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Protein Folding

TIMESCALES FOR PEPTIDE SECONDARY AND TERTIARY STRUCTURE FORMATION

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Recent developments in structured peptide design (for 16- to 20-mers) together with residue-specific ^{13}CO labeling and the discovery of cosolvent effects that produce increased structuring upon warming (*J. Am. Chem. Soc.*, **121**, 9879) have allowed us to measure the time constants for helix and β hairpin formation from the cold-denatured conformer ensemble and the lifetime (3 μsec) of the hydrophobic core of a miniprotein fold. T-jump experiments monitoring the amide-I' band revealed a time constant of 42 ns for helix formation. To date, the experiments on a hairpin forming 16-mer indicate a biexponential growth in the β sheet amide signal with $\tau = 2.6$ μsec and 300 ns. The net extent of β hairpin formation measured in these experiments correlates with NMR measures of the ΔT induced changes in hairpin/coil equilibrium. The fast phase which we detect was not observed in previous indirect measures of β hairpin formation rates (T-jump unfolding studies of fluorophore-tagged peptides, *Nature*, **390**, 196). Efforts to confirm the fast β formation phase are continuing. Several rationales can be suggested for these results (which appear to be inconsistent with an H-bond zippering mechanism after nucleation at the turn locus). ^{13}C-labeling studies in progress should distinguish between the alternative models of the hairpin folding mechanism.

Helical and hairpin peptide ^{13}CO isotopomers are also used to determine temperature dependence of chemical shifts and their FT-IR difference spectra. These provide direct measures of structuring cooperativity and the temperature dependence of ΔG_{fold} at the residue level.

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STUDY ON THE *IN VITRO* OXIDATION/FOLDING OF MAUROTOXIN, A 4 DISULFIDE-BRIDGED SCORPION TOXIN

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Maurotoxin (MTX) is a K^+ channel-acting 34-mer scorpion toxin cross-linked by four disulfide bridges. Its half-cystine pairing pattern was previously investigated by enzyme-based cleavage of the synthetic product (sMTX), and was found to be in an unusual arrangement (of the type C1-C5, C2-C6, C3-C4, and C7-C8) among known scorpion toxins. The natural MTX also adopts this nonconventional disulfide bridge organization. Here we investigated the kinetics and process of *In Vitro* oxidation/folding of reduced sMTX by varying experimentally one of a number of parameters which are likely to affect folding; this includes temperature, pH value, ionic strength, redox potential, peptide concentration, and the presence of dimethylsulfoxide as oxidant. We further assessed the influence of several protein factors on the folding pathway of reduced sMTX, including peptidyl prolyl *cis-trans* isomerase, protein disulfide isomerase, and a mixture of molecular chaperones (GroEL and GroES). During each assay, samples of the oxidation milieu were collected at different times, and the peptide intermediates were blocked by alkylation of their free Cysteinyl. These intermediates were characterized by several methods, such as C18-reversed-phase high pressure liquid chromatography, Edman sequencing, and mass spectrometry analysis. The results obtained from these experiments will be presented and discussed.

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TRANSLOCATION OF PEPTIDE pAntp AND AP-2AL ANALOGUE: EXPERIMENTAL AND THEORETICAL APPROACHES

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The pAntp peptide, corresponding to the third helix of the homeodomain of Antennapedia protein, is internalized by a receptor-independent process into eukaryotic cells and has been successfully used for the intracellular delivery of polar compounds (1). The interaction between the phospholipid matrix of plasma membranes and pAntp is probably involved in the internalization process. However, the mechanism of peptide translocation through the cell membrane remains unclear. We have therefore investigated the translocating ability of pAntp through protein-free phospholipid membrane in comparison to AP-2AL, a more amphipathic analog. We have shown by fluorescence spectroscopy that pAntp peptide does not induce membrane leakage and does not cross the membrane. In contrast, the AP-2AL analog induces the leakage from lipid vesicles and translocates through the membrane. Structural investigations by circular dichroism and ^1H NMR indicate that the lipid-bound form of AP-2AL is more ordered (α-helical) than the lipid-bound form of pAntp. To further understand the difference between the peptide behaviours, we have simulated their insertion in membrane using the IMPALA procedure (2). Calculations indicate that pAntp remains at the surface of the membrane because its insertion into a hydrophobic phase is energetically disfavored. By contrast, AP-2AL penetrates the membrane and adopts an orientation consistent with a translocation mechanism involving the transient formation of a pore. Consideration of the mean hydrophobic potential (MHP) calculated for each peptide indicates that pAntp is not hydrophobic enough to translocate through phospholipid membranes. In conclusion, we have demonstrated that theoretical and experimental approaches can be combined to evaluate the translocation ability of amphipathic peptides. In addition, we report that pAntp does not belong to the amphipathic helical peptide family that normally translocates via pore formation.

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CONFORMATIONAL ANALYSIS OF TRIMERIC COLLAGEN PEPTIDES CROSSLINKED WITH A CYSTINE-KNOT

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Single-stranded collagen peptides consisting of $(Gly\text{-}Pro\text{-}Hyp)_n$ repeats associate in solution to form stable triple-helical homotrimers at room temperature when $n = 10$.[1] This folding process is entropically unfavoured and slow. To partially bypass this entropic penality and to facilitate nucleation of the triple-helix, N- and/or C-terminal crosslinking of collagenous peptides with homotrifunctional templates such as Lys-Lys-Gly, Glu-Glu or 1,2,3-propanetricarboxylic acid to homotrimers has been extensively investigated whereby spacers differing in length and flexibility were applied to allow a staggered alignment of the three chains in the triple-helical fold. More recently, the conformationally constrained Kemp triacid, i.e. *cis,cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid, with its functionalities aligned in parallel orientation was found to represent a very efficient template in terms of triple-helix nucleation and stabilization.[2] Since refolding processes of natural collagen fragments containing cystine-knots are known to occur at high rates, synthetically accessible cystine-knots for crosslinking in regioselective manner both homo- and heterotrimeric collagen peptides in a defined chain raster were designed and realized.[3] To analyze, the effect of N- and C-terminally placed cystine-knots on the stability of the triple helix as well as on the rates of refolding, in the present study related $(Gly\text{-}Pro\text{-}Hyp)_5$ cystine-knot constructs were synthesized and characterized in their conformational properties in view of the potential triple-helix nucleation effect of a correctly placed cystine-knot.

