CHAPTER 10
EXPANDING HORIZONS

Biopharmaceuticals: Extracts to Proteins and Peptides

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In looking over the landscape (and seascape) of natural products chemistry on the 50th anniversary of the American Society of Pharmacognosy (ASP), certain fundamentals still apply. The avid researcher still looks for organisms that have not yet been studied chem-ically and tries to evaluate biological activities with increasingly sophisticated assays. Once a new biologically-active compound is identified and structurally characterized, the next big hurdle is most often how to get enough of it to continue research into its poten- tial utility for human health. Difficulty in obtaining sufficient amounts of many natural products has often resulted in years of delay prior to the development of these agents as potential drugs [e.g., taxol, ecteinascidin, halichondrin]. As the ASP heads into its next 50 years, several strategies going beyond finding new chemistry in unstudied organisms, are the application of modern molecular biology techniques to producing sufficient quantities of new compounds for development. Laboratories now investigate the culturing of previously unculturable microbes, the characterization and genetic engineering of bio-synthetic gene clusters, and conduct metagenomic analyses and express DNA from un-identified microorganisms from sea and soil in *E. coli* or other heterologous organisms.

An approach, which we have pursued is to investigate biologically active peptides and proteins from natural product extracts.[1] Rather than look for novel organisms in toxic lakes or deep-sea vents, we look for novel biological activities in the proteinaceous components of aqueous extracts which have been largely ignored by natural product chemists. The proteins in these extracts provide a rich source of new molecules with unique structures and potent biological activities. In addition, once a new compound is identified, these proteins and peptides can often be readily expressed in *E. coli* and other culturable organisms to supply the amounts of compound necessary for developmental studies. The combination of rich biochemical diversity with excellent possibilities for new discoveries and a ready means for large-scale production makes this area of pharmacognosy a potentially valuable area of research.[2]

Several novel proteins from natural products extracts have been discovered at the NCI by researchers in the Molecular Targets Development Program. This effort began in the mid 1990's with the discovery of the antiviral protein cyanovirin-N (CV-N) by Kirk Gustafson. CV-N was first isolated from aqueous extracts of the Hawaiian cyanobacterium *Nostoc ellipsoporum* based upon its potent anti-HIV activity.[3] CV-N was expressed recombinantly in *E. coli*[4] in quantities that allowed for structural studies by both NMR[5] and x-ray crystallography,[6] which determined that CV-N's structure was unique in the natural world and that its protein fold represented a new superfamily in protein structures. In additional functional studies on CV-N, we defined the molecular targets for its anti-HIV activity as the envelope glycoproteins gp120 and gp41,[7] and determined that its mechanism of action involved preventing viral fusion and entry.[8] CVN is currently being developed as a topical microbicide for the prevention of HIV infection and has shown efficacy in macaque models for topical microbicides.[9] The discovery of CV-N has led to

numerous research studies both structurally and functionally, with over 100 additional manuscripts published since 1997.

Several additional antiviral proteins and peptides have been isolated and characterized at the NCI, including niphatevirin,[10] adociavirin,[11] *Myrianthus holstii* lectin,[12] the circulins,[13] and scytovirin.[14] The majority of these proteins have unique amino acid seq-uences with little homology to known proteins, and as such, represent new pharma-cophores. These discoveries have all been synergized by the knowledge gained in our and other laboratories since the discovery of CV-N. One recent discovery in particular dem-onstrates some of these advances. We originally isolated and characterized the antiviral protein griffithsin (GRFT) in 2005[15] from aqueous extracts of the red alga *Griffithsia* sp. GRFT was shown to specifically bind to gp120 and gp41, and to have picomolar activity in cellular anti-HIV assays. GRFT was quickly produced recombinantly in *E. coli*, and its three-dimensional structure was elucidated *via* x-ray crystallography.[16] GRFT has now been shown to be non-immunogenic in rabbit and rodent studies, to be non-toxic at sys-temic doses of at least 40 mg/kg/day, and to be readily distributed and bioavailable foll-owing subcutaneous injection, as well as possessing the physiochemical qualities neces-sary for a practical microbicide candidate.[17] Additional development studies have shown that GRFT is potently active against all clades of HIV, is non-damaging and non-immunogenic to mucosal tissues and protective against HIV infection of human cervical explant tissues.[18] Finally, we have recently reported on the large-scale agricultural production of GRFT in tobacco plants; we were able to transfer the gene for GRFT into tobacco mosaic virus and utilize that virus to infect *Nicotiana* plants. The infected plants were harvested five days later and were found to have produced approximately one gram of GRFT per kilogram of plant material. The work with GRFT illustrates the full circle of modern pharmacognosy research, whereby the gene for a novel bioactive compound from a New Zealand marine organism is transferred to a bacterium, then to a virus, and finally to one of the oldest medicinal plants ever cultivated in N. America, all within two years. The development of natural product-derived biopharmaceuticals is a nascent area of research with great potential for the discovery of bioactive molecules, and their rapid advancement into useful reagents and lead compounds for drug development.

