No 67

Studies in the Synthesis of Peptide Thioacids and Thioesters

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Peptide C-terminal thioacids and thioesters are key intermediates in a variety of applications, most notably the recently developed native chemical ligation methods for the total synthesis of proteins. So far, they have been prepared mainly by the use of the least prevalent Boc/Bzl solid-phase method, owing to the lability of the thioester bond to strong nucleophiles, such a piperidine, normally used in Fmoc solid-phase method.

Our present research efforts have been focused on the Fmoc-based preparation of peptide thioacids and thioesters (Fig.1) by using trityl-type resins and the non-nucleophilic base 1,7-diazabicyclo [5,4,0] undec-7-ene (DBU). The synthesis route we studied (Fig.1) involves the transformation of Trt-aminoacids to the corresponding thioacids and subsequent attachment on the 2-chlorotrityl, trityl and 4-methoxy-trityl resins. The stability of the thioester bond was studied during the peptide chain elongation either by the stepwise or the fragment condensation method.

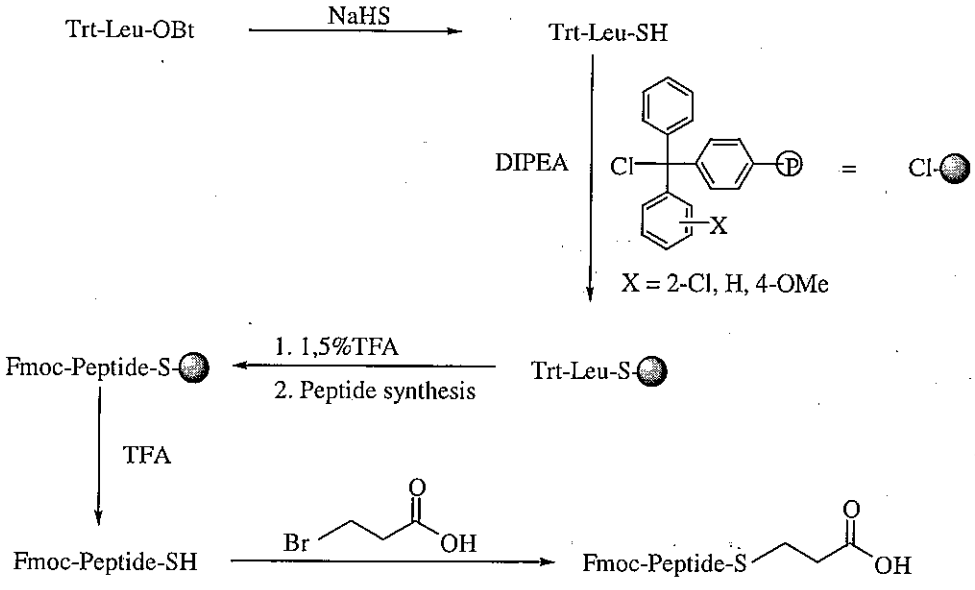

Figure 1

No 68

Solid Phase Synthesis of Cyclic and Linear Peptides Containing Thioether Bonds

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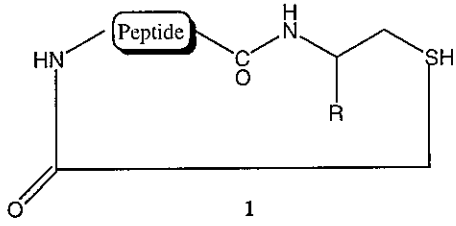
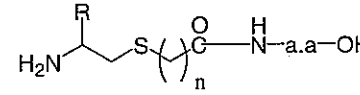
Cyclic and linear peptides containing thioether bonds were synthesized by two methods. In the first method, N-trityl-aminothiols, derived from naturally occurring amino acids were attached

onto trityl-type resins through their thiol function. Then, peptide chains were assembled containing on their N-terminus a haloacid. The cyclic peptides 1 were then obtained by cleaving the haloacylpeptides from the resin and treating them under basic conditions. Alternatively, peptides containing haloacids on their *N*-terminus were solid phase synthesized and transformed to the linear peptides containing thioether bonds 2, using *N*-trityl-aminothiols.



