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Volume II

T. A. V. Subramanian

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Cell Metabolism: Growth and Environment

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Editor

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PREFACE

The economic importance of several microbial metabolites, particularly antibiotics, has provided impetus to research efforts to elucidate the genesis of these compounds. The characterization and identification of biosynthetic routes has greatly facilitated the creation of newer and more efficient microbial products. The main difficulty is the tight degree of metabolic regulation which is frequently encountered in microorganisms, rendering them inefficient producers of metabolites. A certain degree of relaxation of the metabolic regulatory mechanisms is essential for economical production of an abundance of the desired microbial product. In other words, for the microbial cells to serve as efficient producers it is very important to circumvent or overcome the regulatory mechanisms involved. In the competitive world of commercial synthesis and production, simple molecules like glutamic acid and lysine are still made by fermentation rather than by chemical synthesis. About 300,000 tons of citric acid are produced annually from *Aspergillus niger* alone. In addition, microorganisms are extremely useful for carrying out bioconversion processes involved in the production of specific stereo compounds.

Increased biosynthesis of chlortetracyclin by developing mutants of *S. aureofaciens* resistant to the antibiotic has recently been reported. With the developments in biotechnology it is possible now to manufacture biologically important compounds like insulin on a large scale without depending on animal sources. Some of the hydroxylation reactions in steroid biosynthesis which require multistep procedures in chemical synthesis are greatly facilitated by microbial enzymes.

Historically, microbial products have been divided into primary and secondary metabolites. Primary products like proteins, nucleic acids, and carbohydrates are essential for growth and metabolism. Secondary products until recently were believed to have no significant role in the physiology of the producing organism and were considered as metabolic waste products.

As a first step it is necessary to find a wasteful strain which will overproduce and excrete a particular compound. The next stage is to modify the regulatory control of the strain to further increase its inefficiency. An example of such exploitation of microorganisms to waste their precious metabolites is seen in riboflavin production (up to 5000 mℓ/ℓ) by commercial cultures of *E. ashby*.

These two volumes deal with the regulation of metabolism in microbes and plants with respect to differentiation, proteins, nucleic acids, aromatic acids, alkaloids, and other secondary metabolites like aflatoxins and streptomycin.

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THE EDITOR

Dr. T. A. V. Subramanian holds a Ph.D. in Biochemistry from the University of Madras. He has been Head of the Department of Biochemistry, V. P. Chest Institute, University of Delhi, since 1956.

Dr. Subramanian's training and experience, which includes 2 years at Columbia University, New York and 2 years at University of Wisconsin, Madison, is working in areas of biochemistry connected with metabolism of *Aspergillus parasiticus*, metabolism of various strains of tubercle bacilli, and pulmonary surfactants. He has more than 250 publications in these areas. He has been actively engaged in research on various aspects of the metabolism of *Aspergillus parasiticus* for more than 20 years. He has been President of the Society of Biological Chemists (India).

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CELL METABOLISM: GROWTH AND ENVIRONMENT

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Chapter 1

BIOSYNTHESIS OF POLYKETIDES

Ahmed E. Yousef and Elmer H. Marth

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I. INTRODUCTION

Polyketides are a group of biogenically related natural compounds that are usually produced by fungi. Some of the early discoveries about polyketides date back to the turn of the century, but our knowledge about this class of compounds is still far from complete. Polyketides include such highly hazardous compounds as aflatoxins, which have shaped and will continue to shape the agricultural economy in the world for years to come.

Workers in this field have developed a continuously growing list of hazardous metabolites produced by molds, but excluded are organisms from the ever-diminishing list of so-called useful fungi. Hence, researchers working in this field share an important responsibility, and the need exists for intensified work that will lead to a better understanding of fungi and their secondary metabolism.

II. SPECTRUM OF POLYKETIDES

In the broadest sense, polyketides may be defined as those diverse natural products that are related by their common biosynthetic pathway, viz. the acetate-polymalonate route.[1,2] Acetate or other acyl units, such as propionate, benzoate, or cinnamate, can act as chain-initiating moieties, and beside malonate, though less frequently, propionate or butyrate can act as chain-elongating units.[2] This broad definition of polyketides suggests that a wide variety of products such as fatty acids, polyacetylenes, phenols, macrolides, flavonoids, and others are covered by the term. Because of the broad spectrum of compounds included in this definition, many workers in this field prefer to restrict the term to those compounds that are biosynthesized via the acetate-polymalonate pathway, but without obligate reduction of intermediate compounds.[3-5] This restriction sets fatty acids apart as a distinctive group of compounds. This chapter will use the restricted definition of polyketides, with occasional reference to biosynthesis of fatty acids when comparison is necessary. Even in this narrow sense of the term, polyketides are a very diverse group of compounds that vary in complexity from simple tetraketides, such as orsellinic acid, to compounds of elaborate structure, such as the decaketide aflatoxin B_1 (Figure 1).

