P C61 - The motif for prediction of protein fragments able to induce antibody formation in mice.

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It is known that to be immunogenic peptide must contain T-helper epitope, the fragment interacting with T-helper receptor and binding to a class II MHC Several programs for T- helper epitopes prediction are available, but mostly for human MHC. On the basis of the literature data [1, 2] on T-helper epitopes structure and our own results we proposed a simple and efficient method for predicting the structure of peptides stimulating antibody formation in mice of different strains. Using this approach, we selected and synthesized peptide fragments of OpaB and NspA proteins of N.meningitidis and protein E of tick-borne encephalitis virus and studied their immunogenic activity in mice of three haplotypes [3, 4, 5]. Of the 16 peptides selected, 14 induced antipeptide antibody formation in mice of two or three strains, one was immunogenic in one strain and one was inactive.
Besides, to confirm our hypothesis, we have synthesized analogs of immunogenic and non-immunogenic peptide fragments of proteins VP1 from foot-and-mouth disease virus and PorA from N.meningitidis. Study of these peptides has confirmed the correlation between the presence of predicted motif and their ability to induce immune response in mice of different strains.

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P C62 - Synthetic peptides for causative therapy of dilated cardiomyopathy

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Idiopathic dilated cardiomyopathy (DCM) is a progressive heart disease characterised by dilatation of cardiac structures and strongly reduced heart function. Currently, there are no causative therapy options, thus, DCM is one of the principle reasons for heart transplantation
In many patients with DCM heart-reactive immunoglobulins were found. In part, these autoantibodies are directed against extracellular domains of the β_1-adrenoceptor. From immunological studies it is assumed that these β_1-autoantibodies (β_1-aab's) are able to induce, maintain, or promote the course of the disease. Consequently, the elimination of these aab's (e.g. by binding through an immobilised antigen) could improve cardiac efficacy.
The objective of this study was to develop peptides, capable of binding to β_1-aab's and eliminate them from human plasma by immunoadsorption (apharesis) on a sepharose matrix. Therefore, we synthesised peptides which sequences are related to extracellular domains of the β_1-adrenoceptor. The lead compounds were optimised in several steps in view of their biological and physicochemical properties, like ease of synthesis, plasma stability, and coupling efficiency, moreover, peptides coupled to sepharose were examined for their biological activity in inhibiting DCM-autoantibodies.
Coraffin®, a new tool for causative treatment of DCM has been developed on basis of these peptides. In a phase I clinical trial it could been shown that Coraffin® was able to stop the progression of DCM, moreover, cardiac functions of DCM-patients improved.

P C63 - Synthesis of longer, multiple phosphorylated peptides on solid phase applying the H-phosphonate method.

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The phosphorylation of proteins is probably the most important reversible element of the cell regulation. The involvement of tyrosine residue in this process is well known, and a similar controlling mechanism concerning serine/threonine containing proteins was recently discovered. The isolation of phosphorylated peptides from biological sources for functional or conformational studies is usually not feasible, and therefore there is a need for efficient chemical phosphorylation methods. Recently we described an universally applicable method using tert butyl-*H*-phosphonate ammonium salt, which seems to be superior over the phosphoramidite method, especially in case of serine and threonine. Now we wanted to extend it by the investigation of different coupling methods for the incorporation of the phosphate moiety. In additon to the pivaloyl chloride based mixed anhydride coupling the HATU and HBTU/HOAt involved reactions proved to be applicable. In contrast of this, many other common coupling methods have failed.
The model peptides, which were synthesized by the upper method were fragments of different proteins playing important functions in the regulation of the immune system, e.g. FcγIIb, SH2-containing-inositol-5'- phosphatase (SHIP) or Grb2-associated binder-1 (Gab-1) fragments and hybrids in differently phophorylated forms.

P C64 - Effect of flanking regions with D-amino acids on the antibody recognition of a MUC2 epitope

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The high molecular weight mucin glycoproteins, produced by the epithelial cells, can be overproduced and undergycosilated in case of different carcinomas. In consequence the hindered protein backbone in the normal glycoproteins becomes accessible for the immune system. This phenomenon could be useful in tumour diagnosis and immunotherapy. The mucins are built up of tandem repeat units, the sequence of that of the MUC2 mucin found in the gastrointestinal tract is ^{1}PTTTPITTTTTVTPTPTPTGTQT^{23} [1]. Our research group has been studying the epitope structure of the MUC2 repeat unit with the help of a monoclonal antibody, MAb 996 [2], and we have demonstrated that the minimal epitope recognised by this antibody is the PTGTQ sequence [3]. Also we have shown that a β-turn in the N-terminal flanking region facilitates the antibody recognition [4]. We found that the replacement of L-amino acids in the N-terminal flank of the epitope with their D-enantiomer does not significantly influence the antigenicity of the peptide [5]. In this paper we aim to further clarify the effect of D-amino acid substitution on the antibody recognition of the PTGTQ epitope. For this we have modified the C-terminal flanking region of the epitope systematically with 1-3 D-amino acids and both termini. The antigenicity, secondary structure and enzymatic stability of the modified peptides were investigated. The peptides substituted by 0-3 D-amino acids were prepared by solid phase peptide synthesis methodology using Fmoc/tBu chemistry. After cleavage with TFA and purification with RP-HPLC the D-amino acid content was analysed with Marfey's reagent. We studied the antibody binding properties of the peptides in competitive ELISA experiments, where the ability of the peptides to inhibit the antibody binding to a synthetic MUC2 target antigen was measured. We also analysed the enzymatic stability of the D-amino acid containing peptides. Our result indicated that manipulation of the flanking region of the PTGTQ epitope with D-amino acids does not significantly influence the antigenicity, but stabilises the peptides against enzymatic cleavage. This could be useful in designing synthetic vaccines in tumour therapy.

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P C65 - Mice amtipeptide antibodies for detection of protease resistant PrP isoform

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It is known that PrP^{BSE} pathogenic isoform is the infectious agent of prion diseases. The mechanism of BSE pathogenesis includes conformational transitions of normal PrP upon its contact with the pathogenic isoform PrP^{BSE} of sheep or cattle and subsequent accumulation of PrP^{BSE} in the nervous tissue. The endogenous cellular prion protein PrP is expressed by most tissues of the body and is therefore known to be a poor immunogen, probably because of host tolerance. We succeeded in obtaining high titer antibodies against synthetic fragments from four various regions of PrP. Mice of three lines: BALB/c, C57/Bl and CBA/J carrying different haplotypes, were immunized with free peptides without conjugation with high molecular carrier. All fragments were able to induce antibody formation at least in one line of mice. The region including the most immunogenic fragments was the 200–240 region of prion sequence. We have revealed a number of polyclonal antipeptide sera binding selectively to the preparations of brain tissues from BSE infected cattle in immunohistochemical assay. Monoclonal antibodies against one of the immunogenic peptides were also obtained capable of exposing PrP^{BSE} by immunohistochemical analysis.

