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ENZYMOLOGY AND MOLECULAR BIOLOGY OF ALKALOID BIOSYNTHESIS[1]

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Abstract. The biosynthetic pathways leading to the benzophenanthridine alkaloid, sanguinarine, and the indole alkaloids, ajmalicine and ajmaline, have been solved at the level of the individual enzymes involved. The cDNA for two of the key enzymes has been cloned and the enzymes subsequently actively expressed in heterologous organisms.

INTRODUCTION

Ninety-nine percent of the biomass on this planet consists of plants, which corresponds to approximately 1.7 x 10^{12} tons of dry matter.[1] Plants either directly or indirectly support all human and animal life on this globe. In order to survive, plants have developed, in addition to mechanical protection through bark, wood, cork, thorns, etc., a highly sophisticated chemical defense system.[2] Tens of thousands of highly complex and often species-specific organic chemicals formed during evolution serve the purpose, at least in part, of protecting plants from predators.

Among these compounds of chemical defense is the large, nitrogen containing, pharmacologically active group of alkaloids, probably the most chemically diverse of all defense compounds. Within this group, the isoquinoline and the indole alkaloids are of particular interest due to the enormous potential chemical variation of the basic precursor molecules, (S)-norcoclaurine and 3α(S)-strictosidine, which can lead in the case of the isoquinoline derivatives to over 2500 known members and in the case of indole derivatives to more than 1200 known alkaloids. The study of the biosynthesis of these compounds has, in addition to heuristic value, a potential for biotechnological application. In order to understand the biosynthesis of these molecules, the enzymes of specific pathways have to be identified and characterized. Key enzymes should be analyzed at the molecular genetic level in order to clarify the complex regulation of alkaloid biosynthesis in the plant, in particular under conditions of pathogen attack. The expression of plant enzymes (which are often present in minute quantities in plants) in heterologous systems such as bacteria and yeast would allow for detailed biochemical analyses of mechanisms of reaction which are often unparalled in nature and in synthetic organic chemistry. Likewise, heterologous expression would exploit the biotechnological potential of these enzymes for the production of known and new alkaloids for use by mankind.

We provide here two examples which demonstrate that whole pathways of complex alkaloids can be solved at the enzymic level and that enzymes in key locations of these pathways can be heterologously expressed and eventually their regulation studied. The examples given here are the benzo [c] phenanthridine alkaloid, sanguinarine, the biosynthesis of which we have studied in the past years[3-8] and, briefly, the

1) Dedicated to Professor R. Huisgen on the occasion of his 70th birthday.

indole alkaloids, ajmalicine and ajmaline, the biosynthesis of which has been worked by Stöckigt[9-10]. The enzymes involved in the biosynthesis of sanguinarine and ajmaline were identified using plant cell suspension cultures of either isoquinoline (sanguinarine) - forming *Eschscholtzia californica* or indole alkaloid producing *Rauvolfia serpentina*, both of which had been optimized for alkaloid production.

RESULTS AND DISCUSSION

The focus of this first investigation is the isoquinoline pathway leading to sanguinarine, depicted in Fig. 1. (S)-Reticuline is formed by the stereoselective, enzyme catalyzed condensation of dopamine with 4-hydroxyphenylacetaldehyde[11] to yield (S)-norcoclaurine, which is in turn transformed in four consecutive enzyme catalyzed steps into (S)-reticuline, a pathway which has been elaborated *in vitro* and *in vivo*.[12] All

