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Chasing the Key Enzymes of Secondary Metabolite—Biosynthesis from Thai Medicinal Plants

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Abstract: Novel enzymes involved in the biosynthesis of plant secondary metabolites have been discovered in our laboratory from both differentiated plants and *in vitro* cultures of Thai medicinal plants. The discovered enzymes included (1) geranylgeraniol-18-hydroxylase from *Croton sublyratus* containing plaunotol, an anti-peptic ulcer diterpenoid, (2) dopamine-secologanin condensing enzymes from *Alangium lamarckii* containing emetine, an amoebicidal tetrahydroisoquinoline monoterpene alkaloid, (3) lawsone-forming multienzyme complex from *Impatiens balsamina* root cultures containing lawsone, an antimicrobial naphthoquinone, and (4) 1,2-dehydroreticuline reductase from *Papaver somniferum* seedlings containing morphinan alkaloids. Each of these enzymes was isolated, partially purified, characterized and evaluated for its role involved in the biosynthetic pathway of each secondary metabolite accumulated in its producing plant.

INTRODUCTION

The tropical forest of Thailand has been considered as one of the world's regions of origin and diversity of species. It can be viewed as a huge factory producing an enormous diversity of natural products. The complexity and species diversity also represent an important genetic bank from which potential medicinal, agricultural and other commercial products could be derived. Currently, there are research programs that are being carried out in Thailand to screen plants for natural products that are potentially important for medicinal applications. More and more information is available on the biological activities of natural products isolated from Thai medicinal plants. However, very little is known about how plants synthesize these substances, and almost nothing is known about how the synthesis is regulated at the genetic level. The questions on gene expression of plant secondary products are still widely open and are the subject of interest to our research group.

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Since information about enzymes involved in the biosynthesis of secondary metabolites is a prerequisite for metabolic engineering of medicinal plants, we set up a research program with an aim to search for various biosynthetic enzymes of pharmaceutically important compounds found in Thai medicinal plants. Both *in vitro* cultures (e.g. cell cultures, organ cultures etc.) and differentiated plants can be used as starting material of this study although plant cell cultures capable of producing high content secondary products are considered being excellent sources of enzyme systems. For example, *Anchusa officinalis* cell cultures producing high content of rosmarinic acid (refs. 1-3) have been used for searching the entry-point enzymes of rosmarinic acid pathway (refs. 4-7). *Eschscholtzia californica* and *Thalictrum bulgaricum* cell cultures producing benzophenanthridine alkaloids have been used for elucidating the biosynthetic pathways of sanguinarine (for review, see ref. 8), chelirubine (ref. 9) and macarpine (ref. 10). However, differentiated plants and organs have also been used suceessfully for enzymological studies of biosynthetic pathways of secondary products. For example, the discovery of the enzymes geranylgeraniol-18-hydroxylase from *Croton sublyratus* leaves (ref. 11), dopamine-secologanin condensing enzymes from *Alangium lamarckii* leaves (ref. 12), 1,2-dehydroreticuline reductase from *Papaver somniferum* seedlings (ref. 13,14) and lawsone-forming multienzyme complex from *Impatiens balsamina* cultured roots (ref. 15). These studies, which are described here in more detail, show clearly the potential of using the whole plants for elucidating the biosynthetic pathways of secondary metabolites.

GERANYLGERANIOL-18-HYDROXYLASE

Plaunotol (1), the active ingredient of a commercial drug named Kelnac®, is a mucosal protective factor-enhancing antiulcer agent. It was originally found in the leaves of *Croton sublyratus* Kurz. (Thai name : Plaunoi, Euphorbiaceae) (ref. 16), a tropical plant distributed throughout Southeast Asia. Although the structure of plaunotol has been known for almost twenty years, very little is known about its biosynthetic pathway. Based on its structure, however, the biosynthesis of plaunotol in this plant could be simple since the compound is a 18-hydroxy derivative of geranylgeraniol (GGOH), a common precursor of all natural diterpenoids. It is well documented that GGOH is biosynthesized *via* the terpenoid pathway and its immediate precursor is geranylgeranyl diphosphate (GGPP). Therefore, it is reasonable to propose that plaunotol is biosynthesized from GGPP by two steps of enzymatic reactions (Fig. 1). First, GGPP is hydrolysed by a phosphatase enzyme to form GGOH . Second, GGOH is hydroxylated at C-18 position by a specific 18-hydroxylase to form plaunotol. Until now, there has been no report to support this proposed pathway. Due to the availability of *C. sublyratus* plants in Thailand, we decided to use the whole plant as a source for searching the enzyme activity of GGOH-18-hydroxylase (ref. 11). The leaf part was chosen as the material for the study since it accumulates plaunotol and is potentially the site of plaunotol biosynthesis.

