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Drug Discovery and Natural Products: End of an Era or an Endless Frontier?

Jesse W.-H. Li and John C. Vederas*

Historically, the majority of new drugs have been generated from natural products (secondary metabolites) and from compounds derived from natural products. During the past 15 years, pharmaceutical industry research into natural products has declined, in part because of an emphasis on high-throughput screening of synthetic libraries. Currently there is substantial decline in new drug approvals and impending loss of patent protection for important medicines. However, untapped biological resources, "smart screening" methods, robotic separation with structural analysis, metabolic engineering, and synthetic biology offer exciting technologies for new natural product drug discovery. Advances in rapid genetic sequencing, coupled with manipulation of biosynthetic pathways, may provide a vast resource for the future discovery of pharmaceutical agents.

Just over 200 years ago, a 21-year-old pharmacist's apprentice named Friedrich Sertürner isolated the first pharmacologically active pure compound from a plant: morphine from opium produced by cut seed pods of the poppy, *Papaver somniferum* (*1*). This initiated an era wherein drugs from plants could be purified, studied, and administered in precise dosages that did not vary with the source or age of the material. Pharmaceutical research expanded after the Second World War to include massive screening of microorganisms for new antibiotics because of the discovery of penicillin. By 1990, about 80% of drugs were either natural products or analogs inspired by them. Antibiotics (e.g., penicillin, tetracycline, erythromycin), antiparasitics (e.g., avermectin), antimalarials (e.g., quinine, artemisinin), lipid control agents (e.g., lovastatin and analogs), immunosuppressants for organ transplants (e.g., cyclosporine, rapamycins) and anticancer drugs (e.g., taxol, doxorubicin) revolutionized medicine. Life expectancy in much of the world lengthened from about 40 years early in the 20th century to more than 77 years today. Although the expansion of synthetic medicinal chemistry in the 1990s caused the proportion of new drugs based on natural products to drop to ~50% (Fig. 1), 13 natural product–derived drugs were approved in the United States between 2005 and 2007, with five of them being the first members of new classes (*2*).

With such a successful record, it might be expected that the identification of new metabolites from living organisms would be the core of pharmaceutical discovery efforts. However, many pharmaceutical firms have eliminated their natural product research in the past decade. Although more than 100 natural product–based drugs are in clinical studies, this represents about a 30% drop between 2001 and 2008. Is the era of discovery of new drugs from natural sources ending?

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What Challenges Face Drug Discovery from Natural Sources?

Most of the current difficulties can be divided into two categories: the prevailing paradigm for drug discovery in large pharmaceutical industries, and technical limitations in identifying new compounds with desirable activity.

The pharmaceutical environment. The double-digit yearly sales growth that drug companies typically enjoyed until about 10 years ago has led to unrealistically high expectations by their shareholders and great pressure to produce "blockbuster drugs" with more than $1 billion in annual sales (*3*). In the blockbuster model, a few drugs make the bulk of the profit. For example, eight products accounted for 58% of Pfizer's annual worldwide sales of $44 billion in 2007. When such drugs lose patent protection, their sales revenue can drop by 80%. About 25% of the current U.S. drug market will lose patent protection within 4 years (*4*). This will remove more than $63 billion in annual income for pharmaceutical industries by 2014. Competition from generic drug manufacturers, which are generally not involved in drug discovery, accounts for 67% of all prescriptions in the United States and is encouraged by health agencies to reduce costs.

The financial outlook for firms doing drug discovery is further encumbered by extensive litigation, costs of competitive marketing, and increasing expectations for safety both by the public and by regulatory agencies such as the U.S. Food and Drug Administration (FDA). FDA approvals of new drugs reached a 24-year low as of 2007, and drugs approved in Europe have been rejected by that agency. As an example of legal costs, after the withdrawal of the anti-inflammatory drug Vioxx because of a potential increase in risk of heart attack and stroke, Merck had to set aside $970 million to pay for associated legal expenses in 2007, and another $4.85 billion for upcoming U.S. legal claims (*5*). One approach to dealing with rising costs and a dwindling pipeline of new drugs is purchase of other companies that have such resources. Recent examples include the purchase of Wyeth by Pfizer for $68 billion and the acquisition of Schering-Plough by Merck for $41 billion (*6*). Such large sums affect the way drug discovery is done: Firms involved in drug discovery must hit the target not only accurately, but very quickly and very profitably. However, for reasons outlined below, natural product sources are currently not very amenable to rapid high-throughput screening (HTS) for desirable activity as drugs

