Cancer and Stereoselective Solutions

by
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December 4, 1995

Abstract:

To describe the fibronectin molecule and its role in the metastatic process of cancer. To also show the rational behind the structure and the way it was reasoned. To define the method of development for RGD inhibitory molecule through knowledge of past experiments and conclusions. Substituting various amino acid residues into fibronectin like molecules to understand the effect each residue has in the overall stereochemistry and electrochemistry of the fibronectin binding site. Outlining a synthesis for one of more effective inhibiting peptide and the discussion of its binding abilities including receptor site affinity.

Introduction

Cancer is one of the leading killers in the industrialized nations. It stems from one initial growth and then spreads throughout the body to overwhelm the immune system and destroy the systems invaded. Cancers have a metastatic ability, and it is this ability that causes most cancer deaths[1]. If cancers consisted only of primary tumors, then most could be removed surgically or treated with drugs or other chemicals; however, cancers have an ability to move from one area to other unrelated areas of the body and start new growths. If the metastatic process could be slowed, or better yet halted, then a huge advancement in medical science would be made.

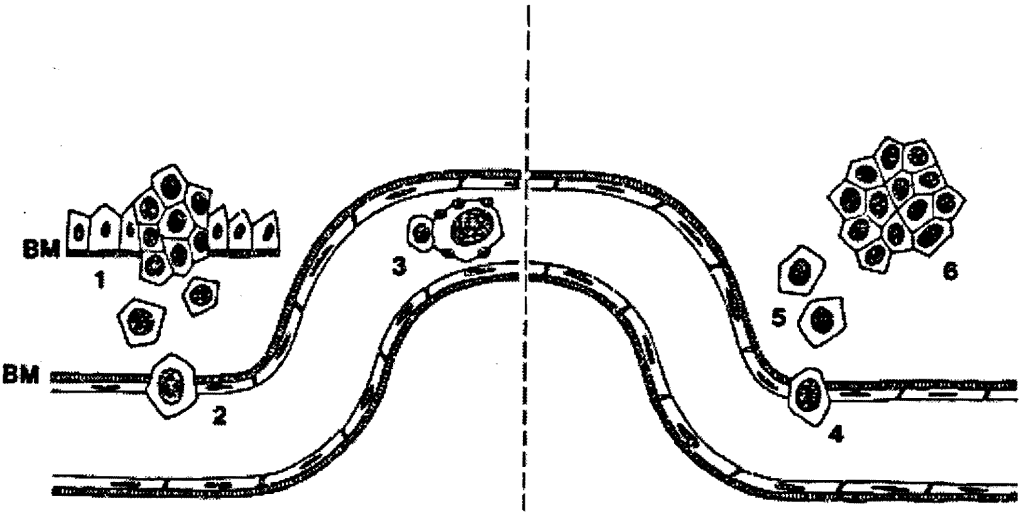
Cancer Metastasis

The metastatic process will be broken down into seven steps for simplicity . It should be noted that these steps are not always independent of one another and may occur simultaneously within the organism. These breakdowns are merely used to make each process more understandable; it is worth noting that if any of these steps are halted, then no secondary growth forms.

A neoplasm of unknown origin (see Figure 1) has some sort of initial (1a) local invasion, the cancerous cells must penetrate the neighboring tissue of the neoplasm. After leaving the neoplasm's environment, the cancerous cells must (1) detach from the primary tumor and survive. The cells may do this either as indivdually or as larger groupings of cells. In order for a dissemination of the metastic cells, the transformed cells must (2) invade local blood vessels and be (3) transported to other areas of the body via the circulatory system. The cells moving in the blood stream then must (4) lodge into the blood vessel wall. Experimentally, it has been shown that the arrest occurs most often in the capillary beds; it is assumed that arrest does not occur often in the larger vessels due to

blood shearing forces that the cells would encounter. The cells lodge at sites distal from the primary tumor in three ways, not always independent of one another. The cells may become physically lodged in a blood vessel or capillary bed due their size versus that of the vessel. Malignant cells may also adhere to blood vessel walls through a specific recognition. Experimentally, it has not been shown that malignant cells have an exclusive mechanism with any specific cell type; however, the cells may display selective adhesion to the blood vessel endothelium of certain organs. The lodgment occurs due to molecular interactions between the surfaces of the malignant cell and the vessel wall; however, the interactions are not exclusive of either adhering cells' surfaces, but rather selective for the surfaces. Once the malignant cells are lodged on the vessel wall, (5) extravasation of the malignant cell out of the circulatory system occur and the transformed cells migrate to a secondary site. Once migration is complete, continued (6) growth [2] must occur.

Figure 1 The Metastatic Process as described above[2]



Fibronectin and the Fibronectin Receptor

Fibronectin is a 440 kD extra cellular matrix protein identified important in the selective attachment step of the metastatic process. Fibronectin is composed of two similar polypeptide chains linked via a disulfide bridge near the carboxyl terminals. Fibronectin is important in the secondary growth of cancer, because it provides an attachment site for the transformed cells free floating in the circulatory system. Fibronectin has a major binding site composed of a three amino acid sequence. Through competitive inhibition, the Arginine-Glycine-Aspartic Acid-(Serine), also known as the RGD(S) sequence, has been proposed to compose the selective binding site.[2]

The fibronectin receptor is glycoprotein complex composed of an alpha and beta subunit, 150 kD and 130 kD, respectively. The fibronectin receptor itself is extremely variable with as many as ten alpha subunits and eight beta subunits. The variability allows the intergrin the ability to be apart of many cellular functions.[3] This variability makes the study of the receptor difficult, so the vast majority of the research is focused on the fibronectin itself.

