CHAPTER II

HISTORICAL

1. BOTANICAL ASPECT OF *IMPATIENS BALSAMINA* L.

Impatiens balsamina Linn. (Fig. 1) belongs to the plant family Balsaminaceae (Touch-Me-Not Family). It has a number of synonyms, including *Balsamina hortensis* DC., *B. impatiens* Hort. and *Impatiens coccinea* Sims. It has several Thai names, such as Thian-ban, Thian-dok, Thian-thai, Thian-suan. The other local names in various countries are Garden Balsam (English); Hosenka (Japanese); Bond Sun Hwa (Korean); Kamantigi (Tagalog); Saungga (Zulu); Suranga (Bikol, Bisaya).

I. balsamina (Bailey, 1963) is a native of India, and now widely cultivated and especially grown ornamentally in shaded parts of gardens. The plants are erect, tender, succulent annual or perennial herb to about 60 cm high; pubescent or nearly glabrous (Fig. 1). Their leaves are alternate; narrowly or broadly lanceolate; 8-10 by 2-3 cm, tapering at the tip and base, consisting of deeply serrate margin and have the glandular petiole. The flowers are clustered in the axils of the leaves and on very short stalk. The color range are from white to dark blood-red, yellowish and spotted. The sepals and petals are similar in color and not easily distinguished. One of the sepals seems to be three and long-spurred. The petals are apparently three, but two of them probably represent two unit petals, thus making five petals. The fruits are explosive long woolly capsules. They have five carpels and very thin partitions, and seeds borne on axile placenta. When the capsules are ripe, a pinch will cause the valves to separate and contract. The seeds are then thrown with considerable force.

I. balsamina is very easy to culture. They are propagated by seeds. The seeds are large and germinate quickly. The plants prefer a rich, sandy loam and must not suffer from moisture.

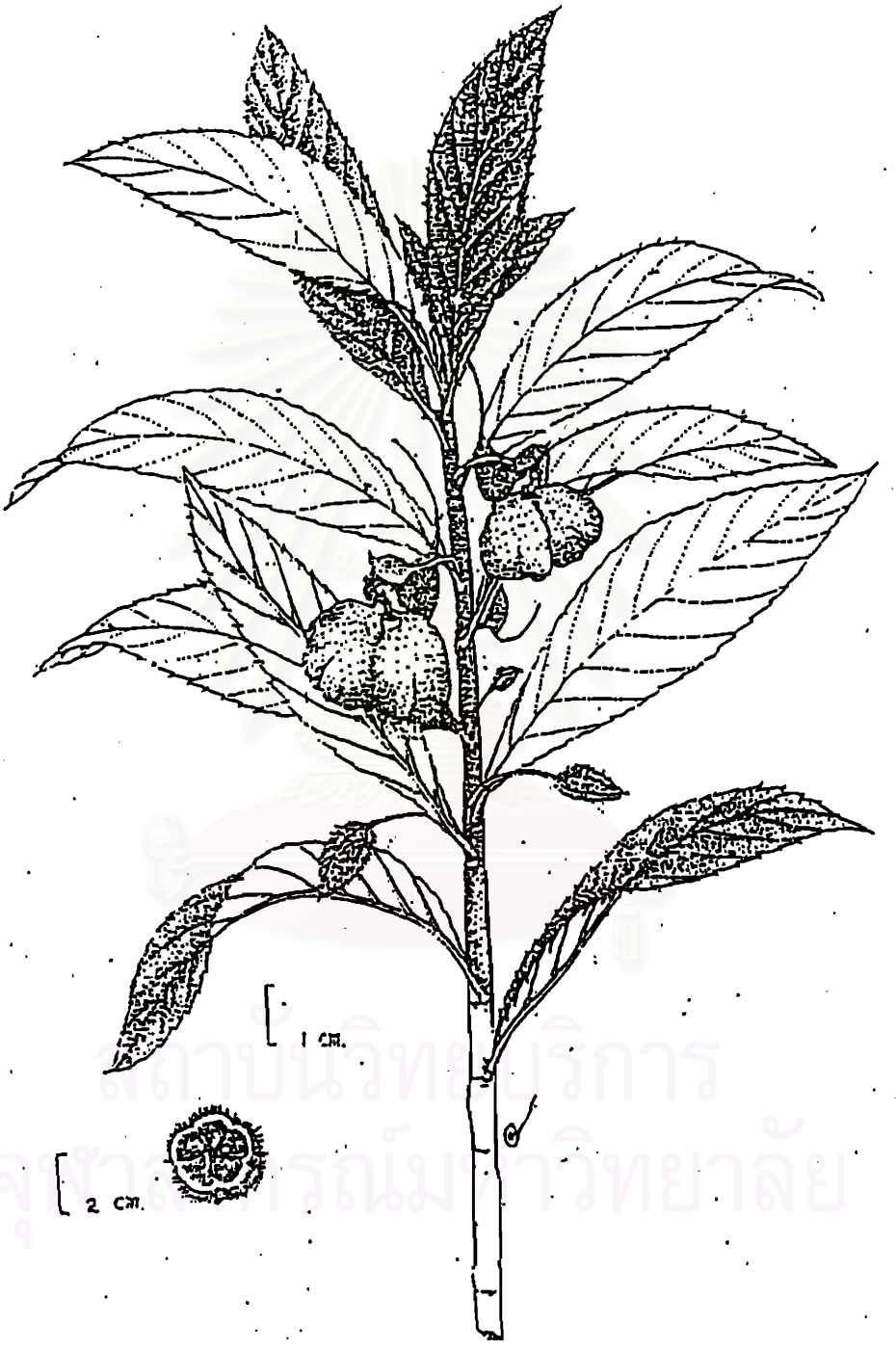

Fig. 1 *Impatiens balsamina* (Balsaminaceae)

2. CHEMICAL CONSTITUENTS OF *IMPATIENS BALSAMINA*

Since 1948, when 2-methoxy-1,4-naphthoquinone was isolated and identified from the flowers of *I. balsamina* (Little *et al.*, 1948), the search for constituents from different parts of *I. balsamina* has continued and has been intensified in recent years. The groups of compounds commonly found in this plant are naphthoquinone, coumarin, phenolic acid, flavonoid, anthocyanidin and steroid. A list of the compounds found in various parts of *I. balsamina* is shown in Table 1.

Table 1 Chemical constituents of various parts of *I. balsamina*

Plant part	Category	Chemical substance	Reference
Leaves	Naphthoquinone	lawsone	Bohm and Towers, 1962
		2-methoxy-1,4-naphthoquinone	Bohm and Towers, 1962
	Phenolic acid	caffeic acid	Bohm and Towers, 1962
		p-coumaric acid	Bohm and Towers, 1962
		ferulic acid	Bohm and Towers, 1962
		gentisic acid	Bohm and Towers, 1962
		p-hydroxybenzoic acid	Bohm and Towers, 1962
		sinapic acid	Bohm and Towers, 1962
	Flavonoid	kaempferol	Weissenboeck *et al.*, 1971
	Coumarin	scopoletin	Bohm and Towers, 1962
Flower	Naphthoquinone	lawsone	Clevenger, 1958
		2-methoxy-1,4-naphthoquinone	Little *et al.*, 1948
	Phenolic acid	*p*-coumaric acid	Mansell *et al.*, 1970
		ferulic acid	Mansell *et al.*, 1970
		hydroxycinnamic acid	Mansell *et al.*, 1970
	Flavonoid	kaempferol	Clevenger, 1958
		myricetin	Clevenger, 1958
		quercetin	Clevenger, 1958

Table 1 (continue)

Plant part	Category	Chemical substance	Reference
	Anthocyanidin	pelargonidin	Clevenger, 1958
		cyanidin	Clevenger, 1958
		peonidin	Clevenger, 1958
		malvidin	Clevenger, 1958
	Enzyme	O-methyltransferase	Mansell and Seder, 1971
		flavonoid-3-β-glucosidase	Boylen *et al*, 1969
Seed	Flavonoid	quercetin	Wellmann, 1975
	Fatty ester	ethyl palmitate	Patra and Chaudhuri, 1988
		ethyl stearate	Patra and Chaudhuri, 1988
		ethyl oleate	Patra and Chaudhuri, 1988
	Baccharane triterpenoid	hosenkol A	Shoji *et al.*, 1983
		hosenkoside A, B, C, D, E,	Shoji *et al.*, 1994a
		hosenkoside F, G, H, I, J, K	Shoji *et al.*, 1994b
	Steroid	β-sitosterol	Dikshit, 1973
		β-amyrin	Mukherjee and Roy, 1956
		α-spinasterol	Mukherjee and Roy, 1956
	Monoglyceride	glycerol-1,9-octadecenoate	Patra and Chaudhuri, 1988

3. USES OF *IMPATIENS BALSAMINA*

I. balsamina has long been used as Thai traditional medicine (Farnsworth and Nuntavan Bunyapraphatsara, 1992). The leaves and roots are used to treat thorn or glass-punctured wounds, felon, chronic ulcers caused by allergic reaction to detergents. The stems are used for the treatment of abscesses and ingrowing nails.

