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Tobacco Chemistry 81.† Biotransformations of (*Z*)-Abienol using Plant Cell Cultures of *Nicotiana silvestris*

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The transformations of (*Z*)-abienol (**1**) effected by cell cultures and cell-free extracts of *Nicotiana silvestris* and by horseradish peroxidase have been examined. Although this compound retarded the growth of the cell cultures in low doses and exhibited a toxic effect in high doses, a fairly rapid conversion was observed when low to moderate doses of cells cultivated under light were used. Of the many transformation products encountered twelve were identified. The major ones of these were (12*R*,13*S*)- and (12*R*,13*R*)-8,12-epoxy-14-labden-13-ols (**2**, **5**), (11*E*)-14,15-bisnor-8-hydroxy-11-labden-13-one (**11**), 12-norambreinolide (**10**), 12,15-epoxy-12,14-labdadien-8-ol (**12**) and 8-drimanol (**13**), and the minor (12*R*,13*R*)-, (12*S*,13*R*)-, (12*S*,13*S*)- and (12*R*,13*S*)-8,13-epoxy-14-labden-12-ols (**6–9**) and (12*S*,13*S*)- and (12*S*,13*R*)-8,12-epoxy-14-labden-13-ols (**3**, **4**). Horseradish peroxidase also transformed (*Z*)-abienol into these products.

Labdanoids were first encountered in tobacco by Giles *et al.*[2–4] some thirty years ago. Subsequent studies have revealed that together with cembranoids and carotenoids they constitute the three major groups of tobacco isoprenoids, all of which have been postulated and shown by biomimetic transformations to give rise to important aroma constituents.[5] In order to try to verify the postulated degradation routes of the diterpenoids, the biotransformations of the two major cembranoids of tobacco, when exposed to plant cell cultures of *Nicotiana silvestris* L. and *Triterygium wilfordii*, have been studied recently.[6–8] The present investigation was undertaken with a view to obtaining information regarding the degradation routes of (*Z*)-abienol (**1**), the assumed predominant precursor of the tobacco labdanoids. It also includes studies of the role of peroxidases in the biotransformations and the effects of added (*Z*)-abienol on cellular viability and on the growth of cell suspension cultures.

(*Z*)-Abienol (**1**), which is synthesised in the trichomes on the surface of the intact tobacco leaves, undergoes a drastic reduction in concentration when the leaf is cured.[9–11] In addition to being an important precursor of many tobacco aroma constituents,[5] it has been shown to possess plant growth regulating[12,13] as well as smooth muscle relaxant properties[14] and to be inhibitory to prostaglandin synthesis (cyclooxygenase) *in vitro*.[14]

The effect of (*Z*)-abienol (**1**) on the cell viability of light cultivated cell cultures and on their growth, when applied in different concentrations, is detailed in Table 1 and Fig. 1. Although methanol, used as solvent, exhibited a certain growth retarding effect, it follows that high doses of (*Z*)-abienol (**1**) (200 and 400 μg, ml^{-1}) had a toxic effect and inhibited the growth of the cells, while low doses (50 μg, ml^{-1}) caused retardation of growth. The growth regulating effect of (*Z*)-abienol observed earlier *in vivo*[12,13] is thus also encountered *in vitro*.

The amounts of (*Z*)-abienol recovered from the cell cultures and from the Murashige-Skoog (MS) medium are given in Fig. 2. It can be seen that cell cultures grown under light degrade (*Z*)-abienol in a time-dependent manner, while neither cell cultures grown in the dark nor the substrate itself do. It seems likely, therefore, that light induces the production of enzymes capable of degrading the exogenous compound.

Twelve degradation products were identified in the extracts of the cells and the growth medium based on GC–MS studies by comparison with spectra and GC-retention times with those of authentic samples. The major components were the (12*R*,13*S*)- and (12*R*,13*R*)-8,12-epoxy-14-labden-13-ols (**2**, **5**), (11*E*)-14,15-bisnor-8-hydroxy-11-labden-13-one (**11**), 12-norambreinolide (**10**), 12,15-epoxy-12,14-labdadien-8-ol (**12**) and 8-drimanol

