Posters: topic D

P 470 Combinatorial Chemistry P 471

COMBINATORIAL SYNTHESIS OF CYCLIC RGD DERIVATIVES BY SOLID PHASE HECK COUPLING

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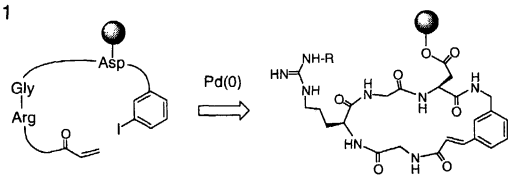
We synthesized a cyclic tetrapeptide derivative using the Heck coupling of acrylic acid amide to a 3-iodobenzyl amine moiety on solid support (Fig.1). The cyclic derivative contains a new 3-substituted cinnamic acid template to construct the rigid cyclic structure and Arg-Gly-Asp (RGD), a tripeptide sequence known to bind to the glycoprotein IIb/IIIa (GP IIb/IIIa). GPIIb/IIIa is a membrane protein expressed on the surface of activated platelets which binds to fibrinogen to cause platelet aggregation.

Palladium(0)-mediated macrocyclization in a DMF/H_2O/Et_3N solvent system was carried out at 37°C for 4 h. The homogeneous product was obtained from the resin in ca 20 % overall yield (calculated from the starting resin). We then investigate cyclization efficiency on solid support in comparison with that in solution phase. The intramolecular cyclization in solution proceeded in proportion to the reaction time, but was relatively slow. In contrast, most of the precursor was converted to the product within 2 h. The results clearly showed that the Pd(0)-mediated intramolecular macrocyclization is a unique reaction especially suitable for solid phase organic synthesis.We also demonstrate that the Heck reaction can be applied to combinatorial library synthesis for designing high affinity ligands of GPIIb/IIIa.

Fig.1



HYDROXYPROLINE-CONTAINING 2,5-DIKETOPIPERAZINES: SYNTHESIS ON DIFFERENT SOLID SUPPORTS AND CHARACTERIZATION BY HR-MAS NMR

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Hydroxyproline-based 2,5-diketopiperazines (DKP) are a new class of heterocyclic molecules which can be considered as potential lead structures for drug discovery. Their solid-phase synthesis was carried out on Ellman polystyrene-resin and conceived to enable a sequence of hydroxyproline-C^{α} alkylation, N-acylation, cyclization and final amide bond alkylation [1]. Three different functional groups were introduced in a combinatorial approach around the bicyclic scaffold. The series of subsequent reactions were performed without control of the diastereoselectivity, therefore generating mixtures of isomers, fully characterized after the final cleavage of the molecules from the resin. In order to detect the ratio between the diastereoisomers deriving from the α-carbon Hyp alkylation, immediately after this step, we tried to monitor the reaction with the high resolution magic angle spinning (HR-MAS) NMR technique. The 1D spectrum was characterized by the presence of broad bands which did not allow a clear assignment of the resonances relative to the different isomers. The linewidth has been associated to the anisotropic magnetic susceptibility induced by the aromatic rings which constitute the polystyrene-based resin [2]. In this communication we present a modified DKP synthesis using a resin containing a cross-linked polyethylene glycol derivative (PEG_{1000}). This solid matrix allows the resolution of the signals and the determination of the ratio between the two linked diastereoisomers by HR-MAS analysis. The resolution of the 1D spectrum is comparable with that of the molecules in solution. The HR-MAS technique becomes an extremely helpful tool in solid-phase organic synthesis. Moreover, modification of the reaction conditions towards a complete stereoselectivity can be more easily studied.

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P 472 P 473

IDENTIFICATION OF NEW ANTAGONISTS OF HIV-1 INFECTIVITY FROM SYNTHETIC COMBINATORIAL LIBRARIES

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The primary event in the infection of cells by human immunodeficiency virus type-1 (HIV-1) is the interaction between the viral envelope glycoprotein gp120 and its cellular receptor CD4. The development of antagonists of HIV-1 replication at the entry level, via blockade of gp120/CD4 interactions and/or gp41 structural changes, therefore represents a potential strategy for HIV-1 treatment. Two recombinant vaccinia virus-based assay systems mimicking the T-cell line-tropism (T-tropic) and macrophage-tropism (M-tropic) have been developed that enable to quantify the fusogenic activity of HIV-1 envelope glycoproteins. Synthetic combinatorial libraries (SCLs) made up of thousands to millions of compounds represent a powerful approach for the development of such antagonists. The strength of SCL approaches relies on the rapid identification of highly active compounds from large pools of individual compounds.

In a first study, three SCLs were screened for their ability to inhibit fusogenic activity mediated by HIV-1 recombinant glycoproteins: a SCL composed of 25,300 imidazol-pyrido-indoles, a SCL composed of 52,900 N-alkylated dipeptides, and a SCL composed of 31,320 N-methyltriamines. Different profiles in activity were observed between the two systems following the screening of these libraries. Following the deconvolution of these SCLs, compounds having separate inhibitory activities in the two tropic systems were identified. For example, compounds with specificity toward the T-tropic system were identified with low micromolar IC_{50}s. The deconvolution to individual HIV-1 antagonists from these SCLs will be presented.

Backbone cyclic CD4 mimetic peptides: Novel anti HIV-1 drug leads

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Prevention of HIV infection of CD4+ T cells could be achieved by inhibition of the CD4-gp120 binding interaction. This may be accomplished using mimetics of either of the two participants of this interaction.

Here we present the design, synthesis and biological activities of a library of backbone cyclic peptidic CD4 mimetics. This library was designed based on the original type II' β-turn composed of residues 40-43 of CD4. Peptides of the library differed only in their conformation, hence making the library an array of scaffolds accommodating the same functional groups at different positions.

The peptides were screened in order to find the scaffold which best mimics the active conformation of the native gp120 binding site of the CD4 protein.

Screening led to the discovery of a backbone cyclic hepta-peptide which inhibited HIV-1 infection of Hela P4 cells with an IC_{50} value of 33μM.

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OPTIMIZATION OF POTENCY, STABILITY AND CELL PERMEATION OF PSEUDOPEPTIDE LEADS FROM SOLUBLE PEPTIDE LIBRARIES AS INHIBITORS OF S-FARNESYL TRANSFERASE.