Gaining Understanding of the RGD-Adhesion Site

Through experimentation, a smaller 75 kD peptide subunit containing the RGDS sequence has the ability to adhere moderately to the transformed cells, while a smaller 11.5 kD peptide fragment containing the RGDS fragment is not very effective in its adherence. From this observation fibronectin was proposed to have two, maybe even three, distinct binding sites for its adherence to metastatic cells.[4]

Trigramin, a snake venom, contains the RGD peptide and inhibits the attachment of transformed cells to fibronectin. From this it was deduced that the concept of two or three binding sites is faulty. The snake venom inhibits attachment yet it shares no relationship with adhesion molecules other than the RGD site. When the trigramin was reduced chemically, there was a loss of inhibitory effect, suggesting the secondary structure of trigramin was important in its binding abilities to the fibronectin receptor. It was shown

that the trigramin had a larger effect on adhesion than the straight GRGDS chain, and the GRGDS chain had a larger effect than that of the reduced trigramin.[5]

Table 1 The effect of trigramin on the adhesion of C32 melanoma cells to fibronectin [a]

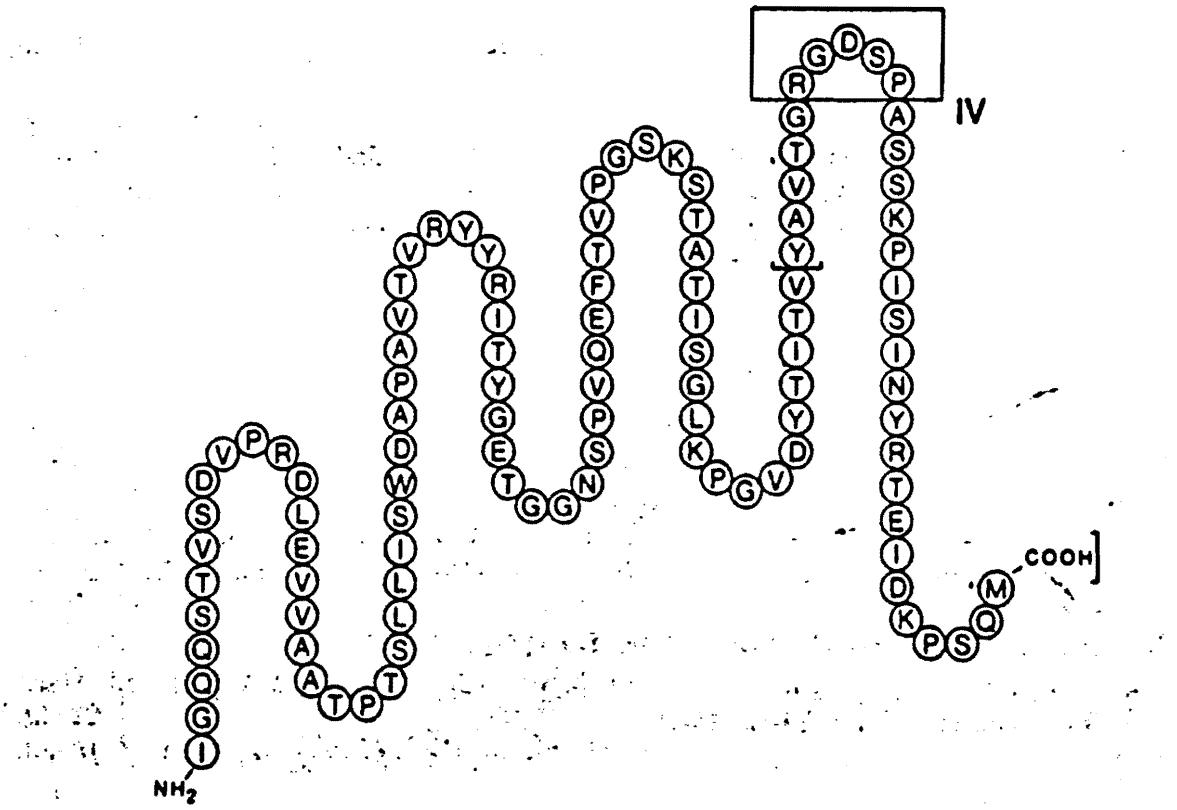
Treatment	Concentration	Adherent cells	Percentage Inhibition
1. No peptide	-----	386 +/- 60	-----
2. Trigramin	0.11 μM	55 +/- 26	86
3. Reduced trigramin	0.55 μM	299 +/- 60	23
4. GRGDS	50 μM	148 +/- 37	62

[a]The method of adhesion assay and value determinations are referenced in Knudsen, et.al.[5]

Predictions of the location of the RGDS adhesion site gives insight into the possible secondary structure of the attachment domain. Located in a hydrophilic portion of the attachment domain, the RGDS sequence may form a ß- turn at a hydrophilic loop at the surface of the molecule. At this external position, the RGDS structure is available to interact with cell surfaces.[6] The integrity of the RGDS sequence has to be maintained to insure activity of the peptide. If the arginine or the aspartic acids are deleted from the sequence, the activity is lost. The activity is also lost if the peptide is cleaved at the arginine, or if the glycine is substituted by a bulky valine residue. Deletions or cleavages cause a shift on the hydrophilic loop and other changes of secondary structure are expected

from the shift. [7] It is noted that a glycine substitution causes a loss of activity without shifting the sequence.

Figure 2 Prediction of secondary structure of 11.5 kD cell attachment domain of fibronectin with attachment signal in a ß turn hydrophilic loop (boxed region).[7]



Several synthetic peptides attached to protein coated plastic beads were tested for their adhesion inhibiting effect. All molecules that inhibited attachment had the RGDS segment, and larger molecules generally had greater inhibitory ability. The decreased activity with decreased size may be due to decreased stability of the smaller molecule or to the small molecule's inability to access the substrate. The RGDS straight chain tetrapeptide was attached to Sepharose beads at the end of a C_6 spacer arm; however, there was no

inhibitory activity displayed. Again, the small size may have limited accessibility, but also the coupling method may not be efficient.[7]

