

Major Indole Alkaloids Produced in Cell Suspension Cultures of *Rhazya stricta* Decaisne

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Eleven main alkaloids were identified from cell suspension cultures of *Rhazya stricta* grown in 4X-medium for 15 days. The alkaloids comprised the five groups *Corynanthe*, *Strychnos*, *Eburnan*, *Secodine*, and *Aspidosperma* and can be regarded as being typical *Rhazya* alkaloids, although the *Strychnos* alkaloid akuammicine has been isolated for the first time from the genus *Rhazya*. The most abundant compound was (+)-1,2-dehydroaspidospermidine (15 mg/l medium) whereas all other constituents were synthesized in about 5–10 times lower amounts. More than 15 further alkaloids were formed as minor components which have not yet been identified. Cultivated *Rhazya* cells have been shown to be one of the richest alkaloid sources from apocynaceous cell suspensions.

The isolated compounds, because of their structural heterogeneity, cannot be presently arranged into a scheme with coherent biosynthetic sequences.

Introduction

Some species of the genera *Catharanthus* and *Amsonia* have already been screened for their alkaloid content as cell suspension cultures. A further example in this series of investigations is *Rhazya stricta* Decaisne from a closely related genus. This plant is a member of the tribe *Alstonieae* belonging to the subfamily *Plumerioideae* of the family *Apocynaceae* (Ganzinger and Hesse [1]). Only two species of *Rhazya* have been so far described, *R. stricta* used above and *R. orientalis* A. DC. *R. stricta* is found in the north-west regions of India and Pakistan and is employed in the indigenous system of medicine as a curative for chronic rheumatism (Chopra *et al.* [2]; Watt [3]). During the last 20 years phytochemical investigations have been widely pursued to establish the alkaloid patterns of both species. Such work resulted in the identification of more than 50 different indole alkaloids (Ganzinger and Hesse [1]; Rahman and Fatima [4]). Some of these compounds were recently demonstrated to exhibit anticancer activity, e.g. tetrahydrosecamines and vallesiachotamine (Mukhopadhyay *et al.* [5]). Because of our interest in the cell-free synthesis and the formation of monoterpenoid indole alkaloids in cellular systems we have determined the major alkaloidal components of cul-

tivated *R. stricta* cells. In this report we describe the identification of eleven compounds, which belong to five alkaloid groups; *Aspidosperma*, *Corynanthe*, *Eburnan*, *Secodine*, and *Strychnos* and we compare the alkaloid patterns of the cell suspensions and the differentiated plant.

Material and Methods

Cell Cultures

Callus cultures were initiated from seedling material of *Rhazya stricta* and were grown on petri-dishes in 4X-medium (Gamborg *et al.* [6]) modified as described by Ulbrich and Zenk [7] and solidified by 1% agar. Cell suspensions were grown for 16 days in 1 l Erlenmeyer flasks (100 rpm, 25 °C, 600 lux) in 4X-medium. 250 g fresh cells (17.3 g weight = 5.7%) were obtained per l medium.

Chromatographic methods

For preparative column chromatography silica gel 60 (particle size < 0.063 mm, Merck) was used with the mobile phase (a) chloroform/methanol/ammonia = 90:10:0.2. Thin layer chromatography (TLC) was carried out on Sil gel plates G/UV₂₅₄ (0.25 mm) obtained from Macherey-Nagel. The following solvent systems were used: (b) ether/xylol/*n*-hexane/ethyl acetate/diethylamine = 5:2:2:1:1, (c) *n*-hexane/ether/diethylamine = 10:10:1, (d) petroleum ether (40–60 °C)/acetone/diethylamine = 8:1:1, (e) petroleum ether (40–60 °C)/acetone/diethylamine =

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7:2:1. Ceric ammonium sulphate (CAS) was used for alkaloid detection during the optimization of TLC systems. HPLC analyses were conducted using a Spectra Physics system equipped with an automatic sample injector (ASI 100, Analytische Biochemie GmbH, Puchheim). A semiprep. column (Nucleosil 7C8, 25 × 1 cm, Macherey-Nagel) and the solvent system acetonitrile/10 mM ammonium carbonate (gradient 50:50 → 99:1 within 50 min) were used. The flow rate was 2 ml/min and alkaloids were detected at 280 nm.

