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Synthesis of a Dityrosine-Linked Protein G Peptide Dimer

Ask Porino Va about the relevance of his research, and he will tell you this: "My project is unique because it concerns the use of organic chemistry in synthesizing potential drugs of the future." Porino plans to continue his education in organic chemistry by attending graduate school, and eventually find his own niche working in industry. His talents aren't limited; when not working in organic chemistry, Porino enjoys playing the guitar and bass. He's also a fan of basketball, backpacking and snowboarding.

Abstract

Beta sheet interactions between proteins are crucial within the body. Beta peptide dimers may be able to cooperatively disrupt undesirable beta sheet interactions. We have synthesized a dityrosine-linked peptide dimer based on the B1 binding domain of Staphylococcal protein G (residues 17-21, LKGET). The monomers were prepared using Solid phase peptide synthesis, and dimerization was achieved through the use of horseradish peroxidase in a pH 9 borate buffer. The dimer was then characterized using HPLC, fluorescence and ^{1}HNMR. This dimer may be able to disrupt the binding of protein G to the Fc domain of immunoglobulin G. This study is concerned with the synthesis and the investigation of secondary structural properties of the protein G dimer mimic [LY*GET]$_2$, where Y* is a dityrosine crosslink. Initial ROESY data shows that the dimer adopts a structure in which the beta protons of tyrosine and the gamma protons of glutamic acid are near each other. Future research plans include scaling up to generate enough dimer for further ^{1}HNMR studies and disruption assays.

Faculty Mentor

Porino is one of a new generation of chemists using organic synthesis to engineer models of biological interactions, atom by atom and bond by bond. Some proteins in the human body form carbon-carbon bonds between tyrosine sidechains. These dityrosine crosslinks exhibit great resiliency towards typical mechanisms of cleavage, either chemical or biological. In spite of their persistent nature, we know virtually nothing about how dityrosines affect local peptide structure or how we might use them to control the shapes and interactions of peptides. Porino's motivation and persistence brought us closer to our long-term goals of understanding dityrosine crosslinks. The frontiers of science and technology continue to expand, but not beyond the reach of UCI's undergraduates. The faculty mentoring system at UCI provides students like Porino with the opportunity to reach those frontiers in a relatively short period of time.

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School of Physical Sciences

Key Terms

- Beta Peptide Dimer
- Beta Sheet Interactions
- Cation Scavenger
- Peptide Secondary Structure
- Protecting Groups
- ROESY NMR
- Side Chains

Introduction

Protein-protein interactions through the edges of beta sheets are crucial in many biological processes. Some of these interactions may lead to harmful effects in the body. Thus, the control of such interactions would be an enormous contribution to medicine. A beta peptide dimer consists of two identical, hydrogen-bonded peptide strands that are connected by a biaryl crosslink (Figure 1A). In a beta peptide dimer, hydrogen bonds between the two inside edges should transmit polarization from one outside edge to the other (Figure 1B). Thus, binding to one outside edge should strengthen binding to the other outside edge. Beta peptide dimers may be able to cooperatively disrupt these beta sheet interactions. This study explores the use of dityrosine cross-links to enforce antiparallel beta structures in peptide dimers. The broader goal of this study is to use the beta sheet interaction of protein G (GB1) and immunoglobulin G (IgG) as a model system for testing the hypothesis that a dityrosine dimer causes cooperative disruption (Figure 2).