† For part 80, see Ref. 1.
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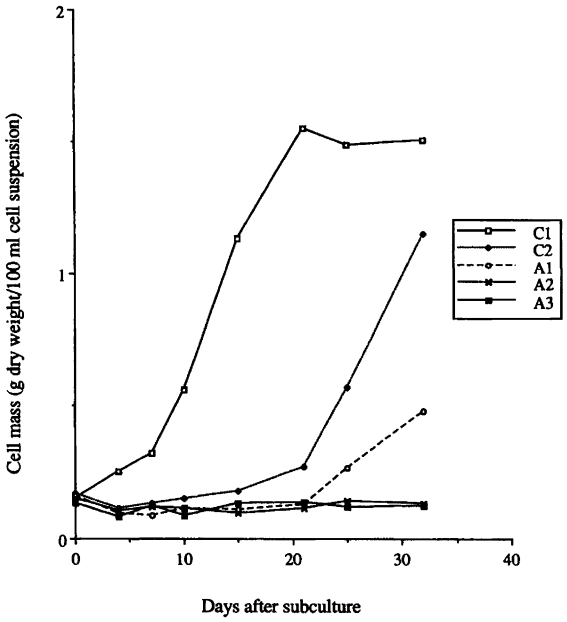


Fig. 1. Effect of exogenous (*Z*)-abienol on the growth of cultures of *N. silvestris* exposed to light. Methanol (2 ml) was added to all cell suspensions (approx. 100 ml) except C1. C1 = Control cell suspension without methanol, C2 = Control cell suspensions containing methanol, A1, A2 and A3 = 5, 20 and 40 mg (*Z*)-abienol, respectively.

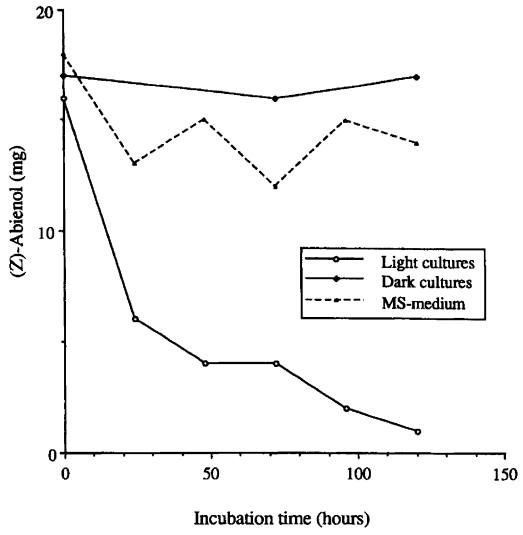


Fig. 2. Recovery of (*Z*)-abienol (20 mg) from cell cultures of *N. silvestris* growing in the dark or on a light/dark cycle, as well as in MS-medium.

(**13**). The minor ones were the (12*R*,13*R*)-, (12*S*,13*R*)-, (12*S*,13*S*)- and (12*R*,13*S*)-8,13-epoxy-14-labden-12-ols (**6–9**) and the (12*S*,13*S*)- and (12*S*,13*R*)-8,12-epoxy-14-labden-13-ols (**3**, **4**), cf., Scheme 1.

