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Natural Products or Not? How to Screen for Natural Products in the Emerging HTS Paradigm

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1. Value of Natural Products: The Screening Perspective

Natural products are undeniably the best source for diversity in chemotype for the discovery of novel therapeutics. One-third of the top selling drugs in the world are natural products or their derivatives (**Fig. 1**). Roughly 60% of the anti-tumor and anti-infective agents that are available commercially or were in late-stage clinical development from 1989–1995 are of natural products origin ***(1,2)***.

Despite the success of natural products as a source for discovery, the interest in natural products as a source of new drugs has experienced highs and lows over the years. Remarkably, it has been estimated that less than 0.5% of the total microbial population in soil samples has ever been tested ***(3)***. Furthermore, a similar low percentage has been tested for other types of natural products, such as plants, microbes from the ocean, and so on. The implication here is that, given the advances allowing access to these new samples, the actual diversity available from natural products has barely been tapped.

New developments that allow the cloning of genomes from "unculturable" microbes allow access to their gene products and gene operons. Use of these new genes in traditional expression systems may result in the production of unique natural products. Plant cell culture is opening up another source of practically unlimited diversity for secondary metabolites, not only those naturally found, but also through modification of the culture conditions, a whole panoply of new secondary metabolites.

Our experience is that samples derived from natural products can be successfully utilized in discovery programs, feeding surprising novelty into the pipeline. Our experience has also taught us that the identification of the active

From: *Methods in Molecular Biology, vol. 190: High Throughput Screening: Methods and Protocols*
Edited by: W. P. Janzen

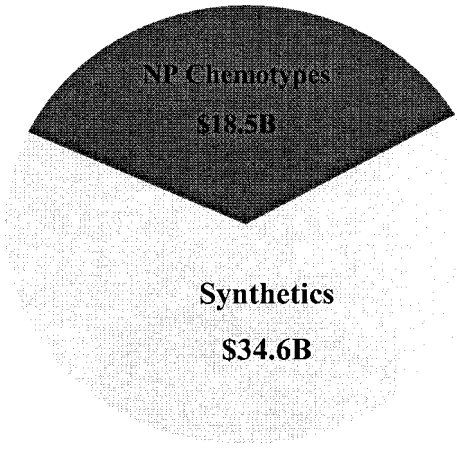


Fig. 1. 1999 Top 25 Prescription Drugs: Schematic representation of 1999's earnings for the 25 top selling prescription drugs, in billions of dollars.

principles present in the natural product samples can be facilitated up front by treatment of the samples prior to screening and by some accommodation in the screening process for the sample type, especially for crude natural product samples (**Fig. 2** and **Subheading 3.1.2.**).

In this chapter, we give details for: 1) the culturing of standard soil organisms and 2) plant preparation to produce samples with an eye toward utilizing this excellent sample source for pharmaceutical screening programs. We also include technological tips on handling the samples to speed the isolation of the active principles found in the screening portion of the discovery program. We also offer some specific, interesting examples to illustrate some of the unique challenges novel natural products offer to drug-discovery programs.

2. Discovery of Pharmacologically Relevant Natural Products from Microbial and Plant Secondary Metabolites

The approach taken in the selection of the microorganisms for investigation of secondary metabolite production is somewhat different from the criteria used for the selection of plants. Actinomycetes and fungi have historically proven to be rich producers of secondary metabolites. These organisms, commonly isolated from soil, can also be found in leaf litter, live plants, and dung. Even among microbiologists there is no consensus as to the optimal conditions for selection of soil. One view is that different microorganisms will be present in different soils because the selection pressures from different environments will lead to microorganisms differing in their physiology and metabolism. The opposite view is

that every microbe is available in the back garden and there is no need to collect soil from all over the world. There are various views on how broad the taxonomic diversity of the microorganisms to be screened for secondary metabolite production should be. However, as the goal is to maximize the diversity of the secondary metabolites produced, we have found the best strategy is to include a broad variety of taxonomically diverse microorganisms.