Dityrosines are found in many naturally occurring long-lived proteins, such as collagen (tendons, cartilage and skin), keratin (fingernails), resilin (insect exoskeleta), and fibroin (wool and spider silk) (Amado et al., 1984). Under photo-oxidative conditions tyrosine and tryptophan residues, found naturally in many peptides, can dimerize to yield rigid biaryl crosslinks. Dityryptophan crosslinks are fluorescent and have the potential to form insoluble aggregates and well-defined secondary structures (Stachel et al., 1998 and 1996). This insolubility is implicated as one of the underlying causes of cataracts (Sell and Monnier, 1995). Dityrosines and ditryptophans are covalent dimers and there is no clear mechanism available for enzymatic cleavage of the central C-C bond (Figure 3). Thus oxidative biaryl crosslinking accumulates over time, compromising the structure and function of long-lived proteins. Furthermore, these highly fluorescent dimers can be used as markers for aging (Giulivi and Davies, 1994).
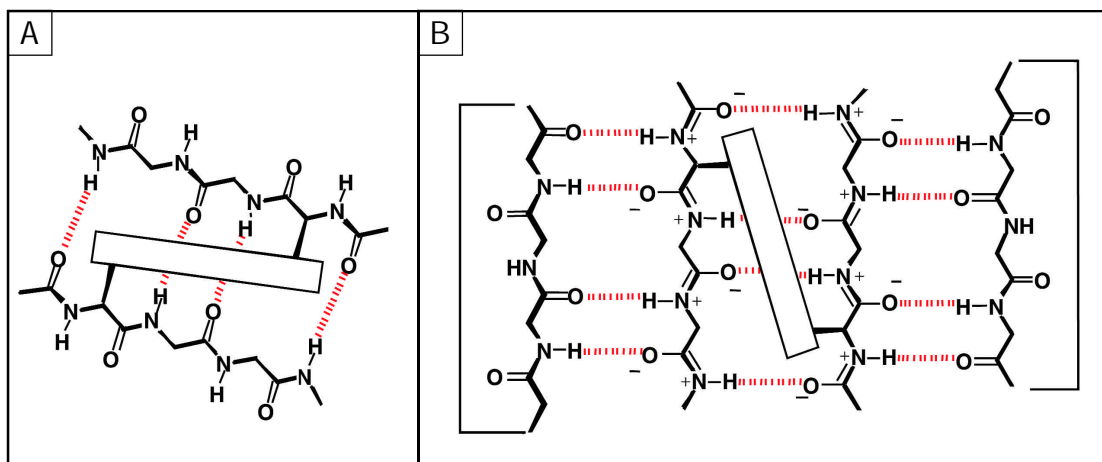

Figure 1

(A) A beta peptide dimer in beta sheet conformation. (B) A beta peptide dimer transmitting polarization from one outside edge to the other. The rectangle represents the biaryl cross-link and the dashed lines indicate hydrogen bonding.
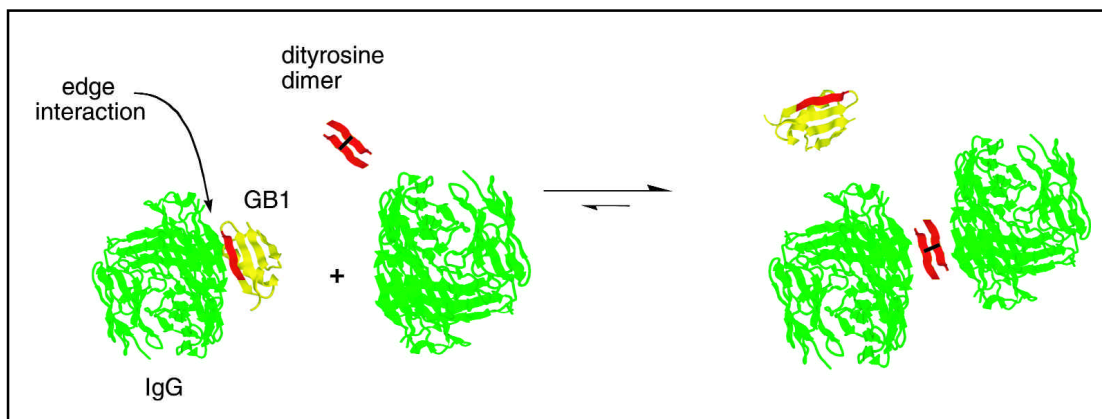

Figure 2

Dityrosine dimers may be able to disrupt undesired beta sheets resulting from edge interactions. The beta sheet interaction of IgG and GB1 will serve as the model system for testing disruption.

The exact effect of dityrosine dimers on controlling peptide secondary structure (the 3D shape that the protein adopts in solution due to features such as beta sheets and gamma turns) is not well understood. However, it is known that ditryptophans, which are similar to dityrosines in that they are both biaryl cross-links, can control local
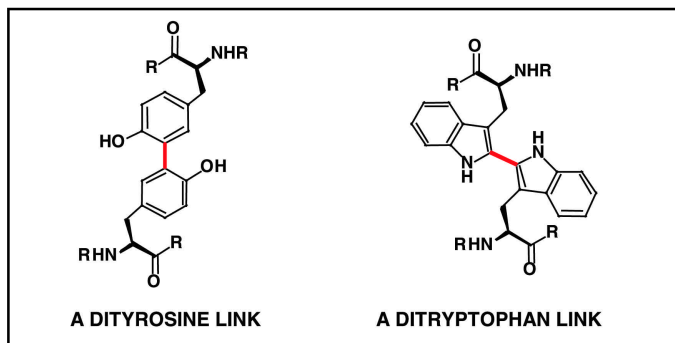
Figure 3
General structure of dityrosine and dityptophan crosslinks. The central C-C bond is shown in red.

peptide conformation. Intramolecular dityptophan crosslinks of the general sequence Trp-Xxx-Trp (Xxx ≠ Gly) can stabilize an inverse gamma turn (90° turn of the peptide strand) in DMSO-d_6 (Figure 4A) (Stachel et al., 1998). Gamma turns are important because they redirect the peptide chain and can ultimately contribute to the specific secondary structures adopted by proteins in solution. Dityptophans can also form antiparallel beta-sheets, which are commonly found in the binding sites of protein-protein interactions (Figure 4B).

