Duvatrienediols in cuticular wax of Burley tobacco leaves

Sai Y. Chang and Claus Grunwald

Department of Agronomy, University of Kentucky, Lexington, Kentucky 40506[1], and Botany and Plant Pathology, Illinois Natural History Survey, Urbana, Illinois 61801[2]

Abstract 4,8,13-Duvatriene-1,3-diol diastereoisomers have been identified in the cuticular wax of fresh Burley tobacco leaves. Their structures were determined by gas–liquid chromatography, mass spectrometry, and infrared and ultraviolet spectroscopy. Butylboronic acid derivatives of the α,β-isomers were separated by gas–liquid chromatography and identified by mass spectrometry. The quantitative determination by gas–liquid chromatography revealed that the duvatrienediols are major components in the cuticular wax of *Nicotiana tabacum*. The duvatrienediol content in young leaves is higher than in old leaves, and in young leaves this compound may account for half of the cuticular wax.

Supplementary key words 4,8,13-duvatriene-1,3-diol • α- and β-isomers • diterpene • cuticular wax • *Nicotiana tabacum* • mass spectrometry • gas–liquid chromatography

In 1962 Roberts and Rowland (1) first isolated from cured Burley tobacco leaves 4,8,13-duvatriene-1,3-diol, a new diterpene. Duvatrienediol exists as the β and α isomers and the ratio of these isomers in Burley tobacco was 1:2. This compound with the unusually large ring resembles cembrene, which has been isolated from the pine tree (2). Since its discovery, the specific localization of duvatrienediol in tobacco tissue has not been documented. Roberts and Rowland (1) isolated the compound from a total leaf extract of dry tobacco and no attempt was made to determine whether this diterpene was an inter- or extracellular component. The purpose of this communication is to present evidence that duvatrienediol is a component of leaf wax and the age of the leaf influences the duvatrienediol concentration. Also presented is a rapid analytical procedure to determine duvatrienediol by GLC.

MATERIALS AND METHODS

Extraction of cuticular lipids

The extraction of cuticular lipids was performed essentially as described by Kolattukudy (3). Approximately 10 g of fresh tobacco leaves (*Nicotiana tabacum* L. cult Burley 21) were dipped in 200 ml of chloroform for 30 sec and excess chloroform was drained from the leaf for 10 sec. As soon as the extraction was completed, an appropriate amount of *n*-eicosane was added as internal standard for quantitative calculations. The chloroform extract was then filtered through Whatman no. 1 filter paper to remove particulate matter. The chloroform extract was concentrated to a smaller volume (ca. 10 ml) under reduced pressure at 50°C, and for routine analysis of duvatrienediol approximately 1 μg was directly injected into the gas–liquid chromatographic apparatus for quantitative determination. Total leaf wax content was determined by taking the chloroform extract to constant weight.

Isolation of α- and β-4,8,13-duvatriene-1,3-diols

The chloroform extract of cuticular lipids was dried under reduced pressure at 50°C. Fifty ml of methanol was added to the residue and then heated for 10 min at 50°C. The methanol solution was cooled to room temperature and all insoluble matter, mostly alkanes, was removed by filtration through Whatman no. 1 filter paper. The clear methanol solution was reduced to approximately 10 ml using a flash evaporator or a stream of nitrogen. The concentrated methanol solution was cooled to 0°C and held at this temperature for one hour. The precipitate formed was removed by filtration and was washed with cold methanol. The clear methanol solution was then evaporated to dryness and 10 ml of hexane–chloroform 1:1 (v/v) was added. If the residue did not dissolve completely, the mixture was warmed at 50°C for a few minutes. The hexane–chloroform solution was applied to a column of silica gel (silica gel 60, 70–230 mesh, 1.8 × 9 cm). The column was washed with approximately 300 ml of hexane–chloroform 1:1 (v/v) and 300 ml of hexane–chloroform 1.2 (v/v). At this point all traces of a migrating yellow band were eluted. Both hexane–chloroform fractions were discarded. Duvatrienediol was then eluted from the column with approximately 400 ml of hexane–chloroform 1:3 (v/v). The duvatrienediol fraction thus obtained was approximately 90% pure as judged by GLC.

