L 37 - What to synthesize? From Emil Fischer to peptidomics

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The driving forces, incentives and strategic targets of peptide synthesis have undergone considerable evolution during the centenary following the pioneer work of Emil Fischer. In those days peptide synthesis was considered as a way to confirm the polypeptide theory of protein structure. The scientific community also expected (naively) that the synthesis will eventually lead to creation of artificial living organisms. Only in 1950-ies, when the first exact amino acid sequences were established peptide chemistry obtained firmer ground and clearly defined targets. Total syntheses of peptide hormones and antibiotics became possible, providing valuable material for elucidating structure-functional relationships and the mechanisms of biological action. In the following years the number of peptides isolated from various biological sources grew with impressive speed and peptides became the most abundant, ubiquitous group of low molecular bioregulators. Design and synthesis of novel peptide based pharmaceuticals became an important area of peptide chemistry. At present we are facing the challenge of analyzing the structures and bioactivities ot total sets of peptides, i. e. peptidoms present in concrete tissues or groups of cells. The results obtained along these lines in the title Institute will be presented in the lecture.

L38 - Molecular machines for protein degradation

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Within cells or subcellular compartments misfolded and/or short-lived regulatory proteins are degraded by protease machines, cage-forming multi-subunit assemblages. Their proteolytic active sites are sequestered within the particles and located on the inner walls. Access of protein substrates is regulated by protein subcomplexes or protein domains which may assist in substrate unfolding dependent of ATP. Four protease machines will be described displaying different subunit structures, oligomeric states, enzymatic mechanisms, and regulatory properties.

References

Proteasome
Groll, M., Ditzel, L., Löwe, J., Stock, D., Bochtler, M., Bartunik, H. D. and Huber, R. (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. Nature 386, 463-471.
Groll, M., Heinemeyer, W., Jäger, S., Ullrich, T., Bochtler, M., Wolf, D. H. and Huber, R. (1999) The catalytic sites of 20S proteasomes and their role in subunit maturation: A mutational and crystallographic study. Proc. Natl. Acad. Sci. USA 96, 10976-10983.
Groll, M., Bajorek, M., Köhler, A., Moroder, L., Rubin, D. M., Huber, R., Glickman, M. H. and Finley, D. (2000) A gated channel into the proteasome core particle. Nature Struct. Biol. 7, 1062-1067.

HslV/HslU
Bochtler, M., Hartmann, C., Song, H. K., Bourenkov, G., Bartunik, H. and Huber, R. (2000) The structure of HslU and the ATP-dependent protease HslU-HslV. Nature 403, 800-805.
Song, H. K., Hartmann, C., Ramachandran, R., Bochtler, M., Behrendt, R., Moroder, L. and Huber, R. (2000) Mutational studies on HslU and its docking mode with HslV. Proc. Natl. Acad. Sci. USA 97, 14103-14108.
Ramachandran, R., Hartmann, C., Song, H. J., Huber, R. and Bochtler, M.(2002) Functional interactions of HslV(ClpQ) with the ATPase HslU(ClpY). Proc. Natl. Acad. Sci. USA 99, 7396-7401.

Tricorn
Brandstetter, H., Kim, J. S., Groll, M. and Huber, R. (2001).Crystal structure of the tricorn protease reveals a protein disassembly line. Nature 414, 466-470.
Kim, J. S., Groll, M., Musiol, H. J., Behrendt, R., Moroder, L., Huber, R. and Brandstetter H. (2002) Navigation inside a protease: substrate selection and product exit in the tricorn protease from Thermoplasma acidophilum. (submitted)
Goettig, P., Groll, M., Kim, J. S., Huber, R. and Brandstetter, H. (2002) Structures of tricorn interacting aminopeptidase F1 with different ligands explain its catalytic mechanism. (submitted)

DegP(HtrA)
Krojer, T., Garrido-Franco, M., Huber, R., Ehrmann, M., and Clausen, T. (2002) Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine. Nature 416, 455-459.

L39 - Synthetic glycopeptides for the development of tumor-selective antigens

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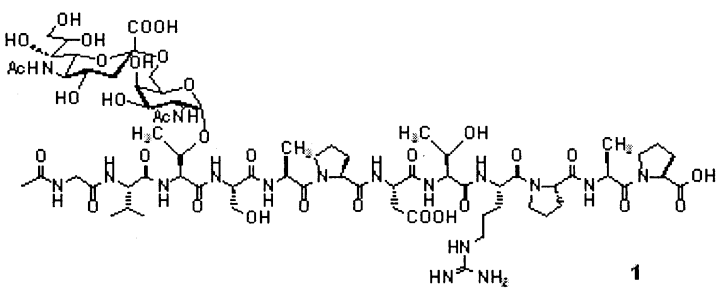
The glycosylation pattern of normal cells and tumor cells is distinctly different in many cases. This holds true, for example, for the polymorphic epithelial mucin MUC1 (episialin). In its tumor-associated form MUC1 carries incompletely developed carbohydrate side chains, in particular, sialyl T and sialyl Tn antigen structures. Because of the short saccharide side chains peptide epitopes, hidden in the MUC1 on normal epithelial cells by large carbohydrate structures, become accessible to the immune system. Therefore, synthetic glycopeptides consisting of tumor-associated saccharide antigens and peptide sequences of the extracellular domain of MUC1, e. g. **1** are promising candidates for the development of antitumor vaccines. These complex glycopeptides are synthesized by solid-phase syntheses using allylic or 2-phenyl-2-trimethylsilyl-ethyl linkers cleavable under neutral conditions and glycosyl amino-acid building blocks containing the suitably protected saccharide side chains. The chemical synthesis of the saccharide and the peptide portions of these complex compounds takes profit of the fundamental work by Emil Fischer in both areas of natural product chemistry, carbohydrates and peptides.



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L40 - Chemical protein synthesis - new methods, new applications for the new century

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In 1906 Emil Fischer set the goal of peptide science as the total chemical synthesis of enzymes, believing these biological catalysts to be proteins (i.e. long polypeptide chains). This prescient ambition remained beyond the reach of synthetic and analytical chemistries until the past few years. With the advent of chemical methods for the ligation of unprotected peptides in aqueous solution and using modern mass spectrometric methods for analytical control, the total synthesis of proteins (including enzymes) has been achieved. Here we will briefly summarize the principles of modern chemical protein synthesis and describe the ligation chemistries that have been developed. Total synthesis has enabled the application of peptidomimetic chemistry to the protein molecule itself to dissect the chemical basis of enzyme catalysis. Future applications include the elucidation of the structure and mechanisms of integral membrane enzymes, the total synthesis of glycoproteins of defined structure, and the application of combinatorial peptidomimetic chemistry to the world of proteins.

L41 - The bold legacy of Emil Fischer

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The bold legacy of Emil Fischer is clearly demonstrated by the content of this symposium and other recent activities throughout the world of chemistry. It is a true testimony to his creativity that we have remained concerned with the syntheses of peptides and peptidomimetics and have added such far ranging themes as biomolecules and biopolymers. In this lecture advances in synthetic methodology, conformational studies and bioassays will be covered using examples from our laboratories. We are investigating the preparation of guanidine containing structures, such as RGD mimetics and guanidinoglycosides. Recently we demonstrated that 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3H)-one (DEPBT) is a novel and useful coupling reagent that allows the formation of amide bonds under mild conditions to access complex structures. Peptide opioid and somatostatin analogs have been synthesized. Some of these cyclic molecules exhibit remarkable biological data. Lastly, the synthesis and biophysical characterization of novel scaffold assembled collagen mimetics will be presented. These biopolymers have potential application as surrogates of natural collagen. The breadth of the lecture clearly derives from the pioneering work of Emil Fischer and all the giants who helped formulate the field of peptide science over the last 100 years. It is my hope that by illustrative examples from current research, this lecture will serve as an appropriate tribute to the creative builders of peptide science.

L 42 - Total synthesis and biological activity of human insulin3 and its monobiotinylated analogue

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The recently identified protein, insulin 3 (INSL3), has structural features that make it a bona fide member of the insulin superfamily. Its predicted amino acid sequence contains the classic two peptide chain (A- and B-) structure with conserved cysteine residues that results in a disulphide bond disposition identical to that of insulin. Recently, the generation of insl3 knockout mice has demonstrated that testicular descent is blocked due to the failure of a specific ligament, the gubernaculum, to develop. As the mechanism by which INSL3 exerts its action on the gubernaculum is currently unknown, we undertook to synthesize human INSL3 and test its action on organ cultures of fetal rat gubernaculum. INSL3 also contains a cassette of residues Arg-X-X-X-Arg within the B-chain, a motif that is essential for characteristic activity of another related member of the superfamily, relaxin. Hence the relaxin-like activity (if any) of human INSL3 was also tested in THP-1 cells which express the human relaxin receptor. The primary structure of human INSL3 was determined by deduction from its cDNA sequence and successfully prepared by solid phase peptide synthesis of the two constituent chains followed by their combination in solution. Following confirmation of its chemical integrity by a variety of analytical techniques, CD spectroscopy confirmed the presence of high β-turn and α-helical content, with a remarkable spectral similarity to synthetic human 2 relaxin. The synthetic human INSL3 was devoid of activity in the relaxin bioassay and, furthermore, it did not augment or antagonize relaxin activity. The human INSL3 did however induce growth of whole organ cultures of fetal rat gubernaculum demonstrating that INSL3 has a direct action on this structure. The preparation of a mono-biotinylated human INSL3 was also successfully achieved and shown to possess similar secondary structural features and bioactivity to the native peptide. It will be a useful tool for INSL3 receptor localization and characterization.

L43 - Novel β-defensins: cysteine-rich peptides of the innate and adaptive immune system

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Defensins represent a steadily growing family of cysteine-rich and cationic peptides about 3-5 kDa in size which are formed from small precursor proteins. In mammals, they are subclassified into the α-, β- and the recently discovered -defensins, based on their characteristic disulfide bond pattern. While α-defensins are predominantly expressed in granulocytes, certain leukocytes and intestinal Paneth cells, β-defensins are often found on epithelial surfaces of the body, such as the tongue, lung and skin. However, each defensin has a distinct pattern of expression. They are inducible by inflammatory factors such as LPS, or can be constitutively expressed. The defensins of both classes each display a specific broad spectrum of antimicrobial activity against Gram-negative and Gram-positive bacteria, and yeasts. Defensins have been shown to act synergistically with other microbicidal peptides or classic antibiotics. While the primary structures are very different, the elements of secondary structure and three-dimensional shape among α- and β-defensins are very similar. In the first instance, defensins have been considered to form a first barrier against pathogens after microbial infection, and the lack of defensin expression has been linked to several diseases, e.g. cystic fibrosis. Thus, defensins are part of the innate immune system. Meanwhile, there is a growing body of evidence that β-defensins in particular augment responses of the adaptive immune system, such as chemotaxis mediated by the chemokine receptor CCR6.
Here, we present an overview of the biology and chemistry of the increasingly explored defensin peptide family. In particular, the β-defensin family including human β-defensins 3 and 4 (hBD-3 and hBD-4) which were recently discovered and investigated in our lab, will be discussed concerning their significant biological features such as gene structure and regulation, mRNA expression, antimicrobial and other functional properties, their isolation and identification by genomics, their chemical synthesis and solution structure.

