1. Introduction

1.1 Primary and secondary metabolism

1.1.1 Introduction

Since prehistoric times man has used plant extracts to heal and to kill. Folklore abounds in references to the use of plant extracts in the healing of a variety of illnesses; examples of applications as agents of death range from that of calabar beans and hemlock as judicial poisons to that of the South American curare arrow poisons [1]. In modern times organic compounds isolated from cultures of micro-organisms, as well as from plants, have been used for the cure of disease (e.g. penicillin and tetracycline antibiotics). These organic compounds from natural sources form a large group known as natural products, or secondary metabolites.

Study of the metabolism, fundamental and vital to living things, has led to a detailed understanding of the processes involved. A complex web of enzyme-catalysed reactions is now apparent, which begins with carbon dioxide and photosynthesis and leads to, and beyond, diverse compounds called primary metabolites, e.g. amino acids, acetyl-coenzyme A, mevalonic acid, sugars, and nucleotides [2, 3]. Critical to the overall energetics involved in metabolism is the coenzyme, adenosine triphosphate (ATP), which serves as a common energy relay and co-operates, like other coenzymes, with particular enzymes in the reactions they catalyse.

This intricate web of vital biochemical reactions is referred to as primary metabolism. It is often displayed usefully in chart form [4], and to the eye appears very much like an advanced model railway layout, not least because of the way primary metabolism proceeds in cycles (e.g. the citric acid cycle). The organic compounds of primary metabolism are the stations on the main lines of this railway, the compounds of secondary metabolism the termini of branch lines. Secondary metabolites are distinguished more precisely from primary metabolites by the following criteria: they have a restricted distribution being found mostly in plants and micro-organisms, and are often characteristic of individual genera, species, or strains; they are

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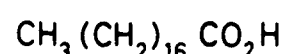
formed along specialized pathways from primary metabolites. Primary metabolites, by contrast, have a broad distribution in all living things and are intimately involved in essential life processes (for further discussion of parts of primary metabolism see Sections 1.1.2 and 5.1). It follows that secondary metabolites are non-essential to life although they are important to the organism that produces them. What this importance is, however, remains, very largely, obscure.

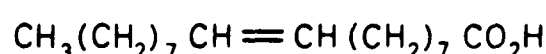
It is interesting to note that secondary metabolites are biosynthesised essentially from a handful of primary metabolites: α-amino acids, acetyl-coenzyme A, mevalonic acid, and intermediates of the shikimic acid pathway. It is these starting points for the elaboration of secondary metabolites which allow their classification, and also their discussion as discrete groups (Chapters 3 to 7). In the remainder of this chapter various aspects of biosynthesis of general importance to the discussion in Chapter 3 and succeeding chapters is reviewed. The first examples of primary and secondary metabolite biosynthesis will be found in Sections 1.1.2 and 1.1.3. Chapter 2 is devoted to a brief discourse on the various techniques used in studying the biosynthesis of secondary metabolites.

1.1.2 Fatty acid biosynthesis [2, 3]

Fatty acids, e.g. stearic acid (*1.1*) and oleic acid (*1.2*), are straight chain carboxylic acids found predominantly as lipid constituents.