Alkaloid isolation

2.5 kg *Rhazya stricta* tissue was freeze dried and extracted twice with 2.5 l methanol for 30 h with stirring. After filtration the organic phases were combined and evaporated under vacuum. The residue was worked up by the procedure reported by Stöckigt and Soll [8]. The resulting crude alkaloid mixture was dissolved in 10 ml chloroform and separated by prep. column chromatography (column 4.2 × 31 cm, 150 g silica gel, flow rate 1 ml/min). 230 fractions (13 ml each) were collected and screened by TLC for alkaloids. Four major fractions (**F**) were obtained by combining fractions No. 6–16 (**F1**), No. 17–35 (**F2**), No. 36–65 (**F3**), No. 121–230 (**F4**). From **F1–F4** the alkaloidal products were isolated by TLC employing the above solvent systems a–e.

Alkaloid identification

The purified alkaloids in methanolic solution were spectroscopically characterized by UV (Perkin Elmer spectrophotometer 551 S), and further examined by mass spectrometry (MS) on a Finnigan MAT 44 S quadrupole instrument (EI-MS, 70 eV, DCI-MS at 150 eV with *iso*-butane as reactant gas) equipped with a MS-Library search program containing the fragmentation patterns of 1800 indole bases. Optical rotation values were determined using a Perkin Elmer 241 polarimeter with chloroform as solvent. In most cases all data, including the chromatographic properties of the isolated indolic bases, were compared with authentic alkaloids.

Results and Discussion

These studies were designed to answer questions, such as whether *Rhazya stricta* cells grown under artificial conditions could provide as rich a source of

indole alkaloids as found in the differentiated plant. It would also be interesting to compare both sources regarding the variability of the basic alkaloid structures formed and to decide whether the culture might be an acceptable tool for future investigations of the enzymatic biosynthesis of special alkaloid types. This strategy has already been proved successful in the study of *Rauwolfia* alkaloid biosynthesis and the first step in those investigations was the determination of the alkaloid composition in *Rauwolfia serpentina* cell suspensions (Stöckigt *et al.* [9]). In addition it was not definitely known whether *R. stricta* cells were able to synthesize alkaloidal metabolites under cell culture conditions.

In a preliminary experiment we extracted *R. stricta* cell suspensions (100 g fwt) and the extract was examined by two-dimensional TLC. The plate was sprayed with CAS-reagent and several CAS-positive spots were revealed (alkaloids). We therefore analyzed 2.5 kg *Rhazya* cells by exhaustive extraction with methanol and subsequent isolation of a crude alkaloid mixture by the method reported earlier [8]. This mixture was further separated on a silica gel column (mobile phase (a)) into four alkaloid-containing fractions (**F1–F4**) from which the individual alkaloids were isolated by TLC.

Using the solvent system (b) fraction **F1** separated into four bands (Rs 1–Rs 4, $R_f = 0.73$, $R_f = 0.61$, $R_f = 0.52$, $R_f = 0.4$ resp.). The less polar compounds Rs 1 and Rs 2 showed at 366 nm a slight blue fluorescence, with CAS an intensive blue colour and UV-maxima at 298 and 328 nm. These data, especially the UV values, are characteristic of *Aspidosperma* alkaloids exhibiting a β -anilinoacrylate chromophore indicative of compounds of the tabersonine series. This suggestion was verified by a MS-analysis including our MS-Library search program which presently contains the MS-data of 1800 indole alkaloids and enables a rapid decision on new or known alkaloids. The compounds were identified as vincadifformine (Rs 1) and tabersonine (Rs 2). The former alkaloid has been reported to occur in plants of *Vinca difformis* as a racemate (Djerassi *et al.* [10] and from *V. minor* the (–)-enantiomer was isolated (Plat *et al.* [11]). Smith and Wahid [12] found (+)-vincadifformine in *R. stricta* plants. Measuring of the optical rotation of the alkaloid from the cell culture clearly agreed with the last result. The isolated tabersonine was of the (–)-enantiomeric series, and is also known to occur in *Rhazya* plants.