L44 - Peptide antagonist and bioactive conformation of Melanin-Concentrating Hormone (MCH), an important regulatory hormone in feeding behavior.

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Melanin-concentrating hormone is a cyclic hypothalamic neuropeptide [NH_2-Asp-Phe-Asp-Met-Leu-Arg-Cys-Met-Leu-Gly-Arg-Val-Tyr-Arg-Pro-Cys-Trp-Gln-Val-COOH cyclo Cys-Cys (7-16)] secreted by hypothalamic cells which plays an important role in energy homostasis and feeding behavior. It is the only known hypothalamic peptide whose disruption results in leanness, hypophagia and a relative increase in oxygen consumption. ICV injection of MCH induces an increase food intake in mice and rats. Transgenic mice overexpressing MCH two- fold in the hypothalamus , gain more weight than WT animals and display higher food intake, especially when given high fat diets. Consequently, a potent MCH antagonist is regarded as potentially useful in therapeutic approaches to obesity management. In a previous publication [1], we described a detailed SAR of MCH and identified Met 8, Arg 11, Tyr 13, as the critical residues for receptor recognition. Based on this information we developed a peptide antagonist to be used as a tool to further evaluate the physiological significance of the MCH/MCH receptor interaction and the relations of receptor sub-types. Furthermore NMR was used to study the conformational aspects of MCH peptides. Complete assignments were made and full sets of NOEs were assessed yielding detailed structures which helped define the position of the key side-chains and allowed a pharmacophore model to be developed. In this model, the important residues are within 10-13 Angstroms of each other, and form a triangle composed of a basic residue and two hydrophobic loci. This is in agreement with the general understanding of the GPCR recognition motif. The pharmacophore model may help in the design of a novel non-peptidic MCH antagonists.

References

[1] W. Danho , J. Swistok, W. Khan, T. Tuitt, A. Aglione, R. Garippa, K. Xu, Y. Chen, Q. Xiang, J. Kochan, and F. Falcioni. *In Peptides: The Wave of the future*. Michal Lebl and Richard Houghten (Editores) American Peptide Society (2001) P.704.

L45 - Growth hormone secretagogues, synthesis and biological evaluation

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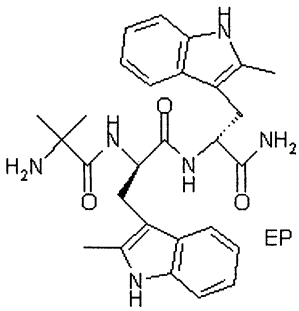
Growth Hormone Secretagogues liberate Growth Hormone acting on receptors distinct from GHRH receptors. In a preliminary study, the hexapeptide Hexarelin His-2-Me-D-Trp-Ala-Trp-D-Phe-Lys-NH_2 was downsized to yield, among others, a tripeptide, compound EP 51389, subcutaneously more potent than Hexarelin in a infant rat model and orally active in dogs and man.
The design, synthesis and biological evaluation of a new and original class of orally bioavailable pseudo-tripeptide analogs will be presented. One of the lead compounds, JMV 1843 was shown to stimulate GH secretion in a infant rat model and in dogs. Administrered in man by i.v. (1.0 µg/kg) it was able to stimulate GH release but not prolactin, ACTH, cortisol, isuline and glucose increases in blood circulation, showing its high specificity. Administered per os in man (0.5 mg/kg) a high GH release was observed and was found to be dose-dependent. These findings made compound JMV 1843 a good candididat for GH therapy replacement.



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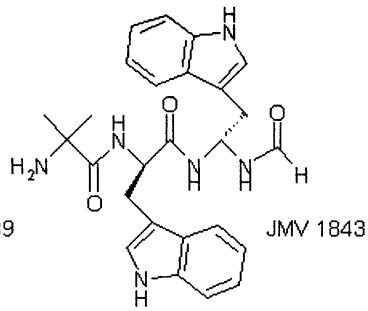

JMV 1843

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L46 - Synthesis and biological activity of a new and highly potent ligand for somatostatin receptors 2, 3 and 5

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Tumor targeting with radiolabelled peptides has been of much recent interest. The prototypes are derivatives of somatostatin (SRIF) like DTPA-octreotide or DOTA-[Tyr]^3-octreotide (DOTA-TOC). Five SRIF-receptor subtypes (sstr) are known and shown to be expressed on different tumors. The above mentioned peptides show high affinity mainly for sstr2 and moderate affinity to sstr5.
We aimed at developing new SRIF-analogs for internal radiotherapy, which recognize different subtypes, to expand the present range of accessible tumors. Using parallel solid phase synthesis, we synthesized 15 new octapeptides based on octreotide, by replacing Phe^3 mainly with unnatural amino acids. DOTA (1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetraacetic acid) was coupled to these peptides and the receptor subtype affinity profile was determined using transfected cell lines with sstr1-5. Internalization was determined using AR4-2J cell lines and biodistribution was studied in a rat tumor model (CA 20948).
Parallel solid phase synthesis allowed quickly to synthesize a range of new SRIF-based radiopeptides. One of them, DOTA-[1-Nal]^3-octreotide (DOTA-NOC), showed high affinity to sstr 2,3 and 5, if complexed with Y(III) or In(III). Surprisingly the sstr2 affinity was almost 4 times higher than the one of [Y]DOTA-TOC and equal to natural somatostatin-28 (SS-28). The rate of internalisation in this series was the highest for [^{111}In]-DOTA-NOC, at 4 h being more than twice than that of [^{111}In]DOTA-TOC. Biodistribution in tumour bearing rats again showed very promising results. The uptake in sstr expressing tissues including the CA 20948 tumour was significantly higher for [^{111}In]-DOTA-NOC compared with [^{111}In]-DOTA-TOC and the tumour-to-kidney ratio was improved by a factor of 2, potentially opening the therapeutic window in a clinical setting.
Parallel solid phase synthesis along with the suitable bioassays is a powerful tool to select new radiopeptides with improved targeting properties. [^{111}In] and [^{90}Y]-DOTA-NOC are very promising radiopeptides for the diagnosis and peptide receptor mediated radiotherapy of a larger range of sstr expressing tumours like thyroid, colon or lung adenocarcinomas which were shown to overexpress SRIF receptor subtypes other than sstr2.

L47 - Discovery and structure-function studies on the cyclotides:applications in drug design

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The cyclotides[1] are a recently discovered family of plant-derived peptides that have a range of exciting applications in drug design and agriculture[2]. They are typically about 30 amino acid in size, contain a head-to-tail cyclised backbone and incorporate three disulfide bonds arranged in a cystine knot topology. In this motif an embedded ring in the structure formed by two disulfide bonds and their connecting backbone segment is penetrated by the third disulfide bond. The combination of this knotted and strongly braced structure with a circular backbone renders the cyclotides completely impervious to enzymatic breakdown and makes them exceptionally stable. This presentation will describe the discovery of the cyclotides in plants from the Rubiaceae and Violaceae families, their chemical synthesis and structural characterization by NMR. Their stability is shown to be critically dependent on the integrity of the cyclic cystine knot (CCK) framework, as synthetic acyclic permutants in which the backbone is cut have greater flexibility. The cyclotides have a diverse range of biological applications, ranging from uterotonic action, to anti-HIV and neurotensin antagonism. Certain plants from which they are derived have a history of uses in native medicine, with activity being observed after oral ingestion of a tea made from the plants. This suggests the possibility that the cyclotides are orally bioavailable. Applications in which the stable framework is used as a peptide delivery template will be described. These involve the grafting of non-native bioactivity onto the stable peptide framework. We have also undertaken extensive studies on the folding mechanisms of the cyclotides. The native peptides were reduced and then monitored by HPLC and NMR during re-folding and a stable two-disulfide intermediate has been isolated. This has provided an insight into the mechanism of formation of the cyclic cystine knot motif. The cyclotides are gene products derived from linear precursor proteins that encode either one, two or three cyclotide domains. Studies on the sequences of cyclotide genes from several plant species have provided an insight into the processing mechanisms that produce circular proteins.

References
[1] Craik DJ, Daly, NL, Bond, T and Waine, C. *J. Mol. Biol.*, (1996) 294, 1327-1336
[2] Craik DJ, Simonsen, S, and Daly NL, *Curr Opin Drug Dev* (in press).

L48 - Discovery of peptido and proteinomimetic drug leads using cyclic peptide spatial libraries

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Cycloscan [1] is a method for the selection of peptido- and proteinomimetic drug leads from backbone cyclic peptide libraries with spatial diversity. In these libraries all the peptides have the same primary sequence but they differ from each other in their conformation. The construction of such spatial libraries is achieved in hierarchical manner using the following diversity elements: the mode of cyclization, the location of the ring along the peptide chain, the ring size and, the ring chemistry. Cycloscan was applied to natural peptides as well as to peptides derived from phage display libraries and to active regions in proteins, leading to highly potent, selective, metabolically stable peptido- and proteinomimetic drug leads. Few examples will be discussed: (i) a backbone cyclic somatostatin analog with physiological selectivity that suppress GH release but not insulin or glucagon [2] (ii) a backbone cyclic penta peptide antagonist derived from the 33 amino acid insect neuropeptide PBAN that inhibit in-vivo pheromone release in Heliothis Pelrigera [3], and (iii) a backbone cyclic proteinomimetic derived from the binding region of HIV-1 gp120 to CD4 that inhibit viral proliferation in cells [4].