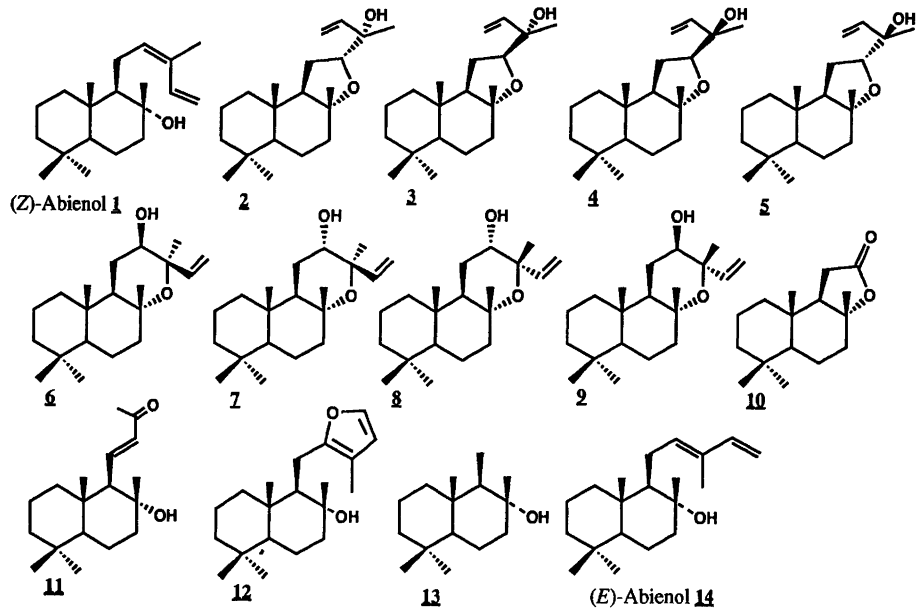
These results lend support to the previously proposed biodegradation routes based on (*Z*)-abienol (**1**) as the predominant and (*E*)-abienol (**14**) as a minor precursor of the degraded labdanoids encountered in tobacco.[5] Thus the biotransformation products **3**, **5**, **6** and **8** seem to arise via epoxidation of the 12,13-double bond in (*Z*)-abienol (**1**) and cyclisation, while **2**, **4**, **7** and **9** are likely to arise from (*E*)-abienol (**14**) by analogous reactions. This suggests that the cell cultures under study are able to convert (*Z*)-abienol (**1**) into (*E*)-abienol (**14**) either prior to or simultaneously with the enzymatic oxidative attack. The reason for this latter interpretation is based on the observation that the last four compounds (**2**, **4**, **7**, **9**) have been encountered as products only when (*E*)-abienol (**14**), but not when (*Z*)-abienol (**1**), has been subjected to epoxidation or singlet oxygen oxidation.[5,15–18] Compound **10** apparently arises from **2–5** by oxidative cleavage of the 12,13-bond and has in turn been postulated to be a precursor of 8-drimanol (**13**), while the third nor-labdanoid encountered (**11**) is apparently formed by way of a singlet oxygen type of attack on the 12,13-double bond in (*Z*)-abienol[17,18] followed by oxidative cleavage of the 14,15-double bond in the resulting doubly allylic alcohol.[5]

Since peroxidases constitute a group of enzymes likely to be involved in the conversion of exogenous compounds in plant cell cultures,[19] the peroxidase activity of the two cell lines examined (cf., the Experimental) were measured. They were found to be 0.63 unit per mg protein for the cell-free extract of cell line X and 0.31 unit for that of cell line Y. Accordingly, (*Z*)-abienol (**1**) was exposed to these cell-free extracts and also to horseradish

Table 1. Cell viability (using fluorescein diacetate) after incubation with (*Z*)-abienol.[a]

t/h of treatment	Concentration of (*Z*)-abienol (μg m^{-1})				
	Control (1)	Control (2)	50	200	400
			(viability)		
3	++++	+++	++	++	++
6	++++	+++	++	+	+
24	++++	+++	++	+	+
48	++++	++	+	±	±

[a] In all experiments, (*Z*)-abienol dissolved in methanol (10 mg ml^{-1}) was added in one batch to growing cultures. An equal volume of methanol was added to each culture except control (1). Viability (100–80%) ++++, (80–60%) +++, (60–40%) ++, (40–20%) +, (20–0%) ±



Scheme 1. Transformed products of (*Z*)-abienol by cell cultures of *N. silvestris* as well as horseradish peroxidase.

peroxidase as well as to the extraction buffer used in these experiments (potassium phosphate buffer). The results, which are summarised in Fig. 3, show that horseradish peroxidase is somewhat more efficient than either of the cell-free extracts and able to convert 63% of the added (*Z*)-abienol during 24 h of incubation. Examination of the biotransformation products derived from the ethyl acetate extract of the horseradish incubation mixture allowed identification of the same twelve metabolites as encountered earlier using the cell suspension culture. The buffer itself did not cause any major change in the amount of (*Z*)-abienol added.

It may be concluded that many of the products previously encountered in *Nicotiana* species, especially in tobacco after post harvest treatment, and resulting from biomimetic oxidations of (*Z*)- and (*E*)-abienol (**1**, **14**) involving peracid[15,16] and singlet oxygen[17,18] oxidations were produced on exposure to cell cultures of *Nicotiana silvestris* L. and to horseradish peroxidase. This in turn implies that peroxidases play an important role in the formation of these compounds in the intact plant as well as during curing and possibly also during ageing.

