ENZYMATIC SYNTHESIS OF AJMALICINE AND RELATED INDOLE ALKALOIDS[1]

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ABSTRACT.—Plant tissue cultures have become an important tool for the investigation of the biosynthesis and its control of natural products. Variant cell clones of *Catharanthus roseus* have the capacity to produce monoterpenoid indole alkaloids in yields equal to or even higher than the differentiated plant from which they are derived. These cell suspension cultures are the source of enzymes for the cell-free biosynthesis of, for instance, ajmalicine (**1**). The enzymatic synthesis of this type of alkaloid involves the condensation of tryptamine (**7**) with secologanin (**5**) to yield strictosidine (**9**) with 3(α)-S-configuration. Highly substrate-specific β-glucosidases transform this precursor into several unstable intermediates en route to 4,21-dehydrocorynantheine aldehyde (**16**) which is, in turn, further metabolized to 4,21-dehydrogeissoschizine (**19**), a suggested important branch point intermediate in indole alkaloid biosynthesis. The precursor of cathenamine (**21**), the key intermediate in corynanthe type alkaloid biosynthesis, is (**19**). After reduction involving reduced pyridine nucleotides, cathenamine (**21**) and its isomers yield ajmalicine (**1**), tetrahydroalstonine (**2**) and 19-epi-ajmalicine (**8**). A biosynthetic scheme which takes into account all the available data is presented (fig. 5).

In the past twenty years, there has been a substantial increase in our knowledge of the structure elucidation and biosynthesis of indole alkaloids. As a result of this extensive research, the structures of over one thousand monoterpenoid indole alkaloids are known (1). This unprecedented structural diversity has led to a great number of biosynthetic hypotheses which were based on radio-tracer experiments using whole plants (see 2). This type of experimental approach, however, has generally not revealed the actual biosynthetic sequence and cannot give information as to the enzymatic and, therefore, the ultimate actual reaction mechanisms. It is generally accepted (3) that the elucidation of such complex pathways is only possible by structure determination of the enzymatically formed intermediates and by characterization of the single enzymes or enzyme systems involved in these reactions. Whole differentiated plants are generally not suited for this type of experiments, since the stationary level of enzymes involved in secondary metabolism seems to be very low, and the high content of phenolics and other undesirable cell constituents tends to inactivate some of these enzymes during the isolation procedures (4).

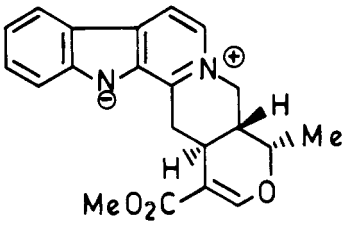
To overcome this general phenomenon, plant cell suspension cultures have been successfully introduced (e.g. 5, 6). Complicated biosynthetic sequences have been subsequently solved at the cell free level, where previous work with differentiated, whole plants, had not been successful.

CELL CULTURE METHODS

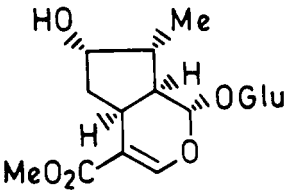
Undifferentiated plants at the level of callus or cell suspension cultures are often considered to contain no secondary metabolites or very limited amounts of secondary metabolites (e.g. 7). However, careful selection of high metabolite-yielding variant strains from callus culture populations enables one to obtain cell lines which may equal, or even surpass in yield (e.g. 8), the levels of secondary pro-

Presented as a plenary lecture at the 20th Annual Meeting of the American Society of Pharmacognosy at Purdue University, West Lafayette, Indiana, July 30–August 3, 1979.

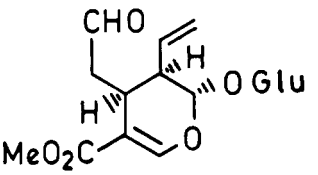
ducts within the original differentiated plant. For the detection of these variant cells either visual, as in the case of pigmented cells (9), or radioimmunoassay (RIA) methods can be employed (e.g. 8, 10). This latter method is presently the most sensitive and most specific analytical tool available for the quantitation of large numbers of samples for the screening of primary and secondary metabolites (11). The semi-automatic RIA methods routinely employed in the laboratory allow the screening of up to 600–800 samples per day per person, which in combination with a recently developed replica method for plant cells (12) gives high yields of disregulated cell clones for the production of secondary metabolites and for subsequent enzymatic work. The genetic or epigenetic stability of some of these strains, as in the case of *Catharanthus*, may prove to be a problem (8, 13). In such cases, constant clonal selection of high-yielding strains may turn out to be a necessity. In our experience, however, these unstable strains have been the exception, and we now have absolutely stable strains of other plant species selected which have not altered their metabolic pattern over a period of 10 years. With the use of these techniques with *Catharanthus* cultures, the maximal yield of the indole alkaloid ajmalicine (**1**) thus far reached has been a little more than 500 mg/liter of medium (13). Other metabolites associated with this indole alkaloid pathway thus far identified in these cultured cells have been tetrahydroalstonine (**2**) (14) and serpentine (**3**) (10) as well as loganin (**4**) and secologanin (**5**). It



Serpentine (3)



Loganin (4)



Secologanin (5)

is worth mentioning that in the case of *Catharanthus*, the highest alkaloid yields have been obtained when the cells were first grown in a medium which allowed vigorous growth and were then transferred into an "alkaloid-production medium" of different nutritional composition (10). Cells of *Catharanthus* in this alkaloid-production medium were routinely grown in 30-liter airlift fermenters (10) and used for the preparation of cell-free extracts for further enzymatic work.

