Natural products – a simple model to explain chemical diversity

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A simple evolutionary model is presented which explains why organisms produce so many natural products, why so many have low biological activity, why enzymes involved in natural product synthesis have the properties they do and why natural product metabolism is shaped as it is.

1 Introduction

"Organic chemistry just now is enough to drive one mad. It gives the impression of a primeval tropic forest, full of the most remarkable things, a monstrous and boundless thicket, with no way to escape, into which one may well dread to enter."†

† Wöhler in a letter written to Berzelius in 1835.

Richard Firn was born in Newcastle-upon-Tyne in 1944 and was educated at the Edinburgh Rudolf Steiner School. Being a farmer's son, he went to Edinburgh University to study agriculture but was increasingly attracted to organic chemistry. During two years of postgraduate study at the Waite Institute, University of Adelaide he became interested in plant physiology, an interest he developed during two years at Wye College, London. Some forays into plant biochemistry were made during 2 years at the Plant Research Laboratory, Michigan State University. He now works at the University of York, puzzled by the results of experiments on plant tropisms and pondering why and how plants and microbes make so many natural products.

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When Wöhler wrote those words he was expressing his despair at the emerging complexity of the composition of natural products. Yet within a few decades, this complexity was tamed by the Kekulé theory of structure. Increasingly confident chemists took up the challenge of determining the structures of ever more complicated natural products. From the late 19th century until today, generations of natural product chemists have applied their skills and intellect to many tens of thousands of molecules made by living organisms, encouraged by a society that values many natural products for their life-giving or life-enhancing properties. By the 20th century, natural products began to attract the interest of some biochemists interested in understanding the way in which compounds were made. In the early 20th century, emerging departments of clinical biochemistry, pharmacology, toxicology, microbiology and cell biology had a few lone workers who found value or fascination in some specific natural products, but the study of natural products as a group was still largely confined to chemistry departments. By the mid 20th century, some cell biologists and physiologists were using a few natural products as experimental tools that influenced or disrupted cell functions in specific ways, for example colchicine, atropine, nicotine, digoxin, *etc*. The discovery of antibiotics gave the study of natural products a great boost in microbiology departments and ensured that natural products remained central to growing pharmaceutical companies. Coming late to the feast, the ecologist waited until the last half of the 20th century before appreciating the key role that natural products play in determining species interactions. Clearly many disciplines, ranging from chemistry to ecology, claim part of the subject of natural products as their own. However, this has resulted in the subject becoming very fragmented, as evidenced by the range of specialist natural product journals. The purpose of this article is to promote a more unified view of natural products. This view is based on an evolutionary perspective. The Darwinian perspective brought to the study of organisms an insight and order that previous classification and scrutiny had simply been unable to provide. We will argue that an evolutionary perspective needs to be adopted by those working on natural products in order to place their own work within a larger, more holistic conceptual framework. Wöhler's "*monstrous and boundless thicket*" is more dense than ever and quite forbidding to strangers. Although there exists excellent information about the composition of that thicket, we would argue that an evolutionary map is needed more than ever.

In this article we shall explain how one simple fact, that potent biological activity is a rare property for any molecule to possess, has caused evolution to shape natural product biochemistry in very distinctive ways. A simple evolutionary model is built around that fact. This model explains why enzymes involved in natural product synthesis have the properties they do, it explains why natural products pathways have the properties they do and why the overall scope of natural product chemistry is as it is.

2 Evolution and natural product diversity

The theory of evolution by natural selection states that the characteristics of organisms arise as a result of selection from among variants in a population. Mutations with characteristics that increase the fitness of an organism in the environment in which they are found, are favoured. Mutations that decrease fitness, directly, or by imposing costs without benefits, will be disadvantaged, and such variants will be lost from the population. Some variants will neither gain nor lose by their new properties hence such variation will be neutral.

Applying these ideas to natural products, it has been widely accepted that the production of any new natural product will increase costs, hence the producer will only have higher fitness if a benefit accompanies such costs. The simplest evolutionary model accounting for natural product diversity thus demands that each natural product retained in a population must have a value to the producers. Organisms might retain for a short time some 'redundant' natural products in their chemistry (products whose production once enhanced fitness but which no longer do so), but natural selection would be expected to continuously prune such dead wood from the thicket. Redundant molecules could, of course, take on a new role as precursors of new generations of compounds that do enhance fitness. However, just how much redundancy could be carried forward was a matter of opinion and depended very much on which part of the subject area one worked. At one extreme, many microbiologists were, until relatively recently, inclined to the view that most microbial natural products had no single function in enhancing the fitness of the producer and they were often regarded as waste products or accidents of metabolism.[1] At the other extreme, many chemical ecologists.[2,3,4] subscribed to the view that natural product diversity was best explained as the consequence of a 'chemical arms race'. The producer gained a fitness advantage by making a biologically active molecule that reduced the fitness of some competitor or natural enemy. Then the evolution of an adaptation/resistance of that other organism drove another round of selection, such that producers making new chemicals were favoured again. Among plant biologists the view that all natural products must have (or once had) a role in increasing fitness was largely unchallenged.[5] Because of the fragmentation of the subject, these two extreme evolutionary scenarios coexisted happily for many years because those working on microbial natural products rarely debated the topic with those working on plant/insect interactions, who themselves rarely met those working on plant/fungal interactions. Even when they were brought together the issue was unresolved.[6] Meanwhile chemists found they had plenty of work simply characterising novel natural products and biochemists could find challenges in isolating and characterising enzymes. Clearly the many groups studying natural products did not feel restricted by the lack of an accepted evolutionary framework, maybe because the old roots of the subject lay deep in chemistry departments where evolutionary theory would rarely be a coffee time topic. However, there is now a simple evolutionary model which seeks to explain why so much chemical diversity is generated by organisms, how such chemical diversity is generated and how that diversity is maintained even in the absence of a current, direct role for a compound.