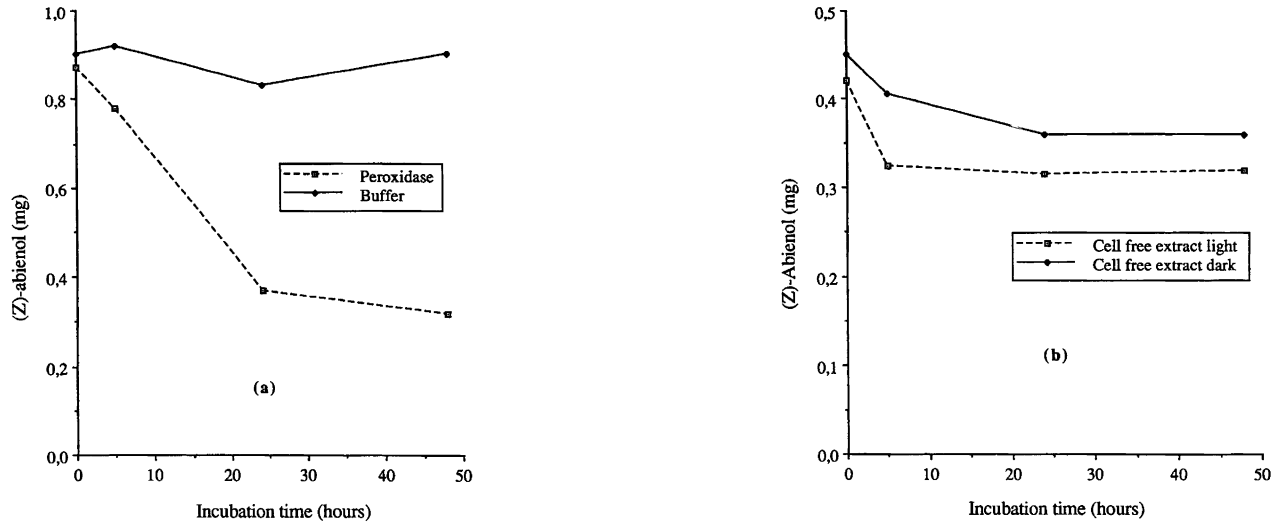


Fig. 3. (*a*) Recovery of (*Z*)-abienol (1 mg) from potassium phosphate buffer as well as from potassium phosphate buffer containing 10 units horseradish peroxidase. (*b*) Recovery of (*Z*)-abienol (0.5 mg) from cell-free extracts of light exposed and light deprived cell cultures of *N. silvestris*.

Experimental

(*Z*)-Abienol was purchased from Helic Biotech Corporation, Richmond, BC, Canada, methylene chloride (HPLC grade, glass-distilled) from FSA Laboratory Supplies, Loughborough, England, dry *N,N*-dimethylformamide (DMF) and *N,O*-bis(trimethylsilyl)trifluoracetamide (BSTFA) with 1% trimethylchlorosilane (*purum*) from Fluka Chemie AG, Buchs, Switzerland, horseradish peroxidase EC(1.11.1.7), hydrogen peroxide, guaiacol, fluorescein diacetate and heptadecanol from Sigma, St Louis, Mo, USA, and ethyl acetate from KEBO, Stockholm, Sweden.

Cell suspension cultures. Two cell lines (X and Y) were produced from calli derived from aseptic *Nicotiana silvestris* leaf material as previously described[7] and subcultured every 3–4 weeks over a period of 36 months on liquid Murashige-Skoog (MS) medium containing sucrose (3 g l^{-1}) and 2,4-dichlorophenoxyacetic acid (1 mg l^{-1}). Cell line X was kept at 24–26°C with a cycle of 8 h darkness and 16 h light (10 W m^{-2}, Philips TML 40 W 29 RS) on a rotary shaker (135 rpm, excenter 25 mm, Infors AG, Bottingen), whereas cell line Y was kept at 23–25°C in permanent darkness on a rotary shaker. The inoculation density of the cells was 1.5 g (dry weight) l^{-1}. Orienting metabolic studies on (*Z*)-abienol were performed by adding 20 mg (*Z*)-abienol to 100 ml of cell suspension in a 250 ml Erlenmeyer flask, whereas experiments aiming at establishing the structures of the metabolites were carried out by adding 100 mg of (*Z*)-abienol to 500 ml of cell suspension in a 1 l Erlenmeyer flask. The growth rate was measured as the increase in dry weight cell mass (freeze-dried) during one culture cycle (32 days). The cell viability was evaluated with the aid of fluorescein diacetate using a previously published procedure.[20]

