Histidine2 of the .alpha.-factor of Saccharomyces cerevisiae is not essential for binding to its receptor or for biological activity

ARTICLE *in* BIOCHEMISTRY · SEPTEMBER 1993

Impact Factor: 3.02 · DOI: 10.1021/bi00083a021 · Source: PubMed

7 AUTHORS, INCLUDING:

Effimia Eriotou
Technological Educational Institute of Ionian…
54 PUBLICATIONS **275** CITATIONS
SEE PROFILE

Jeffrey M Becker
University of Tennessee
290 PUBLICATIONS **6,867** CITATIONS
SEE PROFILE

Fred Naider
City University of New York - College of State…
287 PUBLICATIONS **6,419** CITATIONS
SEE PROFILE

Histidine[2] of the α-Factor of *Saccharomyces cerevisiae* Is Not Essential for Binding to Its Receptor or for Biological Activity†

Yehuda Levin,‡ Ram K. Khare,‡ Greg Abel,§ Dusty Hill,§ Effimia Eriotou-Bargiota,§ Jeffrey M. Becker,§ and Fred Naider*,‡

Department of Chemistry, College of Staten Island, City University of New York, Staten Island, New York 10301, and Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996

Received December 16, 1992; Revised Manuscript Received May 6, 1993

ABSTRACT: Seven His[2] analogs of the *Saccharomyces cerevisiae* [Nle[12]]α-factor, WXWLQLKPGQP(Nle)Y, where X = β-D-thienylalanine, β-L-thienylalanine, 1-D-methylhistidine, 1-L-methylhistidine, 3-D-methylhistidine, 3-L-methylhistidine, and β-3-L-pyridylalanine, were synthesized and purified to homogeneity. Assays were carried out on binding to the α-factor receptor and of biological activity determined by either growth arrest or morphological changes in target cells. In the L-isomer, replacement of the imidazole of histidine by thiophene or 3-pyridyl groups or derivatization of either nitrogen of the imidazole ring by methylation resulted in a 2–100-fold decrease in bioactivity. D-Isomers of the β-thienylalanyl-, 1-methylhistidinyl-, or 3-methylhistidinyl-α-factors did not possess measurable bioactivity with the exception of comparatively low activity of the 3-D-methylhistidinyl and 1-D-methylhistidinyl-α-factors in the morphogenesis assay. In contrast, both active and inactive analogs demonstrated binding affinities 10–20-fold less than that of [Nle[12]]α-factor. These results indicate that the histidine residue of α-factor is not required for binding to the receptor or for biological activity and that bioactivity and binding can be dissociated through the use of pheromone analogs.

Previous structure–activity relationship studies of the *Saccharomyces cerevisiae* α-factor mating pheromone (WHWLQLKPGQPMY) have led to the conclusion that the His residue in position 2 is essential for biological activity. Deletion of this residue resulted in a decrease in activity by a factor of 10^8 and replacement of His by Leu, Phe, and D-His resulted in virtually inactive pheromones (Masui et al., 1977, 1979; Naider & Becker, 1986). Recently, a detailed analysis of the coupling of the *STE2*-encoded α-factor receptor with G proteins showed that the affinity of the pheromone for its receptor at pH 8.0 was almost 1 order of magnitude lower than that at pH 6.0 (Blumer & Thorner, 1990). On the basis of this result, the authors hypothesized that protonation of His[2] of α-factor may stabilize the conformation of the peptide most appropriate for binding. We have shown that alterations at the amino terminus of the pheromone lead to α-factor antagonists. Des-Trp[1]-[Ala[3]]α-factor and des-Trp[1]-[Phe[3]]α-factor were not biologically active but competed with the binding of α-factor to its receptor (Shenbagamurthi *et al.*, 1983; Raths *et al.*, 1988; Xue *et al.*, 1989). Similarly, a truncated pheromone (des-Trp[1],des-His[2]-[Nle[12]]α-factor) was not active biologically but antagonized the activity of native α-factor and competed with the binding of the radiolabeled pheromone with the receptor (Bargiota *et al.*, 1992).

Histidine is often found in the active site of enzymes and is a functional residue in many biologically active peptides. The imidazole side chain of histidine may act as a general acid and/or a general base, may serve as a nucleophilic center, and provides a focal point for hydrogen bonding to water or ligands within the active site of enzymes. Normally, a catalytic function is not ascribed to histidinyl residues in peptide hormones because signal transmission from hormone/pheromone to the cell is believed to occur upon binding of peptide ligands to their cognate receptors. However, histidyl residues in peptide hormones may enter into electrostatic or hydrogen-bond interactions necessary to stabilize the biologically active conformation of the peptide or of the peptide–receptor complex. Thus, it is reasonable that histidine might play an important role in triggering signal transduction during hormone binding. Therefore, we decided to carry out a detailed structure–activity analysis to elucidate the importance of the His side chain for α-factor activity and receptor binding. In this communication we present the synthesis, characterization, and biological activities of seven His[2] analogs of the α-factor mating pheromone.

EXPERIMENTAL PROCEDURES

Materials. All chemicals and solvents used in these studies were reagent grade and were purchased from either Aldrich Chemical Co. or Fisher Scientific. Most of the Boc-protected amino acids were from Bachem Inc., Torrance, CA. Boc-β-(2-thienyl)-L-alanine[1] was purchased from Bachem Inc., and the DL isomer of this compound was prepared starting from the amino acid using di-*tert*-butyl dicarbonate in dioxane/0.5 M NaOH (v/v). Boc-β-(3-pyridyl)-L-alanine was from Peptides International, Louisville, KY. Boc-1-methyl-L-histidine and Boc-3-methyl-L-histidine were the gift of Bachem Inc. The position of the methyl group on the imidazole ring of these compounds was confirmed by 2D NMR spectroscopy.

Peptide Synthesis. All peptides prepared for use in this study contained norleucine (Nle) in place of methionine in position 12 of the α-factor. Nle is isosteric for Met and previous studies have shown that α-factor and [Nle[12]]α-factor have

† This work was supported by Grants GM22086 and GM22087 from the National Institute of General Medical Sciences.

‡ CUNY.

§ University of Tennessee.

[1] Abbreviations: Boc, *tert*-butoxycarbonyl; CZE, capillary zone electrophoresis; Dhp, 3,4-dehydro-L-proline; DMS, dimethyl sulfide; DMSO, dimethyl sulfoxide; FABMS, fast atom bombardment mass spectrometry; HF, hydrogen fluoride; HPLC, high-performance liquid chromatography; Me, methyl; Nle, norleucine; PAM, (phenylacetamido)methyl; TFA, trifluoroacetic acid.