In China, the old Chinese use the powdered seeds of *I. balsamina* to treat difficult labor, to suppress puerperal pain, to act as an emmenagogue, expectorant, and antidote for poisoning from fish. The seeds are also used to soften fish bone while cooking and to stop hiccups. When mixed the powdered seeds with arsenious acid and applied to dental caries, the teeth can be removed easily. The lower part of the stem pounded to juice and added to rice liquor is used as a cure for contusions. It may also be used to wash abscesses and reduce swellings. The dried stems are used for improved circulation and to relieve pain in case of hard labor, leg cramps, and rheumatism. The flowers are mucilaginous and cooling, and are used for the treatment of snake bite, lumbago and intercostal neuragia. They are thought to improve the circulation and to relieve stasis (Lily and Metzger, 1980).

In the Philippines, the leaves are pounded and used in poultices to dissolve felongs. Similarly, the leaves are used in Malaysia for poulticing broken and torn nails. In Bali. the leaves are eaten as food, and in Asia, the flowers and leaves are used as a substitute for Henna (Quisumbing, 1951).

In Indo-China, a decoction of the leaves is used to wash the hair and supposedly to promote its growth. The oil from *I. balsamina* seed may be used for cooking and for burning lamps. It is also suitable for the surface-coating industry (Sastri *et al.*, 1959).

In Korea, the whole plant of *I. balsamina* has been used for the treatment of scrofula, carbuncle and dysentery. (Kang and Moon, 1992).

4. PHARMACOLOGICAL ACTIVITIES OF *IMPATIENS BALSAMINA*

4.1 Antifungal activity

Since *I. balsamina* contains lawsone (2-hydroxy-1,4-naphthoquinone) (Bohm and Towers, 1962), which possesses antifungal activity against *Alternaria*, *Absidia*, *Penicillium*, *Cladosporium* (Steffen and Peschel, 1975; Tripathi, Srivastava and Dixit, 1978; Farnsworth and Cordell, 1976), a study on the crude chloroform and alcoholic extracts of leaves was

carried out. It was reported that both extracts were *in vitro* active against *Trichophyton rubrum*, *T. mentagrophytes* and *Epidermophyton floccosum* which cause ringworm and athlete's foot (Santi Thungsuwan *et al.*, 1985). Furthermore, it has been reported that a simple naphthoquinone derivative, 2-methoxy-1,4-naphthoquinone isolated from *I. balsamina* possessed strong antifungal activity against *Trichophyton rubrum*, *T. mentagrophytes*, *Microsporum gypseum*, *Candida albicans*, *Aspergillus niger*, *Cryptococcus neoformans*, and *Epidermophyton floccosum* (Thatree Phadungcharoen *et al.*, 1988; Kang and Moon, 1992).

4.2 Antibacterial activity

It has been reported that both lawsone and 2-methoxy-1,4-naphthoquinone have antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Pasteurella*, *Escherichia coli*, *Brucella* and *Neisseria* (Kulkarni *et al.*, 1983; Kelkar *et al.*, 1986; Thatree Phadungcharoen *et al.*, 1988; Kang and Moon, 1992). The activity of 2-methoxy-1,4-naphthoquinone on gram-negative was lower than that of gram-positive bacteria (Kang and Moon, 1992). An ether extract of leaves was found to be effective against *Shigella flexneri*, *Sh. sonnei*, *Staphylococcus aureus* and β-hemolytic streptococcus group A. Water and 95% alcoholic extracts of leaves exhibited the same activity on *S. aureus* and β-hemolytic streptococcus (Farnsworth and Nuntavan Bunyapraphatsara, 1992).

4.3 Toxicity assessment

It has been reported that the chloroform extract of *I. balsamina* gave the LD_{50} value of 0.67 gkg^{-1} when given intraperitoneally in mice. The cream preparation, containing 1% of the chloroform extract, potentiated irritation caused by the cream base alone when tested on male rabbits. This effect is potentiated by UV exposure (Santi Thungsuwan *et al.*, 1985).

5. BIOSYNTHETIC PATHWAYS OF PLANT NAPHTHOQUINONES

There are at least four different ways of *in vivo* synthesizing of the naphthoquinones in higher plants (Fig. 2). Among these, three routes involve direct incorporation of shikimic acid into the naphthoquinone nucleus, while the other route utilizes acetic acid, as in polyketide pathway.

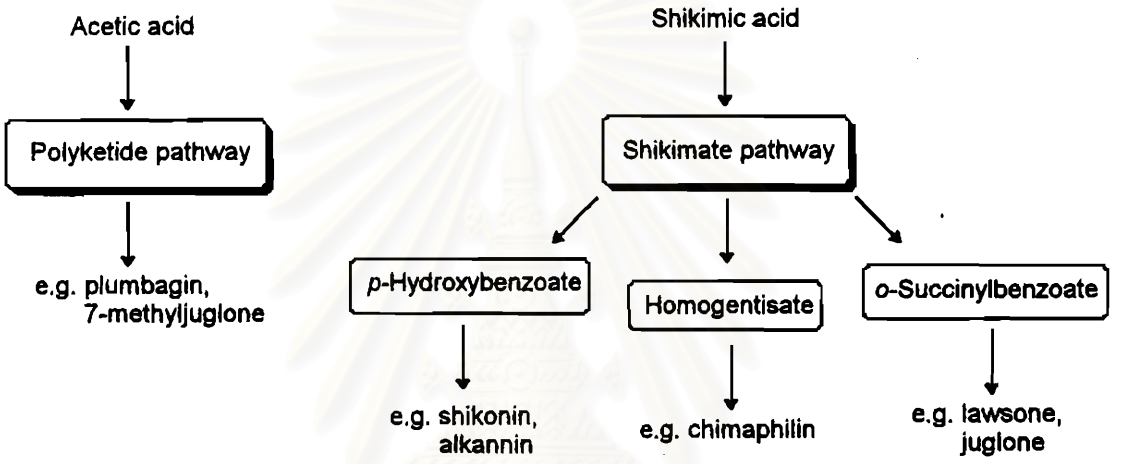

Fig. 2 Biosynthetic routes of plant naphthoquinones

5.1 The shikimate-derived pathways

The three shikimate derived pathways lead to different types of naphthoquinones branch from the main shikimate pathway by passing through their own unique intermediates. The key intermediates of the three different pathways are *p*-hydroxybenzoic acid, homogentisic acid and *o*-succinylbenzoic acid.

In the next section, these so-called "*p*-hydroxybenzoic acid pathway", "homogentisic acid pathway" and "*o*-succinylbenzoic acid " will be described in more details.

5.1.1 The *p*-hydroxybenzoic acid pathway

This biosynthetic route has been discovered in the plants of the Boraginaceae, a family containing the naphthoquinone shikonin and its enantiomer, alkannin. Tracer

experiments (Schmid and Zenk, 1971; Inouye *et al.*, 1979) have demonstrated that their skeletons are biosynthesized through the condensation of *p*-hydroxybenzoic acid with geranylpyrophosphate (GPP) which is itself formed from isopentenyl pyrophosphate (Fig. 3). The biosynthesis of *p*-hydroxybenzoic acid in plants has been demonstrated to proceed from shikimic acid *via* the prephenate-cinnamate pathway by side chain degradation of cinnamic acid (Heide, Floss and Tabata, 1989), although in bacteria, it is directly formed from chorismic acid. Two possible reaction mechanisms of the side-chain cleavage of cinnamic acids was proposed. One is analogous to β-oxidation of fatty acid. An alternative pathway involves a "non-oxidative" side-chain degradation, followed by an oxidation of the resulting benzaldehyde derivative. An enzymatic formation of *p*-hydroxybenzoic acid from *p*-coumaric acid *via* *p*-hydroxybenzaldehyde by "non-oxidative" side-chain degradation rather than a "β-oxidation" has been clearly demonstrated (Yazaki, Heide and Tabata, 1991).

The condensation of *p*-hydroxybenzoic acid with geranylpyrophosphate gives an intermediate, *m*-geranyl-*p*-hydroxybenzoic acid which converted, through decarboxylation, into a key intermediate, geranylhydroquinone (Fig. 3) (Inouye *et al.*, 1979; Yazaki, Fukui and Tabata, 1986), which is subsequently transformed to shikonin derivatives *via* deoxyshikonin (Okamoto, Yazaki and Tabata, 1995).

For the biochemical studies on the regulation of shikonin biosynthesis, it has been found that the enzymatic studies have been hampered by the technical difficulty of detecting the enzymes involved in this pathway. Shikonin derivatives, which have been used for dying silk since ancient times, bind irreversibly to proteins and powerful enzyme inactivators. The attempts to demonstrate the activity of enzymes related to shikonin biosynthesis have, therefore, been unsuccessful. However, it has been reported that the removal of shikonin derivatives from pigment-producing *Lithospermum erythrorhizon* cell suspension cultures before using for the preparation of enzyme has made it possible, for the first time , to detect the activity of *p*-hydroxybenzoate geranyltransferase, a key enzyme in shikonin biosynthesis (Heide and Tabata, 1987a). This key enzyme showed high substrate specificity for both

geranylpyrophosphate and *p*-hydroxybenzoic acid and required the presence of a divalent cation magnesium. Its activity was located in the pellet of 100,000g centrifugation (Heide and Tabata, 1987b).