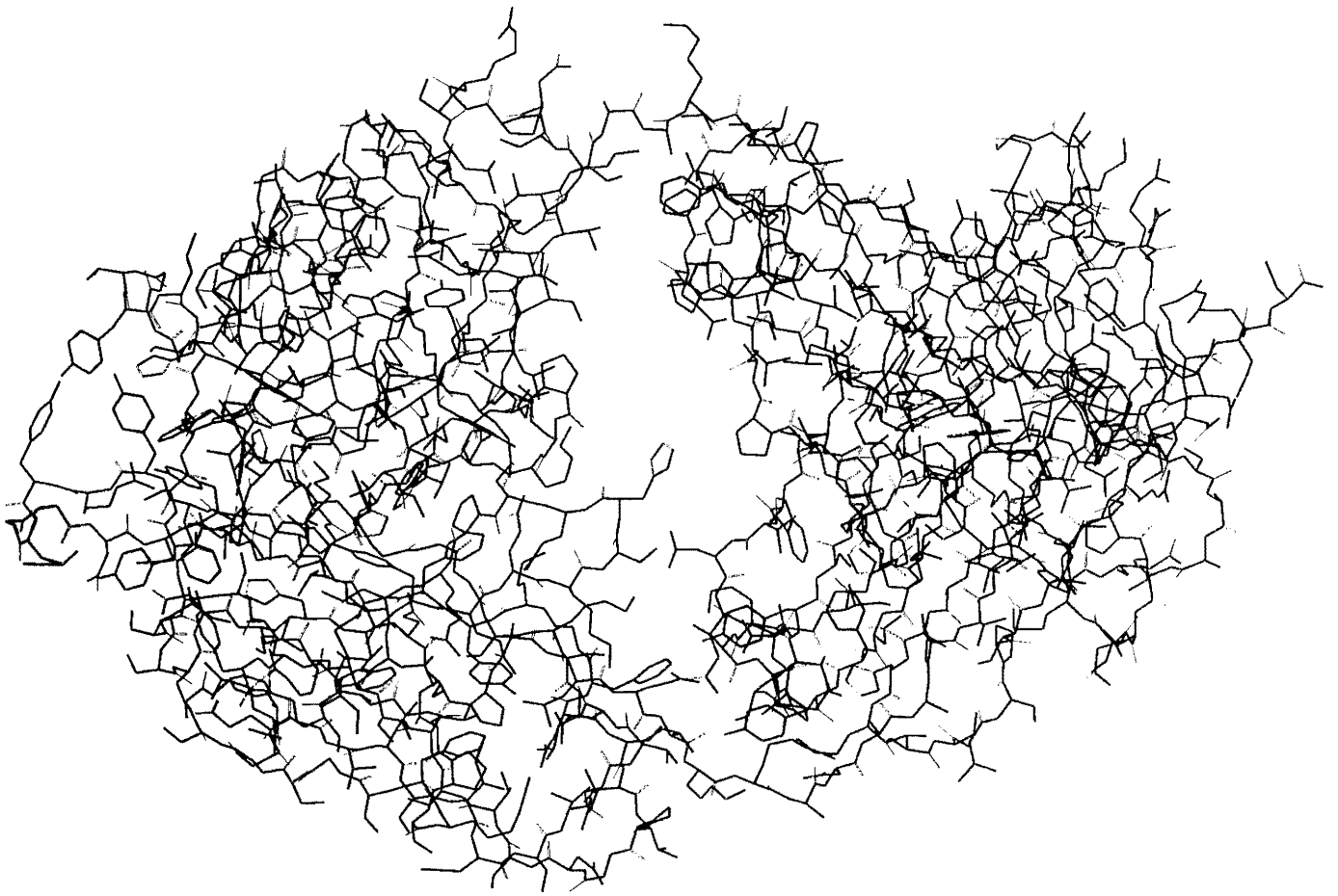
In other experiments, inhibition of cell attachment did not occur with soluble fibronectin or with attainable concentrations with a 30 amino acid sequence. It is presumed to be the low affinity of a single fibronectin molecules to cell surfaces. Large concentrations of the soluble fibronectin fragments are needed to overcome the interactions of extra cellular matrix fibronectins. Smaller more highly soluble peptides are more likely to have activity at lower concentrations because less interaction takes place between the fibronectin fragments and the extra cellular matrix fibronectin.[7] However, testing many different sequences and combinations of sequences show that poly (RGD) molecules have the higher activity on weight basis in the inhibition of adhesions than the oligo peptide or the monomer.[8] CD spectra analysis determined the poly(RGD) sequence is a more ordered sequence than oligo(RGD) or poly(R,G,D), with the sequence of R,G,D being repeatedly rearranged. The CD spectra analysis using a computer program of Chang *et.al.* determined the sequence structure using an average helical segment of 11 residues. The poly(RGD) molecule has the most ß-turns, with ß-turns being predicted in all poly(RGD) regions; thus giving the poly(RGD) sequence the secondary structure most favored by the fibronectin receptor.[6]

A crystal structure has been determined of an OPG2 Fab molecule with a RYD adhesion site that mimics the RGD adhesion site. The entire molecule consists of two protein strands with 214 and 227 amino acids, respectively.[9] From this structure, further study of the binding site and future inhibitor development may follow. The OPG2 Fab molecule is not fibronectin, rather it is a mouse immunoglobulin that shares a similar binding site with fibronectin and other adhesion molecules. The adhesion site in the Fab molecule is an arginine-tyrosine-aspartic acid (RYD) sequence, yet it mimics the RGD sequence found in adhesion molecules. From previous experiments, the structure of the sequence has proven to be extremely important in the activity of the molecule. The OPG2

Fab molecule presents a clear crystalline structure of the binding site, conformation, and secondary structure.[10] The resolution of the Fab molecule is 2 A considered to be impressive for a molecule of this size. The structure of the OPG2 Fab molecule (Figure 3 and Figure 4) was acquired from the Protein Data Bank at the Brookhaven National Laboratory, Upton, New York.[9] The structure was displayed using Sybyl Molecular Modeling Software, version 6.2 Beta, 1995 (Tripos Associates, Inc., St. Louis, Missouri 63144) on a EVS workstation (Evans and Suthreland, Salt Lake City, Utah).

