Lectures and Oral Communications

OC 11 Pasteur Auditorium - Bioactive Peptides OC 12

PEPTIDE MIMOTOPES OF THE NICOTINIC RECEPTOR BINDING SITE

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Peptide libraries allow to select new molecules, defined as mimotopes, able to mimic the structural and functional features of a native protein. Both antigen-antibody and receptor-ligand recognition surfaces can be reproduced by mimotopes. This technology can thus be applied for the development of new reagents which could interfere with the action of specific ligands on their target receptors.
In the present work we have used a combinatorial library approach to produce synthetic peptides mimicking the ligand binding site of nicotinic receptors (AchR). Several different agonist and antagonist molecules can compete with acetylcholine for binding to its specific sites. A sequence of AchR α-subunits was determined as containing at least part of the AchR ligand binding site (1) and synthetic peptides reproducing this region were reported as binding different AchR ligands, including the high affinity snake neurotoxin α-bungarotoxin (α-bgt) (2,3). Nonetheless binding affinity of these peptide was extremely low.
On the basis of amino acid sequence comparison of different α-bgt binding AchR, we designed a 14 amino acid peptide combinatorial library which had five invariant positions, four partially variant and five totally variable positions. Peptides were synthesized using the spot synthesis on cellulose membrane and binding sequences were selected using biotinylated α-bgt. Each variable position was systematically identified and all the possible combinations of best reacting amino acid in each variable positions were tested in subsequent expansions of the combinatorial library.
Best reactive sequences were identified, produced in soluble form and tested in BIACORE to compare their kinetic constants. We identified several different peptides which can inhibit the binding of α-bgt to AchR and which bind α-bgt with an affinity several order of magnitude higher than peptides reproducing receptor native sequences.

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APPLICATION OF NEUROPEPTIDES IN INSECT CONTROL

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Insect neuropeptides play a key role in the regulation of many physiological functions in insects. The varied functional capacities of this group of regulatory substances make neuropeptides a prime target for the development of insecticides and provide new strategies for insect control which can be acquired by antagonists - selective inhibitors, capable of blocking the receptor of the neuropeptide. The conversion of a neuropeptide into an insecticide/insect control agent has to overcome the limitations of linear peptides (lack of selectivity, low metabolic stability and low bioavailability) which make them useless as insecticides, and to provide a solution to the fact that no approach is available to predict *a priori* the structure of a neuropeptide antagonist or the conformation which will lead to a highly inhibitory activity.

In this presentation, we report the development of a novel approach for the generation of a novel type of putative insecticides based on backbone cyclic peptidomimetic antagonists of insects-neuropeptide which overcomes the above limitations. The approach, termed the backbone cyclic neuropeptide based antagonist (BBC-NBA), was applied to the insect pyrokinin/pheromone biosynthesis activating neuropeptide (PBAN) family as a model, and led to the discovery of a potent linear lead antagonist and several highly potent, metabolically stable backbone cyclic peptidomimetic antagonists, devoid of agonistic activity, which inhibited *in vivo* PBAN mediated activities in several moth species and other insects.

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OC 13 Einstein Auditorium - Structural studies - Protein Folding OC 14

CPWR SPECTROSCOPY: A NEW METHOD FOR STUDYING PEPTIDE-INTEGRAL MEMBRANE PROTEIN INTERACTIONS.

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Integral membrane proteins such as seven transmembrane G-Protein Coupled Receptors (GPCRs), Ion Channels, etc. constitute the targets for over 60% of all drugs. The methods for studying these protein receptors in the membrane, and the structural changes that accompany their interactions with ligands, hormones, neurotransmitters, ions, etc., in information transduction have been very limited. We have developed a new method, coupled plasmon waveguide resonance (CPWR) spectroscopy which utilizes the coupling of p- and s-polarized light with plasmons that allow one to monitor structural changes in integral membrane proteins such as GPCRs as they interact with their agonist and antagonist ligands. Thus, one is able to examine independently structure changes that occur in the anisotropic environment of a receptor-membrane bilayer complex both parallel and perpendicular to the membrane normal. From the spectra obtained, we can determine directly the binding constants of the peptide ligands to the receptors, and evaluate conformational changes that accompany these interactions. We have applied this new method to receptors from two different families of G-protein coupled receptors, the human delta opioid receptor and the human glucagon receptor. For the delta receptor, we used a purified receptor, but for the glucagon receptor, we utilized a receptor obtained directly from membranes of a stably transfected cell line. We have obtained binding constants for agonists and antagonists that are consistent with literature values obtained from membrane or whole cell preparations. In addition, we have demonstrated that agonists and antagonists cause different structural changes to their receptors, consistent with a three state model for GPCRs in information transduction. The nature of the structural changes will be discussed, and the potential of the method for time resolved and thermodynamic studies of ligand-receptor interactions including those involving G-proteins will be outlined.

We thank the V.P. Research Office at the University of Arizona and the U. S. Public Health Service for partial support.

2D-NMR AND MOLECULAR MODELLING STUDY OF A PEPTIDE CORRESPONDING TO THE 603-609 DISULFIDE LOOP OF HIV-1 ENVELOPE GLYCOPROTEIN GP41 AND OF ITS ANALOGUES

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Synthetic peptides mimicking epitopes of proteins may have many applications in biology and medecine. However, the low resistance of these peptides to their *in vivo* proteolysis, as well as their bad diffusion through the membrane systems, together with their weak selectivity due to a too large conformational flexibility, remain some of their limiting factors.
Since a few years, analogues have been developed to improve their activity, specificity and stability.
In this work, we will present an example of the use of such analogues in HIV diagnosis.
It has been shown that patients infected by the HIV raised antibodies against the gp41 glycoprotein and more specifically against an area containing the cyclic peptide formed by a disulphide bridge. In order to modulate the size of this loop in relation with the structure-activity relationships, retro-inverso cyclic peptide, analogues involving β-amino acids and several analogues cyclised by side chain lactam bond resulting from Asp (or Glu) and Dap (diamino-propionic acid) or Dab (diamino butyric acid) substituting the disulphide bridge have been synthesized.
A comparative conformational study of these analogues using various NMR experiments (COSY-DQF, TOCSY and NOESY) and restrained molecular modelling will be presented.

NMR INTERACTION STUDIES ON THE ISOLATED APICAL DOMAIN OF GROEL WITH SYNTHETIC PEPTIDES.

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GroEL, one of the best studied chaperone proteins, facilitates the assisted refolding of a variety of proteins in vivo and in vitro. This fact indicates that the interaction is not very specific to the primary sequence of unfolded polypeptides. Recent studies on interactions of GroEL with polypeptides have demonstrated that hydrophobic and negative charged residues on the apical domain of GroEL mainly contribute to this interaction. However, although two crystal structures of the polypeptide-apical domain complex are available, it is still not clear how the domain accommodates non-native proteins on an atomic level.

Here, we have developed a routine system for mapping the polypeptide binding site of GroEL at atomic resolution using a stable isotope labelled apical domain of GroEL and a variety of synthetic peptides.