CELL-FREE BIOSYNTHETIC STUDIES

Using *C. roseus* callus grown on solidified agar, Scott and Lee (15) were the first to demonstrate the formation under cell free conditions of the alkaloids,

geissoschizine (**6**) and ajmalicine (**1**) from tryptamine (**7**) and secologanin (**5**). This achievement clearly marked a breakthrough towards the detailed study of the enzymic formation of this type of alkaloids, and was extended by us using selected high alkaloid-yielding cell suspension cultures of *C. roseus* (14). Three heteroyohimbine alkaloids (ajmalicine (**1**), 19-*epi*-ajmalicine (**8**) and tetrahydroalstonine (**2**)) were formed under cell-free conditions and their production was absolutely dependent on reduced pyridine nucleotides, either NADPH or NADH (14). The soluble enzyme system which synthesized these alkaloids from tryptamine (**7**) and secologanin (**5**) was further characterized by the RIA method with antibodies directed against ajmalicine (**1**) to follow and quantitate the enzymic formation of these compounds (16). The cross reactivity shown by these antibodies also permitted quantitation of the stereoisomers of ajmalicine (**1**), (**2**) and (**8**). This RIA also proved exceedingly useful in determining the overall enzymology of the reaction with regard to pH optimum, time course, substrate specificities, k_m values of substrates, stability of the system and identification of inhibitors (17). This was the first successful attempt in plant science to monitor quantitatively an enzyme-catalyzed, biosynthetic sequence by means of RIA. The major advantages of this type of work are the ease and precision by which quantitative figures can be obtained and the large output capacity with the *semi*-automated procedure. With knowledge of the parameters for the overall reaction, it was possible to investigate further the individual steps in the complex sequence leading to ajmalicine (**1**) and its stereoisomers.

INDIVIDUAL ENZYMATIC STEPS FOR HETEROYOHIMBINE SYNTHESIS

STRICTOSIDINE (**9**).—The formation of heteroyohimbine alkaloids from tryptamine (**7**) and secologanin (**5**) by a crude cell-free extract undoubtedly involves several individual and new enzymes. The first enzyme in the pathway catalyzes the condensation of tryptamine (**7**) with secologanin (**5**) by a Pictet-Spengler type reaction to furnish a glucoalkaloid of the vincoside (**10**) / strictosidine (=isovincoside) (**9**) type. From previous biosynthetic studies, it had been proposed that the exclusive precursor for *Aspidosperma, Iboga* and *Corynanthe* type alkaloids is vincoside (**10**) with 3β(R) configuration (18, 19) and that subsequent inversion at C-3 occured with retention of hydrogen (20). On the other hand, however, strictosidine (**9**) with the proper 3α(S) configuration has been found to occur naturally in *Rhazya stricta* and has been proposed to occupy a crucial position in the biosynthesis of monoterpenoid indole alkaloids (21). The correct stereochemical relationship of the precursor to the products has been a matter of much controversy and confusion (see 2). The availability of enzyme extracts from cell suspension cultures active in alkaloid biosynthesis (14) allowed us to study this question. Use of δ-D-gluconolactone, a general inhibitor of β-glucosidases, inhibited breakdown of the once-formed glucoalkaloid in the enzyme mixture (17), resulting in the accumulation of an intermediate (22–24). This metabolite was isolated and rigorously and unequivocally identified as strictosidine (**9**) with 3α(S) configuration (table 1). The presence of an anomalous acetate signal at δ 1.23 in the nmr spectrum, the cd-measurements and the chemical stability of this compound or derivatives thereof proved the metabolite to be (**9**) (24). Furthermore, dilution analysis of the ^{14}C-labelled enzymatic product, as well as derivative formation and separation of the epimers, showed that only (**9**) and not 3β(R) (**10**) was formed under cell-free

TABLE 1. Comparison of properties of the metabolically formed glucoalkaloidal intermediate with strictosidine (9) and vincoside (10).

	NMR. Anomalous Ac-Signal: δ 1.23 *	CD ** Δε 217nm	Lability in NHE t_2	Dilution anal. ** dpm/μmol
3α(S) Strictosidine	yes	-8.5	no	2.5×10^3
3β(R) Vincoside	no	+10	yes	8
Intermediate	yes	-9.0	no	—

*As lactam tetra acetate.
**As penta acetate.

