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The effect of arsenicals on alkaloid production by cell suspension cultures of *Catharanthus roseus*

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The effect of arsenic compounds on indole alkaloid production by cell suspension cultures of *Catharanthus roseus* was investigated. The analysis of indole alkaloids was achieved by using thermospray liquid chromatography–mass spectrometry (LC MS) which facilitated the rapid screening of alkaloid composition in cultures treated with different arsenicals at different times in their growth cycle.

Treatment with dimethylarsinate (DMA), a non-selective herbicide, has a drastic inhibitory effect on alkaloid production although it is the least toxic arsenical to growth. Tryptamine, an early precursor in the biosynthesis of indole alkaloids, accumulates in cells treated with DMA, indicating that the initial step of condensation of tryptamine with secologanin is inhibited. Treatment with DMA during the early stationary phase of culture growth enhances the accumulation of some alkaloids, although some, such as catharanthine, are suppressed.

The arsenicals arsenate and methylarsonate (MMA) have an inhibitory effect on alkaloid production when applied during the early growth stages. In contrast to MMA and DMA, arsenate has a stimulatory effect on catharanthine production when introduced to the culture during its early stationary phase.

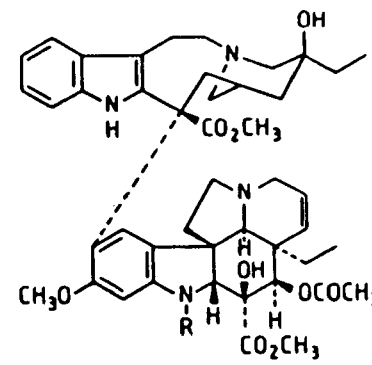
Thus the changes in the pattern of alkaloid accumulation on addition of arsenicals are dependent on the arsenic species and its concentration, as well as the time of application. This variable response indicates that each arsenical has a distinct mode of action on the secondary metabolic pathways of *C. roseus*.

Keywords: Arsenic compounds, alkaloid production, cells, thermospray LC MS

INTRODUCTION

Catharanthus roseus (L.) G. Don, the Madagascar periwinkle, is a well-known medicinal plant belonging to the plant family Apocynaceae. The plant is reported to produce more than 80 monoterpene alkaloids, many of which have important pharmacological activity.[1,2] The most notable of these therapeutic secondary metabolites are the bisindole alkaloids, vinblastine (**1**) and vincristine (**2**), which are used in cancer chemotherapy.[3]

For the last decade, much attention has been focused on the production of secondary metabolites of *C. roseus* by cell culture methods. It is widely recognized that cultured plant cells represent a potential source of valuable phytochemicals and the manipulation of the culture conditions to increase yields should be possible by using cell culture methodology.[4] Forty-three monomeric indole alkaloids have so far been isolated from *C. roseus* cell cultures and some cell lines were found to produce alkaloids at higher



R = CH_3 **Vinblastine (1)**

R = CHO **Vincristine (2)**

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levels than are found in intact plants.[4] The dimeric alkaloids **1** and **2** have not yet been isolated from cell suspension cultures: they have been detected only in callus and organ cultures of *C. roseus*.[5]

The first step in the indole alkaloid biosynthesis is known to be the enzymic, stereospecific condensation of tryptamine (**3**) from tryptophan, with the monoterpene unit secologanin (**4**) from mevalonate. This condensation gives rise to the glucoalkaloid, strictosidine (**5**), as depicted in Fig. 1. The biological conversion of strictosidine into the three major classes of indole alkaloids has been observed. The first step in this sequence is the hydrolysis of strictosidine to remove the sugar moiety. Then, in several steps which are not entirely clear, coryanthe- and strychnos-type alkaloids are first formed, and the latter are then converted to the aspidosperma alkaloids, and finally to the iboga alkaloids.[6]

Changes in the pattern of alkaloid accumulation in cell suspension cultures compared with that in the intact plant suggest that some metabolic pathways are blocked in the culture mode.[7] Substances that facilitate the 'switching' back on of these pathways are known as 'elicitors'.[7] The aim of using elicitors on *C. roseus* cell-suspension cultures is not only to induce the production of

