Biological Methods for Studying the Biosynthesis of Natural Products

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1 Introduction

The interest in pursuing studies of the biosynthesis of natural products that are formed by secondary metabolic pathways evolved from studies of primary metabolism as we began to wonder about the biochemical parallels between these two areas and the reason for the restricted production of metabolites that are often capable of having potent effects on other living organisms. The successful investigation of primary metabolism has depended heavily on the use of isotopically labelled compounds to trace the sequence of events and to define the mechanism of biochemical reactions *in vivo*, an approach that began in 1935 with the use of deuterium to study some aspects of lipid metabolism[1] and has continued unabated, in part, due to the development of increasingly sophisticated methods for the analysis of isotopically labelled compounds.[2] This type of experiment later was overshadowed by metabolic mapping experiments, using auxotrophs, and by studies of the enzymology, using purified proteins *in vitro*, and of the physiology, using mutants and biochemical modulators *in vivo*. During the past decade, the explosive growth in the use of recombinant DNA methods has revolutionized this field by shifting the focus of studies onto the molecular biology and genetics of primary metabolism, as well as greatly facilitating the former types of experiments through an easier availability of scarce proteins and a deeper understanding of the interaction of biochemical systems.

For the field of secondary metabolism, the question naturally arises about its development: has it grown in a parallel way and is it likely to experience an equally revolutionary growth through the application of recombinant DNA methods? The use of isotopes has proceeded in a nearly identical manner, perhaps with even more sophistication,[2] since it has been much harder to study the enzymology *in vitro* and to probe the physiology *in vivo*. This has been partly due to the difficulty in isolating the enzymes of secondary metabolic pathways and in establishing uniformly applicable conditions for the analysis of the complex biochemical systems that often can be viewed only through a narrow window in the organism's lifespan. Such difficulties fortunately are waning, for it is becoming increasingly clear that the use of recombinant DNA methods will overcome problems of this kind in much the same way as for primary metabolism. Therefore, the answer to each of the questions that is posed above has to be affirmative: although the study of secondary metabolism has developed like its counterpart, its growth has been slower in some areas but promises to accelerate with the application of the same methodology that has characterized the other fields of modern biology since the post-1973 revolution in molecular biology.

My purpose in this review is to document the preceding statement by discussing some biological approaches that are useful for investigations of the biosynthesis of natural products. These include biochemical, enzymatic, and genetic methods, taken from work that has been reported in the literature largely since 1980. I give pertinent examples of work from these three areas, with comments on the advantages or limitations of the methodology, rather than attempting to provide a comprehensive survey of the literature. Much of the terminology and general concepts, the *lingua franca* of this scientific field, were given by Brown in 1972.[3] As these have not changed in the intervening thirteen years, I therefore refer the reader to this excellent source for background information, and in the present review discuss only the newer concepts and the terminology that is necessary for an understanding of the biochemical and genetic experiments that have been described.

2 Biochemical Studies with Intact Cells

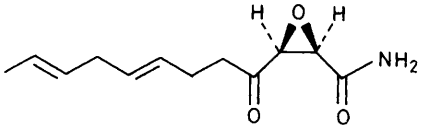
2.1 Protoplasts

Restrictions on the rate or extent of uptake of precursors can sometimes be a problem when using intact cells in biosynthetic experiments, but the difficulty can often be overcome by using protoplasts (which are bacterial, fungal, or plant cells that lack most or all of their cell wall). Protoplasts can also be disrupted by osmotic shock, which permits the isolation of proteins or subcellular organelles under very gentle conditions, and are useful for the transformation of cells with DNA (see Section 4.3). Protoplasts are made by digesting away most of the cell wall with lysozyme (for bacteria) or with other lytic enzymes (for eukaryotes) under conditions that stabilize the resulting sacks of cellular constituents against rupture. Methods for their preparation can be found in the following reviews: bacteria,[4] fungi,[5] and plants.[6]

Examples of the use of protoplasts in studies of the biosynthesis of natural products are aflatoxins,[7] actinomycin,[8] and echinomycin[9,10] (to facilitate the uptake of precursor), and streptomycin[11] and isopenicillin N[12] (to facilitate the preparation of cell-free extracts).

2.2 Inhibitors

Inhibition of some step in a biosynthetic pathway by allowing an organism to grow in the presence of a compound that is known to be an enzyme inhibitor can provide circumstantial evidence for the involvement of similar enzymatic activity *in vivo*, or show which kind of biochemical system has to be functioning for the biosynthesis of the natural product. This approach can be used when a more direct study of the enzyme-



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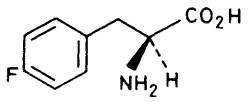

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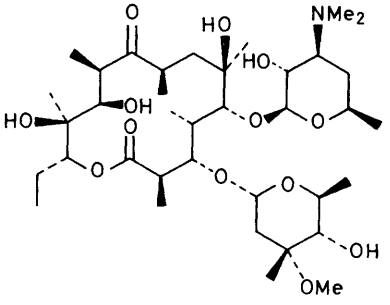

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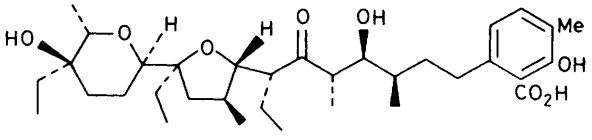

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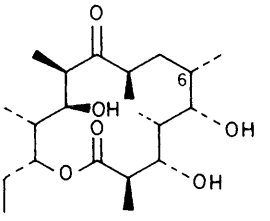
catalysed step or biochemical system is not possible. It suffers from the inability of the experimenter to control the effects that an inhibitor may have at other points in the cellular metabolism and to control what happens to the substrate of the enzyme that is inhibited. Despite being unable to circumscribe the effects of the inhibitor, useful information has been acquired in this way.

Cerulenin (1), which is an antifungal metabolite of *Cephalosporium caerulens*, is a potent inhibitor of the fatty-acid synthases in micro-organisms and in animal tissues.[13,14] It inhibits β-ketoacyl thioester synthetase activity[15] by binding irreversibly to the SH of the peripheral cysteine residue in the active site of the condensing enzyme domain.[16] Since cerulenin also inhibits the biosynthesis of polyketides, which are a class of natural products that are made from simple fatty acids *via* poly-β-ketone intermediates, this has been taken as evidence for the similarity of the enzymes that catalyse the Claisen condensation of acyl thioesters and α-carboxy-acyl thioesters in the two biochemical systems. The active sites of the polyketide- and fatty-acid-condensing enzymes are not identical, however, since the former is more sensitive to (1) than the latter.[4] Nevertheless, cerulenin specifically blocks the formation of the polyketide-derived portion of macrolide antibiotics,[17,18] tetracycline,[19] and candicidin[20] *in vivo* and of naringenin-chalcone synthase[21] and 6-methylsalicylate synthetase[22] *in vitro*, presumably by inhibiting the activity of condensing enzyme. This property of (1) has been exploited in the design of a screening technique for overproducing strains of *Streptomyces peucetius* that make daunorubicin[23] and in the production of hybrid antibiotics (see Section 6).

Fluorinated amino acids can affect the properties of enzymes by being incorporated into their primary structure,[24] thus imparting abnormal properties that are akin to the result of mis-sense mutations,[25] and fluorinated analogues of normal substrates can inhibit the function of the catalytic site of an enzyme.[26] These properties justify studies of the effects of some fluorinated compounds on the biosynthesis of natural products. Packter and co-workers noted that the addition of 4-fluorophenylalanine (2) to cultures of *Aspergillus fumigatus* markedly suppressed the formation of the characteristic acetate-derived phenols and resulted in the secretion of atypical shikimate-derived phenols.[27,28] These effects were different from those that were caused by the addition of cycloheximide, 8-azaguanine, or 5-methyltryptophan, which are known inhibitors of the synthesis of proteins. The effects of (2) could not be explained clearly; however, these and other data were used later to support the proposal that the aromatic [phenol] synthetase could have originated from a dissociated portion of the fungal fatty-acid synthase.[29] The biosynthesis of the macrolide antibiotic erythromycin A (3) was blocked by the addition of 2- or 3-fluoropropionic acid or their ethyl esters to



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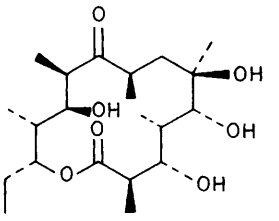

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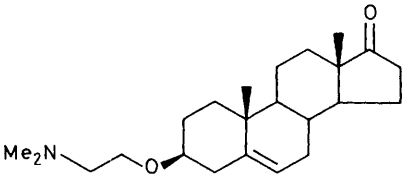

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cultures of *Streptomyces erythraeus*.[30] The blockade of formation of the antibiotic took place without a significant effect on cell growth, and did not result in the accumulation of intermediates that are related to the deoxy-sugars that are characteristic of the biosynthesis of this macrolide. Presumably, the fluorinated propionates blocked the formation of the macrolactone portion of (3), perhaps by inhibiting propionyl-CoA carboxylase, since this enzyme can be inhibited by 3-fluoropropionyl-CoA *in vitro*[31] and is believed to be involved in the biosynthesis of (3).[32] In contrast, the addition of ethyl (2*S*)-2-fluoropropionate to resting cell cultures of *Streptomyces lasaliensis* caused a two- to eight-fold stimulation of the production of the polyether antibiotic lasalocid A (4),[33] which is formed by a polyketide pathway that utilizes acetate, butyrate, and propionate as precursors.[34] This result may have been due to induction of the formation of greater amounts of an enzyme that catalyses one step of the pathway in response to its depletion by the fluoropropionate.

Inhibitors of mono-oxygenases or their associated electron-transport systems can block biosynthetic pathways by disrupting the hydroxylation or epoxidation of intermediates. The conversion of 6-deoxyerythronolide B (5) into erythronolide B (6) thus was inhibited by adding GEB (7) (an inhibitor of an NADPH-specific reductase in the biosynthesis of sterols) to cultures of *Streptomyces erythraeus*.[35] The enzyme

6-deoxyerythronolide-B hydroxylase, which catalyses this conversion *in vitro*, contains a cytochrome *P*-450 prosthetic group and a two-component electron-transport system[36] that presumably is inhibited *in vivo* by GEB. The formation of lasalocid A was found to be blocked *in vivo* by ethyl GEB and SKF525A[33] (another inhibitor of mono-oxygenases), which supports the belief that its biosynthesis involves the epoxidation of a diene intermediate.[37]

Finally, the most widely used inhibitor of cellular secondary metabolism is cycloheximide (8). This compound has often been used to demonstrate that the synthesis of new protein is needed for the biosynthesis of a natural product during the most active period of its formation, as in the case of the phenols of *Aspergillus fumigatus* cited above. If cycloheximide inhibits the formation of a secondary metabolite if it is added at the beginning of production of the metabolite, this usually is interpreted to mean that the enzymes of the pathway are not formed constitutively but are induced by some signal which turns on gene expression; if it does not inhibit the formation of the metabolite, then constitutive formation of the enzymes prior to the production stage is presumed to have occurred. For example, with the *Aspergillus* phenols, it was concluded that orsellinic acid and 2,3,5-trihydroxytoluene were formed by constitutive enzymes and 2,3,4,5-tetrahydroxytoluene and its *O*-methyl derivative by inducible enzymes, based on how cycloheximide affected the production of these compounds.[27]

2.3 Nutritional Regulation

The effects of environmental and nutritional factors on the production of microbial metabolites have been studied extensively, since these are important for their commercial production. Because these effects are more closely related to understanding the regulation of the production of metabolites than to learning how the metabolite is made, I will not discuss this topic in depth. There are several recent reviews of this subject which the interested reader may consult, however.[38–40]

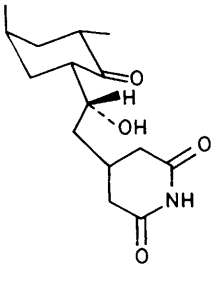
The production of an antibiotic by a micro-organism that is cultured in a laboratory, as a typical example, does not begin until the easily utilizable carbon and nitrogen sources have been depleted below some low threshold level and the rate of growth has declined. The level of phosphate in the culture medium also affects the onset of production of antibiotic; low levels of phosphate usually favour this and high levels usually inhibit it. The work of Dekleva *et al.* about the effects of nutrients on the production of an anthracycline antibiotic is an example of some of these phenomena and how they can be studied.[41] A particularly interesting case is the stimulation of the production of macrolide antibiotics by agents that lower the concentration of ammonium ion in the growth medium.[42,43] In one case, the ability of ammonium ion to repress the production of antibiotic has been attributed to its suppression of the biosynthesis of protylonolide (tylactone) (9), which is the lactone portion of the macrolide tylosin (10).[44] Since there is evidence that, in the biosynthesis of protylonolide, valine can provide several of the carbon atoms of the lactone,[45] it is significant that ammonium ions were found to inhibit the catabolism of valine in the micro-organism that was producing protylonolide.[46]

This limited coverage of the effects of nutrients on the production of microbial metabolites does not mean that it is relatively unimportant to studies of the biosynthesis of natural products. On the contrary, this is an area where much knowledge is needed about the links between changes in nutrient levels and the appearance of secondary metabolites as part of the regulation of the biosynthesis of natural products. At the moment there is little insight into this subject at the molecular level, but this will change soon, through studies of the molecular biology, using cloned genes (see Section 5), and the results will greatly illuminate our understanding of microbial physiology.

