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PRODUCTION OF BISBENZYLISOQUINOLINE ALKALOIDS IN CULTURED ROOTS OF *STEPHANIA CEPHARANTHA*

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Abstract—Roots obtained from the callus and tuber of *Stephania cepharantha* were cultured. These roots produced at least five bisbenzylisoquinoline alkaloids; homoaromoline, aromoline, isotetrandrine, berbamine and cycleanine. In each cell line, the main constituents were always aromoline and berbamine. There was a high, positive correlation between the aromoline and berbamine contents.

INTRODUCTION

Six bisbenzylisoquinoline alkaloids produced by *Stephania cepharantha* (Menispermaceae) have been identified and isolated [1]; cepharanthine (**1**), homoaromoline (**2**), cepharanoline (**3**), isotetrandrine (**5**), berbamine (**6**) and cycleanine (**7**). Some have potent medicinal activities, for example, cepharanthine (**1**) is effective for treating alopecia and berbamine (**6**) is an inducer of leukocytosis [2].

It takes several years to produce tubers large enough to use as the raw material for alkaloid production, but Akasu *et al.* [3] have been successful in culturing the callus of *S. cepharantha*. They reported that the callus of this species produced bisbenzylisoquinoline alkaloids, but not the full spectrum as in the whole plant. Only aromoline (**4**) and berbamine (**6**) were produced. Recently Cassels *et al.* [4] reported the production of aromoline (**4**), isotetrandrine (**5**) and berbamine (**6**) in the calluses of various *Berberis* species. Here we report the production of bisbenzylisoquinoline alkaloids in cultured roots of *S. cepharantha*.

RESULTS AND DISCUSSION

Sterilized segments of *S. cepharantha* tuber, harvested in April, were inoculated on Linsmaier–Skoog (LS) agar medium [5] containing 10^{-5} M α-naphthaleneacetic acid (NAA) and 10^{-6} M benzyladenine (BA). After one month of incubation in the dark at 26°, callus and adventitious roots had formed. The callus was subcultured on the same medium and on LS agar medium containing 10^{-6} M NAA and 10^{-6} M BA. Adventitious roots differentiated when callus was cultured on medium containing 10^{-5} M NAA and 10^{-6} M BA; whereas, shoots differentiated on medium containing 10^{-6} M NAA and 10^{-6} M BA. Roots differentiated spontaneously from the callus irrespective of its age. No adventitious roots were induced, however, when we cultured segments of tuber harvested in the middle of October under the same conditions. The season of harvest of the tuber was significant.

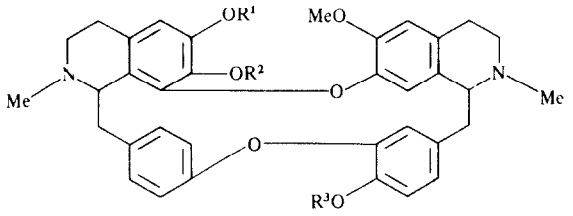
Roots obtained from tuber and from callus were cultured separately on a rotary shaker (85 rpm) in the dark at 26° in liquid media containing various concentrations of indolebutyric acid (IBA). Linsmaier–Skoog and Gamborg B5 [6] basal media were used. In all, 50 cell lines, cultured under the various conditions, were established. The media used are shown in Table 1. In spite of slow growth, root cultures were transferred to fresh media weekly because the culture media gradually turned brown, perhaps because of the secretion of phenolics. After 3 months of successive subculture, the roots were harvested and dried. Alkaloids were extracted from the dried roots with methanol and analysed.

We confirmed that the cultured roots produced at least five bisbenzylisoquinoline alkaloids. Homoaromoline (**2**), isotetrandrine (**5**), berbamine (**6**) and cycleanine (**7**) were identified by comparing their retention times during HPLC and their mass spectra (Table 2) with the respective values of authentic samples. This is the first report to show that homoaromoline (**2**) and cycleanine (**7**) are produced by cultured cells.