All of the studied peptides bind to the apical domain with affinities from μM to mM. We found significant but characteristic differences in the binding modes based on the binding affinity. Strong binding occupies a large region on the binding surface, whereas weak interactions occurred only in one specific area. No non-specific interactions were observed.

SEQUENCE DEPENDENT MEMBRANE UPTAKE OF PRIMARY AMPHIPATHIC SHUTTLE PEPTIDES

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Two families of primary amphipathic peptides issued from the association of a nuclear localization sequence (NLS) with either a signal (SP) or fusion (FP) peptide have been shown to act as efficient carriers facilitating the cellular uptake of drugs or nucleic acids. In order to define the minimal structural requirements which maintain the vector properties of the peptides, it was of major importance to elucidate their membrane crossing process. This process contains three crucial steps *i)*uptake, *ii)*translocation and *iii)*release and we focus, here, on the first one. Based on surface tension and surface potential measurements, we show that the penetration from the subphase into phospholipid monolayers spread at the air-water interface is mainly governed by the hydrophobic sequence. It appears that the aromatic residues strongly participate in the interactions. Also the physical state of the phospholipid (liquid expanded such as DOPG, DOPC or liquid condensed for DPPG and DPPC) interferes with the uptake process since it can or not promote a sequence dependent $\alpha - \beta$ conformational transition of the peptide, depending on the peptide/lipid ratio and the peptide sequence (SP or FP) as detected by Fourier Transform Infra Red spectroscopy. In addition, for the SP-NLS series, the interaction with DOPC induces a phase separation as visualized by Atomic Force Microscopy (AFM). All the above observations confirm the conclusions obtained by analysis of the compression isotherms which revealed a large expansion of the mean molecular area of mixed monolayers. AFM images show that large amounts of peptides leads to the formation of filaments which can be associated with the β-type conformation. For the peptides of the SP-NLS series, we also present the consequences of the introduction of an ionic strength in the subphase on the conformational state of the peptides and on their uptake when the charges of the lipid headgroups are varied. The fact that no lipid induced conformational transition could be detected for the peptides of the FP-NLS series is also discussed in relationship with the ability of various peptides containing hydrophobic sequences derived from SP and FP to adopt helical and/or β-type conformational states.

CATHELICIDIN-DERIVED ANTIMICROBIAL PEPTIDES: *IN VITRO* AND *IN VIVO* ACTIVITY

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Gene-encoded antimicrobial peptides have been identified from extremely diverse organisms, including vertebrates, invertebrates, plants and bacteria. In mammals, such peptides are part of the innate host defence mechanisms and are present in phagocytic cells and mucosal surfaces. Phagocytes store peptides that belong to the defensin (α- and β-defensins) and the cathelicidin families. In recent years, we have identified a number of cathelicidin-derived peptides in various species. These peptides vary significantly by structure, and length and include α-helical, Pro- and Arg-rich, Cys-rich and Trp-rich peptides. They are synthesized at the C-terminus of 15-18 kDa precursors that contain a conserved 'cathelin' N-terminal domain belonging to the cystatin superfamily. We have chemically synthesized and characterized the antimicrobial activity of a number of these peptides and of their fragments and analogues. The *in vitro* activity of members of all the structural classes of cathelicidin peptides has been tested against many antibiotic-resistant clinical isolates and fungi. The results show that the peptides are wide spectrum and that the most active - SMAP-29 – has MIC values in the 0.5-5 μg/ml range against most of the strains tested, including methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *E. faecalis*. SMAP-29 has been further characterized to test its tendency to select resistant mutants and to protect mice i.p. infected with a lethal dose of bacteria. The MIC of the peptide towards *E. coli, P. aeruginosa* and *S. aureus* remains unchanged after 20 passages in the presence of a subinhibitory dose of peptide. At variance, conventional antibiotics (e.g. gentamycin and norfloxacin), under the same conditions, select resistant mutants with a 60-300 fold increase in the MIC values. In *in vivo* experiments with an acute peritonitis model, SMAP-29 at 0.2-0.4 mg/Kg gives an over 90% protection of mice i.p. injected with a lethal dose of MRSA, *P. aeruginosa* or of a capsulated strain of *E. coli*. These results against clinically relevant, antibiotic-resistant bacteria show that cathelicidin peptides are promising compounds for the development of novel anti-infectious agents.

HETEROPOLYMER FOLDING : PROTEINS AND BEYOND

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Proteins perform a wide range of complex chemical functions in natural systems. Nearly all of these molecular operations require the polypeptide chain to adopt a compact and specific folding pattern. We are trying elucidate the origins of protein folding preferences, and to induce analogous folding behavior in unnatural oligomers.

Our protein work focuses on a very common type of secondary structure, the β-sheet. Unlike the other common secondary structures (α-helix and reverse turns), relatively little is known about β-sheet folding behavior, because it is difficult to create appropriate model systems. Within the past few years, we have devised a very effective strategy for generating peptides that adopt predictable β-sheet conformations in aqueous solution. We are using these molecules to study fundamental issues like the stabilizing roles of interstrand sidechain-sidechain interactions and cooperativity. Recently, we have extended this work to create parallel β-sheet models.

We use the term "foldamer" to describe unnatural oligomers with well-defined folding propensities. If we can learn to control foldamer conformations, then it may be possible to endow these molecules with sophisticated functions. Our efforts have focused on β-amino acid oligomers ("β-peptides"). We have found that all three types of secondary structure observed in folded proteins, helix, sheet and turn, are displayed by β-peptides in organic solvents, when properly selected residues are employed. We have begun to generate stable β-peptide secondary structures in aqueous solution, and to use these motifs to try to create β-peptide tertiary structures. Amphiphilic β-peptides that adopt helical conformations in aqueous solution display potent antimicrobial activity; these β-peptides are mimics of host-defense peptides like magainins.

OC 17 Pasteur Auditorium - Immunochemistry - Immunopharmacology OC 18

SYNTHESIS AND BIOLOGICAL EVALUATION OF NEW INTERFERON GAMMA RECEPTOR AGONISTS : EVIDENCE OF AN INTRACELLULAR ACTION

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Intracellular action of IFN-γ has been reported, which recruits a species-independant activation pathway. Recently, we have established that a synthetic lipopeptide derived from the C-terminal extremity of murine IFN-γ was passively delivered to the cytoplasm of intact, non-phagocytic cells. Concomitantly, we have established that this compound was able to reproduce the activities of the recombinant cytokine in both human or murine cells[1].

Modification of peptides by hydrophobic moieties, such as fatty acids, is now widely recognized as a means of enhancing their transport across biological membranes. Lipopeptides have thus found a possible use for the targeting of intracellular receptors[2]. However, most of the available synthetic methods do not allow the modification of peptides by unsaturated fatty acids or cholesterol derivatives. This point is crucial since the nature of the fatty acid is known to have a profound effect upon the interaction with the cell membrane and its alteration.