1



2

No 69

Effect of Retinoic Acid and its Analogues on Tumor Growth and Angiogenesis

E. Kliafa[1], P. Katsoris[2], E. Arsenou[3], S. Nikolaropoulos[3] and E. Papadimitriou[1]

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Retinoic acid (RA) can be regarded as a pharmacological agent that is commonly used for its ability to affect growth and differentiation of a variety of cell types, such as acute promyelocytic leukemic and endothelial cells. In the present work we studied the effect of all-trans RA (ATRA) and its analogues EA136, EA137 and EA4 on the growth of the human promyelocytic HL-60 cells *in vitro*. ATRA decreased the number of HL60 cells. This decrease was mostly evident 96 h after addition of ATRA in the cell culture medium and was significant at concentrations higher than 10^{-5} M. All the analogues tested also decreased the number of HL60 cells with an EC_{50} approximately one order of magnitude lower than that of ATRA. The decreased in the number of cells is probably due to cell death, since ATRA also induced apoptosis of HL60 cells and seemed to decrease the expression of the growth factor HARP (Heparin Affin Regulatory Peptide) in both mRNA and protein levels. Since angiogenesis is important for the growth of hematological malignancies, we furthermore studied the effect of ATRA and its analogues on the formation of new capillaries, in the *in vivo* chicken embryo chorioallantoic membrane (CAM). ATRA and all the tested analogues induced angiogenesis in the CAM and increased the layer of CAM epithelial cells. All analogues were more effective than ATRA. Finally, ATRA also increased the amounts of HARP protein in the CAM. Further studies are in progress in order to elucidate the exact role of ATRA and its analogues in both tumor growth and angiogenesis.

No 70

Design and Synthesis of TRH Analogues

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Thyrotropin Releasing Hormone (TRH) is secreted in pulses from the hypothalamus and it stimulates the pituitary to effect the secretion of the Thyroid Stimylating Hotmone (TSH). The last stimulates the secretion of Thyroxine (T_4) and Triidothyroxine (T_3) by the thyroid gland. Both T_4 and T_3 have a feedback effect on the hypothalamus and the pituitary that reduces TRH and TSH secretion.

TRH is a peptide hormone consisted by three amino acids. Both the N and C terminals of TRH are not *free*, that is, they exist neither as a free

acid nor as a base. The structure of TRH is L-pGlu-L-His-L-Pro-NH_2.

The goal of this research is the synthesis of TRH analogues, with prolonged activity, for possible application in cases of malignant tumours of the thyroid gland. For the achievement of this purpose, a number of TRH analogues was synthesized by substituting the amino acids Histidine and Proline with the non-natural amino acids as nipecotic [Nip], isonipecotic [Inp], pipecolic [Pip], 2-amino-iso-butyric [Aib] and 1,2,3,4-tetrahydro-D-isoquinolic-3-carboxylic [Tic] acids, and in order to obtain: (a) a high-affinity binding between the TRH-receptor and the hormone and (b) the protection of the later against enzymatic degradation. Pyroglutamic acid (pGlu) of TRH structurally keeps its position in the hormone, as the most necessary TRH section for the binding with the TRH-receptor. These TRH analogues were synthesized by solid phase peptide synthesis *via* the Fmoc/tBu methodology. As solid substrate was used the 2-chloro-triphenyl-methyl resin with a Rink Bernatowitz linker. For the purification of the synthesized analogues, at first it was used the gel filtration chromatography method (Sephadex G-10 and 15% solution of AcOH); in a second step, the synthesized analogues of TRH were analyzed by HPLC and gave the expected results on ES-MS.

The above–mentioned analogues of TRH are undergoing biological tests (in collaboration with the Department of Medicine of the University of Patras), in order to confirm the modification results towards prolonged activity and TSH secretion.

No 71

Conformational and Biophysical Studies of AT_1 Non-Peptide Antagonists

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Our laboratory has studied the conformational properties of various AT_1 antagonists present in the market and synthesized by Prof. J. Matsoukas group.

