Poster Presentation Abstracts

P–01

SYNTHESIS OF UNNATURAL AMINO ACIDS FROM SERINE

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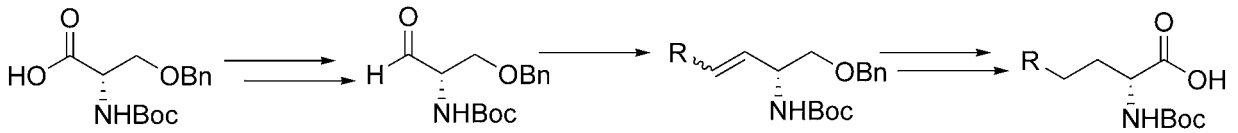
The synthesis of unnatural amino acids is an area of research that has attracted special attention in recent years. Unnatural amino acids play an important role in the design and synthesis of peptide mimetics, enzyme inhibitors and bioactive compounds. Aldehydes obtained from a–amino acids constitute a class of chiral synthons useful in the synthesis of optically active bioactive compounds and, in particular, in the synthesis of unnatural amino acids. Here, we present an efficient route for the synthesis of enantiopure unnatural a–amino acids and 2–amino alcohols starting from serine. Boc–L–Ser(Bn)–OH (**1**) was reduced to alcohol by the mixed anhydride–$NaBH_4$ method and oxidized to aldehyde by treatment with NaOCl/AcNH–TEMPO. Wittig–type olefination reaction of the key intermediate aldehyde **2** with various ylides produced unsaturated derivatives **3**. Hydrogenation, followed by oxidation with NaOCl in the presence of AcNH–TEMPO and tetrabutylammonium hydrogensulphate, as a phase transfer catalyst, gave unnatural amino acids **4**.



1 **2** **3** **4**

P–02

SYNTHESIS OF 2–OXOAMIDES BASED ON LONG CHAIN β–AMINO ACIDS

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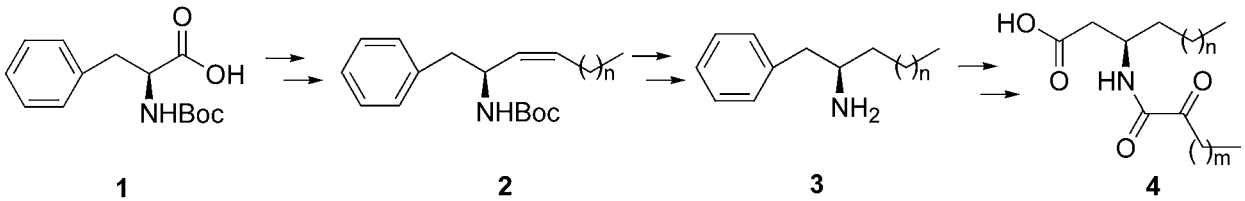
In recent years, compounds containing amine functionalities have attracted much attention, because of their interesting biological properties. Natural α–amino acids may be used as starting materials for the synthesis of chiral amines through modification of the α–carboxy group. For example, enantiopure α–methyl amines have been prepared from various α–amino acids, while 1,2–diamines and triamines have been synthesized from glutamic acid and lysine, respectively. Recently, we have demonstrated that 2–oxoamides based on γ–amino acids are potent inhibitors of human GIVA phospholipase A_2. Here, we report a method for the synthesis of long chain amines **3** starting from Boc–PheOH (**1**). Amines **3** were coupled with 2–hydroxy–acids and the products were directly oxidized to 2–oxoamides **4** using $RuCl_3 \cdot xH_2O/NaIO_4$. The 2–hydroxy–amide group was converted into 2–oxoamide functionality and at the same time the aromatic group was oxidized to carboxyl group. 2–Oxoamides based on long chain β–amino acids are moderate inhibitors of GIVA PLA_2.



1 **2** **3** **4**

P–03

2–OXOAMIDES BASED ON GLUTAMIC ACID AS INHIBITORS OF HUMAN GIVA PHOSPHOLIPASE A_2

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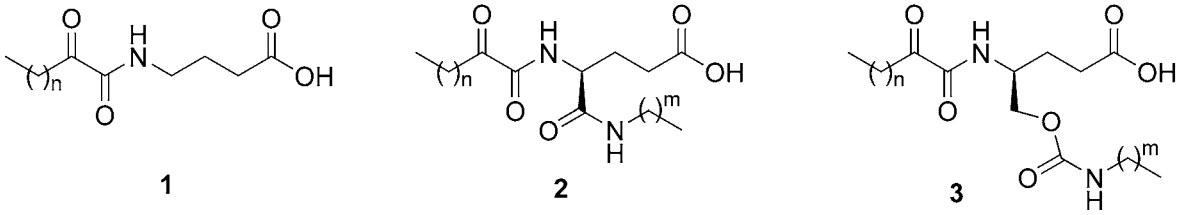
The phospholipases A_2 superfamily consists of a broad range of enzymes defined by their ability to catalyze the hydrolysis of the ester bond at the *sn*–2 position of phospholipids. Among the various PLA_2's, human cytosolic PLA_2 (GIVA PLA_2) is essential for proinflammatory prostaglandin, leukotrienes, and PAF production and therefore, its inhibitors are very attractive targets as agents for treating inflammatory and other diseases. We have recently demonstrated that 2–oxoamides based on γ–aminobutyric acid (**1**) are potent inhibitors of human GIVA PLA_2. Here, we report our studies on 2–oxoamides based on glutamic acid. Two groups of 2–oxoamides, compounds **2** containing an amide group at the α–carboxyl group of glutamic acid and compounds **3** containing a carbamate group at the α–position,. were synthesized. All compounds were tested for their ability to inhibit GIVA PLA_2 in a GIVA PLA_2 specific assay and it was found that compounds **2** with long chains are moderate inhibitors of GIVA PLA_2.



P–04

RESOLUTION OF Boc–PROTECTED 2–AMINO ALCOHOLS BY PORCINE PANCREATIC LIPASE

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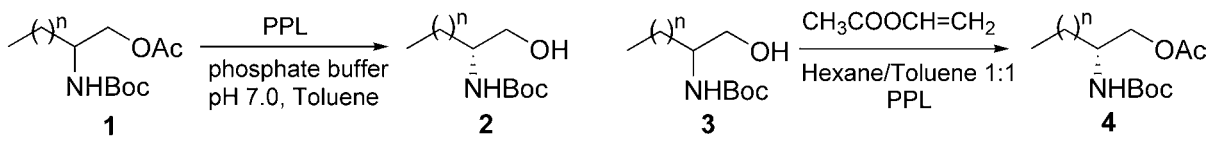
Saturated and unsaturated long chain 2–amino alcohols, which may be considered as sphingosine analogues, have been reported to display various biological activities. They exhibit cytotoxic activity against various cancer cell lines and induce apoptosis through a caspace–3 dependent pathway. In addition, they present *in vivo* antiinflammatory activity and immunosuppressant activity. Here, we present our studies on the resolution of Boc–protected 2–amino alcohols using porcine pancreatic lipase (PPL). Lipases have found wide applications in organic synthesis due to their enantioselectivity, regioselectivity and broad substrate specificity. Among various lipases we have tested for the resolution of amino alcohols, PPL gave the best results. Enantiopure amino alcohols **2** were prepared by the PPL hydrolysis of the racemic acetyl derivatives **1**. Alternatively, racemic amino alcohols **3** were converted into enantiopure acetyl derivatives **4** by treatment with vinyl acetate and PPL. Compounds **2** and **4** were enantiopure as demonstrated by NMR studies and comparison of their specific rotation with those obtained for reference compounds prepared by chemical methods.



P–05

SYNTHESIS OF TETRAZOLE ANALOGUES OF γ–AMINO ACIDS

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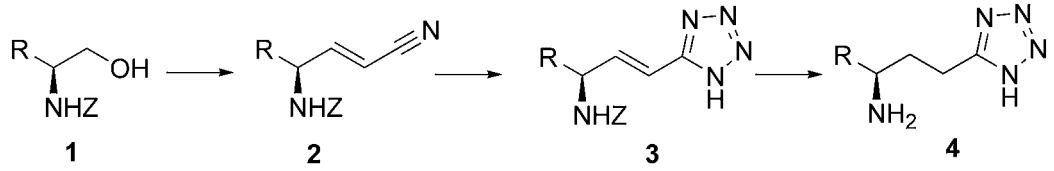
Non–natural amino acids play an important role in the design and synthesis of pharmacologically relevant molecules and peptide mimetics. γ–Amino acids represent a class of special interest since γ–peptides can adopt stable conformations. The tetrazole group is considered isosteric to the carboxyl group and there are several examples in medicinal chemistry where the replacement of a carboxyl by tetrazole lead to products with improved biological properties. Here, we describe a general method for the synthesis of tetrazole analogues of γ–amino acids. Z–Protected amino alcohols **1**, easily prepared from amino acids, were converted into aldehydes by treatment with NaOCl in the presence of AcNH–TEMPO and directly reacted with (triphenylphosphoranylidene)acetonitrile. Treatment of nitriles **2** with NaN_3 and $ZnBr_2$ produced unsaturated tetrazoles **3**, which were converted to compounds **4** by catalytic hydrogenation.



P–06

SYNTHETIC STUDIES TOWARDS THE DEVELOPMENT OF A NOVEL CLASS OF ACITRETIN–TYPE RETINOIDS

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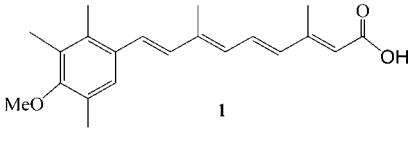
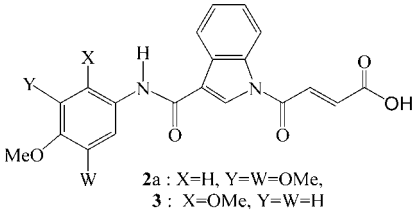
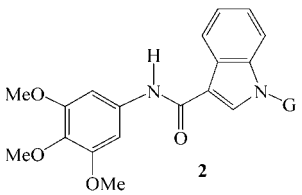
3,4,5–Trimethoxy– and 2,4–dimethoxyaniline, indole–3–carboxylic acid (Inl–OH), the dicarboxylic acids fumaric (Mul–OH), maleic (Mal–OH), succinic (Sul) and phthalic (Phl–OH) and 3–hydroxybenzoic acid (Hbl–OH) have been combined to produce the novel analogs (**2a–f**) and **3** of the aromatic retinoid acitretin (**1**). The related syntheses involved coupling of the *N*–Boc protected Inl–OH with the above mentioned electron–rich anilines, acid–mediated removal of the Boc group, coupling of the thus obtained anilides with the anhydrides or suitable monoesters of the above mentioned dicarboxylic acids or *N*–Boc protected Inl–OH or *O*–triisopropylsilyl protected Hbl–OH in liquid or on solid phase and finally deprotection. The effects of analogs **2** and **3** on metalloproteinases and tissue inhibitors of metalloproteinases (TIMP) production by human articular chondrocytes as well as on ribonucleose P activity from cellular slime molds *Dictyostelium discoideum* were investigated. In preliminary experiments, a dose–dependent inhibition of tRNA maturation by analogs **2a** and **3** was observed, whereas analogs **2a** and **2e** caused an increase of TIMP production by chondrocytes similar to that observed for acitretin.