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Deconvolution of a 331776 member library of tetrapeptides built from 24 amino acids on solid phase resulted in the discovery of several non natural peptide leads with moderate potency in an *in vitro* test of S-farnesyl transferase acitivity. One of the non cysteine-containing leads, *p*-nitrophenyl-L-alanyl-Trp-Phe-His, was chemically optimized to give the proteolytically stable pseudopeptide *p*-F-C_6H_4-$CO(CH_2)_2$-CO-Bta-D-Pheψ[CH_2NH]His-OH (Bta = benzothienyl-L-alanine) with 200 fold potency *in vitro* compared to the original lead and strongly increased half-life both in rat plasma and in the cell incubation medium. The C-terminal ethyl ester of this compound behaved as a prodrug in cell experiments since there was no cellular uptake of the free acid, while the ester hydrolysed back to the C-terminal acid after cell penetration. The method confirmed that original leads can be discovered in large size soluble libraries provided deconvolution relies upon a highly specific assay, and that pharmacological and pharmacokinetic properties of these leads can be optimized by classical peptide, pseudopeptide and peptide mimetic chemistry.

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SUBSTITUTED AND NON-SUBSTITUTED GUANIDINES INTRODUCING DIVERSITY FOR COMBINATORIAL CHEMISTRY

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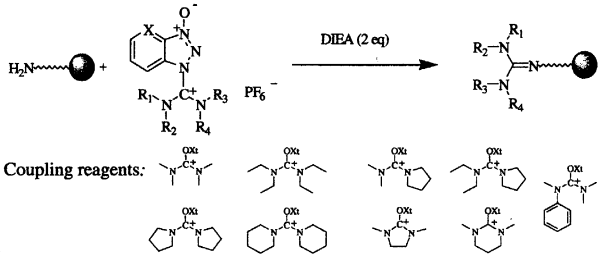
Guanidine functions are an important motif often present in natural products. This moiety is fully protonated on the physiological conditions due to its strongly basic character which is crucial for specific ligand-receptor interactions. Substituted guanidines are also found in many compounds with activity spanning a multitude of therapeutic areas. Consequently, procedures that allow for the preparation of guanidine-derived products with high yield under mild conditions are of great interest in medicinal chemistry.

For the incorporation of the substituted guanidines we describe a new method (see scheme) which involves the use of uronium salt-based coupling reagents used mainly in peptide synthesis. These uronim salts react with amine functions leading to guanidine derivatives with high yields and purities.

As part of our solid phase combinatorial library synthesis program, we desired to introduce substituted and/or non–subsituted guanidine units onto a scaffold on the solid support, a 2,5-diketopiperazine synthesised following an efficient method by using BAL linker.



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SOLID PHASE SYNTHESIS OF RESTRICTED ALANINE SURROGATES

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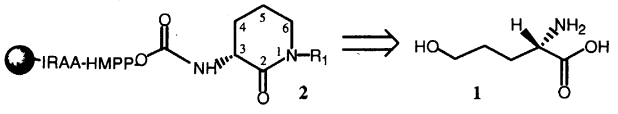
In the context of our studies on the synthesis of conformationally restricted pseudopeptides presenting a 3-aminopiperidin-2-one backbone, we have envisaged the solid phase synthesis of potential enzyme inhibitors with general structure **2**.

For this purpose, (*S*)-5-hydroxynorvaline[1] was linked to the solid support via its N-termini by means of a carbamate function. The resin had been previously functionalized as an active carbonate.[2] The lactam synthesis was envisaged through formation of the N_1-C_2 bond.

The described strategy allows the preparation of small libraries of 3-aminolactams, with diversification of R_1 = basic aromatic moieties, and R_2 = alkyl or alkoxy groups.



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P 477

SEQUENCING OF INDIVIDUAL PEPTIDES FROM COMBINATORIAL LIBRARIES VIA SPECIFIC GENERATION OF CHAIN-TERMINATED SEQUENCES

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Combinatorial peptide libraries are a versatile tool for drug discovery. On-bead assays identify reactive peptides by enzyme-catalyzed staining and sequencing by Edman degradation. Unfortunately, the latter method is expendable, time-consuming and requires free N-termini of the peptides.

Here we present a method of rapid and unambiguous peptide sequencing by utilizing synthesis-implemented generation of termination sequences with subsequent mass spectrometric analysis, as shown by means of a model library.

The basic idea is the introduction of specific terminated peptide sequences during solid phase synthesis („Coding") and determination of the sequence with one particular mass spectrum followed by computer-assisted „decoding".

The algorithm *Biblio* we present here enables the advancement of a concept originally established by Youngquist and Keough (JACS **1995**, *117*, pp 3900-3906). The peptide chain is terminated during synthesis at distinct positions with various reagents with a contingent of about five percent („Capping").

Biblio optimizes the capping pattern in respect of, first, minimizing the necessary capping steps, second, minimizing the number of used capping reagents, third, assuring sequence identity by using the positional given amino acid, N-terminally modified with the desired termination group.

For identification of the sequence of an individual peptide, a single bead is selected and the peptides are cleaved from the bead. The peptide mixture is analyzed by matrix assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry. n + 1 signals are detected, where n is the number of amino acid positions of the library being capped during the synthesis. *Biblio* then decodes the detected masses providing a unique sequence.

Thus, the loss of main peptide can be minimized, which is important for testing in biological systems. Furthermore, this method ensures a greater variability of the peptide library with an increased number of peptides for screening and mass spectrometric analysis than the original concept.

PREPARATION OF INDEXED LIBRARY OF AMIDES AND OLIGOPEPTIDES BY MEANS OF TRIAZINE CONDENSING REAGENT IMMOBILIZED ON CELLULOSE

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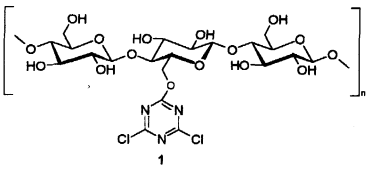
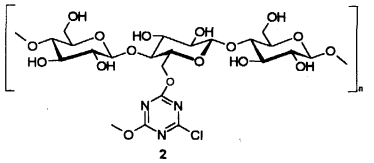
Immobilized triazine condensing reagents **1** and **2** were obtained by the treatment of cellulose with cyanuric chloride or 2,4-dichloro-6-methoxy-1,3,5-triazine.