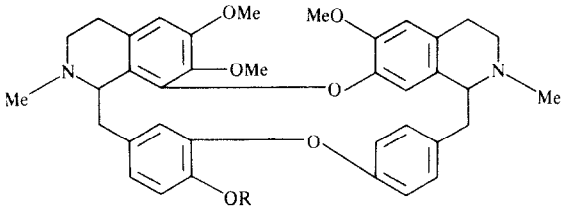
TLC of cultured *S. cepharantha* root extracts indicated that another base, which behaves chromatographically like a bisbenzylisoquinoline alkaloid, was one of the main constituents present. We isolated this base as a powder and identified it as aromoline (**4**) from its R_f value on TLC, melting point and mass spectrum (Table 2). Aromoline (**4**) previously has not been found in *S. cepharantha* plants, only in its callus [3].

In all *S. cepharantha* cell lines, the main alkaloids were aromoline (**4**, 0.1 ~ 1.0% dry wt) and berbamine (**6**, 0.1 ~ 1.0% dry wt), the production of the other alkaloids was less than 1/10 for **4** and **6**.

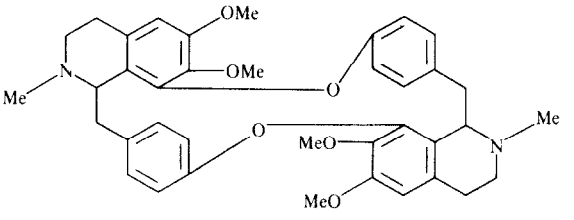
Berbamine and aromoline contents of roots cultured under various conditions are shown in Table 1. In many cultures, berbamine content, to say nothing of aromoline content, was higher than that in tuber. B5 medium was more suitable than LS medium for the root culture of this



1 $R^1 + R^2 = CH_2$, R^3 = Me
2 $R^1 = R^3$ = Me R^2 = H
3 $R^1 + R^2 = CH_2$, R^3 = H
4 R^1 = Me, $R^2 = R^3$ = H



5 R = Me
6 R = H



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plant In both LS and B5 medium, a low concentration (10^{-6} M) of IBA stimulated the production of alkaloids, but root growth was extremely slow (data not shown) A high concentration (5×10^{-5} M) of IBA inhibited alkaloid production Consequently, B5 medium containing 10^{-5} M IBA proved best for alkaloid production but, in this medium as well as in the other media tested, the growth of roots was poor

The correlation between the aromoline and berbamine contents in each culture is shown in Fig 1 Interestingly, the value for the correlation coefficient was very high, $r = 0.89$ This suggests that the production of aromoline (**4**) and berbamine (**6**) in cultured roots depends on the production of a common precursor, *N*-methyl coclaurine [7] The scanty production of the other bisbenzylisoquinoline alkaloids means that the cultured roots have low enzyme activity for the conversion of **4** and **6** to the further-modified, cepharanthine (**1**), cepharanoline (**3**), homoaromoline (**2**) and isotetrandrine (**5**) No aromoline (**4**) has been found in intact plants Therefore, it must be converted very rapidly to the further modified alkaloids in intact plant

Cepharanthine (**1**) and cepharanoline (**3**) could not be identified by mass spectrometry, but their corresponding peaks were detected on HPLC In cultured *S cepharantha* roots, the activity of the methylenedioxy-ring-forming enzyme [8] was extremely low. Methylenedioxy groups are frequently found in the isoquinoline alkaloids. If the methylenedioxy-ring-forming enzyme can be activated, aromoline (**4**) will be converted and full spectrum of bisbenzylisoquinoline alkaloids, i e **1**, **3**, **5**–**7**, should be produced in the cultured system

EXPERIMENTAL

Tissue culture For callus and root induction, sterilized segments of *S cepharantha* tuber (cultivated in Tochigi, Japan) were incubated in the dark at 26 on LS medium containing 10^{-5} M NAA, 10^{-6} M BA, 3% sucrose and 1% agar The calluses formed were maintained under the same conditions with transfer at 3- or 4-week intervals Excised roots were cultured on a rotary shaker (85 rpm) in the dark at 26 in liquid media and subcultured weekly

Extraction of alkaloids Harvested roots were freeze-dried, then powdered and soaked overnight in MeOH This macerated

Table 1 Effect of the basal medium and IBA on alkaloid production in cultured *S cepharantha* roots

Basal medium	IBA concentration (M)	No of cell lines	Alkaloid content (% dry wt) Aromoline (**4**)	Berbamine (**6**)
LS	10^{-5}	5	0.197 ± 0.109	0.156 ± 0.054
	10^{-6}	7	0.392 ± 0.068	0.284 ± 0.059
B5	5×10^{-5}	3	0.176 ± 0.078	0.221 ± 0.028
	10^{-5}	10	0.462 ± 0.254	0.556 ± 0.231
	7.5×10^{-6}	7	0.563 ± 0.231	0.520 ± 0.197
	5×10^{-6}	6	0.444 ± 0.197	0.513 ± 0.149
	10^{-6}	12	0.637 ± 0.241	0.546 ± 0.220
Tuber*			not detected	0.262

