Molecular Click Adventures, a Leap from Shoulders of Giants

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I WOULD LIKE TO START by thanking the Nobel Foundation, the Nobel Prize Committee, and the Royal Swedish Academy of Sciences for awarding the Nobel Prize 2022 to Click Chemistry, and for this incredible honor that I am so proud to share with Professor Barry Sharpless and Professor Carolyn Bertozzi.

I also thank and acknowledge all the many coworkers and collaborators who, over time, have contributed to the discovery, development, and application of copper-catalyzed azide alkyne cycloaddition (CuAAC) Click chemistry.

Most of all I would like to thank my wife Dr. Phaedria Marie St. Hilaire, my children Ajani and Anna, and Anna's mother Ceramist Sandra Davolio, for their great support.

In science, we all stand on the shoulders of giants who have researched before us, and it is important to acknowledge their contributions. My giants include Rolf Huisgen, Klaus Bock, Robert Sheppard, Bruce Merrifield, Kurt Wütrich, Tor Bak, Harry Gray, Hans Paulsen, Raymond Lemieux, Arpad Furka, Kit Lam and many more.

MY PERSONAL JOURNEY TOWARDS TO THE CUAAC REACTION

Nature, and in particular its underlying chemistry, represents to me the amazing beauty and complexity of our existence. This appreciation and fascination with Nature has been with me since early childhood and was inspired and cultivated by my late parents. They dragged me and my siblings through all of Scandinavia's forests and we collected rocks, leaves, butterflies, insects, lichens, you name it. This way they spurred my curiosity for knowledge and understanding. Those images from childhood are strong in my memory. I still remember philosophical thoughts on evolution while submerged in my grandfather's barley fields in what appeared to be a micro-universe of barley, flowers, weeds and insects of all sorts.

Thus motivated, I therefore chose to study engineering and natural sciences, which funneled into a choice between physics, computer science and chemistry. Fortunately, I chose chemistry because chemistry, to me, is everything. Today, with all the global issues, we need chemistry more than ever. I currently see chemistry not only as an education but more as a general education and basic knowledge that everyone should have.

After completing my studies, I worked first within carbohydrates, then peptide chemistry, and eventually, I initiated glycopeptide synthesis at Carlsberg Laboratory to study glycoproteins and their functions. In particular, the peptide world proved to be an incredible cradle of creativity and a source of inspiration for all peripheral sciences. The peptide conferences were true melting pots for serendipitous, interdisciplinary discoveries. It was during investigations at the interface between peptides and organic chemistry that we discovered this amazing CuAAC reaction[1].

THE RISE OF COMBINATORIAL SCIENCE WHICH MADE CLICK NECESSARY

In the early 1990s, in order to mimic bio-selection, a Darwinist approach to chemistry emerged and was termed combinatorial science. In developing this approach, we realized that we do not understand the complexity of molecular interactions and are essentially unable to design active substances. In Combinatorial Science millions of different and putative active molecules are synthesized and presented to a biological selection process for "natural selection" of the best candidates. This approach really required amazing synthesis by quantitative chemical transformations (QCT). In 1997, the Danish National Research Foundation (DG) funded our solid phase combinatorial chemistry (SPOCC) center at Carlsberg Laboratory with the mandate to develop these QCT reactions for combinatorial organic chemistry on our biocompatible polymer matrices for on bead screening.

What is combinatorial chemistry and why were Click reactions so critically important? In combinatorial chemistry, an exponentially growing

