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Natural products and enzymes from plant cell cultures

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Abstract

Plants represent an unlimited source of natural products. Many of the recently detected phytochemicals exhibit remarkable bioactivities, ranging from anticancer activity, phosphodiesterase inhibition to cytotoxicity against HIV-infected cells. Cultivated plant cells produce at their unorganized, dedifferentiated stage secondary metabolites, but in very different amounts in so far as new compounds are concerned. In fact, more than 140 novel natural products are presently known from plant cell cultures, which also include new metabolites formed by biotransformation. The biotransformation capacity of suspended cells is described and recent high yielding transformations, like the formation of arbutin by hydroquinone-transformation with *Rauwolfia* cells are discussed. As an example of alkaloid production by cell suspensions, the pattern of monoterpenoid indole alkaloids of the Indian medicinal plant *Rauwolfia serpentina* Benth. is described and the so far 30 identified compounds are divided into eight groups which are biosynthetically closely related. Some of the key biosynthetic reactions leading to the *Rauwolfia* alkaloids are discussed and an overview of the enzymes involved in the formation of the alkaloid ajmaline and proteins catalyzing side reactions of the ajmaline pathway are given.

Introduction

It is obvious that plants represent a nearly unlimited source of phytochemicals, metabolites of primary and secondary metabolism. Especially the secondary compounds are of major interest because of their different functions and their impressive biological activities ranging from antimicrobial, antibiotic, insecticidal, molluscidal, hormonal properties to highly important pharmacological and pharmaceutical activities. In this context it is interesting to note that, on one hand, an increasing number of investigations are carried out to detect novel bioactive phytochemicals in differentiated plants which could be useful directly in the treatment of a variety of diseases or as starting material for partial syntheses of highly valuable compounds. On the other hand, interest is focused on the formation of such plant products by cell and tissue culture systems in order to understand the biosynthesis of these compounds, to isolate the involved enzymes as biocatalysts and finally to manipulate and to improve the product syntheses in those systems.

New natural products from plants

In a recent survey on natural plant products with biological activity (Stöckigt & Lansing 1992) it became clear that not only new compounds are of great interest. Major research is also carried out to increase the limited supply of some already known, but rare plant products with promising pharmacological activities. One of the presently most discussed examples in this respect is the diterpene alkaloid taxol (1) which was isolated as early as 1971. Taxol has been detected as a trace compound in the bark of an Oregon yew tree (*Taxus brevifolia*) but has also been found later on in other Taxus species e.g. *T. canadensis*, *T. cuspidata* or *T. media*. From 7 kg of dried bark of the yew tree only 1 g taxol can be isolated. This low content and the structural complexity of the compound (unusual tetracyclic structure) were the

main reasons that its structure elucidation took nearly one decade. Most interesting was the observation of the cytotoxicity of taxol (1). In fact the antitumor activity of 1 was detected during the joint program between the United States Department of Agriculture and the National Cancer Institute with the aim to detect novel natural products exhibiting anticancer activity. Further research on this subject has shown the remarkable potential of taxol (1) for the treatment of breast and ovarian cancer and it is believed that this plant constituent is the most important discovery in the area of cancer chemotherapy in the last 20 years. From the pharmacological mechanism of this compound it seems that taxol is a spindle poison like the alkaloids colchicine, vincaleukoblastine, vincristine or the lignan podophyllotoxin inhibiting cell division. The novel mechanism of 1 is, however, the stabilization of micro tubules which prevents their 'dissociation' into tubulin molecules. Within a relatively short time taxol entered the market in USA for cancer treatment. However, the most limiting factor for further clinical studies on the way to a world-wide application of this plant product is the low concentration in *Taxus brevifolia* (Wani *et al.* 1971). It is estimated that about 200 kg taxol are needed per year for clinical treatment of patients suffer from cancer corresponding to nearly one million *Taxus* trees. This fact recently stimulated the intensification of efforts to find alternative sources for this rare drug. Therefore, more than thirty laboratories are involved in the development of a chemical synthesis of 1 (Winkler & Subrahmanyam 1992). Recently the first total synthesis of taxol (1) has been reported (Nicolaou *et al.* 1994). It seems that in the near future an economic total chemical synthesis of the compound will not be possible. The need for a reliable supply has also stimulated other research strategies. One is the development of tissue and cell cultures of Taxus species. Although first reports on the establishment of a culture system have already appeared (Wahl *et al.* 1992), the amounts produced by this culture are still extremely low. However, this system might be useful to explore the so far only partly known biosynthesis of taxol (1). (Strobel *et al.* 1992; Fleming *et al.* 1993). Partial synthesis of 1 or biological active derivatives seem to overcome the problem of taxol shortage. Potier and Guéritte-Voegelein and their co-workers isolated 10-deacetyl-baccatine III from the leaves of an European *Taxus* species (*T. baccata* L.). In sharp contrast to the low amounts of taxol in the bark, deacetyl-baccatine III can be obtained in a yield of 1 g per kg leaves, offering enough material for the partial synthesis of taxol (1)

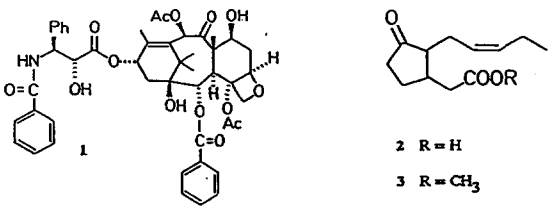


Fig. 1.

