Phytochemistry, Vol 27, No 5, pp 1375 1378, 1988
Printed in Great Britain

0031 9422/88 $3 00+0 00
Pergamon Press plc

COLCHICINE PRECURSORS AND THE FORMATION OF ALKALOIDS IN SUSPENSION-CULTURED *COLCHICUM AUTUMNALE*

KEIICHIRO YOSHIDA, TAKAHISA HAYASHI* and KONOSUKE SANO

Basic Research Laboratory, Central Research Laboratories, Ajinomoto Co Ltd, 1-1 Suzuki-cho, Kawasaki 210, Japan

(*Received* 24 *August* 1987)

Key Word Index—*Colchicum autumnale*, Liliaceae, plant tissue culture, colchicine biosynthesis, phenylalanine ammonia-lyase, *trans*-cinnamic acid 4-hydroxylase; tyrosine decarboxylase, *p*-coumaric acid, tyramine; demecolcine

Abstract—Various precursors of colchicine were added to cell suspension cultures of *Colchicum autumnale* and the formation of colchicine alkaloids was examined. Phenylalanine, tyrosine and methionine had no effect on the formation of colchicine, but *p*-coumaric acid, tyramine and demecolcine increased alkaloid formation. Production of colchicine occurred at maximum level when both *p*-coumaric acid and tyramine were provided together, suggesting a synergistic formation. However, concentrations of *p*-coumaric acid and tyramine were very low in the cultured cells in spite of high levels of phenylalanine and tyrosine Activities of *trans*-cinnamic acid 4-hydroxylase and tyrosine decarboxylase, involved in the biosynthesis of both *p*-coumaric acid and tyramine, respectively, were very low in the callus tissues during growth, although light increased activity of phenylalanine ammonia-lyase. We conclude that *p*-coumaric acid and tyramine are triggers for the formation of colchicine alkaloids in *Colchicum* callus tissues.

INTRODUCTION

Colchicine alkaloids occur in *Colchicum autumnale* cells, and are formed from phenylalanine, tyrosine and methionine (Fig. 1). Tracer experiments have suggested that ring A is derived from tyrosine and ring C from phenylalanine and methionine provides the methoxyl groups [1–6]. *trans*-Cinnamic acid and *p*-coumaric acid have been identified as intermediates from phenylalanine, and tyramine was formed from tyrosine [5, 7]. Androcymbine is known to be a key intermediate on the pathway to colchicine but it has only been obtained from tissues of *Androcymbium melanthioides* [8]. Demecolcine is a closer precursor of colchicine which is metabolized through demethylation and acetylation [9]. The biosynthetic pathway for colchicine has been characterized in tracer experiments using intact *Colchicum* tissues

In the previous paper of this series, we presented the effect of growth substances and nutritional factors on the formation of colchicine in suspension-cultured *Colchicum* cells [10] Levels of colchicine in the callus tissues were about ten times lower than those in intact corms. Further studies on the regulation of colchicine biosynthesis are required in order to increase colchicine production. The present communication examines the effect of several precursors on the formation of colchicine alkaloids together with measurements of several enzymic activities involved in their biosynthesis.

RESULTS

Effects of colchicine alkaloids and their amino acid precursors on the formation of colchicine alkaloids

Since colchicine alkaloids are synthesized from phenylalanine, tyrosine and methionine, levels of amino acids and colchicine alkaloids in cells at their exponential stage were determined by HPLC analysis. Levels of colchicine were very low (90 μM), compared to those of phenylalanine (1 15 mM) and tyrosine (0.7 mM) (Table 1). Furthermore, demecolcine was hardly detected in suspension-cultured *Colchicum* cells This is in full agreement with earlier experiments [10] that callus tissues produce amounts of colchicine alkaloids ten times lower than those in intact corms (0.5–1 mM).

In an attempt to increase the formation of colchicine, demecolcine was administered to suspension-cultured *Colchicum* cells Addition of this compound substantially increased colchicine formation within a few days and maintained a high level during growth (116 μg/g fr. wt). However, addition of colchicine into the medium did not increase the formation of demecolcine and inhibited cell growth. The results confirm that demecolcine is a very close precursor of colchicine and suggest that formation of the latter is an irreversible process. Addition of either phenylalanine (1 mM), tyrosine (1 mM) or methionine (1 mM) alone or in combination to the culture medium did not increase the concentration of colchicine alkaloids. These results also suggest that the trigger for colchicine formation occurs in the biosynthetic pathway between the amino acids and demecolcine.

