Exploration of nature's chemodiversity: the role of secondary metabolites as leads in drug development

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The drug discovery process increasingly requires the availability of large numbers of compounds. Chemodiversity in nature offers a valuable source; for example, secondary metabolites, previously regarded as waste products are now recognized for their resistant activity against pests and diseases. The author discusses some aspects of chemodiversity in plants and the role of secondary metabolites, and explains how exploitation of this resource might be helpful in the optimization of the lead discovery process.

High-throughput screening has become an important tool in drug discovery. However, this technique requires a large number of compounds to be effective; these cannot be supplied by traditional organic synthesis and so two other sources are used: combinatorial chemistry and chemodiversity from nature. This review will discuss some aspects of chemodiversity in plants and the role of secondary metabolites, which might be helpful in the optimization of the lead discovery process.

Nature has developed an enormous diversity during several billion years of evolution. It is currently estimated that there are at least 250,000 different plant species, up to 30 million species of insects, 1.5 million species of fungi and similar numbers of algae and prokaryotes in existence[1]. All of these species coexist in ecosystems and interact with each other in several ways in which chemistry plays a major role – for example, in defence, symbiosis and pollination. In basic terms, these organisms all share a similar biochemistry necessary for a living cell, but in addition to that they produce a wide variety of so-called 'secondary metabolites' that are involved in the interactions between organisms. Considering the number of organisms, and the almost infinite number of interactions possible, it is not surprising that an enormously wide variety of secondary metabolites has evolved within organisms. In 1988 the database NAPRALERT already contained >88,000 secondary metabolites, and every year some 4,000 new ones are reported (N.R. Farnsworth, pers. commun.); thus, there should now be >100,000 known secondary metabolites. This is from the relatively few species so far studied (Table 1). Moreover, most species have been studied for only a certain type of compound, for example alkaloids. Extrapolation of the number of species studied and the number of compounds known suggests that, from all plant species, at least a million different compounds could be isolated. It is clear that nature provides an enormous potential for the discovery of new bioactive compounds.

Most secondary metabolites are derived from just a few building blocks; the acetate C_2-unit (polyketides), the phenylalanine/tyrosine-derived C_9-unit (phenylpropanoids), the isopentenyl diphosphate C_5-unit and some amino acids. In fact combinatorial chemistry is as old as evolution, and this statement is consistent with the

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Table 1. Number of plant species that have been studied phytochemically or for at least one type of biological activity, as on 1 December 1995, in the NAPRALERT database[a]

	Biological activity	Phytochemical
Monocots	1,283	3,721
Dicots	11,924	31,126
Gymnosperms	239	638
Pteridophytes	349	961
Bryophytes	39	457
Lichens	118	625

[a]N.R. Farnsworth, pers. commun.

concept that combinatorial chemistry serves as a synthetic rainforest surrogate[2].

That nature is a potential important source of useful drugs has been recognized since ancient times. This has resulted in the use of a large number of medicinal plants to treat various diseases, and some drugs in Western medicine are based on the traditional use of such drugs. Some are used as pure compounds from the traditional medicinal plant, such as atropine, morphine, quinine and digitoxin, and others as modifications of such compounds, such as aspirin and local anaesthetics. In total, 119 plant-derived compounds are used in Western medicine, and of the world's 25 best-selling pharmaceutical agents, 12 are derived from natural products[3,4]. That means that the hit rate among natural products is apparently much higher than in libraries derived by random synthesis, in which 10,000 compounds are said to be necessary to develop one new drug[5].

With the millions of different species mentioned, what strategies can be followed to discover new leads for drug development? Basically, there are two different approaches: one is screening different materials more or less at random, and the other is to try to prove the efficacy of traditional medicine and to resolve the mode of action.

The first approach involves looking for new compounds for known activities, and only in the case of testing on living cells (e.g. cytotoxicity, antitumour activity, isolated organs or Hippocratic screening) is there a possibility of finding new mechanisms of action. The latter approach might result in finding completely new modes of action, but neither method necessarily leads to novel compounds.