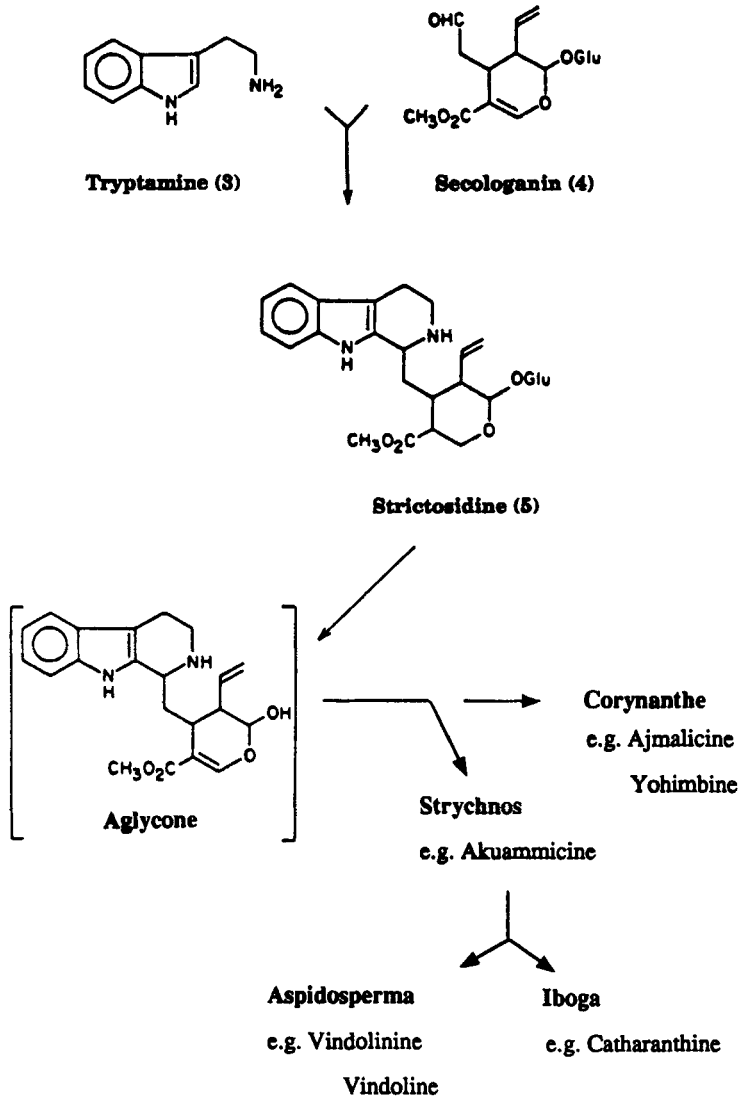
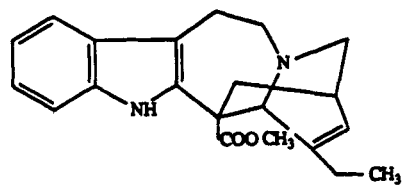
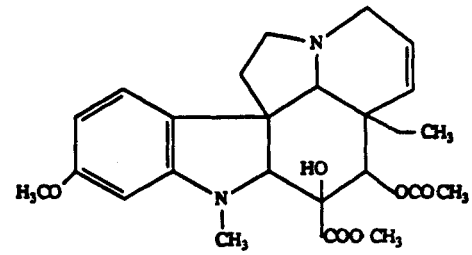


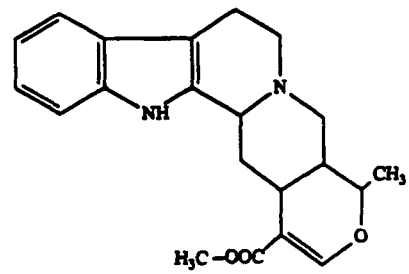
Figure 1 The bisynthetic pathway of indole alkaloids.



Catharanthine (6)



Vindoline (7)



Ajmalicine (8)

valuable bisindole alkaloids, which have not yet been detected in cell cultures, but also to increase the production of monomeric alkaloids, including catharanthine (**6**) and vindoline (**7**) in a shorter period of time.

Several cell lines responded to the addition of a fungal elicitor by the accumulation of tryptamine within 24 h, and catharanthine, ajmalicine (**8**) and other monomeric alkaloids within 72 h.[8] Vindoline or dimeric alkaloids were not detected. The type of fungal homogenate, and the concentration and age of the cell culture at the time of application of the elicitor, all influence the response from each cell line.[8] Similar results were achieved by treatment with abscisic acid, a natural plant-growth regulatory substance.[9]

Increased accumulation of indole alkaloids in *C. roseus* cell suspension cultures was found on treatment with the abiotic elicitor, vanadyl sulphate.[10,11] Both ajmalicine and catharanthine levels showed a 50% increase over control levels on treatment with 25 ppm of vanadyl sulphate, but concentrations over 100 ppm resulted in a drop in alkaloid levels. Cell response to vanadyl sulphate was also found to vary with the cell age at the time of application.

