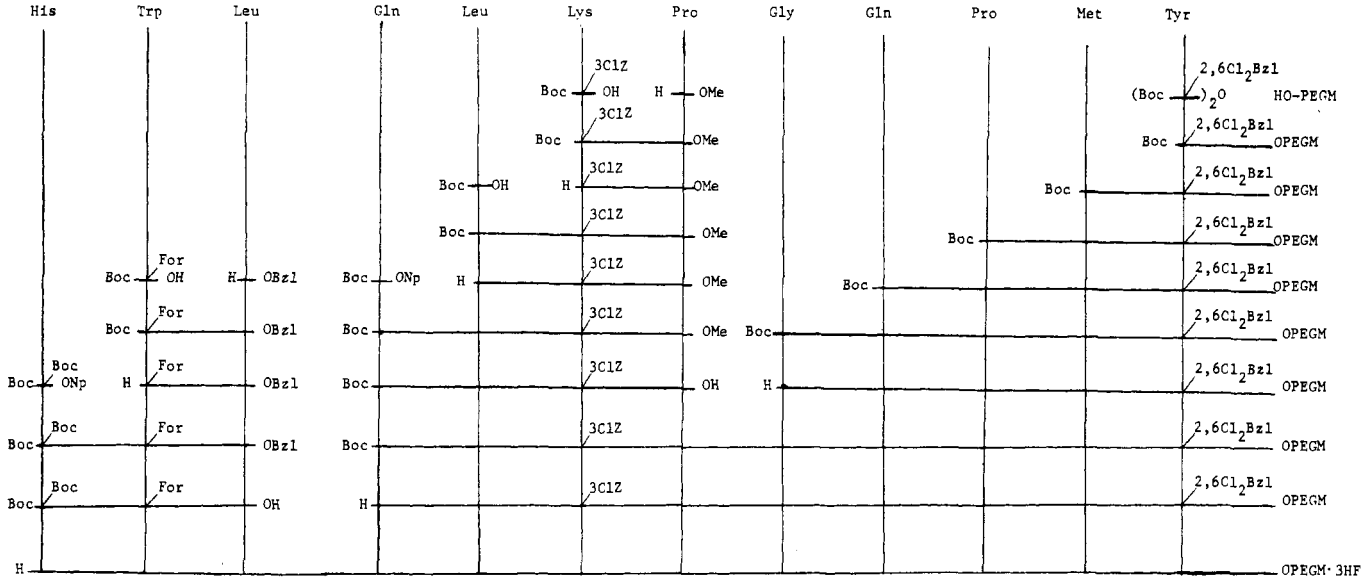
Scheme I: Synthesis of Dodecapeptide α Factor Attached to Poly(ethylene oxide)



mmol) was dissolved in methanol (6 mL), and Pd black (~300 mg) was added. The reaction mixture was treated with 90% HCOOH (0.6 mL) and stirred for 4 h at room temperature. The catalyst was then filtered, the filtrate was evaporated to dryness in vacuo, and the residue was precipitated by addition of ether. The solid obtained was dissolved in DMF (1.5 mL) and treated with *p*-nitrophenyl acetate (32 mg, 0.18 mmol) and NMM (0.036 mL, 0.32 mmol) at room temperature. After 14 h, the DMF was evaporated and the residue triturated with 5% citric acid, filtered, and washed with water and ether. The dry crude product (200 mg) was subjected to acidolysis by using TFA–CH_2Cl_2 (3 mL; 1:1 v/v) containing anisole (0.3 mL). After 30 min the solvent was removed in vacuo, and ether precipitation yielded the crude-free peptide, which was purified by HPLC in MeOH–H_2O–TFA (400:600:0.25 v/v/v): yield 33 mg (11.9% based on protected dodecapeptide); $[\alpha]^{25}_D$ −40° (*c* 0.2, AcOH); R_f (*n*-BuOH–AcOH–H_2O, 4:1:5; upper phase) 0.19; R_f (*n*-BuOH–AcOH–H_2O–Py, 15:3:12:10) 0.61.