This alkaloid, which has an essential function as a biogenetic precursor of vindoline (Scott *et al.* [13]), is a very common alkaloid in cultured cells. Up to now, tabersonine has been isolated from cell suspension cultures of six different species of the family Apocynaceae. The third alkaloid (Rs 3) of the fraction **F1** gave an orange CAS-reaction and the UV-, MS- and chiroptical data were identical with those known for (+)-1,2-dehydroaspidospermidine, which is a constituent of *Rhazya* plants and also of several other members of the Plumerioideae [3].

(+)-1,2-Dehydroaspidospermidine was the major alkaloid of the cell culture and was produced in a yield of about 15 mg per l medium. On a dry weight basis (0.1%) it did not reach the value of 0.21% reported for the differentiated plant [12]. The less polar constituent (Rs 4) of fraction **F1** was rechromatographed using solvent (c) and separated into Rs 4.1 ($R_f = 0.4$) and Rs 4.2 ($R_f = 0.1$). CAS-reaction of the former alkaloid yielded a blue turning greenish colour and, as well as the typical indole chromophore, the MS fragmentation with only one main signal (126 m/z) completely matched that of tetrahydrosecodeine as identified by Cordell *et al.* [14] in *R. orientalis*. Rs 4.2 was identical in every respect with the *Eburnan* alkaloid eburnamonine which was first isolated from *Hunteria eburnea* plants (Bartlett *et al.* [15]). For this alkaloid too the (+)- and (–)-series are naturally occurring. The cell culture yielded the (+)-enantiomer as did the *Rhazya* plant.

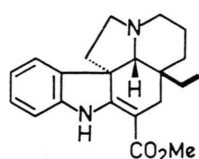
The alkaloid mixture **F2** revealed spots Rs 5 ($R_f = 0.63$) and Rs 6 ($R_f = 0.46$) upon TLC employing the system (d). Both alkaloids, (–)-akuammicine (Rs 5) and (–)-norfluorourarine belong to the *Strychnos* class and they have already been detected several times in cultivated cells of different apocynaceous species. Fraction **F3** rechromatographed using the above system gave Rs 7 and Rs 8 ($R_f = 0.3$ and 0.15 resp.). The MS-Library search suggested for Rs 7 the *Sarpagine* group alkaloids akuamidine and polynuridine. Comparison of the fragment intensities favoured the former alkaloid and the identity was confirmed by co-chromatography with authentic samples. Akuamidine, also called rhazine, had already been detected in *R. stricta* plants in 1962 [16]. A recent pharmacological investigation of this alkaloid apparently indicated a 3-times stronger anaesthetic activity when compared with cocaine (Cordell [17]).

The compound Rs 8 ($M^+ 296 m/z$) showed a MS fragmentation pattern characterized by only a few major peaks at 267, 249 and 226 m/z , which were all superimposable with those described for eburnamonine from *Hunteria eburnea* (Bartlett *et al.* [15]). Determination of the optical rotation indicated, as with eburnamine above, that the corresponding dehydrocompound was of the (+)-series.

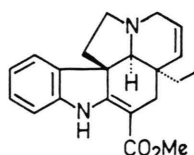
When fraction **F4** was examined by TLC in solvent system (e) two alkaloids were obtained (Rs 9 and Rs 10, $R_f = 0.27$, $R_f = 0.1$ resp.). Rs 9 ($M^+ 498 m/z$, DCI-MS 499 m/z) was identical in every respect with the lactam of strictosidine. The glucoalkaloid strictosidine, which is relatively unstable and undergoes easy lactamization under alkaline conditions, had been identified for the first time from *R. stricta* plants and its biogenetic significance for monoterpenoid indole alkaloids was proposed by Smith [18]. This alkaloid could not, however, be detected in the cultured *Rhazya* cells. The lactam isolated from the cell culture here is most likely *not* an artefact. That was demonstrated by short time extraction of fresh cells with methanol at mild conditions (5 °C) followed by HPLC analysis. Our result is therefore in accord with the finding of De Silva *et al.* [19] that the lactam is a natural constituent of *R. stricta* plants and, in addition, Kurz *et al.* [20] have also recently isolated strictosidine lactam from different cell strains of *Catharanthus roseus* cultures. The second alkaloid of fraction **F4** could be identified as vallesiachotamine and/or its isomer isovallesiachotamine. The former alkaloid is also a constituent of the differentiated plant.

