Investigation of Peptide-DNA Interactions: Synthesis, Characterization and Applications of Lysyl-Fluorophenylalaninamides

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INVESTIGATION OF PEPTIDE-DNA INTERACTIONS:
SYNTHESIS, CHARACTERIZATION AND APPLICATION OF
LYSYL-FLUOROPHENYLALANINAMIDES

by
Teresita T. Ortega

DISSERTATION

Submitted to the Department of Chemistry
of Seton Hall University in partial fulfillment of the requirements for
the degree of Doctor of Philosophy.

December 1998

Mentor: Dr. Richard D. Sheardy
We certify that we have read this thesis and that in our opinion it is adequate in scientific scope and quality as a dissertation for the degree of Doctor of Philosophy.

APPROVED

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Richard D. Sheardy, Ph. D.
Chairman, Department of Chemistry
DEDICATION

To my parents Vivencio and Norma

Sa Aking Mga Magulang - Vivencio at Norma


Nagmamahal at nagpapasalamat,
Marites
ACKNOWLEDGMENTS

The following people have contributed either their faith, time, energy, vision, passion, support or friendship in an important and appreciated way!

Firstly, I would like to thank my mentor Dr. Richard Sheardy for his patience, expertise and guidance. He allowed me great freedom to pursue my research interests. Dr. Sheardy also allowed time for group get-togethers outside his lab (i.e. annual BioPhysics meetings in Baltimore and New Orleans, summer barbecues at the Sheardy homestead), which undeniably enhanced everyone's graduate years at Seton Hall. He and my classmates---Andy Anantha, Dave Calderone, Enrique DiLone, Jennifer Liang, Steve Marotta, and Ben Otokiti formed a unique cohesive research team. Mahrukh Azam, Bitak Bassiri, Rosemary Marques, Tony Paiva and Bryan Vi have continued the same camaraderie. Many a time, we exchanged ideas, shared our woes of graduate research and laughed at our struggles together. I thank all of them for their friendship. I would also like to extend my appreciation to my readers Dr. James Hanson and Dr. Daniel Huchital for their ideas and helpful suggestions. They were able to show me how to look beyond the scope of the biochemist's perspective and find the clarity I needed for the intelligible expression of my work. I am also grateful to Dr. Matthew Petersheim and Dr. John Sowa for their concern and kind advice on my research.

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ABSTRACT

INVESTIGATION OF PEPTIDE-DNA INTERACTIONS:
SYNTHESIS, CHARACTERIZATION, AND APPLICATION OF
LYSYL-FLUOROPHENYLALANINAMIDES

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Our investigation centered on finding an alternate approach for analyzing the precise molecular nature of the localized contacts between peptides and DNA. We first looked at two diastereomeric dipeptide amides L-lysyl-L-phenylalaninamide and L-lysyl-D-phenylalaninamide in thermal denaturation studies with Dickerson’s dodecamer (D12-mer) 5’-CGCGAATTCGCG-3’. The melting temperature (T_m) represents the temperature at which the fraction (α) of single strands is equal to 0.5. This temperature value serves as a measure of the stability of the DNA duplex. The higher melting temperatures reflect the persistence of double-stranded helical structure for a given sequence. No notable differences in the oligomer’s melting temperature were seen in the presence of these dipeptide amides even under low salt conditions. Polynucleotides poly(dA-dT)·(dA-dT), poly(dA)·(dT), and poly(dA-dC)·(dG-dT) were thus selected as DNA models for the denaturation studies. The significant increase in the T_m values for the double-stranded heteropolymers and homopolymer in the presence of the dipeptide amides indicated DNA stabilization.

We then synthesized four monofluorinated analogues of the dipeptide amides above in hopes of using these compounds to serve as labeled peptides in future F NMR work. The fluorinated peptides L-lysyl-p-fluoro-LD-phenylalaninamide and L-lysyl-o-fluoro-LD-phenylalaninamide were characterized by UV spectroscopy, NMR, and circular dichroism (CD). Experimental results from UV, CD and thermal denaturation studies are presented to assess the feasibility of these fluoropeptide derivatives as models for our investigation of peptide-DNA interactions. Thermal denaturation experiments under increasing sodium chloride concentration were also completed to evaluate the electrostatic contributions to the binding of these peptides to DNA. Our data suggests that the fluoropeptides do bind in a similar manner as the L-lysyl-phenylalaninamides, although the binding is slightly diminished. Both thermal denaturation results and preliminary molecular modeling also support that the chirality of the phenylalanine ring dictates the binding of the peptide to DNA.
CHAPTER I
INTRODUCTION

Since cancer is characterized by rapid cell proliferation due to the breakdown of the control mechanism governing DNA replication and transcription, a major focus of current research involves understanding the simple interactions responsible for recognizing and binding of genes by transcription proteins and DNA polymerases. These interactions may include electrostatic interactions, van der Waals, hydrogen bonding and hydrophobic interactions. Because several forces are operating at many sites along the DNA, it is complicated to analyze actual protein-DNA complexes. Major obstacles include their isolation from cells and the preservation of the structural and functional integrity of these complexes for subsequent study. Therefore, some researchers have turned to oligopeptide models for their investigations. From such knowledge, scientists may be able to design DNA-binding ligands that can serve as potential inhibitors of proteins that catalyze the initiation and termination of polynucleotide synthesis. Antibiotics and some oncological drugs react in this fashion by decreasing DNA's ability to unwind and prepare itself for replication and transcription. Examples of antibiotics include Netropsin, Distamycin, and Actinomycin D (Figure 1). Netropsin and Distamycin, known minor groove binders, recognize AT-rich regions via a combination of hydrophobic interactions, hydrogen bonding and electrostatic interactions (Wartell et al., 1974). These cationic drugs not only take part in electrostatic interactions with the polyanionic DNA, but form close ligand-DNA van der Waals contacts and hydrogen bonds in the minor groove (Bailly and Chaires, 1998). Actinomycin D consists of two
major binding components. The pentapeptide lactone rings bind to either side of the DNA helix, while the aromatic phenoxazone moiety inserts between a base pair and binds by hydrophobic interactions. The antibiotic actually covers four to six base pairs along the DNA helix preventing chain elongation during transcription (Muller and Crothers, 1968). The termination of both replication and transcription by antibiotics thus inhibits the generation of more bacterial cells or spread of infection. Anticancer drugs work by similar mechanisms to prevent the rampant growth of cells. Naturally, one significant pursuit for scientists is the discovery and design of DNA-binding compounds that halt either the progression of infection or growth of cancer. Peter Dervan and his colleagues have had promising success in designing polyamides specific for the recognition and binding to the minor groove of DNA (Mrksich, et. al., 1994). Situated as an antiparallel hairpin dimer, their hexapeptide binds to each strand in the minor groove of DNA by hydrogen bonding. The pyrrole, imidazole, and hydroxypyrrole aromatic structures of the polyamide dimer allow special recognition of A•T or T•A base pairs in the minor groove through the lone electron pairs of N3 of adenine (A) and O2 of thymine (T) (Keilkopf, et. al., 1998).
Figure 1: Examples of antibiotics with peptide moieties. Netropsin and Distamycin recognize A-T-base pairs in the minor groove of DNA, while Actinomycin D intercalates between DNA base pairs.
Certain physical features contribute to the uniqueness of DNA. The most common form of DNA found in nature is B-DNA which has two strands that run antiparallel to each other (Figure 2). Each strand consists of a sugar-phosphate backbone with nitrogenous bases attached. The bases are either the purines adenine (A) and guanine (G) or the pyrimidines thymine (T) and cytosine (C). The secondary conformation of double-stranded B-DNA is right-handed helical with internal base pairings (Watson and Crick, 1953). The configuration of the 2'-deoxyriboses is a C2'-endo sugar pucker, specifically, the C2' carbon lies above the pyranose ring as does the base and the C5' carbon. The glycosidic bond between the sugar and base is N9 - C1' for purines and N1 - C1' for pyrimidines. As a result of twists and turns in the helix, two distinct grooves exist in B-DNA—a wider major groove and a narrower minor groove. Although these grooves are of approximately equal depth (8 Å), the minor groove has a width of only 6.0 Å or half the width of the major groove. The helical pitch which describes the spacing between base residues is 3.4 Å. The homogenous, linear array of sugar-phosphates gives rise to a relatively high helical charge distribution due to the two phosphates for every 3 to 4 Å, resulting in a surface charge density of two phosphates per 210 Å². This high charge density provides the molecular basis for non-specific cationic binding via electrostatic interactions. As a long polyanion in solution, DNA has cations associated with it. These counterions (i.e. sodium ions, Na⁺) "condense" along the highly negative backbone (Manning,
The purine-pyrimidine aromatic base pairs are held together by hydrogen bonding. Adenine (A) complements thymine (T); A-T base pairs are bound together with two hydrogen bonds. Guanine (G) complements cytosine (C); G-C base pairs are bound by three hydrogen bonds. Each hydrogen bond is approximately 2.85 Å from the donor atom to acceptor atom. The geometries of A-T and G-C base pairs from points of attachment to the sugars (C1'-C1' distance) are essentially identical at 10.85 Å, hence the double-helix conformation can accommodate all base pair combinations A•T, T•A, G•C, C•G without distortion or loss of symmetry in the helix (Figure 3). For B-DNA, there is an average of ten base pairs per helical twist. Although the purines and pyrimidines are somewhat polar (2.5-7.0 debyes), they are by and large hydrophobic and thus internalized in the helical structure of DNA. The collective strength of the base stacking and hydrogen bonding contributes to the resilience of DNA. The hydrophobic interactions are enhanced by the neighboring base pairs. The distance between base pairs is 3.5 Å, which is equal to the van der Waals radius of planar aromatic compounds and the minimal distance of approach before repulsive nonbonded interactions begin to occur (Cantor and Schimmel, 1980). The edges of the Watson-Crick base pairs contain nitrogen (i.e. N7 of purines and N3 of adenine) and oxygen (i.e. O2 of thymine) atoms that can make hydrogen bonds to protein side chains, water and other molecules. Such structural characteristics can be described as a "lattice of binding sites" (McGhee and von Hippel, 1974).
Figure 2: Structure of B-DNA. (HyperChem Molecular Modeling v. 4.5)
MAJOR GROOVE

Figure 3: Watson-Crick Base Pairing in DNA: G-C and A-T base pairs. Note the potential binding sites in the major (N7 of purines) and minor (N3 of adenine and O2 of thymine) grooves.
LIGAND BINDING

There are three distinguishable binding modes of small molecules and proteins to DNA: external binding to the sugar-phosphate backbone, internal binding to either major or minor grooves of DNA and intercalation. Electrostatic interactions primarily rest on the sugar-phosphate backbone. As previously noted, DNA is a long polyelectrolyte that has many counterions tightly associated with its negatively charged backbone. When basic (i.e. -NH$_3^+$ side chain) amino acids from a peptide or protein bind to DNA, these counterions are displaced and released into the bulk solution or buffer. Amino acid residues arginine and lysine bind and neutralize the phosphates at the site of binding. This binding is non-specific, unlike the binding afforded by hydrogen bonding in the grooves or intercalation between base pairs.

Apart from the steric reasons that certain ligands bind in the major or minor groove, the accessibility of certain hydrogen bond donors and acceptors contributes to the differentiation of recognition sites (Figure 3). Atoms such as nitrogen and oxygen which are strongly electronegative can interact through their lone pairs with protons and bind. This hydrogen bond is electrostatic in nature and is a result of attractive interactions (Stigter and Dill, 1996). In the major groove, the N7 of purines adenine and guanine and the amino group of C4 of cytosine are likely participants in hydrogen bonding. The minor groove has both an N3 of adenine and a C2 carbonyl oxygen atom of thymine accessible for hydrogen bonding. The amino group of C2 in guanine limits the accessibility of N3 of guanine in the minor groove, yet at the same time provides another possible donor for hydrogen bonding. Hence, the trend of AT
preference in the minor groove, while large ligands prefer GC sites of the major groove. For thymine, the presence of a methyl group (–CH₃) in the major groove also contributes to the recognition of sites by ligands. It introduces asymmetry in the major groove, allowing the differentiation between A•T and T•A base pairs. C•G and G•C base pairs should also be distinguishable; however, this is due to the different pattern available for hydrogen bonding and polarization of the base pairs.

Intercalation is the accepted mechanism for the reversible noncovalent binding of compounds with planar aromatic rings as facilitated by hydrophobic interactions (Lerman, 1961). Aromatic amino acid residues like phenylalanine, tyrosine and tryptophan have been shown to engage in stacking interactions with nucleic acid bases. As previously noted, the aromatic phenoxyazone of the antibiotic Actinomycin D inserts itself between base pairs of DNA in a “cardstack fashion” (Cantor and Schimmel, 1980). This insertion or intercalation inhibits the unwinding of helical DNA. Another form of binding is partial intercalation or "non-classical" intercalation first proposed by Brown (1970). It was further defined as “bookmark selection” of DNA binding by Gabbay and his colleagues (Gabbay, et. al., 1973). This type of ligand binding induces bending of the DNA, a common structural distortion measurable by viscometric studies. The effective length of the DNA is shortened thereby causing a decrease in its viscosity. This differs from full intercalation by a ligand where its insertion results in the lengthening of DNA and thus increases the viscosity of DNA. Hydrogen bonding allows the discrimination of certain base sequences or recognition sites, so that intercalation issues a GC preference.
Consequently, charge separation, steric bulk, and hydrophobicity are parameters considered in the investigation of peptide-DNA interactions and by inference, protein-DNA interactions.
Most studies of peptide-DNA interactions have focused on the precise molecular nature of the contacts between peptides and DNA. The first study to combine oligopeptides and DNA for the investigation of DNA recognition involved NMR to determine whether the amino acid residues interact directly with the bases of the nucleic acids (Helene and Dimicoli, 1972). To increase the affinity of the oligopeptides for DNA, the aromatic residues were covalently linked to the basic amino acid lysine (i.e. Lys-Phe, Lys-Trp, Lys-Tyr) to enhance electrostatic interactions. The charge of lysine under physiological conditions of pH 7 is +2 due to the α-amino (pKₐ = 8.95) and ε-amino (pKₐ=10.53) groups. It was shown that stabilization of these complexes through electrostatic interactions involves positively charged lysines and negatively charged adjacent phosphates on the DNA backbone. A series of dipeptides and tripeptides with aromatic residues linked to lysine were also analyzed by NMR and UV to investigate these interactions (Gabbay et. al., 1973). From this work, it was determined that peptide amides stabilized salmon sperm DNA more than the peptides themselves. The C-terminal amide provides an electron-rich oxygen atom from the carbonyl which would lend itself as a hydrogen bond acceptor, and two hydrogen atoms from the amino group to serve as possible hydrogen bond donors. Tripeptides with a second lysyl residue stabilized the duplex more than dipeptides with only one lysyl residue as a result of an additional positive charge to interact with the DNA's phosphates. The sequence of the peptide was also shown to be a factor, since Phe-Lys showed a smaller increase in melting temperature.
(Tₘ) than the counterpart Lys-Phe. No gross alteration in the DNA conformation was observed by the UV and CD spectra for the binding of these peptide amides. The results from Gabbay's work strengthen the hypothesis of "bookmark selection" or partial intercalation (Brown, 1970). Due to steric restrictions which prevent full intercalation, ligands with an indole or phenyl moiety could only partially insert between the base pairs of DNA. This partial insertion resulted in the bending of the DNA's helical structure. Gabbay contended that peptides with basic amino acid residues (i.e. lysine, arginine) bind electrostatically with the phosphate backbone of DNA (Gabbay et al., 1976a, 1976b). The aromatic residues (i.e. tryptophan, tyrosine, phenylalanine) then form intimate contacts with base pairs depending upon the chirality of the α-carbon. Gabbay's theories about partial intercalation have been proven valid by several colleagues throughout the years. He and his colleagues (Patel and Gabbay, 1981; Yen et al., 1982; Sheardy and Gabbay, 1983) presented evidence from UV spectroscopy, CD, and NMR measurements to support this hypothesis. Their work showed that dipeptide amide L-lysyl-L-phenylalaninamide binds more intensely to salmon sperm DNA and poly(dA-dT)·(dA-dT) than its corresponding diastereomer L-lysyl-D-phenylalaninamide, indicating that stereochemistry is indeed a parameter to consider. Gabbay and his colleagues determined the apparent binding constants (Kᵥ) for the dipeptide amides in MES buffer as follows:

\[
\begin{align*}
\text{L-lys-L-pheA} & \quad 1.2 \times 10^{-3} \text{ M for salmon sperm DNA} \\
\text{L-lys-D-pheA} & \quad 1.4 \times 10^{-3} \text{ M for salmon sperm DNA} \\
\text{L-lys-L-pheA} & \quad 4.0 \times 10^{-3} \text{ M for poly(dA-dT)·(dA-dT)} \\
\text{L-lys-D-pheA} & \quad 0.6 \times 10^{-3} \text{ M for poly(dA-dT)·(dA-dT)}
\end{align*}
\]
In hopes of learning the orientation of the aromatic ring of phenylalanine, Sheardy (1979) derivatized the ring with a para-nitro group to serve as a reporter functionality in NMR analysis. The $^1$H NMR results supported the proposed partial intercalation by the L-L peptide amide. The L-L isomer experienced signal broadening and upfield NMR shifts attributed to the disruption of the ring currents of the aromatic protons in the presence of DNA. In an another investigation, diastereomeric tripeptides were monitored by $^{19}$F NMR. L-lysine-fluoro-L-tryptophan-L-lysine and L-lysine-fluoro-D-tryptophan-L-lysine were tagged by the tryptophan's fluorinated indole ring. The results substantiated that the L-lysine-L-tryptophan-L-lysine binds more intensely than its diastereomeric counterpart (Shine and James, 1985). The positioning of the aromatic residue within the peptide's primary sequence was also found to influence its binding to DNA (Robledo-Luiggi et al., 1991). Tetrapeptides like Lys-Phe-Ala-Phe with a N-terminal lysine residue and aromatic residues at the second and fourth positions were analyzed by NMR, CD and viscometric methods. These studies confirmed Gabbay's assertion of stereospecificity of binding, but also expanded the theory to conclude that when two aromatic amino acids are located very close together, both can partially stack between the DNA base pairs and ergo bend the DNA as evident in the decrease in viscosity of the DNA. Robledo-Luiggi also found that when an aromatic group is two or three residues after L-lysine, the interaction with DNA bases is reduced.
RESEARCH PROPOSAL

