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SYNTHESIS, PURIFICATION AND STRUCTURE ACTIVITY RELATIONSHIPS IN THE DODECAPEPTIDE MATING FACTOR OF *SACCHAROMYCES CEREVISIAE*

By

Robert A. Baffi

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1983

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

August 15, 1983
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Fred Naider
Chairman of Examining Committee

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ABSTRACT

SYNTHESIS, PURIFICATION AND STRUCTURE ACTIVITY RELATIONSHIPS IN THE DODECAPEPTIDE MATING FACTOR OF SACCHAROMYCES CERVISIAE

By

Robert A. Baffi

Advisor: Professor Fred Naider

The α-factor is a tridecapeptide, Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr, which mediates the sexual conjugation of a and α haploids of *Saccharomyces cerevisiae*. Five desTrp^{1}-dodecapeptide analogs were prepared in which Trp^{3} was replaced with β-cyclohexylalanine (Cha) while Leu^{6} was substituted by Ala, Val, Ile, Nle or D-Leu. Also synthesized was desTrp^{1},Phe^{3}-dodecapeptide. All peptides were prepared by solution phase techniques utilizing mixed anhydrides or HOBT active esters. Purification to $>98\%$ homogeneity was accomplished by reverse-phase HPLC.

Structure-function relationships for positions 3, 6, 7, 9 and 12 of the des Trp^{1}-dodecapeptides were determined using quantitative bioassays which monitor aberrant cell morphologies (shmoos), increased agglutination, or cell cycle arrest of a-cells. In addition to the 6 peptides whose preparation is reported herein, 18 other dodecapeptides (14, 16, B. J. Kundu unpublished results) were investigated. Replacement of Trp^{3} with Ala or Phe led to inactive analogs which were antagonists for biological activities. Replacement of Trp^{3} with Cha enhanced morphogenic activity 3-fold while decreasing agglutination activity 5-fold. Elimination of the charge on Lys^{7} led to a 3 to 11-fold decrease in morphogenic potency and a 40-fold decrease in agglutination potency. Substitution of Leu^{6} with either Nle, Ile or Val caused a decrease in morphogenic activity of 1 to 10-fold while agglutination potency decreased from 35 to 375-fold. Replacement of Leu^{6} with Ala or D-Leu resulted in inactive analogs which were not antagonists for α-factor activity. These results indicate that size, hydrophobicity and configuration influence the activity of position six analogs.

Substitution of Gly^{9} with D-Ala led to a slight increase in morphogenic potency while the activity of the $L\text{-}Ala^{9}$ analog was reduced 48-fold. Replacement of Met^{12} with Nle resulted in a peptide of equal morphogenic activity.

Competition studies with inactive position 3 analogs demonstrated that agglutination activity could be inhibited with lower concentrations of antagonists than were required to inhibit morphogenic activity. Furthermore, the ratio of morphogenic activity to agglutination activity varied nearly 500-fold for the active analogs. These results indicate a heterogeneous response of a-cells to α-factor and suggest that a-cells contain more than 1 receptor for α-factor.

To My Parents

For All The

Love, Warmth and Comfort

You Have Provided Throughout The Years

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I am also indebted to Dr. P. Lipke for providing laboratory facilities and expertise in the biological aspects of my work. A special thanks to his graduate students and technicians who kept me up to my elbows in clean test tubes and viable yeast cells.

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INTRODUCTION

In Saccharomyces cerevisiae the transition from haploid to diploid occurs through cell fusion between individual cells of opposite mating type called a and α (1). Each mating type secretes a peptide pheromone which induces pre-mating events in cells of the opposite mating type. α-Factor is the pheromone secreted by α-cells. a-Cells treated with α-factor show increased sexual agglutinability (2-4), an altered morphology, (5,6) and inhibition of initiation of DNA synthesis (7). The morphogenesis is a result of continued cell growth and synthesis of a cell wall primed for mating (6,8). The increased agglutinability is due to greater cell surface expression of the agglutinating glycoprotein (4). Conversely, a-cells secrete a-factor, which induces similar changes in α-cells (9,10).

α-Factor can be isolated from culture medium in which α-cells have been grown, and consists of a mixture of 4 peptides based on the tridecapeptide sequence Trp-His-Trp-Leu-Gln-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr (11). The four forms differ in the presence of the N-terminal Trp and in the oxidation state of the Met residue. Three groups have synthesized α-factor. Ciejek et al. (12), and Samokhin et al. (13) reported that the tridecapeptide and dodecapeptide were active in the range of 85-1,000 ng/ml.

Masui et al. (14) claimed activity in the pg/ml range, but the claim remains unsubstantiated. Recently, we reported synthesis and activity of a number of analogs (15). We have found that the tridecapeptide and dodecapeptide are not equipotent, but are active in the range orginally reported by Stotzler, et al., Ciejek et al. and Samokhin et al. (11,12,13).

Structure function relationships have been investigated in our laboratory which demonstrate the effects of amino acid substitution on the potency of mating factors analogs (15,16). The goal of my project was to examine the interrelationship of the amino acid sequence of α-factor with its function as a pheromone in S. cerevisiae. A series of dodecapeptides analogs have been synthesized in which Leu in position 6 has been replaced with Ala, Val, Ile, Nle and D-Leu. Also, Phe was substituted for Trp in position 3 of the α-factor. Quantitative bioassays of these 6 α-factor analogs along with 18 other analogs previously prepared has led to a detailed analysis of the response of a-cells to these pheromones and to the suggestion that a-cells contain more than one receptor for α-factor.

BACKGROUND

The unicellular fungus *S. cerevisiae* is used extensively in industrial microbiology in the preparation of alcoholic beverages and baked products. Genetic manipulation and convenient growth conditions has allowed biochemical studies to elucidate many aspects of its cellular physiology, life cycle and mating processes. For these reasons *S. cerevisiae* is used as a model system to investigate the biochemical basis of developmental and cellular differentiation in eukaryotic organisms.

Segregation of certain enzymatic markers in four-spored asci from diploid cells indicated that *S. cerevisiae* followed the rules of Mendelian inheritance (17). Frequent departure from Mendelian inheritance was observed by Lindegren and Lindegren (18) in which irregular segregation occured, i.e., 4:0, 3:1, 1:3 and 0:4. Lindegren's interpretation of this process as gene conversion following Winkler's (19) theory of recombination was borne out in later studies in *Neurospora* (20) and yeast (21). By utilizing the vegetative cells derived from individual spores, the Lindegrens demonstrated that yeast had two mating types, which were designated *a* and α (21). Cells of mating type *a* mated only with cells of mating type α.

The two haploid cell types of S. cerevisiae are determined by alleles of the mating-type locus MATa for a cells and MATα for α-cells. Cells of opposite mating types mate efficiently, while those of like mating type mate only rarely (22). The a/α diploid formed by the mating of a and α-cells is a third type of cell. a/α Cells are unable to mate with haploid cells or heterogeneous diploids but can be induced to undergo meiosis and sporulation (23). The mating-type locus also controls pheromone production and agglutination. It appears that the mating-type locus codes for regulatory proteins that control expression of other genes coding for mating and sporulation specific process (24,25).

Each haploid cell type secretes a specific oligopeptide pheromone or mating factor (26). These extracellular peptides trigger biochemical changes which prepare each cell for mating. The responses include increased expression of specific cell surface agglutinins (2-4), arrest of cell growth in the G1 phase of the cell cycle (27), and enhanced cell wall synthesis in anticipation of conjugation (6,8).

The response of a-cells to α-factor provides the means for several bioassays for the determination of activity.

Chief among these is a transient morphological change in a cells, an aberrant elongation of the cell which is called shmoo formation after the Al Capp cartoon character (28). These changes are readily observed under the phase contrast microscope (28). Increased agglutination after induction of a-cells with α-factor can be monitored by a co-centrifugation assay which measures a decrease in absorbance due to the settling out of aggregated cells (4). Finally, G1 arrest can be monitored by inhibition of an increase in total cell number by hemacytometer count.

The α-factor is purified from culture medium in which α-haploids have been grown. It was first characterized by Duntze and co-workers (11,28-30). Specific binding of α-factor to a-cells appears to share some features of peptide hormone action in higher eukaryotic systems (31). This binding is dose-dependent, follows saturation kinetics and is first-order with respect to α-factor concentration, whether measured by the appearance of shmoos (32), induction of G1 arrest (33) or by the enhancement of agglutinability (1). Although kinetic evidence appears to support a specific α-factor receptor on a-cells no demonstration of preferential binding to intact a-cells has been reported (32-35). Nonspecific

binding of α-factor to a-cells may be attributed to the negatively charged groups on the cell surface of *S. cerevisiae* interacting with the positively charged α-factor at physiological pH. The phosphomannoprotein polymer which is responsible for the negative charge has also been shown to interact with lipophilic compounds and may associate with hydrophobic regions of the α-factor (36,37). Utilizing ^{3}H-labeled α-factor there was a 3-10 fold increase of α-factor bound to a cells compared to that amount bound to α-cells. Most of this associated α-factor was attributed to non-specific binding to low affinity sites not associated with physiologically relevant responses (36). It has been demonstrated that defects in the specific gene (Ste2) causes a-cells to be both α-factor resistant and sterile presumable due to the lack of α-factor receptors on the cell surface (38).

Besides non-specific binding, another problem in determining α-factor binding is that the effects of α-factor are transient indicating that a-cells have the ability to either remove α-factor from solution or enzymatically degrade it (38-40). It has been demonstrated that ^{125}I - labeled α-factor is chemically altered (34). Studies by various investigators concluded that the chemical alteration was due to proteolytic

cleavage of the α-factor (34,41,42). The primary scission occurs between Leu[6] and Lys[7] (41). The fragments that are produced are not biologically active. This indicates that cleavage results in the inactivation of α-factor leading to recovery of a cells from its effects. Cells of the a mating type containing an sstl mutation degrade ^{3}H-labeled α-factor much slower than the normal strain and appear to lack a membrane bound peptidase. Such mutants are considered super sensitive to the effects of α-factor (43).

Many peptide hormones in other systems appear to influence the physiology of their target cells by either stimulating or inhibiting the activity of membrane-bound adenylate cyclase; therefore, they modulate the intracellular level of 3'-5' cyclic AMP (cAMP). The effects of α-factor on the activity of the membrane-bound adenylate cyclase of S. cerevisiae has been investigated by Liao and Thorner (44). Addition of α-factor was found to inhibit adenylate cyclase of S. cerevisiae (44) in a dose-dependent manner. The integrity of the cell membrane appeared essential for this effect since solubilization of the enzyme with the detergent Triton X-100 abolished inhibition by α-factor (44). The in vitro inhibition of adenylate cyclase activity by α-factor was apparently

specific and appeared to be physiologically relevant to the mode of action of the pheromone *in vivo*. This hypothesis is borne out by several studies. Partial sequences of the α-factor molecule that are biologically inactive *in vivo* did not inhibit adenylate cyclase *in vitro* (44). Heterologous peptides, some of which are hormones known to alter the activity of adenylate cyclases in the membranes of other organisms, had no effect on the yeast enzyme. Artificially elevating the intracellular concentration of cAMP antagonizes α-factor (44). The presence of inhibitors of cAMP - phosphodiesterase activity in the culture medium could prevent the α-factor-induced cell-cycle arrest of *a*-cells. It appears that part of the mechanism of action of α-factor on *a* cells is mediated through an inhibition of adenylate cylase activity, which would lower the intracellular level of cAMP.

It has been borne out that many of the responses of mammalian cells to changes in cAMP concentration are mediated through changes in the enzymatic activity of cAMP-dependent protein kinases (45,46). Yeast cells seem to have a complete cAMP-based regulatory system, which includes two cyclic nucleotide phosphodiesterases, protein kinase activities and a cAMP binding protein that is

apparently a protein kinase regulatory subunit (47-53). The lowering of cAMP concentration presumably caused by α-factor would reduce the activity of cAMP-dependent protein kinase(s) or other specific cAMP-dependent functions and would lower the rate of phosphorylation and dephosphorylation (54-55). It appears that control of cell division and regulation of the gene expression and other metabolic functions required for conjugation may involve proteins whose activites or structural assembly are affected by their degree of phosphorylation.

The ability to synthesize mg quantities of α-factor and analogs of similar sequence has enabled our laboratory to probe the relationship between activity of α-factor and primary sequence substitutions. α-Factor has been synthesized by several groups using either solution or solid phase methods and displays the entire spectrum of biological responses (12-16,30,56). Structure function relationships indicate that some residues are essential for activity while other residues can withstand major modification or replacement (13,15,16,56).

Ciejek et al. (12) and Masui et al (14) reported activity for the synthetic tridecapeptide that differed by a factor of 10^4. Our laboratory undertook the synthesis of the

natural sequence α-factor to verify its activity. Our results were comparable (16) with those of Ciejek et al. (12) and Samokhin et al. (13) who reported activity for the tridecapeptide and dodecapeptide between 85-1,000 ng/ml. Our tridecapeptide at 70 ng/ml is as active as the purified biological α-factor of Duntze et al. (28).

Synthesis of desTrp^{1},Ala^{3}- and desTrp^{1},Cha^{3}-dodecapeptides by Khan et al. (15) served three purposes, first, to determine the importance of the size of the side chain of position 3, second, to simplify the amide proton region of the NMR spectra and third, to compare the activity of desTrp^{1},Ala^{3}-dodecapeptide with the Ala^{3}- tridecapeptide prepared by Masui et al. (56). It was determined that the side chain of position 3 influences activity. Analogs containing the bulky side group of Cha had an activity of 270 ng/ml while those containing the smaller side group of Ala didn't produce any response in a-cells at 500 μg/ml. As expected the amide portion of the NMR spectra was simplified with the removal of the aromatic side chain of Trp. This led to the synthesis of a series of des Trp^{1},Cha^{3} analogs in which Cha^{3}, Leu^{4}, Gln^{5}, Leu^{6}, Gln^{10}, Met^{12}, and Tyr^{13} were individually substituted with the

corresponding α-deuterated amino acid. NMR analysis of these peptides has unequovically determined the chemical shift of the aromatic and amide protons for all the residues in the desTrp^1,Cha^3-peptide (57).
The side chains of Ala and Cha also differ in hydrophobicity. To probe the hydrophobic requirements of the side chain of position 3 desTrp^1,Phe^3 has been prepared. This amino acid has a hydrophobicity in between Ala and Cha.

Shamokhin et al. (13) have demonstrated that Arg can replace Lys in the 7 position of the tridecapeptide without loss of activity. They also suggested that substitution at this position was possible provided that the electrical balance of the peptide was maintained. Shenbagamurthi et al. (16) have synthesized a series of desTrp^1,Cha^3-dodecapeptides in which the Lys in position 7 is either modified or substituted so as to abolish the charge of the epsilon-amino group. The activity of these position 7 analogs are reported in this manuscript.

Structure activity relationships in other positions indicate that the carboxyl terminus of Tyr is necessary for activity since esterification to monomethylpoly-

(ethylene oxide), average M.W. 3,700, resulted in loss of activity (16). Substitution of Met by Nle in the des Trp^{1},Cha^{3},Nle^{12}-dodecapeptide results in high biological activity. This finding is similar to results for the Nle^{12} tridecapeptide synthesized in Masui's laboratory (16,56). It has been demonstrated that when Gly is adjacent to Pro, as is the case in α-factor, that a Type II beta turn is favorable (56). Substitution of Gly by a D residue in a Type II beta turn should therefore be allowed. Analogs in which Gly has been replaced by L-Ala or D-Ala were prepared in order to establish the presence of a Type II beta turn in the secondary structure of the α-factor peptide.

The only substitutions previously reported for Leu in the 6 position was by Masui's laboratory (56). They determined that the activity of the desTrp^{1},D-Leu^{6}-dodecapeptide was 400 μg/ml. Due to the discrepancy in activity of identical sequences synthesized in our laboratory and that of Masui's I have prepared a series of analogs to determine the structural requirements for Leu in position 6. The side chain of Leu consists of four carbons containing a gamma branch. To determine the essential features of the side chain of Leu^{6} several analogs have been prepared in which alterations in configuration, type of branching and size of side chain

have been made. Substituting Leu^6 with its D-isomer allowed us to examine the effect a change in configuration has on biological activity. Replacement of Leu^6 with Nle which contains four carbons in an unbranched side chain or Ile which consists of four carbons in a beta branched side chain gave us the opportunity to correlate biological activity with gamma branching normally found in the side chain of position 6. The relationship between the size of the side chain in this position and biological activity has been probed by substitution with Val which has three carbons in its side chain and is beta branched and Ala which has only one carbon in its side chain.

The goals of this thesis were to distinguish the various responses of a-cells to synthetic α-factor analogs utilizing quantitative bioassays, and to determine the structural requirements at various positions (3,6,7,9,12) of the primary sequence necessary for biological activity.

METHODS AND MATERIALS

ABBREVIATIONS

Ac - Acetyl

Boc - *tert*-butoxycarbonyl

Bu - n-butyryl

n-BuOH - 1-butanol

Cha - ß-cyclohexylalanine

DCC - dicyclohexylcarbodiimide

DMF - dimethylformamide

Dns - 1-dimethylaminonapthalene-5-sulfonyl

EEDQ - *N*-ethoxycarbonyl-2-ethoxy-1-2-dihydroquinoline

EtOAc - ethyl acetate

HOBT - 1-hydroxybenzotriazole

IBC - isobutylchloroformate

La - lauryl

MeOH - methanol

MSA - methanesulfonic acid

Nle - norleucine

NMM - *N*-methylmorpholine

OBzl - benzyl ester

Oc - octanoyl

OMe - methyl ester

ONp - p-nitrophenyl ester

TEA - triethylamine

St - stearoyl

TFA - trifluoroacetic acid

Z - benzyloxycarbonyl.

All amino acids were of the L-configuration unless otherwise specified. All of the amino acid deriviatives were synthesized by me or purchased from Bachem. All the solvents used were of analytical grade supplied by Fischer Scientific, Eastman and Sargent-Welch. The following chemicals were of reagent grade and were used as received: N-methylmorpholine (Aldrich), isobutylchloroformate (Aldrich), anhydrous formic acid (Fluka), 88% formic acid (Baker), 1-hydroxybenzotriazole (Aldrich), methanesulfonic acid (Aldrich), trifluoracetic acid (Sigma), toluenesulfonic acid (Fischer Scientific), [2-(tert-butoxycarbonyloxyimino)-2-phenylacetonitrile] (BOC-ON, Aldrich), N,N' - dicyclohexylcarbodiimide (Aldrich), palladium (II) chloride (Aldrich), triethylamine (Aldrich) N-ethoxycarbonyl-2-ethoxy-1,2, dihydroquinoline (Aldrich), ninhydrin (Aldrich), yeast nitrogen base without amino acids (YNB, Difco Labs) and microtiter plates (Falcon).

The following solvent systems were utilized for thin layer chromatography (TLC) on silica gel plates (Brinkman) for amino acid derivaties and peptides: (A) CH_2Cl_2: MeOH: AcOH (9:1.5:0.5), (B) 1-Butanol:AcOH:H_2O (4:1:5, upper phase) and (C) 1-Butanol:AcOH:H_2O: pyridine (15:3:12:10), and the Rf values are reported as RfA, RfB and RfC. Optical rotations of the peptides were

determined in the solvents indicated in parenthesis while the concentrations are in gms of peptide/100 ml of solvent.

Spots were detected by UV light and visualized by spraying with ninhydrin. Melting points were determined by capillary method and are uncorrected. High performance liquid chromatography (HPLC, analytical) was carried out on 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 through cell. The micro Bondapak C_{18} column (10 μm, 30 cm, 0.39 cm ID) was also from Waters. Sample injections were made with a 25 μl syringe (Hamilton). Filtration of solvents was accomplished using a Pyrex filter holder with a 0.45 μm HA filter (Millipore). All solvents were HPLC grade (Fischer); water was glass distilled. Purity of samples were determined in two solvent systems ($MeOH:H_2O:TFA$ & $CH_3CN:H_2O:TFA$). Generally, a flow rate of 3 ml/min was used which maintained a pressure of 3000-3500 psi. The recorder chart speed was 0.4 in/min. All tests were performed at room temperature. Sample concentrations were 1 mg/ml and volumes of 10-25 μl were injected. Detection was usually at 220 nm. The sensitivity of the UV dectector was set at 0.1 AUFS. A Waters Prep LC/system 500 was used for the purification of the dodecapeptides.

Amino acid analysis were performed at Rockefeller University, New York, or at Hofmann-La Roche Nutley, N.J. Peptides were hydrolized in sealed tubes in 6N HCl at 100° for 24 h. For peptides containing methionine anisole was added as a scavenger. All peptides gave acceptable amino acid ratios (Table 2).

Strains. Haploid strains X2180-1A (a-cells) and X2180-1B (α-cells) were grown in minimal medium containing Yeast Nitrogen Base (2.2 g/l), $(NH_3)_2SO_4$ (4.5 g/l) and glucose (20 g/l). All cells were grown at 25° to mid-log phase.