Preparation of butylboronic acid derivatives and GLC conditions

Butylboronic acid derivatives of duvatrienediol were

Abbreviations: GLC, gas-liquid chromatography; MS, mass spectrometry; IR, infrared; UV, ultraviolet.

[1] Dr. Chang, present address: Department of Internal Medicine, Arizona Medical Center, University of Arizona, Tucson, Arizona 85724.

[2] Dr. Grunwald.

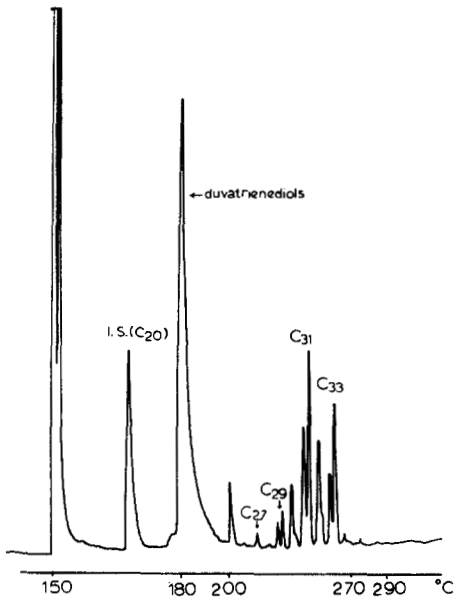


Fig. 1. GLC analysis of cuticular tobacco lipids. For routine quantitative analyses the crude chloroform extract was directly analyzed by GLC with *n*-eicosane as the internal standard. Identified are duvatrienediol and the C_{27}, C_{29}, C_{31}, and C_{33} alkanes.

formed by adding excess butylboronic acid in pyridine and heating the mixture for 15 min at 100°C. The reaction was carried out in a culture tube with a Teflon-lined screw cap (Corning No. 9826).

A F & M 402 gas chromatograph equipped with a hydrogen flame ionization detector and a Hewlett Packard electronic integrator (3370 A) was used. Alkanes, duvatrienediols, and α,β isomers of duvatrienediol butylboronic acid derivatives were separated on a 1 m glass column, 4 mm ID, packed with 1% OV-101 on Gas-Chrom Q (100/120 mesh). Injection port and detector temperatures were maintained at 250°C and 300°C, respectively, and the column temperature was programmed from 100°C to 300°C (4 min initial hold, then 6°C/min).

Identification of α- and β-4,8,13-duvatriene-1,2-diols

Mass spectra of the free diols of the α and β mixture were obtained by GLC-MS. A Varian 1400 gas chromatograph was linked to a Fannigan 1015C mass spectrometer through a glass-jet separator. A 70 eV electron beam was used to obtain all spectra. The gas chromatograph was operated by the program mode described above. The column was 2 mm ID × 1 m packed with 5% OV-101 on Gas-Chrom Q (100/120 mesh). Purified duvatrienediol was also examined by direct probe high resolution mass spectrometry (Hitachi-Perkin Elmer 1 MU-5). These spectra were compared to those obtained by GLC-MS. The butylboronic acid derivatives of the α-β-isomers of duvatrienediol were also examined by GLC-MS.

An infrared spectrum was obtained by coating a thin film of duvatrienediols on a NaCl plate. The spectrometer used

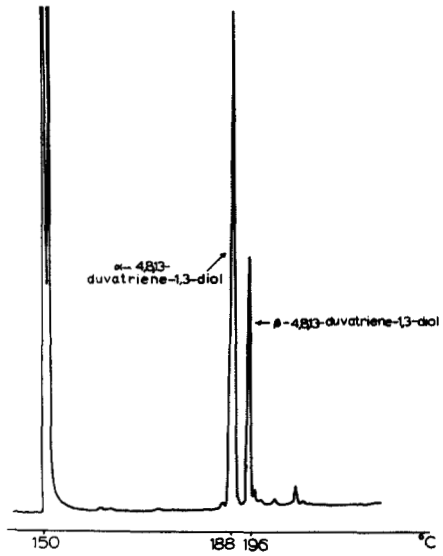


Fig. 2. GLC analysis of α- and β-4,8,13-duvatriene-1,3-diol butylboronate. Purified duvatrienediols were converted into butylboronate before GLC analysis.

was a Beckman model IR-8. A methanol solution of duvatrienediols was scanned for the UV spectrum. The instrument used was a double-beam Coleman-Hitachi-124.

