6-1994

Synthesis of a Hybrid Baldwin Peptide: A Study in the Factors That Influence Helix Formation

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SYNTHESIS OF A HYBRID BALDWIN PEPTIDE: A STUDY IN THE FACTORS THAT INFLUENCE HELIX FORMATION

By

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Submitted in fulfillment
of the requirements for
Honors in the Department of Chemistry

Union College
June, 1994

ABSTRACT

BERGER, JAY STEVEN The synthesis of a hybrid Baldwin peptide: A study in factors that influence helix formation in small peptide chains. Department of Chemistry, Union College, June 1994.

Baldwin has determined the alpha-helix stability in peptides with alternating charged groups. The four peptides used were (i,i+4)AB,(i,i+4)BA,(i,i+3)AB, and (i,i+3)BA, where the A is a positively charged residue (either lysine or arginine) and the B is a negatively charged residue (either glutamic acid or aspartic acid) and the (i,i+4) indicates that the charged groups are spaced four residues apart. The spaces between the charged residues are filled with alanines, which positively influence the formation of an alpha-helix. In all cases the stability of the helix is i+4AB>i+4BA~i+3AB>i+3BA peptide sequences.

In this study, a hybrid i+4/i+3 peptide will be synthesized. It is assumed that this configuration will enhance the formation of the alpha-helix. Previous research has determined that a complete helical turn requires 3.6 amino acid residues. The hybrid peptide will include four residues followed by a second helical turn which will only encompass three residues. It is hoped that by slightly over turning and then under turning the helix, the stability of the overall helix formation will be enhanced.

Acknowledgements:

Union College

N.Y.U.

Peter Kissinger of Bio. Analytical Systems

James Ernst

Dr. L.A. Hull

Dr. and Mrs. Gary Berger

Jay Steven Berger

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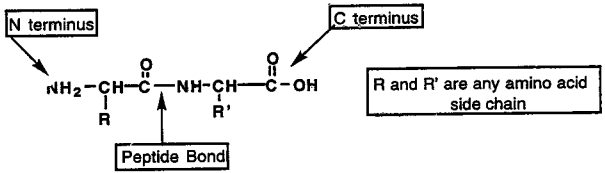
Chapter 1-Introduction:

Historical Background:

For many years, scientists have tried to understand the folding patterns of proteins. However, before the folding patterns of proteins could be explored the actual structure of the protein had to be known. A protein is nothing more than several hundred and even thousands, in some cases, of α-amino acids linked together. The general form of an amino acid is an amine group bonded to the α-carbon of a carboxylic acid. The actual peptide bonds are amide bonds between the amine group of one amino acid with the carbonyl group of the other amino acid.

Structure of a Typical Amino Acid:



Each amino acid is differentiated by its side chain which is connected to the α- carbon. The only amino acid with an unusual structure is proline. In proline, the side chain is three methyl end groups which link the α-carbon to the amine group, forming a ring structure.

In nature, twenty different amino acids account for nearly all of the amino acids presently found. These twenty amino acids have been grouped into six general classes by their side chains. One such grouping system as used by Loudon (1) is:

1) Amino Acids with either a hydrogen or aliphatic hydrocarbon side chain.

2) Amino Acids with an aromatic side chain.
3) Amino Acids whose side chains contain either a SH, SCH_3, or OH group.
4) Amino acids whose side chains contain either a carboxylic acid or amide group.
5) Amino acids with basic side chains.
6) Proline is placed in its own class because of its unique structure.

The actual sequence of the amino acids in the protein is very important in determining the protein's structure and thus activity. As a convention, the naming of peptide chains always starts at the amino terminus or N-terminus, the end with a free amine group, and ends at the carboxy terminus or C-terminus, the end with a free carboxy acid group. The sequence of amino acids in the chain is known as the backbone of the peptide and makes up a majority of the primary structure of a protein. The remaining portion of the primary structure is determined by disulfide bonds between the side chains of two cysteines, which crosslink the two amino acids.

The secondary structure of a protein is the result of hydrogen bonding, constructing three subclass structures. The first subclass of the secondary structures and most important to this study is the α-helix. In this structure, the side chains are looped outside. The hydrogen bonds that stabilize this structure are between the amide functional group on the main chain of one amino acid and the carbonyl oxygen on a residue that is four residues away. The second subclass of secondary structure is the β-pleated sheet. In this structure, a zig-zag conformation is created and held together by hydrogen bonds between the two side by side chains. The third subclass of secondary structure is the random coil, which like its name implies has no determinable pattern. The random coil is important to this study of helix formation, because the peptide chain will enter into an equilibrium between a helical

structure and a random coil structure.

The tertiary structure accounts for all interactions between the functional groups found on the side chains of the amino acids, such as ion pair formations,
which will be used in this study to stabilize the helix. The quaternary structure is defined by the interaction of two or more peptide chains. The combination of the tertiary and quaternary structures depict the exact three dimensional structure of the protein; however, these structures will not be discussed in this study of helix formation.

The α-helix was first proposed as a structure by Pauling (2) in 1955. In the hope of better understanding the formation of an α-helix, scientists tried to determine the physical properties of each residue in the polypeptide chain. The free energy per residue (ΔG_{res}) for the helix was split into both an enthalpy term and an entropy term by the following equation:

$$\Delta G^{o}_{res} = \Delta H^{o}_{res} - \Delta TS^{o}_{res}$$

Schellman (3) estimated that the ΔH_{res} for the hydrogen bonding of the helix to be approximately -1.5 kcal/mol. This data was taken from the dimerization of Urea in water. The entropy term ΔS_{res} was rewritten to -Rln*j* by Schellman (4), where *j* represents the number of equivalent torsional conformations of the peptide backbone. As a result, the entropy term per residue was estimated to be -1.4 kcal/mol, *j* was assumed to be 10. Schellman (4) concluded it is sparingly possible to form a stable, isolated α-helix in a water solvent system. This conclusion is significant because the synthesis of small peptide chains that form α-helices would greatly simplify the study of α-helix formation in

proteins.

The work done by Schellman prompted scientists to ask three basic questions about the formation of an α-helix in a polypeptide. These three questions as stated by Baldwin (5) are: "(a) What is the enthalpy of peptide hydrogen-bond formation in water, and what is the value of ΔH_{res}? (b) What is the actual value of *j*, and how much do side chains contribute to ΔH_{res}? (c) How does the hydrophobic interaction affect the values of ΔH_{res} and ΔS_{res}?" The answers to these questions did not come quickly because only long polypeptide chains could be used to test the factors that encourage or inhibit the formation of an α-helix. The use of short peptide chains would have eliminated many of the problems early researchers faced.

Researchers also addressed the question of why an α-helix forms in another way. Instead of actual experimentation, the question of why an α-helix forms was approached via statistical models. These models first appeared in the late 1950's after Schellman proposed his initial findings. It was hoped that these theoretical models could be used to examine the factors that determine whether or not an α-helix will form. One of the first models is the Zimm-Bragg Theory. In this theoretical model, the first peptide residue in the helix is assigned a statistical weight of (σs), while the second and succeeding residues are each assigned statistical weight of (s). The nucleation parameter (σ) is simply the theoretical possibility that the first residue in the helix was in the correct configuration to initiate the formation of the helix. In contrast, the helix propagation parameter (s) is a measure of \

each residue's ability to enhance or inhibit the formation of an α-helix. Variations of this model also exist, such as the Lifson-Roig model. The Lifson-Roig model differs from the Zimm-Bragg model in the way helicity of the peptide chain is parametered.

In 1959, Zimm et al (6) tested the Zimm-Bragg theory by using polypeptides ranging from 26 to 1500 residues. The chain consisted of a polymer of gamma-benzyl-L-glutamate. The ΔH_{res} was determined by calorimetry. These results were later confirmed by Ackerman(7) who also used calorimetry measurements. The temperature midpoint was the point on the transition curve where the s parameter equals one. ΔH_{res} was compared to the Van't Hoff enthalpy change by the following equation:

$$\Delta H^{o}_{VH} = \Delta H^{o} / \sigma^{1/2}$$

(ΔH^{o} is the calorimetrically determined enthalpy change per residue)

This equation shows that if the nucleation parameter equals 10^{-4} then the cooperative unit must equal 100 residues, where the cooperative unit is the average number of residues in the helical segment of the chain. Fortunately, the first test of the Zimm-Bragg parameters used a nonionizing amino acid in an organic solvent system and the results worked out perfectly when compared to the theoretical results. However, problems arose when an aqueous solvent system was used. The first problem is that both alanine and methionine amino acids are helix-forming but both are also insoluble in water. The second problem was that the amino acids that were soluble were not helix-forming. The third and final obstacle to overcome was the fact that the nucleation parameters of both lysine and glutamic acid were found to be

0.0025.

The next major step in determining why an α- helix forms comes in a set of experiments done by Scheraga (8, 9, 10, 11). Scheraga and his coworkers systematically determined the statistical weight of the propagation parameter for all naturally found amino acids. In the host-guest studies performed by Scheraga, a copolymer that was water soluble and non-ionizing was used as the host residue and the amino acid was used as the guest residue. The interesting result of this study was that most amino acids have a (s) value of about one, meaning that most amino acids are neither helix promoting nor inhibiting. In addition, differences found using an organic solvent versus an aqueous solvent seemed to indicate that β-branched amino acids are helix breakers (12). Lastly, it was also found that the propagation parameter is temperature dependent.

Stellwagen (13) used guest-host studies to determine which parameter affected the formation of an α- helix more. Stellwagen found that the nucleation parameter could range from 0.00001 to 0.0210 depending on which amino acid was used as the guest residue. The propagation parameter was determined to range between 0.51 and 1.47 as the guest residue was changed. Clearly the nucleation parameter has a greater effect in determining if an α-helix will form.

In order to properly study the formation of helices in proteins, a method by which the helix could be detected was needed. One method to determine the existence of an α- helix is to look at the circular dichroism (CD) spectra from 180 nm to 260 nm. An α-helix has two characteristic minima peaks, one at 208 nm and another broader minima peak at 222 nm(14).