2a : X=H, Y=W=OMe,
3 : X=OMe, Y=W=H



b : G=Mal
c : G=Phl
d: G=Sul
e : G=Inl
f : G=Hbl

P–07

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

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

P–08

PREPARATION OF AMINOMETHYL POLYSTYRENE RESINS OF HIGH HOMOGENEITY USING A FERRIC CHLORIDE–BENZOPHENONE COMPLEX AS A CATALYST

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Aminomethyl polystyrene resins were previously prepared under mild conditions using N–(chloromethyl)–phthalimide in CH_2Cl_2 and ferric chloride as a catalyst. We later observed, however, that ferric chloride may contain various contaminants ("dark specks"), which remain insoluble in the reaction mixture and cannot be removed from the polystyrene resin, either by extensive washing, or by repetitive sedimentations and decantations using various solvents, such as CH_3OH, CH_2Cl_2, etc., in an attempt to physically separate the contaminants from the resin. This problem has been solved by using ferric chloride in the form of a complex with benzophenone (molecular ratio, 1:1) as a catalyst. The ferric chloride–benzophenone complex has been formed simply by mixing benzophenone and ferric chloride and shaking until the latter is dissolved in CH_2Cl_2. While the complex is soluble in CH_2Cl_2, the contaminants are insoluble and can therefore be removed by centrifugation. On the other hand, the ferric chloride–benzophenone complex has been proved to be an even milder Friedel–Crafts catalyst and this has allowed us to perform the amidoalkylating reaction under reflux conditions in near quantitative yields. By using the ferric chloride–benzophenone complex as a novel Friedel–Crafts catalyst, aminomethyl polystyrene resins of high homogeneity, controlled loading capacity (0.5–7.3 mmol/g) and excellent swelling properties in most of the widely used solid–phase organic chemistry solvents have been prepared.

P–09

HIGH–YIELD SOLID–PHASE PEPTIDE SYNTHESIS USING A HIGH CAPACITY AMINOMETHYL POLYSTYRENE RESIN IN COMBINATION WITH THE RINK LINKER

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Aminomethylated polystyrene resins of high purity, high homogeneity and high capacity (3.0 – 7.3 mmol/g) were prepared by the amidomethylation reaction using $FeCl_3$ as a Friedel Craft catalyst (Zikos & Ferderigos, Tetrahedron Lett. 36:3741, 1995). As shown, the resin with the highest loading capacity which could be successfully applied to the peptide synthesis was the 5.5 mmol/g one. On this resin an o–Cl–trityl type handle was linked and the bioactive pentapeptide Leu–Enk was synthesized with ~ 60 % final yield (182 mg of peptide / 100 mg of resin, Zikos et al., J. Peptide Sci. 8:S126, 2002). Using the above procedure, the first aminoacid was anchored on the resin in a ~ 60 % yield, as monitored by determining the Fmoc group spectrophotometrically, while the following coupling steps were carried out in near quantitative yields, as monitored with the Kaiser test. The relatively low anchoring yield may be associated with our observation that the resin was shrinked when AcBr (in CH_2Cl_2) was added, in the stage of preparing the trityl bromide polymer. In an attempt to exploit all the active sites present in the aminomethyl resin, we used a different handle molecule, namely the Fmoc Rink linker, and repeated the synthesis of Leu–Enk. Briefly, the synthesis procedure was performed manually, following the Fmoc strategy and the HOBt/DIC activation approach. Coupling was performed by dissolving an excessive quantity (4 mol equiv.) of Fmoc–protected amino acid and HOBt in DMF. The solution was cooled on ice and then DIC (4 mol equiv.) was added. Using this approach, anchoring of the first aminoacid on the resin was carried out quantitatively, as shown by using the Kaiser test and determining the Fmoc group spectrophotometrically. Overall, the Leu–Enk peptide was obtained almost quantitatively (308 mg of peptide / 100 mg of resin) and at > 97 % purity in crude product. The new resin/linker system is currently used in the synthesis of various bioactive peptides, longer than Leu–Enk, in order to further evaluate its potential.

P–10

EFFICIENT Nα–FMOC REMOVAL FROM RESIN BOUND AMINO ACIDS BY 5% PIPERIDINE SOLUTION

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Taking in consideration that piperidine is not only an expensive reagent but is also a controlled substance according to the 92/109/EC recommendation, we tested the efficacy of more dilute solutions of piperidine in Nα–Fmoc removal from amino acids bonded to resin in SPPS. The half lives needed for Fmoc removal when Fmoc–L–Lys (Boc), Fmoc–L–Val, Fmoc–L–Pro or Fmoc–Gln were bound on a chlorotrityl resin, were measured. It was found that the time needed for deprotection depended on the amino acid bound to the resin, with the Lys derivative having the shortest time and the Val derivative the longest one. It was also noticed that the longer the time needed for deprotection, the less differences observed between the 20, 10 or 5% piperidine solutions. We also applied 20, 10, or 5% DMF solutions in the synthesis of H–Ala10–Lys–OH which is a known sterically hindered "difficult" sequence. All three different piperidine solutions tested needed about the same deprotection time without any obvious difference. Finally, we used successfully 5% piperidine solution in the synthesis of acyl carrier protein (65–74) and Leu–enkephaline. In conclusion, 5% piperidine solution in DMF can be used instead of the usual 20% solution in SPPS for Fmoc removal from the resin bound peptide.

P–11

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

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The tendency of intramolecular disulfide bond formation between cysteine residues is strongly dependent on the number of residues separating the two Cys since a small number of spacer amino acids introduces stereochemical constraints. The moiety Cys–X–Y–Cys is the smallest and most highly conserved chelating unit in biological systems. Extensive theoretical and experimental studies have been performed using model peptides of the type (S,S) Cys–X_n–Cys ($n \geq 2$) aiming to establish their conformational profiles and evaluating their tendency to form the disulfide bond. (S,S) cyclic synthetic analogues bearing a single amino acid between the Cys residues were recently reported by our group for the first time. The (S,S) –CDC– scaffold was used to develop constraint anti–aggregatory agents. In this study, we report on the synthesis of numerous (S,S) –CDC–and (S,S) –CRC– cyclic compounds. The disulfide bond formation was performed either on solid support or/and in liquid phase. The acetamidomethyl group was used for protection of the thiol group of Cys and the oxidation was performed by $Tl(tfa)_3$ and/ or iodine. In particular cases, both Boc– and Fmoc– strategies were applied using the MBHA or the Rink Amide AM resin, respectively. In all cases the $Tl(tfa)_3$ oxidizing reagent was more efficient than I_2.

P–12

ISOXAZOLE–PHOSPHINIC PEPTIDES: SOME NEW DEVELOPMENTS

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We reported recently the diastereoselective synthesis–through 1,3–dipolar cycloaddition of in situ prepared nitrile oxides onto dipolarophilic phosphinopeptidic templates–of a novel class of phosphinic pseudopeptides, which incorporate the *isoxazole* ring. Preliminary results showed that some of these heterocyclic pseudopeptides behave as very potent inhibitors of the matrixins. We present here some new developments concerning the synthetic approaches to this class of compounds. Our work deals with the use–in parallel with analogues of natural amino acids–of the biologically important phenyl phosphinic acid at the P_1 position of the peptidic backbone. After the wide diversification of the isoxazole–phosphinic peptides in solution phase, the next step was the development of a solid phase version of this synthetic strategy. To this end, we developed a new protocol (EDC/HOBt mediated coupling) for the coupling of Nα–protected, P–unprotected dipeptidic blocks with solid supported amino acids in a quantitative and diastereoselective manner, since use of the well known adamantyl group as a hydroxyphosphinyl–protecting group proved to be ineffective. Indeed, using the multipin technology (Mimotopes SynPhase PS Lanterns) as a solid support, we succeeded in anchoring dipolarophilic phosphinic pseudopeptides (they bear a triple bond) onto this support. Two kinds of handles were successfully used; the Rink–amide forming handle and the 4–(hydroxymethyl) phenoxyacetamido handle (Wang). Optimization of dipolar cycloadditions onto lantern–embodied phosphinic peptides allowed the convenient preparation of new isoxazole–phosphinic peptides in high yield and purity.

P–13

SYNTHESIS OF NEW PSEUDOPEPTIDIC BUILDING BLOCKS SUITABLE FOR DIVERSIFICATION AT P_1' POSITION OF PHOSPHINIC INHIBITORS

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Phosphinic peptides, also called phosphinic pseudopeptides, represent a class of compounds, in which a standard peptide bond has been substituted by a non–hydrolyzable phophinic acid moiety, {–CO–NH–} → {–P(O)(OH)–CH_2–}. These transition–state analogues are effective enzyme inhibitors for a variety of zink–metalloproteases. The classical approach for synthesizing phosphinodipeptidic blocks involves 1,4–addition of N–protected silyl aminoalkylphosphonites to appropriately substituted acrylates. Such acrylates can be generated *via* alkylation of diethyl malonate followed by saponification and Knoevenagel condensation with formaldehyde. The possibility of introducing alkyl or aryl P_1æ side chains directly into phosphinic building blocks could provide a promising alternative so as to avoid the three–step procedure required for the synthesis of acrylates.

A suitable template for such diversification could be the phosphinic block of type P_1XaaΨ[P(O)(OP_2)CH2]CH($COOP_3$)$_2$ (**1**) which is in fact an α–substituted malonic acid diester. We present herein, a new procedure for synthesizing several blocks of type (**1**), bearing different P_1, P_2 and P_3 protecting groups. Furthermore, the selective deprotection of (1) has also been investigated.

P–14

SYNTHESIS OF NEW PHOSPHINIC SYNTHON, ZPro[P(O)(OH)CH_2]PheOH BASIC UNIT FOR THE DEVELOPMENT OF POTENT ACE2 INHIBITORS

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Angiotensin–converting enzyme 2 (ACE2), a homologue of ACE, is a zinc–carboxypeptidase which represents a new and potentially important target in cardio–renal disease. However, the exact biological role of ACE2 is still unknown. Useful biochemical tools for the elucidation of ACE2 function would be potent synthetic inhibitors that would exclusively target this enzyme. Structure–activity studies have proven that peptide hormone AngII, having the Pro–Phe sequence at C–terminus, is the best known physiological substrate of ACE2 to date. Therefore, using AngII as a structure pattern, we set out to develop phosphinic isosteres of this peptide that would act as transition state analogues. In particular, we envisaged that substitution of the Pro–Phe normal peptide bond by a chemically stable phosphinic bond might lead to potent inhibitors of ACE2. Toward this end, new phosphinic peptide blocks of the type ZPro[P(O)(OH)CH_2]PheOH **(1)** have been synthesized using two different synthetic strategies. According to the classical approach for phosphinic dipeptide synthesis, **(1)** was synthesized via a Michael type addition of the aminophosphinic acid analogue of proline to 1,4 acrylic ester analogue of phenylalanine. Nevertheless, this method provided moderate results and therefore was further modified by addition of alkylphosphinic acid analogue of phenylalanine to the 1–pyrroline trimer. This alternative approach provided a much more convenient and high yielding method to synthesize blocks of type **(1)**.

P–15

EFFORTS TO ESTERIFY CAPTOPRIL WITH POLYETHYLENOGLYCOL

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Recently, the applicability of polymers has been extended to pharmaceutical science. One of these applications concerns the increase of circulation time of drug in the body (half–time) which may lead to improvement of its therapeutic efficacy. One of the major drawbacks of ACE antagonists is their low half–time. Captopril is an *A*ngiotensin *C*onverting *E*nzyme (ACE) inhibitor well known to block the synthesis of Angiotensin II a peptide hormone known to exert vasoconstrictive effects and constitutes a prototype molecule already existing in the market in the series of ACE antagonists. For this reason captopril was coupled with different M_n PEG's in an effort to study the pharmacokinetic properties of captopril/PEG coupling. The reaction was performed in a basic environment (DMAP) and using a condensing agent DIPC. As a first step before pursue pharmacokinetic studies, was to characterize the product of the reaction of captopril with PEG. For the product characterization the spectroscopic methods Nuclear Magnetic Resonance (NMR) and Raman were applied along with *T*hin *L*ayer *C*hromatography (TLC) and Differential Scanning Calorimetry (DSC). The characterization results showed esterification of carboxylate and mercaptane groups of captopril. This prompts us to design a new synthetic pathway for achieving selective esterification.

