MY LIFE WITH NUCLEIC ACID CHEMISTRY

Antonín Holý

In the early sixties, when I joined the oligonucleotide chemistry group in IOCB I knew nothing about nucleic acids at all. I was not particularly fond of biochemistry lessons during the happy days at the faculty and there was nothing to improve this affinity during the days of my Ph.D. study in synthetic organic chemistry. Indeed I was, as a freshener in this field quite confident I would achieve a revolutionary change in this peculiar branch of organic chemistry.

My new boss, (the late) Jiří Smrt asked me to hydrolyze commercial ribonucleic acid by pancreatic ribonuclease A, separate four pyrimidine (Up, Cp)-ending dinucleotides and use them for block-synthesis of oligoribonucleotides by di- or trimerisation. I was left with three problems I never had anything to do with before: use of enzymes in preparative organic chemistry, nucleotide chemistry and nucleotide separation.

IOCB in Prague was at that time one of the three or four places in the world where the oligonucleotide synthesis was cultivated; the others were King's College in London (Colin Reese et al.), Mizuno's group at Hokkaido University in Sapporo (Japan) and the most famous of all, Khorana's group in Madison, Wisconsin, U.S.A. While Khorana was aiming at the secret of the genetic code via synthesis of oligodeoxyribonucleotides, our group and Reese's were contesting with each other in the ribo series. The British may have selected the more difficult chemistry of the ribo series as a challenge, but our reasons were pragmatic: we simply could not afford to pay the price for the 2′-deoxyribonucleosides. Even the ribonucleosides – specifically uridine and cytidine – were for us not easily available at that time. This was the simple reason why we made so much of 6-azauridine chemistry: 6-azauridine was a pyrimidine-like nucleoside which was available in large quantities as a fermentation product. It was heading for approval of its medicinal application. This lack of starting materials lasted several more years, till we succeeded in explaining the Lachema Brno Co. the economical efficacy of ribonucleoside productions to run the simple chemical hydrolysis of RNA and separation of nucleosides on a large scale.

Contrary to the "royal attitude" which consisted in the phosphodiester condensation of the 5′-nucleotide with orthogonally 2′,5′-protected ribonucleoside, the "Prague" approach to stepwise oligoribonucleotide synthesis was based upon phosphodiester condensation of nucleoside 3′-phosphates protected at 2′-hydroxyl group with acidolabile groups and with acetyl at the position 5′, with 2′,3′-O-protected ribonucleosides. And the easiest way to prepare the pure 3′-regioisomers was indeed the cleavage of 2′,3′-cyclic diesters by ribonucleases. I was never able to trace the author of this original approach but evidently it must have been Jiří Smrt himself.

The situation with ribonucleotides was not better: hydrolysis of RNA and separation of nucleotides by ion exchange chromatography was routinely made by our two technicians. The mixtures of regioisomeric uridine and cytidine 2′- and 3′-phosphates were then cyclized by DCC and cleaved by pancreatic ribonuclease to the 3′-isomers. This procedure is relatively easy with pyrimidine nucleotides which are cleavable with pancreatic ribonuclease A, but there was a difficulty with purine nucleotides. Had it not been for the

Japanese scientists (Egami et al.) who just came with description of RNAses T1 and T2 from takadiastase, we might have been growing in the lab carnivorous plants whose digestive juice was reported to contain nonspecific ribonucleases. Instead, we have somehow (most probably with the aid of Professor Šorm, I imagine) managed to get the Czechoslovak Embassy in Tokyo involved and after some time we received, by diplomatic mail, a package containing about half a kilogram of this (for us) valuable dried mycelium. To isolate the acid- and heat-stable enzyme was easy even for a rudimentary synthetic organic chemist.