However, this method is not without problems. For example, the $[\Theta]_{222}$ or $[\Theta]_{208}$ value will correspond when 50% of the sample is helical and 50% is coil, but it cannot distinguish between 50% of the chains being fully helical and the other 50% being fully coil or each chain being 50% helical and 50% coil. In addition, any intermediate stage between the two extremes mentioned above are possible (15). As a consequence, the use of NMR to determine helicity is used to compliment CD data.

NMR spectra make it possible to determine which residues in the chain have helical character and which do not (15). Four proton NMR criteria are used for these measurements: (a) strong NOE's between neighboring peptide NH protons (16), (b) NOE's between a peptide NH and CαH three residues away (17), (c) values of the three-bond $^3J_{\alpha N}$ coupling constants (18), and (d) values of the CαH chemical shifts (19). One reason the use of NMR is so promising is that the peaks of peptides with higher helical content turn out to have well-resolved spectra due to the differences between helical conformations(15).

As already stated, the use of short peptide chains that are helical in nature would greatly simplify the experiments to determine what factors lead to the formation of an α-helix in a protein's secondary structure. Two peptide systems that became valuable to researchers were the C-peptide and the S-peptide. These two peptides are naturally found in ribonuclease A. The C-peptide is the first thirteen residues while the S-peptide is the first twenty residues. Brown and Klee (20) determined that these two peptides showed helical tendencies at low temperature. The helical content of these peptides were determined using CD measurements; however, these experiments

showed that the peptides were only about 25% helical at 0°C. In addition, it was discovered that the C-peptide and S-peptide unfold as the temperature is raised. These results were later confirmed by Bierzynski (21), who used NMR measurements and CD measurements to confirm the existence of an α-helix in a water solvent.

Bierzynski (21) also went on to investigate the pH dependence on the formation of an a helix by the C-peptide. The CD spectra showed a maximal bell shaped curve at a pH of 5, meaning that at a pH of 5 the peptide is most helical. At this pH, it is thought that two ionized side groups are required. One ionized group is thought to the the glutamic acid (pK_a near 3.5) at position 9 in the C-peptide. The other group is the histidine (pK_a near 6.5) at position 12 in the C-peptide. The fact that two ionized groups are thought to be required seems to suggest that specific side-chain interactions are necessary for maximal helical content by the C-peptide. Furthermore, this result that helical content can be measured in short peptides contradicts the host-guest values for the propagation parameter, which predicts that short peptides are not capable of forming an α-helix.

Simultaneous experimentation by Rico (22) and Baldwin (23) showed that certain amino acids can stop helical formation. Both experimenters used NMR to show that the methionine amino acid at position 13 in the S-peptide stops the helical nature of the peptide. In addition, it was determined by free energy simulations that the insertion of proline into the peptide chain terminates the α-helix (24).

Later experiments were performed (25) which confirmed that it is the negatively charged glutamic acid residue at position 2 and the positively

charged histidine residue at position 12 that are necessary for the maximal amount of helical content to be reached. This result is surprising because of the distance between the two amino acids. If the glutamic acid at position 9 was involved then the helix would be stabilized by an ionic interaction between the two amino acids. This interaction is possible because the helix makes one complete turn in about 3.6 nucleotides, placing an amino acid at position 9 directly above the amino acid at position 12. However, substitution experiments (26) show that the glutamic acid residue at position 9 and the histidine residue at position 12 act independently of each other. One possible explanation of this conclusion is that the two amino acids react with the internal dipole moment of the helix, since both are near the terminal ends of the C-peptide.

Baldwin (27) confirmed that the glutamic acid at position 2 interacts with the internal dipole of the α-helix. The lysine with a positive two charge at position one was changed to succinyl alanine with a negative one charge, acetyl alanine with a neutral charge, alanine with a positive one charge, and lysine with a positive two charge. As the first amino acid in the chain became more and more positive, the
helical content of the chain decreased. As a result of this experiment, it was concluded that the charged amino acid near the N-terminus of the chain stabilizes the helix if its charge is opposite in character to the net dipole charge of the α-helix at the N-terminus of the chain. In fact, as the difference between these charged interactions is increased the helix is further stabilized.

In order to further study the factors that determine helix formation such as the intrahelical salt bridges(28), Baldwin created a class of peptides which this study refers to as the Baldwin peptides. In these peptides are

negatively and positively charged residues that are spaced *(i,i+3)* or *(i,i+4)* amino acids apart (29). The charged residues are separated by alanine residues.

Baldwin Peptides:

(i+4) K,E: Ac A K A A A E K A A A E K A A A E A NH_2

(i+4) E,K: Ac A E A A A K E A A A K E A A A K A NH_2

(i+3) K,E: Ac A K A A E A K A A E A K A A E A NH_2

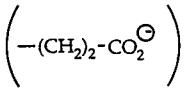
(i+3) E,K: Ac A E A A K A E A A K A E A A K A NH_2

[* NOTE: The *(i+3)* chains have additional alanine residues between the charged residues in order to make the total length of the *(i+4)* and *(i+3)* peptides as close as possible.]

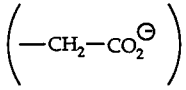
The charged amino acids first used were lysine and glutamic acid. In addition to varying the distance between charged residues, the order of the charged residues was also changed. In order to prevent any complications caused by the ends of the peptide chain being charged, they were blocked. The result of this work was that the *(i,i+4)* E,K is considerably more stable than the *(i,i+3)* E,K peptide. The same is true when the order of charged residues is switched. In addition, the EK peptides are more stable than the KE peptides because of the internal dipole moment of the helix. Many other variation have been done on this system.

One of the first variations on Baldwin's peptides was the replacement of the glutamic acid with aspartic acid. The difference between these two amino acids is the length of their side chains.

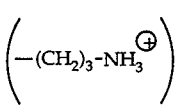
Glutamic Acid:



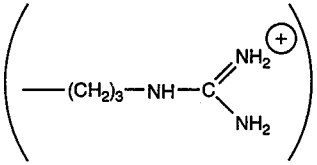
Aspartic Acid:



Lysine:



Arginine



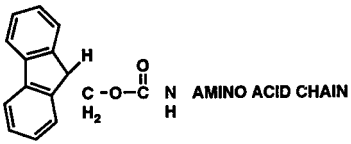
The aspartic acid was held constant when the positive amino acids were exchanged. However, the same tendencies towards the formation of an α-helix were seen as demonstrated by the original Baldwin peptides (30).

Further substitution experiments were performed by Stellwagen and coworkers (13). Stellwagen measured the ΔG_e for a host-guest system. The host chain was acetyl-Y(EAAAK)$_3$A-amide. The guest residue was inserted where the middle alanine at position 9. At a pH of 7.0 and 0°C, the ΔG_e ranged from 0.29 to 0.79 kcal/mol as the guest residue was changed. Stellwagen concluded from this experiment that the changing of one amino acid in the peptide chain can have profound effects on the equilibrium between the helix and coil forms of the peptide.

Synthesis Background:

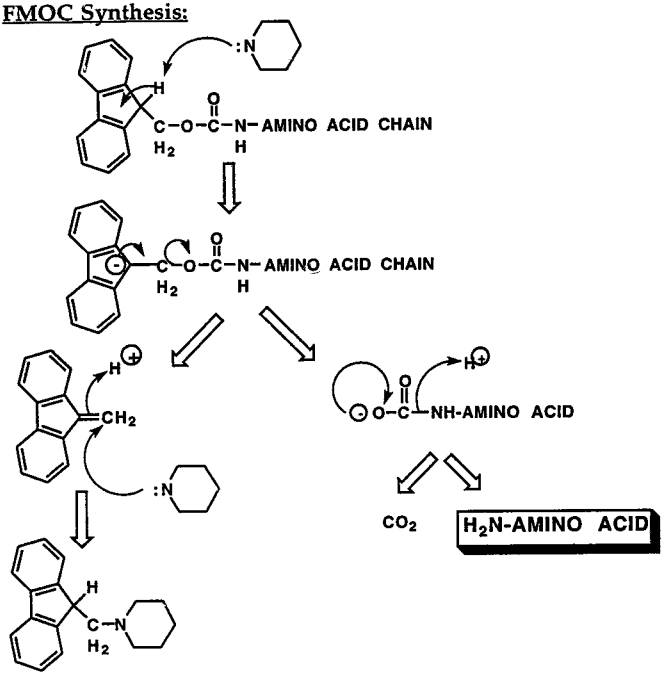
There are several different standard methods for the synthesis of small peptide chains. These synthesis methods are split into two general categories. The first synthesis is known as the FMOC synthesis as described by Jones in

his book Amino Acids and Peptide Synthesis(31). The FMOC group is a dibenzo cyclopentadiene derivative. In addition, a carbonate derivative is also attached to the cyclopentadiene ring. A carboxylate acid links the FMOC group to the amino terminus of the growing amino acid chain. The structure of the FMOC protection group is:

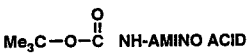


The advantages of the FMOC synthesis is that the amino acids formed are generally stable crystalline substances. The FMOC synthesis method will be used to protect the α- carbons of the amino acid backbone, this fact will be important later in the synthesis of the peptide chain. The mechanism by which the FMOC synthesis follows is detailed below:

FMOC Synthesis:

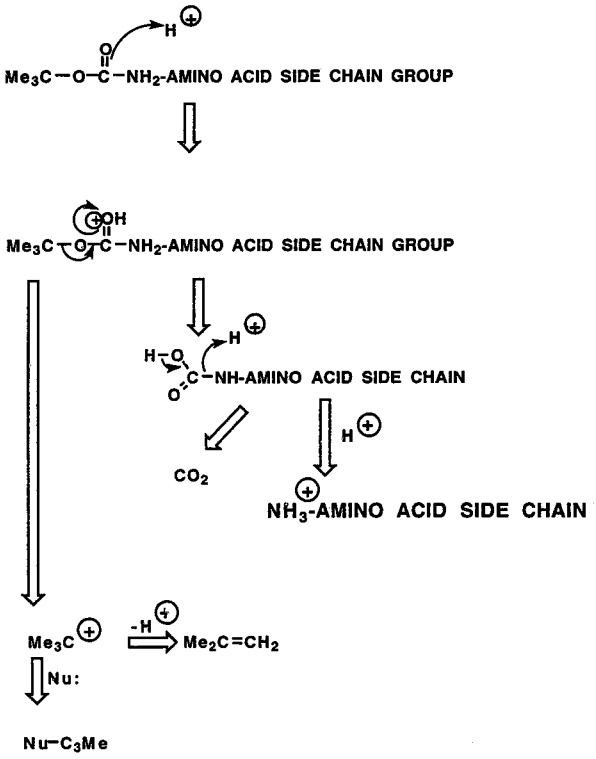


The second synthesis method is the Boc synthesis also described by Jones(31). The Boc protecting group has the following structure:



The mechanism by which the Boc group may be removed is shown below:

Boc Synthesis



These two synthetic methods are said to be orthogonal to each other because of the difference in conditions required for their removal. The FMOC protection group is easily removed by slightly basic conditions. In contrast, the Boc protection group is removed by slightly acidic conditions. As a result,

both synthetic methods can be used in the same synthesis. In this synthesis, the backbone of the peptide chain will be protected by FMOC groups and the functional groups on the side chains will be protected by Boc groups. The coupling of new amino acid residues to the chain will require the removal of the FMOC groups by the addition of base. The functional groups on the side chains will not interfere with the synthesis because they will still be protected by Boc groups.