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Protein Folding

MAUROTOXIN vs Pi1 SCORPION TOXINS: TOWARDS NEW INSIGHTS IN THE UNDERSTANDING OF THEIR DISTINCT DISULFIDE BRIDGE PATTERNS

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Maurotoxin (MTX) is a 34-mer scorpion toxin cross-linked by 4 disulfide bridges. We synthesized MTX and characterized the synthetic product, sMTX. It is active on rat SK channels and blocks rat Kv1.1, Kv1.2, Kv1.3 and *Shaker* B currents in *Xenopus* oocytes, with IC_{50} values of 5, 37, 0.8, 150 and 2 nM, respectively. The disulfide bridge pattern of the toxin was investigated by enzyme cleavage of sMTX. It is in an unusual arrangement (of the type C1-C5, C2-C6, C3-C4, and C7-C8) among known scorpion toxins (natural MTX also adopts this atypical disulfide organization). Two other short scorpion toxins, Pi1 and HsTx 1, were recently characterized. They share from 60-70% sequence identity with MTX but display distinct pairings (of the type C1-C5, C2-C6, C3-C7, and C4-C8). For HsTx 1, the lack of a glycyl in position 32 or 33 does not allow connection between C7-C8, thereby excluding for this toxin –contrary to Pi1- an MTX-like disulfide bridge arrangement. Therefore we focused our study on MTX and Pi1 toxins. We synthesized Pi1 and verified its half-cystine pairings, and studied folding and structure-activity using analogs of Pi1 and MTX. These analogs were point-mutated or possessed a reduced number of disulfide bridges. Our data show that selective point-mutation(s) within MTX (by the residue(s) in homologous position(s) within Pi1) yielded Pi1-like disulfide bridges. This novel organization of the disulfides is accompanied, as in Pi1, with an increased selectivity of the molecules towards Kv1.2 and *Shaker* B channels. Conversely, specific point-mutation(s) of residue(s) within Pi1 could result in an MTX-like pattern of the disulfides in some Pi1 analogs. The results will be discussed in terms of folding/oxidation and pharmacological activity.

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Analytical Methodologies

A novel approach to identify proteins using direct software interpretation of ESI-MS/MS data to determine *de novo* sequences of tryptic peptides

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Known proteins can be rapidly and accurately identified by comparing the MS/MS spectra from peptides in an enzyme digest to entries within protein, genome or EST databases. However, when the protein is not contained within these databases then this simple approach fails. In this case BLAST searching using *de novo* sequence may identify the protein by cross species homology to a different organism. An advantage of this method in combination with tandem mass spectrometry is that often several peptides from the same protein can produce *de novo* sequence and this increases the confidence of the identification.
On line HPLC ESI-MS/MS data obtained on quadrupole hybrid time of flight tandem mass spectrometers (Q-Tof) from multiply charged tryptic peptides can produce spectra containing long stretches of *de novo* sequence. However, to date the interpretation of sequence directly from MS/MS spectra has been time consuming and required specialist knowledge of the fragmentation mechanisms. The high resolution and good mass measurement accuracy in Q-Tof data allows Bayesian computational methods to be applied to directly interpret peptide sequence from this data.
The evaluation of this methodology, using tryptic digests of known proteins, shows the accuracy of sequence interpretation to be in the region of 80 - 90%. Since the BLAST algorithm is error tolerant, 100% sequence accuracy is not required for the method to be useful. In each example using the *de novo* sequence predicted by the MassSeq algorithm to BLAST search protein databases correctly identified the parent protein.

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HIGH-RESOLUTION MAGIC ANGLE SPINNING NMR (HR-MAS) TO STUDY THE BEHAVIOUR OF PEPTIDES IN SOLID-PHASE

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High-resolution magic angle spinning NMR (HR-MAS) is emerging as a powerful non-destructive technique for studying at atomic details resin-bound organic molecules. This technique combines magic angle spinning (MAS) with opportune swelling condition of resin in order to average out the magnetic anisotropy of the sample and to make the detection of proton signals feasible. HR-MAS has been successfully used in our group to study the structure of resin-bound peptides[1,2] and *in situ* solid-phase peptide synthesis.[1,3] Here we report HR-MAS studies to understand the behaviour of peptides in solid-phase. In particular we want to elucidate the function of the neutralised (NH_2) and of the charged (NH_3^+) form both important during the peptide synthesis; the role of the counter-ion when the peptide is in the charged form; the possibility of cation-π interaction between the NH_3^+ and polystyrene resins and the role of the residual water.

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NEW APPROACH OF HRMAS TECHNIQUE USING SOLID STATE NMR METHODS : DETECTION OF DIPOLAR INTERACTIONS BEETWEEN DISTANT NUCLEI.

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High resolution magic angle spinning (HRMAS) technique has opened new avenues for the study of molecules that belong neither to the liquid nor to the solid state but which lie somewhere between the two. The domain of application of this new technique ranges from the study of organic molecules and peptides bound to a solid resin support to polymers, lipids and human and animal tissues. This technique requires the sample under study to have sufficient mobility to greatly average anisotropic interactions. The NMR line-width observed in the spectra of static sample originates mainly from the residual dipolar interactions and variations in the bulk magnetic susceptibility. These remaining interactions can be averaged out by MAS technique leading to spectral resolution approaching that of liquid samples. Thus, High-Resolution multidimensional solution state NMR techniques are practicable and are now routinely used in the analysis and characterization of such compounds. Here we show that the residual static dipolar interaction is high enough to be used as in solid state NMR. We will focus on so-called Rotary-Resonance (RR) and RFDR experiments which belong to the dipolar recoupling techniques family. It is of great interest to consider this interaction under this point of view because the behavior of dipolar recoupling techniques differ from NOE transfers and give rise to structural information unreachable with liquid state NMR experiments like NOESY. For these experiments, longitudinal magnetization is transferred between dipolar-coupled spins by cross-relaxation. In the case of an isolated spin pair, the rate of magnetization transfer is determined by the inverse sixth power of the internuclear distance. For 2D RFDR experiments, longitudinal magnetization is transferred between dipolar-coupled spins by static dipolar couplings. The rate of magnetization transfer is determined by the inverse cube power of the internuclear distance and therefore allows the detection of dipolar interactions between distant nuclei. Theoretical and experimental aspects of dipolar recoupling techniques applied to organic solid phase synthesis will be discussed.

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STUDY OF THE HRMAS TECHNIQUE : ANISOTROPIC BULK MAGNETIC SUSCEPTIBILITIES AND RESIDUAL STATIC DIPOLAR INTERACTION.