It was much more pitoresque to isolate and purify the phosphodiesterase (exonuclease) and 5′-nucleotidase from the snake venom, enzymes commonly used for analytical chemistry of the nucleotides. There was (I think there still is) a breeding of these beasts in the cellar of the Pathology Institute at the Faculty of Medicine and we were getting a fresh snake venom of Bitis gabonica therefrom in gram quantities from a single animal. This snake is much bigger compared to the rattlesnake Crotalus adamanteus which was a common source of the enzymes mentioned. The procedure of "milking" the snake is notorious, and the African snake was its regular and reliable large-scale producer.

The problem of oligoribonucletide synthesis is the stability of their internucleotide linkage. Our approach required very sensitive acidolabile function both at the position 2′ and at the 2′,3-cis-diol group of the ribonucleoside component. For the latter case we developed very acid-sensitive 2,3-O-ethoxymethylene group which was used instead of isopropylidene, while for the 2-hydroxyl we were routinely using the 2-ethoxyethyl group which was easily introduced by an acid-catalyzed addition of ethyl vinyl ether. For the amino grouping at the heterocycle there was the amidine function which was easily introduced by reaction ith dimethylformamide dimethylacetal. This synthetic strategy was ultimately crowned by the synthesis of all sixteen diribonucleoside phosphates we published in a joint paper. I think it is little known that the first trinucleoside diphosphate ever made in the world was (UpUpU) synthesized by Stan Chladek in our lab and described in the Collection.

The separation technique was DEAE cellulose column chromatography in triethylamine bicarbonate. That meant not only the steady access to the concentrated buffer but also liters of water which we distilled in the lab ourselves. I recall the trial to replace the expensive and imported triethylamine in the volatile buffer by trimethylamine. Though we found in a one-day-trial that it would be possible, it was not quite well accepted by the colleagues in the other parts of the Institute. As to the analytical procedures, TLC just began to emerge and one had to prepare the plates himself. There was at that time, and perhaps still for ten more years in nucleotide chemistry hardly anything but paper chromatography. Paper chromatography is an elegant and most sensitive method which also makes it possible to quantify and recover the material. In combination with a preparative chromatography paper and a box which housed six full sheets we had a capacity of several hundred milligrams. We developed specific methods how to elute the identical UV-absorbing bands from some twenty sheets of paper simultaneously. We also had a unique method of loose layer plate chromatography on silica gel containing a luminiscent dye incorporated in the gel made in-house. This technique is still in use in my lab.

I was eagerly reading newly published papers looking for novel mechanisms of action of anticancer drugs based on chemically modified nucleosides and/or the heterocyclic bases their precursors. This topic was well established also in our Institute – the 6-aza and 5-azapyrimidine nucleosides were designed, synthesized and investigated in detail here – and I realized that behind all that is an antimetabolic principle, based in the most cases on an inhibition of enzymes participating in the synthesis of nucleic acids and their precursors de novo and, that this is triggered by the transformation of nucleosides to their phosphomonoester derivatives and additional conversion of the latter to the 5′-di- and triphosphates. I also realized the serious drawback of the nucleoside-based drugs – their decomposition by catabolic enzymes; it not only burdened the patient's body by decomposition products and enforced an application of the drug in a more frequent regimen. That gradually became a obsession. In order to circumvent it I decided to study the principles which controlled the specificity of enzymes towards chemical modification of their natural substrates and I slowly backed out of the oligonucleotide chemistry.