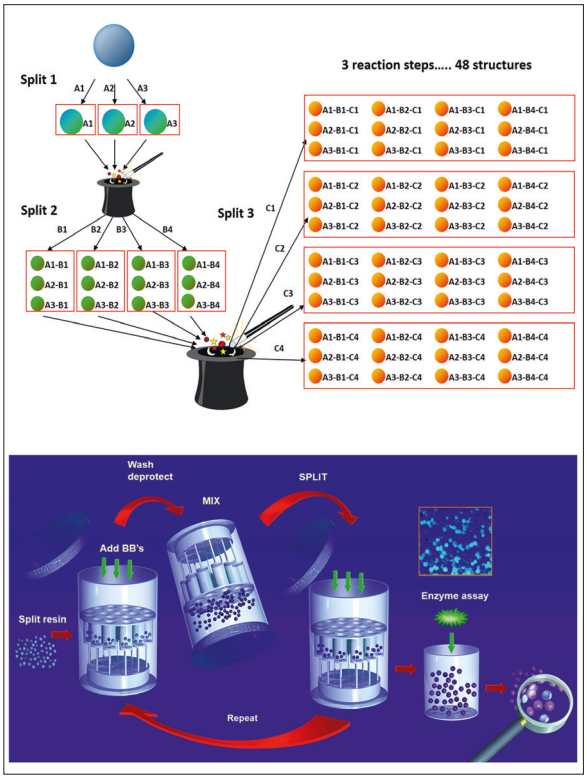


Figure 1. Split-mix combinatorial synthesis and on-bead screening. The resin is divided and coupled in separate wells or columns with different reagents. It is then combined, mixed and prepared for the next reaction. It is re-divided into the wells and coupled with the next set of reagents. This cycle provides an exponential growth in numbers of compounds. In practice this is performed in a Teflon® split-mix reactor that I designed and engineered. This approach required QCT reactions.

number of molecules is synthesized, for example by split-mix approaches or by mixture coupling approaches. Figure 1 illustrates combinatorial chemistry by the split-mix approach in which each resin bead (~400 μm diameter) is used as a separate reaction container. Using this approach with a hydrophilic resin allows assays to be performed on-bead. Synthesized compounds are then only released from the resin for structural analysis after they have shown evidence of the desired, positive activity[2].

During subsequent years after we obtained the SPOCC center, with reference to Star Wars I was mostly known as Dr. Spocc amongst the students at Carlsberg Laboratory, and we had good laughs when Dr. Spocc had to look at Data. At the SPOCC center a large number of successful, new, quantitative chemical transformations (QCT) were established and employed in combinatorial screenings to find enzyme inhibitors and signaling molecules. At the time, we referred to these reactions as QCT or Molecular Lego® as coined by Nobel Laureate, Sir Fraser Stoddart in 1988, and used to characterize molecular assembly by quantitative Diels-Alder reactions.[3]

Importantly, this pointed towards a ten-year period of maturation leading up to the discovery and definition of click chemistry. In the 1990s

worldwide, research had matured and the time was ripe for the development of quantitative and selective Click chemistries, partly motivated by the need for millions of perfectly synthesized compounds for combinatorial science.

THE CUAAC CLICK REACTION DISCOVERY

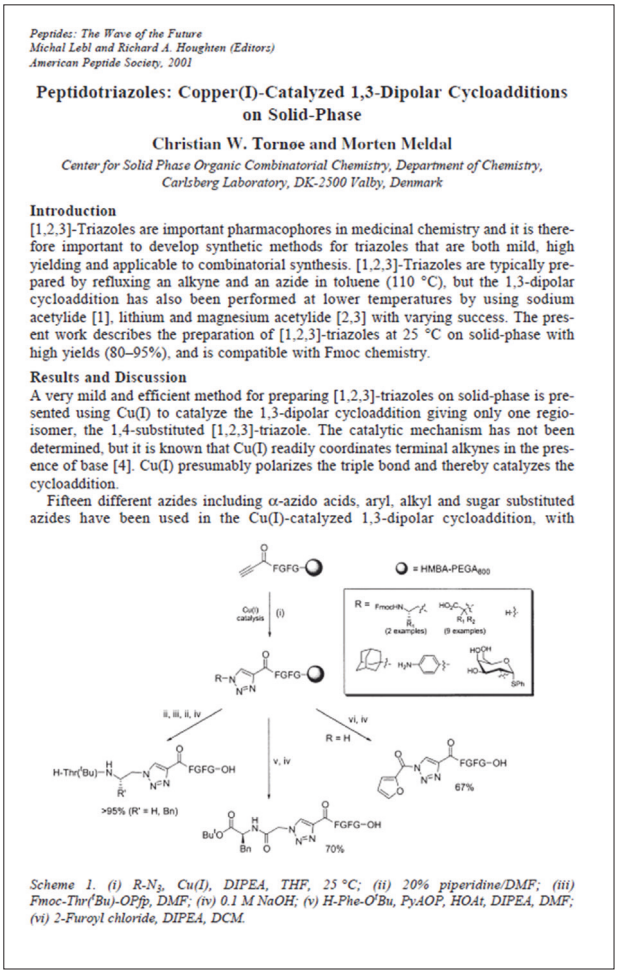
Kolb et al. described a roadmap for Click reactions in 2001, thereby introducing the Click terminology[4]. Click was broadly accepted as a scientific concept and the paper has constituted the important general guidelines for the development of Click reactions. These include CuAAC,[1] SPAAC,[5] Tetrazine-TCO[6, 7] and we may include a SNAr reaction[8] and a traceless Staudinger ligation,[9] but even after 21 years the list is short, indicating the difficulty in finding chemistries that qualify as Click. The original Cu(1) catalyzed triazole formation (CuAAC)[10, 11] may still be considered a crown jewel of Click chemistry.