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High resolution Magic Angle Spinning (HRMAS) provides a powerful tool for the characterization of resin bound molecules, polymer or other soft materials such as lipids or physiological tissues. In this technique, the averaging by MAS of any kind of residual interactions leads to spectra of nearly liquid state quality. Nevertheless typical linewidths obtained by this method are still about three time greater than in real solution samples. Our goal is to understand the role played by all the possible interactions present in those systems formed by organic phases bound to resins. As a result, our findings allow us to point out two relevant interactions : anisotropic bulk magnetic susceptibilities and static dipolar interaction. The first one is found to be mainly responsible for the residual linebroadening : it interacts with the individual nuclear dipoles and thus forms a second order interaction which cannot be averaged by MAS. The solution is then to avoid any aromatic rings in the resin structure. We show how aromatic cycle free resin may drastically improve resolution to obtain liquid-like linewidth. This is of prime importance as it enables the study of larger molecules by HRMAS NMR such as 141-159 peptide of VP1 prótein of Foot and Mouth Disease Virus (FMDV). The case of the residual static dipolar interaction is quite different : MAS fully averages out this interaction. Our purpose is now to take advantage of its presence to obtain informations unreachable in the liquid state. We show that solid-state NMR experiments based on reintroduction of the static dipolar interaction are applicable. Examples dealing with so-called RR and RFDR are shown. Finally, we show that some classical liquid state multidimensional experiments as TOCSY are not so straightforward. Some kind of interference phenomenon between the MAS rate and the radiofrequency pulses occurs in a destructive way. Methods to overcome this problem are given.

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MASS SPECTROMETRY IN ANALYSIS OF PEPTIDE SYNTHESES ON SOLID SUPPORT

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Mass spectrometry is a powerful analytical tool allowing rapid and sensitive structural elucidation of a wide range of molecules issued from solution-, solid- and liquid-phase syntheses.

The relevance of Electrospray Ionization (ESI), Matrix Assisted Laser Desorption Ionization (MALDI) and Secondary Ion Mass Spectrometry (SIMS) to characterize all samples produced daily in peptide chemistry will be illustrated.

First, fully automated high throughput mass spectrometric analyses are required to process the large collections of samples produced by batch syntheses as well as combinatorial strategies. ESI was found the best method since it can be easily implemented on line with liquid chromatography (LC/MS) providing in a single run qualitative and quantitative product assessments.

Second, the challenging direct monitoring of supported reactions on both insoluble and soluble polymers was tackled by mass spectrometry. Resin beads were successfully analyzed by SIMS whereas liquid-phase syntheses carried out on poly(ethyleneglycol) were followed by ESI and MALDI.

POSTSOURCE DECAY (PSD) ANALYSES OF PEPTIDES CONTAINING REDUCED BOND BY MATRIX-ASSISTED LASER-DESORPTION-IONIZATION TIME-OF-FLIGHT (MALDI-TOF) MASS SPECTROMETRY

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Matrix-assisted laser-desorption is known to have several advantages over other methods of ion formation, like the extension of the mass range to over 100 000 Da, high sensitivity (pico or femtomole) and fast analysis. MALDI mass spectrometry has made it possible to obtain information on large biomolecules or synthetic polymers.

Spengler *et al* have demonstrated that analyte ions formed by matrix-assisted laser-desorption ionisation time-of-flight mass spectrometry (MALDI TOF MS) undergo extensive spontaneous decomposition in the field-free region between the source and the reflectron. The resulting fragments can be detected as so-called postsource decay (PSD) spectra[1,2] which can give structural informations of primary structure of peptides.[3] Practical use of this approach provides also valuable sequence information and or confirms additionnal structural features of peptide in enzymatic digests.[4]

Using a MALDI TOF mass spectrometer (BIFLEX III Bruker), we propose here PSD analyses of different pseudopeptides containing reduced bond. Structural informations will be described compare with native peptides.

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Validation of a GC-MS Method for Determination of the Optical Purity of Peptides.

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Measurement of the racemate content is an important aspect of the determination of the purity of peptidic products. One of the most accurate and sensitive methods for the analysis of the optical purity of free amino acids involves derivatization and gas chromatographic separation of the enantiomers using a chiral stationary phase. A technique for the unambiguous quantitation of the racemate content of peptide- or protein-bound amino acids, eliminating the contribution of racemization during hydrolysis, was presented by Frank et al.[1]. The protein or peptide is hydrolyzed in 6N DCl/D_2O, whereby racemization is accompanied by deuterium exchange in the α-C position. By mass spectrometric determination of the relative amounts of deuterium-labeled to non-labeled species of a characteristic ion, the proportion of racemate arising from the hydrolysis could be calculated. Using multiple ion detection, Liardon et al.[2] improved the methodology, deriving a series of equations with which the interference arising from neighbouring ions $(I+1)^+$ and $(I-1)^+$ could be taken into account. Using selected ions and deuterated derivatization reagents, a simplified version ot the latter method was proposed [3] and its automation implemented [4].

In this poster, validation of the method described in [3] for all proteinogenic and several non-proteinogenic amino acids is presented. The validation comprises determination of the following parameters for each amino acid : Precision (repeatability), Intermediate precision, ruggedness, linearity, accuracy, limit of quantitation, limit of detection and selectivity of the chromatographic system. It is demonstrated that in general the method is capable of reliably determining the optical purity down to 0.1% of the unnatural enantiomer. For certain amino acids such as cysteine and amino acids linked to cysteine, this value may increase to 0.4%.

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ZINC(II) BINDING TO CYSTEINE RICH DOMAIN PEPTIDES OF KSR - A CAPILLARY ELECTROPHORESIS STUDY

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We are utilizing affinity capillary electrophoresis (ACE) to measure metal ion interactions with peptides and proteins. The cysteine rich domain of kinase suppressor of RAS (KSR) contains two putative zinc fingers. A 50 residue peptide of this cysteine rich domain was chemically synthesized and purified. With constant concentrations of peptide as a receptor and varying concentrations of zinc(II) as the ligand in the sample buffer and electrophoresis buffer, changes in electrophoretic mobility of the peptide were observed as complexes were formed with zinc(II). Scatchard analysis of the mobility indicates the presence of two types of binding sites for zinc(II). At pH 6.0, one site was shown to bind zinc(II) with a binding constant of $K_b = 2.2 \times 10^5\ M^{-1}$ and the second site has a $K_b = 0.9 \times 10^5\ M^{-1}$. Mutants of the cysteine rich domain were also examined for the ability to bind zinc(II). ACE does not require reverse titration of peptides and the method is straightforward. ACE requires little sample (only nanoliters) and run times are short.