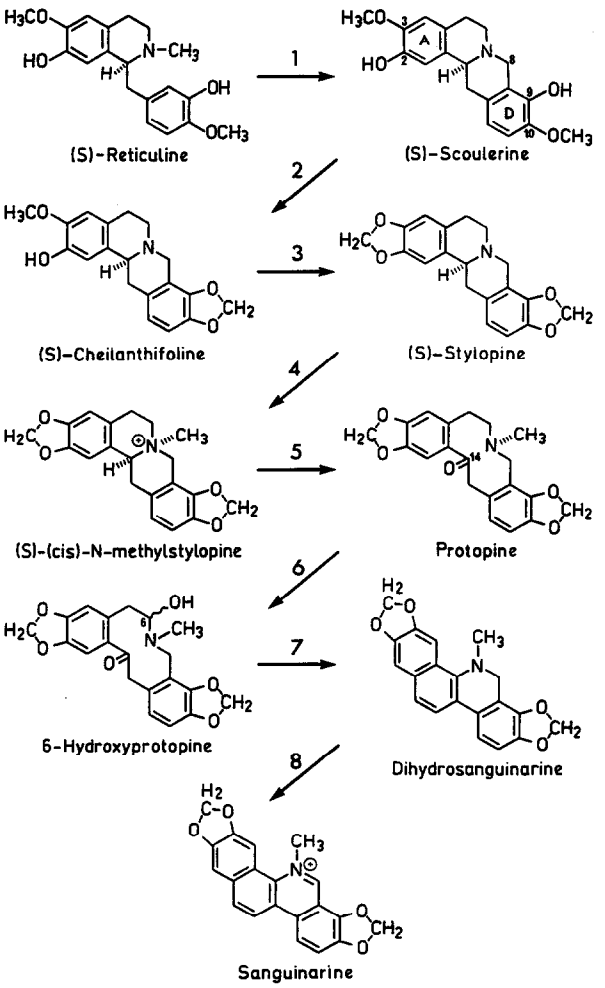

Figure 1. Enzymatically verified biosynthetic pathway leading from (S)-reticuline to sanguinarine.

of these enzymes have been partially purified and characterized in our laboratory[13]. (S)-Reticuline is the major branch point intermediate leading to a vast number of structurally diverse isoquinoline alkaloids.[14] One of the pathways which (S)-reticuline can enter is that leading to the benzophenanthridine alkaloid, sanguinarine. (S)-Reticuline is converted by action of the berberine bridge enzyme [EC 1.5.3.9] (Step **1**) in the presence of O_2 to (S)-scoulerine without apparent cofactor requirement.[3] This enzyme is located in the plant cell within a specific vesicle[3,15] from which it can be released by osmotic shock, suggesting that it is either soluble within the vesicle or very loosely membrane associated. Berberine bridge enzyme catalyzes a classical phenol coupling reaction with the formation of the berberine bridge (C-8); however, whether this mechanism involves a radical coupling or the union of an imminium ion with a phenoxonium ion (Fig. 2) has not yet been clarified. (S)-Scoulerine is, by the action of two highly substrate-specific and stereoselective

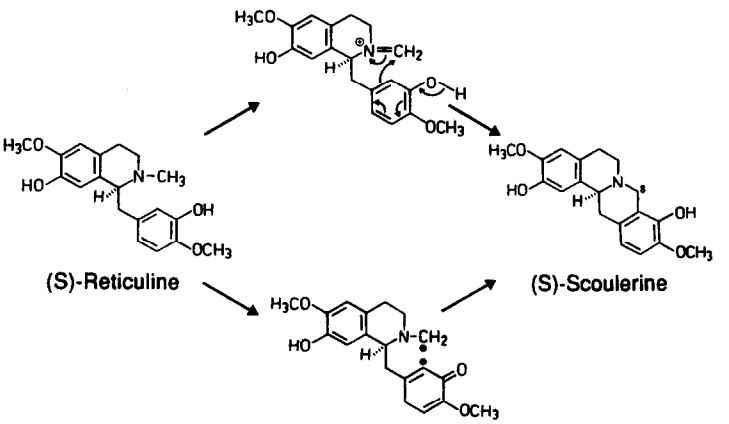

Figure 2. Ionic *versus* radical mechanism of reaction catalyzed by berberine bridge enzyme.