Novel methods were developed for the synthesis of peptides substituted by sensitive fatty acid, which allows the synthesis of hydrazides[3] or hydrazones in good yield and high purity. The reactions were applied to the sequence 95-132 or 113-132 of the murine IFN-γ. A new generation of synthetic agonists were obtained and compared. The biological activities of the different compounds have been evaluated on the basis of a stimulation of MHC-class II expression.

Taken together, our results confirm a potential intracrine activity of endogeneously produced IFN-γ, raising the question of its regulatory activity in producing cells. This work also highlights the existence of a new pharmacological target available for potent therapeutic immunomodulation.

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EFFECT OF LIPOCONJUGATION ON MBP PEPTIDE EPITOPES FOR CD4+ T CELL RESPONSIVENESS

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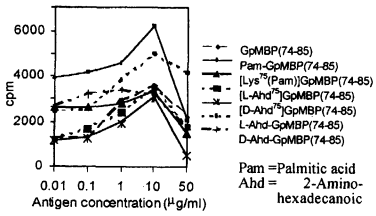
Lipoconjugation of peptide antigens has been used to increase the effectiveness of generation of CD8+ CTL responses. Recently, it has been reported the use of short lipophilic epitopes, increasing the life span of functional presentations to cytotoxic T cells [Gras-Masse, H., *et al., J. Immunol.*, 164 (2000) 900-907]. Very little is known about the effect of lipopeptides on CD4+ T cell response. We now demonstrate the immunoadjuvant effects of lipoconjugation in a CD4+ *in vitro* system.



We investigated the T cell response to lipopeptides of two different epitopes in Lewis rat, GpMBP(74-85) and GpMBP (82-98), obtained introducing Pam as well as Ahd at different positions of the sequences. The two enantiomers of Fmoc-Ahd were synthesized by a large scale method we set up for a series of lipophilic amino acids. We observed a strongly increased CD4+ T cell response with the lipopeptides of GpMBP(74-85) compared to the wild-type peptide, particularly with Pam-GpMBP(74-85) and L-Ahd-GpMBP(74-85) (Figure). As the same results were not observed with lipopeptides of GpMBP(82-98), we tested the stability of the two MBP epitopes to cellular proteases. The *in vitro* digestion by cathepsin D and L, as well as lysosomal fractions of B-LCL cells showed several cleavage sites (all destroying the MHC II binding region) in GpMBP(82-98), while GpMBP(74-85) was remarkably stable to lysosomal proteases. A hypothesis to explain the mechanism increasing the CD4+ T-cell responsiveness is proposed.

Lectures and Oral Communications

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20S STANDARD PROTEASOME VERSUS 20S IMMUNO-PROTEASOME DIGESTION PROFILES OF MHC CLASS I ANTIGEN PRECURSORS

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Most antigenic peptides presented by major histocompatibility complex (MHC) class I molecules are generated during protein breakdown by proteasomes. The 20S proteasome is a barrel-shaped multicatalytic complex composed of four rings (2α and 2β), each of which contains seven subunits, assembled in the order αββα. Among the 7 β subunits of each inner ring, 3 are responsible for the catalytic activities of the 20S proteasome. These activities have been described as been trypsin-like, chymotrypsin-like and PGPH (peptidylglutamylpeptide hydrolyzing). In the presence of γ-interferon (IFNγ), the 3 catalytic subunits of the 20S proteasome are replaced by 3 others with identical cleavage site specificities but different kinetics of proteolysis, leading to different antigen precursor digestion profiles. So far the presence of these subunits has been shown to favor antigen formation, and this proteasome is called immunoproteasome as opposed to "standard" proteasome. However, the precise mechanisms by which proteasomes generate or destroy MHC-restricted antigens remain to be fully elucidated. In this study, we compare the digestion profiles of antigen precursor peptides obtained with "standard" proteasome and immunoproteasome purified from different cell lines (tumor cells and control or IFNγ-treated tumor cells, respectively). Digests are analyzed using liquid chromatography/mass spectrometry to quantify and to identify peptides present in the mixture and cytotoxic T lymphocyte (CTL) assays are performed to test the biological activity. Results show that digestions of VPYGSFKHV antigen precursors by the immunoproteasome lead to major products resulting from the cleavage within the antigen sequence thus preventing antigen formation, and that these digests do not activate CTLs. By contrast, digestions with the "standard" proteasome lead to major products containing the antigen sequence, and activate CTLs. These results show the role of proteasome composition in antigen maturation and thus, the role of cellular environment on CTL activation.

SEQUENTIAL OLIGOPEPTIDE CARRIERS (SOCs): NEW DEVELOPMENTS AND APPLICATIONS.

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A novel class of oligopeptide carriers termed Sequential Oligopeptide Carriers (SOCs) is presented. SOCs are formed either by the Lys-Aib-Gly or the Aib-Lys-Aib-Gly sequential motif. By varying the number of the repeating units, carriers of the general formula either SOC_n-I, $(Lys\text{-}Aib\text{-}Gly)_n$, or SOC_n-II, $(Aib\text{-}Lys\text{-}Aib\text{-}Gly)_n$, where n=2-7, are prepared. The carriers adopt a predetermined secondary structure of 3_{10}-helix, which is given by the presence of Aib, an unnatural amino acid with a known propensity to induce ordered helicoid backbone. This helicoidal structure contributes to the reduction of steric hindrance and conformational restrictions of the carrier, and thus allows antigenic peptides to retain their original structure as confirmed by ^{1}HNMR and molecular modeling studies. Four antigenic sequences were initially selected to assess the utility of SOCs-conjugates, either as antigens in solid-phase immunoassays, or as immunogens in eliciting specific anti-peptide antibodies and producing an immune spreading (the nicotinic acetylcholine receptor, the major surface glycoprotein of Leishmania and the B-cell epitopes of the La/SSB and the Sm autoantigens). Studies on further applications with novel SOCs-conjugates are in progress in our Laboratory with myelopeptides for leukemia treatment. Further developments on the SOCs themselves are also in progress in our Laboratory for introducing adjuvant-conjugated-SOCs as a new generation of SOCs, as well as for anchoring several different, more than one kind, epitopes on the same SOC molecule by selective protection and deprotection of the lysine side chains.

(Grants from GGSRT).

OC 21 Einstein Auditorium - Molecular Design OC 22

STANDARDIZATION OF AGGREGATION GRADE AND NEUROTOXICITY OF β-AMYLOID PEPTIDES.

