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TERPENOID INDOLE ALKALOID BIOTRANSFORMATION CAPACITY OF SUSPENSION CULTURES OF *TABERNAEMONTANA DIVARICATA*

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Key Word Index—*Tabernaemontana divaricata*; Apocynaceae; suspension cultures; indole alkaloids; biotransformation; biosynthesis.

Abstract—Two cell lines of *Tabernaemontana divaricata* derived from the same suspension culture were compared with respect to their biotransformation capacity. One is a high indole alkaloid-producing culture which accumulated mainly *O*-acetylvallesamine. The other cell line biosynthesizes terpenoid indole alkaloids in much lower amounts. Both cell lines were cultured in medium containing either conopharyngine, coronaridine, vobasine or tabersonine. Chemical breakdown was followed in fresh and used culture medium in the absence of cell culture. All the alkaloids investigated underwent chemical transformation. Most of the biotransformation products accumulated by the cultures have been reported to occur in intact plants. Since all the alkaloids added were transformed in the same way by both cultures, the two cell lines seem to have the same biotransformation potential.

INTRODUCTION

Suspension cultures are able to accumulate a variety of secondary metabolites, but often the compounds accumulated differ qualitatively and quantitatively from those found in the parent plants. Biosynthetic studies have revealed that part of the enzymes involved in the formation of compounds in whole plants cannot be detected in suspension cultures. An example is the studies concerning vindoline biosynthesis in *Catharanthus roseus* [1]. The enzymes involved in the last steps of the biosynthesis of vindoline could be detected in plants but not in suspension cultures.

Studies to investigate the factors that determine the pattern and amount of compound accumulated by suspension cultures have focused mainly on their biosynthetic route. This was done by measuring the activity of enzymes involved in the biosynthetic pathway. However, controversial results concerning the correlation between these enzyme activities and alkaloid accumulation have led to the diversification of the studies to subcellular compartmentation, substrate availability and turnover of secondary metabolites.

The terpenoid indole alkaloids accumulated by *Tabernaemontana divaricata* suspension cultures maintained at our laboratory differ both qualitatively and quantitatively from those reported to occur in whole plants of this species [2]. Suspension cultures accumulate mainly *O*-acetylvallesamine (80%), voaphylline (15%) and vallesamine (5%). *O*-Acetylvallesamine and vallesamine have not been reported to occur in *T. divaricata* plants but are found in other species of the genus; voaphylline has been isolated from leaves and flowers of *T. divaricata* [2]. In previous investigations, turnover and chemical breakdown were shown to influence the amount of *O*-acetylvallesamine accumulated by the suspension cultures [3]. The aim of the present study was to investigate whether the suspension cultures are able to metabolize other indole alkaloids which are not accumulated by the cultures. The detection of such activity might indicate that turnover is influencing not only quantitatively but also qualitatively the compounds accumulated. To examine the ability of *T. divaricata* suspension cultures to metabolize indole alkaloids, some of these compounds were added to the culture medium of two cell lines. Four indole alkaloids (coronaridine, conopharyngine, tabersonine and vobasine) of three different biosynthetic classes were chosen for experiments. None of these alkaloids has been detected in the suspension cultures maintained at our laboratory. However, coronaridine and vobasine have been isolated from plants [2] and suspension cultures [4] of *T. divaricata* before. Tabersonine has only been detected in plants [2], while conopharyngine, the 10,11-dimethoxy derivative of coronaridine, has only been reported to occur in other species of this genus [2].

RESULTS AND DISCUSSION

The addition of the alkaloids to the culture medium had no significant effect on dry wt (Fig. 1, Table 1) and fr. wt accumulation, or in the dissimilation curves of both

Table 1. Effect of alkaloid addition to the culture medium on dry wt (mg flask^{-1}) and indole alkaloid accumulation (nmol flask^{-1}) after 19 days of culture

Added alkaloid	Dry wt		Tryptamine		Voaphylline		*O*-Acetylvallesamine	
Cell line:	p*	np†	p*	np†	p*	np†	p*	np†
None	336	320	162	256	50	0	261	0
Coronaridine	351	332	120	197	52	0	342	0
Conopharyngine	337	322	112	141	59	0	361	0
Vobasine	322	321	159	250	84	0	330	0
Tabersonine	347	327	151	143	168	104	271	0

*Producing cell line.
†Non-producing cell line.