cytochrome P-450 containing enzyme complexes (Steps **2** and **3**) in the presence of O_2 and NADPH, transformed into (S)-stylopine.[4,16] It has been proven that the methylenedioxy bridge at ring D (C-9, C-10) is formed first and is followed by that at ring A (C-2, C-3). This was concluded from the unambiguous substrate specificity of both enzymes involved.[16] Again, the mechanism of the methylenedioxy ring closure is not clear and we postulate a sequence[16] as depicted in Fig. 3. The next step (**4** in Fig. 1) is catalyzed by an N-methyltransferase which, in the presence of S-adenosylmethionine, yields (S)-*cis*-N-methylstylopine that was previously identified as a precursor of sanguinarine by *in vivo* feeding experiments.[17] This enzyme is highly stereoselective and only N-methylates tetrahydroprotoberberine alkaloids with the (S)-configuration. In addition, the substitution patterns of rings A and D govern the substrate specificity. For instance, (S)-scoulerine is inactive as a substrate for the N-methyltransferase, while (S)-stylopine shows maximal activity as a substrate. This specificity of the N-methyltransferase also precludes the possibility that N-methylation could occur prior to the formation of the methylenedioxy group in the tetrahydroisoquinoline alkaloid. Step **5** (Fig. 1) involves a microsomal cytochrome P-450 containing, NADPH and O_2 dependent, enzyme complex that stereo- and regiospecifically hydroxylates carbon atom 14 of (S)-*cis*-N-methyltetrahydroproto-berberines to yield the A- and D-ring substituted protopine alkaloids.

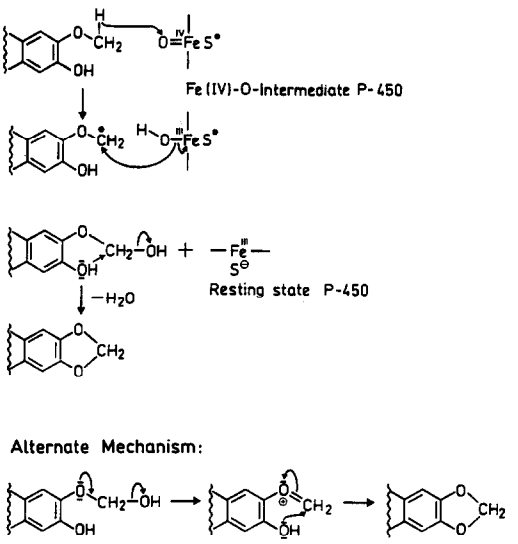

Figure 3. Proposed reaction mechanism of cytochrome P-450 mediated methylenedioxy bridge formation.

The (R)-enantiomer is completely inactive as a substrate for this 14-hydroxylase. As has been postulated previously on the basis of feeding experiments using intact plants, protopine has been demonstrated to be precursory to sanguinarine.[17] Again, the microsomal fraction from *Eschscholtzia* cell suspension cultures yielded a monooxygenase that, in the presence of O_2 and NADPH, hydroxylates specifically the 6-position of protopine (Step **6**). This hydroxylation leads to 6-hydroxyprotopine that spontaneously rearranges (Step 7) to form dihydrosanguinarine. This spontaneous reaction may take place at the enzyme surface. Dihydrosanguinarine is, in the final step (**8**), oxidized by a cytosolic enzyme that, in the presence of O_2, transforms dihydrobenzophenanthridines to benzophenanthridines. Since the five enzymes are known that catalyze the formation of (S)-reticuline from dopamine and 4-hydroxyphenylacetaldehyde, and seven enzymes are now known to catalyze the formation of sanguinarine from (S)-reticuline, a total of twelve enzymes are involved in the biosynthesis of sanguinarine from non-alkaloidal immediate precursors. Four additional enzymes are known (unpublished) which convert sanguinarine to the most highly oxidized benzophenanthridine alkaloid, marcarpine.[17] This is the longest pathway described in detail at the enzyme level for a plant-derived secondary metabolite.