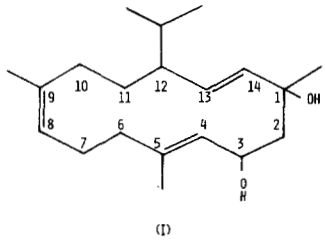
The elemental analysis was performed by Galbraith Laboratories, Inc. (Knoxville, Tennessee).

RESULTS

GLC analysis

A typical crude chloroform extract of cuticular lipids contains alkanes, fatty alcohols, fatty acids, and other compounds. **Fig. 1** shows only the alkanes and fatty alcohols, including duvatrienediol. The fatty acids are not seen, since the sample was not derivatized and the α- and β-isomers of duvatrienediol are not separated under these conditions. But under the same GLC conditions the α- and β-isomers could be separated when their butylboronic acid derivatives were chromatographed (**Fig. 2**). The ratio of β- to α-isomer in the cuticular lipids of tobacco was about 1:2. This ratio is consistent with the original work of Roberts and Rowland (1).

Purified duvatrienediol was subjected to GLC-MS and the spectrum thus obtained indicated 288 as the highest mass (**Fig. 3**). Catalytic hydrogenation of duvatrienediol gave 294 as the highest mass, which indicates three double bonds. Oxidation cleavage of double bonds with permanganate and



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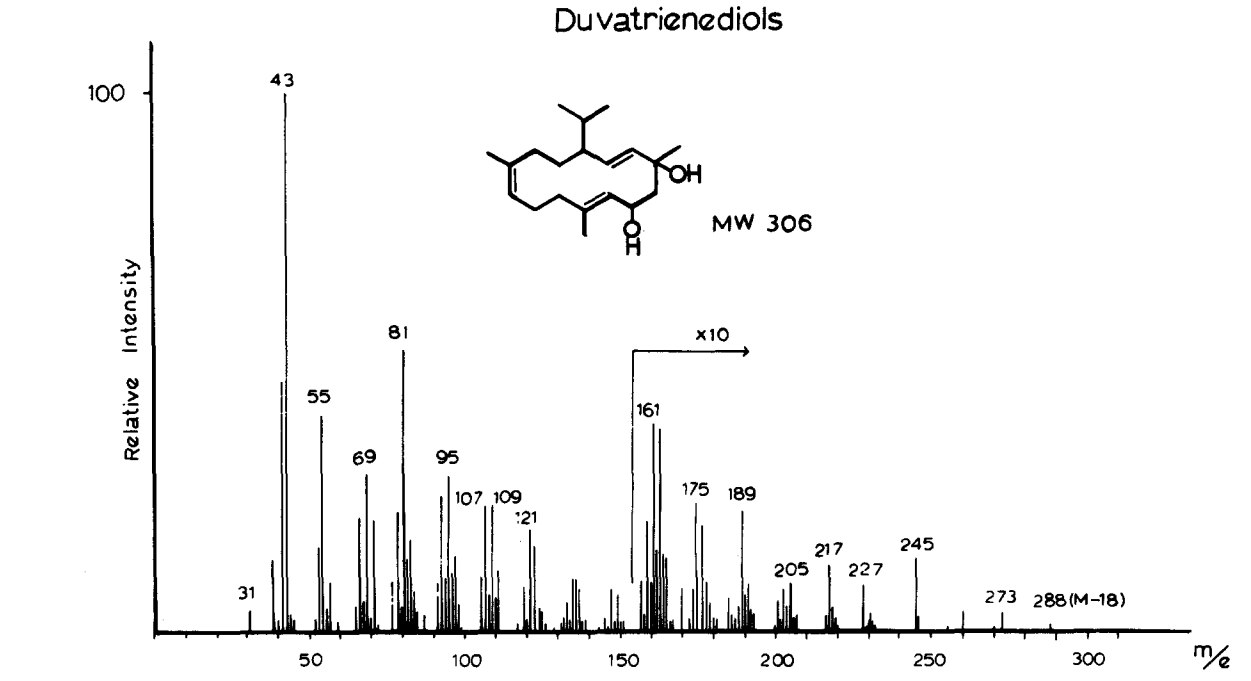