The objective of our research is to find an alternate strategy for the investigation of peptide-DNA interactions. X-ray crystallography and multidimensional high resolution NMR are used in the forefront of this research. However, problems of sample availability and purity are not unusual. It is extremely difficult to isolate pure protein-DNA complexes from cell culture. In addition, the availability of complex instrumentation and software necessary to carry out such experiments is a major hurdle. For crystallographic studies, the question arises whether the interactions are natural or artifacts induced by crystal packing. Analysis of actual protein-DNA complexes for recognition, binding, and structural information is a complex task. For NMR, there are inherent disadvantages such as molecular weight (MW < 30,000 daltons) and solubility limitations. Alternate methods such as $^{19}$F NMR have been used in exploratory efforts. Oligopeptide ligands have been examined as models to mimic localized protein-DNA interactions. Our initial research aspirations involved the use of $^{19}$F and 2-D NMR such as NOESY (Nuclear Overhauser Effect Spectroscopy) and ROESY (Rotating frame Overhauser Effect Spectroscopy) to study the interactions of dipeptide amides with the Dickerson DNA dodecamer which has been definitively characterized by NMR (Patel et. al., 1982). The assignments for the base pairs of this DNA oligonucleotide have already been done using NOESY where through space connectivities of less than 5Å are determined (Hańczyk et. al., 1983). Our proposed method is not to take the place of these more sophisticated analyses, but to complement the information derived from other experimental
approaches to further the study of peptide-DNA interactions.

Our research goals include the following:

1) verify the binding forces between the L-lysyl-phenylalaninamides and DNA
2) synthesize and characterize the monofluorinated analogues of the peptide amides
3) compare the binding of the controls vs. monofluorinated analogues to DNA
4) propose a 3-D explanation via molecular modeling for their binding to DNA.

Our initial research efforts concentrated on the two diastereomeric peptides previously used in Sheardy and Gabbay's studies (1983). These peptides were purchased from Princeton Biomolecules to serve as controls for our experiments---L-lysyl-D-phenylalaninamide (L-lys-D-pheA) and L-lysyl-L-phenylalaninamide (L-lys-L-pheA) (Figure 4). As mentioned above, the amide group provides an additional functionality to enhance the binding to the DNA by hydrogen bonding via the carbonyl oxygen (O····H) or the amino hydrogens (H····O and H····N). Amidation of the carboxy terminus of the peptide has also been shown to be required for the activation of many neuropeptides. Since binding of the peptides to DNA depends upon the nature of the aromatic residue, the stereochemistry of the α-carbon and the primary sequence of the peptides are considered. L-lys-L-pheA and L-lys-D-pheA focus on the stereospecificity that is apparent in the binding of these peptide ligands to DNA. The (N-terminal) L-lysyl residue's α- and ε- ammonium groups bind to the adjacent phosphates across the minor groove of DNA positioning the C-terminal phenylalanine residue for insertion between base pairs as evident for the L-L isomer (Sheardy and Gabbay, 1983).
A series of diastereomeric monofluorinated derivatives were later synthesized to serve as labeled dipeptide amides designed as models for peptide-DNA interactions (Figure 5). Our intentions included using fluorine as a chemical probe in future $^{19}$F NMR experiments to determine the orientation of the phenyl ring upon binding to DNA. Fluorine is very sensitive to its solvent environment, thus allowing the assessment of whether the fluorine-labeled phenyl ring is inserted between the hydrophobic base pairs of DNA or placed away from the DNA helix and situated in the polar buffer environment. These $^{19}$F NMR experiments may also help differentiate between full intercalation and partial intercalation. If the phenyl ring is partially inserted between the base pairs, only the para and meta positions are shielded from the buffer solution. The ortho positions would lie outside in the solution (Figure 6). Four monofluorinated dipeptide amides were isolated and used in our experiments with DNA. The $m$-isomers were not stable, and the corresponding intermediates were difficult to isolate. Consequently, completion of their syntheses was not pursued.
Figure 4: Molecular structures of dipeptide amide controls. The chirality of the α-carbon of phenylalanine dictates the stereospecificity of binding to DNA.
Figure 5: Molecular structures of monofluorinated dipeptide amides: L-lysyl-o, p-fluoro-L-phenylalaninamide and L-lysyl-o, p-fluoro-D-phenylalaninamide.
Figure 6: Schematic illustration of DNA showing partial insertion of the aromatic ring of L-lysyl-L-phenylalaninamide. Under these conditions, only the para and meta positions (H_p and H_m) are shielded from the polar buffer environment (Sheardy, 1979).
Fluorine is the most electronegative atom in the periodic table. Fluorine's free electron pairs may lend themselves as acceptors for hydrogen bonding. The bases—adenine (A), guanine (G), cytosine (C), and thymine (T)—found in DNA are sources of both hydrogen bond donors and acceptors. Could fluorine enhance the binding of the fluoropeptides to the DNA relative to the non-fluorinated peptides? Does the presence of the fluorine change the spatial arrangement of the peptide?

The synthesis of the fluorodipeptide amides involved four major steps (Figure 7): 1) esterification, 2) coupling, 3) amidation and 4) deprotection. The fluorophenylalanine residue that would become the C-terminus of the dipeptide had to undergo esterification to protect the carboxylic group during subsequent coupling (Winitz and Greenstein, 1961). Where necessary, enzymatic resolution of the L/D-fluorophenylalanine esters (Sheardy et al., 1986) was completed using α-chymotrypsin which would digest only the L-isomers by breaking the ester bond, leaving the D-isomers intact for isolation and purification. The mixed carbonic anhydride method (Anderson et al., 1967) was performed to couple the fluorophenylalanine esters with diprotected N, N-diCBZ-L-lysine under anhydrous conditions. This reaction required that the α- and ε-ammonium groups be protected by carbobenzoxyl (CBZ) groups. The activation of the amino acid by a tertiary amine such as triethylamine was completed to abstract the proton from the carboxylic acid group of lysine. The selection of a weak base was important to avoid possible racemization during the coupling step. The reduction in "basicity" of the amine would prohibit the likelihood of another proton abstraction, specifically at the α-carbon. The coupling agent isobutylchloroformate is then reacted with the activated lysine by the
SN₂ mechanism to produce the mixed anhydride. Reaction conditions had to be kept below 0°C in order to stabilize this mixed anhydride. The fluorophenylalanine ester is then added to complete the coupling. The last two steps in the synthesis entail the deprotection of the carboxylic group and amino groups of the dipeptide—amidation of the C-terminus via ammoniolysis and acid hydrolysis using bromic acid/acetic acid to remove the carbobenzoxy groups from the lysine residue (Greenstein and Winitz, 1961).

Characterization of the fluorinated dipeptide intermediates was done after each synthesis step. The final products were then analyzed by ¹H NMR, UV spectroscopy, and circular dichroism (CD). Spectra were compared to the control dipeptide amides and differences noted, presumably due to the substitution of fluorine for one of the protons on the aromatic ring of phenylalanine.

The following DNA sequences served as models for our experiments:

a) Dickerson’s dDNA dodecamer 5’-CGCGAATTCGCG-3’

b) Heteropolymers—poly(dA-dT)•(dA-dT) and poly(dA-dC)•(dG-dT) and homopolymer poly(dA)•(dT).

The double-stranded B-DNA oligomer was synthesized using the automated phosphoramidite method. The self-complementary oligonucleotide was selected to mimic the known regulatory sequence seen in vivo (multi-adenosine-5’-monophosphate thymidine-5’-monophosphate residues). The strand is palindromic; therefore, complicated, overlapping resonances in the NMR spectra are minimized. The decamer version of Dickerson’s DNA model (Dickerson et. al., 1992) was later synthesized to serve as a model in hopes of minimizing the possibility of hairpin formation, yet conserve one helical turn containing a major groove and a minor groove.
1. Ester Synthesis

\[
\begin{align*}
H_2N-CH-COOH & \quad Cl^{-} \quad H_3N^+-CH-COOEt \\
& \quad \text{EtOH/SOCl}_2 \quad + 40^\circ C \\
R & \quad \text{(D,L)} \\
R & \quad \text{Typical Yield 80%-90%} \\
\end{align*}
\]

2. Coupling Reaction

\[
\begin{align*}
\text{CbzNH-CH-COOH} & \quad 1. \text{TEA} \quad \text{CbzNH-CH-COONH-CH-COOEt} \\
R' & \quad \text{2. Isobutylchloroformate} \quad R' \\
R & \quad \text{3. Fluorophenylalanine ethyl ester} \quad R \\
R' = -(CH_2)_4NHCbz & \quad \text{THF, DMF} \quad -15^\circ C \\
\end{align*}
\]

Typical Yield 50%-60%

3. Amidation Reaction

\[
\begin{align*}
\text{CbzNH-CH-COONH-CH-COOEt} & \quad \text{NH}_3 (g) \quad \text{CbzNH-CH-COONH-CH-CONH}_2 \\
R & \quad \text{in CH}_3OH \quad R \\
R & \quad \text{Typical Yield 85%-90%} \\
\end{align*}
\]

4. Deprotection of Amino Groups

\[
\begin{align*}
\text{CbzNH-CH-COONH-CH-CONH}_2 & \quad \text{NH}_3^+-CH-COONH-CH-CONH}_2 \\
R' & \quad \text{HBr/HOAc} \quad R'' = -(CH_2)_4NH_3^+ \\
R & \quad \text{Typical Yield 70%-75%} \\
\end{align*}
\]

Figure 7: The schematic for fluorinated dipeptide amide synthesis including:
1) esterification, 2) coupling, 3) amidation and 4) deprotection.
CHAPTER II
EXPERIMENTAL

2.1 MATERIALS

A. Characterization of Dipeptide Amide Controls. Princeton Biomolecules (Columbus, OH) was selected to synthesize and purify the two diastereomeric dipeptide amides which would serve as controls in our investigation. Princeton Biomolecules was also required to confirm molecular integrity and purity by mass spectrometry and RP-HPLC. Mass spectrometry revealed a peak at 293 m/z for both peptides, an indication of both molecular weight and purity. (With a molecular formula of C_{15}H_{24}N_{4}O_{2}, the expected molecular weight is 291 g/mole or 293 g/mole as the charged peptide.) The peptides arrived as a thin, transparent film. Dr. Michael Shiue of Princeton Biomolecules noted that the peptides were extremely hygroscopic. 

H NMR spectra, both 1-D and 2-D correlation spectroscopy (2-D COSY) were recorded in our laboratories with either a General Electric QE-300 or a Gemini-400 Fourier Transform NMR spectrometer. Chemical shifts were determined relative to the internal standard 2,2,3,3,-d_{4}-3-trimethylsilylpropionate (TSP) in deuterium oxide (D_{2}O). UV spectroscopy was performed on a Hitachi U-2000 dual-beam spectrophotometer. The molar absorptivity value (extinction coefficient) at 260 nm was determined for each dipeptide amide (Figure 8). The calculated value for L-lysyl-L-phenylalaninamide was 70.8 M^{-1} cm^{-1}, while the extinction coefficient for L-lysyl-D-phenylalaninamide was 68.8 M^{-1} cm^{-1}.
Figure 8: Determination of the linearity range and the molar extinction coefficient at 260 nm for L-lysyl-L-phenylalaninamide. As calculated by Beer's Law, the molar extinction coefficient $\varepsilon_{260}$ for L-lysyl-L-phenylalaninamide is $70.8 \text{ M}^{-1}\text{cm}^{-1}$ and $68.8 \text{ M}^{-1}\text{cm}^{-1}$ for L-lysyl-D-phenylalaninamide.
The amino acid L-phenylalanine has three UV absorbance maxima (251.6 nm, 257.5 nm and 263.4 nm under acidic conditions). This absorption of phenylalanine derives from a symmetry-forbidden $\pi-\pi^*$ transition (Cantor and Schimmel, 1980). L-phenylalanine as an amino acid alone has an extinction coefficient of 195 M$^{-1}$ cm$^{-1}$. CD spectra were taken as well to characterize the dipeptide amides and confirm the chirality of the phenylalanine residue, distinguishable by monitoring the molar ellipticity ($\theta$) around 227 nm. The molar ellipticity for L-lysyl-L-phenylalaninamide was +75 mDeg and -80 mDeg for L-lysyl-D-phenylalaninamide.

B. Fluorodipeptide Amide Synthesis and Characterization. All starting amino acids were purchased from either Arcos Organics Co. (Pittsburgh, PA), Sigma Chemical Co. (St. Louis, MO), or PCR, Inc. (Gainesville, FL) and were used without further purification. The fluorophenylalanines were similar to phenylalanine with a $\lambda_{\text{max}}$ near 257 nm. TLC silica gel plates with a fluorescent indicator were purchased from Kodak Chemical Co. (Rochester, NY). Melting points were taken on a Mel-Temp apparatus, and the measurements were uncorrected. $^1$H NMR spectra were recorded with either a Varian XL-200 or a Gemini-400 Fourier Transform NMR spectrometer. Chemical shifts were determined relative to the internal standard trimethylsilane (TMS) for organic solvents or the internal standard 2,2,3,3,-d$_4$-3-trimethylsilylpropionate (TSP) for those in deuterium oxide (D$_2$O). All deuterated solvents were purchased from Cambridge Isotopes (Andover, MA). Polarimetry measurements were completed on a Jasco DP-370 polarimeter.
All characterization work of reaction intermediates and products was completed in our laboratories, while elemental analysis was contracted to Schwarzkopf MicroAnalytical Laboratories, Woodside, New York. The dipeptides and the required intermediates leading up to the final products were analyzed for purity by any of the following methods: RP-HPLC (Yamada et. al., 1990), TLC (Arendt et. al., 1976), NMR or elemental analysis (see Table I). All reactions were known to proceed without racemization as indicated by polarimetry or RP-HPLC results. In some cases, a molecule or two of water of hydration was included for elemental analysis. This is reasonable since some precursors and final compounds are hygroscopic. The estimated net yields ranged from 24% to 37%. Abbreviations used in this section are: 1) THF (tetrahydrofuran); 2) CBZ (carbobenzoxy); 3) DMF (dimethylformamide); 4) TEA (triethylamine).

