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Applied Peptidomimetics: Creating a functional model for Alzheimer's disease research and catalytic activity of small peptides

David A. Cretella
University of Tennessee - Knoxville

Introduction:

Peptides serve a myriad of functions in the human body. They are the backbone of complex structures as well the primary vessel for biological catalysis. The human body is a complex equilibrium of chemical components held in check by the activity and regulation of the infinite number of these peptides catalysts, or enzymes, present at any time. Because this chemical balance is so sensitive, many degenerative diseases are caused by the inaction or faulty action of existing enzymes. Attempting to repair or assist enzymes is an extremely difficult task. This is because peptides are so complex; containing hundreds or thousands of amino acids in a unique and controlled sequence to create the perfect structure for which to catalyze biological reactions. However, each enzyme typically has one site in which it performs its catalytic role. This active site is the focal point of the entire molecule and any subtle change to it can have dramatic effects on its shape and therefore its biological action. This extremely important site is often very small, especially in comparison to the entire peptide. Instead of attempting to alter the complex biological proteins, it is often more effective to create a model of the active site. This model is created with a similar shape and composition of the enzyme active site and can mimic its role. Using this model, we can see the effects of subtle changes and assess the usefulness of synthetically created molecules in relation to the natural enzyme; this is the basis for peptidomimetics. This approach allows for the simplest view of the enzyme in order to better understand its function and different ways to alter it.

Alzheimer's disease (AD) is one of the most prevalent neurodegenerative disorders, affecting nearly 15 million people worldwide and because of its severe symptoms and lack of proven treatment it causes significant stress on the patient's family as well as the patient[1]. The exact pathenogensis of the disease is unclear, what is common in patients with Alzheimer's disease, as well as Huntington's and Parkinson's, is the buildup of plaques within the brain tissue as the disease progresses. Analysis of these plaques has shown no significant relationship between their formation and the degenerative effects of the disease[2]. There is also a growing amount of evidence that suggests that these plaques may be the result of the body's natural defense attempting to fight the disease[3]. This supports the emerging hypothesis that the true toxic agent in AD is the soluble oligomeric form of the Amyloid-β (Aβ) proteins that constitute the plaque buildup in patients. The Aβ proteins found in these plaques are products linked to the cleavage of Amyloid Precursor Protein (APP).

There is experimental evidence that Amyloid β-binding alcohol dehydrogenase (ABAD) is an intracellular target of Aβ and this interaction may play a role in the onset of AD. Under normal cellular conditions, ABAD plays a protective role in healthy neurons. However, in the presence of elevated levels of Aβ, its role appears to be reversed as the occurrence of ABAD- Aβ complexes are enhanced in affected areas of an AD patient's brain[4]. The X-ray structure of the complex shows distortions at the active site and a secondary site, labeled L_D loop[5]. There appears to be a unique insertion point for Aβ at this L_D loop. These results suggest that the ABAD- Aβ complex can be a stepping stone in the understanding of the chain of events that takes place in AD.

The first step in understanding the relationship between ABAD and Aβ is to understand the way in which they bind and how the complex functions. To do this, a

minimal model of the L_D loop of ABAD and the fragment of Aβ that is implicated in binding will be created synthetically. To screen these samples, one of the pieces will be immobilized by attaching it covalently to a micro plate. The other piece, and easily any interesting derivatives, can then be screened and viewed if attached to a fluorophore or another similar labeling molecule. A spacer can be used to aid in the screening process by attaching it to the micro plate and the immobilized molecule; this allows the molecule to be more flexible and easily augmentable during the screening process.

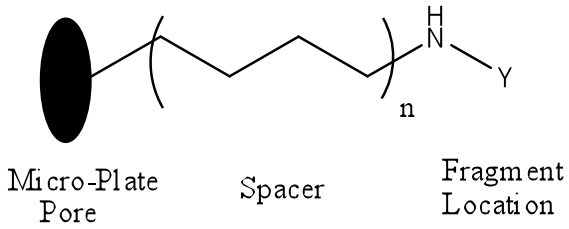
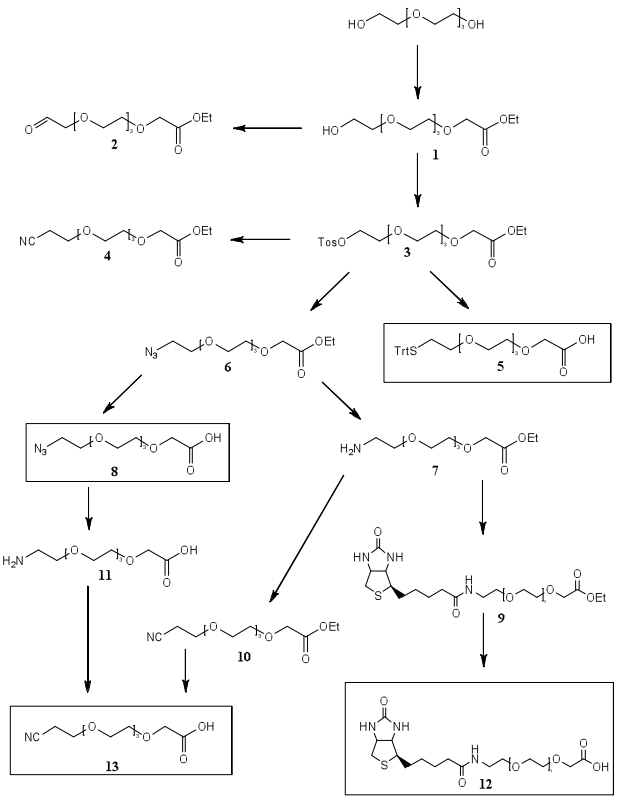