When replacing a residue with tyrosine, two important factors must be considered: the connective nature of dityrosines and the significance of the residue in adding stability to the beta sheet.

The connective nature of dityptophans allows the placement of the tryptophan residue in the center of the monomeric sequence. The distance between the two strands of the dimer is dictated by the length of the biaryl cross-link. A longer biaryl cross-link increases the distance between the two strands and decreases the likelihood of hydrogen bonding. Because the dityptophan cross-link is relatively short, it can be placed in the center of the sequence without compromising hydrogen-bonding distances (Figure 5A). A dityrosine cross-link is much longer than a dityptophan crosslink; therefore, tyrosine must be placed at an off-center position in the monomeric sequence to preserve hydrogen bonding (Figure 5B).

The amino acid side chains of the binding sequence of a beta sheet can play an important role in its stability, and
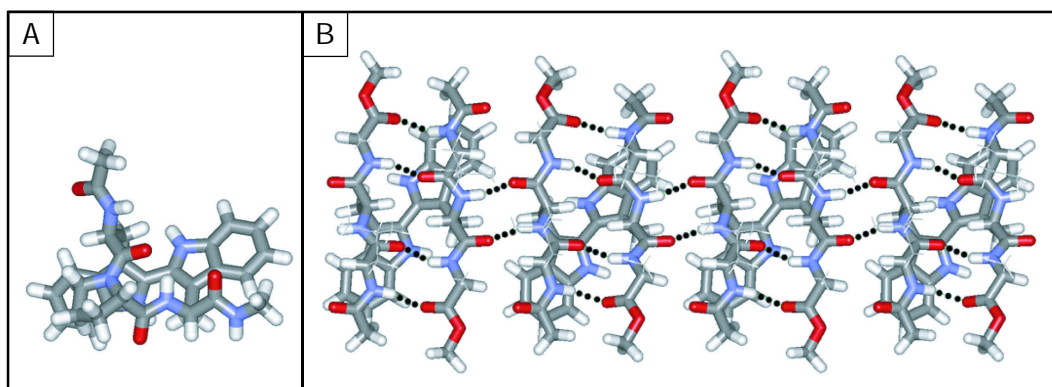
Methods and Discussion

Dityrosine Dimer Design

Using the crystal structure of the relevant portion of protein G (GB1) binding site to immunoglobulin G (IgG), the binding sequence of GB1 was determined to be Leu-Lys-Gly-Glu-Thr. The objective behind the design of the dityrosine dimer was to incorporate a tyrosine crosslink into a monomeric sequence similar to the binding sequence of GB1 and dimerize the resulting monomer. An important issue that had to be considered was which residue of the binding portion of GB1 should be replaced with tyrosine.

Figure 4
(A) Intramolecular dityptophan stabilizing an inverse gamma turn in DMSO. (B) Crystal structure of a dityptophan dimer adopting a beta sheet conformation. Color key: white=H, gray=C, red=O, and blue=N).

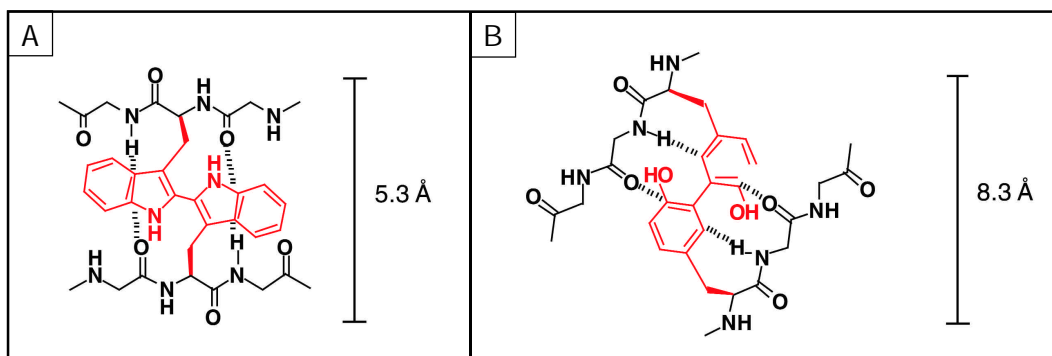
Figure 5
(A) Central placement of the shorter dityptophan cross-link in a peptide dimer. (B) Off-center placement of the longer dityrosine cross-link in a peptide dimer.