(1.1) Stearic acid

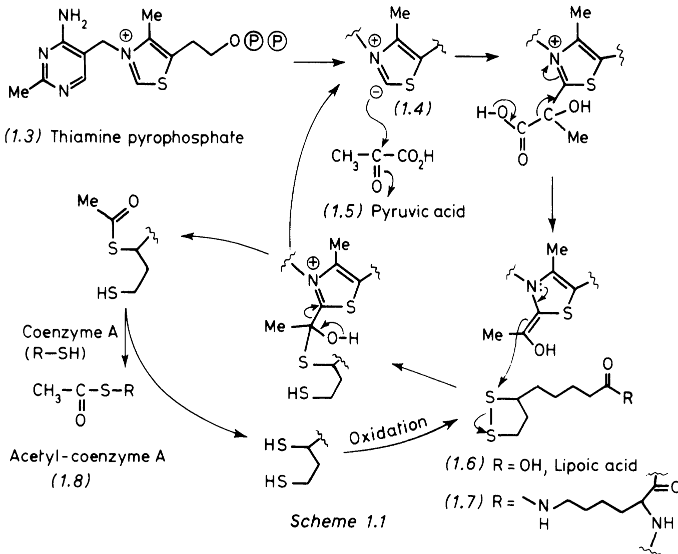


(1.2) Oleic acid

They are primary metabolites formed under enzyme catalysis by linear combination of acetate units. In this they are similar to the polyketides which are secondary metabolites (Chapter 3). Like many primary metabolites, their biosynthesis is understood in intricate detail. Much less detail is generally available on secondary metabolite biosynthesis.

Fatty acids are synthesized in a multienzyme complex from a crucially important primary metabolite, acetyl-coenzyme A (*1.8*). The principal source of acetyl-CoA (*1.8*) is pyruvic acid (*1.5*) and the conversion of (*1.5*) into (*1.8*) involves the coenzymes, thiamine pyrophosphate (*1.3*)* and lipoic acid (*1.6*) (Scheme 1.1). The key to the action of thiamine is the ready formation of the zwitterion (*1.4*) at the beginning and end of the reaction cycle. The lipoic acid (*1.6*) is enzyme linked via the side chain of a lysine residue (*1.7*). The disulphide functionality is thus at the end of a long (14 Å) arm. It has been suggested that this arm allows the lipoate to swing from one

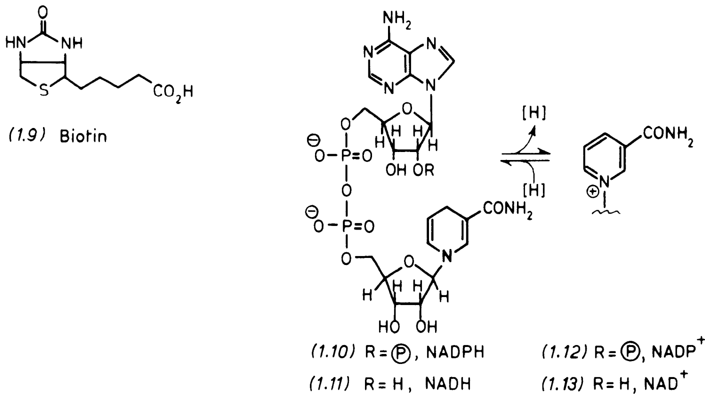
* Ⓟ = phosphate in (*1.3*) and subsequent structures, cf. (*1.10*).



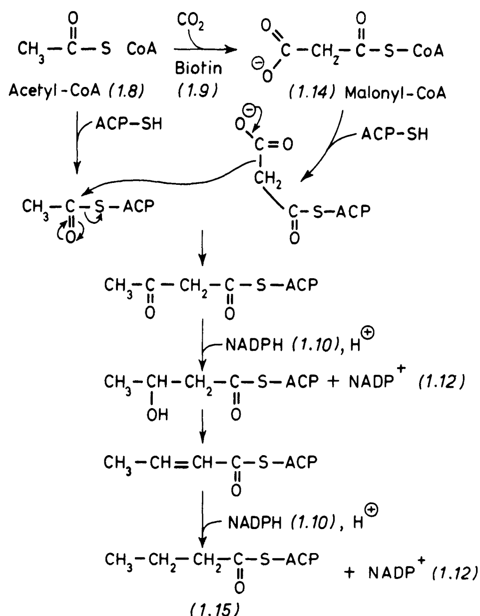
Scheme 1.1

site to another within the multienzyme complex and transfer (and oxidize) the acetyl group [5]. In the sequence shown, pyruvic acid (*1.5*) loses carbon dioxide giving coenzyme-bound acetaldehyde, which is oxidized to the CoA ester of acetic acid.