Bioassays

Morphogenic Activity. The morphogenesis assay was carried out in plastic microtiter plates obtained from Falcon. Each well had 100 µl of medium (pH 5) containing 3×10^2 a-cells and serial binary dilutions of the appropriate synthetic analog. The lowest concentration causing visible morphogenesis after a 4 h incubation at 25° was designated 1 unit of activity.

Agglutination Activity. The agglutination potency was determined by the co-centrifugation assay of Terrance and Lipke (4). a-Cells at a concentration of 3×10^6

cells/ml were incubated with 10-fold serial dilutions of the synthetic analogs. After a 20 min incubation, the a-cells were mixed with α-cells and agglutination was determined in quadruplicate. The concentration inducing half the increase in agglutinability of that induced by 1 unit/ml of biological α-factor was determined from interpolation of dose-response curves.

Cell Cycle Arrest Studies. The inhibition of an increase in total cell number by hemacytometer count was used to determine G1 arrest. a-Cells were incubated at 30° at a cell density of 3×10^6 cells/ml with the appropriate analogs at concentrations 10-fold above morphogenic doses and at agglutination doses. Aliquots were taken every 30 min and sonicated for 4 s before counting under a phase contrast microscope.

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 biological activity 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.

PEPTIDE SYNTHESIS

N-Ac-[α-^{2}H]D,L-Phe. A mixture of $(Ac_2)O$ (22 ml), D_2O (2.5 ml) and phenylalanine (1.65 g, 10 mmol) was refluxed for 2 minutes, at 170°. When the reaction cooled D_2O (2 ml) was added, the solvents evaporated and the residue recrystallizied from EtOAc (58).

Yield 1.44 g (87%); m.p. 183-185°; $[\alpha]_D^{24}$ 0° (c 1,5N HCl).

Preparation of [α-^{2}H],L-Phe. A 0.11M solution of N-Ac-[α-^{2}H]D,L-Phe (2.08 g, 10 mmol) in water was adjusted to pH 7.2 with 25% NH_4OH and Hog Renal Acylase I (5 mg) was added. When the UV absorption of the solution at 220nm had decreased 50%, AcOH (4 ml) was added and the solution chilled to 5°. The phenylalanine was filtered and washed with cold ethanol and then ether and dried (59).

Yield 0.58g (80%) m.p. 250°; $[\alpha]_D^{24}$ -7.2° (c 1, 5N HCl)

Boc-amino acids were prepared with the following general procedure (60). A mixture of amino acid (10 mmol), Boc ON (2.46 g,10 mmol), Et_3N (2.1 ml, 15 mmol), dioxane (5 ml) and H_2O (5 ml) was stirred for 24 h. The reaction mixture was diluted with H_2O (20 ml) and EtOAc (20 ml).

Table 1. Properties of Boc-Amino Acids

Compounds	Yield	RfA	MP °C
Boc-Met	86	0.72	oil
Boc-Met α deuterated	84	0.72	oil
Boc-Gln	55	0.86	116-117
Boc-Gly	82	0.81	84-86
Boc-Lys(Z)	67	0.64	oil
Boc-Leu • H_2O	82	0.67	68-70
Boc-Leu α deuterated • H_2O	85	0.67	67-69
Boc-D-Leu • H_2O	83	0.67	66-69
Boc-Phe α deuterated	86	0.61	80-82
Boc-Cha	88	0.58	oil
Boc-His(Boc)	81	0.74	96-98
Boc-Ala	88	0.70	oil
Boc-Ile	80	0.68	oil
Boc-Val	86	0.69	oil
Boc-Trp	85	0.57	124-127
Boc-Pro	77	0.65	124-128

After separation the organic phase was discarded and the aqueous layer was washed 2 times with Et_2O. The aqueous layer was acidified with 5% citrate and the Boc-amino acid extracted into EtOAc 3 times (30 ml). The EtOAc layer was washed with H_2O 2 times (20 ml), dried with ($MgSO_4$) and evaporated. Table 1 lists the Boc-amino acids prepared in this manner.

The following peptides were prepared by the procedures of Khan et al. (15), I, II, IV, V, VIII, IX, X, XI, XII

Boc-Met-Tyr-OBzl (I). Boc Met (4.98 g, 20 mmol) was dissolved in THF (50 ml) at -15° and was neutralized with N-methylmorpholine (2.2 ml, 20 mmol) and followed by isobutylchloroformate (2.6 ml, 20 mmol). The solution was stirred for 7 min when a precooled solution of Tyr-OBzl Tos (8.8 g, 20 mmol) in DMF (16 ml) was added. The temperature was maintained at -15° with stirring for 30 min and at room temperature for 2 h, the solvents were evaporated *in vacuo*. The residue was dissolved in EtOAc (200 ml) and washed successively with 5% citrate, water, 5% $NaHCO_3$ and water. The organic phase was dried ($MgSO_4$) and evaporated. The residue was crystallized from EtOAc-PE.

Yield 7.70 g (75%); m.p. 98-100°; $[\alpha]_D^{24}$+1.3° (c 0.5,THF); RfA 0.79, RfB 0.87

Boc-Gln-Pro-OBzl (II). A solution containing Boc-Gln-ONp (3.67 g, 10 mmol), Pro-OBzl·HCl (2.42 g, 10 mmol) and HOBT (1.53 g, 10 mmol) in DMF (15 ml) was neutralized with NMM (1.1 ml, 10 mmol) and the mixture stirred at room temperature for 1 h. The solvent was evaporated *in vacuo*, and the residue dissolved in EtOAc. The EtOAc phase was washed successively with 5% citrate, water, 5% $NaHCO_3$ and water. The organic layer was dried ($MgSO_4$) and evaporated. The residue was crystallized from EtOAC.

Yield 3.12 g (72%); m.p. 132-143°; $[\alpha]_D^{24}$-80.46° (c 2, MeOH); RfA 0.71, RfB 0.79.

Boc-Gly-Gln-Pro-OBzl (III). To a solution of Boc-Gln-Pro-OBzl (6.5 g, 15 mmol) in formic acid - CH_2Cl_2 (75 ml, 1:1, v/v) was added MSA (0.98 ml, 15 mmol). Quantitative removal of the Boc group was accomplished in 2 min as indicated by thin layer chromatography. The solvents were evaporated at room temperature *in vacuo* and the residue precipitated from 2-propanol-ether. The dipeptide benzyl ester methane sulfonate after being isolated and dried was dissolved in DMF (22 ml) neutralized with NMM (1.65 ml, 15 mmol) and added to the IBC mixed anhydride formed from Boc Gly (2.63 g, 15 mmol), NMM (1.65 ml, 15 mmol) and IBC (1.95 ml, 15 mmol) in THF (40 ml) at -15°.

After 30 min at -15° and 2 h at room temperature the solvents were evaporated and dried, the residue was dissolved in EtOAc. The organic phase was washed successively with 5% $NaHCO_3$, water, 5% citrate and 10% NaCl. The EtOAc phase was dried ($MgSO_4$) and evaporated. The residue was crystallized from EtOAc-ether.

Yield 5.15 g (70%); m.p. 156-158°; $[\alpha]_D^{24}$-33.4° (c 2,EtOH) RfA 0.68, RfB 0.76.

<u>Boc-Gly-Gln-Pro (IV)</u>. To a solution of III (1.96 g, 4 mmol) in methanol (8 ml), was added Pd-black (300 mg). At room temperature formic acid (88% 0.5 ml) was added and the reaction allowed to stir until thin layer chromatography indicated complete hydrogenolysis. The catalyst was filtered off through Celite and the filtrate evaporated <u>in vacuo</u>. The residue was crystallized from EtOAc-PE.

Yield 1.47 g (92%); m.p. 83-85° ; $[\alpha]_D^{24}$-53.1° (c 2, EtOH); RfA 0.43, RfB 0.53.

<u>Boc-Gly-Gln-Pro-Met-Tyr-OBzl (V)</u>. To a solution of Boc-Met-Tyr-OBzl (2.5g, 5 mmol) in formic acid-CH_2Cl_2 (25 ml, 1:1 v/v) in the presence of anisole (1 ml) was added MSA (0.32ml, 5 mmol).

When quantitative removal of the Boc group was indicated by thin layer chromatography the solvents were evaporated *in vacuo* at room temperature. The residue was precipitated from 2-propanol-ether isolated and dried. To maximize yields, various solvents were used for the formation of the IBC mixed-anhydride of IV including: THF[a], THF-DMF[b] (3:1), HCCl[c] and EtOAC[d]. Since yields were still unsatisfactory the EEDQ[e] mixed anhydride procedure and the HOBT-IBC[f] mixed anhydride active ester in THF-DMF (3:1) were employed. In all cases the pentapeptide was worked-up in the usual manner.

m.p. 196-197°; $[\alpha]_D^{24}$ -71.3° (c 2, MeOH) RfA 0.53, RfB 0.75

Yields

a 0.59 g (15%)

b 1.19 g (30%)

c 0.39 g (10%)

d 0.59 g (15%)

e 1.99 g (50%)

f 2.39 g (60%)

Boc-Pro-Met-Tyr-OBzl (VI). The Boc group of Boc-Met-Tyr-OBzl (5.0 g, 10 mmol) was cleaved by treatment with formic acid-CH_2Cl_2 (50 ml, 1:1, v/v) plus anisole (1 ml). After 10 min the solvents were evaporated and the

residue precipitated from 2-propanol-ether. A solution of the dipeptide benzyl ester methane sulfonate was dissolved in DMF (15 ml), the solution cooled to -15° and neutralized with NMM (1.10 ml, 10 mmol) then reacted with the IBC mixed anhydride formed from Boc-Pro (2.15 g, 10 mmol), NMM (1.10 ml, 10 mmol) and IBC (1.3 ml, 10 mmol) in THF (25 ml) at -15°. The mixture was stirred for 30 min at -15° and at room temperature for 2 h. The solvents were evaporated <u>in vacuo</u> and the residue dissolved in EtOAc and washed successively with 5% citrate, water, 5% $NaHCO_3$ and water. The organic phase was dried ($MgSO_4$) evaporated and the residue was recrystallized from EtOAc-ether.

Yield 5.06 g (78%); m.p. 139-141; $[\alpha]_D^{24}$-46.1° (c 1.3, MeOH); RfA 0.41, RfB 0.67

<u>Boc-Gln-Pro-Met-Tyr-OBzl (VII)</u>. To a solution of Boc-Pro-Met-Tyr-OBzl (2.98 g, 5 mmol) in formic acid-CH_2Cl_2 (25 ml, 1:1 v/v) in the presence of anisole (1 ml) was added MSA (0.32 ml, 5 mmol). When quantitative removal of the Boc group was indicated by thin layer chromatography the solvents were evaporated <u>in vacuo</u> and the residue precipitated from 2-propanol-ether isolated and dried.

The tripeptide benzyl ester methane sulfonate along with Boc-Gln-ONp (1.90 g, 5 mmol) and HOBT (0.77 g, 5 mmmol) were dissolved in DMF (10 ml) and neutralized with TEA (0.7 ml, 5 mmol) and allowed to stir at room temperature for 2 h. The solvent was evaporated in vacuo and the residue dissolved in EtOAc. The organic phase was washed successively with 5% citrate, water, 5% $NaHCO_3$ and water. The organic phase was dried ($MgSO_4$), evaporated and the residue crystallized from EtOAc-ether. Further purification was accomplished by using a Silica 60 column as a stationary phase and EtOAc as the mobile phase.

Yield 3.06 g (83%); m.p.165-168; $[\alpha]_D^{24}$-54.0° (c 1.0, MeOH)
RfA 0.50, RfB 0.70

Boc-Gly-Gln-Pro-Met-Tyr-OBzl (V). To a solution of Boc-Gln-Pro-Met-Tyr-OBzl (2.18 gm, 3 mmol) in formic acid-CH_2Cl_2 (15 ml, 1:1 v/v) in the presence of anisole (0.6 ml) was added MSA (0.21 ml, 3 mmol). When quantitative removal of the Boc group was indicated by thin layer chromotagraphy the solvents were evaporated and the residue precipitated from 2-propanol-ether isolated and dried. The tetrapeptide benzyl ester methane sulfonate was dissolved in DMF (5 ml), the solution was cooled to -15° and neutralized with NMM (0.33 ml, 3 mmol) and allowed to react with the IBC mixed anhydride prepared from Boc-Gly (0.52 g, 3 mmol), NMM (0.33 ml,

3 mmol) and IBC (0.41 ml, 3 mmol) in THF (12 ml) at -15°. The reaction was stirred for 30 min at -15° and for 2 h at room temperature. The solvents were evaporated and the residue dissolved in EtOAc. The organic phase was washed successively with 5% citrate, water, 5% $NaHCO_3$ and water. The organic phase was dried ($MgSO_4$) and evaporated. The residue crystallized from EtOAc-ether. Yield 1.60 g (67%); m.p. 196-197°; $[\alpha]_D^{24}$-71.3° (c 2 , MeOH) RfA 0.53, RfB 0.75

Boc-Lys(Z)-Pro-OMe (VIII). The IBC mixed anhydride prepared from Boc-Lys(Z) (7.6 g, 20 mmol), NMM (2.2 ml, 20 mmol) and IBC (2.6 ml, 20 mmol) in THF (50 ml) at -15° was treated with a solution of Pro-OMe•HCl in DMF (12 ml) precooled to -15° and neutralized with NMM (2.2 ml, 20 mmol). The reaction mixture was stirred at -15° for 30 min and at room temperature for 2 h. The solvents were evaporated *in vacuo* and the residue dissolved in EtOAc and was washed successively with 5% citrate, water, 0.1N NaOH and water. The organic phase was dried ($MgSO_4$) and evaporated. The dipeptide was isolated as an oil. Yield 7.38 g (75%); RfA, 0.85, RfB, 0.91

Boc-Leu-Lys(Z)-Pro-OMe (IX). Boc-Lys(Z)-Pro-OMe (4.91 g, 10 mmol) was treated with formic acid-CH_2Cl_2 (100 ml, 1:1, v/v) containing MSA (0.65 ml, 10 mmol) at room

temperature for 5 min. The solvents were evaporated, and the residue was precipitated from 2-propanol-ether, isolated and dried <u>in vacuo</u> over P_2O_5. The dipeptide methane sulfonate was dissolved in DMF (8 ml), neutralized with NMM (1.1 ml, 10 mmol) at -15°, and reacted with the IBC mixed anhydride prepared from Boc-Leu (2.31 g, 10 mmol),NMM (1.1 ml, 10 mmol) and IBC (1.3 ml, 10 mmol) in THF (25 ml) at -15°. The mixture was stirred for 30 min at -15° and 2 h at room temperature. The solvents were evaporated <u>in vacuo</u> and the residue dissolved in EtOAc and washed successively with 5% citrate, water, 0.1N NaOH and water. The solvent was dried ($MgSO_4$) and evaporated. The tripeptide was isolated as an oil. Further purification was accomplished utilizing a Silica 60 column and CH_2Cl_2: MeOH (98:2 ml) as the mobile phase.

Yield 3.93 g, (65%); RfA 0.82, RfB 0.86.

The following tripeptides were obtained using a procedure analogous to that used for compound IX.

Boc-Nle-Lys(Z)-Pro-OMe (IXa). Yield 4.36 g (72%); RfA 0.81, RfB 0.88.

Boc-D-Leu-Lys(Z)-Pro-OMe (IXb). Yield 4.11 g (68%); RfA 0.83, RfB 0.90.

Boc-Ala-Lys(Z)-Pro-OMe (IXc). Yield 4.45 g (79%); RfA 0.79, RfB 0.82.

Boc-Ile-Lys(Z)-Pro-OMe (IXd). Boc-Lys(Z)-Pro-OMe (4.91 g, 10 mmol) was treated with formic acid-CH_2Cl_2 (100 ml, 1:1, v/v) containing MSA (0.65 ml, 10 mmol) at room temperature for 5 min. The solvents were evaporated, and the residue was precipitated from 2-propanol-ether, isolated and dried *in vacuo* over P_2O_5. The dipeptide methanesulfonate was dissolved in DMF (8 ml) neutralized with NMM (1.1 ml, 10 mmol) at -15°, and reacted with the IBC mixed anhydride-HOBT active ester prepared from Boc-Ile (2.31 g, 10 mmol), NMM (1.1 ml, 10 mmol) and IBC (1.3 ml, 10 mmol) in THF (25 ml) at -15° for 10 min and then 2 eqs. of HOBT (3.1 g, 20 mmol) for an additional 10 min. The mixture was stirred for 30 min at -15° and 2 h at room temperature. Usual workup gave the tripeptide as an oil.
Yield 3.63 g (60%); RfA 0.81, RfB 0.89.

Boc-Val-Lys(Z)-Pro-OMe (IXe). The title compound was obtained using a procedure analogous to that used for compound IXd and isolated as an oil. Yield 2.95 g (50%); RfA 0.81, RfB 0.84.

Boc-Leu-Lys(Z)-Pro(X). To a solution of IX (6.0 g, 10 mmol) in MeOH (30 ml), 1M NaOH (12 ml) was added and the mixture stirred for 3.5 h at 30° at which time TLC system A indicated almost complete saponification. After evaporation of the MeOH *in vacuo* the residue was dissolved in water and extracted with ether. The aqueous phase was then acidified with 5% citrate and the Boc-tripeptide was extracted into EtOAc. The organic phase was washed with water, dried ($MgSO_4$) and evaporated. The residue was crystallized from THF-PE. Yield 4.13 g (70%); m.p. 65-66°; $[\alpha]_D^{24}$ -53.3° (c 2,EtOH); RfA 0.81, RfB 0.91.

The following tripeptides were obtained using a procedure analogous to that used for compound X.

Boc-Nle-Lys(Z)-Pro (Xa). Yield 3.84 g (65%); m.p. 65-68°; $[\alpha]_D^{24}$ -50° (c 1.5, EtOH) RfA 0.74, RfB 0.83.

Boc-D-Leu-Lys(Z)-Pro (Xb). Yield 3.72 g (63%); m.p. 62-64°; $[\alpha]_D^{24}$ -9.0° (c 1.3, EtOH); RfA 0.74, RfB 0.87.

Boc-Ala-Lys(Z)-Pro (Xc). Yield 4.07 g (69%); m.p. 78-80°; $[\alpha]_D^{24}$ -49.9 (c 1.6, EtOH) RfA 0.68, RfB 0.80.

Boc-Ile-Lys(Z)-Pro (Xd). Yield 4.07 g (60%); m.p. 78-80°; $[\alpha]_D^{24}$ -49.9 (c 1.6, EtOH) RfA 0.75, RfB 0.83.

Boc-Val-Lys(Z)-Pro (Xe). Yield 4.19 g (73%); m.p. 63-65°; $[\alpha]_D^{24}$ -51.2 (c2, EtOH); RfA 0.81, RfB 0.93.

Boc-Gln-Leu-Lys(Z)-Pro (XI). The Boc group from Boc-Leu-Lys(Z)-Pro (2.95 g, 5 mmol) was cleaved as described in the preparation of the tripeptide (IX). The tripeptide methane sulfonate was coupled with Boc-Gln-ONp (1.84 g, 5 mmol), neutralized with NMM (1.1 ml, 10 mmol) and accelerated with HOBT (0.77 g, 5 mmol) in DMF (12 ml). After a reaction period of 2 h, the solvent was evaporated *in vacuo* and the residue dissolved in EtOAc and washed with 5% citrate and water. The organic phase was dried ($MgSO_4$) and evaporated *in vacuo*. The residue was crystallized two times from EtOAc-ether.

Yield 2.53 g (69%); m.p. 101-4°; $[\alpha]_D^{24}$ -59.9°
(c 1.8, EtOH) RfA 0.51, RfB 0.68.

The following tetrapeptides were obtained using a procedure analogous to that used for compound XI.

Boc-Gln-Nle-Lys(Z)-Pro (XIa). Yield 2.23 g (62%);
m.p. 99-102°; $[\alpha]_D^{24}$ -58.6° (c 1.6, EtOH);
RfA 0.53, RfB 0.70.

Boc-Gln-D-Leu-Lys(Z)-Pro (XIb). Yield 2.15 g (60%);
m.p. 86-89°; $[\alpha]_D^{24}$ -17.7° (c 3.5,EtOH);
RfA 0.52, RfB 0.68.

Boc-Gln-Ala-Lys(Z)-Pro (XIc). Yield 2.19 g (65%);
m.p. 100-102°; $[\alpha]_D^{24}$ -57.2° (c 1.3, EtOH);
RfA 0.64, RfB 0.65.

Boc-Gln-Ile-Lys(Z)-Pro (XId). Yield 1.97 g (55%);
m.p. 115-117°; $[\alpha]_D^{24}$ -56.2° (c 1.5, EtOH);
RfA 0.61, RfB 0.68.

