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Welcome to 43JPS/PEM4

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It is my great pleasure and honor to welcome you to the *International Conference of 43rd Japanese Peptide Symposium and 4th Peptide Engineering Meeting (43JPS/PEM4)* to be held in Yokohama from November 5 to 8, 2006 (http://peptide-soc.jp/43JPS4PEM.html). The Japanese Peptide Society has organized the annual meeting named Japanese Peptide Symposium, and international symposiums in two or three years' interval. In this year, 43JPS is held as a joint international symposium with PEM4. PEM has been also organized as an international meeting since 1997 by the international committee of this meeting including the late professor Murray Goodman and under the auspices of APS, EPS and JPS. The PEM1 meeting was held in Osaka, Japan, 1997, as a post-symposium of 1st International Peptide Symposium (IPS), Kyoto, with the chair of Dr. Susumu Yoshikawa, Osaka National Research Institute. PEM2 was held in Capri Island, Italy, 2000 by Dr. Ettore Benedetti, University of Naples, and PEM3 was in Boston, USA, 2003, by Dr. Charles Deber, University of Toronto. In PEM, engineering aspects of peptide science and technology have been discussed by a limited number of attendees (ca. 100-200). In 2006, PEM has come back to Japan and is held as a joint international conference with 43JPS.

We, the organizing committee of 43JSP/PEM4, can open this exciting conference in Yokohama, Japan with the main theme of *Peptide Science and Engineering in Chemical Biology*. In the post-genome era, the world-wide research competition has been started to develop novel bio-related science and technology innovations. In these fields, peptide science and technology is one of the major and promising research trends to understand complex peptide and protein events in living organisms. Moreover, new diagnostic and therapeutic researches will be emerged from the chemical biology with various bio-active peptides. The related technologies including biochips, proteomics, and nanobiotechnology have become also very important in the post-genome researches. In this international conference, the jointed meeting bridging science and engineering will be, for the first time, held in Yokohama, Japan. This meeting will be beneficial to develop the international standards in chemical biology of peptide and protein.

The conference will highlight many of the recent developments in the broad area of peptide science and engineering with a particular emphasis on how these advances are being applied to future prospects in chemistry and biology. Specific topics to be covered include *synthetic innovation of peptide & protein, peptide & protein design and engineering, structure-activity relationship for chemical biology, peptide & cell engineering, peptide & drug discovery, peptidomics & proteomics including post-translational modification, peptide library & protein chip, peptide in health & medical science, peptide & nanobiotechnology.* This conference will cover cutting-edge research presented by a wide range of distinguished speakers (53 oral presentations including more than 30 invited talks), as well as poster presentations (more than 230) by scientists from all over the world. In addition, we have many exhibitors and plan the *Young Investigators' Symposium and Competition* with 14 distinguished young scientists.

The proceeding book of 43JPS/PEM4 is published just at the conference date. This is the first trial in the peptide symposiums in the world. I greatly appreciate all authors who have submitted their papers to the proceedings, and the co-editor, Dr. Hitoshi Ishida, Kitasato University, and Mr. Tsuyoshi Hayashi, Proactive Inc., for the hard works for editing. I hope that the book is useful for your understanding the high level sciences in the conference and for your future researches.

The venue for this conference will be at Pacifico Yokohama (Annex Hall) as a world-class integrated convention center in the Minato-Mirai bay area in Yokohama (http://www.pacifico.co.jp/index_e.html). The conference site is located at a convenient place (just south of the metro area of Tokyo, 30 min), and international hotels, the Japanese biggest Chinatown,

many shopping places, and various sights to see including Kamakura (the 12th century historical capital) are located nearby. I am looking forward to seeing you at the international town, Yokohama, Japan, and hope that you enjoy Japanese autumn atmosphere in 2006.

Finally, I greatly appreciate all participants as well as the members of the organizing committee, the programming committee, and the local organizing committee of 43JSP/PEM4 for the efforts and contributions to this international conference. I should express my appreciation to JPS, KPS, APS, EPS, AuPS, CPS, IPS and to many foundations and companies for the supports of the conference.

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Peptides and Sustainable Development

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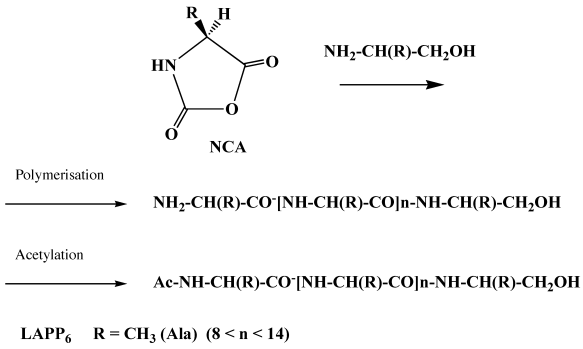
Sustainable development is an ever-increasing problem of our society. In our laboratory in Montpellier, France, several of our research programs are concerned by «green chemistry» and «sustainable development» in relation with peptides. In collaboration with two French chemical companies involved in agriculture, namely «De Sangosse» and «Agronutrition» we have developed a research program that uses peptides as non-toxic, biodegradable materials, respectful of environment. We would like to describe here some of our efforts in the design, synthesis and development of peptides as «elicitors» that can be used in agriculture for the protection of plants against pathogens.