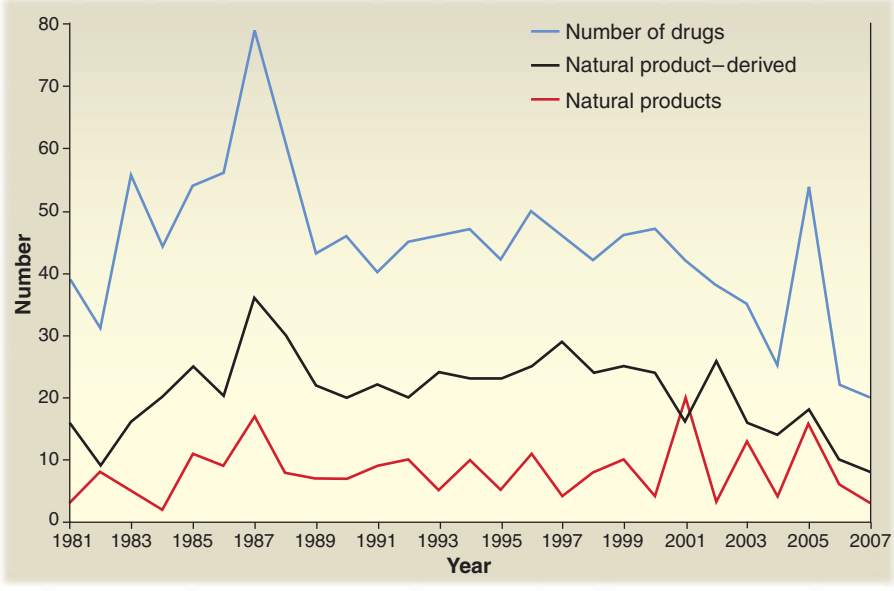

Fig. 1. Number of drugs approved in the United States from 1981 to 2007.

(*7*). Furthermore, in contrast to synthetic libraries, hits from natural sources are likely to have complex structures with numerous oxygen-containing substituents and an abundance of centers of stereochemistry (*8*). This slows the identification process and contributes to problems of supply and manufacture.

Difficulties in discovering natural product drug candidates. Historically, screening of natural materials for biological activity has worked well. Considering only polyketide metabolites, just over 7000 known structures have led to more than 20 commercial drugs with a "hit rate" of 0.3%, which is much better than the <0.001% hit rate for HTS of synthetic compound libraries (*9*). Although the hopes for useful leads from unfocused combinatorial chemistry libraries of mixtures have long since evaporated, pharmaceutical discovery efforts currently favor HTS of massive libraries of pure synthetic compounds. The output has been quite low, but any hits are usually easy to make and modify with simple chemistry. Libraries of pure compounds present in known amounts are also "screen friendly" and accommodate the desire for short timelines in examination of a large number of molecules. The recognition that such libraries are inherently limited prompts increasing interest in "diversity-oriented synthesis" and libraries of "privileged structures" (often based on known drugs or natural frameworks) to produce more complex molecules with a better chance of desirable bioactivity (*10*).