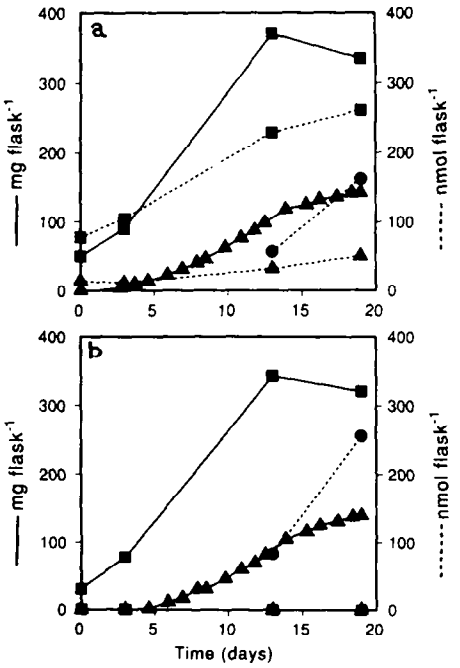


Fig. 1. Time course of dry wt (-■-), *O*-acetylvallesamine (- -■- -), voaphylline (- -▲- -) and tryptamine (- -●- -) accumulation and loss of weight by dissimilation (-▲-) of the control culture of the producing (a) and non-producing (b) cell line. No *O*-acetylvallesamine and voaphylline could be found in the culture of the non-producing cell line.

cell lines (results not shown). The maximum dry wt accumulation occurred on the 14th day after inoculation, while the fr. wt continued to increase.

Although the addition of alkaloids did not affect growth, alkaloid accumulation seemed to have been affected. *O*-Acetylvallesamine accumulation in the producing cell line seemed to be stimulated by the addition of conopharyngine, coronaridine and vobasine when compared to the flasks to which no alkaloid was added. Whether this enhancement was due to induced biosynthesis or reduced turnover is not known. Voaphylline accumulation was enhanced when tabersonine was added to both cell lines and the reason for this will be discussed later. Furthermore, tryptamine accumulation in both cell lines was inhibited by the addition of coronaridine, conopharyngine and the same seemed to occur when tabersonine was added.

Addition of coronaridine

Coronaridine was not stable in fresh culture medium and only a small part of the amount originally added could be detected by the end of the experiment (Fig. 2). Chemical transformation products were found to accumulate; trace amounts of these compounds accumulated in the presence of the cultures.

In the presence of both suspension cultures, the amount of coronaridine per flask also decreased with time. Coronaridine was found mainly in the medium. The

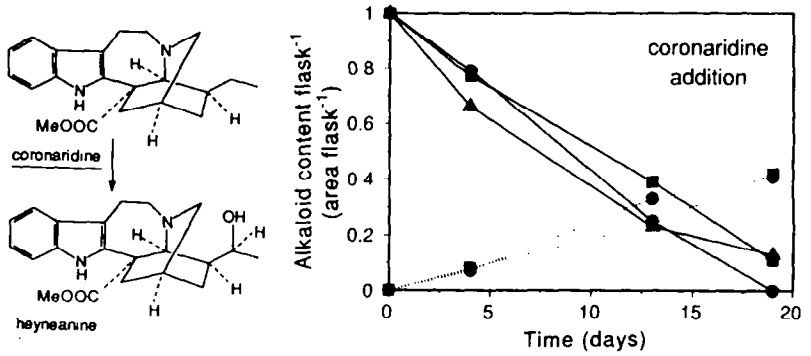
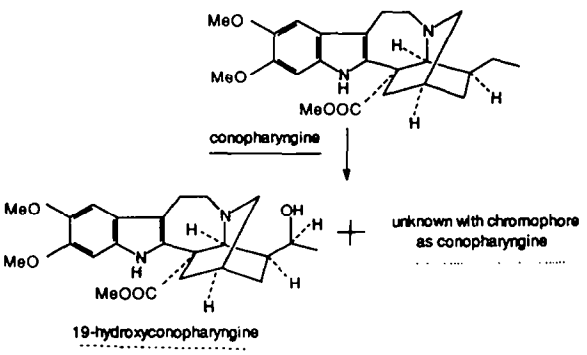


Fig. 2. Chemical stability (▲) of coronaridine in fresh culture medium and time-courses of coronaridine content (——) and its biotransformation product (- - -) in the presence of the producing (■) and non-producing (●) cell lines.