Secondary plant products are regarded as defense compounds directed against phytopathogenic bacteria, fungi and phytophagus animals. Plant cell culture systems are often severely repressed in their biosynthetic capacity for secondary products. However, if a cell culture is challenged by a pathogenic organism, or a cell wall preparation of such a pathogenic organism, the synthesis of secondary products may be drastically increased.[18] Substances triggering this chemical defense system are called elicitors. The plant derived, inducible defense compounds are termed phytoalexins. *E. californica* cell cultures represent a classical case of the elicitor-phytoalexin interaction. If suspension cultures of this plant species are exposed to isolated cell walls of yeast or *Penicillium* , the synthesis of benzophenanthridine alkaloids, among them sanguinarine, is greatly induced.[19] While unchallenged cell cultures contain a low concentration of alkaloids, elicitation by

a yeast cell wall preparation increases drastically the alkaloid yield. Cell dry weight of elicited and unelicited cell cultures remains the same. No depression of growth rate can be observed inspite of the fact that the sanguinarine formed is toxic if applied to microorganisms[20] and could have some inhibitory effect on plant cells during the production phase. Although the specific induction of enzymes of the flavonoid pathway by elicitors has been long known and most of the enzymes and several of the genes involved have been identified,[21] up to now no gene coding an inducible enzyme of alkaloid biosynthesis has been reported.

In order to study the process of elicition of alkaloid biosynthesis in *E. californica* plant cell suspension cultures in more detail, attention was focused on an inducible, vesicular enzyme in the benzophenanthridine alkaloid biosynthetic pathway, the berberine bridge enzyme. The berberine bridge enzyme was purified to apparent homogeneity and amino acid sequences were obtained for both the amino terminal end of the protein and for three tryptic peptides. With the amino acid sequence information obtained in this manner, an oligodeoxynucleotide was designed, synthesized and used to screen an *E. californica* cDNA bank for a berberine bridge enzyme cDNA clone. The purpose of this approach was two-fold. Given a cDNA clone, it becomes possible to study the induction kinetics of the mRNA encoding the berberine bridge enzyme. It also serves as a probe with which to isolate a genomic clone for the berberine bridge enzyme which contains the genetic information concerning exactly how the gene is regulated under conditions of pathogenic attack. Likewise, the cDNA clone can be transferred to a heterologous organism, such as yeast, in order to produce enzymatically active berberine bridge enzyme in large enough quantities to exploit the biotechnological potential of this enzyme for the production of (S)-scoulerine from (S)-reticuline. Once the enzyme is over-expressed in a heterologous system, a detailed analysis of the reaction mechanism (radical *versus* ionic, Fig.2) can begin.

The berberine bridge enzyme cDNA clone, which was isolated from an *E. californica* cDNA bank, contains an open reading frame of 537 amino acids (excluding the amino terminal methionine). The first 22 amino acids from the amino terminus constitute the putative signal peptide which should direct the preprotein to the specific vesicle of tetrahydroprotoberberine alkaloid biosynthesis in which it is accumulated. These twenty-two amino acids are absent from the mature protein as determined from the amino acid sequence obtained for the amino terminus. The mature protein has a molecular mass of 57,334, excluding carbohydrate. Three carbohydrate (N-linked) consensus sequence sites were found in the sequence of the cDNA clone, and one of these sites was confirmed as containing a modified asparagine residue by amino acid sequence analysis. The information obtained from the nucleotide sequence of the cDNA clone for the berberine bridge enzyme confirms that it is compartmentalized and provides the additional information that it is probably a glycoprotein.

Analysis of the elicitation kinetics of benzophenanthridine alkaloids in *E. californica* cell suspension cultures revealed that within 2 h after the addition of an elicitor (cell wall preparation), an increase in the level of berberine bridge enzyme mRNA could be detected (data not shown). Within 4-6 h, the level of berberine bridge enzyme activity increased and, finally, after 8-10 h, an increase in the level of total benzophenanthridine alkaloids accumulated was apparent (Fig. 4). In response to this mimicked pathogenic attack, the level of biosynthetic enzyme and phytoalexin was increased 7.1 fold in 30 h and 6.7 fold in 48 h, respectively. Although the level of enzyme stabilizes after 20 h, alkaloid continues to accumulate for at least 48 h after elicitation.