(Denis *et al.* 1988; Mangatal *et al.* 1989; Potier 1993). Because 1 is only slightly soluble in water (30 mg l^{-1}), derivatives with a more pronounced bioavailability as taxol are of a strong interest. Consequently, the French research group used an intermediate of the hemisynthesis of 1 to develop the taxol derivative Taxotere®. This compound is now in phase II clinical trials and, indeed, it has an enhanced bioavailability and a pharmacological profile which appears to be very similar to taxol. Both, the hemisynthesis of 1 and the development of Taxotere® are presently the most efficient strategies to solve the problem of taxol shortage.

A second example of a known compound with only very recently detected new biological activities is represented by jasmonic acid (2) and even more its methyl ester (3). These rather simple compounds are well known for their properties as plant growth factors with an ubiquitous occurrence in the plant kingdom. Recently they have additionally been identified as highly effective inducers of tendril coiling in *Bryonia dioica* (Falkenstein *et al.* 1991). Moreover, the ester (3) has been found to be released from leaves of an *Artemisia* species after an attack of insects. The volatile compound induces in nearby plants the synthesis of proteinase inhibitors (Farmer & Ryan 1990). It seems quite reasonable that this interspecies process of chemical communication might be one of the defence strategies of plants. In a recent paper, Zenk and co-workers (Gundlach *et al.* 1992) described 2 and 3 as signal transducers in elicitor-induced plant cell cultures. In this study it was observed that endogenous jasmonic acid (2) and its methyl ester (3) accumulate rapidly after the treatment of cultured cells of *Rauwolfia serpentina* and *Eschscholtzia californica* with a yeast elicitor. Addition of methyl jasmonate to a broad range of plant cell suspensions initiated the accumulation of secondary metabolites. Jasmonates are expected to be the chemical signal compounds in the process of elicitation leading to *de novo* gene transcription and finally to the biosynthesis of natural products in cultured plant cells.

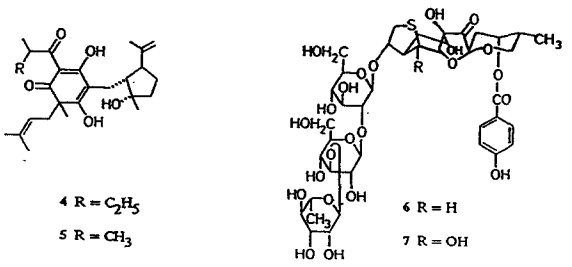


Fig. 2.

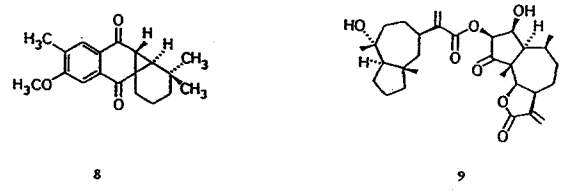


Fig. 3.

Novel phytochemicals from plants exhibiting remarkable bioactivities were detected during a screening for leukotriene D_4 and thromboxane A_2 antagonists which probably could be useful for the development of anti-allergic drugs. From the Chinese plant *Hypericum chinense* the previously unknown compounds chinesin I (4) and chinesin II (5) were isolated. They showed antagonistic activity for both LTD_4 and TXA_2 even in a μM concentration (Tada *et al.* 1991). The broad range of highly active novel plant constituents is also documented by the recently described isolation of breynin A (6) and B (7) leading at very low concentrations (1–20 $\mu g\ kg^{-1}\ day^{-1}$), to an approximately 30% decrease of the blood cholesterol level (Ohkuma *et al.* 1991).

Besides structurally very interesting new compounds for example flavelanone (8) an unusual tetracyclic compound with weak antitumor activity (Endo *et al.* 1991), and arrivacin A (9), a member of an extremely rare group of sesquiterpenoids with potent angiotensin II binding activity (Chen *et al.* 1991), more common types of phytochemicals such as saponins of the oleanan group also show extraordinary pharmacological activity. One example is periandradulcin A. It was isolated as a trace compound from dried roots of *Periandra dulcis* and exhibits potent inhibition of phosphodiesterase (IC_{50} 2×10^{-9} M) (Ikeda *et al.* 1991).

Whereas these phytochemicals are of low molecular weight, some novel more complex natural products reaching molecular weights of approximately 2500 were identified from the plant *Woodfordia fruticosa*. These constituents belong to the group of hydrolyzable tannins occurring as dimers and trimers with antitumor properties (Yoshida *et al.* 1991).

New polypeptides from plant sources seem to be attracting increasing interest. One of the recent examples are some highly active proteins from Cucurbitaceae. These proteins were isolated from rhizomes of *Trichosanthes*, a traditional Chinese medicinal plant which is used for abortion and in the therapy of cancer. Two proteins were isolated, trichosanthin and karasurin. Both are active as an abortivum but trichosanthin also shows immunosuppressive activity and cytotoxicity against HIV-infected cells (Toyokawa *et al.* 1991).

These few examples demonstrate without a doubt that plants are able to produce an enormous variety of phytochemicals with highly impressive biological activities. However, in many cases the limited supply of the drugs is a nearly insurmountable problem, especially for their clinical evaluation. The establishment of cell culture systems might be one possibility for overcoming this supply problem.