P–16

COMPUTATIONAL ANALYSIS OF THE CONFORMATIONAL FEATURES INDUCED IN PEPTIDE ANALOGUES CONTAINING THE (S,S)–CXC– MOTIF

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Cyclization via a disulfide bond is a widely used strategy to design constrained peptide analogues. The CXXC motif, which is the smallest and highly conserved unit in biological systems, has been extensively studied to estimate its propensity to form β–turns. Recently we have reported on the synthesis, activity and conformational preferences of highly constraint RGD analogues containing the (S,S)–CDC– motif. The main goal of this study is to explore the influence of the (S,S)–CXC– motif to the relative orientation of the X amino acid and the X–2 or X+2 residue side chains. The structure of the peptides has been investigated by molecular dynamics methods. Our findings indicate that despite the nature of the X residue, there is a preference for an almost *cis* coplanar orientation of the X and either one of the adjacent to Cysteine residue side chains. The 11–membered cyclic structure does not favor any β–turn conformation while the backbone dihedral angles within the cycle are very constraint. The χ_3 angle of the first Cysteine residue is distributed around $+80^o$ or -100^o. It is concluded that the (S,S)–CXC– motif can be incorporated in peptide analogues in which the *cis* coplanar orientation of the corresponding amino acid side chains is desirable

P–17

DETECTION OF T–CELL EPITOPES AND MODELING OF DQ2 AND DQ7 MOLECULES USING COMPUTATIONAL METHODS

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T–cells recognize antigenic peptide fragments that are derived from protein antigens and are bound to cell surface proteins encoded by genes of the major histocompatibility complex (MHC). The MHC–peptide complex travels to the cell surface where the MHC–II molecule displays the peptide (epitope) to nearby CD4+ T–cells and the immune response is initiated. Determining which peptides bind to a specific MHC molecule is fundamental to understanding the basis of immunity and to developing immunotherapeutics for autoimmune diseases such as Sjogren's syndrome (SS) and Systemic Erythematosus Lupus (SLE). Autoantibodies against La/SSB autoantigen are frequently found is the sera of patient with SS and SLE. The aim of this study was the prediction of MHC class–II binding peptides, T–cell epitopes of La/SSB autoantigen, based on the fact that class II MHC molecules DQ2 (HLA–DQA1*0501/DQB1*201) and DQ7 (HLA–DQA1*0501/DQB1*0301) are strongly associated with SS and SLE, applying computational methods. Initially, models of DQ2 and DQ7 were constructed using as a template the human insulin peptide HLA–DQ8 complex which shows 91% and 96% homology with DQ2 and DQ7 respectively. The Swiss–PdbViewer program was used for the modeling of DQ2 and DQ7 molecules. A sequence pattern common to T cell epitopes was used for the prediction of T cell epitopes as well as the MHCPEP database of MHC binding peptides. The modeled MHC–epitope complexes were subjected to energy minimization using the program X_PLOR 3.1. The quality of the final models was assessed using the Procheck suite of programs and the 'O' program determined the compatibility of the DQ2 and DQ7 models with T–cell epitopes.

P–18

SPECTRAL SIMULATIONS OF STATIC ^{31}P CP NMR MULTILAMELLAR BILAYER SPECTRA IN THE ABSENCE AND PRESENCE OF BIOACTIVE DIPEPTIDE B–ALA–TYR OR GLU

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B–Ala–Tyr and Glu are known to have significant neurological activity. Their mode of action is still unknown and their effects may in part relate to their membrane actions. For this reason static CP ^{31}P–^{1}H experiments with the DPPC (DiPalmitoyl Phosphatidyl Choline) multibilayers with the absence or presence of the dipeptide or the aminoacid. The NMR lineshapes of the three preparations were compared in order to obtain information about the dynamic effects of β–Ala–Tyr and Glu in the realm of the head–group. Glu–containing samples perturbed more the broadline ^{31}P spectrum of DPPC bilayers than β–Ala–Tyr containing samples. The experimental lineshapes were in agreement with fast internal motion and slow overall reorientation of the lamellar structures. A quantitative determination of the orientation of the rotation axis of a relatively small fragment including the phosphate group in the macromolecules was performed in the frame of the CSA (Chemical Shift Anisotropy) tensor and the surface of the lipid monolayer leaflets. Comparison of the structural parameters obtained by the simulations of the experimental lineshapes of the DPPC bilayer samples containing Glu showed indeed a strong interaction of the Glu with the headgroup of the phospholipids and exchange of the aminoacid in the lipid–water interphase. The dipeptide was on the other hand localized not as close to the phosphate as the Glu and furthermore was on the other side of the backbone of the phospholipid, in the edge to the hydrophobic domain of the bilayers.

P–19

BIOPHYSICAL STUDIES ON A NOVEL ANTIHYPERTENSIVE AT1 ANTAGONIST MM1

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MM1 is a bioactive antihypertensive drug synthesized to mimick the C–terminal part of sarmesin. In particular, imidazole group of MM1 mimicks imidazole group of His^6, phenyl group of MM1mimicks Phe group of Phe^8 and lactame amide group of pyrrolidinone the amide group of Phe^8–Pro^7. A superimposition of MM1 with losartan the first AT1 antagonist in the market was sought matching the following equivalent groups: (a) imidazole group of MM1 with imidazole group of His^6; (b) phenyl group of MM1 with phenyl group of Phe^8; (c) lactame amide group of pyrrolidinone with amide bond of Phe^8–Pro^7. The superimposition was excellent (RMS=0.71 Å). The pharmacophore similarity between the two molecules prompted us to investigate their thermal and dynamic effects in DPPC bilayers. The preliminary results show that the two molecules bear similar effects. In particular, at low concentrations they broaden the breadth of pre–transition and at high concentrations abolish the pre–transition and lower the main phase transition temperature. ^{13}C MAS spectra obtained at different temperatures of DPPC bilayers with MM1 showed additional peaks in the aromatic region compared to the corresponding spectra of DPPC bilayers alone at identical temperatures. This is a direct evidence of drug incorporation into DPPC bilayers and the obtained results indicate mobility of aromatic segments in the topography they are located in membrane bilayers.

P–20

3D–QSAR ANTIHYPERTENSIVE MODELS BASED ON 2D–NMR AND COMPUTATIONAL ANALYSIS DRIVEN LOW ENERGY CONFORMERS OF LOSARTAN

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The alignment of molecules in 3D–QSAR CoMFA and CoMSIA is a decisive procedure that usually determines the efficacy of the analysis. The optimum case for the alignment is the existence of X–ray data for the receptor, the ligand or both molecules. When X–ray data are not available, 2D–NMR and computational methods can be applied to provide low energy conformers to be used in the analysis.

Losartan is a non–peptide mimetic antagonist of Angiotensin II. Its bioactive conformation has been derived through 2D–NMR experiments and computational analysis, with the use of different environments that may simulate the biological topography in the active site. Confirmation of the great reliability of that model came later with docking studies of the proposed conformation of losartan with the Angiotensin II receptor. A 3D–Quantitative Structure–Activity Relationships can be performed on SARTANs analogs or mimics of losartan using as template the conformation of losartan derived from various environments This will verify the potency of NMR and computational methods in the conformation determination, indispensable in 3D–QSAR.

P–21

CONFORMATIONAL ANALYSIS USING 2D NMR SPECTROSCOPY COUPLED WITH COMPUTATIONAL ANALYSIS OF VALSARTAN AND ITS DRUG : MEMBRANE INTERACTIONS

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Valsartan is a promising AT1 antagonist that belongs to SARTANs' group. It has structural similarities and differences when compared to prototype losartan. Both molecules have a biphenyltetrazole segment which is a key characteristic among most commercially available antihypertensive AT1 antagonist drugs. Valsartan differs in structure from the prototype losartan when it comes to the lack of imidazole ring. Structural elucidation of valsartan was achieved using 2D NMR spectroscopy. Interestingly, two distinct conformational diastereomers were observed in a wide range of temperatures, which was not the case with prototype losartan. In addition, its interactions with the membrane bilayers were sought using a combination of biophysical methods. Valsartan has shown to exert similar thermal changes at membrane bilayers when incorporated into them at equivalent low concentrations. These effects are summarized to the abolishment of pre–transition temperature and lowering as well as broadening of the main phase transition temperature. At higher concentrations, valsartan and losartan exert different effects attributed probably to their unsimilar way they form domains in the membrane bilayers or reversible transition from a vesicular suspension to an extended peak bilayer network. Biophysical experiments using solid state NMR, Raman spectroscopy and differential scanning calorimetry are under progress in order to obtain more details about their dynamic properties and topography of the drug into membrane bilayers.

P–22

COMPARATIVE THERMAL AND DYNAMIC EFFECTS OF CAPTOPRIL AND ENALAPRIL IN LIPID BILAYERS USING DSC, SOLID STATE NMR AND RAMAN SPECTROSCOPY

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Captopril is of major pharmaceutical importance since it is used as a drug against cardiovascular diseases. Its molecular basis of action is still unknown. For this reason a combination of thermal analysis, solid state NMR and Raman Spectroscopy is used to study its effects on membrane bilayers. In particular, we have used DSC to study the thermal changes caused by captopril in dipalmitoylphosphatidylcholine bilayers. The results showed that the presence of the drug causes broadening of the phase transition, obliterates the pre–transition and decrease of the phase transition temperature. Raman spectroscopy showed that captopril is localized between the acyl chains of DPPC bilayers and exerts mainly hydrophobic interactions. This explains the broadening it causes in phase transitions and small changes in the frequencies referred to the glycerol skeleton of DPPC bilayers. These results may conclude that lateral diffusion of captopril towards receptor must happen in the hydrophobic core of DPPC bilayers. In the future, we will examine if this is a general mechanism exerted by other ACE inhibitors. Similar experiments are under progress for enalapril in order to compare their thermal and dynamic effects in membrane bilayers.

P–23

STRUCTURAL ELUCIDATION AND CONFORMATIONAL ANALYSIS OF A POTENT ALTERED PEPTIDE LIGAND, [Arg^{91}, Ala^{96}] MBP$_{87–99}$, EMPLOYING EXPERIMENTAL ROE CONSTRAINTS COMBINED WITH THEORETICAL CALCULATIONS AND MOLECULAR MODELLING

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Multiple Sclerosis (MS) is an inflammatory, demyelinating disease involving the white matter of the CNS. Its infectious etiology remains unknown but both environmental and genetic factors contribute considerably. A novel approach towards the therapeutic management of MS has been launched by the introduction of antigen–specific therapies that suppress autoreactive T cells. Myelin Basic Protein (MBP) is a constituent of myelin sheath and it has been considered as a candidate autoantigen. Favoring this theory comes the evidence that an MBP peptide analogue has the potential of inducing Experimental Autoimmune Encephalomyelitis (EAE) when injected in Lewis rats. Within the grounds of actively inhibit the disease, the design and use of peptide analogues of MBP is suggested, exploiting the idea of blocking the formation of the trimolecular complex MHC–APL–TCR. Inference to a series of EAE experiments induced by the MBP$_{72–85}$ epitope is that a linear altered peptide ligand (APL), [Arg^{91}, Ala^{96}] MBP$_{87–99}$, holds antagonistic properties. We sought to perform conformational analysis of the APL in order to comprehend the stereoelectronic requirements for antagonistic activity. A combination of NMR spectroscopy and molecular modelling was applied to achieve this aim. ROE data suggest a semicircular conformation, with the backbone of the APL folded and an almost head–to–tail arrangement. Minimized structures generated after a molecular modelling theoretical study supplied conformations akin to the ones generated using distance constraints dictated by 2D ROESY spectroscopy.