III. OUTLINE OF BIOSYNTHESIS OF POLYKETIDES

Biosynthesis of polyketides[1,3,4] involves condensation of an enzyme-bound acyl (mostly acetyl) moiety as the chain-initiating unit with three or more of malonyl (chain-elongating unit). During the condensation, malonyl groups are decarboxylated, adding "C_2-units" to the enzyme-bound ketoacyl intermediate compound. Depending on the number of C_2-units that constitute the fully assembled β-polyketoacyl intermediate compound, polyketides are divided into tetraketides, pentaketides, hexaketides, etc. For the simplest tetraketide, orsellinic acid, the enzyme-bound intermediate compound cyclizes and leaves the enzyme surface without further modification (Figure 2). However, this is not true for most polyketides, which usually undergo various degrees of secondary modifications including alkylation, oxidation, reduction, ring cleavage, etc. These modifications may occur on the enzyme-bound intermediate compound or after the intermediate compound leaves the enzyme surface. It should be emphasized at this point that the scheme just presented is speculative, and is based on a recent version of the so-called "polyketide hypothesis".[6] A full account of the scheme for biosynthesis of polyketides will appear later in this chapter.

IV. METHODOLOGY TO STUDY THE BIOSYNTHESIS OF POLYKETIDES

The study of polyketides, although including peculiar features, seeks answers to the same basic questions asked before any metabolic study is done. The questions to be answered by

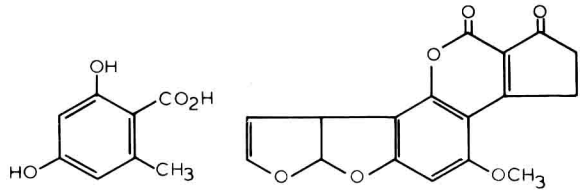

FIGURE 1. Structure of (A) orsellinic acid and (B) aflatoxin B_1.

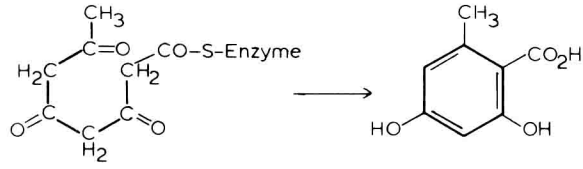

FIGURE 2. Cyclization of orsellinic acid intermediate compound.

such studies are: What are the building blocks? What are the intermediate steps? By which mechanism(s) are these changes brought about? To pursue such a study there should be available: (1) a rich source of biosynthetic activity, (2) the putative precursor, intermediate compound, or an even more remote substrate, and (3) a method to detect and characterize the end product and sometimes the precursor or intermediate compound(s). We will discuss these requirements in more detail as related to studies of polyketides.

A. Biosynthesizing System

To study a biosynthetic route, one first needs a rich source for the biosynthetic activity that leads to the product under investigation. Since most known polyketides are produced by fungi,[5] examples dealing with polyketide-producing organisms will be limited to these organisms. Early studies usually dealt with the entire mold. As knowledge accumulated and hypotheses were made of the kind of biosynthetic activity involved, attempts to isolate the pure enzymes that catalyze various steps of the pathway followed. Enzymatic evidence for the polyketide hypothesis was reviewed by Light[6] in 1970.

1. Growing Cultures

A pure culture of mold is grown on a suitable medium. Minimal media generally work better than highly nutritious subtrates for studies on secondary biosynthesis. This is true because limitation of a nutrient (which is achieved faster in minimal that in highly nutritious media) is thought to trigger secondary metabolism.[3,7] A medium limited in its nitrogen source is probably the one of choice for the synthesis of polyketides.[8] A growing culture may be used as the ultimate synthesizing system or the culture may be further manipulated by one or more of the purification techniques that will be dealt with later. When used as the ultimate biosynthesis system, a growing culture of mold gives only a crude idea about the pathway, and overinterpretation of the results can lead to great ambiguities. Such a study usually gives a preliminary idea about the environmental factors that regulate the in vivo biosynthesis of the metabolite under investigation.

