Rediscovering natural product biodiversity

Discovering untapped natural sources for novel bioactive compounds was the theme of the recent conference entitled *Profiting from biodiversity by leveraging natural product drug discovery*, which took place on 28–29 June 1999 in London. The conference chairman, Tom Simpson (University of Bristol, UK), stated that natural product (NP) drug discovery has been somewhat neglected since the advent of combinatorial chemistry, molecular biology and high-throughput screening (HTS) techniques. However, this conference highlighted that interest is again growing in NP drug discovery as companies are discovering that, even with the advent of these technologies, relatively few new interesting compounds are being generated and there are many novel, potentially important compounds that would not be chemically synthesized using a 'rational' approach to drug design.

Throughout the conference, it was highlighted that ten of the top 20 selling medicines in 1998 were derived from natural products, including many of the angiotensin converting enzyme (ACE) inhibitors, the statins, diclofenac, clarithromycin, conjugated oestrogens, insulin and epoetin. Furthermore, the pencillins and cephalosporins are derived from fungi, steroids, digoxin and opioids from plants and the aminoglycosides from bacteria, demonstrating the importance of these areas as sources of biologically active lead compounds.

Obtaining novel compounds from everyday resources

Robert Nash, from the Institute of Grassland and Environment Research (IGER, Aberystwyth, UK), highlighted that the selection of plants for research based on information on traditional medicinal uses has caused many to overlook a wide structural diversity. Hence, many plants commonly seen in British gardens (e.g. bluebells, daffodils, Irish dandelions) and many product materials commonly used (such as potatoes) contain bioactive metabolites that have not been examined. Nash discussed the great range of compounds that can be obtained by selecting plants that are known to be toxic to animals (bluebells) or that produce substances that are utilized by insects for their protection.

Nash pointed out that plants often contain a wide range of related metabolites that are often missed in crude extracts but that can be accessed by chemical fingerprinting-guided purification. MolecularNature Ltd (Aberystwyth, UK) purify diverse secondary metabolites by using Waters HPLC–PDA/MS Integrity™, GC–MS and Dionex HPLC to select the most interesting compounds, before purifying them using Biotage Flash™ technology. They provide pure (over 90%) dereplicated NPs at known concentrations for screening by other companies, with the advantages of guaranteed resupply and the structures provided within a month of screening.

NP can be obtained from four main sources: plants, bacteria, fungi and animals, with bacteria being the largest source (34%). Thomas Henkel (Bayer AG, Wuppertal, Germany) reported that most natural products have a higher molecular weight than their synthetic counterparts, containing more rings and being generally much more sterically complex. Furthermore, Henkel found that, on comparison of the compounds in a NP database (DNP) with a representative pool of chemical test substances (Synthetics), there was only a 60% homology. Henkel also demonstrated that by comparing the structures of NPs, synthetic compounds and currently used drugs, there was a much higher incidence of O-containing groups, but a much lower incidence of N-, S- and halogen-containing groups in the NP compounds. Furthermore, NP compounds were found to contain more sp^3-hybridized bridgehead atoms. This highlights the diverse range of compounds that can be gained from NPs that would otherwise be missed using synthetic techniques.

Increasing biodiversity by using plant cultures

Angela Stafford (Phytera Ltd, Sheffield, UK) demonstrated the advantages of using plant cell cultures versus whole plants. These include re-accessibility (more than 85% success rate with cryobanking) and that, as more than 12.5% of the world's vascular flora is threatened, the culturing of seeds enables the investigation of rare and endangered species that would otherwise be inaccessible. Furthermore, this method reduces variability caused by seasonal changes and life-cycle phases, and the potential problems if the original taxonomy was wrong, the plant was diseased, or the location is no longer there. Because plant cultures can provide multiple environments to produce hundreds of combinatorial manipulations, they can provide a platform for combinatorial biology approaches. By contrast, whole plant samples lead to fixed chemistry because only one environment can be used at any one time. The possible manipulations include genetic, epigenetic (the use of chemicals to depress areas

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of the genome), elicitation/induction approaches, and the addition of hormones and enzyme inhibitors. To diversify the chemistry and upregulate lesser-expressed compounds, the substrates and the precursors can also be modified.