Effects of trans-cinnamic acid, p-coumaric acid and tyramine on the formation of colchicine alkaloids

trans-Cinnamic acid which is formed from phenylalanine catalysed by phenylalanine ammonia-lyase (PAL) occurs in suspension-cultured *Colchicum* cells at extremely low levels (<2 μM) Addition of *trans*-cinnamic acid ($0.01 \sim 1$mM) into the culture medium inhibited not only the formation of colchicine alkaloids but cell growth

*Author to whom correspondence should be addressed

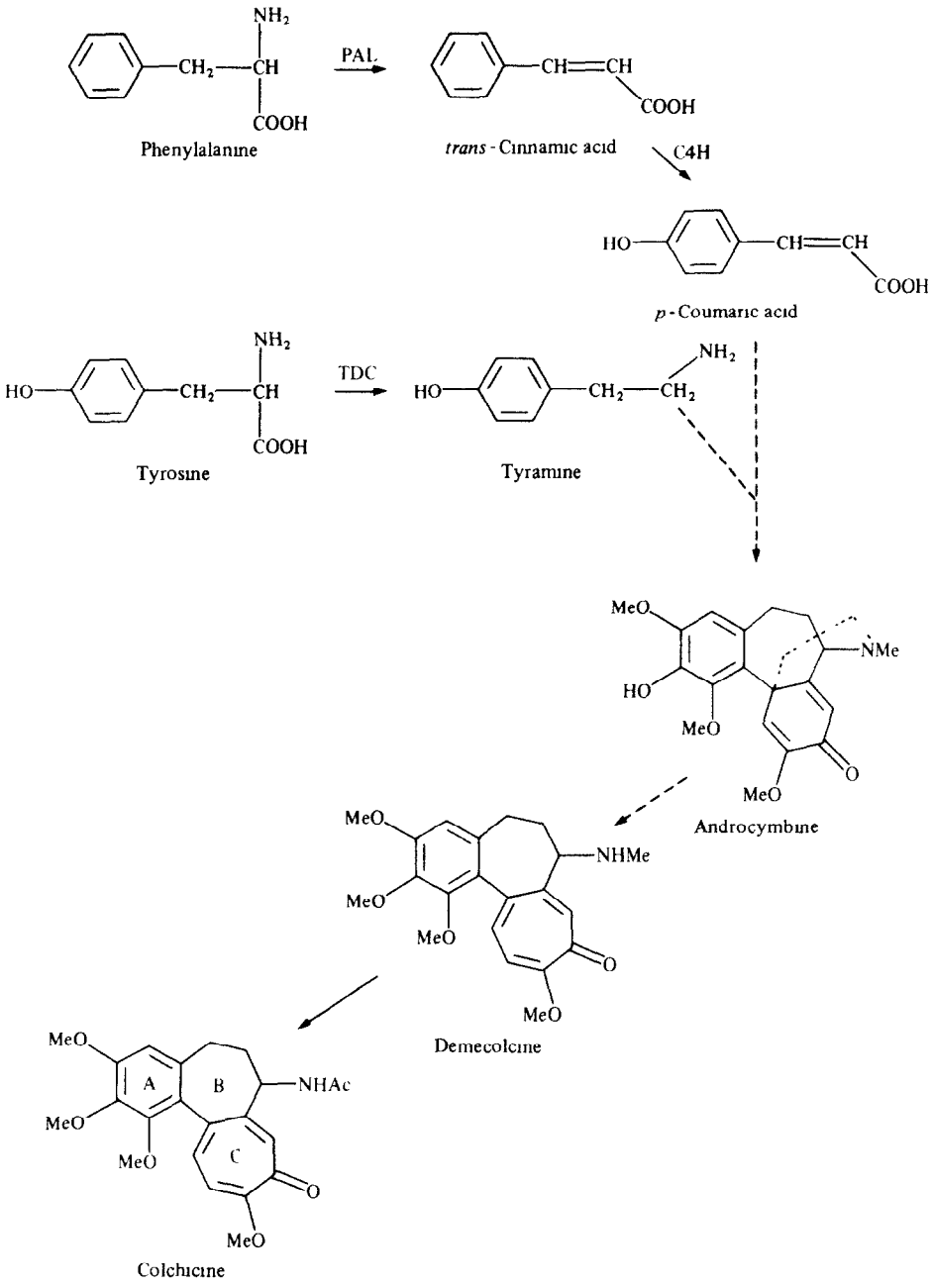

Fig 1 Biosynthetic pathway of colchicine alkaloids

Table 1 Amounts of phenylalanine, tyrosine, methionine and colchicine alkaloids in suspension-cultured *Colchicum* cells