Once the microorganisms have been selected, the cultures can be grown in either liquid or solid conditions in a variety of fermentation media. Each natural products or fermentation group has developed a variety of their own production media. Some media are very specific for the production of one secondary metabolite from a certain microorganism, which usually arises as a result of medium optimization after the active natural product has been identified. Other media are more universal, resulting in a variety of secondary metabolites being detected from taxonomically distinct microorganisms. The more universal media are used for the fermentation of microorganisms for production of secondary metabolites for HTS and are generally considered proprietary to each pharmaceutical or biotechnology company.

There are many considerations for the selection of plants as producers of valuable secondary metabolites ***(4,5)***. One approach is random selection; casting the net wide and choosing plants from different taxonomic groups and different areas. An alternative method would be selective selection of plants using ethnobotanical information in which there has been local use in traditional medicine. An approach that focuses on maximum diversity of the plants would make the selection based on taxonomy, geographical location or compound structural type preferences ***(4)***. A novel approach to plant selection uses the Literature Information Selection Technique (LIST), a computer-based selection method that correlates biological activity, botanical and chemotaxonomic information using the NAPRALERT® database ***(5–7)***. There are many factors that can influence the type and abundance of secondary metabolites produced by plants including seasonal variations, altitude of the collection site, healthy vs diseased plants, plant age, plant part, and soil type. In addition, these variations can make reproducibility of the recollection very challenging. This can often be minimized by collecting sufficient quantities of the plant prior to extraction or by the taking of meticulous notes regarding the collection conditions.

A newer approach to maximizing the secondary metabolites produced by plants is through the use of plant cell culture. A number of companies have started to explore this area. A culture collection is compiled and then using a variety of fermentation techniques these cell cultures can be grown and the secondary metabolites produced can be evaluated for uniqueness and/or biological activity. Using this approach it is possible to produce secondary metabo-

lites that are found in nature, as well as new compounds that would not be produced in the native habitat. Another way to modify the structure of a natural product is through the use of biotransformation.

3. Format of Natural Products as an Integral Part of a Company's Screening Deck

3.1. Crude Extracts: Advantages and Disadvantages

Traditionally microbial secondary metabolites have been screened as crude extracts in high-throughput screening (HTS). This involves growing the organism under a variety of fermentation conditions and then extracting with solvents. Microorganisms have been and still are typically grown in either liquid media (submerged culture) or on solid substrates such as agar or grains. The choice of solvent used for extraction of the secondary metabolites depends on the type of secondary metabolite desired. In order to obtain a broad spectrum of secondary metabolites differing in polarity, a solvent such as methanol can be used for solid fermentations, and butanol can be used for liquid fermentations. Alternatively, resins can be used for extraction of secondary metabolites, with the choice of resin depending on the polarity of the compounds to be extracted. Each natural products group has developed their own fermentation parameters, media, and extraction conditions and as most of this information is not freely available, a general guide is provided below.

3.1.1. Methods for the Preparation of Crude Microbial Extracts

3.1.1.1. Fermentation in Liquid Media

The microorganism is fermented in liquid media for a certain period of time. To the culture is added an equal volume of butanol and the sample mixed for 30 min to 1 h. The sample is then centrifuged and the solvent (top layer) is removed and dried down under a stream of nitrogen. The sample is then re-suspended in the solvent of choice for the screening deck.

3.1.1.2. Growth on a Solid Substrate

The microorganism is grown on a solid substrate such as agar for a certain period of time. To the culture is added a 50% volume of methanol and the sample allowed to stand for 1 h. The liquid is decanted and dried down under a stream of nitrogen. The sample is then re-suspended in the solvent of choice for the screening deck.

3.1.2. Methods for the Preparation of Crude Plant Extracts

The overall procedures for the extraction of plant samples consist of a number of seemingly simple steps. However, careful execution of these steps is

critical to ensure that the resulting sample is suitable for HTS ***(8)***. In most cases plant material is first dried in the atmosphere, either at room temperature or in an oven at no higher than 30°C. The sample should be not be compacted to avoid fungal infections and elevated temperatures. The sample can be ground or frozen with liquid nitrogen and pounded in a chilled mortar. By reducing the particle size, solvent penetration is more efficient and greater yields of secondary metabolites are achieved. There are many approaches to solvent selection depending on the desired metabolites and these are well-covered elsewhere ***(8)***. In general, aqueous-methanol is usually the solvent mixture of choice for the preparation of crude plant extracts. The plant crude extracts at this stage contain tannins, fats, waxes, and chlorophyll and their suitability for use in HTS is debatable.