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Quantitative Determination of R,S,S-Enalapril in Enalapril Maleate

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Determination of the isomeric purity of enalapril maleate concerning the R,S,S-epimere, originated by the common routes of synthesis, is required in the Pharm. Eur. Supplement 2000, with a specification limit of ≤ 1.0%.
In our experience the herein proposed LC method for determination of the R,S,S-epimere is lacking in selectivity/specificity and sensitivity for traces of the R,S,S-enalapril epimere in presence of enalapril maleate.
On basis of an own R,S,S-enalapril maleate standard a reversed phase LC method for separation and quantification of the R,S,S-epimere in enalapril maleate was developed, using a quantitative transformation of the enalapril epimeres to corresponding epimeric diketopiperazines. Detection and quantification limits were found with 0.07 % and 0.12 % R,S,S-enalapril maleate in enalapril maleate, respectively.

The described method is suitable for routine testing of the isomeric purity of enalapril maleate in the range ≤ 0.2 %.

STRUCTURE-ENANTIOSELECTIVITY RELATIONSHIP OF CYCLOHEXAPETIDES FOR CHIRAL CAPILLARY ELECTROPHORESIS

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Synthetic cyclopeptides dissolved in electrolytic solution, can be used as chiral selectors in capillary electrophoresis (CE).

The peptide c-(Arg_1-Lys_2-Tyr_3-Pro_4-Tyr_5-βAla_6) (cYY) was selected from a combinatorial library through a deconvolution process as the best chiral selector in CE for *N*-2,4-dinitrophenyl (DNP) amino acids. In this work, we report NMR studies on the chiral complexes formed between the cyclopeptide and DNP-glutamic acid enantiomers and some synthetic modifications on both cyclopeptide and analytes in order to understand the interactions involved in the chiral recognition.

Molecular Dynamic simulations and Distance Geometry calculations performed on both free cYY and the complexes cYY/ DNP-Glu enantiomers showed that the residue in position 5 is deeply involved in the chiral recognition process. Therefore, the influence on enantioselectivity of the residues Tyr, Phe, 3-nitro-Tyr, *p*-nitro-Phe, Trp and 2-naphthyl-Ala in position five of the cyclopeptide was investigated. In addition, N-benzoyl derivatives of Glu bearing nitro groups on the aromatic ring were synthesised and separated by CE by using the newly synthesised peptides in order to assess the effect of analyte and selector molecular structure on racemate resolution. Aim of the work was to set some basic rules helpful in the design of new CE chiral selectors .

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An Integrated Approach to Automated High Throughput Protein Identification by 2D Gel Electrophoresis and Mass Spectrometry

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Establishing the function of gene products is the major challenge of the post genomic era. The rate-limiting step in this endeavour is the speed with which proteins can be isolated and identified. Separation of proteins from cell lysates or sub-cellular domains by 2D gel electrophoresis is an established method of visualising these complex systems. Mass spectrometry is a powerful method of further characterising these proteins. From the mass spectrum of the enzyme digest of a 2D gel spot, the resulting digest map is compared with the theoretical maps from the databases and the protein identified when these correlate. MALDI-TOF requires a minimal amount of sample, is relatively tolerant to salts and other contaminants arising from the gel and may be configured for automated sample analysis. High sample throughput with automated analyses including data processing and client-server database searching are already available. Our system automatically acquires the data and processes the MALDI mass spectrum into a monoisotopic peak list which is automatically sent to a networked database for protein identification. When proteins are not identified from the MALDI analysis or an ambiguous result is obtained, then further analysis of the sample by Electrospray CapLC-MS-MS is required. The development of a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Micromass, Q-Tof) has facilitated the generation of unambiguous amino acid sequences from the MS-MS analyses of tryptic peptides. These MS-MS spectra can be automatically searched against protein, nucleotide or EST databases, thus enabling protein identification from gel spots, despite non-specific enzymatic cleavage, protein co-migration and post transitional modifications.

INTRAMOLECULARLY QUENCHED FLUOROGENIC PEPTIDE SUBSTRATES FOR CATHEPSIN B

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Cathepsin B is a lysosomal cystein protease of the papain superfamily, that possesses both endopeptidase and carboxydipeptidase activity. While the endopeptydase activity is observed around neutral pH, the digestion by carboxydipeptydase mechanism is restricted rather to acidic conditions with optima of pH 4 - 5. The enzyme is assayed most often by Z-Phe-Arg-NHMec, common fluorogenic substrate for all papain family or by Z-Arg-Arg-NHMec, selective over other lysosomal cystein proteases. The maximal activity of this enzyme with both substrates was found at pH 7.5 - 8, but cathepsin B is very unstable under these conditions and the routine assay is generally performed at pH 6.0. Unfortunately, the enzyme practically does not hydrolyze the mentioned substrates in buffers reassembling lysosomal conditions. We synthesized a new quenched fluorescence substrates designed to assay exopeptidase activity of cathepsin B. The peptide moiety of these substrates is derived from sequence of the binding fragments of cystatin C (natural cysteine proteases inhibitor) and specificity of the active site of cathepsin B. The general structure of our substrates was DABCYL-Arg-Leu-X-Gly-Y-Glu(EDANS)-OH where X = Val, Arg; Y = Trp, Phe, Nal; DABCYL = 4-(4'-dimethylaminophenylazo)benzoil; EDANS = 5[2-(aminoethyl)amino]naphtalene-1-sulfonic acid. The enzymatic cleavage of the substrates took place between Gly-Y residues. The reaction was monitored by the increasing of fluorescence accompanied by releasing Y-Glu(EDANS)-OH fragments. The kinetics of cleavage followed the Michaelis-Menten model. The paper will present k_{cat} and K_m parameters at neutral and acidic conditions.

Acknowledgments: Supported by KBN (Polish State Committee for Scientific Research) research grant.

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DEVELOPMENT OF A NEW ISOTHIOCYANATE-BASED CHIRAL DERIVATIZING AGENT FOR AMINO ACIDS