The results showed that best activity for AT_1 antagonism is achieved if the molecule: (a) forms an aromatic cluster with preferably three aromatic rings involved; (b) the biphenyl ring deviates significantly from the linearity; (c) the alkyl chain is restricted in flexibility due to its interaction with the biphenyl ring.

Further studies are planned to explore in more details the stereoelectronic parameters, which determine the AT_1 bioactivity.

No 72

Epinephrine-Induced Survival and Differentiation of pc12-α_2 Cells is Mediated by PhosphatidylInositol-3 Kinase (PI-3K)

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The three known subtypes of α_2-adrenergic receptor are heptahelical transmembrane receptors coupled to G-proteins (GPCRs) and mediate part of the physiological actions of the hormones-neurotransmitters epinephrine & nor-epinephrine in many tissues including vascular smooth muscle cells, kidneys and the Central Nervous System. PC12 cells are a cell line derived from rat pheochromocytoma, which differentiates to sympathetic neurons in presence of NGF (Nerve Growth Factor), and, for this reason, it constitutes a model system for the study of neuronal differentiation in vitro. Recent studies in our laboratory have shown that PC12 cells expressing, following

transfection, subtypes of α_2-adrenergic receptor (PC12-α_2) differentiate into a neuronal phenotype after exposure to epinephrine through a MAPK-dependent pathway. In the present study we investigated the role of Phosphatidylinositol-3 Kinase (PI-3K) and of the effector molecule Akt/PKB in the epinephrine-mediated survival and neuronal differentiation of PC12-α_2 cells. We have assessed apoptosis by the employment of a DNA fragmentation assay and Western blotting analysis with antibodies against phosphorylated and non-phosphorylated Akt and against the neuronal differentiation marker peripherin in non-differentiated PC12-α_2 cells and epinephrine-differentiated cells in the presence or absence of the inhibitor LY294002. We have found that epinephrine activates Akt/PKB in PC12-α_2 cells and induces peripherin expression during differentiation of these cells, as well as their survival. The expression of peripherin and the survival of PC12 cells are PI3K-dependent, since they are inhibited by LY294002. The present study indicates that epinephrine promotes survival and differentiation of PC12 cells via its α_2 receptors, and these actions are mediated by PI-3K. The results imply that epinephrine might act as a neurotrophic factor in vivo, alone or in combination with other agents.

No 73

Structural Elucidation, Conformational Properties and Interactions of the Toxin Paralysin, β-Ala-Tyr from Larvae of the Gray Flesh Fly with Phospholipid Bilayers Using MAS ^{13}C-NMR Spectroscopy

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Larval extracts of the homometabolous insects (i.e.Neobelleria Bullata-Insecta Diptera) cause immediate paralysis, followed by death, when injected into adult flesh flies. These extracts contain endogenous toxins (which are widely spread over the class of insects), called paralysins, and are present throughout all the development stages, with their concentration gradually increasing from larvae stage over pupation to late pharate adults. Two paralysins soluble in organic solvents and heat stable, were chromatographically purified to homogeneity by Shean-Jaw Chiou et al and they were identified by use of mass spectrometry and nuclear magnetic resonance respectively as β-alanine-tyrosine (β-Ala-Tyr) and as 3-hydroxy-kynurenine (3-HK).

This study aims to explore the stereo-electronic properties responsible for the activity of β-Ala-Tyr, due to its pharmacological importance. Its structure was elucidated using 1D and 2D NMR techniques and the conformational properties were studied using a combination of 2D NOESY spectroscopy and molecular modeling. In addition its dynamic properties in phospholipid bilayers were studied using high-resolution ^{13}C-MAS and DSC technique, in an attempt to investigate its physicochemical interactions with membrane bilayers.