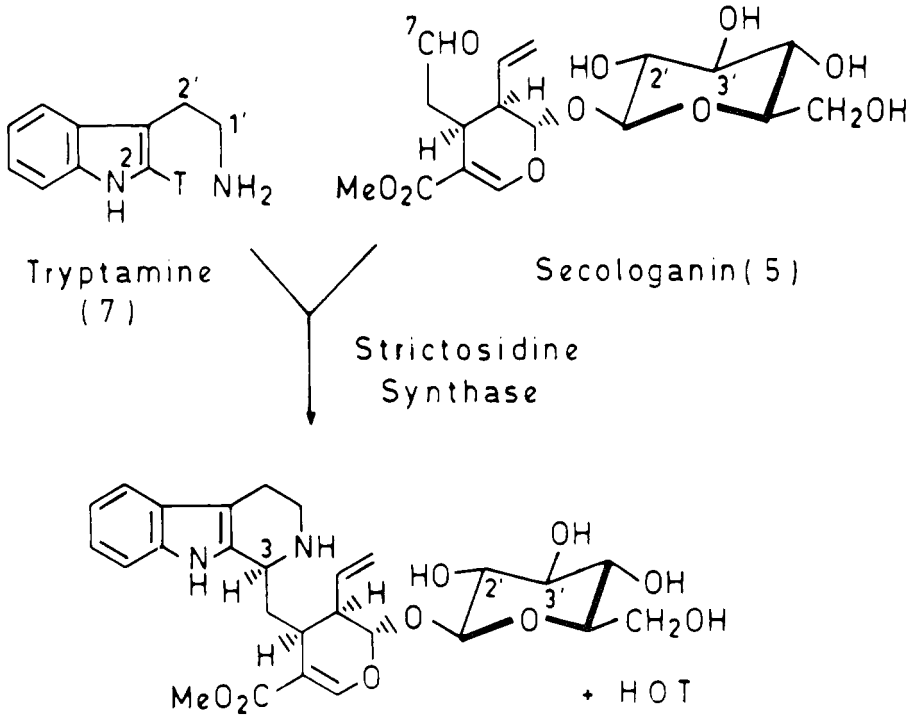


FIG. 1. Reaction sequence catalysed by strictosidine synthase indicating also the assay principle.

conditions (22–24). The enzyme catalyzing the formation of strictosidine (**9**) from (**7**) and (**5**) was first named strictosidine synthetase (23). However, since the term synthetase is reserved for enzymes which catalyze reactions involving nucleotide phosphates, we changed this name to strictosidine synthase (25). With 2-^{3}H-labelled tryptamine as substrate, during the condensation reaction this proton appears in the ambient water (25). This finding was the basis for the development of a simple, convenient and very sensitive assay for this enzyme; the reaction catalyzed by the enzyme and the assay principle are shown in fig. 1. The enzyme was purified about 50-fold to near homogeneity and characterized (26) as shown in table 2. The same enzyme from the same source was also puri-

TABLE 2. Comparison of some properties of crude and purified strictosidine synthase from *C. roseus* suspension cells. Data from (25, 26) and (27).

	Crude cell free extract (25) (45)	Purified enzyme	
		(26)	(27)
Molecular weight	34.000	34.000	38.000 (55.000)
Tryptamine K_m (mM)	5.8	2.3	0.83
Tryptamine V_{max} (nmol/min)	5.7	7.8	n.d.
Secologanin K_m (mM)	2.6	3.4	0.46
Secologanin V_{max} (nmol/min)	1.6	3.3	n.d.
pH Optimum	6.5	6.5	5.0–7.5
Final specific activity (nkat/mg protein)	—	17.7	5.9

n.d. = not determined.

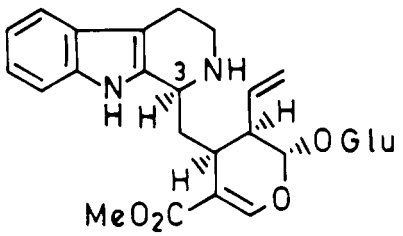
fied by Scott and coworkers (27) using thin-layer chromatography as an enzyme assay. The properties of both preparations are compared in table 2. The k_m values reported by (27) are off by up to one order of magnitude (secologanin); and the *TRIS*-buffer used by these authors is inhibitory to the enzyme (20%). In addition, the concentration (25 mM) of δ-D-gluconolactone used (27) to "completely" inhibit the endogenous β-glucosidases is too low based on our previous experience (17). The reservations therefore limit, the claim for the demonstration and properties of strictosidine synthase presented in (27).

This interesting enzyme was demonstrated to occur in suspension cultures of 15 different species belonging to 9 different genera of the indole alkaloid-producing subfamily *Plumerioideae* of the *Apocynaceae* family, while it is absent in non-indole alkaloid producing plants (26). The properties of the crude enzyme of 8 of these plant species were determined and are shown in table 3. The characteristics of these enzymes from widely differing origins are surprisingly similar, and in every single case strictosidine (**9**) was the exclusive reaction product. No trace of its epimer (**10**) has ever been detected.

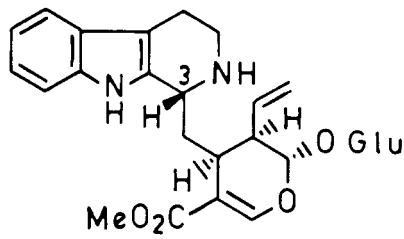
A comparison of strictosidine synthase from *C. roseus* roots, which are the only portion of the plant that contain ajmalicine (**1**), (16) with the enzyme from cell cultures producing the same alkaloid shows that in the former case only 193 pkat/g dwt of enzyme can be detected, while the latter yields 22,400 pkat/g dwt. Again the advantage and the high metabolic state of plant cell cultures, is demonstrated.

TABLE 3. Some properties of strictosidine synthase preparations from different plant cell cultures.

Species	Molecular weight ±3.5%	K_m-values (M)		pH-Optimum	Temp.-Optimum (°C)	Configuration of reaction product	
		Tryptamine	Secologanin			3 α (S)	3 β (R)
Amsonia salicifolia	28 000	4.0×10^{-3}	2.8×10^{-3}	6.5	45	+	0
Amsonia tabernaemontana	33 000	6.9×10^{-3}	3.7×10^{-3}	6.2	45	+	0
Catharanthus pusillus	26 000	1.0×10^{-3}	4.0×10^{-3}	6.9	45	+	0
Catharanthus roseus	34 000	2.3×10^{-3}	3.4×10^{-3}	6.8	45	+	0
Rauwolfia verticillata	33 000	1.9×10^{-3}	4.3×10^{-3}	6.7	45	+	0
Rauwolfia vomitoria	33 000	5.8×10^{-3}	2.1×10^{-3}	6.4	50	+	0
Rhazya orientalis	29 000	2.9×10^{-3}	4.4×10^{-3}	6.0	50	+	0
Voacanga africana	29 000	3.1×10^{-3}	4.2×10^{-3}	6.1	50	+	0