A similar long arm is apparent in biotin (*1.9*), again enzyme bound through a lysine residue. The coenzyme (*1.9*) assists in the carboxylation of acetyl-CoA (*1.8*) with carbon dioxide yielding malonyl-CoA (*1.14*). Exchange of both acetyl-CoA and malonyl-CoA occurs with acyl carrier proteins (ACP) having free thiol groupings. Condensation then occurs between acetyl-S-ACP and malonyl-S-ACP with simultaneous decarboxylation; the carboxylate anion is transferred into the new bond (Scheme 1.2) [6]. Subsequent steps involve reduction, dehydration and double-bond saturation. They



implicate in part the widely utilized reducing coenzyme, NADPH (*1.10*) (Scheme 1.2). The first sequence gives butyryl-S-ACP (*1.15*)



Scheme 1.2

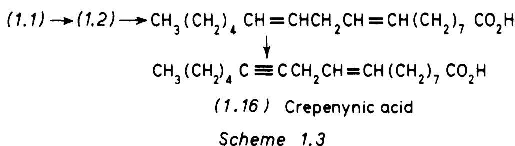
and for the generation of longer chains, as (*1.1*), the sequence, of malonyl-CoA addition, reduction, dehydration and reduction, is repeated the requisite number of times.

In examining the overall conversion of pyruvate into a fatty acid (Schemes 1.1 and 1.2) it is interesting to note the exploitation of particular chemical properties of sulphur: (i), as an easily reduced disulphide (*1.6*); (ii), as an easily oxidized dithiol; and (iii) in reactive thioesters which aid the Claisen-type condensation reactions. Also of crucial importance for the condensation is the use of a malonic acid derivative (*1.14*) as a source of a stable anion. (Further discussion of fatty acid biosynthesis in relation to polyketide formation is taken up in Chapter 3.)

1.1.3 The biosynthesis of polyacetylenes and prostaglandins

The formation of unsaturated fatty acids, e.g. oleic acid (*1.2*), which are also primary metabolites, may occur by at least two routes, one aerobic the other anaerobic. Essentially though, both involve desaturation of a fully saturated fatty acid [2]. Polyacetylenes, e.g. crepenynic acid (*1.16*), which are secondary metabolites, also

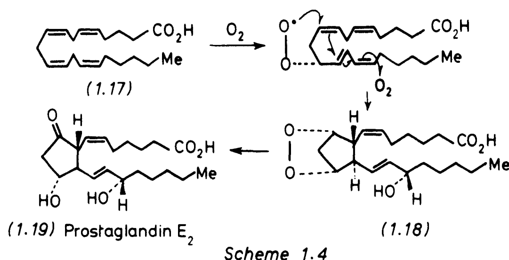
apparently derive by step-wise desaturation of a saturated fatty acid [7]. The path to crepenynic acid (*1.16*) is illustrated in Scheme 1.3.



(*1.16*) Crepenynic acid

Scheme 1.3

Prostaglandins, e.g. prostaglandin E_2 (*1.19*), are physiologically active secondary metabolites found in mammals. The biosynthesis of these compounds also involves an unsaturated fatty acid, e.g. arachidonic acid (*1.17*). Formation of the characteristic prostaglandin skeleton involves the formation of an intermediate endoperoxide (*1.18*). Some of the succeeding steps are indicated in Scheme 1.4 [8–10].



(*1.19*) Prostaglandin E_2 (*1.18*)

Scheme 1.4

It is interesting to note the formation of polyacetylenes (and prostaglandins) from fatty acids, since it is unusual for secondary metabolites to be formed from a fatty acid. The route that dominates the formation of these metabolites is the polyketide one (Chapter 3).

