CHAPTER II

HISTORICAL

1. Botanical Aspects of *Papaver somniferum* L.

The opium poppy (*Papaver somniferum* L.)(Fig. 1) belongs to the family of Papaveraceae or the Poppy Family. Its local names in various areas include Afium (Arabic), Fin (Thai), Hul-gil (Turkish), Opium (English), Pavot (French), etc.(Kirtikar, 1975; Morton, 1977).

The opium poppy is a herbaceous annual 0.6 to 1.2 m tall, with large, soft waxy leaves with dentate margin, sea green in colour and amphexicual. All parts contain white or gray latex. Leaves are alternate, clasping the stem, ovate- to linear-oblong, wavy, irregularly lobed and strongly toothed,7.5 to 25 cm long. Flowers, borne singly at the tips of long usually smooth stalked, are 7.5 to 10 cm wide, have 4 rounded, wavy petals, red or variegated, and a conspicuous, rounded green ovary capped with five- to twelve-rayed stigma which is surrounded by a prominent fringe of yellow stamens 150 to 200 in five concentric circles. The fruit (seed capsule) is globular, 5 to 10 cm wide, crowned with the disk formed by the united stigmas, green when unripe with copious white, grayish, pale- or bright-pink latex. When mature, the capsule turns yellow, then it dries to brown and small aperture below the stigma open releasing a great number of small, kidney-shaped seeds -white, yellowish, gray, lavender, blue, reddish-brown or black (Morton, 1977).

In addition to numerous garden hybrids about four varieties of *P. somniferum* are recognised. These varieties differ in colour of the petal, size and shape of the capsules and colour of seeds, presumbly depending on their geographical locations. For example, the variety *album* is from India, var. *glabrum* from Turkey, and var. *nigrum* and *setigerum* from Europe (Dejei, 1979).

The opium poppy is believed to be native to Europe and Western Asia and subsequently be spreaded to India and China, where began addiction (Dejei, 1979). Presently all legitimate opium culture is conducted under the supervision and regulation

Figure 1 *Papaver somniferum* L. (Papaveraceae)

of the International Narcotic Control Board of the United Nations. The plant can be cultivated in Iran, India, Turky, Bulgaria, Greece, Yugoslavia and Russia under control of the government of these producing countries (Morton, 1977; Lindner, 1985).

In the cultivation, the poppy seeds are spreaded over the field in early autumn, The plants flower in April and May and the capsules mature from June to July. While nearly all parts of the poppy contain a white milky juice or latex, the unripe capsules contain it in most abundance. The collecting of the latex is generally done shortly after the petals and stamens fall. Normally in the afternoon or early morning while the temperature is low, transverse or vertical incision is made into the unripe capsule by means of single or several bladed knife. The white latex exudes and soon harden on the outer surface of the capsule in the brownish, masses which are scraped off in the following day on a wooden tray. Thereafter, the opium is placed on the flat plates to dry under the sun. This lancing may be repeated from three to ten times until the latex cease to flow (Youngken, 1948).

2. The Uses of *Papaver somniferum*

Opium is possibly the oldest known narcotic. It is widely used to relieve pain (Lewis, 1977). The dried whole plant is used in the treatment of dyspepsia, cough, pain, sedative, gastro-intestinal disorders, spasm (Giordano and Levine, 1989). In Afghanistan, the latex of opium poppy is used to insert into vagina for contraceptive effect. In Malaya, it used with *Derris elliptica* root as an abortifacient (Quisumbing, 1951). Poppy heads are used in China for diarrhoeas, and all kinds of fluxes. Opium in combination with other drugs is recommended for the treatment of snake bite and scorpion sting (Kirtikar, 1975).

Since the isolation of morphine by Serturner in 1803, some 40 alkaloids, representing different structural types of isoquinolines, have been isolated from opium (see section 3). The pharmacological activities of these opium alkaloids have been studied (Lindner, 1985).

Morphinan alkaloids, morphine constitutes between 3% to 23% opium. It is marked its strong analgesic action in humans 10 mg given orally will elevate the pain threshold by 50% and a 30 mg dose by 90%. At higher doses, morphine acts as a narcotic and sedative, in contrast, codeine and neopine have only one tenth of the

analgesic activity of morphine. Even low doses of morphine can remove displeasure, anxiety, and the feeling of hopelessness, and can cause euphoria; psychic as well as physical dependence can develop. When morphine is discontinued in humans, signs of deprivation develop in the form of restlessness, excitation, dysphoria, sweating and collapse. Other essential effects of morpine are the elevation of muscle tone in the gastrointestinal tract, thus leading to a depression of peristalsis and a reduction in the stretching of the gastrointestinal tract. As a result there is a reduction in stomach emptying and defecation. The tone of the gall bladder also enhanced. Thebaine devoids of analgesic activity and in some respects is antagonistic and can also cause convulsions. It is not used medicinally by itself (Lindner, 1985).

In the protoberberine alkaloids, berberine contracts uterine muscle and has been used to stop uterine bleeding and to help in childbirth. It also exerts some antiinflammatory activity. Coreximine, also found in opium is closely related to berberine and acts with a positive inotropic effect on dog heart and reduced heart rate. Coptisine has an inhibitory effect on the formation the connective tissue (Lindner, 1985).

Some of the benzylisoquinolines, e.g. papaverine is an excellent spasmolytic drug which relaxes the muscle of blood vessels. Papaverine is combined with atropine in many medicinal preparations used against colic. Noscapine also has spasmolytic effect. The drugs also has a central cough inhibiting effect and in this respect noscapine is not much less effective than codeine. The aporphine alkaloids, magnoflorine acts as a neuromuscular blocking agent and lowers blood pressure in rats and in guinea pigs (Aiyar *et al.*, 1979).

Sanguinarine, also found in low amounts in opium, elevates intraocular pressure and inhibits Na^+/K^+-ATPase similarly to the cardiac glycosides. Several benzophenanthridine alkaloids have antibacterial, cytotoxic or antineoplastic activities (Stermitz *et al.*, 1973).

The pharmacological effects of this complex mixture of alkaloids in opium may in some respects, be more beneficial than the effects of an individual alkaloid. The preparations which contain opium as an active ingredient (Reynold, 1989) include:

- Camphorated Opium Tincture (BP)
- Paregoric (USP)

- Concentrated Camphorated Opium Tincture (BP)
- Compound Camphorated Opium Mixture (BPC 1973)
- Opiate Squill Linctus (BP)
- Pediatric Opiate Squill Linctus (BP)
- Opium Tincture

For the opium seeds, they have been sold as birdseeds and consumed as human food. The seeds are also an important source of oil, which is used for making soap and for making drying oil in paint and vanishes(Morton, 1977; Ulubelen, 1977).

3. Chemical Constituents of *Papaver somniferum*

The opium poppy produces raw opium (latex) which contains more than 40 alkaloids. Among these only six namely, morphine, codeine, thebaine, papaverine, narcotine, and narceine, occur in measurable quantity whereas all others occur only in traces (Lal and Sharma, 1991). Out of the 40 alkaloids, it is believed that there are in fact only 20 alkaloids present in the latex and the others are the metabolites resulted from degradation caused by enzymatic or oxidative reactions.

Chemical constituents isolated from this plant have been reported to include alkaloids, coumarins, triterpenes, chromones, lipids and vitamins. The group of isoquinoline alkaloids is especially the most abundant alkaloids found in this plant. In recent years, there have been several reports on the accumulation of isoquinoline alkaloids in the whole plant and tissue cultures of *P. somniferum*. The list of the alkaloids found in intact opium poppy plant is shown in Table 1 and theirs structures are shown in Appendix I.

Table 1 Isoquinoline alkaloids found in *P. somniferum* (Santav'y, 1979)

Alkaloid Group	No. of Alkaloid	Reference
1.Simple isoquinoline	Hydrocotarnine(1.1)	Lundstrom, 1983
2.Benzylisoquinoilne	1,2-Dehydroreticuline (2.10)	Borkowski *et al.*, 1978
	Codamine (2.1)	Brochmann-Hanssen and Furuya, 1964
	Laudanidine (2.2)	Brochmann-Hanssen and Furuya, 1964
	Laudanosine (2.3)	Brochmann-Hanssen and Furuya, 1964
	Orientaline (2.6)	Preininger, 1986
	Palaudine (2.8)	Proksa *et al.*, 1979
	Papaveraldine (2.9)	Hodkova *et al.*, 1972
	Papaverine (2.7)	Uprety *et al.*, 1975
	Reticuline (2.4)	Brochmann-Hanssen and Neilsen, 1965a
	Tetrahydropapaverine (2.5)	Preininger, 1986
3.Aporphine	Corytuberine (3.1)	Santav'y, 1979
	Isoboldine (3.3)	Brochmann-Hanssen *et al.*, 1967
	Magnoflorine (3.2)	Ikuta *et al.*, 1974
4.Morphinan	10-Hydroxycodeine (4.7)	Brochmann-Hanssen and Neilsen, 1965b
	16-Hydroxythebaine (4.4)	Preininger, 1986
	6-Methylcodeine (4.8)	Brochmann-Hanssen and Neilsen, 1965b
	Codeine (4.5)	Fairbairn and Wassel, 1964
	Codeinone (4.13)	Preininger, 1986
	Morphine (4.11)	Fairbairn and Wassel, 1964
	Morphine N-Oxide (4.12)	Phillipson *et al.*, 1976
	N-oxide of codeine (4.6)	Phillipson *et al.*, 1976
	Neopine (4.14)	Berenyi *et al.*, 1986
	Normorphine (4.9)	Miller *et al.*, 1973
	Oripavine (4.3)	Neilsen *et al.*, 1983
	Pseudomorphine (4.10)	Preininger, 1986
	Thebaine N-Oxide (4.2)	Phillipson *et al.*, 1976

