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DHS Summer Student Project Report

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This work was performed under the auspices of the U.S. Department of Energy by University of California, Lawrence Livermore National Laboratory under Contract W-7405-Eng-48.

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Questions 1 and 2:

Tetanus and botulinum neurotoxins are among the post potent toxins known to man (Montecucco *et al.*, 1995). Produced by the *Clostridium tetani* and *Clostridium botulinum* bacteria, respectively, these toxins concentrate in presynaptic axons and inhibit the release of neurotransmitters leading to paralysis and possibly death. Due to the potency of this lethal class of neurotoxins, we have undertaken a project to develop high affinity ligands that specifically bind to these toxins. Such compounds can have significant implications in both the design of detection systems to monitor for the possible release of these neurotoxins into the public and also the design of possible therapeutics to treat individuals exposed to tetanus or botulinum neurotoxins.

The *Clostridial* neurotoxins are synthesized as 150 kDa proteins that are post-translationally cleaved into N- and C-terminal fragments held together by a single disulfide bond. The tetanus C-terminal fragment (TetC) has been shown to bind specifically to gangliosides present on the neuronal membrane surface and facilitate endocytosis of the toxin (Morris *et al.*, 1980). Once the toxin is internalized in a membrane-bound vesicle, the light chain (N-terminal fragment) translocates to the cytosol where it interferes with neurotransmitter release.

Previous work has demonstrated that various small molecule and peptide-based compounds bind to TetC, albeit in different locations. Among these molecules are the anticancer agent doxorubicin (Dox) and the tripeptides WEY and YEW (Figure 1; Cosman *et al.*, 2002). The crystal structure of botulinum toxin and Dox (PDB code: 1I1E) demonstrates that Dox binds in a surface groove of in C-terminal fragment that is conserved in both botulinum and tetanus toxins. Similarly, YEW has been shown to bind to a second binding site that is highly conserved and

also relatively close to the binding site of Dox. Thus, in our quest to design and synthesize high affinity ligands, we proposed to link Dox and YEW (or WEY) in hopes of creating a bidentate ligand. In theory, such a ligand could have a binding affinity approaching the product of the two binding affinities of the individual ligands.

For my internship project, I was charged with the task of creating libraries of compounds linking Dox and YEW (or WEY) with linkers of varying lengths (Figure 2a). In addition, I was to attach a fluorescein dye to the molecules (Figure 2b) so that they could be used to develop a fluorescence polarization (FP) binding assay. The FP assay will greatly increase the ease with which future ligands can be rapidly screened and binding affinities can be accurately determined.

As a side project, I worked on optimizing the conditions necessary to employ the Huisgen 1,3-dipolar cycloaddition reaction to be able to optimize linker lengths and possibly compound solubility (Huisgen, 1984). This reaction, often termed "click chemistry," utilizes molecules terminally functionalized with either an acetylene moiety or an azide. In the presence of a copper(I) catalyst, the alkyne and azide undergo a step-wise cycloaddition reaction to link the two molecules together via the formation of a 1,4-disubstituted triazole ring (Figure 3; Rostovtsev *et al*., 2002). By varying the length of the tethers between the terminal acetylene or azide and their respective molecules, the overall length of the linker between the two molecules can be "fine tuned" by one carbon unit at a time.

At the completion of my internship I had synthesized conjugates of Doxorubicin and N-acyl-WEY linked together by linkers having 0-2 polyethylene glycol (PEG) linkers. These compounds are currently being used in experiments that employ electrospray ionization mass spectrometry (ESI-MS) to determine whether they bind to TetC with higher affinity than either Dox or WEY alone. I also synthesized the fluorescein tagged versions of the same three molecules. It is expected that these molecules will be used in the near future to develop a fluorescence polarization-based competitive binding assay for TetC and possibly botulinum C-terminal fragment (BotC).

During my internship I also successfully demonstrated the possible utility of using the Huisgen 1,3-dipolar cycloaddition reaction to optimize linker lengths. First, I synthesized 2-azidoethylamine which can be used to attach an azide moiety to any carboxylic acid (e.g. C-terminus of any peptide). Next, I synthesized the starting materials; the linker with 0-2 PEG molecules was functionalized with a terminal alkyne moiety and Ac-WEY was functionalized with a C-terminal azide. Finally, I ran a small scale pilot reaction to determine the proper conditions for the ligation of the two starting materials.

Due to the early timeframe of my internship, I was not going to be present for the Lawrence Livermore National Lab summer intern poster symposium and therefore did not create a poster of my work. Instead, I gave an oral presentation of my work to my supervisors and fellow colleagues in the bioorganic division weekly meeting on July 22, 2005. The publication of this work will be dependent on the results of the mass spectrometry experiments and fluorescence polarization studies.