Boc-Gln-Val-Lys(Z)-Pro (XIe). Yield 2.00 g (57%);
m.p. 94-96° $[\alpha]_D^{24}$ -55° (c 1.7, EtOH);
RfA 0.74, RfB 0.68.

Boc-Leu-Gln-Leu-Lys(Z)-Pro (XII). The Boc group from Boc-Gln-Leu-Lys(Z)-Pro (2.58 g, 3.6 mmol) was cleaved as described in the preparation of the tripeptide (IX). The tetrapeptide methane sulfonate was dissolved in DMF (8 ml), cooled, neutralized with NMM (0.8 ml, 0.72 mmol) and allowed to react with the IBC mixed anhydride prepared from Boc-Leu (0.88 g, 3.8 mmol), NMM (0.42 ml, 3.8 mmol) and IBC (0.47 ml, 3.6 mmol) in THF (7 ml) at -15°. After 30 min at -15° and 2 h at room temperature most of the solvents were removed by evaporation *in vacuo* and the remaining solution poured into 5% citrate. The precipitated solid was washed with water isolated by filtration, dried and precipitated from THF-ether.

Yield 2.1 g (65%); m.p. 114-116°; $[\alpha]_D^{24}$-60.7°
(c 1.2, EtOH); RfA 0.43, RfB 0.72.

The following pentapeptides were obtained using a procedure analogous to that used for compound XII.

Boc-Leu-Gln-Nle-Lys(Z)-Pro (XIIa). Yield 2.2 g (75%); m.p. 121-124°; $[\alpha]_D^{24}$ -59.6°(c 1.4, EtOH); RfA 0.85, RfB 0.76.

Boc-Leu-Gln-D-Leu-Lys(Z)-Pro (XIIb). Yield 1.8 g (62%); m.p. 115-118°; $[\alpha]_D^{24}$ -50.2° (c 1.3, EtOH); RfA 0.61, RfB 0.70.

Boc-Leu-Gln-Ala-Lys(Z)-Pro (XIIc). Yield 1.6 g (57%); m.p. 113-116°; $[\alpha]_D^{24}$ -59.8° (c 1.3, EtOH); RfA 0.50, RfB 0.50.

Boc-Leu-Gln-Ile-Lys(Z)-Pro (XIId). Yield 2.01 g (68%); m.p. 126-130°; $[\alpha]_D^{24}$ -61.4° (c 1.4, EtOH); RfA 0.81, RfB 0.72.

Boc-Leu-Gln-Val-Lys(Z)-Pro (XIIe). Yield 2.13 g (72%); m.p. 106-108°; $[\alpha]_D^{24}$ -57.5° (c 1.6, EtOH); RfA 0.67, RfB 0.72.

Boc-[α-^{2}H]-Phe-Leu-Gln-Leu-Lys(Z)-Pro (XIII). The Boc group from Boc-Leu-Gln-Leu-Lys(Z)-Pro (0.99 g, 1.2 mmol) was cleaved as described for the preparation of the tripeptide (IX). The pentapeptide methane sulfonate was dissolved in DMF (6 ml), neutalized with NMM (0.27 ml, 2.4 mmol) and allowed to react with the mixed anhydride prepared from Boc-[α-^{2}H]-Phe (3.19 g, 1.2 mmol), NMM (0.13 ml, 1.2 mmol) and IBC (0.16 ml, 1.2 mmol) in THF (5 ml) at -15°. The product was isolated and crystallized by the procedure described for (XII).

Yield 0.94 g (80%); m.p. 162-165°; $[\alpha]_D^{24}$-65.1° (c 1.2, MeOH); RfA 0.70, RfB 0.68.

Boc-Cha-Leu-Gln-D-Leu-Lys(Z)-Pro (XIV). The Boc group from Boc-Leu-Gln-D-Leu-Lys-(Z)-Pro (1.88 g, 2.4 mmol) was cleaved as described for the preparation of the tripeptide (IX). The pentapeptide methane sulfonate was dissolved in DMF (12 ml) neutralized with NMM (0.54 ml, 4.8 mmol) and allowed to react with the mixed anhydride prepared from Boc-Cha (0.65 g, 2.4 mmol), NMM (0.26 ml, 2.4 mmol) and IBC (0.16 ml, 1.2 mmol) in THF (10 ml) at -15°. The product was isolated and crystallized by the procedure described for (XII).

Yield 1.94 g (83%); m.p. 123-125°; $[\alpha]_D^{24}$-61.4° (c 1.0, MeOH); RfA 0.76, RfB 0.78.

The following hexapeptides were obtained using a procedure analogous to that used for compound XIV.

Boc-Cha-Leu-Gln-Nle-Lys(Z)-Pro (XIVa). Yield 1.26g (72%); m.p. 179-182°; $[\alpha]_D^{24}$ -62.6°(c 0.9, MeOH); RfA 0.76, RfB 0.79.

Boc-Cha-Leu-Gln-Ala-Lys(Z)-Pro (XIVb). Yield 1.73 g (74%); m.p. 170-173°; $[\alpha]_D^{24}$ -63.0° (c 1.2, MeOH); RfA 0.72, RfB 0.74.

Boc-Cha-Leu-Gln-Ile-Lys(Z)-Pro (XIVc). Yield 2.0 g (86%); m.p. 186-189°; $[\alpha]_D^{24}$ -65.2° (c 1.3, MeOH); RfA 0.75, RfB 0.79.

Boc-Cha-Leu-Gln-Val-Lys(Z)-Pro (XIVd). Yield 1.8 g (78%); m.p. 159-163°; $[\alpha]_D^{24}$ -61.1° (c 0.8, MeOH); RfA 0.72, RfB 0.76.

Boc-His-(Boc) [α-^{2}H]-Phe-Leu-Gln-Leu-Lys(Z)-Pro (XV). The Boc group was cleaved from Boc -[α-^{2}H]-Phe-Leu-Gln -Leu-Lys(Z)-Pro (0.98 g, 1 mmol) as described in the preparation of the tripeptide (IX). The hexapeptide methane sulfonate was dissolved in DMF (6 ml) and added to this solution was Boc-His(Boc)-ONp (0.48 g, 1 mmol) HOBT (0.15 g, 1 mmol) and NMM (0.22 ml, 2 mmol). The reaction was stirred for 2 h, diluted with water (15 ml) and acidified with 5% citrate. The heptapeptide was isolated and purified by precipitation from THF-ether.

Yield 1.07 g (87%); m.p. 182-186°; $[\alpha]_D^{24}$-39.0° (c 1.2, DMF); RfA 0.55, RfB 0.78

Boc-His(Boc)-Cha-Leu-Gln-D-Leu-Lys(Z)-Pro (XVI). The Boc group was cleaved from Boc-Cha-Leu-Gln-D-Leu-Lys(Z)-Pro (1.0 g, 1 mmol) as described in the preparation of the tripeptide (IX). The hexapeptide methane sulfonate was dissolved in DMF (6 ml) and added to this solution was Boc-His(Boc)-ONp (0.48 g, 1 mmol) HOBT (0.15 g, 1 mmol) and NMM (0.22 ml, 2 mmol). The reaction was stirred for 2 h, diluted with water (15 ml) and acidified with 5% citrate. The heptapeptide was isolated and purified by precipitation from THF-ether.

Yield 1.01 g (83%); m.p. 157-159°; $[\alpha]_D^{24}$-35.6° (c 1.4, DMF); RfA 0.59, RfB 0.80.

The following heptapeptides were obtained using a procedure analogous to that used for compound XVI.

<u>Boc-His(Boc)-Cha-Leu-Gln-Nle-Lys(Z)-Pro (XVIa)</u>.

Yield 1.1g (88%); m.p. 212-215°; $[\alpha]_D^{24}$ -32.6° (c 1.1, DMF); RfA 0.61, RfB 0.81.

<u>Boc-His(Boc)-Cha-Leu-Gln-Ala-Lys(Z)-Pro (XVIb)</u>.

Yield 1.00 g(82%); m.p. 195-198°; $[\alpha]_D^{24}$ -30.4° (c 1.5, DMF); RfA 0.55, RfB 0.75.

<u>Boc-His(Boc)-Cha-Leu-Gln-Ile-Lys(Z)-Pro (XVIc)</u>.

Yield 1.1 g (89%); m.p. 203-205°; $[\alpha]_D^{24}$ -34.3° (c 1.0, DMF); RfA 0.60, RfB 0.77.

<u>Boc-His(Boc)-Cha-Leu-Gln-Val-Lys(Z)-Pro (XVId)</u>.

Yield 0.99 g (81%); m.p. 212-215°; $[\alpha]_D^{24}$ -29.7° (c 0.9, DMF); RfA 0.56, RfB 0.73.

His-[α-^{2}H]-Phe-Leu-Gln-Leu-Lys(Z)-Pro-Gly-Gln-Pro-Met-Tyr·3TFA (XVII). Boc-Gly-Gln-Pro-Met-Tyr-OBzl (0.225 g, 0.3 mmol) was treated with a mixture of TFA (1.5 ml), CH_2Cl_2 (1.5 ml) and anisole (0.2 ml) for 30 min at room temperature. The solvents were evaporated *in vacuo* at room temperature and precipitated from 2-propanol-ether isolated and dried *in vacuo* over P_2O_5. The pentapeptide trifluoroacetate was dissolved in DMF (2 ml), neutralized with NMM (0.033 ml, 0.3 mmol) and added to the IBC mixed anhydride prepared from (XV) (0.36 g, 0.3 mmol), NMM (0.033 ml, 0.3 mmol) and IBC (0.039 ml, 0.3 mmol) in a mixture of THF (3 ml) and DMF (1 ml) at -15°. The reaction was stirred for 1 h at -15° and 1 h at room temperature. The solvents were removed and the residue triturated with 5% citrate. The solid was isolated, washed with water and dried. The protected dodecapeptide was precipitated from DMF-ether isolated and dried. To a solution of the protected dodecapeptide (400 mg) in methanol (8 ml), Pd black (300 mg) and 90% formic acid (0.3 ml) were added. After TLC (solvent system B) indicated complete hydrogenation the catalyst was removed by filtration through Celite and the filtrate evaporated to dryness. The residue was dissolved in TFA-CH_2Cl_2 (1:1, 2 ml) containing anisole (0.4 ml) and stirred for 30 min at room temperature. The peptide (400 mg) was precipitated with ether isolated and dried.

The crude peptide was dissolved in MeOH:H_2O:TFA (5 ml 340:660:0.25, v/v) and injected on to a micro-Bondapak 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 analyzed on analytical HPLC and the fractions representing the major peak were pooled and evaporated <u>in vacuo</u> at room temperature. The residue was dissolved in H_2O (8 ml) and freeze dried.

Yield 45 mg (11%); $[\alpha]_D^{24}$-45.2° (c 0.09, AcOH); RfB 0.10, RfC 0.50.

The following dodecapeptides were obtained using a procedure analogous to that used for compound XVII.

His-Cha-Leu-Gln-D-Leu-Lys-Pro-Gly-Gln-Pro-Met-Tyr 3TFA (XVIII). $[\alpha]_D^{24}$ -32.0° (c 0.11, AcOH); RfB 0.26, RfC 0.55.

His-Cha-Leu-Gln-Nle-Lys-Pro-Gly-Gln-Pro-Met-Tyr 3TFA (XVIIIa). $[\alpha]_D^{24}$ -44.0° (c 0.10, AcOH); RfB 0.24, RfC 0.52.

His-Cha-Leu-Gln-Ala-Lys-Pro-Gly-Gln-Pro-Met-Tyr 3TFA (XVIIIb). $[\alpha]_D^{24}$ -25.0° (c 0.12, AcOH); RfB 0.18, RfC 0.48.

His-Cha-Leu-Gln-Ile-Lys-Pro-Gly-Gln-Pro-Met-Tyr 3TFA (XVIIIc). $[\alpha]_D^{24}$ -34.6° (c 0.11, AcOH); RfB 0.23, RfC 0.52.

His-Cha-Leu-Gln-Val-Lys-Pro-Gly-Gln-Pro-Met-Tyr 3TFA (XVIIId). $[\alpha]_D^{24}$ -26.3° (c 0.16, AcOH); RfB 0.23, RfC 0.48.

The final fragment coupling involving the heptapeptide and pentapeptide led to isolation of approximately 400 mg of crude dodecapeptide. After deprotection and purification the yield of peptides greater than 98% pure was 10-20% depending on the purity of the starting crude material.

Table 2. Amino Acid Analysis of Six Dodecapeptides

1 - desTrp^{1},Cha^{3},Ala^{6}

2 - desTrp^{1},Cha^{3},Ile^{6}

3 - desTrp^{1},Cha^{3},Nle^{6}

4 - desTrp^{1},Cha^{3},D-Leu^{6}

5 - desTrp^{1},Cha^{3},Val^{6}

6 - desTrp^{1},Phe^{3}

	1	2	3	4	5	6
His	0.93	1.04	1.06	1.06	1.04	1.03
Cha	1.38	1.14	1.14	1.11	1.09	---
Leu	0.94	0.99	0.95	1.82	0.98	2.02
Gln	1.97	2.06	1.96	2.12	2.08	2.31
Lys	0.96	0.94	0.94	0.95	0.98	1.05
Pro	1.84	2.03	2.09	2.12	1.93	1.92
Gly	1.12	0.98	0.95	0.90	0.94	0.94
Met	0.85	1.04	0.99	1.01	1.01	1.03
Tyr	0.93	0.89	0.95	0.91	0.99	0.92
Ala	1.08	---	---	---	---	---
Ile	---	0.89	---	---	---	---
Nle	---	---	0.97	---	---	---
Val	---	---	---	---	0.97	---
Phe	---	---	---	---	---	0.78

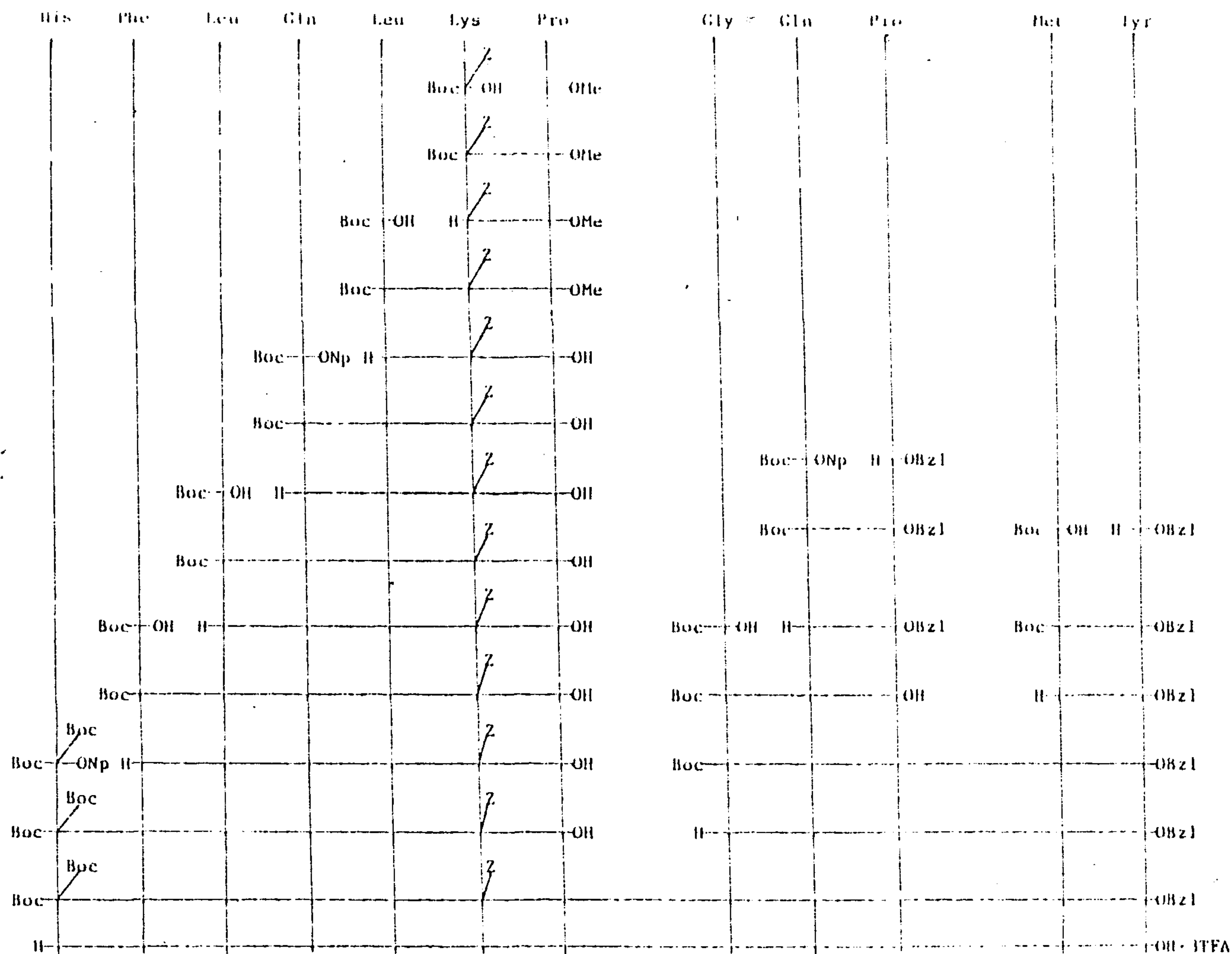
RESULTS AND DISCUSSION

PEPTIDE SYNTHESIS AND PURIFICATION

All the peptides discussed in this manuscript were prepared by the procedures indicated in the Methods and Materials Section. The strategy involved stepwise synthesis of three fragments which were then successively coupled to form the protected dodecapeptide (Scheme 1). Racemization was prevented by utilizing urethane linked protecting groups and all fragment couplings were designed so that Pro was at the C-terminal residue of the activated peptide.

The first fragment prepared was the dipeptide Boc-Met-Tyr-OBzl synthesized in 72% yield, which after being deprotected was coupled to the tripeptide Boc-Gly-Gln-Pro to form the Boc-Gly-Gln-Pro-Met-Tyr-OBzl pentapeptide. Using the isobutylchloroformate mixed anhydride method (61,62) Khan et al. (15) reported an 81% yield for the fragment coupling leading to the pentapeptide. My attempts utilizing similar conditions resulted in a 10% yield. Since this yield was unsatisfactory I proceeded to alter the coupling conditions. It was found that little or no increase in yield occurred on varying the solvent used during formation of the mixed anhydride from tetrahyrdofuran to

Scheme 1. Synthesis of desTrp^{1},Phe^{3}-dodecapeptide
α-factor



His
Phe
Leu
Gln
Leu
Lys
Pro
Gly
Gln
Pro
Met
Tyr
Z
Boc
OH
OMe
H
ONp
OBzl
OH·3TFA

ethyl acetate or chloroform. Experiments performed in a mixed solvent system of tetrahydrofuran:dimethylformamide (3:1) resulted in an increase in the yield to 30%. Since this improvement in yield was not sufficient I then tried the EEDQ mixed anhydride method.

This procedure results in slower formation and rapid consumption of the intermediate mixed anhydride and minimized the possibility of side reactions (62). Utilizing this procedure I was able to increase the yield for the coupling to 50%. The highest yield (60%) was obtained with the IBC mixed anhydride/HOBT active ester method (63). The IBC mixed anhydride/HOBT method is predominately used in the coupling of sterically hindered amino acids. I also employed this procedure in couplings involving both Boc-Ile and Boc-Val to MSA-Lys(Z)-Pro-OMe. In both cases the yields doubled from 30 to 60%. In the tripeptide coupling reaction the increase in yield may be attributed to elimination of wrong way addition in the beta branched and sterically hindered amino acids. The low yields encounted with the isobutylcloroformate mixed anhydride procedure (61,62) during the pentapeptide coupling does not appear to be related to wrong way addition. If this were the case then the EEDQ method should lead to increased wrong way addition due to the less sterically hindered mixed anhydride that is formed.

Several heptapeptide fragments were prepared in a stepwise manner utilizing IBC mixed anhydrides and HOBT accelerated active ester coupling procedures. The dipeptide Boc-Lys(Z)-Pro-OMe was synthesized in 75% yield. After the Boc group was removed by acidolysis the resulting dipeptide salt was divided and coupled to six different Boc-amino acids; Boc-L-Leu, Boc-D-Leu, Boc-Val, Boc-Ile, Boc-Nle and Boc-Ala to form six similar tripeptides. All of these peptides were purified on a Silica 60 column with ethyl acetate: methanol (98:2) as the mobile phase. Analysis of fractions was determined by thin layer chromatography in solvent system A. Fractions that were fluorescent and ninhydrin positive were pooled and isolated. Average yield after purification was 60%.