Very little is known about the biochemical behaviour of arsenicals in terrestrial plants.[12] Although there are reports of the biotransformation of arsenicals in some plant systems, none documents the effect of arsenicals on the secondary metabolism in terrestrial plants.[12] Consequently the influence of arsenicals on a complex metabolic pathway such as alkaloid biosynthesis, which involves a series of enzymes, cannot be predicted. The aim of the present study on *C. roseus* cell suspension cultures is to investigate the effect of arsenicals on the production of indole alkaloids, some of which have important pharmaceutical activity.

Arsenic compounds inhibit several enzymes in biological systems[13,14] and the inhibition by arsenicals is species-dependent. Studies on a variety of isolated enzyme systems suggest that the trivalent arsenicals inhibit an enzyme by interacting with the sulfhydryl groups of the enzyme.[14,15] Other mechanisms of inhibition have also been proposed which may not involve direct reaction between arsenic and the enzyme. Arsenicals could react with the substrate or an intermediate of the reaction, or structurally similar organoarsenicals may competitively inhibit binding of the enzyme to the substrate.[13] Pentavalent arsenate may directly inhibit enzymes by substituting for phosphate in enzyme-catalysed reactions such as phosphorylation.[16] Alternatively, arsenate may be reduced to the trivalent form in the biological system and disrupt enzyme activity.[13]

The response of soybean seedlings to arsenite exposure was found to be identical to heat shock: under both conditions, a new set of proteins known as heat-shock proteins are produced.[17] A similar response, but to a lesser degree, was observed in soybean seedlings on treatment with cadmium.[17] Although the precise role of heat-shock proteins is yet to be established, this action of an arsenical on protein synthesis suggests the possibility that arsenicals may exert an effect on enzymes involved in secondary metabolism in plants.

The present study makes use of thermospray liquid chromatography–mass spectrometry (LC MS) for the analysis of indole alkaloids from cell suspension cultures of *Catharanthus roseus* grown in the presence of arsenicals. The analysis of complex mixtures of non-volatile, structurally similar, polar compounds can be achieved by using this technique with minimal sample manipulation: Auriola *et al.* first reported its application to the analysis of *Catharanthus* alkaloids in 1989.[18] In the present study, the thermospray LC MS technique was used to facilitate the rapid screening of alkaloid compositions in cultures treated with different arsenicals at different times in their growth cycle. A preliminary study has been reported on the effect of arsenicals on the growth of cell suspension cultures of *C. roseus* and on the uptake and biotransformation of arsenicals by these plant cultures.[19]

MATERIALS AND METHODS

Culture methods

Cell suspensions of *C. roseus* used in this study were subcultures of the cell line AC-3 derived from a leaf explant of a mature plant, and were maintained in 1-B5 medium[20] at 26 °C in gyratory shakers at 150 rpm. On the tenth day of growth, cells were transferred to alkaloid production medium (APM)[21] containing known concentrations of arsenic compounds, arsenate, arsenite, methylarsonate and dimethylarsinate as sodium salts. The control cultures did not contain any added arsenic. Each flask was inoculated with 15 cm^3 of the inoculum per 100 cm^3 of medium and was incubated at 26 °C in a gyratory shaker in the absence of light, for an appropriate time before harvesting. Each experiment was carried out in quadruplicate. The cells were harvested by filtration through Miracloth. The fresh weight of cells was obtained before freezing. Dry cell weight was obtained from freeze-dried samples. Fresh cell samples, kept frozen at −20 °C, were used for alkaloid extraction. Speciation of arsenic in the culture media was monitored using hydride generation atomic absorption spectrometry.[19]

The effect of the time of application of the arsenic compounds on alkaloid production was investigated. The arsenic concentrations tested were 3 ppm (0.04 mM) of arsenate, 6 ppm (0.08 mM) of methylarsonate and 20 ppm (0.27 mM) of dimethylarsinate, all below the minimum inhibitory concentration (MIC*) of each arsenic compound.[19] Arsenic solutions were filter-sterilized by using 0.22 μm filter units, and added to the medium at the beginning of growth and after 11 and 22 days of incubation. All cultures were harvested after 29 days of incubation and the alkaloid composition was analysed.