Table 1 (continued)

Alkaloid Group	No. of Alkaloid	Reference
	Thebaine (4.1)	Fairbairn and Wassel, 1964
5.Promorphinan	Salutaridine (5.1)	Wieczorek *et al.*, 1986
6.Protoberberine	Berberine (6.5)	Preininger, 1986
	Canadine (6.1)	Brochmann-Hanssen and Neilsen, 1966
	Coptisine (6.6)	Hakim *et al.*, 1961
	Coreximine (6.7)	Brochmann-Hanssen *et al.*, 1971
	Isocorypalmine (6.3)	Proksa *et.al*, 1979
	Scoulerine (6.2)	Brochmann-Hanssen and Neilsen, 1966
	Stepholidine (6.4)	Brochmann-Hanssen and Richter, 1975
	6-Acetonyl-dihydrosanguinarine (7.3)	Furuya *et al.*, 1972
7.Benzophenanthridine	Dihydrosanguinarine (7.1)	Ikuta *et al.*, 1974
	Norsanguinarine (7.4)	Ikuta *et al.*, 1974
	Oxysanguinarine(7.2)	Ikuta *et al.*, 1974
	Sanguinarine (7.5)	Hakim *et al.*, 1961
8.Protopine	Allocryptopine (8.2)	Hodkova *et al.*, 1972
	Cryptopine (8.3)	Brochmann-Hanssen and Neilsen, 1966
	13-Oxocryptopine (8.4)	Preininger, 1986
	Protopine (8.1)	Brochmann-Hanssen and Neilsen, 1966
9.Phthalidetetrahydro-isoquinoline	5'-O-Demethyl-narcotine (9.3)	Repasi *et al.*, 1993
	Narcotine (9.1)	Preininger *et al.*, 1965
	Narcotoline (9.2)	Proksa and Proksova, 1991
10.Secophthalide-isoquinoline	Narceine imide (10.3)	Proksa *et al.*, 1978
	Narceine (10.2)	Chaudhuri and Thakur, 1989
	Narceinone (10.4)	Chaudhuri and Thakur, 1989
	Nornarceine (10.1)	Preininger, 1986
11.Rhoeadine	Glaudine (11.1)	Preininger *et al.*, 1981
	Papaverrubine C,D (11.3)	Preininger *et al.*, 1981

Table 1 (continued)

Alkaloid Group	No. of Alkaloid	Reference
12.Dimericisoquinoline	Rhocadine(11.2)	Preininger *et al.*, 1965
	O-Methyl ether somniferine (12.2)	Drager and Bick, 1988
	Somniferine (12.1)	Drager and Bick, 1988

It can be seen from Table 1 that the intact opium poppy plant is a good source of medicinally important alkaloids, including thebaine, codeine, morphine, and papaverine. However, field cultivation of this plant has been limited since 1953 to prevent narcotic crime (Yoshimatsu and Shimomura, 1992). As a consequence, tissue culture of *P. somniferum* has been investigated as an alternative way for the production of these alkaloids (Kamo and Mahlberg, 1988).

So far, there have been a number of reports on alkaloid production in callus or suspension culture. The spectrum of the alkaloids accumulated in these cell include aporphine, protopine, benzophenanthridine, phthalideisoquinoline, and benzylisoquinoline type (Staba *et al.*, 1982). Table 2 summerizes various alkaloids produced by in vitro cultures of *P. somniferum*.

Table 2 Alkaloids produced by various in vitro cultures of *P. somniferum*

In vitro Culture	Alkaloid	Reference
Callus	6-Acetonyldihydrosanguinarine	Furuya *et al.*, 1972
	Codeine	Ikuta *et al.*, 1974
	Codeine-N-Oxide	Hsu and Pack, 1989
	Cryptopine	
	Dihydrosanguinarine	
	Magnoflorine	
	Norsanguinarine	
	Oxysanguinarine	
	Protopine	
	Sanguinarine	
	Thebaine	

Table 2 (continued)

In vitro Culture	Alkaloid	Reference
Suspension	Codeine Cryptopine Morphine Narceine Narcotine Papaverine Thebaine	Khanna and Khanna, 1976 Morris and Fowler, 1980 Anderson *et al.*, 1983 Heinstein, 1985 Siah and Doran, 1991
Small cell clusters with "giant cell" and tracheid	Codeine	Tam *et al.*, 1980
Redifferentiated shoots	Codeine Morphine Thebaine	Kamo *et al.*, 1982
Shoot culture	Thebaine	Staba *et al.*, 1982
Callus grown root	Cryptopine	Staba *et al.*, 1982
Embryoid	Codeine Thebaine	Staba *et al.*,1982
Somatic embryogenesis	Codeine Morphine Thebaine	Schuchmann and Wellmann, 1983
Elicitors induction	Sanguinarine	Eilert *et al.*, 1986
Meristemoid	Morphine	Siah and Doran, 1991
Transform root culture with *Agrobacterium rhizogenes* MAFF-03-01724	Morphine Noscapine Papaverine	Yoshimatsu and Shinomura, 1992
Immobilized suspension	Dihydrosanguinarine Norsanguinarine Sanguinarine	William *et al.*, 1992
Transform culture with *A. rhizogenes*	8-Acetonyldihydrosanguinarine 8-Methoxydihydrosanguinarine Cryptopine Dihydrosanguinarine Isothebaine Norsanguinarine	William and Ellis, 1993

Table 2 (continued)

In vitro Culture	Alkaloid	Reference
	Oxysanguinarine Protopine Sanguinarine	

4. Quantitative Analysis of Morphinan Alkaloids in *Papaver somniferum*

The major alkaloids of morphinan group of *P. somniferum* are thebaine, codeine, and morphine, which are distributed in different contents and plant parts. In 1974, Fairbairn *et al* . reported that alkaloids were translocated from stem to capsule during its rapid expansion after petal fall. Thus, it seems that these major alkaloids are metabolized and translocated to the storage site dynamically. It is believed that information on the tissue distribution of morphinan alkaloids within *P. somniferum* plants is important for a better understanding of morphinan biosynthesis and it may also provide insight into the regulation of morphinan pathway.

The methods to quantitate and identify the alkaloids have changed with time and become increasingly more sensitive. From 1972 to 1976 researchers used gas liquid chromatography (GLC), ultraviolet (UV),infrared (IR), or nuclear magnetic resonance (NMR) spectra to identify alkaloids. Since in 1982, high pressure liquid chromatography(HPLC) has been commonly employed(Hodges and Rapoport, 1982a). Recently, there have been two additional techniques, namely radioimmunoassay (RIA) (Hodges and Rapoport, 1982a; Wieczorek *et al.*, 1986) and enzyme immunoassay(EIA)(Yoshikawa and Furuya, 1985) which can detect morphinan alkaloids in the submicrogram-nanogram levels.

Wieczorek *et al.*(1986) analysed the occurrence of the opium alkaloids in dried leaf tissue of various *Papaver* species and reported that *P. somniferum* is the species which contains the highest amount of codeine and morphine. The distribution of the morphinan alkaloids in the living plant (five days after petal opening) is shown in Table 3.

Table 3 The major alkaloids in different organs and latex of *P. somniferum* (Wieczorek *et al.*, 1986)

	Thebaine	Codeine	Morphine
Latex(as g/l)	200	79	430
Leaf(as %dry wt)	0.4	0.6	2.8
Shoot(as %dry wt)	0.76	1.4	3.9

Hodges and Rapoport (1982a) used RIA to screen many calli and found that 45% of the well growing and healthy calli contained morphinan alkaloids. The quantity of each alkaloid produced by callus and suspension cultures is shown in Table 4

Table 4 The content of alkaloids in callus and suspension cultures of *P. somniferum* (Kamo and Mahlberg, 1988)

	Alkaloids			
Type of Culture	Thebaine	Codeine	Morphine	Reference
Callus	0.6-13.1 µg/g dw	0-32.8 µg/g dw	none	Kamo *et al.*, 1982
Callus	0.1-1.15%	0.05-0.28%	0.31-0.83%	Khanna *et al.*, 1978
Callus	2.6-45 µg/g fw	0-34.4 µg/g fw	0-12.5 µg/g fw	Hodge and Rapoport, 1982
Green callus	traces	4.6-100 mg/g dw	none	Yoshikawa and Furuya, 1985
Suspension	0.23-0.50%	0.06-0.26%	0.55-1.58%	Khanna *et al.*, 1978
Suspension	none	0.15% dw	none	Tam *et al.*, 1980
Suspension	no data	0.08-1.44 mg/g dw	0.07-1.40 mg/g dw	Heinstein, 1985

dw= dry weight: fw= fresh weight

Kamo and co-workers (1982) have reported that the biosynthesis of morphinan alkaloids is related to the degree of tissue differentiation. Yoshikawa and Furuya (1985) found that 9 year-old callus and meristemoids did not synthesize morphinan alkaloids. Callus that was green and observed to have trachery elements synthesized codeine as the main alkaloid in addition to the presence of some thebaine.