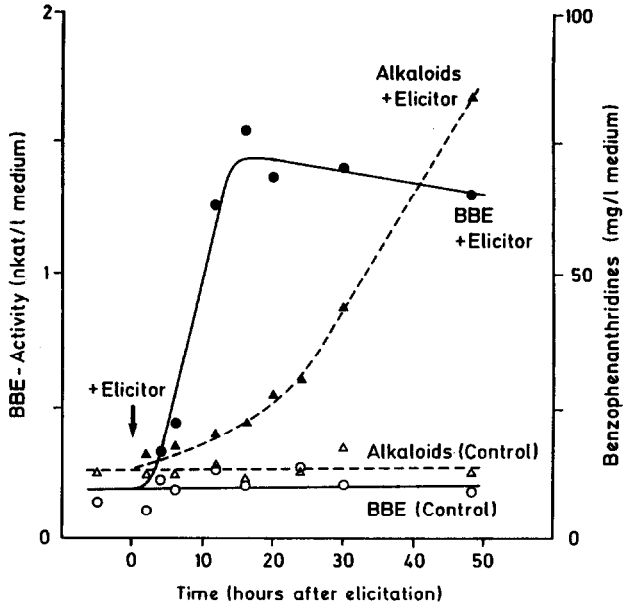

Figure 4. Effect of elicitation on berberine bridge enzyme (BBE) activity and benzophenanthridine alkaloid accumulation in cell suspension cultures of *Eschscholtzia californica*.

As the initial step towards overproduction of the berberine bridge enzyme in a microorganism, the enzyme has been expressed in an enzymatically active form in the baker's yeast, *Saccharomyces cerevisiae*. This represents the second example of an enzyme of alkaloid biosynthesis being actively expressed in a microorganism (the first being strictosidine synthase[22]). The heterologously expressed enzyme was found to be stereospecific, accepting (S)- but not (R)-reticuline as substrate. The enzymatically formed product was characterized to be (S)-scoulerine by mass spectral analysis.

The second pathway described here concerns the formation of 3α(S)-strictosidine, the universal precursor for over 1200 monoterpenoid indole alkaloids formed in higher plants. The fact that the indole alkaloid family contains members of considerable pharmaceutical interest (Fig. 5) makes it a prime target for biotechnological exploitation. The pathway to heteroyohimbine alkaloids has been clarified at the enzyme level[9] and the pathway to ajmaline, involving eleven enzymatic steps, is nearly completely elucidated.[10] The key enzyme in all these pathways is strictosidine synthase that catalyzes a Pictet-Spengler type reaction between the aldehyde function of secologanin and the primary amine of tryptamine (Fig. 6). This cyclization suggests Schiff base formation followed by an electrophilic attack of C-2 of the indole ring. During this attack, the hydrogen atom of C-2 is eliminated. This observation formed the basis for a convenient and sensitive assay of this enzyme using ring [2-^{3}H]-tryptamine as substrate and analysis of the HO^{3}H formed.[23] This enzyme was purified to homogeneity from cell suspension cultures of *Rauvolfia serpentina*,[24] the amino acid sequence was determined for several tryptic peptides, and a cDNA clone for strictosidine synthase was isolated and characterized.[25]