In the SPOCC center, we worked on new peptide technologies and we merged the diversity of the peptide world with reactions from organic chemistry to produce interesting 3-dimensional molecular structures of pharmaceutical interest[12].

This was part of a quest to find new remedies for major chronic diseases including cancer, Alzheimer's and viral infections leading towards medicinal relief in an increasingly aging society. Of particular importance was our combination of peptide chemistry with quantitative and selective intramolecular N-acyl-iminium reactions (INAIC).[13] These transformed readily available diverse peptides into countless multi-heterocyclic drug-like molecules with high structural diversity[14, 15].

In this process, we also developed an outstanding versatile peptide synthesis strategy for the assembly of difficult peptides.[16] This was based on the use of the azido group for amine protection. In turn, azide functionality allowed exceptionally high activation in the peptide coupling reaction. In other words, we could prepare novel, interesting molecules, which were not available in any other way before. The versatile azide group is also used in Click chemistry today.

The SPOCC center worked day and night to develop chemistry that would allow us to combine the world of peptides with organic reactions in a combinatorial fashion. It was an exciting time at Carlsberg Laboratory. It was during this active period of research that our first contribution to Click chemistry was conceived, the discovery of the archetypal CuAAC click reaction[1]. Christian Wenzel Tornøe, was a PhD student at the SPOCC center and his PhD program included preparation of new molecules with our azido acid halide technology. He attempted to react highly activated azido acid chlorides with polymer bound alkynes in presence of copper (1) salts to produce the conjugated keto alkynes. We fought a brave battle to



Peptides: The Wave of the Future
Michal Lebl and Richard A. Houghten (Editors)
American Peptide Society, 2001

Peptidotriazoles: Copper(I)-Catalyzed 1,3-Dipolar Cycloadditions on Solid-Phase

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Introduction

[1,2,3]-Triazoles are important pharmacophores in medicinal chemistry and it is therefore important to develop synthetic methods for triazoles that are both mild, high yielding and applicable to combinatorial synthesis. [1,2,3]-Triazoles are typically prepared by refluxing an alkyne and an azide in toluene (110 °C), but the 1,3-dipolar cycloaddition has also been performed at lower temperatures by using sodium acetylide [1], lithium and magnesium acetylide [2,3] with varying success. The present work describes the preparation of [1,2,3]-triazoles at 25 °C on solid-phase with high yields (80–95%), and is compatible with Fmoc chemistry.

Results and Discussion

A very mild and efficient method for preparing [1,2,3]-triazoles on solid-phase is presented using Cu(I) to catalyze the 1,3-dipolar cycloaddition giving only one regioisomer, the 1,4-substituted [1,2,3]-triazole. The catalytic mechanism has not been determined, but it is known that Cu(I) readily coordinates terminal alkynes in the presence of base [4]. Cu(I) presumably polarizes the triple bond and thereby catalyzes the cycloaddition.

Fifteen different azides including α-azido acids, aryl, alkyl and sugar substituted azides have been used in the Cu(I)-catalyzed 1,3-dipolar cycloaddition, with

Scheme 1. (i) R-N3, Cu(I), DIPEA, THF, 25 °C; (ii) 20% piperidine/DMF; (iii) Fmoc-Thr(tBu)-OPfp, DMF; (iv) 0.1 M NaOH; (v) H-Phe-OtBu, PyAOP, HOAt, DIPEA, DMF; (vi) 2-Furoyl chloride, DIPEA, DCM.