Buds and shoot differentiation was required for morphine alkaloid synthesis (Yoshikawa and Furuya, 1985).

5. The Alkaloidal Storage, Translocation and Metabolism of *Papaver somniferum*

Fairbairn and Djote (1970) have reported that the alkaloids of *P. somniferum* L. occur in active vesicles and that the stem latex is more metabolically active than the capsule latex. Fairbairn *et al.* (1974) reported that the alkaloids are stored in the vacuolar sap rather than being membrane bound, and in this respect the vesicles behave as normal vacuoles. However, it has been shown that the stem latex and vesicles are translocated into the capsule during its rapid expansion after petal fall (Fairbairn *et al.*, 1974). During this time, the morphine itself is being synthesized and metabolized in the vesicle (more rapidly in the stem than in those of the capsule) and the metabolites pass out of the latex into pericarp(Fairbairn *et al.*, 1974).

During the day, there is a marked variation in the cellular content of the three alkaloids, thebaine, codeine, and morphine. Morphine is increased in early morning and decreased at noon. In contrast, codeine and thebaine are increased in the afternoon. This suggests that these alkaloids play an active part in metabolism rather than as a slowly accumulating. Though morphine has been shown to occur as the irreversible end-product of the sequence thebaine-codeine-morphine,its content has been found to decrease markly at certain time during the days (Fairbairn and Wassel, 1964). However, Miller *et al.*(1973) have reported that the morphine is not an end substance but it is changed to normorphine by a demethylation in its degradative pathway. Normorphine has been established as an active metabolite of morphine in *P. somniferum* L. and is subsequently degraded to non-alkaloidal metabolites (Miller *et al.*, 1973). In addition, morphine is rapidly metabolized in the latex into series of compounds some of which are alkaloid-like and others non-alkaloidal,"bound" forms (Fairbairn and El-Masry, 1968). Some of these compounds are transported to the developing seeds and stored in there as large molecules. The bound forms are stored in the seeds and broken down into smaller alkaloid-like substances during the germination.

In conclusion, the morphinan alkaloids in *P. somniferum* are synthesized and stored in the alkaloidal vesicles and translocated to the rapid developing capsule latex.

During this time the biosynthesis of the alkaloids may continue and the degradation of morphine also occurs. The morphine metabolites unlike the alkaloids are translocated out of the capsule latex to the pericarp and stored in the seeds (Fairbairn *et al.*, 1974). Since the morphinan alkaloids are stored in bound form and metabolite, they are not found in the seeds.

Wieczorek and co-workers (1986) have used RIA method to study the formation of opium alkaloids during the germination of *P. somniferum*. They have shown that reticuline and thebaine are formed at the fifth day after germination whereas codeine and morphine can be detected in trace only 6 days after germination. The profile of alkaloid formation in germinated seeds has the same pattern as that found by Williams and Ellis (1989). The alkaloid profile of the aerial tissue at different ages (Fig.2) shows an increase in morphine accumulation from day 35 to day 40, followed by a decrease at day 45. The morphine content is marked risen between day 55 and 80. This increase begin during the bolting period and correlated with extensive stem development followed capsule formation. In the root tissue (Fig.3), morphine attains on days 30 and 35 , followed by a sharp decrease. This rises and falls seems to have been associated with a development shift within roots, as extend branching of the roots. The loss of these alkaloids from the roots is correlated with an increase in the aerial tissue alkaloid content are due to translocation from roots, or to *in situ* degradation in the roots and synthesized in the aerial tissue (Williams and Ellis, 1989).

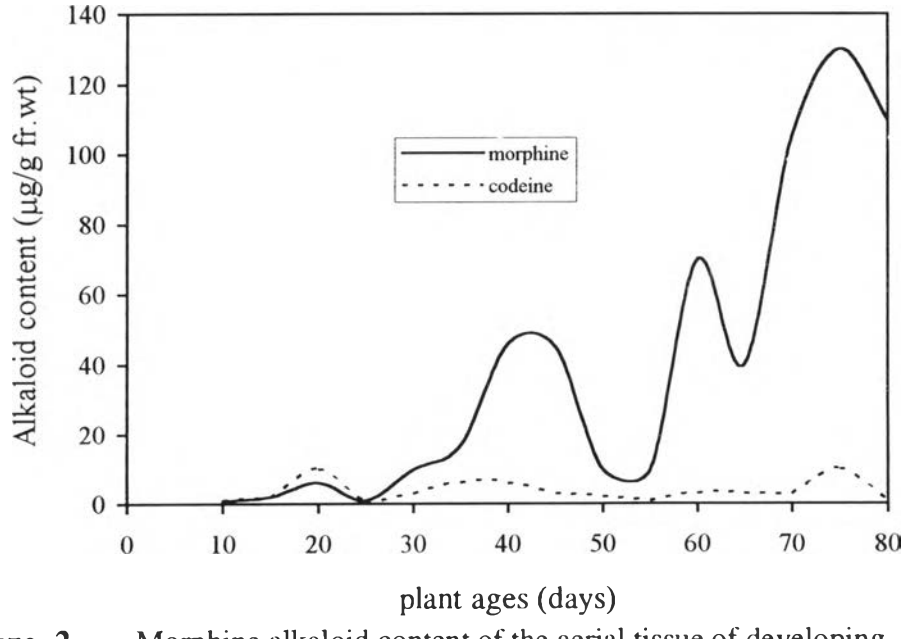


Figure 2 Morphine alkaloid content of the aerial tissue of developing *P. somniferum* plant (Williams and Ellis, 1989)

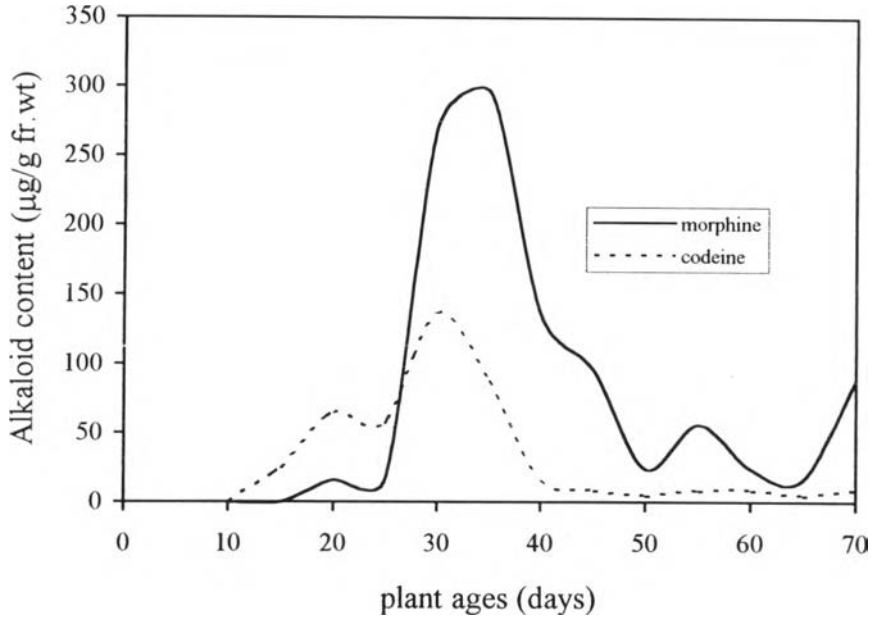
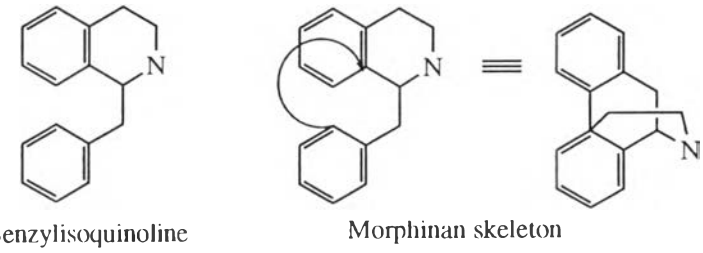


Figure 3 Morphine alkaloid content of the root tissue of developing *P. somniferum* plant (Williams and Ellis, 1989)

6. The Biosynthetic Pathway of Morphinan Alkaloids

Morphinan alkaloids are limited in number and distribution. There are fewer than a dozen which are distributed in genus *Papaver* of the family Papaveraceae and several genera of the Menispermaceae. In terms of structure, the morphinan alkaloids are derived from a benzylisoquinoline skeleton by its additional ring closure (Robinson,1981), as shown below.