3 The Screening Hypothesis – the basic concepts

The Screening Hypothesis, first published in 1991, is a model that is based on a single, simple proposition: potent biological activity is a rare property for any one molecule to possess.[7] Previous models to explain natural product diversity had simply ignored this fundamental fact. The unstated assumption of previous widely accepted models was that every natural product would be serving a role in the producer (or would have once served a role[5]). It was assumed that each addition to a natural product pathway was the result of the chemical arms race and every new molecule was maintained by selection because it enhanced the fitness of the producer. However, this scenario looked highly improbable if each new molecule created as a result of mutation had a very low chance of possessing any useful biological activity. The Screening Hypothesis was the first attempt to build an alternative evolutionary framework which accepted that there must have been very significant constraints to the evolution of natural product diversity if most natural products did not possess potent biological activity of value to the producer. The model proposed that evolution would favour organisms that could generate and retain chemical diversity at low cost. Organisms that make and 'screen' a large number of chemicals will have an increased likelihood of enhanced fitness simply because the greater the chemical diversity, the greater the chances of producing the rare chemical with

a useful, potent biological activity.[8] The model was given the title the Screening Hypothesis to emphasise the analogy with chemical screening programmes carried out by humans, programmes which also need to confront the constraints caused by the low probability of any chemical possessing useful biological activity.

Having recognised the evolutionary constraints, the Screening Hypothesis considered how selection might have favoured individuals which had traits that reduced the magnitude of these constraints. A number of specific metabolic traits were identified that would enhance the generation and retention of chemical diversity at low cost, and it was predicted that these traits would be found in the biochemistry of natural products. This review will summarise the basic tenets of the Screening Hypothesis and it will then identify evidence that has been found which supports each of the main predictions of the model.

3.1 Biological activity is a rare molecular property

Any experienced synthetic chemist asked to make a novel herbicide, insecticide or pharmaceutical product will know that their only chance of success is to make and screen as many chemicals as possible. Humans, with much knowledge and experience, still find it hard to predict whether any given molecular structure will possess any biological activity. This experience stretches back several decades and new evidence is produced on a huge scale every day by those involved in high throughput screening for any form of biological activity. However, does the low incidence of biological activity found in large scale screening trials provide information that needs to be considered when devising models for the evolution of the biochemistries involved in natural product synthesis? It has been argued that 'biological activity' as defined in a commercial screening programme is not equivalent to the types of 'biological activity' relevant to natural selection.[9] It is indeed true that many old commercial screening trials sought dramatic or extreme forms of biological activity, for example a rapid knock down effect for insecticides or a rapid and total kill by a herbicide. It must also be true that an organism could increase its fitness by making a chemical with much more subtle and less dramatic biological activity.[10] However, many modern screening trials, especially for pharmaceutical products, seek much more subtle *in vitro* molecular or biochemical effects, and such trials do not suggest that subtle effects are any more common than crude, lethal effects on whole organisms.[11] Indeed there are good reasons why this might be so. If one screens 1000 different molecules against a single target protein, the chances of potent activity will be low. If the 1000 chemicals are screened against 1000 different target proteins, the chances of any one chemical showing some form of activity will increase. Thus, if one seeks 'biological activity' of a form that could result from one of many forms of inhibition, any of which could reduce fitness ('growth', 'survivorship', *etc.*), screening would be expected to increase the chances of finding such activity compared to screening for a single, specific type of inhibition.

It has also been argued[9] that screening trials seeking biological activity of interest to humans must necessarily ignore much of the world's biological diversity. Most of the opportunities that exist for chemical interactions between species will involve chemicals acting on organisms in ways that are of no value to humans, hence they will be missed by screening trials designed to seek biological activity of value to humans. Might not some form of biological activity be found for any molecule if one looked very thoroughly in enough species? Well maybe, but this is irrelevant because, in the natural world, any organism making a natural product can only gain fitness by influencing the few other organisms with which it interacts.[8] Although most species share environments with numerous other organisms, most of these neighbouring species do not compete for resources or interact directly with each other in a manner that could influence selection. Thus, the evolution of natural product chemistry must be driven by only a few key interactions. There can be no evolutionary significance of organism A making a chemical that is found to have biological activity against organism B that does not even share its habitat nor interact with A. In other words, any screening of natural products by any organism will take place against a limited range of organisms. Hence the human experience of screening trials, where biological activity is sought against a limited range of organisms, is analogous to the type of natural selection occurring in any population.

The argument, to this point, has been focused on using screening trials to find biological activity in collections of synthetic chemicals. One final objection to using evidence from screening programmes to form the basis of an evolutionary model for natural product diversity has been that natural products are 'different' from synthetic molecules – distant echoes of the concept of *vital force* that Wöhler had disproved by experiment in 1828? While it is true that natural products can occupy a larger range of pharmacophore space than most easily made synthetic compounds, this is simply a consequence of the difference in the ways in which natural products and synthetic molecules are made. Enzymes can cause molecular changes by acting on particular atoms in a molecule and such specificity can rarely be matched by chemical reagents. Thus many natural products have delightfully complex structures that are rarely matched by most commercially useful synthetic compounds but there is no clear evidence that the complexity of many natural products is necessary rather than fortuitous. Furthermore, the ingenuity of chemists has blurred this distinction and there is little good evidence that natural products have some unique properties *per se*. The fact that so many large pharmaceutical companies have decreased their commitment to screening plant and fungal extracts in recent years[12] supports this view. The low probability of finding useful chemicals in the natural world is compounded by the fact that, once found, there are often insurmountable problems in devising an economically viable synthetic route to a structurally complex natural product.[13]

The overwhelming evidence from screening programmes is not the only evidence that potent biological activity is a rare property for a molecule to possess. Further evidence comes from studies of protein–ligand interactions. The low probability of any molecule binding with high affinity to any one protein is a consequence of the low probability of any one molecule and any one protein having complementary 3-D charge distributions. Firn and Jones[14] introduced the term *biomolecular activity* to describe the ability of any one molecule to bind to any one protein in a manner which would influence the ability of the protein to carry out its normal function. Being based on physicochemical principles, the term biomolecular activity is more precise in evolutionary arguments than the term biological activity which only has meaning within the context of a specific example.