This review primarily focuses on plants as a source material for drug discovery, but most problems discussed will also apply to other sources, such as microorganisms or insects. However, there are some differences between these source materials. In the case of insects, the major problem is the collection of sufficient amounts of the material for screening. In the use of microorganism sources, it is relatively simple to obtain large amounts of material for further development, when compared with plants. The biochemistry of plants and microorganisms is also quite different. In microorganisms the polyketide pathways in particular are well developed, whereas in plants the terpenoid and phenylpropanoid pathways are predominant. Thus, both plants and microorganisms are interesting and complementary sources for drug discovery programmes. Although, as there are few medical uses described for microorganisms, compounds of microbial origin are usually screened in a random fashion.

Selection of samples

For improved efficiency, various selection criteria can be used to reduce the number of plants to be screened. Important criteria that can be used include: chemotaxonomy, traditional use (ethnopharmacology) or plant ecological observations.

Ecological observations may have originated in antiquity, as many of the traditional uses of plants are probably based upon careful observations; for example, insecticidal preparations that are derived from plants not attacked by insects.

Role of secondary metabolism

For many years plant secondary metabolism has been neglected in science. Secondary metabolites were generally thought to be waste products of plants, without apparent function. Gradually, recognition of the important role of plant secondary metabolites has increased; for example, in terms of resistance to pests and disease. But how may such information be used to find bioactive compounds more efficiently? Unfortunately, knowledge in this field is still limited, and will probably remain so, because the number of possible interactions is almost infinite. During evolution such interactions have resulted in an enormous chemodiversity. Because of limited knowledge of plant secondary metabolism, it will be difficult to identify a single interaction between a plant and, for example, an insect for finding a new bioactive compound. However, based on a more general approach, it is possible to identify those plant materials that are more likely to contain high levels of bioactive compounds. In certain stages of plant life or in different plant tissues there will be a greater need for defence compounds than in others. Fast-growing plants probably expend more energy in growth than in production of high levels of

secondary metabolites. Although there will always be a constitutive level of defence compounds in plants, such as antifeedants or other compounds that help to reduce losses by pests. Young leaves have a higher nutritional value, which means that there is a greater need for defence here than in old leaves to safeguard the future photosynthetic capacity of the plant. For example, in *Cynoglossum officinale* (hound's tongue) the concentration of pyrrolizidine alkaloids is greatest in young leaves and declines with age (Ref. 6 and Van Dam, N.M., PhD thesis, Leiden University, 1995). Moreover, the maximum level coincides with the maximum photosynthetic capacity of the leaves.

In the life-cycle of a plant, it may therefore be expected that a high level of defence compounds occurs in seeds, during germination and in seedlings, as well as in young tissues such as leaves.

In basic terms, three types of defence compounds can be distinguished between: those constitutively expressed in certain cells or tissues; those constitutively expressed but requiring biochemical activation; and those which are inducible. This is illustrated with results from studies on the biosynthesis of terpenoid indole alkaloids and related alkaloids in the *Catharanthus roseus* (Madagascar periwinkle) and *Cinchona* species.

Constitutively produced compounds. The *Cinchona* quinoline alkaloids are present in the tree bark at levels of up to 15% of the bark dry weight. Quinine (Figure 1) was developed as an antimalaria drug, and later its stereoisomer, quinidine, was developed as an antiarhythmic drug.

These alkaloids were found to be produced during the development of the seedling. No alkaloids were present in seeds, but after the small rootlet comes through the seedskin, the enzymes of the alkaloid biosynthetic pathway are rapidly induced and quinoline alkaloids are produced reaching maximal levels only four days later[7,8]. The increase of the alkaloid level is inversely proportional to the amount of feeding by snails (*Deroceras panormytanum*) on the seedlings. Another example are *Ginkgo biloba* (maidenhead tree) seedlings, which contain much higher levels of ginkgolides than the mature plant[9]. Seedlings are thus an interesting source of secondary metabolites.

