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ANTITUMOR ALKALOIDS IN CALLUS CULTURES OF *CEPHALOTAXUS HARRINGTONIA*

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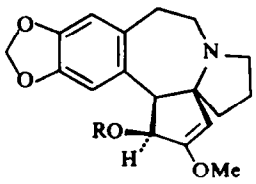
Key Word Index—*Cephalotaxus harringtonia*; Cephalotaxaceae; Japanese plumyew; tissue culture; alkaloids; cephalotaxine esters; homodeoxyharringtonine; structural determination.

Abstract—Leaves and stems of *Cephalotaxus harringtonia* were induced to callus on Murashige and Skoog medium supplemented with additional vitamins, hypoxanthine, naphthaleneacetic acid and kinetin. Analysis by GC–MS showed cephalotaxine and its antitumor esters (harringtonine, isoharringtonine and homoharringtonine) in both callus and medium, whereas deoxyharringtonine was present (in relatively large amounts) only in the medium. The amount of each alkaloid was 2 to 6 times greater at 6 months than at 3 months except for deoxyharringtonine, which was not increased. At 6 months, the total alkaloid production was 1–3% of levels reported for mature trees, but the pattern was the same: 60% cephalotaxine, 40% esters. The pattern of the parent plant was unique (5% cephalotaxine, 80% homoharringtonine, 15% other esters) and may more accurately represent that of fresh plant material. A new cephalotaxine ester, homodeoxyharringtonine, was also detected in the culture medium.

INTRODUCTION

The Japanese plumyew, *Cephalotaxus harringtonia* var. *harringtonia* (Forbes) K. Koch, contains 4 alkaloids with significant antitumor activity in laboratory tests [1, 2]. These alkaloids, harringtonine (1), deoxyharringtonine 2, isoharringtonine (3) and homoharringtonine (4), are all esters of cephalotaxine (5), which is itself inactive.



(1) R = —C(=O)—C(OH)(CH$_2$—CO$_2$Me)—CH$_2$—CH$_2$—CMe$_2$(OH)

(2) R = —C(=O)—C(OH)(CH$_2$—CO$_2$Me)—CH$_2$—CH$_2$—CHMe$_2$

(3) R = —C(=O)—C(OH)(CH—CO$_2$Me)—CH$_2$—CH$_2$—CHMe$_2$

(4) R = —C(=O)—C(OH)(CH$_2$—CO$_2$Me)—CH$_2$—CH$_2$—CH$_2$—CMe$_2$(OH)

(5) R = H

Additional quantities of the active alkaloids are needed to complete preclinical evaluation, but *C. harringtonia* is a slow-growing tree and available supplies have been essentially exhausted [3]. As one possible solution to this problem, we are investigating the production of these compounds in cultured tissues. This initial study shows that *C. harringtonia* tissues can be successfully grown on artificial media and that the same anti-tumor alkaloids are produced in culture as in the intact plant. In addition a new alkaloid, homodeoxyharringtonine, was discovered in the culture medium.

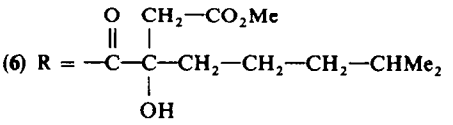
RESULTS

Callus induction required between 7 and 21 days, and was first noticeable along the midrib of the leaf or side of the stem. Young callus tissues were light yellow to yellowish brown in color with no evidence of differentiation. Tissues older than 4 or 5 months were denser and nonuniform in color, ranging from dark brown through yellow or white to green. Root- or shootlike structures occurred only in the older calluses (<2%). Preliminary histological examination shows such structures to have an epithelial layer of cells but little internal differentiation. Callus growth was slow, with a doubling time of *ca* 30 days. Since there were more leaf calluses formed initially and since they grew faster than the stem calluses, the bulk of the cultured tissues in this experiment was of leaf origin.

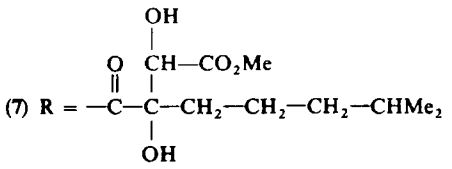
The contamination level during callus initiation was 40% with field-grown plant tissues. The responsible fungi, *Dothiorella* spp. and *Chaetomium* spp., were apparently growing inside the plant protected from the action of the $HgCl_2$ sterilant. When new, laboratory-grown tissues (presumably free of such fungi) were later taken for callus induction, the contamination level was negligible.