Preparation of L-lysyl-o-fluoro-L-phenylalaninamide. Compound (1). The carboxylic group of the amino acid that would subsequently become the C-terminus of the fluorinated dipeptide amide was first protected by esterification. o-Fluoro-L-phenylalanine hemihydrate (Arcos Organics; 0.234 g, 1.22 mmol) was placed in a three-neck flask equipped with a magnetic stir bar, a drying tube, a thermometer and a gas dispersing tube. Approximately 10 ml of ethanol was added to the amino acid. In a chilled Erlenmeyer flask, a mixture of 4 ml thionyl chloride and 5 ml anhydrous ethanol was prepared, then slowly added to the amino acid solution at room temperature until the solid dissolved. Upon dissolution, the flask was heated to 40°C and allowed to stand for four hours. The solvent was then removed by evaporation. The resulting residue was
then dissolved in 15 ml 100 mM sodium bicarbonate solution to establish alkaline conditions to neutralize the product for subsequent extraction with methylene chloride. Pooled organic fractions were dried over sodium sulfate and filtered. The solution was later acidified (pH 2-4) using hydrogen chloride gas so the product would dissolve in methanol. The solid was then recrystallized from methanol/ethyl ether to yield 0.256 g (1.16 mmoles, 95% yield) of o-fluoro-L-phenylalanine ethyl ester. The $^1$H NMR of o-F-L-phenylalanine ethyl ester in D$_2$O showed a 3H methyl triplet at $\delta$1.2 ppm, a 2H multiplet at $\delta$ 3.3 ppm, a 2H methylene quartet at $\delta$ 4.2 ppm, a 1H triplet at $\delta$ 4.4 ppm, and 4H multiplets at $\delta$ 7.2, 7.3, and 7.4 ppm for the aromatic protons. The melting point was 148-150°C.

The ester was then coupled to N, N'-DiCBZ-L-lysine by the mixed anhydride method (Anderson et. al., 1967): N, N'-DiCBZ-L-lysine (Sigma Chemical; 0.376 g, 0.91 mmoles) in 25 ml anhydrous THF was placed in a three-neck flask equipped with a magnetic stir bar, a calcium sulfate drying tube and a gas dispersing tube. A stream of dry argon was passed over the solution to keep the atmosphere free of moisture. The flask was then placed in an ice/acetone bath and 1 mmmole freshly distilled TEA was added to the solution, followed by cold isobutylchloroformate. The solution was allowed to stir for fifteen minutes, after which time a precooled solution of o-fluoro-L-phenylalanine ethyl ester (0.210 g, 0.99 mmoles) in 10 ml of 1:1 THF/DMF was added. Additional 1 mmmole TEA was added, and the reaction was allowed to stir at -15°C for two hours. Upon completion of the reaction, the ice bath was removed. The solvent was then removed by evaporation. The resulting residue was dissolved in 90% ethyl acetate,
and the organic phase was washed twice with saturated NaHCO₃ and three times with 1 M HCl. The organic phase was dried over sodium sulfate, filtered and evaporated. The resulting solid was recrystallized from ethyl ether to yield 0.237 g (0.37 mmoles, 43 % yield) of N, N'-DiCBZ-L-lysyl-o-fluoro-L-phenylalanine ethyl ester. The melting point temperature was determined to be 98-102°C. The ¹H NMR in CDCl₃ showed a broad 10H multiplet at δ 7.4 ppm, a 5H multiplet at δ 7.1 ppm, a 1H doublet at δ 6.5 ppm, a 1H doublet at δ 5.4 ppm; a 4H doublet at δ 5.2 ppm, a 3H quartet at δ 4.2 ppm, a 4H multiplet at δ 3.2 ppm, and a 3H multiplet at δ1.2 ppm.

**Anal.** calculated for C₃₃H₃₈FN₃O₇; C, 65.21; H, 6.32.
Found: C, 65.25 ; H, 6.29.

Deprotection of the C-terminal end of the diprotected dipeptide ethyl ester N, N'-DiCBZ-L-lysyl-o-fluoro-L-phenylalanine ethyl ester was completed by ammoniolysis where the ethyl ester was replaced by an amino group to produce an amide. The diprotected dipeptide ester (0.18 g, 0.30 mmoles) was dissolved in methanol saturated with ammonia gas and allowed to sit in a sealed reaction vessel for 48 hours. After subsequent workup, the sample was dissolved in 50 ml methanol and precipitated via dropwise addition of diethyl ether; the yield was 0.128 g (0.22 mmoles, 75% yield) of N, N'-diCBZ-L-lysyl-o-fluoro-L-phenylalaninamide. The melting point was found to be 180-183°C. The ¹H NMR in DMSO showed a 2H doublet at δ 7.9 ppm, a 10H broad multiplet at δ 7.3 ppm, a 5H multiplet at δ 7.1 ppm, a 2H doublet at δ 5.1 ppm, a 1H multiplet at δ 4.5 ppm, a 1H multiplet at δ 3.9 ppm, a 2H multiplet at δ 2.9 ppm, a 2H multiplet at δ 3.1 ppm, a broad 2H peak at δ 1.5 ppm, a broad 2H peak at δ 1.3 ppm, and
a broad 2H multiplet at \( \delta 1.1 \) ppm.

**Anal.** calculated for \( C_{31}H_{38}FN_4O_6; \) C, 64.34; H, 6.11.

Found: C, 64.05; H, 6.02.

Deprotection of N, N'-diCBZ-L-lysL-o-fluoro-L-phenylalaninamide involved the cleavage of the CBZ groups from the lysyl \( \alpha \)- and \( \varepsilon \)-amino groups from the protected dipeptide amide (Greenstein and Winitz, 1961). The intermediate (0.109 g, 0.19 mmoles) was placed in a three-neck flask equipped with a magnetic stir bar, a calcium sulfate drying tube and a gas dispersing tube. A solution of 30% hydrogen bromide in acetic acid (15 ml) was added to the compound. A stream of dry argon was passed over the solution to keep the atmosphere free of moisture. The solution was stirred at room temperature for 24 hours. The product was worked up using diethyl ether washes to precipitate the final product and remove residual acetic acid/hydrogen bromide. The final product is highly hygroscopic; therefore, all further handling of the compound was completed in an antechamber or glove box. The off-white solid powder would become a tan, gelatinous material upon exposure to air. \( ^1 \)H NMR in \( D_2O \) revealed a 5H multiplet from \( \delta 7.4 \) ppm to \( 7.2 \) ppm, a 1H multiplet at \( \delta 4.6 \) ppm, a 1H multiplet at \( \delta 4.0 \) ppm, a 2H multiplet at \( \delta 3.1 \) ppm, a 2H multiplet at \( \delta 2.8 \) ppm a 2H triplet at \( \delta 1.9 \) ppm, a 2H triplet at \( \delta 1.7 \) ppm and a 2H multiplet at \( \delta 1.4 \) ppm.

**Preparation of L-lysyl-p-fluoro-L-phenylalaninamide, Compound (2).** \( p \)-Fluoro-L-phenylalanine (PCR Organics; 0.967 g, 5.30 mmoles) was placed in a three-neck flask equipped with a magnetic stir bar, a drying tube, a thermometer and a gas dispersing
tube. Approximately 100 ml of ethanol was added to the amino acid. In a chilled Erlenmeyer flask, a mixture of 40 ml thionyl chloride and 50 ml anhydrous ethanol was prepared, then slowly added to the amino acid solution at room temperature until the solid dissolved. Upon dissolution, the flask was heated to 40°C and allowed to stand for four hours. The solvent was removed by evaporation. The resulting residue was then dissolved in 150 ml 100 mM sodium bicarbonate solution to establish alkaline conditions to neutralize the product for subsequent extraction with methylene chloride. Pooled organic fractions were dried over sodium sulfate and filtered. The solution was later acidified (pH 2-4) using hydrogen chloride gas, so the product would dissolve in methanol. The solid was recrystallized from methanol/ethyl ether to yield 1.07 g (5.08 mmol, 96% yield) of p-fluoro-L-phenylalanine ethyl ester. The $^1$H NMR of p-F-L-phenylalanine ethyl ester in D$_2$O showed a 3H methyl triplet at δ 1.2 ppm, a 2H multiplet at δ 3.3 ppm, a 2H methylene quartet at δ 4.3 ppm, a 1H triplet at δ 4.4 ppm, and 4H multiplets at δ 7.1 and 7.3 ppm for the aromatic protons. The melting point corresponded to the literature range of 174-175°C. Polarimetry measurements using a glass cylindrical cell (3.5 x 100 mm) with a 2% solution (w/v) in anhydrous ethanol verified optical purity. The polarimetry result of [α]$_D$ = +31.9 indicated optical purity.

The ester was then coupled to N, N'-DiCBZ-L-lysine by the mixed anhydride method (Anderson et al., 1967): N, N'-DiCBZ-L-lysine (Sigma Chemical; 0.870 g, 2.10 mmol) in 25 ml anhydrous THF was placed in a three-neck flask equipped with a magnetic stir bar, a calcium sulfate drying tube and a gas dispersing tube. A stream of dry argon was passed over the solution to keep the atmosphere free of moisture. The
flask was then placed in an ice/acetone bath and 2.1 mmoles of freshly distilled TEA was added to the solution, followed by 2.3 mmoles of cold isobutylchloroformate. The solution was allowed to stir for fifteen minutes, then a precooled solution of \( p \)-fluoro-L-phenylalanine ethyl ester (0.532 g, 2.52 mmoles) in 10 ml of 1:1 THF/DMF was added. An additional 2.1 mmoles of TEA was added, and the reaction was allowed to stir at -15°C for two hours. Upon completion of the reaction, the ice bath was removed and the solvent was removed by evaporation. The resulting residue was dissolved in 90% ethyl acetate, and the organic phase was washed twice with saturated NaHCO\(_3\) and three times with 1 M HCl. The organic phase was dried over sodium sulfate, filtered and evaporated. The resulting solid was recrystallized from ethyl ether to yield 0.683 g (1.12 mmoles, 54 % yield) of \( N, N' \)-DiCBZ-L-lysyl-p-fluoro-L-phenylalanine ethyl ester. The melting point was 133-134°C. The retention factor (R\(_f\)) from TLC in 2% methanol in chloroform was 0.70 for the dipeptide, indicative of product formation. The \( ^1 \)H NMR in CDCl\(_3\) showed a 3H methyl triplet at \( \delta \) 1.2 ppm, a pair of 2H multiplets at \( \delta \) 1.3 and \( \delta \) 1.5 ppm for the lysyl \( \delta \)- and \( \gamma \)- protons respectively, a 1H multiplet at \( \delta \) 1.7 ppm, a broad 4H multiplet at \( \delta \) 3.1 ppm, a 3H set of overlapping peaks that represent the 2H methylene quartet and 1H lysyl \( \alpha \)-proton at \( \delta \) 4.1 ppm, a 2H multiplet at \( \delta \) 4.8 ppm, a 4H multiplet at 5.1 ppm, a 1H doublet 5.4 ppm representing the \( \alpha \)-amino protons, a 1H doublet at 6.5 ppm representing the e-amino protons, a pair of 2H multiplets at \( \delta \) 6.9 to 7.1 ppm (phenyl ring) and a 10H singlet at \( \delta \) 7.4 ppm (CBZ rings).

**Anal.** calculated for C\(_{33}\)H\(_{38}\)FN\(_3\)O\(_7\): C, 65.21; H, 6.32.

Found: C, 64.94; H, 6.08
Deprotection of the C-terminal end of the diprotected dipeptide ethyl ester N, N'-DiCBZ-L-lysyl-p-fluoro-L-phenylalanine ethyl ester was completed by ammoniolysis where the ethyl ester was replaced by an amino group to produce an amide. The diprotected dipeptide ester (0.484 g, 0.80 mmoles) was dissolved in methanol saturated with ammonia gas and allowed to sit in a sealed vessel for at least 48 hours. After subsequent workup and recrystallization from methanol/diethyl ether, the resulting yield was 0.333 g (0.57 mmoles, 72% yield) of N, N'-diCBZ-L-lysyl-p-fluoro-L-phenylalaninamide. The melting point was 189-193°C. The $^1$H NMR in DMSO showed a 2H doublet at δ 7.9 ppm, a 10H broad multiplet at δ 7.3 ppm, a 5H multiplet at δ 7.1 ppm; a 2H doublet at δ 5.1 ppm, a 1H multiplet at δ 4.5 ppm, a 1H multiplet at δ 3.9 ppm, a 2H multiplet at δ 3.0 ppm, a 2H multiplet at δ 2.8 ppm, a broad 2H peak at δ 1.5 ppm, a broad 2H peak at δ 1.4 ppm, and a 2H broad multiplet at δ 1.2 ppm.

Anal. calculated for C$_{31}$H$_{35}$FN$_4$O$_6$; C, 64.34; H, 6.11. Found: C, 64.59; H, 5.99.

Deprotection of N, N'-diCBZ-L-lysyl-p-fluoro-L-phenylalaninamide involved the cleavage of the CBZ groups from the α- and ε-amino groups of the protected dipeptide amide. The intermediate (0.285 g, 0.49 mmoles) was placed in a three-neck flask equipped with a magnetic stir bar, a calcium sulfate drying tube and a gas dispersing tube. A solution of 30% hydrogen bromide in acetic acid (15 ml) was added to the compound. A stream of dry argon was passed over the solution to keep the atmosphere free of moisture. The solution was stirred at room temperature for 24 hours. The product was worked up using diethyl ether washes to precipitate the final product and
remove residual acetic acid/hydrogen bromide. The final product was highly hygroscopic. All further handling of the compound was completed in an antechamber or glove box. The off-white solid powder would become a tan, gelatinous material upon exposure to air. \(^1\)H NMR in D\(_2\)O revealed a 5H multiplet from \(\delta 7.3\) ppm to \(7.1\) ppm, a 1H multiplet at \(\delta 4.6\) ppm, a 1H multiplet at \(\delta 4.0\) ppm, a 2H multiplet at \(\delta 3.2\) ppm, a 2H multiplet at \(\delta 3.0\) ppm, a 2H triplet at \(\delta 1.9\) ppm, a 2H triplet at \(\delta 1.7\) ppm, and a 2H multiplet at \(\delta 1.4\) ppm.

**Preparation of L-lysyl-o-fluoro-D-phenylalaninamide, Compound (3).** o-Fluoro-D, L-phenylalanine (Sigma Chemical; 7.095 g, 38.7 mmoles) was placed in a three-neck flask equipped with a magnetic stir bar, a drying tube, a thermometer and a gas dispersing tube. Then approximately 100 ml of ethanol was added to the amino acid. In a ice-chilled Erlenmeyer flask, a mixture of 60 ml thionyl chloride and 75 ml anhydrous ethanol was prepared, then slowly added to the amino acid solution at room temperature until the solid dissolved. Upon dissolution, the flask was heated to 40°C and allowed to stand for four hours. The solvent was removed by evaporation. The resulting oil was then dissolved in 150 ml sodium bicarbonate solution to establish alkaline conditions and neutralize the product for subsequent extraction with methylene chloride. Pooled organic fractions were dried over sodium sulfate and filtered. The solution was then acidified (pH 2-4) using hydrogen chloride gas, so the product would dissolve in methanol. The solid was recrystallized from methanol/ethyl ether to yield 7.242 g (34.3 mmoles, 89 % yield) of o-fluoro-D, L-phenylalanine ethyl ester. The \(^1\)H NMR of o-F-D,L-phenylalanine ethyl ester in D\(_2\)O showed a 3H methyl triplet at \(\delta 1.2\)
ppm, a 2H multiplet at δ 3.3 ppm, a 2H methylene quartet at δ 4.2 ppm, a 1H triplet at δ 4.4 ppm, and 4H multiplet at δ 7.3 ppm for the aromatic protons.

