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All About That Amide Bond: The 6th Chemical Protein Synthesis (CPS) Meeting

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Introduction

The sixth biennial Chemical Protein Synthesis Meeting was held June 16th–19th, 2015 in sunny St. Augustine, Florida. In attendance were 93 scientists from 17 countries, representing academia and the pharmaceutical and biotechnology sectors. The meeting featured 33 talks by leaders in the field of protein chemistry, and a lively poster session with over 30 presenters. The organizers, Philip Dawson (The Scripps Research Institute), Seth Horne (University of Pittsburgh), and Stephen Kent (University of Chicago) succeeded in extending this meeting's excellent tradition of bringing together both emerging and established investigators to share their latest discoveries in the broad fields of peptide and protein chemistry. The diverse topics presented ranged from the optimization of peptides as therapeutics and cellular delivery agents and methods to assemble proteins site-specifically modified by ubiquitin, to the intracellular assembly of covalently modified semisynthetic chromatin.

Chemical protein synthesis and semisynthesis are powerful tools that provide access to targets with biologically relevant post-translational modifications (PTMs) in quantities that often remain inaccessible to current molecular biological strategies. Since its introduction by Kent and co-workers in 1994, Native Chemical Ligation (NCL) has proven to be an invaluable component of many protein synthesis efforts.^[1] The simplicity of obtaining full-length proteins with precisely defined PTMs by ligating (from the latin *ligatus*, which means to join) two strategically chosen protein fragments by means of a native amide bond has led to the widespread use of NCL. Many innovative adaptations of NCL, such as small molecules that facilitate ligation and then find use as handles for purification or as activity-based probes, and techniques that convert multi-step ligations to efficient one-pot reactions were a major thrust of the 6th CPS.^[2] Bio-orthogonal strategies such as the popular Huisgen 1,3-dipolar cycloaddition (the *click* reaction),^[3] which permits joining peptides to any number of targets and the incorporation of non-natural amino acids bearing NCL- or click-ready groups were the focus of several presentations.^[4] In combination with classical solid

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Historic setting: The meeting was held in beautiful St. Augustine, FL, founded in 1565 by Spanish settlers.

Award presentation: Stephen Kent presents Richard Payne with the 2015 *MedChemComm* Emerging Investigator Lectureship award. Payne's lecture was entitled "New ligation technologies for the rapid assembly of modified proteins."

Group outing: A brief walk took attendees to Castillo de San Marcos, the oldest fort in the United States, constructed between 1672 and 1695 on Matanzas Bay in St. Augustine.

phase peptide synthesis (SPPS) and well-established recombinant protein production techniques, the exciting technologies described at the 6th CPS promised synthetic access to countless proteins and peptides of interest to the biological and therapeutic research communities.

The meeting began with a warm welcome reception and the keynote address delivered by Ashraf Brik (Technion Israel Institute of Technology, Israel) entitled “Recent developments in the synthesis of unique analogs of free and anchored ubiquitin chains.” Ubiquitin (Ub) is one of many PTMs covalently appended to proteins in living cells. Unlike smaller PTMs such as phosphorylation, methylation, and acetylation, the large size and multiple surfaces of Ub allow it to perform different context-dependent functions. These include signaling a target’s intracellular localization, changing a target’s binding partners, and regulating enzyme activity. A family of over 600 ligase enzymes are involved in the site-specific attachment of Ub to protein targets, but only a handful of these have been characterized thus far. This poses a significant challenge when attempting to obtain ubiquitylated proteins in sufficient amounts for biochemical investigations. Brik’s talk addressed this challenge while describing impressive recent advances by his group in the synthesis of phosphorylated diubiquitin, and studies addressing the effect of this newly discovered ubiquitin modification on the recognition of ubiquitin by the enzymatic machinery responsible for cleaving ubiquitin chains.^[5] In addition, Brik also described efforts on devising new synthetic approaches for the preparation of polyubiquitylated proteins for use in a variety of biochemical and biophysical studies.^[6] His group’s success in appending pre-assembled chains of ubiquitin to the model protein α -globin by a thiol-directed conjugation strategy revealed a general method that should pave the way for mechanistic studies of the roles for protein polyubiquitylation.^[6-7]

