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STRUCTURAL CHARACTERIZATION OF 15-HYDROXYTRICHODIENE, A SESQUITERPENOID PRODUCED BY TRANSFORMED TOBACCO CELL SUSPENSION CULTURES EXPRESSING A TRICHODIENE SYNTHASE GENE FROM *FUSARIUM SPOROTRICHIOIDES*

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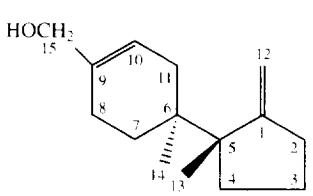
Key Word Index—*Nicotiana tabaccum*; Solanaceae; tobacco; biosynthesis; sesquiterpenoids; trichodiene.

Abstract—Tobacco (*Nicotiana tabaccum*) cell suspension cultures transformed with a gene encoding trichodiene synthase, a sesquiterpene synthase from the fungus *Fusarium sporotrichioides*, produced a novel sesquiterpenoid derived from the *in vivo* production of trichodiene. Mass and nuclear magnetic resonance spectroscopic analyses identified the new compound as 15-hydroxytrichodiene. The *in vivo* hydroxylation of trichodiene by transformant tobacco cell suspension cultures demonstrates that the introduction of a foreign sesquiterpene synthase gene can result in the production of novel sesquiterpenoid metabolites. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

Sesquiterpenoids are a diverse group of natural products. The first biosynthetic step in sesquiterpenoid pathways involves the cyclization of the general isoprenoid pathway intermediate, farnesyl pyrophosphate. This cyclization reaction is catalyzed by a group of enzymes known as sesquiterpene synthases that are responsible for specific sequiterpenoid pathways [1]. Recently, genes encoding sesquiterpene synthases have been isolated from plant [2, 3], fungal [4, 5], and bacterial [6] sources. In a previous study the effects of foreign sesquiterpene synthase gene expression on tobacco sesquiterpenoid metabolism were investigated in transgenic plants carrying a fungal sesquiterpene synthase gene for trichodiene synthase [7]. The trichodiene synthase gene used in this study was obtained from the trichothecene-producing fungus *Fusarium sporotrichioides* [4]. Subsequently, cell suspension cultures derived from the transformed tobacco plants were shown to have acquired the ability to produce a fully functional trichodiene synthase [8]. Analysis of the sesquiterpenoid products of transformed tobacco cell suspension cultures revealed the accumulation of trichodiene and an apparent trichodiene metabolite. In the present paper, we demonstrate that this metabolite is the novel sesquiterpenoid, 15-hydroxytrichodiene (**1**: [*S*-(*R**, *R**)] - 4 - methyl - 4 - (1 - methyl - 2 - methylene - cyclopentyl) - 1 - cyclohexene - 1 - methanol).



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RESULTS AND DISCUSSION

EI-mass spectral analysis of **1** showed a molecular ion of 220, a $[M - H_2O]^+$ ion of 202 and a base peak of 124; mass spectral analysis of 11-hydroxytrichodiene gave the same pattern (Susan McCormick, personal communication). The base peak can be accounted for by the loss of both the cyclopentyl ring ($M = 95$) and a proton from the hydroxyl group on the six-membered ring. Consequently, these data strongly suggested that the trichodiene methabolite is a monohydroxylated derivative of trichodiene. A comparison of the mass spectral data of **1** and 11-hydroxytrichodiene [9] also suggests that the hydroxyl group of **1** is located on the six-membered ring of the trichodiene parent compound.

Initial ^{1}H NMR spectroscopic experiments indicated the absence of a methyl proton from the corresponding trichodiene spectrum [10] and the presence of a resonance at δ 3.77 (*br*). DEPT [11] in combination with

HMQC [12] confirmed that this was due to a methylene group, and chemical shift data suggested that this peak was part of a CH_2OH moiety. Beginning with the methylene group resonance, one-dimensional TOCSY [13] spectra showed connectivity to an alkene resonance of δ 5.45. With longer mixing times, connectivities to the CH_2 groups at positions 7, 8 and 11 were also observed. In the cyclopentyl ring, 1-dimensional TOCSY (starting at one of the alkene peaks at position 12 (δ 4.75)) gave successively, with longer mixing times, signals for its geminal alkene peak (δ 4.99) and the methylenes at positions 2, 3, and 4. HMBC [14] in combination with two-dimensional TOCSY spectra was used to confirm the identity of the quaternary carbons. This was particularly useful for carbons at δ 50.8 and δ 37.5 and their assignment to their respective rings and methyl groups. These results are consistent with previously reported ^{13}C NMR spectra of trichodiene [15] and trichodiene derivatives [16].

