Synthesis of Styrenes through the Biocatalytic Decarboxylation of *trans*-Cinnamic Acids by Plant Cell Cultures

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A novel method for producing styrenes from *trans*-cinnamic acids was developed. When *trans*-cinnamic acid was incubated with plant cell cultures at room temperature, styrene was obtained. 4-Hydroxy-3-methoxystyrene (2a), 3-nitrostyrene (2f) and furan (2g) were synthesized quantitatively.

Key words decarboxylation; styrene; *trans*-cinnamic acid; plant cell culture; enzyme

The chemical methodologies for obtaining styrenes are mainly i) the dehydrogenation of ethylbenzene, ii) the chlorination of ethylbenzene and subsequent removal of hydrogen halide, and iii) the decarboxylation of *trans*-cinnamic acids. Of these methodologies, the decarboxylation of *trans*-cinnamic acids is the most widely used chemical method for preparing styrenes or stilbenes. Typical decarboxylation is carried out by heating under reflux at 200—300 °C for 4—5 h in quinoline in the presence of a Cu powder (Y. >50%). Quinoline is useful as a solvent for the decarboxylation of unsaturated acids because it is basic enough to form the required carboxylate anion and also because it boils at a temperature favorable for decarboxylation. This method, however, needs a high temperature.

The biocatalytic decarboxylation of *trans*-cinnamic acid is the most promising method, because it takes advantage of the mild reaction conditions for preparing styrenes. The known decarboxylative enzymes are mainly as follows: i) pyruvate decarboxylase,[1] ii) oxalate decarboxylase,[2] iii) glutamate decarboxylase,[3] iv) benzoylformate decarboxylase,[4] v) aconitate decarboxylase,[5] and vi) aspartate 4-decarboxylase.[6]

In the case of *trans*-cinnamic acids, β-phenylacrylic acid was decarboxylated by *Aspergillus niger* to give styrene.[7] *Aerobacter* has been found to decarboxylate *trans*-4-hydroxycinnamic acid to the corresponding 4-hydroxystyrene.[8] However, only a few attempts to decarboxylate other *trans*-cinnamic acids by a decarboxylase have been reported.

In recent years, much attention has been paid to the ability of cultured plant cells to enantioselectively transform not only secondary metabolites, but also organic foreign substrates.[9—14] The biotransformation of the exogenous substrates by plant cell cultures can be summarized according to classes of chemical reactions as follows: 1) hydroxylation, 2) oxido-reduction between alcohols and ketones, 3) reduction of the carbon–carbon double bond, 4) glycosyl conjugation, 5) hydrolysis, and 6) miscellaneous reactions.

We have reported synthetic methods by plant cell cultures as follows: 1) the asymmetric reduction of benzoyl pyridines,[10,11,15] 2) asymmetric hydrolysis of (α-acetoxybenzyl)pyridines,[10,11,15] 3) deracemization of racemic alcohols, *i.e.*, 100% conversion of racemic alcohols to the corresponding chiral alcohols,[16—18] 4) dediastereomerization of dibenzylbutanolides, *i.e.* reactions allowing the transformation of two diastereomers into one diastereomer in quantitative yield.[19]

Very recently, we developed a novel method for the decarboxylation of *trans*-cinnamic acids (**1a**—**i**) by plant cell cultures to the corresponding styrenes or furan (**2a**—**i**), as shown in Fig. 1.[20] In this paper, we would like to report our use of plant cell cultures in detail.

In this work, we used suspension-cultured cells which had originally been isolated from *Nicotiana* (*N.*) *tabacum* "Bright Yellow-2", *Daucus* (*D.*) *carota*, *Camellia* (*C.*) *sinensis* and *Catharanthus* (*C.*) *roseus*. These cell cultures (*N. tabacum*, *D. carota*, *C. roseus*) were prepared as described in our previous papers.[10,11,15] *C. sinensis* callus tissues were obtained from the shoot tips after a 4- to 6-week induction period when Murashige and Skoog's (MS) medium[21] was used. The callus was inoculated into liquid Gamborg's B5 medium[22] containing 1.25 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D) as an auxin, 1 mg/l of benzyladenine as a cytokinin and 5% sucrose. Subculturing was performed every 15 d by transferring 10 ml of 2-week-old culture into 80 ml of fresh Gamborg's B5 medium. Incubation was done on a rotary shaker (110 rpm) at 25 °C in the dark.