To isolate the D-isomer, the racemic intermediate (8.040 g, 19.03 mmoles) was enzymatically treated with the addition of 250 mg α-chymotrypsin Type II (Bovine Pancreas, EC 3.4.21.1)(Sheardy et. al., 1986). The resulting workup yielded 3.170 g (15.0 mmoles, 79 % yield). The melting point corresponded with the literature value of 148-150°C. A polarimetry measurement of [α]D = -43.4 indicated optical purity.

The ester was then coupled to N, N'-DiCBZ-L-lysine. N, N'-DiCBZ-L-lysine (Sigma Chemical; 4.708 g, 11.24 mmoles) in 50 ml anhydrous THF was placed in a three-neck flask equipped with a magnetic stir bar, a calcium sulfate drying tube and a gas dispersing tube. A stream of dry argon was passed over the solution to keep the atmosphere free of moisture. The flask was then placed in an ice/acetone bath and 11.4 mmoles of freshly distilled TEA was added to the solution, followed by 12.4 mmoles of cold isobutylchloroformate. The solution was allowed to stir for 15 minutes, then a precooled solution of o-fluoro-D-phenylalanine ethyl ester (2.612 g, 12.37 mmoles) in 20 ml of 1:1 THF/DMF was added. An additional 11.4 mmoles of TEA was added, and the reaction was allowed to stir at -15°C for two hours. Upon completion of the reaction, the ice bath was removed and the solvent was removed by evaporation. The resulting residue was dissolved in 90% ethyl acetate, and the organic phase was washed twice with saturated NaHCO₃ and three times with 1 M HCl. The organic phase was dried over sodium sulfate, filtered and evaporated. The resulting solid was recrystallized
from ethyl ether to yield 0.318 g (0.52 mmole, 4% yield) of N, N'-DiCBZ-L-lysyl-o-fluoro-D-phenylalanine ethyl ester. The melting point was 123-126°C. The Rf value from TLC in 2% methanol in chloroform was 0.65 for the dipeptide, indicative of product formation. The 1H NMR in CDCl3 showed a 3H methyl triplet at δ 1.2 ppm, a pair of 2H multiplets at δ 1.3 and δ 1.5 ppm for the lysyl δ- and γ-protons respectively, a 1H multiplet at δ 1.7 ppm, a broad 4H multiplet at δ 3.1 ppm, a 3H set of overlapping peaks that represent the 2H methylene quartet and 1H lysyl α-proton at δ 4.1 ppm, a 2H multiplet at δ 4.8 ppm, a 4H multiplet at δ 5.1 ppm, 1H doublet at δ 5.4 ppm representing the α-amino protons, a 1H doublet at δ 6.5 ppm representing the ε-amino protons, a pair of 2H multiplets at δ 6.9 to δ 7.1 ppm (phenyl ring) and a 10H singlet at δ 7.4 ppm (CBZ rings).

Anal. calculated for C33H38FN3O7; C, 65.21; H, 6.32.
Found: C, 64.83; H, 6.26.

Deprotection of the C-terminal end of the diprotected dipeptide ethyl ester N, N'-DiCBZ-L-lysyl-o-fluoro-D-phenylalanine ethyl ester was completed by ammoniolysis. The diprotected dipeptide ester (0.255 g, 0.42 mmole) was dissolved in methanol saturated with ammonia gas and allowed to sit in a sealed vessel for 48 hours. After subsequent workup, the sample was dissolved in 50 ml methanol, then precipitated via dropwise addition of ethyl ether; the yield was 0.139 g (0.24 mmole, 57% yield) of N, N'-diCBZ-L-lysyl-o-fluoro-D-phenylalaninanamide. The melting point was 165-168°C. The 1H NMR in DMSO showed a 2H doublet at δ 8.3 ppm, a broad 10H multiplet at δ 7.5 ppm, a 5H multiplet at δ 7.1 ppm; a 2H doublet at δ 5.0 ppm, a 1H multiplet at δ 4.5
ppm, a 1H multiplet at δ 3.9 ppm, 2H multiplets at δ 3.2 ppm, a 2H multiplet at δ 2.9 ppm, a 2H broad peak at δ 1.3 ppm and a broad 2H multiplet at δ 1.0 ppm.

Anal. calculated for C₃₁H₃₅FN₄O₆; C, 64.34; H, 6.11. 
Found: C, 64.22; H, 6.02.

Deprotection of N, N'-diCBZ-L-lysyl-o-fluoro-D-phenylalaninamide involved the cleavage of the CBZ groups from the lysine amino groups of the protected dipeptide amide. The reactant (0.136 g, 0.24 mmoles) was placed in a three-neck flask equipped with a magnetic stir bar, a calcium sulfate drying tube and a gas dispersing tube. A solution of 30% hydrogen bromide in acetic acid (15 mL) was added to the compound. A stream of dry argon was passed over the solution to keep the atmosphere free of moisture. The solution was stirred at room temperature for 24 hours. The product was worked up using diethyl ether washes to precipitate the final product and remove residual acetic acid/hydrogen bromide. The final product is highly hygroscopic, therefore all further handling of the compound was completed in an antechamber or glove box. The off-white solid powder would become a tan, gelatinous material upon exposure to air. ¹H NMR in D₂O revealed a 5H multiplet from δ 7.4 ppm to δ 7.2 ppm, a 1H multiplet at δ 4.7 ppm, a 1H multiplet at δ 3.9 ppm, a 2H multiplet at δ 3.4 ppm, a 2H multiplet at δ 3.0 ppm, 2H triplets at δ 1.6 ppm, δ 1.5 ppm and at δ 1.0 ppm.

Preparation of L-lysyl-p-fluoro-D-phenylalaninamide, Compound (4). To protect the carboxylic group of the amino acid that would subsequently become the C-terminus of the fluorinated dipeptide amide, esterification was completed. p-Fluoro-D, L-
phenylalanine (PCR; 2.088 g, 11.40 mmol) was placed in a three-neck flask equipped with a magnetic stir bar, a drying tube, a thermometer and a gas dispersing tube. Approximately 100 ml of ethanol was then added to the amino acid. In a chilled Erlenmeyer flask, a mixture of 40 ml thionyl chloride and 50 ml anhydrous ethanol was prepared, then slowly added to the amino acid solution at room temperature until the solid dissolved. Upon dissolution, the flask was heated to 40° C and allowed to stand for four hours. The solvent was removed by evaporation. The resulting residue was then dissolved in 150 ml 100 mM sodium bicarbonate solution to establish alkaline conditions and neutralize the product for subsequent extraction with methylene chloride. Pooled organic fractions were dried over sodium sulfate and filtered. The solution was later acidified (pH 2-4) using hydrogen chloride gas, so the product would dissolve in methanol. The solid was recrystallized from methanol/ethyl ether to yield 1.095 g (5.18 mmol, 46% yield) of p-fluoro-D,L-phenylalanine ethyl ester. The $^1$H NMR of p-F-D,L-phenylalanine ethyl ester in D$_2$O showed a 3H methyl triplet at δ 1.2 ppm, a 2H multiplet at δ 3.3 ppm, a 2H methylene quartet at δ 4.3 ppm, a 1H triplet at δ 4.4 ppm, and a 4H multiplet from δ 7.1 to δ 7.3 ppm for the aromatic protons.

To isolate the D-isomer, the racemic intermediate (0.842 g, 1.85 mmol) was enzymatically treated with the addition of 250 mg α-chymotrypsin. The resulting workup yielded 0.286 g (1.26 mmol, 68% yield). The melting point of the D-isomer was 174-175°C uncorrected which matched the melting point reported in literature:

The ester was then coupled to N, N’-DiCBZ-L-lysine. N, N’-DiCBZ-L-lysine
(Sigma Chemical; 0.474 g, 1.14 mmole) in 25 ml anhydrous THF was placed in a three-neck flask equipped with a magnetic stir bar, a calcium sulfate drying tube and a gas dispersing tube. A stream of dry argon was passed over the solution to keep the atmosphere free of moisture. The flask was then placed in an ice/acetone bath, and 1.1 mmole of freshly distilled triethylamine was added to the solution, followed by 1.2 mmole of cold isobutylchloroformate. The solution was allowed to stir for 15 minutes, after which time a precooled solution of \( p \)-fluoro-D-phenylalanine ethyl ester (0.254 g, 1.20 mmole) in 10 mL of 1:1 THF/DMF was added. An additional 1.1 mmole of TEA was added, and the reaction was allowed to stir at -15°C for two hours. Upon completion of the reaction, the ice bath was removed and the solvent evaporated. The resulting residue was dissolved in 90% ethyl acetate, and the organic phase was washed two times with saturated sodium bicarbonate and three times with 1 M hydrochloric acid solution. The organic phase was dried over sodium sulfate, filtered and evaporated. The resulting solid was recrystallized from ethyl ether to yield 0.183 g (0.30 mmole, 26% yield) of \( N, N' \)-DiCBZ-L-lysyl-p-fluoro-D-phenylalanine ethyl ester. The melting point was 153-154°C. The retention factor \( (R_t) \) from TLC in 2% methanol in chloroform was 0.70, thereby indicating product formation and confirming purity. The \(^1\)H NMR in CDCl\(_3\) showed a 3H methyl triplet at \( \delta \) 1.2 ppm, a pair of 2H multiplets at \( \delta \) 1.3 and \( \delta \) 1.5 ppm for the lysyl \( \delta \)- and \( \gamma \)-protons respectively, a 1H multiplet at \( \delta \) 1.7 ppm, a broad 4H multiplet at \( \delta \) 3.1 ppm, a 3H set of overlap peaks that represent the 2H methylene-quartet and 1H lysyl \( \alpha \)-proton at \( \delta \) 4.1 ppm, a 2H multiplet at \( \delta \) 4.8 ppm, a 4H multiplet at \( \delta \) 5.1 ppm, 1H doublet \( \delta \) 5.4 ppm representing the \( \alpha \)-amino protons, a 1H doublet at \( \delta \) 6.5 ppm.
representing the ε-amino protons, a pair of 2H multiplets at δ 6.9 to 7.1 ppm (phenyl ring) and a 10H singlet at δ 7.4 ppm (CBZ rings).

**Anal.** calculated for C_{33}H_{38}F_{N}O_{7}; C, 65.21; H, 6.32.
Found: C, 64.89; H, 5.99.

Deprotection of the C-terminal end of the diprotected dipeptide ethyl ester N, N'-DiCBZ-L-lysyl-p-fluoro-D-phenylalanine ethyl ester was completed by ammoniolysis where the ethyl ester was replaced by an amide. The diprotected dipeptide ester (0.532 g, 0.88 mmole) was dissolved in methanol saturated with ammonia gas and allowed to sit in a sealed vessel for 48 hours. After subsequent workup and recrystallization from methanol/ethyl ether, the resulting yield was 0.337 g (0.58 mmole, 67% yield) of N, N'-diCBZ-L-lysyl-p-fluoro-D-phenylalaninamide. The melting point was 149-154°C. The ^1H NMR in DMSO showed a 2H doublet at δ 8.2 ppm, a 10H broad multiplet at δ 7.5 ppm, a 5H multiplet at δ 7.1 ppm; a 2H doublet at δ 5.0 ppm, a 1H multiplet at δ 4.4 ppm, a 1H multiplet at δ 3.9 ppm, 2H multiplet at δ 3.2 ppm, a 2H multiplet at δ 2.9 ppm, a broad 2H peak at δ 1.3 ppm and a broad 2H multiplet at δ 1.0 ppm.

**Anal.** calculated for C_{31}H_{35}F_{N}O_{6}; C, 64.34; H, 6.11.
Found: C, 64.28; H, 6.01.

Deprotection of N, N'-diCBZ-L-lysyl-p-fluoro-D-phenylalaninamide required the cleavage of the CBZ groups from the amino groups of the protected dipeptide amide. The reactant (0.312 g, 0.54 mmole) was placed in a three-neck flask equipped with a magnetic stirbar, a calcium sulfate drying tube and a gas dispersing tube. A volume of 15 ml of 30% hydrogen bromide in acetic acid was added to the compound. A stream
of dry argon was passed over the solution to keep the atmosphere free of moisture. The solution was stirred at room temperature for 24 hours. The product was worked up using diethyl ether washes to precipitate the final product and remove residual acetic acid/hydrogen bromide. The final product is highly hygroscopic, therefore all further handling of the compound was completed in an antechamber or glove box. The off-white solid powder would become a tan, gelatinous material upon exposure to air. \(^1\text{H}\) NMR in D\(_2\)O revealed a 5H multiplet from \(\delta 7.3\) ppm to \(\delta 7.1\) ppm, a 1H multiplet at \(\delta 4.7\) ppm, a 1H multiplet at \(\delta 3.9\) ppm, a 2H multiplet at \(\delta 3.4\) ppm, a 2H multiplet at \(\delta 2.9\) ppm, a 2H multiplet at \(\delta 2.7\) ppm, a 2H triplet at \(\delta 1.6\) ppm, a 2H triplet at \(\delta 1.5\) ppm, and a 2H multiplet at \(\delta 0.8\) ppm.
TABLE I
SYNTHESIS AND CHARACTERIZATION
OF FLUORINATED DIPEPTIDE AMIDES

1. Esterification to protect -COOH terminus
2. Enzymatic Resolution to isolate D-isomers
3. Characterization of intermediates

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>YIELDS</th>
<th>CHARACTERIZATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{11}H_{14}FNO_{2}</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MW 211.27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>o-F-L-Phe ester</td>
<td>95%</td>
<td>D-isomer confirmed ethyl ester</td>
</tr>
<tr>
<td>(Lot 100495)</td>
<td></td>
<td>150-153 148-150</td>
</tr>
<tr>
<td>o-F-D/L-Phe ester</td>
<td>94%</td>
<td>D-isomer confirmed ethyl ester</td>
</tr>
<tr>
<td>(Lot 052494)</td>
<td>25%</td>
<td>150-153 148-149 -43.1</td>
</tr>
<tr>
<td>o-F-D/L-Phe ester</td>
<td>89%</td>
<td>D-isomer confirmed ethyl ester</td>
</tr>
<tr>
<td>(Lot 022895)</td>
<td>79%</td>
<td>150-153 148-150 -43.4</td>
</tr>
<tr>
<td>p-F-D/L-Phe ester</td>
<td>46%</td>
<td>D-isomer confirmed ethyl ester</td>
</tr>
<tr>
<td>(Lot 080294)</td>
<td>68%</td>
<td>175-177 174-175</td>
</tr>
<tr>
<td>p-F-L-Phe ester</td>
<td>96%</td>
<td>D-isomer confirmed ethyl ester</td>
</tr>
<tr>
<td>(Lot 062294)</td>
<td></td>
<td>175-177 174-175 +31.9</td>
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<tr>
<td>(Lot 010596)</td>
<td>99%</td>
<td>D-isomer confirmed ethyl ester</td>
</tr>
<tr>
<td>p-F-D-Phe ester</td>
<td>92%</td>
<td>D-isomer confirmed ethyl ester</td>
</tr>
<tr>
<td>(Lot 010395)</td>
<td></td>
<td>175-177 175-177 -31.1</td>
</tr>
</tbody>
</table>

Expected Melting Points (Sheardy, 1979)
TABLE I (cont.)