P–24

SYNTHESIS OF A CATHEPSIN–L1 DERIVED SEQUENCE CONJUGATED TO SEQUENTIAL OLIGOPEPTIDE CARRIER AND ^{1}H–NMR CONFORMATIONAL ANALYSIS IN ITS FREE STATE

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The liver flukes *Fasciola hepatica* and *Fasciola gigantica* are causative agents of *fascioliasis* in humans and ruminants, especially cattle, goat and sheep. The disease has been traditionally considered to be an important veterinary disease because of the substantial production and large economic losses in livestock production. Cathepsin cystein proteinases are abundant proteins in Fasciola and form a major component of the material secreted by the parasite. Humoral immune response to cathepsin–L proteases has been used in attempts to develop a reliable test for the diagnosis of *F. hepatica* infections in rats, sheep, ruminants and humans. Nevertheless, in all these attempts, cross–reactivity with other parasites was a major hurdle. In this work we report on the synthesis and conformational analysis of the Cathepsin L1 epitope 110–123 (DKIDWRESGYVTEV) in the free state and conjugated to Sequential Oligopeptide Carrier [Ac–(Lys–Aib–Gly–)$_4$–OH, Ac–SOC$_4$]. This epitope was selected in order to develop a species–specific diagnostic ELISA for *F. hepatica*. The conformational analysis of the epitope in its free state was performed in aqueous solution using 1D and 2D NMR spectroscopy. The numerous and intense NOE effects detected between successive amide protons along almost all the peptide backbone and of type $d_{NN}(i, i+2)$ and $d_{\alpha N}(i, i+2)$ are consistent with a helical type structure.

P–25

COORDINATION PROPERTIES OF Cu(II) AND Ni(II) IONS TOWARDS THE C–TERMINAL PEPTIDE FRAGMENT ELAKHA OF HISTONE H2B AND THE 71–76 PEPTIDE FRAGMENT –TYTEHA– OF HISTONE H4

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Metal ions are essential for the regular operation of the cell. On the the other hand in high concentrations could led to growth of tumors. Even though the molecular mechanism of carcinogenesis is still unclear it is believed that metal binding to histones may play a significant role. In order to reveal more information about the toxicity caused by metals we synthesized the hexapeptide models Ac–GluLeuAlaLysHisAla–am (102–107 fragment of H2B histone) and Ac–ThrTyrThrGluHisAla–am (71–76 fragment of H4 histone) which lie into the nucleosome core and study their interactions with Cu(II) and Ni(II) ions.

The peptides were synthesized by standard SPPS procedures while their coordination properties were examined by means of various potentiometric and spectroscopic techniques (UV/Vis, CD, EPR and NMR). Peptides were found to interact strongly with the metals over a wide pH range (3–11). The imidazole nitrogen atoms of histidine is the primary binding site for both metals while increasing pH leads to the deprotonation and coordination of the amide nitrogens.

The results of this work show that certain protein sequences can strongly interact with toxic metal ions which are capable of altering the chemical or/ and structural properties of the histone octamer.

P–26

COPPER(I) INTERACTION WITH MODEL PEPTIDES OF WD6 AND TM6 OF WILSON ATPase: REGULATORY AND MECHANISTIC IMPLICATIONS

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With the aim to investigate the mechanism of Cu(I) transport by Wilson ATPase (ATP7B), we have studied the interaction of the peptides 2K10p (CH_3CO–Lys–Gly–Met–Thr–Cys–Ala–Ser–Cys–Val–His–Asn–Lys–$CONH_2$), and 2K8p (CH_3CO–Lys–Leu–Cys–Ile–Ala–Cys–Pro–Cys–Ser–Lys–$CONH_2$), part of the sixth metal binding domain (WD6) and the sixth transmembrane segment (TM6) of Wilson ATPase respectively, by means of CD, NMR spectroscopy and homology modeling. In addition, the interaction of Cu(I) with the 2K8p mutants
1s (CH_3CO–Lys–Leu–Ser–Ile–Ala–Cys–Pro–Cys–Ser–Lys–$CONH_2$),
2s (CH_3CO–Lys–Leu–Cys–Ile–Ala–Ser–Pro–Cys–Ser–Lys–$CONH_2$) and
3s (CH_3CO–Lys–Leu–Cys–Ile–Ala–Cys–Pro–Ser–Ser–Lys–$CONH_2$), containing two cysteines in various positions, have been studied with the same methods, in order to understand the role of each cysteine in copper binding. Our studies show that the three cysteine thiolates present in the 2K8p peptide sequence act mainly as bridging ligands for Cu(I) binding, and dithiothreitol acts as an important ligand in Cu(I) ligation by 2K10p and the 2K8p mutants. Formation of oligomeric species has been evidenced for all peptides except 2s. Shift of the equilibrium between the various oligomeric species has been accomplished by reducing the Cu(I):peptide ratio. Significant shifts of proline protons upon interaction with Cu(I) have been observed for all proline containing peptides implying a possible role of proline in facilitating Cu(I) binding. These findings have been further discussed with respect to the molecular basis of copper trafficking and intermolecular interactions.

P–27

SYNTHESIS, CONFORMATIONAL AND IMMUNOLOGICAL STUDIES OF COLLAGEN MODELS: APPLICATIONS IN WORKS OF ART

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Proteinaceous substances have been used as binding media in a variety of works of art as paintings, stone sculptures and wooden statues. Identification of proteins is essential in order to understand ancient technologies, determine the extent of decay and help in future restoration and preservation processes. Sequential polypeptides $(Pro–X–Gly)_n$, where X represents amino residues Val, Lys, Glu, and $(Hyp–Val–Gly)_n$ were prepared and studied as models of collagen, which has been widely used as binding medium in works of art. Polymerization was carried out on the pentachlorophenyl active esters of the appropriate tripeptide unit and molecular–weight range of 10000–20000 was achieved. Tripeptides were synthesized by the solid phase peptide synthesis on a 2–chloro–trityl chloride resin using the Fmoc methodology. Conformational properties of the sequential polypeptides will be evaluated by circular dichroism (CD) studies in aqueous and organic mixtures and compared with type I collagen from calf skin. Immunization experiments in rabbits using collagen and its synthetic models are now in progress. The obtained antibodies will be used for the immunochemical detection of collagen and collagen fragments in artificially and naturally aged samples as follows: development of reference ELISA using artificially coated samples of collagen and models, as well as authentic samples. ELISA plates coated with collagen or collagen models will be "protected" by the anti–collagen antibodies obtained from animal immunizations and then will be subjected to artificial aging. This approach is expected to provide new insights in the conservation processes.

P–28

CONFORMATIONAL PROPERTIES AND INTERACTIONS OF THE TOXIN PARALYSIN B–ALA–TYR AND GLU WITH PHOSPHOLIPID BILAYERS

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Paralysin β–Ala–Tyr, a toxin from the larvae of the grey flesh fly Neobelleria Bullata is known to exert biological activity. Since it causes reduced locomotory activity, paralysis and fast death, it is suggested that it acts on the nervous system. It is also hypothesized that paralysins interfere with some neurotransmitter receptors resulting in drastic changes concerning bio–electrical activity. In particular, it is hypothesized that β–Ala–Tyr acts onto the Glu– receptor. To examine how β–Ala–Tyr and Glu affect membrane bilayers we applied a combination of biophysical methods, DSC, Raman and solid state NMR spectroscopy. Several structural and dynamical properties of the DPPC multilamellar bilayer, with and without the dipeptide or Glu, were compared using high resolution ^{13}C MAS spectra, and simulations of inhomogeneously broadened, stationary (without Magic Angle Spinning) ^{31}P NMR lineshapes, measured under CP (Cross–polarization) conditions. Interpretation of the experimental data indicates that the aminoacid binds in the closest realm of the phosphate in the lipid head. Contrarily, the dipeptide is located deeper in the bilayers. These results were depicted using molecular graphics. In particular, the lowest energy conformations of the two molecules (obtained with the use of ROE distances and X–rays) were positioned inside a simulated DPPC bilayer in accordance with the experimental data.

P–29

STRUCTURAL CHARACTERIZATION BY NMR OF A RHENIUM–BOMBESIN COMPLEX FOR RADIOPHARMACEUTICAL APPLICATIONS

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Radiolabeling of small receptor–avid peptides at specific predetermined chelation sites with radioactive metals has been an effective approach for production of target–specific radiopharmaceuticals for diagnostic and therapeutic applications. Recently, the preparation and study of radiolabeled derivatives of the tetradecapeptide bombesin has become a significant scientific target because bombesin receptors are overexpressed on the cell surface of several cancer tissues. Within this framework, we have synthesized a new pentadecapeptide bombesin derivative properly designed to be labeled with the radioactive isotopes technetium–99m for scintigraphy, or rhenium–186 and rhenium–188 for radiotherapy. The technetium–99m complex has already displayed significant uptake in small cell lung carcinoma and colon cancer tumors developed in nude mice.

In this work we present the synthesis of the non–radioactive rhenium–185/187 complex of our bombesin derivative and its structural characterization by NMR and mass spectrometry. All ^{1}H and ^{13}C resonances of the rhenium–185/187–bombesin complex, as well as of the free bombesin derivative, have been assigned through the combined use of TOCSY, NOESY, HSQC and HMBC experiments. The assigned structure applies to the radioactive technetium–99m and rhenium–186 and rhenium–188 analogues and provides essential information in the process of development of new cancer radiopharmaceuticals.

P–30

NMR, CD, AND MOLECULAR MODELING STUDIES OF HUMANIN, A PEPTIDE AGAINST ALZHEIMER' S DISEASE–RELATED NEUROTOXICITY

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Alzheimer's disease is the most common cause of dementia among the elderly. Recently, a human gene encoding a 24–residue linear peptide, called humanin, that can abolish the death of neuronal cells in Alzheimer's patients was identified through functional expression screening. Although humanin has not yet been isolated from mammalian tissues, immunohistochemical studies indicate that it is a naturally occurring peptide. Humanin is believed to suppress the intracellular mechanisms triggered by familial Alzheimer's disease mutants or β–amyloid without directly affecting β–amyloid production or aggregation offering thus a new perspective for a therapeutic approach to Alzheimer's disease.

Humanin is a rather hydrophobic linear peptide containing a hydrophobic stretch of Leu residues, Leu^{9}–Leu^{10}–Leu^{11}–Leu^{12}, at its N–terminus and a charged area of Lys^{21}–Arg^{22}–Arg^{23} at its C–terminus. A minimal domain for full activity is from Pro^{3} to Pro^{19}.

Humanin was synthesized in our lab, following the Fmoc–solid phase peptide synthesis approach on an in–house prepared o–Cl–trityl–amidomethyl polystyrene resin. The peptide was purified with semi–preparative RP–HPLC and suitably characterized. The solution structure of synthetic humanin was studied in aqueous solutions of different pH as well as in H_2O–TFE mixtures using circular dichroism spectropolarimetry and NMR spectroscopy. These results in combination with molecular modeling studies will be presented.

P–31

^{1}H–NMR CONFORMATIONAL STUDIES OF THE gD [Nle^{11}] 9–22 ANTIGENIC REGION OF HERPES SIMPLEX VIRUS GLYCOPROTEIN D

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Herpes simplex virus (HSV) has two serotypes known as HSV–1 and HSV–2. Glycoprotein D (gD) is an envelope component of both HSV types and is one of the principal targets for the antibody response during an HSV–infection. Therefore, it is a prime candidate for a human subunit vaccine. The strong antigenic [Nle^{11}]analogue (L^{9}KNleADPNRFRGKDL^{22}–NH_2) derived from the gD (9–22) sequence was synthesized in the free form and conjugated to SOC (Ac–[Lys–Aib–Gly]$_4$–OH) and tetratuftsin (H–[Thr–Lys–Pro–Lys–Gly]$_4$–NH_2, T20) carriers. Conformational studies of the gD(9–22) peptide in both free state and conjugated to carriers were performed by 2D–NMR and CD in aqueous solution and mixtures with TFE. Intense NOE cross peaks between almost all the successive NH_i/NH_{i+1} amide protons were observed for the free peptide in water indicating that it adopts a rather well defined conformation. The detection of type $d_{\alpha N}$ (i, i+2) between P^6/R^8, R^8/R^{10} and F^9/G^{11}, type d_{NN} (i, i+2) between N^7/F^9, R^8/R^{10} and G^{11}/D^{13} and type $d_{\alpha N}$ (i, i+3) between P^6/F^9 and R^8/G^{11} suggests the presence of a 3_{10} helix structure in the –P^{6}NRFRGKDL^{14}– segment. A β–turn in the –DPNR– segment seems also to contribute to the structure stabilization of the peptide.