2.4 A-, B-, and C-Factors

The onset of formation of natural products is felt to be part of the developmental biology of organisms which exhibit different stages in their life cycles. In bacteria the production of antibiotics usually does not commence until the organism's cells begin to differentiate,[47] which also is true for the secondary metabolites of plant cells.[48] This is a complex and poorly understood subject for any living organism, yet in the case of bacteria there is a growing awareness that small molecules act as messengers in mediating the onset of production of secondary metabolites and other biological events,[49] something like the roles of pheromones and hormones in more complex living systems. Here again is an area concerning the regulation of the biosynthesis of natural products, but at the most fundamental level, *i.e.* the molecular biology of gene expression. While the precise mechanism by which these messengers function is still unknown, it is informative to review what is known for one system (antibiotic production) as an example of how such work is being pursued.

The best-studied case is A-factor (11), which is an autoregulator that has been discovered in the streptomycin-producing bacterium *Streptomyces griseus* and which, at picomolar concentrations, is capable of inducing the production of aerial mycelia, spores, and antibiotics in strains that lack these characteristics of cellular differentiation.[49,50] A-Factor also causes changes in the levels of several intracellular enzymes,[49] which may be part of the biochemistry of differentiation. Some structure-activity relationships have been reported for A-factor,[51,52] and it recently has been suggested[53] that only a single key enzyme is required for its production in four species of *Streptomyces* that normally do not produce it in detectable amounts (see Section 5). This result implies that the immediate precursors of A-factor are common metabolites in the genus *Streptomyces*.



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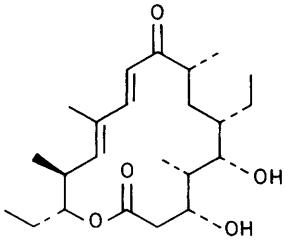

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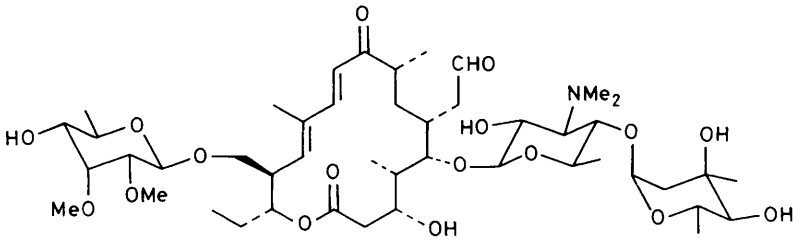

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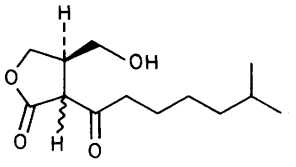

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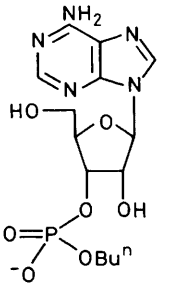

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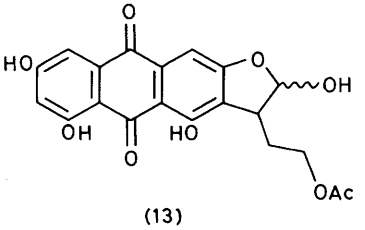

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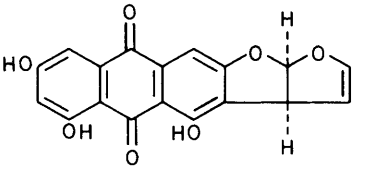

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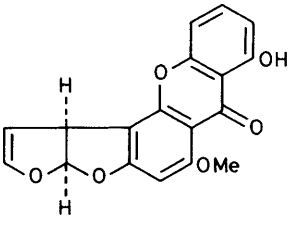

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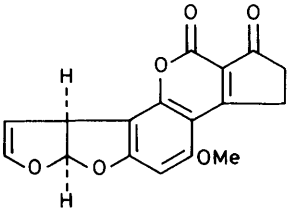

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B-Factor (12) is the most recent autoregulator to be discovered and appears to regulate the production of the ansamacrolide antibiotic rifamycin in a species of *Nocardia*.[54] C-Factor is a third autoregulator of the actinomycetes (but historically the first to be discovered[49]). It is a protein, with a molecular weight of approximately 34.5 kDa, that stimulates cytodifferentiation of a mutant of *Streptomyces griseus*.[55]

The information in this section is typical of what can be learned – and what cannot – about the biosynthesis of natural products by working with intact cells. The limitations of this approach accrue from the difficulty in unravelling the complex biochemical and genetic interactions within cells of living organisms. As in most other situations like this, one assumes that subdivision of the system through dissection of the cellular biochemistry and molecular biology into their individual components is the only way to a deep understanding of the phenomena that are observed. This, consequently, is the reason for including the next four sections in this review.

3 Enzymology

Although it is safe to state that in no case have all of the enzymes that are required for the biosynthesis of any secondary metabolite been isolated and purified to homogeneity, much progress has been made towards this goal in some cases. Yet this requirement has to be met (or, at the least, methods have to be devised for the assay of each enzyme) if one expects to achieve complete understanding of a given biosynthetic pathway. In this section I review several examples of enzymes that have been isolated from bacteria and plants, to highlight what their study has contributed to the field of the biosynthesis of natural products and to reveal some of the technical problems that are associated with such work.

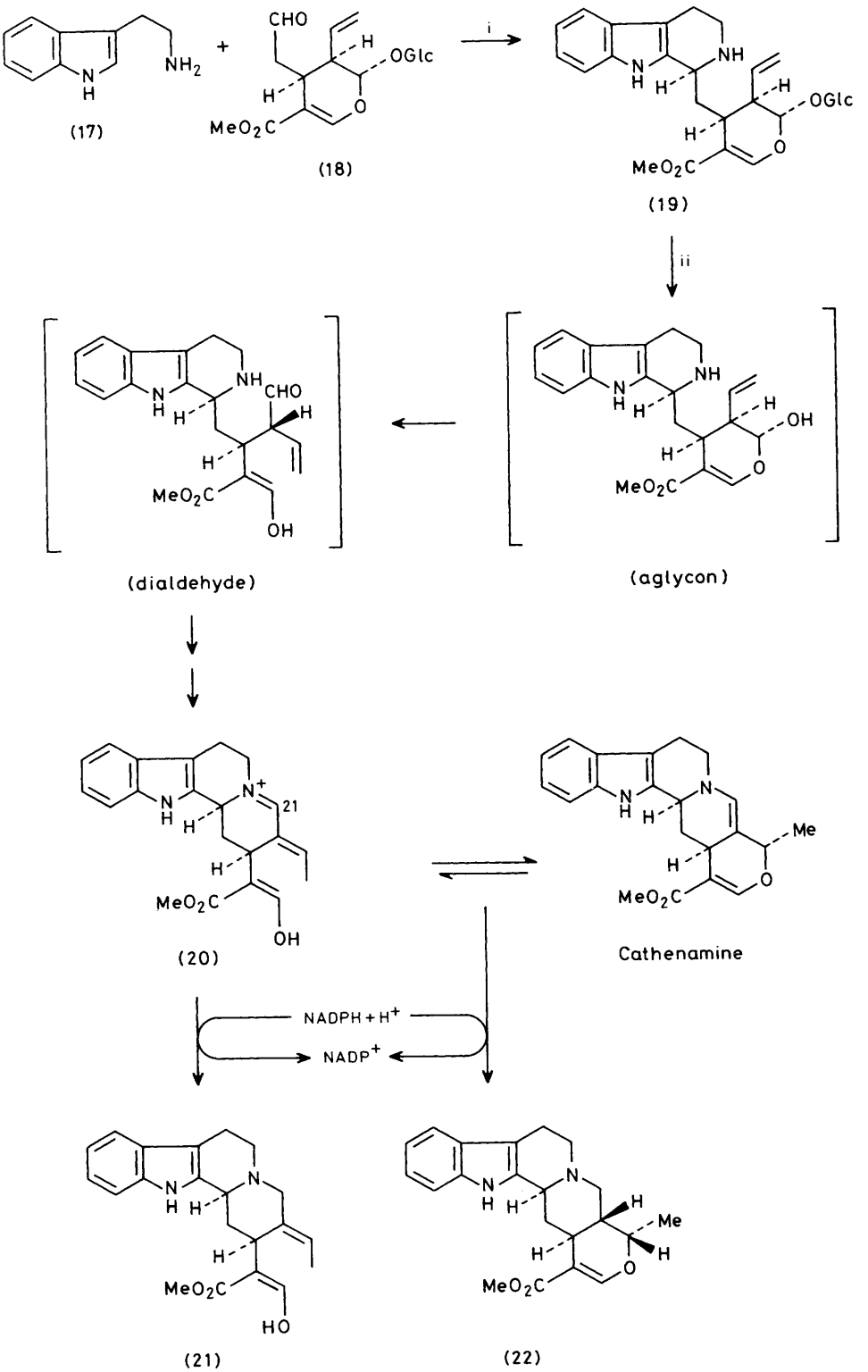
3.1 Aflatoxins

Hsieh and co-workers have described cell-free systems for the conversion of versiconal hemiacetal acetate (13) into versicolorin A (14)[56] and of sterigmatocystin (15) into aflatoxin B_1 (16),[57] which are intermediate steps of the biosynthesis of aflatoxins.[58] The conversion of (13) into (14) did not require oxygen (but the conversion rate was halved under anaerobic conditions) or $NADP^+$, and was inhibited by cysteine and dichlorvos, which these investigators had found earlier to inhibit the biosynthesis of aflatoxins *in vivo* and to cause the accumulation of (13). The activity in the cell-free preparation was soluble and did not require dithiothreitol (DTT), polyvinylpyrrolidone, or glycerol for stabilization (unlike many other cases of the enzymes of secondary metabolism). In this way the authors were able to see chromatographically that several intermediates were involved in the conversion of (13) into (14) *in vitro*.

3.2 Alkaloids

Knowledge about the enzymology of the biosynthesis of alkaloids has progressed rapidly during the past five years, and the use of plant cell cultures has been vital to the success of this work.[59] Examples of the enzymology of two well-studied systems, *i.e.* for the biosynthesis of monoterpenoid alkaloids and of benzylisoquinoline alkaloids, are reviewed here. A comprehensive review of the enzymology of alkaloid metabolism also is available.[60]

The formation of the monoterpenoid indole alkaloid ajmalicine (22) and its isomers from tryptamine (17) and secologanin (18) involves four key intermediates (Scheme 1).[61] Strictosidine synthase has been purified from *Catharanthus roseus* about 50-fold, to near homogeneity, and the values of K_m and V_{max} for secologanin and tryptamine have been established along with the molecular weight and the pH optimum.[62] The presence of 116 times more enzyme in the plant tissue culture than in the roots of the plant shows the advantage of the former. The partially purified enzyme was immobilized on CNBr-activated Sepharose, which markedly increased its thermal stability and permitted the synthesis of strictosidine (19) in

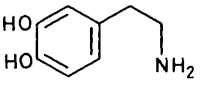


Enzymes: i, strictosidine synthase; ii, strictosidine β-glucosidases I and II

Scheme 1

gram quantities.[63] Two glucosidases were isolated from *C. roseus* which had strict substrate specificity for strictosidine but which differed in their values of K_m and V_{max}, their inhibition by substrate and by D-glucono-1,5-lactone, and their molecular weight (230 and >450 kDa).[64] Reduction of 4,21-didehydrogeissoschizine (20) gives geissoschizine (21), which had previously been reported to play a pivotal role as the precursor of ajmalicine (22) and other types of alkaloids of *C. roseus in vivo*. Yet the study of its metabolism *in vitro* revealed that it is a shunt product of the pathway leading to the other alkaloids[65,66] and enters the mainstream of events by the action of a dehydrogenase.[67] Thus the work with the alkaloid-metabolizing enzymes has clarified the understanding of this early stage of events in the complex biosynthetic pathway to the monoterpenoid alkaloids of *Catharanthus roseus*.