Plant «elicitors» are surface molecules from a plant pathogenic microorganism, which, when applied to host or non-host plants will induce symptoms of resistance reactions typical for the plant-pathogen system studied. Elicitors stimulate the natural defenses of plants[1]. It was recently demonstrated that peptaibols are able to act as plant elicitors[2]. Peptaibols are peptides of usually less than 20 amino acid residues, containing non-natural amino acid, acylated on their N-terminus and having an alcohol function at their C-terminus. They can adopt a helical structure. Based on these observations, we proposed that an elicitor could be a peptide acylated on the N-terminus, having an alcohol function at the C-terminus and able to adopt an helical structure (Figure 1).

Figure 1.

By simple polymerization of N-carboxyanhydrides of α- and β-amino acids, we have synthesized oligomers of amino acids known to favor a helical structure, starting polymerization with the corresponding amino alcohol and acetylating the N-terminus of the oligomer (Scheme 1). Among the series of oligomers we have synthesized, the poly-Ala oligomer (named LAPP6) was among the most potent compound in stimulating the natural defenses of plants[3].



Scheme 1.

The activity of these oligomers was first tested in melon, cucumber, and grapevine leaves by evaluating their ability to stimulate the activity of two enzymes, chitinase and peroxidase. Stimulation of these two enzymes is known to strongly correlate with the resistance of plants to pathogens, and is representative of an «elicitor» activity[2]. The oligomers of amino acids were able to stimulate the enzyme activities at low doses (7-70 mg/L), at a high level. Particularly, LAPP6 was found very potent.

LAPP6 was further tested in grapevine plants infected by the downy mildew fungus *Plasmopara viticola*, in the fields, in three different areas of France, namely Burgundy, Loire Atlantique and Languedoc on different species. The results of these trials confirmed the interesting activity of LAPP6 in reinforcing the natural defenses of grapevine plants and to protect them against *Plasmopara viticola.*

So far, LAPP6 is a very promising oligopeptide elicitor, exhibiting interesting properties. LAPP6 is easy to synthesize, is non-toxic, is biodegradable into non-toxic and eventually assimilable amino acid derivatives. LAPP6 is not expensive to produce and is active at low doses. Further developments of this new class of peptide compounds acting as elicitors that can be used in agriculture are going on in our laboratories, with the goal of developing peptide compounds as agrochemicals for a sustainable development.

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De Novo Design of Membrane-Active Antimicrobial Peptides

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Bacterial resistance to small molecule antibiotics is on the rise and as a consequence, novel approaches to the treatment of microbial infections are urgently required. Antimicrobial peptides provide a viable alternative. Databases currently report close to 1000 sequences for natural antimicrobial peptides and proteins from animals and plants (http://www.bbcm.univ.trieste.it), while several thousand others have been designed *de novo* and produced synthetically[1]. While they differ widely in sequence and structure, cationic antimicrobial peptides (termed CAPs) generally consist of 12-50 residues, approximately 50% of which are hydrophobic, and accordingly have the potential to form an amphipathic α-helical structure when bound to membranes. Cationic antimicrobial peptides are active in the nanomolar to micromolar range and show little target or L- vs. D-residue specificity (their D-enantiomers exhibit similar activity to their L-counterparts), indicating that they interact with achiral components of the cell membrane[2] through a mechanism of physical disruption. Accordingly, bacteria may not easily develop resistance to these substances.

Understanding the mechanism of membrane disruption by a variety of peptides in many different types of membranes is necessary to elucidate the various factors determining the activity of a peptide and to subsequently design therapeutic peptides with the desired potency and selectivity. In prevailing views of CAP mechanisms, peptides interact with the membrane, inducing relocation of the peptide into a position parallel to the membrane at the interface of the hydrophilic head groups and hydrophobic fatty acid chains of the membrane phospholipids. In the so-called "carpet" mechanism[2], peptide-mediated packing defects are introduced into the membrane phospholipids, causing large-scale disruption of the membrane by very dense aggregation of parallel-oriented peptides, wherein the lipid head group imposes strain in the membrane, and membrane permeation is eventually induced at sites where local peptide concentration reaches certain threshold values.