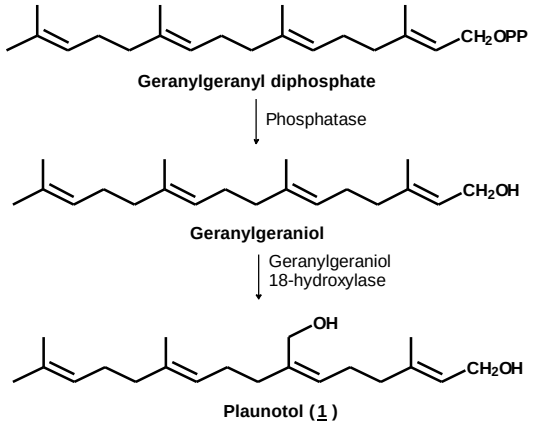

Fig. 1 Proposed biosynthetic pathway of plaunotol

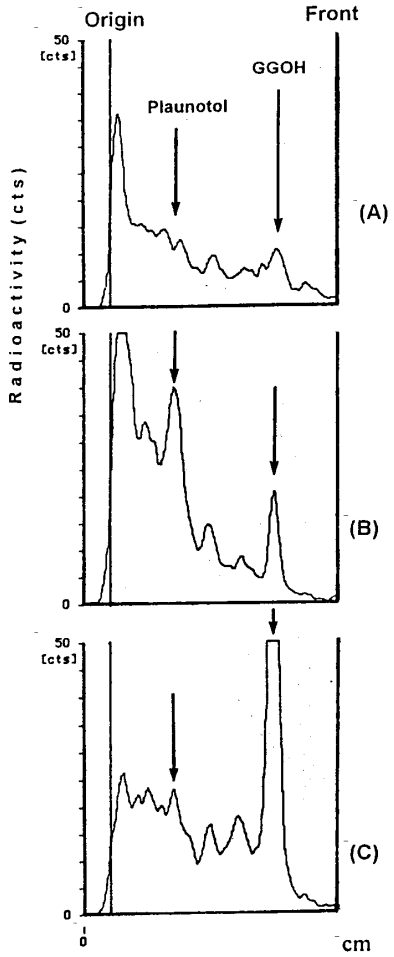

Fig. 2 TLC radiochromatograms of the reaction mix - tures containing [1-^{3}H]GGPP and either 3,000 g pellet (A), 20,000 g pellet (B), or 20,000 g supernatant (C)

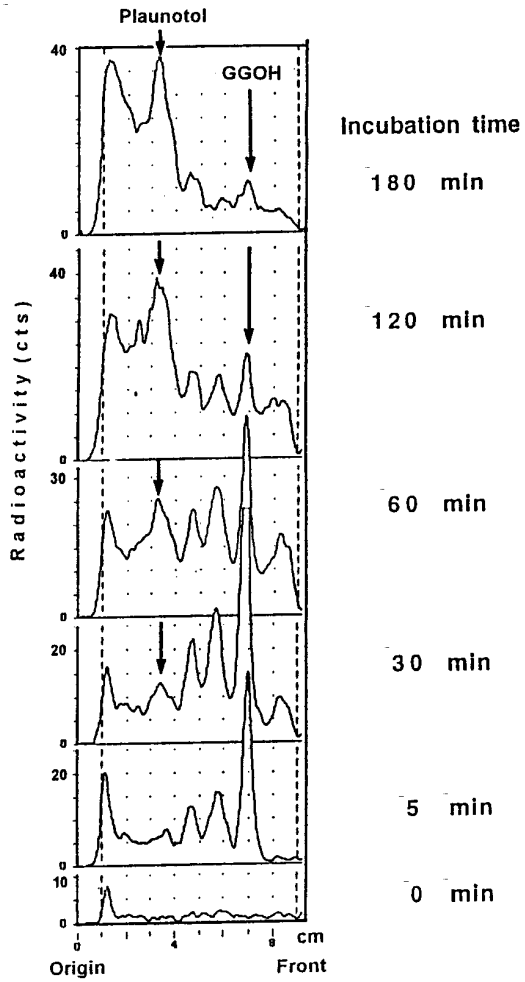

Fig. 3 Time-course of the conversion of [1-^{3}H]GGPP to [1-^{3}H] plaunotol by 20,000 g pellet fraction of *Croton sublyratus* leaves

Enzyme detection using [1-^{3}H]GGPP as substrate revealed that the hydroxylase activity was present primary in the 20,000 g pellet fraction whereas GGPP phosphatase activities were present in both 20,000 g pellet and 20,000 g supernatant (Fig. 2). In order to confirm the proposed biosynthetic sequence of plaunotol from GGPP shown in Fig. 1, a time-course of the conversion of [1-^{3}H] GGPP by the 20,000 g pellet was examined. As shown in Fig. 3, it appeared that [1-^{3}H] GGPP was rapidly converted within 5 min to the major peak of GGOH. The peak increased maximally at 30 min followed by its continuous decrease until almost disappearing at 180 min. Simultaneously, the radioactive peak of plaunotol showed a continuous increase during the whole time-course of the incubation. These results suggested that GGPP in the incubation mixture was first hydrolyzed by the phosphatase enzyme to form GGOH followed by C-18 hydroxylation of GGOH catalyzed by GGOH-18-hydroxylase to form plaunotol and, thus, confirmed the proposed plaunotol biosynthetic pathway shown in Fig. 1.

Subsequently, the technique of TLC-densitometry (ref. 17) was developed for determining the catalytic activity of GGOH-18-hydroxylase. For this technique, non-radioactively labelled GGOH was used as substrate and the reaction was terminated by ether extraction. Both the substrate and reaction product of plaunotol were extracted into the ether phase but subsequently separated by TLC followed by densitometric scanning of the plate (using λ_{210} nm) to produce a chromatogram. Based on the area under plaunotol peak and its stardard curve which showed linearity between 0.5 and 15 nmol plaunotol, the enzyme activity of GGOH-18-hydroxylase could be determined. Using the developed enzyme assay, the formation of plaunotol was found to increase with time and the increased amount of enzyme protein and the reaction product was identified as plaunotol by both GC-MS and IR.