Benzylisoquinoline skeleton

Morphinan skeleton

Winterstein and Trier (1910) had suggested that the benzylisoquinoline system is built up from two units derived from 3,4-dihydroxyphenylalanine (DOPA). Since dopa comes from tyrosine, the investigation consisted of feeding experiments with radioactive labelled tyrosine, dopamine and norlaudanosoline and isolation of

radioactive morphine, codeine and thebaine (Battersby and Harper, 1958; Leete, 1959; Battersby and Binks, 1960; Battersby and Harper, 1960; Battersby *et al.*, 1962; 1964; Leete and Murril, 1964; Battersby and Francis, 1964). When specifically labelled precursors were used, the labelled appeared in the hypothesis. It follows therefore that morphine is biosynthesized from dopamine and 3,4- dihydroxyphenylacetaldehyde, both being derived from tyrosine, and that norlaudanosoline is a likely intermediate. In 1987, the early steps of morphinan biosynthesis was revised. The results of the feeding experiments showed that the morphine skeleton is built up from the condensation product of dopamine with 4-hydroxyphenylacetaldehyde, and therefore norcoclaurine is the true intermediate of this biosynthesis, not norlaudanosoline as hitherto assumed (Loeffler *et al.*, 1987; Stadler *et al.*, 1987; Stadler *et al.*, 1989). Appropriate O- and N-methylation of norcoclaurine affords alkaloids such as reticuline which is converted to morphinandienone skeleton by oxidative coupling of phenol radical (Martin *et al.*, 1967; Hodges and Rapoport, 1982b)

Recently the general pattern of the formation of this fascinating class of compounds has been worked out and proved by feeding labelled precursors to plant or callus (for review, see Zenk *et al.*, 1985). However, this approach of study has led to a number of questions as to the sequence of steps and the identification of the intermediates involved in the formation of these alkaloids. Presently, it is widely accepted that the expected pathway can be confirmed only the demonstration of the presence of enzymes which catalyse individual steps. This kind of work has been carried out only in recent years.

From results of the study, the biosynthetic pathway from tyrosine to morphine can be dissected into two parts: the first part leads from primary metabolite to (S)-reticuline, the second leads from this branch point intermediate ((S)-reticuline) to morphine.

6.1. The pathway from L-tyrosine to (S)-reticuline

Reticuline and its congeners derived from two molecules of tyrosine (I),which derived to two different molecules (Stadler *et al.*, 1987). As early as 1910, Winterstein and Trier suggested that the two molecules of 3,4 dihydroxyphenylalanine (DOPA) might be modified in the plant to yield dopamine and 3,4-dihydroxyphenylacetaldehyde, which could condense and yield norlaudanosoline, as a

isoquinoline alkaloids (Leete, 1959; Battersby and Binks, 1960). However, doubts had been raised whether the tetra-oxygenated (S)-norlaudanosoline was really the first alkaloid precursor to reticuline due to the fact that it was never found to occur naturally. This question was resolved when it was discovered that the tri-oxygenated alkaloid, (S)-norcoclaurine (VI), not a tetra-oxygenated alkaloid, was the precursor to reticuline (X).This compound is built up from the condensation product of dopamine (III) with 4-hydroxyphenylacetaldehyde (IV) by the enzymatic and stereospecific reaction (Fig. 4)(Loeffer *et al.*, 1987; Stadler *et al.*, 1987; 1989). Tyrosine is metabolized *via* tyramine (II) which is converted predominantly to dopamine (III) by hydroxylation and the latter serves as for the upper portion of the benzylisoquinoline system. 4-Hydroxyphenylacetaldehyde (V) is formed from tyrosine (I) by deamination, and subsequent decarboxylation, serves as the lower portion of the benzyl isoquinoline precursor (Zenk *et al.*, 1985; Stadler *et al.*, 1987 ;Frenzel and Zenk, 1990).

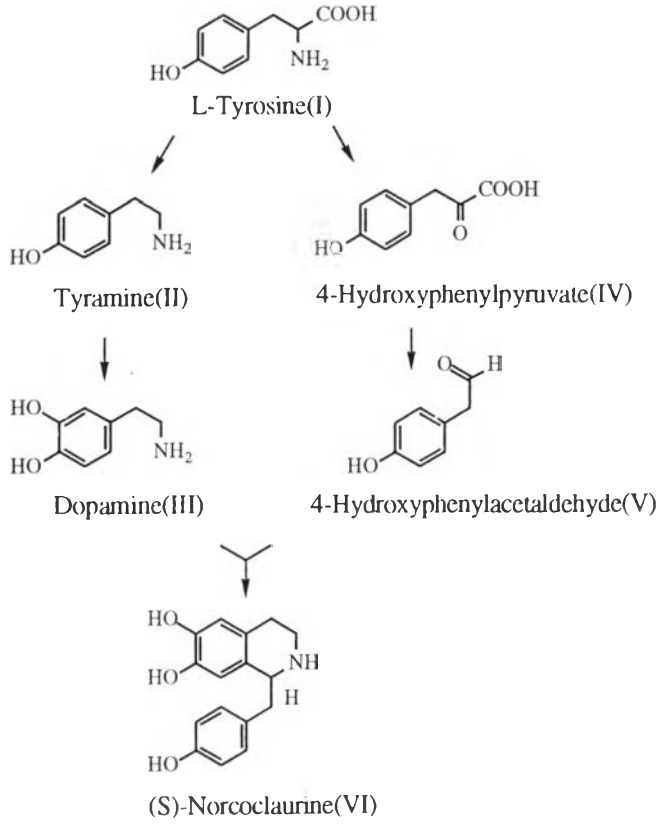


Figure 4 Biosynthetic sequence leading from tyrosine to the building blocks of benzylisoquinoline: dopamine and 4-hydroxyphenylacetaldehyde to yield (S)-norcoclaurine (Frenzel and Zenk, 1990)

The pathway from dopamine(III) and 4-hydroxyphenylacetaldehyde(V) to yield (S)-reticuline(X) is depicted in Fig.5. Both are condensed in an stereospecific manner to (S)-norcoclaurine. 6-O-Methylation yields (S)-coclaurine(VII) which is transformed by N-methylation to (S)-N-methylcoclaurine(VIII), and subsequent 3'-hydroxylation ((S)-3'-hydroxy-N-methylcoclaurine (IX)) as well as 4'-methylation to (S)-reticuline (X), respectively (Frenzel and Zenk, 1990; Loeffler and Zenk, 1990).

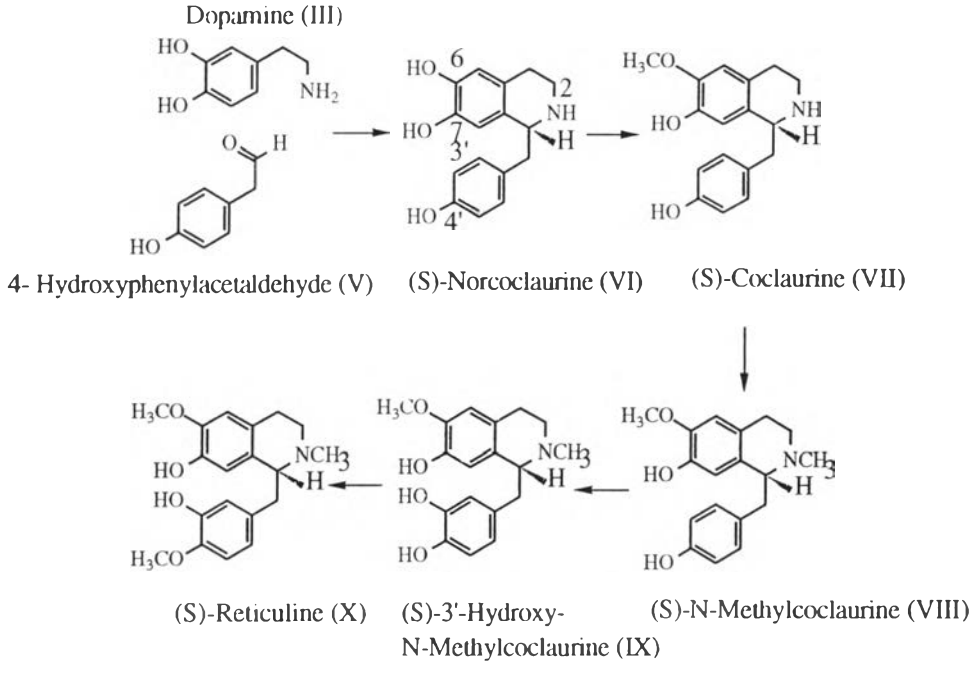


Figure 5 Biosynthesis sequence leading from the dopamine and 4-hydroxyphenylacetaldehyde to (S)-reticuline (Frenzel and Zenk, 1990)

6.2. The pathway from (S)-reticuline to morphine

(S)-Reticuline is the branch point intermediate for almost all of the benzylisoquinoline in higher plants. It has been proposed to be precursor to morphinan alkaloids by precursor feeding experiments(Battersby *et al.*, 1964). (S)-and (R)-reticuline administered to *P. somniferum* were incorporated into morphine alkaloid with about equal efficiency but with substantial loss of ^{3}H at C-1 site (Battersby *et al.*, 1965a). Loffler and co-workers (1990) concluded that the (S)-isomer is subject to complete inversion of configuration via oxidative attack at the asymmetric site. R-Form has the correct configuration to be intermediate of morphinan alkaloid. It exists in oxidation-reduction equilibrium with the (S)-form through the 1,2-dehydro compound,

1,2 dehydroreticuline which is a branch point on the morphinan alkaloid biosynthesis (Battersby *et al.*, 1965a)(Fig. 6).