Synthesis of Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr. Boc-His(Boc)-Trp-Leu-Gln-Leu-Lys(Z)-Pro-Gly-Gln-Pro-Met-Tyr-OBzl (Khan et al., 1981) (0.768 g, 0.4 mmol) was treated with TFA–CH_2Cl_2 (5 mL; 1:1 v/v) in the presence of EDTH (0.3 mL) for 30 min. The reaction mixture was evaporated in vacuo, and the residue was precipitated by ether, filtered, and dried. The resulting dodecapeptide trifluoroacetate was dissolved in DMF (4 mL), neutralized with NMM (0.088 mL, 0.8 mmol), and allowed to react with Z-Trp-ONp (0.193 g, 0.42 mmol) for 18 h at room temperature. The solvent was evaporated in vacuo, the residue triturated with 5% citric acid, filtered, washed with water, and dried (0.834 g, 93%). The crude product (750 mg) was dissolved in methanol (10 mL), Pd black (~400 mg) was added, and the mixture was treated with 90% HCOOH (1 mL). After 4 h of stirring at room temperature additional Pd black (~200 mg) and 90% HCOOH (0.4 mL) were added. The reaction was allowed to proceed for 12 h at which time the catalyst was removed by filtration, the filtrate evaporated in vacuo, and the crude-free tridecapeptide precipitated by ether (600 mg). The above crude material was dissolved in 10 mL of MeOH–H_2O–TFA (450:550:0.25 v/v/v) and injected onto the C_{18} column of the Prep LC/System 500, which had been equilibrated with the same solvent system. The column was eluted, and the fractions were analyzed on analytical HPLC. When the pure tridecapeptide started to elute, the mobile phase was changed to MeOH–H_2O–TFA (490:510:0.25 v/v/v). The main peak was pooled and evaporated in vacuo at room temperature. The residue was dissolved in water (10 mL) and filtered through a microfilter (0.45 μm), and the filtrate was freeze-dried: yield 106 mg (15.4% based on protected tridecapeptide); $[\alpha]^{25}_D$ −34.7° (*c* 0.11, AcOH); R_f (*n*-BuOH–AcOH–H_2O, 4:1:5; upper phase) 0.18; R_f (*n*-BuOH–AcOH–H_2O–Py, 15:3:12:10) 0.54.

Synthesis of Dodecapeptide-Mating Factor Attached to Poly(ethylene oxide). The dodecapeptide α factor was attached to poly(ethylene oxide) by using the liquid phase method for peptide synthesis (Mutter & Bayer, 1980). The general strategy involved attachment of protected tyrosine at its carboxyl group to monomethylpoly(ethylene oxide), average M_r 3700, and stepwise addition up to the pentapeptide, followed by addition of tetrapeptide and tripeptide fragments (Scheme I). Coupling and deprotection were monitored by a colorimetric ninhydrin assay. After final deprotection using anhydrous HF, the peptide-poly(ethylene oxide) conjugate gave the expected amino acid analysis: Glu, 2.00; Gly, 1.06; His, 0.94; Leu, 2.00; Lys, 0.99; Met, 1.00; Pro, 1.92; Trp, 0.84; Tyr, 0.90. Details of the synthesis are given below.

The first amino acid Tyr was esterified to monomethylpoly(ethylene oxide) by allowing the symmetrical anhydride obtained from Boc-Tyr(2,6-Cl_2Bzl) (16 mmol) to react with H-OPEGM (20 g, containing 4 mmol of OH group). The esterification reaction was carried out at 0 °C for 0.5 h, at 25 °C for 20 h, and at 40 °C for 3 h. The polymer was precipitated with ether and recrystallized from ethanol. A spectrophotometric estimation with Boc-Tyr(2,6-Cl_2Bzl) ethyl ester as the standard indicated that more than 98% of the polymer chains were esterified. The remaining OH groups were protected by acetylation (Ac_2O, 3 mL; pyridine, 1 mL; 24 h at 25 °C) in CH_2Cl_2. The resulting Boc-Tyr(2,6-Cl_2Bzl)-OPEGM was recrystallized from ethanol and dried over P_2O_5; yield 20.5 g.

The carboxy-terminal pentapeptide segment of the mating factor attached to poly(ethylene oxide) was synthesized starting with the above polymer by stepwise addition of *N*-acylamino acid active esters in the presence of 1-hydroxybenzotriazole (HOBt). Thus, Boc-Tyr(2,6-Cl_2Bzl)-OPEGM (10 g) was first treated with TFA–CH_2Cl_2–anisole (1.2:1:0.2; 70 mL, 0.5 h at 25 °C). The TFA and CH_2Cl_2 were then evaporated under

reduced pressure, and the polymer was precipitated with ether, isolated, and dried over KOH.

Tyr(2,6-Cl_2Bzl)-OPEGM was then reacted with Boc-Met-OTcp (2.22 g, 2 equiv) in DMF (50 mL) in the presence of HOBt (0.39 g) and NMM (0.28 mL) for 18 h at 25 °C. The resulting dipeptide polymer was isolated by precipitation with ether. The coupling was estimated to be 99% by a ninhydrin spectrophotometric assay. The coupling was repeated with 1 equiv of the active ester to ensure complete acylation of the amino group; in the resulting dipeptide polymer >99.4% coupling was determined by using the ninhydrin method. Since this level of coupling approximated the sensitivity of the spectrophotometric assay, chain elongation was continued by repeating the above sequence of acidolysis and coupling successively to incorporate Pro, Gln, and Gly; the active esters used were Boc-Pro-ONSu, Boc-Gln-ONp, and Boc-Gly-OTcp, respectively.

For completion of the synthesis of the dodecapeptide α-mating factor sequence, the remaining seven amino acids were incorporated by coupling of two segments, Boc-Gln-Leu-Lys(3-Cl-Z)-Pro and Boc-His(Boc)-Trp(Form)-Leu. These partially protected peptides were synthesized by conventional methods.