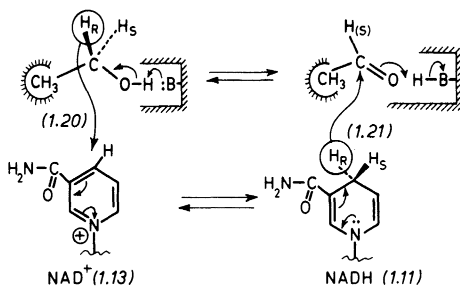
1.2 Stereochemistry and biosynthesis

1.2.1 Chirality and prochirality [11]

It is a common observation that enzymes deal stereospecifically with their substrates; the acceptable substrates must have a particular stereochemistry and the products in turn are formed with a particular stereochemistry (for an illustration of this see particularly Section 5.1). This stereospecificity is associated in many instances with reactions involving chiral centres.

In addition to the stereospecificity of reaction associated with chiral centres there is further stereospecificity to be found in reactions at prochiral centres. The conversion of ethanol (*1.20*) into acetaldehyde (*1.21*) by the enzyme, alcohol dehydrogenase, with NAD^+ as co-enzyme provides a simple illustration. The methylene

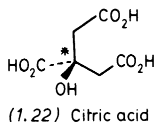
group in (*1.20*) is prochiral*. Oxidation of the ethanol proceeds stereospecifically with removal of the *pro-R* proton from this group. The reverse reaction involves stereospecific addition of a proton to the *re*-face (in this case, the top face as seen by the reader) of the acetaldehyde carbonyl group (i.e. proton removal and addition to the same side of the two molecules). The reaction is, moreover, stereospecific with regard to coenzyme. The interconversion of NADH and NAD^+ involves, respectively, removal of a proton from a prochiral centre and proton addition to form one. Removal and addition again involves the same face of the molecules concerned.



Scheme 1.5

The stereospecific proton removal from (*1.20*) to give (*1.21*) can be understood simply as follows: Imagine that at the active site of the enzyme there is one binding site specific for the ethanol OH and one specific for CH_3. This uniquely locates the molecules on the enzyme surface as shown in (*1.20*). Now imagine that the enzyme/co-enzyme proton removal can only occur physically from above the plane of the paper. This results in unique removal of the *pro-R* proton. There is no way on this model that the *pro-S* proton can be removed. A similar argument can be applied to proton addition to (*1.21*) and to

*The methylene group of ethanol has two identical groups (H) on a tetrahedral carbon atom and two groups different from these and from each other (CH_3, OH), and so is *pro*-chiral: a single change in one of the identical groups (H) makes the centre *chiral*. The identical groups may be distinguished as *pro-R* and *pro-S*. In order to do this the priority of one of the identical groups (H) is raised over the other. If this is done for the hydrogen projecting above the plane of the paper in (*1.20*) then the configuration at the methylene carbon atom becomes *R*. So the hydrogen that is raised in priority is termed *pro-R*. [The reader can try labelling the carboxy-methyl groups in (*1.22*) similarly; answer: (*1.60*).] The acetaldehyde double bond is also prochiral: the two faces of the double bond are not identical (addition may give a chiral product). These faces may be labelled *re* and *si* by following the normal priority rules for chirality (*re* = *R*, *si* = *S*). The face of (*1.21*) viewed from above is *re* (for further discussion see [12]).

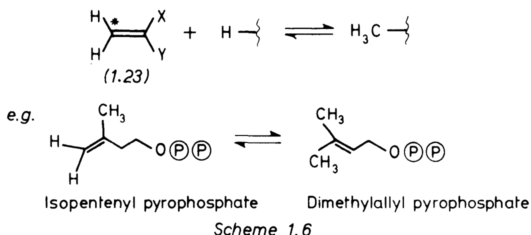


(1.22) Citric acid

proton removal from, and addition to, co-enzyme. [This argument was first applied some thirty years ago to citric acid (*1.22*) in relation to its place in the citric acid cycle. Citric acid has a prochiral centre (*) [13].]

