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BISCOCLAURINE ALKALOIDS IN CALLUS TISSUES OF *STEPHANIA CEPHARANTHA*

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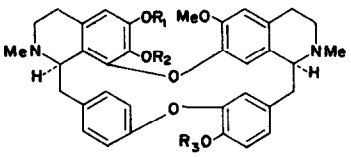
Key Word Index—*Stephania cepharantha*; Menispermaceae; callus tissue; biscoclaurine alkaloids; regulation of alkaloid synthesis.

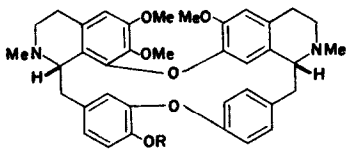
Abstract—Although callus tissues derived from tubers of *Stephania cepharantha* cannot synthesize the main alkaloids of the original plant, cepharanthine and isotetrandrine, they are able to synthesize biscoclaurine alkaloids, berbamine and aromorine, the latter not being found in the original plant. These results suggest that enzymes controlling specific methylation and methylenedioxy group formation are absent from the callus. The maximum content of total alkaloid in the callus tissues subcultured for 9 months was more than 3 times that of original plant. Alkaloid content was affected by addition of various auxins, IAA being most effective.

INTRODUCTION

We previously reported on the sterols, cepharanones, cepharadiones and 7-oxoaporphines from the neutral fraction of *Stephania cepharantha* callus tissue, and that their content was higher than in the original plant [1–3]. The present investigation was undertaken to compare the alkaloid composition of callus tissue with that of the original plant. The effect of auxins on the alkaloid content of the callus was also investigated. The alkaloids in *S. cepharantha*. Hayata have previously been investigated, and several biscoclaurine alkaloids, namely cepharanthine (**1**), homoaromoline (**2**), cepharanoline (**3**), isotetrandrine (**5**), berbamine (**6**) and cycleanine were isolated [4].



(**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

RESULTS

Callus tissues were induced from the tuber of *S. cepharantha* on Murashige and Skoog's agar medium containing 2,4-dichloro-phenoxyacetic acid (2,4-D) or IAA and kinetin. Callus grew as a white compact by adding 1 ppm 2,4-D, 0·1 ppm kinetin (D-medium) or 1–5 ppm IAA, 0·1–1 ppm kinetin in the medium. Differentiation of shoots was observed on the callus under the condition of 1 ppm IAA and 1 ppm kinetin in the medium (IAA-s medium). With light, the number of shoots was increased and green shoot and callus were obtained. Moreover, adventitious roots were differentiated by culturing on the medium containing 5 ppm IAA and 0·01 ppm kinetin (IAA-r medium). The ability to develop shoots was retained through successive subculturing over 3 yr, but the ability to produce roots decreased thereafter, and was lost after further year's subculturing. The above three callus sample and the callus grown on 5 ppm IAA and 1 ppm kinetin contained in the medium (IAA-u medium) were subcultured. Six months later, the callus subcultured on the D-medium was transferred to the other auxin containing medium, 1 ppm α-naphthaleneacetic acid (NAA) and 0·1 ppm kinetin (N-medium), 1 ppm IAA and 0·1 ppm kinetin (I-medium) and basal medium only (B-medium) and subcultured continuously.

The alkaloid fraction obtained from a MeOH extract of fresh callus tissues (2·4 kg, subcultured on N-medium) gave two phenolic alkaloids after chromatographic separation. One was identified as berbamine (**6**), a constituent of the original plant, by comparison with an authentic sample. The second was identified as aromoline (**4**) by comparison with an authentic sample. The TLC pattern of the alkaloids of the callus was simpler than that of the original plant and gave two main and a few minor spots. Aromoline was not observed in the original plant, whereas its main alkaloids, cepharanthine (**1**) and isotetrandrine (**5**), were not detected in callus tissues.

