INSTITUTE of BIOORGANIC CHEMISTRY

POZNAŃ

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INSTITUTE
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BIOORGANIC CHEMISTRY

Polish Academy of Sciences
Poznań 1999

Director: Andrzej B. Legocki

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Address:

Institute of Bioorganic Chemistry
Polish Academy of Sciences
Zygmunta Noskowskiego 12/14
61-704 Poznań, Poland

Telephones & fax:

Director and Secretariat (+48 61) 8528 919
Operator (+48 61) 8528 503
Fax (+48 61) 8520 532
E-mail: ibch@ibch.poznan.pl
http://www.ibch.poznan.pl

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FOREWORD

Over the past three decades life sciences have been transformed, through the development of structural chemistry and molecular biology, from descriptive to reductionist, approaching fundamental questions of living systems. Modern sciences, widely used in industry, agriculture and medicine, have reached a level, which makes a significant impact on the contemporary society.

The Institute of Bioorganic Chemistry of the Polish Academy of Sciences is a modern research unit whose role is to pursue basic research and advanced education on postgraduate level. The Institute connects interdisciplinary research problems of structural chemistry and crystallography with fundamental biological problems on molecular level. Within its internal organization, there are several interacting research groups in each laboratory. Such a structure encourages young scientists and graduate students to early acquire a sense of responsibility. A good number of scientists who graduated from or were trained at our Institute now hold high positions in laboratories all over the world.

The Institute comprises 11 research laboratories and the affiliated Supercomputing and Networking Center.

In 1998, the Institute employed 203 people including 50 staff scientists with Ph.D. degree with 18 professors, 33 Ph.D. graduate students, 64 engineers and technicians and 56 administrative and service personnel. The Ph.D. graduation program takes 4 years combining the research work and a series of specialized lectures. In 1998/99, 46 undergraduate students from the faculties of biology, chemistry, pharmacy and biotechnology of Poznań Universities were working at the Institute's laboratories to complete their M.Sc. degrees.

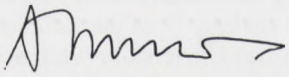
Current scientific interests of the Institute may be summarized as follows:

- chemical synthesis and stereochemistry of modified nucleosides and nucleotides with antiviral and antitumor activity;
- chemical and enzymatic synthesis of nucleic acids and oligonucleotides including structural genes and model oligomers of DNA and RNA;
- chemistry and molecular biology of RNA;
- heteronuclear NMR studies on the conformation of nucleic acid components and nucleic acid-protein interactions;
- structure, organization and expression of plant genes and molecular genetics of plant-microbe interactions;

- transformation of plant cells with foreign DNA and regeneration of transgenic plants with potential practical application including construction of plant-based edible vaccines;
- molecular biology of oncogenes;
- X-ray crystallographic analysis of proteins, nucleic acids and their derivatives;
- modelling and computation methods in molecular biology and chemistry.

Although most scientific activities carried out in the Institute laboratories focus on basic research problems, some of them may become of practical importance. These include synthesis of molecular probes for medical and agricultural diagnostics, analysis of genes BRCA1, BRCA2 and p53 for mutations that are conducive to breast cancer, synthesis of nucleic acids analogs with antiviral and anticancer properties, investigation of naturally occurring symbiotic systems capable of fixing atmospheric nitrogen, transformation and regeneration of higher plants expressing viral antigens with potential vaccinal activity, as well as research concerning applied phytophysiology directly related to agricultural practice. One of the Institute's buildings erected in 1989-1994 serves the local scientific community as the venue of the Science Center of the Polish Academy of Sciences in Poznań with a well-equipped conference center.

Although useful, the reductionist understanding of the structural and biological phenomena is incomplete; the knowledge of the DNA sequence of a gene does not by any means tell us all that we need to know. Transcription, posttranslational events, interactions with the products of other genes, interactions with environment, conformational relationships, are necessary for understanding of fundamental rules of nature. This is a large area and we feel it is our duty to contribute to its further development.



Andrzej B. Legocki
Director of the Institute

Poznań, March 1999

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Aleksander Zamojski	Institute of Organic Chemistry, Polish Academy of Sciences, Warsaw

RESEARCH LABORATORIES

LABORATORY OF NUCLEOSIDE CHEMISTRY

Head: ***Bożenna Golankiewicz***

Staff: *Jerzy Boryski, Piotr Januszczyk, Tomasz Ostrowski, Joanna Zeidler, Andrzej Manikowski, Zofia Adamska, Krystyna Lembicz*

Ph.D. Students: *Daniel Baranowski, Tomasz Gośliński, Tomasz Zandecki*

The research is concentrated upon the synthesis of modified nucleobases, nucleosides and nucleotides together with their structural features, chemical properties and biological activities. The main objects of interest are nucleoside analogs designed to mimic the natural substrates for: i) probing the structural and steric requirements of selected enzymes, ii) enhancing the specificity in enzyme inhibition, iii) developing new antiviral and anticancer agents. Studies in the field of synthesis and reactivity of nitrogen heterocycles also include the stability, solubility and transport characteristics important for the desired activity *in vivo* of the aforementioned analogs.

Current research activities:

- search for new approaches towards synthesis of nucleobases and nucleosides (controlled degradation, rearrangements);
- selective alkylation of nucleosides and nucleotides;
- synthesis and characterization of nucleobase and nucleoside analogs which are potentially and actually biologically active, mainly ring analogs of purine, acyclo-nucleosides and dideoxynucleosides;
- attempts at defining structure-activity relationships in base-modified acyclovir and ganciclovir analogs;
- study on the mechanism and applications of transglycosylation reactions in the nucleoside chemistry;
- determination of the structural factors influencing the stability of glycosylic bonds.

Major recent results

Tricyclic modification of the guanine moiety of acyclovir and ganciclovir

Linking 1 and N^2 positions of the guanine moiety in two potent antivirals, acyclovir and ganciclovir, with an etheno bridge was found to modulate the biological and physical properties of these two compounds. The appended ring alone lowers the activity against HSV-1, HSV-2, VZV and CMV by a factor of 10^2 or more. Further substitutions in that ring enhance the activity and make compounds more selective toward particular viruses. Substitution with phenyl group results in fluorescent analogs which having biological activity similar to that of the parent compounds seem to be promising candidates for noninvasive diagnosis of herpesvirus infections.

Synthesis and selective activity against myxoviruses of some imidazo[1,5-a]-1,3,5-triazine (5,8-diaza-7,9-dideaza purine) derivatives

A variety of imidazo[1,5-a]-1,3,5-triazine derivatives was synthesized by chloro-trimethylsilane/HMDS effected cyclization-rearrangement of 5-acylamino-5-alkyl (aralkyl, omega-benzyloxypolimethylene)-6-amino-2-mercaptopyrimidin-4(5*H*)ones and subsequent transformations of substituents at the 2-, 4-and 8 positions. The compounds were evaluated in broad-spectrum antiviral assays. Simultaneous presence of the benzyl and thio structural units was found to be indispensable for any selective biological activity. Some 2-thio substituted compounds e.g., 8-(4-methylbenzyl)-2-[(4-methyl-benzyl) thio] imidazo[1,5-*a*]-1,3,5-triazin-4-one (1), are specifically inhibitory to influenza A and respiratory syncytial viruses at concentrations below 5 μM.

Lack of anti-influenza A and anti-respiratory syncytial viruses activity of the purine ring counterpart of (1) at concentrations of 750 and 100 μM, respectively, demonstrates that the position of nitrogen atoms within the heterocyclic skeleton is of critical importance.

Transglycosylation reactions of purine nucleosides and their analogs.

Synthesis of the N-glycosylic bond, one of the most important reactions in the nucleoside chemistry, proceeds in two steps: i) the formation of a kinetic glycosylation product, and ii) its rearrangement, called "transglycosylation", to a thermodynamically more stable nucleoside. It has been believed for years that glycosylation of all purine bases takes place in the sequence 3→9, as it has been demonstrated for adenine. However, as shown in our laboratory, 6-oxopurines (e.g. guanine, hypoxanthine) undergo glycosylation in the sequence 7 ⇄ 9, and the site of initial substitution (N7 or N9) depends on the strucure of substrates.

Reaction of 7 ⇄ 9 transglycosylation has found many applications in the nucleoside chemistry, especially for synthesis of acyclonucleosides (e.g. acyclovir, ganciclovir). A variety of aspects related to the mechanism of transglycosylation, i.e. regio- and stereochemistry, reversibility, effects of structural modifications and the role of catalysts, are currently studied in our laboratory.

NUCLEOSIDE CHEMISTRY

KEYWORDS:
nucleoside analogs synthesis • transglycosylation • glycosylic bond stability • antivirals • structure-activity relationships

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LABORATORY OF STRUCTURAL CHEMISTRY OF NUCLEIC ACIDS

Head: ***Ryszard W. Adamiak,***

Staff: *Ewa Biała, Artur Fischer, Katarzyna Kulińska, Tadeusz Kuliński, Joanna Sarzyńska, Grażyna Dominiak, Mariusz Popenda, Danuta Więckowska*
Staff of affiliated NMR Laboratory: *Zofia Gdaniec, Dorota Mnichowska, Elżbieta Pasternak*

Ph.D. Students: *Łukasz Bielecki, Mikołaj Olejniczak*

Undergraduate Students: *Katarzyna Pachulska, Krzysztof Stobrawa, Norbert Oksza Strzelecki*

Major interests of the Laboratory comprise synthesis and chemistry of oligoribonucleotides directed toward elucidation of structure and stereodynamics of RNA, and their interactions with related proteins. Concurrently, new methodologies have been advanced for the design of regioselectively modified oligo(deoxy)ribonucleotide probes (nucleosidic fluorophores, fluorine, NMR-isotopes) for molecular studies of nucleic acids (RNA duplexes and bulges, duplexes modified with fluorescent 1,N^6-ethenoadenine, 2-aminopurine and luminarine analogs) and RNA-proteins interactions.

Until 1985, the major interest of the group was total synthesis of RNA fragments, especially those containing natural, modified and hypermodified nucleosides. Our laboratory pioneered the methods of post-synthetic modifications of oligoribonucleotides as a route to regiospecific modification of RNA. We have described in detail the mechanism of potential side-reactions during oligonucleotide synthesis.

Since our finding of "A"→"Z" RNA transition on oligoribonucleotide level we moved toward structural studies of functionally important RNA domains. The idea is to combine, within the group, the synthetic and structural lines of research in the RNA field. The major methodologies are heteronuclear (^{1}H, ^{13}C, ^{31}P, ^{19}F) NMR, thermodynamics and time resolved spectrofluorimetry. Thanks to the access to in-house supercomputing and networking facilities (Poznań Supercomputing and Network Center) simulation of molecular dynamics is intensively introduced into our research.

Current research activities:

- design and application of regioselectively modified oligoribonucleotides for ^{1}H, ^{13}C and ^{19}F NMR studies of RNA structure, dynamics and interactions with small ligands and recombinant proteins,
- chemistry of nucleoside fluorophores of luminarosine group and their introduction into oligonucleotides,
- studies on the NMR structure and dynamics of RNA domains (RNA duplexes containing alternating CG base pairs, RNA bulge duplexes, TAR RNA HIV-1) and interactions on the RNA-protein interface (^{13}C-[C_5-ribose]labelled TAR RNA and *Tat-1* and *Tat-2* proteins),

- hydration of RNA (functional domains of 5S rRNA, RNA bulge duplexes and P-modified fragments),
- role of 2'-hydroxy function in view of "A → Z" transition in RNA,
- thermodynamics, dynamics *via* time-resolved spectrofluorymetry and MD simulation *in aqua* of modified RNA bulges, TAR RNA HIV-1 and various regions of 5S rRNA.
- chemistry of nucleoside fluorophores of luminarosine group and their introduction into oligonucleotides;
- NMR studies on the structure and dynamics of RNA domains containing alternating CG base pairs in RNA duplexes;
- investigation of the role of 2'-hydroxy function in the "A" → "Z" transition in RNA;
- 2D NMR, time-resolved spectrofluorimetric studies and dynamics of modified RNA bulges within domain of TAR RNA HIV-1;
- simulation of molecular dynamics of DNA duplexes modified with fluorescent, mutagenic 1,N^6-ethenodeoxyadenosine.

Major recent results

RNA synthesis

To study the RNA structure and dynamics by NMR methods a sequence-specific introduction of 1',2',3',4',5'-$^{13}C_5$-ribonucleotide residues into 29-mer of TAR RNA HIV-1 has been elaborated (in cooperation with laboratory of Prof. J. Chattopadhyaya, Uppsala University). Other syntheses are related to preparation of regioselectively modified oligoribonucleotides for ^1H, ^{13}C, ^{19}F NMR and fluorescence studies of RNA domains.

Structure of RNA duplexes containing alternating CG base pairs.