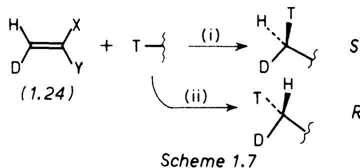
1.2.2 Chiral methyl groups [14]

Enzyme reactions involving methyl groups show none of the stereochemistry associated with prochiral centres as, e.g., the methylene group in ethanol (see above). The hydrogen atoms are indistinguishable (provided they are all one hydrogen isotope, see below) but enzyme-catalysed reactions do occur which involve methyl groups in various ways. Since enzymes are involved the reactions are expected to proceed with a particular stereochemistry. Three main classes of reaction can be identified [14]. By way of illustration we shall briefly examine one of these (Scheme 1.6) (for



Scheme 1.6

further discussion see Section 4.4). Proton addition to C* of the double bond in (*1.23*), with formation of a methyl group, can, in principle, occur from above or below the plane of the double bond. If the three protons involved in the generation of the methyl group are labelled with the three isotopes of hydrogen [^1H, ^2H, and ^3H, indicated in Scheme 1.7 as, respectively, H, D, and T] then the methyl

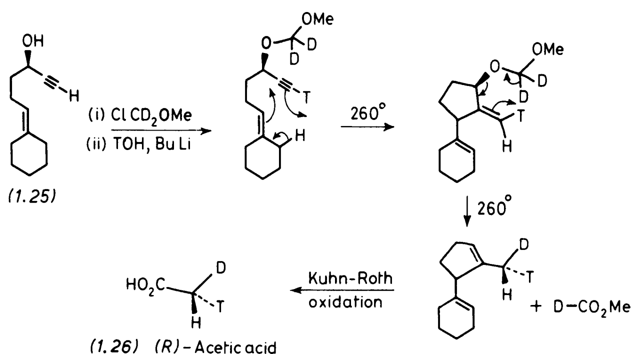


Scheme 1.7

group generated will be chiral. The stereochemical course of the reaction can be deduced, provided that: (i), the chirality (*R* or *S*) of the asymmetric methyl group thus generated can be determined; and

(ii), the substitution of hydrogen isotope around the double bond in (*1.24*) is known, e.g. as shown.

The central analytical problem is to determine the chirality of methyl groups generated or modified in biochemical reactions. The solution is dazzlingly ingenious. First, samples of chiral acetic acid [as (*1.26*)] of known absolute configuration were synthesized. All molecules of acetic acid contained deuterium (and hydrogen) but, as is customary, only very few molecules were also labelled with tritium. Only very few molecules were therefore chiral. Since the analysis is for tritium, however, this does not matter. [Subsequently an economical and supremely elegant synthesis of chiral acetic acid has been developed (Scheme 1.8), which involves two stereospecific

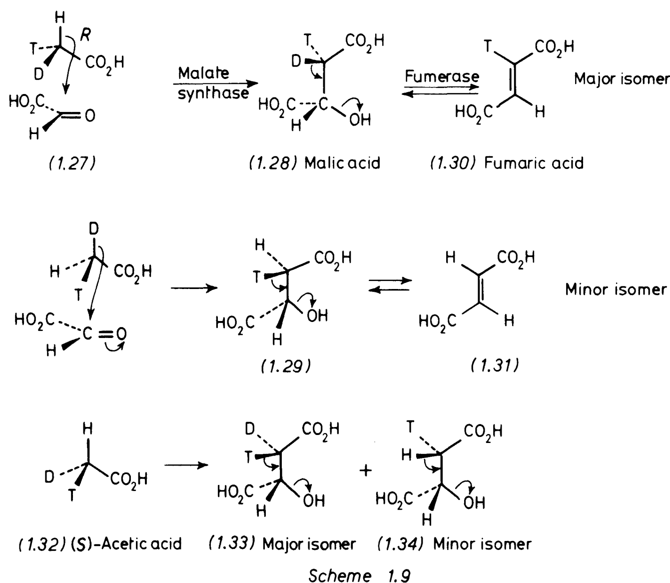


Scheme 1.8

concerted reactions and transfer of the chirality in (*1.25*) into (*1.26*) [15].]