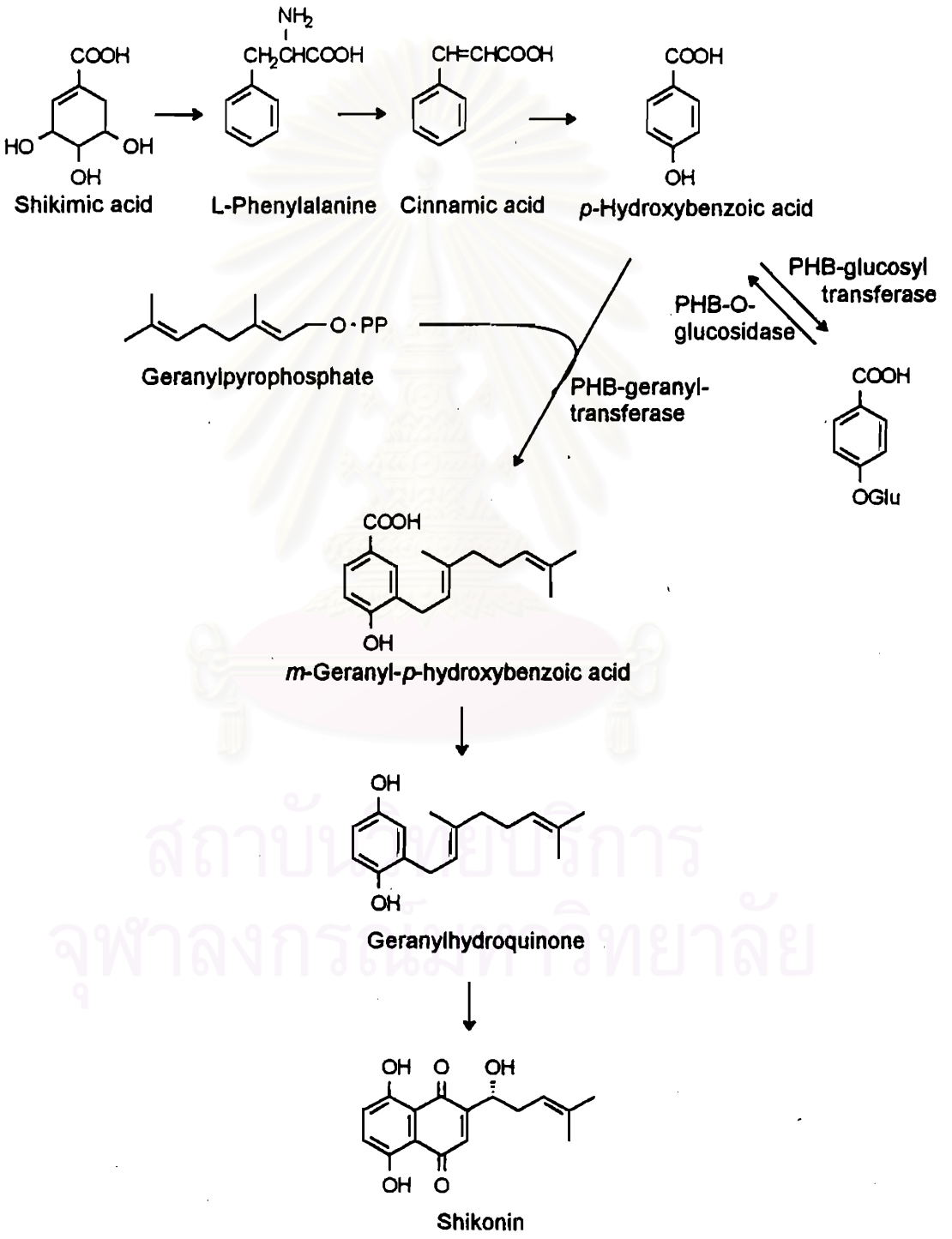

Fig. 3 The biosynthetic pathway of shikonin

Shikonin production has been demonstrated to be closely associated with the development of elongated rough endoplasmic reticulum and the subsequent formation of electron-dense vesicles (0.1-0.2 μm) which appeared to transport shikonin derivatives to the outside of the cell wall (Tsukada and Tabata, 1984). Furthermore, it has been demonstrated that the *de novo* synthesis of *p*-hydroxybenzoate geranyltransferase was inhibited by exposure to light, where neither vesicles nor shikonin was formed by the cells (Heide *et al.*, 1989). It is likely that the vesicles carry this enzyme involved in shikonin biosynthesis. Recent studies have shown that the two enzymes that provide the substrate of *p*-hydroxybenzoate geranyltransferase are soluble enzymes (Yazaki, Heide and Tabata, 1991). Therefore, it may be considered that prenylation of *p*-hydroxybenzoic acid is the first reaction step carried out by a membrane bound enzyme. Moreover, preliminary experiment has suggested that the decarboxylation of *m*-geranyl-*p*-hydroxybenzoic acid, which would yield geranylhydroquinone is also catalyzed by a membrane-bound enzyme (Yamaga *et al.*, 1993). It is interesting that the latter half of the biosynthetic process starting from the prenylation of *p*-hydroxybenzoic acid to shikonin, which is water-insoluble and cytotoxic, is probably carried out in the specific vesicle that also acts the part of transport and secretion of the product through exocytosis (Tsukada and Tabata, 1984).

The detection of *p*-hydroxybenzoate geranyltransferase in *L. erythrorhizon* culture confirms the current concept of shikonin biosynthesis (Inouye *et al.*, 1979). The enzyme catalyses the key step in shikonin biosynthesis, linking precursors of the shikimate pathway and the isoprenoid pathway, which together provide the complete carbon skeleton of shikonin molecule. Interestingly, the activity of this enzyme in shikonin-producing cultures was found to be approximately 35 times higher than in non-producing cultures, suggesting that this enzyme is of regulatory importance in shikonin biosynthesis (Heide and Tabata, 1987b).

In addition, two enzyme activities involving in the formation of *p*-hydroxybenzoic acid from *p*-coumaric acid have also been detected (Yazaki, Heide and Tabata, 1991). One might be an unspecific enzyme activity (e.g. unspecific peroxidase) responsible for the

conversion of *p*-coumaric acid to *p*-hydroxybenzaldehyde. The other is *p*-hydroxybenzaldehyde dehydrogenase, which converts *p*-hydroxybenzaldehyde to *p*-hydroxybenzoic acid.

Recently, it has been reported that one of the regulatory principles of shikonin biosynthesis in *L. erythrorhizon* cultures is the ratio of the activities of *p*-hydroxybenzoate geranyltransferase and *p*-hydroxybenzoate glucosyltransferase (Fig. 3) (Heide, Nishioka, Fukui and Tabata, 1989). This ratio may determine whether *p*-hydroxybenzoic acid is converted into shikonin, which is excreted out of the cell (Tsukada and Tabata, 1984) or into its glucoside, which is likely to be stored in the vacuole. In addition, it has been demonstrated that the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), which converts 3-hydroxy-3-methylglutaryl-CoA to mevalonic acid, correlates with the production of acetylshikonin. This suggests that HMG-CoA reductase may be a regulatory important in the isoprenoid part of shikonin biosynthesis (Gaisser and Heide, 1996).

5.1.2 The homogentisic acid pathway

This biogenetic hypothesis was first developed on the basis of the co-occurence of chimaphilin (2,7-dimethyl-1,4-naphthoquinone) and homoarbutin, in *Pyrola incarnata* (Pyrolaceae). It was assumed that a prenyl derivative of homoarbutin could indeed readily give chimaphilin. Subsequently, it has been reported that the compound corresponding to the assumed prenylated intermediate, 2-methyl-5-isopentenyl-1,4-naphthoquinone was isolated from *P. media* (Burnett and Thomson, 1968). This finding, thus, made the presumptive evidence for the actual formation of chimaphilin from the precursor related to 2-methyl-5-isopentenyl-1,4-naphthoquinone very strong. The experimental proof for this pathway has been adduced by the demonstration of the highly specific incorporation of [2-^{14}C]-mevalonic acid into the methyl group at C-7 of chimaphilin (Bolkart, Knobloch and Zenk, 1968). Simultaneously, it was also shown that the quinonoid ring is formed from tyrosine (Bolkart and Zenk, 1968). The structures of homoarbutin and chimaphilin, and

chimaphilin, and the exclusive labeling of the methyl at C-2 in the latter when [β-^{14}C]-tyrosine is the precursor, suggest a reaction sequence involving conversion of tyrosine into homogentisic acid. This pathway is analogous to the one proposed for the biosynthesis of the methylated benzoquinone ring of plastoquinone and tocopherol with the assumed intermediate role of homoarbutin.