Fig. 3. MS spectrum of duvatrienediol. GLC effluent of crude chloroform extract was scanned directly by MS.

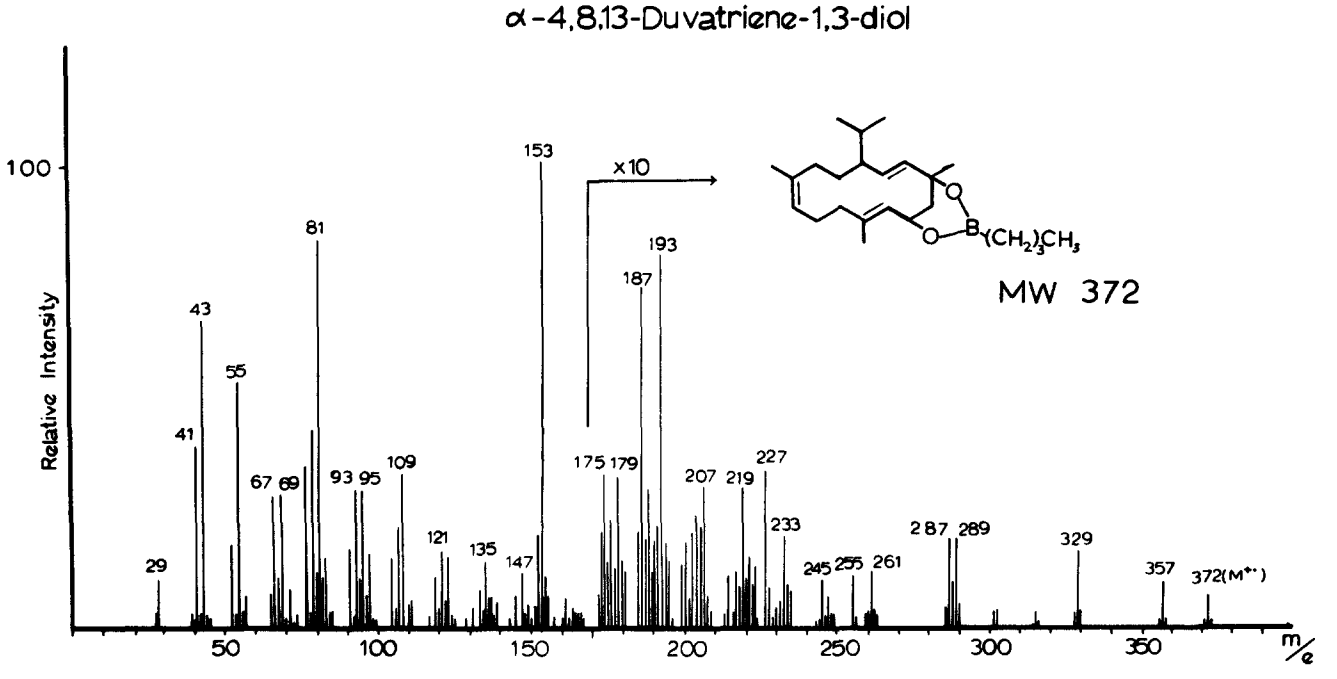

Fig. 4. MS spectrum of α-4,8,13-duvatriene-1,3-diol butylboronate, α- and β-duvatrienediols were separated by GLC and the α- and β-isomers of duvatrienediol were monitored by GLC-MS.

periodate (4) produced three major products. Thus both catalytic hydrogenation and oxidative cleavage provided evidence for three double bonds.