A biodecarboxylative reaction was performed by the following four methods to optimize conditions: A) with freely suspended plant cells in the stationary phase after 10 d of incubation (10 g of cells in 20 ml of a medium); B) with homogenized plant cell culture (10 g) in 0.1 M phosphate buffer solution (pH 6.0, 20 ml); C) with homogenized plant cell culture (10 g) in 0.1 M phosphate buffer solution (pH 6.4, 20 ml); and D) with homogenized plant cell culture (10g) in 0.1 M phosphate buffer solution (pH 7.0, 20 ml). In the case of method A, a substrate (50 mg) was added to the freely suspended *C. roseus* (B-5 medium, pH 5.5), *N. tabacum* "Bright Yellow-2" (MS medium, pH 5.8), *D. carota* (MS medium, pH 5.8), and *C. sinensis* (B-5 medium, pH 5.8). The mixture was shaken at 25 °C in a rotary shaker (110 rpm) in the dark. In the case of methods B, C and D, 10 g of plant cells were homogenized in 20 ml 0.1 M phosphate buffer {B (pH 6.0), C (pH 6.4), D (pH 7.0)}. A substrate (50 mg) was added to the homogenate. The subsequent procedure was the same as for

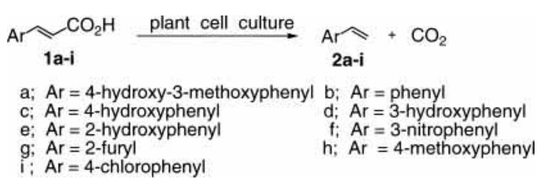

Fig. 1

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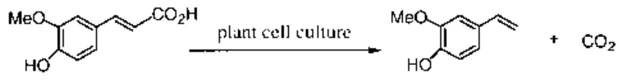
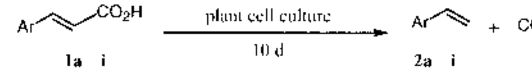
Table 1. Decarboxylation of *trans*-Ferulic Acid (**1a**) with Plant Cell Cultures



Entry	Plant cell culture	Method	Time (d)	Product **2a** C.Y. (%)	Recovery **1a** C.Y. (%)
1	*C. roseus*	A	5	Quant.	0
2	*C. roseus*	B	3	Quant.	0
3	*C. roseus*	C	3	Quant.	0
4	*C. roseus*	D	3	65	20
5	*N. tabacum*	A	5	Trace	84
6	*N. tabacum*	B	5	Quant.	0
7	*N. tabacum*	C	5	Quant.	0
8	*N. tabacum*	D	5	50	25
9	*D. carota*	A	5	0	100
10	*D. carota*	B	5	25	65
11	*D. carota*	C	5	30	65
12	*D. carota*	D	5	0	100
13	*C. sinensis*	A	5	0	100
14	*C. sinensis*	B	5	0	100
15	*C. sinensis*	C	5	0	100
16	*C. sinensis*	D	5	0	100

Table 2. Decarboxylation of Cinnamic Acid Derivatives (**1a**—**i**) with Plant Cell Cultures



Entry	Substrate	Plant cell cultures	Product **2** C.Y. (%)	Recovery **1** C.Y. (%)
1	**1a**	*C. roseus*	Quant.	0
2		*N. tabacum*	Quant.	0
3		*D. carota*	30	65
4	**1b**	*C. roseus*	10	80
5		*N. tabacum*	Trace	86
6		*D. carota*	Trace	85
7	**1c**	*C. roseus*	30	63
8		*N. tabacum*	5	84
9		*D. carota*	32	55
10		*C. sinensis*	Trace	83
11	**1d**	*D. carota*	Trace	86
12	**1e**	*D. carota*	Trace	85
13		*C. sinensis*	Trace	88
14	**1f**	*C. sinensis*	Quant.	0
15	**1g**	*C. sinensis*	Quant.	0
16	**1h**	*D. carota*	Trace	87
17		*C. sinensis*	10	76
18	**1i**	*D. carota*	10	78
19		*C. sinensis*	Trace	82

method A.