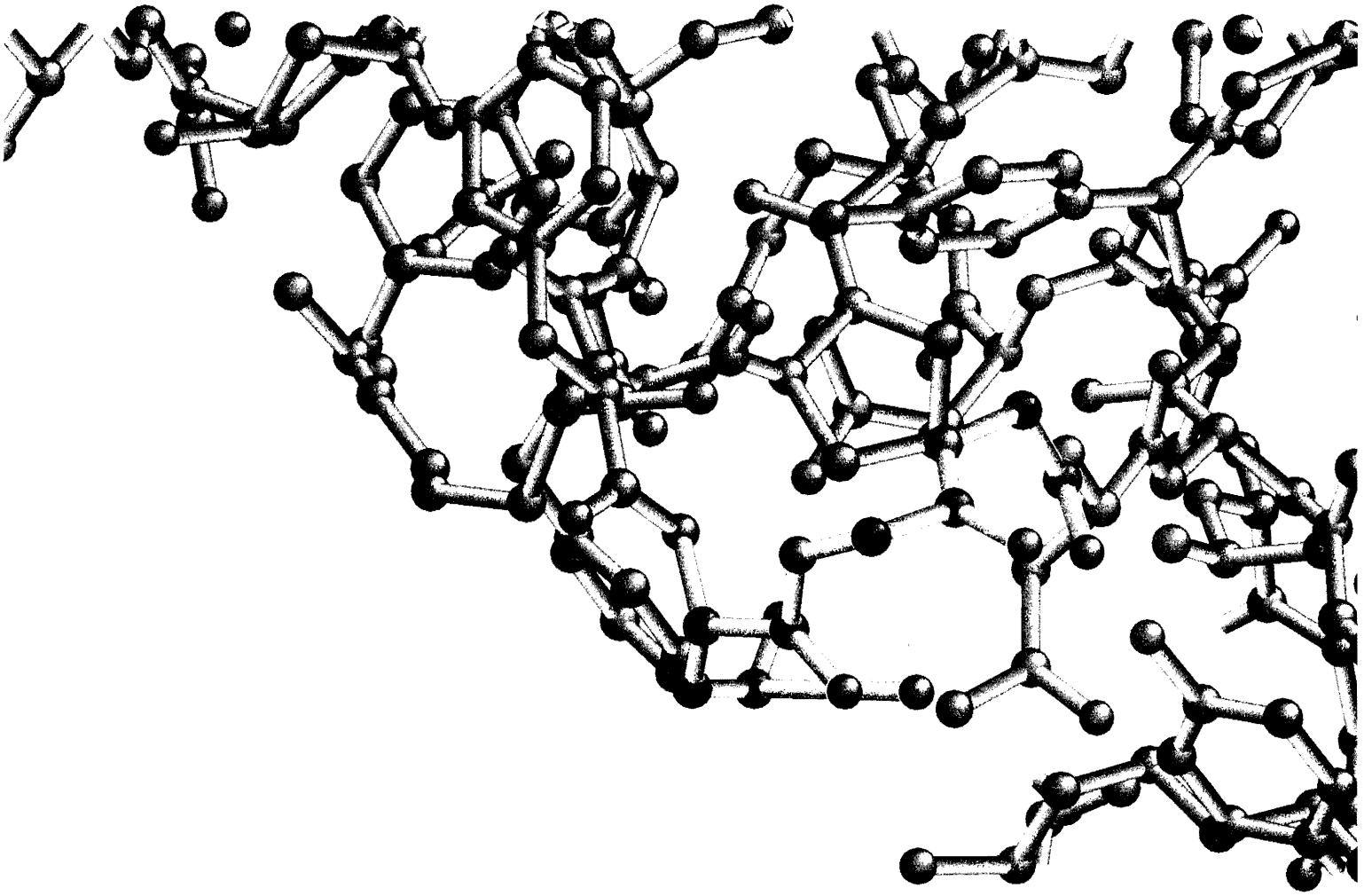
The following is a detailed view of the specific RYD binding site. The binding site has a tyrosine that replaces the glycine of the RGD sequence; however, the backbone stereochemistry of the site is much more important than the effects of the replacement. As can be noted from Figure 3 the RYD binding site is on the external surface of the molecule with little other steric hindrances in close proximity to the site. Figure 4 closely demonstrates the openness of the binding site, as well as the extreme stereoselectivity of the binding site. It can also be noted the pocket behind the RYD sequence open for binding. From this OPG2 Fab crystalline structure, a more directed development of adherence inhibitors may be synthesized in conjunction with other experimental data and molecular modeling.

Figure 3 OPG2 Fab Mouse Immunogloblin with RYD site[a] that mimics RGD site of adhesion molecules



[a] attained from Protein Data Bank, Brookhaven National Laboratory, Upton, New York.

Figure 4 RYD binding site of OPG2 Fab Mouse Immunogloblin[a]



[a] [a] attained from Protein Data Bank, Brookhaven National Laboratory, Upton, New York.

Specific Stereoselective Synthesis

From previous data and knowledge, specific synthetic peptide have been proposed for development. To test the stereochemistry of the binding site, a RGD peptide was synthesized with the residues following the aspartic acid (D) being varied to detect the contribution of each. In the primary RGD sequence the L forms of each amino acid were substituted with their D forms to test the structure stereoselectivity at the binding site. Finally a peptide sequence underwent cyclicazation to prevent flexibility hoping that the affinity of the entire molecule might be increased by a strict conformation. Various residues following the main binding domain in the cyclic RGD were tested, and different activities were noted from each; thus leading to the conclusion that residues following the RGD sequence influence the stereochemistry of the RGD backbone segment itself. From the cyclic molecule, the greatest specificity and adhesive ability was noted. The greater activity was noted from the less flexible conformation, the more stable and specific RGD sequence.[11]

Through specific synthesis for a cyclic pentapeptide, a ß- turn was by changing one of the amino acid residues from a L conformation to that of a D conformation with internal stabilization coming from hydrogen bonding within the peptide backbone.[12] As shown by contrasting Figure 5 and Figure 6, the importance of an amino acid exchanging the L conformation for the D conformation is shown. In Figure 6 there are not the double hydrogen bonding and no ß-turn as demonstrated in Figure 5.