*Harvested in October

Table 2 Mass spectra of bisbenzylisoquinoline alkaloids from *S. cepharantha* root cultures

Compound	M^+		Fragments	
Homoaromoline (**2**)	608 (78 5)*	381 (64.6)	191 (100.0)	
Aromoline (**4**)	594 (69 5)	381 (68.8)	191 (100.0)	174 (73 8)
Isotetrandrine (**5**)	622 (100.0)	395 (52.2)	199 (57 3)	174 (51 7)
Berbamine (**6**)	608 (82.3)	395 (76.8)	198 (100 0)	174 (93 0)
Cycleanine (**7**)	622 (78 6)	312 (53 6)	174 (31.2)	

*m/z (rel. int).

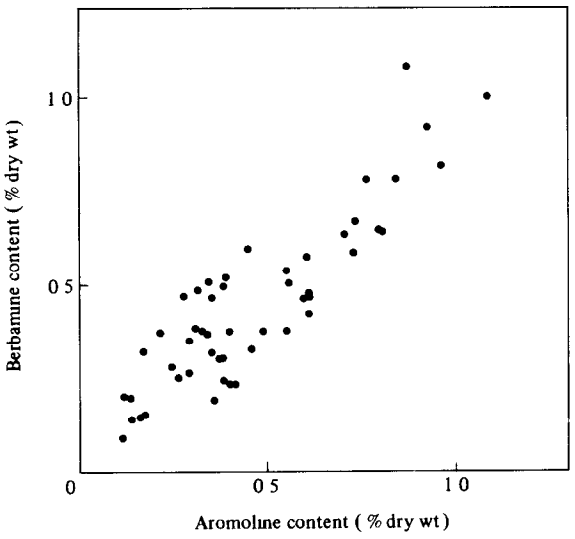

Fig 1 Correlation between aromoline and berbamine contents in *S. cepharantha* root cultures ($r = 0\ 89$)

material was centrifuged for 5 min at 3000 rpm Treatment with MeOH was repeated once more, and the combined MeOH extracts evapd to dryness at 40° The dry residue was dissolved in 2 ml of 3% citric acid, and the acidic aq soln filtered through filter paper into a glass tube and made alkaline (pH 10) with aq NH_3 A 1-ml portion of this alkaline aq soln was loaded onto an Extrelut column (Merck Art 15371) After 10 min, 3 ml of $CHCl_3$ was passed through the column twice. The $CHCl_3$ extracts were combined, then evapd to dryness at 30°. The dry residue was dissolved in 0.5 ml MeOH and put through HPLC and TLC

HPLC. Alkaloid contents were measured with a HPLC (Shimadzu LC-4A) at 35° The stationary phase was Develosil ODS-3 (150 × 4.6 mm) and the solvent 80% MeOH containing 0 2% aq. NH_3. The flow rate was 0.4 ml/min. A short pre-column (30 × 4 6 mm) was placed between the injector and the separation column All the alkaloids were detected by UV at 282 nm The respective R_ts of aromoline, berbamine, homoaromoline, cepharanoline, isotetrandrine, cycleanine and cepharanthine were 10.0, 11 5, 14.7, 15 0, 18 0, 18.8 and 23 8 min Peak areas were calculated with a Chromatopac (Shimadzu CR-2A)

Mass spectrometry Alkaloids were identified by MS following their separation on a HPLC. The mass spectra were recorded with a GC-MS spectrometer (Shimadzu QP-1000) A DI mode with an ionizing energy of 70 eV and an ion source temp of 250° were used

TLC and mp. Extracts from *S cepharantha* roots were separated by TLC on a 0 25 mm silica gel plate (Merck Art. 5715) with a solvent system of $CHCl_3$–MeOH–aq. NH_3 (200 50·1) Alkaloids were located by UV illumination and by spraying the plate with modified Dragendorff's reagent The R_f value of aromoline was 0 33 and of berbamine 0 50 The melting point of aromoline was 176–179° (uncorr)

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