The tripeptide methyl esters were saponified in methanol with 20% excess of 0.1M NaOH at 30° for 3.5 h. The free acids were isolated in 70% yields. Cleavage of the Boc group with an equivalent of methane sulfonic acid (0.1M) in formic acid-methylene chloride 1:1 was utilized to prepare the tripeptides for coupling to Boc-Gln-ONp. These tetrapeptides prepared in 65% yield represented the last stage in the heptapeptide fragment synthesis in which the peptides were isolated in a usual workup involving evaporation of the reactions solvents, dissolution of the peptide in ethyl acetate and extraction with aqueous

solutions. From the pentapeptide through the heptapeptide the solvents were evaporated and the reaction mixture precipitated with 5% citrate. These larger peptides were reprecipitated after isolation from dimethylformamide and diethyl ether. Fragments obtained in this manner were greater than 95% homogeneous on silica thin layers and were used without further purification.

Boc-Cha was coupled to the pentapeptide methane sulfonates in which Leu had been replaced with Ile, Val, Nle, Ala or D-Leu, while Boc-Phe was coupled to the natural sequence pentapeptide. These hexapeptides were prepared in 80% yield. HOBT accelerated coupling of Di-Boc-His-ONp to the hexapeptide methane sulfonates produced the heptapeptides fragments in 85% yield.

Assembly of the protected dodecapeptide involved coupling of the heptapeptide fragment and pentapeptide fragment leading to the protected dodecapeptide was performed by the IBC mixed anhydride method. In this case the mixed anhydride was formed in tetrahydrofuran:dimethylformamide (3:1) and the reaction temperature was maintained at -15° for 1 h. These peptides were deprotected in a two step procedure involving catalytic transfer hydrogenation with Pd black and formic acid followed by treatment with methylene chloride:trifluoracetic acid (1:1) with anisole

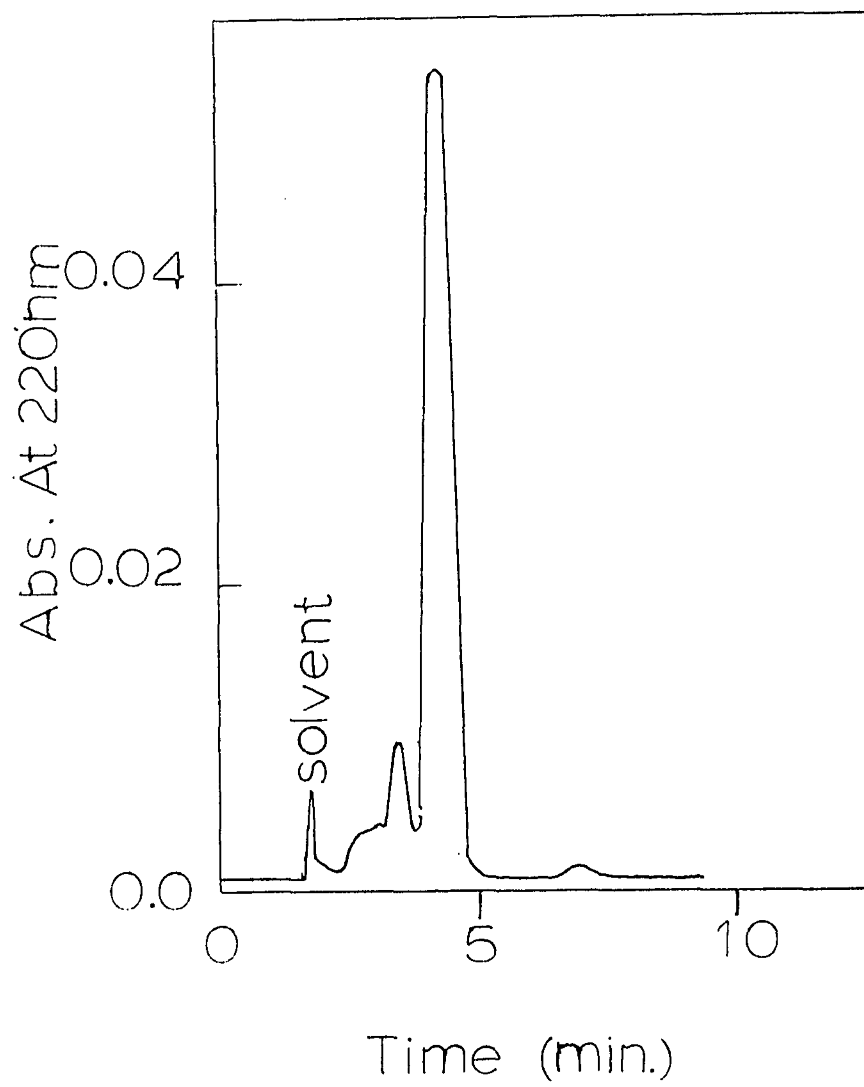
added as a scavenger.

All dodecapeptides were purified by high performance liquid chromatography utilizing step gradients of MeOH:H_2O:TFA mixed solvent system. A typical purification procedure involved injection of 100 mg of a dodecapeptide in 5 ml of MeOH:H_2O:TFA (310:690:0.25 ml) onto a Prep 500 equipped with a micro-Bondapak C_{18} reverse phase column (I.D. 1") equilibrated in the same solvent, at a flow rate of 50 ml/min.

Fractions of 50 ml were collected and analysis done on an analytical HPLC system equilibrated in CH_3CN:H_2O:TFA (200:800:0.25 ml) at a flow of 3 ml/min. After the impurities were eluted the methanol concentration was increased in 2% increments until the desired product was eluted. Fractions of high purity were pooled, the solvents evaporated and lyophilized. Recovery of pure peptide was dependent on the purity of the starting material and was typically 15-20 mg. All peptides were greater than 98% pure as judged in two solvent system using analytical HPLC. Chromatograms of the peptides before and after purification are shown in Figures 1-12.

Figure 1. HPLC of crude desTrp[1],Cha[3],Ala[6]

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System	- $MeOH:H_2O:TFA$ (400:600:0.25 ml)
Flow Rate	- 1.7 ml/min



Abs. At 220nm
0.04
0.02
0.0
solvent
0
5
10
Time (min.)

Figure 2. HPLC of purified desTrp^{1},Cha^{3},Ala^{6}

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System A	- CH_3CN:H_2O:TFA (200:800:0.25 ml)
Flow Rate A	- 3.0 ml/min
Solvent System B	- MeOH:H_2O:TFA (400:600:0.25 ml)
Flow Rate B	- 1.7 ml/min

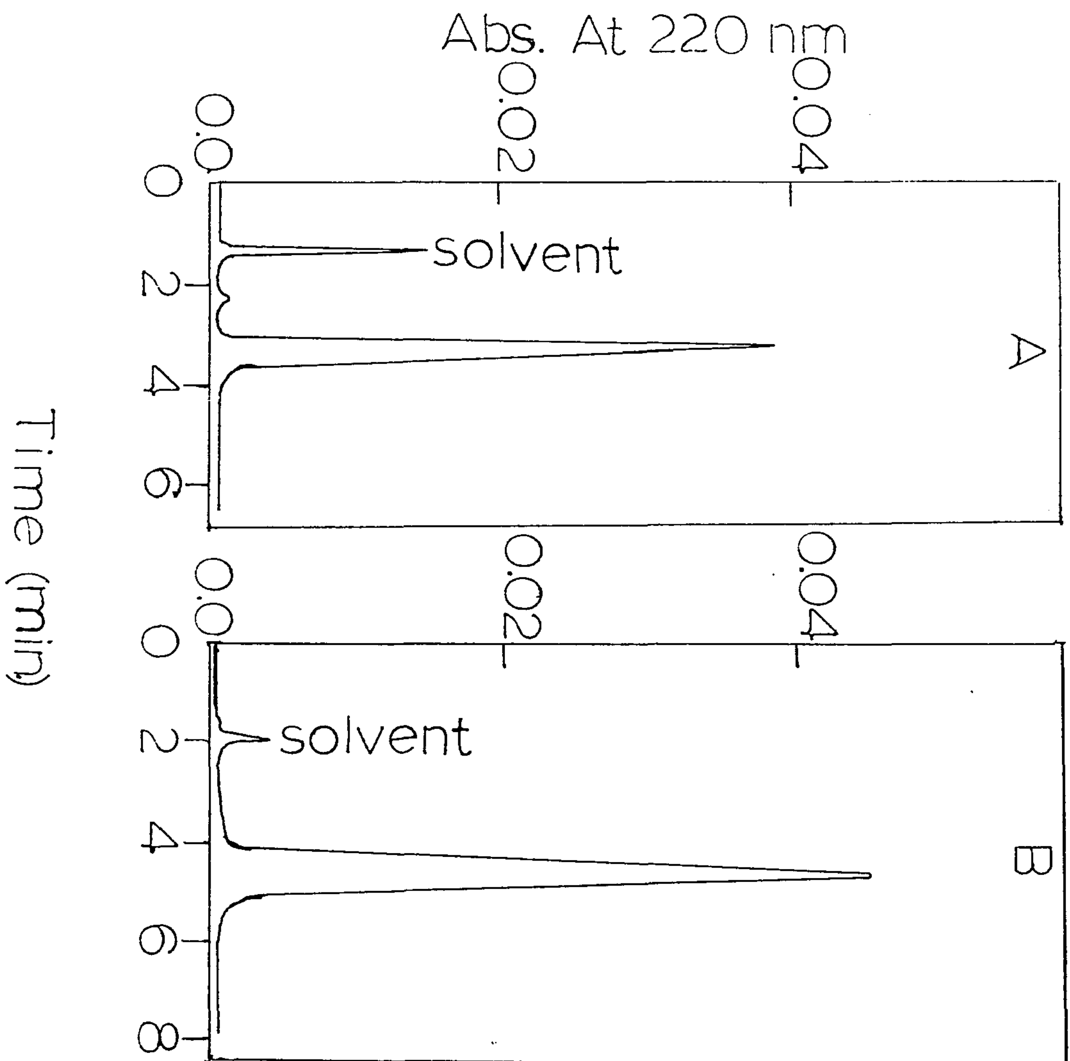

Abs. At 220 nm
0.0
0.02
0.04
solvent
A
0
2
4
6
Time (min)
0.0
0.02
0.04
solvent
B
0
2
4
6
8

Figure 3. HPLC of crude desTrp^{1},Cha^{3},Ile^{6}

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System	- MeOH:H_2O:TFA (400:600:0.25 ml)
Flow Rate	- 1.7 ml/min

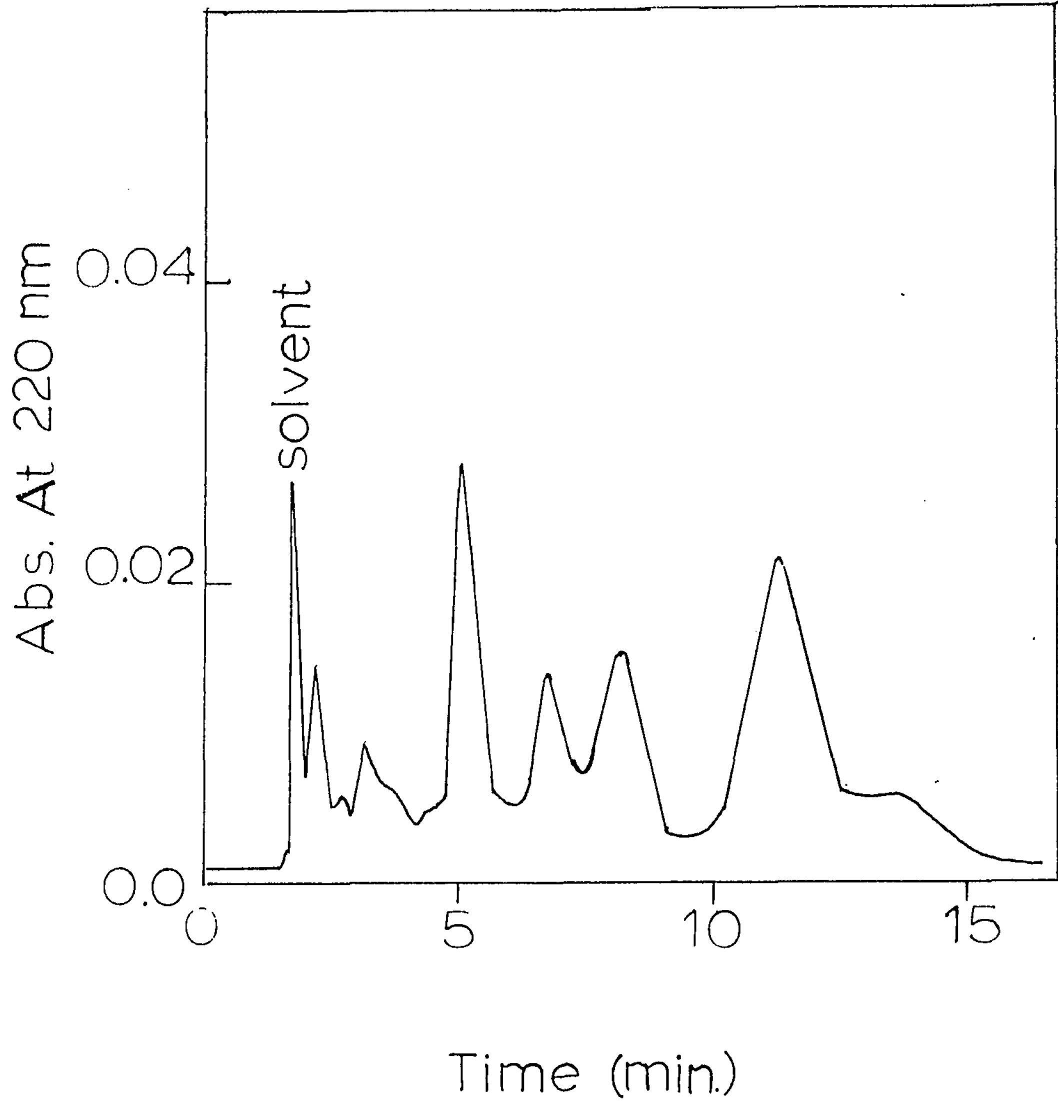
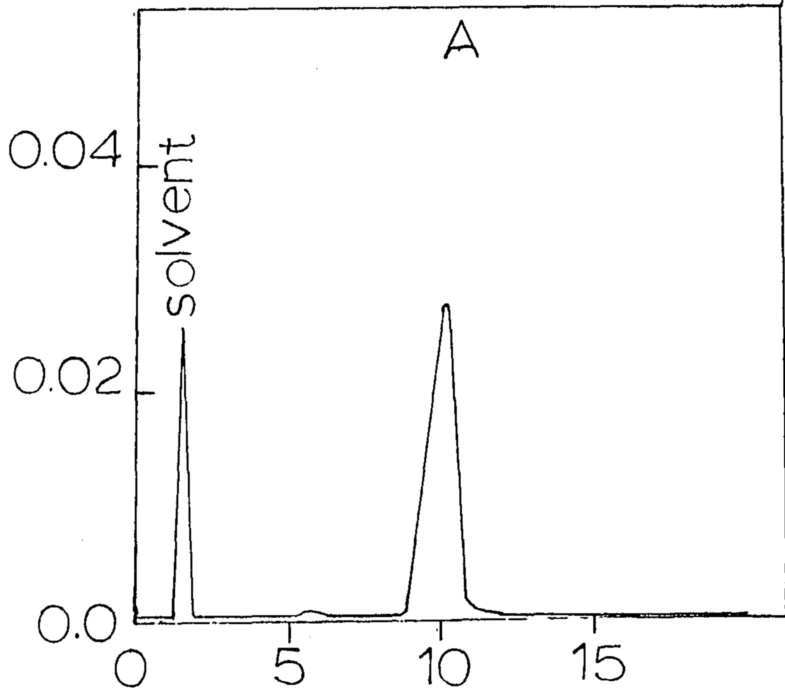

Abs. At 220 nm
0.00
0.02
0.04
solvent
0
5
10
15
Time (min.)

Figure 4. HPLC of purified desTrp[1],Cha[3],Ile[6]

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System A	- CH_3 CN:H_2O:TFA (200:800:0.25 ml)
Flow Rate A	- 3.0 ml/min
Solvent System B	- MeOH:H_2O:TFA (400:600:0.25 ml)
Flow Rate B	- 1.7 ml/min


A
solvent
0.04
0.02
0.0
0
5
10
15

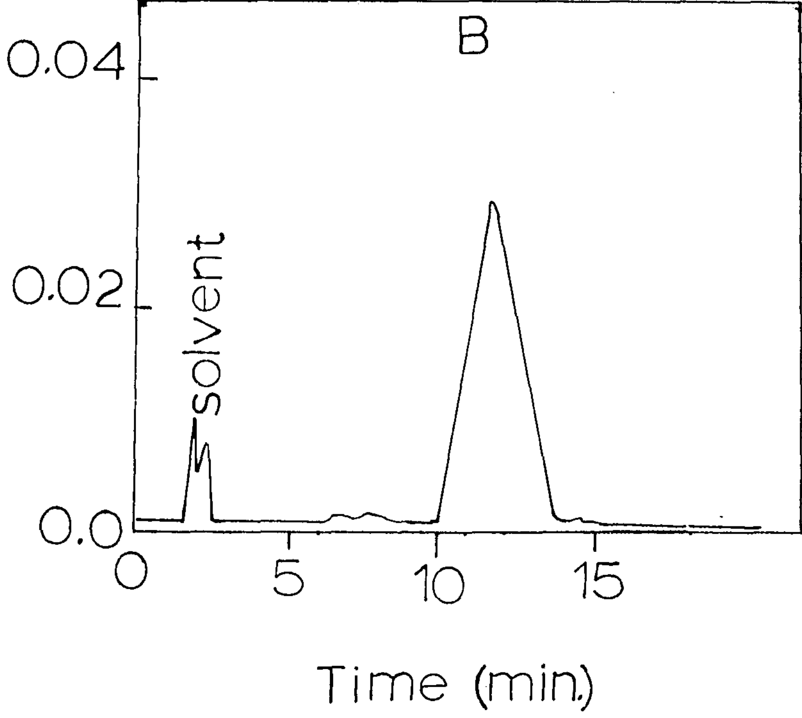

B
solvent
0.04
0.02
0.0
0
5
10
15
Abs. At 220 nm
Time (min.)