P–32

STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE DIMERIZATION SITES OF SOLUBLE GUANYLYL CYCLASE

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Soluble guanylyl cyclase (sGC) is a ubiquitous enzyme that functions as a receptor for nitric oxide. In spite of the obligate heterodimeric nature of sGC, the sequence mediating subunit association has remained elusive. Our initial screening for the interaction site(s) demonstrated that two regions of each subunit, i.e. the regulatory domain and the central region, are involved in heterodimer formation of the most common sGC isoenzyme, α_1/β_1. To precisely map the relevant segments in the β_1 subunit, we constructed multiple N– and C–terminal deletion variants and co–transfected them with full–length α_1 in COS cells. Immunoprecipitation revealed that a sequence segment spanning positions 204 to 408 mediates binding of α_1 to β_1. Fusion of the α_1 dimerization region to EGFP conferred binding activity to the recipient protein. Analysis of deletion constructs lacking portions of the dimerization region identified two distinct sites in β_1 that contribute importantly to its interaction with α_1, i.e. an N–terminal binding site (NBS) covering positions 204 to 244 and a C–terminal binding site at 379 to 408 (CBS). Both NBS and CBS are crucial for sGC function as deletion of either site rendered sGC inactive. We conclude that the dimerization region of β_1 extends over 205 residues of its regulatory and central domains, and that two discontinuous sites of 41 and 30 residues, respectively facilitate binding of β_1 to the α_1 subunit of sGC.

P–33

MISCONCEPTIONS IN STRUCTURAL PEPTIDE CHEMISTRY: AN ANALYSIS FROM A BASIC ORGANIC CHEMISTRY PERSPECTIVE

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The hypothesis and the conclusions of previous ^{17}O NMR studies on the detection of:
I. discrete conformational states in peptides and
II. of both oxygens of the carboxylic group of Boc–[^{17}O]Tyr(2,6–diClBzl)–OH in DMSO–d_6 solution
are reconsidered based on elementary concepts of undergraduate organic chemistry.

It is demonstrated that the ^{17}O shielding time scale is not advantageous compared to that of ^{1}H NMR and, thus, it is not suitable for the detection of discrete hydrogen bonded conformational studies on peptides. ^{17}O NMR spectroscopy is prone to interpretation errors due to formation of ^{17}O labeled impurities during the synthetic procedures. Furthermore, the appearance of two discrete resonances at 340 and 175 ppm of the adove protected amino acid is not now attributed: (a) to the reduction of the intramolecular conformational exchange rate, due to the effect of intramolecular hydrogen bonding of the hydroxy part of the carboxyl with the carbonyl oxygen of the Boc–group, and (b) to the effect of solvent viscosity, suggested in the mentioned study. The cause of this phenomenon is now attributed to a strong hydrogen bonding of the polar proton acceptor solvent DMSO with the carboxy group, which effectively reduces the proton exchange rate, thus becoming slow on the ^{17}O NMR time scale.

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SYNTHESIS AND STUDY OF THE FLUORESCENCE PROPERTIES OF IQFS FOR RENIN CONTAINING THE COMBINATION OF L–H–AMP–OH WITH DNP AT VARIOUS POSITIONS

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The application of Intramolecularly Quenched Fluorogenic Substrates (IQFS) on determination of proteases' activity is advantageous providing a continuous and direct assay. The sensitivity of the assay depends mainly on the kinetic parameters of the substrate, the fluorescence intensity of the donor and the quenching efficiency of the donor–acceptor pair. The quenching efficiency is very much dependent on the distance between the acceptor and the donor. The kinetic parameters of the substrates can be influenced by the type and the position of the fluorescent markers, especially when they are bulky aromatic groups or they are bound on the side chain of amino acids. Thus, a fluorophore providing flexibility on the design of the substrates it would be very useful. L–2–amino–3–(7–methoxy–4–coumaryl)propionic acid (L–H–Amp–OH), being an α–amino acid, is very advantageous over other fluorescent markers, as it can be placed in any position of the peptide chain. Moreover, its Fmoc–derivative can be directly linked to a resin. Taking advantage of these properties we have used L–H–Amp–OH in the synthesis of IQFS for the aspartyl protease renin by solid phase method. As quencher, the 2,4–dinitrophenyl (DNP) group was used, which is known to quench efficiently the fluorescence of 7–methoxy–coumarin derivatives. Selected substrates carrying L–H–Amp–OH at C– or N–terminal and internal positions of the peptide chain and DNP at different distances were studied. We report here the comparison of the spectroscopic properties of these substrates as well as those of the fluorescent peptides liberated upon renin hydrolysis.

P–35

ANTIANGIOGENIC ACTIVITY OF A SYNTHETIC PEPTIDE THAT CORRESPONDS TO THE C–TERMINAL REGION OF HARP

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Heparin Affin Regulatory Peptide (HARP), also known as pleiotrophin, is an 18 kDa growth factor that has a high affinity for heparin. A growing body of evidence indicates that HARP is involved in the control of cellular proliferation, migration and differentiation, and there seems to be a strong correlation between HARP expression and tumor growth and angiogenesis. The identification of HARP domains that are responsible for its biological activity is a matter of investigation of several groups. Several data suggest that the different regions of HARP may exert distinct, or even opposite effects. Recent studies have shown that the last 25 aminoacids of the C–terminal region of HARP are important for HARP binding to its receptor Anaplastic Lymphoma Kinase and the exertion of its angiogenic activity. In the present work, we studied the effects of a synthetic peptide that corresponds to the last 25 aminoacids of the C–terminal region of HARP on endothelial cell functions *in vitro* and on the expression of VEGF receptors. This peptide contains the lysine–rich sequence as well as the entire *a*–helix of the C terminus of HARP and was synthesized by solid phase peptide synthesis techniques, on a 2–chlorotrityl–chloride resin, using Fmoc/t–butyl protection strategies. The peptide decreased the migration of human umbilical vein endothelial cells (HUVEC), while it had no effect on HUVEC proliferation. The peptide also decreased the ability of HUVEC to form capillary–like networks when cultured on matrigel. This inhibitory effect on the endothelial cell functions seems to be, at least partly, due to inhibition of the expression of VEGF receptors. Further studies are in progress in order to clarify the mechanism(s) through which this peptide inhibits angiogenic processes of endothelial cells.

P–36

GLUTATHIONYL–PEPTIDE ANALOGUES FOR THE CHARACTERISATION OF THE BINDING SITE OF MAIZE GLUTATHIONE *S*–TRANSFERASE

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Glutathione *S*–transferases (GSTs; EC 2.5.1.18) are a family of dimeric detoxifying enzymes that catalyse the conjugation of the tripeptide glutathione (GSH) to a variety of endogenous and exogenous electrophiles. The electrophile peptide analogue S–(4–bromo–2,3–dioxobutyl)glutathione (S–BDB–GSH) was shown to be an effective alkylating affinity label for maize glutathione S–transferase I (GST I) expressed in *E. coli*, and was used to probe the substrate binding site of the enzyme. The rate of inactivation of GST I by S–BDB–GSH at pH 6.5 exhibits non–linear dependence on S–BDB–GSH concentration, consistent with the formation of a reversible complex with the enzyme (K_D 217.9±2.1 μM) prior to irreversible modification with maximum rate constants of 0.035 min^{-1}. Protection from inactivation was afforded by the substrate analogue (*S*–nitrobenzyl–glutathione) demonstrating the specificity of the reaction. Structure–function relationships and kinetic analysis of GST I was also carried out by using several alternative substrate analogues including γ–L–glutamyl–L–cysteine, L–cysteinyl–glycine and GSH ethyl ester, L–cysteine and N–acetyl–L–cysteine. The results reveal important protein structural regions that modulate glutathione binding and regulate the induced–fit mechanism of substrate recognition.

P–37

CITRULLINATION OF EAE ANTAGONIST ANALOGUE, CYCLO(87–99) (ARG^{91}, ALA^{96}) MBP$_{87–99}$ WITH PEPTIDYLARGININE DEIMINASE (PAD)

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Multiple sclerosis (MS) is a chronic specific CD4+T–cell mediated disease of the central nervous system (CNS) characterized by local T cell and macrophage infiltrates, demyelination and loss of neurologic function. MS is widely believed to be an autoimmune disease and to be triggered by CNS–specific CD4+T lymphocytes. Posttranslational modifications of proteins often perform a key role in the biological functioning of proteins. Some of these modifications also change the immunogenicity of proteins and peptides and create 'self'–antigens which might induce autoimmune responses. In particular, modifications of arginines within a defined protein context can lead to a specific B–cell immune response. Roughly 30% of Central Nervous System myelin consists of MBP, which is in fact a mixture of different isoforms and isomers. The least cationic isomer of MBP (MBP–Cit$_6$) contains six citrulline residues. This isomer is normally present in MBP of healthy individuals but its level is increased two– to threefold in individuals with a chronic form of MS. In a severe and fulminating form of Multiple Sclerosis 18 of the 19 arginine residues of MBP are converted to citrulline. Peptidylarginine deiminase (PAD) is an enzyme that catalyzes the conversion of peptidyl–arginine into peptidyl–citrulline. This reaction implies the change of the positively charge of arginine side chain into a neutral group In this study, the enzymatic modification of the Arginine into Citrulline of the EAE antagonist analogue, cyclo (87–99) (Arg^{91}, Ala^{96}) MBP$_{87–99}$ which is the immunodominant epitope, was achieved with peptidylarginine deiminase (PAD).

P–38

SCREENING THE EFFECT ON STRUCTURE AND BINDING AFFINITY OF LEUPROLIDE ANALOGUES MODIFIED IN POSITION 6 WITH α,α–DIALKYL AMINO ACIDS

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The regulation of normal mammalian sexual maturation and reproductive function requires the integration and precise orchestration of hormonal regulation at the hypothalamic, pituitary, and gonadal levels. LHRH is a decapeptide synthesized in neurosecretory cells of the hypothalamus and acts upon receptors in the anterior pituitary, where it triggers the synthesis and release of LH and FSH. LH and FSH enter the systemic circulation and bind to receptors in the gonads, where they stimulate steroidogenesis and gametogenesis. LHRH has been the subject of intense structure–activity relationship (SAR) studies aimed at elucidating its mechanism of action and identifying drug candidates (agonists and antagonists), which have been extensively studied in the clinic later on. In this study, we report the synthesis of new LHRH analogues bearing the Fujino modification and substituted at position 6 (Gly^6) by α,α–dialkyl amino acids. We present the conformational analysis in solution of: a) LHRH, b) Leuprolide ([DLeu^6,desGly^{10}]LHRH–NHEt), an agonistic analogue of LHRH widely used in oncology and gynaecology, and c) [Aib^6,desGly^{10}]LHRH–NHEt, using NMR spectroscopy. All these molecules were charecterized by a *U–shape* structure. In addition, the affinities of new analogues for the LHRH–R were determined by competition binding experiments in mouse anterior pituitary αT3–1 cells. Leuprolide showed a high binding affinity, while [Aib^6,desGly^{10}]LHRH–NHEt and [L–Tic^3, Deg^6,DesGly^{10}]LHRH–NHEt bind to the LHRH–R with IC$_{50}$ values comparable to those of native hormone. All other analogues showed inferior binding affinities for the LHRH–R.

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DESIGN, SYNTHESIS AND AFFINITY DETERMINATION OF NEW [Tyr^3]OCTREOTATE ANALOGS

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Somatostatin is a hypothalamic peptide hormone regulating the physiology of a great number of organs and systems, as the pituitary, the pancreas, the gastrointestinal track and the central nervous system, after interaction with specific cell membrane G–protein coupled receptors comprising five subtypes ($sstr_{1-5}$). The overexpression of somatostatin receptors, especially the $sstr_2$, on the surface of neoplastic cells provides the molecular basis for employing metabolically stabilized somatostatin analogs in clinical oncology, i.e. octreotide – [Tyr^3]octreotate (Tate).