One last consideration of the synthesis of these small peptides is the purification of the peptide chains. The synthesis has the inherit problem that a small percentage of the synthesized peptides will contain one to many or one to few amino acid residues. These impurities will be removed by their different retention times on a HPLC column. The purity will then be checked on an electrospray MS to insure the purity of the sample.

The percent helicity of the different peptide chains will be determined by CD measurements. NMR experiments will be used to determine coupling and also restrictions for computer modeling.

Questions:

The work done by Baldwin and others has answered many questions about the factors that influence the formation of an α-helix. However, a few questions arise from Baldwin's work. The first is what effect if any does the difference in length between Baldwin's *(i, i+3)* peptide and the *(i, i+4)* peptide have on the stability of the helix? The second question that arises is what happens if the spacing between the charged groups is not homogeneous like in Baldwin's peptides? The purpose of this study is to try and answer these questions by creating a hybrid Baldwin peptide that combines the *(i, i+3)* and

(i, i+4) spacing.

Chapter 2-Experimental Section:

Abbreviations of Reagents Used:

Pip = Piperdine

DMF = N,N-Dimethylformamide

DIEA = N,N-Diisopropylethylamine

BOP = (Benzotriazol-l-yloxy-tris-(dimethylamino)-phosphonium-hexafluorophosphate)

HOBt = N-Hydroxybenzotriazole

TFA = Trifluoroacetic Acid

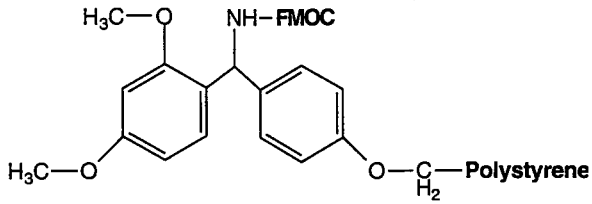
FMOC = 9-Fluorenylmethoxycarbonyl

Boc = t-Butoxycarbonyl

Synthesis:

The synthesis of the small peptide chains used in this study is accomplished using the protocol for a solid-phase FMOC synthesis, which is described below. The overall protocols for the synthesis and cleavage of the peptide are shown at the end of the experimental section. The protocol is a variation from the FMOC synthesis described by John Jones in his book Amino Acid and Peptide Synthesis(31). The actual synthesis is unusual in the fact that the amide form of the peptide is being synthesized.

The resin used in the synthesis is the Rink Amide Resin [4-(2'-4'-Dimethoxyphenyl-FMOC-aninomethyl)-phenoxy resin], produced by Nova BioChem(32). The Structure of the resin support is as follows;

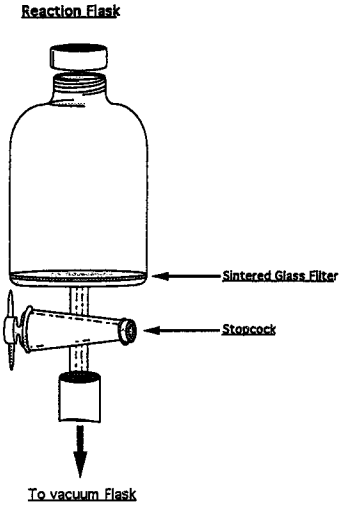


The synthesis of the KE5 peptide was accomplished using 0.5g of resin without complication. The synthesis of the EK5 peptide was later tried using 1.0g of resin; however, the Kaiser test clearly illustrated that the coupling reaction did not go to completion, even after several attempts were made to attach the amino acid were made. As a result, all future synthesis protocols were based on the use of 0.5g of resin. The resin as supplied has a substitution level of 0.39mmol/g, which means approximately 0.195mmol of peptide should theoretically be produced if the reaction of each coupling step goes to 100% completion. In addition, the resin is held together by a polystyrene derived backbone which contains 1% DVB crosslinking.

In the synthesis of the four different peptide chains, four different amino acids are needed and were all supplied by Bachem California. The majority of the peptide chain consisted of alanine residues. The protected alanine residue used is N-FMOC-L-Alanine. The lysine residue had to have both the N-terminus of its backbone protected as well as the amino-terminus of its side chain. The lysine residue used was N-ε-Boc-α-FMOC-L-Lysine. The glutamic acid residue had to have the N-terminus of its backbone protected and the carboxylic acid function group on its side chain protected. The glutamic acid used was N-FMOC-L-Glutamic Acid-γ-t-Butyl ester. The tyrosine residue had to also have the N-terminus of its backbone protected

and the phenol group on its side chain. The tyrosine residue used was N-FMOC-O-t-Butyl-Tyrosine.

The actual synthesis of the peptides took place in the reaction flask which was fitted with a sintered glass filter, allowing easy removal of reagents and soluble side products by suction filtration.



The reaction flask was placed in a Burrel wrist action shaker to increase the reaction rate and to insure a high product yield.

Deprotection:

For the KE5, EK5, EK3.3, EK3.3a, and EK4.0 peptides the amount of resin used was 0.504g, app. 0.5g, app. 0.5g, 0.513g, and 0.509g respectively (Note: EK3.3a is a second synthesis of EK3.3 due to the low purity obtained for the

and the phenol group on its side chain. The tyrosine residue used was N-FMOC-O-t-Butyl-Tyrosine.

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EK3.3 peptide on the first synthesis). The resin was allowed to initially soak for 15min in 15ml of DMF in

order to expand, increasing the surface area of the resin. The FMOC protection group on the resin and on the N-terminus of the resin-peptide complex are removed by addition of app. 15ml of a 30% by volume Pip/DMF solution to the reaction flask, which is shaken for one minute. The solution is removed by suction filtration. A second 15ml portion of the 30% Pip/DMF solution is added to the reaction flask; however, the time of reaction is increased to 10min. After the Pip/DMF solution is removed for a second time the deprotected resin or resin-peptide complex is washed five consecutive times for one minute with 10-15ml of DMF, removing any excess reagents of soluble side products from the reaction flask. To insure complete deprotection, the 30% Pip/DMF solution was freshly prepared every four to five days.

Coupling Step:

In the coupling step an activated amino acid is added to the deprotected resin or deprotected resin-peptide complex. The amino acid is activated by dissolving a five fold mmol excess of the amino acid into the coupling solution, as shown in Table 1.

Table 1:

Amino Acid	Res. M.W.	M.W	mmols	gms of AA added
Lys(Boc)	128.2	468.6	0.975	0.4569
Tyr(tButyl)	163.2	459.5	0.975	0.4480
Glu(tButyl)	129.1	425.5	0.975	0.4149
Ala	71.1	311.3	0.975	0.3035

These concentrations of amino acids are based on 0.5g of resin being used. The mmol amount of each amino acid added is in a five fold excess to the

mmol of peptide that should theoretically be produced, assuming 100% reaction completion. The reagents are also added in a five fold mmol excess as shown in Table 2;

Table 2:

Reagent	M.W	Added Amount	mmol
BOP	442.5	0.431g	0.975
HOBt	135.1	0.132g	0.975
DIEA/DMF	129.25	5.0ml	0.975 of DIEA

The DIEA/DMF solution was made by adding 3.4ml of DIEA with 96.6ml of DMF, making a 1.95mM DIEA solution. The coupling mixture contained 5ml of the DIEA/DMF solution and 5ml of DMF. As a result, the coupling mixture had a 0.975mmol concentration of DIEA, which is a five fold excess of the theoretical amount of peptide being synthesized. The BOP reagent, HOBt reagent, and amino acid are dissolved in the mixture of 5ml of DIEA/DMF solution and 5ml of DMF for 3min, while being shaken. The coupling mixture is added to the resin or resin-peptide complex for approximately one hour; however, this time was usually lengthened when the first amino acid was coupled to the resin.

As with the deprotection step, in the coupling step excess coupling reagents, excess amino acid residues, and soluble side products are removed by five consecutive 15ml washings with DMF for 1min each. In order to insure the coupling reaction went to completion, a few milligrams of resin-peptide complex are removed from the reaction flask and tested for free amino groups by use of a Kaiser test (33). The tested resin-peptide complex is placed in a test tube with two drops of each of the three Kaiser test reagents (0.215M Ninhydrin in n-BuOH, 4.25M Phenol in n-BuOH, and 0.0002M KCN in Pyridine). The reaction took place in a test tube that was placed in a boiling hot water bath for five minutes. If the contents of the test tube had the

slightest blue tint the coupling step was repeated. A new coupling mixture of the amino acid plus the coupling reagents was prepared and allowed to react with the resin or resin-peptide complex for 30 to 45min. It is important to note that the resin or resin-peptide complex was **NOT** deprotected before the amino acid was added for a second time.