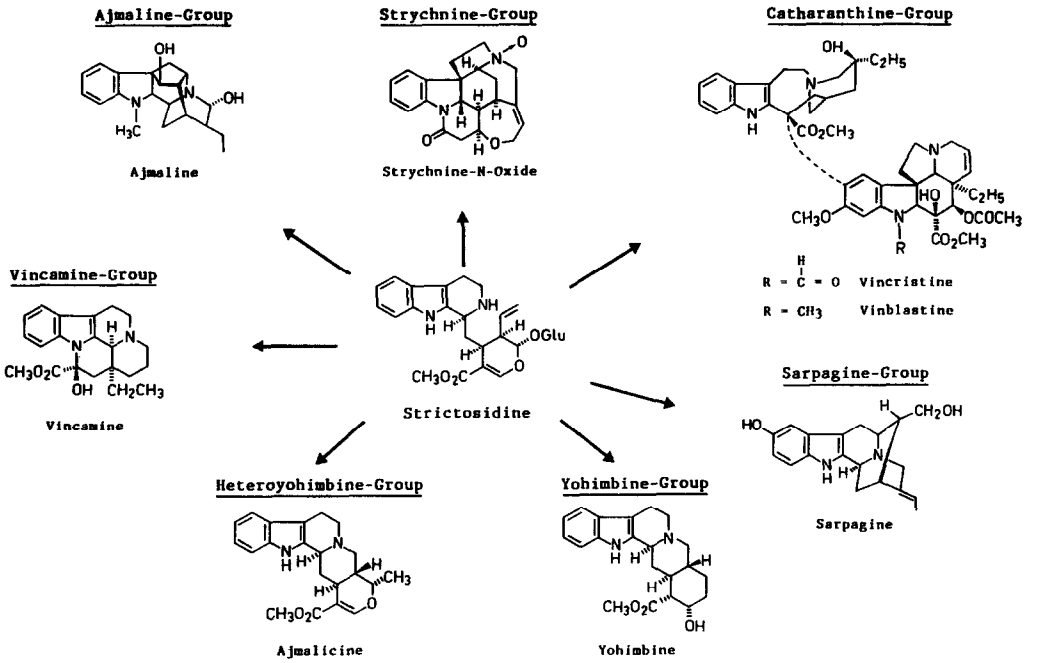

Figure 5. Select pharmacologically active alkaloids biosynthetically derived from strictosidine.

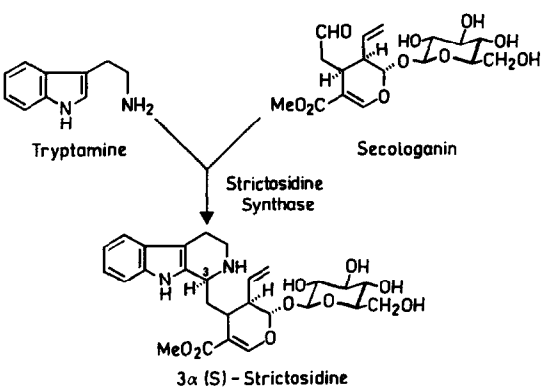

Figure 6. Reaction catalyzed by strictosidine synthase.

Strictosidine synthase has considerable potential for biotechnological application in the production of strictosidine. The enzyme was, therefore, expressed in several heterologous systems in an attempt to maximize the quantitiy of active enzyme which could be produced. The systems which have been investigated to date are bacteria (*Escherichia coli*),[22] yeast (*Saccharomyces cerevisiae*) and insect cells (*Spodoptera frugiperda*). The results of the heterologous expression systems are shown in Table 1. The construction pKSS1 in *E. coli* will produce strictosidine quantitatively when the substrates secologanin and tryptamine are added to the bacteria in either exponential growth or stationary phase. The bacteria take up both precursors and excrete the product, strictosidine, into the medium.[22] This system, as such, is already suitable for the production of unlimited quantities of strictosidine. The vector pJUB5 is a construction which has the gene for ubiquitin[26] fused in front of the gene for strictosidine synthase in an attempt to increase production in *E. coli* . In this example, strictosidine synthase is produced at up to 3% of the total soluble protein of the bacterium. In yeast (pEVP11), the heterologous enzyme comprises 0.005% of the total soluble protein and in insect cells (AcMNPV), >2%. In all systems tested to date, strictosidine synthase is enzymatically active. The latter systems described have potential use in the isolation of large quantities of the enzyme for analysis of the catalytic center. A comparison of the genomic clone for strictosidine synthase from five *Rauvolfia* species (*Rauvolfia serpentina, R. mannii, R. chinensis, R. canescens* and *R. verticillata*) indicates that all of the genes are highly homologous and do not contain introns. A recent report[27] indicates that even in the less related species, *Catharanthus roseus*, the amino acid sequence of strictosidine synthase is 80% identical to that of the *Rauvolfia* enzyme. Preliminary studies of the enzymes of alkaloid metabolism indicate that these catalysts are highly substrate specific, highly conserved, and amenable to heterologous expression in microorganisms and higher eukaryote cell culture for the purposes of biotechnological exploitation and physical biochemical characterization.