Recently we have determined the solution structure of two RNA duplexes of sequence CGCGCG (PDB 1PBM) and 2'-O-methyl-CGCGCG (PDB 1PBL) using 2D NMR methods. The results show details of unique form of over-wounded type duplexes containing consecutive CG base pairs and effect of 2'-O-methylation. In cooperation with Dr. D. A. Adamiak (this Institute) and Dr. Dr. W. Rypniewski and Z. Dauter (DESY Lab. EMBL-Hamburg), the crystal structure of 2'-O-methyl-CGCGCG RNA duplex has been determined (NDB coordinates ARFS26). This duplex structure solved with resolution 1.3 Å, one of the highest in the up-to-date RNA X-ray research, will allow us to compare both crystal and solution RNA structure.

RNA stereodynamics and interactions

X-ray crystallography and NMR spectrometry are the principal methods for the evaluation of structural details of nucleic acids. Those data, when combined with spectrofluorimetry and molecular dynamics, methods allowing to probe fast conformational transitions, a truly stereodynamic picture of molecules might be obtained. One of the major goals in the spectrofluorimetry remained to be of challenge: design of appropriately labelled RNA molecules. Introduction of an emitter/acceptor pair of nucleosidic fluorophores to the interior of the RNA sequence is our ultimate goal.

STRUCTURAL CHEMISTRY OF NUCLEIC ACIDS

KEYWORDS:
nucleic acids chemistry • RNA structure and dynamics • RNA hydration • RNA-protein interactions • NMR ^{1}H, ^{13}C, ^{19}F • spectrofluorimetry • FT-IR • simulation of molecular dynamics

We have designed new fluorescent nucleosidic probes of the luminarosine family (λEm_{max}=530nm, Φ=0.65) and their sequence specific introduction to oligonucleotides. We have found that 1,N^6-ethenoadenine and 2-aminopurine are well matched blue emitters in respect to green-yellow luminarine as acceptor. We have evaluated the photophysical properties (cooperation with Prof. S. Paszyc group from Adam Mickiewicz University, Poznań and Prof. R. Verrall, University of Saskatchewan), thermodynamics and molecular dynamics of DNA duplexes modified with 1,N^6-ethenodeoxyadenosine. As proved by thermodynamics, the 2-amino-purine riboside could be used as a non-invasive fluorescent probe to study dynamics of RNA bulge loops containing adenosines.

TAR RNA HIV-1 was labelled with 2-aminopurine in specified positions to study the structure and dynamics in the hairpin loop region.

Fluorine chemical shifts are extremely sensitive to the nucleus environment thus making fluorine an ideal NMR spin label in the study of nucleic acids interactions with proteins. The small radius of fluorine atom implying little steric hindrance, 100% natural abundance of ^{19}F nucleus, high sensitivity of detection (83,3% of that of ^{1}H) and large chemical shift dispersion are also advantages of using ^{19}F NMR in the study of RNA-protein interactions. 5-Fluorouridine has been regioselectively incorporated into 29 nucleotides long TAR RNA HIV-1 fragments and bulged duplexes by means of phosphoramidate chemical synthesis on solid support. Fluorine labels have been introduced into functionally important bulge and loop regions of TAR RNAs. Currently, the ^{19}F NMR study of interactions of this RNA with magnesium, small ligands and *Tat* proteins is of our interest.

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LABORATORY OF RNA CHEMISTRY AND BIOCHEMISTRY

Head: *Jerzy Ciesiołka*

Affiliated professors: *Maria D. Bratek-Wiewiórowska, Maciej Wiewiórowski*

TEAM OF RNA CHEMISTRY

Head: *Ryszard Kierzek*

Ph.D. Student: *Elżbieta Walczak*

Research in the group has recently focused on many problems of the structure and function correlation of oligoribonucleotides and RNA. Chemistry in the group is not only a tool for the chemical synthesis of the model oligoribonucleotides. We are interested in developing new methods of synthesis, deprotection and purification of nucleosides, nucleotides and oligoribonucleotides. The chemical synthesis of oligoribonucleotides also includes oligomers containing modified nucleotides. All of those oligoribonucleotides and their analogs are used for studies of the catalytic and thermodynamic properties of oligoribonucleotides.

We have been interested in the studies of thermodynamic properties of oligoribonucleotides since 1984, when we started a very productive collaboration with professor Douglas H. Turner (University of Rochester, Rochester, USA) on this subject. At the present moment the group is focused on the influence of uridine modifications on the thermodynamic stability of oligoribonucleotides. These modifications include 5-halogenated and alkylated uridine as well as N-3,5- and 6-methylated uridine. The modified uridines are incorporated into the oligomers at the position of internal and terminal base pairs as well as the 3'-dangling end.

It has been discovered that single stranded oligoribonucleotides can be selectively and quantitatively hydrolyzed to 2',3'-cyclic phosphate and 5'-hydroxyl ended oligomers. This finding has proven that hydrolysis occurs without contribution of ribonucleases and that the presence of several different cofactors stimulates this process. The hydrolysis proceeds via „in line" SN2 mechanism with inversion of configuration at phosphorous atom. Besides, the influence of 5'- and 3'-adjacent fragments of the oligomer on this process was studied.

Current research activities:

- determination of thermodynamic parameters for many structural elements of RNA;
- study of the catalytic activities of selected oligoribonucleotides;
- chemical synthesis of fluorescent oligoribonucleotides as a tool for structural studies;

Major recent results

Thermodynamic stability of 2'-5' oligoribonucleotides versus 3'-5' ones

One of the major questions in prebiotic studies is why nucleic acids have 3'-5' phosphodiester bonds. We have synthesized oligoribonucleotides containing 2'-5' phosphodiester bonds and studied their thermodynamic stability by UV monitored melting. We found that complementary 2'-5' oligoribonucleotides interact to form A-RNA helices. We determined that T_m of 2'-3' oligoribonucleotide is about 40 deg lower than that of an analogous 3'-5' oligoribonucleotide.

Advantages of pyrene 5'- labeled fluorescent oligoribonucleotides

We found that oligoribonucleotides labeled with pyrene at the 5'- end are particularly useful tools for the study of oligoribonucleotide structure and interactions with nucleic acids and proteins. When they form a duplex with the complementary strand, fluorescence increases 5-20 fold. At the same time, the presence of pyrene does not affect the interaction with complementary oligomers, as confirmed by UV and fluorescence detected melting of oligoribonucleotides. The 5' pyrene labeled oligoribonucleotides have been applied to many studies, including studies of structural, kinetic and dynamic interactions with the internal guide sequence of the LSU rRNA self-splicing intron from *Tetrahymena thermophila*.

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RNA CHEMISTRY AND BIOCHEMISTRY
RNA CHEMISTRY

KEYWORDS:
oligonucleotide synthesis • modified oligonucleotides thermodynamics • RNA selfhydrolysis

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TEAM OF RNA BIOCHEMISTRY

Head: ***Jerzy Ciesiołka***

Staff: *Magdalena Alejska, Marek Figlerowicz, Anna Perkowska, Jan Wrzesiński, Nelli Malinowska, Barbara Smólska*

Ph. D. Student: *Michał Łęgiewicz*

Undergraduate Students: *Bartosz Brzezicha, Michał Brzustowski, Anna Urbanowicz*

Metal ions are indispensable components of all biological systems. In many protein enzymes, metal ions do not only play a structural role, but actively participate in catalytic processes. More recently it has been realized that metal ions play a similar dual role in nucleic acids, crucial for catalysis by ribozymes in particular. Site-bound divalent metal ions do not only stabilize the active structure of ribozymes, but also participate directly in catalysis.

Our research is directed toward understanding the role of specific interactions of RNA with metal ions in ribozymes of plant and mammalian origin as well as searching for other RNA molecules in which metal ions play an important structural or regulatory role. In particular, we are interested in the structure, function and possible applications of ribozymes derived from hepatitis δ virus.

RNA structure and RNA-metal ion interactions are being studied using a variety of chemical and enzymatic probes with special emphasis on limited, metal ion-induced RNA hydrolysis. We are currently introducing to our research a new technique, selection-amplification, in order to study the structure and function of hepatitis δ virus ribozyme and selection of novel RNA structures with desired ribozyme activities.

Studies of genetic recombination in RNA viruses and in retroviruses, conducted *in vivo* (with brome mosaic virus – BMV) and *in vitro* (with HIV and MMLV reverse transcriptases), are directed toward understanding the mechanisms that are used by RNA viruses and by retroviruses to rearrange their genomes. The investigation is aimed to determine the role of virus encoded proteins as well as RNA structure in mediating and enhancing of the recombination events. In addition, crystallographic studies of viral replicase proteins are carried out to establish the structural requirements for RNA recombination.

Current research activities:

- synthesis of RNA molecules for biochemical and structural studies by *in vitro* transcription method;
- chemical and enzymatic analysis of RNA structure and RNA-metal ion interactions;
- application of in vitro selection method to study the structure and function of selected ribozymes as well as to search for new RNA activities;
- *in vitro* and *in vivo* studies of the role of RNA structure in genetic recombination in RNA viruses and in retroviruses
- study of the involvement of viral replicase proteins in RNA recombination.

Major recent results

Structural analysis of 5S rRNA by means of Pb^{2+}-induced hydrolysis

We have compared susceptibility of three prokaryotic 5S rRNA and selected 5S rRNA-protein complexes to hydrolysis induced by Pb(II) ions. Lead ions allowed us also to distinguish different conformational forms of 5S rRNA from *E. coli* and from rat liver. More recently, the structure of plant 5S rRNA species from lupin and wheat germ as well as the structure of two RNA fragments that represent domains β and γ of lupin 5S rRNA have been probed by Pb(II) induced hydrolysis. The studies confirmed high sensitivity of the lead hydrolysis method in detecting strong metal ion binding sites, altered conformations of RNA as well as RNA regions of higher conformational flexibility (the studies were carried out in the laboratories of V. A. Erdmann (Free University, Berlin) and W. J. Krzyżosiak (IBCh, Poznań)).

Selection in vitro of Zn^{2+}-binding RNA domains

We have used affinity selection-amplification to select an RNA molecule that binds Zn^{2+} ($K_D \approx 1$ mM), starting from a pool of ribooligonucleotides with 50 randomized positions. An ion binding site is possibly related to the naturally occurring E-loop motif in other RNA molecules. We have reselected Zn affinity, beginning from an RNA pool derived from the truncated Zn-binding RNA with a 23-nt random region. Comparison of selected sequences ($K_D \approx$ 100-400 μM for free Zn^{2+}) with previously known divalent sites suggests three or four small RNA motifs repeatedly found to interact with divalent ions. Such structures may help identify similar divalent sites in sequenced RNAs and serve as substructures for design of functional RNA metallodomains (the research was conducted in the laboratory of Prof. M. Yarus (University of Colorado, Boulder)).

RNA CHEMISTRY AND BIOCHEMISTRY
RNA BIOCHEMISTRY

KEYWORDS:
RNA structure • ribozymes • RNA-metal ion interactions • metal ion-induced RNA hydrolysis • in vitro selection method • RNA viruses • genetic RNA recombination • viral replicase

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LABORATORY OF BIOCONJUGATE CHEMISTRY

Head: ***Wojciech T. Markiewicz***

Staff: *Anna Astriab, Andrzej Grajkowski, Maria Markiewicz, Katarzyna Adrych-Rożek*

Ph.D. Students: *Marcin Chmielewski, Przemysław Godzina, Piotr Kwaśnikowski, Żaneta Ratajska*

Undergraduate Students: *Krzysztof Ozdowy, Anna Szmajda, Joanna Talarek*

The main research interests focus on the elaboration of methods of chemical synthesis of oligonucleotides (DNA, RNA fragments and their analogues) as well as their derivatives. The studied derivatives can result from a chemical or enzymatic conjugation with other types of biomolecules such as proteins (e.g. enzymes, antibodies, toxins etc., their fragments, hybrids) or lower molecular weight molecules with the desired properties. The following properties are taken into consideration: chemical (reactive residues), physical (fluorescent, free radical etc.) and biological/pharmaceutical (e.g. vitamins, enzyme co-factors, enzyme substrates). Studies on both chemical and structural properties of bioconjugates are necessary for their further successful applications. The considered applications comprise use in basic research (e.g. DNA sequencing, gene expression control) and biotechnology (diagnostics, medicine, agriculture).

The above outlined chemical and biological interests form together the main research objective of the laboratory which is the development of a new technology: *Synthetic Oligonucleotide Combinatorial Libraries* (SOCL). A SOCL is a representation of oligonucleotides of a given composition and length permanently bound to a solid support. SOCLs of a dispersed format are studied (one bead of a support – one oligonucleotide sequence). A SOCL technology is a non-biological system of selection of molecules (*chemoselection*) allowing searching for *library elements* strongly interacting with chosen acceptor molecules.

The technologies of *Synthetic Oligomer Combinatorial Libraries* (SOCL) provide an insight into the mechanisms of biomolecular recognition (e.g. gene expression). They are useful in finding new bioactive molecules (e.g. lead compounds for pharmaceutical studies). Results of the above studies will be a subject of computer *molecular modeling*.

