SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P01

SYNTHETIC STUDIES OF MEMBRANE PROTEIN BASED ON LIGATION CHEMISTRY

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In the process of developing a method for the synthesis of membrane proteins we examined a synthetic strategy that involved the use of both the thioester method (Hojo, H., Aimoto, S., Bull. Chem. Soc. Jpn, 64 (1991) 111) and the native chemical ligation method (Dawson, P. E., Muir, T. M., Clark-Lewis, I., Kent, S. B. H., Science, 266 (1994) 776) for a single peptide synthesis. As a model compound we chose the C-terminal region of opioid receptor like 1, ORL1(251-370), which contains two transmembrane and the C-terminal intracellular domains. For the synthesis, we prepared three building blocks, Fmoc-ORL1(251-287)-SR (1) and Fmoc-ORL1(288-328)-SR'(2) and ORL1(329-370) (3). In the preparation of building blocks 1 and 2, Arg5 was introduced to R and R' moieties to enhance the solubility of the peptides. For the coupling of building blocks 2 and 3 via the native chemical ligation method, the reaction conditions, namely, the concentration of detergent and the chemical characteristics of the thiol additive were investigated in detail. For the second condensation via the thioester method, thiosulfonate and Boc groups were introduced to Fmoc-ORL1(288-370) (4) and peptide 1, respectively, to protect the thiol and amino groups. Thiosulfonate groups on the thiol groups were stable in the presence of silver ions, thus permitting the native chemical ligation and the thioester methods to be merged (Sato, T., Aimoto, S., Tetrahedron Letters, 44 (2003) 8085). Building blocks 1 and 4 were successfully condensed in the presence of silver ions to give ORL1(251-370).

P02

MICROWAVE - ASSISTED SYNTHESES OF PEPTIDE ISOSTERE LIBRARIES EMPLOYING AMINO ACYL KETENES

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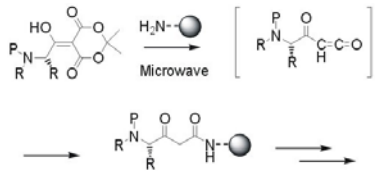
Reagent linkers have been developed as a novel concept allowing for solid phase CC-couplings under very mild conditions.(1,2) In this work, polymer-supported phosphoranes were demonstrated as efficient acylanion equivalents. As the next step we wished to extend the mild, polymer-supported C-acylation chemistry – mimicking the polyketide pathway - towards malonic acid derivatives. For this purpose, Meldrum´s acid was selected as a C-nucleophile in solution and on the solid support. Meldrum´s acid was acylated with amino acids and the racemization of this reaction was minimized. The raw product could be directly transformed into amino acyl ketenes by microwave irradiation, the reactive intermediates were scavenged with polymer-bound nucleophiles. Employing this straightforward approach, various peptide isostere libraries were accessible. Moreover, amino acyl ketenes have been found to be surprisingly general intermediates. References: 1) S. Weik, J. Rademann, Angew. Chem. 2003, 115, 2595-2598; Angew. Chem. Int. Ed. 2003, 42, 2491-2494. 2) J. Rademann, 'Novel polymer- and linker reagents employed for the preparation of protease inhibitor libraries' in: Highlights in Bioorganic Chemistry, H. Wennemers, C. Schmuck (Eds.), Wiley-VCH, Weinheim, 2004, 277-290.



P03

SOLID-PHASE SYNTHESIS OF CYCLODEPSIPEPTIDES. USE OF HEXAFLUOROACETONE FOR PROTECTION/ACTIVATION OF HYDROXYACIDS

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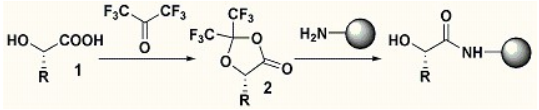
Cyclodepsipeptides are an important class of natural products. Although they exhibit a broad variety of biological activities, their greatest therapeutic potential is as anticancer agents. Thus, some are currently in clinical trial phases. From a topology point of view, natural cyclodepsipeptides can be divided in three classes: head-to-tail, side chain-to-head, and antiparallel dimer. Herein, novel methods for the preparation of representative examples of these cyclodepsipeptides will be discussed. Hexafluoroacetone (HFA), a novel bidentate protecting/activating reagent for a-hydroxy acids 1, constitutes the cornerstone for obtaining head-to-tail cyclodepsipeptides. Dioxolanes 2 are readily available in good yields on reaction of 1 with HFA. In one stereoconservative step, the carboxylic group is activated towards nucleophiles and the a-hydroxy group is protected. The activated carboxylic group of 2 reacts smoothly with the amino function of a peptide resin with concomitant deprotection of the a-hydroxy group, which will be further acylated with the next protected amino acid, thereby yielding the depsipeptide. Side chain-to-head and antiparallel dimer cyclodepsipeptides are prepared by regioselectivity methods based on the concourse of orthogonal-protecting groups (Fmoc, Boc, Alloc/Al, Trt, and the newly developed p-nitrobenzyloxycarbonyl, pNb). Acylation of N-methyl amino acids, which are very common in this kind of compounds, is performed using well established coupling reagents. Final cyclization conditions, which are discussed in each case, minimizes racemization and trifluoroacetylation, an undesirable side-reaction. Examples will include T987 A and B, dolastatin D, thiocoraline, IB01212, and Kahalalides.



P04

INCREASING THE CONFORMATIONAL STABILITY OF ALPHA-CONOTOXIN IMI BY SUBSTITUTION OF DISULFIDE BONDS WITH ISOSTERIC DISELENIDE BONDS

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Disulfide bonds are important structural motifs in may biologically significant peptides which help to maintain the bioactive conformation. However, peptides containing multiple disulfide bonds are conformationally unstable under reducing conditions, such as in a thiol rich environment. We have demonstrated the use of non-reducible diselenide bonds to increase the stability of alpha-conotoxin ImI. Three analogues were investigated with systematic replacement of one ([Sec2,8]-ImI and [Sec3,12]-ImI) or both ([Sec2,3,8,12]-ImI) of the disulfide bonds with isosteric diselenide bonds. A robust and efficient procedure for the synthesis of diselenide containing mutants of conotoxins by Boc-SPPS is described, which avoids side reactions such as beta-elimination and racemistaion. Formation of diselenide bonds in aqueous buffer yielded the correct isomer as confirmed by NMR spectroscopy, which also revealed that each analogue exhibited structure isomorphic to native ImI. Furthermore, full activity at the alpha-7 nAChR was retained, with each analogue demonstrating no significant difference in the EC50. The diselenide analogues exhibited remarkable stability under reducing conditions. These properties make diselenide-containing peptide analogues applicable to the development of disulfide-rich peptide-based drugs that are required to maintain conformational stability in a reducing environment.

SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P05

REVERSIBLY CROSS-LINKED DENDRIMERS - A STRATEGY FOR THE FACILE SYNTHESIS AND COMBINATORIAL VARIATION OF COMPLEX, MULTIVALENT PROTEIN-MIMICS

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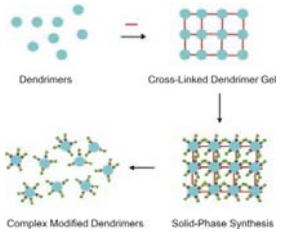
Dendrimers are powerful tools to control biomedical processes including immunization, drug-delivery, transfection, and adhesion. Hitherto, the synthesis of complex derivatized dendrimers in multiple steps is difficult requiring tedious work-up procedures. We have developed a novel concept for the synthesis of peptide-modified dendrimers on the basis of a reversibly cross-linked polymer support.[1] Ultraresins are new high-loading supports constructed of cross-linked polyethylene imine. [2,3] Highly branched polyethylene imine (Mn 10,000) was cross-linked with a silyloxy-tether to yield a rugged, swellable resin. This support was employed efficiently in solid phase peptide synthesis at up to 3 mmol/g loading. After the peptide assembly, the resin was decomposed yielding large, multivalent peptide dendrimers. The complete disintegration of the resin was proven by GPC and NMR. Cellular uptake of these novel protein mimics (Mn 24-53 kDa) was demonstrated in HeLa-cells by living cell confocal fluorescence microscopy. Literature: 1) M. Barth, R. Fischer, R. Brock, J. Rademann, submitted for publication. 2) J. Rademann, M. Barth, 5Angew. Chem., 2002, 114, 3087-3090; Angew. Chem. Int. Ed., 2002, 41, 2975-2978. 3) M. Barth, J. Rademann, J. of Comb. Chem., 2004, in press.



P06

PEPTIDE - OLIGONUCLEOTIDE CONJUGATION. A NEW SYNTHETIC APPROACH

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The objective of this study was to develop a new method for the synthesis of peptide - oligonucleotide hybrids. The syntheses of peptide and oligonucleotide (ODN) are well established, however, they are incompatible with the sequential syntheses of peptide - oligonucleotide conjugates. The major obstacle being that under the conditions required for peptide deprotection, the ODN is not stable. In the present study we have initiated a new strategy for a stepwise synthesis of peptide - oligonucleotide hybrids. The approach is to design appropriate protecting groups for both: the a-amino site and the side chains that can be cleaved under mild conditions. The preferred a-amine protecting group is nitrophenyl sulphenyl group (NPS) that is cleaved by 1M thioacetamide in 3% dichloroacetic acid (DCA). The side chains protecting groups, such as silyl (TIPS, TBDMS or BnSyl) or fluorenyl (Fm, Fmoc), were selected because they can be cleaved by fluoride ion. The nucleotides that were employed are Abz(Fmoc), Cbz(Fmoc), T and Gt-Bu(Fmoc). Instead of the sensitive phosphorimidites commonly used for coupling we have introduced the hydrogen phosphonate residue, which can be coupled by peptide coupling reagents. The assembly of both features was carried out either on Pam resin or Fm resin.

P07

DEUSS: A NEW RESIN FOR SOLID-PHASE SYNTHESIS AND HRMAS NMR CHARACTERIZATION OF SOLID-SUPPORTED MOLECULES

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Introduced by Merrifield in 1963, the polystyrene resin is certainly the most widely used polymer for solid-phase organic and peptide synthesis. In the last decade, however, a big research effort have been made to develop new polymers with alternative physico-chemical properties. For example, biocompatible solid supports with increased polarity, such as Tentagel, PEGA and POEPOP, have been conceived and prepared to improve on-bead biological assays. These polymeric materials proved also to be of great utility for the characterization of the molecules covalently linked to the insoluble matrix using the powerful HRMAS NMR spectroscopy. Some of them greatly improved the quality of HRMAS spectra allowing to reach a resolution very close to the NMR in solution. However, the proton signals of the resin often cover part of the spectrum and overlap with the resonances of the bound molecule. Therefore, the elimination of the matrix peaks from the HRMAS spectrum would result of great advantage. About fifty years ago, deuterated solvents revolutionized the NMR technique since they opened the possibility of acquiring spectra devoid of the contribution of the solvent protons. Having this idea in mind, we have conceived and developed a novel solid support based on a cross-linked perdeuterated polyethylene glycol chain. In this Communication, we present the first water-compatible fully-deuterated resin called DEUSS (perDEUterated Solid Support). DEUSS is suitable for the solid-phase synthesis of peptides, synthetic oligomers and small organic molecules. In addition, this resin allows the easy structural characterization of the linked compounds using the HRMAS NMR spectroscopy.

P08

ORTHOGONALLY-PROTECTED LANTHIONINES AS BUILDING-BLOCKS FOR THE SOLID-PHASE SYNTHESIS OF LANTIBIOTICS

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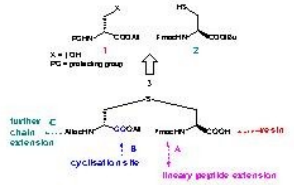
This work belongs to the general approach developed in our laboratory regarding the solid-phase synthesis of side-chain bridged peptides. More precisely, it focuses on the synthesis of peptides containing a thioether bridge, subunits of the class of antibiotics: lantibiotics. For this purpose, the synthesis of the orthogonally-protected lanthionine 3 from serine-derived building blocks 1, and protected cysteine 2, was envisaged. From these building blocks, several approaches have been explored to achieve the synthesis of the desired target in a regio- and stereoselective manner. These approaches required the use of different protecting groups on the serine moiety, or alternatively the use of a direct Mitsunobu reaction using the cysteine 2 as nucleophile. Results of the different pathways will be discussed in detail. The validity of the strategy using orthogonally-protected lanthionine to obtain thioether-bridged peptides has then been illustrated by the successful synthesis on solid support of analogues of the ring C and B of Nisin. These peptides have been obtained and characterised by Mass Spectroscopy and NMR.



SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P09

AN INFLUENCE OF REACTION CONDITIONS ON DISULPHIDE BOND FORMATION EFFICIENCY IN SYNTHESIS OF HIV PEPTIDES

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Numerous examples of biologically active disulphide bond containing peptides comprises hormones, neurotransmitters, defensines, toxins, growth factors and antigenic determinants of viral proteins. In many types of diagnostic systems synthetic antigens possess significant advantages as compared to the natural ones in terms of safety, costs and results reproducibility. However, in restrained terms, disulphide bridge formation may represent a source of specific side reactions reducing the yield and complicating purification of desired product. We have studied an influence of cylization conditions and peptide structure on side products nature and peptide yield in synthesis of gp 36 and gp 41 HIV proteins antigenic determinants. At the stage of disulphide bridge formation there was applied iodine solution in acetic acid, hydrogen peroxide and air oxidation. It was shown that for hydrophobic 35-mer gp 41 fragment, side products formation was due to its high aggregation potential even in 50% of water/acetic acid. In this case an addition of 5 M guanidine chloride solution was efficient both for the increasing of product yield and purity. An efficiency of cyclization process for gp 36 antigenic determinants containing common peptide fragment was strongly dependent on peptide chain length and amino acids composition. Both side products nature and utility of oxidizing reagent was quite different depending on parent peptide structure. In some cases an elongation of peptide chain was accompanied by significant decrease of iodine cyclization efficiency in favour of air oxidation.

P10

REEXAMINATION OF INTRAMOLECULAR HECK METATESIS IN PEPTIDES

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Bar Ilan University/Chemistry, Ramat Gan, Israel

In our search for highly selective alphaV-beta5 peptidomimetic ligands derived from the known RGD sequence we have reexamined the Heck metatesis for obtaining highly constrained peptides. We have both reexamined the solid phase cyclization as well as the cyclization in solution . The reaction has in general a slow kynetics for both SPPS and solution cyclization as demonstrated by HPLC-MS analysis. We have found that the reaction can take between 7-14 days for a complete conversion to the desired products. Different sequences needed different times for completion. New extensions to double loop Heck metatesis will be discussed.

P11

A PLATFORM TECHNOLOGY FOR SYSTEMATIC, AUTOMATED AND HIGH THROUGHPUT PROTEIN EXPRESSION AND PURIFICATION

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Drug discovery and disease scientists increasingly require protein samples fit for purpose in research involving techniques such as crystallography, high throughput screening, antibody production and characterisation, and protein microarrays. This has led to a growing need for new tools to overcome the problems associated with protein expression. This presentation will discuss the design and development of a unique combination of biology, hardware and software tools that enable the entire process of expression vector construction, protein expression and subsequent purification to be automated. The result is a technology enabling systematic and parallel production of many hundreds of purified proteins with minimum hands-on time. A range of unique expression vector systems, which incorporate a series of fusion partners together with affinity tags for subsequent purification to enable optimal soluble protein expression, will also be discussed. The technology integrates a unique information management system for sequence, sample and protein tracking. It is possible to start with PCR products and cDNA clones and plan, schedule, automate and track the whole process of sub-cloning, expression and purification of hundreds of proteins in parallel. The use of the system will be demonstrated with the production of tagged marker and target proteins suitable for use on a protein microarray and breast cancer study.

P12

LANTHIONINE MIMETICS FROM CYSTEINE

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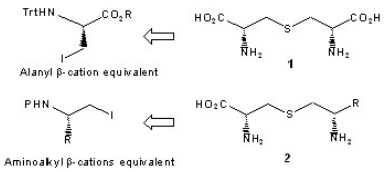
The unusual amino acid lanthionine 1, is the key component of lantibiotic peptides with important biological properties. Incorporation of lanthionine into a peptide results in a cyclic structure, bridged by a thioeter, which cannot be reductively cleaved, and additionally imparts stability to proteolytic cleavage (1). In this context, the synthesis of analogues of bioactive peptides incorporating lanthionine, has received considerable attention in recent years. Here we reported the synthesis of three orthogonally protected (2R)-3-[(2'S)-2'-aminoalkyl]thio-propanoic acid derivatives, 2 that formally contains an amino acids side chain R in the place of one carboxylic group present in natural lanthionine. The synthesis has been realized through the S-alkylation of (L)-cysteine ethyl ester with the chiral N-Boc protected-(L)-beta-Iodoamines (2) obtained from some representative (L)-amino acids, followed by ester hydrolysis and N-alpha-Fmoc-protection. 1)Jack, R.W., Jung, G., Curr. Opin. Chem. Biol., 2000, 4, 310–317 and reference cited therein. 2)Caputo, R.; Cassano, E.; Longobardo, L.; Palumbo, G., Tetrahedron Lett., 1995, 36, 167-168.



SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P13

IODOMETHYLENE DERIVATIVES OF ASPARTIC AND GLUTAMIC ACIDS IN PEPTIDOMIMETIC CHEMISTRY

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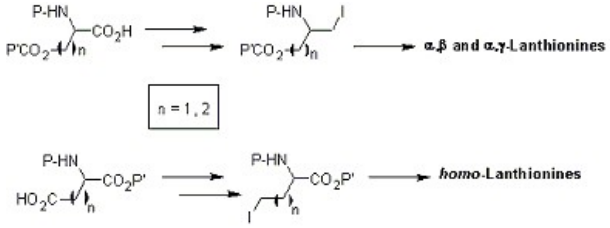
The N-protected beta-Iodoamines obtained from protein amino acids, are new building blocks with promising application in peptidomimetic chemistry [Org. Lett., 2000, 2, 2265]. Here we report the synthesis and the utilization of a series of Iodomethylene derivatives coming from orthogonal protected glutamic and aspartic acid (fig 1). These building blocks, having Boc/Fm protections, has been used in solid-phase thioalkylation of cysteine for the preparation of macrocyclic thioeter of bioactive peptides [Tetrahedron Lett., 2004, 45, 1453]. Also, several other lanthionine like-molecules have been prepared by solution synthesis. The thioalkylation of cysteine with the alpha-iodomethylene derivatives of Asp and Glu furnish the hybrids alpha,beta and alpha,gamma-Lanthionines respectively. Using an appropriate iodomethylene derivatives in side chain, it is possible an easily preparation of homo-Lanthionine



P14

SYNTHESIS OF DIPHENYLALANINE (DIP) AND ITS CYCLOPROPANE ANALOGUE (C3DIP) IN ENANTIOMERICALLY PURE FORM BY HPLC RESOLUTION

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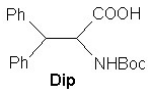
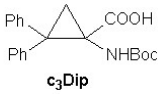
The incorporation of non-natural amino acids into peptides constitutes a strategy widely used in the design of peptide analogues with improved pharmacological properties as well as in structure-activity relationship studies. In this context, the biological significance of phenylalanine, which is located in the pharmacophoric regions of many bioactive peptides and often acts as a key residue in peptide-receptor recognition, has stimulated the synthesis of different phenylalanine surrogates. Among them, diphenylalanine (Dip) bears an extra phenyl group on the beta carbon. This modification confers on it very hydrophobic character, which may favour processes such as crossing membrane barriers and binding to the apolar site of a receptor. Moreover, in the cyclopropane analogue of Dip (that we denote as c3Dip) rotation about the Calpha-Cbeta bond is prohibited and the orientation of the phenyl substituents is therefore fixed, which could bring about additional structural and biological consequences. We have developed very efficient methodologies for the synthesis of Dip and c3Dip starting from easily available materials and through high-yield transformations. Multi-gram quantities of both amino acids, adequately protected to be used in peptide synthesis, have been prepared. In each case, the HPLC resolution of a precursor using a non-commercial polysaccharide-derived chiral stationary phase has allowed us the isolation of enantiomerically pure products.



Dip



c_3Dip

P15

TOTAL CHEMICAL SYNTHESIS OF HEW LYSOZYME

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Hen egg white (HEW) lysozyme is a disulfide-crosslinked secretory enzyme that catalyzes the hydrolysis of specific kinds of polysaccharides found in the cell walls of bacteria. Mature HEW lysozyme has a 129 amino acid polypeptide chain that contains four disulfide bonds and has five alpha-helices, in addition to five beta-strands which are organized in two antiparallel sheets. We are synthesizing HEW lysozyme to study the mechanism of enzyme catalysis using the tools of chemistry. Our synthetic design uses the native chemical ligation (1) of four unprotected peptide segments: (1-29)-thioester, (30-63)-thioester, (64-93)-thioester and 94-129. Peptide segments were prepared by manual SPPS and characterized by LC-MS. Two peptides, (30-63)-thioester and (64-93)-thioester, had small des-Thr and minus 114Da impurities, respectively, which were difficult to purify away from the desired peptides. Syntheses of these two segments were optimized. The ligation reaction of (1-29)-thioester with (30-129) was very slow, because of the Val-Cys ligation site (2). The peptide segment (1-29)-thioester was re-synthesized using Nor-leucine as a C-terminal residue. The total synthesis of lysozyme and the characterization of the synthetic enzyme will be presented 1. Dawson, P., Muir, T., Clark-Lewis, I., Kent, S. Science 266, 776 (1994). 2. Hackeng, T. M., Griffin, J.H., Dawson, P. E. Proc. Natl. Acad. Sci. USA 96, 10068 (1999).

P16

THE 2-AMINO-4-PYRROLIDINOTHIENO[2,3-D]PYRIMIDINE-6-CARBOXYLIC ACID AS N-TERMINAL SURROGATE IN PEPTIDE ANALOGUES

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Research on amino acids has gained enormous popularity in recent years, particularly with the emergence of non-natural analogues as components of molecules with therapeutic potential. The need to replace natural amino acid residuals in peptides with non-proteinogenic counterparts in order to obtain drug-like target molecules has stimulated a great deal of innovation on several fronts. The use of natural peptides as pharmaceuticals suffers from several constraints such as relatively small bioavailability, lack of transportation, or rapid metabolic degradation. The incorporation of or substitution by a hetero ring (or heterocycle) at selected positions along a peptide skeleton acting (or to act) as a conformationally restricted core, has been recently employed to prepare modified peptides (semi-mimetics) with better in vivo efficacy than its natural congener. The replacement of proteinogenic amino acids with unusual compounds has developed into a well-respected method for the study of local conformational states on biological activity and for the preparation of new drug candidates. Our aim was to examine if the new synthesized compound 2-amino-4-pyrrolidinothieno[2,3-d]pyrimidine-6-carboxylic acid (ATPC) is suitable for use in peptide synthesis conditions in order to prepare modified peptides with ATPC as N-terminal. Accordingly, we have coupled 8 representative amino acids with the ATPC and we have shown that ATPC can be incorporated as N-terminal moiety for the preparation of acid, amide or ester peptide derivatives, side-chain protected or not, using Boc or Fmoc methodology, in solution or solid phase peptide synthesis and could be a useful tool as N-terminal surrogate in peptide design and synthesis.

SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P17

SYNTHESIS AND CONFORMATIONAL STUDIES OF A NEW OCTREOTIDE ANALOGUE

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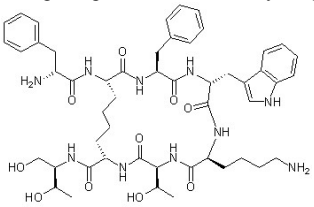
Octreotide is a somatostatin analogue and it is able to inhibit the growth of tumor cells binding to surface somatostatin receptors, mainly the hsst2 subtype. However, it is well known that the disulfide linkage is chemically and metabolically subjected to reducing environments and nucleophilic and basic agents. Thus, the synthesis of dicarba analogues, where the sulfur atoms have been substituted by methylene groups, is greatly encouraged. We designed the synthesis of a dicarba analogue of octreotide by solid phase peptide synthesis (SPPS). As the first step, we prepared the constrained cyclic octapeptide containing a 1,4-disubstituted 2-butene bridge [1]. The amino acid sequence was elongated following the Fmoc/HATU strategy. The cyclization was performed by RCM Grubbs catalyst. The unsaturated peptide was first reduced by using 10% Pd/C in H2 atmosphere affording the desired product 1 in low yield. In a second attempt we reduced the parent cyclooctapeptide under H2 atmospheric pressure using Pd(OH)2/C as a catalyst obtaining in this case an higher yield of the carbacyclic analogue. The saturated cyclooctapeptide 1 was purified by semipreparative RP-HPLC. 1HNMR studies on the conformational structures of the phermacophor portion are currently in progress. [1] Paper submitted.



1

P18

PEPTIDE CYCLISATION BY THIOETHER FORMATION, A COMPARISON OF DIFFERENT METHODS

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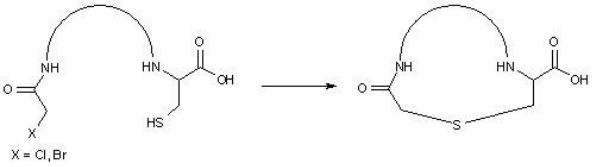
Peptide cyclisation is frequently used to restrict the conformational freedom of a peptide, for example to enhance its capacity for selective binding to a target receptor molecule. Peptides can readily be cyclized by disulfide formation, but these disulfides might not possess the desired stability because they can easily be reduced to the open structures. Cyclisation by reaction of a haloacetyl group with a thiol group in the same peptide is preferred because stable thioether based cyclic structures are obtained. It has been shown before (e.g. [1]) that cyclisation kinetics are sequence dependent. In case intramolecular reaction is slow, multimerization can be a serious side reaction. Here we compare different strategies for this type of cyclisation. As a model system we used several different peptide sequences all having a cystein residue in the C-terminal part and a haloacetyl group at the N-terminus. Several cyclisation strategies were compared and evaluated in terms of reaction speed and purity of the products. a.)Comparison between bromo- and chloroacetyl moieties b.)Comparison between reaction in aqueous and organic media c.)Comparison between solution and solid phase 1. Robey, F.A., J. Peptide Res., 56: 115-120 (2000).



P19

DESIGN AND SYNTHESIS OF CHIRAL CYCLIC ALPHA,ALPHA-DISUBSTITUTED ALPHA-AMINO ACIDS AND ITS PEPTIDES

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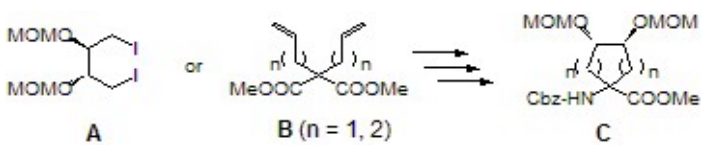
We studied two synthetic route toward chiral cyclic alpha,alpha-disubstituted amino acids, in which the alpha-carbon atom is not a chiral center and the asymmetric centers exist at the side chain. The first synthetic route is as follows: Dimethyl L-(+)-tartrate was converted into a diiodide (A) by conventional procedures, and then dimethyl malonate was alkylated with the diiodide to give a cyclic diester. Monohydrolysis of diester, followed by Curtius rearrangement with DPPA afforded optically active cyclic alpha,alpha–disubstituted alpha–amino acid (C) (n=1). The second route is a chemoenzymatic one. Racemic trans-cycloalkane-1,2-diols were prepared from dialkenyl malonate (B) by Grubbs-reaction, epoxidation, and acidic hydrolysis. Kinetic resolution of the racemic 1,2-diols using Amano PS afforded optically active monoacetates. Monohydrolysis of diesters, followed by Curtius rearrangement with DPPA afforded (C) (n=1, 2). The homooligopeptides composed of amino acids (C) were prepared by solution-phase methods.



P20

PROGRESS TOWARDS A CARBA-ANALOG OF LEUCOCIN A

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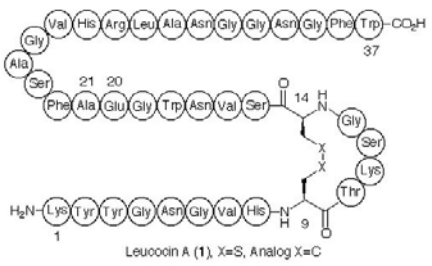
Department of Chemistry, University of Alberta, Edmonton, Canada

It has recently been shown that carba-analogs of disulfide bridge containing peptides such as oxytocin, retain biological activity. In order to extend this methodology, it was applied to a more complex target, namely Leucocin A. (1). The convergent synthesis of 1 was envisaged to require two peptide fragments prepared separately using SPPS. The N terminal portion incorporated allyl glycine residues at positions 9 and 14 so that treatment under ring closing metathesis (RCM) conditions produced the cyclic carba-analog portion of Leucocin A. The C terminal portion (residues 21-37) was prepared with a Cys residue at position 21, and the N terminal unit (residues 1-20) as the C terminal thioester to fulfill the requirements for native chemical ligation. Upon completion of this synthesis, NMR characterization and biological testing will be compared to the parent compound.



SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P21

INTRAMOLECULAR CARBAMOYLIMINIUM ACTIVATED SOLID PHASE PICTET-SPENGLER REACTIONS. TOWARDS MCR AGONISTS

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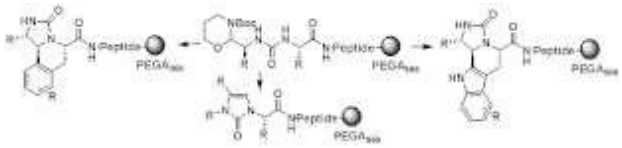
SPOCC-Center, Carlsberg Laboratory, Valby, Denmark

A range of small natural and synthetic peptides containing a His-Phe-Arg-Trp sequence act as non-selective melanocortin receptor (MCR) agonists or antagonists.1 With the aim of preparing libraries of selective MCR agonists, peptides were constrained into peptidic heterocycles via nucleophilic addition to carbamoyliminium intermediates e. g. in the Pictet-Spengler condensation, which has been widely used in the formation of large heterocycles and alkaloid synthesis. This intramolecular Pictet-Spengler condensation stereoselectively form multicyclic compounds. The use of amino aldehyde building blocks containing 3-Boc-1,3-oxazinanes (Box) for aldehyde protection,2 enables coupling to the N-terminal of peptides to form a urea bond. Liberation of the aldehydes on solid support by acid catalysed aldehyde deprotection followed by iminium ion formation and intramolecular Pictet-Spengler condensation provided the multicyclic products in one step. A range of different multicyclic scaffolds was obtained through this method and also peptidic 1,4-substituted imidazolones could be synthesised. As a part of this work new analogs to the Box protecting group for use in solid phase synthesis have been developed. [1] C.Haskell-Luevano, S.Hendrata, C.North, T.K.Sawyer, M.E.Hadley, V.J.Hruby, C.Dickinson, I.Grant, J.Med.Chem. 1997, 40, 2133 [2] T.Groth, M.Meldal, J.Comb.Chem. 2001, 3, 45



P22

PEPTIDE LIGATION FOR THE MIMICRY OF PROTEIN BINDING SITES: COPPER-CATALYZED FORMATION OF [1,2,3]-TRIAZOLES ON SOLID PHASE AND IN SOLUTION

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A crucial step in the generation of assembled or scaffolded peptides for the mimicry of discontinuous protein binding sites is the site-selective linking of peptide fragments with each other, or their attachment to defined sites of a molecular scaffold [1]. We have examined the method for the synthesis of peptidotriazoles, proposed by Meldal et al. [2], for their utility to generate assembled and scaffolded peptides. Peptide precursors containing all 20 proteinogenic amino acids were synthesized on solid phase, and N-terminally modified with azido- and ethinyl-moieties, respectively. Ligation of matching precursors through formation of [1,2,3]-triazoles, on solid phase and in solution, yielded assembled peptides in excellent purity. Scaffolded peptides presenting more than two peptide fragments were generated using this method to attach protected peptide fragments to the support-bound scaffold. [1] R. Franke, C. Doll, V. Wray and J. Eichler, Protein Peptide Lett., 2003, 10, 531-539. [2] C.W. Tornoe, C. Christensen, M. Meldal, J. Org. Chem. 2002, 67, 3057-3064.

P23

SWITCH ON STRUCTURE AND FUNCTION: PEPTIDE PRODRUGS AND BIOSENSORS BY ENZYME-TRIGGERED X, N- ACYL MIGRATION

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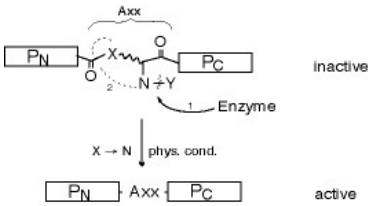
Intramolecular X ? N acyl transfer reactions have recently found considerable attention, notably in peptide ligation, solubilization and prodrug design. In masking the nucleophile within the switch-element (Axx, Figure) connecting N- and C-terminal peptide segments (PN, PC) by enzymatically cleavable protecting groups Y, the evolution of the native peptide bond as key step for the onset of structure and function can be tailored at purpose. Here, we elaborate the use of trypsin-, esterase-, penicillin amidase- and pyroglutamate aminopeptidase- specific cleavage sites in switch-peptides derived from bioactive sequences (angiotensin II, NPY (21-36), amyloid ?, zinc finger protein). As shown by conformational and pharmacological studies (CD, IR, NMR, TEM, receptor binding), the onset of structural changes and biological function in situ by enzyme triggered acyl migration can be modulated over a broad time range, opening interesting perspectives in prodrug design and biosensor technology. [1] M. Mutter, C. Arunan, C. Boyat, S. Dos Santos, B. Mandal, R. Mimna, K. Murat, L. Patiny, L. Saucède, G. Tuchscherer., Angew. Chem., submitted.



P24

INSULIN HEXAMER-HEXAMER BRIDGING WITH BORONATE-CARBOHYDRATE LINKS FOR GLUCOSE-RESPONSIVE INSULIN RELEASE

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Boronates, the anionic form of boronic acids, are known to bind to glucose and other carbohydrates with affinities in the millimolar range, thereby matching the physiological window for glucose fluctua-tions (approximately 2-20 millimolar). Simple boronic acids are however weak acids (pKa >8), and carbohydrate binding is therefore usually observed at alkaline pH only. For use at physiological conditions, the boronic acid pKa must be adjusted by use of e.g. electron-withdrawing groups. Sulfonamides are particularly attractive because they are both strongly electron-withdrawing and may simultaneously function as handles for attachment to e.g. peptides. This presentation will show that insulin derivatized with boronates of suitable pKa can enable glucose-responsive release of insulin from polymers at neutral pH. The response is relatively flat compared to physiological glucose-responsive insulin secretion, but this problem can be helped somewhat by applying insulin as Zn(II) hexamer formulations. Insulin encapsulation in polymers rises however several issues such as biocompatibility, biodegrad-ability, drug stability/denaturation and increase in injecting volume. We will demonstrate circumvention of this issue by engineering insulin hexamers as self-contained polymers via boronate-carbohydrate bridges, thereby enabling the formation of very large, soluble hexamer-hexamer self-assemblies.

SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P25

ON-RESIN UREA CYCLIZATION OF DIAMINES USING TRIPHOSGENE

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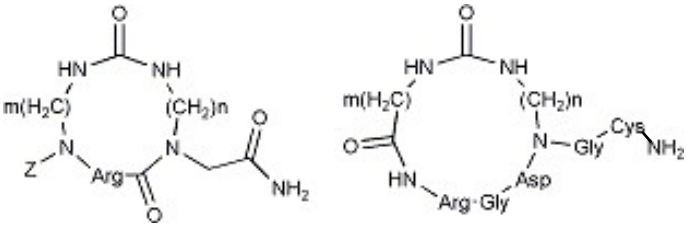
Department of Organic Chemistry, The Hebrew University, Jerusalem, Israel

Cyclic urea has interesting pharmacological properties that have led to their use as inhibitors of a broad set of retroviral proteases including HIV protease. Several synthetic routes starting with diamines reaching the corresponding cyclic urea have been described. Most of these rely on nucleophilic reactions of the amines with phosgene and related compound. The currently used on-resin triphosgen (BTC) mediated urea cyclization leads mainly to diisocynates and starting material. We have developed an improved procedure for on-resin urea cyclization that leads mainly to cyclic urea. The cyclization was performed in two steps. In the first step BTC (1/3 eq.) was used without any base at all. After 2h a base (Et_3N, 2eq) was added and the reaction vessel was shaken for 16h. This procedure led to the formation of clean cyclic urea as characterized by LC-MS and MS-MS. We have prepared both backbone urea cyclic peptides and urea containing macro cyclic molecules with ring size of 11-17 atoms.



P26

SYNTHESIS OF AN ENEDIYNE-BRIDGED PEPTIDE FOR CELL LYSIS

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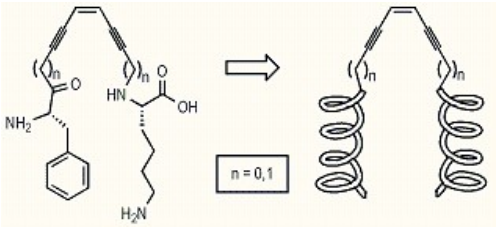
[1]Division of Organic Chemistry and Biochemistry, Rudjer Boskovic Institute, Zagreb, Croatia [2]Institute of Bioagricultural Sciences, Academia Sinica, Taipei, Taiwan

Antibacterial peptides represent a class of small proteins originally found in insects, but now shown to be widely distributed in animals and humans. Their ability of lysing bacterial cells attracts worldwide interest, especially nowadays when we are faced with bacterial resistance toward even the latest generation of antibiotics. Furthermore, many antibacterial peptides show promising anticancer activity without damaging normal eukaryotic cells. The distribution of the net charge of the peptide is recognized to be in direct correlation with biological function. Previous studies have shown that additional cationic residues in synthetic analogs of the antibacterial peptide cecropin B, contribute to higher efficiency in cancer cell lysis, as compared to the natural peptide. Also, conformational studies have confirmed a more compact form for the synthetic than for the native peptides. The decreased flexibility of the bend angle may be one of the factors leading to the higher cancer cell lysis ability. Following this assumption, we focus our interest on the hinge region by preparing models with an enediyne unit bridging two peptide fragments. The presence of a conformationally more defined spacer is expected to influence the pre-organization of the two carrying helices and thereby membrane permeability efficiency.



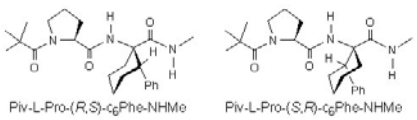
P27

ß-TURN PREFERENCES INDUCED BY THE TRANS CYCLOHEXANE ANALOGUES OF PHENYLALANINE

M. Lasa, P. Lopez, **A.I. Jimenez**, C. Cativiela

Department of Organic Chemistry, Institute of Materials Science of Aragon, University of Zaragoza-CSIC, Zaragoza, Spain

The side-chain groups of certain residues are directly involved in peptide-receptor interactions and are essential for bioactivity. The use of synthetic amino acids with specifically oriented side-chains provides very valuable information about the spatial requirements of the substituents for optimal interaction with the receptor. Moreover, they are of great help to evaluate the interdependence between side-chain and main-chain conformation. The series of 1-amino-2-phenylcycloalkanecarboxylic acids (cnPhe), where the phenylalanine α and β carbons have been connected through an alkylidene bridge, combines the propensity to folded structures associated to α,α-tetrasubstitution with restriction of side-chain mobility. As part of our investigations into the conformational preferences of this family of amino acids in model dipeptides RCO-L-Pro-cnPhe-NHR', we found that the cis cyclohexane analogues of phenylalanine (S,S)- and (R,R)-c6Phe stabilize, respectively, the βI- and βII-turns. The side-chain stereochemistry actually dictates the turn type, whereas the conformations adopted by the analogous peptides containing L-/D-Phe depend on the environmental conditions. We report now the synthesis of the trans cyclohexane analogues of phenylalanine (S,R)- and (R,S)-c6Phe and their incorporation into the peptide sequence above-mentioned. The structure of these peptides, studied in solution by FT-IR and NMR techniques and in the solid state by X-ray diffraction, is compared with that induced by the cis c6Phe derivatives and by L-/D-phenylalanine.



P28

CONFORMATIONAL PREFERENCES OF BICYCLIC PROLINE ANALOGUES WITH INDUCED CHIRALITY AT THE N ATOM

A.M. Gil, E. Buñuel, **A.I. Jimenez**, C. Cativiela

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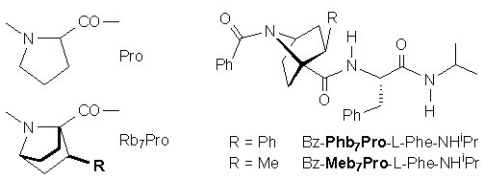
We are involved in a research project devoted to determine the conformational preferences of constrained analogues of proteinogenic amino acids when incorporated into a peptide chain. In this context, we have evaluated the relative stability of the βI- and βII-turns in model peptides RCO-L-Pro-L-Phe-NHR' when phenylalanine is replaced by different constrained derivatives. We report now the structural consequences arising from the replacement of the proline residue by two constrained analogues of bicyclic structure. In these proline surrogates, the flexibility of the pyrrolidine ring has been frozen by linking the α- and δ-carbons through an ethylene bridge. An additional phenyl (Phb7Pro) or methyl substituent (Meb7Pro) has been incorporated at the β-position. The conformation of these peptides has been determined by X-ray crystallography. Remarkably, both compounds adopt a βI-turn, at variance with the L-Pro-L-Phe sequence, which is not able to retain the βI-turn in the solid state. Several unique features of Rb7Pro may contribute to the stabilization of the βI-turn conformation, namely the strong pyramidalisation of the bicyclic amide N, which becomes chiral and acts as a weak hydrogen-bond acceptor, the existence of additional aromatic-NH interactions (R=Ph), and the accessibility of the middle amide hydrogen to intermolecular hydrogen-bonding (R=Me).



P29

SYNTHESIS OF NOVEL FULLERENE AMINO ACIDS AND MULTIFULLERENE PEPTIDES

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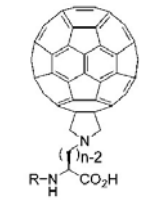
[1]Graduate School of Life Science and Systems Engineering [2]Faculty of Engineering, Kyushu Institute of Technology, Kitakyushu, Japan

Fullerene based molecules have a wide variety of interesting characteristics including nonlinear optical properties, superconductivity and biological properties. Fullerene based amino acids and peptides are interesting targets due to their importance in biological applications and also in structural studies. For the easy incorporation of fullerene moiety into a peptide, we synthesized fullerene functionalised amino acids with spacers of 4 to 6 methylene units from the □-carbon to the nitrogen atom of the fulleropyrolidine. The synthesis of C60-functionalized amino acids was started from recently reported L-α-amino-ω-bromoalkanoic acids [1]. In this presentation, we will describe the details of the synthesis of C60 functionalized amino acid with different methylene spacers (Afn, where n = 6, 7 and 8) and the application of these fullerene amino acids in the synthesis of multifullerene peptides. The attraction of the synthetic approach is the generality of the method for the synthesise of fullerene amino acids with different spacer lengths between the α-carbon and the nitrogen atom of the fulleropyrolidine. These novel amino acids have tremendous potential as building block for the synthesis of biomaterials and peptides with interesting biological properties. References: L. A. Watanabe, B. Jose, T. Kato, N. Nishino, and M. Yoshida, Tetrahedron Lett., 45, 491-494 (2004).



R = Fmoc, Boc n = 6, 7, 8

P30

EXTENDED CHEMICAL LIGATION FOR POLYPEPTIDE SYNTHESIS BY USING A PHOTOREMOVABLE AUXILIARY

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Native chemical ligation is a very useful and convenient method for polypeptide synthesis. This can be performed under neutral conditions in aqueous solutions without the need for protecting groups, although a cysteine residue is required at the condensation site. Recently several groups introduced auxiliaries for the ligation of peptides that permit the condensation without the need for a cysteine residue at the condensation site. These can be removed after ligation by either Zn dust or acid treatment such as trifluorometanesulfonic acid and trifluoroacetic acid. Herein, we describe a photoremovable ligation auxiliary for use in peptide synthesis via the chemical ligation method. As shown in the scheme, peptide 2, which have 2-mercapto-1-(2-nitrophenyl)ethyl (Mnpe) moiety at the N-terminus, was ligated to a peptide thioester 1, to give peptide 3. UV irradiation resulted in the removal of the auxiliary from the peptide.

P31

ETHYLENE GLYCOL BASED PROTECTING GROUPS

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[1]Research Group of Peptide Chemistry,Eötvös L. University, Hungarian Academy of Sciences [2]CF Pharma Ltd., Budapest, Hungary

Polyethylene glycol chains (PEG) have been shown to possess beta-sheet disintegrating properties. They also increase the solubility of the moieties attached. PEG has been previously introduced as a solubilising C- and N-terminal protecting group of carrier bound peptides in SPPS. The attachment of PEG blocks increases the solubility and decreases the aggregation of hydrophobic peptides. Our goal in the development of polyethylene-glycol based protecting groups is to prevent aggregation during the peptide synthesis thus to prevent the appearance of "difficult sequences". Since the use of only one amino acid derivative carrying PEG group in the peptide sequence may not be sufficient for successful solubilization, we prepared ester, ether and urethane type protecting groups bearing triethylene glycol moieties with a well-defined structure. The triethylene glycol monomethyl ether moiety was anchored to 4-hydroxy-benzyl alcohol which was further derivatized to obtain the appropriate benzyl analogue protected amino acid derivatives. The compounds were tested in SPPS with "difficult" sequences to examine the disruption of beta-sheet conformations, and with hydrophobic sequences to prove the increased solubility of the peptides synthesized.

P32

HYDROXYPROLINE-MODIFIED POLY(PROPYLENEIMINE) DENDRIMERS AS CATALYSTS OF ASYMMETRIC ALDOL REACTIONS

G. Kokotos, E. Bellis

Department of Chemistry, University of Athens, Greece

Proline has been proven to be an effective organocatalyst in several powerful asymmetric transformations, such as the aldol, Mannich and Michael reactions. Dendrimers are highly-branched macromolecules, which present complete architectures, precise shapes and functionality. Dendrimers functionalized at the surface have been proposed to fill the gap between homogeneous and heterogeneous catalysis. The aim of this work was to develop dendrimers functionalized at the surface by proline, which would be suitable for the catalysis of asymmetric aldol reactions. (2S,4R)-4-Hydroxyproline has been coupled to the terminal amino groups of poly(propyleneimine) dendrimers through a glutarate spacer. Five generations of dendrimers (first to fifth) were synthesized and were studied as catalysts of aldol reactions. The reaction between acetone and p-nitrobenzaldehyde was used as a model reaction. Using 5% of the second generation modified dendrimer as catalyst, the product of the aldol reaction was obtained in high yield and high enantiomeric excess, comparable to that obtained using proline itself. The advantage of proline-modified dendrimers is that they operate both under homogeneous conditions, and they may also be recovered as if they were operating under heterogeneous conditions.

SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P33

NEW GENERATION OF TRIAZINE-BASED COUPLING REAGENTS USEFUL FOR SPPS

B. Kolesinska[1,2], G. Sabatino[1,3], J. Kolesinska[2], M. Chelli[1,3], P. Rovero[1,4], Z.J. Kaminski[2], A.M. Papini[1,3]

[1]Laboratory of Peptide Chemistry & Biology, Polo Scientifico, Università di Firenze, Sesto Fiorentino, Italy [2]Institute of Organic Chemistry Technical University of Lodz, Poland [3]Dipartimento di Chimica Organica "Ugo Schiff" and CNR-ICCOM [4]Dipartimento di Scienze Farmaceutiche, Università di Firenze, Sesto Fiorentino, Italy

Triazine-based coupling reagents are known to be excellent for amide and ester bond formation in solution [1]. However, their application in SPPS was found less feasible. Preliminary attempts for the application of DMTMM [2] (derived from CDMT and NMM) reported in literature were, unfortunately, not confirmed by other groups. In our hands, re-examination of potential of DMTMM reagent in the synthesis of ACP (64-75), in a batch multiple peptide synthesizer, gave unsatisfactorily results [3]. Recently, substantially improved triazine reagents have been prepared, and we found reasonable to estimate their synthetic value. We compared the performance (in terms of yield, purity of the crude product and extent of racemization) of an entire family of triazine-based coupling reagents (N-triazinylammonium tetrafluoroborates) in the synthesis of ACP (65-74) (ACP: H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH), which is considered as difficult peptide sequence, due to the development of internal secondary structures, using a manual (Advanced ChemTech PLS) and a multiple synthesizer (Advanced ChemTech 396) for SPPS in batch. Acknowledgement: The study was supported by the Polish State Committee for Scientific Research under the Project 4-T09A 189 25, and PRIN 2002 and FIRB 2002, MIUR, Italy. [1] Kaminski, Z. J., Biopolymers 2000, 55, 140-165. [2] Falchi, A.; Giacomelli, G.; Porcheddu, A.; Taddei, M., Synlett, 2000, 275-277. [3] Sabatino, G.; Mulinacci, B.; Alcaro, M. C.; Chielli, M.; Rovero, P., Papini, A. M.; Letters in Peptide Science, 2002, 9, 119-123.

P34

COMBINATORIAL SOLID-PHASE SYNTHESIS OF N-ALKYL HYDROXAMIC ACIDS

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Polymer-supported N-benzyloxy-2-nitrobenzenesulfonamides were N-alkylated using three different routes: via Fukuyama reaction with alcohols, by N-alkylation with alkylbromides, and by Michael addition reaction with α,β-unsaturated carbonyl compounds. The 2-nitrobenzenesulfonyl (Nos) group was cleaved under mild conditions to yield polymer-supported N-alkylated benzyloxyamines. Acylation by carboxylic acids and cleavage with TFA yielded N-alkyl hydroxamic acids.

P35

POLYMER-SUPPORTED N-BENZYL-2-NITROBENZENESULFONAMIDES AS ALTERNATIVE TO ALDEHYDE LINKERS

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Polymer-supported N-benzyl- and N-benzhydryl-2-nitrobenzenesulfonamides were N-alkylated using three different routes: via Fukuyama reaction with alcohols, by N-alkylation with electrophiles, and by Michael addition reaction with α,β-unsaturated carbonyl compounds. The 2-nitrobenzenesulfonyl (Nos) group was then cleaved to yield polymer-supported N-alkylated benzylamines and benzhydrylamines. N-alkylation of polymer-supported 2-nitrobenzenesulfonamide linkers described herein represents an alternative route to reductive amination of aldehyde linkers.

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SOLID PHASE SYNTHESIS ON DUAL LINKER WITH A REFERENCE CLEAVAGE SITE

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Two dual linker systems with specific reference cleavage sites were designed and synthesized to accelerate and simplify development and optimization of reaction conditions for solid-phase synthesis. The dual linker allows simple evaluation of cleavage rate of polymer-supported compounds from the linker and, at the same time, secures that all resin-bound components are cleaved from the solid support.

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DISSECTION OF THE NISIN MODIFICATION AND EXPORT MACHINERY

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Lantibiotics are lanthionine-containing peptide antibiotics. The lantibiotic nisin, encoded by nisA, is produced by some Lactococcus lactis strains. Its five thioether bridges are posttranslationally introduced by two membrane-bound enzymes. NisB dehydrates serines and threonines and NisC couples these dehydrated residues to cysteines thus forming thioether bridges. Subsequently the transporter NisT exports fully modified prenisin and the leaderpeptidase NisP cleaves off the leaderpeptide liberating active nisin. We followed the activity of various combinations of the nisin enzymes by measuring export of secreted peptides using antibodies against the leader peptide and mass spectroscopy for detection. NisP could act independently of the other lantibiotic enzymes and was found to be specific for thioether-ring-containing prenisin. Strikingly, L. lactis expressing the nisBT genes produced dehydrated prenisin without thioether rings and a dehydrated form of a non-lantibiotic peptide. In the absence of the biosynthetic NisBC enzymes, the NisT transporter was capable of excreting unmodified prenisin and fusions of the leader peptide with non-lantibiotic peptides. Our data show that NisT specifies a broad spectrum (poly)peptide transporter that can function either in conjunction with or independently from the biosynthetic genes. NisT secretes both unmodified- and partially or fully posttranslationally modified forms of prenisin and non-lantibiotic peptides. These results open the way for production of a wide range of peptides with increased stability or novel bioactivities.

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CHEMICAL SYNTHESIS AND APPLICATION OF C-TERMINALLY 5-CARBOXYFLUORESCEIN-LABELED THYMOPENTIN AS A NOVEL FLUORESCENT PROBE

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The ligand-receptor interactions have been demonstrated for a number of polypeptide hormones by showing specific binding of radiolabeled hormone with the receptor molecule. Although there have also been a few attempts at using ligands tagged with fluorescent markers, the use of fluorescent receptor probe has nevertheless remained somewhat limited, in part because of the notorious difficulty of tagging receptor ligands with fluorophore without affecting their biological properties and the lack of sensitivity. Thymopentin (TP5) is a synthetic pentapeptide fragment, composed of Arg-Lys-Asp-Val-Tyr, which corresponds to position 32–36 of thymic polypeptide thymopoietin. It has pleiotropic actions, including the induction of early T cell differentiation, regulation of immune responses, and regulation of neuromuscular transmission. Previous chemical modification experiments suggested that there was an absolute requirement for the N-terminal structure, especially Arg at position 1, to maintain biological activity of TP5. According to this structure-activity relationship, we designed and synthesized the C-terminally 5-carboxyfluorescein-coupled TP5 (TP5-FAM) as a fluorescent probe for thymopoietin receptor. TP5-FAM binding to human lymphoid cell lines, MOLT-4 cells, resulted in a significant increase in fluorescence anisotropy, and the binding is specific and saturable (Kd=30 μM). TP5 and human splenin are almost equipotent inhibitors of TP5-FAM binding to the thymopoietin receptor, but porcine secretin did not show the competitive inhibition of TP5-FAM binding to MOLT-4 cells. Thus, we demonstrated that TP5-FAM is a potent, selective ligand that is useful for the study of the binding and functional characteristics of the human thymopoietin receptor.

P39

OVERXPRESSION OF N-TERMINAL ACTIVE SITE OF HUMAN ANGIOTENSIN CONVERTING ENZYME (ACE 361-468) IN E. COLI CELLS

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Angiotensin converting enzyme (ACE) has a critical role in cardiovascular function by cleaving the carboxy terminal His-Leu dipeptide from Angiotensin-I to produce a potent vasopressor octapeptide, Angiotensin-II. ACE is a gluzincin Zn-mettalopeptidase. There are two isoforms of ACE, the somatic and the testicular one. The somatic is present in all human cells except the testis cells, where the testicular isoform is produced. The difference between the two types is that, the somatic form has two active sites, at the N- and C-terminal respectively while the testicular has only one, almost identical to the somatic C-terminal active site. Here we report, for the first time, the overexpression in bacteria, and purification of a 108 aa peptide which corresponds to an extended domain of the human somatic N-terminal active site of ACE (Ala361-Gly468). The DNA sequence, encoding the Ala361-Gly468 protein fragment, was subcloned into the pET-3a expression vector at NdeI/BamHI restriction sites. The resulting plasmid used to transform competent ER2566 cells. The transformed cells were used to inoculate M9 minimal medium, and the induction of the expression of recombinant protein fragment occurred using 0,5mM of IPTG. The protein was isolated from the inclusion bodies by chromatography techniques. The recombinant protein fragment has a molecular weight, measured by ESI MS, of 12419 kDa which is in consistence with the theoretical calculation based on the DNA sequence. The resulting peptide will be studied in solution by NMR spectroscopy in order to suggest possible coordination models of the zinc ion in the native enzyme.

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SOLID PHASE SYNTHESIS OF CYCLIC PEPTIDES WITH A GUANIDINE BRIDGE

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Cyclization through covalent linkage between two side-chains has proved to be efficient in producing peptide analogues with improved biological properties, such as metabolic stability, potency and receptor selectivity. Cyclization can be achieved by the formation of a disulfide bond, an amide bond, etc… We were interested in synthesizing peptides bridged through a guanidine function, thus implicating an arginine side-chain. In addition to the global constraint afforded to backbone by cyclization, it would also limit the mobility of the arginine side-chain while keeping its basic function. Bridging has been performed on solid support after peptide assembly to take advantage of the pseudo-dilution effect, and was achieved between the side-chains of two ornithine residues, each being placed at one end of penta- or hexapeptides. Our strategy followed the classical steps of guanidine formation: i) deprotection of the delta-amino group of one ornithine and conversion into isothiocyanate; then deprotection of the second ornithine and reaction of the free delta-amino group with the isothiocyanate to afford a thiourea bridge; ii) S-methylation of thiourea; iii) guanidinylation using ammonium acetate. Cleavage of the resin generated the guanidine-bridged cyclic peptides, which have been characterized by HPLC, mass spectrometry and 1H NMR. Finally, guanidinylation using secondary amines such as morpholine will also be presented.

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EFFICIENT SYNTHESES OF AZABICYCLO[X.Y.0]-ALKANE PEPTIDOMIMETICS FROM PYROGLUTAMATE AND HOMOPYROGLUTAMATE

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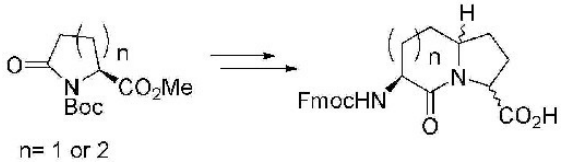
Azabicyclo[X.Y.0]-alkane aminoacids (AZABIC, Figure) are conformationally rigid dipeptide mimics that constrain three backbone dihedral angles within a fused bicyclic framework1. The growing use of these dipeptide units in structure-activity relationship studies of biologically active peptides has created a demand for new, efficient methodology for their synthesis. We are interested in employing AZABIC mimetics in SAR studies of peptide-based inhibitors of oncogenic signal transduction proteins. We report here an efficient synthesis of 3-(Fmoc-amino)-azabicyclo[4.3.0]-nonane-2-carboxylate (n = 1) and its homologue 3-(Fmoc-amino)-azabicyclo[5.3.0]-decane-2-carboxylate (n = 2). Boc-pyroglutamate or Boc-homopyroglutamate is cleaved with a vinyl Grignard reagent to produce acyclic g- or d-vinyl ketones. Michael addition of N-diphenylmethylene glycine tert-butyl ester to the vinyl group produces diamino dicarboxylate precursors, which, on hydrogenloysis, undergo double cyclization to give the fused bicylic ring system. Acidolysis of the tert-butyl-based protecting groups followed by treatment with Fmoc-OSu results in Fmoc-protected dipeptide mimetics ready for solid phase synthesis. In this presentation we will give details of the syntheses and discuss the stereochemistry of the products. References: 1. Hanessian, S.; McNaughton-Smith, G.;Lombart, H.-G.; Lubell, W.D. Tetrahedron 1997, 53, 12789. (b) Gillespie, P.; Cicariello, J.; Olson, G. L. Biopolymers, 1997, 43, 191; (c) Eguchi, M.; Kahn, M. Mini Reviews in Medicinal Chemistry, 2002, 2, 447.



P42

NEW INSIGHTS IN PEPTIDE SYNTHESIS CATALYZED BY LIPASES

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This work aimed to determine experimental conditions and reliable information to be used in peptide synthesis catalyzed by lipases. Two C. cylindracea lipases (CCL) and two porcine pancreatic lipases, a purified (pPPL) and a crude (cPPL), were characterized by PAGE-SDS and activity in olive oil. CCLs presented higher purities and activities than PPLs. Trypsin (T) and a-chymotrypsin (a-CT) were identified among the cPPL contaminants. A systematic study of Ac-YG-NH2 synthesis catalyzed by CCLs and PPLs was then performed. The optimized conditions were: 0.05M Ac-Y-OEt, 0.5M G-NH2, 50mg/mL cPPL, mixture of n-hexane with Tris-HCl buffer 0.5M, pH 8.0, (8/2,v/v), 37oC and 300 rpm (~90% in 5 min). As secondary hydrolysis occurred in long-lasting reactions, cPPL was treated with TPCK and had the a-chymotryptic activity extinguished. The synthesis conditions cited above were also the most suitable for the synthesis of Z-DG-NH2. A further systematic investigation aiming to determine which Z-amino acids-OMe (Z-aa-OMe) are good substrates for cPPL was conducted. The results indicated the following preference: (K, R, H)>(F, Y)>(D, E, N, S, T, L). The natures of the ester and Na-blocker affected ester hydrolysis since among some esterified Na-acyl-D, Z- or Boc-D-OBzl were the best substrates. Surprisingly, Z-group removal occurred in a few ester hydrolyses. This side reaction was not catalyzed by the cPPL contaminants T and a-CT. Incubation of cPPL with 11 of the 20 Z-aa tested provided Z-group removal initial rates that followed the order: Y>F>S>N>K>H>W>L>M>R>I. Incubations of cPPL and with some Z-peptides are now being undertaken.

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SULFHYDRYL PROTEASE-CATALYZED PEPTIDE SYNTHESIS USING CARBAMOYLMETHYL ESTERS AS ACYL DONORS

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We have recently found the superiority of the carbamoylmethyl (Cam) ester as an acyl donor in the kinetically controlled peptide bond formation mediated by a-chymotrypsin [1]. Thus, major drawbacks associated with protease-catalyzed peptide synthesis – a narrow substrate specificity and the secondary hydrolysis of a growing peptide – can be overcome by employing this particular ester. We have also reported the utilization of the Cam ester for the peptide synthesis catalyzed by some microbial serine proteases [2,3]. Along these lines, we have investigated the peptide bond formation mediated by sulfhydryl proteases such as papain using the Cam ester as the acyl donor. Acetonitrile with low water content (2% by volume) was found to be the solvent of choice. As in the Bacillus licheniformis protease-catalyzed couplings [3], a significant effect on the coupling efficiency was observed of the pH of the buffer solution from which the immobilized papain on Celite was prepared: high peptide yields were obtained with the immobilized enzyme prepared from a buffer solution of pH 8–10. Moreover, this protease was stable even at 50 C and the elevated temperature sometimes ameliorated the coupling efficiency. Thus, the procedure employing the Cam ester as the acyl donor proved to be useful for the sulfhydryl protease-catalyzed peptide synthesis. 1) T. Miyazawa et al., J. Chem. Soc., Perkin Trans. 1, 2001, 82; ibid., 2001, 87; ibid., 2002, 390; ibid., 2002, 396. 2) T. Miyazawa et al., Biocatal. Biotrans., 21, 93 (2003). 3) T. Miyazawa et al., Lett. Pept. Sci., 9, 173 (2002).

P44

(S)-PYROGLUTAMIC ACID BASED DESIGN AND SYNTHESIS OF NEW ANTIHYPERTENSIVE AGENTS

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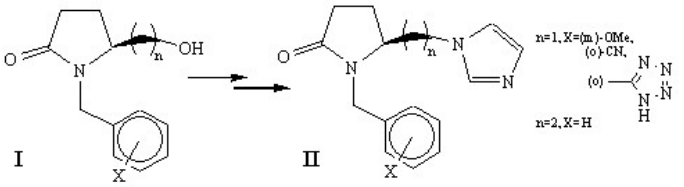
AT1 antagonists constitute a new generation of drugs for the pressure regulation and are designed and synthesized to mimic the C-terminal segment of Angiotensin II (Ang II). Conformational analysis of Ang II and its derivatives as well as the AT1 antagonists belonging to SARTANs class of molecules led to the design and synthesis of (5S)-1-benzyl-5-(1H-imidazol-1-ylmethyl-)-2-pyrrolidinone, with considerable bioactivity (71% compared to the drug losartan). Novel analogs were synthesized as follows: The alcohol I (for n=1) derived from the appropriate N-substituted methyl (S)-pyroglutamate after reduction, was activated to the tosylate and reacted with lithium imidazole to give II. The o-cyano-benzyl derivative was converted to the tetrazole, under appropriate conditions. The alcohol I (for n=1) produced alcohol I (for n=2) after elongation by conversion to aldehyde (Moffat oxidation) and further to alkene with Wittig reaction using the ylide (Ph3P=CHCOOMe). After hydrogenation, the methylester was converted to a new alcohol by LiBH4, which was finally activated to the tosylate and reacted with lithium imidazole. The bioactivity of the new analogs is under investigation.



SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P45

INVESTIGATION OF THE SYNTHESIS OF LONG ANGIOTENSIN II AND BRADYKININ G PROTEIN-COUPLED RECEPTORS FRAGMENTS

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[1]Biophysics, Universidade Federal de São Paulo [2]Biochemistry - Chemistry Institute, Universidade de São Paulo, Brazil

Following our previous studies of peptide receptor fragments [Eur.J. Org. Chem. (2002) 21, 3686], the AT1 AII receptor RIQDDC18PKAGRHSYI-(X)-IQLGVIHDC274KISD fragment that seems to mimic the putative region of the AII molecule binding were synthesized through Fmoc strategy. In this case X was either the polar (KG)3 or the apolar aminoundecanoic spacers. By using appropriate coupling conditions given by solvation data furnished by swelling studies of peptide-resin beads, a final yield of about 20% were achieved in both synthesis. Of note, a severe incomplete Fmoc group removal was detected at position 25 (Ser) and was only overcome by replacing the (2:8) piperidine/DMF for the (2:4:4) piperidine/DMF/toluene solution. As regard to the BK B2 receptor, the (66-97) VAEIYLGNLAGADLILASGLPFWAITIANNFD segment was synthesized deliberately in heavily substituted 2.6 mmol/g MBHAR. To our knowledge this synthesis strategy represents one of the most challenging condition applied so far in the SPPS field, as a very high 92% peptide-content is achieved at the end of synthesis. As expected, severe difficulties in couplings occurred during the peptide chain assembly but mainly at the 5-13 and 20-27 regions. Solvation studies of peptide-resins at regular intervals indicated significant variation in the solvation of beads throughout the peptide growth. Accordingly, ESR studies of peptide-resins labeled with paramagnetic amino acid TOAC also confirmed that the use of strong electron donor solvent system reduced the level of difficulty thus allowing the achievement of an acceptable synthesis yield of about 15%.

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SYNTHESIS OF THE BIS-CYSTINYL-FRAGMENT 225-232/225'-232' OF THE HUMAN IGGL HINGE REGION

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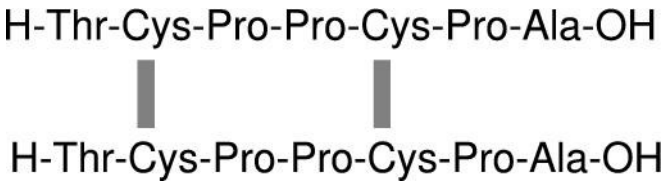
Iocb Cas Cr, Prague, Czech Republic

The main chemical structure of immunoglobulins consists of four polypeptide chains, two identical heavy chain and two identical light chains, which are held together by covalent bonds (i.e. the disulfide bridges) and non covalent interactions. The disulfide bridges linking the heavy chains are generally located in the midlle region of the molecule (225-232/ 225'-232'),which acts like a swivel point and has therefore been named the hinge region. In IgGl the two heavy chains are crosslinked in the hinge region by two disulfide bridges forming a double-chain bis-cystinyl cyclic peptide in parallel alignment. Abundance of proline residues imparts rigidity for this structure. Hinge peptide was prepared by Boc strategy on soluble polyethylene glycol (PEG) carrier without or with enzymatically cleavable linker with intention to use this compact structure as core molecule in the synthesis of immunogens – like conjugates.



P47

CONVERGENT SOLID-PHASE APPROACH PERFORMED IN A CHLOROFORM-PHENOL MIXED SOLVENT: SYNTHESIS OF AMYLOID BETA-PEPTIDES AS EXAMPLES WITH A DIFFICULT SEQUENCE

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A mixture of chloroform and phenol (v/v, 3/1) capable of dissolving almost all sparingly soluble protected peptides could be successfully applied to the synthesis of peptides with severe solubility problems in a solution employing a maximum protection strategy with Boc chemistry. In the case of synthesis of amyloide beta-peptides (Abs), however, the efficiency of reprecipitation to purify the desired protected peptides was extremely hampered by the remaining carboxyl components and their phenyl ester derivatives, partially generated during the coupling in chloroform-phenol, due to their insolubility in the ordinary organic solvents. Even after the HF procedure, contamination of these segments interfered with the purification using RP-HPLC because they caused seeding of amyloid formation, resulting in a low yield of the final product. To overcome these obstacles, we tried to employ a convergent solid-phase peptide synthesis performed in chloroform-phenol for the synthesis of Abs since the excess sparingly soluble protected segments which remain are readily removable by washing with chloroform-phenol after completion of the coupling. Each segment is prepared by solid-phase assembly on a base-labile N-[9-(hydroxymethyl)-2-fluorenyl] succinamic acid linker and detached from the resin by treatment with 20% triethylamine in DMF to obtain it in the form of a fully protected segment with a free alpha-carboxyl group. Next, the Ab molecule was constructed by sequential assembly of segments in chloroform-phenol onto the resin-bound C-terminal segment. Each segment condensation reaction proceeded smoothly when 1.5-2 eq of the respective segment, DIC and HOAt or 6-Cl-HOBt were used.

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A SOLID-PHASE SYNTHESIS OF TETRAAZAMACROCYCLES FROM CYCLOTETRAPEPTIDES

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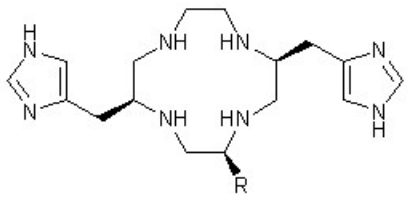
The synthesis of chelating ligands based on poly-azamacrocycles is a relevant goal for their considerable potential in diagnostic and therapeutic medicine as MRI contrast agents and as radionuclides vehicles. In particular, 1,4,7,10-tetraazacyclododecane (cyclen), owing to its remarkable complexation properties, has received considerable attention for the synthesis of functionalized pendant-armed derivatives. In order to develop an efficient synthesis of cyclen derivatives, we investigated a solid-phase approach involving the reduction of head-to-tail cyclotetrapeptides, following our previously proposed scheme [(a) Sabatino G., Chelli M., Mazzucco S., Ginanneschi M., Papini A.M. Tetrahedron Lett. 1999, 40, 809–812; (b) Alcaro M.C., Orfei M., Chelli M., Ginanneschi M., Papini A.M. Tetrahedron Lett. 2003, 44, 5217–5219]. The amide bonds were reduced on-resin by BH3 THF, obtaining the corresponding core-substituted tetraazacyclododecanes that were recovered after cleavage from the resin. From the four tetracyclopeptides, namely c(HGHX) where X = G [Ref. b], K, E, D, we obtained reduction products reported in figure. These compounds are currently under investigation for their ability to complex Cu(II).



R = H, -$(CH_2)_4NH_2$, -$(CH_2)_3OH$, -$(CH_2)_2OH$

SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

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SELECTION OF THE BEST TRIAZINE-BASED CONDENSING REAGENT FOR SPPS

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Several coupling reagents currently used in peptide synthesis belongs to the abundant group of phosphonium and iminium-guanidinium salts. The majority of them make use of excellent leaving group properties of the benzotriazole or azabenzotriazole moiety. Efficient coupling reagents should be useful not only for amide bond formation in peptides, but widely for ester or amide bonds occurring in numerous other organic compounds of biological interest such peptoids, β-lactams. The reagent should operate in stoichiometric quantities, be soluble and stable in most of the solvents. It should function efficiently in solution as well as in SPPS, to get high purity crude products and minimize racemization of the products. The last, but not least, after coupling it should be completely removed by a convenient and easy procedure (such as extraction). The aim of this study was to evaluate the efficiency of a new family of triazine based coupling reagents. We compared the triazine-based coupling reagents with the commonly used phosphonium, uronium (iminium) salts, and carbodiimides. In this study we focused our attention on the performance (in terms of purity of the crude and extent of racemization) of a series of coupling reagents using manual, and multiple peptide synthesizer in batch (APEX396), testing the solid phase synthesis of ACP(65-74), which is a good example of difficult peptide sequence, because of the development of internal secondary structures. Acknowledgement: The study was supported by the Polish State Committee for Scientific Research under the Project 4-T09A 189 25, and PRIN 2002, FIRB 2002, MIUR, Italy.

P50

A NOVEL AND FLEXIBLE METHOD FOR THE PREPARATION OF PEPTIDE HOMO- AND HETERODIMERS FUNCTIONALIZED WITH AFFINITY PROBES, REPORTER MOLECULES AND CHELATING LIGANDS

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The construction of artificial proteins or multimerized peptide scaffolds requires an efficient method for coupling peptides with diverse linkers, spacers and reporting groups. For a number of problems under study in our laboratories we wished to employ and optimize dimeric constructs of lead peptides for chemical and biological studies. We developed a method in which two peptides can be efficiently coupled by using bis-succinimidyl glutarate as the tethering group. This method provides sufficient flexibility to afford complete control of the position of attachment of each peptide, thus allowing the preparation of CA-CB, CA-NB, CB-NA and NA-NB dimers (where CA and NB refer to the C-terminus and N-terminus of peptides A and B respectively). For dimers of the form peptide A-linker-tether-peptide B, where the tether is the glutaryl group, our method is compatible with the introduction of a wide variety of linker functionality for joining the peptides so that factors such as the hydrophilicity, length and rigidity of the linkage could be systematically varied to optimize the binding and physical properties of the constructs. Thus, we provide a general and stepwise procedure for incorporating two similar or different peptides via their C or N terminus which allows attachment of unnatural linkers and reporting groups.

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SYNTHESIS OF USEFUL BUILDING BLOCKS FOR PEPTIDE SYNTHESIS: A COMPARATIVE STUDY OF DIFFERENT COUPLING REAGENTS FOR ESTER AND AMIDE BONDS

M.C. Pozo-Carrero[1,2], B. Kolesinska[1,3], G. Sabatino[1,4], M. Chelli[1,4], P. Rovero[1,5], Z.J. Kaminski[3], A.M. Papini[1,4]

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The enormous diversity of building blocks useful for the synthesis of modified peptides imposes the development of more and more efficient coupling reagents for the formation of ester and amide bonds. Polymer-supported protected amino-acid esters are an important class of building blocks and active esters (to be used directly for peptide bond formation) are also functional for other compounds, such as macrolide precursors. Since 1955 carbodiimides, azides, active esters, anhydrides, phosphonium or iminium salts have been, and are still being extensively used. The encouraged results of research for the most effective coupling reagents caused a rapid and flourishing expansion of peptide chemistry and, as a consequence, promoted the progress in other areas of organic synthesis [1,2]. In this communication, the versatility of several classic coupling reagents and the new triazine based coupling reagents have been evaluated for amide or ester bond formation, anchoring Fmoc-amino acids to resins, and for the preparation of useful building blocks including esters (allyl or pentafluorophenyl esters) and amides (glycosylated Asn residues). It is expected that improving the efficiency of ester and amide bond formation would benefit also synthesis of other organic compounds of biological interest such as peptoids, oligocarbamates, β-lactams, benzodiazepines, diketopiperazines, hydantoines. Acknowledgement: The study was supported by the Polish State Committee for Scientific Research under the Project 4-T09A 189 25, and by PRIN-2002 and FIRB-2002, MIUR, Italy. [1] F. Albericio, et al , Organic Preparations and Procedures International (2001), 33(3), 203-303 [2] F. Albericio et al , Methods Enzymol., 289, 104 (1997).

P52

AMINO ACIDS AS COMPONENTS OF POLYMERIC HYDROGELS

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Polymeric gels are the most versatile class of materials and have changed our day-to-day lives over the past several decades. Advances in polymer science have led to the development of several novel applications of these materials, including drug deliver systems (DDS), construction of sensors and artificial muscles. In specific environmental conditions, polymeric hydrogels are known to exist in two phases: swollen and collapsed. The volume transition between the phases may depend on temperature, pH, ionic strength and other factors. This transition may occur continuously or discontinuously. It has been observed that hydrogels containing positively and negatively charged groups are very sensitive to the changes in pH. Amino acids are very interesting compounds in such purpose. We synthesized acrylic derivatives of basic amino acids to use them as the copolymers in polymeric hydrogels. The obtained gels contained positively or negatively charged groups depending on pH. The syntheses of amino acids were accomplished in solution. As the starting compounds Orn and Lys with blocked amino groups were selected. At first the methyl esters were synthesized, and then the side amino groups were deblocked. The couplings of acrylic acid and amino acid esters were done with DIC as the coupling reagent. The carboxylic and α-amino groups were deblocked and the derivatives were obtained as trifluoroacetate salts. The polymer gels were prepared by radical polymerization of N-isopropylacrylamide, N,N' methylenebisacrylamide and various amounts of the acrylic derivatives of amino acids. Then the studies of phase transition were performed as a function of temperature and pH.

SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P53

SYNTHESIS OF CONSTRAINED ANALOGUES OF TRYPTOPHAN

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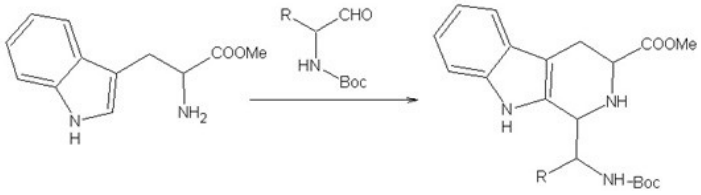
Aromatic groups of amino acids are often key element of pharmacophores in many peptide ligands binding to their receptors. A number of peptidomimetics motifs based on aromatic amino acids (Trp, Tyr, Phe) have been developed for restriction of local conformation of bioactive peptides. The Pictet – Spengler reaction has been widely applied to transform protein amino acids into their conformationally constrained analogues. The reaction between aromatic amino acid and aldehydes (excluding formaldehyde) creates additional chiral center. We have investigated the Pictet – Spengler reaction of tryptophan methyl ester and α-amino aldehydes. To obtain these compounds N-methyl-N-methoxy amides of amino acids were reduced by lithium aluminium hydride (LiAlH4) to the corresponding aldehydes. Then the synthesis of constrained analogues of tryptophan methyl ester was performed. Constrained analogues of tryptophan could be used as an additional rigid element in cyclic peptides. In this communication the results of incorporation of constrained analogues of tryptophan into peptide chain will be also presented.



P54

P1-SITE DIVERSITY IN PEPTIDE ISOSTER LIBRARIES VIA SMOOTH CC-COUPLINGS ON LINKER REAGENTS

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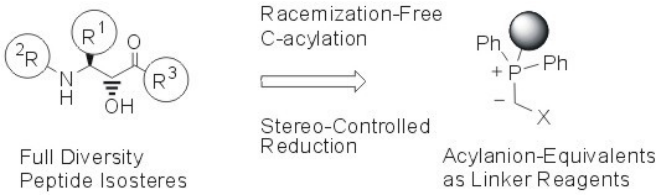
Whereas the combinatorial decoration of preformed isosteric building blocks is the state-of-the-art in protease inhibitor design, diversity in the central P1-position has been difficult to obtain so far. We have developed reagent linkers as a novel concept allowing for solid phase CC-couplings under very mild conditions.(1,2) The strategy was employed to construct several types of peptide isosters (including norstatines, diaminopropanols, and statines) on solid support. All available amino acids could be introduced in the P1-position of peptide isosteres with ease. Thus, without building block synthesis peptide isostere libraries were generated with full variation of all isosteric positions. Moreover, the C-acylations proceeded racemization-free and were followed by stereo-controlled reductions. With the new concept, scrutinizing the effect of P1-site diversity was facilitated considerably. A library of P1-variants was synthesized and screened against several aspartate proteases of biomedical significane including plasmepsin, HIV-protease, and cathepsin D. References: 1) S. Weik, J. Rademann, Angew. Chem. 2003, 115, 2595-2598; Angew. Chem. Int. Ed. 2003, 42, 2491-2494. 2) J. Rademann, 'Novel polymer- and linker reagents employed for the preparation of protease inhibitor libraries' in: Highlights in Bioorganic Chemistry, H. Wennemers, C. Schmuck (Eds.), Wiley-VCH, Weinheim, 2004, 277-290.



P55

ADVANTAGES OF MICROWAVE ASSISTED PEPTIDE SYNTHESIS

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In our continuing efforts to optimize the synthesis and purification of peptides, we implemented microwave assisted peptide synthesis in our laboratories. Short cycle times, the use of less excess of reagents and the overcoming of aggregation enabled us to synthesize peptides faster and purer than with conventional methods.

P56

A COMPARATIVE STUDY ON NEW NOT AZABENZOTRIAZOLE-BASED COUPLING REAGENTS FOR SPPS

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An optimal coupling reagent should be valid for a wide variety of peptide sequences. It should be soluble and stable in the solvents used for peptide synthesis, efficient in terms of yield, minimize the racemization, and it should be inexpensive. The most commonly used coupling reagents in SPPS are based on azabezotriazole derivatives with aminium/uronium or phosphonium salts. We previously reported a comparative study of new 6Cl-HOBt based coupling reagents using an automatic solid phase peptide synthesizer [1]. In this work, we compare the performance (in terms of yield, purity of the crude product and racemization) of a series of not azabenzotriazole-based coupling reagents using a manual (Advanced ChemTech PLS), or a multiple peptide synthesizer (Advanced ChemTech 396) in batch, testing the solid phase synthesis of ACP(65-74) (ACP: H-Val-Gln-Ala-Ala-Ile-Asp-Tyr-Ile-Asn-Gly-OH), which is a good example of difficult peptide sequence, because of the development of internal secondary structures. For these studies we selected: TOTU (kindly provided from Luxembourg Industries), new triazine-based coupling reagents [2], and the most commonly used ones. Syntheses were performed using a Fmoc/tBu strategy. In order to emphasize the differences between the compared coupling reagents, we reduced the excesses of the acylating mixture and used a single coupling procedure. Acknowledgement: The study was supported by the Polish State Committee for Scientific Research under the Project 4-T09A 189 25, and PRIN 2002 and FIRB 2002, MIUR, Italy. [1] Sabatino, G., et al. Letters in Peptide Science, 2002, 9, 119-123. [2] Kaminski, Z. J., Synthesis, 1987, 917-920

P57

SYNTHESIS OF CIS-PRO-TYPE ALKENE DIPEPTIDE ISOSTERE USING SMI2

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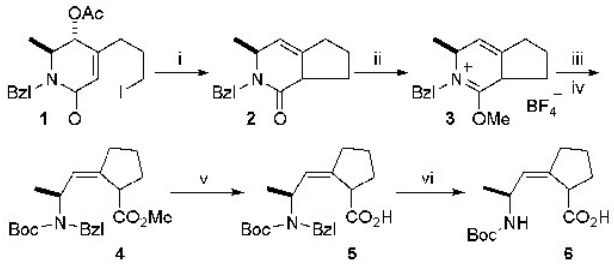
Cis-/trans-equilibrium of Xaa-Pro peptide bonds exerts great influence on both the structure and function of peptides and proteins. Generally, it is difficult to define whether cis- or trans-geometries are involved with bioactive conformations. Restriction of Xaa-Pro conformation with (Z)- or (E)- alkene units could provide one promising means for studying the biologically active geometries. We have synthesized Pro dipeptide isosteres using organocopper-mediated reaction. In our protocol, (Z)-alkene isosteres have remained to be synthetic targets. In this study, we have developed new synthetic methodologies for (Z)-alkene-type Pro isosteres, where SmI2 is utilized as a key reagent. As shown in Scheme, γ-acetoxy-α,β-unsaturated-δ-lactam 1 possessing alkyl halide moiety was reduced to Sm(III) dienolate followed by intramolecular cyclization to yield bicyclic lactam 2. Resulting compound was converted to desired (Z)-alkene isostere 6 corresponding to cis-Ala-Pro unit by a sequence of reactions consisting of formation of imidium salt 3, acid hydrolysis, Boc-protection, and Birch reduction.

Scheme. Synthesis of cis-Pro-type Alkene Isostere



Reagents: (i) SmI_2-DMPU in CH_3CN; (ii) $Me_3O{\cdot}BF_4$; (iii) 0.5 M HCl aq.; (iv) $(Boc)_2O$; (v) 1 M KOH aq.; (vi) Na-NH_3.

P58

CHEMICAL SYNTHESIS OF THE C-TERMINAL REGION OF THE OPIOID RECEPTOR LIKE 1

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Synthesis of the C-terminal region of the opioid receptor like 1 (ORL1), one of the G protein coupled receptors is described. Based on ligation strategy, we synthesized ORL1(251-370), which contains the sixth and the seventh transmembrane domains and the C-terminal intracellular domain. Two ligation chemistries, the native chemical ligation method and the thioester method, were applied to the synthesis since a combination of these two chemistries is known to be applicable to the synthesis of a single polypeptide (Sato, T. and Aimoto S. Tetrahedron Letters, 2003, 44, 8085-8087). For the synthesis, we prepared a peptide ORL1(329-370) (1) and peptide thioesters, Fmoc-ORL1(288-328)-SR (2) and Fmoc-ORL1(251-287)-SR' (3). R and R' contain a sequence of arginine to enhance the solubility of the peptides. During the coupling of peptide 1 and peptide thioester 2, the reaction conditions used in the native chemical ligation method were investigated in detail. As a result, Fmoc-ORL1(288-370) (4) was effectively synthesized under conditions in which the detergent concentration was set below the critical micelle concentration and the ionic thiol was used as an additive. We then introduced a thiosulfonate group and a Boc group to protect the side chain thiol groups and the amino groups, respectively, on peptide 3 and 4. These building blocks were condensed by the thioester method, in the presence of silver ion, HOObt and DIEA. The reaction was monitored by SDS-PAGE, and the purification was achieved by size exclusion chromatography to give the desired product.

P59

SYNTHESIS OF MACROCYCLIC PEPTIDES WITH AZO-BRIDGES ON SOLUBLE AND/OR INSOLUBLE POLYMERIC CARRIERS.

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Synthesis of macrocyclic peptides becomes an attractive field for drug developement due to their conformational rigidity, biological stability and additional ?cavity? properties. Many of them are used or intended to use as anti-microbial, anti-viral and anti-parasitic drugs. Although synthesis of azo-bridged cyclic peptides was described in eightieth by Siemion et al(1), the side chains cyclization is possible only after peptide detachment from the resin. On the other hand synthesis of such peptides on a polymeric carriers is essential for high-throughput screening and drug development. The azo compounds with one free carboxyl group were prepared from two ortogonaly protected amino acids in solution. Linear precursors were synthesized either on polyethylene glycole 6000 by Boc strategy or on Wang resin by Fmoc strategy. Allyl ester protection from second carboxyl was removed and peptides were cyclized by amide-bond formation. Acknowledgment: This work was supported by Grant Agency of Czech Republic grant no. 203/04/1421 and project of Acad. Sci. CR no. Z 4055905. 1. Siemion, I. Z., Szewczuk, Z., Herman, Z. and Stachura, Z.: Mol. Cell. Biochem. 34, 23 (1981).

P60

ENZYMATIC PEPTIDE SYNTHESIS USING INVERSE SUBSTRATES: BIOCATALYSTS INVESTIGATIN IN INDUSTRIAL WASTE

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Protease have frequently been used catalyst as racemization-free peptide bond formation, but it is sometimes difficult to find a highly effective new enzyme pertinent to be porpose. For the screening of the appropriate enzymes, formation reaction of Boc-L-Ala-L-Ala-pNA from N-tert-butyloxycarbonyl-L-alanine p-guanidinophenyl ester (Boc-L-Ala-OGp) (inverse substrate) and L-alanine p-nitroanilide (L-Ala-pNA) was proposed. In our previous work, it was shown that p-guanidinophenyl esters behave as specific substrates for trypsin and trypsin-like enzymes and allow the specific introduction of an acyl group carrying a non-specific residue into the enzyme active site. The characteristic features of inverse substrates suggested that they are useful for enzymatic peptide synthesis. Recently, we demonstrated successful application of peptide condensation using inverse substrates with chum salmon trypson from industrial waste (pyloric caecae from chum salmon) as catalyst. Herein, we describe the application of biocatalysts from mid-gut gland of Japanese giant scallop (Patinopecten Yessoensis) and residue of squeeze a wine dry for the peptide condensation reaction proposed as above. Both biocatalysts were concluded to be effective for the coupling reaction.

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AN IMPROVED SYNTHESIS OF BETA-AMYLOID PEPTIDES FOR IN VITRO AND IN VIVO EXPERIMENTS

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Beta-amyloid peptides (Aß) are the primary components of senile plaques found in the brains of Alzheimer's patients. Aß petides (l-40, l-42, 4-42, 5-42) have fibrillar structure in the plaques. Knowledge of the structure of ß-amyloid is essential for understanding the abnormal assembly of Aß. The peptides undergo a conformational change to form very stable water-insoluble fibrills. Producing synthetic Aß and preparing suitable solutions for both in vitro and in vivo studies still remained serious tasks for the chemists due to variations in the starting conformation and assembly state of Aß. In our study,we introduce an improved method for Aß synthesis using anisole during solid-phase synthesis with Fmoc-protocol. The use of anisole improves the swelling of the resin and behaves as a scavanger during reaction. For the final cleavage of Aß peptides from the resin, a coctail has been used which contains 95% of trifluoroacetic acid, 2.5% of dithiothreitol and 2.5% of water. In order to prevent aggregation, peptides were immediately purified on a preparative HPLC column without lyophilization after the final cleavage. The purified and lyophilized peptides were redissolved in distilled water and the pH of the solution was adjusted to 7.0 with diluted ammonia solution.After the second lyophilization from the neutral solution, the Aß peptides show good aggregation properties according to electron microscopic studies. We prepared Aß (l-42, l-40, 4-42, 5-42) peptides. All of these peptides were neurotoxic in MTT test. The above method is advantageous for the preparation of Aß peptides for physiological and biophysical studies.

P62

DESIGN AND SYNTHESIS OF CYCLIC ALPHA,ALPHA-DISUBSTITUTED ALPHA-AMINO ACIDS: 1-SUBSTITUTED 4-AMINOPIPERIDINE-4-CARBOXYLIC ACIDS

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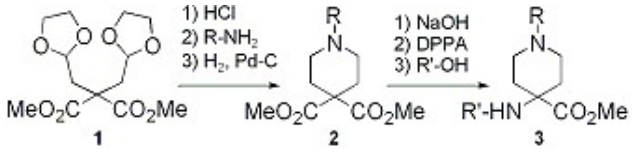
alpha,alpha-Disubstituted alpha-amino acids are non-proteinogenic amino acids, and their characteristic properties such as chemical stability, metabolic stability, and conformational restriction attract many organic, peptide, and medicinal chemists. We have already reported an asymmetric synthesis of alpha-ethylated alpha,alpha-disubstituted alpha-amino acids, and conformational study of their peptides. Besides acyclic alpha,alpha-disubstituted alpha-amino acids, cyclic alpha,alpha-disubstituted alpha-amino acids, in which the side-chain of amino acids constructs a cycloalkane-ring, have been reported. Among cyclic alpha,alpha-disubstituted alpha-amino acids, 4-aminopiperidine-4-carboxylic acid (Pip), which is an achiral amino acid bearing a gamma-nitrogen atom, has been focused by organic and peptide chemists. However so far, the Pip derivatives have just been prepared from piperidone derivatives by the Strecker, or Bücherer-Bergs methods. We designed and synthesized a new class of achiral and chiral 1-substituted 4-aminopiperidine-4-carboxylic acids. The Pip derivatives were prepared as follows: Bisalkylation of dimethyl malonate with 2-bromomethyl-1,3-dioxolane gave a diester (1). The acetal moiety in the diester (1) was removed by acidic conditions, and the crude dialdehyde was condensed with various amines, and subsequent hydrogenation yielded cyclic diesters (2). Hydrolysis of monoester, followed by Curtius rearrangement with diphenylphosphoryl azide afforded various achiral and chiral Pip derivatives (3).



P63

A GENERAL SYNTHETIC APPROACH TO SIDE-CHAIN BRIDGED PEPTIDES: SOLID-PHASE SYNTHESIS USING ORTHOGONALLY PROTECTED BIS-AMINO ACIDS

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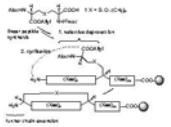
Peptides containing unusual side-chain to side-chain bridges, such as aliphatic, ether and thioether bridges, are of increasing importance. Such bridges are key features of biologically active peptides such as the lantibiotics, and have also been used to design conformationally constrained and metabolically stable, mimics of naturally occurring peptides. Most synthetic approaches to such peptides involve the synthesis of a linear peptide, followed by activation and joining of two side-chains. This requires the activation chemistry to be compatible with the underlying peptide, and it may be difficult to achieve the desired regio- chemo- and stereoselectivity. We have developed a powerful alternative approach. Bis-amino acids 1 are first synthesised, with the required bridge and stereochemistry already in place, with one amino acid protected for conventional Fmoc-based synthesis and with the other orthogonally protected (Allyl/Aloc). This can be incorporated into the desired peptide using conventional techniques: to form the bridge, the Aloc/allyl groups are deprotected and the peptide cyclised on-resin, revealing a free N-terminus for further chain extension. We illustrate this approach by the synthesis of thioether-bridged cyclic peptides (subunits of the lantibiotics) and ether-bridged peptides (as beta-turn mimics).



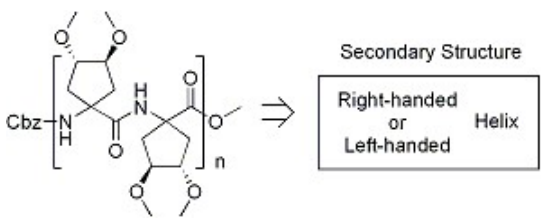
P64

CONTROLLING THE HELICAL SCREW SENSE OF OLIGOPEPTIDE BY ALPHA-AMINO ACID SIDE-CHAIN CHIRALITY

M. Tanaka[1], Y. Demizu[1], M. Doi[2], M. Kurihara[3], H. Suemune[1]

[1]Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka [2]Osaka University of Pharmaceutical Sciences, Osaka [3]Division of Organic Chemistry, National Institute of Health Sciences, Tokyo, Japan

Oligopeptides composed of proteinogenic L-alpha-amino acids, such as alanine, valine, and leucine form a right-handed (P) alpha-helix because of the asymmetric center of the alpha-carbon atom. Among proteinogenic L-alpha-amino acids, threonine and isoleucine exclusively possess an additional asymmetric center at the side-chain besides the alpha-carbon atom. However so far, it has not been clear how the side-chain chirality affects the secondary structure of their peptides. We designed and synthesized a chiral cyclic alpha,alpha-disubstituted alpha-amino acid, that is, (3S,4S)-1-amino-3,4-dimethoxycyclopentanecarboxylic acid [(S,S)-Ac(5)c(dOM)], in which the alpha-carbon atom is not a chiral center but the asymmetric centers exist at the side-chain gamma-carbon. We prepared homooligopeptides composed of optically active (S,S)-Ac(5)c(dOM), and studied their conformation in solution and in the crystal state. The effect of alpha-amino acid side-chain chirality on the secondary structure of oligopeptides will be presented.



SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

P65

SYNTHESIS OF HIGHLY CONSTRAINT (S,S) –CDC- AND –CRC- CYCLIC PEPTIDES ON SOLID SUPPORT AND IN LIQUID PHASE

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The tendency of intramolecular disulfide bond formation between cysteine residues is strongly dependent on the number of residues separating the two Cys since a small number of spacer amino acids introduces stereochemical constraints. The moiety Cys-X-Y-Cys is the smallest and most highly conserved chelating unit in biological systems. Extensive theoretical and experimental studies have been performed using model peptides of the type (S,S) Cys-Xn-Cys (n³2) aiming to establish their conformational profiles and evaluating their tendency to form the disulfide bond. (S,S) cyclic synthetic analogues bearing a single amino acid between the Cys residues were recently reported by our group for the first time [Stavrakoudis et al., Biopolymers, 2001, 56, 20-26]. The (S,S) –CDC- scaffold was used to develop constraint anti-aggregatory agents. In this study, we report on the synthesis of numerous (S,S) –CDC-and (S,S) –CRC- cyclic compounds. The disulfide bond formation was performed either on solid support or/and in liquid phase. The acetamidomethyl group was used for protection of the thiol group of Cys and the oxidation was performed by Tl(tfa)3 and/ or iodine. In particular cases, both Boc- and Fmoc- strategies were applied using the MBHA or the Rink Amide AM resin, respectively. In all cases the Tl(tfa)3 oxidizing reagent was more efficient than I2.

P66

NEW METHOD FOR THE SYNTHESIS OF MEMBRANE PROTEIN MODELS USING LIPID BILAYER ASSISTED MEMBRANE PEPTIDE LIGATION

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Membrane proteins including 7-transmembrane G-protein-coupled receptors (7TM-GPCRs) play critical roles in converting an extracellular stimulus to an intracellular signaling. Chemical synthesis of membrane proteins could allow the proteins to be modified by informative amino acids or moieties, which provides one potential approach for elucidating the structural basis of their function. In this study, we report the synthesis of membrane protein model with two transmembrane domains (TMDs) and one extracellular loop (ECL) derived from CXCR4, chemokine receptor (7TM-GPCR), using a lipid bilayer assisted membrane peptide ligation (Figure). Since the CXCR4 has one Cys residue in each of three ECLs, these positions (Leu-Cys, TMDs 2-3; Ile-Cys, TMDs 4-5; Gly-Cys, TMDs 6-7) were selected as the ligation sites. Lipid/peptide mixed film was hydrated with phosphate buffer containing tris(carboxyethyl)phosphine followed by addition of thiophenol to initiate chemical ligation. Attempted ligations (TMD 2 + 3, TMD 4 + 5 and TMD 6 + 7) afforded the desired membrane protein models in satisfactory yields (> 80%).

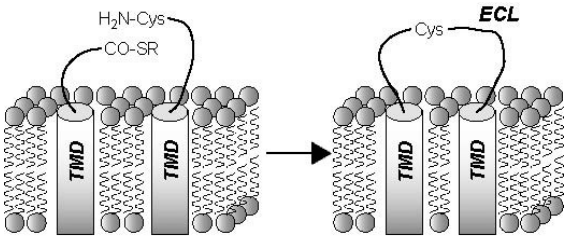

Fig. Synthetic Strategy of Membrane Protein Models

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CROSS-METATHESIS REACTIONS OF AMINO ACID-DERIVED COMPOUNDS

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Over the past decade metathesis has emerged as an important and useful reaction in synthetic organic chemistry. Of particular interest is cross metathesis (CM), in which two alkyl groups from different substrates are coupled together to form a new olefin product. The use of biological substrates in CM reactions could lead to important applications in catalytic antibody and vaccine development, and could be used to mimic natural cross-peptide linkages in biological settings. In our studies alkyl-substituted lysine and cysteine compounds have been coupled to various organic coupling partners in CM reactions. Effects of varying the alkene chain lengths (M) on the amino acid substrate have been investigated, as well the use of dipeptide-based substrates. Biological examples such as protected sugars, fatty acid-derivatives, and long chain protected alcohols (X) have been coupled onto amino acid scaffolds. Results indicate that this strategy could prove useful in the attachment of target molecules to peptide and protein entities. Related examples have also been investigated using solid-phase chemistry.

P68

STRUCTURAL AND ORIENTATION CHARACTERS OF POLYPEPTIDE BRUSHES

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Polymer brushes are the systems composed from macromolecules bonded by one of their terminal ends with polymer surfaces. We have synthesized and investigated two exemplars of polypeptide brushes. The first cast of polypeptide brushes consisted from polypeptides in a-helical conformation to exterior part of which hydrocarbons of different length were bonded. Orientation and conformation characters of such kind grafted polymer in block, liquid crystalline and isotropic states were studied. The second cast of polypeptide brushes consisted from polypeptide bonded to planar silicon plates previously modified with gold atoms was prepared with the use of two approaches. The first approach included additional modification of gold silicon plates with the spacers, amino acid derivatives of cystamine so as ortho- and para-diamine diphenyl sulfide and disulfide. The polypeptides were grafted on silicon surface as a result of amino acids (Ala or g-benzyl-glutamate) N-CA polymerization at the amino groups of the spacers. The second approach included touch-down on gold silicon plates of polypeptides previously prepared by polymerization of N-CAs in solution with the use of the same spacers as initiators of polymerization. The structural and orientation characters of polypeptide brushes prepared by N-CA polymerization at interface and by touch-down of polypeptides previously prepared in solution will be compared with that of for the polypeptides in solution. This work was supported by RFBI grants # 03-03-32709 and # 04-03-33032.

SYNTHETIC APPROACHES TO BRIDGES IN PEPTIDES

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IMPROVED REGIOSELECTIVE DISULFIDE BOND SYNTHESIS OF HUMAN RELAXIN 3, A NOVEL BRAIN-SPECIFIC MEMBER OF THE INSULIN SUPERFAMILY

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Relaxin is a peptide hormone of the insulin superfamily being composed of two short chains (A and B) linked by three disulphide bonds. Until recently, only two human relaxin genes were known (H1 and H2) whereas non-primate species had one gene (eg. mouse; M1). With the availability of the human genome sequence, we have identified a novel human relaxin gene (H3) in the Celera Genomics database. The peptide sequence retains all the key structural features of a relaxin peptide. Surprisingly, efforts to prepare H3 relaxin by simple combination of the S-reduced A- and B-chains were unsuccessful. This necessitated the development of Fmoc-based regioselective disulfide-directed solid phase synthesis. Preliminary efforts were hampered by substantial Trp side reaction during the final disulfide bond formation step. These were eliminated by the careful positioning of the three S-protecting groups and subsequent order of formation of disulfide bonds. This highlights the importance of sequence dependent effects in such strategies. Overall yield of synthetic H3 relaxin was nearly 15%. Use of various relaxin bioassays indicates that synthetic H3 relaxin is an authentic relaxin but has 10-50 fold lower activity than H2 relaxin. Further studies showed it to bind to, and activate, native relaxin G-protein coupled LGR7 receptor. More recently, it has been shown that mouse 3 relaxin expression levels are very high in discrete areas of the brain. This latter observation together with the remarkable conservation of the relaxin 3 peptide indicates it may be an important new neuropeptide.

P70

SYNTHESIS OF EFRAPEPTIN C AND EFRAPEPTIN ANALOGUES

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Efrapeptins are a class of sequence-homologous peptides that are synthesized by the fungus tolypocladium niveum and show antibiotic properties and inhibit ATPases. Furthermore efrapeptins are active against the malaria pathogen plasmodium falciparum. The peptaibiotic efrapeptin C contains several Aib residues and an unusual cationic head group at the C-terminus derived from leucinol, while the N-terminus is acetylated. It was synthesized chemically for the first time by solid phase methods. The approach allows for the synthesis of a series of analogues required for biological and conformational studies. The incorporation of the Aib residues succeeds smoothly using the highly reactive alpha-azido isobutyryl chloride followed by reduction of the resulting azido peptidyl resin to the amino peptidyl resin. Additionally, D6-Aib residues have been incorporated into strategic positions of efrapeptin C in order to obtain distance constraints involving the undeuterated Aib residues as insufficient NOE data may be obtained in the case of Aib-rich peptides. Investigation of side chain distances should facilitate structure determination in solution by NMR methods.

P71

TOTAL SYNTHESIS OF THE PROPOSED STRUCTURE OF ONCHIDIN

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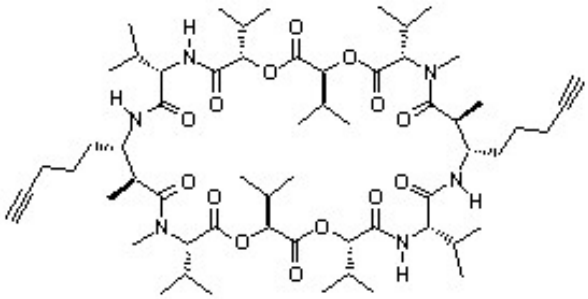
The first total synthesis of a molecule possessing the stereochemistry proposed for onchidin is described. The structures synthesized appear to be different from that of the marine natural product.



P72

THE CHEMICAL SYNTHESIS OF HUMAN AND MOUSE INSULIN-LIKE 6

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Insulin-like 6 (INSL-6) or relaxin/insulin-like factor 1 (RIF-1) is a newer member of the insulin super-family. The solid-phase syntheses of human and mouse INSL-6 of the predicted sequences based on the possibly enzymatic cleavage sites from the preprohormone had been performed. Using the modified method as described in the synthesis of human INSL-3 (Bullesbach and Schwabe, Biochemistry 1999, 38 3037-3078), the Cys(Acm)-7, Cys(tBu)-20- A(GYSEKCCLTGCTKEELSIACLPYIDF) and Cys(Acm)12- B chains (ELSDISSARKLCGRYLVKEIEKLCGHANWSQFR) of the human INSL-6 were obtained by solid-phase method. Formation of the intra S-S bond (A-6 to A-11) of the A chain and the inter-disulfide bridges (A-20 to B-24 and A-7 to B-12) of the A- and B-chains were carried out either by iodine oxidation or S-2-pyridylsulfenyl activation selectively. Purification of the peptide by preparative HPLC gave pure human INSL-6 with corrected molecular ion with mass spectrum.
The mouse INSL-6 with the A-chain (GFADKCCVIGCTKEEMAVACLPFVDF) and the B-chain (EEEEESRPRKLCGRHLLIEVIKLCGQSDWSR) was also synthesized and purified as described in the preparation of human INSL-6.

P73

SYNTHESIS AND BIOLOGICAL EVALUATION OF LINEAR AND CYCLIC DOTA-MSH ANALOGS FOR MELANOMA TUMOR TARGETING

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Peptides labeled with gamma- or beta-emitters have become important clinical tools for tumor diagnosis and radiotherapy. A major obstacle in wider-spread application of internal radiotherapy for treatment of tumor metastases is the high non-specific accumulation of the radiolabeled peptides in central organs such as the liver and the kidneys, the latter also serving as excretory route for the radiopeptides. In order to test the factors that govern the extent of non-specific accumulation by the kidneys on the one hand and the uptake by the tumor lesions on the other, we have synthesized and compared in vitro and in vivo several linear and cyclic peptides based on the structure of the melanoma targeting candidate [Nle4, Asp5, D-Phe7]-?-MSH4-11, containing the metal chelator DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) for radiometal incorporation. One of the aims was to assess the effect of cyclization of the peptides on the tumor-to-kidney ratio of radioisotope accumulation. The other aim was to study specific enzymic break points or the role of the position of the chelator in the different peptide molecules. Although marked improvements on the tumor-to-kidney ratio could be achieved, neither cyclization nor the other alterations abolished kidney uptake, demonstrating that both factors, an optimized radiopeptide ligand structure as well as novel inhibitors for blocking the non-specific transport of the radiopeptides through proximal tubular cell membranes in the kidneys are required before internal radiotherapy can be applied more generally or with fewer side-effects.

P74

PEPTIDE CONJUGATES - TOWARD TARGETED PHOTODYNAMIC THERAPY

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Targeting of drugs specifically to tumor cells has become a desired goal of many studies aiming to improve related therapies. Various human tumors are hormone dependent or hormone responsive and contain relevant hormone receptors. Receptors for peptide hormones such as gonadotropin-releasing hormone (GnRH) and vasoactive intestinal peptide (VIP) are often over-expressed on various cell lines, including ovarian, prostate, pancreas, endometrium and breast cancer cell lines. This present study was directed toward conjugation of cytotoxic molecules to these hormone 'carriers' with the hope to significantly improve cancer treatment. Thus, several derivatives of VIP and GnRH (agonists as well as antagonists) were coupled to cytotoxic molecules such as hypericin and helianthrones derivatives, quinone derivatives (mainly anthraquinones), and phorphrin derivatives (mainly chlorine e6). The ability of the peptide conjugates to produce reactive oxygen species (ROS), upon irradiation (i.e. application to Photodynamic Therapy, PDT) or enzymatic reactions was examined by employing electron spin resonance (ESR) studies. Generation of these reactive species might be a precondition for the manifestation of anti tumor activity. The affinity and specificity of conjugates for the specific receptors was also studied by different biological assays. The cytotoxicity towards cancer cells is currently being tested compared to their unconjugated cytotoxic molecules.

P75

MASS SPECTROMETRIC CHARACTERISATION OF THE LECTIN-LIKE DOMAIN OF HUMAN TNF-ALPHA

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Tumour Necrosis Factor-alpha (TNF-alpha) is a protein secreted by activated macrophages and lymphocytes which induces hemorrhagic necrosis of certain tumours in vivo, initiated by binding to high-affinity receptors. It also has a lectin-like affinity of unknown molecular specificity and interacts with certain parasites, e.g. inhibiting the development of Trypanosomes. The trypanolytic and lectin-like affinity is mediated by the a TNF-alpha domain, called TIP-domain (amino-acid sequence CQRETPEGAEAKPWY), which is functionally and spatially distinct from the receptor binding sites. In our study, we synthesised different linear and cyclic TIP-peptides and investigated their proteolytic stability in human plasma, showing that these peptides are extremly stable against proeolytic digestion, because of binding to specific plasma proteins, possibly glycoproteins. Using ESI-FTICR mass spectrometry, we examined the interaction of these peptides with two carbohydrates, chitobiose and cellubiose. The experiments demonstrated, that exclusively chitobiose is forming a noncovalent complex with TIP-peptides but not cellobiose.

P76

INVESTIGATIONS INTO THE C-TERMINAL MODIFICATION OF SOMATAOSTATIN ANALOGS

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Somatostatin receptor (SSTR) selective peptides, in particular octreotide and octreotate have found widespread application as transporters for radioisotopes in nuclear medicine. The notable similarity in the traditional SPPS of octreotides is that they are all functionalised at the N-terminal. The main reason for this is that the C-terminal amino acid, threonine, is usually loaded onto the resin via its carboxylic acid moiety, thus, blocking this terminal for manipulation whilst still being attached to the resin. The aim of this work was to investigate the potential of conjugates of Tyr3-octreotate linked at the C-terminal end of the peptide. In order to access the C-terminal, N-alpha-Fmoc-L-threonine allyl ester was synthesized and loaded onto Ellman's dihydropyran resin. The linear octapeptide Fmoc-D-Phe-Cys(Acm)-Tyr(tBu)-D-Trp(Boc)-Lys(Boc)–Thr(tBu)–Cys(Acm)–Thr(THP-resin)-Oallyl was synthesised stepwise using an Fmoc protocol. After cyclization and removal of the allyl ester protecting group, this peptide could be selectively functionalised at the C- or N-terminal. To allow the determination of structure activity relationships for the C-terminal modification of Tyr3-octreotate, receptor affinity profiles of the C-terminal modified Tyr3-octreotate derivatives (IC50) were obtained from competition binding experiments with 125I-TOC on rat cortex membranes. The biodistribution was studied in tumor bearing rats. In the somatostatin receptor positive tumor CA20948 the accumulation rate of the 125I labeled peptides was relatively low as compared to N-terminally conjugated derivatives.

P77

SYNTHESIS AND EVATLUATION OF INTERCALATING PEPTIDE CONJUGATES FOR THE ENDORADIOTHERAPY

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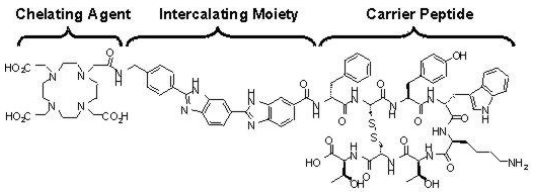
When conjugated to 'therapeutic' nuclides such as 90Y, somatostatin receptor binding peptides have been shown to be successful for the therapy of receptor expressing tumours. The effectiveness could be further increased with conjugates which enable the nuclear targeting of the low energy emitting radioactive isotopes. The bis-benzimidazole dyes, Hoechst 33258 and 33342, have been shown to bind to the minor groove of DNA in A-T rich regions. Auger-electron-emitting radionuclides can be extremely radiotoxic and produce extensive DNA damage. Auger electron-emitting radioisotopes, such as 111In and 67Ga are known to be highly cytotoxic when localised in cell nuclei due to highly localised energy deposition. The bis-benzimidazole intercalating moiety was prepared using variations on the literature methods and coupled under normal SPPS conditions to the carrier peptide, Tyr3-octreotate. Next the chelating agent (DOTA) needed to be attached to the intercalating moiety. First, the free amine derivative was prepared by its treatment with trifluoroacetic acid. This was coupled in solution to DOTA tris-t-butyl ester and coupled to the Tyr3-octreotate using SPPS. These conjugates can be efficiently labelled with radionucildes as exemplified with 111In. Using fluorescence microscopy, the cellular uptake of these conjugates was investigated.



P78

DISCOVERY OF PEPTIDE BASED FIBRIN TARGETED THROMBUS MR IMAGING AGENTS

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Thrombus imaging is an area of significant clinical importance. Previous approaches employed fibrin-specific monoclonal antibodies or radiolabelled peptides which had limited success for imaging DVT. We are interested in developing compounds with sufficient fibrin affinity and selectivity to be incorporated into a fibrin-targeted thrombus magnetic resonance contrast agent. Small peptides offer many advantages over monoclonal antibody targeting methods including more favorable antigenic and pharmacokinetic properties. We describe the design, synthesis and evaluation of peptides and peptide-based metal conjugates that selectively bind to fibrin. Phage display screen using a X1X2CX3X4X5X6X7CX8X9 library against fibrin and DD(E) yielded a family of peptides with the consensus sequence X1X2CX3YYGTCLD. The fibrin-binding affinity of one such representative peptide 1 (LPCDYYGTCLD-NH_2) was optimized using medicinal chemistry approaches such as determination of minimum fragment, Ala scan, D-amino acid scan, and unnatural amino acid substitutions. Linear peptide sequences were synthesized on Rink resin by Fmoc strategy and cyclization was carried out using thallium trifluoroacetate. DTPA was coupled to 1 followed by chelation with gadolinium to give prototype thrombus MR imaging agent 2.

P79

A BRIDGE BETWEEN NATURAL PRODUCTS TO CANCER DRUGS: TOWARD SUBCLASS SELECTIVE CYCLIC-PEPTIDE-INHIBITORS OF HISTONE DEACETYLASES

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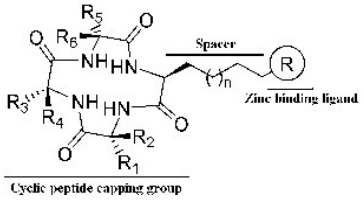
Inhibitors of histone deacetylases (HDACs) have been recognized as attractive therapeutic targets for anticancer, antifungal, antiviral and antiinflamatory treatment. HDAC inhibitors played an important role in discovering HDACs and in elucidating the functions of HDACs [1]. Cyclic tetrapeptide-based HDAC inhibitors, such as CHAPs and SCOPs provided information about the factors leading to HDAC inhibitory activity and selectivity [2]. The structural diversity at the periphery of the catalytic sites of different members of the HDAC family showed the importance of cyclic peptides as capping group in the inhibitor. In order to develop specific inhibitors of different HDACs, we used different cyclic peptides such as cyclic tetrapeptides with different combinations of amino acids with different chirality patterns and also with diverse terminal functional groups such as hydroxamic acid, retrohydroxamic acid, electrophilic ketones, benzamides and sulfhydryl groups. The inhibitors with different functional groups and the same scaffold provided information about the interactions in the active site of HDACs. The next generation of HDAC inhibition research will lead to the discovery of highly selective inhibitors of each member or sub-class of HDAC family. References: 1. M. Yoshida, A. Matsuyama, Y. Komatsu, N. Nishino, Curr. Med. Chem., 10, 2351-2358 (2003). 2. N. Nishino, B. Jose, S. Okamura, S. Ebisusaki, T. Kato, Y. Sumida, M. Yoshida, Org. Lett., 5, 5079-5082 (2003).



P80

FUNCTION OF CORAZONIN IN REGULATION OF INSECT ECDYSIS

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Insect development from larva to adult depends on the repeated shedding of old cuticle through a highly stereotyped behaviour called ecdysis. A successful ecdysis requires the precise co-ordination of behaviour with the developmental changes that occur late in a moult. This co-ordination involves two sets of peptidergic cells: the peripherally located Inka cells, which produce pre-ecdysis triggering hormone (PETH) and ecdysis triggering hormone (ETH), and the brain neurosecretory cells, which synthesize eclosion hormone (EH). Blood-borne EH acts on the Inka cells to cause the release of PETH, ETH, which in turn act on the CNS to initiate specific phases of the ecdysis. We report that corazonin, a highly conserved neuropeptide of widespread occurrence in insects, is another factor necessary in the regulation of ecdysis. Injection of corazonin into pharate larvae elicits release of PETH, ETH from Inka cells, which induce precocious pre-ecdysis and ecdysis. In vitro studies with isolated Inka cells show that corazonin induces PETH, ETH secretion at physiological concentrations (25-100 pM). Corazonin circulates in the hemolymph at 20 min prior to natural pre-ecdysis onset at concentrations ranging from 20-80 pM, and then declines over next 30-40 min. These findings support the role of corazonin signalling in initiation of the ecdysis, and we propose a new model for peptide-mediated interactions between Inka cells and the CNS. Corazonin controls calcium-mediated initial release of PETH, ETH from Inka cells, while EH induces depletion of Inka cell peptides 30 min later, through elevation of calcium and cGMP.

P81

LANSOPRAZOLE RAISES SOMATOSTATIN, CALCITONIN GENE-RELATED PEPTIDE AND SUBSTANCE P IN HUMAN PLASMA

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Lansoprazole, a proton pomp inhibitor (PPI), widely used in treatment of peptic ulcer, gastroesophageal reflux disease and eradication of Helicobacter Pylori. Lansoprazole has reported a PPI with mucosal protective action, but the mechanism other than inhibition of gastric acid secretion is not well understood. So we examined the effects of lansoprazole on plasma levels of gastrointestinal peptides (somatostatin, gastrin, motilin, substance P and calcitonin gene-related peptide (CGRP)). Lansoprazole at a dose of 30 mg or placebo was orally administered in five healthy male volunteers aged 25-30 years. Venous blood samples were taken before and at 30, 60, 90, 120, 180, 240 and 300 min after administration. Plasma peptide levels were measured using sensitive enzyme immunoassays. Compared with the response of the placebo treated group, lansoprazole causes significant ($p<0.05$) increase in somatostatin-, substance P- and CGRP-immunoreactive substance (IS) at 120 min, 90-240 min, and 120 min, respectively. Lansoprazole had no effect on plasma motilin-IS levels compared with the placebo. In this study, lansoprazole raises somatostatin-IS levels and inhibites increase in gastrin-IS levels. Furthermore, lansoprazole raises CGRP-IS and substance P-IS levels. Recently, capsaicin-sensitive afferent nerves play an important role in gastric mucosal defensive mechanisms. Capsaicin stimulates afferent nerves and enhances the release of CGRP and substance P in the stomach. Therefore, lansoprazole might have inhibition gastric acid secretion but also gastroprotective action via capsaicin-sensitive afferent nerves.

P82

α AND ß-ASPARTYL PEPTIDE ESTERS FORMATION VIA ASPARTIMIDE RING OPENING

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A well-documented problem in the synthesis of aspartic acid- containing peptides is the aspartimide formation. This undesirable reaction has been proved to occur under both acidic and basic conditions and is dependent on the β-carboxyl protecting group, the acid or base used during the synthesis as well as the peptide sequence. Hydrolysis of the aminosuccinimide yields a mixture of α- and β-aspartyl peptides. On the other hand, cyclization of an aspartyl residue to succinimide in the polypeptide chain is particularly useful in the diketopiperazine formation. The mechanism for this reaction is an intramolecular attack of the peptide amino terminus on the aminosuccinyl carbonyl group. In a previous study, we demonstrated, for the first time, that treatment of the Asu-containing peptides with methanol in the presence of 2 % DIEA results in α- and β -aspartyl methyl ester peptides [Kostidis et al. Tetrahedron Letters 2003,44, 8673-8676]. Taking advantage of these results, we decided to test the aspartimide ring opening conditions using different types and concentration of alcohols (primary and secondary) and bases (DIEA, collidine, 4- Pyrolidino pyridine, 1- Methyl- 2- Pyrolidone, Piperidine) at different temperatures and times of reaction. These experiments were performed both in solution and on solid phase just after completion of the peptide sequence. The best results were obtained with DIEA while the aspartimide ring opening with secondary alcohols was realized only at high temperature values.

P83

FOS-RELATED ANTIGEN 1 (FRA-1) IN THE RAT AMYGDALA MIGHT BE INVOLVED IN THE ACQUISITION AND CONSOLIDATION OF EMOTIONAL MEMORIES: DENSITOMETRY AND STATISTICAL ANALYSIS

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Memory consolidation is thought to involve a series of molecular events requiring transcriptional gene regulation, which eventually ends in long-term changes at the synapse. We found by Western Blot analysis, a decrease in phosphorylated extracellular-signal regulated kinase 1 and 2 (ERK 1/2) in amygdala protein extracts from Conditioned Taste Aversion (CTA) trained animals at 1 hour and 6 hours after conditioning. Western Blot analysis also showed a decrease in the levels of cAMP Responsive Element Modulator Protein (CREM), at 1 hour after conditioning. We did Western Blot analysis for the transcription factor Fra-1 using protein extracts of CTA trained animals sacrificed at different time-points. The highest levels of Fra-1 were at early and late time-points, such as 5 minutes and 30 minutes, and 6 hours after CTA training, respectively. In order to make statistical analysis by One Way ANOVA, we performed a triplicate of Fra-1 immunodetection, and performed densitometric analysis of the data. We believe that the molecular events modulating the generation of a strong emotional memory, such as the one generated in CTA, is involved in other learning experiences or psychoemotional disorders involving limbic system structures, such as the amygdala. Most, if not all of psychoemotional disorders, involve associative relations between different events, similar to the associative learning involved in CTA.

P084

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ENANTIOSEPARATION OF BETA-AMINO ACIDS

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The past decade has seen a growing interest in b-amino acids, which are important intermediates for the synthesis of compounds of pharmaceutical interest and can be used as building blocks for peptidomimetics. Oligomers of β-amino acids (β-peptides) fold into compact helices in solution. Recently, a novel class of β-peptide analogues adopting predictable and reproducible folding patterns (foldamers) was evaluated as a potential source of new drugs and catalysts. Studies on synthetic β-amino acids can be facilitated by versatile and robust methods for determining the enantiomeric purity of starting materials and products. High-performance liquid chromatography (HPLC) is one of the most useful techniques for the recognition and/or separation of stereoisomers including enantiomers. The aim of the present work was to evaluate HPLC methods for the separation of enantiomers of 3-amino-substituted unusual β-amino acids (β-3-homo amino acids) such as 3-amino-3-phenylpropanoic acid, β-5-phenyl-pentanoic acid, β-4-(3-methylphenyl)-butyric acid, β-4-(4-methylphenyl)-butyric acid, β-4-(4-chlorophenyl)-butyric acid, β-4-(2-naphthyl)-butyric acid, β-4-(3-pyridyl)-butyric acid, β-4-(4-pyridyl)-butyric acid, β-3-(2-furyl)propanoic acid, β-3-(3-furyl)propanoic acid, β-3-(2-thienyl)propanoic acid and 3-amino-3-(3-thienyl)propanoic acid. The effects of different parameters on selectivity, such as the nature of the organic modifier, the mobile phase composition, the flow rate and the structure of the analytes are examined and discussed. The separation of the stereoisomers was optimized by variation of the chromatographic parameters. The efficiency of the different methods and the role of molecular structure of the derivatized analytes in the enantioseparation were noted. The elution sequence of the enantiomers was determined in most cases.

P085

FORMULATION OF AN INJECTABLE IMPLANT FOR PEPTIDE DELIVERY AND STUDYING THE EFFECT OF POLYMER MOLECULAR WEIGHT ON ITS RELEASE BEHAVIOR

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Purpose: Injectable implants, as a novel drug delivery systems, look very promising in peptide delivery. These systems are usually injectable polymeric solutions that solidify after injection, and release their drug in a controlled manner. the main problem related to these systems is usually their burst effect. It was decided here to study the effects of polymer molecular weight on release behavior of a leuprolide acetate (LA) containing injectable implant. Methods: Poly (lactide-co-glu=ycolide) 50:50 with molecular weights of 12000 and 48000 were used in this investigation. Polymeric solutions containing 3%(w/w) LA were prepared and their release behavior were studied using a home- made especially -designed diffusion cell. The cells allow studying release behavior the system in both liquid and solid phases. Results: Results showed that molecular weight affects burst effect significantly. However, the steady-state rate of the release looked nearly the same, regardless of molecular weight(MW). The amount of drug released at first 24 hours for lower MW 32%+- 0.485%(X+SD,n=3), was significantly ($p<0.05$) higher than that of higher MW, 13% +- 0.078% (X+SD, n=3) . This should be due to increased polymer chain interactions and therfore, reduced diffusion coefficient of drug in higher molecular weight polymer.

P086

CYCLIC RGD-PEPTIDES WITH AROMATIC PHOSPHONATE-ANCHORS FOR STIMULATED CELL ADHESION ON TITANIUM

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Cyclic RGD-peptides of the type c(RGDf[NMe]V) are high affinity ligands for the alpha-v-beta-3 integrins [1]. These peptides stimulate osteoblast adhesion, cell proliferation, and support ingrowth of bone implants in vivo when immobilized on biomaterials [2]. Phosphonic acid groups can be used for anchoring RGD-peptides on titanium [3]. We present a set of cyclic RGDfK-peptides conjugated with an aromatic scaffold bearing two phosphonic acid groups. Different spacer length and numbers of anchor molecules were used. We describe the synthesis of the conjugates, and the results of cell adhesion tests. [1] Sulyok, G.A.G., Gibson, C., Goodman, S.L., Hölzemann, G., Wiesner, M., Kessler, H., J. Med. Chem. 44, 1938 (2001). [2] a) Kantlehner, M., Schaffner, P., Finsinger, D., Meyer, J., Jonczyk, A., Diefenbach, B., Nies, B., Hölzemann, G., Goodman, S.L., Kessler, H., ChemBioChem. 1, 107 (2000). b) Kantlehner, M., Finsinger, D., Meyer, J., Schaffner, P., Jonczyk, A., Diefenbach, B., Nies, B., Kessler, H., Angew. Chem. Int. Ed. 38, 560 (1999). [3] Meyer, J.; Nies, B.; Dard, M.; Hoelzemann, G.; Kessler, H.; Kantlehner, M.; Hersel, U.; Gibson, C.; Sulyok, G.. WO 2002013872 A1, (2002).