When *trans*-ferulic acid (**1a**) was subjected to plant cell culture in a medium, 4-hydroxy-3-methoxystyrene (**2a**) was given quantitatively as shown in Table 1. In the case of *C. roseus*, **1a** was quantitatively decarboxylated to **2a** not only with method A, but also with B and C (entries 1, 2, 3). But, in the cases of *N. tabacum*, the decarboxylation proceeded quantitatively with only method B and C (entries 6, 7). In the case of *D. carota*, the decarboxylation proceeded with only method B and C in 25—30% yield (entries 10, 11). In this biodecarboxylation, *C. sinensis* had no ability to convert **1a** to **2a** (entries 13—16).

In these experiments, we have succeeded in the biodecarboxylation of **1a** with homogenized plant cell culture in 0.1 M phosphate buffer solution (pH 6.4) [method C].

Next, we attempted the decarboxylation of other *trans*-cinnamic acids [*trans*-cinnamic acid (**1b**), 4-hydroxycinnamic acid (**1c**), 3-hydroxycinnamic acid (**1d**), 2-hydroxycinnamic acid (**1e**), 3-nitrocinnamic acid (**1f**), 2-furancarboxylic acid (**1g**), 4-methoxycinnamic acid (**1h**), and 4-chlorocinnamic acid (**1i**)] using method [C], as shown in Table 2. The decarboxylation of **1f** and **1g** with *C. sinensis* gave 3-nitrostyrene (**2f**) and furan (**2g**) quantitatively (entries 14, 15). In the case of **1c**, 4-hydroxystyrene (**2c**) was given in 30—32% yield by *C. roseus* or *D. carota* (entries 7, 9). But, in the case of **1d** and **1e**, decarboxylated products, 3-hydroxystyrene (**2d**) and 2-hydroxystyrene (**2e**), were obtained in trace quantities by *D. carota*. (entries 11, 12). In the case of **1b**, **1h** and **1i**, the corresponding products, styrene (**2b**), 4-methoxystyrene (**2h**) and 4-chlorostyrene (**2i**), were given in low chemical yields (entries 4, 17, 18). These styrenes (**2b**,[23] **2f**,[24] **2h**[24] and **2i**[24] and furan **2g**[25] were chemically synthesized by the decarboxylation of *trans*-cinnamic acids (**1b**, **1f**—**i**) in the presence of a copper powder in quinoline at 185—195 °C for 2—4 h (Y. >50%). A major advantage of our method is that the decarboxylation with plant cell culture proceeds mildly at room temperature.

Next, it was reported that *E*-α-phenylcinnamic acid (**1j**) was decarboxylated in the presence of $2CuO \cdot Cr_2O_3$ in quinoline at 230 °C to afford *Z*-stilbene in 75% yield.[26] Then, we tried the decarboxylation of **1j** with plant cell cultures. But *C. roseus*, *N. tabacum*, *D. carota* and *C. sinencis* cell cultures had no ability to decarboxylate **1j**.

Thus, we have developed a novel method for producing styrenes from *trans*-cinnamic acids through the biocatalytic decarboxylation by plant cell cultures. Studies are now in progress to shorten the reaction time.

Experimental

Thin layer chromatography (TLC) was performed on silica gel (Kieselgel $60F_{254}$ on aluminum sheets, Merck). All compounds were located by spraying the TLC plate with a 10% solution of phosphomolybdic acid in ethanol and heating it on a hot plate. Preparative TLC was performed on preparative layer chromatography plates (Kieselgel $60F_{254}$ 2 and 0.5 mm, Merck). Column chromatography was performed on silica gel (Kieselgel 60, 70—230 mesh, Merck).