References
[1] Gilon and Hornik US Patent 6,117,974 (2000)
[2] Affargan et al. *Endocrinology 142*, 477 (2001)
[3] Zeltser et al. *J. Pepide Res 58*, 275 (2001)
[4] Cohn et al. in preparation

L49 - Determining the pharmacophore structure of substrates of the Mammalian Peptide Transporter (PEPT1) by molecular modeling and 3D-QSAR investigations

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PEPT1 is a transmembrane protein that transports di- and tripeptides and peptidomimetics such as β-lactam antibiotics across the luminal membrane of mammalian small intestinal cells. The system is a symporter energized by a transmembrane H^+ gradient. The three-dimensional structure of PEPT1 is still unknown and will not be available in the near future. As long as this situation persists, hypotheses about the active site structure depend on two types of experiments, namely, the functional analysis of chimeric mammalian peptide transporters and molecular modeling approaches using substrate affinity information. The latter strategy pursued in our group relies on *(i)* the active analog approach, *(ii)* the comparison with crystal structures of bacterial peptide binding proteins complexed with their ligands and *(iii)* 3D-QSAR analysis.
A conformational analysis of more than 60 dipeptides, dipeptide derivatives and β-lactam antibiotics covering a broad range of K_i values was performed. Small and relatively rigid dipeptides that can adopt only few conformations revealed two structures shared by all dipeptides and derivatives. The similarity of one of these conformations to the X-ray structure of di- and tripeptide/oligopeptide binding protein complexes is striking. Therefore, we chose this conformation as a model for the bioactive conformation and alanylalanine as the template.
Based on distance comparison using the DISCO module of SYBYL it was possible to superimpose the suggested bioactive conformations of 65 different, arbitrarily chosen dipeptides and derivatives by their hydrogen-bond donor and acceptor properties. For the subsequent 3D-QSAR analysis two methods were employed: The widely used ***comparative molecular field analysis*** (CoMFA) and the recently introduced ***comparative molecular similarity indices analysis*** (CoMSIA). In all cases, statistically significant CoMFA and CoMSIA models were generated. In general, q^2 values of 0.722 for CoMFA (using 6 components) and 0.636 for CoMSIA (using 10 components) were found. The CoMSIA approach highlights those regions in the substrate structure that are responsible for the differences in selectivity. The steric contour map of the present model indicates two small favorite regions for the side chain of the first amino acid and one large favorite region for the side chain of the second amino acid. Preferred electrostatic regions involve the carboxyl group and C-terminal side chain. Favorite hydrophilic regions are located at the peptide bond and at the side chain of the second amino group. As expected, one contour map in the acceptor field surrounds the carbonyl oxygen of the peptide bond suggesting its important role in hydrogen bonding toward PEPT1. On the basis of spatial arrangements of the various field contributions, it is now possible to design and predict the affinity of novel substrates or inhibitors.

L50 - Identification of GLGPRPLRF-NH_2 (AF9) as a peptide activating C39E6.6, the *C. elegans* GPCR implicated in social behavior in worms

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The C39E6.6 gene (also called *npr-1*) encodes a *C. elegans* GPCR, resembling vertebrate NPY-receptors. A single amino acid mutation, F215V, in the third intracellular loop of NPR-1 has been shown to dramatically influence feeding behavior in the worm (*Cell* 94, 679, 1998). It has been postulated that food is likely to regulate secretion of a neuropeptide that acts through the NPR-1 receptor. However, NPR-1 has remained an orphan receptor to date because its activating neuropeptide had not been identified. We cloned *npr-1* and transiently transfected it into CHO cells. Membranes from the transfected NPR-1/CHO cells were used in a GTPγS assay for detecting possible functional receptor activation in response to various invertebrate FMRF-amide-related peptides (FaRPs). When routine conditions were used for cell cultures and transfections, no receptor activation was observed. However, by modifying cell growth parameters, NPR-1 became functional as reflected in a dose-dependent stimulation of [^{35}S]GTPγS binding to cell membranes after treatment with GLGPRPLRF-NH_2 (AF9). AF9 was the only peptide, out of the over 100 FaRPs tested, that activated NPR-1. This peptide is encoded on a putative *C. elegans* precursor gene *flp21* and is also present in other parasitic nematodes (e.g. *A. suum*). Significant differences in functional responses to AF9 were noted in the F215-NPR-1/CHO and V215-NPR-1/CHO membranes, which could explain, at the molecular level, the observed phenotype differences in the mutant *C. elegans*.

L51 - Synthesis and screening of a positional scanning library based on the Bowman-Birk reactive site

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Bowman-Birk Inhibitors (BBI) are small proteinaceous serine inhibitors found in many plants. BBIs have two inhibitory regions, each comprising a nine-residue disulfide constrained loop. These loops have the general sequence $CTP_1S\varepsilon P\varepsilon\varepsilon C$ (ε being any amino acid residue). The exact sequence determines the inhibitory specificity and peptides with K_i values in the low nanomolar region have been synthesised as a result of the synthesis and screening of bead libraries based on a general template sequence. In this work, the four sublibraries of a positional scanning library have been synthesised, each containing 331776 components. The peptide libraries were constructed based on the sequence of the reactive site loop of the BBI, and randomising the four positions P_4, P_2, P_1 and $P_{2'}$. The libraries have the general sequence XCXXSXPPQCY (Where X = mixture of 24 amino acids).
The sublibraries have been assayed for inhibition of several serine proteinases including Bovine Trypsin and Chymotrypsin, Porcine Pancreatic Elastase and Human Leukocyte Elastase. Distinct amino acid specificities have been discovered at the four positions tested.
Individual peptides generated from the library were synthesised and tested. Results will be discussed.

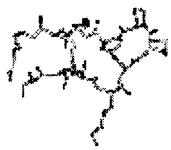

Fig. 1 - Tube diagram of soybean BBI.

Fig. 2 - Truncated loop SCTKSMPPKCR of trypsin inhibitor BBI

L52 - Combinatorial approaches: a new tool to search for highly structured β-hairpin and monomeric-α-helical peptides

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We present a combinatorial approach to evolve both, stable β-hairpin [1] and monomeric α-helix [2] in linear peptides. Starting with de novo-designed linear peptides that showed structure population below 30%, we selected in both cases four positions to build up a combinatorial library. Deconvolution of the libraries using circular dichroism reduced the library complexity to defined sequences. Circular dichroism and NMR of the defined peptides resulted in the identification of a 14-aa-long peptide (for the β-hairpin) and 17-aa-long peptide (for the monomeric α-helix) that in plain bufferes solutions showed a percentage of structure higher than 70%. Our results show how combinatorial approaches can be used to obtain highly structured peptides that then we use as templates in which functionality can be introduced.

References

[1] Pastor, M.T., López de la Paz, M., Lacroix, E., Serrano, L. and Pérez-Payá, E. (2002) *Proc. Natl. Acad. Sci. USA* (in press)
[2] Pastor, M.T., Salgado, J., Mingarro, I., Serrano, L., Ferrer-Montiel, A. and Pérez-Payá, E. (submitted)

L53 - Peptides, proteins and PNAs delivery into mammalian cells mediated by the Chariot peptide

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The transfer into living cells of macromolecules, which monitor or modify molecule-specific intracellular processes, provides an efficient way to study the temporal or spatial regulation of protein systems that underlie basic cellular functions. Several methods have been developed for this purpose. Each of the methods has its characteristic advantages and disadvantages with respect to cell viability, transfer efficiency, general applicability and technical requirements. Up to date, none of these techniques (microinjection, electroporation, membrane permeabilization by using chemical reagents or bacterial toxin) are of sufficient flexibility to cover all possible applications and demands of macromolecule delivery into living cells. In order to overcome these problems, we have designed a short amphipathic vector peptide, called Chariot, which efficiently delivers a variety of peptides and proteins including antibodies, into several cell lines in less than 1 hour, without the need of prior chemical cross-linking or denaturation steps. Chariot forms stable complexes with peptides and protein through non covalent interactions and promotes their delivery into cells in a fully biologically active form. In addition, this peptide vector presents several advantages for protein therapy, including stability in physiological buffer, lack of toxicity and sensitivity to serum. Chariot has been now extended to new delivery applications and allowed the rapid and efficient transfer of PNA (Peptide Nucleic Acid) into mammalian cells. In this study, we used an PNA antisens targeting the RNA messenger of cyclin B1, a protein required for the G2/M transition of the cell cycle. The antisens effect was monitored by FACS analysis of treated cells which were arrested in the G1 phase and the inhibition of the mRNA expression as well as the corresponding protein was controlled by northern and western blot analysis. We believe that the Chariot technology would be extremely profitable for screening novel therapeutics peptides, proteins and PNA *in vivo* and constitutes a very promising approach which overcomes the mean drawback encountered with current strategies.

L54 - Conceptual expansion of protein internalization mediated by arginine-rich peptides

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Basic peptides such as HIV-1 Tat-(48-60) and *Drosophila* Antennapedia-(43-58) have been reported to have the ability to translocate through the cell membranes and to bring exogenous proteins into the cells. Although these peptides are highly basic and rich in arginine or lysine residues, they are able to cross the cell membranes to reach the nucleus within a few minutes. It was reported that these peptides even internalized at 4 °C where a typical endocytosis pathway should be considerably suppressed. We have found that various arginine-rich peptides, including such peptides as HIV-1 Rev-(34-50), flock house virus (FHV) coat protein-(35-49) as well as octaarginine $(Arg)_8$, have a translocation activity very similar to Tat-(48-60) [1]. Using $(Arg)_n$ (n=4-16) peptides, we also demonstrated that there would be an optimal number of arginine residues (n~8) for the efficient translocation. Quantification and time-course analyses of the cellular uptake of the above peptides by mouse macrophage RAW264.7, human cervical carcinoma HeLa and simian kidney COS-7 cells revealed that Rev-(34-50) and $(Arg)_8$ had a comparable translocation efficiency to Tat-(48-60) [2]. Internalization of Tat-(48-60) and Rev-(34-50) was saturable and inhibited by the excess addition of the other peptide. Typical endocytosis and metabolic inhibitors had little effect on the internalization. These results strongly suggest the presence of a ubiquitous internalization mechanism for arginine-rich oligopeptides. Here, we will discuss the translocation mechanisms of these peptides and introduce our new approaches of intracellular protein delivery using arginine-rich peptides.

