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Account

Natural Product Chemistry: From Plants to Human

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Conventionally, natural product chemistry deals with the isolation, characterization, and synthesis of compounds from plants. It a broad sense, natural product chemistry may include all fields of biochemistry. Two projects are presented to illustrate the expanded definition of natural product chemistry. One deals with phosphatidylinositol (PI) and the key enzyme involved in its metabolism, PI-specific phospholipase C. The other project is on the structure-function relationship of human tumor suppressor p16.

The conventional meaning of natural product chemistry is the isolation, characterization, and synthesis of chemical compounds from plants. This is also an area of historical strength in the Department of Chemistry at the National Taiwan University. Professor Y. S. Cheng has contributed significantly to this strength. It was in her laboratory that I started my research career in chemistry, working on the isolation and characterization of natural products. In 1972, as an undergraduate student, we published the first scientific paper in my life, entitled "Terpenes and Sterols of *Cunninghamia Konishi*".[1] Two years later we published another paper entitled "Air Oxidation of α-Terpineol",[2] which used α-terpineol (Fig. 1) to address the problem of air oxidation during the isolation of natural products.

In a broad sense, natural products may include all chemicals that are produced by all living organisms, not just plants. Thus natural product chemistry could be broadly defined as the chemistry of life processes. One can ask how the natural products are formed; this subfield is called "biosynthesis", which bridges traditional chemistry and biochemistry. On the chemical side of biosynthesis one only asks what are the precursors of a natural product. On the biochemical side, one can ask what are the enzymes that are involved in each biosynthetic step. This brings us into biochemistry. If we then proceed to isolate the enzyme involved in each step, and to study how the enzyme functions, then we get into the field of "enzymology". If we further pursue the structure of the enzymes or other biological macromolecules, then we enter the new field "structural biology". Just like organic chemists like to make analogs of natural products, biochemists today like to make analogs of enzymes (i.e., mutants), by manipulating the gene of the enzyme. This brings us into the field of molecular biology. Today it is not uncommon to perform a "total synthesis" of a protein through molecular biology techniques: gene synthesis or gene cloning followed by expression.

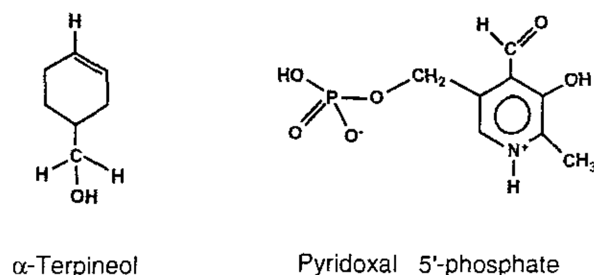

Fig. 1. Structures of α-terpineol and pyridoxal 5′-phosphate.

The processes and fields mentioned above could apply to plants as well as other organisms, including microorganisms and mammals, and human being. The subjects could also include carbohydrates, lipids, nucleotides, nucleic acids, secondary messengers, neurotransmitters, etc. The training in traditional natural product chemistry I received in Professor Cheng's laboratory had seeded my interest in the broadly defined natural product chemistry, and prepared me for my research career in the chemistry of life processes.

My graduate research dealt with pyridoxal 5′-phosphate (PLP, structure in Fig. 1), a common natural product in all living organisms. PLP functions as a "coenzyme" in that it assists many enzymes to carry out different types of catalysis. One of the key questions addressed was why (and how) the simple PLP structure can function in different types of reactions. Experimental evidence was obtained to support that different types of enzymes control their specificity by controlling the conformation of the C_4-C_4' bond of PLP at the active site of the enzyme.[3,4] Another problem investigated was the stereochemistry of enzyme-mediated

Dedicated to Professor Yu-Shia Cheng on the occasion of her 65th birthday.

proton transfers in the reactions catalyzed by PLP-dependent enzymes.[5]

Since I started my independent research career, my research has evolved from conventional bioorganic chemistry into the structure-function relationship and the chemical mechanism of several enzymes: 5′-nucleotidase, adenylate kinase, phospholipase A_2, Phospholipase C, phospholipase D, acetyl coenzyme A synthetase, DNA polymerase β, and phosphatidylinositol-specific phospholipase C (PI-PLC). The substrates of these enzymes include different types of "natural products": nucleotides, phospholipids, and DNA. The experimental approaches used include organic synthesis of substrates and substrate analogs, purification of enzymes, enzyme kinetics, NMR determination of enzyme structures, cloning and expression of enzymes using molecular biology techniques, etc. The experimental problems vary greatly due to the very different types of substrates and reactions. However, the underlying approaches are within the broad boundary of natural product chemistry - in a broad definition.