Studies of *Oligonucleotide* SOCL concern their possible applications for the development of new methods of fast sequencing of DNA.

Current research activities:

- search for new protecting groups, solid supports (3'-end labeling supports) for the chemical synthesis of DNA, RNA fragments and their analogues; DNA sequencing;

- synthesis of SOCLs, their applications in chemoselection and sequencing of nucleic acids; the application in studies of triplex DNA;
- synthesis of polyaminonucleosides and polyaminooligonucleotides and their studies by a SOCL approach;
- synthesis of new biotinylating reagents and use of biotinylated oligonucleotides with magnetic supports;
- chemical synthesis of oligonucleotides for biochemical and structural studies as well as for diagnostic purposes (IL-2 gene, hybridization probes);
- applications of the polymerase chain reaction (PCR) for synthesis of longer nucleic acids fragments, e.g. synthesis of structural genes, allele specific PCR, PCR products as hybridization probes, etc;
- development of new algorithms useful in DNA sequencing by hybridization (SBH);
- applications of a phage display approach using phagemids for preparation of semi-synthetic combinatorial libraries of fragments of antibodies.

Major recent results

DNA sequencing using 3'-end labeled primers

3'-End labeled oligodeoxynucleotides with free 3'-hydroxyl group (labeled at 3'-end base moiety) were found to be effective primers for DNA polymerases used in DNA sequencing by the dideoxy approach. A new type of supports for the chemical synthesis of such primers was developed in order to facilitate the synthesis of labeled primers by the automate DNA synthesis. The supports are loaded with nucleosides carrying appropriate modifications or their precursors.

2'-O-Ribosylribonucleosides, minor nucleosides of tRNA

A new type of minor nucleosides found in initiator methionine tRNAs from eukaryotic organisms (yeast, plants), 2'-O-ribosyladenosine and 2'-O-ribosyl-gua-nosine, was synthesised for the first time. The synthesis of those nucleosides involved the glycosylation reaction of N-protected 3',5'-O-(tetraisopropyldisiloxane-1,3-diyl)-ribonucleosides with 1-O-acetyl-2,3,5-tri-O-benzoyl-β-D-ribofuranoside in the presence of tin tetrachloride. Cytidine and uridine analogs were also obtained. All new nucleosides were characterized by ^{1}H and ^{13}C NMR. 2'-O-Ribosyluridine was obtained in a crystalline form and its structure was determined by X-ray crystallography. All four new minor nucleosides were transformed into 5'-O-di-methoxytritylated 3'-phosphoramidites. A dimer, ArpG, with a 2'-O-ribosyladenosine unit, was synthesized and its structure in aqueous solution was studied by NMR and molecular dynamics. 5''-Phosphates of 2'-O-ribosylribonucleosides were obtained.

Application of SOCL in studies of triplex DNA

A synthetic oligonucleotide combinatorial library (SOCL) containing 15-mer G/T randomised region was prepared on beaded polystyrene support and used in search for oligonucleotides capable to form triplex complexes with double stranded

BIOCONJUGATE CHEMISTRY

KEY WORDS:
RNA/DNA chemical synthesis • protecting groups • synthetic oligonucleotide combinatorial libraries (SOCL) • magnetic support • interleukin-2 • DNA-sequencing • hybridization probes

probe containing a sequence of the promoter of human epidermal growth factor receptor gene (HER2).

Diagnostics of Thrombotic and Haemorrhodic Disorders

An improved method for identification of Factor V Leiden mutation by the PCR method using sequence specific primers with an additional mismatch at the 3'-end penultimate position was developed and applied in clinical studies of over 200 patients. These studies were performed in collaboration with the Academy of Medicine in Poznań.

Algorithms for DNA sequencing by hybridisation (SBH)

New sequential and parallel algorithms for sequencing of DNA by hybridization (SBH) were developed in collaboration with mathematicians from the Poznań Supercomputing and Networking Center.

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LABORATORY OF PLANT MOLECULAR BIOLOGY

Head: ***Andrzej B. Legocki***

Staff: *Joanna Jeleńska, Józef Kapusta, Jan Podkowiński, Michał Sikorski, Tomasz Stępkowski, Paweł Stróżycki, Alina Kasperska, Iwona Femiak, Hanna Glapiak*

Ph.D. Students: *Jacek Biesiadka, Luiza Handschuh, Olesia Lisowa, Tomasz Pniewski, Żaneta Zaborowska*

Undergraduate Students: *Magdalena Czaplińska, Barbara Długoszewska, Marek Fiksa, Bożena Grabowska, Kacper Jankowski, Agnieszka Kisiel, Katarzyna Miedzinska, Ewa Sobieszczuk, Arkadiusz Trąbka, Joanna Wachowiak*

Naturally occurring symbiotic systems between legumes and *Rhizobium* species provide interesting models to study controlling mechanisms of plant gene expression. As a result of these endosymbiotic interactions, nitrogen-fixing root nodules are developed with significant modifications in gene expression of both symbionts – host plant and microorganism.

A basic model chosen by the laboratory is the agriculturally important lupin plant-*Bradyrhizobium* sp. (*Lupinus*) symbiotic system. The availability of genetic material (cDNA and genomic libraries, cloned genes, bacterial mutants) allowed an analysis of the expression of lupin symbiotic genes at different stages of interaction using the molecular as well as cytological tools. Plant transformation systems provide an important approach for testing the *in vivo* expression of recombinant DNA. These systems were introduced to recognize the function of lupin sequences in transgenic plants: tobacco, *Lotus corniculatus*, potato, pea, lettuce and to distinguish whether they can be expressed in the nonsymbiotic conditions. The important methodologies which are also applied to the characterisation of the symbiotic genes are structure alignments and elucidation of X-ray structures of *in vitro* overexpressed proteins.

A long term goal of the laboratory is to understand the complexity of the symbiotic response and to recognize to which extent it represents a variation of the pathogenic response.

A new line of research in the laboratory deals with the construction of plant-based edible vaccines against certain viral diseases. This research concerns transformation of selected plants with T-DNA-based recombinant vectors to obtain organ-specific expression of desired viral antigens in transgenic plants. In this manner, efficient, inexpensive and easy-to-administer vaccines against certain viruses as well as pathogens might be obtained. Currently, the investigations are focused on Hepatitis B Virus for human and Classic Swine Fever Virus for animals.

Current research activities:

- identification, expression analysis and structure determination of plant genes involved in effective symbiosis;
- genetic analysis of nodulation region of *B.* sp. (*Lupinus*) controlling synthesis of the Nod factor – a key morphogen for the initiation of root nodule meristem.
- phylogenetic analyses of the plant genes and bacterial nodulation genes to elucidate co-evolutionary correlations of symbiotic systems;

- X-ray analysis of plant proteins involved in nodule morphogenesis and biological fixation of nitrogen;
- molecular links between symbiotic and pathogenic interactions using model of PR10 proteins;
- iron metabolism in symbiotic and nonsymbiotic systems;
- transformation and regeneration of legumes and nonlegumes for obtaining constitutive as well as organ-specific transgenic expression;
- construction of transgenic plants expressing viral antigenes for preparation of edible vaccines.

Major recent results

Identification, organization and expression of lupin symbiotic genes

A number of plant genes expressed during symbiotic interactions of *Lupinus luteus-Bradyrhizobium* sp. (*Lupinus*) were selected from lupin cDNA and genomic libraries and their transcriptional activation during effective symbiosis development was analyzed by Northern hybridization.

Two genes whose expression is down-regulated during nodule formation were identified in roots of uninfected plants (*Llpr10.1a* and *Llpr10.1b*). The deduced amino acid sequences showed that both genes encode for polypeptides exhibiting high similarity to known pathogenesis-related and stress-induced proteins of PR10-class from other plants. These similarities suggest their common function in plant-microbe interactions. The repression of *PR10* genes in developing nodule might be crucial for the establishment of functional symbiosis (in mature nodule the defense system is turned off). It is postulated that the expression of PR10 in lupin represents the link between symbiotic and pathogenic responses. As far as the function of these proteins is concerned, it has been shown that PR10 overexpressed *in vitro* reveals ssRNase activity. Its tertiary structure has been recognized to 2,5 Å resolution showing high similarity to birch pollen allergen *Betv1*.

One of the first plant genes activated during symbiosis is ENOD40. Short peptide identified as a product of the ENOD40 gene expression as well as its expression pattern suggest that this gene plays a regulatory role in morphogenesis. LlENOD40B genomic clone was isolated and its sequence revealed characteristic conserved motifs. Conformation studies of lupin ENOD40 transcript to approach the puzzle of its regulation are under way.

The major factors affecting the biological processes and controlling the efficiency of symbiotic nitrogen fixation are oxygen and iron. Genes coding for plant leghemoglobins and ferritins, i.e. proteins regulating levels of these components, are investigated in this respect. The structure of lupin leghemoglobin promoters appearing different than that of other legumes as well as an unusual pattern of expression of ferritin genes in lupin nodules make this model especially attractive.

Cloning and sequencing of the nodulation genes from B. sp. *(Lupinus)*

Isolation of the nodulation region of *B.* sp. (*Lupinus*) provided insight into the genetics of lupin nodulation. The nucleotide sequence of two nodulation clusters, of 50 kb in total have been determined. All *nod* genes whose products control either N-methylation (*nodS*), carbamylation (*nodU*), fucosylation (*nodZ*) or 4-O-acetylation of fucose (*nolL*) have been identified. The presence of additional regulatory circuit en-

KEYWORDS:
plant genes • recombinant proteins • organ-specific gene expression • symbiotic nitrogen fixation • plant transformation

coded by the product of *nodW* gene as well as the organization of *nodC-nodZ* resembles *B. japonicum*. However, a comparison of the nucleotide or amino acid sequences indicates their phylogenetic distinctness from the nodulation genes in other *Bradyrhizobium* strains. It is assumed that lupin and the whole tribe *Genisteae* are a very ancient group of legumes. The observed dissimilarity of the *nod* functions might be a reflection of the coevolution of lupins and their microsymbionts.

Plant transformations and plant-based edible vaccines

The idea to develop an edible vaccine comes from the need to construct cheap, safe and feasible vaccine to assure immunization on a global scale. Gastro-intestinal tract is programmed to generate immunological response against antigen and pathogenic organisms present in food, therefore edible vaccine produced in plants appears to be one of the most effective ways for both human and animal immunization.

Transgenic lettuce (*Lactuca sativa* L.) and yellow lupin (*Lupinus luteus* L.) carrying hepatitis B surface antigen have been engineered *via* genetic transformation. The presence of antigenic protein in transgenic plant tissue was proved with ELISA test. Transformed lupin callus and lettuce plants have been used afterwards for oral immunization of mice and human volunteers. In the blood of animals as well as people specific anti-HBs antibodies have been detected, confirming elicitation of immunological response. It was found that following oral immunization IgG antibodies have been mainly present in the blood serum. This fact indicated that systemic immunity have been induced.

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LABORATORY OF DNA SYNTHESIS

Head: ***Adam Kraszewski***

Staff: *Jadwiga Jankowska, Barbara Imiołczyk, Michał Sobkowski, Izabela Zagórowska*

Ph.D. Students: *Jacek Cieślak, Marzena Szymczak, Małgorzata Wenska*

Studies on the chemistry of nucleoside H-phosphonate mono- and diesters. These studies are directed toward improvement of synthesis and modification(s) of the oligonucleotides which are going to be used as molecular probes detected with nonradioisotopic systems and also as antisense oligonucleotides. Recent studies have been focused on the reactions of nucleoside H-phosphonate mono- and diesters with bifunctional reagents (amino alcohols, diamino alkanes, dihydroxy alkanes, tioalcohols) to produce nucleotide units or oligonucleotides bearing modified phosphate residue.

Current research activities:

- elaboration of new and effective methods of chemical synthesis of DNA fragments;
- studies on reaction mechanisms involved in the chemical synthesis of DNA;
- chemical modifications of oligonucleotides addressed to biological studies e.g. inhibition of enzyme activity, oligonucleotide molecular probes and antisense oligonucleotides.

Major recent results

Modified nucleotides

On the basis of aryl nucleoside Ḣ-phosphonates developed in our laboratory a versatile method of synthesis of nucleoside H-phosphonamidates has been elaborated. Studies on properties of these compounds delivered new data essential for the synthesis of the so called P-N antisense oligonucleotides, compounds which for the last three years have been intensively investigated in antisense therapy.

New simple methods for synthesis of nucleoside phosphorothio- and phosphorodithioates have been developed in our laboratory. These compounds were tested as enzyme inhibitors and showed promising biological activity.

Molecular probes and antisense oligonucleotides.

Currently, molecular probes and antisense oligonucleotides are commonly accepted as modern tools in molecular biology studies and genetic diagnostics.