References
[1] Futaki et al., *J. Biol. Chem.*, 276, 5836-5840 (2001)
[2] Suzuki et al., *J. Biol. Chem.*, 277, 2437-2443 (2002)

L55 - A glycopeptide detecting autoantibodies in multiple sclerosis: from a diagnostic kit toward a selective therapeutic strategy

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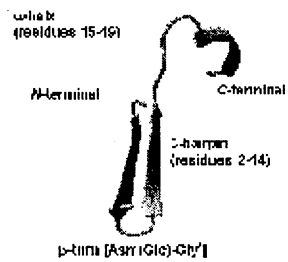
In most of the autoimmune diseases and particularly in Multiple Sclerosis (MS), an inflammatory, demyelinating disease of the central nervous system, the antigens responsible of the pathogenesis of the autoimmune response are not completely recognized. The difficult identification may be due to post-translational modifications (such as lipidation and/or glycosylation) present in protein antigens. We previously reported that the introduction of the simple β-D-glucopyranosyl moiety on an Asn residue (but not on Ser or Hyp) at position 31 of the immunodominant epitope hMOG(30-50) (unique site of glycosylation of MOG, one of the putative autoantigens for MS) led to the first synthetic antigen able to identify autoantibodies (auto-Abs) in a high percentage of MS patients. The Ab titre correlates with disease activity in sequential studies of MS patients.
NMR analysis in water/hexafluoroacetone solution showed limited conformational differences between the immunologically active glycosylated peptides and their inactive unglycosylated counterparts. Thus, the specific auto-Ab recognition is most likely driven by direct interactions of the Ab binding site with the Asn-linked sugar moiety and not with the MOG peptide sequence. Our data let us to assess that in MS, auto-Ab recognition is strictly correlated with glycosylated and specific epitopes for Asn(Glc). Taken together, these results were relevant for a rational design by molecular modelling of the first selective Ab ligand CSF114 [Figure] to be used as a synthetic marker, in the development of a prototype diagnostic kit, based on ELISA, able to detect pathogenic auto-Abs in MS patients sera. Validation of the proposed kit is in progress. In the context of the "Regional Coordination Centre for Plasma Filtration and Immunoadsorption", since January 2002, the Hospital Unit Careggi (Firenze, Italy) has been following-up, for 12 months, 150 MS patients. Starting from these observations, we decided to move toward the development of an innovative immunotherapy based on Therapeutic Plasma Filtration (TPF). TPF is a method for removing unwanted substances from the blood, such as Abs. The ability to remove Abs from the blood, using nonspecific Protein A columns, has led to the use of TPF as a therapy for neurological conditions in which autoimmunity is believed to play a role. Up to now, a specific TPF was never undertaken for MS, because no specific antigen was recognized. For that reason the optimised glycosylated antigen anchored to a biocompatible resin in specific immunoaffinity columns will be used in a TPF for patients affected by MS, to block the detected auto-Abs with a pathogenetic role.



L56 - Substance P conversion to bioactive fragments - an important pathway for the neuromodulatory effect of the undecapeptide

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The biosynthesis and inactivation processes of neuroactive peptides are shown to include several enzymatic steps. In addition to enzymatic processing and degradation several neuropeptides have been shown to undergo enzymatic conversion to fragments with retained or modified biological activity. This has been shown for e.g. opioid peptides, tachykinins as well as for peptides belonging to the angiotensin system. Sometimes the released fragment shares the activity of the parent compound. However, in many cases the conversion reaction is linked to a change in the receptor activation profile, i.e. the generated peptide fragment is shown to act on and stimulate a receptor not recognised by the parent peptide. This presentation describes a typical example of this, namely the tachykinin substance P (SP), which is shown to be converted to the bioactive fragment SP(1-7), a heptapeptide which mimic some but opposes other effects of the parent peptide. SP(1-7) may be generated by the action of substance P endopeptidase (SPE) or by neutral endopeptidase (NEP). Studies have shown that SP exhibits binding preference for the NK-1 receptor, which is not recognised by SP(1-7). SP is known to facilitate the influx of pain signals by acting at the spinal level, while SP(1-7) exhibit antinociceptive effects. In peripheral inflammation, using a rat model, SP is shown to induce vasodilatation, an effect, which according to our present result is opposed by SP(1-7). In morphine dependent mice SP is shown to potentiate the withdrawal reaction, whereas the heptapeptide is shown to attenuate this response. In this presentation we also show that SP(1-7) attenuates several signs of opioid withdrawal also in rats. We further found that the SP fragment affects both glutamate and dopaminergic pathways in brain areas related to the functional anatomy of opioid reward. It thus seems that the activity of SP can be modulated by its bioactive SP(1-7) fragment, which can be formed through enzymatic conversion of the parent peptide. This observation appears to represent an important regulatory mechanism that is shown to occur also among many neuroactive peptides, a phenomenon generally not acknowledged.

L57 - ITF1697, an agent with novel specific anti-ischemic properties

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ITF1697 [G(Et-N_α)KPR] is a modified tetrapeptide whose sequence derives from one of the degradation products of human C-reactive protein, the prototype acute phase reactant. ITF1697 has increased stability *in vivo* ($t_{1/2}$ in humans, 1.8 hr) and is well tolerated in toxicity studies (mice, rats and monkeys) and in Phase I clinical trials. ITF1697 is extremely efficacious in experimental models of myocardial ischemia (I) and ischemia and reperfusion (I&R). For example, in rats subjected to permanent regional ischemia, the drug (1-10 μg/kg, i.v. bolus) reduced dose dependently the duration and incidence of rhythm disturbance and mortality (8% vs 50% in controls). In acute myocardial infarction in the rabbit, ITF1697 (25-100 μg/kg/hr, i.v. infusion) reduced mortality by 80% and restored ECG traces to normal. In a further model of canine I&R, the peptide (50 μg/kg/hr, i.v. infusion) reduced the degree of myocardial necrosis and neutrophil infiltration of the myocardium by 50% and dramatically improved global myocardial contractility indicating restoration of left ventricular function.
Myocardium protection by ITF1697 is due to inhibition of microvasculature damages as shown by intravital microscopy of the hamster cheek pouch microcirculation subjected to I&R. In this model, ITF1697 (0.1-100 ug/kg/hr, iv infusion) completely prevented capillary occlusion and blood cell plugging. It also maintained unaltered the structure of capillaries and strongly inhibited microvasculature permeability, the adhesion of leukocytes to the endothelium and the expression of adhesion molecules. In this model, ITF1697 was well tolerated (absence of bleeding) and more efficacious than therapeutic doses of Reopro or Integrilin, two RGD-based anti-platelet drugs. Thus ITF1697 protects tissue microcirculation and has a novel mechanism of action. A Phase II trial evaluating its efficacy in primary percutaneous transmural coronary angioplasty has been recently completed (Dec. 2001).

L 58 - Helicomimetic cyclic peptides that inhibit steroid receptor coactivator interactions represent a novel approach for transcriptional regulation

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The discovery of a recurring LXXLL motif among more than 20 nuclear coactivators has suggested an alternative approach for the control of steroid-mediated transcription. Such control might provide an alternative therapy for such diseases as breast and prostate cancer. Since the productive binding of the LXXLL-containing coactivator proteins to the ligand binding domain (LBD) of the nuclear receptor involves a helical peptide, we investigated various types of side chain to side chain cyclic peptides to stabilize that conformation while retaining the inhibitory activity of some linear parent peptides. We found that disulfide bridged peptides were more active than lactams, and the best appear to contain D-Cys^{i} to L-Cys^{i+3} bridges. In fact one of our best analogs, an octapeptide, displays a K_i of 12 nM, compared to a K_i of 720 nM for a standard linear 14-mer peptide. Additional SAR studies have been performed in which ring size has been varied using homocysteines in place of cysteine, and conformational constraints have been introduced through D and L penicillanine replacements. An X-ray structure of one of our most potent analogs ("PERM-1") with the estrogen receptor LBD confirm the helical nature of the bound peptide analog. In contrast, the solution structure as assessed by NMR and CD studies suggests only at best a partially helical conformation. These compounds are being optimized for improved transport and delivery to the nuclear compartment and could represent an alternative approach to cancer therapy, in place of standard steroid antagonists such as tamoxifen or raloxifen.

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L59 - Selective inhibition of the chymotrypsin-like activity of the 20S proteasome by 2-aminobenzylstatine derivatives

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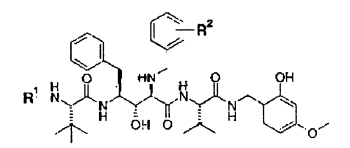
The proteasome is an intracellular multicatalytic protease complex that is involved in the ubiquitin (Ub)-dependent and Ub-independent degradation of proteins implicated in critical intracellular regulatory cascades (e.g. mitotic cycle, cell growth and viability, antigen presentation or inflammatory response). The proteolytic activity of this enzyme occurs in a 700-kDa barrel-shaped core structure known as the 20S proteasome, which consists of four stacked rings arrayed in an $\alpha_7\beta_7\beta_7\alpha_7$ manner. Substrate specificity studies have revealed at least three distinct peptidase activities for the 20S proteasome: chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolytic (PGPH) activities. Our specific target in the search of novel cytotoxic and antiproliferative agents is the chymotrypsin-like activity of the 20S proteasome. Modulation of this enzymatic activity by β-subunit-specific proteasome inhibitors may convey an anti-tumor effect by induction of cell cycle arrest and apoptosis in tumor cells. Early inhibitors of the 20S proteasome were relatively non-specific compounds and used for *in vitro* studies of the ubiquitin/proteasome-dependent degradation pathway. The inherent drawbacks of these covalent inhibitors (e.g., non-target specific, too reactive or unstable) prompted us to search for alternative subunit-specific proteasome inhibitors. Recently, we have described the identification by high throughput screening (HTS) of our in-house compound archive of a series of 2-aminobenzylstatine derivatives that inhibit non-covalently the chymotrypsin-like activity of the 20S proteasome in an *in vitro* enzyme assay (e.g. compound 1; Figure 1). This new structural class shows good selectiviy over the trypsin-like and post-glutamyl-peptide hydrolytic activities of the 20S proteasome (for compound 1, $IC_{50} > 20$ μM). We have utilized a structural model of the human proteasome in complex with a 2-aminobenzylstatine derivative to guide the optimization process of these β-subunit-specific proteasome inhibitors. In this communication, we shall present our medicinal chemistry strategy that has resulted in a further improvement in *in vitro* 20S proteasome inhibition (e.g. compound 2, Figure 1). The modular modifications introduced have no effect on the 20S proteasome specificity profile of the inhibitor. Compound 2, which is one of the most potent derivatives identified, still shows good selectiviy over the trypsin-like and post-glutamyl-peptide hydrolytic activities of the 20S proteasome ($IC_{50} > 20$ μM).



Entry	R^1	R^2	IC_{50} (μM)
1	CO_2CH_2Ph	4-OCH_3	0.9
2	C(O)CH-(1 Naphthalene)	3,4,5-$(OCH_3)_3$	0.007

Fig. 1 - Representative examples of 2-aminobenzylstatine derivatives and inhibition of the chymotrypsin-like activity of the 20S proteasome.