The method of analysis developed is for acetic acid and involves the use of two enzymes. Chiral acetic acid [as (*1.26*)] is irreversibly condensed as its CoA-derivative with glyoxylic acid (*1.27*), using the enzyme malate synthase, to give malic acid (*1.28*). The condensation occurs with loss of hydrogen isotope by a primary kinetic isotope effect ($k_H > k_D > k_T$). This means that loss of H is favoured over loss of D which is in turn favoured over loss of T. The result is a high retention of tritium.

By loss of deuterium (^{2}H) or protium (^{1}H) two samples of *radioactive* malate are formed: (*1.28*) as major product by loss of H, and (*1.29*) as minor product from (*R*)-acetic acid by loss of ^{2}H (D); (*1.33*) and (*1.34*) are, respectively, the major and minor radioactive products from (*S*)-acetic acid (*1.32*). (The malate synthase reaction is now known to proceed with inversion of configuration. Since the method of analysis depends on simply getting one result for *R*-acetic acid and the opposite for the *S*-isomer this does not matter for the purposes of *assay*.)



Scheme 1.9

The malic acid is isolated and incubated with fumarase until no further loss of carbon-bound tritium results. [The enzyme catalyses *trans* removal of water, as (*1.28*) ⇄ (*1.30*).] For the samples of malate derived from (*R*)-acetic acid, the minor product (*1.29*) loses TOH. For the material derived from (*S*)-acetic acid it is the major product (*1.33*) which loses TOH. So for (*R*)-acetic acid a higher retention of tritium (ca. 75%) is observed in the fumarase equilibration and for (*S*)-acetic acid a lower value (ca. 25%). With the method of analysis established for samples of acetic acid of known chirality it can be used to determine the configuration of samples of acetic acid of unknown chirality derived from biological reactions (for examples see Section 4.2 and 4.4, and [14]).

1.2.3 *Hydroxylation at saturated carbon atoms* [2]

An oft encountered reaction in secondary metabolite biosynthesis is hydroxylation at saturated carbon atoms. These reactions are mediated by a mixed-function oxidase (or mono-oxygenase) i.e. an oxidase which uses molecular oxygen for hydroxylation, according to the equation: substrate-H + O_2 + XH_2 → substrate-OH + H_2O + X.

These hydroxylations are stereospecific proceeding with retention of configuration [11, 16] (examples are cited in following Chapters). This is the expected result for an electrophilic substitution at saturated carbon, i.e. attack at the point of highest electron density which is the sigma bond itself [17].

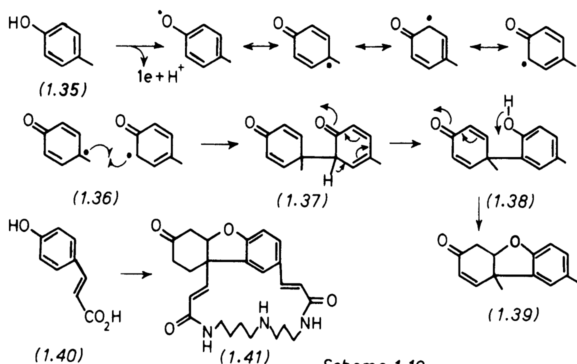
1.3 Some reactions of general importance in secondary metabolism

It will be convenient to discuss under this heading three quite different reaction types which will be mentioned again in succeeding chapters. Two concern aromatic compounds only, the third is of widespread significance.