P C66 - The effect of non-native flanking sequences on antibody recognition and solution conformation of PTGTQ epitope from mucin-2

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A minimal epitope PTGTQ of tumour associated mucin-2 glycoprotein was identified by means of a monoclonal antibody, mAb 996 raised against a synthetic mucin-derived 15-mer peptide-conjugate [1, 2, 3]. Now we report on our findings on the effect of non-native flanking sequences on the recognition by mAb 996 and the conformation in solution. For this nonapeptides were prepared with elongation of PTGTQ epitope core by two alanine residues at both termini (AA-PTGTQ-AA). Substitutions of alanine in the position next to the epitope core at the N-and C-terminal flanks AX-PTGTQ-AA [3] and AA-PTGTQ-XA, respectively were executed by all the other proteinogenic amino acids (X) except Cys. Two arrays of nonapeptides- 19 peptides each - were synthesized by parallel SPPS using Boc/Bzl methodology. The binding properties of the peptides were studied in competitive ELISA using mAb 996. Apart from the AT-PTGTQ-AA peptide corresponding to the native mucin sequence four other peptides were recognized with similar efficiency by the antibody [3]. The identity of amino acids (X) in the first position adjacent to the C-terminus (AA-PTGTQ-XA) had also pronounced effect on the binding of monoclonal antibody to the epitope core. In this case peptides containing Trp or other aromatic side-chains seem to have stronger immunoreactivity than the peptide comprising the native amino acid in the X position. To clarify the relation between the conformation in solution and the antibody-recognition, secondary structure of peptides has been analyzed by circular dichroism and Fourier transform infrared spectroscopic methods.

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P C67 - Bovine milk component PP3 as a source of antimicrobial peptides: identification of an active peptide using a predictive approach.

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In milk and colostrum, various components that offer protection against infections have been identified. These antimicrobial factors can be divided in two groups: specific factors that are represented by the immunoglobulins from the mother and non-specific factors. In this last group, lactoferrin and lactoperoxydase, two antibacterial milk proteins, are the most studied. Several peptides are also components of this group and are naturally present in milk or can be generated by enzymatic cleavage of milk proteins. It must be noted that almost all the major proteins from bovine milk (caseins α, β, κ, α-lactalbumin and β-lactoglobulin) encrypt antimicrobial peptides within their sequence. The procedure used in the studies that identified active bovine milk peptides was always the same: the peptide fractions obtained after proteolysis were separated by chromatography techniques and screened for antimicrobial potency. In this work, the approach was completely different since we used structural parameters to predict the presence of putative antibacterial peptides in a protein. Antibiotic peptides encompass a wide variety of structural motifs but the major class is composed of cationic peptides that fold into amphipathic α-helix. Other structural parameters are important for the peptide activity as the hydrophobic moment, the charge and the angle subtended by the hydrophilic/hydrophobic helix surfaces. The sequence of bovine component PP3 (135 amino acid residues) was analysed using secondary structure prediction methods and taking into account all the pre-cited parameters. It appears that the proteins contains at least two distinct domain. The first and longest one covers the N-terminal part (residues 1-97) and is largely negatively charged. No amphipathic character can be detected. The second domain (residues 98-135) is positively charged and displays a clear amphipathic character assuming an α-helical structure. Therefore we hypothize that this domain could provide antibacterial peptides. Since the reduction of this zone to the 113-135 fragment maintains the structural parameters then, this 23-mer peptide was chosen for antibacterial test. Liquid inhibitory-growth experiments against various bacterial strains were carried out using the 113-135 synthetic peptide. The peptide was mainly active against Gram-positive bacteria. However, the bacterial growth was not completely inhibited except for *S. thermophilus*. In that case, the minimal inhibitory concentration (MIC) of the peptide was of 10 μM and the minimal lethal concentration (MLC) was equal to twice the MIC (20 μM). No hemolysis of human red blood cells was detected for peptide concentrations below 200 μM (about ten times higher than the MLC) indicating that the peptide would be non-toxic in this concentration range. These results indicate that the predictive approach was successful although a bactericidal activity was observed only against a non-pathogenic strain. The good MLC value observed is however promising and we will further investigate the peptide activity against other pathogenic strains.

P C68 - Structural features, antimicrobial and membrane properties of microcin E492.

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Microcin E492 (MccE492) is an antimicrobial peptide secreted by *Klebsiella pneumoniae*. Recombinant *Escherichia coli* VCS257 carrying the genetic determinants of MccE492 production and immunity (plasmid pJAM229) were engineered to secrete higher amounts of active microcin in the culture medium [1]. A channel-forming mechanism was proposed for MccE492 based on studies performed with a partially purified microcin [2]. In an attempt to better characterize MccE492 structure and mode of action, we optimized a protocol of production and purification of MccE492 secreted by the recombinant *E. coli* strain cultured in minimum medium (M63-glucose). MccE492 was purified to homogeneity as controlled by silver-stained SDS-PAGE and MALDI-TOF-MS, with an extraction yield of 1.9 mg per liter of culture.
The mature form purified from the culture supernatant corresponds to an anionic and hydrophobic (poly)peptide with a molecular mass at m/z 7887.5 (MALDI-TOF-MS). The first five N-terminal amino acids (Gly-Glu-Thr-Asp-Pro-) together with aminoacid composition showed that it is generated from the precursor molecule by elimination of a 19-residue leader sequence. Preliminary CD conformational studies showed that MccE492 adopts either a random coil structure in aqueous solution or a partly α-helical structure in the presence of 0.3% SDS, that suggests it could gain its active form in contact with membranes. The spectrum of activity was determined against Gram-positive, Gram-negative bacteria as well as yeast and filamentous fungi. MccE492 was found to be specifically active against *Enterobacteriaceae* with MIC values ranging from 20-80 nM on *E. coli* to 2-8 μM on *K. pneumoniae*. This activity was bactericidal against all the tested strains. At high peptide-concentrations (5-10 μM), MccE492 permeabilized *E. coli* ML35 outer and inner membranes, as indicated by a β-galactosidase assay. Nonetheless, no leakage of encapsulated carboxyfluoresceine could be observed from neutral or negatively-charged liposomes when treated with MccE492. Comparison of the MccE492 concentrations needed for growth inhibition and permeabilizing activity on *E. coli* ML35 indicates that the bactericidal activity does not result exclusively from membrane disruption, but might involve a more complex mechanism.

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P C69 - Polymyxin B nonapeptide analogs: biological evaluation and molecular modeling

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Polymyxin B nonapeptide (PMBN) is a cationic cyclic peptide derivative generated by enzymatic processing from the naturally occurring antibacterial peptide, Polymyxin B [1]. PMBN is able of increasing the permeability of the outer membrane (OM) of Gram-negative bacteria towards hydrophobic antibiotics, due to its binding to the bacterial lipopolysaccharide (LPS). The permeability increasing activity of PMBN is highly specific stemming from its unique architecture [2,3]. The present study was aimed at evaluating the significance of the hydrophobic segment of PMBN, i.e. DPhe^5-Leu^6. Hence, two analogs were synthesized by substitution of DPhe^5 with DTrp and DTyr and evaluated for their ability to bind cell-free LPS and to increase bacterial OM permeability. The experimental results have reveled that [DTyr^5] PMBN posse a significantly reduced LPS-affinity and biological activity, i.e. increasing of OM permeability and capacity to inhibit of TNFα and IL-6 release from LPS stimulated immune cells. [DTrp^5]PMBN exhibits similar affinity to cell-free LPS and moderate reduced OM permeability capacity compared with PMBN. The results have demonstrated the crucial role of the hydrophobic segment of PMBN in effecting biological activity. In attempt to understand the above mentioned results molecular modeling was performed based on NMR structure of the complex PMB/LPS [4]. The following assumptions were taken (i) one to one ratio of LPS-peptide complex and (ii) no major changes at the peptide's backbone due to the above substitutions. The calculated minimum energies of the complex LPS-peptide were found to correspond qualitatively to the hydrophobic order of the amino acid residues, i.e. Phe>Trp>Tyr. The energy is lowest for [DTyr^5]PMBN than [DTrp^5]PMBN and finally for PMBN ([DPhe^5]PMBN). Thus, the above findings were opposed to the experimental ones. This discrepancy may be attributed to oversimplification of the modeling study's assumptions.