For both microbial and plant samples, the use of solvents extracting the majority of the secondary metabolites, regardless of their polarity, satisfies the purists who are concerned with losing some of the components as a result of further processing. The disadvantage is that the crude extract is complex and de-convoluting (dereplication) of the hit is a time-consuming process. All natural product groups have their own procedures for dereplicating an activity from a crude extract ***(9)***. It is usually necessary to perform bioassay-guided fractionation, which relies on additional resource from screening personnel at a time when the screen may no longer be running, extending the timelines for the program (*see* **Fig. 2**). Dereplication almost always requires some sort of separation step resulting in separation of compounds. This can be done by gravity flow chromatography, fast protein liquid chromatography (FPLC), high-performance liquid chromatography (HPLC), etc. The samples are collected and correlated to regions of the chromatography profile. Once the active region of this profile is determined, the UV/visible spectrum of the peak(s) are compared with a database available either commercially or developed in-house ***(10)*** to determine if it is a known compound. There are a number of commercial databases that are capable of performing a search based on UV/visible spectrum and molecular weight for the identification of microbial products. These include Bioactive Natural Products Database (Berdy), which lists 23,000 compounds; Antibase™ Database containing 21,000 microbial metabolites; Kitasato Microbial Chemistry Database with 16,000 entries; and the DEREP Database containing 7,000 compounds with a planned expansion to 15,000. Although molecular weight is the most definitive information, the UV/visible absorption data can often result in an exact match. Difficulties can arise when there are nuisance compounds present such as fatty acids, detergents, and pigments. These can cause a false-positive result, usually slow down the isolation process because of their presence in very low amounts, and may be masked with other compounds ***(2)***. Historically, it has been through the use of crude

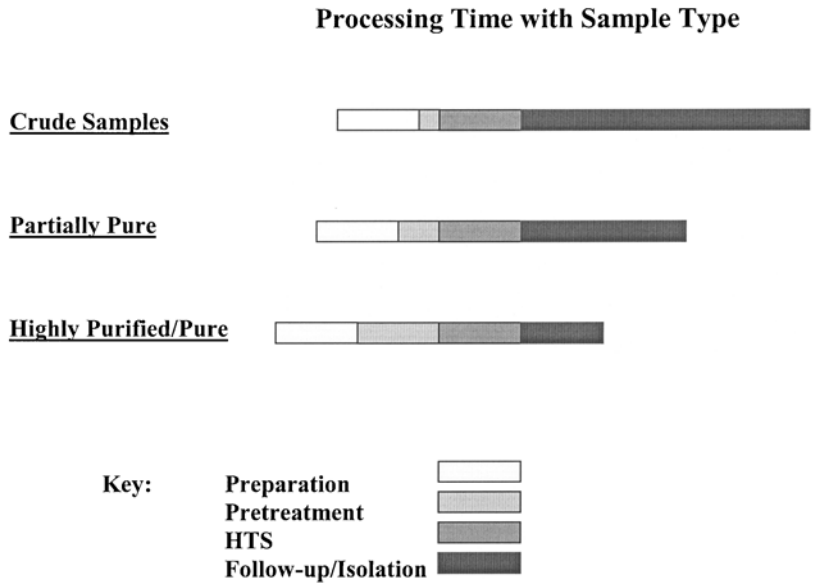


Fig. 2. Schematic showing timelines for screening programs utilizing natural-products samples treated variously prior to entering the screening stream.

extracts as screening samples that the natural products present as drugs in the marketplace today were discovered. However, the screening environment at the time of their discovery was very different from the present screening paradigm, which requires much shorter timelines and the need to know the chemotype at an early stage in the screening process.