Table 1

Heterologous expression of strictosidine synthase

Vector Construct	Activity (nkat l^{-1})	Relative Activity	%Soluble Protein
pUC18SS1.10/DH5[1]	0.45	1	0.0002
pKSS1/SG935[1]	140	330	0.06
pJUB5/SG935[1]	6600	14600[4]	3
pEVP11/DH484[2]	90	200	0.005
AcMNPV/Sf9[3]	900	2000	> 2

1. *Escherichia coli* 2. *Saccharomyces cerevisiae* 3. *Spodoptera frugiperda*
4. Projected activity after cleavage of ubiquitin.

EXPERIMENTAL

Plant cell cultures. Plant cell suspension cultures were provided by the cell culture laboratory of this Institute. Cells were routinely grown in 1 l Erlenmeyer flasks containing 250 ml Linsmaier-Skoog medium[28] from 7-14 days at 23 °C. Flasks were maintained on a gyratory shaker (100 rpm) in diffuse light (750 lux). Cells were harvested by suction filtration and immediately shock frozen in liquid nitrogen. Cells were then stored at -20 °C and used as an enzyme source. In the indicated cases, cell suspension cultures of *E. californica* (strain BB) were elicited as previously described[19] with 10 mg yeast elicitor preparation per 250 ml medium.

Enzyme assays. All enzyme assays were performed as previously described: berberine bridge enzyme,[3] (S)-scoulerine- and (S)-stylopine-synthase,[4,16] S-adenosyl-L-methionine:(S)-tetrahydroprotoberberine-*cis*-N-methyltransferase,[5] (S)-*cis*-N-methyltetrahydroprotoberberine-14-hydroxylase,[6] protopine-6-hydroxylase,[7] dihydrobenzophenanthridine oxidase,[8] strictosidine synthase.[23]

Benzophenanthridine alkaloid analysis. Benzophenanthridine alkaloid analysis was performed as previously described.[19]

cDNA clone isolation. Messenger RNA was isolated from elicited cell suspension cultures of *E. californica* as described previously.[25] cDNA was prepared with a cDNA synthesis kit from Pharmacia according to the manufacturer's instructions. The cDNA was ligated into *Eco* RI restricted, dephosphorylated λ gt11 arms, and packaged by standard procedures.[29] Positive clones were initially identified by hybridization to a synthetic deoxyoligonucleotide (40 mer based on the amino acid sequence of a berberine bridge enzyme tryptic peptide) as previously described,[25] followed by complete nucleic acid sequencing.

Genomic clone isolation. Genomic DNA was isolated from cell suspension cultures of *Rauvolfia serpentina*, *R. mannii*, *R. chinensis*, *R. canescens* and *R. verticillata* according to standard protocols.[30] *Eco* RI restricted genomic DNA was ligated into *Eco* RI restricted λ Charon 4A, and packaged as above. Positive clones were identified by hybridization to the cDNA clone for strictosidine synthase[25] and subsequently sequenced as above.

Characterization of (S)-scoulerine produced by heterologously expressed berberine bridge enzyme. [N-$^{13}C^2H_3$]-(S)-Reticuline (1 mg in 500 µl CH_3OH) was administered to heterologously produced berberine bridge enzyme (10 pkat) in 40 mM glycine, pH 9.0 in a total volume of 7.3 ml. The reaction mixture was incubated at 37 °C for 2 h. The reaction was stopped by addition of 300 µl Na_2CO_3, pH 8.5, followed by extraction with 2 x 3 ml ethyl acetate. The enzymatic product was purified by thin layer chromatography (CH_2Cl_2:CH_3OH:NH_4OH (90:9:1)), eluted with CH_3OH, and identified as [8-$^{13}C^2H_2$]-(S)-scoulerine by mass spectral analysis. Scoulerine standard MS (EI) $^m/_z$ 327 (M^+); [8-$^{13}C^2H_2$]-(S)-scoulerine MS (EI) $^m/_z$ 330 (M^+).