Convincing evidence for the involvement of homogentisic acid and aglycone of homoarbutin (toluquinol) has been obtained (Bolkart and Zenk, 1969). It has been demonstrated that [6-^{14}C]-shikimic acid, fed to *Chimaphila*, yields chimaphilin with 50% of the activity in position 2, the anticipated result if the substrate is converted to 2,6-labelled tyrosine with subsequent shift of the side chain to give homogentisic acid (Fig. 4). Since labelled homogentisic acid was likewise very specifically incorporated, and its decarboxylation product, toluquinol, was active (if weakly) when the labelled tyrosine was fed, the biosynthesis of chimaphilin can be established as shown in figure 4.

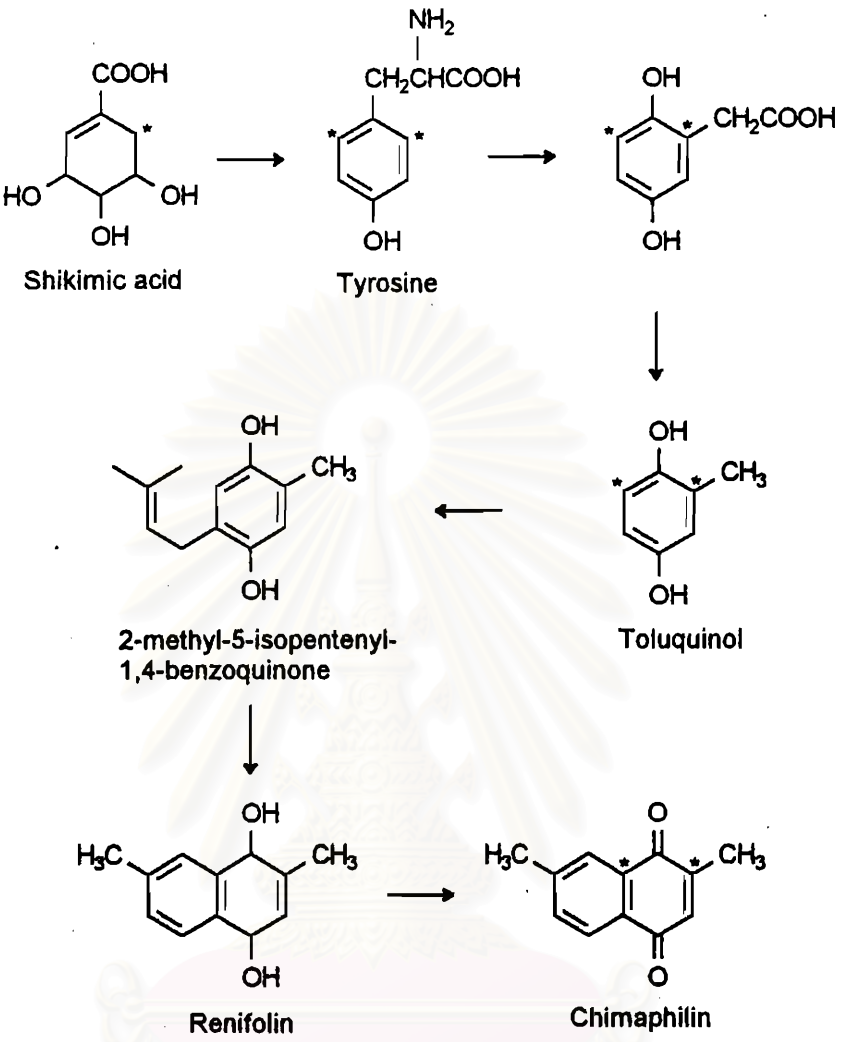

Fig. 4 The proposed biosynthetic pathway of chimaphilin

5.1.3 The o-succinylbenzoic acid pathway

This pathway has been demonstrated as a route for the biosynthesis of bacterial menaquinones and of some plant naphthoquinones and anthraquinones. It involves incorporation of shikimic acid and the intermediate, o-succinylbenzoic acid, into the naphthoquinones (Fig. 5). The plant napthoquinones expected to be synthesized by this route are phylloquinones, e.g. vitamin K_1, distributed in higher plants, lawsone presented in *Impatiens balsamina* and juglone presented in *Juglans regia*.

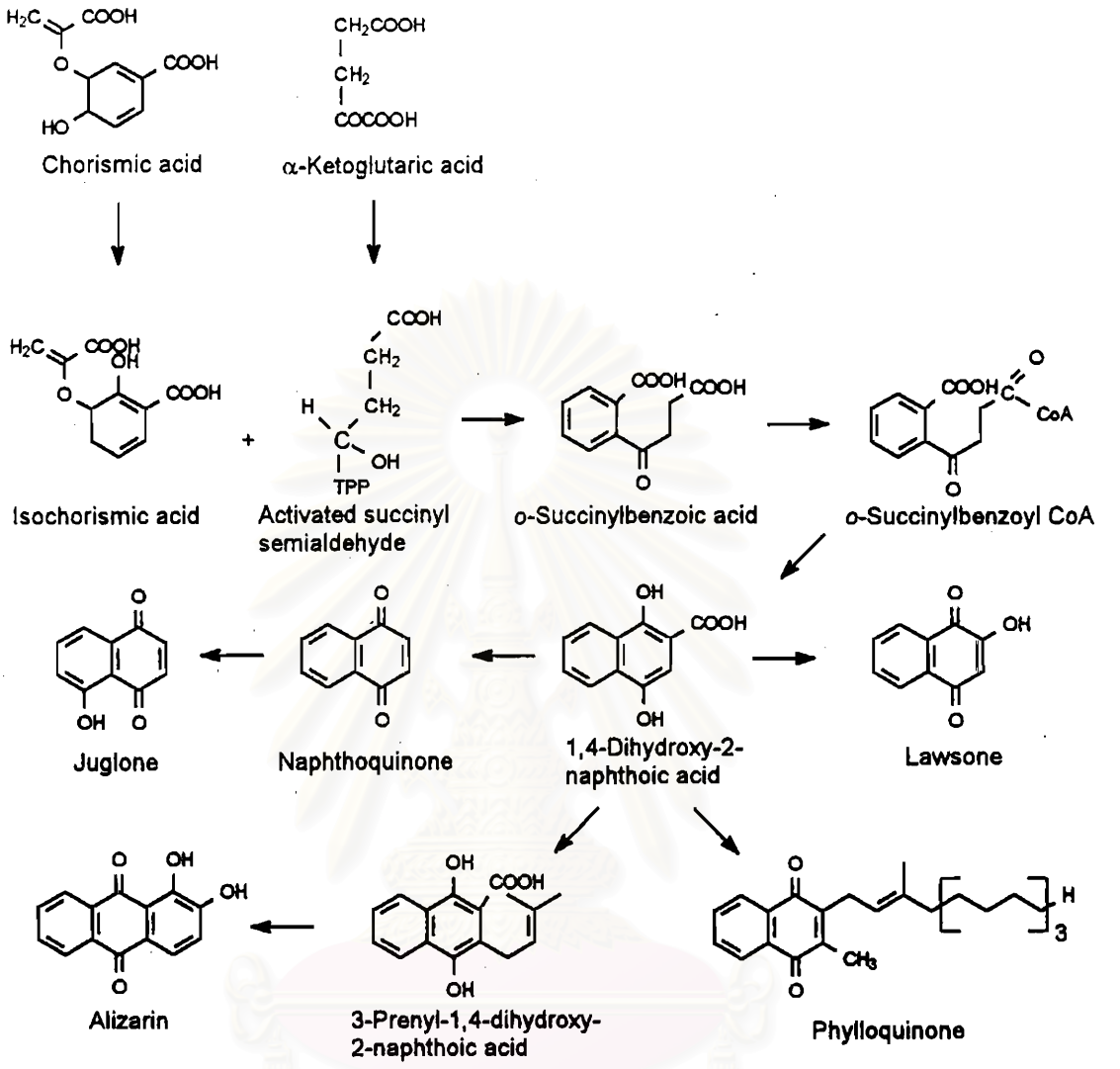

Fig. 5 The proposed biosynthetic pathway of naphthoquinones and anthraquinones by *o*-succinylbenzoate pathway

Experiments with [1,6-$^{14}C_2$] and [3-^{3}H]-shikimic acid establish that lawsone is derived from shikimate with C-1 and C-2 appearing at the naphthoquinone ring junction. The carboxy group of shikimic acid is retained on naphthoquinone formation, and thus accounts for seven of the ten nuclear carbon atoms in the molecule. The remaining C-3 unit has been identified as having its origin from glutamic acid or its transamination product, α-ketoglutaric acid (Campbell *et al.*, 1971; Grotzinger and Campbell, 1974). The very efficient incorporation of *o*-succinylbenzoic acid into plants and bacterial naphthoquinones suggests

that this compound is a later intermediate derived from shikimic acid and α-ketoglutaric acid (Dansette and Azerad, 1970). Further corroboration comes from the identification of radioactive *o*-succinylbenzoic acid in *I. balsamina* following the feeding of [U-^{14}C]glutamic acid (Dansette and Azerad, 1970; Grotzinger and Campbell, 1974). In addition, it has been reported that *o*-succinylbenzoic acid has been isolated from anthraquinone producing cell suspension cultures of *Galium mollugo* (Simantiras, Schmidt and Leistner, 1991).