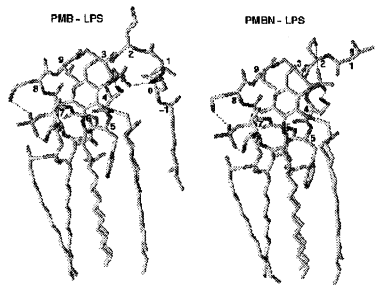

Fig. 1 - The structure of PMB-LPS and PMBN-LPS complex

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P C70 - *In vitro* activity of cathelicidin peptides against fungal clinical isolates

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The need for safe and effective antifungal drugs increases in parallel with the expanding number of immunocompromised patients at risk for invasive fungal infections. The emergence of fungal pathogens resistant to current therapies further compounds the dearth of antifungal agents. Currently available compounds act on targets also found in mammalian cells, which may result in toxicity or an adverse drug interaction. It is therefore imperative to find antifungal agents that are not toxic to mammalian cells. A promising source of such compounds is the variety of gene encoded antimicrobial peptides that are widespread in living organisms as part of the innate immune defenses. In mammals, a major source of these peptides are circulating phagocytic leukocytes and epithelial cells of the skin and mucosal surfaces. In recent years we have identified and characterized a number of novel myeloid antimicrobial peptides from various mammalian species. They are derived from precursors named cathelicidins that are characterized by a highly conserved pre-prosequence. The mature cathelicidin peptides instead vary significantly in sequence, structure, and lenght and include alfa-helical, Pro- and Arg-rich, Cys-rich, and Trp-rich peptides. To select the most promising candidate(s), the *in vitro* antifungal activity of representative members of the α-helical (SMAP-29, BMAP-27, and BMAP-28), Cys-rich (PG-1) and Trp-rich (indolicidin) groups was determined against more than 70 clinical isolates from immunocompromised patients. They included *Candida* spp., *Cryptococcus neoformans*, *Pichia etchellsii* and *carsonii*, *Rhodotorula rubra*, *Saccharomyces cerevisiae*, *Aspegillus* spp., *Penicillium* spp. and *Kloeckera apis*. The minimum inhibitory concentration (MIC) values against these fungi were determined by using the microdilution susceptibility test performed according to the guidelines of the NCCLS. PG-1 showed the broadest spectrum of activity, with MIC values in the 1-8 microM range of concentration for most of the strains tested, followed by SMAP-29 and BMAP-28, which are particularly active against *C. neoformans*. The killing kinetics of some of the peptides was determined against *C. albicans* and *C. neoformans* by counting the survivors after dilution and plating. The lethal effect against fungi is rapid with a decrease from 2 to 4 log in the colony forming units within 30-120 min for *C. albicans*, and 5-60 min for *C. neoformans*, depending on the peptide concentration. Experiments aimed at finding early effects on fungal cells demonstrated that the peptides induce a rapid release of intracellular ATP into the culture medium, indicating an effect on the membrane permeability properties. The release of ATP is followed by alteration of the fungal surface, as shown by SEM.
The activity against clinically relevant fungi indicates that cathelicidin-derived peptides are promising compounds for the development of novel anti-infectious agents.

P C71 - Interaction of antimicrobial peptides with liposomes containing simple analogues of lipid A

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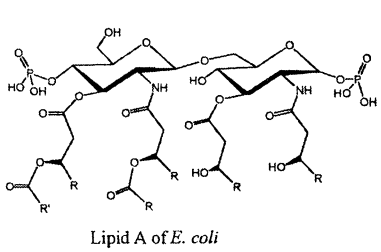
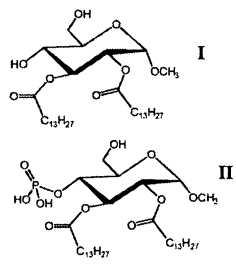
Many studies utilized lipid bilayers (liposomes) of various phospholipid composition as a model of the cytoplasmatic membrane to elucidate the molecular mechanisms of membrane specificity and permeabilization. In contrast, little is known about the interaction of cationic peptides with the negatively-charged cell walls to which the peptides are expected to bind before reaching the ultimate target. In the case of Gram-negative bacteria the outer leaflet of the outer membrane is mainly built up from polyanionic lipopolysaccharides (LPS) consisting of polysaccharide moiety and a covalently linked moiety called lipid A. The limited number of studies concerning LPS-peptide interactions showed that magainins perturb the lipid organization of the membrane and that, probably, the lipid A moiety is the binding site [1,2]. By using phosphatidylcholine (PC) liposomes doped with compounds **I** or **II**, as simple lipid A analogues, we investigated how the conformation of antimicrobial peptides and the properties of the phospholipid bilayer are affected by modifications in the glycolipid structure. Compound **I** was prepared in 3 steps starting from the methyl - glucopyranoside. The isopropylidene acetal was used for the temporary protection of the hydroxyl groups on C6 and C4, to achieve selective O-acylation on positions 2 and 3 of the sugar. Selective silylation of the deprotected 6-OH group, followed by 4-O-phosphorylation with diphenylchlorophosphate yielded, after removal of the protecting groups, the glycolipid **II**. Mixed vesicles of PC and compound **I** or **II**, were prepared by sonication and used to investigate, by CD measurements, the conformational changes induced on magainin 2, an antimicrobial peptide belonging to the class of amphipathic -helical peptides, and on two Pro-Arg rich peptides, apidaecin Ib and drosocin. The effect of peptides on the barrier property of the different bilayers was also investigated by the dye leakage assay.



Lipid A of *E. coli*



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P C72 - Biological activities of amino acids and peptide-functionalized cholic acid derivatives

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Combinatorial chemistry, in conjunction with solid phase synthesis, is proving a powerful tool for the discovery of biological active molecules, receptors and catalysts. As noted by several groups, the steroid nucleus has special attractions as a starting point for scaffold design and synthesis. It is rigid, readily accessible, versatile in terms of substitution patterns, and has been intensively studied by synthetic chemists over many years.
The bile acids, such as cholic acid and derivatives are especially interesting. Their co-directed functionality suggests the presentation of arrays of structural units to protein surfaces, binding sites or substrates.
Now we describe a simple method for the synthesis of various cholates. A series of condensation products of cholic acid with amino acids and peptides were prepared and tested in vitro for antimicrobial activity.
Chromatographic, spectral, and optical properties of the molecules have been investigated.

P C73 - Design and synthesis of indolicidin analogues with enhanced positive net charge and amphipathicity

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Indolicidin, a cationic antibacterial tridecapeptide amide [1] (1) isolated from the granules of bovine neutrophils, has been found to possess antimicrobial activity against a broad range of pathogens including bacteria, fungi, and enveloped viruses, however its nonselective toxicity may restrict its therapeutic utility.
In order to obtain peptides with selective activity we carried out the secondary structure prediction studies of indolicidin and maintaining the same length (13 amino acids), designed analogues with the increased number of positively charged amino acid residues and with the enhanced amphipathicity.