Although homoerythrina alkaloids and others were present in the culture extracts, only the cephalotaxine

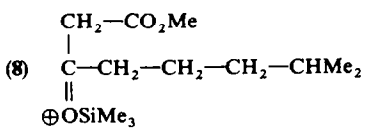
alkaloids were quantitated. Identification of the alkaloids was by GLC R_t on OV-101 and Dexsil 300 and by their GC–MS spectra [4]. On OV-101 the alkaloids eluted at lower temperatures but gave *ca* the same RR_t values. For the esters of cephalotaxine, OV-101 gave a better separation with less column bleed, which facilitated characterization of peaks by MS. Cephalotaxine itself was contaminated by an unidentified compound on OV-101 and was therefore characterized on Dexsil 300. Each cephalotaxine alkaloid in the plant and callus extracts gave the characteristic MS fragmentation pattern and M^+ reported in ref. [4]. In addition, a compound was detected by GC–MS in the 6-month culture medium (MS data were not obtained for the 3-month medium) which had the expected characteristics of a new alkaloid, homodeoxyharringtonine. We propose structure (6) for this new compound by analogy to the



other alkaloid esters present in *C. harringtonia*, but our data do not rule out an acid moiety with a straight side chain, or an alternate type of branched chain. Homodeoxyharringtonine (6) has not previously been reported but would be expected, together with homoisoharringtonine (7), from biogenetic considerations. The concentration in the medium was 8 μg/kg fr. wt.



Homodeoxyharringtonine elutes just before isoharringtonine when OV-101 is substituted for Dexsil 300 in the GLC method [4]. The R_t for the homodeoxy-compound is 7.5% longer than for the deoxy-compound, which is comparable to the 8.1% difference between homoharringtonine and harringtonine. The MS of homodeoxyharringtonine shows the characteristic cephalotaxine ions at *m/e* 141, 150, 266, 282, 298 and 314. In addition, it shows the M^+ at *m/e* 601 and also an ion at *m/e* = 259, attributable to structure (8), which correspond in intensity to similar ions at *m/e* 587 and 245 for deoxyharringtonine. Homoisoharringtonine may well have been overlooked in this study if present in small amounts because its M^+ (*m/e* 689) would have been obscured by column bleed.



The alkaloid concentrations in the youngest portion of stem and leaves from the small plant used to initiate callus cultures are compared in Table 1 to those reported earlier [5, 6] for stems plus roots from relatively mature plants. Not only is the total amount of alkaloids higher than might be expected for such young plant material, but the pattern of alkaloid metabolism is entirely different. Unesterified cephalotaxine has been the major component in all previous studies [4–6], ranging from 57 to 83% of the cephalotaxine-alkaloids. In the present study, the bulk of the cephalotaxine appeared as homoharringtonine.

Table 1. Alkaloid concentrations in parent plant compared to those of previous studies

Alkaloid	Alkaloid concentration (mg/kg)		
	Parent plant	Previous studies	
		ref. [6]	ref. [5]
Cephalotaxine	16	860	390
Deoxyharringtonine	0–1	24	—
Harringtonine	25	64	12
Isoharringtonine	34	98	32
Homoharringtonine	280	61	37
Total	355	1107	471

In tissue cultures established from this young plant, cephalotaxine and its esters were found in both the callus and the agar medium, except for deoxyharringtonine which was detected only in the medium (Table 2). At 3 months, the other alkaloids were more concentrated in the fresh callus tissue than in the medium; but by 6 months, the reverse was true. The higher medium vs callus levels at 6 months could not be explained solely by concentration of the medium with time, since the wt loss between 3 and 6 months was only 40% (23% for callus growth and 17% by evaporation), and therefore must represent active secretion by the tissues.

Total production of cephalotaxine alkaloids (callus plus medium amounts) roughly paralleled the increase in dry wt of callus; hence, the alkaloid/callus ratio was *ca* constant: 10.1 mg/kg at 3 months and 11.1 at 6 months. These levels were 1–3% of the concentrations in cephalotaxin plants (Table 1). Comparing the amount per culture vial at 6 months to that at 3 months, dry wt increased 210%; cephalotaxine, 440%; harringtonine, 350%; isoharringtonine, 580%; and homoharringtonine, 220%. Deoxyharringtonine, on the other hand, either remained constant or decreased by 20%.