The easiest available compounds for such a study were indeed monomers – mononucleotides and their 2′,3′-cyclic analogues, modified both at the base, at the sugar moiety and/or the phosphate group, eventually dinucleoside monophosphates. Just at that time, I discovered an original synthetic procedure which made it possible to convert any cis-diol grouping containing compound into its appropriate cyclic phosphate which worked equally well both in micro and macro scale. I soon possessed a collection of some 40–50 base- and sugar-modified nucleotides and related compounds, including the 2′,3′-cyclic nucleotides and began to systematically investigate the specificity of enzymes towards the chemical modification of the heterocyclic base and the sugar moiety of the modified nucleosides and nucleotides as substrates mainly in the catabolic reactions. In addition to the enzymes which cleave nucleoside linkage (phosphorylases) and aminohydrolases I focused mainly on nucleolytic enzymes cleaving the phosphomono- and diester linkages in nucleotide derivatives. During that time, I created quite a collection of modified substrates which was easily applicable to the structure–activity studies of enzymes and their characterisation. Enzymes both of already established character as well as those from often peculiar sources (bull semen RNAse, 5′-nucleotidase from pollen of Brassica sp., ribonucleases isolated from diverse microorganisms) were characterised by means of this collection, often with the aid of the scientists themselves who came to my lab to scrutinize the substrate specificity of their enzymes.

The gradually collected data on the effect of chemical modification upon the enzyme activity brought me to the conclusion that the stability against catabolic enzymes can be achieved solely by such a chemical modification which would make the cleavage structurally impossible. It resulted in the concept of minimum necessary resemblance to the (sugar moiety of) parent nucleoside molecule structure and, ultimately, to the 2,3-dihydroxypropyl analogues of nucleosides. One of these compounds, the (S)-enantiomer of the adenine derivative showed a plethora of biological activities. It became the target of our investigation for nearly a decade and is still being used as a versatile tool for studying the effects of biological methylations.

In May 1975 there was a conference in Göttingen which was organized by my great friend, the late Karl-Heinz Scheit with whom and young Fritz Eckstein I spent three months

of my only postdoctoral stay in life. The group of Fritz Cramer at the MPI für experimentelle Medizin was an important site of pilgrimage for nucleic acid chemists in Europe. I was preceded by Mikolajczyk from Poznan, by (the late) Tsuji Hata from Yokohama and followed by Matthias Sprinzl, Frank Seela and others. At this meeting I met for the first time Erik De Clercq, a young Belgian M.D. who came there to get acquainted with the chemists and to find someone willing to collaborate in looking for antivirally active compounds. Erik has by far the best chemical mind among the M.D.'s I ever met; he correctly estimated the potential of nucleosides as antimetabolites acting against cellular parasites. This encounter developed into a friendship which has had a decisive role in my professional life. It also enabled me to meet and make friends with Erik's numerous collaborators from Belgium. Was it perhaps an omen that the first foreign scientist I ever worked with in a nucleic acid chemistry lab in IOCB was also a Belgian, later the famous guru of plant genetics, Mark Van Montague?

Ivan Votruba managed over the years to build up the novel topics in IOCB – a molecular pharmacology. We had occasionally collaborated on several topics already before but since the discovery of DHPA and its mode of action which consists in binding upon SAH-hydrolase where it replaces adenosine, this collaboration never ceased. We have jointly developed an affinity chromatography which allowed us to isolate very pure enzyme from a few milligrams of any tissue homogeneate in one step. The enzyme was then used for the in vitro search of inhibitors – derivatives of DHPA with modified heterocyclic base and side chain with hydroxyl, amino, sulfanyl, carboxy groups of diverse configuration. That study resulted in another lead structure – eritadenine. This carboxylic group containing acyclic nucleoside analogue is contained in the Japanese mushroom shitake (Lentinus edodes shitake) – an elixir of life by traditional medicine. The antiviral activity of this compound is higher than that of DHPA, with a similar spectrum of selectivity. Also its simplified analogue, one carbon atom shorter 3-(adenin-9-yl)-2-hydroxypropanoic acids (AHPA) are nearly equally active. While DHPA has all the characteristics of a revesible inhibitor of SAH hydrolase, eritadenine and AHPA are very potent irreversible inactivators causing a drop of the enzyme level to the "core value" within several seconds – just like in a titration. In principle, the methylation inhibitors must act in every rapidly proliferating system. Following an application of such an effector to the biological system it is possible to view what is going on step-by-step after the methylation was inhibited. We have used it to inhibit the growth of plant roots, spermatogenesis in male mice, oogenesis in insects, etc. In chicken embryos, it is possible to visualize the sequence of events leading ultimately to a teratogenic effect.