Figure 2. *Front* page from the first reporting of the CuAAC Click reaction at the American Peptide Symposium in San Diego in June 2001.

make this reaction work. However, Christian was about to give up this approach, because he consistently got a side-reaction instead of the expected keto-alkyne. When we analyzed the byproduct in detail, we found that the 1,4 substituted 1,2,3-triazole had formed quantitatively and, importantly, at ambient temperature and with complete 1,4-regio-selectively. This occurred leaving the highly reactive acid chloride untouched. Surprisingly, the reactive acid chloride could subsequently be used in other difficult acylation reactions. It was immediately realized that this regio-selective copper[1] catalyzed 1,2,3-triazole reaction had extraordinary properties[10] and was in fact just what we had been looking for in the SPOCC center. From that moment, we focused on the Click reaction and Christian W. Tornøe and his fellow student Caspar Christensen proudly presented the results at the American Peptide Symposium in San Diego in 2001 (Figure 2).[1]

Today, we have two toolboxes, the large one used by pharma in medicinal chemistry and by the materials industry, and a smaller Click toolbox. The large toolbox contains generally nonselective reactions requiring multiple, orthogonal protecting schemes and consumes large amounts of solvents. The Click toolbox is a completely complementary toolbox with click reactions. New true click reactions are quite difficult to find and as indicated by the fact that the box only contains three reactions, and maybe a few new candidates.

WHAT IS THE CUAAC CLICK REACTION?

But what is the Click reaction? A click reaction is a quantitative reaction that can be used to combine different macromolecular functions into a single molecular entity that has the collective ability of the individual parts it is composed of (Figure 3). We can express or synthesize functional protein and peptide building blocks. Figure 3 illustrates a putative example of a single molecular robot comprising three different functionalities, an apoptosis-promoting molecule, a single-chain antibody and a cell-penetrating peptide. These are combined with Click reactions to give the combined molecule new abilities, which the single molecules did not have e. g. in fighting cancer.

Mechanistically, the CuAAC Click-reaction is quite interesting and most important to the mechanism is the high affinity and multinuclear interaction of terminal alkynes with copper in the oxidation state (1)[17, 18].

The CuAAC Click reaction is initiated by π-coordination of a Cu(1) atom to the triple bond. This in turn greatly acidifies the terminal proton to the extent that it can be removed by a simple amine. The proton is then replaced with a Cu(1) atom binding to the terminal electron pair and a second Cu(1) atom forms a π-bond to the alkyne. During the next critical transformation, the second Cu(1) atom strips the π-electrons away from the secondary carbon of the alkyne and the two Cu(1) atoms form a Cu-Cu bond and become equivalent[19]. In a more or less concerted fashion, this highly reactive com-

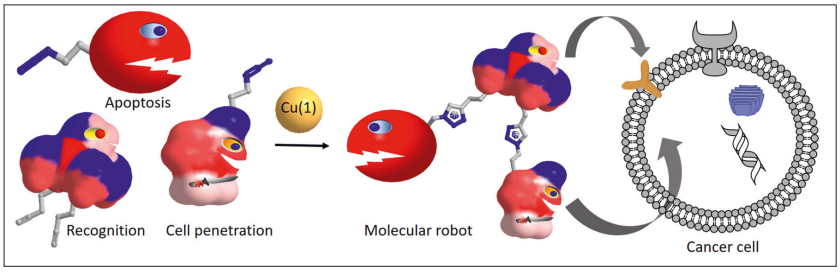

Figure 3. Animated molecular robot composed by clicking a functional apoptosis-promoting molecule with a single-chain antibody and a cell-penetrating peptide for recognizing, entering a cell and facilitating apoptosis.