Our laboratory has for several years been studying the sequence-dependent conformations and oligomeric states of *de novo* designed transmembrane peptides to systematize the contributions of hydrophobicity and helicity to peptide-membrane interactions[3]. We subsequently noted that their sequences and positive character broadly resembled a variety of naturally-occurring CAPs. Key features of our designed peptides were the consecutive non-amphipathic stretch of hydrophobic core residues, and the multi-positively charged Lys/Arg in either segregated (all basic residues at the N- or C-terminus), or separated forms (basic residues at both termini). The Lys or Arg tags solubilize the hydrophobic peptides in aqueous media[4]. When the average hydrophobicity of the peptide full core segment is above an experimentally determined 'threshold' value based approximately above that where X = Ala[3] − the peptides spontaneously insert into micellar membranes with accompanying formation of α-helical structures. One series of high antimicrobial potency is typified by the 17-residue sequence KKKKKKAAFAAWAAFAA-NH_2, wherein the Lys charges are typically grouped at the N-terminus, and a hydrophobic core of only 11 residues. This series of peptides was found to be highly effective against a wide variety of Gram-positive and Gram-negative bacteria, and yeast, with minimum inhibitory concentrations (MICs) in the range of 4-128 μM or 8-256 μg/mL, but display no hemolytic activity against rabbit or human red blood cells up to relatively high concentrations (325 μM or 650 μg/ml)[5]. From these results, we realized that targeting of membrane-active antibiotics to the invaders (bacteria) instead of to the host (mammalian cells) must originate in the significant differences in the lipid compositions of these two systems. *E coli* membranes are highly anionic, and contain about 75% phosphatidyl- ethanolamine (PE), 20% phosphatidylglycerol (PG), and 5% of other components, including cardiolipin. The anionic head groups of PG + cardiolipin in particular render this membrane susceptible for association with positively charged species such as CAPs. In contrast, the mammalian erythrocyte membrane (outer leaflet) typically contains 33% phosphatidylcholine, 18% sphingomyelin, 9% PE, along with 25% cholesterol, and their outer leaflets consist exclusively of zwitterionic (neutral) head groups.

Using lipid vesicles prepared from bacterial and mammalian lipid mixtures, we indeed found that the

designed CAPs readily inserted into bacterial membranes, but did not insert into mammalian membranes. Experimental indication for such bacterial membrane insertion and evidence of peptide selectivity for bacterial vs. mammalian cell membranes is shown in Fig. 1, where we display measurements of fluorescence emission intensity and blue shifts (or lack thereof) in the λ_{max} of the Trp probe incorporated into the hydrophobic core of peptides in this library. As shown in Fig. 1, the gray bars indicate that blue shifts of Trp λ_{max} values of 17-23 nm occur when bacterial lipid mixtures are used (LUV-bact). Mixtures corresponding to human red cell plasma membranes (LUV-RBC (outer) (small black bars) are essentially inert to the CAPs. Only the relatively hydrophobic all-D W17-6k-(4L) inserts in zwitterionic lipids (dark gray bars) and only in the absence of cholesterol.

These findings supported an initial electrostatic membrane interaction step in which bacterial membranes attract and bind peptides onto the anionic bacterial surface (modeled in Fig. 2) similar to the "carpet mechanism" – but in this case followed by the further 'sinking' of the core segment to a depth of *ca*. 2.5 - 8 Å into the membrane, as derived from Trp fluorescence measurements in conjunction with doxyl labeling of lipids[6]. We note that the synthetic

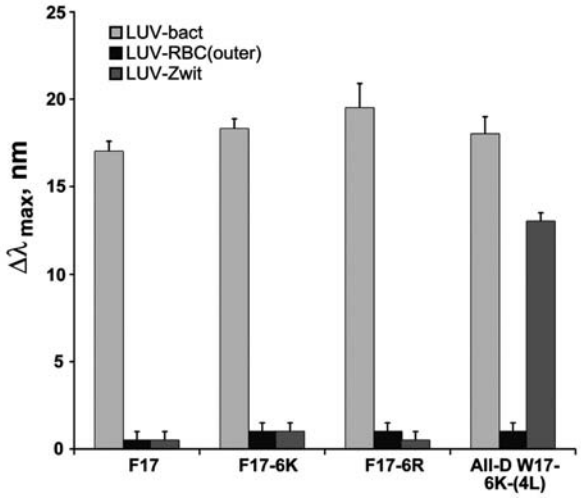

Figure 1. Blue shifts in wavelength maxima emission ($\Delta\lambda_{max}$) of Trp fluorescence in large unilamellar vesicles (LUVs) prepared from bacterial lipid mixtures (LUV-bact) or mammalian red blood cell mixtures (LUV-RBC(outer)). Peptides are: F17 = KKKAAAFAAWAAFAKKK-NH_2; F17-6K = KKKKKKAAFAAWAAFAA-NH_2; F17-6R = RRRRRRAAFAAWAAFAA-NH_2; all-D W17-6K-(4L) = D-isomer of KKKKKKALWALWLAWLA-NH_2. The LUV-Zwit mixture consists of 0% anionic lipids, 100% zwitterionic lipids, and no cholesterol. Temperature=44 ℃ to maintain lipid fluidity. Adapted from[6].

CAPs contain an uninterrupted stretch of hydrophobic residues conducive to membrane insertion while the majority of natural CAPs such as magainin have amphipathic sequences that would appear to promote anchoring only into the bacterial membrane surface *per se*. This relatively deep insertion of the designed peptides – likely parallel to the membrane surface[6] – may facilitate movement of the peptides at right angles to the long axes of the lipid chains, and consequently interfere with lipid packing. Due to the presence of AxxxA sequence motifs that promote close approach of helices in membranes[7], the peptides are believed to penetrate bacterial membranes in dimeric form, as modeled in Fig. 2, which produces larger particles that may have enhanced destructive power.