Boc-Gly-Gln-Pro-Met-Tyr(2,6-Cl_2Bzl)-OPEGM (1.8 g, containing 0.415 mmol of the peptide) was deblocked at the amino terminus with TFA–CH_2Cl_2–anisole (1.2:1:0.2; 14 mL, 0.5 h at 25 °C), and the pentapeptide polymer was isolated by precipitation from Et_2O. The dried polymer was reacted with Boc-Gln-Leu-Lys(3-Cl-Z)-Pro (0.625 g, 0.83 mmol) in DMF (20 mL) by using DCC (0.17 g, 0.83 mmol) in the presence of HOBt (0.25 g, 1.66 mmol) and NMM (0.1 mL). The coupling was carried out at 0 °C for 2 h and at 25 °C for 20 h, and the product was isolated by precipitation. After the coupling reaction using 1 equiv of Boc-Gln-Leu-Lys(3-Cl-Z)-Pro was repeated, the protected nonapeptide polymer was recrystallized from ethanol and dried (yield 2.2 g, coupling >99% by ninhydrin assay).

The nonapeptide polymer (1.8 g, containing 0.335 mmol of the peptide) was treated with TFA–CH_2Cl_2–anisole (1.2:1:0.2; 20 mL, 40 min at 25 °C). The deblocked peptide polymer was dissolved in DMF (18 mL) and reacted with Boc-His-(Boc)-Trp(Form)-Leu (0.52 g, 0.76 mmol) by using DCC (0.16 g, 0.76 mmol) in the presence of HOBt (0.2 g, 1.3 mmol) and NMM (0.1 mL). The reaction was carried out at 0 °C for 1 h and at 25 °C for 22 h. The peptide polymer was isolated as usual, and the coupling was repeated with 1 equiv of tripeptide fragment. The ninhydrin assay indicated 98.8% coupling at this step of the synthesis.

The Boc, 3-Cl-Z, and 2,6-Cl_2Bzl groups were cleaved from the fully protected dodecapeptide polymer (0.4 g) by treatment with liquid hydrogen fluoride–anisole [HF–anisole (9:1) for 45 min at 0 °C]. The deblocked dodecapeptide polymer was isolated after evaporation of the HF and recrystallized from ethanol. Analysis of ultraviolet spectrum of the conjugate indicated that the formyl protecting group on the tryptophan residue was missing in the final product. There was, therefore, no need to subject the dodecapeptide–OPEGM conjugate to piperidine treatment. The above dodecapeptide–OPEGM conjugate was used in biological studies without further purification.

Cleavage of Peptide Fragments from the Poly(ethylene oxide) Support. The goal of the liquid phase synthesis was preparation of α factor attached to a macromolecular protecting group. Although coupling and cleavage reactions were close to quantitative based on a spectrophotometric assay, we attempted to assess the homogeneity of the peptide–OPEGM conjugates at various stages of synthesis. This was accomplished by cleaving peptide fragments and examining their purity by using HPLC analysis.

Table I: Cleavage of Peptides from Poly(ethylene oxide)

peptide polymer	yield (%)[a]
Hydrazinolysis	
Boc-Tyr(2,6-Cl_2Bzl)-OPEGM	89
Boc-Met-Tyr(2,6-Cl_2Bzl)-OPEGM	79
Boc-Pro-Met-Tyr(2,6-Cl_2Bzl)-OPEGM	78
Boc-Gly-Gln-Pro-Met-Tyr(2,6-Cl_2Bzl)-OPEGM	86
Saponification	
Boc-Tyr(2,6-Cl_2Bzl)-OPEGM	60
Boc-Met-Tyr(2,6-Cl_2Bzl)-OPEGM	80
Boc-Pro-Met-Tyr(2,6-Cl_2Bzl)-OPEGM	78

[a] The indicated peptide-polymer conjugate was treated as described under Materials and Methods. The yield is based on the dry weight of recovered peptide after cleavage from the peptide polymer conjugate. All calculations assume that coupling reactions are quantitative (see text).

(*1*) *Hydrazinolysis* was carried out in ethanol by using 98% hydrazine hydrate. In a typical experiment, Boc-Gly-Gln-Pro-Met-Tyr(2,6-Cl_2Bzl)-OPEGM (1.1 g) was dissolved in ethanol (10 mL) and treated with hydrazine hydrate (0.15 mL) for 18 h at 25 °C. The solvent was evaporated and the residue treated with water (20 mL), and the precipitate was isolated, washed with water, and dried; yield 0.20 g.

(*2*) *Saponification* of the peptide polymer was carried out as follows. In a typical experiment, Boc-Met-Tyr(2,6-Cl_2Bzl)-OPEGM (0.6 g) was dissolved in 0.25 N NaOH (2 mL) and the solution kept at 25 °C for 1.5 h. Acidification with 0.1 N HCl deposited a solid, which was isolated and dried; yield 0.07 g (80%). Yields for saponification or hydrazinolysis using the above conditions are summarized in Table I.