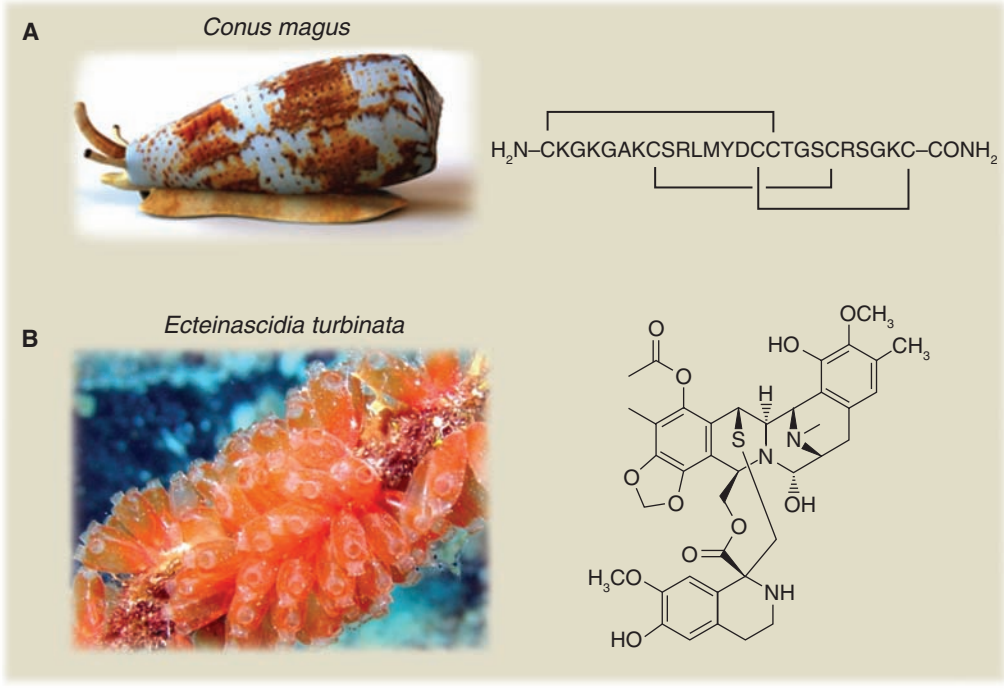

Fig. 2. Marine sources of drugs. (**A**) Ziconotide (Prialt) from *Conus magus*. Abbreviations for amino acids: A, Ala; C, Cys; D, Asp; G, Gly; K, Lys; L, Leu; M, Met; R, Arg; S, Ser; T, Thr; Y, Tyr. (**B**) Trabectedin (Yondelis) from *Ecteinascidia turbinata*. [Image of *C. magus* from (*45*), reprinted by permission of Macmillan Publishers Ltd.; image of *E. turbinata* by Florent Charpin, reprinted by permission of *Florent's Guide to the Tropical Reefs* (http://reefguide.org)]

HTS of natural sources also presents a variety of difficulties. Problems of reliable access and supply, especially with respect to higher plants and marine organisms, are compounded by intellectual property concerns of local governments and the Rio Convention on Biodiversity (*11*). Seasonal or environmental variations in the composition of living organisms can cause problems with initial detection of active compounds as well as subsequent repetition of assays or purification. Loss of source is also possible: Current extinction rates for natural species of higher plants are estimated to be a factor of 100 to 1000 over natural background (*12*). It has been suggested that 15,000 out of 50,000 to 70,000 medicinal plant species are threatened with extinction (*12*). Even if supply is easy and guaranteed, the initial extract of the natural material usually consists of a complex mixture after fractionation. It may contain only very small quantities of a bioactive substance, often as a mixture with structurally related molecules. The initial concentration of an interesting compound may be too low to be effectively detected by HTS, or the assay may be obscured by poor solubility or by fluorescent or colored contaminants. The key compound may be unstable in the mixture. A further complication can be synergistic (or antagonistic) activity of two constituents that may then diminish or disappear upon separation. For example, a number of bacteriocins (antimicrobial peptides from bacteria) must function as two-component systems to display full activity (*13*). Finally, considerable time is often required to complete structural characterization to determine whether the molecule is already known.

A prevailing sentiment in many pharmaceutical organizations is that screening of natural product sources is a difficult effort with a high probability of duplication; that is, the result may be a known compound that cannot be patented. However, fewer than 1% of microorganism species are easily cultured, and perhaps fewer than 15% of higher plant species have been examined for bioactivity (*14*). Certain insects and other animals have been targeted for specific bioactivities, such as toxins (*15*), but are not generally subjected to HTS efforts. Clearly the biological resource is there, but access and examination are problematic, especially if there is pressure for a short time frame for discovery of new leads.

What Tools Are Emerging to Enhance and Accelerate Drug Discovery from Natural Sources?