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Combinatorial Biosynthesis

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"During World War II countless lives were saved through the use of the antibiotic penicillin, a natural product of a mold. However, the drug became widely available only after a method was developed to mass-produce it from a selected and genetically altered strain of the mold. University of Wisconsin bacteriologist Kenneth B. Raper isolated a productive organism, botanist John F. Stauffer genetically modified it, and biochemists William H. Peterson and Marvin Johnson developed submerged fermentation techniques to produce penicillin in quantity. The early wholesale cost of 100,000 units dropped from twenty dollars to three cents by the end of the war." This plaque on the University of Wisconsin-Madison campus commemorates the UW-Madison contributions to the early development and mass-production of penicillin and highlights, in my opinion, combin-atorial biosynthesis in the broadest definition working at its best. Since these early days, culminating with the seminal work of Sir David Hopwood and colleagues on cloning and heterologous expression of the actinorhodin biosynthetic gene cluster for the production of novel "hybrid" natural products in the early 1980s, combinatorial biosynthesis as a field has come to embrace both traditional and contemporary methods of strain improve-ment, metabolic engineering and all aspects of genetic manipulations used to engineer the production of the natural products and their analogs.

Combinatorial biosynthesis (CB) depends on four prerequisites to realize its full potential in natural product production and structural diversity. These criteria include: (i) the avail-ability of the genes encoding the production of a particular natural product or family of natural products, (ii) genetic and biochemical characterizations of the biosynthetic machinery for the targeted natural products to a degree that CB principles can be rationally applied to engineer their biosynthesis, (iii) expedient genetic systems for *in vivo* manipulation of genes governing the production of the target molecules in their native producers or heterologous hosts, and (iv) production of the natural products or their engineered analogs in quantities appropriate for detection, isolation, and structural and biological characterization.

Since the cloning of the first biosynthetic gene cluster in the early 1980s, databases have now expanded to include more than 200 billion base pairs of sequence information from 240,000 named organisms; the number of base pairs in the GenBank doubles about every 18 months. The observation that biosynthetic, resistance, and regulatory genes encoding natural product production in bacteria are often clustered within one region of the microbial chromosome has greatly simplified annotation of genes with respect to natural product biosynthesis. Whole-genome sequencing has also revealed that there are far more biosynthetic gene clusters than there are known metabolites for a given organism, suggesting that the biosynthetic potential for natural products in micro-organisms has been greatly under-explored by traditional methods of natural product discovery. This finding has inspired conceptual breakthroughs and technological innovation to discover these so-called "cryptic" natural products. A variety of pathway-specific strategies have also been developed and refined to clone characteristic DNA sequences that can ultimately be used to identify the biosynthetic machinery for families of natural products from various organisms. Biosynthetic machineries of representative members of all major natural product classes have been cloned. It is reasonable to assume that access to the genetic information for natural product production is no longer the limiting factor for CB.