Despite many efforts, preparation of the above type of modified oligonucleotides is still difficult and often employs multistep procedures and expensive chemicals. The main goal of the studies in our laboratory is to simplify these methods by elaboration of a "new chemistry" which should allow the use of simple, commercially available chemicals. Three new efficient methods of functionalisation of oligonucleotides, prepared to attachment of reporter group(s) have been found. The first one is based on phosphonylation of the 5' hydroxyl group of oligomer with pyrophosphonic acid followed by coupling with unprotected amino alcohol using standard coupling reagents (pivaloyl chloride or cyclic phosphochloride). The second one employs "nucleotide like" synthons (aminoalkyl H-phosphonate monoesters) which were joined to the oligomer in standard procedures. In the third method of functionalisation, the N-protonated amino alcohol is attached to 5'- (2-cyanoethyl)H-phosphonate residue via oxidative coupling and forms specifically phosphoester bond. It is worthy of note that all chemicals used in the above methods of modification are commercially grade and do not require additional preparation for further synthesis.

It is also worth noticing that all procedures of modification were carried out on an oligomer attached to the solid support and that this process was controlled by a computer. This makes it possible to apply the above chemistry in the most commonly used oligonucleotide synthesisers.

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DNA SYNTHESIS

KEYWORDS:
oligonucleotide synthesis • modified nucleotide • modified oligonucleotide • oligonucleotide molecular probes • antisense oligonucleotide analogs

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LABORATORY OF tRNA BIOCHEMISTRY

Head: ***Jan Barciszewski***

TEAM OF tRNA BIOCHEMISTRY

Head: ***Jan Barciszewski***

Staff: *Mirosława Barciszewska, Małgorzata Giel-Pietraszuk, Maciej Szymański, Eliza Wyszko, Marzanna Deniziak, Iwona Witkowska, Iwona Gawrońska, Elżbieta Wieczorek*

Ph.D. Students: Monika Kamińska, Monika Skotnicka, Joanna Szepeta

Undergraduate students: *Karolina Czubak, Grażyna Kryszak, Michał Mielcarek, Adrian Morawiec, Jolanta Skalska, Agnieszka Ślisińska, Grzegorz Żukociński*

The scientific interest of the laboratory is to understand the recognition mechanism between nucleic acids and proteins. Therefore, a long time ago we chose tRNA and aminoacyl-tRNAs (AARS) as well as 5S rRNA and transcription factor IIIA from plants as the objects of our studies. We have sequenced many plant tRNAs, 5S RNAs and found some of their interesting properties. Recently our attempts to clone plant TFIIIA and glutaminyl-tRNA and methionyl-tRNA synthetase genes have proven successful.

Major recent results

The new tertiary model of 5S rRNA from plants

Over the last several years, 15 primary structures of plant 5S rRNAs have been determined. They showed high homology. We have noticed that nucleotides 34-37 and 85-88 are totally conserved. Using the Zuker algorithm, a secondary structure of plant 5S rRNA has been calculated. The single stranded fragment (85)GGGU(88) offers the possibility of the tertiary bonds formation with the (34)CCCA(37) sequence. Proposed long range hydrogen-bond interactions have been confirmed by digestion with RNaseH, α-sarcin, NMR measurements and other data.

Interactions of plant 5S rRNA with zinc fingers of TF IIIA

Xenopus laevis transcriptional factor IIIA (TF IIIA) forms the 7S RNP complex with plant 5S rRNA. To analyze the contribution of a particular zinc-finger domain (ZF) of TF IIIA to specific recognition of 5S rRNA, we synthesized a series of the ZF peptides containing 3-9 of zinc finger domains. We showed that the strongest complex is formed with the ZF6. The obtained data support the elongated tertiary structure model of 5S rRNA.

Cloning of plant glutaminyl-tRNA synthetase

The primary structure of the GlnRS gene is 2880 nucleotides and it is the first GlnRS from plants to be known on a gene level. It contains KMSKS and HIGH sequences, which are characteristic of all aminoacyl-tRNA-synthetases. GlnRS from

Lupinus luteus shows high homology (47%) to other known GlnRS. The N-terminus GlnRS contains lysine rich peptide of sequence KPKKKKEKPAK, which could serve as a nuclear localization signal.

Tertiary structure of systemin

Using two dimensional NMR spectroscopy, the tertiary structure of systemin (Ala-Val-Gln-Ser-Lys-Pro-Pro-Ser-Lys-Arg-Asp-Pro-Pro-Lys-Met-Gln-Thr-Asp) was analyzed. From these data and molecular calculations we concluded that the peptide could adopt a Z-like β-sheet structure, which had previously been found in various DNA-binding proteins. Using DNA-cellulose affinity column chromatography we showed that systemin strongly binds to DNA.

Effects of high pressure on the function of nucleic acids

We showed that phenylalanine specific tRNA of *E. coli* as well as the yellow lupin methionine initiator $tRNA^{Met}$ can be charged under high pressure of 6 kba specifically with phenylalanine and methionine respectively, in the absence of specific aminoacyl tRNA-synthetases. The estrification reaction takes place at the 3'end of tRNA molecule. On the basis of circular dichroism spectra, we showed that the conformation of tRNA at high pressure slightly differs from its original A-RNA form. We think that at high pressure a unique tertiary structure of tRNA creates an active center which catalyzes ester bond formation. If so, the structure of the aminoacid stem of tRNA may determine charging specificity of tRNA.

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tRNA BIOCHEMISTRY

KEYWORDS:
tRNA • 5S rRNA • structure and function • aminoacyl-tRNA sythetase • systemin

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TEAM OF PROTEIN BIOSYNTHESIS

Head: ***Tomasz Twardowski***

Staff: *Hanna Górnicka-Holeczek, Krystyna Ludwiczak*

Ph. D. Students: *Maciej Astriab, Beata Madej, Joanna Smól*

Undegraduate Student: *Mariola Legieć, Marcin Urbanek, Olimpia Witczak, Magdalena Kwiatkowska, Piotr Solarczyk*

Research conducted by the Team of Protein Biosynthesis concerns the regulatory mechanisms of protein biosynthesis in the plant system and the structure-function correlation of rRNA. The antisense strategy is one of the most important research tools used by us as the experimental approach. A better understanding of the molecular mechanism of protein biosynthesis may appear of significant importance for the future development of agriculture. We pay special attention to the function of proteins responsible for the plant reaction to biotic and abiotic stress, e.g ferritin (protein responsible for iron housekeeping) and lysine decarboxylase (LDC, the key enzyme for alkaloids metabolic pathway). These projects are correlated with the experiments concerning the functioning of elongation factors EF1 and EF2-proteins responsible for the binding of AA-tRNA to ribosomes and elongation of polypeptide chain. The very important subject of our experiments is the role of rRNAs in correlation with their active structures.

Legislation and public perception of biotechnology are separate aspects of our activity.

Current research activities:

- correlation of structure and function of rRNA during protein biosynthesis in the plant system;
- study of molecular properties of plant stress proteins: lysine decarboxylase and ferritine;
- the analysis of the regulatory mechanism at the translational level with antisense oligonucleotides as a tool;
- consultancy in the area of legislation, biosafety and public perception of Polish biotechnology.

Major recent results

Biosynthesis of plant ferritin

The mechanism of biosynthesis of plant ferritin and the phytosanitary function of this protein have been intensively studied. Based on our experiments, we presented a hypothesis concerning translational regulation of plant ferritin synthesis.

Secondary metabolites

A characteristic feature of plants is their ability to synthetize a wide range of natural products, the so-called secondary metabolites, including alkaloids. These low molecular weight components are essential for plant's self-defense system. We have tested the allelochemical properties of some alkaloids: inhibition of bacterial growth, germination and growth, toxicity, etc. In order to find the potential molecular target of these metabolites of protein biosynthesis we studied their action during the essential steps of protein synthesis. Lysine decarboxylase has recently been purified to homogeneity and studied as the key enzyme of chinolizidine alkaloids biosynthesis pathway.

Structure-function relations of RNA

In order te determining the functional structures of ribosomal RNAs we took advantage of the strategy of antisense oligonucleotides. We tested a fragment of large rRNA of plant ribosomes, which is characterized by highly conservative primary and secondary structures. This approach allows us to present the role in correlation with function of the so-called alfa-sarcin domain of large ribosomal RNA and loop „C" of 5S rRNA during the elongation step of the polypeptide chain synthesis in plant system. The alfa-sarcin domain of the LrRNA (the stem-loop structure) plays the key function during elongation of the polypeptide. At this domain the elongation factors interact with ribosomal components. The activity and availability of this struc-ture with specific conformation determines the activity of ribosomes: speed and error of translation. We presented data for correlation of the action of this domain and of loop „C" of 5S rRNA.

Our methods and approaches are also used for interpretation of experimental observations concerning e.g. the mechanism of drug action.

Legislation of biotechnology

We are also engaged in the problems of bioinformation, intellectual property, legislation and public perception of biotechnology, as well as in general analysis of Polish biotechnology. Our activities include publishing a Polish journal dedicated to biotechnology „BIOTECHNOLOGIA" (editor-in-chief: T. Twardowski), under the auspices of the Polish Biotechnology Committee. In 1993, we organized a meeting:

tRNA BIOCHEMISTRY
PROTEIN BIOSYNTHESIS

KEYWORDS:
regulation of translation – plant system • antisense oligonucleotides • natural products biotechnology: legislation • biosafety • public perception

„The Other Face of Biotechnology", dedicated to the specific problems of biotechnology: intellectual property rights, biosafety and biohazard, public perception. Accordingly, in 1995 we organized an international conference „Agrobiotechnology"; where the application of basic and applied sciences for agricultural purposes was discussed.

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LABORATORY OF CANCER GENETICS

Head: ***Włodzimierz J. Krzyżosiak***

Staff: *Daniel Michałowski, Teresa Dymarek-Babś, Barbara Bekasiak*

Ph.D. Students: *Anna Jasińska, Piotr Kozłowski, Marek Napierała, Krzysztof Sobczak*

Undergraduate Students: *Marta Birak, Łukasz Borzęda, Sławomir Jakubowski, Bożena Marszałek, Mateusz de Mezer*

The activity of this laboratory is focused on two areas of biomedical research: I. genetic predisposition to breast cancer, II. pathogenesis of human neurological diseases caused by trinucleotide repeat expansions.

As for as breast cancer is concerned selected groups of women from the local population at risk are screened for predisposing mutations in BRCA1 and BRCA2 genes. The carriers of different common polymorphic variants of these genes are also analysed in relation to cancer risk. Close attention is paid to increasing the efficiency of genetic technologies used for mutation detection.

The research concerning the hereditary neurological diseases is concentrated on the role of RNA level effects in cytopathogenesis. Specific RNA structures formed by normal and expanded trinucleotide repeats are investigated in order to establish a structural basis for normal and pathogenic function of the repeated sequences.

Earlier efforts of the laboratory to improve methods of RNA structure probing in solution are continued. They are now directed towards the analysis of regulatory regions in selected mRNAs of biomedical interest.

Current research activities:

- analysis of BRCA1 and BRCA2 genes in women with breast cancer or at increased familial risk of breast and/or ovarian cancer;
- search for efficient scanning method of screening large genes for mutations which takes advantage of PCR with fluorescently labelled primers and combines the SSCP and heteroduplex analysis;
- structural studies of regulatory elements located in 5'-UTR of BRCA1 mRNA, using lead cleavages and a battery of other chemical and enzymatic probes;
- structure probing of triplet repeat regions in transcripts of genes implicated in myotonic dystrophy, fragile X syndrome and other diseases caused by dynamic mutations;
- development of new RNA labelling and structure probing technologies as well as approaches to the analysis of heterogeneous RNA structures.

Major recent results:

Improvements in PCR

We have optimised the PCR conditions to make the process faster and economical. When short DNA fragments are to be amplified, the time of denaturation, annealing and extension steps can be as short as 1 s each, and the yield of PCR product is still high, sufficient for many types of analyses. The PCR can even be done in a reaction volume as low as 1 µl. The recommended volume, 2.5 µl or 5 µl, allows significant savings in the laboratory budget especially in the case of laboratories which use PCR frequently and on a large scale.

More efficient mutation detection method

We have shown that the SSCP technique when combined with heteroduplex analysis is highly efficient in detecting DNA mutations. As it is amenable to multiplexing and to analysing PCR products obtained from pooled genomic DNA samples, the PCR-SSCP-HDX is a method of choice for low cost screening of large genes for unknown and dispersed mutations.

Search for BRCA1 mutations

Among 122 women with positive, in most cases moderate, family history of breast and/or ovarian cancer, 34 unselected breast cancer tissue specimens and 80 controls, we have identified three different novel BRCA1 mutations, two novel rare BRCA1 variants and five independent cases of the same 12bp insertion-duplication in intron 20. Our study shows that in order to understand the significance of different BRCA1 variants and mutations a more population-oriented research is needed, involving women with less profound family history of breast and ovarian cancer.

