The Logic of Biologically Active Small Molecules: Amazing Ability of Microorganisms

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In this review article, I will outline my way of thinking about biologically active small molecules. The structure of liposidomycin B from *Streptomyces* species resulted in my initial sense that a structure tells its function. A biologically active small molecule may save directly or indirectly a number of people. Even if the molecule has not been used as a therapeutic agent, it can be used as a useful chemical probe for dissecting a living cell into different biochemical pieces. Such biologically active small molecules derived from microorganisms have been primarily found in cultivable microorganisms that make up only 1% of total microbes in nature. Discovery of novel growth factors, zincmethylphyrin, zinc coproporphyrin, and coproporphyrin enabled laboratory cultivation of previously uncultured *Leucobacter* sp. These findings might expand the possibility for further discovery of novel therapeutic agents or chemical probes.

Key words: Liposidomycin, tautomycin, tautomycetin, zincmethylphyrin, uncultured *Actinobacteria*.

When I read an article written in English, my eyes scan left and right from the top of the page to the bottom to obtain information. On the other hand, in the case of a book written in traditional Japanese style, my eyes scan up and down from the right of the page to the left while recognizing pictographs (Chinese characters) and two kinds of symbols (Hiragana and Katakana). The relationship between structure and function of a small molecule is somewhat similar to that between language and information. Some researchers say that the chemical structure is like a face and it is used to distinguish it from other compounds. But I do not agree with this way of thinking. The chemical structure of a molecule is like a sophisticated pictograph written in an international language (structural formula) which implies functional information about the molecule.

Synthetic perspective helps scientist gain insight into the function of biologically active small molecule

In 1974, I received a liberal arts education at Hokkaido University and decided to further education in the Department of Agriculture. The reason for choosing the Department of Agriculture was due to the information that their students can handle microorganisms. But organic chemistry seemed more attractive than microbiology so I started my career as a synthetic chemist. My favorite game was to imagine a target molecule for total synthesis and move it in my mind. My first work was stereoselective synthesis of (±)-palitantin.[1,2)] Prior to starting the research, Dr. Ichihara, my first lab mentor, handed me a synthetic strategy written on paper and said, “Think about tactics by yourself.” After completing the synthesis of palitantin for my master thesis, he said, “Since you become a doctoral student, you should set up a

strategy for the synthesis of frenolicin." For my doctoral dissertation, I achieved the total syntheses of (±)-nanaomycin A, (±)-frenolicin[3] and plumbagin using the retro-Diels-Alder reaction.[4] I was second author on all the paper because of the philosophy of Dr. Ichihara. I said to him "Although you showed frenolicin as the synthetic target, I did everything including selection of nanaomycin and plumbagin as target molecules, synthetic strategies, tactics, and actual syntheses…, and I have written these papers, too." Dr. Ichihara told me very quietly, "This is the American style. You should use your own philosophy when you became a mentor." I understood the American style after becoming a mentor. Not all things, including acquisition of research fund, research place, and infrastructure can be done alone. In addition, this might be an educational consideration for fueling the doctoral students' ambitions. My science mentor in the Sakamura laboratory was Dr. Ichihara but Dr. Sadao Sakamura was the principal professor. Besides Assistant Professor Ichihara, there were three other research associates. Professor Sakamura was not only my mentor in science but also in philosophy. Even though I was the second author in all my original papers,[1-4] I acquired a Ph.D. from Hokkaido University while learning about both science and philosophy. Dr. Sakamura's laboratiory was an academic incubator for students with freedom, except for a few points, and produced many excellent professors, scientists, and leaders in the world.

In 1980, I joined the Grieco Laboratory immediately after it moved to Indiana University Bloomington from the University of Pittsburgh. Professor Paul A. Grieco graduated from Boston University and went to Columbia University where he studied in the Laboratory of Professor Gilbert Stork. After receiving his Ph.D. from Columbia University, he took a postdoctoral fellowship with Professor E. J. Corey at Harvard. Professor Grieco was one of the active stars in

the field of organic chemistry and had worked with many Japanese postdoctoral fellows who later became famous professors or leaders. After a year and a half as a postdoctoral research associate in his laboratory, I found I could not present my ideas to Dr. Grieco due to the hesitancy peculiar to the Japanese and his very busy schedule also dissuaded me. Because of my personal difficulties, it was not always a happy time, but the Bloomington era was one of my most important times in my life. At a party held for Dr. Grieco during the Paul A. Grieco Symposium in 2009,[5] 29 years later, I made a speech about him and the Japanese Grieco Family presented him a gift. Upon this occasion, he approached me and hugged me.