HPLC Purification of Peptides. All synthetic peptides were purified by using HPLC on a preparative μBondapak C_{18} reversed-phase column (Waters Prep LC/System 500) with methanol–water–trifluoroacetic acid mixtures as the mobile phase. In a typical procedure 125–500 mg of crude peptide was loaded on the preparative HPLC column and eluted by using a step gradient. The use of the volatile trifluoroacetic acid in the mobile phase was essential in order to obtain sharp peaks and eliminated the need for desalting of the final product. All peptides were >98% homogeneous as judged by analytical HPLC in two different mobile phases and gave one ninhydrin positive, UV positive spot on silica thin layers using two different eluent systems. A typical HPLC profile for the crude and purified peptide is shown in Figure 1 for the Cha^2-D-[α-^{2}H]Ala^8-dodecapeptide analogue. Further details on the chromatographic procedures will be reported elsewhere.

Other Procedures. Amino acid analyses were performed at the Weizmann Institute of Science, Rehovot, Israel, or at Hoffmann-La Roche, Inc., Nutley, NJ. Peptides were hydrolized in sealed tubes in 6 N HCl at 100 °C for 24 h. For peptides containing methionine, anisole was added as a scavenger. All peptides except the N^{α}-Dns-His^1 analogue gave the expected ratios within ±5%. For the N^{α}-Dns-His^1 analogue all amino acids in the amino-terminal fragment gave higher than expected ratios. The results were consistent with a minor contamination by the N-terminal heptapeptide. The presence of this impurity was confirmed by HPLC. Since the heptapeptide exhibited no biological activity, its presence at very low amounts in the assay did not affect the results.

Biological Assay of α-Factor Activity. The ability of various analogues to cause morphological changes in *a*-type cells

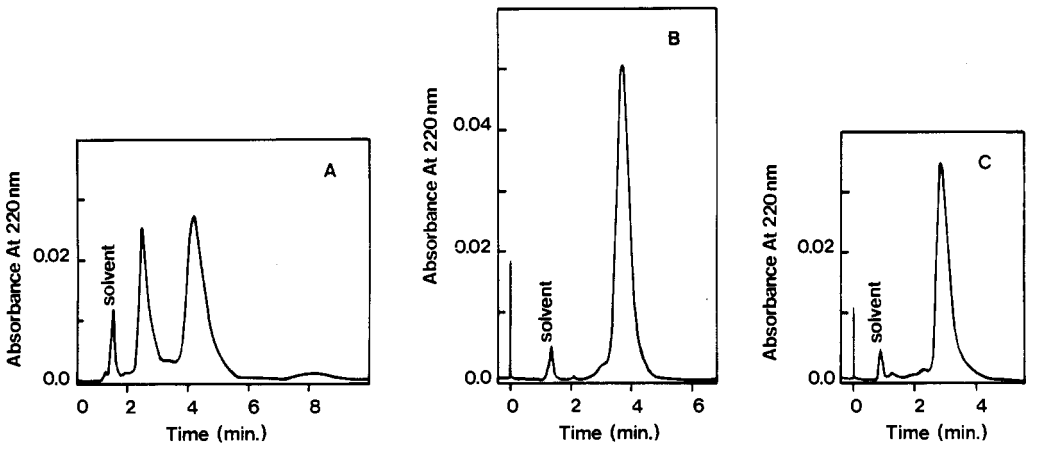


FIGURE 1: High-pressure liquid chromatography of Cha^2-D-[α-^{2}H]Ala^8-dodecapeptide mating factor. Chromatography was run on a μBondapak C_{18} reversed-phase column by using a flow rate of 2 mL/min. (Panel A) Crude peptide prior to purification. Mobile phase CH_3OH–H_2O–CF_3COOH (460:540:0.25). (Panel B) After purification by preparative HPLC. Mobile phase CH_3OH–H_2O–CF_3COOH (460:540:0.25). (Panel C) After purification CH_3CN–H_2O–CF_3COOH (120:380:0.1).

Table II: Properties and Biological Activity of Dodecapeptide Mating-Factor Analogues, His^1-Trp^2-Leu^3-Gln^4-Leu^5-Lys^6-Pro^7-Gly^8-Gln^9-Pro^{10}-Met^{11}-Tyr^{12}

compound	$[\alpha]^{25}_D$	R_f(A)[a]	R_f(B)[a]	mean lowest active concn[b] (ng/mL)
synthetic tridecapeptide[c]	−34.7° (*c* 0.11, AcOH)	0.18	0.54	50
synthetic dodecapeptide	−38.4° (*c* 0.17, AcOH)	0.12	0.41	800
Cha^2-	−34.7° (*c* 0.28, AcOH)	0.13	0.43	250
Cha^2-Orn^6-	−52.3° (*c* 0.22, AcOH)	0.18	0.60	2900
Cha^2-Nle^6-	−41.0° (*c* 0.20, AcOH)	0.22	0.63	2900
Cha^2-ε-Ac-Lys^6	−40.0° (*c* 0.20, AcOH)	0.19	0.61	1000
Cha^2-ε-biotinyl-Lys^6-	−21.9° (*c* 0.20, AcOH)	0.14	0.72	3300
Trp^2-ε-(Dns-Gly)-Lys^6	−32.9° (*c* 0.27, AcOH)	0.25	0.60	800
Cha^2-D-[α-^{2}H]Ala^8	−23.3° (*c* 0.35, AcOH)	0.11	0.61	200
Cha^2-Nle^{11}-	−48.3° (*c* 0.30, AcOH)	0.17	0.54	250
α-Dns-His^1-Cha^2-	−78.0° (*c* 0.20, AcOH)	0.30	0.73	700
Phe^2-	−45.2° (*c* 0.09, AcOH)	0.10	0.50	>5 × 10^5

[a] Solvent systems for TLC: (A) 1-butanol–AcOH–H_2O (4:1:5, upper phase); (B) 1-butanol–AcOH–H_2O–pyridine (15:3:12:10). [b] Represents the average of at least four independent trials. [c] Trp on α end of His.