Search for BRCA2 mutations

Analysis of the BRCA2 gene in 144 women with positive family history of breast and ovarian cancer, among them 58 breast cancer patients, revealed three novel frameshift mutations, two novel missense mutations and two novel common polymorphisms. Interestingly, one of the frameshift mutations was identified in three families and the possibility of their common origin is being investigated.

Novel type of RNA structure

While analysing the CUG repeat region of DMPK RNA which is implicated in pathogenesis of myotonic dystrophy, we found that the repeats form a novel type of hairpin structure. The stem in the (CUG)n hairpins is a relaxed duplex structure, the stability of which increases with the repeat length. In addition to having the quasistable stem the (CUG)n hairpins were shown to be „slippery". They showed several different alignments of the repeated sequence, giving the impression of RNA slippage on the CUG repeats.

CANCER GENETICS

KEYWORDS:
breast cancer susceptibility genes • DNA mutation screening technology • SSCP-heteroduplex analysis • trinucleotide repeat expansion diseases • RNA repeats in pathogenesis • RNA structure probing • metal ion-induced hydrolysis

Analysis of heterogeneous RNA structures

Human FMR-1 mRNA fragments containing the CGG repeats and AGG interruptions show high propensity to form different stable conformers under conditions of structure probing. Two alternative approaches designed to obtain structural information specific for individual conformers have been developed, and the stable conformers have been shown to be a single, distorted hairpin and a bifurcated Y-shaped structure. Structural role of the AGG interruptions in transcripts has been revealed.

Modified protocol for RNA structure probing

Extended protocol has been proposed for RNA structure probing in solution using biochemical approach. It includes a test for RNA sequence homogeneity and a test for molecularity and number of stable conformers. The protocol is recommended to follow when approaching structural analysis of any new RNA fragment of potential functional significance.

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LABORATORY OF PHYTOCHEMISTRY

Head: ***Krzysztof Gulewicz***

Staff: *Maciej Stobiecki, Przemysław Wojtaszek, Wojciech Wysocki, Danuta Ciesiołka, Małgorzata Zielińska*

Ph.D. students: *Paweł Bednarek, Mariola Piślewska*

Undergraduate students: *Małgorzata Kulik, Magdalena Rapp, Maciej Buśko, Adam Burzyński, Jarosław Majewski*

Research carried out in the laboratory focuses on the problems within two areas of plant biology: 1) the identification and biological activity of lupin natural products, and their use for ecological agriculture, and 2) the mechanisms of plant responses to pathogenic or symbiotic microorganisms, with lupin plants as a model system.

Bitter lupin plants can be utilized as a valuable source of proteins and other natural products. However, high content of quinolizidine alkaloids makes their use possible only after some kind of processing. During our studies, a strategy for complete utilization of lupin plants has been developed, where seeds of bitter lupin plants are debittered (= their alkaloids removed) and used as a source of protein in livestock fodder. The extract remaining after the debittering process reveals several interesting biological activities, and could be used e.g. as a yield promoting agent, or as a source of some biologically and pharmacologically active compounds with or without biotechnological processing. Finally, the straw remaining after the lupin seed harvest is used for composting, and the preparations thus obtained can be used for biological plant protection.

Another field of interest includes the plant-microorganisms interactions, particularly plant responses to pathogenic and/or symbiotic microorganisms. Two major aspects of these responses are studied: 1) changes in plant secondary meta-bolism in response to pathogenic attack or at the first stages of legume-*Rhizobium* interactions, and 2) the role of plant cell walls, with particular emphasis on plant cell wall glycoproteins, in both types of interactions. Lupin plants or cell cultures are used as model systems, and defense responses are induced with the use of fungal elicitor molecules or some abiotic factors. Two major groups of secondary metabolites are analyzed, namely phenolic compounds (phenolic acids and isoflavonoids), and quinolizidine alkaloids. We hope that multidirectional correlation of data should enable us to get insight into the important differences between plant responses to pathogenic and symbiotic microorganisms.

Current research activities:

- isolation and characterization of biologically active compounds from plants;
- studies on the potential biotechnological use of lupin extract;
- use of lupin compost preparations in biological plant protection;
- analysis of changes in the secondary metabolism of plants in response to pathogenic and symbiotic microorganisms;
- determination of the role of plant cell wall (glyco)proteins in interactions with microorganisms.

Major recent results

Secondary metabolites from plant extracts

Phenolic compounds and quinolizidine alkaloids play important roles in plant defence against herbivores or in interactions with microbes. These low molecular weight compounds are also main components of the extract obtained as a result of debittering of high alkaloid lupin seeds. In the course of our studies we elaborated an efficient procedure for isolation of the secondary metabolites with fractions highly enriched with compounds from a given class as end products. The central point of this procedure is the solid phase extraction on silica gels modified with octadecyl groups for the phenolic compounds purification, and with benzenesulfonic acid for the isolation of quinolizidine alkaloids. Mixtures extracted in this way are further characterized by GC-MS or LC methods.

Yield promoting activity of lupin extract

The extract obtained after the debittering of high alkaloid lupin seeds was initially treated as a waste product. However, long-term studies on its use in agriculture indicated its potential importance as a yield promoting agent. The lupin extract was used as a spray on cultures of 14 different crops (cereals, root crops, and vegetables). It was demonstrated that such treatment promoted the growth and development of plants. No negative effects of the extract spray were noted. The studies performed on rats also indicated that the spray improved the biological value of proteins from the tested plants.

Exudation and accumulation of isoflavonoids

The formation of legume-*Rhizobium* symbiosis is initiated by the exchange of signalling molecules. Depending on the plant species, flavonoids or isoflavonoids, exuded by roots, are major inducers of nodulation. On the other hand, the initiation of nodulation is sensitive to various external stimuli, such as the level of nitrogen in the soil or presence of plant growth regulators. We have analyzed the exudation and accumulation of free and conjugated phenolic compounds by lupin (*L. albus*) roots.

PHYTOCHEMISTRY

KEYWORDS:
lupin natural products • ecological agriculture • plant-microbe interactions • plant defence reactions • secondary metabolites • cell wall proteins

We demonstrated that isoflavonoids were the major phenolics present both in roots and in root exudates. The most important finding, however, was the demonstration that together with the increase of the concentration of nitrogen, regardless of its form (NO_3^-, NH_4^+ or urea), the exudation of isoflavonoids decreased. This indicated the existence of possible additional controls in the regulation of the nodulation process.

Lupin phytoalexins

Plants react to pathogenic infection with a broad array of defence responses. The best characterized reaction is the production of low molecular weight antimicrobial compounds, termed phytoalexins. In legume plants these molecules are mainly isoflavonoids with the pterocarpan-type structures. However, up to now no data have been available on the presence and structure of phytoalexins in lupin plants. Using the cut lupin cotyledon assay and the elicitor preparation derived from the yeast cell walls, we demonstrated that the major phenolic compound exuded in response to elicitation was simple isoflavone, present constitutively in lupin tissues – 2'-hydroxygenistein. No isoflavonoids of pterocarpan-type structure were found thus opening the possibility that in lupins simple isoflavones may act as phytoalexins.

Glycoproteins of the plant extracellular matrix

Plant cell walls together with the intercellular spaces form an apoplast – a central element in the interactions of plants with the environment. As most of the cell wall proteins are highly posttranslationally modified, their biochemical characterization and ultrastructural localization are of particular importance. Exocellular (glyco)proteins play an important role in plant defense responses against invading pathogens. A small subset of glycoproteins becomes immobilized in the walls due to oxidative formation of cross-links with other wall components, and the putative mechanism of this phenomenon has been demonstrated. Three major pathogenesis defense-related wall proteins have been identified in lupin cell culture and their biochemical and cytochemical characterization is now in progress.

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CENTER FOR BIOCRYSTALLOGRAPHIC RESEARCH

Head: ***Mariusz Jaskólski***

Staff: *Dorota A. Adamiak, Grzegorz Bujacz, Marian Gawron, Mirosław Gilski, Mieczysława Kluge,*

Ph.D. Students: *Dominika Borek, Robert Janowski*

Undergraduate Students: *Karolina Michalska, Piotr Paryzek*

X-Ray crystallography has been the major source of information about the 3D structure of macromolecules. Typically, crystallographic studies of the structure of a macromolecule are divided into several separate steps. First, the biomolecule under study has to be obtained in crystalline form (often a success-limiting step). Next, the crystals are exposed to X-rays and the diffraction pattern is recorded. The diffraction pattern is then the basis for the structure determination process. The Center is equipped and prepared for carrying out all the steps necessary for macromolecular structure determination, from protein purification and crystallization, through diffraction experiments, to structure evaluation and analysis. Biomolecules studied in the Center include both proteins and oligonucleotides. Some of the projects are rooted in the Institute of Bioorganic Chemistry (ferritin, napin, oligonucleotides) while others are based on extramural collaboration (myoglobin, retroviral proteins, asparaginases, S-adenosylhomocysteinase, TNF-α muteins). The Center was created in 1994 through financial support from the Foundation for Polish Science.

Current research activities and major results

Crystallographic studies of bacterial and fungal L-asparaginases

L-Asparaginases, which hydrolyse asparagine to aspartic acid and ammonia, have been found in some mammals, fungi, plants and bacteria. For many years, the enzymes from bacteria (*Escherichia coli*, EcAII, and *Erwinia chrysanthemi*, ErA) have been in clinical use for the treatment of acute lymphoblastic leukemia. Seventeen years of crystallographic effort culminated in 1993 in the determination of the structure of EcAII by a team which included one of our current members (MJ). This project became the leading theme of our crystallographic studies and is currently sponsored by the Howard Hughes Medical Institute (USA). Recently, we have crystallized and solved the structure of several point mutants of EcAII and of a new crystallographic form of ErA. The structures should shed more light on the active site of the enzyme which is formed

by a T-K-D triad (reminiscent of the active site of serine proteases) and on the enzymatic mechanism. Further plans include crystallographic studies of L-asparaginases from eukaryotic sources (yeast). The ultimate goal is to design new mutants with better therapeutic values.

Crystallographic studies of retroviral integrase

In a collaborative project with Dr. A. Wlodawer (NCI-FCRDC, USA) and Dr. A.M. Skalka (FCCC, USA), we have determined a high resolution structure of the catalytic domain of Avian sarcoma virus (ASV) integrase. The retroviral integrase is essential for the viral life cycle as it incorporates viral DNA into the host genome. Therapies directed at its inactivation could help control retroviral diseases, such as AIDS. The ASV integrase active site has been characterized in its apo form and in complexes with divalent metal cations which are necessary cofactors during viral integration.

Site-directed mutational and crystallographic studies of myoglobin

Our structural studies of genetically engineered mutants of porcine myoglobin are carried out together with Dr. A. J. Wilkinson (Univ. of York, U.K.) to explore the determinants of ligand binding, in particular the role of polarity and steric effects. Recently, we have determined the crystal structure of a double mutant, H64V/V68H, in which two distal residues, essential for ligand binding, are swapped. The structure of this mutant (which is defective in O_2 but not CO or NO binding) shows that not only the chemical nature but also precise spatial disposition of the residues is of crucial importance. The structure of another mutant, V68N, shows the molecular basis (extra hydrogen bond formed by the mutated residue) of its increased O_2 *vs.* CO differential affinity.

Crystallographic studies of S-adenosylhomocysteinase

We have successfully crystallized S-adenosylhomocysteinase (SAHase) isolated from *Lupinus luteus* (Prof. A. Guranowski, Agricultural Univ., Poznań). SAHase is a hydrolytic enzyme involved in the control of cellular methylation processes. We plan to identify the SAHase gene and to design an efficient expression system. Further, we plan to overproduce the protein as a selenomethionyl derivative and to solve its crystal structure using multiwavelength anomalous diffraction.

Crystallographic studies of cysteine proteases and their inhibitors

Several serious diseases related to tissue degeneration, such as muscular dystrophy, are linked to abnormalities in the function of cysteine proteases. There is a variety of natural inhibitors of cysteine proteases, ranging from sizable proteins (rystatin superfamily) to relatively small molecules, such as E-64. The latter compound, which is among the most potent inhibitors, contains an oxirane ring which reacts with the catalytic thiol group of the enzyme to form an irreversible covalent bond. Together with our collaborator, Prof. Z. Grzonka (Univ. of Gdańsk), we are interested in structural details, at atomic level, of the complexes formed between papain (a cysteine protease) and various

KEYWORDS:
protein crystallography • macromolecular crystallization • myoglobin • L-asparaginase • retroviral integrase • retroviral protease • S-adenosyl-homocysteinase • ferritins • napin • TNF-α • pre-tRNA • oligonucleotides

inhibitors. In one project, we are trying to grow X-ray crystals of papain inhibited with unmodified human cystatin. In another study, we have determined a high resolution structure of a covalent complex between papain and an irreversible synthetic inhibitor based on E-64.