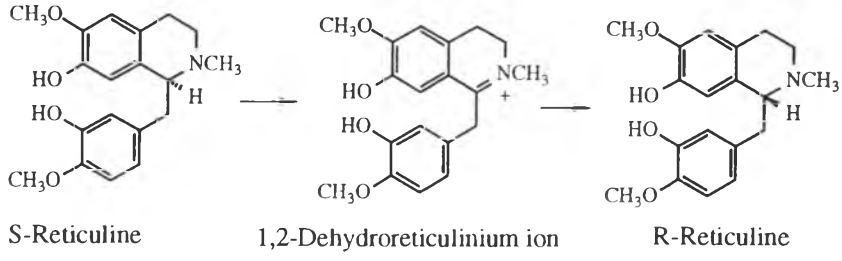


Figure 6 The conversion of (S)- to (R)-reticuline *via* 1,2-dehydroreticuline

Then, (R)-Reticuline is attacked by a phenol oxidase yielding a biradical which is stabilized by the formation of the dienone (+)-salutaridine (C-12/C-13 bond is formed)(Barton *et al.*,1965; 1967; Luckner, 1990). (+)-Salutaridine is reduced stereoselectively to (7S)-salutaridinol followed by the presented to close pentacyclic ring, to yield thebaine (Zenk *et al.*, 1989; Lotter *et al.*,1992). The conversion of thebaine to codeine is investigated by initial demethylation to neopinone, followed by rearrangement to codeinone which is reduced to codeine (Parker *et al.*, 1972). The final step is morphine formation by demethylation of codeine (Fig 7).

7. Review of Enzymes Involved in the Morphinan Alkaloid Biosynthesis

It is presently accepted that the powerful support for any biosynthetic pathway, as well as detailed information on the reaction involved may be gained by isolation, purification and characterization of enzymes which catalyse individual biosynthetic steps (Herbert, 1981). Tracer work only suggests probable biosynthetic sequence; the ultimate proof needs in vitro work of the isolated purified enzymes to give more reliable information on the actual biosynthetic sequence.

Enzymes of some general metabolic pathways have been determined in the latex of *P. somniferum*. A number of enzymes involved in the glyoxylic acid and tricarboxylic acid cycles have been found namely, aconitase, isocitrate dehydrogenase, succinate dehydrogenase, fumarase, malate dehydrogenase and isocitrate lyase (Antoun and Roberts, 1975).

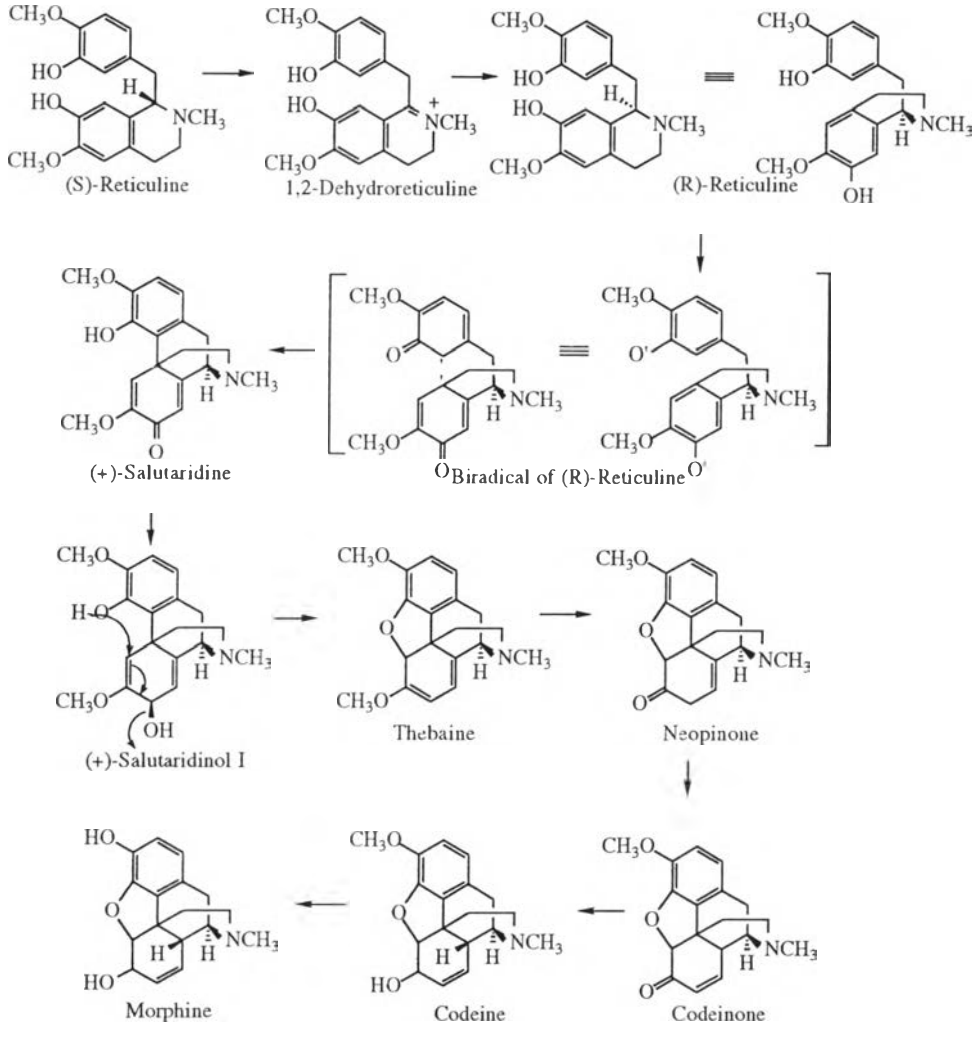


Figure 7 Proposed biosynthetic pathway of morphine in *P. somniferum* (Luckner, 1990)

For the biosynthesis of morphine, the complete sequence of morphine biosynthetic pathway, at the enzymes level, leading from the primary metabolite L-tyrosine to the end product morphine has been solved (De-Eknamkul and Zenk, 1990; Frenzel and Zenk, 1990; Gerardy and Zenk, 1990: Lenz and Zenk, 1994). The initial steps of (S)-reticuline biosynthesis involves the two enzymes, tyrosine decarboxylase and tyrosine aminotransferase, to act on tyrosine. In one way, tyrosine decarboxylase changes L-tyrosine to tyramine which is then converted to dopamine by another enzyme tyramine hydroxylase. In another way, tyrosine is changed to 4-hydroxyphenylpyruvate by tyrosine aminotransferase, and then 4-

hydroxyphenylpyruvate is converted to 4-hydroxyphenylacetaldehyde by 4-hydroxyphenylpyruvate decarboxylase (Fig.8)(Hara *et al.*, 1994).

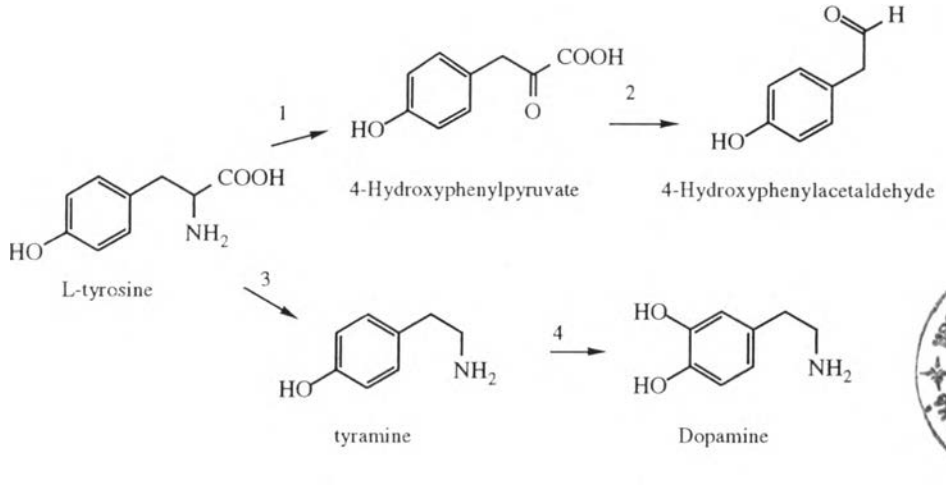


Figure 8 The enzymes involved in the initial steps of (S)-reticuline biosynthesis
1. tyrosine aminotransferase, 2. 4-hydroxyphenylpyruvate decarboxylase, 3. tyrosine decarboxylase, 4. tyramine hydroxylase