The high selectivity of these peptides for non-mammalian membranes, combined with their activity toward a wide spectrum of gram-negative and gram-positive bacteria and yeast – while retaining water solubility – represent significant advantages of this class of peptides. The long-term goal of the current research program is thus the development of novel therapeutics. However, in order to aid in the treatment of chronic *P. aeruginosa* infections that occur, for example, in the lungs of cystic fibrosis patients, it will be necessary to design CAPs that can evade the highly anionic exopolysaccharide alginate matrix that is secreted upon the colonization of bacteria into "biofilms"[8]. Success here will bolster our command of the underlying mechanism(s) as to how bacteria resist our interventions, and how we can best develop future strategies to outwit them.

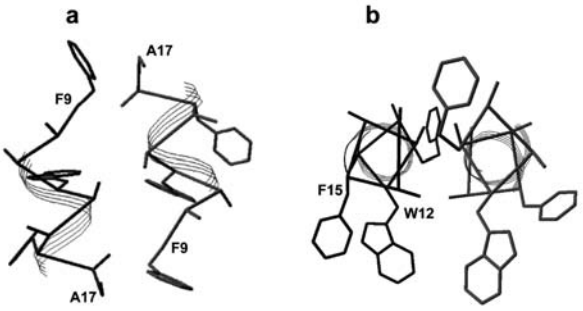

Figure 2. Energy-minimized dimer models showing the interaction surface between antiparallel helical segments from the hydrophobic core of the cationic antimicrobial peptide F17-6K. (a) side view; (b) end view. The model was generated using the global conformational search software CHI with idealized helices corresponding to peptide residues 9-17 (FAAWAAFAA). A dielectric constant of 1 was used in the calculation. Adapted from[6].

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Antimicrobial Peptide Research at the Research Center for Proteineous Materials

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Antimicrobial peptides are being increasingly recognized as potential candidate antibacterial drugs in the face of the rapidly emerging bacterial resistance to conventional antibiotics in recent years. The action of antimicrobial peptide is mediated by a direct interaction with cell membranes and a common feature of these interactions is the induction of cationic amphipathic secondary structure following binding of the peptides to the membrane surface. The mechanisms of action of antimicrobial peptides are known to differ depending on the peptides, namely carpet, toroidal or barrel-stave model. Among the more potent antibiotics reported so far are small bioactive peptides like cecropin A (CA), magainin 2 (MA), melittin (ME), HP (2-20), PMAP-23, SMAP-29 and plant defensin. These antimicrobial peptides possess a potent antibiotic activity against bacteria, yeast and even certain enveloped viruses. At present, a large number of antimicrobial peptides are reported and their antibiotic activities are related to joining of phosphatide membrane of target cell, and forming of ion channel or pore in cell membrane which leads to cell lysis by destroying cell membrane.

Peptide research is one of the major research activities focused at the Research Center for Proteineous Materials (RCPM), Chosun University, Korea. RCPM is a Center of Excellence designated and supported by the Korean Government. The peptide research in RCPM is being carried out mainly by the Peptide Engineering Lab (Kyung-Soo Hahm, Ph. D.: kshahm@chosun.ac.kr and Yoonkyung Park, Ph. D.: y_k_park@chosun.ac.kr). The research has been focused on antimicrobial peptides ranging from natural sources and synthetic designed peptides. The antimicrobial peptides obtained from natural sources include Potide-G[1], Potide-J[2], Potamin-1[3] and Potamin-2 from various potato tubers, Bacillin from bacteria, Nidulin and AN-1 from fungi, and HAF-1 from human amniotic fluid. Synthetic peptides were designed as analogs including hybrid peptides, CA-MA, CA-ME, HP-MA[4,5] and HP-ME[6] and analogs of HP (anal 3), CA-MA (P5), PMAP-23 (P6) and HP-ME (anal 6).

Structure-activity relationship studies carried out using peptides including HP (2-20) and its analogue 3, CA-MA analogue P5 and PMAP-23 and its analogue P6 of antibacterial and antifungal activities. HP (2-20) is the antimicrobial sequence derived from N-terminus of *Helicobacter pylori* Ribosomal Protein L1 (RPL1). Several analogues with amino acid substitutions were designed to increase or decrease only the net hydrophobicity. In particular, the substitution of Trp for the hydrophobic amino acid, Gln and Asp at position 17 and 19 of HP (2-20) (HPA3) caused a dramatic increase in antibiotic activity without a hemolytic effect. These results indicated that the hydrophobic region of peptides is prerequisite for its effective antibiotic activity and may facilitate easy penetration of the lipid bilayers of the cell membrane[7,8,9,10].

CA-MA analogues were designed to increase not only net positive charge by Lys-substitution but also hydrophobic helix region by Leu-substitution from CA (1-8)-MA (1-12) hybrid peptide (CA-MA). In particular, CA-MA analogue P5 (P5), designed by hinge region (GIG → P)-substitution, Lys- (positions (4, 8, 14, 15) and Leu- (positions 5, 6, 12, 13, 16, 17, 20) substitutions, showed an enhanced antimicrobial and antitumor activity without hemolysis. This P5 may have a potential as a specific pharmacological agent, or as a model for the study of the net positive charge, hydrophobicity-antibiotic relationship of peptides[11,12,13].