Strictosidine(9)
(=Isovincoside)



Vincoside(10)

GLUCOSIDASES AND STRICTOSIDINE METABOLITES.—It was clear when the glucoalkaloid was discovered as a metabolite in indole alkaloid biosynthesis, that the glucose moiety of this intermediate (strictosidine (**9**)) would be enzymatically removed at a relatively early stage in the biosynthesis. Commercial β-glucosidase at pH 5.5 was indeed capable of hydrolyzing **9**, **10** and their derivatives and these reactions are of considerable interest in biomimetic conversions (28, 29). It is known that mild hydrolysis of strictosidine (**9**) yields vallesiachotamine (**11**) and iso-vallesiachotamine (**12**) (30), and a very convenient way to prepare these compounds is by the incubation of the glucoalkaloids (**9**, **10**) with commercial β-glucosidase overnight at pH 5 (31). Since the overall conversion of strictosidine (**9**) to the heteroyohimbine derivatives (11) is inhibited by the rather specific glucosidase inhibitor, δ-D-gluconolactone (17), there was good evidence for the presence of a β-glucosidase involved in indole alkaloid biosynthesis in *C. roseus* cell-free extracts. The participation of *non-specific* glucosidases was suggested by workers (32) when this reaction sequence was carried out with *C. roseus* callus extracts as the experimental plant material. For it was reported that in this plant, non-specific glucosidases hydrolyzed *p*-nitrophenylglucoside (a chromogenic substrate for unspecific glucosidases) and that these were, in turn, involved in the formation of indole alkaloids (32). A simple gel-filtration step suggested that these *p*-nitrophenylglucoside-splitting, unspecific glucosidases are a part of an "ajmalicine synthetase", and quite a number of speculations concerning this finding were published (32). However, a specific assay system using the true substrate stricto-

sidine (**9**), led to the discovery (33) of two highly-specific glucoalkaloid β-glucosidases named strictosidine-β-D-glucosidase I and II which disproves the above claims. An "ajmalicine synthetase" (32) does not exist! The two specific glucosidases were easily separated (by DEAE-cellulose chromatography) from each other and from unspecific glucosidases which also occur in *C. roseus*. The specific glucosidases occur in cell cultures of several indole alkaloid-containing plants of the family *Apocynaceae*, while they are absent in cells from unrelated families which are devoid of these alkaloids. It is of interest to note that in cell-free extracts of *C. roseus* cell suspension cultures actively synthesizing indole alkaloids, the formation of vallesiachotamine (**11**) and its isomer (**12**) was never observed. This indicates that there is a basic difference between the specific glucosidases and the unspecific ones, as predicted (35) prior to the discovery of the strictosidine synthase (33).

β-D-Glucosidases capable of hydrolyzing the glucosides of secondary plant products have previously been understood to act mainly on metabolic end products. The strictosidine glucosidases, however, are involved in an essential and initial step of a rather complicated biosynthetic sequence; this requires a high degree of specificity of these enzymes towards their substrate, and the properties of the two glucosidases from *C. roseus* are in full accord with these criteria (33).

Removal of the glucose moiety of strictosidine (**9**) by specific β-glucosidases leads to a series of highly reactive intermediates. The aglycone (**13**) most likely opens to a dialdehyde (**14**) (19) which then cyclizes to a carbinolamine (**15**) (3) (figure 2).

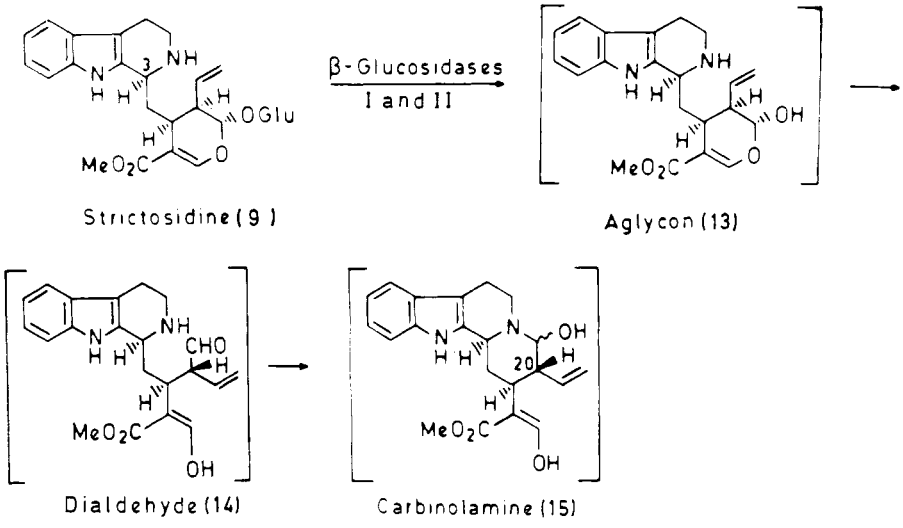


FIG. 2. Hydrolysis of strictosidine (**9**) by specific glucosidases I and II and rearrangement of strictosidine aglycon.

4,21-DEHYDROCORYNANTHEINE ALDEHYDE.—Since it was known that the removal of the glucose moiety of secologanin (**5**) leads not to the expected aglycone but rather to a bicyclic hemiacetal (34), it was expected that hydrolysis of strictosidine (**9**) under physiological conditions would not lead to the strictosidine aglycone (**13**), nor indeed to the highly transient dialdehyde (14) (19). This dialdehyde (**14**), under physiological conditions, will certainly undergo cyclization to a carbinolamine (**15**) which, upon further transformations, yields the precursors for the heteroyohimbine alkaloids (**1**, **2**, **8**).