I left the Grieco Lab and went on a one-month journey at the end of 1981. The small plane from Indianapolis International Airport shook like a rural bus and managed to land at the JFK International Airport in an intense snow storm. From there, I used airplanes and car rentals to travel south along the east coast of the USA. Through New York, Washington, Florida, Key West, and then west through New Orleans, Las Vegas, Grand Canyon, San Francisco, and Los Angels.

New chemotypes of biologically active small molecules

The Antibiotic Laboratory in RIKEN, The Institute of Physical and Chemical Research was the place where I worked since April 1, 1982. The Head Scientist in the lab, Dr. Kiyoshi Isono said to me “You and I are equivalent as scientists, I will delegate all the chemistry of this lab to you.” During this time, I was also able to fulfill the minimal obligation as a husband. The presence of my wife, two newly born daughters and one son spiritually supported my motivation to continue scientific work. New chemotypes of biologically active small molecules were discovered in the lab. Neopeptins[6,7] acted as inhibitors of fungal cell wall biosynthesis,

ascamycin[8-10] was a *Xanthomonus* specific antibiotic, cationomycin[11-13] was a polyether ionophore antibiotic produced by *Actinomadura azurea*, liposidomycin B[14] was an inhibitor of peptidoglycan biosynthesis, tautomycin[15-17] and tautomycetin[18] were antifungal antibiotics which were later put into practical use as biochemical reagents. Reveromycin A[19] also was utilized as a biochemical reagent and a candidate drug for osteoporosis, respinomycins[20,21] was novel groups of anthracycline antibiotics, epiderstatin[22-24] was discovered as a glutarimide antibiotic, and so on.

Forty-three years after the structural determination of penicillin, the structure of liposidomycin B was uncovered in 1988.[14] The structure suggested it was a potent inhibitor against phospho-MurNAc-pentapeptide translocase (MraY), an essential membrane enzyme for bacterial cell wall biosynthesis[25] (Fig. 1a). After the structural determination of liposidomycin B, I worked with Dr. James A. McCloskey in the University of Utah for one month as a Japan-US cooperation scientist to finish a detailed paper for structure elucidation of liposidomycins, a class of complex lipid nucleoside antibiotics.[26] First day, I proposed *in situ* charge-remote fragmentation analysis of the fatty acid moiety of liposidomycin A with LiOH. Professor McCloskey told me “You are a chemist. Can you with John make the experiment success?” John M. Gregson was a Ph.D. student at McCloskey Lab. John and I made a reaction tube from a glass capillary and processed 5-15 μg of liposidomycin A with 5 μL of 35 mM LiOH in the tube. When the resulting hydrolysate was mixed with glycerol, the FAB mass spectrum exhibited ions from the saponified fatty acid. Collision activation of a dilithiated ion, suitable for charge-remote fragmentation, produced a nice mass spectrum which confirmed the positions of the two double bonds unambiguously.[26] This method may be applicable for diagnosis of

hyperglyceridemia and other fatty acid related diseases. John Gregson and Dennis R. Phillips, another Ph.D. student at McCloskey Lab became my friends. Professor McCloskey was a very nice person not only as a scientist but also as a human being. He was an excellent educator, too.

The structure of liposidomycin B was used as the structure for the Anti Nuclear Energy Bacteria (ANEB), which weaken Godzilla in a science fiction film "Godzilla *vs.* Biollante" released in 1989. In the real world, the 4-butylaniline amide derivative of anhydrodeacylliposidomycin,[14] CPZEN-45, derived from caprazamycins at Microbial Chemistry Research Center, has been recognized as a candidate drug for extensively drug-resistant (XRD) tuberculosis (Fig. 1b).[27,28]

<Fig. 1>

Research on biologically active small molecules gives insight into organic chemistry

In 1995, I was appointed Professor of Biotechnology Research Center, Toyama Prefectural University to set up a brand-new laboratory. Professor Hideaki Yamada, the first Director of the Biotechnology Research Center, told me, "Please contribute to the industry through your research work. The research work that contributed to the industry will remain in history." One of my first jobs was to recruit excellent young researchers and new graduate students through my scientific network. Fortunately, Dr. Noriyuki Nakajima as Assistant Professor and Dr. Nobuyasu Matsuura as Research Associate joined the laboratory. Another important mission was to design my laboratory so that graduate students and researchers could work comfortably in both chemistry and biology related to biologically active small molecules. In order to put my research team on the right track, I set short, medium, and long-term goals.