therefore influence the formation of the beta sheet. Important side chains of GB1 are the protonated amine of lysine (Lys) and the deprotonated carboxylic acid of glutamic acid. These charged side chains might interact with sites of opposite charge on IgG, which would add stability to the beta sheet. Close examination of the crystal structure revealed that these

Figure 6
Replacement of Lys in GB1 with Tyr and dimerization of the resulting monomer to obtain the dityrosine peptide dimer.

interactions do not occur and that either Lys or Glu could be replaced without impacting the beta sheet stability. Lysine was arbitrarily replaced and the target monomeric sequence became Leu-Tyr-Gly-Glu-Thr. Dimerization of this monomeric sequence yielded the dityrosine peptide dimer (Figure 6).
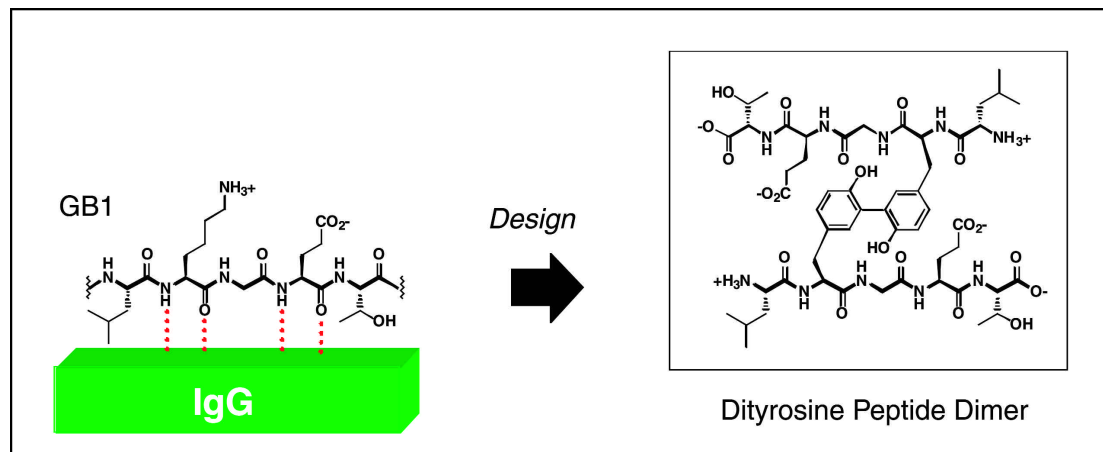
Solid Phase Peptide Synthesis
Solid phase peptide synthesis (SPPS) was employed for the synthesis of the monomer because of the efficiency of the technique. In SPPS, an insoluble resin bead is attached to a linker, which is attached to the C-terminus of the peptide. Fmoc-amino acids are coupled to the N-terminus of the peptide on the resin bead. The Fmoc group is a protecting group, a removable piece of the molecule that prevents a functional group on the peptide from participating in undesired side reactions. After the removal of the Fmoc protecting group, the next Fmoc protected amino acid is coupled. This procedure was repeated, attaching and deprotecting each amino acid one by one, until the desired peptide was made (Figure 7). The insoluble bead affords an efficient way to separate the peptide from the excess reagents used in the couplings by rinsing the beads.