This work reports on the synthesis and $sstr_2$–binding capabilities of a series of new [Tyr^3]octreotate analogs, namely: [D–Tyr^1,]Tate, [D–TyrO(Et)^1]Tate, [D–Phg^1]Tate, [Asp^1]Tate, [Phg^3]Tate, [Tic^3]Tate, [D–Phg^1,Phg^3]Tate, [D–Tic^4]Tate, [Aib^5]Tate, [Lys^8]Tate, [Asp^0,Lys^8]Tate and [Asp^8]Tate.

The analogs were synthesized on the acid–sensitive 2–chlorotrityl chloride resin applying Fmoc/^{t}Bu methodology. The affinities of new analogs for the $sstr_2$ were determined during receptor binding assays in $sstr_2$–positive rat acinar pancreatic AR42J cell membranes using [^{125}I–Tyr^3]octreotide as the radioligand.

During this structure–affinity relationship study the effects of polarity and charge of amino acids in positions 1 (D–Phe), 3 (Tyr), and 8 (Thr) in the peptide backbone (Tate) were investigated. In addition, the impact of β– turn reinforcement in the region 3–6 and N–terminal elongation by Asp residues on peptide affinity for the $sstr_2$ was explored.

P–40

EVALUATION OF THE STRUCTURE–ACTIVITY RELATIONSHIP OF RGD PEPTIDES BASED ON ARG AND ASP SIDE CHAINS ORIENTATION

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The binding of the integrins to the Arg–Gly–Asp (RGD) motif, as the primary recognition sequence of their ligands, have been extensively studied due to the versatility of the integrins and their RGD ligands. Structure–activity studies of RGD–containing peptides and proteins have revealed that the specificity of an integrin for its RGD ligands is strictly correlated to their conformational features. Three criteria have been proposed for the evaluation of the structure–activity relationship: i) the distance between the charged centers, ii) the distance between the Arg C$^\beta$ and Asp C$^\beta$ atoms and iii) the pseudo–dihedral angle, formed by the Arg C$^\zeta$, Arg C$^\alpha$, Asp C$^\alpha$ and Asp C$^\gamma$ atoms, defining the orientation of the Arg and Asp side chain. In this study we designed, synthesized and studied the peptides
Ac–S**RGD**VG**R**AibGK(Ac)AibG–OH (**1**),
Ac–S**RGD**VG**Nle**AibGK(Ac) AibG–OH (**2**),
Ac–S**NleGD**VG**R**AibGK(Ac)AibG–OH (**3**) and
Ac–**R**Aib**GD**AibG**R**AibGK(Ac)AibG–OH (**4**). A comparative conformation–activity study was performed between the peptides **1**–**4** and strongly constrained cyclic (S,S) –CDC– bearing compounds which cover a wide range of platelets aggregation inhibition potency. The fulfillment of the –45°≤pseudo–dihedral angle≤+45° criterion was proved to be a prerequisite for an RGD compound to exhibit inhibitory activity and the latter increases in combination with the longer the distance between the charged centers of Arg and Asp.

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RGD PEPTIDES CONTAINING SALICYLIC ACID DERIVATIVES WITH ANTIPLATELET ACIVITY *IN VITRO*

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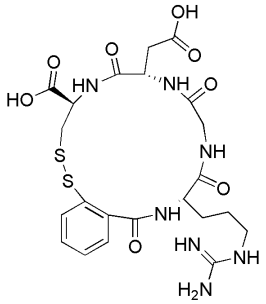
Our efforts are focused on the synthesis of RGD analogs incorporating salicylic acid derivatives at the N–terminal amino group of the tripeptide Arg–Gly–Asp (Arg–Gly–Asp). Some of them have shown satisfactory effect against human platelet aggregation *in vitro* and important specificity for the Gp Ib receptor, a glycoprotein receptor existing on the platelet surface and responsible for their attachement to the blood vessel wall.

In the present study the synthesized linear and cyclic RGD peptides contain the 5–amino–2–hyroxy benzoic acid and the 2–thiosalicylic acid in their sequence. Their antiplatelet activity results will be discussed in details during the forum.



P–42

MAPPING THE BINDING SITES OF THE GPIIIa SUBUNIT OF THE PLATELET RECEPTOR GPIIb/IIIa

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Extensive studies utilizing a combination of immunological, biochemical and mutational approaches have been performed for determining the interacting sites of the GPIIIa subunit with fibrinogen. Although some disagreements have appeared in literature for the proposed regions, the GPIIIa 211–222 region is generally accepted as a ligand–binding site on the receptor. In a previous study, we managed to determine the binding regions within the GPIIIa subunit, which are involved in aggregation process, using synthetic 20–peptides, covering the extracellular region of GPIIIa subunit. From the biological assays, we concluded that some of the 20–peptides included in the GPIIIa: 289–356, 385–440, and 589–644 regions exhibit inhibitory activity, which could characterize them as possible fibrinogen interacting regions. In this work, we present the synthesis and the anti–platelet properties of various peptides derived from the GPIIIa 589–644 region. The GPIIIa (211–222) peptide was used as control peptide in the biological assays. It was found that the peptides GPIIIa (211–222) and GPIIIa (618–630) at a final concentration of 1mM exhibit the highest inhibitory effect on stimulated with ADP human platelet aggregation and binding of FITC–Fg to activated GPIIb/IIIa receptor.

P–43

THE ROLE OF THE GAPL[309–312] SEQUENCE AND E[315] MOIETY OF THE GPIIb SUBUNIT ON PLATELET AGGREGATION

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The platelet membrane glycoprotein GPIIb/IIIa is the most proeminent of integrin adhesion receptors that mediates platelet aggregation by binding fibrinogen. Potential ligand contact sites on integrin GPIIb subunit have been identified by chemical cross–linking approaches, site–directed mutagenesis and peptide studies. The GPIIb 294–314, GPIIb 300–312 and GPIIb 313–332 regions have been proposed to be implicated in ligand binding. The GAPL sequence found on residues 309–312 in GPIIb has also been predicted by the anticomplementary hypothesis to be a fibrinogen binding site on GPIIb subunit. In addition, within the sequences of the proposed binding sites, the variant E^{315} D^{315} has been reported. Taking adventage from these results, we aim at evaluating the contribution of the GAPL sequence in fibrinogen binding as well as the role of the amino acid at the position 315 (E^{315}). To this aim, we synthesized a series of GPIIb 313–324 derived analogues incorporating also the GAPL sequence. From the inhibition studies of human platelets aggregation we conclude that the GAPL sequence does not affect the inhibition potency of the GPIIb 313–320 peptide, whereas substitution of E^{315} by D^{315} enhances the anti–aggregatory activity of the analogue.

P–44

CONTRIBUTION OF EACH AMINO ACID MOIETY WITHIN THE GPIIb/IIIa 313–320 SEQUENCE TO ITS ANTIPLATELET PROPERTIES

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The GPIIb/IIIa receptor, which is a member of the integrin family, is the most abundant receptor in the surface of platelets and can interact with a variety of adhesive proteins including fibrinogen, fibronectin and Von Willebrand factor. Fibrinogen binding on GPIIb/IIIa is an event essential for platelet aggregation and thrombus formation. Mapping of the fibrinogen binding domains on α_{IIb} subunit suggested the sequence 313–332 as a putative binding site. This region was restricted to sequence GPIIb 313–320 (YMESRADR) using synthetic octapeptides overlapping by six residues. The YMESRADR octapeptide inhibits ADP stimulated human platelets aggregation and binds to immobilized fibrinogen. In this study, we used the Ala scanning methodology within the sequence 313–320 aiming to evaluate the contribution of each amino acid to the antiplatelet properties of this region. We found that although deletion of the –YM– sequence in the peptide ESRADRKL resulted in a lower anti–aggregatory activity of the analogue compared to YMESRADR, substitution of either Y or M by A does not affect the activity of the parent octapeptide. The –RADR– motif seems to be the most essential for the biological activity of the GPIIb 313–320 site.

P–45

PEPTIDES DERIVED FROM CYTOPLASMIC REGION OF THE $\alpha_{IIb}\beta_3$ SUBUNITS CAN INHIBIT PLATELET AGGREGATION

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The platelet integrin receptor $\alpha_{IIb}\beta_3$ is composed of a two–chain a subunit bound non–covalently to a single–chain β–subunit. The C–terminal cytoplasmic tail of each subunit consists of 20 amino acid residues in α_{IIb} and 47 residues in β_3. The conformational switch necessary for ligand binding to $\alpha_{IIb}\beta_3$ is regulated by intracellular molecules that bind to the cytoplasmic tails of the integrin or by integrin–associated membrane proteins. In addition in vivo charge swapping mutation studies suggested that α_{IIb} and β_3 tails have a direct site of interaction between α_{IIb}(R995) and β_3(D723) which is related to the affinity state of the receptor. Peptides derived from the cytoplasmic tail sequences can specifically induce or block $\alpha_{IIb}\beta_3$ activation in platelets. The aim of this work is to develop peptide analogues based on the cytoplasmic tail sequences of both α_{IIb} and β_3 subunits that could inhibit platelet thrombus formation by specifically disrupting the inside–out signaling pathway. To this end, suitably modified peptides have been synthesized and tested for their ability to inhibit the activation and expression of $\alpha_{IIb}\beta_3$ and platelet aggregation in vitro. The pathway of their action is now under evaluation.

P–46

ANTI–PLATELET PROPERTIES OF CYCLIC (S,S) –CDC–AND (S,S)–CRC– CONTAINING PEPTIDES

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The final common step in platelet aggregation and thrombus formation is the binding of the plasma protein fibrinogen (Fg) to the receptor GPIIb/IIIa of the activated platelet. It has been shown that two copies of the Arg–Gly–Asp (RGD) sequence (95–97 and 572–574) of the fibrinogen α chain contribute to the recognition and binding event. RGD peptides inhibit platelet aggregation antagonizing fibrinogen binding through their interaction with the receptor. In a previous study we have shown that the cyclic scaffold (S,S –CDC– induces a favorable orientation of the R and D side chains leading to very potent anti–aggregatory agents. In this work we report on the antiplatelet properties of various (S,S)–CDC– and (S,S) –CRC–) containing analogues. The peptides were found to be potent inhibitors of aggregation of stimulated by ADP, Thrombin, TRAP and Collagen human platelets, whereas the IC_{50} values of inhibition of the fibrinogen binding to washed platelets were 10 to 25 times lower than in the preceding case. Contrary to RGDS, some of the reported analogues did not inhibit the binding of the ligand mimetic monoclonal antibody PAC–1 to activated platelets, providing evidence for a different, compared to RGD peptides, mechanism of action.

P–47

THE La/SSB PHOSPHORYLATED ANTIGENIC DETERMINANTS AND THEIR CONTRIBUTION IN THE AUTOIMMUNE RESPONSE OF PATIENTS WITH SS AND SLE

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The autoimmune response in patients with Sjogren's Syndrome (SS) is mainly directed against four discrete B cell epitopes 145–164, 289–308, 349–368 of La/SSB a conserved cellular phosphoprotein of 47KD. Among the four, well defined, phosphorylation sites of La/SSB, two are located within the 349–368 epitope (Thr–362 and Ser–366), one within 301–318 (Thr–302) and the fourth one between them (Ser–325). Over the last years, phosphorylation of proteins has been proposed as the most important post–translational event that may potentiate the autoimmune response by altering the immunogenicity, the conformation and the cell distribution of the phosphorylated proteins, resulting thus in the breakdown of the immune tolerance. With the aim to investigate the potential role of phosphorylation in the conformational characteristics (^{1}HNMR and MD studies) and the autoimmune response of the La/SSB epitopes in SS patients sera (ELISA assays), we have synthesized and studied the phosphorylated epitopes:
(359–368)–P:Ac–GKKT(PO_3H_2)KFAS(PO_3H_2)DD–NH_2,
(346–368)–P:Ac–AQPGSGKGKV–QFQGKKT(PO_3H_2)KFAS(PO_3H_2)DD–NH_2 and
(301–318)–P: Ac–VT(PO_3H_2)WEVLE– GEVEKEALKKI–NH_2, with respect to their non–phosphorylated counterparts. Their cell distribution was also investigated by confocal microscopy.