4. Coupling of N, N'-diCBZ-L-Lysine to Fluoro-Phenylalanine Ethyl Ester

5. Characterization of intermediates

<table>
<thead>
<tr>
<th>DIPEPTIDE</th>
<th>YIELD</th>
<th>CHARACTERIZATION</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Melting Point Rr</td>
</tr>
<tr>
<td></td>
<td></td>
<td>°C</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>C_{33}H_{38}F_{3}N_{3}O_{7}</th>
<th>MW 607.75g/mole</th>
<th></th>
<th></th>
<th>%C</th>
<th>%H</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Cbz_{2})-L-Lysine-</td>
<td>26%</td>
<td>153-154</td>
<td>confirmed</td>
<td>0.70</td>
<td>64.89</td>
<td>5.99</td>
</tr>
<tr>
<td>p-F-D-PheOEt (Lot 112994)</td>
<td>26%</td>
<td>153-154</td>
<td>confirmed</td>
<td>0.70</td>
<td>64.89</td>
<td>5.99</td>
</tr>
<tr>
<td>(Lot 110795)</td>
<td>33%</td>
<td>152-154</td>
<td>p-isomer?</td>
<td>65.09</td>
<td>6.24</td>
<td></td>
</tr>
<tr>
<td>(Cbz_{2})-L-Lysine-</td>
<td>54%</td>
<td>133-134</td>
<td>confirmed</td>
<td>0.70</td>
<td>64.94</td>
<td>6.08</td>
</tr>
<tr>
<td>p-F-L-PheOEt (Lot 111294)</td>
<td>54%</td>
<td>133-134</td>
<td>confirmed</td>
<td>0.70</td>
<td>64.94</td>
<td>6.08</td>
</tr>
<tr>
<td>(Cbz_{2})-L-Lysine-</td>
<td>4%</td>
<td>123-126</td>
<td>confirmed</td>
<td>0.65</td>
<td>64.83</td>
<td>6.26</td>
</tr>
<tr>
<td>o-F-D-PheOEt (Lot 101695)</td>
<td>4%</td>
<td>123-126</td>
<td>confirmed</td>
<td>0.65</td>
<td>64.83</td>
<td>6.26</td>
</tr>
<tr>
<td>(Cbz_{2})-L-Lysine-</td>
<td>43%</td>
<td>98-102</td>
<td>confirmed</td>
<td>0.65</td>
<td>65.25</td>
<td>6.29</td>
</tr>
<tr>
<td>o-F-L-PheOEt (Lot 121295)</td>
<td>43%</td>
<td>98-102</td>
<td>confirmed</td>
<td>0.65</td>
<td>65.25</td>
<td>6.29</td>
</tr>
</tbody>
</table>

Elemental Analysis Results provided by Schwarzkopf Microanalytical Labs, NY.
6. Deprotection of -COOH via conversion to amide by ammoniolysis

<table>
<thead>
<tr>
<th>DIPEPTIDE AMIDE</th>
<th>YIELD</th>
<th>CHARACTERIZATION</th>
<th>Melting Point</th>
<th>NMR</th>
<th>Elemental Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CBZ)₂-L-Lysine-p-F-D-PheAmide</td>
<td>67%</td>
<td>confirmed</td>
<td>149-154</td>
<td></td>
<td>64.28</td>
</tr>
<tr>
<td></td>
<td>337 mg</td>
<td></td>
<td></td>
<td></td>
<td>6.01</td>
</tr>
<tr>
<td>(CBZ)₂-L-Lysine-p-F-L-PheAmide</td>
<td>72%</td>
<td>confirmed</td>
<td>189-193</td>
<td></td>
<td>64.59</td>
</tr>
<tr>
<td></td>
<td>333 mg</td>
<td></td>
<td></td>
<td></td>
<td>5.99</td>
</tr>
<tr>
<td>(CBZ)₂-L-Lysine-o-F-D-PheAmide</td>
<td>57%</td>
<td>confirmed</td>
<td>165-168</td>
<td></td>
<td>64.22</td>
</tr>
<tr>
<td></td>
<td>139 mg</td>
<td></td>
<td></td>
<td></td>
<td>6.02</td>
</tr>
<tr>
<td>(CBZ)₂-L-Lysine-o-F-L-PheAmide</td>
<td>75%</td>
<td>confirmed</td>
<td>180-183</td>
<td></td>
<td>64.05</td>
</tr>
<tr>
<td></td>
<td>128 mg</td>
<td></td>
<td></td>
<td></td>
<td>6.02</td>
</tr>
</tbody>
</table>

7. Deprotection of lysyl-NH₂ groups by acid hydrolysis (HBr/HOAc)

<table>
<thead>
<tr>
<th>FLUORO-DIPEPTIDE AMIDE</th>
<th>UV SPECTRA</th>
<th>NMR SPECTRA</th>
<th>CD SPECTRA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peaks</td>
<td></td>
<td>Peaks</td>
</tr>
<tr>
<td>L-lysyl-o-fluoro-L-phenylalaninamide, #1</td>
<td>262 nm; 268 nm</td>
<td>confirmed L</td>
<td>+225 nm</td>
</tr>
<tr>
<td>L-lysyl-p-fluoro-L-phenylalaninamide, #2</td>
<td>264 nm; 270 nm</td>
<td>confirmed L</td>
<td>+227 nm</td>
</tr>
<tr>
<td>L-lysyl-o-fluoro-D-phenylalaninamide, #3</td>
<td>262 nm; 268 nm</td>
<td>confirmed D</td>
<td>-225 nm</td>
</tr>
<tr>
<td>L-lysyl-p-fluoro-D-phenylalaninamide, #4</td>
<td>264 nm; 270 nm</td>
<td>confirmed D</td>
<td>-227 nm</td>
</tr>
</tbody>
</table>
C. DNA Oligomer Synthesis. Each self-complementary DNA strand was synthesized on a 10 μM column by the automated phosphoramidite method (Caruthers, 1985) carried out by the Applied Biosystems 380B DNA synthesizer (Foster City, CA). The synthesis is done with the growing nucleotide chain attached to a solid Controlled Pore Glass (CPG) support so that excess reagents that are in liquid phase can be removed by filtration (Figures 9-12). As the starting material, the solid support is derivatized with the nucleoside that will be the 3'-hydroxyl end of the product. The nucleoside is bound to the solid silica support through a long spacer arm attached at the 3'-hydroxyl via an ester bond, which is base labile and allows for removal of the DNA from the support. All free silanol groups are capped to prevent side reactions. The 5'-hydroxyl is blocked with a dimethoxytrityl (DMTr-) group. Thus, automated DNA synthesis builds the oligonucleotide chain 3' to 5'. The use of the solid support is essential to the automation of DNA synthesis as it eliminates the need for purification steps between base additions.

The first step of the synthesis cycle is treatment of the derivatized solid support with 10% trichloroacetic acid (TCA) in methylene chloride to remove the trityl group and free up the 5'-hydroxyl for the addition reaction. The next step, activation, created a highly reactive nucleoside derivative that reacts with the hydroxyl group. This intermediate is created by simultaneously adding the phosphoramidite derivative of the next nucleoside and a weak acid, tetrazole, to the reaction chamber. The tetrazole protonates the nitrogen of the phosphoramidite, making it susceptible to nucleophilic attack. Since this intermediate is so reactive, the addition reaction is complete in less than 30 seconds at room temperature. The added phosphoramidite is blocked at the 5'.
hydroxyl end with the dimethoxytrityl group. The exocyclic amines on adenosine (A) and cytosine (C) are protected by a benzoyl group, and the exocyclic amine on guanosine (G) is protected by an isobutryl group. Thymidine is already unreactive, and therefore does not need a protecting group.

The next step, capping, terminates any chains that did not undergo addition. Since the unreacted chains have a free 5'-hydroxyl, they can be terminated or capped by acetylation. This reaction is accomplished with acetic anhydride and dimethylaminopyridine. Since the chains that reacted with the phosphoramidite in the previous step are still blocked with the dimethoxytrityl group, they are not affected by this step. Although capping is not required for DNA synthesis, it minimizes the lengths of the impurities and thus facilitates purification by RP-HPLC.

The internucleotide linkage is then converted from the phosphite to the more stable phosphate by oxidation. Iodine is used as the oxidizing agent and water as the oxygen donor. This reaction is complete in less than 30 seconds. After the oxidation step, the dimethoxytrityl group is removed and the cycle is repeated until chain elongation is complete. At this point, the oligonucleotide is still bound to the support and has protecting groups on the phosphates and the exocyclic amines of the bases A, G, and C. To produce biologically active DNA, the methyl groups on the phosphates are removed by a 30-minute treatment with thiophenol. The chain is then cleaved from the support by a one-hour treatment with concentrated ammonium hydroxide. After the solution containing the DNA is removed from the instrument, the protecting groups on the exocyclic amines of the bases are cleaved by a 18-hour treatment with concentrated
18-hour treatment with concentrated ammonium hydroxide at 55° C. Once the treatment is complete, the ammonium hydroxide is removed by evaporation, and the DNA sample is ready for purification by reverse-phase HPLC.
Figure 9: The schematic of DNA synthesis via the phosphoramidite method.

Step 1: Detritylation of initial nucleotide which becomes the 3' end of the sequence to be synthesized.

Step 2: Activation of phosphoramidite.

Step 3: Addition of next nucleotide (phosphoramidite) in sequence.
STEP 4: CAPPING of UNREACTED CHAINS

DMTr -- O

(98 -100%)

H₂C

Base

O

CH₂CH₂CN

O

O

OH

H₂C

Base

CH₂

Base

CH₂

Base

LINKER

LINKER

CPG SUPPORT

CH₃

CH₃

CH₃

CH₃

Figure 10: Schematic of DNA synthesis (cont.).
Step 4: Capping of unreacted chains or failure sequences.
Figure 11: Schematic of DNA synthesis (cont.).
Step 5: Oxidation of trivalent phosphorus phosphite.

Steps 1 thru 5 are repeated until desired DNA sequence is complete.
Figure 12: Schematic of DNA synthesis (cont.).
Step 6: Removal of cyanoethyl chain from phosphates.
Step 7: Cleavage from column support and deprotection of bases.
**DNA Coupling Efficiency Determination.** The coupling efficiency for the synthesis was determined by the dimethoxytrityl assay. After each completed coupling, the sequencer is programmed to collect the released dimethoxytrityl groups into 15 ml test tubes. These fractions are then diluted with 0.1 M toluenesulfonic acid/acetonitrile, and their absorbances measured at 498 nm in 1.0 cm cuvettes. The overall yield is calculated by comparing the absorbance of the last fraction versus the absorbance of the first fraction. The stepwise yield can also be determined, once the overall yield is calculated.

Overall Yield = \[ \frac{A_{498 \text{ last fraction}}}{A_{498 \text{ first fraction}}} \times 100\% \]

Stepwise Yield = \((\text{Overall Yield})^{\frac{1}{N-1}}\)

where \(N\) = number of couplings

**DNA Purification.** For short DNA sequences, reverse-phase HPLC instead of the standard gel electrophoresis method is used to purify the strands of interest. To remove any failure sequences and protecting groups, each strand is subjected twice to RP-HPLC on a C-18 silica gel column and eluted with a solvent gradient of triethylammonium acetate/acetonitrile system. The ammonium hydroxide in the above DNA sample is evaporated by vacuum with several additions of ethanol to azeotrope water. Volumes of triethylamine were also added to keep the DNA basic to minimize detritylation during evaporation. The failure sequences have hydroxyl groups at both ends as a result of the removed linker at the 3' ends and the removed acetyl cap at the 5' ends which have no dimethoxytrityl group. The dried white residue is then reconstituted in 0.1 M triethylammonium acetate buffer and filtered through a 0.45µm Millex-HA filter (Millipore Corp.) to remove any particulates before HPLC analysis.
The first purification entails the separation of the tritylated DNA (TR-DNA) from capped failure sequences and excised protecting groups of the exocyclic amines. Using Water's MicroBondapak C-18 (Type 8MBC18) Plastic Radial-Pak cartridge column, the DNA oligomer of interest was collected under the following HPLC conditions:

Acquisition Software: BASELINE (version 2.1)
Pumps: Model 510
Detector: Lambda-Max Model 481 set at \( \lambda = 280 \) or \( 260 \)nm
Column: Waters µBondapak C-18, 19 mm x 150 mm, 10 µm; 500 psi
Waters RCM 8 x 10 Radial-Pak HPLC (analytical run)
Waters RCM 25 x 10 Radial-Pak HPLC (prep run)
Mobile Phase A: 0.1 M triethylammonium acetate with 5% acetonitrile
Mobile Phase B: Acetonitrile
Solvent Gradient Profiles for the first HPLC purification:
TR-DNA-A 10-30% B over 15 minutes
TR-DNA-P10 for 10 µmole synthesis Prep HPLC Run 10-25% B over 40 minutes

The HPLC analytical run is done before a prep HPLC run to produce a chromatographic profile of the sample (Figure 13). After collection of the major tritylated DNA peak, another analytical HPLC run is completed on the major fraction isolated to confirm correct fraction collection. The collected sample is then transferred to a round-bottom flask for evaporation via vacuum to remove water and salts.
Figure 13: HPLC chromatogram of the first DNA purification run of DMTr-D12-mer (TR-DNA-A gradient). A) failure sequences, B) protecting groups of the exocyclic amines and C) DMTr-DNA of interest.
After the first purification, the tritylated DNA is reconstituted in water. An analytical HPLC run (DNA-ANAL) with a 50 μl injection is performed while the remaining sample is treated with approximately 2 ml of 0.4 M acetic acid to detritylate the DNA. After 15 minutes of acid treatment, the sample is then analyzed by HPLC to monitor the progress of deprotection. Another 2 ml of acetic acid is added after 30 minutes. Detritylation of the strands is complete within an hour. Once detritylation is complete, the sample is neutralized with ammonium hydroxide to pH 8. Most of the free dimethoxytrityl alcohol is removed by extraction with diethyl ether. After removal of the ether by evaporation, the detritylated DNA is redissolved in 0.1 M triethylammonium acetate buffer and filtered as before. A final reverse-phase HPLC preparative run (DNA-PREP) removes any dimethoxytrityl alcohol from the oligonucleotide. Product purity is checked by performing another analytical HPLC run.

After fraction isolation, the DNA sample is transferred to a new round-bottom flask for evaporation to dryness. The resulting residue is reconstituted in water then placed in Spectra-Por tubing (MWCO 1000) for dialysis against 1.0 M sodium chloride, then water to effectively remove salts and any small molecular impurities. The triethylammonium salt is also converted to sodium salt during this step. DNA is then transferred to small eppendorf tubes for lyophilization. Prior to any studies, the oligomer is dissolved in buffer, heated at 85°C for two minutes to completely separate strands and allowed to slowly cool for proper annealing.
D. **Reagents and Buffers** Phosphoramidites were purchased from Applied Biosystems, Inc. (Foster City, CA) or Cruachem (Dulles, VA). HPLC grade solvents were obtained from Aldrich (St. Louis, MO). The polynucleotides were purchased from Pharmacia Biotech (Piscataway, NJ). As mentioned above, L-lysyl-phenylalaninamides were synthesized by Princeton Biomolecules (Columbus, OH). Solutions and buffers were made from water that had been deionized and filtered through a Millipore Milli-Q Water Reagent System (Bedford, MA). All buffers were filtered again through 0.22 micron cellulose acetate membranes.

E. **Sample Preparation.** Oligomers were reconstituted in the buffer of interest. Initially, 10 mM sodium phosphate pH 7, with 2.5 mM EDTA and 100 mM sodium chloride was used. The EDTA is typically added to remove divalent cations which would inhibit degradation by DNases. The concentration of the oligomers were quantitated optically by using the molar extinction coefficient per mole of strand or 1200 bp M⁻¹ cm⁻¹ at 260 nm (Fasman, 1975). DNA homopolymers and heteropolymers were purchased from Pharmacia Biotech. Poly(dA-dT)•(dA-dT) had a λ_max at 262 nm with a molar extinction coefficient of 6600 L M⁻¹ cm⁻¹. Poly(dA-dC)•(dG-dT) had a λ_max at 258 nm with a molar coefficient of 6500 L M⁻¹ cm⁻¹. Poly(dA)-(dT) had a λ_max at 260 nm with a molar extinction coefficient of 6000 L M⁻¹ cm⁻¹. DNA oligomers were heated to 95°C, then allowed to slowly cool to room temperature. Ligands at the predetermined concentrations were added then incubated either overnight or for fifteen minutes before melt experiments. The concentrations of the peptide controls were calculated by using the extinction coefficient as determined at 260 nm. Each fluorinated peptide was
transferred into a tared eppendorf tube then lyophilized to remove any residual solvents from the syntheses. Samples were reconstitued in water to a final stock concentration of 100 mg/ml for each fluorinated peptide.
2.2 METHODS

A. NMR Analysis. The chemical nature of each dipeptide intermediate prescribed the deuterated solvent used for NMR analysis. All deuterated solvents were purchased from Cambridge Isotopes (Andover, MA). Chemical shifts were determined relative to the internal standard trimethylsilane (TMS) for organic solvents or the internal standard 2,2,3,3,-d 4 -3-trimethylsilylpropionate (TSP) for those in deuterium oxide (D 2 O). 2-D 1H COSY was completed to establish the intramolecular connectivities for L-lysyl-L-phenylalaninamide. By examining the crosspeak data, the connectivities within two to three bonds from α-carbon were determined. 1H NMR spectra, both 1-D and 2-D correlation spectroscopy (2-D COSY) were recorded in our laboratories with either a General Electric QE-300 or a Gemini-400 Fourier Transform NMR spectrometer.