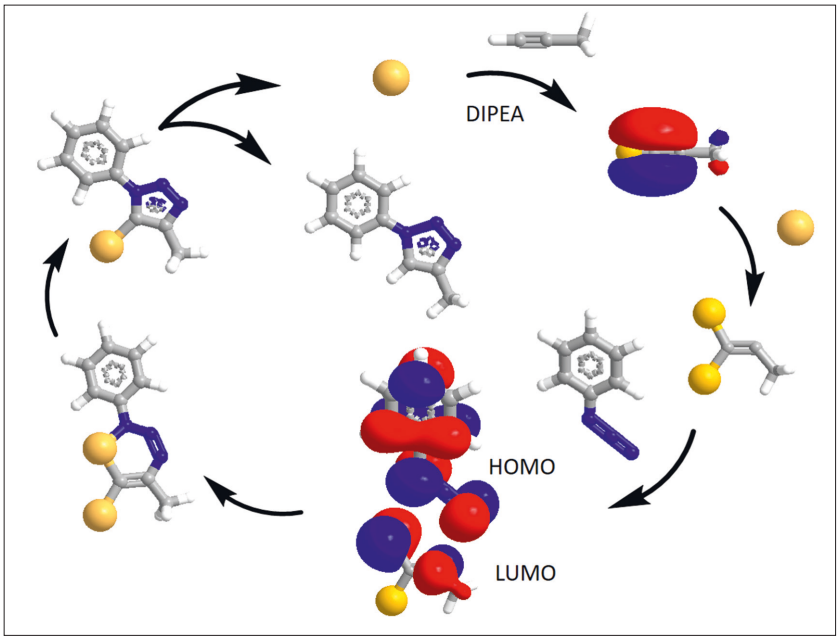

Figure 4. A mechanistic explanation for the efficiency of the Click reaction involves the tight multinuclear coordination of Cu(1) ions, first to the terminal σ- and then π-electrons at the primary acetylide carbon providing a LUMO for the transfer of electron density from the ideally positioned HOMO of the electron rich azide through regio-selective coordination to the Cu(1) atom with the imine lone pair.

plex coordinates the imine lone-pair of the azide and delivers the terminal azide lone-pair to the electrophilic secondary carbon of the alkyne, providing the extreme regio-selectivity of the reaction (Figure 4).

The intermediate metallocycle loses one Cu(1) atom and rapidly collapses to the stable triazole while still carrying a Cu atom at the previously terminal alkyne carbon. This is, in principle, a nucleophilic metallo-organic compound that can be reacted with electrophiles or simply be protonated to release Cu(1) and generate the target triazole. The reaction mechanism is supported by countless kinetic studies, by intermediate trapping and by crystal structures of complexes containing the mechanistic intermediates. One of the caveats of CuAAC is the maintenance of an efficient concentration of Cu(1) throughout the reaction without disproportionation or oxidation.

This explanation illustrates how the copper salt facilitates the triazole formation by interacting with the electrons of the alkyne and the azide to increase the rate of reaction 10,000,000-fold.

One of the lesser known caveats of the CuAAC Click reaction is the very aggressive Cu(1)–Cu(2) redox cycle of the catalyst (Figure 5). Cu(1) reacts with oxygen and produces a number of very reactive oxygen species (ROS) and Cu(2). When performing the click reaction in the presence

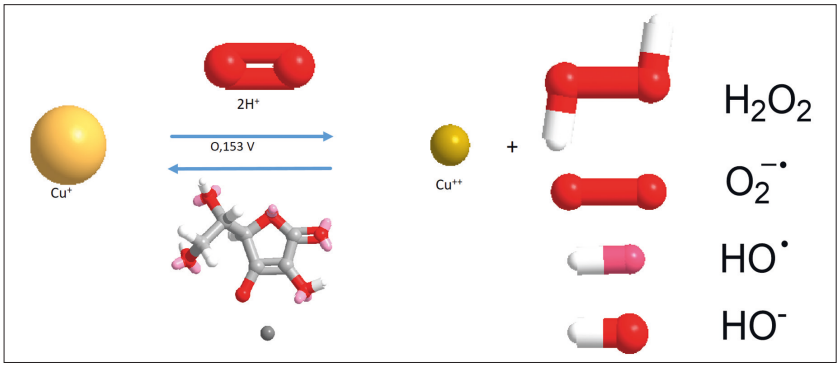

Figure 5. The aggressive redox cycle of the click reaction performed in the presence of oxygen and producing reactive oxygen species (ROS)

of atmospheric oxygen, a large amount of reducing agent is required to reduce Cu(2) back to Cu(1). While small inert molecules may survive the reactive oxygen species produced in the redox process, larger, more complex biomacromolecules are destroyed by these. We therefore developed catalysts and completely anaerobic methods for macrocyclizations through which we are able to maintain the catalytic amount of Cu(1) needed during the entire biomolecular click conjugation.