3.1.2. Methods for Screening and Evaluating Crude Natural Product Samples

In order to run natural product crudes as samples in screens, a very careful evaluation for tolerance in the screen is required. This entails running 2,000–5,000 samples through the screen at several concentrations to establish the concentration for the HTS. The goal is to run the crude at the highest possible concentration tolerated by the screen; this allows any particular molecule to be run at the highest possible concentration. The dilution series data are treated to generate a normal curve by evaluating how many samples were active at any given level, so, x-axis representing signal and y-axis representing test sample value relative to control. The curve that is the tightest normal distribution will be the concentration to choose. This enables the screener to have a high level of confidence that outlying points are genuine actives and that nonspecific effects are not too interfering.

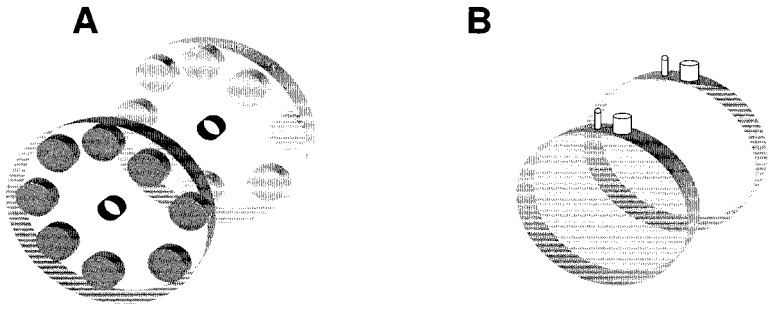


Fig. 3. Schematic of equilibrium microdialysis apparatus. **(A)** Plexiglass rotating cells containing eight walls. **(B)** Schematic of a single well with dialysis film shown between the open halves. One side is loaded with the solvent and natural product, and the other side loaded with vehicle. Entry and exit holes are shown for each half-well and the well are loaded by syringe or sequencing gel-loading tips. These openings are then sealed and the cell is rotated until equilibrium is reached.

Obviously, before committing resources to isolating active principles from crude extracts one might want to eliminate high molecular weight entities. We developed a method that can estimate the molecular weights of the active materials in the milieu of the crude extract, avoiding wasting resource effort on preliminary isolation studies (**Fig. 3**) ***(11)***. Using overnight equilibrium microdialysis, and testing both the dialysate and the filtrate samples, one can very easily determine activity on the two sides of a dialysis membrane with a given cutoff. For routine use in evaluating natural product crude samples, either a 1200 or a 3500 MWCO membrane was utilized.

3.2. Partially Purified Extracts: Advantages and Disadvantages

A number of groups prepare partially purified mixtures of microbial secondary metabolites as a means of providing a less complex sample for screening, but not excluding the more minor components. Partially purified mixtures can be obtained by processing the crude extract using either resins or solvents that will separate the components of the mixture based on their polarity ***(12)***.

3.2.1. Methods for Preparation of Partially Purified Microbial Extracts

The fermentation is extracted with a polar solvent as described earlier. The butanol extract is then applied to an HP-20 cartridge followed by step gradient dilution, first with 100% water, and then with increasing amounts of methanol, and finally with methanol and acetone to generate essentially water soluble, polar, moderately polar, and nonpolar fractions. These different fractions can be submitted for screening ***(10)***. An alternative approach is to use a solvent

spartitioning protocol which will also separate components based on their polarity ***(12)***. The advantage of these approaches is that the sample is less complex and more concentrated, and therefore there is a greater chance of detecting the relevant biological active entities. The length of time needed for the isolation of the active entity is somewhat reduced, and for the purists there is less chance of overlooking a component from the original mixture than if further manipulation was done.

3.2.2. Methods for the Preparation of Partially Purified Plant Extracts

3.2.2.1. General Procedure to Obtain a Detannified Chloroform Extract from a Plant Methanolic Extract

The plant is extracted with methanol as described earlier. The resulting aqueous-methanol extract is then defatted with a nonpolar solvent such as hexane or petroleum ether. The hexane layer (containing the fats, waxes, and chlorophylls) is discarded and the aqueous-methanol layer is evaporated and partitioned against chloroform. This results in an aqueous extract that is then washed with 1% aqueous NaCl and dried over anhydrous Na_2SO_4, and a chloroform extract that has been "detannified." Both of these extracts can be submitted for HTS ***(8)***.