Ile-Leu-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Arg-Arg-NH_2 (1)	+4*)
Ile-**Lys**-Pro-Trp-Lys-Trp-Pro-Trp-Trp-Pro-Trp-Ala-Arg-NH_2	+5
Ile-**Lys**-Pro-Trp-Lys-Trp-Pro-Trp-**Lys**-Pro-Trp-Arg-Arg-NH_2	+6
Ile-**Lys**-Pro-Trp-Lys-**Lys**-Pro-Trp-**Lys**-Pro-Trp-Arg-Arg-NH_2	+7
Lys-Lys-Pro-Trp-Lys-Trp-Pro-**Lys-Lys**-Pro-Trp-Arg-Arg-NH_2	+8
Ile-**Lys-Lys**-Trp-Lys-**Lys**-Pro-Trp-**Lys-Lys**-Trp-Arg-Arg-NH_2	+9

*) net positive charge

It is well known that the presence of tryptophan suggests a potential for side reactions. Van Abel R.J. et al. [2] were developed the conventional method of indolicidin synthesis using Fmoc-technology. According to literature data all indolicidin analogues were obtained by this technology also. We synthesized indolicidin and its analogues by means of the stepwise Boc-strategy, using the various set of protective groups for Trp (Form, Mts) and Arg (Mts, NO_2). As a result we found their optimal combination [Trp(Form), Arg(NO_2)] and prepared the aim peptides with a good yields and purity. All synthetic analogues showed *in vitro* activity against gram-negative and gram-positive bacteria comparable to that of indolicidin or better but did not exhibit any hemolytic activity.

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[2] Van Abel R.J et al *Int. J. Pept. Prot. Res.* 1995, 45(5), 401-409.

P C74 - Amphiphiles containing steroid lipophilic groups

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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 an 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 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 amino acids and dipeptides as shielded biological agents were obtained.

P C75 - Antimicrobial activity ff SMAP-29 against clinically relevant anaerobic bacteria

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SMAP-29 (RGLRRLGRKIAHGVKKYGPTVLRIIRIA-NH_2) is a 28 residues peptide first identified in sheep myeloid cells as a member of the cathelicidin family of antimicrobial peptides [1]. This linear peptide undergoes a transition from random coil to α-helical conformation in the presence of chemical agents (TFE, SDS) mimicking a hydrophobic environment. Edmundson wheel representations of the peptide sequence suggest a nearly perfect segregation of the hydrophobic and hydrophilic/cationic amino acid residues. SMAP-29 was shown to exert a potent, rapid and broad-spectrum activity against a variety of aerobic bacteria, also including antibiotic-resistant clinical isolates, and fungal species (Travis et al., Infect. Immun. 2000, 68, 2748). This activity appears to be mediated by the ability of the peptide to rapidly permeabilize the cytoplasmic membranes of target microorganisms. Despite a wealth of published information on the *in vitro* activity of SMAP-29, no data on the effects of this peptide on anaerobic bacteria have thus far been reported. In this study, the peptide was assayed against reference and clinical anaerobic strains, also including strains carrying antibiotic resistant mechanisms. The bacteriostatic and bactericidal activities were determined by means of broth microdilution susceptibility test and time-killing experiments, respectively, and the killing mechanism was investigated using a two-color fluorescent permeabilization assay and scanning electron microscopy. Several reference strains of *Bacteroides fragilis*, *Prevotella* and *Porphyromonas* (gram-negative) and *Clostridium perfringens* (gram-positive), and most clinical isolates were inactivated by SMAP-29 at 1 – 2 μM peptide concentration. In all cases, the bactericidal concentrations were only 1.5 – 2-fold higher than those bacteriostatic. These values are similar or only slightly higher then those required for inactivation of aerobic bacteria, thus confirming the antimicrobial potency of SMAP-29. The bacterial cells were almost completely permeabilized (90-100%) within two hours of exposure to SMAP–29 in a peptide concentration range between MIC and MBC. The morphology of the peptide-treated bacteria confirmed that the envelope is the primary target of the bactericidal activity of SMAP-29. Thus, despite structural differences in the composition of the membrane components, both aerobic and anaerobic bacteria share surface patterns which are targeted by SMAP-29.

Reference
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P C76 - Thionins from *pyrularia pubera*: synthetic and structural studies

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In the course of our studies on antimicrobial peptides we have focused our attention on the thionins, the first plant antimicrobial peptides whose activity against pathogens was demonstrated *in vitro*. Thionins were first isolated from wheat seeds and play an important role in the innate immunity of plants together with several other families of cysteine-rich peptides. Mature thionins are 45-47-residue peptides with 3 or 4 internal disulfide bridges. Our specific target was the thionin from *Pyrularia pubera* (Pp-THPY, Fig. 1). We developed an efficient synthesis of Pp-THPY based on the Fmoc/tBu protection scheme and using Trt to protect the 8 Cys residues. Following chain assembly, the peptide was deprotected and cleaved from the resin by TFA acidolysis and purified by RP-HPLC to homogeneity. Oxidative folding at high dilution of the octathiol in NH_4AcO, pH 7.8, in the presence of GnHCl and reduced and oxidized glutathione provided the target peptide in acceptable yields and purity. Analytical data (MALDI-TOF MS) agreed with the presence of 4 internal disulfides. Determination of Cys connectivity, presently under way, was complicated by the resistance of folded Pp-THPY to most proteases. However, the synthetic material coeluted with an authentic sample of thionin from *Pyrularia pubera* and was as active as the natural peptide against a panel of representative bacterial and fungal plant pathogens such as *Clavibacter michiganensis, Fusarium oxysporum* and *Botritys cinerea*. In an attempt to improve/expand the spectrum of activity of Pp-TPHY we have started an analogue synthesis program. A first interesting result is that replacement of Asp32 by Arg (Pp-THPY(Arg)) causes a substantial (6- to 10- fold) increase in activity against Gram negatives such as *Rhizobium meliloti* and *Xanthomonas campestris*. These expanded antimicrobial properties, which can be related to the increase in cationic character in one of the two a helices of Pp-THPY, will be discussed in the light of structural data for both Pp-TPHY and the Pp-TPHY(Arg) mutant.

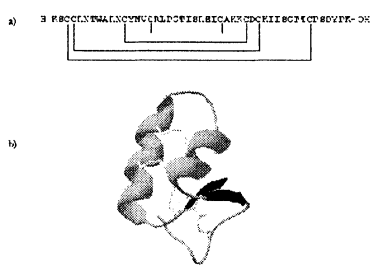

Fig. 1 - Amino acid sequence (a) and 3D structure (b) of P. pubera thionin (Pp-THPY)

P C77 - Sequence specificity of the serine protease, factor Xa

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Factor Xa, a mammalian protease, operates at the convergence of the extrinsic and intrinsic blood coagulation pathways and is a prime target for the development of new selective anticoagulants. Intimate knowledge of the peptide sequence most preferred by Factor Xa provides insight into the enzyme's physiological role, its interactions with natural inhibitors and allows more effective design of potent selective anticoagulants. The specificity of Factor Xa for residues N-terminal to the cleavage point in a peptide (the non-prime side) has previously been investigated. We are interested in the specificity of the enzyme for substrate residues C-terminal to the scissile bond (the prime side of the substrate). We have used fluorescence quenched peptide probes and mutant variants of Factor Xa to gain insight into the sequence specificity of this enzyme at prime sites.
The probes used in these studies are of the type shown in Figure 1. They contain an N-terminal 2-aminobenzoyl (Abz) group as a fluorophore and a penultimate 2,4-dinitrophenyl (Dnp) derivatised lysine as an intramolecular quencher. Cleavage of the substrate probe by Factor Xa stops the intramolecular quenching of the fluorophore and the resulting fluorescence can be measured. This allows determination of the rate of hydrolysis and the corresponding Km and Kcat values. We have synthesised various peptide substrates on a small scale using solid phase techniques. Mimotopes' Macrocrowns with an hydroxymethylphenoxy-acetic acid (HMPA) linker have been utilised to enable deprotection of side groups and cleavage of the peptide from the solid phase as the free acid in one step. A series of peptides of the sequence Abz-Val-Gly-Pro-Arg--Ser-Phe-Leu-Leu-Lys(Dnp)-AspOH with varying amino acids at the P_1', P_2' and P_3' sites, have been obtained and tested with bovine Factor Xa. Also, three peptides based on the sequences of the physiological substrates of Factor Xa have been synthesised and tested with human plasma-derived, wild type and mutant recombinant human Factor Xa. The synthesis of these probes and the results obtained will be described. It is hoped this information will significantly advance the development of inhibitors with high affinity and selectivity for Factor Xa and lead to the ultimate aim of novel anticoagulants for both prophylactic use and the treatment of acute disorders.