L60 - Design, synthesis and activity of chloromethyl ketone inhibitors of Gingipains

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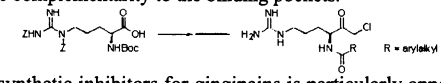
Periodontal diseases represent a group of infection associated with chronic inflammation of the gingiva, destruction of the periodontal tissue and alveolar resorption, eventually leading to exfoliation of teeth. *Porphyromonas gingivalis*, a Gram-negative, anaerobic bacteria has been implicated as the essential etiologic agent of this pathology. It secretes high level of cysteine proteases, referred respectively as gingipains R and K. Gingipains act as key virulence factors of *P. gingivalis* degrading many proteins of human connective tissue and plasma, and thus disrupting a number of normally tightly controlled pathways [1]. The most effective strategy in construction of inhibitors for cysteine proteases is synthesis of peptidyl substrate analogues bearing a moiety able to bind covalently to the thiol residue in the active site and thus to block its crucial function in the process of hydrolysis. Chloromethyl ketones, diazomethyl ketones, fluoromethyl ketones, acyloxymethyl ketones and epoxides belong to this type of irreversible cysteine inactivators [2]. The question of high specificity and affinity towards the certain enzyme can be addressed to the computer-aided design of the side chain substituents exhibiting steric and electrostatic complementarity to the binding pockets.



The design of synthetic inhibitors for gingipains is particularly appealing since these proteases are not inactivated by host inhibitors and in fact degrade such proteins. Their properties are unique within the cysteine protease family and due to this specificity they represent the promising targets for the development of novel types of antibiotics [3]. In this work we describe the results of our study on chloromethyl ketones derivatives of lysine and arginine. The study has been undertaken in order to evaluate the usefulness of computer-aided procedures for the development of new inhibitors of gingipains. The design of the compounds, their syntheses and activity are presented, the limitations of the synthetic methods and stability of the compounds are discussed.

References
[1] Genco, C. A.; Potempa, J.; Mikołajczyk-Pawlińska, J.; Travis, J. *Clin. Infect. Dis.* 1999, *28*, 456-465. Graves, D.; Jiang, Y.; Genco, C. A. *Curr. Opin. Infect. Dis.* 2000, *13*, 227-232.
[2] Otto, H. H.; Schirmeister, T. *Chem. Rev.* 1997, *97*, 133-171. Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* 2000, *43*, 305-341.
[3] Travis, J.; Potempa, J. *Biochim. Biophys. Acta*, 2000, *1477*, 35-50. Supuran, C. T.; Scozzafava, A.; Mastrolorenzo, A. *Exp. Opin. Ther. Patents*, 2001, *11*, 221-259.
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L61 - Inhibitors of aminopeptidase P incorporating β-amino acids

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Aminopeptidase P – APP (EC 3.4.11.9) is a metallopeptidase which is present in tissues, particularly the brushborder membranes of kidneys and lungs. The precise role of APP is not clear but it is thought to be involved in cardiovascular function due to its ability to inactivate bradykinin, a potent vasodepressor and cardioprotective peptide. To confirm the function of APP, there is a need for specific and potent inhibitors.
Recent reports suggest that incorporation of β-amino acids in a peptide sequence stabilises the scissile peptide bond preventing cleavage by enzymes. -amino acids are similar to -amino acids with the exception of an additional carbon atom in the -amino acid backbone. The increase in the length of amino acids enables the scissile bond to move away from the active site of the enzymes. In this study, a range of peptides incorporating -amino acids were synthesized and tested for inhibitory activity against the bacterial form of APP. This was chosen as the initial target, since bacterial APP has similar substrate specificities to mammalian APP and the x-ray crystal structure is known and can be used for modeling studies.
All peptide inhibitors displayed competitive inhibition against the cleavage of bradykinin. -amino acid incorporations into certain peptides resulted in enhanced inhibitory potency, with some compounds exhibiting nanomolar Ki's. Molecular modelling suggested that the more potent compounds are accommodated more closely at the active site. These inhibitors can now be used to further define the physiological role of mammalian APP and perhaps in the long term act as lead molecules in the development of therapeutic agents in the treatment of cardiovascular diseases.

L62 - Miraziridine A: a three-in-one protease inhibitor

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Marine organisms represent an interesting source for biological active compounds. Recently, the isolation and structure elucidation of miraziridine A (Figure 1), a tetrapeptide from the marine sponge *Theonella* aff. *mirabilis* has been reported [1]. The structure analysis revealed the presence of three elements that possibly could inhibit proteases belonging to three different classes: (i) cysteine proteases are irreversibly inhibited by (2*R*,3*R*)-aziridine-2,3-dicarboxylic acid as well as by the α,β-unsaturated carbonyl moiety present in the vinylogous arginine, (ii) aspartyl proteases are inhibited reversibly by statine as transition state analog, and (iii) trypsin-like serine proteases should be inhibited by the vinylogous arginine as a backbone mimic of arginine residues. Therefore, among the great variety of protease inhibitiors isolated from natural sources this particular tetrapeptide appears to be unique due to its putative three functions in one molecule.
To analyse the features of this interesting compound, miraziridine A was synthesized in solution by a convergent strategy. The analytical data for the synthetic miraziridine A are in quite good agreement with those reported for the natural product. The inhibitory properties of the synthetic miraziridine A against representative cysteine-, aspartyl-, and serine proteases will be discussed.

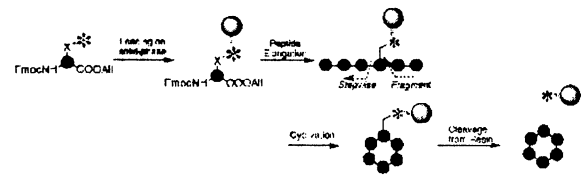

Fig. 1 - Structure of Miraziridine A

Reference
[1] Nakao, Y., Fujita, M., Warabi, K., Matsunaga, S. and Fusetani, N. *J. Am. Chem. Soc.* 2000, *122*, 1046210463.

L63 - Novel synthetic approaches to potent HIV-1 protease inhibitors and their prodrugs

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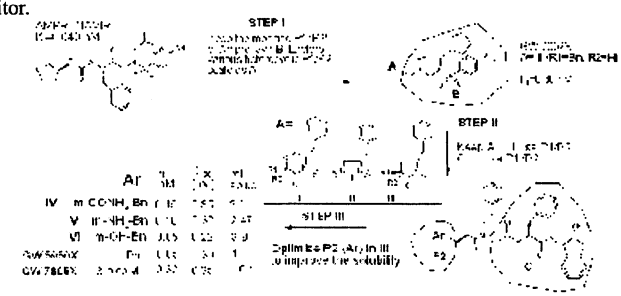
The Highly Active Antiretroviral Therapy (HAART), which employs cocktails of reverse transcriptase and HIV-1 protease inhibitors (PI) such as Amprenavir and Indinavir, has dramatically reduced the number of AIDS-related deaths. Nonetheless, there is a continued need for new PIs with improved potency, viral resistance, safety profile and solubility (and thus decreased pill burden). Along these lines, GlaxoSmithKline and Vertex laboratories have recently disclosed potent protease inhibitors GW 7652X and GW 5950X, both hybrids of Bn-pyrrolidone III as a P1/P2 moiety and of P1'/P2' moieties of Amprenavir and Indinavir (*Bioorg. Med. Chem. Lett.* 1998, 8, 3637-3642; *Bioorg. Med. Chem. Lett.* 2000, 10, 1159-1162). In an effort to further optimize these novel PIs, we have developed novel synthetic methodologies towards mono-, di-substituted and spiro I, as well as towards the related scaffolds I-II. Initially (STEP I), the newly synthesized scaffolds I-III were coupled with the Amprenavir-like P1'/P2' fragment B, providing many potent PIs such as the sub-nanomolar GW 7652X. Next (STEP II), optimization of the P1'/P2' moiety, while retaining the best P1/P2 scaffold identified from STEP I (III, R1=Bn, R2=H), allowed us to identify GW 7805X, a potent hybrid of III and of the P1'/P2' moiety of Indinavir (C). Step III was focused on improving the compound solubility, which reduces the pill burden, while maintaining the potency. We discovered that meta-substituted Bn-III or 3-picolyl, in combination with C gave potent and soluble PIs, exemplified by IV-VI and the GW 7805X. This presentation will focus on new synthetic approaches to peptide mimetics I-III, and properties of new PIs, such as GW 7805X, which uniquely combine both high potency and improved solubility. This unexpected property will be rationalized based on the structure of HIV-1 protease co-crystallized with the inhibitor.



L64 - Structural determinants of antiviral and signaling functions in the CC chemokine RANTES: generation of HIV-1 inhibitory peptides with anti-inflammatory properties.

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Selected chemokines act as natural HIV antagonists due to their ability to block or downregulate critical viral coreceptors, such as CCR5 or CXCR4. Due to the inherent difficulties in obtaining structural information on G-protein-coupled receptors, an alternative strategy for the rational design of effective inhibitors is based on the fine mapping of the HIV-inhibitory determinants of their chemokine ligands. The primary determinants of CCR5 recognition and anti-HIV function of RANTES, the most effective anti-HIV CC-chemokine, were identified by peptide scanning. Synthetic peptides derived from the N-loop region not only blocked HIV-1 envelope-mediated cell fusion and infection in a dose-dependent fashion, but also antagonized RANTES-elicited chemotaxis, thus formally demonstrating that the antiviral and signaling functions can be uncoupled. Alanine scanning mutagenesis of full-length recombinant RANTES confirmed that the N-loop contains critical residues for the antiviral and receptor-activating functions. Structural analysis showed that such residues participate in the formation of a large, solvent-exposed hydrophobic surface that also includes a β1-strand cluster of aromatic residues. To validate the hypothesis that this hydrophobic patch is the major receptor interface of RANTES, we generated peptides encompassing both the N-loop and the β1-strand aromatic cluster. Such peptides were significantly more effective than peptides based on the N-loop alone. The mapping of the HIV-inhibitory determinants was further refined by alanine scanning of the most effective N-loop/β1-strand peptide. Interestingly, we found that our peptides are biologically active only in a dimeric form, which is consistent with the prevalent molecular form of the complete chemokine at physiological pH. Of relevance for the development of potential therapeutic approaches, a retroinverted RANTES peptide mimetic, based on D-amino acids and thus resistant to protease-mediated digestion, maintained both HIV- and chemotaxis-antagonistic functions. These data open new perspectives for the rational design of effective HIV antagonists.