The pattern of alkaloid biosynthesis in the callus cultures is compared to whole plant data in Table 3. The distribution of alkaloids produced in culture at 3 months is remarkable because of the high levels of deoxyharringtonine present. This alkaloid generally

Table 2. Alkaloid concentration based on fresh weight of callus and medium

Alkaloid	Concentration (μg/kg)			
	3 Month culture		6 Month culture	
	Callus	Medium	Callus	Medium
Cephalotaxine	50	13	40	144
Deoxyharringtonine	0	18	0	25
Harringtonine	8	1	tr	11
Isoharringtonine	11	4	11	48
Homoharringtonine	15	4	0.5	27

Table 3. Relative amounts of cephalotaxine alkaloids in callus and *C. harringtonia plants*

Alkaloid	Percent of total cephalotaxine alkaloids			
	Callus plus medium		Parent plant	Previous data ref. [4]
	3 Months	6 Months		
Cephalotaxine	38.0	58.5	4.6	57.2
Deoxyharringtonine	38.4	8.8	—	1.9
Harringtonine	3.0	3.8	6.9	4.7
Isoharringtonine	9.9	19.2	9.4	25.4
Homoharringtonine	10.7	9.7	79.1	10.8

comprises less than 3% of the cephalotaxine alkaloids in *C. harringtonia* [4, 6] and was present in only trace amounts in the parent plant. At the end of 6 months, the relative amounts of alkaloids per vial were quite different from the parent plant, being high in cephalotaxine and low in homoharringtonine, but strongly resembled the pattern found in earlier studies [4–6] of mature plants.

Preliminary analytical results from several other *C. harringtonia* cultures grown on different media were similar to those presented here but were generally higher. Lack of replication due to the large amount of tissue required for a single analysis prevents a quantitative comparison at this time; however, a qualitative difference should be noted. Several of the calluses did not produce harringtonine in detectable amounts and in one experiment homoharringtonine was produced at 5 months but not at 3 months. It remains to be seen whether this represents variability between cultures or metabolic control exerted by the medium. In all such callus tissues, isoharringtonine was synthesized in relatively large amounts and deoxyharringtonine was also present in large amount in the only other culture medium analyzed so far.

DISCUSSION

Since *C. harringtonia* is a gymnosperm, several components from successful gymnosperm media were incorporated in the present study to enhance the chances of tissue growth. These ingredients included hypoxanthine [7, 8] and protein hydrolysate [9], the latter being known to promote alkaloid formation in *Scopolia japonica* [10] and *S. parviflora* [11]. Similarly, NAA was chosen over 2,4-D because of its favourable effect on alkaloid formation in *Nicotiana tabacum* [12] and three other species [13, 14]. Murashige and Skoog's [15] salt mixture proved better for the growth of *C. harringtonia* than that of White [8], contrary to the findings of Steinhart, Standifer and Skoog [9] with another gymnosperm, *Picea abies*. Whether or not Murashige and Skoog's medium is actually better for alkaloid production remains an interesting question, particularly in view of the finding of Nettleship and Slayter [16] that NH_4NO_3 inhibited alkaloid formation in *Peganum harmala*.

Many plants produce alkaloids in tissue culture, but quite often the compounds produced are not typical of the parent plant [14, 17, 18]. The fortunate similarity that we observe between mature plants and callus tissues may be due to slow tissue growth that would tend to favor secondary metabolism. Organogenesis, which has been associated with secondary metabolites [13, 16, 19–21], occurred very rarely in this study, although preliminary histological examination showed some tissue differentiation and gross differences in callus color were observed.

Alkaloid deposition in tissues of *C. harringtonia* plants has not been studied thoroughly. It is not known whether specialized storage cells are normally present, but if the alkaloids are uniformly distributed throughout the entire plant, specialized structures may not be required for their synthesis or storage. The absence of a requirement for specialized structure would favor production in culture.

In terms of relative alkaloid content, the parent plant used in these studies differed markedly from its derived callus tissue and from whole plants studied earlier. This difference cannot be explained on the basis of the portion of the plant sampled (i.e., leaves plus stem) because alkaloid ratios are essentially identical for stems plus roots [5, 6], leaves, roots, seeds and whole plants [4]. Several other possibilities will be explored in future work. The high homoharringtonine level in the parent plant is encouraging for it demonstrates that, at least in the intact plant, alkaloid biosynthesis can be directed to favor the most potent antitumor ester. It will be a challenge to reproduce those conditions in culture.