Enzyme characterization showed that GGOH hydroxylase had a pH optimum at 5.0. For enzyme stability, interestingly, the enzyme activity appeared to increase about 3-fold when the 20,000 g pellet fraction was heated for 30 min prior to be put into the reaction mixture. The reason behind this observation is still not clear. It might be possible that some-heat labile inhibitors of the enzyme are present in the cell-free extract. Upon heating, the inhibitors might be destroyed whereas the hydroxylase enzyme which is presumably a very complex structure remains active. For coenzyme requirement, the hydroxylation was found to depend on NADPH which was the best electron donor tested. NADH could also substituted for NADPH with the activity about 70% of NADPH. However, the hydroxylation of GGOH by the cell-free extract could also be detected in the absence of these cofactors with the activity about 54% of NADPH. Study on substrate specificity of GGOH-18-hydroxylase showed that the enzyme was highly specific to the diterpene GGOH (C-20). No hydroxylation reaction was observed with the shorter carbon-length substrate of either sesquiterpenoid, farnesol (C-15) or monoterpenoid, geraniol (C-10). Thus, there can be no doubt that this highly substrate specific 18-hydroxylase enzyme catalyses the last step of the biosynthetic pathway of plaunotol. The discovery of GGOH-18-hydroxylase leads to a potential of plaunotol synthesis by biotechnological techniques such as biotransformation by the enzyme either in its free or immobilized form.

LAWSON-FORMING MULTIENZYME COMPLEX

Lawsone (2-hydroxy-1,4-naphthoquinone 2) and Me-lawsone (2-methoxy-1,4-naphthoquinone 3) are the two main antifungal naphthoquinones found naturally in *Impateins balsamina* L. (ref. 18).

Biosynthetically, it has been proposed based on feeding experiments that lawsone is formed in plant *via* 2-succinylbenzoate, a key intermediate arised from glutamate and chorismate (ref. 19) (Fig. 4). However, none of the enzymes involved in the formation of the naphthoquinones have been found in plants. This prompted us to investigate the biosynthetic pathway of lawsone and its derivative Me-lawsone. We first examined for a suitable enzyme source by establishing various types *in vitro* cultures of *I. balsamina* cultures (ref. 20). It was found that the root cultures of *I. balsamina* could produce a number of natural products, mostly belonging to the chemical groups of coumarins and napthoquinones including lawsone and Me-lawsone (ref. 21). This indicated that the biosynthetic pathways of both naphthoquinones and coumarins were actively operated in the root culture and thus it was suitable for being used for studying the biosynthetic enzymes involved in the formation of these compounds.

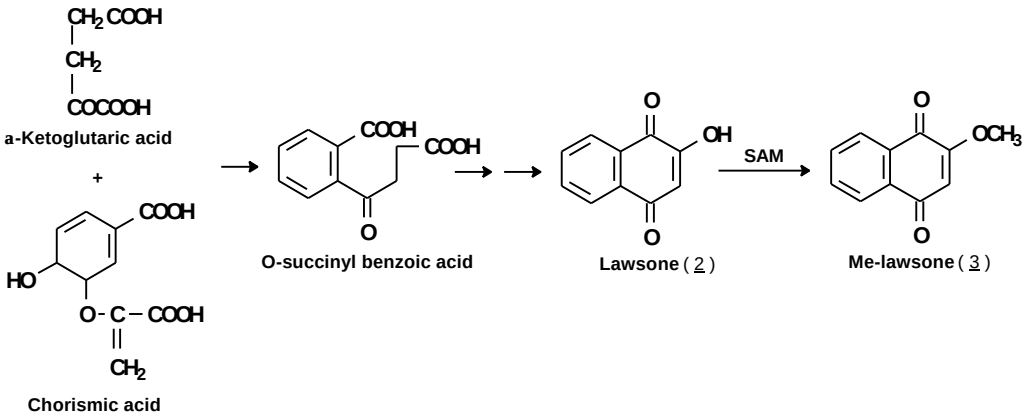

Fig. 4 Proposed biosynthetic pathway of lawsone in *Impatien balsamina*

In order to confirm the biosynthetic pathway of lawsone and Me-lawsone proposed in Fig. 4, *in vivo* feeding experiments were carried out using [^{14}C-U]-α-ketoglutarate as precursor. In doing this, the labelled precursor was fed to the one-week-old and four-week-old root cultures. After 3 days of the feedings, it was found that the added [^{14}C-U]-α-ketoglutarate was taken up by the old cultured roots (76%) more effectively than the younger one (40%). Both root cultures were then harvested, extracted and the resulting crude extracts were analyzed for their radioactive patterns by TLC-radioscanning. As shown in Fig. 5, the ethanolic extract prepared from the young roots showed essentially the radioactive peak of lawsone (Fig. 5A) whereas the crude extract from the old roots showed mainly the radioactive peak of Me-lawsone (Fig. 5B). These suggested that the radiolabelled precursor taken up by the young root cultures were incorporated directly into lawsone and the precursor taken up by the old root cultures were incorporated directly into Me-lawsone. In both cases, no significant detection of radiolabelled intermediates in the reaction mixture. This may be due to the presence of the enzyme O-methyltransferase (ref. 22) which methylates lawsone to form Me-lawsone which is active only in the old root cultures.