The availability of genomic information and genes has significantly facilitated the deciphering of biosynthetic logic and machinery as a whole, as well as the dissection of enzyme reaction mechanisms responsible for individual transformations. The past two decades have witnessed exponential growth in the discovery of novel biosynthetic pathways, chemistry, and enzymes and in the biochemical characterization of individual enzymes for the biosynthetic machinery of all major classes of natural products. The remarkable ability to produce soluble and functional enzymes in large quantities, coupled with advances in automation and resolution of X-ray crystallography, have dramatically accelerated the pace at which biosynthetic enzyme structures are solved. This was most beautifully exemplified by the recently solved structures of fatty acid synthase, polyketide synthase, and nonribosomal peptide synthetase, as well as a myriad of other enzymes from diverse natural product biosynthetic machineries. Equally impressive are the other emerging technologies, such as FTMS with remarkable precision and resolution to follow enzyme-bound intermediates, and cryo-electron microscopy to directly visualize, hence localize, large biosynthetic enzyme complexes *in situ*. It is now possible to predict the biosynthetic machinery and to design combinatorial biosyntheses for the major classes of natural products by knowing their origin and inspecting their structures.

Establishing an expedient genetic system for *in vivo* manipulation of the biosynthetic machinery for a targeted natural product is of paramount importance for CB. Although not practical, empirical circumstances may dictate that a genetic system be developed for each natural product producer. However, a more desirable goal would be the establishment of a limited number of model heterologous hosts whereby the designed genetic engineering experiments can be carried out for any natural product or family of natural products. A single, "universal" host, suitable for all classes of natural products from all natural sources, is not likely to be found. Advances towards the development of a suite of expression systems for specific natural product classes are known. For instance, natural products of actinomycetes origin have been heterologously expressed in several *Streptomyces* hosts. Significant progress has also been made in developing *E. coli*, *Myxococcus xanthus, Bacillus subtilis* and *Pseudomonas putida* as general hosts for natural product production and engineering. By and large, however, there remain many challenges and few solutions to the development of practical genetic systems for the heterologous production of many important natural products.

Two historical shortcomings of natural products research are the small quantities of materials available from nature and the difficulty of their chemical total synthesis and structural diversification. CB offers promising solutions to both problems. It is now possible to dramatically increase natural product titers by deregulating their biosynthetic machinery. Progress has also been made in producing natural products of plant or other origin in model microbial hosts, thereby enabling their mass-production by fermentation. Rational engineering of biosynthetic machinery for natural product structural diversity has been very successful. The engineered novel products can be produced by recombinant organisms that are amenable for large-scale fermentation, although how to improve their yield remains a considerable challenge.

The principles of combinatorial biosynthesis were evident in the early days of natural products research. Recent advances in genomics, pathway elucidation, enzyme catalysis and emerging technologies fuel the growth of the field with speed and precision, ensuring that combinatorial biosynthesis will play an increasingly important role in natural products research.

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Drug Discovery and the Expanding Role of Endophytes

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Pharmaceutical science was born from man's need and will to survive by searching the natural world for things that would relieve pain and suffering and restore health. Being a land creature, the most diverse and readily available natural resources were members of the plant kingdom. Even today, many indigenous peoples throughout the world, have developed their own pharmacopeias, most of which rely on higher plants as the healing source. Certain practices of these peoples have provided useful and rewarding clues in the search for new medicines.[1] The best example of this is the use of willows by certain natives to reduce tooth and head pain via the chewing of small limbs of these trees. Thus, the discovery of aspirin can be attributed to the practices of native people who, probably, through trial and error, found willows as a source of pain relief.

In time, rationale was developed that pointed to the direction of other creatures having benefits to the health and well-being of human kind. Certainly one of the best-known of these is the lab fungus (*Penicillum notatum*), from which penicillin was derived. Other promising microbes (*Streptomyces* spp.) found in soil environments soon had the developing pharmaceutical industry looking at every soil type on the planet for newer sources of antimicrobial activity and biological diversity. It turns out that in the frantic search for soil microbes with pharmaceutical potential, a major reserve of biological activity that was generally overlooked were the microbes that inhabit plants. These microbes are known as endophytes, and they live within the tissues (between the cells) of plants. They cause no overt tissue damage, thus producing no symptoms and, in fact, there is no overt evidence that they are even present in the plant. All plants seem to possess endophytic associations. However, it appears that plants living in the wet tropics have more endophytes than plants in polar or desert regions of the world. The exact role of these endophytic microbes in the plant has yet to be defined, but it has been suggested that they may be providing protection against insects and attack by parasitic microbes.