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The application of chiral derivatizing agents (CDAs) was the first widely used method for the enantiomeric separation of chirally active molecules in liquid chromatography. The popularity of indirect resolution procedures involving pre-column derivatization of enantiomers with a CDA and subsequent separation of the diastereomers formed on achiral HPLC columns does not seem to have decreased, despite the appearance of highly effective chiral stationary phases. The CDAs introduced for this purpose include many isothiocyanate based compounds [1]. The isothiocyanate group is selective towards primary and secondary amines under mild conditions, the protection of other free functional group is not required, and the thiourea derivatives produced are very sensitive to UV detection. However, the commercially available reagents are sometimes rather expensive or available in only one enantiomeric form.
The present paper describes the synthesis of a new isothiocyanate-type CDA, and its application for the separation of selected α- and β-amino acid enantiomers. (1*S*,2*S*)- or (1*R*,2*R*)-1,3-Diacetoxy-1-(4-nitrophenyl)-2-propylisothiocyanate ((*S,S*)- or (*R,R*)-DANI) was obtained after a straightforward two-step synthesis starting from the trifunctional (1*S*,2*S*)- or (1*R*,2*R*)-2-amino-1-(4-nitrophenyl)-1,3-propanediol [2]. The reagent was found to be stable without decomposition for several months.
Derivatization of amino acids was carried out in alkaline solution. The pH of solution was adjusted with triethylamine and the effect of pH on the yield of derivatization was investigated. Other parameters effecting the yield of derivatization were also optimised: temperature of derivatization, molar ratio of reagent/analyte.
Analyses were performed on reversed-phase columns. Mobile phase consisted of aqueous trifluoroacetic acid or sodium acetate buffer as inorganic part, while organic modifiers were methanol or acetonitrile.
The analytes were proteinogenic and unusual α- and β-amino acid enantiomers. The applicability of this new CDA in the separation of amino acids is demonstrated.

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Impurity detection in solid phase organic chemistry : scope and limits of HR MAS NMR

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We evaluate the possibilities and limits of HR MAS NMR to detect and quantify side products during solid phase synthesis, using a model system where Pyroglutamate and Glutamate are coupled in a well defined ratio [1]. Resins swollen in deuterated and protonated solvent are studied. Use of the LED sequence [2] eliminates the peaks due to the use of protonated solvents, but sensitivity is decreased, and differential losses of magnetization might lead to a biased population estimation. As all sample workup steps are eliminated, this technique will be helpful in detecting minority species in solid phase combinatorial chemistry, and its application at the different steps of the reaction might lead to the early detection of otherwise unidentifiable active components.

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Analysis of Cyclohexapeptides with ESI-FT-ICR-Mass Spectrometry

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The increasing number of compounds synthesized by combinatorial organic synthesis (high-throughput-synthesis) [1,2,3], may be examined rapidly with respect to their pharmaceutical properties by means of "high-throughput-screening". To avoid bottlenecking with respect to the chemical analysis of these compounds, it is necessary to implement "high-throughput-analysis"..For this purpose, FT-ICR-MS is predestined because of its combination of high to ultrahigh resolution, very high mass accuracy, high speed and high sensitivity.
This is exemplified here with cyclohexapeptide libraries. Here the measurements of peptides synthesized by combinatorial chemistry can be performed automatically with one measurement each 2 minutes [4]. To avoid the time-consuming manual evaluation of the numerous signals a respective automation is also possible. We were able to show that peptides produced by combinatorial organic synthesis can be automatically indentified by FT-ICR-MS with a very high accuracy also in complex mixtures. Furthermore it could be shown that by coupling of ESI-FT-ICR-MS with Micro-HPLC it is possible to resolve isobaric cyclopeptides over the time. A fragmentation was performed so that the study of the primary structure of cyclopeptides was possible. Moreover the potential of these technique in mass spectrometry is shown by performing single bead analysis [5] of not yet cycled peptides for verification of the synthesis.

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SYNTHESIS OF NEW LIGAND-EXCHANGE CHIRAL STATIONARY PHASES DERIVED FROM *N*-NONYL- (*S*)-AMINOALCOHOL SELECTORS AND THEIR APPLICATION TO RESOLUTION OF RACEMIC AMINO ACIDS BY HPLC.

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Chiral ligand-exchange chromatography (LEC) has been widely employed in resolution racemic α-amino acids since the pioneering work of Davankov and co-workers in the late 1980s. The separation of the analytes in LEC is based on formation of tertiary complexes: analyte - divalent metal cation - chiral selector. Three chiral phases used in the present study were prepared by loading N-nonyl-(*S*)-aminoalcohol onto octadecyl-silica support: N-nonyl-(S)-Tyrosinol [C_9-(*S*)-Tyr-ol], N-nonyl-(*S*)-3,4-Dihydroxyphenylalaninol [C_9-(*S*)-DOPA-ol], N-nonyl-(*S,S*)-2-Amino-1-phenyl-1,3-propanediol [C_9-(*S,S*)-APP-ol]. The surface concentration of chiral selectors determined according to the breakthrough method was in the range of 0.78-0.82 μm m^{-2}. A good enantioselectivity (α) in the range of 1.17 to 1.46 was observed. The enantioselectivity of the chiral columns in resolution of amino acid enantiomers was influenced by the constitution of aminoalcohol molecules - the chiral selectors of CSP. The column contained C_9-(*S*)-DOPA-ol as a selector showed the greatest enantioselectivity. In this molecule two hydroxyl groups linked to the phenyl residue in the side chain of the aminoalcohol molecule ortho and para are potentially able to form additional bonds with copper (II) ions and therefore the C_9-(*S*)-DOPA-ol selector creates more stable complex. The lowest resolution factors - efficiency was observed on the column containing C_9-Tyr-ol. The same elution order of enantiomers was observed for all the CSPs, independently of the applied selector - aminoalcohol molecule. All the phases formed more stabile complexes with *(R)*-enantiomers, and consequently (*S*)-enantiomers were eluted from the column before *(R)* isomers. In order to establish the thermodynamics of the stereodiscriminating process, the effect of temperature on the chromatographic parameters was investigated. The resolution selectivity depended on the concentration of Cu (II) ion, pH of the mobile phase and concentration of a organic mobile phase modifier.

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A rapid method for estimating the binding of ligands to ELISA microwells.

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This report presents a rapid and simple assay for estimating to what extent the surface of ELISA microwells is coated by a ligand of choice such as, for example, proteins, peptides, hormones, polysaccharides and nucleic acids. The method also provides a practical approach for defining the conditions required for optimal coating, such as ligand concentration, coating buffer, temperature and duration of coating and also for evaluating the efficiency of the reagents used to saturate the ELISA microwells. The important advantage of this procedure is that, in contrast to conventional ELISA procedure, the detection of the microwell-adhered ligand is not achieved by using an antibody. It is therefore the solution of choice when, as is often the case, no primary specific antibody is available. The test consists of three steps: first the ligand is allowed to adsorb to the microwells. Second, alkaline phosphatase is added to bind to any residual microwell surface not occupied by the ligand. Finally, substrate is added and the resulting color reaction is measured. Light absorbancy is inversely correlated with the level of ligand adherence. The results obtained by this method match those of direct ligand quantitation, as evaluated by a regular ELISA procedre.