highest per cent of coronaridine in the biomass was 25% for the non-producing cell line in the stationary phase. While coronaridine was being transformed, the cultures of both cell lines accumulated one compound having an indole chromophore. GC-MS analysis of extracts showed a $[M]^+$ of m/z 354 which corresponds to that of coronaridine plus 16 mu and a mass spectrum similar to that reported for heyneanine [5], the 19-hydroxy derivative of coronaridine. Analysis of reference compounds by GC-MS and HPLC coinjection confirmed the identity of the biotransformation product to be (19*S*)-heyneanine. This alkaloid heyneanine has been reported to occur in root bark of *T. divaricata* [2] and suspension cultures [6] before. No (19*R*)-heyneanine was detected, although it has also been reported to occur in plants of this species.

Transformation of coronaridine to (19*S*)-heyneanine was dependent on the presence of cells and occurred throughout the period investigated. No significant accumulation of (19*S*)-heyneanine was observed after the medium was separated from the biomass. Coronaridine and (19*S*)-heyneanine were both unstable in the culture medium used (Table 2); the same chemical transformation products (comparison of retention times) of coronaridine found in fresh culture medium accumulated in used medium. Part of the decrease in the amount of coronaridine is, thus, due to chemical breakdown and the amount of (19*S*)-heyneanine accumulated does not correspond directly to the total amount of coronaridine transformed by the cells.



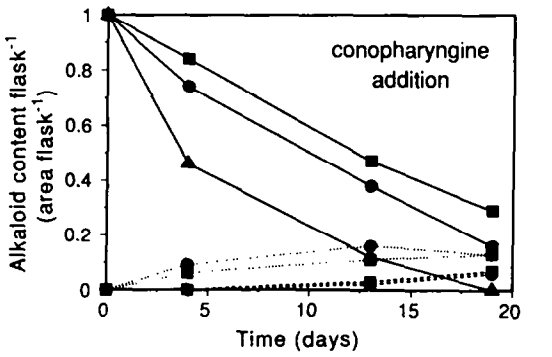


Fig. 3. Chemical stability (▲) of conopharyngine in fresh culture medium and time-courses of conopharyngine content (——) and its biotransformation products (– – –) in the presence of the producing (■) and non-producing (●) cell line.

Addition of conopharyngine

Conopharyngine was not stable in fresh culture medium. At the end of the experiment no conopharyngine could be detected in the flask without cell culture (Fig. 3). Three chemical transformation products accumulated which had a similar chromophore to conopharyngine, one of them having a similar retention time to that of conopharyngine. These compounds could not be detected in the presence of the cells.

In the cultures of both cell lines, conopharyngine was found mainly in the medium. The highest per cent of conopharyngine in the cells was 20% during the stationary phase. Conopharyngine was not stable in the presence

Table 2. Stability of added alkaloids and their biotransformation products in fresh and used culture medium separated from the cultures at two time intervals (days of culture)

	% of alkaloid still present at the time of harvest					
	Producing line		Non-producing line		Fresh medium	
Alkaloid Time interval	4–13	13–19	4–13	13–19	4–13	13–19
Coronaridine	79	96	75	98	35	56
19-Hydroxycoronaridine	87	102	80	114	—	—
Conopharyngine	67	79	nd*	87	25	0
19-Hydroxyconopharyngine	np†	93	nd	110	—	—
Unknown1	78	83	nd	88	—	—
Vobasine	87	np	89	np	90	92
Tabernaemontanine	np	91	np	92	—	—
Dregamine	np	95	np	85	—	—
Tabersonine	np	np	np	np	42	56
Voaphylline	54	93	76	86	—	—

*Not determined.
†Not present.

of the suspension cultures and the two cell lines seemed to transform conopharyngine in a similar way. Biotransformation products accumulated while the amount of conopharyngine decreased. Two compounds having a similar chromophore to conopharyngine were detected in the culture medium and biomass. Attempts to identify them by GC-MS were unsuccessful, probably due to their instability under the conditions used. HPLC coinjection with reference compounds allowed the identification of one of the compounds. In both HPLC systems used, (19*S*)-hydroxyconopharyngine coeluted with the minor biotransformation product accumulated. The other probable hydroxylation product of conopharyngine, 3-hydroxyconopharyngine was not detected; the structure of the main product remains unknown.