Figure 5 RGDFV cyclic amino acid with all residues in L conformation except the Phe (F) group

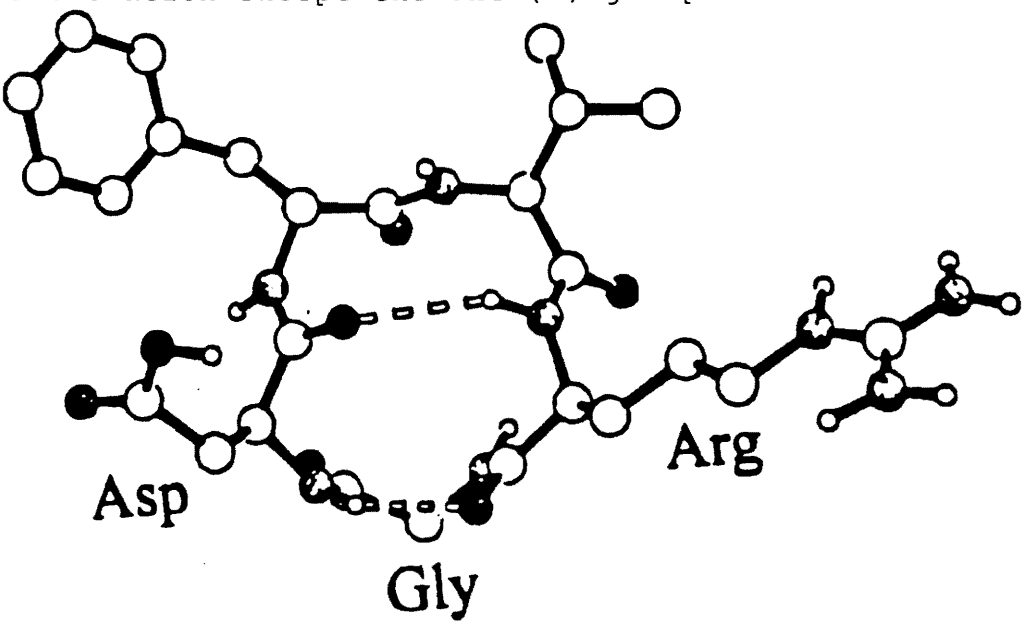
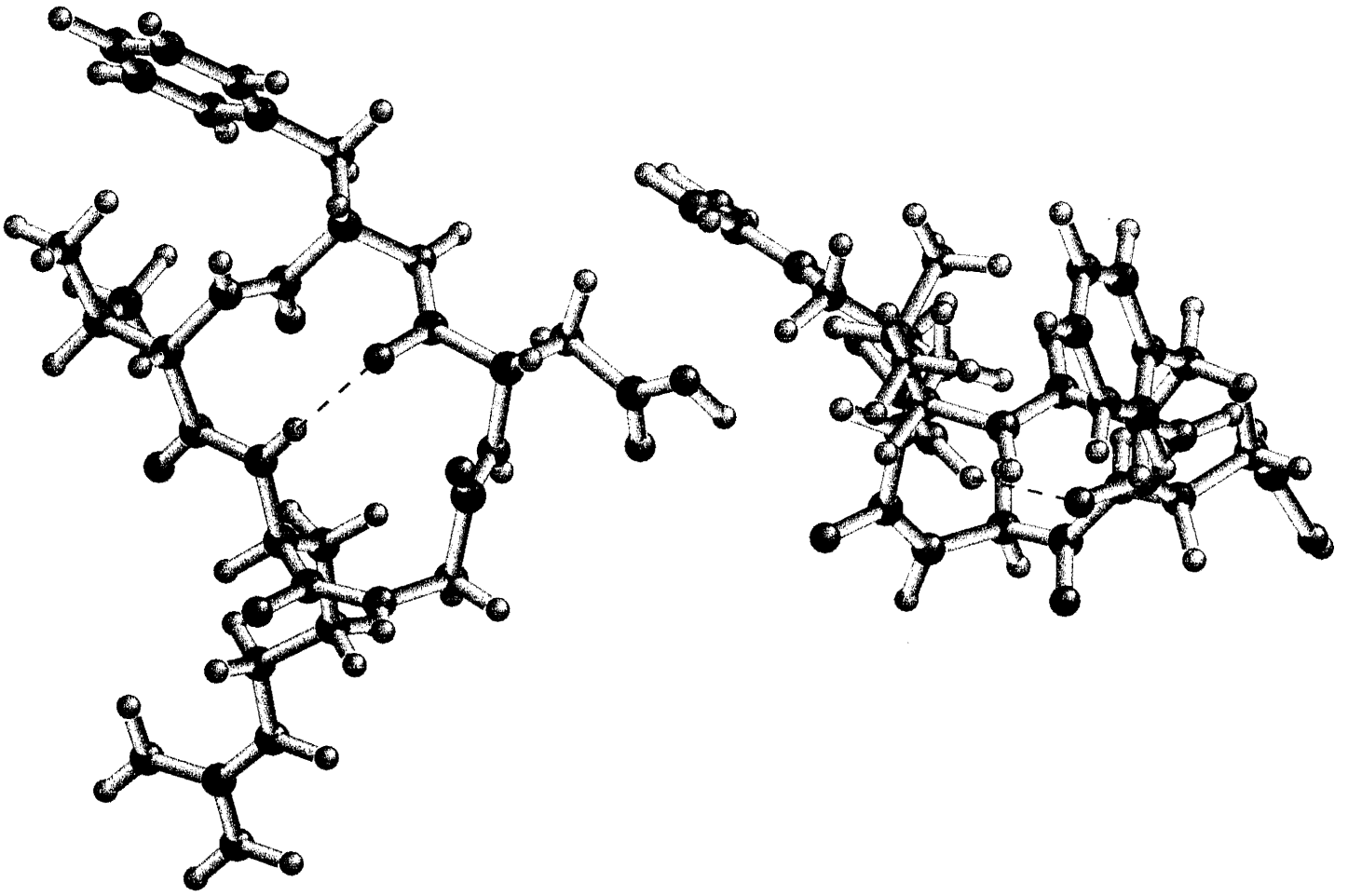