P–48

DEVELOPMENT OF AN (AVIDIN–BIOTIN) ENZYME IMMUNOASSAY FOR THE BIOACTIVE PEPTIDE AcSDKP

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The tetrapeptide AcSDKP (Seraspenide, Goralatide) is a physiological regulator of hematopoiesis (blocking the transition from G_0/G_1 to S phase in hematopoietic stem cells), which has a protective effect on subjects undergoing anticancer chemotherapy. AcSDKP immunoassays are useful laboratory tools, which may help elucidate the peptide biochemical origin and biological functions as well as facilitate its clinical use. In this work, we describe the development of an (avidin–biotin) immunoassay for determining AcSDKP. In the immunoassay developed, biotinylated AcSDKP is indirectly immobilized onto microwells that have been pre–coated with avidin. The microwells are then incubated with suitably diluted anti–AcSDKP rabbit antiserum in the presence of standard solutions of synthetic AcSDKP (1 ng/ml to 10 μg/ml). In a following step, an enzyme (horseradish peroxidase, HRP) labeled – second antibody (anti–rabbit IgG/HRP) is incubated with the microwells, while 2,2′–azinobis–(3–ethyl–2,3–dihydrobenzothiazole–6–sulfonic acid) diammonium salt (ABTS) is then added, incubated with the microwells, and the optical absorbance (405 nm) is finally measured. AcSDKP was synthesized following the Fmoc solid–phase strategy on an in–house prepared o–Cl–trityl– amidomethyl polystyrene resin . Synthetic AcSDKP was biotinylated following a well established protocol, in which a commercially available long–chain biotin active ester (sulfo–NHS–LC–biotin) was used. Anti–AcSDKP rabbit antisera were developed by immunizing New Zealand white rabbits against a conjugate of synthetic AcSDKP with the carrier protein keyhole limpet hemocyanin, which was prepared according to the glutaraldehyde method. Optimization of the assay characteristics is currently in progress, aiming at the reliable analysis of biological samples.

P–49

COMPARISON OF [^{99m}Tc–N_4^{0-1},ASP^0]TATE AND [^{111}In–DOTA^0]TATE IN VITRO AND IN TUMOR BEARING LEWIS RATS

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Today OctreoScan® is the agent of choice in the scintigraphic detection and staging of sstr$_2$–positive (somatostatin subtype receptor 2) tumors, while other more affine somatostatin analogs, like [^{177}Lu–DOTA^0]Tate (Tate=(D)Phe–c(Cys–Tyr–(D)Trp–Lys–Thr–Cys)–Thr–OH), are used in sstr$_2$–targeted radionuclide therapy. At the same time the availability of a sstr$_2$–affine radiotracer based on ^{99m}Tc is highly desirable, due to the superior nuclear properties and cost effectiveness of this radionuclide. We have recently developed [N_4^{0-1},Asp^0]Tate, a cyclic somatostatin nonapeptide analog functionalized at the N–terminal with an open chain tetraamine chelator for stable binding of ^{99m}Tc. In this study, we compared [^{99m}Tc–N_4^{0-1}, Asp^0]Tate with [^{111}In–DOTA^0]Tate in the same sstr$_2$–positive cell lines and in tumor bearing Lewis rats. Both radiopeptides were obtained after labeling in high yields and high specific activities in a pure form. In *in vitro* receptor autoradiography experiments in rat cortex slices the two conjugates showed a comparably high affinity binding to the sstr$_2$. The radiopeptides internalized efficiently in sstr$_2$–positive cells (CA20948, AR42J) at 37°C in a time and dose dependent manner, while remaining at the extracellular compartment of sstr$_2$–negative cells. After injection in male Lewis rats bearing CA20948 tumors, both [^{99m}Tc–N_4^{0-1},Asp^0]Tate and [^{111}In–DOTA^0]Tate showed a high and receptor–specific uptake in the sstr$_2$–positive tissues (pancreas, adrenals, pituitary) and in the receptor–positive rat pancreatic CA20948 tumors, while a rapid body clearance via the kidneys into the urine was found. These favorable characteristics illustrate the suitability of [^{99m}Tc–N_4^{0-1}, Asp^0]Tate and [^{111}In–DOTA^0]Tate in the targeted diagnosis and staging of sstr$_2$–positive tumors in man employing either ^{99m}Tc or ^{111}In radionuclides.

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FIRST EXPERIENCE WITH [^{94m}Tc–N_4^0]TATE AS A PET TRACER IN THE DETECTION & STAGING OF SSTR2–POSITIVE TUMORS

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While ^{99m}Tc remains the ≤gold standard≤ of diagnostic nuclear medicine using SPET methodology (single photon emission tomography), ^{94m}Tc constitutes an alternative radionuclide for PET applications (positron emission tomography). ^{94m}Tc was produced on a biomedical cyclotron via a ^{94}Mo(p,n)^{94m}Tc nuclear reaction. ^{94m}Tc is a positron emitter (72% β^+ E_{max}= 2.47 MeV) with a $t_{1/2}$= 52.5 min and two accompanying photons (28% EC 0.871 (94), 1.868 (5.7) MeV). The main radionuclidic impurity at EOB (approximately 7%) is ^{94g}Tc ($t_{1/2}$= 4.88 h,11%β^+ E_{max}= 0.82 MeV, 89%EC 0.703 (100) and 0.871 (100) MeV). The high quantitative ability of PET makes ^{94m}Tc an attractive radionuclide, particularly in clinical oncology when an estimate of the number of receptors is needed for therapy planning. During our recent work, we have developed a series of cyclic somatostatin octapeptide analogs functionalized at the N–terminus with open chain tetraamines for stable binding of ^{99m}Tc. [^{99m}Tc–N_4^0]Tate in particular, has proven a very promising radiotracer in the scintigraphic detection of sstr$_2$–positive tumors (somatostatin subtype receptor 2), both in animal models and in patients. In this study, we present our initial experience with [^{94m}Tc–N_4^0]Tate as acquired during cell and animal model experiments. The radiopeptide internalized efficiently and specifically in cells induced to be sstr$_2$ positive with an adenoviral vector. Athymic nude mice were implanted with two axillary tumors followed by direct intratumoral injection of the adenovirus encoding for sstr$_2$ or a control adenovirus. Two days after infection, the mice were injected i.v. with [^{94m}Tc–N_4^0]Tate and imaged using a microPET scanner. The [^{94m}Tc–N_4^0]Tate selectively localized in the sstr$_2$+ tumor while showing a favorable *in vivo* clearance profile. This promising data has prompted further studies on [^{94m}Tc–N_4^0]Tate and other peptides as candidates in the targeted tumor diagnosis in combination with PET.

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COMPARATIVE PRECLINICAL EVALUATION OF [^{99m}Tc]DEMOBESINS 3–6 IN THE TARGETED IMAGING OF GRP–R–POSITIVE NEOPLASMS

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The aim of this study is the comparable preclinical evaluation of a series of new bombesin (BB) analogs labeled with ^{99m}Tc as candidates in the targeted *in vivo* imaging of GRP–R–positive neoplasms in combination with SPET (single photon emission tomography). Synthesis of the new analogs was performed on the solid support after coupling the respective Boc–protected tetraamine precursor to the N–terminal of the corresponding peptide chain. After deprotection and purification the following conjugates were isolated: Demobesin 1 (N_4^0–Pro^1,Tyr^4]BB), Demobesin 2 (N_4^0–Pro^1,Tyr^4,Nle^{14}]BB), Demobesin 3 ([$N_4'^0$]BB(7–14)) and Demobesin 4 ([$N_4'^0$,Nle^{14}]BB(7–14)). Labeling of Demobesins 3–6 with ^{99m}Tc was completed under mild conditions, leading nearly quantitatively to the respective radiopeptides ([^{99m}Tc]Demobesins 3–6) in high specific activities. The new analogs exhibited a high affinity binding to the GRP–R in human prostate cancer cell (PC–3) membranes with relevant IC_{50} and K_d values in the lower nM – sub–nM range. Furthermore, in *in vitro* receptor autoradiography experiments in human cancer samples Demobesins 3–6 showed a high affinity binding to the GRP–R, a lower affinity to the NMB–R and no affinity to the BB_3–R. The radiopeptides internalized efficiently in living PC–3 cells at 37°C. After injection in athymic mice bearing human PC–3 xenografts, [^{99m}Tc]Demobesins 3–6 showed a high and specific uptake in GRP–R–positive tissues as well as in the experimental tumors. The [^{99m}Tc]Demobesin 3 και 4, in particular, were rapidly excreted over the kidneys into the urine showing a low portion of hepatobiliary excretion. These favorable characteristics reveal the potential usefulness of [^{99m}Tc]Demobesins 3 and 4 in the targeted diagnosis and staging of GRP–R–positive neoplasms in man.

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CELL & ANIMAL MODEL SELECTION IN THE EVALUATION OF CLINICALLY RELEVANT BOMBESIN–BASED RADIOPEPTIDES: PITFALLS – CONSIDERATIONS

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Currently three mammalian members of the bombesin receptor family have been identified, the NMB–, GRP– and the BB_3–R that are structurally related and belong to the superfamily of the G–protein coupled receptors. Of particular clinical relevance is the GRP–R due to its expression in high incidence and density in many frequently occuring human tumors, like prostate and breast cancer. Several recent studies propose the use of radiolabeled BB analogs in the GRP–R–targeted imaging and radionuclide therapy of cancer. However, as GRP–R interspecies homology is not always preserved, only rational selection of GRP–R–positive cell and animal models can justify extrapolation of laboratory data into clinically meaningful information. In this study, we compared two BB analogs [^{111}In]–Z070 ([^{111}In–DOTA$^{0-1}$,PEG$_4^0$,(D)Tyr^6,(β)Ala^{11},Thi^{13},Nle^{14}]BB(6–14)) and [^{99m}Tc]Demobesin 1 ([^{99m}Tc–N_4^{0-1},bzlg^0,(D)Phe^6,Leu–NHEt^{13}, *des*–Met^{14}]BB(6–14)) in cell lines of man (human prostate adenocarcinoma PC–3) and rat (rat acinar pancreatic carcinoma AR42J) origin. While binding affinities of tested peptides were found comparably high in the AR42J membranes, Demobesin 1 showed a >20fold higher binding affinity in the human PC–3 membranes as compared to ([^{111}In])–Z070. Furthermore, after coinjection of both radiopeptides in athymic mice bearing a double tumor model including a rat AR42J (left flank) and a human PC–3 (right flank) xenograft, [^{99m}Tc]Demobesin 1 showed a higher uptake in the PC–3 tumor, whereas [^{111}In]–Z070 showed a higher uptake in the AR42J tumor. The above results are suggestive of interspieces differences in the homology of the GRP–R between rat and man. Therefore, the use of the rat AR42J cell line / tumor model in the evaluation of radiolabeled BB analogs may lead to erroneous conclusions in respect to their clinical usefulness in patients with GRP–R–positive tumors.