3.2 The evolutionary constraints resulting from biomolecular activity being a rare property

Why would the low probability of a chemical possessing biomolecular activity be problematic to the widely accepted 'chemical arms race' model? Simply put, it would be very hard to explain the evolution of long, complex biochemical pathways leading to natural products. If the chances of each chemical in a simple linear pathway possessing potent, specific biomolecular activity is very low, then at every stage of evolution that could extend the pathway, the chances of the pathway leading to a product that enhances the fitness of the producer would be very low. Furthermore, mutations that result in the loss of the pathway will be more frequent than mutations that extend the pathway.[15] This is because mutations in **any** of the enzymes in a

pathway will cause loss of functionality of the pathway if the product of the final enzyme is the raison d'être of the pathway (Fig. 1). Furthermore, mutations at most positions along any one gene will reduce the activity of the enzyme coded for by that gene, while the opportunity for gain of function will be restricted to smaller regions of the gene. Since addition of functionality at the last step is an inherently low probability phenomenon, selection will favour those individuals who have reduced their costs by no longer making the now redundant chemicals that compose the earlier steps in the pathway. Thus the improbability of making a sequence of biologically active compounds is compounded by the high probability that there would be a loss of redundant diversity at frequent intervals. Although it can be argued that extremely improbable events do happen by chance in evolution, another conundrum arises if one accepts this improbable scenario. Why do so few natural products possess potent biologically activity? The earlier models explaining the evolution of natural products predicted that organisms should possess a relatively small number of highly active natural products. The reality is that many organisms produce a large number of compounds for which no clear evidence of function has been presented.

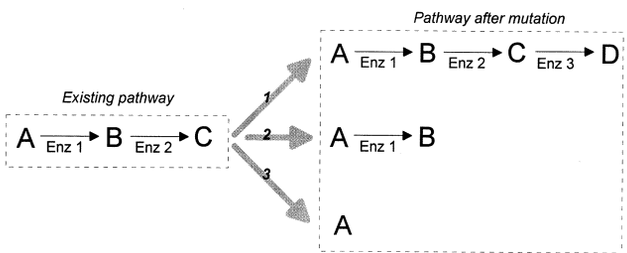

Fig. 1 The probability of a mutational event extending or reducing a biochemical pathway. Take the example of a 2 step synthesis of natural product C. Once the production of C no longer enhances the fitness of the producer, mutational events can extend (1) or trim back (2,3) the pathway. The chances of a new enzyme activity arising that can produce D is very small and the chances of D being biologically active in a way that enhances the fitness is very small. The chances of events such as 2 or 3 arising are significantly higher than chances of 1 because many mutations in the genes coding for Enz 1 or Enz 2 will abolish enzymatic activity and give some cost savings. Hence the more common mutations that result in cost savings will tend to be selected in the short term.

3.3 Predicted ways of reducing the constraints imposed by biomolecular activity being a rare property

If one accepts that biomolecular activity is a rare property for any one molecule to possess, and if the probabilistic arguments challenge the traditional evolutionary models to explain natural product diversity, then a new evolutionary scenario is needed to explain natural product diversity. The Screening Hypothesis provides such a scenario. This model simply argued that, during the evolution of natural product producing capacity, variants which possessed metabolic traits that reduced the very poor odds of making molecules with potent biomolecular activity would have been favoured. The simplest strategy that was predicted was that organisms would have evolved metabolic traits that favoured the generation of chemical diversity – the more new chemicals made in a new variant after mutation, the greater the chances of any one of these chemicals possessing potent, useful biomolecular activity that could enhance the fitness of the producer. Secondly, it was predicted[7] that metabolic traits that would favour the retention of chemical diversity, even if the production of many of the current crop of chemicals did nothing to enhance current fitness, would have evolved – new chemical diversity is more easily generated in an organism that has an existing richness of chemical diversity. It was the similarity of these strategies to those adopted by humans when they are seeking useful biological activity that caused us to name the new model the *Screening Hypothesis*. Humans, faced with the very low probability that any chemical they make will be useful, improve the odds by generating and screening as much chemical diversity as they can, hence the development of combinatorial chemistry. Humans also retain as much of their existing chemical diversity as possible, even though the majority of it has no value in any one screen; the huge libraries of synthetic compounds are a resource even though most will never have a value. The human experience of seeking exploitable biomolecular activity also points to another factor that the Screening Hypothesis of natural product diversity considers: economics. There will always be a trade-off between the benefits that generation and retention of chemical diversity give and costs that are incurred by generating and retaining products of no current value. The organism, or organisation, that can reduce these costs most will thrive at the expense of its competitors. However, an individual member of a species that gains fitness by exploiting the cost saving available through the deletion of a whole, newly redundant, pathway risks being unfit in the long term because they have sacrificed the ability to generate some new chemical diversity at a future date. The evolution of the mammalian immune system is another example of how the low probability of generating molecules with specific, potent biological activity can shape a biological strategy. The immune system is a highly economical way of generating and screening chemical diversity and it is accepted that most antibodies that are made are redundant.