Figure 6 RGDFV cyclic amino acid with all residues in L con conformations

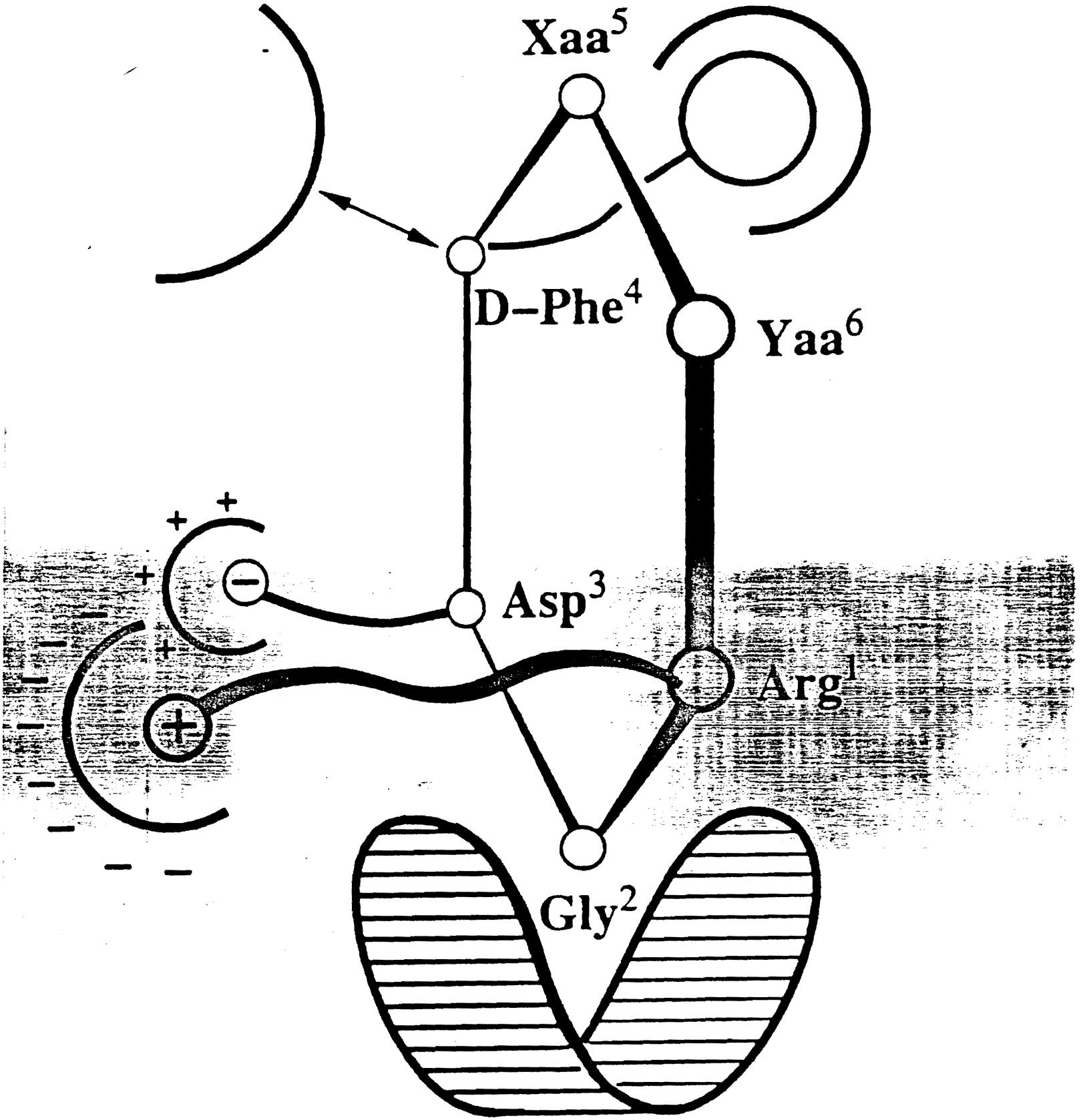
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Using molecular dynamic simulations, Gurrath *et al.*(13) refined the pentapeptide synthesis to also include hexapeptide analogues to describe more comprehensively the structure/activity relationship. Cyclic molecules are used to introduce conformational constraints required of receptor binding and activity; six membered rings are synthesized to test their inhibitory activity versus those of the tested five member cyclic rings. It was found that the six membered rings were less active and less selective than the five member cyclicpeptide. The correlation between structure and activity indicate that it was ring contraction, not the position of the RGD section in a hydrophilic loop, that increased the affinity for adhesion.[13] RGD positioning within the fibronectin's ß-turn no longer explained the importance of the cyclic compound, rather ring contraction appears to be important. Again both conclusions validate one another to a degree; however, the work of Gurrath showed that simply a turn was not the only requirement needed to synthesize an effective inhibitor.

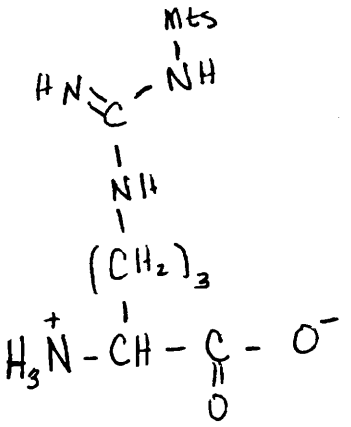
Through experimentation and various substitution, the positive and negative side chains of the arginine and aspartic acid are found to be necessary functionalities for binding. Equally important is the unmodified glycine spacer amino acid.[13] Shown in Figure 7, positive and negative charges are needed for strong affinity to the receptor site. Glycine is important to the binding capability of the RGD sequence, since a large side chain from the glycine position may cause receptor repulsion in a streically demanding binding pocket. A narrow binding cleft is postulated and any large substituents would not allow tight binding, if binding occurs at all. All steric modifications of the arginine or aspartic acid lead to reduced binding efficiencies. Some studies have shown the ability to bind such molecules, yet this binding is probably due to a compensation of the torsional freedom in the amino acid side chains themselves.