P087

CONVENIENT ENZYMATIC METHOD TO SYNTHESIZE PEPTIDES P-NITROANILIDES IN ORGANIC MEDIA

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Synthesis of oligopeptides containing different chromogenic and fluorogenic moieties, which are substrates for proteases of different types, is one of the main sectors of modern peptide chemistry. Chemoenzymatic production of such a peptide derivatives in organic media is the favorable way in most cases. We compare product yields in typical synthesis of Z-Ala-Ala-Leu-Phe-pNA in acetonitrile/DMF/water media (6/3.5/0.5) catalyzed by suspension of native subtilisin, suspension of noncovalent complex subtilisin with poly(acrylic acid) (PAA) and subtilisin covalently immobilized on cryogel of poly(vinyl alcohol). In first two cases high yields was reached within 2 hours at [E]/[S] = 1/5000. Considerable acceleration of reaction rate was not observed when enzyme was modified by complexation with PAA. In spite of slightly lower [E]/[S] ratio in reactions catalyzed by immobilized subtilisin (1/1000) its superior stability and possibility of biocatalyst reuse made this modified enzyme very promising for catalysis of peptide synthesis. p-Nitroanilides of amino acids were amino components and N-protected tripeptides with free -COOH group were carboxyl components; hydrophobic (Leu, Phe in P1 and in P1') and hydrophilic (Glu, Lys, Thr in P1 and Asp, in P1') amino acid residues were tested in different reactions catalyzed by immobilized enzyme. Multifunctional amino acids have no protection groups in side chain. It was shown that Leu-Phe, Leu-Asp, Glu-Phe, Glu-Asp, Lys-Phe, Lys-Asp, Phe-Phe and Thr-Leu bonds could be formed under subtilisin catalysis. Quantitative yields of all products within short reaction time were obtained in all cases. This work was supported by RFBR grant 03-03-32847 and INTAS grant 01-0673.

P088

STUDIES ON THE SYNTHESIS OF PEGYLATED INSULIN DERIVATIVES

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The site-specific pegylation constitutes one of the most efficient ways to improve the physicochemical, immunological and pharmacological properties of important pharmaceutical peptides and proteins. So far, the majority of PEG conjugations are performed in recombinantly produced proteins with poorly defined poly(ethylene glycol) derivatives. The aim of our studies is to develop an efficient synthetic strategy to produce human insulin conjugated with precisely defined PEGs. As a first approach, we prepared fully protected fragments spanning the entire length of A- and B-insulin chains, using Fmoc/tBu chemistry on 2-chlorotrityl resin. Alternatively, A- and B- chains were synthesized by the conventional SPPS, for comparison. Cys side-chains were protected by the Acm, Trt, or Mmt group. The selective formation of intrachain and interchain disulfide bonds was investigated. A second approach involves the convergent assembly of the human mini-proinsulin sequence and subsequent folding, to take advantage of the conformationally assisted formation of the correct disulfide bonds.

P089

NOVEL TOOLS TO PREPARE PEPTIDE FRAGMENTS FOR CHEMICAL LIGATION BY USING FMOC STRATEGY

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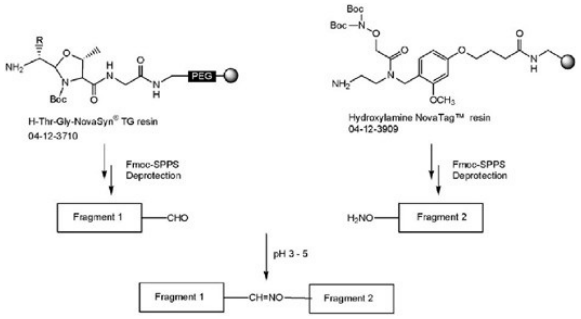
Chemoselective ligation is a powerful technique to synthesize large peptides, cyclic peptides and MAPs from unprotected peptide fragments in aqueous solution, based on the selective formation of particular covalent bonds. Typical peptide side-chain functionalities can be present. H-Thr-Gly-NovaSyn® TG resin (04-12-3710) and Hydroxylamine NovaTag™ resin (04-12-3909) were used as tools to prepare peptide fragments for oxime ligation using Fmoc standard procedures [1-3]. The protected aldehyde fragment was synthesized on N-Boc protected H-Thr-Gly-NovaSyn® TG resin via an oxazolidine formation [4]. The hydroxylamine fragment was directly obtained using the novel N,N-Bis-Boc protected Hydroxylamine NovaTag™ resin. We will present syntheses of peptide fragments and their ligation by oxime bond formation. [1] K. Rose, et al. (1994) JACS, 116, 30. [2] J. Shao & J. P. Tam (1995) JACS, 117, 3893. [3] F. Wahl & M. Mutter (1996) Tetrahedron Lett., 37, 6861. [4] N. J. Ede, et al. (2000) J. Pept. Sci., 6, 11.



P090

OPTIMIZATION OF SYNTHESIS OF VARIOUS PEPTIDES CATALYZED BY IMMOBILIZED SUBTILISIN 72

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Kinetic study of Z-Ala-Ala-Leu↓Phe-pNA synthesis catalyzed by subtilisin 72 immobilized on poly(vinyl alcohol) cryogel was carried out in anhydrous DMF/MeCN mixtures. The initial rates were estimated over the range of reagent concentrations 0.5-200 mM. It was shown that the higher concentrations of reagents were used, the greater yields of product were obtained. Using of immobilized subtilisin as a catalyst under these conditions enables to synthesize in high yields (76-98%) a series of di-, tri- and tetrapeptides, containing chromophores. Methyl and carbamoyl (Cam) esters of peptides containing Leu, Ala, Arg, Gly in the P1 position were employed as acylating components in these reactions. As amino components a number of p-nitroanilides of amino acids were used with Leu, Phe, Glu in the P1' position. Influence of the carboxyl group activation was investigated in Z(Boc)-Leu-OMe(OCam) + Phe-pNA reaction, the yield of the reaction reached 98% in 0.5 h when Boc-Leu-OCam was used as acyl donor. It should be noted that peptides with a free carboxyl group were found to be efficient as acylating components in some cases. Special attention has been called to prove stereospecificity retaining. The effectiveness of the optimized method was demonstrated by preparative synthesis of chromogenic substrate Z-Ala-Ala-Leu-pNA (100% yield after 2 h). This work was supported by RFBR grant №03-03-32847 and INTAS grant №01-0673.

P091

AUXILIARIES FOR COMBINATORIAL INTRAMOLECULAR STAUDINGER LIGATIONS

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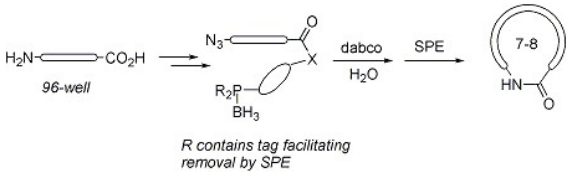
Small cyclopeptide like medium-sized lactams (7- and 8- membered) are promising drug-like molecules. To date, intensive biological screening of small cyclopeptides is hampered because of their difficult synthetic accessibility. Direct ring-closure of the precursor dipeptides made up of linear alpha- and beta-amino acids or of two linear beta-amino acids, providing 7-membered [1,4]-diazepane-2,5-diones or 8-membered [1,5]-diazocane-2,6-diones, is especially difficult. Besides the unfavored ring-size, the trans arrangement of the amide bond prevents the required spatial positioning of the mutually reactive terminal amine and activated carboxy groups for ring-closure. Recently, we developed the intramolecular Staudinger ligation as a powerful ring-closure method to cyclise small peptides that are reluctant to ring-closure.[1] On the poster several new auxiliaries for the Staudinger ligation will be presented which were designed for solution phase combinatorial approaches to 7- and 8- membered N-lactams from peptides. We focus on the synthesis of auxiliaries containing basic tags that enable simple removal by acidic solid-phase extraction (SPE). [1] O. David, W.J.N. Meester, H. Bieraugel, H.E. Schoemaker, H. Hiemstra, J.H. van Maarseveen, Angew. Chem. Int. Ed., 2003, 42, 4373-4375.



Scheme 1 : Parallel intramolecular Staudinger ligations

P092

PHOTO-ACTIVATED VASOACTIVE PEPTIDES

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Human endothelin-1 (hET-1) and urotensin-II (hU-II) are potent vasoactive peptides involved in a variety of biological processes. In order to gain a better knowledge of the molecular mechanism driving the different biological phenomena of these two vasoconstrictor hormones, some masked analogues, becoming biologically active only upon UV photolysis, were developed. Those compounds, known as ²caged², are derivatized with a photolabile 4,5-dimethoxynitrobenzyl group. Thus, libraries of caged analogues of hET-1 and hU-II were obtained by solid phase peptide synthesis based on the Fmoc chemistry. For these syntheses, special chemical approaches were used: (i) derivatization of selective Fmoc-amino acids prior to their incorporation in the peptide chain, (ii) targeted chemical modification on the residue side chain during peptide synthesis and (iii) post-synthesis chemical derivatization. After purification and analytical characterization, all caged peptides were evaluated pharmacologically with the rat thoracic aorta ring and/or guinea-pig lung parenchyma strip bioassays. All caged analogues showed a marked reduction of their affinity for their respective receptors, especially [Lys(DMNB)8]hU-II and [Glu(DMNB)10]hET-1. After UV irradiation, all native biologically active peptides were recovered. In situ photolysis was also performed and revealed that this technology was compatible with an experimental system using animal tissues. This study showed that we developed an efficient method for the preparation of caged peptides. The ulterior use of these photo-activated vasoactive peptides will facilitate the molecular characterization of intracellular biological processes.

P093

A NEW STRATEGY TOWARDS A HIGH YIELD SYNTHESIS OF THE ETB RECEPTOR ANTAGONIST BQ-788

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The synthesis strategy of the first selective ETB receptor antagonist, BQ-788 (N-(cis-2,6-dimethylpiperidinocarbonyl-g-methylleucyl-D-1-(methoxycarbonyl)-tryp tophanyl-D-norleucine sodium salt) was reported by He et al. (JOC, 60, 8262, 1995), who used a classical way of N-elongation via urea type linkage done with phosgene. Coupling of N-(cis-2,6-dimethylpiperidine) to the activated N-terminal amino acid and then in a convergent manner (N→C-elongation) building in a 13 steps synthesis process the final peptidoid at a yield of 27 %. To date, BQ-788 is one of the most predominant tool to identify ETB-receptors dependent responses in over 550 publications in the last 10 years. In order to improve both yield and to reduce the number of steps in synthesis BQ-788, we have adopted a Boc-strategy in solution for assembling the peptidoid from C-terminal p-nitrobenzyl ester amino acid. The carbamination on the side chain of tryptophan was constructed on Boc-D-Trp-D-Nle-OBzl(NO2). The final product was achieved using a one pot procedure for highly hindered trisubsituted urea link with triphosgene and sodium iodide. Our strategy using 7 steps allowed an overall 53 % yield of BQ-788 which has been subsequently NMR- and MS-identified. Finally, synthesized BQ-788 at a 1 mg/kg dose abolished the pressor responses to a selective ETB agonist IRL-1620 (1 nmol/kg) in anesthetized CD-1 mice. Overall our strategy offers an elegant and more efficient alternative towards the synthesis of the selective ETB receptor antagonist BQ-788. This project is financially supported by the Canadian Institutes for Health Research.

P094

INDUSTRIALLY ATTRACTIVE SYNTHESIS OF ENANTIOPURE FUNCTIONALISED UNNATURAL AMINO ACIDS

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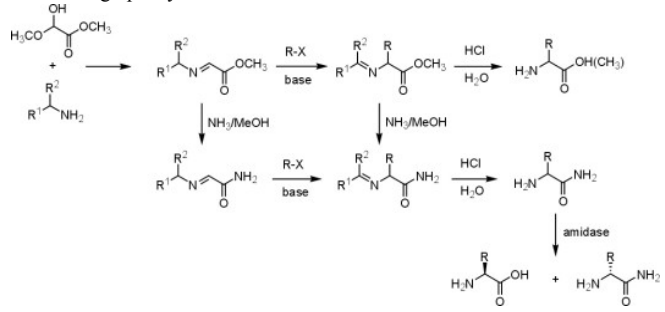
In addition to the natural amino acids, there is an increasing demand for enantiopure unnatural amino acids with target-oriented functionalised side-chains. With increasing complexity of the side-chain new synthetic methods are needed to prepare these amino acids. In this paper we describe for the first time a new industrially attractive alternative for the alkylation of benzophenone imines of glycine derivatives as developed by O'Donnell.(1) This new method makes use of the readily available glyoxylic acid esters (optionally as its hemi-acetal) and a,a-disubstituted methylamines, like benzhydrylamine, (racemic) a-methylbenzylamine or i-propylamine as starting materials.(2) Imine formation with these reagents proceeds smoothly and in high yield. Subsequent alkylation of these imino esters gave after hydrolysis Ca-substituted amino acid (esters). Alternatively, the imino esters can be easily transferred into the imino amide derivatives and subsequently alkylated. The amino acid amides thus obtained can be resolved using the DSM amidase technology. In this way both enantiomers of the a-amino acid (amide) can be obtained in high purity.



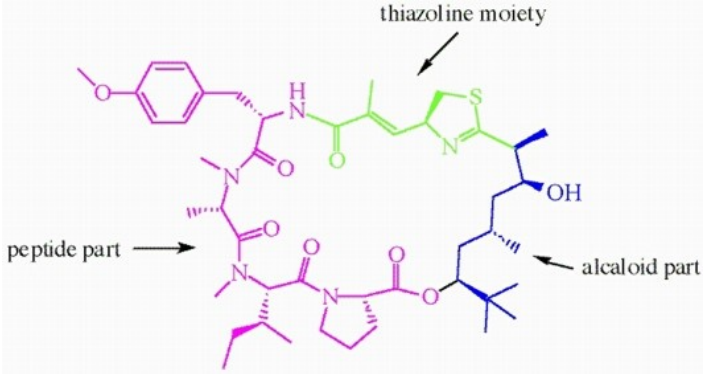
P095

TOWARDS THE TOTAL SYNTHESIS OF APRATOXINE A

A. Gilles[1], L. Rocheblave[1], D. Stien[2], E. Vivès[3], J. Martinez[1], **F. Cavelier**[1]

[1]Lapp Umr 5810 Cnrs-Umi-Umii, Montpellier [2]Cnrs, Ups 2561, Cayenne [3]Umr 5124 Cnrs-Umii, Montpellier, France

Apratoxine A is a natural compound isolated from a marine cyanobacterium. This molecule exhibits subnanomolar in vitro cytotoxicity against human tumor cell lines [1]. Our purpose was to synthesize this natural peptolide [2,3] to study its activity mechanism, which is still unknown so far. We will use our synthetic scheme to prepare analogues for structure-activity relationship study. In this communication, we describe our approach to the synthesis of apratoxin A. References : [1] Luesh, H.; Yoshida, W. Y.; Moore, R. E.; Paul, V. J.; Corbett, T. H. J. Am. Chem. Soc. 2001, 123, 5418. [2] Chen, J.; Forsyth, C. J. J. Am. Chem. Soc. 2003, 125, 8734. [3] Zou, B.; Wie, J.; Cai, G.; Ma, D. Org. Lett. 2003, 5, 3503.



P096

MICROWAVE ENHANCED SOLID PHASE PEPTIDE SYNTHESIS

J.M. Collins, E.E. King, J.J. Lambert, G.R. Greene, J.T. Hegeman, J.D. Ferguson

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With the growth in biological peptide research it is important to have a way of efficiently creating high quality peptides. Traditional solid phase peptide synthesis is plagued with the difficulties of aggregation leading to slow and incomplete synthesis. Microwave energy has proved to be an efficient energy source for enhancing peptide synthesis. In this paper we show the use of microwave synthesis on longer and more difficult 30-50mer peptides using Fmoc chemistry. Racemization and other side reactions were explored and found minimized. In addition, the use of alternative reagents was explored and found possible. Using an automated microwave peptide synthesizer complete cycle times of 15 minutes were possible for each amino acid addition. With microwave we were able to create higher purity peptides with much faster synthesis time then compared to traditional methods.

P097

BASE-INDUCED GLUTARIMIDE FORMATION DURING FMOC-BASED SPPS

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Contrary to aspartimide formation, reports describing glutarimide formation during Fmoc-based SPPS have rarely appeared in the literature [1]. During our work on the aspartimide problem [2], we studied the Glu3 –analogues of the well-established model peptide Val-Lys-Asp3-Xaa-Tyr-Ile (Xaa = Gly, Arg(Pbf) or Cys(Acm)). Glu showed a much lower tendency towards base-catalyzed cyclization than Asp, thus, OtBu g-carboxy protection efficiently prevented this side reaction. Less hindered Glu side chain protecting groups such as OAll are less effective. Prolonged base treatment of resin-bound Val-Lys(Boc)-Glu(OAll)-Gly-Tyr(tBu)-Ile mimicking repetitive Fmoc cleavage with piperidine or DBU yielded glutarimide as the main product, precluding the use of OAll in a Glu-Gly situation without further precautions. Again (as in many other cases), Hmb backbone protection is the key solution to the problem. The motifs Glu(OAll)-Arg(Pbf) and Glu(OAll)-Cys(Acm) are less sensitive, but by no means inert towards bases. On the other hand, Glu(OPp) turned out to be slightly less stable than Glu(OtBu). This makes the phenylisopropyl ester an excellent choice if the g-carboxy protecting group has to be removed selectively for postsynthetic modifications of the Glu side chain in a base-sensitive vicinity. Detailed results will be presented in our poster.

1. J.A. Kates & F. Albericio, Lett. Pept. Sci. 1 (1994) 213.
2. M. Mergler, F. Dick, B. Sax, C. Stähelin & T. Vorherr, J. Pept. Sci. 9 (2003) 518.

P098

DIORASSP: DIOSYNTH RAPID SOLUTION SYNTHESIS OF PEPTIDES

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Ever-increasing pressure is being imposed upon the pharmaceutical industry to reduce time-to-market for new drugs. Time-to-market, from the API-manufacturer's point of view, comprises the development of synthesis routes for new compounds, the scale-up of the ensuing processes and their validation c.q. registration. An additional challenge lies in the eventual manufacturing of API's of increasingly higher and reproducible purity in a commercially competitive way, which is environment-friendly and compatible with ever more stringent guidelines regarding cGMP. These incentives prompted Diosynth's R&D Peptides Department to a thorough revaluation of the two classical approaches of peptide synthesis, resulting in the development of a novel method for the synthesis of peptides, called DioRaSSP. It combines the advantages of the homogeneous character of classical solution-phase synthesis with the generic character and the amenability to automation inherent to the solid-phase approach. Intermediates of a DioRaSSP process are not isolated. DioRaSSP is further characterized by the application of a new quenching procedure for residual activated carboxylic compounds. A considerable number of protected peptides have been synthesized at Diosynth. Purities and yields are generally high, as exemplified by the synthesis of a protected human Insulin octapeptide fragment, which was obtained in 98% purity and 85% yield in a first 7 days' trial. Several processes according to DioRaSSP have been directly scaled-up after a preliminary feasibility study on a laboratory scale, achieving reproducible results in terms of both yield and impurity profile. Diosynth is currently implementing the first fully automated solution-phase peptide synthesizer.

P099

'ONE-POT' PREPARATION OF N-PROTECTED AMINO ACIDS VIA THE AZIDE METHOD

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In the last decade, the N-fluorenylmethyloxycarbonyl (Fmoc) and N-allyloxycarbonyl (Alloc) groups have become important tools in peptide chemistry. The use of the powerful chloroformate reagents for their introduction can lead to the formation of protected dipeptides as side products. Even when the relatively hindered Alloc-Val-OH was prepared, 14% of the corresponding dipeptide was obtained. The use of the Bolin method, involving the in situ preparation of the trimethylsilyl amino acid derivatives and their subsequent reaction with the chloroformate, is tedious and impractical for large scale. Furthermore, the succinimide derivative is not practical when small amounts of complex amino acids are to be prepared, because the N-hydroxysuccinimide formed can contaminate the final product. Herein, a convenient and clean method for the preparation of Fmoc and Alloc amino acids is discussed. This method is particularly attractive due to the fact that the reaction sequence Fmoc/Alloc-chloride to Fmoc/Alloc-azide to Fmoc/Alloc-amino acid can readily be carried out in one-pot. A further advantage is the minimization of by-products, these being easily removed in the work-up. Finally, this strategy minimizes the formation of dipeptides that are difficult to remove by crystallization. Thus, Fmoc and Alloc amino acids are obtained in high yield (60-80%) and purity as evidenced by thin-layer chromatography, high-performance liquid chromatography and other analytical methods.

P100

BETA-ALANINE A NEW IMPURITY FOUND IN COMMERCIAL BATCHES OF FMOC-AMINO ACIDS DERIVATIVES

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beta-Alanine a new impurity found in commercial batches of Fmoc-amino acids derivatives beta-Ala has been found as an impurity in commercial batches of Fmoc-Ala-OH. During a routine solid phase synthesis of a peptide, beta-Ala was incorporated into the peptide structure, and formed a new impurity, which was impossible to reduce to an acceptable level in the product using the registered purification procedure. The synthesis and purification procedures for this drug substance has been developed at PolyPeptide Laboratories AB several years ago and several production scale batches have been produced since then. The impurity pattern for all these batches was similar and all impurities at levels ³0.1% have been isolated and identified. However, during the purification of the above-mentioned batch, the presence of a new impurity was observed. This impurity was detected as a well-separated peak using the in-process analytical HPLC system and its level was monitored to be 0.5% (HPLC height %). Attempts to reduce the content of the impurity to an acceptable level of 0.2% (HPLC height %) were unsuccessful. The impurity was isolated and analysed by different analytical methods. The results obtained proposed that the structure was an endo Ala analogue of the mother peptide. In addition several possible analogues were synthesised in order to confirm the suggested structure of impurity isolated. The specific batch of Fmoc-Ala-OH used for the synthesis of the peptide was re-analysed and the presence of Fmoc-beta-Ala-Ala-OH was confirmed. Analytical results for the presence of beta-Ala in other Fmoc-amino acids derivatives will be presented.

P101

IMMOBILISATION OF ALDO/KETO REDUCTASES BY PROTEIN LIGATION STRATEGIES

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New strategies for the specific enzyme immobilisation play a key role in the development of biosensors and in the emerging research field of bioelectronics today. The major drawback of common immobilisation methods is the lack of any specific site directed position of the enzyme on a surface. This may lead to randomly orientated enzymes and subsequently limited activity. The aim of this work is to selectively activate enzymes at their C-terminal position in order to allow specific immobilisation. We chose AKR1A1, an enzyme of the aldo/keto reductase superfamily, as subject for the synthesis of an artificially monolabeled redoxprotein. AKR1A1 is a monomeric enzyme of 324 amino acids and has a well-characterized (α/β)-barrel structure. It catalyzes the NADPH dependent reduction of aliphatic/aromatic ketones and aldehydes. To produce monofunctionalized enzyme we applied the strategy of Expressed Protein Ligation (EPL). Accordingly, we used the IMPACT®-system and cloned the aldo/keto-reductase as fusion protein with an additional intein/chitin binding domain. Through intein mediated splicing we could produce C-terminal thioester of the AKR1A1. In the next step, the thioester was coupled to Cys-modified TENTAGEL-beads by native chemical ligation, which led to C-terminally attached enzymes on microspheres. The enzyme activity was proven to be intact as shown by cyclovoltametry. Therefore the functionalized beads were embedded in PCS-hydrogel on a sensor chip. Further strategies for specific enzyme immobilisation and the suitability of the subsequent analytical steps will be described as well as the potent scope of artificial aldo/keto reductase systems based on the AKR1A1.

P102

DABCYL PLUS(TM), A HYDROPHILIC SUBSTITUTE FOR DABCYL

Z.J. Diwu, X.H. Tong, **A.L. Hong**

AnaSpec, Inc., San Jose CA, USA

Peptide substrates are important to study the activity of proteases, many of which are used in high throughput screening of compound libraries to obtain drug leads. DABCYL/EDANS is a FRET pair that is often used to label these peptide substrates. DABCYL increases the hydrophobicity of these peptide substrates, rendering some of them water insoluble. We have designed a molecule to replace DABCYL, in order to alleviate this problem. This molecule, which we named DABCYL Plus(TM), increases the water solubility of peptide substrates. Its excitation and emission wavelengths are close to those of DABCYL. These data will be presented. Utility of this molecule will also be presented by comparing the activity of peptide substrates labeled with DABCYL Plus(TM)/EDANS and DABCYL/EDANS, respectively.

P103

PEPTIDE SYNTHESIS ON FLUOROUS SUPPORT

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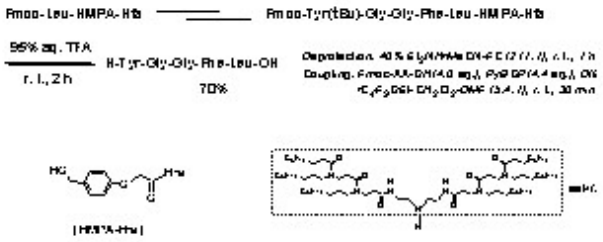
Recently, fluorous chemistry has been studied in several fields [1-3]. A fluorous (highly fluorinated) solvent is immiscible in an organic solution, and a fluorous compound partitions out of the organic phase and into the fluorous phase. Therefore, a fluorous compound is readily separated from nonfluorinated compounds by a simple "fluorous/organic" extraction. In this study, the synthesis of Leu-enkephalin by an Fmoc-strategy based on fluorous chemistry was demonstrated. Fmoc-Leu-OH was esterified onto new fluorous support HMPA-Hfa in 94% yield. Fmoc group was cleaved by 40% Et2NH solution, and the coupling was carried out using PyBOP. A 4-fold excess of the amino acid derivative was used in each coupling reaction. The peptide having a fluorous support was treated with TFA containing 5% H2O to cleave the peptide from the fluorous support and remove the side-chain protecting group. After partition between C4F9OEt□EtOAc (2:1) and water, the desired peptide was extracted into the water layer. After purification of the water layer by RP-HPLC, the Leu-enkephalin was provided in 70 % yield. [1] D.P. Curran, Science, 275 (1997) 823.[2] T. Miura et al Org. Lett., 3 (2001) 3947; T. Miura et al Angew. Chem. Int. Ed., 42 (2003) 2047; T. Miura et al Tetrahedron Lett., 44 (2003) 1819.[3] Chem. Commun., (2003) 972.



P104

NEW ARGININE AND HOMOARGININE DERIVATIVES FOR PEPTIDE SYNTHESIS

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We have recently described a method for the synthesis of new guanidinylation reagents [1], N,N´-bis(ortho-chloro-Cbz)-S-methylisothiourea (1) and N,N´-bis(ortho-bromo-Cbz)-S-methylisothiourea (2). These reagents turned out to be very efficient in the transformation of amines to urethane protected guanidines. Now we report the preparation of several derivatives of arginine and homoarginine by guanidinylation of α-protected Orn and Lys. The products were obtained in high yield and purity. In order to assess the utility of these derivatives for peptide synthesis, analogues of dynorphin containing two or three Arg residues were synthesized. These syntheses were compared to those with the use of Boc-Arg(Tos)-OH. A comparison of the products in terms of yield and purity indicated that the new derivatives were superior to Boc-Arg(Tos)-OH. 1 and 2 were also used for the efficient transformation of Orn and Lys containing peptides to Arg and Har containing peptides, respectively. Reference [1] Gers T., Kunce D., Markowski P., Izdebski J., Synthesis 2004, 37-42.

P105

2-CHLORO-4,6-DIBENZYLOXY-1,3,5-TRIAZINE AND N-(3,5-DIBENZYLOXY-1,3,5-TRIAZINYL-1)AMMONIUM TETRAFLUOROBORATES AS NEW, HIGHLY EFFICIENT COUPLING REAGENTS FOR PEPTIDE SYNTHESIS IN SOLUTION

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In the search for new triazine coupling reagents bearing improved stability and increased solubility in organic solvents, we prepared 2-chloro-4,6-dibenzyloxy-1,3,5-triazine by treatment of cyanuric chloride with benzyl alcohol. Taking advantage of modular structure of triazine based coupling reagents we prepared, in the presence tertiary amines, the entire family of N-(3,5-dibenzyloxy-1,3,5-triazinyl-1)ammonium chlorides and tetrafluoroborates, and compared their features. We found the chlorides instable, and therefore efficient as coupling reagents only when prepared in situ, directly in the activation stage. On the other hand, all the tetrafluoroborates were found stable and useful for amide bond formation in solution.
Acknowledgement: This work was supported by the State Committee for Scientific Research (KBN) grant 4-T09A 189 25.

P106

N-TRIAZINYLAMONIUM TETRAFLUOROBORATES AS NEW, HIGHLY EFFICIENT COUPLING REAGENTS FOR PEPTIDE SYNTHESIS IN SOLUTION

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Triazine based coupling reagents were found useful in the syntheses in solution of amides, esters, peptides and carboxylic acid anhydrides, affording high yield of products even in the case of less reactive, sterically hindered substrates. In order to take full advantages of triazine "superactive esters" participating in above mentioned condensations as highly efficient acylating intermediates, we attempted to improve the activation stage of carboxylic function by modification of the structure of the reagent. The modular structure of triazine reagents allowed the fine tuning of the properties to reach the given synthetic goal. Tetrafluoroborates of quaternary N-(3,5-dimethoxy-2,4,6-triazinyl-1)ammonium salts derived from 2-chloro-4,6-dimethoxy-1,3,5-triazine and N-methylmorpholine, quinuclidine, DABCO, and/or M-methylpiperidine were obtained and used for preparation of peptides and esters in solution. All of them were found efficient as condensing reagents and gave Z-, Fmoc- and Boc- protected peptides in 79-93% yield. We proved the participation of triazine "superactive ester" as reactive intermediates in the condensations by its isolation and identification in the model experiment involving relatively stable triazine esters of benzoic acid. We found the new, improved triazine coupling reagents more versatile in synthesis because of the increased stability and increased solubility in most of organic solvents. Acknowledgement: This work was supported by the State Committee for Scientific Research (KBN) grant 4-T09A 189 25.

P107

SPPS OF THE HIGHLY AMYLOIDOGENIC, 67-RESIDUE PROTEIN HUMAN PRO-ISLET AMYLOID POLYPEPTIDE (PROIAPP) AND THE MUTANT (C13S, C18S)PROIAPP

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Pancreatic amyloid is found in the beta cells of more than 95% of type II diabetic patients and its formation is associated with the pathogenesis of this disease. Islet amyloid consists of fibrillar aggregates of islet amyloid polypeptide (IAPP). IAPP is a 37 residue polypeptide that is stored as a 67-residue propeptide (ProIAPP) in the granules of the beta cells and is co-secreted -following ProIAPP processing- with insulin. We have previously devised a method for the efficient recombinant expression and purification of ProIAPP (Krampert, et al., Chem. & Biol. (2000)). We have shown that recombinant ProIAPP has typical biophysical properties of a strongly aggregation-prone and amyloidogenic protein. However, the role of ProIAPP, its conformational and amyloidogenic properties, and its processing in the formation of pancreatic amyloid has not been yet clarified. The SPPS of aggregation-prone polypeptide sequences is often accompanied with synthetic difficulties. Here, we present our protocols for the automatic solid phase synthesis of ProIAPP and its mutant (C13S, C18S)ProIAPP. Both proteins were synthesized by Fmoc/tBu chemistry. Our synthetic procedures resulted in crude products of very good purity. Following only one HPLC purification step, pure proteins were obtained in high yield. The protocols applied and the process of the automatic solid phase synthesis -its progress followed by HPLC/MALDI analysis at various chain lengths- and the purification of the two 67-residue polypeptides will be presented. Following upscaling, our synthetic protocols may find application for the industrial synthesis of ProIAPP and/or other ProIAPP-related aggregation-prone sequences.

P108

A WATER-SOLUBLE N-PROTECTING GROUP, 2-(4-SULFOPHENYLSULFONYL)ETHOXYCARBONYL GROUP, AND ITS APPLICATION TO SOLID PHASE PEPTIDE SYNTHESIS IN WATER

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Solid phase peptide synthesis has many advantages comparing to classical solution synthesis and has led not only to automatic peptide synthesis but also to the development of combinatorial synthesis. The procedure of solid phase synthesis is simple but requires large amount of organic solvents. Disposal of used organic solvents is an important environmental problem, we aimed to perform peptide synthesis in water. Previously we reported preparation of water-soluble 2-[phenyl(methyl)sulfonio]ethoxy- carbonylamino acids and their application to solid phase peptide synthesis. Here we designed a water-soluble N-protecting group, 2-(4-sulfophenylsulfonyl)ethoxycarbonyl (SPS) group, and its application to solid phase peptide synthesis in water Synthetic SPS amino acids are water-soluble and Leu-enkephalin was synthesized in water by the solid phase method using SPS amino acids.

P109

EFFICIENT PEPTIDE PURIFICATION BY HPLC - EFFECT OF PORE SIZE, PARTICLE SIZE AND CHEMISTRY

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Reversed phase high performance liquid chromatography is an invaluable tool for the analytical and preparative separation of peptides and proteins. The availability of different pore sizes and particle sizes have made alkyl-bonded silica gel media the economical choice for both analytical and preparative separations. Although the surface area decreases with pore size, wide pore material has become a popular material for many separations. This is unfortunate since the choice of the wrong pore size results in poor chromatography, low resolution and inferior separations. The appropriate choice of pore size and particle size also plays an important role in preparative separations where resolution and yield are required. This study shows how pore size and particle size affects resolution and throughput on the various alkyl ligand such as C18, C8, C4, Phenyl.

P110

SEMI-SYNTHESIS OF HUMAN GHRELIN: COMBINATION OF RECOMBINANT PEPTIDE SYNTHESIS WITH CHEMICAL SYNTHESIS OF O-ACYLATED FRAGMENT (1)

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The use of a recombinantly expressed peptide segment in combination with a chemically synthesized one is a very powerful tool for the production of a wide variety of polypeptides modified with phosphorlylation, glycosylation, etc. Using this methodology, we have succeeded in establishing the semi-synthesis of human ghrelin. Ghrelin is a natural ligand for the growth hormone (GH) secretagogue receptor. The peptide has a fatty acid acyl chain C8:0 at Ser3, which is necessary for GH releasing activity. The N-terminal fragment of ghrelin containing the octanoyl modification was synthesized chemically, and the 21 amino acid-residue C-terminal fragment was produced recombinantly. A recombinant fusion protein containing ghrelin (8-28) was expressed in E. coli. The fusion protein consisted of truncated E. coli β-galactosidase followed by the target peptide with a junction designed to allow cleavage with OmpT and Kex2 proteases. Thus, treatment of the fusion protein with OmpT resulted in generation of the C-terminal portion of ghrelin accompanied by a 13 amino acid-long linker peptide at its N-terminus. The amino groups of the peptide were then protected by a t-butoxy carbonyl group. After purification by reversed-phase chromatography, the peptide was cleaved by Kex2 to release [Lys16,19,29,24(Boc)]ghrelin(8-28) with a free N-terminus amino group. The peptide was then coupled with the chemically synthesized [Nα-Boc, Ser2,6(tBu)]ghrelin(1-7). Finally, the condensed peptide was deprotected with trifluoroacetic acid to obtain human ghrelin (1-28). Ghrelin prepared by this method exhibited the same activity of GH release in vitro as that of fully synthetic ghrelin.

P111

SOLID PHASE SYNTHESIS OF ARGININE-CONTAINING PEPTIDES AND THEIR INHIBITORS THROUGH A SIDE-CHAIN ANCHORING APPROACH

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Attachment of an amino acid to a solid support through its side-chain is sometimes necessary to take advantage of an alpha-carboxylic group available for diverse modifications, including incorporation of a fluorophore for the preparation of fluorogenic substrates. Contrary to most other amino acids, anchoring arginine side-chain to a resin requires the use of a supplementary linker. In order to avoid the usually multi-step synthesis of such linker as well as its difficult attachment to the guanidinium group, we developed a simple method where the guanidinium group is constructed on a Rink amide resin. Our strategy followed the classical steps of guanidine formation: i) Addition of the resin amino group to an isothiocyanate derivative of ornithine, producing N-omega-linked thiocitrulline; ii) S-methylation of thiourea; iii) guanidinylation using ammonium acetate. Cleavage of the resin generated the arginine-containing compound, the amino group of the resin becoming part of the guanidine. After presenting the general procedure, we will demonstrate the usefulness of this method by the synthesis of a series of fluorogenic substrates for trypsin-like serine proteases, which were obtained in high yield and purity. Our strategy also allows to generate from the same precursor different substituted arginine derivatives, including Nomega-methyl- and Nomega-ethyl-arginines. The ability to prepare such analogs together with the intermediates thiocitrulline and S-methyl-isothiocitrulline makes this method also a powerful tool for combinatorial solid-phase synthesis of NO synthase inhibitors.

P112

USE OF FMOC-AMINO ACID CHLORIDES IN SOLID PHASE SYNTHESIS OF PEPTIDES AND

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Here is reported on the method we have developed for solid phase synthesis of peptides and peptidomimetics with difficult aminoacid sequence. Through this procedure we have reacted Fmoc-protected proteinogenic aminoacyl chlorides with sterically hindered N-alkylated aminoacid peptidyl resin or poorly nucleophilic amino function to produce peptide bond in good to quantitative yield avoiding the occurrence of any detectable racemization (LC / MS). In order to show part of the work carried out we report the structures of some molecules synthesised as model structures in our laboratories (Fig 1). All sequences described have been prepared on commonly used for solid phase synthesis resins.

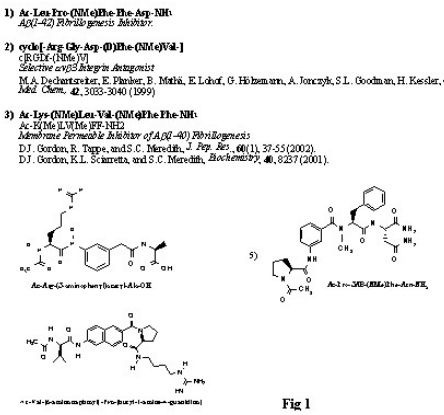

Fig 1

P113

SEMI-SYNTHESIS OF HUMAN GHRELIN: COMBINATION OF RECOMBINANT PEPTIDE SYNTHESIS WITH CHEMICAL SYNTHESIS OF O-ACYLATED FRAGMENT (2)

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Ghrelin is a 28-residue peptide with an n-octanoyl modification at the hydroxyl group of the 3rd Ser, which is essential for growth hormone-releasing activity. Chemical synthesis can readily produce the peptide, but is estimated to be economically inefficient. Also, posttranslational modifications such as fatty acid acylation cannot be achieved by recombinant production in prokaryotic cells. Therefore, we have established a semi-synthesis route by combining chemical synthesis of the N-terminal fragment and recombinant expression of the C-terminal fragment. To avoid possible racemization upon coupling with the C-terminal portion of ghrelin, protected ghrelin(1-7), which has a Pro residue at position 7 of the fragment, was selected as the fragment to be prepared by chemical synthesis. We attempted several synthetic routes as well as protecting groups for the Ser residues. The best result was obtained via protection with tBuMe2Si ether at the 3rd Ser and tBu ether at the 2nd and 6th Sers. Starting from Pro-2-chlorotrityl-resin, the peptide backbone was successively elongated by Fmoc chemistry, giving Boc-Gly-Ser(tBu)-Ser(tBuMe2Si)-Phe-Leu-Ser(tBu)-Pro-2-chlorotrityl-resin. After treatment of the peptide-resin with TBAF followed by acylation, the acylated peptide-resin was treated with diluted TFA to give the desired fragment with high purity by one precipitation. In the course of this approach, the conditions of each reaction were optimized. After coupling with the C-terminal fragment, ghrelin was obtained by deprotection followed by purification. This methodology would be applied to the production of other peptides including specific residual modifications.

P114

NOVEL HIGH LOAD POLYETHER BASED RESINS AND THEIR APPLICATION IN SYNTHESIS OF 'DIFFICULT PEPTIDES'

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Novel high load polyether based resins and their application in synthesis of “difficult peptides” Roice Michael, Patrik Gavelin, Nicola Rehnberg, Monica Ramos, and Ib Johannsen Versamatrix A/S, Valby, Denmark A new generation of oxetane – PEG based resins is presented and their application for synthesis of difficult peptides is discussed. The new resins avoid the known limitations of highload polystyrene based resins (Merrifield resins) with respect to network structure, solvent access and biocompatibility. At the same time the new oxetane resins offer loading in excess of 2 mol/kg and a very high chemical stability. Results of difficult peptides including ACP (65-74) fragment and deca-Alanine using the new resin as well as applications within large-scale synthesis and affinity separations will be discussed.

P115

RATIONAL DESIGN OF TRYPSIN VARIANTS WITH IMPROVED SYNTHETIC PROPERTIES

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The narrow substrate specificity of proteases generally limits the choice of amino acids between which a peptide bond can be synthesized. While for the carboxyl component the restricted enzyme specificity can be overcome by the use of substrate mimetics,[1] no universal approach exists for broadening the specificity of the biocatalyst towards the amino component. In the present contribution we describe the suitability of site-directed mutagenesis to improve the binding of originally less specific amino components to the enzyme’s active site, which was found to be crucial for efficient synthesis. Starting from the trypsin variant K60E, D189K, which is already known as a promising catalyst with reduced proteolytic activity,[2] further mutations were introduced forming an artificial metal binding site within the S’ subsite of the biocatalyst. The resulting enzyme variant shows a significantly altered specificity towards the amino component. Especially peptide sequences for which the wild-type enzyme is originally rather non-specific are efficiently recognized by the optimized trypsin variant. Ester hydrolysis studies using a library of substrate mimetics could further prove a high activity of the variant towards peptide 4-guanidinophenyl esters enabling its use as coupling reagent for peptide synthesis. Model peptide ligation reactions finally verified the improved acceptance of originally non-efficient peptide sequences resulting in an increase of product yields from about 20% (wild-type enzyme) up to nearly 100% (enzyme variant). [1] F. Bordusa, Chem. Rev. 102 (2002), 4817. [2]K. Rall, V. Cervsky, F. Bordusa. In: Peptides 2000, (J. Martinez, J.-A. Fehrentz, Eds.), EDK, Paris, France, pp. 339, (2001).

P116

SYNTHETIC STRATEGIES TO BACKBONE CYCLIC PHOSPHOTYROSINE CONTAINING PEPTIDES

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Cellular signal transduction pathways that are initiated by transmembrane receptor tyrosine kinases are strongly depending on protein-protein-interactions mediated by small protein modules such as SH2 and SH3 domains. In the course of our study on ligands for the N-terminal SH2 domain of protein tyrosine phosphatase SHP-1 conformationally restricted peptides have been designed and preselected by molecular modeling and docking methods using Insight II. For our backbone cyclic candidates, the phosphotyrosine residue was predicted as suitable position participating in ring formation. In principle, there are two synthetic strategies for the preparation of phosphotyrosine containing peptides - either by postassembly (global) phosphorylation or by incorporation of a phosphorylated tyrosine (pTyr) building block. The latter is generally preferred due to the availability of differentially protected pTyr building units and the high yield and quality of the phosphopeptides which can be achieved using this approach. However, for the preparation of our backbone cyclic phosphopeptides the synthesis of N-functionalized phosphotyrosine was required. This building block could be successfully synthesized by applying phosphoramidite chemistry to the free hydroxyl group of Na-functionalized tyrosine, but due to several synthetic steps and essential purification steps the preparation is rather difficult. Therefore, we have tested different synthetic ways to find the most effective procedure leading to backbone cyclic peptides containing an internal phosphotyrosine residue.

P117

SYNTHETIC PEPTIDE PURIFICATION - CHOICE OF HPLC MEDIA AND OPERATING CONDITIONS FOR MAXIMUM SELECTIVITY AND CAPACITY

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The choice of HPLC media for the purification of synthetic peptides can have a significant effect on the production economics. It is essential that high sample loads can be used so the media must exhibit excellent selectivity/resolution and good capacity. Most purification work is carried out using silica based materials with alkyl bonded ligands but they are limited to a relatively narrow pH operating range. By using rigid polymeric materials with chemical stability from pH 1 to 14, it is possible to extend the eluent options. Data will be presented to show how using high and low pH eluents can optimise the resolution/selectivity. To maximise capacity it is essential that the correct pore size is chosen. Data will be presented to show how the particle/pore size and pore size distribution of the PLRP-S media influences the capacity of the material for a given linear velocity. The effect of linear velocity for the various pore sizes will also be determined. Guidelines will be developed for the optimum porosity/linear velocity needed to achieve the maximum capacity for synthetic peptide purification.

P118

A SHORT SYNTHESIS OF 2'-DEOXYMUCIGINEIC ACID

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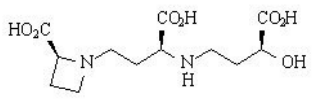
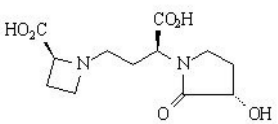
2'-Deoxymugineic acid (DMA, 1), isolated from the roots of gramineous plants, promotes uptake of Fe3+ in plants by chelating iron. It contains two reduced peptide bonds. A total of four syntheses are reported in literature. Two of which have utilized reductive alkylation of amine via Schiff's base and two others have exploited the reduction of the amide bond via thioamidation with Lawesson's reagent. In addition, 1 has been assembled from left to right or right to left. The main problem in the synthesis of DMA has been the formation of lactam (2). In this presentation, we report a short and efficient synthesis of DMA via reductive alkylation of azetidine-2-carboxylic acid. The -COOH function of azetidine-2-carboxylic acid was not protected. Other –COOH groups in the molecules were protected as benzyl esters. This combination allowed the purification of benzyl-protected DMA by HPLC. The benzyl protecting groups were hydrogenolyzed in the final step to avoid lactam 2 formation.



1



2

P119

COMPARISON OF PYBOP, HCTU AND TCTU AS ACTIVATORS IN PARALLEL SPPS

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Successful peptide synthesis strongly depends on proper activation of the incoming amino acid during coupling. Many reagents have been proposed over the years including e.g. carbodiimides and symmetrical or unsymmetrical anhydrides and active esters. Since the late 80s several new uronium- and phosphonium-based activators have been introduced which allow in-situ activation in the presence of a tertiary amine as a base. PyBOP has been our choice for routine synthesis over the years because of its high solubility in DMF and NMP allowing concentrations of acylating species of up to 0.3 M. Recently HCTU and TCTU have been introduced and several reports describe these compounds as very efficient. Here we report the parallel SPPS of 24 different peptides (9-30 AA) using PyBOP, HCTU or TCTU as a coupling reagent, in either NMP or NMP/DMSO as a solvent. After synthesis peptides were cleaved with TFA, precipitated with ether and lyophilized. Comparison of the effectiveness of the various activators was evaluated by determination of the purity of the crude peptides obtained using RP-HPLC and MALDI-TOF mass spectrometry. Our results compare the usefullness of the various activators in parallel SPPS where stability of the compounds in solution and sensitivity of the activated amino acids in an open system are important parameters. This study does not necessarily reflect the possible effectiveness of the activators in other types of synthesis.

P120

USE OF AZIRIDINES DERIVED FROM AMINO ACIDS IN PSEUDOPEPTIDE SYNTHESIS

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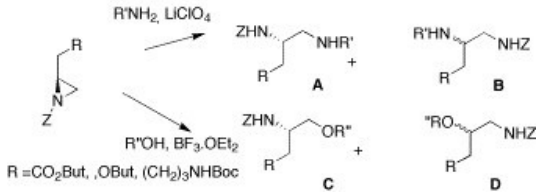
In connection with our program of analog synthesis of the tetrapeptide AcSDKP[1], we were led to study the reactivity of aziridines derived from amino acids. Aziridines as activated species have often been used in ring-opening reactions with N-, O-, S-, or C- nucleophiles. However, aziridines derived from amino acids have scarcely been utilized except for α-carboxylalkyl aziridine obtained from serine and could be valuable synthons to prepare pseudodipeptides. The ring-opening reactions with N- and O-nucleophiles have been studied with aziridines derived from aspartic acid, serine ans lysine. These aziridines were prepared from the corresponding β-amino alcohols. Satisfactory yields and good regioselectivity were observed with N–nucleophiles (R'NH_2 , with R'= -CH_2Ph, -$CH_2CH{=}CH_2$, -OCH_2Ph, -$NHCO_2CH_2$Ph) using LiClO4 as an additive, yielding β-amino acids (A). In contrast, no selectivity was obtained when treating aziridines with alchol (R" = CH_2Ph, $CHMeCO_2Me$, C :D = 1 : 1) adding BF3-etherate in catalytic amount. Use of solid supports to improve the regioselectivity or microwaves to increase the speed of the reaction has also been explored. The ring-opening reaction of an aziridine, with a suitably protected amino acid, provided a good way to prepare pseudodipeptides. Iterative use of this reaction yielded pseudopeptides resistant to proteolysis. [1] Gaudron, S.; Adeline, M.-T.; Potier, P.; Thierry, J J. Med. Chem, 1997, 40, 3963-3968.



P121

COUPLING OF PEPTIDE FRAGMENTS BY PROTEASES SPECIFIC FOR NON-CODED AMINO ACIDS

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Forced by successful enzyme, medium and substrate engineering methods, proteases have gained in importance as regio- and stereospecific catalysts in organic synthesis.[1] Especially for applications based on their native hydrolysis activity, proteases are generally recognized as normal bench reagents. In principle, these strategies allow also for the alteration of the enzyme specificity and minimization of undesired proteolytic cleavages, which are the main drawbacks when proteases are used for peptide synthesis. However, even the most impressive examples published so far are handicapped by the deficiencies of catalysts; i.e. their restricted substrate specificities and the permanent risk of proteolytic side reactions. In summary, proteases appear to be far away from being perfect catalysts for universal and flexible peptide synthesis. Inevitably, this conclusion holds true for classical approaches, but is wrong when considered as a general rule. In fact, the combined use of substrate mimetics,[2] solid phase peptide synthesis,[1] and proteases specific for non-coded amino acids[3] broaden the synthetic scope of the enzymatic method significantly. Due to its non-specificity for coded amino acids the latter allows for the coupling and selective modification of native polypeptides without the risk of proteolytic cleavages. Selected original examples including the ligation of peptide fragments and the incorporation of biophysical probes to polypeptides will be presented. [1] F. Bordusa, Chem. Rev. 102 (2002), 4817. [2] F. Bordusa, D. Ullmann, C. Elsner, H.-D. Jakubke, Angew. Chem. Int. Ed. 36 (1997), 2473. [3] Y. Asano, H. Ito, T. Dairi, Y. Kato, J. Biol. Chem. 271 (1996), 30256.

P122

PSEUDOPROLINE DIPEPTIDES IN FMOC-SOLID PHASE PEPTIDE SYNTHESIS

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Oxazolidine dipeptides were introduced by Mutter et al. [1-2] as reversible proline mimetics for modulation of protein structure. The proline-like moiety is generated by formation of an oxazolidine ring between the alpha-amino and the side chain hydroxy groups of Ser or Thr with an aldehyde or ketone. Such pseudoprolines dipeptides are highly effective at preventing aggregation during solid phase synthesis as they disrupt the formation of beta-sheets and helical conformations. They have been used with great effect to prepare long peptides/small proteins [3], cyclic peptides [4], or to disrupt structure as synthetic proline analogues [5]. The incorporation of pseudoproline dipeptides has been found to lead to overall improvements in acylation and deprotection kinetics, resulting in better yield, purity and solubility of crude products and easier HPLC purification with higher amounts of isolated products. In this talk we will examine the rationale for the 'pseudoproline' effect, and attempt to draw guidelines for their effective use from synthetic data. Recent examples of the successful application of pseudoproline dipeptides in the synthesis of long and difficult peptides will be presented. The novel use of these derivatives in enantiomerization free fragment condensation will also be discussed. [1] T. Haack & M. Mutter (1992) Tetrahedron Lett. 33, 1589. [2] M. Mutter et al. (1995) Pept. Res. 8, 145. [3] P. White et al. (2004) J. Pept. Res. 10, 18. [4] N. Schmiedeberg & H. Kessler (2002) Org. Lett. 4, 59. [5] A. Wittelsberger et al. (2000) Angew. Chem. Int. Ed. 39, 1111.

P123

ONE POT SYNTHESIS OF AZOLE CARBOXIMIDAMIDES AND GUANIDYLATION OF AMINES

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Amidino (carboximidamide) group transfer reagents [1-5] are valuable synthons for preparation of guanidines from amines both in solution and on the solid support. Derivatives of 1H-pyrazole-carboximidamides are selective inhibitors of inducible nitric oxide synthase. In this study we present two simple and efficient, eco-friendly methods for preparation of 1H-pyrazole-(PyC) and 1H-benzotriazole-carboximidamides (BtC). Both methods are superior to previously reported ones in terms of high yields, facile work-up, short reaction times and easy scale-up. The use/cost of the solvents have been minimized. The purity of resulting BtCs was sufficiently high to be used in one pot procedure for guanidylation of amines, including amino acids and peptides, both in solution and on the solid supports. In addition, both BtC and side product from guanidylation, are well soluble in organic solvents, which allows the reaction procedure to be accomplished with easy work-up [4]. The crystal structure of N,N-dimethyl-benzotriazole-1-carboximidamide, a reagent for conversion of ornithine to N^{ω},N^{ω}-dimethylarginine (ωDma), was resolved. References 1. Berrnatowicz, M. S.; Wu, Y.; Matsueda, G. R., J. Org. Chem., 57, 2497-2502, 1992. 2. Katritzky, A. R.; Parris, R.; Allin, S.; Steel, P. J., Synth. Commun., 25, 1173-1186, 1995. 3. Musiol, H. J.; Moroder, L., Org. Lett., 3, 3859-3861, 2001. 4. Draeger, G.; Solodenko, W.; Messinger, J.; Schoen, U.; Kirschning, A., Tetrahedron Lett., 43, 1401 – 1404, 2002. 5. Katritzky, A.R.; Rogovoy, B.V.; Cai, X.; Kirichenko, N.; Kovalenko, K.V., J. Org. Chem. 69,309-313, 2004.

P124

FALSE RESULTS OF THE KAISER (NINHYDRIN) TEST AFTER THE FMOC-DEPROTECTION OF CERTAIN AMINO ACIDS COUPLED TO PROLINE DURING SPPS

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The Kaiser (ninhydrin) test, which detects primary amino groups, is one of the most popular qualitative tests used in Organic Chemistry. This test has been widely used in SPPS, especially for monitoring the presence of free amine after deprotection (dark blue colour) as well as for checking completeness of the successive coupling steps (yellow colour). Although the Kaiser test is generally reliable, it is known that in some cases it can lead to false results. More specifically, according to the literature, the test does not yield the typical dark blue colour with serine, asparagine, aspartic acid or with arginine and cysteine when their lateral chains are deprotected. In our lab we have observed that during the SPPS of various peptides on various resins, the Kaiser test does not lead to dark blue colour after the Fmoc-deprotection of some amino acids, when they are coupled to a proline residue. In this work, we have coupled the 19 natural amino acids –suitably site-protected, if necessary– to proline, which was anchored to the Rink amide resin. For comparison reasons, we have also directly anchored the above amino acids to the same resin. As shown, false results (very slight blue colour) were often obtained after the Fmoc-deprotection of certain amino acids, such as asparagine, histidine, serine and arginine, when they had been previously coupled to proline. Especially when aspartic acid was coupled to the proline residue, complete absence of blue colour was observed. The above findings are currently under evaluation.

P125

IDENTIFICATION OF NEW AUTOANTIGENS IN MULTIPLE SCLEROSIS BY GLYCOPEPTIDE LIBRARIES

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The glycopeptide CSF114(Glc) [1] is characterising, for the first time, an antibody-mediated Multiple Sclerosis (MS), the most known demyelinating autoimmune disease of the central nervous system. In fact, CSF114(Glc) is, up to now, the first synthetic antigen able to detect specific antibodies (Abs) by ELISA, on sera of MS patients. Moreover, we demonstrated that auto-Abs can be detected only using glycosylated antigens, and that specific epitopes for Asn(Glc) (possibly conformational ones) are recognized. The relevant epitope recognized by the auto-Abs should contain the Asn(Glc) moiety, and the amino acid sequence is important for a correct epitope accessibility. The Asn(Glc) epitope is present at position 7 of CSF114(Glc), and it is exposed at the best because of its position on the tip of a beta-hairpin structure present between the residues 2 and 14. In order to possibly improve the epitope accessibility in CSF114(Glc), we synthesized a glycopeptide library focused on amino-acid diversity at the level of the beta-turn position. The glycopeptide library was synthesised using the split-and-mix approach, and the screening assays were performed by competitive ELISA, comparing the auto-Ab titre to CSF114(Glc) in MS patients' sera and controls. [1] Papini, A.M., Rovero, P., Chelli, M., and Lolli, F. (2002) PCT/EP02-06767 20020619. Priority: IT 2001-FI114 20010622.

P126

COMBINATORIAL SELECTION OF PEPTIDES SPECIFIC FOR THE EPITOPES OF PAI-1 NEUTRALIZING MABS

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Monoclonal antibodies (Mabs), with their unique specificity and selectivity, are convenient tools for studying the mechanism of action of plasminogen activator inhibitor 1 (PAI-1). Murine anti-PAI-1 Mabs (MA-56A7C10 and MA-55F4C12) that interact with different epitopes have been chosen as displacement agents in the combinatorial screening for peptides mimicking known intermolecular mechanisms of inactivation of PAI-1. Two phage display libraries, a fUSE5/15-mer library and a f88-4/Cys6 constrained library, with inserts encoding random peptides on the pIII and pVIII coat proteins have been employed to select ligands competing with MA-55F4C12 and MA-56A7C10. For the first step, acid elution was used to collect all phage able to bind to immobilized PAI-1. In two subsequent rounds of selection, phage were eluted with increasing concentrations of the corresponding Mabs (0.2 and 2.0 micromolar at round II, 0.02 and 0.2 micromolar at round III). Finally, a single elution with 0.5 micromolar Mabs was used for elution in round IV. Phage clones from the outputs of round IV have been amplified, sequenced, and tested for competition with the corresponding Mabs. This phage display-based combinatorial selection of peptides specific for known epitopes of PAI-1 neutralizing Mabs will generate new specific ligands for modulating PAI-1 activity in vivo.

P127

FLUORESCENTLY LABELLED PROTEIN LIBRARIES

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Recently, the generation of libraries of peptides, peptidomimetics, or other small organic molecules in combination with high-throughput screening (HTS) has become a well-established method for the production of new pharmacological leads. In our laboratory, we have been using the powerful split-mix synthesis strategy [1] for random combinatorial synthesis and the simple and elegant string synthesis for the parallel synthesis of peptide libraries [2]. Presently, to increase the sensitivity of the subsequent binding assays, fluorescent reagents were used for the labeling of target proteins. After immobilization onto the surface of highly porous, hydrophilic solid supports, the proteins were reacted with a variety of fluorescent reagents (FITC, DTAF, Dansyl chloride, FAM). The immobilization and the labeling reactions were monitored by high-performance analytical methods (e.g. HPLC, SDS-PAGE, capillary zone electrophoresis, MEKC). Different mass spectrometric (ESI, MALDI) techniques were used in order to evaluate the coupling efficiency, and to determine the number of the attached labeling groups. Application of fluorescent labeling for functional analysis of the proteome will be shown. [1] Á. Furka, F. Sebestyén, M. Asgedom, G. Dibó, 14th Int. Congress Biochem., Prague, 1988., Int. J. Peptide Prot. Res. 1991, 37, 487–493. [2] Á. Furka, G. Dibó, N. Gombosuren, Curr. Drug Disc. Technol. 2004, in press.

P128

FACILE SOLID-PHASE SYNTHESES OF SOLUBLE AND RESIN-BOUND PEPTIDE ALDEHYDES, APPLYING WEINREB AMIDE AND BAL CHEMISTRIES

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Peptide aldehydes are important synthetic targets because they may serve as enzyme inhibitors, and they may also be useful intermediates for further elaboration of bioactive substituents. The present work reports on how on-resin Weinreb amide reduction can be used to generate peptide aldehydes, either released into solution concomitant to reduction, or retained on the support. In one method, methoxylamine is introduced by a reductive amination reaction onto BAL-PEG-PS resin. Further acylation is carried out to extend a peptide chain, and final cleavage to provide a C-terminal peptide aldehyde is achieved by treatment with $LiAlH_4$. In a second method, the N-terminus of a peptidyl-resin is acylated, using either a cyclic anhydride or a dicarboxylic acid, following which the Weinreb amide is created by coupling of N,O-dimethylhydroxylamine. In this case, treatment with $LiAlH_4$ results in the peptide with an aldehyde-containing N-terminal appendage being retained on the solid support. Because all steps are carried out on-resin without the need to prepare building blocks in solution, these newly developed methods should be particularly advantageous, by comparison to prior ones,[1-3] for the preparation of many-membered combinatorial libraries of peptidomimetics and small organic molecules. References: 1. Fehrentz, J.-A.; Paris, M.; Heitz, A.; Velek, J.; Liu, C.-F.; Winternitz, F.; Martinez, J. Tetrahedron Lett. 1995, 36, 7871. 2. Gosselin, F., Van Betsbrugge, J., Hatam, M., Lubbel, W.D. J. Org. Chem. 1999, 64, 2486. 3. Guillaumie, F.; Kappel, J.C.; Kelly, N.M.; Barany, G.; Jensen, K.J. Tetrahedron Lett. 2000, 6131.

P129

PEPTIDE MIMOTOPES FOR DIAGNOSIS OF EPSTEIN-BARR VIRUS SELECTED FROM A RANDOM PEPTIDE LIBRARY DISPLAYED ON PHAGE

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Epstein-Barr virus (EBV) is the causitive agent of infectious mononucleosis, a disease that affects more than 90% of the world's population. ELISA is the most sensitive method for diagnosis and current diagnostics use preparations of EBV whole cell extracts to capture IgM or IgG antibodies in patients' serum. The objective of this study was to evaluate whether peptides corresponding to immunodominant antigenic regions of EBV may replace solid phase antigens in EBV diagnostics. The use of peptide epitopes as diagnostic antigens is advantageous as it allows focus on single specificities and avoids the unimportant epitopes present in crude extracts. In addition peptides of high quality can be cheaply and reproducibly produced. Using monoclonal antibodies we selected 4 peptides that mimic different EBV epitopes from a random peptide library. The peptides showed no apparent similarity to EBV proteins but were shown to mimic its binding activity. To assess their diagnostic value we screened a panel of 62 individual EBV-IgM sera for reacivity with the peptides. For all the peptides there was a clear distinction between the EBV-positive and negative samples, resulting in 100% specificity. For the IgM-positives, the sensitivity of detetection was F1 (88%), A3 (85%), gp125 (66%) and A2 (54%). If any combination of the peptides was used the sensitivity of detection would increase, the best combination was for gp125 and A3 with maximum sensitivity of 97.5.%. We conclude that specific combinations of peptide mimotopes could replace the use of complex virion extracts used in current serodiagnostics for EBV infection.

P130

ON THE FREQUENCY OF FOLDED POLYPEPTIDES IN A RANDOM SEQUENCE PRODUCTION BY PHAGE DISPLAY

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Synthesis of libraries of small peptides with random amino acid sequences, followed by a selection procedure that yields new compounds with the desired properties has become a relatively widely exploited area of research. This work is focused on the development of a method for the isolation of folded proteins which should have a defined and specific three-dimensional structure at room temperature in aqueous solution but that, a priori are not subject to preconceived restraints as to their specific structure or to the presence of a certain phenotypic trait. The motivation to investigate this problem comes from the intention to explore the realm of all-possible folded proteins. Extant proteins have been produced by Nature in a long selective evolutionary process. Simple calculations led us to believe that it is indeed very unlikely that Nature tried all possible amino acid combinations, even for relatively short peptides. The development of an experimental procedure that could distinguish between unfolded and folded peptides is described in this project and the concept that folded proteins are more protected against digestion by a protease than unfolded polypeptides was taken as a basis for this study. Mass-spectroscopy studies will be carried out to characterize the amino-acid sequence and the possible folding. The final idea is to amplify, isolate and purify folded random peptides and screen them on biomedical applications.

P131

SOLID-PHASE SYNTHESIS OF PEPTIDE BASED PHOSPHINE LIGANDS: TOWARDS COMBINATORIAL LIBRARIES OF HIGHLY SELECTIVE PEPTIDO-PHOSPHINE TRANSITION METAL CATALYSTS

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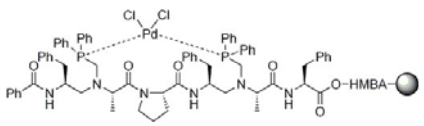
In Nature enzymes achieve their excellent efficiency and selectivity in the catalysis of biological processes through a combination of binding affinity and delicate catalytic machinery. Hence, it is obvious to mimic nature in the design of folded peptidic catalysts for asymmetric synthesis. In order to exploit a selection process to identify efficient transition metal binding catalysts with such properties, synthesis of combinatorial catalyst libraries on solid support is crucial. Resin bound catalysts based upon phosphine functionalised peptides would combine the excellent catalytic properties known from phosphine transition metal complexes with the selectivity and possibly increased reactivity imposed by binding to the folded peptide scaffolds. The presented new methodology can be used for the solid-phase combinatorial synthesis of peptide based phosphine ligand libraries. Solid supported peptide scaffolds possessing secondary amines were synthesised using commercially available Fmoc-protected amino acids and readily available Fmoc-protected amino aldehydes in standard solid-phase peptide synthesis combined with reductive alkylations. Phosphine moieties were introduced by phosphinomethylation of the free secondary amines, immediately prior to complexation with transition metals. The synthesised ligands and transition metal complexes thereof, were characterised by ^{31}P NMR, HPLC, and ESMS. To demonstrate the catalyst properties of the resin bound complexes, cross coupling reactions were successfully performed.



P132

COMBINATORIAL SYNTHESIS OF RHENIUM-CYCLIC SOMATOSTATIN ANALOGS USING THE NOVEL PEPTIDE BACKBONE METAL-CYCLIZATION (BMC) METHOD

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A novel approach for the combinatorial synthesis of backbone-derived metal-cyclic peptide libraries is presented. In this approach the metalo cyclic peptides are prepared from their linear precursors through complexation of a metal atom via two hemi-chelating arms, located on the peptide backbone. Thus, cyclization and metal-labeling of the peptides are achieved simultaneously. The diversity of such libraries can result from several parameters including: the position of the chelating arms on the peptide backbone, the nature of their donor atoms, the stereochemical configuration of the ligands and the length of the linkers connecting them to the peptide backbone. In the present study the last two parameters were varied systematically for the successful preparation of a library, composed of 48 backbone-to-end rhenium-cyclic somatostatin analogs. The linear pre-cyclic precursors of this library were synthesized on solid support, and after cleavage from the resin, peptide cyclization was performed in solution by hemi-chelation of rhenium as the monooxorhenium(V) species. The library thus obtained contained peptides with different ring size and different streochemical compositions of the hemi-chelator metal binding site, and, accordingly, peptides with different conformations. All rhenium complexes exhibited high to moderate in vitro binding affinities towards cloned human somatostatin receptor subtype 2 (hsstr2). Five rhenium-cyclic peptides were found to be most potent with IC_{50} values between 1 and 3 nM making them excellent leads for further development of tumor diagnostic and therapeutic radiolabeled agents. We report the synthesis, characterization and in vitro binding studies of these novel backbone rhenium-cyclic somatostatin analogs.

P133

AUTOMATED POWDER DISPENSING: FASTER AND EASIER MANUAL AND AUTOMATED PEPTIDE SYNTHESIS

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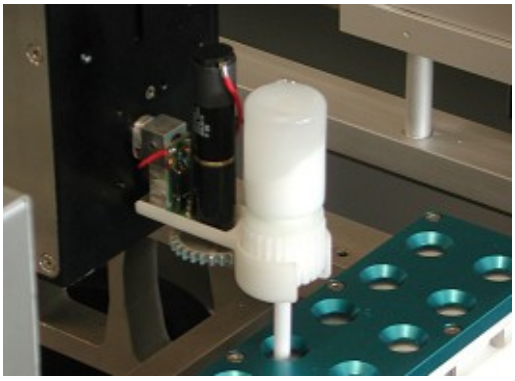
Chemspeed Ltd., Augst, Switzerland

Peptide synthesis, more than any other any field in organic chemistry, requires multiple weighing steps : aminoacids, coupling reagents, resins... Handling powders raises several risks such as weighing mistakes, picking of the wrong building block, contamination of the stock reagent etc. This presentation demonstrates the efficiency of automated solid dosing [1] , for the dispensing of solid reagents, the filling of reactors and cartridges, and the preparation of stock solutions. As a conclusion, we will present a peptide synthesis carried out on a synthesizer combining automated powder dispensing and liquid handling. [1] Michael Dechantsreiter, Thomas Schnieder, Michael Schneider, Chemspeed Application Note N 015.



P134

A NOVEL STRATEGY FOR HIGH-THROUGHPUT SCREENING

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Since the introduction of the split–mix synthesis strategy [1], combinatorial chemistry has revolutionized the drug discovery process. By the middle of 1990s, high throughput screening (HTS) systems were able to test thousands of compounds a day. A considerable portion of screening experiments is based on specific binding of the compound of interest to a target macromolecule (e.g. receptors, enzymes, antibodies, nucleic acids). In the post-genomic era, the number of the pharmacologically important targets obtained from the proteome has been growing at an unprecedented rate. Thus, there is a high demand for faster and more efficient high-throughput screening technologies. An obvious solution is to use high-density plates and to miniaturize the assay format. In spite of all these efforts, HTS is still one of the bottlenecks in the drug discovery process. Facing this challenge, we present a chemical genetic approach for the efficient screening of the pool of target macromolecules with small molecule libraries. [1] Á. Furka, F. Sebestyén, M. Asgedom, G. Dibó, 14th Int. Congress Biochem., Prague, 1988.; Int. J. Peptide Prot. Res. 1991, 37, 487–493.

P135

PAOLA CONCEPT FOR DIRECTED SPLIT-AND-POOL COMBINATORIAL SYNTHESIS

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Partitioning of pooled particles (Paola) for the directed split-and-pool combinatorial solid-phase synthesis eliminates both tagging and individual handling of solid support during the synthesis. In addition to molecular weight, retention times on reversed phase HPLC are suitable inherent markers that can be used for compound identification and simple detection of unexpected products. The Paola concept has been reduced to practice and simple personal chemistry tools have been designed.

P136

STRUCTURAL STABILIZATION OF THE HYAP65 WW DOMAIN WHILE RETAINING ITS ACTIVITY: AN ITERATIVE SYSTEMATIC TRANSFORMATION PROCESS BASED ON SPOT-TECHNIQUE

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WW domains are protein-protein interaction modules composed of about 35-50 amino acids. They are the smallest naturally occurring, monomeric, triple-stranded, anti-parallel beta-sheet protein domains. The WW domain binds proline-rich ligands with dissociation constants in the range of 1-50 mM. The wildtype hYAP65 (human Yes kinase-associated protein) consist of 57 amino acids, and a truncated version (44 aa) can be efficiently synthesized either by SPPS on resin or by SPOT synthesis (1). NMR- and CD measurement, showed that folding of the domain is only complete if the ligand EYPPYPPPPYPSG is added. It has been reported (2) that the structure of hYAP65 WW domain could be stabilized via a mutating process but unfortunately the ligand binding activity of the structural stabilized domain is dramatically decreased. We will demonstrate that the structural stability of the hYAP65 WW domain could be increased by a transformation process while retaining its ligand binding affinity. A set of double substitution analogs (two positions were exchanged by each natural amino acid) were synthesized as a cellulose-bound array and subsequently probed for binding. Based on the known NMR structure, different positions in the hYAP65 WW domain sequence were selected for double exchange. Best binding analogs were identified and subsequently melting points of those domains were determined by CD measurements. Domains with the highest melting points were chosen for the next stabilization step. (1) Livia Otte et al., Protein Science, 2003, 12, 491-500 (2) Xin Jiang et al., Protein Science, 2001, 10, 1454-1465

P137

KINOME PROFILING FOR STUDYING SIGNAL TRANSDUCTION ON PEPTIDE MICROARRAYS

J.T.H. Joore[1], **M.A.J. Kriek**[1], P. van Dijken[1], S.H. Diks[2], K. Kok[2], T. O'Toole[2], D.W. Hommes[2], M.P. Peppelenbosch[2]

[1]Pepscan Systems B.V., Lelystad [2]Laboratory for Experimental Internal Medicine, Academic Medical Center, Amsterdam, The Netherlands

The DNA array technique allows the analysis of the genome and transcriptome of cells and has revolutionised the study of the molecular biology. Currently no techniques exist that allow parallel assessment of the intracellular metabolism. We synthesized arrays of peptides with consensus sequences for different kinases on glass (PepChip Kinase Arrays). By using cell lysates and in vitro phosphorylation of these PepChip Kinase Arrays, we were able to analyse the kinome (the specific activity of different kinases present in the cell) of peripheral blood-derived human mononuclear cells before and after stimulation with lipopolysaccharide (LPS). Using this system, we then investigated the effects of semapimod, an anti-inflammatory compound, on the kinome of LPS-stimulated cells. Though this drug has demonstrated remarkable clinical efficacy in steroid-refractory Crohn's disease, its mechanism of action is not understood. We have now identified MEK kinase c-Raf as a relevant target and thus conclude that array technology is a viable and promising strategy to analyse cellular metabolism.

P138

DESIGN AND SYNTHESIS OF A PEPTIDE CONSTRUCT MIMICKING THE P23 PROTEIN USING OXIME LIGATION

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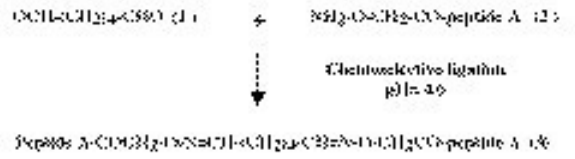
COPI, COPII protein coats are macromolecular assemblies that transiently cover the cytosolic face of vesicules. They specifically interact with the cytosolic part of transmembrane "cargo" proteins such as p23, p24, thereby favoring their incorporation into transport vesicles. p23 interacts directly with subunits of the COPI coats influencing its cycle of assembly/disassembly [1]. To assess this influence in a reconstituted system, we have designed a peptide construct (3) as a dimer scaffold. Each monomer (peptide A of 24 amino acids) contains the cytosolic tail and part of the transmembrane segment of p23. Three extra N-methylated leucine were added at the N-terminus to avoid the presence of NH group not involved in an a-helical hydrogen bond. An aminoxyacetic acid (Aoa) was introduced at the very N-terminus of the peptide A to allow dimerization by oxime ligation. The key step of our approach consisted of a one-pot double oxime ligation of the Aoa-peptide A (2) with the homobifunctionnal linker (1) bearing two aldehydes. Aoa-peptide A (2) was synthesized by SPPS with a satisfactory yield in spite of the well-known coupling difficulties of N-alkylated amino acids. No overacylation was observed on the Boc-Aoa. The double oxime bond step was directly performed with the crude peptide (2) and the target construct (3) was purified by HPLC and characterized by ESI/MS. Fluorescent studies performed with lipidic vesicles showed that the target construct interacted fastly and efficiently into membranes. [1] Sohn K et al., J Cell Biol., 1996, 135,1239-48.



P139

ANALYSIS OF PROTEASE AND PEPTIDASE ACTIVITY ON PEPTIDE MICROARRAYS

J.T.H. Joore, **G.J. Ligtvoet**, P. van Dijken, J. Creemers

Pepscan Systems BV, Lelystad, The Nethrelands

Proteases play key roles in many diseases, including cancer, inflammatory diseases and congenital diseases. We have developed a peptide microarray for analysis of single purified proteases and protease activity in complex mixtures such as serum or tissue lysates. The peptide microarray contains peptide substrates for a wide range of proteases. Digestion of these peptides is detected using fluorescent methods and the digestion pattern is analyzed. With these microarrays, unique insights in protease activity and specificity can be obtained. Examples will be shown and discussed in this presentation.

P140

SIMPLE AND EFFICIENT SYNTHESIS OF NEW TRIFUNCTIONAL TEMPLATES

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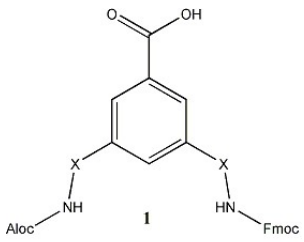
Max Planck Research Unit for Enzymology of Protein Folding, Halle/S., Germany

Bivalent ligands can better imitate biological substrates than their single constituents and bind to the two separated receptor epitopes. Taking advantage of this, the one bead two compounds combinatorial chemistry approach has found several applications in modern drug design and lead discovery. Peptide libraries synthesized on such templates are already successfully used in screening for protease inhibitors, catalysts, and artificial receptors. Several trifunctional templates 1 have been synthesized starting from commercial available 3,5-diamino-benzoic acid and trimesic acid. The phenyl ring is used as a rigid scaffold between the two amino groups that are orthogonally protected with Fmoc and Aloc protective groups. The distance between the two peptide arms has been varied using aliphatic linkers of different length. These rigid templates were attached to several polymer supports via their carboxyl function and used for synthesis of diverse peptide pairs and cyclic peptides. The influence of templates on secondary structures of linear and cyclic peptides in different solvents has been studied by CD and NMR spectroscopy.



1

X=(CH_2)n, $CONH(CH_2CH_2O)_2CH_2CH_2$

P141

ENABLING TECHNOLOGIES FOR THE FUTURE OF PEPTIDE SYNTHESIS AND SCREENING

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Genomics has brought with it one of the most daunting challenges to bioscience – the determination of the structure, function and expression of all the corresponding proteins that are encoded within the genome sequences. The expanding field of proteomics has the potential to make a significant impact by making the drug discovery process more predictive and accelerating the development of effective drugs. The power of molecular biology techniques has resulted in the increasingly prevalent use of proteins for consumer and therapeutic applications. Solid phase peptide synthesis is particularly useful when large numbers of analogues in relatively small quantities are required as in structure function studies on hormones, growth factors, antibiotics and other biologically active peptides or for determining the antigenic epitopes of peptides. The PEPSY system aids in the design and planning of peptide libraries and can synthesise up to 864 peptides in parallel. In order to prepare plates for screening the MOSS platform was developed for its high throughput, reliability and its ease of integration into the existing infrastructures. CLEVA "X" was designed for sample distribution and reformatting of 96, 384 and 1536 well plates. This system is also linked to cooled and humidity controlled storage facilities. The generation of such a bewildering number of peptide arrays in the modern laboratory requires new and innovative approaches to the management of the associated research databases. All of Zinsser Analytics' systems store the data in an internal database that is compatible with standard systems, such as, MS Excel, Oracle etc.

P142

SYNTHESIS OF AN ARRAY COMPRISING 551 VARIANTS OF THE FBP28-WW PROTEIN DOMAIN

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WW domains are protein-protein interaction modules composed of 35-50 amino acids. They are the smallest naturally occurring, monomeric, triple-stranded, anti-parallel beta-sheet protein domains. The WW domain binds proline-rich or proline-containing ligands with dissociation constants in the range of 1 to 50 mM. Recently we have shown, that the WW domains can be classified into six groups according to their preferences for ligand recognition. The binding motifs are PPx(Y/poY), (p/f)P(p,g)PPpR, (p/f)PPRgpPp, PPLPp, (p/y)PPPPP, and (poS/poT)P (1). Furthermore, we predict that the FBP28 (formin binding protein) WW domain (2) will bind preferable to the XPPPPPX motif. We will demonstrate the ligand recognition preference of the FBP28 WW domain by probing a cellulose-bound peptide array for binding in order to validate our prediction. Additionally, we will show a complete substitutional analysis of the FBP28-WW domain synthesized as a cellulose-bound peptide array and subsequently probed for binding to the ligand. Those investigations contribute to a better understanding of structure-function relationship of the FBP28 WW domain/ligand interaction. Furthermore, we will demonstrate an optimized FBP28 WW domain synthesis protocol for SPPS on resin and SPOT synthesis, including the use of pseudo-prolines as building blocks, advantageous for both technologies. In order to verify the successful synthesis of the domain, HPLC and mass-spectrometry analysis will be presented for both technologies. (1)Livia Otte et al., Protein Science, 2003, 12, 491-500 (2)Maria J. Macias et al., Nature Structural Biology, 2000, Vol. 7, Num 5, 375-379

P143

A NOVEL UNIVERSAL MULTICLEAVABLE RESIN TEMPLATE FOR SYNTHESIS SCREENING AND IDENTIFICATION OF PEPTIDE AND PEPTIDE AMIDE LIBRARIES

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Many multifunctional resins for multiple release applications suffer from complicated handling and/or incompatibility of the cleaved by products with the assay system. We have developed a universal multiple release resin template which allows to synthesize either free peptides or peptide amides. The release was performed by acid treatment under very dedicated conditions. Acid treatments of the resin release the peptides stepwise from the resin. The acid can easily be removed from the cleaved products by evaporation. No additional complicated tagging is necessary for peptide identification because a small portion of the product is immobilized on the resin for follow up on bead identification. Model peptides and peptide amides were synthesized by this method, identified, quantified and tested in a functional assay system.

P144

MASS SPECTROMETRY AND EDMAN SEQUENCING ANALYSIS OF AFFINITY SELECTED HISTIDINE TAGGED PEPTIDES

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We are interested in identifying histidine containing peptide tags capable of binding to nickel chelators immobilized on a two-dimensional surface with affinities that are dependent on the structure of the chelator. Identification of such orthogonal chelator/peptide pairs will allow nano-scale manipulation of histidine tagged proteins on surfaces composed of arrays of the chelators. The chelators consist of bi-, tri-, or tetra- repeats of the nickel-nitrilotriacetic acid (NTA) moiety. Libraries of peptides that have histidine residues separated by non-histidine spacer residues were incubated with glass slides coated with the different nickel chelators, and the bound peptides and washed at various stringencies and then eluted and analyzed by MALDI-TOF mass spectrometry. Additionally, the mixture of eluted peptides has been sequenced by Edman degradation. Initial results show that phenylalanine as spacer residue is more favourable than glycine or arginine residues. Within decapeptides, the histidines should be placed in the center of the peptide sequence, whereas the presence of non-histidine residues at the ends is tolerated. In some cases, bound peptide collections have been resolved into individual peptide species by MALDI-TOF MS and then sequenced by collision induced fragmentation (MALDI-TOF MS/MS). The sensitivity of MALDI-TOF analysis proved to be sufficient for the application of peptide mixtures having a diversity of more than 100 peptides.

P145

METHOD FOR PREPARING A RANDOMIZED LIBRARY OF DEHYDRATED AND THIOETHER BRIDGED PEPTIDES

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Lantibiotics are lanthionine-containing antibiotic peptides. Their lanthionine rings are post-translationally introduced by enzymes that dehydrate serines and threonines and couple the dehydrated residues to cysteines to form thioether rings. The goal of this study is to make use of this post translational process to produce peptides with thioether bridges. Thioether bridges are known to stabilize peptides against proteolysis and to introduce structure which can greatly increase specific binding of peptides to receptors. The nisin modification machinary of Lactococcus lactis is used for generating a library of peptides with dehydrated residues and / or thioether rings. A randomized library has been constructed on a genetic level. The sequence encoding the nisin leader peptide is followed by a sequence encoding a random hexapeptide containing the essential Thr/Ser and Cys residues which make up the thioether ring. The genetic constructs are expressed in a L. lactis strain which also expresses the nisin modification genes [1]. The results learn us more about substrate specificity of the involved modification enzymes. The system can be used for searching new peptides with increased receptor binding and stability. Kuipers et al. (2004) J. Biol. Chem. 279:22176-22182.

P146

SOLID PHASE SYNTHESIS OF 4-METHYLCARBOXY-1,4-BENZODIAZEPINE-2,5-DIONES AS BUILDING BLOCKS FOR PEPTIDE SYNTHESIS

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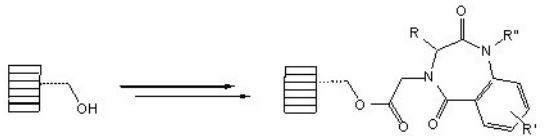
1,4-benzodiazepine-2,5-diones are well known pharmacophores exhibiting a wide scope of biological activities [1], but are also described as constrained templates [2] such as dipeptide mimics. Our goal was to develop a new route to 1,4-benzodiazepine-2,5-diones as building blocks easy to use in automated synthesis. As a purpose to expand quickly the chemical diversity of such templates, we explored solid phase strategies. Numerous synthetic pathways leading to benzodiazepinones have been described in literature. However, none lead to building blocks bearing a free carboxylic acid function and eventually, a protected amino function suitable for peptide synthesis. Strategic choice of the linker system and optimisation steps of the synthesis performed on Synphase Lanterns and polystyrene resin will be discussed. Finally, the analytical results of a focussed library will be presented as well as an alternative route to straightforward generate the benzodiazepinone moiety during the course of SPPS. [1] Bock, M.G.; Dipardo, R.M.; Evans, B.E.; Rittle, K.E.; Whitter, W.L.; Veber, D.F.; Anderson, P.S.; Freidinger, R.M. J. Med. Chem. 1989, 32, 13 [2] McDowell, R.S.; Gadek, T.R.; McGee, L.R.; Webb, R.R.; Venuti, M.C. J. Am. Chem. Soc. 1994, 116, 5077



R"= Alk or $(CH_2)_n$-NH-Fmoc

P147

PHOTOCHROMIC SPIROPYRAN-CONTAINING PEPTIDES FOR A NOVEL PROTEIN DETECTING SYSTEM

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In the postgenome era, a number of biochemical technologies to uncover cellular events have been increasingly emerging. Protein chip is one of the most promising technologies to solve some difficulties encountered. Although there are several requirements to develop practical protein chips, such as development of capture agents, active immobilization, and efficient detection. Thus, we have designed fluorophore-containing short-peptide libraries with the secondary structures (loop and α-helix) and demonstrated characterization of proteins of interest using the "protein fingerprint" technology based on changes in the fluorescence intensity resulting from protein–peptide interactions [1–3]. With such a developing protein–detection system in hand, another microenvironment-sensitive labeling group incorporated into the peptides has been exploited. Herein, we report the first example of the site–specific incorporation of a photochromic spiropyran molecule into peptides and characterization of proteins using such spiropyran–containing peptides with the photochromism depending on the binding properties between proteins and peptides. [1] M. Takahashi, K. Nokihara, H. Mihara, Chem. Biol. 2003, 10, 53–60. [2] K. Usui, M. Takahashi, K. Nokihara, H. Mihara, Mol. Div. 2004, in press. [3] K. Usui, T. Ojima, M. Takahashi, K. Nokihara, H. Mihara, Biopolymers 2004, in press.

P148

PROTEIN-DETECTION MICROARRAYS USING STRUCTURE-BASED DESIGNED PEPTIDE LIBRARIES

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As advances in genome-wide sciences, the protein microarrays have been promising robust technologies providing a high-throughput detection for proteins of interest. To realize such a practical protein-detection system, peptide microarrays have been constructed using designed peptides with various secondary structures [1-3]. Additionally, we established peptide libraries with functional groups including phosphorylated or glycosylated amino acids to increase the diversity of such an array system and to apply to the focused proteomics. Initially, the fluorescent probes and suitable scaffolds of peptides were examined for the effective detection of proteins. Then, peptide libraries consisting of totally ca. 500 peptides with secondary structures were constructed with systematic replacement of residues. Using these libraries, various proteins were characterized to give their own 'protein fingerprints', which correlated with the recognition properties of the proteins. Furthermore, to develop an easy and high-throughput detection system, various micro protein-detection methods including a micro-well method were demonstrated. Throughout this study, it has been demonstrated that the peptide microarray is one of useful approaches for protein-chips technology. With further improvement of micro/nano-detection systems, a practical protein-chip technology will be accomplished. [1] Takahashi, M., Nokihara, K., and Mihara, H., Chem. Biol., 10, 53-60, 2003. [2] Usui, K., Takahashi, M., Nokihara, K., Mihara, H., Mol. Divers., in press. 3. Usui, K., Ojima,T, Takahashi,M., Nokihara, K and Mihara, H., Biopolymers, 76, in press.

P149

STUDY ON SHORT PEPTIDE LIGANDS BINDING TO INSULIN RECEPTORS BY QUANTUM DOTS LABELING

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The important and complex physiological activity of insulin depends on its binding to the insulin receptor (IR) [1]. In this study, we took the Chinese hamster ovary cells over-expressing insulin receptors (CHO-IR) as targets to select peptide ligands of IR from a phage display random hexapeptide library. Assayed with cell-ELISA, we found five peptide ligands could compete for insulin binding and have Kd values in the micromolar range. In the mouse adipocyte lipogenesis assays, three of peptides exhibit antagonist effect, and the other two peptides exhibit partly agonist effect in millimolar concentration. In addition, the positive phage clones and the five peptide ligands conjugated with quantum dots (QDs) can specifically label the CHO-IR. We observed competitive binding between QD570-peptides (peptides conjugated with QDs; emission maximum of QD 570 nm) and QD620-insulin, and also between unlabeled peptides and QD620-insulin. According to the change of fluorescence intensity and color in the cell image, we can observe the difference of binding affinities for each peptide. The QD labeling method is consistent compared with the result of ELISA assay. In recent years, QDs have emerged as a new class of potential and promising fluorescent probe for many biomedical applications, especially for protein signaling and cell imaging , and our study have explored the application of QDs in characterizing the short peptide insulin analogs. [1]Renuka, C. P., Ku-chuan, H., James, R . B., et al. J. Biol. Chem., 277, 22590 (2002).

P150

PEPTIDE POOLS OF MAMMALIANS, INSECTS AND PLANTS: TISSUE SPECIFICITY AND COMMON PRINCIPLES OF FORMATION

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Systematic study of structure and biological activity of peptide components of tissue extracts and biological fluids allowed to formulate a concept of tissue-specific peptide pools. The basic features of these peptide pools are: (i) tissue specificity, (ii) quantitative and qualitative stability of the composition and (iii) its dependence on the tissue state. These peptides are formed as a result of the proteolytic degradation of tissue proteins by a specific and regulated system of tissue enzymes. The probable biological role of this system is maintaining of tissue homeostasis. This concept was formulated on the basis of data obtained earlier for mammalian tissue extracts [Karelin A.A., et al., FEBS Lett. 428 (1998) 7-12]. In present work we compare the peptide pools isolated from mammalian (rat and bovine) tissue extracts, human blood, insect hemolymph and green plant tissue extracts. The samples were subjected to the chromatographic separation under standard conditions, the individual peptides were isolated, quantified and sequenced. We confirmed the earlier established common principles of composition of peptide pools: stability and tissue specificity, total peptide amount of about 0.1% of tissue weight, 100-200 predominant components, mainly the fragments of basic tissue-specific proteins. Furthermore, the experimental approach used in our work was found to be the effective tool for peptidomic studies.

P151

A SPECIAL CELLULOSE MEMBRANE SUPPORT FOR THE COMBINATORIAL AND PARALLEL SYNTHESIS OF PEPTIDE LIBRARIES SUITABLE FOR THE SC2-TYPE MANUFACTURING OF HIGH DENSITY MULTI-PURPOSE CHEMICAL MICRO-ARRAYS

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We have recently developed a new process for the manufacturing of high density multi-purpose chemical micro-arrays which we call SC2 (Spotting Compound Support Conjugates). It involves the stepwise solid phase assembly of peptides or peptide mixtures permanently anchored on a particular type of support material which can be dissolved post synthesis to yield soluble peptide-support conjugates suitable for the direct spotting on planar surfaces such as a glass microscope slide by respective pikoliter pipetting devices [1]. Starting from a commercially available cellulose paper, we have designed a membrane support, which is particularly suited for the SC2 process. It provides a non-hydrolyzable primary amino functionality, it is resistant to all chemical and mechanical operations required for peptide assembly, but it readily dissolves in reagent mixtures containing more than 50 % trifluoroacetic acid. The new support can be utilized as planar synthesis support for the solid-phase assembly of chemical compounds by several types of combinatorial and parallel synthesis techniques such as the Filter Disc method [2,3], the SPOT-synthesis [4] or the Cut&Combine method [5]. This material will open the further miniaturization, automation and integration of high throughput synthesis & biological screening processes for immunological, functional genomics, proteomics and drug discovery studies. [1] Beutling et al. (2004) Patent applied. [2] Frank, R. et al. (1983) Nucleic Acids Res. 11, 4365-4377. [3] Frank, R. and Döring, R. (1988) Tetrahedron, 44, 19, 6031-6040. [4] Frank, R. (1992) Tetrahedron, 48, 9217-9232. [5] Dittrich, F., Tegge, W. and Frank, R. (1998) Bioorg. Med. Chem. Lett., 8, 2351-2356.

P152

FUNCTIONALISED β-AMINO ACIDS

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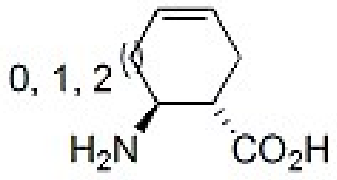
Molecules that have their conformation constrained by a constituent cycle impart important physical and chemical properties to biopolymers derived from them. For example, the α-amino acids proline and hydroxyproline are responsible for the secondary structure and hence function of collagen - one of nature's vital scaffolding proteins. Structurally related β-amino acids are an important class of biological molecules that, when incorporated into peptides, adopt predictable and reproducible folding patterns. However, unlike α-peptides, they are proteolytically stable, therefore provide a potential source of new drugs and catalysts. The peptidomimetic group at the University of Canterbury has established a new and versatile approach to the synthesis of novel five, six and seven-membered cyclic β-amino acids (1) utilising ring closing metathesis (RCM). The functionalisation of these compounds will be presented and discussed.



1

P153

IODINATED DESOCTAPEPTIDE INSULIN

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The biologically inactive fragment of insulin, desoctapeptide insulin, is used mainly in the semisynthesis of new insulin analogues. Enzymically catalyzed condensation of analogues of the C-terminal B23-B30 part of the peptide chain with desoctapeptide insulin produced analogues suitable for studying the effect of structure on their biological activity, such as the lowering of glucose level, glucose transport or stimulation of the incorporation of radioactive thymidine into DNA. Other new insulin derivatives were prepared by attaching biologically active opioid peptides or chromogenic aminopeptidase substrates to the carboxyl group of arginine in position 22. We also prepared a specifically labelled monoiodo-Tyr B26 analogue of human insulin. When insulin was used as an acceptor, iodination occured in the Tyr A14 position. Under similar conditions, using lactoperoxidase catalysis for the incorporation of iodine, we obtained a monoiodo derivative of desoctapeptide insulin. The semisynthetic condensation of octapeptide B23-B31, having iodinated tyrosine in position B26, with iodinated desoctapeptide insulin makes it possible to prepare human insulin labelled in two defined positions. All new insulin derivatives were analyzed and characterized by HPLC, capillary electrophoresis and mass spectrometry. The work was supported by the Grant Agency of the Academy of Sciences of the Czech Republic (IBS 4055303), by the Grant Agency of the Czech Republic (203/02/1469) and by Research Project AV0Z4055905 of ASCR.

P154

OSCILLAPEPTIN J, A NEW DEPSIPEPTIDE GRAZER TOXIN FROM LAKE ZÜRICH

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Mass occurrences of cyanobacteria frequently observed in mesotrophic and eutrophic lakes cause severe problems in reservoirs and lakes used as drinking water supplies, recreation areas, and watering places for livestock. The most common cyanobacterium in deep prealpine freshwater lakes in Central Europe is the red filamentous Planktothrix rubescens, formerly known as Oscillatoria rubescens D.C. This cyanobacterium accumulates to very high densities in Lake Zürich (Switzerland) and forms a dense layer at a depth of 10.5-12.5 m in the second part of the year. The collapse of such blooms liberates high amounts of toxins into the water. Oscillapeptin J has been isolated[1] from such biomass and the complex structure was solved by HPLC-MS, FTICR-MS, chiral GC-MS and 2D-NMR. 3-Amino-6-hydroxy-2-piperidone (Ahp), sulfated glyceric acid, N-methyl-tyrosine, and allo-isoleucine are constituents of this heptapeptide lactone. Enzyme inhibitor studies have shown that oscillapeptin J is a protease inhibitor. In addition, we have isolated and characterized another protease inhibitor from the blue-green coloured Microcystis cyanobacterium. This new depsipeptide is structurally related to the cyanopeptolins. [1] J.F. Blom, B. Bister, D. Bischoff, G. Nicholson, G. Jung, F. Jüttner, R. D. Süssmuth, J. Nat. Prod. 2003, 66, 431-434.

P155

HOW TO OPTIMIZE SCREENING OF THE PEPTIDES AFFECTING CELL PROLIFERATION?

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The ability to affect cells proliferation rate, and as a consequence, to change cell number may be considered as one of the common features of the mammalian peptides. Change of cell number may be a result of several processes: stimulation or inhibition of cell proliferation, cell differentiation and cytolysis. It allows to apply this parameter as a primary characteristic of biological activity of a given compound. Such approach is widely used both in fundamental and applied research. These methods may be divided into 2 groups: (1) direct methods (i.e., those based on direct cells count) and indirect ones (i.e., based on the determination of total quantity of cell material, level of enzyme activity, protein or nuclear acids synthesis). This work is devoted to review and analysis of both strong and weak points of different approaches to investigation of proliferative, antiproliferative and cytotoxic activities in cell cultures, as well as to estimation of application limits of these methods. As an application area of a given test directly depends on both the study objective and the mechanisms of action realized by a given substance, the algorithm of selection of methods optimal for resolution of different tasks will be suggested. The suggested approaches to identification and characterization of peptide (as well as of non-peptide) compounds are easy and relatively cheap, they allow rapid obtaining a large amount of information using low quantities of testing material and permit performing the majority of key studies in vitro, i.e., according to the international regulatory requirements.

P156

ACRETOCIN, AN EFRAPEPTIN-ANALOGUE PEPTAIBIOTIC FROM ACREMONIUM CROTOCINIGENUM

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Peptaibiotics are fungal polypeptides containing a high proportion of Aib (alpha-aminoisobutyric acid) with antibiotic properties. The mold Acremonium crotocinigenum (CBS strain 217.70) was cultured in a malt extract medium. Fermentation was conducted in twelve 2-L shake flasks, each containing 400 ml medium, for 8 days at 24 C. The peptaibiotic, here named acretocin, was isolated from the culture broth using chromatography at XAD adsorber resin followed by Sephadex LH-20 gel filtration. Comparison of acretocin and efrapeptin (isolated from Tolypocladium spp.) by thin layer chromatography revealed almost identical RF-values. Further, chiral gas chromatography after total hydrolysis provided similar amino acid compositions of acretocin and efrapeptin. In acretocin all amino acids are of the L-configuration with the exception of D-isovaline (D-Iva). In efrapeptin, however, Iva is of the L-configuration. A structural characteristic of both peptaibiotics is the heterocyclic C-terminus. Analytical HPLC of acretocin and efrapeptin provided similar elution profiles. The individual peptides of acretocin were isolated with semi-preparative HPLC and the fractions were analyzed by direct infusion electrospray ionisation mass spectrometry (ESI-MS) as well as on-line analytical HPLC – ESI-MS. The molecular masses of the acretocin peptides were analyzed to 1604 Da, 1618 Da, 1632 Da, 1646 Da and 1660 Da, whereas the molecular masses of efrapeptin components were measured to 1606 Da, 1620 Da, 1634 Da, 1648 Da and 1662 Da. From the data the sequences of eight peptides representing acretocins are presented.

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NEW SEQUENCES OF SUZUKACILLIN PEPTAIBOLS FROM TRICHODERMA VIRIDE

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Sequences and amino acid exchange positions of several suzukacillins produced by the mold Trichoderma viride (strain 63 C-I) have been reported [1]. Thin layer chromatography (chloroform/MeOH, 70/30, v/v) showed that crude suzukacillin (SZ) comprises two groups of peptides, named SZ A (RF = 0.27) and lipophilic SZ B (RF = 0.74). The crude suzukacillin was dissolved in chloroform/MeOH and acetone was added. The crystalline precipitate formed consisted of SZ A, whereas the mother liquid represented a mixture of SZ A and SZ B. Crystalline SZ A was analysed by HPLC using a Kromasil KR 100 column (150 mm x 4.6 mm ID, particle size 3.5 µm) together with a binary gradient consisting of acetonitrile/methanol/water (38/38/24, v/v/v) and acetonitrile/methanol (50/50, v/v). The suzukacillin A peptides were fractionated by semi-preparative HPLC on a Spherisorb ODS-column (250 mm x 8 mm ID, particle size 3 µm) using the eluents described above. Eighteen fractions were isolated each of which was subjected to sequencing using RP-HPLC coupled with ESI-MSn. Gas chromatography of total hydrolysates of fractions of suzukacillin A on Chirasil-L-Val revealed the "L"-configuration of chiral components with exception of D-Iva. Isomeric L-Val and D-Iva were detected in each fraction analysed. From the data the sequences of major peptides of suzukacillin A were deduced and correlated with those already reported. [1] Katz, E., Aydin, M., Lucht, N., König, W.A., Ooka, T., Jung, G., Liebigs Ann. Chem., 1041-1062 (1985)

P158

SEQUENCES OF SHORT CHAIN PEPTAIBOL ANTIBIOTICS TRICHOBRACHINS

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In a previous communication we had reported on the isolation, separation and structur determination of trichobrachins (TB) from the culture broth of the mold Trichoderma longibrachiatum (CBS, strain 936.69) [1]. Trichobrachin was separated by thin-layer chromatography (TLC) into three groups of different hydrophobicities named TB I, TB II and TB III. The structures of peptides of TB I and TB II could be established, whereas structures of TB III components were partially elucidated. In order to complete and repeat the work, Trichoderma parceramosum (CBS, strain 936.69; synonym T. longibrachiatum) was cultured in shake flasks in malt extract medium. Mycelia were removed by filtration and crude trichobrachin was isolated from combined culture broths using XAD- and Sephadex LH-20 chromatography. Preparative TLC on silica gel (chloroform/methanol/glacial acetic acid/water (80/20/2/1, v/v/v)) yielded again the three peptide groups TB I, TB II and TB III. Analytical HPLC of each group on a Kromasil KR 100 column (150 mm x 4.6 mm ID, 3.5 µm particle size) using a binary gradient provided microheterogeneous peptaibols. The sequences were determined using analytical HPLC coupled with electrospray ionisation tandem mass spectrometry (LC-ESI-MSn). Sequences of major peptides of TB I and TB II could be confirmed. Here we report on the structures of peptides TB III, representing extremely microheterogeneous short chain peptaibols (molecular masses < 1200 Da). [1] Brückner, H., Kripp, T., Kieß, M., in: Giralt, E., Andreu, D. (eds), Peptides 1990, Escom, Leiden: 347-349

P159

RAPID AND SELECTIVE SCREENING METHOD ON PEPTAIBIOTICS/PEPTAIBOLS USING LC-ESI-MS(N)

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Peptaibiotics are defined as a family of fungal peptides containing a high proportion of the nonproteinogenic amino acid Aib (alpha-aminoisobutyric acid) and exerting biological activities. N-acetylated members of this group containing a C-terminal 1,2-amino alcohol are defined as peptaibols. Lipopeptaibols are acylated with a fatty acid at the N-terminus, and aminolipopeptides contain unusual heterocyclic residues at N- and/or C-termini. For screening experiments filamentous fungi were grown on agar plates. The mycelia were treated with a mixture of organic solvents, and the peptides extracted were adsorbed on a Sep-Pak C-18 cartridge. The peptides were eluted with methanol and analysed by HPLC on a Kromasil KR 100 column (150mm x 4.6 mm ID, 3.5 µm particle size) using a binary gradient consisting of acetonitrile/methanol/water (32/32/36, v/v/v) and acetonitrile/methanol (50/50, v/v). Eluting peptides were sequenced partially on-line using ESI-MS(n) thus providing molecular ions and more or less complete series of characteristic fragment ions. From the mass differences (Δm) of these fragment ions the presence of the marker amino acid Aib, characterised by Δm = 85 Da, as well as other constituents could be deduced. An advantage of the LC-MSn screening is that a single Petri dish provides sufficient diagnostic information on peptaibiotics production. Using characteristic N- and C-terminal mass fragments, among peptaibiotics and subgroups such as peptaibols and lipopeptaibols could be distinguished. Moreover, as almost 300 peptaibiotics are known, the fragment pattern made possible the judgement whether or not structures are novel or identical or related to structures already known. Examples are presented.

P160

YEAST NUCLEOPEPTIDE COMPLEXES AND THEIR BIOLOGICAL EFFECT

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Endogenous peptide regulators isolated from various tissues of superior organisms have attracted much attention over the last few years. It is known that peptides and low molar mass proteins that bind specifically to nucleic acids and belong to the group of nonhistone proteins of chromatine regulators that function in developing cells. In this study complexes of nucleic acids and acidic nuclear peptides were isolated from cells of Saccharomyces cerevisiae. These complexes could induce the regeneration of culture cells of Saccharomyces cerevisiae and they were active toward human T-lymphocytes. Cross-flow microfiltration through track-etched membrane was applied to separate this high molar mass component from low molar mass components in the solution. Gel permeation chromatography and electrophoresis were used to study the molar masses and the composition of the components of the retentate mixture obtained after filtration. It was shown that only the high molar mass nucleopeptide complex (1430 kg/mol) possessed the biological activity.

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ISOLATION AND CHARACTERIZATION OF PSALMOPEOTOXIN I AND II, TWO NOVEL ANTIMALARIAL PEPTIDES FROM THE VENOM OF THE TARANTULA PSALMOPOEUS CAMBRIDGEI

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Two novel peptides which inhibit in vitro the erythrocytic stage of Plasmodium falciparum infection were identified from the venom of the Trinidad chevron tarantula, Psalmopoeus cambridgei. The two peptides, named Psalmopeotoxin I (PcFK1) and Psalmopeotoxin II (PcFK2), are 33- and 28- amino acid peptides with three disulfide bridges that belong to the Inhibitor Cystine Knot superfamily. The cDNAs encoding both peptides were cloned, nucleotide sequence analysis showed that both peptides are synthesized as a signal peptide and a pro-peptide that is cleaved at a basic doublet before secretion of the mature peptide. The IC50s of PcFK1 and PcFK2 for P. falciparum growth inhibition were 1.59 ± 1.15 and 1.15 ± 0.95 μM, respectively. Two peptides affect the parasite development cycle. PcFK1 but not PcFK2 adsorbs strongly to uninfected erythrocytes, however, the peptides do not exhibit significant hemolytic activity over 10 μM. Interestingly, PcFK1 and PcFK2 do no affect growth or viability of human epithelial cells, nor do they display antifungal or antibacterial activity at 20 μM. Electrophysiological recordings in isolated frog and mouse neuromuscular preparations revealed that the peptides do not affect neuromuscular transmission nor quantal transmitter release at 9.3 μM. The overall results suggest that PcFK1 and PcFK2 interact specifically with infected erythrocytes, possibly through distinct receptors or channels of the red blood cells. PcFK2 is not toxic for erythrocytes, human epithelial cells, or neuromuscular junctions, and do not adsorb non-specifically to healthy erythrocytes, raising the prospect that it could be used as potential new antimalarial drug.

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VENOMS PROTEOMICS: NOVEL APPROACHES FOR DRUG DISCOVERY

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Natural bioactive compounds are usually characterized on the basis of a particular observed biological activity. In a complementary approach, we focus on a backward strategy going from structure to function. To this end, we use a combination of genomics (sequencing of cDNA from venom glands), proteomics (systematic identification and characterization of the venom components) and bio-computing (the development of a unique biological and biochemical database on venomous animals and their venoms). Proteomic strategies applied to animal venoms will be illustrated through several examples. The direct analysis of crude venoms using on-line LC-ESI-MS, MS/MS and/or MALDI-TOF-MS in combination with related micro- or nano-technologies allows fast peptide and protein identification and characterization. For example, complete molecular mass mapping by MALDI-TOF-MS of hundreds of venoms could be carried out leading to the identification of novel candidates as bioactive peptides. These techniques (referred as "venomics") have also been successfully applied to the complete mapping of honey bee (Apis mellifera) venom, the discovery of a novel family of sarafotoxins (endothelin-type peptides) or bradykinin-potentiating peptides (BPP's) from snake venoms. The proteomic analysis of crude Conus textile venom allowed a full mapping of the toxin profile (referred as "toxinome") together with the identification, isolation and characterisation of novel conotoxins. We believe that our unique combination of mass spectrometric techniques coupled to in silico data and text mining strategies is a straightforward approach to discover and characterize novel bioactive toxins or enzymes from animal venoms or other natural sources.

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UBIQUITOUS PEPTIDE SEQUENCES CONTROLLING CELL PROLIFERATION

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Norwegian Authors demonstrated that cells from different tissues produce tissue-specific peptides active in the control of cell proliferation. The structure of peptides inhibiting specifically the growth of epidermal, hepatoma , colon carcinoma and neuroblastoma cells has been described (K.Elgjo et al Progr. Mol. Subcell. Biol. 20,143;1998). Our and other laboratories reported results according to which peptides isolated from the chromatin of animal and vegetal tissues exert a dose-dependent inhibition on the proliferation of several tumor cells "in vitro" (L1210, HL60, HeLa cells) (G.L. Gianfranceschi et al Biol Chem 338,31;1999) In this context a main question is: are all the controlling proliferation peptides tissue-specific or could the presence of some ubiquitous molecules be demonstrated? We performed many FIB+ and electron spray mass spectrometry data on the peptide fractions purified from bull spermatozoa, trout testis, seminal plasma, pea and wheat sprouts. An accurate comparative analysis of the mass data is in progress to see if some sequences are present in all the studied tissues. Preliminary results show that peptides with MH+ 571/2 and 635 are widely distributed in the active peptide fractions. The mass spectra analysis is performed with a recently patented automatic combinatorial method that carries out the computation of all amino acid sequences compatible with a given spectrum data. The possible sequences of these compounds are automatically obtained by considering the mass of ions that are potential breakdown products. The structures supported by more significant breakdown products will be synthesised to check the activity in the control of cell proliferation.

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CYSTEIN-RICH ANTIFUNGAL SMALL PEPTIDE FROM THE POTATO TUBERS (SOLANUM TUBEROSUM L CV. JOPUNG)

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A novel antifungal protein (17 kDa), which was active (MIC; 6. 25 μg/ml) against Candida albicans, Trichosporon beigelii and Saccharomyces cerevisiae, a yeast fungus, has been purified from the potato tubers of Solanum tuberosum L cv. Jopung using gel filtration and HPLC. The amino-terminal amino acid sequences of the purified protein from HPLC were determined as NH_2-Leu-Pro-Ser-Asp-Ala-Thr-Leu-Val-Leu-Asp-Gln-Thr-Gly-Lys-Glu-Leu-Asp-Ala-Arg-Leu. The sequence showed a 75% homology with a serine protease inhibitor and the protein indeed showed inhibitor activity against chymotrypsin, pepsin and trypsin. In addition, the protein was subjected to partial acid digestion using HCl in order to isolate active peptide, and a peptide (Potide-J) that was active against Candida albicans (MIC; 6. 25 μg/ml) was isolated. The amino acid sequence of Potide-J was determined as, Ala-Val-Cys-Glu-Asn-Asp-Leu-Asn-Cys-Cys and found no homology with known antifungal peptides. Therefore the new antifungal peptide, Potide-J with MW of 1083.1 Da could be a lead peptide for the development of commercially applicable antibiotic agent.

P165

AN EFFICIENT METHOD FOR PARALLEL SYNTHESIS OF MULTIFUNCTIONAL MOLECULES IN THE SCAVIDIN MEDIATED CELL MEMBRANE TRANSPORT

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Multifunctional molecules contain several different chemically and/or biologically active sites. They may consist of targeting, internalisation, protease or acid sensitive, nuclear localizing sequences and tracer moieties. Multifunctional molecules provide a tool for basic biological research such as selective cell surface binding, membrane transport and intra-cell distribution. We have synthesized a set of multifunctional molecules in order to study Scavidin mediated transport, which can be used as an effective carrier for prodrugs [1.] Synthesized molecules consisted of biotin, biotinidase resistance group, Cathepsin B (CB) sensitive sequence, self-immolative spacer, and the tracer. Molecules were synthesized and labeled on solid phase using simultaneous C to N and N to C directed synthesis methodology, and selectively protected building blocks. Fluorescent tracer was chosen as a model in functional prodrug designing. The protease sensitivity of the molecules was tested using small scale affinity chromatography. After CB treatment, biotinylated molecules were bound to 50 mg of streptavidin-Sepharose, mixed and centrifuged. Fluorescence was measured from the remaining supernatant, and the signal level correlates to the amount of released FITC moiety. Our synthesis strategy provides an efficient method for parallel synthesis of different prodrug variations and the testing of their biological activity in vitro. [1] P. Lehtolainen et.al., J. Biol. Chem., 277, 8545-8550 (2002).

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NOVEL PEPTIDE INHIBITORS OF HUMAN KALLIKREIN 2 (HK2)

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Human kallikrein 2 (hK2) is a serine protease produced by the secretory epithelial cells in the prostate. Because hK2 activates several factors participating in proteolytic cascades that may mediate metastasis of prostate cancer, modulation of the activity of hK2 is a potential way of preventing tumor growth and metastasis. Furthermore, specific ligands for hK2 are potentially useful for targeting and imaging of prostate cancer. We have used enzymatically active recombinant hK2 captured by a monoclonal antibody exposing the active site of the enzyme to screen phage display peptide libraries. Using libraries expressing 10 or 11 amino acids long linear peptides we identified six different peptides binding to hK2. Three of these peptides were shown to be specific and efficient inhibitors of the enzymatic activity of hK2. Amino acid substitution analysis revealed that motifs of 5-7 amino acid were required for the inhibitory activity of the peptides. These peptides are potentially useful for treatment and targeting of prostate cancer. The peptides are also of potential utility for development of immunopeptidometric assays for hK2, which is promising marker for diagnosis of prostate cancer.

P167

SYNTHESIS AND CHEMOTACTIC ACTIVITY OF CONJUGATES CONTAINING FOR-MET-LEU-PHE CHEMOTACTIC PEPTIDE

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Chemotactic responsiveness is a basic character of a wide variety of motile subpopulations of cells belonging to different tissues/organs of vertebrates. Selective accumulation of specific cell population achieved by chemotactic compounds might have a new approach in drug targeting. In this study we describe the synthesis of a new group of conjugates consisting of a tetratuftsin oligopeptide derivative [TKPKG]4 as carrier and a chemoattractant tripeptide formyl-methionyl-leucyl-phenylalanine (fMLF) or its fNleLF derivative. The chemotactic activity of Ac-[TKPK(X)G]4-NH_2 type conjugates (where X is chemotactic peptides) was studied on various cell lines including macrophages (J774), monocytes (THP-1) and MRC fibroblasts as well as on Tetrahymena pyriformis cultures. The effect of these conjugates were compared with that of ovalbumine attached fMLF and respective control compounds (tetratuftsin, ovalbumine, fMLF-OH, and fMLF-NH_2), also with the branched chain polypeptide-fMLF conjugates. Some of the conjugates were chemoattractant on THP-1 monocytes, but had no effect on the other cells. Three-party conjugates containing cytotoxic drug (Y-GFLG-K(Y-GFLG)-[TKPK(X)G]4-NH_2, where Y is the cytotoxic compound connected to the carrier through an enzyme labile spacer and Y is the chemotactic peptide) were also prepared. Our results show, that chemotaxis provides a new possibility of cell-type and carrier/ligand specific targeting, defined as “chemotactic drug-targeting”. Aknowledgement: This work was supported by grants of the Hungarian Research Fund (OTKA, T043576, T032533 and T032425).

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BACKBONE MODIFIED PEPTIDE LIGANDS IN TRANSITION METAL CATALYSIS

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The present work explores the potential of solid phase combinatorial peptide chemistry for transition metal catalysis to form catalysts with enzyme like properties of stereo-, regio- and even substrate selectivity. A series of transition metal donors within the peptide backbone was synthesised on a high swelling PEGA-resin, using standard solid phase peptide coupling techniques. The synthetic strategy gave access to both mono- and bidentate ligand systems providing a folded structure around the central transition metal, with different steric congestions and bite-angles. Changing the number of incorporated amino acids between the two donors facilitated variation of the parameters of the catalyst. Both donor systems were complexed to palladium by simple treatment with Pd(OAc)2 and other transition metals in THF at elevated temperature to provide a range of novel catalysts for organic transformations

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NOVEL BACKBONE AMIDE LINKERS FOR SOLID-PHASE PEPTIDE SYNTHESIS

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Handles (linkers) with an aldehyde functionality allow anchoring of substrates by convenient reductive amination and have become widely used tools for solid-phase synthesis. In this approach the growing peptide chain can be anchored through a backbone amide, thus providing easy access to C-terminal modified and cyclic peptides. This Backbone Amide Linker (BAL) concept was first implemented in a tris(alkoxy)benzyl system, which allowed release of final products by treatment with conc. trifluoroacetic acid (TFA). By far most acid-labile handles for SPPS have used a very limited set of core structures, i.e., benzyl, benzhydryl, or trityl. An indol based handle has also been reported. Very recently, we have described naphthalene and thiophene based handles. Novel BAL-type handles with new properties continue to be of significant interest. Here we describe our efforts towards synthesis and evaluation of new handles based on naphthalene and thiophene core structures. We have synthesized a trisalkoxynaphthaldehyde handle and applied it in solid-phase synthesis. This new structure exhibited a clear peri-effect next to the aldehyde functionality.

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NOVEL NATRIURETIC PEPTIDES FROM THE VENOM OF THE AUSTRALIAN WESTERN TAIPAN

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Natriuretic peptides (NP) function as vasorelaxing hormones and contain a conserved seventeen amino acid loop generated by a single disulfide bond. We have recently discovered three novel natriuretic peptides (TNPa, TNPb and TNPc) from the venom of the Western taipan Oxyuranus microlepidotus. They were identified by mass spectrometry and RP-HPLC analysis and consist of 35-39 amino acid residues considerably longer than mammalian forms. They also differ from other NP groups (eg ANP/BNP) through replacement of invariant residues within the seventeen-member ring structure and by inclusion of proline residues in the C-terminal tail. Synthetic access to these three TNP toxins has been established. In this paper we will describe the selectivity and potency of these peptides and specific mutants on selected tissues, cells expressing guanyl cyclase receptors and whole animal studies.

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PRODUCTION OF PEPTIDES BY CELL CULTURES: IS IT ACTIVE SECRETION OR PASSIVE EXCRETION?

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The presence of peptide material in the conditioned medium of the primary culture of the erythrocytes were established earlier (Ivanov et al, 2000). The erythrocytes were incubated in PBS at 37 C for 4 hours. After that the erythrocytes were pelleted by centrifugation and the supernatant was used for peptides isolation. As a result, 37 peptides were isolated and sequenced. The isolated peptides were identified as hemoglobin and beta-actin fragments. The supernatant of the erythrocyte culture did not contain the material present in the lysate of red blood cells. Moreover, the protein fragments identified in the lysate were not found in the supernatant. Study of dynamics of peptides accumulation in the culture medium demonstrated that this process is saturable, i.e., peptides content in the supernatant became constant at hour 2. Addition of 3% glucose to the culture medium lead to reliable increase of peptide material content and prolonged the release of peptide material over 4 hours the components composition being similar to that obtained without glucose addition. The major part of the isolated peptides was shown active in cell cultures. All components except neokyotorphin-related peptides inhibit tumor cells proliferation. The specificity of the peptides release from red blood cells will be discussed. The results obtained for the erythrocytes will be compared with those demonstrated in the framework of the study of peptides production by K562 human erythroid leukemia cells.

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USE OF LANTIBIOTIC ENZYMES FOR SYNTHESIS OF THIOETHER BRIDGES IN A BROAD RANGE OF PEPTIDES

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Thioether bridges can play an essential role in peptide activities and protect peptides against proteolytic degradation. Insertion of thioether bridges in peptides can dramatically increase their efficacy in vivo. Thioether-bridge-containing antibiotic peptides, so called lantibiotics, are produced by various Gram-positive bacteria. The pentacyclic lantibiotic nisin is produced by the food grade organism Lactococcus lactis. Nisin synthesis and export involves the dehydration of serines and threonines by NisB, their consecutive coupling to cysteines via the cyclase NisC, and its subsequent translocation by NisT. This study focuses on the exploitation of the nisin enzymes by inserting thioether bridges in therapeutic peptides. We have followed the activity of combinations of the nisin enzymes by measuring export of recombinantly expressed and secreted peptides using antibodies against the leader and mass spectrometry for detection. We here report that a broad range of non-lantibiotic peptides genetically fused to the C-terminus of the nisin leader peptide or (parts of) prenisin are modified and thereafter exported out of the cell via NisT. By using semi-randomized oligonucleotides leader peptide fusions can be generated. This allows the generation of thioether (poly)peptide libraries which can be used for screening for peptides with desired properties. The results demonstrate the feasibility of biological production of a wide variety of thioether-bridged (poly)peptides with increased stability or novel bioactivities.

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FUNCTIONAL STUDIES OF ADIPOKINETIC PEPTIDES IN PYRRHOCORIS APTERUS (HETEROPTERA, INSECTA) - RHYTHMICITY OF THEIR CONTENT AND DEPENDENT PHYSIOLOGICAL PARAMETERS

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Insect neuropeptides belonging to the adipokinetic hormones (AKH) are typical stress hormones that mobilise energy sources (lipids, carbohydrates and/or certain amino acids depending on insect species). Recently, two octapeptide members, Pyrap-AKH (pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-NH_2, Kodrík et al., Insect Biochem Mol Biol 30: 489, 2000) and Peram-CAH-II (pGlu-Leu-Thr-Phe-Thr-Pro-Asn-Trp-NH_2, Kodrík et al., Peptides 23: 583, 2002) have been identified in the firebug Pyrrhocoris apterus. In this study we compared the patterns of rhythms of the AKH content in CNS and haemolymph (using a competitive ELISA), adipokinetic response to AKH, and locomotory activity in adult macropterous females of the bug, reared under long-day photoperiod (LD 18:6 h) and those transferred to constant darkness. We found positive correlation between the rhythms of AKHs content in CNS, adipokinetic response to AKHs, and locomotory activity rhythms in LD females, and persistence of these rhythms under conditions of constant darkness, which demonstrates endogeneity of the rhythmicity of all these phenomenons. The present study is the first report demonstrating endogeneity of AKH content in CNS in insects and extends our knowledge about chronobiology of neuroendocrine regulation of behavioural and physiological functions in this heteropteran species. This study was supported by grant No. A6007202 from the GA of the Czech Acad. Sci. (DK) and grant No 206/03/0016 from the Grant Agency of the Czech Rep. (RS).

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INFLUENZA A HEMAGGLUTININ C-TERMINAL ANCHORING PEPTIDE. MS IDENTIFICATION AND APPROACH TO STRUCTURAL STUDY

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Influenza A hemagglutinin (HA) is a major envelope glycoprotein mediating viral and cell membrane fusion. While structure of the bromelain released HA ectodomain (including HA1 chain and residues 1-175 of HA2 chain) has been solved by X-ray crystallography [1,2], there is no 3D information for a C-terminal anchoring peptide. This peptide (176-221 HA2) includes the transmembrane domain (HA TMD), which has a crucial role at a late stage of the fusion. To isolate the highly hydrophobic 176-221 HA2 peptide, bromelain-shaved subviral particles of influenza A/X-31 strain were subjected to extraction with chloroform-methanol (2:1). By MALDI-TOF MS we could detect in the organic phase of these extracts several mass peaks in a range of 3500 to 6000 m/z. Such peaks were absent in the analogous spectra got for intact virus particles and for the bromelain preparation. Partial N-terminal amino acid sequencing helped us to deduce a number of putative peptides, which corresponded to the MALDI-TOF masses and could be fragments of the C-terminal anchoring peptide. The fragments had different N- and C-termini and were acylated heterogeneously. Applying tritium bombardment technique [3] to these fragments will open an opportunity for 3D structural investigation of the influenza HA TMD in situ. [1] Wilson I.A. et al. (1981) Nature, 289:366-373. [2] Bullough P.A. et al. (1994) Nature, 371:37-43. [3] Shishkov A.V. et al. (1999) Proc. Natl. Acad. Sci. USA, 96:7827-7830. This work was supported by RFBR #02-04-48655 and by ISTC #2816.

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TYROSINE-CONTAINING PEPTIDES MEASUREMENT IN ENDOGENEOUS INTOXICATION DIAGNOSTIC

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Development of endogeneous intoxication in various diseases is accompanied by the accumulation of toxic metabolites in blood. Endogeneous intoxication diagnostic is usually a common content of peptides in a blood plasma measurement. Tyrosine-containing peptides (TCP) measurement has been shown to be a more sensitive and specific marker of endogeneous intoxication. We have performed a study in 55 patients with pulmonary tuberculosis, to evaluate whether TCP measurement might provide a better diagnostic of endogeneous intoxication than common content of peptides. According to clinical date, three groups of patients with different degrees of the endogeneous intoxication syndrome were distinguished. TCP and common content of peptides were measured by UV absorbance at 290 and 280 nm accordingly of blood after protein removal. In patients of 1st group the TCP and common content of peptides values were respectively 158 % and 148 % relative to the control group (healthy people). In 2nd group, the parameters were 284 % and 173 %. In 3rd group the TCP and common content of peptides values were 368 % and 187 % respectively. These results suggest that plasma content of TCP is more sensitive marker than common content of peptides to early stages of endogeneous intoxication syndrome development and in monitoring its progression.