APPLICATIONS OF CUAAC CLICK CHEMISTRY

Applications of CuAAC pursued at the SPOCC and CECB (Center for Evolutionary Chemical Biology) centers include peptide bond mimetics, structural control by triazole bridging in macromolecules, ligation of functional molecules to macromolecules, surface modifications and finally, multifunctional molecular robots. Some of these applications are illustrated below.

Click chemistry was used in combinatorial synthesis of enzyme inhibitors. The sandfly-transmitted parasite *Leishmania mexicana*[20] causes cutaneous ulcers and if untreated, death occurs in the majority of cases. Leishmaniasis was, at the time, endemic in large regions of South America and treatments for the disease were urgently needed. The parasite has an important cysteine protease that we targeted. We employed the Click reaction to produce a molecular library of inhibitor molecules with the triazole as the key inhibitory element, and which replaced a central peptide bond in a one-bead-two-compounds combinatorial library of protease inhibitors[21]. The library was constructed by split-mix synthesis on 200,000 beads of our biocompatible PEGA-resin[20]. Each bead contained a single putative inhibitor of this essential protease Cysteine Protease2.8, from the parasite and a protease activity indicator, also called a FRET or fluorescence quenched substrate. In the absence of inhibitor, the activated protease

cleaved the substrate within 1 h resulting in brightly fluorescent beads. However, in beads containing an active inhibitor, the protease activity was inhibited, and the bead remained dark. This allowed us to isolate beads containing active inhibitors of the *Leishmania mexicana* protease. Inhibitor structures were analyzed by mass spectrometry and resynthesized. The inhibitory activity was measured, and a decent Ki of 76 nM could be measured. The triazole prevented peptide bond cleavage of both inhibitor and substrate. Thus, using the CuAAC triazole chemistry in a molecular library we could identify an inhibitor (H-Gly-RTr-ClF-Leu-Thr-Ile-Ser-Arg-Gly-NH_2) of the parasites' functionally important enzyme.

We soon realized that the selectivity and quantitative nature of the CuAAC reaction provided a unique opportunity to perform macrocyclizations of protected and even unprotected macromolecules to stabilize a particular bioactive conformation[22]. The structural variety of building blocks possible with the CuAAC reaction provided the opportunity to efficiently mimic the important disulfide bonds used by Nature to maintain structural integrity in both peptides and proteins. However, in contrast to the disulfide bond, the novel disulfide mimicking triazole provided complete stability to metabolic processing and facilitated studies under physiological conditions.

We used the CuAAC technology to produce a range of cyclic ligands for the melanocortin 1–5 receptor system (MC) with the aim of producing MC3/4 receptor selective ligands based on the core motif of the natural ATCH peptide. The melanocortin receptors are involved in activities as diverse as appetite control (obesity), memory/cognition, growth, puberty change, sexual dysfunction, mood, and skin pigmentation. A single vector approach to expression of both melanocortin receptors and YFP upon CREB – activation was developed and employed in analysis of receptor activation. We were able to synthesize the ligands in high purity and establish the importance of individual sub-sites for the receptor selectivity. Our best ligand had 300-fold selectivity towards the obesity regulating receptor MC4R over the closely related MC5R and MC3R (Figure 6) and could constitute an excellent starting point for the development of obesity regulation drugs.

Our next example is with the Japanese horseshoe crab. We used the efficient CuAAC reaction to establish several triazole bridges simultaneously to form multi-cyclic peptides[23]. As a model, we used the antimicrobial peptide Tachyplesin 1, from the Japanese horseshoe crab, famous for its efficient innate immune system, which has enabled this alien-looking animal to exist virtually unchanged for 400,000,000 years. The animal uses a unique set of molecules to trap and imprison foreign bacteria, which are then killed with antimicrobial compounds. For this unique property of its blue blood, the animal is, unfortunately, being bled on an industrial scale for the detection of bacteria in human blood. Tachyplesin 1 is a peptide