Capping Step:

The synthesis of the peptide chain completed by capping the N-terminus of the last added amino acid with an acetyl group. The capping step is similar to the coupling step in that the FMOC protection group on the resin-peptide complex must first be removed in the deprotection step. The difference between the coupling step and the capping step is the substitution of acetic anhydride for the amino acid. In addition, the HOBt and BOP reagents are not required, but their addition did not cause any obvious complications. The capping of the KE5, EK5, EK3.3, and EK4.0 peptides all had the HOBt and BOP reagents added in their capping steps. Acetic anhydride has a molecular weight of 102.09g/mol and a five fold mmol excess of 0.975mmol is wanted so 92µl of acetic anhydride was added to 10ml of DMF. The capping solution is shaken for three minutes and then added to the resin-peptide complex and allowed to react for one hour. The capping reagents are removed by five consecutive washing steps of 15ml of DMF for one minute each. The resin-peptide complex is then tested for free amino groups by using the Kaiser test.

Cleavage Step:

The resin-peptide complex is first washed five consecutive times with

15ml of DMF for one minute each. The resin-peptide complex is then dried completely in a vacuum desiccator over night. The KE5 peptide was cleaved by using a 95/5% by weight TFA/Phenol solution. Approximately 35ml in total of the TFA solution is needed for the cleavage step. The other four peptide chains were cleaved using a 90/5/5% by weight TFA/Anisole/Thioanisole solution. The total weight of the TFA solution was assumed to be 50g because this calculation prepared approximately 35ml of solution. The amounts added of each reagent is shown in Table 3.

Table 3:

Reagent	Volume(ml)
TFA	30.41
Anisole	2.51
Thioanisole	2.36

15ml of the cleavage mixture was added to the resin-peptide complex and stirred with a magnetic stir bar for two hours at room temperature in the hood.

After the two hours the resin support is removed from the solution by suction filtration through a glass sintered filter. The peptide chain, on the other hand is dissolved in the filtrate. The resin is washed three times with 5ml portions of the TFA solution to insure all of the peptide synthesized is transferred to the filtrate. The filtrate is then evaporated down to approximately 10ml on a rotovap with a dry-ice trap. The peptide is removed from the filtrate by the addition of 125ml of cold, dry ether. The ether was also a new unopened can of anhydrous ether that was stored in the refrigerator over night before use. The ether was added dropwise over about a five minute period. The precipitate formed almost immediately but was kept in the ether overnight in the refrigerator. The peptide precipitate was

kept in the ether overnight in the refrigerator. The peptide precipitate was removed from the ether solution by suction filtration through a glass sintered filter. The precipitate was washed by three portions of 5ml of cold, dry ether. The precipitate was than dried completely in a vacuum desiccator with a liquid nitrogen trap overnight. The product yields of the different peptides is shown in Table 4.

Table 4:

Peptide	Total Weight Collected(mg)	Theoretical Weight	Percent Yield
KE5	138	145	96
EK5	137	145	95
EK3.3	224	418	54
EK3.3a	390	418	93
EK4.0	360	418	87

Analysis of Peptide:

The presence of the KE5 and EK5 peptides were confirmed by using the Diode Array UV Spectrometer. The samples were dissolved in a 1% AcOH solution. The spectra of the peptides were compared to the spectra of pure L-Tyrosine sample. The spectra were taken over a wavelength range of 180nm to 350nm. The spectra taken of the KE5 peptide were taken at pH's of approximately 3, 7, and 10. The spectra of the EK5 peptide were taken at pH's of approximately 3, 7, and 12. In addition, the spectra of anisole, thioanisole, and phenol at these different pH's were taken. The presence of the peptide was confirmed by the similarity between how the peptide sample reacted to the different pH conditions and the pure L-Tyrosine sample reacted.

NMR Peak Assignments:

Peak assignments were made on non-purified samples of the EK5 and KE5 peptides by using Proton COSY spectra to determine the peaks on the 1D

Proton spectra. The NMR spectra were taken on a 200 MHz Varian Gemini NMR. The same experiments plus several other 2D experiments were done on non-purified samples of the EK3.3a and EK4.0 peptides. However, these spectra were taken on a 500MHz NMR at NYU.

Molecular Weight Confirmation:

The purity of the EK5 and KE5 peptides were assumed to be pretty good by the relatively clean NMR spectra that were obtained. The purity of the EK3.3, EK3.3a, and EK4.0 peptides were checked on an electrospray mass spectrometer at NYU.

Purification of Peptide:

The EK3.3 peptide was not purified due to its poor purity shown by its electrospray mass spectrum. The EK3.3a peptide was purified by using a gradient HPLC purification technique, conditions shown in Table 5.

Table 5: Conditions of Gradient HPLC Purification of EK3.3a Peptide

Time	Water%	Acetonitrile%
0	95	5
15	95	5
20	90	10
67	90	10
75	20	80
95	20	80
100	95	5

The water reservoir used contained 1% acetonitrile to kill any bacteria that might grow in the water and 0.1% TFA to maintain a slightly acidic environment for the peptide. The acetonitrile reservoir also contained 0.1%TFA to maintain a slightly acidic environment. The flow rate was set at 3ml/min. The column used was a semi-prep reverse phase C-8 column with

a 10 micron packing. The EK3.3a peptide was dissolved in ethanol at a concentration of 40mg/1 ml EtOH. The injection loop on the HPLC was increased to a volume of 200µl. The purification of the EK3.3a peptide was accomplished by 6 collections of 200µl injections. The UV detector used for detecting the peptide was set to 275nm. After each collection, the samples were collected individually and sealed in test tubes. The samples were put immediately
into the refrigerator for storage. The column was cleaned after each collection when the acetonitrile percentage was raised to 80% and allowed to remain there for 20min. The retention time of the EK3.3a peptide under these conditions was approximately 56min. All of the collections were combined and placed in a 500ml round bottom flask. The solvent was removed by evaporating off the solvent on a rotovap with a dry-ice trap. In addition, the evaporation of the solvent was aided by placing the sample flask in a 40°C water bath. The peptide was extracted from the flask using several 1ml portions of distilled water. The water was removed by freezing the sample in dry-ice and placing it in a vacuum desiccator being pumped through a liquid nitrogen trap overnight. The sample tube was wrapped in aluminum foil to prevent the loss of radiant heat. This purification method yielded a purified sample of 7mg, which is a percent yield of 14.6%.

The EK4.0 peptide was purified by using isocratic HPLC purification techniques. The water reservoir and acetonitrile reservoir are the same used in the purification of the EK3.3a peptide. The isocratic conditions of the purification runs were 90% water and 10% acetonitrile. The same flow rate, column and injection loop that were used in the purification of the EK3.3a peptide were used in the purification of the EK4.0 peptide. Originally, 75mg of

peptide was dissolved in approximately 15.5ml of 1% AcOH. The EK4.0 peptide did dissolve in approximately 2ml of 1% AcOH; however, the viscosity of the solution was that of maple syrup. Instead the EK4.0 peptide was dissolved in ethanol at a concentration of 20mg/1 ml EtOH. The retention time of the EK4.0 peptide was approximately 40 minutes. The purification of the EK4.0 peptide was accomplished by 10 collections of 200µl injections. The UV detector used for detecting the peptide was set to 275nm. After each collection, the samples were collected individually and sealed in test tubes. The samples were put immediately into the refrigerator for storage. After every
three collections the column was washed by running an 80% Acetonitrile, 20% water mixture through the column for thirty minutes. All of the collections were combined and placed in a 500ml round bottom flask. The removal of the solvent follow the same conditions used for the EK3.3a peptide. This purification method yielded a purified sample of 4.8mg, which is a percent yield of 12%.

Determining the Concentration of Peptide Solution for CD Measurements:

The concentration of the EK4.0 peptide solution was determined by taking absorption measurements on the Diode Array UV Spectrometer. The Spectrometer was set to take the absorption at 276nm, had an integration time of 3.0 seconds, and had a 0.5 second delay before taking the spectrum. A special cuvette was used that held only 1ml of solution. The peptide stock solution was prepared by dissolving the 4.8mg of purified peptide in 1ml of distilled water, making theoretically a roughly 2mM solution. In addition, a 50ml of a 7M Guanidine-HCl (QHCl) buffer solution was prepared. Before the

buffer solution was used it was filtered through a 2 micron filter. The blank consisted of 857µl of 7M QHCl and 123µl of distilled water. Following the scan of the blank, 20µl of the stock solution was added to the blank and mixed. The absorption of this solution was taken five times and later averaged. Next, an additional 20µl of stock solution was added to the blank and again the absorption of the solution was taken 5 times and averaged. This procedure was repeated three more times, but the results of the third measurements were not used in the calculation.

Table 6: UV Data for CD Prep

Measurement	Amount of Peptide	Absorbance	Concentration
1	20µl	0.0110	0.379mM
	40µl	0.0236	0.415mM
2	20µl	0.0110	0.379mM
	40µl	0.0229	0.403mM
3	20µl	0.0113	0.390mM
	40µl	0.0256	0.450mM
Average			**0.403mM**

Protocol for Peptide Synthesis

(starting with 0.5g FMOC-AA-Polymer 0.39mmol/g = 0.195mmol)
Fmoc-AA = _____ Sequence # = _____
Done by:_____ Date__/___/__

Wash I:
_____1. wash 100% DMF (15ml for 1 min. five times)a.___b.___c.___d.___e.___
_____1a. Kaiser test (safe to leave overnight).