Cleavage and Deprotection
Once the entire peptide was synthesized and the last deprotection was performed, the peptide was cleaved from the resin. During resin cleavage, the three side chain protecting groups (Ot-Bu) were removed simultaneously, generating a large amount of tertiary carbocations. Triisopropyl silane (TIS) and water were used as cations during the removal of side chain protecting groups (Figure 8). These cations would otherwise participate in potential side reactions with any nucleophilic sites of the peptide. Purification of the monomer was achieved by High Performance
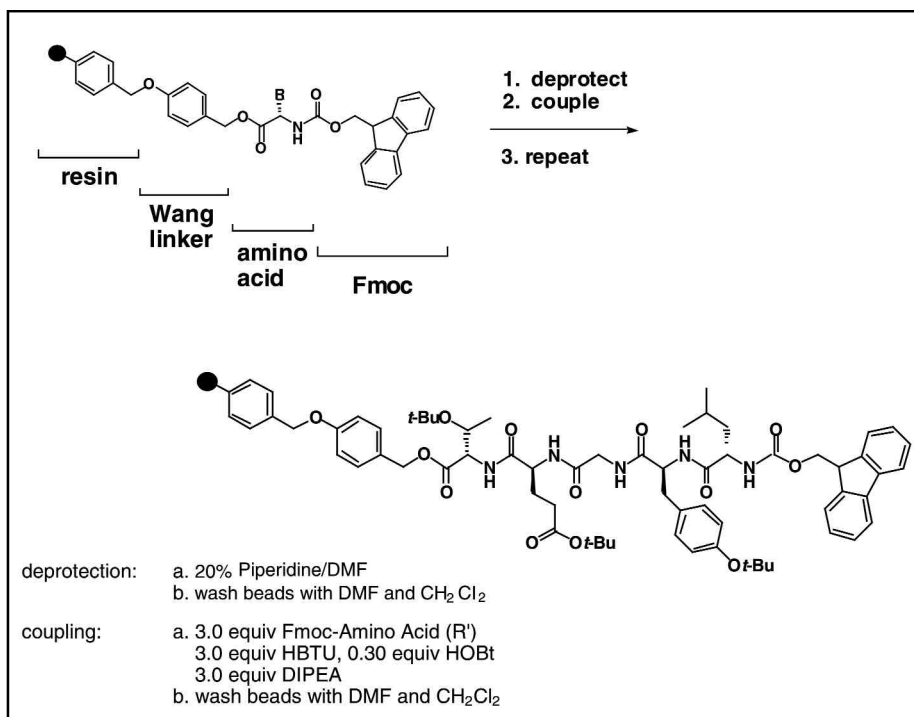

Figure 7
The monomeric sequence of Leu-Tyr(Ot-Bu)-Gly-Glu(Ot-Bu)-Thr(Ot-Bu) was synthesized using the general deprotection and coupling cycle of SPPS.

Liquid Chromatography (HPLC).

Enzymatic Peptide Dimerization
Dityrosines can be prepared under a variety of conditions. For example, dimerization can occur via oxidative coupling with reagents such as VOF$_3$. Alternatively, dimerization can occur via reductive coupling utilizing potassium hexacyanoferrate(III). These methods can dimerize simple tyrosines, but fail to dimerize full-length peptides. The most effective full-length peptide dimerization methods use enzymes. Dimerization of the monomer employed the enzyme horseradish peroxidase (HRP) in a pH 9 borate buffer (Eickoff et al., 2001).

HRP, a relatively low molecular weight peroxidase, has a heme cofactor, an Fe(III) center bound to a porphorin ring that is in turn attached to HRP's peptidic structure. Hydrogen peroxide can react with this heme to generate the ferryl (Figure 9), which can directly or indirectly, via HRP's peptidic structure, react with the tyrosine residue of the monomer to generate phenoxy radicals (Figure 10). After the coupling of two phenoxy radicals and tautomerization, the complete protein G peptide dimer is formed (Figures 11 & 12).

During dimerization the yield can be depleted by the formation of many side products. For example, trimers can form from further enzymatic coupling of a dimer and a monomer. Therefore, it was necessary to monitor the reaction every 30 min by HPLC so that the reaction could be quenched before the rate of side product formation exceeded the rate of dimerization. After 3.5 h, the reaction was quenched (Figure 13). The excess borate salts, present in the reaction after the evaporation of water, were removed by dialysis. The crude dimer was then purified using HPLC.
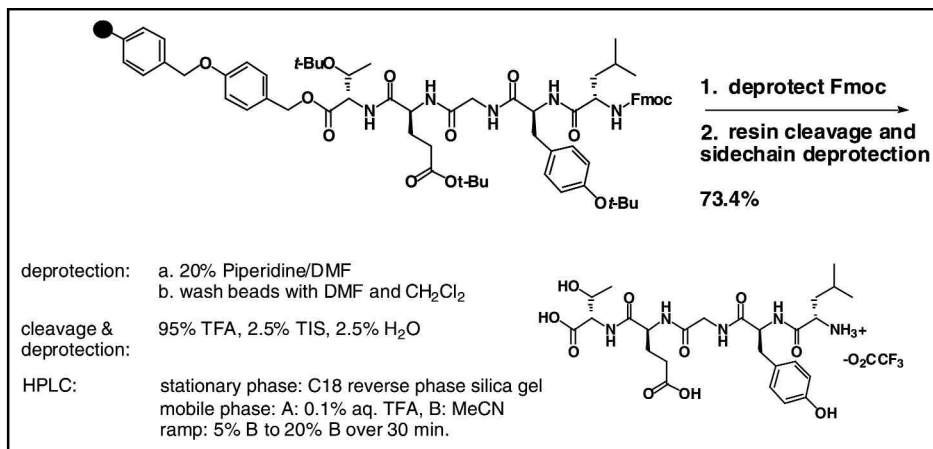