(shmoo assay) was determined. *S. cerevisiae* 2180-1A (*a*-mating type) was grown at 30 °C in 100 mL of yeast nitrogen base without amino acids (YNB; Difco Labs) containing 2% glucose. Overnight cultures were then used in the shmoo assay by adding 100 μL of cells to 100 μL of the analogue solution in plastic test tubes. Serial dilutions were performed and the percent morphologically abnormal cells after a 4-h incubation at 30 °C and 200 rpm determined. In other experiments serial dilutions were performed in microtiter plates and the end point was determined as the last well in which shmoos were observed after a 3-h incubation. All active analogues also induced agglutination between *a* and α cells.

In competition assays the biologically active mating factor was present at a constant concentration in all wells of the microtiter plates, and the competitor was serially diluted. Competition was judged by the ability of the competitor to eliminate the morphological aberration caused by the biologically active mating factor. In other experiments the competitor was added to yeast cells which had been incubated with biologically active mating factor for a fixed amount of time. The change in percent aberrant cells with time was then determined.

Results

Synthesis of Mating Factor Analogues. Using the procedures described under Materials and Methods we have synthesized the tridecapeptide α factor, the dodecapeptide α factor, and 10 analogues of the dodecapeptide α factor. In the synthesis of the tridecapeptide α factor, the octapeptide Boc-Trp-His-Trp-Leu-Gln-Leu-Lys(Z)-Pro was obtained by deblocking Boc-His(Boc)-Trp-Leu-Gln-Leu-Lys(Z)-Pro with TFA and subsequent reaction with Boc-Trp using the mixed anhydride procedure. Attempts to couple the above octapeptide to the pentapeptide Gly-Gln-Pro-Met-Tyr-OBzl by the mixed anhydride procedure were unsuccessful. So that this difficulty could be circumvented, the protected dodecapeptide Boc-His(Boc)-Trp-Leu-Gln-Leu-Lys(Z)-Pro-Gly-Gln-Pro-Met-Tyr-OBzl was deprotected by acidolysis and the resulting peptide was reacted with Z-Trp-ONp. This strategy provided the desired tridecapeptide in reasonable yield. All deprotected peptides were chromatographically pure and gave expected amino acid ratios. A summary of their chemical properties and biological activity is given in Table II. The biological activities reported in the table are those found in the microtiter plate assay and reflect the minimum concentration of mating factor at which shmoos are observed. The value for the tridecapeptide (10–50 ng/mL) is close to the highest activity reported for the natural purified mating factor (Duntze et al., 1973). The Cha^2 analogue had an activity of 200–250 ng/mL, and the Trp^2 analogue had an activity of ~800 ng/mL.

In addition to the free peptide prepared by standard techniques, the dodecapeptide α factor was also synthesized attached to poly(ethylene oxide) by using the liquid-phase procedure. Synthesis by this approach went in high yield and could be accomplished at a rate of one coupling per day. The yield at all coupling steps was at least 98.8%, and the final

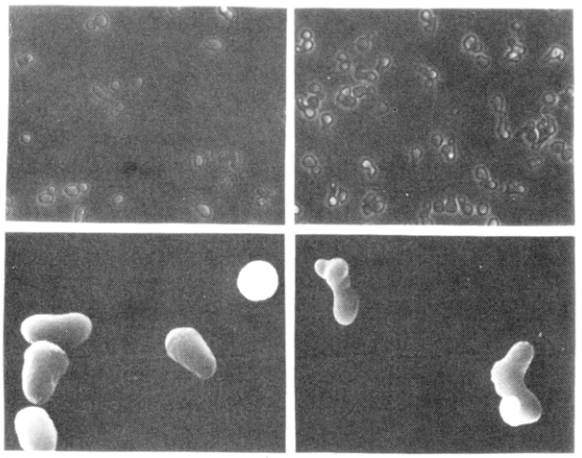


FIGURE 2: Morphology of *S. cerevisiae* 2180-1A incubated with tridecapeptide or dodecapeptide mating factors. (Upper left panel) Phase contrast photomicrograph of cells incubated with 1.25 μg/mL tridecapeptide. (Lower left panel) Scanning electron photomicrograph of cells from upper left. (Upper right panel) Cells incubated with 1.25 μg/mL Cha^2-dodecapeptide. (Lower right panel) Scanning electron photomicrograph of cells from upper right. For scanning EM studies cells were fixed for 2 h with 4% glutaraldehyde and vacuum coated with gold on palladium. The tridecapeptide treated cells are shown 1750×, and the Cha^2-dodecapeptide treated cells are shown 1400×. For all experiments 2×10^6 cells/mL were incubated with pheromone at 30 °C for 4 h.