The first important study was brought about by happenstance that the amide-alcohol

precursor of (±)-epideratatin was synthetically converted to an unexpected nitrile-aldehyde under modified Swern oxidation conditions.[23] The method, using the activated DMSO conditions,[29] led to the first conversion of trifluoroacetamide to trifluoroacetonitrile (bp –64 °C) at –78 °C. Thus, our group attempted the in-situ trap of benzylalcohol to trifluoroacetonitrile at –78 °C, but the formation of imidate was almost nonreproducible and benzaldehyde was the major by-product. I told a graduate student who had conducted the experiment, "Why not add DBU?" Addition of a strong base, 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU) was effective for the preparation of benzyl-trifluoroacetimidate. The one-pot procedure proved to be operationally simple and useful for the preparation of various trifluoroacetimidates or perfluoroimidates.[30] All these trifluoroacetimidates are more stable and easier to handle than corresponding trichloroacetimidates. Benzyl, 4-methoxybenzyl (MPM), and 3,4-dimethoxybenzyl (DMPM) trichloroacetimidates can be replaced by corresponding stable trifluoroacetimidates.[31] 3,3-Sigmatropic rearrangement of allyl trifluoroacetimidate or Lewis acid-catalyzed rearrangement of 2,3-epoxy trifluoroacetimidates is a useful conversion method to obtain corresponding trifluoroacetamide,[32] which can be smoothly converted into amine. The stereochemistry of liposidomycin B deduced from NOE and conformational analysis was confirmed by syntheses of diazepanone ring model compounds[33,34] by using the above developed methods. Organic chemistry is useful for the study of biologically active small molecules, and such research adds insight into new organic chemistry, and the new organic chemistry is also useful for further research on biologically active small molecule (Fig.2).

<Fig. 2>

In the laboratory, we discovered (+)-indocarbazostatin which has a positive Cotton effect, and (–)-indocarbazostatin B having a negative atropisomeric chirality, from a culture broth of *Streptomyces* sp.[35-37] In addition, an efficient screening system for the Maillard reaction inhibitor from natural product extracts was established. When the fluorometric analysis of fluorescent material based on advanced glycation end products (AGEs) was applied to screening for Maillard reaction inhibitor from plant extract, a graduate student encountered a quenching effect in most of the natural product extract tested. I told him, "Why not add TCA to remove such quenching and autofluorescent materials from the reaction mixture?", and it worked.[38] Dr. Akiko Saito joined my laboratory as JSPS Fellow and she had achieved excellent synthetic studies for proanthocyanidin.[39-41] My role was to simply provide hints, "Why not screen Lewis acids?[39,40] Why not connect two parts with a linker?[41]" Not only faculty members and a postdoctoral fellow have left from the lab, many graduates from the Biotechnology Research Center, Toyama Prefectural University have also left and are active in academic or industry fields.

Tautomycetin, specific inhibitor of protein phosphatase type 1

In 2003, I left Toyama and moved to Sapporo as a Professor in the Graduated School of Agriculture, Hokkaido University, which might be the last chance I have to teach at my alma mater. In the laboratory, research on lignin, pulp, extractives from wood and its extended fields of their research had been conducted. Beside the studies on various biologically active small molecules which I have found, I proposed two new projects. One is creation of artificial lignin and another is exploratory research for biologically active small molecules from forest using the

198 entire assemblage of organisms such as trees, shrubs, herbs, microorganisms, and animals. Dr.
199 Takao Kishimoto, Research Associate, finally synthesized β-*O*-4 type artificial lignin comparable
200 to milled wood lignin in 2006. The research work was published and was featured as centerfold
201 in the *Organic & Biomolecular Chemistry.*[42] He was appointed Associate Professor of Toyama
202 Prefectural University in 2007. Dr. Yasumitsu Uraki, Assistant Professor, raised the theme of
203 artificial cell-wall creation-research, and working together with a graduate and an
204 undergraduate students, they succeeded in making a honeycomb structure from bacterial
205 cellulose produced by *Gluconacetobacter xylinus.*[43] His research was highly appreciated and he
206 was promoted to Professor and given his own laboratory. Tulip is the prefecture flower of
207 Toyama, and Dr. Kazuaki Shoji of Toyama Agricultural Center found antimicrobial activity in
208 water extract of the tulip anthers. I undertook elucidation of the active product and soon found
209 that it was 6-tuliposide B.[44] This compound was a known compound, but it was unstable under
210 various deprotection conditions, so that total synthesis could not be achieved by the other
211 research groups. Therefore, I decided to synthesize tuliposide B and clarify the structure-activity
212 relationship on the compound in the laboratory of Hokkaido University. Kengo Shigetomi who
213 was an undergraduate student expressed a strong desire to achieve this goal. Together we
214 completed the first total synthesis of 6-tuliposide B,[45] and structure-activity relationship
215 study.[46,47] We gained evidence that the putative adduct of 6-tuliposide B with UDP-*N*-
216 acetylglucosamine (UDP-GlcNAc) inhibits the growth of MurA-overexpressing *E. coli* which
217 shows resistance to fosfomycin (Fig. 3).[48] Shigetomi became a talented faculty member of the
218 laboratory after completing his doctoral degree and subsequent postdoctoral fellowship.
219