PMAP-23 is a 23-mer peptide derived from Porcine Myeloid. Several analogues, with amino acid substitutions, were designed to increase the net hydrophobicity by Trp-substitution at positions 10, 13 or 14 at the hydrophilic face of PMAP-23 without changing the hydrophobic helical face. The analogue P6 showed an enhanced fungicidal activity and antitumor activity. The results suggested that the increase of hydrophobicity of the peptides correlated with fungicidal activity[15,16,17].

Structure-mechanism relationship studies were confirmed by HPA3, CA-MA analogue P5 and melittin. The result showed that HPA3 has a higher affinity than HP(2-20) for microbial cell wall and membrane components. Transmission electron micros-

copy (TEM) and scanning electron microscopy (SEM) revealed that HPA3 formed pores via peptide oligomerization in a manner similar to the toroidal pore-forming mechanism of melittin, whereas HP(2-20) showed the barrel-stave model (submitted).

In addition, the effects of melittin on various cell wall components and vesicles of various lipid compositions are being studied and obtained results that the oligomeric state of melittin varied between tetramers and octamers during the formation of toroidal pores. TEM revealed that melittin formed pores via peptide oligomerization and the toroidal pores composed of 7- to 8-nm diameter rings that encircled 3.5- to 4.5-nm diameter cavities on zwitterionic lipid vesicles[18].

Designing novel antibiotic peptides leads to HPA3 and P5 which showed potent antibacterial activity against both Gram-positive and Gram-negative bacteria based on minimal inhibition concentrations defined by the National Committee for Clinical Laboratory Standards (NCCLS). Against more than 10 clinically isolated antibiotic-resistant strains in the presence of 0, 150 or 300 mM NaCl, these peptides retained strong activity against all bacteria, yet showed no hemolytic activity or cytotoxicity against the HaCaT human keratinocyte cell line (submitted).

An effort was also given in order to produce antimicrobial peptides in large quantities by using surface display method. *Lactobacillus casei* was used for the purpose, and the mass-cultured cells with antimicrobial peptides attached on the surface are currently being tested as commercial products including animal feed.

In conclusion, the Peptide Engineering Laboratory, RCPM is dedicated to develop peptide materials as commercial products including antibiotics, agrochemicals, food additives, cosmetics, etc, by acquiring intellectual property rights (about 50 patents were filed or obtained during last 6 years), technology transfer and actively working with industrial partners or academic collaborators of both domestic and international institutions.

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Elaboration of Biologically Active Peptides

Dawei Ma

During the past decades, we have appreciated that peptides serve as the ideal bridges to connect chemical and biological sciences. In the Post-Genome Era, peptides will be able to play more important role for this connection. Thus, active discussions by scientists working in different fields will be of benefit for prompting the peptide research. The 43rd Japanese Peptide Symposium will provide another excellent chance for such discussion. It is my great pleasure to introduce you our recent progress on the peptide-related investigations.

Cyclic depsipeptide halipeptins were isolated from the sponge *Haliclona* species by Gomez-Paloma and co-workers. These compounds displayed potent anti-inflammatory activity and their action mode might be different with current anti-inflammatory drugs, thus making halipeptins as ideal leads for the discovery of novel anti-inflammatory agents. Following the success in total synthesis of halipeptin A,[1] we recently developed a more efficient protocol to assemble this class of natural products, which highly relied on using methylation of aspartates to elaborate the required *N*-methyl-δ-hydroxyisoleucine residue.[2]

Apratoxin A is a cyclodepsipeptides isolated from the marine cyanobacterium *Lyngbya majuscula*. This compound was shown to be cytotoxic against LoVo and KB cancer cell lines with IC_{50} values ranging from 0.36 to 0.52 nM *in vitro*, being most cytotoxic among several cyclodepsipeptides discovered from the marine cyanobacterium. We have discovered a flexible route to this compound and its analogues.[3] An oxazoline analogue of apratoxin A showed a slightly low potency against HeLa cell proliferation in comparison with apratoxin A. However, a C40 demethylated oxazoline analogue of apratoxin A displayed much low cytotoxicity, while C37-epimer and C37 demethylation product of this new analogue were inactive. These results suggest the two methyl groups at C37 and C40 as well as the stereochemistry at C37 are essential for the potent cellular activity of oxazoline analogue of apratoxin A. Further biological analysis revealed that both synthetic apratoxin A and its oxazoline analog inhibited cell proliferation by causing cell cycle arrest in the G1 phase.

A number of natural cyclopeptides bear a diaryl ether moiety, which include monocyclic tripeptides such as K-13 and OF4949-I to OF4949-IV, bicyclic bouvardins, and RP-664536, as well as complex polycyclic antibiotics such as vancomycin, teicoplanin and chloropeptins. The Ullmann-type coupling reaction between aryl halides and protected tyrosine or related amino acid derivatives, could afford the desired diaryl ethers with suitable amino acid moieties, in a straightforward and economic manner. However, racemization often occurred when traditional Ullmann reaction conditions were employed. We found that *N*, *N*-dimethylglycine could promote Ullmann-type diaryl ether formation, leading to completing the reaction at 90 ℃. By combination of this ligand effect and a newly discovered *ortho* substitution effect caused by the acetoamido groups, we revealed that CuI-catalyzed diaryl ether synthesis between L-phenylalanine-derived aryl halides and L-tyrosine derivatives took place at room temperature.[4] Importantly, this reaction also worked in a macrocyclization manner, thereby providing a facile approach to assemble diaryl ether embodied cyclopeptides.