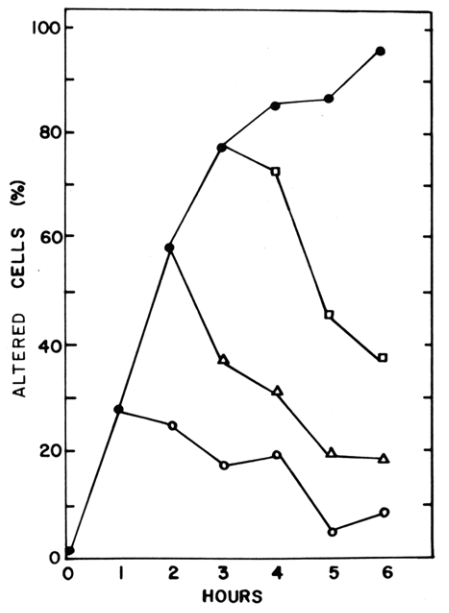


FIGURE 3: Effect of Ala^2-dodecapeptide α factor on the biological activity of Cha^2-dodecapeptide α factor. *S. cerevisiae* 2180-1A (*a*-mating type) was incubated with Cha^2-dodecapeptide (2.5 μg/mL) in the presence and absence of the Ala^2-dodecapeptide (100 μg/mL), and the percent of aberrant cells was determined microscopically. (●) Cha^2-dodecapeptide alone; (○) Ala^2-dodecapeptide added 1 h after Cha^2 analogue; (△) Ala^2-dodecapeptide added 2 h after Cha^2 analogue; (□) Ala^2-dodecapeptide added 3 h after Cha^2 analogue.

deprotected peptide gave the expected amino acid ratios. In order to evaluate the homogeneity of the peptide–OPEGM conjugate we attempted to cleave various intermediates from poly(ethylene oxide) using hydrazinolysis. Despite reports to the contrary in the literature (Mutter & Bayer, 1980), this treatment resulted in degradation of the poly(ethylene oxide) support. Specifically saponification of Boc-Pro-Met-Tyr-OPEGM yielded a homogeneous product as judged by HPLC (data not shown). Treatment of the same peptide–poly(ethylene oxide) conjugate with hydrazine in ethanol resulted in a product which exhibited multiple peaks on HPLC analysis. Similar results were obtained for the analogous di-, tetra-, and pentapeptide. Since saponification results in the isolation of homogeneous peptides in good yield (Table I), the multiple peaks obtained after cleavage by hydrazinolysis do not reflect peptide purity. We conclude that hydrazinolysis cannot be routinely applied to the release of peptides from poly(ethylene oxide) and that our synthesis of α factor by the liquid-phase method resulted in a highly homogeneous peptide. The dodecapeptide–OPEGM conjugate had no biological activity at equivalent peptide concentrations of 100 μg/mL.

Biological Activity of Dodecapeptide Analogues. Five dodecapeptides differing in position 6 all cause shmooing of *a* cells of *S. cerevisiae*. A 4-fold variation in activity (800–3000 ng/mL) was found, and the most active analogue was 16 times less effective than synthetic tridecapeptide. Microscopic investigation suggested that the shapes assumed by yeast cells treated with the dodecapeptide analogues differed somewhat from those observed after *S. cerevisiae* 2180-1A was treated with either synthetic tridecapeptide or biological α factor. Scanning electron micrographs show that at similar concentrations the tridecapeptide leads to shmoo-like morphologies (Figure 2, left panels) whereas all dodecapeptides result in severely distorted morphologies (Figure 2, right panels). Nevertheless all dodecapeptides examined caused cessation of cell growth, G-1 arrest, and increased agglutinability of *a* cells. It is clear that significant chemical alterations can be tolerated in the side chain of residue 6 without abolishment of biological activity. Furthermore cells incubated with position-6 analogues for long periods of time (~10 h) start to recover from G-1 arrest and resume budding.

In addition to the mating factor activity of position-6 analogues α-Dns-His^1-Cha^2-dodecapeptide, Cha^2-Nle^{11}-dodecapeptide, and Cha^2-D-Ala^8-dodecapeptide all cause shmooing by *S. cerevisiae* 2180-1A. The Cha^2-Nle^{11} and Cha^2-D-Ala^8 analogues have activities (~200 ng/mL) which are almost identical with that of the Cha^2-dodecapeptide, suggesting that neither a methionine in position 11 nor glycine in position 8 is essential for activity. The observation that α-Dns-His^1-Cha^2-dodecapeptide has mating factor activity suggests that a free α-amine is not necessary for recognition by the mating-factor receptor. In contrast to the above analogues, Phe^2-dodecapeptide did not cause shmooing of *S. cerevisiae* at concentrations as high as 500 μg/mL. Similarly, we have shown previously that Ala^2-dodecapeptide has no α-factor activity (Khan et al., 1981).