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CHROMATIN PEPTIDES INDUCE G2/M ARREST IN HELA CELLS BY A P53 INDEPENDENT PATHWAY

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A family of close related peptides, found to be associated to the chromatin of eukaryotic and prokaryotic cells, are involved in the control of gene expression and cell proliferation. At present the effector sequence is unknown. Treatment of HeLa cells with chromatin peptides from wheat bud results in inhibition of cell proliferation and in a G2/M cell cycle block. The nuclear accumulation of the regulatory complex cdc2-cyclin B has also been observed. Since the cdc2-cyclin B kinase activity has the major role in regulating the G2/M transition, its accumulation was further investigated. The data show that in treated cells the complex accumulate in an inactive state in the G2 phase of the cell cycle (its activity is 3-4 times lower than in the corresponding control cells). We then checked the involvement of the G2/M damage checkpoint pathway in inducing the cdc2-cyclin B activity inhibition. This pathway prevents cells from entering mitosis if genome is damaged. The expression levels of the active chk1 kinase and of the inactive cdc2 subunit were measured in G2 phase cells. These levels are higher in treated cells when compared in those found in G2 control cells. However the expression of phosphorylated p53 is decreased by the treatment. These data suggest that the chromatin peptide fraction, according to their ability in inhibiting DNA synthesis, probably induces DNA damage. The overall effect results in G2/M arrest by p53-independent cascade of the checkpoint control pathway.

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STRUCTURE OF A NOVEL CIRCULAR PROTEIN FROM VIOLA TRICOLOR: THE FIRST FLORAL CYCLOTIDE DISCOVERED

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The cyclotides are a novel group of backbone-cyclised plant peptides found in great abundance in various species of the Rubiaceae and Violaceae plant families. These intriguing peptides are approximately 30 amino acids in length, contain three disulfides arranged into a cystine knot motif and display unusual thermal, chemical and biological stability. Only four members of the cyclotide family have been structurally characterised. These structures reveal a rigid molecule consisting of a three-stranded beta-sheet that provides the compact fold for the formation of the cystine knot. The spacing of the cystine residues that make up the cystine knot is conserved across all characterised cyclotides, with variation occurring in backbone segments that connect the outer edges of the knot. In this work a cyclotide was discovered in the flowers of V. tricolor that at the time was the largest cyclotide discovered. The increased size affected only one solvent exposed loop, which at ten amino acids is the largest inter-cystine segment discovered in the cyclotides. Intriguingly this loop has also been distinguished in other cyclotides as the processing site for the as yet unidentified cyclising mechanism. In order to understand the effect of increased loop size on the compact cyclotide fold, vital for the engineering of novel bioactivities to this framework, the three-dimensional structure of this peptide was determined using NMR spectroscopy. In addition the gene structure was elucidated so as to determine the N and C termini of the linear precursor and hence provide clues to the mechanism of cyclotide cyclisation.

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THE SOLID-PHASE INTRAMOLECULAR N-ACYLIMINIUM PICTET-SPENGLER REACTION AS A CROSSROAD TO A PLETHORA OF NOVEL HETEROCYCLIC PEPTIDES

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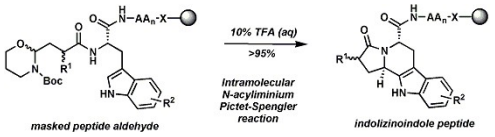
In the present investigation, peptide aldehydes undergo intramolecular condensation reactions with the amide N of a solid-supported peptide backbone, thus forming a cyclic N-acyliminium ion, which may append a second ring via Pictet-Spengler cyclization with the indole moiety of a neighbouring tryptophan. The formation of the new C-C bond proceeds quantitatively in a highly stereoselective fashion (purity > 95%, dr > 20:1), thus providing a highly desired tetracyclic ring system anchored to a peptide backbone. By introducing substituents in the aldehyde building block (R1) and on the indole core of tryptophan (R2), a range of diverse indolizinoindole containing peptides are conveniently obtained in one synthetic step from masked aldehyde peptide substrates, which are readily available via standard peptide coupling procedures. In addition to indoles, the methodology has been extended to a range of reactive heterocyclic ring-systems as C-nucleophiles incorporated in amino acid side-chains, such as furanes, thiophenes and benzothiphenes, thus providing a simple, unique platform for diversity-oriented synthesis of peptides containing pharmacologically interesting tri- and tetracyclic scaffolds in a combinatorial library format. The Pictet-Spengler cyclization of less reactive mono-, di-, and trisubstituted phenylalanine derivatives with diverse sets of electron withdrawing and donating substituents and naphthylalanines works equally well in the reaction sequence, notably with excellent yields and stereoselectivities.



P179

FUNCTIONAL AND STRUCTURAL ANALYSES OF PEPTIDES BINDING TO HUMAN KALLIKREINS HK2 AND HK3

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Human kallikreins are a subgroup of the serine protease enzyme family. The human prostatic kallikreins hK3 (PSA), and more recently human grandular kallikrein, hK2, are widely used tumor markers for prostate cancer diagnosis. Several peptide sequences with specific binding to PSA and hK2 have been identified by using phage display peptide libraries. Small, cyclic peptides, have high affinity to active form of PSA and they have been shown to increase the serine protease activity of PSA [1]. PSA has been shown to inhibit endothelial cell proliferation being a candidate anti-angiogenesis factor [2]. Thus, ligands modulating PSA enzyme activity are potential molecules in cancer therapy. The level of hK2 in aggressive prostate cancer has shown to be increased. Found hK2 binding peptides are effective and specific hK2 protease inhibitors providing another option for therapy. We have studied the functionality of the kallikrein binding peptides using amino acid replacement studies in order to determine the amino acid residues important for the activity of the peptides and the 3D structure of the peptides by NMR spectroscopy [3]. According to the replacement studies we were able to increase the biological activity of the hK2 binding peptide by single point mutation. Replacement studies together with structure analyses support the design of the optimal structure of kallikrein binding peptides. [1] Wu et al. Eur. J. Biochem. 2000; 267: 6212-6220 [2] Fortier et al. J. Natl. Cancer Inst. 1999; 91: 1635-1640. [3] Pakkala et al. J. Pept. Sci. In press.

P180

IDENTIFICATION OF NOVEL CYSTEINE-RICH PEPTIDE TOXINS FROM MACROTHELE GIGAS BY SIMPLE RANDOM SCREENING OF THE VENOM GLAND CDNA LIBRARY

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One of the most prevalent spider toxin is a multi-cysteine peptide with a potent and unique inhibitory or modulatory activity on ion channels. However, sequence determination and precise pharmacological analysis frequently failed or restricted because of difficulties in obtaining plenty venom materials. We previously reported the isolation and characterization of of six novel peptide ion channel blockers, Magi 1-6, from the venom gland of a spider, Macrothele gigas. In a present study, we ide tified further ten novel multi-cysteine peptides, namely, Magi 7-16 from the same spider, by simple random screening of the venom gland cDNA library prepared from only five venom glands. Random screening of ca. 300 independent cDNA clones led to detection of approximately 200 open reading frame encoding a single copy of multiple cysteines-containing peptide toxin-like sequence. Sequence analysis of the deduced amino acid revealed that ten novel multi-cysteine peptide, Magi 7-16, as well as previously characterized Magi 1-6, were encoded by each clone. MALDI-TOF Mass analysis of the crude venom detected all mass numbers of the cross-linked forms of Magi 7-16, confirming that Magi 7-16 are undoubtedly present in the venom. Moreover, pharmagological assays showed diverse lethal or paralytic activities of these peptide toxins on mice and insects. These results suggest that Magi 7-16 serve as toxic components. In combination, our data showed that simple random cDNA screening is a promising method for identification of novel peptide toxins from a small amount of venom gland samples.

P181

NOVEL INSECT PEPTIDES AND PROTEINS THAT REGULATE PHENOLOXIDASE ACTIVITY DURING METAMORPHOSIS OF HOUSEFLY MUSCA DOMESTICA

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Melanogenesis is considered to be crucial in insects for their growth and maintenance of life such as sclerotization of cuticle in the course of metamorphosis and also for defense by clotting foreign pathogens. This cascade requires activation of prophenoloxidase (proPO) that catalyzes an initial and rate-limiting step in complex reactions: oxidation of catecholamines to quinones. Activity of PO in the hemolymph of housefly dramatically changes especially in the stages of morphological transition. The mechanism of PO generation by proteolytic cascade has been well elucidated; however, regulation of activated PO is not unclear except for our finding of PO inhibitor POI [1]. POI is a unique endogenous peptide that inhibited PO activity with 10-9 [M] order, and has a cystine motif virtually identical to conotoxin GVIA [2]. POI strongly inhibited PO activity in vitro, however, in vivo content of POI was considerably lower than the expected amount to suppress the actual PO activity. In this study, we performed a 'natural pull-down method' to identify the actual PO regulator(s). In this procedure, target peptides were recovered from crude extracts escaping severe interference by the presence of contaminating peptides without any knowledge of their physicochemical characters. By applying this procedure, we found novel PO regulatory peptides and proteins in the housefly that are strongly linked to the dramatic changes of PO activity during morphological transitions. [1] Daquinag, et al (1995) Proc. Natl. Acad. Sci. USA 92, 2964-8. [2] Daquinag, et al. (1999) Biochemistry 38, 2179-88.

P182

IMMUNOCYTOCHEMICAL MAPPING OF INSECT FMRFAMIDE- AND TACHYKININ-LIKE PEPTIDES IN THE IXODID TICK RHIPICEPHALUS APPENDICULATUS

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Antisera against insect FMRFamide and tachykinin were used to investigate distribution of immunoreactive cells in the central nervous system (CNS) and salivary glands of the tick Rhipicephalus appendiculatus using immunocytochemistry on wholemounts and sections. FMRFamide-like immunoreactive cells are widely distributed in various regions of the synganglion. Immunoreactive material was also found in the type I acinus of the salivary glands. Tachykinin-like immunoreactivity was found in two major neurons and their axons on the dorsal site of the synganglion. The imunoreactivity was also observed in the type II acinus of the salivary glands. We also determined developmental changes of these immunoreactive cells and structures during attachment and feeding of ticks. Our data suggest that these peptides may control important functions during attachment and feeding of ticks. This is the first report of tachykinin-like peptides in the synganglion and tachykinin-like and FMRFamide-like peptides in salivary glands in ixodid tick.

P183

CHARACTERISATION OF ACCESSORY GLAND AND HAEMOLYMPH PROTEINS IN ADULT MALES OF PYRRHOCORIS APTERUS (HETEROPTERA, INSECTA) AND THEIR ENDOCRINE CONTROL

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Insect accessory glands (AG) are an important part of male reproductive system and are responsible for a fluid secretion controlling various functions of insect reproduction and behaviour. The amount of the total proteins in AG of the bug Pyrrhocoris apterus (L.) increased with age of adult male. Extirpation of endocrine glands corpora allata producing juvenile hormone (JH) decreased the levels of the total proteins and PL 15.2 immunomarker in AG and induced quantitative changes in the protein pattern. Electrophoresis revealed that AG of allatectomized males are characterised by a decrease in the amount of 53, 41, 35 and 19 kDa proteins. Haemolymph of allatectomized males contained higher quantity of 77 and 21 kDa proteins, and lower quantity of 34 kDa protein when compared with controls. Application of juvenile hormone (JH) analogue enhanced the quantity of allatectomy-suppressed proteins and decreased the quantity of allatectomy-stimulated proteins. The 53, 41 and 35 kDa polypeptides detected in the wall and secretion of AG, were identified as glycoproteins with presence of glucose, mannose and/or the N-acetylglucosamine. The presence of two different male seminal fluid esterases in the spermathecae of fertilised females indicates their transfer to females during mating. The study showed that AG maturation in adult males of P. apterus and the pattern of the proteins synthesised in this tissue is regulated by JH. This study was supported by grant No. A 6007301 from the Grant Agency of the Czech Acad. Sci.

P184

PEPTIDE NANOTUBES: DESIGN, SYNTHESIS AND CHARACTERIZATION

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Peptide nanotubes have been found to be biologically relevant transmembrane ion channels. Based upon the work of Ghadiri we have modeled peptide nanotubes with different ring sizes and hydrophobic properties. Alternating L and D amino acids were used to create flat, stable ring shaped structures. These rings were then stacked one above the other to generate the nanotube. This work was done using InsightII modeling software. The behavior of the peptide nanotubes in vacuum, water and lipid medium was investigated using molecular dynamic simulations with Gromacs potential, under conditions of constant temperature and volume (NVT). Picosecond level simulations reveal that the structure is stable, both mechanically and thermally, in water as well as in lipid medium. These structures were synthesized, using Boc solid phase synthesis protocol. They were then characterized both in water and lipid environment by Atomic Force Microscopy, Fourier Transform infrared spectroscopy, H1 NMR and mass spectrometry.

P185

SYNTHESIS OF CHIRAL ORTHOGONALLY PROTECTED ALPHA-HYDRAZINOESTERS AND N-AMINODIPEPTIDES IN SOLUTION AND ON SOLID PHASE

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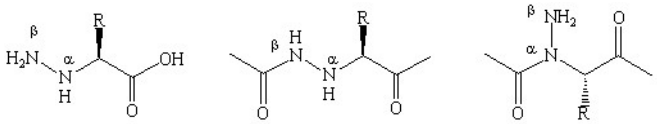
Among the large variety of pseudopeptide structures currently investigated in organic and medicinal chemistry, relative little attention has been devoted to hydrazino- and N-amino peptides because of difficulties in obtaining the chiral alpha-hydrazinoacid precursors ; morever, in the special case of the preparation of N-aminopeptides, the coupling reaction (involving the alpha-N atom of the hydrazine moiety of an alpha-hydrazinoacid) give generally poor yields. Recently, we described a new and original method allowing the preparation in liquid phase of othogonally protected alphahydrazinoacids and N-amino pseudodipeptides using chiral hydroxyesters involved in the Mitsunobu reaction. Herein, we described the first solid phase synthesis of N-alpha, N-beta bisprotected hydrazinoacid derivatives and the preparation in liquid and in solid phase of N-aminopseudopeptides. As an application, these pseudopeptidic moities have been introduced in the target molecule Ala-Ala-Pro-Val-Ala-Ala derived from a fragment of the alpha1-PI inhibitor of Human leucocyte elastase (HLE).



alpha-hydrazinoacid **hydrazino peptide** ***N*-amino peptide**

P186

NEW PEPTIDE (DUP-1) TARGETS PROSTATE CARCINOMAS IN VIVO

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Prostate cancer is one of the most frequent cancer in males and a leading cause of death from cancer worldwide. Peptides as tracers with high sensitivity and specificity for prostate tumor cells would be beneficial for diagnosis and therapy. We used a phage display peptide library consisting of 10^9 different peptide 12 aa long motifs to select a peptide specifically binding to the prostate carcinoma cell line DU-145: the peptide DUP-1 (DU-145 binding peptide) with the sequence FRPNRAQDYNTN. In vitro binding studies showed that DUP-1 binds to DU-145 and PC-3 prostate tumor cells. This binding can be competed up to 95% with unlabeled DUP-1. The peptide did not bind to primary endothelial cells HUVEC. The ID50 value on DU-145 was approx. 50 µM. The kinetics of the binding was rapid and showed a peak at 10 min. In vitro internalization studies with FITC-labeled DUP-1 demonstrated in a pulse-chase experiment that the peptide was internalized into the tumor cells in endocytotic vesicles. Preliminary stability experiments demonstrated that DUP-1 was degraded rapidly in serum. Biodistribution studies using radiolabeled DUP-1 with s.c. transplanted DU-145 and PC-3 cells in nude mice revealed the targeting potential of DUP-1. A high accumulation rate of up to 7 % of the injected does per gram tumor tissue could be observed, lower concentrations were observed in all of the organs except for the kidneys, from where the peptide is rapidly cleared.

P187

NEUROPEPTIDE Y EXPRESSION IN THE NORADRENERGIC NEURONS PROJECTING TO THE SUBMANDIBULAR GLAND IN THE SHEEP. A COMBINED RETROGRADE TRACING AND IMMUNOHISTOCHEMISTRY STUDY

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Sympathetic neurons projecting to the ovine submandibular gland (SMG) from the superior cervical ganglion (SCG) and middle cervical ganglion (MCG) were identified using retrograde tracing with fluorescent dye (Fast Blue). Antibodies to tyrosine hydroxylase (TH), dopamine beta-hydroxylase (DβH) and neuropeptide Y (NPY) were used to determine the immunochemical characteristics of SMG-innervating sympathetic neurons. Immunohistochemistry combined with the retrograde tracing revealed that the population of SMG-projecting neurons consist of four distinct sub-populations but taking into account their possible different physiological properties only three major sub-populations can be distinguished. The vast majority of neurons in both ganglia are noradrenergic in nature (co-express TH and DβH). All examined TH-immunoreactive (IR) neurons also show immunoreactivity to DβH. Sub-population of noradrenergic neurons can be divided into NPY-IR and non-NPY-IR. Noradrenergic neurons expressing NPY may act as vasoconstrictors. The second sub-population of SMG projecting neurons in the ganglia studied consists of non-noradrenergic neurons (containing NPY, but not TH). It is known that this kind of neurons may play a vasodilatory role. In both examined ganglia the third sub-population consists of non-TH-IR and non-NPY-IR neurons of unknown physiological function. Since no DβH immunoreactivity was found in any of TH– neurons these nerve cells can also be regarded as non-noradrenergic. It is possible that some of neurons of the second as well as the third sub-population are cholinergic and some of them are non-noradrenergic/non-cholinergic in character. * This work was supported by the research grant 3 P06K 019 25 from the Polish State Committee for Scientific Research.

P188

PEPTIDERGIC INNERVATION OF THE PROSTATE IN THE MALE SHEEP

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Double immunohistochemistry was used to determine the occurrence and distribution pattern of nerve fibers immunoreactive to calcitonin gene-related peptide (CGRP), substance P (SP) and galanin (GAL) in the prostate of the male sheep. Tissue samples were cut with cryostat into 15 □m sections. Slides were studied for the co-existence of SP and CGRP, or SP and GAL. Slides were observed with a laser scanning confocal microscope. Additionally, control stainings in which the primary antibodies were either omitted, or replaced by normal non-immune sera were performed. Control sections shows no immunoreactivity. CGRP- and SP-IR nerve fibers in the prostate of the male sheep were located mainly in the connective tissue in the vicinity of the glandular epithelium. It was found that the majority of SP-IR nerve fibers contained immunoreactivity to CGRP. The areolar connective tissue of the prostate gland also contained rare GAL-IR nerve fibers. The majority of them were also SP-immunoreactive. In the prostate of the male sheep the presence of CGRP was found in the majority of SP-IR nerve fibers. This may suggest the sensory function of the nerve fibers mentioned, but the influence of secreted CGRP and SP on the functions of the prostate cannot be excluded. The role of GAL-IR nerve fibers in the regulation of functions of the prostate is unclear. It is known that GAL acts as an anti-nociceptive factor in the nervous system which may explain the presence of SP in the majority of GAL-IR nerve fibers in the prostate of the male sheep.

P189

RETRO-INVERSO PEPTIDES ARE HIGHLY POTENT NEUROPROTECTIVE AGENTS AGAINST THE NEUROTOXICITY OF BETA-AMYLOID IN ALZHEIMER'S DISEASE

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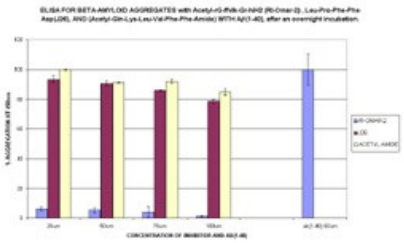
There is a great deal of evidence that aggregation of the main causative agents in Alzheimer's Disease, the 40-42-residue peptides beta-amyloid damage neuronal cells, leading to cognitive decline as the disease progresses. Possible therapeutic drugs are agents that prevent b-amyloid aggregation, and ensuing neurotoxicity. We have found that peptides comprising the reverse of the focal sequence 16-21 in beta-amyloid, in the D-configuration, framed by arginine residues, are more potent than peptides of the native sequence 16-21 (L-configuration) in the inhibition of aggregation of both beta-amyloid 1-40 and 1-42, as measured by an ELISA assay which detects the aggregation of epitopes. Moreover, the retro-inverso peptides prevent cell death of SH-SY5Y human neuroblastoma cells in culture, by blocking apoptosis initiated by aggregated beta-amyloid.



P190

OXYTOCIN IN MODELS OF PROLONGED PHYSICAL ACTIVITY IN RATS

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The neuropeptide oxytocin belongs to the factors activated by physical and mental stress stimuli. In addition, some reports suggest possible role of oxytocin in the control of cardiovascular functions. This study was designed to verify whether oxytocin release would be changed in two models of cardiovascular activation induced by increased physical activity. Adult Sprague Dawley rats were exposed to either voluntary or forced running for three weeks. Voluntary running rats were housed in cages with a free access to a running wheel attached. Artificially moving wheels with regulated speed were used for forced running with gradually increasing time periods from 15 – 60 min daily. Oxytocin levels in plasma and posterior pituitary were analyzed by radioimmunoassay. No changes in plasma oxytocin were observed after both intensive voluntary running and the forced exercise, however, the content of oxytocin in the posterior pituitary decreased in response to voluntary running. No parallel changes in vasopressin concentrations were found. Plasma rennin activity tended to increase in voluntary running rats. Both forms of physical exercise used failed to induce a significant cardiac hypertrophy. The percentage of epididymal fat significantly decreased in voluntary but not in forced running groups, which corresponds to higher total work load during voluntary running. Oxytocin levels did not show a significant correlation with changes in body fat. Thus, decreased levels of oxytocin in the posterior pituitary in rats induced by intensive voluntary running do not seem to be directly related to the physical activity performed. Supported by SP 51/0280800/0280802.

P191

MODULATION OF THE CONFORMATIONAL BEHEVIOR OF A–β–(25-35) BY INTERACTION WITH NATURAL COMPOUNDS

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The major components of neuritic plaques found in Alzheimer disease (AD) are peptides known as amyloid β-peptides (A-β-peptides). The A-β-(1-42) is the most prone to aggregation and is produced in larger quantities. A-β-(25-35), sequence GSNKGAIIGLM, is a synthetic derivative of amyloid β-peptide, that is highly toxic and forms fibrillar aggregates typical of β-amyloid. Like the A-β-(1-42), A-β-(25-35) undergoes a conformational transition from a soluble, alpha-helical form to aggregated fibrillary β-sheet structures which are neurotoxic (1). Since it retains both the physical and biological properties of A-β-peptides it can be used as a suitable model of full-length peptides, for testing inhibitors of aggregation and toxicity. The design of inhibitors of aggregation is one of the strategies to overcome the Alzheimer disease. It is well known that several substances are able to interact with A-β-peptides affecting the α-helical/β-sheet transition rate. Many oligopeptides have beta-breaking activity since they are able to disaggregate the beta fibrils. Furthermore several natural compounds are shown to be endowed with protective activity against the amyloid toxicity. Nicotine is one compound that was reported to inhibit the conversion of several synthetic fragments of A-β-peptide from its soluble into insoluble form (2). Presently we investigated by Circular Dichroism and NMR spectroscopy the effects on the conformations of A-β-(25-35) due to the interactions with several different natural compounds known to interfere with amyloid neurotoxicity. References 1.Lorenzo, A. and Yankner, B.A. (1994) Proc. Natl.Acad.Sci. U.S.A. 91, 12243. 2. Moore S.A. et al , Biochemistry (2004) 43, 819-826

P192

AGGREGATION STUDIES OF ß-AMYLOID (1-42) IN INTERACTION WITH MODIFIED OF ß-AMYLOID PEPTIDE FRAGMENTS

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One of the most common causes of senile dementia is Alzheimer's disease (AD). Formation of amyloid plaques and cerebrovascular deposits is pathological characterization of this disease. β-amyloid peptides (Aβ) derived from the amyloid precursor protein (APP) are the major components of the deposits core. Amyloid peptides have 39 to 43 amino acids residues, however the form 1-42 occurs predominantly in amyloid plaques. It has been recognized that changes of conformation and aggregation of other types of proteins could cause other neurodegenerative diseases. During the studies of Aβ derivatives it was shown that sequence of Aβ 25-35 is the shortest fragment that exhibits large β-sheet fibrils and retains the toxicity of full length peptide. Our previous research showed that the aggregation of the whole β-amyloid peptide is significantly reduced in the presence of shorter fragments of β-amyloid peptide (including Aβ 25-35 fragment) and prion protein (PrP). This communication presents aggregation studies using HPLC of the whole β-amyloid peptide in the presence of short, modified β-amyloid peptide fragments corresponding to β-amyloid 25-35, 26-35, 27-35, 28-35 and doubled 31-35 fragments.

P193

ELECTRON MICROSCOPIC STUDIES ON BETA-AMYLOID AGGREGATION

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The fibril formation of Aß[1-40] and Aß[1-42] peptides in the brain has a key role in the pathomechanism of Alzheimer`s disease. Numerous studies have proven that the aggregation of Aß peptides can influence their biological effect. Based on the results of in vitro and in vivo experiments, monomeric Aß peptides considered to be less toxic, than the oligomers or highly aggregated Aß fibrils. Several methods, like quasielastic light scattering (QLS), small-angle X-ray scattering (SAXS) or adsorption of Congo-red or Thioflavine-T on the aggregates, have been used for characterising the aggregational grade of Aß peptides. In addition, transmission electron microscopy (TEM) has been successfully used for the visualisation of the fibrils. We have used this method to examine Aß-aggregation and the possible disaggregating effect of short peptides. According to our results, synthetic Aß[1-42] shows a rapid aggregation in aqueous solution. We found, that after the post-synthetic isolation of this peptide, the water-insoluble fraction contains large Aß-clusters with a diameter of 1-2 µm. An Aß-fragment analogue, LPYFD amide, which expressed a definite neuroprotective effect against Aß[1-42] both in vitro and in vivo, was also examined in TEM experiments, where 5 equivalents of this peptide were not able to prevent the fibril-formation of Aß[1-42]. These results prove that LPYFD amide and similar molecules can act not only as beta sheet breakers (BSBs), but they are also able to influence the effect of Aß[1-42], by covering the surface of the fibrils, and by preventing the interaction of the aggregates with cell membrane proteins.

P194

STUDIES ON STRUCTURE-ACTIVITY RELATIONSHIPS OF THE NEUROPROTECTIVE TRIPEPTIDE GLY-PRO-GLU (GPE)

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[1]*Instituto de Química Médica (CSIC)* [2]*Centro de Química Orgánica Manuel-Lora Tamayo (CSIC)* [3]*Departamento de Farmacología, Universidad de Navarra, Madrid, Spain*

There is evidence that Gly-Pro-Glu (GPE), the N-terminal sequence of insulin-like growth factor (IGF-1), shows neuroprotective properties, both in vitro and in different animal models of neurodegenerative diseases [1,2]. Although the mode of action of this tripeptide remains unknown, it has been reported that GPE inhibits glutamate binding to the N-methyl-D-aspartate (NMDA) receptors [1,2]. Our current interest in exploring novel strategies for the development of effective neuroprotective drugs, directed our attention to the search of GPE mimics, which could be used as pharmacological tools for gaining insight into the mode of action of this tripeptide. To this aim, a series of modified analogues of GPE has been prepared, as first step to determine the essential structural requirements for biological activity. These modifications involve: a) Replacement of L-Pro and L-Glu with D-amino acids; b) Variation of the length of the Glu side chain; c) Derivatization at N-and/or C-terminus, and d) Incorporation of conformational restrictions. The effect of these structural modifications on the in vitro biological activity of GPE will be presented. [1] V. R. Sara, C. Carlsson-Skwirut, T. Bergman, H. Jörnvall, P. J. Roberts, M. Crawford, L. Nilsson Hakansson, I. Civalero, A. Norberg, Biochem. Biophys. Res. Commun., 1989, 165, 766-771. [2] S. V. Sizonenko, E. S. Sirimanne, C. E. Williams and P. D. Gluckman, Brain Res. 2001, 922, 42-50 367-368 and references therein.

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CHIMERIC STRATEGIES FOR THE DELIVERY OF CYTOTOXIC PEPTIDES TO ASTROCYTOMA

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The cell-type specific delivery of cytotoxic agents can be achieved using a peptidyl address motif that binds protein targets selectively expressed at the cell surface. We are currently using this strategy to develop chimeric constructs that target tumour cells including astrocytoma and medulloblastoma. As examples, we have synthesized a range of chimeric peptides consisting of cytotoxic moieties (peptide and non-peptide) linked to the C-terminal heptapeptide of gastrin (G7; H-AYGWMDF-NH_2), a sequence that binds a novel receptor on U373 MG astrocytic tumour cells [1]. Therapeutic advantages of this non CCK1/CCK2 receptor binding site include the inhibition of malignant astrocytoma cell motility without the growth promoting effects of gastrin [1]. To compare the cytotoxicity of peptide chimeras, we determined changes in cellular viability using MTT conversion assays. Our data indicate that chimeric peptides rapidly (<8hours) reduced the viability of U373 MG cells and this action was both time- and concentration-dependent. Moreover, as a chimeric amino-terminal extension, the G7 address motif modified and enhanced the cytotoxicity of both mastoparan (H-INLKALAALAKKIL-NH_2) and D(KLAKLAK)2, peptides that are known to stimulate necrosis and/or apoptosis of eukarytoic cells. In conclusion, hybrid G7 chimeras display pharmacodynamic properties that differ from their composite components and may be useful probes to study and manipulate other aspects of astrocytoma biology. [1] Pannequin,J. et al (2002) J.Pharmacol.Exp.Ther. 302:274-282.

P196

CONFORMATIONAL STUDIES (FT-IR, CD, NMR) OF 11-28 FRAGMENT OF ß-AMYLOID PEPTIDE AND ITS E22K MUTANT

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Cerebral amyloid angiopathy (CAA) due to β-amyloid (Aβ) formation is one of the key pathological features of the Alzheimer disease (AD). In hereditary AD CAA is linked to mutations at positions 21-23 within the Aβ sequence. To gain more insight into conformational changes and aggregation of the Aβ peptide and its E22K mutant, we have studied their behaviour by means of CD, FT-IR and NMR techniques. As working with full length Aβ peptides is difficult due to their aggregative properties in our studies we have used the 11-28 fragment of both peptides, which is reported to have amyloidic properties. By analysing CD spectra we have followed conformational changes of both peptides in time at different pH and in the presence of sub-micellar and micellar concentration of SDS. We have also studied the influence of cyclodextrin on the peptide conformational changes. To study solvent effects on aggregation process of Aβ peptides, we used FT-IR spectroscopy. The peptides were studied in hexafluoroisopropanol and D2O mixtures. To verify the obtained results, CD spectra were registered in the same conditions and analysed. The secondary structure of the Aβ mutant in solution (with SDS) was also estimated on the basis of NMR. The CD and IR experiments revealed that the peptides studied have a tendency to adopt helical structure in hydrophobic environment. The NMR data indicate a helical structure of the Aβ mutant in the K16-S26 fragment of the peptide studied. The work was supported by the UG grant (DS/8350-4-0131-4).

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DES-LEU-PAGA ANALOGUES - SYNTHESIS AND EFFECT ON THE EXPERIMENTALLY INDUCED IMPAIRMENT OF SPATIAL MEMORY IN MULTIPLE T-MAZE TEST IN RATS

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Humanin, 24 amino acid peptide, first published 4 years ago, and its analogues have been shown to protect neuronal cells against death induced by various Alzheimer's disease genes and amyloid-β-peptides in vitro; recently the structures of the active core corresponding to the sequence Pro3-Pro19 and of hyperactive analogues were published (e.g. [Ala4,Ala6,D-Ser14]humanin3-19, PAGA), which show an activity in vitro in 10000fold lower concentrations than humanin. An analogue [Gly14]humanin has also been shown to be potent in vivo. It was able to reverse learning and memory impairment induced by scopolamine in mice. We have also reported positive in vivo effects of [Gly14]humanin and des-Leu-PAGA, another analogue of humanin, using different behavioral method, i.e. multiple T-maze test in rats. Here we report the synthesis of several analogues of des-Leu-PAGA having replaced Leucine in positions 9 or 10 or ll by Tertleucine and effect of the analogues on the 3-quinuclidinyl benzilate-induced (QNB, 2 mg.kg-1, i.p) impairment of spatial memory in multiple T-maze test in rats. All substances were administered intraperitoneally. Cognitive functions were tested before drug application and then 15 minutes, 24 hours and 7 days following the QNB injection. Controls were tested at the same time intervals after physiological saline or peptide administration alone. Number of entries into wrong arms, and passage times through the maze were recorded. Statistical analysis was performed with Statistica software ´98 Edition. 'The work was supported by the Grant Agency of Czech Republic No. 305/03/1100'

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SEPARATION OF HUMANIN PEPTIDE DERIVATIVES BY CAPILLARY ELECTROPHORESIS USING ION PAIR INTERACTIONS AND SUPRAMOLECULAR COMPLEX FORMATION

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Humanin (HN), Met-Ala-Pro-Arg-Gly-Phe-Ser-Cys-Leu-Leu-Leu-Leu-Thr-Ser-Glu-IIe-Asp-Leu-Pro-Val-Lys-Arg-Arg-Ala, natural peptide recently discovered in human brain, is important neuroprotective peptide which could effectively suppress neuronal cell death caused by a wide spectrum of familial Alzheimer's disease (AD). Several derivatives of HN show much higher biological activity, for example [G-14]-HN. In this work, capillary electrophoretic behavior of [G-14]-HN and several derivatives was studied. Various background electrolytes and different additives were examined using fused silica or coated capillaries. Ion association between positively charged peptides and several anions was studied and the interaction e.g. with phosphate was established. The most efficient separation was achieved in slightly acid solutions by forming ion associates or supramolecular complexes.

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PREDICTION OF PROTONATION CONSTANTS OF HUMANIN-LIKE PEPTIDES

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Capillary electrophoretic separation is very powerful technique for the analysis of peptides. Good separation results however depend on many factors including the kind of background electrolyte, its composition, concentration and pH. Choosing the suitable pH value is the key factor. However, pKa values for peptides are not easy to determine experimentally, especially when low amounts of analytes are available. In this work, we applied theoretical prediction to estimate protonation constants of a neuroprotective peptide humanin fragments and analogues in order to elucidate how the charge of peptides is changed with pH. The results of modeling using pKa-computing program ACD/pKa DB are compared with the experimental values. The distribution diagrams of various acid-base forms of peptides as a function of pH, as well as changes of theirs effective charges with pH are calculated. From these diagrams conclusions concerning electrophoretic behavior of the peptides are drawn and discussed. Knowledge of protonation constants and of the diagrams is important to optimize capillary electrophoretic separation of peptides. Examples of peptide separations are demonstrated. Acknowledgment This work was supported by the Grant Agency of the Czech Republic, grant Nr. 305/03/1100.

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DELTA SLEEP INDUCING PEPTIDE (DSIP) AS STRESS-PROTECTIVE FACTOR

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DSIP is an endogenous neuropeptide with reporting sleep-promoting and extra sleep actions. A wide range of evidences confirmed beneficial effects of this peptide under experimental stress models. Based upon these findings DSIP-related drug Deltaran® was developed and registered in 1998 in Russia. However, specific underlying mechanisms of DSIP action continue to present many open questions. Recently we investigated the influence of DSIP and Deltaran on oxidative phosphorylation and ATP production in rat brain mitochondria. DSIP or Deltaran application led to the significant enhancement of coupling between the oxidation and phosphorylation and prevented the reduction of respiratory activity under hypoxia. These effects are probably attributed to DSIP antioxidant and membrane-tropic action. The influence of DSIP on the intensity of lipid peroxidation prompted us to investigate the interaction of DSIP with cellular membranes by means of spin probes. Previously we have detected DSIP–induced significant alterations in dynamic structure of human blood cells membranes. In order to evaluate a putative correlation between biological activity and membrane-tropic properties of the neuropeptide we have compared the action of DSIP together with some biologically active and inactive analogues on dynamic structure of erythrocyte membranes. Preliminary data obtained are consistent with the suggestion that membrane-tropic effects of DSIP might have a direct relation to activity of this regulatory peptide in vivo.

P201

HPLC SEPARATION OF NEUROPROTECTIVE PEPTIDES. GENERAL APPROACH TOWARDS OPTIMIZATION: EXPERIMENTAL DESIGN COMBINED WITH ARTIFICIAL NEURAL NETWORK

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Humanin peptide and its derivatives were found to defend the neural cells against pathological proteins which cause Alzheimer´s disease. Although HPLC is frequently applied in peptide separation, large variability in peptide properties does not allow to use always the same separation conditions. Even slight changes in mobile phase composition may lead to dramatical changes in separation patterns. Optimization is needed and a general optimization procedure is required. Separation and retention behaviour of synthetic humanin derivatives and similar peptides by HPLC was studied. Since these peptides have similar structure and closed physico-chemical properties, the influence of mobile phase composition (TFA concentration, pH and acetonitrile content), as well as column temperature was carefully investigated. With respect to a high number of factors influencing the separation, it might be difficult to reach optimal conditions. Recently, we have developed in our laboratory general optimization approach based on the combination of Experimental Design (ED) and Artificial Neural Networks (ANN). This ED-ANN is based on sophisticated design of experiments and then the optimal conditions are predicted with an artificial intelligence method. This ED-ANN approach was applied to optimization of neuroprotective peptides separation. After the optimal conditions were found, fractions of peptides were collected and the purity of individual fractions was checked using off-line Matrix Assisted Laser Desorption/Ionization Time Of Flight Mass Spectrometry (MALDI-TOF MS). Concluding, ED-ANN approach was proved to be highly effective and can be used to optimize any separation method. Keywords: humanin derivatives; HPLC; experimental design; neural network

P202

THE AMINOTERMINAL SUBSTANCE P (SP) METABOLITE SP(1-7) AND THE MECHANISM FOR ITS MODULATATORY EFFECTS ON THE SP RESPONSE

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In addition to proteolytic processing and degradation, several neuropeptides are shown to undergo enzymatic conversion to fragments with retained or modified biological activity. The released fragment can act on and stimulate receptor sites not recognized by the parent peptide. One example is substance P (SP), which is converted to the bioactive metabolite SP(1-7), which mimicks some but opposes other effects of the parent peptide. E.g., SP receptor (NK-1) agonists are shown to increase opioid tolerance and withdrawal, effects which are counteracted by SP(1-7). We here report recent studies on the mechanism by which SP(1-7) exerts these effects. Using a rat model the effects of the heptapeptide on dopamine (DA) release and DA receptor density in mesolimbic pathways were examined using microdialysis and autoradiography techniques, respectively. We also report the identification of specific binding sites for the heptapeptide in the brain as well as spinal cord. It was found that SP(1-7) induces a release of DA during withdrawal in morphine dependent rats. It also affected the density of the DA receptor D1 and D2. An inverse correlation between DA levels and D1 receptor density was found in nucleus accumbens. Also, specific binding sites for SP(1-7) in VTA, as well as the spinal cord were identified. These sites exhibited only weak binding for NK-1 and NK-3 agonists. Thus, it appears that the modulatory effects seen for the SP fragment are mediated through activation of unique sites for the heptapeptide.

P203

RECOGNITION OF THE AMYLOID PEPTIDE F4-Y10 EPITOPE IS ZINC-DEPENDENT

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The amyloid peptide A-beta is the main component of senile plaques deposited within the cerebral tissue in Alzheimer's disease (AD). Zinc binding to A-beta has been described as either critical for its pathogenic aggregation or protective against A-beta toxicity. A high content of Zn2+ has been detected within amyloid plaques. We have previously shown that the A-beta N-terminal region constitutes an autonomous zinc binding domain and proposed R5, H6, H13 and H14 as the zinc binding residues. Furthermore, this domain contains the F4-Y10 epitope recognized by mouse antibodies able to dissociate amyloid fibrils. In order to assess the influence of zinc binding to A-beta on the epitope recognition we here investigated by ELISA experiments and mass spectrometry the recognition of Biotin-G5-1-16 and Biotin-G5-1-10, two peptides sharing the A-beta N-terminal domain sequence, by monoclonal antibodies recognizing the F4-Y10 epitope, in absence and in presence of Zn2+ or other cations (Cu2+, Co2+, Ni2+). Zn2+ ions increased the recognition of Biotin-G5-1-16, while they had no effect on Biotin-G5-1-10 recognition. Other metal cations did not influence the epitope recognition for both peptides. These observations illustrate a zinc binding-induced conformational change of the A-beta N-terminal region, which results in a better accessibility of the F4-Y10 epitope. The high content of Zn2+ detected within amyloid plaques here appears not to hinder, but rather to favor their interaction with anti-A-beta antibodies raised against A-beta N-terminal region, thus opening the door for AD therapeutic approach by active or passive immunization.

P204

CARBOXY-TERMINAL FRAGMENTS OF AMYLOID PRECURSOR PROTEIN MODULATE CELLULAR SIGNAL TRANSDUCTION IN ALZHEIMER'S DISEASE BRAIN

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Amyloid precursor protein (APP) represents an ubiquitous transmembrane glycoprotein with a large N-terminal ectodomain, a single membrane-spanning domain, and a short cytoplasmatic carboxy-terminus (CT). APP can function both as a membrane-anchored receptor-like molecule and as a secreted derivative that acts upon other cells. The cytoplasmatic CT of APP is suggested to regulate APP metabolism and functions in normal and Alzheimer's disease (AD) brain. The influence of three C-terminal sequences and of transmembrane domain from amyloid precursor protein on the activity of G-proteins and of the coupled cAMP-signalling system in the postmortem Alzheimer's disease and age-matched control brains was compared. 10 microM APP(639-648)-APP(657-676) (PEP1) causes a 5-fold stimulation in the [35S]-GTPgammaS-binding to control hippocampal G-proteins. APP(657-676) (PEP2) and APP(639-648) (PEP4) showed less pronounced stimulation whereas cytosolic APP(649-669) (PEP3) showed no regulatory activity in the [35S]-GTPgammaS-binding. PEP1 also showed 1.4-fold stimulatory effect of on the high-affinity GTPase and adenylate cyclase activity in control membranes, whereas in AD hippocampal membranes the stimulatory effect of PEP1 was substantially weaker. The PEP1 stimulation of the [35S]-GTPgammaS-binding to the control membranes was significantly reduced by 1.5 mM glutathione, 0.5 mM antioxidant N-acetylcysteine and, in the greatest extent, by 0.01 mM of desferrioxamine. In AD hippocampus these antioxidants revealed no remarkable reducing effect on PEP1-induced stimulation. Our results suggest that C-terminal and transmembrane APP sequences possess receptor-like G-protein activating function in human hippocampus and that abnormalities of this function contribute to AD progression.

P205

CAPILLARY ZONE ELECTROPHORESIS OF PROTEINS AND NEUROPROTECTIVE PEPTIDES

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Biocompatibility of inner surface of capillaries in CE is critical for separation and detection of proteins and peptides. The problems come from adsorption of the peptides (proteins) on the surface because biomolecules are sensitive to changes in ionic strength, pH, and/or temperature of the electrolyte, etc. Therefore, modification of the fused silica capillary surface is often necessary. Procedures for capillary coating described in literature are usually complicated and time consuming. We optimized three types of coating procedures suitable for protein and peptides separation: a/ polyethyleneimine (PEI) b/ glycidoxy propyl trimethoxy silane (GPTMS) c/ amino propyl silane (APS) Composition, concentration and pH of several background electrolyte were tested, namely citrate, phosphate and epsilon-caproate. The capillary surface was always characterized by determination of the electroosmotic flow. The conditions were optimized by running a standard mixture of proteins and then applied to [G14]-humanine and its derivatives.

P206

CHOLINESTERASE INHIBITORY ACTIVITIES OF THE SCORPION MESOBUTHUS GIBBOSUS VENOM PEPTIDES

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In the present study, crude venom of the scorpion Mesobuthus gibbosus was isolated and purified by the Sephadex G-50 gel filtration and HPLC separation. Two of the five fractions, obtained from the Sephadex G-50 filtration and detected as lethal on mice and Musca domestica larvae in in vivo toxicity tests were independently subjected to the HPLC separation. Only one of 7 fractions obtained from the HPLC separation of the fraction 5 was found to be extremely letal. SDS-PAGE analysis of the crude venom and its chromatographic fractions demonstrated that crude venom was consisted of peptides with molecular weights of 6500-210 000 Da while its neurotoxic fraction appeared as a single band of 28 000 Da. Two bands of 6200 and 22 000 Da in SDS-PAGE, respectively, suggesting that it might be consisted of two chains attached by a disulfide bridge. Crude venom and its neurotoxic fractions significantly and specifically inhibited acetylcholinesterase (AChE) in human erythrocytes with the appearent Ki values of 0.90±0.022 , 0.86±0.017 and 0.78±0.018 mg venom protein/ ml, respectively, in a reversible and non-competitive manner. None of the fractions inhibited the butyrylcholinesterase (BChE) of human plasma and erythrocytes. Kinetic data indicated that cholinesterase inhibitory peptides of Mesobuthus gibbosus venom might be interacted with the enzyme at an alternative binding region possibly close to the peripheral site in the catalytic gorge of the enzyme molecule.

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INTERACTION OF AGGREGATED AMYLOID BETA PEPTIDE WITH TRITON-SOLUBLE PROTEINS

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The extracellular accumulation of amyloid-beta (Aß) in neuritic plaques is one of the characteristic hallmarks of Alzheimer's disease. Aß is a 37-43 -amino acids peptide. By virtue of its structure, Aß is able to bind to a variety of biomolecules, including lipids, proteins and proteoglycans. Conversion of Aβ to fibrillar Aβ increases his binding to lipids and proteins. The binding of the various forms of Aß (soluble or fibrillar) to plasma membranes has been studied as regard the direct toxicity of Aß on neurons, and the activation of a local inflammation phase involving microglia. The aim of the present study is to identify the membrane proteins involved in the Aß peptide-cell membrane interaction. Our approach consists of (i) extracting membrane proteins by detergents from rat synaptosomes; (ii) selecting fAβ binding proteins by co-precipitation of the detergent-soluble proteins with fAβ ; (iii) analyzing by 1D, 2D-BAC or 2D-IEF electrophoresis the pattern of the co-precipitated proteins; (iv) identifying the purified proteins by mass spectrometry. Our first results have shown that it is possible to analyze a large number of proteins co-precipitated with fAβ by 1D and 2D electrophoresis. Different mitochondrial, cytoplasmic or plasmatic proteins have been proved to be co-precipitated with fAß. In order to focus on cell membrane proteins, we are performing a better membrane preparation. This work should allows us to identify membrane proteins which interacts with Aß peptide for a better understanding of Alzheimer's disease and development of news therapies.

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NEUROPROTECTIVE EFFECTS OF SMALL PEPTIDES IN ALZHEIMER'S DISEASE

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ß-Amyloid peptides (Aß) play central role in the etiopathology of Alzheimer's disease (AD). Aß[1-40] and Aß[1-42] peptides show neurotoxic and synaptotoxic effects if aggregated. These peptides are hydrolized in the brain by proteases like insulin degrading enzyme and neprilysin. During this metabolic process, short peptide fragments will be formed. We have synthesized a series of short Aß fragments and their analogues and studied their in vitro and in vivo effects. MTT test, measurements of Ca2+- influx and changes of membrane potential were used in differentiated neuroblastoma cell culture (SH-SY5Y). In vivo electrophysiology using multibarrel electrodes, and behavioral experiments (Morris water-maze, T-maze and radial maze) in rats have been applied for studying the CNS effects of the short Aß fragments and their analogues. We have found that four types of short Aß-fragments possess neuroprotective activity: Aß[5-8], Aß[17-21], Aß[31-35], and Aß[38-42]. These fragments and several analogues protect the neurons from the toxic effect of Aß[1-42] both in vitro and in vivo. Three different mechanisms might be responsible for the neuroprotective effect: competitive inhibition on membrane proteins, ß-sheet breaking (BSB) activity, and prevention of Aß-membrane protein interaction by covering the active surface of Aß aggregates with short peptides. The latter compounds (Amyloid Surface Covering Molecules, ASCOM) can not disintegrate Aß clusters, howerer, they show full protection against Aß[1-42], both in vitro and in vivo experiments. One of the best compounds (LPYFD-amide) might serve as a lead for design putativ drugs for AD-treatment.

P209

HEXAPEPTIDES TGENHR AND TQVEHR INDUCE LEUKEMIA CELL DIFFERENTIATION AND EXHIBIT NEUROPROTECTIVE PROPERTIES

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Homological regions were discovered in a primary structure of cell differentiation factors HLDF (Human Leukemia Differentiation Factor) and PEDF (Pigment Epithelium-Derived Factor). The ability of these factors to induce differentiation of human promyelocytic leukemia cells (line HL-60) is determined by six-residued fragments within their homological regions. The hexapeptides TGENHR and TQVEHR corresponding to those fragments of HLDF and PEDF, respectively, induce differentiation of HL-60 cells as well as factors. To identify signal transduction pathways activated by these peptides, we studied their effects on activity of the major second messenger biosynthesis enzymes. Adenylate cyclase activity was inhibited by TGENHR but unaffected by TQVEHR; this was observed both in intact cells and isolated cell membranes. Neither TGENHR nor TQVEHR affected phosphatidylinositol-specific phospholipase C activity, but TQVEHR prevented activation of this enzyme by aluminum tetrafluoride anions which are known to stimulate Gq proteins. Besides that, TGENHR protects HL-60 cells from apoptotic death upon hypoxia-like state caused by sodium azide, while TQVEHR does not show such effect. At the same time, the both peptides exhibit neuroprotective properties in vivo on Purkinje cells of rat vermis cerebelli preventing their degeneration upon hypoxia-like state caused by sodium azide application. Neuroprotective effect was also demonstrated on a behavioral level. The formation of conditional fear did not appear if hypoxia-like state of vermis cerebelli was induced. Both peptides are able to recover this behavioral reaction. The habituation of animals to intensive sound stimuli in such conditions was recovered by TGENHR but not TQVEHR.

P210

ANALYSES OF ENKEPHALIN AND DALARGIN ANALOGUES AND FRAGMENTS BY CAPILLARY ZONE ELECTROPHORESIS

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Dalargin, hexapeptide H-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH, is a synthetic analogue of natural opioid hormone dynorphin. Dalargin was tested both in human and in veterinary medicine, e.g. when fish were treated for a short time with a solution of dalargin (cca 1µg/ml), they endured stress from cooling, oxygen insufficiency and fluctuation of pH; consequently, they grew better. In order to obtain peptides with higher potency and metabolic stability, a number of dalargin analogues were prepared by solid phase synthesis. The peptide structure was altered by the methylation of the peptide bond nitrogen, substitution of leucine in position 5 by bulky amino acids such as tertiary leucine or neopentylglycine, modification of the C-terminal arginine carboxyl group and by combining these changes. Dalargin fragments corresponding to products of enzymatic cleavage were also obtained. Purity of synthetic peptides was checked by capillary zone electrophoresis (CZE); peptides were analyzed as cations in acidic background electrolytes (pH 2.25; 2.5) and/or as anions in weakly alkaline background electrolyte (pH 8.1). Analyses were carried out in a home-made apparatus equipped with fused silica capillary (I.D. 50µm, effective/total length 19/30 cm) and UV-photometric detector at 206 nm. Picomole to femtomole amounts of peptides in nanoliter sample volume were sufficient for their qualitative and quantitative analyses and for development of appropriate separation conditions of free-flow electrophoresis, which was used for preparation of peptides in high purity degree necessary for their biological tests. Supported by grant no. S405 5006 of GA ASCR, grant no. 203/02/1469 of GACR and Research Project AV0Z4055905 of ASCR.

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THE MEMBRANE AFFINITY OF ANTIMICROBIAL BETA-PEPTIDES

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Antimicrobial peptide action is mediated by a direct interaction with cell membranes. A common feature of these interactions is the induction of cationic amphipathic secondary structure following binding of the peptides to the membrane surface. Since selective binding to different phospholipids is central to the design of non-hemolytic antimicrobial peptides, the affinity of the peptide for the membrane surface is a critical factor in the cell-lytic process. We have developed a sensitive method based on surface plasmon resonance (SPR), which allows the real-time measurement of peptide binding to phospholipid membranes [1]. We have used this SPR method to measure the membrane affinity of β-17, a novel β-amino acid based antimicrobial peptide and the scrambled β-17 - a non-antimicrobial β-peptide analogue [2] and compared these properties with the membrane binding of the well-characterised antimicrobial peptides magainin and melittin. Interaction studies were performed with a series of phospholipid mixtures which mimic either mammalian cells (high in phosphatidylcholine and cholesterol) or microbial cells (high in phosphatidylethanolame or phosphatidylglycerol). Results revealed significant differences in the membrane affinity of the β-peptides compared to melittin and magainin and the present study thus has implications in the design of new specific antimicrobial peptides. 1. H Mozsolits et al, Biochim Biophys Acta, 1512 (2001) 64-76. 2. E A Porter, X Wang, HS Lee, B Weisblum & S H Gellman, Nature 404 (2000) 565.

P212

LPS- AND LTA-NEUTRALIZING ACTIVITIES OF ALPHA-HELICAL CATHELICIDIN PEPTIDES

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This study was designed to investigate the anti-endotoxin and anti-lipoteichoic acid (LTA) activities and therapeutic efficacy of alpha-helical cathelicidin-derived antimicrobial peptides. Binding of these peptides to purified lipopolysaccharide (LPS) was measured using the Limulus chromogenic assay. The ability to inhibit the production of nitric oxide (NO) and the release of TNF-alpha by LPS- or LTA-stimulated murine macrophages RAW 264.7 was determined using the Griess reagent and ELISA, respectively. All the peptides under study (BMAP-27 and 28, SMAP-29) were found to bind LPS in the low micromolar range and to completely inhibit the LPS procoaugulant activity at approximately 10 microM peptide concentration. The production of NO by RAW cells stimulated with 100 ng/ml LPS for 24 hours was fully inhibited at 2-4 microM peptide concentration. This effect was dose- and time-dependent. The addition of peptides up to 60 minutes after cell incubation with LPS still resulted in significant reduction in NO production. All peptides also decreased the NO production elicited by 1 microg/ml LTA from S. aureus in vitro, and protected from lethality in rat models of septic shock. These results suggest the capability of these peptides to neutralize the proinflammatory effects of LPS and LTA and point to their potential use for the treatment of septic shock.

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IDENTIFICATION AND CHARACTERIZATION OF NEW ANTIMICROBIAL COMPOUNDS FROM FLESHFLY NEOBELLIERIA BULLATA

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World Health Organization stated that resistance to antimicrobial agents is a serious public health problem. Levels of resistance have been increasing at an alarming rate (resistance towards tetracyclines has increased from 0% in 1948 to a 98% in 1998). Today 1500 people die each hour from an infectious disease, half of these children under five years of age. These findings have stimulated a sustained search for new potent antimicrobial agent against drug resistant strains. Insects are the largest and the most diverse group of living animals on earth. They have potentially been confronting all microorganisms. As a result, they have evolved powerful defense system, thus representing vast source of novel potential therapeutics. We chose a fleshfly Neobellieria bullata for identification and characterization of new promising molecules, peptides or proteins, which participate in immunity response against microbial infections. The hemolymph of the third-instar larvae of Neobellieria bullata was used for isolation. The larvae were injected with bacterial suspension of E. coli or Staph. aureus and the hemolymph was collected 24 hours later. The hemolymph was separated into crude fractions, which were subdivided by chromatography. RP-HPLC isolated fractions were characterized by UV-VIS spectroscopy, amino-acid analysis, mass spectroscopy (MALDI), SDS-PAGE, tryptic digests and by N-terminal sequencing. We found out significant antimicrobial activities against E.coli and Staph. aureus in a few isolated peptides and proteins.

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CANDIDACIDAL EFFECTS OF LL-37 AND HISTATIN 5

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In this study, we compared the candidacidal mechanism of two antimicrobial peptides present in human saliva, histatin 5 and the cathelicidin LL-37. We showed that both LL-37 and histatin 5 kill the oral yeast Candida albicans at comparable concentrations (approx. 1 μM). Using confocal microscopy we found that LL-37 associated with the cell membrane of the yeast, whereas histatin 5 translocated over the cell membrane and targeted to the mitochondria. Furthermore, LL-37 caused severe damage to the cell membrane, as demonstrated by freeze fracture EM, and caused leakage of ATP. The membrane disturbing activity of LL-37 was confirmed using liposomes as a membrane model system. It was found that LL-37 induced leakage of trypsin encapsulated in liposomes. On the other hand, histatin 5 caused only little damage to C. albicans membranes, and no trypsin leakage from liposomes was seen. Apparently, LL-37 has a high affinity for membranes, which is in line with the cationic and amphipathic characteristics of this peptide. Upon binding to the cell membrane, LL-37 causes gross disturbance resulting in loss of cellular compounds. Histatin 5, which is only weakly amphipathic, transmigrates over the cell membrane, apparently without causing permanent membrane damage.

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INTERACTION OF ACYLATED ANTIMICROBIAL PEPTIDES WITH ARTIFICIAL MEMBRANE SYSTEMS

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Interest in biophysical studies on the interaction of antimicrobial peptides and lipids has strongly increased because of the rapid emergence of antibiotic-resistant bacterial strains. Antimicrobial peptides exert their effects on cells largely by interacting with the lipids of their cell membranes and are able to distinguish between bacterial and eukaryotic membranes. Hydrophobicity of the peptides is one of the crucial determinants that affects function and specificity of peptides that interact with membranes. In the following work peptides have been investigated which are acylated analogs of a lactoferrin fragment. Attachment of a lipophilic chain into a peptide sequence allows the modulation of peptide hydrophobicity and affinity with membranes. These so called lipopetides have various lipophilic chains at the N-terminus which cause different bilayer destabilizing properties. This allows us to gain information about the role of chain length, position of the branched acyl group within the lipophilic chain and length of the side chain. The effects of lipopeptides have been tested on model membrane systems consisting of either dipalmitoyl-phoshatidylcholine (DPPC) as major component of mammalian cell membranes or dipalmitoyl-phoshatidylglycerol (DPPG) and dipalmitoyl-phoshatidylethanolamine (DPPE) as components of bacterial membranes. The effects on model membranes were determined by microcalorimetry, monolayer - techniques and X-ray diffraction. Supported by grant ANEPID (QLK2-CT-2002-01001) from the EC.

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THE IRON-TRANSPORTER FHUA IS THE RECEPTOR OF THE MICROCIN J25 ANTIMICROBIAL PEPTIDE AT THE BACTERIAL MEMBRANE

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Microcins are gene-encoded antimicrobial peptides secreted by Enterobacteriaceae. Microcin J25 (MccJ25) is a unique 21-residue lasso-type peptide presenting a side chain to backbone cyclization between Glu8 and the N-terminus. The embedded ring is threaded by the C-terminal tail of the molecule, thus forming a noose-like feature. The highly potent activity and narrow spectrum of activity of MccJ25 (CMI ranging from 1 to 600 nM against enterobacterial strains) argue for a receptor-mediated mechanism of action. In early studies, bacteria resistant to MccJ25 were reported to display mutations in the multifunctional outer membrane protein FhuA, the high-affinity transporter of iron-ferrichrome and the receptor for phages T5, T1 and f80. In this study, we have demonstrated by size-exclusion chromatography that MccJ25 binds directly to FhuA. This interaction was shown to prevent binding of phage T5 to its receptor and subsequently inhibit the ejection of the phage DNA, which normally occurs upon binding of the phage to FhuA. We compared MccJ25 to the thermolysin-cleaved variant t-MccJ25 in which the 8-18 β-hairpin region is disrupted. From our results, thermolysin cleavage both results in a 100 fold dropped antibacterial activity of the peptide and induces a loss of interaction between MccJ25 and FhuA. Therefore we propose that FhuA is the receptor of MccJ25 at the bacterial outer membrane and that the interaction implicates the 8 to 18 residues.

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LINEAR ANALOGUES OF GOMESIN: DESIGN AND STRUCTURE-ACTIVITY RELATIONSHIP

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Gomesin (Gm) is a potent antimicrobial peptide isolated from hemocytes of the spider Acanthoscurria gomesiana. It contains two intramolecular disulfide bridges Cys2,15 and Cys6,11 (pGlu-CRRLCYKQRCVTYCRGR-NH_2). NMR studies showed that gomesin presents a beta-hairpin structure. The aim of this work was to develop linear active analogues that lack the cysteines. Thus, the cysteines were replaced by Ser or Thr and the Gln9 was substituted by D- or L-Pro to evaluate their effects on peptide folding. The analogues were synthesized by SPPS on a MBHAR using the t-Boc strategy, purified by RP-HPLC and characterized by CE, AAA and LC/MS. Antimicrobial assay revealed that [Ser2,6,11,15, Pro9]-Gm showed to be 16-fold less active than the gomesin against M. luteus and inactive toward E. coli and C. albicans. On the other hand, [Ser2,6,11,15, D-Pro9]-Gm was 8 to 32-fold less potent than gomesin against M. luteus and E. coli, respectively, and inactive on C. albicans. Interestingly, [Thr2,6,11,15, D-Pro9]-Gm was just 2 to 8-fold less active than gomesin in the three microorganism strains. CD studies of the analogues [Ser2,6,11,15, Pro9]-, [Ser2,6,11,15, D-Pro9]-, [Thr2,6,11,15, D-Pro9]-Gm, performed in the presence of SDS micelles displayed spectra of an unordered, tendency to adopt a turn and a beta-turn structures, respectively. These findings indicate that: i) peptide secondary structure is important for the expression of the antimicrobial activity; ii) D-Pro residue contributed to beta-turn formation; iii) Thr residues seems to be more efficient than Ser to promote the interaction between adjacent strands, stabilizing peptide folding. [Supported by CNPq and FAPESP]

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IDENTIFICATION OF PEPTIDES AND PEPTIDOMIMETICS THAT NEUTRALIZE BACTERIAL ENDOTOXINS

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The shock septic is a leading cause of mortality in septic patients, and no specific drugs are as yet available for its treatment. Thus, there is a need to identify new leads in order to increase the number of active molecules at the pre-clinical stages that may be develop in efficacious and safe LPS-neutralizing molecules. We have developed a dual strategy to identify such LPS-neutralizing molecules. First, we use peptides derived from the binding regions of known LPS-binding proteins as scaffolds to introduce modifications at the amino acid level that after structure-activity relationship studies generate highly active peptides. Second, the screening of peptoid combinatorial libraries allowed us to identify active molecules with both in vitro and in vivo ability to neutralize LPS and therefore its toxic effects.

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CALORIMETRIC STUDIES OF THE INTERACTIONS OF TWO HYBRID PEPTIDES (CECROPIN/MELITTIN) WITH LIPOSOMES

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The continued emergence of microbial strains resistant to conventional antibiotics is driving an intense search for new active structures. Eukaryotic antimicrobial peptides seem to provide an alternative and unique mode of action, and are thus very promising candidates in this direction. The molecular basis of their action on pathogen membranes is not fully understood and needs to be further investigated, in order to design and produce competent drugs. In this regard, the use of liposomes as model biomembranes can be a very useful approach. We have been studying the interaction of two cecropin A - mellitin hybrids, CA(1-8)M(1-18) and CA(1-7)M(2-9), with liposomes by a variety of techniques, namely calorimetry, cicular dichroism and light scattering. Here we present results from isothermal titration calorimetry, obtained at 35 ºC. The peptides were prepared by Fmoc/tBu solid phase synthesis methods, purified by reverse phase liquid chromatography and characterized by HPLC, amino acid analysis and MALDI-TOF mass spectrometry. LUV's from DMPC, DMPG and their (3:1) mixture were used as model membranes. From the calorimetric titration of liposome suspensions onto peptide solutions (or the reverse) the thermodynamic parameters for the interaction of each peptide with the different model membranes were derived. The results are discussed and compared with those previously obtained by the other techniques, in an attempt to further understand the mechanism of action of these peptides. Acknowledgments: Thanks are due to FCT for financial support to CIQUP (MB, PG, GB) and to CRUP for financial support through Portuguese-Spanish bilateral agreement E-29/03 (DA, PG).

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FUNGAL CELL MEMBRANE-LYTIC PEPTIDE: ACTIVITY AND MECHANISM

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HP (2-20) (AKKVFKRLEKLFSKIQNDK) is the antimicrobial sequence derived from N-terminus of Helicobacter pylori Ribosomal Protein L1 (RPL1). In order to develop novel antibiotic peptides useful as therapeutic agents, potent antibiotic activities against bacteria, fungi and cancer cells without a cytotoxic effect are essential. To this end, several analogues with amino acid substitutions were designed to increase or decrease only the net hydrophobicity. In particular, the substitution of Trp for the hydrophobic amino acid, Gln and Asp at position 17 and 19 of HP (2-20) (Anal 3) caused a dramatic increase in antibiotic activity without a hemolytic effect. The fungal-lytic mechanism of Anal 3 was investigated by confocal laser scanning microscopy (CLSM) and was visualized by using TEM of artificial liposome and Candida albicans disrupting activity test. Furthermore, in vitro binding assay was performed using common microbial cell components such as lipopolysacchride (LPS), peptidoglycan, β-1,3-glucan, cellulose and chitin. These results indicated that the hydrophobic region of Anal 3 is prerequisite for its effective antibiotic activity and may facilitate easy penetration of the cell target site.

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ANALOGUES OF THE ANTIBACTERIAL PEPTIDE RRWWRF-NH_2

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The hexapeptide RRWWRF-NH_2, (1) is a potent antimicrobial peptide isolated from combinatorial hexapeptide libraries. To investigate the importance of tryptophan and arginine in the antibacterial activity of (1) we prepared analogues in which tryptophan was selectively replaced with a phenylalanine and /or arginine with lysine. The analogues were purified and tested for antibacterial activity against both S. aureus ATCC 25923 and E. coli ATCC 25922. In general, we found that tryptophan and arginine are sensitive to substitution. However, we also prepared dimer and dendrimer analogues of (1) which displayed interesting antibacterial properties. The most active compound was the dimer analogue KKFFKFKKFFKF-NH_2 which showed MIC values of 4 and 4-8 μg/mL against E. coli and S. aureus, respectively.

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ALA-SCAN STUDY OF THE ANTIBACTERIAL PEPTIDE ANOPLIN

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Anoplin is a decapeptide amide, GLLKRIKTLL-NH_2, active against Gram-positive and Gram-negative bacteria with little hemolytic activity towards human erythrocytes. We report a structure-activity study of anoplin based on an Ala-scan. The analogues were tested against both S. aureus ATCC 25923 and E. coli ATCC 25922 and four potent antibacterial analogues were identified. Antibacterial activity and selectivity of the analogues varied considerably, reflected both by the average hydrophobicity (GRAVY) change and a high degree of correlation (r = 0.960) with retention-time (RP-HPLC). Generally, increased hydrophobicity resulted in improved activity against Gram-positive and Gram-negative bacteria. The selectivity could either be reversed or altogether eliminated. The work presented here contributes to the understanding and future design of potent antibacterial analogues of anoplin.

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STRUCTURE-ANTIBACTERIAL ACTIVITY RELATIONSHIP IN SERIES OF SELECTED CYSTAPEP 1 ANALOGUES

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Cysteine protease-inhibiting proteins of the cystatin superfamily can inhibit the replication of certain viruses and bacteria. Previously, we have found that some peptidomimetics based upon the N-terminal binding fragment of human cystatin C possessed bactericidal properties [1, 2]. The widest antibacterial spectrum and the highest activity against several clinically important Gram-positive pathogens, including multi-resistant staphylococci, was displayed by the compound, called Cystapep1, of the structure: Z-Arg-Leu-NH-CH(iPr)-CH_2-NH-Cin (where Cin = cinnamoyl). The presence of an unsubstituted, carbocyclic aromatic system on the end of both the X- and Y-acyl groups in Cystapep 1 analogues, X-Arg-Leu-NH-CH(iPr)-CH_2-NH-Y, is absolutely required for their antibacterial activity. Herein, the synthesis and structure-antibacterial properties relationship for two series of Cystapep 1 analogues: X-Arg-Leu-NH-CH(iPr)-CH_2-NH-Cin and Z-Arg-Leu-NH-CH(iPr)-CH_2-NH-X (where X = Ar-$(CH_2)_n$-CO- or Ar-CH=CH-CO-) are presented. We have found that a double bond in the acyl group on the C-terminus and an urethane-type bond on the N-terminus are not necessary for saving the antibacterial spectrum and the activity. An appropriate length of both, the peptide fragment and the X acyl groups seems to be important requirement. Furthermore, the discussed analogues do not require any chemically reactive groups in their structure for displaying bactericidal effects and might therefore be more suitable than e.g. diazomethylketones for development into clinically useful drugs. Acknowledgements: This work was supported by KBN, grant BW. References: 1. Kasprzykowski, F. at al.,APMIS, 108, 473-481 (2000); 2. Jasir, A. et al.,APMIS, 111, 1004-1010 (2003).

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SYNTHESIS AND BIOLOGICAL ACTIVITY OF INDOLICIDIN ANALOGUES CONTAINING W-AMINO ACIDS

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Antibacterial peptide indolicidin possesses the extremely wide spectrum of antimicrobic activity and attracts the attention as a suitable base compound for design of the new antibiotics generation. One of the possible directions of design is to develope indolicidin analogs containing unnatural ω-amino acids instead of natural α-amino acids. As it is known such peptides are capable of forming the second structures similar to natural peptides, but in contrast to the latter they are more resistant to proteolysis. For the reason of SAR investigation novel indolicidin analogs containing β-alanine, γ-aminobutyric (Abu), δ-aminovaleric (Ava) and ε-aminocaproic (Acp) acids instead proline residues were synthesized: Ile-Leu-X-Trp-Lys-Trp-X-Trp-Trp-X-Trp-Arg-Arg-NH_2, where X=Pro (indolicidin); β-Ala (II); γ-Abu (III); δ-Ava (IV); ε-Acp (V). All peptide were prepared by solid-phase method using Boc-technology and the combination of such arginine and tryptophan derivates as Arg(NO_2) and Trp(Form), that as it was shown earlier is optimal for indolicidin analogs synthesis. Peptides were purified (HPLC) and identified (MS). The peptides obtained showed in vitro activity against gram-negative (E.coli, Klebsiella) and gram-positive (S.aureus) microorganisms comparable to that of indolicidin. Hemolytic activity of analogs III-V was compared with that of indolicidin and analog II was less active in this test.

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INVESTIGATION OF THE CYTOTOXICITY OF A SERIES OF ANTIMICROBIAL PEPTIDES ON INTESTINAL EPITHELIAL CELLS IN VITRO

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Antimicrobial peptides (AMP) are a diverse group of proteinaceous compounds ranging in size, complexity and activity. A common factor between many of these peptides is cationicity, and hence activity is believed to be concentrated on polyanionic prokaryotic cell membranes. However the cationic and amphipathic nature of these peptides suggests that there are potential eukaryotic targets. Some AMPs exhibit potent cytotoxicity in eukaryotic cells. Low selective toxicity for prokaryotic membranes may be an issue in the development of therapeutic AMPs. The current study evaluates in vitro cytotoxicity of a range of antimicrobial peptides from both prokaryotic and eukaryotic origin on both mucous-secreting and non-mucous-secreting gut epithelial cells. These cells are robust epithelial cells that encounter many xenobiotics and hence any cytotoxicity observed is likely to translate to cytotoxicity in many other cell types. The MTT assay was carried out to evaluate cell viability. The LDH assay and neutral red assay were carried out to evaluate the effect of AMPs on the integrity of eukaryotic plasma membranes. The in vitro effect of peptides on intestinal epithelial tight junctions was demonstrated using transmembrane epithelial resistance. The concomitant effect of peptides on the ultrastructure of epithelial cells was evaluated by transmission electron microscopy. This study has shown that certain antimicrobial peptides exhibit cytotoxicity, and suggests that the mechanism of action may be a loss of plasma membrane integrity. The evaluation of potential cytotoxicity and the underlying mechanisms of toxicity of these AMPs will allow novel peptides to be designed that exhibit greater selective toxicity.

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EFFECTS OF THE ANTIMICROBIAL PEPTIDE TEMPORIN L ON CELL MORPHOLOGY, MEMBRANE PERMEABILITY, AND VIABILITY OF ESCHERICHIA COLI

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Antimicrobial peptides are produced by all organisms in response to microbial invasion and are considered as promising candidates for future antibiotics. Many of them interact with the bacterial membrane and increase its permeability. However, it is not clear whether this is the lethal step for the killing mechanism. To address this issue, we studied the interaction of the antimicrobial peptide temporin L, isolated from Rana temporaria frog skin secretions with Escherichia coli by using fluorescence, confocal, and electron microscopy. With regard to fluorescence microscopy, we applied, for the first time, a triple staining method based on the fluorochromes 5-cyano-2,3-ditolyl tetrazolium chloride, 4',6-diamidino-2-phenylindole, and fluorescein isothiocyanate. This technique enabled us to identify, in the same sample, both living and total cells, as well as bacteria with altered membrane permeability. We found that temporin L increases the permeability of the bacterial inner membrane in a dose-dependent manner without destroying the cell's integrity. At low peptide concentrations, the inner membrane becomes permeable to small molecules but does not allow the killing of bacteria. However, at high peptide concentrations, larger molecules, but not DNA, leak out, which results in cell death. Very interestingly, in contrast to many antimicrobial peptides, temporin L does not lyse E. coli cells but rather, forms ghost-like bacteria, as observed by scanning and transmission electron microscopy. Overall, this study demonstrates the advantage of using the triple fluorescent approach combined with microscopical techniques to explore the mechanism of membrane-active peptides in general.

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MEMBRANE TOPOLOGY AND MECHANISM OF PORE FORMATION OF THE ANTIBIOTIC LIPOPEPTIDE TRICHOGIN GA IV

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Trichogin GA IV is a natural, Aib-containing peptide, belonging to the lipopeptaibol family. Despite its remarkable antibiotic activity, its mechanism of action is still debated. Recently, we have shown that trichogin self-associates into oligomers both in water and in the lipid phase, and that bilayer permeabilization is caused by peptide aggregation in the membrane (1). Here we report new photophysical studies performed on synthetic fluorescent analogues of trichogin. Energy-transfer experiments indicate that the peptide readily translocates across the membrane. Fluorescence quenching measurements, performed with water soluble quenchers and with quenchers positioned in the membrane at different depths, allowed us to determine peptide insertion and orientation in the lipid bilayer. These studies were carried out as a function of peptide concentration in order to correlate peptide topology with membrane permeabilizing activity. To clarify the molecular details of peptide-induced membrane leakage, giant unilamellar vesicles (diameter 10-100 μm) were prepared by electroformation. Direct visualization of the giant liposomes by fluorescence microscopy shows that leakage is caused by pore formation rather than membrane micellization, and that it is size selective. Furthermore, leakage experiments performed with different lipids indicate that permeabilization is independent on membrane viscosity, ruling out an ion-carrier mechanism, at least in the concentration range investigated. On the basis of the foregoing results a model of the mechanism of membrane perturbation by trichogin will be discussed. 1) L. Stella, C. Mazzuca, M. Venanzi, A. Palleschi, M. Didonè, F. Formaggio, C. Toniolo, and B. Pispisa. Biophys. J. 2004, 86: 936-945.

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ALANINE SERIES OF THE ANTIMICROBIAL PEPTIDE GOMESIN: A STRUCTURE-ACTIVITY RELATIONSHIP STUDY

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The antimicrobial peptide gomesin (Gm) was isolated from hemocytes of the tarantula spider Acanthoscurria gomesiana. It contains four cysteines performing two disulfide bridges: Cys2-15 and Cys6-11 (pGlu-CRRLCYKQRCVTYCRGR-NH_2), being effective against several Gram-positive and Gram-negative bacteria strains, fungi and yeast. However, it exhibits some hemolytic activity. Recent NMR studies have shown that the structure of gomesin consists in a well-resolved two-stranded antiparallel beta-sheet connected by a noncanonical beta-turn. The aim of this work was to evaluate the contribution of each amino acid residue in the antimicrobial and hemolytic activities of gomesin. Thus, each of its 14 amino acids residues was replaced by alanine. Peptide syntheses were performed by SPPS. They were purified by RP-HPLC and characterized by CE, AAA, LC/MS and CD. Their antimicrobial activities were determined against B. megaterium, E. coli and C. albicans at physiological salt concentration. All analogues showed to be as active as gomesin except for [Ala5]-, [Ala7]-, [Ala9]-, [Ala10]-, [Ala12]- and [Ala14]-Gm that presented a significative decrease in the antimicrobial activity mainly against C. albicans. These results indicated that the presence of the amino acids Gln9, Arg10 in the beta-turn motif and residues Leu5, Tyr7, Val12 and Tyr14 were essential for the maintenance of the gomesin potency. No obvious correlation could be found between the biological activity and the secondary structural features inferred from the CD studies. These findings suggest that the hydrophobic residues, and the turn region are important for the peptide interaction with the microorganism membranes. [Supported by CNPq and FAPESP]

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SYNTHESIS, BIOLOGICAL ACTIVITY AND CONFORMATIONAL STUDIES OF GOMESIN

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The antimicrobial peptide gomesin (Gm), isolated from hemocytes of tarantula spider Acanthoscurria gomesiana, is very effective against several pathogenic microorganisms. The peptide (pGlu-CRRLCYKQRCVTYCRGR-NH_2) contains four cysteines performing two disulfide bridges in positions 2/15 and 6/11. In order to study its interaction with the microorganism membranes, we examined the conformational behavior of some analogues in different environments that mimic the biological membrane/water interface. The analogues were synthesized by SPPS and purified by RP-HPLC. The peptides were characterized by AAA and LC/MS. Their antimicrobial activities were evaluated by a liquid growth inhibition assay against C. albicans, B. megaterium and E. coli. EPR studies were performed with analogues containing TOAC as spin probe {[TOAC1]-Gm (I) and [TOAC1, Ser2,6,11,15]-Gm (II)}. Fluorescence studies were done with [Trp7]-Gm (III), [Trp9]-Gm (IV), [Ser2,6,11,15, Trp7]-Gm (V) and [Ser2,6,11,15, Trp9]-Gm (VI). CD studies were also conducted with all analogues and gomesin. From our results we concluded that: i) the incorporation of TOAC or the replacement of Tyr7 or Gln9 by Trp caused no reduction on the antimicrobial activity of gomesin; ii) the analogues I-III presented a beta-hairpin conformation identical to that of the native gomesin in all environments used, while the linear analogues IV-VI presented an unordered conformation; iii) all analogues strongly interacted with SDS, but not with LPC micelles, corroborating with previous results that the first step of the gomesin killing mechanism on bacteria is an electrostatic interaction with the lipid bilayer causing the disruption of the internal membrane. [Supported by FAPESP, CAPES and CNPq]

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ROLE OF LOOP 7 OF PROTEIN P2 FROM HAEMOPHILUS INFLUENZAE IN THE INFLAMMATORY RESPONSE

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In the mosaic of antigenic components present on Gram-negative surface, the outer membrane proteins (OMPs) could play a crucial role in the pathway associated with bacterial infections. The importance of immunodominant surface exposed loops of OMPs is the focus of recent studies and the ability of Gram-negative bacteria to cause recurrent infections is in part attributable to antigenic variability in all surface exposed loops of OMPs. Recently, it has been demonstrated that surface exposed loops of Haemophilus influenzae type b P2 porin play an important role in the inflammatory and immunological response. The loops L5, L6 and L7 corresponding to the amino acid sequences of variable loop regions facing the cell exterior and thus more probably involved in the initial interaction with the host cell, are able to activate the MEK1-MEK2/MAPK cascade in a manner similar to the entire porin P2. In this study we analised in detail the peptides corresponding to loop L5, L6 and L7 in order to determine if they were able to induce the production of cytokines and we used an alanine scanning mutagenesis approach to identify residues critical for the activation of the MAPK cascade and for the production of cytokines. The results obtained showed that only the loop L7 is able to significantly activate the MEK1-MEK2/MAPK cascade and to induce the release of IL-6 and TNF-alpha.

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THE ANTIBACTERIAL ACTIVITY OF TEMPORIN A AND ITS ANALOGUES AGAINST STRAINS USED FOR THE EVALUATION OF PRESERVATIVES

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The peptide antibiotics are secreted by most organisms, protecting them against microbial infections. Owing to the low toxicity of endogenous antimicrobial peptides, particularly high hope is attributed to the possibility to use them as biopharmaceuticals in modern chemotherapy of infectious diseases. In the future they can be used for preservation of medicines, cosmetics and food as well. In this work we present the solid-phase synthesis of Temporin A (FLPLIGRVLSGIL-NH_2), two its analogues and retro-Temporin A. Additionally we show the evaluation of their antibacterial activity against reference strains proposed for the study of preservatives by European Pharmacopeia. The peptides were synthesized manually using a fluorenyl-methoxycarbonyl (Fmoc) chemistry. Microorganisms (Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli) obtained from American Type of Culture Collection (ATCC) were used. This work was supported by State Committee for Scientific Research (KBN), Grant 3 P05F 04124.

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ISOLATION AND CHARACTERISATION OF PROTEOLYTIC ANTIMICROBIAL ACTIVITY FROM BIFIDOBACTERIA

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Bifidobacteria represent a species that has been considered to impart beneficial effects when present in the gut flora of an individual. Among the attributes claimed to be associated with Bifidobacteria is an improved immune system, hypocholestrolaemic effects, reduced gastrointestinal disorders and improved digestion (Saarla et al, 2000.) An uncharacterised mixed culture collection at the Institute Technology Tallaght was screened for the presence of Bifidobacterial strains. Pure cultures grown on Raffinose-Bifidobacterium media (Hartemink et al., 1996) reduced the pH of the medium upon growth and showed the characteristic morphology of a bifurcated Y forms or club-shaped rods in the Gram stain. Potent antimicrobial activity was detected in the supernatant from fermentations of one of the selected strains using the plate diffusion assay with Micrococcus luteus as the marker strain. The antimicrobial activity was lost totally when incubated in the presence of □-chymotrypsin and Proteinase K indicating that it is of a proteinacious nature. Although Bifidobacteria sp. are know to be heterofermentative, producing both lactic and acetic acids, the antimicrobial activity was shown not to be correlated with pH. The antimicrobial activity seems to be wide spectrum showing activity against both Gram positive and Gram negative strains. References: Hartemink, R., Kok, B.J., Weenk, G.H. and Rombouts, F.M., 1996. J. Microbiol. Methods 27, pp. 33–43. Saarela, M., Morgensen, G., Fonden,R., Matto, J., Mattila-Sandholm, T., 2000. J. Bacteriol. 84 No. 3 pp 197-215

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DESIGN OF NOVEL ANTIBACTERIAL PEPTIDES BASED ON SHORT, PROLINE-RICH NATIVE SEQUENCES

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Pyrrhocoricin and drosocin, representatives of the short, proline-rich antibacterial peptide family kill bacteria by inhibiting DnaK-mediated protein folding. While the amino-terminal half of pyrrhocoricin carries the DnaK-binding domain, the carboxy-terminus is responsible for penetration into bacterial and host cells. Mid-chain proline residues are positioned to maintain structural integrity of the peptide and avoid changes in the predominant mode of action. Indeed, minor sequence modifications, designed to improve the membrane-penetrating ability of the peptide, result in a shift from DnaK inhibition to membrane disintegration. However, overly ambitious membrane activities of antibacterial peptides lead to toxicity to host cells. From clinically significant pathogens, pyrrhocoricin exhibits the strongest activity against Escherichia coli, Salmonella typhimurium, Klebsiella pneumoniae and Haemophilus influenzae, strains belonging to the Gram-negative Enterobacteriaceae family. Pyrrhocoricin's selectivity to the susceptible strains can be explained by sequence alterations in the D-E helix fragment of the target protein DnaK. Using a series of biochemical assays, we identified Asp3, Tyr6, Leu7 and Arg9 as pyrrhocoricin's putative contact points to the E. coli DnaK D-E helix. This information is currently applied to find improved pyrrhocoricin analogs to treat mammalian infections caused by pyrrhocoricin- and drosocin susceptible strains, and completely new derivatives directed against bacteria non-susceptible to the native peptides.

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ANTI PROSTATE TUMOR ACTIVITY DETECTED IN VITRO AND IN VIVO BY A CELL SELECTIVE MEMBRANE-ACTIVE D,L-AMINO ACID CONTAINING PEPTIDE

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Native, membrane active lytic peptides were recently found to be highly potent against cancer cells. However, their in vivo use is limited due to the loss of their function, mainly because of enzymatic degradation. We designed a 15-mer all-L-amino acid (aa) lytic peptide and its diastereomer (composed of D- and L-aa), both of which kill similarly prostate cancer cells in vitro. The L-aa peptide was active on all types of cells and had high hemolytic activity. In contrast, the diastereomer selectively targeted prostate cancer cells compared with non-tumor cells, and was devoid of hemolytic activity. Most importantly, a complete growth arrest of prostate cancer tumor progression was observed in mice model only when treated with the diastereomer. This effect was accompanied by a significant decrease in prostate-specific antigen (PSA) serum levels. We found a necrotic-like mechanism of killing due to swelling and rupturing of the prostate cancer cells as observed by using fluorescent confocal microscopy and histological studies. Using fluorescent markers and labeled peptides we found that the steps involved in the killing process include: (i) binding to distinct areas on the cytoplasmic membrane; (ii) a significant membrane destabilization; and only after the peptide reaches a threshold concentration there is (iii) a rapid membrane permeabilization which is followed by cell lysis. Since the diastereomer is acting in vivo in a highly destructive mechanism, it may become a new agent for the prevention of prostate cancer.

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STRIPED BASS HEPCIDIN: SYNTHESIS, SOLUTION STRUCTURE AND BIOLOGICAL ACTIVITY

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Hepcidin is a small, cysteine rich, antimicrobial peptide hormone found in many vertebrate species ranging from fish to humans. This peptide hormone is primarily involved in regulating iron uptake and sequestration and is strongly associated with juvenile hereditary hemochromatosis. However, hepcidin is strongly over-expressed as part of the initial acute phase response to infection, potentially controlling microbial growth by sequestering iron from the bloodstream. In hybrid white striped bass, hepcidin expression is upregulated nearly 5000 fold in the liver in response to infection. We have successfully synthesized this 21-residue peptide, (GCRFCCNCCPNMSGCGVCCRF), containing 4 disulfide bonds using an Fmoc/tBu SPPS strategy. Folding conditions were established for optimal formation of the biologically active form of the molecule. The solution structure of the peptide was determined using NMR. Like human hepcidin (Hunter et al., (2002) J. Biol. Chem. 277: 37597-37603), bass hepcidin contains 4 disulfide bonds, including a rare vicinal disulfide bridge between two adjacent cysteine residues. The peptide consists of a distorted two-stranded antiparallel beta sheet connected by a flexible turn that is non-planar with the beta sheet, giving the molecule a convex surface. The position of the disulfide bonds alternate from one side to the other side of the beta sheet. The molecule has an amphipathic character, with a hydrophobic convex surface and concave positively charged surface. This amphipathic character is relatively common amongst the antimicrobial peptides. Bass hepcidin did not directly bind ferric iron, as shown by NMR and presumably regulates iron uptake and release via a signaling cascade.

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STRONG SYNTHETIC INHIBITORS OF ANTHRAX LETHAL TOXIN

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Anthrax, which was thought to be practically eradicated from the western countries thanks to the extensive use of antibiotics and to the widespread vaccination of recipient animals, is going back to be a disquieting problem for its use as a biological weapon. A possible therapeutic approach for anthrax treatment is by new anti-toxin molecules which can interfere with the formation of the toxic complex (Lethal Toxin) responsible of the lethal action of Bacillus anthracis. Here we present the production of anti-anthrax synthetic inhibitors derived from competitive selection of a phage peptide library. These inhibitors interfere with the formation of the Lethal Toxin by inhibiting binding of Protective Antigen (PA) to Lethal Factor (LF). Different 12 mer sequences were identified by phage selection and used to design peptide sub-libraries that were synthesized on solid phase in dendrimeric form. Iterative screening of peptide sublibraries for PA-LF inhibition brought to selection of 3 dendrimeric peptides that can inhibit LF binding to PA in ELISA and also the cytotoxicity induced by anthrax toxin on J774 A.1 cells. These peptide inhibitors are at least ten times better in terms of IC50 than lead dendrimeric peptides and several hundred times better than peptides selected from the phage library. This IC50 is compatible with in-vivo use for the development of specific drugs for the treatment of Anthrax lethal symptoms. Moreover, our peptide inhibitors are extremely resistant to blood proteolytic activity.

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N-DERIVATIZED ANALOGUES OF INDOLICIDIN AND [PHE4,6,8,9,11] INDOLICIDIN WITH ENHANCED ANTIBACTERIAL ACTIVITY

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Indolicidin, ILPWKWPWWPWRR-NH_2, is a 13-mer antimicrobial peptide found in bovine neutrophils (1). The peptide has an unusually high content of tryptophan accounting for 38% of the total amino acid residues. Indolicidin is active against Gram-positive and Gram-negative bacteria, protozoa, fungi and HIV. Furthermore, it is cytotoxic towards mammalian erythrocytes. [Phe4,6,8,9,11] indolicidin displays antibacterial activity but is less hemolytic as compared with the parent peptide. In continuation of our study of indolicidin (2), we report the synthesis and biological activity of analogues of indolicidin and [Phe4,6,8,9,11] indolicidin, carrying a hydrophobic group attached to the N-terminus. The modifications include: fatty acids, non-standard amino acids and N-substituted glycines (peptoids). The indolicidin and [Phe4,6,8,9,11] indolicidin analogues were tested for antibacterial activity against S. aureus and E. coli. Furthermore, the cytotoxicity towards mammalian erythrocytes was assessed by a hemolytic activity assay. Several N-terminal modified analogues of indolicidin and [Phe4,6,8,9,11] indolicidin were active against both S. aureus and E. coli. However, the hemolytic activity was also increased. (1) Selsted, M.E. et. al. (1992) J. Biol. Chem. 267, 4292-4295. (2) Ryge, T.S. et. al. J.Peptide Res. (2004) Submitted.

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UNDERSTANDING THE MOLECULAR INTERACTIONS OF THE ANTIMICROBIAL PEPTIDE TRITRPTICIN WITH BIOMIMETIC SYSTEMS: FROM MOLECULAR SELF-ASSEMBLY TO ION CHANNEL ACTIVITY

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Natural or designed amphipathic peptides have a growing importance both from the biophysicochemical standpoint and for novel bionanotechnological applications. The cationic peptide tritrpticin (TRP3, VRRFPWWWPFLRR) has a broad antimicrobial spectrum, acting against Gram-positive and Gram-negative bacteria, as well as some fungi, while also displaying hemolytic activity. TRP3 properties were examined by means of a variety of techniques. Ellipsometry, contact angle, and atomic force microscopy data demonstrated that TRP3 adsorbs on silicon wafers forming thin films (1.2-2.4 nm) that modify the native surface wettability, making it compatible for lipid interaction. The data showed that TRP3 has a pronounced surfactant-like activity. The interaction of TRP3 with acidic lipid monolayers showed a concentration-dependent increase of surface pressure and surface potential. We also investigated the activity of TRP3 and its N-terminally labeled TOAC derivative (TOAC0-TRP3) in planar lipid bilayers (BLM) and were able to demonstrate, for the first time, their ion channel-like activity. Both TRP3 and TOAC0-TRP3 formed large conductance channels (hundreds to thousands and tenths to hundreds of picosiemens, respectively) both at positive and negative potentials. The predominant ion channel activity was characterized by very regular and discrete current steps of uniform amplitude, which exhibited relatively long residence times (of the order of seconds). Occasionally, multiple conductance steps were observed, indicating the simultaneous presence of more than one open pore. These results should contribute to the understanding of the molecular interactions and mechanism of interaction of

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STRUCTURE-ACTIVITY RELATIONSHIP STUDY ON BAC7(1-35), A SYNTHETIC FRAGMENT OF THE PRO-RICH ANTIBACTERIAL PEPTIDE BAC7

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Bac7, a cationic, 60-residue peptide originally isolated from bovine neutrophils, is a member of the Pro-rich family of antimicrobial peptides, that acts predominantly against Gram-negative bacteria with a non-membranolytic mechanism. Chemical synthesis of numerous Bac7 fragments showed that the N-terminal fragment 1-35 displays an activity comparable to that of the natural peptide. To investigate the mechanism of action of this peptide, several C- and N-terminal truncated forms of Bac7(1-35), as well as its all-D enantiomer, were synthesized. Killing kinetics experiments showed that the all-D Bac7(1-35) showed a greatly reduced potency and kinetics of bactericidal action. Similarly, shortening at the N-terminus (e.g., removal of the first four residues) reduced both the potency and the killing kinetics. Conversely, C-terminal truncation was better tolerated, at least up to a certain peptide length. The capacity of some of these Bac7 fragments to enter Escherichia coli cells was investigated by using immunogold electron microscopy. Results indicate that Bac7(1-35) rapidly penetrates the bacterial cells (within 5 min) and localizes into the cytoplasm. On the contrary, the 5-35 N-terminal truncated fragments and the all-D enantiomer cross the bacterial membrane with a much slower kinetics, which correlates with the killing kinetics. The pretreatment of bacteria with the uncoupler 2,4-dinitrophenol determined a dramatic decrease of L-Bac7(1-35) penetration into the cytoplasm and a parallel decrease in bacterial killing. Interestingly, this effect was not observed with the all-D peptide. Results are discussed in terms of a different mechanism of action of L-Bac7(1-35) with respect to its all-D enantiomer.

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EFFECT OF THE HUMAN ANTIMICROBIAL PEPTIDE LL-37 ON MODEL MEMBRANES MIMICKING BACTERIAL AND MAMMALIAN CELL MEMBRANES

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The human peptide LL-37 is considered to play an important role in immune defence participating in the inflammatory process by several means. Apart from its antimicrobial action the peptide was shown to be chemotactic, angiogenic and to act on cancer cells. Unlike other antimicrobial peptides that act selectively on bacterial cells, LL-37 exhibits cytotoxic effects against both bacterial and mammalian cells. Eucaryotic and procaryotic membranes differ markedly in their complexity and lipid composition. Zwitterionic choline phospholipids (phosphatidylcholine and sphingomyelin) constitute the major lipids in the outer leaflet of mammalian membranes whereas the predominant phospholipids in bacterial cytoplasmic membranes are phosphatidylethanolamine and negatively charged phosphatidylglycerol. In this study, the action of LL-37 on phospholipid monolayers and liposomes mimicking bacterial and mammalian membranes was observed. LL-37 inserted into monolayers composed of DPPG and DPPC, resulting in altered characteristics of the compression-isotherm. In accordance to these monolayer data, perturbation of DPPG and DPPC bilayers was observed in calorimetic experiments, indicating no pronounced preference of LL-37 for one or the other lipid. In both cases the pretransition was abolished and the chain melting transition was markedly affected in its thermodynamic characteristics such as transition temperature, cooperativity and enthalpy. In PE liposomes action of LL-37 was found to depend on carbon chain characteristics showing negligible effects on disaturated DPPE. These results are in line with in vitro studies revealing cytotoxic activity of LL-37 to bacterial but also to eucaryotic cells.

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6-NITROTRYPTOPHAN AND 6-AMINOTRYPTOPHAN CONTAINING ANALOGS OF INDOLICIDIN

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Indolicidin is an antimicrobial natural peptide with an unique amino acid sequence that contains five tryptophan residues: Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH_2. It is likely that indolicidin antibacterial activity to certain extent is caused by so-called cation-π interactions between a cationic moiety (Lys, Arg) and the π-face of tryptophan aromatic ring. Introduction of various electron donating and electron accepting substituents into indole tryptophan rings may influence this interaction and result in the increase of the specific activity. So, 6-nitro-Trp and 6-amino-Trp containing indolicidin analogs with total or partial substitution of five Trp-residues in natural molecule were synthesized using Fmoc solid phase method. Starting Fmoc-NO2-Trp was obtained by modified procedure [1]. Amino-Trp containing indolicidin analogs were prepared by reducing of corresponding nitro-peptides by means of various reagents (SnCl2, TiCl3, catalytic hydrogenation). All analogs were tested with respect to their antibacterial and hemolytic activity. Incorporation of nitro-tryptophan into natural molecule may be used as intrinsic photoaffinity labelling probe for more detail investigation of peptide-cell membrane interactions also.

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FLUORESCENCE AND EPR STUDY OF THE INTERACTION BETWEEN THE ANTIMICROBIAL PEPTIDE INDOLICIDIN AND ITS N-TERMINALLY LABELED TOAC ANALOGUE AND MODEL MEMBRANES

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The cationic antimicrobial peptide indolicidin (ILPWKWPWWPWRR-NH_2, IND) interacts with membranes, causing disruption. However, its detailed molecular mechanism of action is still unknown. This study aims at providing further insight into this process. Fluorescence and EPR were used to investigate the binding of IND and of its N-terminally TOAC-labeled analogue (TOAC0-IND) to model membranes (zwitterionic or negatively charged detergent micelles and phospholipid bilayers). The fluorescence intensity of both peptides increased and the wavelength of maximum emission decreased upon binding to all model membranes. Similar wavelength shifts were observed for both peptides suggesting that membrane binding occurred in a similar manner. EPR spectra of TOAC0-IND also evinced the peptide-membrane interaction. The spectra of the peptide bound to micelles displayed lineshapes narrower than in bilayers, as usually observed for lipid spin probes. A two-component spectrum was obtained in bilayers, one due to membrane-bound peptide and the other due to peptide free in the aqueous phase, allowing the calculation of partition coefficients. Peptide-induced changes in order and mobility within the aggregates were assessed by the EPR spectra of incorporated lipid spin probes. The spectra showed that the order increased and the mobility decreased upon IND binding to micelles and bilayers at low peptide:lipid molar ratios. The spectral changes indicated that IND affected micelle organization to a greater extent, probably due to the looser packing in these structures, which facilitates the peptide insertion. The data demonstrate that both electrostatic and hydrophobic interactions play a role in IND binding to membranes. Financial support: FAPESP and CNPq.

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CHARACTERIZATION OF THE CELL-PENETRATING MECHANISM OF A PROLINE-RICH ANTIMICROBIAL PEPTIDE BY USING MODEL MEMBRANES

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Antimicrobial peptides are a first-line defense against microbial invasion. Besides direct antimicrobial activity, they mediate other biological effects connected with inflammation and wound repair. The interaction of these peptides with host cells is object of intense research. Proline-rich antimicrobial peptides cross the membrane of mammalian cells without causing membrane damage or other cytotoxic effects. We have analyzed the cell-penetrating properties of a proline-rich peptide (Bac7(1-35)) on cultured mammalian cells and artificial phospholipid bilayers (PC/cholesterol). Quantification of the fluorescence associated to cells following treatment with fluorescently labeled peptides under different experimental conditions (various time lengths, low temperature, ATP depletion), suggests that translocation of Bac7(1-35) is not receptor- or energy-dependent. The ability of Bac7(1-35) and of a truncated analog of this peptide to interact with phospholipid bilayers has been measured by Surface Plasmon Resonance and polarized ATR-FTIR. The results obtained indicate a different capability of the two molecules to interact with the phospholipid membrane.

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LACTOFERRAMPIN: A NOVEL ANTIMICROBIAL PEPTIDE IN THE N1-DOMAIN OF BOVINE LACTOFERRIN

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Bovine lactoferrin (bLF) is a multifunctional protein. After digestion in the stomach with pepsin a peptide, called lactoferricin B, is liberated from the N1-domain. This peptide has activity against several microorganisms and is more potent than the whole protein. We investigated whether additional antimicrobial domains were present in lactoferrin, based on common features of antimicrobial peptides. A second putative antimicrobial domain in the N1-domain of lactoferrin, designated lactoferrampin, was identified. This novel peptide indeed exhibited candidacidal activity, which was substantially higher than the activity of lactoferrin. Furthermore, lactoferrampin was active against Bacillus subtilis, Escherichia coli, and Pseudomonas aeruginosa, but not against the fermenting bacteria Actinomyces naeslundii, Porphyromonas gingivalis, Streptococcus mutans and Streptococcus sanguis. Whereas the candidacidal activity of lactoferrampin was comparable to lactoferricin, the specificity towards bacteria was different. Notably, the fact that lactoferrampin is located in the N1-domain in close proximity to lactoferricin suggests that both domains play a concerted role in membrane-mediated activities of lactoferrin.

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STRUCTURAL STUDIES IN SOLUTION OF FLUORESCENT ANALOGUES OF TRICHOGIN GAIV BY SPECTROSCOPY AND MOLECULAR MECHANICS

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Antimicrobial peptides exert their biological action through permeabilization of the cell membrane. As they are of relevant pharmacological interest as potential new antibiotic agents, the 3D-structural motifs determining their biological activity are currently actively explored. We have studied the conformational features in solution and the membrane activity of fluorescent analogues of trichogin GA IV. The newly synthesized compounds are: Fmoc-Toac-Gly-Leu-Aib-Gly-Gly-Leu-Aib-Gly-Ile-Leu-OMe(F0T1)
Fmoc-Aib-Gly-Leu-Toac-Gly-Gly-Leu-Aib-Gly-Ile-Leu-Ome(F0T4)
Fmoc-Aib-Gly-Leu-Aib-Gly-Gly-Leu-Toac-Gly-Ile-Leu-OMe(F0T8)
In these analogues the fluorescent Fmoc group (the donor molecule) replaces the natural octanoyl moiety, and a Toac residue, a nitroxide-based fluorescence quencher,1 was inserted at different positions along the peptide chain, replacing the natural Aib residues. The investigated peptides populate a set of ordered conformations involving 310/(-helical segments and some locally disordered forms at the level of the flexible Gly-Gly sequence. The conformational equilibria were characterized by CD, time-resolved fluorescence experiments and molecular mechanics calculations in different solvents and in media mimicking the membrane phase. Interestingly, the membrane perturbing activity of the fluorescent analogues investigated is not affected by the substitution of the octanoyl moiety with the Fmoc group, as shown by release experiments in lipid bilayers. Results concerning the localization of the fluorescent analogues in the lipid phase will be also presented. 1. B. Pispisa, C. Mazzuca, A. Palleschi, L. Stella, M. Venanzi, M. Wakselman, J.-P. Mazaleyrat, M. Rainaldi, F. Formaggio, and C. Toniolo, Chem. Eur. J. (2003), 9, 4084-4093.

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STRUCTURAL DISSECTION OF THIONIN REVEALS MINIMAL ACTIVE MOTIF

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Pyrularia pubera thionin (PpTH) was one of the first eukaryotic peptides for which antimicrobial activity was demonstrated and a defensive role postulated [1]. Thionins are complex peptides, 45-47 residue-long and 4 disulfide bonds, with a high rate of sequence homology and a well conserved secondary structure that consists of one b-sheet and two antiparallel amphipathic a-helices. Recently we showed that strategic mutations on the PpTH sequence could significantly enhance its activity against specific types of bacteria [2]. We now describe how substantial structural dissection of this thionin leads to a minimalist motif with antimicrobial activity. Peptides reproducing different elements of the secondary structure of PpTH have been designed and evaluated against several representative plant pathogens; in parellel, conformational studies by circular dichroism upon binding to phospholipid liposomes have been performed. Our results clearly show that the central amphipathic double helix of PpTH is the key feature for its antimicrobial activity, and that disulfide bonds play a crucial role stabilizing a characteristic folding which is clearly related to the ability of this peptide to bind and disrupt microbial membranes. [1] Fernandez de Caleya, R., Gonzalez-Pascual, B., García-Olmedo, F. and Carbonero, P. Appl. Microbiol. 23, 998 (1972). [2] Vila-Perelló, M., Sánchez-Vallet, A., Molina, A. and Andreu, D. FEBS Lett. 536 , 215-219 (2003).

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TRYPTOPHAN-RICH ANTIMICROBIAL PEPTIDES; THE MECHANISM OF ACTION OF TRITRPTICIN AND PUROINDOLINE-DERIVED PEPTIDES

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Several naturally occurring antimicrobial peptides contain a high percentage of Trp residues, such as indolicidin and tritrpticin. We have recently shown that similar ~15-residue Trp-rich peptides derived from the wheat protein puroindoline, also act as potent antimicrobial peptides. In all cases the activity is diminished when the Trp residues are replaced by smaller hydrophobic residues. This suggests that the unique membrane binding properties of the Trp sidechain play a crucial role in the activity. We have used micro-calorimetry and fluorescence spectroscopy to study the binding of these peptides and several analogs to model membranes containing different phospholipids headgroups: PG, PC, PE. Our results indicate that binding of these basic peptides to the negatively charged PG headgroups is quite strong, while the binding to zwitterionic PC headgroups is relatively weak. This runs parallel with their activity, PG is abundant in bacterial membranes, while PC is abundant in eukaryotic membranes. However, in vesicle leakage assays, no obvious correlation could be observed between the leakage activity of a peptide and its antimicrobial potency. This indicates that the mode of action is more complex than simple membrane perturbation and probably involves inhibition of intracellular targets, eg RNA or DNA. NMR was used to determine the structures of micelle-bound peptides; again no correlation could be found between their secondary structures and the antimicrobial activity. Thus the deleterious effects of these peptides on the bacterial membrane and the intracellular targets are jointly responsible for the bacteriostatic activity. (Supported by the Alberta Heritage Foundation for Medical Research)

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EVOLUTION OF PRIMATE ß-DEFENSINS: EFFECT OF STRUCTURAL VARIATIONS IN MODULATING ANTIMICROBIAL AND SIGNALLING ACTIVITIES

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β-Defensins are a family of small (30-40 residues), cationic Cys-rich antimicrobial peptides that have an important role in mammalian host defence. β-Defensin sequences are highly variable, apart from six conserved cysteines. All determined 3D structures are however similar, based on an anti-parallel β-sheet core and N-terminal α-helical segment, so that folding depends mainly on disulfide bridging. The evolution of these molecules, in response to selective pressures coming from the environment in relation to their antimicrobial and immune functions, was thus relatively free of structural constraints. A detailed analysis of primate sequences indicates neutral evolution for β-Defensin 1 congeners, positive selection for variation in β-defensin 2 congeners and strong conservative selection in β-defensin 3. We have synthesised selected β-Defensin from human (hBD-1, -2 and -3), from macaque (mfaBD-1 and -2) and from gibbon (hcoBD3), as well as analogues, using optimised SPPS and folding protocols, to investigate the structural factors that might be responsible for these different patterns of evolution. The tested peptides showed a broad but differing spectrum of activity against Gram-positive and Gram-negative bacteria as well as fungi in low concentration medium (5% v/v), and this activity was differentially sensitive to the salt concentration. Killing kinetics and membrane permeabilisation kinetics of reference Gram-positive and Gram-negative bacteria indicated that the mode of action may vary in a manner dependent on both structure and charge. Results are discussed in terms of possible roles of both the direct antimicrobial activity, and activity as signalling molecules, in determining their evolution.

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SYNTHESIS AND APPLICATION OF A NEW CELL-PENETRATING CATHEPSIN D SUBSTRATE

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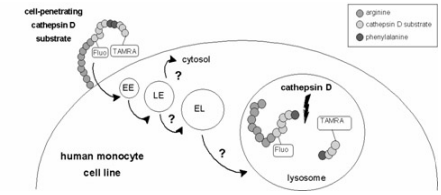
We describe the synthesis of a cell-penetrating peptide substrate for Cathepsin D (CatD). The substrate consists of the highly cationic sequence (RRRRRRRRR) which has been described as a cell-penetrating peptide (CPP) and the CatD specific peptide substrate KAPISFFELGK which is cleaved by CatD between the aromatic phenylalanine residues. We have synthesized the peptide Ac-RRRRRRRRRK(TAMRA)APISFFELG-ω-K(Fluo), consisting of the N-terminal cell penetrating peptide and the C-terminal substrate. The two lysine residues flanking the CatD substrate are labelled with the two fluorophores 5(6)-carboxytetrymethylrhodamine (TAMRA) and 5(6)-carboxyfluorescein (Fluo). The fluorophores adjacent to the substrate form intramolecular dimeres so that the two fluorophores within the intact peptide show almost no fluorescence emission. After cleavage of the peptide substrate with CatD the fluorophores are no longer quenched and the emission of both fluorophores can be detected. In this study we have investigated the fluorescent properties of the synthetic peptide substrate by in vitro digestion experiments as well as its application for in vivo imaging in human monocytes using confocal laser scanning microscopy (CLSM) and flow cytometry.



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THE SYNTHESIS AND BIOSENSING OF CYCLIC PEPTIDES MIMICKING CARBOHYDRATE RECOGNITION SEQUENCES

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Carbohydrates are important substructures in biological systems. They play a tremendous role in cells as glycoproteins, glycolipids or acidic proteoglycans and are found at the cell surface or in the extracellular matrix. Carbohydrates are involved in many biological processes like immune defense and regulation, cell-cell interaction, cell adhesion or metastasis. In the nervous system they are bound to many recognition molecules, which regulate modification of synaptic activity, cell interactions during development, and regeneration of nerve connections after damage in adults. Since many of the carbohydrate substructures are not very abundant in natural sources, and because of their often very complex structure and the difficulties in synthesis, it is desirable to find peptides which are able to mimic their carbohydrate counterparts resulting in highly affine surrogates. The potential utility of peptides as carbohydrate mimetics, for instance as vaccines, has been demonstrated in immunological studies. In our studies we investigated the synthesis of conformationally restricted cyclic peptides to discover molecules with a high affinity to antibodies against neural carbohydrate recognition sequences. These sequences are involved in neurite outgrowth and regeneration of nerves for example. The linear peptide precursors were synthesized by SPPS on Barlos-resin by Fmoc-tactics, cyclisation under high dilution conditions with a two-channel syringe pump and finally deprotection. Affinity studies were carried out with surface plasmon resonance measurements on a BIACORE® 3000 biosensor. Highly affine peptides were also tested in neurite outgrowth experiments.

P251

STRUCTURE-FUNCTION RELATIONSHIPS OF THE DI/TRI-PEPTIDE TRANSPORTER OF THE YEAST SACCHAROMYCES CEREVISIAE: A MODEL SYSTEM FOR STUDYING SUBSTRATE RECOGNITION AND REGULATION OF MEMBRANE PROTEINS INVOLVED IN PEPTIDE TRANSPORT

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The integral membrane protein Ptr2p transports di/tri-peptides into yeast and is representative of PTR peptide transporters found in all organisms studied to date. The power of yeast molecular biology serves to make this system extremely useful as a model for studying the flux of peptides across cellular membranes. The 5th transmembrane domain (TM5) contains a motif (FYING) conserved in all PTR family members ranging from yeast to human. Ala-scanning mutagenesis on TM5 was performed to investigate its role in tranporter function. All mutant receptors, except one, were functionally expressed in the membrane. The preference of the remaining 21 mutants for different peptide substrates was determined by growth assays and radioactive peptide accumulation. Eleven mutant receptors exhibited a clear preference for particular peptides for growth and uptake. Mutations within the conserved motif (I251, N252 and G254) resulted in the most striking changes in substrate preference. A recent phosphoproteome analysis using mass spectrometry identified four phosphorylated serines in Ptr2p in the cytoplasmic N- and C-termini. To investigate the functional role of these phosphoserines, we created a series of mutants where serine was converted to alanine to mimic unphosphorylated serine. Immunoblot analysis of the membrane fraction showed greater accumulation of mutant Ptr2p in the membrane compared to wild-type indicating that phosphorylation regulates its half-life by providing a signal for removal from the cytoplasmic membrane. These studies should help guide the design of substrates targeted for cellular uptake and reveal strategies to enhance or inhibit transporter function.

P252

THE STUDY OF COMBINATION ANTP-P16 PEPTIDE WITH CONVENTIONAL CHEMOTHERAPEUTIC AGENTS IN VITRO

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As was shown previously chimeric peptide that consist the fragment of Antp and p16 protein (84-106) accumulate in different cell types and has apoptotic effect. The aim of investigation: to analyse combined effect of chimerical cell penetrating peptide Antp-p16 with conventional chemotherapeutic agents: taxol, 5-FU and etoposide in vitro. Methods: cytotoxic effect and level of apoptosis in A549 and 293 cell lines were measured by flow cytometry after 24h of agent addition. The tested concentrations were 0.1 – 5mkM for chemotherapeutic agents and 1 – 10 mkM for peptide. Results: We show that combined effect of p16-Antp peptide with etoposide and Antp-p16 with 5-ftoruracil was additive (cytotoxic and apoptotic) for both cell lines. The usage of taxol with the peptide has multiplicative effect on apoptotic level in 293 and A549 cell lines. Interesting, that combinations of 0.1 mkM taxol with 5 mkM p16-Antp or 1.0 mkM taxol with 5 mkM p16-Antp can exact the same level of apoptosis.

P253

THE IMPORT MECHANISM OF CATIONIC CELL-PENETRATING PEPTIDES AND ITS IMPLICATIONS FOR THE DELIVERY OF PEPTIDE INHIBITORS OF SIGNAL TRANSDUCTION

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Recently, it was shown that cationic cell-penetrating peptides (CPPs) enter cells by endocytosis[1]. In this contribution we demonstrate evidence supporting an import of cationic CPPs along the retrograde pathway[2]. In addition we address the relevance of the endocytic uptake for the delivery of inhibitors of intracellular signal transduction by CPPs. In MC57 fibrosarcoma cells the inhibitors of endosomal acidification chloroquine and bafilomycin A1 abolished the release of the peptides into the cytoplasm. Furthermore, brefelding A, a compound interfering with trafficking in the trans-Golgi network inhibited peptide uptake. In contrast, nordihydroguaiaretic acid, a drug that stimulates the retrograde movement of Golgi stacks and trans-Golgi network to the ER, promoted a cytoplasmic localization of Tat-peptides in Hela cells. The presence of intact peptides was demonstrated by mass spectrometry of cell lysates. The relevance of the import mechanism for the bioavailability of functional peptides was confirmed using a peptide that interferes with signalling in apoptosis. Cells were loaded with fluorescein-labelled analogues of the peptide alone or a penetratin fusion construct either by electroporation or by penetratin-mediated uptake. Electroporation achieved a much higher activity than penetratin-mediated import demonstrating that for the cytoplasmic delivery of functional peptides the mode of delivery and the biological activity are strongly interconnected. 1. J.P. Richard et al., J.Biol.Chem. 278, 585-590 (2003). 2. R. Fischer, K. Köhler, M. Fotin-Mleczek, R. Brock, J.Biol.Chem. (2004).

P254

DIFFERENTIAL PLATELET RESPONSES MEDIATED BY ALPHAIIB BETA3 MEMBRANE PROXIMAL-DERIVED PEPTIDES AND THEIR ROLE IN PLATELET ACTIVATION

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The integrin aphaIIbbeta3 plays an important role in thrombus formation by permitting the crosslinking of adjacent platelets by fibrinogen molecules following platelet activation. It has previously been demonstrated that the alpha-subunit membrane-proximal KVGFFKR motif plays an integral role in integrin activation. However, how this activation process actually occurs still remains unclear. Previous work within our laboratories demonstrated the ability of a lipid-modified cell-permeable peptide, palmitylated-KVGFFKR peptide (Pal-FF) to induce platelet aggregation equivalent to that of thrombin. It is speculated that Pal-FF can compete for endogenous cytoplasmic sequences, thereby displacing the hypothetical tether, which constrains the integrin in its quiescent state. Alanine scanning identified the central GFFK residues as critical for biological activity. A library of palmitylated 7-mer peptides was then prepared by substituting F-residues with various amino acids. The importance of the side chains at positions 4 and 5 has been investigated by modifying their steric, chiral and hydrophobic properties. Pal-F4F5, Pal-F4A5 and Pal-A4F5 peptides were capable of initiating platelet aggregation, thromboxane synthesis and extensive tyrosine phosphorylation of intracellular signalling molecules, comparable to that of thrombin. The double alanine-substituted peptide (Pal-A4A5) exhibited a minimal agonist effect but inhibited thrombin-initiated bi-directional alphaIIbbeta3 signalling. This antagonist profile applied to platelet aggregation, integrin activation, alpha-granule secretion, thromboxane synthesis and the phosphotyrosine profile. These results highlight the differential platelet responses mediated by membrane proximal-derived peptides. They demonstrate a requirement for both the phenylalanine residues to be present to preserve a resting integrin and demonstrate the Pal-A4A5 peptide as a potential inhibitor of integrin function.

P255

STUDIES OF CELL-PENETRATING PEPTIDES WITH FLUORESCENT CORRELATION SPECTROSCOPY

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Due to the cell membrane barrier, only a limited variety of compounds enters the intracellular milieu. Hydrophilic compounds are especially hindered, unfortunately, excluding potential drugs. A novel group of transport vectors, cell-penetrating peptides (CPPs), is able to enhance the uptake of hydrophilic molecules, however, there is no satisfactory explanation for the mechanism of internalization so far. Fluorescence correlation spectroscopy (FCS) was introduced in early 1970 but has recently become useful to bioscience due to increase in sensitivity. It is a single-molecule detection method in a tiny confocal volume (0.2 fL) which allows the study of fluorescently labeled ligand with receptors. We labeled the CPPs Tat(48-60), transportan 10 and penetratin with the fluorophore Alexa 488 and studied their uptake into HeLa cells and Bowes melanoma cells with FCS. For displacement studies and calculations of specific bindings on the cell membrane, the cells (ca. 4000) were exposed to 100 nM – 1.0 µM concentrations of the labeled peptides and unlabeled peptides. The experiments were carried out at 4 C, 15 C, 26 C, and 37 C for kinetics studies. The peptides are seen inside the cells after ca. 1 min and inside the nucleus shortly after.

P256

CELL UPTAKE PROPERTIES OF CIS-γ-AMINO-L-PROLINE OLIGOMERS

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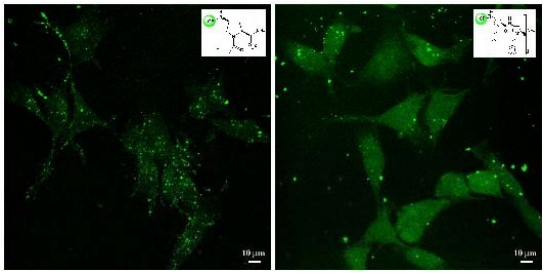
A large number of natural peptides based on distinct protein sequences show good cell-uptake capacity in eucaryotic cells. Positive charges and hydrophobicity are the main determinants of good cell uptake. The mechanism involved in cell uptake of peptides is unclear. Although endocytosis is the most common, penetration across the membrane for cationic peptides may also occur. Proline-rich peptides have good cellular uptake capacity. Herein, the capacity of a new γ-peptide family derived from the cis-γ-amino-L-proline to translocate across the cell membrane is discussed. Structure-internalization relationship studies were performed using several γ-aminoproline peptides in COS-1 and HELA cells. Cytotoxicity assays, confocal microscopy and quantification using fluorimetry and flow cytometry were used to elucidate the uptake behaviour of the distinct γ-peptides. Our results (Fig. 1) indicate that these peptides have an excellent cellular uptake efficiency. Figure1. Confocal images of two distinct peptides based on the cis-γ-amino-L-proline monomer. Conditions: 10 micromolar of peptide, incubate 2 hours in COS-1 cells.



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EFFECT OF SILAPROLINE SURROGATE IN A PRO-RICH CELL PENETRATING PEPTIDE

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Silaproline (Sip) is a proline analogue in which its gamma-methylene carbon is replaced by a dimethylsilyl group. It is highly lipophilic since its partition coefficient is 14 times greater than that of Pro [1] so it should favour the interaction with an amphipathic environment as is the cell membrane. In this context, we have recently reported a new family of amphipathic Pro-rich cell penetrating peptides (CPPs) [2,3]. The goal of the present study was to examine the influence of a silaproline residue on the peptide conformation and translocation properties of these Pro-rich CPPs. A proline residue from the original sequence (VRLPPP)3 was exchanged by a silaproline analogue resulting in VRLPPSip(VRLPPP)2. The effect of this modification on the secondary structure and aggregation properties of the Pro-rich CPP was studied by two different biophysical techniques CD and TEM. The internalization through the cell membrane of HeLa cells of this new silaproline-containing derivative was also studied by confocal microscopy and flow cytometry. [1] F. Cavelier, B. Vivet, J. Martinez, A. Aubry, C. Didierjean, A. Vicherat, M. Marraud, J. Am. Chem. Soc, 124, 2002, 2917-2923. [2] J. Fernández-Carneado, M. J. Kogan, S. Castel, E. Giralt, Angew. Chemie Int. Ed., 2004, in press. [3] J. Fernández-Carneado, M. J. Kogan, S.Pujals, E. Giralt, Biopolymers (Pept. Sci)., 2004, in press.

P258

IN VITRO AND IN VIVO APPLICATIONS OF DOUBLY-LABELLED FLUORESCENT CELL-PENETRATING PEPTIDES

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Peptide-mediated import attracts growing attention as a delivery technology. Recently, cell-penetrating peptides have been demonstrated to be taken up by endocytosis (1;2). The revision of the import mechanism raises key questions on the cytoplasmic release of the CPPs and their possible degradation by endolysosomal proteases. In order to address the structural integrity of cationic CPPs along the endocytic pathway we synthesized a penetratin analogue terminally labelled with tetramethyl-rhodamine and fluorescein using our newly developed procedure (3). In buffers the fluorescence of both fluorophores is almost completely quenched due to intramolecular dimer formation of the dyes. Upon in vitro proteolysis the fluorescence of both dyes was enhanced dramatically. The doubly-labelled penetratin was readily taken up into cells. Different subcellular localization of both dyes detected by confocal laser scanning microscopy were indicative of an intracellular proteolytic break-down. However, incubation of the peptide with synthetic phospholipid vesicles also led to an increase of fluorescence. In order to avoid ambiguities in the interpretation of cellular peptide stabilities caused by the environment dependence of its fluorescence properties we performed fluorescence emission measurements in whole cell lysates. In summary, our results indicate that the doubly-labelled penetratin analogue will be useful to study (i) intracellular routing, (ii) intracellular peptide stability and (iii) peptide-membrane interaction of CPPs. [1]J.P. Richard et al., J.Biol.Chem. 278, 585-590 (2003). [2]R. Fischer, K. Köhler, M. Fotin-Mleczek, R. Brock, J.Biol.Chem. (2004). [3]R. Fischer, O. Mader, G. Jung, R. Brock, Bioconjugate Chem. 14, 653-660 (2003).

P259

CATIONIC CELL-PENETRATING PEPTIDES MODULATE CELLULAR SIGNAL TRANSDUCTION BY PROMOTING RECEPTOR INTERNALIZATION

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Cationic cell-penetrating peptides (CPPs) have been widely used as a delivery vector for the non-invasive import of different cargos into eukaryotic cells. Recent works demonstrated an endocytosis-based uptake mechanism of cationic CPPs. CPPs exploit the endocytic machinery to reach the cytoplasm. However the endocytic pathway is critically involved in signal transduction events originating from cell membrane receptors. Therefore we investigated the influence of CPPs on the Tumor Necrosis Factor Receptor1 (TNF-R1)-mediated signal transduction. We found that cationic CPPs strongly promote TNF-R1 internalization. Induction of receptor internalization was dependent on the peptide concentration and occurred immediately after peptide addition. Laser scanning microscopy data demonstrated co-localization of CPPs and TNF-receptor molecules within the endocytic compartments. FACS-analysis revealed that incubation of HeLa cells with 20 µM penetratin peptide for 1 h significantly reduced the number of cell membrane associated receptor molecules. The stimulation of TNF-R1 normally leads to NF-kappaB dependent gene induction and can also induce apoptosis in the presence of cycloheximide. However both TNF responses were inhibited in Hela cells pre-incubated with penetratin peptide. Cationic CPPs also interfere with Fas- and TNF-R2-mediated signalling pathways indicating strongly side effects of these peptides on signal transduction events. Interestingly, in case of Toll-like receptor 4 (TLR4), CPP-mediated receptor internalization did not lead to the inhibition of signalling but was sufficient to induce TLR4 dependent signal transduction even in the absence of its ligand lipopolysaccharide. Our data demonstrate for the first time, that cationic cell-penetrating peptides interfere with different signalling pathways via induction of receptor internalization.

P260

ARGININE-RICH PEPTIDES: THE STRUCTURES AND MANNERS OF INTERNALIZATION

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Using the HIV-1 Tat peptide as a membrane permeable carrier vector, successful delivery of various proteins into cells to control cellular function have been reported. We have shown that various arginine-rich peptides, including that derived from HIV-1 Rev protein and even oligoarginine peptides can also serve as carrier vectors [1]. The internalization mechanism of these vector peptides and their protein conjugates was previously regarded as one away from endocytosis, but the recent reevaluations have been elucidating that a certain endocytosis is involved in the intracellular delivery using these peptides. Here we show that the uptake of octaarginine (R8) peptide by HeLa cells was significantly suppressed by a macropinocytosis inhibitor, ethylisopropylamiloride (EIPA), suggesting that macropinocytosis plays a crucial role in the internalization of the peptides. Real time imaging of the cells treated with R8 peptide revealed the active movement of endosome-like structures. Chain length dependency of oligoarginine peptides was observed in the internalization efficiency and the contribution of macropinocytosis. Considerably less susceptibility to EIPA for the cellular uptake of penetratin, which is also a representative carrier peptide, was observed. Although penetratin and R8 peptides have basic characteristics in common, the above facts suggested that the manners of internalization seem to be considerably different from each other. Based on these findings, the effects of carrier peptide structures on the intracellular delivery of cargo molecules will be discussed. [1] Futaki. S., et al., (2001) J. Biol. Chem. 276: 5836.