Coronaviruses–the family of viruses that causes the common cold– gained widespread recognition when the deadly severe acute respiratory syndrome, familiarly known as SARS, killed at least 800 people in 2003. Research efforts to design antiviral agents to combat coronaviruses intensified after the SARS epidemic and have focused mostly on just this virus. But because coronavirus sequences and structures mutate so quickly, a challenge is to find wide-spectrum vaccines, since a vaccine targeting one strain would likely be ineffective against another. In collaboration with Professor Rao, we recently discovered a class of inhibitors that can target several coronaviruses.[5] These compounds bind to the structurally conserved substrate-binding region of the main protease, and therefore showed effectiveness to all coronaviruses tested, which include new coronavirus strains that cause conjunctivitis, bronchiolitis, and pneumonia.

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Biophysical Studies of Peptide-Protein Interactions: Targeting the Grb7-SH2 Domain

Jackie Wilce

The development of peptide ligands for targeting specific proteins is a challenging and rewarding area of research. Peptides offer the chemical versatility required for forming specific and high affinity interactions and will continue to be utilised for probing the biological role of their target as well as providing new drug leads. When conducting such research it is highly advantageous to understand the basis for the affinity and specificity of the interaction – as is now possible using a combination of molecular biological and biophysical techniques. The **43rd Japanese Peptide Symposium** provides an excellent forum for the discussion of peptide-protein interactions, and it is an honour to be able to summarise some of the efforts that have been made in targeting SH2 domains and to present our work on the biophysical characterisation of peptide-protein interactions as applied to targeting the Grb7-SH2 domain.

SH2 domains are modules of ~100 amino acid residues which bind with high affinity and specificity to phosphotyrosine (pY) containing peptide sequences. They are present in many proteins and play a critical role in cellular signalling pathways by via their interaction with activated receptor tyrosine kinases at their pY sites. This initiates downstream events that control cell growth and proliferation as well as numerous other cellular activities[1].

Being at the junction of critical cellular events, they are an attractive target in the design of therapeutics targeting diseased cells. Designing competitive ligands to these domains has been problematic, however, due to the requirement of pY for high affinity binding to the SH2 domain. These problems include the susceptibility of the phosphate group to phosphatases, the poor cell permeability of phosphorylated peptides and the lack of specificity displayed by inhibitors designed to solely target the pY binding pocket[2].

Consequently, recent work has focused on the identification of high affinity, non-phosphorylated SH2 domain inhibitors. One such approach has been applied to the development of a non-phosphorylated inhibitor of the Grb7-SH2 domain. Grb7 is an attractive therapeutic target as it is overexpressed in breast, oesophageal and gastric cancers and may contribute to the invasive potential of cancer cells[3,4]. The Grb7-SH2 domain was thus subjected to a peptide phage display library to identify non-phosphorylated peptides with a high binding affinity[5]. This produced seven cyclic non-phosphorylated peptides that were able to bind to the Grb7 SH2 domain with good specificity. Interestingly, the cyclic structure of these peptides (which were cyclised via a disulphide bond) was required for binding to the Grb7 SH2 domain. Subsequent experiments found that a truncated, thioether-cyclised analogue of G7-18, termed G7-18-No Arms Thioether (G7-18NATE; Figure 1), was able to inhibit the association of Grb7 with members of the ErbB receptor family and focal adhesion kinase (FAK) as well as attenuate cell migration[4,5]. G7-18NATE is the first cyclic, non-phosphorylated peptide inhibitor identified for Grb7 and represents an attractive starting point for the design of therapeutics targeting Grb7.

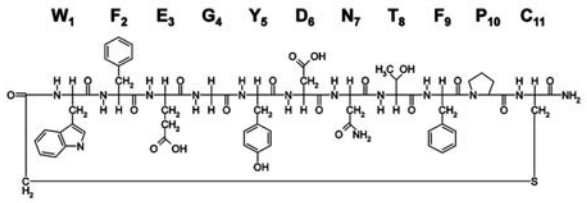

Figure 1. Chemical structure of the cyclic, non-phosphorylated peptide G7-18NATE.

In our laboratory we have undertaken to characterise the interaction between the Grb7-SH2 domain and the G7-18NATE peptide using biophysical techniques. The G7-18NATE peptide was prepared using Fmoc chemistry and cyclized via thioether bond formation between a chloroacetylated N-terminus and the free thiol of the cysteine at the C-terminus of the peptide as previously reported[5]. The Grb7-SH2 domain (Grb7 amino acid residues 415-532) was produced as a glutathione-S-transferase fusion protein, overexpressed in an *E. coli* expression host and purified using a combination of glutathione affinity chromatography and cation exchange chromatography[6]. In the following paragraphs I have outlined some of the techniques used to measure the affinity of the interaction and obtain structural information for the peptide:protein complex.