As plants naturally have to deal with stresses such as excessively high or low levels of sunlight, temperature, water, salt and nutrients, as well as infection and predation, another method of inducing the production of different compounds is by exposing the plants to stresses. Stafford demonstrated a number of unique small plant-derived molecules induced by oxidative stress, which can be stimulated naturally by, for example, photosynthesis, wounding, infection and pollution. This early fractionation of the extracts before screening has been found by Phytera to reduce the problems of traditional NP extracts such as the production of complex mixtures with overlapping chemistry that then require significant post-screening separation and analysis work-up.

Combinatorial genomics to isolate novel compounds from marine organisms

Marine organisms are an excellent source of bioactive substances as they release molecules for communication as well as antimicrobial and cytotoxic molecules for defending themselves against infection and predation. Stafford pointed out that, as with plants, there are similar advantages with culturing marine organisms, such as access to microorganisms from previously unmined marine habitats and rapid re-access and re-growth on use of standard microbial storage conditions.

However, there are also several problems with culturing organisms, including the induction of variability through culture conditions, classification by phenotype means only 5000 species of bacteria have been described, and only organisms that can be cultured can be used. However, many microorganisms have been deemed 'non-culturable'. David Manyak (Oceanix Biosciences, Hanover, MD, USA) therefore described new biotechnological methods that classify organisms by molecular genotyping using 16S rRNA as the gold standard, and that could have the potential to identify 50,000–100,000 species. The environmental sample is lysed and the nucleic acids extracted using *in situ* hybridization. These nucleic acids are then amplified and cloned using dot-blot hybridization to produce rRNA sequences that can then be compared with a database. Manyak explained that the use of rRNA had several advantages, including the production of a sufficient length of RNA (16S subunit = 1500 nucleotides), a high copy number (1000–100,000 molecules per cell), no lateral gene transfer reported, highly conserved regions and variable sequence regions.

'Non-culturable organisms', which by definition are novel sources for bioactive compounds, can be isolated by altering the culture media to enable growth. Culture media that mimic natural marine substrates can enhance culture growth, and reverse HTS can then be used (the screening of one or two compounds through 30–40 different assays). Isolation of genes or gene clusters leads to the expression of enzymes or enzyme pathways and has the potential for recombination and the production of unique molecules. By contrast, as full NP synthetic pathways can be transferred using environmental genomes or chromosomes (for example, through the use of liposomes to get the DNA into culturable hosts), expression of multiple enzymes and enzyme pathways is seen and can produce higher quantities of unique molecular structures and recombination.

Valerie Berman (Wyerth-Ayerst Research, Pearl River, NY, USA) pointed out that whilst it is now recognized that there is an important and untapped source of bioactive compounds in the oceans, it is also important to research the marine environment itself. This research would then provide a better insight into the physiological requirements of marine microorganisms and hence, a better understanding of appropriate growth conditions for these organisms.

Bioactive metabolites produced from microbial fermentation

Many potential therapeutic products from microbial fermentation are produced as secondary metabolites, and appear to be formed as a result of encountering other organisms, producing metabolites that either kill or harm other organisms, or are signal compounds involved in mating. Hence, to get organisms to produce these metabolites and to maximize the potential chemical diversity, they need to be grown in various nutrient-limited media. For example, media that are deficient in C have been used to produce penicillins, those that are P-limited produce cephalosporins and vancomycin and those that are N-limited can produce carbapenems.

Michael Bushell from the University of Surrey (Guilford, UK) demonstrated the importance of a high O-availability to many organisms, especially the actinomycetes. Bushell demonstrated that many culture conditions only allow O-transfer on a small scale (25 ml in 250 ml culture volume only produces an oxygen transfer rate of 166 h^{-1}, whereas *Streptomyces* requires 1883 h^{-1}). If the media is O-limited then it will not be P-, N- or C-limited, leaving the hit-rate too low to be able to identify significant improvements. Bushell showed that the ability to get an adequate concentration of oxygen into the media depended on both the design of the vessel used and the biomass concentration in the culture. High cell concentrations are often necessary to produce a sufficiently high yield of active compounds to allow their detection in screens, but if cell concentrations are too high, O-limitation can occur.