1.3.1 Oxidative coupling of phenols [18–20]

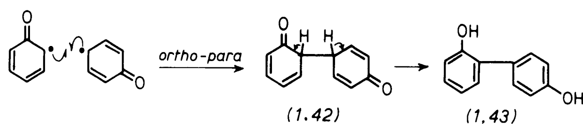
Phenols are biosynthesized essentially in two ways. One is along the polyketide pathway starting with acetyl-CoA (Chapter 3), the other along the shikimic acid pathway (Chapter 5). A phenol, or indeed any benzenoid compound, so formed may be at a terminus in biosynthesis or be involved in the formation of other metabolites. Of general importance in this regard is the coupling together of two phenolic residues, e.g. (*1.35*) → (*1.38*). A firm mechanistic base was given to this process by the quite brilliant recognition that this bond forming could occur by inter- or intra-molecular coupling of two mesomeric radicals [as (*1.36*)] formed by the one-electron oxidation of each of a pair of phenols. Carbon–carbon bond formation can only occur, according to this hypothesis, *ortho* or *para* to the phenolic hydroxy groups. Numerous studies on the biosynthesis of various phenolic compounds (see Sections 6.2.2, 6.3, 6.4 and 3.3) have demonstrated the overall correctness of this hypothesis: coupling is always *ortho* or *para* to phenolic hydroxy groups (occasionally C—O—C bonds may be formed); a hydroxy group must always be present on each of the aromatic rings (*O*-alkylation, for instance, blocks the coupling reaction). In one exceptional case (Section 6.3.5) it has been found that coupling only occurs if one of the aromatic rings bears *two* hydroxy functions.

Chemical conversion of *p*-cresol into the ketone (*1.39*) provides a simple illustration of the hypothesis (Scheme 1.10), in which coupling occurs between the position *ortho* to one phenolic hydroxy group



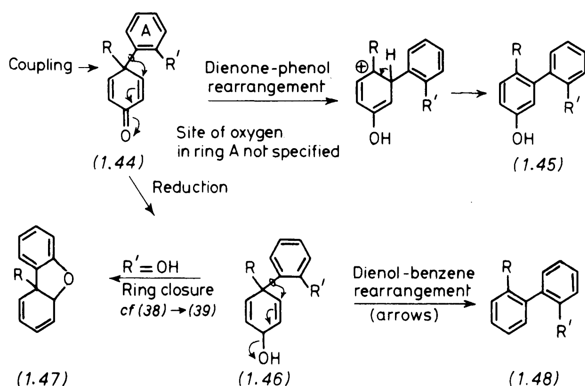
Scheme 1.10

and the position *para* to the other. Aromaticity is regained by proton loss from the coupling sites(s) [as (*1.37*) → (*1.38*) and (*1.42*) → (*1.43*)]. In the formation of (*1.38*) only one ring can become



aromatic in this way; the methyl group in the other ring secures it in the dienone form (*1.38*). Such dienones have been isolated from living things. They may react in the way shown for (*1.38*) → (*1.39*). A strikingly close analogue of (*1.39*) is the plant alkaloid lunarine (*1.41*). It arises plausibly from two molecules of *p*-hydroxycinnamic acid (*1.40*) along a pathway similar to that shown for (*1.39*). [In support, phenylalanine, a precursor for (*1.40*), is incorporated into lunarine (*1.41*) [21].]

Instead of undergoing a ring-closure reaction of the type just described, dienones may rearrange *in vivo* [as (*1.44*) → (*1.45*)] and thus regain aromaticity (the 'dienone-phenol' rearrangement). Reduction gives dienols [as (*1.46*)] which either rearrange [as (*1.46*) → (*1.48*) ('dienol-benzene' rearrangement)] or undergo ring-closure reactions of the type (*1.46*) → (*1.47*). An outstanding example of the latter reaction involving a dienol is found in the biosynthesis of morphine (Section 6.3.4), and of the former in the biosynthesis of isothebaine (Section 6.3.2). (The various possibilities are summarized in Schemes 1.10 and 1.11; *ortho-para* coupling is illustrated; similar schemes can be written for *ortho-ortho* and *para-para* coupling.)



