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The formation of benzophenanthridine alkaloids

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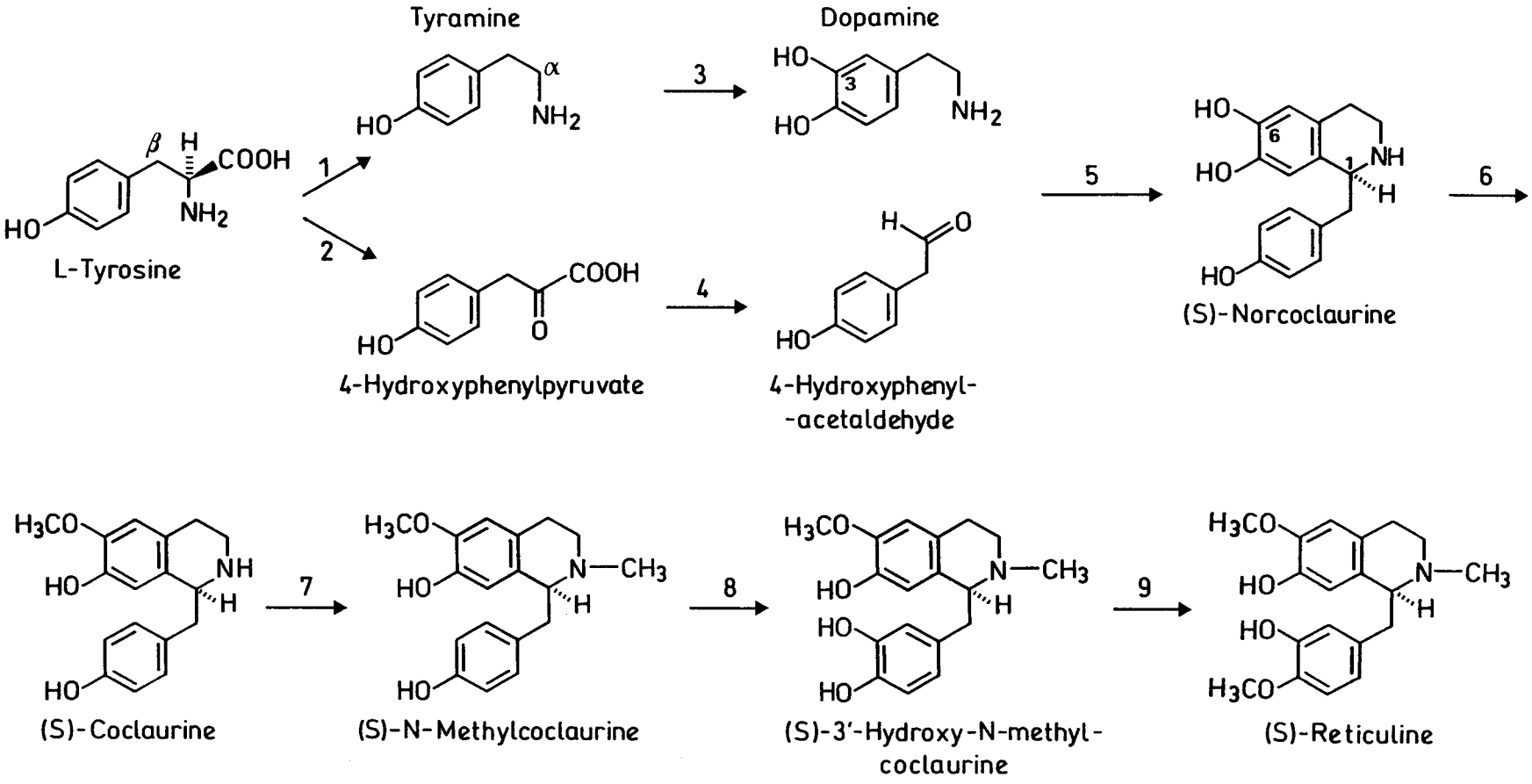
Abstract: Isoquinoline alkaloids are a major group of pharmacologically important compounds. The universal precursor to the majority of this alkaloid class is (S)-reticuline. The biosynthesis of this important intermediate starting from the primary metabolite, L-tyrosine, has been completely solved at the enzyme level. One of the most diverse structures in the class of isoquinoline alkaloids are the benzo[c]phenanthridines. The most highly oxidized member is macarpine, an alkaloid produced in considerable quantity in cell suspension cultures of *Eschscholtzia californica*. This plant source was used to isolate all of the enzymes involved in this pathway. Twelve steps are necessary for the transformation of (S)-reticuline to macarpine, eleven of these are enzyme catalyzed, one is spontaneous.