Competition between Various Pheromones. Ala^2-dodecapeptide and Phe^2-dodecapeptide, which do not elicit mating-factor-like responses from *S. cerevisiae* 2180-1A, were competed with synthetic tridecapeptide or Cha^2-dodecapeptide. It was found that both of these analogues prevented biologically active mating factor from causing the formation of shmoos. For the synthetic tridecapeptide, competition by Phe^2-dodecapeptide is observed at a ratio of 15/1 or greater whereas competition by the Ala^2 analogue requires a ratio of 2500/1. The Phe^2 analogue competes with Cha^2-dodecapeptide at a ratio of 5/1 whereas competition by the Ala^2-dodecapeptide is observed at a ratio of 40/1 or greater. It should be noted that competition by these analogues is additional proof that they do not cause shmooing in yeast, as competition is determined by the reversal of aberrant growth. As seen in Figure 3 the reversal of the effect of Cha^2-dodecapeptide occurs almost immediately upon addition of Ala^2-dodecapeptide. When cells treated with 2.5 μg/mL of the Cha^2 analogue are incubated for 1–6 h, the percent of morphologically aberrant cells increases from 20–40% after

1 h to up to 85–95% at 4–6 h. Addition of 100 μg/mL of the Ala^2-dodecapeptide after 1, 2, or 3 h results in an immediate decrease in the number of shmoos, and within 5 h after addition, the percent affected cells is close to control values. The shape of the recovery portion of the curve is similar regardless of whether the Ala^2 analogue is added after 1, 2, or 3 h. In contrast to the Ala^2 and Phe^2 analogues, the dodecapeptide–PEGM conjugate did not compete against biologically active mating factor.

Discussion

Sexual conjugation of *a*- and α-mating types of *S. cerevisiae* provides an ideal model system for investigation of cell–cell interactions. This yeast is well characterized genetically and is simple to grow in the laboratory. It therefore possesses unique advantages in comparison to complex mammalian cells. Study of mating in *S. cerevisiae* may allow insights into the mechanism of action of peptide hormones since this process is triggered by diffusable peptides.

Previous studies on structure–function relationships in the tridecapeptide α factor reported that almost any change in amino acid composition resulted in a marked decrease in mating-factor activity (Masui et al., 1979). In contrast several analogues that we synthesized had activities within 1 order of magnitude of that of the synthetic tridecapeptide with the natural α-factor sequence. Although several laboratories have reported the properties of chemically modified derivatives of α factor, we note that virtually all reports on the effect of amino acid replacement are from one laboratory (Masui et al.). In addition the biological activity of 6 pg/mL reported by this group reflects a biological activity at least 3 orders of magnitude greater than that reported for the homogeneous biological preparation of α factor (Duntze et al., 1973) or by other synthetic attempts (Ciejek et al., 1977; Samokhin et al., 1979). The biological activity of our synthetic tridecapeptide, 10–50 ng/mL, compares favorably with that for biological mating factor and is slightly higher than that from other syntheses. Since all of our analogues were >98% homogeneous in two HPLC systems and gave correct amino acid ratios and the expected 300-MHz NMR spectra, we fell that it is reasonable to conclude that the comparative activities which we report are an accurate representation of the biological activity of the dodecapeptides.

It is interesting to note that in the microtiter plate assay more dodecapeptide (800 ng/mL) possessing the natural sequence was required for activity than homologous tridecapeptide (50 ng/mL). This observation differs from the results of previous studies (Masui et al., 1977; Ciejek et al., 1977). Careful analysis revealed that the dodecapeptides cause the formation of markedly different yeast morphologies in comparison to equal concentrations of the tridecapeptide (Figure 3). Thus it is possible that Trp^1 influences both the biological activity of α factor and the nature of the response of yeast to the peptide pheromone. We note, however, that changes in morphology are far removed on a molecular level from pheromone binding. Indeed differences in cellular morphology caused by different mating factors could reflect (1) different pathways of morphological change, (2) different affinities for the receptor, or (3) different relative rates of inactivation of the pheromones by proteolysis. Therefore, some caution may be required in the interpretation of our experimental findings.

Samokhin and co-workers showed that the lysyl residue of the tridecapeptide pheromone could be replaced by arginine without loss of activity (Samokhin et al., 1979). They suggested that substitution at this position was possible provided that the electrical balance of the peptide was maintained. Our findings with the various dodecapeptide analogues show that neither a free α-amine nor ε-amine is required for biological activity of this pheromone. This result conflicts with recent conclusions (Thorner, 1980) that free amino groups are necessary for a mating-factor-like response. In these analyses α factor treated with acetic anhydride (Lipke, 1976) was reported to lose (10^6 reduction) biological activity. Although structure determination on derivatives was not conducted, it is possible that acetic anhydride caused multiple acylation of the mating factor, thus resulting in loss of activity. The variations in activity of the position-6 analogues show no simple relationship to polarity or size of the side chain. It is known that the biological activity of α factor, as judged by the shmoo assay, reflects both the inherent affinity of this molecule for the receptor and the rate of degradation of the pheromone. Chemical modification may be expected to affect either of these parameters. Until detailed binding studies with specific derivatives are carried out and an analysis of relative proteolysis rates can be conducted, it is not possible to interpret the molecular basis for the influence of chemical modification on mating-factor activity.