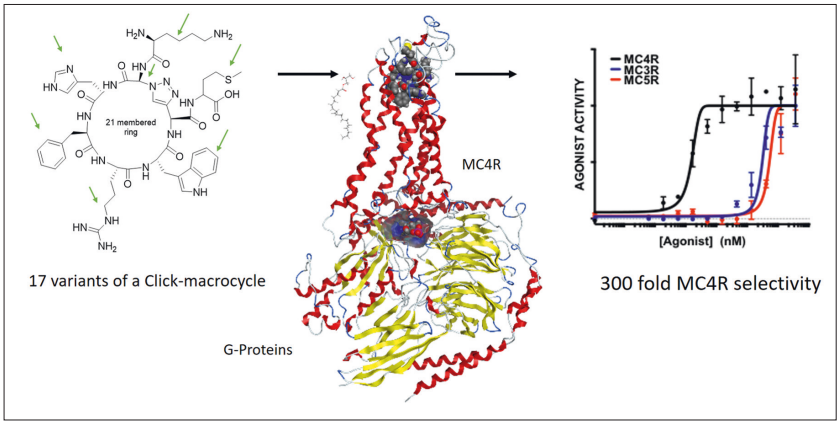

Figure 6. The CuAAC-click reaction was used to produce a set of macrocyclic molecules containing the core binding motif for melanocortin receptors, and a 300-fold selective MC4R ligand was obtained.

β-hairpin bridged by two disulfide bonds. We efficiently replaced these SS bridges with triazole bridges and the products were at least as active as Tachyplesin 1. Furthermore, in bacteria where Tachyplesin 1 was inactive, probably due to disulfide reduction, the triazole analog was fully active (Figure 6). Structural NMR studies allowed us to suggest a mechanism of action in which the hydrophobic antibiotic property is initially hidden by peptide dimerization to form a globular complex with arginines at the surface hiding away the lipophilic parts in the interior.

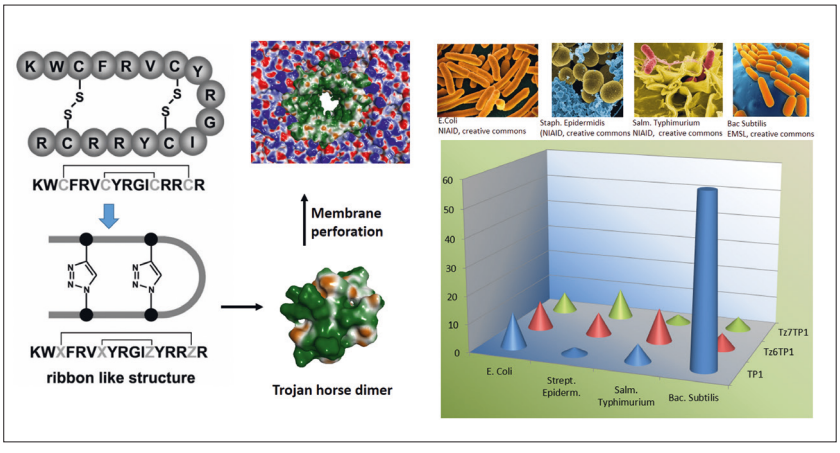

Figure 7. The antibiotic Tachyplesin 1 beta-hairpin from the Japanese horseshoe crab was equipped with two triazole-bridges and the mimetics were fully active even against Tachyplesin 1 resistant Bacillus subtilis. NMR-studies revealed the Trojan horse mechanism of the antibiotic peptide involving formation of a dimer.

Upon interaction with negatively charged cell membranes, the dimer unfolds and forms pores that affect cell lysis. Thus, we have verified that we can maintain bioactivity with triazole mimetics of disulfide bonds. We currently use click reactions to maintain the structural design of unnatural, but functional proteins. If successful and selective proteins can be obtained, we aim to present the very first therapeutic enzymes for use in medicine.