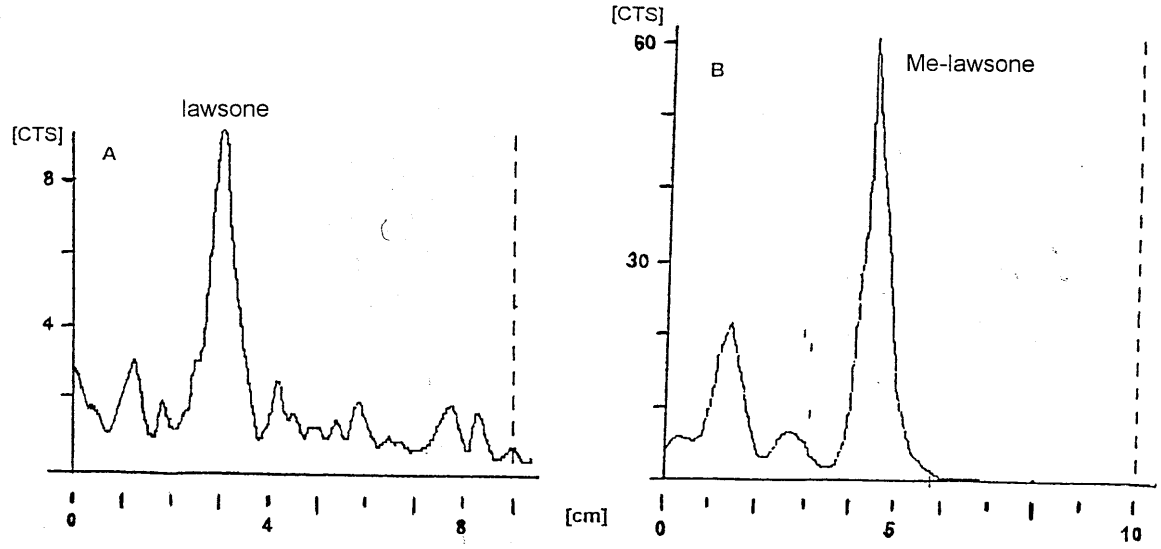

Fig. 5 TLC-radiochromatograms obtained from *in vivo* feeding experiments of [^{14}C-U]α-ketoglutarate to (A) one-week-old and (B) four-week-old cultured roots of *Impatiens balsamina*.

To search for the enzymes involved in the biosynthetic pathway of lawsone and Me-lawsone, a cell-free extract of the four-week-old *I. balsamina* root cultures was prepared and examined for their enzyme activities. The results showed interestingly that incubation of the crude cell-free extract with [^{14}C-U]α-ketoglutarate in the presence of CoASH, ATP and Mg^{2+} led to a rapid formation of radioactively labelled Me-lawsone (Fig. 6). This suggested that the complete enzymes involved in the biosynthesis of Me-lawsone were present in the cell-free extract. It was also observed that no radiolabelled intermediates were detected in the TLC-radiochromatogram. This confirms our assumption that the enzymes of lawsone and 2-methoxy-1,4-naphthoquinone are organized as an multienzyme complex.

In order to confirm that the enzymes responsible for lawsone and Me-lawsone biosynthesis are organized as an enzyme complex system, the technique of gel filtration was introduced. The purpose of this experiment was to observe whether the enzyme activity of lawsone biosynthesis disappeared or still remained after the enzyme solution was subjected to gel filtration chromatography. In this experiment, the crude cell-free extract was first subjected to ultracentrifugation (100,000g, for 30 min). The resulting supernatant and pellet fractions were both assayed for the enzyme activity. It was found that only the 100,000g supernatant fraction converted the labelled α-ketoglutarate rapidly into Me-lawsone. Therefore, the 100,000g supernatant part was used for the next step of gel filtration (Superose 12 column). The eluted profile of protein showed a major single peak eluted closely with the void volume. Enzyme assay of all the fractions also showed that the enzyme activity was present only in the fractions of the same major protein peak (Fig. 7). These results confirm our assumption that the enzymes involved in lawsone biosynthesis are organized as a high-molecular-weight enzyme complex.

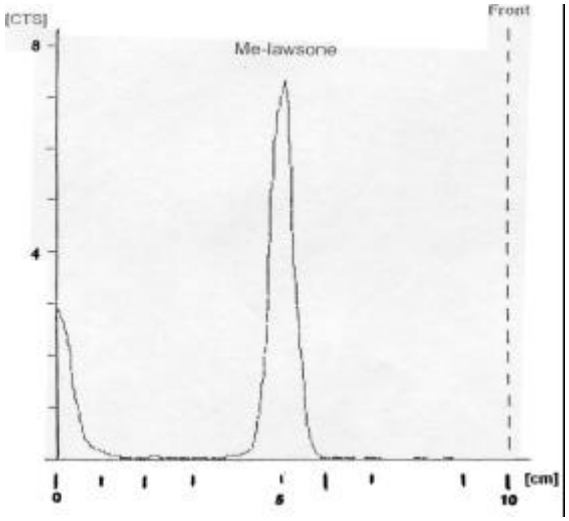

Fig. 6 TLC-Radiochromatogram showing the con -version of [^{14}C-U]α-ketoglutarate into 2-methoxy-1,4-naphthoquinone by cell-free extract of *I. balsamina* root cultures