Although most of the conopharyngine and its transformation products were found in the culture medium, accumulation was dependent on the presence of cells, since no significant accumulation of these products was observed after the culture medium and the biomass were separated. Conopharyngine, (19*S*)-hydroxyconopharyngine and the unknown biotransformation product were not stable in the culture medium separated from the cells, though stability increased with increasing age of the culture from which the medium had been isolated (Table 2). Part of the decrease in the amount of conopharyngine per flask is, thus, due to chemical degradation and partly to biotransformation. Since the biotransformation products also were not stable, their accumulation curve does not include the total amount biotransformed.

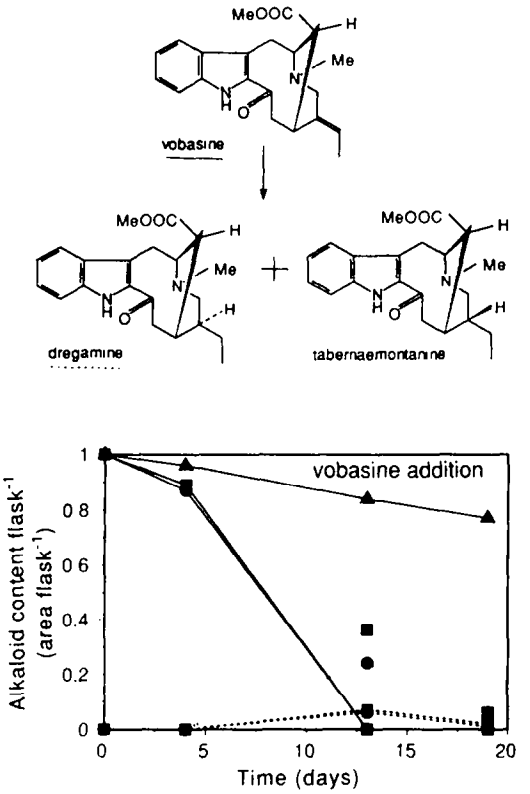


Fig. 4. Chemical stability (▲) of vobasine in fresh culture medium and time-courses of vobasine content (—) and its biotransformation products (.....) in the presence of the producing (■) and non-producing (●) cell line.

Addition of vobasine

Compared to the other alkaloids added, vobasine was quite stable in fresh culture medium. After 19 days, only *ca* 25% of the vobasine originally present in the medium had undergone chemical degradation, but no degradation products were detected by HPLC-UV (Fig. 4).

For both cell lines vobasine was found mainly in the culture medium. The transformation rate was much higher than that observed in the absence of cells and no vobasine could be detected at the 13th day of culture. The HPLC-UV chromatograms showed at least one compound having the same chromophore as vobasine. GC-MS analysis of the extracts revealed the presence of two products having a $[M]^+$ of *m/z* 354, which corresponds to that of vobasine plus 2 mu. These compounds had mass spectra similar to those reported for tabernaemontanine and dregamine. The identity of the transformation products was further confirmed by HPLC coinjection with reference compounds. The separation of vobasine and tabernaemontanine achieved in both HPLC systems and by the GC system used was poor, but determination of which compound was present could be carried out by GC-MS, monitoring at *m/z* 180 and 182. Although the two C-20 epimers were present, the amount of tabernaemontanine detected was always *ca* four–five times higher than the amount of dregamine. Both compounds were found mainly in the culture medium. Tabernaemontanine and dregamine have been found in several parts of plants of *T. divaricata* before [2] and tabernaemontanine has been reported to occur in suspension cultures [6]. To our knowledge there is no report on the occurrence of dregamine in suspension cultures of this species.

Transformation of vobasine to tabernaemontanine and dregamine was dependent on the presence of cells, since no further accumulation was found in the culture media separated from the cells. Like the other biotransformation products, these compounds were also not stable in used culture medium (Table 2) and so the amounts accumulated do not correspond to the total amount biotransformed. Unlike the other biotransformation products, the amounts of tabernaemontanine and dregamine in the cultures increased, but then decreased. Chemical instability cannot explain the decrease in the amounts of these compounds between the 13th and 19th day of culture; they must have been further metabolized by the cells to compounds which cannot be detected by the HPLC-UV system used.