Deblock:
____ 2. 30% Pip/DMF (15ml for 1 min, one time)
____ 3. 30% Pip/DMF (15ml for 10 min, one time)
Wash II:
____ 4. wash 100%DMF (15ml for 1min. 5 times) a. ___ b. ___ c.___ d.___ e.___
Coupling: Fmoc-AA = _____ Sequence # = _____
____ 5. 5eq. (0.975 mm) of BOP (0.431 g), HOBt (0.132 g), FMOC-AA (_____), weigh into flask
____ 6. Add to bottle 5 mL DIEA/DMF, 0.195 mm/mL (5 eq of DIEA) and 5 mL of DMF
____ 7. Swirl & dissolve for 3 mins
____ 8. Add solution to resin and let sit from 30-60min.
start time_____stop time_____

Repeat (go to **Wash I**, step 1)

Protocol for Peptide Cleavage:

Cleavage:

1. Wash and Dry resin
2. Add 15ml 95/5% TFA Phenol let sit with stirring for 2hr

Wash:

3. Filter TFA solution with vacuum filtration and stinted glass funnel into 500ml
   round bottom flask
4. Wash resin 3x 5ml 90/5/5% TFA/Anisole/Thioanisole mixture and filter TFA
   solution to rest of TFA solution

Evaporation:

5. Evaporate TFA solution down to ~10ml on rotovap with dry ice acetone trap

Precipitation:

6. Add drop wise a ~125ml of cold dry ether
7. Place in refrigerator over night
8. Filter precipitate from ether with sintered glass filter
9. Wash 500ml flask and precipitate 3x with cold ether
10. Remove precipitate and place in vacuum desiccator

Chapter 3-Results and Discussion:

Peptides Synthesized:

In this study, four structurally different peptides were synthesized. The structure of the peptide chains are as follows:

EK 5 PEPTIDE:
Ac-Y-E-A-A-K-NH_2

KE 5 PEPTIDE:
Ac-Y-K-A-A-E-NH_2

EK 3.3 PEPTIDE: (HYBRID BALDWIN PEPTIDE)
Ac-Y-E-A-A-K-A-A-E-A-A-A-K-A-A-E-A-A-K-NH_2

EK 4.0 PEPTIDE: (i, i+4 PEPTIDE)
Ac-Y-E-A-A-A-K-A-E-A-A-A-K-A-E-A-A-A-K-NH_2

Common to all four peptide structures is a tyrosine residue at position 1. The tyrosine residue contains an aromatic ring which serves as an UV marker for the peptide chain. The UV marker is important in the purification step of the peptide chain. The one problem the tyrosine residue introduces is the fact that guest-host studies have shown that tyrosine destabilizes helix formation(34). As a result, the tyrosine residue is placed at the very extreme of the N-terminus in hope of minimizing its destabilizing effect. In future studies, the tyrosine residue maybe moved one alanine residue away from the chain if it is determined that it is in fact destabilizing the helix. The four peptide structures also contain a high concentration of alanine residues. The first and most important reason for the high concentration of alanine residues is the fact that guest-host studies have shown that alanine stabilizes the formation of a helix(28). Secondly, the side chain of alanine consists entirely of one methyl group, which does not interfere with the ion-pairing

between the side chains of the lysine and glutamic acid residues.

In the hope of better stabilizing the helix, Baldwin and other researchers have made modifications to the peptide chains. The first modification is capping the N-terminus of the peptide by attaching an acetyl group. The acetyl group serves three purposes. The first is that it prevents the addition of unwanted amino acid residues to the N-terminus of the peptide during the cleavage step. The second reason is to prevent the protonation of the α-amine group, which would destabilize the helix by placing a positive charge at the positive net dipole moment of the helix. A third reason for the acetyl group is that it can act as a hydrogen bond acceptor, making the chain act as if it has more than 18 amino acids by further stabilizing the α-helix by additional hydrogen bonding. The second modification is the addition of an amine group to the C-terminus by synthesizing the amine form of the peptide. The amine group prevents the deprotonation of the carboxylic acid, which would destabilize the helix by placing a negative charge at the negative net dipole moment of the helix. In addition, the amine group can act as a hydrogen bond donor, stabilizing the α-helix through hydrogen bonding(29).

The two smaller synthesized peptides each contain five amino acids and are identical at positions 1,3, and 4. The difference between the EK5 and KE5 peptides is the order in which the lysine and glutamic acid residues appear in the peptide backbone sequence. In the EK5 peptide, the glutamic acid is at position 2 and the lysine is at position 5. In the KE5 peptide the lysine is at position 2 and the glutamic acid is at position 5. Studies have shown that the EK positioning stabilizes the helix better because it compliments the net dipole moment of the helix better. The two short

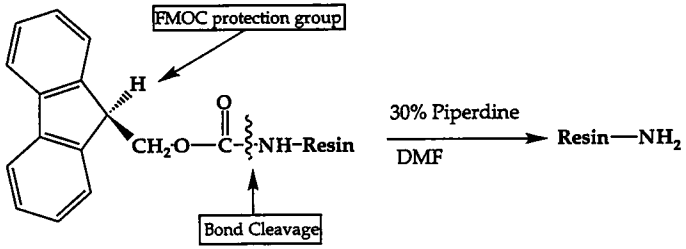
peptides were synthesized for two reasons. The first reason was to test our synthesis method for any complications. The second reason was to enable a system which the 200MHz NMR at Union College could be used to determine parameters for the computer modeling of the larger peptides by James Ernst. In addition, the NMR studies done on the small peptides was able to determine the assignments of many of the hydrogens in the peptide.

The EK4.0 peptide is analogous to the *(i, i+4)*EK Baldwin peptide. The EK4.0 peptide was synthesized so that the results of this study could be compared to the results of previously done studies on similar peptide structures. The EK3.3 peptide is designed to answer the questions of how the overall length of the peptide and heterogeneous spacing between the charged amino acids effects the overall helicity of the peptide. As a result, the EK3.3 and EK4.0 peptides both contain 18 amino acid residues, insuring the length of the peptide does not enhance the helix stability of one peptide over that of the other. Furthermore, the two peptides contain the same number of alanine, glutamic acid, and lysine residues. In the EK3.3 peptide the spacing between the first ion-pair group is of the *(i, i+3)* variety. The spacing between the second ion-pair group is of the *(i, i+4)* variety. The spacing between the third ion-pair group returns the *(i, i+3)* variety of the first ion-pair group. The reason why the spacing between charged groups is changed is based on X-ray data which shows that it takes approximately 3.6 residues to make one complete helical turn(35). As a result, it is thought that the EK3.3 peptide will be slightly under turned, then over turned, and then under turned again. The differences in the extent of turning should make the helix more compact and therefore more stable than the EK4.0 peptide.

Synthesis-Deprotection Step:

The first step in the synthesis is the deprotection of the resin. The FMOC protection group is removed by the addition of a 30% by volume Pip/DMF solution. Piperdine is a secondary amine base which is basic enough to cleave the FMOC group from the resin by the following pathway(31):

FMOC Deprotection Step:



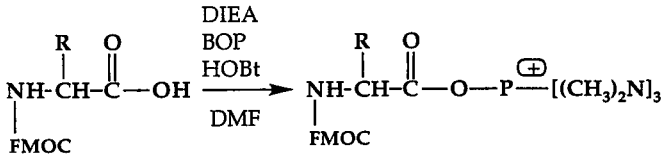
Important to note about the resin is its 0.39mmol/g substitution level. In this study, 0.5g of resin is used in each synthesis. As a result, there are 0.195mmol of free amine groups on the resin that can result in peptide chain growth. The amount of free amine groups on the resin determine the amount of reagents and amino acids that are allowed to react with the resin. Lastly, it is important to mention that the FMOC group leaves as a salt formed with the piperdine which is soluble in DMF and is easily removed by several washing with DMF and suction filtration. The deprotection of a previously coupled amino acid is analogous to the steps taken to deprotect the resin.

Activation Step:

Before the amino acid is coupled to the free amine group on the resin it

must be activated, by reaction with HOBt and BOP reagents in a 0.975mM DIEA/DMF solution. The activation pathway is as follows(31):

Activation of Amino Acid:

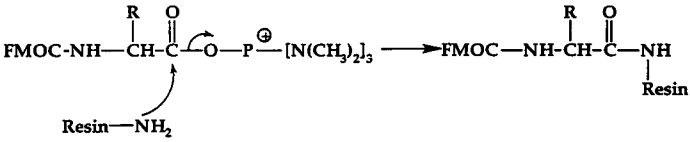


The amount of amino acid and reagents added is 0.975mmol, which is a five fold excess over the amount of free amine groups on the resin that are open to react. A five fold excess is used in order to insure the coupling step goes as close to 100% completion as possible. Amino acid and activation reagents are allowed to dissolve and react for three minutes while shaken on the Burrel wrist action shaker. The obvious question is why the amino acid must first be activated before being coupled to the resin or resin-peptide complex? The non-activated amino acid will couple but at a much slower rate, increasing the time needed for the addition of one amino acid. Nevertheless, the main reason the amino acid is activated is not to save time but to prevent the peptide chains being synthesized from being racemized.

Coupling Step:

The activated amino acid in the coupling mixture is added to the resin and allowed to react for approximately one hour. The coupling pathway is as follows(31):

Coupling Reaction Step:



In order to insure complete reaction the reaction flask is shaken to agitate the resin, increasing the reaction area of the resin. It is important to note the amine base uses in DIEA and not piperdine. DIEA is used because it is a tertiary amine base that is not basic enough to remove the FMOC protection group from the amino acid being coupled to the resin or resin-peptide complex. However, if the time needed for the coupling step had to be increased because the amino acid had not been previously activated a greater percentage of amino acids being coupled may lose their FMOC protection groups synthesizing peptide chains that contain the wrong number of amino acids, which could greatly effect the peptide's ability to form a stable helix. Excess reagents and amino acids are removed by several washing with DMF and suction filtration.

Kaiser Test:

The extent of the coupling step is monitored by using a Kaiser test, which tests for free amine groups that exist on the resin or resin-peptide complex(33). If free amine groups in very low concentration exist on the resin or resin-peptide complex the test solution will turn a blue color. If the result is a blue color the coupling step is repeated. However, the resin or resin-peptide complex must not be deprotected if the coupling step is repeated. If the coupling step is repeated after the resin-peptide complex is

deprotected the amino acid will wrongly couple with the previously coupled amino acids, producing peptides with the wrong number of amino acid residues. The Kaiser test insures that the coupling step goes to nearly 100% completion, insuring a good product yield. Nevertheless, the Kaiser test destroys the portion of the resin or resin-peptide complex tested so the percent yield is slightly lowered even if every coupling step went to 100% completion.