In an attempt to intercept this sequence of reactions and to trap one of the expected aldehyde precursors, the *C. roseus* enzymes were allowed to act on strictosidine (**9**) as substrate under physiological conditions but in the presence of 10^{-1} M BH_4^- (35). It was expected that any of the aldehydes formed under these conditions would be reduced to alcohols and the reaction intercepted at that point. Large scale enzyme incubations (1.6 liter, containing ca. 2 g of protein) were conducted, and resulted in the isolation of two compounds in a 1 : 1 ratio (fig. 3), expected to be stereoisomers. The products were identified as

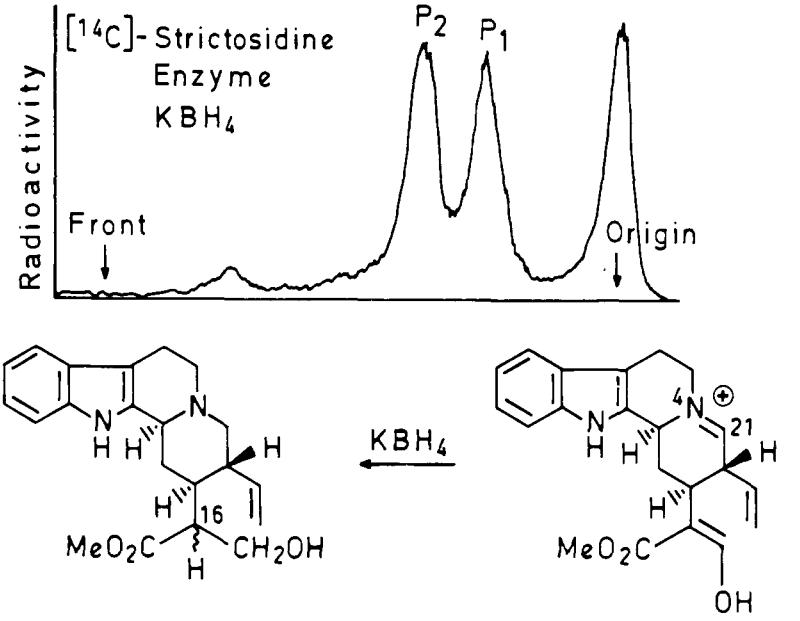


FIG. 3. Scanning record of a thin layer chromatogram of an incubation of [^{14}C]-strictosidine with a *C. roseus* crude enzyme extract in the presence of BH_4^-. The products formed P_1 and P_2 were identified as (**17**) and (**18**).

sitsirikine (**17**) and 16-episitsirikine (**18**). Formation of these isomers under the specified reaction conditions demonstrates that the postulated dialdehyde (**14**) is so highly reactive, that it cannot be trapped by BH_4^-, but rather undergoes intramolecular closure of ring D to yield 4,21-dehydrocorynantheine aldehyde (**16**). This compound, along with several others, had been hypothesized (19), as an intermediate in indole alkaloid biosynthesis. The involvement of this intermediate (**16**) in the biosynthesis of the monoterpenoid indole alkaloids is strongly suggested by this reduction *in situ* with BH_4^-, to yield (**17**) and (**18**).

4,21-DEHYDROGEISSOSCHIZINE.—4,21-Dehydrocorynantheine aldehyde (**16**), must be considered an early intermediate in the biosynthesis of heteroyohimbine alkaloids. In the subsequent steps of this biosynthetic sequence, and particularly in the formation of the heterocyclic E-ring system, one has to assume that the C–18, 19 vinyl group of the iminium intermediate (**16**) isomerizes to form a double bond between C–19 and C–20, thereby rendering the molecule susceptible to

subsequent ring closure. In an excellent piece of work, Kan-Fan and Husson (36) isolated large quantities (3.45 g per kg of dry leaves), of this postulated (37) intermediate, 4,21-dehydrogeissoschizine (**19**), from a plant endemic to New Calidonia, *Guettarda eximia* (*Rubiaceae*). The isolate (**19**) underwent biomimetic reactions (36) which closely reflect the biosynthetic expectations of an intermediate following (**16**) in the natural sequence. Thus, (**19**) was transformed into geissoschizine (**6**), 17-OH-19-epi-cathenamine (**20**), cathenamine (**21**) and vallesiachotamine (**11**) and isovallesiachotamine (**12**) (figure 4).