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PLASMIN GENERATES BIOLOGICALLY ACTIVE PEPTIDES OF THE GROWTH FACTOR HARP

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Heparin–affin regulatory peptide (HARP) is a growth factor with an approximate molecular weight of 18kDa. The secondary structure of the molecule contains two termini that are lysine–rich and two central domains forming β–sheets (N– and C–domain). In previous studies, our team has shown that HARP stimulates angiogenesis both *in vitro* and *in vivo*. Furthermore, we have shown that plasmin cleaves HARP *in vitro*, generating five peptides. These peptides correspond to different parts of the HARP molecule and contain either one or both central domains. The purpose of this study was to clarify whether and how the plasmin–generated HARP peptides affect angiogenesis. Initially, we studied their effect on angiogenic processes of human umbilical vein endothelial cells (HUVEC). All five HARP peptides stimulate HUVEC adhesion. The peptides that contain either the N– or the C–domain induce the migration of HUVEC, as well as their differentiation and tube formation on matrigel. On the contrary, the peptides that contain both domains of the molecule have an inhibitory effect on both migration and differentiation of HUVEC. When we studied their effect on angiogenesis *in vivo,* using the chicken embryo chorioallantoic membrane (CAM) assay, we found that all plasmin–derived HARP peptides increased the number of vessels. Further studies are in progress in order to clarify the physiological significance of these data.

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DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL AND POLYCLONAL ANTIBODIES AGAINST ESTROGEN RECEPTORS α AND β

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Accurate quantification of estrogen receptor alpha (ERα) and beta (ERβ) protein levels is important for breast cancer prognosis and treatment. To facilitate the assessment of ERs proteins levels, we have developed site–directed monoclonal and polyclonal antibodies against ERα and ERβ. Sites of high antigenicity and low evolutionary conservation between human and mouse were selected in the N–terminal domain of ERα and the N– and C–terminal domains of ERβ, with the C–terminal ERβ domains selected as to differentiate between ERβ and its non–hormone–binding isoform β2. Mouse monoclonal antibodies (mAbs) were raised against synthetic peptides coupled to KLH, whereas polyclonal antibodies (pAbs) were raised in rabbits against GST–chimeras coding for relatively larger portions encompassing the antigenic ERα and ERβ peptides. The specificity and sensitivity of anti–ERα and ERβ mAbs and pAbs were assessed by Western blotting using pure ER proteins and extracts of cells transfected with the respective ER c–DNAs or expressing the endogenous ERs. Sandwich ELISAs for research and/or clinical applications were developed and immunohistochemical assays are being set up using these antibodies. The sensitivity and specificity of anti–ERα and ERβ mAbs and pAbs will likely allow for a better understanding of ER physiology and pharmacology in normal and cancer cells as well as for the development of new diagnostic tools for breast cancer prognosis and treatment.

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LARGE–SCALE EXPRESSION AND PURIFICATON OF N–ACTIVE SITE OF HUMAN ANGIOTENSIN CONVERTING ENZYME (ACE) IN *ESCHERICHIA COLI*

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Angiotensin–I Converting Enzyme (ACE) is a member of the gluzincin family zinc metalloproteinases that contains two highly homologous catalytic domains at the N– and C–terminal. Both domains catalyze Ang II formation and bradykinin degradation. ACE has a critical role in cardiovascular function by cleaving the carboxy terminal His–Leu dipeptide from angiotensin I to produce the potent vasopressor octapeptide, angiotensin II. There are two isoforms of ACE that are transcribed from the same gene in a tissue–specific manner. In somatic tissues it exists as a glycoprotein composed of a single, large polypeptide chain of 1,277 amino acids containing both catalytic domains, whereas in sperm cells it is a lower–molecular–mass glycoform of 701 amino acids containing only the C– catalytic domain. Here we report, for the first time, the overexpression in bacteria, and purification of an ^{15}N and ^{13}C labeled 108 aa peptide which corresponts to an extended domain of the human somatic N–terminal active site of ACE (Ala361–Gly468). The DNA sequence, encoding the Ala361–Gly468 protein fragment, was subcloned into the pET–3a expression vector at NdeI/BamHI restriction sites. The resulting plasmid used to tranform competent ER2566 cells. The tranformed cells used to inoculate M9 minimal medium, and the induction of the expression of recombinant protein fragment occured using 0,5mM of IPTG. The protein isolated from the inclusion bodies by chromatography technics. The recombinant protein fragment has a molecular weight, mesured by ESI MS, of 12419 kDa which is in consistence with the therotical calculation based on the DNA sequence. The resulting peptide has the potentiality of suggesting possible coordination models of zing ion, in the native enzyme by studying its structure features in solution through NMR spectroscopy.

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IN VIVO EXPRESSION OF A PEPTIDE ENCODING THE THIRD INTRACELLULAR LOOP OF THE δ OPIOID RECEPTOR IN INTACT CELLS ALTERS ERK ACTIVATION MEDIATED BY THE OPIOID RECEPTORS

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The activation of the family of mitogen–activated protein kinases (MAPK), also known as extracellular signal regulated protein kinases (ERK), has been classically associated with growth factor receptors containing intrinsic receptor tyrosine kinase activity. Recently, many G protein coupled receptors (GPCRs), including all three opioid receptors, have been demonstrated to activate the MAPK signaling cascade. MAPK activation by GPCRs may allow for plasma membrane receptor systems to influence diverse cellular processes, ranging from the regulation of neuronal survival to cell differentiation and gene expression. Opioid receptors belong to the large superfamily of G protein coupled receptors (GPCRs) and modulate a variety of physiological responses in the nervous system via coupling to multiple patterns of G proteins. In order to design and develop analogues that could potentially block or activate selectively in vivo the interactions of these receptors with G proteins or other downstream signaling components we constructed a minigene encoding the third intracellular loop of the murine δ–opioid receptor (pDORi3). "Minigene" plasmid vectors are constructs, designed to express relatively short polypeptide sequences following by their transfection into mammalian cells. The presence of pDORi3 in HEK293 cells stably expressing μ and δ opioid receptors resulted in a significant inhibition in ERK activation by DAMGO and DSLET respectively. Moreover functional assays have indicated that the presence of pDORi3 in the activated receptor results in inhibition of PLC and adenylyl cyclase activities. Supplementary studies of DAMGO stimulated [^{35}S]GTPγS binding indicated that the presence of pDORi3 also interferes in G protein activation. These observations underlie the significance of the third intracellular loop for signal transfer of opioid receptors. The specificity by which the third intracellular loop peptide interferes in opioid receptor mediated signaling suggests the feasibility of developing drugs that might exert an inhibitory or stimulatory effect at the level of receptor–G protein interface rather than at the level of ligand–receptor binding.

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ASSESSMENT OF GROWTH–PROMOTING ACTIVITY AND THERMOSTABILITY OF HUMAN RECOMBINANT INTERLEUKIN –1β(rhIL–1β) PRODUCED FROM *E. COLI* CELLS PRIOR TO FORMULATION

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Human interleukins (IL) are major class of cytokines that regulates several hematopoietic and lympoid cellular functions. We have already cloned, expressed and purified several human cytokines including IL–1β from geneticall engineered E.Coli TB–1 cells using the pMAL C2 fusion vector purification system. In this study, we evaluated the growth promoting activity of rhIl–1β in cultures of human leukemia K562 cells and assessed its thermostability in an effort to optimize conditions for formulation. Cells were incubated with varying concentrations of rhIL–1β and pulsed labelled witη 3H–thymidine for 2hrs. The incorporation of radiolabelled thymidine into DNA of K562 cells was measured. Moreover, rhIl–1β was assessed for thermostability at various times (from one week to six months) under different pH conditions (pH 4.0, 6.3, 7.4 and 8.0) and temperatures (4°, 22°, 37°, 44° and 65°) . The physical integrity was asssessed by SDS–PAGE electrophoresis. Our results have shown the following:(α) rhIL–1β increased DNA synthesis by six–fold over control untreated cells at concntrations as low as 0.01 μg/ml. The growth promoting activity was dose–dependent and reached its maxium value at 0.1μg/ml. Preincubation of rhIL–1β at increasing temperatures caused an inversely proportional loss of biological activity. At temperatures > than 44° rhIL–1β was decayed under acidic (pH 4.0) but not under neutral and slightly alkaline conditions. Incubation of rhIL–1β for 3 to 6 months at temperatures equal or lower than 22° did not lead to substantial loss of physical and biological activity. These data incidate that we have produced biologically potent rhIL–1β that is maintained quite stable upon incubation at relatively low temperatures for long time in aqueous buffered soultion. However, rhIL–1β exhibited thermosusceptibility at temparatures higher than 22°. These results indicate optimal formulation conditions.

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A NEW STRATEGY FOR ANTI–CANCER VACCINE DESIGN: COMBINED APPLICATION OF CD8 AND CD4 EPITOPES OF MAGE–3 TUMOR ANTIGEN

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Anti–MAGE–3 cytotoxic responses have been detected in many tumor patients and MAGE–3 CD8 epitopes, as well as CD4 epitopes have been identified but these responses are not accompanied by tumor regression. In order to improve cancer vaccine strategy, we propose the design and preparation of constructs that will encompass both CD8 and CD4 epitopes from tumor antigen MAGE–3 and test them for their potency to elicit anti–tumor responses. For this reason a synthetic carrier SOC_4 (Sequential Oligopeptide Carrier) was synthesized on a Wang resin by the Fmoc methodology. Lysine has been introduced as Fmoc–Lys(Alloc)–OH in positions 1 and 7, and as Fmoc–Lys(Mtt)–OH in positions 4 and 10. This orthogonal protection allows the synthesis of two different epitopes on the Lys–$N^{\varepsilon}H_2$ groups. After removal of the Mtt group (1.8% TFA in DCM), the CD8+ epitope (168–176) was synthesized on the Lys–$N^{\varepsilon}H_2$ groups by the Fmoc strategy. The synthesis of the second epitope CD4 (281–295) was performed by the Fmoc methodology after the catalytic removal of Alloc group by $Pd(PPh_3)_4$. Cleavage of the SOC_4–constructs from the resin was realized by TFA. Purification was performed by HPLC (analytical, preparative) and the final product was identified ESI–MS. Construct will be further subjected to in vitro testing for evaluation of its ability to stimulate T cells (from Tumor Infiltrating Lymphocytes and from PBMCs) of melanoma patients using autologous dendritic cells pulsed with the peptide as APCs and its ability to generate T–cell immune responses against autologous tumor cells (lysis of tumor cells, cytokine production in response to tumor cells).

SERPIN INSERTION PEPTIDES INHIBIT HIV–1 PROTEASE

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Peptides derived from the termini of HIV Protease (PR) show inhibition of PR and act as "dimerization inhibitors" targeting the interface. The segments of one monomer fit dove–tailed into an open pair of parallel N– and C–terminal segments of a second PR monomer, forming a *stable anti–parallel β–sheet*. A similar case is the insertion of segments into the parallel β–sheet of serpins after activation cleavage. Serpin insertion peptides were therefore tested whether they inhibit PR, using a chromogenic substrate as described [1].

Ac–TEASAATAVKITLL–OH	Antichymotrypsin	38 μM	IC50
Ac–SEAAASTAVVIAGR–OH	Antithrombin	38 μM	IC50
Ac–FLEAIP–Nle–E–OH	α1–Antitrypsin	63 μM	IC50
Ac–AMFLEAIP–Nle–E–OH	α1–Antitrypsin	25 μM	IC50
Ac–AAGAMFLEAIP–Nle–E–OH	α1–Antitrypsin	30 μM	IC50
Ac–TEAAGAMFLEAIP–Nle–E–OH	α1–Antitrypsin	27 μM	IC50
Palmitoyl–Tyr–Glu–Thyronine–OH	(reference, dimerization inhibitor [1]	0.005 μM	(Ki–dim)

While some anti–PR peptides interfere also with β–sheet proteins (β–secretase, Alzheimer Aβ aggregates), serpin insertion peptides (courtesy A.J. Schulze) also inhibit PR. This may explain why α1–AT inactivates PR [2]. Such interactions may be ~1000x stronger if amino acids mutate to 'anchor residues'. Can mutated or PR cleaved endogenous proteins explain AIDS long–time survivers (dimerization inhibition)? [1] Schramm, H.J. *et al.*, Biol. Chem., 380 (1999) 593; [2] Cordelier, P. and Strayers, D.S., B.B.A. 1638 (2003) 197.

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