The importance of the Kaiser test was seen in the synthesis of both the EK5 and EK3.3 peptides. The EK5 peptide was originally based on a synthesis method that called for the use of 1.0g of resin. However, the Kaiser test showed that the reaction area of the resin was too small to allow an acceptable product yield, because the Kaiser test indicated incomplete reaction. As a result, the amount of resin used in synthesis of the larger peptides was kept at 0.5g. Future studies could try using a reaction flask with a larger diameter which would spread the resin out more, increasing the reaction area of the resin. In addition, the rate at which the reaction flask is agitated and the amount of reagents used could be increased, but these changes did not work with the reaction flask used in this study. It was later determined during the synthesis of EK3.3 peptide that the slightest blue color demands that the coupling step be repeated, due to the low purity of the first EK3.3 peptide synthesized.

Capping Step:

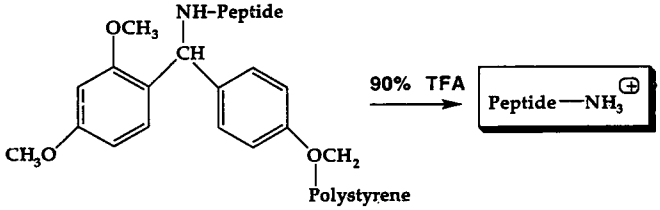
Before the amino acid is cleaved from the resin support, the tyrosine residue which is the last amino acid added to the peptide is capped with an acetyl group. The capping step is analogous to the coupling step. The

differences between the two steps are that acetic anhydride is used instead of an amino acid and the acetic anhydride cannot cause racemization, meaning it does not need to be activated by the addition of HOBt and BOP reagents. However, the synthesis of the EK5, KE5, EK4.0 peptides included the use of HOBt and BOP reagents in their capping steps with no obvious problems occurring.

Cleavage Step:

The cleavage step in this study serves two purposes. The first is to remove the peptide chain from the resin support. The second is to remove the t-Butyl derived protection groups on the lysine, tyrosine, and glutamic acid side chains. The peptide is cleaved from the resin by placing the resin-peptide complex in 15ml of TFA and allowed to react for two hours. The pathway of the cleavage step is as follows(36):

Cleavage of Peptide from Resin Support:



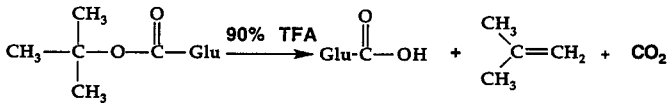
The actual synthetic method used for the cleavage of the KE5 peptide is slightly different from the protocol used on the other peptides. The cleavage mixture used with the KE5 peptide contained 5% phenol and 95% TFA. The other cleavage mixtures used on the other peptides contained 5% anisole, 5%

thioanisole, and 90% TFA. The phenol, anisole, and thioanisole act as ion and free radical scavengers. Without the scavengers the ions and free radicals produced as side products of the cleavage step could interfere with the cleavage or attack the peptide at the tyrosine residue. The use of TFA is important because the form of the peptide that is isolated is the mono trifluoroacetic salts for the EK5 and KE5 peptides and the tri trifluoroacetic salts for the EK3.3 and EK4.0 peptides.

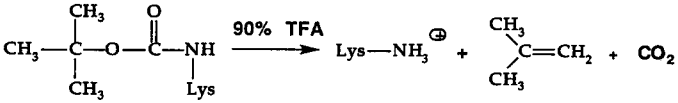
In addition, the cleavage step also deprotects the functional groups found on the side chains of the lysine, tyrosine, and glutamic acid residues. The deprotection of these side chains is accomplished by the following pathways:

Cleavage of Side Chain Protection Groups:

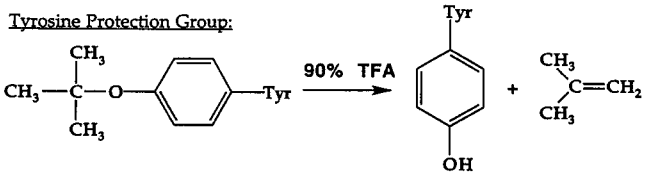
Glutamic Acid Protection Group:



Lysine Protection Group:



Tyrosine Protection Group:



The side chains of these amino acids are protected during the synthesis

because their t-Butyl derived protection groups are labile only under acidic condition, while the synthesis to this point has been carried out under basic conditions. The side chains are protected in order to prevent amino acids from coupling to them during the synthesis producing a branched peptide chain.

The percent yield of each peptide is shown below:

Percent Yields of each Peptide:

Peptide	Total Weight Recovered	Percent Yield
EK5	137mg	95%
KE5	138mg	96%
EK3.3	224mg	54%
EK3.3a	390mg	93%
EK4.0	360mg	87%

The low percent yield of the EK3.3 peptide seems to indicate that several different peptides of lower weight were synthesized instead of the one 18 amino acid long chain. This hypothesis is later confirmed by analysis with electrospray MS.

Confirmation of Shorter Peptides:

The unique UV spectrum of the aromatic ring of the Tyrosine residue was used to confirm the presence of the peptide. The UV spectra of the KE5 peptide at pH's of 3,6, and 10 from 190nm to 350nm were compared to the spectra of monomer tyrosine acid, phenol acid, and phenol neutralized, shown in Figure 1 of the appendix. The spectra confirmed that the isolated product from the cleavage contained a tyrosine residue and was not caused by the collection of phenol from the cleavage step. However, this technique did not confirm the presence of the entire peptide chain or the purity of the sample. These questions were answered by NMR analysis done by James

Ernst(37).

The UV spectra of the EK5 peptide at pH's of 3, 6, and 12 from 190nm to 350nm were compared to the spectra of monomer tyrosine, thioanisole, and anisole, shown in Figure 2 of the appendix. The spectra confirmed that the isolated product from the cleavage contained a tyrosine ring and not the cation scavengers from the cleavage step. The peptide sequence and relative purity were determined as with the KE5 peptide by NMR analysis by James Ernst(37).

Molecular Weight Confirmation of Larger Peptides:

The more complex EK3.3, EK3.3a, and EK4.0 peptides could not be confirmed using the UV detection system used with the shorter peptides. Instead, the relative purity of the larger peptides was determined by analysis by electrospray mass spectrometry. This technique is a non-fragmenting procedure in which the cationic form of the sample is analyzed. As a result, the mono, di, and tri-protonated form of the peptide are detected. The determination of the peptide's molecular weight must take into consideration the use of trifluoroacetic acid in the cleavage step because the tri-trifluoroacetic salts were the isolated product from the cleavage. As a result, the neutral form of the peptide has a molecular weight of 1776. The mono-protonated form of the peptide has a molecular weight of [(1776+1)/1]=1777. The di-protonated form of the peptide has a molecular weight of [(1776+2)/2]=889. The tri-protonated form of the peptide has a molecular weight of [(1776+3)/3]=593.

The electrospray mass spectrum of the EK3.3 peptide, Figure 3 of the appendix, shows multiple peak of relatively the same intensity from a

molecular weight of 500 to 900, indicating that multiple lower weight peptides were synthesized. As a result, the purity of this sample is fairly low. As a consequence of the low purity of the EK3.3 peptide it was discarded and resynthesized under the name EK3.3a. In order to insure that the purity of the EK4.0 or EK3.3a peptides would be of an acceptable level, it was decided that the activating and coupling reagents be remade every four to five days to prevent their decomposition. In addition, the slightest blue color given by the Kaiser test demanded that the amino acid be recoupled before the synthesis was continued.

The electrospray mass spectrum of the EK3.3a peptide, Figure 4 of the appendix, confirmed a relatively high purity of the wanted material. This spectrum showed the unique pattern expected for the peptide. The two most intense peaks are found at molecular weights of approximately 889 and 590, which represent the di and tri-protonated forms of the peptide respectively. The spectrum illustrates that the most favored form the peptide when ionized is the di-protonated form. However, the spectrum also shows peaks that correspond to the molecular weights of peptides that contain one to many, one to few, or two to few alanine residues; however, these peaks represent a small impurity in the sample. The appearance of these peaks is not disturbing because the majority of the peptide consists of alanine residues and the small impurity they cause can easily be removed by HPLC purification.

The electrospray mass spectrum of the EK4.0 peptide, Figure 5 of the appendix, also showed the unique pattern that would be expected for this peptide. The characteristic peaks are at molecular weights of approximately 890 and 590, which correspond to the di and tri-protonated forms of the

peptide. Again the spectra shows the peaks that correspond to the impurity of peptides that contain one to many, one to few, or two to few alanine residues. However, this impurity like the impurity of the EK3.3a peptide should be easily removed by HPLC purification.

HPLC Purification of EK3.3a and EK4.0 Peptides:

The impurities were removed from the peptides by their different retention times on a HPLC column. The peptides had to be purified so CD measurements could be taken, even though, the electrospray mass spectra of the peptides showed that the samples were relatively pure. In order to make the number of collections as low as possible that would give 20mg of material, a few modifications were made to the HPLC. The injection loop was increased from a 20µl volume to a 200µl volume. The column also had to be increased from an analytical column with a 3 micron packing to a semi-prep column with a 10 micron packing. The water and acetonitrile reservoirs were poisoned with 0.1% TFA for two reasons. The first was to keep the peptide in an acidic environment, keeping its retention time constant. In addition, the TFA acts like a poison killing any bacteria that might try to live in the water reservoirs.

The HPLC purification technique was first tried on the EK4.0 peptide. Initially, 75mg of peptide was dissolved in 2.0ml of 1% acetic acid (AcOH); however, the result was a solution with a viscosity of maple syrup that could not go through the HPLC. As a result, the solution had to be diluted with 1% AcOH to a total volume of 15.5ml. Originally, it was thought that the high viscosity of the peptide was due to the peptide's helical nature in solution. However, it is now thought that the high viscosity of the solution was a result

of aggregation between peptide chains.

The dilution of the peptide stock solution to such low concentration caused several problems. The first problem encountered was the small amount of material that could be collected from each HPLC run, increasing the number of collections needed. The second problem the low concentration of peptide caused was that its peak could not be determined from the chromatograph, as shown in Figure 6 of the appendix. Electrospray mass spectrometry was used to determine which of the two most likely peaks was the peptide. Figure 7 shows that only the electrospray mass spectrum of peak two correlates to the characteristic spectrum of the EK4.0 peptide. In order to solve the dilution problem, the solvent used to dilute the peptide was changed to ethanol. This solvent allowed 20mg of peptide to be dissolved in 1ml of ethanol, at this concentration the peptide peak was obvious and easily collected as seen by Figure 8 of the appendix. The solvent 2,2,2-trifluoroethanol was also tried, but the much higher cost of this solvent suggested the use of ethanol, since it worked equally as well.