Proteases constitute a conundrum in biology since they are required to process and activate other proteins while their proteolytic activity, at the same time, can be poisonous for cells because of their ability to destroy the proteins of the cells. Proteases, particularly those with a broader specificity, can process a wide range of proteins including those needed for cell survival and they frequently even self-destroy. Their activity during expression is mostly controlled by a pro-peptide that binds in the active site and inactivates the enzyme by self-inhibition until there is a need for activity, at which point the pro-peptide is removed, frequently by self-activation. In Biotech, we would like to produce enzymes at a large scale by overexpression in *E. coli* or other expression systems. However, the expression and activation of proteases is very cumbersome to repeat artificially. For these reasons the expression of the active proteases usually gives very low yields, and cells expressing proteases are not thriving. In order to circumvent this problem, Christian Kofoed, a Ph.D. student at the CECB center, split the tobacco etch virus (TEV) protease in two halves (domains) around the active site[24]. Each half was expressed in high yields in *E. coli* using Amber stop codon suppression technology to incorporate an alkyne in one half and azide in the other. Both were according to molecular modeling, positioned close to the terminals where the two halves would have been joined in the full-length protease. When expressed, neither of the two domains showed any protease activity. However, when the two halves were mixed in a 1:1 ratio and upon equilibration subjected to CuAAC click conditions, a quantitative formation of the protease occurred. Satisfyingly, when we measured the proteolytic activity of the triazole-linked protease it was at least as active as the native protein (Figure 8). This could be a great general way to produce proteolytic enzymes that currently cannot be prepared industrially by full-length expression.

CONCLUDING REMARKS

On an important note, I would like to emphasize the importance of serendipity in all great research, realizing that almost all groundbreaking discoveries have a major element of serendipity. Great discoveries are not fabricated behind office desks; rather, they are usually surprising and are achieved by an integral combination of ideas, experimental design, and keen experimental observation of the unexpected. This has profound implications for the requirement for scientists to be able to change the

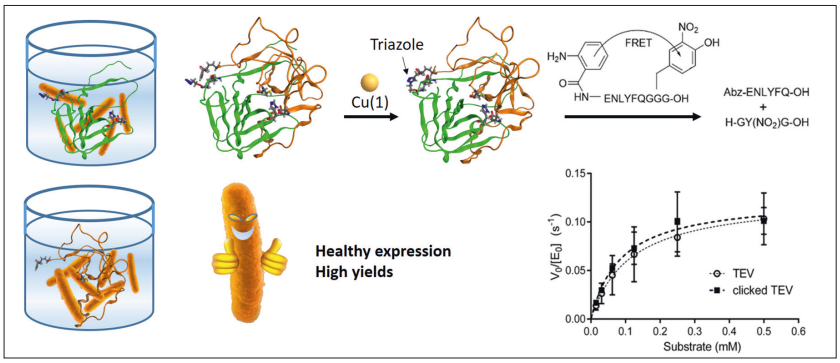

Figure 8. Proteases which cannot be expressed in useful yields due to inherent proteolytic activity in the cells causing toxic effects by host protein processing can be expressed as benign fragments containing azide and alkyne and upon folding, they can be clicked to form the fully active protease.

direction of research instantaneously and to operate freely. I hope this realization could trigger a different way of funding for research, one which is based on recent peer reviewed results, rather than blue sky ideas fitted to suit a particular research area call.

The Nobel Prize in Chemistry is more important than ever. It inspires, justifies, and provides direction in a challenged world. Chemistry is omnipresent; in fact, everything around us including you and me, life itself and all associated processes is chemistry. Thus, chemistry is more than an education; it is a conceptual route to understanding our existence and the beautiful complexity of Nature. We need chemistry to solve many global issues and live life in a sustainable manner. We need chemistry to understand the importance of sharing the resources of our world with all our fellow human beings in a responsible way. Chemistry is important for all crafts and for academic and industrial enterprises alike. Chemistry may even provide a societal framework supporting proper political decision-making and ethical considerations. Let our young study chemistry from an early age with this perspective. Let us therefore induce a great sense of curiosity, for our young to eventually make groundbreaking discoveries, required for solving the problems of their future.

Finally, although in science we all stand on the shoulders of giants, it is the hard work and intellect of every-day (not-yet giants) friends and colleagues working together that creates monumental breakthroughs in research. I am greatly indebted to so many and my sincerest thank you to all my coworkers, collaborators, funding institutions, DG, Carlsberg Laboratory and University of Copenhagen. Much appreciation goes to my family and friends, in particular to Klaus Bock, Christian W Tørnoe and Mikael Bols.

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