Novel chemical strategies for protein synthesis

Canonical NCL involves a chemoselective reaction between a C-terminal thioester on one polypeptide fragment and an N-terminal cysteine on another. The efficient preparation of an activated thioester at the C-terminus of a peptide following SPPS remains a challenge. To address this, Ryo Okamoto (Osaka University, Japan) presented an approach in which a peptide is *S*-thiocarbonylated at cysteine and subsequently treated with *N*-acetylguanidine. The resulting peptidyl-*N*-acetylguanidine is readily converted to an α -thioester, or is suitable for direct use in ligation reactions containing the alkyl thiol additive sodium 2-mercaptoethanesulfonate (MESNa).^[8] Oleg Melnyk (University of Lille, France) introduced the selenium analog of the SEA group, the bis(2-selenylethyl)amino (SeEA) moiety, as a latent selenoester. In a ligation mixture containing multiple peptide fragments, alkylthioester, SEA, and SeEA functionalities can be activated specifically, and in sequential order, by controlling the reductive power of the ligation mixture, allowing for elegant one-pot assemblies of cyclic or branched peptides.^[9]

One-pot, multi-segment NCL was also addressed by Thomas Durek (The University of Queensland, Australia), who described the synthesis of disulfide rich, venom-derived peptides and characterization of their structures.^[10] Richard Payne (The University of Sydney, Australia) delivered the 2015 MedChemComm Emerging Investigator Lectureship

on the development of general synthetic routes toward building blocks for thiol-derived amino acids and their utilization in ligation reactions. Post-ligation conversion to a natural amino acid is often achieved by metal-free radical-mediated desulfurization,^[11] which is unfortunately incompatible with the common NCL thiol catalyst, 4-metcaptophenylacetic acid (MPAA). In order to combine ligation and desulfurization into a one-pot process, Payne employed 2,2,2-trifluoroethanethiol (TFET) as the NCL thiol additive to achieve ligation rates near that afforded by MPAA without any detrimental effect on the desulfurization reaction.^[2b] An alternative one-pot ligation-desulfurization method was presented by Christian Hackenberger (Humboldt Universität zu Berlin, Germany) and demonstrated on the unstructured Tau protein, which is closely associated with Alzheimer's disease pathology.^[12] Hackenberger's method employed a photocleavable biotin tag at the C-terminus of the Tau ligation product as a traceless purification handle that is compatible with free radical desulfurization on streptavidin-conjugated resins.

An on-resin ligation and purification strategy was presented by Agnès Delmas (CNRS-Orleans, France). Following SPPS, a peptide fragment is linked to a water-compatible resin for a series of click triazole ligations.^[13] Importantly, the resin linker is compatible with the ligation chemistry as well as enzymatic glycosylation of the protein ligation product, MUC1, yet can still be cleaved efficiently in the presence of sensitive glycosidic bonds. This is an exciting development for the rapid synthesis of glycosylated proteins. Serine/threonine ligation, an alternative to thiol-based NCL, was presented by Xuechen Li (The University of Hong Kong, Hong Kong).^[14] In this method, a peptide generated by traditional 9-fluorenylmethoxycarbonyl (Fmoc-) SPPS bearing a C-terminal salicylaldehyde ester was reacted with a second peptide bearing an N-terminal serine or threonine residue. Acidolysis of the formed *N,O*-benzylidene acetal at the ligation site generated the native serine or threonine. The high abundance of serine and threonine in proteins, relative to cysteine, provides significantly more options when selecting the site of chemical ligation.

Elucidating functions of post-translational modifications

The synthesis of post-translationally modified target proteins or peptides is necessitated when recombinant or *in vitro* enzymatic methods do not yield product in the quantity, purity, or site-specificity required for biochemical or biophysical assays. Use of the ligation auxiliary 2-aminoxyethanethiol to site-specifically append ubiquitin to the ϵ -amine of lysine was presented by Champak Chatterjee (University of Washington, USA).^[2a] Surprisingly, retention of the ligation auxiliary in ubiquitylated substrates conferred stability to the ~100 deubiquitylating enzymes (DUBs) in cellular lysates, which may be useful in lysate pull-down experiments with these compounds. Huib Ova (Netherlands Cancer Institute, Netherlands) presented a novel and unexpected property of a subset of deubiquitylating enzymes. His group has developed active-site directed probes for DUBs that employ terminal alkynes. The alkyne probe displays unexpected reactivity with the active site cysteine of DUB proteases to form a vinyl thioether that covalently traps the DUB.^[15] Such activity based probes are invaluable for determining the substrate specificity of enzymes, even within complex mixtures, and are especially useful to identify enzymes responsible for the addition and removal of PTMs, many of which remain unidentified.