Plant cell suspension cultures as a source for natural products

It is now more than 90 years ago since plant cells were separated for the first time from differentiated plant tissue and kept alive under artificial conditions. However, it took many decades to find appropriate nutrition media for the cultivation of these cell systems. The detection and application of phytohormones, auxins and cytokinins, was the prerequisite for efficient cell growth and cell division. Meanwhile plant cell culture methodology was improved to a stage, where the establishment of such a culture system became possible on principle for any plant species. Rare and slowly growing plants of subtropical or tropical origin are a particulary useful target for the establishment of cell culture systems. The initiation of a cell culture starting with a surface sterilized plant part (leaves, stems, roots) can take very different times. In our case the time necessary for this process can vary between 8 weeks and several years. After this, however, using relatively simple equipment and laboratory conditions (rotary shakers, Erlenmeyer flasks, controlled temperature) such material can be obtained in a 5–20 kg scale (fresh weight) within only few weeks. A cell density of about 400–500 $g\ l^{-1}$ (fresh weight) medium can be reached

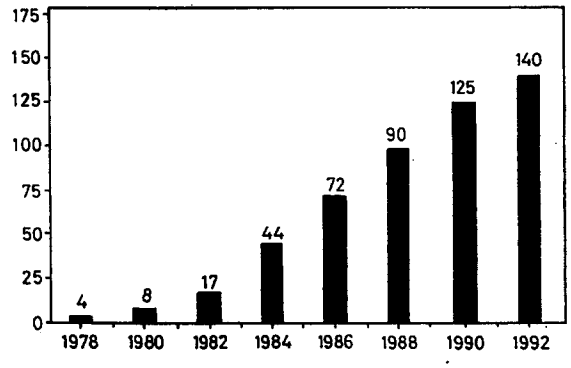


Fig. 4. Novel secondary metabolites isolated from plant cell and tissue cultures.

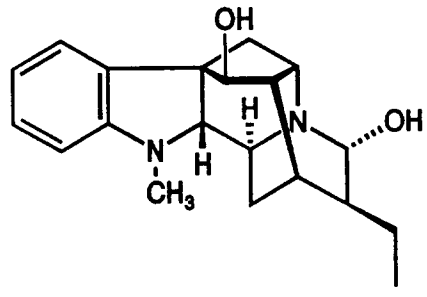


Fig. 5.

with a well established cell culture. This cell material can be easily separated from the nutrition medium by suction filtration, the cells can be stored frozen for an unlimited time at -30 °C and used for the isolation of secondary metabolites or enzymes involved in biosynthesis or biotransformation. From the biotechnological point of view bioreactor facilities have been developed which allow the production of plant cells on the scale of 10–75 m^3. Whereas a number of examples exist showing that production rates for known natural products in culture exceed that of the differentiated plant, it must also be realized that compounds with a high commercial value often are not produced by cultured cells. As shown in Table 1 there is a number of cell culture systems producing secondary metabolites at a gram per litre scale varying from 1.2 g up to 7.0 g of *de novo* synthesized compounds. Of these, the naphthoquinone shikonin is commercially produced by the Mitsui Company (Fujita *et al.* 1982) and the alkaloid berberine which is pharmaceutically useful because of its antiseptic activity accumulates in surprisingly high amounts of 7 g l^{-1} (Fujita & Tabata 1987).

Besides the production of specific natural metabolites, the plant material itself and its ingredients with its pharmaceutical activity are also the target for production under bioreactor conditions. One of the best examples is the production of Ginseng as a food additive. Ginseng roots are produced mainly in north-eastern China and Korea and are in use for their activity against gastroenteric disorders, modulation of blood circulation or as a tonic and adjuvant preventing disorders.

Bioreactor facilities with a two-ton culture capacity have been used for Ginseng production in Japan resulting in an average of 19 g l^{-1} of cell material (dry weight). The analysis of this material indicated that the culture product (Ginseng tissue, not cell suspensions) contained basically the same chemical constituents as that of the differentiated plant. Since 1988 this material has been commercially produced by the Nitto Denko Corp. and is applied as an additive for tonic drinks, wines, soups, herbal liquors etc. (Ushiyama 1991).

Novel natural products from cell and tissue cultures

Although the capacity of cultivated plant cells for the production of natural products has been a matter of intensive discussion in recent years it can be concluded from a previous review that, probably without many exceptions, all groups of secondary metabolites are generated by such cell systems (Ellis, 1988). As revealed in Table 2, ten groups of compounds belonging to phenylpropanoids were detected in cell cultures, ten groups of alkaloids are synthesized in cultivated cells in addition to five groups of terpenoids and three of quinones.

This result of the phytochemical analysis of cultivated plant cells is remarkable because systematic investigations concerning the pattern of secondary metabolites of a given culture have been carried out only sporadically. This, in fact, is also the case for novel natural products from culture systems which are not produced or have not been detected yet as constituents of differentiated plants. Nevertheless, the number of new phytochemicals isolated and identified exclusively from cultivated cells is continually increasing. Since our previous update on 'Novel Natural Products from Plant Cell and Tissue Culture' (Ruyter & Stöckigt 1989) in which we described 85 new compounds, the number of novel structures increased to about 140. Figure 4 indicates the drastic increase during the last few

Table 1. Examples of natural products formed by plant cell suspension cultures in remarkable yields