Two projects are described briefly here to extend the "modern natural product chemistry" from plants to bacteria to human. The first project is the study of PI-PLC from bacteria; the second deals with human tumor suppressor p16. Both projects are ongoing in our lab and the following descriptions are meant to be introductory rather than comprehensive.

The bacterial PI-PLC catalyzes the conversion of phosphatidylinositol (PI) to inositol 1,2-cyclic phosphate (IcP) and diacylglycerol, followed by a slow ring opening of IcP to inositol 1-phosphate (IP), as shown in Fig. 2. This seemingly simple reaction is central to many complicated metabolic pathways and is thus important to biological regulations.[6] We have investigated this reaction in several different aspects: substrate specificity, stereospecificity, kinetics, and use of site-directed mutagenesis to identify key catalytic residues in the active site of the enzyme. In this particular project the synthesis of substrates and substrate analogs is a major challenge due to the presence of multiple hydroxyl groups with specific configurations on the inositol ring. We have developed systematic procedures to synthesize various analogs of PI, IcP, and IP.[7] Some of the substrate analogs synthesized are shown in Fig. 3.

In our most recent work,[8] the stereochemistry of the reaction was studied by using the stereo isomers of DPPsI, which are converted by PI-PLC to *trans* and *cis* isomers of IcPs (structures are shown in Fig. 3). By a combination of kinetic assays and ^{31}P NMR analysis, we have calculated that the wild-type enzyme shows a stereoselectivity ratio of 160,000:1 in favor of the R_p isomer versus the S_p isomer of DPPsI. To the best of our knowledge, this is the highest stereoselectivity toward R_p and S_p isomers of phosphorothioate analogs among those quantitatively measured and reported. This result is particularly intriguing since the catalysis by bacterial PI-PLC does not involve a divalent metal ion. One would expect that the metal-phosphate interaction is a likely mechanism for the observed stereoselectivity.

The stereoselectivity of PI-PLC toward the two diastereomers of DPPsI, coupled with site-directed mutagenesis, was then used to probe the catalytic role of an active residue arginine-69. The activity of the R69K mutant (in which the residue arginine-69 was replaced by lysine) toward DPPI decreased by three orders of magnitude relative to the activity of wild-type PI-PLC. This suggests that arginine-69 is critically important for catalysis since the change of the side chain structure from arginine to lysine is very small as shown in Fig. 4. However, the kinetic data alone do not suggest how the side chain of arginine-69 interacts with the substrate. DPPsI was used to probe this question. The activity of R69K decreased by four orders of magnitude toward (R_p)-DPPsI; however, the activity of R69K toward (S_p)-DPPsI is comparable to that of the wild-type PI-PLC. Consequently, the stereoselectivity R_p/S_p is relaxed

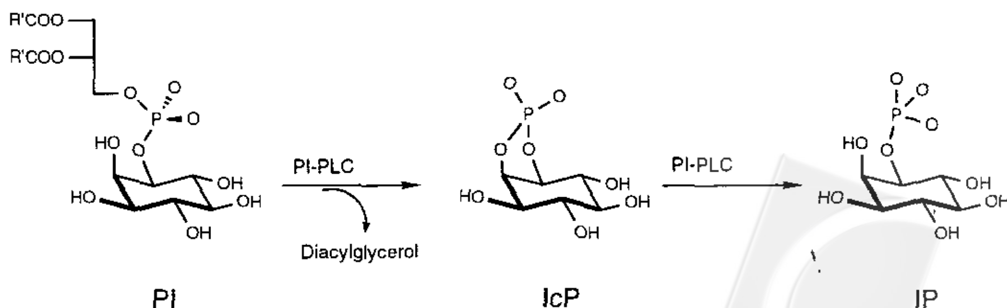

Fig. 2. Conversion of Phosphatidylinositol to IcP and IP Catalyzed by PI-PLC.