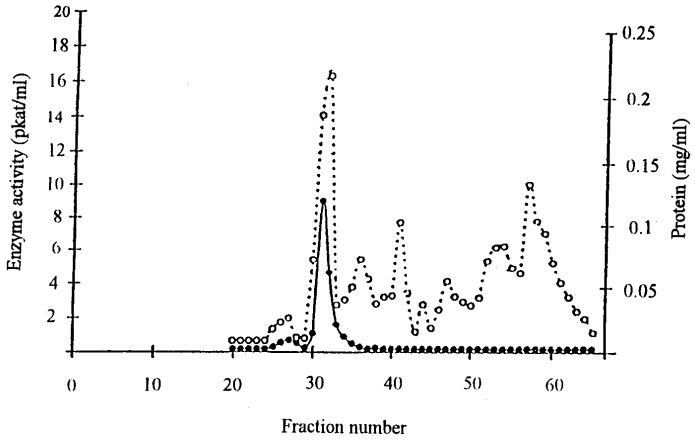

Fig. 7 The elution profile of enzyme activity (o) and protein (- - o - -) on Superose 12 gel filtration column

With the partially purified enzyme preparation, the effect of some cosubstrates and cofactors involved in the catalytic activity of enzyme complex was examined. As shown in Table 1 it was found that the degree of substrate conversion of the labelled a-ketoglutarate to Me-lawwone was not affected by the addition of OSB into the incubation mixture, suggesting that OSB could not reach the enzyme complex. On the other hand, the decrease in the radioactivity of Me-lawsone was observed by the addition of unlabelled lawsone into the incubation mixture. This suggested that the labelled lawsone could be diluted by the added unlabelled lawsone prior to the step of methylation by the enzyme O-methyltransferase. Therefore, the enzyme O-methyltransferases seems to be loosely bound to the enzyme complex due to the ability of the unlabelled lawsone in interfering the formation of Me-lawsone in the methylation step.

TABLE 1 Relative Incorporation of [^{14}C]α-Ketoglutarate into Me-lawsone by the Partially Purified Enzyme Preparation of *I. balsamina* Root Cultures

Condition	Relative enzyme activity (%)
Complete incubation	100.0
boiled enzyme	0.2
+ OSB	105.2
+ benzoic acid	100.1
+ lawsone	53.4
Incomplete incubation	
- CoASH	60.6
- ATP	36.0
- $MgCl_2$	43.7

In the absence of CoASH or ATP, or Mg^{2+}, the radioactivity of Me-lawsone, compared with the complete incubation , was also decreased. This suggested that CoASH, ATP and Mg^{2+} were also required as cosubstrate or cofactor in the biosynthesis of lawsone and Me-lawsone. This study showing channeling of the intermediates of lawsone biosynthesis in *Impatiens balsamina* root cultures represents a rare example of biosynthetic enzymes of plant secondary products.

DOPAMINE-SECOLOGANIN CONDENSING ENZYMES

Alangium lamarckii Thw. (Alangiaceae) is a tropical medicinal plant that is widely distributed throughout India and Southeast Asia. The plant contains a number of alkaloids and nitrogenous glucosides, most of which are characterized by the presence of a tetrahydroisoquinoline-monoterpene skeleton in the molecules. Biosynthetically, it has been proposed based on feeding experiments that secologanin is condensed with dopamine in a Pictet-Spengler manner to form two epimers, namely (1*R*)- deacetylipecoside (4) and (1*S*)-deacetylisoipecoside (5) (Fig. 8) (ref. 23). The (*R*)-epimer of deacetylipecoside, is presumably converted further to alangiside-type glucosides wherease the (*S*)-epimer of deacetylisoipecoside is transformed in *A. lamarckii* not only to the alkaloids but also to the isoalangiside-type glucosides. Before this work, the proposed biosynthetic scheme has not been investigated at the enzymatic level. This study, therefore, aims to evaluate the pathway by searching for the activities of the first enzymes catalyzing dopamine-secologanin condensation (ref. 12).