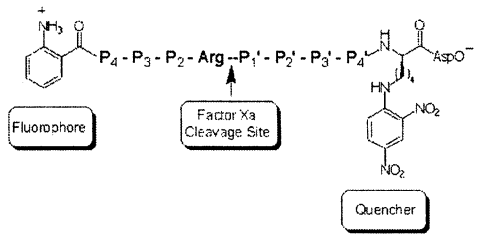

Fig. 1 - Design of Peptide Probes: To determine sequence specificity, varying amino acids are substituted at the prime sites.

P C78 - Down regulation of T-cell activation by synthetic dipeptidyl peptidase IV inhibitors with the N-terminal MXP sequence

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Dipeptidyl peptidase IV (DP IV, CD26, EC 3.4.14.5) is expressed on various cell types, including T cells. As an ectopeptidase DPEIV removes N-terminal dipeptides from oligopeptides with protonated N terminus if the penultimate amino acid is proline or alanine. It has been shown that DP IV plays a key role in the the regulation, differentiation and growth of lymphocytes.
Synthetic DP IV inhibitors as well as the human immunodeficiency virus–1 Tat(1-86) protein (HIV-1) suppress DNA synthesis of antigen- as well as mitogen stimulated peripheral blood mononuclear cells (PBMC) and T cells. Recently we could show that the N-terminal nonapeptide Tat(1-9) (MDPVDPNIE) inhibits DP IV activity and DNA synthesis in a comparable extent to Tat(1-86) if it is used in a 20-fold higher concentration. Further synthetic peptides containing the N-terminal MXP sequence also inhibit DP IV and therefore suppress T cell growth.
Studying the effect of amino acid exchanges in the N-terminal three positions of the Tat(1-9) sequence, we found that tryptophan in position 2 of Tat(1-9) strongly improves DP IV inhibition.
Also other peptides such as the N-terminal nonapeptides of Met-IL-2 and Met-G-CSF exerting poor DP IV inhibition, were converted into much better DP IV inhibitors by exchange the amino acid in position 2 for tryptophan. These facts emphasize the importance of the N-terminal MWP structural motif for DP IV inhibition. Kinetic studies demonstrated that amino acid exchange at different positions of Tat(1-9) (parabolic mixed type of inhibition) can result in changing the inhibition type. Data base searches revealed the thromboxane A2 receptor (TXA2-R) as a membrane protein extracellularly exposing N-terminal MWP. TXA2-R is expressed within the immune system on antigen–presenting cells, namely monocytes. The N-terminal nonapeptide TXA2-R(1-9) inhibits DP IV and DNA synthesis and IL-2 production of tetanus toxoid-stimulated PBMC. Moreover, TXA2-R(1-9) induces the production of the immunsuppressive cytokine transforming growth factor-1. These data suggest that the N terminus of TXA2-R is a possible endogenous inhibitory ligand of DP IV with N-terminal MWP structural motif and may modulate the immune response via DP IV inhibition.

P C79 - Design and characterization of a synthesized hybrid inhibitory miniprotein

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The third domain of turkey ovomucoid inhibitor (OMTKY3) inhibits a broad spectrum of serine proteases. The peptide sequence corresponding to the binding loop of OMTKY3 was optimized which respect to affinity and specificity to porcine pancreatic elastase (PPE). For this peptide libraries were synthesized on cellulose using the spot technique. The optimized inhibitory peptide shows a high affinity and specificity but was not stable against proteolytic attack. This peptide was built into the binding loop of a squash-type inhibitor. The family of squash-type inhibitors comprise of about 30 amino acids and have a stable and rigid structure fixed by three disulfide bridges. Most of these inhibitors are specific for trypsin. The new hybrid inhibitor HEI-TOE I consists of 28 amino acids and shows the same specificity as the optimized peptide. The K_i value against PPE was determined to $9.8*10^{-8}$ M. The complex formed by HEI-TOE I and PPE was crystallized and the structure determined. The structure of the hybrid inhibitor shows the same fold as the parent squash-type inhibitor. The hybrid miniprotein was considerably more stable against proteolytic cleavage than the inhibitory peptide.

P C80 - Peptide-based protease inhibitors of the hepatitis C virus full-length NS3 protein (protease-helicase/NTPase).

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The Hepatitis C Virus (HCV) NS3 protease is inhibited by both classical serine protease inhibitors with an electrophilic serine-trap functionality in the P1 position and, uniquely, by its N-terminal cleavage products which have a free -carboxylate in the P1 position. Peptide-based inhibitors of these two types and, additionally, bioisosteres thereof have been prepared (Fig. 1). Synthesis of the P1 residues and the subsequent synthesis of the C-terminal modified peptide inhibitors using N- to C-directed (inverse) SPPS will be presented. The inhibitory potency of all these inhibitors, including a small library of tetra and tripeptides, evaluated in our in vitro assay system comprising the native bifunctional full-length NS3 (protease-helicase/NTPase) protein will be shown and compared.

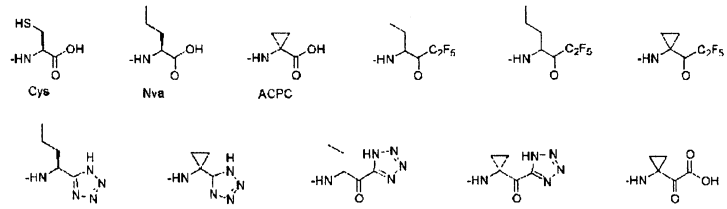

Fig. 1 - P1-building blocks used in the inhibitor sequences Suc-D-Glu-Leu-Ile-Cha-P1 and Suc-Asp-D-Glu-Leu-Ile-Cha-P1.

P C81 - Synthesis of TMC-95A analogs as reversible inhibitors of eukaryotic proteasome

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The proteasome is a multienzyme complex that plays a key role in intracellular non-lysosomal protein degradation. Since it regulates the central functions of cells, it is involved in various pathophysiological processes and thus, selective proteasome inhibition could possibly be of therapeutic relevance in cancer, malaria, Alzheimer, HIV and inflammatory or immune diseases. Recently, TMC-95A, a novel natural product from the fermentation broth of Apiospora montagnei Sacc. TC 1093, was found to be a surpringly strong reversible inhibitor of the chymotrypsin-like activity of the proteasome [1]. X-ray crystallographic analysis of the yeast proteasome/TMC-95A complex [2] enabled us to identify its binding mode and to derive the minimum core structure (Figure 1) as template for the design of new proteasome inhibitors [3]. To facilitate the synthesis of this cyclic tripeptide containing the bis-aryl moiety, a synthesis on resin will be presented that allows iterative replacements of the R^1, R^2 and R^3 residues to address inhibition of the three active sites of eukaryotic proteasome with chymotrypsin-, trypsin- and peptidylglutamyl hydrolase-like activities.