Key words: ELISA; alkaline phosphatase; peptide; binding; saturation; coating; method;

PEPTIDE SCANNING APPROACH TO THE STUDIES OF PROTEIN-PROTEIN INTERACTIONS IN MONOOXYGENASE SYSTEMS

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[b] *University of California at Irvine, Irvine, USA*

Studies of protein-protein interaction mechanisms and mapping of sites responsible for these interactions are among the most important and complex problems in modern protein chemistry.

Method of peptide scanning (PEPSCAN) that includes parallel solid phase peptide synthesis followed by the analysis of peptide binding activity by immunoassay, was used for mapping linear sites responsible for protein-protein interactions. The work was carried out on a liver microsomal monooxygenase system, composed of cytochrome _450 2_4, NADPH-cytochrome _450-reductase and cytochrome b5, and a bacterial monooxygenase system from pseudomonas putida, composed of cytochrome _450cam, putidaredoxin, and putidaredoxin-reductase.

The use of PEPSCAN approach, which is widely applied for antigenic protein mapping, is proven by the fact that interaction of an antigen with an antibody is a special case of protein-protein interactions.

Short overlapping peptides, covering the complete aminoacid sequence of putidaredoxin and membrane fragments of both cytochrome b5 and cytochrome _450 2_4, were synthesized on polyethylene pins and tested for their abilities to bind the corresponding redox partner proteins. A special experimental procedure was developed for the studies of pin-linked peptide interactions with the proteins. Peptide-bound proteins were detected by ELISA.

Results of redox partner binding studies of cytochrome b5 and cytochrome P450 2B4 transmembrane fragment-derived peptides confirmed the nonspecific character of interactions of these sites upon complex formation between these proteins. In contrast to that, seven linear hexapeptide fragments were revealed by PEPSCAN in putidaredoxin molecule that can bind cytochrome P450cam. These data were confirmed by the optical biosensor technique studies of the inhibition of P450cam-putidaredoxin interaction with PEPSCAN-synthesised cleavable peptides.

This work was supported by grants from RFBR _ 98-04-48684 and NATO CRG No.974288.

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LASA SPECIFICITY : USE OF CE AND LSIMS FOR STUDYING HYDROLYSIS OF ELASTIN PEPTIDES

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Pseudomonas aeruginosa LasA belongs to the family of ß-lytic endopeptidases which cause cell lysis of other bacteria by cleaving specific peptide bonds within the cell wall peptidoglycan network. Besides its staphylolytic activity, LasA may hydrolyse insoluble elastins and enhance the elastolytic activity of various elastases including *Pseudomonas* and human leukocyte elastases which are involved in tissue damaging associated with bacterial infection and cystic fibrosis.

To elucidate the substrate specificity of LasA and considering the high glycine content of both elastin and *Staphylococcus* peptidoglycan we have studied the ability of LasA to hydrolyse three-glycine-containing tropoelastin pentapeptides or analogues.

Capillary electrophoresis (CE) has proved to be the most suitable method to determine which peptides are hydrolysed and to calculate the catalytic ratios k_{cat}/K_M from initial rate measurements. As an example, 59 $min^{-1}.mmol^{-1}.L.$ is the value obtained for the best substrate of LasA, LGGGA.

Liquid Secondary Ion Mass Spectrometry (LSIMS) and Collision Activated Dissociation-Mass analysed of Ion Kinetic Energy (CAD-MIKE) spectra have been useful to ascertain the specificity of LasA towards GG↓X sequences (X : Gly, Ala, Phe, Tyr).

Similar cleavage sites have been demonstrated in sequences 19-35, 642-656 and 637-657 from human tropoelastin presuming that pentapeptides are good models for studying the specificity of elastolytic enzymes.

Reference :

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THE MASS SPECTRAL ANALYSIS OF N-TERMINAL AND SIDE-CHAIN PROTECTED PEPTIDES

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Analysis of N-terminal and side-chain protected peptides has previously been performed using Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF). However, due to matrix effects, inconsistent mass data has been obtained resulting in inaccurate mass determinations. This poster describes an analytical method employing Electrospray Ion Trap mass spectometry (ESI-IT MS) for the accurate determination of molecular weights of N-terminal and side-chain protected peptide fragments. By utilizing a high performance liquid chromatography buffer system with a basic buffer range, deprotonation of the C-terminal carboxyl group of protected peptides can be achieved. This enables analysis of the monovalently charged species by ESI-IT MS for protected peptides with an average mass < 2000 mass units. For protected fragments with an average molecular weight >2000 mass units, removal of the 9-fluorenyl-methoxycarbonyl (FMOC) group by ionization energy resulted in a monovalently or divalently charged species that could be analyzed. The analysis of the unprotected peptides containing the FMOC group by ESI-IT MS indicated that the mass data obtained was accurate. This approach to mass analysis of protected peptides yields accurate data, which is easily interpreted over the previously used MALDI-TOF approach.

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UNRAVELLING THE AUSTRALIAN FUNNEL-WEB SPIDER COMPLEX VENOM COCKTAIL.

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The Australian Funnel-web spiders are a group of venomous arachnids restricted to the south eastern coast of Australia. These spiders carry a formidable reputation worldwide for their aggressive nature and the extreme toxicity of their venom to humans. This reputation is supported by the thirteen recorded deaths resulting from the envenomation by the male Sydney Funnel-web spider before the development of an anti-venom in 1980.

The Australian Funnel-web spiders are currently classified into two genera comprising 35 characterised species. The complex array of peptidic components in the venoms of these spiders remains largely uncharacterised. Here we present the results of a study to characterise some of the more pertinent peptide components comprising the venom of several species of Funnel-web. Using a technique combining reversed-phase high performance liquid chromatography (RP-HPLC) and reversed-phase high performance liquid chromatography/electrospray mass spectrometry (RP-HPLC/ES-MS), together with molecular biology rapid amplification of cDNA ends (RACE) methods, the study revealed numerous new families of peptide. We show that similar leader peptides are involved in the production of vastly different mature peptide toxin sequences and present evidence of post-translational C-terminal trimming of the mature sequences.

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OXY- AND SULFOGUANIDINO ANALOGUES OF HISTAMINE. SYNTHESIS AND BIOLOGICAL ACTIVITY

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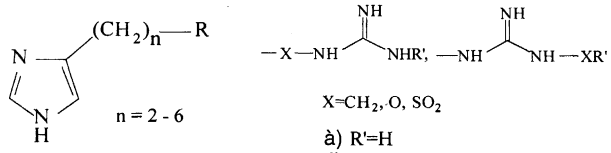
The histamine is an endogenous substance with neurotransmitter and neuromodulator functions in the organism. Its antagonists are used in the therapy of allergic diseases and inflammatory reactions and as antiulcer drugs.
The limited potentialities of the antihistamine therapy together with the increasing number of the people suffering from allergic diseases give rise to the design and synthesis of new histamine analogues as a perspective area in the chemistry of therapeutic drugs.
Additionally, the guanidine functionality has been found in many natural products and guanidine-containing molecules are found to be a critical part of many biological processes.
On the other hand, similar compounds, being a part of bigger structures (for instance peptides), can imitate the molecules of already known AT II-receptor antagonists.