The EK3.3a peptide was also dissolved in ethanol but at a concentration of 40mg/1ml of EtOH, decreasing the number of collections needed in half. The chromatograph of this collection is Figure 9 of the appendix.

The retention time differences seen between the chromatagraph of the EK4.0 peptide that was dissolved in 1% AcOH and the EK4.0 peptide that was dissolved in ethanol indicates a problem. The cause for the difference in retention times was caused by the filter in the water reservoir being clogged, decreasing the percentage of water that goes through the column. As a result, the composition of the mobile phase was really more non-polar than expected, decreasing the retention time on the column for the peptide.

Removal of HPLC Mobile Phase from Peptides:

Following the purification of the peptide by HPLC, the peptide had to be isolated from the mobile phase. The majority of the mobile phase was removed from the EK4.0 and EK3.3a peptides by using a rotovap with a dry-ice trap. The precipitate left in the flask was extracted with several milliliters of distilled water. All moisture was removed from the peptide by subliming the sample in a vacuum desiccator with a liquid nitrogen trap. The amount of purified EK3.3a and EK4.0 peptide isolated was 7.0mg and 4.8mg respectively, giving percent yields for the purification step of 12% and 4.3% respectively.

UV Concentration Determination for CD Measurements:

The amounts of material isolated after the purification were much lower than expected; although, the CD measurements only need 4mg of material for accurate measurements to be taken. However, the UV measurements at 276nm determined that the concentration of the stock peptide solution contained 0.4mmol of material. The needed concentration of the solution had to contain 2.0mM of material that could be accurately diluted to 0.5mM by addition to a KF buffer; however the stock solution already was under the 0.5mM concentration. As a result of this experiment, it is shown that the original stock solution must be made much more concentrated and that the addition of more material is needed before CD measurements can be taken. Time restraints prohibit the CD prep work from being done in this study and therefore will have to wait for a future study to be completed.

Conclusion:

Unfortunately, the needed CD measurements could not be taken at this time, meaning the questions this study intended to answer cannot be answered at this present time. However, this study did show that the synthetic method used produces high yields of the needed peptides. In addition, the conditions needed for the purification and later techniques were worked out so the CD measurements can be done in the future.

Future Work:

Further purification by collections of the EK3.3a and EK4.0 peptides on the HPLC are needed before samples for CD measurements can be made. In addition, other peptide derivatives of the Baldwin peptides are to be synthesized and analyzed. The future peptides might have the following configurations:

(i+4, i+3, i+4); (i+3, i+3, i+4); and/or (i+4, i+3, i+3).

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Appendix of Figures:

Figure 1-UV Confirmation of KE5 Peptide:

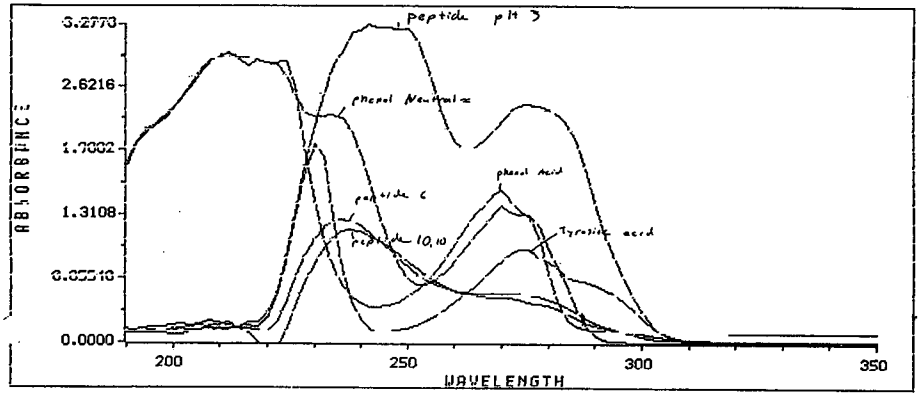
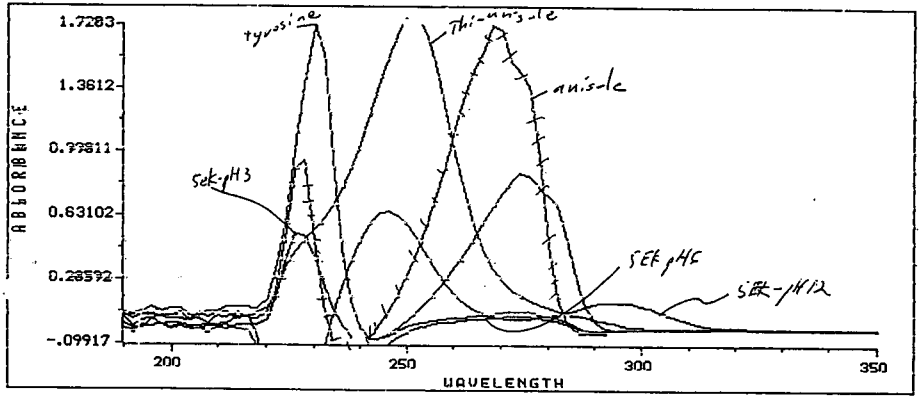


Figure 2-UV Confirmation of EK5 Peptide:



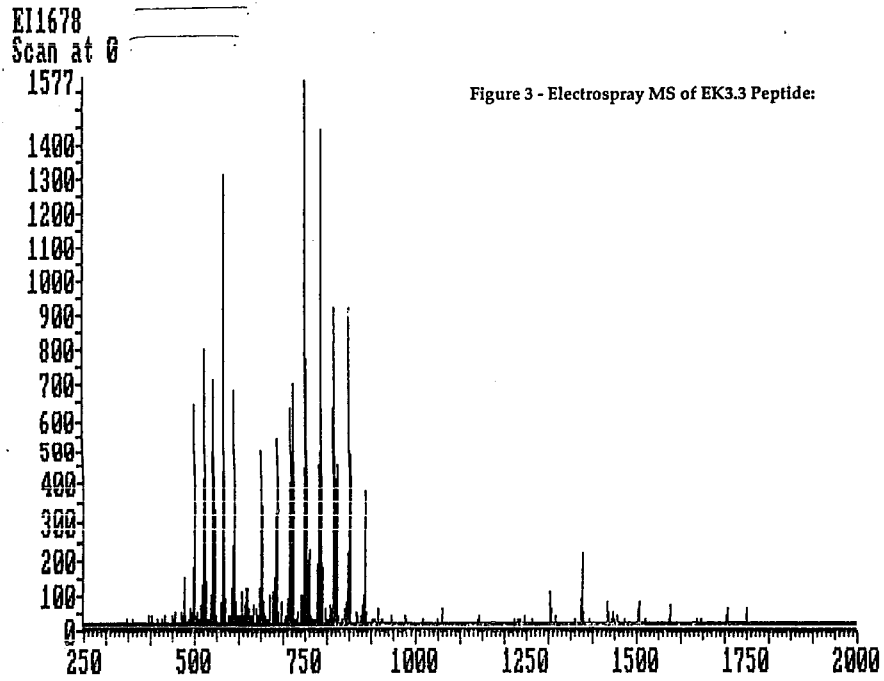


Figure 3 - Electrospray MS of EK3.3 Peptide:

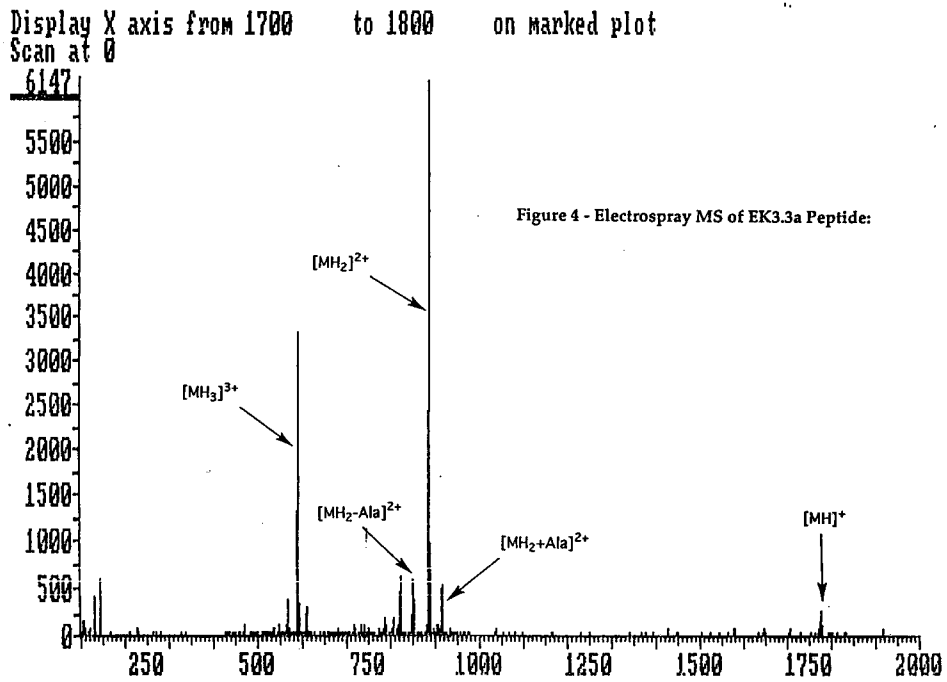
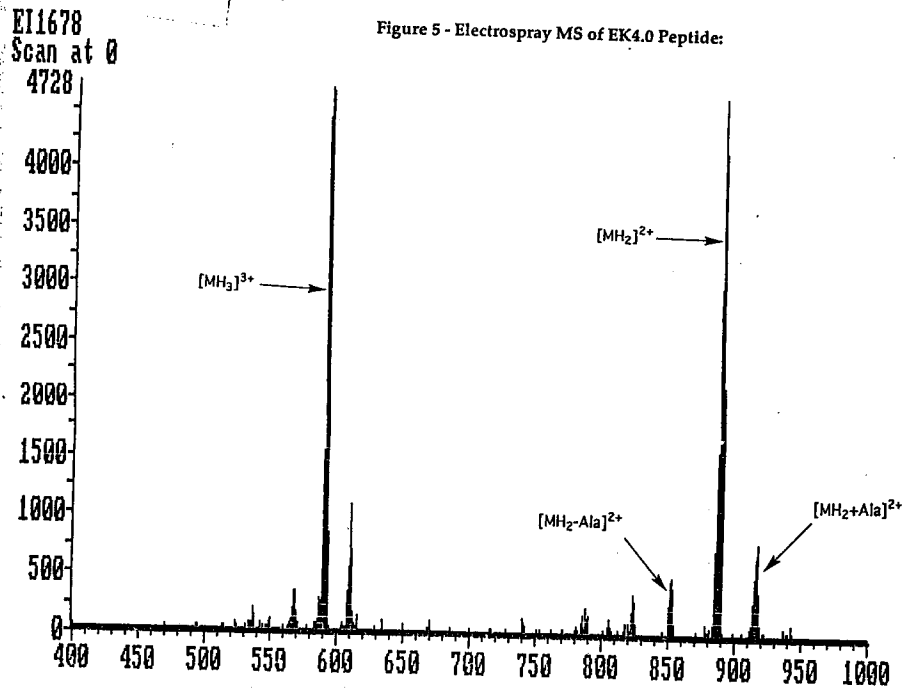