Nature's pathways to complex secondary products, expecially alkaloids, have attracted for a long time the interest of scientists in the chemical fields (e.g. 1). However, it was not until the 1960's that a picture of the biosynthesis of these compounds began to emerge, largely speculative, based on isotopically labelled precursor feeding experiments to differentiated plants (2). It became quite clear at that time that the question of how these metabolites are truly formed was to identify and characterize the enzymes involved in the biosynthesis of these natural products (3). This aim has largely been achieved for select groups of compounds in the past two decades through the consequent use of plant cell suspension cultures to chase the biosynthetic enzymes of the plant kingdom (4). The rational behind biosynthetic studies these days is at least threefold: for one, the aesthetics of learning how nature with its protein catalysts created a repertoire of synthetic methods, far ahead of synthetic organic chemistry; Second to provide chemists with ideas for biomimetic synthesis and thirdly, and most important, to provide (catalytic) targets for gene technological manipulation. Therefore the knowledge of a biosynthetic pathway is an absolute prerequisite for the isolation of the genes and regulatory elements underlaying these reactions. No doubt, enzyme and gene technology will soon become available on a larger scale, which will have a major impact on the production of agriculturally and medicinally useful plants. It has been predicted that the second "revolution" in gene technology will concern low molecular weight compounds, including alkaloids.