Crystallographic studies of $(C \cdot G)_n$ RNA duplexes

Our studies of RNA oligonucleotides are done in collaboration with Prof. R.W. Adamiak from the Institute of Bioorganic Chemistry. After several years of effort, we finally managed to grow X-ray quality crystals of a self-complementary O2'-methylated RNA duplex, CGCGCG. Together with Drs. W. Rypniewski and Z. Dauter (EMBL, Hamburg), we solved the crystal structure to very high resolution (1.3 Å) from synchrotron diffraction data and demonstrated that the hexamer exists in type A double-helical form.

Crystal chemistry of nucleic acids constituents

Our interest in crystal chemistry of nucleosides is rooted in a long standing collaboration with Prof. M.D. Bratek-Wiewiórowska and Prof. M. Wiewiórowski of the Institute of Bioorganic Chemistry. In our earlier studies we discovered phosphate salts of nucleosides in which phosphate-sugar hydrogen bonds mimic the ester links in polynucleotide chains and described the formation of $C^+ \cdot C$ base pairs in 2'-deoxycytidine hemiphosphate. More recently, we have solved the crystal structure of two new forms of 2'-deoxycytidine, a nucleoside once thought to be an unrewarding compound for crystallization.

Crystallization of proteins and nucleic acids

In addition to those mentioned above, there are several other projects which are currently in the crystallization stage.

- plant ferritin (iron storage protein) from *L. luteus*
- napin (seed storage protein) from *Brassica napus*
- TNF-α muteins
- pre-tRNA (immature tRNA) from *Arabidopsis thaliana*

As the Center was established only in 1994 the selected publications mainly include papers by its staff members from their previous research carried out elsewhere.

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POZNAŃ SUPERCOMPUTING AND NETWORKING CENTER

Head: **Scientific Director:** ***Jan Węglarz***
Technical Director: ***Maciej Stroiński***
Chairman of the Council of Users: ***Jacek Rychlewski***

Department Managers:
Artur Binczewski (Network Department)
Krystyna Lewandowska (Administration Department)
Cezary Mazurek (Internet & Network Services Department)
Norbert Meyer (Supercomputing Department)
Sławomir Niwiński (Scientific Applications Department)

Staff: *Krzysztof Bober, Maciej Bocian, Krzysztof Brodecki, Joseph Cancellaro, Michał Chmielewski, Włodzimierz Dymaczewski, Dariusz Ferenc, Paweł Flotyński, Bartosz Gajda, Marcin Garstka, Bartłomiej Grenda, Henryk Gołębiewski, Adam Gowdiak, Piotr Grzybowski, Tomasz Jabłoński, Maciej Jarkowski, Michał Kosiedowski, Mirosław Kupczyk, Marcin Lawenda, Bartosz Lewandowski, Elżbieta Łobaza, Danuta Marek, Rafał Mikołajczak, Małgorzata Miller, Jarosław Nabrzyski, Zbigniew Ołtuszyk, Tomasz Ostwald, Leszek Pisarek, Wiktor Procyk, Michał Przybylski, Juliusz Pukacki, Tomasz Rajtar, Maciej Rutkowski, Krzysztof Stanecki, Paweł Stencel, Anna Strumnik, Ewa Surmanowicz, Tomasz Szewczyk, Sebastian Szuber, Szymon Trocha, Andrzej Waligóra, Piotr Wierzejewski, Paweł Wolniewicz, Michał Zawirski*

Poznań Supercomputing and Networking Center (PSNC) was founded at the end of 1993 by the State Committee for Scientific Research (KBN). Due to the decision of the Conference of Rectors of the City of Poznań, PSNC was affiliated to the Institute of Bioorganic Chemistry of the Polish Academy of Sciences (PAS). Main fields of activities of PSNC cover: operating the Poznań Metropolitan Area Network (POZMAN) and national scientific network POL-34, computations in a metacomputer environment, integration of scientific research on computing methods, promotion of new high performance computing and networking technologies.

POZMAN is the double technology, FDDI and ATM network. It consists of 190 km fiber optic cable connections with 20 NetBulider II routers and 7 ATM switches. The network connects all the major scientific institutions in Poznań. POZMAN offers the following services: access to metacomputer including vector, scalar and scalable parallel high performance computations, e-mail services, anonymous FTP, UseNet News and WWW services. It has connections with the main telecommunication operators in Poland: TP S.A., TEL-ENERGO, PKP, TELBANK, NASK.

Based on TEL-ENERGO SDH network, PSNC has built a national broadband ATM 34 Mbps network called POL-34. This network connects the main MANs in Poland, enabling them to effectively use Internet services, connect to supercomputing centers and access advanced telematic services.

The current architecture of a metacomputer consists of systems with shared memory architecture (scalar: SGI Power Challenge, vector: Cray J916, Cray Y-MP EL), distributed memory (IBM SP-2), a massively parallel Cray T3E-900, data managing systems and visualization laboratory. The installed systems have the following characteristics:

- Cray Y-MP EL – 4 processors, 512 MB operating memory, 20GB disc memory, compatible with other Y-MP systems,
- Cray J916 – 16 processors, 4GB operating memory, 167 GB disc memory: compared to the Y-MP EL system, it has a greater possibility of scalability, a faster central processing unit, faster access to operating memory, and additional network interfaces,
- SGI Power Challenge, XL – 12 processors R8000, 1GB operating memory, 20GB disc memory,
- IBM SP-2 scalable 15 nodes system containing 1 wide node (POWER2 processor 66MHz, 128 MB operating memory, 18GB disc memory) and 14 thin nodes (POWER2 processors 66MHz, 64 MB operating memory, 1GB disc memory),
- Cray T3E-900 – 8 processing elements with 128 MB operating memory each, 38GB disc memory)
- AUSPEX NS 7000/500 – network file server used to export files to other computers due to NFS protocol. It supports the disc memory of Cray and SGI supercomputers,
- Archives containing an ATL ACL 2640 automated tape library, HP 165 ST magneto-optical disc library together with the UniTree management system,
- Visualization laboratory – widens the range of possible services rendered in PSNC introducing pre-processing and post-processing graphical elements and visualization of the results of the conducted computations. The visualization laboratory consists of SGI Onyx2 Infinite Reality2, SGI Onyx Reality Engine 2, 4 Indigo 2 XZ, Indigo 2 Extreme, 2 Indy S.C. stations, Indy PC and Challenge S WEBForce.

The research interest of the Poznań Supercomputing and Networking Center covers:

- the development of algorithms for parallel and distributed processing in various branches of science and technology;
- the development of algorithms for integrated management of systems and networks together with their environment;
- studies of the communication complexity of parallel and distributed algorithms;
- the development of broadband telematic services.

AND NETWORKING CENTER

KEYWORDS:
metropolitan area network • FDDI network • ATM network • metacomputer • distributed processing • vector processing • parallel computing • multimedia.

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RESEARCH PROJECTS

Grants from State Committee for Scientific Research (KBN)

M. D. Bratek-Wiewiórowska	Crystal engineering of DNA and RNA fragments.	Aug. 1995 – Jun. 1998
J. Kapusta	Regeneration *in vitro* of selected *Lupinus* species for genetics and agriculture.	Sep. 1995 – Feb. 1998
J. Barciszewski	Application of the structural studies of proteins to the analysis of their functions. Mechanism of plant resistance and properties of plant storage proteins.	Jan. 1996 – Dec. 1998
M. Sikorski	Molecular aspects of plant response to stress and their biotechnological application	Jan. 1996 – Dec. 1998
T. Twardowski	Activity of ribozymes and antisense oligomers in plant translation system. Structure-function correlation of nucleic acids.	Jan. 1996 – Dec. 1998
R. Kierzek	The influence of uridine modification on the thermal stability of the RNA duplexes.	Apr. 1996 – Dec. 1998
R. Kierzek	The model ribozymes. The structure and self-hydrolytic properties of single-stranded oligoribonucleotides.	Aug. 1996 – Jul. 1999
W. J. Krzyżosiak	Techniques used for detection of dispersed mutations in large genes. The BRCA1 gene example.	Jul. 1996 – Jun. 1999
W. J. Krzyżosiak	Structure of human mRNA fragments containing trinucleotide repeats.	Jul. 1996 – Jun. 1999
W. J. Krzyżosiak	Analysis of BRCA1 mRNA structure.	Jul. 1996 – Jun. 1999

T. Stępkowski	The role of the NOD factor from *Bradyrhizobium* sp. (*Lupinus*) in determination of morphogenetic processes occuring during nodule development in lupin.	Jul. 1996 – Jun. 1999
R. W. Adamiak	TAR element of the retroviral HIV-1 RNA. Chemistry, stereodynamics and interaction with recombinant *Tat* protein.	Jan. 1997 – Dec. 1999
J. Boryski	Application of transglycosylation method to stereoselective synthesis of biologically active ribonucleosides, 2'-deoxy-β-ribonucleosides and their modified analogs.	Jan. 1997 – Dec. 1999
J. Ciesiołka	Ribozymes of hepatitis *delta* virus.	Jan. 1997 – Dec. 1999
M. Figlerowicz	Structural requirements of genetic recombination in RNA viruses	Jan. 1997 – Dec. 1999
W. J. Krzyżosiak	Technology of genetic test for hereditary predisposition to breast and ovarian cancer	Jan. 1997 – Dec. 1998
W. T. Markiewicz	Development and applications of technologies of dispersed and integrated combinatorial oligonucleotide libraries.	Jul. 1997 – Jun. 2000
P. Wojtaszek	Biochemical characterization, ultrastructural localization, and molecular analysis of selected defence-related proteins from white lupin.	Jul. 1997 – Jun. 2000
P. Stróżycki	Molecular basis of iron management in plant tissues.	Jul. 1997 – Jun. 2000
J. Podkowiński	ENOD40 – signal oligopeptide, a new regulator of plant morphogenesis.	Jul. 1997 – Jun. 2000
W. J. Krzyżosiak	Studies on polymorphism of BRCA1 and BRCA2 genes and its association with breast cancer risk.	Oct. 1997 – Sep. 2000
A. Kraszewski	Aryl nucleoside H-phosphonates – novel derivatives of controlled reactivity in oligonucleotide and their analogues synthesis.	Jan. 1998 – Dec. 2000

J. Barciszewski	CDNA cloning, expression and properties of methionyl-tRNA synthetase.	Jan. 1998 – Dec. 1999
W. T. Markiewicz	Chemical synthesis of oligonucleotides carrying 2'-O-ribosyl units.	Jul. 1998 – Jun. 2000
W. T. Markiewicz	Semi-synthetic combinatorial libraries of fragments of antibodies (Fab) and their applications.	Jan. 1999 – Dec. 2000
J. Kapusta	Plant based edible vaccine against *Hepatitis* B.	Jan. 1999 – Dec. 2001
R. W. Adamiak	Conformational analysis of modified DNA and RNA duplexes by molecular dynamics simulation methods.	Jan. 1999 – Dec. 2000
B. Golankiewicz	Synthesis of second generation tricyclic nucleosides for antiviral and gene anticancer therapy.	Jan. 1999 – Dec. 2001
T. Twardowski	Molecular properties of plant ferritin; correlation between biological functions and potential application.	Apr. 1999 – Dec. 2000

GRANTS FROM FOREIGN SOURCES

Commission of European Communities

T. Twardowski	Biotechnology and the European public.	Jul. 1996 – Jul. 1999

French-Polish Biotechnology Center

M. Barciszewska, J. Barciszewski, A. B. Legocki and coworkes.	Nine projects on isolation and cloning of methionyl-tRNA synthetase, transcription mechanism of plant polymerase III and characterization of plant genes and their expression during symbiosis.	Jul. 1997 – May 1999

Howard Hughes Medical Institute (USA)

M. Jaskólski	Crystallographic studies of antitumor amidohydrolases.	Aug. 1995 – Aug. 1999

Fogarty International Research Collaboration Award (FIRCA)

R. Kierzek	Folding RNA with Modified Oligonucleotides	Feb. 1999 – Jan. 2002

LIST OF KEYWORDS

STAFF

Professors

Adamiak, Ryszard W. 9, **17**, 59, 66, 67	adamiakr@ibch.poznan.pl
Barciszewska, Mirosława 9, **41**, 68	mbarcisz@ibch.poznan.pl
Barciszewski, Jan 9, **41**, 65, 67, 68	jbarcisz@ibch.poznan.pl
Boryski, Jerzy 9, **13**, 66	jboryski@ibch.poznan.pl
Bratek-Wiewiórowska, Maria D. 9, **21**, 59, 65	mdbw@ibch.poznan.pl
Ciesiołka, Jerzy 9, 21, **25**, 66	ciesiolk@ibch.poznan.pl
Golankiewicz, Bożenna 9, **13**, 67	bogolan@ibch.poznan.pl
Gulewicz, Krzysztof 9, **53**	krysgul@ibch.poznan.pl
Jaskólski, Mariusz 9, **57**, 68	mariusz@krystal.amu.edu.pl
Kierzek, Ryszard 9, **21**, 65, 68	rkierzek@ibch.poznan.pl
Kraszewski, Adam 10, **37**, 67	akad@ibch.poznan.pl
Krzyżosiak, Włodzimierz J. 10, 26, **49**, 65, 66	wlodkrzy@ibch.poznan.pl
Legocki, Andrzej B. 10, **33**, 68	legocki@ibch.poznan.pl
Markiewicz, Wojciech T. 10, **29**, 66, 67	markwt@ibch.poznan.pl
Rychlewski, Jacek 10, **61**	rychlew@man.poznan.pl
Twardowski Tomasz 10, **45**, 65, 67, 68	twardows@ibch.poznan.pl
Węglarz, Jan 10, **61**	weglarz@man.poznan.pl
Wiewiórowski, Maciej 10, **21**	