In parallel, I was performing studies on modification of the phosphate monoester residue; unspecific phosphomonoesterases and 5'-nucleotidases are important enzymes of nucleotide catabolism. As 5'-nucleotides are synthesized from the nucleosides by the action of cytosol enzymes (e.g. nucleoside kinases) as the key-step for the consecutive reactions catalyzed by nucleotide kinase and nucleoside diphosphate kinase leading ultimately to NTP analogues, the importance of such enzymes is certainly self-explaining. Various interesting types of molecules were synthesized and widely investigated in this connection, many of which were later exploited by other authors with (or without) the proper reference to the primary source. Among them, e.g. nucleoside H-phosphonates ("phosphites"),

methylphosphonates, hydroxymethylphosphonates (their enzymatic degradation compared to resistence of their methylphosphonate congeners was first to indicate the importance of oxygen atom presence in the vicinity of phosphorus), aminomethylphosphonates (the 2'-isomers cleave in an intramolecular non-enzymatic reaction in the presence of high protein concentration the nucleosidic linkage under liberation of the heterocyclic base).

General conclusions which resulted from this complex investigation were rather simple: (a) while the anabolic reactions are usually very specific and do not stand much of structural modifications of the base, the enzymes of the catabolic pathways were not much influenced by the structure alteration, particularly not by those taking place at the base. (b) The first-generation nucleoside-based antimetabolites which were designed on the principles of maximum similarity to natural metabolites of the cell must on principle suffer from limited catabolic stability. (c) Phosphorus-modified nucleotide analogues must obey the rule of isopolarity. (d) Catabolic stability must be warranted by such modifications of linkages which make the cleavage a priori impossible.

The first lot of compounds whose samples went in 1976 by mail from Prague to Erik De Clercq in Leuven contained six compounds which I selected as typical representatives of structural classes of analogues I was at that time working with. Unbelievable as it may seem to be, two of these synthetic compounds were antivirally active. In addition to DHPA which acts best against minus stranded RNA viruses (evidently due to the block of capping at the 5'-end of viral mRNA) and whose activity was duly discovered at that time, the activity of the present favourite compound against hepatitis B, L-thymidine, remained undetected – not only was there no in vitro test for anti-HBV activity available at that time, but the virus itself was still waiting to be isolated and characterised. L-thymidine went back to the shelf and stayed there forgotten for nearly thirty years.

In those days I had absolutely no knowledge of viruses, their life cycle and pathological manifestations and I presume that many of my contemporaries in the profession must have felt the same. After all, this knowledge was at that time rather scarce anyway. The pharmaceutical industry paid it but a formal interest compared to the effort in cytostatics, etc. There was indeed a general knowledge of Bayer's aspirin for treatment of flu and quinine for fever depression, but the general hope was given to vaccination – it was just past the time of successful smallpox irradication and there was interferon coming as a new star. Thus, though at that time the future renown antivirals idoxuridine, vidarabine, trifloridine and ribavirine already existed, their use, if any, was substantially limited. Acyclovir came just in time (1975) to cope with the immediate danger of genital herpes epidemy by HSV-2 and antivirals became a hot issue. We performed a placid research on SAH-hydrolase, its inhibition and on DHPA, focusing on its introduction to the market. It has eventually succeeded and the Duvira® gel which was approved in Czechoslovakia with the indication of herpes labialis (cold sore) became a shelf drug.