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by four orders of magnitude, from 1.6×10^5 for the wild-type enzyme to 16 for the R69K mutant. Such a large change in the stereoselectivity toward DPPsI is strong evidence that the side chain of Arg-69 interacts directly with the phosphate moiety of phosphatidylinositol. Since the effect is mainly on the rate of catalysis, the interaction should occur at the transition state.[8]

The second project deals with the structure-function relationship of human tumor suppressor p16. In this case, the "natural product" p16 is a protein with a molecular weight of ca. 16,000. The protein was discovered in 1993 and its function has been uncovered only in the last couple of years.[9] It is now known that p16 regulates cell growth by inhibiting a key enzyme called cyclin-dependent kinase 4 (cdk4). When the function of p16 is impaired by mutation, the cell cycle loses a negative regulator and the cell growth proliferates - resulting in the formation of cancer.[10]

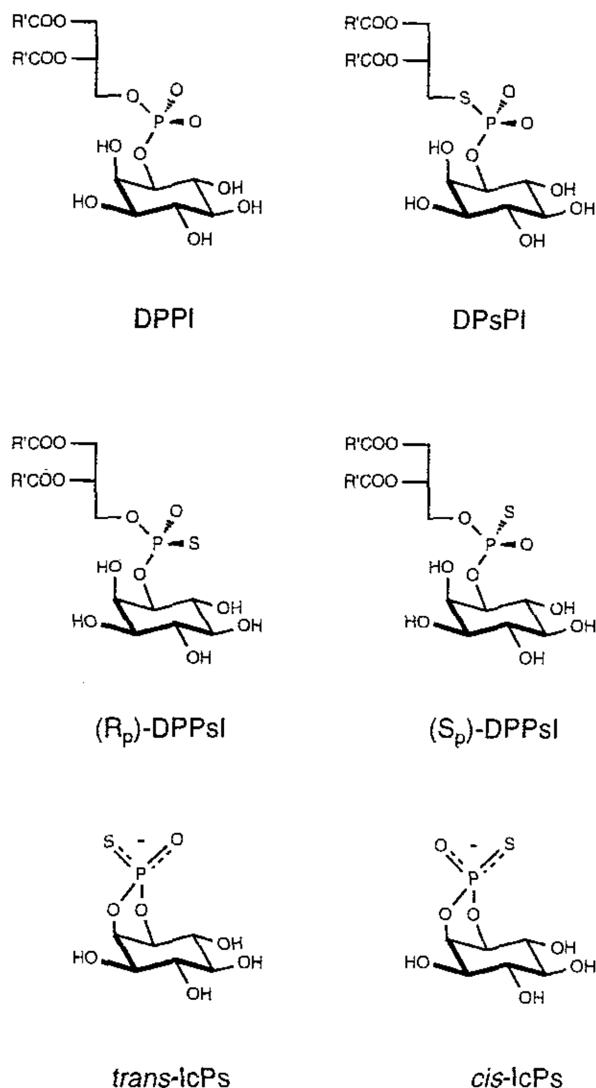

Fig. 3. Structures of DPPI and the substrate analogues used in this study. Abbreviations: IcPs, inositol 1,2-cyclic phosphorothioate; DPPI, dipalmitoyl-phosphatidylinositol; DPPsI, 1,2-dipalmitoyl-*sn*-glycero-3-thiophospho-1-*myo*-inositol; DPsPI, (2*R*)-1,2-dipalmitoyloxypropanethiophospho-1-D-*myo*-inositol.