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UPTAKE OF CELL-PENETRATING PEPTIDES IS DEPENDENT ON THE PEPTIDE-TO-CELL-RATIO RATHER THAN ON PEPTIDE CONCENTRATION

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Little information is available concerning the influence of cell-state on CPP uptake and degradation of labelled peptides. Particularly degradation could seriously affect uptake as well as intracellular distribution of the label. Here, the influence of the cell-culture age, peptide-to-cell-ratio and energy depletion on uptake and degradation of the cell-penetrating peptide (CPP) MAP was investigated. At given peptide concentration and incubation volume the intracellular concentration of peptide increased with lower cell number. At given cell number, doubling of the incubation volume increased intracellular peptide concentration to a similar extent as a doubling in incubation concentration.. No influence of the peptide/cell ratio was found for the cellular uptake of peptide nucleic acid (PNA), or a non-amphipathic MAP analogue, investigated in parallel for comparison purposes. Energy depletion resulted in significantly reduced quantities of intracellular fluorescence label. However, we show that this difference is mainly due to a membrane impermeable fluorescent-labelled degradation product, which is lacking in energy-depleted cells. The mechanism of its generation is not likely to be endosomal degradation of endocytosed material, as it is not chloroquine sensitive. With view at the practical aspects of using CPPs, this means that the peptide/cell ratio has at least the same importance for the uptake of CPPs as the used peptide concentration

P262

STRUCTURE-ACTIVITY RELATIONSHIP STUDY OF THE CELL-PENETRATING PEPTIDE PVEC

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Cell-penetrating peptide pVEC is derived from murine vascular endothelial cadherin. It has previously shown that pVEC is able to translocate across various types of cellular membranes, without causing any noticeable disturbance in its structure. In order to define the amino acid residues responsible for the translocation through the cell membrane, the structure-activity relationship study of pVEC was performed. Together with the alanine-substituted peptides, the retro-pVEC, D-isomer, and scrambled sequence of pVEC are included for comparison. The uptake of fluorescently labeled peptide analogues was quantified in non-fixed Bowes melanoma cells with or without the presence of endocytosis inhibitors. Herein we show that the alanine substitution of the six respective N-terminal amino acids decreases the uptake significantly while substitution in C-terminus tends to increase the translocation properties. The internalization abilities of retro-pVEC and D-isomer are comparable to pVEC, while scrambled sequence did not internalize in any noticeable extent. The presence of endocytosis inhibitors did not have any noticeable effect to the internalization of peptides. The data we present show that the charge distribution in N-terminal hydrophobic part is substantial for efficient cellular translocation.

P263

MECHANISMS OF NON-COVALENT PEPTIDE MEDIATED CELLULAR DELIVERY OF THERAPEUTICS: INFLUENCE OF THE CONFORMATION

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Two different N-acetylated and C-ter cysteamide cell-penetrating peptides, MPG (GALFLGFLGAAGSTMGAWSQPKKKRKV) and Pep-1 (KETWWETWWTEWSQPKKKRKV) were shown to promote non-endosomal intracellular delivery of non-covalent bound cargos, namely nucleic acids and proteins, respectively. In order to identify the peptide mediated internalization pathway, we undertook conformational investigations of both peptides with and without associated cargos and checked the conformational consequences of the presence of phospholipids. From the conformational point of view, Pep-1 behaves differently from MPG. CD analysis revealed a transition from a non-structured to a helical conformation upon increase of the concentration while MPG remained non-structured. Determination of the structure by NMR showed that in water, its α-helical domain extends from residue 4 to 14. CD and FTIR indicated that Pep-1 adopts a helical conformation in the presence of phospholipids while MPG is in a β-sheet form. Adsorption measurements performed at the air-water interface were consistent with the helical form. Pep-1 did not undergo conformational changes upon formation of a particle with a cargo peptide. In contrast, we observed a partial conformational transition when the complex encountered phospholipids. For MPG, interactions with nucleic acids generated a partial folding into β-sheet which was more pronounced in the presence of lipids. Electrophysiological measurements showed that both peptides, whether associated or not with their cargo, can induce transmembrane ionic currents. Therefore, we propose that the membrane crossing processes involve formation of transient transmembrane pore-like structures. For Pep-1, these are based on the association of helices while MPG induces formation of a β-barrel structure instead.

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CELL PENETRATING PEPTIDES IN CELLULAR MICROARRAYS

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Peptide microarrays have become an important tool for highly parallel and miniaturized profiling of humoral immune responses1. In order to extend the microarray technology for profiling of cellular immune responses, we developed a cell chip for the localized transfer of antigenic cell penetrating peptides into antigen presenting cells (APC). Peptides were fluorescently labelled to monitor peptide release and uptake. Nanoliter volumes of peptides were spotted onto a hydrogel composed of a polyethylene glycol (PEG) matrix followed by incubation with APCs. For this purpose peptides have to be released from the substrate and taken up into the cells for processing and presentation by major histocompatibility complexes (MHC). Localized cellular uptake was achieved for peptides immobilized by non-covalent interactions with the PEG matrix. To increase the uptake efficiency, peptides were conjugated to the cell-penetrating peptide penetratin. Several different conjugates of the H-2Kb-restricted epitope SIINFEKL were tested with this system. The uptake that depended on the peptide composition was compared by fluorescence microscopy. The uptake of antigenic peptides from the substrate provides a range of options for the analysis of antigen processing. The cell chip will enable the profiling of T cell activities for epitope identification and immune diagnostics using minimum amounts of reagents and cells in future applications.

P265

SENSITIVITY OF HUMAN CARCINOMA CELL LINES TO A CYTOTOXIC CELL PENETRANT MIMETIC OF P21

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The disruption of proliferating cell nuclear antigen (PCNA) has been demonstrated to result in the inhibition of cell growth. In this study, a cytotoxic cell penetrant mimetic of the cyclin dependent kinase, p21, was used to disrupt PCNA function to cause the apoptosis of human carcinoma cells. PCNA is heavily implicated in DNA replication and repair, acting as a vehicle for numerous protein-protein interactions. A consensus sequence, QXX(I/L/M)XX(F/H/D)(F/Y), known as the PCNA interacting protein (PIP) box, has been identified within several proteins including polymerase d and p21. It is via the PIP box sequence that many interactions with PCNA are thought to occur. DNA replication can be inhibited by the interaction of p21 with PCNA when DNA damage occurs. DNA fidelity is ensured by prevention of cell cycle progression by the action of p21. A chimeric peptide construct (P10-Tat) was developed based on the p21 PIP box sequence known as P10 and utilises a fragment of the HIV-1 replication protein, Tat, to enable the construct to be nuclear and cell penetrating. This study shows the comparative cytotoxicity of P10-Tat upon the human carcinoma cell lines MCF7 (breast carcinoma), SW480 (colon adenocarcinoma), U373 MG (glioblastoma astrocytoma) and U251 MG (glioblastoma). Significant cytotoxic effects, believed to be attributable to the disruption of PCNA function, were observed with the P10-Tat peptide.

P266

SYNTHESIS AND IN VITRO ACTIVITY OF ARG OLIGOPEPTIDE CONJUGATES WITH ANTITUMOUR AND ANTIMICROBIAL DRUGS

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The use of oligopeptides with intracellular transporting ability to translocate covalently attached drugs across the cell membrane has a great potential for improved therapies. Arg-based oligopeptides derived from the Arg-rich Tat protein domain are considered as one of the most efficient delivery agent [1]. In order to study the effect of oligoarginine on the cargo-related functional activity several conjugates were prepared. These conjugates were synthesised by solid phase methods or by solution conjugation using various linkages (amide, thioether or disulfide). Among the cargo components an antitumour/antimicrobial drug, methotrexate, an antimicrobial peptide (the cecropin A - melittin hybrid CA(1-7)M(2-9) [2], H-KWKLFKKIGAVLKVL-NH_2) and ferrocenecarboxylic acid were used. After HPLC purification and ESI-MS identification comparative studies were performed to determine the antitumour or antimicrobial properties of the free and oligoarginine attached compounds. Supported by the Hungarian Research Fund (OTKA No. T043576 and TS 44742), the Ministry of Culture (NKFP 047/2001), and Hungarian-Spanish Intergovernmental Programme (4/2001) [1]Tung, C.H., R. Weissleder, R. (2003) Arginine containing peptides as delivery vectors. Advanced Drug Delivery Reviews 55: 281–294 [2]Rivas, L., Andreu, D. (2003) in Pore forming peptide and protein toxins (Menestrina, G., Dalla Serra, M., Lazarovici, P., eds) Taylor & Francis, London, pp. 209-259.

P267

STUDY OF THE ELECTROSTATIC INTERACTION BETWEEN CHARGED LIPIDS AND PEPTIDES AT THE MEMBRANE SURFACE USING MOLECULAR DYNAMICS SIMULATION

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It was experimentally proved that many peripheral proteins use electrostatic interactions to bind to biological membranes. The aim of this paper is to analyze the lipids-peptides electrostatic interactions at the surface of the cell membrane using a model at the atomic level and the simulation of the molecular dynamics technique. There are in the literature some recent papers which study this problem but they use a phenomenological approach for the electrostatic interactions. This is correct for longer distances between peptides and membranes but at the interface membrane-solution the phenomenological approach is incorrect. In order to reveal the importance of the electrostatic interactions for the peptide binding we used two membranar systems; one of them contains only electrically neuters lipids palmitoyl-oleoyl-phosphatidyl-choline (POPC) and a positive charged peptide (protonated glutamate and arginine) and the other system contains electrically neuters lipids (POPC), negatively charged lipids palmitoyl-oleoyl-phosphatidyl-serine (POPS) and the same peptide. In the paper we present the steps used for building the systems and we compare for the two membranar systems the results of the molecular dynamics simulation for 1 ns. We also compare our results with those obtained using the phenomenological approach.

P268

MIMICKING OF HES-1 BY A CELL-PENETRATING PEPTIDE INDUCES NEURONAL DIFFERENTIATION IN HUMAN STEM CELLS

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Transduction across biological membranes is one of the key elements in cell related science and pharmaceutical research. Several methods have been developed to decrease the side effects and increase the cellular uptake of bioactive molecules and one novel delivery method is a peptide carrier system based on cell penetrating peptides. Although cell-penetrating peptides have been shown to increase the cellular uptake of several molecules both in vivo and in vitro, the transduction pathway remains unknown. Various peptide sequences with different properties have been proposed to have membrane translocating activity and in this study we compare a selection of the most well known cell-penetrating peptide sequences and their utility as delivery vectors for an antisense-oligo in a GFP based reporter system.

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INTERNALISATION OF SIRNA BY CELL PENETRATING PEPTIDES AND DOWN-REGULATION OF GALANIN RECEPTOR TYPE 1

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The recent discovery that small double stranded RNA can specifically silence gene expression has received a lot of attention. It is called RNA interference (RNAi) and is mediated by short interfering RNA (siRNA), which cause degradation of homologues single stranded mRNA. RNAi holds great potential as a basic research tool and as therapeutic agent. So far delivery to cells has mostly involved lipid based transfection agents in vitro or hydrostatic pressure in vivo. In order to introduce a new delivery method for siRNA, the fact that siRNA can be modified at some positions without significant loss of efficiency has been used to link them to cell penetrating peptides (CPP). The siRNA has been modified in three different ways: by addition of a thiol group, extending the sense strand to produce an overhang and introduction of a loop structure between the sense and antisense strands. The CPPs Transportan 10 and pVEC have been used to deliver the modified siRNA. This has been done by formation of a disulfide bridge and by coupling a PNA sequence to the CPP, which can hybridise to the loop and overhang structure on the modified siRNA. All the complexes are internalised by bowes melanoma cells as indicated by fluorescein labelling of siRNA. Coupling of siRNA and CPP by a disulfide linker show down-regulation of galanin receptor subtype 1 at mRNA and protein levels.

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CROTAMINE IS AN EFFICIENT IN VITRO AND IN VIVO DNA DELIVERY PEPTIDE FROM THE VENOM OF RATTLESNAKE CROTALUS DURISSUS TERRIFICUS

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Crotamine is a small lysine- and cysteine-rich protein of 42 amino acids from the venom of the South American rattlesnake. We showed, in the micro molar range it proved non-toxic to any of the cell cultures and animals tested. In vitro, as well as, in vivo Cy3-conjugated crotamine can penetrate within a few minutes into different cell cultures and murine tissues. In the cytoplasm, crotamine specifically associates with centrosomes, thus allowing to follow the process of centrioles duplication and separation. In the nucleus, it appears on the chromosomes at S/G2 phase, when centrioles start division as shown by fluorescent confocal laser scanning microscopy. In metaphases, crotamine binds to the chromosomes. Crotamine is capable to bind to pEGFP-N1 plasmid, which contained the enhanced version of the green fluorescent protein (EGFP) reporter gene. Peptide - DNA binding was demonstrated by means of gel retardation assay. We showed that peptide – DNA condensates possessing a calculated charge ratio (NH4+ : PO4-) 3:1 and 6:1 were efficiently internalized (~100%) in vitro by different murine cell lines: fibroblasts (3T3); melanoma (B16); embryonic stem cells (USP-2) and human carcinoma cells (HCT116).The condensates were injected intraperitoneally into mice and a strong green fluorescence was observed in different tissues after 3, 24 and 48 hours. Moreover, strong fluorescent signal was shown in more than 50 % of murine bone marrow cells and other tissues 30 days after injection. Thus, we demonstrated that crotamine is a novel nonviral efficient in vitro and in vivo DNA delivery system.

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THE LIQUID PHASE SYNTHESIS OF CELL PENETRATING PEPTIDES - OLIGONUCLEOTIDES CONJUGATES

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Covalent conjugates of cell penetrating peptides with different molecules, in particularly with oligonucleotides, have attracted an attention during last years as potential therapeutic agents. Two main strategies have been proposed for preparing peptide-oligonucleotide hybrid molecules, i.e. the solid phase synthesis of the whole conjugate and the assembling of peptide and oligonucleotide fragments in the liquid phase. The incompatibilities of the peptide and oligonucleotide chemistries lead to a restricted number of sequences that can be prepared by the solid phase strategy. The liquid phase fragment conjugation strategy could avoid these limitations, because oligonucleotides and peptides are synthesized separately, and then they are combined; nevertheless, this approach has also some problems mainly associated with the solubility of the reaction components. The present work was aimed on the elaboration of the direct oligonucleotide and peptide coupling via the phosphoramide bond in aqueous, aqueous-organic and organic media. Two methods were used for this synthesis: phosphorylation of a peptide amino group by an oligonucleotide terminal phosphate 1-hydroxybenzotriazole ester in aqueous media or condensation of phosphate and amino groups in presence of triphenylphosphine, 2,2'-dithiopyridine and 4-dimethylaminopyridine in organic media. Peptides derived from the third helix of the homeodomain of Antennapedia, the influenza envelope hemagglutinin subunit, melittin and polymyxin B were chosen to improve antisense oligonucleotide penetration inside cells. Several oligonucleotides, including an 18-mer antisense oligodeoxyribonucleotide complementary to an internal coding region of the reporter gene of the green fluorescent protein (GFP) were used for the conjugate synthesis. This work was supported by the RFBR grant 03-04-48927.

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CONJUGATION OF PEPTIDE OR PROTEIN MOLECULES WITH QUANTUM DOTS

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Semiconductor quantum dots (QDs) are light-emitting nanoparticles [1].In recent years, quantum dots are emerging as promising alternatives to chemical fluorophores and visible fluorescent proteins molecular probes[2].Several recent publications have demonstrated the successful application of bioconjugated quantum dots in cell biology for labeling cells and macromolecular constituents of cells. To make quantum dots useful in molecular and cellular imaging applications, elaboration of strategies for the conjugation or adsorption of molecules of interest is needed for these nano-particles. In this paper, we studied conjugation conditions of QDs to peptide or protein molecules. We tried to give optimal reaction time and pH for the conjugation reactions. We employed HPLC for fast and simultaneously detection and purification of the QDs-tagged molecules from the reaction mixture. In addition, we modified the conjugation reaction conditions to enhance the fluorescent properties for QDs-tagged peptides. The QDs-tagged peptide or protein molecules generated and purified by these strategies give superior results in cell labeling. [1] Bruchez, M. Jr et al. Semiconductor nanocrystals as fluorescent biological labels. Science 281, 2013-2015 (1998) . [2] Chan,W.C.W. et al. Luminescent QDs for multiplexed biological detection and imaging. Curr. Opin. Biotechnol. 13, 40-46(2002).

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TARGETING OF QUANTUM DOT-SIGNAL PEPTIDES BIOCONJUGATES IN LIVING CELLS

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Semiconductor quantum dots (QDs) that conjugate with bio-recognition molecules have recently attracted widespread interest in biology and medicine. QDs bioconjugates that are highly luminescent and stable are thought to have potential as novel intravascular probes for both diagnostic and therapeutic purposes. These bioconjugates raise new possibilities for studying genes, proteins and drug targets in single cells, tissue specimens and even in living animals. We set out to explore the feasibility of signal peptides targeting in cells by using QDs. We show that 3-Mercaptopropyl acid-stabilized CdTe nanoparticles coated with nuclear targeting signal (NTS) and endoplasm targeting signal (ETS) accumulate selectively in organelles of different cell lines after endocytosis; furthermore the cells remained stably labeled for over a week as they were maintained in the culture. These results encourage the construction of more complex bioconjugates with capabilities not only for targeting in living cells, but also for diagnostics and drug delivery. References: 1. Xingyong Wu, Hongjian Liu, Jianquan Liu, Kari N. Haley, Joseph A.Treadway, J. Peter Larson, Nianfeng Ge, Frank Peale, and Marcel P. Bruchez Immunofluorescent labeling of cancer marker with quantum dots Nature Biotechnology (2003) 21, 41-46 2. CHEE-KAI CHAN, and DAVID A JANS Using nuclear targeting signals to enhance non-viral gene transfer Immunology and Cell Biology (2002) 80, 119–130.

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TRANSCELLULAR PASSAGE OF CELL-PENETRATING PEPTIDES

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The cell- barriers are essential for the maintenance and regulation of the microenvironments of the human body. Cell-penetrating peptides have simplified the delivery of bioactive cargoes across the cell plasma membrane. Here, the passage of three cell-penetrating peptides: transportan and the transportan analogue, transportan 10, and penetratin, across a Caco-2 human colon cancer cell layer in vitro was investigated. The peptides were internalised in the epithelial Caco-2 cells as visualised by indirect fluorescence microscopy and quantified by fluorometry. Studies of peptide outflow from cells show that the peptides were in equilibrium across the plasma membrane. Moreover, the ability of the peptides to cross a Caco-2 cell layer was tested in a two chambered model system. After 120 min, 7.0%, 2.8% and 0.6%, respectively, of added transportan, transportan 10 and penetratin were detected in the lower chamber. However, both transportan and transportan 10, reversibly decreased the transepithelial electric resistance (TEER) of the barrier model with a minimum after 60 min with 46% and 60% of control, respectively. Penetratin did not affect the cell layer to the same extent. Although, transportan markedly increased the passage of ions, the paracellular flux of 4.4 kDa fluorescein labelled dextran was limited. In conclusion, the obtained results indicated that the transportan peptides passed the epithelial cell layer mainly by a mechanism involving a transcellular pathway.

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MEMBRANE INTERACTIONS OF PEPTIDES DERIVED FROM THE N-TERMINUS OF UNPROCESSED PRION PROTEINS, COMPARISONS WITH PENETRATIN AND MELITTIN

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We studied the membrane perturbation effects of peptides with sequences derived from the N-terminus of mouse and bovine prion proteins (mPrPp and bPrPp, respectively). The mPrPp has earlier been demonstrated to become incorporated into various cell lines and function as a cell-penetrating peptide (CPP). By use of confocal fluorescence microscopy, we observed that also bPrP translocates through the plasma membrane of live HeLa cells. The membrane interaction of the PrP-derived peptides was studied and compared with effects of penetratin, a classical CPP, and the pore forming peptide melittin. Induced secondary structures, changes in membrane order, and leakage in phospholipid vesicles of varying charge densities were studied, as well as haemolysis in red blood cells. Unlike the α-helical melittin, penetratin and the two prion peptides undergo an α-to-β structural conversion when interacting with membranes of increasing negative surface charge densities. The structural conversion is concomitant with an increase in the degree of membrane order and leakage caused by those peptides. The prion peptides exhibit a significant degree of membrane perturbation and induced leakage at μM concentrations even at the low surface charge densities, similar to melittin, suggesting pore formation. The degree of haemolysis caused by the peptides followed the same order as was observed with the induced leakage effects on the vesicles: penetratin, bPrP, mPrP and melittin, most effective. The behavior of the prion-derived peptides shows that uncleaved signal peptides in the PrPs may play a role for the neurotoxic effects and membrane catalysed structure conversions of the proteins.

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ENHANCEMENT OF INTRACELLULAR GENE DELIVERY BY A DERMASEPTIN-DERIVED KARYOPHILIC CELL PENETRATING PEPTIDE

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Over the past few years, the discovery that a number of small peptides, defined as cell penetrating peptides (CPPs), are able to efficiently cross cell membranes through a mechanism that avoids lysosomal degradation, and the ability shared by several of these peptides to accumulate inside the nucleus, have been enthusiastically considered of key interest for the development of new therapeutic approaches, including gene therapy. Despite the extensive research on the unusual ability of these peptides to translocate across cell membranes, the mechanism of their internalization remains poorly understood. Reports that peptide penetration is a consequence of artifactual observations caused by a redistribution of surface-bound peptides upon cell fixation, and results of criterious reevaluations of the cellular uptake of these peptides that are consistent with the involvement of an endocytic process, have recently added great controversy to this field. Recently, the S4(13)-PV karyophilic cell penetrating peptide was described by Hariton-Gazal et al. (2002). The main goals of the present work were to evaluate the ability of this peptide to penetrate and accumulate inside the nucleus of live cells, as well as its capacity to enhance intracellular/nuclear gene delivery. Analysis of the internalization of the peptide into cells under conditions in which different internalization pathways are selectively compromised, clearly demonstrated that the uptake mechanism is distinct from endocytosis. Additionally, the biological performance of different peptide-DNA conjugates was evaluated in terms of their capacity to deliver DNA into the cell nucleus and of the subsequent efficacy of transgene expression.

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CONFORMATIONAL SOLUTION STUDIES IN DIMETHYL SULFOXIDE OF A PROTEGRIN PEPTIDE IB-367 USING NMR SPECTROSCOPY

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A synthetic analogue of antimicrobial cationic peptide protegrin-1 IB-367, is a 17-amino-acid peptide with amidated C-terminus (RGGLCYCRGRFCVCVGR-NH_2). It is thought to form an antiparallel β-sheet, with two intramolecular disulfide bridges between C5 – C14 and C7 – C12 residues. The IB-367 displays broad spectrum of an antimicrobial activity and rapid killing of pathogens by interaction with their cell membrane. The proper conformation of peptide is responsible for the entry of IB-367 into the microbial cell membrane and as the consequence, for the catalytic activity. In this study we investigate the solution structure of IB-367 in dimethyl sulfoxide (DMSO-d6) using 2D NMR spectroscopy and theoretical methods. The solution structure of IB-367 was established using the NOE spectroscopy. The peptide was found to occur as one stable isomer with all-trans geometry of the peptide bonds. For distance-geometry and simulated-annealing calculations the XPLOR program was used. The results of our researches were compared to published information about protegrin-1 and the other analogues in order to propose a new, cheap in synthesis peptide with the similar to the IB-367 antimicrobial activity. This work was supported by State Committee for Scientific Research (KBN), Grant DS/8372-4-0138-4.

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INTERNALIZATION OF CELL-PENETRATING PEPTIDES IN PLANT CELLS

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Mammalian cells are protected from their chemical environment by the cell membrane, which excludes the majority of molecules that are not actively imported by living cells. The delivery of large, hydrophilic molecules to the cytoplasm and nucleus of mammalian cells is problematic due to their poor plasma membrane permeability. In the last few years there has been a development of cell-penetrating peptides (CPPs), peptides that can enter mammalian cells and carry cargo molecules with molecular weights several times their own weight over the plasma membrane. We have studied and compared the uptake of fluorescein labelled CPPs, transportan, TP10 and penetratin, in plant cells and mammalian cells. The plant cells used were N. tabacum protoplasts and the mammalian cells were Bowes melanoma. The cells were exposed to a 5 μM peptide solution for 30 min and the internalized peptides were visualized by microscopy and quantified by fluorimetry measurements. Transportan has the highest uptake of the examined peptides in both mammalian cells and plant cells with an internalization of 13.0 % of added fluorescence in Bowes melanoma cells and 13.8 % of added fluorescence in N. tabacum protoplasts. The internalization of CPPs in plant cells opens up a new and better method for genetic modification of plants. By coupling conjugated peptides, oligonucleotides, plasmids and nucleic acids to CPPs can it be possible to effectively transport them inside the plant cells.

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THE INFLUENCE OF TWO ENVIRONMENTAL PEPTIDES, VALINOMYCIN AND CEREULIDE, TO HUMAN NK CELLS

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Two related cyclic peptides, valinomycin and cereulide, are produced by bacteria found in food or/and environment. Valinomycin is produced by several species of Streptomyces, whereas cereulide is produced by Bacillus cereus strains, frequently present in food, such as rice or beans. Valinomycin is commonly isolated from moisture-troubled buildings, where occupants suffer from respiratory infections. These lipophilic ionophoric toxins passively penetrate through biological membranes into tissues and cells, causing health hazard. There are no known methods to eliminate these stable toxins from food or environment. We have earlier showed that valinomycin and cereulide are toxic especially to normal human NK cells as compared with T cells, monocytes or cultured cell lines, and detected as reduction of the NK activity, mitochondrial swelling, increased apoptosis and diminished cytokine production. These toxic effects were seen with physiologically relevant concentrations. We have now measured cytokine production of peripheral blood lymphocytes in the presence of valinomycin. When primed with IL-4, the valinomycin-exposed lymphocytes of different individuals produced highly different amounts of IL-4. Roughly, donors could be divided into strong producers, intermediate producers and non-producers. It will be interesting to study whether the differences in IL-4 production reflect to degree of symptoms in individuals exposed to valinomycin in moisture-troubled buildings or individuals exposed to cereulide, coming from food.

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DIFFERENT WAYS FOR MAGNETIC RESONANCE IMAGING TO REACH THE CELL NUCLEUS

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Until now, the use of gadolinium contrast agents in Magnetic Resonance Imaging (MRI) was limited to the extracellular space. We demonstrate several novel methods with which to accumulate gadolinium-complexes into the cytoplasm and finally into the cell nucleus. The gadolinium-complex was attached to different FITC-labeled transmembrane transport peptides and peptides faciltating the nuclear uptake. Magnetic Resonance Imaging (1.5 and 3 Tesla field strength), FACS-Analysis, Confocal Laser Scanning Microscopy (CLSM) and ICP-MS were performed to filter out those conjugates with the best cellular and nuclear import characteristics in 9 different human and rat cell lines (glioma, bone marrow, colon carcinoma). By use of magnetic resonance imaging, Gd3+ was detected in all cell lines still after 10 minutes. The nuclear signal, which has lasted for almost four weeks, was confirmed with CLSM. In contrast the cytoplasmic signal was washed out already after a few hours. Due to the long-term and bright staining of the cell nuclei, our conjugates seem to be promising MRI contrast agents with which to label migrating stem cells. Additionally they could serve as a platform for future contrast agents with which to measure the activity of nuclear DNA-repair enzymes before chemotherapy.

CELL PENETRATING PEPTIDES

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PROTEIN TRANSDUCTION PROPERTIES OF CELL-PENETRATING PEPTIDES

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Cell penetrating peptides have been applied for cellular delivery of a wide variety of biologically relevant macromolecules last years. Though their mechanism of internalization is still not known and under debate, a method enabling cellular delivery of a functional and intact cargo is of high importance. Whether there exists one common internalization mechanism or different peptides use divergent pathways has to be examined. We assessed the internalization kinetics of fluorescein-labeled avidin complexed with biotin-tagged cell penetrating peptides: penetratin (pAntp), pVEC, Tat(48-60) and transportan in live HeLa cells by flow cytometry and in cell lysates by fluorescence spectrometry. Protein delivery efficiency of pTat and transportan was about 10-fold higher compared to pAntp and pVEC when detected by flow cytometry and 2-3 fold by spectrofluorimetry. The difference between the results of two methods could stem from entrapment of peptide-protein complexes into endocytic structures and reduced emission of fluorescein in acidic vesicles of live cells. This may also suggest that transportan and pTat after entering cells could escape from vesicular structures and translocate to cytoplasm more efficiently than pAntp and pVEC. Cellular transduction of avidin reached maximum in about 12 h for three peptides but transportan, which was not saturable even in 24 hours. In live cells transportan-avidin complexes did not localize in vesicles containing Alexa 594-labeled transferrin or other endosomal/lysosomal markers suggesting clathrin-independent uptake mechanism. Substantial colocalization with cholera toxin B was detected by fluorescence microscopy suggesting involvement of cholesterol-rich plasma membrane subdomains in transportan-mediated protein uptake.

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TOWARDS THE DESIGN OF PORE-FORMING PEPTIDES

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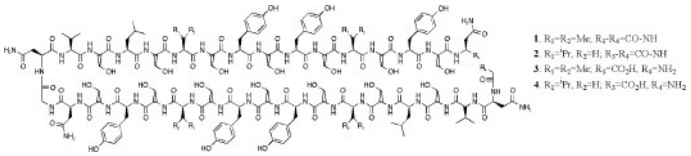
Statistical exercises to determine amino acid preferences at specific positions in beta-hairpins of beta-barrel membrane proteins have allowed the design of a cyclic peptide template that inserts into synthetic membranes at low peptide:lipid ratios (≥1:3000) to form channels that are at least 1 nm in diameter (Ref 1). In current work we are probing the structure of the pores formed by these peptides in membranes by Atomic Force Microscopy (AFM), laser tweezing of liposomes (Ref 2), and linear dichroism in order to ascertain their key structural features; namely the diameter of the pores, and their angle with respect to the membrane normal. These data will allow us to generate models for these structures based on natural pores in the PDB. Ultimately we intend to examine pore activity and structure as a function of peptide sequence, with the aim of designing pores with tailored properties. Reference 1: John M. Sanderson and Sarah Yazdani, Chem. Commun., 2002, 1154. Reference 2: John M. Sanderson and Andrew D. Ward, Chem. Commun., 2004, submitted.



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TISSUE SPECIFIC PEPTIDES: ALTERNATIVE MECHANISMS OF NEGATIVE CELL PROLIFERATION CONTROL

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As was reported earlier, tissue specific peptide pools are composed by fragments of functional proteins. Extensive study of the peptides effects on cell proliferation has shown that the inhibitory components strongly dominate both in their number and efficacy, which allowed to postulate for them the role of negative tissue growth regulators. Representatives of 4 main structure families (hemorphins, two families of beta-actine fragments, short acidic peptides), being non-hemolytic at high doses (up to 0.1 mM) and exhibiting low effect in normal cells, are highly effective in suppressing tumor cell proliferation in a mode resembling classic opioid peptides (reversible cell cycle arrest followed by temporary resistance of the cells to the further action of the agent). Hemorphins bind to opiate receptors, while for other peptides no receptors on cell surface were found. The ability of the inhibitory peptides from different families to induce cross-resistance speaks of the junction of their signaling pathways at distal steps. Using fluorescent labeling (FTC, Dansyl), we have shown that one of the most active inhibitory peptides, the short acidic peptide 11.2 enters a tumor cell and within 15 min binds to intracellular target located in the cytoplasm in the nucleus proximity. The shown diversity of the targets triggering the same events (reversible delay of cell proliferation without cell damage) may speak of the universality of such mechanism of tissue growth control in the organism.

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SYNTHESIS OF FLUORESCEIN LABELED PEPTIDES FOR SPECIFIC CELL TARGETING STUDIES

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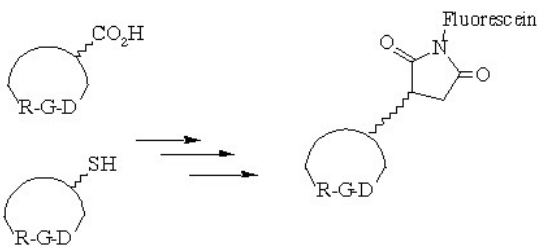
Since RGD peptides (R = arginine; G = glycine; D = aspartic acid) have been found to promote cell adhesion 20 years ago [1], this short sequence has been largely used for academic studies and medicinal applications. Our purpose is based on the labeling of small cyclic peptides containing the RGD sequence to examine cellular uptake and specific cell targeting of the fluorescein cargo. In this communication, synthesis of cyclic peptides specifically labeled on acidic or thiol containing side chains with different fluorescein maleimide derivatives will be described. Such labeling strategies with several synthetic peptides will allow extensive studies to rationally evaluate the parameters governing either cellular internalisation or cell selectivity. References: [1] Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule Pierschbacher MD, Ruoslahti E., (1984) Nature, 309, 30-33.



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SYSTEMATIC ANALYSIS OF CELL PENETRATING PEPTIDES TO DELIVER SIRNA MOLECULES

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We have performed a systematic analysis of two classes of cell penetrating peptides; hydrophobic peptides versus positively charged hydrophilic peptides to deliver siRNA. Studies were performed with peptides linked to the 5' end of the siRNA moiety or by complex formation by mixing of the two components. The capacity of these conjugates to translocate across the plasma membrane and to inhibit gene expression was also studied. The introduction of a viral fusogenic peptide to mediate escape of peptide-siRNA conjugates from endosomes and localize in the cytosol was also investigated. In addition, the de novo design of novel cell penetrating peptides with enhanced delivery capabilities will be discussed.

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CELLULAR UPTAKE OF PNA AFTER CONJUGATION WITH POSITIVELY AND NEGATIVELY CHARGED μ-HELICAL AMPHIPATHIC, ß-SHEET FORMING AND UNSTRUCTURED PEPTIDES

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In order to examine structural influences on the ability of peptides do deliver peptide nucleic acids (PNA) into intact cells we investigated the cellular uptake of PNA conjugated with various peptides showing different structure forming properties, charge and size. The cellular uptake was assessed by means of fluorescence-activated cell sorting (FACS), confocal laser scanning microscopy (CLSM) and capillary electrophoresis with laser-induced fluorescence detection (CE-LIF). The uptake into various cell types of the PNA-peptide conjugates assessed in this way differed not more than by an order of magnitude, irrespective of strongly different structural properties of the peptide moieties [widely unstructured: RQIKIWFQNRRMKWKK (penetratin); μ-helical amphipathic, positively charged: KLALK LALKA LKAAL KLA; μ-helical amphipathic, negatively charged: ELALE LALEA LEAAL ELA; ß-sheet forming: DPKGDPKGVTVTVTVTVTGKGDPKPD]. After energy depletion the conjugates with the acidic helical amphipathic and the ß-sheet forming peptides exhibited enhanced internalization related to that observed under normal conditions, whereas in the other cases the uptake was depressed. These findings infere the involvement of nonendocytic energy dependent besides energy independent and endocytic transport processes. The effects were found differently pronounced with various cell types, which promises a possibility of achieving a cell selective cargo delivery by exploiting differently expressed active transport systems.

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A NOVEL METHOD FOR PEPTIDE-MEDIATED DELIVERY OF PEPTIDE NUCLEIC ACIDS TO THE NUCLEUS

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Peptide nucleic acids (PNA) are uncharged DNA mimics, having a pseudopeptide backbone that makes them very stable in biological fluids. PNAs binds complementary RNA and DNA with high affinity and specificity. These qualities make PNAs leading agents among antisense and antigene agents We have developed a delivery system that shows efficient uptake and gene silencing. Several PNAs were conjugated to different LS (localization signal) peptides and a fluorescense marker. Uptake of PNA in living cells was studied by inverted fluorescense microscopy, confocal fluorescense microscopy and flow cytometry in different cancer cell lines. Results show that the efficiency of cellular uptake is highly dependent upon the net charge of the molecule. In contrast to earlier studies, our PNAs were not translocated directly into the nucleus, rather endocytosed. To overcome this barrier, we used a method termed photochemical internalization (PCI). PCI is based on photosensitizers, such as TPPS2a (disulfonated tetraphenylporphine), which localize in endocytic membranes and upon light exposure produce damage, thus causing the contents to be relased. After PCI treatment, PNAs were shown to be efficiently re-localized into the nucleus, regardless of the specific localization signal used. As a model system, the metastasis-relevant S100A4 gene was efficiently knocked down, as demonstrated by reduced aprotein levels in three different cell lines. Our results show a dose- and time-dependent inhibition of the S100A4 gene activity. In summary, the combination of conjugated PNA and PCI is a promising new method for efficient PNA delivery to the nucleus with resultant gene silencing.

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SELF ORGANISATION OF β-PEPTIDE HELICES BY NUCLEOBASE PAIRING

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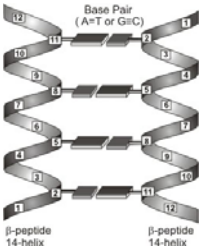
Helical tertiary structures are fundamental structural motifs of most of the natural proteins. They are widely involved in recognition and binding process between proteins or proteins and nucleic acids. β-Peptides can adopt a stable and defined helical secondary structure with as few as six amino acids [1]. In a β-peptide 14-helix side chains are oriented spatially in such a manner that every i and i+3 residues are oriented on the same side of the helix. Residues with the ability for molecular recognition, like nucleobases lead to reversible organisation of the helices towards tertiary structures (Figure). According to this model β-nucleo amino acids were incorporated into the β-peptides in every third position by solid phase peptide synthesis [2]. Self organisation of a variety of β-peptide helices by base pairing will be reported. The evidence of helix formation derived from CD spectra will be described. Furthermore, thermal stability (Tm) of the base pairing and thermodynamic stabilities of the duplex will be discussed. References: [1] a) R. P. Cheng, S. H. Gellman, W. F. Degrado, Chem. Rev. 2001, 101, 3219. b) D. Seebach, J. L. Matthews, Chem. Commun. 1997, 2015. [2] A. M. Brückner, P. Chakraborty, S. H. Gellman, U. Diederichsen, Angew. Chem. Int. Ed. 2003, 42, 4395.



P289

INVESTIGATION OF ALANYL-PNA AGGREGATION

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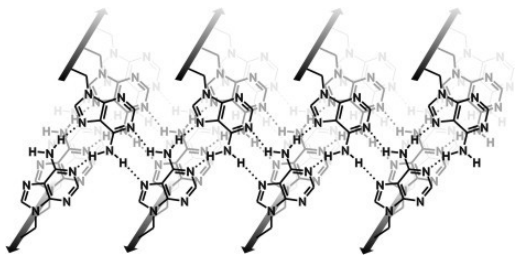
Oligomers based on a regular peptide backbone with nucleo amino acids of alternating configuration are called alanyl-peptide nucleic acids (alanyl-PNA)[1]. Their aggregates are based on the formation of hydrogen bonds between the nucleobases, as well as base stacking and hydrophobic interactions. Different types of aggregation can be obtained through sequence selection and homology. Homology of PNA can be performed by side chain homology in which the distance between backbone and nucleobases is enlarged by adding one or two methylene groups to give homoalanyl- and norvalyl-PNA[2]. Another type of homology is to extend the distance between the side chains using β-amino acids to give β-homoalanyl-PNA[3]. Complexes like dimers, hexamers or even higher ordered structures such as band like or plane like aggregates (Figure) can be designed[3]. Temperature dependent UV spectroscopy and CD spectroscopy indicate some kind of aggregation. To confirm these noncovalent complexes FT-ICR mass spectrometry is performed. [1] U. Diederichsen, Angew. Chem. Int. Ed. Engl. 1996, 445; [2] U. Diederichsen, N. J. Diezemann, E. Vockelmann, D. P. Weicherding, manuscript in preparation; [3] U. Diederichsen, H.W. Schmitt, Angew. Chem. 1998, 110, 312.



P290

PYRROLIDINE-BASED PEPTIDE NUCLEIC ACIDS WITH VARIOUS MAIN CHAINS

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Four stereoisomers of oxy-peptide nucleic acids and N-methylated peptide nucleic acids containing conformationally-restricted pyrrolidine rings in the main chains were synthesized and investigated for binding to DNA.

PEPTIDE NUCLEIC ACIDS

P291

TOPOLOGY AND SECONDARY STRUCTURE OF D, L - ALTERNATING PEPTIDES WITH RESPECT TO ZN(II) COORDINATION

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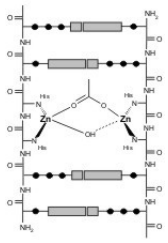
Responsible for efficient catalytic activity of metalloenzymes are active site cooperation of metal ions and amino acid side chains[1]. Furthermore metal binding sites incorporated in peptides achieve stabilization of secondary structures[2]. In order to prove the catalytic potential and cooperativity of Zn(II) centres, the assembly, orientation, and structural features of D,L-alternating peptides with respect to their topological evidence were examined. First D,L-alternating peptides with aromatic side chains were studied. X-ray studies of a tyrosine oligomer have shown that two right handed helices dimerize as antiparallel helices with all side chains oriented to the outside[3]. Incorporation of histidine in these helices gave metal binding sites which lead to stronger aggregation of the dimers. Alanyl peptide nucleic acids[4] provide a second environment for Zn(II) binding sites (Figure). The linear and well defined topology of alanyl-PNA represents an excellent tool in order to organize Zn(II) ligands within a hydrophobic environment surrounded by nucleobase pairs. The distance and the amount of Zn(II) coordination sites can be fine tuned by using side chain homology of alanyl-PNA. The D,L-alternating oligomers containing metal binding sites were examined regarding stability and catalytic activity. [1] J. E. Coleman Curr. Op. Chem. Biol. 1998, 222. [2] M. Albrecht, P. Stortz, R. Nolting Synthesis 2003, 9, 1307. [3] E. Alexopoulos, A. Küsel, I. Usón, U. Diederichsen, G. M. Sheldrick, manuscript in preparation [4] U. Diederichsen, Angew. Chem 1996, 35, 445.



P292

CONFORMATION OF FUSOGENIC PEPTIDES AND AN ANTISENSE OLIGONUCLEOTIDE IN COMPLEX IN THE PRESENCE OF MICELLES

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The elucidation of the mechanism of the translocation of antisense oligodeoxynucleotides (ODN)s is an important step in evaluating their therapeautic applications. Cationic peptides or lipides, fusion peptides, etc. are considered as possible candidates to deliver hydrophilic molecules into the cytosol. The complete mechanism of translocation is still mostly unknown. One main point is that whether any particular secondary structure has to be induced on the surface of the membrane in order to allow translocation. In present work we analyzed the conformation of different carrier peptides and an antisense ODN by circular dichroism (CD) spectroscopy in solution and in the presence of anionic lysolipid micelles. The following peptides were studied: (i) the third helix of Antennapedia homeodomain, pAntp-(43-58,(ii) its analogues where Try48 and Try50 were replaced by Phe, (iii) the amphiphilic 364-407 sequence of the Flock-house virus FHV gamma peptide and its N- (364-384)and C-terminal (390-407) sequences. The antisense ODN used in these experiments was that targeted towards the green fluorescent protein (GFP) mRNA with the following sequence d(GAG CTG CAC GCT GCC GTC). The most significant conformational changes were observed in [ODN-pAntp-(43-58)] and [ODN-FHV(390-407)] complexes. The pAntp-(43-58) forms a strong complex with ODN through electrostatic interaction resulting in profound changes in the conformation of both the peptide and the ODN. In the case of FHV C-terminal peptide the complex formation occurs without altering the structure of ODN, and the changes in the peptide CD spectrum reflect the insertion of the complex into the micelle.

P293

TARGETING OF PNA TO A CYTOPLASMIC VIRUS

D. Musumeci[1], G. Roviello[1], M. Valente[1], E. Bucci[1], C. Pedone[1], R. Palumbo[1], D. Capasso[1], M. Schmidtke[2], R. Zell[2]

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Peptide nucleic acids (PNA) are among the most powerful oligonucleotides analogues, with a N-(2-amino-ethyl)-glycine unit replacing the sugar-phosphate backbone. They show strong and sequence specific hybridization to complementary single stranded DNA and RNA as well as to double stranded DNA by triplex formation. In addition, this type of DNA mimic is not degraded by nucleases and proteases and was shown to have both good antisense and antigene activity. Recently we have reported the design, synthesis and characterization of a PNA able to bind specifically to a control region of Coxsackievirus. In particular, the target system is the 5´-nontranslated region (5´-NTR) of Coxsackievirus B3 (CVB3), which is an important human pathogen. Regulatory regions often are unique in sequence and/or structure, suited for specific recognition with a low risk of nonspecific side effects. The 5´-NTR of CVB3 is a key player in the assembly of the viral replication complex which contains also viral and host cell proteins. We have demonstrated that the synthesised PNA binds to the target RNA disrupting its native structure and inhibits the interaction with the RNA of a key viral protein, the protease 3Cpro. We report here the PNA entrance in HeLa cells and its antiviral activity.

P294

SYNTHESIS OF POLYFUNCTIONALIZED PYRROLO[3,2-D]PYRIMIDINES: INTERCONNECTING PEPTIDE AND NUCLEOBASE CHEMISTRY

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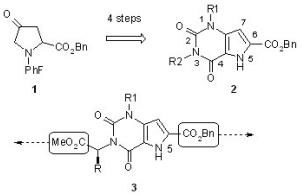
We have recently developed a novel method for synthesizing 7-deazapurines with variation of the pyrimidine ring nitrogen substituents. Benzyl 2,4-dioxo-2,3,4,5-tetrahydro-1H-pyrrolo[3,2-d]pyrimidine-6-carboxylates 2 were prepared in four steps in 37-55% isolated overall yields from 4-oxo-N-(PhF)proline benzyl ester 1. In view of creating nucleobase-peptide hybrid molecules, this protocol was further evaluated using amino acid-derived building blocks. This yields pyrrolo-[3,2-d]pyrimidines of type 3 which can be functionalized at the two orthogonally protected acid functions, and by alkylation of N-5. A first example hereof is disclosed in this poster.



PEPTIDE NUCLEIC ACIDS

P295

MOLECULAR RECOGNITION OF PNA-DNA HYBRIDS FORMED BY CHIRAL AND ACHIRAL PNAS BY DUPLEX INTERACTIONS WITH AGGREGATES OF A CYANINE DYE

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Peptide nucleic acids are mimics of DNA which hybridize to complementary DNA, RNA or PNA strands according to Watson-Crick rules. We recently introduced a modified 'chiral box' PNA containing three D-Lys side chains in the middle of the sequence, which showed a great improvement in the mismatch recognition [1]. The symmetrical 3,3'-diethylthiadicarbo-cyanine dye has been demonstrated to bind to achiral PNA-DNA duplexes resulting in an instantaneous color change from blue to purple, providing a simple visual indication for PNA hybridization [2]. In this work we present studies made on achiral and 'chiral box' PNA probes in the presence of the cyanine dye. The interaction to the different PNA-DNA duplexes and the binding specificity have been evaluated by UV and CD. We have studied different experimental conditions in order to minimize the intrinsic binding affinity of the dye to the PNA strands alone, by diminishing the polarity of the environment or by introducing competing negatively charged molecules. From these preliminary experiments, the high sequence specificity of the 'chiral box' PNAs is confirmed, allowing to find the best experimental conditions in order to rapidly and visually detect the presence or the absence of a specific DNA sequence. [1] Sforza, S. et al., Eur J. Org. Chem. 2000, 2905; Corradini, R. et al. J.Mol.Rec. 2004, 17, 76. [2] Smith J.O. et al., J. Am. Chem. Soc., 1999, 121, 2686; Wilhelmsson L.M. et al., Nucleic Acids Res., 2002, 30, e3; Komiyama M. et al., J.Am.Chem Soc., 2001, 123, 9612.

P296

SYNTHESIS AND STRUCTURAL STUDIES OF MUCIN-LIKE GLYCOPEPTIDES

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Mucins are cell surface or secreted proteins, rich in alpha-O-GalNAc-linked glycosylated serine and threonine residues. Mucins and mucin glycoprotein domains serve a wide range of biological and pathological functions. Different conformations of a glycoprotein, modified glycosylation patterns, and sequences have a significant influence on their functions, which are still not fully understood. To elucidate the structure-function relationships in the post-translational glycotransferease reactions that generate the glycoprotein, a series of glycopeptide enzyme substrates are being prepared by chemical synthesis for NMR conformational analysis. Fmoc-Thr(alpha-Ac3-D-GalN3)-OPfp and Fmoc-Ser(alpha-Ac3-D-GalN3)-OPfp are prepared by Koenigs-Knorr condensation of Fmoc-Thr-OPfp and Fmoc-Ser-OPfp with 3,4,6-Tri-O-acetyl-2-azido-2-deoxy-alpha-D-galactopyranosyl bromide or 3,4,6-Tri-O-acetyl-2-azido-2-deoxy-beta-D-galactopyranosyl chloride [1]. These building blocks are being incorporated by SPPS methods into glycopeptides based on the sequence $PTTTPLKNH_2$, with permutations of glycosylation on the T residue (e.g. PT(alpha-D-GalNAc)TTPLKNH_2). As there is a profound organizational effect on the peptide backbone with a-linked glycans [2], our NMR studies of these glycopeptides, taken with the knowledge of their behavior as enzymatic substrates [3], will provide a basis for understanding the enzymatic selectivity responsible for specific patterns of glycosylation, and more generally the conformational preferences of mucin motifs. [1] Lemieux R. U., et al. Can. J. Chem., 1979, 57, 1244. [2] Coltart, D. M., et al. J. Am. Chem. Soc., 2002, 124, 9833. [3] Takeuchi, H., et. al. Eur. J. Biochem., 2002, 269, 6173. Supported by NIH Grant GM 66148

P297

SYNTHESIS AND BIOLOGICAL APPLICATIONS OF COMBINATORIAL NEOGLYCOPEPTIDE ARRAYS

M.R. Carrasco, M.D. Bernardina, R.T. Brown, K.A. Rawls, O. Silva

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We have developed a robust method for the synthesis of small combinatorial neoglycopeptide arrays. One of a set of novel N-alkylaminooxy amino acids is incorporated into a peptide by standard solid-phase peptide synthesis methods, and the resulting N-alkylaminooxy side chain of the unprotected peptide can be reacted chemoselectively with a large variety of native reducing sugars. Thus, with a small set of peptides, one can quickly generate a large set of neoglycopeptides. We are now using our synthesized neoglycopeptide arrays to explore biologically-relevant questions regarding the effect of attached sugars on peptide structure and function. Here we present details of the optimized synthetic procedures and our current progress in structure/function studies.

P298

LIPOPHILIC DERIVATIVES OF OCTREOTATE FOR THE TUMOR SELECTIVE TRANSFECTION WITH LIPOSOMES

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The transfection of human cells is predominantly achieved by the use of modified viruses. These demonstrate a small infection efficiency and no selectivity for tumor tissue. Now the question arises whether analogs of somatostatin are suitable for the selective transport of liposomal particles for gene therapy. As an optimized carrier system cationic liposomes shall be modified with peptides, which bind to the somatostatin receptors on tumor cells. For this purpose somatostatin receptor affine peptides are synthesized and linked to a lipophilic moiety. These peptides can be integrated into plasmid containing liposomes. Depending on the structure of the membrane-bound peptides the influence of the selective transfection in somatostatin receptor positive tissues is to be examined in vitro and in an animal model. Different peptides with modifications of the lipid moiety (fatty acids or a lysine spacer comprising two fatty acid derivatives) and the spacer (polyethylene glycol) were synthesized by Fmoc solid phase peptide synthesis. The products are purified by RP-HPLC and characterized by size exclusion chromatography and MALDI TOF. The peptide can be inserted for into cationic liposomes containing for example N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammoniumchloride (DOTMA).

P299

SYNTHESIS AND BIOLOGICAL ACTIVITY OF THE EXTRACELLULAR IG DOMAIN OF EMMPRIN CARRYING VARIOUS CARBOHYDRATE CHAINS

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Emmprin is a glycoprotein located on the surface of tumor cells. It stimulates nearby fibroblasts to produce matrix metalloproteinases (MMPs)1), which are the key enzymes for tumor invasion and metastasis. Thus, the analysis of emmprin's function in detail will provide a clue to suppress tumor metastasis. However, as in other glycoproteins, the biological study of emmprin is hampered by the difficulties associated with microheterogeneity at the carbohydrate portions. As the functional site of emmprin is reported to be the extracellular first immunoglobulin (Ig)-like domain (34-94), which retains one N-glycosylation site at Asn44, the Ig domains (34-94) carrying various structurally distinct carbohydrates at Asn44 would be good models to study the role of emmprin as well as of its carbohydrate. In this study, we synthesized the first Ig domains (34-94) carrying various carbohydrates at Asn44 by the thioester method2) and their biological activity was measured. We also measured the CD spectrum of these domains to estimate the effect of the carbohydrate on the conformation of the Ig domain. References 1) Nabeshima, K., Kataoka, H., Koono, M. and Toole, B. P. (1999) in Collagenases (Ed.: Hoeffler), R. G. Landes Company, Texas, pp. 91-113. 2) a) Hojo, H., Watabe, J., Nakahara, Y., Nakahara, Y., Ito, Y., Nabeshima, K., Toole, B. P. (2001) Tetrahedron Lett. 42, 3001-3004. b) Hojo, H., Haginoya, E., Matsumoto, Y., Nakahara, Y., Nabeshima, K., Toole, B. P., Watanabe, Y. (2003) Tetrahedron Lett. 44, 2961-2964.

P300

THE CHEMICAL AND ENZYMATIC STABILITY OF THE GLYCATION PRODUCTS DERIVED FROM OPIOID PEPTIDES

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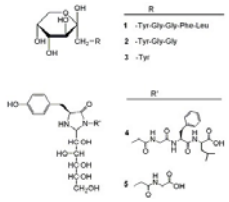
Nonenzymatic glycation is posttranslational modification of peptides and proteins by sugars, which, after a cascade of reactions, leads to the formation of a complex family of irreversibly changed adducts implicated in the pathogenesis of human diseases. The work reported here was stimulated by the finding that hyperglycemia induces impaired functioning of the endogenous opioid system thus contributing to the worsening of diabetic complications. In order to better understand the metabolic and chemical behavior of the products formed by glycation of the opioid peptide, leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu), and of structurally related fragments, compounds 1-5 were prepared. The influence of N-glycation on the enzymatic and chemical stability of the parent peptide compound was studied in 80% human serum and phosphate buffer, pH 7.4. The results obtained in human serum indicated that the rates of degradation of the studied compounds depend on the structure and length of the peptide moiety. Amadori compound 1 and imidazolidinone 4 were slowly degraded by dipeptidyl carboxypeptidase(s) which cleave the Gly-Phe bond leading to compounds 2 and 5, respectively, as the main metabolites. The hydrolysis pattern of the studied Amadori (1-3) and imidazolidinone compounds (4, 5) in phosphate buffer and in human serum was not the same and appears to be specific for each substrate.



P301

NON-COVALENT STABILIZATION OF A BETA-HAIRPIN PEPTIDE INTO LIPOSOMES

D.W.P.M Löwik, J.T. Meijer, P.J.H.M. Adams, J.CM. van Hest

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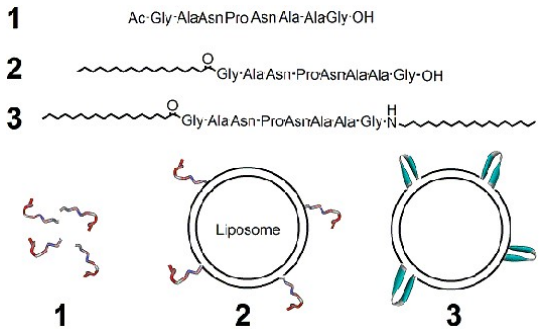
A novel non-covalent approach to stabilize secondary structures of peptides is presented, employing a liposome bilayer as a scaffold. Oligopeptides capable of forming e.g. beta-hairpins or helices can be modified on both the N- and C-terminus with hydrophobic moieties, allowing the peptides to be anchored at both ends in the dynamic bilayer, as shown schematically below. Our synthetic strategy allows us to introduce a variety of N- and C-terminal hydrophobic modifications on any peptide entirely on the solid phase. This in our view simple approach, results in stabilization of the folding pattern without much interference with the dynamic character of the peptide, contrary to covalent methods used to impose secondary structure. These peptides structures have been tested for their ability to mimic a folded peptide as found in nature in its native protein.



P302

A CYCLIC SIALO-GLYCOPEPTIDE LIGAND FOR SIALOADHESIN SYNTHESIZED ON GENERALLY APPLICABLE PHOTOLABILE LINKERS

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Solid-phase synthesis has been applied successfully in the preparation of peptides and glycopeptides. Crucial for an overall synthesis strategy towards a glycopeptides is the selection of an appropriate linker for the attachment to the support. In the solid phase synthesis of sialo-glycopeptides both acidic and basic conditions are needed to provide sufficient orthogonal protection [1]. Common acid and base labile linkers, cleave under such conditions. To circumvent this problem a handle, which cleaves under different mild conditions, e.g. photolysis, may be employed. The currently most advanced photolabile linkers are the activated O-nitrobenzyl linkers developed by Holmes [2]. However, these linkers suffer from only being applicable for terminal amides or carboxylic acids. In the present work these linkers have been developed to facilitate more general use for linking to heteroatoms in the peptide. Embedded in the presentation of a cyclic sialoglycopeptide synthesis, the concepts of a Photolabile backbone Linker (PhoB), as well as a photolabile Tryptophan linker, are introduced. Their general applicability will be exemplified by synthesis of small molecule libraries as well as in the synthesis towards a sialo-glycopetide. This constrained cyclic glycopeptide was designed from a crystal structure as a high affinity ligand for sialoadhesin. Its structure was modelled to fit the active site of Siglec1, based on crystal structures of sialoadhesin complexed to a promising hit from a sialic-glycopeptide library [1]. [1] K.M. Halkes, P.M.S. Hilaire, P.R. Crocker, M. Meldal J. Comb. Chem. 5, 2003, 18-27 [2] C.P. Holmes, J. Org. Chem. 62, 1997, 2370-2380.

P303

β-HAIRPIN GLYCOPEPTIDES AS MIMETIC AUTOANTIGENS IN MULTIPLE SCLEROSIS

B. Mulinacci[1,2], I. Paolini[1,2], A. Carotenuto[3], B. Mazzanti[1,2], M. Pazzagli[1,4], M. Chelli[1,2], F. Lolli[1,5], P. Rovero[1,6], A.M. Papini[1,2]

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The glycopeptide CSF114(Glc) [1], containing a beta-D-glucopyranosyl residue linked to an Asn residue, is the first synthetic antigen able to detect specific antibodies (Abs) by ELISA on sera of patients affected by Multiple Sclerosis (MS). We are demonstrating that CSF114(Glc) could mimic a glycosylated autoantigen responsible of an antibody-mediated MS. CSF114(Glc) is characterized by a beta-hairpin structure between the residues 2 and 14, with a beta-turn type I'. We synthesized beta-hairpin sequences containing Asn(Glc) fixed and glycopeptides without secondary structure [2], and we showed that a beta-hairpin and the length of the glycopeptides play a fundamental role in exposing at the best the epitope Asn(Glc) in the solid-phase conditions of ELISA (SP-ELISA). To optimize the length of the synthetic mimetic antigen to be used in SP-ELISA, we synthesized several glycopeptides, all containing Asn(Glc), shortened at both termini to find out the minimal glycopeptide sequence. To study the role of the turn in the auto-Ab recognition, we synthesized linear and cyclic CSF114(Glc) analogues with the residues GN instead of NG (to get a beta-turn type II'), and with the consensus sequence GNXT. Moreover, we introduced also D amino acids in position 8, to obtain different beta-turn. The glycopeptides and the corresponding unglycosylated sequences were tested in SP-ELISA for auto-Ab recognition, in comparison to CSF114(Glc). [1] Papini, A.M., Rovero, P., Chelli, M., and Lolli, F. (2002) PCT/EP02-06767 20020619. Priority: IT 2001-FI114 20010622. [2] Mulinacci, B., et al. In E. Benedetti and C. Pedone (Eds.), Peptides 2002, Ziino, Naples, Italy, 388-389 (2002).

P304

CLICK AND LIGATION CHEMISTRY FOR THE SYNTHESIS OF GPI-ANCHORED MEMBRANE PROTEINS

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Cell membrane proteins are anchored to the lipid bilayer by single or multiple insertion of transmembrane helices or by post-translational lipidation such as fattyacylation of amino and thiol groups, isoprenylation of cysteine residues and rather commonly even by C-terminal amidation with glycosylphosphatidylinositols (GPI). Although the synthesis of complex GPIs were recently accomplished [1,2], methods for their selective grafting to C-termini of proteins are still not available. Taking advantage of the well established native ligation chemistry for assembly of semisynthetic proteins, we have elaborated a new approach for a GPI-type C-terminal lipidation of proteins by exploiting the copper-(I)-catalyzed 1,3-dipolar cycloaddition of terminal alkynes to azides [3], i.e. the highly selective click chemistry [4]. For this purpose dimyristoylphosphatidylethanolamine was converted to the azide by metal-catalyzed diazo transfer reaction from triflyl azide. The azide was used in synthetic model peptides for 1,2,3-triazole formation with the alkyne side chain of C-terminal propargylglycine (Pra). Both selective lipidation of Pra and ligation of the model peptides via their N-terminal Cys residues with peptide thiol esters in aqueous-organic media as well as in micelles confirmed the efficiency of this new synthetic strategy for the semi-synthesis of C-terminally lipidated proteins. [1] Xue, J., Shao, N., Guo, Z., J. Org. Chem. 68 (2003) 4020. [2] Pekari, K., Schmidt, R.R., J. Org. Chem. 68 (2003) 1295. [3] Huisgen, R. in 1,3-Dipolar Cycloaddition Chemistry (Padwa, A., ed.), Wiley, New York, 1984, pp. 1-176. [4] Rostovtsev, V.V., Green, L.G., Fokin, V.V., Sharpless, K.B., Angew. Chem. Int. Ed. 114 (2002) 2708.

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SYNTHESIS AND STRUCTURAL INVESTIGATIONS OF O-GLYCOSYLATED B-AMINO ACIDS AND B-PEPTIDES

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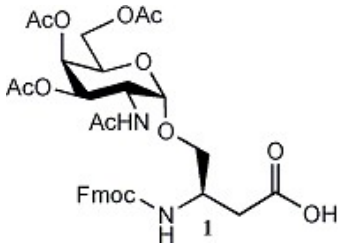
Oligomers of b-amino acids, so-called b-peptides, represent a new and exciting type of peptidomimetic foldamers. These molecules form more stable secondary structures than their natural analogues, and are also stable towards proteolytic degradation.[1] Glycopeptides includes a carbohydrate moiety which most often affects the structure and the biological activity of the peptide.[2] This has made glycopeptides interesting as tools in medicinal chemistry and chemical biology. To study the influence of glycosylation on b-peptides, the amino acid Fmoc-GalNAc-b-HSer-OH (1) has been synthesised and incorporated into a b-heptapeptide. The poster presentation will describe the synthesis and the structural studies of this novel type of peptidomimetic foldamers. [1]Frackenpohl J.; Arvidsson, P.I.; Schreiber, J.V.; Seebach, D., The Outstanding Biological Stability of b- and γ-Peptides toward Proteolytic Enzymes: An In Vitro Investigation with Fifteen Peptidases. ChemBioChem, 2001, 2, 445-455. [2] Chan, W.C.; White, P.D. Fmoc Solid Phase Peptide Synthesis- A practical approach, Chapter 11



P306

OPTIMIZATION OF SYNTHETIC STRATEGIES TO GLYCOSYLATED AMINO ACIDS

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Protein glycosylation plays an important role in the synthesis and biological activity of glycoproteins involved in autoantibody recognition, because they are exposed at the cellular membranes. Glycopeptide templates carrying several glycans and showing molecular mimicry of complex oligosaccharides between the host and pathogen, can be assembled using a building-block or a convergent approach. In the chemical synthesis of N-linked glycopeptides and glycopeptidomimetics, it was demonstrated that the most versatile methodology is the stepwise approach, in which protected glycosylamine is coupled to Fmoc-protected Asp-residue to give Fmoc-Asn(Sug)-OR to be used in peptide synthesis. A general strategy for this synthesis was developed and successfully applied to the synthesis of CSF114(Glc) analogues, up to now the first synthetic antigens that allow the detection of specific antibodies (Abs) in sera of patients affected by Multiple Sclerosis (MS) [1]. Our aim is the optimization of the crucial elements for this synthetic strategy adopted for a large scale the preparation of the target molecules. In particular, synthesis of sugar mimetics (i.e. pseudo imino-C-disaccharides), orthogonal protection of polyhydroxylated natural and imino sugars for SPPS, and conjugation of carbohydrate moieties to the side chain of Fmoc-Asp-OPfp are reported. The efficiency and potential versatility of this approach will be demostrated introducing the new glycosylated amino acids in beta-hairpin peptide sequences used to detect, by ELISA, Ab titre in MS patients. [1] Papini, A.M., Rovero, P., Chelli, M., and Lolli, F. (2002) PCT/EP02-06767 20020619. Priority: IT 2001-FI114 20010622.

P307

AN INNOVATIVE APPROACH TO THE SYNTHESIS OF N-GLYCOPEPTIDES

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N-Glycosylation is a co-traslational modification having a deep influence on protein folding. Glycosylation is often a difficult task in the solid phase synthesis of glycopeptides. They have been usually prepared using building blocks containing 'N-linked' carbohydrates obtained by coupling a glycosyl moiety with an Asn residues orthogonally protected for the solid phase strategy. In previous studies, we demonstrated that shortened sequences of CSF114(Glc) [1] were able to detect specific antibodies by SP-ELISA on sera of patients affected by Multiple Sclerosis [2]. Therefore, we propose innovative synthetic strategy to obtain glycopeptide sequences incorporating, in solid phase, suitable unglycosylated amino acids able to couple the sugar, before or after cleavage of the peptide from the resin. We selected two different synthetic pathways: 1) a chemoselective ligation approach leading glycopeptide mimetics, based on N(alpha)-Fmoc amino acid with a methoxyoxyamino side-chain protected as O-methyloxime for SPPS; 2) a convergent approach based on an Asp residue tridimensionally protected to allow glycosylation in solid phase. The insertion of the sugar in a late step requires low amounts of material and avoids exposure of the oligosaccharides to acidic conditions. The development of such a strategy could provide a flexible tool for the preparation of glycopeptides. [1] Papini, A.M., Rovero, P., Chelli, M., and Lolli, F. (2002) PCT/EP02-06767 20020619. Priority: IT 2001-FI114 20010622. [2] Mulinacci B., et al. in Peptide Revolution: Genomics, Proteomics & Therapeutics 2003, in press.

P308

RECOMBINANT SYNTHESIS OF THE RNASE A FRAGMENT 40-124 VIA PROTEIN SPLICING

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Protein splicing in conjunction with native chemical ligation opens many new possibilities in protein design and protein investigation. Usually, recombinantly synthesized proteins carry an N-terminal methionine. This is in contrast to the requirements for native chemical ligation, where C-terminal thioesters are coupled with protein fragments containing an N-terminal cysteine. This segment approach overcomes the limitations associated with the solid phase synthesis of peptides larger than 50 amino acids. We expressed the RNase A fragment 40-124 in an intein-system to serve as a building block for uniformly glycosylated RNase B. To obtain this fragment with an N-terminal Cys we cloned the DNA containing the sequence of the RNase A 40-124 into the pTWIN-1 vector. After overexpression of the 34.4 kDa fusion protein in E. coli K12 (B ER2566) inclusion bodies were found, which were solubilized in a strong detergent. The denaturated inclusion bodies were refolded using an optimized buffer under reducing conditions and the splicing reaction was induced by a pH-shift to 7.0. The key reaction was controlled by SDS -PAGE and resulted in the expected 25.1 and 9.3 kDa fragments. Finally, the fragment RNase 40-124 was concentrated by ultrafiltration and purified by RP-HPLC.

P309

GLYCOPEPTIDE DETECTION AND CHARACTERISATION WITH FAIMS AND LC-MS/MS USING A CAPLC Q-TOF SYSTEM

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High field asymmetric wave ion mobility spectrometry (FAIMS) separates ions in the gas phase based on species dependent changes in mobility observed at high electric fields. In addition, when a FAIMS device, such as the Ionalytics SELECTRA is fitted to the source of an electrospray mass spectrometer it causes a reduction in the spectral noise and allows a high transmission efficiency of ions due to an ion focusing mechanism, often resulting in an increase in signal compared to ESI without the FAIMS. The experimental parameter that reflects changes in ion mobility at high vs. low electric fields is called the compensation voltage. Therefore the selection of the desired ion species is achieved with the selection of the CV value associated with that species. Site specific analysis of protein glycosylation is fundamental to understanding protein function and the condition of cells or tissue. A tryptic digest of a glycoprotein can be analysed by LC MS, however the glycopeptide signal is often weak compared to the peptides, due to the heterogeneity of the carbohydrate moieties and the significant increase in mass. This work explores the gas phase ion separation of Glycopeptides using the FAIMS device. Our aim is to present results from the use of CapLC / FAIMS / Q-TOF to analyse a bovine fetuin tryptic digest

P310

CARBOHYDRATE-PEPTIDE MODEL SYSTEMS: TOOLS FOR STUDYING NON-ENZYMATIC GLYCATIONS OF PEPTIDE HORMONES IN VIVO

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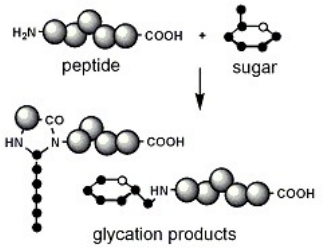
The non-enzymatic reaction of the amino groups of proteins, lipids and nucleic acids with reducing sugars (the Maillard reaction) results in the formation of rearranged and dehydrated adducts collectively termed advanced glycation end-products (AGEs). Because of the complexity and widespread patho-biochemistry of AGEs, the study of the Maillard reaction represents one of the most growing areas of research today. In order to gain better insight into the mechanism and products of the non-enzymatic glycation network, our work is focused on investigating structure-reactivity relationships of different monosaccharides (Glc, Man, Gal) in the Maillard reaction by using Leu-enkephalin and its shorter N-terminal fragments as starting peptide material. Model reactions were performed under different conditions using RP-HPLC as well as NMR and MS techniques for monitoring, separation and identification of the glycation products. We will demonstrate that the model established herein results in the formation of two different types of glycation products. In addition to Amadori compounds, novel carbohydrate-substituted imidazolidinones were formed through intramolecular cyclization of the initial Schiff base. The formation of these products provides a mechanism for generating Maillard reaction intermediates under physiological conditions and insights into the damage to endogenous peptides as a result of glycation by reducing sugars in vivo.



P311

PEPTIDES OF 13-METHYL-12-(ALPHA-L-ARABINOPYRANOSIL)-9-BROMO-INDOLO [2,3-A]PYRROLO[3,4-C]CARBAZOLE-5,7-DIONE

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Indolo[2,3-a]carbazoles and thier pyrrolo[3,4-c]-anellated variants represent a highly interesting class of natural and synthetic compounds as potential anticancer agents. The target enzymes for N-glycosides of indolo[2,3-a]carbazole derivatives are protein kinase C, CDK kinases and topoisomerases. Recently, it has been shown that pseudopeptide residues which are linked to indolo[2,3-a]pyrrolo[3,4-c]carbazole aglycone modify the range of its cytostatic activities. In our present studies we propose a method for the synthesis of peptides which contain the end NH_2-group blocked with carbazole.The solution of 13-methyl-12-(2,3,4-tri-O-acetyl-alpha-L-arabinopyranosil)-9-bromo-indolo[2,3-a] furano[3,4-c]carbazole-5,7-dione and a hydrochloride of the corresponding peptide in DMF at the presence of TEA was heated in the autoclave for 2 hrs at 130oC. During this procedure the nitrogen of the aminogroup built in the 5-member ring of carbazole to give peptide derivatives of the glycoside of indolo[2,3-a]pyrrolo[3,4-c]carbazole-5,7-dione (CAR). Per-O-acetylated CAR-Lys(Z)-Arg(NO2)-OMe, CAR-Lys(Z)-Arg(NO2)-NH_2, CAR-Lys(Z)-Thr-OMe, CAR-Arg(NO2)-Lys(Z)-Thr-OMe were deacetylated with K2CO3 in absolute methanol.The prepapative TCL was used for the isolation of compounds, and their structures were confirmed with the data of NMR-spectra and the elemental analysis. CAR-Peptides were studied in vitro on human leucose cell culture Jurkate(wt), and showed IC50 more than 10 micromoles.

P312

GHRELIN ACYLATION STRONGLY INFLUENCES ITS BINDING TO ALBUMIN AND LIPID BILAYERS

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Purpose: To investigate the effect of acylation on the ghrelin binding to (i) bovine serum albumin (BSA) and to (ii) a model of lipid bilayers i.e. large unilamellar liposomes. Methods: Radioiodinated Tyr4-human ghrelin (4hG) and Tyr4-human desacylghrelin (4hGNA) at 5 µg/mL were used to assess the binding to BSA (45mg/mL) and to liposomes (lipid/peptide concentration ratio 2000:1) using a dialysis chamber system. The radioactivity was measured using a gamma?counter. Human ghrelin (hG) and human desacyghrelin were used to assess the binding to liposomes (lipid/peptide concentration ratio 65.5:1). The liposome/peptide mixture was incubated and then ultracentrifugated. The supernatant was quantified at 210 nm following a RP-HPLC separation. Results: At 37 C the 4hG was bound at 49.1±2.8% to BSA whereas 4hGNA was significantly less bound (18.8±1.1%, $p<0.001$). Using the dialysis chamber system we found that at 4 and 25 C the 4hG was bound, respectively, at 81.7±1.5% and 85.0±0.4% to liposomes whereas 4hGNA was only bound at 40.5±6.0% ($p<0.005$). Using the ultracentrifugation method, we found that at 4 C and 37 C, hG was bound to liposomes at 95.3±1.5% whereas hGNA was only bound at 19.4±3.2% ($p<0.001$). Conclusion: Our data clearly showed that the acylation of ghrelin greatly enhances its binding to BSA as well as to the lipid bilayers even though the absolute binding levels differ with the method. The interaction mechanisms of human ghrelin and human desacylghrelin with the lipid bilayers will be further investigated.

P313

STUDIES ON GLYCATION OF PEPTIDES ON THE SOLID SUPPORT

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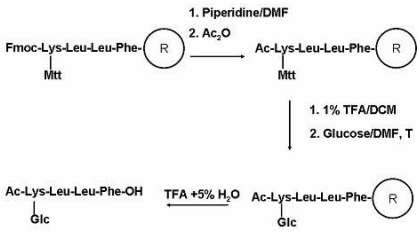
Glycation, the nonenzymatic reaction of protein modification by reducing sugars, is involved in diabetes, aging, and other pathological processes. This reaction is also important in food systems. Most data available on the glycation concern proteins. There are few reports only on preparation of glycated peptides and their biological and conformational properties. Peptides modified by glucose and other sugars are potentially useful research tools for studying allergenic properties of glycated proteins, for determination of the level of glycation of proteins, and as model systems for investigation of the mechanism of advanced glycation products formation. In this communication a simple procedure for glycation of peptides on the solid support compatible with Fmoc strategy is proposed. After completing synthesis of peptide on Wang resin, the side chain amino group of Lys is directly glycated with solution of glucose in DMF. Glycated peptide is cleaved and purified by standard techniques. Communication discusses the influence of reaction conditions on the yield of a product and stability of glycated peptide under cleavage conditions.



P314

SOLID PHASE SYNTHESIS OF GLYCOPEPTIDE THIOESTERS AS BUILDING BLOCKS FOR A UNIFORM RNASE B GLYCOPROTEIN

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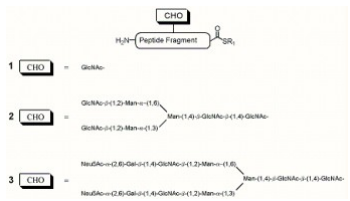
Glycoproteins play an important role in many biological processes and their synthesis has attracted much interest in recent years. Many methods have been developed to obtain glycoproteins, the most flexible being the native chemical ligation of chemically synthesized or recombinant fragments. The fragments containing the carbohydrate moiety are usually obtained chemically by solid phase methodology and the incorporation of the glycosylated amino acid into the peptide chain can be carried out using partially or fully protected carbohydrates. Ribonuclease (RNase) is an enzyme, which occurs in vivo both in the glycosylated (RNase B) and the non-glycosylated (RNase A) form. To investigate the total synthesis of uniformly glycosylated RNase B by native chemical ligation, two fragments are required. Besides the non-glycosylated RNase fragment 40-124, a glycosylated thioester of the RNase 1-39 fragment is also needed. To obtain this fragment three approaches were tested: The full length 1-39 glycopeptide was first synthesized using a GlcNAc-Asn building block (1). In a second approach the shorter fragment 29-39 was obtained inserting a chemically synthesized complex N-glycan (2) or a biantennary complex-type sialyl undecasaccharide (3). The syntheses were carried out by solid phase using a Safety Catch linker. After the activation of the linker, the glycopeptides were then released as a thioester.



P315

SOLID-PHASE SYNTHESIS OF OLEFIN-CONTAINING HTLV-1 PROTEASE INHIBITORS USING HORNER-EMMONS REACTION

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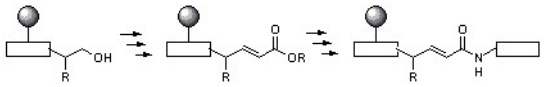
The introduction of an olefin structure into a peptide backbone is a promising approach for inducing conformational change, and thus is an important design consideration in peptidemimetics. In this paper, we determined for the first time that the olefin-containing peptide functions as an effective inhibitor for the human T-cell leukemia virus type-1 (HTLV-1) protease, for which few inhibitors have been reported to date. Because most syntheses of olefin-containing peptides were achieved by the conventional liquid-phase reactions and no general solid-phase procedure has been reported, we first established a conventional procedure for the solid-phase synthesis of olefin-containing peptides using Horner-Emmons reaction as a key reaction (Fig). The use of CLEAR support was critical to force the multi-step reactions on the solid support to completion. The established procedure can be easily applied to the combinatorial synthesis of a variety of olefin-peptide libraries. Using the olefin-peptide libraries containing the substrate sequence for the HTLV-1 protease, the specific substrate recognition of the protease was then clarified. Based on the results obtained above, a novel type of the protease inhibitor was prepared by the replacement of the scissile amide bond with an olefin bond. Thus, we found for the first time that the olefin peptidemimetic containing a part of the substrate sequence functions as an effective inhibitor for HTLV-1 protease.



P316

SYNHESIS AND BIOLOGICAL EVALUATION OF AN NONPEPTIDIC MIMETIC OF THROMBIN RECEPTOR

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Thrombin is a plasma serine protease that plays a critical role in hemostasis, as well as in thrombotic disorders and atherosclerosis. Proteinase-activated receptors are a recently described, novel family of seven-transmembrane G-protein-coupled receptors. Four PARs have now been identified PAR1, PAR2, PAR3 and PAR4. Thrombin receptor is activated by a novel mechanism in which thrombin cleaves its receptor's amino-terminal extension to reveal a new amino terminus that functions as a tethered peptide ligand. Synthetic thrombin receptor activating peptides (TRAPs) comprising only the first five amino acids (SFLLR) of tethered ligand, have been shown to be sufficient to possess thrombin mimetic activity. Recent structure activity studies in this laboratory have shown that a cluster of the two groups (phenyl, guanidine) together with an adjacent primary amino group is important for expression of maximum biological activity by thrombin receptor-derived peptides. In our effort to develop TRAP non-peptide mimetics useful in drug therapy we have synthesized a low molecular weight non-peptide mimetic (CIN3) which carries the pharmacophoric features of Phe and Arg residues, present in the active pentapeptide SFLLR. Compound CIN3 has been tested in rat aorta relaxation assay and it was found to stimulate the endothelial thrombin receptor in a concentration - dependent manner.

P317

A NOVEL BIOACTIVE INHIBITOR OF PROPROTEIN CONVERTASE FURIN THAT CONTAINS A BETA-TURN INDUCING ENEDIYNE AMINO ACID SCAFFOLD

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Furin is a member of mammalian subtilase called Proprotein Convertases (PCs). PCs are responsible for the tissue-specific cleavage of inactive protein precursors at the general motif (K/R)-(X)n-(K/R)↓, (where n= 0, 2, 4 or 6 and X is usually not a Cys), generating a large diversity of bioactive proteins and polypeptides in an exquisitely regulated manner. Furin has been implicated in various highly infectious diseases including ebola, avian Hong Kong, possibly SARS viruses and bacterial pathogenesis like anthrax and aerolysin. Owing to these findings we became interested to develop potent and specific inhibitors of furin with therapeutic potentials. Our approach is primarily based on the prodomain sequence of furin which is inhibitory to the cognate enzyme. Herein, for the first time we report that incorporation of a beta-turn inducing aromatic enediyne amino acid (Eda) moiety at the scissile P1-P1' peptide bond of a pro-domain furin peptide (residue 98-112) led to the generation of a potent furin-inhibitor (I) with IC50 <100 nM. This is compared with full length 83-mer furin prodomain and other prodomain peptides made synthetically. A comparison of secondary structures of all peptides by CD study and their furin inhibitions will be presented. Anti-protease and bioactivity of these inhibitors using specific viral and bacterial fluorogenic peptides will be demonstrated. Funds: CFI, NSERC. Q98-Q-V-A-K-R-R-T-K-R-Eda-D-V-Y-Q-E112 (I); Eda = 1-[1'-Amino 3'-yne] 2-[1''-carboxy 3''-yne] benzene.

P318

SYNTHESIS OF CRF-R2 SELECTIVE PEPTIDE LIGANDS BY USING THE SPOT SYNTHESIS TECHNIQUE

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Corticotropin-releasing factor (CRF) is being synthesized in the hypothalamus and regulates the secretion of adrenocorticotropic hormone (ACTH) from the posterior pituitary. As an important stress hormone it is involved in regulatory activities of both the central and peripheral systems. CRF and Urocortin-I (Ucn-I) are endogeneous agonists of the two receptor subtypes, CRF-R1 and CRF-R2, whilst Urocortin-II (Ucn-II) has been described as the first CRF-R2 selective agonist.

CRF

SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA-NH_2.

Ucn

DDPPLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV-NH_2.

Ucn-II

VILSLDVPIGLLRILLEQARYKAARNQAATNAQILAHV-NH_2.

A conspicuous difference between the selective and nonselective agonists consists in the underlined N-terminal tetrapeptide within a highly conserved region which was subject, therefore, of systematic replacement study to understand the molecular basis of the Ucn-II selectivity. Syntheses were carried out using the SPOT technique with Fmoc chemistry on cellulose membranes. The peptides synthesized and characterized by LC-MS. Biological activities of the analogs were determined by using an HTS assay. Results and Discussion Replacing the LTFH motif in Ucn-I by the corresponding VPIG of Ucn-II led to a highly selective agonist of CRF-R2, suggesting the VPIG motif to be essential, an idea that was checked by a positional scan, replacing each position by the other 19 proteogenic amino acids. Peptide arrays prepared by the SPOT technique became quite popular tools for studying numerous aspects of molecular recognition. Here, we show that the method permits rapid and highly parallel synthesis of even longer biologically active peptides. The quantity and quality was sufficient for structure-activity relationship studies even on membrane-bound proteins such as peptide receptors.

P319

DESIGN AND SYNTHESIS OF ANALOGUES OF 4-N-HYDROXY-L-2,4-DIAMINOBUTYRIC ACID AS POTENTIAL INHIBITORS OF GLUTAMINE SYNTHETASE

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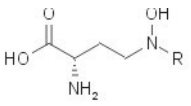
Glutamine synthetase (GS) is a key enzyme in nitrogen metabolism in cell [1]. This enzyme catalyses conversion of glutamate to glutamine in presence of ammonium ion and ATP. Inhibition of this enzyme in plants causes total degradation of nitrogen metabolism and death of organism. Thus, inhibitors of GS are potent total herbicides. Moreover glutamine synthetase is considered as a good target in tuberculosis treatment, because inhibition of GS blocks cell wall biosynthesis and subsequently causes death of patogen — Mycobacterium tuberculosis. It is also known that GS plays significant role in brain and the level of its activity is related to development of several diseases, namely: schizophrenia, Parkinson's disease, Alzheimer's disease and Huntington's disease. 4-N-Hydroxy-L-2,4-diaminobutyric acid — strong inhibitor of GS was selected to be a lead compound [2]. It was docked into active site of GS and this structure was used for design several new potential inhibitors. LUDI program from Insight 2000 package was applied for this purpose. From several designed compounds 8 structures were chosen for synthesis. They were synthesized in enantiomerically pure forms starting from the appropriately protected 4-N-hydroxy-L-2,4-diaminobutyric acid. References: 1.a) D.L. Purich, Advances in Enzymology and Related Areas of Molecular Biology, 72, 1998, 9-42; b) D. Eisenberg, H.S. Gill, G.M.U. Pfluegl, S.H. Rotstein, Biochem. Biophys. Acta 1477, 2000, 122-145. 2.S. Fushiya, K. Maeda, T. Funayama, S. Nozoe, J. Med. Chem. 31, 1988, 480-483.



R= $COCH_3$, $CONH_2$, $COCH_2NH_2$, $COCH(CH_3)NH_2$, $COCH_2CH_2NH_2$, $CH_2CH_2NH_2$, $CH_2CH_2CH_2NH_2$

P320

DESIGN AND SYNTHESIS OF NOVEL INHIBITORS OF GINGIPAINS

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Proteolytic activity of gingipains, secreted by bacterium Porphyromonas gingivalis - human gingiva pathogen, is responsible for the development of major periodontitis symptoms. Being involved in processes crucial for bacterium growth and proliferation, these enzymes seem to be an attractive target for the design of new class of antibiotics. Previously we reported N-acylated chloromethyl ketone derivatives of arginine and lysine as model compounds to examine structural and chemical properties of the S2 pocket of gingipains [1]. Structures of the P2 substituents were generated by computer aided molecular modeling methodology. On the basis of kinetic assays data the selected ones, exhibiting the highest affinity, were chosen as conservative P2 residues for further studies. However, derivatives containing chloromethyl ketone moiety are not the optimal to apply in terms of potential drug development. They are easily inactivated in vivo reacting with numerous oxygen and sulfur nucleofiles. Thus, the other selectively alkylating moieties have been designed to bind covalently to the thiol group of the gingipain catalytic cysteine. The most interesting seem to be epoxides allowing optimization the interactions of the inhibitor with both Sn and Sn' subsites of the enzymes. In this work we present the structure-based design and synthesis of novel inhibitors of gingipains, including acyloxymethyl ketones or variously substituted epoxides. References [1] Mucha, A.; Grembecka, J.; Białas, A.; Stachowiak, D.; Otlewski, J.; Potempa, J. In Peptides 2002, (Eds. E. Benedetti and C. Pedone), Napoli 2002, 988-989. Acknowledgements This work is supported by KBN, grant 6 P04B 025 21.

P321

REPLACEMENT IN POSITION 12 OF MCD PEPTIDE LEADS TO THE FORMATION OF AN ANTAGONIST OF IGE BINDING

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Mast cell degranulating (MCD) peptide is a bicyclic basic peptide, which binds with high affinity to mast cell receptors resulting in histamine release. These properties are comparable with those of IgE, the immunoglobulin thought to be the main cause of histamine release in allergic reactions. In order to determine the structure-activity relationship of MCD peptide, many analogs of MCD peptide including an "Ala-scan" have been synthesized. The purpose of these studies was to find inhibitors of IgE. The most promising inhibitor was the [Ala12] analog where the proline residue was replaced with alanine. This analog had virtually no intrinsic activity but had a high affinity for the mast cell receptor as quantified by fluorescence polarization. Indeed in a competitive binding assay, the IC50 for the [Ala12] analog was comparable with the IC50 for IgE. Furthermore [Ala12]MCD inhibited IgE-mediated mast cell degranulation measured in a beta-hexosaminidase assay. Monte Carlo simulations using the primary hydration shell approach will be performed to study the structural changes that convert MCD peptide into an inhibitor.

P322

A 6 KD PEPTIDE FROM PLANT ORIGIN INHIBITS THERMOLYSIN AND MATRIXINS

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The existence of metalloproteinase inhibitors of small molecular mass in the dry quiescent cotyledons of Lupinus albus has been previously reported by our group. In the present work we purified a metalloproteinase peptide inhibitor by a single step of affinity chromatography. Since this inhibitor strongly inactivated thermolysin (a bacterial metalloendopeptidase), this enzyme was utilised as a ligand coupled to a Sepharose matrix. The proteins specifically bound to thermolysin at pH 7.5 were further eluted by lowering the pH to 2.8. Only the protein fraction eluted at pH 2.8 contained the inhibitory activity and that was further purified by reverse-phase HPLC in a C4 column. The inhibitory activity of thermolysin was eluted in a single protein peak which, after analysis by Tricine-SDS-PAGE, revealed a protein band with molecular mass ca. 6 000. In the absence of a reducing agent, the same electrophoretic pattern was observed, suggesting that this peptide do not contain disulfide bonds. The inhibition constants (Ki) were determined towards different metalloproteinases: thermolysin, a bacterial enzyme, matrix-metalloproteinase-type 9 (MMP-9), a human matrixin and a lupin metalloproteinase matrixin-like by a very sensitive continuous spectrofluorimetric assay. The determined Ki values were in the nanomolar range. Since there are no known inhibitors of plant origin for matrixins this 6 kD peptide may constitute an important tool for structural and functional studies. (D. Carrilho received a scholarship Praxis XXI/BD/11135)

P323

SYNTHESIS OF 2-OXOAMIDES BASED ON δ-AMINO ACIDS AND STUDY OF THEIR ACTIVITY ON HUMAN GIVA PLA2

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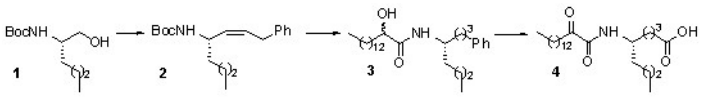
The Group IVA cytosolic phospholipase A2 (GIVA PLA2) is a particularly attractive target for drug development since it is the rate-limiting provider of proinflammatory mediators. We have previously reported the discovery of novel 2-oxoamides that inhibit GIVA PLA2 [Kokotos et al. J. Med. Chem. 2003, 45, 2891-93]. Here, we present our results on the synthesis of 2-oxoamides based on δ-amino acids. Compound 4 was synthesized starting from Boc-norleucinol (1) as depicted in the Scheme. Compound 4 is a potent inhibitor of human GIVA PLA2.



P324

STRUCTURE-ACTIVITY RELATIONSHIP STUDIES AND CONFORMATIONAL ANALYSIS OF NOVEL ANTIRETROVIRAL PEPTIDES DERIVED FROM FELINE IMMUNODEFICIENCY VIRUS GLYCOPROTEIN GP36

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Feline immunodeficiency virus (FIV) provides a valuable animal model by which criteria for antiviral vaccines and drug development can be investigated. We have previously shown that C8, a conserved stretch of few amino acids (Trp770-Ile777) containing three Trp residues of the membrane-proximal ectodomain of FIV transmembrane glycoprotein gp36, displayed potent in vitro anti-FIV activity. Ala scan experiments showed that the Trp residues are crucial for the antiviral activity. Further evidence suggests that C8 antiviral action was own to the ability to bind an N-terminal segment of gp36 that becomes available following FIV interaction with substrate cells. Finally, we have shown that a retro-inverso analogue of C8 maintains crucial conformational features and, most importantly, displays a remarkable serum stability. Preliminary data indicate that riC8 exerts in vivo a strong antiviral activity in infected cats.(1, 2) In the attempt to develop a full structure-activity relationship study, quantifying the contribution of factors such as steric hindrance, aromatic properties and distances among the single Trp residues, we synthesized and tested a series of C8 analogs where the Trp residues were substituted by different natural and non natural amino acids. Furthermore a second series of C8 analogs was synthesised where the amino acids between the Trp residues were substituted by alkylic spacers of variable length. A wide NMR conformational analysis of these products was undertaken in order to correlate their conformational properties to the antiviral activity. 1)Giannecchini et al J.Virology 77, 6, 3724-3733, (2003) 2) D'Ursi et al. J. Med. Chem, 2003 May 8;46(10):1807-10.

P325

SYNTHESIS OF POTENTIAL INHIBITORS OF HIV-1 PROTEASE DIMERIZATION

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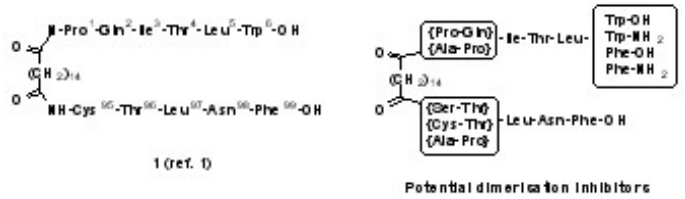
Compounds type 1 have been reported to be good inhibitors of the dimerisation of the HIV-1 protease [1], a process which is necessary for the development of the virus. We have designed a collection of derivatives of compound 1, in which we replace the dipeptides on the hinges for our 3-aminolactam synthons [2], which are beta-turn mimetics. The preparation of a new Fmoc-{Ser-Thr}, and the synthesis of the inhibitors will be discussed. [1] R. Zutschi, J. Franciskovich, M. Shultz, B. Schweitzer, P. Bishop, M. Wilson, J. Chmielewski, J. Am. Chem. Soc., 1997, 119, 4841-4845. [2] a. Fernández, M.; Diez, A.; Rubiralta, M.; Montenegro, E.; Casamitjana, N.; Kogan, M.; Giralt, E.; J. Org. Chem., 2002, 67, 7587-7599. b. Estiarte, M.A.; Rubiralta, M.; Diez, A.; Thormann, M.; Giralt, E.; J. Org. Chem., 2000, 65, 6992-6999. Piró, J.; Rubiralta, M.; Giralt, E.; Diez, A. Tetrahedron Lett., 2001, 42, 871-873.



P326

INHIBITION OF PLASMEPSINS BY SUBSTRATE-BASED PEPTIDOMIMETICS

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Malaria is a devastating disease that affects nearly half of the world's populations, causing about 2 million deaths per year. In addition, the periodic chills and fever associated with malaria cause nearly 300 million clinical cases each year, thus impacting both quality of life and the economy of many countries. The parasites causing malaria in man, Plasmodium falciparum, P. vivax, P. ovale, and P. malariae, use aspartic peptidases known as plasmepsins to cleave hemoglobin into fragments, which are further processed by cysteine and metallopeptidases. In addition, other members of the aspartic peptidase family are encoded by the parasite genome. Our studies are designed to evaluate a number of compounds as potential inhibitors of this group of enzymes. In this poster, we will present new data on the Ki values of peptidomimetic inhibitors with eight different plasmepsins. These inhibitors were developed from studies of the substrate specificity of the plasmepsins.

P327

SYNTHETIC MIMICRY OF DISCONTINUOUS PROTEIN BINDING SITES FOR THE EXPLORATION AND MODULATION OF PROTEIN FUNCTION

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German Research Centre for Biotechnology, Braunschweig, Germany

The design and generation of molecules, which, due to their specific molecular architecture, are capable of mimicking the binding and/or functional sites of natural proteins, represents a promising strategy for the exploration an understanding of protein structure and function. The functional and binding sites of proteins are often not localized in short, continuous stretches of the amino acid sequence, but rather in sequentially distant fragments of the molecule, which are brought into spatial proximity by protein folding. Synthetic molecules aimed at mimicking such discontinuous protein binding sites should therefore also be sequentially discontinuous. The synthetic basis of this concept are assembled and scaffolded peptides, which are more likely than linear, consecutive amino acid sequences to adopt structures capable of mimicking discontinuous protein binding sites. Recently, we have introduced a versatile strategy for the generation of structurally diverse scaffolded peptides (1). Now we present the utility of this strategy for the synthetic mimicry of discontinuous binding sites of a range of biomedically relevant proteins, including interaction domains, cytokines, as well as viral and bacterial virulence factors. (1) R. Franke, C. Doll, V. Wray and J. Eichler, Protein Peptide Lett., 2003, 10, 531-539.

P328

VERSATILE PHOSPHORANES: ACCESS TO VARIOUS ISOSTERE STRUCTURES FOR PROTEASE INHIBITION VIA SMOOTH C-C-COUPLINGS ON POLYMER SUPPORT

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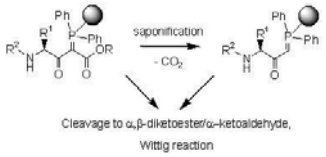
Norstatine, diaminopropanol and statine isosteres incorporated in a peptidic environment represent major structural units for aspartate protease inhibition. Recently we demonstrated the application of polymer cyanophosphoranes in the assembly of α-hydroxy-β-amino esters and amides (norstatines). [1, 2] We now present a further development of our phosphorane approach: By changing to polymer carboxylatophosphoranes diaminopropanols and several other substructures of high relevance in medicinal chemistry are within reach, i.e.α,β-diketoesters, α-ketoaldehydes and α,β-unsaturated ketones. Carboxylatophosphoranes derived from bromo acetic acid esters which can be acylated readily play the key role in the synthetic strategies. We show the oxidative cleavage to peptidic diketoesters as well as to ketoaldehydes after a saponification/decarboxylation step on the solid support. The latter offer the possibility of derivatization by reductive amination to diaminopropanols. Furthermore Wittig reactions with peptidic α-keto ylides are demonstrated. Literature: 1) S. Weik, J. Rademann, Angew. Chem. 2003, 115, 2595-2598; Angew. Chem. Int. Ed. 2003, 42, 2491-2494. 2) J. Rademann, 'Novel polymer- and linker reagents employed for the preparation of protease inhibitor libraries' in: Highlights in Bioorganic Chemistry, H. Wennemers, C. Schmuck (Eds.), Wiley-VCH, Weinheim, 2004, 277-290



P329

BEYOND THE BOUNDARIES OF SECONDARY STRUCTURE: DEVELOPMENT OF A BETA HAIRPIN TO MIMIC AN ALPHA HELIX

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Generally, functional mimicry aims at minimizing a large protein epitope to a smaller binding entity that retains the conformational features of the original epitope. The approach described here goes beyond the boundaries imposed by the common secondary structural elements by replacing an alpha-helical ligand with a cyclic beta-hairpin mimetic. Many strategies have been undertaken over the last few years to induce tumor-suppressor activity of p53. A recent approach relies on inhibition of Mdm2 protein. Starting from the structure of Mdm2 in complex with the p53 transactivation domain, computer-aided modeling led to the design of a first potential p53-mimetic. A SPR-based inhibition assay revealed that the designed peptide was effectively able to bind Mdm2, although more than 100 times less strongly than the natural sequence. Alanine scanning allowed the identification of residues critical for binding. The site on Mdm2 involved in the interaction with the mimetic was localized by NMR chemical shift mapping (HSQC). Subsequently, SAR and binding site mapping analyses provided the tools for the rational design of libraries, which led to the step-wise optimization of this lead compound. At the end, the anti-Mdm2 potency of the original mimetic was improved by 1000 fold. NMR studies proved that some of these compounds adopt a stable beta-hairpin in solution and the structure of the complex between Mdm2 and one beta-hairpin p53-mimetic was obtained by X-ray crystallography. Based on these findings, we believe that this type of mimicry may be of general use in the design of novel regulators of protein-protein interaction.

P330

DEVELOPMENT OF MOLECULAR AGENTS FOR PROTEASOME TARGETING

P. Furet[1], P. Imbach[1], V. Guagnano[1], M. Noorani[2], J. Koeppler[1], K. Laumen[2], M. Lang[1], P. Fuerst[2], J. Roesel[1], J. Zimmermann[1], **C. Garcia-Echeverria**[1]

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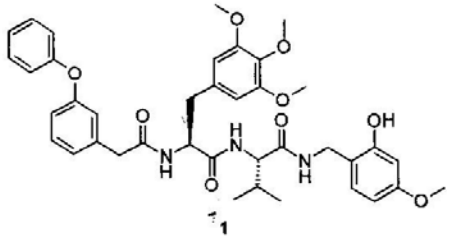
The involvement of the proteasome in the degradation of critical intracellular regulatory proteins has suggested the potential use of proteasome inhibitors as novel therapeutic agents in cancer. Recently, we have reported the optimization of 2-aminobenzylstatine derivatives that inhibit non-covalently the human proteasome. This work has been expanded with the structure-based design of a new class of non-covalent inhibitors. These new inhibitors are able of modulating the subunit-specific proteolytic activities of the proteasome in ways not previously possible. For example, compound 1 inhibits the chymotrypsin-like peptidase activity of the proteasome with an IC50 value of 15 nM, and shows at least 1000-fold selectivity over the trypsin-like and post-glutamyl-peptide hydrolytic activities of this enzyme. It blocks proteasome activity in cultured cells (IC50 = 19 nM) and potently inhibits the proliferation of different tumour cell lines (IC50< 100 nM). The design and synthesis of this new class of peptide-based proteasome inhibitors will be presented in this communication.



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P331

MAPPING ENZYME-INHIBITOR INTERACTIONS BY MULTIDIMENSIONAL NMR OF DOUBLE-LABELED PEPTIDES

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SGCI (Schistocerca gregaria chymotrypsin inhibitor) is a potent inhibitor of bovine and arthropod trypins, whereas the closely related SGTI (Sch. gregaria chymotrypsin inhibitor). The solution structure of the inhibitors has been determined previously by NMR spectroscopy [1]. The internal dynamics of SGCI and SGTI has also been studied using 15N-labeled peptides and we found that the dynamical behavior of the two inhibitors differ significantly [2]. The aim of our present study is to get insight into the enzyme-inhibitor interaction in detail. NMR titration experiments with the 15N-labeled peptides showed that the majority of the signals shifted in a way that prevented unambiguous assignation of the complexed inhibitor. Therefore, we turned to the production of double-labeled (15N, 13C) peptides allowing the use of triple-resonance experiments necessary for signal assignment. The additional labeling also makes possible the detailed investigation of the alteration of side-chain conformation, contacts and dynamics. [1] Zoltán Gáspári, András Patthy, László Gráf & András Perczel: Comparative structure analysis of proteinase inhibitors from the desert locust, Schistocerca gregaria. Eur J Biochem., (2002) 269: 527-537. [2] Borbála Szenthe, Zoltán Gáspári, Attila Nagy, András Perczel & László Gráf Same fold with different mobility: backbone dynamics of small serine protease inhibitors from the desert locust, Schistocerca gregaria Biochemistry, in press

P332

THE STERICAL REQUIREMENTS FOR THE ACTIVITY OF THE MYCOBACTERIA PHAGOCYTOSIS INHIBITORS

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It follows from our synthetic and theoretical studies that a proper spatial localization of the guanidine and carboxyl groups within inhibitor molecules plays a crucial role in the activity of Mycobacteria phagocytosis inhibitors. These groups should be situated at the distance of about 9.2 Å [1]. In this study a several series of peptide inhibitors, like analogs of the RGDVY fragment of the HLA-DQ protein and of the GRGD fragment of fibronectin (Gly-Arg-(Gly)n-Asp-Val, n=1-4), systemin and cecropin fragments, and the palindromic sequences of the GRGDVVNGRG type [2], as well as the series of w-guanidino acids of the formula H2N-C(NH)-NH-(CH_2)n-COOH, n=1-5, were used. The above conclusions were confirmed by molecular modeling of some of the peptides studied, and the X-ray analysis of the compounds belonging to the guanidino acid series. References: [1]. I. Z. Siemion et al., Peptides, 2003, 24, 1109-1125. [2]. I. Z. Siemion et al., Polish J. Chem., in press.

P333

DESIGN, SYNTHESIS AND BIOLOGICAL CHARACTERIZATION OF OPTICALLY ACTIVE INHIBITORS OF GAMMA-SECRETASE AND NEUROMEDIATORS

M.G. Georgieva[1], L.T. Vezenkov[1], T.C.B. Ivanov[1], G.I. Ivanova[2], V. Mitev[3]

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The Alzheimer's disease (AD) is a neurodegenerative illness, which affects millions of people worldwide. The AD is characterized by progressive dementia of memory, intellectual and speech disturbances. We have already synthesized in our previous work derivatives of the 2,3,4,5-Tetrahydro-7, 8-dimethoxy-1-phenyl-1H-3-benzazepine containing dipeptides /N- (3,4-dichlorophenyl)-Ala-Phe-OH, N- (3,4-dichlorophenyl)-Ala-Val-OH, N- (3,4-dichlorophenyl)-Ala-Leu-OH, N- (3,4-dichlorophenyl)-Ala-Gly-OH/. In the present work we propose a new approach to the creation of inhibitors of gamma-secretase, by synthesizing optically active derivatives of the benzazepine containing the same peptide fragments as mentioned above and other. By the synthesis of these compounds we intend to combine two important pharmacological effects for the treatment of the disease: inhibition of the gamma-secretase by the peptide fragments and increasing of the level of the neuromediators dopamine, noradrenaline and serotonine in brain by inhibition of their uptake in neurons by the benzazepine heterocycle. We proved the later effect in our earlier publication. We intended to research the action of the stereo chemical factors on the inhibition of the gamma-secretase of these compounds. Synthetic, biochemical and pharmaceutical data will be discussed.

P334

INHIBITION OF AMYLOID FIBRIL FORMATION BY PEPTIDE ANALOGUES MODIFIED WITH ALPHA-AMINOISOBUTYRIC ACID

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The formation of amyloid fibrils is associated with major human diseases of unrelated origin, including Alzheimer's disease, Parkinson's disease, Prion disorders, and Type II diabetes. Amyloid fibrils are highly ordered supramolecular structures, self-assembled by a single protein or peptide. An increasing body of evidence supports the hypothesis that the assembly of amyloid fibrils is a central factor in the development of clinical symptoms of the diseases. Much effort is therefore directed towards the development of potent amyloid formation inhibitors. A key feature in the process of amyloid formation is transition into beta-sheet structure. Therefore, the use of beta-breaker elements is a promising direction in the development of fibrillization inhibitory drugs. Previous work had demonstrated that incorporation of beta-breaker elements into short peptides composed of the recognition sequence of the amyloidogenic protein, results in inhibition of amyloid formation. Here, we introduce a novel inhibition strategy using the non-coded alpha-aminoisobutyric acid (Aib) as a beta-breaker element. Aib, which is an alpha-methylated alanine, has an extremely limited conformational space. Its conformational constrains are significantly higher compared to the commonly used beta-breaker proline, as reflected by the allowed torsion angles viewed by the Ramachandran plot. Peptide fragments of the intermolecular recognition domain within the islet amyloid polypeptide (IAPP), the main constituent of pancreatic deposits in Type II diabetes, were modified with Aib. The modified peptides completely lost their amyloidogenic potential. Furthermore, the modified peptide showed a powerful inhibitory effect on the formation of amyloid fibrils by the full length IAPP.

P335

DESIGN, SYNTHESIS AND STUDY OF PEPTIDES ABLE TO RECOGNIZE THE HYDROPHYLIC SURFACE OF P53 TETRAMERIZATION DOMAIN: COMBINATORIAL OPTIMIZATION

M. Martinell[1], X. Salvatella[1], M. Vilaseca[2], M. Gairí[1], **E. Giralt**[1,2]

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Proteins often perform their functions as part of networks regulated by protein-protein interactions and the design of specific inhibitors of these interactions will lead to new approaches for the treatment of important diseases. Synthetic peptides, thanks to their large chemical diversity at the side-chain level, provide a good opportunity for the design of protein-specific ligands, including those addressed to highly hydrophilic surface-patches (1,2). We report here the design and synthesis of a series of peptide ligands able to recognize a highly hydrophilic patch located on the surface of the tetramerization domain of the tumor suppressor protein p53 (3). The design of the lead peptide has been assisted by molecular modeling. The structure has been refined using a combinatorial approach. The interactions have been studied by the combined use of fluorescence spectroscopy, NMR, surface plasmon resonance and isothermal titration calorimetry. We suggest that this recognition event could be used to interfere with the interaction of p53 with other proteins or DNA, and/or to modulate the stability of the domain, and therefore the stability of the whole protein. [1] Haack, T., Peczuh, M.; Salvatella, X.; Sánchez-Quesada, J.; de Mendoza, J.; Hamilton, A.D. and Giralt, E. J. Am. Chem. Soc. 12, 11813 (1999). [2] Orner, B.; Salvatella, X.; Sánchez-Quesada, J.; de Mendoza, J.; Giralt, E. and Hamilton, A.D. Angew. Chem. Int. Ed. 41, 117 (2002). (3) Salvatella, X.; Martinell, M; Gairí, M.; Mateu, M.G.; Feliz M.; Hamilton, A.D.; de Mendoza, J. and Giralt, E. Angew. Chem. Int. Ed. 43, 196 (2004).

P336

EXPRESSION AND STRUCTURAL STUDIES ON THE PEPTIDE INHIBITOR OF THE HEPATITIS C VIRUS HELICASE

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HCV is a human pathogen that represents a serious epidemiological threat. The non-structural protein 3 (NS3) of Hepatitis C virus (HCV) of the Flaviviridae family, is one of the most promising targets for anti-HCV therapy because of its multiple enzymatic activities, such as RNA-stimulated nucleoside triphosphatase (NTP-ase), RNA helicase and serine protease. The helicase activity is indispensable for viral replication in vivo, presumably unwinding double-stranded replication intermediates or regions, allowing RNA amplification. Our studies centred on a peptide inhibitor of the NS3 protein that corresponds to a highly conserved arginine-rich sequence of domain 2 of HCV helicase. Preliminary results suggest that the mechanism of inhibition could involve direct binding of the peptide to an active site of the enzyme. To confirm this hypothesis and elucidate the mechanism of reaction so as to create potent and specific inhibitors of HCV replication, we chose to analyse the kinetics of the peptide-helicase binding by NMR. To this end we designed a construct that made it possible to label the peptide with 15N and 13C in Escherichia coli and to purify it for NMR studies. Mass spectroscopy analysis confirmed the proper size of the synthesised peptide. CD and NOESY spectra obtained for the unlabeled peptide show that it has a random coil structure. According to the 1H-15N HSQC spectrum, all amide resonances including arginine, are resolved and assigned. NMR measurements using the relaxation filtered technique reveal that the peptide binds to the full-length helicase. Further studies with separately expressed domains 1 and 2 of the helicase demonstrated that the peptide binds only to domain 1.

P337

USEFULNESS OF ALLOPHENYLNORSTATINE-DIMETHYLTHIOPROLINE SCAFFOLD TO ASPARTIC PROTEASE INHIBITORS

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Aspartic proteases are involved in the processing of functional peptides and proteins, and are essential for the sustenance of life of all living beings from virus to mammals. Inhibition of aspartic proteases such as HIV protease, HTLV-1 (human T-cell leukemia virus type-1) protease, plasmepsin, human renin, and beta-secretase is considered to be targets for therapeutic agents of AIDS, ATL/ HAM (adult T-cell leukaemia/ HTLV-1 associated myelopathy), malaria, hypertension, and Alzheimer's disease, respectively. In our HIV protease inhibitor study, we have developed a series of substrate-based inhibitors containing allophenylnorstatine [Apns; (2S,3S)-3-amino-2-hydroxy-4-phenylbutyric acid] with a hydroxymethylcarbonyl isostere as an ideal transition state mimic [1]. Among them, the tripeptide KNI-272 containing Apns-thiazolidine-4-carboxylic acid (Thz) at P1-P1' position was a highly selective and superpotent HIV protease inhibitor. KNI-272 also exhibited potent antiviral activities with low toxicity. Further, dimethylthioproline [Dmt; (R)-5,5-dimethyl-1,3-thiazolidine-4-carboxylic acid] was designed as a chimeric structure of Thz and Val. The introduction of Dmt at P1' position remarkably increased affinity against the enzyme, and resulted to generate highly potent small sized dipeptide type inhibitors. Since HIV protease recognize several substrates, the optimised Apns-Dmt scaffold may be useful for other aspartic proteases which have similar substrate recognition at P1-P1' position. Therefore, we expanded this scaffold to development of plasmepsin II and HTLV-1 protease inhibitors. As a result, in both cases, the Apns-Dmt was effective at P1-P1' position, and the affinity of Dmt containing inhibitors was better than that of Thz. Reference [1] Y.Kiso, H.Matsumoto, S.Mizumoto, T.Kimura, Y.Fujiwara, and K.Akaji. Biopolymer, 51, 59-68 (1999).

P338

MECHANISM OF THE SUBSTRATE RECOGNITION OF PEPTIDYLARGININE DEIMINASE V

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Peptidylarginine deiminase V (PADV) catalyzes the deimination of the guanidinino group of an Arg residue of various peptides and proteins, such as nucleophosmin/B23 and histones, resulting in the production of a citrulline residue in a variety of granulocytes [1,2]. This post-translational modification by PADV is closely associated with cell-specific stages of granulocyte differentiation. It has recently been reported that the protein deimination/citrullination also plays a role in rheumatoid arthritis [3]. Here we report on the mechanism of the substrate recognition and a specific inhibitor. We synthesized various analogs of Bz-Arg in which the guanidinino group was modified, in order to investigate the substrate specificity of PADV. Modification of the guanidinino group and the lack of a carboxyl group completely resisted citrullination. The results suggest that both imino and carboxyl groups are important for the molecular recognition of PADV. In addition, it should be noted that these synthetic analogs specifically inhibited the deimination of Bz-Arg by PADV. In order to investigate the catalytic mechanism of PADV, an N-terminal peptide (H4-10) of histone H4, a candidate for the citrullinated site in histone H4, was chemically synthesized and incubated with PADV in the presence of 18O-labeled water. Mass spectrometry provided convincing evidence, showing that the reaction proceeds hydrolytically. 1) Hagiwara, T., et al., Biochem. Biophys. Res. Commun. 290, 979-983 (2002) 2) Nakashima, K., et al., J. Biol. Chem., 277, 49562-49568 (2002). 3) Suzuki, A., et al., Nature Genetics, 34, 395-402 (2003).

P339

ASSEMBLED PEPTIDES MIMICKING THE DISCONTINUOUS BINDING SITE OF THE MENA-EVH1 DOMAIN

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The mammalian Enabled (Mena) VASP homology 1 (EVH1) domain is a protein-protein interaction module of proteins implicated in actin-based cell motility, and has a binding specificity for the sequence motif -FPPPP-. As shown by the crystal structure of Mena-EVH1 in complex with the peptide ligand FPPPP [1], the primary contact residues of Mena-EVH1 for this interaction (Y16, W23, and F77) are located in sequentially distant parts of the protein. As part of our ongoing effort to explore the synthetic mimicry of discontinuous protein binding sites, we have generated a range of assembled peptides by connecting fragments covering the contact residues of Mena-EVH1 via a thioether linkage. The distance of the fragments within the assembled peptides was varied using spacer amino acids with different backbone lengths. The affinities of these peptides to a peptide ligand for Mena-EVH1 were determined using a parallel competitive binding assay, and were found to depend on the orientation of the fragments within the assembled peptides. The results of this study will be presented. [1] Prehoda, K.E., Lee, D.J., and Lim, W.A. 1999. Cell 97:471-480.

P340

INHIBITION OF L-PHENYLALANINE AMMONIALYASE BY PHOSPHONIC ACID ANALOGUES OF PHENYLGLYCINE

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Phenylalanine ammonia lyase is a key enzyme of the synthetic pathway of lignins, coumarins, quinones, flavonoids and plant defence substances. It catalyzes the conversion of L-phenylanaline into trans-cynnamic acid. Therefore, inhibitors of this enzyme might be useful plant growth regulators as well as tools for studying physilogical processes connected with this metabolic pathway. In this work we present a set of over twenty inhibitors of the enzyme, which are phosphonic acid analogues of phenylglycine and rationalization of their inhibitory activity based on three-dimensional structure of the active-site of L-phenylalanine ammonia lyase. The structure of this site was obtained by means of molecular modelling based on the already known structure of realted L-histidine ammonia lyase. Additionally maps of electrostatic potential of the inhibitors were constructed and show that the inhibitory potency of the phosphonic acid analogues of phenylglycine are strongly dependent on electrostatical potential of aromatic ring. The origin of that phenomenon is discussed in terms of interactions of this portion of the molecule with active-site amino acids site groups.

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DESIGN AND SYNTHESIS OF HIGHLY POTENT CELL-PERMEABLE PEPTIDOMIMETIC INHIBITORS OF ALZHEIMER'S ß-SECRETASE (BACE1)

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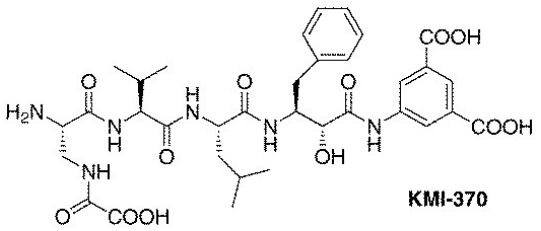
Accumulation of ß-amyloid peptide(Aß) in the brain is a major factor in pathogenesis of Alzheimer's disease (AD) and Aß is produced from ß-amyloid precursor protein (APP) by protease. ß-Secretase, (BACE1) which is an aspartic protease, catalyzes the first step in Aß production and is considered to be an important drug target for AD. Therefore, we designed and synthesized a novel class of substrate-based BACE1 inhibitors containing phenylnorstatine as an effective transition state mimic [1]. Among them, a small-sized KMI-370 [2] exhibited highly potent inhibitory activity (IC50=3.4 nM). Also, KMI-370 inhibited dose-dependently the secretion of sAPPß from BACE1-HEK293 cells (IC50=200nM). Furthermore, we synthesized several stable inhibitors and structure-activity relationships have been studied. References [1] D. Shuto, et al. Bioorg. Med. Chem. Lett., 13, 4273-4276 (2003). [2] T. Kimura, et al. Bioorg. Med. Chem. Lett., 14, 1527-1531 (2004).



KMI-370

P342

STUDIES TOWARDS A NEW FOOT AND MOUTH DISEASE ANTIVIRAL AGENT

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During its replication cycle FMDV translates a long polyprotein, this is subsequently cleaved into smaller proteins which constitute a complete FMDV particle. Of the total 14 cleavages, 10 of these are carried out by a particular FMD enzyme, namely FMDV 3C protease. I have undertaken work towards a 3C protease peptide inhibitor based on its natural cleavage sequences. If successful, this inhibitor will prevent viral replication and potentially provide a new FMDV antiviral agent.

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2-OXOAMIDES BASED ON γ-AMINO ACIDS INHIBIT HUMAN GIVA PHOSPHOLIPASE A2 AND PRESENT IN VIVO ANTIINFLAMMATORY ACTIVITY

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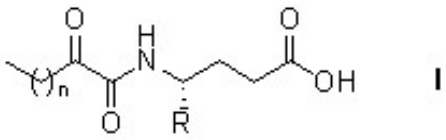
The phospholipase A2 superfamily consists of a broad range of enzymes defined by their ability to catalyze the hydrolysis of the ester bond at the sn-2 position of phospholipids. Among the various PLA2s, human cytosolic PLA2 (GIVA PLA2) is essential for proinflammatory prostaglandin, leukotrienes and PAF production, and therefore its inhibitors are very attractive targets as agents for treating inflammatory and other diseases. Taking into consideration the mechanism of the enzyme´s catalytic action and the structure of the substrate, we rationally designed inhibitors containing the 2-oxoamide group as the reactive functionality and a free carboxyl group mimicking the negative charge of phospholipids. We have prepared a variety of 2-oxoamides of the general structure I. Long chain 2-oxoamides based on γ-amino-butyric acid, γ-leucine and γ-norleucine are potent inhibitors of GIVA PLA2. Such inhibitors act through a fast and reversible mode of inhibition in vitro and demonstrate potent in vivo antiinflammatory and analgesic activity.



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INTERMOLECULAR INTERACTIONS IN THE AQUEOUS PROLINE-LEUCINE SYSTEM. A VOLUMETRIC STUDY

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The comprehension of intermolecular interactions of water with building blocks of living matter is of crucial importance to understand physiological processes. Thermodynamic characterization of proteins in aqueous solutions is a macroscopic tool often used to complement microscopic structural information. As the chemistry of life is mainly occurring in aqueous medium, the role of hydration in biological systems is essential in modulating their structural stability and functional activity. It has been argued that the behavior of small peptides, specially chosen as model compounds for the more complex protein molecules, is very useful to understand their structural features in aqueous medium. Only a few macroscopic observable properties are sensitive to solute hydration in diluted solutions. Molar isobaric expansion is an example among them. In this work we present an experimental volumetric study based on a set of density data at eight different temperatures between 283.15 and 318.15 K in the system Proline-Leucine+Water. Accurate density measurements were obtained for several diluted solutions with molality ranging from 0.005 to 0.012 approximately. A DMA 60/602 Anton Paar vibrating tube densimeter with a temperature control of ±0.01 K has been used. Limiting partial molar volumes and isobaric expansions were afterwards derived. Insight on solute-solute-solvent intermolecular interactions has been obtained using the present results jointly with literature data from other parent thermodynamic studies.

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ASCORBIC ACID-PEPTIDES DERIVATIVES AS TYROSINASE INHIBITOR

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We have previously prepared kojic acid-peptide library by using 2-chlorotrityl chloride (CTC) resin, screened them for tyrosinase inhibitory activity, and confirmed that kojic acid-peptide conjugates were very active, but not toxic [1]. Based on these results, we attempted to synthesize ascorbic acid-peptide conjugates, which is assumed to have longer storage stability than pure ascorbic acid and to have improved tyrosinase inhibitory activity. In order to connect the ascorbic acid to the N-terminal of the peptides via a urethane bond, ascorbic acid was converted to an activated form using CDI after blocking 5- and 6-hydroxy group of the ascorbic acid as an acetonide. The resulting product was precipitated as white powders. By using this activated ascorbic acid, we have prepared ascorbic acid-peptide library by the same method as above, and tested their tyrosinase inhibitory activity. We found that several ascorbic aicd-tripeptides, which are hydrophobic, revealed the best tyrosinase inhibitory activity as the kojic acid analogue. The inhibitory mechanisms of those tri-peptide derivatives for their excellent tyrosinase inhibitory activity will be discussed. Reference [1] Kim, H. Y.; Choi, J. H.; Cho, J. K.; Kim, S. Y.; Lee, Y. S. Bioorganic & Medicinal Chemistry Letters 2004, in press.

P346

DISCOVERY OF A NEW CLASS OF POTENT CYCLIC PENTAPEPTIDE ANTAGONISTS OF THE GRB2-SH2 DOMAIN BY THE UTILIZATION OF 3'-SUBSTITUTED TYROSINE

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Src homology 2 (SH2) domains are protein modules that mediate intracellular protein-protein interactions in signal transduction pathways. The growth factor receptor bound protein 2 (Grb2) is an SH2 domain-containing docking module that participates in the oncogenic actions of certain receptor tyrosine kinases. So intervention of the Grb2-SH2 domain association with pTyr-containing segment on activated receptors provides an attactive approach to develop new anti-proliferative agents. By the first utilization of tyrosine derivatives bearing electron-donating or electron-withdrawing group at the 3'-positon of tyrosine, we discovered a new class of cyclic pentapeptide inhibitors of Grb2-SH2 domain, among which the 3'-amino-Tyr containing sulfoxide-cyclized analog exhibited low-nanomolar Grb2-SH2 domain binding affinity (IC50 = 65 nM), the most potent peptide inhibitor with only 5 a. a. binding motif, Gla-L-(3'amino-Tyr)-Ac6c-N, free of pTyr or pTry mimics. We also developed new strategies to synthesize globally protected tyrosine derivatives with 3'-subtituent, e.g. amino, hydroxy, nitro, methoxy or carboxy, suitable for solid phase peptide synthesis. The electronegativity and free active hydrogen endowed by the 3'-substituent of the Tyr were found to modulate the binding of the resulting cyclic pentapeptide with the Grb2-SH2 domain. The enhancement in potency could be ascribed to the increased aromatic ring p interaction with the Arg86 and/or Arg67 side chain and the additional hydrogen-bond interactions within the pTyr binding pocket. This potent small peptidomimetic provids a novel template for the development of non-pTyr containing Grb2-SH2 domain antagonists as potential anticancer agents.

P347

TRIACYLGLYCEROLS BASED ON 2-(N-TERT-BUTOXYCARBONYLAMINO)-OLEIC ACID ARE POTENT INHIBITORS OF PANCREATIC LIPASE

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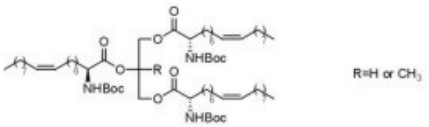
Human pancreatic (HPL) and gastric (HGL) lipases are essential enzymes for efficient fat digestion. The hydrolysis of dietary triacylglycerols by these enzymes to form monoacylglycerols and free fatty acids is a necessary step for fat absorption by the enterocytes. Therefore, potent and specific inhibitors of digestive lipases are of interest, because they may find applications as anti-obesity agents. Here, we present the synthesis of a novel class of potent human pancreatic lipase (HPL) inhibitors, based on the non-natural amino acid 2-(N-tert-butoxycarbonylamino)-oleic acid, which was esterified with glycerol and 2-methyl-glycerol. (S)-2-Amino-oleic acid was prepared starting from methyl or tert-butyl (2S)-2-[bis(tert-butoxycarbonylamino)]-5-oxopentanoate. The esterification reaction was carried out using either DCC in the presence of DMAP or the amino acid symmetric anhydride in the presence of DMAP. The ability of the triolein analogues to form stable films at the air/water interface was studied. The inhibition of human digestive lipases by the compounds synthesized was studied by the monolayer technique and the novel triolein analogues were found to be potent inhibitors of HPL.