Natural product	Class of compound	Plant species	Yield ($g \times l^{-1}$)	Authors (year)
Berberine	Isoquinoline alkaloid	*Coptis japonica*	7.0	Fujita *et al.* (1897)
Rosmarinic acid	Phenylpropanoid	*Coleus blumei*	5.6	Kesselring (1985)
Shikonin	Naphthoquinone	*Lithospermum erythrorhizon*	3.5	Fujita *et al.* (1982)
Anthraquinones	Quinones	*Morinda citrifolia*	2.5	Zenk *et al.* (1975)
Ajmaline	Indole alkaloid	*Rauwolfia serpentina*	2.0	Vollosovitch (1988)
Coniferin	Phenylpropanoid	*Linum flavum*	2.0	van Uden *et al.* (1992)
Raucaffricine	Indole alkaloid	*Rauwolfia serpentina*	1.6	Schübel *et al.* (1989)
Cinnamoylputrescin	Cinnamic acid derivative	*Nicotiana tabacum*	1.5	Schiel *et al.* (1984)
Shikimic acid	Cyclohexen carbonic acid	*Galium molugo*	1.2	Amrhein *et al.* (1980)

Table 2. Groups of natural products which were so far isolated from tissue and cell suspension cultures of high plants

I. Phenylpropanoids	II. Alkaloids	III Terpenoids	IV. Quinones
1. Anthocyanins	1. Acridines	1. Carotenoids	1. Anthraquinones
2. Coumarins	2. Betalaines	2. Monoterpenes	2. Benzoquinones
3. Flavonoids	3. Quinolizidines	3. Sesquiterpenes	3. Naphthoquinones
4. Hydroxycinnamoyl derivatives	4. Furoquinolines	4. Diterpenes	
	5. Harringtonines	5. Triterpenes	
5. Isoflavonoids	6. Isoquinolines		
6. Lignans	7. Indoles		
7. Phenalenones	8. Purines		
8. Proanthocyanidins	9. Pyridines		
9. Stilbenes	10. Tropane alkaloids		
10. Tannins			

years of newly detected metabolites, not known from whole plants.

It must, however, be mentioned that a number of these new phytochemicals also come from biotransformation experiments with plant tissue cultures - a result pointing to the variety of applications using cultivated cells for the biosynthesis of novel metabolites. Some of the recent examples of novel alkaloids from cell suspension cultures came from our group. The analysis of crude methanol extracts of cell suspension cultures of the Indian medicinal plant *Rauwolfia serpentina* by Rotation Locular Counter Current Chromatography (RLCC) revealed four highly polar alkaloids which turned out to be so far unknown glucosides of ajmaline (10) and its derivatives (Ruyter *et al.* 1988). These alkaloids were named raugIucines and their structures are shown later in this article. The same number of a new series of indole alkaloids, named raumaclines, were isolated and identified from this *Rauwolfia* culture system during a co-operation with the Institute of Pharmaceutical Chemistry of the Chiba University (Japan). The group of raumacline alkaloids will also be discussed separately later.

Two additional novel monoterpenoid indole alkaloids were isolated and spectroscopically identified from cell suspensions of the medicinal plant *Aspidosperma quebracho blanco* Schlecht (Apocynaceae) (Aimi *et al.* 1991). From the freeze-dried cells of the culture grown for 14 days under standard conditions the new 3-oxo-14,15-dehydrorhazinilam (11) and aspidochibine (12) were isolated as minor compounds. Whereas 11 is an undescribed derivative of rhazinilam (13), aspidochibine (12) represents a novel structural class of the quebrachamine series. 2D-NMR techniques and Nuclear Overhauser Effect spectroscopy were a prerequisite for delineating the struc-

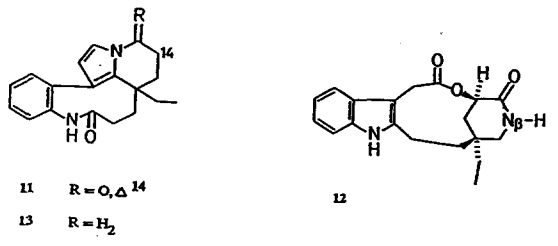


Fig. 6.

ture of 12. From the biosynthetic point of view the formation of this alkaloid would be a highly interesting subject to investigate. However, the observed rates of synthesis in this culture system which do not exceed amounts of 1 mg alkaloid per litre of medium are insufficient to start investigations of the biosynthetic pathway. The examples of natural products described here demonstrate again that dedifferentiated plant systems frequently lead to novel metabolites exhibiting a completely new skeleton, as already described for the voafrines (Stöckigt *et al.* 1983).

Of new compounds isolated from cultivated plant cells, novel polysaccharides also need to be mentioned. In addition to three known polysaccharides from cultivated *Echinacea* cells which have been demonstrated to be immunomodulators, a second cell system has been established (from *Arnica montana* plants) producing two polysaccharides (Puhlmann *et al.* 1991). One of these is an arabino-3,6-galactan-protein with a Mr of 95–100 kDa and represents one of the immunologically most active plant polysaccharides, stimulating at 6 μg ml^{-1} excretion of TNF (tumor necrosis factor). These polysaccharides which are excreted into the nutrition media of the cell suspension cultures might become particularly interesting from the economic point of view, because their production can now be carried out on a 75 m^3 scale (Rittershaus *et al.* 1989).