o-Succinylbenzoic acid had been reported to arise from chorismic acid, α-ketoglutaric acid, and thiamine pyrophosphate, presumably *via* the succinic semialdehyde-thiamine pyrophosphate, derived from decarboxylation of α-ketoglutarate (Fig. 5) (Meganathan, 1981; Meganathan and Bentley, 1983). Evidence has been presented that this decarboxylation is not a function of the α-ketoglutarate dehydrogenase complex, but is carried out by a separate activity (Marley, Meganathan and Bentley, 1986). This clearly confirmed by the separation of the two enzyme activities by chromatography. Subsequently, it has been reported that 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate, an expected intermediate prior to *o*-succinylbenzoic acid has been synthesized by cell-free extract of *menD*$^{+}$ *E. coli* (Emmons, Campbell and Bentley, 1985). The enzyme 2-Succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase has also been detected (Popp, Berliner and Bentley, 1989). Furthermore, it has been demonstrated that at least two intermediates in the conversion of isochorismate to *o*-succinylbenzoic acid were formed by cell-free extracts from anthraquinone producing cell suspension cultures of *Galium* species and from phylloquinone producing cell suspension culture of *Morinda lucida* (Simantiras and Leistner, 1991). One of these intermediates is likely to be identical to 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate. The structure of the second intermediate is as yet known. A third intermediate may also occur.

More recent studies have shown in both microorganism and plant systems that the substrate for biosynthesis of *o*-succinylbenzoic acid is isochorismate, which is derived from chorismate by the action of isochorismate synthase (isochorismate hydroxymutase)

(Weische and Leistner, 1985; Simantiras and Leistner, 1989). Recently, it has been reported that isochorismate synthase has been detected in protein preparations of various anthraquinone producing cell suspension cultures of plants belonging to the family Rubiaceae (Leduc, Petra and Leistner, 1991), and of *Flavobacterium* (Margarethe *et al.*, 1993). The enzyme preparation from *Escherichia coli* has now been shown to catalyze the formation of *o*-succinylbenzoic acid from α-ketoglutarate and isochorismate in the presence of thiamine pyrophosphate. It has also been demonstrated that cell-free preparations from the vitamin-K-producing bacteria *E. coli* and *Aerobacter aerogenes* (*Klebsiella pneumoniae*) contained enzyme *o*-succinylbenzoate synthase which produces *o*-succinylbenzoic acid when incubated with isochorismate and α-ketoglutarate in the presence of thiamine pyrophosphate (Weische, Johanni and Leistner, 1987a). Chromatographic separation showed that an enzyme activity separated from the fractions contain *o*-succinylbenzoate synthase could decarboxylate α-ketoglutarate. This activity is provisionally referred to as the decarboxylating "subunit" or decarboxylating activity of *o*-succinylbenzoate synthase. Both the subunit and the holoenzyme have been characterized (Weische, Garvert and Leistner, 1987b). Incubation of the decarboxylating fraction with these substrates gives succinic semialdehyde. After treatment of the incubation mixture with phosphorylase, the obtained product is believed to be the thiamine adduct of succinic semialdehyde.

The biosynthetic pathway from *o*-succinylbenzoic acid to naphthoquinones proceeds, in most cases, *via* 1,4-dihydroxy-2-naphthoic acid (Meganathan and Bentley, 1979), and requires activation to an unstable mono-coenzyme A ester (Heide and Leistner, 1981). This had been identified as the "aromatic" ester rather than the "aliphatic" ester (Heide, Arendt and leistner, 1982). Further refinement of procedures has led to a reversal of assignment for the position of esterification, and the intermediate has now been confirmed to be "aliphatic" CoA ester of *o*-succinylbenzoic acid (Fig. 5). Synthetic samples of "aliphatic " and "aromatic" CoA ester were incubated with naphthoate synthase fraction from both *E. coli* and *Mycobacterium phlei*. Whereas "aromatic" CoA ester was not converted, "aliphatic" CoA

ester gave good yields of 1,4-dihydro-2-naphthoic acid without any requirement for the cofactor (Kolkmann *et al.*, 1982). The error in the earlier studies was traced back to the fact that the enzymically formed *o*-succinylbenzoic acid mono-CoA ester consisted of both "aliphatic" (85%) and aromatic" (15%) CoA esters, and that the former ester was considerably less stable. Moreover, it has been demonstrated that enzyme preparations from *M. phalli*, *E. coli* and the plant *Gallium mollugo* are all capable of giving coenzyme A ester when incubated in the presence of *o*-succinylbenzoic acid, ATP, coenzyme A, and Mg^{2+} (Kolkmann and Leistner, 1987a). Comparison with the synthetic CoA esters (Kolkmann and Leistner, 1987b) has confirmed this intermediate to be the "aliphatic" CoA ester. Minor amounts of the "aromatic" CoA ester and the diester were also produced, but these were not enzymatically convertible to 1,4-dihydronaphthoic acid, whereas the "aliphatic" CoA ester was smoothly transformed.

Recently, *o*-succinylbenzoate:coenzyme A ligase, an enzyme catalyzing the formation of *o*-succinylbenzoate-CoA ester, has been purified and characterized from *M. phlei* (Sieweke and Leistner, 1991), and *Galium mollugo* cell culture (Sieweke and Leistner, 1992). It has been shown that the plant and the bacterial enzymes are completely different with respect to specificity, molecular weight and mechanism of activation.

5.2 The polyketide pathway

The biosynthesis of polyketides (Manitto and Sammes, 1981; Luckner, 1990) is of minor significance in plants, but is particularly well developed in bacteria, fungi and lichens. Polyketides are formed by multifunctional proteins, from a starter molecule, in most case acetyl CoA. The starter molecule reacts with several molecules of malonyl CoA, methylmalonyl CoA or ethylmalonyl CoA, as extended units, with loss of the free carboxyl group of the latter. The synthesis of polyketides resembles the formation of fatty acids, in terms of the precursors used and the mode of their alignment. In both cases the formation of linear chains proceeds by the addition of C_2 units. In contrast to the formation of fatty acids, however, the biosynthesis of polyketides proceeds without obligatory reduction of

intermediates. Most poly-β-ketoacids are probably formed. Such compounds are very reactive since they contain both active methylene groups (potential nucleophiles) and carbonyl groups (potential electrophiles). They seem to be attached to the core unit of the enzyme complex, stabilized by hydrogen bonding or by chelation of their semienolates with metal ions held by the enzymes. The spatial arrangement of the poly-β-ketoacid leads to intramolecular reactions, either by aldol condensation, i.e. the reaction of carbonyl groups with acidic CH_2-groups, or Claisen condensation, i.e., the reaction of the ester group at the head of molecule with one of the acidic CH_2-groups. The actual type of the condensation depends on the nature of the polyketide synthetase in question and the spatial arrangement of the poly-β-ketoacid directed by the enzyme.

The polyketide route to the naphthalene nucleus and consequently of naphthoquinones, is well established in microorganisms but it appears to be quite rare in higher plants, where other biosynthetic pathways are preferred. Plumbagin (2-methyljuglone) and 7-methyljuglone are the first naphthoquinones in higher plants showed to be formed according to the polyketide pathway (Manittoand Sammes, 1981; Herbert, 1989; Luckner, 1990). It has been demonstrated that the two naphthoquinones synthesized by plants of the *Drosera* and *Plumbago* genera, arise from a hexaketide (Fig. 6) rather than the shikimic acid route as occurs for juglone and menadione (Durand and Zenk, 1971). Plumbagin, by its striking structural similarity with juglone and menadione, was first expected to be formed *via* the shikimate pathway by C-methylation of juglone in the 2-position. Feeding experiments with young shoots of *Plumbago europaea* L., however, showed that neither [7-^{14}C]-shikimate, [$^{14}CH_3$]-L-methionine, [β-^{14}C]-DL-tyrosine, [ring-1-^{14}C]-DL-phenylalanine, nor [5-^{14}C]-DL-mevalonic acid was incorporated into plumbagin to a significant extent. In contrast, [1-^{14}C]-, [2-^{14}C]-acetate and [2-^{14}C]-malonate labelled this naphthoquinones heavily. This fact suggests that the plumbagin is formed by the well known polyketide pathway. In addition, it has been confirmed by the chemical degradation of the labelled plumbagin, either from [1-^{14}C]- or [2-^{14}C]- acetate, that the polyketide pathway is the route used in the biosynthesis of plumbagin. Similar results have been obtained by labelled acetate feeding to different

Droseraceae species and degradation of labelled plumbagin. The co-occuring 7-methyl-juglone in *Drosera* plants is also formed *via* the polyketide route (Durand and Zenk, 1971).