Figure 1. Visual Representation of the modeling style, the same structure can be employed using the labeling model in place of the micro plate pore.

In a related experiment, the catalytic activity of a small tetra peptide will be examined and compared to its naturally occurring counterpart. Metalloporteins are enzymes that catalyze a wide range of chemical reactions with relevance to medicinal chemistry as well as alternative energy sources. In the human body, enzymes often use the aid of copper(II) or Iron(II) to catalyze oxidation reactions. Evidence of this is seen in the active site of enzymes such as laccases, catecholases, and monooxygenases. In each case, the most common metal coordinating amino acid is histidine. It has been demonstrated that a small functional model of these complex enzymes can still have catalytic activity[6]. Their results suggest that a simple tetra peptide with residues His-His-Gly-His can be used as a starting point in experimenting with the catalytic activity of these small peptides. While their experiment only encompassed biological oxidation, the same principle catalyst may be used to assist oxidation of other substrates. As noted before, a subtle change in the model's composition can have dramatic effects on its catalytic ability. Towards this end, artificial residues containing 1, 2, 4-triazoles as histidine replacements were synthesized and incorporated into the tetra peptide sequences in hopes that this substitution can diversify the possible substrates of the catalytic peptide. The resulting peptide analogs will then be screened for their ability to complex copper and iron.

Experimental:

Tetra Ethylene Glycol (TEG) Spacers



Synthesis of the TEG spacers was done using classic liquid phase synthesis starting with tetra ethylene glycol itself. They were synthesized in a way that there can be variable functional groups on one end of the molecule but retain the carboxylic acid end on the other to facilitate attachment of the peptide sequences.

Beginning with TEG, molecule **1** is created using ethyl diazoacetate. Molecule **2** is then synthesized by oxidizing the alcohol. From molecule **1**, the alcohol can be converted to a Tosyl group to yield molecule **3**. The Tosyl group can then be displaced by a cyanide group, a trityl group, and an azide to yield molecules **4**, **5,** and **6** respectively. Molecule **6** can then be reduced to yield an amine (molecule **7**), or saponified to replenish the carboxylic acid necessary for peptide formation (molecule **8**). The amino group of molecule **7** can then be converted to either a nitrile or a biotin group yielding molecules **10** and **9** respectively. Saponification of molecules **9** and **10** yielded molecules **12** and **13.** Molecule **8** could also be reduced to an amine (molecule **11)** and then converted to a nitrile (Molecule **13**) as well.

The major products are highlighted with boxes. These all possess the carboxylic end and an opposing functional group that facilitates attachment to many micro plate resins or other functional pieces. Molecule **5** was a focus point for the current micro plates in use because its trityl group reacts with maleimide freely to form a covalent disulfide linkage. The biotin and azide groups form a similar covalent interaction with other micro plates.

Tetra Peptide Synthesis

The three triazole analogues were created modeling the structure of histadine.

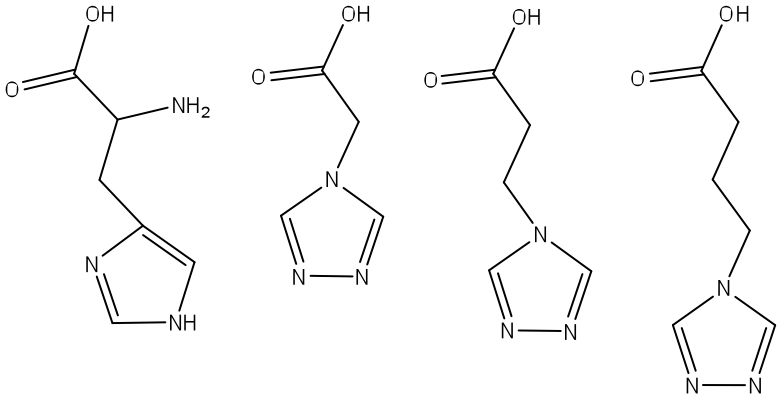
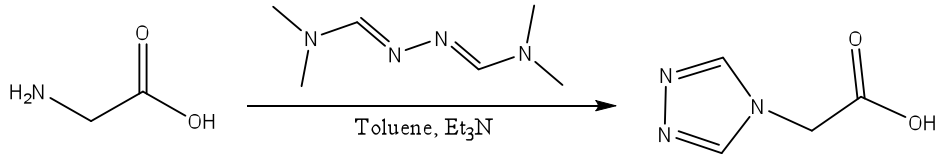