In vivo biotransformations with plant cells

The biochemical capacity of cultivated plant cells to perform specific biotransformations under *in vivo* conditions has been well known for many years. Up till now a whole range of reactions has been observed including, for example, esterification, oxidation, reduction, hydroxylation, and glycosylation. In all cases the stereo- and regioselectivity expressed by the *in vivo* process is the enormous advantage of such a system, even if the transformation rates are in general still relatively low. For instance cell cultures of *Nicotiana tabacum* reduce the monoterpene menthone under formation of (*S*)-menthol, but do not accept the isomer (*iso*-menthone). Cell suspensions of *Euphorbia characias* or *Glycine max.* convert exogenous added geraniol into nerol and geranial, respectively as major transformation products. The observed transformation rates, however, are only in the range of mg per litre culture medium (Carriere *et al.* 1989). In addition, the biotransformation of digitoxigenin by cell suspension cultures of *Strophanthus intermedicus* turned out to be very efficient in producing a whole variety of hydroxylated derivatives or glucosylation products (Kawaguchi *et al.* 1989) which, however, again were found only in trace amounts. In contrast, cardiac glycosides can be biotransformed with cell suspensions of *Digitalis lanata* in relatively high yields (Table 3). Isolated and optimized cell strains were found to be able to convert β-methyldigitoxin into the more valuable β-methyldigoxin by hydroxylation at the C-12 position of the substrate. In fact biotransformation rates of up to 0.8 g l^{-1} of medium were measured after 20 days even under bioreactor conditions (20 l reactor) (Alfermann & Reinhard 1988). Most detailed investigations exist, however, on *in vivo* glucosylations of phenolic compounds. As shown in Table 3 extensive work has been carried out on the glucosylation of the flavonoid umbelliferone or the hydroxybenzoic acid (Alfermann & Reinhard 1988), salicylic acid. A special interest seems to be concentrated on conversion of hydroquinone by different cell systems like *Datura innoxia*, *Catharanthus roseus* or *Rauwolfia serpentina*. These cell cultures transform hydroquinone into its β-D-glucoside, named arbutin. Arbutin is a natural product occurring in leaves of many different plants e.g. *Pyrus communis*, *P. serotina* (bear leaves), *Vaccinium vitis-idaea*, *V. myrtillus* (blueberry), *Bergenia crassifolia* or in leaves of the medicinal plant *Arctostaphylos uva-ursi* (syn. *Arbutus uvaursi*). Arbutin containing plant extracts are widely used in traditional and modern medicine because of their urethral disinfectant activity. Recently this compound has attracted much interest as an additive in cosmetics, because it is an efficient suppressor of melanin biosynthesis in human skin (Akiu *et al.* 1988).

Although arbutin can be relatively easy prepared chemically in a three step procedure (Shiseido 1936), a one step bioconversion seems still to be of biotechnological interest. In a recent publication S. Inomata and co-workers from the Basic Research Laboratories of

Table 3. Examples of biotransformations of exogenous added compounds by cell suspension cultures

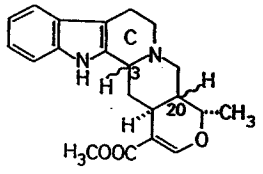
Added compound	Cell suspension culture	Biotransformation product	Yield	Authors (year)
Geraniol	*Euphorbia characias*	Nerol	0.1 g × l^{-1} × 2 h^{-1}	Carriere *et al.* (1989)
β-Methyldigitoxin	*Digitalis lanata*	β-Methyldigoxin	0.8 g × l^{-1} × 20 d^{-1}	Alfermann & Reinhard (1988)
Umbelliferone	*Perilla frutescens*	Umbelliferone-β-D-glucoside	1.6 g × l^{-1} × d^{-1}	Tabata *et al.* (1976)
Salicylic acid	*Mallotus japonicus*	Salicylic acid-β-D-glucoside	1.2 g × l^{-1} × d^{-1}	Tabata *et al.* (1976)
Salicylic acid	*Mallotus japonicus*	Salicylic acid-β-D-glucoside	1.1 g × l^{-1} × 14 d^{-1}	Umetani *et al.* (1990)
Hydroquinone	*Datura innoxia*	Arbutin	0.7 g × l^{-1} × 6 d^{-1}	Tabata *et al.* (1976)
Hydroquinone	*Datura innoxia*	Arbutin	7.1 g × l^{-1} × 3 d^{-1}	Suzuki *et al.* (1987)
Hydroquinone	*Catharanthus roseus*	Arbutin	9.2 g × l^{-1} × 4 d^{-1}	Inomata *et al.* (1991)
Hydroquinone	*Rauwolfia serpentina*	Arbutin	18 g × l^{-1} × 7 d^{-1}	Lutterbach & Stöckigt (1992)

the Shiseido Company described the *in vivo* formation of arbutin from hydroquinone with a maximum yield of 9.2 g l^{-1} medium as the only compound formed within 4 days by cultivated *Catharanthus* cells. This conversion rate might eventually compete economically with the chemical synthesis (Inomata *et al.* 1991)

When we investigated this bioconversion with a cell suspension of the Indian medicinal plant *Rauwolfia serpentina* Benth. using a nutrition medium optimized for the production of the glucoalkaloid raucaffricine, we observed production rates of arbutin up to 18 g l^{-1} medium after 7 days of continuous hydroquinone feeding. Although this is by far the highest value of a natural product formed by a plant cell culture system, we believe that the productivity of our *Rauwolfia* culture can still be significantly enhanced (Lutterbach & Stöckigt 1992).

The alkaloid profile of cell suspensions of *Rauwolfia serpentina* Benth

After a long term optimization of growth and production rates of alkaloids, a stable cell culture system of *Rauwolfia serpentina* Benth. was established. During this process and over several years we determined the pattern of monoterpenoid indole alkaloids of this culture. We have isolated and identified so far 30 alkaloids belonging to different subgroups. This cell suspension is phytochemically one of the most detailed investigated, serving as an example of the broad capacity of *in vitro* cultivated cells for the biosynthesis of phytochemicals.