The benzylisoquinoline pathway provides commercially important alkaloids, *e.g.* berberine, codeine, thebaine, and morphine. It is significant, therefore, that key enzymes of this pathway have been isolated and partially purified from plant tissue cultures by Zenk and co-workers. The first enzyme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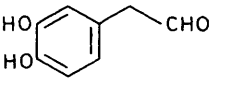
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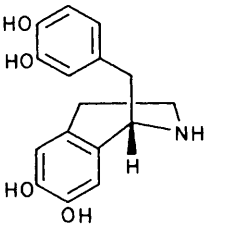
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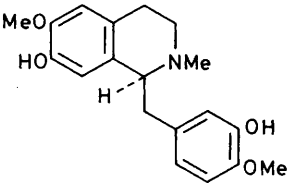
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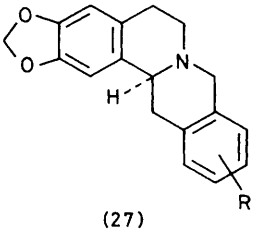
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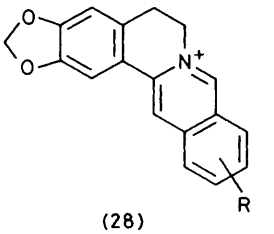
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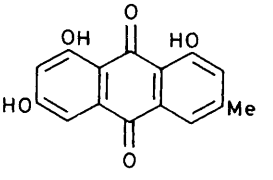
(R = OH or OMe)

of the pathway, (*S*)-norlaudanosoline synthase, which catalyses the conversion of dopamine (23) and of (3,4-dihydroxyphenyl)acetaldehyde (24) into 1*H*-(*S*)-norlaudanosoline (25), has been purified about 40-fold from cell suspension cultures of *Eschscholzia tenuifolia*.[68] Its molecular weight, pH optimum, and values of K_m and V_{max} for the two substrates were established, but the most interesting finding was that there are four isoenzymes, differentiable by polyacrylamide gel electrophoresis (PAGE) and their isoelectric points, that catalyse the same reaction. While the reason for the presence of multiple forms of the enzyme remains unknown, the results of this work invalidated an earlier claim that (*S*)-norlaudanosoline was formed by the condensation of dopamine and (3,4-dihydroxyphenyl)pyruvate followed by decarboxylation and reduction. Subsequent work by this group uncovered two kinds of methyltransferase (one of which acts on both enantiomers of norlaudanosoline) that are involved in the formation of reticuline (26), which is the central and branch-point intermediate of metabolism of benzylisoquinolines in plants,[69] and the enzymes that catalyse the formation of (*S*)-tetrahydroprotoberberines (27)[70] and protoberberines (28).[71] Several properties of each enzyme were described and, for the latter two, it was reported that they were contained exclusively in an organelle that could be isolated from the cells by sucrose density-gradient centrifugation and that differed in density from mitochondria, protoplastids, and other known organelles of plant cells.

3.3 Anthraquinones

Metabolites that are derived from anthraquinones (which are made by the polyketide pathway) are widely distributed in fungi and plants. One of these metabolites is geodin (31), which is made from emodin (29). Sankawa and co-workers have purified two enzymes from *Aspergillus terreus* that act on intermediates of the biosynthesis of geodin. Emodin 1-*O*-methyltransferase was purified 89-fold in a two-step procedure, using chromatography on blue Sepharose and on Sepharose 6B.[72] The use of the former separation method was predicated on the knowledge that *S*-adenosyl-L-methionine (SAM) is a co-substrate for this enzyme; thus the protein should have a site that has an affinity for the nucleotide-like ligand that is attached to the blue Sepharose. This technique, by its speed and selectivity, circumvented the extreme instability of the enzyme in crude preparations. Besides the molecular weight, Michaelis constants, and pH optimum, the most significant property that was reported for this enzyme was its strict specificity for the natural substrate when compared with seven other compounds that are closely related to emodin. Dihydrogeodin oxidase [a blue copper protein which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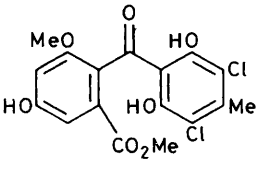
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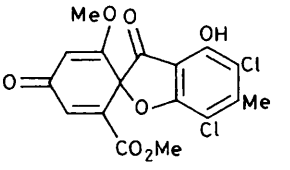
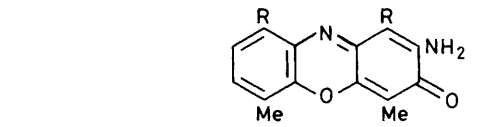

(31)

oxidatively couples dihydrogeodin (30) to form (+)-(31)] was purified to homogeneity by a nine-step procedure, including the use of chromatofocusing, and was found to contain sixteen copper atoms per molecule and to consume 0.5 mole of oxygen per mole of (+)-(31) that is produced.[73] This is the first example of the complete purification of an enzyme that catalyses the oxidative coupling of a phenolic natural product.

3.4 Antibiotics

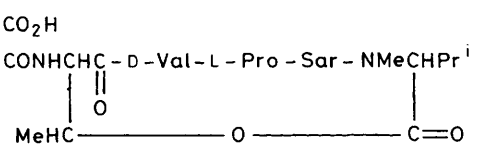
Since many secondary metabolites have antibiotic activity, there are a large number of examples that I could discuss in this section. To restrict my coverage within reasonable limits I have selected five examples of bacterial and fungal antibiotics, the majority of which represent cases where an enzyme has been purified to homogeneity.

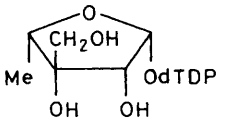
Actinomycin D (33) is a well-known microbial secondary metabolite and the first antitumour antibiotic to be isolated from a micro-organism. It is biosynthesized from five amino acids and 3-hydroxy-4-methylanthranilic acid, which is produced by the degradation of tryptophan to 3-hydroxyanthranilic acid followed by methylation at C-4. The aromatic acid can be dimerized oxidatively to form actinosin (32)[74] or it can first be coupled to a pentapeptide, giving actinomycinic acid, which then is dimerized to (33).[75] Phenoxazinone synthase, which catalyses the formation of (32), has been purified from *Streptomyces antibioticus* to homogeneity by Choy and Jones.[76] Affinity chromatography with 3-hydroxyanthranilic acid that was bound to Sepharose 4B was



(32) R = CO_2H

(33) R = CONHCHC – D-Val – L – Pro – Sar – NMeCHPr^i





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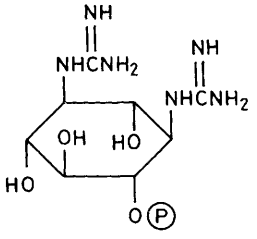
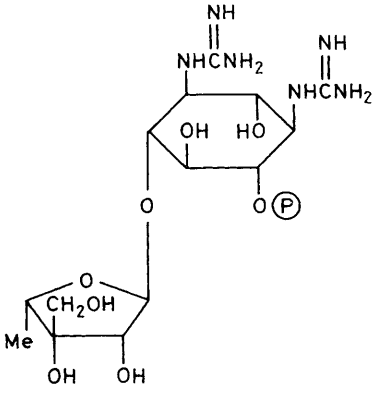


(35)

(dTDP = thymidine – 5′ – diphospho)



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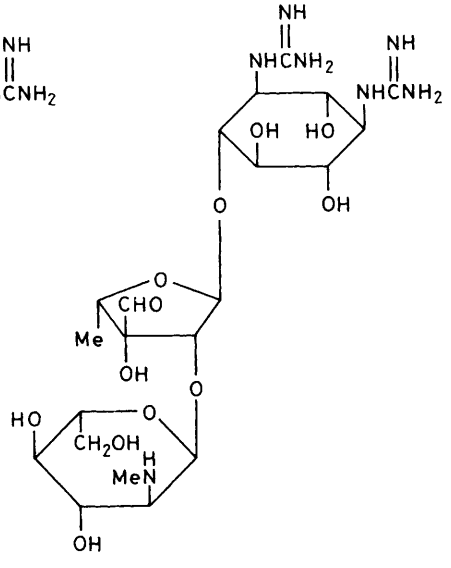


(37)

an important purification step at an early stage in their isolation procedure. Two forms of this enzyme were found: a large form, with a molecular weight of 799—919 kDa, and a small form, of 237 kDa. Denaturing polyacrylamide gel electrophoresis showed that these forms were composed of a subunit of 97 kDa. It was not established that the same subunit was present in both forms of the enzyme (but see refs. 159 and 160 for more recent information), but antibody that had been raised to the combined forms, or just to the large form, cross-reacted equally with both forms, suggesting that the two forms have some common antigenicity. Both forms catalyse the formation of (32), in a reaction that involves the consumption of 1.5 mole of oxygen per mole of (32) that is produced. The authors also noted a decline in the amount of the small form, with an increase in the amount of the large form, as the cultures aged.

Streptomycin (37) is an aminocyclitol (aminoglycoside) antibiotic and was one of the first antibiotics to be isolated from a streptomycete during the pioneering investigations of Selman Waksmann. It also is prominent as an antibiotic for which many of the 28 to 30 enzymes that comprise its biosynthetic pathway can be assayed in cell-free preparations or as partially purified proteins.[77,78] During the past six years, Grisebach and co-workers have described enzymes that catalyse the second and third stages of the biosynthesis of streptomycin. These workers purified dTDP-L-dihydrostreptose synthase 50-fold from *Streptomyces griseus* by using buffers that contained diphenylcarbamyl chloride and phenylmethanesulphonyl fluoride (PMSF), washing mycelial cells with 1M-KCl, and precipitating crude cell extracts with polyethyleneimine (for the inhibition and removal of protease activity).[79] (The presence of proteases is a common problem with cellular extracts from species of *Streptomyces*.) They also circumvented the lack of convenient sources of dTDP-4-keto-6-deoxy-D-glucose and dTDP-4-keto-L-rhamnose (which are substrates for dTDP-L-dihydrostreptose synthase) by using a protein-free extract of *E. coli* Y-10; this mutant is blocked in rhamnose biosynthesis but has the enzymes that are needed for the formation of these two compounds. The product of the synthase *i.e.* dTDP-L-dihydrostreptose (34), is transferred onto streptidine 6-phosphate (35) to give *O*-α-L-dihydrostreptosyl-(1→4)-streptidine 6-phosphate (36) by an enzyme from *S. griseus* that these workers purified to near homogeneity by procedures including affinity chromatography on a streptidine

6-phosphate/Sepharose column.[80] This enzyme has a molecular weight of 63 kDa (composed of two apparently identical subunits, of M_r 35 kDa), requires Mn^{2+} or Mg^{2+} for activity, and is stabilized by the presence of streptidine during its purification. Finally, a cell-free system for the conversion of (36) into dihydrostreptomycin 6-phosphate, which is the penultimate intracellular precursor of (37), has been described by the same group.[81]

The biosynthesis of antibiotics that contain a β-lactam ring as part of their structure has attracted much interest, due to the medical and commercial importance of these compounds. Thus it is not surprising that the knowledge about the enzymology of the biosynthesis of β-lactam antibiotics is well advanced. So much has been accomplished in this area that I can mention only a few salient aspects; readers may consult several recent reviews for further information.[82–84]

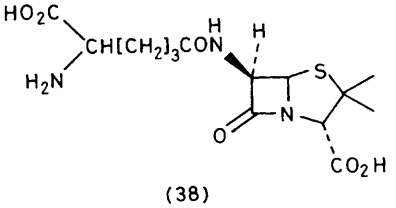
After it was shown that the tripeptide L,L,D-α-aminoadipylcysteinylvaline was the immediate precursor of isopenicillin N (38) in cell-free systems,[85,86] the enzyme that catalyses the cyclization, *i.e.* isopenicillin-N synthetase, was purified to homogeneity from *Cephalosporium acremonium, Penicillium chrysogenum,* and *Streptomyces clavuligerus* by four research groups.[87–90] One group, who used rapid high-performance liquid chromatography as an essential part of their isolation scheme, reported the presence of three forms of this enzyme, distinguishable by h.p.l.c. elution time but not by molecular weight, which they believed were either products of limited proteolysis of the principal protein (M_r 41 kDa) or true isoenzymes.[87] The other research groups, who used more conventional isolation methods, did not report finding multiple forms of this enzyme. An interesting property of this enzyme is its ability to catalyse the formation of a wide variety of β-lactam structures,[91,92] which may provide a practical biochemical route to useful analogues of the pencillins and cephalosporins.[91,93] [It is tempting to imagine that this lack of specificity will be found to be true for other enzymes of secondary metabolism, yet it is more likely that it is a special property of the enzymatic mechanism for the conversion of the tripeptide into (38).] Note also that the tripeptide had been promulgated as the precursor of (38) for more than twenty years; yet this could not be proven until the cell-free system, thence purified isopenicillin-N synthetase, became available.