0006-2960/93/0432-8199$04.00/0

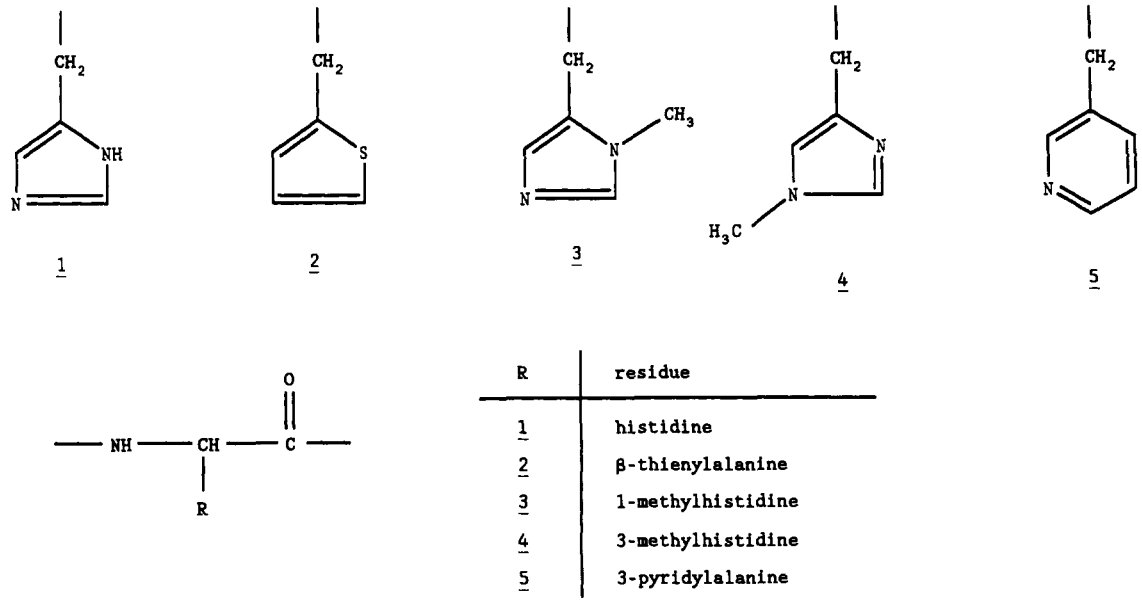

FIGURE 1: Structures of side chain of position 2 residue in α-factor and α-factor analogs used in this study.

identical activity (Raths *et al.*, 1988). Replacement of Met by Nle results in an improved peptide synthesis and peptides which are more stable on storage. The His[2]-α-factor analogs were synthesized by starting with a Boc-Tyr(2-BrZ)-(phenylacetamido)methyl (PAM) resin and building up the peptide chain as described previously (Xue *et al.*, 1989). The chain was extended by the consecutive addition of suitably protected derivatives of Nle, Pro, Gln, Gly, Pro, Lys, Leu, Gln, Leu, and Trp. The resulting undecapeptide–resin conjugate was dried down and portions of this identical resin were then used to prepare the various position 2 analogs. During solid-phase synthesis there is always a danger that small impurities with high biological activity can be isolated (Lifson *et al.*, 1988; Ewenson *et al.*, 1990). We believe that our synthetic strategy should result in a series of peptides wherein any impurities that are carried through the purification procedure should be similar in the different analogs. Thus, differences in biological activity found for the His[2] analogs should reflect the influence of the position 2 side chain and not spurious impurities. The chemical structures of the position 2 side chains are shown in Figure 1.

All residues were coupled using either diisopropylcarbodiimide or diisopropylcarbodiimide/hydroxybenzotriazole activation. Coupling was monitored using the Kaiser test (Kaiser *et al.*, 1970), and coupling reactions were repeated to ensure completeness. Boc protection was used for the α-amino group, and 2-chlorobenzyloxycarbonyl and formyl protection was used for the Lys and Trp residues, respectively. The Boc group was usually deprotected using 45% TFA and 2% DMS in CH_2Cl_2. However, 4 N HCl in dioxane was employed for deprotection of BocGln. In general 0.167 mmol of resin was used in the preparation of each analog. The final peptides were cleaved from the PAM resin using HF/anisole (9:1) at O °C for 1 h. Peptides were extracted from the resin using 25–30% acetic acid and purified by preparative reversed-phase high-performance liquid chromatography (HPLC). The yield of purified peptide ranged from 13% to 48%.

Reversed-Phase HPLC. The final purification and characterization of the His[2] analogs was carried out on a Waters Instruments HPLC equipped with two Model 510 pumps, a Model 481 or Model 490 variable-wavelength detector, and a U6K injector. Preparative separations were carried out on 19-mm × 300-mm C_{18} reversed-phase column; analytical runs were performed on a 3.9-mm × 300-mm C_{18} reversed-phase column and a 3.9-mm × 300-mm CN reversed-phase column. Separations were accomplished using acetonitrile/water/trifluoroacetic acid gradients.

Thin-Layer Chromatography, Ultraviolet Spectroscopy, and Polarimetry. All peptides gave one ninhydrin-positive spot on silica thin layers using both butanol/acetic acid/water (4:1:2) and butanol/acetic acid/water/pyridine (15:3:12:10) as mobile phase. The UV spectra of all peptides in distilled water were measured using a Varian Instruments DMS-300 UV–visible spectrophotometer. As judged by the presence of the 287.5-nm absorption shoulder and the calculated extinction coefficient, all analogs had two intact Trp residues. The molar extinction coefficient of the [Nle[12]]α-factor was determined to be 10 576 M^{-1} cm^{-1} at 287.5 nm using absorption measurements and elemental nitrogen analysis. This value was used to calculate the actual concentration of all pheromone solutions used for bioassays and for measurement of the specific rotation. Rotations were determined using a Jasco Dip-370 digital polarimeter and are reported for solutions in distilled water.

Mass Spectrometry and NMR Spectroscopy. The molecular weight of all peptides was determined using fast atom bombardment mass spectrometry (FABMS) on a VG ZAB EQ mass spectrometer equipped with a flow FAB ion source and an Ion Tech fast atom gun at the University of Tennessee. The FABMS values are reported to the nearest 0.1 mass unit and are monoisotopic values. NMR spectra were recorded on either a 200-MHz FT/NMR (IBM/Bruker) or a AMX 500-MHz spectrometer (Bruker instruments). Spectra were recorded in DMSO-d_6.

Capillary Zone Electrophoresis and Amino Acid Analysis. Amino acid analysis and capillary zone electrophoresis were performed by the peptide group at Hoffman LaRoche Inc., Nutley, NJ. Amino acid analyses were run using a Beckman Instruments Model 121M amino acid analyzer. The amino acid analysis showed that neither the starting Boc-1-methylhistidine nor the product peptide contained as much as 1% free histidine. Capillary zone electrophoresis was performed on a Spectra PHORESIS 1000 instrument (Spectra Physics, San Jose, CA) equipped with a fast-scanning variable-wavelength UV–vis detector. The fused-silica capillary with polyimide outercoating (70-cm × 75-μm i.d.) was coiled in a

cartridge thermostated by circulating air. Sample injection was by electromigration at 15 kV for 1–2 s, and separation was performed at a constant 25 kV, 25 °C. The capillary was washed with running buffer (3–5 min) immediately prior to injection and treated with 0.1 M NaOH (3–5 min) followed by water (3–5 min) between runs. The 50 mM phosphate running buffer was prepared by mixing equimolar solutions of NaH_2PO_4 and H_3PO_4. Separations were carried out at pH 2.5 for the [1-L-MeHis^2,Nle^{12}]α-factor and the [3-L-MeHis^2,Nle^{12}]α-factor and at pH 5.5 for the [3-L-Pydala^2,Nle^{12}]-α-factor. Approximately 1 ng of peptide was injected on the column.