Ph.D. Research Associates

Astriab, Anna **29**	astriab@ibch.poznan.pl
Adamiak, Dorota 18, **57**	dorota@tryptophan.ibch.poznan.pl
Alejska, Magdalena **25**	magdaale@ibch.poznan.pl
Biała, Ewa **17**	biala@ibch.poznan.pl
Fischer, Artur **17**	marekf@ibch.poznan.pl
Figlerowicz, Marek 9, **25**, 66	afischer@ibch.poznan.pl
Gawron, Marian **57**	mgawron@ibch.poznan.pl
Giel-Pietraszuk, Małgorzata **41**	giel@ibch.poznan.pl
Gdaniec, Zofia **17**	zgdan@ibch.poznan.pl
Grajkowski, Andrzej **29**	agrajko@ibch.poznan.pl
Imiołczyk, Barbara **37**	agata@ibch.poznan.pl
Jankowska, Jadwiga **37**	jadwigaj@ibch.poznan.pl
Januszczyk, Piotr **13**	pioczyk@ibch.poznan.pl
Jeleńska, Joanna **33**	jelenska@ibch.poznan.pl
Kapusta, Józef **33**, 65, 67	jozefk@ibch.poznan.pl
Kulińska, Katarzyna 7, **17**	kasiak@ibch.poznan.pl
Kuliński, Tadeusz **17**	tadkul@ibch.poznan.pl
Manikowski, Andrzej **13**	manimani@ibch.poznan.pl
Markiewicz, Maria **29**	marmar@ibch.poznan.pl
Michałowski, Daniel **49**	damm@ibch.poznan.pl
Ostrowski, Tomasz **13**	tostr@ibch.poznan.pl
Perkowska, Anna **25**	annaper@ibch.poznan.pl
Podkowiński, Jan **33**, 66	jantsp@ibch.poznan.pl
Sarzyńska, Joanna **17**	asias@ibch.poznan.pl
Sobkowski, Michał **37**	msob@ibch.poznan.pl

STAFF

Professors

Adamiak, Ryszard W. 9, **17**, 59, 66, 67	adamiakr@ibch.poznan.pl
Barciszewska, Mirosława 9, **41**, 68	mbarcisz@ibch.poznan.pl
Barciszewski, Jan 9, **41**, 65, 67, 68	jbarcisz@ibch.poznan.pl
Boryski, Jerzy 9, **13**, 66	jboryski@ibch.poznan.pl
Bratek-Wiewiórowska, Maria D. 9, **21**, 59, 65	mdbw@ibch.poznan.pl
Ciesiołka, Jerzy 9, 21, **25**, 66	ciesiolk@ibch.poznan.pl
Golankiewicz, Bożenna 9, **13**, 67	bogolan@ibch.poznan.pl
Gulewicz, Krzysztof 9, **53**	krysgul@ibch.poznan.pl
Jaskólski, Mariusz 9, **57**, 68	mariusz@krystal.amu.edu.pl
Kierzek, Ryszard 9, **21**, 65, 68	rkierzek@ibch.poznan.pl
Kraszewski, Adam 10, **37**, 67	akad@ibch.poznan.pl
Krzyżosiak, Włodzimierz J. 10, 26, **49**, 65, 66	wlodkrzy@ibch.poznan.pl
Legocki, Andrzej B. 10, **33**, 68	legocki@ibch.poznan.pl
Markiewicz, Wojciech T. 10, **29**, 66, 67	markwt@ibch.poznan.pl
Rychlewski, Jacek 10, **61**	rychlew@man.poznan.pl
Twardowski Tomasz 10, **45**, 65, 67, 68	twardows@ibch.poznan.pl
Węglarz, Jan 10, **61**	weglarz@man.poznan.pl
Wiewiórowski, Maciej 10, **21**	

Ph.D. Research Associates

Astriab, Anna **29**	astriab@ibch.poznan.pl
Adamiak, Dorota 18, **57**	dorota@tryptophan.ibch.poznan.pl
Alejska, Magdalena **25**	magdaale@ibch.poznan.pl
Biała, Ewa **17**	biala@ibch.poznan.pl
Fischer, Artur **17**	marekf@ibch.poznan.pl
Figlerowicz, Marek 9, **25**, 66	afischer@ibch.poznan.pl
Gawron, Marian **57**	mgawron@ibch.poznan.pl
Giel-Pietraszuk, Małgorzata **41**	giel@ibch.poznan.pl
Gdaniec, Zofia **17**	zgdan@ibch.poznan.pl
Grajkowski, Andrzej **29**	agrajko@ibch.poznan.pl
Imiołczyk, Barbara **37**	agata@ibch.poznan.pl
Jankowska, Jadwiga **37**	jadwigaj@ibch.poznan.pl
Januszczyk, Piotr **13**	pioczyk@ibch.poznan.pl
Jeleńska, Joanna **33**	jelenska@ibch.poznan.pl
Kapusta, Józef **33**, 65, 67	jozefk@ibch.poznan.pl
Kulińska, Katarzyna 7, **17**	kasiak@ibch.poznan.pl
Kuliński, Tadeusz **17**	tadkul@ibch.poznan.pl
Manikowski, Andrzej **13**	manimani@ibch.poznan.pl
Markiewicz, Maria **29**	marmar@ibch.poznan.pl
Michałowski, Daniel **49**	damm@ibch.poznan.pl
Ostrowski, Tomasz **13**	tostr@ibch.poznan.pl
Perkowska, Anna **25**	annaper@ibch.poznan.pl
Podkowiński, Jan **33**, 66	jantsp@ibch.poznan.pl
Sarzyńska, Joanna **17**	asias@ibch.poznan.pl
Sobkowski, Michał **37**	msob@ibch.poznan.pl

Sikorski, Michał 10, **33**, 65	mmsik@ibch.poznan.pl
Stępkowski, Tomasz **33**, 66	sttommic@ibch.poznan.pl
Stobiecki, Maciej 10, **53**	mackis@ibch.poznan.pl
Stróżycki, Paweł **33**, 66	spprom@ibch.poznan.pl
Szymański, Maciej 41	mszyman@ibch.poznan.pl
Wojtaszek, Przemysław **53**, 66	przemow@ibch.poznan.pl
Wrzesiński, Jan **25**	wrzesinj@ibch.poznan.pl
Wyszko, Eliza **41**	wyszkoe@ibch.poznan.pl
Zagórowska, Izabela **37**	izag@ibch.poznan.pl
Zeidler, Joanna **13**	joandler@ibch.poznan.pl

Ph.D. Graduate Students

Astriab, Maciej **45**	astriabm@ibch.poznan.pl
Baranowski, Daniel **13**	danibari@ibch.poznan.pl
Bednarek, Paweł **53**	bedek@ibch.poznan.pl
Bielecki, Łukasz **17**	bielecki@ibch.poznan.pl
Biesiadka, Jacek **33**	jabies@ibch.poznan.pl
Chmielewski, Marcin **29**	maro708@ibch.poznan.pl
Cieślak, Jacek **37**	jcieslak@ibch.poznan.pl
Deniziak, Marzanna **41**	marden@ibch.poznan.pl
Godzina, Przemysław **29**	diament@ibch.poznan.pl
Gośliński, Tomasz **13**	tomigos@ibch.poznan.pl
Handschuh, Luiza **33**	luizahan@ibch.poznan.pl
Jasińska, Anna **49**	aniaj@ibch.poznan.pl
Kamińska Monika **41**	gregor@ibch.poznan.pl
Kozłowski, Piotr **49**	eryk@ibch.poznan.pl
Kwaśnikowski, Piotr **29**	pkk@ibch.poznan.pl
Lisowa, Olesia **33**	olesia@ibch.poznan.pl
Łęgiewicz, Michał **25**	michall@ibch.poznan.pl
Madej, Beata **45**	madej@ibch.poznan.pl
Napierała, Marek **49**	napmarek@ibch.poznan.pl
Olejniczak, Mikołaj **17**	mikolaj@ibch.poznan.pl
Piślewska, Mariola **53**	marpis@ibch.poznan.pl
Pniewski, Tomasz **33**	tompniew@ibch.poznan.pl
Popenda, Mariusz **17**	marpop@ibch.poznan.pl
Ratajska, Żaneta **29**	jeanette@ibch.poznan.pl
Skotnicka Monika **41**	mona@ibch.poznan.pl
Smól, Joanna **45**	jsmol@ibch.poznan.pl
Sobczak, Krzysztof **49**	ksobczak@ibch.poznan.pl
Szepeta, Joanna **41**	szepelia@ibch.poznan.pl
Szymczak, Marzena **37**	mszym@ibch.poznan.pl
Świderska, Anna **33**	annarudz@ibch.poznan.pl
Walczak Elżbieta **21**	ewalczak@ibch.poznan.pl
Wenska, Małgorzata **37**	mwen@ibch.poznan.pl
Witkowska, Iwona **41**	iwit@ibch.poznan.pl
Zaborowska, Żaneta **33**	zanetaz@ibch.poznan.pl
Zandecki, Tomasz **13**	tezande@ibch.poznan.pl

Sikorski, Michał 10, **33**, 65	mmsik@ibch.poznan.pl
Stępkowski, Tomasz **33**, 66	sttommic@ibch.poznan.pl
Stobiecki, Maciej 10, **53**	mackis@ibch.poznan.pl
Stróżycki, Paweł **33**, 66	spprom@ibch.poznan.pl
Szymański, Maciej 41	mszyman@ibch.poznan.pl
Wojtaszek, Przemysław **53**, 66	przemow@ibch.poznan.pl
Wrzesiński, Jan **25**	wrzesinj@ibch.poznan.pl
Wyszko, Eliza **41**	wyszkoe@ibch.poznan.pl
Zagórowska, Izabela **37**	izag@ibch.poznan.pl
Zeidler, Joanna **13**	joandler@ibch.poznan.pl

Ph.D. Graduate Students

Astriab, Maciej **45**	astriabm@ibch.poznan.pl
Baranowski, Daniel **13**	danibari@ibch.poznan.pl
Bednarek, Paweł **53**	bedek@ibch.poznan.pl
Bielecki, Łukasz **17**	bielecki@ibch.poznan.pl
Biesiadka, Jacek **33**	jabies@ibch.poznan.pl
Chmielewski, Marcin **29**	maro708@ibch.poznan.pl
Cieślak, Jacek **37**	jcieslak@ibch.poznan.pl
Deniziak, Marzanna **41**	marden@ibch.poznan.pl
Godzina, Przemysław **29**	diament@ibch.poznan.pl
Gośliński, Tomasz **13**	tomigos@ibch.poznan.pl
Handschuh, Luiza **33**	luizahan@ibch.poznan.pl
Jasińska, Anna **49**	aniaj@ibch.poznan.pl
Kamińska Monika **41**	gregor@ibch.poznan.pl
Kozłowski, Piotr **49**	eryk@ibch.poznan.pl
Kwaśnikowski, Piotr **29**	pkk@ibch.poznan.pl
Lisowa, Olesia **33**	olesia@ibch.poznan.pl
Łęgiewicz, Michał **25**	michall@ibch.poznan.pl
Madej, Beata **45**	madej@ibch.poznan.pl
Napierała, Marek **49**	napmarek@ibch.poznan.pl
Olejniczak, Mikołaj **17**	mikolaj@ibch.poznan.pl
Piślewska, Mariola **53**	marpis@ibch.poznan.pl
Pniewski, Tomasz **33**	tompniew@ibch.poznan.pl
Popenda, Mariusz **17**	marpop@ibch.poznan.pl
Ratajska, Żaneta **29**	jeanette@ibch.poznan.pl
Skotnicka Monika **41**	mona@ibch.poznan.pl
Smól, Joanna **45**	jsmol@ibch.poznan.pl
Sobczak, Krzysztof **49**	ksobczak@ibch.poznan.pl
Szepeta, Joanna **41**	szepelia@ibch.poznan.pl
Szymczak, Marzena **37**	mszym@ibch.poznan.pl
Świderska, Anna **33**	annarudz@ibch.poznan.pl
Walczak Elżbieta **21**	ewalczak@ibch.poznan.pl
Wenska, Małgorzata **37**	mwen@ibch.poznan.pl
Witkowska, Iwona **41**	iwit@ibch.poznan.pl
Zaborowska, Żaneta **33**	zanetaz@ibch.poznan.pl
Zandecki, Tomasz **13**	tezande@ibch.poznan.pl