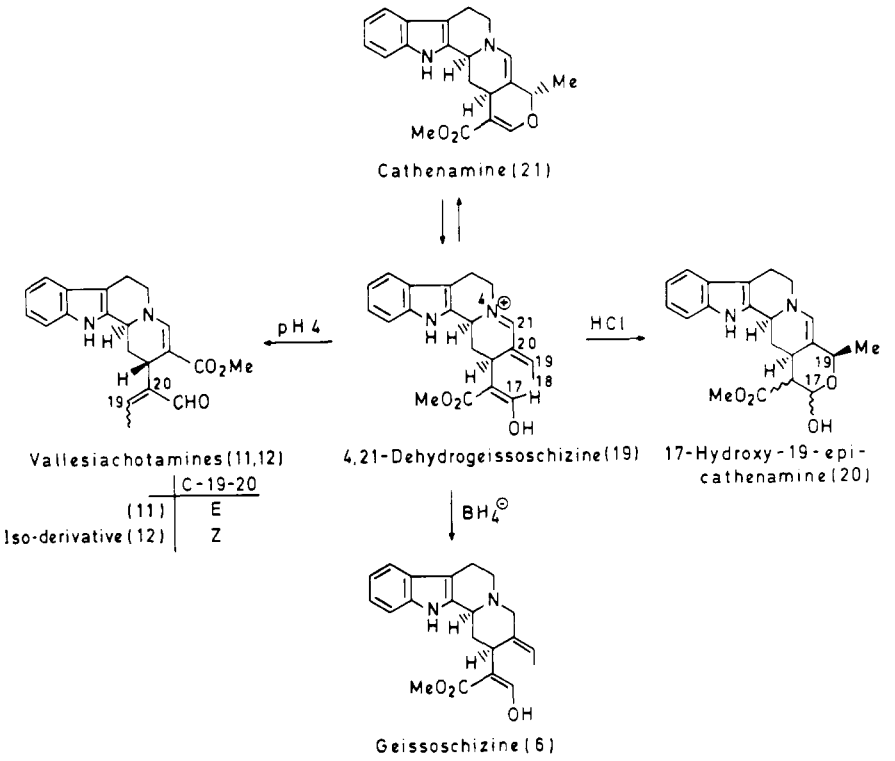


FIG. 4. Biomimetic conversion of 4,21-dehydrogeissoschizine (**19**) into biogenetically related alkaloids (after (36)).

4,21-Dehydrogeissoschizine (**19**) was, therefore, tested as a substrate for heteroyohimbine alkaloid formation with enzyme preparations from *C. roseus* cell cultures in the presence of NADPH (38). Under these conditions, transformation of (**19**) into the three heteroyohimbine derivatives (**1**, **2**, **8**) was achieved. Omission of NADPH from the reaction mixture allowed formation of the previously recognized intermediate, cathenamine (**21**). The enzymic behaviour of this compound (**19**) suggested intermediacy in heteroyohimbine biosynthesis. In order to establish (**19**) as an *obligatory* intermediate in the biosynthetic sequence, [^{14}C]-tryptamine (**7**) and secologanin (**5**) were allowed to be transformed in the presence of a ten-fold (with respect to (**7**)) excess of unlabelled (**19**), enzyme and NADPH. The specific activity of the alkaloids (**1**, **2**, **8**) formed under these conditions was ten times less than that of the control experiment which was performed in the absence of (**19**). This demonstrates conclusively that tryptamine (**7**) is channelled

through (**19**) in the course of heteroyohimbine alkaloid formation. Furthermore, short term incubation of [^{14}C]-tryptamine (**7**) and (**5**) in the presence of unlabelled 4,21-dehydrogeissoschizine (**19**) led to the accumulation of radioactivity in (**19**). The postulated intermediate (**19**) is therefore formed under cell-free conditions from distant precursors and establishes (**19**) as an obligatory intermediate in the formation of the heteroyohimbine alkaloids. The enzyme catalyzing the formation of cathenamine (**21**) from 4,21-dehydrogeissoschizine (**19**) was named cathenamine-synthase (38). We can anticipate that this new intermediate (**19**) occupies a crucial position at the branch point in the biosynthesis of *Iboga, Aspidosperma* and *Corynanthe* type alkaloids (38).

CATHENAMINE.—Crude cell-free extracts of *C. roseus,* freed from low molecular weight compounds by dialysis, when incubated in the presence of [^{14}C]-tryptamine (**7**) and secologanin (**5**) led to the accumulation of an unknown compound which was clearly demonstrated to be a precursor of the heteroyohimbine alkaloids (**1**, **2**, **8**) *in vivo* and *in vitro* (14). This compound was isolated in pure form from large scale enzyme incubation mixtures and was rigorously identified as 20, 21-didehydroajmalicine (**21**) by chemical and spectroscopic means (37). Because of the enamine structure and its isolation from cell-free extracts of *Catharanthus,* the compound was given the trivial name cathenamine. This compound was also isolated in good yield from dried leaves of *Guettarda eximia* (39). Cathenamine (**21**), either enzymatically generated or isolated from *Guettarda,* was reduced to a mixture of ajmalicine (**1**), tetrahydroalstonine (**2**) and 19-epi-ajmalicine (**8**) in the presence of the reduced nucleotide NADPH (NADH) and cell-free extracts of *Catharanthus.* Since ajmalicine (**1**) is not converted into its isomers under cell-free conditions, a stereochemical control should occur in the cathenamine stage as previously suggested (37).

At that time an interconversion was rationalized, which would proceed *via* an equilibrium of cathenamine (**21**) with a mixture of the iminium form of cathenamine (**22**) and, by retro-Michael ring opening, 4,21-dehydrogeissoschizine (**19**). Each of these postulated compounds has recently been obtained and characterized. From the level of deuterium incorporation during the reduction of cathenamine (**21**) to tetrahydroalstonine (**2**) with $NaBD_4$, in the presence of D_2O, the occurrence of the enamine and iminium ion forms of cathenamine could be demonstrated (40). These two forms could be interconverted, depending on the presence or absence of sulfate ions. Both the enamine (**21**) and iminium (**22**) forms of cathenamine were also observed under cell-free conditions using enzymes of *Rauwolfia verticillata* cell cultures and the interconversion of cathenamine (**21**) and 4,21-dehydrogeissoschizine (**19**) has been demonstrated (36). It might be argued that biosynthetic reduction to ajamalicine (**1**) and tetrahydroalstonine (**2**) occurs from the equilibrium of the enamine (**21**) and iminium (**22**) forms of cathenamine, while the opening of ring E of (**21**, **22**) and subsequent stereochemical rearrangement of C–19 prior to ring closure and reduction could lead to 19-epi-ajmalicine (**8**). This rearrangement yielding 19-epi-cathenamine (**23**) was indeed observed in nmr-studies of cathenamine (**21**) (41), and (**23**) was recently isolated and partially characterized after glucolysis of strictosidine (**9**) using commercial glucosidase (42). Purified samples of both alkaloids (**21**, **23**) were readily interconverted, in both aqueous and organic solvents.