However, not all secondary metabolites in plants are found in seedlings; and again, *Cinchona* serves as an example. In further studies on the role of alkaloids in this species, plants of ~6 months old were investigated for the sites of alkaloid biosynthesis and the particular alkaloids

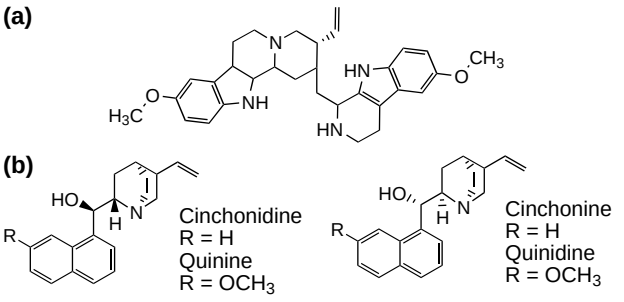

Figure 1. *Indole (a) and quinoline (b) alkaloids in* Cinchona *species; examples of secondary metabolites that have medical applications.*

present[10,11]. The greatest biosynthetic activity was found in young leaves and stem tips, and a somewhat lesser activity was present in the lower parts of the stem and the roots. The alkaloid levels were highest in young leaves and were of the cinchophylline-type – semidimeric indole alkaloids, structurally distinct from the quinolines (Figure 1). These alkaloids act as antifeedant agents against larvae, such as *Spodoptora exigua*[10,11]; the biosynthetically-related isoquinoline alkaloid emetine has a similar activity. Previously these alkaloids were shown to have antimicrobial activity[12].

Although seedlings and young leaves appear to be a good source of bioactive compounds, in older leaves, other compounds can be found. These include breakdown products formed from the major compounds present in the young leaves. For example, in the young leaves of *C. roseus*, strictosidine (Figure 2) is the major alkaloid, whereas in the older leaves this alkaloid is not found; instead, compounds such as vindoline, catharanthine, anhydrovinblastine and, as minor constituents, vinblastine and vincristine are present. Thus, depending on the age of the harvested plant material, considerable differences may occur in the spectrum of compounds present.

Biochemically activated compounds. In addition to constitutively expressed compounds that have a protective effect because of their biological activity, there are other compounds in plants that in themselves have no activity but can, in combination with other factors, become active. Probably the best known example is provided by the cyanogenic glycosides, which in combination with glucosidases yield the highly toxic hydrogen cyanide[13]. There are examples (for a review concerning antifungal compounds, see Ref. 14) that show this to be a new potential area for

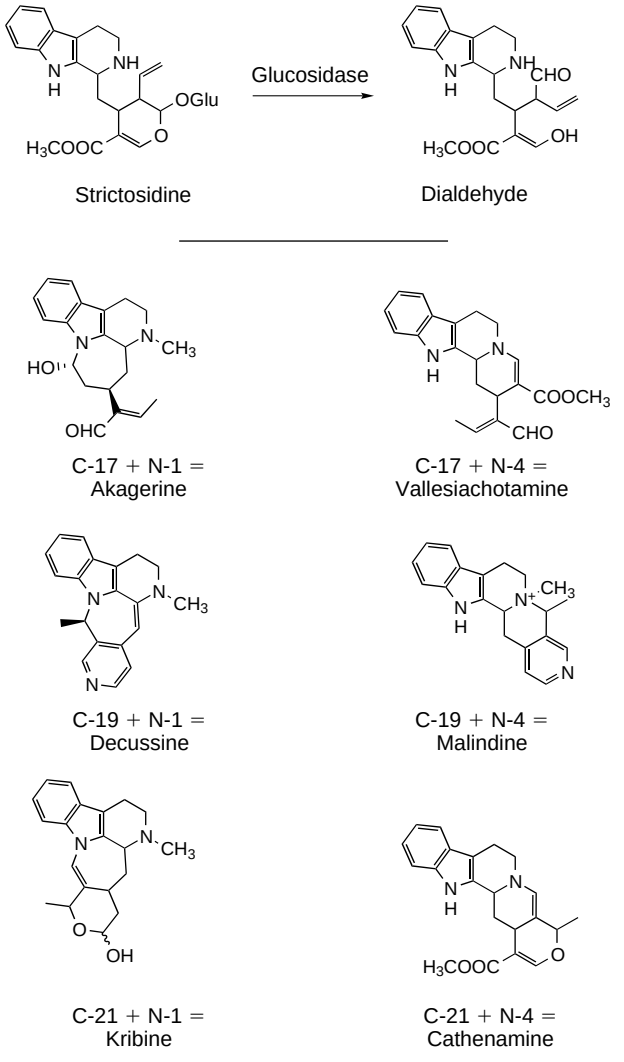

Figure 2. *Strictosidine glucosidase converts strictosidine into a reactive dialdehyde. Each of the aldehyde groups can subsequently react with any of the amine groups, resulting in a wide variety of indole alkaloid skeletons. The most common reactions are shown.*