Extraction and analysis of alkaloids

The cells were suspended in methanol and homogenized using an UltraTurrax homogenizer. The resulting cell suspensions were sonicated for 1 h before filtering off the residue. The extracts were subjected to the standard extraction procedure previously published by Kutney *et al.*[22]

The HPLC system consisted of Waters M45 and M510 pumps coupled to a Waters automated gradient controller. The sample was introduced via a Waters U6K injector. A Waters M418 variable-wavelength UV detector and associated Waters QA-1 data system were used for detection. When necessary, fractions were collected with a Gilson Microfractionator.

Two reversed-phase columns were used in alkaloid separation. When using a Waters μ-Bondpak C_{18} [3.9 mm (i.d.) × 30 cm] steel column, isocratic elution with water–acetonitrile (60:40) containing 0.1% (v/v) triethylamine as modifier at a flow rate 1 $cm^3\ min^{-1}$ typically gave a good separation. Use of a Phenomenex Bondclone [3.9 mm (i.d.) × 30 cm] steel column required modification to the mobile phase. Water–acetonitrile (54:46) containing 0.15% (v/v) triethylamine was used at a flow rate of 1 $cm^3\ min^{-1}$. Detection was typically at 280 nm.

The chromatographic conditions used for thermospray liquid chromatography–mass spectrometry (LC MS) were as described for HPLC. A Waters M510 pump was used for solvent delivery at 0.9 $cm^3\ min^{-1}$ and the samples were injected

with the aid of a Rheodyne Model 7125 injector (loop volume 20 μl). An ammonium acetate (1 M) solution was added to the solvent stream after the column by using a Waters 6000A pump at a flow rate of 0.1 $cm^3 min^{-1}$. A Vestec Kratos thermospray system was interfaced to a Kratos MS 80 RFA double-focusing mass spectrometer. The thermospray probe temperature was 120 °C and the ion source temperature was 220 °C. Dilute solutions of poly(ethylene glycol) polymers, which afford MNH_4^+ ions, were used for calibration.

RESULTS AND DISCUSSION

The growth of *C. roseus* cell cultures in APM containing various concentrations of arsenic compounds was monitored. The variation in dry cell weight of cultures with the initial concentration of arsenicals [arsenate, arsenite, methylarsonate (MMA) and dimethylarsinate (DMA)] in the media is illustrated in Fig. 2. The minimum inhibitory concentration (MIC), the lowest concentration tested at which growth in inhibited, is a useful indicator of the tolerance of microorganisms to various inhibitors.[23] In principle, MIC values of arsenicals with respect to *C. roseus* cell cultures could be estimated from plots of the type shown in Fig. 2. However, because the onset of inhibition of the growth of *C. roseus* cell cultures is not distinct, we prefer to use the descriptor MIC*, defined as the concentration of the arsenic species at which the biomass of the culture is 50% less than that of the control culture into which no arsenical is added.

The toxicity of the four arsenicals in APM and 1-B5 media can be compared by using the estimated MIC* values (expressed as arsenic), which are listed in Table 1. Concentrations above 3 ppm of arsenate inhibits growth in APM, as seen from the dry cell weight after 21 days of growth, whereas in 1-B5 medium the MIC* of arsenate is estimated to be 5 ppm at the stationary phase. Similarly, a greater toxicity effect of other arsenicals is evident in *C. roseus* cell suspension cultures grown in APM compared with the 1-B5 medium.

The higher toxicity response in APM in comparison with 1-B5 medium may be related to the differences in the nutrient composition or the growth characteristics of the cultures in the two media, or a combination of both. For example, the phosphate concentration in APM is 0.5 mM,