Figure 3. From left to right, Histadine and the three variable 1,2,4 Triazole analogues

These analogues were each created using a one step synthesis following the general reaction scheme of



The three starting materials were glycine, beta alanine, and gamma-amino butyric acid and the reactant used in this experiment was an azene compound created separately and purified as a hydrochloride salt of the above compound. In each case, three equivalents of triethyl amine were used and the reaction proceeded for two days at 80° Celsius, being monitored by thin layer chromatography (TLC) (Ninhydrin Stain). After sufficient conversion is noted by TLC, the reaction mixture is filtered and washed with hexane and ethyl ether. Converting the terminal amine of these compounds into the triazole creates a different structure than the natural occurring histidine which has its ring on a side chain and retains a terminal amino group. This conversion provides a much easier route of synthesis than attempting to retain both a terminal amino group and a side chain triazole and it brings the functionality of a triazole into the molecule in order to assess the effect of its replacement.

The four residue peptides were created by solid phase peptide synthesis using Fmoc-amino acid (Fmoc = 9-fluorenylmethoxycarbonly) methodology. Rink Amide resin was used as the solid support. Starting with the solid support, each residue was coupled with the aid of HBTU, HOBt, and N,N-diisopropylethlamine in NMP. At each stage the residue's protecting group was removed using piperdine then the coupling solution was added and allowed to couple for 1-3 hours. The residues were added sequentially following the general order of Histidine-Glycine-Histidine-X, where X is one of the three triazoles or histidine in the control. The simplest route was to synthesize one large amount of HGH resin then separating it to add the four different final pieces.

Results/Discussion:

TEG Linker Synthesis

The linkers synthesized from TEG were all purified to the point of being one spot by TLC and verified by DART mass spectrometry. Purification at each step was completed by silica gel chromatography. Specific emphasis was placed on the creation of molecule **5** for the current micro plates and also molecule **8.** Molecule **8** has potential in being used not only as a linker for micro plates, but also for covalent attachment to other molecules following the general "Click" reaction. In this reaction, an azide, such as the spacer molecule contains, attaches itself to an alkyne or nitrile under aqueous conditions to form a ring. This attachment has many applications, and can be used for the attachments of peptide models as well as other small molecules that may have activity with the Aβ peptide. It can also be used for attachment of molecules that may aid or inhibit catalytic activity.

Upon completion they were incorporated into various peptide sequences that had possible activity with piece Aβ implicated in activity. This was often done with the aid of a peptide synthesizer; in this case the spacer of choice was dissolved in NMP just as any amino acid residue and added via the same coupling sequence via the carboxylic acid end. This attachment was verified by MALDI mass spectrometry and HPLC.

Tetra Peptide Synthesis

Synthesis of the proposed triazole analogues proved to be difficult. The reaction conditions listed initially were used and produced satisfactory results by TLC and by DART mass spectrometry. However, the presence of an unknown contaminant persisted through the initial reaction conditions as well as further recrystallization. While it did not appear in the DART spectrometry, the contaminated was identified to behave similarly to triethylamine by GCMS and NMR. While normally, it would not persist through methanol crystallization, it may have done so in the form of an ionic salt with the triazole product. After separation of the two proved to be extremely difficult, the triazole monomers were created again using no triethyl amine and were refluxed in toluene for three days at 90º Celsius. After this period, the reaction mixture was evaporated and washed with ethanol; this ethanol was then collected and evaporated to yield a yellow oil. This process again yielded the product, yet it remained impure showing the masses of a larger byproduct likely from incomplete reaction with the azine.

The peptides created in this experiment were done without the difficulty noted in the monomer synthesis. Using the above techniques the His-Gly-His peptide as well as the His-Gly-His-His control peptide was created and verifed by MALDI mass spectrometry and HPLC. Taking small amounts of the His-Gly-His peptide, the triazole-triethyl amine salt compounds were added in an attempt to couple. This process did not appear to work as the resulting product was one peptide by HPLC but the corresponding mass could not be verified.

Conclusion:

The linkers synthesized for attachment of molecules to current micro plates proved to work well for their role. They were easily incorporated at the end of peptide sequences and worked as an attachment device for small molecules. This will aid in the further testing of not only Aβ peptide but a wide range of chemicals that may be useful in understanding and altering the function of peptides related to Alzheimer's disease. The triazole monomer synthetic strategy must be adjusted if the testing of their catalytic activity is to be done. Because of the nature of contamination, it was very difficult to purify the molecules and it wasn't possible to make any observation of activity. Another realm of future research would be to abandon the simple modification of the terminal amine of an amino acid monomer and attempt to covalently modify the side chain of an amino acid. This would allow for the triazole to be incorporated at any location in the catalytic peptide rather than only at the end.

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David Cretella
Dr. Shane Foister

University of Tennessee
Chemistry Department
1420 Circle Drive
Knoxville, TN 37919