lead finding. Moreover, this phenomenon shows that the 'prodrug' concept could have been learnt from nature. Strictosidine, an intermediate in the terpenoid indole alkaloid biosynthesis in several plant species[15], serves as an example. Studies on the distribution of different alkaloids in *C. roseus* showed that young leaves accumulate high levels of the glucoalkaloid strictosidine in the vacuole (Refs 16,17 and Luijendijk, T.J.C., PhD thesis, Leiden University, 1995). Its aglucone has, after ring opening, two reactive aldehyde groups, which can couple with each of the two amine functions in the molecule, resulting in various types of indole alkaloid skeletons (Figure 2). Surrounding the vacuole are high levels of a specific glucosidase (Ref. 16 and Luijendijk, T.J.C., PhD thesis, Leiden University, 1995). For strictosidine itself no antifeedant or antimicrobial activity could be demonstrated, but in combination with a glucosidase it has strong antimicrobial activity (Ref. 18 and Luijendijk, T.J.C., PhD thesis, Leiden University, 1995). Such 'prodrug-type' phytoanticipins (antimicrobially active compounds already present in the plant before infection) offer another interesting, and as yet unexplored, target for screening. Treating plant material with enzymes such as glucosidases before extraction and screening might result in the formation of new active compounds (e.g. aglucones or products derived from them) not found in untreated plant material.

Biochemically induced compounds. Certain compounds are only present following biochemical induction of secondary metabolism. Upon infection with microorganisms or viruses, plants react in a highly complex way with different local and systemic reactions. There can be direct induction through signal compounds (elicitors) from the infecting microorganisms, or from the breakdown products of plant cell walls. Such local induction at the site of infection results in the production of endogenous signal molecules that induce a systemic reaction. Salicylic acid and jasmonic acid are examples of such endogenous signal molecules. The methyl esters of these compounds can even serve as signal compounds between plants[19]. The response to elicitors includes, among others, the induction of plant-specific secondary metabolism pathways, resulting in the production of phytoalexins. The phytoalexins are defined as 'antimicrobial compounds that are synthesized and accumulated by a plant after infection with microorganisms'; thus, in a healthy plant these compounds are not found. Each plant species produces a specific set of phytoalexins, which comprise many different types of compounds including sesquiterpenes, triterpenes, isoflavones, anthraquinones, coumarins and stilbenes[20,21].

Consequently, a plant infected with microorganisms might produce a different spectrum of secondary metabolites than a healthy plant. For example, in material of *Phytophthora cinnamomi*-infected *Cinchona ledgeriana* trees from plantations in Africa, anthraquinones could be detected (Figure 3) together with alkaloid levels that were lower than normal[22,23].

As phytoalexins, by definition, have antimicrobial activity, they form an interesting target for searching for antibiotics

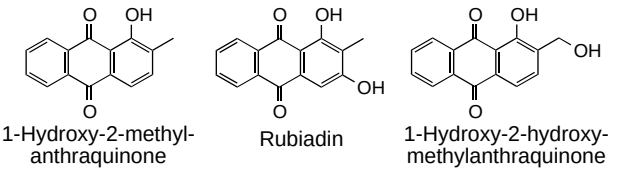

Figure 3. *Examples of anthraquinones formed in* Cinchona *cell cultures after elicitation.*

and antitumour compounds. Plant cell cultures induced by jasmonic acid or fungal elicitors should provide an excellent source for such a screen.

As phytoalexin biosynthesis is inducible, the collection of plant material must take into account the possibility that diseased plants might have different activity profiles than healthy plants. It also means that by studying only healthy plants, the enormous potential of phytoalexins as a source of bioactive compounds will be missed.