1



2

For the reagents **1** and **2** immobilized on the standard Whatman filter paper, three independent methods determined maximum loading of the carrier $10*10^{-6}$ M/cm^2. The activation and coupling procedures involving **1** and **2** were elaborated. The extraction of the final products from the solid support gave chromatographically homogeneous amides and oligopeptides in 60-99% yield.
The described coupling procedure was applied for the preparation of indexed library of amides and oligopeptides positioned checker-wise on the cellulose plate.

The study was supported by the Polish State Committee for Scientific Research under the Project 3 T09A 029 16.

CELLULOSE-BOUND PEPTIDE LIBRARIES AS TOOLS FOR KINASE DRUG DISCOVERY PROGRAMS

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Cellulose-bound peptide libraries generated by the SPOTS technology (1) have been extensively applied to characterize ligand receptor interactions (2,3), but their use to monitor enzymatic activity is less frequently described (4). We have developed the **PhosphoSpots** technology offering rapid and uncomplicated solutions for characterization of protein kinases and generating novel kinase substrate peptides. Moreover, uniquely designed peptide libraries enable the de novo generation of effective kinase substrate peptides without knowledge of endogenous substrate protein sequences. These peptides are ideally suited for the use in HTS systems screening for novel kinase inhibitors due to reduction of the amount of protein kinase needed per assay, the higher stability compared to protein substrates and due to cost effective production.
The development of de novo kinase peptide substrates is achieved by applying knowledge-based and combinatorial cellulose-bound peptide libraries (4,5). One of the peptide libraries we have designed is derived from 800 Ser/Thr phosphorylation sites. Tests of this library type with different protein kinases reveal characteristic phoshorylation patterns for most of the kinases, which may relate the kinase to a kinase family or can be used for characterization of the kinase in a cellular context. Different applications of the **PhosphoSpots** technology will be discussed.

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P 480

Arrays of peptides & carbohydrate molecules on self-assembled monolayers and their application in blood serology.

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The quality of the information which can obtained in a biochip experiment is primarily dependent on the structure and purity of the sensors being used to gather information. Because the information content and phenotype association of the different information biomolecules increase in the series DNA < RNA < Protein < Saccharide we have taken an interest in the development of biochips with higher information content, by arraying besides nucleic acids and protein also oligosaccharides - Glycochips.
We have invented[1] a technology which permits us to immobilize up to 10^{11} saccharides per spot, in highly reproducible (SD 2.4%) manner. The technology is based on the production of a isotropic immobilization surface by self-assembled-monolayers of biotinylated long-chain alkylthiols adsorbed to a thin-layer of 24 carat gold and the saturation of this surface with streptoavidin. We have use this chemical defined layer to immobilize a library of biotinylated branched and non-branched carbohydrates corresponding to relevat human blood cells surface antigens. The chip was used as substrate for blood group determination and for glycosidade activity.

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4-AMINO-3-(AMINOMETHYL)BENZOIC ACID : A NOVEL AMINO ACID FOR PSEUDOPEPTIDE SYNTHESIS AND COMBINATORIAL CHEMISTRY

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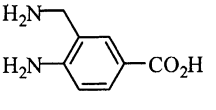
4-Amino-3-(aminomethyl)benzoic acid (AmAbz) is a novel non-natural amino acid of interest for peptidomimetic constructions as a rigid equivalent of dipeptides, or for the synthesis of cyclic or branched pseudopeptides. It may also find applications in combinatorial chemistry owing to its scaffold structure. The first synthesis of AmAbz will be reported. It involves three steps (63% overall yield) from 4-aminobenzoic acid including regioselective amidomethylation to introduce the aminomethyl group on the aromatic ring. The three distinct functionalities of AmAbz were shown to be conveniently discriminated when the derivatives AmAbz(Boc), AmAbz(Fmoc), and Fmoc-AmAbz(Boc) were prepared or subsequently used in coupling experiments or solid phase synthesis of branched pseudopeptides.



AmAbz

Posters: topic D

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Combinatorial Chemistry

Novel High Loaded Spacer Polymers Peptide Synthesis and Combinatorial Chemistry

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Normally on polystyrene type resins the functionality is directly introduced at the aromatic ring and the reactive sites are located directly on the polymer matrix where one can expect inhomogenous kinetic behavior. Spacer molecules, separating the reactive sites from the crosslinked matrix, increase the chemical homogeneity and the kinetic rates. In polystyrene resins the functionality is normally introduced via a benzyl function on the aromatic backbone. Benzyl type derivatives suffer from instability toward harsh acidic conditions. To overcome shortcomings of benzyl type polystyrene resins and take advantage of spacers, we have developed a series of novel polystyrene resins having either ethyl-, butyl- and hexyl- alkyl spacer chains or oligoethylene glycols of various molecular masses as spacers (PS A OEG resin) to separate the reactive sites from the matrix. Comparison studies with other resins show clearly the spacer effect in chemical synthesis of small molecule libraries and peptides or by their usage as scavanger resins. The resins can be tailored to the application and high resin capacities are achieved. On the PS A OEG resins we have synthesized a hydantoine library using combinatorial methods. Yields and purities are very comparable to those which we have obtained by using TentaGel resins. High loaded TentaGel resins show capacities of 0.4 – 0.6 mmol/g whereas the PS A OEG resins have increased capacities with up to 1 mmol/g. A comparison of PS-ethyl and PS-butyl modified resins in a side by side experiment results in an overall increase of app. 10 % in respect to yield and purity for the PS butyl resin. Solid state 13 C and 1H MAS NMR spectroscopy show increased mobility of the spacers for both resin types resulting in narrow line width of the signals for resin attached compounds. Physicochemical properties are investigared and a variety of derivatives are synthesized and applied to show the advantage of this novel supports in peptide synthesis, organic synthesis of nonpeptidic molecules and for it's application as scavanger resins.