14	H-3α,	H-20β
15	C = arom.,	H-20β
16	C = arom.,	H-20α
17	H-3β,	H-20β

Fig. 7.

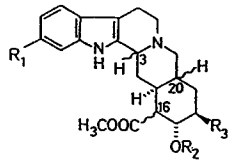
In the following sections we will briefly describe the alkaloids isolated thus far from *Rauwolfia serpentina* cell suspension culture.

Alkaloids with the Heteroyohimbine Skeleton

Of the heteroyohimbine alkaloids, the typical *Rauwolfia* components were detected: ajmalicine (=raubasine) (14), its dehydroderivative serpentine (15), an isomer of serpentine, alstonine (16) and 3-iso-ajmalicine (17) (Stöckigt *et al.* 1981). In contrast to the *Rauwolfia* root bark in which ajmalicine (14) and serpentine (15) are the primary alkaloids, they occur in cultivated cells only in extremely small amounts.

The Yohimban Alkaloids

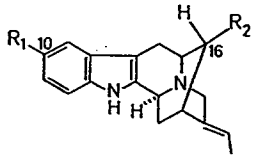
Only two representatives of the yohimban skeleton were detected in the cultivated cells, yohimbine (18) and reserpine (19). In fact, the capability of the cells to synthesize reserpine (19) was lost after 2 years of cultivation, an effect which is frequently observed with the production of secondary metabolites by cultivated plant cells.



18 H-3α, H-16,20β, $R_1 = R_2 = R_3 = H$

19 H-3β, H-16,20α, $R_1 = OCH_3$, $R_2 = CH_3$, R_3 = trimethoxybenzoyl

Fig. 8.



20 $R_1 = OH$, $R_2 = CH_2OH$

21 $R_1 = H$, $R_2 = CH_2OH$

22 $R_1 = H$, $R_2 = CHO$

Fig. 9.

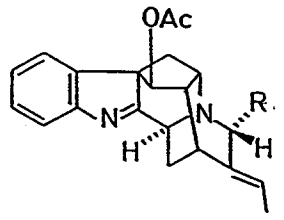
The formation of 19 was also reported in *Rauwolfia* tissue systems some years ago (Yamamoto & Yamada 1986). To our knowledge there is no culture system available at the present time showing significantly higher production of reserpine (19) when compared to the differentiated plant.

The Sarpagan Alkaloids

Within the Apocynaceae family the relatively small alkaloid subgroup of sarpagans consists of about 45 members (Koskinen & Lounasmaa 1983). Under normal growth conditions the cell culture forms only a very limited number of sarpagan alkaloids as minor components. As several alkaloids of this subgroup also serve as biogenetic precursors of other *Rauwolfia* alkaloids, these intermediates were detected only in low concentrations. Up to now the following 3 indole bases have been isolated from the cell suspension: sarpagine (20), its 10-deoxyderivative (21) and vellosimine (22). Vellosimine differs from 10-deoxysarpagine (21) only by an aldehyde function instead of a CH_2OH group at C-16. Vellosimine (22) is in fact a precursor of 21 as shown by the isolation of the appropriate reductase converting 22 into 21 (Pfitzner & Stöckigt 1983). Therefore 22 can be isolated from the cell culture only as a trace compound.

The Indolenine Alkaloids

As just mentioned for the sarpagan group, the alkaloids which belong to the indolenine type might also be involved in the formation of the ajmalan skeleton and therefore should appear in the cells in small amounts. We have been able to isolate and to identify only 3 compounds of this alkaloid group represented by vinorine (23), vomilenine (24) and its glucoside, raucaffricine (25). Whereas vinorine (23) is in fact a trace alkaloid of the culture, vomilenine (24) is biosynthesized in the higher milligram range, if optimized conditions are used. Raucaffricine (25), however, can be produced up to an amount of 1.6 grams per litre medium (Schübel *et al.* 1989), so that its production exceeds that of the plant 70-fold (Ruyter *et al.* 1991)[1].



23 R = H

24 R = OH

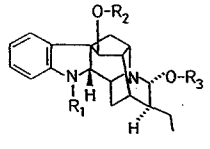
25 R = O-Glc

Fig. 10.

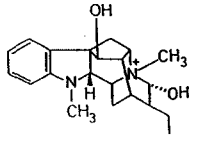
The Ajmalan Alkaloids

The most prominent member of the ajmalan subgroup consisting of eight alkaloids is without doubt the antiarrhythmic ajmaline (10). In contrast to the other mentioned *Rauwolfia* alkaloids, except vomilenine (24) and raucaffricine (25), 10 is one of the major components in cell cultures as well as in the roots of the differentiated plant. Under optimal growth conditions 10 accumulates after 18 days in amounts of up to 0.3 g per litre of medium, corresponding to 0.9% of the dry cells. This value is comparable with that reported for ajmaline concentrations in plants (0.8%) (Sahu 1978). In addition to ajmaline (10), a range of structurally very related alkaloids like the Nα-norcompound, norajmaline (26), its acetyl derivative,17-*O*-acetylnorajmaline (27), 17-*O*-acetylajmaline (28) and N_β-methylajmaline (29) were isolated, but in significantly lower yields. N_β-Methylajmaline (29) which was already known as a synthetic derivative of ajmaline (10) (Anet *et al.* 1954), was now isolated as a natural product from the cultured cells. Three further compounds can be derived from the mentioned ajmalan alkaloids by glucosylation, however, in extremely low

[1] During the last year the high accumulation rates of 25 are continuously decreasing.