Biological Assays of α-Factor Analogs. The biological activity of the [His^2]α-factor analogs was assayed using a growth arrest assay (Halo) as described previously (Raths *et al.*, 1988). Activity was examined using wild type (2180-1A) and supersensitive (RC629[*sst1*]), and temperature-sensitive (50B^{ts}) mutants of *S. cerevisiae*. The *sst1* mutation results in the loss of the *BAR1* protease, a secreted enzyme, which cleaves the α-factor (Chan & Otte, 1982). Strains 2180-1A and 50B^{ts} secrete *BAR1* protease, but strain 50B^{ts} contains a mutant *STE2* gene that encodes an α-factor receptor inactivated by incubation of cells at 34 °C. At 23 °C the STE2 receptor of strain 50B^{ts} is fully functional.

In separate experiments the ability of nonactive analogs to compete with the activity of the parent ([Nle^{12}]α-factor) was analyzed by placing the analog on a disk which was about 15 mm from a second disk containing the parent compound. Antagonism was evidenced by the appearance of bean-shaped zones of inhibition for the α-factor (Bargiota *et al.*, 1992). The effects of pheromone analogs on the cellular morphology of strain RC629 were measured as previously described (Bargiota *et al.*, 1992).

Binding Competition Assay. Competition of bound tritiated [Nle^{12}]α-factor by unlabeled α-factor analogs was measured by the following protocol. *S. cerevisiae* strain 4202-15-3 (*MATa cry1 bar1-1 ade2-1 his4-580 lys2 tyr1 SUP4-3*) was grown in YM-1 medium (yeast extract, 5 g/L; peptone, 10 g/L; yeast nitrogen base without amino acids, 6.7 g/L; adenine, 0.01 g/L; uracil, 0.01 g/L, succinic acid, 10 g/L; sodium hydroxide, 6 g/L; and glucose, 10 g/L, at a final pH of 5.8) overnight at 30 °C and 120 rpm to 1×10^7 cells/mL and harvested by centrifugation (6000*g* for 10 min at 4 °C). The resultant pellet was washed twice with ice-cold YM-1+*i* medium (YM-1 containing 10 mM NaN_3, 10 mM KF, and 10 mM *p*-tosyl-L-arginine methyl ester) and resuspended to 1.25×10^9 cells/mL in YM-1+*i*. Final assay concentrations of the unlabeled competitors were between 3×10^{-5} and 1×10^{-9} M as determined from the A_{287} for each peptide from extinction coefficients calibrated for [Nle^{12}]α-factor from quantitative elemental analysis. The reaction was started by the addition of 100 μL of analog/[^{3}H]-α-factor mix (120 μL of appropriate unlabeled α-factor analog mixed with 120 μL of labeled [Nle^{12}]α-factor, 9.3×10^{-7} M, 9.24 Ci/mmol) to 400 μL of cell suspension. Reaction initiations were staggered by 2 or 3 min such that each binding reaction could be processed at 30 min with ample time for the manipulations involved. After the reaction was started, two 20-μL samples were removed and counted to determine the total amount of radioactivity present. Duplicate reactions were started 3 min after initial reaction for each analog tested. At 30 min, two 200-μL portions of cell suspension were added separately to 2 mL of YM-1+*i* and filtered over 1% bovine serum albumin-presoaked GN-6 Metricel filters (Gelman Sciences, Inc., Ann Arbor, MI). The reaction tubes were then rinsed twice with 2 mL of ice-cold YM-1+i medium, with each rinse filtered over the same filter. Finally, each filter was then rinsed twice with 2 mL YM-1+*i* medium. The nonspecific binding of labeled α-factor in the absence of cells was less than 100 cpm. Over the course of the reaction, tubes were vortexed approximately every 5 min. The filters were counted in 5 mL of Budget-solve counting cocktail (Research Products International Corp., Mt. Prospect, IL). All assays and manipulations were done in siliconized borosilicate tubes or vials. The effective concentration of the competitor at which α-factor binding is 50% of the maximal is the EC_{50}. This value for each competitor was determined by visual inspection of the binding curve. K_D was calculated from the EC_{50} by the method of Cheng and Prusoff (1973). The binding curve for α-factor has been previously done by the infinite dilution experiment by us and others (Raths *et al.*, 1988; Blumer *et al.*, 1988; Jenness *et al.*, 1986) to show that there is no evident cooperativity in the binding between α-factor and its receptor.

Tritiated [Nle^{12}]α-factor was prepared as previously described (Raths *et al.*, 1988). Briefly, α-factor containing dehydroproline (Dhp) in place of proline in positions 8 and 11 and norleucine at position 12 in place of methionine was synthesized by standard methods of solid-phase peptide synthesis. [Dhp^8,Dhp^{11},Nle^{12}]α-factor was tritiated by Amersham Corp. by the T-3 method involving reduction by tritium gas. The crude tritiated peptide was purified to homogeneity by HPLC as previously described (Raths *et al.*, 1988). Purity and specific activity were monitored by TLC, HPLC, amino acid analysis, and biological activity. In our laboratory, we demonstrated complete reduction of the two dehydroproline residues in [Dhp^8,Dhp^{11},Nle^{12}]tridecapeptide using hydrogen gas and palladium black in methanol in 2 h as judged using 400-MHz NMR and monitoring resonances associated with the double bond of DHP (data not shown). The FABMS spectrum of the reduced peptide indicated conversion to [Nle^{12}]α-factor.

RESULTS

Synthesis and Characterization of His^2 Analogs. The synthesis of the His^2 analogs of the α-factor was accomplished efficiently using the solid-phase approach on a PAM resin. In general, crude peptides were isolated in 80–95% yield after HF cleavage and contained one or two dominant products. The purified peptides were isolated using reversed-phase HPLC. In the case of the β-thienylalanine analog, the crude product prepared from the DL amino acid mixture exhibited nearly equal quantities of the diastereomeric isomers (Figure 2A). These were readily separated by preparative HPLC to yield both the D and the L isomers. The latter compound eluted first from the HPLC column. An independent synthesis starting with the L isomer of β-thienylalanine gave a product with an elution time identical to that of the first eluted product from the synthesis using the DL mixture. Analysis of the biological activities of these analogs confirmed the identity of the [β-L-thienylalanyl^2]α-factor (see below).