3.4 Predictions about the properties of the individual processes leading to new natural products

The Screening Hypothesis predicted that there would be several 'ground rules' evident in natural product metabolism, which together or in combination would help the producers of natural products generate and retain chemical diversity, and which would help reduce the costs of retaining the overall capacity to produce the rare chemical whose production clearly enhanced the fitness of the producer. In order to generate and retain chemical diversity, processes that favour the production of multiple products, instead of the more usual single products considered to be the norm of biochemical processes, were predicted. Two possible ways of generating and retaining chemical diversity were proposed (each of which will be considered in more detail later):

- using enzymes with broad substrate specificity
- exploiting the fact that many chemical reactions give multiple products.

3.5 Predictions about the properties of combined processes leading to new natural products

At the next level of organisation, the combination of these individual processes into 'pathways', there would inevitably be consequences of the selection of individual processes that could give rise to multiple products. The Screening Hypothesis predicted that the overall shape and scope of metabolism would be expected to differ significantly from that found in 'primary metabolism'. The following general features were predicted to be common in pathways leading to natural products:

- branched pathways

branched pathways give a cost saving because the processes used up to the branch point are shared costs. An enzyme with a broad substrate tolerance has a greater potential to participate in a branched pathway hence such pathways would be favoured,

- matrix pathways

if enzymes with a broad substrate tolerance were favoured, the sequence in which several such enzymes might be used could be varied. The use of the same enzyme to produce different products at different places in the matrix clearly gives a cost saving. The fact that the same product can be produced by more than one route gives a robust means of increasing the chances

of retaining chemical diversity even after loss of one enzyme by cost saving selection,

• combined pathways

clearly by combining the products of two formerly distinct pathways to generate new chemical diversity there is a cost saving and a potential to carry chemical diversity forward.

If organisms evolved with these biochemical traits, the model predicted further implications for the regulation of such metabolism and the relationship between natural product composition and function.

Having laid out the basic framework of the Screening Hypothesis, each of the main predictions will be now considered in a little more detail before the evidence in favour of the model is considered in some depth.

4 Evidence consistent with the predictions of the Screening Hypothesis

4.1 Enzymes with broad substrate tolerance will be commonly found to be involved in natural product biosynthesis

An enzyme that can act on more than one substrate to give multiple products is a cost effective means of generating chemical diversity and the use of such an enzyme also enhances retention of chemical diversity. As long as one of the products being produced enhances the fitness of the producer, the genes coding for the overall process will be favoured by selection and chemical diversity will be retained (Fig. 2).

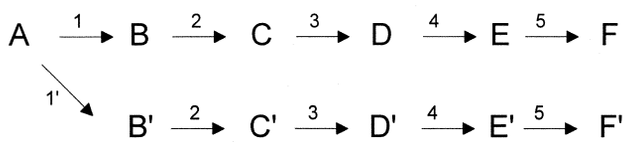

Fig. 2 The generation and retention of chemical diversity. A linear pathway exists which uses 5 enzymes to produce 5 products, B-F. Duplication of the gene coding for enzyme 1 occurs. A mutation in that gene then gives rise to a new enzyme 1′ which produces B′. If the other enzymes possess a broad substrate tolerance, 4 other new products will also be made and the 6 enzymes will be capable of producing 10 products.

In the early 1990s, predicting that some enzymes involved in natural product production might have a broad substrate tolerance seemed provocative. Even when evidence in favour of this prediction was marshalled some years later,[8] the reception was still cool and counter-arguments were advanced.[9,16] However, the proposition that some enzymes involved in natural product biosynthesis will have a broad substrate tolerance is now firmly supported by experimental evidence gained for every major natural product pathway.

This perceptual change comes partly from the adoption of new molecular tools but also from improved methods of chemical analysis which simplify the analysis of trace amounts of natural products. With the benefit of hindsight, it now appears that some experimental approaches to the exploration of enzyme specificity were very prone to finding what was sought. Guided by biochemical dogma that enzymes must be substrate-specific (dogma that largely arose from studies conducted with enzymes involved in 'primary metabolism'), and armed with only a limited range of substrates available for testing, and with only limited means of measuring low rates of conversion, it was hardly surprising that many reports of substrate tolerance were incomplete and tended to find the 'expected' specificity. However, once the number of genes in organisms began to be determined, some researchers[17] noted that there seemed to be rather too few genes to account for the amount of chemical diversity supposedly generated by the one enzyme/one product dogma. The ability to express genes coding for enzymes involved in natural products synthesis in exotic organisms enabled a more thorough study of enzyme specificity to be undertaken. Many such studies, which will be discussed in the next section, have revealed the broad substrate tolerance predicted by the Screening Hypothesis. It is now indisputable that many enzymes involved in natural product biosynthesis are indeed 'promiscuous'. Not only does this accord with the predictions of the Screening Hypothesis, it is now possible to speculate why evolution has resulted in this condition. There are reasonable arguments that much biochemical inventiveness[18] results from gene duplication and subsequent mutation of one gene copy such that the substrate specificity is changed in that copy.[19] Clearly if the mutated gene is to remain in the population, the individual carrying it must not have lost fitness and if the variant is to thrive it must indeed have gained fitness. Such increased fitness could only result from the new gene product acting on some substrate to produce a new molecule. Fitness depends on the properties of the new molecules that are made – the production of genes and the production of enzymes always incur costs and those costs must be equalled or exceeded by the utility of the product of the enzyme if the mutated gene is to survive. A mutated gene that codes for a new enzyme with a very narrow substrate tolerance has only a very low chance of being beneficial because the chance of *any* new molecule being made by that enzyme is low. However, a mutated gene that codes for an enzyme with a broad substrate tolerance has a higher chance of producing at least one product that enhances the fitness of the producer. Thus enzymes with a narrow substrate tolerance may be the result of selection narrowing the initial broad substrate specificity of a new enzyme and narrow substrate tolerance should not be regarded an inherent property of all new enzymes. The selection pressures that operate in different areas of metabolism will vary, hence it is predictable that substrate tolerance will vary. In 'primary' metabolism, judging by what is currently known about the narrow substrate tolerance of many enzymes, there would appear to have been strong selection to narrow substrate tolerance. In the case of natural product metabolism there would appear to have been much less selection for narrow tolerance, indeed the converse may be true – mutations which narrow the substrate specificity may provide small short term gains but large long term costs.