No 74

Synthetic Analogue Approach to Bleomycins' Antitumor Antibiotics: Synthesis and Characterization of the Gallium(III) Complex of a Bleomycin Analogue

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Bleomycins (BLMs) are a family of glycopeptide antitumor antibiotics and are clinically used against several types of cancer. Therapeutic action of BLM takes place when it forms a metal

complex with ferrous ion which activates molecular oxygen, thereby inducing the degradation of DNA. This BLM metal complex is also known to oxidize simple organic molecules like olefins. Since gallium(III) resembles iron(III) in certain respects (e.g., ionic radii: 62 and 65 ppm respectively) and since gallium salts have antitumor activity, the Ga^{III}-BLM complex could also have similar activity. The reaction of the peptide ligand H_2L, N-(2-(4-imidazolyl)ethyl)pyridine-2-carboxamide, which resembles part of the metal-chelating section of bleomycins, with $Ga(acac)_3$ in acetone has afforded the tetranuclear complex $Ga_4(acac)_4L_4 \cdot 3H_2O$. The structure and characterization of this synthetic Ga^{III}-BLM analogue are reported and the complex will be tested for its activity in tumor cells.

No 75

α_{2A} Adrenergic Receptors(AR) Activate MAPK in Renal Proximal Tubule Cells

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α_2 ARs are an heterogenous class of receptors comprising 3 subtypes (α_{2A}, α_{2B}, α_{2C}) encoded by distinct genes and having different pharmacological properties, chromosomal localization, tissue distribution and possibly signal transduction properties. All 3 subtypes have been found to be expressed in the human and rat kidney, but their precise expression and their specific physiological role in renal function remains unclear.

The α_{2B} subtype specifically, which is expressed principally in the proximal tubular cells, has been demonstrated to be of great importance for the mediation of the regulatory actions of catecholamines on gleomerular filtration and Na^+ and water excretion and renin release.

Stimulation of α_2 ARs in the proximal tubule increases Na^+/HCO_3^- reabsorption by activating the Na^+/H^+ exchanger type 3 isoform, which is expressed in the brush border membrane of the tubular cells. Furthermore, reabsorption of di/tripeptides in the proximal tubule is controlled by two systems of transport PEPT-1 and PEPT-2.

Recent studies in some epithelial cellular systems expressing these transporters have shown that stimulation of α_2 ARs activates transepithelial transport of antibiotics by PEPT-1. The purpose of the present research project is to investigate the mechanism by which α_2 ARs activates these transport systems. To this end, we have been using LLC-PK1 cells permanently cotransfected with the α_{2B} human AR and the transporter PEPT-1 or PEPT-2. Ligand binding experiments have shown substantial level of expression (1pmol/mg of protein). As a first step, we have been investigating the activation of MAPK by α_2ARs. Stimulation of these cells by the α_2-specific agonist UK 14304 caused an increased ERK1/2 phosphorylation, which correlated with enhanced MAPK activity and nuclear translocation. MAPK activation was inhibited by pertussis toxin and partially by pretreatment of the cells with the PI3 kinase inhibitor LY 294002. Additional studies will be necessary to clarify the role of MAPK stimulation in the activation of the transporters PEPT-1 and PEPT-2, given the pharmacological importance of these two molecules as they are responsible for the reabsorption of a series of drugs, including β-lactam antibiotics and iostatic molecules like AZT.

No 76

Selective Clean-up Applicable to Lemon Acetonitrile Extracts for the Determination of Carbendazim Thiabendazole and O-phenylphenol by HPLC with UV Detection

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Nowadays, the use of agrochemicals is considered very important for the safe growth of almost all cultivated plants. Their application is crucial for the rise of agricultural production. However, pes-

ticide residues contaminate human beings, water, animals and soil. FAO (Food and Agriculture Organization), WHO (World Health Organization), the Environmental Protection Agency (EPA) of the United States and the European Community have introduced Maximum Residue Limits (MRL's) in order to protect the environment and the people from pesticides harmful effects.

The two Benzimidazoles, Carbendazim (MBC) and Thiabendazole (TBZ) and the phenolic compound o-Phenylphenol (OPP) are important systemic fungicides. They are used pre- or postharvestly for the protection of several crops, such as: grapes, lettuce, citrus fruits, bananas, tomatoes, cereals and many more.