L65 - Rational engineering of a CD4 mimic, powerful inhibitor of HIV-1 infection and potential component of an AIDS vaccine

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The human immunodeficiency virus type-1 (HIV-1) avoids immune surveillance by exposing hypervariable and heavily glycosylated regions on its exterior envelope, while the conserved surfaces used to bind entry cellular receptors are either in recessed cavities or exposed only after binding to CD4, the main entry receptor. Although the structure of CD4 and the HIV-1 envelope gp120 glycoprotein in complex with CD4 have been reported, they have not been exploited yet for the development of new HIV-inhibitory molecules. We have exploited such structural information and rationally designed a CD4 miniprotein (27 amino acids long) that mimics the core of the CD4 site interacting with gp120 and displays a native-like affinity in gp120 binding, nanomolar antiviral potency and effective exposure of cryptic conserved gp120 epitopes to neutralizing antibodies. Engineering was structure-guided. First, the functional β–hairpin 35-46 of CD4 was selected for being reproduced, since this region is central in gp120 binding. Then, a small scorpion toxin, scyllatoxin, presenting a structurally similar β–hairpin was selected as host scaffold, and eight solvent-exposed residues of the CD4 β–hairpin were introduced at homologous positions in the host mini-protein scaffold. The chimeric mini-protein, CD4M3, was produced chemically and found to specifically inhibit the binding of CD4 to gp120 with 40 μM IC_{50}, by competitive ELISA. Next, the CD4M3 miniprotein was improved by increasing the structural and functional resemblance of its binding site with that of CD4. An NMR study and Ala-scanning approach suggested five mutations that were incorporated in a new CD4M9 miniprotein, resulting in a 100-fold increase in gp120 binding affinity. Finally, a potent mini-CD4 with *bona fide* CD4-like properties was designed by optimizing miniprotein interactions with the viral envelope gp120 protein. Thus, the three-dimensional structure of the CD4M9 miniprotein was determined by NMR, and a model of the miniprotein in complex with gp120 was computed by using the crystallographic structure of the gp120-CD4 complex as a template. Analysis of the computed complex suggested additional five mutations that were incorporated into an optimized mini-protein, CD4M33, which exhibited a further 100-fold increase in gp120 affinity to 4 nM Kd, identical to that of native sCD4. The optimized CD4M33 miniprotein, by binding to the exterior HIV-1 envelope with high affinity, potently inhibits a broad range of HIV-1 isolates, including primary CCR5-tropic and dual-tropic patient isolates that are generally resistant to inhibition by soluble CD4. In addition, the optimized CD4 miniprotein unmasks conserved epitopes of HIV-1 envelope that are cryptic on the unbound envelope glycoprotein, suggesting that immunization with such miniprotein - gp120 complex should elicit broad-spectrum neutralizing antibodies. Thus, this mini-CD4 could be exploited as a component of novel vaccine formulations, in association with envelope proteins. The use of the CD4 miniprotein should also avoid potential autoimmune phenomena that may be associated with the use of human CD4 for complex formation. This work demonstrates that it is possible to rationally engineer a miniprotein with desired biochemical properties that may be useful for the treatment or the prevention of a major human disease.

L66 - Development of focused tyrosine kinase inhibitory library and testing against MDR

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Signal transduction therapy has become recently a leading area of modern drug research aiming to inhibit and modulate the pathomechanism based validated target molecules in intercellular and intracellular signaling. Tyrosine kinases are essential components of the phosphorylation-dephosphorylation cascade which plays key role in the signaling of cell proliferation. Therefore search for novel tyrosine kinase inhibitory lead compounds has got into the front line of antitumor drug research worldwide, and quite a few (more than thirty) such compounds are in clinical development phase now. We have designed and synthesized a large set of genuinly new potential PTK inhibitors which showed inhibitory activity on EGFRTK, PDGFRTK, HER2, IGF and VEGF. Most of the synthesized compounds were designed like ATP analogues, several structures resembles to known small peptide substrate analogues. The compounds were synthesized in solution phase by parallel synthesis. Structure validation and quantitive determination was made by spectroscopic methods (NMR, IR, LC-MS). The compounds were tested in biochemical and cellular assays and we have found that in several cases the difference in the activity in the cellular and biochemical assays can not be explained only with the differences in bioavailability ("ADME parameters"). We have checked several newly developed tyrosine kinase inhibitors from the literature (e.g. pyrrolo-pirimidines, quinazolines and Gleevec) as well and got similar results. In order to explain these unpredictable differences in activity, we have studied the interaction of these molecules with multidrug transporter proteins. These plasma membrane glycoproteins are present in phisiological barriers and in a large variety of tumor cells which can alter the bioavailability of tyrosine kinase inhibitors both at the systemic and the cellular levels. The two key proteins, MDR1 (P-glycoprotein) and MRP1 (multidrug resistance protein 1) were used in our studies since these are believed to be involved in cancer multidrug resistance. We have measured the MDR1 and MRP1 ATP-ase activity in isolated insect cell membranes and determined the multidrug resistance activity of the selected tyrosine kinase inhibitors in a calcein accumulation assay. We have found that several tyrosine kinase inhibitors examined show a direct interaction with MDR1 and MRP1 with large variability in the transporter selectivity and specificity. We could not correlate the interaction with common physico-chemical properties like lipophilicity therefore we suggest that high-throughput enzyme assays and cellular assays with the multidrug transporters should be used in the early drug development phase in order to predict the possible clinical resistance based on this mechanism.

L67 - Conformationally constrained epitope mimics of the HIV gp41 envelope protein

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New treatments and an effective vaccine are greatly needed against infection from Human Immunodeficiency Virus (HIV). The HIV envelope glycoproteins in particular, gp120 and gp41, which mediate the viral fusion process, are important targets for both therapy and prophylaxis. They are the target of neutralizing Abs and therefore have a key role for a humoral component in a vaccine. However eliciting a broadly protective humoral response has proven so far challenging because very little of the envelope spike surface appears accessible to antibodies and the immunogenicity is low. Binding of gp120/gp41 to the cell-surface receptor CD4 and to a coreceptor, such as CCR5 or CXCR4, triggers a series of conformational changes in the gp120/gp41 oligomer that ultimately lead to formation of a trimer-of-hairpins structure in gp41. In this structure, the so-called pre-fusion intermediate, the amino- and carboxyl-terminal regions of the protein are brought into close proximity, causing the viral and cellular membranes to merge. Drugs were developed to interfere with this rearrangement, some of which are already in clinical trials. The conformational changes in gp41 also result in the exposure of regions, which were completely buried in the viral spike which are promising targets for a neutralizing antibody response. During the natural infection these gp41 epitopes may be present for a too short time to raise a protective humoral response. We have now designed conformationally constrained epitope mimics by grafting gp41 sequences onto small protein domains and presentation scaffolds. These epitope mimics represent portions of gp41 in the pre-fusion intermediate. We used near-UV CD and NMR to establish that our molecules display excellent *structural* mimicry to the conformational gp41 epitopes. Moreover, a subset of the mimetics showed antiviral activity.
We believe that this approach will be key to overcome the intrinsic inefficiency of the humoral response during the HIV natural infection.

L68 - Lead structure for active immunisation against Alzheimer's Disease (AD) upon elucidation of a plaque-specific epitope recognised by therapeutically active antisera from transgenic AD mice

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Immunisation of transgenic mouse models of Alzheimer's disease using amyloid-beta peptide ($A\beta_{42}$) reduces both the Alzheimer's disease-like neuropathology and impairments in the spatial memory in these mice. The mechanism underlying these improvements remains unclear. We have recently found that antibodies generated during therapeutically effective immunisation with $A\beta_{42}$ inhibit Aβ-fibril formation, disaggregate preformed fibrils, and abrogate *in vitro* cell death elicited by Aβ. Using selective proteolytic digestion of the antigen from the immune complex (epitope excision) in combination with high resolution Fourier-transform ion cyclotron resonance-mass spectrometry (FTICR-MS), we have identified the active, plaque-specific epitope [1]; the epitope structure isolated and identified from mouse plaque supernatant and pellet, and from synthetic $A\beta_{42}$ is identical. Mass spectrometric analysis of Aβ-peptides from plaques confirm the presence of $A\beta_{42}$ with highest abundance, with ca. 20 % and 5 % of $A\beta_{40}$ and $A\beta_{38}$, respectively. These results suggest that antibodies to the specific recognition structure are effective modulators of Aβ-induced neurotoxicity; they also demonstrate the efficiency of mass spectrometry as a molecular tool for elucidation of epitopes. Knowledge of the mechanism and the Aβ epitope recognised by therapeutically active antibodies provides the basis for the design of molecular mimics for vaccination therapy; of specific immunisation antigens that minimise side effects induced by antibodies recognising other domains of the amyloid precursor protein (APP); to create human monoclonal antibodies with antigen-binding sites designed to recognise only this specific epitope of Aβ. Based on this epitope we have synthesised and evaluated lead structures for AD immunisation, the biochemical properties of which are completely decoupled from Aβ toxicity.

L69 - Specific blocking of anti-idiotypic antibodies for unmasking the anti-La/SSB response in Sjogren's syndrome patients

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Autoantigen La/SSB is molecular target of humoral autoimmunity in patients with primary Sjogren's Syndrome (pSS) and Systemic Lupus Erythematosus (SLE)[1]. Synthetic peptide analogues of its major antigenic determinants were prepared (pep). Based on the "molecular recognition" theory [2], complementary peptides (cpep), derived from anti-parallel readings of the non-coding strand of La/SSB DNA encoding its antigenic determinants, were constructed (Fig. 1). These peptides specifically recognized by anti-La (+) sera and by isolated anti-cpep specific IgG, were subjected to enzymatic degradation with pepsin. Anti-cpep F(ab')2 were found to specifically recognize a common idiotype (Id), located within or spatially close to the antigen combining site of anti-La/SSB (anti-pep) antibodies. Homologous and cross-inhibition experiments further confirmed this relation. Immunizations of BALB-c mice with pep led to the development of anti-pep followed by the production of anti-cpep antibodies 10 days later and vice-versa. The anti-idiotypic antibodies were also found to mask anti-La/SSB antibodies (91% inhibition). A procedure to overcome this anti-idiotypic interference in La/SSB detection was developed: upon heating of the sera for dissociation of the Id – anti-Id complexes, the anti-Id antibodies were blocked with cpep and the anti-La/SSB reactivity was recovered. Application of this method to anti-Ro positive/anti-La/SSB "negative" sera showed that virtually all the anti-Ro/SSA positive autoimmune sera also possess anti-La/SSB antibodies.

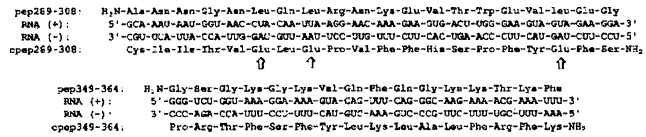

Fig. 1 – Design of complementary peptides cpep289-308 and cpep349-364 by the antisense approach.

References
[1] Sakarellos-Daitsiotis, M., Tsikaris, V., Sakarellos, C., Vlachoyiannopoulos, P.G., Tzioufas, A.G., Moutsopoulos, H.M., *Vaccine*, 2000, 18, 302.
[2] Araga, S., Xu, L., Nakashima, K., Vilain, M., Blalock, J.E., *FASEB J.*, 2000, 14, 185.