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It is generally accepted that β-amyloid peptides (Aβ) are neurotoxic and cause dementia and Alzheimer's disease. However, Aβ have neurotrophic effect in young animals. These peptides are neuromodulators in monomeric form. β-amiloids have a very high tendency for the formation of β-pleated sheet structure and aggregation. We have found that about $2x10^{-5}$M is the critical concentration for the aggregation of Aβ [1-42] in aqueous solution. As the monomeric form is not toxic only the aggregated one, it is necessary to standardize the Aβ aggregation grade for biological experiments. Aβ [1-42] exists in monomeric form in dimethyl sulfoxide, or hexafluoro isopropanol solution (FT-IR studies), also in $5x10^{-3}$ M concentration. Rapid dilution with buffers to 10^{-9}-10^{-10} M concentration save the monomeric form for biological experiments. Dilution to 10^{-4}- 10^{-5} M concentration with buffers results in rapid aggregation and precipitation within some hours. The first aggregation products are amorphous, diffusible clusters; however, they are already neurotoxic. The aggregation process can be investigated by FT-IR spectroscopy or capillary electrophoresis. Diffuse Aβ aggregates interact with neuron membranes. According to our experiments, ^{3}H-labeled Aβ-peptides enter the neurons by endocytosis in 30 minutes, and they might be bound to mitochondrial membranes and the endoplasmic reticula. Aggregated amyloid preparation of physiological concentration can be made by dilution of the aggregated Aβ cluster-system from 10^{-4} to 10^{-9}-10^{-10} M concentration with buffers; dilution with aqueous buffers never causes deaggregation. Slow aggregation at 10^{-4} M concentration gives fibril formation; these fibrils are neurotoxic in the CNS but not diffusible. "Ageing" process (fibril formation) of Aβ peptides using Ca^{2+} ion containing buffers for aggregation at 37 °C can cause peptide bond cleavage by the Asn^{27} residue.

CHIMERIC TASP MOLECULES AS POTENTIAL INHIBITORS IN E-SELECTIN-MEDIATED CELL ADHESION

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The construction of protein-like folding motifs as structurally stable scaffolds for the introduction of 'function' represents one of the major objectives in protein de novo design. Topological templates as versatile tools for inducing and stabilizing secondary and tertiary structures allow to bypass the well-known folding problem of linear polypeptides and offer a way to mimic native packing topologies by the template-directed self-assembly of helical and/or β-sheeted peptide blocks to protein-like folding units [M. Mathieu et al., Angew. Chem. Int. Ed. Engl. **37**, 2990 (1998)]. In conceptually separating structure and function, we have designed and synthesized a chimeric 4-helix bundle TASP derived from the ROP (repressor of primer) protein and the cell adhesion glycoprotein E-selectin aimed at inhibiting an early stage in cell adhesion processes, in particular leukocyte adhesion. To obtain a stable 4-helix bundle structure, the core of the antiparallel homodimeric ROP protein has been redesigned to ensure optimal internal packing and thus, a defined bundle structure, independently of the structurally non-related binding surface of E-selectin to be grafted. In a second step, crucial residues on the surface of E-selectin covered upon binding to its counterreceptor PSGL-1 have been matched onto the surface formed by two neighboring helices in the TASP molecule, including the Ca^{2+} complexing site. As a special feature, the resulting TASP molecule contains two identical surfaces each of which resembles the binding site as found in native E-selectin comprising residues either involved in ligand recognition or calcium binding. The antiparallel attachment of the helices was achieved via chemoselective ligation using a two-step procedure, i.e. selective thioether bond formation with orthogonally protected Cys residues in the template and the Br-CH_2CO- moiety at the N- or C-terminus of the helices. CD, NMR and molecular dynamics simulations that confirm the proposed bundle structure as well as cell adhesion assays on the potential inhibitory effect of the chimeric TASP will be presented. In summary, surface mimetics of this kind allow for systematic studies of *structure-function* relationships and molecular recognition processes representing a valuable asset to protein design and mimicry.

THE CYSTINE-STABILIZED β-SHEET MOTIF IS A COMMON AND USEFUL MOTIF FOR PROTEIN ENGINEERING

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One attractive way to build new active molecules is to use a stable protein scaffold as a framework on which to graft active sites or recognition fragments. Interestingly, stable and easy to synthesize scaffolds have been observed in small disulfide-rich proteins. In these proteins, only few different motifs have been recognized and are observed in several protein families with very diverse origins and functions.

Folding studies and structure comparisons led us to the conclusion that the elementary structural motif in the squash trypsin inhibitors is made up of a small triple-stranded β-sheet and of only two disulfide bridges. This motif, that we call the Cystine-Stabilized β-Sheet (CSB) motif, is present with minor variations in about 25 different protein families including enzyme inhibitors and cofactors, toxins, antimicrobial and antihemostatic peptides, a race-specific elicitor, growth factors, lectins, and various other peptides isolated from plants.

Focusing on the CSB motif, we will:

- Describe its specific sequence and structure features, and its differences with the related but imprecise terms knottins, cystine knots or T-knots.
- Present a new example of a macrocyclic peptide containing the CSB motif..
- Show that the CSB motif is an autonomous folding unit that retain a high thermal stability when isolated from a protein.
- Present an application that uses the CSB motif as a structural platform on which we grafted a foreign recognition site.
- Present a new structure-specific computer method, designed to predict potential stabilizing mutations, and its application to peptides based on the CSB motif.

Conformational analysis of peptides neutralising LPS pathogenicity - NMR and molecular modelling study

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One of the major ligands recognised by the innate immune system is lipopolysaccharide (LPS), a common constituent of Gram-negative bacterial outer membranes. Its lipid A moiety is responsible for triggering a cascade of defence reactions and, at higher levels, septic shock. In an effort to understand the molecular motifs of lipid A specific binding by peptides we are investigating the complexes of LPS and peptides that neutralise LPS pathogenicity by NMR and molecular modelling. These peptides are

- natural polycationic peptides (polymyxins) and
- fragments of proteins that bind LPS, e.g. the lipid-binding protein (LBP) and limulus-anti-LPS factor (LALF).

We have determined the LPS-bound structures of polymyxin B and E with the aid of the transferred NOE effect and proposed a model of the complex [1]. We have employed a similar methodology with a 16 residue fragment of LALF (LALF-16) that neutralizes LPS in vitro [2]. The preliminary model of the bound structure of LALF-16 is compared with polymyxin B and E. Both structures display a grouping of polar and non-polar residues giving them an amphiphilic character.

The results will contribute to the understanding of lipopolysaccharide recognition at the molecular level and will serve in structure based anti-septic drug design.

[1] P. Pristovšek and J. Kidrič, *J. Med. Chem.* 42, 4604(1999).

[2] C. Ried, *et al, J. Biol. Chem.* **271**, 28120 (1996).

Lectures and Oral Communications

L 07 — Tuesday Afternoon: Pasteur Auditorium — L 08

RECEPTOR ACTIVITY MODIFYING PROTEINS

Steven M. Foord
Receptor Systems Unit, GlaxoWellcome Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire, SG1 2NY. United Kingdom.

The calcitonin peptide family currently comprises calcitonin (CT), the calcitonin gene related peptides (CGRP), amylin, and adrenomedullin (ADM). The peptides share significant amino acid homology but show discrete biological activities. All four peptides activate just two receptors, the calcitonin receptor (CTR) and the calcitonin receptor-like receptor (CRLR). Both receptors are controlled by RAMPs (Receptor Activity Modifying Proteins). They are type I transmembrane proteins of about 140 amino acids. RAMPs determine whether the CTR binds (and responds to) CT, CGRP or amylin. CTR alone produces a CT receptor. CTR expressed with RAMP1 adopts a different pharmacology, binding and responding to CGRP and amylin. CTR expressed with RAMP3 adopts a different pharmacology again, binding and responding to amylin. In contrast CRLR does not appear at the cell surface at all unless a RAMP is co-expressed. RAMPs enable CRLR to respond to either CGRP (RAMP1) or ADM (RAMP2 or RAMP3).