The 11 alkaloids described comprise the major indole bases of *Rhazya* cell suspension grown in 4X-medium (Scheme). Except for (+)-1,2-dehydroaspidospermidine which was synthesized under the applied culturing conditions at about 15 mg/l medium, the concentration of most of the other alkaloids was about 5–10 times lower. Optimum cell production was reached after a growth period of 15 days (Fig. 1). After that a drastic decrease of the cell dry weight was observed. This effect obviously was correlated with the sucrose content of the medium, because at the end of the cell growth period the carbon source was completely consumed.

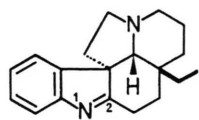
When the time course of alkaloid production was followed for the *Strychnos* type compound akuammicine and the *Aspidosperma* type corresponds tabersonine and vincadifformine similar results were obtained. Maximum alkaloid formation was found



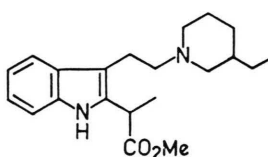
(+)–Vincadifformine



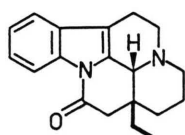
(–)-Tabersonine



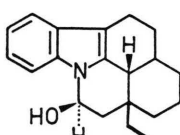
(+)–1,2-Dehydroaspidospermidine



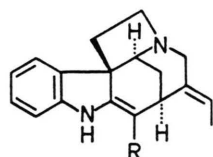
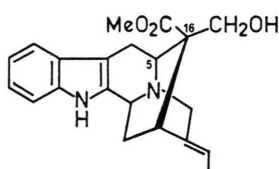
(–)-Tetrahydrosecodine



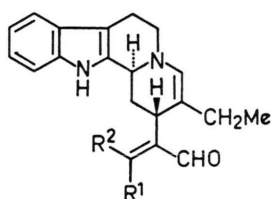
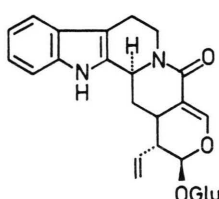
(+)–Eburnamonine



(+)–Eburnamine

(–)-Akuammicine
(R = CO₂Me)(–)-Norfluorocurarine
(R = CHO)

Akuamidine

Vallesiachotamine or Isovallesiachotamine
(R¹ = CH₃, R² = H) (R¹ = H, R² = CH₃)

Strictosidine lactam

Scheme. Major indole alkaloids produced in cell suspension cultures of *Rhazya stricta* Decaisne.

between the 10th and 16th days and then declined due to the lysis of cells. The *Strychnos* alkaloid was produced first, but its content decreased at about the 10th day whereas the synthesis of the *Aspidosperma* alkaloids vincadifformine and tabersonine was still in the logarithmic phase reaching an optimum between the 14th and 16th days. These observations are of particular interest in comparative studies to obtain

the optimum yields of individual alkaloids which exhibit significant turnover rates during the growth cycles of the cells. In this context it should be noted that akuammicine is the only alkaloid of the *Rhazya* cell culture which has not so far been found in the differentiated plants. Its 10-hydroxy derivative sewarine has been isolated several times from the above plant-source and we suggest that akuammicine

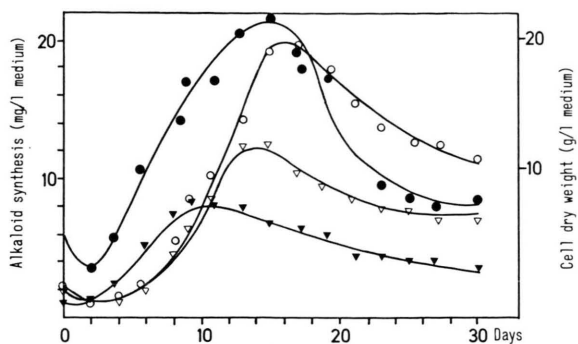


Fig. 1. Time course of the change of cell dry weight and alkaloid formation in cell suspension cultures of *R. stricta*. Cell dry weight (●—●). Tabersonine (○—○), Vincadifformine (△—△), Akuammicine (▲—▲).