One intriguing aspect of the biology of endophytes is the possibility that, over time, the endophyte may have assumed some of the genetic information of the plant and begun to make some of the same products as the plant. In fact, some of these compounds have turned out to be phytohormones, and other products normally associated with the higher plant. Related to pharmaceutical discovery is the seminal observation that certain fungal endophytes of yew (*Taxus* spp.) also make the important anticancer drug paclitaxel,[2] and other important active compounds of higher plants, such as campthothecin and vincristine, also have their endophytic microbial counterparts. This of course raises the question of what the source might be of the cathartic effects of plants as observed by native peoples. Is the active ingredient from the plant, or from microbes found therein?

In my experience, it is generally the case that novel biology can represent novel chemistry. Novel chemistry, with appropriate biological activity can immediately lead to intellectual property, publication, and product licensing. Thus, the discovery of novel microbes is the key goal in the search process. Also, since endophytes as a group already have established an association with another eukaryotic organism, the likelihood of finding bioactive compounds having significant toxicity seems much reduced, in contrast to products from other microbes.

The search for endophytes can be a daunting exercise, because they originate in plants, and the botanical world is diverse. It is important to develop a rationale to provide the best opportunities to isolate novel endophytic microorganisms. Creative and imaginative strategies must be used to expediently narrow the search for interesting endophytes displaying bioactivity. The following are some reasonable hypotheses governing this plant selection strategy: Plants from unique environmental settings; that have an ethno-botanical history; that are endemic, have an unusual longevity, or that have occupied a certain ancient land mass, such as Gondwanaland; and plants growing in areas of great biodiversity also have the prospects of hosting endophytes with great biodiversity.[1] Some examples of compounds and microbes having promise for their pharmaceutical potential are:

Endophytic streptomycetes: Nearly every endophytic streptomycete that has been found in the world's rainforests possesses a different 16S rDNA similarity to those 800 + streptomycete sequences found in GenBank. Many of the products of these endophytes are also new to science including kakadumycin, the munumbicins, and others in various stages of characterization.[3,4]

***Muscodor* species:** This new endophytic fungal genus is characterized by each species producing one or more biologically active volatile antibiotic or other volatile compound having biological activity. The volatiles of several species are active, on relatively short-term exposure, to some of the world's nastiest plant and human pathogens, including *Pasturella pestis*, *Xanthomonas citri*, *Mycobacterium* spp., *Salmonella* sp. *Escherichia coli* and *Staphylococcus* sp.[5] The concept of aromatherapy may eventually take on a new meaning to pharmaceutical science, especially with the prospect that lung and bronchial infections may be treatable with the volatiles of *M. albus*.

***Volutella* sp.:** As an endophyte of *Pteromischum* sp., this fungus makes volutellin A, which is not only antifungal, but is immunosuppressive. Its antifungal activity parallels that of cyclosporin A, and its immunosuppressive activity is in the same range of this same well-known compound. However, volutellin A has no detectable toxicity to human blood cells, in contrast to cyclosporin A.[6]

While many barriers present themselves in a jungle search for endophytes, including the necessary permit forms, the potential to get a nasty parasitic disease, and the likelihood of running into a poisonous snake or spider; the search is always productive.

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Genomics/Metagenomics – Impact on the ASP

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The potential of genomics in pharmacognosy is broad and varied, but it is fair to say that many of the discoveries in this field have not surfaced in ASP meetings. Perhaps the first widely used genomic application directly affecting pharmacognosy research was the acceptance of 16S rRNA as the definitive taxonomy indicator for microbes, yet most of the science behind this, and its application, involved more specialized societies. Two major genomics breakthroughs in the early 90s were the understanding of the modular nature, co-linearity and domain specificity of PKS Type Is[1] and the modular nature of NRPSs.[2] In the latter case, however, the specificities of the amino acid-determining adenylating domains were not elucidated until about 2000.[3] The genomics governing the biosynthesis of 2° metabolites has expanded to include new routes to bioactive natural products, including the production of "unnatural natural products", combinatorial biosynthesis, and meta-genomics, and additionally a super-sensitive genomic screen detects novel metabolites in a classical bioactivity directed paradigm.