Bushell and colleagues have devised a mechanism for the slow release of oxygen from the media by using miniaturized dissolved O-electrodes that can be used with microtitre plates. Even if the culture grows slowly, the media will release and/or absorb oxygen slowly ensuring the culture will not become O-limited, despite reaching high biomass concentrations. Bushell further suggested using wells made of O-permeable plastics and baffles to produce turbulence in the well. Organisms that require very high levels of oxygen can then be used as indicator organisms (such as vancomycin-producing organisms).

Compatibility of natural product drug discovery with HTS

Many organisms are not suitable for high-throughput screening (HTS) techniques, especially the slow-growing organisms. Furthermore, timelines for chemical optimization of NPs leads has shortened and hence, many pharmaceutical companies have stopped researching NPs. However, Kai Bindseil (Analyticon, Potsdam, Germany) discussed a technique called MEGAbolite that he believes combines the production of high structural-value compounds with a high chance of finding leads, high-quality test results and no need for dereplication. This technique involves pre-screening of the profiling sample with a fast high-performance liquid chromatography (HPLC)-gradient (8–9 minutes). The sample is put through Biotage Flash to remove the polar end of the extract (normally containing sugars) and the lipophilic end (normally containing fatty acids). The pure compounds are then extracted from the mixtures using SEPBOX™, which involves liquid chromatography (LC), followed by solid-phase extraction (SPE), then LC, and then SPE again. This whole process is repeated until a purity of over 75% has been reached for at least 5 mg of final product. The compounds are then identified using mass spectroscopy (MS) and quantified using evaporative-light scattering detection (ELSD) with fast +/− switching.

Further complications of using HTS for NP drug discovery include the production of unspecific hits, mixtures of compounds being produced that have antagonistic or synergistic effects, high rates of redundancy and the long duration of the follow-up process whilst waiting for the results of biological assays. However, Hubert Haag (Boehringer Ingleheim, Vienna, Austria) demonstrated the effectiveness of SPE in improving the ratio of secondary metabolites, reducing the possibilities of synergistic and antagonistic actions of mixtures by screening 'cleaner' compounds and concentrating extracts by up to 20–30-fold. Furthermore, SPE can increase recovery rates by up to 70–100%, the most polar compounds being lost through the column. Specificity of compounds can then be increased by the use of HPLC fractionation followed by time-of-flight MS (TOF–MS). Haag showed that peak-guided isolation of compounds was quicker than using the classical bioassay-guided isolation techniques.

Conclusions

This conference therefore highlighted both the large number of successfully marketed compounds that were originally derived from natural products, and the vast range of potentially biologically active compounds that remain unknown. The conference further highlighted that even with the advent of high-throughput screening techniques and combinatorial chemistry, natural product drug design still has its place in drug discovery and can work together to enhance lead optimization.

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In short…

The biotechnology and pharmaceutical industries have been found to lead all other industry sectors in virtually every aspect of patent ownership and use. The survey of 349 leading European companies (19.4% of which were from the biotechnology or pharmaceutical sectors) by **Derwent Information** (London, UK) examined the way in which companies access patent information.

One of the main findings of the survey was that pharmaceutical and biotechnology companies are now using a two-tiered process to source patent information. They are first accessing the growing number of Web sites offering free patent advice to give them a general overview. Only then are they accessing more definitive information from the fee-based services.

Although 84% of the pharmaceutical/biotechnology company respondents use the Internet to source information compared to the survey average of 68%, the majority of all respondents (80%) did make it clear that they would not be happy to make business-critical decisions purely on the basis of the free information. In fact, 17% of respondents claimed their use of fee-based services had increased. One reason for the concern over the use of the Internet sites was that there is not enough security on the sites.

For a copy of the research paper entitled *Managing Patent Information: an Emerging 2-tiered Approach to Business Information*, please contact The Marketing Department, Derwent Information, 14 Great Queen Street, London, UK WC2B 5DF. fax: +44 171 344 2901, e-mail: ajoyce@derwent.co.uk