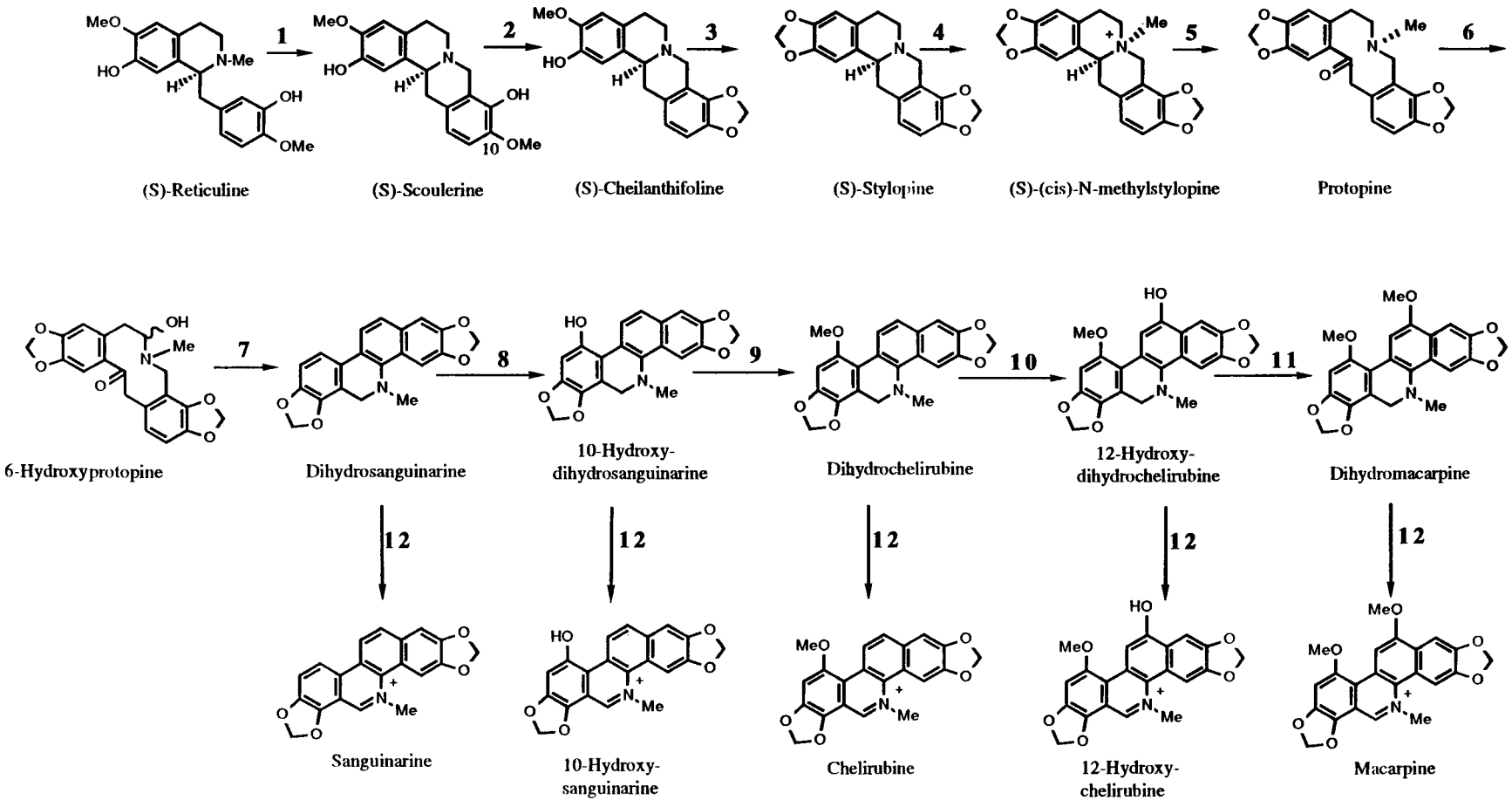
Within the class of alkaloids, the isoquinoline alkaloids are of particular interest, due to their enormous potential chemical variations of the basic precursor molecules and on the other hand comprises some of the most important drugs for therapy and euphoria (e.g. morphine and its chemical derivatives, papaverine, berberine, dimeric bisbenzylisoquinolines). Of about 29 alkaloids used as pure compounds in western medicine today, six are benzylisoquinoline derived compounds (5). Now what is the common precursor to benzylisoquinolines? No doubt, (S)-reticuline. This compound was predicted on theoretical grounds to be the biogentic precursor of, for instance, morphine (6) even prior to its discovery in nature (7). The next question to be asked is, how is (S)-reticuline biosynthesized in the plant. Visually dissecting the then-known benzylisoquinoline alkaloids, Winterstein and Trier (8) and later Robinson (9) came to the conclusion that all of these alkaloids were derived from a simple tetrahydroxylated benzylisoquinoline named norlaudanosoline. These authors also had already suggested that this molecule might arise by condensation of dopamine with 3,4-dihydroxyphenylacetaldehyde to afford the tetraoxygenated base norlaudanosoline (8,9). This proposal was put to test when radiolabelled tracer molecules became commercially available. Satisfactory incorporation of the labelled tracer molecule, norlaudanosoline, was found to occur into different classes of benzylisoquinoline alkaloids (10, 11) which seemingly verified the old Winterstein and Trier



Scheme 1: Enzymatically verified biosynthetic pathway from L-tyrosine to (S)-reticuline, the precursor of a multitude of benzylisoquinoline alkaloids.