Figure 5. HPLC of crude desTrp[1],Cha[3],D-Leu[6]

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System	- MeOH:H_2O:TFA (400:600:0.25 ml)
Flow Rate	- 1.7 ml/min

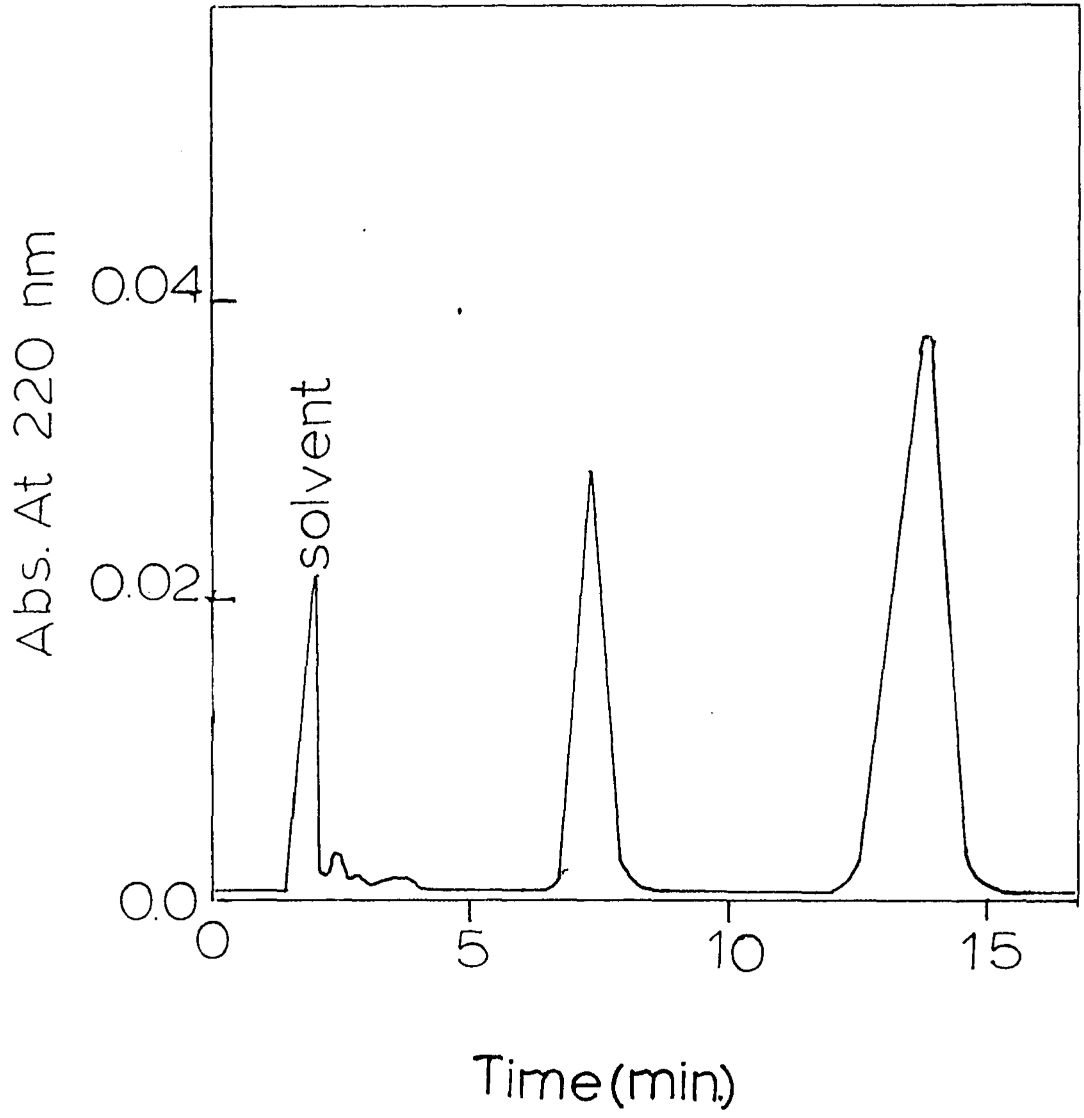
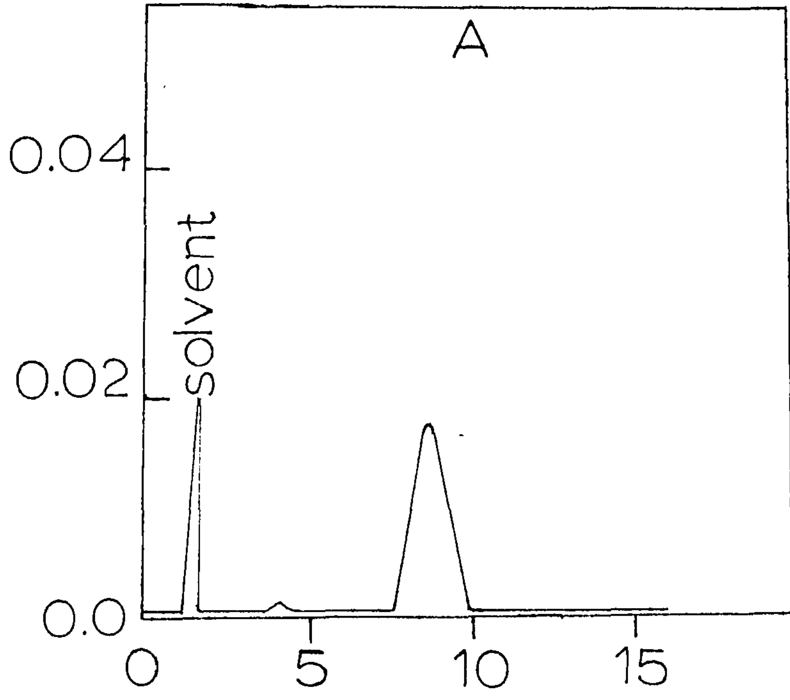

Abs. At 220 nm
0.04
0.02
0.0
solvent
0
5
10
15
Time (min.)

Figure 6. HPLC of purified desTrp^1,Cha^3,D-Leu^6

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System A	- $CH_3CN:H_2O:TFA$ (200:800:0.25 ml)
Flow Rate A	- 3.0 ml/min
Solvent System B	- $MeOH:H_2O:TFA$ (400:600:0.25 ml)
Flow Rate B	- 1.7 ml/min


A
Abs. At 220 nm
0.04
0.02
0.0
solvent
0
5
10
15

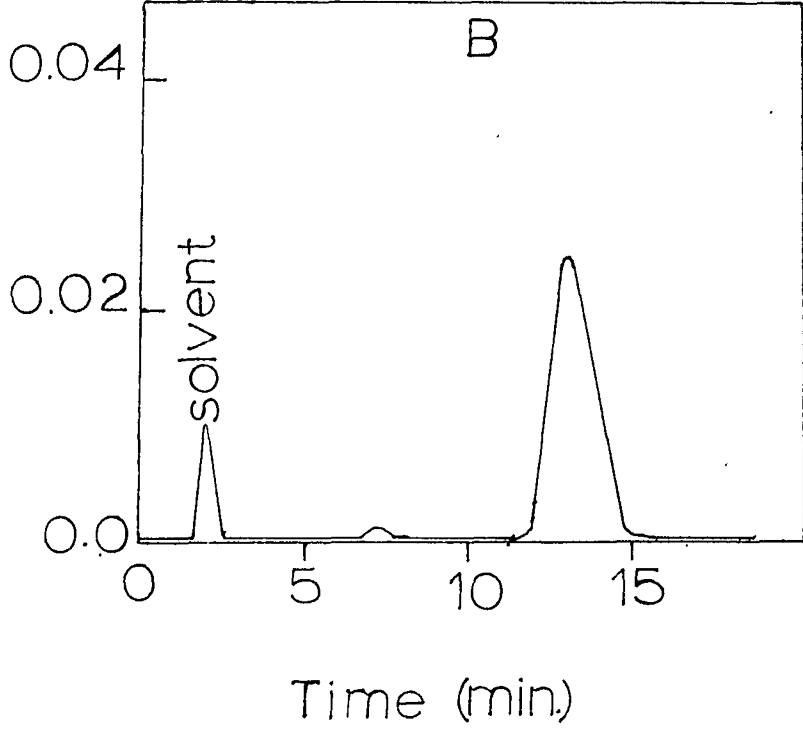

B
0.04
0.02
0.0
solvent
0
5
10
15
Time (min.)

Figure 7. HPLC of crude desTrp[1],Cha[3],Nle[6]

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System	- MeOH:H_2O:TFA (400:600:0.25 ml)
Flow Rate	- 1.7 ml/min

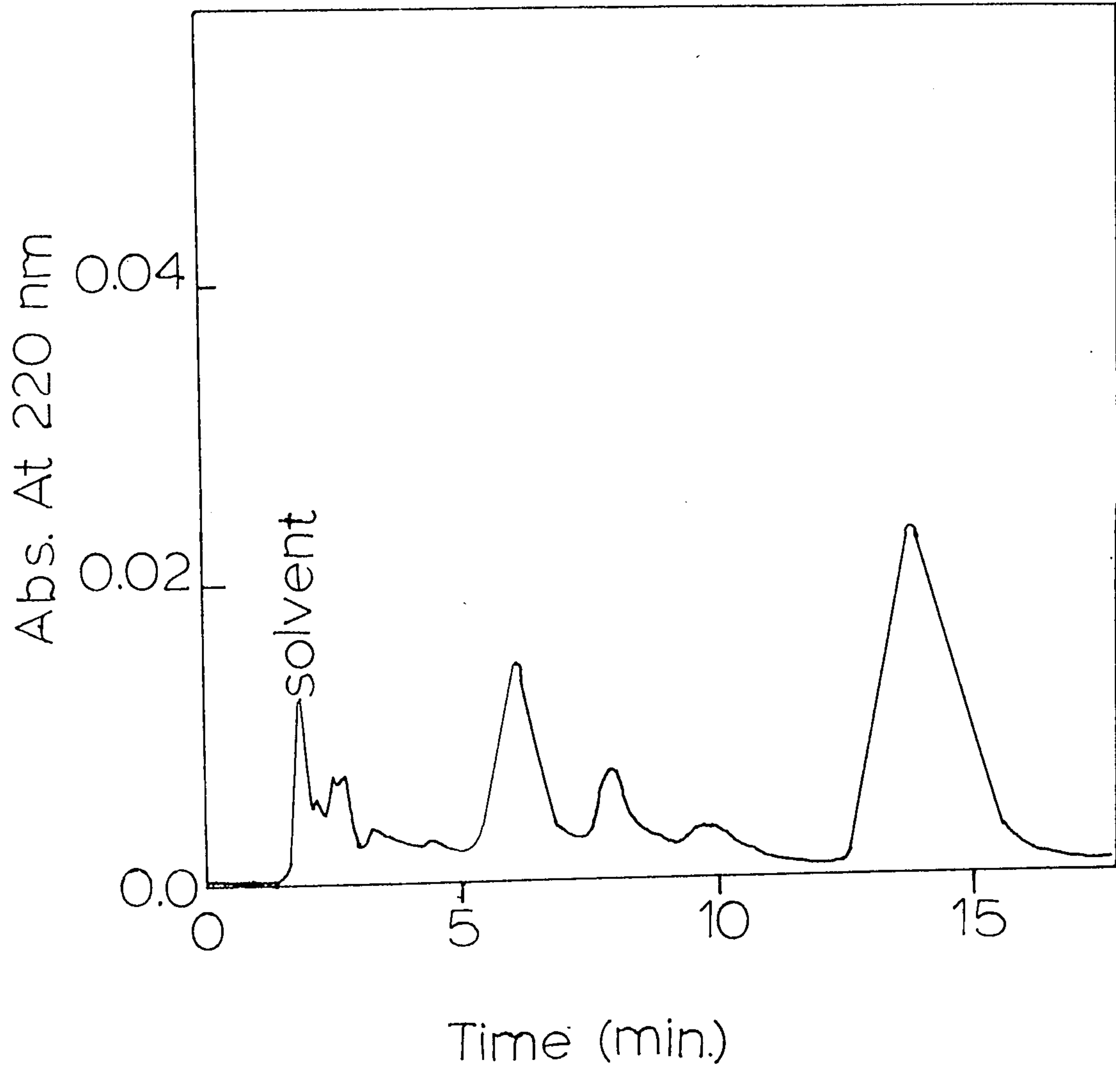
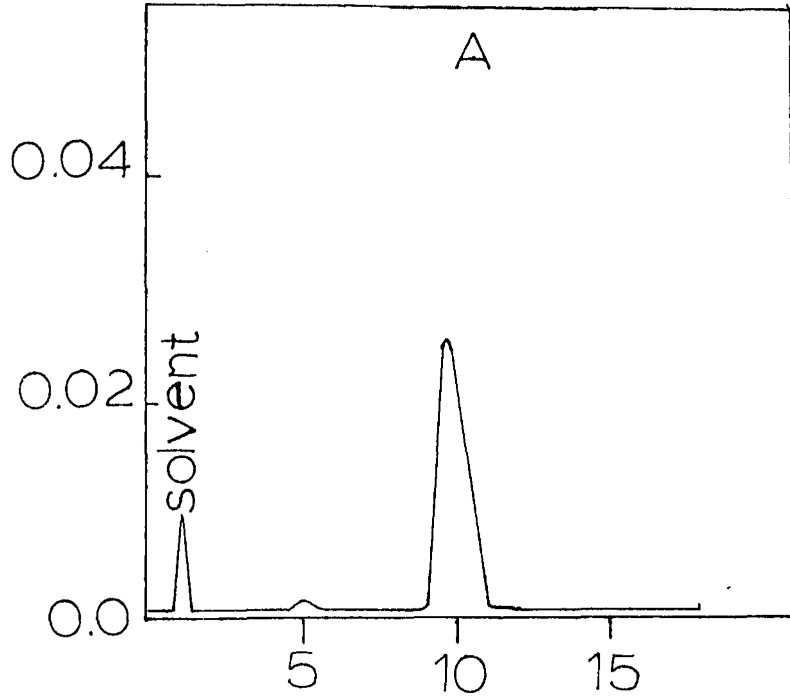

Abs. At 220 nm
0.0
0.02
0.04
solvent
0
5
10
15
Time (min.)

Figure 8. HPLC of purified desTrp^1,Cha^3,Nle^6

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System A	- CH_3CN:H_2O:TFA (200:800:0.25 ml)
Flow Rate A	- 3.0 ml/min
Solvent System B	- MeOH:H_2O:TFA (400:600:0.25 ml)
Flow Rate B	- 1.7 ml/min


A
0.04
0.02
0.0
solvent
5
10
15

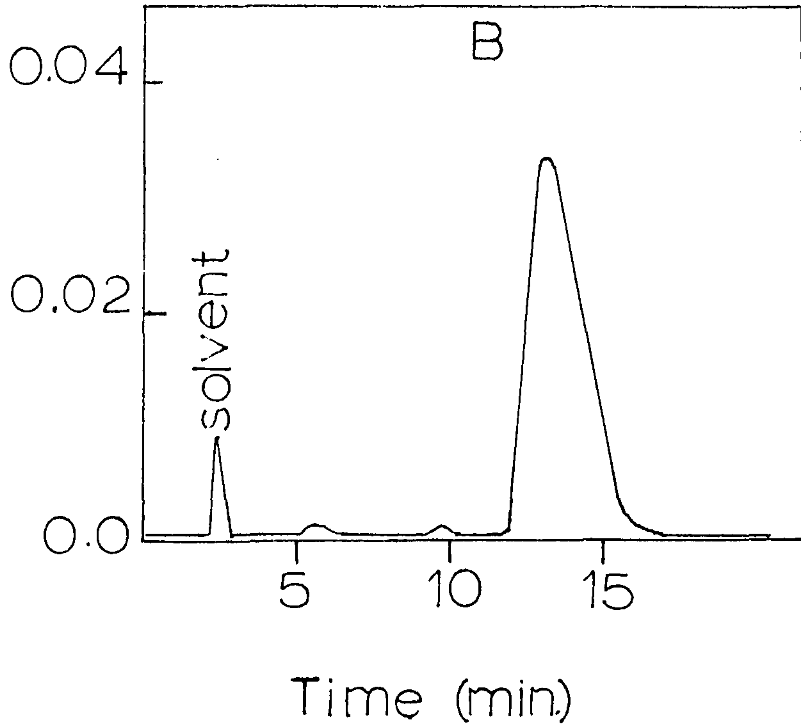

B
0.04
0.02
0.0
solvent
5
10
15
Abs. At 220 nm
Time (min.)

Figure 9. HPLC of crude desTrp[1],Phe[3]

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System	- MeOH:H_2O:TFA (400:600:0.25 ml)
Flow Rate	- 1.7 ml/min

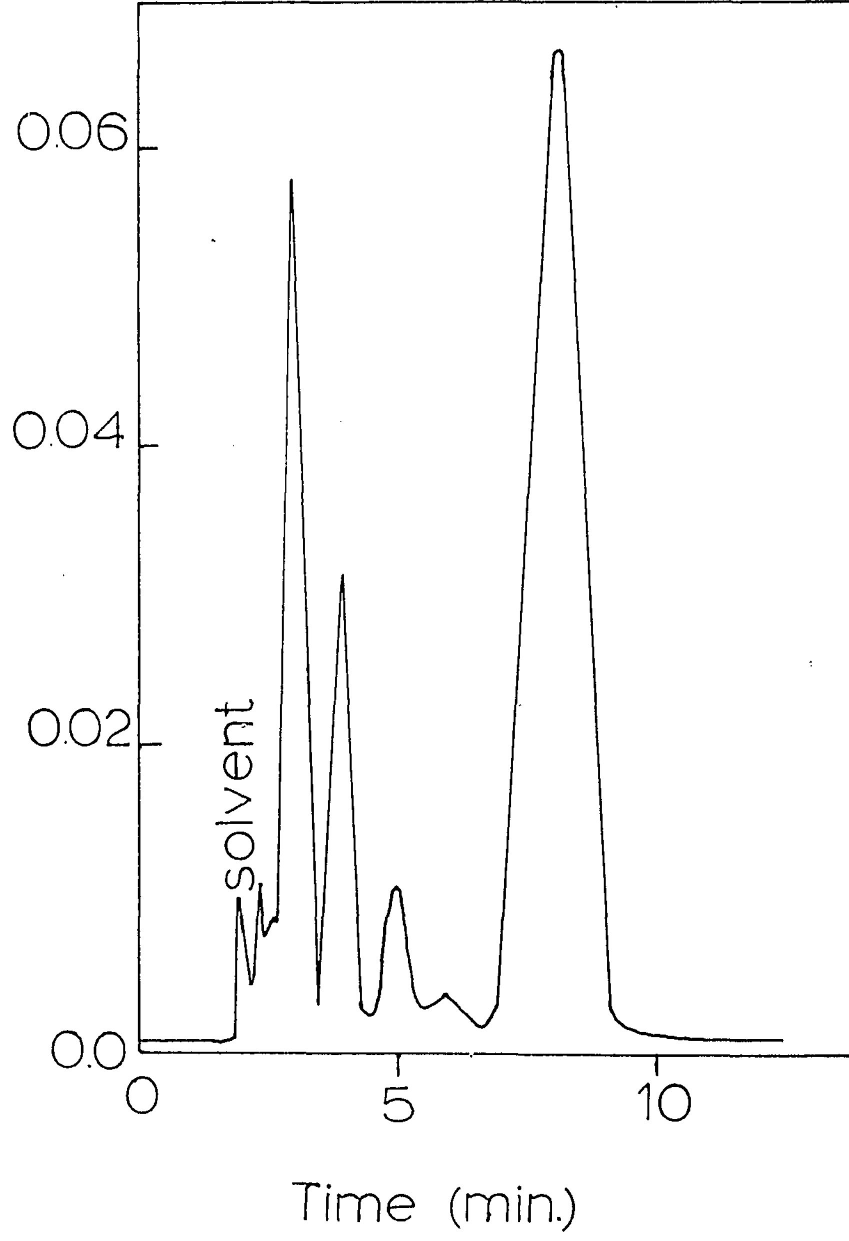

Abs. At 220 nm
0.0
0.02
0.04
0.06
solvent
0
5
10
Time (min.)

Figure 10. HPLC of purified desTrp^1,Phe^3

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System A	- $CH_3CN:H_2O:TFA$ (200:800:0.25 ml)
Flow Rate A	- 3.0 ml/min
Solvent System B	- $MeOH:H_2O:TFA$ (400:600:0.25 ml)
Flow Rate B	- 1.7 ml/min