Our goal is to understand the structure of p16 and its mechanism of action. Such information could then be used to design p16 mimetics that can serve as candidates of anti-tumor drugs. Using molecular biology techniques we have successfully cloned the gene encoding p16 from HeLa cells and overexpressed it in *Escherichia coli*. The protein was then purified and shown to function like the native protein. We then used two, three, and four dimensional NMR experiments to determine the structure of p16. In order to perform these NMR experiments, the protein needs to be labeled with C13 and N15 isotopes. Such isotope labeling is possible only *via* molecular biology techniques - by feeding the bacteria with N15-ammonium sulfate and/or uniformly labeled C13-glucose. Fig. 5 shows an example of a two-dimensional NMR spectrum of p16 uniformly labeled with N15. Each cross-peak in Fig. 5 comes from a NH proton of the peptide backbone. As shown by the figure, almost all cross-peaks have been assigned. To date the total NMR assignments have been completed and secondary structures have been determined;[11] the determination of the tertiary structure is in progress.

We have also constructed a number of p16 mutants that have been identified in tumor cells. These mutants have been characterized for their structural integrity (by use of NMR), conformational stability (by use of CD), and func-

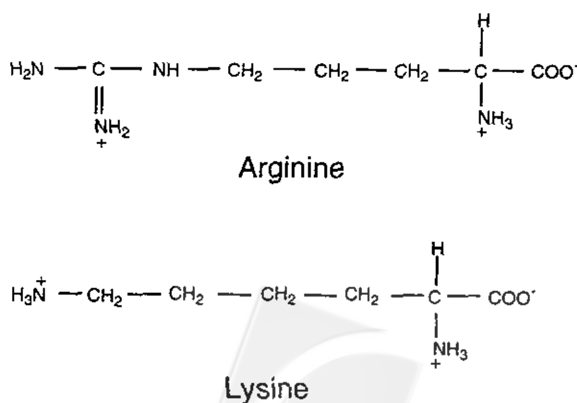

Fig. 4. Structures of arginine and lysine.

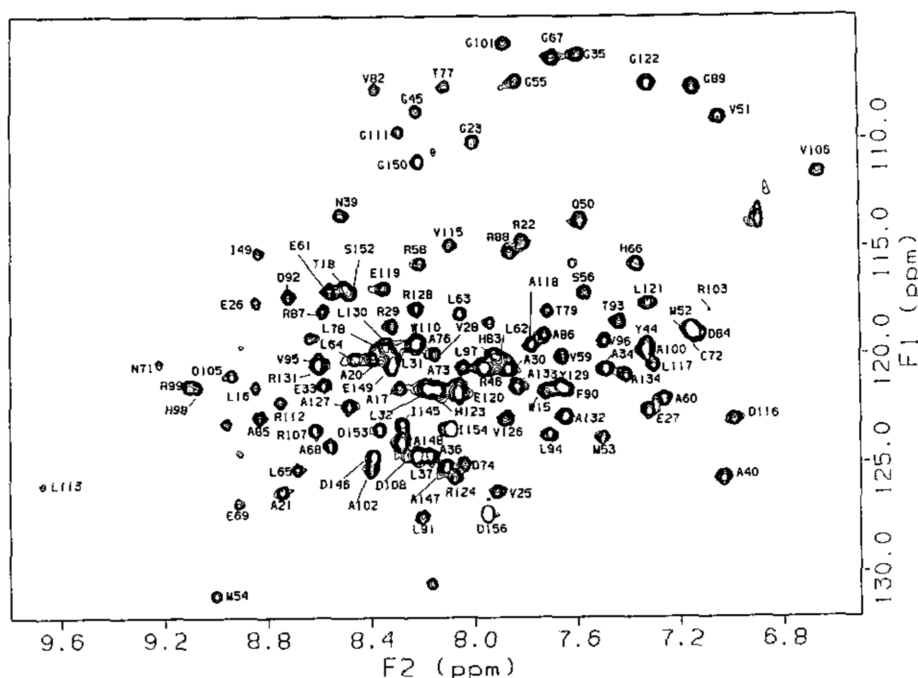

Fig. 5. The ^{15}N-^{1}H HSQC spectrum of p16 at 600 MHz. The assignments are labeled by one-letter code of amino acids accompanied by a sequence number.

tional changes (by assaying its ability to inhibit the activity of cdk4).[11]

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Key Words

Natural product; Phosphatidylinositol-specific phospholipase C; Tumor suppressor p16; Pyridoxal 5′-phosphate; Stereochemistry.

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