Figure 8
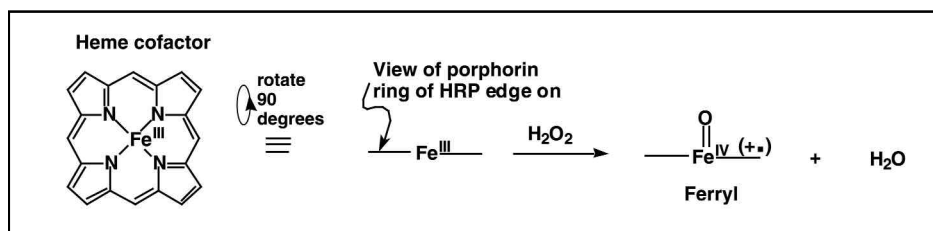Cleavage, deprotection, and HPLC purification of the monomer
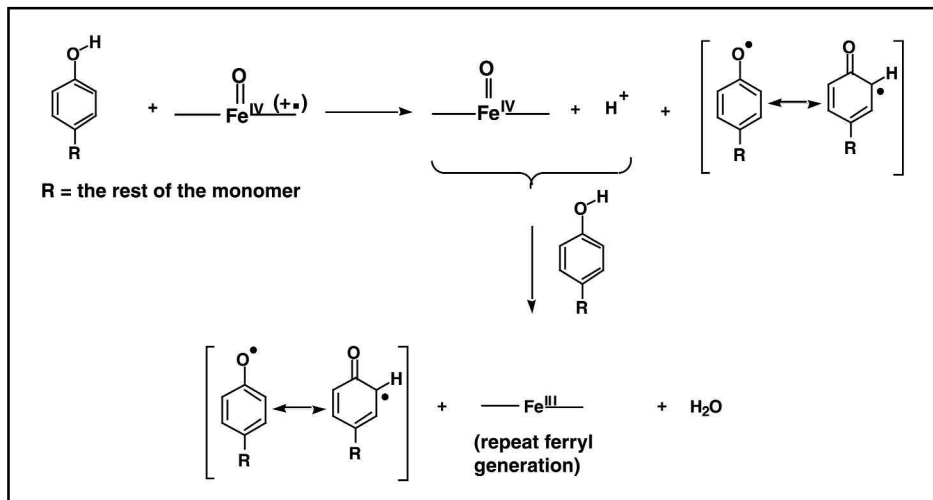