<Fig. 3>

An important biological-work was the discovery of the function of tautomycetin as a specific inhibitor of protein phosphatase type 1 (PP1).[49,50] The main experiments were carried out by Shinya Mitsuhashi who was previously a graduate student in the laboratory of Professor Kunimi Kikuchi of the Institute for Genetic Medicine, Hokkaido University. Professor Kikuchi wanted to investigate the efficacy and specificity of tautomycetin as a protein phosphatase inhibitor, so he had been conducting joint research with me. After completing a doctoral degree, Dr. Mitsuhashi joined my laboratory as a postdoctoral fellow and later became Project Lecturer at Hokkaido University.

When I determined the structure of tautomycin,[15] it was clear to me that the molecule must have potent inhibitory effects against protein phosphatase because of structural similarity with okadaic acid which was just uncovered to be a potent inhibitor of protein phosphatase.[51] Protein phosphatase type 2A (PP2A) was the primary target of okadaic acid, while tautomycin was a dual inhibitor for PP1 and PP2A with a slight bias to PP1.[52] Tautomycetin having very similar structure to tautomycin was found to be the only inhibitor specific to PP1 at low concentrations.[49,50] The discovery opened the door to treatment strategies for PP1-mediated immune disorders, cancer or neurological disorder via understanding the distinguishable roles of PP1 and PP2A, two major serine/threonine protein phosphatases in human cells.[53-55] For example, sephin 1, a selective inhibitor of PP1 holoenzyme containing growth arrest and DNA damage-inducible protein (GADD34), attenuated expression of stress-inducible gene products.[56] The approach is one of the several attempts to develop PP1-targeted therapeutics

for neurological disorders such as amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS) in which circadian rhythm may be involved. Thus, appropriately modulating PP1 activity could lead to new treatments for neurological disorders in Minkowski space, a combination of three-dimensional space and one dimension of time (Fig. 4).[55)]

<Fig. 4>

Further efforts for discovery of biologically active small molecules helps the development for new therapeutic strategy

Rediscovery of mycophenolic acid as a latent agonist of PPARγ[57)] led to development of many interesting inhibitors against histone deacetylase (HDAC),[58)] human IMPDH,[59)] and *Trypanosoma congolense* IMPDH.[60)] The study on *Tc*IMPDH in turn led to the identification of *T. congolense* guanosine 5'-monophosphate reductase (*Tc*GMPR).[61)] The structurally new biologically active small molecules first discovered in the laboratory of Hokkaido University are (+)-indocarbazostatin C and (–)-indocarbazosatin D, inhibitors of NGF-induced neuronal differentiation, isolated from a culture broth of *Streptomyces* sp.[62)] Another important new compound is (+)-epogymnolactam, an autophagy inducer, from *Gymnopus* sp.[63,64)]

Microorganisms, especially *Actinobacteria,* have been considered to be treasure houses of biologically active compound resources, but recently it seems difficult to discover structurally new compounds. However, researchers have only discovered novel compounds from cultivable microorganisms that account for 1% of the total microorganisms. If you can cultivate previously uncultured microorganisms, accounting for the other 99% of microorganisms, the possibility to

discover new biologically active small molecules will expand. We discovered zincmethylphyrins and coproporphyrins released by α-proteobacterial strain *Sphingopyxis* sp. GF9 as novel growth factors for an uncultured actinobacterial strain *Leucobacter* sp. ASN212.[65] I proposed a general growth mechanism for uncultured *Actinobacteria* having noncanonical heme biosynthesis pathway but it lacks the early canonical pathway (Fig. 5).[66,67] Human beings have lived with microorganisms. Although intestinal bacteria are the best example of this, mitochondria that produce ATP using heme retain some of the genes homologous to those of α-*Proteobacteria* internal symbiotic to archaea far past. The symbiotic relationship between α-proteobacterial strain GF9 and actinobacterial strain ASN212 was reminiscent of the birth of eukaryotes that occurred only once 1.5 – 2.0 billion years ago. These findings are not only important from scientific perspectives but also for practical applications point of view for discovering new natural compounds.