P348

P3 AND P4 POSITION ANALYSIS OF THE PEPTIDE VINYL ESTHER DERIVATIVE PROTEASOME INHIBITORS

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The 26S proteasome is a multicatalytic protease complex which plays an essential role in intracellular protein degradation. Inner 20S core exhibits three distinct hydrolytic activities: post-acidic, trypsin-like and chymotrypsin-like respectively associated with the active sites of beta1, beta2 and beta5 subunits (1). The development of proteasome inhibitors into novel therapeutic agents represents a stimulating approach in the treatment of many disease states (2). We recently reported on the identification of a number of tripeptidic sequences derivatized with arecoline derivatives, active against chymotrypsin-like subunit(3,4). Also, we have designed a series of peptide-based derivatives, selective for trypsin-like activity, were C-terminal leucyl vinyl ester has been considered as a potential substrate for the catalytic threonine. Now, we present an analysis of the P3 and P4 position of vinyl ester peptide derivatives by means oligolibraries generated by solid phase synthesis. P3 Hmb-Xaa-Gln-LeuVE P4 Xaa-Val-Gln-LeuVE Hmb = 3-hydroxy-2-methylbenzoyl; Xaa = all natural amino acids (except cysteine) We rapidly identified some vinyl ester derivatives able to efficently interact with 20S proteasome, with a remarkable selectivity for beta2 subunit with an IC50 < 0.01 microM and having favourable pharmacokinetic properties. 1) Ciechanover A. EMBO J. 1998, 17, 7151-7160; 2) Kisselev A.F., Golberg A.L. Chemistry & Biology 2001, 8, 739-758; 3) Marastoni M. et al. J. Med. Chem. In press. 4) Marastoni M; et al. Bioorg. Med. Chem. Lett. in press.

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STRUCTURE-ACTIVITY RELATIONSHIPS AND CELLULAR ACTIVITY OF PHOSPHOPEPTIDE-BASED INHIBITORS OF STAT3

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Signal transduction and activator of transcription 3 (Stat3) mediates signals from the IL-6 family of cytokines and various growth factors, is constitutively activated in a variety of tumors (e.g breast, head and neck, prostate), and is a target for anti-cancer drug design [1]. Stat3 becomes activated by phosphorylation of Tyr705 and dimerizes by reciprocal interactions between SH2 domain of one molecule and the phosphotyrosine of the second. The dimer translocates to the nucleus and initiates transcription of anti-apoptotic genes resulting in cancer cell proliferation. To disrupt Stat3 activity we have embarked on the development of peptidomimetic inhibitors targeted to the SH2 domain. A lead peptide, acetyl-Y(p)LPQTV-amide (1), was found which exhibited an IC50 value of 150 nm [2]. SAR studies have revealed a number of important peptide-protein contacts, e.g. pY+1 backbone NH and the pY+3 Gln side chain NH_2 protons and the fact that the Leu-Pro peptide bond is trans. This work has lead to high affinity peptidomimetics with IC50 values of ca 100 nM in a fluorescence polarization assay. Analogues of peptide 1, when attached to the hydrophobic membrane transporting sequence AAVLLPVLLAAP, inhibit Stat3 activity in cells in culture. Stat3 translocation to the nucleus and expression of a luciferase reporter gene were reduced in IL-6 stimulated HepG2 and HepB3 hepatoma cells. References [1] Yu, H.; Jove, R. Nature Rev. Cancer. 2004, 4, 97. [2] Ren, Z., Cabell, L.A., Schaefer T.S., McMurray, J.S. Biorg. Med. Chem. Lett. 2003, 13, 633.

P350

STRUCTURAL DETERMINANTS FOR 20S PROTEASOME INHIBITION BY TMC-95A

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The yeast 20S proteasome (CP) contains in duplicate three proteolytically active sites that are non-covalently inhibited by the natural product TMC-95A (Fig. 1, 1) with distinct affinities [1]. A correlation of the X-ray structures of GP complexed with 1 [2] and the synthetic analogues 2a and 2b with their inhibition potencies allowed to factorise the structural determinants mainly involved in the protein-ligand interaction and to evaluate their entropic/enthalpic contributions. [1] Koguchi, Y., Kohno, J., Nishio, M., Takahashi, K., Okuda, T., Ohnuki, T., Komatsubara, S., J. Antibiot. 53 (2000) 105-109. [2] Groll, M., Koguchi, Y., Huber, R., Kohno, J., J. Mol. Biol. 311 (2001) 543-548.

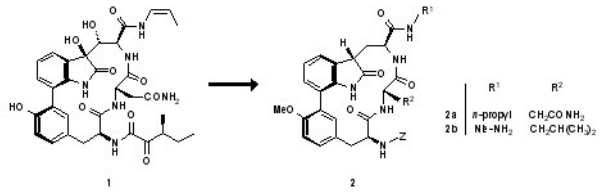

*Fig. 1. Structure of TMC-95A (**1**) and related synthetic analogues (**2a** and **2b**).*

P351

THE EFFECT OF DIGUANIDYL- AND DIAMINO-ALKANES ON THE DIMERIZATION OF HUMAN CYSTATIN C

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Human cystatin C (HCC), a natural protein inhibitor of the cysteine proteases, shows a tendency to dimerization. This process is particularly easy in case of the L68Q HCC mutant and might lead to formation of amyloid deposits in brain arteries. The structure of the HCC monomer consists of a core with a five-stranded antiparallel β-sheet wrapped around a central α-helix (Figure 1). The β-sheet structure contains four acidic amino acid residues with the carboxyl groups facing outwards. According to the proposed mechanism (3D domain swapping) the dimerization of HCC precedes the disruption of the noncovalent bonds between β2 and β3 strands and separation of the β1, α-helix and β2 fragments from the remaining part of the β-sheet. Our purpose was to find low-molecular ligands of monomeric HCC that can prevent its dimerization. We synthesized salts of diguanidylalkanes [$NH_2C(=NH)NH(CH_2)nNHC(=NH)NH_2 \times 2HNO3$] and di(trimethylammonium)alkanes [$(CH_3)3NI(CH_2)3NI(CH_3)3$] where n = 5, 7 and 1. We expect these compounds to interact with Asp, and Glu carboxyl groups of β-sheet, "strapping" appropriate β strands, which leads to stiffening of the HCC monomer. In this study the ability of the obtained alkane derivatives to inhibit the dimerization of HCC were verified using gel and agarose electrophoresis methods. This work was supported by the University of Gdańsk.

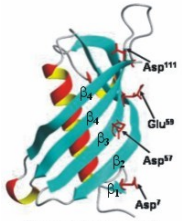

Figure 1.Structure of the monomeric HCC

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DETECTION OF PPII-HELIX-LIKE STRUCTURES IN SHORT PROLINE-CONTAINING PEPTIDE INHIBITORS OF THE CELL-SURFACE PROTEASE DIPEPTIDYL PEPTIDASE IV

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[3]*Department of Internal Medicine, Institute of Immunology, Otto-Von-Guericke-University, Magdeburg, Germany*

The type II transmembrane serine protease dipeptidyl peptidase IV (DP IV), also known as CD26 or deaminase binding protein, is involved in the activation and proliferation of T lymphocytes. DP IV inhibitors, such as the HIV-1 Tat protein and other peptides containing the N-terminal sequence MXP suppress DNA synthesis and production of immunostimulatory cytokines. Replacement of the amino acid at position 2 of Tat(1-9) (Asp2) and of other MXP-containing peptides by the hydrophobic tryptophan strongly improves inhibition of DP IV-catalyzed substrate degradation. The N-terminal nonapeptide of the thromboxane A2 receptor (TXA2-R) MWPNGSSLG inhibits DP IV as potent as Trp2-Tat(1-9) (MWPVDPNIE). To examine whether there are basic changes in the backbone conformations of the nonapeptides containing the N-terminal MWP motif, we studied the conformations by using NMR spectroscopy and molecular modeling. The structure of Trp2-Tat(1-9) is flexible with the propensity to form fairly rigid conformations along the residues Pro3 to Pro6. The close inspection of the phi, psi values indicates that this peptide adopts a left-handed polyproline II helix (PPII) around the mid segment region covering Trp2 to Pro6. The solution conformations of TXA2-R(1-9) turned out to be highly flexible, although the NMR data show certain similarities to those of Trp2-Tat(1-9). The inherent flexibility of TXA2-R(1-9) makes definitive conformational conclusions difficult. PPII-helix-like structures were also found for the parent peptide Tat(1-9) as well as for the well known DP IV substrates neuropeptide Y and pancreatic polypeptide suggesting that they might represent a favoured structural class for the interaction with DP IV.

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SYNTHESIS AND ACTIVITY OF PHOSPHINIC TRIPEPTIDE ANALOGUES AS INHIBITORS OF CATHEPSIN C

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Cathepsin C or dipeptidyl peptidase I (DPP I, E.C. 3.4.14.1) is a lysosomal cysteine protease representing the most numerous papain-like clan, expressed throughout the animal and plant kingdom as well as in viruses and bacteria. Cathepsins are promising drug targets as their key proteolytic activities have been implicated in degenerative, invasive and immune system related disorders. Like other lysosomal cysteine proteases, DPP I is involved in non-specific intracellular protein degradation. Beyond this function, it is recognized to be crucial for activation of a number of granular serine proteases, granzymes A and B, cathepsin G, neutrophil elastase and chymase. It has been also suggested to be involved in several pathological disorders, like Duchene muscular dystrophy and basal cell carcinomas. Mutation in DPP I gene have been shown to result in autosomal-recessive palmoplantar keratoderma syndromes. In this work we present development of phosphinic tripeptide analogues of general formula Gly-Xaaψ[$P(O)(OH)CH_2$]-Gly as inhibitors of cathepsin C. The target compounds were synthesized by addition of methyl acrylate to the appropriate phosphinic acids followed by the N-terminus elongation using mixed anhydride procedure. The elongation step has been demonstrated to be a suitable method for lengthening of such pseudopeptides without requirement of hydroxyphosphinyl protection. The title compounds appeared to be moderate inhibitors of cathepsin C. However, although designed as transition state analogues, they surprisingly exhibited non-competitive mode of binding to the enzyme. Differences in mechanism of inhibition has been observed comparing C-terminal acids to the corresponding esters.

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STRUCTURE-BASED DESIGN OF CHLOROMETHYL KETONE INHIBITORS SPECIFIC FOR THE SN SUBSITE OF GINGIPAINS

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Chloromethyl ketones represent a class of irreversible inhibitors of cysteine proteases able to bind covalently to the thiol residue present in the active site and thus to block its crucial function in the process of hydrolysis of the peptide bond. Although very reactive versus various nucleophiles and therefore easily inactivated in physiological condition, they seem to be suitable model compounds to verify the usefulness of computer aided molecular modelling to design cysteine protease inhibitors. In this work we present the application of methods implemented in the computer programme Ludi/Insight II and the crystal structure of gingipain R complexed with D-Phe-Phe-ArgCH_2Cl or homology model of the catalytic domain of gingipain K to optimise the structure of inhibitors of these enzymes. The exquisite specificity of S1 binding pocket of gingipains towards arginine or lysine residues was verified versus their heteroaromatic analogues Het-GlyCH_2Cl. Structural and chemical properties of the S2 pocket compounds were examined using two series of compounds: R-ArgCH_2Cl and R-LysCH_2Cl. Finally, dipeptide analogues of general formula R-Phe-LysCH_2Cl were used to explore selectivity of hydrophobic S3 pocket of gingipain K, presumably able to bind bulky P3 aromatic residues. Acknowledgements This work is supported by the Polish Committee of Scientific Research (KBN, grant 6 P04B 025 21).

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EMERGING BIOSENSOR TECHNOLOGIES FOR THE EARLY DETECTION OF CANCER

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Cancer is often not detected until tumour cells have invaded surrounding tissues, and metastasized to other organs. More than 60% of patients presenting with colon, lung, breast and ovarian cancer have metastases at the time of presentation (1). In such cases, current treatment regimes are ineffective, and the prognosis poor. The early detection of cancer is therefore essential for improved treatment and management of the disease. We will discuss the potential of two emerging technologies: Magnetically Amplified Luminescence (MAL) (3) and Surface Plasmon Resonance using the BIAcore (4). MAL uses the amplified detection of DNA or proteins using rotating functionalised magnet particles with electrogenerated chemiluminescence as a read out. SPR measures changes in refractive index as binding occurs at, or near to, a sensor surface. Telomerase, a ribonucleoprotein that stabilises and extends telomeres of eukaryotic chromosomes (5), and hence regulates cell proliferative potential and lifespan, can be used as a target in both applications. Expression of telomerase is turned off early in embryogenesis, and in most normal somatic cells it is undetectable. However, telomerase is expressed in >85% of tumours, and hence could be a useful tumour marker (6) (1)Wulfkuhle et al, Nature Reviews Cancer, 3, 267, 2003 (2)Etzioni et al. Nature Reviews Cancer, 3, 1, 2003 (3)Weizmann et al, J.Am.Chem.Soc., 125, 3452, 2003 (4)Maesawa et al, Nucleic Acids Research, 31, E4, 2003 (5)Blackburn, Nature, 350, 569, 1991 (6)Saldanha et al, Anal. Biochem., 315, 1, 2003.

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STUDY OF A PUTATIVE WW-DOMAIN IN THE GOODPASTURE ANTIGEN BINDING PROTEIN

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Goodpasture disease is an autoinmune human disorder caused by the accumulation of autoantibodies against the alpha 3 chain of collagen IV. Goopasture antigen binding protein (GPBP), is a Serine/Threonine kinase that could phosphorilate the alpha 3 chain and therefore could be related with pathogenicity. We have identified a putative WW-like domain in the sequence of GPBP. This type of domains have been implicated in the stablishment of protein-protein interactions and present a well defined structrure, arranged in three hydrogen-bonded anti-parallel β-strands. The most usual ligands for this domains are proline rich regions of proteins and certain WW domain –ligand interactions could be also regulated by ligand phosphorylation. In fact recently, one of this domains has been identified in a tyrosin protein kinase receptor and it seems to play a key role in a regulatory manner. The synthesis and structural characterization of the identified WW domain of GPBP will be presented and its putative biological role will be discussed.

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NEW DERIVATIVES OF PICOLINIC ACID WITH ANTICONVULSANT ACTIVITY

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We have previously found, that several benzylamides of amino acids and heterocyclic acids show anticonvulsant activity (Pharmazie, 55, 27 (2000), Chem. Pharm. Bull., 49, 629 (2001)). Our last research demonstrated that picolinic acid benzylamide (Pic-BZA) is the most effective anticonvulsant (Prot. Pept. Lett., 10, 475 (2000)). However, its half life time is rather short. In the present study, looking for compounds with prolonged time of action we synthesized a series of analogs of Pic-BZA substituted with fluorine, methyl or meyhoxy group in different positions of the phenyl ring. Lipophilicity of the all synthesized Pic-BZA derivatives was moderate, what suggested their good biological barrier penetration. To predict the receptor affinity of the synthesized compounds we superimposed their molecular structures on the structure of the highly active Pic-BZA. This tests showed a close structural similarity of the all synthesized compounds to Pic-BZA. Anticonvulsant activity of the obtined compounds was tested in mice and in rats in Anticonvulsant Screening Project (ASP) of NINDS. The compounds were classified into three classes of activity. The highest activity showed picolinic acid fluorobenzylamides. ED50 of the most effective Pic-FBZA is 14,7 mg/kg (MES) and PI about 3,4 against MES (rats, ip.). Two another fluorobenzylamides were numbered into the second class of activity. Preliminary tests of stability showed, that fluorobenzylamides are decomposed in liver and half life time is prolonged to about 2 hours. Acknowledgements This investigation was supported in part by the State Committee for Scientific Research (Grant 6 P05F 027 21).

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NEW STABLE RENIN INHIBITORS WITH TWO PSEUDODIPEPTIDIC UNITS

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Majority of the synthesized peptidic renin inhibitors showed poor bioavailability. Therefore other structures were also searched. A distinguish nonpeptidic inhibitor is aliskiren (Biochem. Biophys. Res. Commun., 308, 698)2003)). Looking for inhibitors with better pharmacokinetics we previously obtained several compounds(Chem. Pharm. Bull., 50, 850 (2002)). Now we synthesized four new inhibitors: HCl.Phe(4-OMe)-MeLeu-(SS)-ACHPA-6-Ahx-Iaa; HCl.(SS)-AHPPA-(SS)-ACHPA)-6-Ahx-Iaa; HCl.(SS)-ACHPA-(SS)-ACHPA-6-Ahx-Iaa; HCl.(SS)Sta-(SS)-ACHPA-6-Ahx-Iaa. We assumed, that linear, hydrophobic fragment -6-Ahx-Iaa is able to fit to hydrophobic S2'-S3'portion of the renin cleft and that hydrophobic interaction of 6-Ahx with the S2' site can force affinity of the ACHPA portion to the active site of renin at the S1-S1' positions. Then the second pseudodipeptide can fill the hydrophobic cavity including S3-S2 portion of renin. The inhibitors containing two pseudodipeptidic units have one peptide bond between them and the second one coupling 6-Ahx. One additional peptide bond couples Iaa with the basic structure of the inhibitor. Molecular weight of the inhibitors is low (about 600-700 g/mol). Also lipophilicity is rather low. These properties seem to provide good bioavailability of the inhibitors. Activity determination in vitro of four synthesized inhibitors showed IC50 in limits of 10(-5)-10(-6) M/l. They are stable to chymotrypsin. Ackowledgements This investigation was supported in part by the Warsaw Medical University (Grant FW 22 W 03).

PEPTIDIC INHIBITORS AND PROTEIN-PROTEIN INTERACTIONS

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STRUCTURE, DYNAMICS AND RATIONAL DESIGN OF SMALL CHYMOTRYPSIN INHIBITORS

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Solution structure of small (35-residue) serine protease inhibitors were determined by NMR spectroscopy. One of the inhibitors, SGCI (Schistocerca gregaria chymotrypsin inhibitor), is an excellent inhibitor of both mammalian and arthtropod enzymes, whereas the other, SGTI (trypsin inhibitor), is a specific inhibitor of crayfish and shrimp trypsin, inhibiting the bovine protease only moderately. The two types of inhibitors exhibit very similar 3D-fold stabilized by a different network of residue-residue interactions [1]. Structural models of SGCI of different size were designed and obtained using convergent peptide synthesis [2]. Proton-deuterium exchange experiments as well as 15N-relaxation studies reveal that their dynamic properties of the molecules differ greatly, suggesting that the biochemical specificity can be attributed to the observed dynamical features of these inhibitors [2]. NMR mapping of the residues involved in contacts with the proteases both in SGCI and SGTI as well as ab intio calculations addressing the enzyme-inhibitor interactions were completed. These results open new insights in the understanding of protein-protein interactions. [1] Z. Gáspári, A. Patthy, L. Gráf, A. Perczel Eur. J. Biochem., 269, 527 (2002). [2] B. Szenthe, Z. Gáspári, Z., A. Nagy, A. Perczel, L. Gráf Biocmeistry (2004) in press. [3] Z. Mucsi, Z. Gáspári, Gy. Orosz, A. Perczel Protein Eng. 16, 673 (2003).

P360

PRIMED SITE PROBING OF PAPAIN-LIKE CYSTEINE PROTEASES

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Epoxysuccinyl peptides of the structure R-HN-Leu-(2S,3S)-tEps-OH are potent irreversible inhibitors of papain-like cysteine proteases [1]. Their binding mode is well characterized by X-ray crystallography [2]. The leucine side chain interacts via hydrophobic contacts with the well defined S2 pocket, which is a feature shared by all papain-like cysteine proteases. Due to the common binding mode, this type of inhibitor represents a privileged structural element suitable for probing the S1' and S2' pocket of members of the enzyme superfamily. By loading the carboxyl group of the probe head with dipeptides, the structural requirements for an optimal interaction with the primed sites can be determined in a systematical manner. Consequently, to probe the S1' pocket, we have synthesized by a solid phase approach 18 libraries of the structure R-HN-Leu-(2S,3S)-tEps-Xxx-Yyy-OH. At position Xxx one of the 20 natural occurring amino acids except cysteine and methionine is present, whereas position Yyy is defined by an isokinetic mixture (cysteine and methionine excluded). By this approach, we have investigated representative members of the papain superfamily. [1] Powers, J. C., Asgian, J. L., Ekici, Ö. D., James, K. E., Chem. Rev. 2002, 102, 4639-4750. [2] Matsumoto, K., Mizoue, K., Kitamura, K., Tse, W.-C., Huber, C. P., Ishida, T., Peptide Science 1999, 51, 99-107.

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PEPTIDE BINDING TO S100A4 PROTEIN

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S100A4 or Mts-1 is an 11.5 kDa calcium-binding protein. It is overexpressed in many cancers cells, and is a well-established marker of tumor progression, invasion, and metastasis formation. Up-regulation of S100A4 expression was also reported for several pathologies associated with activated endothelium such as ocular neovascularization, artheriopathy, neurodegeneration, and various inflammations including Rheumatoid arthritis. In this communication we describe a peptide binding to S100A4. The peptide sequence was found using phage-displayed peptide library. The phage selection has been done on S100A4 fused with GST, immobilized on glutathione-sepharose beads, in the conditions of MTS1-dimer formation. The synthetic peptide binds specifically to S100A4 with a nanomolar affinity, determined by fluorescent assays. The peptide-protein binding is calcium-dependent. We also report a synthesis of a conjugate of the peptide with an amphiphilic block copolymer, Poloxamer 407. The conjugate is capable of forming micelles with a hydrophobic core, and it is tested as a model drug delivery system.

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ACCURATE MASS ANALYSIS OF GLYCOPROTEIN VARIANTS BY ELECTROSPRAY IONISATION, ORTHOGONAL ACCELERATION TIME-OF-FLIGHT MASS SPECTROMETRY AND MAXIMUM ENTROPY

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Over the past 15 years electrospray ionisation (ESI) combined with mass spectrometry has proven to be a very powerful combination for the determination of intact protein mass. Algorithms have also been developed to deconvolute the multiply charged spectra of intact proteins onto a true mass scale. Maximum Entropy (MaxEnt 1TM), being the most powerful. The inactive glycoprotein mutant Populus tremula x tremuloides Xyloglucan Endotransglycosylase 16A (PttXET16A E85A) was introduced into a Waters Micromass LCT PremierTM (Waters, MS Technologies, Manchester UK) at 5uL/min from a 250uL gas-tight syringe, at a concentration 10pmol/uL. Within the PttXET16A solution, horse heart myoglobin was added as an internal standard. Multiply protonated peaks of myoglobin were used to recalibrate the spectral data. Maximum Entropy was used to generate an accurate deconvoluted mass spectra for the glycoprotein studied. In terms of RMS ppm errors, the data presented is comparable to the errors obtained during the accurate mass analysis of compounds whose molecular weight is below Mr 1000. With such high mass accuracies, one can therefore, calculate, the presence of other post-translational modifications such as phosphorylations, sulphations, oxidations, disulphide bonds and possible discrepancies with submitted database sequences. Using the combination of electrospay ionisation, oaTOF and Maximum Entropy we can accurately mass measure intact proteins. Of 3 out of the 4 glycoforms analysed we have obtained mass accuracies better than an RMS of 3ppm. Two out of 4 were sub 1ppm RMS.

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ANALYSIS OF NONCOVALENTLY ASSOCIATED PROTEIN COMPLEXES BY ELECTROSPRAY ORTHOGONAL ACCELERATION TIME-OF-FLIGHT MASS SPECTROMETRY

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Mass spectrometry has allowed denatured proteins and non-covalently assembled macromolecular protein complexes to be accurately mass measured. Therefore, allowing one to determine the presence of possible amino acid substitutions, post translational modifications, protein-subunit and protein-ligand stoichiometry. The transfer of noncovalently associated protein-protein complexes from solution to the gas phase generally results in the formation of ions possessing relatively few charges and as such m/z values are often above 3000. Some charged species as high as m/z 9,000-10,000 1 and m/z 20,000 2 have been reported. Such mass ranges would be unachievable on a standard triple quadrupole or ion trap instrument. This is why orthogonal acceleration Time-of-flight (oa-TOF) is the ideal analyzer for this application. Additionally, electrospray ionization is a very gentle form of ionization, thus enabling the intact transfer into the gas phase and detection of large multiprotein structures with little or no fragmentation. Here we show the detection of several, different, non-covalently associated protein-protein complexes by ESI oa-TOF. The influence of source and analyzer parameters on the transmission of these complexes has also been investigated and will be discussed. The macromolecular complexes have masses of 300kDa, and multiple charged species in the ranges of m/z 7000-8000. 1. Van Berkel WJH, Van Den Heuvel RHH, Versluis C & Heck AJR Protein Science 2000 9 435–439.

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PSEUDOPEPTIDIC APPROACH TO CONTROL THE PROTEOLYTIC ACTIVITY OF HUMAN LEUCOCYTE ELASTASE (HLE) IN INFLAMMATORY PROCESSES

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Human leukocyte elastase (HLE, EC 3.4.21.37) is involved in the pathogenesis of pulmonary emphysema, chronic bronchitis and rheumatoid arthritis. Inhibitors from different chemical classes have been described [1, 2]. We further investigated the effect on HLE of newly synthesized pseudopeptides. We have introduced carbonylhydrazone psi[CO-NH-N=], ketomethyleneimino psi[CO-CH=N] and ketomethyleneamino psi[CO-CH_2-NH] pseudopeptide moieties in the target molecule Ala-Ala-Pro-Val-Ala-Ala derived from a fragment of the alpha1-PI inhibitor. The sterically constrained carbonylhydrazone- and ketomethyleneimino-peptides are not recognized by HLE. On the other hand, the ketomethyleneamino pseudopeptide is flexible and binds better to a catalytic site of the HLE. The synthesis of the pseudopeptide moiety Fmoc-Val-CHO consists in the action of diazomethane on Fmoc-Valine isobutyl carbonate at very cold temperature, followed by mild oxidation with dimethyldioxirane. The glyoxal, thus obtained, is then condensed on the growing peptide in the presence of $NaBH_3CN$ leading to the ketomethyleneamino link. We started NMR studies of the inhibitor in the presence of elastase. From the first results, it appears that the flexibility of the pseudopeptidic inhibitor is necessary to fit to the catalytic site of the HLE. 1. Reboud-Ravaux M. (2001) J. Soc. Biol. 195 (2), 143-50. 2. Vanderesse R., Thévenet L., Marraud M., Boggetto N., Reboud-Ravaux M. and Corbier C. (2003) J. Peptide Sci. 9, 282-299.

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INTRODUCTION OF RADIOLABEL INTO CONSTRUCTS DESIGNED TO BIND COVALENTLY AND INHIBIT ACETYLCHOLINESTERASE

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We seek acetylcholinesterase (AChE) inhibitors that will bind to a peripheral site and exclude toxic organophosphorylating agents from the acylation site while interfering minimally with essential acetylcholine passage. Our initial goal is to screen inhibitors after tethering them covalently to the sulfhydryl group of a cysteine residue introduced at the peripheral site by site-specific mutagenesis (H287C). Inhibitor synthesis first involves preparation of cyclic peptides with our previously developed solid-phase approach (?SPPS) that includes on-resin cyclization and subsequent cleavage from the resin by HF. The tether 8-amino-3,6-dioxaoctanoic acid (mini-PEG) is then attached in amide linkage to an amino group on the peptide, and a thiol-reactive methanethiosulfonyl (MTS)-propionyl substituent is inserted at the end of this linker. When applied with a cationic ligand in place of the cyclic peptide, this strategy leads to tethered inhibition of AChE. Confirmation of enzyme attachment is greatly facilitated by incorporation of a radiolabel in the construct. We introduce the label through reductive alkylation of the N-epsilon-amino group of a peptide lysine side chain with [3H]formaldehyde and $NaCNBH_3$. However, the radiolabeling must precede introduction of the labile MTS group in the last step of the synthesis. Preliminary reactions with unlabeled alkylation reagents indicate that the peptide must be in excess of formaldehyde to provide a sufficient yield of the mono-methylated derivative. Subsequent acylation of this secondary amino group with the OPfp ester of Fmoc-mini-PEG proceeds at a rate comparable to that of residual nonalkylated peptide, while the di-methylated byproduct is excluded from further processing.

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NEW FLUOROGENIC SUBSTRATES FOR THE LYSOSOMAL PROTEASE CATHEPSIN S

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Cathepsin S (CatS) is a lysosomal cysteine protease and has been found to be predominantly expressed in antigen presenting cells. Findings that CatS is responsible for critical steps in antigen presentation leads to the development of specific inhibitors which may be beneficial for the treatment of various autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. Up to now only few substrates for CatS are described. Most of them show very low specificity especially to the related CatL. Therefore, we synthesized several peptide substrates and characterized them by in vitro digestion with CatS. The generated cleavage products were separated by RP-HPLC followed by mass spectrometry. Based on x-ray crystallographic data we initially focused on the P2 position. Interestingly, mutations of the P2 peptide site gave a relatively clear picture; only the 4 amino acids V, L, M and C are readily cleaved. In addition, to some extent phenylalanine also allows cleavage of the substrate. Peptides with other amino acids, including isoleucine, at P2 are stable to digestion with CatS. Based on these experiments we exchanged the amino acid in position P3, P1, P1´and P2`. This allowed us to design completely new substrates for CatS with excellent specificity. A detailed study of the properties of these substrates and comparison with other lysosomal cysteine proteases will be presented. To show the properties in the biological system we tested the substrates in lysates of APCs and also in subcellular compartments like endosomes and lysosomes where CatS is highly enriched.

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DETERMINATION OF OPTICAL PURITY OF N-BENZYLOXYCARBONYL PROTECTED AMNINOPHOSPHONATES BY MEANS OF CAPILLARY ELECTROPHORESIS AND 31P NMR SPECTROSCOPY WITH APPLICATION OF CYCLODEXTRINS

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Aminophosphonic and aminophosphinic acids represent a class of compounds attracting considerable interest because of their diverse and useful biological activities, which usually derive from their competition versus amino acids for the active centre of an enzyme or other cellular receptor. N-Benzyloxycarbonyl protected aminophosphonates and aminophosphinates are the essential intermediates for synthesis of phosphorus-containing peptide analogues which resemble the tetrahedral transition state of the amide bond hydrolysis. They are able to coordinate the zinc atom present in the active sites of metalloproteases and to block its function in the process of hydrolysis. Thus, their chemistry continues to attract considerable interest and provides a wide variety of potent inhibitors of proteases. It is well recognized that the biological activity of organic compounds is strongly dependent on their stereochemistry. The preparation of well defined, optically pure peptidomimetics based on phosphorus may be achieved by the use of enantiomeric forms of N-benzyloxycarbonyl aminophosphonates or aminophosphinates as substrates. Thus, a simple and versatile method for determination of optical purity of these acids is strongly desirable. The detailed studies concerning capillary electrophoresis and 31P NMR spectroscopy enantiodifferentation of N-benzyloxycarbonyl protected alpha-aminophosphinic and alpha-aminophosphonic acids and their monoesters with various commercially available cyclodextrins are presented. The stoichiometry and geometry of inclusion complexes of cyclodextrin with selected compounds were studied in some detail. Molecular modeling was additionally applied to confirm the proposed modes of complexation. On the basis on obtained results from different techniques the mechanism of resolution is postulated.

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A POLAR RESIDUES MOTIF (QXXS) RATHER THAN A GXXXG MOTIF DRIVES THE ASSOCIATION OF ESCHERICHIA COLI ASPARTATE RECEPTOR TRANSMEMBRANE DOMAIN

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Protein-protein interaction and recognition within the membrane are involved in many vital cellular processes. However, our knowledge of the factors that control these interactions within the membrane milieu is very limited. Previous studies have shown a role for polar residues in the assembly of synthetic peptides in vitro and the association of de-novo designed TM helices in vivo. Here we examined, for the first time, the involvement of polar residues in the dimerization of a biological TM domain in its natural environment. For this purpose we grafted the N-terminal TM domain of the Escherichia coli aspartate receptor (Tar-1) within the ToxR assembly system. Replacement of the two original polar residues (Gln and Ser), located within the Tar-1, to two non-polar residues (Gly and Ile) created a GxxxG dimerization motif within this TM domain. The low dimerization ability of this mutant demonstrated the role of QxxS motif in the dimerization of the Tar TM domain and implied that the presence of a GxxxG motif is not always sufficient to promote dimerization. A Tar-1 transmembrane (TM) domain analog peptide had a dominant-negative effect on the dimerization ability of Tar-1. However a mutant peptide in which the two polar residues were substituted for non-polar residues showed only marginal inhibition ability. These results demonstrate that the assembly of the Tar-1 homo-dimer is mediated by the presence of the polar motif QxxS and that a Tar-1 homologues peptide can inhibit this dimerization by disruption of the proper association of the TM domains.

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SMALL LINEAR AND CYCLIC RGD PEPTIDES CONTAINING SALICYLIC ACID DERIVATIVES AND THEIR ANTIPLATELET ACTIVITY IN VITRO

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The combination in the same molecule of dipeptide or tripeptide amides, containing amino acid(s) of RGD sequence and salicylic acid derivatives at their N-terminal amino group have shown satisfactory effect against human platelet aggregation in vitro and important specificity for the Gp Ib receptor, a glycoprotein receptor on the surface of the platelets. In an attempt to synthesize more potent inhibitors of platelet aggregation, we tried out the synthesis of linear and cyclic RGD analogs, containing thiosalicylic acid, 5-methyl salicylic acid, 5-amino salicylic acid, 2-methoxy benzoic acid, etc. The syntheses of the new analogs were carried out by using SPPS. The synthesized compounds were purified by RP-HPLC and lyophilised to give fluffy solid, identified by FT-IR, NMR and ES-MS spectra. These compounds were tested for inhibitory activity on human platelet aggregation in vitro, by adding common aggregation reagents to citrated platelet rich plasma (PRP). The aggregation was determined using a dual channel electronic aggregometer by recording the increase of light transmission. Their specificity for the Gp receptors was checked by using flow cytometry with monoclonal antibodies against Gp Ib, Gp IIb/IIIa, Gp IIIa and GMP140 receptors. The IC50 values of the synthesized and tested compounds and the flow cytometry results will be discussed in details. References 1. Sarigiannis Y., Stavropoulos G., Liakopoulou-Kyriakides M. and Makris P.E., Peptides: (Proceedings of the 27th European Peptide Symposium), Sorrento, Italy, 2002, p.606 2. Sarigiannis Y., Stavropoulos G., Liakopoulou-Kyriakides M. and Makris P.E., Letters in Peptide Science, 9, 101-109, 2002

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INHIBITION OF CATHEPSIN L BY PROPEPTIDE-DERIVED EPOXYSUCCINYL PEPTIDES

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Since the discovery of the natural product E-64 (Agm-Leu-(2S,3S)-tEps-OH) in 1978, the class of epoxysuccinyl peptides has received great attention as irreversible inhibitors of papain-like cysteine proteases [1]. Many of these compounds are potent inhibitors but do not show substantial selectivity for an individual member of the papain superfamily. Due to the high degree of structural homology, it is difficult to gain selectivity exploiting only interactions within the active site cleft. The crystal structure of procathepsin L [2] shows that the propeptide binds along the active site cleft in a direction opposite to the substrate. The amino acids Phe-78 and Gln-79 of the propeptide occupy the S2 and S3 pocket of the protease respectively, whereas the remaining C-terminal part of the propeptide (amino acids 80-96) makes contacts with the protein surface. Because of these structural features the amino function of Phe-78 is ideally suited to connect propeptide fragments of increasing length with (2S,3S)-oxirane-2,3-dicarboxylic acid. The obtained series of epoxysuccinyl peptides were used to study the influence of this remote sites on selectivity and inhibitory potency. [1] Powers, J. C., Asgian, J. L., Ekici, Ö. D., James, K. E., Chem. Rev. 2002, 102, 4639-4750. [2] Coulombe, R., Grochulski, P., Sivaraman, J., Menard, R., Mort, J. S., Cygler, M., EMBO J. 1996, 15, 5492-5503.

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PYROGLUTAMATE FORMATION OF PENTAPEPTIDES QXNAD

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The endogenous pentapeptide QYNAD was found at elevated levels in the cerebrospinal fluid of patients with multiple sclerosis or Guillain-Barré syndrome. The peptide blocks voltage-gated sodium channels at very low concentrations (Nature Medicine, 2000, 6, 808). QYNAD was prepared by fully automated solid phase peptide synthesis using 9-fluorenylmethoxycarbonyl/tert.-butyl chemistry on chlorotrityl-resin. During storage of the peptide in solution cyclization to form an N-terminal pyroglutamate was observed followed by hydrolysis of the pyroglutamyl to a glutamyl residue. For HPLC separation of the product and side products we used a "Hypercarb" column with a separation phase made from porous graphitic carbon which is well suited for the separation of polar compounds. A series of peptides was prepared to investigate the influence of the amino acid in position 2 on pyroglutamate formation. Significant differences in storage stability have been observed with respect to sequence, pH and temperature.

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PROGRESS IN THE PREPARATION OF PEPTIDE ALDEHYDES VIA A POLYMER SUPPORTED IBX OXIDATION AND SCAVENGING BY A THREONYL RESIN

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A novel and efficient strategy for the parallel preparation of pure peptide aldehydes by solid phase synthesis combined with polymer-assisted solution phase synthesis followed by purification with an aldehyde scavenger resin is reported. The procedures include the oxidation of peptide alcohols with the mild and selective IBX-polymer reagent[1] and the purification of the resulting peptide aldehyde by a capture-release procedure using a threonine moiety attached to aminomethyl resin. This three-step method delivered peptide aldehydes in an excellent purity with a satisfying yield and conservation of the optical integrity of the C-terminal residue to a high degree. Representative examples show a high compatibility with the use of common side-chain protecting groups. Several new protease inhibitors were found and tested in caspase-1 and -3 assays in comparison to known inhibitors. [1] G. Sorg, A. Mengel, G. Jung, J. Rademann, Angew. Chem. Int. Ed. 2001, 40, 4395 4397; Angew. Chem. 2001, 113, 4532 4535.

P373

PREPARATION AND EVALUATION OF THROMBIN INHIBITORS CONTAINING CYA

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Thrombin is considered to play a major role in . Among other functions thrombin is crucially involved in the formation of fibrin and platelet aggregates. Under pathophysiological conditions where the capacity of the natural thrombin inhibitor, antithrombin III (ATIII), is depleted, the thrombin generation and its action needs to be controlled. Therefore, many research groups have adressed their attention on the elaboration of synthetic thrombin inhibitors. Most inhibitors are base on the peptide sequence D-Phe-Pro-Arg or they contain highly basic moieties, such as the amidino group (example: NAPAP ?-naphthylsulfonyl-Gly-D,L-4-amidinophenylalanine-piperidine). The objective of this work has been to design and to synthesize a new dipeptide Mtr-Cya-D-4-Hydroxyamidino-Phe-Pip and Mtr-Cya-D-4-amidinophenylalanine-Pip and to evaluated their biological properties.

P374

PEPTIDE - PEPTOID HYBRID PROTEASE INHIBITORS BASED ON TRYPSIN INHIBITOR SFTI-1 ISOLATED FROM SUNFLOWER SEEDS: CHEMICAL SYNTHESIS, KINETIC AND STRUCTURAL PROPERTIES

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The design of new protease inhibitors is often based on modifications of naturally occurring (endogenous) inhibitors. The SFTI-1 inhibitor isolated from sunflower seeds is the smallest, endogenous member of the Bowman – Birk protease inhibitor family. Due to its small, 14 amino acid size, compact structure and easy chemical synthesis, it was chosen as a model peptide inhibitor to incorporate the N-substituted glycine residues into the native SFTI-1 peptide sequence. By modification of key, specificity determining position amino acid residue – Lys5 (P1 – according to Schechter and Berger notation) of SFTI-1 a new class of hybrid, peptide – peptoid inhibitors of antitrypsin, antichymotrypsin and antielastase activity was obtained. Such hybrid inhibitors exhibit improved protease resistance and possess considerable activity. According to our knowledge, there are no reports in the literature considering the application of N-substituted glycine residues (peptoid monomers) as the mimetics of natural amino acids, which are used to replace them in the native peptide protease inhibitors. The chemical synthesis, kinetic parameters and structural properties data of several SFTI-1 analogues are presented. This work was supported by the University of Gdansk Grant.

P375

SPECIFIC AND MEMBRANE PERMEABLE INHIBITORS OF CGMP-DEPENDENT PROTEIN KINASE

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cGMP-dependent protein kinase, the closest relative of cAMP dependent protein kinase, acts downstream of NO mediated cellular signalling pathways. It is involved in smooth muscle relaxation and neuronal synaptic plasticity. We have developed highly specific, membrane permeant peptide blockers of cGMP-dependent protein kinase utilizing affinity selection from peptide libraries [1,2]. The inhibitory peptides facilitate the disentanglement of the interconnected regulatory pathways of cAMP and cGMP dependent protein kinases. We now report on further inhibitory sequences, their kinetic behaviour, cellular uptake, and their influence on cell morphology. The inhibitory peptide D-DT2 entirely composed of D amino acids showed a significant potentiation of PKG inhibition over the L amino acid sequence with an inhibition constant of 500 pM. Resistance against tryptic degradation makes D-DT2 a valuable tool to study the role of PKG over a wide range of incubation times under in vivo conditions. The membrane permeable inhibitors DT2 and DT3 induce the arrestment of the cell cycle of various mammalian cell lines after doubling of the cell nucleus. 1. Dostmann, W.R.G., et al., Proceedings of the National Academy of Sciences of the United States of America, 2000. 97(26): p. 14772-14777. 2. Tegge, W., et al. in Peptides: The wave of the future. 2001. San Diego, California, USA: Proceedings of the Seventeenth American Peptide Symposium, p. 191-193.

P376

SOLID-PHASE SYNTHESIS OF HYDANTOINS AS CASPASE INHIBITORS

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Apoptosis is a key process in a wide variety of biological systems, including normal cell turnover, the immune system, embryonic development and metamorphosis. Inappropriate apoptosis is involved in many human pathologies, including neurodegenerative diseases. Designs based on papain structure have given potent peptidic and pseudopeptidic inhibitors, , but with physical-chemistry and pharmacokinetic characteristics that limit their use to the intravenous treatment of acute episodes of some diseases. Non-peptidic inhibitors could solve this problem, but the few that have been synthesized to date present a low selectivity. Here we discuss a new family of papain inhibitors based on a hydantoin ring as scaffold. In silico studies demonstrated that the heterocycle fits into the active center of the enzyme, where its carbonylic moieties form hydrogen bonds with residues of the protein, which confers an improved interaction of the rest of the enzyme with the compound. A first generation of compounds provided us with an apropriate skeleton and a second generation was synthesized bearing in mind these results. The in vitro evaluation, using recombinant caspase-3 and a fluorescent substrate, was performed. 1)D.S. Karanewsky et al., Bioorg. Med. Chem. Lett., 1998, 8, 2757. 2)M.Garcia-Calvo et al., Cell Death and Differentiation, 1999, 6, 362. 3)C.W. Scout et al., J. Pharmacol. Exp. Ther., 2003, 304, 433.

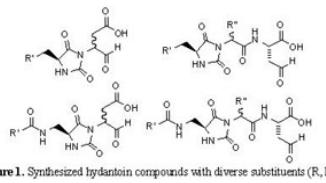

Figure 1. Synthesized hydantoin compounds with diverse substituents (R, R', R")

P377

DESIGNED A POWERFUL CD4 MINIPROTEIN, USEFUL TO DISCOVER INHIBITORS OF HIV-1 TARGETTING THE GP120 GLYCOPROTEIN.

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Using structural information on CD4-gp120-17b complex, we designed a 27 amino acid miniprotein, CD4M33, which reproduces the hot spot of CD4 surface binding gp120, presents optimal interaction with gp120 and binds HIV-1 and viral envelopes with native-like affinity. CD4M33 inhibits infection of primary isolates and induces gp120 conformational changes that expose conserved cryptic epitopes, thus it is a small-size functional CD4 substitute. CD4M33 was labeled with fluorescein and used in polarisation assays to determine binding equilibrium constants of a panel of HIV-1 envelope glycoproteins in solution. CD4M33 contains a biphenylalanine(Bip)-23 residue, which is equivalent to CD4 Phe-43 and, in a CD4M33-gp120 computed complex, engages the gp120 "Phe-43 cavity". To better understand how CD4M33 binds gp120 structure, we synthesized a CD4M33F23 mutant, which contains a shorter Phe-23 side chain, and a gp120S375W mutant, which presents the "Phe-43 cavity" filled by the larger W375 side chain. ELISA or Biacore experiments showed that CD4M33 bound gp120S375W with thousand-fold lower affinity, while CD4M33F23 bound with nanomolar affinity, thus demonstrating that CD4M33 Bip-23 side chain engages the "Phe-43 cavity". Thus, fluorescent CD4M33 or CD4M33F23 can be used to distinguish molecules that can bind to the CD4 binding site or "Phe-43 cavity" of HIV-1 envelope glycoproteins. By using fluorescent CD4M33 and CD4M33F23, a sensitive fluorescent polarization assay has been developed and used in a competition assay and 384-well microplate format to determine dissociation constants of different gp120 ligands and screen small molecule libraries to discover new gp120 ligands, potential inhibitors of HIV-1 entry.

P378

BIOSENSOR-BASED BIOMOLECULAR INTERACTION ANALYSIS: THE USE OF 2-[PHENYL(METHYL)SULFONIOLETHYLOXYCARBONYL] (PMS)-SERINE AS A NOVEL REAGENT FOR SURFACE DERIVATISATION

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Surface plasmon resonance-based biosensors provide a sophisticated and discriminating means of probing biomolecular interactions. Specific ligands such as peptides and proteins can be immobilized onto the sensor surfaces by a number of chemistries including covalent attachment via amine, thiol or aldehyde chemistry, capture via biotin-avidin/neutravidin interaction or the use of specific tags [1]. However, whilst each of these immobilization strategies has advantages and disadvantages, in many cases the chemistry used leads to surface heterogeneity. There is therefore a requirement for developing new chemistries that enable derivatisation onto sensor surfaces via selected amino acids in a unique orientation. A new water-soluble N-protecting group, 2-[phenyl(methyl)sulfoniolethyloxycarbonyl (Pms), has been recently been described and successfully used in solid phase peptide synthesis under aqueous conditions [2]. We have efficiently coupled Pms-Ser-OH in situ onto a BIAcore biosensor using a carboxymethyldextran surface by (1) amine coupling of the Pms-Ser-OH using NHS/EDC chemistry, (2) blocking of any residual activated surface with ethanolamine, (3) N-deprotection with 5% sodium bicarbonate solution, (4) conversion of the serine into an aldehyde by reaction with sodium periodate. This surface can then be reacted specifically, rapidly and efficiently with N-terminal Cys-containing peptides to form a stable thiazolidine bond. This provides a simple means of aqueous surface derivatization of an N-terminal cysteine-containing peptide or protein in a regiospecific orientation. Examples of the use of this chemistry will be given. 1. Nice, E. and Catimel, B. BioEssays, 21, 339, 1999. 2. Hojo, K., Maeda, M. and Kawasaki, K. J. Peptide Sci., 7, 615, 2001.

PEPTIDIC INHIBITORS AND PROTEIN-PROTEIN INTERACTIONS

P379

DESIGN AND SYNTHESIS OF A NOVEL CASPASE INHIBITOR

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Caspases are essential for apoptosis, which appears to contribute to the occurrence of many ailments. Therefore, caspase inhibitors are promising therapeutic candidates for the treatment of inflammation, cancers and neurodegenerative disorders including Alzheimer's disease. Today, many designed and potent caspase inhibitors were developed, while still have some deficiencies to be overcome to act as effective and practical drugs, such as instability, low bioavailability and poor pharmacological profiles. It was reported that reversed peptide not only displayed stability toward enzymatic degradation but also improved bioavailability and potency. Therefore, we designed a novel caspase inhibitor based on wide-spectrum inhibitor Z-VAD-CHO. In this design, we replaced L-amino acid with D-amino acid, which resulted in all the amide bonds reversed. However, C-terminal aldehyde group remained. Therefore, it still belongs to reversible inhibitors. In this paper, the synthesis of the inhibitor molecule was presented and the inhibitory activity of the synthetic compound was tested by fluorescent activity assay.

P380

APPLICATION OF COMBINATORIAL CHEMISTRY METHODS FOR SELECTION POTENT SUBSTRATES AND INHIBITORS OF SERINE PROTEINASES

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The field of combinatorial peptide chemistry has emerged as a powerful tool in the study of many biological systems. This strategy allows synthesising a large number of molecules and selection the most potent compound(s) from library of compounds. Here we report a simple method for solid phase synthesis and deconvolution of the tetrapeptide library of chromogenic substrates of bovine beta-trypsin with 5-amino-2-nitrobenzoic acid (Anb5, 2) as a chromophore. All four positions were modified by the same eleven natural amino acids. The most active substrates were resynthesised, and their kinetic parameters (kcat, KM, kcat/KM) with bovine beta-trypsin were determined. We describe also the synthesis of peptide library based on the active acyclic analogue of SFTI-1 [1], the smallest known naturally occurring peptidic trypsin inhibitor isolated from the sunflower seeds. P1 and P4' position (according to Berger Schechter notation) were modified. The peptide library synthesized was screen against bovine beta-trypsin, alfa-chymotrypsin and human leukocyte elastase. Both peptide libraries were synthesized using portioning-mixing method. The method for selection the most active inhibitors was described previously [2]. References [1] Zablotna E. et al., Biochem. Biophys. Res. Commun., 2002, 292, 855-859. [2] Kazmierczak K. et al, Biochem. Biophys. Res. Commun., 2003, 310, 811-815. Acknowledgements. This work was supported by University of Gdansk. Grants No 8000-5-0225-3 & 8000-5-0324-3 and by the State Committee for Scientific Research (KBN) Grant No 1007/T09/2003/24.

P381

DIRECT STUDIES OF GPCR SIGNAL TRANSDUCTION EVENTS USING PLASMON-WAVEGUIDE RESONANCE SPECTROSCOPY

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Understanding structure-function relationships and mechanisms of signal transduction in GPCRs is becoming increasingly important taking into account their central roles as pharmacological targets. Their integral membrane nature and rather low natural abundance present many challenging problems. Using a recently developed technique, plasmon-waveguide resonance (PWR) spectroscopy, we have been able to directly measure the binding between the hDOR and its G-protein effectors in real time. This highly sensitive technique can directly monitor changes in mass density, conformation and orientation occurring in thin proteolipid films without requiring labeling protocols, and allows the direct determination of binding constants and other biophysical properties in a system very close to the receptor's natural environment. Using this technique we have immobilized the human δ-opioid receptor (hDOR) in a solid-supported lipid bilayer and investigated the binding of G-proteins to this receptor when pre-bound with different types of ligands. We have found that both the affinity of G-protein sub-types (Gi{alpha}1, Gi{alpha}2, Gi{alpha}3, and Go{alpha}) towards the receptor and the ability to undergo GDP/GTP{gamma}S exchange are highly dependent on the nature of the ligand that is pre-bound to the receptor, leading to great diversity of downstream signaling. We have been able, using this technique, to monitor the conformational changes of the receptor upon ligand binding in the presence and absence of G-proteins that is, in the high and low affinity states of the receptor. The results provide new insights into GPCR functioning and signal transduction in the hDOR, and illustrate a powerful new protocol for drug development involving GPCRs.

P382

THE 2-BENZAZEPIN-3-ONE STRUCTURE AS A BETA-TURN MIMIC: SYNTHESIS AND CONFORMATIONAL ANALYSIS

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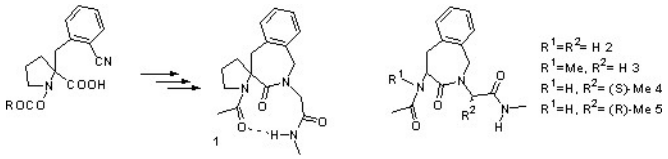
Freidinger gamma-lactams have been proposed to induce turn structures in peptides.[1] A stronger bias has been obtained by using spiro-lactams.[2] We now report the synthesis of a novel turn mimic 1 based on the spiro-benzazepin-3-one skeleton, starting from alfa-(o-cyanobenzyl)Pro. NMR analysis revealed that the model N-acetyl, N'-methylamide preferentially adopts a turn conformation with an intramolecular hydrogen bond. In contrast, neither the N-H or N-methyl-benzazepine-Gly analogues 2,3, nor the homochiral benzazepine-Ala 4, nor the heterochiral benzazepine-D-Ala 5 showed any sign of turn structures. The use of this new turn mimic in endomorphin peptides is also reported.[3] [1] Freidinger,R.M.; Veber,D.F.; Perlow,D.S.; Brooks,J.R.; Saperstein,R. Science 210(4470) 656-8 (1980) [2]Ward,P., Ewan, G.B., Jordan, C.C., Ireland, S.J., Hagan, R.M., Brown, J.R., J. Med. Chem. 1990, 33, 1848 [3] poster presented by Cs. Tömböly



P383

SECONDARY STRUCTURES OF BETA-PEPTIDES: THE UNNATURAL BEHAVIOUR OF BETA-SHEETS

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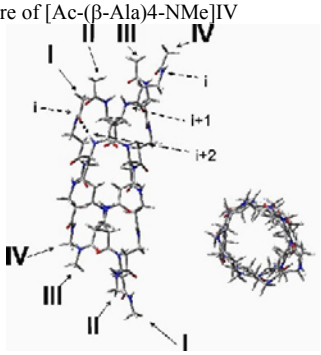
Structural properties of beta-peptides are in the focus of current research on unnatural oligomers.[1] However, most of the experimental observations were made on beta-peptides containing various sidechains, thus the driving force of secondary structure folding could not be fully understood. After providing information on the conformational properties of beta-amino acids [2], and helices of beta-peptides, we intend to explore the beta-sheet folding affinity of beta-peptides. Modelpeptides with 2-4 parallel strands were built using Ac-(beta-Ala)2-4-NHMe. beta-sheets of these beta-peptides were investigated at different levels (RHF/3-21G, B3LYP/6-311G++(d,p)//RHF/3-21G) of theory. Our results show that these strands are able to form highly ordered super-beta-sheets (Figure1). Finally, we compared secondary structures of alfa- and beta-peptides. [1]. Apella D. H., Christianson L. A., Klein D. A., Powell D. R., Huang X., Barchi J. J. Jr., Gellman S. H., Nature 1997, 387, 381-384 [2]. Beke T., Csizmadia I. G., Perczel A., J. Comp. Chem. 2004, 25, 285-307
Figure 1: I+2 structure of [Ac-(β-Ala)4-NMe]IV



P384

IDENTIFICATION OF OPIOID RECEPTOR-LIGAND INTERACTIONS USING STRUCTURALLY RELATED DELTA OPIOID RECEPTOR AGONISTS AND ANTAGONISTS AND MOLECULAR MODELING

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Opioid receptors (δ, μ, κ) and their endogenous ligands (enkephalins, endorphins, dynorphins and endomorphins) are located in the CNS and are eminent for nociception. Furthermore, synthetic opioid therapeutics were developed for immunosuppression, treating cocaine addiction, autism and Tourette's syndrome. These findings have led to the development of compounds specifically targeted for opioid receptors as potential treatments for a number of human illnesses that span beyond pain control without the malevolent side-effects associated with opioid compounds. Major challenges facing opioid ligand design include limited information regarding the mechanism of ligand-receptor association and the absence of bioactive conformations. To investigate putative ligand receptor interactions we developed a molecular model of the δ-opioid receptor based on the structure of bovine rhodopsin and docked structurally related δ-opioid agonists and antagonists into putative binding sites. Previous docking studies of peptides containing the Dmt and Tic pharmacophores led to a hypothesis for distinct regions of binding for δ-agonists and antagonists. To further explore this hypothesis we examined the docking of structurally related agonist and antagonist alkaloids including BW373U86 (δ-agonist) and DPI 2505 (δ-antagonist), both 3-(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-hydroxybenzyl)-N-alkyl-N-arylbenzamides, agonist and antagonist 14-alkoxy-substituted indolo- and benzofuromorphinan derivatives, and SIOM (δ-agonist) and BSINTX (δ-antagonist), spiroindan derivatives and compared the results with those of two peptides, H-Dmt-Tic-CH_2-Bid (δ-agonist) and H-Dmt-Tic-CH_2-CH_2-Bid (δ-antagonist). Selection of ligand receptor partners was based on site-specific mutagenesis, chimeric studies and results from previous docking studies. Peptide derivatives adopted different binding partners than the alkaloid derivatives. Agonist derivatives displayed interactions with aromatic and hydrophobic regions of the δ-opioid receptor.

P385

STRUCTURE/FOLDING RELATIONSHIPS FOR CONOTOXINS

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An unprecedented molecular diversity among an estimated 50,000 conotoxins presents a specialized version of the "protein folding problem". Conotoxins are grouped into families, which share the same three-dimensional conformations stabilized by characteristic patterns of disulfide bonds. However, individual conotoxins from the same family differ significantly in their primary amino acid sequence, posing the question of how diverse peptide sequences can be folded into identical structures. In this study, we investigated folding determinants of three-disulfide containing conotoxins from the same family. Conotoxins GIIIA, PIIIA and SmIIIA were synthesized and oxidized in the presence of glutathione. Oxidative folding kinetics and thermodynamics differed for all three conotoxins. For GIIIA, slower folding kinetics was accompanied by higher folding yields, whereas SmIIIA exhibited a fast, but low-yield, "scrambled" folding. In contrast, fast folding of PIIIA resulted in higher yields. We investigated the contribution of tertiary and electrostatic interactions to these three different folding mechanisms. The folding properties of GIIIA, PIIIA and SmIIIA could be correlated with their primary amino acid sequences, in particular with a distribution of hydroxyprolines and positively charged lysine and arginine residues. Our results indicate that diversity of conotoxins sequences is reflected by heterogeneity of in vitro folding mechanisms, all leading to the same three-dimensional conformation.

P386

INTERACTION OF CAM AND CAM-BINDING PEPTIDES: FLUORESCENCE AND CD SPECTRAL PEPTIDE CHANGES AS A PROBE OF THE PEPTIDE-PROTEIN COMPLEX FORMATION IN THE PRESENCE OF DIFFERENT IONS

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Calcium-free Calmodulin (ApoCaM) contains two globular domains connected by a flexible central linker. Each domain contains two well-defined helix-loop-helix EF-motifs that are responsible for calcium binding. Upon binding, the calcium ions organize and stabilize the four-domains structure inducing large conformational changes: in this active form CaM can bind to its numerous target regulatory proteins. Since most of them are large and multimeric proteins, the CaM-protein complexes are usually simulated with template peptides. To date, the only known structures of CaM/substrate complexes involve Ca++-CaM and peptides derived from kinases, for which a common binding mechanism is observed. In fact, all these binding peptides have slightly regular secondary structure when free in aqueous solution, but become helical when complexed with Ca++-CaM. The aim of this study is the set-up of a simple methodology to identify and characterize the altered conformational states induced by ions different from calcium. To this purpose we study the fluorescence and CD peptide spectral changes induced by the formation of the complex between the synthetic peptide corresponding to the sequence 577-602 of skMLCK, M13 [Ikura, M. et al., 1992, Science 256, 632-638], in the presence of different ions. This study has been supported by MURST – Programma Strategico "Post-genoma" – Legge 449/97.

P387

UT RECEPTOR LIGANDS. SWITCHING FROM AGONIST TO ANTAGONIST ACTIVITY

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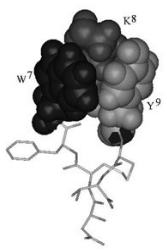
Urotensin II (U-II) is a disulfide bridged undecapeptide recently identified as the ligand of an orphan G protein-coupled receptor (UT). Human U-II (hU-II) has been described as the most potent vasoconstrictor compound identified to date (Ames, R.S et al. Nature 1999, 401, 282-286). We have recently identified both a superagonist of hU-II termed P5U (Grieco P. et al. J. Med. Chem. 2002, 45, 4391-4394) and the compound termed Urantide, which is the most potent UT receptor antagonist since now described (Patacchini R. et al. Br. J. Pharmacol. 2003, 140, 1155-1158). Our previous conformational studies showed that hU-II and its analogues with agonist activity adopt a well defined type II' beta-hairpin structure in anisotropic SDS membrane-like environment (Carotenuto A. et al. J. Med. Chem. In press). This structural arrangement allows a tight contact among the side chains of the indispensable residues Trp-7, Lys-8,.and Tyr-9 in order to obtain full agonist activity (Figure). We have now investigated, by spectroscopic and computational methods, the conformational behavior in micelles of recently synthesized U-II analogues with partial-agonist and antagonist activities. We derived a structure based model to explain the agonist-antagonist functional switching of these ligands.



P388

TARGETING LIPOSOMES WITH ANGIOGENIC-HOMING PEPTIDES

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In vivo phage display techniques have identified peptides that bind to receptors specifically present on the angiogenic endothelium involved in tumour pathogenesis. Exemplary peptide constructs(RGD, YIGSR, and NGR sequence containing motifs) have been shown to guide conjugates of doxorubicin(dox), tumour necrosis factor and dox-loaded liposomes to the tumour site in vivo. As of yet, the structural and chemical interaction is less known. Our focus is to study the synthesis and biophysical characteristics of the tumour-homing sequence, NGR , tethered to the surface of a liposome in such a way as to optimise the interaction of targeted liposomes with the targeted receptor, aminopeptidase N/CD13. We here present a synthesis for a cyclic peptide ligand that is optimised for conjugation to polymers which extend from lipid anchors embedded in the liposomal bilayer. Successful constructs are confirmed by RP-HPLC/MALDI-MS, and characterised by 2D NMR. We intend to use this scheme to show that variations in the sequence and spacial distribution of a membrane will affect the liposomes in vitro binding to aminopeptidase N/CD13 expressing endothelial cells. Our understanding of a defined peptide construct will introduce the concept of chemoselectivity to targeting liposomes. It is hoped that this will evolve into rational design of targeted liposomes for use as therapeutics against solid tumours.

P389

THE ORGANIZATION AND ASSEMBLY OF A PRION PEPTIDE AGGREGATE

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An understanding of the mechanism of assembly of β-sheet aggregates is prerequisite for describing the development of fibrils and plaques in protein misfolding diseases. Studies of model peptides have been valuable for elucidating the kinetics and thermodynamics of alpha helix formation, but the details of β-sheet formation remain to be determined. In this poster, we report a temperature-dependent isotope edited infrared spectroscopic study of a fourteen residue model peptide which forms an extended β-sheet in solution. The peptide sequence is derived from the putative helical region closest to the N-terminus of the Syrian Hamster prion protein (109-122). Upon initial preparation of a sample, the peptide rapidly forms a kinetically-trapped intermediate which slowly converts to a thermodynamically stable arrangement. Using IR spectra of specific singly 13C labeled derivatives of this peptide, we have determined that this thermodynamically favored conformation is an antiparallel β-sheet with a 3-residue overhang at the N-terminus, so that residue 117 is aligned in all strands. Once in this stable conformation, repeated heating results in a reversible conformation change from a β-sheet to a helix/coil structure. Variable temperature and time-resolved IR studies of these labeled peptides have been used to measure thermodynamics and kinetics the conformation changes from β-aggregate to helix/coil, resulting in a complete map of the energy landscape of β-sheet formation. We have measured these thermodynamic parameters as a function of the structure of the residue 117, allowing us to assess the importance of side chain length and branching on the packing involved in β-sheet formation.

P390

FLUORESCENCE STUDIES FOR MONITORING BINDING INTERACTION BETWEEN PEPTIDE LIGANDS AND THEIR RECEPTOR FRAGMENTS

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The interaction between a peptide ligand and its membrane receptor, or the receptor fragment responsible for binding, can be monitored by fluorescence spectroscopy. We will report few examples concerning the study of the interaction between the CCK8 peptide or its new analogs with the CCKA-R 1-47 N terminal fragment and between the same CCK8 peptide and its newly designed analogs with the CCKB-R 352-379 third loop fragment. The fluorescence emission measurements are obtained in micellar environment due to the presence of SDS or DPC, in which the receptor fragments adopt the right conformation for ligand binding. The fluorescence study permits fast monitoring of binding interaction and allows us to evaluate affinity constants for the complexes CCKA-R(1-47)/CCK8, CCKA-R(1-47)/cycloCCK8 and CCKB-R(352-379)/CCK8. The apparent dissociation constant1,2, Kd, were 255 nM for CCKA-R(1-47)/CCK8, 120 nM for CCKA-R(1-47)/cycloCCK8, and 50 mM for CCKB-R(352-379)/CCK8. The results confirm that the receptor fragments chosen on the basis of previously reported structural studies3,4, represent reliable model systems to monitor the ability of CCK8 or newly synthesized CCK analogs to bind the cholecystokinin receptors. 1 R.Ragone, S. De Luca, D. Tesauro, C. Pedone, G. Morelli, Biopolymers, 2001, 56, 47-53. 2 S. De Luca. R.Ragone, C. Bracco, G. Digilio, L. Aloj, D. Tesauro, M. Saviano, C. Pedone, G. Morelli, ChemBioChem, 2003, 4, 1176-1187. 3 M. Pellegrini, D. Mierke, Biochemistry, 1999, 38, 14775-14783. 4C. Giragossian, D. Mierke, Biochemistry, 2002, 41, 4560-4566.

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CHARACTERIZATION OF CYSTINYL AMINOPEPTIDASE BY [125I]ANGIV BINDING AND CATALYTIC ACTIVITY

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Angiotensin IV (AngIV), a fragment of the cardiovascular peptide AngII was shown to improve memory acquisition, long-term potentation, and to have vascular and renal actions. In combination with high affinity binding sites for labeled Ang IV, the concept of an AT4 receptor was therefore proposed. Cystinyl aminopeptidase (EC3.4.11.3), a membrane-associated zinc-dependent metallopeptidase of the M1 family, has recently been found to display high affinity for Ang IV and was proposed to represent the putative AT4 receptor. We present evidence for the presence of endogenous cystinyl aminopeptidase in membranes from Chinese hamster ovary (CHO-K1) cells by binding studies with [125I]AngIV and by measuring the cleavage of L-leucine-p-nitroanilide. The equilibrium dissociation constant of [125I]AngIV in saturation binding studies (Kd = 0.90 nM) was similar to the value (Kd = 0.70 nM) calculated from the association and dissociation rates. Binding was displaced with high potency by the "AT4 receptor" ligands (AngIV > divalinal1-AngIV ~ LVV-hemorphin-7 ~ LVV-hemorphin-6 > Ang(3-7) > AngIII > Ang(4-8)) but not by AT1/AT2 receptor antagonists. Enzymatic activity in CHO-K1 cell membranes was competitively inhibited up to 94 % by AngIV and other "AT4 receptor" ligands (AngIV > AngIII ~ divalinal1-Ang IV ~ Ang(3-7) ~ LVV-hemorphin-7 > Ang(4-8) ~ LVV-hemorphin-6). High affinity binding of [125I]Ang IV required the presence of metal chelators. Peptides such as AngIV and LVV-hemorphin-7 displayed higher potency in the binding studies as in the enzyme assay. These pharmacological properties match with those previously reported for the recombinantly expressed human cystinyl aminopeptidase in embryonal kidney cells.

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TRANSMEMBRANE ELEMENTS 3, 6 AND 7 FORM THE LIGAND BINDING POCKET OF THE TYPE 1 ANGIOTENSIN II RECEPTOR

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Determination of the binding environment of peptide ligands in their cognate G-protein coupled receptors is essential for establishing the ligand-bound receptor structure. Indeed, the determination of several contact points can provide the key biochemical restraints necessary for the accurate modeling of the receptor-ligand complex. In this context, we have developed and applied the Methionine Proximity Assay (MPA) to the human type 1 receptor (AT1) of angiotensin II (AngII). An extensive series of X→M mutants of hAT1 were constructed with individual Met residues introduced into the 7 transmembrane elements (TM's) of the receptor. These receptor mutants were then photolabeled with 125I-[Sar1,Bpa8]AngII (Bpa = p-benzoyl-L-phenylalanine), a high-affinity photolabel of the AngII-receptors. Benzophenone containing photolabels like Bpa have pronounced selectivity for thioethers and therefore incorporate preferentially into Met residues in the target protein. CNBr cleavage (selective for Met residues) of the labelled receptor mutants revealed ligand contacts in residues 112 (TM3), 249 & 253 (TM6), and 293 to 297 (TM7). Parallel experiments with a non-Met selective, carbene-generating photoprobe were then undertaken. After a new stereo-specific synthesis of p-[3-(trifluoromethyl)-3H-diazirin-3-yl]-L-phenylalanine (Tdf), a further high-affinity photoprobe of AngII was synthesized: 125I-[Sar1, Tdf8]AngII. Photolabeling experiments with this photoprobe confirmed the new contacts in TM3 and TM6, besides the previously established contacts in TM7. The combined results allowed for the development of a three-dimensional model of a ligand bound receptor structure of the hAT1 receptor where the ligand is bound in an extended conformation and parallel to the transmembrane helices. Supported by the Canadian Institutes for Health Research.

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ANTI-HIV C-TERMINAL SDF-1 DERIVED PEPTIDES: A CONFORMATIONAL STUDY

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Human immunodeficiency virus (HIV-1) enters target cells by the interaction of viral envelope glycoprotein, gp120, with cell surface CD4 molecule and either CXCR4 or CCR5 that are G-protein-coupled chemokine receptors. Stromal cell-derived factor-1 (SDF-1), a member of the chemokine superfamily, is the only known ligand of CXCR4. Apart from its physiological functions, SDF-1 has the unique and selective capacity to inhibit entry of HIV strains (X4) that use CXCR4 as coreceptor. The blocking of the virus entry by the occupation of the coreceptor with synthetic peptides, mimicking the parent protein, has potential antiviral applications. SDF-1 is produced in two forms, namely SDF-1a (68 a.a.) and SDF-1b (72 a.a.), differing by four additional C-terminal aminoacids in the SDF-1b sequence. Both SDF-1a and SDF-1b have been shown to inhibit the infection of CD4+ cells by X4 HIV-1 strains. The mechanism by which SDF-1 interferes with the virus entry into cells is still unkown. Several studies have shown that the N-terminal region of SDF-1 is mainly involved in the chemokine's antiviral activity. Despite such observation, SDF-1b, which has an extra C-terminal trait, has an antiviral activity stronger than SDF-1a. In order to investigate at a molecular level the role played by the SDF-1 C-terminal region, two peptides reproducing the C-terminal regions of SDF-1a (residues 49-66) and SDF-1b (residues 49-70) have been synthesized. Strikingly, the C-terminal fragment of SDF-1b showed X4 HIV-1 inhibitory activity, whereas the C-terminal fragment of SDF-1a showed no activity. A conformational analysis of both peptides is here presented.

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ORDERING OF THE 'PEPTIDE-LIKE' C-TERMINUS OF DUTPASE UPON BINDING OF SUBSTRATE ANALOGUES: A HETERONUCLEAR NMR STUDY

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Heterotrimeric dUTPase, the indispensable enzyme maintaining the dUTP/dTTP ratio in the cell, has a flexible C-terminal 'tail' essential for catalytic activity. Although the whole enzyme cannot be studied by conventional NMR techniques due to its size (about 3 x 190 residues), the C-terminus gives rise to distinguishable resonances of poor dispersion, indicative of an unordered state. Titrating the Drosophila enzyme with its product dUMP and also the substrate analogues dUDP and alpha,beta-imino dUTP causes the 'disappearing' of several resonance peaks in the 15N-1H HSQC spectrum. This is interpreted as conformational rearrangement of the corresponding segment of the C-terminal part, namely, it becomes ordered and tumbles together with the 'core' enzyme, hence the relevant resonance peaks are no longer detectable by NMR. Assignment of these peaks revealed that the involved residues lie in the conserved nucleotide-binding motif characteristic of the enzyme family (motif 5). Analysis of changes in signal intensities during the titration experiments suggest allosteric behavior of the Drosophila enzyme. Kinetic experiments consonantly indicate positive cooperativity in substrate binding to the enzyme. Besides demonstrating the applicability of routine NMR techniques in this system, implications on the mechanism of Drosophila dUTPase are also discussed.

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NONGENOMIC EFFECTS OF PROGESTERONE ON THE CALCIUM SIGNALING OF G-PROTEIN COUPLED PEPTIDE RECEPTORS

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We have previously observed that progesterone attenuates the signaling of the following G-protein coupled peptide receptors: B2 bradykinin receptor, cholecystokinin B receptor, V1 vasopressin receptor, and oxytocin receptor, in addition to the M3 muscarinic acetylcholine and histamine receptors. In all cases, the effect of progesterone was fast and reversible. The effect was more cell type-specific than receptor-specific and was not mediated by changes of the membrane fluidity. Progesterone also inhibited non-receptor mediated calcium responses induced by AlF4– and thapsigargin suggesting that progesterone acts at a later step of the signal transduction cascade. However, the precise molecular mechanisms that underly these actions are unknown. Recently, we found that progesterone and other steroid hormones are able to induce rapid cellular calcium responses themselves. Among the steroid hormones tested, progesterone, pregnenolone, and 5ß-dihydroprogesterone induced the strongest calcium responses. These calcium responses were also observed in a calcium-free environment suggesting that intracellular stores are involved. The calcium responses were not blocked by inhibitors of phospholipases C or adenylate cyclase. Thapsigargin, an inhibitor of the Ca2+-ATPase of the endoplasmic reticulum, blocks calcium responses induced either by progesterone or by peptide. Overall, these observations lead us to the assumption that progesterone attenuates the signaling of G-protein coupled receptors via calcium depletion of the endoplasmic reticulum.

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ANALYSIS OF THE FOLDING PATHWAY OF A MODEL BETA-HAIRPIN PEPTIDE

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Beta-hairpin structures are a ubiquitous structural feature found in many proteins and biologically active peptides. This structural motif has been found to play multiple roles in protein-protein recognition, protein-bacterial endotoxin interactions and protein and peptide biological activity. Most of the available knowledge on beta-hairpin formation and stability has been obtained from some sequences from natural protein that fold partly into a beta-hairpin conformation outside of their original protein context. We earlier designed from a conformationally restricted library (Pastor, M., López de la Paz, M, Lacroix, E, Serrano, L. and Pérez-Payá, E., 2002, PNAS 99, 614) a monomeric 14-aa-long beta-hairpin peptide that in plain buffered solutions showed a percentage of beta-hairpin structure higher than 70%. In the present study we examine the influence of the hydrophobic face on the cooperativity and on the folding process in general of our model beta-hairpin peptide.

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PROBING THE CONFORMATION OF PROLINE-RICH PEPTIDES ATTACHED TO GOLD SURFACES

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Proline in transmembranal helical segments is thought to play specific roles in membrane protein structure and function [1]. Polyproline oligomers exist in two distinct conformations. In organic solvents, they adopt a conformation known as polyproline I (PPI), a right-handed helix while in aqueous solvents, they adopt the conformation known as polyproline II (PPII), a left-handed helix. The transition from PPI to PPII implies a considerable increase in the long dimension of the helix that changes from 1.9 to 3.1 Å per residue [2]. In the last years, the reasons why single-molecule analysis is essential for studies of intracellular protein systems such as cell signaling system have been discussed [3]. The research presented here aims to mimic in the highly specialized local environment of a single peptide molecule to explore the principles that would govern its function. Proline rich peptides were end-grafted onto gold surfaces to study its conformational transition among PPII to PPI at the solid-water interface. The conformational transition from PPII to PPI was induced by change from water to propanol. The process of the conformational transition was followed by atomic force microscopy. We are confident that the results obtained will be useful for the study of the behavior of single molecules in their biological environment. [1] Deber, Ch and Thieren, A. G., Nature Struct. Biol., 2002, 9, 318-319. [2] Crespo, L. et al. J. Am. Chem. Soc. 2002, 124, 8883-8889. [3] Sako, Y. et al. Cell Structure and Function, 2002, 27, 357-365.

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PHARMACOPHORE MODELS FOR SUB-TYPE SELECTIVE ANALOGS OF SOMATOSTATIN BASED ON NMR STRUCTURE DETERMINATIONS

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The three-dimensional NMR structures of six sst1-selective and five sst4-selective analogs of somatostatin (SRIF, H-Ala1-Gly2-c[Cys3-Lys4-Asn5-Phe6-Phe7-Trp8-Lys9-Thr10-Phe11-Thr12-Ser13-Cys14]-OH) were determined to arrive at the consensus bioactive conformation of these analogs at their respective receptors. For sst1-selective analogs, the conformations indicate that the backbones have a hairpin-like structure similar to that of the sst2-selective analogs. This scaffold retains a unique arrangement of the side chains of DTrp8, IAmp9, Phe7 and Phe11 or mITyr11. For sst4-selective octapeptide analogs, with the basic sequence, H-c[Cys3-Phe6-Xxx7-Yyy8-Lys9-Thr10-Zzz11-Cys14]-OH, with Xxx7 being Phe/Ala/Tyr, Yyy8 being Trp/DTrp/D-threo-ß-Me2Nal/L-threo-ß-Me2Nal and Zzz11 being Phe/Ala, the NMR conformations suggest that the backbones do not have the usual ß-turn (1) reported in the literature for the sst2-selective analogs (2). Instead, the conformations have a unique arrangement of the side chains of Yyy8, Lys9 and Phe6 or Phe11, which form the binding motif for sst4-selectivity. In both cases, the binding motifs for sst1 and sst4 selectivity are necessary and sufficient to explain the binding affinities of all the analogs studied and are distinct from the existing model suggested for sst2 selectivity (2). References: 1. C. R. R. Grace, S. C. Koerber, J. Erchegyi, J. C. Reubi, J. Rivier, R. Riek. 2003 J. Med. Chem. 18:5606-5618. 2. G. Melacini, Q. Zhu, G. Osapay, M. Goodman. 1997 J. Med. Chem. 40:2252-2258.

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HUMAN INTEGRIN AVB5: HOMOLOGY MODELING AND LIGANDS DOCKING

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The concept that tumor grow and its spread are dependent on the formation of new blood vessels has sparked great interest in identifying protein targets, which inhibit vessels formation and are amenable to small-molecule discovery. Untill now, great attention has been paid to the role of av integrins, in angiogenesis. Peptidic and non-peptidic av integrin ligands are currently being evaluated in phase I and II clinical trials for patients with late-stage cancer. The reported crystal structures of the extracellular domains of the avb3 integrin in its unligated state and in complex (1) with the peptide cyclo(-RGDf[NMe]V-) (2) have prompted us to build a three-dimensional model for the human avb5 integrin by means of homology modeling. In an attempt to provide a preliminary rationalization, at the molecular level, of ligand selectivity towards the two av integrins, the refined avb5 model has been used to explore the landscape of the interactions between this integrin and avβ3/avb5 dual and avb3-selective ligands (3). We found that, in the RGD binding site of the avb5 receptor, a partial "roof" composed mainly from the Specificity Determining Loop residues Tyr179 and Lys180 is present and hampers the binding of compounds containing bulky substituents in the proximity of the carboxylate group.(1)Xiong, J.-P.; Stehle, T.; Zhang, R.; Joachimiak, A.; Frech, M.; Goodman, S. L.; Arnaout, M. A. Science 2002, 296, 151-155. (2)Dechantsreiter, M. A.; Planker, E.; Mathä, B.; Lohof, E.; Hölzemann, G.; Jonczyk, A.; Goodman, S. L.; Kessler, H. J. Med. Chem. 1999, 42, 3033-3040.

P400

POSSIBLE COMMON STAGES IN GUSTATORY SENSES GENERATION MECHANISMS FOR DIFFERENT SUBSTANCES

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Previously we suggested a possible mechanism of distant signal transmission from peptides to cell receptors in the water medium based on soliton mechanism. The mentioned mechanisms can explain some other manifestations of biologically active substances effect. The sense of sweet on tongue receptors may be caused by substances totally different in chemical structures and conformations: sugars, cyclic structures (saccharine), peptide compounds (aspartam) etc. There is no general theory explaining the generation of gustatory senses on molecular level, but there must be common characteristics for different compounds causing a similar effect in corresponding taste receptors that evoke the sense of sweet. These could be common characteristics of soliton signal in the water medium, transmitted distantly and carrying the information signal from ligand molecules (sugar, saccharine, aspartam) to target cells. Previously, we found that parameters of temperature dependence of infra-red spectra of substances' water solutions in near and far areas can indirectly confirm the characteristics of soliton signal. We studied these indices for water solutions of saccharose, saccharine, aspartam and other small peptides. It was revealed that in case of substances causing the sense of sweet the parameters of temperature dependence of infra-red spectra are distinctly similar, which prove the similarity of generated water soliton signals registered by taste receptors. For non-sweet or sour substances temperature dependences of infra-red spectra look totally different. Thus, soliton theory of signal transmission to target cells can explain the mechanisms of generation of similar gustatory senses for substances that differ strongly in terms of composition.

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SYNTHESIS AND INVESTIGATION OF NEUROPEPTIDE Y ANALOGUES CONTAINING ARTIFICIAL PRO-XXX DIPEPTIDE MIMETICS

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Neuropeptide Y (NPY) is a 36-amino acid peptide amide and binds to the so-called Y-receptors. Its structure has been characterized by CD and NMR spectroscopy. The most dominant element is the C-terminal alpha-helix that spans amino acid residues 12-36. This helix has been reported to be important for receptor binding and contact to cell membranes. The residues 1-10 form a polyproline helix with highly conserved proline residues at positions 2, 5 and 8, followed by a loop structure from residue 9-12. The role of the polyproline helix is probably receptor subtype dependant. It has no relevance for binding of NPY to the Y2 receptor subtype since the truncated analog NPY(13-36) shows full activity. In contrast, the N-terminal segment is of high importance for the binding of NPY to Y1 and Y5-receptor subtypes, as NPY 3-36 is inactive at these receptor subtypes and [F7,P34]-NPY is a Y1-receptor preferring ligand. In order to further study the importance of the polyproline helix we introduced a conformational constrained fluorescent Pro-Xxx dipeptide mimetic in positions 2/3, 5/6, 8/9 and combinations thereof via solid phase peptide synthesis (SPPS) by using Fmoc/tBu strategy. The resulting peptides have been investigated in cell lines selectively expressing the Y1, Y2, Y4 and Y5-receptor, respectively. Different methods including CD and fluorescence quenching have been applied to investigate the conformation and the interaction of receptor and ligand. These novel peptides that contain a modified polyproline helix clearly indicated the importance of the Pro residues for ligand binding.

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COMBINATORIAL SYNTHESIS AND COMPUTATIONAL DESIGN OF PROTEINS ASSEMBLED FROM β-HAIRPIN PEPTIDES

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The TASP concept is efficient for the chemical synthesis of proteins from peptide building blocks. Although a large number of four-helix bundle proteins with a variety of cofactors has been assembled from amphipathic helices only a few synthetic proteins with all β-structure have been reported. To expand the possibility for synthetic bioactive conformations we have designed and synthesized proteins from short peptides with β-hairpin structures.These are β-sandwiches with two β-sheets of four β-strands each. The amino acids of the hydrophobic contact surfaces of the two sheets have been designed by a computational approach. This includes the dead end elimination algorithm and the non-bonding term of the Charmm force field to select amino acid side chain conformations. The hydrophilic surface of the protein was rationally designed using positve excess charges and stabilizing salt briges to minimize the aggregation. The best hits have been synthesized in a combinatorial way on solid support [1] and screened for folding stability. CD and FTIR spectroscopy confirm the β-sheet structure. Fluorescence from Trp as a function of [GuHCl] indicates a stable and rapidly reversible fold. Following a similar strategy we have also designed β-barrel structures with six and eight strands. Proteins with β-barrel structures provide a variety of functions. Their folding indicates conformations different from other proteins. A strategy for their complete de novo synthesis will be discussed. [1] Rau, H.K., DeJonge, N., Haehnel, W. (2000) Angew. Chem. Int. Ed. 39, 250.

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INTERACTION WITH MEMBRANE MODEL SYSTEMS OF SYNTHETIC PUTATIVE FUSION PEPTIDES DERIVED FROM HEPATITIS G VIRUS E2 PROTEIN

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Infection of eukaryotic cells by enveloped viruses requires the fusion of the viral and plasma or endosomal membranes. The interaction with the target membrane has been thought to involve a hydrophobic stretch of about 15 residues called “the fusion peptide”[1]. The overall genomic organization of the hepatitis G virus (HGV/GBV-C) is similar to that of hepatitis C virus (HCV) and other members of the Flavivirus family. Recently, a stretch of conserved, hydrophobic amino acids within the envelop glycoprotein of HCV has been proposed as the virus fusion peptide. However, the mode of entry of GBV-C/HGV into target cells is at present unknown. Therefore, we have initiated the first steps towards the definition of putative sequences of HGV/GBV-C structural proteins that will be able to insert into the target cell membrane causing local destabilization of the lipid bilayer, necessary to catalyze the fusion process. In the present work, sequences derived from E2-protein have been selected by means of semiempirical methods (i.e. Wimley&White scale and Chou&Fasman prediction algorithm) and then synthesized manually following solid-phase methodologies. Their ability to induce perturbations in model membranes has been analysed by a variety of biophysical studies following procedures previously described [2]. Moreover, the secondary structure when inserted into lipid bilayers has been studied by CD and FTIR to observe if the peptides share properties similar to those reported for known fusion peptides. [1] Peisajovich SG and Shai Y, BBA (2003), 1614:122-129 [2] Rojo N, Gómara MJ, Alsina MA & Haro I, J.Peptide Res (2003), 61:318-330

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DESTABILIZING AND STABILIZING AMYLOID AGGREGATE FORMATION OF THE PRION PEPTIDE PRP106-126

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The 106-126 fragment of the human prion protein (PrP106-126, KTNMKHMAGAAAAGAVVGGLG) is known to be able to form neurotoxic amyloid aggregates. This is of interest as the pathogenic conformation of the prion protein is associated with neurotoxicity. We studied the amyloid aggregate forming behaviour (Thioflavin T flourescence) of the unprotected PrP106-126 peptide and a number of closely related molecular variants, including the alpha-carboxamide, the oxidised peptide, a conformationally relaxed form of the peptide and a dimeric form of the peptide. Furthermore the effect of various dendrimers on amyloid formation by PrP106-126 was studied. It was found that oxidation lead to reduced amyloid aggregate formation. The peptide carboxylate formed amyloid aggregates more readily than the carboxamide form of the peptide, indicating that the negative charge of the peptide carboxylate stabilises aggregate formation. A conformationally stabilised dimer of the peptide only aggregated when in its amide form. These findings indicate that the aggregation takes place between electrostatically stabilised antiparallel peptide molecules. The inherent structure-forming ability of the peptide was also found to be important for its amyloid behaviour as evidenced by a decrease in aggregate formation by a conformationally relaxed PrP106-126 variant. Dendrimers were found to perturb amyloid formation in a reaction that was dependent on the presence of positive charges on the dendrimer surface indicating that such charged dendrimers affect amyloid by acting as a denaturant, solubilising the amyloid.

P405

IDENTIFYING THE 6-HELIX BUNDLE DOMAIN IN SARS CORONAVIRUS GLYCOPROTEIN S

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The goal was to identify the heptad repeat regions, denoted HR-N and HR-C, in the spike glycoprotein of SARS-coronavirus that undergoes a conformational change (typical of type I viral fusion proteins) from their native state to a 6-helix bundle state (trimer of dimers) which mediates fusion of viral and host membranes. Our coiled-coil prediction algorithm STABLECOIL identified the two coiled-coil regions, HR-N (residues 882-1011) and HR-C (residues 1147-1185) which are separated from each other by ~140 residues. Using synthetic truncation analogs of the HR-C region we identified the minimal length required for coiled-coil folding to 35 residues (1151-1185). This region formed a trimeric coiled-coil with a thermal denaturation midpoint of 33 degrees C. To determine the region of HR-N that forms a complex with HR-C a peptide mapping approach (thirteen 35-residue overlapping synthetic peptides) was used along with the following criteria: induction of a-helical structure; an increase in thermal stability; size-exclusion chromatography to isolate complexes; reversed-phase chromatography to identify the ratio of HR-N and HR-C peptides in the complexes; SDS-PAGE and analytical ultracentifugation to determine the masses of the complexes and selective disulfide formation to form parallel and antiparallel heterostranded peptides to determine helix orientation in the complexes. These results showed the formation of a 6-helix bundle with a 1:1 peptide ratio of HR-N and HR-C peptides in an antiparallel orientation with complexes as stable as 76 degrees C. The implications of these results for the design of peptide inhibitors will also be discussed.

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SYNTHESIS AND BIOPHYSICAL PROPERTIES OF THE TRANSMEMBRANE SEGMENTS OF THE MITOCHONDRIAL UNCOUPLING PROTEINS

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Uncoupling proteins (UCPs) in animal and plant tissues are a subfamily of the mitochondrial inner-membrane carrier proteins. In recent years, there has been an increasing interest in the possible roles of UCPs in cellular functions, such as heat generation, regulation of certain metabolic pathways and control of superoxide production. It has been assumed that the principal function common to all UCPs is the uncoupling of respiration from ATP synthesis by reducing the proton-motive force through dissipation of proton gradient across the inner-mitochondrial membrane. However, despite partial homology in their primary sequences, functions of UCPs can be dissimilar. Most of the structure-function studies on UCPs have been dedicated to the heat-generating uncoupling protein UCP1, found in brown adipose tissues. The structure-function relationship in other UCPs is not well understood. In an attempt to study the membrane conformation and ion-conducting properties of UCPs, we have designed and synthesized all of the six transmembrane segments of the ubiquitous human-UCP2. Using biophysical techniques such as circular dichroism spectroscopy and single-channel recording patch-clamp method, it has been shown that one of these transmembrane segments in UCP2 (TM2) forms stable ion channel structures in lipid bilayers, and may be the essential domain for voltage-dependent channels of UCP2. We have further synthesized TM2 segments of human-UCP1 and UCP3, to compare their biophysical properties with those of TM2 in UCP2. The effect of the membrane environment and activators and inhibitors of UCP activities on biophysical properties of these membrane-interacting peptide segments are the main aspects of this study.

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SYNTHESIS OF PYRIDYL 4-HELIX BUNDLE CARBOPROTEINS FOR METAL-BINDING AND ADSORPTION TO AU SURFACES

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Mapping of structure and function of proteins adsorbed on solid surfaces is important in many contexts. Electrochemical techniques based on single-crystal metal surfaces and in situ scanning probe microscopies (SPM) have recently opened new possibilities for studies towards detection at the single molecule level. De novo design of model proteins has evolved in parallel and holds promise for test and characterization of protein folding and for new tailored protein structural motifs. We have previously described 4-helix bundle carboproteins, which are the first members of a class of peptide-carbohydrate chimeras. These carboproteins were assembled on tetravalent monosaccharides. A thiol-functionalized carboprotein was shown to form self-assembled monolayers on Au surfaces and was extensively studied by scanning tunnelling microscopy (STM). Here we report the synthesis of novel pyridyl carboproteins assembled on di- and tetravalent templates using pyridyl-functionalized peptides. These new carboproteins range in size from 4 to 16 kDa. The pyridyl moieties provide sites for selective binding of metals, thus the new carboproteins are potential metalloproteins. The folding and metal-binding properties of these carboproteins was studied. The thiol-functionalized carboproteins were adsorbed to Au surfaces and studied by STM.

P408

CONTRIBUTION OF FLEXIBLE N-TERMINAL RESIDUES TO THE BIOLOGICAL ACTIVITY OF INSECT CYTOKINE, GROWTH-BLOCKING PEPTIDE (GBP)

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Growth-blocking peptide (GBP) is a 25 amino acid cytokine isolated from Lepidopteran insects that exhibits various biological activities, such as proliferation of a few kinds of cultured cells and stimulation of a class of insect immune cells (plasmatocyte). The tertiary structure of GBP consists of a well-structured core and disordered N and C termini. Our previous studies revealed that not only the core structure of GBP but the particular residues in the unstructured N-terminal region, such as Glu1 and Phe3, were also essential for the plasmatocyte-stimulating activity. In this study, a number of GBP mutants targeting the unstructured N-terminal residues were constructed to gain more detailed insight into the mode of interaction between the N-terminal region and receptor. Alteration of the backbone length of linker region between the core structure and N-terminal domain reduced the plasmatocyte-stimulating activity. The substitutions of Gly5 or Gly6 in this linker region with more bulky residues, such as Phe and Pro, also remarkably reduced this activity. We conclude that the receptor interaction of GBP depends upon the relative position of the N-terminal domain to the core structure, and therefore the backbone flexibility of Gly residues in the linker region is necessary for adoption of a proper conformation suited to receptor binding. Additionally, antagonistic experiments using deletion mutants confirmed that both the core and the N-terminal domain of GBP were required for receptor binding, and furthermore Phe3 is a binding determinant of the N-terminal domain.

P409

A DE NOVO DESIGNED A-TO-B TRANSITION PROTEIN, AMYLOIDGENESIN, WITH AN AMYLOIDOGENIC PROPERTY

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To investigate the mechanism of fibril formation associated to several amyloidogenesis, including Alzheimer's disease and prion disease, we have designed and synthesized a small globular protein, amyloidgenesin, composed of a central core-forming hydrophobic oligoleucine and four amphiphilic α-helical and / or β-structure sequences. This small protein has a monomeric α/β structure in buffer solution at pH 7.4, while under elevated temperature or in the presence of a chemical denaturant it undergoes irreversible α−to-β conformational transition rapidly to form amyloid protofilaments and then fibril clusters. It forms the narrow unbranched and Congo red-stained fibril structure displaying a green biferefringence, characteristic of amyloidogenic proteins. In the room temperature, the seeding of a solution containing amyliod-like fibrils into an intact solution induces the α-to-β transition slowly to form amyloid-like fibrils. On the contrary, a designed protein, SGP-5A , having oligoalanine in central core did not show any amyloidogenic property, indicating that the appropriate hydrophobic core is crucial for amyloid fibril formation. The present de novo designed proteins should provide useful models not only for studying the mechanism of amyloidogenesis, but also for designing potential inhibitors to the conformational disease induced from amyloidgenetic proteins.

P410

TOWARDS GELSOLIN AMYLOID FORMATION

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Mutated forms D187N and D187Y, of an actin-binding-and-severing six-domain protein gelsolin are the cause of Finnish amyloidosis disease. The abnormally cleaved gelsolin fragments G173-243 and/or G173-202 are supposed to be involved in the amyloid formation. Fragments G173-243 and G173-202 were cleaved from the crystal structure of gelsolin (1DON). The fragment G173-243 comprises alfa-helix and beta-structure, while the fragment G173-202 has only beta-structure. Fragment G173-243 was immersed in a water box and subjected to molecular dynamics (MD) simulation in AMBER 5.0 force field, using NTP protocol, T 298 K. After 1154 ps of MD run the G173-243 partly lost its alfa-helical structure thus suggesting that G173-243 could probably transfer to beta-structure and take part in the amyloid formation. Some possible amyloid strand structures were built from the G173-202 units, minimized and subjected to MD at 313 K, NTP protocol, which lead to hydrogen bond formations between the beta-structure fragments G173-202. Afterwards the newly created strands were immersed in water boxes, and MD at 313K, was performed to check the stability of strands. * This work was supported by NATO Collaborative Linkage Grant LST.CLG.976647.

P411

ALPHA-HELICAL STRUCTURE OF VASOACTIVE INTESTINAL PEPTIDE IS ESSENTIAL TO ITS BIOLOGICAL FUNCTIONS

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Vasoactive intestinal peptide (VIP) is one of the major peptide transmitters in the central and peripheral nervous systems, being involved in a wide range of biological properties in organisms including metabolic processes, exocrine and endocrine secretions, cell differentiation, and smooth muscle relaxation. The conformational properties of vasoactive intestinal peptide include the N-terminal randomized structure and the C-terminal long alpha-helical structure. We have previously observed that the N-terminal random coil structure plays a crucial role in the receptor-selectivity. Here, to clarify how the formation of the alpha-helix plays a role in its biological functions, we chemically synthesized VIP analogues modified at the C-terminus, mid-chain, and N-terminus of the alpha-helical region, and evaluated the relationship between their alpha-helical contents and their biological activities including relaxant effects on murine stomach and receptor-binding activities. C-terminally truncated analogues of VIP resulted in a significant decrease in the relaxant effect, receptor-binding activity, and the helical content in proportion to the peptide length. In addition, disruption of the mid-chain and the N-terminus in the alpha-helical stretch by oxidation of Met17 and deletion of Thr11 also inhibited biological activities. These findings suggest that the presence of alpha-helical structure forming in 14 amino acid residues between position 10 and 23 in VIP is essential to its biological functions.

P412

THE INFLUENCE OF RADICAL FORMATION ON THE CONFORMATION OF THE AMYLOID β PEPTIDE FRAGMENT (25-35)

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In Alzheimer's disease, reduction of either Cu2+ or Fe3+ ions and the resultant oxidation of amyloid beta (Aβ) to its radical cation could lead to formation of stable alpha-C-centered radicals at either Gly29 or Gly33. The subsequent delocalization of the unpaired spin to peptide bonds, both in the N- and C-terminal direction, would lead to a conformational change in the peptide chain which subsequently causes an overall conformational transition leading to protein aggregation. Therefore, the overall structural consequences of the β-conformation of the critical glycyl residues of Aβ(25-35) were studied using molecular dynamics simulations. Simulations (40.2 ns) were carried out on four structures for the 25-35 fragment obtained from the Brookhaven Protein Data Bank using entry codes 1AML, 1BA4, 1HZ3 and 1IYT. Additional structures were created by adjusting the conformations of Gly29 and Gly33 to β-sheet. Results indicate that the conformation of both Gly33 and Gly29 have significant impact upon the secondary structure of the Aβ(25-35) peptide. During simulations, the initial alpha-helices were transformed to β-turns and most β-sheet and β-bridge secondary structure characteristics of the peptide were observed when simulations started with either glycyl residue in β-conformation. Involvement of a radical mechanism in conformational change was further supported when CD spectroscopy revealed more β-sheet character in the conformation of Aβ(25-35) in the presence than in the absence of Cu2+.

P413

DENDRIMERIC PEPTIDES FOR TUMOR TARGETING

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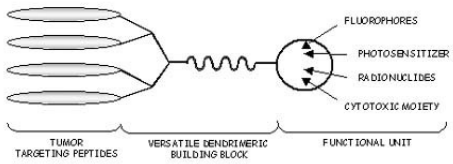
We reported that synthesis in a MAP dendrimeric form dramatically improves the half-life of bioactive peptides, due to acquired resistance to protease and peptidase activity (1). Moreover, dendrimeric peptides retain the full biological activity of native peptides. An important possible development of the protease-resistant MAPs is the application to tumor therapy and in vivo imaging, where specific regulatory peptides can be used in a MAP form as tumor targeting agents. The MAP peptidyl core, onto which peptides can be built, offers linking units for coupling of functional moieties such radioisotope chelators, cytotoxic drugs, photosensitizing and dye agents for tumor treatment or diagnosis. We synthesised in MAP dendrimeric form different regulatory peptides whose receptors are overexpressed in different tumors. Targeting molecules include neurotensin (NT), a 13-mer peptide that binds to the related receptors overexpressed in several neuroendocrine human tumors. We assessed that the MAP form of neurotensin, and particularly of its short analogue NT8-13, fully retain biological activity of the monomeric peptide and acquire a much higher stability to blood peptidases. 1- Bracci L. et al. JBC, 2003, 278: 46590-46595.



P414

SYNTHESIS OF BETA-SHEET MIMETICS BASED ON A 4-DEOXY-PYRROLO[3,2-D]PYRIMIDINE SCAFFOLD

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Extended conformations such as beta-sheets are common elements of protein structure important in amyloid fibril formation, protein:protein interactions, and the design of protease inhibitors (1). A wide range of peptidomimetics have been developed to mimic beta-strands; however, few preserve both the potential hydrogen bonding interactions of the backbone amide bonds and the orientation of side chains in conformations similar to native peptides. Monte Carlo conformational analysis suggests that a 4-deoxy-pyrrolo[3,2-d]pyrimidine scaffold will mimic the hydrogen bonding elements as well as the backbone and side chain geometry of native beta sheet structures. We recently completed the synthesis of a 4-deoxy-pyrrolo[3,2-d]pyrimidine scaffold in 6 steps starting from 4-hydroxyproline. Our approach features the annulation of the pyrimidine onto a 4-amino pyrrole intermediate (2, 3). A range of diversity elements can be conveniently incorporated at the 1- and 4- positions of the 4-deoxy-pyrrolo[3,2-d]pyrimidine scaffold. Our presentation will discuss this synthesis, progress towards incorporation of this scaffold into peptides and conformational analysis. References: 1. Maitra, S., and Nowick, J. S. (2000) in The Amide Linkage: Structural Significance in Chemistry, Biochemistry, and Materials Science (Greenberg, A., Breneman, C. M., and Liebman, J. F., Eds.), John Wiley and Sons. 2. Marcotte, F.-A., and Lubell, W. D. (2002) Org. Lett. 4, 2601-03. 3. Marcotte, F.-A., Rombouts, F., and Lubell, W.D. (2003) J. Org. Chem. 68, 6984-87.

P415

DIMETHYLTYROSINE (2',6'- DIMETHYLTYROSINE) ANALOGUES OF BIPHALIN AND RELATED COMPOUNDS'

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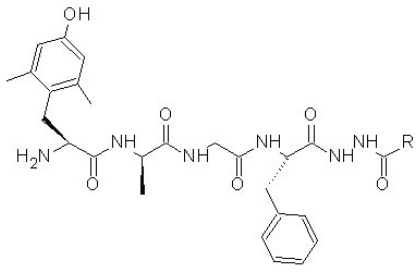
Biphalin (Tyr-D-Ala-Gly-Phe-NH-NH<-Phe<-Gly<-D-Ala<-Tyr) is a dimeric peptide with high affinity to all three types (μ, δ, κ) of opioid receptors. This compound is a hybride of two opioid active tetrapeptide fragments of enkephalin analogues connected with hydrazide bridge. Biphalin is a potent antinociceptive compound when administered intravenously and very potent when administered intrathecally. These promising results have stimulated extensive structure-activity studies of biphalin. Recent study of biphalin shows that the full dimeric structure is not required for high biological potency. Elimination of a tripeptide from one "arm" of biphalin does not reduce opioid receptor affinities significantly. It was shown by others that replacement of Tyr by DMT (2',6'-dimethyltyrosine) increases binding affinity and biological activity of many of opioid analogues. Therefore it was interesting to evaluate the effect of replacement of DMT for Tyr in biphalin and active compounds related to biphalin, with the general structure shown below. The communication will report the synthesis and receptor affinity differences of analogs with DMT and Tyr.



P416

BACKBONE MODIFICATION OF CYCLIC PENTAPEPTIDE ANTAGONISTS OF CXCR4

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Cyclic pentapeptides that inhibit the CXCR4 chemokine receptor and infectivity of the X4-strain of HIV were reported by Fujii et al.[1]. The most potent was c[Arg-Arg-Nal-D-Tyr-Gly] designated FC131. We have prepared FC131 and nine analogs in which the glycine residue was replaced by Ala, D-Ala, NMe-Ala, NMe-D-Ala, Pro, D-Pro, Pip, D-Pip, and Aib to further constrain the cyclic peptide backbone. Peptides were prepared either by solid phase synthesis of linear precursors, followed by cyclization in solution, or by on-resin cyclization while attached through the side chain phenol of the D-Tyr residue. Overall yields by either procedure were in the 40-50% range.

After purification by HPLC and characterization, peptides were bioassayed for their ability to inhibit HIV X4-strain infectivity of cells expressing the CXCR4 receptor with an analogous assay for CCR5 receptors as control.

Two analogs, c[Arg-Arg-Nal-D-Tyr-D-Ala] and c[Arg-Arg-Nal-D-Tyr-NMe-D-Ala], retained significant activity. Conformational analysis showed that the glycine replacement by these more constrained residues stabilized a distinct β-turn centered at Arg-Arg with a reverse γ-turn centered on the NMe-D-Ala residue. NMR studies are underway to confirm the calculations. These observations provide a basis for design of even more constrained analogs in order to define the CXCR4-bound conformation of the peptide. 1. Fujii, N., et al. Angew Chem Int Ed Engl, 2003. 42:3251-3253.

P417

THE MULTIPLE CYSTINE-CONTAINING HEAD DOMAIN OF MINICOLLAGEN FROM HYDRA NEMATOCYSTS FOLDS OXIDATIVELY INTO A COMPACT GLOBULAR STRUCTURE

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Most multiple cystine-containing bioactive peptides such as hormones, neurotransmitters, growth factors, enzyme inhibitors, and toxins are known to refold in high yields into the native topoisomer under optimized oxidative conditions, although biosynthetically these peptides are products of post-translational processing of larger prefolded precursor forms [1,2]. Even small subdomains of proteins are capable of refolding correctly when the sequence-encoded structural information in the excised fragments suffices for the thermodynamically controlled correct oxidative refolding. Generally, stabilization of preferred ordered structures such as alpha-helices or beta-sheets by defined disulfide-crossbridgings represents the driving force in such processes. From the sequence composition of minicollagen from Hydra nematocysts a larger stretch of collagen-type triple helix followed by a poly-proline II helix can be foreseen, while the N- and C-terminal proline- and cysteine-rich domains are not consistent with predictable ordered structures [3]. Surprisingly even the short synthetic 24-membered C-terminal domain refolds in the presence of redox buffer (GSH/GSSG, 9:1) mainly into a single isomer whose disulfide connectivities were unambiguously assessed by the NMR structural analysis. A possible correlation between 3D-structure and correct folding will be addressed. [1] Tamaoki, H.; Miura, R.; Kusunoki, M.; Kyogoku, Y.; Kobayashi, Y.; Moroder, L. (1998) Prot. Engin. 11, 649-659. [2] Kimura, T. In: Houben-Weyl, Methods of Organic Chemistry, Synthesis of Peptides and Peptidomimetics (Goodman, M.; Felix, A.; Moroder, L.; Toniolo, C., eds.) Georg Thieme Verlag, Stuttgart, E 22b, 2002, 142-161. [3] Engel, U.; Pertz, O.; Fauser, C.; Engel, J.; David, C. N.; Holstein, T. W. (2001) EMBO J. 20, 3063-3073.

P418

IN SITU NUCLEATION AND DISRUPTION OF SECONDARY STRUCTURES USING X, N-ACYL MIGRATION

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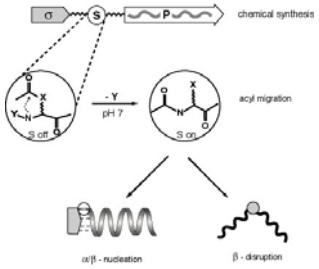
A novel concept for the in situ nucleation and disruption of secondary structures is presented. To this end, we assemble a modular system ('switch-peptide') consisting of a conformational induction unit σ, a switch element S and a target peptide P (Figure). As a major feature, S allows to activate ('switch on') the conformational impact of σ upon P by triggering (change of pH, enzymatic cleavage of Y) intramolecular X → N acyl migrations at physiological conditions. In making use of the large pool of synthetic devices for controlling peptide structure, we explore the concept for the in situ nucleation (σ = templates, N/C-caps) and disruption (σ = peptidomimetics, pseudo-prolines) of helices and β− sheets, monitored by CD, IR and SPR. As shown for protein-derived oligopeptides, the induction of conformational transitions of type β-sheet ('recognition state') to α-helix or random-coil ('functional state') offers new perspectives in reversing conformational processes of utmost therapeutic relevance. [1] M. Mutter et al., Angew. Chem., submitted.



P419

MULTICOMPONENT AGGREGATES CONTAINING THE CCK8 BIOACTIVE PEPTIDE AND GD COMPLEXES AS TARGET-SPECIFIC MRI CONTRAST AGENTS

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Mixed micelles are obtained in aqueous solution by using two amphiphilic monomers: the first monomer contains one or two C18 hydrophobic moieties bound to the C-terminal cholecystokinin octapeptide amide (CCK8) while the second monomer contains the same C18 hydrophobic moieties bound, through a lysine residue, to the DTPAGlu chelating agent complexing a Gd(III) ion. A physico-chemical characterization, by surface tension measurements, FT-PGSE-NMR technique and fluorescence quenching, of mixed C18DTPAGlu(Gd)/C18CCK8 micelles is presented. Structural data are obtained by Small-Angle Neutron Scattering technique. The structural charecterization indicates a spheric arrangment of the micelles with an external shell of 20 Å and the inner core of 18 Å. An aggregation number of about 60 monomers is found. Both the DTPAGlu(Gd) complexes and the CCK8 peptides point toward the external surface. Relaxivity measurements indicate that the micellar aggregates, containing the Gadolinium complexes, show high relaxivity parameters ($R1p = 18.7\ \mu M^{-1}s^{-1}$) with a large enhancement with respect to the isolated DTPAGlu(Gd) complex. Mixed micelles are also obtained using two C18 hydrophobic tails for each monomer. In this case the mixed micelles are larger than those containing only one C18 hydrophobic moiety for each monomer and display a rod-like form. Anyway, the structural characterization and the relaxometric behavior, indicates that both these kind of mixed micelles are promising candidates as target-specific MRI contrast agents. In fact the supramolecular aggregates contain a bioactive molecule able to drive to specific place, e.i. where tumor tissues are present, and a large amount of paramagnetic Gadolinium ions.

P420

BIOSYNTHESIS AND BIOPHYSICAL STUDIES OF FRAGMENTS OF A G PROTEIN COUPLED-RECEPTOR : APPROACHES TO STUDY THE STRUCTURE OF MEMBRANE PROTEINS

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G protein-coupled receptors (GPCRs), found in a wide range of organisms, mediate cellular responses to extracellular signals such as physical stimuli, hormones, and neurotransmitters. Biophysical studies on GPCRs are hampered by difficulty in crystallizing these molecules and conducting NMR on intact receptors in the presence of vesicles. We applied a reductionist approach to membrane receptor structure and examined the biophysical properties of peptides representing fragments of Ste2p, a GPCR for alpha-factor, a tridecapeptide from the yeast Saccharomyces cerevisiae. Receptor fragments were prepared by synthetic, native chemical ligation and biosynthetic approaches. A multidomain fragment containing the cytosolic tail, the seventh transmembrane domain and the third extracellular loop of Ste2p was biosynthesized as a fusion protein. The fusion protein (MW =21,510 Da) was purified to near homogeneity as judged by HPLC and its MW verified by MS. Approximately 100 mg of fusion protein per liter of culture was obtained in rich medium and 30 to 50 mg of this protein was purified from minimal medium. The 73-residue peptide was released from the fusion protein by CNBr and purified by HPLC. 14N- and 15N-labeled forms of this multidomain peptide were isolated in ~10 mg quantities. CD analysis was performed in TFE/water, DPC and PPG micelles and in DMPC/DMPG bilayers. The peptide integrated into the detergent micelles and the bilayers and the various domains behaved independently. A high resolution structure of this peptide is currently being determined using NMR spectroscopy.

P421

BIOLOGICAL ACTIVITIES OF CYCLIZED PEPTIDES DERIVED FROM LAMININS

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[1]Graduate School of E.E.S., Hokkaido University, Sapporo [2]School of Pharmacy, Tokyo University of Pharmacy and Life Science, Tokyo [3]Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan [4]NIDCR, National Institutes of Health, Bethesda MD, USA

Laminins, basement membrane glycoproteins, consist of three distinct chains, α, β, and γ. So far, five α chains have been identified. The α chains contain a C-terminal globular domain (G domain) consisting of five tandem modules (LG1-LG5), and are tissue- and/or developmental stage-specifically expressed. Previously, we have found that A3G75 and A4G82 sequences, located on LG4 module of the α3 and α4 chains, had strong cell attachment activity. The LG modules consist of a 14-stranded β-sheet (A-N) sandwich structure, and A3G75 and A4G82 are located in the homologous loop region between β-strands E and F in the LG4 module. We synthesized the homologous peptides of this region in the α1-α5 chains (EF-1-EF-5), and evaluated their biological activities. EF-1 (DYATLQLQEGRLHFMFDLG) interacts with cells through α2β1 integrin and EF-4 (DFMTLFLAHGRLVFMFNVG) interacts with cells via syndecan-2. Since EF peptides are located in the loop region, we focused on the importance of the loop structure for biological activities with a series of cyclized EF peptides. Cyclized EF-1 showed a stronger cell attachment activity via α2β1 integrin than linear EF-1, and more strongly inhibited fibroblast attachment on EF-1 than linear EF-1. Similarly, cyclized EF-4 and its derivative peptides showed enhanced cell attachment activity and inhibited binding of recombinant α4 G domain protein to heparin-Sepharose beads better than the linear peptides. These results indicate that the biological activities of the EF peptides highly depend on the loop structure, and this loop region is playing a crucial role in the biological activities of the LG4 module.

P422

NOVEL PHOTOSWITCHABLE AMINO ACIDS

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Our research program aims to develop novel photoswitchable amino acids and analogs to reversibly control the structure and function of biomolecules using light. So far the azobenzene chromophore has been investigated intensively for use in biological systems. For future applications the development of new classes of photoisomerizable compounds is of importance. Among the demanding requirements these compounds have to fullfill the thermal stability of the photoisomeric states is of considerable interest in view of structural analysis and biological studies. Novel fulgimide-, azobenzene- and hemithioindigo-based α-amino acids and ω-amino acids will be presented, that appear to meet these requirements. The photochromic properties of the photoswitches have been characterized by UV/Vis- and 1H-NMR spectroscopy.

P423

DESIGN OF PEPTIDIC INHIBITORS OF LIPOPOLYSACCHARIDE (LPS) USING THE EXCHANGE-TRANSFERRED NOE

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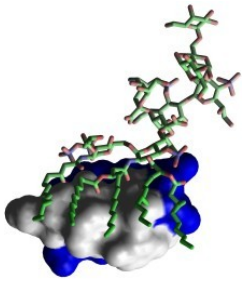
LPS induced Gram-negative sepsis and septic shock remain lethal in up to 60 % of cases, and LPS antagonists that neutralize its endotoxic action are the subject of intensive research. The interaction of LPS with its cognate binding proteins has been structurally elucidated in the single case of the X-ray crystallographic structure of LPS in complex with the integral outer membrane protein FhuA from E. coli K-12 (1). Another independent source of structural information are solution structures of peptides in complex with LPS (2) that can be determined using the transferred NOE effect. We use molecular docking methods (3) to derive models of LSP-peptide complexes. These models serve as basis for rational drug design of peptides with altered or enhanced anti-endotoxic properties. The peptides are tested in vitro for biological activity in order to confirm (or reject) the predicted structural model of action, and serve to refine the model. References: (1) Ferguson et al., Science 1999, 282, 2215. (2) Pristovšek & Kidrič, J. Med. Chem. 1999, 42, 4606. (3) Pristovšek & Kidrič, in Drug discovery and design : medical aspects; Matsoukas, J.; Mavromoustakos, T.; Eds; IOS Press: Amsterdam, 2002; pp 161-166 Figure 1: Model of the LPS : LF16 peptide complex (LPS as sticks model).



P424

THE THIRD INTRACELLULAR LOOP OF THE A1B-ADRENERGIC RECEPTOR: STRUCTURAL FEATURES AS DERIVED BY NMR AND MD CALCULATIONS

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The adrenergic receptors (ARs) mediate the physiological effects of catecholamines and are members of the G-protein-coupled receptors (GPCRs) superfamily that share the common structural feature of a single polypeptide chain with seven a-helical transmembrane-spanning domains (TMDs) connected by alternating intracellular and extracellular loops. The TMDs cooperate in forming a ligand binding pocket, whereas cytoplasmic portions mediate the coupling to specific G-proteins. We have focused our investigations on the alpha1B-AR subtype. Studies with chimeric receptors have demonstrated that discrete sequences in the third intracellular loop (i3) are crucially involved in selective receptor/G-protein coupling and activation (1,2). A model of the receptor has been developed (3,4) but it remains only a qualified prediction of the actual tertiary structure. We have therefore synthetized fragments corresponding to residues 225-242, 236-260 and 262-295 and determined their solution structures using NMR-derived distance constraints. We modeled the structure of the whole i3 loop (residues 225-295) by means of MD calculations using the secondary structure elements experimentally observed in the isolated fragments. The results show that the specific domains adopt a helical conformation with a peculiar arrangement of some charged side-chains. These findings support the hypothesis that the process of GPCR/G-protein coupling and activation involves selective helix-helix interactions. References: (1) Cotecchia S. et al. (1990) Proc. Natl. Acad. Sci., 87, 2896-2900. (2) Cotecchia S. et al. (1992) J. Biol. Chem., 267, 1633-1639. (3) Scheer A. et al. (1996) The EMBO Journal, 15, 3566-3578. (4) Fanelli F. et al. (1999) Proteins: Structure, Function and Genetics, 37, 145-156.

P425

DRUG AFFINITY COMPLEX (DAC); A VERSATILE PEPTIDE DELIVERY PLATFORM TECHNOLOGY

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Endogenous peptide hormones are responsible for regulating a multitude of metabolic signals and thus, constitute important templates for drug design. However, few peptides have been commercialized due primarily to their rapid elimination and proteolytic degradation. As a chemical alternative to other methods and with the objective of prolonging the peptide's systemic presence, we have developed various stable, long-acting synthetic peptide analogs using our propriety DAC technology. The versatility of the technology lies in its ability to provide any peptide with the chemical means of bioconjugating covalently to circulating albumin in vivo via HSA's free thiol Cys34 and thus improves bioavailability, extends compound half-life while retaining the peptide's potency. The thiol moiety of the Cys34 is part of a hydrophobic pocket and is mainly found in its nucleophilic anionic state. Therefore, we synthesized using an Fmoc methodology/direct attachment, peptides to which we incorporated an electrophilic maleiidopropionic acid moiety responsible for the covalent attachment of the peptide to HSA's Cys34. Herein we present, Dynorphin A (1-13), GLP-1(7-36), ANF (99-126), Insulin, GRF (1-29), peptides DACs along with the resulting albumin bioconjugates. In addition, we present the in vitro binding along with the pharmacokinetic, pharmacodynamic and stability profile of both the DAC and corresponding preformed albumin conjugates. The results from the various studies demonstrate repeatedly that we have been able to developed DAC-analogs that maintained the endogenous characteristics in vitro and in vivo, while exhibiting an extended pharmacokinetic profile of endogenous peptide hormones.

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APPLYING PEPTIDE DESIGN TO SUPRAMOLECULAR ENGINEERING

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An ability to engineer functional materials from the bottom up would impact on nano-scale science and technology, which could result in the fabrication of nano-structured materials. Mimicking natural peptide assemblies holds particular promise as these exhibit exquisite selectivity and specificity to adopt a wide variety of structures. Our work has focused on assemblies based on coiled-coil peptides of de novo design. Here we present two examples from our work: self-assembling peptide linker and nanofibre. The linker – dubbed "belt-&-braces" – comprises three leucine-zipper peptides in which one peptide, "the belt", templates the co-assembly of the other two, half-sized peptides, "the braces". We exemplified this principle as follows: the brace peptides were bound to colloidal gold nanoparticles; when mixed these conjugates gave dispersed nanoparticles; however, addition of the belt led to the organization of the particles into networks with precise nanoscale separations. The nanofibre is based on the concept of sticky-ended assembly; two complementary leucine-zipper peptides make a staggered dimer with sticky ends to propagate longitudinal fibre assembly. We have extended this work with new concepts for fibre shaping (FiSh) and fibre recruiting (FiRe). Specifically, we have engineered peptides that by co-assembling with the sticky-ended peptides 1) introduce branches and kinks into the fibres and 2) recruit active biomolecules on fibre surfaces.

P427

PEPTIDES DERIVED FROM CYTOPLASMIC REGION OF THE INTEGRIN PLATELET RECEPTOR AS ANTI-AGGREGATORY AGIENTS

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The platelet receptor αIIbβ3 plays a critical role in the process of platelet aggregation and thrombus formation. αIIbβ3 is an integrin which is composed of a two-chain α subunit bound non-covalently to a single-chain β-subunit. The C-terminal cytoplasmic tail of each subunit consists of 20 amino acid residues in αIIb and 47 residues in β3. The conformational switch necessary for ligand binding to αIIbβ3 is regulated by intracellular molecules that bind to the cytoplasmic tails of the integrin or by integrin-associated membrane proteins. In addition in vivo charge swapping mutation studies suggested that αIIb and β3 tails have a direct site of interaction between αIIb(R995) and β3(D723) which is related to the affinity state of the receptor. More specifically, extensive studies have shown that peptides derived from the cytoplasmic tails of αIIbβ3 receptor can maintain a low-affinity state of the receptor or induce its activation, in platelets. The aim of this work is to develop peptide analogues based on the cytoplasmic tail sequences of both αIIb and β3 subunits that could inhibit platelet thrombus formation by specifically disrupting the inside-out signaling pathway. To this end, suitably modified peptides have been synthesized and tested for their ability to inhibit the activation and expression of αIIbβ3 and platelet aggregation in vitro. The pathway of their action is now under evaluation.

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EVOLUTION OF PROTEIN FOLDING PATHWAYS WITHIN THE SUBTILASE FAMILY OF SERINE PROTEASES

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While biological conformations are usually the most thermodynamically stable, there are examples wherein proteins fold into kinetically stable conformers. That kinetic stability is observed in a select few suggests thermodynamically driven folding processes are more efficient, while kinetically driven processes maybe selected for specific functional advantages. Since primary sequences encode all information necessary for folding, whether sequences are selected for particular folding pathways remains unknown. Using the highly conserved subtilase family, we demonstrate that adaptive evolution of protein sequences dictates the selection of folding pathways. Intracellular (ISPs) and extracellular (ESPs) serine proteases, two subgroups within subtilases share ~50% sequence identity, ~90% conserved hydrophobic cores, similar structures, topologies and catalytic activities. Using subtilisin E and ISP1 from Bacillus subtilis as models, we establish that ISPs fold into their global minima over one million times faster using folding pathways and mechanisms that are different from kinetically trapped ESPs, which require N-terminal propeptides to function as dedicated intramolecular chaperones. The dramatic differences in folding rates are attributed to specific surface residue changes within ISPs, which alter the interface that interacts with the intramolecular chaperones. Hence, although the hydrophobic core and topology are the driving force, our results suggest that surface residues can significantly influence folding and, their positive selection dictates folding pathways, mechanisms, and the choice between kinetic versus thermodynamically stable folds.

P429

MOLECULAR MODELLING STUDY OF FREE-ENERGY LANDSCAPE OF OXYTOCIN AND ATOSIBAN

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Oxytocin, a regulatory peptide of a number of physiological processes, occurs in water solutions in two different conformations that are in dynamic equilibrium. To study this phenomenon we used molecular modelling combined with multivariate statistical data analysis. First, an ensemble of possible conformations of oxytocin was generated using simulated annealing method. Resulting conformations were ranked into two distinct families of conformations based upon measurements of 3D parameters for each simulated structure and pre-selection of key parameters using skewness and kurtosis of their distribution. Ranking of individual conformations into families was carried out by the K-means clustering method. This procedure approved the existence of two basic conformations of the oxytocin molecule, whereas only one basic conformation could be identified for its antagonistically acting analogue Atosiban. Using techniques that combine molecular mechanics with continuum electrostatics approach, these conformation families were analyzed in term of their free energies. The results together with the potential physiological role of conformational duality will be discussed.

P430

CONTRIBUTION OF THE GAPL[309-312] SEQUENCE AND E[315] MOIETY OF THE CD41 SUBUNIT ON PLATELET AGGREGATION

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The platelet membrane glycoprotein GPIIb/IIIa (CD41/CD61) is the most prominent of integrin adhesion receptors that mediates platelet aggregation by binding fibrinogen. Potential ligand contact sites on integrin GPIIb subunit have been identified by chemical cross-linking approaches, site-directed mutagenesis and peptide studies. The GPIIb 294-314, GPIIb 300-312 and GPIIb 313-332 regions have been proposed to be implicated in ligand binding [Taylor et al., J Biol. Chem., 1992, 267, 11729-11733; Biris et al., Eur. J. Bioch., 2003, 270, 3760-3767] The GAPL sequence found on residues 309-312 in GPIIb has also been predicted by the anti-complementary hypothesis to be a fibrinogen binding site on GPIIb subunit. In addition, within the sequences of the proposed binding sites, the variant E315→D315 has been reported. Taking advantage from these results, we aim at evaluating the contribution of of the GAPL sequence in fibrinogen binding as well as the role of the amino acid at the position 315 (E315). To this purpose, we synthesized a series of GPIIb 313-324 derived analogues incorporating also the GAPL sequence. From the inhibition studies of human platelet aggregation, we conclude that the GAPL sequence does not affect the inhibition potency of the GPIIb 313-320 peptide, whereas substitution of E315 by D315 enhances the anti-aggregatory activity of the analogue.

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DESIGN, SYNTHESIS AND CHARACTERIZATION OF ß-SHEET-FORMING PEPTIDES: THE FBP 28 WW DOMAIN

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The WW domains, named according to the two conserved tryptophan residues, function as noncatalytical domains of signalling proteins. The WW modules adopt highly twisted, antiparallel three-stranded ß-sheet structures. The small size make them interesting model systems in order to analyze ß-sheet folding and stability. Our initial attempts to synthesize the FBP 28 domain, GATAVSEWTEYKTADGKTYYYNNRTLESTWEKPQELK, by SPPS failed. However, the application of two pseudoproline units in position E27-S28 and K17-T18 and the substitution of D15 by E and N, respectively, in order to circumvent Asp-Gly-succinimide formation had an impressive impact on the synthesis. Substitution of D15 by E or N may influence the interaction at the turn and thus the stability of the entire domain. However, NMR analysis of the N15 peptide reveals close structural similarity with the wt, albeit the Tm value points to a slight destabilization. Former NMR studies indicated that beside the formation of hydrophobic clusters and backbone interactions also H-bonds between side chains of polar amino acids or with the backbone may contribute to the stabilization of the twisted ß-sheet structure (Marcias, M. J., et al. (2000) Nature Struct. Biol. 7, 375; Jäger, M., et al. (2001) J. Mol. Biol. 311, 373). Therefore, we synthesized a set of mutants, characterized by insertion of H-bond-forming side-chains (e.g. Gln). The Tm values demonstrate that the Gln-carboxamide groups may have a remarkable influence on the folding in FBP 28 WW domains. Furthermore, the ability of the synthesized mutants to recognize proline-rich motifs was analyzed by surface plasmon resonance measurements.