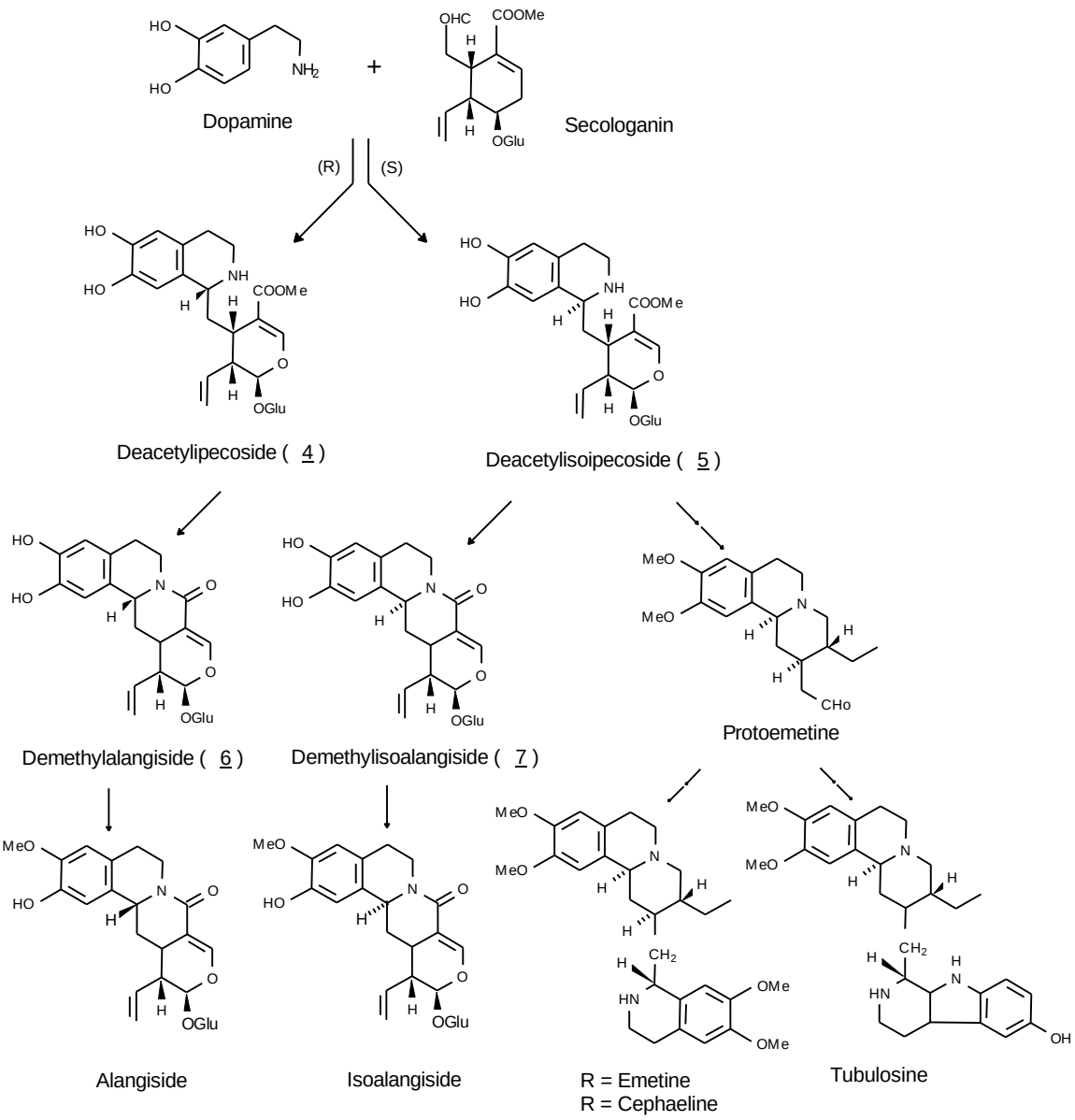

Fig. 8 Proposed biosynthetic pathways of tetrahydroisoquinoline-monoterpene alkaloids and glucosides in *Alangium lamarekii*

When a crude enzyme extracts prepared from the leaves of *Alangium lamarckii* was incubated with dopamine and secologanin under the condition of pH 7.5, a reaction product appeared in the incubation mixture as detected by the technique of TLC-densitometry. The resulting chromatograms showed that, during a 2-hr incubation, there was a rapid increase of the alkaloidal reaction product which occurred simultaneously with a decrease of the substrate dopamine (Fig. 9). It should be noted that during the first 60 min there appeared an increase of two peaks close to each other around an *Rf* value of 0.75. The lower Rf-value peak seemed to increase more rapidly than the one with the higher Rf-value. After 60 min, however, only one big

peak was observed. These results suggested that there might be more than one reaction product generated in the reaction mixture during the incubation.

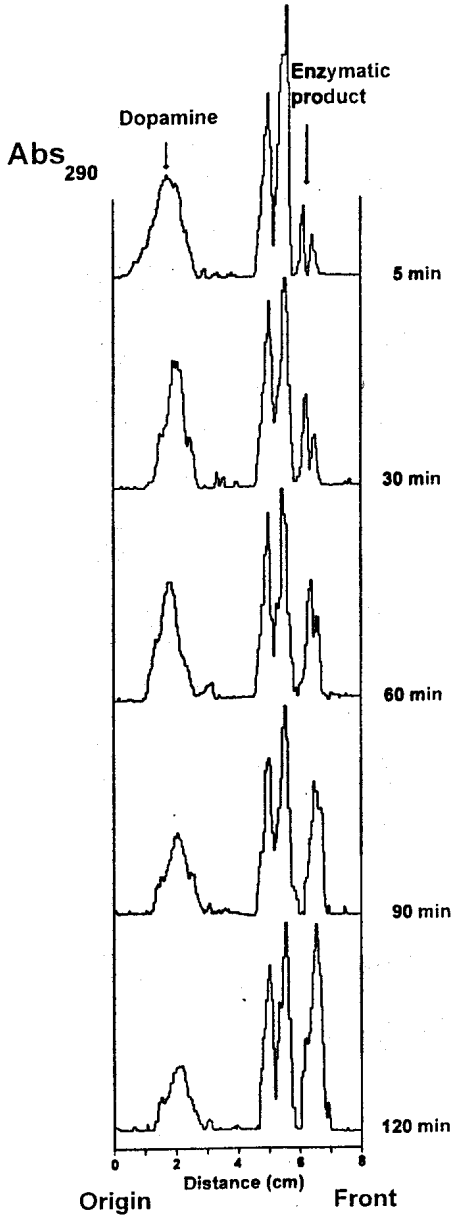

Fig. 9 TLC-densitometric chromatograms showing time-course of the enzymatic dopamine-secologanin condensation by *Alanglum lamarckii* crude enzyme extract