Cultivation of *N. tabacum* "Bright Yellow-2" Cells *N. tabacum* "Bright Yellow-2" was subcultured every 7 d by transferring 1.3 ml of a 1-week culture into 80 ml of MS medium containing 2,4-D (0.2 mg/l) and 3% sucrose (pH 5.8) on a rotary shaker (110 rpm) at 25 °C in the dark.

Cultivation of *C. roseus* Cells Suspension cells of *C. roseus* were subcultured every 7 d by transferring a 1-week culture (8 ml) into B5 medium (80 ml) containing 2,4-D (1mg/l) and 2% sucrose (pH 5.5) on a rotary shaker (110 rpm) at 25°C in the dark.

Cultivation of *D. carota* Cells Suspension cells of *D. carota* were subcultured every 7 d by transferring a 1-week culture (8 ml) into MS medium (80 ml) containing 2,4-D (2 mg/l) and 3% sucrose (pH 5.8) on a rotary shaker (110 rpm) at 25 °C in the dark.

Preparation of *C. sinensis* Callus Callus tissues were prepared from seedlings of *C. sinensis*. The seedlings were rinsed with EtOH (30 s) and NaOCl (2% aqueous solution), followed by washing with sterile distilled H_2O (×5), and were inoculated onto MS medium containing 2,4-D (10 mg/l) as an auxin and 3% sucrose with 0.8% agar at 25 °C. The shoot tips after a 4- to 6-week induction period were transferred into 10 ml of fresh B5 medium (10 ml) containing 2,4-D (1.25 mg/l) and 5% sucrose. Incubation was done in a rotary shaker (110 rpm) at 25 °C in the dark. The first subcultures took place at 2 to 3 weeks. To this culture, fresh B5 medium (70 ml) containing 2,4-D (1.25 mg/l) and 5% sucrose was added. Subculturing was performed every 14 d by transferring 10 ml of 1-week-old culture into fresh B5 medium (80 ml) containing 2,4-D (1.25 mg/l) and 5% sucrose.

Biotransformation of Substrates with Method (A) A substrate (**1a**—**j**) (50 mg) was added to the freely suspended *C. roseus* (B-5 medium pH 5.5), *N. tabacum* 'Bright Yellow-2' (MS medium, pH 5.8), *D. carota* (MS medium, pH 5.8), and *C. sinensis* (B-5 medium, pH 5.8). The culture was incubated at 25°C in a rotary shaker (110 rpm) in the dark. At the conclusion of the reaction, the incubation mixture was filtered, and the filtered cells were washed with CH_2Cl_2. The filtrates were extracted with CH_2Cl_2, and the combined organic layer was washed with brine, dried over $MgSO_4$ and concentrated *in vacuo*. The residue was subjected to column chromatography on SiO_2 with CH_2Cl_2. The reaction time and the chemical yield are listed in Tables 1 and 2.

2a (Ar-CH_x=$CH_A$$CH_B$): ^{1}H-NMR: 3.92 (3H, s, OCH_3), 5.11 (1H, d, H_B, J_{BX}=8.9 Hz), 5.58 (1H, d, H_A, J_{AX}=17.8 Hz), 6.63 (1H, q, H_X), 6.81—6.95 (3H, m, Ph). ^{13}C-NMR: 55.87 (OCH_3), 108.03 (Ph), 111.43 (=CH_2), 114.34 (Ph), 120.05 (Ph), 130.26 (Ph), 136.62 (–CH=), 145.62 (Ph), 146.58 (Ph).

Biotransformation of Substrates with Method (B, C, D) Ten grams of plant cells were homogenized in 20 ml 0.1 M phosphate buffer [method B: pH 6.0, method C: PH 6.4, method D: PH 7.0] . A substrate (50 mg) was added to the homogenate. The subsequent procedure was the same as for method A.

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