Improvements in assays to evaluate known enzymes that act on PTMs were also described. Dirk Schwartz (University of Tübingen, Germany) presented his group's research on histone deacetylases (HDACs), which are key regulators of epigenetics and chromatin activity. They have established new chemical probes, based on methodologies of modern peptide and protein chemistry, for interrogating the substrate selectivity and composition of endogenous HDAC complexes. The utility of their approach was demonstrated in determining the preference of HDAC8 for deacetylation at specific lysine residues in the C-terminus of p53.^[16] Another PTM that garnered much attention for its complex cellular functions and synthetic challenges was protein glycosylation. Matthew Pratt (University of Southern California, USA) focused on O-linked β -N-acetyl glucosamine (O-GlcNAc). O-GlcNAcylation is the addition of the single monosaccharide N-acetyl glucosamine to intracellular proteins in metazoans and plants. Pratt's group used cutting-edge semisynthesis to prepare α -synuclein site-specifically modified with a physiologically relevant O-GlcNAc modification. They demonstrated that this modification has a dramatic inhibitory effect on the aggregation and toxicity of the protein, which has important implications for ensuring the persistence of α -synuclein O-GlcNAcylation in Parkinson's disease.^[17]

Glycosylated proteins have long been difficult targets for the synthetic community,^[18] due to the diverse nature of glycosidic linkages, monosaccharide composition and polysaccharide chain lengths, and the formation of branched chains. Christian Becker (University of Vienna, Austria) and Carlo Unverzagt (University of Bayreuth, Germany) described tackling these challenges with two different strategies that have expanded chemical access to glycosylated proteins. The Becker group recently reported a synthetic method for glycosylated protein ligation based on a PEGylated photocleavable auxiliary, and demonstrated its utility by generating a library of site-selectively O-glycosylated MUC1 variants with different glycosylation patterns.^[19] Carlo Unverzagt shared his group's progress toward the extremely challenging semisynthesis of biologically active glycoforms of human IL-6 by NCL of recombinant and synthetic glycoprotein fragments. Key to their success was an efficient refolding method that gave rapid access to libraries of IL-6 glycoforms in high yield and purity.^[20] Ratmir Derda (University of Alberta, Canada) discussed the assembly and screening of genetically-encoded libraries of chemically modified peptides on the surface of the bacteriophage M13. Using this high-throughput approach, his team discovered hybrid peptide-carbohydrate ligands that specifically bind to carbohydrate-binding proteins (lectins).^[21]

Dissecting biomolecular interactions with protein chemistry

In addition to interrogating PTMs, chemical protein synthesis has also been applied to directly investigate protein-protein interactions (PPIs) and to facilitate structure-activity studies of PPI inhibitors. Wuyuan Lu (University of Maryland School of Medicine, USA) discussed his use of protein semisynthetic techniques to investigate the basis of high-affinity peptide inhibition of the p53-MDM2/MDMX interaction,^[22] and presented recent work on the inhibition of this interaction by stress-induced MDM2/MDMX phosphorylation. By synthesizing the site-specifically phosphorylated p53 binding domains of MDM2 and MDMX, Lu's group was able to show that phosphorylation at a site different from that previously identified is in fact responsible for disrupting binding. Yaowen Wu (Chemical

Genomics Centre of the Max Planck Society, Germany) described a novel protein N-to-N and C-to-C ligation strategy to trap weak interactions, in which small molecules with thiol or thioester moieties can link two protein termini only when weak interactions hold the proteins in close proximity. Using this strategy, his group prepared a covalent complex between the yeast Rab family GTPase Ypt1 and Rab GDP dissociation inhibitor (GDI) that mimics the transient intermediate of the prenylated Ypt1-GDI interaction. This served as a substrate for GDI-displacement factor (GDF), which revealed insights into the mode of action of GDF.^[23]

Tom Grossmann (Chemical Genomics Centre of the Max Planck Society, Germany) presented his groups' efforts to use PPIs to drive efficient chemical ligation. A domain of the CREB binding protein (CPB), capable of binding two different peptide sequences simultaneously, served as a template to align the N-termini of peptidyl ligands and increase the rate of a ligation reaction between them by 6200-fold.^[24] This concept was then applied to selectively label proteins in a cell lysate.