Several interlocking phases of exploration of natural sources can be considered: access to the biological resource, appropriate screening of that resource to locate a useful activity, analysis of the structure of the key compound, generation of analogs for optimal activity, and production of the target drug.

Biological resources. Traditionally, soil bacteria (especially actinomycetes), fungi, and higher plants were main sources for drug discovery. Pharmaceutical firms increasingly abandoned screening of microorganisms after 1990 because of decreasing success rates. Common antibiotics such as streptomycin occur in ~1% of soil actinomycetes and display activity in screens; this activity masks interesting new antimicrobials, which may be produced at a frequency of less than 1 in 10 million fermentations. A solution pursued by Cubist Pharmaceuticals is to massively increase the number of fermentations (to many millions per year) while miniaturizing their size, using calcium alginate beads as the containers (*16*). This approach is coupled to an assay with engineered *Escherichia coli* strains that are resistant to common well-known antibiotics. However, despite the urgent need to find new antibiotics effective against life-threatening organisms resistant to current therapy, many pharmaceutical firms do not develop such drugs. Sales and "blockbuster potential" are limited by the short duration of treatment with antibiotics relative to other drugs, such as cholesterol-lowering or hypertensive agents, which are consumed daily for prolonged periods and relieve

symptoms rather than provide a cure. Nonetheless, development costs, standards for safety, and requirements for limited side effects are similar for both antibiotics and "long-term" drugs.

Although plants remain a major source of new drugs, with 91 compounds in clinical trials as of late 2007, cyanobacteria (*17*) and marine organisms (*18*) have been actively investigated in recent decades, especially for neurotoxic and cytotoxic compounds. Ziconotide (Prialt), a peptide toxin from cone snails (Fig. 2A), was approved in 2004 for treatment of chronic pain resulting from spinal cord injury. A tropical sea squirt has yielded the cytotoxic trabectedin (Yondelis) (Fig. 2B), which is approved in Europe since 2007 for treatment of advanced soft-tissue sarcoma (*19*). Useful organisms may exist in extreme environments, such as at great sea depths (*20*), in thermal vents, or in salt lakes. An appealing example is the identification of haloduracin, a two-component lantibiotic (lanthionine-containing peptide antibiotic) from *Bacillus halodurans*, which grows at an extreme pH of >9.0 (*21*). A well-known lantibiotic is nisin A, which is used to preserve food and is very active against Gram-positive bacteria resistant to conventional antibiotics. However, nisin's therapeutic potential is blocked by its instability at neutral pH or above. Van der Donk and co-workers reasoned that base-stable lantibiotics may be produced by bacteria growing in alkaline environments (*21*). Using bioinformatics, they found haloduracin, which can survive pH ranges well above that of human serum. Although haloduracin's solubility is limited, it provides a basis for development of new lantibiotics with drug potential.

It may initially appear that there are few unexplored locations to look for natural sources of drug candidates, but enormous numbers of species have remained unexamined. There are claims that more than 99% of all bacteria cannot be cultured, and that in marine environments there may be 3.7×10^{30} microorganisms, many of which may produce fascinating natural products as drug candidates (*22*). To address this, in 1998 the concept of metagenomics was proposed to look at genes and their function in samples obtained directly from the environment (*23*). This field has exploded as faster and cheaper gene sequencing is becoming available (*24*) in combination with the ability to rapidly sort cells from the environment and efficiently clone genes in improved vectors (*25*). Together with automated screening techniques, the metagenomic approach could afford access to the pool of 99% of unexamined microorganisms. However, at present most pharmaceutical firms do not appear to be undertaking efforts in this direction for drug discovery.