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REFERENCES

1. Bolin, B. *Science* **1977**, *196*, 613-615.
2. Ames, B.N.; Gold, L.S. *Angew. Chem.* **1990**, *102*, 1233-1246 and literature cited therein.
3. Steffens, P.; Nagakura, N.; Zenk, M.H. *Phytochemistry* **1985**, *24*, 2577-2583.
4. Bauer, W.; Zenk, M.H. *Tetrahedron Lett.* **1989**, *30*, 5257-5260.
5. Rueffer, M.; Zumstein, G.; Zenk, M.H. *Phytochemistry* **1990**, *29*, 3727-3733.
6. Rueffer, M.; Zenk, M.H. *Tetrahedron Lett.* **1987**, *28*, 5307-5310.
7. Tanahashi, T.; Zenk, M.H. *Phytochemistry* **1990**, *29*, 1113-1122.
8. Schumacher, H.-M.; Zenk, M.H. *Plant Cell Reports* **1988**, *7*, 43-46.
9. Stöckigt, J. *GIT Fachz. Lab.* **1988**, 608-615.
10. Stöckigt, J.; Schübel, H. *NATO ASI Series* **1988**, *H18*, 251-264.
11. Schumacher, H.-M-; Rueffer, M.; Nagakura, N.; Zenk, M.H. *Planta Medica* **1983**, *48*, 212-220.
12. Stadler, R.; Zenk, M.H. *Liebigs Ann. Chem.* **1990**, 555-562.
13. Frenzel, T.; Zenk, M.H. *Phytochemistry* **1990**, *29*, 3505-3511.
14. Cordell, G.A. **1981** "Introduction to Alkaloids: A Biogenetic Approach," John Wiley & Sons, Inc.
15. Amann, M.; Wanner, G.; Zenk, M.H. *Planta* **1986**, 310-320.
16. Bauer, W.; Zenk, M.H. *Phytochemistry* **1991**, in press.
17. Takao, N.; Kamigauchi, M.; Okada, M. *Helv. Chim. Acta* **1983**, *66*, 473-484.
18. Examples given in: "Biology and Molecular Biology of Plant-Pathogen Interactions" *NATO ASI Series* ***1986***, *H1*.
19. Schumacher, H.-M.; Gundlach, H.; Fiedler, F.; Zenk, M.H. *Plant Cell Reports* **1987**, *6*, 410-413.
20. Dzink, J.L.; Socransky, S.S. *Antimicrob. Agents Chemother.* **1985**, 663-665.
21. Hahlbrock, K.; Scheel, D. *Annu. Rev. Physiol. Plant Mol. Biol.* **1989**, *40*, 347-369.
22. Kutchan, T.M. *FEBS Lett.* **1989**, *257*, 127-130.
23. Treimer, J.F.; Zenk, M.H. *Eur. J. Biochem.* **1979**, *101*, 225-233.
24. Hampp, N.; Zenk, M.H. *Phytochemistry* **1988**, *27*, 3811-3815.
25. Kutchan, T.M.; Hampp, N.; Lottspeich, F.; Beyreuther, K.; Zenk, M.H. *FEBS Lett.* **1988**, *237*, 40-44.
26. Butt, T.R.; Jonnalagadda, S.; Monia, B.P.; Sternberg, E.J.; Marsh, J.A. Stadel, J.M.; Ecker, D.J.; Crooke, S.T. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 2540-2544.
27. McKnight, T.D.; Roessner, C.A.; Devagupta, R.; Scott A.I.; Nessler, C.L. *Nucl. Acids Res.* **1990**, *18*, 4939.
28. Linsmaier, E.M.; Skoog, F. *Physiol. Plant* **1965**, *18*, 100-127.
29. Maniatis, T.; Fritsch, E.F.; Sambrook, J. **1982** "Molecular Cloning: A Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
30. Murray, M.G.; Thompson, W.F. *Nucl. Acids Res.* **1980**, *8*, 4321-4325.