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MICROWAVE-ASSISTED PARALLEL SYNTHESIS OF AMINOACIDS USING POLY(ETHYLENE GLYCOL) AS SOLVENT AND POLYMERIC SUPPORT

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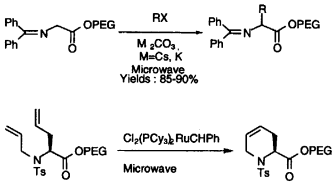
Microwave activation has become a widely used technique in organic synthesis for the preparation of new molecules.[1] This technique has been applied to the parallel synthesis of small organic molecules on an insoluble polymer (solide-phase organic synthesis or SPOS).[2] Recently we have developed the synthesis of aminoacids and peptidomimetics supported on a soluble polymer (poly(ethylene glycol) (PEG)).[3] We now report that a substrate supported poly(ethylene glycol) can react under microwave activation in alkylation or ring closing metathesis reactions. In these cases the melted PEG serves also as solvent promoting the reaction without the need for an extra solvent.



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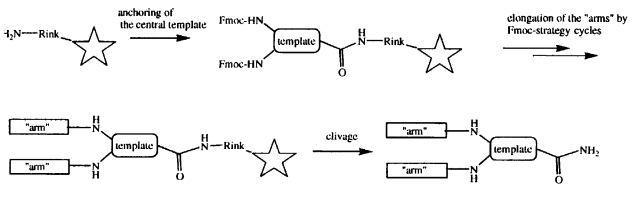
SOLID SUPPORTED PARALLEL SYNTHESIS OF DIMERS LIBRARIES

Gilles Subra[a], Muriel Amblard[a], Philippe Durand[b], Sylvianne Komesli[b], Patrice Renaut[b] and Jean Martinez[a], [a]L.A.P.P., Montpellier, France [b]Laboratoires Fournier, Daix, France.

As a purpose of discovering agonists of TGF-β (transforming growth factor beta), we designed focused libraries of dimeric and tetrameric compounds. In this approach, we followed the hypothesis that such molecules should be able to close up TGF-β's receptors subunits, leading to the transmission of biological signal. We chose the Multipin technology as a tool to synthetise several hundreds of discrete compounds without any automation. The core of the compounds is a diaminoacid template wich is linked to the Synphase crown by a Rink amide type linker.



Both the arms and the central template of these molecules had been modulated. The synthesis and the biological results of some libraries will be presented ans discussed.

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PROTEINCHIP® ARRAYS: A RAPID METHOD FOR SCREENING COMBINATORIAL PEPTIDE MIXTURES

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Although the backbone of drug-lead discovery involves high-throughput screening of large compound libraries, traditional sources of leads such as natural products or endogenous ligands can be important contributors to this process. However, rapid minimization of the protein or peptide ligands and identification of side chains contributing to binding can often be a time-consuming procedure. Towards this goal, we have developed a Surface-Enhanced-Laser-Desorption-Ionization-Mass Spectrometry (SELDI-MS)-based assay to rapidly screen protein-protein interactions. In particular, peptides with sequences derived from a surface exposed protein, PAM, of *S. pyogenes* have been screened on a ProteinChip® array containing a covalently-linked kringle domain of plasminogen. Initially His^{12}, which was identified as a putative binding residue from ^1H Nuclear Magnetic Resonance (NMR) chemical shift analysis, was replaced with 8 additional side chains. The mixture of 16-mer peptides was incubated on the kringle-linked ProteinChip® and then washed with buffer. The major peak corresponded to the His-containing peptide, with minor peaks from the $H^{12}R$, $H^{12}K$ and $H^{12}W$ variants. A SELDI-monitored competition assay using the native forms of the 16-mer and a larger wild-type fragment (30-mer) allowed the relative affinities of these peptides to be determined. This approach was validated using Isothermal Titration Calorimetry (ITC). With the overall goal of elucidating the mechanism by which *S. pyogenes* binds to plasminogen and utilizes the proteolytic nature of plasmin for bacterial invasiveness, we have applied the above screening method to rapidly identify the kringle-binding region on the peptide. The general applicability of this method and its ease of implementation make it a potentially attractive choice for high-throughput screening of peptides and smaller compounds libraries.

EVOLUTIONARY PRINCIPLES FOR GENERATING PROTEIN MIMETICS : DIRECTED ASSEMBLY OF PEPTIDE LOOPS ON TOPOLOGICAL TEMPLATES

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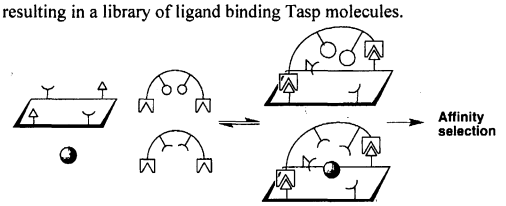
Institute of Organic Chemistry, University of Lausanne, Switzerland.

The use of topological templates proves to be a versatile concept in protein design and mimicry[1,2]. In separating the structural and functional part of a protein receptor, the attachment of ligand binding peptide loops to regioselectively addressable template molecules gives access to protein mimetics (Tasp) exhibiting essential features of native receptors[3]. In exploring novel methodologies for the reversible condensation of peptide libraries to chemoreactive templates, the principles of combinatorial chemistry are applied for generating a new class of protein mimetics. To this end, topological templates, e.g. cyclic decapeptides of type c[proGlyLys(Y1)AlaLys(Y2)]$_2$ are reacted with a library of peptide loops, e. g. X1-Xaa$_6$-X2 (X1, X2, Y1, Y2 = chemoselectively addressable groups) in the presence of ligands, e.g. metal ions (Figure), resulting in a library of ligand binding Tasp molecules.



Due to the reversibility of the assembly process, the ligand directed selection of metal coordinating peptide loops results in the formation of Tasp of maximal metal binding affinity. The potential of this concept will be exemplified for the ligand directed assembly of a number of metalloprotein mimetics, focussing on the elucidation of the chemical and structural parameters of the evolutionary process.