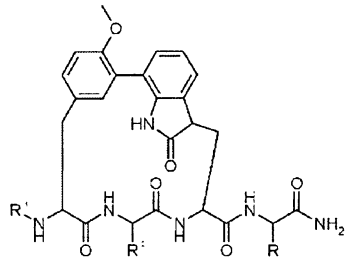

Fig. 1 - The minimal skeleton of TMC-95 A for proteasome inhibition.

References
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[3] Kaiser, M.; Groll, M.; Renner, C.; Huber, R.; Moroder, L. (2002) *Angew. Chem. Int. Ed.*, in press.

P C82 - Structure-based design and synthesis of cathepsin K inhibitors

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Cathepsin K appears to be the major enzyme responsible for organic matrix degradation during bone resorption by the osteoclasts. Successful inhibition of the enzyme holds great promise for the treatment of osteoporosis. We have shown previously that human cystatin C, the natural thioprotease inhibitor present in most tissues, strongly inhibits mineral mobilisation and bone matrix degradation [1]. Additionally, we have found that some low-molecular peptidyl aldehydes, structurally based upon the cysteine protease interacting segments of selected cystatins show the inhibitory activity against cathepsin K [2]. This time, the synthesis and biological evaluation of another group of putative cathepsin K inhibitors are presented. Various cystatins possessing the sequence X_1-X_2-Leu-Gly in their P_4-P_1 sites play a role of the lead compounds in our designing study.

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P C83 - Chemical synthesis and kinetic study of the smallest naturally occurring trypsin inhibitor SFTI-1 isolated from sunflower seeds and its analogues

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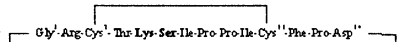
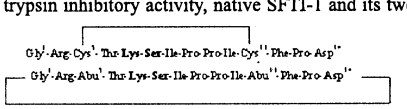
Trypsin inhibitor SFTI-1 isolated from sunflower seeds [1], is the smallest naturally occurring plant peptidic serine proteinase inhibitor. Its head-to-tail cyclised peptide structure is additionally stabilised by a C^3 - C^{11} disulfide bridge.



The crystal structure of SFTI-1 in complex with bovine -trypsin shows both sequence and conformational similarity to the trypsin reactive loop of the Bowman-Birk family [2] of serine proteinase inhibitors (BBI). SFTI-1 however, is unique being monofunctional (inhibits only one proteinase at a time), significantly shorter than other inhibitors belonging to this family (typically 60-70 amino-acid residue) and the only one found to be cyclic. In addition, its trypsin inhibitory activity is considerably higher than that of other members of the Bowman-Birk family inhibitors. SFTI-1 also inhibits other proteinases (chymotrypsin, elastase and thrombin), but with lower affinity [1]. Because of the small size and strong activity this peptide turned out to be a very attractive template for design and chemical synthesis of new proteinase inhibitors with the potential use as therapeutic agent. In order to investigate the influence of the cyclic nature of SFTI-1 and disulfide bridge on the trypsin inhibitory activity, native SFTI-1 and its two analogues (I and II) were synthesised:



All peptides were synthesised by the solid phase method using Fmoc-chemistry. Association equilibrium constants (K_a) of all three peptides with bovine β-trypsin were determined by the Green-Work method developed in the lab. of M. Laskowski, Jr. The results obtained indicate that all peptides studied display inhibitory activity towards bovine β-trypsin. Both peptides - native SFTI-1 and the analogue II with the disulfide bridge inhibit trypsin with very high potency (the association equilibrium constants: $1.1 \cdot 10^{10}\,M^{-1}$ and $9.9 \cdot 10^{9}\,M^{-1}$, respectively). Analogue I has a weaker affinity towards bovine -trypsin ($K_a = 4.6 \cdot 10^{9}\,M^{-1}$) and additionally demonstrate temporary inhibition (enzyme regains activity after prolonged incubation time). In order to state proteolytic stability of these inhibitors, rates of hydrolysis were determined. The results obtained indicate that head-to-tail cyclisation of inhibitor molecule is less important for activity, and especially stability, than formation of the internal disulfide bond.

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P C84 - Peptidomimetic aspartic protease inhibitors: general use of the hydroxymethycarbonyl scaffold as a transition-state isostere

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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 β-secretase is considered to be targets for therapeutic agents of AIDS, ATL (adult T-cell leukemia), malaria, hypertension, and Alzheimer's disease, respectively. Based on the substrate transition state, we designed and synthesized a novel class of human renin inhibitors and HIV protease inhibitors containing the hydroxymethylcarbonyl (HMC) isostere (Fig. 1). Among them, the tripeptide KNI-272 (Fig. 2) was a highly selective and superpotent HIV protease inhibitor. KNI-272 exhibited potent in vitro and in vivo antiviral activities with low cytotoxicity. The NMR, X-ray crystallography and molecular modeling studies showed that the HMC group in KNI-272 interacted excellently with the aspartic acid carboxyl groups of HIV protease active site. The hydroxyl group makes one hydrogen bond to Asp125 oxygen and the carbonyl oxygen makes a hydrogen bond to the protonated oxygen of Asp25 in the essentially same manner as the transition state (Fig. 1). Thus, the HMC isostere is an ideal transition-state mimic [1]. We further expanded this concept to development of inhibitors of plasmepsin II and HTLV-1 protease. Plasmepsin II is a key enzyme in the life cycle of the *Plasmodium* parasites responsible for malaria, a disease that afflicts more than 300 million individuals annually. Since plasmepsin II inhibition leads to starvation of the parasite, it has been acknowledged to be an important target for the development of new antimalarials. We identified and characterized high affinity plasmepsin II inhibitors containing HMC isostere. Among them, KNI-727 inhibited plasmespin II with a Ki of 70 nM and a 22-fold selectivity with respect to the highly homologous human enzyme cathepsin D [2]. KNI-727 also exhibited relatively potent activity against HTLV-1 protease. These results indicate that the HMC isostere is generally applicable for design of aspartic protease inhibitors as an effective transition-state mimic.

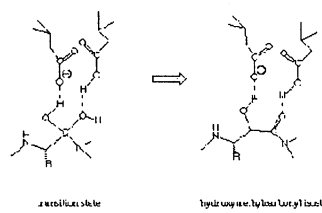

Fig. 1 - Transition state and hydroxymethylcarbonyl isostere

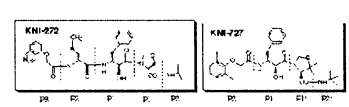

Fig. 2 - Structure of KNI-272 and KNI-727

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P C85 - Identification of the angiotensin IV receptor as insulin-regulated aminopeptidase: enzyme inhibition by AT_4 ligands