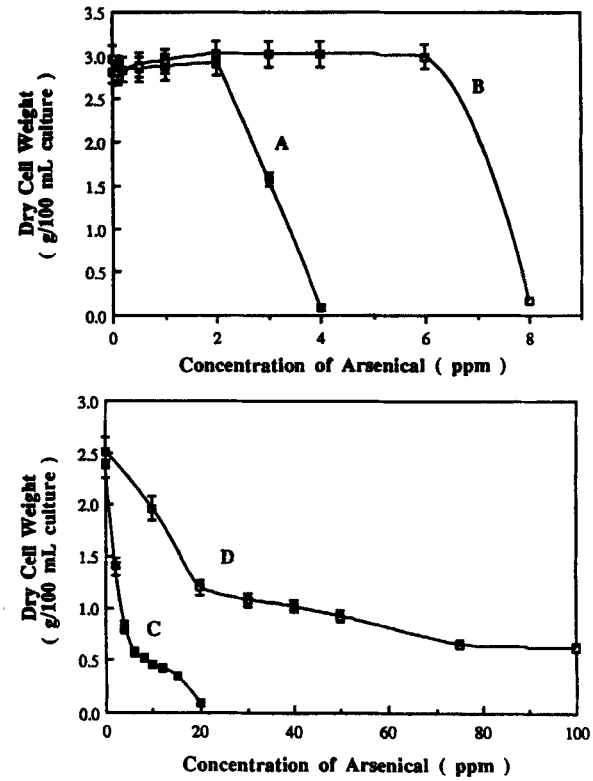


Figure 2 The variation of dry cell weight of *C. roseus* cultures with the concentration of the arsenical in the APM medium. The dry cell weights were obtained after 23 days of growth in the APM medium containing different concentrations of the arsenicals. A, arsenate; B, arsenite; C, MMA; D, DMA.

whereas it is 1.1 mM in 1-B5 medium. The lower phosphate concentration in APM may lead to an increased uptake of arsenate because phosphate

Table 1 The minimum inhibitory concentration (MIC*) values of arsenicals for *C. roseus* cell suspension cultures in the standard 1-B5 and APM media[a]

	Minimum inhibitory concentration, MIC* [ppm (mM)]	
Arsenic compound	1-B5 medium	Alkaloid production medium (APM)
Arsenate	5 (0.07)	3 (0.04)
Arsenite	10 (0.13)	7 (0.09)
Methylarsonate	8 (0.11)	3 (0.04)
Dimethylarsinate	50 (0.67)	20 (0.27)

[a] The MIC* values (expressed as arsenic) of arsenicals in APM are estimated from data presented in Fig. 2. The MIC* values in 1-B5 media are presented in a previous publication by Cullen *et al.*[19]

is a competitive inhibitor of arsenate uptake. It should be noted that arsenate is rapidly reduced to arsenite by *C. roseus* cultures.[19] Presumably, the process involves arsenate uptake, reduction and arsenite discharge. Moreover, the longer lag phase in APM may result in lower cell density in the culture during the first stages of growth. The resultant higher arsenical concentration per cell may result in the higher toxicity.

Application of thermospray liquid chromatography–mass spectrometry for the analysis of indole alkaloids

Thermospray LC MS facilitated the rapid screening of cell extracts for the presence of a variety of indole alkaloids. Both retention time and mass spectral information aid in the identification of the alkaloid. As thermospray LC MS is a soft ionization technique, the spectra are primarily composed of molecular adduct ions with minimal fragmentation.

In the thermospray LC MS analysis of a standard indole alkaloid, catharanthine, the total ion chromatogram shows an impurity (peak A) eluting after 5 min and catharanthine (peak B) eluting around 15 min. The mass spectrum of peak B shows a protonated molecular ion peak, MH^+ (*m/z* 337), at the base peak. No significant fragment ions or ammonium adduct ions are present.

Similar LC MS spectra are observed for several monomeric indole alkaloids. Ajmalicine elutes around 15 min and the mass spectrum consists only of the peak at *m/z* 353 assigned to the MH^+ ion. Similarly, the LC MS spectra of vindolinine, epivindolinine and vindoline all contain a single peak (*m/z* 337, 337 and 457, respectively) corresponding to the MH^+ ion of each alkaloid.

In the LC MS analysis of a dimeric alkaloid, anhydrovinblastine, the thermospray mass spectrum shows some fragmentation where peaks corresponding to the two component monomeric alkaloids (*m/z* 337, MH^+ of catharanthine; and *m/z* 457, MH^+ of vindoline) are observed in addition to the prominent molecular ion at *m/z* 809. Previously, Auriola and coworkers[18] reported more extensive fragmentation for another dimeric alkaloid, vinblastine, on thermospray LC MS analysis. This anomaly can be attributed to differences in thermospray mass spectral conditions. A key difference is the post-column addition of the electrolyte, ammonium acetate, in the present study, whereas addition of ammonium acetate prior to the LC column separation was employed in the previous study. Moreover, the changes in thermospray interface conditions such as vaporizer and ion-source temperature and the probe position may give rise to different fragmentation patterns.