The available evidence suggests that the entire RAMP interacts with their cognate receptors to change their conformation and so their pharmacology but their amino termini appear to be a primary determinant of their activity. This information has been derived from the study of chimeric RAMPs, the amino terminus of RAMP1 being associated with the transmembrane and carboxyterminal domains of RAMP2 (and vice versa).

RAMPs provide a mechanism whereby cell can change their sensitivity from one peptide to another. They appear to effect this change within hours and irrespective of whether the receptors are expressed from native genes or via artificial constructs. We do not know whether RAMPs are equilibrium with their receptors or only interact with de novo receptors.

RAMPs are expressed in most cell lines and tissues and probably interact with other receptor systems.

MAPPING OF THE CCK-A AND CCK-B/GASTRIN RECEPTOR BINDING SITES

Daniel Fourmy, INSERM U531, IFR 31, CHU de Rangueil, 31403 Toulouse Cedex 4, France.

CCK-A and CCKB/gastrin receptors are two distinct G protein coupled receptors which mediate central and peripheral effects of two structurally related neuropeptides, cholecystokinin and gastrin. The knowledge of the binding site(s) of these receptors represents an important challenge which may serve to understand their activation or inactivation processes and to design or optimize ligands. Binding sites of the two receptors were mapped using site-directed mutagenesis, pharmacochemistry and molecular modeling. Numerous amino acids of both the CCK-A and CCK-B/gastrin receptors were mutated. Wild-type and mutated receptors were analyzed after transfection in eucaryotic COS-7 cells. Ability of the mutated receptors to couple to phospholipase-C was considered as representative of their functionality. Several key amino acids of the CCK-A receptor binding site for CCK as well as their partners residues on CCK were identified. We demonstrated that Trp39 and Gln40 at the top of TM I interact with the N-terminal portion of CCK. Met195 and Arg197 of the second extracellular loop were shown to interact with the sulfated tyrosine of CCK. Asn333 and Arg336 at the top of TM6 were demonstrated to pair with the C-terminal amide and the penultimate Asp of CCK, respectively. Several of these key amino acids are involved differentially in the binding sites of peptide and nonpeptide agonists/antagonists. Using a similar strategy, we identified several amino acids of the CCK-B/gastrin receptor which are important for the high affinity binding of CCK. Interestingly, we showed that His207 of the CCK-B/gastrin receptor which is the corresponding basic aminoacid of Arg197 of the CCK-A receptor interacts with the penultimate Asp residue of CCK. Our data indicate that binding sites of CCK-A and CCK-B/gastrin receptors involve distinct aminoacids located in similar regions, however, CCK is docked differently in the two binding sites.

Grant supports :ARC, Région Midi-Pyrénées.

OC 25 — Pasteur Auditorium - Ligation Chemistry - Protein Modification - Analytical Methodologies — OC 26

A NOVEL GENERATION OF GLYCOMIMETICS FOR THE TARGETING OF DENDRITIC CELLS THROUGH THE MANNOSE RECEPTOR

Cyrille Grandjean[a], Gerhild Angyalosi[b], Véronique Pancré[b], Hélène Gras-Masse[a], Claude Auriault[b], Oleg Melnyk[a]*
[a] UMR 8525, [b] UMR 8527 Institut de Biologie de Lille, Institut Pasteur de Lille

Dendritic cells play a crucial role in the control of immunity, and are efficient antigen presenting cells able to initiate and modulate the immune response.[i] The selective targeting of these cells represents an important issue in the context of synthetic vaccines design. Dendritic cells possess a membrane receptor, the mannose-receptor, which is able to bind and internalize antigens functionalized by several D-mannose residues.[ii] Thus, the selective targeting of this receptor requires the preparation of multivalent ligands such as cluster glycosides or glycodendrimers. However, the cost associated with the large scale synthesis of these ligands precludes their incorporation in a synthetic vaccine.

Based on structural studies, we have developed a novel generation of mannose bioisosteres for the selective targeting of dendritic cells through the mannose receptor.[iii] The pyranose ring was replaced by a cyclohexane ring substituted by a carboxyl group, which allowed the anchoring of the mimics using standard activation protocols. We will describe the structural hypotheses of the study, and the synthesis of various dendrimers functionalized at their surface by the bioisosteres. The ligands were elaborated using solid phase and chemical ligation methodologies, thus allowing the size or the charge properties of the dendrimer to be easily modulated. We will also disclose the labelling of the glycomimetics with fluoresceine, and the selective internalization of the labelled compounds by dendritics cells, as followed by FACS analysis.

[i] Blanchereau, J. et al. Nature 1998, 392, 245-252.
[ii] Avrameas, A. et al. Eur. J. Immunol. 1996, 26, 394-400.
[iii] Grandjean, C. ; Rommens, C. ; Gras-Masse, H. ; Melnyk, O. Tetrahedron Lett. 1999, 40, 7235-7238.
Grandjean, C. ; Rommens, C. ; Gras-Masse, H. ; Melnyk, O. J. Chem. Soc. Perkin Trans. 1 1999, 2967-2975.
Grandjean, C. ; Rommens, C. ; Gras-Masse, H. ; Melnyk, O. Angew. Chem. Int. Ed. 2000, 39, in press

NEW AMINO ACIDS WITH SIDE-CHAIN OXYAMINO AND ALDEHYDE FUNCTIONS, AND THEIR USE IN CHEMOSELECTIVE LIGATION

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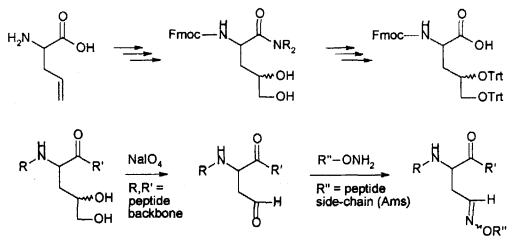
In chemoselective ligation, unprotected peptides are coupled to other peptides or substrates via complementary reactive groups. The reaction between an oxyamine and an aldehyde, to yield an oxime, is one of several useful ligation methods. Among other, peptide dimers, glycopeptides and cyclopeptides can be prepared by this strategy. Since amino acids with oxyamino and/or aldehyde functions are not common, the required functional groups are often introduced at a peptide terminus or on a lysine side-chain by acylation.

We report here the preparation and utilization of two new amino acids with build-in oxyamino and aldehyde side-chain functions. The oxyamine is O-aminoserine (Ams), protected as Fmoc-Ams(Boc). This building block is compatible with standard peptide build-up and standard deprotection with TFA. Since aldehydes are rather reactive in TFA, we choose to mask the aldehyde group as a side-chain diol and generate the aldehyde function in a separate step using sodium periodate. The new amino acid L-2-amino-4,5-dihydroxy-pentanoic acid (Ada) was prepared from L-allylglycine and protected in a synthesis of 6 steps overall. The synthesis was complicated by the tendency of the intermediate γ-hydroxy carboxy derivative to form a γ-lactone.