3.2.2.2. General Procedure to Obtain a Precipitate of Water-Soluble Compounds such as Saponins from Plants

The ground plant is directly extracted with chloroform and the extract concentrated to dryness. The gummy residue is then treated with 80% aqueous-methanol, concentrated, and extracted with water-saturated with butanol. To the butanol phase is added ethyl acetate and precipitation of the crude saponins occurs ***(8)***.

3.2.2.3. General Procedure to Obtain Alkaloidal Extracts from a Plant Methanolic Extract

The methanolic crude extract is treated with 1% aqueous HCl and partitioned against diethyl ether. The diethyl ether phase can be concentrated and contains neutral compounds. The aqueous acid phase is alkalinized with NH_4OH controlling the pH and then partitioned with chloroform. The chloroform extract contains the primary, secondary, and tertiary alkaloids and the aqueous extract contains the quaternary alkaloids, and both of these extracts can be submitted for screening.

3.2.3. Method for Preparation of Partially Purified Microbial and Plant Extracts

Supercritical Fluid Extraction (SFE) is being used as a general extraction strategy for a wide range of metabolites from plant and microbial samples ***(13)***. This approach offers many advantages in that it leads to lower solvent usage, controllable selectivity, cleaner extracts, and less thermal degradation as compared to conventional solvent extraction and steam-distillation methods. The essential components of a typical SFE include a carbon dioxide source, a pump, an extraction vessel, a restrictor, and an analyte-collection vessel, and various SFE systems are commercially available ***(13)***. Into the extraction vessel is placed the matrix to be extracted, which could be plant material or fermentation broth. Some preliminary sample preparation may be required, for instance grinding, drying, or even wetting. If the sample is a fermentation broth it must be immobilized onto a solid support, since SFE is generally unsuitable for liquid samples because of difficulties in handling two phases under pressure. The sample is then extracted generally with at least three extraction-cell volumes, in static, dynamic, or re-circulating modes. The extraction cell is usually an oven so that the temperature can be controlled. As the supercritical carbon dioxide passes through the restrictor, the change in pressure in the restrictor causes the pressure of the supercritical fluid to decrease. The analyte is then swept into an on- or off-line collection device. With on-line collection, an analytical instrument such as a gas chromatography (GC), SFC, or HPLC is connected, and the analyte is then analyzed. For the purposes of preparing partially purified mixtures of plant and microbial crude extracts, an off-line collection would be used.

The advantage of using SFE is the removal of deleterious nuisance compounds such as tannins, waxes, and fats from plant material, and fatty acids from microbial fermentations ***(13)***. These compounds can give rise to false positive results and mask truly active components, and therefore their removal is of value in the preparation of samples for HTS. The disadvantage is that this procedure is somewhat lengthy and would require some level of automation to be used for numerous samples.

3.3. Relatively Pure Compounds: Advantages and Disadvantages

The logical step in moving away from crude or even partially purified extracts is to produce relatively pure compounds that can be an integral part of the screening deck. There are a number of sources of relatively pure or pure natu-

ral products that are commercially available for screening. In addition a number of pharmaceutical companies have developed their own "purified natural product deck," the information about which is proprietary.