Protein chemistry in living cells

Techniques that permit the manipulation of proteins within intact cells are useful to study complex processes that may not be accurately recapitulated *in vitro*. One such process is the biochemical *crosstalk* between epigenetic PTMs, or *marks*, in chromatin.^[25] Tom Muir (Princeton University, USA) described the use of ultrafast split inteins, single-turnover enzymes that catalyze the process of protein ligation (also called protein splicing), to incorporate ubiquitylated histone H2B into chromatin in live cells.^[26] With this technique, his group was able to show that ubiquitylation of H2B leads to an increase in methylation at lysine 79 in histone H3.

Kathrin Lang (Technische Universität München, Germany) discussed genetic code expansion in *E. coli* and mammalian cells to incorporate non-natural amino acids bearing strained alkene or alkyne moieties into proteins.^[27] These groups, which react rapidly with tetrazine conjugates by an inverse-electron-demand Diels-Alder cycloaddition, were utilized to fluorescently label proteins *in vivo* for imaging by super-resolution microscopy techniques. Gonçalo Bernardes (University of Cambridge, UK) described the successful construction of artificial ruthenium dicarbonyl metalloproteins that spontaneously release biologically functional carbon monoxide (CO) in live cells and mice.^[28] Bernardes also discussed studies utilizing albumin artificial metalloproteins that accumulate and selectively deliver CO to tumors in mice, which permitted investigation into the poorly understood anti-cancer properties of toxic CO.

Ned Budisa (Technische Universität Berlin, Germany) shared his group's exciting work towards the evolution of synthetic cells with alternative biochemical building blocks.^[29] In a long-term evolution experiment, tryptophan auxotrophic *E. coli* were gradually forced to adapt to an alien environment by reducing the concentration of tryptophan and maintaining a constant concentration of new substrate, L- β -(thieno[3,2-b]pyrrolyl)alanine. The experiment yielded bacteria capable of using this new amino acid for all its cellular processes. Not only does this open the way to engineering new spectral, thermodynamic, and pharmacological

properties into existing proteins, but also showcases the potential of *E. coli* in potentially colonizing hostile environments.

Chemical synthesis of mirror-image peptides and proteins

Due to their protease resistance and low immunogenicity, proteins and peptides containing mirror-image α -D-amino acids are attractive as therapeutic agents. With this in mind, Tilman Hackeng (Maastricht University, Netherlands) presented efforts to develop a D-peptide inhibitor of tissue factor pathway inhibitor (TFPI), a protein that inhibits normal blood clotting and leads to uncontrolled bleeding in hemophilia.^[30] Lei Liu (Tsinghua University, China) presented work on the use of his group's peptide C-terminal acylhydrazide method to synthesize photo-caged chemokines and D-amino acid containing mirror-image versions of immune check-point proteins.^[31] These synthetic proteins were used for the study of cell migration under spatiotemporal control and toward the development of D-peptide antagonists for cancer immune therapy.

A subset of cellular proteins can only adopt the correct folded structure with the assistance of chaperone proteins such as GroEL and Hsp90.^[32] Given their opposite chirality to natural proteins, it is possible that D-proteins may require the mirror-image version of naturally occurring molecular chaperones to fold correctly. Michael Kay (University of Utah School of Medicine, USA) addressed this question by investigating the ability of the GroEL/ES chaperone complex to fold a mirror-image protein.^[33] His group synthesized the 312-residue protein DapA, which requires GroEL/ES-mediated folding, in both L- and D-diastomeric forms and determined that, surprisingly, GroEL/ES can fold both diastereomers. Such detailed mechanistic studies support a non-specific hydrophobic interaction model for chaperone activity, and provide a valuable tool to correctly fold D-proteins for mirror-image drug development and synthetic biology applications.

Protein and peptide therapeutics with unusual topologies

N-to-C-terminally conjugated peptides, or cyclic peptides, are biologically active, thermally stable and resistant to enzymatic degradation, which makes them attractive as therapeutic agents. David Craik (The University of Queensland, Australia) presented a method to generate cyclotides, small disulfide rich cyclic peptides that may serve as scaffolds for bioactive molecules.^[34] Craik's group utilized Sortase A, a bacterial transpeptidase, to cyclize a variety of small disulfide-rich peptides ranging in size from 14 to 29 amino acids and containing up to three disulfide bonds. One feature of cyclotides that is particularly appealing is that they occur naturally in numerous plant species including the pea, potato, and grass families.^[35] A pleasant vision for the future of pharmaceutically active cyclotides may indeed be their production in current cash crops, although several issues including dosage and bioavailability would need to be addressed prior to commercialization. Les Miranda (Amgen, USA) presented efforts towards engineering selective analogs of the GpTx-1 tarantula venom peptide antagonist of sodium channel $\text{Na}_v1.7$, an important channel found in pain-sensing neurons. Positional alanine scanning of the GpTx-1 peptide, which contains three disulfide linkages in the inhibitory cystine knot (ICK) motif,^[36] led to

the identification of an analog that is nearly 10-fold more potent and over 1000-fold more selective than wild-type GpTx-1.^[37]