The finding that Cha^2-Nle^{11}-dodecapeptide has high biological activity is consistent with conclusions on the Nle^{12} analogue of the tridecapeptide (Masui et al., 1979). The D-Ala^8 substitution was expected to result in a conformation similar to that for the native sequence. Pro-Gly sequences are often found in β turns and are known to assume a type II structure (Venkatachalam, 1968). Since in the type II β-turn replacement of Gly by a D residue is permitted, we expected that the D-Ala^8 analogue might exhibit biological activity. In fact it was among the most active analogues tested. In this context a preliminary NMR study concluded that the mating factor contains three β turns in water (Higashijama et al., 1979), although we believe that this suggestion requires further experimental verification.

The finding that the Phe^2-dodecapeptide was not biologically active was somewhat surprising. In a previous report (Khan et al., 1981) we concluded that position 2 of the dodecapeptide required a large hydrophobic side chain to elicit a mating-factor-like response from *S. cerevisiae*. The competition studies show that both the Ala^2-dodecapeptide and the Phe^2-dodecapeptide can prevent synthetic tridecapeptide and the Cha^2-dodecapeptide from causing yeast cells to shmoo. It is not unreasonable to conclude, therefore, that the Ala^2 and Phe^2 analogues bind to the α-factor receptor. If this is true it appears that specific side chains play a dominant role in triggering morphogenesis. In particular the side chain of residue 2 in the dodecapeptide must be especially important in this respect, and it appears that neither a methyl nor a benzyl group is sufficient to trigger activity.

In contrast to the Ala^2- and Phe^2-dodecapeptides, which are by themselves inactive but can compete with biologically active α factors, the PEGM-dodecapeptide was neither active in the morphogenesis assay nor competed with activity of either the Cha^2-dodecapeptide or the synthetic tridecapeptide. It appears that a free carboxyl terminus is important for both biological activity and binding to the α-factor receptor. This conclusion is consistent with previous studies which found that Tyr^{13} esterification decreased activity of the tridecapeptide α factor (Thorner, 1980). On the basis of the present study, however, it is not possible to conclude whether the effect of PEGM is due to the chemical modification of the carboxyl terminus or the size of the macromolecular protecting group. Previous studies have shown that biotin conjugated to ferritin inhibited biotin uptake into yeast sphaeroplasts (Bayer et al., 1978) and

trimethionine attached to poly(ethylene oxide) competed with $(Met)_3$ transport into *S. cerevisiae* (Naider et al., 1980). Therefore under certain conditions small molecules bound to polymers presumably can interact with receptors on the yeast surface. Since the ϵ-amine of Lys^6 can be modified without loss of activity, it is a reasonable site for conjugation of α factor to a macromolecule. Future attempts with poly(ethylene oxide) will be directed toward attachment at the Lys^6 side chain.

The results reported in this study show that rather large fluorescent groups can be attached to α factor without eliminating biological activity. α factor tagged with fluorescent groups or biotin can be used to localize the α-factor receptor by fluorescence and electron microscopy procedures and to isolate the α-factor receptor by affinity chromatography. Studies utilizing these approaches are currently in progress in our laboratories.

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Registry No. Factor α_1 (*Saccharomyces cerevisiae*), 59401-28-4; factor α_2 (*Saccharomyces cerevisiae*), 59401-29-5; Cha^2-dodecapeptide, 78395-56-9; Cha^2-Orn^6-dodecapeptide, 84303-22-0; Cha^2-Nle^6-dodecapeptide, 84280-04-6; Cha^2-ϵ-Ac-Lys^6-dodecapeptide, 84280-05-7; Cha^2-ϵ-biotinyl-Lys^6-dodecapeptide, 84280-06-8; Trp^2-ϵ-(Dns-Gly)-Lys^6-dodecapeptide, 84280-07-9; Cha^2-D-Ala-dodecapeptide, 84280-08-0; Cha^2-Nle^{11}-dodecapeptide, 84280-09-1; α-Dns-His^1-Cha^2-dodecapeptide, 84280-10-4; Phe^2-dodecapeptide, 84280-11-5; Boc-Tyr(2,6-Cl_2Bzl)-OPEGM, 84280-12-6; Boc-Met-Tyr(2,6-Cl_2Bzl)-OPEGM, 84280-13-7; Boc-Pro-Met-Tyr(2,6-Cl_2Bzl)-OPEGM, 84280-14-8; Boc-Gln-Pro-Met-Tyr(2,6-Cl_2Bzl)-OPEGM, 84280-15-9; Boc-Gly-Gln-Pro-Met-Tyr(2,6-Cl_2Bzl)-OPEGM, 84280-16-0; Boc-Gln-Leu-Lys(3-Cl-Z)-Pro-Gly-Gln-Pro-Met-Tyr(2,6-Cl_2Bzl)-OPEGM, 84280-17-1; Boc-His(Boc)-Trp-(Form)-Leu-Gln-Leu-Lys(3-Cl-Z)-Pro-Gly-Gln-Pro-Met-Tyr(2,6-Cl_2Bzl)-OPEGM, 84280-18-2; His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr-OPEGM, 84280-19-3.

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