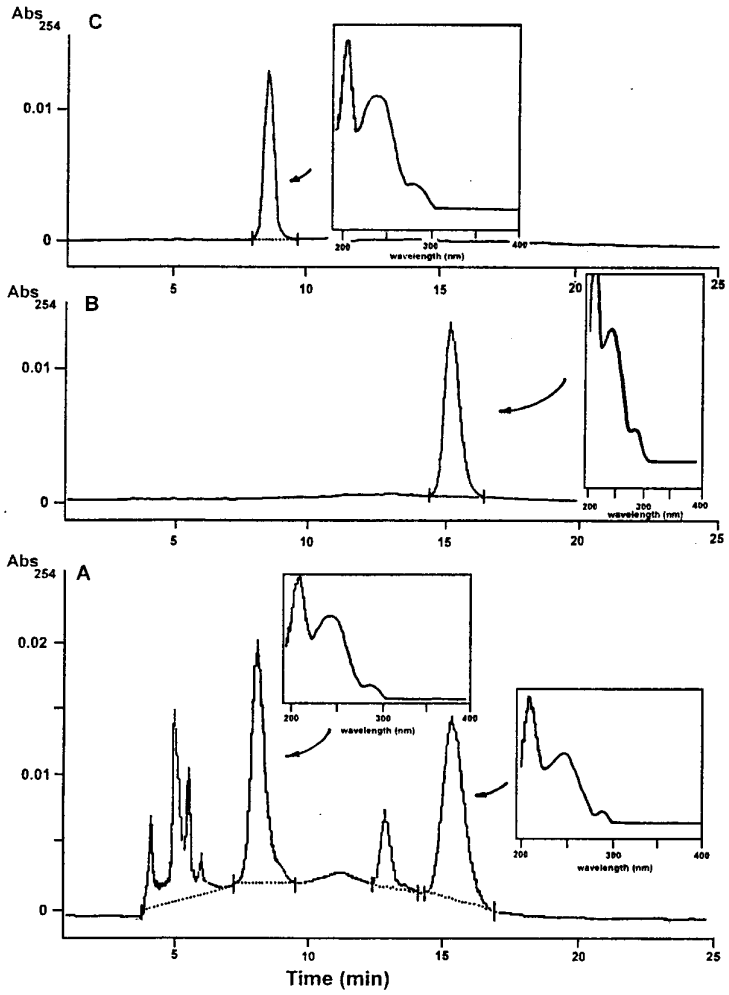

Fig. 10 Identification of the reaction products obtained from enzymatic dopamine-secologanin condensation by HPLC equipped with photodiode array detector. (A) HPLC chromatogram and UV-absorption spectra (inset) of the reaction products ; (B) of authentic demethylisoalangiside ; and (C) of authentic demethylalangiside.

Identification of the reaction product was performed by both HPLC connected to a photodiode array detector and LC-MS. Upon HPLC analysis, the product preparation showed the presence of two major peaks that co-migrated with authentic demethylalangiside (6) (*Rt* = 8.3 min) and demethylisoalangiside (7) (*Rt* = 15.6 min) (Fig. 10). The UV-absorption spectrum of each peak produced by the photodiode array detector was also identical with its authentic spectrum (Fig. 10, inset). With LC-MS, both peaks showed their CI mass spectra with $[M+H]^+$ at *m/z* 492, corresponding to the molecular mass 491 of, again, both demethylalangiside (6) and demethylisoalangiside (7). These results clearly showed that there was an enzyme-catalyzed condensation of dopamine and secologanin occurring in the reaction mixture. The immediate condensation products were likely to be (1*R*)-deacetylipecoside (4) and (1*S*)-deacetylisoipecoside (5) which were converted rapidly to 6 and 7, respectively. This spontaneous cyclization has been reported recently by Itoh *et al*. (ref. 24).

In summary, We have found two novel enzyme activities involved in the condensation of dopamine and secologanin in the cell-free extracts prepared from the leaves of *Alangium lamarckii*. The extracts condensed dopamine and secologanin rapidly at pH 7.5 to form both (1*R*)-deacetylipecoside and (1*S*)-deacetylisoipecoside which were converted spontaneously to demethylalangiside and demethyliso-alangiside, respectively. The discovery of these two enzyme activities in *A. lamarckii* suggested that the naturally occurring (*S*)- and (*R*)-forms of various tetrahydroisoquinoline-monoterpene alkaloids and nitrogenous glucosides found in this plant are determined by the first enzymatic step of dopamine and secologanin condensation which yields either the (1*R*) or the (1*S*)-condensation product.

1,2-DEHYDRORETICULINE REDUCTASE

Both the (*S*)- and (*R*)-enantiomeric forms of reticuline are known to be the biosynthetic precursors of morphinan-type alkaloids. Of the two enantiomers, it is the (*R*)- form of reticuline that corresponds in absolute stereochemistry to the configuration found at that particular chiral centre in this alkaloid group. Therefore, isomerization of (*S*)-reticuline to its (*R*)-counterpart was postulated (ref.25) (Fig. 11).