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Angiotensin IV (Ang IV) is a hexapeptide fragment of angiotensin which displays a distinct pharmacological profile, and its central administration results in the enhancement of memory retention and retrieval. The effects of Ang IV have been attributed to its binding to a unique receptor, the AT_4 receptor, which to date has eluded purification and/or cloning. We have recently isolated the AT_4 receptor from bovine adrenal membranes and identified it as insulin-regulated aminopeptidase (IRAP). The binding characteristics of Ang IV and its analogues to IRAP expressed in HEK 293T cells were identical to those seen in adrenal membranes. Furthermore, the distribution of IRAP immunoreactivity in the mouse brain matches that of specific Ang IV binding. Expressed IRAP readily cleaved the N-terminal residues of vasopressin, oxytocin, and met-enkephalin, and to a lesser extent, neurotensin and a-neo-endorphin. Amongst the angiotensin peptides, Ang I and Ang II were resistant to cleavage, while Ang III and Ang IV were slowly cleaved; however, the level of peptidase activity was equivalent to that seen with mock-transfected HEK 293T membranes. The AT_4 ligands LVV-hemorphin-7, Nle^1-Ang IV, and Divalinal-Ang IV were also resistant to cleavage by IRAP. However, all the AT_4 ligands could inhibit IRAP, as determined using the fluorescent substrate Leu-b-naphthylamide (K_i's: Ang IV, 125 nM; Nle^1-Ang IV, 165 nM; LVV-hemorphin-7, 550 nM; Divalinal-Ang IV, 2.2 μM; compared to AVP, ~ 22 μM). Lineweaver-Burk analysis indicates that both Ang IV and Divalinal-Ang IV are competitive inhibitors of IRAP, although previously proposed as AT_4 agonist and antagonist, respectively. In conclusion, it seems likely that the physiological effects of Ang IV are the consequence of the protection of other bioactive peptides from IRAP cleavage, although this mechanism remains to be confirmed, and the peptide substrates involved are yet to be identified.

P C86 - Bioactive peptides as potential substrates for enteropeptidase

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Enteropeptidase (enterokinase EC 3.4.21.9), catalyzing trypsinogen activation, exhibits unique properties for high efficiency hydrolysis of the polypeptide chain after the N-terminal tetraaspartyl-lysil sequence. This makes it a convenient tool for gene engineering research, for the processing of a variety of fusion proteins containing this sequence. The use of enteropeptidase, and activation peptide, obtained via EP-catalyzed processing of trypsinogen, acquires a great significance for medical research devoted to the undesirable premature activation of proenzymes in acinar cells of the pancreas. Such activation leads to a most serious disease - pancreatitis. The duodenopancreatic reflux of enteropeptidase causes its penetration in the blood flow and to disorders. Therefore, it is important to study the degradation of hemoglobin fragments. While the development of a method for testing the activation peptide of trypsinogen in the pancreas tissue, acsites liquids, urine and blood plasma is necessary for recognition pancreatitis at an early stage of the disease. Enteropeptidase can hydrolyse peptide bonds formed by carboxyl groups of Lys or Arg residue in corresponding peptides if less than four negative charged amino acid residues are in substrate P_2-P_5 positions. Kinetic parameters for enteropeptidase hydrolysis for three substrates of this type: human angiotensin II DR-VYIHPF (AT) and cattle hemoglobin β-chain fragments (hemopoietic peptides isolated previously from the bone marrow extract [1]) LTAEEK-A (Hb 2-8) and MLTAEEK-AA (Hb 1-9) were determined. K_m values for all these substrates (~10^{-3} M) are an order of magnitude higher than corresponding values for typical enteropeptidase artificial peptide or fusion protein substrates with full enteropeptidase linker -DDDDK- (k_m~10^{-4} M). k_{cat} values for ATand Hb 2-8 are similar and not great (~30 min^{-1}). The resultant hydrolytic efficiency of such substrates did not exceed 1% of the corresponding value for typical enteropeptidase peptide or artificial protein substrates. But one additional amino acid residue at both N- and C-terminus of Hb 2-8 results in a drastic increase of hydrolysis efficiency: k_{cat} value for Hb 1-9 is 1510 min^{-1}. Hb 1-9 hydrolysis is only three times less effective than the hydrolysis of the fusion protein with full enteropeptidase linker. Thus, besides its unique natural substrate trypsinogen, enteropeptidase has the ability to sufficiently specifically hydrolyse in vitro several biologically active peptides.

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P C87 - The synthesis of aminomethyl substituted azaphenylalanine derivatives as thrombin inhibitors

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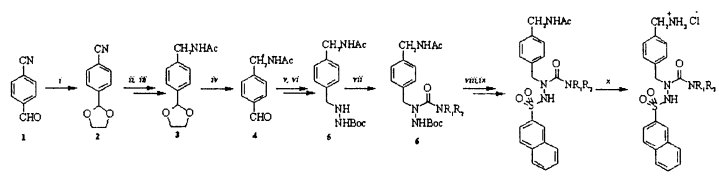
The serine protease thrombin is a key enzyme in the control of blood coagulation and displays numerous other effects in the organism. Thus a potent and selective thrombin inhibitor could be beneficially used in the treatment of several cardiovascular disorders. Most compounds reported to date have low oral bioavailability due to the presence of highly basic functional group such as guanidine or amidine that interacts with Asp189 at the bottom of selectivity pocket of thrombin. We decided to prepare aminomethyl analogues of the compounds already published by our group [1,2], in which the azaphenylalanine fragment was also incorporated as an isosteric substitution for phenylalanine. The replacement of amidine moiety with aminomethyl group resulted in reduced basicity [3,4], on the other hand with the formation of azatides the center of chirality is removed and the enzymatic hydrolysis of peptide bond is avoided [5]. We present herein the synthesis of aminomethyl derivatives **8**, starting from tert-butyl 2-{4-[(acetylamino)methyl]benzyl}-1-hydrazinecarboxylate (**5**) that was obtained by condensation of *N*-(4-formylbenzyl)acetamide (**4**), prepared by a three-step reaction from 4-cyanobenzaldehyde, and *tert*-butyl carbazate.



i. $HO(CH_2)_2OH$, p-TsOH, toluene; ii. $LiAlH_4$, THF; iii. Ac_2O; iv. 90% HCOOH; v. NH_2NHBoc, EtOH; vi. H_2/Pd, MeOH; vii. triphosgene, NHR_1R_2, DIEA, CH_2Cl_2; viii. HCl, AcOH; ix. naphthalene-2-sulfonyl chloride, DIEA, CH_2Cl_2; x. 4M HCl

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P C88 - New renin inhibitors with pseudodipeptide unit in P1-P1 prim and P2 prim-P3 prim positions

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A series of four new potential inhibitors has been synthesized. The structures of the compounds were designed in such a way as to produce agents resistant to enzymatic degradation, metabolically stable and possibly potent. All positions of the 8 - 13 fragment of the human angiotensinogen were occupied by unnatural units. They were unnatural amino acids [Phe(4-OMe) and MeLeu or MePhe] in positions P3 and P2 and pseudodipeptides [statine, Sta, (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid or AHPPA, (3S, 4S)-4-amino-3-hydroxy-5-phenylpentanoic acid] in positions P1-P1 prim and P2 prim-P3 prim . Both N- and C- terminl functions of the inhibitors were blocked (with t-Boc and ethyl ester substituents respectively). Such blocked peptides are unpolar and therefore they were expected to penetrate well across the biological barriers. Hydrophobicity of the inhibitors evaluated as a log P value, calculated by a computer method, was within the limits of 6.1 - 6.6. It is rather a moderate lipophilicity and therefore we hoped for rather a good intrinsic membrane penetration. The presence of hydroxyl group in molecules of both Sta and AHPPA could improve the solubility of the inhibitors. It could be also important for biological activity. All peptides were obtained by the carbodiimide method in solution and purified by column chromatography. Their purity and identity was confirmed by TLC, HPLC and 1H NMR. The resistance to enzymatic degradation was assayed by determination of stability against chymotrypsin activity after incubation for 4 h at 37 °C degree at pH 6.9. The potency was measured in vitro by a spectrofluorimetric method (assay of Leu-Val-Tyr-Ser releasef from the N-acetyltetradecapeptide substrate by renin in the presence of the tested inhibitor). All inhibitors were stable to chymotrypsin. Their IC_{50} (M/l) values were: $9.6 \cdot 10^{-4}$, $1.6 \cdot 10^{-5}$, $1.0 \cdot 10^{-5}$ and $1.0 \cdot 10^{-5}$ respectively.