A set of peptides containing these new amino acids have been prepared and used in ligation with the formation of homo- and hetero-dimers, glyco- and cyclopeptides.



Lectures and Oral Communications

OC 27

Pasteur Auditorium - Ligation Chemistry - Protein Modification - Analytical Methodologies

OC 28

SUBNANOMOLAR-SCALE METHODS FOR DERIVATIZATION OF PEPTIDES FOR SEQUENCE ANALYSIS BY MALDI-TOF MS.

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Matrix assisted laser desorption/ionisation (MALDI) mass spectrometry is universaly used for peptide mass analysis and has emerged as a powerful method for protein identification. Individual peptides are completely sequenced in-line using PSD MALDI-TOF MS, even in a complexe mixture. The main problem is the difficulty in interpreting the complicated PSD spectrum which has several types of product ions.

We report procedures that trace primary amines. Using $CO(OSu)_2$ derivatization in aqueous acetonitrile, the N-terminal residue was transformed into the corresponding hydantoin and ε-NH_2 group in lysin into isocyanate. Other reagents, including carbonyl diimidazole, failed to provide adequate substitutions Derivatization and analysis was carried out without material transfer or purification at subnanomolar quantities. Several illustrative prototype peptide hormones (angiotensin, PACAP, ACTH...) are presented. This method facilitates peptide identification after enzymatic digestion and PSD sequence interpretation by MALDI-TOF mass spectrometry.

QUANTITATIVE ANALYSIS OF COMPLEX PEPTIDE REACTION SYSTEMS BY MASS SPECTROMETRY

Peter J. A. Weber[a,b], Asad Chavochi-Negad[b], and M. Reza Ghadiri[b]
[a]University of Leipzig, Institute of Biochemistry, Talstraße 33, 04103 Leipzig, Germany
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The quantitative study of mixtures or multicomponent reaction systems is very challenging since most of the existing methods are either limited to the analysis of a small number of species in solution, or the method development and sample-analysis is very time-consuming. To be able to analyze complex reaction systems based on the templating effect of coiled-coil peptides[1,2], and thereby to discover novel self-organizing networks we have sought to evaluate the ability of matrix-assisted laser desorption/ ionization mass spectrometry (MALDI-MS) for multicomponent reaction profile analyses. MALDI-MS measurements typically depend strongly on external parameters like the sample preparation method, the type of matrix, and the deposition procedure. Furthermore, intrinsic characteristics of the peptides like size, amino acid composition and even secondary structure are known to cause significant changes in signal intensities. In addition, a number of intrinsic properties of the mixtures, e.g. the suppression of the signals of less abundant species, are known to influence the measurements. Despite these parameters, we were able to show that the simultaneous quantitative MS-analysis of up to 16 different peptides is easily achievable by using a single internal standard and a new matrix system. Through detailed studies of a variety of matrices in combination with several co-matrices to analyze a carefully selected peptide mixture we found that the novel combination of 2–mercaptobenzothiazole with tris(2–carboxyethyl)phosphine yields similar ionization intensities despite huge differences in amino acid composition and aggregation state of the peptides employed. In addition, the matrix system provides strong signals even in the presence of large amounts of sample buffer and other contaminants. The significance of the system for the quantitative analysis of peptide mixtures was confirmed by the good agreement of the MALDI-MS data and HPLC control experiments for several multicomponent reactions of different complexity. The results raise the prospect of further applications of MALDI-MS for the studies of reaction systems of even higher complexity as well as fast quantitative analyses in the field of proteomics and combinatorial chemistry.

[1]D.H. Lee; K. Severin; Y. Yokobayashi; M.R. Ghadiri *Nature* **1997**, *390*, 591.
[2]K. Severin; D.H. Lee; J.A. Martinez; M.R. Ghadiri *Chem.-Eur. J.* **1997**, *3*, 1017.

OC 29

Total Synthesis of Ion Channel Proteins by Native Chemical Ligation

Gerd G. Kochendoerfer, Gryphon Sciences, South San Francisco, CA 94080

Ion channels are critical regulators of many fundamental biological processes and have been implicated in illnesses ranging from neurological disorders to heart disease. However, the molecular mechanisms by which ion channel proteins assemble and function is only poorly understood. This is largely due to the inadequacy of recombinant DNA-based expression methods in producing large quantities of ion channel proteins for biophysical and structural studies. We have overcome these problems by applying the power of total chemical protein synthesis in the production and systematic analysis of this important family of proteins. For instance, we have applied the native chemical ligation approach to the total chemical synthesis of the native influenza A virus M2 protein, **a 97-residue channel-forming tetrameric integral membrane protein** that is a key component in influenza pathogenesis.[Kochendoerfer, G.G., Salom, D., Lear, J.D., Wilk-Orescan, R., Kent, S.B.H., DeGrado, W.F. (1999)*Biochemistry*; 38 (37); 11905-11913 (1999)]. The same approach has been utilized to gain access to even larger ion channel proteins from other disease causing organisms, including the native 151-residue MscL protein of *M. tuberculosis*. Synthetic access to ion channel proteins significantly expands the type and scope of questions that researchers can ask in their endeavor to develop drugs as well as understand the fundamental process of ion channel protein assembly and conduction.

Lectures and Oral Communications

OC 30 — Einstein Auditorium - Chemistry of Amino Acids — OC 31

Functional α-H- and α,α-disubstituted amino acids based on target oriented side-chain design.

Quirinus B. Broxterman, Bernard Kaptein, Theo Sonke, Hans E. Schoemaker

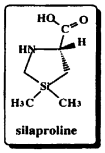
DSM Research ; Section Organic Chemistry & Biocatalysis; P.O.Box 18; 6160 MD Geleen; The Netherlands

One of the DSM processes to make enantiomerically pure amino acids consists of the following elements :
1) A wide array of α-H- and α,α-disubstituted amino acid amides are prepared either by Strecker synthesis from the corresponding ketones or by phase transfer catalysed alkylation of N-benzylidene-alaninamide.
2) These amides are resolved into the L-acid and the D-amide by enzymatic resolution using the amidases from *Pseudomonas putida*, *Mycobacterium neoaurum* or *Ochrobactrum anthropi*.
A large variaty of side-chain substitution patterns are accepted by these amidases as substrates. Enantiomerically pure amino acids containing functional side chains which are designed for target oriented action can therefore be synthesized.
In a number of collaborations with academic partners, we explore the potential of these amino acids. This is illustrated with the following results.
For example, α-methyl substituted phenylglycine is used for the preparation of ampicillin analogues. Similarly, α-methyl substituted phenylalinanine has been used to prepare the corresponding aspartame analogue. The α-methyl substituted amino acids are also used to influence the secondary structure of oligo-peptides, e.g., changing from an α-helix into a 3_{10}-helix. Isotopic labelling is used to study the biosynthesis of peptaibol antibiotics. In addition, long-chain amino acids are used to mimic membrane bound lipoproteins. Amino acids containing unsaturated side-chains have been used in ruthenium-catalysed methathesis and palladium-catalysed cyclisation reactions towards conformationally constrained cyclic amino acids. The functional amino acids can also be used to prepare constrained ligands for (catalytic) asymmetric synthesis.