Appropriate screening. It is likely that natural products represent privileged structures for drug discovery. This suggestion is supported by the fact that there are a limited number of protein folds known, and that natural products must bind to some of these in order to be biosynthesized and to fulfill their inherent function in the producing organisms. Hence, many of them may be structurally favored to bind to enzymes or protein receptors. However, as described above, the complexity of the initial natural extract can make it unfavorable for HTS. The ideal approach to overcome this obstacle would be automated separation of all constituents in the organism into individual components, coupled with full spectroscopic identification prior to HTS. Although this is not yet achievable, Ireland and co-workers (*26*) recently automated fractionation of crude extracts of natural materials from marine sources using desalting followed by high-performance liquid chromatography (HPLC) on highly efficient monolithic columns. This was in turn coupled to mass spectrometric analysis and collection on HTS plates. The process produces highly purified samples in 96-well plates, which are generated as replicates for initial screening as well as for a material archive. It is claimed that typically only three compounds per well are observed (*26*). Testing of a 15,360-member library of this type against hamster cell lines allowed identification of novel compounds with antitumor potential selective for breast cancer despite the co-occurrence of general cytotoxins in the same extract.

The efficiency of HTS can be greatly enhanced by using the best target to achieve "smart screening." One approach mentioned above is the use of organisms resistant to common known antibiotics, as used by Cubist. An innovative alternative was developed at Merck to identify broad-spectrum antibiotics (Fig. 3) (*27*). It uses a two-plate assay, in which one plate has *Staphylococcus aureus* bearing a plasmid that produces antisense RNA to a key fatty acid synthase enzyme (e.g., FabF) and the other is a *S. aureus* control plate without capability to produce the antisense RNA. The antisense RNA causes degradation of the mRNA at the 5′ end for the key enzyme, thereby enhancing the organism's sensitivity for inhibitors of that particular protein catalyst. This approach enables more sensitive detection of activity, and also permits identification of individual targets in complex systems through parallel screening with different antisense RNAs. The hit rate for such screening of more than 250,000 natural product extracts was high (0.3%). More important, it identified a new target, bacterial fatty acid synthesis, and novel antibiotics, platensimycin A and platencin.

Increasingly, multiple targets are being investigated with the use of cells. An interesting example is single-cell screening of inhibitors of phosphorylation (kinase) signaling pathways using flow cytometry (*28*). This phosphospecific flow cytometry (phosphoflow) makes multiple quantitative measurements of phosphorylation levels of different signaling proteins by measuring specific fluorescently labeled antibodies that recognize them after phosphate at-

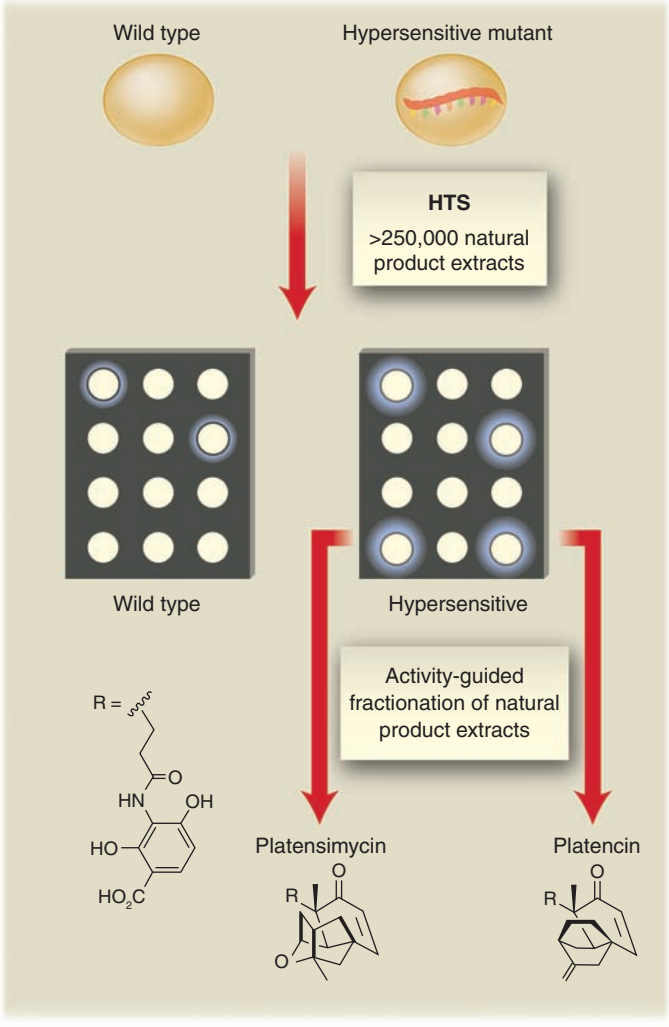

Fig. 3. HTS using differential-sensitivity whole-cell two-plate agar diffusion (the Merck platensimycin assay). The strain expressing antisense RNA to FabH or FabF enzymes of fatty acid biosynthesis is hypersensitive to inhibitors of those proteins. The approach identified two new antibiotics, platensimycin and platencin.