During the synthesis of the 1-methylhistidine- and 3-methylhistidine-containing analogs, we observed significant heterogeneity in the crude products. Two major products, in unequal ratio, appeared in the chromatograms of the peptides cleaved from the PAM resins (Figure 2B,C). We suspected that the observed heterogeneity was the result of racemization of the urethane-protected methylhistidines during activation and coupling. Significant racemization has been reported for 1- and 3-alkylhistidines during carbodiimide-activated addition to growing peptide chains (Stewart & Young, 1984;

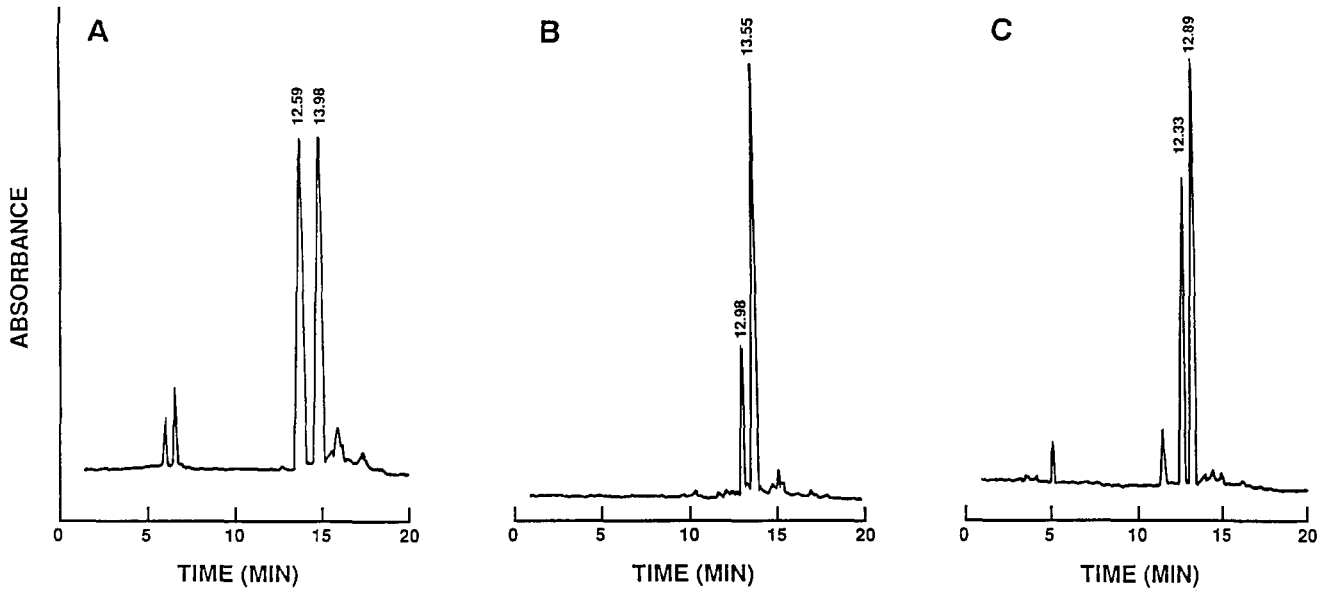

FIGURE 2: (A) HPLC of crude product from synthesis of [β-DL-thienylalanyl^2,Nle^{12}]α-factor. (B) HPLC of crude product from synthesis of [1-L-methylhistidinyl^2,Nle^{12}]α-factor. (C) HPLC of crude product from synthesis of [3-L-methylhistidinyl^2,Nle^{12}]α-factor. Chromatography was performed on a C_{18} column using the following gradients: (A) water/CH_3CN/TFA from 70:30:0.025 to 30:70:0.025 over 20 min; (B and C) water/CH_3CN/TFA from 80:20:0.025 to 40:60:0.025 over 20 min.

Windridge & Jorgensen, 1971). It is also possible that the starting Boc-methylhistidines were not optically pure. This possibility was not tested due to the small amount of these derivatives available to us. In either case it was reasonable to assume that the smaller peaks in panels B (~21%) and C (~40%) were due to the incorporation of the D isomer. This conclusion is consistent with the results of our biological assays (see below).

The position 2 analogs were characterized for homogeneity using gradient elution on reversed-phase C_{18} columns (Figure 3) and a reversed-phase CN column (data not shown). Similar results were obtained on both columns; however, much sharper peaks were obtained on the C_{18} column. The peptides ranged from 96.4% homogeneous ([1-L-MeHis^2,Nle^{12}]α-factor; IV) to 99.5% homogeneous ([3-L-PydAla^2,Nle^{12}]α-factor; VIII). Most importantly, from the perspective of this study, by comparison of retention times and the coinjection of [Nle^{12}]-α-factor (Figure 3, panels E and H), the HPLC analysis allowed the following conclusions: (1) The [β-L-thienyl-Ala^2,Nle^{12}]α-factor contains less than 0.1% material with a chromatographic mobility equal to that of the [Nle^{12}]α-factor. (2) The [β-D-thienylAla^2,Nle^{12}]α-factor contains a maximum of 0.5% of the homologous L-isomer. (3) The [1-D-Me-His^2,Nle^{12}]α-factor does not contain a measureable amount of the homologous L-isomer or of [Nle^{12}]α-factor. (4) The [3-D-MeHis^2,Nle^{12}]α-factor contains approximately 1% of an impurity with a mobility equal to that of the corresponding L-isomer. (5) The similar retention times of the [1-L-Me-His^2,Nle^{12}]-, [3-L-MeHis^2,Nle^{12}]-, and ([3-L-PydAla^2,Nle^{12}]-α-factor analogs and [Nle^{12}]α-factor (Table I) makes it impossible to prove using HPLC that these samples do not contain the parent pheromone. However, it is proven that the small impurities found in the other analogs are not the [Nle^{12}]α-factor.

To gain additional insights into the homogeneity of the [1-L-MeHis^2,Nle^{12}]-, [3-L-MeHis^2,Nle^{12}]-, and [3-L-Pyd-Ala^2,Nle^{12}]α-factors, these compounds were subjected to capillary zone electrophoresis. Under the conditions used, excellent separation of the above peptides and the [Nle^{12}]α-factor were obtained (data not shown). The results indicate that the [3-L-PydAla^2,Nle^{12}]α-factor contains less than 1% of the parent compound and that the [1-L-MeHis^2,Nle^{12}]α-factor and [3-L-MeHis^2,Nle^{12}]α-factor have approximately 3% of an impurity with an electrophoretic mobility similar to that of the [Nle^{12}]α-factor.