Scheme 1.11

1.3.2 *Hydroxylation of aromatic substrates* [22, 23]

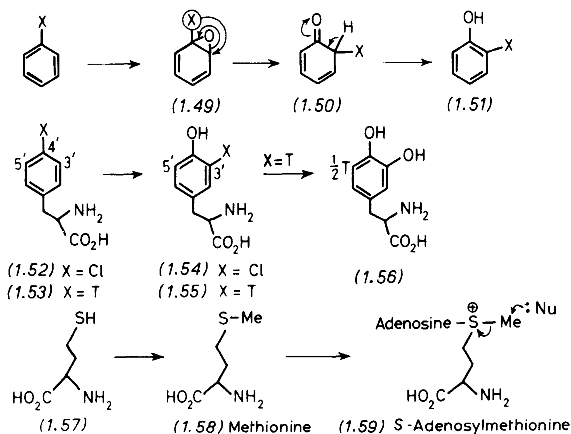
It has been found generally in a variety of living systems that hydroxylation of aromatic substrates involves utilization of molecular

oxygen and proceeds *via* an arene oxide [as (*1.49*)]. Collapse of this arene oxide to give (*1.51*) occurs with a 1,2 shift of the substituent X (e.g. X = ^{1}H, ^{2}H, ^{3}H, Cl). In the hydroxylation of 4′-chlorophenylalanine (*1.52*) proton loss occurs from (*1.50*) to give 3′-chloro-4′-hydroxyphenylalanine (*1.54*) as the major product. For X = ^{3}H (T) or ^{2}H (D) loss of hydrogen isotope occurs from (*1.50*) in accord with a primary isotope effect ($k_H > k_D > k_T$). Thus hydroxylation of [4′-^{3}H]phenylalanine (*1.53*) gives the α-amino acid tyrosine (*1.55*) with a high retention (ca. 90%) of tritium at C-3′. A lower retention is observed for [4′-^{2}H]phenylalanine (ca. 70%) as a reflection of the different isotope effects. This 1,2-shift in aromatic hydroxylation is called the NIH shift (named after the National Institutes of Health, U.S.A., where the rearrangement was discovered).

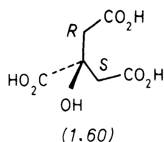
Subsequent hydroxylation of tyrosine [as (*1.55*)] to give dopa [as (*1.56*)] occurs without NIH shift, i.e. loss of substituent X, although another arene oxide is probably implicated. For the conversion of [4′-^{3}H]phenylalanine (*1.53*) → (*1.55*) → (*1.56*), ca. 90% tritium is retained after the first hydroxylation. Half of this is lost in the next reaction because there are two equivalent (C-3′ and C-5′) sites, each bearing half of the residual tritium, which are available in (*1.55*) for oxygenation and consequent loss of tritium.

1.3.3 Methylation [2]

C-, *O*-, and *N*-methylations are encountered frequently in the biosynthesis of secondary metabolites. All appear to involve nucleophilic substitution on the *S*-methyl group of *S*-adenosyl-L-methionine (*1.59*) (Scheme 1.12). In biosynthetic feeding experiments it is normal to use [*methyl*-^{14}C]methionine [as (*1.58*)] as a precursor for methyl groups; reaction *in vivo* with ATP and Mg^{2+} affords (*1.59*). Particu-



Scheme 1.12



(1.60)

larly in early studies formic acid was used to test for C_1 units in biosynthesis. It serves as a source for methyl groups via *N*-formyltetrahydrofolic acid which suffers reduction to give *N*-methyltetrahydrofolic acid; the methyl group is then transferred to L-homocysteine (*1.57*) to give L-methionine (*1.58*).

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Further reading: [2] and [3].

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