Use of a growing mold culture to study the biosynthesis of polyketides (and probably the other secondary metabolites) could be more informative if the kinetics of mold growth and of production of the metabolite are clearly demonstrated.[9-11] By this means, segregation

between the two overlapping events, growth and secondary biosynthesis, is possible. The growing culture may be fed a labeled precursor or intermediate compound, and the sequence of incorporation of the label into various putative intermediate compounds and the end product is followed. By this technique (called kinetic pulse labeling), the sequence of appearance of intermediate compounds can suggest an overall biosynthetic pathway. Forrester and Gaucher[12] effectively used this procedure during their study on the biosynthesis of patulin.

Usually, liquid media are used to grow molds in studies on the biosynthesis of polyketides. Cultures could be incubated with or without agitation during the course of growth and secondary biosynthesis. The use of fermentors helps to control growth conditions and produces well-phased cultures.

2. Resting Cultures

In a growing mold culture, primary metabolism may severely modify results obtained from the study of secondary metabolism and dilute the label in the final secondary metabolite. To prepare a resting culture, the mold is first grown in a suitable medium until the biosynthetic system is well developed. The mycelium is then transferred to a nitrogen-free medium where growth is arrested. This procedure was used to study the regulation and biosynthetic inhibition of some polyketides.[13,14] The mold suspended in the resting medium could be fully capable of producing the ultimate metabolite or production could be inhibited at a certain step in the biosynthetic pathway through the use of a mutant or a specific enzyme inhibitor. For example, a blocked mutant and dichlorvos-treated *Aspergillus parasiticus* were used to elucidate the biosynthetic pathway of aflatoxin in resting cultures.[15] The intermediate compounds formed immediately before the metabolic block may accumulate in quantities sufficient for isolation and detection. Also, incorporation of an advanced intermediate compound, beyond the blocked step, may be monitored by resumption of the production of the metabolite.

3. Cell-Free Extracts

A further step toward a purer biosynthetic system is to prepare a cell-free extract from a mold culture. The consistent production of polyketides by cell-free extracts was, in many instances, difficult to achieve. This may be related, among other reasons, to the mechanical disruptive technique employed to prepare cell-free extracts.[16] Alternative procedures using no or less severe mechanical disruption were proposed.[16,17] These milder techniques involve enzymatic lysis of the fungal cell walls to set free the fungal protoplast from which the cell-free extract can be easily prepared.

4. Pure Enzymes

Pure enzymes capable of converting different putative intermediate compounds to the metabolite to be investigated constitute unambiguous proof of the biosynthetic pathway. With many polyketides, this goal is extremely difficult to achieve. This difficulty manifests itself by the instability and variability of activity between different enzymatic preparations.[6] However, several enzymes with various degrees of purity have been prepared from polyketide-producing fungi.

B. Precursors or Intermediate Compounds

A carbohydrate such as glucose can be catabolized by mold cells to produce acetate units, which can be viewed as the building blocks of polyketides. Externally supplied acetate also can be incorporated into polyketides. Although the actual biosynthetic route involves malonate as well, the in vivo biosynthesis of polyketides can proceed with acetate being the only precursor supplied. This is true because interconversion of acetate and malonate is a rapid process.[4]

An isotopically labeled precursor of the putative intermediate compound is fed to whole mold cells or added to cell-free extracts to demonstrate specific incorporation of the material into the metabolite being studied. Labeling with ^{14}C was extensively applied to biosynthetic studies of polyketides and labeling with ^{3}H was occasionally employed. Currently, ^{13}C-labeling, accompanied by nuclear magnetic resonance (NMR) spectroscopy, provides a valuable technique for biosynthetic studies of secondary metabolism.