Real examples of the concepts just outlined are being reported regularly and it is clear that examples can be found in the majority of major natural product biosynthetic routes. It must be emphasised that the evidence presented never comes from studies that have explicitly sought to test the predictions of the model; instead the model was being probed fortuitously.

Monoterpenoids

In plants, a study[20] of monoterpene production in *Mentha* showed that a single gene mutation of Spearmint produced a plant with a mix of chemicals that were characteristic of Peppermint. The mutant hydroxylation enzyme added a hydroxyl to a 3- position in a cyclohexene ring, whereas the wild type caused a 6-hydroxylation. The subsequent enzymes in the pathway accepted the new 3-hydroxy substrates produced by the mutant to give an array of new products. The appearance of an unexpected novel product in the mutant suggests that a further elaboration of one of the newly created products by some unidentified secondary metabolism enzyme, from another pathway, generated further chemical diversity. This is a fine example of how a single gene mutation can generate several new products and how new diversity can be propagated in unexpected ways.

Diterpenoids

One of the most extensively studied classes of diterpenoids are the gibberellins, well known for their biological activity in plants. However, why do plants and fungi produce so many gibberellins, given that the majority of these possess very low biological activity? Surely in the case of a compound of such great importance to plants, selection should have reduced such

apparently wasteful redundancy? We would argue that gibberellins are made by a pathway that was evolved to generate and retain chemical diversity and that even though few gibberellins would classically be regarded as 'primary metabolites', selection has still favoured enzymes that retain a broad substrate tolerance. A good example of this tolerance is the GA3 gene of *Arabidopis* which encodes *ent*-kaurine oxidase, an enzyme that catalyses three steps of gibberellin biosynthesis.[21] Later in the pathway there are more enzymes with a broad substrate tolerance. For example, GA 20-oxidase can convert both GA_{12} to GA_9 and GA_{53} to GA_{20} and GA 2-oxidase can convert GA_1, GA_4, GA_9 and GA_{20} to their corresponding 2ß-hydroxy derivatives.[22]

Triterpenoids

The major sterols of plants and fungi have either a methyl or ethyl substitution at C-24, with the alkyl carbons coming from *S*-adenosylmethionine-dependent transmethylation. The methyl or ethyl substitution is the result of either a single or two sequential single carbon additions. Recent studies have shown that *Arabidopis* contains three sterol methyltransferase genes (SMT) which are involved in C-24 substitution and it has been found that each of the enzymes can perform two sequential substitutions. It was concluded[23] "that C-24 SMT, like other enzymes in sterol biosynthesis, can be somewhat promiscuous". Interestingly, in the *smt1* mutant, the expected massive reduction in some C-24 substituted sterols was not found and it was postulated that SMT2 and SMT3 enzymes substitute as "impostors" – further evidence of metabolic flexibility.

Tetraterpenoids

The enzymic flexibility of enzymes involved in carotenoid biosynthesis has been thoroughly explored in bacteria.[24,25] Enzymes have been found that can catalyse multiple sequential steps in a pathway (*e.g.* CrtI is responsible for 4 desaturation steps between phytoene and lycopene in *Erwinia herbicola*) or two or more non-sequential steps (*e.g.* CrtA, CrtC or CrtD in *Rhodospirillum rubrum* or *Rhodobacter sphaeroides*).

Phenylpropanoids

By studying the incorporation of various possible precursors (cinnamic acid, caffeic acid, *p*-coumaric acid and ferulic acid) into phenylphenalenones, it was shown that there is some flexibility in the enzymes involved in the biosynthesis of phenylphenalenones because a range of products were formed.[26]

The final stage of lignin synthesis is the oxidation of cinnamyl alcohols catalysed by a peroxidase. In tomato, the TPX1 gene codes for a peroxidase that is not only responsible for the synthesis of lignin but also of suberin.[27] Further evidence for substrate tolerance has come from studies of tobacco lignin biosynthesis.[28]

Alkaloids

The very elaborate chemical structures of many alkaloids have been considered as evidence that plants must possess enzymes of great specificity.[29] However, it has been known for some years that *Nicotiana* will incorporate some non-natural putrescine or nicotine analogues into pyrroline alkaloids, hence it is clear that some alkaloid producing enzymes must have some substrate tolerance.[30] Extending these studies, Boswell *et al.* fed a range of analogues of *N*-methylputrescine and tropinone to root cultures of *Nicotiana* and *Brugmansia* and found that many were incorporated into novel alkaloids.[31] The authors concluded that "a considerable degree of plasticity exists in substrate specificity of many of the enzymes in alkaloid biosynthetic pathways". A study[32] of some of the enzymes confirmed that some of the enzymes "will accept alien substrates to varying degrees". It would appear that the inference that great enzyme specificity must underlie the structural complexity of many alkaloids is insecure.