The old ideas were not quite forgotten and some time later we began reviving the search for nucleotide analogues resistent to the dephosphorylation. There was a general belief that nucleotides cannot get across the cellular membrane due to their polar character but a few contemporary papers vaguely hinted at a remote possibility that such a process could take place but is imperceptible owing to the massive dephosphorylation which takes place at (in) the membrane. It was evident that with the above knowledge of requirements for

interference with nucleotide recognizing enzymes, there were two candidate structures left: phosphothioates and „C"-phosphonates. The first group was investigated primarily by F. Eckstein and there was no indication of their in vivo activity in the literature aimed at either transformed cells or at cellular parasites. I also suspected that such compounds perhaps might not be quite stable under the in vivo conditions. I had made the 5′-deoxy-5′-phosphonouridine already in 1967 by the simplest possible method, Arbuzov reaction of 5′-iodo-5′-deoxyuridine with trialkyl phosphite. Inhibitory activity in vitro was not overwhelming, possibly due to a steric incompatibility. The fact, that the UpU analogue I had made from it resisted completely the pancreatic ribonuclease while it was easily cleaved by the snake venom exonuclease was corroborating my expectations.

Keeping now in mind the importance of oxygen atom in the vicinity of phosphorus I decided to put this atom now in the nearest position, i.e. next to alpha-carbon and got thus from the phosphoric ester grouping (=P–O–C–) to its isomeric phosphonomethyl ether (=P–C–O–). These two types of compounds were isopolar and it was to be hoped that the C–P and C–O linkages would stand the attack by enzymes in the cytoplasm.

I was working with my Ph.D. student, I. Rosenberg and between ourselves we soon had a whole group of such compounds derived from different ribonucleosides. And the preliminary tests of enzyme inhibitory activity on several 5′-nucleotidases showed that our theory was correct. The new 5′-nucleotide analogues were not only stable against dephosphorylation by these enzymes but, on the other hand, acted as their strong inhibitors in vitro. They resisted the action of E. coli phosphomonoesterase as well. Masahiko Nishizawa a Japanese postdoctoral student, proved that the ribonucleoside 5′-phosphophosphonomethyl ether (analogues of NDP) combined with natural substrates, though generally inhibiting the polymerisation reaction, can themselves be incorporated into the polynucleotide chain synthesized de novo by polynucleotide phosphorylases.

However, none of the 5′-phosphonomethyl ethers derived from natural ribonucleosides showed any cytostatic activity in tissue cell cultures. That was still feasible, but the synthesis of phosphonomethylether of biologically active nucleosides (6-azauridine, 5-bromo-2′-deoxyuridine, adenine arabinoside) which were also devoid of any cell growth supressing activity, seemed to definitely bury the last hopes. However, serendipity did its job again and we decided to link the phosphonomethyl residue to the racemic DHPA which was available in sufficient quantity on our bench. In 1986, the thus-obtained mixture of two enantiomeric pairs of 2′- and 3′-regioisomers found in due time among other compounds its way to KU Leuven for antiviral screening and the excited reaction therefrom soon marked the beginning of ANP era. One of the four components was namely 2′-phosphonomethyl ether of (S)-DHPA, (S)-enantiomer of HPMPA, a novel lead compound which is very active against DNA viruses and – as has been shown later, also against higher cellular parasites – protozoa (Plasmodium, Leishmania, Trypanosoma spp., etc.).

The events which followed possessed all the features characteristic for such discoveries: application for patent protection, animal experiments to confirm the activity, preliminary tox studies, search for a license partner, search for related compounds with a similar or improved activity… The history of license and compound development was recently described elsewhere (Nature Drug Discovery). It is not the subject of this history, though some of its milestones are certainly worth of a detailed description. What must not be

forgotten, is the great effort and contribution of my collaborators – earlier Ph. D. students: Ivan Rosenberg, Hana Dvořáková, Dana Hocková (née Amblerová), Marcela Krečmerová, Michal Hocek, Petr Alexander, Mirek Otmar and Michal Česnek. John Martin, Norbert Bischofberger and Bill Lee of Gilead Sciences exhibited great belief in the compounds and invested an enormous effort to turn them from bench chemicals to shelf drugs. They and their numerous collaborators have really opened the era of acyclic nucleoside phosphonates in medicinal chemistry.