One source of pure natural products is the Natural Product Pool ***(14)*** the creators of which expect to gain major impact in current lead discovery efforts in the pharmaceutical industry in Germany. In this endeavor, the samples are organized in a 96-well microtiter plate format containing 1 mg quantities of each compound together with a database covering the chemical/physical data, biological activities known to date, references, and suppliers. The collection contains natural products as well as carefully selected compounds from chemical synthesis in order to achieve maximum structural diversity. In order to minimize dereplication, special emphasis has been placed on selection of compounds that are biologically active secondary metabolites, although there are new compounds and compounds with patent applications pending. As of June 1997, the Natural Product Pool comprised approx 1700 compounds from microoganisms, plants, marine or aquatic sources, animal sources, natural-product derivatives, and synthetic compounds. The compounds range in molecular masses from 200–400 daltons, and to ensure structural diversity the pool contains representative compounds from most biosynthetic pathways together with classification of the compounds according to chemotype. A number of groups have formed a collaboration to construct this collection of compounds including GBF, Analyticon, and the Universities of Gottingen and Tubingen, among others. The compounds are then commerically available to industrial users, which have included Asta Medica, Boehringer Ingelheim, Boehringer Mannheim, E. Merck, Schering, and AgrEvo.

Another approach to the construction of a natural-product deck of relatively pure or pure compounds is to use novel microbial and plant sources. The microorganisms could be cultured under a variety of conditions based on the experiences of the natural products group. Extracts of both plants and microbes could be prepared in a manner similar to those described earlier. The natural product group at Glaxo Wellcome has moved away from random HTS of uncharacterized extracts, towards smaller targeted sample sets. Prefractionation of these samples takes place prior to screening where appropriate in order to be more compatible with the changes in HTS within the company ***(15)***. At Glaxo Wellcome a high-throughput prefractionation program has been implemented that utilizes preparative reverse-phase high-performance (or high pressure) liquid chromatography (RP-HPLC) to fractionate crude extracts. The resulting UV absorbance chromatogram is visually examined and discrete components are selected for inclusion in the sample library, bulking fractions where appro-

priate. The fractions for submission for HTS include those with sufficient weight and apparent purity by the HPLC/UV ***(15)***.

Using preparative HPLC as a means of fractionating the crude extract means that nuisance compounds such as chlorophyll can be eliminated due to their characteristic UV and retention time. In addition for the fractions submitted to the screening deck there are chemical characteristics associated with each fraction that can be used as an aid in either dereplication or as a guide to further isolation work if the biological activity merits.

A commercially available system for separating natural products is the SEPBOX ***(16)***. The system was designed with five major specifications: that the total process should provide almost pure compounds in under one day, be completely automated, cover the entire spectrum of compound polarities, provide isolated compounds of sufficient material for structure elucidation, and be able to accommodate all extract types ***(16)***. With the SEPBOX, extracts are absorbed on reverse phase materials placed in a pre-column to allow continuous elution based on polarity. The main separation on reverse-phase material of medium polarity produces 18 fractions. Each of these fractions is further fractionated using five to six separation materials. Compounds eluted from these columns are collected into tubes or 96-well plates. Special techniques are required to trap the highly polar fractions. Light-scattering detection is used to characterize the compounds ***(16)***.

The advantages of spending time to generate relatively pure or pure natural product compounds in a deck is that the biological data such as potency, IC_{50} values, and specificity is much more meaningful, and allows for comparison with data from synthetic compounds early in the screening process. As shown schematically in **Fig. 2**, the current screening paradigm using pure or nearly pure natural products provides for faster speeds, not only in the initial screening, but also in the subsequent follow up work. With the timelines demanded by a modern HTS program, there is not usually the luxury of having a screen wait for a long period of time while the structure of a natural product is determined, before deciding which of the synthetic or natural product compounds are worth following further. By having the natural-product samples relatively pure, this time can be significantly reduced. Ensuring that the natural products samples are structurally characterized to a degree similar to the other compounds in the deck will enable a larger number of targets to utilize this sample type. In addition, with the move to greater miniaturization of the assay volumes in screens, sample purity will become more of an issue as crude mixtures will not be compatible with the submicroliter volumes that will come from moving from 384-well plates to 1536- and 3456-well plates.

The disadvantages of this approach is that the majority of the purification work is done prior to submission to screening without the guarantee of there being any biological activity. In the past using crude extracts there was detection of biological activity in the sample of interest and this guided the selection of natural product samples to follow. However, with the ever-increasing number of screens that are run each year in the pharmaceutical industry, a deck of sufficient quantities of purified natural products could see hundreds of targets, and therefore warrant the effort. Although using this approach means that fewer organisms and plants end up being included, incorporation of a chemical prescreening step prior to the preparative HPLC stage results in only the most promising extracts being prepared.