Mohamed Marahiel (Philipps-University Marburg, Germany) presented his work with lasso (or lariat) peptides that are proteolytically and thermally stable bacterial natural products with therapeutic potential. The unique defining structural feature of lasso peptides is a macrolactam ring formed via an isopeptide linkage between the peptide N-terminus and an aspartate or glutamate side-chain. The C-terminal tail is threaded through the macrolactam ring and held in place by sterically bulky residues above and below the plane of the ring. Marahiel discussed his group's latest findings on lasso peptides, their biosynthesis, and their physicochemical properties, including the proposed mechanism through which the precursor peptide is enzymatically processed into a mature lasso peptide.^[38] He also presented new analytical tools to assess and prove the lasso topology without the requirement for complete structural elucidation.

Rob Liskamp (University of Glasgow, UK) discussed his group's work in the synthesis of protein mimetics consisting of discontinuous epitopes connected to a central scaffold.^[39] He described the efficient convergent synthesis of scaffolds presenting cyclic peptides, which may be used as individual protein mimics or as targeted synthetic vaccines. Repetitively branched structures, called dendrimers, also provide a scaffold on which to present multiple therapeutic ligands. Paul Alewood (The University of Queensland, Australia) discussed dendrimers developed in his group that incorporate the $\alpha 7$ -nicotinic receptor blocker α -conotoxin ImI (α -ImI).^[40] The dendrimers were synthesized by a Cu(I)-catalyzed cycloaddition of azide-modified α -ImI to an alkyne-modified poly-lysine dendron. Alewood demonstrated that the multimeric display of conotoxins significantly enhances potency against a receptor relative to the monomeric ligand.

Improving the stability and delivery of protein-based biologics

Protein-based biologics must circulate in the bloodstream long enough to achieve their therapeutic goals. Hence, resistance to proteolysis and diminished renal clearance are two highly desirable properties. Toward these goals, the modification of proteins with long-chain polyethylene glycol (PEGylation) is a well-established strategy that significantly increases their half-lives in biological fluids.^[41] However, the precise position in a protein where PEG may be appended is typically only empirically determined. Joshua Price (Brigham Young University, USA) presented a thought-provoking structure-guided method for predicting sites on a protein that may confer stabilization upon PEGylation.^[42] This methodology correctly predicted a location in the Src kinase SH3 domain where PEGylation led to conformational stabilization and a concomitant increase in resistance to proteolysis. Understanding the principles that govern site-specific stabilization of proteins by PEGylation will undoubtedly have a tremendous impact in the rapidly growing biologics industry.

Product homogeneity and manufacturing efficiency are two key factors in the successful commercialization of biologics. With this in mind, Anouk Dirksen (Pfizer Inc., USA) highlighted the significant improvements that were made in Development to the conjugation

process of a FGF21 protein-antibody conjugate in order to make it commercially viable.^[43] This conjugate reached clinical trials for metabolic disease indications. Thorough evaluation and greater understanding of the key process parameters, focused on enhancing the stability and quality of reaction intermediates, greatly improved the overall yield and final product purity. This allowed for a significant reduction in the consumption of materials such that the projections made for a commercial process were exceeded.

Many protein and peptide based drugs must cross the cell membrane before reaching their intended targets, and methods to cross this barrier were also presented. Bradley Pentelute (Massachusetts Institute of Technology, USA) described the use of protective antigen (PA) from anthrax toxin to deliver a D-polypeptide cargo into the cytosol of mammalian cells.^[44] When conjugated to the C-terminus of the PA-binding domain of anthrax lethal factor, LF_N, the cargo was efficiently delivered and able to disrupt protein-protein interactions in cancer cells leading to their apoptosis.

Outlook

The 6th CPS meeting emphatically highlighted the seemingly endless potential of peptide and protein chemistry when applied to understanding and controlling complex biological processes. As an enabling tool that provides facile access to naturally occurring proteins and their non-natural analogs, chemical protein synthesis will no doubt continue to inspire many new mechanistic investigations and rational engineering approaches in the years ahead. We look forward to hearing about these at the 7th CPS meeting to be held in Haifa, Israel.

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