(8) biogenetic hypothesis. These tracer experiments later were supported by enzymatic investigations, which showed that in cell cultures of various *Papaveraceae*, an enzyme is present which stereoselectively condenses dopamine and 3,4-dihydroxyphenylacetaldehyde (as well as other substituted aldehydes) by a Pictet-Spengler-condensation to yield the (S)-configurated assumed alkaloid precursor 12,13). One of the unresolved questions was the fact that precursor feeding experiments with labelled DOPA or dopamine into a multitude of alkaloids showed that only the isoquinoline and not the benzylic portion of these tetraoxygenated molecules were labelled while tyrosine labelled both halves (2). These experiments proved that the two C_6-C_2 units derived from tyrosine differ from one another. The intermediacy of the purported base, norlaudanosoline, in the construction of benzylisoquinoline alkaloids was seriously questioned for the first time when a common biogenetic origin was shown for the trioxygenated bases of the coclaurine type and reticuline in *Annona reticulata* plants (14). The question of the pathway leading from L-tyrosine to (S)-reticuline was solved when it was demonstrated that the label from (S)-[1-^{13}C]norcoclaurine and (S)-[1-^{13}C]coclaurine was highly and non-randomly incorporated into (S)-reticuline in benzyltetrahydroisoquinoline-producing plant cell cultures of *Berberis stolonifera, Eschscholtzia californica* as well as *Peumus boldus* (15). From these surprising incorporation data observed in vivo, it was possible to conclude that (S)-norcoclaurine is stereoselectively metabolized to (S)-reticuline via (S)-coclaurine, (S)-N-methylcoclaurine and (S)-3'-hydroxy-N-methylcoclaurine. Neither norlaudanosoline, its 6-0-methyl ether nor (S)-norreticuline are intermediates in this important metabolic route. In the meanwhile, all enzymes involved in this transition from tyrosine to (S)-reticuline are in hand and published (16). The following enzymes are listed in the order of their biosynthetic sequence: 1) (S)-norcoclaurine synthase (previously called (S)-norlaudanosoline synthase, 2) S-adenosyl-L-methionine: (R,S)-norcoclaurine-6-O-methyltransferase (previously called (R),(S)-norlaudanosoline-6-O-methyltransferase), 3) S-adenosyl-L-methionine: (R,S)-coclaurine-N-methyltransferase (previously called S-adenosyl-L-methionine: norreticuline N-methyltransferase), 4) (R,S)-N-methylcoclaurine-3'-hydroxylase (most likely a phenolase), 5) S-adenosyl-L-methionine: 3'-hydroxy-N-methyl-(S)-coclaurine-4'-O-methyltransferase. A total of five hitherto not known enzymes is involved in the transformation of dopamine and 4-hydroxyphenylacetaldehyde into (S)-reticuline (Scheme 1). Two enzymes (1 and 5) are absolutely stereoselective yielding or acting only on the (S)-configurated benzylisoquinoline alkaloid. Three of the enzymes (1, 2, 3) had to be renamed in light of the above evidence that the trihydroxylated and not the tetrahydroxylated alkaloids as assumed previously are the true precursors. The sequence of O- and N-methylation steps is strictly governed by the sequential action of stereoselective and non-selective enzymes affording the bisphenolic dimethylether, (S)-reticuline, with the potential for further complex modifications. The clarification of the (S)-reticuline pathway was the result of the combination of in vivo ^{13}C NMR, and in vitro enzymatic studies. It should be noted, that the compound (S)-N-methylcoclaurine of the gerneral reticuline pathway is an important branchpoint intermediate. This compound, together with its (R)-congener, is oxidized by a newly discovered cytochrome P-450 enzyme from *Berberis* cellcultures to give rise to the bisbenzylisoquinoline alkaloid, berbamunine (17), a member of a large family (18) of differentially substituted and coupled dimers.