C. End Product Analysis

Metabolic studies can be confined to identifying and/or quantifying the metabolite under investigation or can be expanded to localize certain labeled atom(s) in that metabolite. The degree of sophistication depends on the questions to be answered by the study. Studies on the environmental and regulatory factors of secondary metabolism may require mere quantitation of the metabolite, which does not have to be labeled. Elucidation of biosynthetic pathways, however, usually requires feeding a labeled precursor and determination of the specific incorporation of the label into the product. Detection of the label in the product may be enough to implicate a precursor or intermediate compound in the biosynthetic pathway. Understanding the underlying mechanism, however, requires localization of the label in the product either by chemical degradation (used with ^{14}C-labeling) or NMR spectroscopy (when ^{13}C-labeling is used). About 1 decade ago, double-labeling of precursors with ^{13}C was successfully used as a powerful technique in biosynthetic studies of polyketides.[18] The success of this technique is related to the fact that only those pairs of adjacent ^{13}C atoms will show carbon-carbon spin-spin coupling that can be revealed by NMR spectroscopy. This method gave interesting information about the folding patterns of the polyketides.[18]

V. PHASES IN THE BIOSYNTHESIS OF POLYKETIDES

It is generally agreed[1,4,5,19] that the biosynthesis of polyketides includes two main phases:

1. Chain assembly and cyclization, where condensation of acetate and malonate units gives a β-polyketoacyl intermediate compound. This phase is supposedly carried out on the surface of an enzyme complex. Cyclization stabilizes the intermediate compound that leaves the enzyme surface by hydrolysis.
2. The secondary transformation phase, where less specific enzymes take over the biosynthetic process. This phase is responsible for the great diversity observed among polyketides. During this phase, the enzyme-free intermediate compound undergoes one or more of the following reactions: methylation, reduction, oxidation, chlorination, or ring cleavage.

A. Chain Assembly and Cyclization

Although many attempts were made to purify and characterize polyketide synthetases, none of these attempts resulted in a completely pure preparation. Purification of 6-methylsalicylic acid synthetase is an outstanding example of these efforts. Dimroth and coworkers[20] obtained that enzyme from *Penicillium patulum* and then purified it 100-fold. Their preparation, however, was not consistent with that prepared by Light.[6]

6-Methylsalicylic acid synthetase from both preparations proved to be a multienzyme complex, as is true for fatty acid synthetase. Also, like fatty acid synthetase, 6-methylsalicylic acid synthetase is inhibited by the sulfhydryl group inhibitors, iodacetamide and *N*-ethylmaleimide[20] The pH-dependent inhibition with *N*-ethylmaleimide and the pH-independent inhibition with iodacetamide imply that 6-methylsalicylic acid synthetase contains two kinds of sulfhydryl groups.

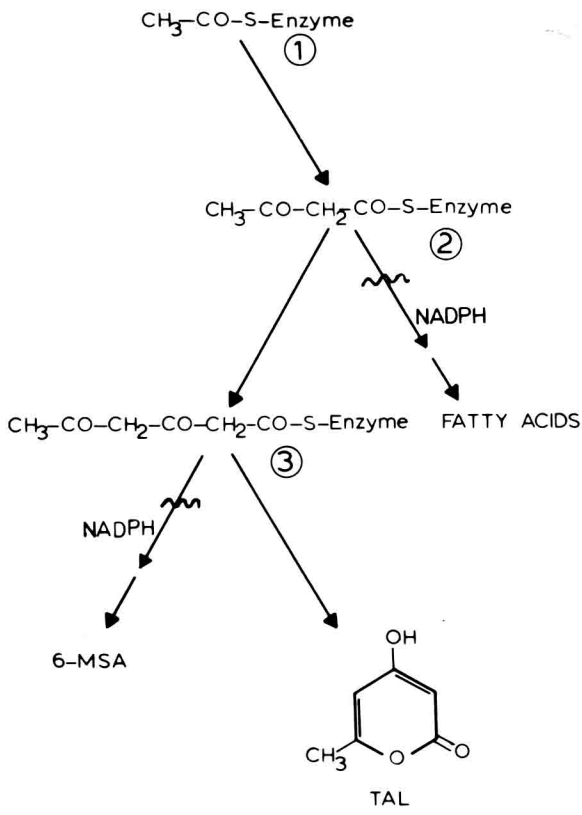

FIGURE 3. Biosynthesis of triacetic acid lactone by fatty acid synthetase and polyketide synthetase in the absence of NADPH. (1) Enzyme-bound acetate; (2) enzyme-bound acetoacetate; (3) enzyme-bound diketoacyl, TAL = triacetic acid lactone, 6-MSA = 6-methylsalicylic acid. ~~ = biosynthetic block through lack of NADPH.