tachment. The effect of natural product libraries can be rapidly and quantitatively measured on single cells. Cell-based assays have also recently been used to find rapamycin analogs that lack the drug's usual immunosuppressive activity but protect nerve cells in models of stroke (*29*).

Analysis of the structure of active compounds. Structure elucidation on small quantities of material has been greatly assisted by advances in mass spectrometry and multidimensional nuclear magnetic resonance (NMR) spectroscopy. It is beyond the scope of this review to discuss the details of these techniques. However, automated coupling of mass spectrometry to separation by HPLC and HTS library creation clearly accelerates the identification of known compounds and possible hits. Cryoprobes for NMR spectroscopy have greatly enhanced sensitivity and have markedly reduced the amount of material required for analysis; for smaller compounds (molecular weight 200), useful proton spectra require 2 μg of material and carbon correlation spectra can be obtained on 0.2 mg (*30*). Comparison of NMR signal positions to corresponding databases of known compounds can hasten dereplication (i.e., recognition of known compounds). Efforts are also under way to automatically capture substances from HPLC separations by solid-phase extraction and then elute directly into an NMR cryoprobe for analysis.

Generation of analogs. Traditionally, structure-activity relationships in bioactive natural products were examined by simple chemical transformations. For example, methylation of the side chain of the cholesterol-lowering agent lovastatin produces simvastatin (Zocor) (Fig. 4), an improved drug that had sales in excess of $4.3 billion in 2006 (before loss of patent protection). The developing understanding of secondary metabolite biogenesis allows use of biosynthetic enzymes or genetically altered organisms to generate derivatives of drugs. Tang and co-workers used an esterase (LovD), which normally attaches the side chain of lovastatin, to make a series of analogs, including simvastatin (*31*). The esterase normally uses a 2-methylbutyrate attached as a thioester to a large protein as its acylating agent for the corresponding alcohol. However, a variety of simple thioesters could be substituted to give efficient conversion to many analogs.

Combinatorial biosynthesis, especially using modular polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs), holds great promise (*32*). PKSs and NRPSs are large multidomain enzymes that sequentially condense short fatty acids and α–amino acids, respectively. They resemble assembly lines for making a metabolite by chain elongation and functional group transformation, and can be altered to make new compounds. However, the results of mutation are frequently unpredictable, and the levels of product formation can be very low. The growing understanding of linkers between individual domains in these proteins as well as protein-protein interactions between domains may solve this problem. One approach to overcoming difficulties resulting from introduction of a modified domain is directed evolution to increase production by the chimera. Only three rounds of mutagenesis and screening of modest libraries (10^3 to 10^4 clones) of an NRPS domain were sufficient to give substantial improvement of production of an isoleucine-containing analog of andrimid (Fig. 4) having better antibiotic properties (*33*). In a different example, direct mutation of a PKS domain combined with inactivation of a gene responsible for post-assembly oxidation gave nystatin analogs (Fig. 4) with improved antifungal activity and lower toxicity (*34*). Inactivation of a post-PKS P450 that oxidizes a methyl to the C16 carboxyl in nystatin, coupled with mutation of the enoyl reductase activity in the NysC PKS module, affords methyl derivatives with a conjugated heptaene moiety (as opposed to an interrupted hexaene). The best compounds are effective against disseminated candidosis in mouse models and are considerably less toxic than amphotericin B. However, such generation of new analogs using combinatorial biosynthesis can require extensive time and effort, and is currently not matched to the requirements of HTS.