is the immediate precursor of sewarine and might therefore be isolatable from *Rhazya* seedlings which have not as yet been investigated for their alkaloid content. All the other alkaloids found in the cultured cells are typical *Rhazya* alkaloids. They belong to the

five skeletal groups *Corynanthe*, *Strychnos*, *Eburnan*, *Aspidosperma*, and *Secodine*. Three other groups, which are well known from *Rhazya*, rha-zinilams, picralines and dimeric alkaloids were not detected as major components in our cell system. A HPLC-analysis of the crude alkaloid mixture (Fig. 2) demonstrated that more than 15 additional alkaloids were present as minor components. The *Rhazya* culture therefore provides a productive source of indole alkaloids comparable with cell suspensions of *C. roseus* and *R. serpentina*. A further investigation concerning these trace compounds is necessary to decide whether all the alkaloid groups found in the intact plant are also synthesized by the cultivated cells. Application of the HPLC-system of Fig. 2 in combination with automatic peak collection should largely simplify such experiments. Preliminary work with different nutrient media showed that at least a 2–3 times enhancement of alkaloid content was possible using an alkaloid production medium (Zenk *et al.* [21]). After an optimization of the culture conditions especially by regulating the sucrose concentra-

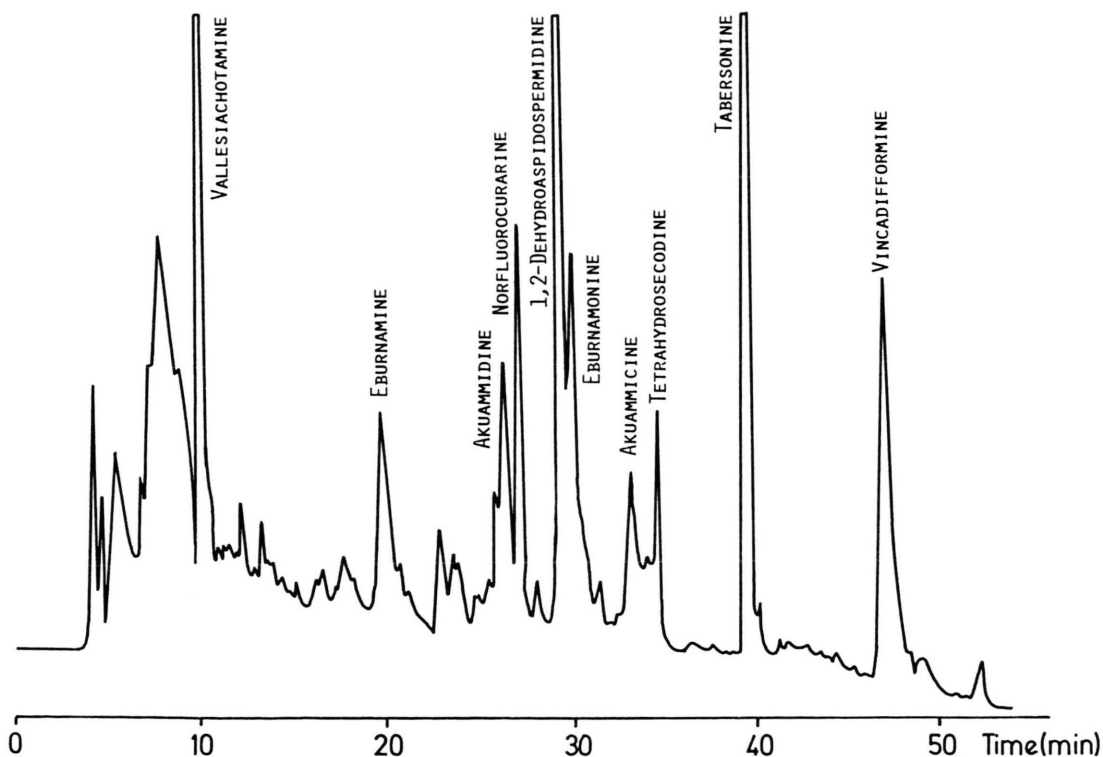


Fig. 2. HPLC-separation of a crude alkaloid mixture isolated from *R. stricta* cell suspensions.

tion, fresh cell material of only some kg should be sufficient to perform the isolation and identification of the remaining trace alkaloids.

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