In the absence of the reduced coenzyme NADPH, 6-methylsalycilic acid synthetase converts acetyl-CoA and malonyl-CoA into triacetic acid lactone[20] (Figure 3). From this experiment, Dimroth and colleagues[20] concluded that reduction of the β-polyketoacyl intermediate (of 6-methylsalicylic acid) occurs at the triacetic acid level and before the final condensation with malonyl-CoA takes place (Figure 3). Working with fatty acid synthetase from yeast, and in the absence of NADPH, Yalpani[21] also obtained the same metabolite (triacetic acid lactone) (Figure 3).

Dimroth et al.[22] demonstrated further similarities between 6-methylsalicylic acid and fatty acid synthetases. Both systems exhibited malonyl-CoA carboxylase activity upon treatment with iodoacetamide. 6-Methylsalicylic acid synthetases had obvious specificity toward acetyl-CoA; when it was replaced with propionyl-CoA or other higher homologue, the rate of reaction dropped significantly. Like fatty acid synthetase, 6-methylsalicylic acid synthetase has acyl transferase activity; the enzyme transacylated the acetyl moiety of acetyl-CoA to pantetheine.

Other polyketide synthetase systems may also be similar to fatty acid synthetases. Sjöland and Gatenbeck[23] found that replacement of acetyl-CoA with propionyl-CoA in the alternariol biosynthesizing system dramatically decreased the reaction rate. Fatty acid synthetase and polyketide synthetase, however, differ in the extent of reductive steps they exert on intermediate compounds. With fatty acid synthetase, every condensation of the malonyl group

(i.e., elongation of the chain by a C_2-unit) is accompanied by two reduction steps that require two molecules of NADPH.[24] For comparison, synthesis of 6-methylsalicylic acid requires the condensation of three malonyl units, but only one reduction step is required.[20]

1. Polyketide Synthetase and Chain Assembly

The close analogy between the two systems, fatty acid synthetase and polyketide synthetase, may warrant extrapolating the well-characterized mechanism involved in fatty acid biosynthesis to that of polyketides. The hypothetical scheme that will be presented derives its main features from those proposed by Lynen[25,26] and Bu'Lock[3] with modifications based on more recent findings about polyketide synthetase[27] and yeast fatty acid synthetase.[28]

Analogous to fatty acid synthetase, polyketide synthetase is a multifunctional protein thatgenerally carries out the following enzymatic activities: (1) transacylation of acetyl and malonyl species, (2) condensation of acetyl, β-polyketoacyl, or reduced β-polyketoacyl moieties with the elongating malonyl units, (3) reduction of β-polyketoacyl intermediate compounds, and (4) dehydration of the reduced intermediate compound. Furthermore, the multifunctional protein carries two sulfhydryl groups where acyl groups can be attached: one of a cysteine residue at the site of condensing activity (Enzyme-SH) and the other on a flexible arm of 4′-phosphopantethiene that is directly attached to the protein (Pantethiene-SH). The polyketide synthetase can be viewed as a multifunctional protein of more than one subunit (probably two, analogous with yeast fatty acid synthetase) rather than a multienzyme complex. The enzymatic activity of the system is distributed between these subunits.

The first step of polyketide synthesis is the transfer of acetyl and malonyl moieties from acetyl-CoA and malonyl-CoA to the Enzyme-SH and Pantethiene-SH groups, respectively. That transfer is mediated and catalyzed by the acyl transfer activity of the multifunctional protein. With the help of the flexible arm (about 20 Å in length), the malonyl group is at juxtaposition on the condensation activity site where the acetyl group is attached. At this site, decarboxylative acylation takes place, wherein C_1 of the acetyl groups is covalently linked to C_2 of the malonyl moiety with the release of CO_2 (Figure 4).

The decarboxylative acylation is facilitated by a coordination between a metal ion attached to the active site and oxygen atoms of the carbonyl or carboxyl groups of the reactants. At this point, the acetoacetyl moiety is attached to both the arm and the active site through thiol ester linkage and metal chelation, respectively.