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P C89 - Thrombin inhibitors with an azaphenylalanine scaffold: potency enhancements

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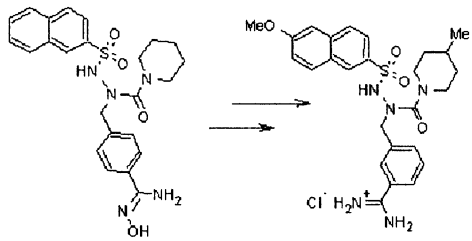
Thrombin plays a major role in thrombosis, which is one of the leading causes of cardiovascular disease and morbidity in developed societies. Not surprisingly, the development of low molecular weight thrombin inhibitors has become the subject of extensive research [1].
In the course of our ongoing research programme directed towards the design and synthesis of thrombin inhibitors, we have incorporated an azapeptide scaffold into the central part of the argatroban-like thrombin inhibitor structure [2,3]. 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. Incorporation of the azapeptide scaffold can favorably alter the inhibitor properties with regard to stability, enzymatic degradation, absorption and transport [4].
By systematic structural modifications for this series we have identified optimal groups for achieving nanomolar potency, that led us to potent inhibitors of thrombin with Ki values up to 11 nM.



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P C90 - Diastereoselective synthesis of RXP 407, a potent pseudopeptidic inhibitor of ACE-I, able to differentiate between its two active sites

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Phosphinic peptide inhibitors are powerful tools in the study of the function of many zinc metallopeptidases. The development of combinatorial chemistry of phosphinic peptides has led to the discovery of both highly potent and selective inhibitors of this important class of enzymes.
By screening phosphinic peptide libraries, we recently discovered the pseudopeptide Ac-Asp-$_{(L)}$Pheψ(PO_2-CH_2)$_{(L)}$Ala-Ala-NH_2, called RXP 407, which is a highly potent inhibitor of angiotensin I converting enzyme. This pseudopeptide is able to differentiate the two ACE active sites, with a dissociation constant three orders of magnitude lower for the N-domain of the enzyme (K_i = 12 nM) than for the C-domain. The reported synthesis of this novel selective inhibitor is not diastereoselective, making thus unavoidable the isolation of optically pure diastereoisomers by means of HPLC. Stimulated by the fact that this inhibitor is not only highly potent and selective toward ACE N-terminal active site, but also metabolically stable *in vivo*, we have focused our attention on developing a diastereoselective synthesis of this compound. Such a synthetic approach is crucial, since *in vivo* experiments require large quantities of optically pure inhibitor, and HPLC purification would not facilitate our experimental schedule.
Herein, we report the diastereoselective synthesis of the phosphinic pseudotetrapeptide RXP 407. This synthetic method consists of 10 steps and affords the target compound as a pure diastereoisomer. Starting from enantiomerically pure Cbz$_{(L)}$PhePO_2H_2 and under standard procedures, we easily prepare the pseudotetrapeptide Cbz-Asp(OBu^t)-$_{(L)}$Pheψ[P(O)OH-CH_2]$_{(L/D)}$Ala-Ala-NH_2. Careful titration of the mixture of the peptide isomers, with diluted hydrochloric acid, in a water-ethyl acetate biphasic system allows the quantitative separation of the two diastereoisomers of the peptide, by fractional crystallization. Final tert-butyl ester deprotection and removal of Cbz-group by catalytic hydrogenation, followed by pyridine-catalysed acetylation of the free N-terminus, affords the desired inhibitor.
The above described method has been applied in our laboratory and allows for the rapid and easy preparation of this phosphinic inhibitor in large quantity for *in vivo* experiments.

P C91 - Galanin receptor ligands and antisense oligonucleotides in the study of hippocampal galanin receptors

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Galanin, has been discovered in 18 different species, always as linear peptide. Among the 29 or 30 amino acid residues, the N-terminal 15 amino acids are the same in all species. Differences can be found only in the C-terminal parts. We have examined the secondary and tertiary structures (CD, FTIR, NMR) mainly of rat and human galanin and some larger fragments. These peptides do not belong to any other family of peptides. Galanin has a great number of biological functions, some of which may be important in future therapy of depression, cognitive and feeding disorders. Galanin is a strongly hyperpolarizing neuropeptide in the hippocampus. It suppresses LTP in the CA1 and CA3 and arcuate gyrus, acting at galanin type 1 and galanin type 2 G-protein-coupled receptors. We have synthesized a series of galanin fragments with varying receptor subtype specificity as receptor ligands, by different solid-phase synthetic methods, and examined their effects on LTP and seizure activities. Antisense oligonucleotides of PNA-type have also been employed to knock down the expression of galanin receptor subtypes in vivo, in order to determine their contribution to the regulation of hippocampal excitability.

P C92 - The Interaction of dDAVP analogues with human platelet vasopressin receptors

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A series of dDAVP -([8-D-arginine]deaminovasopressin) analogues with modifications in positions 2 and 8 were synthesized [1,2]. Tyrosine in position 2 was replaced by L or D p-alkyl phenylalanine or by stereoisomers of tyrosine analogues and D-arginine was substituted by D-homoarginine.The substances had a remarkably different profile of biological properties as compared with dDAVP (decreased antidiuretic potency, strong inhibitory effect in the uterotonic assay, etc.). Some of the peptides were also tested for their ability to activate Factor VIII and release the tissue platelet activator [3,4]. We present results of the interaction of dDAVP analogues with the platelet vasopressin receptors. [8-L-Arginine]vasopressin (AVP) caused a concentration dependent aggreagation in human heparinised platelet rich plasma, the IC_{50} value for AVP was 30 12 nM (Student´s t-test). dDAVP decreased the AVP induced aggregation response and its IC_{50} value was 660 84 nM. [8-D-homoarginine] deaminovasopressin was a better inhibitor with a IC_{50} value of 370 56 nM. Table I summarizes the inhibitory properties of [8-D-homoarginine]deaminovasopressin analogues in AVP induced platelet aggregation. Both structural modifications contributed to stronger inhibition of AVP induced platelet aggregation. Modifications in position 2 of [8-D-Har] deaminovasopressin resulted in peptides which bound to the AVP platelet receptor even more strongly than AVP.

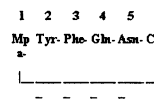

Table I. - Inhibitory effects of dDAVP analogues on AVP induced platelet aggregation in human heparinised platelet rich plasma

Peptide	IC_{50} (nM)	Peptide	IC_{50} (nM)
dDAVP [8-D-arginine] deaminovasopressin	660 ±84	[2-L-pBiPha, 8-D-Har] deaminovasopressin	39 ±0.6
[8-D-Har] deaminovasopressin	370 ±56	[2-D-pBiPha, 8-D-Har] deaminovasopressin	92 ±11
[2-L-pMePha, 8-D-Har] deaminovasopressin	Not tested	[2-L-Tyr(OMe), 8-D-Har] deaminovasopressin	14 ± 4
[2-D-pMePha, 8-D-Har] deaminovasopressin	0.1 ±0.05	[2-D-Tyr(OMe), 8-D-Har] deaminovasopressin	23 ±6

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