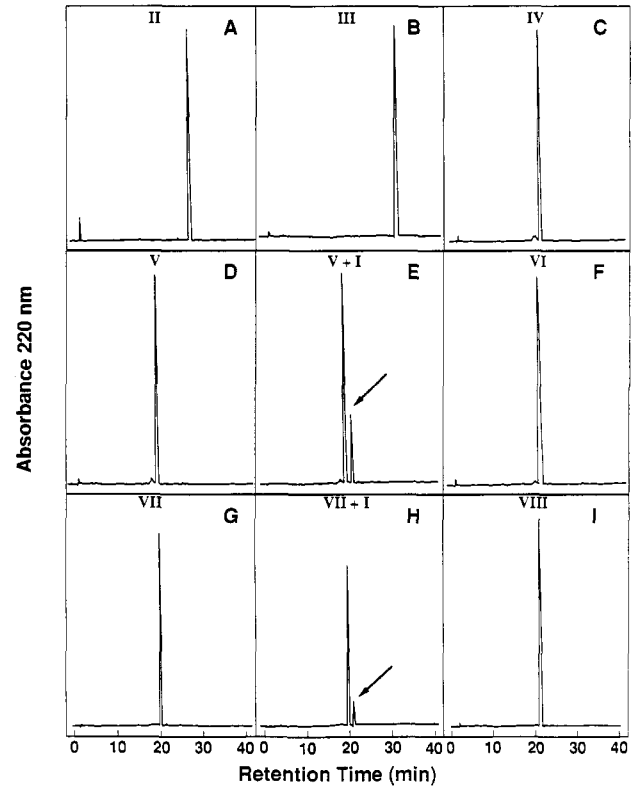

FIGURE 3: HPLC of purified peptides. (A) [β-L-thienylAla^2,Nle^{12}]α-factor. (B) [β-D-thienylAla^2,Nle^{12}]α-factor. (C) [1-L-MeHis^2,Nle^{12}]-α-factor. (D) [1-D-MeHis^2,Nle^{12}]α-factor. (E) [1-D-MeHis^2,Nle^{12}]α-factor and [Nle^{12}]α-factor (the arrow indicates the [Nle^{12}]α-factor peak). (F) [3-L-MeHis^2,Nle^{12}]α-factor. (G) [3-D-MeHis^2,Nle^{12}]α-factor. (H) [3-D-MeHis^2,Nle^{12}]α-factor and [Nle^{12}]α-factor (the arrow indicates the [Nle^{12}]α-factor peak). (I) [3-L-PydAla^2,Nle^{12}]α-factor. Chromatography was run on a C_{18} column using a gradient of water/CH_3CN/TFA from 80:20:0.025 to 50:50:0.025 over 35 min.

Table I: Analytical and Physical Chemical Characteristics of [His^2]α-Factors

no.	peptide	FABMS[a] calcd	FABMS[a] obsd	specific rotation [α]	K'[b]	R_f(A)[c]	R_f(B)[c]
II	[β-L-thienylAla^2,Nle^{12}]α-factor	1681.9	1681.5	−47.5	9.47	0.57	0.76
III	[β-D-thienylAla^2,Nle^{12}]α-factor	1681.9	1681.6	−59.6	11.00	0.58	0.77
IV	[1-L-MeHis^2,Nle^{12}]α-factor	1679.9	1680.0	−41.3	7.06	0.46	0.73
V	[1-D-MeHis^2,Nle^{12}]α-factor	1679.9	1679.6	−88.6	6.59	0.46	0.75
VI	[3-L-MeHis^2,Nle^{12}]α-factor	1679.9	1679.7	−51.5	7.28	0.43	0.73
VII	[3-D-MeHis^2,Nle^{12}]α-factor	1679.9	1680.0	−67.3	6.68	0.49	0.77
VIII	[3-L-PydAla^2,Nle^{12}]α-factor	1676.9	1676.7	−45.4	7.14	0.51	0.72

[a] FABMS are monoisotopic values and refer to [M + H$^+$]. [b] Chromatography was carried out on a C_{18} reversed-phase column using a water/CH_3CN/TFA gradient from 80:20:0.025 to 50:50:0.025 over 35 min. [c] R_f(A) = butanol/AcOH/H_2O (4:1:2); R_f(B) = butanol/AcOH/H_2O/pyridine (15:3:12:10).

Table II: Biological and Biochemical Properties of [His^2]α-Factors

no.	peptide	halo[a] 2180	halo[a] RC629	halo[a] 50B^{ts}	shmoo[b] μg/mL	antagonist[c]	binding[d] ($K_D \times 10^{-7}$ M)
I	[Nle^{12}]α-factor	1.0	0.05	0.5	0.1	–	0.2
II	[β-L-thienylAla^2,Nle^{12}]α-factor	5.0	5.0	5.0	0.5	–	7.4
III	[β-D-thienylAla^2,Nle^{12}]α-factor	>10	>50	>10	>100	yes	6.5
IV	[1-L-MeHis^2,Nle^{12}]α-factor	5.0	0.5	1.0	0.1	–	3.7
V	[1-D-MeHis^2,Nle^{12}]α-factor	>10	>50	>10	20	yes	4.1
VI	[3-L-MeHis^2,Nle^{12}]α-factor	5.0	0.5	1.0	0.5	–	3.5
VII	[3-D-MeHis^2,Nle^{12}]α-factor	>10	>50	>10	5.0	yes	7.1
VIII	[3-L-PydAla^2,Nle^{12}]α-factor	5.0	0.10	1.0	0.1	–	3.7

[a] Growth arrest was determined by the formation of halos in lawns of the three strains (2180, RC629, and 50B^{ts}) represented. The value shown is the minimal amount of peptide (micrograms applied to disk) that caused a halo. [b] Cell morphogenesis was determined for RC629 by the formation of shmoo-shaped cells in response to peptide. The value shown in the table is the amount of peptide causing formation of shmoo-shaped cells in 40–50% of the cell population. [c] Antagonism was determined by competition for halo formation by peptide analogs. See Figure 5 for a representation of this assay. [d] K_D was determined by the method of Cheng and Prusoff (1973).

The molecular weight of all peptides was determined using fast atom bombardment mass spectrometry. All peptides had the expected molecular ion within ±0.4 mass unit (Table I). The FABMS spectrum of the [3-L-PydAla^2,Nle^{12}]α-factor gave a major peak at approximately 1677 amu but was completely flat at 1664 and 1665 amu. The latter molecular ion would be expected for the [Nle^{12}]α-factor. Similar results were obtained for the [1-L-MeHis^2,Nle^{12}] and [3-L-MeHis^2,Nle^{12}] analogs.

To gain additional evidence on the structure of the methylhistidine analogs, 200-MHz NMR spectra were recorded for solutions of Boc-1-MeHis and Boc-3-MeHis in DMSO solution. Both of these derivatives exhibited a sharp singlet at 3.53 ppm which was absent in Boc-His. This singlet is consistent with an *N*-methyl substituent. In addition, NOESY spectroscopy indicated that both the C2H and C4H protons were close in space to the NCH_3 protons in Boc-3-methylhistidine, whereas only the C2H proton was close to the NCH_3 protons in Boc-1-methylhistidine. Most importantly, solutions of the [1-L-MeHis^2,Nle^{12}] and [3-L-MeHis^2,Nle^{12}] analogs and their D-containing homologs also exhibited a large resonance near 3.5 ppm in DMSO. The presence of small amounts of bound water in these peptides makes it impossible to conclude definitively that this resonance is due to the *N*-methyl group. However, taken together with the FABMS and amino acid analysis results on these compounds, the NMR results provide evidence that the tridecapeptides contain intact methylhistidine.