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INFLUENCE OF BACKBONE MODIFICATION ON THE HYBRIDIZATION POTENCY OF PEPTIDE NUCLEIC ACIDS (PNAs)

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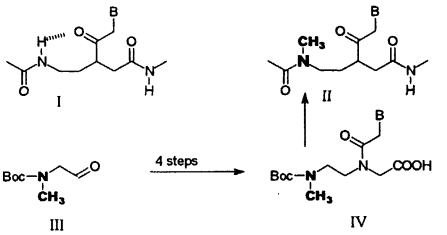
Peptide nucleic acids (PNAs) were developed as an oligonucleotide mimic in which the deoxyribose or ribose phosphate backbone of DNA/RNA has been replaced by (2-aminoethyl)glycine units (Nielsen et al., 1991). PNAs bind sequence-specifically to DNA and RNA with surprisingly high affinity. The lack of electrostatic repulsion may account for the increased hybridization properties of PNAs. However, on the basis of molecular mechanics calculations (e.g. Torres and Bruice, 1996) it was also suggested that backbone amide protons may be involved in hydrogen bonds (formula I), thus



contributing to duplex stabilization. In order to evaluate the possible significance of backbone amide protons for the hybridization potency, we synthesized N-methylated PNAs (II) via the corresponding monomers (IV), which were obtained from amino aldehyde (III). Hybridization studies show that backbone-methylated PNAs hybridize effectively with PNA and DNA, although the T_m values have been found to be diminished by 1-3 °C per modified monomer residue.

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TRITIUM LABELING OF AN OXYTOCIN ANTAGONIST

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A potent Oxytocin antagonist (OTA), (Cyclo S^1-S^6) (S)Pmp-D-Trp-Ile-Gln-Asn-Pen-Pro-Arg-Gly-NH2 (I), in which (S)Pmp is ß,ß-(3-thiapentamethylene)-ß-mercaptoapropionic acid, is under study as a potential inhibitor of preterm labor. In connection with studies on its pharmacokinetics and pharmacodynamics, it was deemed desirable to label this OTA with tritium. To this effect we prepared (S)Pmp-D-Trp-Ile-Gln-Asn-Pen-(3,4-dehydro-Pro)-Arg-Gly-NH2 (II) and verified that I and II could be separated by HPLC. However, catalytic tritiation of II under a variety of conditions including variation in pH, solvent and catalyst type led to disappearance of II without any formation of I. Probably, the presence of 3 sulfur atoms in this peptide leads to scission of any of the numerous S-C bonds precluding the formation of product. Hence,we had to resort to an indirect method. The tripeptide Boc-(3,4-dehydro-Pro)- Arg(Tos)-Gly-NH2 was made by solid phase peptide synthesis (SPPS) and, after treatment with TFA to temove the Boc group, the (3,4-dehydro-Pro)-Arg(Tos)-Gly-NH2.HCl was subjected to catalytic tritiation, yielding (3,4-ditritio-Pro)-Arg(Tos)-Gly-NH2 with high specific activity (37 Ci/mmole). We also prepared, (S)Pmp-D-Trp-Ile-Gln-Asn-Pen by SPPS, purified it by HPLC and characterized it by amino acid analysis and FABMS. Coupling of (S)Pmp-D-Trp-Ile-Gln-Asn-Pen was unsuccessful when the free tripeptide was used, but was successful when the protected tripeptide was used. Coupling with (3,4-ditritio-Pro)-Arg(Tos)-GlyNH2 gave (S)Pmp-D-Trp-Ile-Gln-Asn-Pen-(3,4-ditritio-Pro)-Arg(Tos)-Gly-NH2. Best yields were obtained when this intermediate was purified by HPLC and then the Tos group removed by treatment with Na in liquid ammonia, and the resulting peptide disulfide was oxidized by potassium ferricyanide. Purification of the reaction product by HPLC gave the desired I in specific activity (37 Ci/mmole) suitable for our studies.

NOVEL CONCEPT OF RESIN POLYMERIZATION: PREPARATION OF BIFUNCTIONALIZED POLYETHYLENE GLYCOL (PEG) BASED RESINS BY REDUCTIVE AMINATION

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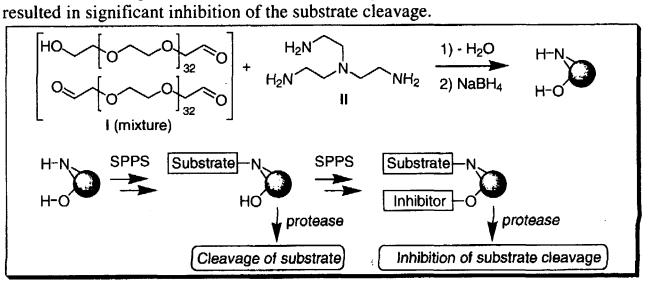
A chemically inert PEG-based resin was synthesized by reductive amination of a mixture of mono- and bis-aldehyde PEG-1500 **I** and the branched cross-linker tris(2-aminoethyl)amine **II**. This unique concept of resin polymerization yields a polar resin with two functionalities present (OH and NH). Resin batches with an OH loading between 0.33 and 0.80 mmol/g and an NH loading between 0.88 and 0.24 mmol/g were obtained by varying the monomer composition in the polymerization mixture. The bifunctionlized supports showed excellent swelling properties in an broad range of solvents ranging from water to dichloromethane. Furthermore, the resins were shown to be able to withstand typical harsh chemical conditions for weeks.

To demonstrate their usefulness in peptide library generation and screening, an octapeptidic substrate for the 27 kDa protease Substilisin Carlsberg was first assembled through the NH functionality. Incubation of the resin with the protease revealed that the resin was indeed permeable by the enzyme. Next, a decapeptidic inhibitor for this protease was assembled through the hydroxy functionality which resulted in significant inhibition of the substrate cleavage.



Posters: topic E

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Miscellaneous

NEW INSIGHT INTO THE STEREOSPECIFICITY OF THE INTESTINAL H^+/PEPTIDE SYMPORTER

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The H^+-gradient-dependent symport system PEPT1 actively transports di- and tripeptides and a variety of peptidomimetics across the brush border membrane of intestinal epithelial cells.
In this study we investigated the interaction of a number of dipeptides and dipeptide derivatives in LL-, LD-, DL- and DD-configuration with PEPT1. The syntheses of the compounds were performed according to standard procedures of peptide chemistry. We applied two different experimental approaches: First, we determined the apparent affinity constants of the substrates/inhibitors by measuring their ability to inhibit competitively the [^{14}C]Gly-Sar uptake in Caco-2, a cell line which constitutively expresses the human peptide transporter PEPT1. In a second approach, hPEPT1 was expressed in *Pichia pastoris* yeast cells and the [^{3}H]-D-Phe-L-Ala uptake was measured in a competition assay.
From our results we conclude: PEPT1 shows a high specificity for the L-form of substrates. The D,L-configuration is tolerated in many cases and preferred compared to the L,D-configuration. The affinity is often completely abolished, when both amino acids are in the D-configuration. However, in contrast to current knowledge, we identified some dipeptides with D,D-configuration that interact with PEPT1 as substrates and/or inhibitors with appreciable affinity constants. Among them are dipeptides containing a side chain protected diaminocarbonic acid.