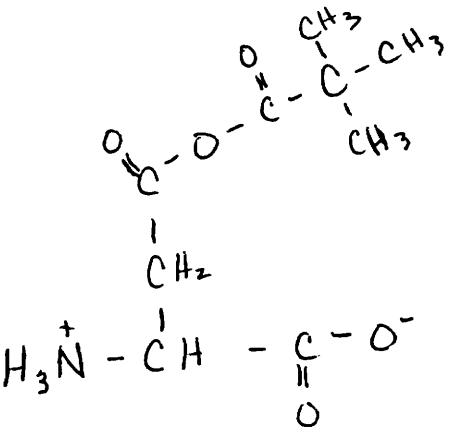
Figure 7 Electrical and steric requirements for binding



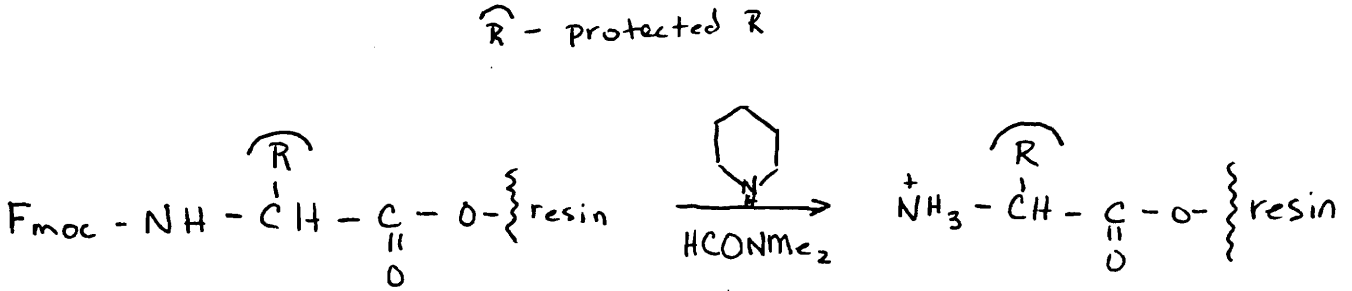
Synthesis of cyclic RGDDFV pentapeptide

The synthesis was carried out on a Sasrin® resin solid phase using 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr) as the arginine guanidino function protecting group, and *t*-butyl ester as the aspartic acid ~~the~~ side chain protector. No other protectors are needed for the remaining side chains.

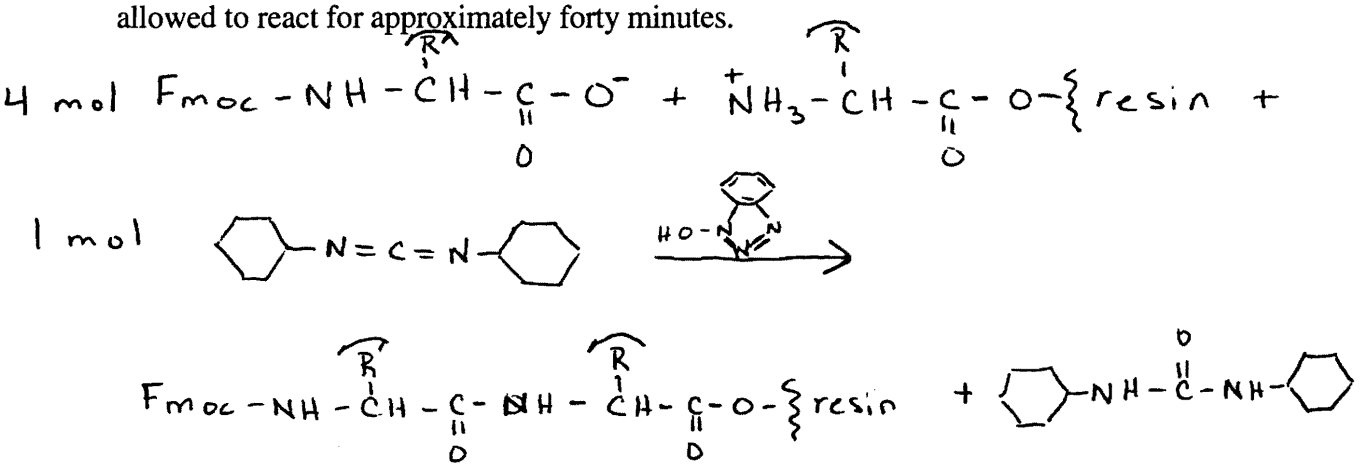




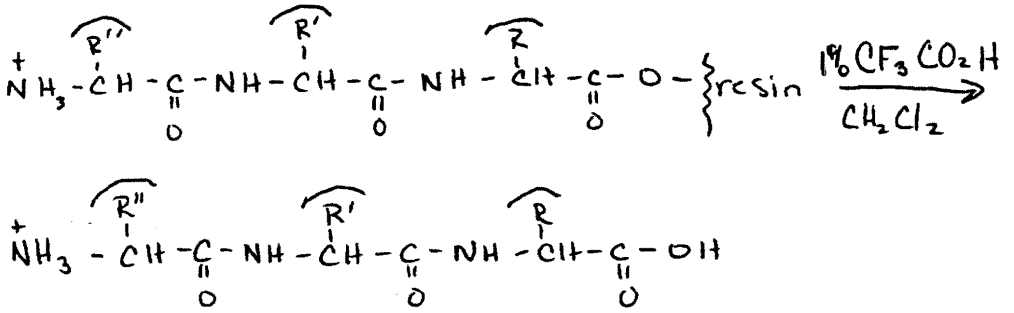
In the synthesis the washings are important to insure that the peptide is made up of only added material not of any waste material from previous steps. The first step of the synthesis is two washings for three minutes with $HCONMe_2$, (N,N-dimethylformamide). The second step is prewashing in 20% piperidine in $HCONMe_2$ for three minutes also. A deprotection of the amino acid derivative is carried out in 20% piperidine in $HCONMe_2$ once allowing the reaction to proceed for fifteen minutes, followed by ten, three minute washings in $HCONMe_2$.