Volatile esters

Strawberries produce in excess of 300 chemicals that contribute to their odour,[33] with volatile esters being the most attractive to humans. The biosynthesis of volatile esters has been explored recently using a DNA microarray approach to identify genes associated with odour production. A gene coding for a strawberry alcohol acyltransferase (SAAT) was identified and found to code for an enzyme that had relaxed substrate specificity. Although the enzyme was most efficient acting on medium chain alcohols, it showed activity against 14 of the 20 alcohols tested. Furthermore, it accepted a wide range of different acyl-CoAs as substrates. It accepted acyl-CoAs up to C_{10}, branched acyl CoAs and aromatic acyl-CoA when assayed with either 1-propanol or 1-butanol.[34] Rose petals release 400 volatiles and acetyl-coenzyme A: geraniol/citronellol acyltransferase has been characterised which shows a relaxed substrate tolerance and hence, can produce multiple products.[35]

Polyketides

The polyketide pathway in *Streptomyces* shows very considerable metabolic flexibility due to relaxed substrate tolerance.[36,37,38] Gene replacement studies show that the introduction of new individual modules into the pathway results in the synthesis of a wide range of new structures because the new products made by the introduced enzyme are accepted by many other enzymes that carry out later transformations.[39]

Clearly the last 10 years have yielded considerable evidence in support of a central prediction of the Screening Hypothesis – that many enzymes involved in natural product synthesis will possess a broad substrate tolerance. We are not alone in reaching this conclusion. A recent review[40] of the specificity in enzymes involved in plant biochemistry, a review apparently unbiased by any underlying evolutionary theory, was confident in its conclusion that "further analysis ··· will confirm that multifunctional enzymes are ubiquitous in the plant kingdom".

4.2 Reactions giving multiple products will be found

The Screening Hypothesis postulated that the use of enzymes that produce more than one product, or the incorporation of non-enzymic reactions into secondary metabolic pathways, would be advantageous for generating and retaining chemical diversity at low cost. At the time of the prediction, the best evidence for such ways of generating chemical diversity was the existence of non-enzymic reactions that follow the wounding of a plant which give rise to chemical diversity at a time and place when it is most needed. However, it is now clear that this predicted strategy serves not only wounded plants but also healthy ones.

Plants seem to have evolved to benefit from the fact that some enzymes not only possess an ability to create unstable intermediates but that the enzymes can also influence the way in which the unstable intermediates rearrange to produce stable products. In such enzymes, selection can operate not only on substrate choice but also on products made. This combined flexibility is best illustrated in the terpenoid pathway where there are now many examples of enzymes capable of producing multiple products.[41] The Screening Hypothesis argued that such flexibility would enhance the generation of chemical diversity at low cost and would be very effective at retaining chemical diversity – only one of the multiple products needs to confer a benefit on the producing organism for selection to have an opportunity to favour that variant.

Monoterpenoids

A study of the members of the Tpsd gene subfamily in Grand Fir (*Abies grandis*) found five monoterpene synthases (ag6, ag8,

ag9, ag10 and ag11) that were capable of producing multiple products.[42] A further interesting example of the generation of multiple monoterpene products comes from a study of monoterpenes in Common Sage (*Salvia officinales*), where the search for a (+)-bornyl diphosphate synthase and a (+)-pinene synthase led to the suggestion that both these activities reside in a single enzyme.[43]

Sesquiterpenoids

The most striking evidence for the ability of enzymes to produce multiple products comes from a study of two sesquiterpene synthases in Grand Fir (*Abies grandis*). One enzyme (δ-selinine synthase) produced 34 different compounds from a single substrate and another (γ-humulene synthase) produced 52 products from its precursor.[44] Similar flexibility is shown by limonene synthase which has been shown to produce multiple products in isotopically-sensitive branching experiments and cDNA cloning.[45,46] In tomato (*Lycopersicon esculentum*), the sesquiterpene synthase germacrene C synthase also produces multiple products.[47]

Clearly the chemistries available within any one natural product pathway will determine the ability of an organism to exploit the opportunity to produce multiple products and the fact that the examples given come from the terpenoid pathway suggest that this strategy could be less universal than the selection of enzymes with broad substrate tolerance. The production of multiple products by an enzyme makes the naming of the enzyme somewhat difficult. Naming the enzyme after the major product produced seems logical but we would suggest that such a convention is arbitrary and even misleading. The product that enhances fitness of the organism need not be the major product and evolution is most likely selecting the overall properties of the enzyme (and indeed the overall pathway), not the one product. A convention to denote the ability of an enzyme to produce multiple products would seem to be desirable.

4.3 Evidence for matrix or grid metabolic pathways

As is the case in many hierarchical systems, a property at a lower level can shape properties at a higher level. Thus the low probability of a chemical possessing potent biomolecular activity determines the selective pressures that operate on the evolution of enzymes involved in natural product synthesis with the result that enzymes with a broad substrate tolerance are favoured. It is predictable that such selection of substrate tolerant enzymes will have a profound effect on the shape of the pathways to which they contribute. Linear or cyclical pathways, the norm if all the enzymes act only on one substrate to produce one product, would not be expected because enzymes capable of acting on more than one substrate would be expected to facilitate branching pathways, and at the extreme, to participate in a matrix grid. It is predicted that there would indeed be some selective pressure to shape natural product pathways in this manner because of the potential for cost savings. Using an enzyme twice in a linear sequence gives some cost savings but using just a few enzymes in a matrix grid provides even greater cost savings and a greater robustness against loss of chemical diversity (Fig. 3).