This work was supported by the *Land Sachsen-Anhalt Kultusministerium, grant* 2880A/0028G and by the Fonds der Chemischen Industrie.

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INVESTIGATION OF NON-SPECIFIC CLEAVAGE OF A RECOMBINANT HUMAN MONOCLONAL ANTIBODY DURING PAPAIN DIGEST

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Hydrophobic interaction chromatography is used in determining the purity of a recombinant humanized monoclonal antibody. Heterogeneity caused by the isomerization of Asp L32 residue on the N-terminal portion of the light chain is further increased due to the dimeric nature of the molecule, resulting in antibodies having two, one or zero L-Asp L32 forms. To overcome this complexity, hydrophobic interaction chromatography (HIC) assay was developed to analyze the molecule after it has been fragmented by digestion with Papain. Papain cleaves the monoclonal antibody (Mab) molecule on the C-terminal side of His-H228 to yield two Fab fragments and a Fc fragment. (1). HIC elutes these fragments using a high ionic strength buffer with more hydrophilic Fab portion eluting first followed by the various heterogeneous fragments and then the Fc portion of the molecule. However, a peak was always noticed eluting before the main peak-containing Asp L32, which increased when the pH of the digestion buffer was lowered below pH 7. The peak was attributed to the Fab fragment resulting from a non-specific cleavage between Ser-H28 and Ile-H29 residue. We think that due to N to O acyl migration the peptide bonds adjacent to Serine and Threonine are likely to rearrange reversibly to ester bonds[1], favored by the lower pH, reversible over pH7.0. Since Papain has also the esterase activity, it can hydrolyze any ester bond, lowering the amount of ester shifting the equilibrium to the right for more ester formation and subsequent hydrolysis.
A peptide sequence, **GYSITSGY** corresponding to the portion of the protein sequence covering the non-specific cleavage site was synthesized. The peptide was subjected to Papain digest at pH 6.2 and 8.2, which showed interesting cleavage products at Serine and Threonine supporting our hypothesis. The reactivity was also checked with Chiroscreen_ -EH, a kit containing cross linked esterases and lipases. The results of this work will be presented.
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P 492

SOLID PHASE SYNTHESIS OF THYMOSIN BETA-15 AND INVESTIGATION OF ITS BIOLOGICAL ACTIVITY IN THE CHORIOALLANTOIC MEMBRANE ANGIOGENESIS MODEL

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We report here the Fmoc solid-phase synthesis of thymosin beta-15 (a 44 amino acid polypeptide recently detected in human prostate tumours) using a self-made *p*-cyanotrityl resin, according to the amino acid sequence predicted from full-length cDNA cloning analysis. Crude thymosin beta-15 (TB15) was purified with semi-preparative reversed-phase HPLC on a PrepNovaPak C18 column. The analytical reversed-phase HPLC chromatogram (60 % AcN/H_2O/TFA gradient in H_2O/TFA eluent) of the purified product showed a single peak at 16.13 min. The overall yield obtained was high (45 %). The amino acid analysis of TB15 (PICO TAG method, Waters) was in accordance with that expected from the *c*DNA cloning analysis. The purified synthetic product was finally analyzed with MALDI-TOFF mass spectral analysis and the molecular mass obtained (*Mexp* = 5,173.52 Da) was identical to the average molecular mass theoretically calculated.
The effect of the synthetic TB15 on the angiogenesis process was investigated using the chick chorioallantoic membrane (CAM) as an *in vivo* model. Increasing concentrations (0.05, 0.1, 1, 5 or 20 μg/10 μl) of TB15 were placed on sterile plastic discs, the discs were dried, placed inverted on the CAM surface of fertilized eggs (six eggs were used for each TB15 concentration) and incubated for nine days. On each egg, discs containing control material were placed one-centimeter away from the test ones. The eggs were incubated for two more days, the CAM was flooded with 10% buffered formalin, the discs were removed, a large area around them was cut off, placed on a glass slide and underwent microscopy for measuring the number of blood vessels present. According to the results obtained statistically significant differences in vascular density were observed between certain test and control discs, TB15 exibiting a positive effect on angiogenesis at concentrations higher than 1 μg/10 μl/disc.

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MOLECULAR DYNAMICS OF THE GELSOLIN 150-169 BINDING TO PHOSPHOINOSITIDE LIPIDS

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[a]Latvian Institute of Organic Synthesis, Riga, Latvia; [b]Faculty of Chemistry, University of Gdansk, Gdansk, Poland; [c]Institute of Medicine and Engineering, University of Pennsylvania, Philadelphia, PA, USA