B. Circular Dichroism Studies. Circular dichroism (CD) was used to confirm the chirality of the phenylalanine residue for each dipeptide amide. The L-L isomers gave an intense positive peak at approximately 230 nm, while the L-D isomers gave a negative peak at the same wavelength. Since CD is sensitive to local interactions, it is quite useful for examining any structural changes due to the binding of small molecules to DNA. Using the CD spectropolarimeter AVIV-62A DS, (AVIV Associates, Inc. Lakewood, NJ) spectra were collected on the samples prepared for the DNA melting experiments. Each spectrum was usually taken as a single scan with a time averaging constant of 2.0 seconds and a 1.0 nm step size. After the blank (10 mM sodium cacodylate, pH 7 buffer) was subtracted, the zero offset was checked against 320 nm
where no absorbance occurs. AVIV software version 4.1t was used to smooth the curve by least squares polynomial fit.

C. UV Spectroscopy and Optical Melting Studies. UV absorbance readings and spectra were obtained at 25°C, unless noted otherwise. UV absorption of nucleic acids is largely due to the purine and pyrimidine bases. The transition dipoles associated with various electronic transitions of the nucleic acid bases lie in the planes of the aromatic rings (Cantor and Schimmel, 1980). This alignment of the π-electrons induces a reduction of the molar absorptivity, thus hypochromicity results for double-stranded DNA. Programmed melting of each DNA model was monitored by either a Gilford Response II UV/Vis spectrophotometer (Ciba-Corning, Oberlin, OH) or Beckman DU-640 UV/Vis spectrophotometer (Fullerton, CA). Each instrument included a thermostated cell holder used to facilitate thermal denaturation. The resulting melt profile provides a means for the determination of the melting temperature $T_m$, which represents the temperature at which the fraction ($\alpha$) of single strands is equal to 0.5. The temperature was typically ramped from 25°C to 95°C at ca. 0.3°C/min. The DNA absorbance was recorded every 0.1°C after ten consecutive identical readings of the thermostat holder by the spectrophotometer. A wavelength scan (240 nm to 350 nm) for each sample was recorded before and after the "melting" of DNA. In the case for the experiments involving DNA oligomers, each DNA sample was allowed to slowly cool and reanneal, so that another wavelength scan could be taken to compare with the initial scan for the sample taken prior to thermal denaturation. The difference in absorbance of the DNA sample before the denaturation and after the reannealing of each DNA
oligomer duplex should not exceed 2%. If the system suitability parameter is not met, the experiment was discarded and repeated. An “all-or-none” model or two-state system of duplex to single-strand, was assumed for all the DNA thermal denaturation experiments. The melting temperature (T_m) is defined as the midpoint of this transition. In order to work with the raw experimental data from the Gilford and Beckman spectrophotometers, the melt profiles were transferred to an external PC as an ASCII file via Procomm Software version 2.4.1 (Datastorm Technologies, Inc. Columbia, MO). The ASCII files were then sorted and relabelled in order to be analyzed by two software programs GOMELT and GODIFF (Turbo-Basic, Borland International) for melting temperatures and thermodynamic parameters (Breslauer and Marky, 1987). GODIFF measured transition temperatures by calculating the maximum point (T_max) of the differential curve for the melting curve. GOMELT used baselines at the lower T_1 and upper T_2 regions of the melting curve to select the midpoint of the transition or the point of inflection for T_m. The Beckman DU-640 had similar software internally that calculated via 2-point average (i.e. GOMELT) or first derivative (i.e. GODIFF) methods. The preferred analysis depended upon the thermal transition. Long sequences of DNA melt in a cooperative manner resulting in a sharp phase transition that would ensure the reliability of the calculation for T_m by the first derivative analysis. However for small oligomers like D12-mer and D10-mer, the thermal transition was less concerted because of end effects, such that DNA oligomers of less than 50 base pairs are affected by the opening and closing of their ends during denaturation. If baselines could not be drawn for GOMELT analysis, an alternate mean to calculate T_m was the GODIFF method.
GODIFF had its own limitations. Small errors from noise greatly affected the calculation therefore the option to eliminate local pseudo maxima attributed to noise was done. GODIFF uses an NPI or number of points interval to calculate the first derivative. A higher interval results in a more smoothed differential curve. Values for NPI can be set at intervals of ten degrees through the range from 50 to 100. Due to instrumental limitations (temperature control range of 4°C to 95°C), the upper T₂ region was manually extended past 95°C when necessary in order to draw proper straight baselines for Tₘ determinations.

Because phenylalanine absorbs between 230 nm and 270 nm, blanks that consisted of peptide in water were prepared for each DNA/peptide sample. A separate set of experiments with varied DNA concentration was completed to confirm that the melting temperatures were not concentration-dependent. In addition, peptide saturation experiments (with peptide concentrations of 20x; 40x; and 50x the DNA concentration) were also completed to determine the ratio of peptide-to-DNA that was needed to ensure that the peptide was fully bound for subsequent studies.

D. HPLC. DNA oligomers were purified with a Radial-Pak reverse-phase C-18 HPLC column. The tritylated sequences of interest were separated from failure sequences and protecting groups by a trityl-select RP-HPLC method using triethylammonium acetate buffer (0.1 M, pH 7) in which the amount of acetonitrile, CH₃CN varied 15% to 35%. Acetic acid (0.4 M) detritylated the strands within one hour. Most of the free dimethoxytrityl alcohol was removed by extraction with diethyl
ether. A final RP-HPLC preparative run (8%-20% CH$_3$CN) removed any residual dimethoxytrityl alcohol from the pure strands of oligonucleotides.

The protected dipeptide intermediates were checked by isocratic RP-HPLC (Yamada et al., 1990). Using a Vydac C-18 (Catalog #201HS5415) Protein & Peptide column, the protected dipeptide intermediates N, N-diCBZ-L-lysyl-p-fluoro-L/D-phenylalanine ethyl ester were analyzed under the following HPLC conditions:

Acquisition Software: Perkin-Elmer LC Analyst Version 2.3
HPLC System: Perkin-Elmer LC Analyst System
Detection: $\lambda = 214$ nm
Column: Vydac C-18, 4.6 mm x 150 mm, 5 $\mu$m; 90 $\AA$ at 30°C
Mobile Phase: 60% Methanol in Water at 1 ml/min

E. Molecular Modeling. Molecular modeling was done using the HYPERCHEM Software, version 3.0, (Hypercube Inc., Ontario, Canada). The peptides were each constructed and spatial differences due to the chirality of phenylalanine noted. Structures were rendered for 3-D visualization of the molecules and geometrically minimized to show proposed arrangements of the peptide binding to DNA. Such 3-D visualization allows depth perception of structures and evaluation of potential structural changes as a result of ligand binding.
CHAPTER III
RESULTS AND DISCUSSION

Buffer Selection. Common pH 7 buffers include N-[2-Hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid] “HEPES”, 2-[N-Morpholino] ethanesulfonic acid “MES”, Piperazine-N,N’-bis[2-ethanesulfonic acid] “PEPES” and Tris[Hydroxymethyl] aminomethane “Tris”. Tris is a poor choice since the pH is affected by temperature. Phosphate buffers have certain inherent properties such as the advantage of phosphate ions over the DNA’s sugar-phosphate backbone for the encounter with charged ligands. An alternative buffer system is sodium cacodylate, the sodium salt of dimethylarsinic acid. For our studies, the buffer 10 mM sodium cacodylate, pH 7 was used.

Fluoropeptide Amide Characterization and Comparison with Control Peptide Amides. Comparisons of NMR spectra for the L-lysyl-phenylalaninamides and the monofluorinated derivatives suggested that these compounds were structurally similar. Slight changes in the NMR resonances for the monofluorinated dipeptide amides are attributed to the presence of electron-rich fluorine. The proton resonances lied in the following areas: the aromatic proton region of δ 7 to δ 8 ppm, at δ 4.0 and δ 4.6 ppm, at δ 3.0 and δ 3.3 ppm, and the alkyl region of δ 1 to δ 2 ppm (Figure 14). 2-D COSY crosspeaks revealed that the following resonances are attributable to the lysine alkyl chain: δ 1.9 ppm for β-CH₂⁻, δ 1.7 ppm for δ-CH₂⁻, and δ 1.4 ppm for γ-CH₂⁻. The resonances at δ 3.0 ppm and δ 3.2 ppm are due to the lysyl ε-CH₂⁻ and phenylalaninyl β-CH₂⁻ respectively, while resonances at δ 4.0 ppm and δ 4.6 ppm are for the α-protons of the lysyl and phenylalaninyl residues respectively (Figure 15).
Figure 14: $^1$H NMR spectrum of L-lysyl-L-phenylalaninamide.
Figure 15: 2-D COSY NMR spectrum of L-lysyl-L-phenylalaninamide for the elucidation of intramolecular connectivities.
The NMR spectrum for the L-L control resembled the NMR spectra for the L-L monofluorinated analogues. The resonances in the alkyl region were separate and distinct for the L-L isomers. However in the NMR spectra of the L-D isomers, a different pattern in this same region was evident. This allowed easy distinction between the L-L peptides and the L-D peptides. From the NMR spectra, the L-L isomers (Figure 16) could also easily be distinguished from L-D isomers (Figure 17) because of the downfield chemical shifts for particular NMR resonances. The resonances attributed to lysine’s alkyl groups, α-CH- and ε-CH₂-, and phenylalanine’s α-CH- and β-CH₂- protons were shifted downfield in the L-L isomers due to the deshielding of the protons by either a nearby nitrogen atom or the ring currents of the aromatic ring.

Fluorine interacts with the other atoms within the molecular structure of the peptide amide. Fluorine’s non-bonding electrons cause shifts in absorption bands via the extension of the conjugation or π-electron system for the aromatic ring in phenylalanine, thus causing a bathochromic (red) shift in the UV spectra for the monofluorinated analogues (Figure 18). The electronic energy levels of the chromophore move closer together, therefore less energy is required to produce the π-π* transition. As a result, the wavelength of light absorbed becomes longer. The L-lysyl-phenylalaninamides have absorbance maxima at 252 nm, 257 nm, and 264 nm. The ortho-derivatives have maxima at 262 nm and 268 nm, while the para-derivatives’ maxima are at 264 nm and 270 nm respectively. UV spectra of the dipeptide amides did not change within the range of 25° to 95°C.
CD spectra revealed only one CD band for each peptide amide located between 215 to 240 nm which is usually attributable to the amide chromophore. However, for our CD data, the band is a result of the aromatic residue (Figure 19). Aromatic side chains like phenylalanine, tryptophan and tyrosine typically have bands around 224 nm (Krittanai and Johnson, 1997). The direction of the ellipticity distinguishes the stereochemistry of the molecule. L-L isomers have positive bands, while negative bands characterize the L-D isomers. Also note that the spectral bands of the lysyl-fluorophenylalaninamides were less intense than the controls.
Figure 16: $^1$H NMR spectra of monofluorinated L-L isomers: L-lysyl-o-fluoro-L-phenylalaninamide and L-lysyl-p-fluoro-L-phenylalaninamide.
L-LYSYL-\textit{o}-FLUORO-D-PHENYLALANINAMIDE

Figure 17: $^1$H NMR spectra of monofluorinated L-D isomers: L-lysyl-\textit{o}-fluoro-D-phenylalaninamide and L-lysyl-\textit{p}-fluoro-D-phenylalaninamide.
Figure 18: UV Spectra of Dipeptide Amides: Phenylalanine has three maxima at 252 nm, 257 nm, and 264 nm resulting from the aromatic ring's π-π* transitions. The UV spectrum for L-lysyl-L-phenylalaninamide is similar to the spectrum of L-lysyl-L-phenylalanine purchased from Sigma (not shown). The presence of the fluorine substituent on the phenyl ring results in a bathochromic (red) shift for the fluorinated peptides. The maxima for the ortho-isomers are 262 nm and 268 nm, while the maxima for the para-isomers are 264 nm and 270 nm.
Figure 19: Circular Dichroism (CD) spectra of a) L-lysyl-phenylalaninamides, b) L-lysyl-p-fluorophenylalaninamides, and c) L-lysyl-α-fluorophenylalaninamides confirming chirality of the phenylalanine residue.
CD Studies of DNAs and DNA-peptide Complexes. CD spectroscopy measures a compound’s optical activity which arises from structural asymmetry. As a consequence, CD is sensitive to changes in secondary structure. Native B-DNA typically has a minimum at 248 nm that reflects the purine and pyrimidine bases of the nucleotides. By noting the intensity of this CD band, one may monitor the base stacking of helical DNA. The maximum located at 264 nm reflects information about the sugars in the nucleotides. CD spectra are influenced by DNA’s sequence because of the resulting base-to-base interactions. The CD spectrum of poly(dA-dT)·(dA-dT) shows global right-handed B-DNA stacking of bases as determined by Patel and Canuel (1976). According to other scientists, poly(dA-dT)·(dA-dT) adopts an “alternating B-DNA conformation” with adenosine adopting C3’ endo sugar puckers, unlike the standard C2’ endo configuration seen in classical B-DNA (Klug et al., 1979). Poly(dA-dT)·(dA-dT) has a helical pitch of 32.9 Å, in contrast to the classical helical pitch of 33.8 Å. In the presence of dipeptide amides, the CD spectra reveal no gross changes in secondary structure for poly(dA-dT)·(dA-dT) (Figures 20 and 21).

The CD spectrum of poly(dA)·(dT) is also quite different from classical B-DNA. The CD spectrum of poly(dA)·(dT) reveals a peculiar secondary structure as evident by the maxima at 260 nm and 280 nm and an intense minimum at 245 nm (Figures 22 and 23). The secondary structure of poly(dA)·(dT) is rather inflexible or conformationally stiff, making this DNA polymer resistant to change (Aktipis and Martz, 1974). Notwithstanding, poly (dA)·(dT) is still considered B-like, but has narrower minor grooves due to its altered helical bending. The C3’ endo phosphates are moved closer
together in poly(dA)•(dT) resulting in an interphosphate distance of 5.9 Å, while in classical B-DNA the C2' endo phosphates are 7.0 Å apart. The altered grooves in poly(dA)•(dT) suggest that the accessibility for ligands is distinct from native DNA. Since poly(dA)•(dT) is relatively rigid, the CD spectra reveal no changes in secondary structure of the homopolymer in the presence of dipeptide amides.