Last year, in our laboratory, a very simple and rapid method was developed for the determination of these three fungicides in lemons. In the present work, an effort to improve this method has been made. Lemon homogenates were extracted more efficiently in acidified acetonitrile (CH_3CN/TFA 99,5:0,5 v/v). The extracts were successfully purified and pre – concentrated by a Solid Phase Extraction (SPE) procedure using Waters Oasis HLB cartridges. In addition, the three fungicides were separated on a Supelcosil LC-18 column, eluted with a mixture of acetonitrile/water/ammonium hydroxide (30% solution) (39:60,5:0,5 v/v/v) and detected using a UV-detector at 254nm.

No 77

Synthetic RGD Peptides Incorporating Salicylic Acid Derivatives and their Antiplatelet Activity *in vitro*

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RGD (Arg-Gly-Asp) sequence is a structural element of proteins belonging to the extracellular matrix like fibrinogen, fibronectin, osteopontin, vitronectin, etc. Extensive research studies have revealed the property of RGD containing peptides to inhibit platelet aggregation and thrombus formation by interferring with fibrinogen-platelet receptor GP IIb/IIIa.

We have previously reported that combination in the same molecule of dipeptide amides, containing amino acid(s) of RGD sequence with salicylic or acetylsalicylic acid moiety, at their N-terminal amino group, have shown inhibitory activity on human platelet aggregation.

In view of these results we have synthesized a series of RGD analogs incorporating salicylic acid derivatives by conventional solution techniques and/or by solid phase.

The synthesized compounds were purified by RP-HPLC [Nucleosil C18RP], lyophilised to give fluffy solids and identified by FT-IR, ^{1}H NMR and ES-MS spectra and tested for inhibitory activity on human platelet aggregation *in vitro*, by adding common aggregation reagents (collagen, ADP, thrombin) to citrated platelet rich plasma (PRP). The aggregation was determined using a dual channel electronic aggregometer by recording the increase of light transmission. The IC_{50} values of the synthesized and tested compounds will be discussed in details.

(Grant 2468 from Research Committee of the University of Patras)

No 78

Does Nitric Oxide Lies Upstream or Downstream of HARP? NO Alters HARP Presence and Biological Effects

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Heparin Affin Regulatory Peptide (HARP) is a 18-kDa heparin-binding polypeptide that together with midkine constitute a two-member family of heparin-binding growth factors. HARP is highly

expressed in developing tissues and in several primary human tumors and seems to play a key role in cellular growth and differentiation. Nitric Oxide (NO) is an important bioactive agent and signaling molecule that mediates a variety of physiological actions and contributes to the pathogenesis of a variety of disorders, including cancer. NO is endogenously produced by a family of enzymes known as NO synthases (NOS). In the present work, we studied the effects of HARP on the production of NO by two different types of tumor cells, C6 rat glioma cells and GH3 rat adenocarcinoma cells. Additionally, we investigated whether NO plays a role in the expression of HARP by the cell types mentioned above. HARP did not affect the production of NO by C6 cells. However, inhibition of NOS increased the expression of HARP at both mRNA and protein levels and decreased C6 cell proliferation. Exogenous HARP also decreased the proliferation of C6 cells. In contrast, HARP increased NO production by GH3 cells, whereas it had no effect on cell proliferation. Inhibition of NOS increased the amounts of HARP protein in the extracellular space of GH3 cells. Further work is in progress in order to elucidate the exact pathway that links NO and HARP, as well as the exact role that HARP plays in the functions of the two tumor cell types.

No 79

Attachment and Cleavage of Monosaccharides onto Resins of the Trityl- and Benzhydryl-type

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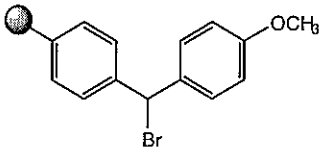
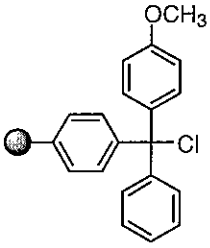
Sugar-based combinatorial libraries are very important for the development of new drugs. Therefore resins, suitable for the attachment of sugars and the cleavage thereof of the solid-phase synthesized sugar derivatives are of interest. In our manuscript we describe the conditions for the loading of the trityl- and benzhydryl-type [1a, 1b, 1c] resins by sugar derivatives. Methods for the determination of the obtained loading and conditions of cleavage from the resins under the mildest possible conditions were studied.