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MAPPING THE FIBRINOGEN BINDING SITES OF THE PLATELET RECEPTOR USING SYNTHETIC PEPTIDES DERIVED FROM THE ß3 SUBUNIT

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Extensive studies utilizing a combination of immunological, biochemical and mutational approaches have been performed for determining the interacting sites of the GPIIIa subunit with fibrinogen. Although some disagreements have appeared in literature for the proposed regions, the GPIIIa 211-222 region is generally accepted as a ligand-binding site on the receptor. In a previous study, we managed to determine the binding regions within the GPIIIa subunit, which are involved in aggregation process, using synthetic 20-peptides, covering the extracellular region of GPIIIa subunit [Tenente et al. Proceedings of 26th EPS, p.757]. From the biological assays, we concluded that some of the 20-peptides included in the GPIIIa: 289-356, 385-440, and 589-644 regions exhibit inhibitory activity, which could characterize them as possible fibrinogen interacting regions. In this work, we present the synthesis and the anti-platelet properties of various peptides derived from the GPIIIa 589-644 region. The GPIIIa (211-222) peptide was used as control peptide in the biological assays. It was found that the peptides GPIIIa (211-222) and GPIIIa (618-630) at a final concentration of 1mM exhibit the highest inhibitory effect on stimulated with ADP human platelet aggregation (% inhibition: 39.7±7.6 and 34±8.5 respectively) and binding of FITC-Fg (82±18% and 71±20% respectively), but failed to significantly inhibit the binding of PAC-1 (26±9% and 17±8% respectively) to activated GPIIb/IIIa receptor.

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PROBING THE TWO-STEP, TWO-SITE BINDING MODEL OF FAMILY B G PROTEIN-COUPLED RECEPTORS

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A tentative model has been proposed for the binding of Family B GPCRs to their peptide ligands. The N-terminus (Nt) of the receptor may initially associate with the C-terminus of the peptide bringing it to a second site involving the extracellular loops and the 7-helical bundle where additional interactions provide the stabilization energy that leads to activation. Peptides of the glucagon family are most divergent at the C-terminus and highly homologous at the N-terminal portion of the molecule. The Nt of Family B receptors may function as the first line of selectivity filters that restrict access of inappropriate ligands to an activation site in the central core. To test this model, most of the Nt was removed from glucagon and GLP-1 receptors. The idea was to expose the putative activation core and stimulate it directly with peptide ligands. We also constructed receptor chimeras in which most of the Nt of GR was replaced by glucagon 1-29 or glucagon 1-15; and correspondingly, in GLP-1R, the Nt was replaced with GLP-1, GLP-1(7-21), or GLP-1(22-37). Tethering the peptides to the receptor should position the peptides in the proper topology for activation bypassing the first step. We interchanged the entire Nt of GR and GLP-1R and the Nt of rat and goldfish GR. Adenylyl cyclase activity of the mutant receptors were compared to that of native receptor. Our results reveal differences in the activation of Family B receptors that may be the key to regulating selectivity.

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MOLECULAR DYNAMICS STUDY OF THE EFFECT OF GABU INSERT ON THE CONFORMATIONAL BEHAVIOR OF THE MULTIPLE ANTIGEN GLYCOPEPTIDES DURING THE SOLID-PHASE SYNTHESIS

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We used molecular dynamics (MD) techniques to examine structural differences taking places during synthesis of two classes of tetravalent Multiple Antigen Glycopeptides (MAGs) that differ only by the GABU insert in the structure of the oligolysine core. Each of the selected intermediates of the synthesis was modeled, subjected to the 2ns run in N,N'-dimethylformamide (DMF) and geometrically characterized. We characterized: a) distances of free, or extended termini from the anchor, b) interatomic distances between free or substituted N termini, c) radius of gyration and d) spatial distribution of molecular density. A detailed conformational analysis of 16 glycodendrimers shows a distinct behavior of the inserted vs. non-inserted constructs already during the first steps of the modeled synthesis. It suggests that the character as well as the length of the insert has a major impact on the spatial characteristics and behavior of the dendritic molecules. The inserts can increase, in principle, a tendency of dendrimers to establish high-density core, which is similar to the effect of higher generation.

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SYNTHESIS, PROTON AND COPPER(II) COMPLEXES OF A NOVEL POLYHISTIDINE TYPE LIGAND

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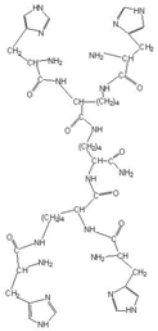
Histidines play an essential role in binding of biological metal ions, either in small or macromolecular chelating molecules, e.g. in metalloenzymes. Therefore the low molecular weight polyhistidine type ligands are of potential importance for use as model substances. A novel branched oligopeptide type ligand (as shown in figure) was prepared by solid phase peptide synthesis. The ligand excerts eight primary protonation sites. As the pH potentiometric titrations revealed, the pK values for the deprotonation processes of these donor groups are close to each other indicating no or weak interaction among them. In addition to amine and imidazole nitrogens, in the presence of copper(II) ions the amide nitrogens may loose protons, as well. Combined potentiometric, spectrophotometric and CD spectroscopic methods were utilized to investigate the speciation and the structure of the copper(II) complexes formed in aqueous solution.



P436

THIOXOPEPTIDE BONDS AS A SPECTROSCOPIC PROBE IN POLYPROLINE HELICES

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The left-handed polyproline II 310- helix (φ=-83 , ψ=+158 , ω=180 ; 3.1 Å/proline) is an essential ligand feature recognised by different regulatory protein domains like Src homolog (SH3) and WW. These domains specifically bind proline-rich motifs with low affinities (Kd = 1 to 200 μM), a property that is necessary for transient signalling interactions.(Nguyen et al., Science 1998) It is known that poly-L-proline can undergo a reversible and cooperative cis/trans isomerisation from a left-handed (ω=180) to a right handed helix (form I; ω=0) in certain solvent systems. A detailed kinetic analysis of the cis/trans isomerisation during poly L proline folding increases our understanding for the molecular basis of folding in biological macromolecules. During conformational changes within poly-L-proline peptides physical and chemical characteristics are hardly to assign because of low differences in the respective signals. For this reason we have introduced the isosteric and photo-switchable thioxopeptide bond into poly L-proline by single O/S substitution at a defined peptide bond.(Shalaby et al., Journal of Organical Chemistry 1996) The unique optical UV- and CD-spectroscopic properties of this extraordinary backbone label allows the exact monitoring of a single peptide bond during conformational transition between polyproline I and II or a photo induced cis/trans isomerisation. From this approach we can derive a model which allows us to study the influence of individual peptide bonds on the stability and dynamics of the polyproline secondary structures.

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THE N-TERMINUS OF NEUROHYPOPHYSIAL PEPTIDE HORMONE RECEPTORS: ITS ROLE IN HORMONE BINDING, LIGAND SELECTIVITY AND RECEPTOR ACTIVATION

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Defining how agonist:receptor interaction differs from that of antagonist:receptor interaction and understanding the mechanisms of receptor activation are fundamental issues in cell signalling. The V1a vasopressin receptor (V1aR) is a member of a family of related G-protein-coupled receptors (GPCRs) that are activated by neurohypophysial peptide hormones, including vasopressin (AVP) and oxytocin (OT). Using alanine-scanning, we have identified that an arginyl in the distal N-terminus of the V1aR is critical for binding agonists but not antagonists. Moreover, this arginyl is strongly conserved throughout the sub-family of Family A GPCRs which comprises the AVP/OT receptors. Substitution of this arginyl by all other 19 encoded amino acids showed an absolute requirement for arginyl at this position and revealed that the N-terminus constitutes part of the activation switch of the receptor. Molecular modelling of this N-terminal region revealed that Arg46 resides within a defined alpha-helical structure which forms a proximal extension to the first transmembrane domain. We have now identified a second residue within this N-terminal segment which is also critical to both agonist binding and receptor activation. The effect of re-positioning both of these functionally-important residues has been evaluated. We have also demonstrated the importance of the helical nature of the N-terminus by introducing a proline residue to disrupt the structure. Our findings explain why this region is conserved throughout the evolutionary lineage of the neurohypophysial peptide hormone receptor family of GPCRs.

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RAPID INHIBITION OF INSULIN BINDING BY GLUCOSE IN RAT LIVER PLASMA MEMBRANES

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Insulin is the main regulator of glucose uptake in several tissues. In insulin resistant state the tissues do not respond to insulin sufficiently and consequently they face an elevated glucose concentrations. Hyperglycemic conditions lead to protein modification by mechanisms of glycation and glycoxidation. Chemical modification of proteins by glucose in vitro is rather slow; measurable effect at diabetic levels of glucose is obtained after several days of incubation. We studied the effect of preincubation of rat liver plasma membranes at 37 ˚C with different concentrations of glucose (10, 40, 120 mM) on ability of insulin receptor to bind insulin. Insulin binding was performed at 20˚C for 2 h in 100 mM Tris-HCl, 2 mM N-ethylmaleimide, 4 mM EDTA, 0.1% BSA, pH 7.4 buffer. Surprisingly, we noticed significant inhibition of insulin binding already after 20 min preincubation at 40 mM of glucose. Reduction of insulin binding was both time and concentration dependent. D and L-glucose were equivalently effective. Mannitol displayed the same effect as glucose. Fructose and arabinose acted significantly only at 120 mM concentration. Inhibitors of protein modification such as lysine (5 mM), aminoguanidine (20 mM) and pyridoxamine (10 mM) were ineffective in inhibiting the glucose action. Our results suggest different pathway for glucose effect on insulin receptor from those described so far for chemical modification of proteins by glucose. This work was supported by grant of VEGA 2/3190/23 and in part by grant of IBS4055303 GA AV CR.

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ISOSELECTIVE INHIBITORS - A NOVEL TOOL FOR ENZYME MECHANISM BASED QSAR

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Understanding enzyme mechanism at the molecular level is of high priority both as a fundamental science and for a variety of practical applications, especially in the field of medicinal chemistry. Many of the drugs that are currently in use or at different stages of development are enzyme inhibitors. Nevertheless, till today there is not a single enzyme whose catalytic mechanism is fully understood at atomic level. Chymotrypsin, probably the best studied enzyme, is a good example, were the fundamental principles of the driving force of its catalytic machanism is still under intensive dispute. Modern molecular modeling methodologies based on molecular and quantum mechanical approaches are not useful for mechanistic studis of many enzymes whose 3D structure is unknown. We present here a novel combined experimental/theoretical approach, serving the dual purpose of enzyme mechanism studies and drug design. It utilizes experimental kinetic binding trend in a series of inhibitors to identify the reaction stages that determine this trend. In turn, this trend analysis clarifies mechanistic details of the native enzyme catalytic reaction. This approach does not require any 3D structural information.

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D,L CYCLIC PEPTIDES AS IMMUNOMODULATORY AGENTS

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We have investigated a linear peptide (LP) as an immunomodulatory agent. LP consists of nine amino acids, the sequence of which has been derived from the T-cell antigen receptor (TCR)α transmembrane region. LP is able to inhibit IL-2 production in T-cells following antigen recognition. Extending these studies in vivo, LP, given subcutaneously or intraperitoneally, significantly reduced the induction of T-cell mediated inflammation in animal models with adjuvant induced arthritis, allergic encephalomyelitis and delayed type contact hypersensitivity. However, there are limitations in delivering linear peptides to the desired site in an intact and biologically active form. To overcome this problem we have designed a number of D-, L-cyclic peptides to improve stability and biological activity. The ability of these peptides to inhibit T cell activation were tested using the antigen presenting assay and each peptide demonstrated a dose responsiveness, although only C1 inhibited IL-2 production appreciably. Cell viability and proliferation assays determined that C1's activity is not due to toxicity. 2H and 31P NMR studies of C1 at 10% concentration in DMPC/DMPG phospholipid membranes show that the peptide orders the lipid bilayer, suggesting a transmembrane orientation. Here we report biological and biophysical studies carried out on C1 and compare its efficacy to LP.

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STRUCTURE-ACTIVITY RELATIONSHIP OF RGD MIMETICS WITH 2H-1,4-BENZOXAZINE-3(4H)-ONE SCAFFOLD

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Development of fibrinogen receptor antagonists has been one of most promising areas of antithrombotic research over the last decades[1]. The minimal sequence in many natural ligands of the receptor required for binding was found to be RGD (Arg-Gly-Asp). A free carboxylic group and a basic functionality are the key pharmacophore elements of the fibrinogen receptor antagonists, mimicking the aspartate ß-carboxylate and the arginine guanidinium group [2]. Several orally active RGD mimetics targeted against the receptor have been converged to clinical trials but have proven to be out of desired clinical benefits. The proposed explanation for the mentioned failure is their poor pharmacokinetic profile. We have prepared several RGD mimetics based on the 2H-1,4-benzoxazine-3(4H)-one scaffold and have evaluated their anti-aggregatory activities as well as affinity towards fibrinogen and vitronectin receptors. In contrast to the generally accepted model, compounds containing alkyl esters as aspartate mimetics inhibited platelet aggregation more potently than corresponding free acids. The latter, however, elicited higher affinity towards fibrinogen receptor than corresponding alkyl esters. We outline the rational behind the choice and design of these peptide mimetics and present some hypothesis in terms of receptor binding and platelet aggregation. [1]R. M. Scarborough, D. D. Gretler, J Med Chem. 43 (2000) 3453-3473.J. A. Zablocki, S. N. Rao, D. A. Baron, D. L. Flynn, N. S. Nicholson, L. P. Feigen, Curr. Pharm. Des. 1 (1995) 533-558. [2]I.Ojima, S. Chakravarty, Q. Dong, Bioorg. Med. Chem. 3 (1995) 337-360.

P442

PLANT PEPTIDE HORMONE PHYTOSULFOKINE (PSK-A) AND ITS ANALOGUES

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In our further studies on structure / activity relationship in the plant peptide hormone PSK-α (H-Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln-OH), we performed the synthesis of a series of analogues modified at position 1 or 3, such as: [H-Phe(4-Cl)1]- (1), [H-Phe(4-Br)1]- (2), [H-Phe(4-F)1]- (3), [H-Phe(4-I)1]- (4), [H-Phg(4-$NHSO_2CH_3$)1]- (5), [H-Tyr(PO_3H_2)1]- (6), [H-D-Tyr(PO_3H_2)1]- (7), [H-Phg(4-OPO_3H_2)1]- (8), [Phe(4-Cl)3]- (9), [Phe(4-F)3]- (10), [Tyr(PO_3H_2)3]- (11), [D-Tyr(PO_3H_2)3]- (12), [H-Phe(4-Cl)1, Phe(4-Cl)3]- (13), [H-Phe(4-F)1, Phe(4-F)3]- (14), [H-Phe(4-Br)1,Tyr(3-SO_3H)3]- (15), [H-Phe(4-F)1, Tyr(3-SO_3H)3]- (16), [H-Phe(4-I)1, Tyr(3-SO_3H)3]- (17), [H-Tyr(3-SO_3H)1, Phe(4-Cl)3]- (18), [H-Tyr(3-SO_3H)1, Phe(4-F)3]- (19), [H-Tyr(PO_3H_2)1, Tyr(PO_3H_2)3]- (20), [H-D-Tyr(PO_3H_2)1, D-Tyr(PO_3H_2)3]- PSK-α (21). Peptides were synthesized by solid phase method according to the Fmoc procedure on a Wang-resin. Free peptides were released from the resin by 95% TFA in the presence of EDT. All peptides were tested by competitive binding assay to the carrot membrane using 3H-labeled PSK according to the Matsubayashi et al. test. Among the tested peptides, analogue (1), (4) and (9) retained 30% of the native peptide activity.

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SOLID-PHASE PREPARATION OF GALANINS AND FRAGMENTS AND THEIR CONFORMATION STUDIES. MODIFIED METHOD OF AVP PREPARATION, EFFECTS OF RAT GALANIN(S) ON AVP AND OT EXCREATION ON WATER METABOLISM REGULATION

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We have synthesized and already published full sequences of human(h.), chicken (ch.), bovine (b.) and rat(r.) galanins(GALs),a number of their had published/unpublished fragments.The conformations(with CD-, NMR-studies) of h.GAL,r.GAL, some of their fragments have been studied by us, too. Only the unpublished syntheses and conformation results and the modified Argvasopressin(AVP), oxytocin(OT) syntheses will be published here. GALs have already a lot of hormonal and neuropeptide effects [1]. Some of the mentioned GALs, their fragments have been studied and published continously during the last 10 years by us and by other cooperation-partners, too [1]. Therefore now, we are going to publish only our latest hormonal/neurological results; especially the inhibitory effect of r.GAL and h.GAL on dopamine induced increased OT and enhanced AVP secretions in r. hypophyseal tissues cultures. We have found some explanations for both OT and AVP releases [2].

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P444

ACTIVITY AND STRUCTURAL CHANGES OCCURED IN HORSERADISH PEROXIDASE IN SEVERAL PH VALUES

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The pH induced activity and structural changes of HRPC have been investigated, using kinetic, circular dichroism and fluorescence spectroscopy studies. The systematic studies on the stability of HRP are important, since their biotechnological applications require them to function under extremes of pH and/or temperature; furthermore there is a lack of systematic studies providing information on the impact of pH on kinetic and structural parameters of this enzyme. The Trp fluorescence and the kinetic constants were found dependent on the pH. Above pH 9 and under pH 6, the enzyme exhibited structural phase transitions as revealed by a decrease in the fluorescence intensity, while circular dichroism studies in this range of pH showed almost no variation in the CD ellipticity at 222 nm. The reduced catalytic activity of enzyme in the range of 6 > pH > 9 is correlated to the pH induced unfolding and ionization or protonation of key protein residues. Results from our thermal studies on MHRP at pH 5, suggested that melting of the tertiary structure to a pre-molten globule form takes place at 55°C, which is much lower than the temperature (Tm = 75.5°C) at which depletion of heme from the heme cavity takes place. The melting of the tertiary structure of MHRP at pH 5 associated with a pKa of ~ 5 indicting that this phase possibly involves breaking of the hydrogen bonding network of the heme pocket which is responsible for keeping the heme group inside it.

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SOLID-PHASE SYNTHESIS OF AZA-BETA-3-PEPTIDES VIA FMOC-STRATEGY AND ITS APPLICATION IN THE SYNTHESIS OF RGD MIMETICS

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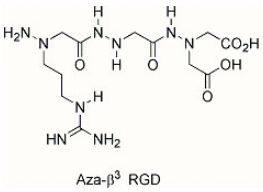
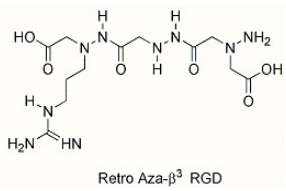
A particular interesting class of oligomeric peptidomimetics is formed by aza-beta-3-peptides, which consist of N-substituted acid hydrazino-acetic monomers or aza-beta-3-amino acid residues (aza-beta-3-aa). A solid phase synthesis method for aza-beta-3-peptides is presented using their Fmoc derivatives. This monomer method allowed the monitored synthesis of relatively large quantities of pure aza-beta-3-peptides as well as the translation of, in principle, any peptide into the corresponding aza-beta-3-peptides. The required Fmoc-substituted aza-beta-3-amino acids were accessible by convenient synthesis, and a number of monomers including those containing side chains with functional groups have been synthesized. The method was exemplified by the solid-phase syntheses of aza-beta-3-peptides of RGD-mimetics. First investigations about possible secondary structure of such aza-beta-3-peptides will be described.



Aza-β^3 RGD



Retro Aza-β^3 RGD

P446

MEMBRANE LIPID-PEPTIDE CHARGE INTERACTIONS USING SURFACE PLASMON RESONANCE AND T-CELL ANTIGEN RECEPTOR PEPTIDES

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The T-cell antigen receptor (TCR) is a multisubunit structure, composed of at least seven transmembrane proteins, that recognize antigens in the context of MHC-encoded proteins on the surface of antigen presenting cells. This trimolecular interaction consisting of antigen-MHC and TCR is of paramount clinical importance and has been the focus of intense research over a number of decades. As part of our ongoing studies on the mode of action of immunomodulating peptides derived from the transmembrane region of the TCR, we report here the binding properties of these hydrophobic peptides on model membranes using surface plasmon resonance. The di-basic “core” peptide was found to bind to both zwitterionic and anionic model membranes. By contrast, switching one or both of the basic residues to acidic or neutral residues, the position of the charged amino acids in the sequence and the number of hydrophobic amino acids between the charged residues, were found to effect binding. These results are in agreement with in vitro T cell stimulation assays and in vivo adjuvant induced arthritis models, showing that amino acid charge and their location is essential for peptide activity and protective effect on T-cell mediated arthritis respectively. In conclusion, the cationic nature of the peptides leading to initial electrostatic membrane binding followed by hydrophobic peptide–lipid interactions, as shown by binding kinetics, are critical steps to membrane attachment and insertion. These peptide-membrane interactions correlate well with the expression of biological effect.

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EFFECT OF THE ERYTHROCYTE MEMBRANE STATE ON THE GRAMICIDIN S - INDUCED HAEMOLYSIS OF ERYTHROCYTES

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Gramicidin S (GS) is a cyclic decapeptide of primary structure [cyclo-(Val-Orn-Leu-D-Phe-Pro)2] secreted by Bacillus brevis. Activity of GS was demonstrated against Gram-negative and Gram-positive bacteria and also against some pathogenic fungi. Unfortunately, GS is rather non-specific in its actions and also exhibits a high haemolytic activity, limiting its therapeutic usage. It was hypothesized that the lipid-peptide interaction could induce (i) the attenuation of the chemiosmotic potential by loss of protons or ions into the environment or (ii) by the inhibition of respiratory enzymes found in the bacterial cell membrane. In the present work we studied the effect of erythrocyte membrane state on the GS-induced haemolysis of erythrocytes in vitro. To modify the membrane state we used a number of physical and chemical factors: temperature, salt stress, detergents, lipid oxidation, different content of cholesterol in the membrane. We have shown that the haemolytic activity of GS strongly depends on the membrane state, at that its activity depends more on the state of membrane lipids than of proteins. We have found that some factors influencing the membrane state can attenuate and even completely suppress the haemolytic activity of GS. On the basis of our result we attempted to make a prediction in which direction modification on GS molecule should be made in order to decrease its haemolytic activity.

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SYNTHESIS AND CONFORMATIONAL ANALYSIS OF HIGHLY N-METHYLATED SOMATOSTATIN ANALOGUES

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Somatostatin, a tetradecapeptide, is a major endocrine hormone with multiple physiological actions which are modulated by one or more of the five known G-protein-coupled receptor subtypes: sst1-sst5 [1]. The biological role as well as the cellular distribution of each receptor subtypes is far from being completely understood. For this reason, the search for synthetic analogues of somatostatin which exhibit selective affinities for the five receptors subtypes is of considerable basic and therapeutic interest. In the past, conformational restriction of the side chain groups and the peptide backbone has yielded the most interesting results. Consequently we were interested in the potential effects of one or many N-methylation of peptide bond NH groups on binding affinity of some somatostatin analogues. N-Methyl amino acids are well-known to increase pharmacokinetically useful parameters such as membrane permeability, proteolytic stability, and conformational rigidity [2]. Full N-methyl scans of somatostatin cyclopeptidic agonists were aided by the introduction of N-Me group during regular solid phase peptide synthesis. A new series of 32 Veber peptide (c[Phe-Pro-Phe-D-Trp-Lys-Thr]) [3] and 16 octreotide (NH_2-D-Phe-c[Cys-Phe-D-Trp-Lys-Thr-Cys]-Thr-ol) [4] analogues have been synthesized and their conformation studied by NMR. The effect of one or many N-methylation on the biological activity and on the structure of octreotide and Veber peptides will be presented. [1] Patel, Y.C. Front. Neuroendocrinol. 20, 157 (1999). [2] Fairlie, D.P.; Abbenante, G.; March, D.R. Curr. Med. Chem. 2, 654 (1995). [3] Veber, D.F. et al. Nature 292, 55 (1981) [4] Bauer, W. et al. Life Sci. 31, 1134 (1982).

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NEUROPEPTIDE ANALOGUES LABELLED WITH THE RADIONUCLIDES Tc-99M AND Re-188 FOR TUMOUR DIAGNOSIS AND THERAPY

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G-protein coupled receptors with high affinity to neuropeptides are known to be over-expressed on tumour cells and are thus an interesting target for tumour diagnosis and therapy. However, the rapid in vivo metabolism of the peptides is a severe drawback which can be overcome replacing natural amino acids by non-natural ones. We have synthesised accordingly series of neurotensin (NT) and bombesin (BBS) analogues. These peptides have a great potential in the treatment of pancreas tumours and prostate or breast cancers, respectively. For labelling with the 99mTc(CO)3 and 188Re(CO)3 moiety a chelator is needed (tridentate chelators bring about optimal pharmacokinetics). Thus, retro[N(alpha)-carboxymethyl-histidine], short (N(alpha)His)Ac, was linked to the N-terminus of the binding part of the NT and BBS analogues. These inert metal carbonyl complexes (oxidation state +I) remained stable under in vivo conditions. Some of the peptide analogues (20 NT and 40 BBS analogues were prepared) preserved the high affinity and persisted clearly longer intact in plasma (see table) than the natural peptides (T1/2 < 0.5 h). Biodistribution data in nude mice with tumour xenografts showed results which encouraged the start of clinical studies with a few patients.

Table: Receptor affinity (Kd) and stability in plasma ($T_{1/2}$) of some ^{99m}Tc labelled peptides

	Kd [nM]	$T_{1/2}$ [h]
NT analogues:		
(NαHis)Ac-Arg-(ΨCH$_2$NH)-Arg-Pro-Tyr-Tle-Leu	0.52	>100
(NαHis)Ac-Arg-(N-Me)-Arg-Pro-Tyr-Tle-Leu	0.50	>100
BBS analogues:		
(NαHis)Ac-Gln-Trp-Ala-Val-Gly-His-Cha-Met-NH$_2$	0.02	16
(NαHis)Ac-Gln-Trp-Ala-Val-Gly-His-Leu-Nle-NH$_2$	0.09	6
(NαHis)Ac-Gln-Trp-Ala-Val-Gly-His-Cha-Nle-NH$_2$	0.8	16

Tle=tert-Leucine, Nle=norleucine, Cha=Cyclohexylalanine

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ENKEPHALIN TETRA- AND PENTAPEPTIDE ANALOGUES CONTAINING ACETYL GROUP IN THE 2-POSITION. SYNTHESIS AND INVESTIGATION OF BIOLOGICAL ACTIVITY IN VIVO AND IN VITRO

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Enkephalins - natural ligands of opioid receptor have attracted considerable attention since their discovery, most notably, with respect to the appearance of the new principal approach in analgesic design. A series of acetylated enkephalin analogues with branched and linear structure of peptide chain based on the common tetrapeptide enkephalin sequence - Tyr-D-Orn-Gly-Phe have been synthesized by classical methods of peptide chemistry in solution. Investigation involved determination of analgesic activity in the 'tail pinch' method following intracisternal and intravenous administrations to mice, affinity constants for an opiate receptor site and potency in the GPI and MVD bioassays. Acetylation in the 2-position of the tetrapeptide enkephalin sequence turned out to be a successful modification for high opioid activity. Analogues demonstrated a remarkable increase of analgesic activity and duration of the action exceeded that of [Leu5] enkephalin or non-acetylated correlates. The acetylation of the peptide side chain enhanced the potency on the GPI and MVD by about one order. The compounds were more potent than [Leu5] enkephalin in displacement of [3H]Naloxone from rat brain membranes. The results obtained justify further work with those and similar substances. We wish to acknowledge the Grant Agency of Malardalens University for financial support to complete the research.

STRUCTURE ACTIVITY RELATIONSHIP STUDIES

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INVOLVEMENT OF NITRIC OXIDE IN THE ANTINOCICEPTIVE EFFECTS OF TYR-MIF-1 FAMILY OF PEPTIDES IN RATS

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The members of the Tyr-MIF-1 family include MIF-1, Tyr-MIF-1, Tyr-W-MIF-1 and Tyr-K-MIF-1 have been isolated from bovine hypothalamus and parietal cortex of human tissue. Tyr-MIF-1 and Tyr-W-MIF-1 are able to interact with opioid receptors with a higher potency at mu sites than at delta and kappa sites. Tyr-MIF-1 exerts its antinociceptive action by binding to mu-opioid receptors as well as to its specific non-opiate receptors in the brain. Tyr-K-MIF-1 has been isolated from human brain cortex and appears to bind to Tyr-MIF-1 specific sites and to its own sites. Nitric oxide (NO) appears to play a role a variety of biological events in the central nervous systems. The SIN-1 induced hyperalgesia after ith. or i.c.v. administration in rats. Recent research has highlighted the role of No in central nociception. The aim of our study was to investigate involvement of nitric oxide in the antinociceptive action of endogenous Tyr-MIF-1, Tyr-W-MIF-1 and Tyr-K-MIF-1 peptides during acute pain (paw-pressure test) in rats. The results showed that Tyr-MIF-1 (1mg/kg, i.p.), Tyr-W-MIF-1 (1mg/kg, i.p.), Tyr-K-MIF-1 (1mg/kg, i.p.) and L-arginine (L-arg, 1mg/kg, i.p.) exerted the antinoinociceptive effects. L-arg, the NO synthesis precursor, inhibited the co-administration of L-NAME with each of peptides. SIN-1 (3-morpholino-sydnonimine, 100 microg i.c.v.), a donor of NO, naloxone (1mg/kg, i.p.) and methylene blue (MB, 500 microg/paw), an inhibitor of activation of guanylyl cyclase, decreased the antinociceptive effects of Tyr MIF-s peptides. Our results suggest the involvement of nitric oxide in the antinociceptive action of Tyr-MIF-1, Tyr-W-MIF-1 and Tyr-K-MIF-1.

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ENKEPHALINS AND SELECTIVE ANALOGUES: MU- AND DELTA-DEPENDENT OPIOID RECEPTOR INTERACTIONS

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Effects of mu-selective (DAMGO and N-Me-[L-Phe4]-dalarginamide) and delta-selective (Cys-sulfonamide2-Leu5-enkephalin and Cys-sulfonamide2-Met5-enkephalin) enkephalin analogues on spontaneous contractile activity of guinea-pig myenteric plexus-longitudinal muscle preparations were examined and compared with those of endogenous Leu5-enkephalin and Met5-enkephalin. Delta-selective Cys2-containing analogues and endogenous enkephalins exerted both relaxatory and contractile effects in this tissue, while mu-selective produced only pronounced relaxatory responses. Both phases revealed different concentration range which was as follows: 0.1 nM – 1 mM for the relaxatory phase and 1 mM - 100 mM for the contractile phase. The further pharmacological analysis show that: (i) relaxation was sensitive to opioid antagonist naloxone (50 nM), tetrodotoxin and adrenoblockers (phentolamine plus propranolol) and resistant (50 %) to atropine; (ii) the contraction was sensitive to naloxone (> 100 nM), but resistant to TTX; the presence of adrenoblockers highly potentiated the contractile responses. The data obtained suggest that: (i) biphasic effects of endogenous enkephalins and their selective mu- and delta-analogues on the spontaneous activity of guinea-pig ileum are mediated by neurogenic (mu-dependant) and smooth-muscle (delta-dependant) opioid receptors; and (ii) delta-selective Cys2-containing analogues potentiate the neurogenic contractile responses of longitudinal muscle isolated from guinea-pig ileum .

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A GENERAL METHOD FOR THE SCREENING OF PEPTIDOMIMETIC LIBRARIES BY ELISA BASED TYROSINE KINASE ASSAY

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A non-radioactive ELISA-based tyrosine kinase assay was applied for the screening of new PTK inhibitors. The assay is suitable to use different tyrosine kinases depending on the biological properties of the peptidomimetic library to be tested. The method based on the phenomenon that all the applied components are the same (e.g. the substrate, antibody, colorimetric determination) only the specific recombinant kinase enzyme (EGF-R, PDGF-R, VEGF-R2) is different. At the same time the assay is suitable to test big number of samples in parallels. We have designed and synthesized a large set of new potential PTK inhibitors. Most of the synthesized compounds were designed to act on the ATP-binding site, while several others are related to peptidomimetic structures. Analysing the structures of several ATP analog inhibitors revealed that most of the potent inhibitors contained a condensed bicyclic heteroaryl moiety where the pyrimidine ring seems to be crucial. For the synthesis of condensed bicyclic heteroaryl cores containing a pyrimidine ring, the ortho-cyanoarylamines are very practical building blocks. We synthesized more than 100 ATP-binding site targeted molecules from ortho-cyanoarylamines in solution phase using parallel synthetic methods. The resulting compounds and intermediates were characterized via MS and NMR spectroscopy and analyzed by HPLC for purity.

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STRUCTURE-ACTIVITY RELATIONSHIPS AND STRUCTURAL CONFORMATION OF A NOVEL UROTENSIN II-RELATED PEPTIDE

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Urotensin II (UII), a cyclic peptide initially isolated from the urophysis of teleost fish, has been described as the most potent vasoconstrictor peptide identified so far. Subsequently, it has been shown that UII is the endogenous ligand of the orphan receptor GPR14. The existence of a paralogue gene encoding a UII-related peptide (URP, ACFWKYCV) has been recently reported in rodent and human. URP exhibits high binding affinity and high potency to increase Ca2+ mobilization in GPR14-transfected cells, suggesting that the biological effects previously attributed to UII could be exerted, at least in part, by URP. To study the structure-activity relationships of URP, a series of URP analogues has been synthesized and each analogue was tested for its binding affinity on GPR14-transfected cells and its contractile activity in a rat aortic ring bioassay. Alanine substitution of each amino acid significantly reduced the binding affinity and the contractile activity of the peptides, except for the Ala8-substituted analogue that retained biological activity. Most importantly, D-scan revealed that [D-Trp4]URP abrogated and [D-Tyr6]URP partially suppressed the UII-evoked contractile response. [Orn5]URP, which had very low agonistic efficacy, was the most potent antagonist in this series. NMR spectroscopy and molecular dynamics revealed that URP exhibited a single conformation characterized by an inverse γ-turn comprising residues Trp-Lys-Tyr. These data suggest that the triade Trp-Lys-Tyr has a critical importance in the biological activity of URP. These results should prove useful for the rational design of non peptidic agonists and antagonists of GPR14.

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A STUDY ON ANIONIC AND CATIONIC HOMOCONJUGATION EQUILIBRIA MODELLING ACID-BASE INTERACTIONS IN SIDE CHAIN OF BIOMOLECULES BY USING POTENTIOMETRIC AND AB INITIO METHODS

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Acid-base and hydrogen bonding equilibria including anionic and cationic homoconjugation reactions belong to the most important and frequent chemical processes, as well as play an important role in living systems. To examine such interactions, the homoconjugating acetic acid – acetate and phenol – phenolate anionic systems and n-butylammonium cation – n-butylamine, imidazole cation – imidazole and 4(5)imidazole cation – 4(5)imidazole cationic systems were studied in polar protophobic aprotic non-aqueous solvent - acetonitrile using the potentiometric-titration method. The systems under considerations were designed to model acid-base and hydrogen-bonding phenomena that involve acid and basic amino-acid side chains in proteins. To perform main goal of this study in both solvents studied the pKa values, anionic and cationic homoconjugation constant values were determined. On the basis of the obtained constant values the influence of solvent properties on acid–base equilibria has been discussed. Experimental studies were supplemented by theoretical ab initio studies at the RHF (Restricted Hartree Fock) and MP2 (Møller-Plesset) levels on energetic parameters of the protonation, and homoconjugation reactions studied. To estimate solvation effects self-consistent reaction field (SCRF) and polarizable continuum (PCM) models were applied. The Polish Scientific Research Council (KBN) under grant No. 7 T09A 160 21 financially supported this work.

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A STUDY ON MOLECULAR HETEROCONJUGATION EQUILIBRIA MODELLING ACID-BASE INTERACTIONS IN POLIPEPTYDES BY USING POTENTIOMETRIC AND AB INITIO METHODS

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Acid-base reactions, the formation of hydrogen bonds and proton transfer within the bond belong to the most important and frequent chemical processes. Almost each reaction in organic chemistry involves a complete or partial proton transfer. Acid-base equilibria are also crucial for stabilization of biomolecules and their functioning structures. To study such interactions potentiometrical measurements in two polar protophobic aprotic non-aqueous solvents acetonitrile and dimethyl sulfoxide have been carried out for 10 molecular heteroconjugating acid-base systems containing acetic acid (AcA) modelling the C-terminal carboxylic group and the side chain of aspartic and glutamic acid; phenol (PhOH) modelling the phenolic group of the tyrosine side chain; imidazole (Imid) and methylimidazole (MeImid) modelling the histidine side chain; isopropylamine (iso-Prop) modelling the ornithine side chain; methylguanidine (MeGua) modelling the guanidine group of arginine, and n-butylamine (n-But) modelling the lysine side chain. The systems under considerations were designed to model acid-base and hydrogen-bonding phenomena that involve acid and basic amino-acid side chains in proteins. Experimental studies were supplemented by theoretical ab initio studies at the RHF (Restricted Hartree Fock) and MP2 (Møller-Plesset) levels on energetic parameters of the protonation, and homoconjugation reactions studied. To estimate solvation effects self-consistent reaction field (SCRF) and polarizable continuum (PCM) models were applied. The Polish Scientific Research Council (KBN) under grant No. 7 T09A 160 21 financially supported this work.

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STRUCTURAL MODIFICATIONS ON [TYR3]OCTREOTATE: EFFECTS ON SSTR2-BINDING AFFINITY

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The hypothalamic peptide somatostatin exerts regulatory effects on its target cells in the pituitary, the pancreas or the gastrointestinal tract of mammals. Its actions are mediated by high affinity receptors located on the surface of target cells. Five receptor subtypes (sstr1–sstr5) are known so far, all members of the superfamily of G-protein coupled receptors. Somatostatin has been attracting a great deal of attention lately due to the massive expression of somatostatin receptors, particularly sstr2, in neuroendocrine tumors. The advent of synthetic somatostatin analogs has rather recently allowed the exploitation of the somatostatin/somatostatin receptor system in clinical oncology. In this study, a small library of novel [Tyr3]octreotate analogs was synthesized on the acid-sensitive 2-chlorotrityl chloride resin following Fmoc-tBu methodology. These analogs have undergone single or double amino acid substitutions in positions 1 (D-Phe) and 3 (Tyr) or alternatively in positions 4 (D-Trp) and 5 (Lys) of the parent peptide. The effects of above modifications on the binding affinity of new analogs to the sstr2 were studied by competition binding assays in rat acinar pancreatic AR42J cell membranes using [125I-Tyr3]octreotide as the radioligand and [Tyr3]octreotate as reference compound. This structure – activity relationship study revealed that while non-polar amino acid residues at positions 1 and/or 3 were well tolerated by the sstr2, substitutions leading to ?-turn reinforcement in the sensitive 3 – 6 region caused a total loss of sstr2-affinity. Further studies are currently in progress to unravel the main factors operating during interaction of somatostatin cyclic octapeptide analogs with the sstr2.

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SYNTHESIS AND BIOLOGICAL ACTIVITIES OF NEW ANALOGUES OF [MPA1, D-TYR(OET)2]OXYTOCIN

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Oxytocin (OT), a physiologically important nonapeptide hormone and neurotransmitter containing a 20-membered tocin ring and an acyclic tripeptide tail, regulates several physiological functions, such as milk ejection, uterine contractions, vascular and cardiac relaxation etc. Hundreds OT analogues have been the subject of extensive pharmacological investigations. Potent peptide inhibitors of OT action have been synthesized with the aim of using them for arresting preterm labor. Literature data suggested that the C-terminal tripeptide sequence (especially the proper orientation of the C-terminal glycine carboxamide) appear to be critical for obtaining high potency OT agonists. The configuration and hydrophobicity of the aromatic amino acid in position 2 are important for the antagonistic activity. On the basis of these findings, we designed and synthesized new analogues of [Mpa1, D-Tyr(OEt)2]oxytocin having the α-helix inducing amino acid 2-aminoisobutyric acid (Aib) and/or the unnatural amino acid D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (D-Tic) in positions 7 or 9 or penicilamin (Pen) in positions 1 or 6. The new analogues were synthesized by Fmoc solid phase methodology utilizing a 2-chlorotrityl chloride resin as solid support bearing a Rink-Bernatowitz linker to provide the peptidic amide and DIC/HOBt as coupling agents. The analogues were tested for their potency in two pharmacological tests, i.e. rat uterotonic in vitro test in the absence or in the presence of 1mM magnesium ions, and in the pressor test on phenoxybenzamine treated male rats. All the new analogues showed strong antioxytocic activity in the uterotonic test in vitro (pA2 7.9 – 8.4) and no pressor activity.

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INFLUENCE ON BINDING AFFINITY OF LEUPROLIDE ANALOGUES MODIFIED IN POSITION 6 WITH α,α-DIALKYL AMINO ACIDS

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The pivotal role that LHRH and its analogues play in the modulation of reproductive functions have attracted considerable scientific interest because of their usefulness in the treatment of endocrine-based diseases such as prostate and breast cancer, endometriosis and precocious puberty. LHRH has been the subject of intense structure-activity relationship (SAR) studies aimed at elucidating its mechanism of action and identifying drug candidates (agonists and antagonists), which have been extensively studied in the clinic later on. Agonistic analogues of LHRH, represented by Leuprolide ([DLeu6,desGly10]-LHRH-NHEt), have been widely used in oncology and gynaecology for nearly two decades. In this study, we report the synthesis of new LHRH analogues bearing the Fujino modification and substituted at position 6 (Gly6) by α,α-dialkyl amino acids (Aib, Deg). In addition, the affinities of new analogues for the LHRH receptor were determined by competition binding experiments in mouse anterior pituitary αT3-1 cells. Leuprolide showed a high binding affinity, while [Aib6,desGly10]LHRH-NHEt and [L-Tic3,Deg6,desGly10]LHRH-NHEt bind to the LHRH receptor with IC50 values comparable to those of native hormone. All other analogues showed inferior binding affinities for the LHRH receptor.

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STRUCTURE-ACTIVITY STUDIES OF NOVEL CONFORMATIONALLY CONSTRAINED RGD CYCLIC PEPTIDES

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RGD analogs have been extensively studied as antagonists of alpha V integrins. These integrins, as physiological role, mediate their angiogenic effect through the binding to specific proteins of the extracellular matrix; therefore, they represent an interesting therapeutic target for potential inhibitors. Cyclic peptides containing the RGD sequence are an exciting class of integrins antagonists; a great number of analogs have been extensively studied and the most promising developed as drugs [1]; one of them reached now the phase II clinical trial [2]. We synthesized a number of RGD cyclopeptides incorporating a C alpha, alpha-trifluoromethylamino acid (Tfm-AA) into the cyclic motif, with the purpose to examine the effect on the conformation and activity. Some representative members of our collection were chosen and their receptor affinity and cell adhesion was compared with a parent compound lacking the Tfm substitution. Preliminary biological data indicate higher affinity for the Tfm-AAs. In this presentation, we will discuss the relationships between activity and conformation of this new class of potent alpha V beta 3 integrin antagonists. The preferred conformations were determined by NMR. [1] R. Haubner, D. Finsinger and H. Kessler, Angew.Chem.(1997) 36, 1374-1389 [2] J.W. Smith, Current Opinion in Investigational Drugs, (2003) 4, 741-746.

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STRUCTURE ACTIVITY RELATION SHIP OF SHORT ANALOGUES OF ANTISTASIN (ATS) AND GHILANTENS (GLS) INCLUDING BASIC AMINO ACIDS IN 109 POSITION

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ATS and GLS are protein isolated from salivary glands of Mexican leech Haementeria officinalis and South American leech Haementeria ghilianii, respectively [1, 2]. They both manifest anticoagulant activity by inhibiting of factor Xa. The recombinant ATS (rATS) has IC50 = 20nM [3]. The differences between sequences 109 to 116 of rATS and natural GLS are such that in the position 110 Arg residue is replaced by Lys and in the position 115 the Ile residue is replased by Val. There isn't data for the biological activity of sequence 109-116 of GLS. In order to investigate the biological activity of this sequence of GLS, the role of the hydrophilic amino acids Arg and Lys on the 109 position, and the role of basic group in 109 position, on the anticoagulant activity analogues of ATS and GLS including Arg, Lys, Orn and Creatin in the N-terminus and its amides were synthesized. SPPS method and Fmoc-strategy were used. The anticoagulant activity and inhibitor constants against wild range of serine proteinase such as Factor Xa, thrombin, plasmin and s.o. will be discussed. [1] Nutt E., Gasic T. et al, J. Biol. Chem., 1988, v. 263, N 21, 10162-10167 [2] Blankenship D., Brankamp R., 1990, v. 166, N 3, 1384-89 [3] Vezenkov L., Danalev D., Grigorova B., Coll. Symposium series, v. 6, 2003.

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NOVEL EXTENDED AND BRANCHED N-TERMINAL ANALOGS OF VIP

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Vasoactive intestinal peptide (VIP) is a prominent neuropeptide exhibiting a large spectrum of biological actions in mammals. VIP's effects are mediated through high affinity interaction with two receptors: VPAC1 and VPAC2. VPAC1 and VPAC2 are preferentially coupled to Gαs protein that stimulates increases in adenylate cyclase. In order to elucidate the physiological role of VIP, several antagonists have been developed. All the potent VIP antagonists synthesized have modifications in the N-terminal domain of the peptide. Thus, it is suggested that the N-terminal domain of VIP is responsible for the peptide's activity. In view of the above, we examined the effect of multiplication of the N-terminal domain of the VIP ligand, on the VIP receptor (VPAC1) binding and cAMP activation. The multiplication of the VIP N-terminal was performed through extending or branching methodology. We created several VIP analogs carrying multiplication of the N-terminal domain of VIP. Circular dichorism (CD) analysis revealed that these peptides maintained similar helicity to VIP in organic environment. The analog receptor binding and activation of HT29 cells expressing VPAC1 was examined. A VIP branched analog that was slightly more efficacious as compared to native VIP towards VPAC1 – related cAMP production was discovered. It is concluded that two branched N-terminal VIP sequences are superior in recognizing VPAC1 as compared to two N-terminals in tandem. Supported by the Lily and Avraham Gildor Chair.

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NEW POTENT B2 BRADYKININ ANTAGONISTS ACYLATED ON THEIR N-TERMINALS

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We previously reported that acylation of the N-terminus of several known B2 antagonists with various kinds of bulky acyl groups has consistently improved their antagonistic potency in rat blood pressure assay. On the other hand, our earlier results seem to suggest that effects of acylation on the contractility of isolated rat uterus depend substantially on the chemical character of the acyl group, as we observed that this modification may either change the range of antagonism or even transform it into agonism. Bearing all this in mind, we designed three new analogues of bradykinin by modifying the moderately potent B2 antagonist, previously synthesized by Stewart's group (D-Arg-Arg-Pro-Hyp-Gly-Thr-Ser-D-Phe-Thi-Arg). New analogues where obtained by acylation of the N-terminus of the above peptide with butanedioic acid, 12-aminododecanoic acid and 4-aminobenzoic acid in order to confirm if either the positive or the negative charge on the N-terminal and of the peptide is responsible for the transformation of activity. Our results may be of value in the design of new B2 agonists and antagonists. The activity of analogues was assessed on blood pressure and uterotonic in vitro tests.

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NEW BRADYKININ ANALOGUES MODIFIED WITH 1-AMINOCYCLOHEXANE-1-CARBOXYLIC ACID

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In previous studies, we demonstrated that the D-amino acid in position 7 of bradykinin analogues, which was considered necessary for B2 antagonism, may be replaced by a suitable L-amino acid residue (N-Me-phenylalanine) or sterically restricted dipeptide unit, namely –Phe–Phe , a fragment which was prepared by introducing -CH_2-CH_2- bridge, spanned two subsequent peptide nitrogens. In the present work we selected the 1-aminocyclohexane-1-carboxylic acid (Acc) in order to reduce the flexibility of the peptides by imposing a sterically constrained residue limiting conformational freedom, thus forcing the peptide backbone and side chains to adopt specific orientations. We decided to use as a model compound, B2 antagonist previously synthesized by Stewart's group, [D-Ary0,Hyp3,Thi5,8,D-Phe7]BK, and modify this peptide by placement in positions 6,7 or 8 of Acc. Knowing that acylation of the N-terminus of several known B2 blockers with various kinds of bulky groups has consistently improved their antagonistic potency in rat blood pressure assay, we designed the next three analogues by acylation of N-terminus of the peptides mentioned above, with 1-adamantaneacetic acid (Aaa). Finally, we found it interesting to check how substitution of Phe8 in bradykinin with Acc and the combination of this modification with acylation will change the pharmacological properties of this hormone. The activity of the new analogues was assayed on isolated rat uterus and in rat blood pressure tests

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RAMAN OPTICAL ACTIVITY OF MALARIA-VACCINE CANDIDATES FROM THE NPNA FAMILY

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Raman Optical Activity (ROA) is the difference in intensity of the right and left circularly polarized Raman light scattered by a chiral molecule. This effect was predicted and its existence demonstrated by Barron L. D. in 1973. Improvements in experimental techniques have extended the field, allowing studies of proteins, nucleic acids and viruses. A particularly important experimental improvement has been the cancelation of offsets by substracting the spectra measured for the actual chiral molecule and of its virtual optically generated enantiomer (Hug W., 2002). ROA yields new information about the structure, hydration, and dynamics of molecules. We present the ROA analysis of the tandemly repeated pentapeptide sequence (NPNA)5 and of the two cyclic parent peptides containing either (2S,3R)-3-aminoproline or (2S,3R)-4-aminoproline at position 6 linked by an amide bond to a glutamate at position 16 (Pfeiffer B. et al, 2003). These peptides mimic epitopes in the central repeat region (NPNA)~37 of the major surface protein of the invasive sporozoite stage of Plasmodium falciparum, the Plasmodium species known to cause malaria. The three almost identical ROA spectra present surprising similarities with the ROA spectrum of the bovine b-casein protein, being dominated by a strong positive band centered at 1320 cm-1, assigned to the PPII-helical conformation. Other similarities are ROA signals which are observed at ~1670, 1455, 1260 and 1215 cm-1. The presence of ROA or Raman signals typical for cis or trans proline are discussed.

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SYNTHETIC PEPTIDES ABLE TO MODULATE THE INTERACTION OF AKAP121 WITH MITOCHONDRIA

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Protein phosphorylation and dephosphorylation play a central role in the regulation of cellular functions in response to change in external stimuli. Phosphorilation is mediated by different kinases that are ubiquitous in cells. One the best characterized protein kinase is the c-AMP-dependent kinase A (PKA). The subcellular localization of this enzyme is maintained through its association with A-kinase anchoring proteins (AKAPs). AKAPs represent a group of functionally related proteins, classified by their ability to interact with PKA inside cells. AKAP proteins are able to bring PKA to different membrane and cellular organelles and thus can mediate cAMP signalling events such as development, differentiation, cell survival and cell progression. In particular AKAP 121 localizes PKA on mitochondria and endoplasmic reticulum and mediate the protective effect of cAMP on cell survival. It was demonstrated that the targeting of AKAP 121 to the outer membrane of mitochondria both in male germ cells and in transfected heterologous cells is mediated by the first 30 N-terminal residues (MT) that are predicted to form a highly hydrophobic alpha-helical wheel. Starting from the data available in the literature we have developed a new class of highly selective peptides that interfere with the biological function of the PKA scaffold protein AKAP121. Most likely, these peptides compete and inhibit anchoring of cAMP PKA to the outer wall of mitochondria and doing so, profoundly influence survival and reduce cAMP cytoprotective effects.

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ANALOGUES OF ARGININE VASOPRESSIN MODIFIED IN POSITION 2 AND 3 WITH CONFORMATIONALLY CONSTRAINED DIPEPTIDE FRAGMENTS

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Previously, we reported that introduction of -Phe-Phe fragment, dipeptide having $–CH_2–CH_2–$ link bridging two nitrogens, into AVP molecule resulted in loss of all activities checked, although, the same change applied to [Mpa1]AVP (dAVP) gave weak pressor antagonist. However, spectacular results were obtained by placing this unit in positions 2 and 3 of two V1a antagonists, namely [Cpa1]AVP and [Cpa1,Val4]AVP, (where: Cpa is 1-mercaptocyclohexaneacetic acid), as we observed a significant increase of anti-V1 potency and selectivity. In this study we described the synthesis and some pharmacological properties of ten new analogues of arginine vasopressin (AVP) containing conformationally constrained dipeptide fragment in the N-terminal part of their molecules. We replaced amino acid residues in positions 2 and 3 of AVP and some of its agonistic analogues with –Phe-Phe and D-Phe-D-Phe. All new peptides were tested for vasopressor and antidiuretic activities. Four peptides, having –Phe-Phe , with pA2 values ranging from 5.96 to 7.21 turned out to be weak or moderately potent V1a antagonists. As far as the peptides designed by D-Phe-D-Phe substitution are concerned, this modification alone, or in combination with other changes, resulted in complete removal of all activities checked. Our results supplied new information about structure – activity relationship of AVP analogues. As some of these are unexpected, it points to the need for caution when extrapolating previously known effects of modifications used to analogues having conformationally constrained fragments in their molecules.

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INFLUENCE OF ENANTIOMERS OF 1-NAPHTHYLALANINE IN POSITION 2 OF VAVP AND DVAVP ON THEIR PHARMACOLOGICAL PROPERTIES

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Previously we reported that the hindering effect caused by the bulky naphthyl moiety of L-1-naphthylalanine (L-1-Nal) or D-1-naphthylalanine (D-1-Nal) in position 2 of arginine vasopressin (AVP) or deamino arginine vasopressin (dAVP), had a significant impact on bioactive conformations of molecules and thus influenced their interaction with V1a, V2 and oxytocic receptors. In this study, we described the synthesis and some pharmacological properties of four new analogues of AVP. Two peptides are substituted in position 2 with L-1-Nal or its D-enantiomer and in position 4 with valine. In the further two compounds we combined the above modifications with deamination, e.g. placement into position 1 of 3-mercaptopropionic acid residue (Mpa). All new peptides were tested for vasopressor and antidiuretic activities. We also estimated the uterotonic activities of these compounds in vitro. All urine samples prior and after peptide administration were analyzed for electrolytes excretion. All analogues are potent oxytocin antagonists. One of them, namely [L-1-Nal2,Val4]AVP, which appears practically not to interact with V1a and V2 receptors, is exceptionally selective oxytocin antagonist which has potential as a tocolytic agent. As far as electrolytes excretion is concerned, we observed for two peptides statistically significant decrease of concentration of Ca2+ , Na+ and K+. All new analogues may constitute valuable tools for studies on the physiological roles of oxytocin. Moreover, our results offer new possibilities in the design of new potent and selective oxytocin blocers.

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THE INFLUENCE OF ENZYMATIC HYDROLYSIS ON FOAMING PROPERTIES OF WHEY PROTEINS

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Another use of whey proteins in food industry is highly requested. One of the best known methods of modifying whey proteins is enzymatic hydrolysis by which is possible to produce a wide range of hydrolysates with desired properties. Enzymatic hydrolysis of whey protein concentrate (WPC) by various proteinases (Alcalase; Flavourzyme; Neutrase; Protamex) obtained from commercial sources was performed. Powdered WPC was diluted on protein concentration 4 % (w/v) and 8 % (w/v) and batch hydrolysis with E/S = 1/100 was performed in stirred reactor. The degree of hydrolysis (DH) was monitored by the OPA method during the course of hydrolysis. Foaming properties of hydrolysates and their molecular mass distribution was investigated. The molecular mass distribution was monitored by SEC. Comparison of separate data obtained was made and relationship between molecular distribution profiles of hydrolysates and the change of their foaming properties characteristic was investigated and discussed. The stability of hydrolysate foam decreased with increasing DH in most cases except the Alcalase hydrolysates which were estimated as the most suitable for subseqent use in food industry from the view of technological parameters.

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NPY AND ITS [D-TRP34] ANALOG: CHARACTERIZATION BY 2D NMR AND FLUORESCENCE

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Neuropeptide Y (NPY) family of hormones exhibits a wide spectrum of central and peripheral activities mediated by six G-protein coupled receptor subtypes denoted as Y1,Y2,Y3,Y4,Y5, and Y6. We have shown that a single substitution of NPY with D-Trp at position 34 imparts Y5 selectivity and this analog, [D-Trp34] NPY, on central administration can mimick the orexigenic activity of NPY in rats (Peptides 21:393;2000). To investigate the structural changes that imparted Y5 selectivity, we have compared the solution structures of [D-Trp34] NPY and NPY using 600 MHZ NMR. 2D NMR studies of NPY and its analog, [D-Trp34] NPY in DMSO and H2O were performed on a Varian 600 MHz. The sequential assignments for NPY and its analog were derived from COSY and TOCSY. These sequential assignments were used to identify cross peaks in the NOESY of NPY and its analog. NOE distances were used as a restraint in molecular modeling of the two polypeptides. Fluorescence studies to observe energy transfer from Tyr to Trp was performed which provided a center to center distance between Trp34 and Tyr's. Initial model was built using X-ray derived structure of avian pancreatic polypeptides (PDB code: 1PPT). The restraint from fluorescence and NOE data was used in molecular dynamics simulation to derive the final structure of NPY and its analog. GROMACS was used to derive the structure. The derived model was also used for docking with Y receptors to derive the specific binding of [D-Trp34] with Y5 receptor.

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BIO-FUNCTIONNALIZATION OF MICRO AND NANOPARTICLES

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The field of biomaterials has been the subject of important researches in the last few decades, and developments in scopes such as biocatalysts, biosensors, and diagnostics are in progress. We were interested in the development of methods for the functionnalisation of silica microparticles and zeolites nanoparticles for biological applications. Nanosized microporous zeolite crystals could provide additional interesting properties and could be used as host for small drug molecules or contrast agents. We present here the syntheses of semicarbazide functionalised silicas. These materials could allow the chemoselective anchoring of peptides modified by alpha-oxo aldehyde moieties. The reactivity, the selective bonding and the non specific interactions of the modified silicas with chemical molecules or peptides were investigated. Functionalized colloidal zeolite nanoparticles have been prepared by grafting organosilane or phosphonate reagents at their surface. Further complex functionalization with various bio-molecules or proteins can be accomplished by appropriate chemical reaction with these grafted functions (primary amino groups or semicarbazide). The present study reports immobilization of Cytochrome C or biotin at the surface of the zeolite nanoparticles. Studies of the nanoparticles reactivity with functionalized peptides are also in progress. The obtained colloidal suspensions have been characterized by complementary techniques providing information on the size distribution, morphology and porosity of the particles. These results showed that microporous zeolite-type nanoparticles are good candidates for biologically or pharmaceutically functionalized systems that could find applications, for example, in immunodiagnostics and drug delivery.

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GALLIUM-LABELED DOTA-ALPHA-MSH ANALOGS FOR PET IMAGING OF MELANOMA METASTASES

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As both melanotic and amelanotic melanomas overexpress receptors for α-melanocyte-stimulating hormone (α-MSH, receptor name: MC1R), radiolabeled α-MSH analogs are potential candidates for melanoma diagnosis and therapy. The aim of this study was to develop positron emitter-labeled α-MSH analogs suitable for PET imaging of melanoma metastases which will serve as leads for future clinical application of MC1R-mediated internal radiotherapy of these tumors using alpha-, beta- or Auger-electron emitters. Several short linear α-MSH analog, such as [Nle4, Asp5, D-Phe7]-α- MSH4-11 (NAPamide), were conjugated to the metal chelator DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) to enable radiometal incorporation. In a structure-activity study, the peptides were first labeled with 111In, 67Ga and the short-lived positron-emitter 68Ga and then evaluated in vitro for MC1R binding and bioactivity using melanoma cells, followed by in vivo testing for tumor selectivity using mouse models of primary and metastatic melanoma. Biodistribution experiments, PET imaging studies and autoradiography of tissue sections from organs colonized with melanoma metastases demonstrated that several of the new DOTA-MSH analogs led to higher tumor and lower kidney uptake than any of the previously published peptides, making it possible now to start a clinical evaluation. The selectivity of DOTA-MSH targeting was also demonstrated by visualizing the skin primary melanomas as well as lung and liver melanoma metastases with tissue section autoradiographs after systemic injection of [67Ga]DOTA-NAPamide and by PET imaging studies using [68Ga]DOTA-NAPamide. In conclusion, radiolabeled MSH peptides have now become available with which melanoma metastases can be specifically targeted.

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THE STRUCTURE AND SYNTHETIC CAPABILITIES OF A SMALL CATALYTIC PEPTIDE FORMED UNDER PREBIOTIC CONDITIONS BY A SUBSTRATE-DIRECTED MECHANISM

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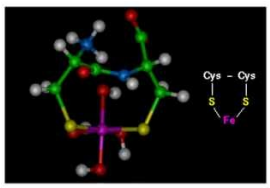
Life depends on biocatalysts – the enzymes, which perform and regulate all biochemical reactions in the living cells. During the prebiotic era, when the complex DNA/RNA genetic system and the ribosomal machinery were not yet available, catalysts, essential for the evolution of life, should have consisted of much simpler molecules, formed by simple chemical reactions. We have shown experimentally that substrate-templating could serve as a mechanism for synthesis of catalytic peptides by the formation of a catalytic metallo-dipeptide, Cys2-Fe+2, around the substrate o-nitrophenyl galactopyranoside (ONPG)(Kochavi et al, J. Mol. Evol. 45, 342-351, 1997). This dipeptide was capable of catalyzing the hydrolysis of ONPG at a specific activity lower only 1000 fold than that of beta galactosidase. In the following work we have examined the structure of the catalyst and its complex with ONPG by molecular modeling techniques and proposed the mechanism of the substrate-dependent formation of the catalyst and its mode of action. In addition, we have demonstrated the ability of Cys2-Fe+2, in its matrix-bound form, to catalyze the formation of ONPG from D-galactose and o-nitrophenol under anhydrous conditions.



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ANALOGUES OF A POTENT OXYTOCIN ANTAGONIST HAVING A UREIDO GROUP IN THE AMINO ACID SIDE CHAIN IN POSITION 4 OR 5

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The oxytocin analogue (Cyclo S1-S6) (S)Pmp-D-Trp-Ile-Gln-Asn-Cys-Pro-Arg-Gly-NH_2, PA, in which (S)Pmp is ß,ß-(3-thiapentamethylene)-ß-mercaptopropionic acid, is a potent antagonist of oxytocin uterotonic action. In preliminary studies, position 4 of PA was substituted with citrulline (Cit), or Orn(Carb) in which Carb is carbamoyl. In the rat uterotonic in vitro assay, [Cit4]PA, had uterotonic antagonistic activity unchanged from that in PA. However, when the same modification was performed in position 5, [Cit5]PA showed a drop in antagonist potency more than ten times that of PA. The analogue [Pen6]PA, which is a very highly potent oxytocin antagonist with potential usefulness in the control of preterm labor was similarly modified. Gln in position 4 was substituted with Lys, Orn, Dab and Dap, and the side chain amino groups were carbamoylated, which resulted in the Lys(Carb)4, Cit4, Dab(Carb)4 and Dap(Carb)4 analogues. In the case of position 5, Asn was substituted with Orn, Dab and Dap and the side chain amino groups were again carbamoylated to the respective, Cit5, Dab(Carb)5 and Dap(Carb)5 analogues. The new analogues were tested for their potency in the uterotonic in vitro test and the most potent antagonists were also tested for their pressor and antidiuretic activities. In general, our biological studies indicate that for some of the substituents at position 4, the introduction of the carbamoyl group is accompanied with substantial retention of antagonistic potency. For the substituents at position 5, there was a more significant loss of potency. Potency and specificity of these analogues in these bioassays will be discussed.

P475

BICYCLIC ANALOGUES OF A POTENT OXYTOCIN ANTAGONIST

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The oxytocin analogue (Cyclo S1-S6) (S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Arg-Gly-NH_2, (or ANTAG), in which (S)Pmp is ß,ß-(3-thiapentamethylene)-ß-mercaptopropionic acid, is a potent antagonist of oxytocin in the in vitro uterotonic assay, and a potential inhibitor of preterm labor. We have previously reported bicyclo analogues (cyclo 1-9)[(HN)Pmp1, Gly9]-ANTAG in which (HN)Pmp is ß,ß-(3-iminopentamethylene)-ß-mercaptopropionic acid and (Cyclo 4-9)[Lys4, Gly9]ANTAG, both of which were very potent in the uterotonic in vitro assay and also showed increased selectivity. We report the synthesis and biological properties of other bicyclic derivatives, including: cyclo 1-4 and cyclo 1-5 lactams, as well as other bicyclic lactams involving position 4 and 5 and the tail end of ANTAG, in attempts to delineate some of the steric requirements for the design of more potent and specific antagonists of oxytocin suitable for the inhibition of preterm labor and for studies on receptor specificity. The biological potency was determined in the rat uterotonic in vitro assay, and in the case of the most potent antagonists, we also tested their activities in the rat pressor and antidiuretic tests. Potency and specificity of these analogues in these bioassays will be discussed.

P476

BETA-TURN AND 3-10-HELICAL PEPTIDES AS TEMPLATES FOR ENANTIOSELECTIVE CATALYSIS: ASYMMETRIC ACYLATION OF ALCOHOLS

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In a recent series of papers S. J. Miller and coworkers were able to show that His(p-Me)-based, terminally protected peptides are potent catalysts of the asymmetric acyl transfer reaction, useful for the kinetic resolution of alcohols. In a structure supporting solvent one of the most active compounds, an Aib-containing tetrapeptide, is folded in a doubly intramolecularly H-bonded beta-hairpin motif incorporating a type-II' beta-turn conformation. In this work we have expanded the study of the Miller tetrapeptide by examining a set of analogues and shorter sequences (dipeptide amides), characterized by chiral C-alpha-tetrasubstituted alpha-amino acids [Iva, (alphaMe)Val, (alphaMe)Phe, (alphaMe)Phg] of diverging bulkiness and optical configuration. Peptide synthesis in solution, conformational analysis by FT-IR absorption and 1H NMR techniques, and screening of catalytic activity as well have been performed. Our results confirm the close relationship between the beta-hairpin 3D-structure and the catalytic activity of the peptides. A tetrapeptide analogue [Boc-L-His(p-Me)-D-Pro-L-(alphaMe)Val-L-Phe-OMe], slightly more selective than the Miller compound, has been found. However, the terminally protected, industrially more appealing, dipeptide amides are poorly effective.

P477

THE STRUCTURE-ACTIVITY RELATIONSHIP OF DMT-NH-X ON OPIOID RECEPTOR AFFINITY

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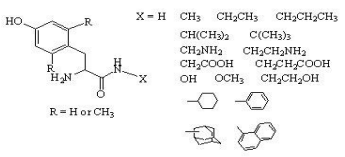
2',6'-Dimethyl-L-tyrosine (Dmt) plays a very important role in the development of opioid receptor ligands. All of the opioid ligands containing Dmt have higher binding affinity to opioid receptors and more potent bioactivity than the corresponding Tyr1 opioid ligands. We found that H-Dmt-NH_2 exhibited moderate binding affinity to the mu-receptor (Ki = 112 nM); however, H-Dmt-OH did not bind to opioid receptors indicating that this amino acid (Dmt) represented the smallest component of an opioid ligand that interacts with opioid receptors. To explore novel opioid ligands based on only the Dmt residue, we modified the carboxylic group with various amine, aliphatic, aromatic or saturated ring substituents (Fig. 1). In this presentation, we present data on the opioid receptor binding profiles of a series of Tyr-NH-X and Dmt-NH-X compounds. Fig. 1 Structures of Dmt-NH-X compounds.



P478

GLYCOGEN PHOSPHORYLASE INHIBITORS AS POTENTIAL DRUGS FOR THE TREATMENT OF TYPE 2 DIABETES

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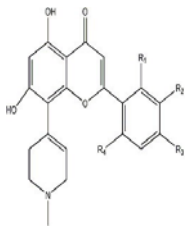
Inhibitors of Glycogen phosphorylase (GP) have been proposed as a therapeutic strategy for improving glycaemic control in type 2 diabetes mellitus[1,2]. In this work we wish to extend our knowledge on molecular recognition of small molecules by using the inhibitor flavopiridol as scaffold. Flavopiridol binds at the inhibitor site of GP (with a Ki value of 1 mM) and exhibits synergistic inhibition with glucose[3]. It is intercalated between the two aromatic residues Phe285 and Tyr613 and its ring system forms several contacts with GP. Here, we describe a 5 step synthetic procedure for 8 potential inhibitors of GP, analogues of flavopiridol, where the phenyl ring has been decorated with various substituents and the hydroxyl group of the piperidinyl ring is not present. NMR, mass spectrometry, kinetic and crystallographic data will be presented. [1]Treadway, J.L., Mendys, P. & Hoover, D.J. (2001). Expert. Opin. Investig. Drugs 10, 439-454. [2]Oikonomakos, N.G. (2002). Curr. Protein Pept. Sci. 3, 561-586. [3]Oikonomakos, N.G., Schnier, J.B., Zographos, S.E., Skamnaki, V.T., Tsitsanou, K.E. & Johnson, L.N. (2000). J Biol. Chem. 275, 34566-34573.



i. R1=Cl, R2=R3=R4=H; ii. R2=Cl, R1=R3=R4=H;
iii. R3=Cl, R2=R1=R4=H; iv. R1=F, R2=R3=R4=H;
v. R1=Br, R2=R3=R4=H; vi. R1=I, R2=R3=R4=H;
vii. R1= R4=Cl, R2=R3=H; viii. R1=R2=R3=R4=H

P479

COMBINATION CANCER CHEMOTHERAPY WITH A SINGLE AGENT: BRADYKININ PEPTIDE ANTAGONISTS AND THEIR MIMETICS

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Lung and prostate cancers are major health problems worldwide. Treatments with standard chemotherapy agents are relatively ineffective. Combination chemotherapy gives better treatment than a single agent because the drugs can inhibit the cancer in different pathways, but new therapeutic agents are needed for the treatment of both tumor types. Our bradykinin (BK) antagonists offer advantages of combination therapy in one compound. These promising multitargeted anticancer compounds selectively stimulate apoptosis in cancers and also inhibit both angiogenesis and matrix metalloprotease (MMP) action in treated tumors in nude mice. Our highly potent, metabolism-resistant bradykinin antagonist peptide dimer, B9870 [SUIM-(DArg-Arg-Pro-Hyp-Gly-Igl-Ser-DIgl-Oic-Arg)] (Igl = a-(2-indanyl)glycine; Oic = octahydroindole-2-carboxylic acid) and its mimetic, BKM-570 [2,3,4,5,6-pentafluorocinnamoyl-(O-2,6-dichlorobenzyl)-L-tyrosine-N-(2,2,6,6-tet ramethyl-4-piperidyl)amide] are superior to the widely used but toxic chemotherapeutic drugs cisplatin and taxotere [1-3]. In certain combinations they act synergistically with standard anti-cancer drugs. Due to its structure and biological activity, BKM-570 is an attractive lead compound for derivatization and evaluation for lung and prostate cancer drugs. Syntheses and structure-activity relationships of new analogs will be discussed. These compounds show an exciting new approach to combined cancer therapy. (Supported by grants HL-26284 and CA78154 from the U.S. NIH.) [1] Gera, L., Chan, D.C., Helfrich, B., Bunn, P.A. Jr., York, E.J. and Stewart, J.M., In 'Peptides 2000'. Edited by J. Martinez and J.-A. Fehrentz. EDK, Paris, 2001, pp. 637-638. [2] Chan, D.C., Gera, L., Stewart, J.M., Helfrich, B., Zhao, et al., Clin. Cancer Res., 8: 1280-1287, 2002. [3] Stewart, J.M. Curr. Pharmaceut. Design, 9: 2036-2042, 2003.

P480

MECHANISM OF BASE EXCISION DNA REPAIR AS REVEALED FROM THE STRUCTURE OF ENDONUCLEASE-VIII/DNA COVALENT INTERMEDIATE MECHANISM OF BASE EXCISION DNA REPAIR AS REVEALED FROM THE STRUCTURE OF ENDONUCLEASE-VIII/DNA COVALENT INTERMEDIATE

G. Golan[1], D.O. Zharkov[2], R. Gilboa[1], A.S. Fernandes[2], S.E. Gerchman[3], J.H. Kycia[3], R.A. Riege[2], A.P. Grollman[2], G. Shoham[1]

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Endonuclease-VIII of E. coli is a DNA repair enzyme that excises oxidized pyrimidines from DNA. EndoVIII shares significant sequence homology with E. coli Fpg, a DNA repair enzyme that excises oxidized purines from DNA. The catalytic mechanism of EndoVIII and Fpg is thought to be similar, involving base removal followed by two sequential ?-elimination steps. EndoVIII has close human homologs, making the structural and biochemical analysis of this enzyme critical for the understanding of cancer-related DNA damage processes and their potential prevention. We have recently determined the 3D structure of EndoVIII covalently crosslinked to DNA. In the resulting structure of this trapped reaction intermediate Pro1 is covalently bound to the DNA via the sugar of the damaged base, revealing for the first time the detailed interactions of EndoVIII with damaged DNA. EndoVIII consists of two domains, connected by a short flexible polypeptide. The space between the two domains creates the DNA-binding cleft. Two DNA binding motifs, the Helix-2turn-Helix and the Zinc-finger motifs, contribute to non-specific binding of DNA by EndoVIII. The DNA in the complex is kinked, and the deoxyribitol moiety is everted from the duplex into an active site. Leu70 fills the resulting space in the bound helical DNA. Two conserved glutamic acid residues, Glu2 and Glu5, are suggested to be involved in protonation of the heterocyclic oxygen. Molecular modeling, analysis of amino acid conservation and site-specific mutagenesis of critical amino acids suggest a site for recognition of the damaged base and form the basis for a proposed mechanism.

P481

STRUCTURAL EVIDENCE FOR SIGNIFICANT INTER-DOMAIN FLEXIBILITY OF THE DNA REPAIR ENZYME ENDONUCLEASE-VIII

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Endonuclease-VIII of E. coli is a DNA repair enzyme that excises oxidized pyrimidines from DNA. The 3D structure of EndoVIII in its complex with DNA has been recently determined, indicating the amino acids important for DNA binding and catalytic activity, and suggesting a general catalytic mechanism. Nevertheless, the structure of the free enzyme has not been analyzed until recently. Based on information from the DNA-bound enzyme structure, several site-specific mutants have been produced and tested for activity and specificity against substrates. We have now determined the 2.8 Å resolution structure of the DNA-free EndoVIII (WT), as well as the structure of two less-reactive mutants, EndoVIII-E2A (2.3 Å res.) and EndoVIII-R252A (2.1 Å res.). The three structures of the free enzyme are practically identical, demonstrating that the mutations did not affect the overall structure of the protein in its DNA-free form. All three structures show, however, a significant conformational change, compared to the protein structure in its Enzyme-DNA complex, reflecting an inter-domain rotation of about 50° between the free and the DNA-bound enzyme. This conformational transformation involves the exchange of specific sets of hydrogen bonds in the loop connecting the two domains, resulting in “open” (DNA-free) and “closed” (DNA-bound) forms of the enzyme. Such inter-domain flexibility has not been reported for any other base excision repair enzyme, and may present the first evidence for a DNA-induced conformational change of a DNA repair enzyme. These results may be used to simulate non-specific enzyme-DNA binding, the first step necessary for DNA damage detection.

P482

UROTENSIN-II PEPTIDE AGONISTS AND ANTAGONISTS: A COMPREHENSIVE SAR STUDY

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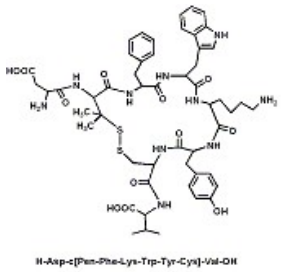
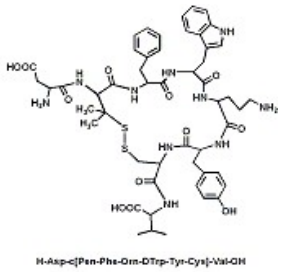
Urotensin II (hU-II), a potent vasoconstrictor, is found in diverse species, including human. U-II C-terminal cyclic heptapeptide portion (CFWKYCV), which is essential for the biological activity, has been highly conserved in evolution from fish to mammalians. Several biological studies indicate that hU-II is the most potent mammalian peptide vasoconstrictor reported to date, and it appears to be involved in the regulation of cardiovascular homeostasis and pathology. Recently, we have reported the first superagonist (P5U) and full antagonist (UrantideTM) at UT receptor (Figure 1) [1,2]. These compounds represent valid tools to elucidate the physiological roles played by U-II. In this communication we report an extensive structure-activity study aimed to shed light on the most important substructural features responsible for agonist/antagonist activity. The study here reported will be an important guide in effort to design therapeutically useful Urotensin analogues. [1]Grieco, P.; Carotenuto, A.; Campiglia, P.; Zampelli, E.; Patacchini, R.; Maggi, C.A.; Novellino, E.; Rovero, P. J. Med. Chem., 45, 4391-4394, (2002). [2]Patacchini, R.; Santicioli, P.; Grieco, P.; Rovero, P.; Novellino, E.; Maggi, C.A. Br. J. Pharmacology, 140, 1155-1158, (2003).



H-Asp-c[Pen-Phe-Lys-Trp-Tyr-Cys]-Val-OH

P5U



H-Asp-c[Pen-Phe-Orn-DTrp-Tyr-Cys]-Val-OH

URANTIDE

P483

THE STEREOSELECTIVITY OF COGNITION-ACTIVATING EFFECT EXERTED BY PGLU-ASN-NH_2 - N-END DIPEPTIDE FRAGMENT OF THE MAJOR VASOPRESSIN METABOLITE

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Nonapeptide vasopressin is known to control both peripheral and central functions, moreover, the regulatory role vasopressin plays in CNS is expressed as its influence upon learning and memory. Vasopressin proteolysis in brain results in the formation of hexapeptide pGlu-Asn-Cyt-Pro-Arg-Gly-NH_2 which produces a potent and selective action upon memory [Burbach J.P.H. et al., 1983]. As we have reported previously [Gudasheva T.A. et al., 1985],certain pyroglutamyl-containing dipeptides which were designed as analogues of the classical nootropic Piracetam, produce the specific cognition-enhancing activity when used at low doses. We have suggested the key role of the N-end pyroglutamyl-containing dipeptide fragment plays in the main vasopressin metabolite action on memory. Developing this idea we have the diastereomeres of pGlu-Asn-NH_2 were synthesized and assessed for the mnestic activity in a passive avoidance step-through paradigm in rats. The dipeptides were synthesized using the method of activated esters, their diastereomeric purity was confirmed by NMR spectroscopy. Studying the effect the synthesized diastereomeres produce on learning and memory it was shown that the diastereomere L-pGlu-L-Asn-NH_2 with the naturally occurring configuration used at a dose of 0.1 mg/kg intraperitoneally improved learning by 44% as compared to maximal possible effect. Diastereomere L-pGlu-D-Asn-NH_2 used at the same dosage was found to reduce learning by 36%. The diastereomere D-pGlu-L-Asn-NH_2 produced no activity. The dipeptide pGlu-Asn-NH_2 was shown to improve stereoselectively learning and memory, thus indicating the valuable role that N-end dipeptide fragment of the main metabolite of vassopressin plays in the cognition enhancing effects of the latter.

P484

A NOVEL GENERATION OF PEPTIDIC BRADYKININ B1 RECEPTOR ANTAGONIST BEARING LIPIDIC MOIETY.

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The therapeutic potential for the use of a selective bradykinin (BK) B1 receptor antagonist has been supported by recent studies demonstrating that activation of inducible B1 receptor subtypes plays an important role in pain and inflammation. We have previously synthesized a series of potent and selective peptidic BKB1 antagonists, which included R-715 (AcLys[D-ß-Nal7, Ile8]desArg9BK). We used that peptide as a scaffold to prepare a new generation of BKB1 receptor antagonists bearing short lipidic acyl side chains (C6-C12) at the N-terminal position. This modification brought important changes in both physical and pharmacologic properties of the previous generation of antagonists. Herein, we will describe the preparation of such BKB1 receptor antagonists and discuss the impact of various chain lengths on the biological activity in various in vitro pharmacological preparations.

P485

AN OVERVIEW OF ELECTRON TRANSFER BETWEEN HEME PROTEINS OF CYTOCHROME C SUPER FAMILY: COMPARISON BETWEEN THEORY AND EXPERIMENTS

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Cytochrome c, an ancient protein, responsible for transferring individual electrons in the cellular machinery, gathering electrons from cytochrome bc1 complex and delivering it to cyt-c oxidase complex keeping the entire work of energy production running smoothly. Functional role of the prosthetic group along with the electron-electron transfer property, is of crucial importance for technological applications of cytochrome c as a biosensor has been the raison d'être for our study. Cyt-c', used to detect NO, was employed as a probe for the biosensor design along with cyt c (from many organisms like yeast, horse heart) was chosen as the probe for our investigation. To ransack this process and building up the protein complex we used protein docking software's like GRAMM and HEX with further evaluation by GROMACS potential. The electron transfer mechanism in the protein complexes were evaluated on the basis of their hydrophobic packing, proximity of the hemes and gain of hydrogen in place of the water and lipid molecules, finally calculating the free energy to procure the best stable model. MOLMOL was used to calculate the electrostatic potential around the heme in the presence and absence of other proteins and also varying the ionic state of Fe. In parallel, we conducted experiments on current v/s voltage behavior of the complexes on gold-plated surfaces using cyclic voltagramms. The electrochemical behavior of the complex obtained from cyclic voltagramms was compared with the theoretical value that helps us design better mediators for potential applications in the field of bio-sensors.

P486

STARBURST PEPTIDES CONTAINING HIV-1 TAT (48-60): POSSIBILITY OF THEIR USE AS CARRIERS FOR DNA DELIVERY INTO CELL

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The improvement of nonviral gene transfer systems is overwhelmingly important for the development of gene therapy. Among the carriers of DNA, basic peptides containing multiple functional domains are most promising. It is due to effective condensation and protection of DNA from enzymatic degradation, cell targeting and nuclear localisation of required gene. HIV-1 Tat (48-60) fragment (GRKKRRQRRRPPQ) is known to be able to penetrate membranes of cell and to transfer into the nucleus. We synthesized dimer of HIV-1 Tat (48-60) N2 ([GRKKRRQRRRPPQ]2K-Ahx-C(Acm)) using alpha- and epsilon-amino groups of lysine by classical SPPS approach. This dimer was modified with ligand GRGDS for specific interaction of peptide with integrins on the surface of cell (RN2, [GRGDSGRKKRRQRRRPPQ]2K-Ahx-C(Acm)). N2 as well as its analogue RN2 were used for the synthesis of starburst tetramer by disulfide bond formation between C-terminal residues of cysteine (N4 {[GRKKRRQRRRPPQ]2K-Ahx-C}2 and RN4 {[GRGDSGRKKRRQRRRPPQ]2K-Ahx-C}2 respectively). CD spectra showed changes of secondary structure of the peptides under transition dimer – tetramer. The conformation of DNA changed in presence of the peptides as well. All the peptides were able to protect DNA from the action of DNase. Hemolytic assays revealed the absence of influence of the peptides on the membranes of erythrocites, unlike more hydrophobic lysine peptides modified with capric acid [1]. Particularities of synthesis of peptides and their properties will be discussed. Reference: [1] Gurjanov I., Korol'kov V., Vlasov G., Kiselev A., Lesina E., Baranov V., Avdeeva E., Vorobjev V. Peptides 2002. Proc. 27th EPS. P. 764-765

P487

LIGAND BINDING TO STRUCTURALLY RELATED SH2 DOMAINS: A COMPUTATIONAL AND BIOLOGICAL STUDY

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The PTPases SHP-1 and SHP-2 are cytosolic proteins which contain one catalytic subunit (PTP domain) and two SH2 domains (tandem SH2 domains). Whereas SHP-1 is mainly expressed in hematopoietic cells and acts as negative regulator in different cell signaling pathways, the ubiquitous SHP-2 may act as a negative regulator for receptor function or as a positive effector for downstream signaling. According to structural similarities their SH2 domains have been placed into a distinct subgroup within SH2 domain-containing proteins [1]. The N-SH2 domains play an important role for the catalytic activity of both enzymes. In the inactive state the N-SH2 domain blocks the PTP domain. Upon phosphotyrosine (pY) peptide binding the N-terminal SH2 domain undergoes a conformational change thereby releasing the active site of the catalytic domain which subsequently is accessible for pY-substrates. Using the consensus sequence of the N-SH2 domain of SHP-1 [2] and a natural high affinity ligand from the RTK Ros (pY2267) [3] we have derived constrained linear and differentially cyclized peptides which have been designed and preselected by means of computational studies. The synthesized ligands have been tested for their ability to activate SHP-1 and SHP-2 using p-nitrophenylphosphate as substrate. Furthermore, we determined the binding affinities using surface plasmon resonance and CD spectroscopy in order to further characterize the recognition requirements of these structurally related SH2 domains. [1] Songyang, Z., et al. (1993) Cell, 72, 767-778. [2] Beebe, K.D., et al. (2000) Biochemistry, 39, 13251-13260. [3] Keilhack, H., et al. (2001) J. Cell Biol., 152, 325-334.

P488

INTESTINAL PEPTIDE TRANSPORTER PEPT1: NEW ASPECTS OF THE TRANSPORTER-LIGAND INTERACTION

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Due to its broad substrate specificity, the intestinal peptide transporter PEPT1 is capable of transporting small dietary peptides, modified amino acids and dipeptides as well as various drugs and prodrugs. As the number of new peptide and peptidomimetic drugs are rapidly increasing, the peptide transport system has gained attention as a possible drug delivery system for small peptide-like compounds. However, rational design of peptidomimetic drugs/prodrugs requires a precise knowledge of the structural requirements of a substrate in order to understand its interaction with the transporter. Up to now, the three-dimensional structure of PEPT1 has not yet been elucidated. Therefore, such information has been obtained from extensive structure-affinity/transport studies using different experimental methods and model systems. From these investigations a great number of generally valid results concerning the structural features required for the interaction of a ligand with the intestinal peptide transporter PEPT1 have been obtained. Nevertheless, some crucial points need to be clarified. Based on previous results we have determined the affinities of a series of supplementary modified amino acid derivatives and dipeptides for PEPT1, containing w-amino carboxylic acids or derivatives of them with or without additional charged or uncharged side-chain residues. These new substrate-affinity studies supported by molecular modelling investigations might provide more detailed insight into specific ligand-transporter interactions relevant for future rational drug design.

P489

STRUCTURE-ACTIVITY RELATIONSHIP OF A NEW ANTI-MICROTUBULE DIKETOPIPERAZINE PHENYLAHISTIN AND ITS DERIVATIVES

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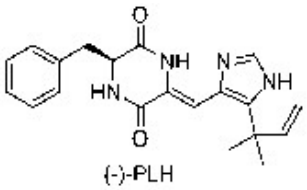
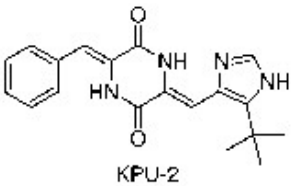
The introduction of anti-microtubule agents such as taxanes and vinca alkaloids has revolutionized the treatment of cancer and improved the survival time of patients. However, after long-term treatment, tumors become resistant to these drugs. Hence, there is a significant need for developing novel anti-microtubule agents. One of such candidates is a natural cyclic-dipeptide, phenylahistin (PLH), consisting of Phe and unique isoprenylated dehydroHis, having a colchicine-like anti-microtubule activity. Based on the observations that olefin structure at the dimethylallyl group could be reduced without decreasing the activity and the branched geminal dimethyl group was important for the activity [1], and the information that α,β-dehydrophenylalanine derivative increased the activity [2], we synthesized about 100 derivatives, evaluated their biological activities and studied the structure-activity relationship. From these studies, a highly potent cytotoxic derivative KPU-2 (NPI-2358) with an IC50 value of ~10 nM for HT-29 cells and its derivatives with a small substituent on the phenyl ring were developed. The active conformation required for the activity was also studied. These highly potent diketopiperazines would be developed as new cancer drugs with advantages in resistance, toxicology and medical economics of current drugs. References [1] Kanoh, K., Kohno, S., Katada, J., Takahashi, J., Uno, I., Hayashi, Y. Bioorg. Med. Chem. 7, 1451 (1999). [2]Kanzaki, H., Yanagisawa, S., Kanoh, K., Nitoda, T. J. Antibiotics 55, 1042 (2002).



{-}-PLH



KPU-2

P490

CONFORMATIONAL AND INHIBITORY ACTIVITY CHANGE OF ELASTASE INHIBITOR FROM SEA ANEMONE UPON EXCHANGE OF A CYS4-CYS34 BOND FOR A CYS6-CYS31 BOND

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An elastase specific inhibitor from the sea anemone(AEI) strongly inhibits porcine pancreatic elastase(PPE). AEI has three disulfide bridges in a molecule composed of 48 amino acid residues and is classified as a member of "non-classical" Kazal-type inhibitors by the arrangement of the disulfide bridges pattern. Most of "non-classical" Kazal-type inhibitors have a cystine-stabilized alpha-helical (CSH) motif in the sequence but AEI has not. Thus, we chemically synthesized the disulfide variant of AEI, Cys4Ala/Leu6Cys/Gly31Cys/Cys34Ala, having the CSH motif upon exchange of a Cys4-Cys34 bond for a Cys6-Cys31 bond. Then, we examined the effect of exchange of the disulfide bond on the conformation and the inhibitory activity of AEI. The disulfide variant lost most of the inhibitory activity against PPE. The solution structure of this variant was then determined by two-dimensional nuclear magnetic resonance methods, which showed that it has the characteristic scaffold of a typical Kazal-type inhibitor such as OMSVP3 consisting of a central alpha-helix and a three-stranded antiparallel beta-sheet. However, the N-terminal loop of the variant has drawn the central helix to form the newly introduced Cys6-Cys31 bond. In the previous study, we reported that the disulfide variant of OMSVP3 having a CSH motif lost most of its inhibitory activity toward PPE, accompanying with some conformational change in the N-terminal loop (1). These results suggest that the N-terminal loop conformation of this kind of inhibitor may closely correlate to the inhibitory activity against PPE. [1]Hemmi, H. et al. Biochemistry 42, 2524-2534 (2003).

P491

INTERACTIONS OF HANTAVIRUS ENVELOPE GLYCOPROTEIN DOMAINS AND TRANSMEMBRANE TAILS

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The rodent carried hantaviruses cause hemorrhagic fever with renal syndrome (HFRS) in the Eurasia and hantavirus pulmonary syndrome (HPS) in the Americas. We use hantaviruses as a model, enveloped/membranous virus for identification of protein domains as antiviral and protective immunity or immunotherapy targets. We have earlier used random phage-displayed peptide libraries and identified peptides, which inhibit virus infection. The peptides that bound to a virus neutralization site inhibited infection up to 60 % and targeted a conformational integrin-binding domain. We are currently developing live virus biopanning strategies to integrin domains. In the present study we have also analysed interactions of glycoprotein transmembrane tails with the viral nucleocapsid. The virus envelope protein domains of interest include MMP-domain mimicry, Zinc-finger type fold and an ITAM motif.

P492

MODEL CYCLIC PEPTIDES TO INVESTIGATE THE TRP-MEDIATED PHOTOREDUCTION OF DISULFIDE BONDS IN PROTEINS

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Irradiation with ultraviolet light may reduce or even abolish the biological activity of proteins. The damage is initiated through photon absorption of the chromophore amino acids, for example the indole part of tryptophan. The structure of goat α-lactalbumin is stabilized by four S-S bridges and they are in close contact with Trp-residues situated at parts of the different parts of the molecule. During our physico-chemical studies of native goat α-lactalbumin we observed structural modification upon its irradiation at 280 or 295 nm. Mass spectroscopic data presented evidence that these conformational changes are caused by cleavage of disulfide bonds [1], however a direct e- transfer from the excited indole part to the disulfide bond can be realized. For evaluating the Trp-mediated photolysis by near-UV light a number of cyclic penta- hexa- and heptapeptides were synthesized by solid phase peptide synthesis to alter the distance between the indole ring of Trp and S atoms of the disulfide bridge. To clarify the role of Trp in the light-induced degradation we synthesized modified sequences, where Trp was changed to Phe and Val. To optimize the ring closure we used several cyclization methods. Present study tries to make a clear distinction between disulfide bond cleavage induced by light absorption of Trp and other less specific light-induced processes. [1]Vanhooren et al, Biochemistry 41, 36, 11035-11043 (2002). The research was supported by OTKA Grant T 037719 (ZS.M.), FWO-Flanders G-0180-03(I. H.) and Reanal Fine Chemical Co.

P493

AMPHIPHILIC PEPTIDE ENGINEERING: CHARGE DELOCALISATION AND THE DESIGN OF NOVEL MASTOPARAN ANALOGUES

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The formation of an amphiphilic helix is a major determinant of the biological activities of the mast cell degranulating tetradecapeptide mastoparan (MP; H-INLKALAALAKKIL-NH_2). To assess the impact of charge delocalisation within the hydrophilic face of MP, we synthesised novel analogues using sequence permutation and arginine-substitution at positions 4, 11 and 12. Comparative bioassays determined cytotoxicity, b-hexoseamindase secretory efficacy and peptide-activated ERK1/2 phosphorylation. The mono-substitution of individual lysine residues with arginine produced differential changes to the indices of cytotoxicity and secretion indicating that these conservative substitutions are compatible with both membrane translocation and the selective binding and activation of intracellular proteins. More profound changes to the predicted hydrophilic face of MP, resulting from the relocation or substitution of additional lysyl residues, enhanced both the cytotoxicity and secretory efficacy of novel peptides. Significantly, the more amphiphilic peptide [Lys5,Lys8,Aib10]MP, including a-aminoisobutyric acid (Aib) to increase the propensity for helix formation, was identified as both the most cytotoxic and the most potent secretagogue of all the peptides compared in this study. Charge delocalisation within the hydrophilic face of MP analogues was also compatible with peptide-induced activation of ERK1/2 phosphorylation. Our data indicate that charge delocalisation is a suitable strategy to engineer more potent analogues of MP that differentially target intracellular proteins.

P494

DESIGN AND SYNTHESIS OF NEW CHOLIC ACID CONTAINING AMPHIPHILES

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Cell membranes inhibit the entry of many classes of biological active highly hydrophilic and/or charged molecules, e.g. proteins, certain peptides, DNA, oligonucleotides. One way to facilitating the transport of compounds across the biological membranes is to synthesize molecules that mimic the structure of umbrella, i.e. molecules that can cover and attached biological active agent and protect it from an incompatible environment. In this work a cholic acid was used to prepare a bi-walled molecular umbrella. The carboxyl groups of the both steroid units were coupled to the terminal amino groups of the derivatized with ethylene diamine or spermidine glutamic acid. Attachment of the biologically active agent to the remaining amino groups of the glutamic acid yields a molecular umbrella. The model compounds containing dipeptides (Tyr-Arg) and enkephalins as shielded biological agents were obtained. Morphological transformations of model membranes (giant unilamellar vesicles) were investigated in order to elucidate mechanisms of permeabilization co-operating on the level of induced reorganization of the membrane.

P495

A NOVEL CIS-PEPTIDE BOND MOTIF INDUCING TYPE VI BETA-TURN. SYNTHESIS AND BIOLOGICAL EVALUATION OF ENKEPHALIN AND MORPHICEPTIN ANALOGUES CONFORMATIONALLY RESTRICTED AT C-TERMINUS

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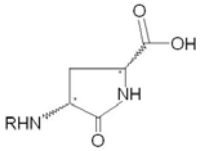
We have published already synthesis and biological evaluation of four analogues of Leu-enkephalin amide, modified in positions 2-3 by four stereoisomers of 4-aminopyroglutamic acid (1, R=H) residues [1]. Our cis-peptide bond motif 1 is a hybrid of glycine and alanine (for R=H). This feature limits its utilization as a replacement for most dipeptide sequences suspected of existing in cis-conformation during receptor recognition. In order to convert compound 1 (R=H) into more valuable tool for probing existence of particular peptide bond in cis-conformation, we have synthesized morphiceptin and Leu-enkephalin analogues [2], containing N-terminal mono-alkylated derivatives of 1 (R=p-benzyloxybenzyl) through reductive alkylation reaction performed on solid support [3]. Following this idea we have attempted the synthesis of analogues with N-benzylated motif (R=benzyl) inserted as a replacement for Phe-Aaa dipeptide unit. Synthetic strategy as well as biological activity of analogues will be discussed. References [1] Kaczmarek, K. et al., Acta Biochim. Pol. 2001, 48, 1159-1163. [2] Kaczmarek, K. et al., Peptides 2002 (Proceedings of the 27th European Peptide Symposium), Edizioni Ziino, Napoli, Italy 2003, p.158. 3.Szardenings, A.K et al., Org. Chem. 1996, 61, 6720-6722.



1

(2S,4R), (2R,4S) - trans
(2S,4S), (2R,4R) - cis

P496

NESTED CHEMICAL LIBRARY OF KINASE INHIBITORS AND PHARMACOPHORE MODELLING

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Kinase inhibitors are in the front line of modern drug research where mostly three technologies are used: HTS of random libraries, 3D design based on X-ray data, and focused libraries around limited number of new cores. In our novel Nested Chemical Library(TM) (NCL) focused libraries around 98 cores are used to generate a pharmacophore model. We have established a unique proprietary kinase inhibitory chemistry with small focused sublibraries around each core. Structures are stored in a big unified SQL database along with measured and calculated physicochemical and ADMET properties. Kinase assay data obtained for the selected compounds from NCL can provide enough biological data for rational computerized design of new analogues based on our Pharmacophore model generating 3DNET4W(TM) QSPAR approach. New analogues are designed on the basis of bioisosteric changes using validated standard reaction schemes. New potential hit molecules are filtered by their ADMET properties, druglikeness and patentability. Thousands of molecular descriptors are calculated for hit compound structures and fed with 3DNT4W(TM) QSPAR program system. The program builds abstract pharmacophore (QSAR) models from validated, significant descriptors selected by sequential or genetic algorithms. Enhanced MLR, PLS, ANN, LLM methods are optional and can be combined with PCA. Pharmacophore models are validated by external validation, (double blind study) the validation set is not known for the program. After about five research cycles with this approach, with a good and reliable biochemical kinase assay in hand we can generate a lead candidate against a particular target molecule.

P497

PROTEOLYTIC STABILITY OF NEUROPEPTIDE Y ANALOGUES WITH HIGH BINDING AFFINITY FOR Y1 AND Y2 RECEPTORS

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It has recently been indicated that there is a putative role of NPY in breast tumours. The expression of the two best-investigated NPY receptor subtypes, Y1 and Y2 in this tissue shows predominant occurrence of the Y1 receptor subtype (Cancer-Research -- Reubi et al. 61(11): 4636). In order to investigate the usefulness of NPY analogues in tumour diagnosis, we determined the proteolytic stability of selected analogues in human blood plasma. NPY analogues were synthesised by Fmoc/t-Bu solid-phase strategy. Prior to the cleavage of peptides from resin, they were labelled with 5(6)-carboxyfluorescein (CF) at the N-terminus while in another set of the same analogues, the CF group was introduced at the side chain of Lys4. For proteolytic studies, micromolar quantities of these CF-NPY analogues were incubated with plasma at 37 C at various time intervals till 24 hours, and digestion of peptides was determined by HPLC. The cleavage products were identified by Maldi-TOF mass spectrometry and characteristic segments were monitored. This analysis showed that full-length [F7,P34]NPY analogues, which show high binding affinities for Y1 receptors are stable in plasma up to 12 hours, while the centrally-truncated analogues, e.g., Ahx(8-20)NPY and Ahx(5-24)NPY, which show high binding affinities for Y2 receptors are only stable up to 2 hours. Furthermore, the data suggest that analogues labelled at N-terminus are more stable compared to their counterparts containing the CF label at Lys4 side chain. Accordingly we conclude that N-terminally modified radiolabelled NPY analogues might have promising characteristics for future applications in nuclear medicine and tumour therapy.

P498

STRUCTURE AND FUNCTION OF RIBOSOME RECYCLING FACTOR

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Ribosome recycling factor with a molecular weight of about twenty thousands plays an essential role in the protein biosynthesis of prokaryote. NMR and X-ray analyses revealed that this molecule has an L-shaped structure consisted of two domains; domain I is in a triple helical bundle structure and domain II is in a β/α/β sandwich structure, which is very similar to that of t-RNA in size and shape. The triple helical bundle is unique because each helical strand in straight stands side by side contrary that in usual helical bundle each chain is twisted each other to form a loose left-handed helix. We investigated the stability of this unique helical bundle. Then we investigated the intra-molecular movement of the molecule focusing on the mutual spatial arrangement of these two domains by NMR and molecular dynamics calculation and showed furthermore that this movement is inevitable to express its biological activity. Binding assay by surface plasmon resonance and affinity chromatogram showed domain I is bound to 50S subunit of ribosome using the cationic cluster formed by three Arg residues on domain I. In order to identify the binding spot on ribosome, cryo-electron microscope analysis and cross-linking experiment on their complex were carried out. Finally we proposed a mechanism of the ribosome recycling in a cycle of peptide synthesis on ribosome in bacterium cell. In the connection of a rational drug design of an antibiotic as an inhibitor of the factor, we will discuss the structure-function relationship of ribosome recycling factor.

P499

SYNTHESIS AND BIOACTIVITY OF PEPTIDES RELATED TO CARNOSINE

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Histidine containing natural dipeptide L-carnosine is active antioxidant agent. However, L-carnosine appeared to be uneffective in vivo, due to the presence specific hydrolytic enzymes such as carnosinase. Therefore, a series of peptides were synthesized in order to confer resistance to enzymatic hydrolysis and also to improve the penetration through membranes. Carnosine analogues containing His residue in position 1 or 2 of peptide chain were prepared (I-VIII): b Ala-His-OMe (I), Boc-b Ala-His-OMe (II), Oleyl-b Ala-His-OH (III), Boc-Pro-His-OMe (IV), His(Bom)-Pro-OMe (V), Boc-His-Tyr-OEt (VI), Z-His-Pro-OMe (VII), cyclo(HisPro) (VIII). Analogues II-VII contain tert-butyloxycarbonyl, benzyloxycarbonyl, benzyloxymethyl and oleyl moieties. C-terminal carboxyl was blocked by alkyl ester derivatives (I, II, IV-VII). The synthesis of histidine containing protected peptides was performed by a classical procedure in solution using the method of activated esters. Peptide IV was used as starting compound for preparing cyclo(His-Pro) (VIII) after deprotection IV and by its cyclization in the presence of 0.1 M acetic acid in i-butanol. The peptides were purified by column chromatography on Silicagel and characterized by amino acid analysis of acid hydrolyzates and by tlc. Preliminary analysis revealed that the peptides prepared posessed antioxidant activities. This work was supported by the Russian Foundation for Basic Research (grant 03-04-49133).

P500

THE RELATIVE ORIENTATION OF THE ARG AND ASP SIDE CHAINS IN RGD PEPTIDES: A KEY CRITERION FOR EVALUATION THE STRUCTURE-ACTIVITY RELATIONSHIP

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The binding of the integrins to the Arg-Gly-Asp (RGD) motif, as the primary recognition sequence of their ligands, have been extensively studied due to the versatility of the integrins and their RGD ligands. Structure-activity studies of RGD-containing peptides and proteins have revealed that the specificity of an integrin for its RGD ligands is strictly correlated to their conformational features.

Three criteria have been proposed for the evaluation the structure-activity relationship: i) the distance between the charged centres, ii) the distance between the Arg Cβ and Asp Cβ atoms and iii) the pseudo-dihedral angle, formed by the Arg Cζ, Arg Cα, Asp Cα and Asp Cγ atoms, defining the orientation of the Arg and Asp side chain. In this study we designed, synthesized and studied the peptides Ac-SRGDVGRAibGK(Ac)AibG-OH (1), Ac-SRGDVGNleAibGK(Ac)AibG-OH (2), Ac-SNleGDVGRAibGK(Ac) AibG-OH (3) and Ac-RAibGDAibGRAibGK(Ac)AibG-OH (4). A comparative conformation-activity study was performed between the linear peptides 1-4 and strongly constrained cyclic (S,S) -CDC- bearing compounds which cover a wide range of platelets aggregation inhibition potency. The fulfillment of the –45º < pseudo-dihedral angle < +45º criterion was proved to be a prerequisite for an RGD compound to exhibit inhibitory activity. Moreover, once this criterion is accomplished, the longer the distance between the charged centres and/or between the Arg and Asp Cβ atoms, the higher is the anti-aggregatory activity.

P501

INFLUENCE OF 1-AMINOCYCLOPENTHANE-1-CARBOXYLIC ACID IN POSITION 2 OR 3 OF AVP AND ITS ANALOGUES, ON THEIR PHARMACOLOGICAL PROPERTIES

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Conformationally constrained analogues of bioactive peptides have awakened growing interest in studies of their structure-activity relationship. Restrictions can be imposed by means of constraints, such as the formation of cyclic structures within the peptide backbone, or the reduction of peptide flexibility by introducing amino acids with limited conformational freedom, which in turn, results in specific orientations of the peptide backbone and its side-chains. It is believed that Tyr2 of arginine vasopressin (AVP) is involved in initiating the pressor response to AVP while, Phe3 seems to play a role in the recognition of this hormone. Recently, we replaced the residues in positions 2 or 3 of AVP and some of its agonistic and antagonistic analogues, with 1-aminocyclohexane-1-carboxylic acid (Acc). We demonstrated that Acc2 modification selectively changed the activities of the analogues, whereas Acc3 modification has been found to be deleterious for interaction with all three neurohypopheseal hormone receptors. Considering all this, we decided to replace with 1-aminocyclopenthane-1-carboxylic acid (Apc) the residues in position 2 or 3 of AVP and some of its agonistic and antagonistic analogues. We decided to check how diminishing of the ring of Acc residue will change the pharmacological properties of analogues. We inserted Apc in order to reduce the flexibility of the peptides by imposing a sterically constrained residue limiting conformational freedom. The proposed modification should also enhance the resistance of the resulting peptides to enzymes. All analogues were tested for pressor, antidiuretic and uterotonic in vitro activities.

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HIGHLY POTENT 1-AMINOCYCLOHEXANE-1-CARBOXYLIC ACID SUBSTITUTED V2 AGONISTS OF ARGININE VASOPRESSIN (AVP)

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The synthesis of arginine vasopressin (AVP) stimulated not only the fields of synthetic chemistry and peptide endocrinology, but also the structure-activity relationship studies of this hormone, and particularly the search for analogues with both high and specific activities. It's believed that Tyr2 of AVP is involved in initiating the pressor response to AVP, while Phe3 seems to play a role in recognition of this hormone. It's well known that deamination is the most effective of the individual changes in AVP which lead to enhanced antidiuretic activity, whereas inversion of configuration Arg8 results in analogues with distinctly increased specificity of antidiuretic action. Recently, we replaced the residues in positions 2 or 3 of AVP and some of its agonistic and antagonistic analogues, with 1-aminocyclohexane-1-carboxylic acid (Acc). Acc2 modification has been shown to selectively modulate the activities of the analogues, whereas Acc3 modification has been found to be deleterious for interaction with all three neurohypopheseal hormone receptors. Bearing all this in mind, we obtained new analogues modified with Acc2 or Acc3. All the peptides were tested for pressor, antidiuretic and uterotonic in vitro activities. Again, Acc2 modification has been shown to selectively modulate the activities of the analogues. Four of the compounds were highly potent antidiuretic agonists, with different pressor and uterotonic activities. On the other hand, the 3-substituted counterparts failed to exhibit any of the activities. Only analogue: [Mpa1,Acc3,Val4,D-Arg8]VP exhibited antidiuretic activity matching that of AVP, yet, unlike AVP, it was fairly selective and inactive in the pressor and uterotonic tests.

P503

STRUCTURE-BASED DESIGN, SOLID PHASE SYNTHESIS AND BIOLOGICAL CHARACTERIZATION OF HUMAN MONOCYTE CHEMOATTRACTANT PROTEIN 1 (MCP-1) AND ITS ANALOGS

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Monocyte chemoattractant protein 1 (MCP-1), a member of the CC family of chemokines, is a 76 amino acid protein, with two disulfide bridges. It has a role in the activation of inflammatory cells (including monocytes, macrophages, fibroblasts and lymphocytes) and has been implicated in disease states including chronic obstructive pulmonary disease, atherosclerosis and oncology. The objective of the work was the design and synthesis of novel analogs of MCP-1 in order to better characterize receptor interactions and as tools to generate monoclonal antibodies to be used as human therapeutics. Based on the known structure of MCP-1, several amino acids were predicted to be distal from the putative receptor-binding domain. Analogs at these positions were designed and synthesized using both single and double coupling FMOC protocols. The folded proteins were purified by reversed phase HPLC and affinity chromatography using an immobilized mouse anti-human MCP-1 monoclonal antibody (mAb). The final products were extensively characterized. Using a panel of six mouse anti-human MCP-1 monoclonal antibodies, synthetic and recombinant human MCP-1 (rhMCP-1) gave identical binding profiles using surface plasmon resonance. Biological characterization was performed in competition radio-ligand binding and chemotaxis assays. In these assays, synthetic MCP-1 and the analogs competed equally well as the recombinant MCP-1 with 125I-rhMCP-1 for binding to the CCR2 receptor on Thp-1 cells. Synthetic MCP-1, the analogs and recombinant MCP-1 were comparable in their ability in inducing Thp-1 chemotaxis. These results demonstrate the utility of chemical synthesis as a complementary strategy to recombinant expression for the preparation of small proteins.

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SYNTHETIC ASTIN ANALOGUES KILL CELL BY AN APOPTOTIC MECHANISM

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Astins, a family of natural antitumor cyclopentapeptides, consist of a 16-membered ring system containing a unique beta,gamma-diclorinated proline [Pro(Cl2)], involved in a peptide bond with a cis conformation and the presence of several uncoded aminoacid residues (1). Although the antineoplastic activity of the natural astins has been screened in vitro and in vivo, the mechanism of action has never been thoroughly investigated. We have prepared new astin-related cyclopeptides, differing from the natural product because of the presence of some non-proteinogenic aminoacid residues, such as Aib, Abu, -S(beta 3)-hPhe and the presence of the peptide bond surrogate (-SO_2-NH-) (2). These new cyclic astins inhibit the growth of tumoral cell lines, whereas acyclic astins do not. Cyclic astins induce apoptosis in an human tumor cell line and this phenomenon seems to be associated with caspases activation (3). The specificity of caspase activity has been determined by the action of specific caspase inhibitors. Our data show that the new synthetic cyclic astins are toxic for tumoral cells and a possible mechanism of their antineoplastic action is proposed. References: [1]Morita, H., et al.(1995) Tetrahedron 51, 1121-1132 [2]Rossi, F., et al. (2004) Journal of Peptide Science 10, 92-102 [3]Budihardjo, I., et al. (1999) Annu Rev Cell Dev Biol. 15, 269-290.

P505

STRUCTURAL PROPERTIES OF TANDEM CCP MODULES FROM THE COMPLEMENT PROTEIN C1R

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The complement system, part of the innate immune system of higher animals, is crucial for a proper defense mechanism against pathogens. Among the complement proteins, a module composed of about 65 amino acids, typically forming two to four consecutive units, is widely distributed. This module, termed CCP (complement control protein), has two disulfide bridges (1-3; 2-4 pattern or abab) [1]. C1s is a multidomain enzyme of the complement system that cleaves two protein substrates (C4 and C2). It was shown that the catalytic efficiency of its serine protease domain is greatly enhanced by the two CCP modules attached to it [2]. A supposed role for the tandem CCPs in C1s and in the related C1r is positioning the terminal serine protease for effective contact with their substrates [3]. Thus, we have aimed to determine the structure and backbone dynamics of the tandem CCP modules from the C1r enzyme by heteronuclear NMR spectroscopy. A rather flexible tetrapeptide linker region (Lys–Ile–Lys–Asp) between the modules is long enough to raise the possibility for altering the relative positions of the two domains, therefore contributes to the proteolytic activity of C1r. [1] Reid KBM, Day AJ. Immunol Today 1989 10(6):177-180. [2] Rossi V, Bally I, Thielens NM, Esser AF, Arlaud GJ. J Biol Chem. 1998 273(2):1232-1239. [3] Kardos J, Gál P, Szilágyi L, Thielens NM, Szilágyi K, Lőrincz Z, Kulcsár P, Gráf L, Arlaud GJ, Závodszky P. J Immunol. 2001 167(9):5202-5208.

P506

COMPATIBILITY OF PEPTIDE-PROTEIN INTERACTION ANALYTICS IN STRUCTURE-FUNCTION ANALYSIS OF MICROBIAL HOST INTERACTIONS, EXPERIENCES AND PRACTICES

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We employ membrane anchored Spot peptide arrays, phage-displayed peptide libraries, phage and yeast-hybrid protein arrays and assays, and surface plasmon resonance interaction kinetics as structure-function complement in (i) identification of antigenic sites for serodiagnostics of virus infections, (ii) molecular mimicry of antibody responses to viral and cellular proteins and (iii) invation and evation mechanisms in viral and bacterial infections. Compatibility of peptides in description of protein-protein interactions are described by examples from hantavirus neutralization site, immuno-dominant epitope of parvovirus, complement factor H binding to Borrelia outer surface proteins and interactions of hantavirus structural proteins.

STRUCTURE ACTIVITY RELATIONSHIP STUDIES

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STRUCTURE-ACTIVITY RELATIONSHIP OF AMINOGLYCOSIDE-ARGININE PEPTIDO MIMETICS TARGETING HIV-1 GP120-CXCR4 BINDING STEP

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We have recently designed and synthesized aminoglycoside-arginine conjugates (AACs) as potential anti HIV-1 agents. AACs exert a number of activities related to Tat antagonism. We here present a new set of AACs, conjugates of neomycin B, paromomycin and neamine with different number of arginines (1-6), their (a) uptake by human T-cell lines, (b) antiviral activities, (c) competition with monoclonal antibody (mAb) 12G5 binding to CXCR4, (d) competition with SDF-1α binding to CXCR4, and (e) competition with HIV-1 coat protein gp-120 cell penetration. Our results point that the most potent AACs is the hexa-arginine neomycin B conjugate (NeoR6), the other multi-arginine-aminoglycoside conjugates are less active, and the mono-arginine conjugates display the lowest activity. Our studies demonstrate that, in addition to the core, the number of arginines attached to a specific aminoglycoside, are also important in the design of potent anti-HIV agents. HIV-1 isolates resistant to NeoR6 have mutations in the C3, V4 and C4 regions of gp120, and in the 'heptad repeat' 2 (HR2) region of gp41. These finding strongly suggest that AAC obstruct HIV-1 replication by interfering with the fusion step, dependant of both conformational changes in gp120 following CD4 and CXCR4 interaction, and in gp41 induced by HR1 and HR2 interaction. The AACs may thus play an important role not only as HIV-1 RNA binders but as a novel family of HIV-1 fusion inhibitors.

P508

DMT, THE PROGENITOR OF POTENT OPIOID BEHAVIOR: EVOLUTION OF BIFUNCTIONAL OPIOID MIMETIC COMPOUNDS

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Dmt (2′,6′-dimethyl-L-tyrosine)-Tic (1,2,3,4-tetrahydroisoquinoline-3-carboxylate) or Dmt in opioid peptides and analogues (dermorphin, deltorphin, dynorphin, endomorphin-2), 3,6-bis-[Dmt-NH-(CH_2)n]-2(1H)pyrazinone or bis-[Dmt-NH]-(CH_2)n opioid mimetics yielded high affinity compounds and potent bioactivities. A third substituent at the C-terminus of Dmt-Tic with a linker and/or N-alkylation played major roles in determining opioid receptor selectivity, agonism and antagonism. Substitution of -COOH with amino acids, aromatic, heteroaromatic or heteroaliphatic groups produced analogues with delta-agonism/mu-agonism and delta-antagonism/mu-agonism profiles while maintaining Ki(delta) < 0.2 nM as delta-selectivity varied with changes in Ki(mu). Aromatic or heteroaromatic groups enhanced mu-opioid properties. Third amino acid derivatives affected delta-antagonism (pA2 = 6.9-10.07); however, H-Dmt-Tic-Glu-NH_2 [Ki(mu)/Ki(delta) = 22,620] became a delta-agonist (IC50 = 2.5 nM), which was one tenth of H-Dmt-Tic-NH-CH_2-Bid (pEC50 = 9.90). N-Alkylation converted the comparable Ph derivative from a delta-/mu-agonist into a potent delta-antagonist (pA2 = 9.47). Whereas H-Dmt-Tic-Gly-NH-CH_2-Ph (delta-antagonist/mu-agonist) was 40% as active as morphine, naloxone reversible, and active as a delta-antinociceptive agent. Whereas bis-[Dmt-NH]-pyrazinone and -alkyl compounds depended on the chain length to produce high mu-affinity [Ki(mu) < 0.5 nM] and potent mu-agonism (IC50 = 1-5 nM) with in vivo biological potency. Intracerebroventricular, subcutaneous and oral administration provided evidence of blood-brain barrier transit without derivativization. On the other hand, bis-[Dmt-Tic]-pyrazinone or –alkyl derivatives had Ki(delta) = 0.10-0.16 nM and Ki(mu) = 1.8-5.7 nM. Data provide evidence that (i) Dmt-Tic-NH-(R)-R' specified delta-/mu-opioid receptor interaction, (ii) (R)-R' defined increased mu-affinity, and (iii) Dmt enhanced interaction to all opioid receptors, presumably by reinforcing or stabilizing pi-pi bonding in a ligand-bind site.

P509

IDENTIFICATION OF AN UNUSUAL CLEAVAGE SITE FOR THE PROLYL ENDOPEPTIDASE PEP : INVESTIGATION OF THE BREAKDOWN OF THE OCTADECANEUROPEPTIDE ODN

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Prolyl endopeptidase (PEP), a serine protease involved in many neurological processes, preferentially cleaves proline-containing peptides such as oxytocin, α-MSH and substance-P at the carboxyl-side of proline residues. PEP can also specifically hydrolyze post-alanine bonds, a type of cleavage that is thought to contribute to the formation of the β-amyloid peptide. The octadecaneuropeptide (ODN, QATVGDVNTDRPGLLDLK) is generated by proteolytic cleavage of diazepam-binding inhibitor. ODN exhibits multiple behavioral and neurobiological activities but the mechanism involved in its inactivation is currently unknown. The presence of both proline and alanine in the ODN sequence led us to investigated in vitro the effect of PEP on the breakdown of ODN and related peptides. Incubation of ODN with recombinant Flavobacterium PEP generated two compounds that were identified by MALDI-TOF mass spectrometry as ODN(3-18) and ODN(5-18), in addition to [pGlu1]ODN which was spontaneously produced during the incubation. Paradoxically, we did not observe the formation of the ODN(1-12) or ODN(13-18) fragments. The specific PEP inhibitor S17092 markedly reduced the degradation of ODN. Incubation of Nα-bobipy-ODN (a fluorescent ODN-derivative) and [Ala2]ODN(11-18) (a C-terminal fragment analogue that corresponds to the biologically active core of ODN) with PEP showed the same S17092-sensitive post-alanine and/or post-valine cleavages, while [pGlu1]ODN was not catabolized by the enzyme. Taken together, these data reveal that PEP preferentially cleaves the Ala2-Thr3 bond of ODN but, surprisingly, cannot hydrolyze the Pro11-Arg12 bond. In addition, this study shows for the first time that PEP can specifically cleave a post-valine bond in a neuropeptide.

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NEW SYNTHESIS OF CIS-5-TERT-BUTYL-L-PROLINE, A CIS PROLINE MIMETIC. INCORPORATION IN AN OCTAPEPTIDE ANALOGUE OF THE ENDOZEPINE ODN AND NMR STUDY

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The octadecaneuropeptide ODN has been originally characterized as an endozepine, i.e. an endogenous ligand of benzodiazepine receptors (BZ-R). However, we have demonstrated that, in astrocytes, ODN increases [Ca2+]i through activation of a GPCR pharmacologically different of BZ-R. SAR studies have shown that the C-terminal octapeptide (OP) is the shorter analogue of ODN that retains full activity, and that [DLeu5]OP is a weak antagonist of the ODN-GPCR. Recently, we have designed a selective antagonist, the cyclo1-8[DLeu5]OP. Structural analysis by 2D-1H-NMR and molecular modelling showed that cyclo1-8[DLeu5]OP adopts two equimolar conformations resulting from a trans/cis isomerization of the Arg-Pro bond. To obtain information about the conformation required for antagonistic activity, cis-5-tert-butylproline (cistBuPro), a cis-amide inducer, was incorporated into [DLeu5]OP. CistBuPro was efficiently prepared by a reaction involving addition of low-valence tert-butyl-cuprate to an aminal derived from N-Boc-Pro-OMe. The mixture of diastereoisomers was separated after TFA treatment affording the (2S,5R)tBuPro-OMe. Saponification and Nα-protection generated Fmoc-cistBuPro-OH suited for automatic peptide synthesis. Incorporation of cistBuPro in place of Pro2 into [DLeu5]OP led to [cistBuPro2,DLeu5]OP which presented a 967.6 [MH]+ value. Conformational analysis using NOESY experiment, revealed a main correlation between Hα-Arg1 and Hα-cistBuPro2, and a minor one between Hα-Arg1 and Hδ-cistBuPro2 characteristic of a cis- and trans-amide bond, respectively. NH-Asp1 signal area of each conformer led to a cis/trans ratio of 71/29. The present data show that the incorporation of cistBuPro enhances the steric interactions favoring the cis-amide population. Biological analysis of [cistBuPro2,DLeu5]OP may open new vistas for the design of novel antagonists of ODN-GPCR.

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DEVELOPMENT OF μ-OPIOID RECEPTOR LIGANDS BY USING UNIQUE L-TYROSINE ANALOGUES

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Opioid studies revealed that the replacement of the N-terminal tyrosine in opioid peptides with 2', 6'-dimethyl-L-tyrosine (Dmt) enhanced both opioid receptor binding affinity and functional bioactivity. In order to develop potentially more potent opioid ligands, it would be necessary to study the structure-activity relationship of the aromatic alkyl groups on the Tyr residue. In this study, six L-tyrosine analogues were developed: 2'-monomethyl-tyrosine (Mmt), 2'-ethyl-6'-methyl-tyrosine (Emt), 2'-isopropyl-6'-methyl-tyrosine (Imt), 2', 6'-diethyl-tyrosine (Det), 2', 6'-diisopropyl-tyrosine (Dit) and 2', 3', 6'-trimethyl-tyrosine (Tmt). There were stereo-selectively synthesized (e.e. > 94.5 %) according to the method of Dygos [1] and their bioactivity was evaluated by incorporation into endomorphin-2 (EM-2: YPFF-NH_2). A NMR study in DMSO showed that the Xaa-Pro amide bond in our [Xaa1]-EM-2 analogues except [Mmt1]-EM-2 adopted the cis form. The μ-opioid receptor binding assay revealed that the new these analogues except [Dit1]-EM-2 have a similar or higher binding affinity compared to [Dmt1]-EM-2 (Kiμ= 0.15 nM). The δ-opioid receptor binding assay indicated that all the [Xaa1]-EM-2 analogues had higher binding affinities than EM-2 but lower affinity than [Dmt1]EM-2. In the in vitro functional bioactivity assays (GPI and MVD), all our synthetic EM-2 analogues exhibited μ-agonist activities, which were weaker than that of [Dmt1]-EM-2 yet most of them exhibited greater bioactivity than EM-2 in both assay systems. The in vivo analgesic activity of [Det1]-EM-2 in comparison to [Dmt1]-EM-2 and EM-2 is currently being investigated. [1] J. H. Dygos, et. al. Synthesis, 1992, 741-743

P512

IMPACT OF TETRAZOLE CIS-AMIDE BOND SURROGATE ON METALLOPEPTIDES

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The 1,5-disubstituted tetrazole ring has been proposed as a surrogate for cis-amide bonds, making it a valuable tool in the design of conformationally constrained peptidic receptor probes [1]. Insertion of tetrazole in a peptide chain influences the conformation of that chain, encouraging the beta-turn structure in aqueous solution. Since this bent conformation appears to be of critical importance in many biological processes we have undertaken study to find further evidence for the possible stabilization by metal ions of bent conformations of peptide chains. Hence, in this work we present potentiometric and spectroscopic data including CD and EPR results demonstrating that 1,5-disubstituted tetrazole ring is an unique element modifying chelating abilities of peptides towards copper(II) ions. The tetrazole enkephalin, β-casomorphin and deltorphin analogues have been the targets of the investigation. The position of the tetrazole ring system in the peptide backbone plays a critical role in the metallopeptide molecule stabilization. The insertion of the tetrazole between amide groups leads to the stability of metallopeptide system and to a very effective peptide chelating agent [2, 3]. These findings can serve as important information for modeling the biologically relevant peptide-metal binding sites. References 1. Zabrocki J.; Dunbar, J. B., Jr.; Marshall, K. W.; Toth, M. V.; Marshall, G. R. J. Org. Chem. (1992), 57, 202-209. 2. Łodyga-Chruścińska E.; Micera G.; Sanna D.; Olczak J.; Zabrocki J.; Kozłowski H.; Chruściński L. J Inorg Biochem. (1999), 76, 1-11. 3. Chruścińska E.; Micera G.; Sanna D.; Olczak J.; Zabrocki J. Polyhedron (2001), 20, 1915-23.