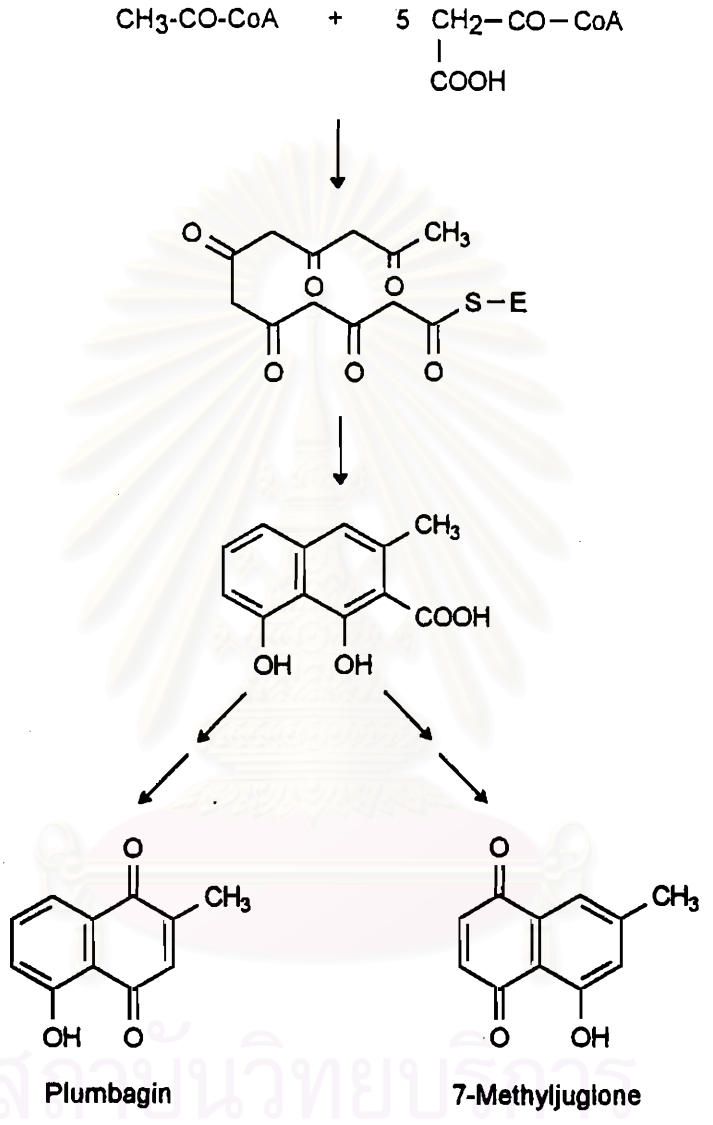

Fig. 6 The proposed biosynthetic pathways of some polyketide-derived naphthoquinones

6. GENERAL CONSIDERATION ON SECONDARY METABOLITES

Organic compounds derived from higher plants may be classified as either primary or secondary metabolites. Primary metabolites are substances that are widely distributed in nature, occurring in one form or another in virtually all organisms. In higher plants, such compounds are often accumulated in seed and vegetative storage organs, such as rhizomes

and tubers. These compounds are needed for general growth and physiological development because of their role in basic cell metabolism. In contrast, secondary metabolites are biosynthetically derived from primary metabolites, but are more limited in distribution in the plant kingdom, usually being restricted to a particular taxonomic group (species, genus, family, or closely related group of families). Secondary metabolites are usually not distributed uniformly within the whole plant. Some are restricted to specific organs, e.g., the root or seed, others to specific tissues such as the epidermis. The secondary metabolites have no obvious roles in a plant's primary metabolism, since they are non-nutritive and are thus not directly essential for growth. The secondary compounds, however, often play ecologically significant roles in how plants deal with their environment, and are therefore important in their ultimate survival (Balandrin and Klocke, 1988).

A characteristic feature of higher plants is their capacity to produce a large number of secondary metabolites. For a long time these compounds were regarded as waste products which had interesting structures and, in many cases, exhibited biological activities (Wink, 1990). However, a rapid increasing of experimental evidence indicates that most secondary metabolites are (a) defense against herbivores (insects, mollusces, vertebrates); (b) defense against microorganism (viruses, bacteria, fungi); (c) display metabolic or further ecological functions such as UV-protection, attraction of pollinating or seed-dispersing animals, chemical adaptations to environmental stresses. Thus, since plant secondary metabolites serve basically to combat infectious disease, to aid in weed aggressiveness, and to discourage herbivores (in addition to roles as attractants for pollinators), they are by definition biologically and/or physiologically active compounds. As a consequence, a number of these compounds are economically important, serving as pharmaceuticals, aromatics, fragrances, stimulants, colors, or pesticides.

Secondary metabolites are generally metabolically expensive to produce and accumulate, and are therefore frequently presented in plants in much smaller quantities than are primary metabolites. However, in nature the accumulation of large quantities of secondary metabolites is not usually required, since these compounds have been selected,

by means of evolution, for relative potent biological activity. In addition, secondary metabolites, in contrast to primary metabolites, tend to be biosynthesized in specialized cell types and at distinct developmental stages, making their extraction, isolation, and purification difficult. As a result, secondary metabolites that are used commercially as biologically active compounds are generally higher value-lower volume products than are primary metabolites. In addition, many secondary metabolites are chiral molecules and this often presents an obstacle to their chemical synthesis. Therefore, biological product of these compounds is usually a more economic alternative. In order to overcome the problems and restrictions that are inherent in intact plant production, plant tissue cultures have been investigated as a possible source of valuable compounds (Banthorpe, 1994).

7. SECONDARY PRODUCTS FROM PLANT CELL AND TISSUE CULTURES

As the natural habitats for wild plants become endangered, and environmental and geopolitical instabilities make it difficult to acquire certain plant derived chemicals, it may become critical to develop alternative source of important natural plant products. There has been considerable interest in investigating the potential of plant cell cultures as an alternative to traditional agriculture for the industrial production of secondary plant metabolites (Heinstein, 1985; Neumann, Braz and Reinhard, 1985).

Many of the secondary metabolites have already been produced by plant cell culture technique and the versatility of cultures to produce a range of secondary products is illustrated in Table 2. Cultures from a group of taxonomically diverse higher plants have yielded a range of compounds including quinones, phenylpropanoids, isoprenoids, alkaloids. Yet despite the undoubted capability of plant cell cultures to produce secondary compounds, it has proved difficult to harness this capability to industrial processes.

Cell suspension systems, however, could be used for the large-scale culturing of plant cells from which secondary metabolites can be extracted. The principal advantage of this technology is that it may ultimately provide a continuous, reliable source of natural

Table 2 Selected examples of useful secondary products from plant cell and tissue cultures

Class of compound	Compound	Plant species	Culture type	Reference
Terpenoid	ginsenosides R_{b1}, R_{g1}	*Panax ginseng*	suspension	Furuya, 1988
	limonene	*Pelargonium fragans*	callus culture	Sauerwein *et al.*, 1992
	chrysanthemic acid	*Chrysanthemum cinerariaefolium*	callus culture	Sauerwein *et al.*, 1992
	dihydroleucodin	*Artemisia douglasiana*	callus culture	Sauerwein *et al.*, 1992
	geranylgeraniol	*Croton sublyratus*	suspension	Kitaoka *et al.*, 1989
	sclareol	*Salvia sclarea*	suspension	Sauerwein *et al.*, 1992
	cycloartenol	*Nicotiana tabacum*	suspension	Furuya, 1988
	terpinolene, terpinen-4 ol	*Thuja occidentalis*	suspension	Ellis, 1988
	α-pinene	*Pinus radiata*	callus culture	Ellis, 1988
	quassin	*Picrasma* sp.	callus and suspension	Banthorpe, 1994
Cardenolide	digitoxin	*Digitalis purpurea*	suspension	Furuya, 1988
	digoxin, digitoxin	*Digitalis lanata*	liquid culture	Ohlsson *et al.*, 1983
Alkaloid	nicotine, anabasine	*Nicotiana tabacum*	suspension	Furuya, 1988
	morphine, codeine	*Papaver somniferum*	suspension	Furuya, 1988
	sanguinarine	*Papaver somniferum*	suspension	Eilert *et al.*, 1985
	berberine, coptisine	*Coptis japonica*	suspension	Furuya, 1988
	caffeine	*Coffea arabica*	suspension	Furuya, 1988
	canthin-6-one	*Ailanthus altissima*	suspension	Anderson *et al*, 1983
	atropine	*Atropa belladonna*	hairly root	Kamada *et al.*, 1986
	hyoscyamine, scopolamine	*Duboisia leichhardtii*	roo culture	Endo and Yamada, 1985
	hyoscyamine	Solanaceous spp.	hairy root	Sauerwein *et al.*, 1992
	emetine, cephaeline	*Cephaelis ipecacuanha*	callus and root	Sauerwein *et al.*, 1992
	catharanthine	*Catharanthus roseus*	suspension	Banthorpe, 1994
	quinine, quinidine	*Cinchona ledgeriana*	root culture	Anderson *et al.*, 1982
	reserpine	*Rauwolfia serpentina*	suspension	Yamamoto and Yamada, 1986
Phenolic	anthraquinones *via* O-succinyl-benzoic acid	*Morinda citrifolia*, *Galium* spp.	suspension	Zenk et al., 1975; Bauch and Leistner, 1978
	shikonin	*Lithospermum erythrorhizon*	suspension	Fujita *et al.*, 1985
	tannins	*Geranium thunbergii*	suspension	Sauerwein *et al.*, 1992
	anthocyanins	*Euphorbia millii*	suspension	Sauerwein *et al.*, 1992
	rosmarinic acid	*Anchusa officinalis*	suspension	Wanchai De-eknamkul and Ellis, 1984
Steroids	diosgenin	*Dioscorea deltoidea*	suspension	Banthorpe, 1994
Lignan	podophyllotoxin	*Podophyllum hexandrum*, *Linum flavum*	suspension	Van-Uden, 1993

products, e.g. tobacco biomass produced in 20,000 l fermenters over a continuous period of 66 days resulted in a product of uniform quality which is independent of climatic or geographic factors (Hashimoto et al., 1982). In addition, compounds from tissue cultures may be more easily purified because of simpler extracts and absence of significant amount of pigments, thus possibly reducing production and processing costs.