The minimal fragmentation of monomeric indole alkaloids during thermospray LC MS analysis limits the information available for identification of the alkaloids, and it is not possible to differentiate between two isomers with the same retention time as only molecular-weight information is available. But this feature is an advantage in evaluating the purity of LC peaks. Because monomeric indole alkaloids produce only MH^+ ions under these conditions, a scan giving rise to a spectrum containing several significant peaks is indicative of the presence of several compounds of the appropriate molecular weights. Lack of fragmentation also enhances the sensitivity of detection of alkaloids.

Quantitative information from thermospray LC MS is limited. The intensities of peaks observed in the thermospray mass spectrum do not necessarily reflect the proportions of different alkaloids present because the optimum thermospray conditions such as vaporizer and ion-source temperatures are not identical for all alkaloids. Quantitation is possible by establishing a calibration curve for each alkaloid of interest relative to the intensity of an internal standard added to each sample.

LC MS analysis of *C. roseus* cells grown in APM

The total ion chromatogram (TIC) of the alkaloid extract from *C. roseus* cells grown in APM for 22 days and mass-spectral scans of two peaks in the TIC are depicted in Fig. 3. The assignments are summarized in Table 2.

The major component in peak B, Fig. 3, eluting at 4.0 min has *m/z* 339. It can be assigned to perivine, an aspidosperma alkaloid, on the basis of its molecular weight. Peak D contains several components of *m/z* 341, 371, 353 and 387 which could be $(MH)^+$ peaks of several unassigned alkaloids. Peak F eluting at 8.8 min contains a compound of *m/z* 355. This can be assigned to yohimbine†, a corynanthe alkaloid, on the basis of its molecular weight. Retention times data are not available. This tentative assignment is denoted by the dagger (†). The other possibilities for this

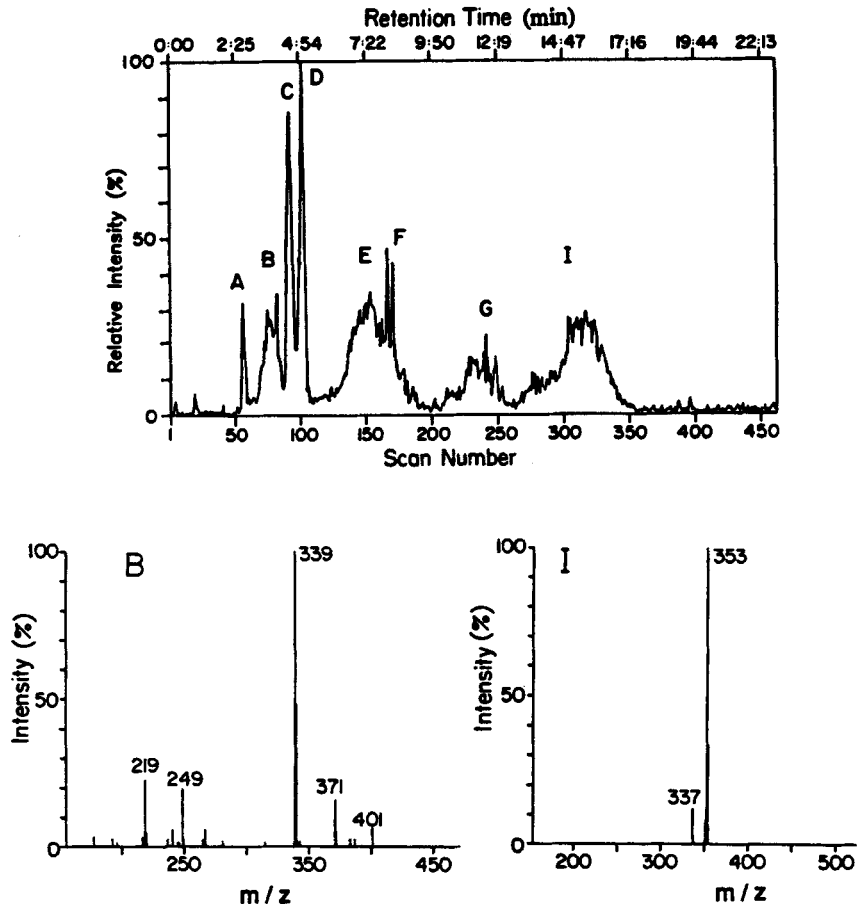


Figure 3 Thermospray LC MS analysis of alkaloid extracts from *C. roseus* cells grown in APM for 22 days. (a) Total ion chromatogram; (b) mass spectrum of peak B; (c) mass spectron of peak I.

compound are sitsirikine and isositsirikine, both coryanthe alkaloids. The major component in peak G, eluting at 12.0 min, has *m/z* 323. This can be assigned to akuammicine† on the basis of its molecular weight. The other components of *m/z* 353 and 385 are not assigned.