Figure 4 - Electrospray MS of EK3.3a Peptide:

Figure 5 - Electrospray MS of EK4.0 Peptide:



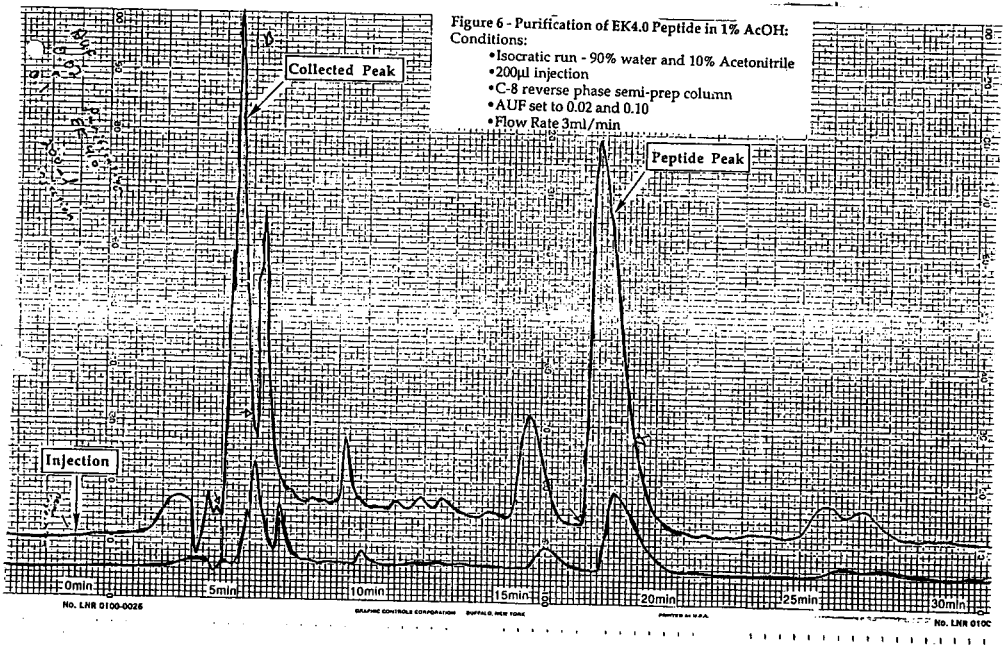


Figure 6 - Purification of EK4.0 Peptide in 1% AcOH:
Conditions:

- Isocratic run - 90% water and 10% Acetonitrile
- 200µl injection
- C-8 reverse phase semi-prep column
- AUF set to 0.02 and 0.10
- Flow Rate 3ml/min

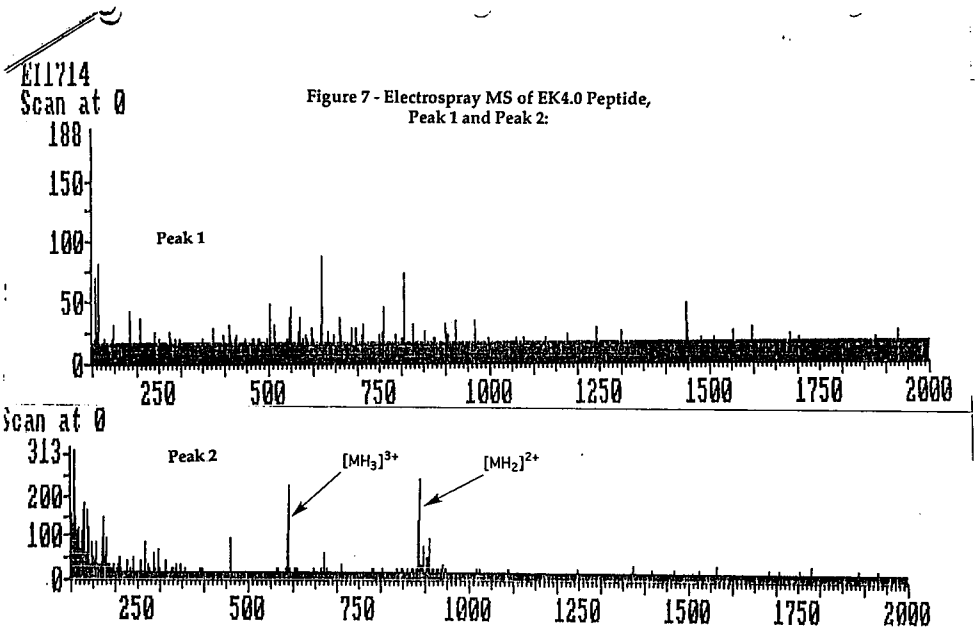


Figure 7 - Electrospray MS of EK4.0 Peptide, Peak 1 and Peak 2:

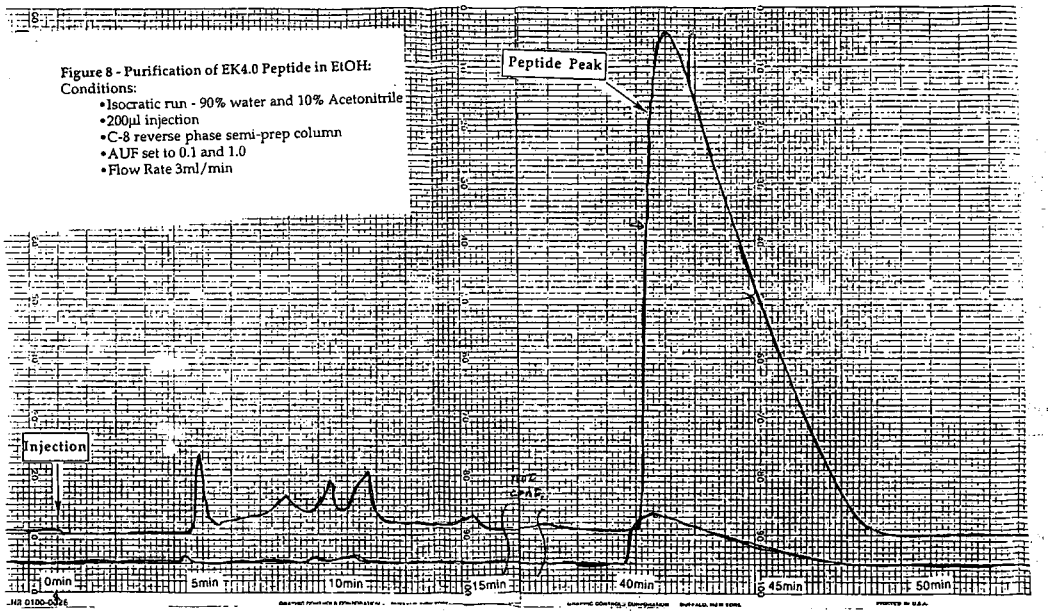


Figure 8 - Purification of EK4.0 Peptide in EtOH:
Conditions:

- Isocratic run - 90% water and 10% Acetonitrile
- 200µl injection
- C-8 reverse phase semi-prep column
- AUF set to 0.1 and 1.0
- Flow Rate 3ml/min

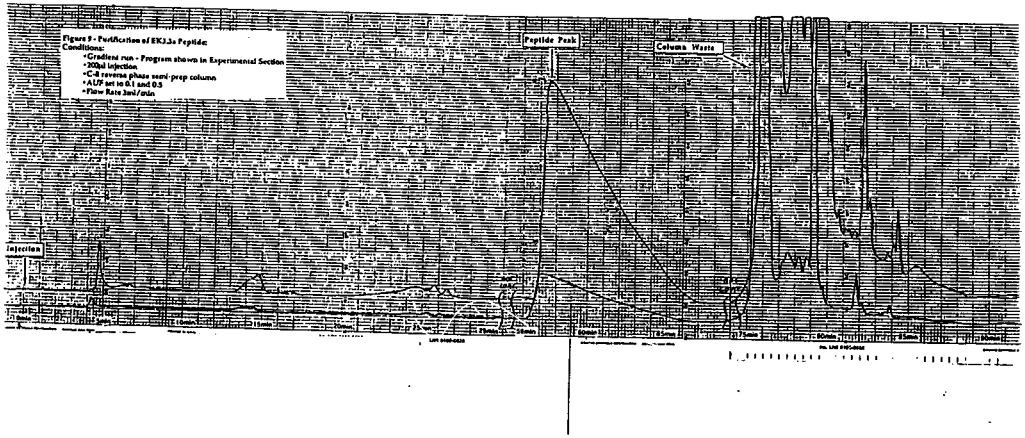


Figure 9 - Purification of EKL3a Peptide:
Conditions:
•Gradient run - Program shown in Experimental Section
•200µl injection
•C-8 reverse phase semi-prep column
•AUF set to 0.1 and 0.5
•Flow Rate 3ml/min
Peptide Peak
Column Waste
Injection