The basic technologies for obtaining high yields of specific secondary metabolites from large-scale cultures are still being developed. In some instances two-stage media are used, a growth medium to produce biomass as quickly as possible is followed by a production medium which is used to encourage formation of secondary metabolites. The composition of the media is often critical for secondary compound production, e.g. cell suspension cultures of *Lithospermum erythrorhizon* grown in Linsmaer-Skoog medium fail to produced shikonin whereas White's medium yield shikonin (Fujita and Tabata, 1987). The development of stable high-yielding cell line is a prerequisite for any commercial process designed to produce specific secondary compounds. In an attempt to increase productivity, prolong production, protect sensitive cells, or to release products, plant cell cultures have been immobilized on a variety of inert supports (Rhodes, 1985; Brodelius, 1986; Yeoman, 1986).

In higher plants, high concentrations of secondary metabolites tend to accumulated in specific cell types at specific developmental stage. One of the reasons why some cell cultures fail to accumulate secondary compounds that are present in the parent plants may be due to the lack of storage facilities, e. g. alkaloids of *Papaver* species are located in a latex system, essential oil may be found within glandular trichomes. Although some plant cell cultures fail to accumulate secondary compounds, some of them can produce higher product levels than the intact plant. Thus, in some cases expression of a biosynthetic pathway is evidently better in cultured cells. It has been, however, demonstrated that when morphological differentiation is induced in those cell cultures, product level increases distinctly (Lindsey and Yeoman, 1983; Hagimori, Matsumoto and Mikami, 1984; Yoshikawa

and Furuya, 1985). Thus, a possible approach to increase yields of secondary products is to allow differentiation of tissue by production of shoot or root cultures.

8. ACCUMULATION OF SECONDARY PRODUCTS BY ORGANIZED PLANT CULTURES

The differentiation of plant tissue *in vitro* to produce callus or cell suspension cultures is usually accompanied by an apparent loss of ability to accumulate secondary metabolites. There are several given reasons for that non-accumulation, such as (i) the lack of gene expressions in unspecialized cells that control the essential steps in the biosynthetic pathway; (ii) the diversion of substrate away from secondary product formation; (iii) the inoperation of transport mechanisms by which potentially toxic end-products may be removed from the biosynthetic site; (iv) the unavailability of storage sites in which secondary metabolites would normally be sequestered; (v) the unregulated catabolism of synthesized product.

The differentiation of unorganized tissue, however, often leads to the partial or complete restoration of the capability to accumulate secondary metabolites. Furthermore, organ-specific differentiation enables biomass production, and actually expresses both their biosynthetic and accumulative potential towards the target compounds.

The basic techniques required to initiate and maintain excised root cultures were established as early as the 1930s and further modifications and refinements in methodology were reviewed by Butcher and Street (1964). The shoot cultures may be developed from axillary meristem or shoot tips grown on solid or liquid media supplemented with suitable plant growth regulators (Heble, 1985). Alternatively, shoot cultures may be obtained by the induction of organogenesis on submerged callus-aggregates by appropriate choice of media composition and environmental conditions (Charlwood and Moustou, 1988).

Although the root cultures of more than 70 species from 21 families of angiosperms and gymnosperms has been established, little attention has been paid to the production of secondary metabolites in these systems. More recently work, however, has extended the

types of metabolites accumulated in root cultures to include alkaloids, terpenoids, steroids, and phenolic compounds (Flores, Hoy, and Pickard, 1987). Some secondary metabolites produced by cultured roots and shoots are shown in Tables 3 and 4, respectively. As an ability to increase the rate of biomass production within such cultures increase (perhaps by transformation with *Agrobacterium*), organized cultures may also become appropriate for commercial production of selected, high-value secondary products.

In short-terms, the use of organized cultures to study the biosynthesis, transport, storage, and turnover of natural products presents an attractive proposition. For a wide range of natural products, particularly those that are synthesized or stored in specialized structures in the intact plant, organized cultures may provide the best available model system for biochemical and physiological studies.

Table 3 Examples of the useful secondary metabolites accumulated in various root cultures.

Plant source	Accumulated compound	Yield cf. with intact plant	Reference
Atropa belladonna	hyoscyamine, scopolamine	1 x cf. root	Hartmann *et al.*, 1986
Calystegia sepium	hyoscyamine, scopolamine	8 x cf. root	Jung and Tepfer, 1987
Datura stramonium	hyoscyamine	-	Hashimoto *et al.*,1986
Duboisia leichhardtii	hyoscyamine, scopolamine	0.8 x cf. root	Endo and Yamada, 1985
Hyoscyamus albus, H. niger	hyoscyamine, scopolamine	up to 3 x cf. root	Hashimoto *et al.*, 1986
Catharanthus roseus	ajmalicine, catharanthine	1 x cf. root	Endo *et al.*, 1987
	vinblastine	much lower cf. plant	Miura *et al.*, 1987
Amsonia elliptica	pleiocarpamine	-	Sauerwein *et al.*, 1992
Cinchona ledgeriana	quinine, quinidine, cinchonine, cinchonidine	much lower cf. bark	Hay *et al.* 1986
Cephaelis ipecacuanha	cephaeline, emetine	1 x cf. root	Teshima *et al.*, 1988
Papaver somniferum	codeine, cryptopine, thebaine	much lower cf. plant	Staba *et al.*, 1982
Stephania cepharantha	aromoline, berbamine, cycleanine, homoaromoline	higher cf. tuber	Sugimoto *et al.*, 1988
Senecio eruciifolius	senecionine, seneciphylline, intergerrimine, eruciifoline A, B	1 x cf. root	Toppel *et al.*, 1987
Centranthus ruber	valepotriates	1 x cf. root	Violon *et al.*, 1984
Zingiber officinale	geranial, neral, 6-gingerol	much lower cf. rhizome	Charlwood *et al.*, 1988
Digitalis purpurea	digitoxin	1 x cf. root	Rucker *et al.*, 1983
Solanum chacoense	dehydrocommersonine	none in tuber	Zacharius and Osman, 1987
Linum flavum	5-methoxypodophyllotoxin, coniferin	10 x higher cf. leaf	Berlin *et al.*, 1988
Lobelia inflata	lobeline	-	Ishimaru *et al.*, 1992
Swertia japonica	amarogentin	-	Sauerwein *et al.*, 1992
Sanguisorba officinalis	tannins	-	Sauerwein *et al.*, 1992

Table 4 Examples of the useful secondary metabolites accumulated in various shoot cultures.

Plant source	Accumulated compound	Yield cf. with intact plant	Reference
Atropa belladonna	hyoscyamine, scopolamine	0.07 x cf. plant shoot	Benjamin *et al.*, 1987
Datura innoxia	hyoscyamine, scopolamine	0.07 x cf. leaves	Hiraoka and Tabata 1974
Catharanthus roseus	ajmalicine, catharanthine, vindoline	1 x- 8 x cf. plant	Hirata *et al.*, 1987
	3',4'-anhydrovinblastine	0.3 x cf. leaves	Endo *et al.*, 1987
	vinblastine	lower cf. leaves	Miura *et al.*, 1988
Rauwolfia serpentina	ajmalicine, ajmaline, yohimbine	1.4 x cf. young shoot	Roja *et al.*, 1987
Cinchona ledgeriana	quinine, quinidine, cinchonine	0.2 x cf. leaves	Chung and Staba, 1987
	cinchonidine	0.01 x cf. bark	Chung and Staba, 1987
Papaver somiferum	thebaine, codeine, morphine	much lower cf. plant	Kamo *et al.*, 1982
Chrysanthemum cinerariaefolium	pyrethrin, cinerin, jasmolin	-	Zieg *et al.*, 1983
Digitalis lanata	digitoxin	0.4 x cf. leaves	Lui and Staba, 1979
	odoroside H, odorobioside G, verodoxin	not present in adult plant	Seidel and Reinhard, 1987
D. purpurea	digitoxin	0.1 x cf. leaves	Hagimori *et al.*, 1980
Dioscorea composita	diosgenin	0.7 x cf. tuber	Datta and Datta, 1984
Yucca schidigera	sarsasapogenin, smilagenin	0.3 x cf. mature rhizome	Kaneda *et al.*, 1987
	markogenin, samogenin	0.66 x cf. mature rhizome	Kaneda *et al.*, 1987
	gitogenin, neogitogenin	0.54 x cf. mature rhizome	Kaneda *et al.*, 1987
Pelargonium fragans	pinene, sabinene, farnesene, carvone, cadinene	0.5 x cf. leaves	Charlwood and Moustou, 1988
P. tomentosum	menthone, isomenthone	0.5 x cf. leaves	Charlwood and Moustou, 1988
Solanum nigrum	solasodine	0.35 x cf. leaves	Bhatt *et al.*, 1983
Citrus paradisi	naringin	0.18 x cf. seedling leaves	Barthe *et al.*, 1987
Pimpinella anisum	anethole	0.056 x cf. shoot	Reichling *et al.*, 1988
Lippia dulcis	hernandulcin	-	Sauerwein *et al.*, 1992
Mentha citrata	linalool, linalyl acetate	-	Sauerwein *et al.*, 1992