SILAPROLINE: A SURROGATE OF PROLINE. INFLUENCE ON PEPTIDE CONFORMATION AND BIOACTIVITY

Florine Cavelier[a], Bertrand Vivet[a], André Aubry[b], Claude Didierjean[b], André Vicherat[c], Michel Marraud[c] and Jean Martinez[a], [a]Laboratoire des Aminoacides, Peptides et Protéines, UMR 5810, CNR-UM1-UM2, Université Montpellier 2 Place E. Bataillon, 34095 Montpellier Cedex 05, France. [b]LCM3B, ESA-CNRS 7036, Université Henri Pointcaré, BP 239, 54509 Vandœuvre les Nancy Cedex, France. [c]LCPM, UMR-CNRS 7568, ENSIC INPL, BP 451, 54001 Nancy Cedex, France.

Proline analogues are of great interest considering the importance of such a residue in reverse-turn structures. Here we report the synthesis of silaproline (Sip) derivatives in both optically pure forms. These proline analogues may also be useful as solubilizing building blocks, considering the high lipophilicity of silyl groups. In addition, replacing proline with this unnatural amino acid, presumably stable towards proteolytic enzymatic degradation, should increase the bioavailability of related peptides.
Silaproline was synthesized in both optically pure forms by diastereoselective alkylation of a chiral glycine equivalent using Schöllkopf's *bis*-lactim ether method. This new proline surrogate was incorporated in several peptides. For example, Sip has been introduced in Pro-containing bioactive peptides to compare their stability and activity. Sip was also substituted for Pro in model peptides to investigate the structural consequences of such modification by using X-ray, IR and NMR. The conformational and biological results will be discussed with reference to those for the parent Pro-containing peptides.



silaproline

OC 32 — OC 33

DIASTEREOSELECTIVE SYNTHESIS OF β²-AMINO ACIDS

Rachel Ponsinet[a,b], Gérard Chassaing[a] and S. Lavielle[a]
[a] UMR CNRS 7613, Chimie Organique Biologique, UPMC, tour 44-45, 4 place Jussieu, 75252 Paris Cedex 05, France.
[b] present address : Dyson Perrins Lab., South Parks Road, Oxford OX1 3QY, GB.

Most of the syntheses of β-amino acids described in the literature deal with either β³-amino acids, i.e., substituted in β position to the carboxylic acid or α,β-disubstituted amino acids. As part of an ongoing project concerning the synthesis of non natural amino acids, we have now developped diastereoselective syntheses of β²-amino acids. Two strategies have been envisaged.
The first one (Fig. 1) corresponds to a diastereoselective carboxylation α to a nitrile, precursor of the amino function, the first step involves the preparation of a chiral electrophile, obtained by reacting Oppolzer's sultam with triphosgen. Enantiomerically pure β²-phenylglycine has been obtained. But, this procedure cannot be extended to the preparation of the other β²-amino acides.
The second strategy (Fig. 2) is based on the alkylation of a chiral precursor derived from β-alanine. The stereochemistry of the side-chain is governed by the chiral auxilliary (Oppolzer's sultam). The stragegy leads to β²-Phe, β²-Ala, β²-Leu with excellent enantiomeric excess. The key step implies to use electrophiles reactive at low temperature (≤ - 45°C) to prevent the enolate from eliminating.

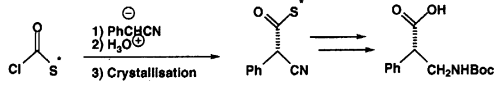


Figure 1

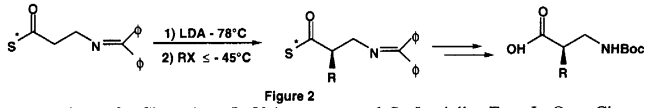


Figure 2

R. Ponsinet, G. Chassaing, J. Vaissermann and S. Lavielle. Eur. J. Org. Chem., 2000, 83-90.
R. Ponsinet, G. Chassaing, S. Lavielle.Tetrahedron : Asymmetry, 1998, 9, 865-871.

A NOVEL GROUP OF PEPTIDIC BIOLOGICAL RESPONCE MODIFIERS

ElenaYu. Blishchenko, Andrei A. Karelin and Vadim T. Ivanov
Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117871 Moscow, RUSSIA

Several hundreds of endogenous peptides ranging from 45 to 2 amino acid residues in length have been isolated from various tissue extracts and sequenced. In contrast to the majority of classical peptide hormones, neurotransmitters and neuromodulators, the peptides studied in that work are formed by *in vivo* proteolysis of proteins with well-established functions, such as hemoglobin, actin, cellular enzymes, etc. The sets of these peptides in tissues are tissue-specific on one hand and conservative in a given tissue at normal conditions on the other. Their levels are sensitive both to pathologies due to alterations of tissue metabolism and to prolonged physiological changes. More than a hundred of such peptides have been evaluated for bioactivity *in vitro* and the majority of them was shown to inhibit or stimulate proliferation, to induce cytolysis of tumor cells or to restore proliferation of normal cells treated with drugs *in vitro*[1-3].
In the present work a more detailed study was carried out of the patterns of action of tissue-specific peptides at cellular level. The typical mechanism of action of the growth inhibitory peptides both in tumor and normal cells is due to the reversible arrest of proliferation. The latter leads to the ability of such peptides to protect the rapidly dividing cells from the toxicity of chemiopreparations. Both the antitumor activity and the cell-protective effect in the case of application of chemiopreparations have been confirmed *in vivo*. The latter results point to an important biological role of growth inhibitory peptides in the organism. On the other hand, the growth-stimulatory peptides were shown to restore proliferation of normal cells after treatment with chemiopreparations or in the case of growth factors deprivation. They also accelerate the rate of cell division at intermediate cell density, which can be interpreted as a tissue restoration model. We believe that these peptides replace growth factors in the case of their deficiency and participate in tissue regeneration processes. On the basis of the data obtained, we suggest that tissue-specific peptides participate in regulation of cell number in tissues, i.e., in maintenance of tissue homeostasis.

1. Karelin A.A. et. al., *Neurochem. Res.*, 1999, 24, 1119-1126.
2. Ivanov V.T., et. al., *Pure Appl. Chem.*, 1998, 70, 67-74.
3. Ivanov V.T., et. al., *Pure Appl. Chem.*, (in press) (2000).

ARE β-AMINO ACIDS γ-TURN MIMETICS ?

Norbert Sewald, Annett Müller, Frank Schumann, Mario Koksch,[a] Faculty of Chemistry, University of Bielefeld, FRG; [a]Medical Clinic I, University of Leipzig, FRG.

Homo-oligomers of β-amino acids adopt predictable and reproducible folding patterns, e.g. helices [1]. β-Amino acid incorporation considerably modifies the skeleton atom pattern and the hydrogen bond pattern of peptides resulting in a modulation of physiological activity and increased metabolic stability. However, the issue whether peptides containing both α- and β-amino acids display new types of secondary structures has not yet been addressed systematically [2]. Hence, very little information on conformational preferences of peptides modified by β-amino acids is available.