The *Stephania* callus tissues subcultured under the various conditions were analyzed for alkaloid content and the results are shown in Table 1. Callus growth was relatively poor, but the amount of alkaloids was higher than in the original plant. Undifferentiated callus showed the highest alkaloid content, in the case of culturing in the IAA-s medium. There was no significant difference in alkaloid content between pure callus, callus with differentiated shoot, and callus with differentiated root.

Table 1. Alkaloid content of callus, differentiated callus and original plant of *Stephania cepharantha*

Origin	Fresh wt (g/flask)	Dry wt (g/flask)	Alkaloid content (mg/g dry wt) fraction a	fraction b
Callus				
IAA-s medium*	3·36	0·22	14·2	8·7
IAA-s medium†	3·31	0·24	10·2	9·8
IAA-s shoot‡	—	—	4·6	11·5
IAA-r medium¶	2·36	0·15	11·3	6·3
IAA-u medium	4·98	0·28	7·8	3·9
Original plant				
tuber	—	—	1·5	6·7
aerial part	—	—	0·3	0·7

Subcultured for 9 months, grown in the dark for 8 weeks at 25°. * Undifferentiated callus; † shoot differentiated callus; ‡ differentiated shoot; ¶ root differentiated callus.

Alkaloid content decreased with increasing callus growth.

Callus cultured in the presence of various auxins for about 3 yr were analyzed in Table 2. Callus growth and water content were higher than in the IAA callus, but the alkaloid content was extremely low. There were no significant differences between the various auxins. Alkaloid content was highest in callus cultured on I-medium and lowest in callus grown on auxin-free medium.

DISCUSSION

The callus tissues derived from the tuber of *Stephania cepharantha* can synthesize biscoclaurine alkaloids, but the constituents formed are different from those in original plant; this is now a well known phenomenon. Only two compounds are formed; aromaline and berbamine. Aromoline (**4**), which has been only found in *Daphnandra* species (Monimiaceae) [5–7] and *Thalictrum thunbergii* (Ranunculaceae) [8], is a possible intermediate in the synthesis of cepharanthine (**1**), homoaromoline (**2**) and cepharanoline (**3**). Berbamine (**6**) is similarly a possible intermediate in the formation of isotetrandrine (**5**). The major alkaloids of the original plant, **1** and **5**, were not detected in any callus tissue. Thus the callus apparently lacks some specific enzymes for methylation and methylenedioxy group formation.

EXPERIMENTAL

Tissue culture. For callus induction, a cylinder of tissue (from tuber cultured in Ibaragi prefecture, Japan) was aseptically excised with a cork borer, cut into disks, and was then placed on the Murashige and Skoog's basal medium. The basal medium was supplied with various concentration of IAA and kinetin or 1 ppm of 2,4-D and 0·1 ppm of kinetin (D-medium) and 3% of sucrose and 0·7% of agar. The pH of the medium was adjusted to 5·8 before autoclaving. Callus tissues were subcultured in the same medium for callus induction at 4 week intervals, at 25° in the dark. A part of callus cultured on the D-medium (6 month old) was transferred to the other auxin-containing medium instead of 2,4-D, i.e. 1 ppm of NAA (N-medium) and 1 ppm of IAA (I-medium) and also to auxin- and kinetin-free medium (B-medium), and these calluses were subcultured at 5 weekly intervals.

Table 2. Alkaloid content of callus on different auxin-containing media

Medium*	Fresh wt (g/flask)	Dry wt (g/flask)	Alkaloid content (mg/g dry wt) fraction a	fraction b
D-medium	16·2	0·57	1·53	1·20
I-medium	19·9	0·74	2·21	1·56
N-medium	14·7	0·59	2·11	1·06
B-medium	20·3	0·57	1·41	0·62

Subcultured for 3 yr, grown in the dark for 6 weeks at 25° * for key, see Experimental.