3.4. Flow for UHTS

In order to gain an appreciation of the differences the sample type for natural products makes on the timelines in the screening process, the different approaches are compared (**Fig. 2**). It is apparent that for crude extracts from both plants and microorganisms time has to be spent to deconvolute this incredibly complex mixture. At one end of the spectrum, with crude extracts the time is spent after the identification of biological activity, whereas at the other end of the spectrum for relatively pure samples the majority of the work is done prior to the sample being screened. Although there may be a strong need for the natural products group to generate samples that satisfy their needs in terms of how inclusive the sample is, the ultimate destination of that sample, and the environment in which it will be screened has to be kept in mind to ensure that the sample is compatible, and this may require some compromise on the preparation and content of the sample.

4. Examples

Are there unique attributes for natural-products identified as screening hits? Yes.

Special attributes for natural-products identified in HTS programs include: 1) unanticipated and unusual mechanisms of action, 2) the molecular diversity may be synthetically inaccessible, even semisynthetically. Below are examples of (1) and (2). Another clear advantage is the frequent occurrence of analogues, i.e., related compounds found as secondary metabolites in the same culture fermentation.

Examples of (1) are the "activators" of glucokinase, Glucolipsin A and B *(17)*. Glucose phosphorylation influences circulating blood glucose levels, making glucokinase regulation one of the principle points for therapeutic intervention in diabetes. A trivial way to "activate" glucokinase would be to remove a negative allosteric effector from the assay. These two nov el natural products

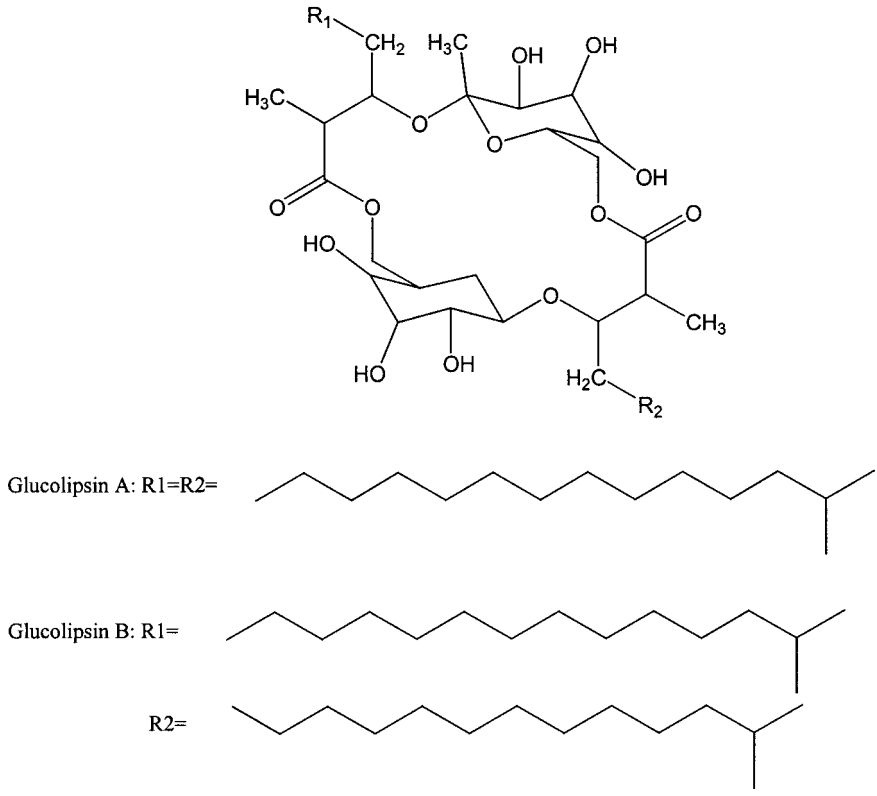
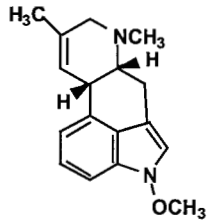


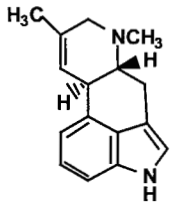
Fig. 4. Structures of two novel natural products that "activate" glucokinase.

exhibit a sequestering activity for one type of negative effector molecules for glucokinase, fatty acyl CoA esters (**Fig. 4***)*.