Addition of tabersonine

In fresh culture medium, tabersonine was continuously degraded, but no transformation products were detected in the HPLC-UV system used. In the presence of

cultures, tabersonine was transformed at a much higher rate than that of the control. In the culture of both cell lines, no tabersonine was detected in the cells nor in the culture medium at any of the sampling times. In the culture of both cell lines, two products accumulated in the culture medium, one having a chromophore similar to that of tabersonine and the other with an indole chromophore; the latter also occurred in the cells. The first compound was observed only at the first sampling point, while the other was found throughout the culture period. The compound having an indole chromophore had the same retention time as voaphylline which is accumulated by the culture of the producing cell line. In the cells, a third transformation product was detected having an indole chromophore.

GC-MS analysis of the biomass and culture medium extracts of the non-producing cell line confirmed the identification of one of the products as voaphylline. Since voaphylline is also present in control cultures of the alkaloid-accumulating cell line, part of the amount of this compound accumulated in the flasks to which tabersonine was added is not a consequence of its addition. Voaphylline here is considered to be a biotransformation product of tabersonine, although, unlike the other biotransformation products identified, it is the result of several reaction steps. The biosynthetic route of voaphylline through tabersonine has been suggested before [2],

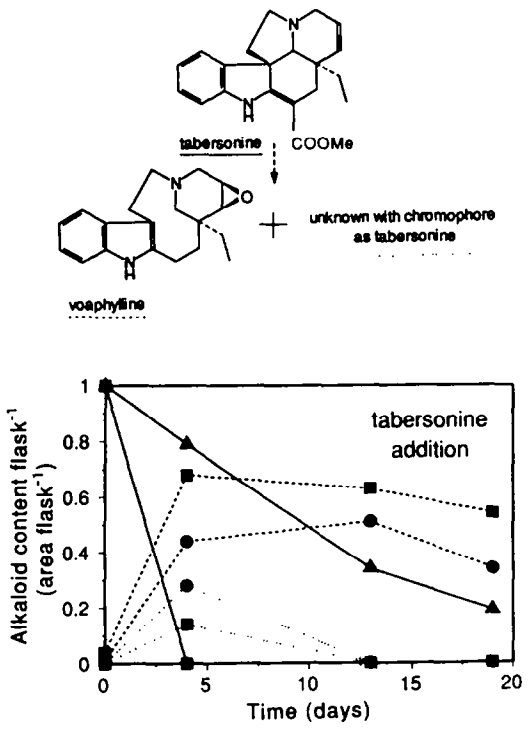


Fig. 5. Chemical stability (▲) of tabersonine in fresh culture medium and time-courses of tabersonine content (——) and its biotransformation products (·····) in the presence of the producing (■) and non-producing (●) cell line. The per cent of areas given has been corrected for the biotransformation products of tabersonine in order to achieve a molar relation.

but a final confirmation would require the identification of the intermediates and/or addition of a labelled precursor. *Catharanthus roseus* suspension cultures are able to transform tabersonine to the C-14, C-15-epoxy derivative lochnericine [7], the epoxide having the inverse stereochemistry to that of voaphylline and also lochnerinine, the 11-methoxy derivative of lochnericine. The other compound having a chromophore similar to that of tabersonine is probably an intermediate of the transformation of tabersonine to voaphylline; the structure of this compound remains unknown.

Transformation of tabersonine to voaphylline was dependent on the presence of cells. Compared to the total amount of terpenoid indole alkaloids accumulated by the control culture of the producing cell line during the first four days of culture (*ca* 30 nmol), the amount of voaphylline formed from the added tabersonine during the same period was very high (*ca* 200 nmol). Both tabersonine and its transformation products were not stable in the used culture medium (Table 2); the compound with the chromophore similar to tabersonine was completely broken down. Thus, not all the transformation of tabersonine was due to biotransformation and not all the compound biotransformed was accumulated.

As expected, none of the alkaloids added was stable in culture medium [8]. The stability of some of the alkaloids differed in new and used medium indicating that the medium composition, pH and/or other factors have an influence on their stability. While the amount of the alkaloid decreased, chemical transformation products were often observed to accumulate. Most of these products were not found in the presence of the cultures, probably because they are further metabolized by the cells. Instead, other compounds accumulate which are considered to be the biotransformation products of the added alkaloids, sine they are only formed in the presence of the cells. This will include compounds derived from the chemical degradation with subsequent biotransformation or the other way around.