The [3-L-PydAla^2,Nle^{12}]α-factor was studied at 400 MHz in DMSO solution. The spectrum of this peptide lacked a singlet resonance at 8.95–9.1 ppm due to the His C_2H which has been observed in all samples of α-factor analogs known to contain histidine. Furthermore, when [Nle^{12}]α-factor was added to [3-L-PydAla^2,Nle^{12}]α-factor the C_2H resonance at 8.95 was readily observed (data not shown). The combination of the NMR, FABMS,and CZE results suggests that the pyridinylalanine analog does not contain the parent pheromone.

The analytical data and the strategy used for synthesis (see Experimental Procedures) allow us to conclude that the differences in activity of the position 2 analogs reflect the influence of the position 2 side chain and not the presence of either the parent pheromone or spurious impurities. The activities found for the L-analogs are 10%–50% of that of the [Nle^{12}]α-factor. This cannot be accounted for by the maximum 3% impurity found in analogs IV and VI. The results of these syntheses show that under our coupling conditions Boc-1-methylhistidine and Boc-3-methylhistidine may be prone to racemization. Interestingly, in contrast to the results with the methylhistidines, the synthesis of the β-(3-pyridylalanine) analog did not result in appreciable racemization. The conclusions of the chromatographic and spectroscopic analyses are in harmony with the relative activities found for the β-thienylalanine- and methylhistidine-containing peptides in the halo assay (see below).

Biological Activity of Analogs. The biological activity of peptides I–VIII (Table II) was determined using both wild-type and supersensitive strains. None of the peptides tested were active against strain 50B^{ts} at the restrictive temperature (34 °C). This was true for both active and inactive analogs and indicates that the effects seen with all active analogs were due to interaction with the α-factor receptor. In general we found that the relative activity of the His^2 analogs was similar in different strains. Peptides III, V, and VII (D-containing analogs) showed no activity in the halo assay against strains 2180-1A, RC629 and 50B^{ts}. At high concentration peptide VII (at 5 μg/mL) and peptide V (at 20 μg/mL) induced morphogenesis of strain RC629, whereas the β-D-thienylalanine-containing peptide had no activity in the morphogenesis assay at concentrations up to 100 μg/mL (Figure 4). We believe that the small activity observed for the D-methyl-

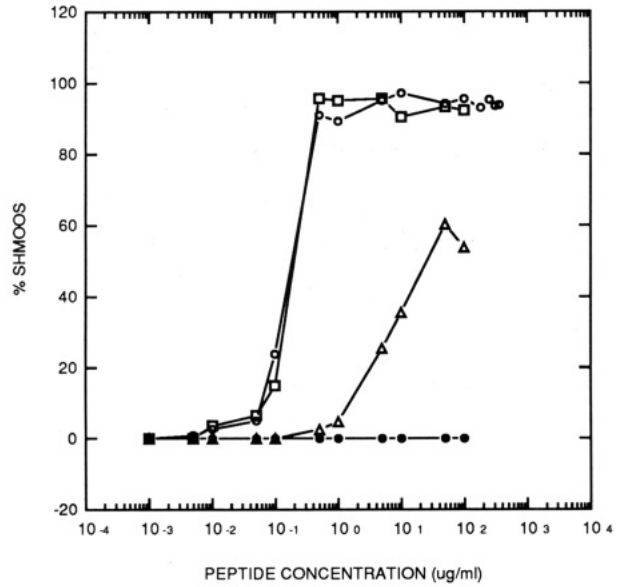

FIGURE 4: Effects of [Nle[12]]α-factor and various position 2 substituted analogs on cellular morphology of *S. cerevisiae* RC629. The symbols represent [Nle[12]]α-factor (O), [3-L-PydAla[2],Nle[12]]α-factor (□), [3-D-MeHis[2],Nle[12]]α-factor (△), and [β-D-thienylAla[2],Nle[12]]α-factor (●) at the indicated concentrations.

histidine-containing peptides reflects the 0.5%–1.0% of the L-isomer that may be present in these samples. Our conclusion is that all of the D-analogs are antagonists and are not the weak agonists that might be inferred from the morphogenesis studies (see below). Replacement of the imidazole ring with a thiophene ring (peptide II versus peptide I) resulted in a 5–100-fold drop in activity, and methylation of either imidazole nitrogen (peptides IV and VI) resulted in a 5–10-fold decrease in activity, whereas replacement of the imidazole ring with a pyridyl moiety (peptide VIII) resulted in only a 2–5-fold decrease in activity.

Peptides III, V, and VII, which were inactive up to 50 μg/disk in the halo assay against all strains, antagonized the activity of the native pheromone using RC629 as the test organism (Figure 5). The bean-shaped halos observed for the α-factor when the competing peptide was applied at a 5–20-fold excess for the β-thienylalanyl analog (III) and the 1-methylhistidinyl (V) and 3-methylhistidinyl (VII) analogs, are indicative of a competitive antagonist (Scannell & Pruess, 1974). The nature of the inhibition of activity is supported by direct binding competition studies using radioactive pheromone (see below).

Competition with Binding to the Receptor. The ability of the His[2] analogs to bind to the α-factor receptor was determined directly using competition binding assays with radioactively labeled [Nle[12]]α-factor. Binding competition curves for all analogs were parallel to those measured for the parent pheromone, and complete displacement of the agonist was achieved at high concentration (Figure 6). The competition assays indicate binding constants, K_D, of (3.5–7.4) × 10^{-7} M for the position 2 analogs (Table II). These are approximately 1 order of magnitude lower than the K_D observed for the parent pheromone. The K_D values found for the biologically active and the inactive pheromones were within a factor of 2 under the assay conditions used.

DISCUSSION

Histidine residues in peptides and proteins have been associated with significant influences on biological activity. In addition to the well-documented cases of the involvement

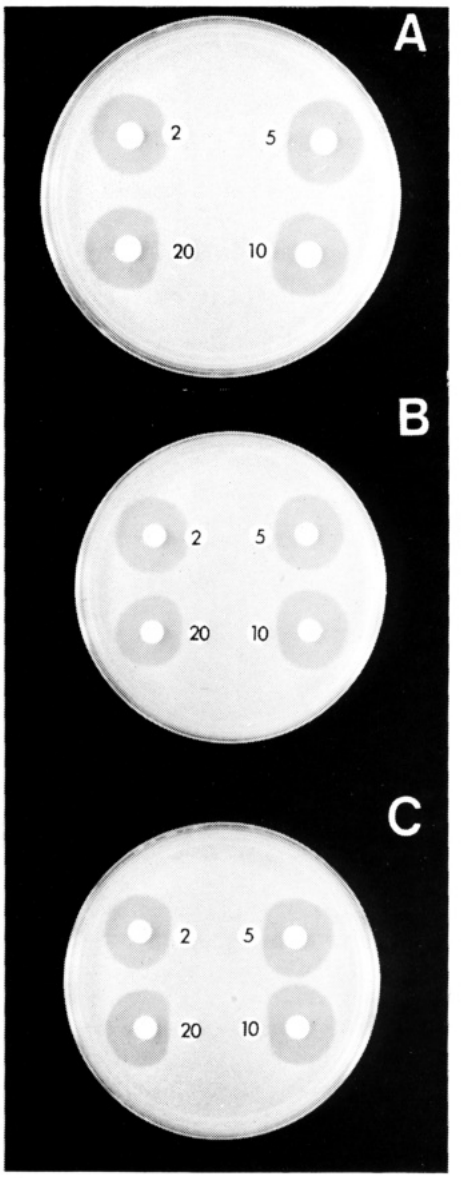

FIGURE 5: Effect of various amounts of position 2 α-factor analogs on α-factor activity. [Nle[12]]α-Factor (0.5 μg) was placed on the disk which appears at the center of growth arrest shown by a halo of no growth in the lawn. Analogs (panel A = [1-D-MeHis[2],Nle[12]]α-factor; panel B = [3-D-MeHis[2],Nle[12]]α-factor; panel C = [β-D-thienylAla[2],Nle[12]]α-factor) at 2-, 5-, 10-, and 20-fold the amount of [Nle[12]]α-factor were placed on disks at the halo periphery as shown in the figure. Antagonism is shown by reversal of growth arrest.