Enzyme extraction. The cell mass obtained by filtration of a 12 day old cell suspension through a Miracloth filter (Calbiochem Corp.) was frozen in liquid nitrogen and homogenised in a precooled mortar together with potassium phosphate buffer of pH 6.1 (1 ml, g^{-1} of fresh weight cells), 1% polyvinyl polypyrrolidine and sand. The homogenate was filtered through a Miracloth filter and centrifuged at 3000*g* for 20 min at 4°C to give a supernatant, which was used for measuring the peroxidase activity employing an established procedure.[21] The activity of both cell lines (X and Y) was measured and expressed in standardised units (Sigma Chem. Co), where one unit is defined as the amount of enzyme required to convert 1 mg of substrate (pyrogallol) into product (purpurogallin) in 20 s. The calibration curve required for quantification was obtained with the aid of horseradish peroxidase.

Bioconversion of (Z)-abienol. (*Z*)-Abienol (**1**) was exposed in separate experiments carried out in duplicate to suspension cultures as well as cell-free extracts (enzyme extracts) from both cell lines and to horseradish peroxidase. A solution of this compound in methanol (10 mg ml^{-1}), was sterilised by filtration through a 0.2 μm Acrodisc filter and added in one batch to 12 day old cell cultures, which were kept on a rotary shaker for a period of 0–120 h. Similarly, a small amount (20 μl) of a filtered solution of (*Z*)-abienol in methanol (50 mg ml^{-1}) was added to the cell-free extracts (980 μl) containing hydrogen peroxide (20 mM) and kept on rotary shaker for a period of 0–48 h. The latter procedure was also used when carrying out experiments with horseradish peroxidase (10 enzyme units per mg substrate).

After termination of a cell suspension experiment, the culture medium (100 ml) was filtered through Miracloth and the cell mass washed with ethyl acetate (100 ml). The filtrate was extracted twice with ethyl acetate (150 ml) and the ethyl acetate phases combined, dried over Na_2SO_4, filtered and evaporated to dryness. The cell residue was homogenised in ethyl acetate (150 ml) using an Ultra Turrax T25 (Janke & Kunkel GmbH & Co, IKA Laboratory Technology, Germany, impeller destination S25-25F), filtered (miracloth) and the ethyl acetate phase dried (Na_2SO_4), filtered and concentrated to dryness. Each residue was dissolved in ethyl acetate (4 ml), mixed with the internal standard (heptadecanol, 1 mg) and an aliquot of the resulting mixture subjected to trimethylsilyl derivatization. The ethyl acetate was evaporated off under a stream of nitrogen and the residue treated with DMF (400 μl) and BSTFA (100 μl) for 30 min at 76°C before being subjected to GC analysis.

The enzymatic reactions were stopped by addition of 6 M HCl (10% v/v) and the mixture extracted twice with ethyl acetate (3 v/v of the mixture). The combined extracts were dried (Na_2SO_4), filtered, evaporated to dryness, and the residue dissolved in ethyl acetate and subjected to GC analysis.

Gas chromatography. The quantitative analyses were performed on a Varian GC 3400 equipped with a fused silica column (50 m × 0.32 mm) coated with SE-54 (0.25 μ phase layer, Orion Analytical, Espoo, Finland) and a flame ionisation detector. Following a procedure previously described by Severson *et al.*,[22] the initial temperature was set to and kept at 130°C for 3 min and then increased by 4° min^{-1} to 300°C, where it was maintained for 60 min. Quantification was accomplished by comparing the area under the peak due to the internal standard and that due to (*Z*)-abienol.

Gas chromatography–mass spectrometry. Low resolution mass spectra were obtained using a Kratos MS25RFA instrument interfaced with a Data General Eclipse S/280 computer operated by DS90 software and connected to a Carlo Erba Mega 5160 gas chromatograph equipped with the aforementioned column and programmed as mentioned above. The spectra obtained were compared with those of the reference compounds, as were the retention times.

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