The action of gelsolin, an actin-severing protein, is regulated by Ca^{2+} and phosphoinositides. Gelsolin 150-169 fragment (G150-169) KHVVPNEVVVQR-LFQVKGRR are responsible for the binding of this protein to actin and to the cellular signal transmitters - phosphatidylinositol 4,5-bisphosphate (PIP_2) clusters.
Four low energy structures of G150-169 were found using the electrostatically driven Monte Carlo (EDMC) method with the ECEPP/3 force field including surface solvation. All of the structures are largely α-helical. The global minimum structure of G150-169 (with energy 20 kcal/mol lower than the alternative conformations) was subjected to a 1533 ps molecular dynamics (MD) simulation in a periodic water box with counter ions.
Lipid bilayer built from dimyristoylphosphatidylcholine (DMPC) and four PIP_2 molecules, was equilibrated in a 300 ps MD simulation. The dynamically equilibrated G150-169 structure was placed in the periodic lipid-water box on 7.5 Å distance from the PIP_2 lipid and docked to it by 1251 ps of MD simulation. The binding of G150-169 with a PIP2 molecule occured after 980 ps of MD simulation via hydrogen bonding of the positively charged Lys^1 side chain and the N-terminus of G150-169 with the oxygens of PIP_2 phosphate groups. After 65 ps the hydrogen bonds have been broken, and formed again after 24 ps, while one of the arachidonate tails of a PIP_2 molecule was aprroaching G150-169. On the 1108th ps of the MD run two of the four PIP_2 molecules of the cluster left the lipid bilayer: an arachidonate tail of a PIP_2 was hydrophobically bound to the Val^4 residue of G159-169, while the stearate tail of another PIP_2 molecule interacted with the Val^3 and Pro^5 residues and partly also with the Val^8 residue of G150-169, suggesting that the binding of PIP_2 molecules to gelsolin is not only electrostatic, but also hydrophobic.
Parallely to the docking calculations, the MD simulation of the PIP_2 lipid was continued. After 1512 ps MD of simulation all fatty acid tails of the PIP_2 molecules remained in the lipid bilayer. Most of them still formed a cluster, the two phoshphoinositide heads of PIP2 molecules were hydrogen bonded, while the other two tended to repulse. It can therefore be concluded that the disruption of the lipid occurs on the influence of gelsolin, which interacts with PIP_2 molecules both electrostaticaly and hydrophobically thus pulling them out from the lipid bilayer.

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Miscellaneous

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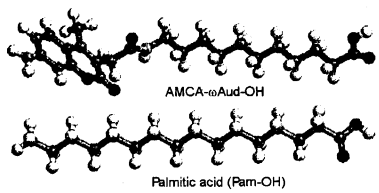
A NEW FLUORESCENT PROBE TO MIMIC LIPOPHILIC MOIETIES FOR INTERACTION STUDIES OF BIOACTIVE LIPOPEPTIDES WITH MEMBRANE MODELS

Elisa Peroni,[1] Gabriella Caminati,[1] Piero Baglioni,[1] Mario Chelli,[2] and Anna M. Papini,[2] from [1]Dipartimento di Chimica; [2]Dipartimento di Chimica Organica "Ugo Schiff" and CNR CSCEA, Università degli Studi di Firenze, I-50121 Firenze, Italy.

We previously demonstrated that the lipopeptide Pam-GpMBP(74-85) increases the *in vitro* CD4+ T cell proliferative response in Lewis rats immunized with the immunodominant epitope GpMBP(74-85), compared to the lipid-free wild type peptide. This was observed only with epitopes stable to cellular proteases [Vergelli, M., *et al.*, in preparation]. Lipoconjugation may favour the internalization of the peptide by APCs as suggested by the increase of CTL [Gras-Masse, H., *et al., J. Immunol.*, 164 (2000) 900-907] and CD4+ T cell response in the presence of lipopeptides. We hypothesized that the lipopeptide bypasses MHC II binding on the cell surface (typical of MBP) because of the presence of the lipophilic moiety. Antigens are then loaded on newly generated MHC II molecules, increasing the efficiency of antigen presentation. In order to define Pam localization in cellular membranes, we studied phospholipidic vesicles as biomimetic models. We used FRET to study the interaction of AMCA-labelled peptides with a quencher, *i.e.* BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) localized into the bilayer or at the water/bilayer interface. We demonstrated that Pam-[Lys^{75}(AMCA)]GpMBP(74-85) was not an efficient tool to determine the exact position of the lipophilic moiety owing to its distance from Pam. In fact, FRET experiments gave analogous results with [Lys^{75}(AMCA)]GpMBP(74-85). So, we designed and synthesized the new fluorescent probe AMCA-ωAud-OH, mimetic of the palmitoyl moiety. FRET studies on AMCA-ωAud-GpMBP(74-85) demonstrated the insertion of the palmitoyl moiety into the bilayer. Moreover, we propose our synthetic approach to synthesize lipophilic fluorescent probes with alkyl chains of different length as building-blocks to obtain fluorescent labelled molecules.



New data on generation of hemoglobin derived peptides by erythrocytes

Marina M. Philippova, ElenaYu. Blishchenko, Andrei A. Karelin and Vadim T. Ivanov

Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117871 Moscow, RUSSIA

Earlier we have shown that hemoglobin is being proteolytically degraded within human erythrocytes giving rise to a set of relatively long (>30 amino acid residues) peptides. Further proteolysis takes place at the red blood cell membrane followed by release of shorter peptides (<20 amino acid residues) into the surrounding medium [1,2].

In this work we continued the study of hemoglobin fragmentation. It was found that primary splitting is carried out by an unknown enzyme at neutral pH. The resultant peptides partially dissociate from the tetrameric hemoglobin globule. Acidification of the erythrolysate dissociates the tetramer and activates an aspartate protease that produces an additional set of peptides [3].
We also studied formation of hemoglobin derived peptides during the interaction of human red blood cells with murine macrophages. That process models normal phagocytosis of aging erythrocytes *in vivo*. The set of peptides obtained during that interaction showed significant correlation with the sets of hemoglobin fragments found earlier in various mammalian tissues [4].
The contribution of different pathways of hemoglobin proteolysis to *in vivo* formation of tissue-specific sets of endogenous hemoglobin fragments will be discussed.

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4. Karelin A.A., Philippova M.M., Karelina E.V., Strizhkov B.N., Grishina G.A., Nazimov I.V., Ivanov V.T. *J. Peptide Science*. **4**, 211 (1998)

P 496

Synthesis of glutathione amide, the main low molecular weight thiol of *Chromatium* species

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Glutathione amide disulfide reductase (GAR) from *Chromatium* species is a novel flavoprotein-disulfide-oxidoreductase that catalyzes the reaction

$$NADH + H^+ + GASSGA \rightarrow NADP^+ + 2\ GASH\ [1,2].$$

The enzyme is peculiar, not only because it displays specificity for a glutathione derivative, but also because it utilizes NADH instead of NADPH as a reductant. The synthesis of GASH, and subsequently GASSGA is necessary, not only for the characterization of the *Chromatium gracile* GAR, but also for the comprehension of the complete GASH-metabolism, especially since the recent discovery of a probable GASH-dependent peroxidase coding sequence immediately upstream the GAR gene [3].
The predominant organic thiol found in photoheterotrophically grown *Chromatium* species is GASH, whereas photoautotrophically grown *Chromatium* species produce GASSH as the predominant form [2]. This finding is remarkable because biological persulfides are believed to be very unstable under most conditions, decomposing chemically into elemental sulfur and the corresponding thiol [4,5]. Brüser and collaborators [6] propose that GASH is able to accept a terminal thiol group from periplasmic polysulfane chains, thereby transporting sulfide from the periplasmic space into the cytosol. This hypothesis suggests that after transport into the cytoplasm, sulfide has to be reductively released by a kind of heterodisulfide reductase. Whether GAR displays such an activity will be assayed, using the synthetic GASSH.