The structural analysis of 15-hydroxytrichodiene presented in this report provides the first evidence of a biochemical modification *in planta* of a terpenoid product from a transgenic enzyme. Since trichodiene is foreign to tobacco cells, the production of 15-hydroxytrichodiene in transgenic tobacco cell suspension cultures suggests that the metabolism of trichodiene may involve a tobacco enzyme(s) with broad substrate specificity. The only sesquiterpene hydroxylases that have been characterized in tobacco are those which are involved in the production of capsidiol from 5-epi-aristolochene [17]. At present, the enzyme(s) involved in the hydroxylation of trichodiene is unknown. The biosynthesis of trichodiene and 15-hydroxytrichodiene in several different transformant tobacco cell suspension cultures has been described recently [8]. The data presented in the current report, demonstrate that the introduction of a foreign sesquiterpene synthase gene can result in the accumulation of novel sesquiterpenoid metabolites.

EXPERIMENTAL

General. NMR spectroscopic experiments were performed on Varian VXR-500 Spectrometer. Pulse programs were taken either from the standard pulse sequence library or from the Varian NMR user library.

Plant material. Tobacco cells were grown in a liquid medium of MS salts, Gamborg's B-5 vitamin mixture, 3% sucrose, 1 mg/l Mes (pH 5.8), and 1 mg/l 2,4-D. Cell cultures were subcultured on a weekly basis. Tobacco cells were used for experiments 3–4 days after subculturing. Cell suspension cultures were generated from callus cultures grown on the same media described above with 0.7% agar. The callus cultures were generated from sterilized seed from third generation individual cell lines of transgenic tobacco plants. Tobacco plants (*Nicotiana tabaccum* cv. Petite Havana) were transformed with the entire coding sequence of trichodiene synthase from *Fusarium sporotrichionides* and the CaMV (35S) promoter [7].

Extraction and isolation. 15-Hydroxytrichodiene was present mainly within transformed tobacco cells rather than in the culture media. Therefore, both the culture cells and the culture medium were extracted. Although untreated transgenic cell cultures will accumulate 15-hydroxytrichodiene (data not shown), treatment with cellulase increases the accumulation of 15-hydroxytrichodiene [8]. Fourteen hours after treatment of 300 ml of transformed tobacco cell cultures with cellulase R10 (Karlan Research Products, Santa Rosa, CA) (0.5 μg ml^{-1} of culture), the cells were harvested by suction filtration using a single sheet of pre-moistened Miracloth and immediately homogenized in 100 ml of $CHCl_3$–MeOH (2:1) using a mortar and pestle. The homogenate was filtered through Miracloth, and the retentate was washed with a fresh 20 ml portion of $CHCl_3$–MeOH (2:1). The organic filtrates were combined with the filtrate from the cell suspension culture. Chloroform (150 ml) was added to the combined filtrates, and the solution was shaken vigorously. After the phases had separated, the aqueous phase was re-extracted with 150 ml of $CHCl_3$. The organic extracts were pooled, dried over anhydrous sodium sulfate and evapd under red. pres. This process was repeated such that approximately 6 l of cellulase-treated cell suspension culture was extracted. The pooled extracts were purified by flash chromatography on a 1.0 cm × 15 cm silica gel column. Portions (100 ml) of 1, 2, 3, 5, 7, 10, 15, 20% EtOAc in hexane were applied sequentially to the column, and 20 ml frs were collected. Frs containing 15-hydroxytrichodiene were identified by TLC analysis. 15-Hydroxytrichodiene has an R_f of 0.6 on silica gel TLC plates developed with hexane–EtOAc (1:1) and gives a purplish-red color after spraying with 1% vanillin (w/v) and 6% concd H_2SO_4 (v/v) in MeOH and heating at 110°C for 3 min. Frs which contained 15-hydroxytrichodiene were pooled and evapd under red. pres. The residue was further purified by HPLC. HPLC sepns were performed at room temp. with a 4.6 mm × 250 mm silica column (Alltech, Deerfield, IL). The mobile phase of hexane–tetrahydrofuran (9:1) was pumped through the column at a flow rate of