P513

LANTHANIDE(III) LABELED PEPTIDES IN DIFFERENT APPLICATIONS BASED ON TIME-RESOLVED FLUORESCENCE LANTHANIDE CHEMISTRY

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As non-radioactive labels the lanthanide(III) chelates with their unique fluorescent properties have found increasing applications in diagnostics, research and high throughput screening. Because of their ability to give strong, long decay-time luminescence, they are ideal labels for assays requiring high sensitivity. The DELFIA technology is known to be very sensitive technique due to its heterogeneousity. Lanthanide(III), dissociated with DELFIA enhancement solution, creates highly fluorescent complexes which are measured in a detection instrument having TRF-option, i.e. VICTOR. The homogeneous technology with fluorescent lanthanide(III) chelates is applied with TruPoint platform for example in protease assays. SignalClimb technology of TruPoint protease substrates is based on TR-FQA, time-resolved fluorescence quenching assay. DELFIA is applied here with Europium-labeled peptides designed to be used in ligand-reseptor binding assays. Reseptor binding is analysed by detecting the labeled ligand after washing of the unbound Eu-ligand. We introduce our Eu-chelates to peptides as modified amino acid building blocks during automated peptide synthesis. Saturation and displacement curves shown in poster presentation proves our results to be compareable to radioligands. In homogeneous TruPoint application the peptide is dual-labeled with fluorescent europium chelate coupled to one end and a quencher of europium fluorescence coupled to the other end. When the sample displays protease activity, the europium chelate and the quencher will be separated as the substrate is cleaved. The increase in time-resolved europium signal is measured. TruPoint Caspase-3 substrate is one of our model assays in detection of proteases.

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SYNTHESIS OF METABOLICALLY STABLE 99MTC-LABELLED NEUROTENSIN(8-13) ANALOGS FOR TUMOR DIAGNOSIS

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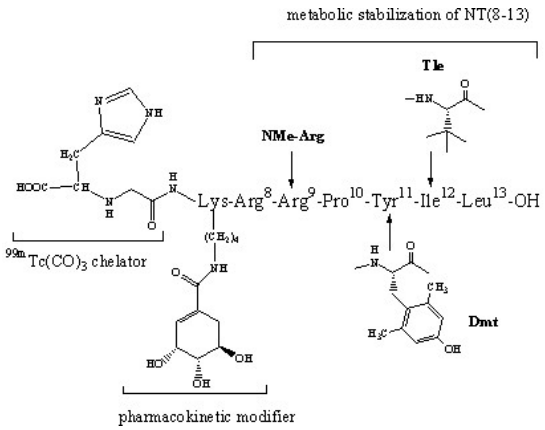
Tumor diagnosis using the SPECT technique is based on the delivery of a gamma-emitter (99mTc) into the tumor cell using a specific targeting molecule. Since pancreas carcinoma overexpress neurotensin (NT) receptors, we have developed new NT(8-13) analogs for ductal pancreatic carcinoma diagnosis. The half-life of NT(8-13) of ~3 min, has been increased to >24h in plasma by a combination of NMeArg, and the bulky side chains of Dmt and Tle. The retro-[N-alfa-carboxymethyl-histidine] moiety, short (N-alfa-His)Ac, functions as an efficient chelator of 99mTc(CO)3, and shikimic acid has been conjugated to a Lys side chain to reduce kidney accumulation. The whole construct has been prepared on solid-support. The most promising analog is currently undergoing clinical trials.



P515

ENZYMATIC DEGRADATION OF CARBAMOYL-NOCICEPTIN ANALOGUES

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Nociceptin, a 17-amino acid peptide (FGGFTGARKSARKLANQ) has been identified as a major metabolite of pronociceptin and also as the natural endogenous ligand of ORL1 receptor [1]. Nociceptin is degraded by aminopeptidases in plasma to yield Noc(2-17)-OH and smaller fragments. N-terminal truncation leads to complete loss of activity. Structure-activity experiments suggest that Noc(1-13)-OH is the minimum sequence required for receptor binding considering that C-terminal amidation may protect the peptide against degradation [2]. We have synthesized Noc(1-13)-NH_2 and its carbamoyl analogues at the N-terminal part of the peptides. We investigated the in vitro metabolic stability of these analogues in rat brain homogenate [3] and also in the presence of aminopeptidase M (leucine aminopeptidase, type-S, from porcine kidney microsomes, EC 3.4.1.1.2.). Peptides were incubated with the enzyme or brain homogenate up to 24 hr. The metabolic stability of the analogues was compared with that of Noc(1-13)-NH_2. Samples were analysed by high-performance liquid chromatography and degradation products were identified by electrospray ionization mass spectrometry. This work was supported by grand from the Hungarian Research Foundation (OTKA T 033078) and the Ministry of Education (Medichem, NKFP 1/047). [1] Reinscheid R.K., et al. (1995) Science 270: 792-794; Meunier J.-C., et al. (1995) Nature 377: 532-535 [2] Lars Terenius, et al. (2000) Peptides 21: 919-921 [3] Prókai L., et al. (1998) J. Chromatogr. A 800: 59-68.

P516

INVESTIGATION OF STRUCTURE-REACTIVITY RELATIONSHIPS IN THE ACYDOLYSIS OF ALPHA,ALPHA-DIALKYL GLYCINE DERIVATIVES

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N-Acyl-N,alpha,alpha-trialkyl and N-acyl-alpha,alpha-dialkyl glycines suitable for incorporation into peptide chains can be obtained in fair to good yields by acydolysis of Ugi-Passerini adducts.[1] Preliminary investigations were carried out with a set of eight model compounds resulting from combination of different substituents in three of the four substituent sites within the adducts. These showed that the nature of the final cleavage product depends not only from the reaction conditions but also from the structure and bulkiness of the various substituents[2] and were corroborated by kinetic measurements performed at 25 ºC.[3] We now present activation thermodynamic results obtained with the same set of compounds at various temperatures and the results of the kinetic investigation of a set of derivatives of five different alpha,alpha-dialkyl glycines at 25 ºC. The results from the former set allowed (i) establishing the reaction pathways connected with the competitive and consecutive reactions involved in the process and also detecting the formation of at least two intermediate oxazolonium derivatives and (ii) describing the effect of temperature in terms of an Arrhenius-type equation. Those from the second set of compounds were subjected to a Taft multiparametric treatment. In both cases it was possible to evaluate polar and steric substituent effects, including those related to the amino acid side chains. [1] H.L.S. Maia et al., Org. Biomol. Chem., 1 (2003) 1475; [2] Ibid., ibid., 1 (2003) 3804; [3] Ibid., in E. Benedetti and C. Pedone (eds.), "Peptides 2002", p. 966.

P517

ANALOGS OF THE VASOPRESSIN VASODILATING PEPTIDE D(CH_2)5[D-TYR(ET)2,ARG3]VAVP WITH STRIKINGLY ENHANCED HYPOTENSIVE AGONISM

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We reported a new class of vasopressin (VP) peptides which exhibit selective hypotensive agonism [1]. The parent peptide, d(CH_2)5[D-Tyr(Et)2,Arg3]VAVP(A) which has the following cyclic structure: (CH_2)5C(S)-CH_2-CO-D-Tyr(Et)-Arg-Val-Asn-Cys-Pro-Arg-Gly-NH_2 (with disulfide bridge between positions 1 and 6) exhibits a vasodepressor Effective Dose (ED) = 4.66 micrograms/100g. (ED, is the dose that produces a vasodepressor response of 5 cm2 AUC (area under the curve) in the 5 min period following injection of test peptide). We subsequently reported analogs of (A) which exhibit hypotensive potencies 2 to 9 fold greater than (A) [2-4]. The most promising of these, the Lys7,Lys8,Eda9 analog (B) (where Eda = ethylenediamine) was modified at positions 2, 4, 6 and 9, to give analogs which are substantially more potent than (A). Two peptides, the D-Tyr(iPr) (iPr = isopropyl) and D-Tyr(nPr) (nPr = normal propyl) analogs of (B), with ED values of 0.15 and 0.18 microgram/100g, are respectively 31 and 26 times more potent than (A). These findings offer promising clues to the design of more potent hypotensive agonists and of antagonists of the putative vasodilating VP receptor, which mediates the hypotensive response. References [1] Chan, W.Y., et al. Brit. J Pharmacol. 125, 803-811 (1998). [2] Manning, M. et al. Peptides 1998 (eds. S. Bajusz and F. Hudecz). Akademiai Kiado, Budapest 1999, pp.56-57. [3] Manning, M. et al. J Peptide Sci. 5, 472-490 (1999). [4] Cheng, L.L. et al. Peptides: The wave of the Future (eds. M. Lebl and R.A. Hougthen). American Peptide Society 2001, pp.978-979.

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STRUCTURE ACTIVITY RELATIONSHIP STUDIES STUDIES OF A NOVEL CLASS OF TETRAPEPTIDE INHIBITORS OF PLATELET AGGREGATION

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We found that backbone-modified tetrapeptides of general formula X-(+)-Pro-(+), where X is a neutral residue and (+) a positively charged residue, inhibit the aggregation of human platelets in vitro and in vivo. The mechanism of action involves inhibition of ATP release and thromboxane synthesis from activated platelets. A structural study was carried out under physiological conditions where the influence of side chain substitution and backbone modification on conformation was examined by Circular Dichorism, Fluorescence and NMR spetroscopies. It was thus found that the tetrapeptides exhibit well defined secondary structures at neutral pH. The dominating conformations are characterised by a bent around the (+)-Pro sequence which is not stabilised by hydrogen bonds. The influence of substitution on the inhibitory activity of these peptides was then measured in the assay of ADP-induced platelet aggregation and the STRUCTURE ACTIVITY RELATIONSHIP STUDIES thus derived used to design and synthesise peptide-mimetic analogs of the (+)-Pro-(+) tripeptide. Finally, a correlation between the anti-platelet activity of these peptides and their ability to bind membrane phospholipids is presented.

P519

SYNTHESIS, STRUCTURE AND ANTITUMOUR ACTIVITY OF GNRH-III DERIVATIVES

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GnRH-III (Glp-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2) isolated from sea lamprey is a naturally occurring GnRH analogue. Cell proliferation of GnRH receptor-positive breast cancer cells was suppressed by GnRH-III in vitro [1]. However, it did not exert significant endocrine activity suggesting selective antitumor activity of this peptide. In this study we prepared GnRH-III modified by Cys at the ε-amino group of 8Lys a conjugation site applied in previous studies [2]. The Cys-derivatives (GlpHWSHDWK(H-Cys)PG-NH_2 and GlpHWSHDWK(Ac-Cys)PG-NH_2) were used for the synthesis of homodimers containing disulfide bridge as well as for conjugates, in which GnRH-III analogue was attached to tetratuftsin derivative [TKPKG]4 as carrier molecule. Cell proliferation of the compounds on GnRH receptor-positive human breast (MDA-MB-231, ZR-75-1) and colon carcinoma cell lines (HT-29) was studied. Differences in receptor binding and antitumour activity of dimers ([GlpHWSHDWK(Ac-Cys)PG-NH_2]$_2$ and [GlpHWSHDWK(H-Cys)PG-NH_2]$_2$) were observed. To explain these findings we have studied the conformation of the dimers by CD spectroscopy in water, TFE and their 1:1 (V/V) mixture and also by NMR (2D NOESY and TOCSY) spectroscopy. These results indicate structural alterations especially around the His resiudes. [1] Mező, I. et al. J. Med. Chem. 40, 3353-3358 (1997) [2] Pályi, I. et al. Drugs of the Future 26, 51-59 (2001) Aknowledgement: This work was supported by grants from Hungarian National Science Fund (OTKA No. T032425, T043576) and from the Hungarian Ministry of Education (Medichem NKFP 1/047)

P520

DESIGN, SYNTHESIS AND BIOLOGICAL EVALUATION OF LEPTIN FRAGMENTS

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Leptin is involved in the control of energy homeostasis, in the food intake and weight maintenance. In an attempt to identify in the leptin molecule regions responsible for its bioactivity, we have synthesized and tested six peptide fragments {Ac-hLEP2-26-NH_2 (I), Ac-hLEP27-50-NH_2 (II), Ac-hLEP51-67-NH_2 (III), Ac-hLEP71-94-NH_2 (IV), Ac-[Ser96]-hLEP95-119-NH_2 (V), Ac-hLEP120-143-NH_2 (VI) and their correspondent dimeric form which were generated by a disulfide bridge formation. The fragments were designed on base of the three-dimensional structure of the native leptin. In fact fragments I, III, IV and VI encompass fragments of the native protein that present an alpha–helical structure. Peptide [D-Leu4]-OB3 [Grasso,P. et al. Regulatory Peptides 101, 123, 2001] and its human sequence analogue were also synthesized and studied. Herein, we report the peptide synthesis by SPPS and their chemical characterization (LC/ESI-MS, HPLC, CE and AAA). They were evaluated, comparatively to native leptin, by their ability to interact to leptin receptor present in HP-75 cells using cytosensor microphysiometry. Our results indicated that the fragments IV and V and the peptide [D-Leu4]-OB3 and its human sequence analogue were recognized by leptin receptor present in HP-75 cells and that two of their amino acids residues seems to be important for such recognition. These amino acids are: Ser77 and Leu80 of the fragment IV and Ser95 and Leu98 of the fragment V. Although the peptide fragments design needs refinement, this kind of approach may offer the basis for the development of leptin-related compounds with potential application in human obesity. [Supported by FAPESP and CNPq]

P521

SYNTHESIS AND PROPERTIES OF NEW POTENTIAL RECEPTORS FOR AMINO ACIDS BASED ON AMINOPHOSPHONATE AND AMINOPHOSPHINATE COMPOUNDS

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The last decades of studies on phosphonate properties towards amino acids bindings have revealed that phosphonate group appeared to be selective binder for guanidino- and other strongly cationic entities e.g.: ammonium group of amino acids. The use of phosphonate function gives the possibility of the regulation of molecule charge either by its selective esterification or by the choice of proper phosphorous derivative. This should directly influence electrostatic interactions between guest and host molecule as well as formation of networks of hydrogen bonds. Simultaneous use of the protonated amino group (ammonium group) gives a good possibility of hydrogen binding donor and positively charged point for electrostatic attractions for negatively charged parts of guest molecules. In our work we have taken a challenge of synthesis of enatiomericaly pure aminophosphonates and aminophosphinates designed as new receptors for amino acids. For this purpose we have synthesized bisaminophosphonic and bisaminophosphinic receptors based on aryl skeleton. The use of both phosphonate and amino group located into carefully tailored cavity should result in synthetic receptors for certain amino acids. Additionally enantioselective binding might be received upon the use of enantiomers of these aminophosphonates. Acknowledgements: This work was financially supported by the Polish Ministry of Scientific Research and Information Technology (grant no. 4 T09A 033 25).

P522

A DUAL SCREENING OF CONFORMATIONAL DEFINED PEPTIDE LIBRARY BASED ON THE OLIGOMERIZATION DOMAIN OF p53

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p53 is a key protein involved in cancer suppression, and has been considered an interesting target for oncology research. This protein in response to different stress conditions, such as DNA damage, hypoxia, etc, increases its cellular concentration followed by its activation, which leads to cell cycle arrest or to apoptosis. Activated p53 prevents damaged cells from dividing therefore p53 is designed as a tumour-suppressor protein. The primary structure of p53 contains four domains: a N-terminus domain (1-94), a DNA-binding domain (110-286), a tetramerization domain (326-355) and a C-terminus domain (363-393). For its tumour suppressor activity, p53 has to adopt a tetrameric structure that enables it to bind to DNA. The structure of the tetramer, can be defined as a dimmer of dimmers, each monomer adopts a α-helix with a short β-strand at the N-terminus. In this work we present the design, synthesis and analysis of a conformational defined p53 mimetic library based on the oligomerization domain. The library has three randomized positions at the solvent exposed b-strand. The objective are the study of he influence of solvent exposed residues in the folding pathway and the designed of a synthetic minimized transcriptional factor based on p53 tetramerization domain.

P523

ANGIOTENSIN II AND BRADYKININ ANALOGUES CONTAINING THE PYRROLIDINE-BASED PARAMAGNETIC AMINO ACID POAC : BIOLOGICAL ACTIVITY AND EPR STRUCTURAL INVESTIGATION

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This report initiated a structure-activity investigation of vasoactive AII and BK analogues containing the 2,2,5,5-tetramethypyrrolidine-N-oxyl-3-amino-4-carboxylic acid (POAC) spin label [Chem. Pharm. Bull. (2001) 49, 1027]. Paralleling previous studies with a piperidine-type spin probe TOAC (Biopolymers, in press), POAC0-BK, POAC3-BK and POAC1-AII were synthesized. The two N-terminally labeled derivatives maintained partially the biological potency as occurred with TOAC-derivatives whereas the mid-chain labeled POAC3-BK partner was completely devoid of activity. EPR spectra of these paramagnetic peptides indicated a lack of dependency to the pH of the medium. The two amino-terminal labeled analogues presented slightly greater chain motion than that of the internally labeled derivative, indicated by rotational correlational time around 4 x 10^{-10}s against 6 x 10^{-10}s, respectively. Moreover in considering the midpoint of the curve when the isotropic hyperfine splitting are plotted as a function of the pH of the medium, the pKa values of about 7 could be estimated for the POAC0-BK and POAC1AII amine functions. Structural differentiation between peptides was observed when TFE was added. In contrast to POAC-attaching BK derivatives where the expected and progressive immobilization occurred as a functions of the increase in the viscosity, the POAC1-AII showed abrupt variation in the motion when the medium reached about 50%TFE. These initial findings suggested differentiated structural features between these two peptides.

P524

DESIGN, SYNTHESIS AND IN VITRO EVALUATION OF NEW AZAPEPTIDE INHIBITORS OF SERINE PROTEASES

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Trypsin-like serine protease, especially thrombin and factor Xa, are enzymes that take part in the control of blood coagulation. A therapeutically useful inhibitors should be potent, selective and orally bioavailable. Compounds with such properties could be used in the prevention and treatment of several cardiovascular disorders. The derivatives of the amino acid sequence D-Phe-Pro-Arg as in a peptidomimetic argatroban are the most studied group among thrombin inhibitors. In our laboratories we prepared analogues in which the para substituted phenylalanine part of argatroban (rigid mimic of arginine) was replaced by azaphenylalanine. This modification resulted in the loss of a stereogenic center and the change on overall conformation. The metabolic stability of compounds is also increased. Our previous results indicated that proline is not an optimal fragment for binding in proximal pocket of the active site of both serine proteases. Azepane significantly reduces the inhibitory constants of compounds and was therefore incorporated in the scaffold. In the part of a molecule that binds in the distal pocket of serine proteases arylsulfonamido group in argatroban was replaced by a series of aryl- and arylalkylcarboxamido groups to enable us to study the SAR of serine proteases inhibitors. The results of in vitro evaluation of newly synthesized molecules has shown a marked decrease in activity against trombin that was counterbalanced by a greatly enhanced activity against factor Xa, making these derivatives interesting lead compounds for dual thrombin, factor Xa inhibitors.

P525

EFFECTS OF STRUCTURAL ANALOGUES OF ENKEPHALIN ON THE THERMOTROPIC BEHAVIOR OF MODEL LIPID MEMBRANES

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The influence of three groups of structural analogies of enkephalin on the thermotropic behavior of multilamellar vesicles (MLVs) composed of dipalmitoyl phosphatidyl glycerol (DPPG) and of a 4:1 mixture of dipalmitoyl phosphatidyl choline (DPPC) with DPPG was studied by differencial scanning microcalorimetry. The first group of enkephalin analogues were halogenated in their thyrosine, the second group were modified in their primary structure, and the third were both Tyr-halogenated and with a modified primary structure. The thermotropic behavior of the MLVs formed in presence of enkephalins indicates the possible set of variables liable for interactions from weak association of the peptides to the lipid interface up to their insertion into the hydrophibic zone of the lipid bilayer. This set is isomorphic to the established notion of weakly interacting with the lipid peptides to bind to receptors from the water phase and peptides strongly interacting with the lipid to enter the receptor from within the hydrophobic phase.

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SYNTHESIS OF MODIFIED AT POSITIONS 1 AND 3 ANALOGUES OF ENDOGENOUS TETRAPEPTIDE TYR-MIF-1

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The N-terminal Tyr1 ring is typical for endogenous opiate peptides and it is considered to be responsible for the opiate action. To understand more about correlation between modification of Tyr-sceleton and the biological activities of these tyrosine-like compounds, several peptide analogues with combined substitutions, based on the structure of the naturally occurring Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH_2) have been synthesized. In this study we present a series of new analogues in which: 1) in purpose to change the basicity and hirality, the L-Tyr1-residue has been modified with different electrophylic and nucleophylic substituents - D-Tyr(Me), Nα-MeTyr, Tyr(Cl2); 2) on the other hand, following our previous work, the position 3 has been performed with unnatural aminoacids - S-substituted cysteinsulfonamides (sLeu, sIle, sNle, sLys). The synthesis is carried out in solution according to the general procedure of the peptide synthesis by stepwise – chain building method. The obtained peptides are subjected of pharmacological investigation giving evaluation their possibility of binding affinity and μ- selectivity.

P527

VISUALIZING THE INTERACTIONS OF ENKEPHALINS WITH MODEL MEMBRANES

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Enkephalins have their own protein receptors in biological membranes. This is maybe one reason for relatively restricted amount of work on interactions with the lipid component of cell membranes. A second restriction ensues from the established notion that enkephalins' binding to lipid membranes is one of weak affinity and low specificity. Further, it was accepted that enkephalin binding requires negative electric charge on the lipid surface. We synthesized 4 enkephalin derivatives along with the naturally occurring Leu5-enkephalin, and investigated the morphologic transformations of giant unilamellar vesicles from various lipid mixtures, along with the phase transitions, upon local microinjection of enkephalins. Our results revealed a plethora of various transformations, such as: adhesion of adjacent vesicles; formation of dense lipid aggregates; vesiculisation; cycles of exo-/endocytosis; permeabilization for the peptide; fluidization. Type and range of the effects depend on: charge density; type of charged lipid; lateral structure of the lipid bilayer. With pure lecithin no effects were observable. But with composite membranes of neutral lipids we observed for the first time strong interactions in terms of profound transformations. Type and range of the transformations varies for the derivatives of enkephalin. Steric and charge effects could be responsible for different transforming activity. The mode of morphologic transformations implies an important role for the enkephalin-lipid interactions in processes such as intracellular and transmembrane trafficking of enkephalins, and at least mediatory role of these interactions in the signaling functions of enkephalins.

P528

SYNTHESIS AND STUDY OF NEW INTRAMOLECULARLY QUENCHED FLUOROGENIC SUBSTRATES FOR HIGHLY SENSITIVE RENIN DETERMINATION

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Renin is involved in the first and rate-limiting step of the renin-angiotensin system, which plays a central role in the regulation of blood pressure and electrolyte homeostasis. Excessive secretion of renin can cause hypertension and exacerbate the abnormalities that occur in other diseases, such as congestive heart failure. Therefore, the development of specific and potent renin inhibitors has been an interesting area for pharmaceutical and clinical research. For potency evaluation and mechanistic characterisation of tight-binding inhibitors, highly sensitive renin substrates are required. We have synthesized 16 intramolecularly quenched fluorogenic substrates (IQFS) for a sensitive, direct and continuous measurement of renin activity. The synthesis was performed totally by solid phase technique and the design of the substrates was based on the N-terminal sequence of human angiotensinogen. The combination of the fluorescent amino acid L-2-amino-3-(7-methoxy-4-coumaryl)propionic acid (L-Amp) and the quencher 2,4-dinitrophenyl (DNP) group, placed at various positions in the sequence, was used. Most of the new substrates exhibited good kinetic parameters and satisfying increase in fluorescence upon hydrolysis. The best one, with the sequence DNP-Lys-His-Pro-Phe-His-Leu-Val-Ile-His-L-Amp-OH, was found to be a highly active renin substrate, having a kcat/Km value of 350 000 M-1s-1, 94% quenching efficiency and strong product fluorescence. When compared with a commercially available renin substrate, based on EDANS/DABCYL combination, the sensitivity is improved 20-fold, allowing as little as 2.4 ng/ml (0.06 nM) of renin to be detected in a continuous assay and short incubation time.

P529

STRUCTURE-FUNCTION RELATIONSHIP OF ANALOGUES OF PTH(1-11) CONTAINING A COMBINATION OF AIB AND ALPHA-METHYL-VAL

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The N-terminal 1-34 fragment of paratyroid hormone (PTH) is fully active in vitro and in vivo and it can reproduce all biological responses characteristic of the native intact PTH. Recent studies [1] have demonstrated that analogues of PTH(1-11) fragment with helicity enhancing substitutions yielded potent analogues of PTH(1-34). To further investigate the role of α-helicity on bioactivity, in the present work we synthesised and conformationally and biologically characterised the following PTH(1-11) analogues containing sterically hindered and helix-promoting Cα-tetrasubstituted amino acids, Aib and αMeVal. The following peptides were studied:

I [(αMe)Val2, Aib3, Nle8, Arg11]-rPTH(1-11)NH_2
II [Aib1, (αMe)Val2, Nle8, Arg11]-rPTH(1-11)NH_2
III [(aMe)Val2, Aib4, Nle8, Arg11]-rPTH(1-11)NH_2
IV [Aib1, (αMe)Val2, Aib3, Nle8, Arg11]-rPTH(1-11)NH_2
V [Nle8, Arg11]-rPTH(1-11)NH_2

The peptides have been synthesised by SPPS employing Fmoc protected amino acids. To maximize the total yield we combined the HBTU/HOBt/DIPEA and the acyl fluoride coupling methods. [2] The later was used for the incorporation of the Ca-tetrasubstituted amino acids. Results obtained by CD and NMR experiments and molecular dynamics calculations demonstrate that the substitution with Ca-tetrasubstituted amino acids led to the enhancement of the helical conformation. In TFE/water solutions, analogue V, used as reference for PTH(1-11), presents a stable α-helical segment spanning the sequence from Ile5 to His9. Analogues I -IV show a higher preference for the helical structure which comprises the sequence 2-9. Biological tests are underway to establish the correlation between helicity and bioactivity. [1] Tsomaia N., et al., Biochemistry 2004, 43, 690-699 [2] Carpino L. A., et al., Acc. Chem. Res. 1996, 29, 268-274.

P530

AN "ATCUN MOTIF" CONFERS METALLOPEPTIDE NATURE TO THE PEPTIDE/HORMONE HEPCIDIN-25

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Hepcidin(Hepcd) is a circulating 20-25 amino acids cystein-rich peptide which exhibits a broad range of antimicrobial activity against bacteria and fungi. A key role of Hepcd in regulation of mammals iron metabolism is proposed and absence of its expression is related to the pathogenesis of the hemochromatosis. The NMR solution structures of the Hepcd-25 and -20 show that both peptides form a distorted b-sheet but whereas hepcidin-20 exists as a monomer in solution, hepcidin-25 readily aggregates. The NMR spectra of the aggregates indicate an interface that involves the first five residues of Hepcd-25. A striking feature of this Hepcd-25 N-terminal pentapeptide, is the presence of the motif Asp-Thr-His. This site correspond to an "ATCUN motif" a potential binding site for Cu(II) and Ni(II). To study this potential metal-binding site, we synthesized two peptides matching the first five amino acids of the Hepcd-25 and Hepcd-20 respectively and performed a spectroscopic study. The binding of Cu(II) to Hepcd-25 peptide shows an absorption maximum at 525 nm at a pH range 5.0–10.5 whilst the visible spectra of Ni(II) binding clearly shows an absorbance maximum at 420 nm at a pH range 6.0-10.5. The results demonstrate that the visible spectra are characteristic of the ATCUN motif whereas the visible spectra of the binding of Cu(II) and Ni(II) to Hepcd-20 peptide demostrate an aspecific metal-binding. These results strongly evidence the metallopeptide nature of the Hepcd-25 and may help to a better understanding of the structural and physiological features of this molecule.

P531

SYNTHESIS AND BIOASSAYS OF PROTHROMBIN-DERIVED PEPTIDES

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Thrombin, a plasma serine protease, plays a key role in various disorders such as arterial thrombosis, atherosclerosis, restenosis, inflammation and myocardial infarction. Inhibition of this enzyme has become a major therapeutic target in the treatment of cardiovascular diseases. However, there are a number of questions that still need answers with respect to the nonproteolytic functions of thrombin. In the present study, the modified prothrombin peptides TP-H7, TP-L13, and TR-6 were produced and studied using of a various techniques for synthesis, purification, and characterisation, including by application high-pressure liquid chromatography, mass spectrometry, and bioassays. To evaluate cardioprotective effects of peptides, the models of experimental myocardium infarction and heart rhythm disturbances on the anaesthetised rats were used. Pre-treatment by TP-H7 significantly reduced the incidence of ventricle fibrillation, a summary duration of the heart rhythm disturbances, and lethality induced by ischemia-reperfusion. TP-L13 also decreased overall rat mortality, although less efficiently, whereas in animals treated with TR-6 effects were similar to those in the control group. Although TP-H7 and TP-L13 did not protected from myocardium ischemia during occlusion, both compounds prevented arterial blood pressure decrease and contributed more significant functional recovery during reperfusion. These results demonstrate for the first time that the small peptide fragments derived from prothrombin possess cardioprotective properties on ischemia-reperfusion induced heart failure on rats and might be used as a starting point to develop new cardioprotective drugs. Acknowledgements This study was supported by the NATO Collaborative Linkage Grant 979856, the Latvian Science Council Grants and National Scientific Program.

P532

ANALYSIS OF INFORMATIONAL STRUCTURE OF PROTEIN AMINO ACID SEQUENCES AND OBTAINING LARGE BIOLOGICALLY ACTIVE PEPTIDES OF HUMAN 1-CYS PEROXIREDOXIN

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The new method of the analysis of informational structure of protein amino acid sequences permits to reveal hierarchically organized elements of informational structure and their associations (IDIC-associations) corresponding to separate domains in proteins spatial structure. This theoretical approach is extremely convenient for investigation of protein functional organization because according to modern conceptions protein single functional activity is corresponded to the spatial domain in protein three-dimensional structure. Basis of the method is a new description of primary polypeptide structure using «informational units», instead of traditional amino acid residues. Based on the statistical data of frequencies of «informational units» occurrence in sets of unhomological protein sequences this approach allows to expect a structure of the sequence «informational importance» and build the diagrams (IDIC-diagrams) serving as the description of protein domain organization. The method was applied to idenntify PrxVIhum separate domains and to find its biologically active peptides. The goal for PrxVIhum investigation is its perspective application as a component of antioxidant medical composition. Two large PrxVIhum fragments were obtained. Protective and antioxidant properties were investigated in vitro in the generating H2O2 model systems, by the methods used earlier for the analysis of the whole Prx molecule. The method of analysis of informational structure of a sequence was shown to allow to elucidate functionally independent sites in polypeptide chain and coud be used for searching and revealing biologically active fragments of proteins especially with unknown spatial structures.

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PEPTIDE 'TOOLKIT' FOR COLLAGEN-PLATELET STUDIES

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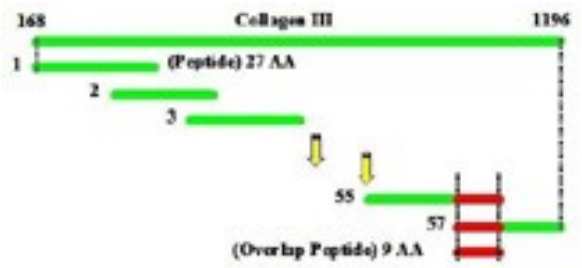
The triple-helical structure of collagen can be exploited in short collagen-like peptides by inserting a stretch of Gly-Pro-Hyp or Gly-Pro-Pro triplets before and after a specific collagen binding sequence; this is referred to as the “host-guest” strategy. Collagen-related peptides (CRPs) and GFOGER peptides have been shown to induce platelet activation through GpVI and to act as antagonists of platelet recognition of collagen through α2β1, respectively. We have prepared a synthetic “toolkit” to allow us first to map and then define the collagen III motifs responsible for recognition of receptors and other proteins which bind collagen, and to probe for unknown collagen receptors. We have cut the 163-1196 collagen III sequence into 27-AA sequences with a 9-AA overlap which gives us 57 peptides. To ensure a triple helical structure, we use the “host-guest” strategy adding on each extremity the GPC-(GPP)5- unit. We obtain in this way, GPC-(GPP)5-(GXX’)9-(GPP)5-GPC-NH_2 peptides of 63 AA. The first results have shown the capacity of some peptides to bind platelets. This overlapping process allowed us to determine the short sequences responsible for the activations. This project is a collaboration between several laboratories in the world. The integrins α2β1 and α1β1 are the first targets explored in the laboratory followed by GpVI and CD36. Abroad, the targets to be explored will be, Von Willebrand factor, Matrilins, GpV,DDRs,Decorin, Opticin, Fibrin, Fibronectin, MMP, etc.



P534

NEW POTENT LIGANDS FOR THE RECEPTOR OF NOCICEPTIN-ORPHANIN FQ

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Nociceptin/Orphanin FQ (N/OFQ), the heptadecapeptide ligand and its specific target, the orphan receptor ORL1, recently named NOP, is considered a new neuropeptidic system that can contribute novel therapeutically useful agents for pain and other diseases of the central nervous and other systems. Numerous N/OFQ-related peptides were prepared and tested: they have contributed to establishing that the N-terminal tetrapeptide, N/OFQ(1-4) is critical for the message and the highly cationic C-terminal, N/OFQ (5-17) is important for the address domain. Moreover, the pharmacological spectrum of N/OFQ has been changed from agonism to partial agonism by the reduction of the peptide bond Phe1-Gly2 (in (F/G)-N/OFQ) and to antagonism by replacing Phe1 with Nphe (as in (Nphe1)-N/OFQ). Further progres has been made by changing Phe4 to pFPhe or adding two cationic residues, Arg14-Lys15 in place of Leu14-Ala15. From these findings, a series of 9 new ligands were prepared to improve potencies and understand which modification (alone or in combination) will favour agonism, partial agonism or antagonism. When tested in biological assays (mouse vas deferens, guinea pig ileum) as well as in cell transfected with human NOP, or in vivo (in the tail withdrawal and other assays), all conpouds were very active and mantained the pharmacologic spectra of the resective references. The most potent compounds were those with the combined changes in positions 4and 14-15, namely (pFPhe4,Arg14,Lys15)N/OFQ-NH_2, (F/G-pFPhe4,Arg14,Lys15)N/OFQ-NH_2 and the antagonist (Nphe1,Arg14,Lys15)N/OFQ-NH_2, without pFPhe in 4. They show long duration of action in vivo and appear to be promising new agents as analgesics and aquaretics.

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CHEMICAL SYNTHESIS AND BIOLOGICAL PROPERTIES OF PEPTIDES CONTAINING NUCLEOSIDES IN THE SIDE CHAIN

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In recent years peptides bearing natural or non-natural organic structures have found an increasing number of applications as research tools for molecular and cell biology. These new biomolecules may display several novel properties, such as: fluorescence emission, catalytic activity, altered hydrophobicity or bioaffinity, resistance towards biodegradation or ability to carry metal ions. Nowadays the preparation of new amino acids or peptides containing non-coded amino acids and different kinds of peptidomimetics or organic structures is one of the main goals of organic and medicinal chemistry. In our studies we have designed and synthesized some galanin and Tat peptide analogues modified with L-Lys containing nucleosides (adenosine, guanosine, uridine and cytidine) in the side chain. An acid-labile isopropylidene group was used to protect the 2'- and 3'-hydroxy groups of D-ribose. The 5'-hydroxy groups of 2',3'-O-isopropylidene-protected nucleosides were oxidized to the corresponding 5'-carboxylic acids using stoichiometric amounts of [bis(acetoxy)-iodo]benzene (BAIB) and catalytic amounts of 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO). Obtained 2',3'-O-isopropylidenenucleoside-5'-carboxylic acids were coupled with epsilon-amino group of L-Lys introduced into amino acid sequence of galanin and Tat peptide. Such modifications may provide more information about molecular mechanisms of ligand-protein or ligand-nucleic acid interactions. In the case of galanin analogues, this may lead to finding high-affinity antagonists of galanin receptors. This work was partly supported by a grant no. BW-8000-5-0189-4 from the University of Gdansk.

P536

EVALUATION OF TYROSINE CONFORMATIONAL RESTRICTION IN PTK PEPTIDE SUBSTRATES

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Phosphorylation is one of the main post-translational modifications that regulate many cellular processes such as cell cycle, growth and differentiation. It is not surprising therefore that altered functions of individual protein kinases underlie numerous pathological conditions with special reference to neoplastic growth. Therefore, the development of reagents suitable for the specific monitoring and/or inhibition of individual protein kinases is a crucial goal in the field of signal transduction and related pathologies. We have previously demonstrated that the replacement of the phosphorylatable tyrosine included in the EDDEYEEV peptide-substrate, with its gauche (+) or trans restricted analogues yielded substrates selective for Syk tyrosine kinase. These data confirm the hypothesis that the shape of biologically active peptides is strongly determined by the side chain orientation of the target residue. Side chain flexibility allows the molecule to adopt many conformations in solution, but only one fits into the enzyme active site. Although the peptide-analogues undergo conformational changes during the interaction with the kinase, the catalytic domain prefers the sequence, whose favourable conformation adopted in solution is close to the most suitable for the phosphorylation process. In these conditions, additional energy is not required for the side chain conformational rearrangements dictated by enzyme-peptide interaction. A new series of EDDEYEEV analogues, in which the phosphorylatable tyrosine was replaced by β-methyltyrosine, 5-hydroxy-2-aminoindan-2-carboxylic acid, 6-hydroxy-2-aminotetraline-2-carboxylic acid, or 6-hydroxy-tetrahydro isoquinoline-3-carboxylic acid were synthesised and analyzed. The side chain orientations of different tyrosine analogues determined by 1H-NMR and 13C-NMR experiments were correlated with the phosphorylation efficiency of different PTKs.

P537

4-S-FLUORO-L-PROLINE RESIDUE TO ENHANCE POLY-PROLINE TYPE II HELIX BINDING CONFORMATION OF SH3 BINDING PEPTIDES

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Eukaryotic signal transduction involves the assembly of transient protein-protein complexes mediated by modular protein-protein interaction domains. Small molecules that selectively disrupt these interactions can be used to dissect complex signalling pathways and most importantly to combat genetic diseases. Specific proline-rich sequences with consensus core motif PxxP adopt the conformation of poly-proline type II helix (PPII) upon binding to SH3 domains. For short proline-rich peptides, little or no ordered secondary structure is usually observed before binding interactions. The association of a proline-rich peptide with the SH3 domain indicates unfavourable binding entropy likely due to a loss of rotational freedom on forming the PPII helix. Our aim is to stabilize PPII helix by replacing proline residues of the HPK1 proline-rich decapeptide, PPPLPPKPKF (P2), with the 4-S-fluoro-L-proline (fp) at different positions (PfpPLfpPKfpKF, PPfpLPfpKPKF and PfpfpLfpfpKfpKF). The interactions of the fp-peptides with SH3 domain of HS1 protein were analysed quantitatively by Non-Immobilized Ligand Interactions Assay by Circular Dichroism (NILIA-CD). CD thermal transitions of the P2 analogues were measured to correlate their propensity to adopt PPII helix with their affinity for SH3.

P538

FLORESCENCE LABELLING OF PEPTIDES WITH 9-ISOTHIOCYANATOACRIDINE

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Considering the immense biochemical importance of peptides, it is desirable to detect minute of amounts of these molecules by extremely sensitive methods. Fluorescence labelling offers a chemical method for this purpose. Peptides labelled with fluorescent dyes are useful in a variety of biological screens including fluorescent polarization, time-resolved fluorescence and FRET assays. The labeling agent 9-isothiocyanatoacridine has a readily available reactive functional group –NCS which can spontaneuosly react with amino group and produce thiourea. This reaction utilized e.g. the Eddman degradation of peptides [1]. The reaction of 9-isothiocyanatoacridine with triglycine proceeds according the following equations (Fig.1) : We have investigated by spectrophotometry the kinetic of these reactions at four various temperatures, wavelenght = 417 nm. Fluorescence emmission spectra of labelled triglycine has 1,5 fold higher fluorescence than the starting isothiocyanate. The results of kinetic and fluorescence measurements confirm that 9-isothiocyanatoacridine [2] might be used as a new fluorescent labeling marker for peptide and proteine structures. Acknowledgements. This study was supported by the grant No. 1/1274/04 and 1/0432/03 from the Slovak Grant Agency. References: [1]Eddman, P., Acta Chem. Scand., 7-700, 1954, [2]Sabolová, D. et al., J., Collect Czech. Chem. Commun. 59, 1682-1689, 1994.

P539

COLLAGEN MODELS:SYNTHESIS, CONFORMATIONAL STUDIES AND APPLICATIONS IN WORKS OF ART

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Proteinaceous substances have been used as binding media in a variety of works of art as paintings, stone sculptures and wooden statues. Identification of proteins is essential in order to understand ancient technologies, determine the extent of decay and help in future restoration and preservation processes. Sequential polypeptides (Pro-X-Gly)n, where X represents amino residues Val, Lys, Glu, and (Hyp-Val-Gly)n were prepared and studied as models of collagen, which has been widely used as binding medium in works of art. Polymerization was carried out on the pentachlorophenyl active esters of the appropriate tripeptide unit and molecular-weight range of 10000-20000 was achieved. Tripeptides were synthesized by the solid phase peptide synthesis on a 2-chloro-trityl chloride resin using the Fmoc methodology. Conformational properties of the sequential polypeptides will be evaluated by circular dichroism (CD) studies in aqueous and organic mixtures and compared with type I collagen from calf skin. Immunization experiments in rabbits using collagen and its synthetic models are now in progress. The obtained antibodies will be used for the immunochemical detection of collagen and collagen fragments in artificially and naturally aged samples as follows: development of reference ELISA using artificially coated samples of collagen and models, as well as authentic samples. ELISA plates coated with collagen or collagen models will be "protected" by the anti-collagen antibodies obtained from animal immunizations and then will be subjected to artificial aging. This approach is expected to provide new insights in the conservation processes.

P540

PRODRUGS ACTIVATED BY INTRAMOLECULAR CYCLIZATION: DIPEPTIDE ESTERS OF PARACETAMOL

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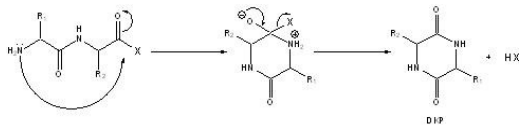
Paracetamol is an analgesic drug commonly considered as innocuous. However, its metabolism in the liver yields a toxin that causes cell death [1, 2]. The development of pro-drugs of paracetamol appears as a way to circumvent this problem, while improving other features, such as oral bioavaliability. We have been working on pro-drugs activated by intramolecular cyclization based on dipeptide carriers [3], since dipeptides can release the parent drug through diketopiperazine formation (Figure 1). Despite the advantages of pro-drugs favouring enzymatic drug release, this can be strongly affected by biological variability. Therefore, chemical mechanisms for drug delivery become attractive. We thus present a mild and simple method for the synthesis of dipeptide esters of paracetamol, and the corresponding in vitro kinetics of drug release at physiological conditions. Figure 1. release of HX through diketopiperazine formation. [1] Potter, W. et al., 1973, Pharmacol. Exp. Ther. 187: 203-210. [2] Nelson, S. et al. 1980, Biochem. Pharmacol. 29: 1617-1620. [3] Gomes, P. et al , Tetrahedron, 2003, 59, 7473-7480. Thanks to FCT (Portugal) for financial support to CECF (RM), CIQUP (PG) and through grant SFRH/BD/9272/2002 (CS).



P541

POTENT ENKEPHALIN AND DELTORPHIN ANALOGUES LACKING AN N-TERMINAL PHENOLIC HYDROXYL GROUP

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In opioid peptides, the N-terminal Tyr and the Phe at position 3 or 4 are important structural elements that interact with the opioid receptors. Recent structure-activity studies revealed that the introduction of 2', 6'-dimethyltyrosine (Dmt) in place of Tyr1 is a promising way to greatly enhance receptor affinity and biological activity. However the Dmt1-substitution generally has resulted in low receptor selectivity. We have recently demonstrated that 2', 6'-dimethylphenylalanine (Dmp) is an effective surrogate for both Tyr1 and Phe3 in mu opioid receptor-selective ligands [1, 2]. In the present study, we report analogues of delta opioid receptor-selective ligands, enkephalin (ENK: Tyr-Gly-Gly-Phe-Leu) and deltorphin II (DLT: Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2), containing Dmp at position 1, and their receptor binding and biological activities in comparison with the parent peptides. In the receptor binding assay, both [Dmp1]DLT and [Dmp1]ENK bound to the delta receptor with high affinity and selectivity and were nearly as effective as the corresponding Tyr1-peptides. The potency of the Dmp1-peptides on the MVD and GPI assays correlated well with the receptor binding date These results were in contrast to the tendency of corresponding Dmt1-peptides to have poor receptor selectivity. Taken together with the results with the mu receptor ligands, it is possible to conclude that Dmp can mimic the N-terminal Tyr in opioid peptides and the Dmp1-peptide is superior to the corresponding Dmt1-peptide in its receptor selectivity. References [1]Sasaki, Y. et al., Bioorg. Med. Chem., 11, 675-678 (2003). [2]Ambo, A. et al., Bioorg. Med. Chem. Lett., 13, 1269-1272 (2003).

P542

DEVELOPMENT OF LINEAR AND CYCLIC NMU-8 ANALOGS WITH SELECTIVITY FOR THE NEUROMEDIN RECEPTORS R1 AND R2

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Neuromedin U-23 (NMU), a 23-amino acid peptide found in many species, and its C-terminal octapeptide NMU-8 have been shown to affect various physiological functions. Recently, two subtypes of the NMU receptor, NMU-R1 and NMU-R2, from rat and human have been cloned. Both are members of the family of G-protein coupled receptors and both display a distinct anatomical distribution. Structure-activity relationship studies of NMU-8 (Tyr-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2) were performed to further characterize the involvement of the two receptors in mediating various pharmacological effects and to develop receptor selective agonists and antagonists as pharmacological tools. Peptides were screened against human and rat receptors in binding and functional assays. Peptide modifications performed included sequence truncations, multiple amino acid scans, incorporation of conformational backbone and side chain constraints and peptide cyclization. The guanidino group in the side chain of position 7 and a C-terminal amide were the only absolutely essential structural features for binding and activity at both receptors. Substitution of Arg5 by various non-basic residues or amino acids with extended aromatic ring systems in position 2 resulted in NMU-R2 selective analogs. In contrast aromatic amino acids in the third position yielded NMU-R1 selective peptides. Based on the SAR of linear analogs and the conformational analysis of the NMU-R2 selective cyclic analog Tyr-Phe-Leu-Phe-Arg-c(Lys-Arg-Asp)-NH_2, a pharmacophore model was developed and used for the mining of in-house and commercial databases to identify small drug-like organic molecules. Surprisingly, the SAR studies resulted in the discovery of a new class of potent k opioid antagonists.

P543

PROBING DNA-PEPTIDE INTERACTION FORCES ON THE SINGLE MOLECULE LEVEL

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Interactions between proteins and DNA are of eminent importance to all living organisms with the scope of these contacts ranging from rather unspecific binding to highly specific molecular recognition in the case of e.g. transcription factors. The transcription activator PhoB binds to the phosphate box in the promotor region of the phosphate regulon pho. PhoB from E. coli consists of a phosphorylation domain in the N-terminal region (1-127) and a DNA binding domain in the C-terminal region (128-229). The DNA-binding domain of PhoB structurally belongs to the family of „winged helix-turn-helix" proteins with the topology b1-b2-b3-b4-a1-b5-a2-a3-b6-b7. The amphiphilic helix a3 is the DNA recognition epitope with the two helices a2 und a3 being separated by a loop. A series of amphiphilic helical peptides was designed and synthesized. Binding of these peptides to DNA has been examined with surface plasmon resonance (SPR, Biacore) and – on the single molecule level – in atomic force spectroscopy studies (AFM). Single peptides with the amino acid sequence of helix a3 bind to an appropriate DNA fragment. Central goal of this project is to reveal molecular recognition phenomena between DNA and protein epitopes and to characterize them both macroscopically and on the single molecule level.

P544

SYNTHESIS OF VARIOUS PYRAZINONE RING-CONTAINING OPIOID MIMETICS AND STUDIES ON THEIR STRUCTURE-ACTIVITY RELATIONSHIPS

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Our aim is to develop novel opioid mimetics that are more potent and less addictive than morphine. Previously we demonstrated that the pyrazinone ring-containing opioid mimetic 3,6-bis[3'-Dmt-aminopropyl]-5-methyl-2(1H)-pyrazinone (A) exhibited potent antinociceptive activity following i.c.v., s.c. and p.o. administration. Boc-Dmt-OH was coupled through the amino function of pyrazinone, followed by removal of the Boc group to give 3-[4'-Dmt-aminobutyl]-6-[3'-Dmt-aminopropyl]- 5-methy-2(1H)-pyrazin one (B). Compound (B) bound to μ-opioid receptors with high affinity (Ki = 0.021 ± 0.002 nM) and less to δ-opioid receptors (Ki = 22.6 ± 5.9 nM) with a selectivity (Kiδ/Kiμ) of 1076. We synthesized various 3-[Dmt-aminoalkyl]-6-[Dmt-aminoalkyl]-5- methy-2(1H)-pyrazinone (I~IV). Compound (IV) bound to μ-opioid receptors with high affinity (Ki = 0.065 ± 0.003 nM) and to δ-opioid receptors (Ki = 1.46 ± 0.21 nM; Kiδ/Kiμ = 22.5.). In this presentation, the structure-activity relationship of pyrazinone ring-containing opioid mimetics will be discussed.

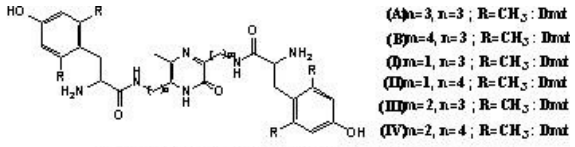

Fig.1 Structures of various pyrazinone derivartives

P545

THE STRUCTURAL BASIS FOR THE CATALYTIC ACTION OF BASE-EXCISION DNA-REPAIR ENZYMES

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In all living organisms, genomic DNA is continuously monitored by an array of DNA repair enzymes, in order to detect, remove and repair various DNA damages. The most common such pathway is characterized by base excision repair (BER), involving an initial catalytic action of DNA glycosylases. It remained to be clarified how these enzymes efficiently locate a single damaged base embedded in the vast excess of DNA, and how they selectively recognize and repair only specific damages. We have recently examined two related superfamilies of DNA glycosylases, the Fpg-superfamily and the EndoIII-superfamily. The two families have no sequence similarity but they overlap in substrate specificity. Two enzymes of each have been selected for detailed study in order to understand the relationships between sequence, structure, specificity and mechanism. Endonuclease-VIII and Fpg belong to the Fpg-superfamily. They share significant sequence homology but differ significantly in their damaged base target (oxidized T/C for EndoVIII, oxidized G/A for Fpg). Similarly, Endonuclease-III and hOgg1 belong to the EndoIII-superfamily, with sequence homology but different targets (oxidized T/C for EndoIII, oxidized G/A for Ogg1). Structural studies of these enzymes and their complexes with DNA demonstrated that the overall 3D structure of EndoVIII and Fpg is similar, while their base recognition pocket is different. Similar pattern was suggested for EndoIII and hOgg1. These studies indicated that all four enzymes share a similar mode of action, involving DNA distortion, damaged base eversion and sugar ring-opening by an amine-nucleophilic attack. The general biochemical significance of these results is discussed.

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SYNTHESIS AND ANTITUMOR ACTIVITY OF MODIFIED SOMATOSTATIN ANALOGUES

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The N. N. Blochin CRC of RAMS carries out the search of new antitumor agents among hypothalamic hormone analogues. The antitumor activity of such compounds is determined by inhibition of release of some steroid and protein hormones enhancing neoplastic growth. Besides, analogues of hypothalamic peptides were shown to act directly on the tumor. These drugs have the advantage of low toxity and high selectivity of their antitumor action. Synthetic analogues of hypothalamic hormones demonstrate high resistance to tissue enzymes wich is due to addition of non-natural aminoacids (D-Trp, p-di[2-chloroethyl]aminophenylalanine) to the peptide chain. This paper presents data concearing synthesis and study of biological activity of modified somatostatin analogues. Somatostatin analogues were synthesized by classical methods of peptide chemistry. Some of these compounds were demonstrated to inhibit secretion of the growth hormone, insulin, prolactin, glucagon and had cytotoxic activity on human ovarian carcinoma cell culture. Two of them inhibited the growth of breast adenocarcinoma Ca-755, melanoma B-16 and lewis lung carcinoma in mice (tumor growth inhibition, TGI = 80-90%) and increased of life span (ILS = 15-25%). Thus, the search of antitumor agents among cytotoxic peptide hormone analogues seems very promising.

P547

ANALOGUES OF ARGININE VASOPRESSIN (AVP) MODIFIED IN THE N-TERMINAL PART OF THE MOLECULE WITH BETA-HOMOPHENYLALANINE

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It is believed that the tyrosine in position 2 of arginine vasopressin (AVP) is involved in initiating the pressor response to AVP, while the phenylalanine in position 3 seems to play a role in the recognition of this hormone. However, in 1997 we synthesized several analogues of AVP modified in position 3 with L-1-naphthylalanine and results obtained suggested that position 3 is important also for intrinsic activity. In recent years, the design and synthesis of analogues of various naturally occurring compounds modified with beta-amino acids have acquired growing importance, as such analogues often exhibit interesting pharmacological properties. In the present study, as a continuation of our efforts to elucidate the role of positions 2 and 3 in AVP and its analogues, we designed and synthesized two peptides modified in these positions with beta-homophenylalanine (beta-Hph). In the next four analogues we combined these changes with substitution of Cys1 with 3-mercaptoproponic acid (Mpa) or 1-mercaptocyclohexaneacetic acid (Cpa). Cpa1 modification is commonly used to bring antagonistic activity to an analogue, whereas deamination of position 1 (Mpa) is well known as the most effective individual change in AVP which leads to enhanced antidiuretic activity. An additional stimulus to our studies came from our earlier results describing AVP analogues substituted in N-terminal part with N-benzylglycine, an amino acid which may be considered to be an analogue of Phe, in which the aromatic side chain is moved from C-alpha to nitrogen. All peptides were tested for pressor, antidiuretic and uterotonic in vitro activities.

P548

SYNTHESIS OF NEW N-SUBSTITUTED CINNAMOYL DERIVATIVES OF AMINO ACIDS

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New amides of ferulic acid with unprotected phenolic group and natural and unusual amino acids have been prepared in 50-75% yields by direct condensation in the presence of N,N-diisopropylcarbodiimid, HOBt and DIPEA. All compounds have been characterized by their UV, 1H-NMR and MS spectra. The antioxidant activity of the synthesized amides has been studied.

P549

INFLUENCE OF EACH AMINO ACID MOIETY WITHIN THE 313-320 CD41 DERIVED SEQUENCE TO ITS ANTIPLATELET PROPERTIES

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The GPIIb/IIIa (CD41/CD61) receptor, which is a member of the integrin family, is the most abundant receptor in the surface of platelets and can interact with a variety of adhesive proteins including fibrinogen, fibronectin and Von Willebrand factor. Fibrinogen binding on GPIIb/IIIa is an event essential for platelet aggregation and thrombus formation. Mapping of the fibrinogen binding domains on αIIb subunit suggested the sequence 313-332 as a alleged binding site. This region was restricted to sequence GPIIb 313-320 (YMESRADR) using synthetic octapeptides overlapping by six residues [Mitsios et al., Eur. J. Bioch., 2004, 271, 855-62]. The YMESRADR octapeptide inhibits ADP stimulated human platelets aggregation and binds to immobilized fibrinogen. In this study, we used the Ala scanning methodology within the sequence 313-320 aiming to evaluate the contribution of each amino acid to the antiplatelet properties of this region. We found that the substitution of Y, M or E by A does not affect the activity of the parent octapeptide, while the substitution of S substantially reduces it. The –RADR- motif seems to be the most essential for the biological activity of the GPIIb 313-320 site.

P550

ENDOMORPHIN-2 ANALOGUES WITH A NOVEL SPIROLACTAM TYPE BETA TURN MIMETIC

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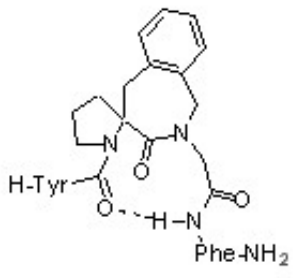
Endomorphin-2 (H-Tyr-Pro-Phe-Phe-NH_2) is a highly potent and selective agonist acting at m opioid receptors [1]. Although this tetrapeptide is highly flexible, its conformational analysis revealed the formation of certain secondary structural elements, such as different β- and γ-turns [2]. In one type of the β-turns an intramolecular hydrogen bond was also present. The importance of the formation of a β-turn was emphasized when the β-turn mimetic 4,7-dioxo-hexahydro-pyrazino[1,2-a]pyrimidine scaffold was used to mimic endomorphins [3]. This bicyclic scaffold mimics a type III 4'1 β-turn with retention of the bioactivity of endomorphin. A novel β-turn mimetic with spirobenzazepinone scaffold was recently prepared and its structure was examined with NMR methods [4]. A Boc-protected derivative compatible with SPPS was synthesized and then this spirobenzazepinone ring system was incorporated into the endomorphin-2 sequence. The effect of this structural modification on the bioactivity of endomorphin-2 is examined by radioligand binding assay. This new analogue will give further information on the importance of the β-turn as a structural element of endomorphins to achieve high μ opioid receptor affinity and selectivity. The preparation of this modified endomorphin-2, its receptor binding data and its agonist/antagonist properties will be presented. [1] Zadina, J.E. et al. Nature 386, 499-502 (1997). [2] Leitgeb, B. et al. Biopolymers, 68, 497–511 (2003). [3] Eguchi, M. et al. J. Med. Chem. 45, 1395-1398 (2002). [4] Poster presented by D. Tourwé at this meeting.



P551

NOVEL ENDOMORPHIN ANALOGUES WITH MU-OPIOID AGONIST AND DELTA-ANTAGONIST PROPERTIES USING DIMETHYL-TYROSINE AND ALICYCLIC BETA-AMINO ACIDS

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Endomorphins (endomorphin-1: Tyr-Pro-Trp-Phe-NH_2, and endomorphin-2: Tyr-Pro-Phe-Phe-NH_2) are potent and selective mu-opioid receptor agonists [1]. In order to improve their biological activity and stability against proteolytic enzymes, we designed and synthesized novel analogues with unnatural amino acids such as 2'6'-dimethyltyrosine (Dmt), in position 1 and 2-aminocyclopentanecarboxylic acid (Acpc) /2-aminohexanecarboxylic acid (Achc) in position 2. Competitive radioreceptor binding assay indicated that the endomorphin-2 analogues had high affinity for mu- and delta-opioid receptors in mouse bain membranes. Interestingly, while the Dmt-endomorphin-2 analogues stimulated [35S]GTPgS binding in recombinant cell membranes expressing the human mu-opioid receptors, they exhibited neutral antagonist or inverse agonist effects in recombinant cells expressing the human δ-opioid receptors. Thus, the Dmt-endomorphin-2 analogues appeared to behave as mixed mu-agonist/delta-antagonist. Acute intracerebroventricular injection with Dmt-endomorphin-2 and Dmt-Acpc-Phe-Phe-NH_2 produced potent analgesia in mice tail flick test. Replacement of Pro by alicyclic beta-amino acids increased significantly the resistance to proteolytic degradation in rat brain preparation. Supported by NKFP grant (27/2001). [1] Zadina, J.E. et al. Nature 386, 499-502 (1997).

P552

NOVEL MODIFICATIONS OF THE DMT-TIC PHARMACOPHORE PROVIDE NEW PEPTIDOMIMETIC μ-OPIOID ANTAGONISTS

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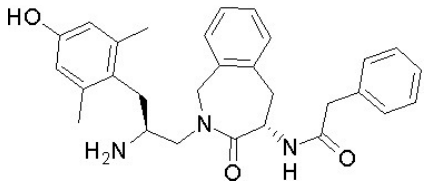
The use of the Dmt-Tic pharmacophore has resulted in peptide derivatives having a wide range of agonist/antagonist properties for the mu- and delta-opioid receptors.[1] C-terminal substitutions of this dipeptide provided further potent analogs. [2,3] Rather few modifications of the heterocyclic ring system of aromatic substitutions of Tic are allowed without resulting in a loss of potency. We have designed a new peptidomimetic based on the tetrahydro-2-benzazepin-3-one heterocycle to replace the Tic ring, and having a reduced Dmt amide bond. The four stereoisomeric peptidomimetics were prepared and their receptor affinity and selectivity as well as their activity in the GPI and MVD bioassays was determined. The compounds turned out to be fairly potent mu-antagonists, confirming the important role of the Tic-conformational constraint for antagonism. [1] P.W. Schiller et al. Biopolymers (Peptide Sci.) 51, 411-425 (1999) [2] D. Pagé et al. J. Med. Chem. 44, 2387-2390 (2001) [3] G. Balboni et al. J. Med. Chem. 45, 713-720 (2002).



P553

ANTI-PLATELET PROPERTIES OF CYCLIC (S,S) -CDC-AND (S,S)-CRC- CONTAINING PEPTIDES

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The final common step in platelet aggregation and thrombus formation is the binding of the plasma protein fibrinogen (Fg) to the receptor GPIIb/IIIa of the activated platelet. It has been shown that two copies of the Arg-Gly-Asp (RGD) sequence (95-97 and 572-574) of the fibrinogen α chain contribute to the recognition and binding event. RGD peptides inhibit platelet aggregation antagonizing fibrinogen binding through their interaction with the receptor. In a previous study we have shown that the cyclic scaffold (S,S –CDC- induces a favorable orientation of the R and D side chains leading to very potent anti-aggregatory agents [Stavrakoudis et al., Biopolymers, 2004, 56, 20-26]. In this work we report on the antiplatelet properties of various (S,S)-CDC- and (S,S) –CRC-) containing analogues. The peptides were found to be potent inhibitors of aggregation of stimulated by ADP, Thrombin, TRAP and Collagen human platelets, whereas the IC50 values of inhibition of the fibrinogen binding to washed platelets were 10 to 25 times lower than in the preceding case. Contrary to RGDS, some of the reported analogues did not inhibit the binding of the ligand mimetic monoclonal antibody PAC-1 to activated platelets, providing evidence for a different, compared to RGD peptides, mechanism of action.

P554

COMPUTATIONAL ANALYSIS OF THE CONFORMATIONAL FEATURES INDUCED IN PEPTIDE ANALOGUES CONTAINING THE (S,S)-CXC- MOTIF

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Cyclization via a disulfide bond is a widely used strategy to design constrained peptide analogues. The CXXC motif, which is the smallest and highly conserved unit in biological systems, has been extensively studied to estimate its propensity to form b-turns. Recently we have reported on the synthesis, activity and conformational preferences of highly constraint RGD analogues containing the (S,S)-CDC- motif [Stavrakoudis et al., Biopolymers, 2001, 56, 20-26]. The main goal of this study is to explore the influence of the (S,S)-CXC- motif to the relative orientation of the X amino acid and the X-2 or X+2 residue side chains. The structure of the peptides has been investigated by molecular dynamics methods. Our findings indicate that despite the nature of the X residue, there is a preference for an almost cis coplanar orientation of the X and either one of the adjacent to Cysteine residue side chains. The 11-membered cyclic structure does not favor any β-turn conformation while the backbone dihedral angles within the cycle are very constraint. The χ3 angle of the first Cysteine residue is distributed around +80º or -100º. It is concluded that the (S,S)-CXC- motif can be incorporated in peptide analogues in which the cis coplanar orientation of the corresponding amino acid side chains is desirable.

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SYNTHESIS OF CYCLIC PEPTIDES WITH BINDING AFFINITY FOR MC-4 RECEPTOR

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The melanocortin receptors (MC-1R to -5R) are involved in a wide range of physiological functions. The endogenous MC-Rs agonists ACTH and alpha-MSH contain a common sequence, His-Phe-Arg-Trp, which is an essential core for their biological activities. Bendnarek et al. [1] reported cyclo(CO-CH_2-CH_2-CO-His-D-Phe-Arg-Trp-Dab)-NH_2 (Dab=2,4-diaminobutyric acid) exhibited a potent agonist activity at hMC-4R. Our aim was to examine the ring size-binding affinity relationship of cyclic peptides, and extend that structure to design non-peptide agonist for MC-4R. In this presentation, we deal with the synthesis of cyclo(His-D-Phe-Arg-Trp-Z) (Z=omega-amino acids) and their binding affinities at MC-R4. The protected linear peptides were built up on a resin. After cleavage of protected peptides from a resin, formation of the lactam bridge was performed in solution. Binding affinity of peptides were measured with a [125I]NDP-alpha-MSH displacement assay in human recombinant HEK-293 cells. The IC50 values were 0.33, 0.25 and 0.67 microM for a 21-membered, a 20-membered and 19-membered cyclic peptide, respectively, while an IC50 of NDP-alpha-MSH was 0.11 nM. Reduction of the lactam ring to 17-membered decreased the affinity to an IC50 of 6.0 microM. Furthermore, the IC50 of a 16-membered cyclic peptide was more than 10 microM. The data suggest that the positioning of (His-D-Phe-Arg-Trp) sequence is necessary for the efficient interaction with MC-R4, and that proper ring-size contributes to the formation and stabilization of the ligand-receptor complexes. Secondary structure analysis of cyclic peptides by CD spectroscopy will be presented. [1] Bednarek, M. A. et al. (2001) Biochem.Biophys.Res.Commun., 286, 641-654.

P556

DEGRADATION OF ACTIVE ANALOGUES OF INSECT OOSTATIC DECAPEPTIDE

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Degradation of environmentally safe C-terminus truncated analogues of the decapeptide H-Tyr-Asp-Pro-Ala-Pro6-OH (Aed – TMOF), having proved oostatic activity with different insect species, was analyzed in ovaries and hemolymph of flash fly Neobellieria bullata. Using two analogues with the highest oostatic activity, i.e. the pentapeptide H-Tyr-Asp-Pro-Ala-Pro-OH(5P) and tetrapeptide H-Tyr-Asp-Pro-Ala-OH(4P), degradation products were followed by LC-MS or radio-HPLC. For radiochromatographic analysis, the peptides were labeled separately in different positions, [3H]Tyr1 or [3H]Pro3 or [3H]Pro5, using either catalytic exchange for tyrosine labeling or catalytic tritiation of the relevant sequences having in position 3 or 5 dehydroproline. Such different labeling made possible sensitive detection of degradation products in detail. Degradation of peptides was followed after incubation of the peptides (10nmols in 100µl of physiological solution) for different time intervals (1-90 min) at room temperature with hemolymph or ovarian homogenate, in the absence or in the presence of protease inhibitor. The hemolymph and the ovaries were collected from 4 days old females shortly before the experiment. Comparison of both chromatographic separation methods showed that using radio-HPLC we achieved higher detection sensitivity so that more degradation products were detected. In the case of both peptides 4P and 5P, the main degradation products were the des- Tyr1 peptides. The halflife in the hemolymph and ovaries was approximately 60 min and 5-30min, respectively. Another shortened sequences as well as metabolic product of tyrosin were also detected.

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SYNTHESIS OF TETRA- AND TRIPEPTIDE ANALOGS OF SUBSTANCE P FRAGMENTS AND THEIR PEPTOID-PEPTIDE HYBRIDS. STUDY OF THEIR ANTIRPOLIFERATIVE ACTIVITY IN VITRO

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Synthetic peptides are under investigation as possible anti-tumor agents. The Substance P (SP) analog [D-Arg1, D-Phe5, D-Trp7,9, Leu11]SP (antagonist D) and the C-terminal analog [Arg6, D-Trp7,9, MePhe8]SP6-11 (antagonist G) inhibit the tumor growth and cell proliferation of Small Cell Lung Cancer (SCLC) in vitro and in vivo [1,2]. In the present study a series of tetra- and tripeptide analogs have been synthesized, based on the sequence of antagonist G, using the stepwise synthesis or the fragment condensation method either in solution or in SPPS. Also a series of tetra- and tripeptoid-peptide hybrids [3] have been synthesized corresponding to the above peptide analogs. All the analogs were purified (HPLC) and identified (ESI-MS). The peptoid-peptide hybrids are oligomeric peptidomimetics containing the N-substituted glycine residue. This incorporation in peptide chains has been proved to improve their stability against proteases. Thus, in our case, the analogs have incorporated the peptoid monomer [-N(CH_2-Ph)-CH_2-CO-] (NPhe) instead of the corresponding amino acid residue of Phe. The in vitro antiproliferative activity of the above analogs was studied against the breast (T47D, SK-BR-3) and prostate (PC-3) cancer cell lines. The method used was the determination of 3H-Thymidine incorporation in the DNA of proliferating cells. References [1]Woll, P.J. and Rozengurt, E., Cancer Res., 50 (1990) 3968. [2]Cummings, J., MacLellan, A.J., Jones, D.A., Langdon, S.P., Rozengurt, E., Ritchie, A.A. and Smyth, J.F., Annals Oncol., 6 (1995) 595 [3]Kruijtzer, J., Hofmeyer, L., Heerma, W., Versluis, C. and Liskamp, R., Chem. Eur. J., 4 (1998) 1570.

P558

MIMICKING HEMOPROTEINS: A NEW SYNTHETIC METALLOENZYME BASED ON A FE(III)-MESOPORPHYRIN ACTIVE SITE FUNCTIONALIZED BY TWO CONFORMATIONALLY CONSTRAINED DECAPEPTIDES

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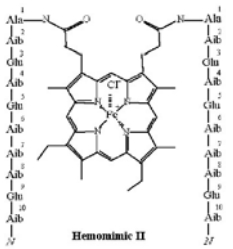
Model systems mimicking the catalytic properties of enzymes may help to understand the structural features of the system leading to chiral discrimination in electron transfer reactions. We present here a de novo designed artificial enzyme (Hemomimic II) based on a Fe(III)-mesoporphyrin IX (FemP) group covalently linked to two decapeptide chains. Each decapeptide comprises six Aib residues that force the peptide to attain a helical conformation and three Glu residues to improve the solubility of the catalyst in aqueous solutions. Fluorescence Energy Transfer experiments coupled with molecular mechanics calculations allowed to determine the most probable structure in solution, which shows a compact, protein-like arrangement of the peptide chains around the FemP group. The catalytic properties of Hemomimic II were tested in the oxidation of L- and D-Dopa by H2O2 (25ºC, pH=7.5). The enantiomeric substrates show similar catalytic rate constants, but different equilibrium constants.for the formation of the diastereomeric non covalent adducts L-Dopa Fe(III)mP and D- Dopa Fe(III)mP.



STRUCTURE ACTIVITY RELATIONSHIP STUDIES

P559

PLURAL FORMS OF FGL FRAGMENT OF NCAM: STRUCTURE AND INTERACTION WITH SPECIFIC ANTIBODIES

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Plural forms of peptides give the possibility to enhance biological activity of peptides. To study reasons responsible for that we have prepared fragment NCAM (Neuronal Cell Adhesion Molecule which fulfill important functions in the coarse of growth and fusion of axons in the process of neuronal system formation), namely pentadecapeptide EVYVVAENQQGKSKA (FGL-I), its dimer - FGL2(a,e)-Lys-X-Cys(SAcm)-A-NH_2 (FGL-II) and tetramer forms - FGL2(a,e)2-Lys-X-Cys-(A-NH_2)-S-S-(A-NH_2)-Cys-X-Lys(a,e)FGL2 (FGL-III), where X is alanine or e-aminohexanoic acid. Investigation of secondary structure of FGL-I by CD method has shown that in water FGL-1 has predominantly beta-structure (more 50 %) but in TFE no beta-structure but alpha-helical structure forms. At once FGL-II and FGL-III preserve in TFE great part of its original beta-structure. Stabilization of beta-structure in peptides FGL-II and FGL-III is a result of "near-by interaction" of two peptide sequences, having primordial beta-structure, connected with N-alpha- and N-epsilon- amino groups of lysine. Remarkable that there is no additional stabilization effect when we compare FGL-II and FGL-III in TFE. The effect of "near-by interaction" of peptides was also elicited in the coarse of SPPS of the peptides so as in the coarse of their interaction with anti-FGL-I antibodies.

P560

STUDY ON FLUORINE- AND/OR BORON-CONTAINING AMINO ACIDS AND THEIR RELATED COMPOUNDS AS TOOLS FOR DIAGNOSIS AND TREATMENT OF CANCER

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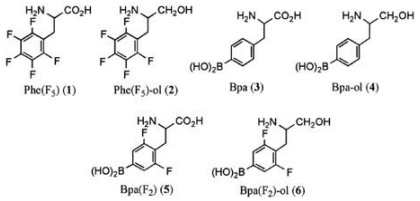
Dipeptides containing 3-(4-fluorophenyl)alanine [Phe(F)] seem to be selectively transferred into some kinds of tumor cells through the oligopeptide transporter. This fact suggests that the magnetic resonance imaging (MRI) based on 19F NMR measurement of the Phe(F)-containing peptides internalized into the tumor cells may be accessible as a promising means for diagnosis of cancer. From the standpoint of the treatment of brain cancer or melanoma, the boron neutron capture therapy (BNCT) based on the interaction of boron-10 (10B) isotope and neutron has been highly noted in recent years. In order to develop more practical tools for MRI and BNCT, we first synthesized a series of dipeptides or dipeptide mimics composed of fluorine- or boron-containing compounds such as 3-(2,3,4,5,6-pentafluorophenyl)alanine [Phe(F5)] (1), 3-(2,3,4,5,6-pentafluorophenyl)alaninol [Phe(F5)-ol] (2), 3-(4-boronophenyl)alanine (Bpa) (3), and 3-(4-boronophenyl)alaninol (Bpa-ol) (4). Next we designed and synthesized the novel compounds containing both fluorine and boron atoms in a single molecule such as 3-(4-borono-2,6-difluorophenyl)alanine [Bpa(F2)] (5) and 3-(4-borono-2,6-difluorophenyl)alaninol [Bpa(F2)-ol] (6). In the present paper we focus on the syntheses, 19F NMR, and tumor cell killing effect of various compounds related to 1 ~ 6.



P561

REPLACEMENT OF THE TYR1 HYDROXYL GROUP IN A CYCLIC ENKEPHALIN ANALOGUE WITH VARIOUS SUBSTITUENTS RESULTS IN POTENT OPIOID AGONISTS

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The cyclic enkephalin analogue H-Tyr-c[D-Cys-Gly-Phe(pNO2)-D-Cys]NH_2 is a highly potent opioid agonist with an IC50 of 35 pM in the guinea pig ileum (GPI) assay. The Phe1-analogue of this peptide showed 370-fold lower agonist potency (IC50 = 13 nM) in the GPI assay, indicating the importance of the Tyr1 hydroxyl group in the interaction with the mu receptor. In the present study we examined the effect of various substituents introduced in the para-position of the Phe1 residue of H-Phe-c[D-Cys-Gly-Phe(pNO2)-D-Cys]NH_2 on opioid agonist potency. The p-aminophenylalanine1- and p-nitrophenylalanine1-analogues displayed 2-fold and 3-fold potency increases, respectively, in the GPI assay, whereas the potency of the p-cyanophenylalanine1-analogue was unchanged. Interestingly, introduction of a methyl substituent produced a 6-fold increase in mu agonist potency. On the other hand, the p-carboxyphenylalanine1-analogue was only weakly active, most likely due to the presence of the negative charge. Twelve-fold and 26-fold potency increases were observed with the p-acetylphenylalanine1- and the p-carbamoylphenylalanine1-analogues, respectively. The latter compound showed an IC50 of 0.5 nM in the GPI assay and represents the most potent enkephalin analogue lacking the Tyr1 hydroxyl group reported to date. Parallel potency behavior was observed in the delta receptor-representative mouse vas deferens assay. These results indlicate that various substituents introduced in the para-position of Phe1 enhance opioid activity via hydrogen-bonding or hydrophobic interactions with the receptor. (Supported by grants from the NIH (DA-04443) and CIHR (MOP-5655)).

P562

THE ROLE OF THE SPATIAL POSITION BETWEEN AROMATIC RESIDUES AND THE CATIONIC SECTOR IN MEMBRANE-ACTIVE ANTICANCER PEPTIDES

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Idealized helical peptides (KAAKKAA)3 with three Trp residues located opposite to cationic sector displayed no anticancer activity, while peptides with three Trp located adjacent to cationic sector exhibited high anticancer activity, and high tumorcell-specificity versus normal cells. The active peptides induced leakage in liposomes composed of a mixture of negatively charged phosphatidylserine and zwitterionic phosphatidylcholine (POPC:POPS.) None of the peptides induced leakage in liposomes composed of POPC only. Tumor cells with an enhanced expression of phosphatidylserine on their outer leaflet were more sensitive to the active peptides than cells with a low phosphatidylserine expression. The results indicated a correlation between phosphatidylserine expression and peptide-induced cell cytotoxicity. Fluorescence experiments revealed that Trp residues were deeper inserted into the hydrophobic environment of the membrane of negatively charged vesicles than in neutral membranes. Binding-isothermes were obtained by titrating fixed concentrations of peptides with POPC:POPS liposomes, measuring change in fluorescence emission. CD data indicated that the two active peptides were more helical in POPC: POPS solution than the inactive peptide, suggesting that helicity propensity is important for anticancer activity. This was supported by NMR data showing that an active peptide had a rigid helical structure, whereas the inactive peptide showed a less degree of helical propensity with a more flexible N-terminus. The spatial position between cationic sector, involved in an electrostatic interaction with phosphatidylserine, and Trp residues seem to be important for an anticancer activity by helical amphipatic peptides.

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SEQUENCE-DEPENDENT INTERACTION BETWEEN AMPHIPHILIC HELICAL PEPTIDES AND DNA

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PhoB is a transcriptional activator that binds to the phosphate box in the promoters of the phosphate-regulon pho. It contains two functional domains, an N-terminal phosphorylation domain and a C-terminal DNA-binding domain, that recognizes a TGTCA sequence. The DNA-binding domain is a member of the family of winged helix-turn-helix proteins with the topology b1-b2-b3-b4-a1-b5-a2-a3-b6-b7. It is composed of three a-helices, a1 and a2, linked by a b-sheet, that are flanked by two antiparallel β-sheets, an N-terminal four-stranded β-sheet and a C-terminal hairpin, that interacts with the short β-sheet connecting α1 and α2 to form a three-stranded b-sheet. Helix α3 is the recognition helix. It contains several basic aminoacids on one side of the helix that interact with the major groove.[1] rl key while clicking in order to select more than one option. Video VHS Data Projector (Computer Projection) OverheThe synthesis of C-terminally modified PhoB 190-209 [E. coli], containing the α3-helix, was performed by loading 1,8-diamino-3,6-dioxaoctane to 2-chlorotritylchloride resin, coupling the first amino acid manually and using standard Fmoc/tBu SPPS procedures to complete the synthesis. Several amino acids that are known to be involved in the DNA-binding were replaced by alanine to lower the affinity to DNA, and some amino acids, that do not participate in DNA-binding were replaced with arginine to increase the affinity.[2] All peptides were purified by RP-HPLC using acetonitrile/water/TFA gradients, and the binding activities were studied using AFM and SPR technics.

P564

SYNTHESIS AND IN VITRO ACTIVITY OF HEPTAPEPTIDE OXYTOCIN ANTAGONISTS

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A Ferring's oxytocin antagonist, [D-Tyr(Et)2,Thr4,Orn8]dOT, atosiban, has been approved in Europe for the treatment of pre-term labour. Since atosiban has proven very efficacious in the clinic, a program was initiated to discover new peptides with improved pharmacological properties. The project resulted in the follow-up drug candidate, carba-6-[D-Trp2,aIle4,MeOrn7]dOT-(1-7)-ol, barusiban. The compound showed a significant improvement in potency and selectivity at the OT receptor in vitro, and in potency and duration of action in vivo as compared to atosiban. As part of the program, a series of analogs of barusiban modified in positions 2 and/or 4 was synthesized. Several methods to obtain the peptides were examined and the following procedure turned out to be the most efficient one: Commercially available Fmoc-Orn(Z)-OH was reduced to the corresponding alcohol, which was subsequently attached to chloride 2-chlorotrityl resin. The Fmoc group was replaced with the o-NBS group and the resulting resin-bound sulfonamide was N-methylated under Mitsunobu reaction conditions. The o-NBS group was then removed, and the desired peptides were synthesized using Fmoc chemistry. Cleavage from the resin afforded partially protected peptides, which were cyclized with PyBOP in DMF. Finally, the Orn side chain amino function was deprotected, and the crude peptides were purified by HPLC. The peptides were tested in vitro and IC50 and EC50 values were determined at the human OT and related receptors. Several analogs of barusiban with lipophilic residues at position 4 and/or the D-Nal residue at position 2 were found to be potent, selective, and efficacious OT antagonists.

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ANALOGUES OF ENDOMORPHIN-2 MODIFIED WITH 4-ALKYL-PSEUDOPROLINES IN POSITION 2

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The opioid peptides, endomorphins (endomorphin-1: Tyr-Pro-Trp-Phe-NH_2 and endomorphin-2: Tyr-Pro-Phe-Phe-NH_2), with high affinity and selectivity for the m-opioid receptor were discovered by Zadina et al. in 1997 [1]. For their potency, efficacy, and selectivity they were proposed as the endogenous mu-opioid receptor ligands. In order to assess the steric influence on opioid binding activity and selectivity we have synthesized analogues of endomorphin-2 with the proline residue in position 2 replaced with 4-methyl-pseudoproline derived from [R] and [S] alpha-methylserine. Tyr-[S]-MeSer[psipro]-Phe-Phe-NH_2 Tyr-[R]-MeSer[psipro]-Phe-Phe-NH_2 4-Alkylpseudoprolines[2] derived from alpha-alkyl-alpha-hydroxymethyl amino acids are structurally similar to proline, but besides the oxygen atom in the third position, they have a bulky substituent at the C-4 atom of the oxazolidine ring. In this presentation the synthetic strategy of new endomorphin-2 analogues, as well as biological activity will be evaluated. References: [1]J.E. Zadina, L. Hacker, L. Ge, A.J. Kastin, Nature 386, 499 (1997) [2]J. Katarzyńska, S. Jankowski, K. Huben, M.T. Leplawy, J. Zabrocki, Peptides 2002, Proc. 27th Eur. Peptide Symp., Edizioni Ziino, Napoli, Italy, 160 (2002). Supported by The Polish State Committee for Scientific Research (KBN), Grant 4 T09A 065 22.

P566

SHORTENED INSULIN ANALOGS: MARKED CHANGES IN BIOLOGICAL ACTIVITY RESULTING FROM REPLACEMENT OF TYRB26 AND N-METHYLATION OF PEPTIDE BONDS

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More than 142 million people all around the world suffer from diabetes mellitus; according to the World Health Organization this number is expected to double by 2025. More than 90% of these patients suffer from diabetes type 2, which may be caused by mutation in insulin receptor, defect in interaction between insulin and its receptor, or fault in insulin signalling downstream cascade. Recent studies have emphasized the importance of the C-terminal beta-strand domain of the insulin B-chain. This domain plays an important role in the self-association of insulin and in interaction of insulin with its receptor. Our study with new shortened insulin analogues focused on the triplet of aromatic amino acids PheB24-PheB25-TyrB26. All analogues were destetrapeptide(B27-B30)insulin-amides and were synthesized by trypsin-catalyzed coupling of desoctapeptide(B23-B30)insulin to synthetic tetrapeptides. We combined a single amino acid replacement in the position B26 with N-methylation of the peptide bond in various positions. Tyrosine, originally in position B26, was replaced by an amino acid with bulky side chain (Phe or His). The peptide bond between residues B24-B25 or B25-B26 was N-methylated and the resulting insulin analogues were compared with respective analogues without N-methylation. The effect of modifications was followed by two types of in vitro assays, i.e. by the binding to the insulin receptor of rat adipose plasma membranes and by the stimulation of the glucose transport. The analogues with histidine in position B26 were super potent and [NMeHisB26]destetrapeptide(B27-B30)insulin-amide is the analogue with the highest binding affinity of all insulin analogues described so far.

P567

THROMBIN INHIBITORS BUILT ON AN AZAPHENYLALANINE SCAFFOLD

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The central role in coagulation processes makes thrombin an important target for therapeutic agents designed for thrombus prevention. Orally active thrombin inhibitors have been reported recently, for example, the most advanced oral thrombin inhibitor ximelagatran. However, an ideal, clinically useful direct thrombin inhibitor is still a high priority in medicinal chemistry research. In earlier studies we have identified a novel series of noncovalent inhibitors built on conformationally restricted azaphenylalanine scaffold.[1-3] In this type of compounds, the α-carbon of the original peptidomimetic structure was replaced with nitrogen and the stereogenic center of the central amino acid was omitted, with the result that the overall conformation was changed.[4] In this report we describe synthesis, SAR and a binding mode of thrombin inhibitors with azaphenylalanine scaffold, which are based on amidoxime prodrug principle. [1]Zega A, Mlinšek G, Šolmajer T, Tschopp T, Stegnar M, Urleb U. Thrombin inhibitors built on an azaphenylalanine scaffold. Bioorg Med Chem Lett; 2004; 14: 1563-1567. [2]Zega A, Mlinšek G, Šepic P, Golič-Grdadolnik S, Šolmajer T, Tschopp T, Steiner B, Kikelj D, Urleb U. Design and Structure-activitv relationship of thrombin inhibitors with an azaphenylalanine scaffold: Potency and selectivity enhancements via P2 optimization. Bioorg Med Chem 2001; 9: 2745-2755. [3]Zega A, Trampuš-Bakija A, Fortuna M, Stegnar M, Tschopp T, Steiner B, Urleb U. Novel thrombin inhibitors with azaphenylalanine scaffold. Pharmazie 2001; 56, 638-685. [4]Gante J. Azapeptides. Synthesis 1989; 405-413.

P568

ANALYSIS OF PEPTIDES IN MOULD CHEESE DURING RIPENING

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The aim of this study is a isolation of oligopeptides from blue-veined cheese during different stages of ripening and sugests a method which allows their determination, especially electrophoresis. Extraction by water was used to isolate nitrogen compounds and the obtained extract was further fractionated and recleaned. First, high molecular peptides and proteins were precipitated by methanol, second, the methanol-soluble fraction was further fractionated (SPE, gel permeation chromatography). The fractions obtained by this procedures were then analysed using capillary electrophoresis and SDS-PAGE. The obtained results indicate that this procedure, described above, is applicable for isolation of oligopeptides from cheese allowing also to determine individual peptides. This is necessary in particular for monitoring of formation and origin of bitter peptides in cheese, which can influence negativelly final flavour of cheese.

P569

ANALOGUES OF NOCICEPTIN/ORPHANIN FQ.SYNTHESIS AND BIOLOGICAL ACTIVITY

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Nociceptin/orphanin FQ is a neuropeptide that selectively interacts with the opioid-like receptor 1 (ORL1 or OP4), a novel member of the opioid receptor family. The nociceptin-ORL1 system has been reported to modulate several biological functions. In-vitro studies have shown that the fragments N/OFQ(1-9)NH_2 и N/OFQ(1-5)NH_2 are inactive on electrically-stimulated preparations from rat, mouse and rabbit vas deferens. Previously, we have proved that the substitution of Lys with Orn in the molecule of N/OFQ(1-9)NH_2 leads to a slight but significant decrease of the evoked smooth-muscle contractions by high peptide concentrations [1]. The present study aims at synthesis of a series of new NC analogues and evaluation of their biological activity. The influence of the Lys-residues was assessed by their consecutive or complete substitution with Orn-residues. In order to estimate the impact of Ala and to increase the stability towards enzyme degradation, we synthesized the peptides [βAla7]N/OFQ(1-13)NH_2, [βAla11]N/OFQ(1-13)NH_2 and [βAla7,βAla11] N/OFQ (1-13)NH_2. Two analogues of N/OFQ(1-9)NH_2 were also synthesized, by chain prolongation by inserting Trp or Ile between Arg8 and Lys9. Thus, the sequence Arg-Trp-Lys and Arg-Ile-Lys was achieved. This sequence is present in a series of highly active hexapeptide ligands of the NOP-receptor, identified via combinatory library [2]. The peptide analogues were synthesized by SPPS, Fmoc-strategy. The structure-activity relationships of the new-synthesized peptides are discussed. [1]E.Naydenova, V.Jivkova, Y.Dobrinova, L.Vezenkov, R.Zamfirova, S.Todorov Compt. Rend. Acad.Bulg. Sci., 57, 1, (2004) 39-42, [2] G.Calo, R.Bigoni, A.Rizzi, R.Guerrini, S.Salvadori, D.Regoli Peptides, 21, (2000) 935-947.

P570

DESIGN, SYNTHESIS, CHARACTERIZATION AND CONFORMATIONAL STUDIES OF NEW POTENT ATHEROPROTECTIVE AMPHIPATHIC α-HELICAL PEPTIDE MODELS OF APOA-I

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Epidemiological studies have shown an inverse correlation of high density lipoprotein (HDL) and apolipoprotein (apo) A-I levels with occurrence of atherosclerotic events. Because of its antiatherogenic properties, apoA-I and amphipathic peptide mimetics have been the subject of intense studies.
We now report on the design, synthesis, characterization and conformational analysis of two amphipathic apoA-I models: Ac-ESK(Palm)KELSKSW10SEM13LKEK(Palm)SKS-NH_2 (1) and Ac-ESK(Palm)KELSKSM10SEW13LKEK(Palm)SKS-NH_2 (2). Positively (Lys) as well as negatively (Glu) charged residues distributed at positions i,i+3/i,i+4 constitute the polar face, while the lipid associating domain of the amphipathic a-helix consists of Met, Phe, Leu, Trp residues and palmitoyl groups. Fluorescence experiments confirmed our initial design. The synthesis was performed on Rink amide resin according to Fmoc-strategy, using an orthogonal system of protections. During purification with RP-HPLC three fractions (A,B,C) of each model were isolated with molecular mass 2648, 2743 and 2888 Da respectively. The MS findings were further confirmed by NMR spectroscopy. In 40% CD3CN, the aggregates of 1B, 2B, 2C in aqueous solutions , were dissociated. NOESY spectra of 1B and 2B showed that only the N-terminal 1-10 sequence was structured, while the intense NOE cross-peaks between almost all of the successive NHi/NHi+1 and various weak long range interactions dNN(i,i+2), dαN(i,i+3) and dαN(i,i+4) suggest the presence of α-helix in 2C. Based on the NMR findings, the modeling of the above compounds is also presented. CD studies revealed that 2C, in phospholipid environments, is characterized by a strong α-helix in accordance to NMR findings and its ability to inhibit LDL oxidation in vitro.

P571

NMR DETERMINATION OF PEPTIDE/MINIPROTEIN FOLDING RATES

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There is a great deal of current interest in the folding rates of small proteins and peptide models of elements of protein structures. The limited data to date indicates that some small proteins may fold in the 20–100 microsec time range while the time scales for helix formation and beta-hairpin formation are faster, 200-300 nsec and 1–20 microsec, respectively. There is one report of a miniprotein with multiple elements of secondary structure and a hydrophobic core with a folding time as short as 4 microsec. This system was a moderately stable representative of the Trp-cage fold motif. Recent data, for more stable Trp-cage species, suggest sub-microsec folding times can be achieved with this system. While there are excellent methods for determining folding times in the 100 microsec to 100 msec range, the options are more limited at the faster times associated with secondary structure elements and miniproteins. The latter typically rely on IR- or fluorescence-detected changes after nsec temperature or pH jumps, experiments that are technically demanding and thus unlikely to be applied to a series of mutants in order to ascertain whether a stabilizing mutation operates by increasing the folding rate or by decreasing the unfolding rate. We have found many instances in which simple "dynamic NMR" experiments can, in fact, probe exchange in the 0.4–20 microsec time domain for peptide conformational transitions. We will report mutational effects on folding rates, obtained using this method, for Trp-cage species, beta-beta-alpha miniproteins, and beta-hairpins.

P572

NON-COVALENT OLIGOMERS OF ADAN, AND ABRI, DETECTED BY ION-SPRAY MASS SPECTROMETRY, ARE INVOLVED IN GENETIC NEURODEGENERATIVE DISEASE

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Abri and Adan peptides are 34-residue peptide fragments derived from mutated form of the BRI gene on chromosome 13, and deposit in th brains of individuals with familial dementia and paralysis. We synthesised Abri and Adan in the reduced and oxidised, cyclic forms and showed that fresh solutions of the oxidised forms, which contain non-fibrillar oligomeric species, are toxic to neuronal cells in culture. Oligomers of AbriOx and AdanOx were found to be present in solutions of oxidised Abri and Adan, and in precipitates of AdanReduced at pH 7.4 by electrospray mass spectrometry. Oligomeric forms were analysed by using a Thermo LCQ Deca XP. A peak in Adan was found at m/z of 1625. High resolution zoom scan of ion at m/z = 1625 showed that the Δm between isotopic peaks is ~0.2 Da which indicates a dimeric species (MW = 8124) at charge state 5+., Moreover, ms2 of this species showed the appearance of a monomeric triple-charged species at m/z = 1355. Aged preparations of the reduced form of Adan showed the same non-covalent dimer peaks, and a covalent intramolecular dimer of 7+ charge at m/z of 1162. The results show that ESI-mass spctrometry has great potential for analysis of non-covalent oligomeric amyloids which are pathogenic in a number of human neurodegenerative diseases, and do not survive ionisation conditions in other types of ms sources.

P573

PREFERRED CONFORMATION OF TERT-LEUCINE

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Tle (tert-leucine or C-alpha-tert-butylglycine) is a Leu-isomeric, bulky, highly hydrophobic alpha-amino acid. A theoretical conformational study predicted a limited conformational space available to Tle. The preferred conformations are semi-extended or extended. To date, no detailed experimental conformational data are available for peptides heavily based on alpha-amino acids tetrasubstituted at C-beta, such as Tle. We synthesized a homo-oligopeptide series based on L-Tle, produced by DSM, and a variety of co-oligopeptides of L-Tle in combination with either Aib or Gly, to the hexamer level. Their conformational preferences have been assessed in solution (by FT-IR absorption, 1-H NMR, and CD) and for a few peptides in the crystal state (by X-ray diffraction) as well. Our conformational analysis of the Aib/L-Tle peptides established that L-Tle can accomodate, although with some difficulties, in a regular, right-handed 3(10)-helix. Conversely, the longest Gly/L-Tle peptides adopt a strongly intermolecularly H-bonded, antiparallel beta-sheet conformation. It is more difficult to unambiguously define the preferred conformation of the L-Tle homo-peptides. In CDCl3 and in the crystal state they tend to extensively form intermolecular H-bonds, which are however weaker that those typical of a regular beta-sheet. In MeOH and TFE solutions the homo-hexapeptide is essentially unordered, but, upon addition of water, its molecules tend to pack by virtue of hydrophobic interactions.

P574

STUDIES ON THE RACEMIZATION (EPIMERISATION) OF AMINO ACIDS AND PEPTIDES IN THE COURSE OF THE MAILLARD REACTION

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We had recently demonstrated [1] that heating of protein L-amino acids together with an excess of reducing sugars lead to the formation of relatively large amounts of the corresponding D-amino acids. It was postulated that amino acids bonded in intermediates formed in the course of the Maillard reaction are susceptable to racemization. In oder to test the hypothesis we have synthesized stable intermediates (Amadori compounds, fructosyl-L (and D)-amino acids and investigated the effect of amino acid side chain, chirality and temperature on racemization. Short peptides were included in the study. An unexpected high degree of epimerization was detected, in part, on brief heating of the model compounds. [1] Brückner H, Kirschbaum J, Pätzold R, in Peptides 2002 Edizioni Ziino, Bendetti E, Pedone E, (Eds.) (2002) 54-55

P575

HETEROLOGOUS EXPRESSION, CHARACTERIZATION AND STRUCTURAL STUDIES OF A HIGHLY HYDROPHOBIC PEPTIDE FROM THE HIV-1 CAPSID PROTEIN (P24)

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Proteins from inner core of HIV-1 are involved in crucial processes for viral infectivity such as assembly, disassembly, maturation, and life cycle of the virus. p24 is the major capsid protein of HIV and is initially expressed as part of the gag polyprotein. The association of gag proteins to the cell inner-membrane surface initiates virus assembly and induces budding from the host cell membrane, thus, p24 play an active structural role both as part of the Gag protein and in its mature form. In this sense, we have chosen a peptide from C-terminal region, TLRAEQASQEVKNWMTETLLVQNA, incorporated the additional residues (AMA), named rp24-3, which is part of the major region responsible for protein dimerization. This peptide possibly interacts with different molecules by hydrophobic contacts. The linear peptide and its cyclic variant were produced by recombinant strategy in Escherichia coli. The gene fragments were obtained by the synthetic gene approach and inserted into pET 32a to produce fusion proteins in the soluble form. The expression products were purified by Ni-affinity chromatography and submitted to proteinase cleavage. Subsequently, a reverse phase chromatography on C18 column was performed. The purity was checked by mass spectrometry and the sequence by Protein Amino-terminal Sequence Analysis. The secondary structure of the linear peptide was investigated by circular dichroism showing that it is differently structured in water and in buffer. The addition of methanol above 70% caused significant changes in the spectra. Its fluorescence spectra showed that the tryptophan is in a partially buried environment in all solutions.

P576

DESIGN, SYNTHESIS AND STRUCTURAL STUDIES OF CYCLIC ALPHA-CONOTOXINS

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Alpha-conotoxins are a family of disulfide-rich peptides isolated from the venom of cone snails, carnivorous snails from tropical marine environments. These peptides specifically antagonise nicotinic acetylcholine receptors which are implicated in a range of diseases including Parkinson's disease, Alzheimer's disease, depression and small cell lung carcinoma. However, like all peptides, conotoxins exhibit poor bioavailabilty and are vulnerable to proteolytic cleavage that diminishes their therapeutic potential. Clues as to how to achieve peptide stabilisation have come from recent research in our group on a particularly stable group of peptides from plants, the cyclotides. This family of peptides feature a cyclic backbone and three conserved disulfide bonds that result in a very stable structure. As a consequence, the cyclotides show remarkable stability against heat and enzymatic degradation and it is the cyclic backbone that is a major factor in conferring this stability. Therefore, to improve stability, we proposed that for alpha-conotoxins it may be feasible to form a bridge between the N and C terminii of the peptide backbone without perturbing the structure or activity of the molecule. Here, we describe the design, synthesis and NMR structures of several cyclic alpha-conotoxins and discuss the effect of cyclisation on stability and biological activity.

P577

CONFORMATIONAL ANALYSIS AND BINDING EPITOPE DETERMINATION, BY STD-NMR SPECTROSCOPY, OF THE MIR AND VICE B REGIONS OF ACHR SUBUNITS

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The nicotinic acetylcholine receptor (AChR) from fish electric organs and vertebrate skeletal muscles is a transmenbrane glycoprotein composed of five homologous subunits with stoichiometry a2bgd forming the cation channel. Monoclonal antibodies (mAbs) to the AChR have proved to be excellent tools for studying the molecule, as well as, the autoimmune disease myasthenia gravis, which is caused by anti-AChR autoantibodies. The majority of mAbs are directed against the a67-76 extracellular main immunogenic region (MIR). The very immunogenic cytoplasmic epitope, which contains the b subunit 380-391 phosphorylation site (VICE-b) is also the target of some mAbs. Various MIR and VICE-b analogues exhibit different affinity for the mAbs. A recently introduced technique, called saturation transfer difference (STD) NMR, provides additional information whether some peptide fragments are in direct contact with mAbs. Moreover the intensity of STD signals can be directly correlated with their binding affinity. We report now on the results of the STD studies of a few MIR and VICE-b analogues in the presence of mAbs. Taking into account all of the gathered data, the conformational features of these analogues, which have been previously studied by using transferred NOESY experiment and molecular dynamics calculations, will be discussed. This work was supported by the AFM, the CNRS and the Quality of Life program of the EU.

P578

CONFORMATIONAL DEPENDENCE OF THEORETICAL AND EXPERIMENTAL ALANINE CHEMICAL SHIELDING VALUES: AN COMPARATIVE STUDY

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Chemical shielding values have been determined for the all atoms of the backbone homoconformers, beta-turn and polyprolin-type structures as well as antiparallel and parallel beta-sheets of For-L-(Ala)n-NH_2 (n=1-12) model peptides using the GIAO-RHF formalism with the 3-21G(d), 6-31+G(d) and the 6-311++G(d,p) basis sets. Two-dimensional chemical shift plots have been generated using all nuclei of all residues. We found that nine regions, corresponding to major conformational clusters could be separated from each other by means of HA-CA, HA-CB and CA-CB chemical shift-chemical shift plots. Comparing the computed chemical shielding values with experimental chemical shifts of alanine we obtained moderately good agreement. Thus, we were also able to determine the nine backbone conformers of alanine by the help of the experimental chemical shielding values. The standard deviations of the measured chemical shifts for all conformers are considerably high therefore some conformers overlap with others. Nevertheless, the backbone conformations of most residues can be determined, if the chemical shielding data of the neighbouring residues are known. Experimental chemical shielding values of an apolar (valine), an aromatic (phenylalanine), a polar (serine) and two charged (glutamic acid and lysine) amino acids were also examined. We found that their HA-CA, HA-CB and CA-CB chemical shift-chemical shift plots are similar to the plots obtained in the case of alanine. Thus, our results can be applied for all amino acids. In conclusion, the secondary structure elements of the proteins can be reliably identified by means of CA, CB and HA chemical shifts.

P579

CONFORMATIONAL PROPERTIES OF A PEPTIDE-BRIDGE BETWEEN TUBULIN AND CELLULAR MEMBRANE

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The bulk of cellular tubulin is cytoplasmic, but a significant fraction is embedded in, or firmly associated with, the plasma membrane and other membranes. 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNP) has been identified as a possible linker protein for microtubules to the plasma membrane. (1) The CNP is both prenylated and palmitoylated, providing hydrophobic domains for membrane intercalation and showing both cytoplasmic and membrane localization. It was demonstrated that the synthetic 13-aa peptide dubbed A81 (SRKGGAMQICTII) has a key role in the CNP bridge-function since it contains the prenylation site responsible of the link with plasma membrane and it is able to promote microtubule assembly. To shade light on the conformational peculiarities which make possible the interaction between the 13-mer CNP C_terminus and the cell membrane we have undertaken a CD and NMR structural investigation of A81 in several different environments. We chose mixture of water and fluorinated solvent to test the conformational property of the peptide in an isotropic environment with stabilizing conformational properties. Then we used two different media generally known as membrane mimicking environments: SDS and DPC in water micellar solutions. The comparison of A81 structural properties in these two micellar environments points out interesting differences in the membrane mimicking capacity of the two solvents and on the specificity of the interaction of A81 with membranes. 1) Bifulco M., Laezza C., Stingo S., Wolff J., PNAS 1999, 4, 1807-1812

P580

ELECTRON TRANSFER ACROSS AIB OLIGOPEPTIDES

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Long-range electron transfer (ET) in proteins relies on the efficiency of the peptide chains to support charge transfer processes. Therefore, understanding how the electron tunneling between a donor (D) and an acceptor (A) separated by model peptide bridges takes place is a relevant issue. Although a variety of studies have outlined interesting features of the ET processes, there are still aspects that remain elusive. The role of the secondary structure is one of them, mainly because of the poor conformational control of the peptide bridges so far employed and/or the absence of intramolecular hydrogen bonding. We have recently started a research programme aimed at unraveling the ET dynamics across well-defined peptide bridges. As peptide bridges we chose alpha-aminoisobutyric acid (Aib) homo-oligomers, which are known for their propensity to form rigid 3-10-helices because of the tetrasubstitution at the alpha-carbon and the resulting restricted torsional freedom. We studied the dissociative ET from the D end (phthalimide or para-cyanobenzamide radical-anion donors) of the peptide to the A end (a peroxide). The ET results, obtained as a function of the number of Aib units of the peptide bridge, point to a very delicate balance between distance increase and concomitant intramolecular hydrogen-bond formation.

P581

INDUCED AXIAL CHIRALITY IN THE BIPHENYL CORE OF LINEAR AND CYCLIC BIP AND β2,2-BIP SHORT PEPTIDES

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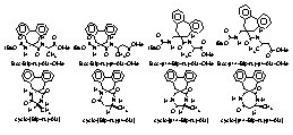
Bip and β2,2-Bip are atropoisomeric, conformationally labile amino acid residues with interconverting, non isolable (R) and (S) enantiomers. We have reported previously that in linear dipeptides with Ala, Val, Leu, Phe, MeVal and MeLeu residues at the C-terminal position of Bip, the onset of an equilibrium between two diastereoisomeric conformers with unequal populations could be observed by CD and 1H NMR. We have now expanded the study of the Bip linear dipeptides to a larger series of Bip/(L)-Ala, Bip/(L)-β3-Ala, β2,2-Bip/(L)-Ala and β2,2-Bip/(L)-β3-Ala linear and cyclic dipeptides, all synthesized by solution methods. In most cases, low temperature 1H NMR spectra showed closer populations of the two diastereoisomeric conformers for the β2,2-Bip peptides vs the Bip peptides. The CD bands of several compounds were too weak below 220 nm to be meaningful, and otherwise reflected an induced axial chirality lower for β2,2-Bip vs Bip, lower for β3-Ala vs Ala, and lower for the cyclic Bip dipeptides vs their linear counterparts.



P582

SPECTROSCOPIC STUDIES ON THE METAL ION BINDING LOOP OF A-LACTALBUMIN

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The strong and selective binding of Ca2+ to a large number of proteins is closely related to the regulation of many physiological functions. A good understanding of the selective impact of Ca2+ essentially implies the unraveling of determinants that contribute to Ca2+ affinity. Rationally designed low-molecular-weight rigid model compounds that contain the key liganding amino acids are very useful in such study. α-Lactalbumins are small, globular calcium-binding milk proteins. The molecule consists of two lobes: one lobe mainly contains α-helices, the other one is rather β-sheet-like [1]. The lobes are connected by a loop which is an aspartic acid rich decapeptide (-K79FLDDDLTDD88-): its Asp82, Asp87 and Asp88 bind to Ca2+ with side-chain carboxyl groups. The other two Asp-residues (Asp83, Asp84) are not involved in complex formation with Ca2+ but significantly contribute to the local charge. We synthesized (by solid phase peptide synthesis) a number of cyclic peptides of LA binding loop using different linkers in the ring closure and modified sequences (changing Asp to Ala) within series of the same ring size to study the influence of the ring size and the excess of the negative charges upon Ca2+ binding. This work reports the potential calcium binding of models using microcalorimetry (ITC), circular dichroism (CD) and Fourier transform infrared (FTIR) spectroscopies. [1] Calcium binding proteins, Volume II, Biological functions; CRC Press, Inc., (2000) 79-116. The research was supported by OTKA Grant T 037719 (Zs. M.), FWO-Flanders G-0180-03 (I. H.) and Reanal Fine Chemicals Co.

P583

FIRST DIRECT OBSERVATION OF C=O ... H-N INTRAMOLECULAR HYDROGEN BONDS IN 3(10)-HELICAL PEPTIDES

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The recently developed NMR pulse sequences for the direct measurement of scalar coupling through H-bond provides a useful tool for the observation of the H-bonding network in labeled peptides and proteins. We synthesized the following series of 5- or 6-peptides containing the strongly helicogenic D-(alphaMe)Val and Aib residues: Z-D-13C'-(alphaMe)Val-(Aib)2-D-15N-(alphaMe)Val-Aib-OtBu (I), Z-Aib-D-13C'-(alphaMe)Val-(Aib)2-D-15N-(alphaMe)Val-Aib-OtBu (II), Z-[D-13C'-(alphaMe)Val]2-(Aib)2-D-15N-(alphaMe)Val-Aib-OtBu (III), Z-13C'-Gly-(Aib)2-D-15N-(alphaMe)Val-Aib-OtBu (IV), and Z-Aib-13C'-Gly-(Aib)2-D-15N-(alphaMe)Val-Aib-OtBu (V). The X-ray structure of peptide (I) was solved and a regularly developed, left-handed 3(10)-helix found. We measured quantitatively the 3h-J-NC' scalar couplings through H-bonds. With the exception of peptide (IV), for all oligopeptides a signal indicating a H-bond typical of the 3(10)-helix was observed. For peptide (IV), containing a Gly residue at its N-terminus, it was not observed any signal. When an extra Aib residue was added at the N-terminus [peptide (V)], the peak corresponding to the H-bond between Gly(2) and (alphaMe)Val(5) was clearly observed. Finally, peptide (III) was designed to allow a direct detection of hydrogen bond either for the 3(10)- or the alpha-helical structure. Only the signal indicating the presence of the 3(10)-helix was seen, unequivocally demonstrating the propensity of these peptides for such type of folding.

P584

MONITORING THE INTERACTION BETWEEN THE HIV-1 GP120 V3-LOOP AND THE N-TERMINAL CCR5 PEPTIDE CONSTRUCTS THROUGH NMR SPECTROSCOPY

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The main cell population affected by the HIV-1 infection belongs to the CD4+ T-lymphocyte family. Recent evidence indicates that the majority of these cells that die due to HIV-1 are not actually infected by the virus. Instead, these cells are being led to programmed cell death after activation of apoptotic mechanisms by the virus or its components. A potential mechanism by which the virus appears to deregulate the physiological function of these cells during the process of antigen presentation has been identified, using synthetic peptides. Ionic interactions between the V3 domain of the HIV-1 coat glycoprotein gp120 and the N-terminal of the chemokine receptor CCR5 play a prominent role in this process [Baritaki S., Zafiropoulos A., Sioumpara M., Politis M., Spandidos D.A., Krambovitis E. BBRC 298: 574-580 (2002)]. NMR spectroscopy has been applied in order to probe the structural and physicochemical determinants of the interaction between peptides that represent the crown of the V3 loop of the HIV-1 gp120 and the N-terminal of the chemokine receptor CCR5. Conformational studies of the peptides, analysis of their interaction during titration experiments and Kd determination, have been performed. Although V3 peptide seems to be flexible even at low temperature the free CCR5 22-mer construct is giving rise to numerous NOEs at 286-278K. Preliminary data analysis indicates a weak-type interaction between V3-CCR5 (Kd in the range of 0.1-0.2 M) and suggests a remarkable role for a 7-residue CCR5 N-terminal domain and particularly for Tyr3, which is the first among the four CCR5 tyrosines.

P585

SOLUTION STRUCTURE ELUCIDATION OF A BOWMAN-BIRK INHIBITOR FROM LENS CULINARIS BY MEANS OF 1-H NMR AND MOLECULAR DYNAMICS CALCULATIONS

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Bowman-Birk inhibitors, particularly abundant in legume seeds, have recently been taken into consideration for their beneficial activity as anti-inflammatory and tumour-preventing agents [1]. We have isolated and purified from lentil seeds one peptide belonging to this class of inhibitors by several chromatographic steps including Trypsin Affinity Chromatography (TAC). The isolated protein has a high anti-trypsin and anti-chymotrypsin activity, as measured from kinetic studies, and corresponds to 14% of total inhibition activity of the crude extract. Several trypsin inhibitors with different activities have actually been isolated from lentil [2]. This cysteine-rich protein (67 amino acid residues)is highly homologous to other Bowman-Birk Inhibitors (Pisum sativum: 85%; Glycine max: 53%). We report on our CD and 1H-NMR studies performed on the purified protein. We have been able to produce an almost complete sequential assignment by means of two-dimensional homonuclear correlation techniques (2D-COSY-DQF, 2D-TOCSY and 2D-NOESY). We identified long-range interactions that confirm the characteristic beta-sheet-rich folding. In particular, the anti-chymotryptic region between Cys-38 and Cys-50 was fully characterised by short- and long-range NOEs. The folding has been modelled by homology with other inhibitors of known X-Ray and solution structures [3,4] with the aid of the observed long-range interactions. The model was subsequently optimised by nanosecond-scale molecular dynamics calculations performed with the explicit inclusion of solvent molecules. References: [1] Kennedy,A.R. (1998) Pharmacol. Ther., 78 (3), 167-209 [2] Weder,J.K.P., Kahleyss,R. (2003) J. Agric. Food Chem., 51, 8045-8050 [3] Catalano,M., Ragona,L., Molinari,H., Tava,A., Zetta,L. (2003) Biochemistry, 42, 2836-2846 [4] Werner,M.H., Wemmer,D.E. (1992) Biochemistry, 31, 999-1010

P586

SYNTHESIS AND CONFORMATIONAL STUDY OF (BIN)N AND (β2,2-BIN)N HOMOPEPTIDES BASED ON ATROPOISOMERIC α- AND β-AMINO ACIDS DERIVED FROM 1,1'-BINAPHTHYL

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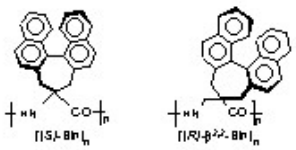
The exploitation of Cα,α-disubstituted glycines with well-defined stereochemical properties have greatly enhanced the scope of peptide design by imposing local restrictions on backbone conformation. Oligomers of β-amino acids have also emerged in the past few years as capable of forming highly stable secondary structures. In this connection, inter alia we have previously shown that Bin, a novel Cα,α-disubstituted glycine possessing only an axial chirality, behaves as a 310-helix inducer and can give rise to efficient rigid helical peptide fluorophores. Here, we present our results on the synthesis and a conformational study of homo-α-peptides based on (S)-Bin as well as of linear and cyclic homo-β-peptides based on (R)-β2,2-Bin. Spectroscopic experimental analyses of the [(S)-Bin]n homo-peptides in solution performed by CD, FT-IR absorption, and VCD, supported by VCD calculations, identified the most largely populated secondary structures as right-handed b-turns which eventually evolve to 310-helices in the highest oligomers. In contrast, it is hard to determine the secondary structure of the [(R)- β2,2-Bin]n peptides from their CD and FT-IR spectra.



P587

CONFORMATIONAL STUDIES ON GLYCOSYLATED ASPARAGINE-OLIGOPEPTIDES BY NMR SPECTROSCOPY AND MOLECULAR DYNAMIC CALCULATIONS

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Despite protein glycosylation represents one of the most significant post-traslational events and is involved in important biological processes such as cellular and molecular recognition, intercellular communication, cell control and growth, the consequence of glycosylation on the conformational and molecular properties of proteins are not yet fully understood. In order to study the effect of the sugar moieties on the peptide conformation, three series of homo oligomers of increasing chain length: Boc-(Asn)n-NHMe (n = 2,4,5), Boc-(GlcN2'Ac-β-Asn)n-NHMe (n = 2,4,5,8) and Boc-[GlcN2'Ac(Ac)$_3$-β-Asn]n-NHMe (n = 2,4,5) were synthesized and investigated by NOE experiments and Molecular Dynamic Calculations (MD). Sequential NOEs and medium range NOEs, including the (i,i+2) interactions, were detected by ROESY experiments and quantified. The inter-proton distances are higher than those typical of β-turn structures. Owing to the large conformational motions expected for linear peptides, MD simulations were performed without NMR constraints, with explicit water and by applying different treatments of the electrostatic interactions. In agreement with the NOE results, the simulations showed, for all peptides, the presence of both folded and unfolded structures. The existence of significant populations of β-turn structures can be excluded, but two families of structures were more frequently recognized. One family with sinusoidal or S-shape forms, and an other one of large turns, together with some more or less extended conformations. The results achieved in water and in DMSO are compared and discussed, together with the effect of glycosylation. Acknowledgments Financial support from the MIUR (Cofin 2002 and 2003) is gratefully acknowledged.

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FIRST OBSERVATION OF SEVEN HELICAL BUNDLE FORMATION IN CRYSTAL STRUCTURE OF HELICAL PEPTIDE SCAFFOLDS CONTAINING DEHYDROPHENYLALANINE RESIDUE

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De novo designed peptide based super secondary structures can provide scaffolds for the incorporation of functional sites as in proteins. Weak interactions leading to the self-association of helices of similar screw sense have been probed by the structure determination in crystals of two peptides. Circular Dichroism studies also reveal the preferential formation of right-handed 310- helical conformation in both the peptides. The crystal structures of the two peptides have been determined to atomic resolution and refined to R-factor of 8.15%and 4.01% respectively. Our aim was to critically analyze the packing of the helices in the solid state so as to design super secondary structural motif such as two, three and four helical bundles based on the helix-helix interactions. An interesting finding is that a packing motif could be identified in both the structures, in which a given 310 -helix is surrounded by six other helices reminiscent of transmembrane seven helical bundles. The outer helices are orientated either parallel or antiparallel to central helix. The parallel helices interact through C-H…O and C-H…Pi hydrogen bonds while the antiparallel helices are interacting via C-H…Pi hydrogen bonds. Common to both the structures is the hydrophobic interaction between the pairs of leucine residues. The packing of the helices in the solid state seem to provide clues for the design of super secondary structures such as two, three or four helix bundles by connecting them through suitable linkers such as tetraglycine segments.

P589

CYCLIC PEPTIDES AS AFFINITY LIGANDS IN FACTOR VIII PURIFICATION

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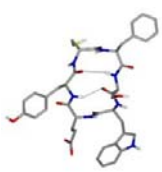
Haemophilia A, one of the most common bleeding disorders, is the result of an inherited deficiency of Factor VIII, a protein involved in the blood coagulation cascade [1]. For medical treatment, patients are given doses of Factor VIII derived from either blood plasma or recombinant cells. Factor VIII is usually concentrated by affinity chromatography, employing either monoclonal antibodies or oligopeptides as ligands [2,3]. We report the synthesis and evaluation of short peptide sequences showing good Factor VIII binding properties as well as improved proteolytic stability. Immobilisation is achieved by selective linkage of a cystein thiol function on an epoxide functionalized resin. Furthermore, we examined the structure of two cyclic peptides binding Factor VIII by NMR and distance-geometry calculations. Structure activity relationships will be presented.[1] Bolton-Maggs, P. H. B.; Pasi, K. J. Lancet 361, 1801 (2003). [2] Amatschek, K; Necina, R.; Hahn, R.; Schallaun, E.; Schwinn, H.; Josic, D.; Jungbauer, A. J. High Resol. Chromatogr. 23, 47 (2000). [3] Klinge, J.; Ananyeva, N.M.; Hauser, C. A. E.; Saenko, E. L. From Basic Science To Clinical Practice 28, 309 (2002).



P590

TRUE FOLDING OF CONFORMATIONALLY CONSTRAINED ß-PEPTIDES: CHAIN LENGTH-DEPENDENT SECONDARY STRUCTURE

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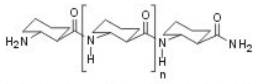
The β-peptides are currently attracting increasing interest by virtue of the wide range of their potential applications, due to their propensity to adopt compact ordered conformations. The β-peptides containing cyclic side-chains are among the most thoroughly studied models in foldamer chemistry. Homo-oligomers built up from trans-ACHC monomers having protecting groups at both ends are known to form a highly stable left-handed 14-helix, which in fact is so stable that it is always predominant when the chain is longer than three monomers. This finding raises the question of whether these oligomers exhibit real folding or whether the conformational space is too constrained to allow partly folded or other stable secondary structures. In order to answer this question, the potential energy hypersurface of trans-ACHC homo-oligomers without protecting groups (Scheme) were probed by calculating the conformational energies of the theoretically possible 10-, 12- and 14-helices at ab initio HF/6-311G** level. The calculations revealed the 14-helix to be the most stable structure for 4 and 5, whereas for 3 the 10-helix is the preferred conformation. Experimental work was performed to test this calculated trend. The β-peptides in question were synthesized on a solid support, and their structures were characterized by NMR methods. The results were found to be in good accordance with the prediction. This chain length-dependent secondary structure formation proves that the studied oligomers undergo a true folding process. The explanation is the balance between the local steric and the remote H-bond contributions to the conformational energy.



1, n = 0; 2, n = 1; 3, n = 2; 4, n = 3; 5, n = 4

P591

CONFORMATIONAL ANALYSIS OF EFRAPEPTIN C

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The efrapeptins are a class of peptide antibiotics produced by the fungus Tolypocladium niveum and other members of this species. They are inhibitors of F1-ATPase and also active against the malaria pathogen Plasmodium falciparum. Currently, six closely related sequence analogues (efrapeptin C-G) are known . They are rich in a,a-dialkylated amino acids, contain one b-alanine and three pipecolic acid residues . The N-terminus is acetylated, while the C?terminus bears an unusual cationic headgroup derived from leucinol and DBN . Conformational analysis of efrapeptin C was started to derive structure-activity relationships for this kind of peptides. The conformation of efrapeptin C in solution was first studied in the unpolar solvent 2,2,2-trifluoroethanol which provides basic information about the secondary structure and dynamics in solution. The MD simulations show 310-helical regions between the amino acids Pip-1 to Aib-5 and Aib-9 to Aib-15. These two helices are linked by a highly flexible region between Leu-6 and Gly-8. The presence of the nonproteinogenic amino acids Aib and Pip complicates the interpretation of the NMR data and reduces the amount of restraints for the MD simulations, especially for the N-terminal amino acids Pip-1 to Aib-5. Additionally, problems in assigning the methyl groups of Aib and Pip occur. New conformational studies were performed in DMSO-d6 to approve a complete assignment. Also efrapeptin C analogues with 2H-labeled Aib residues at definite positions were synthesized to improve the structural information for the N-terminal region (Pip-1 to Aib-5) in efrapeptin C .

P592

NMR STUDIES OF THE N-TERMINAL SEGMENT OF S100C/A11 PROTEIN

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The S100C/A11 protein is a member of the S100 family of calcium-binding proteins. S100C/A11 was first identified in chicken gizzard smooth muscle; it has also been referred to as calgizzarin. About 15 proteins have been found thus far in the S100 family, all of which are relatively small proteins with a molecular mass of approximately 10 kDa. S100 family proteins have a pair of EF hand motifs, and their activities are modulated by Ca++ concentrations. We have recently reported that S100C/A11 is phosphorylated in the cytoplasm of human fibroblast cells reached confluence, and is then translocated to the nuclei. The S100C/A11 localized in nuclei remarkably inhibits DNA synthesis with increases in cyclin-dependent kinase inhibitors such as p21(CIP1/WAF1), which may play an important role in the induction of growth arrest and cell differentiation. Moreover, these functions of S100C/A11 require the phosphorylation at Thr10 and Ser94 in advance. Interestingly, not only full length of S100C/A11 protein but also truncated peptide, for example, the peptide including the N-terminal 19 residues, undergoes the phosphorylation by incubation in cell extracts. In the present study, we prepared unphosphorylated and phosphorylated peptides including the initial 19 amino acids and analyzed their conformations using NMR and CD spectra. While both two peptides adopted helical conformation in the presence of TFE, some differences in their stability and the ability of dimerization were found. Our results may indicate the significance of the changes induced by the phosphorylation.

P593

PREDICTION OF HELICAL SCREW SENSE OF OLIGOPEPTIDES CONTAINING CHIRAL ALPHA,ALPHA-DISUBSTITUTED ALPHA-AMINO ACIDS: COMPUTATIONAL STUDY

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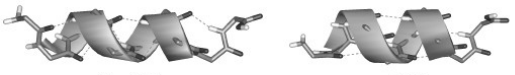
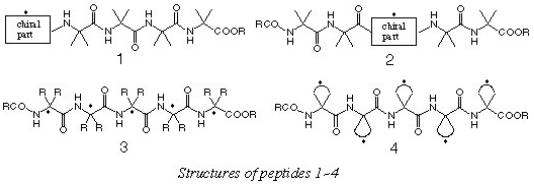
Computational simulation of the conformation of peptides presents an interesting challenge to predict the conformation for the design of functionalized and bioactive molecules. Peptides comprising non-proteinogenic amino acids (alpha,alpha-disubstituted alpha-amino acids, beta-amino acids, gamma-amino acids, etc.) are currently attracting attention mainly because of their stable secondary structure. Recently, we have shown the Monte Carlo conformational search using MacroModel is useful for conformational study of oligopeptides prepared from alpha,alpha-disubstituted alpha-amino acids. Here we report computational simulation using conformational search calculation could predict the helical screw sense of oligopeptides 1~4 containing chiral alpha,alpha-disubstituted amino acids.



3_{10}-helix α-helix

Typical helical structures of peptide



1 2 3 4

Structures of peptides 1-4

P594

STRUCTURAL STABILITY STUDIES OF THE β2-L1-β3 FRAGMENT USING NMR SPECTROSCOPY - INVESTIGATIONS OF THE HCC DIMERIZATION MECHANISM

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Human Cystatin C (hCC) is a natural protein inhibitor of cysteine proteases and is involved in the formation of amyloid fibrylls[1]. Formation of dimer, presumably the first stage of hCC oligomerization, results from 3D swapping of the N-teminal domain[2]. It is initiated by partial unfolding of the monomeric cystatin, resulted from conformational change of a hinge region (Fig 1.).The hinge element of hCC is L1 loop of β hairpin structure consisting of β2, β3 strands and L1 loop[2]. The initiation factor of the hinge fragment straightening is unknown. It was suggested that the main cause is the presence of distortions in the L1 loop backbone[3]. In this case the native-like secondary structure of peptide of the hinge region sequence would not be conserved in the solution. In order to test the hypothesis we conducted the NMR studies of structural stability of the hCC fragment, consisted of L1 loop with β2 and β3 strands. [1] Olafsson et al. J.Exp.Clin.Invest. 7,70 (2000) [2] Janowski et al. Nat.Struct.Biol. 8,4 (2001) [3] Staniforth et al. EMBO J. 20,17 (2001)

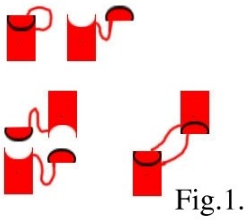

Fig.1.