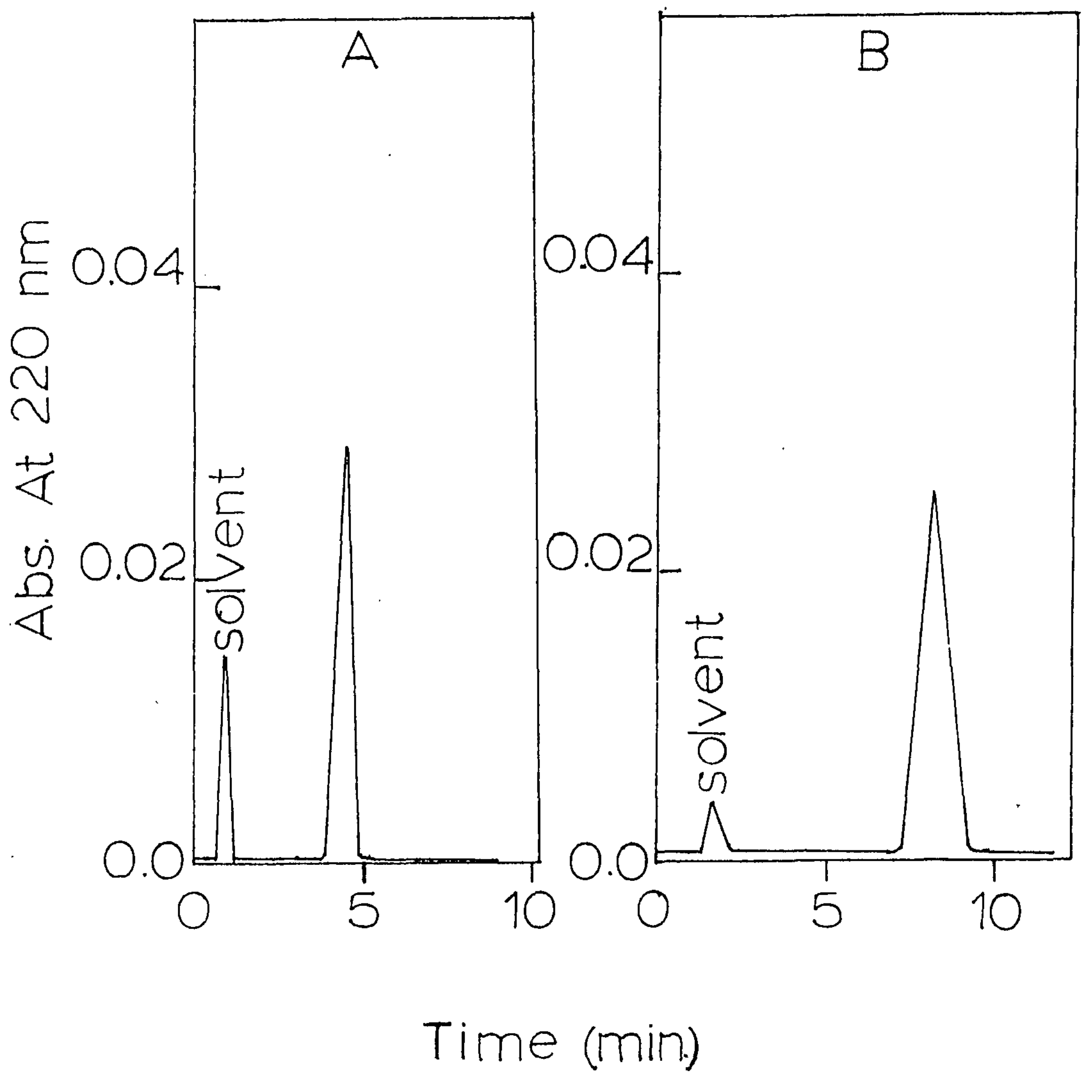
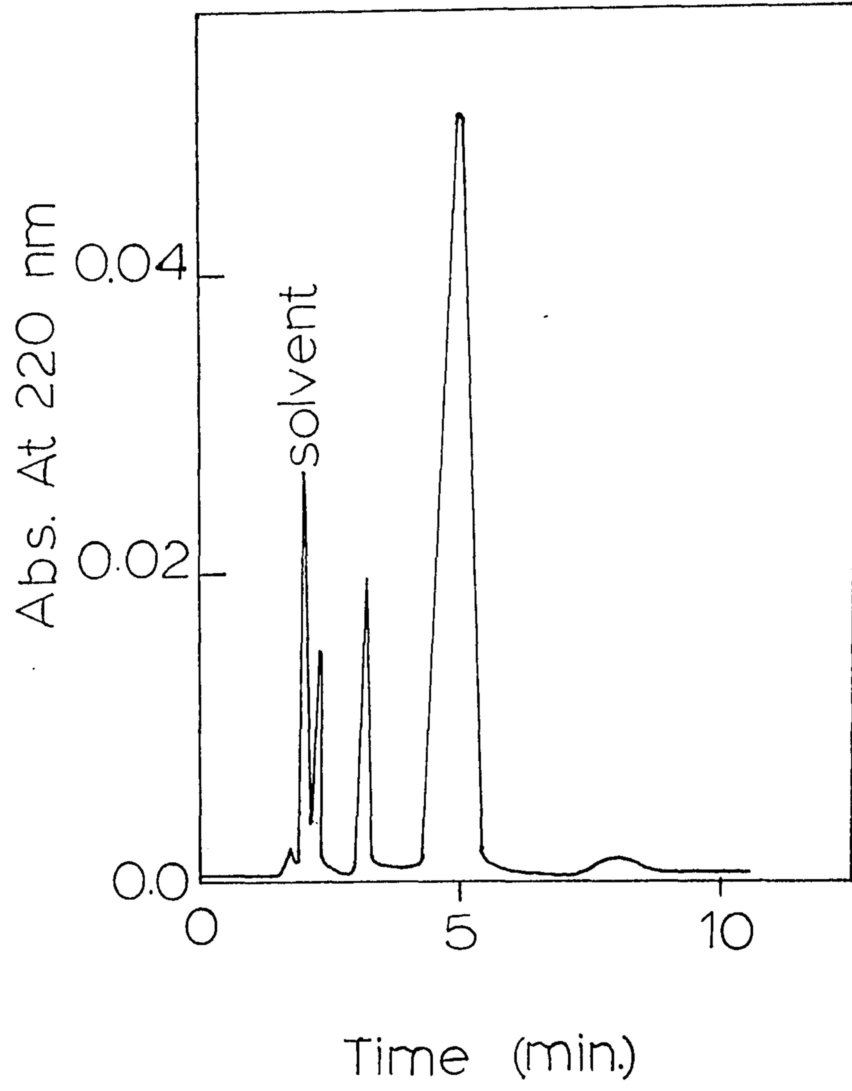

A
B
Abs. At 220 nm
0.04
0.02
0.0
solvent
solvent
0
5
10
Time (min)

Figure 11. HPLC of crude desTrp[1],Cha[3],Val[6]

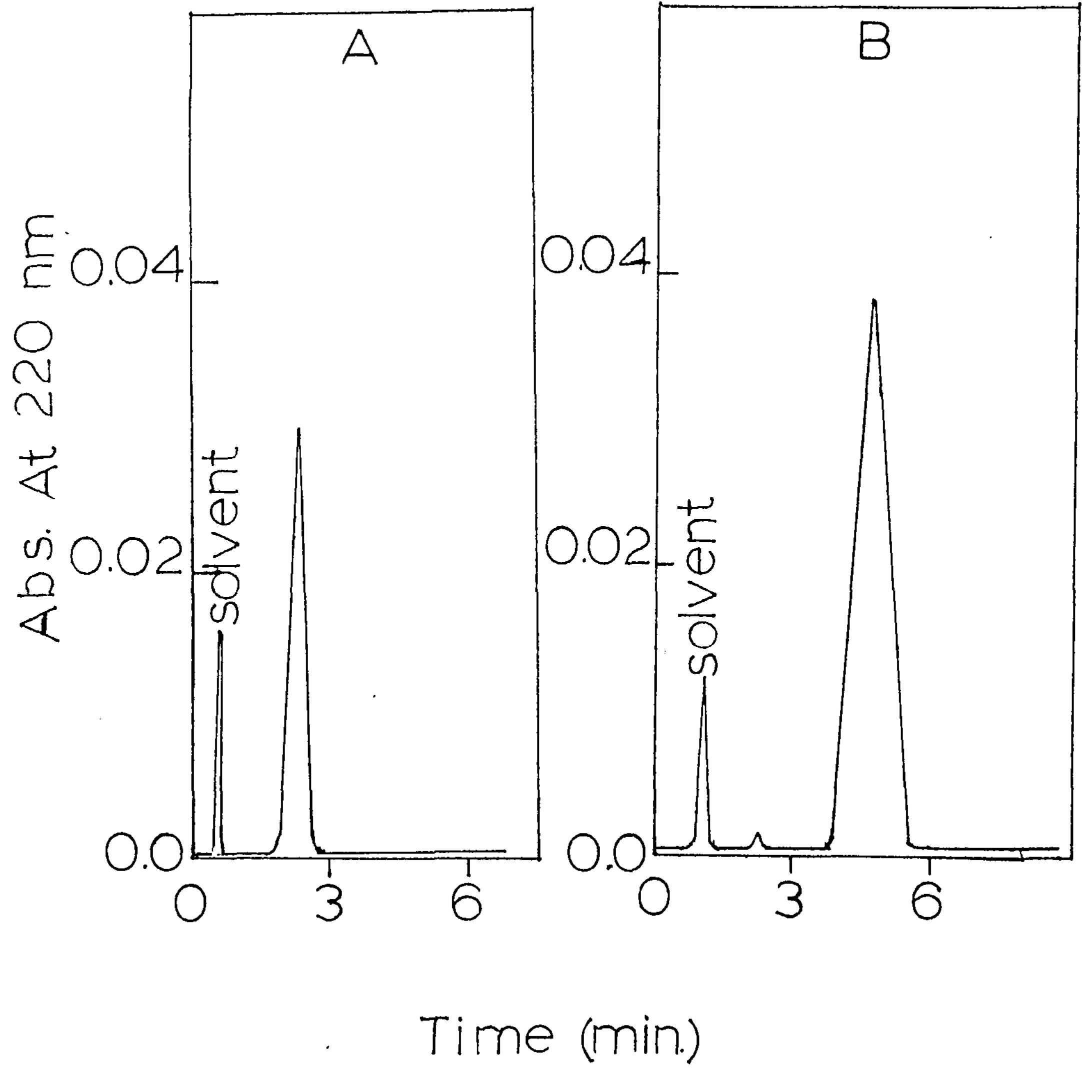
Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System	- MeOH:H_2O:TFA (400:600:0.25 ml)
Flow Rate	- 1.7 ml/min



Abs. At 220 nm
0.04
0.02
0.0
solvent
0
5
10
Time (min.)

Figure 12. HPLC of purified desTrp^{1},Cha^{3},Val^{6}

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System A	- CH_3CN:H_2O:TFA (200:800:0.25 ml)
Flow Rate A	- 3.0 ml/min
Solvent System B	- MeOH:H_2O:TFA (400:600:0.25 ml)
Flow Rate B	- 1.7 ml/min


A
B
Abs. At 220 nm
0.0
0.02
0.04
solvent
0
3
6
Time (min.)

BIOLOGICAL RESPONSES ASSOCIATED WITH α-FACTOR

The biological assays utilized in this study were the basis for relating the response of a-cells to the structural changes in our α-factor analogs. Morphogenesis which is not essential for mating represents the most easily observable phenomenon associated with α-factor activity (26). The endpoint in this assay represents the concentration of α-factor present in the last well of the microtiter plate which contains morphologically altered a-cells. Enhanced agglutinability between a and α-cells provides a mechanism for fusion of the cells during the mating process. The concentration of α-factor analog which induces half maximal agglutination when compared with that induced by 1 unit/ml of biologically isolated mating factor was taken as the potency value for that particular analog (4). In this assay the endpoint for agglutination was not used since it was not statistically different from the negative controls. In both assays the results were very reproducible. Arrest of the cell cycle of a-cells in the G1 phase of DNA synthesis after exposure to α-factor allows the cells to synchronize their cycles prior to mating (39). This assay which was monitored by deviation from log linear growth

provided information relevant to the extent and duration of growth arrest.

Morphogenic activities of various α-factor analogs have been investigated. The synthetic peptides had potencies ranging from 50 ng to 12 μg per unit of activity. As stated previously, one unit of activity represents the lowest concentration which causes shmoo formation in the microtiter plate assay. The natural sequence tridecapeptide was the most potent species by a factor of four and was comparable to the homogeneous biological preparation of α-factor (27). Scanning electron micrographs of a - cells exposed to synthetic tridecapeptide shows shmoo-like morphologies while all active dodecapeptides analogs including the natural sequence dodecapeptide resulted in severly distorted morphologies as shown in Figure (13).

All of the morphogenically active analogs of α-factor tested also induced increased agglutinability of a-cells. The active analogs had dose response curves as represented in Figure 14. It was determined that the analogs induced half maximal agglutinability over a wide range of concentrations. The natural sequence was sixteen fold more potent than the next most active analog.

Standard errors and ranges for both morphogenic and agglutionation assays are illustrated in Figure 15. The reported values for the morphogenesis assay represents the antilog of the mean log concentration for 3 to 21 separate determinations on each peptide. The concentration inducing an increase in agglutinability half that induced by 1 unit/ml of biological α-factor was determined from interpolation of dose-repsponse curves. Two to four separate experiments were performed for each analog, and the half-maximal doses averaged as logarithms.

Figure 13. Electron micrographs of induced *S. cerevisiae*

Upper Panels *a*-cells induced with natural sequence tridecapeptide

Lower Panels *a*-cells induced with natural sequence dodecapeptide

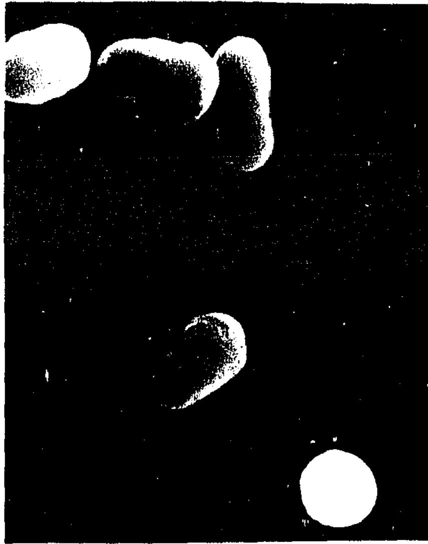
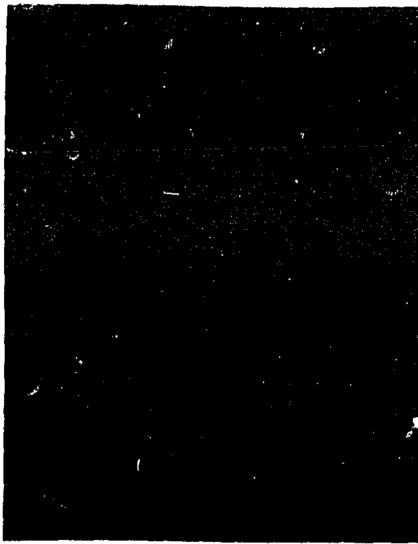



Figure 14. Dose Dependent Agglutination

1 - Natural Sequence Tridecapeptide

2 - desTrp^{1},Cha^{3}

3 - desTrp^{1},Cha^{3},Ile^{6}

4 - desTrp^{1},Cha^{3},Val^{6}

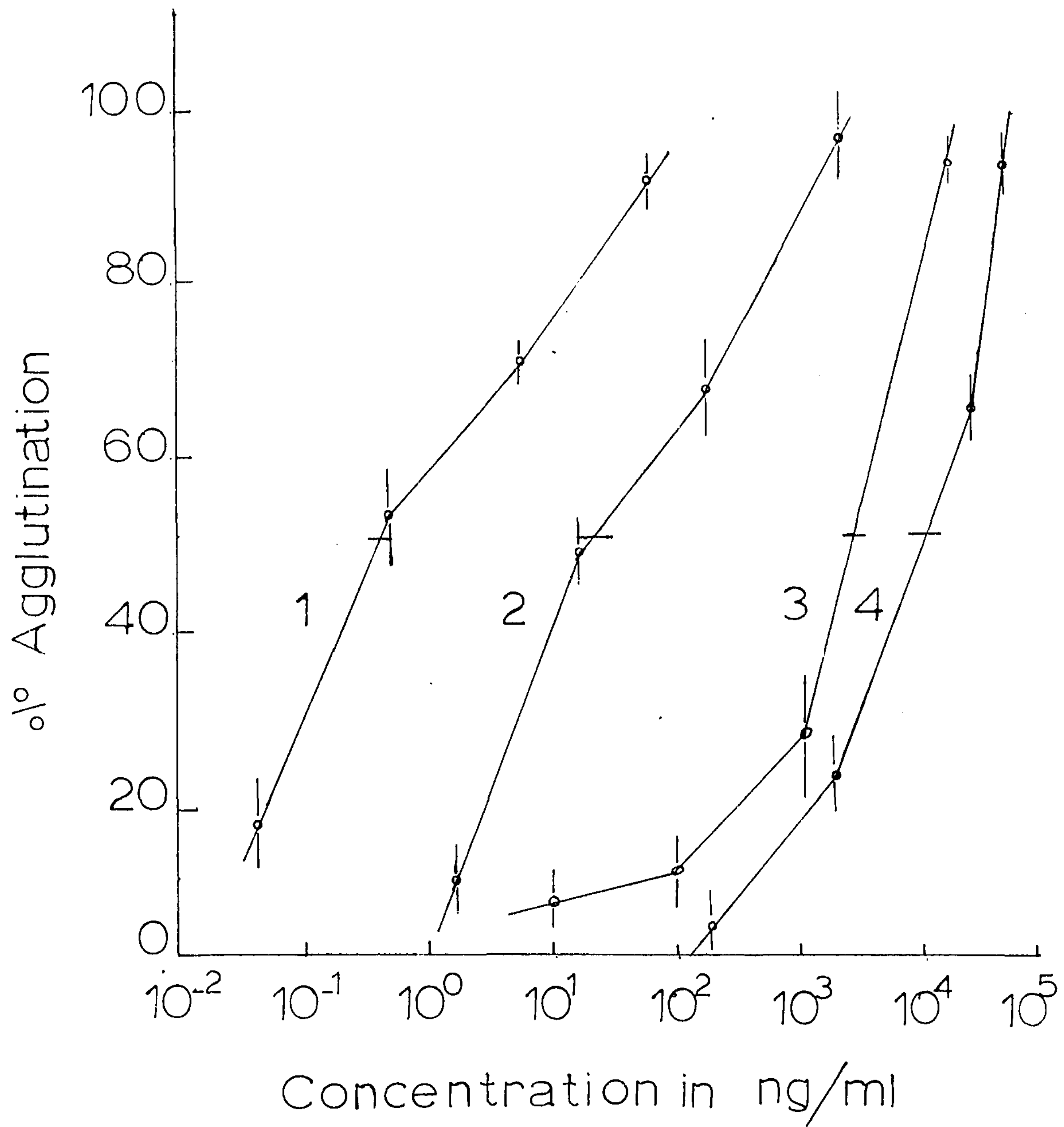

100
80
60
40
20
0
% Agglutination
1
2
3
4
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
10^{5}
Concentration in ng/ml

Figure 15. Potencies of α-Factor Analogs

1 - Tridecapeptide

2 - Dodecapeptide

3 - $desTrp^{1},Cha^{3}$

4 - $desTrp^{1}$,(N-α-Dns)His^{2},Cha^{3}

5 - $desTrp^{1},Cha^{3},Orn^{7}$

6 - $desTrp^{1},Cha^{3},Lys^{7}$(Dns-Gly)

7 - $desTrp^{1},Cha^{3},Nle^{6}$

8 - $desTrp^{1},Cha^{3},Lys^{7}$(Ac)

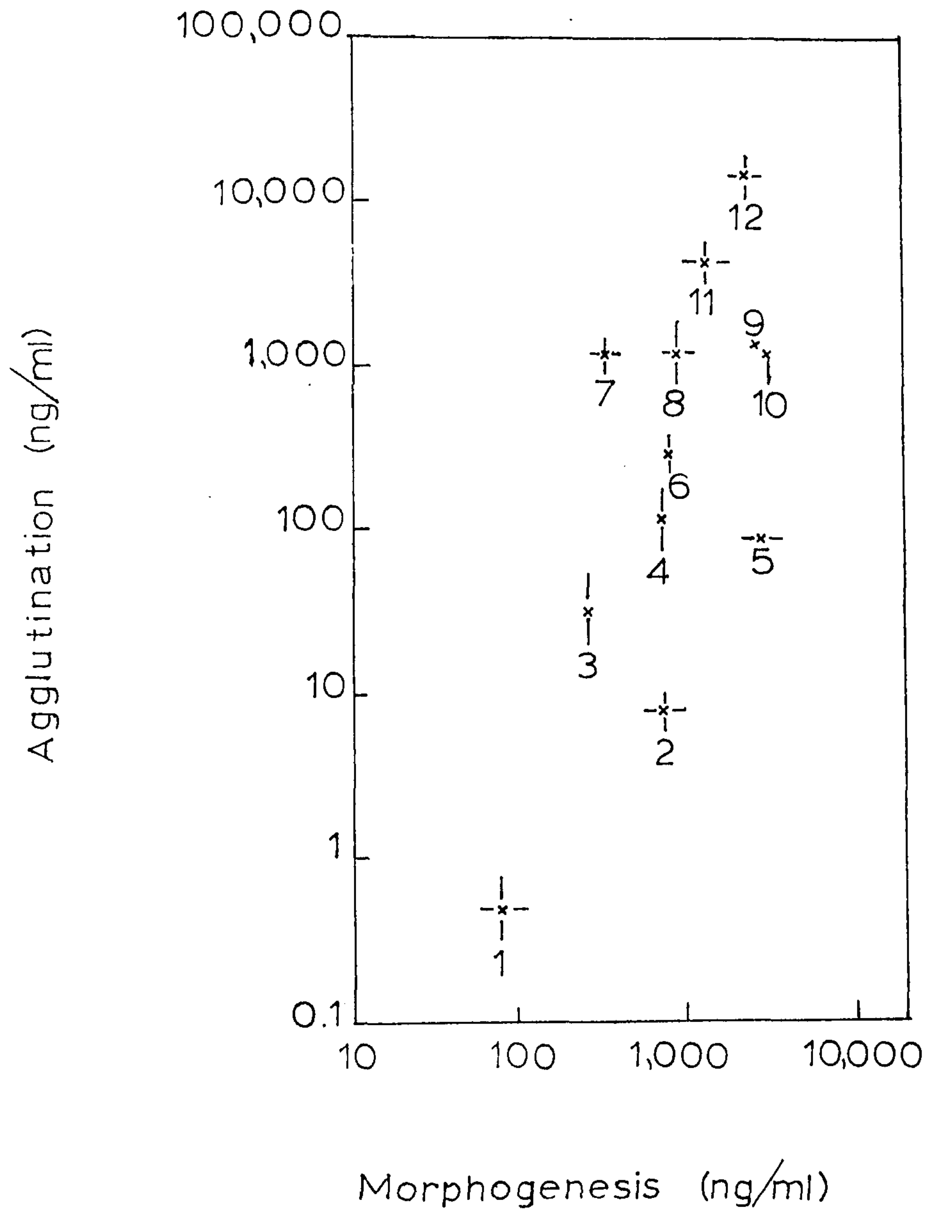
9 - $desTrp^{1},Cha^{3},Lys^{7}$(Biotinyl)

10 - $desTrp^{1},Cha^{3},Nle^{7}$

11 - $desTrp^{1},Cha^{3},Ile^{6}$

12 - $desTrp^{1},Cha^{3},Val^{6}$

The error brackets represent the standard error determined from the mean log concentrations of each analog for both activities.