Table 1. ^{13}C (125 MHz) and ^1H (500 MHz) spectral data of 15-hydroxytrichodiene from one- and two-dimensional experiments (in benzene-d_6 as solvent at 35°)

C	^{13}C (δ)	DEPT	^1H (δ)
1	136.5	C	
2	39.0	CH_2	2.23 *dd*, 2.12 *m*
3	37.6	CH_2	1.47 *m*, 1.73 *m*
4	28.1	CH_2	1.32 *m*, 1.24 *m*
5	50.8	C	
6	37.5	C	
7	23.6	CH_2	1.89 *m*, 1.98 *d*
8	23.5	CH_2	1.69 *m*, 1.51 *m*
9	159.6	C	
10	121.7	CH	5.45 *s*
11	33.0	CH_2	1.98 *d*, 1.69 *d*
12	107.4	CH_2	4.7 *s*, 4.90 *s*
13	24.2	CH_3	0.99 *s*
14	18.2	CH_3	0.83 *s*
15	67.1	CH_2	3.77 *br*

1.0 ml min $^{-1}$. The effluent was monitored at 210 mm. Peaks corresponding to 15-hydroxytrichodiene (retention time of 11 min) from separate HPLC runs were collected. The HPLC solvent was evaporated under a stream of N_2 prior to structural analysis. 15-Hydroxytrichodiene (60 μg) was isolated from 300 ml cellulase-treated transformed tobacco cell suspension culture.

^{1}H and ^{13}C NMR data are presented in Table 1. GC-MS (EI), *m/z* (rel. int.): $[M]^+$ 220 (2.0), 202 (1.0), 189 (5.0), 124 (100), 107 (85), 95 (75), 91 (70), 79 (92), 67 (52), 55 (65).

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REFERENCES

1. Croteau, R. and Cane, D. E. (1985) *Methods Enzymol.* **110**, 383.
2. Chappell, J. (1995) *Plant Physiol.* **107**, 1.
3. Back, K. and Chappell (1995) *J. Biol. Chem.* **270**, 7375.
4. Hohn, T. M. and Beremand, P. D. (1989) *Gene* **79**, 131.
5. Proctor, R. H. and Hohn, T. M. (1993) *J. Biol. Chem.* **268**, 4543.
6. Cane, D. E., Sohng, J. K., Lamberson, C. R., Rudnicki, S. M., Wu, Z., Lloyd, M. D., Oliver, J. S. and Hubbard, B. R. (1994) *Biochemistry* **33**, 5846.
7. Hohn, T. M. and Ohlrogge, J. B. (1991) *Plant Physiol.* **97**, 460.
8. Zook, M. N., Hohn, T. M., Bonnen, A., Tsuji, J. and Hammerschmidt, R., *Plant Physiol.* (in press).
9. McCormick, S. P., Taylor, S. L., Plattner, R. D. and Beremand, M. N. (1989) *Appl. Environ. Microbiol.* **55**, 2195.
10. Van Middlesworth, F. L. (1986) *J. Org. Chem.* **51**, 5019.
11. Doddrell, D. M., Pegg, D. T. and Bedall, M. R. (1982) *J. Magn. Reson.* **48**, 323.
12. Summers, M. F., Marzilli, L. G. and Bax, A. (1986) *J. Am. Chem. Soc.* **108**, 4285.
13. Griesinger, C., Otting, G., Wuethrich, K. and Ernst, R. R. (1988) *J. Am. Chem. Soc.* **110**, 7870.
14. Bax, A. and Summers, M. F. (1986) *J. Am. Chem. Soc.* **108**, 2093.
15. Savard, M. E., Blackwell, B. A. and Greenhalgh, R. (1989) *J. Nat. Prod.* **52**, 1267.
16. Hesketh, A. R., Gledhill, L., Bycroft, B. W., Dewick, P. M. and Gilbert, J. (1993) *Phytochemistry* **32**, 93.
17. Whitehead, I. M., Threlfall, D. R. and Ewing, D. F. (1989) *Phytochemistry* **28**, 775.