Isothermal titration calorimetry (ITC) was used to measure the affinity of the interaction between the peptide and protein. This involved the stepwise addition of peptide solution to the protein and precise measurement of the heat evolved or absorbed during the binding event. In the case of the G7-18NATE:Grb7-SH2 interaction the reaction pulses

were negative indicating that the binding reaction is exothermic (i.e heat was released upon binding). The heat produced upon each addition is proportional to the amount of peptide bound and the data can be analysed to determine the association constant, stoichiometry and enthalpy of the binding reaction.

NMR spectroscopy is an ideal tool for examining the structure of the peptide in and out of the presence of the protein. As long as the peptide remains soluble in solution at high concentration (100 μM to mM), NMR spectra can readily be acquired and used determine structural and dynamic properties of the peptide. The chemical shifts of the αH resonances and $^{3}J_{NH\text{-}\alpha H}$ coupling constants are a preliminary indication of whether the peptide has a tendency to exist in an α-helical or extended secondary structure. The sensitivity of the NH chemical shift to temperature changes is an indication of the relative protection of the NH from solvent – often as a result of hydrogen bonding. Most importantly, the measurement of NOEs (Nuclear Overhauser Effect), provides the distance restraints between protons that can be used to define the three dimensional structure of the entire peptide. Such information may be useful for understanding the structural basis for a peptide-protein interaction.

It is, however, most often the case that a small peptide does not exist in one rigid conformation, but rapidly interconverts between many structural species. A cyclized peptide is clearly more restricted, but still may not adopt a single preferred structure. When characterising a peptide-protein interaction, it may be of greater interest to determine the structure of the peptide when bound to the protein. Here the exchange-transferred NOE spectroscopy (et-NOESY), also known as transfer NOE spectroscopy (trNOE) can be employed. A typical et-NOESY experiment involves the acquisition of a 2D-NOESY spectrum for the ligand in the presence of sub-stoichiometric amounts of its macromolecular binding partner. Under these conditions the ligand can exist in either the free or bound states, with the relaxation and hydrodynamic properties of the peptide-protein complex very different to those of the free peptide. In particular, the tumbling correlation time of the receptor bound ligand (τ_B) is significantly greater than that of the unbound ligand (τ_F) and as a result NOEs build up faster for the ligand in the bound state. Depending upon the kinetics of the interaction (i.e. if the rate of exchange is fast relative to the magnetisation life-time in the unbound state) the NOEs that built up in the bound peptide state are transferred to the free peptide state and can be detected at the free ligand resonances using the usual NOESY experiment. In order to satisfy the above condition the bound and free states need to be in fast exchange which typically corresponds to an equilibrium dissociation constant greater than 10^{-6} M.

NMR spectroscopy can also be used for examining the effect on the protein that the peptide has upon binding. The commonly used NMR method involves chemical shift perturbation mapping to identify protein binding interfaces. This method involves noting how the ^{15}N-^{1}H heteronuclear single-quantum coherence (HSQC) spectrum of the ^{15}N-isotopically labelled protein is affected by the addition of its unlabelled binding peptide. In the ^{15}N-^{1}H HSQC experiment, cross-peaks arise from ^{15}N and ^{1}H nuclei that are covalently linked, hence with the exception of proline and often the N-terminal amino acid, each amino acid in a proteins primary sequence is represented by at least one cross-peak. The ^{15}N and ^{1}H frequency at which the signal occurs is sensitive to the surrounding chemical and electronic environment of the nuclei. A change in this environment caused for example by binding to a peptide ligand will perturb the position of the cross-peak. As long as global conformational changes do not occur upon binding, mapping the residues that show significant chemical shift perturbation onto a known structure of the protein can help with identification of the interaction interface and subsequent modelling of the protein-ligand complex.

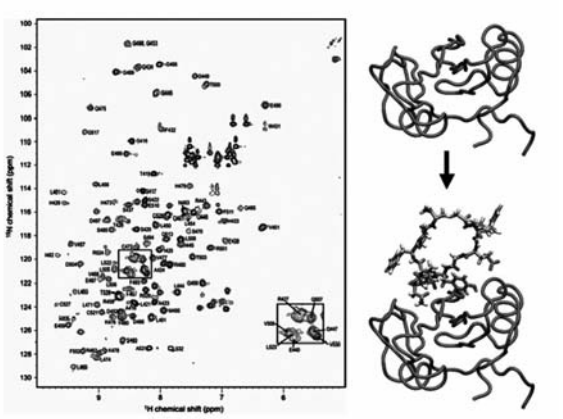

Figure 2. ^{1}H-^{15}N-HSQC spectrum of Grb7-SH2 domain used for mapping peptide interactions.

We have applied all of the described techniques to the G7-18NATE:Grb7-SH2 interaction and in this way both delineated the structural features of the G7-18NATE peptide as well as the peptide binding site on the surface of the Grb7-SH2 domain. This has allowed us to better understand the basis of the protein-peptide interaction, and provided insight into ways in which the peptide may be developed to improve its affinity and specificity to its target.