The products dopamine and 4-hydroxyphenylacetaldehyde are then served as precursor of (S)-reticuline biosynthesis (see Section 6 and Fig. 9). Both compounds are condensed stereospecifically to form the first intermediate (S)-norcoclaurine by the enzyme norcoclaurine synthase (Schmacher *et al.*, 1983). (S)-Norcoclaurine is methylated at the C-6 position in the presence of SAM by (S)-adenosyl-methionine:(R),(S)-norcoclaurine-6-O-methyltransferase (Rueffler *et al.*, 1983), to yield (S)-coclaurine. (S)-Coclaurine is then methylated at N-position by S-adenosyl-methionine:(R),(S)-coclaurine-N-methyltransferase. The N-methylcoclaurine is hydroxylated at 3'-position to form (S)-3'-hydroxy-N-methylcoclaurinet (Frenzel and Zenk, 1990; Loeffler and Zenk, 1990). The N-methylcoclaurine can be further metabolized *via* hydroxylation and peripheral modification to (S)-reticuline and its derivatives. This O- and N-methyltransferases showed a low order of stereoselectivity in that they could methylate both the (R)- and (S)-enantiomers (Brochmann-Hanssen *et al.*, 1975; Frenzel and Zenk, 1990). With the cell cultures of *Berberis stolonifera* (Loeffler and Zenk, 1990) introduced the *meta*-hydroxyl group into N-methylcoclaurine to yield 3'-hydroxy-N-methylcoclaurine. It shows a relatively broad substrate specificity with the similar capability of hydroxylating tyrosine, tyramine, (R),(S)-coclaurine and (R),(S)-N-methylcoclaurine (Loeffler and Zenk, 1990). The final methylation reaction is mediated by (S)-adenosyl-methionine:3'-hydroxy-N-methyl (S)-

coclaurine-4'-O-methyltransferase, a regio- and stereoselective enzyme, which involves the transfer of the SAM-methyl group to 4'-hydroxy group of 3'-hydroxy-N-methylcoclaurine, thus yielding the central intermediate of isoquinoline alkaloid metabolism in plants: (S)-reticuline (Frenzel and Zenk, 1990).The reticuline biosynthetic pathway is now fully discovered. The complete pathway sequence and enzymes in each step from L-tyrosine to (S)-reticuline is depicted in Fig. 9.

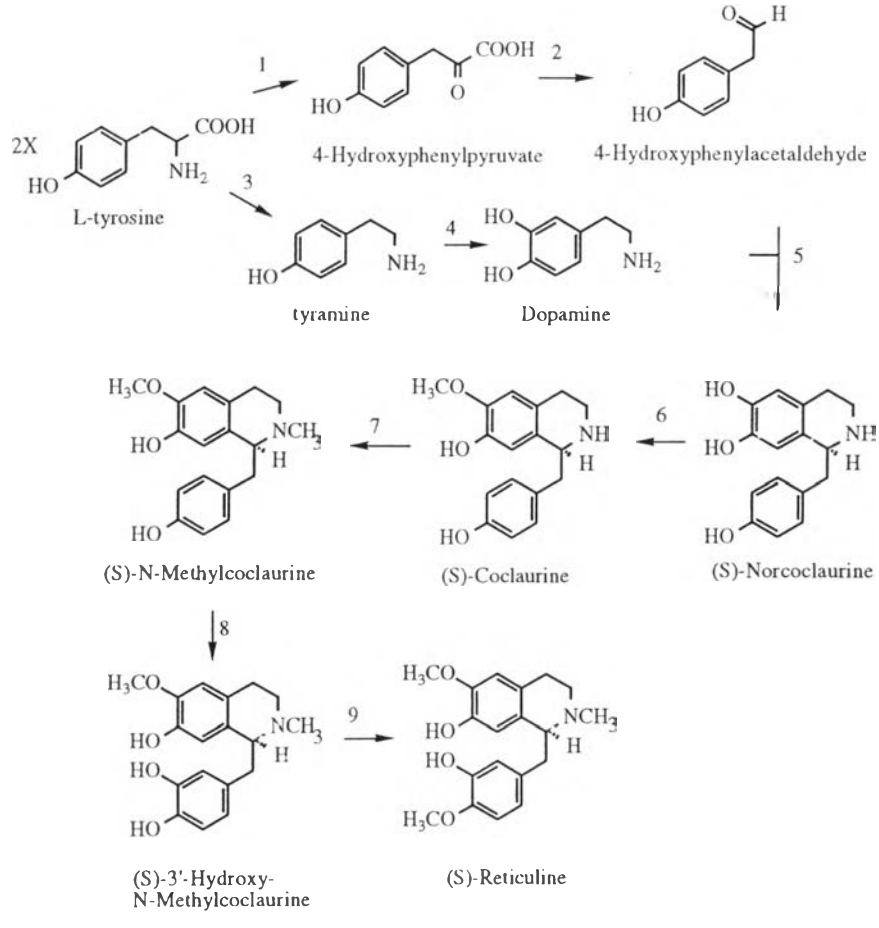


Figure 9 Various enzymes involved in (S)-reticuline biosynthesis

1. tyrosine aminotransferase, 2. 4-hydroxyphenylpyruvate decarboxylase, 3. tyrosine decarboxylase, 4. tyramine hydroxylase, 5. norcoclaurine synthase, 6. 6-O-methyltransferase, 7. N-methyltransferase, 8. 3'-hydroxylase, 9.4'-O-methyltransferase

The next step of morphine biosynthesis is the conversion of (S)-reticuline to (R)-reticuline. The enzymatic racemization of (S)-reticuline, which is essential to the biosynthesis of morphinan alkaloids, is very substrate specific (Brochmann-Hanssen *et al.*, 1982). Recently, it has been shown that the pathway leading to the morphinan

alkaloids is set in motion by 1,2 dehydroreticuline reductase (De-Eknamkul and Zenk,1990; 1992). 1,2-Dehydroreticuline reductase is a cytosolic enzyme. It requires NADPH as cofactor and present only in morphinan alkaloid-containing plants (De-Eknamkul and Zenk, 1992). The key step in the morphinandienone skeleton is the formation of the crucial C-12/C-13 bond by intramolecular phenol oxidative coupling to give salutaridine. This reaction is catalysed by a cytochrome P-450-linked enzyme, salutaridine synthase (Hodge and Rapoport, 1982; Zenk *et al.*, 1989; Gerardy and Zenk, 1993a). Next step in the pathway to morphine is the stereoselective reduction of the keto group of salutaridine to yield salutaridinol with (7S)-configuration(Lotter *et al.*, 1992). The enzyme catalysing this latter step is called, salutaridine: NADPH 7-O-oxidoreductase (Fig.10)(Gerardy and Zenk, 1993b). The transition of salutaridinol to thebaine involves the closure of the oxide bridge between C-4 and C-5 of (7S)-salutaridinol. The enzyme is named acetyl-coenzyme A: salutaridinol-7-O acetyltransferase which catalyses the reaction depicted in Fig.11.

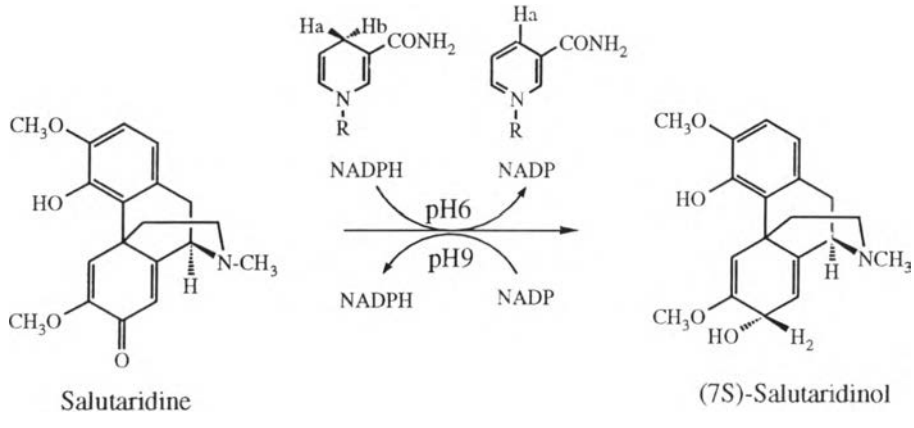


Figure 10 The reaction catalysed by salutaridine: NADPH-7-O-oxidoreductase indicating the stereochemistry of the hydride transfer (Gerardy and Zenk, 1993)

Only (7S)-salutaridinol is transformed to thebaine *via* salutaridinol-7-O-acetate, which at slight alkaline pH values, spontaneously rearranges to thebaine by closing of the oxide bridge (Lenz and Zenk, 1994). Thebaine conversion to neopinone was established by Parker *et al.* (1972). Neopinone is then spontaneously isomerized to codeinone, which is subsequently reduced to codeine by the enzyme codeine:NADP oxidoreductase (Furuya *et al.*, 1978; Hsu, 1981; Corcete and Yeoman, 1987; Luckner, 1990; Gollwitzer, 1993). The final step, codeine is demethylated to morphine

(Hsu and Pack, 1989). In all the biosynthetic pathway from L-tyrosine to morphine involves 16 enzymes, 14 of which have been isolated and characterized. However, two of the reactions steps occur spontaneously: those of the formation of thebaine from 7-O-acetylsalutaridinol and the isomerization of neopinone to codeinone. The overall sequence reaction from (S)-reticuline to morphine is shown in Fig. 12. The list of catalysing enzymes in each step is presented in Table 5, and the list of properties of some enzymes which were purified and characterized, is concluded in Table 6.