References

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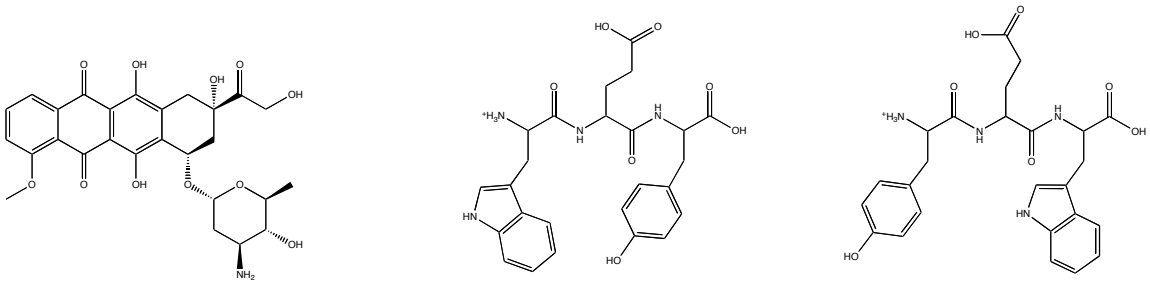
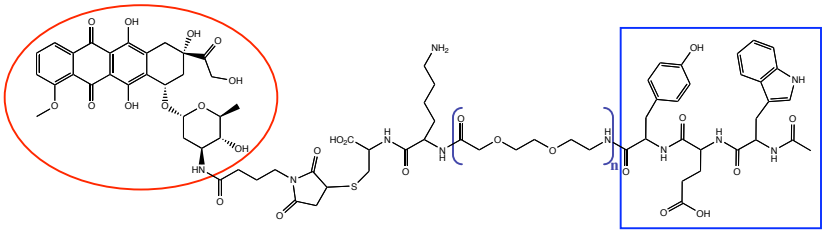
Figure 1. Known ligands of tetanus and botulinum neurotoxins.



(Doxorubicin) (WEY) (YEW)

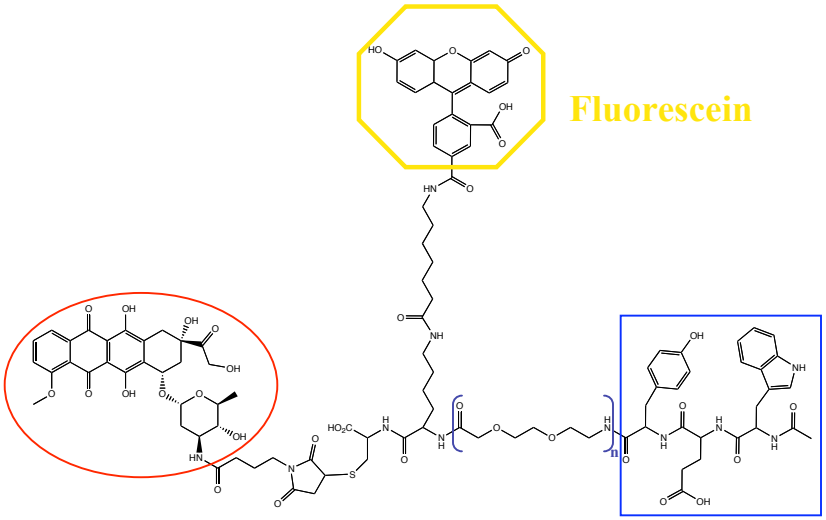
Figure 2. Dox/Ac-WEY conjugates with and without fluorescein, and having variable linker lengths.

a.



Dox **Ac-WEY**

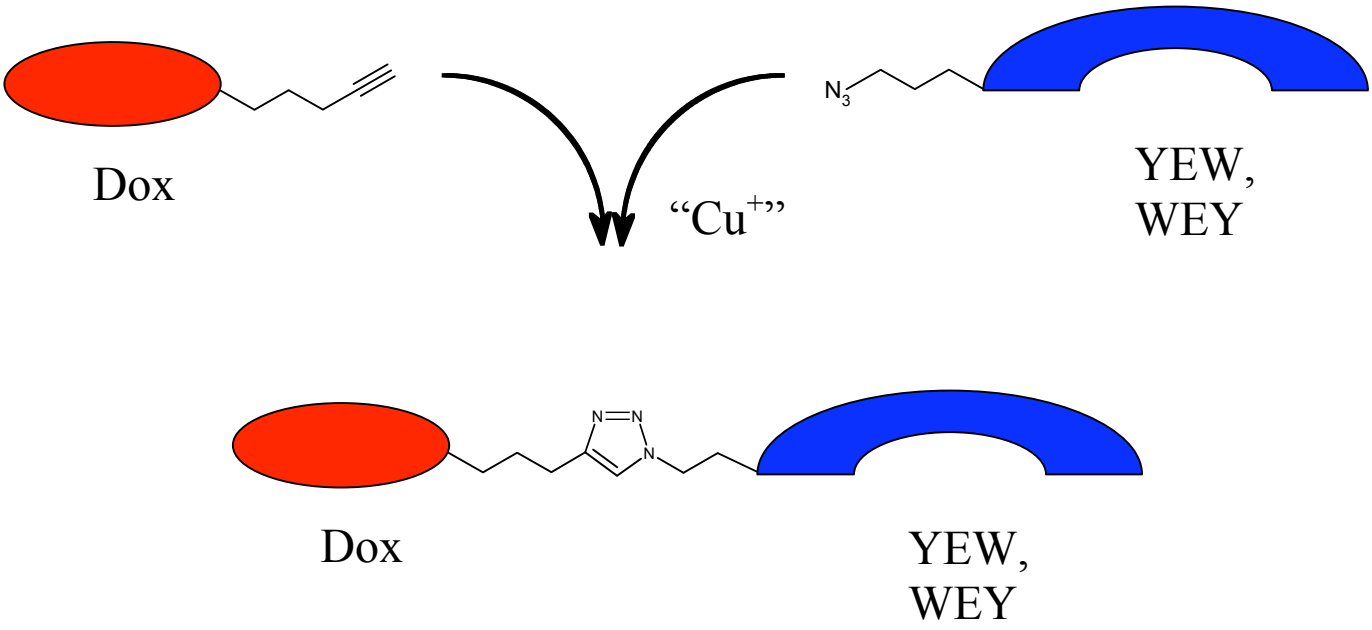
b.