Figure 9
Ferryl Generation

Figure 10
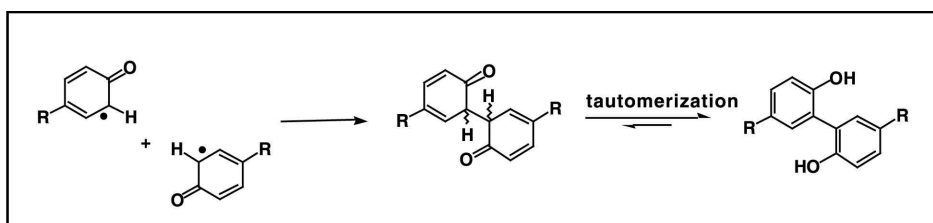Phenoxy Radical Generation

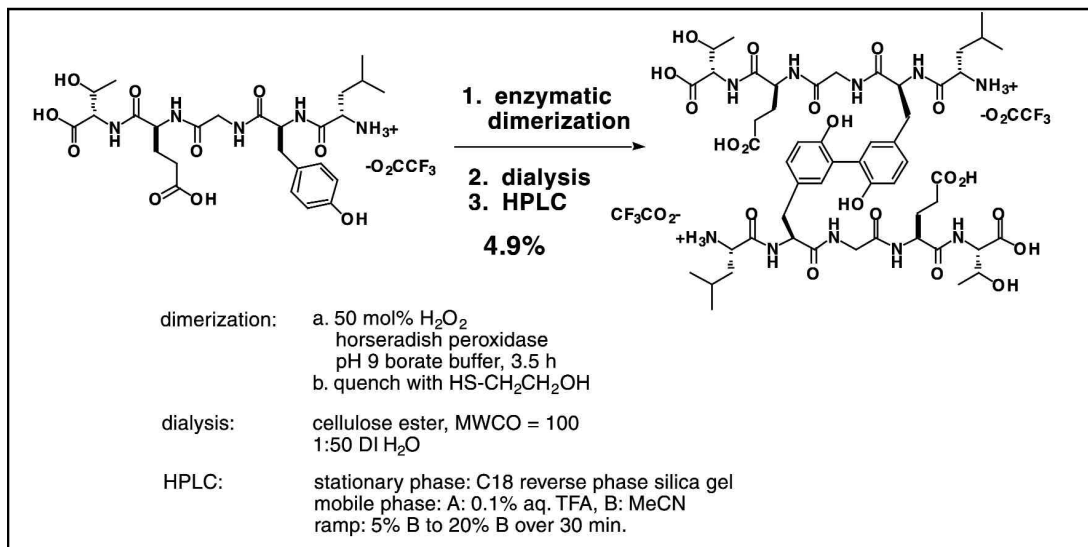Figure 11
Dityrosine Formation

Figure 12
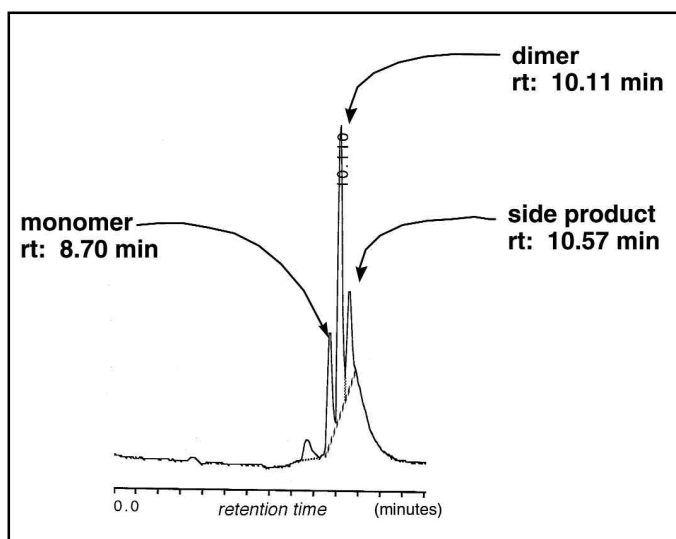Enzymatic dimerization of the monomer

Figure 13
Chromatograph of the dimerization reaction after 3.5 h. (ramp 0-100% B in 30 min).

Physical Analysis of the Peptide Dimer

The protein G peptide dimer had an electrospray molecular ion of 1161.87 m/z. Its reverse phase HPLC retention time was 27.2 min when ramping 5-20% B in 30 min. Fluorescence detection was used at excitation and emission wavelengths of 320 and 400 nm, respectively.

The ^{1}HNMR of the dimer exhibited the expected doublet, singlet and doublet signals (protons j, k and i, Figure 14) corresponding to the aromatic protons of the dityrosine cross-link. The integrated values of these signals were 4, 2 and 4 as expected in the dimer. This was in stark contrast to the ^{1}HNMR of the monomer, which has only two dou-

blets in the same region, each integrating to 2. The solvent was a pH 4 D_2O/acetic-d3 acid-d1/sodium acetate-d3 buffer.

If the dimer was indeed locked in beta sheet conformation, the beta protons (protons h, Figure 14) of tyrosine should have separate signals due to their different environments. However, the two beta protons are equivalent, which suggests that the rotations of the C-C bonds around them are relatively fast on the ^{1}HNMR time scale. Furthermore, ROESY NMR data (a proton NMR that indicates which protons are close to each other), shows that the beta protons of tyrosine and the gamma protons of glutamic acid are near each other (protons h and q, Figure 14). This signal would be expected in a beta sheet conformation. However, since it is possible that the dimer can be in a conformation other than a beta sheet and still yield the q-h proton signal, and because the h protons are equivalent, it was concluded that the dimer was not in beta sheet conformation.