Isopenicillin N is epimerized at the α-position of its aminoadipyl side-chain, then ring-expanded to deacetoxycephalosporin C (39), from which cephalosporin C (40) results by hydroxylation and acetylation. The enzyme activities for ring-expansion and hydroxylation could not be separated in a nearly pure protein fraction from *C. acremonium*; thus it was concluded that these two reactions are catalysed by a single, bifunctional protein with a molecular weight of 31 kDa.[94] In *S. clavuligerus*, however, these two activities were easily separable into two proteins, with M_r 29 kDa and 26.2 kDa, respectively.[95] This is the first example of a major difference between the shared portions of a biosynthetic pathway that exists in prokaryotes and eukaryotes.

The macrolide antibiotics, typified by erythromycin A (3) and tylosin (10), are biosynthesized by pathways that involve the formation of a macrolactone and deoxy- or deoxyaminosugars, which then merge to create the characteristic macrolide structure. Possibly because of the number of enzymes that is required (although the biosynthesis of streptomycin has an equivalent complexity) or the requirement for large catalytic proteins, with their inherent instability (but this is also true of the oligopeptide antibiotics), the understanding of the biosynthesis of macrolides is not as well advanced as in the cases discussed above. Therefore, I will describe only two examples of purified enzymes and direct the reader to recent reviews for additional information.[96–100]

In the biosynthesis of erythromycin A, the formation of the macrolactone 6-deoxyerythronolide B (5) is succeeded by its hydroxylation at C-6 to form erythronolide B (6). A cell-free system for this oxidation has been described by Corcoran and Vygantas.[35,36] Their preliminary evidence shows that a protein of M_r 46 kDa, containing a cytochrome *P*-448 prosthetic group, and at least two other proteins for electron transport are required *in vitro* to couple the oxidation of substrate to the consumption of NADPH. We have examined this system further and, by purifying the cytochrome-*P*-448-containing protein to homogeneity, have found that an industrial strain of *Streptomyces erythraeus* that had been selected for its greater production of erythromycin A contains about five-fold more of this enzyme than a wild-type strain (A. Shafiee and C. R. Hutchinson, unpublished results). Of more interest, however, is the presence in the same strain of two or more forms of this protein, which could be separated by chromatography on hydroxyapatite {based on their ability to catalyse the hydroxylation of (9*S*)-[9-^{3}H]-9-dihydro-6-deoxyerythronolide B}. We have not determined yet if these forms are true isoenzymes. Since the titre of this enzyme reaches its maximum coincident with the period of biosynthesis of antibiotic by *S. erythraeus*,[36] and the production of erythronolide B can be increased markedly by adding propanol to the fermentation medium of a wild-type strain,[101] it will be important to determine if the synthesis of 6-deoxyerythonolide B hydroxylase is inducible *in vivo* by 6-deoxyerythronolide B.

It has been noted that in an industrial strain of *Streptomyces fradiae* that produces about three-fold more tylosin than the wild-type strain, the level of macrocin (41), which is the



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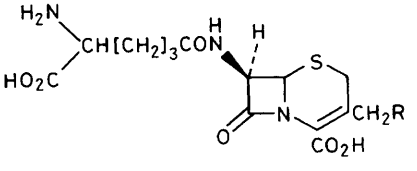
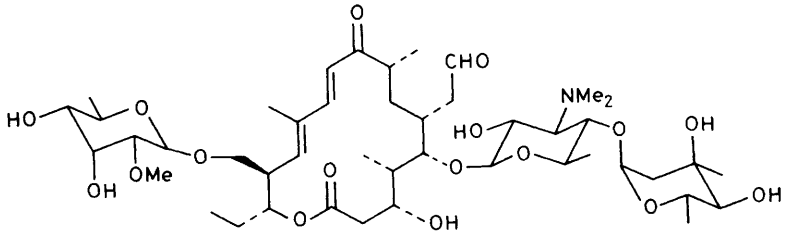


(39) R = H

(40) R = OAc



(41)

intermediate that is methylated *in vivo* to give tylosin (10), was much higher than in the wild-type strain even though the titre of the macrocin *O*-methyltransferase was elevated at least two-fold.[102] This may be due to the ability of (41) and some other intermediates of the biosynthesis of tylosin to inhibit this enzyme *in vitro*, but the authors also suggest that the level of macrocin *O*-methyltransferase is rate-limiting for the production of tylosin. Thus it was considered to be important to purify this enzyme, for a detailed study of its properties. This has been accomplished recently, to reveal the following things. It is an acidic protein (pI of 4.5), composed of two subunits (M_r 33 kDa and 65 kDa), and it requires a metal ion for maximal activity. The Michaelis constants of 64 and 24 μmol dm^{-3} for *S*-adenosyl-L-methionine and macrocin, respectively, and other kinetic data from experiments with these substrates and the inhibitor 2‴-*O*-demethylmacrocin are consistent with an Ordered Bi Bi mechanism for the enzymatic reaction, with SAM as the first substrate to be bound and *S*-adenosyl-L-homocysteine as the second product that is released.[103]

The peptide antibiotics represent a diverse group of microbial secondary metabolites whose biosynthesis takes place non-ribosomally. The enzymology of the formation of these antibiotics has been studied for more than fifteen years and is particularly fascinating for what it has revealed about the properties of multicatalytic-site proteins.[104] Since this topic has been thoroughly reviewed recently,[105,106] I will mention the highlights of gramicidin-S synthetase and tyrocidin synthetase[107] only as an example of what is generally true for all of the peptide antibiotics.

Gramicidin S (42) is assembled by gramicidin-S synthetase, which is composed of two proteins; these are the light enzyme (100 kDa) and the heavy enzyme (280 kDa). The light enzyme accepts L-phenylalanine, activates it by the transient formation of its adenylate *via* ATP (perhaps simultaneous with its epimerization to D-phenylalanine), and then uses D-phenylalanine to initiate the synthesis of a pentapeptide. The heavy enzyme accepts and activates the L isomers of proline, valine, ornithine, and leucine, and attaches them onto the amino-group of D-phenylalanine *in sequo*. Gramicidin S is then formed by dimerization of this pentapeptide through attachment of the amino-group of L-leucine to the carboxyl carbon of D-phenylalanine. The heavy enzyme contains one mole of pantetheine, which is used successively to transfer the growing peptide (bound as a thioester, *via* the cysteamine portion of pantetheine) to each amino acid that also is bound by a different (peripheral) thioester to the enzyme surface. The evidence that all of these transformations take place with enzyme-bound intermediates by means of multicatalytic-site proteins is very convincing. Furthermore, work with tyrocidin synthetase, which is composed of three large proteins (of M_r 100, 230, and 440 kDa), has shown that the two larger proteins can be resolved into subunits (of M_r *ca* 70 kDa) that are believed to correspond to the regions (domains) that hold the individual, thioester-bound amino acids. From these and other data, the concept has evolved that the amino-acid sequence of peptide antibiotics is dictated by the spatial order of the amino-acid-binding sites on the enzymes, with the pantetheine residue serving as the tether by which each amino acid can be picked up for addition to the growing oligopeptide. This idea is modelled on the biosynthesis of fatty acids, but differs in its provision for the addition of different amino acids in each chain-growing step rather than of the same (two-carbon) unit, as occurs in the formation of fatty acids. It also imagines that the size of the oligopeptide is limited by the physical properties of the enzyme that catalyses the assembly; *i.e.*, that at some point the oligopeptide becomes too large for sufficient stability or fidelity, and this limits the length of the peptide which can be assembled by one multicatalytic-site protein. Perhaps this is the reason that the alternative process of protein assembly *via* mRNA and ribosomes evolved.

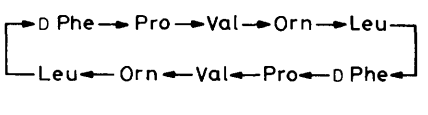
3.5 Flavonoids

The flavonoids are widely distributed in plants and serve useful functions as insect attractants and natural defensive agents.[108] Because of this and their role in plant pigmentation (as anthocyanins), the study of flavonoid metabolism has attracted much interest. Using cultured cells of parsley, most of the enzymes that catalyse the formation of flavonoid glycosides have been isolated and are available for study,[109] but I will discuss only one of them, naringenin-chalcone synthase, since there is information about this enzyme at the protein, mRNA, and DNA levels.

When dark-grown cell suspension cultures of parsley (*Petroselinum hortense*) are irradiated with ultraviolet light, the three enzymes of general phenylpropanoid metabolism (Group I) are rapidly induced, together with the enzymes (approximately thirteen) of the flavonoid glycoside pathway (Group II). Two of the enzymes of Group I are found in all plants and the third, 4-coumarate–CoA ligase, plays a central role in directing intermediates into the various pathways of flavonoid metabolism, of which the enzymes of Group II are one example. Naringenin-chalcone synthase, which catalyses the formation of naringenin chalcone (44) from 4-coumaroyl-CoA (43) and three molecules of malonyl-CoA, is the key enzyme in flavonoid biosynthesis and has been purified to homogeneity from parsley cells.[110] Since the ultraviolet-irradiated cells make large quantities of this enzyme,[111] its purification required only standard procedures except for the need to remove phenolic materials by treating the crude cell homogenate with an anion-exchange resin and polyethylenimine before subsequent purification steps. The most notable properties of this enzyme are its small size (two identical subunits, of M_r 42 kDa), the lack of pantetheine in its primary structure, and its ability to catalyse the formation of products that contain one or two two-carbon units less than naringenin chalcone (44)[112] or of analogues of (44)[113] under certain conditions *in vitro*. This and other information[109] has resulted in the proposal that naringenin-chalcone synthase and the enzyme that is important for the synthesis of fatty acids in higher plants, *i.e.* 3-oxoacyl-[acyl-carrier-protein] synthase, have a functional relationship and a common origin, perhaps by evolution of the former from the latter through gene duplication.[113]

3.6 Terpenes

Progress in studying the enzymology of the biosynthesis of terpenes has been slow, largely due to difficulties in isolating sufficient amounts of enzymes and to the need for considerable chemical work to prepare substrates and to identify products. Although none of the enzymes that is involved in the formation of terpenoid secondary metabolites has been purified to homogeneity on a large enough scale for detailed study, there is a growing understanding of the isolation techniques which can be used successfully and of the mechanisms of the enzymatic reactions.[114]



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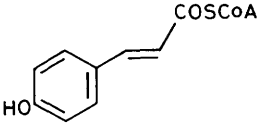

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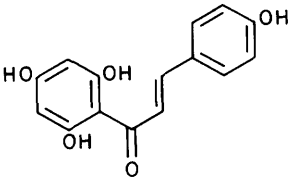

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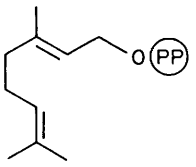

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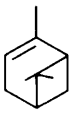


(46)

One of the best examples in this area is a pair of soluble enzymes from the sage *Salvia officinalis* which each catalyse the conversion of geranyl diphosphate (45) into either (+)- or (−)-α-pinene (46) and stereochemically related cyclic monoterpenes.[115] Gambliel and Croteau reported low recoveries of activity from enzymes that had been partially purified by a four-step fractionation scheme that included the use of chromatofocusing, yet they were able to show that the cyclization activities for the two enantiomerically related sets of products co-purified with the geranyl pyrophosphate : pinene cyclases I and II. This is the most conclusive evidence to date that multiple products can be synthesized by a single monoterpene cyclase, and points to the need for further study to learn how this occurs through the balance of chemical and enzymatic factors.