HETEROYOHIMBINE ALKALOIDS.—It is now assumed that relatively unspecific, $NADPH-H^-$ -(or $NADH+H^+$)- dependent (14) reductases act, under physiological conditions, on the intermediates (**21**), (**22**) and (**24**) and reduce these to

the heteroyohimbine derivatives (**1**), (**2**), and (**8**), respectively, (see figure 5). These reductases have not yet been isolated and characterized. It should be noted, however, that investigations of the specificity of different *C. roseus* cell lines indicated that there is definitely a strain specificity to accumulate different amounts of the three heteroyohimbine derivatives (45). Furthermore, enzymatic

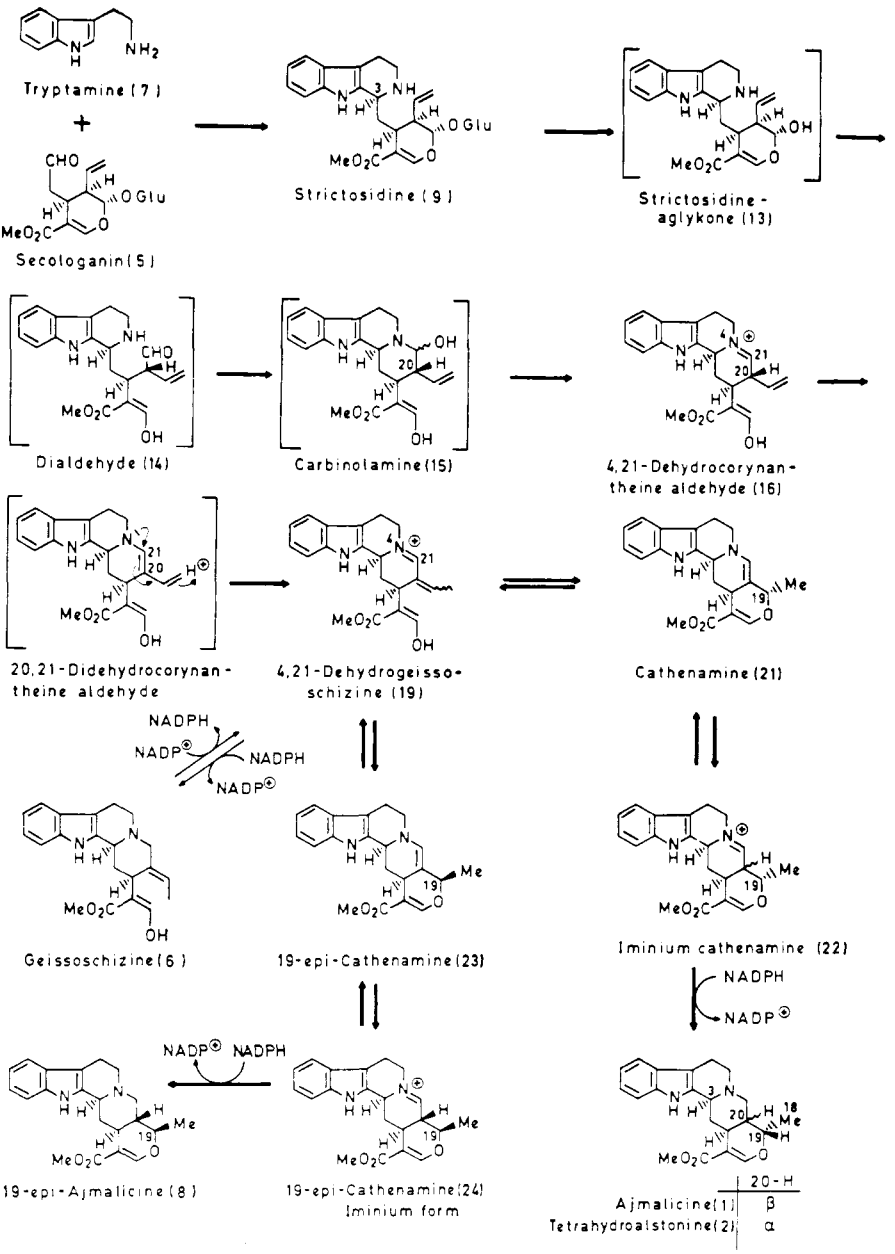
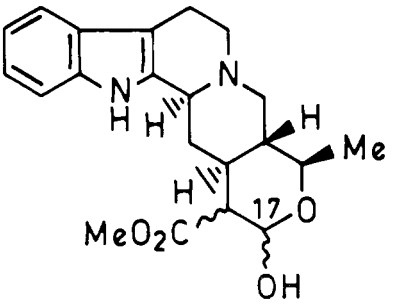
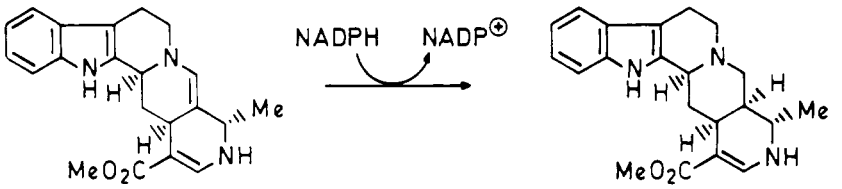