Relatively little is known of the biosynthesis of cephalotaxine esters. A proposal that cephalotaxine itself might arise from a homoerythrina precursor was advanced by Powell [6], and supported by the data of Schwab, Chang and Parry [22]. Recently Parry, Sternbach and Cabelli have found [23] that the acyl moiety deoxyharringtonine is biosynthesized from L-leucine by the same mechanism as that involved in the homologation of L-valine to L-leucine. Extension of this scheme through one more cycle would provide the acid moiety of the homodeoxyharringtonine discovered in our studies, assuming this to have the structure (**6**) proposed. Presumably the acyl groups of the other ester are derived from these deoxy-acids. It is not known, however, whether cephalotaxine is esterfied by a non-specific ester synthetase that adds a variety of acyl substituents, or whether certain of the esters arise from a common ester precursor. The early biosynthesis of large amounts of deoxyharringtonine, normally a minor constituent of the alkaloid fraction, is consistent with the hypothesis that it is a precursor for harringtonine and isoharringtonine. The existence of homodeoxyharringtonine permits a similar pathway for homoharringtonine and for homoisoharringtonine should the latter be found in *C. harringtonia* tissues.

EXPERIMENTAL

Plant material. A young *C. harringtonia* tree *ca* 30 cm tall was planted in 2:1 peat moss–sandy loam in a 17 cm diameter clay pot and grown under fluorescent light (1500 lx for 16 hr day) at 25 ± 3°. Shortly after planting, several branches were removed for callus initiation. After 9 months, the upper tier of branches and associated main stem were removed and cut into 2–3 mm portions for analysis.

Callus initiation. Branches were sterilized *in vacuo* 15 min with 0.1% $HgCl_2$ soln containing 1 drop detergent per 250 ml, rinsed twice in H_2O and cut into 5 mm pieces. Callus formation

and subsequent growth was on Murashige and Skoog's complete medium [15] supplemented with 1 g enzymic protein hydrolysate, 25 mg hypoxanthine, 0.5 mg riboflavin, 0.5 μg vitamin B_{12}, 10 μg biotin, 1 mg folic acid and 1 mg pantothenic acid per l. Growth factors were 10 mg NAA and 1 mg kinetin per l. Tissues grown under ordinary laboratory lighting at 25 ± 3° and transferred to fresh medium at roughly 3 month intervals have been successfully maintained under these conditions for over 2 years. Callus formation was also induced on a medium with a modified White's salt mixture [8] but callus growth was slower.

Alkaloid analysis. The fresh plant sample was cut into 2–3 mm pieces and all samples were dried to constant wt at 80°. The dried callus samples were then pulverized with a mortar and pestle. Extraction and partial purification of the alkaloids was according to the procedure of ref. [5] except for the omission of the countercurrent distribution step. To minimize emulsion problems, the agar medium was first diluted with an equal vol. of H_2O, then adjusted to pH 9 and extracted ×3 with $CHCl_3$. Centrifugation facilitated phase separation. The combined $CHCl_3$ extracts were then extracted ×3 with 2% citric acid, and the citric acid soln further purified according to the original method. Individual alkaloids were identified and quantitated by the GC–MS method of ref. [4] using OV-101 in addition to Dexsil 300. RR_t data below are with Me lignocerate as internal standard for both columns. MS data were obtained by GLC on Dexsil 300 for cephalotaxine and OV-101 for the other alkaloids.

Cephalotaxine. RR_t 0.55 Dexsil 300, MS *m/e* 387 (M^+, 54), 372 (M^+-15, 21), 314 (3), 298 (53), 282 (9), 266 (14), 238 (20), 73 (100).

Deoxyharringtonine. RR_t 1.76 Dexsil 300, 1.80 OV-101, MS *m/e* 587 (M^+, 10), 556 (M^+-31, 3), 314 (4), 298 (100), 282 (6), 266 (8), 245 (5).

Homodeoxyharringtonine. RR_t 1.93 OV-101, MS *m/e* 601 (M^+ 10), 570 (M^+-31, 4), 314 (7), 298 (100), 282 (10), 266 (5), 259 (5), 173 (4), 155 (5), 150 (10), 141 (5), 73 (38).

Isoharringtonine. RR_t 1.88 Dexsil 300, 1.99 OV-101, MS *m/e* 675 (M^+, 8), 314 (3), 298 (100), 282 (4), 266 (7), 234 (5).

Homoharringtonine. RR_t 1.97 Dexsil 300, 2.29 OV-101, MS *m/e* 689 (M^+, 8), 314 (6), 298 (100), 282 (3), 266 (7), 201 (4).

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