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CELLULAR DELIVERY OF OLIGONUCLEOTIDE-PEPTIDE CHIMERAS AND PRELIMINARY PHARMACOLOGICAL STUDIES BY MALDI-TOF SPECTROMETRY.

Eric Vivès, Manuel Sauveplane, Jean-Jacques Vasseur[a] and Bernard Lebleu, EP2030, Institut de Génétique de Montpellier, 1919 route de Mende, 34293 Montpellier cedex 05, France.
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Poor cellular uptake of oligonucleotides (ONs) and their fast extracellular degradation by exonucleases remain critical steps in routine antisense strategy. Several methods for enhancing the cellular bio-availability and/or the stability of ONs have been developped. Along this line several peptides were recently shown to translocate rapidly and efficiently across the plasma membrane by a yet unknown mechanism. Such translocating peptides could be an interesting alternative for ON intracellular vectorization. We focused our study on a short peptide (13 mer) belonging to the basic domain of the HIV tat protein. We have established that this tat derived peptide efficiently promoted the uptake of chemically linked oligonucleotide or peptide. Along the same line several publications recently reported the internalization of entire proteins fused to this peptide both *in vitro* and *in vivo*. In our study a 19 mer ON was chemically linked to the Tat peptide through disulfide bridge formation. Cellular internalization and intracellular reduction of the disulfide bridge was monitored by MALDI-TOF spectrometry. Additionnally, MALDI-TOF spectrometry allowed to study the intracellular rate of degradation of the ON.
Results presented here will mainly focus on the cellular internalization of the chimeric molecule, the reduction state of the disulfide bridge linking the ON to the peptide, and the intracellular stability of the oligonucleotide itself once detached from the delevery peptide in the cell. In addition attempts to compare the biological activity of an ON vectorized by the short Tat peptide will be compared to other ONs delivery strategies.

PSEUDOPROLINES IN DRUG DESIGN : CYCLOSPORIN C DERIVATIVES AS A NOVEL CLASS OF ACTIVE SITE INHIBITORS OF CYCLOPHILIN A

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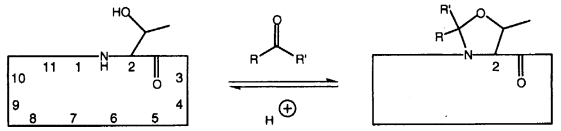
We have shown previously that serine or threonine containing peptides can be reversibly converted to oxazolidine (pseudoproline, ΨPro) containing analogues featuring novel chemical and structural properties[1]. In particular, the temporary induction of a cis-amide bond into the peptide backbone as well as the introduction of functional substituents (R,R') at the C2'-position of ΨPro, offers a versatile way for modulating the biological and pharmacological properties of peptides. Here we present the direct insertion of various ΨPro-systems into the immunosuppressive cyclic undecapeptide Cyclosporin C (CsC, Figure) and their inhibitory properties towards cyclophilin A (CypA). Despite of the remarkable constrain induced by ΨPro, the derivatives retain differential binding properties to CypA, as demonstrated by enzymatic studies on the inhibition of the cis/trans isomerase activity of CypA. Conformational studies by NMR revealing a close correlation of the nature of the C2'-substituents on the structure-function relationship of the various ΨPro-containing CsC derivatives will be discussed in view of the development of novel CypA inhibitors.



CsC $Thr^2(\Psi^{R,R'}{}_{pro})$-CsC

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BIOACTIVE PEPTIDES OBTAINED FROM NATURAL LIBRARY

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Many kinds of bioactive peptides have been isolated from enzymatic digests of proteins which had not been regarded as precursors for them. These include opioid peptides, complement C3a agonists, fMLP agonist and so on. Usually, they are small in molecular weights and low in specific activity. However, some of them exhibit obvious physiological effect even after oral administration while most endogenous bioactive peptides are orally ineffective. Thus, hydrolysate of proteins by various proteases can be regarded as a sort of natural random library for bioactive peptides.

Many opioid peptides having YPX sequence have been isolated from protein digests; X is aromatic amino acid residue in β-casomorphin (YPFPGPI) and hemorphin (YPWTQ). On the other hand, X is aliphatic residue in gluten exorphin C (YPISL), neocasomorphin (YPVEPF) and rubiscolin (YPLDLF), which were isolated by us from digests of wheat gluten, β-casein and tea protein, respectively. Among them, rubiscolin was selective for δ-receptor.

Casoxin C (YIPIQYVLSR) and oryzatensin (GYPMYPLPR), which were isolated as anti-opioid peptides from tryptic digests of κ-casein and rice albumin, respectively, proved to be complement C3a agonists. Similar complement C3a agonist peptides having the hydrophobic residue-X_1-Leu-X_2-Arg sequence were also obtained from tryptic digests of serum albumin, ovalbumin, hemoglobin, histone and so on. We established the concept of natural library from these examples because the probability of obtaining the sequence to satisfy above conditions from pentapeptide library made from random DNA library may be rather high; it's calculated to be 1/3 x 6/64 x 6/64 = 1/340. Interestingly, these complement C3a agonists exhibited anti-analgesic and anti-amnesic activities after intracerebro-ventricular administration.

Soymetide-4 (MITL) which was derived from soybean β-conglycinin as an immunostimulating peptide showed a weak affinity for fMLP receptor. Besides stimulating phagocytosis by polymorphonuclear leukocytes, the peptide suppressed alopecia induced by etoposide, the cancer chemotherapy agent, after oral administration.

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