Despite these breakthroughs, their influence on the science reported at ASP meetings or published in *J. Nat. Prod.* was slim until near 2000. In 1998 (Orlando meeting), S. Gould described much of the gene locus encoding the biosynthetic enzymes for production of pyoluteorin [first known example of a eubacterial (*Pseudomonas fluorescens*) type I aromatic polyketide],[4] and G. Ashley described technology being exploited at Kosan BioSciences for production of novel macrolides by engineering the genes responsible for the biosynthesis of erythromycin. In 2000 (Seattle), Dick Hutchison received the Research Achievement Award (RAA) for work on the genetics/biosyntheses of polyketides and the manipulation of PKSs to effect combinatorial biosynthesis, while Ben Shen, in receiving the newly named. Matt Suffness Young Investigators (MSYI) Award, spoke on the biosynthesis of bleomycins, an excellent model for NRPS/PKS hybrids.[5] In 2002 (New Brunswick), A.Vulpanovici (Gerwick group) described studies on Phormidolide, including the use of ketosynthase-domain conserved motif probes to search for genes encoding the "HMG-CoA synthase-like PKS modifying enzymes". Dick Moore's (U. Hawaii) RAA lecture included studies on the biosynthesis of apratoxin, the product of an NRPS/PKS, and of the Nostopeptolides, where the genes *nspE* and *nspF* code for the enzymes to convert leucine into 2S,4S-4-methylproline. Posters described the cloning of the gene cluster for Mitomycin C (L.C. Chang, Sherman group), and use of PCR-based DNA amplification methods in the QA of herbal preparations. (S. Crockett, Khan group). At Chapel Hill (2003), J. Nicholson discussed the application of NMR-metabonomics to functional genomics, toxicology and clinical diagnostics, and R. Trethewy described use of MS/MS to examine the metabolic profiles of mutant and wild-type *Arabidopsis*. M. Zabriskie's group reported using specific NRPS and PKS probes to screen marine cosmid libraries to discover a novel NRPS/PKS biosynthetic locus from a sponge. E. Schmidt, speaking in memory of John Faulkner (posthumously given the 2003 RAA), recalled how John's interest in biosynthesis led him into molecular biology and the study of the genomics of biosynthesis, particularly in marine organisms. Seichi Matsuda, a MSYI Awardee, spoke on terpenoid biosynthesis, the enzymes involved, evolutionary relationships, and site-specific mutagenesis.

The meeting in Phoenix (2004) featured the exploding role of genomics in pharmacognosy. The RAA recipient, Jon Clardy, spoke on "A DNA-based Approach to Natural Products", addressing the heterologous expression of extracted environmental DNA for the discovery of novel 2° metabolites, and MSYI Awardee, Jörn Piel, described the heterologous expression of the pederin biosynthetic gene cluster from the beetle *Paederus fuscipes*, and of related compounds from the sponge *Theonella swinhoei.* A symposium on Structure-based Biosynthesis included talks on terpenes (J. Chappell), Type III polyketide synthases (J. Noel), protein engineering of PKSs (C. Khosla), and macrocyclic peptides biosynthesis (M. Marahiel). Contributed oral papers covered the analysis of the genetic loci encoding the biosynthesis of aminocoumarins and the resultant production of over 50 new antibiotics by combinatorial biosynthesis (S-M Li & L. Heide);[6] heterologous cloning of some of the biosynthetic genes for the capreomycidine residue of the tuberoactinomycins and their use to elucidate the biosynthesis from arginine (X.Yin & M. Zabriskie);[7] the paradox of phenyl acetate primer units in cyanobacterial NRPS/PKS systems (M. Moffitt/Brad Moore group),[8] and phosphopantetheinyl transferases involved in priming these NRPS/PKSs (J. Copp & B. Neilan);[9] identification of the biosynthetic genes for equisetin as the first iterative PKS-NRPS hybrid identified in a fungus (*Fusarium heterosporum*) (J. Sims/ Schmidt group);[10] a shunt mevalonate to fatty acid synthesis pathway in the myxobacterium *Stigmatella aurantiaca* leading to the branched starter units (T. Mahmud).[11] Other oral contributions discussed the effects of the introduction of different cDNAs, for enzymes in the pathway to benzylisoquinolines, into explants using *Agrobacterium* (S. Frick);[12] development of a toolbox of functionally characterized biosynthetic genes derived from plants (A. Goossens);[13] discovery of a novel antifungal via genomic screening of actinomycetes, and the use of the genomic information to assure novelty prior to fermentation, and as an integral part of the structure determination (J. McAlpine);[14] a metabolomics approach, using multivariant analysis to examine the entire spectrum of metabolites from plants, as a means to discover novel drugs, prodrugs, and synergies (R. Verpoorte). Some 200 posters were featured in a "Biotechnology, Biosynthesis, Biological Assays, Phytochemistry, and Pharmacology" session, and included a range of subjects, including: a description of the genetic biosynthetic potential of myxobacteria, cyanobacteria and marine proteobacteria (G. König); a Favorskii-like carbon rearrangement in the biosynthesis of enterocin (L. Xiang); heterologous expression of two *Fusarium* trichothecene P450 genes (S. McCormick); keto-reduction timing in aromatic polyketide assembly (J. Kalaitzis); biosynthesis of saframycin MX1 from *Myxococcus xanthus* (J. Lee); molecular genetics of saponin biosynthesis in *Saponaria vaccaria* (D. Meesapyodsuk).