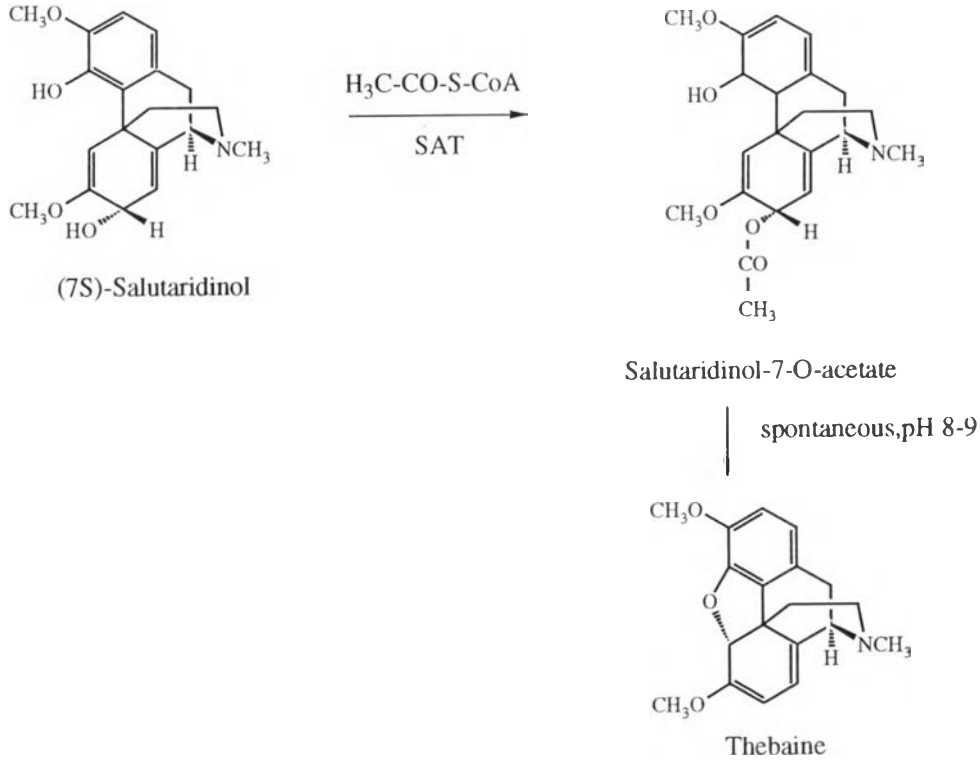


Figure 11 Reaction catalysed by acetyl coenzyme A: salutaridinol-7-O-acetltransferase (SAT) and subsequent spontaneous allylic elimination at pH 8-9 to thebaine (Lenz and Zenk, 1994)

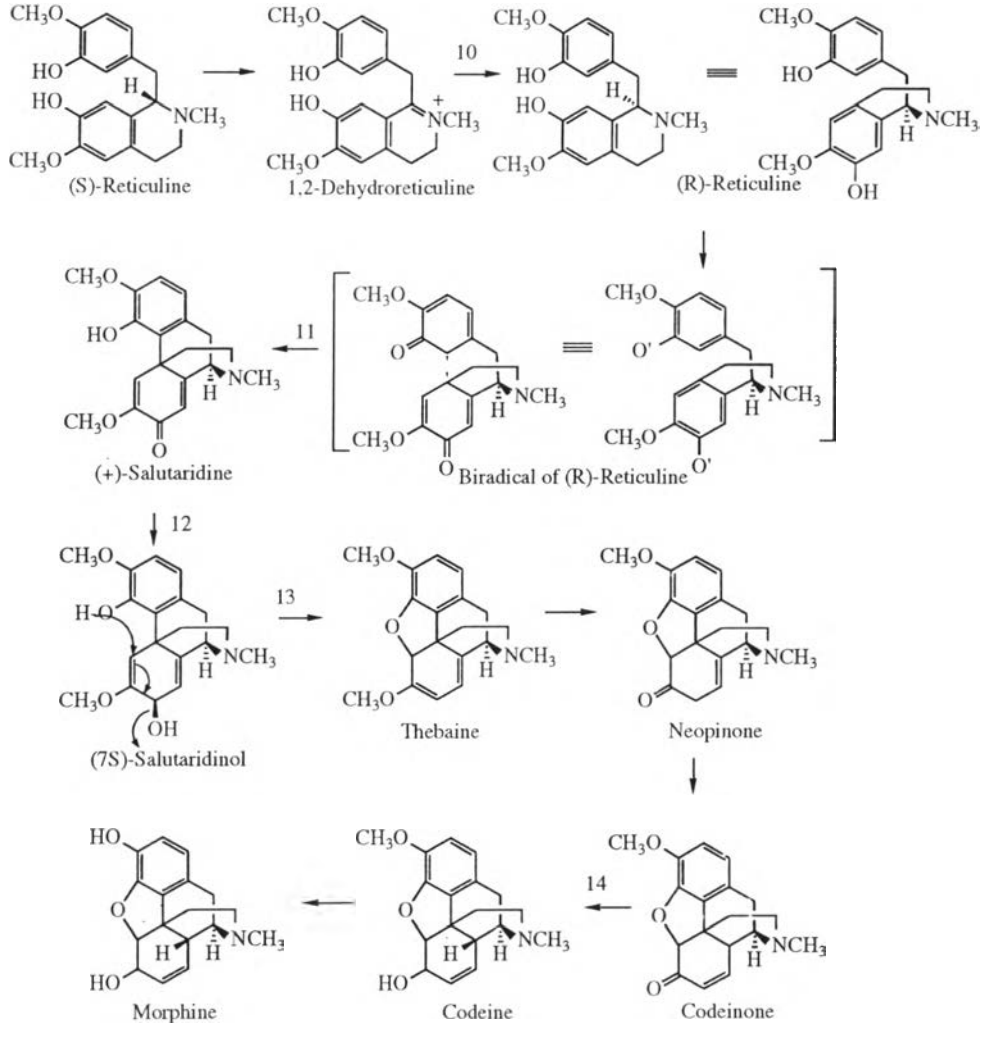


Figure 12 Various enzymes involved in morphine biosynthesis:

10. 1,2-dehydroreticuline reductase, 11.salutaridine synthase,

12.salutaridine: NADPH-7-O-oxidoreductase, 13.salutaridine-7-O-acetyltransferase,

14. codeine: NADP-oxidoreductase

Table 5 List of enzymes involved morphinan alkaloid biosynthesis

No.	Name of enzyme
1	Tyrosine aminotransferase
2	4-Hydrxoyphenylpyruvate decarboxylase
3	Tyrosine decarboxylase
4	Tyramine decarboxylase
5	Norcoclaurine synthase
6	6-O-Methyltransferase
7	N-Methyltransferase
8	3'-Hydroxylase
9	4'-O-Methyltransferase
10	1,2-Dehydroreticuline reductase
11	Salutaridine synthase
12	Salutaridine:NADPH-7-O-oxidoreductase
13	Salutaridinol-7-O-acetyltransferase
14	Codeine: NADP-oxidoreductase

Table 6 Some properties of the some enzymes which were purified and characterized

Name of enzyme	pH (opt.)	T °C (opt.)	Molecular weight (kD)	Stereo-specificity
Norcoclaurine synthase	7.8	40	15.5	yes
6-O-Methyltransferase	7.5	35	47	no
N-Methyltransferase (isoform)	6.8, 7.4	35,40	60-78	no
3'-Hydroxylase	6.0	20-30	60	no
4'-O-Methyltransferase	8.3	35-40	40	yes
1,2-Dehydroreticuline reductase	8.5	30	30	yes
Salutaridine synthase	7.5	25	-	yes
Salutaridine: NADPH-7-O-oxidoreductase	6-6.5	9-9.5	52	yes
Salutaridinol-7-O-acetyltransferase	7,9	47	50	yes

8. 1,2-Dehydroreticuline

8.1. Structure and chemical properties

1,2-Dehydroreticuline exists as a natural product and its role has provided a means of interconverting the two enantiomeric forms of (R)- and (S)-reticuline (Battersby *et al.*, 1965b; Borkowski *et al*, 1978). A cytosolic NADPH-dependent enzyme, 1,2-dehydroreticuline reductase, has been found to catalyse this reaction (De-Eknamkul and Zenk, 1990). 1,2-Dehydroreticuline is an benzylisoquinoline alkaloid, the structure of it is shown in Fig. 13. Its chloride salt has a formula of $C_{19}H_{22}NO_4Cl$ and molecular weight of 327 (Borkowski *et al.*, 1978). Its chemical name is 3,4 dihydro-1-(3'-hydroxy-4'-methoxybenzyl),-6-methoxy-2-methylisoquinoline-7-ol (He, 1993).

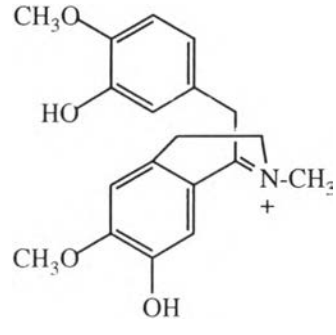


Figure 13 The chemical structure of 1,2 dehydroreticulinium ion (Borkowski *et al*, 1978).

1,2-Dehydroreticulinium chloride is a stable salt. Its melting point is 190-200°C (decomposed). The infrared spectrum shows a band at 1630 cm^{-1} which is the characteristic of conjugate iminium salt. Its ultraviolet spectrum(λ_{max} 250, 323 nm) (Battersby et al., 1965b) is similar to the UV spectra of dihydroisoquinoline(Bill and Noller, 1948). The NMR spectrum confirms its iminium salt character by the appearance of the C-9 methylene hydrogen as a two-hydrogen singlet at 4.40 ppm (Borkowski *et al.*, 1978).