The acetoacetyl moiety is internally transacylated to the Enzyme-SH site, and the Pantethiene-SH site is freed for a new malonyl group. The elongation of the ketoacyl moiety goes on and every time a new malonyl is reacted with the protein-bound ketoacyl intermediate compound, its chain length increases by two carbon units. Metal chelation makes feasible the folding of the β-polyketoacyl intermediate compound to a stable conformation, which dictates the specificity of the cyclization process that follows. It is possible that the geometry and position of the metal ion relative to the active center as well as the geometry of the active site itself determine the stereostructure of the final polyketide. The electronic delocalization that should accompany metal chelation with the enolizable carbonyl groups of the polyketoacyl may ease the electrophilic substitution that occasionally happens at the methylene groups of the polyketide intermediate compound.

Involvement of a metal in the proposed mechanism is supported by some experimental evidence. In their model system, Kobuke and Yoshida[29] noted the importance of magnesium in the condensation of malonate and acetate. With another model system for the biosynthesis of polyketides and fatty acids, Scott et al.[30] found that the role of magnesium was the control of *C*-acetylation over possible *O*-acetylation of acetate derivative. A scheme for chain assembly is shown in Figure 4.

As indicated before in the 6-methylsalicylic acid synthetase system, the absence of NADPH resulted in an unstable intermediate compound that went through lactonization and was

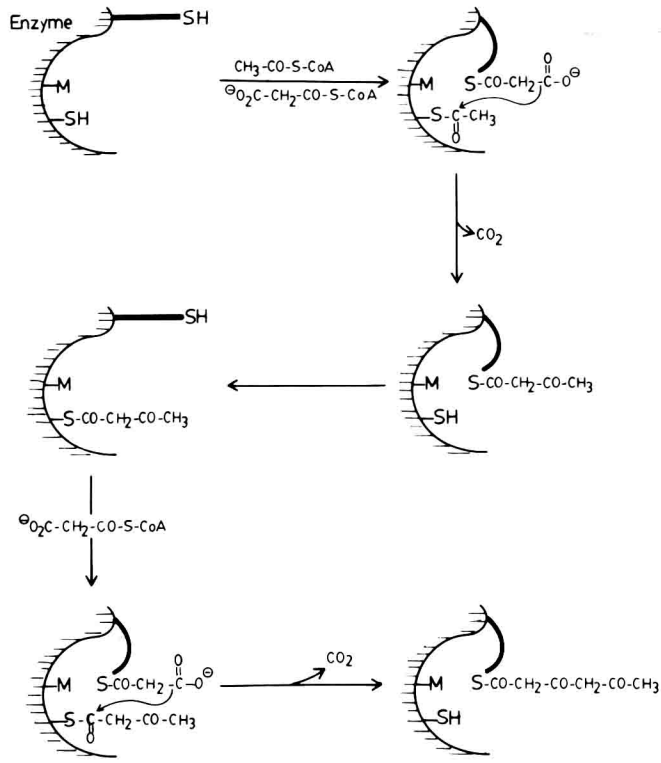

FIGURE 4. Chain assembly on polyketide synthetase. Note the involvement of two –SH groups; one is directly attached to the active site and the other is attached to a flexible arm of 4′-phosphopantethiene. Note also the presence of a metal ion (M) attached to the active center. Metal chelation is not illustrated to simplify the drawing.

released from the enzyme at an early state of chain assembly.[20] This may indicate the importance of the reduction step and probably the subsequent dehydration (giving a double bond) in stabilizing the intermediate compound. Other polyketide synthetases (e.g., that of alternariol biosynthesis), however, do not require NADPH, and the intermediate compound is not reduced. Metal chelation in this instance may play a significant role in stabilizing those intermediate compounds.

B. Secondary Transformation

As discussed earlier, synthesis of the first free polyketide intermediate compound is assumed to take place on the surface of a multifunctional protein. Modification of the intermediate compound is accomplished by several enzymes. Unlike in the previous phase, a greater number of enzymes of secondary transformation were purified and studied. However, confusion about the route that these intermediate compounds follow still exists, and may be greater than that encountered in the previous phase. The lack of absolute specificity of many enzymes of this phase, and failure to define the obligatory intermediate compounds of several steps led to what Bu'Lock described as a "metabolic grid".[31] It simply means that it is possible to demonstrate the incorporation of putative intermediate "E" into a product "C" without "E" being a normal obligatory intermediate compound of the route from A to C:

$$\begin{array}{ccccc} A & \rightarrow & B & \rightarrow & C \\ & & \uparrow\downarrow & & \\ D & \rightarrow & E & \rightarrow & F \end{array}$$