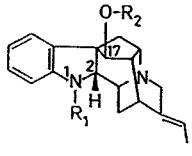


26	R_1 = H,	R_2 = H	R_3 = H
27	R_1 = H,	R_2 = Ac	R_3 = H
28	R_1 = CH_3,	R_2 = Ac	R_3 = H
30	R_1 = CH_3,	R_2 = H	R_3 = Glc
31	R_1 = CH_3,	R_2 = Ac	R_3 = Glc
32	R_1 = H,	R_2 = Ac	R_3 = Glc



29

Fig. 11.



33	R_1 = CH_3,	R_2 = H
34	R_1 = H,	R_2 = H
35	R_1 = H,	R_2 = Ac
36	R_1 = CH_3,	R_2 = Ac

Fig. 12.

concentrations (< 10^{-3}% of dry weight). They are called rauglucine (30), 17-*O*-acetylrauglucine (31) and its norderivative (32) (Ruyter *et al.* 1988). The structures of the ajmalan alkaloids allow them to be arranged in a plausible biogenetic sequence which was finally proved by isolation of the appropriate enzymes.

The Tetraphyllicine alkaloids

A very similar situation to that just described for the ajmalan group also exists for the alkaloids named tetraphyllicines. These alkaloids are derived most probably by the ajmaline pathway because of the similarity with the pathway intermediate vinorine (23). Of the tetraphyllicines, the cell culture forms tetraphyllicine (33), nortetraphyllicine (34) and both acetyl derivatives (35, 36).

The Raumacline akaloids

Another alkaloid subgroup are the raumaclines. They represent a novel type of *Rauwolfia* alkaloids which are formed after feeding a *Rauwolfia serpentina* cell culture with ajmaline (10). In cell cultures grown under normal conditions these alkaloids do not occur. The assumption that the raumaclines are transformation products of 10 was proved by using radioactive labelled ajmaline (10). Until now, four raumacline-type alkaloids have been isolated from the tissue and also from the medium of this cell culture. Their structures were elucidated by spectroscopic methods and chemical syntheses. In addition, suaveoline (37) (Majumdar *et al.* 1972) which shows the same skeleton type as the raumaclines has also been detected. The major transformation products of ajmaline (10) are raumacline (38) and its N_β-methylderivative (39) (Polz *et al.* 1990). In addition, 19-hydroxy-N_β-methylraumacline (40) and 6α-hydroxyraumacline (41) are formed, though in much lower concentrations (Endreß *et al.* 1992; Takayama *et al.* 1992). 6α-Hydroxyraumacline (41) is the first alkaloid of this new subgroup which belongs to the rather small group of monoterpenoid indole alkaloids oxidized at the C-6 position. In comparison, the C-19 position is more frequently the target of oxidative reactions. For example alstonerine (42), a representative of the macroline alkaloids, shows a C-19-oxo-function (Cook *et al.* 1969).

The raumaclines are structurally related to the macroline-type alkaloids, so that during their biosynthesis the carbon skeleton has to be changed from the ajmalan type to the raumacline type. The single steps of this transformation have been recently clarified. An oxidative bond cleavage between C-7 and C-17 can be imagined, leading to the skeleton type of a sarpagan alkaloid which is the intermediate on the pathway to the raumacline. The enzyme which catalyses the bond cleavage was isolated, but others which are responsible for further transformations, like N_β-methylation or hydroxylation, are not yet known (Obitz *et al.* 1995). The hydroxylations at C-6 and C-19 are probably secondary transformations of raumacline, but at the moment it cannot be excluded that ajmaline (10) is oxidized before the raumacline carbon skeleton is formed.

The Glucoalkaloid Strictosidine

The glucoalkaloid 3α(*S*)-strictosidine (43) is one of the most important key intermediates leading to all classes of monoterpenoid indole alkaloids and even to the group of cinchona alkaloids (Nagakura *et al.* 1979). Because of this universal biogenetic role, 43 is only detectable in trace amounts under normal growth and medium conditions. In *Rauwolfia* cells grown for 18 days in a modified alkaloid production medium, stric-

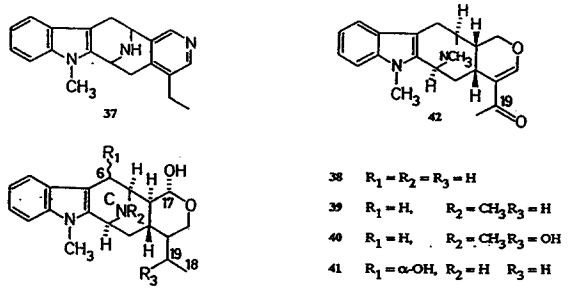


Fig. 13.

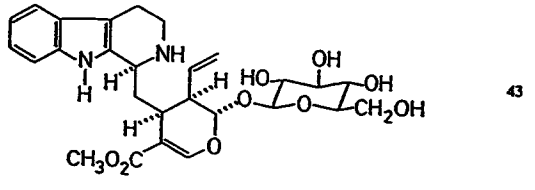


Fig. 14.

tosidine (43) accumulates in a higher mg range per litre nutrition medium indicating the influence of medium components on alkaloid biosynthesis and offering a direct way to manipulate a biosynthetic sequence. The immobilization of strictosidine synthase, however, allows a very simple preparation of the glucoalkaloid in the higher gram scale (Pfitzner & Zenk 1982), a method often used for labelling and feeding experiments with strictosidine (43).