Diterpenoids

The major human food sources are cereal crops and considerable gains were made in productivity when it was found that plants with a dwarf stature gave higher grain yields. Given that gibberellins are important in controlling plant height, it is not surprising that a very large and sustained effort has been made in trying to understand gibberellin biosynthesis. This effort has yielded a very large amount of information about the properties of the individual enzymes that contribute to gibberellin synthesis and how the structures are interconverted. As predicted

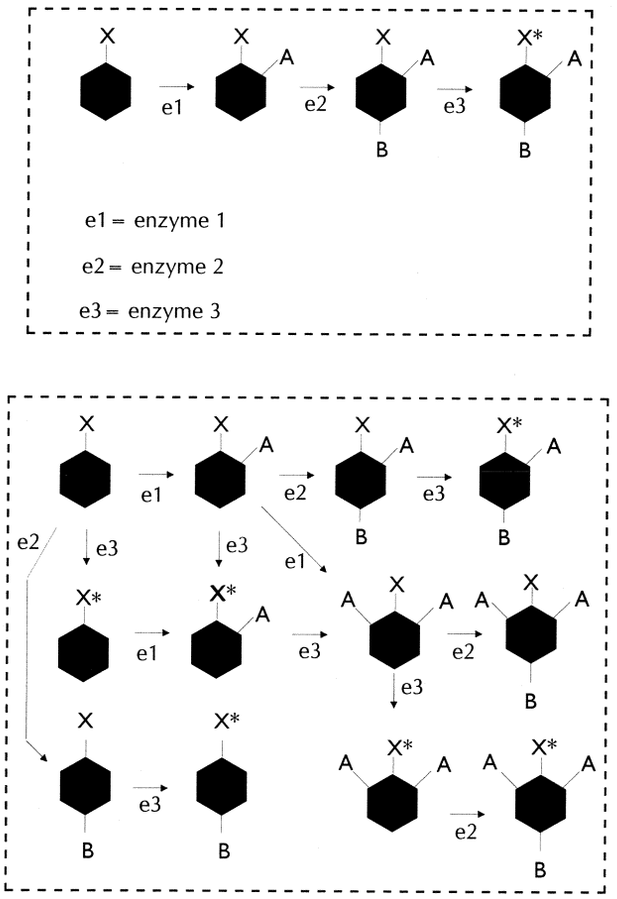

Fig. 3 The advantages in matrix grid in generating and retaining chemical diversity. The upper panel is a schematic 3 step conversion using highly substrate specific enzymes giving 3 products. The lower panel illustrates how a broad substrate tolerance can allow 11 products to be created by three enzymes if they possess a broad substrate tolerance and how there might be more than one route to any compound.

by the Screening Hypothesis, a small number of enzymes generate considerable diversity using matrix conversions.[41,48,49]

Tetraterpenoids

One of the nicest examples of the efficient use of enzymes in a matrix comes from a study of carotenoid biosynthesis in the marine bacterium *Agrobacterium aurantiacum.* ß-carotene was converted by 2 enzymes (the products of genes *crtW* and *crtZ*) into 9 different carotenoid products in a matrix of sequential conversions.[50]

Anthocyanins

The great diversity of flower colours is being probed at a molecular level and studies reveal matrix conversions. For example, in petunia flowers, 3 enzymes (F3H, F3′5′H and F3′H) can produce 5 different products (eriodictyol, pentahydroxyflanone, dihydromyricetin, dihydroquercetic and dihydrokaempferol) from naringenin.[51] Studies of the flavonoid 3-*O*-glucosyltransferase (3-GT) in *Perilla* provide evidence for its role in a metabolic grid.[52]

Lignin

A scheme for monolignol biosynthesis has been proposed,[53] where the three enzymes CAOMT (caffeic acid *O*-methyl transferase), CCoAOmt (caffeoyl-coenzyme A *O*-methyl transferase) and hydroxycinnamate CoA ligase have sufficient substrate tolerance that they can each act on more than one substrate to create a matrix of transformations.

Glucosinolates

In excess of 100 glucosinolates are known.[54] This diversity of aliphatic glucosinolates found in some plants has resulted in a proposal that a grid of conversions using a limited number of enzymes is involved.[55]

5 The overall shape of metabolism leading to natural products – why are there so few major pathways leading to natural products?

The final hierarchical level to consider is the overall extent of natural product metabolism. Given the very considerable chemical diversity within any one major group of natural products, why are there so few major pathways? Two of the possible explanations deserve consideration. Firstly it could be argued that the chemistries of each of the major groups of natural products have characteristics (which may be different for each major group) that favour the generation and retention of chemical diversity. This is an argument that chemists may like to consider as the authors are ill equipped to participate in it. However, the second explanation is an evolutionary one and can be considered in terms of the concepts already discussed. It could be argued that the more similar the products of natural product metabolism are to the structures used in the primary metabolism of the producer, the more likely it is that self inhibition will result. The argument that the chances of a variant within a population producing a new molecule with biomolecular activity is very low has largely been drawn without reference to biological activity in the producer itself. However, should the rare molecule with potent biomolecular activity arise, the most likely target that will be influenced by that molecule will be a protein within the producer. The logic behind this statement is as follows. Firstly, under most circumstances, unless the new molecule is produced within some specialised structure that isolates the molecule from the producer to a large extent (for example a glandular hair in a plant), the highest concentration that is achievable will be within the producer. Secondly, many potential target proteins will be present in the producer at a high concentration. Consequently it is predictable that many novel natural products that possess some biomolecular activity will have a high probability of decreasing the fitness of the producer. Variants that produce such self inhibitory compounds will be rapidly lost from the population. During the early evolution of natural product biochemistry the problem must have been especially acute because new molecules that are but one step away from the major metabolic pathways of the cell will have had a structure that would have been very similar to some vital metabolic intermediates. The chances of such molecules acting as substrate or allosteric inhibitors with detrimental effects would have been quite high. However, suppose such a new molecule is made whose production increases fitness. Any further enzymic transformation of that product will then produce molecules that have a decreased chance of mimicking major metabolites and the risk will be reduced at every new incremental step of the newly emerging pathway. In other words, once a pathway producing natural products begins to elongate, that pathway will have a higher chance of producing new chemical diversity that can increase the fitness of the producer than a new pathway branching from primary metabolism. The fact that there are only a few major pathways leading to natural products can be interpreted as a consequence of this constraint.