Conclusion

Beta peptide dimers with biaryl cross-links have the potential to disrupt protein-protein interactions. Binding to one outside edge of a beta peptide dimer should cooperatively enhance binding to the opposite outside edge by the transmission of polarization between them. Dimers can be made to target specific beta sheet interactions by incorporating a dityrosine cross-link into the monomeric sequence of the binding site. A dityrosine cross-link is longer than a ditryptophan crosslink, and therefore must be placed in an off-center position in the monomeric sequence to preserve hydrogen-bonding distances between the peptidic strands of the dimer. SPPS is an efficient and systematic way to obtain monomers of specific sequences. Enzymatic peptide dimerization using HRP is an effective way to couple tyrosine residues in full length peptides. Proton NMR analysis of the dimer did not yield enough evidence to definitively confirm beta sheet conformation. However, this does not mean that in the presence of protein G and IgG the dimer will not adopt beta sheet conformation and potentially disrupt the IgG-GB1 beta sheet interaction. A dis-
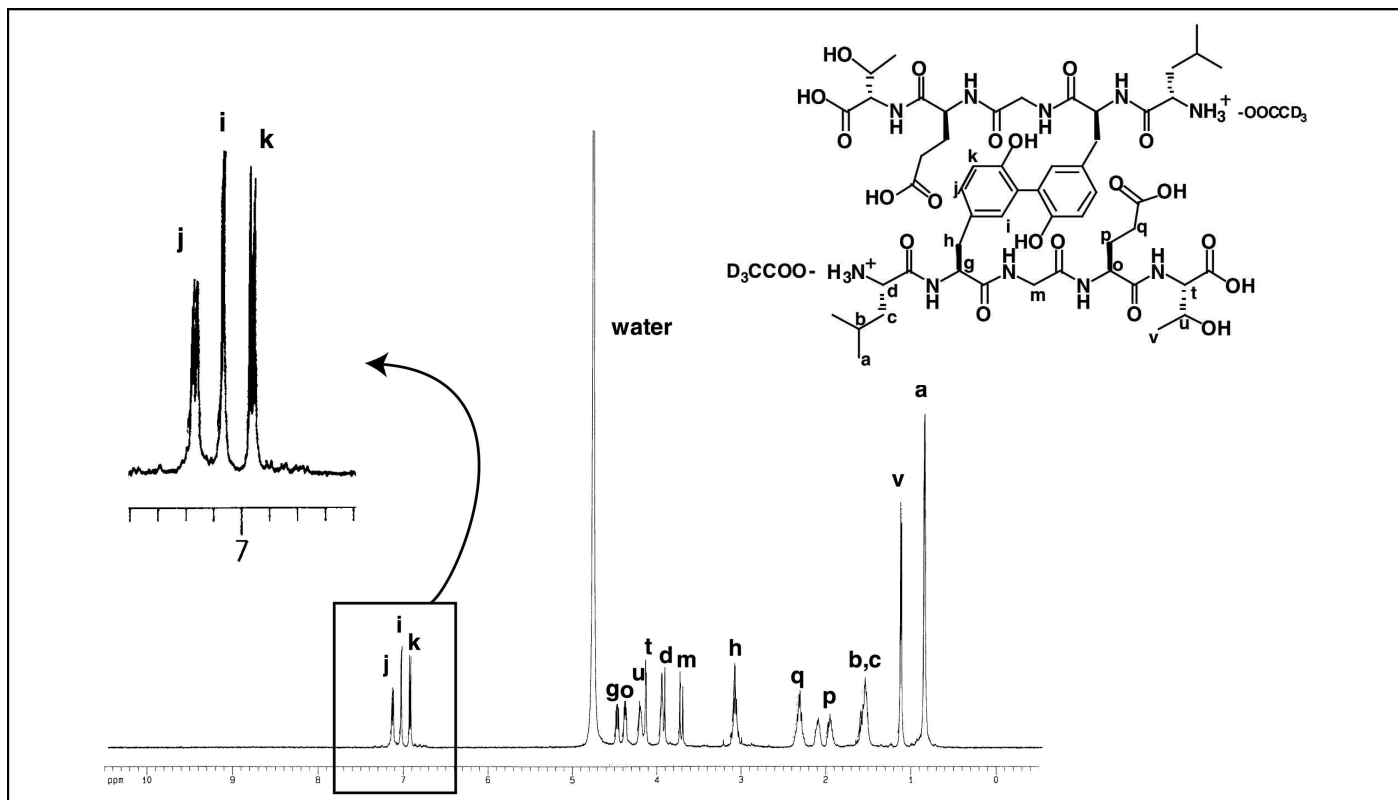
Figure 14
Proton NMR of the protein G peptide dimer.

ruption assay is currently being developed to test this theory. Binding to IgG can ultimately induce beta sheet conformation in the dimer. The control of protein-protein interactions and the biological processes that they dictate will lead to tremendous advances in medicine.

Acknowledgements

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