Let us turn now to the enzymic formation of one of the more complex (S)-reticuline-derived alkaloids, the benzophenanthridines. Benzo[c]phenanthridine alkaloids are a specific group of isoquinoline alkaloids which occur only in higher plants and are constituents mainly of the *Papaveraceae* family. A cell culture system has been worked out in our laboratory using *Eschscholtzia californica* as the experimental plant system, which produces copious amounts of highly oxidized alkaloids especially if the culture is treated with a sterilized cell wall preparation of microorganisms, a so-called elicitor. Using this cell culture, the complete pathway leading from (S)-reticuline to the most highly oxidized member of the benzophenanthridine alkaloid family, macarpine, was worked out in our laboratory. The initial steps in the general pathway were made clear by the pioneering work of Battersby (19) and Takao (20) and their coworkers. Starting with (S)-reticuline, the benzophenanthridine pathway is opened by the vesicularly contained berberine bridge enzyme which gives rise to (S)-scoulerine (step 1, Scheme 2). This berberine bridge enzyme was purified to homogeneity from *E. californica*, cloned (21) and exactly characterized. This enzyme is housed in a specific vesicle with a density of $\rho = 1.14\ g \cdot ml^{-1}$. It is absolutely specific for (S)-reticuline. (R)-Reticuline tested as a substrate is absolutely inactive. (S)-Scoulerine is the reaction product and stoichiometric amounts of H_2O_2 are also generated. The enzyme is absolutely dependent on the presence of O_2 but no soluble cofactor is necessary. The product (S)-scoulerine has to leave the vesicle in which it was formed, diffuse within the cell towards the microsomes and in the presence of NADPH and O_2 two methylendioxybridges are formed in two consecutive steps: (S)-cheilanthifoline from (S)-scoulerine (step 2) and (S)-stylopine from (S)-



Scheme 2: Enzymatically verified biosynthetic pathway from (S)-reticuline to the benzophenanthridine alkaloid, macarpine.

cheilanthifoline (step 3). Use of [9-OC^{3}H$_3$]- and [3-OC^{3}H$_3$]-labelled scoulerine and cheilanthifoline, respectively, offered a convenient assay for both of these enzymes (22). Both cytochrome P450-enzymes show maximal activity at pH 8. Both enzymes were surprisingly substratespecific. Only (S)-scoulerine or (S)-cheilanthifoline was transformed. Absolutely no reaction was observed with tetrahydroprotoberberine analogues with an (R)-configuration or any of the other derivatives tested in either enantiomerically pure (S)- or (RS)-forms. Stylopine and other (S)-configurated tetrahydroprotoberberine alkaloids like (S)-canadine can be subjected to N-methylation at the expense of SAM which generates the *cis*-N-methyl-derivatives of tetrahydroprotoberberine, which has been elegantly shown to be the precursor for the benzophenanthridines in vivo.(20) This methyltransferase was partially purified, and is of about 78 kD. This enzyme (step 4, Scheme 2) appears to be cytosolic and was named S-adenosyl-L-methionine: (S)-tetrahydroprotoberberine-*cis*-N-methyltransferase (23). This enzyme opens the benzophenanthridine pathway. The (S)-*cis*-N-methyl-stylopine acts as substrate for another cytochrome P-450 hydroxylase which oxidizes the substrate compound to protopine (step 5), the lead alkaloid of the *Papaveraceae* (24). This microsomal cytochrome P-450 NADPH-dependent enzyme hydroxylated stereo- and regio-specifically carbon atom 14 of (S)-*cis*-N-methyl-tetrahydroprotoberberine. It is a typical monooxygenase and the enzyme is inhibited by cytochrome P-450 inhibitors as well as by carbon monoxide. The most critical step in the biosynthesis of benzophenanthridine alkaloids is the enzymatic opening of the B-ring of protopine. In an elegant piece of work, Tanahashi (25) in our laboratory succeeded in the synthesis of 6-[^{3}H]protopine which was used as a substrate for the purported hydroxylase. It was assumed that during the hydroxylation of the 6-position of protopine the ring would open and possibly rearrange either enzymatically or spontaneously. This goal was achieved by using [6-^{3}H]protopine as a substrate and measuring the release of tritium using cell-free preparations of *E. californica*. Indeed a microsomal preparation could be found which catalyzed the hydroxylation of [6-^{3}H]protopine with the concomitant formation of [11-^{3}H]dehydrosanguinarine and HOT. The hydroxylation proved strictly dependent on NADPH as reduced cofactor and on molecular O_2. The monooxygenase was inhibited by the classical cytochrome P-450 inhibitors and CO. The hydroxylase was induced by a fungal elicitor about 8-fold after challenging the plant cell culture with the glucoprotein. The hydroxylase is specifically present only in those plant species which produce benzo[c]phenanthridine alkaloids in culture (25). No evidence has ever been obtained in this enzyme work for a stable intermediate between protopine and dehydrosanguinarine. Therefore we assume that a single enzymatic hydroxylation at C-6 of protopine leads to the spontaneous rearrangement of this molecule to yield dihydrosanguinarine (steps 6 and 7, Scheme 2). Obviously the protein pocket of the active center of the 6-hydroxylase (step 6) provokes this rearrangement which proceeds in a quantitative manner.