Production of target compounds. The traditional approach to optimal production of drugs by microorganisms is to "mutate and screen" for strain improvement, as was done in the 1940s for penicillin. This methodology can readily yield enhancement of drug production by two to three orders of magnitude, and in some cases by four to five orders of magnitude (*35*). At present, this tactic is difficult to beat, and molecular biological manipulations have been more successful only in isolated cases. An interesting example is the improvement of production of doramectin (Dectomax) (Fig. 4), an antiparasitic agent in the avermectin family (*36*). The initial production strain was a mutant of *Streptomyces avermitilis* lacking a dehydrogenase and an O-methyl transferase. This strain can be fed cyclohexane carboxylic acid as the initiator to give doramectin

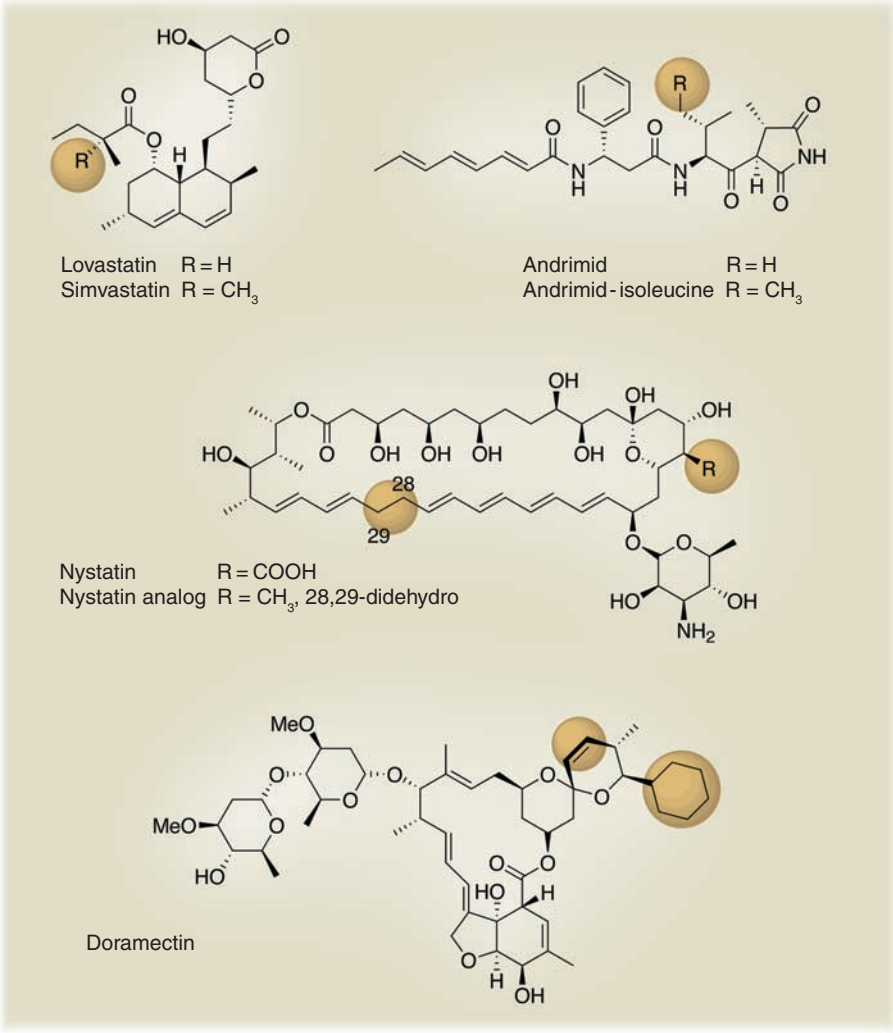

Fig. 4. Drug analogs produced by modification of biosynthetic genes. Shaded areas indicate sites of altered structure; Me, methyl.

together with a side product having a hydroxyl at C-23 instead of a double bond. DNA shuffling of the *aveC* gene and screening gave a new gene that encoded 10 amino acid mutations and reduced the unwanted contaminant for an overall improvement of production by a factor of 23. Natural products, including those from bacteria, can of course also be made or improved synthetically. For example, a large amount of effort has gone into synthetic analogs of epothilone B, a promising anticancer drug (*37*).