9. PLANT CELL AND TISSUE CULTURES AS SOURCE OF NOVEL PHYTOCHEMICALS AND ENZYMES

It is obvious that plants represent a nearly unlimited source of primary and secondary metabolites. Especially the secondary metabolites are of major interest because of their different functions and their impressive biological activities ranging from e.g. insecticidal, antimicrobial, antioxidant, hormonal properties to highly important pharmacological activities. Recently, an increasing number of investigations are carried out to detect novel bioactive biochemicals in intact plants which could be useful directly in the treatment of a variety of diseases or as starting material for partial synthesis of highly valuable compounds. On the other hand, interest is focused on the formation of such plant secondary products by plant cell and tissue culture systems in order to understand the biosynthesis of these compounds, to isolate the involved enzyme as biocatalysts and finally to manipulate and improve the product synthesis in those system.

9.1 Novel secondary products from cell and tissue cultures

Cell and tissue cultures often accumulate the secondary products different quantitatively in their spectrum from the parent plant and, in some cases, qualitative changes also occur. These might be due to metabolic blocks or the level of trace metabolites being amplified by the lack of normal controls on rates of formation and degradation. If genetic aberrations are excluded, it must be presumed that the ability to synthesize any novel metabolites must be present in the parent tissue even if this ability is not expressed under normal growing conditions (Banthorpe, 1994).

An example of unexpected metabolism in plant tissue culture is *Andrographis paniculata* (Overton, 1977), where the intact plant synthesizes a group of diterpenoids with an ent-labdatriene skeleton, as typified by andrographolide. The callus culture, however, produces a group of sesquiterpenoids, base on γ-bisabolene, typified by paniculide C. It has been reported that dimeric alkaloids, voafrine A and B, isolated from cell suspension cultures of *Voaconga africana* Stapf, have not previously been found in plants (Stockigt *et al.*, 1983).

These new alkaloids originates from the dimerization of the monomeric alkaloid tabersonine. The coumarins isofraxidin and scopoletin occur in plant tissue cultures of a variety of the Compositae but again are either absent or at low levels in the intact plants. This may be a marker for the family and represents the remnants of a primitive metabolism that is negligible *in vivo* and is expressible only when the later-developed complex patterns of metabolism can not be achieved in plant tissue cultures (Banthorpe and Brown, 1989).

Pharmacological screening of various cell cultures led to the isolation of several new natural products. Through opiate receptor binding studies, two new alkaloids pericine and pericalline were isolated from cell suspension cultures of *Picralima nitida* (Arens *et al.*, 1982). Cell suspension cultures of *Plagiorhema dubium* have been reported to yield the anti-inflammatory compound, dedihydrodiconiferyl-alcohol-α-D-glucoside (Arens *et al.*, 1985). The new prenylated flavonoids, podoverines A, B and C, have been isolated from cell mass of *in vitro* cultures of *Podophyllum versipelle* (Arens *et al.*, 1986).

It has been reported that colchicine-treated suspension cultures of *Valeriana wallichii* yielded a series of new valepotriates (Becker *et al.*, 1984). These new compounds have the basic iridoid skeleton, but the substitution pattern differs from the pattern in known valepotriates of the intact plants. Feeding of tryptophan to *Cinchona* or *Phaseolus* spp. led to the formation of alkaloids with the β-carboline skeleton that had not been reported in these genera (Veliky and Barber, 1975).

The number of new phytochemicals isolated and identified exclusively from cultivated cells is continually increasing. In 1989, 85 new compounds were reported and the number of novel structure increases to about 140, in 1992 (Stockigt, *et al.*, 1992). It must, however, be mentioned that a number of these new phytochemicals also come from biotransformation experiments with plant tissue cultures. This points to the variety of applications using cultivated cells for the biosynthesis of novel secondary products, and also indicates that cell and tissue cultures now have a good chance of being used as a promising screening source of new drugs for the pharmaceutical industry.

9.2 Plant cell and tissue culture as source of enzymes

In fact, the exact molecular reasons why *de novo* synthesis of many secondary metabolites is lacking or unsatisfactory low in cultured cells are unknown. Nevertheless, currently there are many pieces of evidence showing that the corresponding biosynthetic enzymes are not present or not well expressed in these cultures. Absence of *de novo* synthesis may be due to the absence of all biosynthetic enzymes or to the lack of only one or a few enzymes. For instance, the lack of serotonin biosynthesis by *Peganum* cell cultures could clearly be traced to the lack of tryptophan decarboxylase (Berlin and Sasse, 1988), but these nonproducing cell cultures were able to transform large amounts of tryptamine to serotonin. Thus, even nonproducing cultures may be used for specific biotransformation or as a source of enzyme of a seemingly unexpressed pathway. The enzymes of a blocked pathway that are present and highly active in a nonproducing culture has to be tested in each case. The most famous example in this field is the 12-β-hydroxylation of β-methyldigitoxin to β-methyldigoxin by *Digitalis lanata* cell cultures which are unable to produce cardiac glycosides *de novo* (Alfermann, Spieler and Reinhard, 1985).

It has been shown that plant cell cultures metabolize a great variety of exogenously supplied substrates (Reinhard and Alfermann, 1980). Consequently, interesting enzymes may also be isolated from plant cells. However, without knowing which interesting enzymes can be found in cell cultures, a discussion of the enzymatic potential of plant cell cultures remains speculative. The potential of *de novo* synthesis of products from many culture systems is known. Instead of wasting too much time with improving total product levels of such systems by conventional techniques, it seems more appropriate to check whether these cultures contain interesting enzyme activities. The cell cultures are indeed suitable for the detection of novel enzyme activities, as has been best demonstrated for flavonoid biosynthesis (Hahlbrock and Grisebach, 1979) and for isoquinoline and monoterpenoid indole alkaloids (Zenk, 1980; Zenk *et al.*, 1985).

Plant cell and tissue cultures have also provided material for numerous studies on the mechanism of secondary product biosynthesis using either direct feeding techniques, cell-free extracts or purified enzymes. The implicit and reasonable assumption is that the routes in culture are the same as those in the parent plants. Plant cell and tissue cultures offer a number of advantages over intact plants for these studies. They are relatively easy to establish and maintain under strictly controlled nutritional and environmental conditions. They can be grown in either small containers, e.g. 250 ml Erlenmeyer flasks, or large fermentors, and thus the amount of biomass are sufficiently available as needed. As cell suspension cultures often consist of a relatively homogeneous population of cells, they can be rapidly and uniformly exposed to exogenously added chemical agents, that often a difficult task when dealing with the intact plants. Finally, because of their limited degree of cell differentiation cell cultures generally display simple metabolic composition.

In spite of these advantages, the use of plant cell and tissue cultures for metabolic studies has so far been limited, mainly because of the special metabolic characteristic imposed by the usual culture environment. In the case of secondary metabolism, the cultures of many species do not produce significant amount of the compound characteristic of intact plants. This may sometimes be due to the loss of genetic information in the cultured cells. It is more likely that the failure of cultured tissue to produce the pattern or level of secondary metabolites typical of the parent plant is a consequence of the specific physiological and morphological state of cultured tissue.

In numerous plant cell cultures, the biosynthetic pathways of secondary metabolites can be expressed at a level and rate much higher than intact plant. Such cell cultures are therefore valuable experimental system to elucidate the biosynthesis and enzymology of their secondary metabolites, for example, the biosynthetic studies of shikonin in cell suspension culture of *Lithospermum erythrorhizon* (Yazaki *et al.*, 1986; Heide and Tabata, 1987b). Use of cell-free extracts from *Berberis* suspension culture allowed elucidation of the stereo and regiospecificity of the steps from reticuline to columbarine, berberine, palmatine and jatrorrhizine (Ruefler and Zenk, 1986).