If a plant accumulates sanguinarine, then its precursor, dihydrosanguinarine, is subsequently oxidized by the known enzyme dihydrobenzophenanthridine oxidase (26) to afford the fully aromatized quarternary benzo[c]phenanthridine sanguinarine (26, 27). If, however, the plant species produces macarpine in addition, as it is the case, for instance, in *E. californica* cell cultures, two subsequent hydroxylation and two methylation steps have to follow. In additional work, again using *E. californica* cell suspension cultures it was possible to detect two additional cytochrome P-450 enzymes: dihydrosanguinarine-10-hydroxylase (step 8) and S-adenosyl-L-methionine: 10-hydroxydihydrosanguinarine-10-O-methyltransferase (step 9). Both enzymes were partially characterized and found to be highly substrate specific (28). Both enzymes are specific only for the dihydro-derivatives, the oxidized members of the sanguinarine family are not transformed at all. Recently, the two enzymes transforming dihydrochelirubine to macarpine were also discovered in our laboratory using suspension cultures of *Thalictrum bulgaricum* and *E. californica* (29). The enzymes were designated dihydrochelirubine-12-hydroxylase (step 10) and S-adenosyl-L-methionine: 12-hydroxydihydrochelirubine-12-O-methyltransferase (step 11). Again both enzymes were found to be highly substrate specific and only the dihydro-compounds were substrates for both enzymes. The final step in macarpine synthesis is then the oxidation of dihydromacarpine to macarpine by dihydrobenzophenanthridine oxidase (step 12) (26, 27). This enzyme oxidizes a variety of dihydrobenzophenanthridine molecules and it is most likely a copper containing enzyme (27). The enzyme is only active in the presence of molecular oxygen and H_2O_2 is formed as a reaction product. This terminal reaction concludes the pathway from (S)-reticuline to macarpine which involves a total of 11 highly specific enzymes and 1 spontaneous rearrangement (step 7). Out of these 11 enzymes, 6 catalysts are cytochrome P-450 enzymes which are highly specific and seem to catalyze only one reaction each. Since the pathway from tyrosine to (S)-reticuline is also known at the enzyme level (see above), the conversion of L-tyrosine to macarpine involves a total of 19 enzymes which are now all

known and at least partially characterized. Only one cDNA encoding one of these enzymes has been cloned yet (21). This pathway leading to macarpine is probably the longest which has ever been elucidated at the enzyme level for any given secondary product.

The knowledge of these pathways will also give a hint as to the regulation of secondary products within plants. Attention should be drawn to the recent finding that one of the signal compounds in controlling the formation of secondary products, including benzophenanthridine alkaloids, in plant cell cultures is jasmonic acid (30, 31). The combination of enzymology with molecular biology will be expected to eventually clarify the mode of regulation of secondary compounds (32).

A start has been made to unravel the first complete pathway to an alkaloid from the primary metabolite (L-tyrosine) to the final product (macarpine). No doubt other pathways will follow. This biosynthetic knowledge will provide molecular biologists with new targets in their aim to modify the productivity of plants towards these pharmacologically active molecules. Plant enzymes expressed in microorganisms can possibly be used to establish biotechnological processes for the production of some of these desirable molecules. A new area for secondary plant products will emerge which will consist of the combination of phytochemistry with biochemistry, molecular biology and biotechnology. This subject, we predict, will have considerable impact on agriculture and on the supply of desirable plant products for society.

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Dedicated to Professor Wolfhart Rüdiger on the occasion of his 60th birthday.

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