Having in mind these data we aimed to synthesize new analogs of histamine containing sulfo- and oxyguanidino groups:



à) R'=H
á) R'=Boc, Cbz, Bzl

Herein we report a general, high yielding solid - phase method for the synthesis of histamines.

EFFICIENT SOLID PHASE SYNTHESIS AND BIOLOGICAL EVALUATION OF DESTRUXIN A AND RELATED ANALOGS

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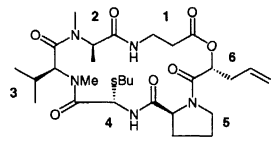
[a]Jerini Bio Tools GmbH, 12489 Berlin, Germany; [b]FMC Corporation, Agricultural Products Group, P.O. Box 8, Princeton, NJ USA 08543.



Destruxins are a class of cyclic depsipeptides originally isolated from the fungus *Oospora destructor*. The increasing interest in the insecticidal destruxins and related analogs inspired the development of an efficient stepwise solid phase synthesis protocol and the exploration of the biological effects of structural modifications to Destruxin A. Destruxin A was previously not available by solid phase procedures due to the following synthetic difficulties: i) unknown solid phase procedure for the incorporation of 2-hydroxy-pent-4-enoic acid and formation of an unusual depsipeptide bond, ii) incorporation of sterically hindered *N*-alkylated amino acids and lability of resulting *N*-alkyl amides during acidolytic cleavage, iii) high tendency of *N*-alkyl amino acids to form diketopiperazines at the dipeptide stage and iv) limited suitability of *C*-terminal residues for final cyclization. A solid phase synthesis strategy was developed circumventing the anticipated problems by means of a pre-synthesized depsidipeptide building block, selection of an appropriate *C*-terminus for resin linkage and cyclization, application of highly reactive coupling procedures and an optimized cyclization strategy. Established conditions were applied to the synthesis of 17 analogs varied at positions **2, 3** and **4** which were subsequently tested towards insecticidal activity by determinating LD_{50} -values upon injection into *heliothis virescens* larvae.

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SYNTHESIS OF TUFTSIN ANALOGUES CONTAINING N$^{\omega}$-HYDROXY-L-ARGININE DERIVATIVES

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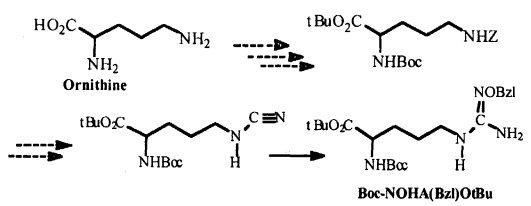
Tuftsin is a natural tetrapeptide (H-Thr-Lys-Pro-Arg-OH) which is an integral part of the C_H2 domain within the F_c fragment of the heavy chain of human immunoglobulin G, located in position 289-292. Tuftsin is enzymatically cleaved from its carrier molecule, *leukokinin*, via the action of *Tuftsin-endocarboxypeptidase* and *Leukokininase*. This molecule possesses a wide spectrum of biological activities associated with immune system functions. It is able to enhance all phagocytic cells (granulocytes and monocytes/macrophages) functions such as phagocytosis and motility, and to stimulate macrophages immunogenic response such as the antigen processing.



Tuftsin exhibits also very important antimicrobial, antiviral and anti-cancer activities. To optimize biological activity of tuftsin, we decided to incorporate in the tuftsin sequence a new α-amino acid, N$^{\omega}$-hydroxy-L-arginine (**NOHA**) which has an important role in the nitric oxide (**NO**) biosynthesis. NO produced by mammalian cells is a very powerful antimicrobial agent. On the other hand, tuftsin enhances also macrophages NO production. The syntheses of nor-NOHA and homo-NOHA containing tufsin analogs will be described. Their biological activity is under study.

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OXYTOCIN ANTAGONISTS CONTAINING NOVEL CONFORMATIONALLY CONSTRAINED AMINO ACIDS IN POSITION TWO

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Analogues of an oxytocin antagonist (Mpa-Xxx-Ile-Gln-Asn-Cys-Sar-Arg-Gly-NH_2) containing Pentafluorophenylalanine (Pff), 2-Naphthylalanine (Nal), Indanylglycine (Igl), 2,4-Dimethyl-phenylalanine (Dmf), 2,4,6-trimethyl-phenylalanine (Tmf) with D or L configurations in position 2 were synthesized, and their receptor bindings were tested on isolated guinea-pig uterus and rat kidney inner medulla plasma membranes. The peptides were synthesized in the solid phase by using racemic mixtures of Boc-(D,L) Dmf, Boc-(D,L)Tmf, Boc-(D,L)Igl or optically pure enantiomers of Boc-Pff , Boc-2-Nal and Boc-2-Igl. After liquid HF cleavage, in case of the racemic derivatives, the resulting crude diastereomeric mixtures were folded by various methods and separated by means of RP-HPLC. The determination of the absolute configuration of the Dmf and Tmf containing analogues are in progress. The binding to the oxytocin receptor in several cases were even higher than the parent hormone (oxytocin), although the V_1 receptor affinities were only slightly decreased, therefore the OT/V_1 receptor selectivity proved not enough high in most cases. However, the real pharmacological value of these analogues can be evaluated after *in vivo* measurements of their inhibition of uterine motor activity, which is in progress. Interestingly, several constrained phenylalanine containing analogues previously proved to be valuable in vivo antagonists[1], while these analogues displayed far lower oxytocin receptor affinities than the upper amino acid containing peptides[2]. This is further evidence of the crucial role of the amino acid residue in position 2 for receptor binding and antagonistic properties.

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EFFECTS OF SYNTHETIC GALANINS (GALs) AND THEIR FRAGMENTS ON THE CONTRACTILE ACTION AND ON THE RELEASE OF ACETYLCHOLINE (ACh) IN RAT BRAIN: SOME STRUCTURE-ACTIVITY RELATIONSHIPS.