4 Genetic Methods

Since the formation of natural products is governed ultimately by the genes that encode the information for the production of appropriate enzymes and its regulation, it is desirable to explore the genetic basis of the biosynthesis of natural products. In the next three Sections I discuss some genetic methods and their use in investigations of natural products, primarily those made by bacteria. The intention is to show applications of these techniques rather than to review the concepts and methodology of bacterial or plant genetics and recombinant DNA work; therefore, I have limited my explanation of genetics and molecular biology to only what is required for a rudimentary understanding of these matters in the context of the examples that are presented. Readers should consult the excellent monograph by Old and Primrose[116] for a more detailed discussion of the concepts underlying genetic engineering in addition to any up-to-date, introductory textbook of genetics, *e.g.* those by Lewin[117] and by Watson, Tooze, and Kurtz.[118]

4.1 Mutation

Besides being the experimental cornerstone of bacterial genetics, the mutation of micro-organisms has been a mainstay of their industrial use, principally to create high-yielding strains, to direct the metabolic output towards one particular product, or to create other desirable properties, such as heat tolerance, assimilation of a special nutrient, or resistance to environmental toxins and to bacteriophages. For biosynthetic studies, the main goal of creating mutations has been to produce strains that have metabolic blocks or high titres of pathway enzymes. This facilitates the identification of biosynthetic intermediates, if these accumulate in the blocked mutant and are not further metabolized to shunt products, and the isolation of enzymes of the biosynthetic pathway. Unfortunately, knowledge about the art of mutagenesis is not widely disseminated, since it often becomes proprietary information in the companies where it is most frequently practiced. Hopwood[119] and Baltz[120] have written useful reviews about methods of mutagenesis and there is a comprehensive account of the effects of mutagenizing antibiotic-producing bacteria.[121] Mutagenesis protocols also can be found in standard laboratory manuals.[122,123,138]

Since it is difficult to find literature that exemplifies all of the facets of mutagenesis, I offer the following comments as a distillation of our limited experience with the construction of blocked mutants of streptomycetes. Conditions for the growth and storage of the organism which do not result in significant loss of the ability to produce metabolites upon repeated culturing must be developed and a convenient biological or chemical assay for the production of metabolites that has the necessary sensitivity and selectivity must be devised. Growth on a solid medium and bioassay has been more practical in our hands than growth in a liquid medium and thin-layer chromatography of the metabolites. Since the blocked mutants often appear with a frequency between 0.1 and 1%, it is desirable to screen batches of 1000 colonies when evaluating the result of mutagenesis. A combination of different mutagenetic treatments should be used (but not at the same time!) rather than just one kind, based on experience[119] and the chemistry of mutagenesis.[124] Mutants are usually picked from the 1 to 0.1% group that survives treatment with the mutagen (this depends on the type of mutagen that is used[120]), to minimize the presence of strains that carry multiple mutations. After picking colonies that represent independent mutational events,[119] it is best to allow the mutants to pass through one haploid growth cycle [to ensure that all progeny are derived from one and the same genome (*i.e.* are homozygous)] and to transfer mutants serially a few times, to test the stability of the mutation. For organisms (*e.g.* the streptomycetes) that have a morphologically complex life cycle, it is very important to choose blocked mutants for further study which appear to be normal in all other respects; otherwise the metabolic blockade may be due to pleiotropic effects of the mutation rather than to the lack of an enzyme for a particular pathway. Once a set of blocked mutants has been prepared, they can be classified in several ways (accumulated metabolites, cosynthesis, *etc.*), as exemplified by the work of Seno and Baltz with mutants of the tylosin-producing bacterium *Streptomyces fradiae*[125] and of Weber *et al.* for mutants of *Streptomyces erythraeus*.[126]

Mutation, of course, is a function of what happens to the DNA of the organism. It therefore is useful to be cognizant of the chemistry[124] and biology[127] of the mutation process for an appreciation of why I have made some of the preceding generalizations. Changes in the sequence of nucleic acids of DNA ultimately cause the mutations, and these result from mistakes in DNA repair, which are natural (but rare) events but which may also be induced. As replication of DNA occurs with high fidelity, alterations of sequence that occur during replication are not the main reason for the observed mutations. Some mutations stem instead from the erroneous pre- and post-replication repair of the nucleic acids in the DNA of an organism that have been affected because it has been treated with a mutagen. The post-replication events that are known as the SOS functions in *Escherichia coli* (a metaphor to the universal signal for distress), which are induced in response to damage to DNA, are particularly error-prone. These biochemical systems can result in transversions (*i.e.* substitution of a pyrimidine for a purine) or transitions (*i.e.* interchange of one purine or pyrimidine with another of the same type) as point mutations, in the addition or deletion of one nucleic acid residue as frameshift mutations, or in the deletion of segments of the DNA. These alterations to DNA then block the production of an enzyme or the activity of its regulatory element by interfering with gene expression (nonsense, frameshift, and polar mutations) or result in the production of a malfunctional gene product (mis-sense mutations). The single-base-change mutations are repairable at observable frequencies, but the deletions are not, which is why mutants can exhibit different stabilities. Moreover, these effects vary, depending on how the mutagen affects the nucleic acids,[124] which is why different mutagens should be used in constructing a set of blocked mutants. Finally, some mutations, largely those that are due to rearrangements of the DNA, are easily induced and very unstable. These can be encountered if bacteria are treated with acridine or acriflavine dyes, undergo regeneration of protoplasts, are grown under stressful conditions, or are just serially cultured.[128] Such mutations often have pleiotropic

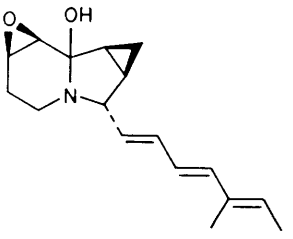
properties and normally are undesirable, yet in a recent instance,[129] treatment of a polyether-producing strain with ethidium bromide gave mutants that lacked aerial mycelia and which produced a low yield of the polyether antibiotic, but which also produced a second antibiotic, with a quite different structure.

4.2 Recombination

Genetic recombination is the breakage of DNA and the reunion of two or more segments of DNA into a different arrangement than was present originally. This can occur with cellular genomes and with the smaller independent replicons of plasmid and viral DNA. The mechanisms of recombination and mutagenesis differ in many respects,[117] with recombination being the event by which organisms exchange information at the DNA level. The artificial recombination of DNA *in vitro* is the keystone of the process of gene cloning and therefore the foundation of genetic engineering. Enzymes that cut DNA at specific sites (restriction endonucleases) and join the 3′- and 5′-ends of DNA (ligases) can be used to build molecules of DNA with endless structural variations. This enables the structural and functional analysis of genes, their juxtaposition in different arrangements, and thereby the construction of living organisms with properties that are quite different from those of their parents. In a sense, this is evolution speeded up many-fold; conversely, the process promises to elucidate how present living organisms evolved.

Genetic recombination is important to biosynthetic studies of natural products for two reasons. The first is its role in the development of strains, where the goal is to create new organisms which have acquired the best properties of their parental strains. These properties might be growth on a certain nutrient, better genetic stability, or tolerance to a certain pH, among the many desirable possibilities. Work towards such goals is currently proceeding empirically, through the use of natural or artificial mating processes,[130] but is likely to become a rational endeavour as more information about the molecular biology of cellular biochemistry becomes available. The second reason for the importance of genetic recombination is its use in the search for new natural products, which comes from the idea that random recombination of genomic DNA may result in the production of new secondary metabolites. Thus by artificially accelerating evolution, it is expected that valuable metabolites will be found at a higher frequency than by screening the current population of living organisms. This seems sensible intuitively, but there is not yet enough evidence for a valid judgement.

An example from the field of antibiotics illustrates the use of genetic recombination in the production of a new natural product. Protoplasts can be made to fuse by treatment with polyethylene glycol under hypertonic conditions,[4-7] and in the case of streptomycetes this results in more or less random genetic recombination at high frequency.[131,132] Fusion of protoplasts from non-antibiotic-producing mutants of two species of *Streptomyces*[133] resulted in production of the antibiotic indolizomycin (47),[134] which was structurally unrelated to any of the known secondary metabolites of the two parental strains. The data do not prove that recombination actually took place in this instance, nor can it be said that the production of (47) was caused by the recombination of genes



(47)

from the two species, as it could have resulted instead from the expression of normally silent genes (from either parent) in the fusant; I favour the latter explanation. Nonetheless, this case invites further study to learn what did happen and justifies continued pursuit of the approach. (See also the case of iremycin in Section 6.)

4.3 Gene-cloning Methods

The insight that led to the construction of chimeric DNA molecules (*i.e.*, artificially made molecules that have an uncertain function), which was enabled by the discovery and isolation of site-specific restriction endonucleases three years earlier, and the introduction of the chimeras into bacteria[135] gave birth to the technology of recombinant DNA and the gene-engineering industry. Gene cloning in any context uses the same strategy. A vector, *i.e.* a small DNA molecule (a plasmid or a viral genome) that is able to carry DNA into the host cell and which is capable of autonomous self-reproduction (replication), is chosen that has the following minimal properties. It must be easy to isolate, in microgram amounts, from the host organism; it must have at least one unique site for the insertion of foreign DNA (*i.e.*, DNA that was not originally part of the vector) that can be cut by a restriction endonuclease but which is in a region that does not interfere with the replication of the vector or with other functions that are essential for its existence; and it must carry a gene whose product signals the presence of the vector in the host cell (usually by making the cell resistant to some normally lethal substance). In the simplest case, the appropriate restriction enzyme is used to cut the vector and the foreign DNA (*e.g.*, pieces of a bacterial genome) into pieces of a suitable size for insertion into the vector. The vector and the foreign DNA inserts are joined (by treatment with a DNA ligase), thus creating a population of molecules that have a random structure with respect to the size and the sequence of the DNA in the case of shotgun gene cloning. These vector–foreign DNA constructs (chimeras) are introduced into the host cell by one of several means, the presence of the chimera in the cell being ascertained by selecting for expression of its gene that confers resistance to a lethal antibiotic, for example, and the population of host cells is then screened for a property that results from expression of a gene in the DNA that has been inserted. The process (and the result) of introducing the DNA into host cells is called transformation for circular (plasmid) or linear DNA, transfection for viral genomic DNA, and transduction for DNA that is carried within an intact virus. In the most general sense, a cell is said to be transformed whenever it has been genetically modified by the introduction of purified DNA. A clone, therefore, is the genetically identical progeny of a cell into which has been introduced a unique piece of DNA, containing a gene that imparts some specific property (the phenotype) to it. Any cell that contains this gene then has the corresponding genotype, but this may not be evident phenotypically under all situations.