<Fig. 5>

Cobalt, copper, or nickel complex of coproporphyrin I inhibited the viability of uncultured *Leucobacter* sp. ASN212.[66] Given high rates of antimicrobial resistance among *Actinobacteria* and *Firmicutes* and the observation that knock-out of *hemQ* in *Staphylococcus aureus* resulted in a small colony variant phenotype,[68] the development of HemQ or HemH inhibitor might be useful in the treatment of infectious disease caused by drug-resistant strains of *Mycobacteria*, *Listeria*, *Staphylococcus* species,[69] and diseases relating to the abundance of *Firmicutes* in the human gut microbiome.[70]

Before finishing this article, I would like to introduce a book that has encouraged me since

1975. The book contains the signature of Professor Akira Suzuki, a disciple of Dr. Herbert C. Brown, and is still on my bookshelf. At the end of the book “Boranes in Organic Chemistry” written by Professor Brown, it says “Tall oaks from little acorns grow.”[71)]

Conclusion

Biologically active small molecules of interest are also isolated from plants,[72,73)] but many of the novel compounds having specific molecular target have been discovered from cultures of microorganisms. Even within my narrow experience, I caught a glimpse of the amazing capability of microorganisms. It was a coincidence that I made the choice to use microorganisms, but the Microorganism Utilization has been inevitable as the use of my own name, Makoto Ubukata. A new biologically active small molecule may change our scientific perspective, and contribute to the survival and welfare of mankind and my dreams may seem as a faraway star but they are not just dreams. When I think of scientific matters, my cranial nerve-cell mitochondria produce a lot of ATP through the electron transport chains. I have thought about the logic of biologically active small molecules using such ATP, one of the most important biologically active small molecules for all living things. Only several hints for understanding the logic have been shown in this article (see Graphical Abstract). I am always hungry for creative things and am still making effort to fully understand them. Chance favors only the prepared mind. An important finding may be found in negative data. Don’t throw away your data even if you got an unexpected result. Work hard and think flexibly.

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Author contributions

The author has read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the author.

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Figure Legends

Graphical Abstract

517 Microorganisms, plants, and other organisms have uniformity as living things. Among them,
518 microorganisms have genetic diversity of secondary metabolite biosynthesis and its epigenetic
519 diversity.
520
521 **Figure 1.** Liposidomycins, inhibitors of bacterial peptidoglycan synthesis. Notes: (a)
522 Liposidomycin B mimics the transition state between UDP-MurNAc pentapeptide and P-lipid,
523 and inhibits phospho-*N*-acetylmuramoyl-pentapeptide-transferase (MraY), an essential enzyme
524 for bacterial cell wall synthesis. (b) Determination of diene double bond positions in the lipid
525 moiety of liposidomycin A, and derivatization of anhydrodeacylliposidomycin to CPZEN-45, as a
526 drug candidate for extensively drug-resistant (XRD) tuberculosis.
527
528 **Figure 2.** Trifluoroacetimidates are more stable and easy to handle than trichloroacetimidates.
529 Notes: During the synthetic study of epiderstatin, a new conversion method of the amide to the
530 nitrile was discovered. Using a one-pot synthesis of trifluoroacetimidate, the stereochemistry of
531 liposidomycin was confirmed.
532
533 **Figure 3.** Analogs designed from the putative UDP-GlcNAc-tulipalin B adduct or itself provide a
534 new type of inhibitors which are effective for fosfomycin-resistant pathogenic bacteria by
535 blocking UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) and subsequent enzymes in
536 peptidoglycan biosynthesis.
537
538 **Figure 4.** Structural similarities and differences between okadaic acid, tautomycin, and

tautomycetin. Notes: The biological researches using tautomycetin opened the door for understanding the role of PP1 which could be a molecular target for treatment of neurological disorders such as amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), schizophrenia and other mental illnesses.

Figure 5. Zincmethylphyrins and coproporphyrins, novel growth factors released from *Sphingopyxis* sp. enable laboratory cultivation of previously uncultured *Leucobacter* sp. Notes: Protoheme is essential for redox reactions involving electron transport chains that generate electrochemical proton gradients which drive the synthesis of adenosine triphosphate (ATP).