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GAL is a 29/30-amino acid residue neuroendocrine peptide that is present in specific neuronal systems in the brain spinal cord. It is also found in neurons and neuroendocrine cells in a variety of peripheral tissues. GALs have been shown to coexist with other hormones and neurotransmitters in various brain regions. We have synthesized human, pig, rat, bovine and chicken GALs and a number of N- and C-terminal larger and smaller fragments by solid-phase synthesis. The crude peptides were purified by RP-HPLC and the purities were controlled. Most of the GALs were examined as concerns their contractile effects on the smooth muscle in the rat, guinea pig and dog ileum and these effects were compared with those of standard rat and pig GALs, substance P and ACh. Neurochemical, histochemical,, and immunohistochemical observations revealed that the elements of the cholinergic system are reduced, whereas the number of GAL-positive nerve fibers is significantly increased in Alzheimer's disease (AD). The number of excitatory cholinergic neurons in the basal forebrain is reduced and the number of inhibitory GAL-containing nerve fibers is increased in AD. It is therefore suggested that GAL may be involved in the etiology of the disease. In our studies, we compared the effects of GALs on the basal release of ACh and that evoked by K^+ or electrical stimulation in different areas of the CNS of the rat. The biological findings concerning the contractile effects of different GALs on the smooth muscle and the ACh release effects of the same GALs were compared as structure-activity relationships. This comparison (among others) clearly showed that the two actions of GALs occur on different GALR subreceptors. The results demonstrate that porcine and human GALs and some C-terminals fragments can modulate the *in vivo* release of ACh. GALs are definitely regulatory peptides. They act as neurotransmitters and neuromodulators. Supported by OTKA (T 030526, T 022683, T 26624 and T 019306).

SINGLE MUTATION IN STROMA CELL-DERIVED FACTOR-1α ABROGATES BINDING TO HEPARIN.

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Chemokines are small proteins which interfere with the HIV entry in cells. This entry is mediated by interactions between CD4 / Chemokines receptors and the viral envelope GP120. Furthermore, biological properties of chemokines are believed to be influenced by their association with glycosaminoglycans.
We have already identified one heparin-binding site on the chemokine Stroma cell-Derived Factor 1α (SDF-1α), as a cluster of 3 basic residues (1). Looking closer to the SDF-1α / heparan sulfates interaction, we synthetised 10 new proteins, each bearing a single mutation. Their interaction with heparin was measured by (i) passing through an heparin column and (ii) by using the surface plasmon resonance technology (Biacore). The results revealed that for two of the mutated proteins, one single mutation is sufficient to drastically reduce their affinity for heparin. These two basic amino-acids are located in the previously identified heparin biding site. None of the other mutation affected so drastically the binding to heparin, confirming that we have identified the main heparin binding site of SDF-1α.

(1)Stromal cell-derived factor-1α associates with heparan sulfates through the first ß-strand of the chemokine.
A. Amara, O. Lorthioir, A. Valenzuela, A. Magerus, M. Thelen, M. Montes, J.L. Virelizier, M. Delepierre, F. Baleux, H. Lortat-Jacob and F. Arenzana-Seisdedos
The Journal of Biological Chemistry, 1999, *274(34)*, 23916-23925

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PYROGLUTAMIC FRAGMANTS OF OXYTOCIN (OT), VASOPRESSIN (AVP) AND DESMOPRESSIN WITH *L*-ORNITHINE IN POSITION 6

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Fragments of oxytocin and vasopressin (OT and VP) that are produced in the brain by the action of enzyme systems have a higher effect on learning and behaviour than the natural hormones. The common feature of the hexa- or pentapeptides is their N-terminal pyroglutamic acid residue, while the C-terminal residue is either glycine amide or the penultimate amino acid (i.e. leucine or arginine). If the peptides are administered on the periphery, their transport into the brain is limited. In an attempt to improve the ability of the peptides to penetrate the blood-brain barrier, we prepared analogues of OT, VP and Desmopressin, in which the cysteine residue in position 6 was substituted by L-ornithine.
The general formula of the peptides is:

pGlu – Asn – Orn – Pro – X

where **X** is ***Leu – GlyNH$_2$***, ***Arg – GlyNH$_2$*** *or* ***D-Arg – GlyNH$_2$***.

The peptides were synthetized on methylbenzhydrylamine resin according to Merrifield. The course of the reaction was followed using Kaiser's test. After the reaction had been completed, the peptide as well as the protecting groups were split off by liquid hydrofluoric acid, the product was extracted by 20% acetic acid, purified by semipreparative RP-HPLC, and characterized by MS and capillary electrophoresis. The influence of the Desmopressin fragment on the behaviour of rats was tested in a T- or Y-shaped maze. Amnesia was evoked by the anticholinergic compounds scopolamine and 3-quinuclidynylbenzylate. When the peptide was administered in daily doses of 50g/kg for 5 days prior to the application of the anticholinergic compounds, it lowered amnesia significantly and the animals recalled learned reactions better. Further peptides with modifications of the amino group of L-ornithine are being synthetized and characterized.

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EXAMINING THE INFLUENCE ON CONFORMATION AND BIOLOGICAL ACTIVITY OF PENICILAMINE^6-5-TERT-BUTYLPROLINE^7 IN OXYTOCIN ANALOGUES

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In the context of our program to study the relationship between prolyl amide conformation and peptide activity, we introduced 5-*tert*-butylproline (5-*tert*-BuPro) into a series of oxytocin analogues. The steric interaction between the *tert*-butyl and Cys^6 residues augmented the Cys^6-5-*tert*-BuPro^7 amide *cis*-isomer population. Furthermore, analysis of the biological activity of the 5-*tert*-BuPro^7-oxytocin analogues provided additional support for the hypothesis that the prolyl amide geometry may differentiate between agonist and antagonist activity. Attempting to augment the steric interactions that favor the prolyl *cis*-isomer population, we have now begun to explore the equilibrium of the penicillamine^6-5-*tert*-butylproline^7 motif *en lieu* of the Cys^6-Pro^7 portion of the peptide hormone. Conformational analysis, using 2D NMR experiments (TOCSY and ROESY), has shown that penicillamine (Pen) *N*-terminal to 5-*tert*-butylproline can augment the population of the amide *cis*- isomer. Comparison of *N*-Fmoc-(S-*p*-MeOBn)Pen-5-*tert*-BuPro-OAllyl and *N*-Fmoc-(S-Tr)Cys-5-*tert*-BuPro-OAllyl have revealed an augmentation of 14% in *cis*-amide population (from 64% for the Cys dipeptide to 80% for the Pen dipeptide). These preliminary results have prompted us to synthesize Pen^6-5-*tert*-BuPro^7-oxytocin analogues and their proline counterparts on solid support. Synthesis, conformational as well as biological analysis of these peptides will be presented.