Precursor	μM
Phenylalanine	1150
Tyrosine	700
Methionine	79
Demecolcine	0
Cochicine	90

Table 2 Effects of colchicine alkaloids and their ami acid precursors on colchicine alkaloid formation

Additive (1 mM)	Demecolcine	Colchicine
	μg/g fr wt	
None	0	36 4
Phenylalanine	0	22 4
Tyrosine	0	15 4
Methionine	0	17 0
Demecolcine		116 0
Colchicine	0	

Table. 3 Effects of phenylalanine and tyrosine metabolites on growth and colchicine alkaloid formation

Additive	Fr wt of callus (mg/flask)	Content	
		Demecolcine (μg/g fr wt)	Colchicine (μg/g fr wt)
None	525	0	36 4
trans-Cinnamic acid	120	0	1 4
p-Coumaric acid	460	0	72
Tyramine	440	0	90
p-Coumaric acid + Tyramine	480	0	220

(Table 3) This may be due to the toxicity of *trans*-cinnamic acid to the cells reported previously [11].

p-Coumaric acid which is formed from trans-cinnamic acid catalysed by *trans*-cinnamic acid 4-hydroxylase (C4H) also occurs at extremely low levels (<2 μM). Addition of p-coumaric acid (1mM) increased colchicine formation to levels twice as those of control cultures (Table 3). Tyramine which is the tyrosine metabolite catalysed by tryosine decarboxylase (TDC) is found in *Colchicum* cells at low levels (0.8 μM). Addition of tyramine (1 mM) increased colchicine formation to levels three times higher than controls (Table 3) These results strongly suggest that *p*-coumaric acid and tyramine are significant precursors for the biosynthesis of colchicine alkaloids Formation of colchicine occurs at maximum rate when both *p*-coumaric acid and tyramine are provided together, indicating that one enhances the effect of the other This implies that concurrent conjugation of two metabolites is required during the synthesis of colchicine alkaloids These results suggest that the biosynthesis of *p*-coumaric acid and tyramine is rate limiting to colchicine formation in suspension-cultured *Colchicum* cells

Changes in enzyme activities

Several enzymic activities which may be involved in the biosynthesis of *p*-coumaric acid and tyramine were determined in cells grown with or without light PAL and C4H activities are required for the biosynthesis of *p*-coumaric acid from phenylalanine. Levels for these enzyme activities were 0.3×10^{-3} units/mg protein and 3×10^{-6} units/mg protein, respectively. The level of C4H activity was extremely low (about 10^{-6} units/mg protein) compared to those in intact parsley and carrot cells [12–14]. The pH optima for PAL and C4H activities in *Colchicum* callus enzyme preparation were 8.8 and 6 0, respectively There is no report on the levels of TDC activity in higher plants, but bacterial TDC activity contains usually 10^{-3} units/mg protein [15] which is much higher than that in *Colchicum* callus tissues (10^{-9} units/mg protein) The activities of PAL increased markedly only in the light-irradiated (9000 lx) cells at one day but declined soon to the basal level. This is in full agreement with earlier observations [12, 13] that PAL is specifically induced on irradiation of suspension-cultured plant cells. However, the activities of C4H did not increase in the irradiated cells. Prolonged cultivation with light did not evoke the activities, although C4H can be induced by light irradiation in suspension-cultured parsley cells [12, 13]. These results suggest that the metabolism of phenylalanine is blocked at the pathway between *trans*-cinnamic acid and *p*-coumaric acid by low levels of C4H activity. Also, the activities of TDC did not increase in light-irradiated cells. These assays (Table 4) after light irradiation were consistent with the low levels of colchicine alkaloid formation in light irradiated suspension-cultured *Colchicum* cells [10] The results showed that levels of both C4H and TDC activities are extremely low in the cultured *Colchicum* cells and, thereby, the biosynthesis of *p*-coumaric acid and tyramine is probably rate limiting for the formation of colchicine alkaloids.