The mass spectrum of the peak I eluting between 14 and 15 min shows the co-elution of two components of *m/z* 353 and 337 (Fig. 3). The former is assigned to ajmalicine and the latter to catharanthine, based on both retention time data and molecular weight information. Thus LC MS provides strong evidence for the presence of a minor concentration of catharanthine in the 22-day-old *C. roseus* cell suspension.

C. roseus cells grown for 29 days show a wider spectrum of alkaloids than cells grown for 22 days. After 29 days, cells still copntain perivine† (aspidosperma) and ajmalicine and yhohimbine† (coryanthe). Akuammicine†, the only strychnos alkaloid detected, is also present after 29 days. Vindolinine and epivindolinine, both aspidosperma alkaloids, are present in 29-day-old cells although they are not detected after 22 days of growth. Catharanthine (iboga) is found at a much higher concentration. The production of catharanthine appears to increase during the time between 22 and 29 days, whereas the content of ajmalicine diminishes. This provides evidence for the sequential formation of alkaloids; coryanthe and strychnos alkaloids are produced earlier in the growth cycle and are followed by aspidosperma alkaloids and finally the iboga alkaloids.

Previous work by Auriola *et al.*[18] first demonstrated the feasibility of using thermospray LC MS for screening alkaloids in cell suspensions of *C. roseus*. The present study documents a more extensive investigation into the changes in the alkaloid production pattern in *C. roseus* by means of thermospray LC MS.

Table 2 LC MS analysis of alkaloid extracts from *C. roseus* cells grown in APM for 22 days

Peak	Retention time (min)	*m/z*	Peak assignment
A	2.6	339, 371	?
B	4.0	339	Perivine†[a]
C	4.3	192	?
D	4.6	341, 371 353, 387	(MH)† peaks of several alkaloids
E	7.2	371	?
F	8.8	355	Yohimbine†[b]
G	12.0	323 (major) 353, 385	Akuammicine†[c]
I	14.0–15.0	353 (major) 337 (minor)	Ajmalicine[b] Catharanthine[d]

† The assignment is based only on the molecular weight information from thermospray LC MS results. [a] Aspidosperma class. [b] Corynanthe class. [c] Strychnos class. [d] Iboga class.

Effect of arsenic compounds on alkaloid production

Treatment of cultures of *C. roseus* with dimethylarsinate (DMA), a non-selective herbicide, during the early growth stages results in drastic inhibition of alkaloid production, but the cell culture growth is not affected as cell yields are higher than for cells treated with other arsenic compounds. The culture appearance also is not affected and is comparable to that of the control.

The common feature found in all cultures treated with DMA on day 0 or day 11 of the growth cycle is the accumulation of tryptamine (**3**), an early precursor in the biosynthesis of indole alkaloids. It is detected as an early-eluting peak on HPLC separation and accounts for about 40% of the total peak area when analysed soon after extraction. However, the identification of tryptamine can be hampered by its slow conversion into *N*-acetyltryptamine. This product of the base-catalysed *N*-acetylation of tryptamine, in the presence of ethyl acetate, was detected and identified by using thermospray LC MS as well as preparative thin-layer chromatography and HPLC separation followed by NMR and MS.

Tryptamine accumulation has been observed in *C. roseus* cell suspension cultures treated with other elicitors. These elicitors include fungal homogenates[8] and abscisic acid.[9] Treatment with vanadyl sulphate on day 5 of growth resulted in tryptamine accumulation, but not when treatment is on day 10.[10,11]

Elevated tryptamine levels in cells can result from increased tryptamine production associated with the stimulation of the enzyme tryptophan decarboxylase (TDC). However, the net suppression of alkaloid accumulation in these cultures suggests that the next step, condensation of tryptamine with secologanin (**4**), is most likely to be inhibited. The strictosidine (**5**) produced in this step is the precursor to all three groups of alkaloids produced by *C. roseus* plant systems (Fig. 1).