In Portland, ME (2007), talks covered the use of mutants to generate biosynthetic analogs of NRPS/PKS products of *Stigmatella aurantica*, and the genetics and enzymology of biosynthesis of the C7N aminoglycoside moiety of Cetoniacutone A by heterologous expression of the bio-synthetic cluster in three strains of *S. lividans* (Mahmud group); the discovery of platensimycin using a new screen in which fatty acid synthesis is turned down via antisense RNA for FabF, hence sensitizing the test organism to inhibitors of this enzyme (Merck group).[15]

Clearly genomics and metagenomics are at the forefront of many of the scientific disciplines that make up pharmacognosy and feature increasingly in the Society's meetings and publications.

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Microbial Involvement in Secondary Metabolite Production

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Introduction

Over the centuries, the source of any compound from Nature was considered to be that of the organism from which it was first isolated. Thus up to roughly the last twenty or so years, secondary metabolites were almost always prefaced in reports and/or titles by a modifier such as "plant (insert taxon) metabolites…, or marine invertebrate (insert taxon) sourced...", and over the years, relatively complete schema have been derived in the literature that developed the concept of "chemotaxonomy", whereby analyses of the metabolite patterns from given organism(s) were used to assign organisms to specific taxons. However, chemists working predominately with compounds isolated from marine invertebrates began to ask "what is/are the actual producer(s) of these metabolites that we are finding?" as the structures being elucidated were close to materials that had been reported from microbes, and in one famous example described further below, from an Amazonian beetle, yet these had been isolated from diverse marine invertebrates. So what could be the link between these discoveries? As will be shown, the fundamental relationships appeared to be common microbial gene clusters.

The Pederine-Mycalamide Linkage (Beetles to Sponges!)

When the extremely potent series of compounds exemplified by mycalamides A and B were reported from a *Mycale* sponge species by the Blunt & Munro group (note that another entirely different structural series, also named as mycalamides, were reported by Fusetani *et al.* the same year from the same genus), their relationship to the previously reported beetle toxin, pederine was obvious. Over the next ten to fifteen years, a series of over 35 compounds that were obviously related to pederine were reported; these included ring opened variants first reported by the Pettit group as irciniastatins and then rediscovered/redescribed by the Crews group as psymberins the following year, but do not include the psymberin-pederine hybrids recently synthesized by De Brabender's group. However, the most important advances were those made by Piel (and later coworkers on two continents), where, having first reported that pederine was produced by a commensal pseudomonad in blister beetles of the genus *Paederus,* he proceeded to demonstrate that using the same gene probes, the clusters were present in the onnamide producing sponge *Theonella swinhoei* (onnamide being a close relative of mycalamide). Piel has continued working in this area and recently published, in conjunction with Hentschel, an analysis of common PKS-gene clusters found in microbial consortia in marine sponges.