1a



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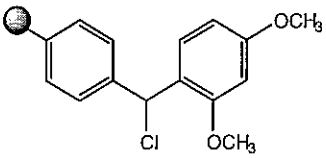

2a

No 80

Total Synthesis of Medicinally Interesting Linear, Branched & Conformationally Restricted Polyamines Using Amino Acids as N-C_n Synthons

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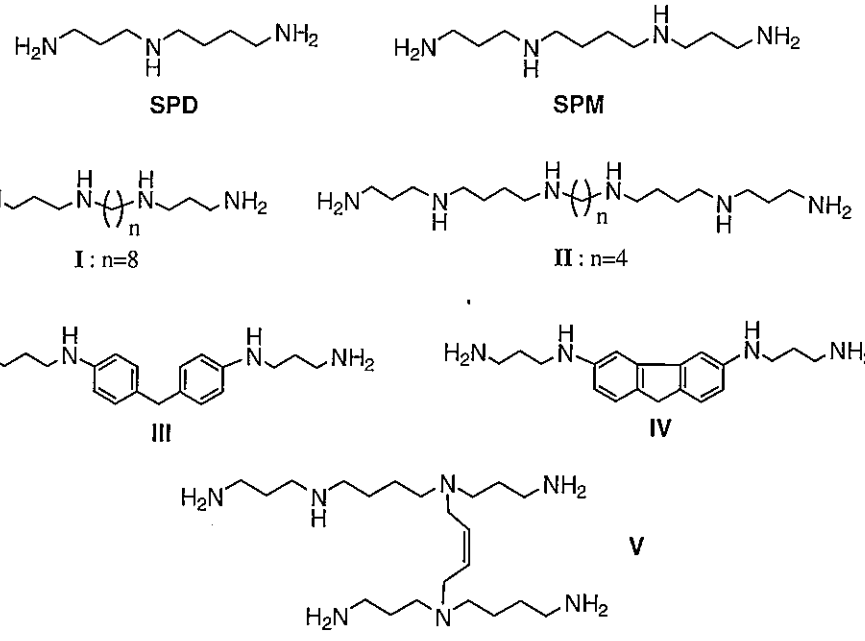
Linear polyamines (PAs): (a) of the types 3.n.3 (e.g. *I*) and 3.4.n.4.3 (e.g. *II*) and (b) incorporating aromatic rings as conformational restraints (e.g. *III* and *IV*), as well as branched PAs (e.g. *V*) of the spermidine(*SPD*)-spermine (*SPM*) type were readily obtained using the isolable 'active' esters

Trt-βAla-OSu and Trt-βAla-γAba-OSu and suitable α,ω-diamines to build the PA skeleton and α,ω-dicarboxylic acids to bridge the PA moieties.



No 81

Induction of Sialic Acid to Proteins for Immunoreactivity Studies

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Sialic acids comprise a family of about 40 derivatives of a monosaccharide with 9 carbon atoms, neuraminic acid (5-amino-3,5-dideoxy-D-Glycero-D-Galacto-2-nonulopyranos-1-onic acid) (Fig.1). They occur in all animal and sometimes in bacterial cells, mainly at the extracellular membrane. Their exposure to molecules and cells, because of their position and their strong anion charge (pK_a value about 2), contributes to their involvement in important cellular functions such as the binding and transfer of positively charged compounds and the arrangement of glycoproteins. Sialic acids are also antigenic determinants and receptor components of the cellular membrane. They also act as a biological mask by covering the antigenic regions and preserving glycoproteins in blood circulation. Based on this masking effect theory we considered it would be interesting to investigate the reduction of immunogenicity, of different proteins, cause by sialic acid induction. Our aim is the induction of native and modified sialic acid derivatives to proteins in order to study the immunoreactivity modulation in their presence and absence.

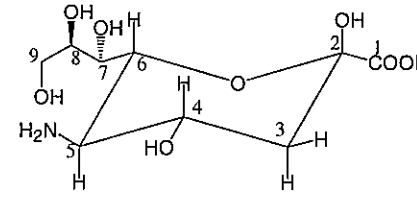

Figure 1