Extraction and identification of alkaloids. The fresh callus (2·4 kg in fr. wt, 106 g in dry wt) subcultured on N-medium (18 month old) and thereafter grown for 7 weeks was harvested and homogenized 3× in MeOH and filtered. Residue was refluxed 2× with MeOH. Extracts were combined and concentrated to dryness. Residue was dissolved in 3% citric acid soln and extracted with $CHCl_3$ × 3. The aq. layer was made alkaline to pH 10 with 10% NH_4OH and extracted with $CHCl_3$ repeatedly. After drying, the $CHCl_3$ extract was evaporated, and the alkaloid fraction obtained. TLC gave 2 main spots (Si gel G(Merk), solvent a, $CHCl_3$–MeOH (7:3), R_f, 0·50, 0·37; b, NH_4OH satd C_6H_6–MeOH (8:1), R_f, 0·28, 0·25 and several minor spots (a, 0·27, 0·22, 0·07; b, 0·22, 0·19, 0·09), reagent; Dragendorff. The alkaloid fraction was chromatographed over Si gel and gradient eluted with $CHCl_3$–MeOH. From the $CHCl_3$–MeOH (19:1) fraction were obtained 2 main alkaloids and they were rechromatographed by preparative TLC. Two alkaloids, berbamine and aromoline were obtained as powders. The above alkaline soln was immediately made acid to pH 4 with conc. HCl, and a small amount of quarternary alkaloid was obtained by using the ammonium reineckate precipitate method. TLC (Si gel G, solvent; BuOH–AcOH–H_2O (4:1:2)) 0·10 (main), 0·52, 0·64. *Berbamine*; powder, mp 168–173°, $[\alpha]_D$ + 97° ($CHCl_3$), UV, λ_{max} (EtOH), nm, (log ε): 284 (3·79), IR, ν_{max} cm^{-1}: 3570 (OH), NMR, δ, ppm ($CDCl_3$): 2·25, 2·53 (3H, *s*, 2 × NMe), 3·08, 3·54, 3·70 (3H, *s*, 3 × OMe), 5·95 (1H, *s*, C-8′), MS, *m/e*: 608 (79%, M^+), 607 (50%), 485 (2%), 417 (7%), 395 (68%), 381 (34%), 198 (100%). *Aromoline*; powder, mp 177–181°, $[\alpha]_D$ + 328° ($CHCl_3$), UV, λ_{max} (EtOH), nm, (log ε): 285 (3·98), IR, ν_{max} cm^{-1}: 3570 (OH), NMR, δ, ppm ($CDCl_3$): 2·45, 2·49 (3H, *s*, 2 × NMe), 3·53, 3·64 (3H, *s*, 2 × OMe), 5·62 (1H, *s*, C-8′), MS, *m/e*: 594 (85%, M^+), 593 (72%), 487 (7%), 403 (7%), 382 (100%), 368 (55%), 192 (57%).

Quantitative analysis of alkaloids. All callus tissues were dried at 50° for 2–3 days. Dried calluses were powdered and refluxed in MeOH ×3 for 5 hr. After evaporating extract to dryness, residue was dissolved in 3% citric acid soln and extd with $CHCl_3$ ×3. The aq. layer was made alkaline to pH 10 with 10% NH_4OH and extd with $CHCl_3$ ×3. $CHCl_3$ layer was extd with 1 N NaOH. And alkali layer was added with excess of NH_4Cl and extd with $CHCl_3$ and evaporated to dryness after drying (fraction a). The above $CHCl_3$ layer was extd with citric acid soln and above procedure was repeated again and evaporated after drying (fraction b). Analysis was by measurement of wt or by photometric method and was calculated as berbamine (fraction b) and aromoline (fraction a). Standard curves were made by measuring maximum absorption (284 nm) of authentic samples.

Quantitative analysis of alkaloids in original plant and of differentiated shoots from callus. Tuber (1 kg in dry wt, water content 74·4%), aerial part (100 g in dry wt, water content, 93·7%) harvested in October and differentiated shoots (17 g in dry wt, water content, 92·3%) were dried at room temp. and were analyzed by the method described above.

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