Cryptic (Silent) Gene Clusters in Microbes

One of the major concerns over many years of microbial fermentation as a method of producing beneficial secondary metabolites (e.g., antibiotics and anticholestemics) is "why do we not find more/different metabolites when we ferment microbes?" Although many years of effort have gone into optimizing production organisms, still some microbes would not produce consistently, even after use of techniques reviewed by Zahner in 1982. When the first antibiotic producing Streptomycete genome sequence, that of *S. avermitilis* was published by Omura *et al*. in 2001, the authors identified over 20 "gene clusters" with the potential to produce previously unknown agents. A year later, the Hopwood group published the sequence of *S. coelicolor* and again, over 20 clusters were identified, though only a handful of metabolites had ever been isolated from fermentation broths of this organism. The potential for use of these techniques in analyzing genome sequences was amply demonstrated by workers at Ecopia (now Thallion) in Laval, Canada, who devised a technique whereby they were able to identify and interrogate previously unrecognized secondary metabolite clusters in a large number of actinomycetes. Using their technique, they described the identification and production of a previously unrecognized antifungal agent from *Streptomyces aizunensis* NRRL B-11277, and mentioned that, on average, over 12 unrecognized clusters were in every Actinomycete they had investigated. These cryptic clusters are not only in bacteria but also in at least one fungus, *Aspergillus nidulans*, in whose genome over 40 secondary metabolite clusters have been identified by Keller's group.

Plant Metabolites

Although there have been sporadic reports suggesting that a number of "plant secondary metabolites "might have a microbe involved, with an excellent example being paclitaxel production by *Taxomyces adreanae*, the major argument against this idea was that the yields were so low when fermented. Well, if the control mechanisms of production are not known, this would be expected. Over the last decade however, there have been reports in the literature that show that the vinca alkaloids, camptothecin, and podophyllotoxin have all been isolated from fermentation of commensal fungi isolated from the producing plant, albeit at low levels. These fungi, however, are not grown under optimal fermentation conditions, though are free of carried over materials. One can argue that the field is at a similar stage to that of the marine actinomycetes prior to the work of Fenical and Jensen, and the work of Keller on control of *Aspergillus* metabolism may show the way to approach these problems. Certainly, the identification of bacteria that utilized PKS Type III gene clusters to produce chalcones (the so-called plant chalcone synthase), would also demonstrate that compounds once thought to be from plants may well be from a commensal microbe or from co-metabolism.

In Conclusion

What has become evident is that Mother Nature has used and reused genetic information in all Domains and Kingdoms. No longer (except when isolating from an axenic microbe) can one be assured that the organism you are using is the source. As a final example demonstrating the reason for the "axenic" comment, recently Hertweck's group in Germany demonstrated that rhizoxin, one of the early antimitotic agents, was actually produced by an endophytic bacterium (*Burkholderia rhizoxina*) in the fungus, not by the *Rhizopus microsporus*. Finally, the potential number of novel secondary metabolites still to be discovered is incalculable, but will be massive in size, and will require a multitude of skills including genomic analyses on a vast scale and identification of methods of rapidly expressing gene clusters in homo-and heterologous hosts.

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The Power of Total Synthesis and Combichem

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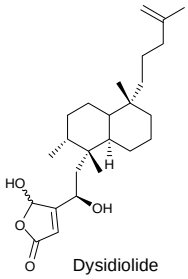
Total Synthesis: The total synthesis of complex natural products has long challenged top synthetic chemistry groups worldwide, and has led to dramatic advances in the field of organic chemistry.[1] It has led to a revision of original published structures, with notable examples being the marine-derived antitumor compounds, palmerolide[2] and diazonamide A.[3] As all pharmacognosists know, the isolation of sufficient supplies of an exciting, novel bioactive compound can be a serious limiting factor in furthering development, and our 'synthesis' colleagues have been coming to the rescue with the design of efficient approaches to the synthesis and structural modification of many challenging drug targets. This focus on devising economically feasible synthetic strategies is a very welcome development for both clinicians conducting clinical trials and patient populations. An excellent example is the marine-derived anticancer agent discodermolide, where total synthesis based on strategies developed by Amos Smith and Ian Patterson provided sufficient quantities for thorough clinical trials, which unfortunately were terminated due to lack of objective responses and toxicity.[4] Another strong plus of the synthetic process is the frequent identification of a sub-structural portion of the molecule bearing the essential features necessary for activity (the pharmacophore), leading in some cases to the synthesis of simpler analogs having similar or better activity than the natural product itself. Prominent among these is the marine derived antitumor agent, halichondrin B, where total synthetic studies showed that all or most of the potency resided in the righthand half of the molecule of the parent compound, and the analog, E7389 (Eribulin) is currently in Phase III clinical trials.[5] In some instances, clinical trials of the original natural product may fail, but clinical trials of totally synthetic analogs continue. Thus, while trials of the marine-derived anticancer agent, dolastatin 10, were terminated, the synthetic analog, TZT-1027 (auristatin PE or soblidotin) is in Phase II clinical trials.[6]