Fluorescein

Dox **Ac-WEY**

Figure 3. Schematic representation of the use of the Huisgen 1,3-dipolar cycloaddition to optimize linker lengths.



Question 3:

My internship at Lawrence Livermore National Laboratory (LLNL) has broadened the scope of possible post-doctoral and career placement options. Before coming to LLNL I had always assumed that I would earn my Ph.D. in Medicinal Chemistry and then find a job with a pharmaceutical company such as Pfizer or Eli Lilly. I believed that outside of an academic environment, my talents and training would only be suitable for industry. However, my experience at LLNL has demonstrated to me that would also be well suited for a career at a national laboratory.

During the past 10 weeks, I have had a chance to learn about and discuss the various research projects that are being undertaken in the Chemistry and Material Science division at LLNL. I am astounded by the wide range of projects being investigated and by the numerous collaborations that are involved in each project. I had previously assumed that LLNL would be the same as other national laboratories and have little or no organic and bioorganic chemistry projects. My assumptions were wrong.

I have also had the chance during my internship to attend numerous lectures on topics ranging from bioforensic science to bioaerosol mass spectrometry. These projects, which are directly associated with national security, have aspects to which I would be perfectly suited to study. The DHS sponsored briefings that I attended every week also increased my knowledge of the issues facing DHS in the era to come. I was particularly interested by discussion on the Biodefense Knowledge Center and by the National Atmospheric Release Advisory Center. Before my internship, I was unaware of the existence of both of these projects.

I was also extremely impressed by my tour of the National Ignition Facility (NIF). This project, although far from completion, is utterly astounding both in size and engineering. In addition, the implications that this project could have for the scientific community are extremely exciting.

Question 4:

Over the past 11 months, I have been extremely impressed by the speed with which the Department of Homeland Security has organized and refined the DHS Scholars and Fellows program. For such a new program it is amazing to me that everything has run so smoothly.

The most influential part of this program has been the summer internship. As afore mentioned, I was previously unaware of the scope of research topics being investigated by the national laboratories. I was first introduced to the types of projects being studied when I attended the informational meeting last November in Washington, D.C.. This meeting was well structured and extremely informative. I definitely made choosing a national laboratory in which to do my internship a much easier process.

While at LLNL, the DHS sponsored weekly briefings were very informative about the topics that DHS is concerned about. It also awakened me to the endless possibilities for national security related research projects that have yet to be addressed. I believe that if I were to accept a job at a national laboratory after earning my Ph.D., I would certainly be able to find a research project that would meet my expectations and those of DHS.

Nevertheless, I believe that the DHS Scholars and Fellows program still has room to improve. Most importantly I believe that DHS should review its policies about scholars and fellows accepting other sources of income. Obviously, it would be inappropriate for students to accept income that bears extra responsibilities that would reduce their commitment to DHS. However, there are other scholarships and Fellowships that have no extra responsibilities tied to them. For instance, the University of Michigan College of Pharmacy offers a competitive fellowship worth $3-6,000 that is based solely on the grades earned by graduate students in their first year and a half. It seems shortsighted on the part of DHS to limit the amount of income students can receive for such scholarships and fellowships. This is not to say that DHS is being at all frugal with the stipends that they offer. However, for me, being married and living in Ann Arbor (a more affluent city than some), I know that additional merit based income would be a welcomed gift and reward for the hard work that I put into my graduate career.

Question 5:

Finally, I believe that DHS should work to improve the amount of synthetic organic and bio-organic chemistry that is performed in the national laboratories. Although, LLNL is making an impressive effort to increase that amount of chemistry being performed, other national laboratories I believe are still lacking in this area. In the past decades, nuclear chemistry has been a number one priority. However, in the present day, biological and chemical warfare is an ever increasing threat. Without the foundation provided by organic and bio-organic chemists, I fear that DHS will be handicapped in its efforts to counter these new and emerging threats.