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L70 - Matrix-scan as effective tool to map discontinuous epitopes

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Biological life is dependent on specific recognition of molecules by other molecules, which are often proteins. The specific interaction-site (epitope) between proteins is composed of 7-30 amino acids. A continuous stretch of amino acids is called a linear epitope, however, the majority of epitopes are composed of amino acids that are close in space, but far apart in the linear amino acid sequence. These epitopes are referred to as discontinuous epitopes. Here we describe a systematic tool for mapping discontinuous epitopes. The concept behind this socalled Matrix-Scan is to use peptide constructs that mimic discontinuous interaction sites. A single part of an interaction site usually does not have sufficient affinity to the binding partner to allow its detection. Therefore, the different parts of the discontinuous interaction site were combined into one molecule. The Matrix-Scan uses large arrays of peptide constructs that contain all combinations of two or more sequences derived from the protein antigen, that are covalently linked in all kinds of spatial orientations. These arrays contain the peptide constructs that combine the different parts of the interaction sites that are normally (far) apart in the primary sequence. As an example all possible 32.761 (combination of all 13-mers:181*181) overlapping 26-mer peptide constructs of human Follicle Stimulating Hormone (hFSH) were synthesized and screened with antibodies. In short, peptides containing a bromoacetyl group were coupled to a thiol functionalised polypropylene surface to form a stable thioether bond. To these surface-coupled peptides all peptides were coupled again through spotting with gridding pins. The whole matrix-scan was incubated with anti-hFSH antibodies. After washing an incubation of a second anti mouse antibody conjugated to alkaline phosphatase was performed. After washing the bound enzyme caused fluorescent product signals which were quantified. Figure 1 shows a detail of all 32.761 hFSH 26-mer peptide constructs that were tested.

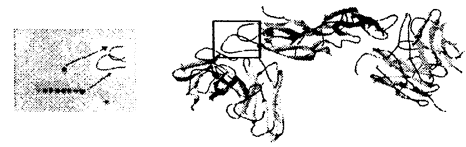

Fig. 1. Example of an identified discontinuous epitope of FSH. The left part shows a small part of the whole Matrix-Scan with positive spots of 26-mer construct peptides corresponding to a discontinuous epitope (the spot right below is a control), covering the hFSH-β regions IAIEKEECRFCI and VYETVRVPGCAHHADSLYTYPVATQ. The right part shows the X-ray structure of the similar protein human choriogonadotropin (hCG) bound to two different antibodies. Our results on hFSH nicely match with this homologous discontinuous epitope (in square) on hCG [1].

References
[1] Tegoni, M; Spinelli, S; Verhoeyen, M; Davis, P; Cambillau, C. (1999). *J.Mol.Biol.* 289: 1375-85.

L71 - Lipid-core-peptides for vaccination; structure-activity relationship.

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Synthetic peptides require immunopotentiation if they are to be effective vaccines. Currently available vaccine adjuvants are associated with various problems, including toxicity, hypersensitivity and short-term effects. We have developed vaccine delivery systems, which offers a possibility for immunisation without the need of a traditional adjuvant. The systems are based on lipoamino acids coupled to multiple copies of peptides via polylysin (LCP) or carbohydrate (LCI) cores. These systems represent carriers, which highly versatile in their physio-chemical and biological properties (increased lipophilicity and membrane-binding effects). Outstanding immunological data were obtained applying the systems in many cases. The immunogenicity was found to be much greater for synthetic peptides from Group A Streptococci (GAS) incorporated into the systems, than when given with Freund's adjuvant. We have previously demonstrated complete protection of mice from systemic GAS infection following immunisation with an M protein-based multi-epitope vaccine construct.[1] The efficacy of the construct, however, required administration in CFA, which is not suitable for human use. This current study has investigated the LCP system as an alternative delivery strategy for a GAS vaccine, and has demonstrated the immunogenicity of different GAS-based LCP constructs in mice. Moreover, our data demonstrated the immunogenicity of these constructs in the absence of any additional adjuvant. These findings indicate the potential use of the adjuvant/carrier systems in the delivery of a synthetic GAS vaccine with self-adjuvanting properties, with a view to the development of a mucosal-based vaccine for human application.

Reference
[1] Brandt, E.R.; Sriprakash, K.S.; Hobb, R.I.; Hayman, W.A.; Zeng, W.; Batzloff, M.R.; Jackson, D.C.; Good, M.F. Nature Medicine 2000, 6, 455-9.

L72 - Mobility, flexibility and inhibition of peptide bond formation

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The atomic structures of the two ribosomal subunits from eubacteria, Thermus thermophilus and Deinococcus radiodurans, and of the large subunit from Haloarcula marismortui were determined at various functional states, under close to and far from physiological conditions. Further crystallographic investigations on ribosomal subunits in complexes with antibiotics, substrate analogues and cellular factors highlighted region of a high mobility, revealed the mechanisms of selected steps in protein biosynthesis, suggested how antibiotics may inhibit this process and illuminated elements that may confer selectivity for clinically relevant antibiotics. Comparative studies indicated that several functionally relevant features are extremely flexible, so that under specific conditions they become disordered, suggesting that the induction of disorder in functionally relevant features is the strategy ribosomes exploit to minimize peptide-bond formation under less optimal conditions. Among the flexible features is the perturbing arm that serves as a gate for the exiting tRNA, the region that hosts the GTPase activity, and the intersubunit bridges that are formed upon the association of the entire ribosome. One of these bridges connects the peptidyl-transferase active center in the large subunit and the decoding region in the small one, and may serve as a spanner that assists translocation of tRNA molecules between the A- and P- sites.
Analysis of the binding modes of substrate analogues rationalized the suggestion that the peptidyl transferase center serves as a template for proper positioning of tRNAs rather than participating in the catalytic events associated with peptide bond formation. The ribosomal components constructing the frame for the accurate orientation of the tRNA molecules include rigid as well as flexible RNA regions and may involve a few ribosomal proteins.

L73 - Oxidative folding process of amaranthus α-amylase inhibitor

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Oxidative protein folding comprises two main processes: i) formation of native disulfide bridges, sometimes via reshuffling and non-native intermediates, and ii) recovery of the native tertiary structure (conformational folding). We have characterised in detail the oxidative folding of a small cysteine knot protein, Amaranthus alpha-amylase inhibitor[1,2] (AAI, 32 amino acids, 3 disulfide bridges) with RP-HPLC, electron spray ionization mass spectroscopy (ESI-MS), ^{1}H NMR and photo-chemically induced dynamic nuclear polarization (photo-CIDNP).
Using the acid-trapping technique and RP-HPLC the disulfide intermediates of the folding process were isolated and their disulfide pairings determined by combining enzymatic digestion and LC-MS.[3] Five species with three non-native disulfide bonds were observed on the folding process of which the most abundant, main folding intermediate (MFI), was discovered to have a very rare structural element: a vicinal disulfide bond. The tertiary folds of the species present along the folding process have been elucidated using equilibrium and real-time NMR and photo-CIDNP[4], an NMR method, which probes the surface accessibility of the aromatic side chains (Tyr-21, Tyr-27, Tyr-28, Trp-5). The equilibrium spectra of purified species were compared with real-time spectra taken at various time intervals along the folding pathway. And the findings were consistent with RP-HPLC and ESI-MS real-time measurements.
The molecular dimensions of the native protein and MFI species were compared by performing NMR pulsed field gradient diffusion measurements. Even though native and MFI possess different disulfide pairings, their sizes are indistinguishable within the experimental error. Additional results from 2D NMR experiments led to the conclusion that the MFI state of AAI is compact and well-ordered, with one major and at least one minor conformation. The results give a comprehensive picture of oxidative folding as a contemporary search for the correct fold and the correct disulfide pairing. MFI may play a role similar to that of the molten globule state of larger proteins by constraining the peptide chain to a smaller number of conformations that can be rapidly funnelled towards the native state.

References
[1] Chagolla-Lopez, A., Blanco-Labra, A., Patthy, A., Sanchez, R. & Pongor, S. (1994) *J Biol Chem* 269, 23675-80.
[2] Lu, S. & Pongor, S. et al (1999) *J Biol Chem* 274, 20473-8.
[3] Cemazar, M. et al, in preparation (2002).
[4] Maeda, K., Lyon, C. E., Lopez, J. J., Cemazar, M., Dobson, C. M. & Hore, P. J. (2000) *J Biomol NMR* 16, 235-44.

L74 - Chemical shifts, the ultimate test of polypeptide folding cooperativity

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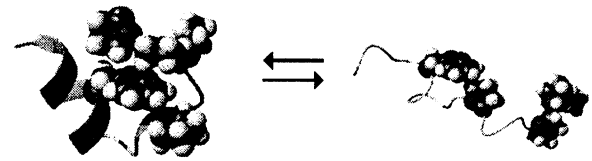
Of all NMR parameters, chemical shifts are the most exquisitely sensitive to structure. For peptides and partially folded states of small proteins, conformers are typically in rapid equilibrium, as a result chemical shifts are the linear population-weighted average over the ensemble of conformers that is sampled. Since the chemical shifts associated with fully-disordered polypeptide states (random coil reference values) have been defined, the thermodynamics and cooperativity of structuring equilibria can be assessed from the magnitude and temperature dependence of chemical shift deviations (CSDs) associated with structuring. Examples illustrating these principles will be drawn from studies of peptide structuring transitions (the thermal stability of local hydrophobic clusters; α-helix, β-hairpin and 3-stranded β sheet formation), miniprotein fold optimization, and other efforts to assess the cooperativity of folding for polypeptides in the peptide/protein borderlands. Although the random-coil/α-helix transition is the most thoroughly studied structuring phenomenon of peptides, experimental measures of cooperativity are lacking and the driving force for helix formation in short peptides is ill-defined. We have employed temperature and solvent effects on both Hα and ^{13}C=O CSDs to obtain new insights into the local and global effects of N- and C-capping and the effects of hydrophobic residue substitutions. For designed β-hairpins and β-sheets, chemical shift analysis (using HN and Hα CSDs) can provide unambiguous measures of the folded fraction. In addition, the H-bonding register can be determined and equilibria between alternative turn loci can be detected using these methods. ^{13}C=O shifts may provide additional insights regards structuring details. The use of chemical shifts in fold optimization and as probes for melting studies will be illustrated with two series of miniproteins. Here also chemical shifts have an advantage over CD (and most other spectroscopic measures) since multiple sites are probed and this provides a direct measure and new definition of "cooperativity". The 19-20 residue Trp-cage miniproteins [*Nature Struct. Bio.*, in press] display Tm values between 30 and 61°C; independent of ΔG_U, the chemical shift criterion reveals cooperative folding. The high temperature CSDs also serve to define a residual hydrophobic cluster that is partially populated in the unfolded state ensemble.