Drugs with complex structures from higher plants present a challenge if they are found in small concentrations. An example is the antitumor agent taxol (paclitaxel), which occurs in the bark of the pacific yew tree *Taxus brevifolia* (*38*). Initially it was predicted that treatment of ovarian cancer and melanoma cases in the United States would require the destruction of more than 360,000 yew trees annually. However, a semisynthetic route to taxol from 10-deacetylbaccatin, which was isolated from the needles of the European yew *T. baccata*, was developed that averted the devastation of trees.

In the past decade, taxol has been commercially produced by Bristol-Myers Squibb by plant cell fermentation (PCF) technology. However, for a number of other drugs there are substantial difficulties in getting sufficient production by PCF (*39*). An alternative approach is heterologous expression of the biosynthetic pathway in yeast or bacteria, frequently with modification of the genes to optimize production (synthetic biology). A well-known example is the effort to produce the antimalarial drug artemisinin in *E. coli* and yeast (*40*). Artemisinin from *Artemesia annua* is a sesquiterpenoid that is effective against multi–drug-resistant *Plasmodium* species but is expensive for Third World patients. The Keasling group (*40*) has engineered *E. coli* to produce its precursor, artemisinic acid, in concentrations of up to 300 mg per liter. This required extensive work, including engineering a mevalonate pathway to produce ample amounts of precursor for isoprenoid synthesis, optimization of expression of amorphadiene synthase (the key terpene cyclase), and incorporation of a modified version of amorphadiene oxidase (the P450 that converts amorphadiene to artemisinic acid). The work demonstrates that many complex natural products from plants can be engineered into heterologous hosts for fermentative production.

What Are the Future Prospects for Natural Product Drugs?

With the current framework of HTS in major pharmaceutical industries and increasing government restrictions on drug approvals, it is possible that the number of new natural product–derived drugs could go to zero. However, this is likely to be temporary, as the potential for new discoveries in the longer term is enormous. Access to rapid and inexpensive genome sequencing via 454 sequencing (*41*) or single-molecule real-time (SMRT) (*42*) methods will fully enable metagenomics for unculturable organisms. It will also uncover "silent pathways" in plants (*43*) to afford access to a large collection of new products and biocatalysts. It will allow preservation of any threatened species, through cataloging of its genetic blueprint, and may permit recovery of extinct organisms. Estimates for the total number of living species range from 2 million to 100 million, with one claim of 30 million species just for insects. Hence, the number of biosynthetic products and enzymes remaining to be examined is huge. Systems biology could eventually map the likely metabolism for most species. Such a library of biochemical transformations could be a magnificent tool for the design and generation of new products. Just as synthetic chemists currently plan total syntheses of a target compound using established reagents and well-precedented transformations, synthetic biologists will be able to call on vast arrays of enzymes to rationally make complex molecules. Directed evolution and site-specific mutation can optimize the desired activity of such proteins. Rapid gene sequencing of individual humans will enhance the development of personalized medicine—the use of an individual's DNA sequence as a basis for drug selection (*44*). This could satisfy the current expectations for high levels of safety by predicting side effects and assisting the correct choice of therapeutic drugs. New gene-mapping techniques will facilitate speedy diagnostic tests to determine causes of illness, including infections. This could lessen the indiscriminate use of antibiotics and thereby reduce the development of bacterial resistance to such drugs.

To achieve the potential of facile genome sequencing, the development of robust platforms is essential for heterologous expression of genes of novel biosynthetic pathways. Expression of biosynthetic enzymes often requires considerable effort. Problems of codon usage and optimization (requiring gene synthesis), of protein localization and modification, and of metabolite toxicity to the producing organism are only a few of the difficulties. The solutions will involve modifications of organisms that are already widely used (e.g., *E. coli*, *Saccharomyces cerevisiae*). For example, enhancement of primary precursor production can be coupled with programmed control of promoters and modifications to enhance the stability of foreign proteins. Active export of toxic target metabolites could potentially be engineered using drug resistance transporters. It is likely that such expression platforms will initially be demonstrated in academic laboratories prior to use in pharmaceutical industry. Although the current industry model for drug discovery does not favor natural products, the resource is so vast as to seem unlimited, and these emerging tools will provide exhilarating discoveries leading to new medicines.

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