6 Some other consequences of the Screening Hypothesis

6.1 Primary *vs.* secondary metabolism – redundant terms?

Many of those working on natural products have never been enthusiastic about the term secondary metabolites to describe the class of chemicals that interest them. There is now a good reason not only to reject the term secondary metabolism but also to reject the concept of primary metabolism. A more generalised evolutionary model to explain the properties of metabolism has been devised of which the Screening Hypothesis is a part. This general model is based on the concept that selection will operate to shape biochemistry on the basis of the properties of the molecules being produced by enzyme activity. The fitness of any variant producing a new enzymatic activity will be the sum of the fitness that results from possessing the unique chemical mix that the variant's enzymes produces. Because the fitness contribution that any chemical makes depends on the properties of the chemical, and because there are at least three main property classes, a very complex optimisation will occur over evolutionary time. The main property classes are: component properties (typical examples being primary metabolites which largely serve a role as being a necessary part of an overall pathway and the main property they possess is an ability to serve the needs of the enzyme that acts on them), physicochemical properties (for example lipids, where many similar chemicals share physicochemical properties and variation in the overall lipid mix is tolerated because each molecule contributes to that overall mix) and the biomolecular activity. It can be shown that the selection pressures that operate in pathways creating chemicals with component properties (much of what was called primary metabolism) will result in severe canalisation and narrow substrate tolerance. In pathways leading to chemicals whose physicochemical properties contribute to fitness (for example lipids, many pigments, waxes, *etc.*), there will be a low selection pressure to narrow substrate tolerance. In pathways leading to biomolecular activity the arguments outlined in this paper will apply. This overall evolutionary model provides a more flexible and dynamic view of biochemical evolution. For example, the selection pressures operating on a pathway could change over evolutionary time. The isoprenoid pathway leads to pigments, to compounds with high biomolecular activity that act against other organisms, and to molecules with high biomolecular activity that are vital for the functioning of the producer organism. Using traditional terminology, it is impossible to assign the isoprenoid pathway to primary or secondary metabolism. The new model explains why attempts at such an assignment were certain to fail because biochemical evolution simply did not work that way.

7 Which natural products should be a priority for chemists?

Finally, can the evolutionary model be of practical help to the natural product chemist? Hundreds of thousands of natural products are thought to exist in plants and microbes, the majority still not characterised. Clearly many chemists have made choices as to which chemicals should be characterised first but what were the criteria used for this selection? The most abundant? The most biologically active? The greatest commercial potential? The easiest to characterise? The criteria for choice for characterisation will have changed with time because the analytical techniques and resources available. As long as chemists accepted the old dogma that organisms only made natural products which served a role, there was a justification for selecting any natural product – they were surely all important? However, if the Screening Hypothesis is valid, more care is needed in justifying an interest in any particular molecule. Once it is accepted that many (the great majority?) natural products are made despite of the fact that they do not possess potent biomolecular activity, how does one know which ones are worthy of attention?

7.1 The most abundant?

Surely the natural products made in the largest amounts must be the most important to the producer? There must be costs to producing products in large amounts for molecules that produce little apparent current benefit. But, on the other hand, if that major constituent is converted to a very much

smaller amount of a chemical with much more potent biomolecular activity by an enzyme with low efficiency, the production of the large amount of 'precursor' might be cost effective. Thus there remains a logic in determining the structure of natural products that occur in large amounts but maybe consideration needs to be given to the possibility that such compounds are not important end products but might be important intermediates. There are some nice recent examples in the insect chemoperception literature of very minor components being much more important than the numerous plant-derived molecules that occur at much higher concentrations.[56]

7.2 The most biologically active?

Given the great difficulty in defining what one means by the term biological activity, it is clearly hard to be sure that any biological activity of a natural product found by humans corresponds to the biological activity that is selected for by the producer. It seems very probable that many forms of biological activity that do serve the producer of the natural product are simply too subtle for humans to appreciate at the early stages of the exploration of the natural products in any species. The Screening Hypothesis can offer no rules to guide the investigator, it merely cautions the investigator to consider that most natural products will possess no biological activity of value to the producer and any biological activity found could well be fortuitous.

7.3 Those with the greatest commercial potential?

This is the easiest selection criteria to defend because there is no reference point with regards to the needs of the producer organism and the form of biological activity being sought is unequivocally defined by the economics of human society. The Screening Hypothesis does suggest how the rules of natural product metabolism might most usefully be exploited by humans to generate chemicals of value.[13] Extending the concept of combinatorial biochemistry,[57,58] and using innovative culture methods for microbes,[59] new chemical diversity can be created or revealed. Chemists will be needed to fully define the capabilities of enzymes involved in natural product transformations. The chemists can characterise all the products being made by any one enzyme from their natural substrates and they can logically explore the substrate tolerance of any one enzyme by making and presenting to the enzyme some novel substrates. However, more fascinating is the potential to generate even more chemical diversity by feeding synthetic substrate analogues to organisms with known substrate tolerances. Natural product chemists will not only have a much bigger arena to explore but they will be able to extend that arena virtually infinitely in very productive ways.

8 Summary

We would hope that Wöhler would have found the evolutionary model expounded in this article helpful in navigating the thicket of natural products. The model provides a few general rules that are independent of the particulars of any chemical, any enzyme and any pathway, general rules that may help investigators reach their own objectives more efficiently. The model is an opportunity to restore some unity to the study of natural products because, being based at the lowest functional level (a physicochemical constraint), it has implications for all higher levels of organisation. Thus chemists, biochemists, molecular biologists, and biologists of many flavours can all contribute to the evaluation of the model once they appreciate that they are studying processes that can be viewed within a consistent, credible evolutionary framework. The isolation of the stakeholders that has characterised the study of natural products in the past should not be allowed to continue.

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