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L75 - Lipid-bound structure and phospholipid selectivity of mesentericin Y105 a bacteriocin from leuconostoc mesenteroïdes and its Trp substituted synthetic analogues.

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Mesentericin Y105 (Mes), a 37 residue-long bacteriocin which integrity is required for biological activity on Listeria (Fleury et al. *J.Biol.Chem.* 1996), and two single-substituted Trp/Phe analogues were synthezised to address the structure of the toxin bound to lipids mimiking its putative membrane target. The toxin structure and orientation in phospholipids are adressed by polarized IRFT (PMIRRAS) in situ at the air-water interface, and by intrinsic fluorescence of W18 and W37. At a few tens of nM, i.e. conditions closed to those required for anti-listeria activity, Mes forms films at the air-water interface. When injected in the subphase of a lipid monolayer Mes inserts on negatively charged phospholipids (PS and PG) but affinities for lecithins (PC) are an order of magnitude weaker. Such interactions are reversible according to pH and ionic strength.
PMIRRAS analysis allows to conclude that Mes does not change significantly the lipid acyl chains order. Conversely the analysis of the peptide amide bands indicates the folding into an antiparallel β-sheet and an α-helix for Mes bound to PG monolayers, in agreement with NMR data on related bacteriocins bound to lyso PC micelles (Wang et al. *Biochemistry* 1999). According to pH the relative orientation between the β-sheet and the amphipathic helix changes. Intrinsic fluorescence indicates that peptides in buffer are structureless with W18 and W37 fully exposed. On binding to lipid vesicles W18 is strongly deshydrated, then probably embedded into the acyl chains, W37 very sensitive to the protonation of the Cterm has a versatile location according to pH.
From these data, a new model for bacteriocins at a lipid interface is proposed and discussed in relation with their mode of action, it emphasizes the role of the C-terminal amphipathic helix which could be prolongated up to W37. This residue, mandatory for the biological activity of Mes, locks the helix orientation at interface. According to the carboxyl group charge the helix lies either flat or is more embedded at low pH values

L76 - Artificial remodeling of gp41-C34 peptide leads to effective HIV fusion inhibitor with high anti-HIV-1 activity

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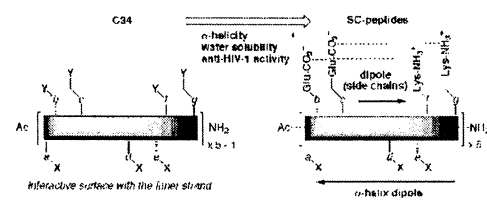
An inhibitor capable of blocking an HIV-1-cell fusion step represents one potential candidate for an anti-HIV-1 drug. Recent disclosure of HIV-1-entry mechanism provides two-types of inhibitors for blocking HIV-1-entry into host cells. One is antagonist for chemokine receptors which HIV-1 utilize as coreceptors for membrane fusion mediated by the envelop glycoprotein complex (gp120/gp41). The other is a molecule targeting the following events which involve the formation of a fusogenic trimer-of-hairpins structure of gp41 ectodomain. The trimer-of-hairpins takes a bundle structure of six α-helices consisting of inner three-stranded coiled-coil formed by the gp41 N-terminal α-helical region and outer three α-helices, packed in a manner antiparallel to the inner strand, derived from the gp41 C-terminal membrane proximal region (C-region). The packing of the C-regions onto the inner strand brings the viral and cell membrane into close proximity, thereby prompting the membrane fusion. Several groups have designed C-peptides derived from the C-region, and evaluated their anti-HIV activity. Among these C-peptides, C34 forms a stable 6 helix bundle with an N-region derived peptide (N36) and exhibits its anti-HIV-1 activity at nM level, indicating that the C34 could serve as a potential candidate for HIV fusion inhibitor. However, we encountered the problems during the large-scale preparation of C34 due to less soluble property of C34. In this study, we present the design and synthesis of a more soluble C34 variant (referred to as SC-peptide) based on the concepts as follows: (1) maintain the amino acid residues critical for the interaction with the inner coiled-coil formed by the N36; (2) replace the residues, locating at the solvent accessible site in the 6 helix bundle consisting of N36 and C34, with Glu or Lys capable of promoting α-helix formation as well as enhancing the solubility to aqueous solution. Synthetic SC-peptides with enhanced α-helicity and water solubility exhibit higher anti-HIV-1 activity than parent C34.



Schematic representation of design of SC-peptides

L 77 - Mechanism of membrane permeabilization by the lipopeptaibol trichogin GA IV and its fluorescent analogues

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Lipopeptaibols are members of a unique family of membrane active peptides, characterized by a linear sequence of 6-10 amino acid residues, a high proportion of the helix-inducing Aib, a N-terminal fatty acyl group, and a C-terminal 1,2-amino alcohol. Trichogin GA IV is the main component of this family, having the following sequence: *n*-Oct-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Lol, where *n*-Oct is *n*-octanoyl and Lol is leucinol. This peptide is able to remarkably modify membrane permeability, displaying both antibacterial and hemolytic activities.
A series of [Leu^{11}-OMe] trichogin analogues marked with fluorescent probes (fluorene and azulene) were synthesized and characterized both in solution and membrane phase. In the following table Aal denotes β-(1-azulenyl)-L-alanine and Fmc the fluorenyl-9-methylcarbonilic group.

			3				7			10			
n-Oct	Aib	Gly	**Aal**	Aib	Gly	Gly	Leu	Aib	Gly	Dab(Boc)	Leu	OMe	**(A3)**
n-Oct	Aib	Gly	Leu	Aib	Gly	Gly	Leu	Aib	Gly	**Dab(Fmc)**	Leu	OMe	**(F10)**
n-Oct	Aib	Gly	**Aal**	Aib	Gly	Gly	**Dab(Fmc)**	Aib	Gly	Ile	Leu	OMe	**(A3F7)**
n-Oct	Aib	Gly	**Aal**	Aib	Gly	Gly	Leu	Aib	Gly	**Dab(Fmc)**	Leu	OMe	**(A3F10)**

Circular dichroism, IR absorption and NMR data show that in methanol solution the peptide backbone of the analogues maintains the ordered, intramolecularly H-bonded structure of the natural peptide (Fig. 1). These spectra are consistent with a mixed $\alpha/3_{10}$ helical conformation.

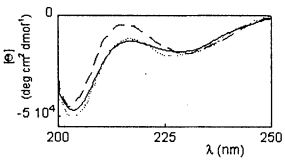

Figure 1: far uv CD spectra of [Leu^{11}-OMe] trichogin (solid line, T=25 °C) and of fluorescent analogue F10 (broken lines T=10 and 40 °C) in methanol solution. Molar ellipticity is expressed in terms of polymer concentration.

Fluorescence studies on methanol solutions of analogues A_3F_7 and A_3F_{10} demonstrate that fluorene fluorescence is quenched by azulene by a dipole-dipole energy transfer mechanism, that depends on relative probe orientation and distance. Fluorescence experiments as a function of temperature, viscosity and solvent composition were performed and compared with computational results, to obtain the most relevant structural features of the compounds examined. The interaction of the trichogin analogues with unilamellar vesicles was also studied. Their permeabilizing activity is comparable to that of the natural peptide, confirming the absence of significant structural perturbations induced by the fluorophores. Experiments of fluorophore accessibility to external and internal quenchers and intramolecular energy transfer studies yielded information on the structure of the membrane-bound peptide.On the basis of both experimental and theoretical results, a model of the trichogin mechanism of action will be presented.

L78 - The second extracellular loop E2 of the human NK-1 receptor is photolabelled by photoreactive analogs of Substance P. Photoaffinity labeling and modelisation studies.

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Substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH_2) the undecapeptide tachykinin interacts with the NK-1 receptor, a G-protein coupled receptor, which has been cloned in different species. Binding experiments with SP analogs in tissues and in NK-1 receptor transfected cells have shown that two types of non-stoechiometric binding sites with distinct pharmacological profiles are associated with the NK-1 receptor (NK-1M and NK-1m). The topology of the Substance P binding site within the human NK-1 receptor transfected in CHO cells has been analyzed by photolabeling studies. Both photoreactive undecapeptide and C-terminal hexapeptide analogs of SP have been used. The photoactivatable probe p-benzoylphenylalanine (pBzl)Phe has been introduced first in position 8 of SP and then in different positions of the undecapeptide from position 4 to the C-terminal methionine. All these analogs were substituted with an aminopentanoyl spacer and a biotinylsulfone (altogether : Bapa) at the N-terminus to achieve purification of the complex[1] formed after photoirradiation at 365 nm. Combined trypsin and endo-Glu C enzymatic digestions of the complex and MALDI-TOF mass spectrometry analysis led to the following results. Firstly, full-length or C-terminal analogs of SP modified by (pBzl)Phe in position 8 were shown to interact with the same domain of the hNK-1 receptor, I^{173} MPSR^{177} in the second extracellular loop (E_2) of the human NK-1 receptor. Further digestion with carboxypeptidase Y led to the identification of $T^{173}MP^{135}$. Cyanogen bromide treatment of the covalent complex demonstrated that Bapa^0[(pBzl)Phe^8]SP or Bapa-Lys[(pBzl)Phe^8, Pro^9, Met(O_2)]SP (7-11) was covalently linked[2] to the methyl of Met^{174}. Secondly, with the photoactivable SP analogs modified by (pBzl)Phe in position 4, 6, 9, 10 or 11[3] the photolabeling appeared reversible, the only peak observed in the MALDI-TOF mass spectrometry analysis was the corresponding free peptide. The analog modified in position 7 was a too weak competitor (K_iνμM). With Bapa^0[(pBzl)Phe^5]SP the irreversible bond occurred also within the T^{173} MPS^{176} domain. Models of the conformation of this crucial E_2 loop in the human NK-1 receptor were generated using two strategies : one based on homology with bovine rhodopsin and the other one based on the solution conformation preferences on a synthetic peptide AcY^{168}STTTETMPSRVVSMIEWPEHPNKIYEKVY^{196}-NH_2 corresponding to this E_2 loop[4]. All these results will be discussed.

References

[1] S. Girault, G. Chassaing, G. Bolbach, J.C. Blais and A. Brunot. Analytical Chemistry, 1996, 68, 2122-2126.
[2] S. Girault, S. Sagan, G. Bolbach, S. Lavielle and G. Chassaing. Eur. J. Biochem., 1996, 240, 215-222.
[3] E. Sachon, S. Girault-Lagrange, G. Chassaing, S. Lavielle, S. Sagan. J. Peptide Res., 2002, 59, 232-240.
[4] O. Lequin, G. Bolbach, F. Frank, O. Convert, S. Girault-Lagrange, G. Chassaing, S. Lavielle, S. Sagan. J. Biol. Chem., 2002, in press.