Chemotaxonomy

Although the number of plant species that has been studied is rather limited, from the available data it is possible to identify that the occurrence of certain types of secondary metabolites is usually restricted to a few families or even genera. This knowledge can be applied in different ways. When an interesting lead is found, and either a new (richer) source of the compound or related structures are sought, chemotaxonomy can point to related plant species to screen. However, within a family, the regulation of the biosynthesis of related compounds can be quite different; for example, the anthraquinones in *Cinchona* are only found after infection, whereas in *Rubia* they are normal root products and in *Morinda citrifolia* (Indian mulberry) they are found throughout the plant[24].

Chemotaxonomy can also be used as a negative indicator; for example, if a cytotoxic compound with no value as lead has been found in several related species, screening further related species could be stopped. For the identification of active compounds, knowledge of chemotaxonomy can be helpful; the most important source of information on chemotaxonomy is the work of Hegnauer[25].

Ethnopharmacology-based approach

In Europe, several phytopharmaceuticals are still used for their traditional purpose. In most cases it has not been possible to connect the activity of these preparations to a single compound. However, from various double-blind studies of these medicinal plants, it is clear that they do have some activity[26]. In addition, the past success in finding new compounds from traditional European medicines (e.g. atropine, morphine, digitoxin and salicylic acid) and traditional Chinese medicines (e.g. artimisinin) shows that traditional use is a major indicator of activity. It is questionable whether screening of such traditional drugs by means of *in vitro* assays, be it at the organ- or molecular-level, is the most effective approach to validation. A well-controlled clinical study is possibly more useful, as activity found in such a study can be directly translated to the utility of the traditional medicine. In developing countries the use of traditional medicines is of great importance, as it gives the population access to drugs. In some Asian countries, studies are made to validate the use of such medicines, and in several countries, standardized traditional medicines are produced and made available to patients.

Isolation of the active compound(s) in traditional medicines for which the efficacy has been proven, might result in interesting products. This has been demonstrated by Shaman Pharmaceuticals (San Francisco, CA, USA) who have specialized in identifying the active components of medicinal plants from the Amazonian rain forest[27]. However, it cannot be excluded that the activity of some traditional medicines is caused by the combination of certain compounds, which means that no single active compound can be isolated.

Extraction methods

Depending on the solvent used in the extraction method, different quantities and types of compound can be extracted from plant material. In the literature, many kinds of extraction schemes are described: some highly selective for a certain group of compounds, others very general, using an array of consecutive steps going from nonpolar (e.g. petroleum ether) to highly polar (e.g. methanol, water) solvents. In a programme screening for antitumour compounds, scientists at the National Cancer Institute compared a series of extraction methods and found that, with dichloromethane–methanol (1:1), all known plant antitumour compounds were extracted [McCloud, T.G. *et al.* (1985) poster presented at *The American Society of Pharmacognosy Meeting*, Chapel Hill, NC, USA]. In a study to assess the chances of finding bioactive compounds in plant cell cultures, we have compared several extraction solvents by testing the extracts in approximately 40 different

receptor-binding assays. From the results it was concluded that by using only two different extraction methods, one with toluene and another with ethanol[28], all of the bioactive compounds observed using all the different extraction methods could be isolated. Given that certain compounds can be biochemically activated, treatment of plant material with glucosidases or acidic hydrolysis may increase the chances of finding bioactive compounds.

With all extraction methods, several primary metabolites will be extracted and usually in larger quantities than the secondary metabolites. In nonpolar extracts for example, steroids and lipids will be present in large amounts, whereas in polar extracts sugars will be the major components. As most test systems are performed under aqueous conditions, the resolubilization of compounds extracted with nonpolar solvents can be a problem.

In crude plant extracts, various compounds may be present that interfere with biological assays. Phenolic compounds, in particular, may hamper receptor-binding and enzyme assays by binding to, and inactivating, proteins[29]. Some phenolics, like tannins, can be present in relatively high concentrations in some plant tissues, although various methods, such as treatment with XAD_2, PVPP or adsorbents that bind aromatic compounds, can be used to overcome this problem. However, caution should be taken with such an approach, as the important compounds podophyllotoxin and salicylic acid would probably have never been found in such procedures. In other words, leads could be missed.