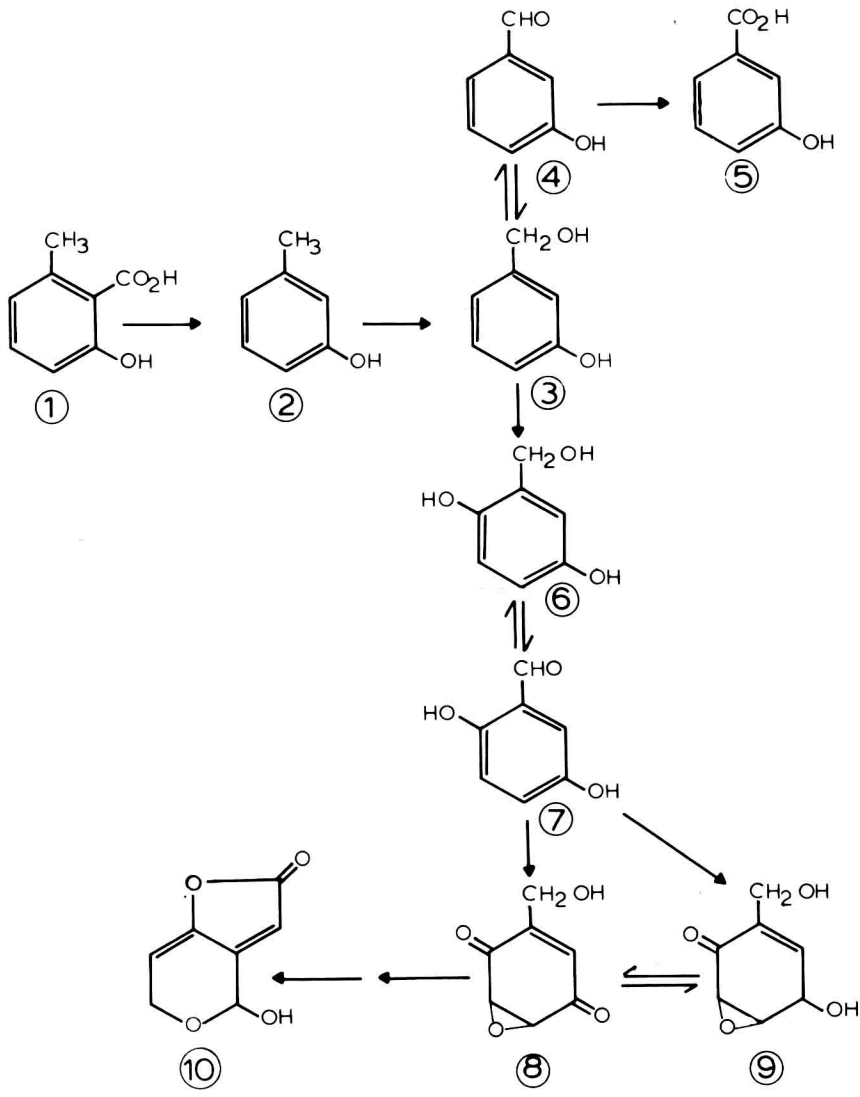

FIGURE 5. Conversion of 6-methylsalicylic acid into patulin. Note the involvement of a metabolic grid. (1) 6-Methylsalicylic acid, (2) *m*-cresol, (3) *m*-hydroxybenzyl alcohol, (4) *m*-hydroxybenzaldhyde, (5) *m*-hydroxybenzoic acid, (6) gantisyl alcohol, (7) gentisaldhyde, (8) phyllostine, (9) isoepoxydon, (10) patulin.[32,59]

The lack of specificity of some of the enzymes can cause the interconnecting of several pathways, giving more than one route for production of a single metabolite. This resulted in ambiguities in many proposed polyketide pathways and led to emphasis on such expressions as "obligate intermediate compounds", "derailments", and "co-metabolites". An example of a metabolic grid (Figure 5) is demonstrated by processes involved in the conversion of 6-methylsalicylic acid into patulin.

In the following paragraphs, we will discuss some of the important reactions of the secondary transformation phase and the enzymes that are involved in the reactions.

1. Reduction and Oxidation

In addition to reduction of the protein-bound β-polyketoacyl derivatives, biosynthesis of some polyketides may include further reduction steps. Oxidation, however, is the most