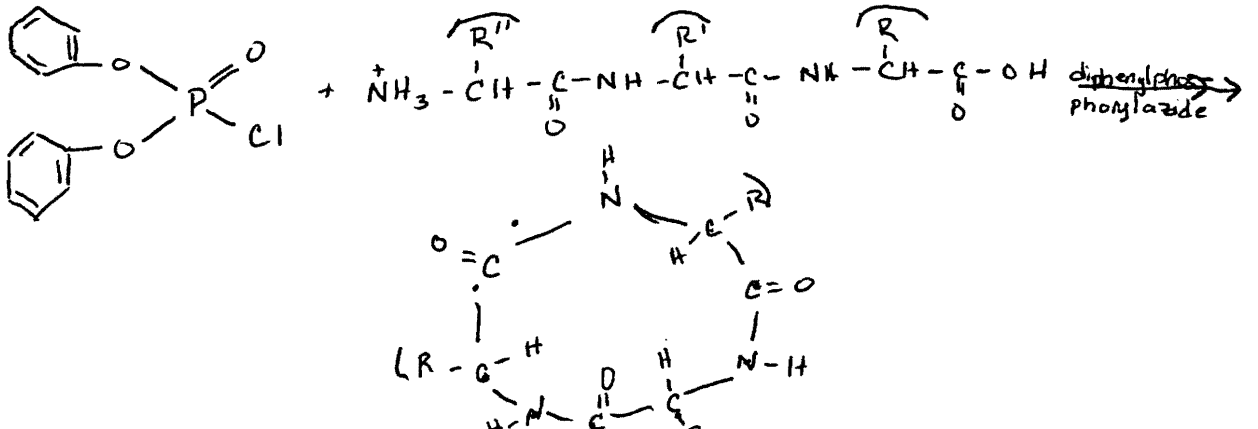
The coupling of the amino acid is four moles of Fmoc (9-fluorenylmethoxycarbonyl) protected amino acid derivative, and one mole of the coupling reagent of 1,3-dicyclohexylcarbodiimide 1-hydroxybenzotriazole, with C_4H_6ONMe (1-methyl-2-pyrrolidinone) as the basic component in $EtN(iPr)_2$ (N,N-diisopropylethylamine). It is allowed to react for approximately forty minutes.



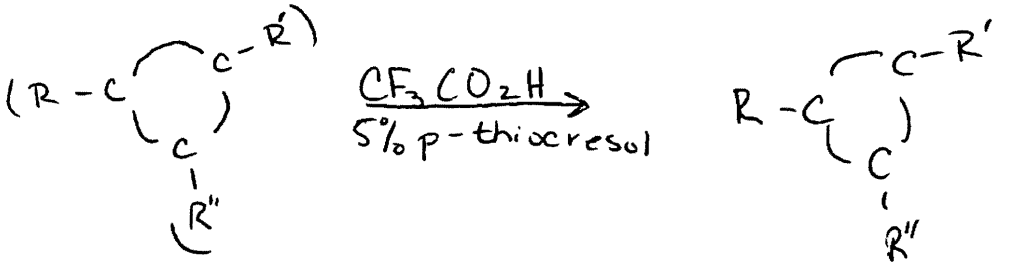
This is followed by ten, three minute washings in $HCONMe_2$ followed by two, three minute washings in dichloromethane. The entire procedure is repeated until the length of the chain is produced. The chain is cleaved from the resin using 1% trifluoroacetic acid in dichloromethane (1:1:3). Under these conditions the side chain protecting groups are stable.



The cyclicazation was performed in N,N-dimethylformamide with 1.5 moles of amino acid chain in one mole diphenlyphosphorylazide at high dilution for four days.



The pH was maintained at 8.5- 9.0 by additions of triethylamine. The side chain protecting groups were removed with trifluoroacetic acid and a scavenger of 5% *p*-thiocresol.



The peptide was purified in high performance liquid chromatography with a reverse phase C_{18} 3-μm material using a gradient of water/CH_3CN/trifluoroacetic acid (95 : 5 : 0.2 to 20 : 80 : 1.0) over a fifteen minute period. The peptides were characterized by fast atom bombardment mass spectroscopy, amino acid analysis and various NMR techniques.[13]

Future Developments

With continued work and development, cyclic pentapeptides may have a place in cancer treatment one day. Further refinement of the binding site stereochemistry is important, as well as, the fragments' affinity to the fibronectin receptors of the cancer cells.

References

(1) Fidler, I.J. In *Metastasis;* Bock, G.; Whelan, J., Eds.; John Wiley and Sons Chichester, 1988; p. 1.

(2) Evans, C. W. *The Metastatic Cell;* Chapman and Hall: London, 1991; pp. 170-186; pp.364-375.

(3) Albelda, S. M. *Adhesion Molecules;* Academic: London, 1994; Chapter 4.

(4) McCarthy, J. B.; Hagen, S. T.; Furcht, L. T.; *J. Cell Biol.* **1986**, *102,* 179-188.

(5) Knudsen, K. A.; Tuszynski, G. P.; Huang, T-F.; Niewiarowski, S.; *Expl. Cell Res.* **1988**, *179,* 42-49.

(6) Murata, J.; Saiki, I.; Ogawa, R.; Nishi, N.; Tokura, S.; Azuma, I.; *Int. J. Peptide Protein Res.* **1991**, *38,* 212-217.

(7) Pierschbacher, M.D.; Ruoslahti, E.; *Nature* **1984**, *309*, 30-33.

(8) Saiki, I.; Iida, J.; Murata, J.; Ogawa, W.; Nishi, N.; Sugimura,K.; Tokura, S.; Azuma, I.; *Cancer Res.* **1989**, *49*, 3815-3822.

(9) Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

(10) Kodandapani, R.; Veerapandian, B.; Kunicki, T. J.; Ely, K. R.; *J. Biol. Chem.* **1994**, *270*, 2268-2273.

(11) Pierschbacher, M.D.; Ruoslahti, E.; *J. Biol. Chem.* **1987**, *262*, 17294-17298.

(12) Aumailley, M.; Gurrath, M.; Müller, G.; Calvete, J.; Timp, R.; Kessler, H.; *FEBS Lett.* **1991**, *291,* 50-54.

(13) Gurrath, M.; Müller, G.; Kessler, H.; Aumailley, M.; Timpl, R.; *Eur. J. Biochem.* **1992**, *210*, 911-921.

(14) Müller, G.; Gurrath, M.; Kessler, H.; *J. Comput.-Aided Mol. Design,* **1994**, *8,* 709-730.