of the imidazole moiety of histidine in the active site of many enzymes (Fersht, 1985; Walsh, 1979), histidinyl residues of peptide ligands have also been shown to influence binding to protein receptors. In a series of thorough experiments it was shown that the His residue of the S-peptide (residues 1–20) of ribonuclease A influenced both binding of S-peptide to S-protein (residues 21–124) and the activity of the resulting peptide–protein complex (Dunn *et al.*, 1974; Van Batenburg *et al.*, 1977).

A number of studies on histidine-containing hormones have measured effects of histidine deletion, replacement, or modification on the resulting bioactivity and receptor binding of the analog. Deletion of the N-terminal His[1] of vasoactive intestinal peptide (VIP) gave an analog with comparable diminution of biological and binding activity compared to the native 28 amino acid hormone (O'Donnell *et al.*, 1991). In contrast, replacement of histidine by alanine resulted in a 100–200-fold decrease in bioactivity, but binding was dimin-

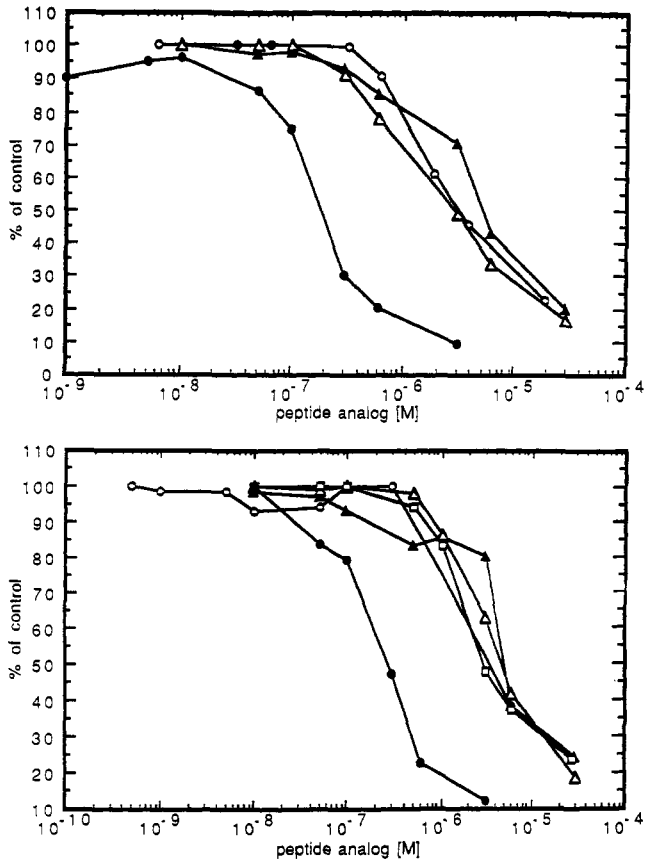

FIGURE 6: Competition binding of [^{3}H-Nle^{12}]α-factor by unlabeled [Nle^{12}]α-factor and position 2 analogs. Top panel: The curves represent [^{3}H]α-factor plus [Nle^{12}]α-factor (●), [3-D-MeHis^2,Nle^{12}]α-factor (▲), [1-L-MeHis^2,Nle^{12}]α-factor (O), or [3-L-MeHis^2,Nle^{12}]α-factor (△). Bottom panel: The curves represent [^{3}H]α-factor plus [Nle^{12}]α-factor (●), [1-D-MeHis^2,Nle^{12}]α-factor (O), [β-L-thienylAla^2,Nle^{12}]α-factor (▲), [β-D-thienylAla^2,Nle^{12}]α-factor (△), or [3-L-PydAla^2,Nle^{12}]α-factor (□). The data shown are the means of triplicate determinations, with each curve representative of two separate experiments.

ished only 3-fold (Bolin *et al.*, 1990). Replacement of histidine by norvaline in the tripeptide hormone TRH (thyrotropin-releasing hormone) gave an analog with reduced binding to the high-affinity receptor and total loss of thyrotropin-releasing capacity (Vonhof *et al.*, 1990). Investigations on angiotensin II showed that His^6 could be replaced by Gln but not Asn with retention of full bioactivity, leading the authors to conclude that the Gln side chain is an isostere for the imidazole of His (Pottorf *et al.*, 1990). It is clear that histidine can have a marked influence on hormone activity and that in certain cases bioactivity is correlated with binding whereas in others these two characteristics are dissociable.

In a previous study a pH-dependent shift in α-factor binding affinity was reported, and it was concluded that protonation of His^2 influenced binding (Blumer & Thorner, 1990). The results reported herein clearly indicate that the imidazole side chain of the His^2 residue is not essential for biological activity of α-factor. Specifically, replacement of this moiety with a thiophene or 3-pyridyl ring led to analogs with $^1/_{100}$–$^1/_2$ the biological activity of the parent pheromone. In addition the NH of the imidazole ring could be changed to a *N*-methyl group with significant retention of activity (Table II). It is possible that the loss of activity observed for the thiophene-containing peptide (which cannot be protonated) or the lower activity observed for the *N*-methylhistidinyl analogs (which are protonatable) may reflect the importance of protonation for pheromone-induced responses. Nevertheless, it is clear from the data in Table II that biological activity is not totally dependent upon either protonation or the NH of the imidazole ring. Furthermore, the similarity of the K_D's of the thienylalanine analogs (nonprotonatable side chain) to those for all other compounds examined in this study (which contain protonatable side chains) provides evidence that protonation of His^2 is not essential for binding.

It is interesting that change in the chirality of residue 2 from L to D results in a marked (at least 50–100-fold) loss in activity. For all of the D-diasteromers the nearly complete loss of activity is supported by the observation that these inactive analogs antagonize the activity of the [Nle^{12}]α-factor. Indeed, all of the His^2 analogs bind to the receptor and exhibit binding constants similar to those of active homologs. Therefore, we conclude that antagonism can be attributed to displacement of the agonist from the receptor.