Poly(dA-dC)•(dG-dT) is likewise B-like, but altered from the classic Watson-Crick B-DNA. The helical pitch is much smaller at 24.2 Å, and there are only eight base pairs per helical turn (Leslie et. al., 1980). For the most part, the presence of either L-lysyl-phenylalaninamides or L-lysyl-fluoro-phenylalaninamides produced little or no conformational change for each respective DNA polynucleotide. Only a slight decrease in the molar ellipticity (θ) was seen upon the peptides’ binding to DNA (Figures 24 and 25). However in the case for L-lysyl-L-phenylalaninamides and the respective fluorinated derivatives, the CD spectrum changed for poly(dA-dC)•(dG-dT), with a shift of the bands to longer wavelength. The experiment was repeated twice with fresh samples, but the results were consistent. Usually the breaking of hydrogen bonds or pairing of bases causes a red shift in band location (Hashizume and Imahori, 1967). However this implies a destabilization of the DNA duplex and should be evident in our thermal denaturation studies by lower T_m values. Similar CD spectral results for poly(dA-dC)•(dG-dT) were obtained by McAfee and his coworkers (1996) in their binding studies of the Sac7d protein. Produced by the hyperthermophile Sulfobolobus acidocaldarius, this DNA-binding protein belongs to a series of small basic proteins ranging from 7000 to 10,000 daltons in molecular weight. This protein also contains
several lysine residues in its primary structure. McAfee and his coworkers have
surmised that Sac7d induces a structural transition in the DNA such as bending or
unwinding. Since different base-base interactions are observed in repeating
polynucleotides, any DNA conformational change induced by ligand binding will result
in CD changes in the long-wavelength region, and such an effect is exaggerated with
repeating polynucleotides like poly(dA-dC)•(dG-dT). Theoretical calculations indicate
that the CD band at 275 nm is most sensitive to the helix unwinding angle and the base
pair propeller twist (Johnson et. al., 1981), and such correlation has been established
experimentally (Baase and Johnson, 1979).
POLY(dA-dT)(dA-dT) with L-LYSYL-PHENYLALANINAMIDES

Figure 20: CD spectra of poly(dA-dT)-(dA-dT) alone and in the presence of L-lysyl-phenylalaninamides.
Figure 21: CD spectra of poly(dA-dT)-(dA-dT) alone and in the presence of a) L-lysyl-o-fluorophenylalaninamides, and b) L-lysyl-p-fluorophenylalaninamides.
Figure 22: CD spectra of poly(dA)-(dT) alone and in the presence of L-lysyl-phenylalaninamides.
Figure 23: CD spectra of poly(dA)$\cdot$(dT) alone and in the presence of a) L-lysyl-$o$-fluorophenylalaninamides, and b) L-lysyl-$p$-fluorophenylalaninamides.
Figure 24: CD spectra of poly(dA-dC)(dG-dT) alone and in the presence of L-lysyl-phenylalaninamides.
Figure 25: CD spectra of poly(dA-dC)-(dG-dT) alone and in the presence of a) L-lysyl-o-fluorophenylalaninamides, and b) L-lysyl-p-fluorophenylalaninamides.
Thermal Denaturation Studies. Hypochromism is observed in UV spectra of DNA taken at 25°C compared to spectra taken at 75°C. At low temperature, the paired and stacked structure restricts the bases, allowing their transition dipoles to interact which reduces the absorbance. At high temperature the strands are fully separated; the bases linked to the sugar-phosphates move freely and display an unperturbed absorbance spectrum (Cantor and Schimmel, 1980). In our first set of thermal denaturation experiments, the free acid form of L-lysyl-L-phenylalanine (Sigma Chemical Co., St. Louis, MO) was added to Dickerson's dodecamer in the following DNA:peptide ratios: 1:1 (excess peptide), 5:1, and 50:1 (fully-bound peptide). The buffer system was 10 mM sodium phosphate, pH 7, with 2.5 mM EDTA and 50 mM sodium chloride. Patel and his coworkers (Patel, et. al., 1982) monitored the same melting transition for the dodecamer alone by NMR and by differential scanning calorimetry (DSC). The resulting T_m was 72°C. The optical thermal denaturation studies performed in our laboratories from 15° to 90°C were monitored at 270 nm. This wavelength was selected because of the preponderance of G-C base pairs in the dodecamer's sequence. No T_m difference was seen between the DNA control (T_m = 60°C) and the DNA-peptide solutions. These findings were not surprising since the free acid form of the dipeptide is not as strong a ligand for DNA as the dipeptide amide. In addition, the phosphate buffer system may interfere with binding because of the excess of phosphate anions in solution. An alternate buffer system for studying intercalation is 10 mM sodium cacodylate, at pH 7 which reduces the nonspecific outside mode of binding (Feigon et. al., 1984). The cacodylate anion does not compete for sodium ions in solution as strongly as the phosphate anion.
Thermal denaturation experiments with control L-lysyl-phenylalaninamides and the DNA dodecamer (D12-mer) were then done using the following buffer system---10 mM sodium cacodylate, pH 7, with 10 mM magnesium chloride (for duplex stability of the oligomer) and 50 mM sodium chloride. Again, no $T_m$ difference was seen between the DNA control ($T_m = 55^\circ$C) and the DNA-peptide solutions. However, the sample with DNA and ethidium bromide (2:1) had a higher $T_m$ of 62$^\circ$C as expected for this classical intercalator. NMR analysis of the D12-mer with ethidium bromide compared to the dodecamer alone supported this experimental result. The imino resonances (12-14 ppm) experienced signal broadening when ethidium bromide was added to the DNA. The next set of denaturations was performed in 50 mM sodium chloride in hopes of reducing the disruption of electrostatic binding. Denaturation experiments on the dodecamer alone in cacodylate buffer, with sodium chloride concentrations ranging from 10 mM to 50 mM, verified that $T_m$ was attainable and that the DNA was stable at such low salt concentrations. Unfortunately, even at 10 mM sodium cacodylate, pH 7, with 10 mM magnesium chloride and no sodium chloride, there was still no significant difference in the melting temperature of the DNA dodecamer in the presence of either dipeptide amide controls.

Another DNA model with the sequence 5'-GCGAATTTCGC-3' was synthesized for study and to repeat the melting experiments above. Results show that $T_m$ is only 53$^\circ$C for this decamer. In the presence of peptide, no differences in the melting temperature were evident. Presumably, the presence of Mg$^{+2}$ cations blocked any potential binding by the dipeptide amides. Therefore the buffer was changed to 10 mM.
sodium cacodylate, pH 7 with sodium chloride. Unfortunately, the oligomers did not serve as good models for the melting studies as evident in the lack of significant differences among the melting temperatures for the control and the DNA-peptide solutions. Different polynucleotide sequences were then examined for potential use as our DNA models. DNA melts in 10 mM sodium cacodylate, pH 7 with 10 mM sodium chloride (in the absence of peptide) were performed to assess whether $T_m$ values were attainable. Out of the six heteropolymers and homopolymers tested, only poly(dA-dT)·(dA-dT), poly(dA)·(dT), and poly(dA-dC)·(dG-dT) resulted in monophasic melting curves with marked baselines. Poly(dG-dC)·(dG-dC) and poly(dG)·(dC) had melting temperatures which did not fall below 95°C, presumably because of the additive strength of the hydrogen bonding for G-C base pairing. Hence, these DNA polymers were not used for our experiments. Thermal denaturation of poly(dA-dG)·(dC-dT) revealed a melt curve with two transitions, thus a biphasic melt (pseudo-$T_m$ values at 41°C and 68°C) and inappropriate for our studies. Consequently, subsequent melt experiments were pursued using only poly(dA-dT)·(dA-dT), poly(dA)·(dT), and poly(dA-dC)·(dG-dT) as the DNA models of choice because of their two-state transitions. Each polynucleotide had a different maximum wavelength as evident in the collected UV spectra (Figure 26). Therefore each set of experiments were monitored at the unique wavelength for that particular polynucleotide. The presence of the peptide did not change the relative absorption increases seen upon denaturation of duplex DNA (Figure 27). The increase in absorption is a function of temperature as seen in a standard melt curve (Figure 28). The degree of peptide binding was evaluated by thermal denaturation
experiments where the $T_m$ of the polynucleotide in the absence and presence of the peptide was determined (see Table II, Table III, and Table IV). The increase in the melting temperature ($T_m$) for the double-stranded heteropolymers and homopolymer in the presence of the dipeptides is indicative of DNA stabilization.

All peptides, including the lysyl-fluorophenylalaninamides, stabilized the duplex state (see Table II, III, and IV). At low sodium concentration (10 mM), the addition of peptide greatly increased the stability of the DNA helix. Our data shows that there is an approximate 20° increase for $T_m$ in the presence of L-lysyl-phenylalaninamides. The stabilization was less dramatic for the polynucleotides in the presence of the monofluorinated analogues, however they still increased the values of $T_m$ by at least 10°. As the concentration of sodium is increased, the stabilizing effect of the peptide becomes less dramatic. Our studies confirmed Gabbay’s finding for poly(dA-dT)*(dA-dT) in that the L-L isomers stabilized the DNA polymer more so than the L-D isomer (Figure 29). Surprisingly, our studies revealed that the L-L isomers did not stabilize poly(dA)*(dT) and poly(dA-dC)*(dG-dT) over the L-D isomers (Figures 30 and 31). This is possibly due to the variable helical states of these polynucleotides relative to the usual B-DNA conformation seen in natural DNA. Natural DNAs like calf thymus and salmon sperm DNA were used in the research of Helene (1972) and Gabbay (1973) respectively. Their results showed that the L-L isomers stabilized duplex more so than the L-D isomers. Indeed our $T_m$ results did show a slight difference between the diastereomers’ effect on the stabilization of duplex DNA. However, in some of the thermal denaturation experiments with lysyl-fluorophenylalaninamides, no significant difference
in the stabilization could be seen between the diastereomers. The difference in the
dimensions of the secondary structures of poly(dA)•(dT) and poly(dA-dC)•(dG-dT) may
account for these results.
Figure 26: UV spectra of polynucleotides used as DNA models: poly(dA-dT)-(dA-dT), poly(dA)-(dT), and poly(dA-dC)-(dG-dT).
Figure 27: UV spectra of poly(dA-dT)·(dA-dT) "before" and "after" melting: 
a) absence of peptide, b) in the presence of L-lysyl-p-fluoro-L-phenylalaninamide, 
and c) in the presence of L-lysyl-p-fluoro-D-phenylalaninamide. Note the 
hyperchromicity as a result of the unstacking of the base pairs in the DNA.
THERMAL DENATURATION of
POLY (dA-dT)(dA-dT)
IN 10 mM SODIUM CACODYLATE, pH 7 AND 25 mM NaCl

Figure 28: Example of an optical melt curve: Absorbance vs. Temperature
Figure 29: Optical melt profiles for poly(dA-dT)-(dA-dT).
Figure 30: Optical melt profiles for poly(dA)-(dT).
POLY (dA-dC)(dG-dT)
IN THE PRESENCE OF
L-LYSYL-PHENYLALANINAMIDES
IN 10 mM SODIUM CACODYLATE, pH 7 AND 25 mM NaCl

Figure 31: Optical melt profiles for poly(dA-dC)-(dG-dT).
# TABLE II
## INFLUENCE OF PEPTIDES ON THE THERMAL DENATURATION OF POLY (dA-dT)·(dA-dT)

### MELTING TEMPERATURE \( T_m (^\circ C) \)

<table>
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<tr>
<th>SAMPLE</th>
<th>10 mM</th>
<th>20 mM</th>
<th>35 mM</th>
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<td>DNA Control</td>
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<td>47.3</td>
<td>52.5</td>
<td>57.5</td>
<td>62.6</td>
</tr>
<tr>
<td>w/ L-L-PheA</td>
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<td>62.6</td>
<td>62.0</td>
<td>62.7</td>
<td>63.1</td>
</tr>
<tr>
<td>w/ L-D-PheA</td>
<td>60.6</td>
<td>61.1</td>
<td>61.0</td>
<td>62.4</td>
<td>63.9</td>
</tr>
<tr>
<td>w/L-p-F-L-PheA</td>
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<td>58.1</td>
<td>58.6</td>
<td>60.3</td>
<td>63.3</td>
</tr>
<tr>
<td>w/L-p-F-D-PheA</td>
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<td>55.8</td>
<td>57.5</td>
<td>60.7</td>
<td>64.7</td>
</tr>
<tr>
<td>w/L-o-F-L-PheA</td>
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<td>56.4</td>
<td>56.5</td>
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<td>63.4</td>
</tr>
<tr>
<td>w/L-o-F-D-PheA</td>
<td>53.6</td>
<td>54.5</td>
<td>56.5</td>
<td>59.8</td>
<td>65.0</td>
</tr>
</tbody>
</table>

\( T_m \pm 0.3^\circ C \)

[DNA] = 0.15 mM

Peptide in Excess (40× or 6.0 mM)

Buffer: 10 mM Sodium Cacodylate, pH 7

Monitored at 262 nm from 25°C - 75°C
### TABLE III

**INFLUENCE OF PEPTIDES ON THE THERMAL DENATURATION OF POLY (dA)·(dT)**

**MELTING TEMPERATURE $T_m (^\circ C)$**

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>10 mM</th>
<th>20 mM</th>
<th>35 mM</th>
<th>60 mM</th>
<th>110 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Control</td>
<td>49.2</td>
<td>55.6</td>
<td>59.3</td>
<td>63.9</td>
<td>69.7</td>
</tr>
<tr>
<td>w/ L-L-PheA</td>
<td>68.4</td>
<td>66.4</td>
<td>68.0</td>
<td>68.2</td>
<td>71.2</td>
</tr>
<tr>
<td>w/ L-D-PheA</td>
<td>69.4</td>
<td>69.8</td>
<td>69.2</td>
<td>69.2</td>
<td>72.3</td>
</tr>
<tr>
<td>w/ L-p-F-L-PheA</td>
<td>60.6</td>
<td>61.0</td>
<td>61.4</td>
<td>64.9</td>
<td>70.2</td>
</tr>
<tr>
<td>w/ L-p-F-D-PheA</td>
<td>63.4</td>
<td>62.8</td>
<td>63.4</td>
<td>66.9</td>
<td>71.7</td>
</tr>
<tr>
<td>w/ L-o-F-L-PheA</td>
<td>60.9</td>
<td>60.7</td>
<td>61.5</td>
<td>64.9</td>
<td>70.1</td>
</tr>
<tr>
<td>w/ L-o-F-D-PheA</td>
<td>59.4</td>
<td>61.2</td>
<td>62.6</td>
<td>66.0</td>
<td>71.4</td>
</tr>
</tbody>
</table>

$T_m \pm 0.3^\circ C$

[DNA] = 0.15 mM
Peptide in Excess (40x or 6.0 mM)
Buffer: 10 mM Sodium Cacodylate, pH 7
Monitored at 260 nm from 25°C - 75°C
TABLE IV
INFLUENCE OF PEPTIDES
ON THE THERMAL DENATURATION OF
POLY (dA-dC)·(dG-dT)

MELTING TEMPERATURE  Tm (°C)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>[Na⁺] 10 mM</th>
<th>[Na⁺] 20 mM</th>
<th>[Na⁺] 35 mM</th>
<th>[Na⁺] 60 mM</th>
<th>[Na⁺] 110 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Control</td>
<td>69.6</td>
<td>76.2</td>
<td>80.5</td>
<td>85.0</td>
<td>90.3</td>
</tr>
<tr>
<td>w/ L-L-PheA</td>
<td>86.0</td>
<td>84.4</td>
<td>84.4</td>
<td>86.2</td>
<td>91.4</td>
</tr>
<tr>
<td>w/ L-D-PheA</td>
<td>87.1</td>
<td>85.0</td>
<td>85.6</td>
<td>87.5</td>
<td>90.4</td>
</tr>
<tr>
<td>w/L-p-F-L-PheA</td>
<td>79.0</td>
<td>79.8</td>
<td>82.3</td>
<td>85.5</td>
<td>90.4</td>
</tr>
<tr>
<td>w/L-p-F-D-PheA</td>
<td>79.5</td>
<td>81.7</td>
<td>84.8</td>
<td>88.7</td>
<td>93.8</td>
</tr>
<tr>
<td>w/L-o-F-L-PheA</td>
<td>81.0</td>
<td>79.3</td>
<td>82.1</td>
<td>85.6</td>
<td>91.2</td>
</tr>
<tr>
<td>w/L-o-F-D-PheA</td>
<td>80.8</td>
<td>80.5</td>
<td>82.1</td>
<td>88.1</td>
<td>93.7</td>
</tr>
</tbody>
</table>

Tm  ± 0.3°C
[DNA] = 0.15 mM
Peptide in Excess (40x or 6.0 mM)
Buffer: 10 mM Sodium Cacodylate, pH 7
Monitored at 258 nm from 45°C - 95°C
According to the polyelectrolyte theory of Manning (1969), sodium ions establish tight local associations to highly-charged DNA. This phenomenon gives rise to the counterion condensation model where DNA is treated as a long, cylindrical polyion in solution that experiences small ion screening interactions and counterion binding along its surface. The concentration of sodium ions around the surface of DNA has a great influence on the successful encounter of charged ligands. The charged peptides must overcome this mobile sodium ion layer in order to come into contact with the phosphates and bind to the DNA backbone. The peptides displace the sodium ions at the site of binding. The concentration of displaced counterions is determined in order to evaluate the binding of these charged ligands to DNA.