Apart from such denaturing compounds, other compounds may interfere with biological assays through nonselective binding. For example, it was found that linoleic acid binds nonselectively in the adenosine receptor assay. As this compound is quite widespread throughout the plant kingdom, it would mean that many false positives would be found when screening for this particular activity. To identify such false positives rapidly, as well as activity by other well-known compounds (e.g. tryptamine and GABA), a reproducible preseparation method as a first step after extraction would be necessary. Such an approach has the advantage that it would enable the recognition of fractions that contained known or false-positive compounds. Moreover, it would result in the enrichment of trace compounds, thus increasing the chances of finding new leads, and also dilute any phenolic compounds over the various fractions, thus helping to overcome the problems of assay interference.

Rapid screening requires the availability of a library of reference compounds and methods for simple identification of active compounds from crude extracts. The coupling of HPLC with diode array detection and mass spectrometry, and recently with NMR, is thus another important tool for high-throughput screening[30]. This is particularly true if these methods can be coupled with on-line activity measurements – a technology that is presently being developed (Oosterkamp, A.J., PhD thesis, Leiden University, 1996).

Conclusion

The examples described underline the enormous chemodiversity in plants and other organisms worthy of further exploration. Several approaches are possible, but all suffer from certain drawbacks. These can be overcome, but often it means that certain groups of compounds are less likely to be present in the final fractions for screening of biological activity.

Young plant tissues and seedlings are an interesting source for finding bioactive compounds, as they often contain relatively high levels of secondary metabolites. However, there is no indication regarding the type of activity these compounds may have, other than the probability of an antifeedant or insecticidal function. Any type of pharmacological activity might be found, such as in a random screening of plant materials. Screening seedlings of *Ginkgo* and *Cinchona* with the appropriate bioassays (i.e. PAF-inhibition, antimalaria and antiarhythmic) would have resulted in the discovery of important drugs (i.e. ginkgolides, quinine and quinidine). However, by screening only the young leaves of *C. roseus*, vinblastine and vincristine would not have been found. A major advantage of screening seedlings is that it is an easily reproducible system. Elicited plant-cell cultures are an important source for screening for antiviral, antitumour and antimicrobial activity.

Traditional use is another approach to discovering bioactive compounds, and it may offer a significant reward, as validation of traditional medicine is of great importance to enable human access to affordable medicines.

A series of closely related compounds is usually present in the 'chemical defences' of plants, rather than a single example of a compound type. The plant then has the advantage of protection against an array of organisms. This process is analogous to combinatorial chemistry, but with evolution as the selection method. Resistance against a

broad range of compounds with similar structures is probably less likely to occur.

The various compounds produced may also display synergism in their biological activity[31–38]. This property may be of interest for drug development, though it contrasts with the present paradigm of using single, pure chemical entities.

One of the problems in screening plants for bioactive compounds is the variability of the raw material. Variability can be caused by different genotypes, seasonal and diurnal variation, and phenotypic differences between the younger and older parts of the plants. Moreover, an infected plant might contain a completely different set of compounds that are not normally found in a healthy condition.

To optimize the chances of finding new leads through high-throughput screens, preseparation of extracts of selected plant parts, seedlings and (elicited) plant-cell cultures would seem to be a promising approach.

The application of plant genetic engineering has meant that a new and exciting possibility is close to becoming reality – the production of novel compounds by metabolic engineering or 'recombinatorial biochemistry'. In microorganisms this approach is already being explored for the polyketide pathway[39]. Such an approach also seems possible in plants. Most plant secondary metabolites are derived from only a few building blocks but, after the formation of the basic skeletons, each plant species has different, highly specific enzymes that modify these compounds – this creates the great chemodiversity. By, for example, the introduction of heterologous genes encoding oxidative enzymes, such as cytochrome P450 enzymes, new derivatives might be formed. The possibility of transforming plant cell-suspension cultures by co-culturing with *Agrobacterium tumefaciens*, containing constructs that harbour a gene encoding the desired enzyme is, in this connection, an interesting option.

Plants offer an enormous potential for developing new drugs. Chemical ecology and traditional use can identify plants or parts of a plant that may be interesting sources of bioactive compounds. Whereas plant biotechnology offers the possibility for the production of interesting compounds and even the potential of generating novel compounds.

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