Although gene cloning is not as simple in practice as is implied by the discussion above, it is a powerful way to isolate genes, and its success depends only on the availability of a suitable vector and in finding a way to detect the gene that is being sought amongst the many other clones that are of no interest to the investigator. Several strategies for gene cloning are reviewed in the monograph by Old and Primrose.[116] I shall mention only those used in the streptomycetes, since these are important for understanding the studies of gene cloning that are discussed in Section 5. Two reviews are available by Hopwood and his colleagues[136,137] about vectors and cloning strategies for streptomycetes. They have also recently produced an indispensible laboratory manual in which they describe this and related topics in the genetics of streptomycetes.[138]

There are now several different kinds of vectors that can be used to introduce DNA into species of *Streptomyces*, all of

which have been developed from indigenous plasmids or bacteriophages of this genus. pIJ61 is a typical plasmid vector and replicates at about 4–5 copies per bacterial genome (*i.e.* it is a vector that has a low copy number).[137] This vector contains genes that are capable of conferring resistance to thiostrepton and neomycin, which are antibiotics that inhibit the growth of most strains of *Streptomyces*, but it is currently known to have only a narrow host range. The gene for resistance to neomycin has a unique site (the cloning site) into which the insertion of foreign DNA results in its inactivation; hence the result is called insertional inactivation. Transformed host cells that contain the vector into which foreign DNA has been inserted can be identified as being resistant to thiostrepton but sensitive to neomycin, whereas the undesired ones are resistant to both antibiotics. This property is very useful since construction of the DNA chimeras usually results in less than 100% with inserts (20–80% is typical). This strategy illustrates the most important attribute of a good vector for use with streptomycetes: an ability to be screened easily for the presence of cloned DNA. Since the most interesting genes of streptomycetes encode properties that are not essential for bacterial growth (and therefore their presence cannot be selected directly), anything which reduces the laborious work of screening is a boon.

pIJ702, which is a plasmid that has a high copy number (40–300 copies per host cell) and which can be inserted into a wide range of hosts, represents another vector which facilitates gene cloning.[139] It carries the gene for resistance to thiostrepton (which is a valuable attribute because very few strains of *Streptomyces* are naturally resistant to thiostrepton) and the gene for tyrosinase in a small replicon (5.7 kb of DNA; 1 kb = 1000 nucleic acid bases). Tyrosinase is the enzyme from *Streptomyces antibioticus*[139] that catalyses the oxidation of dihydroxyphenylalanine to melanin, and its gene has three cloning sites which cause insertional inactivation. The result of this is readily apparent, for the thiostrepton-resistant transformants with DNA inserts are not black, in distinct contrast to those without inserted DNA. This result is easily visible, of course, only if the host strain does not normally produce melanin yet does produce it when it has been transformed with pIJ702.

KC301 and KC400 are vectors that have been made from the bacteriophage ϕC31, which attacks a wide range of hosts; they are used somewhat differently than the plasmids.[137] KC301 imparts resistance to thiostrepton to its host and can accommodate about 4.8 kb of inserted DNA. When it is introduced into a host by transfection or transduction, it integrates into the genome (chromosome) of the bacterial host by recombination between a specific 'attachment site' on the DNA of the phage and a corresponding site on the chromosome of the host (this is called lysogeny), resulting in only one copy of the vector per host cell. KC400 and its derivatives[140,141] can accept 1.8 to 9 kb of inserted DNA, but in some of these vectors this can occur only by loss of the genes for resistance to antibiotic or for the repressor (which codes for a protein that is involved in maintaining lysogeny). The most important property of KC400 and its derivatives is the inability to lysogenize a host directly, since they lack the attachment site that is required for recombination with the genome. Lysogeny results in expression of the gene for resistance to antibiotic; thus the only way in which these vectors can lysogenize a host and impart resistance to an antibiotic to it is by homologous recombination between their DNA insert and the genome of the host (or between the DNAs of two bacteriophages, if the host is a lysogen of ϕC31 or one of its derivatives). The Campbell mechanism of homologous recombination[142] results in duplication of the DNA insert and can cause inactivation of a gene if the insert is internal to the gene, *i.e.* if it does not include the signals that commence and terminate the expression of that gene. This property of the vectors that have been derived from KC400 has been exploited in the powerful technique of mutational cloning that, in principle, enables the isolation of any gene which has a detectable phenotype without first having to isolate a strain that contains a mutation in the gene, since mutants result directly from the gene disruption that accompanies lysogeny.[143] It also provides an expedient way to obtain DNA fragments for use as probes in screening gene libraries (*i.e.* collections of randomly constructed clones representing an entire genome) for the genes that are of interest, since the phage vector that contains the 'mutating' DNA fragment can be recovered easily from the mutated lysogen.

Forms of each of these types of plasmid and phage vectors are available that also contain sequences of nucleic acids that permit their replication both in species of *Streptomyces* and in *Escherichia coli*; such vectors are 'shuttle vectors'.[137,138] Representative examples, besides the vectors mentioned above, are ones that have been constructed by researchers at Eli Lilly and Company, such as pFJ105[144] and pHJL197.[145] It must be borne in mind that very few genes of *Streptomyces* are expressed in *E. coli*, and shuttle vectors therefore are seldom used for the functional analysis of genes of *Streptomyces* in *E. coli*. Their main value is to facilitate manipulations of recombinant DNA and mutagenesis (including transposon mutagenesis: *e.g.*, ref. 152), using the many techniques that are available for *E. coli*.[146,147]

Cloning strategies can take many forms, depending on the phenotypic properties of the gene that is to be cloned and the information that is available about its product.[116] With species of *Streptomyces*, the genes for production of antibiotics have been most often sought and, being non-essential (under laboratory conditions), usually have been found in one of two ways. The first approach requires, through a shotgun cloning experiment, the complementation of a mutation that has resulted in a specific metabolic block in the biosynthetic pathway. In this method a random collection of DNA inserts is used, ideally, to represent the entire genome of the wild-type strain, and its success depends on one of these inserts providing the genetic information that is malfunctioning or absent in the mutant (complementation). Even when the insert is an incomplete gene or provides only a partial transcript, the shotgun cloning method can work well enough because crossing-over between the DNA insert and resident DNA can restore the phenotype that is sought. It also works well even when the intermediates of the pathway are not freely diffusible and does not require prior knowledge of their structures unless the gene(s) for a particular metabolic function is desired. On the other hand, since the preparation of a suitable mutant collection is empirical and laborious, and all of the biosynthetic genes may not have a distinguishable phenotype, shotgun gene cloning is not without its drawbacks. Nonetheless, this approach has been used the most, as evident by the examples in Section 5.

The second approach depends on the fact that an organism must be resistant to the antibiotic that it makes if it is to continue to grow, and that genes for resistance to its own antibiotic are known to be contiguous with the genes for their biosynthesis in several, if not all, cases. Since resistance to an antibiotic is a selectable genetic property, it is comparatively easy first to clone the gene for resistance (because the cells of a normal sensitive host will not grow in the presence of the antibiotic unless they have been transformed with the gene for resistance to it), then to use it as a probe to isolate larger pieces of DNA that are contiguous with it, or simply to clone large pieces of DNA in a sensitive host by direct selection for resistance to the antibiotic. While this approach seems easier than the shotgun method, there are occasionally some practical problems, stemming from the lack of sufficient knowledge about antibiotic-resistance properties or a convenient selection agent and, less so, from the instability of vectors that contain large DNA inserts. Until it is tested extensively, I cannot predict if it will become the method of choice. Note, however, that it cannot supplant the use of blocked mutants, since they are still needed to identify and to study the specific genes of a pathway. Two other cloning strategies, based on the use of

messenger RNA (mRNA) to prepare cDNA clones (see Section 5 for an example) or the use of protein sequence data to make oligonucleotide DNA probes for locating specific genes in gene libraries,[116] are available in principle but have not yet been used in practice for genes of *Streptomyces*. This is due only to the comparative difficulty in identifying specific mRNAs of *Streptomyces* and in isolating samples of enzymes of the purity that is needed for protein microsequencing.

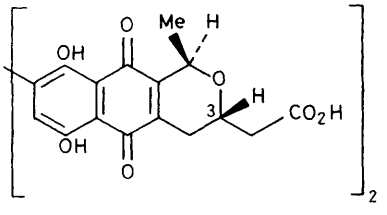
5 Gene Cloning

In this section I discuss examples of genes that govern the production of antibiotics by species of *Streptomyces* only, for these illustrate best how typical genes for the biosynthesis of natural products can be cloned and studied. One example of a plant gene is included, as a bellwether of the possibilities for cloning the genes for formation of secondary metabolites from plants. Previous reviews [137,148,149] on the subject of gene cloning in *Streptomyces* have been produced by Hopwood and co-workers.

Since resistance to antibiotics is directly selectable and *sine qua non* for the production of antibiotics, such genes were the first to be cloned from streptomycetes and have had major importance in the development of cloning vectors. The gene for resistance to methylenomycin A from *Streptomyces coelicolor* was the first to be cloned[150] and now there are many examples in the literature; for instance, the cloning and characterization of genes that encode resistance to erythromycin, neomycin, thiostrepton, and viomycin by Hopwood and his collaborators.[151,152] Further discussion of this subject is omitted because it is not directly important to the main topic of this section.

Streptomyces coelicolor, which is the organism that was used in the pioneering studies of the genetics of *Streptomyces* by Hopwood and his co-workers,[149] produces four different types of secondary metabolites which have measurable antibiotic activity, two of which are also pigments. Thus it is not surprising that this bacterium was the source of the first genes for natural products to be cloned.

All of the genes for the biosynthesis of actinorhodin (48), which is a pigment that has properties that make it suitable as an indicator of pH, have been cloned in a low-copy-number vector (pIJ922)[153] as one large (*ca.* 32 kb) segment of DNA.[154] This accomplishment is a landmark for two reasons: it showed that the genes for production of actinorhodin are clustered in one contiguous region of the bacterial genome, which had been demonstrated at low resolution from the results of earlier studies of the genetics of its production by *S. coelicolor*,[149] and it established that these genes could be expressed in another streptomycete following its transformation with the *act* DNA (gene descriptors usually are three-letter acronyms). Consequently, the observations imply that the expression of genes for the production of antibiotics is not species-specific (this has been verified in several other cases; see below) and that genes for the biosynthesis of polyketides, like those for actinorhodin, are present as gene clusters (it is too soon to tell if this is true for all secondary metabolites of *Streptomyces*), which is consistent with current thinking about the relationship between the enzymology of the biosynthesis of polyketides and fatty acids. The work that is reported in ref. 154 furthermore highlights some technical considerations which may be general for gene



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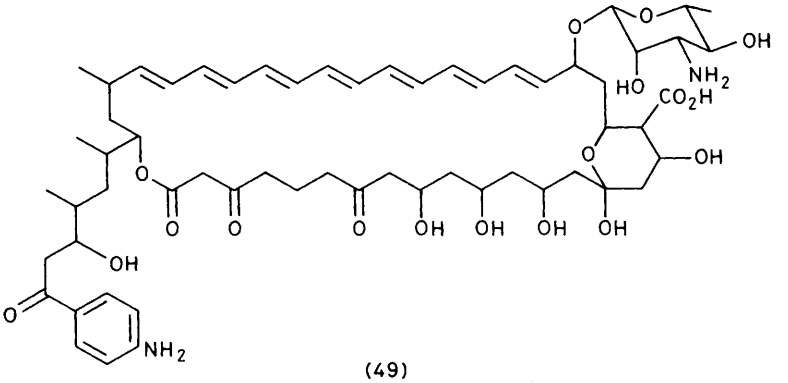
cloning in species of *Streptomyces*. Vectors such as pIJ922 (containing large DNA inserts which impart a final size of almost 60 kb) appear to be stable in the strains that have been transformed with them, whereas multicopy vectors such as pIJ702, which have large DNA inserts, may not exhibit high stability. Stability, in this context, means an infrequent loss of the insert DNA, such losses arising from recombination within the chimera or between the chimera and the genome of the host. Therefore, it may not be absolutely necessary to use recombination-deficient streptomycete hosts, which would minimize or avoid the loss of homologous DNA by analogy to the use of *recA* mutants of *E. coli*, when shotgun cloning DNA that had been obtained from the same species in which the blocked mutant was constructed.

The biosynthetic pathway to actinorhodin has not been fully elucidated, although it surely must resemble those for the formation of other isochromanequinone antibiotics.[155] Japanese workers have reported that they cloned a 4.3 kb segment of the DNA from *S. coelicolor* that encodes the production of a brown pigment whose chemical and spectral properties suggest that it is an intermediate or shunt-product of the pathway to actinorhodin.[156] Since the pigment was only produced if the DNA fragment was inserted into pIJ41 (which is the parent of pIJ61) downstream from a functional promoter sequence (*i.e.* a region at the 5′-end of a gene where a RNA polymerase binds, to initiate transcription, and which is thus vital for gene expression), the cloned segment did not apparently have its own promoter. Horinouchi and Beppu then used this fact to develop a plasmid which can be used to clone and study promoter sequences from *Streptomyces*.[156] This is an important development for the following reasons. Since the expression of many genes (and hence the level of their products) of prokaryotes is controlled transcriptionally, the 'promoter probe' plasmid will enable semi-quantitative assay of the function of the promoter by monitoring the production of pigment. In organisms such as species of *Streptomyces* this control may be exerted through RNA polymerases that recognize two types of promoters: vegetative ones, which function throughout the growth period, and developmental ones, which function only during the period when cell growth has slowed greatly and cell differentiation has begun, according to Westpheling *et al.*[157] The production of a pigment or antibiotic is a typical property of the latter kind of promoter; therefore the plasmid could be useful in studies of how such pathways are regulated.