P595

CONFORMATIONAL VARIABILITY OF DEHYDROPEPTIDES WITH THE DEHYDRO-PHE AND DEHYDRO-ALA RESIDUES: A CD AND NMR STUDY

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During studies on cathepsin C inhibitors we investigated a series of DAla- and DPhe-containing dehydropeptides. It was found from CD studies that p-nitroanilides (pNA) of four of them: Boc-Gly-DAla-Phe-pNA (I), Boc-Gly-Gly-DAla-Phe-pNA (II), Gly-DPhe-Gly-Phe-pNA (III), and Boc-Gly-DPhe-Gly-Phe-pNA (IV) change gradually their conformation in solution on standing. Time-dependence of the CD spectra of those peptides in methanol, acetonitrile, and trifluoroethanol was measured. It was found that I and II change their conformations in all three solvents used, while for III and IV this occurs in methanol and trifluoroethanol only. These changes start to show a day after dissolving of the peptides and they take place on a day-after-day basis. The largest changes in the CD spectra were observed for the pNA moiety in I and II, and for pNA and DPhe in III and IV. This indicates that these regions of the peptides studied undergo principal conformational changes. The NMR results confirm the CD observations but they differ distinctly as far as the rate of conformational changes is concerned. First changes in the NMR spectra were detected only 3 weeks after solutions of the peptides were prepared. The largest changes in the NMR spectra are connected with the t-butyl group orientation against the rest of the molecules.

P596

EFFECT OF COUPLING OF FUNCTIONAL PEPTIDES WITH THE SIGNAL PEPTIDE ON THEIR CONFORMATIONS

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Recently we were studying the influence of peptide fragments of the Fas and TNF-R1 receptors death domains on the inhibition of apoptosis. Twelve peptides, encompassing the 225-243 and 270-288 regions of Fas and the 401-419 region of TNF-R1, were synthesized. Death domains are intracellular parts of Fas and TNF-R1 so their peptide fragments must penetrate the cell to evoke a biological effect. To place a peptide in the cell signal peptides are used. It is important for a signal peptide not to influence the conformation of the functional peptide and thus its biological activity. In this connection we synthesized another 12 peptides in which the functional peptides were coupled to the signal peptide of the Kaposi's sarcoma. We wanted to check if the both parts of hybrid peptides retain their conformations. CD spectra of 12 functional peptides, 12 hybrid peptides, and the signal peptide in methanol were measured. The CD spectra of the hybrid peptides were compared with the sum of the CD spectra of the functional and signal peptides. An identity or a close similarity of spectra of the hybrid peptides and the sum of spectra of their components indicates that the functional and signal peptides retain their initial conformations. CD spectra of 12 functional and 12 hybrid peptides were also measured in trifluoroethanol and water.The signal peptide could not be measured in these two solvents because of its poor solubility. Still, some conclusions on the subject studied could be drawn here from another approach as well.

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SYNTHESIS AND INVESTIGATIONS OF NEW ACTIVE PEPTIDES CONTAINING CONVERTED SEQUENCES OF NATURAL CYCLOLINOPEPTIDE X: NMR, CD, AND BIOLOGICAL STUDIES

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Cyclolinopeptide X (CLX) is a natural peptide isolated from flax which plays presumably the defence role in plant seeds. Its sequence cyclo(XPPFFILL) is able to convert to a new hexapeptide cyclo(XPPILL) after oxidative X ring breaking and exclusion of the FF dipeptide moiety. Linear analogs of the above new cyclic sequence of the general formula XPPILL, in which the X residue is substituted by γ–aminobutyric acid, lysine, N-methylproline and the glycine-N-methylglycyl dipeptide moiety were synthesized. The NMR and CD measurements show that conformational equilibria of these peptides in solution are dominated by unordered structures. The preliminary investigations of two analogs (X = γ-aminobutyric acid, glycyl-N-methylglycyl dipeptide) showed their strong antiproliferative activity against lymphocytes induced to proliferation by known mitogen concanavalin A. The inhibition of mitogenic activity of concanavalin A by these peptides is comparable to that revealed by cyclosporine A (CsA). This result strongly supports our observation of the oxygen induced convertion of the parent structure of CLX to a smaller cyclic hexapeptide containing a new peptide bond. This new peptide forms active polycondensate which may be preventive against the pathogen cell development in plants. The results of our investigations on this group of peptides will be presented.

P598

PEG-AMYLIN FRAGMENTS AS MODELS FOR CONFORMATIONAL STUDIES

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Islet amyloid peptide (IAPP) has been referred to as the main component of the fibrous protein aggregates present in the pancreas of patients with severe hyperglycemia.[1] Such amyloid plaques are a characteristic histopathological marker in over 90% of all cases of type-2 diabetes.[2] Mature IAPP is a 37-residue peptide synthesized in the pancreas and it co-localizes with insulin in beta-cell dense core secretory granules. Due to its co-secretion with insulin, IAPP has been suggested to play some role in regulating the blood level glucose by controlling insulin release.[3] The spectroscopic characterization of human amylin and its related amyloidogenic fragments in water solution is a difficult task due to the low solubility. Since the conjugation of polyethylene glycol is known to tune the solubility of certain highly hydrophobic proteins and peptides, we have synthesized two novel PEG conjugated peptide derivatives of the human amylin 17-29 amino acid sequence that is considered the molecular region responsible of the formation of the insoluble amyloid fibrils. The two peptides differ only for the position of the attached PEG moiety being at the C-terminus in one derivative and at the N-terminus in the other. By means of spectroscopic techniques (CD and NMR), furthermore, we were able to characterize the structural features of the important amylin fragment and understand more about its conformational behaviour. [1] E. L. Opie, J. Exp. Med. 1997, 397. [2] P. Westermark, Amyloid, 1997, 47. [3] M. Kogire, J. Ishizuka, J. C. Thompson, G. H. Greeley, Jr, 1991, Pancreas, 6, 459.

P599

SPECTROSCOPIC INVESTIGATIONS ON MODEL CYCLIC PEPTIDES CONTAINING ß-AMINO ACIDS

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The continuously growing interest in the design of biologically active peptides led to the development of molecular tools capable of inducing a required three-dimensional structure in conformationally flexible peptides. The properties of peptides strongly depend on their folded conformation, from which β- and γ-turns are two important groups of regular/ordered secondary structures. Characterization of turns is usually achieved by combined FTIR and CD spectroscopic studies, however, spectroscopic detection of γ-turns and their distinction from β-turns is often difficult. This shows the importance of studies on model compounds known to preferentially adopt γ-turns, such as small cyclic peptides containing β-amino acids. Recently, vibrational circular dichroism (VCD) spectroscopy is one of the most promising techniques in the conformational analysis of peptides and proteins. This work reports systematic FTIR and VCD and CD spectroscopic studies in different solvents on model cyclic tetrapeptides of type cyclo(β-Ala-Xxx-β-Ala-Yyy) (Xxx, Yyy = Ala, Pro) forming two γ-turns (centered at Xxx and Yyy) stabilized by C7 H-bonds in the main conformer. Cyclic tetrapeptide cyclo(Ala-Pro-Phe-Gly) and its analogues containing β-homo amino acids were also investigated. The comparison of their spectra suggests that inclusion of β-homo amino acid residues induces a preference for γ- or pseudo-γ-turn structures. Detailed conformational analysis is given on the basis of experimental and computed VCD spectra, the assignment of vibrational bands being based on normal mode calculations performed at 6-31G(d)/B3LYP level. Acknowledgements: This work was supported by the Hungarian Research Fund OTKA (grants T037719 to Zs.M., T034866 to M.H. and T047186 to E.V.).

P600

CONFORMATION AND DYNAMICS OF THE 'HINGE' PEPTIDE - A POTENTIAL CARRIER FOR ANTIGENIC SEQUENCES

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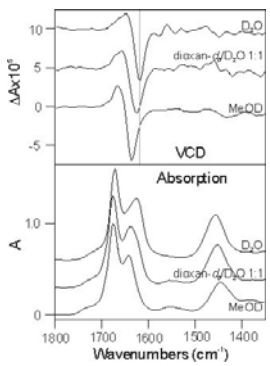
The combination of NMR analysis, relaxation times, electronic (ECD) and vibrational (VCD) circular dichroism was used in concert with a molecular simulation study of the target molecule, a parallel cyclic dimer of H-Thr-Cys-Pro-Pro-Cys-Pro-Ala-Pro-OH with two disulphide bridges. The peptide is a part of the central sequence of human immunoglobulin IgG1 and we consider its utility as potential carrier of synthetic antigenic sequences. The molecule (see VCD spectra in the figure) appears to be very rigid with the dominant conformation of the polyproline II type, regardless of sequence modification or external conditions. In the dimer it forms an interesting S-S connected double PII helix.



P601

PROBING THE CONFORMATION OF A POTENT ALTERED PEPTIDE LIGAND, [ARG91, ALA96] MBP87-99: A 2D SPECTROSCOPIC AND MOLECULAR MODELLING STUDY

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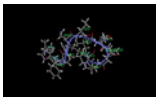
Multiple Sclerosis (MS) is a myelin-specific CD4+ T-cell mediated autoimmune disease that is triggered by unknown exogenous agents, in individuals with a specific genetic background. A novel approach towards the therapeutic management of MS has been launched by the introduction of antigen-specific therapies that suppress autoreactive T cells or switch the immune response from Th1 to Th2. Myelin Basic Protein (MBP) has been considered as a candidate autoantigen, supported by evidence depicting that the epitope MBP72-85 has the potential of inducing Experimental Autoimmune Encephalomyelitis (EAE) when injected in Lewis rats. Within the grounds of actively inhibit the disease, the design and use of peptide analogues of MBP is suggested, exploiting the idea of blocking the formation of the trimolecular complex MHC–APL–TCR. Inference to a series of EAE experiments induced by the MBP72-85 epitope is that a linear Altered Peptide Ligand (APL), [Arg91, Ala96] MBP87-99, holds antagonistic activity. We sought to perform conformational analysis of the APL in order to comprehend the stereoelectronic requirements for antagonism. A combination of 2D NMR spectroscopy and molecular modelling was applied to achieve this aim. ROE data suggest a semicircular conformation, with the backbone of the APL folded and an almost head-to-tail arrangement. Energy minimized structures generated after a molecular modelling theoretical study, supplied conformations akin to the ones generated using distance constraints dictated by 2D ROESY spectroscopy.



P602

STRUCTURAL STUDIES OF HIS-, GLY-, AND TYR-RICH DOMAINS IN PLANT GLYCINE-RICH PROTEINS (GRPS)

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Plant glycine-rich proteins (GRPs) are roughly classified into two classes; RNA-binding glycine-rich proteins and structural cell wall components like hydroxyproline-rich glycoproteins (HRGPs) or proline-rich proteins (PRPs). The structure and function of these proteins are unknown still. A group of GRPs such as ozone-inducible proteins (OI2-2 and OI14-3) from Atriplex canescens, GRPF1 from Fagus sylvatica, and GRP1 and GRP2 from Cicer arietinum contain repetitive domains that are His-, Gly-, and Tyr-rich. From circular dichroism (CD) and nuclear magnetic resonance (NMR) for synthetic peptides corresponding to sections of the sequences of these proteins, we recently found that the GRPs bind copper ion. In order to obtain 3D - structure of the copper ion complexes, we performed 2D-NMR for the His-, Gly-, and Tyr-rich repetitive domains in the absence and the presence of copper ion

P603

CROWNED (L)-Cα-METHYL-DOPA AND (L)-β3-DOPA AS POTENTIAL HELIX INDUCERS IN α- AND β-PEPTIDES

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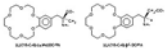
Crowned Cα,α-disubstituted amino acids, as well as crowned β-amino acids with well-defined stereochemical properties, could stabilize helical conformations by imposing local restrictions on backbone conformation in peptide frameworks, allowing the assembly of structurally rigidified architectures of bis-crown as well as polymeric crown compounds. Here, we report the synthesis and a conformational study of peptides based on (L)-[crown]-(αMe)DOPA with [crown]= [15-C-5] or [18-C-6] or [benzo-24-C-8], as well as the synthesis of the corresponding (L)-[crown]-β3-DOPA derivatives. Peptides based on (L)-[crown]-(αMe)DOPA, combined with either (L)-Ala, or Aib and (L)-Ala, or Aib and Gly residues, up to the hexamer level, with two crowned residues at the i and i+3 positions of the main chain, were synthesized by solution methods. Their conformational analysis by combined FT-IR absorption, 1H NMR and CD methods showed a remarkable bias for right-handed β-turn and 3_{10}-helix structure formation. In the same manner, peptides based on (L)-$[OBz]_2$-β3-DOPA and (L)-β3-DOPA, combined with either (L)-β3-Ala or (1S,2S)-Achc were synthesized in view of conformational studies, as models for future crown-carrier β-peptides.



P604

SYNTHESIS, STRUCTURE AND DEAMIDATION OF NGR-CYCLOPEPTIDES

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Beside the RGD sequence, peptides containing the NGR tripeptide are also integrin receptor ligands, although with a much lower affinity. In our work we have addressed the question whether cyclisation of linear peptides containing the NGR cell adhesion sequence leads to a less flexible conformation and at the same time to an increased alpha5 beta1 integrin receptor selectivity. The head to tail cyclisation of LNGRV and LNGRv caused only a marginal change in their integrin receptor binding capacity contrary to the RGD-peptides. This was shown by the limited effect and selectivity on the adhesion of endothelial cells to ECM components. The structure determination of the two cyclopeptides by NMR and MD, semiempirical and ab initio methods revealed that in solution both are very flexible and take on multiple stable conformations. This flexibility and the presence of the Asn-Gly peptide bond enhance succinimide ring formation leading to the hydrolysis of Asn. It has been demonstrated that c(LNGRV) both in solution and during storage suffered deamidation, and as the isoaspartyl-peptide may co-eluate with the asparginyl-peptide in the course of HPLC analysis, MS measurement is necessary to check the purity of peptides containing the NGR sequence. Our stability investigations raise the question whether in in vivo experiments the NGR motif or its hydrolysis product is effective? This work was supported by Hungarian Research Fund OTKA T037761 and EORTC-Drug Discovery Committee mini project.

P605

SYNTHESIS OF THE CATHEPSIN-L1 110-123 EPITOPE CONJUGATED TO SEQUENTIAL OLIGOPEPTIDE CARRIER AND 1H-NMR CONFORMATIONAL ANALYSIS IN ITS FREE STATE

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The liver flukes Fasciola hepatica and Fasciola gigantica are causative agents of fascioliasis in humans and ruminants, especially cattle, goat and sheep. The disease has been traditionally considered to be an important veterinary disease because of the substantial production and large economic losses in livestock production. Cathepsin cystein proteinases are abundant proteins in Fasciola and form a major component of the material secreted by the parasite. Cathepsin L1 was shown to be one of the most important antigen in fascioliasis, especially the epitope 110-123. Humoral immune response to cathepsin-L proteases has been used in attempts to develop a reliable test for the diagnosis of F. hepatica infections in rats, sheep, ruminants and humans. Nevertheless, in all these attempts, cross-reactivity with other parasites was a major hurdle. In this work we report on the synthesis and conformational analysis of the Cathepsin L1 epitope 110-123 (DKIDWRESGYVTEV) in the free state and conjugated to Sequential Oligopeptide Carrier [Ac-(Lys-Aib-Gly-)4-OH, Ac-SOC4]. This epitope was selected in order to develop a species-specific diagnostic ELISA for F. hepatica. The conformational analysis of the epitope in its free state was performed in aqueous solution using 1D and 2D NMR spectroscopy. The numerous and intense NOE effects detected between successive amide protons along almost all the peptide backbone and of type dNN(i, i+2) and dαN(i, i+2) are consistent with a helical type structure.

P606

AN USEFUL METOD FOR MONITORING OF PEPTIDE SYNTHESIS ON SOLUBLE POLYMER SUPPORT

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Polyethylene glycols (PEG)s have been successfully used as polymer soluble supports in the synthesis of peptides, oligosacharides and oligonucleotides (1).The synthesis is carried out in homogeneous solution while purification is performed by precipitation of the polymer. They also offer certain advantages over insoluble polymers in terms of analysis and products monitoring because the molecules anchored to soluble PEG can be fully characterized in both the solid and solution state by NMR, IR, CD, Raman spectroscopy. Hovewer, even during the synthesis it would be advantageous to watch the peptide chain growth quantitatively. For that reason, we developed new method for monitoring of loading level and synthesis on polymer soluble support.The method is based on densitometric evaluation of paper electrophoresis of the corresponding compounds bound to PEG. Prior the evaluation, the specific chemical reagens are used to detect PEG derivatives. The accuracy of this method is comparable to other analytical methods like amino acid analysis, elementary analysis, NMR; the advantage is a possibility of evaluate the course of synthesis without interupting the reaction process. (1) Bayer, E.; Mutter, M. Nature (London) 1972, 237, 512.

P607

DIFFERENT EFFECTS OF 4-HYDROXYPROLINE AND 4-FLUOROPROLINE ON THE THERMAL STABILITY OF COLLAGEN TRIPLE HELIX

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The collagen triple helix is a characteristic structural motif found in proteins. CD, analytical ultracentrifugation and DSC analyses of collagen model peptides, (Pro-Pro-Gly)10, (Pro-HypR-Gly)10, (Pro-fProR-Gly)10 and (fProS-Pro-Gly)10 (HypR: 4(R)-hydroxyproline, fProR: 4(R)-fluoroproline, fProS: 4(S)-fluoroproline), suggested that Hyp and fPro have different effects on the stability of the triple helices according to their arrangements and stereochemistries at the 4 position; the increased stability of (Pro-HypR-Gly)10 is ascribed to the enthalpic effects whereas that of (Pro-fProR-Gly)10 or (fProS-Pro-Gly)10 is achieved through the entropic ones. We considered that high enthalpy change of (Pro-HypR-Gly)10 should be arisen from an extensive hydration of Hyp in the triple helix because that (Pro-HypR-Gly)10 has the higher degree of hydration than (Pro-Pro-Gly)10 has already been known by the X-ray analyses of the peptides containing HypR. However, there was no evidence that this explanation is applied to the solution. Therefore we measured partial specific volumes of collagen model peptides and compared them with the calculated and intrinsic molecular volumes. This result confirmed that the assumption about the high degree of hydration of (Pro-HypR-Gly)10 in the solution. In contrast, it was revealed that (Pro-fProR-Gly)10 and (fProS-Pro-Gly)10 have the lower degree of hydration than (Pro-Pro-Gly)10. This means that these two peptides have the smaller enthalpy change of the transition than that of (Pro-Pro-Gly)10. To interpret the high thermal stability of fPro-containing peptides, it is inevitable to reduce an entropic change due to a decrease in the amount of water molecules attached to the peptide.

P608

SYNTHESIS AND THERMAL STABILITY OF NEW COLLAGEN MODEL PEPTIDES CONTAINING 4-HYDROXYPROLINE AND 4-FLUOROPROLINE

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It has been known that the substitution of Pro of collagen model peptide (Pro-Pro-Gly)10 by 4-hydroxyproline (Hyp) or 4-fluoroproline (fPro) affects the thermal stability of the triple helical structure. The series of the synthesis and characterization of model peptides with single substitution of (Pro-Pro-Gly)10 by Hyp or fPro had been done. For example, we reported that (fProS-Pro-Gly)10 forms a triple helix whereas (fProR-Pro-Gly)10 does not.1) However, the effects of the double substitutions of (Pro-Pro-Gly)10 by Hyp or fPro have not been investigated yet. Therefore we systematically synthesized these model peptides and investigated their physico-chemical properties with circular dichroism (CD), analytical ultracentrifugation and differential scanning calorimetry (DSC) measurements. The results indicated that both (HypS-HypR-Gly)10 and (fProS-fProR-Gly)10 form a triple helix at 4 C but their transition temperatures are unexpectedly lower than those of (Pro-HypR-Gly)10 and (Pro-fProR-Gly)10, respectively. Furthermore, it was revealed that (HypR-HypR-Gly)10 forms a triple helix and its thermal stability is as high as that of (Pro-HypR-Gly)10. These imply that the additivity of the effects of the substitution on the thermal stability of the triple helical structure is not valid. The syntheses and characterizations of other peptides such as (fProR-fProR-Gly)10 will be also presented. 1) Doi, M., Nishi, Y., Uchiyama, S., Nishiuchi, Y., Nakazawa, T., Ohkubo, T. and Kobayashi, Y., J. Am. Chem. Soc., 123, 9922 (2003).

P609

THE CHIRALITY OF THE SIDE GROUPS IN POLYPEPTIDES FOLLOWED BY VIBRATIONAL CIRCULAR DICHROISM

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Poly-γ-benzyl-L-glutamate (PBLG) and poly-β-benzyl-L-aspartate (PBLA) posses right and left handed α-helix, respectively, in the CDCl3 solutions. The both structures are clearly demonstrated in the vibrational circular dichroism (VCD) spectra by the pattern having opposite signs in the amide I and II regions. Besides these characteristic peptide bands, the characteristic vibration of the ester groups at 1737 cm-1 is observed in IR absorption and VCD for the both polypeptides. The corresponding VCD signal differs for the both polypeptides. This difference may be caused by different distances of the ester groups from the a-helix backbone, which is shorter for PBLA and longer for PBLG. Therefore, the chirality of the PBLA backbone is transferred more easily to the side chain groups. Acknowledgement Grants MSM 223400008 from the Ministry of Education, Youth and Sports of the Czech Republic and 203/02/0328 from Grant Agency of the Czech Republic supported the work.

P610

METAL ION DRIVEN TURN CONFORMATION IN TWO HISTIDINE-CONTAINING SHORT PEPTIDES

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Recent studies have pointed out that the design of metal-binding sites and their incorporation into appropriately designed oligopeptides can be successfully used to template or stabilise well-defined three-dimensional structures.1 This currently active research field has made possible to achieve remarkable results especially in the conformational control and self-assembly of peptide structures and therefore represents a promising approach to model active sites of metalloproteins in de novo designed peptide architectures. Examples of exchange labile and inert systems, as well as use of natural and unnatural metal ligands in the formation of organised, metal ion associated alpha-helical secondary structures, are documented.1,2 In contrast, metal ions stabilising reverse turn conformations are less reported. Reverse turns play an important role in the molecular architecture of proteins and may serve as a site for molecular recognition processes.3 An interesting question is whether metal ions, bound either to the backbone amide and side chain functional group(s), have a turn-stabilising effect. Here we report a comparative spectroscopic study on the copper(II) and Zinc(II) complexes formed with two histidine-containing hexapeptides appropriately designed to undergo a metal dependent beta-turn conformational transition. The study has been carried out by CD, NMR, ESR and UV-vis spectroscopic methods. Moreover, ESI-MS has been used to directly determine the stoichiometry of the metal complexes. References 1.T. Sasaki et Al., in Comprehesive Supramolecular Chemistry Vol. 4 Y. Muratami Ed.,1996, 193. 2.J.P. Schneider et Al. Chem. Rev., 1995, 95, 2169. 3.G. D. Rose et Al. Adv. Prot. Chem., 1985, 37, 1.

P611

THE FUSION-ACTIVE COMPLEX OF SEVERE ACUTE RESPIRATORY SYNDROME (SARS) CORONAVIRUS

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The causative agent of a recent outbreak of an atypical pneumonia, known as severe acute respiratory syndrome (SARS) has been identified as a novel Coronavirus (CoV) [1]. Fusion of SARS-CoV with the host cell is mediated by the envelope spike (S) protein, within which two regions have been identified, characterized by the presence of 4,3 heptad repeats (HR). We have studied a large set of recombinant and synthetic peptides derived from the predicted HR regions of the S protein by proteolysis protection analysis, CD, and size-exclusion chromatography. Our data show that the HR1 and HR2 of SARS-CoV associate into a stable, antiparallel 6-helical complex, with structural features typical of class I fusion proteins. This complex is also strikingly similar to the recently solved fusion-active complex of the murine CoV (MHV) [2], the closest relative of SARS-CoV [1]. Notably, HR1 forms an inner trimeric coiled coil which is stable also in the absence of interaction with HR2, showing high resistance to proteolysis, and a fully reversible CD thermal denaturation curve. Our conclusions are further supported by preliminary X-ray crystallographic data. Since inhibitors binding to HR regions of fusion proteins are efficacious against many viruses, our results may help in the design of SARS therapeutics. 1. Stadler, K., Masignani, V., Eickman, M., Becker, S., Abrugnani, S., Klenk, H.-D., and Rappuoli, R. Nat Rev Microbiol 1, (2003) 209. 2. Bosch, B.J., van der Zee, R., de Haan, C.A.M., and Rottier, P.J.M. J. Virol. 77, (2003) 8801.

P612

CYCLOTIDES: NOVEL PEPTIDIC PESTICIDES FOR PLANT PROTECTION

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Cyclotides are a unique family of cysteine–rich plant peptides that are resistant to proteolytic degradation and extreme temperature and pH conditions. This chemical stability is due to their cyclic backbone and knotted disulfide topology, termed the "cyclic cystine knot", where two disulfide bridges and their connecting backbone forms a ring that is threaded by a third disulfide bond. They also have a wide range of biological activities including uterotonic, anti-microbial, anti-tumour, and anti-HIV activities. A recent report that the prototypic cyclotide kalata B1 has insecticidal activity suggests that they function as defence molecules in plants. In this presentation, the discovery and characterisation of new cyclotides and the potential use of cyclotides as novel pesticides for plant protection will be discussed. New cyclotides from the African plant, Oldenlandia affinis, were discovered, thus completing the "cyclotide fingerprint" for the above-mentioned plant. The methodology developed in the isolation and sequencing of these macrocyclic disulfide-rich plant peptides should be applicable for the characterisation of more complex cysteine–rich proteins. Based on interesting preliminary bioassay data, the NMR structure and solution behaviour of the cyclotide kalata B5 was determined and compared to previously reported cyclotides. A range of cyclotides were tested and found to have biological activities against some major agricultural pests. The insecticidal, molluscidal and nematicidal activities of cyclotides against Helicoverpa armigera (cotton bollworm), Pomacea canaliculata (golden apple snails), and Caenorhabditis elegans and plant-parasitic nematodes, respectively, demonstrate the potential for cyclotides to be utilised in plant protection as agrochemicals or in transgenic crops.

P613

SOLUTION STRUCTURE OF THE HUMAN ENDOSTATIN FRAGMENT EH6-49 BY 1-H NMR AND MOLECULAR DYNAMICS CALCULATIONS

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Endostatin, produced by cleavage of collagen XVIII, is a potent endogeneous inhibitor of angiogenesis. We have recently synthesized peptides based on the endostatin primary structures with the same level of anti-angiogenic activity [1]. The possibility to obtain synthetic short anti-angiogenic peptides opens the way to the rational design of mutants more suitable to clinical applications. The molecular basis of the biological activity of endostatin and synthetic fragments is not at present clear. One mechanism has been suggested with endostatin binding to heparane sulphate [2]. Another hypothesis relates to integrin binding [3]. We report here on our conformational analysis of the human endostatin peptide Eh6-49. The solution structure has been determined by 2D-NOESY experiments on the peptide dissolved in water/trifluoroethanol. About 350 NOE interactions were quantitated at three different mixing times and used for the structure determination, based on a full relaxation-matrix approach. The NMR-derived structure was refined by ns-scale molecular dynamics calculations with the explicit inclusion of water molecules. References: [1] Chillemi F. Francescato P. Ragg E. et al., J. Med. Chem., 46, 4165-72 (2003); [2] H. F. Blackhall, C.L.R. Merry, M. Lyon, G.C. Jayson, J. Folkman K. Javaherian and J.T. Gallagher Biochem. J.,375, 131-9 (2003); [3] A. Sudhakar et al., Proc. Natl. Acad.Sci USA,100, 4766-4771 (2003).

P614

PEPTIDES FROM THE N-TERMINAL DOMAIN OF A PORE-FORMING TOXIN, STICHOLYSIN II. CONFORMATION AND ACTIVITY

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The pore-forming toxin sticholysin II (StII) produced by the sea anemone Stichodactyla heliantus belongs to the actinoporin protein family. The N-terminal domain of these proteins is required for interaction with membranes. To investigate the role of StII´s N-terminal domain in membrane binding and in the molecular mechanism of hemolysis, peptides of different size containing residues from the portion corresponding to amino acids 1 to 35, were synthesized. In some, leucine was replaced by tryptophan for fluorescence studies. All peptides were hemolytic, albeit to a lesser extent than the whole protein. Moreover, peptides lacking the 1-14 hydrophobic stretch were less active than those carrying this sequence. Circular dichroism spectra showed that, while the more hydrophobic peptides underwent aggregation in aqueous solution, the more hydrophilic ones displayed random conformation. In the presence of trifluoroethanol and upon binding to micelles, the peptides showed a propensity to acquire □-helical conformation, in agreement with theoretical predictions for the sequence comprising residues 14 to 26. The acquisition of □-helical conformation was less pronounced in the presence of phospholipid bilayers. Fluorescence studies demonstrated that StII´s first twelve residues penetrate more deeply into the bilayer, whereas residues 14-26 are located more superficially, in agreement with the predicted amphipathic nature of the helix formed by these residues. The results corroborate the existing hypotheses for the role of the N-terminal domain in the molecular mechanism of membrane insertion. Financial support: FAPESP and CNPq.

P615

MEMBRANE INTERACTIONS OF ANTIMICROBIAL PEPTIDES FROM AUSTRALIAN TREE FROGS

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Antimicrobial peptides from the dorsal skin glands of Australian tree frogs are being studied in phospholipid membranes. These peptides disrupt the cell membranes of Gram-positive but not Gram-negative bacteria and bind strongly to anionic 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG) phospholipid membranes but not to zwitterionic 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) membranes. Solid-state NMR and CD spectroscopy were used to study the interaction of these antimicrobial peptides with phospholipid membranes. As well as maculatin 1.1 and caerin 1.1, which contain a proline residue, the antibiotic peptides aurein 1.2 and citropin 1.1, without a proline in their sequence, have been studied. 31P NMR results reveal an effect on the phospholipid headgroups when the peptides interact with DMPC/DHPC (dihexanoyl-phosphatidylcholine) bicelles and aligned DMPC multilayers. 2H NMR showed a small effect of the peptides on the acyl chains of DMPC in bicelles or aligned multilayers, suggesting interaction with the membrane surface for the shorter peptides and partial insertion for the longer peptides. 15N NMR of selectively labeled peptides in aligned membranes and oriented CD spectra indicated an helical conformation with axis ~ 50 deg. to the bilayer surface at high peptide concentrations. The peptides did not appear to insert deeply into PC membranes, which may explain why these positively charged peptides preferentially lyse bacterial rather than eucaryotic cells. Results of NMR studies of these peptides in mixed DMPC/DMPG bilayers will be described.

P616

TOWARDS MODULAR ALPHA-HELICES: COMFORMATIONALLY STABLE CYCLIC PENTAPEPTIDES AS ALPHA-TURN MIMETICS

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α-Helices account for ~30% of protein structure. Often only a few α-helical turns of exposed protein surfaces are recognized by other proteins, DNA or RNA.1 In isolation, such helical segments could be valuable biological probes and drug leads, however the corresponding short peptides (=15 residues) do not form thermodynamically stable α-helices in water. Long helical peptide sequences have often been stabilised with covalent side chain bridges (disulfide, hydrazone, lactam, aliphatic). A pentapeptide is the minimal a-helical unit - an α-turn. We have found that a cyclic pentapeptide bridged via Lys(i) and Asp(i+4) side chain cyclization forms a stable α-turn in water. These cyclic pentapeptides can be considered modules, that when joined back to back give highly structured a helices that are remarkably stable to temperature (5-65¡C), denaturants (8M Gua.HCl), and proteolysis (trypsin). Synthetically, the modular approach presents several advantages over other bridging strategies, and permits variation to 60% of its component amino acids. We describe a promising new generic approach to a-helix mimicry.

P617

CONFORMATIONAL STUDIES OF TWO BRADYKININ ANALOGUES BY USING TWO-DIMENSIONAL NMR TECHNIQUES

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Bradykinin (BK) is a nonapeptide, known to mediate some vital processes such as hypotension, smooth muscle, edema and cell growth. Its activity is mediated by specific receptors, classified pharmacologially as B1 and B2 subtypes. B2 receptors require the entire BK sequence for recognition, whilst B1 receptors recognize and bind only des-Arg8-bradykinin. Clinical studies have shown that most relevant effects caused by BK are functions of the B2 receptors. In this cnnection, the structure of two B2 antagonists, Aaa-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-DPhe-(N-Bzl)-Gly-Arg and Aaa-DArg-Arg-Pro-Hyp-Gly-Thi-Ser-(2-Nal)-Thi-Arg, was determined in a dimethyl sulfoxide solution by two-dimensional NMR spectroscopy and theoretical conformational calculations. The Electrostatically Driven Monte Carlo (EDMC) method with the ECEPP/3 force field was used in conformational search. The applied algorithm consists of the following three steps: (i) search for the conformational space in order to find conformations with reasonably low energy, (ii) simulation of the NOE spectrum and vicinal coupling constants for each of the low energy conformations, and (iii) determining the statistical weights of the conformations by means of the maximum entropy method in order to obtain the best fit of the averaged NOE intensities and coupling constants to the experimental quantities. Bearing in mind the finding, that it is impossible to describe conformational preferences of short peptides, such as bradykinin, by one structure only, a set of conformations with the highest statistical weights, constituting about 75 % of the ensamble, was presented for each peptide.

P618

CONFORMATION OF THE AVP ANALOGUES SUBSTITUTED WITH N-METHYLPHENYLALANINE AT POSITIONS 2 AND 3 IN DIMETHYL SULFOXIDE SOLUTION DERIVED FROM NMR SPECTROSCOPY

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The choice of solvent is a very important problem in conformational investgations by using NMR spectroscopy. Small peptides, such as vasopressin, generally adopt the random-coil conformation in aqueous solution and their backbones exhibit considerable flexibility. Some of organic solvent, such as DMSO, mimick the envirinment of biological membrane and stabilize a preferred conformation. In this paper, we studied the conformations of four AVP analogues substituted at positions 2 and 3 with all possible combinations of N-methylphenylalanine enantiomers, in DMSO. Earlier investigations showed that characteristic features of these peptides is their considerable mobility, this resulting in a variety of conformers precluding structure elucidation. The structure in DMSO tend to be more rigid than those in aqueous solution. The major species of analogues with the same N-methylphenylalanine enantiomers at both positions, ([MePhe2,3]AVP and [DMePhe2,3]AVP), possess a cis peptide bond between the modified residues, as in the case in aqueous solution. The synthesis of [MePhe2, DMePhe3]AVP afforded two products, (a) and (b), which had identical molecular ions and similar retention times on HPLC. In the ROESY spectra in DMSO, in contrast to those in aqueous solution, the exchange cross-peaks between M HaPro7 and m1 HaPro7 for [MePhe2, DMePhe3]AVP (a) and (b), suggests cis/trans isomerization of the Cys6-Pro7 peptide bond. In the case remaining analogues, neither in DMSO nor in aqueous solution, these cross-peaks were missing in ROESY spectra. In our opinion a comparison of the structures in both solvents would better contribute to designing with definite pharmacological properties.

P619

NMR MODELS OF THE ANGIOTENSIN-I CONVERTING ENZYME ZN(II) CATALYTIC SITES : THE BASIS FOR A STRUCTURAL STUDY ON THE ENZYME-SUBSTRATE INTERACTION

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The Angiotensin-I Converting Enzyme (ACE) is a gluzincin Zinc Metallopeptidase and constitutes one of the major partners of the Renin-Angiotensin System. Inhibition of ACE enzymatic activity is strongly coupled with blood pressure regulation and for this reason it was considered as one of the major challenges, against hypertensive disease and congestive heart failure. ACE catalyzes the hydrolysis of the Angiotensin-I (AI) carboxy-terminal dipeptide His-Leu and trasnforms AI to the vassopressor octapeptide Angiotensin-II (AII). ACE is encountered in two distinct forms in humans, the somatic and the testis. These differ from the structural point of view, mainly in size (1306 and 732 AA for somatic and testis isoform, respectively) bearing 2 and 1 Zn(II)-containing catalytic sites, respectively. ACE inhibitors have been designed over the past 30 years using other Zn Metallopeptidases (Carboxypeptidase A, Thermolysin, etc.) as templates for drug design. Only recently the X-ray structure of testis ACE has been determined. The reconstitution of the the two catalytic site peptide bearing the ACE amino acid sequence (synthesized using Fmoc/tBu chemistry on 2-chloro trityl resin), has been performed by ZnCl2 addition. The 3D solution structures of these constructs have been studied using high-resolution multinuclear NMR spectroscopy [Spyroulias, G.A.; Galanis, A.S.; Pairas, G.; Manessi-Zoupa, E.; Cordopatis, P. Current Topics in Medicinal Chemistry, 4, 403-429, (2003)]. The NMR models of the two ACE catalytic sites set the basis for understanding the different substrate binding specificity and study their interaction with physiological substrate, such as Angiotensin I, as well as with various inhibitors.

P620

IN VIVO PHARMACOKINETIC AND METABOLIC STUDIES OF PEPTIDE DRUGS BY MASS SPECTROMETRY

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An increasing number of polypeptides and proteins are used as therapeutics or are in drug development. This requires appropriate tools for metabolic, tissue distribution, pharamacokinetic and pharmacodynamic studies, in vivo, in animal models or directly in Human for both endogenous and exogenous proteins. We first discuss analytical methods for fast, precise and sensitive measurements of peptide hormone levels in body fluids and tissues using LC-MS and LC-MS/MS strategies. For example, the level of lutheinizing hormone-releasing hormone and its analogues such as buserelin, goserelin or leuprolide can be determined in 50-200 ul plasma or serum samples. Following a simple extraction protocol, after addition or not of an internal standard, an on-line LC-MS/MS analysis allows us to reach a typical lower limit of detection in the range of 0.1 to 1.0 ng/L (sub-pM concentrations). A second approach named Isotope Dilution Assay (IDA) is illustrated with insulin, C-peptide and proinsulin. IDA allows more accurate and sensitive detection of proteins or peptides by mass spectrometry. This method involves the use of target protein analogues labeled with stable isotopes (such as deuterium or 15N). These analogues that are authentic and non-radioactive, have different molecular masses and will act as internal standards allowing precise quantification on the basis of the relative intensities of the observed signals (principle of isotope dilution). IDA is not susceptible to errors arising from immunological dosages. Furthermore, this method can clearly discriminate endogenous and injected forms, thus allowing for simultaneous in vivo quantitative investigations of both endogenous and injected compounds.

P621

DERIVATIZATION OF PEPTIDES FOR CAPILLARY ELECTROPHORESIS WITH LASER INDUCED FLUORESCENCE AND OFF-LINE MALDI TOF DETECTION

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Capillary Electrophoresis with Laser Induced Fluorescence detection (CE-LIF) is a powerful tool for separation and sensitive determination of fluorescent compounds. Biologically active compounds, such as amino acids, peptides and proteins, usually have to be derivatized using a fluorescent dye with appropriate spectral characteristics matching the wavelength of the excitation laser to enhance limit of detection. In this work, various derivatizing rhodamine dyes were studied for use with diode pumped Nd:YAG frequency doubled excitation laser (532 nm). New method of derivatization was suggested and tested on model peptides (including neuroprotective humanin-like peptides). Derivatization conditions such as pH, time and temperature of derivatization as well as separation conditions were optimized for all studied dyes and techniques using artificial neural networks (ANN). Additionally to CE with LIF detection, separation of derivatized peptides was also performed by CE with off-line detection using Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI TOF MS).

P622

CHIROSPECTROSCOPIC CHARACTERIZATION OF PEPTIDE 3-10-HELIX IN AQUEOUS SOLUTION

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The aim of this work was to extensively characterize a strongly right-handed biased, 3-10-helical peptide in water using the currently most popular chirospectroscopic techniques, namely electronic CD (ECD), vibrational CD (VCD), and Raman optical activity (ROA). To this end we synthesized by solution methods a water-soluble, terminally blocked heptapeptide based on five markedly helicogenic, C-alpha-tetrasubstituted alpha-amino acids L-(alphaMe)Nva and two strongly hydrophilic ATANP {2-amino-3-[1-(1,4,7-triazacyclononane)]-L-propanoic acid} residues at positions 2 and 5. FT-IR absorption and NMR analysis in deuterated chloroform and aqueous solutions of the heptapeptide and two side-chain protected synthetic precursors confirmed our working hypothesis that all oligomers are folded in stable 3-10-helical conformations. Based on these findings, we exploited this attractive, all-L heptapeptide as a chiral reference compound for a detailed ECD, VCD, and ROA characterization of the right-handed 3-10-helix in aqueous solution. This investigation is instrumental for an expansion of our knowledge of the standard spectroscopic patterns characteristic of the classical, long-range, ordered secondary [alpha-helix, beta-sheet, poly(Pro)n II] structures of polypeptides and proteins.

P623

1H-NMR CONFORMATIONAL STUDIES OF THE [NLE11] 9-22 ANTIGENIC REGION OF HERPES SIMPLEX VIRUS GLYCOPROTEIN D

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Herpes simplex virus (HSV) has two serotypes known as HSV-1 and HSV-2. Glycoprotein D (gD) is an envelope component of both HSV types and is one of the principal targets for the antibody response during an HSV-infection. Therefore, it is a prime candidate for a human subunit vaccine. The strong antigenic [Nle11]analogue (L9KNleADPNRFRGKDL22-NH_2) derived from the gD (9-22) sequence was synthesized in the free form and conjugated to SOC (Ac-[Lys-Aib-Gly]4-OH) and tetratuftsin (H-[Thr-Lys-Pro-Lys-Gly]4-NH_2, T20) carriers. Conformational studies of the gD(9-22) peptide in both free state and conjugated to carriers were performed by 2D-NMR and CD in aqueous solution and mixtures with TFE. Intense NOE cross peaks between almost all the successive NHi/NHi+1 amide protons were observed for the free peptide in water indicating that it adopts a rather well defined conformation. The detection of type daN (i, i+2) between P6/R8, R8/R10 and F9/G11, type dNN (i, i+2) between N7/F9, R8/R10 and G11/D13 and type daN (i, i+3) between P6/F9 and R8/G11 suggests the presence of a 310 helix structure in the -P6NRFRGKDL14- segment. A beta-turn in the -DPNR- segment seems also to contribute to the structure stabilization of the peptide.

P624

SLOW CONFORMATIONAL EXCHANGE OF SHORT LINEAR PEPTIDES IN SOLUTION

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The search of conformational features of peptides in solution is of great importance especially for evaluating the conformational-activity relationship. It is generally accepted that short linear peptides are found in very fast conformational exchange in solution. The structures of these peptides derived from NMR spectroscopy are, therefore, believed to represent only population-weighted averages over all rapidly interconverting conformations of an ensemble in a given solvent at ambient temperature. The search for discrete conformations has so far been limited to low temperatures, especially when sharp NMR resonances are detected at room temperature. Our recent studies have indicated that a solvent induced slow conformational exchange can take place in solutions of short linear peptides (Biris et al. Biopolymers 2003, 69, 72-89; Tsikaris et al. Tetrahedron Lett. 2000, 41, 8651-8654; Tsikaris et al. Biopolymers 2000, 53, 135-139). In this work a NMR-based strategy that would allow us to identify directly discrete conformational states of short linear peptides, differentiate them from the average one, and gain new insight into their folding process will be presented. Various examples of linear peptides, which adopt stable conformation in solution even at high temperature values, will be given.

P625

POLYPEPTIDE STRUCTURAL CHANGES AS CONSEQUENCE OF A NON-COVALENT ASSOCIATION TO PORPHYRINS. VCD STUDY

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Effect of conformational polypeptide changes caused by non-covalent interaction between polypeptides and appropriate porphyrins was studied by the combination of vibrational circular dichroism (VCD) and electronic circular dichroism (ECD) spectroscopies. It was shown that such interaction influences structure and chirality of both agents vice versa. The structure of the polypeptide part of complexes was tuned by variation of physico-chemical properties of solutions. It was shown that the interaction with porphyrins prevents the precipitation of poly-L-glutamic acid even at strong acidic pH. Figure shows the VCD spectra of the complexes composed of poly-L-glutamic acid and the porphyrin derivative that posses either α-helical or random coil conformations. Besides, the mixture of both conformations was observed in specific condition. Ackowledgement. Grants MSM 223400008 from the Ministry of Education, Youth and Sports of the Czech Republic and 203/02/0328 from Grant Agency of the Czech Republic supported the work.

P626

PEPTIDE SELF-ASSEMBLED MONOLAYERS ONA GOLD SURFACE

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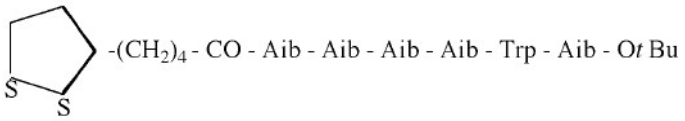
Peptide foldamers are a new class of conformationally constrained compounds, which are able to attain specific secondary structures. Our idea is to use helically ordered oligopeptides to obtain self-assembled monolayers (SAMs) on a gold surface, presenting superior properties in terms of nanomolecular organization of the monolayer. To this purpose, we synthesized a new peptide-based compound having the molecular formula shown in the figure below. The lipoic acid was introduced at the N terminal to exploit the S-Au affinity for binding the peptide to the gold surface. The presence in the peptide sequence of several Aib residues forces the peptide in a 310-helical conformation, as detected by CD measurements in ethanol solution. A Trp residue was also inserted in the peptide sequence as an intrinsic fluorescent probe. The high propensity of the peptide to bind to the gold surface was characterized by Quartz Crystal Microbalance and Cyclic Voltammetry measurements. STM imaging of the modified Au surface in ultra-vacuum conditions reveals the formation of an 'holes' pattern, whose height line profile corresponds to Au monoatomic steps (≈2Å). The nature of the holes, commonly observed in alkanethiol SAMs, has been attributed to depressions in the top layer of the Au surface, originating from an etching process promoted by the peptide binding.



P627

STRUCTURE AND BINDING OF THE COMPLEX BETWEEN A SYNTHETIC C-TERMINALLY TRUNCATED HEVEIN AND CHITOOLIGOSACCHARIDES: DEFINING THE MINIMUM HEVEIN DOMAIN WITH MEASURABLE AFFINITY

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Plants respond to pathogenic attack by producing defense proteins able to bind reversibly to chitin which is a structural component of fungal cell walls or invertebrate exoskeletons. A number of those defense proteins share a highly conserved Gly, Cys-rich common structural pattern organized around a four disulfide core known as hevein domain or chitin binding motif [1]. The motif is found in lectins or wheat germ agglutinins, in addition to hevein itself or its natural variant pseudo-hevein. Its small size make it an excellent model system for the study of protein-carbohydrate recognition [2]. Further motivation for the study of hevein domains comes from the role of hevein as major latex allergen and from the antifungal activity of class I chitinases or Ac-AMP antimicrobial peptides, all of them bearing hevein motifs. We have synthetically modified key residues of hevein domains, with the goal of outlining the minimum scaffold capable of effective chitooligosaccharide binding. A first step in this direction is a C-terminally truncated, 32-residue version of hevein (HEV32), for which an efficient solid phase synthesis has been developed. The 3D structure of this modified lectin in both its free and chitin trimer (GlcNAc)3-bound state has been studied in water solution by NMR and molecular dynamics simulations, and the thermodynamics of the binding process has been characterized both by 1H-NMR and fluorescence methods. [1] Rodriguez A, et. al. J Cryst Growth 1986;76:710-714. [2] Espinosa JF, et. al. Eur. J. Biochem. 2000, 267:3965-3978

P628

NMR STRUCTURE DETERMINATION OF A CARBOXY-TERMINAL BAND-3 PEPTIDE BOUND TO ALDOLASE

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Band-3 is the predominant protein in the human erythrocyte membrane (~1 million copies per cell). It consists of a 55-kDa membrane spanning domain, an N-terminal 43-kDa cytoplasmic domain, and a C-terminal 33-residue cytoplasmic tail. The 55-kDa helical transmembrane domain acts as a carbonate/chloride anion exchanger; while the extreme N-terminal region is known to bind and regulate the activities of several glycolytic enzymes. The C-terminal tail of Band-3 has been shown to bind carbonic anhydrase II specifically. Tyrosines 8 and 21 in the N-terminal domain can be phosphorylated by the tyrosine protein kinase Syk. This abolishes the binding of glycolytic enzymes, increasing the glycolytic flux. The structure of a 15-residue fragment containing Tyr 8 complexed with aldolase or glyceraldehyde-phosphate-dehydrogenase has been determined previously, where the Tyr sidechain is located in a small hydrophobic cluster in the bound peptide that stabilizes an overall turn structure. Phosphorylation of Tyr 8 would make the turn unstable and thereby prevent the binding. The Tyr 902 residue in the C-terminal tail is flanked by similar acidic amino acids as Tyr 8 and 21. Therefore we studied the binding of a 16-amino-acid peptide containing Tyr 902 to aldolase. Using exchange-transferred NOESY NMR, a loop structure was calculated for the fragment bound to aldolase. In this new structure, Tyr 902 is situated at the turn of the loop. The phosphorylation of this Tyr is not likely to interfere with the loop formation but may interfere directly with binding to the enzyme (supported by Alberta Heart and Stroke Foundation).

P629

MATRIX ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRY IDENTIFICATION OF FLUORESCENTLY LABELED PEPTIDES FOR OPTIMIZATION OF LASER INDUCED FLUORESCENCE DETECTION

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Laser induced fluorescence detection (LIF) is an effective instrumentation for the sensitive determination of biologically important molecules, such as peptides and proteins, after Capillary Electrophoresis (CE) separation. Since the native fluorescence of these compounds is often low and/or an expensive laser is required for their excitation, labeling of the analytes with a fluorescent dye is often necessary. An optimization of the derivatization step is required to achieve the lowest detection limits. In this work we use CELIF and CE – matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) to investigate derivatization of model peptides with rhodamine-based reagents, which are compatible with the wavelength of our diode pumped Nd:YAG excitation laser (532 nm). A subatmospheric deposition interface [1] is used for off-line coupling of CE with MALDI MS. While the LIF detection mode is needed for the sensitive detection, the MALDI MS analysis can reveal the mass of analyte. Thus, MALDI-TOF Mass Spectrometry is used for identification of the derivatization products, the number of fluorescent labels per peptide, free reagent and unlabeled peptides. The further structural information can be obtained from post source decay (PSD) analysis of selected parent ions. The combined CE–MS analysis can improve the identification of electropherogram data and verify the optimal derivatization procedure. This work was supported by the Grant Agency of the Czech Republic, grant # 203/03/0515. Reference 1. T. Rejtar, Ping Hu, P. Juhasz, J. M. Campbell, M. L. Vestal, J. Preisler and B. L. Karger, J. Proteome Res., 1, (2002), 171-178.

P630

FRET STUDY OF QD PROTEIN BIOCONJUGATES

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Quantum dots (QDs) have exhibited significant advantages over traditional fluorophores as fluorescent probes in biological staining and diagnostic[1]. From the HPLC elution curves, we found that the fluorescence intensity of QD-BSA is significantly stronger than that of free QDs. This phenomenon could be explained by Forster resonance energy transfer (FRET)[2]. The wide absorption spectrum of QDs provides the necessary qualification for FRET. This was supported by three groups of experiments. (I) Under the same condition, the fluorescence intensity of QD-BSA and free QDs were measured by spectrofluorophotometer separately, and the QD-BSA is significantly stronger than the free QDs. In the conjugation reaction, different concentration of BSA was used to conjugate with QDs. The fluorescence of the QD-BSA conjugate increased correspondingly with the increase of BSA concentration. (II) QDs were conjugated with BSA by electrostatic attraction. The fluorescence spectrum of QD-BSA solution remained unchanged when the pH value was higher than the pI of BSA; when the pH was lower than the pI of BSA, the conjugation is prohibited, which resulted in much weaker fluorescence intensity. (III) Bio-conjugates were made between QDs and different proteins in separate reactions. The Different fluorescence intensity of these bio-conjugates further demonstrated the existence of FRET. [1]Thomas M.Jovin, Nature Biotechnology, 21,32(2003) [2]Kara A.S., Zhang Z.S., Philip A, C., Anal.Biochem. 317,226(2003)

P631

ENANTIOMERICALLY PURE, CYCLIC, CHIRAL, SPIN-LABELLED B-AMINO ACIDS: TRANS-POAC AND CIS/TRANS-B-TOAC

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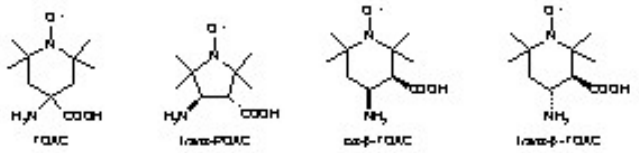
We have been interested by the prospect of creating spin-labelled cyclic b-amino acids that could induce helical structures in b-peptides, different in nature from the 310-helices induced in a-peptides by the Ca-tetrasubstituted TOAC residue, and that could be obtained in enantiopure form. Here, we report the synthesis and the resolution of trans-POAC and of both cis- and trans-b-TOAC amino acids. Both enantiomers of cis-H-b-TOAC-OMe were obtained through amination of 3-carboxymethyl-1-oxyl-2,2,6,6-tetramethyl-4-piperidone with either (R)- or (S)-a-methyl-benzylamine, reduction of the resulting enamine with NaBH3CN/AcOH, and removal of the chiral auxiliary from the separated highly predominant cis diastereoisomers. An X-ray diffraction analysis of the (1'S,3R,4R) N-alkylated b-amino ester intermediate allowed us to assign the absolute configuration at C3 and C4. Equilibration of the cis diastereoisomers to the trans ones led to each enantiomer of trans-H-b-TOAC-OMe. Resolution of the known racemic trans-Fmoc-POAC-OH was achieved through its (aR) binaphthyl mono-esters. All the enantiomers of both POAC and cis/trans-b-TOAC were obtained with >99 % ee, as shown by chiral HPLC.



P632

SYNTHESIS AND CONFORMATIONAL STUDIES OF DIASTEREOMERS OF TRI- AND TETRAPEPTIDES CONTAINING ALPHA-PHENYL-ALPHA-(2-PYRIDYL)GLYCINE

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The availability of structurally rigid peptides is of value on biochemical and biophysical studies. alpha,alpha-Disubstituted glycines are of particular importance in developing small, acyclic, conformationally restricted peptides. Recently, we have synthesized tripeptides containing alpha,alpha-di(2-pyridyl)glycine (2Dpy) (1) and revealed that 1 adopts a unique conformation based on two intramolecular hydrogen bonds between 2Dpy-NH and a pyridine nitrogen and between AA3-NH and another pyridine nitrogen, and, further, 1 is able to self-assemble in the presence of a Cu(II) ion. We have also prepared tripeptides containing alpha-phenyl-alpha-(2-pyridyl)glycine (2Ppg) (2) and found that 2 adopt beta-turn conformation stabilized by two intramolecular hydrogen bonds between 2Ppg-NH and a pyridine nitrogen and between AA3-NH and C=O of Z group [1]. In order to ascertain the contribution of a pyridine nitrogen to intramolecuar hydrogen bonding and conformational preference, we have investigated synthesis, chromatographic separation and conformational studies of diastereomers of tri- and tetrapeptides containing 2Ppg. Tripeptides [Z-AA1-(L/D)-2Ppg-AA3-OMe (3): AA1 = Gly, Aib, L-Ala, L-Val, Leu; AA3 = Gly, Aib] have well prepared by the Ugi reaction, and various tetrapeptides have synthesized by coupling of Z-L-AA or L-AA-OMe to N-terminal or C-terminal of 3, respectively. By comparing with the corresponding peptides containing alpha,alpha-diphenylglycine (Dph), which mostly adopt C5-conformation, it was clarified that an intramolecular hydrogen bonding associated with a pyridine nitrogen makes 2Ppg-containing peptides prefer beta-turn conformation. References [1]. T. Yamada et al., Peptide Science 1999, 303 (2000), Peptide Science 2000, 305 (2001); L. Di Costanzo et al., Dalton Trans., 787 (2003).

P633

SOLUTION STRUCTURE OF ISTX, A MALE SCORPION TOXIN FROM O. MADAGASCARIENSIS

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The novel sex-related potassium channel inhibitor IsTX, a 41-amino acid peptide residue, was isolated from the venom of male O.madagascariensis. IsTX is classified, based on its sequential homology, in the scorpion short toxin family alpha-KTx6. The alpha-KTx6 family contains a single alpha-helix and two beta-strands connected by four disulfide bridges and binds to and apamin-sensitive Ca2+-activated K+ channels. Two-dimensional NMR techniques revealed that the structure of IsTX contains a cysteine-stabilized alpha/beta-fold. The three-dimensional structure of IsTX is similar to those of alpha-KTx6 toxins. IsTX has much lower affinities toward voltage-gated potassium channels at micro-molar concentrations. Other toxins block them at nano- or pico-molar concentrations. In order to investigate the activity-structure relationship, the geometry of sidechains and electrostatic surface potential maps were compared with others. As a result of the comparison of the primary structures, Lys27 of IsTX was conserved at the same position in other toxins of the same family. The lysine of the beta-sheet region, the most critical residue for binding to potassium channels, inserts into the channel pore. However, IsTX has fewer basic residues to interact with acidic channel surfaces than other potent toxins. MALDI-TOF MS analysis clearly indicated that IsTX was found in male scorpion venom, but not in female. This is the first report that scorpion venom contains sex-related compounds. IsTX may explain why male scorpions have low potency peptide toxins.

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OSMIUM-LABELED PEPTIDES. ELECTROCHEMICAL, MASS SPECTROMETRIC AND ELECTROPHORETIC ANALYSIS

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Because of increasing interest in structure-activity relationships, the probes elucidating peptide and protein structure are under scope of a number of studies. Osmium tetroxide – bipyridine reagent (Os,bipy) has been applied as a probe for the DNA structure. The aim of this work was to elucidate the mechanism of peptide derivatization using Os,bipy and to examine the properties of osmium-modified peptides by means of various electrochemical techniques, such as cyclic voltammetry and differential pulse stripping voltammetry as well as Capillary Zone Electrophoresis (CZE) and Matrix Assisted Laser Desorption-Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS). Peptides with different number of tryptophan residues were derivatized and analyzed. It was found that only those which contain tryptophan residues produce characteristic electrochemical signals. The most negative signal was of a catalytic nature. Femtomole amounts of these peptides were detectable by means of an adsorptive transfer stripping methods. Using CZE it was shown that tryptophan in peptides forms an osmate ester with Os,bipy, which was monitored as a specific shift in peptide migration times and a change in UV-Vis spectra after the derivatization. The presence of osmium atom in the complex was confirmed using MALDI-TOF MS. We propose a new method of peptide derivatization which can be monitored by the above mentioned methods. Os,bipy can react with peptides under conditions close to physiological and can be expected to become a probe of protein structure. Acknowledgement: This work was supported by Grant Agency of the Czech Republic, projects No. 204/03/0566 and 305/03/1100.

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AFFINITY-BASED TAGGING OF PROTEIN FAMILIES WITH REVERSIBLE INHIBITORS

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In proteomics, sensitive detection and quantification are conventionally done using different staining methods. However, they are non-selective and often difficult to compare and to reproduce.[1] As a matter of principle it is possible to tag certain proteins covalently with a specific ligand (peptide or organic molecule) bearing a reporter group prior to two-dimensional separation. Subsequently the protein in question can easily be identified, e.g. fluorimetrically, and quantified. Especially tagging of enzymes has been achieved simply by using irreversible enzyme inhibitors (suicide inhibitors).[2,3] We extended the applicability of this strategy towards the use of reversible enzyme inhibitors.[4] A probe for the mechanism-based detection of serine/threonine kinases was synthesized: the kinase inhibitor H-9 [5] was attached via a polar linker to a photoreactive group (p-benzoylphenylalanine, Bpa) and to a reporter group (carboxyfluorescein). The kinase binding ability of modified H-9 was further proved by BIAcore experiments. This concept is suited for many different classes of proteins and may facilitate the discovery of new members of a protein family. [1] F. Lottspeich, Angew. Chem. Int. Ed. Engl. 1999, 38, 2476-2492. [2] Y. Liu, M. P. Patricelli, B. F. Cravatt, Proc. Natl. Acad. Sci. USA 1999, 96, 14694-14699. [3] D. Greenbaum, K. F. Medzihradszky, A. Burlingame, M. Bogyo, Chem. Biol. 2000, 7, 569-581. [4] M. C. Hagenstein, J. H. Mussgnug, K. Lotte, R. Plessow, A. Brockhinke, O. Kruse, N. Sewald, Angew. Chem. Int. Ed. Engl. 2003, 42, 5635-5638. [5] H. Hidaka, M. Inagaki, S. Kawamoto, Y. Sasaki, Biochemistry 1984, 23, 5036-5041.

P636

EVOLUTION OF THE INVERTEBRATE OXYTOCIN/VASOPRESSIN SUPERFAMILY PEPTIDE

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Members of oxytocin/vasopressin (OT/VP) superfamily widely distribute in invertebrates as well as vertebrates, and play key roles as signaling molecules in the regulation of reproduction and osmoregulation. We have revealed that the common octopus, Octopus vulgaris, has two members of OT/VP superfamily as in vertebrates, octopressin (OP) and cephalotocin (CT), an observation made for the first time in invertebrate. The OP- and CT-precursors were composed of a signal peptide, a nonapeptide, and a neurophysin domain, the typical structural organizations of the superfamily precursors. Genomic analysis revealed that OP and CT genes lack introns and consist of a single exon in their protein-coding regions, which is unlike the 2 intron-3 exon structures in most vertebrates and Lys-conopressin in Lymnaea stagnalis. Each single copy of those genes was present in the octopus genome. Moreover, the promoter regions of OP and CT genes exhibited several putative regulatory elements including GRE, CRE, and AP-2 binding sites which are commonly found in those of vertebrate VP genes despite the distinct tissue distribution of the OP and CT mRNA. These results suggest that the OP- and CT-genes originate via gene duplication, and expression of the OP and CT gene is regulated by different transcription factors from vertebrates. It can be also presumed that the OT/VP superfamily peptide evolved from a common ancestral gene in invertebrates and vertebrates, but the octopus OT/VP gene evolved through duplication, while gene regulation was established through different evolutionary lineages from those vertebrate counterparts.

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IDENTIFICATION OF A RECEPTOR FOR ANNETOCIN, INVERTEBRATE OXYTOCIN/VASOPRESSIN SUPERFAMILY PEPTIDE

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Annetocin is structurally related to oxytocin/vasopressin (OT/VP) family peptides, which have been isolated from earthworms, Eisenia foetida. Annetocin-injection into earthworm induces the egg-laying behavior. In this paper, we report the identification of an endogenous receptor for annetocin (AnR). The deduced AnR precursor includes seven transmembrane domains and typical sites for G-protein coupled receptor and displays high sequence similarity to OT/VP receptors. A functional analysis of the AnR expressed in Xenopus oocytes demonstrated that AnR activates calcium-dependent signal transduction upon binding to annetocin. In situ hybridization experiment indicates that AnR gene is expressed specifically in the nephridium located in the clitellum region. This result leads to the presumption that annetocin induces the egg-laying behavior through the action in the nephridium. Furthermore, determination of genomic sequence indicates that promoter region of annetocin gene is more similar to that of VP than OT. This result suggests that annetocin was derived from ancestral vasopressin gene.

P638

PEPTIDOMICS STUDY OF DIABETIC MOUSE MODELS REVEALS CHANGES IN PEPTIDE HORMONE LEVELS AND PRECURSOR PROCESSING

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A spontaneous mutation in the carboxypeptidase E (CPE) causes a slowly developing obesity and mild diabetes in homozygous Cpefat mice. CPE removes C-terminal basic amino acids of peptide hormones preprocessed by prohormone convertases. In contrast to Cpefat/Cpefat leptin-mutated Lepob/Lepob develop transient hyperinsulinemia and later severe diabetes. In this study we describe a peptidomic profiling approach of pancreatic peptide hormones and their precursors which allows for the qualitative and quantitative analysis of peptide levels in two mouse models suffering from severe obesity. Peptides were extracted from tissues and those with a molecular mass below 15 kDa were analysed by a combination of liquid chromatography, mass spectrometry and bioinformatics. Sequencing of differentially regulated peptides in Cpefat/Cpefat mice revealed that the ratio of the relative abundance of prohormones versus their corresponding peptide hormones was significantly altered. Here, we describe for the first time sequential steps in prohormone processing in a comprehensive analytical approach. In particular, the levels of proinsulin and c-terminally extended peptides were increased, whereas the level of active insulin remained unaltered. In contrast in Lepob/Lepob the levels of active peptide hormones were significantly increased. 75% of the differentially regulated peptides were classified as functionally related to the exocrine/endocrine pancreas. The differential display of single gene affected animals leads to specific detection of gene-related phenotypic changes. Supported by German BMBF 'BioChance' Program

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EXPRESSION AND FUNCTIONAL CHARACTERIZATION OF A NEW CYP2C9 VARIANT

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CYP2C9 is polymorphic enzyme in human liver and metabolizes 10% of therapeutically important drugs, such as warfarin with a narrow therapeutic index. By now 6 kinds of human CYP2C9 allelic variants have been found. CYP2C9*1 is the wild-type protein and the other variants showed reduced intrinsic metabolic activity. Recently we found a new CYP2C9 variant from a Chinese poor metabolizer of lornoxicam. It is a leu90Pro substitution in the encoded protein. This special person simultaneously has the new CYP2C9 and CYP2C9*3 alleles occurred on the different chromosome. His clearance of lornoxicam and tolbutamide is much slower than one with CYP2C9*3 homozygote. In order to further study its catalytic activity in vitro, the new variant CYP2C9 (leu90Pro change) was stably expressed in CHL cells. The kinetic studies of the new variant were carried out using tolbutamide and lornoxicam as substrates. The results showed that the new CYP2C9 variant decreased catalytic activity of hydroxylase. It suggests that the substition from Leu to Pro in 90th may induce the conformation change of CYP2C9 protein in relation with lower enzyme activity.

P640

IMMUNE FOCUSING TO MAJOR ALLERGENS PREVENTS THE RECOGNITION OF MINOR ANTIGENS AND ALLERGY FORMATION

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Natural allergens are complex mixtures. They do not replicate inside the body and thus the concentration of individual proteins is very low. On the contrary, pathogens are able to replicate after invasion by this mean increasing significantly the local concentration of antigens. Immune response to pathogens is focused to dominant antigens whose peptides presented in excess, while allergic response is probably not focused due to the low concentration of allergen proteins. The aim of this work was to determine whether it is possible to focus allergic response to a single major allergen. Allergy to A. fumigatus (Af) fungus was induced in mice by immunizations with Af crude extract, which contains around 100 proteins. Immune focusing to a major allergen Asp f 2 was fulfilled before allergy induction by immunization of mice with the protein or with its dominant B-cell epitopes 140-154 and 254-268 expressed in the yeast virus-like particles. The number of recognized proteins was analyzed by Western blotting. Immunization of mice with Af crude extract in the control group resulted in the production of IgG able to recognize most bands on the blot membrane. On the contrary, Asp f 2 immunized mice subjected to allergy induction protocol produced IgG, which recognized only two bands on the blot corresponding to Asp f 2 and some other protein. Immune focusing to Asp f 2 resulted also in the decreased IgE production and lung inflammation. Thus, the results demonstrated that immunization with major allergens could prevent allergy progression.

P641

HIGHLY IMMUNOGENIC AND BROADLY RECOGNIZED HIV DERIVED CTL EPITOPE MIMICS

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With the still progressing human immunodeficiency virus (HIV) pandemic, the need for an effective and safe HIV vaccine is more than ever urgent. HIV-specific cytotoxic T lymphocytes (CTLs) are known to play an important role in the initial clearing and control of chronic HIV. In the aim toward the development of an HIV vaccine, we have used peptide combinatorial libraries and a biometrical analysis to identify CTL epitope mimics that stimulate CD8+ CTL response more efficiently than the natural infection. The Gag p17-derived epitope SL9 was selected for these studies since about 71% of HLA-A2-positive individuals have circulating T-cells that recognize this epitope. Thus, fourteen nonapeptides were identified that are up to one order of magnitude more effective than SL9 in stimulating T cell clones isolated from different seropositive patient, as determined by a cytotoxic assay. More importantly, the mimics also stimulate gammaIFN release in PBMCs isolated from HIV-1 seropositive patients. Using transgenic mice, we have demonstrated that a number of these epitope mimics are able to generate de-novo T cell responses more effectively than the native SL9 epitope and are highly cross reactive with the original peptide sequence. Overall, these studies demonstrate that using peptide library approaches it is possible to engineer peptide vaccine candidates with enhanced immunogenicity and broader recognition by multiple T cell receptors. The approach and results will be discussed.

P642

SYNTHESIS AND IN VITRO EFFECT OF PEPTIDE CONJUGATES FROM 38 KDA PROTEIN OF M. TUBERCULOSIS ON T-CELL PROLIFERATION AND IFN-G PRODUCTION

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The 38 kDa protein of M. tuberculosis is a major secreted antigen provoking specific immune response, therefore related epitope peptides can be considered as potential diagnostics. Peptide H-350DQVHFQPLPPAVV362-NH_2 is an immunodominant and genetically permissive epitope of the 38 kDa protein, exhibiting peripheral blood mononuclear cells (PBMC) stimulatory activity from over 60% of infected or BCG-vaccinated individuals. For the present studies two peptides (H-350DQVHFQPLPPAVV362-NH_2 (38G), H-351QVHFQPLPPAVV362-NH_2 (38Q)) were prepared by solid phase synthesis on MBHA resin using Boc/Bzl strategy. The homogeneity and the primary structure of peptides were checked by analytical RP-HPLC, amino acid analysis, and ESI-MS. Peptide 38G was conjugated to branched polypeptide with polylysine backbone (poly[Lys(Seri-DL-Alam)] (SAK), tetratuftsin derivative (H-[Thr-Lys-Pro-Lys-Gly]4-NH_2), lysine dendrimer (H-Lys-Lys(H-Lys)-Arg-Arg-b-Ala-NH_2) and KLH carrier via thioether bond formation [1]. 38Q and its conjugate with sequential oligopeptide carrier, Ac-[Lys-Aib-Gly]4-OH) (SOC) were also synthesised. PBMC from 20 healthy PPD positive donors were stimulated with the free peptide alone or with peptide conjugates containing an equimolar amount of peptide, or with a mixture of free peptide and carrier. We have measured cell proliferation and the IFN-g production. We found that conjugation of peptide 38G and/or 38Q enhanced specifically human T-cell proliferation and IFN-g production. [1] G. Mező, E. de Oliveira, D. Krikorian, M. Feijlbrief, A. Jakab, V. Tsakaris, C. Sakarellos, S. Welling-Wester, D. Andreu, F. Hudecz, Bioconjugate Chem. 14, 1260-1269 (2003)

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DEVELOPMENT OF AN PEPTIDE-BASED ELISA FOR THE DETECTION OF TWO TYPES OF AUTOIMMUNE DERMATOSES

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Bullous pemphigoid (BP) and pemphigoid gestationis (PG) are organ-specific autoantibody (AAB)-induced skin diseases. The main target for these AAB's is BP180, an transmembrane glycoprotein and part of a protein-network, linking epidermis and dermis. The 16th non-collagenous stretch of the BP180 ectodomain (NC16A) contains epitopes recognized by BP and PG sera and serum levels of AAB's against NC16A correlate with disease activity. The NC16A-domain encompasses 76 aa and is localized adjacent to the transmembrane region of BP180. Several findings indicate that the major immunodominant region spans a region within the N-terminal portion of NC16A between aa 507-528. Currently, BP/PG AAB's are detected by reactivity of patients serum in an NC16A-based ELISA. [Zillikens,D. et al., J Invest Dermatol. 1997;109:679]. Here, NC16A is expressed as gluthathione-S-transferase (GST) fusion protein. The use of GST-NC16A, however, may induce unspecific antibody-binding to the GST-portion of the fusion protein or to lipophilic domains on NC16A which may generate false results. Therefore, the present study aimed at the development of a peptide-based ELISA system facilitating the detection of BP/PG AAB's with high specificity. By epitope-mapping, we identified 2 main immunodominant regions within NC16A. Based on these findings, a peptide-based ELISA was established and compared with the GST-NC16A ELISA. Our novel method, which used BSA as blank for unspecific AAB binding, proved to be more sensitive and more specific for the detection of BP/PG AAB than the previous ELISA. This novel ELISA system should be most useful for the diagnosis and evaluation of disease activity in BP and PG.

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IDENTIFICATION OF A CD14-DERIVED PEPTIDE WITH LPS NEUTRALIZING ACTIVITY

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LPS is highly potent immune stimulatory component of the cell wall of Gram-negative bacteria. Low concentrations in blood lead to moderate immune responses in healthy humans, whereas higher concentrations may induce massive release of cytokines, resulting in life threatening disorders like septic shock. The cellular response to LPS is mediated by Toll-like receptor 4 (TLR4) and accessory molecules. TLR4 belongs to a class of receptors involved in the response of the innate immune system to bacterial components. Compounds interfering with the LPS induced immune response hold great potential for the therapy of septic shock. We reasoned that the LPS-binding proteins may provide a source of functional domains for the development of LPS-neutralizing agents. For this reason, synthetic 20mer peptides covering the whole 356 amino acid human LPS-binding protein CD14 were synthesized. Peptides were tested for their potential to neutralize the LPS-dependent induction of IL-8 expression in human myelomonocytic THP-1 cells. One peptide corresponding to amino acids 81-100 exhibited strong antagonistic activity due to its ability to bind to LPS, probably representing a portion of the LPS binding site of human CD14. A detailed analysis of the structure-activity relationship attributed this interaction to a set of residues that are highly conserved among CD14 proteins from different species. Based on the results from an alanine-scan, several analogues were synthesized to improve the solubility and antagonistic activity.

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FAMILY OF EW-PEPTIDE DRUGS

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Two dipeptide analogs: L-Glu-L-Trp (1) and gamma-D-Glu-D-Trp (2) have been registered in Russia as immunomodulator Thymogen (1) and immunosuppressor Thymodepressin (2). These two drugs have exhibited reciprocal activities in vivo. The polypeptide fraction has been initially separated by the preparative HPLC from the crude thymus homogenate and a number of individual Trp-containing dipeptides have been purified and sequenced. Dipeptide L-Glu-L-Trp was the most active in the majority of in vitro and in vivo tests. In the process of SAR studies the "signal" role of L-Glu-L-Trp in the immune response has been observed, as well as the critical role of indolyl-side chain of Trp. It was discovered that two particular enantiomeric structures: L-Glu-L-Trp (gamma-L-Glu-L-Trp) and D-Glu-D-Trp (gamma-D-Glu-D-Trp), possess reciprocal immunological effects in vivo. D-isomers cause blockage of the immunocompetent cell proliferation, thus suppressing immuno- and hemopoesis in vitro and in vivo. Structure–functional in vitro studies have been performed in different models to determine the biological target of these peptides. Manifestation of in vitro effects as well as the influence of precise chemical and optical structures on biological activity of different EW- peptide analogs will be discussed. The experiments were conducted on blood neutrophiles, monocytes, thymic epithelial cells, lymphocytes, thymocytes, endothelial cells of human blood vessels and newborn blood cord. The dose dependence data of (1) and (2) on such activities as cytokines production, the processes of stimulation and suppression of apoptosis, and proliferation of immunocompetent cells will be presented and discussed.

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DESIGN AND SYNTHESIS OF A POTENT CYCLIC ANALOGUE OF MYELIN BASIC PROTEIN MBP87-99 EPITOPE: INHIBITION OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

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Experimental Autoimmune Encephalomyelitis (EAE) is a demyelinating disease of the Central Nervous System (CNS) and is an animal model of Multiple Sclerosis (MS). In the present study, a cyclic peptide analogue cyclo(87-99) [Ala91, Ala96] MBP87-99 based on the human Myelin Basic Protein epitope (MBP87-99) (Val87-His-Phe-Phe-Lys91-Asn-Ile-Val-Thr-Pro96-Arg-Thr-Pro99) has been designed and synthesized and was found to be a strong suppressor of EAE induced by guinea pig MBP74-85 epitope. Structure-activity studies have shown that Lys91 and Pro96 residues are important for encephalitogenicity. The synthesis of linear protected peptide was carried out by the Fmoc/tBu methodology utilizing 2-Chlorotrityl-Chloride resin. Each coupling was accomplished by the use of Fmoc protected amino acids in the presence of N,N'- diisopropylcarbodiimide (DIC) and 1-Hydroxybenzotriazol (HOBt) as coupling reagents. The cyclization was achieved using O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium tetrafluoroborate (TBTU), 1-hydroxyl-7-azabenzotriazole and 2,4,6 collidine in dry dimethylformamide (DMF) as solvent. These conditions were proved to be optimal for highest yield and free racemization product. The final cyclic analogue was purified by High Performance Liquid Chromatography (HPLC) and its structure was confirmed by Electron Spray Ionization Mass Spectroscopy (ESI-MS).

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SOLID PHASE SYNTHESIS OF IL-2 RECEPTOR (IL-2R) EXTRACELLULAR DOMAIN BETA CHAIN EPITOPES: INHIBITION OF T-CELL ACTIVATION

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Interleukin-2 binds to its high affinity receptor (IL-2R) on the surface of T-cells. This binding initiates a signal transduction cascade leading to lymphocyte proliferation and secretion of IL-2. Thus, IL-2R is an attractive target for the selective inhibition of autoreactive T-cells in autoimmune diseases and alloreactive T-cells in transplantation. Molecules that interfere with the binding of IL-2 to its high affinity receptor can block the proliferation of activated T-cells and the resulting secretion of IL-2. Given that IL-2 binds to its receptor via the extracellular domain of the β chain of the complex IL-2R, synthetic peptides mapping epitopes of this domain recognized by anti-IL2Rβ chain monoclonal antibodies (mAbs), were synthesized and tested for their ability to prevent binding of IL-2 and inhibit the proliferation and cytokine secretion of mitogen-stimulated peripheral blood T-cells. Solid phase methods were applied for the synthesis of two linear peptides with a primary structure corresponding to the epitopes M107-E118, Y178-Q199 of the extracellular domain of the IL-2Rβ chain. PHA-induced T-cell proliferation was significantly inhibited by the M107-E118 peptide in 500nM concentration. The amount of IL-2 in the culture supernatants was decreased by 56,65%. The peptide Y178-Q199 had a similar effect on cell proliferation but did not inhibit significantly IL-2 secretion by the PHA-activated cells, and flow cytometry showed that activation indicators were not suppressed. Experiments with T-cells isolated from autoimmune patients are under way to investigate whether these peptides and especially M107-E118 are promising for further development as putative inhibitors of T-cell activation in disease states.

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SCAFFOLDED PEPTIDES FOR THE MIMICRY OF THE DISCONTINUOUS BINDING SITE OF HIV-GP120 FOR CD4

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Virus entry of HIV-1 into the host cell is initiated by specific interaction of the HIV-1 glycoprotein gp120 with the receptor protein CD4 on lymphocytic T-cells. Synthetic molecules mimicking the gp120 binding site for CD4 are promising candidates for synthetic vaccines against HIV-1. Based on the resolved crystal structure of the complex of gp120 with an extracellular domain of CD4 (1), the fragments of gp120 making up its sequentially discontinuous binding site for CD4 could be identified. We have used a recently introduced strategy for the generation of structurally diverse scaffolds (2) to design and synthesize a range of scaffolded peptides, in which the fragments making up the gp120 binding site are presented in a non-linear and discontinuous fashion through molecular scoffolds of different size and conformational flexibility. The affinities of these peptides to CD4 were evaluated in a competitve binding assay. The results of this study will be presented. 1P.D. Kwong, R. Wyatt, J. Robinson, R.W. Sweet, J. Sodroski and W.A. Hendrickson (1998), Nature 398, 648-659. 2 R. Franke, C.Doll, V. Wray and J. Eichler (2003), Protein Peptide Lett. 10, 531-539.

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THE EFFECT OF CHEMICAL STRUCTURE AND STEREOCHEMISTRY ON INTERACTIONS OF IMMUNOMODULATORY PEPTIDES WITH LIPID BILAYERS IN LIPOSOMES

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Adjuvants are compounds that are used to enhance the immune response of the host to an antigen. Among the others the peptidoglycan fragments were recognized as potent immunomodulators especially low molecular weight fragments that are mostly devoid of the toxic properties characteristic for large peptidoglycans, but still retain marked immunomodulating activity. Our studies concern the peptidoglycan monomer, GlcNAc-MurNAc-L-Ala-D-isoGln-meso-DAP(wNH_2)-D-Ala-D-Ala (PGM), the natural compound originating from the Brevibacterium divaricatum peptidoglycan, its semisynthetic derivatives (Boc-Tyr-PGM and (Adamant-1-yl)-acetyl-PGM) and synthetic adamantyltripeptides D- and L-(adamant-2-yl)-Gly-L-Ala-D-isoGln. All examined compounds are a water-soluble, non-toxic and non-pyrogenic substances and their chemical structure has been completely defined. Biological activity of the prepared compounds has been continuously investigated and reported1. In order to slow down their hydrolytic inactivation and thus achieve a prolonged biological action they were incorporated into liposomes2. The interaction of incorporated compounds with phospholipids in liposomal bilayers was investigated by electron paramagnetic resonance spectroscopy. The spin labelled fatty acids, n-doxyl-stearic acids (n=5,7,16) were used for investigating the interactions3. The entrapment of the Boc-Tyr-PGM and adamantyltripeptides affected the motional properties of all spin labelled lipids, while the entrapment of PGM and (Adamant-1-yl)-acetyl-PGM had no effect. 1.J. Tomašić, I. Hanzl-Dujmović, B. Špoljar, B. Vranešić, M. Šantak, A. Jovičić, Vaccine 18 (2000) 1236. 2.R. Frkanec, D. Travaš, M. Krstanović, B. Halassy Špoljar, Đ. Ljevaković, B. Vranešić, L. Frkanec and J. Tomašić, J. Lip. Res., 13 No. 3-4.(2003) 279. 3.R. Frkanec, V. Noethig Laslo, B. Vranešić, K. Mirosavljević and J. Tomašić, Biochim. Biophys. Acta, 1611 (2003) 187.

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DIAGNOSTIC VALUE OF CHIMERIC AND CYCLIC SYNTHETIC PEPTIDES OF SEQUENCES BELONGING TO NS4 AND NS5 PROTEINS OF HEPATITIS G VIRUS

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Hepatitis G virus (HGV/GBV-C) is a recently discovered RNA virus belonging to the Flaviviridae family. The prevalence rate of this agent in individuals with frequent exposure to blood products is about 15-30%. Moreover, recent studies suggest that this infection in HIV-positive people is associated with prolonged survival, by this reason it will be interesting to find an easy tool to diagnose this virus [1]. Synthetic peptides have been used in diagnostic systems for various diseases. However, the main drawback of this approach is that peptides representing B-cell epitopes are poorly recognised by antibodies. There is a trend toward using chimeric peptides, including linear, branched or cyclic, to improve the sensitivity and specificity of the assays [2]. In this work, new putative epitopes located in non-structural proteins of HGV/GBV-C were synthesized in solid-phase for their use in immunoassays. Moreover, we synthesized two linear chimeric peptides incorporating monomeric sequences in both possible orientations separated by a spacer of three glycine residues. On the other hand, to induce or stabilize the bioactive conformation of peptides, we prepared head-to-tail and side chain-to-head cyclic peptides. Chimeric and cyclic HGV/GBV-C constructs are being antigenically studied by ELISA using different panels of human sera. Furthermore, CD and FTIR have been used in conjunction to characterize the conformational changes therein with synthetic constructs that could explain their different antigenicity. [1] Stapleton, T.J., Sem. Liver Disease, 2003, 23, 137-148. [2] Gómara, M.J., Riedeman, S., Vega, I., Ibarra, H., Ercilla, G., Haro, I., J. Immunol. Methods, 2000, 234, 23-34.

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A SYNTHETIC GROUP A STREPTOCOCCAL VACCINE OF HIGH PURITY AND BROAD PROTECTIVE RANGE

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Short synthetic peptides generally do not elicit effective antibody responses upon immunization, with the individual peptide epitope sequences. In animal studies, synthetic peptides often need to be coupled to larger proteins, such as bovine serum albumin, in order to provide strong antibody responses to the peptide epitope/s of interest. Due to the potentially harmful side effects in humans, this strategy is not generally acceptable for use in humans. Chemical ligation offers a new approach to the synthesis of high molecular weight synthetic peptides with high purity and reproducibility. We synthesized a group A streptococcal (GAS) vaccine which contains three different GAS M protein peptide epitopes. We targeted two of eight common aminoterminal serotypic epitopes, the sequences of which were derived from GAS isolates obtained from Australian Aboriginal patients and a conserved region epitope in order to generate a vaccine with broad strain coverage. The individual peptide epitopes were synthesized by solid phase peptide synthesis. After purification and characterization the three peptides were coupled into a single well characterized construct by native chemical ligation. Upon immunization of mice, the vaccine formulation, which contained the pure ligated construct and Complete Freund's Adjuvant, elicited high levels of specific antibody responses to all three epitopes without the need for protein carriers. Development of such a synthetic vaccine ensures high purity and reproducibility of the final vaccine formulation. The synthesis of the three epitope containing construct by native chemical ligation will be discussed in details.

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FAST HIGH RESOLUTION CHROMATOGRAPHY OF PEPTIDE LIBRARIES AND DIGESTS ON SILICA-BASED CAPILLARY MONOLITHS DIRECTLY COUPLED TO ES-FTICR-MS

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Complex peptide libraries with more than 1 000 different molecules or complex enzymatic digests can be screened within less than 20 min using a sophisticated set-up for highest resolution chromatography and on-line mass detection. Silica-based capillary monoliths with bimodal pore size distribution provide the unique combination of high separation efficiency (corresponding to that of a packing of 2 µm spheres), low pressure drop (like that of a packed bed of 10 µm spheres) and a high surface area [1,2]. This combination cannot be realized with any type of a particulate stationary phase material and has the following advantage for capillary LC/MS analysis: - Time efficient gradient elution - Excellent separation efficiencies - No need for expensive ultra-high pressure equipment - Ease of handling in comparison with packed capillaries - Dead volume-free LC-FTICR-MS coupling possible without loss of ionization efficiency by direct electrospray out of the monolith. Silica monoliths enable the rugged and sensitive high-throughput analysis of complex peptide mixtures. 1. Leinweber et al., Rapid. Commun. Mass Spectrom. 2003, 17, 1180-1188. 2. J. Mass Spectrom. 2004, in print.

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MULTIBRIDGED MICROBIAL PEPTIDES: BIOSYNTHESIS AND DIRECTED VARIATION

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Microbial peptide bridge builders possess smart enzymatic tool boxes with almost an unlimited potential for cyclizing and connecting structurally diverse peptides. In collaboration with genetic microbiologists our group elucidated the structures and the biosynthetic steps of various classes of multibridged peptide antibiotics. We then used the gained knowledge to prepare smart peptide libraries via genetic modifications of the producer strains. Two different biosynthetic principles shall be discussed in the context of applications: firstly, the natural bridge formation via posttranslational modification of ribosomally synthesized precursors and secondly, the bridge formation in peptides synthesized by multienzyme clusters. Lantibiotics: the structural genes of precursor peptides have been successfully modified producing new lantibiotic variants with several thioether bridges, which exhibit higher bioavailability and enzymatic stability. Vancomycin type glycopeptide antibiotics: genes for the biosynthesis of distinct amino acids involved in biaryl- and diarylether bridges were deleted and altered amino acids were fed to yield for example fluorinated and brominated tetracyclic variants.

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DEVELOPMENT OF ANTI-THYMOSIN BETA-4 IGY-ANTIBODIES AND THEIR PRELIMINARY APPLICATION TO THE IMMUNOSTAINING OF HUMAN BREAST CANCER CELLS

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Aiming at the investigation of the diagnostic significance of Tβ4 levels in cancer cells, we had previously developed polyclonal anti-Tβ4 IgG-antibodies, while we have recently developed anti-Tβ4 antibodies belonging to the immunoglobulin Y class (IgY-antibodies). Laying hens were immunized against synthetic Tβ4 (100 µg/injection), which had been either conjugated to keyhole limpet hemocyanin (Tβ4-KLH) or not. Hens were also immunized against the synthetic fragments Tβ4[1-14], Tβ4[31-43] and Tβ4[38-43] (25 µg/injection), conjugated to KLH. Injections were given every 3 weeks. The eggs were collected and divided into groups of 5 to 10, according to the date of collection. The IgYs were isolated from the egg yolk as previously described. Highly pure IgYs were obtained (~100 mg/egg). When non-conjugated Tβ4 was used as immunogen, no anti-Tβ4 IgY-antibodies were developed, while anti-Tβ4 IgY-antibodies of very low titre were developed when the synthetic fragments of Tβ4 conjugated to KLH were used as immunogens. Immunization with Tβ4-KLH led to anti-Tβ4 IgY-antibodies of relatively high titer (highest titer: 2nd - 4th booster injection). Cross-reactivity of the anti-Tβ4 IgY-antibodies for synthetic Tβ10 and synthetic Tβ15 was non-detectable. The above antibodies were used in a Tβ4 - ELISA (1 - 250 µg/mL). In preliminary experiments, the anti-Tβ4 IgY-antibodies were applied to the immunostaining of human breast cancer cells (MDA-MB-231 cell line). Immunoblotting of protein extracts obtained from the above cells is currently in progress, while immunostaining and immunofluorescence visualization of cells obtained from human breast tumours after surgery has been also envisaged.

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PLASMODIUM FALCIPARUM N-TERMINUS MSP-1 SIDE-DIRECTED REDUCED AMIDE PSEUDOPEPTIDES INDUCE PROTECTION OF AOTUS MONKEYS AGAINST MALARIA

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Antigen structure modulation represents an approach towards designing subunit malaria vaccines. The alpha carbon stereo-chemistry of a specific epitope, as well as its backbone topochemistry, was thus assessed for obtaining chemically modified malarial immunogens. Based on solid-phase pseudopeptide chemistry strategies, a variety of pseudopeptides derived from the N-terminus MSP-138-61 Plasmodium falciparum epitope were synthesized; these included all-L, all-D, partially-D substituted, all-Pse-[NH-CO]-Retro, all-Pse-[NH-CO]-Retro-inverso and Pse-[CH_2NH] surrogates. We demonstrate that specific recombinant-MSP-134-469 fragment binding to Red Blood Cells (RBC) is specifically inhibited by non-modified MSP-142-61, as well as by its V52-L53, M51-V52 reduced amide surrogates and partial-D substitutions in K48 and E49. In vivo tests revealed that reduced amide pseudopeptide immunized Aotus monkeys induced neutralizing antibodies specifically recognizing the MSP-1 N-terminus region. Some of the K48-Pse-[CH_2NH]-E49 immunized animals were protected upon experimental challenge with an infectious dose of parasite.

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SYNTHESIS, ANTIBODY RECOGNITION AND ENZYMATIC STABILITY OF LINEAR, CYCLIC AND BRANCHED POLYPEPTIDES CONTAINING A ß-AMYLOID PLAQUE SPECIFIC EPITOPE

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Peptide antigens used to generate site-specific antibodies to proteins are of interest in the development of vaccines. Based on the identification of Aß(4-10) (FRHDSGY) as the predominant B-cell epitope recognised by therapeutically active antisera from transgenic Alzheimer's disease (AD) mice, we synthesized and characterized linear, cyclic and branched polypeptides containing the Aß4-10 epitope that could be used as possible vaccines for Alzheimer's disease. In branched constructs tetratuftsin derivative (Ac-[TKPKG]4-NH_2) was applied as a carrier and the epitope was attached to it by chemical ligation (thioether bond) or built up directly by stepwise synthesis on solid phase. Structures and molecular homogeneity of all polypeptide conjugates were ascertained by HPLC, MALDI- and ESI-FTICR- MS, and secondary structures characterised by CD spectra. Comparative binding studies of these constructs with a mouse anti-Aß(1-17) monoclonal antibody were performed by direct ELISA, and the binding affinities correlated with the conjugate structures. Because peptide stability to protease degradation could have important implications to elicit an immune response, we investigated the stability of all conjugates in the presence of trypsin and human plasma proteases, by monitoring the degradation reactions by MS and HPLC. High stabilities were determined in the case of branched polypeptides in comparison with the stabilities of the carrier and the Aß(4-10) epitope. The presence of oligo-alpha and ß-alanine flanking regions significantly influenced the enzymatic stabilities of linear and cyclic epitopes and of branched conjugates. The use of polypeptides protected against degradation should enable the optimization of immunization protocols for AD vaccines.

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NEW ANALOGS OF HEXAPEPTIDE PRO-GLU-VAL-LYS-GLU-LYS.DESIGN, SYNTHESIS AND BIOLOGICAL ACTIVITY

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Coxsackieviruses (Enterovirus genus) cause wide range of diseases: meningitis, myocarditis, acquired diabetes, etc. There are two predominant types of antiviral compounds active against enteroviruses: the first blocks the hydrophobic pocket and the viral uncoating [1], the second inhibits the synthesis of viral RNA. The hexapeptide Pro-Glu-Val-Lys-Glu-Lys induces cross-reactive immune responses.[2] On the other hand guanidine and flufenamic acid inhibit antiviral activity.[3] We synthesized hybrid structure, including the above mentioned peptide and flufenamic acid or creatin residues, in order to investigate: 1.The role of flufenamic acid and creatin residues on biological activity; 2.The influence of glutamic residues by obtaining them as γ-benzyl esters; 3.The effect of C-terminal carboxy function by replacing it with CONH2 (amid function). The products was tested for antiviral activity by two-step procedure as follows: I. The agar-diffusion plaque inhibition test; II.The CPE inhibition test in microplates; The structure- activity relationship will be discussed. [1]. Otto, M. J., Fox,M.P.,Fancher,M.J., Kuhrt, M.F., Diana, G.D.,McKinlay, M.A.(1985) Antimicrob. Agents Chemother. , 27: 883-886 [2]. Marttila J, Juhela S, Vaarala O, Hyoty H, Roivainen M, Hinkkanen A, Vilja P, Simell O, Ilonen J., Virology. 2001 May 25; 284(1):131-41 [3]. Caliguiri , L.A., and I. Tomm,(1968) Virology , 35:405-417

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SYNTHESIS AND IMMUNO-CHARACTERIZATION OF BRANCHED POLYPEPTIDE CONJUGATE AS VACCINE FOR HSV-1 VIRUS

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Herpesviruses are ubiquitous pathogens of all vertebrates. Herpes Simplex Virus Type I (HSV-1) is one of at least eight herpes viruses that persistently infect man. Like all herpes viruses, HSV encodes a large number of glycoproteins, three of these, designated glycoproteins B (gB), gD and the gH/gL heterodimer, are absolutely required for virion infectivity and for virus induced cell-cell fusion. The aim of this work is to evaluate the efficiency of a HSV-1 vaccine constructed on a lipid core and using peptides from the four glycoproteins involved in virus entry. The lipidic polylisine core peptide (LPC) system represents a potentially safe option for vaccine delivery in humans and gives the possibility to incorporate multiple copies of different glycoprotein peptides. The solid phase synthesis was performed following a Boc strategy for the LPC and a Fmoc strategy for the peptides. The LCP core incorporates lipoamino acid coupled to a branched three lysine system containing up to two different antigenic peptides and is uniquely designed to incorporate antigen, carrier and adjuvant in a single molecular entity. Potential antigenic regions were predicted according to the following criteria: (a) regions of high antigenic propensity according to different prediction methods based on hydrophobicity, flexibility and accessibility profiles; (b) regions predicted to form surface loops; (c) regions of high sequence variability and/or containing insertion/deletion sites comparing sequence of HSV and other a-herpesviruses; (d) regions containing potential T-cell epitopes. We gratefully acknowledge support by EU under contract no.QLK2-CT-2002-00810

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BIOCONJUGATES OF SYNTHETIC PEPTIDE EPITOPS OF FOOT-AND-MOUTH DISEASE VIRUS WITH POLYELECTROLYTES AND THEIR IMMUNOLOGICAL ACTIVITY

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We have conjugated chemically synthesized peptide antigens of 40-60, 140-160 and 135-160 amino acid sequences of immunogenic VP1 capsid protein of a type foot-and-mouth disease virus (FMDV) to poly (N-isopropylacrylamide-co-acrylic acid), poly (N-vinylpyrrolidone-co-acrylic acid) and bovine serum albumin (BSA) in hydrated reversed micelle systems by using carbodiimide, and the effects of the mol mass ratio of components on the formation of polymer-peptide and BSA-peptide conjugates have been studied. It was shown that the peptide molecules in the structure of conjugate particles are practically exposed to the solution and the conjugate species can be represented rather as a macromolecule of a segmented (bloc) copolymer in which the hydrophobic blocks alternate with hydrophilic ones. The covalent binding of BSA with peptide molecules leads to the formation of soluble bioconjugates with a complicated structure and heterogeneous charge composition. These BSA-peptide conjugates are formed stable electrostatic and Cu+2-induced nonstoichiometric polycomplexes (PEC) with polycations and polyanions in neutral water. A single immunization of mice with conjugates as well as PEC without an adjuvant evoked increased specific immune response to peptides, which practically did not depend on the present of protein molecules in the composition of bioconjugates and the chemical nature of polyelectrolyte carriers. This different peptide containing polymeric immunogens were used in vaccinating guinea pigs for estimation of the potency against FMDV and dose dependent high protection was achieved. Such a modulated system is attractive for application as a novel immunogenic system in vaccine technology.

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RECEPTOR-LIGAND ASSAYS WITH SYNTHETIC PEPTIDES DERIVED FROM THE PLASMODIUM FALCIPARUM MAEBL PROTEIN IN THE SEARCH OF NEW MALARIA VACCINE CANDIDATES

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Erythrocyte binding proteins from Plasmodium falciparum are normally localized in the surface and organelles of parasite's merozoites. These proteins are mapped by employing radio-labeled-peptides covering the entire protein sequence in order to find the so-called high activity binding peptides (HABPs) to red blood cells (RBCs). This methodology has allowed us to identify peptides that belong to different proteins from P. falciparum, most of them able to inhibit the red globule invasion in vitro. It has been also possible to physical-chemically characterize the interaction of these high binding peptides with their target cells as a receptor-ligand interaction. In addition, some to these peptide sequences are recognized by sera from malaria patients and in this way demonstrating the existence of important B-cell epitopes involved in generation of certain immune response. As an example of the most recent works here we present the study with synthetic peptides that cover the whole sequence of the Pf-MAEBL protein. MAEBL is an erythrocyte binding protein located in the rhoptries and on the surface of mature merozoites, being expressed at the beginning of schizogony and has been suggested is involved in processes of invasion of the P. falciparum of its target cells.

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THE IMMUNE PROTEOME: IMPACT OF HLA POLYMORPHISM ON LIGAND SPECIFICITY, STRUCTURE AND FUNCTION

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The HLA B44 family is an important family of HLA molecules that are found at high frequency in all populations (e.g. ~ 24% of Caucasians). We have examined the implications of naturally occurring polymorphism between three HLA B44 family members (HLA B*4402, B*4403 and B*4405). These alleles differ from each other by 1-2 amino acids yet they display very different behavior in terms of antigen presentation. Compared to HLA B*4402 and B*4403, HLA B*4405 is able to present antigen more rapidly and exhibits independence on the chaperone tapasin for antigen loading and surface expression. This is not due to acquisition of higher affinity ligands or enhanced thermostability of the complex. A proteomics based analysis of the peptides naturally presented by these alleles demonstrates that despite significant repertoire overlap, important differences are observed in ligand selection by each allele (1). X-ray crystallographic structures of each allele bound to the same natural ligand demonstrate the subtle structural basis of ligand specificity and results of receptor polymorphism on the conformation of the complex. Finally we demonstrate that these differences have profound biological outcomes in terms of induction of alloresponses and intracellular behavior of the complexes during their maturation and trafficking to the cell surface (1,2).
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MIXED MONOLAYERS OF DPPC/POLY-LYSINE CONSTRUCTS

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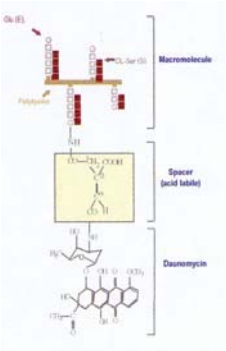
Lysine polymers are frequently used as carriers for biologically active molecules and drugs. Among them, the association of cytostatics with poly-lysine derivatives has shown promising results as far as reduction of toxicity and increase in efficacy In this paper the physicochemical characteristics of one polymeric construct as well as its corresponding daunomycin derivative (Scheme I) are described. Properties studied are related to their ability to spread on aqueous surfaces forming monomolecular layers. Besides, their interaction with membrane models has been determined in mixed monolayers with the main component of biological membranes (phosphatidyl choline). The energies involved in this process are small thus indicating an ideal miscibility. These results suggest a safe interaction of these macromolecules with biological membranes



P663

SEQUENTIAL OLIGOPEPTIDE CARRIERS (SOC) OF IMMUNOGENIC PEPTIDES WITH A BUILT-IN VACCINE ADJUVANT: APPLICATION TO RECONSTITUTED MODELS OF ACHR, LA/SSB AND SM AUTOANTIGENS

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We combined specific B and T cell epitopes for three different antigens, a 'promiscuous' T cell epitope that could overcome genetic restrictions, and an "adjuvant" that could enhance and prolong humoral and cellular immune response, all anchored to a multivalent synthetic carrier, (trying to form an immunogen and consequently an effective vaccine). IL-1β(163-171), previously reported that is devoid of all pro-inflammatory effects of IL-1β but which maintain immunostimulatory activity of the intact cytokine, was added to the N-terminus of the SOCn carrier, (Lys-Aib-Gly)4 a carrier successfully applied in our laboratory for anchoring antigenic/immunogenic peptides. A promiscuous T cell epitope deriving from TT (Tetanus toxoid)(593-599) was also covalently attached to the C-terminus of SOCn, as universal immunogen, resulting in the final modified carrier IL-1β(163-171)-SOC4-TT(593-599). B and T cell epitopes derived from AChR, the main target of autoantibodies in myasthenia gravis and the La/SSB and Sm antigens, against which is directed the majority of autoantibodies in patients with Sjögren's and Systemic Lupus Erythematuous were coupled to the Lys-NεH2 groups of the modified SOCn carrier. The obtained constructs were administered in mice and rabbits following either the complete/incomplete Freund's adjuvant protocol or without any adjuvant. Induction of specific autoantibodies recognizing the priming constructs, as well as their cognate immunogens was detected in ELISA assays, even when immunization occurred without adjuvant, while production of antibodies against the carrier itself was negligible. It is concluded that IL-1β(163-171)-SOC4-TT(593-599) is a promising multifunctional carrier for developing human vaccines.

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DEVELOPMENT OF A NEW SENSITIVE IMMUNOASSAY FOR THE DETECTION OF CARDIAC TROPONINS IN SERA OF PATIENTS WITH CARDIOVASCULAR DISEASES

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Troponin complex plays an important role in regulating skeletal and cardiac muscle contraction. It consists of three different subunits (I, C, T) each of which is responsible for different functions. Cardiac troponins are released in the bloodstream during irreversible cardiac muscle damage and they are being detected in patients' sera 4-6 hours after the cardiovascular incident. The development of biological assays for the detection of cardiac troponins is based on the application of specific antibodies against the whole complex or individual subunits. The high homology between cardiac and skeletal muscle troponins, establish the problem of cross-reactions. This fact combined with the need of signifying improvement of the method's sensitivity enforces the search of more sensitive and specific reagents. In order to contribute in this field, we utilized homology computer software to determine regions of cardiac troponin I and T, which present the minimum homology compared to the skeletal isoforms. From the entire regions that have been determined, we have chosen the 19-31 (RRRSSNVRAYATE) and 118-131 (TKNITEIADLTQKI) regions of cardiac troponin I isoform for production of antibodies. To this aim the selected peptide sequences were conjugated to Sequential Oligopeptide Carrier (SOC), Ac-(-Lys-Aib-Gly-)4-OH. The constructs have been synthesized by solid phase peptide synthesis method using the Boc/Bzl strategy, purified by semi-preparative HPLC and identified by mass spectrometry (ESI-MS). Rabbits immunizations with the above analogues gave high titer immune responses. Evaluation of the specificity and affinity of the produced antibodies is now in progress.

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P10 RESIDUE IMPORTANCE IN ANTIGEN ANCHORING TO CLASS II MHC MOLECULE

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Class II major histocompatibility complex (MHC) proteins bind peptide antigens and present them at the cell surface for recognition by CD4+ T cells. X-ray crystal structures reveal peptides bound in an extended conformation, with pockets in the peptide-binding site spaced to accommodate peptide side chains. In structures of HLA-DR variants, pockets within the overall peptide-binding site are found at the P1, P4, P6, and P9 positions, with smaller pockets at P3 and P7. These positions are consistent with experimentally determined peptide binding motifs, which indicate peptide sequence preferences at these positions. A recent study of HLA-DR52a binding to an integrin peptide suggests a role for the P10 position in peptide binding. To investigate this phenomenon, a crystal structure for HLA-DR1 bound to a similar peptide AAYSDQATPLLLSPR was obtained. The structure shows that the peptide binds in the usual, polyproline type II conformation, with a shallow shelf or pocket that accommodates the P10 residue. The residues that line the P10 pocket, b57 and b60, are polymorphic within human MHC class II proteins. These residues potentially could influence peptide-binding specificity to HLA-DR. To investigate this phenomenon, mutations were introduced at these positions into HLA-DR1. Two series of peptides (AAYSDQTLLLXSPR and (AAYSDQTPLLXSPR) containing all natural amino acids (X) at P10 were synthesized and tested for binding to HLA-DR1 and HLA-DR1 mutants. The results suggest that MHC polymorphism does effect sequence preferences at the P10 position, and additionally reveal a preference in all variants tested for tryptophan at this position.

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PREPARATION OF GLYCO-REPRICA PEPTIDES WITH PHAGE-DISPLAYED PEPTIDE LIBRARY AND THEIR FUNCTIONS

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Phage peptide library technology was used to prepare peptides which mimic the shape of carbohydrate using monoclonal antibody (Mab) against ganglioside antigen. The peptides selected by this procedure showed similar characteristic properties or functions as those of the antigen ganglioside. We proposed to call the peptides glyco-replica peptides. Four peptides were obtained from a 15 mer peptide library displayed on phage envelopes by biopanning with a Mab, 4F6 specific for GD3 ganglioside. By immunization with GD3P4, one of the GD3-replica peptides showing the highest affinity to 4F6, we could produce anti-GD3 antibodies. A Mab, 3D2 obtained by immunization with the peptide showed high binding potency to GD3 by ELISA and by TLC-immunostaining as well as binding to the human melanoma cells expressing GD3 ganglioside by flow cytometry. Comparative analysis of amino acid sequences of the variable regions of heavy and light chains showed only a few amino acid substitutions between 4F6 and 3D2. The present data indicate that the GD3P4 peptide is processed in a certain size and exposed on the antigen-presenting cells with a molecular shape quite similar to that of the GD3 ganglioside epitope. This results support the mechanism of initiation of some autoimmune diseases that are caused by infectious diseases.

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SYNTHESIS OF CELL-PERMEABLE FLUORESCENT LIPO-PHOSPHOPEPTIDES

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Phosphopeptide synthesis has proved to be an invaluable method for the investigation of the rule of phosphorylation/dephosphorylation in cell regulation and signal transduction. While synthesis of peptides phosphorylated on Tyr is usually quite feasible, several side-reactions hamper the synthesis of phosphopeptides containing Ser/Thr. Recently we described a universally applicable method using tert butyl-H-phosphonate ammonium salt, which seems to be superior to the phosphoramidite method, especially in case of serine and threonine. Using these achievements, the preparation of phosphopeptides came easier, but one problem still remained. For functional investigations, the cell permeability of the above derivatives is crucial. In the last decade numerous "protein transduction domains" were described. One of them corresponds to an oligoarginine chain acylated with fatty acid at the N-terminus. According to our goals we worked out a chimeric system containing phosphorylated fragments of the Gab1 protein (Grb2 associated binder), which plays important role in the regulation of the immune system and the above oligoarginine containing transporter molecule. In addition to this, for the functional investigations fluorescent labeled molecules were coupled to these peptide derivatives. The resulting cell-permeable lipopeptide constructs acting as "protein transduction domain" were capable of transporting functionally active molecules into the living cells. Several of them could cause tyrosine phosphorylation in the target cell in a dose and time dependent manner.

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LIPOSACCHARIDES FOR DRUG AND VACCINE DELIVERY

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To induce high antibody responses when administering low molecular weight synthetic peptides, conventionally peptides must be conjugated to a carrier protein (KLH, BSA). High antibody titres have been attained by coupling immunogenic peptides to a multiplying core (eg polylysine) and a lipophilic anchor (lipoamino acids; LAAs), to form a Lipid-Core-Peptide (LCP) system. The long alkyl chains of the LAAs are capable of protecting labile synthetic peptides from enzymatic attack. The LCP system has the capacity to form particulates and can be incorporated into liposomes providing a further avenue for oral vaccination. The LCP system is self adjuvanting, so the antigen, carrier and the adjuvant exist in the same molecular entity. It is particularly important, since currently no human compatible mucosal adjuvant system exists for oral immunisation. Group A streptococci (GAS) are amongst the most common and widespread of human pathogens. They cause a wide range of infections, including acute rheumatic fever, rheumatic heart disease and acute glomerulonephritis. Protection against GAS infection is mediated predominantly by opsonic antibodies directed against the surface M protein, the major virulence factor of GAS.1 The design, development and delivery of a vaccine against GAS infection will be described. The vaccine construct contains three variable and one constant domain from the GAS surface M protein which is mainly responsible for immunity against GAS infection 1.Brandt, E.R. et al. Nature Medicine 2000, 6, 455-9.

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INTERACTIONS OF INTEGRINS WITH CHEMICALLY MODIFIED PEPTIDES

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Integrins are cell surface heterodimer receptors, that consist of noncovalently bound a and b subunits. They play an important role in many physiological and pathophysiological processes involving cell-cell and cell-extracellular matrix interactions. Integrins are involved in leukocyte migration, adhesion and infiltration during inflammatory processes and participate also in differentiation, embryogenesis and development. alpha4beta1 integrin (VLA-4, very late antigen-4; CD49d/29) is expressed mainly on T and B lymphocytes, monocytes, eosinophils, basophils and also on numerous cell lines derived from malignant tumours. It interacts with two natural ligands, VCAM-1 (vascular cell adhesion molecule 1) and the alternately spliced CS1 region of fibronectin. Inhibitors of these interactions may be useful in treatment of various inflammatory diseases. Integrins may interact with their natural ligands through minimal sequence, for example Arg-Gly-Asp (RGD) or Leu-Asp-Val (LDV). It has been shown, that VCAM-1 and CS1 region of fibronectin bind to alpha4beta1 integrin by different signalling sequences, QIDSPLN and LDV respectively. An aspartic residue within the integrin binding epitope of VCAM-1 (transmembrane counter-receptor of the immunoglobulin super family) is positioned in a prominent beta-turn loop, which connects two beta-strands (the CD-loop). Cyclic peptides mimicking the binding epitope of VCAM-1 were synthesized by SPPS (solid phase peptide synthesis). D-amino acids and beta-amino acids were used as inducers of secondary structure. A series of peptides was analyzed with respect to biological activity. A cell adhesion assay using Ramos cells has been established to investigate the biological properties of the cyclic peptides.

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PEPTIDE MOTIF OF LIPOPEPTIDES RECOGNIZED BY TOLL-LIKE RECEPTORS

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Lipoproteins from mycoplasma and their synthetic S-(2,3-dipalmitoyloxy-propyl)-cysteinyl-peptide analogues are strong immune modulators that activate early host responses after infection. These diacylated lipopeptides induce signalling in cells of the immune system through Toll-like receptor TLR2/TLR6 heterodimers. To investigate the influence of the peptide sequence on biological activity a combinatorial lipohexapeptide collection was prepared by fully automated solid phase peptide synthesis and Fmoc/tBu chemistry. By screening this collection in an in vitro IL-8 induction assay with the human monocytoid cell line THP, we systematically evaluated the potential of 19 proteinogenic amino acids in all sequence positions of the peptide moiety to induce cytokine release by interaction with TLR. Amino acids favourable and unfavourable for biological activity were listed in an activity pattern and used as a base for the definition of individual lipopeptides. Unexpectedly most Pam2Cys-lipopeptides induce IL-8 except those lipopeptides with a proline residue next to the lipoamino acid. Fluorescently labelled derivatives of active and inactive lipopeptides were prepared and investigated for interaction with cellular membranes by fluorescence microscopy.

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HYPER BRANCHED POLY(AMINO ACIDS): SYNTHESIS, STRUCTURE AND INTERACTION WITH DNA

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The creation of new types of biodegradable and biocompartible polymer carriers for biologically active compounds bonding and targeted delivery is now one of the important problems in the fields of preparation and medical use of artificial vaccines and in gene therapy. We have developed quick, one stage method of hyper branched homopoly(L-lysine) synthesis in the process of catalytic reductive removing of the N-epsilon-carbobenzyloxy-blocking group from N-epsilon-carbobenzyloxy-L-lysine N-carboxy-anhydride by hydrogen in the presence of activated palladium. In analogous conditions the mixture of two N-carboxy-anhydrides, one of which is N-epsilon-carbobenzyloxy-L-lysine N-carboxy-anhydride and the others are gamma-tert-butyl-L-glutamate N-carboxy-anhydride or alanine N-carboxy-anhydride, the hyper branched heteropoly(amino acids) were prepared. Investigation of their structure by light scattering and hydrodynamic methods has shown that polymers have compact symmetrical sphere like structure which is different from that of stick like structure of linear poly-L-lysine. CD investigation of hyper branched poly(peptides) has allowed us to determine the secondary structure of hyper branched homo- and heteropoly(amino acids). It was shown that homopoly(L-lysin) has predominantly random coil and beta-structures whereas secondary structure of heteropoly(l-lysins) depends on the structure of additional amino acids (alanine or L-glutamic acid) and solvent used (H2O, TFE, 0.01 N HCl or 0.01 n NaOH). The secondary structure of hyper branched poly(amino acids) was compared with that of liner poly-L-lysine. Ability of hyper branched poly(amino acids) to interact with DNA investigated with the help of CD method will be presented and discussed.

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PROTECTIVE SYNTHETIC FRAGMENTS OF NS1 NON-STRUCTURAL TICK-BORNE ENCEPHALITIS VIRAL PROTEIN

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Twelve 16-27 membered fragments of the NS1 non-structural tick-borne encephalitis viral (strain Sophyin) protein were designed according to the previously formulated selection rules [1] and synthesized. Most of these peptides were able to induce antipeptide antibodies in Balb/c, C57/Bl or CBA/J mice in a free state without conjugation with a protein carrier. 5 peptides were able to induce protective immunity in Balb/c mice against lethal challenge with tick-borne encephalitis virus. Antibodies against only one peptide with the 37-55 amino acid sequence were able to react with the NS1 protein of the tick-borne encephalitis virus. In adoptive transfer experiments sera from mice immunized with the 37-55 protecte 60% of animals from lethal infection. Therefor the protective effect of peptide immunization is due to antibodies raised against that peptide. We considered 37-55 NS1 peptide as a perspective tick-borne encephalitis vaccine component and antibodies against that peptide as a drug lead for treatment of patients with tick-borne encephalitis. [1] Volpina O.M., Titova M.A., Zhmak M.N., Koroev D.O., Oboznaya M.B., Volkova T.D., Ivanov V.T. Russian J. of Bioorg. Chemistry, (2002), 28, 387-395.

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CELL-ACTIVATION THROUGH TOLL-LIKE RECEPTORS: TLR6 DEPENDENCE OF TRIACYLATED LIPOPEPTIDES

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Lipoproteins are part of the outer membrane of Gram negative bacteria, Gram positive bacteria, Rhodopseudomonas viridis, and mycoplasma. They are characterized by the N-terminal unusual amino acid S-(2,3-dihydroxypropyl)-L-cysteine (Dhc) acylated by two or three fatty acids (Ac2Cys or Ac3Cys). Ac3Cys contains two ester-bound and one amide-bound fatty acid moiety, Ac2Cys contains only two ester-bound fatty acid moieties and a free N-terminus. Synthetic analogues of the N-terminal part of these lipoproteins constitute potent immunoadjuvants in vivo and in vitro. Cell activation by lipoproteins and shorter lipopeptides is induced through their interaction with Toll-like receptor 2 (TLR2). TLR2 forms heterodimers with either TLR1 or TLR6 to attain specificity for a given stimulus. Diacyl lipopeptides like macrophage activating lipopeptide from Mycoplasma salivarium (FSL-1, Pam2Cys-GDPKHPKSF) or Mycoplasma fermentans (MALP-2, Pam2Cys-GNNDESNISFKEK) were described to require TLR2 and TLR6 for signalling, whereas triacylated synthetic compounds like Pam3Cys-SK4 are able to activate immunocompetent cells independently of TLR6 mainly through TLR2/TLR1 heterodimers. TLR6 appears to confer the ability to discriminate between the N-terminally acylated and not acylated lipopeptides. A systematic approach was performed to investigate the structural influence of the amide-bound fatty acid moiety of triacylated lipopeptides on the TLR2/TLR6 dependent activation of murine B-lymphocytes. Therefore Pam2Cys-GNNDESNISFKEK and lipopeptides Ac3Cys-GNNDESNISFKEK differing in length and character of the amide-bound fatty acid moiety were synthesized and tested for their ability to stimulate TLR6 wild-type and TLR6 deficient murine B-lymphocytes.

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COMPUTATIONAL STUDY OF THE INTERACTION OF GLUTEN PEPTIDES WITH COELIAC DISEASE-ASSOCIATED HLA-DQ2 MOLECULE

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Coeliac disease is sustained by an abnormal intestinal mucosal T-cell response to wheat gliadin and it is strongly associated with HLA class II molecules encoded by DQA1*0501/DQB1*02 (DQ2) or DQA1*03/DQB1*0302 (DQ8). The in vitro stimulatory activity of gliadin is increased after treatment with tissue transglutaminase (tTG) which catalyses the deamidation of specific residues of glutamine to glutamic acid that can serve as anchors for binding to DQ2 as well as to DQ8 molecules. In our work, we have predicted by homology modelling the three-dimensional structure of HLA-DQ2 molecule, the most frequent in celiac patients, and simulated its complex with some gluten peptides by superimposition and energy minimization on the basis of the experimental structure of DQ8-insulin B9-23 complex (PDB code: 1JK8). Moreover, we have evaluated the energies of interaction for each peptide/DQ2 complex and simulated the deamidation of specific peptide glutamines. When a single glutamine residue in the anchor positions is exchanged with glutamic acid, the energy of interaction for the peptide-DQ2 complex appear more favourable. The detailed analysis of the DQ2 peptide binding motif evidenced peculiar features that may explain why this molecule, but not other HLA class II molecules, has the preference for negatively charged residues in specific anchor positions. This finding gives an explanation at molecular level of the experimental results reported in literature. We are applying this strategy to evaluate the effects of other peptide modifications as well as the interaction of DQ2 with other peptides.

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BIOPEP DATABASE AS THE SOURCE OF INFORMATION ABOUT BIOACTIVE PEPTIDE SEQUENCES

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In the recent years bioinformatics had become a very popular discipline applied in contemporary biological sciences. Currently we can observe the massive growth of computer databases, which are suitable tool in prediction of the protein function from its sequence, searching evolutionary similarities between proteins etc. Despite the structural and functional similarities between proteins - they may be also a valuable source of bioactive peptides including antihypertensive, opioid, antithrombotic, immunomodulating and other activities. We characterised about 150 protein sequences being the precursors of bioactive peptides. To achieve this purpose we applied a highly specialised BIOPEP database [http://www.uwm.edu.pl/biochemia], which was designed in our Chair. Currently our database contains 150 protein and 1516 bioactive peptide (possessing about 44 activities) sequences. Thanks to additional database of proteolytic enzymes it is possible to design proteolytic processes to check the potential release of bioactive peptides from their protein precursors. It may be meaningful in designing the functional food and in the nutraceutical industry. The evaluation of protein value as the source of bioactive peptides was based on the following criteria: the profile of potential biological activity of protein (i. e. type and location of bioactive fragment in protein sequence), the frequency of the occurrence of bioactive fragment in protein chain (A) and potential biological activity of protein (B). All above-mentioned criteria are detaily defined in „Record operations", one of the options available in BIOPEP. Above-mentioned criteria of protein evaluation can facilitate designing of proteolysis in aspect of gaining desirable or "removing" non-desirable bioactive fragments.

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AFFINITY PROTEOMICS AND MASS SPECTROMETRIC EPITOPE ANALYSIS: POWERFUL TOOLS FOR IDENTIFICATION OF VACCINE LEAD STRUCTURES AGAINST ALZHEIMER'S DISEASE

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High resolution and high selectivity mass spectrometric approaches for the identification of molecular recognition structures of mono- and polyclonal antibodies have been developed in our laboratory as key prerequisites for vaccine design and targeting. Using combinations of selective proteolytic digestion of immune complexes and MS-peptide mapping (epitope excision) these methods have been applied to molecular epitope identications of peptide and protein antigens, from combinatorial mixtures and in proteome analysis. Using partial, overlapping epitope excision, mass spectrometric "affinity-proteomics' is a new approach providing unprecedented selectivity for direct protein identification from complex biological mixtures. The potential of these MS methods is illustrated by the elucidation of a ß-amyloid plaque-specific epitope recognised by therapeutically active antisera from transgenic Alzheimers' disease (AD) mice upon immunisation with the neurotoxic Aß(1-42) polypeptide. The N-terminal core epitope, Aß(4-10) is independent of the ß-amyloid structure, aggregation properties and neurotoxicity, thus providing a lead structure for the development of AD vaccines. First vaccine conjugates encompassing this epitope have been synthesized and their immunogenic properties evaluated. An epitope-affinity microcolumn was developed from the biotinylated Aß(4-10) containing a flanking D-pentaglycine spacer and employed (i) for antibody-proteomics, and (ii) for the isolation of paratope binding structures of the therapeutically active antibody, using high resolution FTICR mass spectrometry. The use of the epitope-specific affinity column and paratope-mapping by FTICR-MS provided the identification of two specific variable heavy- and light chain paratope peptides, respectively.

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DETECTION OF T-CELL EPITOPES AND MODELING OF DQ2 AND DQ7 MOLECULES USING COMPUTATIONAL METHODS

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The MHC-II/peptide complex interacts with CD4+ to initiate the immune response. Autoantibodies against La/SSB autoantigen are frequently found in the sera of patient with Sjogren's Syndrome (SS) and Systemic Lupus Erythematosus (SLE). The aim of this study was the prediction of MHC class-II binding peptides, T-cell epitopes of La/SSB autoantigen, based on the fact that class II MHC molecules DQ2 (HLA-DQA1*0501/DQB1*201) and DQ7 (HLA-DQA1*0501/DQB1*0301) are strongly associated with SS and SLE, applying computational methods. Initially, models of DQ2 and DQ7 were constructed using as a template the human insulin peptide HLA-DQ8 complex which shows 91% and 96% homology with DQ2 and DQ7 respectively. The Swiss-PdbViewer program was used for the modeling of DQ2 and DQ7 molecules. A sequence pattern common to T cell epitopes was used for the prediction of T cell epitopes as well as the MHCPEP database of MHC binding peptides. The modeled MHC-epitope complexes were subjected to energy minimization using the program X-PLOR 3.1. The quality of the final models was assessed using the Procheck suite of programs and the 'O' program determined the compatibility of the DQ2 and DQ7 models with T-cell epitopes.

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EROP-MOSCOW, A DATABASE OF THE NATURAL OLIGOPEPTIDES

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At present we are familiar with several thousand endogenous oligopeptides involving in the functioning of the nervous, immune, endocrine, and other regulatory systems. They are extracted from more than 1000 biological species of all the kingdoms/empires (animals, plants, fungi, bacteria, viruses). We have developed a specialized database of this substances containing from 2 to 50 amino acid residues named EROP-Moscow (Endogenous Regulatory OligoPeptides). The database contains more than 5,000 entries (with a total of more than 100,000 amino acid residues) of fully referenced different regulatory oligopeptide data including primary structures, posttranslational modifications, functions, location, biological species, taxons, molecular mass values (experimental and calculated), pI, etc. Links to the accessible protein database SwissProt/TrEMBL and the biomedical database PubMed have been created. These entries have been compiled from scientific publications and public databases, cleaned of errors and enriched with additional structural, functional and some other information. EROP-Moscow has a set of different search tools that allow users to extract data and perform specific queries. Except for this it has special statistic pages containing summarized data on sequence sizes, amino acid composition, functional classes of oligopeptides, biodiversity, taxons, literature sources and some other data. The structural and functional diversity of the natural oligopeptides can be demonstrated using EROP-Moscow data. The grouping (structural-functional family formation) of regulatory oligopeptides provides a basis for extending and clarifying the existing structural and functional classification. The EROP-Moscow database is accessible via the Internet at: http://erop.inbi.ras.ru. This work supported by the Russian Foundation for Basic Research (Grant No 02-07-90175).