The cultures used in the experiment have been shown to differ in their biosynthetic abilities [9]. Nevertheless, their biotransformation capacity was similar for all the alkaloids added; all compounds were biotransformed to the same extent in the same way. As was the case for the precursors, the biotransformation products also were not stable in the culture medium.

Unspecific oxidation products of the added alkaloids expected to occur by the action of peroxidases were not detected. In fact, metabolism of vobasine by the cultures was complete; in previous incubations with horseradish peroxidase [10] vobasine was very stable. The type of biotransformation observed was specific for the class of alkaloid added. Coronaridine and its 10,11-dimethoxy derivative, conopharyngine, were hydroxylated at C-19 (same stereochemistry). Vobasine underwent reduction of the double bond to give both dregamine and tabernaemontanine. Tabersonine underwent a series of modifications which included epoxidation, ring-opening and decarboxylation (not necessarily in this order). What all these modifications have in common is the fact that the

added alkaloids were transformed to a compound known to occur in the plants of this species (except conopharyngine which has not been detected in plants of *T. divaricata*). Whether this ability is induced by the addition of the alkaloid or is constitutive is a subject for further investigation. Nevertheless, it is clear that cell cultures are able to metabolize a variety of indole alkaloids. Whether compounds, e.g. tabersonine and vobasine, are not accumulated by suspension cultures due to rapid metabolism or a lack of biosynthesis will be a subject for further studies.

EXPERIMENTAL

Two cell lines of *T. divaricata* (L.) R. Br. ex Roem. et Schult were selected. Both were originally derived from the same suspension culture and were kept under identical culture conditions (MS medium [11] without growth regulators, gyratory shakers at 100–120 rpm, 25°, 1500 lux). One is the highest alkaloid-accumulating cell line maintained at our laboratory, the other is below the detection limit. These cell lines have been previously shown to differ in their biosynthetic capacity [9].

Source and purity of alkaloids. Conopharyngine, vobasine and tabersonine were taken from the collection of reference compounds available in our laboratory. Coronaridine was a kind gift from T. Taesotikul. Before addition to culture media all alkaloids were analysed by HPLC-UV; the main peak accounted for at least 95% of the total area.

Addition of alkaloids. Alkaloids were dissolved in previously sterilized culture medium and distributed between 100-ml Erlenmeyer flasks (20 ml per flask) as described before [9]. To determine initial alkaloid contents of the different media, 200 μl was analysed by HPLC-UV as described below. Initial amounts per flask of coronaridine and tabersonine were calcd by inj. of ref. compounds and were 650 and 312 nmol, respectively. The amounts of conopharyngine and vobasine were estimated by the reported ε [12] to be 350 and 750 nmol, respectively.

Inoculation procedure. Cells used as inocula were obtained from the stock cultures described above. A precisely weighed amount, *ca* 1.6 g (fr. wt), of biomass was inoculated in each 100-ml Erlenmeyer flask containing 20 ml of culture medium with or without the added alkaloid. To controls, no cells were added. During expts, flasks were maintained like stock cultures, except that the flasks were closed with silicon stoppers instead of cotton plugs to allow the determination of dissimilation curves.

Sampling procedure. The developmental stage of cultures was monitored daily by dissimilation curves [13]. A flask of each of the treatments was harvested at the beginning (day 3) and end of the growth phase (day 13), and in the stationary phase (day 19). Culture medium and cells were sepd through a glass filter with the aid of vacuum and the cells washed × 3 with 10 ml of distilled H_2O. Biomass was stored at −20° before freeze-drying, and freeze-dried biomass was stored at the same temp. until analysis. Culture medium was stored at −20° until analysis (0–4 days after harvesting).

Alkaloid stability in used culture medium. Culture medium was sepd from biomass under aseptic conditions at 2 different times during the culture period. One part of the collected medium was used to determine the alkaloid content and the other part was kept under normal culture conditions until the time of harvest.

Analysis. For quantification purposes, alkaloids were extracted from the biomass as described before [14]. The culture medium was centrifuged and injected directly into the HPLC system. Cell extracts and media were analysed by HPLC-UV as described in ref. [15]. Analyses were monitored at 220, 280, 310 and 340 nm. Ref. compounds were coinjected either into the HPLC system described above or into a second one [16]. The eluent was slightly modified and consisted of 0.1 M ammonium acetate buffer–MeCN (21:29); the pH was adjusted to 7.2. For GC-MS identification [15], extracts from the culture medium were made as described in ref. [14].

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