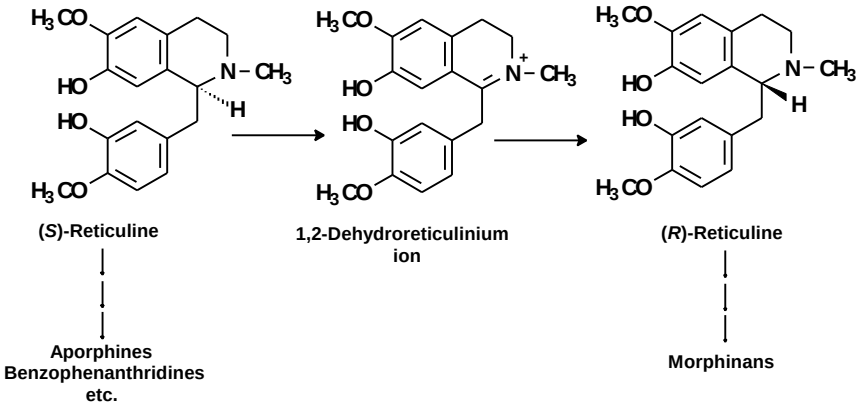

Fig. 11 Proposed isomerization of (*S*)-reticuline to (*R*)-reticuline and their involvement in *Papaver* alkaloid biosynthesis

This proposed isomerization of reticuline has evantually been confirmed by Zenk's group who demonstrated the presence of 1,2-dehydroreticuline reductase enzyme in *Papaver somniferum* seedlings (refs. 13,14).

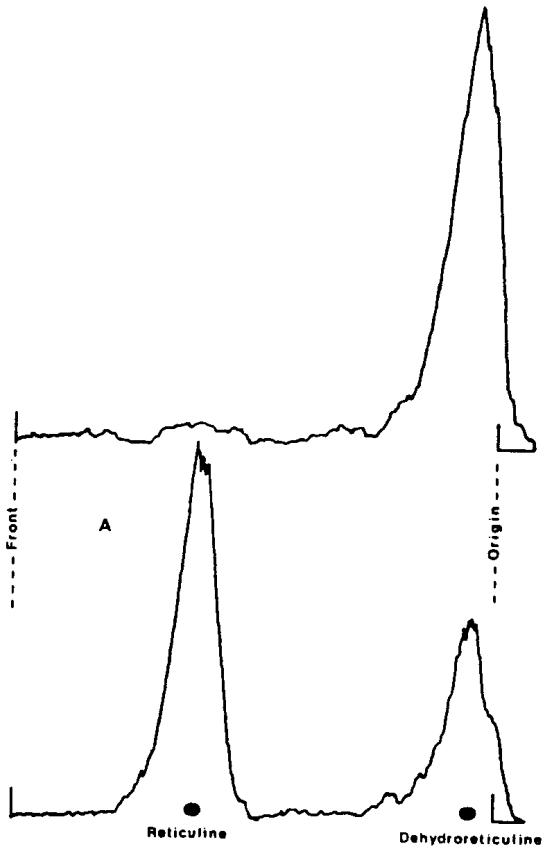

Fig. 12 Radioscans of (A) complete standard assay mixture containing [N-$^{14}CH_3$]-1,2-dehydroreticuline

As shown in Fig. 12, the incubation of [N-$^{14}CH_3$]-1,2-dehydroreticuline with crude proteinaceous extracts of five-day-old *Papaver somniferum* seedlings in the presence of NADPH at pH 8.5 resulted in the rapid appearance of a radiolabelled product chromatographically indistinguishable from authentic reticuline. The product was unequivocally identified as reticuline by mass spectral analysis and the absolute configuration of reticuline was further examined by employing radioimmunoassay and circular dichroism and was clearly shown to have (*R*)-configuration.

The reductase was subsequently purified to about 1400-fold purification with 22% recovery of the enzyme activity (ref. 14). Based on the developed purification scheme, a large-scale purification of 1,2-dehydroreticuline reuctase was performed and the purified enzyme preparation has been subjected to amino acid sequencing. Characterization of the enzyme revealed an apparent molecular weight of 30,000, a pH optimum at 8.5 and a temperature optimum at 30°C. Its activity was strictly dependent on NADPH. The enzyme exhibited a high

specificity towards both the substrate 1,2-dehydroreticuline and the coenzyme NADPH with a relatively low affinity for both compounds. Absolutely no reduction was detected with 1,2-dehydronorreticuline or 1,2-dehydrococlaurine. These results support the original proposal that the transformation of the (*S*)-benzylisoquinoline to the (*R*)-enantiomer takes place at the reticuline and not at the norreticuline level. We proposed the name 1,2-dehydroreticuline reductase (NADPH) for this stereospecific novel enzyme.

A survey for the reductase activity in both differentiated plants and cell cultures of some families showed that the enzyme was present only in morphinan-alkaloid containing plants (ref. 14). Among some members of the Papaveraceae tested, only *P. somniferum* and *P. bracteatum* displayed activity. In the cell cultures of both species which, however, did not produce these alkaloids, no enzyme activities could be detected. Therefore, there can be no doubt that this highly species- and substrate- as well as stereospecific enzyme catalyses the provision of (*R*)-reticuline for the formation of morphinandienone alkaloids also possessing the (*R*)-configuration at the corresponding chiral center.

SUMMARY

These four examples from our research works at Chulalongkorn University, Bangkok demonstrate the usefulness of enzymological techniques in elucidating the biosynthetic enzymes of secondary metabolites found in medicinal plants. We believe that the complete understanding of secondary metabolism at the enzyme level is a prerequisite for metabolic engineering of medicinal plants, which potentially leads to yield improvement of pharmaceutically important secondary products.

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