As the DNA helix denatures to single strands, cations are released. As single strands, the DNA has a lower charge density, therefore fewer sodium ions are condensed. In other words, a decrease in axial charge density reduces the requirement for local accumulation of counterions near the polyion. Hence, counterions are released into the bulk solution, an entropically favored process. At low concentrations of sodium chloride (< 400 mM), there is a linear relationship between melting temperatures and sodium chloride concentration (T_m vs. log [Na+]). From the resulting line, one can use the slope to evaluate the binding in the following equation (Record et. al., 1978):

\[
\frac{\delta T_m}{\delta \log [\text{Na}^+]} = \frac{2.303RT_m^2}{\Delta H} \Delta n
\]  

(1)

The enthalpy term \( \Delta H \) is obtained from analysis of the optical thermal denaturation data. A large degree of error (± 7.6 kcal/mole) is found in the determination of \( \Delta H \) because of the dependence on baselines drawn relative to the lower
and upper extremes of the melt curve. Using the GOMELT program where the molecularity assumption is two (duplex to single-strand), enthalpies of denaturation were determined. To minimize the error, several slope calculations near the transition midpoint were done with $\Delta t$ of $2^\circ$, $4^\circ$, $6^\circ$, $8^\circ$, and $10^\circ$, and the enthalpies were averaged. By manipulating Equation 1, the $\Delta n$ term is determined. This value represents the release of counterions upon denaturation of DNA. This term is referred to as the differential ion binding term and denotes the difference between the number of sodium ions condensed onto the double-helix vs. the number of sodium ions condensed onto the single strands. Because the peptide itself exudes an ionic charge of +2 under our experimental conditions, graphs of $T_m$ vs. log I where I represents the ionic strength of the solution were drawn (Figure 32). The differences in $\Delta n$ for the polynucleotide with each peptide amide indicate subtle differences in the binding of each as revealed by the release of sodium ion (see Table V, Table VI, and Table VII). For poly(dA-dT)*(dA-dT), the binding of the L-L isomers is clearly different from the respective L-D isomers as suggested by $\Delta n$. The $\Delta n$ values for the L-D isomers, both controls and the fluorinated analogues, are twice as high as the values for the L-L isomers. The other two polynucleotides, poly(dA)*(dT) and poly(dA-dC)*(dG-dT), present a different trend where the $\Delta n$ values for L-L vs. L-D isomers do not vary as much, but subtle differences are seen between the controls vs. fluorinated peptides. For AT-rich DNA at pH 7 in sodium chloride, the slope is 8.9 (Record, et. al., 1990). Our results of 8.4 and 9.3 fall in line with Record’s findings.
Figure 32: a) Graph of $T_m$ vs. log [Na$^+$] for poly(dA-dT)(dA-dT) alone and b) Graph of $T_m$ vs. log $I$ for poly(dA-dT)(dA-dT) in the presence of peptides. The slope of the line is used to calculate $\Delta n$ the differential ion binding term.
### TABLE V
EVALUATION OF PEPTIDE-DNA BINDING via DIFFERENTIAL ION BINDING TERM for POLY (dA-dT)•(dA-dT)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Tm (°C)</th>
<th>ΔH (kcal/mole)</th>
<th>Δn</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Alone</td>
<td>39.3</td>
<td>156.7</td>
<td>8.4</td>
</tr>
<tr>
<td>w/ L-L-PheA</td>
<td>62.1</td>
<td>163.7</td>
<td>0.5</td>
</tr>
<tr>
<td>w/ L-D-PheA</td>
<td>60.6</td>
<td>170.6</td>
<td>2.0</td>
</tr>
<tr>
<td>w/L-p-F-L-PheA</td>
<td>59.0</td>
<td>170.3</td>
<td>3.1</td>
</tr>
<tr>
<td>w/L-p-F-D-PheA</td>
<td>55.5</td>
<td>183.3</td>
<td>6.5</td>
</tr>
<tr>
<td>w/L-o-F-L-PheA</td>
<td>56.9</td>
<td>169.5</td>
<td>4.2</td>
</tr>
<tr>
<td>w/L-o-F-D-PheA</td>
<td>53.6</td>
<td>186.3</td>
<td>8.1</td>
</tr>
</tbody>
</table>

[DNA] = 0.15 mM  
Peptide in Excess (40x or 6.0 mM)  
Buffer: 10 mM Sodium Cacodylate, pH 7  
Monitored at 262 nm from 25°C - 75°C
TABLE VI
EVALUATION OF PEPTIDE-DNA BINDING
via DIFFERENTIAL ION BINDING TERM
for POLY (dA)•(dT)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>Tm (°C)</th>
<th>ΔH (kcal/mole)</th>
<th>Δn</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA Alone</td>
<td>59.3</td>
<td>209.7</td>
<td>9.3</td>
</tr>
<tr>
<td>w/ L-L-PheA</td>
<td>68.0</td>
<td>160.4</td>
<td>2.0</td>
</tr>
<tr>
<td>w/ L-D-PheA</td>
<td>69.2</td>
<td>181.7</td>
<td>1.6</td>
</tr>
<tr>
<td>w/L-p-F-L-PheA</td>
<td>61.4</td>
<td>234.3</td>
<td>7.8</td>
</tr>
<tr>
<td>w/L-p-F-D-PheA</td>
<td>63.4</td>
<td>264.3</td>
<td>7.8</td>
</tr>
<tr>
<td>w/L-o-F-L-PheA</td>
<td>61.5</td>
<td>199.6</td>
<td>6.5</td>
</tr>
<tr>
<td>w/L-o-F-D-PheA</td>
<td>62.6</td>
<td>206.7</td>
<td>8.3</td>
</tr>
</tbody>
</table>

35 mM Na⁺

SAMPLE

[DNA] = 0.15 mM
Peptide in Excess (40x or 6.0 mM)
Buffer: 10 mM Sodium Cacodylate, pH 7
Monitored at 260 nm from 25°C - 75°C
### TABLE VII
EVALUATION OF PEPTIDE-DNA BINDING via DIFFERENTIAL ION BINDING TERM for POLY (dA-dC)•(dG-dT)

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>$T_m$ (°C)</th>
<th>$\Delta H$ (kcal/mole)</th>
<th>$\Delta n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM Na$^+$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA Alone</td>
<td>69.6</td>
<td>226.2</td>
<td>9.8</td>
</tr>
<tr>
<td>w/L-L-PheA</td>
<td>86.0</td>
<td>179.8</td>
<td>3.5</td>
</tr>
<tr>
<td>w/L-D-PheA</td>
<td>87.1</td>
<td>236.5</td>
<td>3.3</td>
</tr>
<tr>
<td>w/L-p-F-L-PheA</td>
<td>79.0</td>
<td>214.1</td>
<td>8.9</td>
</tr>
<tr>
<td>w/L-p-F-D-PheA</td>
<td>79.5</td>
<td>206.7</td>
<td>10.5</td>
</tr>
<tr>
<td>w/L-o-F-L-PheA</td>
<td>81.0</td>
<td>203.4</td>
<td>8.0</td>
</tr>
<tr>
<td>w/L-o-F-D-PheA</td>
<td>80.8</td>
<td>183.6</td>
<td>9.0</td>
</tr>
</tbody>
</table>

[DNA] = 0.15 mM  
Peptide in Excess (40x or 6.0 mM)  
Buffer: 10 mM Sodium Cacodylate, pH 7  
Monitored at 258 nm from 45°C - 95°C
Molecular Modeling. As cited earlier, the CD spectra of the dipeptide amides reveal different secondary structures imposed by the chirality of the phenylalanine residue. Preliminary molecular modeling of the L-lysyl-phenylalaninamides seems to support such evidence (Figure 33). Using HYPERCHEM's table of amino acids, the L-L isomers and L-D isomers of the controls and monofluorinated derivatives were constructed in the β-sheet protein conformation and energetically minimized to render the 3-D representation. The aromatic ring in each case was situated differently. As illustrated in Figure 33, the position of the ortho-fluorine is affected. The 3-D models for the control diastereomers were used in docking exercises with poly(dA-dT)•(dA-dT) to evaluate any binding differences due to the chirality of the phenylalanine residue. The 3-D schematic representation shows the stereospecific electrostatic binding of the N-terminal L-lysyl α- and ε-amino groups of L-lysyl-L-phenylalaninamide and L-lysyl-D-phenylalaninamide to adjacent phosphates along the DNA. The specificity of the peptide dictates the positioning of the C-terminal phenylalanine aromatic ring either towards the helix (L-L isomer) or away from the helix (L-D isomer).
Figure 33: 3-D schematic representation of L-lysyl-\(\alpha\)-fluorophenylalaninamides drawn using HYPERCHEM molecular modeling software. Note the spatial differences due to the chirality of the phenylalanine residue.

[Atom colors: black = carbon; cyan = hydrogen; red = oxygen; yellow = fluorine]
CHAPTER IV
SUMMARY AND CONCLUSIONS

Once successfully synthesized, the lysyl-fluorophenylalaninamides proved to be as hygroscopic as the lysyl-phenylalaninamides, making them difficult to characterize by traditional means (i.e. melting point determinations and elemental analysis). Characterization of the peptide amides were therefore accomplished using NMR (\(^1\)H and COSY), circular dichroism and UV spectroscopy. Chirality of the phenylalanine residue was readily distinguishable by CD and NMR, while UV spectra provided evidence of the presence of fluorine on the phenyl ring.

The polymorphism of DNA has long been recognized (Leslie et al., 1980). DNA can undergo different transitions from classical B-DNA to the other extreme of dehydrated A-DNA (Arnott and Hukins, 1972). Although A-DNA is also a right-handed helix, the helical dimensions and grooves are very different from B-DNA (Figure 34). These different DNA configurations may account for the distinct binding patterns we see in our studies. These “A-tracts” as represented by poly(dA)-(dT) are commonly seen as non-expressed DNA sequences that may have some relevance on the expression of genes. Circular dichroism confirmed that no significant alteration in the secondary conformation of poly(dA-dT)-(dA-dT) and poly(dA)-(dT) occurs upon the binding of both the L-lysyl-phenylalaninamides and the monofluorinated derivatives to these polynucleotides, as they remained B-like. However CD spectra revealed a different case for poly(dA-dC)-(dG-dT). More CD work must be done to confirm the reason for the bathochromic shift for poly(dA-dC)-(dG-dT) in the presence of L-L isomers. It would
be interesting to examine the primary sequence of the Sac7d protein for any patterns similar to our lysyl-phenylalanine structure for the lysine-aromatic amino acid (tryptophan or tyrosine) pattern that may result in intercalation. Full intercalation will cause some helical unwinding thus providing an explanation for the CD spectral results seen by us and McAfee's group for our respective systems. Regardless, no secondary changes were noted for poly(dA-dC)-(dG-dT) in the presence of the L-D isomers.

Thermal denaturation studies revealed that all dipeptide amides, including the monofluorinated analogues, stabilized the duplex state of DNA. However, the lysyl-fluorophenylalaninamides did not stabilize DNA as strongly as the lysyl-phenylalaninamides as evidenced by the lower T_m values obtained. Experiments with increasing sodium chloride concentrations showed a salt dependence for the binding of these dipeptide amides to DNA. This dependence on the ionic strength of the buffer solution verifies that the binding forces between the L-lysyl-phenylalaninamides and DNA are predominantly electrostatic. There is a strong indication that the monofluorinated analogues are also binding primarily by electrostatic interactions. Different T_m values for the polynucleotides in the presence of either the L-L or L-D controls suggest that chirality of the phenylalanine residue does influence the binding to DNA. In addition, the results from molecular modeling seem to indicate that the diastereomeric dipeptide amides bind in accordance to the chirality of the aromatic residue. The rendered 3-D models (Figure 35) show that the L-L isomer's phenyl ring is inserted into the B-DNA helix, while the L-D isomer's phenylalanine residue falls
behind the duplex. When the molecule is viewed from the other side of the DNA helix, the phenyl ring is seen protruding away from the helix and into the buffer solution. Future experiments designed to evaluate the extent of intercalation need to be worked out.
Figure 34: B-DNA compared to the conformation of A-DNA. Both represent extremes in the many polymorphic configurations DNA can exist. Although both are right-handed helical structures, each has different groove dimensions and helical characteristics.
Figure 35: 3-D representation of the stereospecific binding of the control peptides (in green) to poly (dA-dT)•(dA-dT) drawn using HYPERCHEM molecular modeling software: L-lysyl-L-phenylalaninamide (left) and L-lysyl-D-phenylalaninamide (right) [Atom colors: black = carbon; cyan = hydrogen; red = oxygen; yellow = fluorine]
An example of a protein that binds mainly by nonspecific interactions is the *E. coli* lac repressor protein. The binding decreases ten times when the solution’s ionic strength is increased by 30% (0.15 M to 0.2 M). This demonstrates the importance of electrostatic interactions and counterion release on the stability of this protein-DNA complex at low salt. This also suggests that the physiological ionic environment must be considered in the analysis of complexes involved in the control of gene expression (Record *et al.*, 1985). It has also been shown that this protein binds 1000 times more tightly to poly(dA-dT)·(dA-dT) than to calf thymus DNA (Riggs *et al.*, 1972). Consequently, this serves as an indication that the protein regulates gene expression by binding to signal DNA fragments similar to the alternating purine-pyrimidine sequence above.

The lingering question remains as to what determines whether the aromatic residue fully intercalates or only partially intercalates. Wilson and his coworkers (1992) reported that the polarizability of the planar aromatic ring system determines the extent of interactions with DNA base pairs. They examined small molecules that had a short cationic chain attached to aromatic ring systems to evaluate the importance of stereoelectronic effects on DNA binding. The direction and magnitude of the polarization of the aromatic ligand make it more likely to interact with the negative potential found in the DNA grooves and base pairs.

Ultimately in order to definitively describe the orientation of the peptide amide’s aromatic residue in its binding to DNA, NMR has to be done. In reference to our initial hopes for 19F NMR work, the lysyl-fluorophenylalaninamides are to be used as molecular probes in order to determine whether the ortho- and/or para- positions are inserted into
the hydrophobic center of DNA. $T_m$ and $\Delta n$ results from our studies indicate that the L-lysyl-fluorophenylalaninamides may not be suitable models for the study of peptide-DNA interactions because of the influence of fluorine on their binding to the polynucleotides. 2-D NMR experiments with control peptides L-lysyl-L-phenylalaninamide and L-lysyl-D-phenylalaninamide combined with D10-mer may serve as as better system to analyze. By using DNA oligomers that have already identified proton resonances, one can obtain NOE's to see the interconnectivities between the protons of the peptide amide and DNA. NMR can also provide the opportunity to confirm the mode of binding by examining the imino proton region. Upfield shifts of both A-T and G-C imino protons and some signal broadening are indicative of intercalation, due to shielding of the protons by the ring currents of the ligand. As for groove binding, downfield shifts of the A-T resonances result from deshielding of the protons. No spectral changes in the imino proton region suggest nonspecific outside binding to DNA.

Another area of studies involve obtaining calorimetric enthalpies, and thus using the data to calculate differential ion binding terms. This can be done using differential scanning calorimetry (DSC) to measure molar heat capacities for the denaturation of DNA. Heat capacity as a function of temperature is unique for a DNA sequence according to its base composition (Esposito et. al., 1997) Previously such experiments were difficult due to sample requirements, however with the availability of new instrumentation that allows microvolumes of DNA samples to be used, the experiments are now possible. Results from DSC can be compared to our results presented here as obtained from differential UV-absorption technique.
Our research was able to further illustrate the importance of particular DNA sequences in gene expression. The structural variations serve as recognition signals for DNA-binding proteins, thus bringing on the initiation or termination of transcription. Our peptide models with their inherent differences give rise to subtleties in their binding to the DNA duplex. The understanding of the fundamentals concerning the localized contacts collectively accomplished by non-covalent interactions opens the world of possibilities for taking advantage and applying such knowledge to design similar peptides for the manipulation of gene expression.
REFERENCES


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