8.2 Synthesis of 1,2-dehydroreticuline

In 1965, Battersby and co-workers have reported the synthesis of 1,2-dehydroreticuline fin order to morphine alkaloid biosynthesis. 1,2-Dehydroreticuline is

synthesized from the constitution of freshly prepared silver chloride and the solution of 1-(3-benzyloxy-4-methoxybenzyl)-6-methoxy-7-benzyloxy-3-4-dihydroisoquinoline methyliodide to yield *OO*-dibenzyl-1,2-dehydroreticuline chloride. Then, acid catalysed debenzylation of the product afforded the stable 1,2 dehydroreticuline chloride.

About 15 years later, Borkowski and co-workers synthesized 1,2-dehydroreticulinium ion, and established as an intermediate in morphinan alkaloid biosynthesis. The initial step of its total synthesis proceeded from vanillin to 3-benzyloxy-4-methoxy phenylacetic acid, while another precursor, 4-benzyloxy-3-methoxyphenylacetonitrile was reduced to amine using sodium borohydride and cobalt chloride in methanol. The amine and 3-benzyloxy-4-methoxyphenylacetic acid were condensed to amide when refluxing in xylene. The amide then form quantitatively when acid and amine were refluxing toluene with $POCl_3$, gave the iminium chloride in 95% yield. The iminium chloride was treated with methyl iodide in methanol, to give the methiodide was treated with excess freshly prepared silver chloride with a yield 92% in aqueous methanol, yield the methochloride. Finally, debenzylation of the methochloride was performed in refluxing ethanolic HCl to give pure 1,2-dehydroreticulinium chloride (Borkowski *et al.*, 1978). The total synthesis of 1,2-dehydroreticulinium chloride is summarized in Fig.14.

The authentic 1,2-dehydroreticulinium ion was prepared by 7 stages of chemical synthesis (as mentioned above), had low specific activity with enzyme which catalysed its reaction. The synthesis can also be carried out enzymatically either which norreticuline or reticuline as substrate. First, (S)-reticuline was synthesized from (S)-norreticuline using S-adenosyl-L-methionine and the enzyme N-methyltransferase isolated from *Berberis stolonifera* cell cultures. 1,2-Dehydroreticuline was then synthesized from (S)-reticuline by using the partially purified (S)-tetrahydroprotoberberine oxidase (STOX) enzyme from the same cell culture. The catalysed steps are shown in Fig.15 (Amann and Zenk, 1987).

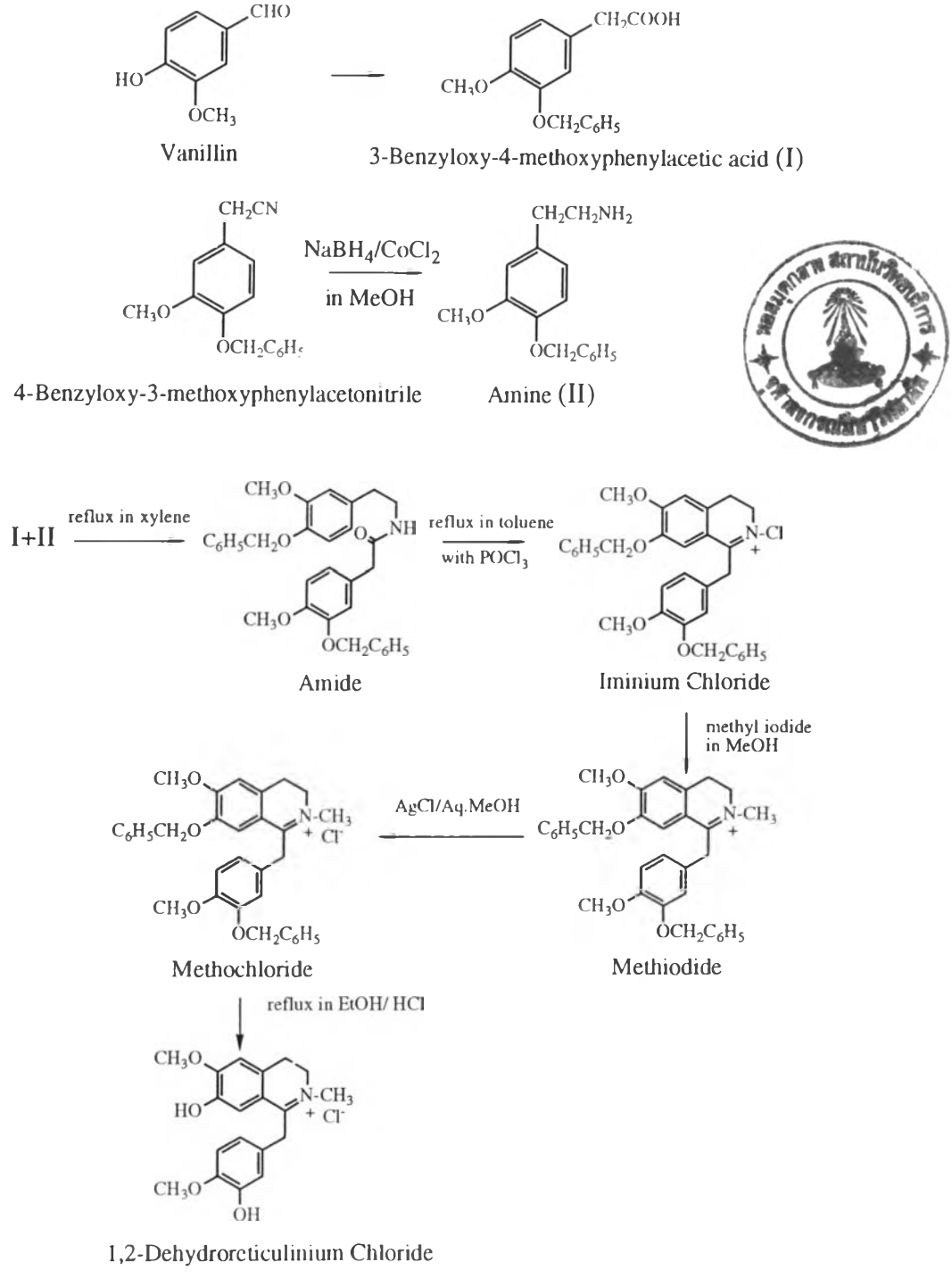


Figure 14 The total synthesis of 1,2-dehydroreticulinium chloride

(Borkowski *et al.*, 1978)

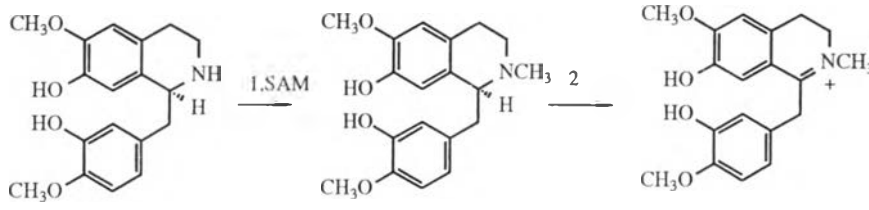


Figure 15 The synthesis of 1,2 dehydroreticuline by two enzymatic steps from (S)- norreticuline (1=N-methyltransferase; 2=S-tetrahydroprotoberberine oxidase)

In *Berberis stolonifera* cell culture, STOX catalyses the dehydrogenation of (S)-tetrahydroprotoberberine to their corresponding 1,2-dehydro analogues. However, for this process the rate of oxidation of (S)-reticuline is approximately 1% of that of (S)-norreticuline(Amann *et al.*, 1988). Therefore, another alternative method of 1,2-dehydroreticuline synthesis was proposed. (S)-Norreticuline was first oxidized by 0-70% ammonium sulfate precipitated enzyme fraction from *Berberis stolonifera* cell cultures to yield 1,2-dehydronorreticuline followed by a step of chemical methylation using methyl iodide to form 1,2-dehydroreticuline (De-Eknamkul and Zenk, 1992)(Fig.16).

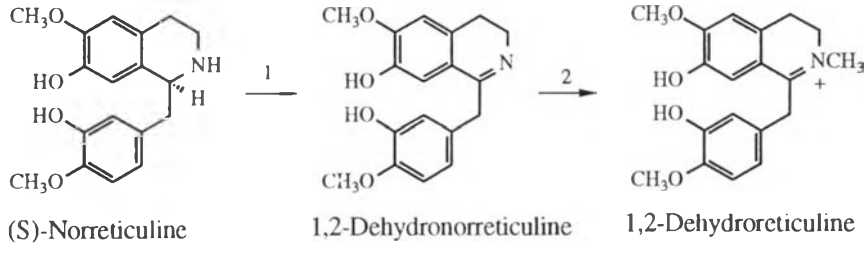


Figure 16 The synthesis of 1,2 dehydroreticuline from (S)-norreticuline by enzymatic and chemical reaction, respectively (1=S-tetrahydroprotoberberine oxidase, 2=methyl iodide)