FIG. 5. Biosynthetic sequence for the formation of heteroyohimbine alkaloids based on enzymatic experiments.

experiments with cell-free extracts of these individual strains resulted in widely differing proportions of the heteroyohimbines (**1**), (**2**), (**8**). Therefore, the ratio of (**1**):(**2**):(**8**) = 1:2:0.5 determined (14) is specific for one individual *C. roseus* cell culture strain. We have found cell lines which, under cell-free conditions, produce almost exclusively (**2**) or in other cases only (**8**) and still others where intermediate levels accumulated. This situation may be a reflection of either an enzymatic catalysis of the equilibrium of (**21**), (**22**) and (**23**), or it may point to the existence of different and substrate-specific reductase enzymes. However, some degree of non-specificity of the reductase system has already been observed (3), since it was shown that 17–OH–19-epi-cathenamine (**20**) (46) is reduced under cell-free conditions and in the presence of NADPH, to 17–OH–19-epi-ajmalicine (**25**), a compound hitherto not observed in *Catharanthus* (3). Another artifact, namely *N*-cathenamine (**26**), was reduced by the reductase system to the *N*-analogue of (**2**), (**27**) (44).



17-OH-19-epi-Ajmalicine(25)

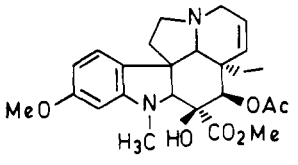


N-Cathenamine(26) N-Tetrahydroalstonine(27)

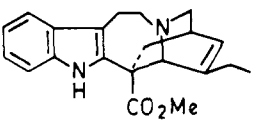
A mechanistically most important observation has been made and presented by Stöckigt (43, 38, 3). During the enzymatic biosynthesis of the heteroyohimbine alkaloids (**1**, **2**, **8**) in the presence of D_2O, a total of two deuterium atoms are incorporated, one each at C–18 and C–20. This observation was later confirmed (42). Moreover, it was shown (38) that with 4,21-dehydrogeissoschizine (**19**) as substrate for heteroyohimbine biosynthesis in the presence of D_2O, only one deuterium atom (at C–20) is incorporated (38). This is most interesting, from a mechanistic point of view, in that it fully supports the suggested biosynthetic pathways (38) and shows that no isomerization takes place when (**19**) is reached, which would lead to (**16**) or its 20, 21-isomer. Furthermore, this finding proves (**19**) to be the immediate precursor of (**21**) in the biosynthetic pathway. The synthesis of the heteroyohimbine alkaloids in the presence of $H_2{}^{18}O$ has been independently studied (42, 44) and found *not* to lead to a labelling of the hetero-

cyclic oxygen atom. This clearly demonstrates that 4,21-dehydrogeissoschizine (**19**) exists in the enolized form exclusively and that the ring oxygen in the heteroyohimbine alkaloids is of *intra*molecular origin. The enzymic biosynthesis of heteroyohimbine alkaloids as presently determined is shown in figure 5.

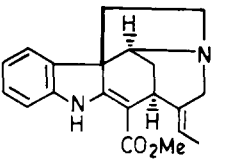
Geissoschizine (**6**), which was already implied as a precursor of ajmalicine (**1**) in the first cell-free system (15), has been unequivocally ruled out to be a precursor of ajmalicine by Stöckigt (47), in spite of the fact that in a recent publication it was again claimed and firmly believed that geissoschizine (**6**) plays a role in the intermediary metabolism leading to ajmalicine (**1**) (48). Geissoschizine (**6**) is only indirectly involved, in that it is first converted into 4,21-dehydrogeissoschizine (**19**), which is transformed to cathenamine (**21**) by the action of cathenamine synthase and is subsequently reduced to (**1**), (**2**), (**8**). This indirect involvement of geissoschizine (**6**) also explains the observation that, by *in vivo* tracer experiments, geissoschizine (**6**) was found to be incorporated into ajmalicine (**1**), vindoline (**28**), catharanthine (**29**) and akuammicine (**30**) (49). The intermediacy



Vindoline (28)



Catharanthine(29)



Akuammicine(30)

of geissoschizine is, however, only an indirect one and it is now quite clear that geissoschizine (**6**) is only indirectly involved in the biosynthesis of the above alkaloids (3, 50). This deduction demonstrates once again the superiority of the enzymatic studies over the conventional *in vivo* tracer feeding experiments.

CONCLUSIONS

In conclusion, we may infer from the above findings that, in general, the previous scheme of heteroyohimbine alkaloid biosynthesis and the underlying precursor studies have not led to a determination of the true sequence of events. In our opinion, extension of the ongoing enzymatic work and purification and characterization of the enzymes involved will lead to the clarification of the biosynthetic pathway. However, the enzymatic studies should not be conducted by an "organic chemist" sort of approach (% incorporation, etc.), but requires a basic knowledge of enzymology and biology. It is our firm belief that only the cooperation of biologists, chemists and enzymologists will lead to the complete understanding of one of Natures most fascinating areas, the formation and regulation of complex secondary plant products, which itself may eventually lead to the biotechnological synthesis of chemotherapeutic agents.

ACKNOWLEDGMENTS

It is my great pleasure to acknowledge the devoted work of all members of the Bochum laboratory. My sincere thanks are also to Dr. Geoffrey A. Cordell for his comments and suggestions and to Mr. Pablo Jourdan for his help in the preparation of the English version of this manuscript and to the Bundesminister für Forschung und Technologie, Bonn, for financial support.

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