Bibb and co-workers have constructed a different type of plasmid for use in the identification and semi-quantitative analysis of promoter sequences that occur in *Streptomyces*.[158] This vector contains a promoterless *cat* gene which encodes the production of a chloramphenicol-inactivating acetyltransferase. Only when a DNA segment that contains a promoter sequence is inserted on the 5′-end of this gene can it be expressed, and confer resistance to chloramphenicol to a host strain. This promoter-probe vector thus can be used to identify the promoter regions of cloned genes, but only if the promoter functions early enough in the life cycle of the host to prevent excessive background growth of the non-transformed, chloramphenicol-sensitive cells. It also can indicate the relative strength of promoters by the level of drug resistance that is imparted to the host strain. Both of these applications have been demonstrated recently by Bibb *et al.* in a study of several of the genes for resistance to antibiotics in *Streptomyces*.[159]

Genes that encode the production[160] of A-factor (11) and indirectly control its production[161] have been cloned from streptomycetes. In the former case, a 1.1 kb fragment of DNA was found later[53] to be sufficient for the production of A-factor in four different streptomycetes which do not produce (11) or contain DNA that is homologous with this fragment. The latter case is intriguing since strains of *S. lividans* and *S. coelicolor* which had lost the ability to produce actinorhodin, A-factor, and a red pigment regained all three properties when transformed with a *ca.* 2 kb segment of DNA that had been



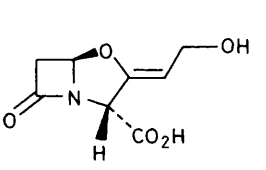
(49)

cloned from *S. coelicolor*.[161] This piece of DNA therefore must positively control the formation of all three substances in some way.

Gil and Hopwood[162] have cloned a gene that provides *p*-aminobenzoic acid (PABA) for the biosynthesis of folic acid by *S. griseus* and possibly also provides the *p*-aminoacetophenone portion of the polyene macrolide candicidin (49). They used both the insertional inactivation property of pIJ41 to locate clones that contained inserted DNA and the correspondence that exists between resistance to sulphonamides and the production of PABA, which is known to cause over-production of the latter, to facilitate identification of the *pab* clone. A 4.5 kb piece of cloned DNA complemented a *pab* mutation of, and imparted resistance to sulphonamides to, a strain of *S. lividans*. It also complemented *pabA* and *pabB* mutations of *E. coli*, but only after a portion of the *Streptomyces* DNA had been spontaneously deleted, so that expression of the *pab* gene could occur from a promoter sequence of *E. coli*.

DNA that is complementary to the naringenin-chalcone synthase mRNA of parsley has been cloned in *E. coli* by Hahlbrock and co-workers.[163] This was possible because ultraviolet irradiation of dark-grown parsley cells causes a rapid increase in the concentration of mRNA for naringenin-chalcone synthase; hence, the total mRNA from induced cells could be used for the identification of DNA that had been prepared from it *in vitro* and cloned in *E. coli*. This type of cloning did not give the natural plant gene which encodes naringenin-chalcone synthase, but a copy of it (cDNA) from which the introns (which are the non-translated sequences of eukaryotic DNA) were missing, due to the way in which such cloning is done. Total mRNA from the induced cells was used as a substrate for reverse transcriptase, which is the enzyme that can produce single-stranded DNA from an RNA template. The second, complementary strands of DNA were then synthesized (by treating the DNA copies with DNA polymerase I) and ligated into pBR322, this being the most popular vector for gene cloning in *E. coli*. Clones in the latter host which contained cDNA that was complementary to the mRNA from induced cells, but not from non-induced cells, first were identified, then screened for the production of a protein that reacts with naringenin-chalcone-synthase-specific antiserum after translation of their DNA *in vitro*. The resulting cDNA clone had a 1.5 kb insert that contained all of the coding sequence for naringenin-chalcone synthase.[164] The availability of this cDNA and others that were prepared similarly[165] permitted the discovery that the synthesis of secondary metabolites in parsley in response to stress conditions (irradiation with ultraviolet light or challenge by fungal elicitors), which has been implicated as a major defence response of higher plants, is due to activation of genes, resulting in transient increases in the rates of transcription of the associated genes.[163,166]

Two segments of DNA that complement a mutation in the biosynthesis of clavulanic acid (50) have been cloned from *Streptomyces clavuligerus* by the Beecham group, using pIJ702.[167] This represents the first report of cloned DNA from



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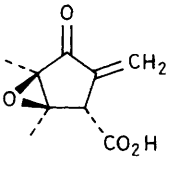

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the commercially important biosynthetic pathways to β-lactam antibiotics.

A group at the Eli Lilly company has established a second landmark in this field by their cloning of all of the genes for biosynthesis of erythromycin A (3).[168] This was accomplished in *Streptomyces lividans* by the use of a cosmid vector, which was made from plasmid and phage components and which could accept a DNA insert of *ca* 35 kb (cosmid vectors are described and defined in ref. 116), that could function in *Streptomyces* and *E. coli*. With such large inserts, the complete *Streptomyces* genome is represented by only about 1000 independent clones, which greatly reduces the screening effort. Colonies of *E. coli* that contained the cosmid with random DNA inserts from *S. erythraeus* [which is the species from which erythromycin A (3) was first isolated] were screened for the presence of the gene for resistance to (3),[152] to identify clones which also might contain the genes for biosynthesis of erythromycin A. (It had already been shown, by genetic mapping experiments, that the *ery* genes are clustered.[126]) It was very rewarding to discover consequently that one of the clones that carries the gene for resistance to erythromycin A also conferred the ability to produce erythromycin A when plasmid DNA that had been isolated from it was transferred into *S. lividans*. This resulted from the presence of an insert (of *ca*. 35 kb) of the DNA of *S. erythraeus* in the vector; this amount of DNA is surprisingly small when one realizes that about 40 catalytic events are needed theoretically for the biosynthesis of erythromycin A. The presence of the *ery* genes in this insert was confirmed by its ability, and by the ability of specific DNA fragments that could be sub-cloned from it, to complement three types of *ery* mutation when transformed into blocked mutants of *S. erythraeus* (P. Treadway, R. H. Baltz, and C. R. Hutchinson, unpublished results). This work exemplifies the value of cosmid vectors when using the gene for resistance as the essential tool for cloning the genes for the biosynthesis of antibiotics.

Mutational cloning is the keystone of the approach that has been used by Chater and Bruton to clone the genes that govern the formation of methylenomycin A (51), which is another antibiotic of *Streptomyces coelicolor*.[143] The methylenomycin A (*mmy*) genes are carried by a large plasmid, SCP1, rather than being part of the chromosome of *S. coelicolor*, and this fact was used to simplify the mutational cloning of *mmy* genes. DNA from an SCP1-containing strain of *Streptomyces parvullus* was inserted into KC400 *in vitro* and the constructs were transfected into *S. lividans* to give plaques (*i.e.* the partially cleared areas on an agar culture plate that are produced by lysis of bacterial

colonies within which the phage is growing) that contained KC400 with a random assortment of DNA inserts that were <6 kb long. When *S. lividans* that contained SCP1 was transduced to viomycin resistance with this population of phages, only those that contained insert DNA from SCP1 were able to lysogenize this host because the rest either had no DNA insert or only contained inserts of the DNA of *S. parvullus*, which, being essentially non-homologous with the DNA of *S. lividans*, could not recombine with the host at a significant frequency. Thus the majority, if not all, viomycin-resistant colonies contained DNA of SCP1, and 278 independent colonies were screened for the absence of the ability to produce methylenomycin A due to insertional inactivation. Nine such clones were found, and their genotype was designated *!mmy!*, to distinguish them from *mmy* mutations that had been produced in the customary way. These clones *in toto* represented about 7 kb of *mmy* DNA and three different types of *!mmy!* mutations, based on their cosynthetic properties.

The gene for phenoxazinone synthase (PHS) has been cloned from *Streptomyces antibioticus* by Jones and Hopwood[169] in an elegant study that also uncovered two other unrelated DNA fragments that have the surprising ability to cause the production of this enzyme by *S. lividans*.[170] The PHS structural gene was cloned in pIJ702 by assaying transformants of *S. lividans* for the production of PHS activity *in vitro*. Three different DNA fragments were isolated, but only the one of 2.4 kb produced PHS in a coupled transcription–translation system of a streptomycete *in vitro*,[171] and thereby was identified as the structural gene for PHS. The other two DNA fragments (1.8 and 4.3 kb) did not show this property (although they did encode the formation of other polypeptides), yet they conferred the ability to produce PHS on *S. lividans* if they were introduced into it by transformation. As the PHS that was produced from these latter two clones was indistinguishable from the purified PHS that has been described in Section 3 of this review, the authors concluded that these two fragments could activate an endogenous PHS gene of *S. lividans* which normally is silent or expressed at very low levels. Besides this unexpected outcome, it was observed (as anticipated) that cells of *S. lividans* that had been transformed with pIJ702 that contained the 2.4 kb fragment produced a higher level (about five-fold) of PHS than *S. antibioticus*, presumably due to an increase in gene dosage from the high-copy-number vector.

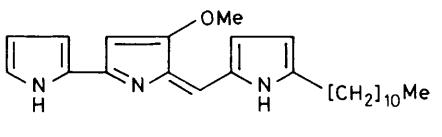
The first gene for biosynthesis of an antibiotic that was cloned[172] was one that directs the formation of an *O*-methyltransferase that is involved in the formation of the prodigiosin derivatives (52) and (53), which are the red pigments of *Streptomyces coelicolor*.[173] In this instance, the complementation, by DNA that was carried in pIJ702, of a double mutant that was defective in the formation of the two pigments of *S. coelicolor* was evident by the appearance of the characteristic red coloration that is due to (52) and (53) among essentially colourless colonies of the bacterium. (A similar advantage was enjoyed in the cloning of genes for actinorhodin, when blue colonies were sought.) More recent work on this system[174] has provided over 21 kb of the genomic DNA that complements *red* mutations of four classes and some information about the properties of the *O*-methyltransferase. This enzyme seems to exist in two forms, of M_r >5 MDa and 49 kDa, respectively, as deduced from studies of cell-free extracts that had been prepared from protoplasts and mycelia.

The final example of cloning of genes for the production of an antibiotic concerns tetracenomycin C (56), which is a pigmented anthracycline metabolite of *Streptomyces glaucescens*[175] that has significant antitumour activity.[176] By complementation of blocked mutants of *S. glaucescens*, a total of 24 kb of DNA has been cloned in pIJ702 that contains all of the genes for the formation of (56) (H. Motamedi, E. Wendt-Pienkowski, S. Yue, and C. R. Hutchinson, unpublished results). When *S. lividans* or two other streptomycetes were transformed by the plasmid from one of the clones that contained a 12.4 kb DNA insert, the red pigments (54) and (55) were produced in large quantity. The presence of pIJ702 with any of the cloned DNA in blocked mutants of *S. glaucescens* did not result in increased yields of (56), however, which means that the normal mechanism by which the production of (56) is regulated can overcome the effect of an elevated gene dosage.

6 Novel Antibiotics produced by Genetic Means: Hybrid Antibiotics and Others

In the previous sections of this review I have discussed examples of what can be learned about the biosynthesis of natural products by using biochemical and genetic methods; in this section I present an interesting use of this information which may have commercial importance. It concerns a desire to increase the pace of discovery of new natural products by the application of methods by which the genetic constitution of organisms can be altered faster than the rate of spontaneous mutation but which are more directed than the outcome of random mutagenesis; it also concerns the basis for the specificity of biochemical interactions, or the relative lack thereof in the case of pathways to secondary metabolites. Its output is hoped to be the creation of antibiotics that are structural hybrids or simple derivatives of known compounds and that have valuable biological activity. This is a commendable goal, yet will be difficult to reach until we truly are able to engineer organisms (or only proteins) to do whatever is desired. In the meantime, the construction of hybrid antibiotics is being pursued empirically, as illustrated by the three ways discussed below. Some of the early results look quite promising and certainly justify continuation of this approach.