The finding that both active and inactive analogs bind to the pheromone with similar affinities implies that the biological activity of the position 2 analogs cannot be correlated solely with receptor occupancy. Furthermore our results show that pheromone binding and signal transduction can be uncoupled. Studies on glucagon (Unson *et al.*, 1991) and human chorionic gonadotropin (Willey & Leidenberger, 1989) also led to the conclusion that receptor binding and biological activity are dissociable. In all these cases uncoupling was achieved by modification, replacement, or deletion of a histidine residue. In the present case of α-factor, the side chain of histidine plays an extremely crucial role in the information transduction induced by the pheromone. The ability of this side chain to trigger the mating cascade reflects both the specific atoms present in the side chain and their stereochemical orientation. Changing the chirality of the side chain from L to D is sufficient to cause significant loss of activity without significant loss in the ability of the pheromone to bind to the receptor.

The individual atoms of the imidazole ring appear to mediate the transduction of the pheromone signal. Thus, changing the two nitrogens of the His side chain to a sulfur and a carbon results in a 100-fold decrease in activity. The five-membered ring of the His side chain is not absolutely essential since it can be replaced by the six-membered ring in the β-pyridylalanine analog with only a 2–5-fold decrease in activity. Furthermore, the steric bulk of the side chain can be increased by methylation without total loss in activity. It appears that the 3-nitrogen of the imidazole side chain may be especially important for high pheromone potency. Thus, deletion of this nitrogen, as in [Phe^2]α-factor (Masui *et al.*, 1979) or in the β-thienylalanine analogs, results in a marked drop in activity, whereas retention of this nitrogen, as in the 3-pyridylalanine analog, leads to an analog with high activity.

The emerging picture includes the influence of a variety of electronic and steric factors in triggering the response pathway via the side chain of residue 2. Additional analysis of this side chain might prove especially fruitful in clarifying the atomic basis for mating factor activity. However, there remains the possibility that some of the changes caused by modification of the position 2 side chain are due to overall conformational effects on the pheromone or on the resulting pheromone–receptor complex. Therefore, we are currently carrying out conformational analysis of α-factor analogs using high-resolution NMR spectroscopy in both organic and aqueous solvents.

ACKNOWLEDGMENT

We thank David Miller and Shu-Hua Wang for technical assistance and help in the preparation of figures. We also

thank Dr. Arthur Felix for running the amino acid analysis and capillary zone electrophoresis and John Gounarides and Wei Yang for NMR analyses. FAB-mass spectrometry was carried out at the University of Tennessee Mass Spectrometry Center funded by the Science Alliance of the Tennessee Center for Excellence and by NSF Chemical Instrumentation Program (Grant CHE-86-09251). We are grateful to Dr. Michael Verlander and Bachem, Inc., for the gift of the protected methylhistidines used in peptide synthesis.

REFERENCES

Bargiota, E. E., Xue, Chu-Biao, Naider, F., & Becker, J. M. (1992) *Biochemistry 31,* 551–557.

Blumer, K. J., & Thorner, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87,* 4363–4367.

Blumer, K. J., Reneke, J. E., & Thorner, J. (1988) *J. Biol. Chem. 263,* 10836–10842.

Bolin, D. R., Cottrell, J. M., O'Neill, Garippa, R. J., & O'Donnell, M. (1990) in *Peptides: Chemistry, Structure, and Biology* (Rivier, J. E., and Marshall, G. R., Eds.) pp 208-210, ESCOM Press, Leiden, The Netherlands.

Chan, R. K., & Otte, C. A. (1982) *Mol. Cell. Biol. 2,* 11–20.

Cheng, Y., & Prusoff, W. H. (1973) *Biochem. Pharmacol. 22,* 3099–3108.

Dunn, M. B., Dibello, C., Kirk, K. L., Cohen, L. A., & Chaiken, I. M. (1974) *J. Biol. Chem. 249,* 6295–6301.

Ewenson, A., Marcus, S., Becker, J. M., & Naider, F. (1990) *Int. J. Pept. Protein Res. 35,* 241–248.

Fersht, A. (1985) *Enzyme structure and mechanism,* 2nd ed., W. H. Freeman, New York.

Jenness, D. D., Burkholder, A. C., & Hartwell, L. H. (1986) *Mol. Cell. Biol. 6,* 318–320.

Kaiser, E., Colescott, R. K., Bossinger, C. D., & Cook, P. I. (1970) *Anal. Biochem. 34,* 595–598.

Lifson, J. D., Hwang, K. M., Nara, P. L., Fraser, B., Padgett, M., Dunlap, N. M., & Eiden, L. E. (1988) *Science 241,* 712–716.

Masui, Y., Chino, N., Sakakibara, S., Tanaka, T., Murakami, T., & Kita, H. (1977) *Biochem. Biophys. Res. Commun. 78,* 534–38.

Masui, Y., Tanaka, T., Chino, N., Kita, H., & Sakakibara, S. (1979) *Biochem. Biophys. Res. Commun. 86,* 982–987.

Naider, F., & Becker, J. M. (1986) *CRC Crit. Rev. Biochem. 21,* 225–248.

O'Donnell, M., Garippa, R. J., O'Neill, N. C., Bolin, D. R., & Cottrell, J. M. (1991) *J. Biol. Chem. 266,* 6389–6392.

Pottorf, R. S., Ripka, W. C., Chiu, A. T., & Wong, P. C. (1990) in *Peptides: Chemistry, Structure, and Biology* (Rivier, J. E., and Marshall, G. R., Eds.) pp 312–313, ESCOM Press, Leiden, The Netherlands.

Raths, S. K., Naider, F. R., & Becker, J. M. (1988) *J. Biol. Chem. 263,* 17333–17341.

Scannel, J. P., & Pruess, D. L. (1974) in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins,* (Weinstein, B., Ed.) Vol. 3, pp 189–244, Marcel Dekker, New York.

Shenbagamurthi, P., Baffi, R., Khan, S. A., Becker, J. M., & Naider, F. (1983) *Biochemistry 22,* 1298–1304.

Stewart, M. J., & Young, J. D. (1984) *Solid Phase Peptide Synthesis,* Pierce Chemical Co., Rockford, IL.

Unson, C. G., MacDonald, D., Ray, K., Durrah, T. L., & Merrifield, R. B. (1991) *J. Biol. Chem. 266,* 2763–2766.

Van Batenburg, O. D., Voskuyl-Holtkamp, I., Schattenkerk, C., Hoes, K., Kerling, K. E. T., & Haringa, E. (1977) *Biochem. J. 163,* 385–387.

Vonhof, S., Feuerstein, G. Z., Cohen, L. A., & Labroo, V. M. (1990). *Eur. J. Pharmacol. 180,* 1–12.

Walsh, C. (1979) *Enzymatic Reaction Mechanisms,* W. H. Freeman, New York.

Willey, K. P., & Leidenberger, F. (1989) *J. Biol. Chem. 264,* 19716–19729.

Windridge, G. C., & Jorgensen, E. C. (1971) *Intra-Sci. Chem. Rep. 15,* 375–386.

Xue, C.-B., Bargiota, E. E., Miller, D., Becker, J. M., & Naider, F. (1989) *J. Biol. Chem. 264,* 19161–19168.