100,000
10,000
1,000
100
10
1
0.1
Agglutination (ng/ml)
10
100
1,000
10,000
Morphogenesis (ng/ml)
1
2
3
4
5
6
7
8
9
10
11
12

POSITION 3 ANALOGS

Four position 3 dodecapeptide analogs exhibited markedly different k' values utilizing a micro-Bondapak C_{18} column equilibrated with $CH_3CN:H_2O:TFA$ (200:800:025ml) (Figure 16, Table 3). The different retention times can be explained in terms of differences in the hydrophobicity of these peptides. When Trp in position 3 is replaced with the smaller and less hydrophobic amino acids Ala or Phe, activity in both morphogenesis and agglutination is abolished. In contrast when Trp is replaced by the more hydrophobic amino acid Cha an increase in morphogenic activity from 720 to 270 ng/ml is observed, while a decrease in agglutination activity is observed. In the agglutination assay the natural sequence dodecapeptide induces half maximal response at 8 ng/ml while the $desTrp^1,Cha^3$-dodecapeptide requires 35 ng/ml as shown in Table 3.

Antagonism of Morphogenesis Activity

The $desTrp^1,Ala^3$- and $desTrp^1Phe^3$-dodecapeptides, which do not elicit shmoo formation in _a_ cells, were competed with synthetic tridecapeptide and

desTrp^{1},Cha^{3}-dodecapeptide (Table 4). It was found that both of these analogs prevented biologically active mating factor from causing shmoo formation in a-cells. For the synthetic tridecapeptide, competition by the des Trp^{1},Phe^{3}-dodecapeptide is observed at a ratio of 15:1 or greater while competition by the desTrp^{1},Ala^{3}-dodecapeptide required a ratio of 2500:1 or greater. For the desTrp^{1},Cha^{3}-peptide competition by desTrp^{1},Phe^{3}-dodecapeptide is observed at a ratio of 5:1 or greater while competition by the desTrp^{1},Ala^{3}-dodecapeptide required a ratio of 40:1 or greater. These competition studies are further proof that these analogs are not biologically active. The results with the dodecapeptides differ with those of Masui et al. (56) who reported activity of the Ala^{3}- and Phe^{3}-tridecapeptide at 0.1 ng/ml.

Antagonism of Agglutination Activity

The desTrp^{1},Ala^{3}- and desTrp^{1},Phe^{3}-dodecapeptides, which do not induce increased agglutination of a cells were competed with desTrp^{1},Cha^{3}-dodecapeptide. It was found that both of these analogs prevented biologically active mating factor from inducing increased agglutinability of a-cells (Figure 17). For the

Table 3. Position 3 Analogs: k' Values and Biological Activities of Dodecapeptide-α-Factor Analogs

Peptide	k'	Morphogenesis Activity ng/ml	Agglutination Activity ng/ml
desTrp[1],Ala[3]	1.4	NA[a]	NA[b]
desTrp[1],Phe[3]	4.6	NA[a]	NA[b]
desTrp[1],	7.2	720 (12)[c]	8 (2)[c]
desTrp[1],Cha[3]	13.1	270 (12)[c]	35 (2)[c]

a = Not Active at 500 μg/ml

b = Not Active at 50 μg/ml

c = Number of Independent Trails

Figure 16. HPLC of Four Position 3 Dodecapeptides

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System	- CH_3CN:H_2O:TFA (200:800:0.25 ml)
Flow Rate	- 3.0 ml/min

1 - $desTrp^1,Ala^3$

2 - $desTrp^1,Phe^3$

3 - $desTrp^1$

4 - $desTrp^1,Cha^3$

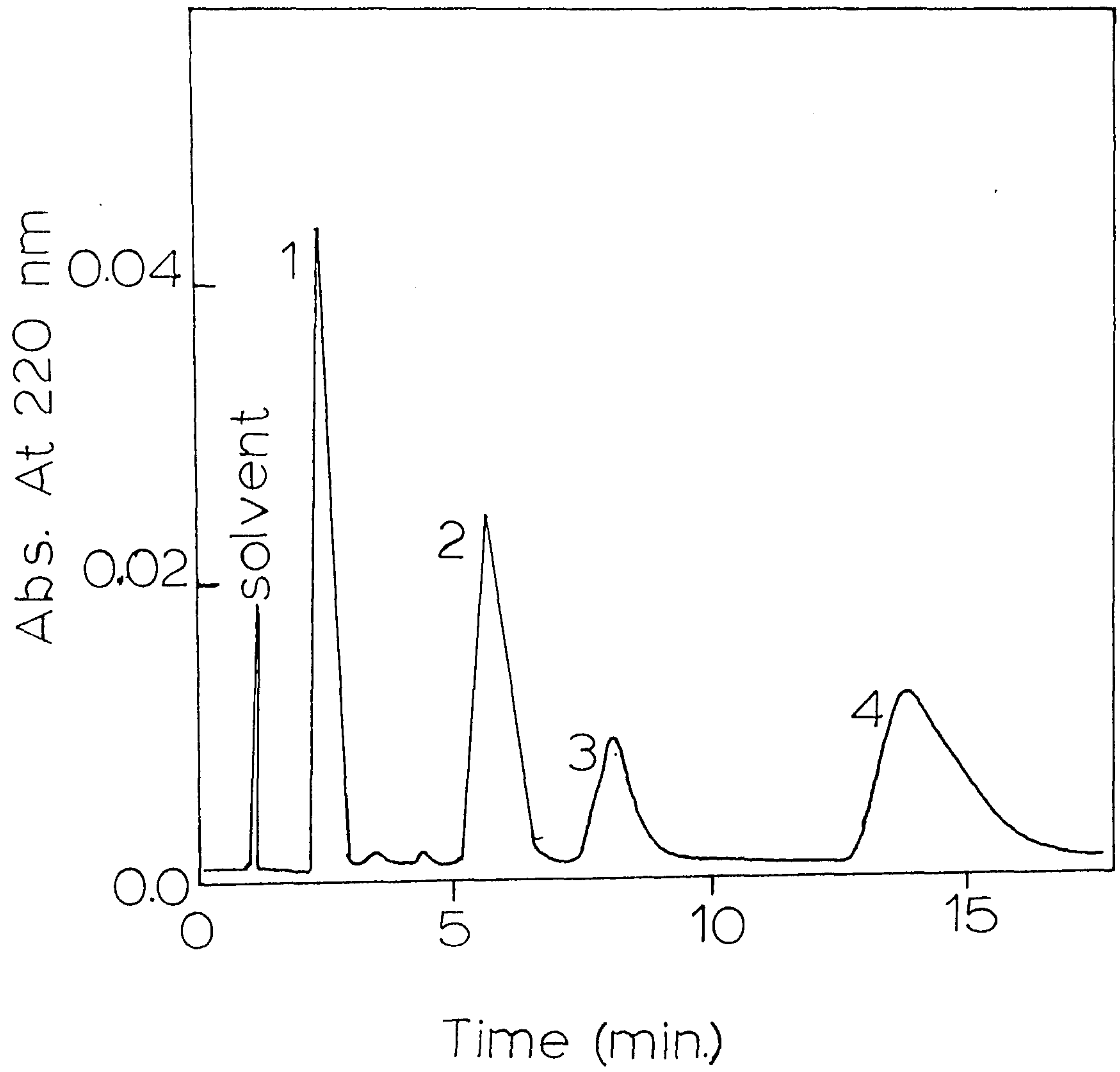

Abs. At 220 nm
0.04
0.02
0.0
solvent
1
2
3
4
0
5
10
15
Time (min.)

Table 4. Competitive Inhibition of Morphogenesis by Non-active α-Factor Analogs

MINIMUM RATIO FOR COMPETITION OF MORPHOGENESIS

Peptides	Ratio
desTrp^{1},Phe^{3} vs desTrp^{1},Cha^{3} [a]	5:1
desTrp^{1},Ala^{3} vs desTrp^{1},Cha^{3} [a]	40:1
desTrp^{1},Phe^{3} vs Tridecapeptide[b]	15:1
desTrp^{1},Ala^{3} vs Tridecapeptide[b]	2,500:1
desTrp^{1},Cha^{3},Ala^{6} vs desTrp^{1},Cha^{3} [a]	NC
desTrp^{1},Cha^{3},D-Leu^{6} vs desTrp^{1},Cha^{3} [a]	NC

NC = No competition at ratios of 500:1

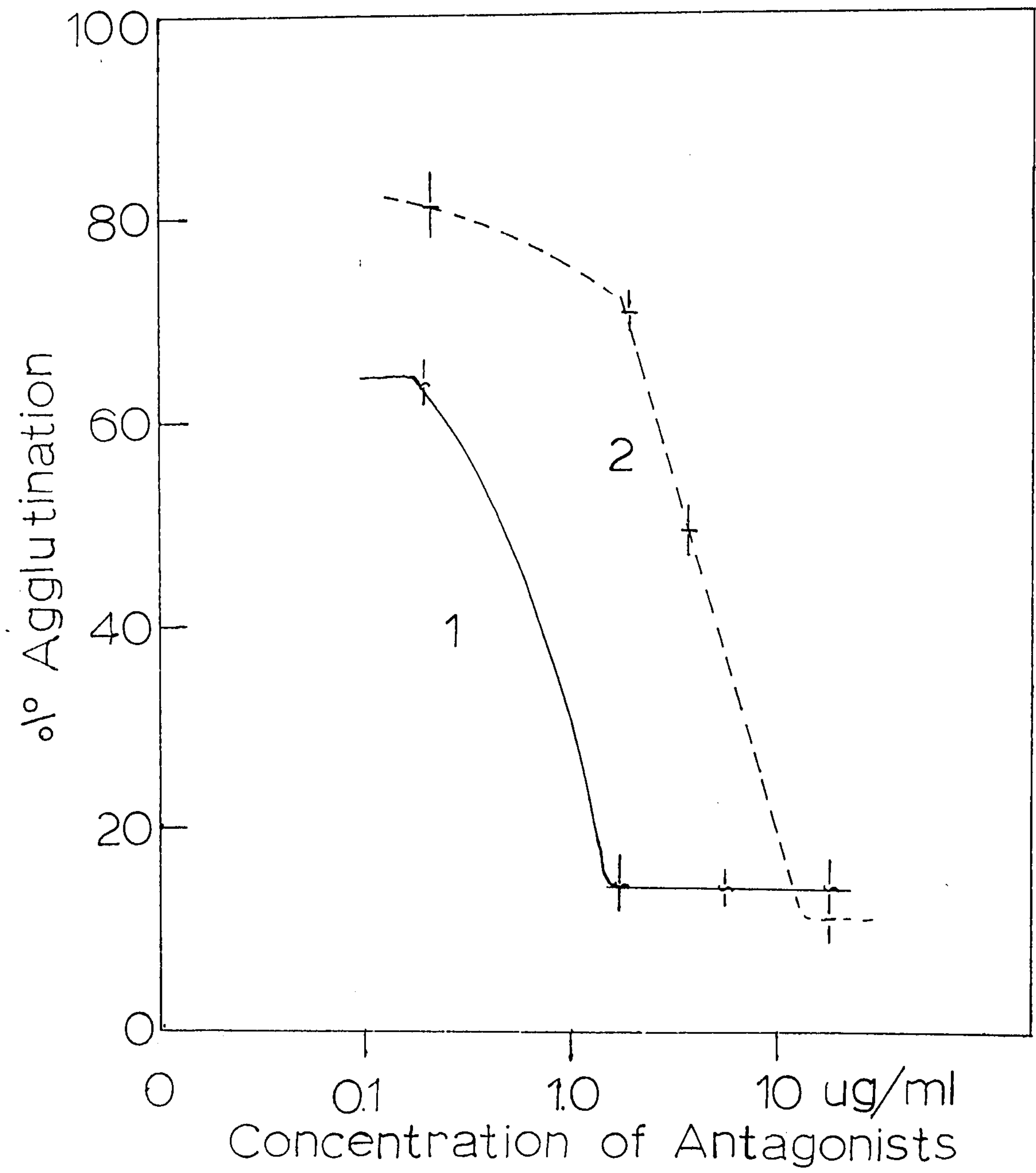
a = Concentration of 2.5 ug/ml

b = Concentration of 500 ng/ml

Figure 17. Competitive Inhibition of Agglutination by Non-active α-Factor Analogs

1 - desTrp^{1},Phe^{3} vs desTrp^{1},Cha^{3} (2.5 ug/ml)

2 - desTrp^{1},Ala^{3} vs desTrp^{1},Cha^{3} (2.5 ug/ml)


100
80
60
40
20
0
% Agglutination
2
1
0
0.1
1.0
10 ug/ml
Concentration of Antagonists

desTrp^{1}, Phe^{3}-dodecapeptide competition is observed at a ratio of 1:1 or greater while competition by the desTrp^{1},Ala^{3}-dodecapeptides requires a ratio of 4:1 or greater. Both of these inactive analogs are more potent antagonists of agglutination than they are of morphogenesis. These competition studies demonstrate that the antagonists can bind to the receptor without triggering biological responses in a-cells.

POSITION 6 ANALOGS

Analysis of the k' values of position 6 dodecapeptide analogs (Table 5) and of comparative HPLC chromatograms (Figure 18,19) demonstrate that as expected the hydrophobicity of the peptide increases as the side chain of residue 6 is changed from methyl to butyl. The desTrp^{1},Cha^{3},Ala^{6}-analog being the least hydrophobic followed by the desTrp^{1},Cha^{3},Val^{6}-analog and then the desTrp^{1},Cha^{3},Ile^{6}-analog. There appears to be other reasons besides chain length which affect retention on the micro-Bondapak C_{18} support. The desTrp^{1},Cha^{3}, Leu^{6}-analog has a greater k' than the desTrp^{1},Cha^{3}, Ile^{6}-analog. This may be attributed to the increase in hydrophobicity of the Leu side chain due to gamma branching when compared to the beta branching in Ile. Since the branching in the side chain of Leu occurs further away from the peptide backbone it can better interact with the column support. This same reasoning may be used to explain the greater k' value when Nle replaced Leu^{6}. Substitution of D-Leu for L-Leu lead to a slight increase in k'. Since the difference in these amino acids is in configuration and not in size, branching or overall hydrophobicity the strength of the interactions of these

diastereomeric peptides with the column may reflect differences in their conformations.

Analysis of the biological activities of position 6 dodecapeptides analogs by the two different bio-assays indicates that these substitutions affect the agglutination activity more than the morphogenic activity. The gamma branched side chain of Leu in the six position was found not to be essential for activity (Table 5). Substitution with the unbranched amino acid Nle leads to nearly identical activity in morphogenesis with a concomitant 35-fold reduction in agglutination potency. When the beta branched Ile is substituted for Leu the morphogenic potency decreases six fold while agglutination potency is reduced by a factor of 130. Substitution with Val leads to a ten fold reduction in morphogenesis activity and a 375-fold decrease in agglutination activity. Replacement of Leu with either Ala or D-Leu abolishes activity for both assays. This finding is in contrast to Masui et al. (56) who reports activity for the desTrp^{1},D-Leu^{6}-dodecapeptide at 400 μg/ml. The desTrp^{1},Cha^{3},Ala^{6}- and desTrp^{1},Cha^{3}, D-Leu^{6}-dodecapeptides, which do not elicit shmoo formation or induce increased agglutinability in a-cells

Figure 18. HPLC of 5 Position 6 Analogs

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System	- Gradient from 15-30% CH_3CN over 40 min
Flow Rate	- 1.5 ml/min

1- $desTrp^1,Cha^3,Ala^6$

2- $desTrp^1,Cha^3,Val^6$

3- $desTrp^1,Cha^3,Ile^6$

4- $desTrp^1,Cha^3,D\text{-}Leu^6$

5- $desTrp^1,Cha^3,Nle^6$

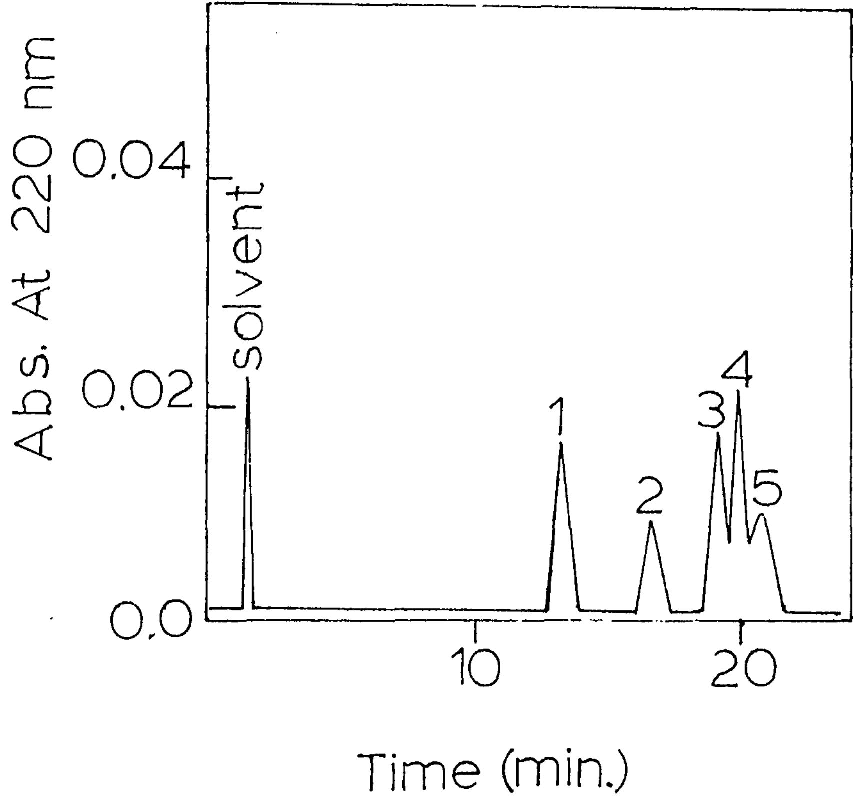

Abs. At 220 nm
0.04
0.02
0.0
solvent
1
2
3
4
5
10
20
Time (min.)

Figure 19. HPLC of 6 Position 6 Analogs

Column	- Micro-Bondapak C_{18}
AUFS	- 0.1
Chart Speed	- 0.4"/min
Wavelength	- 220 nm
Solvent System	- MeOH:H_2O:TFA (400:600:0.25 ml)
Flow Rate	- 0.5 ml/min

1- desTrp^1,Cha^3,Ala^6

2- desTrp^1,Cha^3,Val^6

3- desTrp^1,Cha^3,Ile^6

4- desTrp^1,Cha^3,L-Leu^6

5- desTrp^1,Cha^3,D-Leu^6

6- desTrp^1,Cha^3,Nle^6

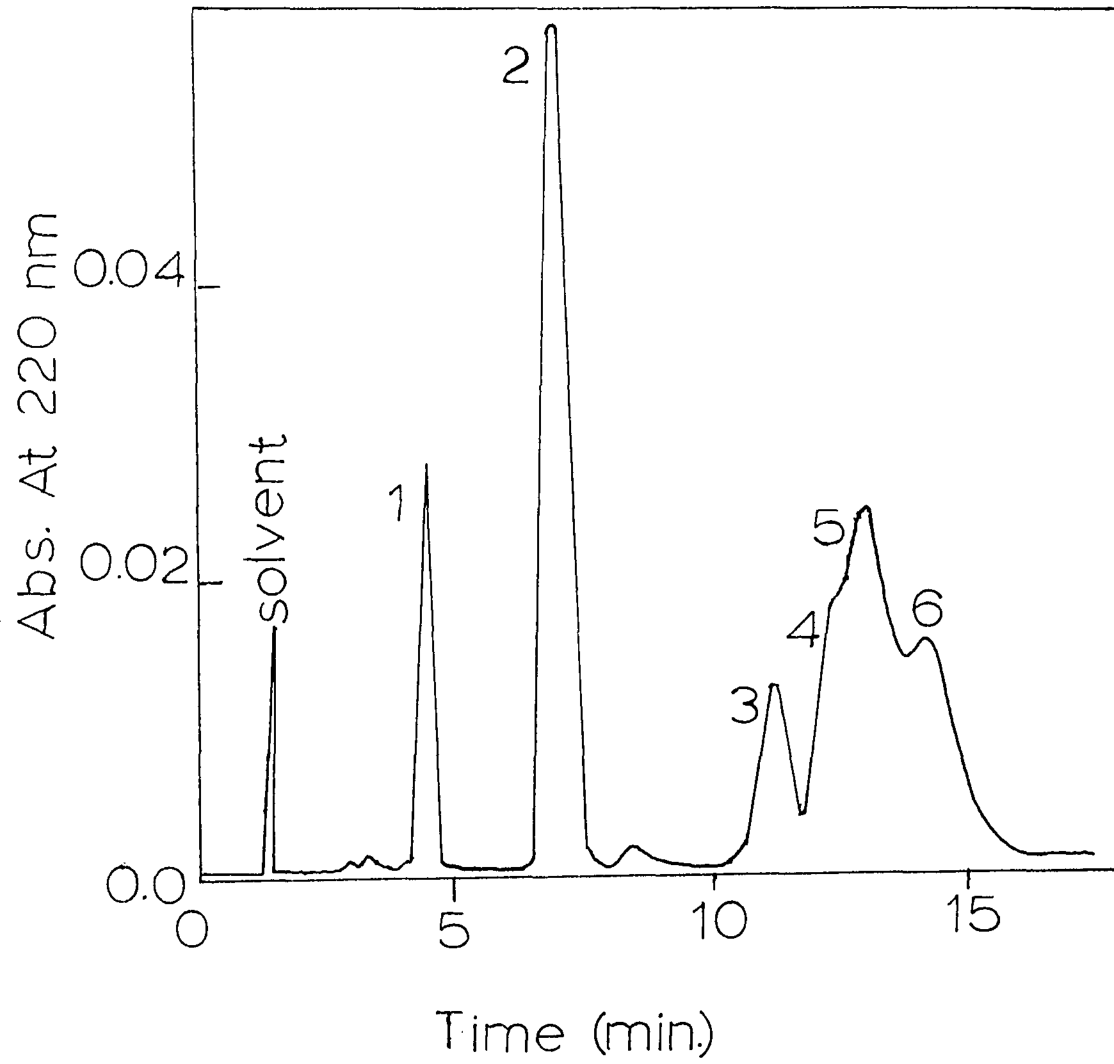

Abs. At 220 nm
0.0
0.02
0.04
solvent
1
2
3
4
5
6
0
5
10
15
Time (min.)