Cyclic RGD peptides have been modified using β-amino acids and the biological activity has been examined. The amino acid sequence Arg-Gly-Asp (RGD) plays a major role in cellular binding and recognition phenomena (e.g. blood platelet aggregation, tumour cell adhesion, osteoporosis). The cyclic penta- and hexapeptides c-RGDfV [3] and c-RGDfVG selectively inhibit binding of different proteins containing the RGD triad to integrins [4]. Peptide selectivity between the fibrinogen receptor ($\cdot_{IIb,3}$) and the vitronectin receptor ($\cdot_{v,3}$) strongly depends on the local conformation of the pharmacophoric RGD sequence. The known correlation of solution structure and receptor selectivity [4] renders this class of peptides excellent model systems for probing the consequences of peptide modification using β-amino acids in terms of biological activity (integrins $\cdot_{IIb,3}$ and $\cdot_{v,3}$) and solution structure (NMR/MD).

A strategy for the synthesis of cyclic peptides was adapted for on-resin backbone cyclization of the peptide [5], allowing multiple or combinatorial syntheses. A dramatic change of the activity was found in some cases [5]. In several cases β-amino acids exert strong conformational bias and are found in the central position of an extended "pseudo"-Á-turn.

1. López-Carrasquero, F. et al., *Macromolecules*, 29 (1996) 8449; Appella, D.H et al, *Nature*, 387 (1997) 381; Seebach, D. et al., *J. Chem. Soc., Chem. Commun.* 1997, 2015.
2. Lombardi, A. et al., *Biopolymers*, 38 (1996) 693; Graf von Roedern, E. et al., *J. Am. Chem. Soc.*, 118 (1996) 10156.
3 One-letter-code; f = D-Phe
4. Aumailley, M. et al., *FEBS Lett.*, 291 (1991) 50; Haubner, R. et al., *Angew. Chem.*, 109 (1997) 1440.
5. Müller, A. et al., *Lett. Pept. Sci.*, 4 (1997) 275.

DIVERSITY OF POST-TRANSLATIONAL MODIFICATIONS OF PROTEINS PROVED BY MASS SPECTROMETRY

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Post-translational modifications of numerous proteins in eucaryotes are crucial for biological functions including protein-protein interaction, metabolic regulation, specific recognition of effectors, etc. This implicates importance of the elucidation of the structures of these modifications, which involve phosphorylation, glycosylation, lipidation etc. Mass spectrometry is a method of choice for the characterization of biomolecules, particularly because of its high sensitivity along with increased accuracy in molecular mass determination. Accurate mass provides a molecular formula in some cases, while tandem mass spectrum obtained by low- or high-energy collision-induced dissociation provides considerable structural information. Hitherto, we have developed various procedures using mass spectrometry for elucidating the structures of post-translational modifications of proteins, which include blood coagulation factors, GTP-binding protein, core histone etc. These experiments resulted in the finding of various kinds of post-translational modifications that are important for biological activities of modified proteins. Here we explain the procedure for identifying the structures of post-translational modifications using various techniques of MS and show some new structures.

Protein Ectodomain Shedding

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The proteolytic release of the extracellular domain of cell surface transmembrane proteins, also known as protein ectodomain shedding, has been recently recognized as an important aspect of the regulation of cell-cell interactions. Since ectodomain shedding affects to structurally and functionally diverse proteins, such as transmembrane growth factors, growth factor receptors, cell adhesion molecules or ectozymes, its functional consequences are of broad interest.

The *metalloprotease-disintegrin* TACE (*Tumor necrosis factor-Alpha* (TNF-α) Converting Enzyme), initially identified because of its TNF-α processing activity, has been recently shown to be also responsible for the shedding of an heterogeneous group of proteins, indicating that TACE plays a central role in protein ectodomain shedding. This group of TACE substrates includes the ligand for the epidermal growth factor receptor pro-Transforming Growth Factor Alpha (proTGF-α) and the Alzheimer disease-related protein beta-Amyloid Precursor Protein (βAPP). The mechanisms that regulate the activity of TACE, the best characterized of which involves the protein kinase C (PKC), are currently under investigation. At least in the case of proTGF-α, compartimentalization seems to be an effective way to modulate its shedding.

Despite its large substrate specificity, PKC activated metalloprotease-disintegrins do not seem to be the only mechanism involved in protein ectodomain shedding. It has been recently shown that the growth factor receptor HER-2 can release its ectodomain by a mechanism that is likely mediated by matrix metalloproteases. In summary, due to its extent and broad functional implications, protein ectodomain shedding has attracted the attention of cell biologists; the elucidation of the mechanism that regulate the metalloproteases involved in this process seems to be the next significative step in this field.

SOLID-PHASE SYNTHESIS OF CYCLIC AND POLYCYCLIC PEPTIDES

Alethea B Tabor

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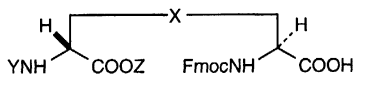
The cyclisation of bioactive linear peptides *via* side-chain linkages not encountered in nature is of considerable importance in medicinal chemistry, as the position and methods of cyclisation may be fine-tuned to produce a conformationally constrained peptide.[1] We are developing a solid-phase approach to the synthesis of cyclic peptides where the side-chains are linked *via* alkyl or thioether bridges. Our strategy involves the synthesis of double-headed amino acids,[2] orthogonally protected using both allyl/allyloxycarbonyl and photolabile transient protecting groups, and the incorporation of these into cyclic and polycyclic peptides using solid-phase methods. The synthesis of the amino acids, their incorporation into cyclic peptides and structural studies on the resulting peptides will be described.



X = S or aliphatic
Y, Z = allyl or photolabile protecting groups

1. A. F. Spatola *et al.*, *J. Med. Chem.*, **39**, 3843 (1996): G. Ösapay et al., *J. Med. Chem.*, **40**, 2241 (1997).
2. M. J. I. Andrews, A. B. Tabor, *Tetrahedron Lett.*, **38**, 3063 (1997).

COPPER(II)-CONTAINING RACEMIZATION SUPPRESSORS AND THEIR USE IN SEGMENT COUPLING REACTIONS

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One of the most serious challenges in the field of peptide synthesis is the prevention of racemization during couplings. In our efforts to develop processes for the large scale synthesis of peptides, we have discovered and investigated several different reagents that may have utility as racemization suppressors. Some of these reagents include the copper (II) complexes of 1-hydroxybenzotriazole ($Cu(OBt)_2$), 3-hydroxy-1,2,3-benzotriazin-4(3H)-one ($Cu(OOBt)_2$), and 1-hydroxy-7-azabenzotriaxole ($Cu(Obt)_2$). With the use of $Cu(OBt)_2$, we have been able to control the level of racemization in model coupling reactions at 0.5% and below. We will describe the preparation of these reagents as well as their application in the coupling of peptide segments.