DISCUSSION

When both *p*-coumaric acid and tyramine were provided together, colchicine formation occurred at a maximal rate (Table 3), similar to that in intact corms [10] Therefore, it may be concluded that *p*-coumaric acid and tyramine are triggers for the alkaloid production. In fact, levels of C4H and TDC activities were extremely low compared with those in other plant tissues [13–15], although during growth PAL activity was similar to that in other plant cells [13] PAL could be induced by light but C4H and TDC could not (Table 4) Our results are in full agreement with a previous paper [10] showing that light irradiation did not increase the formation of colchicine alkaloids

Thus, several precursors were added to culture medium and callus tissues were cultured in the medium for four weeks. The formation of colchicine alkaloids was increased steadily during growth for *ca* six weeks High levels of colchicine accumulation were only observed within a few days after addition of demecolcine but not

Table 4 Light-induced activities of PAL, C4H and TDC

Enzyme assayed	Light-induced activity		
	0 day	1 day	3 day
	activity in the light/activity in the dark		
PAL	1	10	2
C4H	1	1	1
TDC	1	0.5	1

by addition of amino acid metabolites during early stages of cultivation. *p*-Coumaric acid and tyramine were toxic to cell growth and inhibited colchicine formation at concentrations above 10 mM Also, demecolcine and colchicine were highly toxic for callus cells at concentrations above 10 mM The results suggest that these precursors not only serve as colchicine intermediates but play an important role of cell growth in the metabolism of *Colchicum* cells

Only trace amounts of demecolcine were detected in *Colchicum* callus tissues during cultivation Since colchicine is formed from demecolcine (Table 2), demecolcine turnover is probably very high in cultured *Colchicum* cells However, demecolcine occurs in *Colchicum* corms in amounts equivalent to those of colchicine. It seems that high levels of colchicine accumulation induce the formation of demecolcine or metabolic levels of colchicine alkaloids change in cultured *Colchicum* cells

Colchicoside which is a glucosylated colchicine could be formed from colchicine However, colchicoside appeared only at low levels (2 μg/g fr wt in medium containing 1 mM demecolcine, data not shown), where the amount of colchicoside formed was at most 2 μg/g fresh weight Neither demethyl colchicine nor demethyl demecolcine were detected in the cultured tissues Further studies are now in progress on the formation of glucosyl colchicine

EXPERIMENTAL

Culture conditions Callus tissues induced from flower shoots of *C autumnale* were cultured on the Murashige & Skoog medium [16] modified as in ref [10] *Ca* 125 mg fr wt of cultured calluses were inoculated into a 50 ml Erlenmeyer flask containing 10 ml of test medium, after which the calluses were cultured at 25° on a rotary shaker in the dark for 4 weeks. All expts were performed at the same growth stage of cells

Materials. Colchicine, demecolcine, *trans*-cinnamic acid, *p*-coumaric acid and tyramine were purchased from Sigma Amino acids were obtained from our company stocks

Enzyme preparation Suspension-cultured *Colchicum* cells (500 mg) were ground in 1 ml of 0 1 5 M sodium phosphate buffer (pH 6 8) in a glass homogenizer The homogenate was centrifuged at 1000 *g* for 10 min and the supernatant used as the enzyme prepn.

Enzyme assays. PAL activity was assayed by the method of ref [17] The incubation mixt contained 10 mM phenylalanine, 50 mM sodium borate (pH 8 8) and the enzyme prepn (*ca* 0 1 mg protein) in a total vol of 100 μl The mixt was incubated for 1 hr at 30° and the reaction terminated by adding 5 μl of 3M HCl The mixt was centrifuged at 17000 *g* for 15 min to eliminate the denatured proteins and the amount of *trans*-cinnamic acid was determined by HPLC on Lichrosorb RP-18 Analysis was carried out using MeCN–MeOH–50 mM Na phosphate buffer (pH 6), (4 1 15). Detection was by *A* at 290 nm. One unit of the enzyme is defined as the amount that catalyses the synthesis of 1 μmol of *trans*-cinnamic acid/min at 30°