Table 5. Position 6 Analogs: k' Values and Biological Activities of Dodecapeptide-αFactor Analogs

Peptide	k'	Morphogenesis Activity ng/ml	Agglutination Activity ng/ml
desTrp^{1},Cha^{3},Ala^{6}	2.00	NA[a]	NA[b]
desTrp^{1},Cha^{3},Val^{6}	4.93	2,650 (3)[c]	12,000 (3)[c]
desTrp^{1},Cha^{3},Ile^{6}	6.53	1,500 (3)[c]	5,200 (3)[c]
desTrp^{1},Cha^{3},L-Leu^{6}	7.00	270 (12)[c]	35 (2)[c]
desTrp^{1},Cha^{3},D-Leu^{6}	7.47	NA[a]	NA[b]
desTrp^{1},Cha^{3},Nle^{6}	8.13	270 (6)[c]	1,200 (3)[c]

a = Not Active at 500 μg/ml

b = Not Active at 50 μg/ml

c = Number of Independent Trails

were competed with the desTrp^{1},Cha^{3}-dodecapeptide (Table 4). It was found that neither of these dodecapeptides prevented biologically active mating factor from causing shmoo formation or inducing increased agglutination at 500 fold excess. This was in contrast to the position 3 analogs desTrp^{1},Ala^{3}- and desTrp^{1},Phe^{3}-dodecapeptides which acted as antagonists for both activities.

POSITION 7 ANALOGS

Analysis of the biological activities of position 7 dodecapeptide analogs by the two different bio-assays demonstrates that the epsilon-amino group of Lys is not essential for either activity (Table 6). Elimination of the charge of Lys by substitution with Nle resulted in a 10-fold reduction in morphogenic activity with a 43-fold decrease in agglutination. Masking of the charge of Lys with an acetyl group lead to 4-fold decrease in morphogenic potency with a 35-fold decrease in agglutination. Modification of Lys with a biotinyl group led to a 13-fold decrease in morphogenesis and a 37-fold decrease in agglutination. Moving the charge farther away from the backbone of the peptide by coupling a Dns-Gly group to Lys^7 resulted in a peptide of indentical morphogenic potency and 38-fold decrease in agglutination potency. In contrast moving the charge closer to the peptide backbone by substituting Orn (which has one less methylene in its side chain than Lys) results in a 12-fold decrease in morphogenic activity and only a 3-fold decrease in agglutination potency. Morphogenic potency only was determined for modification of Lys with fatty acids of increasing length. Coupling of a butanoic or octanoic acid to Lys resulted in activity similar to the

Table 6. Position 7 Analogs Biological Activities

Peptide	Morphogenesis Activity ng/ml	Agglutination Activity ng/ml
desTrp^1	720 (12)^a	8 (2)^a
desTrp^1,Cha^3	270 (12)^a	35 (2)^a
desTrp^1,Cha^3,Orn^7	2,800 (4)^a	90 (3)^a
desTrp^1,Cha^{3}Lys^7(Ac)	900 (4)^a	1,300 (3)^a
desTrp^1,Cha^3,Lys^7(Biotinyl)	3,100 (5)^a	1,300 (2)^a
desTrp^1,Lys^7(Dns-Gly)	700 (5)^a	120 (4)^a
desTrp^1,Cha^3,Nle^7	2,700 (5)^a	1,500 (2)^a
desTrp^1,Cha^3,Lys^7(Bu)	800 (3)^a 2.9 $\pm$ 0.01^b	ND
desTrp^1,Cha^3,Lys^7(Oc)	800 (3)^a 2.9 $\pm$ 0.11^b	ND
desTrp^1,Cha^3,Lys^7(La)	6,000 (3)^a 3.8 $\pm$ 0.04^b	ND
desTrp^1,Cha^3,Lys^7(St)	NA at 500ug/ml	ND

NA = Not Active

ND = No Determination

Bu = (C_3H_7CO)

Oc = ($C_7H_{15}CO$)

La = ($C_{11}H_{23}CO$)

St = ($C_{17}H_{35}CO$)

a = Number of Independent Trails

b = Mean of the log concentrations $\pm$ Standard Error

acetyl derivative (900 ng/ml). When the size of the fatty acid was increased to dodecyl the morphogenic activity was reduced by 24-fold. Modification with stearic acid abolished activity indicating that only a group of certain size can be tolerated in the position 7 side chain, however this analog was not soluble in medium and may have precipitated from solution during the assay. Abolishing the charge on Lys resulted in an average drop in agglutination potency of 39-fold.

OTHER POSITIONAL ANALOGS

Activity of desTrp^{1}(N-α-Dns)His^{2},Cha^{3}-dodecapeptide demonstrates that the alpha amino group was not needed for activity (Table 7). Nevertheless this substitution led to a decrease in activity of 3-fold in both morphogenesis and agglutination assays. Substitution of Met with Nle in position 12 of the desTrp^{1},Cha^{3},Nle^{12}-dodecapeptide had no effect on morphogenesis. Replacing Gly with D-Ala in position 9 of the desTrp^{1},Cha^{3},D-Ala^{9}-dodecapeptide resulted in a slight increase in morphogenic activity. Substitution of Gly^{9} with L-Ala resulted in a 48-fold reduction of activity to 11,700 ng/ml. The findings with the D-Ala^{9} and L-Ala^{9} analogs would be consistent with the hypothesis that Gly in position 9 is involved in a Type II beta bend (64).

Cell Cycle Arrest

In order to determine if cell cycle arrest was more closely related to morphogenesis or induction of increased agglutinability, we compared the natural sequence tridecapeptide which is our most potent analog in both assays with the desTrp^{1},Cha^{3},Lys^{7}(Ac)-dodecapeptide which is equipotent for both activities. For the tridecapeptide we used concentrations which induced half

Table 7. Biological Activities of Other Positional Analogs of α-Factor Analogs

Peptide	Morphogenesis Activity ng/ml	Agglutination Activity ng/ml
Trideca	70 (21)[a]	0.5 (2)[a]
desTrp^{1}	720 (12)[a]	8 (2)[a]
desTrp^{1},Cha^{3}	270 (12)[a]	35 (2)[a]
desTrp^{1},(N-α-Dns)His^{2},Cha^{3}	700 (5)[a]	120 (4)[a]
desTrp^{1},Cha^{3},D-Ala^{9}	175 (3)[a] 2.2 ± 0.04[b]	ND
desTrp^{1},Cha^{3},L-Ala^{9}	11,700 (3)[a] 4.1 ± 0.09[b]	ND
desTrp^{1},Cha^{3},Nle^{12}	270 (3)[a] 2.4 ± 0.1[b]	ND

ND = No Determination

a = Number of Independent Trails

b = Mean of the Log Concentration ± Standard Error

Figure 20. Cell Cycle Arrest

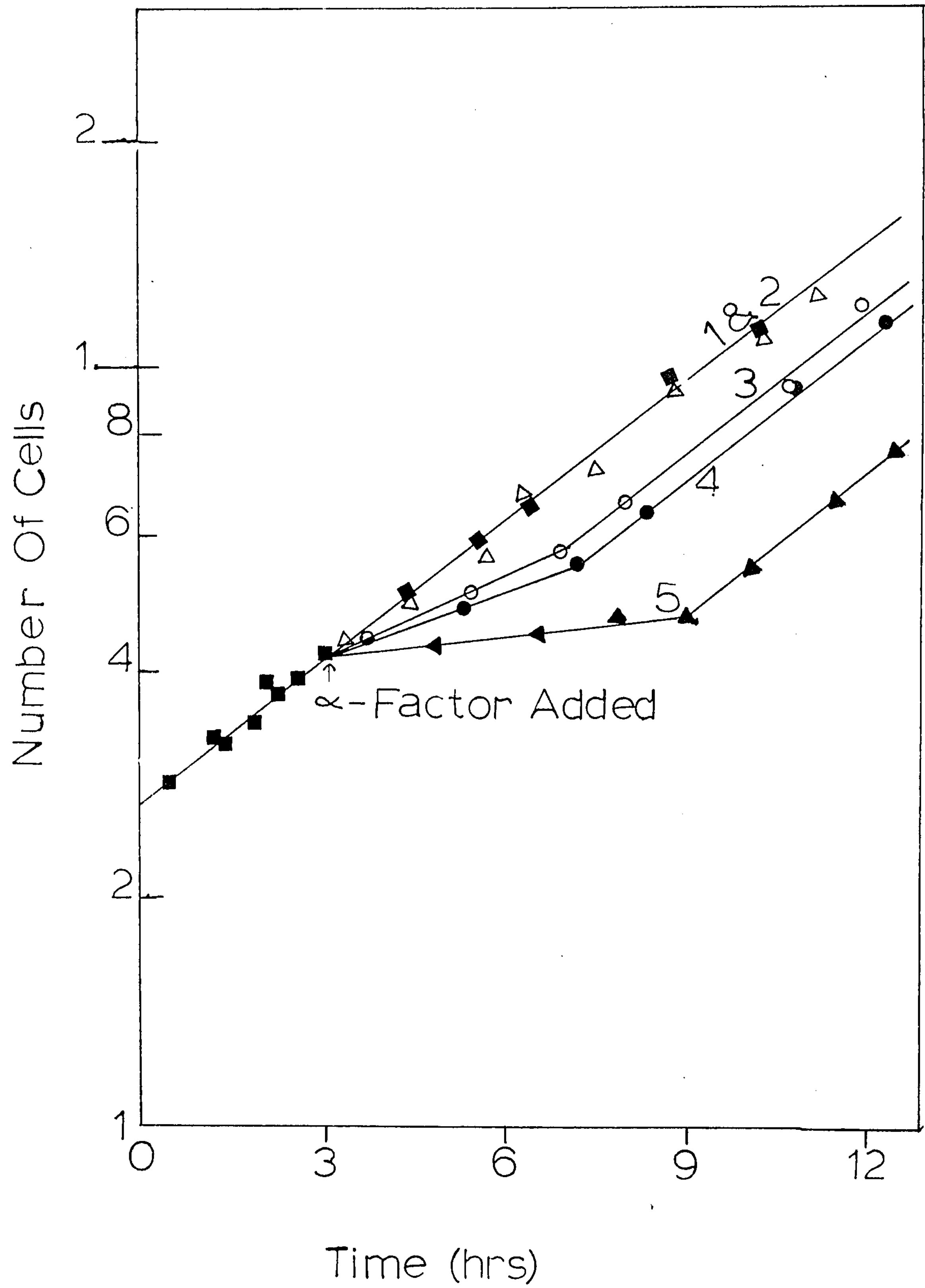
1 - Uninduced Cells (Control)

2 - Natural Sequence Tridecapeptide at Agglutination dose

3 - $desTrp^{1},Cha^{3},Lys^{7}(Ac)$ at 10x Morphogenic dose

4 - $desTrp^{1},Cha^{3},Lys^{7}(Ac)$ at 50x Morphogenic dose

5 - Natural Sequence Tridecapeptide at 10x Morphogenic dose



Number Of Cells
2
1.
8
6
4
2
1
0
3
6
9
12
Time (hrs)
1&2
3
4
5
α-Factor Added

maximal agglutination and ten times the morphogenic dose. For the desTrp^{1},Cha^{3},Lys^{7}(Ac)- dodecapeptide we used concentrations ten and fifty times the amount needed for morphogenesis. The results (Figure 20) demonstrate that agglutination doses of tridecapeptide were not sufficient to cause cell cycle arrest. Although cell cycle arrest required morphogenic doses, the length of growth arrest was not proportional to dose.

To better assess the effects of amino acid substitution, deletion or modification on the potency of various dodecapeptides a ratio of morphogenic potency to agglutination potency was employed (Table 8). The various analogs can be divided into groups that are better agglutinogens [natural sequence trideca-and dodecapeptides, desTrp^{1},(N-α-Dns)His^{2},Cha^{3}, des Trp^{1},Cha^{3}, desTrp^{1},Cha^{3},Orn^{7} and desTrp^{1}, Lys^{7}(Dns-Gly)] or those that are better morphogens (all active position 6 analogs) and those that are close to equipotent [desTrp^{1},Cha^{3},Nle^{7}, desTrp^{1},Cha^{3}, Lys^{7}(Biotinyl) and desTrp^{1},Cha^{3}Lys^{7}(Ac)].

Table 8. Ratio of Biological Activities of α-Factor Analogs

Peptide	Ratio of Concentration Causing Shmoo Formation to Concentration Causing Half Maximal Increase of Agglutination
Trideca	140
desTrp^{1}	90
desTrp^{1},(N-α-Dns)His^{2},Cha^{3}	6.0
desTrp^{1},Cha^{3}	8.0
desTrp^{1},Cha^{3},Orn^{7}	30.0
desTrp^{1},Cha^{3},Nle^{7}	1.8
desTrp^{1},Cha^{3},Lys^{7}(Ac)	0.7
desTrp^{1},Cha^{3},Lys^{7}(Biotinyl)	2.5
desTrp^{1},Lys^{7}(Dns-Gly)	5.8
desTrp^{1},Cha^{3},Nle^{6}	0.2
desTrp^{1},Cha^{3},Ile^{6}	0.3
desTrp^{1},Cha^{3},Val^{6}	0.2
α-Factor from Culture Medium	65

CONCLUSIONS

Implicit in life is control. Regardless of its level of organizational complexity, no living system can exist without precise mechanisms for controlling its various activities. Thus, an understanding of cellular physiology requires not only a knowledge of the basic processes but also the mechanisms which control them. Sexual conjugation of a- and α-mating types of S. cerevisiae provides an ideal model system for investigation of cell-cell interactions. This system shares several features of peptide hormone systems in more complex mammalian cells. Utilizing assays sensitive to changes in morphogenesis, agglutination and cell cycle arrest of yeast cells we have been able to differentiate the fundamental processes involved in mating of yeasts in terms of comparative interaction of molecules of known structure.

Structure - function relationships of morphogenic and agglutination activities indicate a heterogeneous response of a-cells to α-factor. We observed conditions under which either response could be induced without the other. For the majority of analogs low concentrations and short exposure time (20 s) (per. comm. K. Terrance) led to increased agglutinability without effecting morphogenesis. The major exception occured with position six analogs which were more potent morphogens, as indicated

by a potency ratio less than 1.0. By making single amino acid substitutions we were able to preferentially enhance one activity over the other. An example of enhanced morphogenic activity was demonstrated by substituting Cha for Trp in position 3. Subsitution of Nle for Leu in position six showed similar morphogenic potencies while agglutination activity decreased 35-fold. Elimination of the charged group in position 7 analogs decreased morphogenesis activity anywhere from 3 to 11-fold while agglutination activity decreased 40-fold on the average.

We have found several examples of dodecapeptide analogs where changes in morphogenic activity do not correlate with changes in agglutination activity. The tremendous variations in potencies are illustrated in Figure 15 and Table 8. Competition experiments show that antagonism occurs at different ratios in the two bio-assays (Table 4, Figure 17). These results represent the first report of an α-factor analog with antagonisitc properties. Cell cycle arrest studies infer that the cells can be stalled in the G1 phase of DNA synthesis at morphogenic doses and not necessarily at agglutination doses (Figure 20). These results indicate that there is a heterogeneous response of <u>a</u>-cells to α-factor, which is inconsistent with a single

receptor responding in a simple manner. Therefore, we propose that there must be two or more α-factor receptors.

There are several lines of evidence which indicate that differential degradation of α-factor analogs is not responsible for the observed heterogeneous response of a-cells to α-factor. Cells which lack barrier factor, an enzyme which cleaves -factor at the amide bond between Leu^6 and Lys^7 are known as sst 1 mutants (43). The stability of α-factor in the mutant strain RC-629, an sstl mutant, is substantially greater than in the parential strain RC-618. The relative potency of the pheromone is virtually unaffected in the mutant strain (per comm P. Lipke). Degradation thus appears to reflect the abiltiy of a-cells to recover from the effects of α-factor and is not related to the respone mechanism.

α-Factor destruction is crucial for recovery of a-cells from cell cycle arrest (42). Preliminary degradation studies (J.M. Becker per. comm.) indicate that the natural sequence synthetic tridecapeptide is cleaved the fastest. This peptide is the most potent pheromone. At ten times morphogenic doses it arrests a-cells in G1 for 6 h. The $desTrp^1,Cha^3,Lys^7(Ac)$ dodecapeptide is equipotent in the two assays. a-Cells given different concentrations

(10x and 50x morphogenic doses) of this analog recovered from the effects of cell cycle arrest in 3.5 h. These results indicate that although morphogenic concentrations of a particular analog may be needed to cause cell cycle arrest, the enhanced length of arrest time for the tridecapeptide when compared at equal morphogenic dose cannot be due to slower degradation.

All of the active position 6 analogs induce morphogenesis at lower concentrations than are needed to induce enhanced agglutination. Since morphogenesis requires longer exposure than does the agglutination response, these analogs must not be degraded quickly at active concentration. Competition studies demonstrate that the receptor(s) has a high affinity for the antagonist des Trp^{1},Ala^{3}-dodecapeptide. It prevents enhanced agglutination of a-cells at 4:1 ratio against $desTrp^{1},Cha^{3}$-dodecapeptide. This concentration is not sufficient to inhibit morphogenesis which requires a 40:1 ratio of antagonist to active α-factor. Degradation studies have shown that the $desTrp^{1},Ala^{3}$-dodecapeptide is not cleaved by barrier factor (per comm. J. Becker). Therefore the difference in concentrations needed to compete against $desTrp^{1},Cha^{3}$ for the two activities is not related to preferential degradation of either α-factor analog but represents a heterogeneous response of a-cells

to different concentrations of α-factor.

Most of the goals of my thesis have been attained. The synthesis and characterization of five position six analogs and one position three analog has led to knowledge concerning the size, hydrophobicity and configuration of these amino acid residues which are most suitable for biological activity. In addition, structure-function relationships for the side chains in positions 7, 9 and 12 have been determined. The differential response of a-cells to α-factor analogs along with competition studies and cell cycle arrest experiments provide the basis of a model for the mode of action of α-factor which involves multiple receptors.

Preliminary circular dichroism studies on $desTrp^{1},Cha^{3}$-dodecapeptide in trifluoroethanol indicates that this peptide contains a non-random secondary structure. Comparative spectra with position 7 analogs in which the positive charge of Lys is eliminated show a shift in the maxima and a change in the molar elipticity. We believe that the $Pro-Gly^{9}$ unit of α-factor may be involved in a Type II beta turn. Investigations on position nine analogs show that replacement of Gly with D-Ala gives a peptide with nearly identical morphogenesis activity and CD spectra.

Substitution with L-Ala results in a peptide of drastically lower activity and markedly different CD spectra. Our collaborators at the University of Tennesse have undertaken degradation studies. Preliminary results indicated that various active α-factor analogs have different half-lifes. It also appears that inactive α-factor analogs are degraded very slowly or not at all. The facts presented in this thesis form a basis for comparison of future degradation studies and conformational analysis, with biological activity of the various analogs. Hopefully these comparisons will provide insights into the relationship between chemical structure and the response of yeast cells to the α-factor pheromone.

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