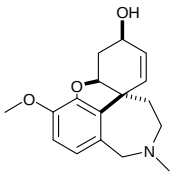
Nicolaou et al. stated it well - *"Today, natural product total synthesis is associated with prudent and tasteful selection of challenging and preferably biologically important target molecules; the discovery and invention of new synthetic strategies and technologies; and explorations in chemical biology through molecular design and mechanistic studies. Future strides in the field are likely to be aided by advances in the isolation and characterization of novel molecular targets from nature, the availability of new reagents and synthetic methods, and information and automation technologies."* [1]

Diversity-Oriented Synthesis, Privileged Structures and Combinatorial Chemistry: In a recent review of the impact of natural products on drug discovery and development during the period 1981-2006, we noted that the expected surge in productivity due to the introduction of combichem. did not materialize, and the number New Chemical Entities (NCEs), including

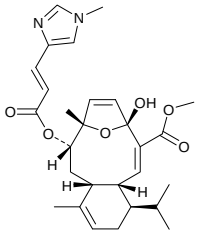
biologics and vaccines, from available data hit a 24-year low of 25 in 2004.[7] However, as noted in a recent article entitled "*Rescuing Combichem. Diversity–oriented Synthesis (DOS) aims to pick up where traditional combinatorial chemistry left off*", the use of natural product-like scaffolds for generating meaningful combinatorial libraries has been increasingly emphasized, and "*natural product-like compounds produced in DOS have a much better shot at interacting with the desired molecular targets and exhibiting interesting biological activity*".[8] This approach is exemplified by the work of the Schreiber group, who have combined the simultaneous reaction of maximal combinations of sets of natural-product-like core structures ("latent intermediates") with peripheral groups ("skeletal information elements") in the synthesis of libraries of over 1000 compounds bearing significant structural and chiral diversity.[9] Detailed analyses of active natural product skeletons enables the identification of relatively simple key precursor molecules to use as building blocks in combinatorial synthetic schemes, permitting the study of structure-activity relationships. The generation of small libraries, as exemplified by the work of the Waldmann group and others in the synthesis of molecules such as epothilone A, dysidiolide, galanthamine and psammaplin, has been reviewed.[10]



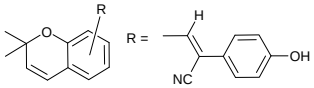
Dysidiolide



Galanthamine



Sarcodictyin



Benzopyrans

The use of an active natural product as the central scaffold in the generation of large numbers of analogs for structure-activity studies, the so-called parallel synthetic approach, is exemplified by the syntheses around the sarcodictyin scaffold.[11] Likewise, the use of natural products as leads for combinatorial synthetic strategies, embodied in the concept of "privileged structures",[12] is illustrated by the application of solid-phase synthetic methods to the optimization of benzopyrans with a cyanostilbene substitution to yield compounds that are effective against vancomycin-resistance bacteria.[13]

\iThus, as eloquently stated by Wilson and Danishefsky, "*We would chance to predict that even as the currently fashionable "telephone directory" mode of research is subjected to much overdue scrutiny and performance-based assessment, organic chemists in concert with biologists and even clinicians will be enjoying as well as exploiting the rich troves provided by nature's small molecules*".[14]

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