This condensation step can be blocked as a result of two processes: (a) DMA inhibits the activity of the enzyme strictosidine synthase, either by acting as an inhibitor of the enzyme or by preventing its synthesis; or (b) DMA has an inhibitory effect on at least one of the steps involved in the production of secologanin. Further studies are in progress to investigate these possibilities. The isolation of secologanin, a non-alkaloidal glucoside, will require a different extraction/isolation procedure.[24]

When *C. roseus* cells are treated with DMA on day 22, tryptamine accumulation is not detected. This observation fits in with the notion that DMA blocks alkaloid production early in the pathway; by day 22 of growth, tryptamine condensation has already taken palce, and thus the addition of DMA does not have any profound overall effect on alkaloid accumulation. Several coryanthe, strychnos and aspidosperma alkaloids are detected which are also found in the control. But a notable absence is that of catharanthine. Catharanthine is produced to a large extent only after day 22. Thus, DMA added on day 22 can effectively stop catharanthine production. This indicates that DMA interferes with the pathway leading to catharanthine. A metabolite of *m/z* 355 [tentatively assigned to yohimbine† (corynanthe)] is found to accumulate in these cells; this accumulation may be the result of a blockage of a step leading to catharanthine, an iboga alkaloid.

Another arsenical that has been investigated, methylarsonate (MMA), is a widely used selective herbicide,[12] and low concentrations of this methylarsenical have an inhibitory effect on growth of cell suspension cultures of *C. roseus* (Fig. 2). The cell alkaloid production is also suppressed when MMA is added to the medium at the beginning of growth, either on day 0 or day 11 of growth. Cells harvested after 22 days of growth contain a few alkaloidal metabolites which are assigned to ajmalicine and yohimbine†, both corynanthe alkaloids. After 29 days, ajmalicine is

completely absent but several other alkaloidal metabolites are detected, including antirhine† (*m/z* 297), which were present in the control culture at low concentrations. The failure to detect ajmalicine in cells after 29 days of growth could indicate that it is present at very low concentrations or it is transformed or catabolized inside the cells over time. Cell lysis can also release alkaloids into the medium, resulting in a lower intracellular concentration of alkaloids.

Cells treated with MMA on day 22, and analysed after 29 days of growth, contain a variety of alkaloids including catharanthine (iboga class), all at low concentrations. The metabolite of *m/z* 297 is again present in these cells. Another alkaloid that consistently appeared in MMA-treated cells is horhammericine† (*m/z* 369). It is not present in the control culture but has been previously reported in *C. roseus* cultures.[4]

The overall low intracellular concentration of alkaloids in MMA-treated cells can be attributed to cell lysis releasing alkaloids into the residual medium. This is supported by the low cell yield (both fresh and dry cell weights) of cultures treated with methylarsonate on day 11 as well as on day 0. Cell lysis could be indicated by the turbid appearance of the spent medium.

Of the arsenicals studied, arsenate is the most toxic to the cell suspension cultures of *C. roseus*.[19] Treatment with arsenate at the beginning of growth has a suppressing effect on alkaloid production. Overall, alkaloid content decreases after 22 or 29 days of growth compared with the control, and only a few alkaloids observed in the control are detected. They are perivine†, akummicine† (strychnos) and ajmalicine (corynanthe). The production of catharanthine is completely suppressed in these arsenate-treated cells.

Application of arsenate on day 11 of growth has an even greater inhibitory effect on alkaloid production compared with day 0 application. Many of the alkaloids observed in the control are absent in this culture. Only lochnerine† (*m/z* 325) and antirhine† (*m/z* 297) can be assigned, of those that are present in the control culture. By day 11, the cell culture is in its growth phase.

Arsenate treatment on day 22 does not have a suppression effect on alkaloid production. Alkaloid production has already commenced by day 22 and arsenate may even have a stimulatory effect on the production of specific alkaloids. This is most obvious in the increased peak area of the later-eluting peak J, where ajmalicine and catharanthine co-elute.

In concluison, we have demonstrated that the addition of arsenicals to the alkaloid production medium, APM, changes the pattern of alkaloid accumulation in the *C. roseus* cell line under investigation. The effect is dependent on the arsenic species, and its concentration, as well as on the stage of the growth phase at which the cells were treated. This variable response indicates that these arsenicals have a distinct mode of action on the secondary metabolic pathway of *C. roseus* cell suspension cultures and that it is not simply a stress response. We were unable to establish the elicitation of dimeric alkaloids in this cell line of *C. roseus*, on treatment with the three arsenicals arsenate, MMA and DMA at different times in the growth cycle.

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