The enzymes involved in the biosynthesis of *Rauwolfia* alkaloids

One of the major and most interesting alkaloids of *Rauwolfia* is without doubt the anti-arrhythmic ajmaline (10). The understanding of the biosynthesis of 10 was previously based only on few *in vivo* feeding experiments, indicating for instance that ajmaline is derived from the amino acid tryptophan and the monoterpenoid loganin. These few experimental findings did not allow to form a comprehensive picture of the ajmaline biosynthesis.

In recent years we have studied the enzymology of the biosynthesis of sarpagan and ajmalan alkaloids using the above described cell suspension of *R. serpentina*. The major part of the metabolic pathway determined thus far can be summarized as follows:

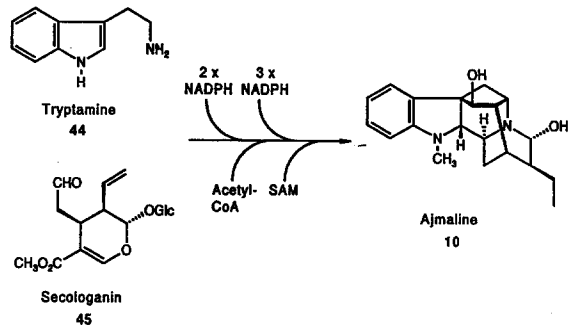


Fig. 15. Cofactor dependency of the overall biosynthesis of ajmaline (10) from tryptamine (44) and secologanin (45).

Starting with tryptamine (44) and secologanin (45) *five* reactions are dependent on NADPH, *one* reaction on acetyl-CoA and *one* reaction depends on *S*-adenosylmethionine (SAM). In addition, several reaction steps of the biosynthesis of 10 are cofactor independent (Fig. 15).

In this rather complex biogenetic route at least eleven enzymes are involved, all of them have been detected during the last decade and all of them are novel biocatalysts. The characterization of these enzymes showed in general high substrate specificity. Obviously each enzyme is not just responsible for one particular reaction, the catalyst also occurs only in those plant cell cultures or differentiated plants which bear the appropriate enzyme substrates *and* enzyme products. In fact, the distribution of these enzymes within plant families and genera is strongly correlated with the occurrence of the appropriate monoterpenoid indole alkaloids. Table 4 summarizes the isolated enzymes of which exclusively one, strictosidine synthase, is purified to homogeneity, its gene has been successfully cloned and the synthase heterologously expressed (Kutchan *et al.* 1988; 1994).

In addition to the metabolic sequence leading to ajmaline (10) several side products and enzymes of side reactions were recently detected. They can be summarized as shown in Table 5.

From the point of view of product synthesis in cultivated *Rauwolfia* cells the enzyme converting vomilenine (24) into raucaffricine (25) seems to be the most important (Fig. 16). Raucaffricine (25) accumulates as the major alkaloid during cell growth and reaches values which are 70 times higher than the raucaffricine content of *R. serpentina* roots (Ruyter *et al.* 1991). Although the enzyme responsible for the reverse reaction - the hydrolysis of raucaffricine (25) - is also well

Table 4. Major enzymes which catalyze the biosynthesis of ajmaline (10)

1. Strictosidine-Synthase	6. Vinorine-Hydroxylase
2. Strictosidine-β-Glucosidases	7./8. Vomilenine-Reductase/s
3. Sarpagan-Bridge-Enzyme	9. 17-*O*-Acetyl-Ajmalan-Esterase
4. PNA-Esterase	10. Ajmalan-17-*O*-Acetyl-Transferase
5. Vinorine-Synthase	11. SAM:Ajmalan-*N*α-Methyltransferase

Table 5. Enymes which catalyze side reactions of the biosynthesis of ajmaline (10)

12. UDPG:Vomilenine-Glucosyltransferase
13. Raucaffricine-β-*D*-Glucosidase
14. Vomilenine-Oxidoreductase
15. Perakine-Reductase
16. Vellosimine-Reductase
17. 10-Deoxy-Sarpagine-Hydroxylase*

*This enzyme has not yet been detected.

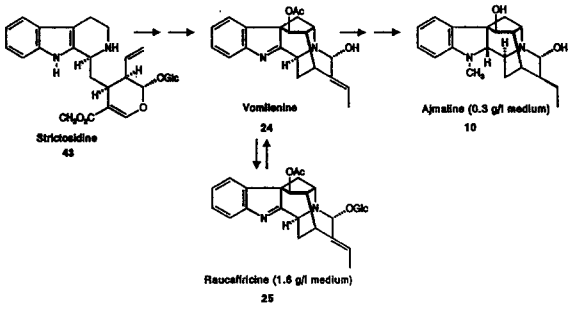


Fig. 16. Formation of raucaffricine (25) - the major side reaction of the ajmaline pathway.

known, its real metabolic function is not yet completely understood. Future work has to concentrate on these side reactions, especially to increase the cellular production of ajmaline (10). As raucaffricine (25) biosynthesis results in a decrease of the ajmaline pathway intermediate vomilenine (24), inhibition of the raucaffricine (25) forming enzyme might eventually lead to a significant enhanced ajmaline (10) synthesis.

If this goal can be reached, the question still needs to be answered whether ajmaline (10) which is then expected to accumulate in much higher amounts will be stable or will be metabolized by the *Rauwolfia* cells. Work on this is now in progress.

Acknowledgements

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