The pathways of secondary metabolism are not as tightly controlled and their enzymes not as fastidious as those of primary metabolism; thus it is not surprising to find more than one route in the pathway to a given natural product, or more



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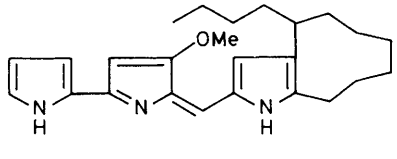

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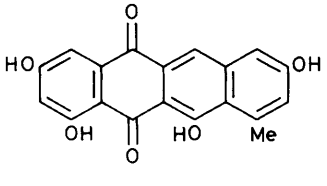

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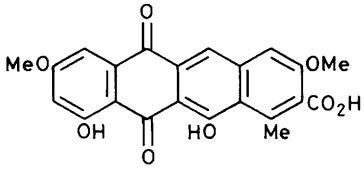

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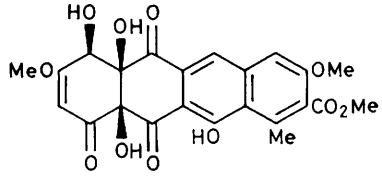


(56)

than one type of product associated with an enzyme. These observations suggest that new, 'unnatural' compounds might be formed if an organism is given analogues of the known intermediates of a pathway to natural products, which is the foundation of mutasynthesis. Because Daum and Lemke reviewed this subject in 1979,[177] I mention only one recent example.

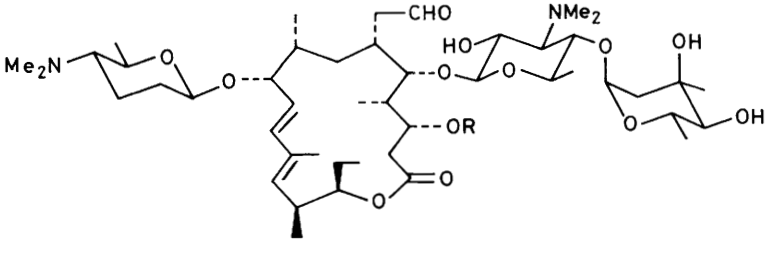
There are three ways through which mutasynthesis can be accomplished: by addition of analogues of the intermediates of a pathway to the normal producer, to a blocked mutant of it, or along with an inhibitor of some step of the normal pathway. The first approach is the least successful since the analogue must compete with the normal substrate of an enzyme that catalyses a reaction of a pathway and may do so poorly, thus resulting in a low yield of the desired analogue of the usual natural product. The second method requires that the enzymes that catalyse stages beyond the metabolic blockade in the pathway function normally to transform the substrate analogue into the corresponding product analogue. Since the normal product is not formed by the mutant, there is less competition for the substrate and therefore a better chance that the desired product will be formed. As the necessary blocked mutant may not be available, this approach is complemented by the third, where the biochemical inhibitor can produce a situation like a blocked mutant but without first having to make the mutant strain.

The third approach cannot replace the use of mutants since there are few specific inhibitors of biosynthetic pathways that are suitable for use with intact cells (see Section 2.2). Cerulenin (1), however, can be used for the mutasynthesis of antibiotics in this way if they are made by the polyketide pathway. Omura and co-workers have demonstrated its usefulness in their preparation of the chimeramycins (57), which are 'hybrids' of tylactone (9) and the deoxyamino-sugars that occur in spiramycin (58) and which have valuable antibiotic activity.[178] Other examples where cerulenin has been used for similar purposes are cited in their paper.

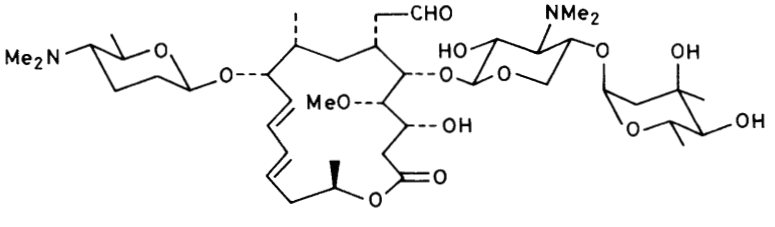
A successful mating of two antibiotic-producing strains involves, by definition, the recombination of their genomic DNAs. Since mating is operationally a simple process, this approach to creating novel antibiotics can be explored in a straightforward way, but there is an important limitation. There are barriers to exchange of information at the DNA level between different species of most of the organisms that produce antibiotics (arising from low structural homology of DNAs and from the presence in the members of each species of restriction enzymes that recognize non-self DNA) that can prevent interspecific recombination. Nonetheless, interspecific mating has been applied successfully in one case, and resulted in the production of a new antibiotic. Non-producing, genetically marked strains of *Streptomyces hygroscopicus* IMET JA6599 (which normally makes turimycin, a macrolide) and of *Streptomyces violaceus* IMET JA6844 (which normally makes violamycin, an anthracycline) were mated to give 90 recombinants, which were identified by their growth on selective media. Three of these recombinants produced a new antibiotic,[179] which was later identified as the anthracycline iremycin.[180] The production of iremycin was apparently caused by some effect of the mating process, but it cannot be said that this resulted from recombination of DNAs, nor is iremycin truly a hybrid antibiotic since none of its structural features are present in the turimycins.

Intraspecific mating of strains of *Nocardia mediterranei* resulted in the production of new ansamycin antibiotics that are related to the rifamycins.[181,182] This is much more probably a result of the recombination of DNAs than the case above, since the genome of strains that have been derived from the same species will be largely homologous, but again these compounds were not true hybrid structures.

The first example of the production of a truly hybrid antibiotic is the work of Hopwood and collaborators,[183] who capitalized on the close structural relationships between the three isochromanequinone antibiotics actinorhodin (48), medermycin (59), and granaticin (60) to create dihydrogranatirhodin (61) and mederrhodin A (62). Close inspection of structures (61) and (62) shows that C-3 of (61) is epimeric with C-3 of (60) but that the stereochemistry at C-3 of (61) is identical with that at C-3 of (48); (62) has an extra hydroxyl group at the position corresponding to C-8 of (59), where a hydroxyl group is also present in (48). These changes resulted from 'cooperation' between gene products in the pathways to actinorhodin and granaticin, or to actinorhodin and medermycin, when the strains that normally produce (59) or (60) were transformed with the *act* gene cluster or subportions thereof. Both (48) and (59) were produced when *Streptomyces* sp. AM-7161 carried all of the *act* genes, and (62) appeared only when a partial *act* clone was introduced into this host; in distinct contrast, (61) was produced to the almost total exclusion of the normal antibiotics when *S. violaceoruber* Tü22 carried the *act* gene cluster. Therefore, compounds (61) and (62) represent true hybrid antibiotics that have been created by genetic engineering.



(57) R = H or COMe



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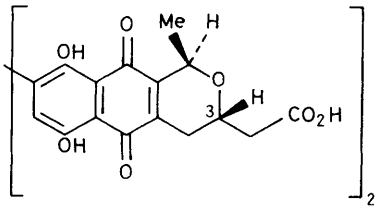

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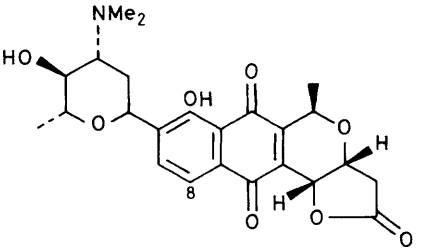

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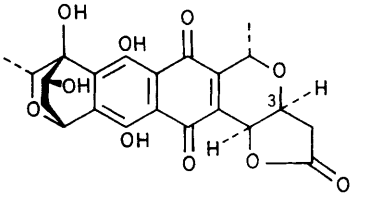

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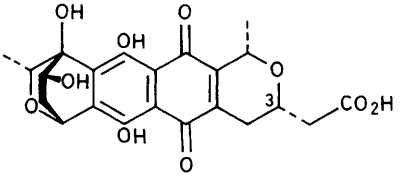

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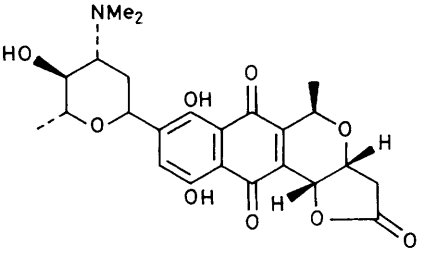

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7 Summary and Acknowledgments

In this review of some biological methods that are useful for studying the biosynthesis of natural products I have intertwined the discussion of methodology with examples of its use, taken from the recent literature. By illustrating fruitful applications of these methods I have strived to highlight their utility to researchers who might wish to make use of them. It is clear from my presentation that enzymological studies are bearing much fruit, and that suitable techniques of gene cloning are available and have great promise for rapidly accelerating the pace at which new knowledge will be acquired in this field.

The preparation of this review would not have been possible without the stimulation of research support from the National Institutes of Health (GM 25799, GM 31925, CA 35381) and fellowships from the John Simon Guggenheim Foundation and Fulbright Program of the Council for the International Exchange of Scholars. I am particularly grateful to David A. Hopwood, Keith F. Chater, and Mervin J. Bibb for their advice and encouragement during a sabbatical leave in the Department of Genetics, John Innes Institute, Norwich, England in 1983, and to David Hopwood for critical reading of the manuscript for this review.

8 Addendum

Two research groups have cloned genes from *Streptomyces griseus* that encode information that is needed for the formation of streptomycin (37).[184,185] In both laboratories the researchers used assays for the enzymes of the biosynthetic pathway to streptomycin to characterize the phenotypes of blocked mutants,[186,187] and thereby to identify the particular *str* genes which complemented the *str* mutations. As noted for several other cases cited in Section 6, the results of this work showed that the genes for the production of this antibiotic and for self-resistance to it are clustered in the genome of *Streptomyces griseus*.

The gene from the fungus *Cephalosporium acremonium* that encodes the formation of isopenicillin-N synthetase has been cloned by researchers at the Eli Lilly Company.[188] Their work represents the first use in the field of natural products of an oligonucleotide, whose nucleotide sequence corresponded to a portion of the *N*-terminal amino-acid sequence of the enzyme, to probe a gene bank, made in *Escherichia coli*, for DNA with a sequence that is homologous to the oligonucleotide probe. The clone that was identified in this way was shown to contain the desired gene, based on the observation that isopenicillin-N synthetase was formed when the correct DNA segment was isolated from the clone and was expressed in *E. coli* (after suitable genetic engineering to provide the nucleotide sequences that are necessary for expression of the gene in this bacterium).

Researchers at the Technical University in Berlin have cloned the genes for the production of the ornithine-activating fragment of gramicidin-S synthetase 2 from *Bacillus brevis*[189] and of tyrocidin synthetase 1 from the same bacterium.[190] They used antibodies to gramicidin-S synthetase (which could cross-react to the two proteins) to identify *Escherichia coli* clones that produced either protein by virtue of a special 'expression vector' which permitted the expression of the genes of *B. brevis* in *E. coli*. In this case and in the work on isopenicillin N, alternative strategies of gene cloning, *e.g.* complementation of mutations, were not feasible.

Finally, Uchiyama and Weisblum have made an interesting speculation regarding the propensity of streptomycetes to produce a diverse number of natural products in their recent paper on the nucleotide sequence of the gene for resistance to erythromycin, which was cloned from *Streptomyces erythraeus*.[191] They noted that the nucleotide sequences that signal the termination of gene expression have a much lower probability of occurrence in genes of *Streptomyces* than in those of other genera of bacteria. As one consequence, they predicted that it should be easier for the streptomycetes to manufacture new proteins by small changes in the nucleotide sequence of a gene, since there would be less chance that random mutation would be non-productive and would lead to sequence alterations which could not be expressed as functional proteins. Hence:[191] "the environment of the *Streptomyces* genome can support a 'molecular foundry' in which experimental genes and their respective proteins are cast with high efficiency." This would be just the attribute that was required for the diverse secondary metabolism of the streptomycetes to have evolved by natural processes.

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