C4H activity was assayed by the method of ref [18] The incubation mixt contained 330 μM tetrahydrofolic acid, 167 μM $NADP^+$, 3 3 mM glucose 6-phosphate, 0 05 units glucose 6-phosphate dehydrogenase and 1 mM *trans*-cinnamic acid and the enzyme prepn (*ca* 0 2 mg protein) in a total vol of 100 μl The reaction was carried out at 30° for 20 min and was terminated by adding 5 μl 3M HCl. After centrifugation, the amount of *p*-coumaric acid produced was determined by HPLC using the column described above Analysis was carried out using MeCN–MeOH–50 mM Na phosphate buffer (pH 6), (1 1 18) One unit of enzyme is defined as the amount that catalyses the synthesis of 1 μmol of *p*-coumaric acid/min at 30

TDC activity was assayed by the method of ref [15] combined with the analytical method of ref [19] The incubation mixt contained 1 mM tyrosine, 0 1 M acetate buffer (pH 5 6) and the enzyme prepn (*ca* 0 5 mg protein) in a total vol of 500 μl Incubation was at 30° for 10 min and the reaction was stopped by boiling the mixt After centrifugation, the supernatants were passed through a Cellex P column and the amines isolated fluorescence-labelled with fluorescamine The amount of tyramine was determined by HPLC using the column described above The fluorescence intensity was monitored at an emission wavelength of 490 nm with the excitation wavelength at 390 nm The mode of sepn used was a linear gradient between 45 to 80% MeOH in 0 5M NH_4Cl, 0 175 M Na benzensulphonate and 30 mM HOAc (pH4) One unit of enzyme is defined as the amount that catalyses the synthesis of 1 μmol of tyramine/min at 30

General methods Amino acids were analysed by HPLC using a *O*-phthalaldehyde-labelling system Protein was determined using the Bio-Rad Protein assay kit

Acknowledgements—We thank Dr T Furuya (Kitasato University, Japan) for his valuable advice and Director K Tahara of this laboratory for his encouragement We also thank Dr H Flores (Louisiana State University, U S A) for his helpful comments during the final preparation of this paper

REFERENCES

1 Leete, E and Nemeth P E (1960) *J Am Chem Soc* **82**, 6055
2 Leete, E (1963) *J Am Chem Soc* **85**, 3666
3 Battersby, A R and Raynolds, I I (1960) *Proc Chem Soc* 346
4 Battersby, A R and Herbert, R B (1964) *Proc Chem Soc* 260
5 Battersby, A R, Binks, R and Yeowell, D A (1964) *Proc Chem Soc* 86
6 Leete, E and Nemeth, P E (1961) *J Am Chem Soc* **83**, 2192
7 Robinson, T (1968) in *The Biochemistry of Alkaloids*, p 75 Springer, New York
8 Battersby, A R and Herbert, R B (1965) *Chem Comm* 228
9 Barker, A C, Battersby, A R, Macdonald, E, Ramage, R and Clements, J H (1967) *Chem Comm* 390
10 Hayashi, T, Yoshida, K and Sano, K (1988) *Phytochemistry* **27**, 1371
11 Mizukami, H Konoshima, M and Tabata, M (1977) *Phytochemistry* **16**, 1183
12 Hahlbrock, K, Ebel, J, Ortmann, R, Sutter, A, Wellmann, E and Grisebach, H (1971) *Biochim Biophys Acta* **244**, 7
13 Hahlbrock, K, Knoblock, K H, Kreuzaler, K, Potts, J R M and Wellmann, E (1976) *Eur J Biochem* **61**, 199
14 Ozeki, Y and Komamine, A (1985) *Plant Cell Physiol* **26**, 903
15 Epps, H M R (1944) *Biochem J* **38**, 242
16 Murashige, T and Skoog, F (1963) *Physiol Plant* **15**, 473
17 Zimmermann, A and Hahlbrock K (1975) *Arch Biochem Biophys* **166**, 54
18 Russell, D W and Conn, E E (1967) *Arch Biochem Biophys* **122**, 256
19 Kai, M, Ogata, T, Haraguchi, K and Ohkura, Y (1979) *J Chromatog* **163**, 151