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In Memories of Professor R. Bruce Merrifield

Yoshiaki Kiso

I was profoundly shocked by an unexpected e-mail from Professor George Barany of The University of Minnesota that Professor Bruce Merrifield passed away on May 14th, 2006. This sad news quickly spread all over the world by public and scientific media, through Chemical & Engineering News, Nature, and the likes.[1-4]

I had been and am still very much impressed by his excellent scientific achievements. In the 8th American Peptide Symposium held in Tucson, Arizona, on 1983, I presented a paper concerning a deprotection method based on the Push-Pull mechanism, and had the privilege of having an intense discussion with him about peptide chemistry. In the fall of 1984, I sent Professor Merrifield a congratulatory telegram when I heard a most welcoming news that he would be awarded The Nobel Prize in Chemistry. This was the first time for me to make a direct personal contact to him[5]. Without much delay, I received a hand-written "thank you" letter from him. From then on, our friendship grew closer as one of my former graduate students, Dr. Makoto Yoshida, joined Professor Merrifield's group as a postdoctoral fellow (Fig. 1),

I was once invited by Professor Merrifield to give a lecture on peptide synthesis at Rockefeller University (Fig. 2), and had the opportunity to visit his laboratory as my family and I sojourned at Abby Aldrich Guest House at Rockefeller University. In the fall of 1992, I traveled with him and his wife all the way from the Chinese International Peptide Symposium held in Hangzhou, China, to the 2nd JASPEC (Japan Symposium on Peptide Chemistry) held in Shizuoka, Japan (Fig. 3).

The Merrifields visited Kyoto Pharmaceutical University where Professor Merrifield delighted us with a lecture entitled, "Solid Phase Peptide Synthesis"

Fig. 1. Dr. Yoshida (left) and Professor Merrifield in the laboratory at Rockefeller University.

Fig. 2. In the office of Professor Merrifield at Rockefeller University. The photographs between us are that of Dr. Bergmann (left), Dr. Du Vigneaud (center) and Dr. Zervas (right).

Fig. 3. At the dinner table during the 2nd Japan Symposium on Peptide Chemistry. From left to right, Dr. Makoto Yoshida (Kyowa Hakko Co.), Dr. Tomishige Mizoguchi (Tanabe Pharmaceutical Co.), myself, Professor and Mrs. Merrifield, Professor Garland Marshall (Washington University), and Professor John Stewart (Colorado University).

Fig. 4. At Kyoto Pharmaceutical University

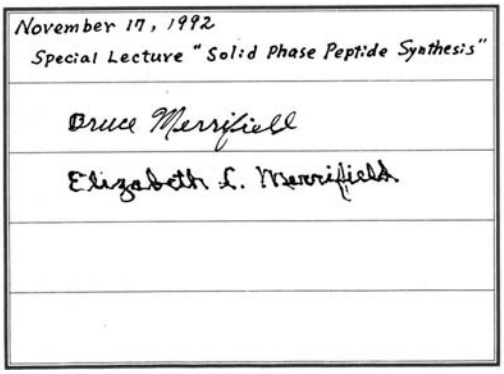

Fig. 5. Autograph of Professor and Mrs. Merrifield.

Fig. 6. Enjoying an excursion with my graduate students during The 16th American Peptide Symposium held in 1999.

Fig. 7. Scenes from a video entitled, "Peptide and Protein Synthesis: Origin and Development".

(Figs. 4 and 5).

I really enjoyed our many reunions at various scientific meetings and symposia, where we exchanged greetings and scientific discussions (Fig. 6).

In 2001, student researchers graduating from Merrifield's laboratory made a one-and-a-half hour video entitled, "Peptide and Protein Synthesis Origin and Development". Professor Merrifield enjoyed it so much that he sent me a copy attached to a handwritten letter. (Fig. 7)

In 2004, I was awarded the "Cathay Award" by The Chinese Peptide Society, where Professor Merrifield served as the Chairman of the Selection Committee of the award. Later I heard from somebody that he highly praised my contributions to peptide science.

Through my friendship with Professor Merrifield, I always felt his gentle, amiable personality, his devotion and mutual understanding of his wife Elizabeth, and a deep, sincere love for science[6]. I would like to express my most heart-felt condolence on the early lost of Professor Bruce Merrifield.

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【Symposium & Meeting】

International Conference of 43rd Japanese Peptide Symposium/
4th Peptide Engineering Meeting
(43JPS/PEM4)
"Peptide Science and Engineering in Chemical Biology"
Yokohama, Japan, November 5-8, 2006
http://peptide-soc.jp/

2nd International Symposium on Biomolecules and Related Compounds
(A satellite meeting of 43JPS/PEM4)
Kyoto, Japan, November 10-12, 2006
http://www.kyoto-phu.ac.jp/2nd_isb/

Membrane-Permeable Peptides: Chemistry, Biology and Therapeutic Applications
(A satellite meeting of 43JPS/PEM4)
Kyoto, Japan, November 10-11, 2006
http://www.scl.kyoto-u.ac.jp/%7Ebfdc/cpp-eng.html

20th American Peptide Symposium
Montreal, Canada, June 26-30, 2007
http://www.americanpeptidesociety.com/

4th International Peptide Symposium/2nd Asia-Pacific International Peptide Symposium/7th Australian Peptide Symposium
Cairns, Australia, October 21-26, 2007
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