Computer Scientists

Binczewski Artur **61**	artur@man.poznan.pl
Bober Krzysztof **61**	bobik@man.poznan.pl
Bocian Maciej **61**	maciejb@man.poznan.pl
Brodecki Krzysztof **61**	kbrod@man.poznan.pl
Cancellaro Joseph **61**	joec@man.poznan.pl
Chmielewski Michał **61**	bfis@man.poznan.pl
Dymaczewski Włodzimierz **61**	dymaczew@man.poznan.pl
Ferenc Dariusz **61**	ferenc@man.poznan.pl
Gajda Bartosz **61**	gajda@man.poznan.pl
Garstka Marcin **61**	marcinga@man.poznan.pl
Gowdiak Adam **61**	zupa@man.poznan.pl
Grenda Bartłomiej **61**	grenda@man.poznan.pl
Grzybowski Piotr **61**	piotrgrb@man.poznan.pl
Jabłoński Tomasz **61**	tomjab@man.poznan.pl
Kosiedowski Michał **61**	kat@man.poznan.pl
Kupczyk Mirosław **61**	miron@man.poznan.pl
Lawenda Marcin **61**	lawenda@man.poznan.pl
Lewandowski Bartosz **61**	bartekel@man.poznan.pl
Niwiński Sławomir **61**	niwinski@man.poznan.pl
Mazurek Cezary **61**	mazurek@man.poznan.pl
Meyer Norbert **61**	meyer@man.poznan.pl
Mikołajczak Rafał **61**	fuji@man.poznan.pl
Nabrzyski Jarosaw **61**	naber@man.poznan.pl
Ołtuszyk Zbigniew **61**	neevor@man.poznan.pl
Ostwald Tomasz **61**	leon@man.poznan.pl
Pisarek Leszek **61**	leszekp@man.poznan.pl
Procyk Wiktor **61**	wiku@man.poznan.pl
Przybylski Michał **61**	michalp@man.poznan.pl
Pukacki Juliusz **61**	pukacki@man.poznan.pl
Rajtar Tomasz **61**	ritter@man.poznan.pl
Rutkowski Maciej **61**	mrut@man.poznan.pl
Stanecki Krzysztof **61**	stanecki@man.poznan.pl
Stencel Paweł **61**	paulus@man.poznan.pl
Stroiński Maciej **61**	stroins@man.poznan.pl
Surmanowicz Ewa **61**	es@man.poznan.pl
Szewczyk Tomasz **61**	tomasz@man.poznan.pl
Szuber Sebastian **61**	sebach@man.poznan.pl
Trocha Szymon **61**	konserwa@man.poznan.pl
Waligóra Andrzej **61**	bronek@man.poznan.pl
Wierzejewski Piotr **61**	wierzej@man.poznan.pl
Wolniewicz Paweł **61**	pawelw@man.poznan.pl

Technicians

Adamska Zofia **13**
Adrych-Rożek Katarzyna **29**

Computer Scientists

Binczewski Artur **61**	artur@man.poznan.pl
Bober Krzysztof **61**	bobik@man.poznan.pl
Bocian Maciej **61**	maciejb@man.poznan.pl
Brodecki Krzysztof **61**	kbrod@man.poznan.pl
Cancellaro Joseph **61**	joec@man.poznan.pl
Chmielewski Michał **61**	bfis@man.poznan.pl
Dymaczewski Włodzimierz **61**	dymaczew@man.poznan.pl
Ferenc Dariusz **61**	ferenc@man.poznan.pl
Gajda Bartosz **61**	gajda@man.poznan.pl
Garstka Marcin **61**	marcinga@man.poznan.pl
Gowdiak Adam **61**	zupa@man.poznan.pl
Grenda Bartłomiej **61**	grenda@man.poznan.pl
Grzybowski Piotr **61**	piotrgrb@man.poznan.pl
Jabłoński Tomasz **61**	tomjab@man.poznan.pl
Kosiedowski Michał **61**	kat@man.poznan.pl
Kupczyk Mirosław **61**	miron@man.poznan.pl
Lawenda Marcin **61**	lawenda@man.poznan.pl
Lewandowski Bartosz **61**	bartekel@man.poznan.pl
Niwiński Sławomir **61**	niwinski@man.poznan.pl
Mazurek Cezary **61**	mazurek@man.poznan.pl
Meyer Norbert **61**	meyer@man.poznan.pl
Mikołajczak Rafał **61**	fuji@man.poznan.pl
Nabrzyski Jarosaw **61**	naber@man.poznan.pl
Ołtuszyk Zbigniew **61**	neevor@man.poznan.pl
Ostwald Tomasz **61**	leon@man.poznan.pl
Pisarek Leszek **61**	leszekp@man.poznan.pl
Procyk Wiktor **61**	wiku@man.poznan.pl
Przybylski Michał **61**	michalp@man.poznan.pl
Pukacki Juliusz **61**	pukacki@man.poznan.pl
Rajtar Tomasz **61**	ritter@man.poznan.pl
Rutkowski Maciej **61**	mrut@man.poznan.pl
Stanecki Krzysztof **61**	stanecki@man.poznan.pl
Stencel Paweł **61**	paulus@man.poznan.pl
Stroiński Maciej **61**	stroins@man.poznan.pl
Surmanowicz Ewa **61**	es@man.poznan.pl
Szewczyk Tomasz **61**	tomasz@man.poznan.pl
Szuber Sebastian **61**	sebach@man.poznan.pl
Trocha Szymon **61**	konserwa@man.poznan.pl
Waligóra Andrzej **61**	bronek@man.poznan.pl
Wierzejewski Piotr **61**	wierzej@man.poznan.pl
Wolniewicz Paweł **61**	pawelw@man.poznan.pl

Technicians

Adamska Zofia **13**
Adrych-Rożek Katarzyna **29**

Bekasiak Barbara **49**	
Ciesiołka Danuta **53**	
Dominiak Grażyna **17**	
Dymarek-Babś Teresa **49**	
Femiak Iwona **33**	iwofem@ibch.poznan.pl
Gawrońska Iwona **41**	iwonag@ibch.poznan.pl
Gilski Mirosław **57**	mirek@ibch.poznan.pl
Glapiak Hanna **33**	
Kasperska Alina **33**	alinakas@ibch.poznan.pl
Kluge Mieczysława **57**	
Ludwiczak Krystyna **45**	
Lembicz Krystyna **13**	
Malinowska Nelli **25**	
Michalski Zbigniew	michalsk@ibch.poznan.pl
Mnichowska Dorota **17**	
Pasternak Elżbieta **17**	
Smólska Barbara **25**	
Wieczorek Elżbieta **41**	
Więckowska Danuta **17**	
Wysocki Wojciech **53**	
Zielińska Małgorzata **53**	

Administration

Adamczyk Elżbieta	eada@ibch.poznan.pl
Cudzińska Marzena	
Dąbkiewicz Małgorzata	
Gołaś Danuta	danutag@ibch.poznan.pl
Górnicka-Holeczek Hanna **45**	
Kaczmarek Danuta	
Kaczyńska-Kubiak Jolanta	
Kopińska-Figlarz Maria	
Kosicki Roman	
Kozakiewicz Elżbieta	
Krajewski Ryszard	
Lewandowska Krystyna **61**	
Łobaza Elżbieta **61**	krysia@man.poznan.pl
Marek Danuta **61**	ela@man.poznan.pl
Miler Małgorzata **61**	danka@man.poznan.pl
Muszyńska Urszula	malgos@man.poznan.pl
Ostach Paweł	ulka@ibch.poznan.pl
Ostój-Sędziak Zofia	
Sobczak Elżbieta	
Słowińska Ewa	
Stępkowska Joanna	joastep@ibch.poznan.pl
Strumnik Anna **61**	anna@man.poznan.pl
Szubińska Grażyna	grazyna@ibch.poznan.pl

Bekasiak Barbara **49**	
Ciesiołka Danuta **53**	
Dominiak Grażyna **17**	
Dymarek-Babś Teresa **49**	
Femiak Iwona **33**	iwofem@ibch.poznan.pl
Gawrońska Iwona **41**	iwonag@ibch.poznan.pl
Gilski Mirosław **57**	mirek@ibch.poznan.pl
Glapiak Hanna **33**	
Kasperska Alina **33**	alinakas@ibch.poznan.pl
Kluge Mieczysława **57**	
Ludwiczak Krystyna **45**	
Lembicz Krystyna **13**	
Malinowska Nelli **25**	
Michalski Zbigniew	michalsk@ibch.poznan.pl
Mnichowska Dorota **17**	
Pasternak Elżbieta **17**	
Smólska Barbara **25**	
Wieczorek Elżbieta **41**	
Więckowska Danuta **17**	
Wysocki Wojciech **53**	
Zielińska Małgorzata **53**	

Administration

Adamczyk Elżbieta	eada@ibch.poznan.pl
Cudzińska Marzena	
Dąbkiewicz Małgorzata	
Gołaś Danuta	danutag@ibch.poznan.pl
Górnicka-Holeczek Hanna **45**	
Kaczmarek Danuta	
Kaczyńska-Kubiak Jolanta	
Kopińska-Figlarz Maria	
Kosicki Roman	
Kozakiewicz Elżbieta	
Krajewski Ryszard	
Lewandowska Krystyna **61**	
Łobaza Elżbieta **61**	krysia@man.poznan.pl
Marek Danuta **61**	ela@man.poznan.pl
Miler Małgorzata **61**	danka@man.poznan.pl
Muszyńska Urszula	malgos@man.poznan.pl
Ostach Paweł	ulka@ibch.poznan.pl
Ostój-Sędziak Zofia	
Sobczak Elżbieta	
Słowińska Ewa	
Stępkowska Joanna	joastep@ibch.poznan.pl
Strumnik Anna **61**	anna@man.poznan.pl
Szubińska Grażyna	grazyna@ibch.poznan.pl

Szubińska Maria	
Trajnerowicz Katarzyna **61**	kasia@man.poznan.pl
Wojciechowski Marek	marwoj@ibch.poznan.pl
Zielińska Żaneta	zanziel@ibch.poznan.pl

Service

Balczak Józef
Bzdurska Stanisława
Dzięcioł Jerzy
Flotyński Paweł **61**
Gołębiewski Henryk **61**
Grellus Mirosława
Gromanowski Mariusz
Jezierski Mieczysław
Korybalska Lilianna
Kosicka Barbara
Kóska Arleta
Kubacka Iwona
Lecko Paweł
Mager Alicja
Matuszak Jan
Michalska Katarzyna
Mnich Zenon
Nowak Jerzy
Podeszwa Aleksandra
Reinke Iwona
Rucińska Bożena
Siebert Zofia
Sulska Elżbieta
Szułczyńska Janina
Szułczyński Andrzej
Szubińska Błażena
Tajszner Katarzyna
Thiel Renata
Wilandt Elżbieta
Węgrzeniejewski Marek

Szubińska Maria	
Trajnerowicz Katarzyna **61**	kasia@man.poznan.pl
Wojciechowski Marek	marwoj@ibch.poznan.pl
Zielińska Żaneta	zanziel@ibch.poznan.pl

Service

Balczak Józef
Bzdurska Stanisława
Dzięcioł Jerzy
Flotyński Paweł **61**
Gołębiewski Henryk **61**
Grellus Mirosława
Gromanowski Mariusz
Jezierski Mieczysław
Korybalska Lilianna
Kosicka Barbara
Kóska Arleta
Kubacka Iwona
Lecko Paweł
Mager Alicja
Matuszak Jan
Michalska Katarzyna
Mnich Zenon
Nowak Jerzy
Podeszwa Aleksandra
Reinke Iwona
Rucińska Bożena
Siebert Zofia
Sulska Elżbieta
Szułczyńska Janina
Szułczyński Andrzej
Szubińska Błażena
Tajszner Katarzyna
Thiel Renata
Wilandt Elżbieta
Węgrzeniejewski Marek

Edited by:
Bożenna Golankiewicz, Jerzy Boryski
Hanna Górnicka-Holeczek, Zbigniew Michalski, Andrzej Wójtowicz

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Scientific Publishers OWN
Polish Academy of Sciences
Wieniawskiego 17/19, 61-713 Poznań
tel.: (48-61) 8528503 fax: (48-61) 8520671
e-mail: own@man.poznan.pl

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Edited by:
Bożenna Golankiewicz, Jerzy Boryski
Hanna Górnicka-Holeczek, Zbigniew Michalski, Andrzej Wójtowicz

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tel.: (48-61) 8528503 fax: (48-61) 8520671
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