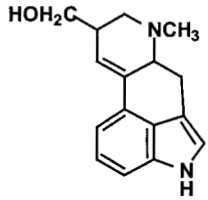
A novel member of the ergot alkaloid family, 1-methoxy-5R,10S-agroclavine, was found to be a very selective and potent inhibitor of the Lck tyrosine kinase ***(18)***. Inspection of the two-dimensional line drawings for 1-methoxy-5R,10S-agroclavin, and alkaloids such as 5R, 10R-agroclavine, and lysergol, compounds very closely related to LSD or lysergic acid diethlyamide, suggest a structure/activity relationship (**Fig. 5**). Neither 5R,10R-agroclavin or lysergol, which have a trans C/D ring juncture, showed any inhibitory activity toward the Lck tyrosine kinase, nor did they inhibit the autophosphorylation of related kinases. Further studies led to the complete structural elucidation of 1-methoxy-5R,10S-agroclavin. **Figure 6** compares the 3-D structures by line drawing of 1-methoxy-5R,10S-agroclavin and 5R,10R-agroclavin. The drastic three-dimensional structural difference because of the opposite C/D ring junctures may



1-methoxy-5R,10S agroclvin

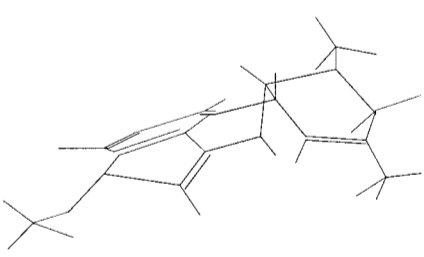


5*R*,10*R* agroclavine

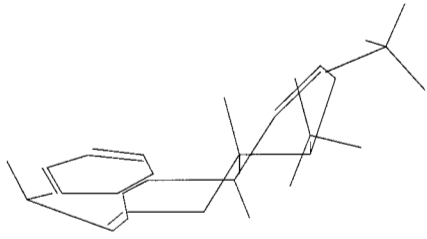


lysergol

Fig. 5. Line drawings of 1-methoxy-5R, 10S-agroclavin, and related ergot alkaloids.



1-methoxy-5R, 10S agroclavin



5R, 10R agroclavin

Fig. 6. Three-dimensional structure of 1-methoxy-5R, 10S-agroclavin, and 5R, 10R-agroclavin.

contribute to the observed enzyme specificities of these compounds. This sort of ring configuration represents a significant challenge synthetically and semi-synthetic methods were not found viable.

5. Conclusions

So, natural products or not? This chapter reviewed methods for incorporating natural products as samples in HTS programs, both historical and current, including the emerging uHTS approaches. A limited discussion of the value of natural products as a source for discovery of unusual and surprising compounds was included.

It is our conviction that the potential for drug discovery from natural product sources has only begun to be utilized. In the long term, the most successful HTS programs will almost certainly be utilizing this source of samples and will undoubtedly feed compounds with interesting and unusual profiles into the pipelines of the pharmaceutical developers.

Acknowledgments

The authors wish to acknowledge the critical review of the manuscript by Salvatore Forenza and Elizabeth Hall. We would also like to thank Salvatore Forenza for **Fig. 1**. Additionally, we would like to acknowledge other members of the BMS Natural Product Research community, including Li-Ping Chang, Lynda S. Cook, Regina D. Ezekiel, Donald R. Gustavson, Robert M. Hugill, Raouf A. Hussain, Kin S. Lam, Grace A. McClure, Robert W. Myllymaki, Edward J. Pack, Christopher J. Poronsky, Jenny Qian-Cutrone, Y.-Z. Shu, Tom Ueki, and Judith A. Veitch.

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