Duvatrienediol was converted into its butylboronic acid derivative and GLC analysis of the product gave two peaks; the highest mass for each peak was 372 (**Fig. 4**). The corrected molecular weight thus obtained is 306. The mass of 288 is not the molecular ion, but an ion created through the loss of one H_2O (18 amu) from the molecular ion of 306. The *m/e* of 153 (Fig. 4) is caused by the 1,3-hydroxyl groups of the butylboronic acid derivatives, indicative of at least two hydroxyl groups per molecule. Calculations of the $^{12}C/^{13}C$ ratios of *m/e* 270 and 288 revealed a carbon number of 20.

The IR spectrum had a strong hydroxyl band at 3330 cm^{-1}

TABLE 1. Duvatrienediol and normal hydrocarbons in cuticular waxes from fresh leaves of 10-week-old Burley 21 tobacco

	Cuticular Wax	% Weight in Cuticular Waxes	
	mg/g fr wt	*Alkanes*[a]	*Duvatrienediol*
Top leaves	1.44	17.5	46.0
Middle leaves	1.35	12.3	14.9
Bottom leaves	1.43	8.9	8.0

[a] C_{27}, C_{29}, C_{31}, and C_{33}.

and a weak band at 1665 cm^{-1}. The UV spectrum did not show absorption above 220 nm. These data are indicative of nonconjugated double bonds. The elemental analysis revealed that carbon, oxygen, and hydrogen accounted for 100% of the molecule. Thus, an empirical formula of $C_{20}H_{34}O_2$ was assigned.

All data obtained indicated that the compound isolated from cuticular lipids was 4,8,13-duvatriene-1,3-diol. Both the α- and β-isomers were identified, and the data are consistent with the findings of an earlier report (1).

Quantitative analysis of tobacco leaves for duvatrienediols

Quantitative GLC analysis of cuticular wax isolated from fresh 10-week-old Burley tobacco leaves from various stalk positions revealed that young leaves contained larger quantities of duvatrienediol than did old leaves (**Table 1**). Extracts of 6-day-old Burley 21 seedlings however, did not contain any duvatrienediol. Quantitative values for alkanes (C_{27}, C_{29}, C_{31}, and C_{33}) give a measure of the relative concentration of duvatrienediol in surface waxes. The concentration of alkanes was higher in younger leaves. Three cultivars of *N. tabacum* (Burley 21, Burley 37, and Kentucky 16) were examined and all contained similar amounts of duvatrienediol. Chloroform extracts from *N. glutinosa* leaves, however, did not contain duvatrienediol.

Better than 97% of the duvatrienediol could be extracted with a 30-sec dip in chloroform. Tobacco leaves that had their surface wax removed contained only trace amounts of duvatrienediol. Essentially, no additional duvatrienediol could be recovered even after grinding leaves in chloroform and extracting the homogenate for 6 hr. Change in fatty acid composition was used as a marker for extraction of surface lipids.

DISCUSSION

The composition of surface waxes of plants has not been thoroughly defined although it is considered to consist mainly of higher alkanes (C_{27} to C_{31}) and branched or normal long chain fatty alcohols (4–6). The present study of fresh tobacco leaves indicates that two duvatrienediol isomers are major components of leaf surface waxes, and that they occur at higher concentrations than do the alkanes (Table 1).

Duvatrienediol is an unusual macrocyclic diterpene but its localization and physiological importance and function have not been established. The surface lipids of plants can be extracted quantitatively by dipping leaves in chloroform for short periods and such techniques have revealed that duvatrienediol is part of the leaf waxes. A 10-sec dip in chloroform removed 90% of the radioactively labeled surface lipids from broccoli leaves which were fed [1-^{14}C] acetate (3). A change in fatty acid composition can also be used to distinguish between the removal of surface lipids and of intracellular lipids. The major fatty acid in fresh tobacco is linolenic acid (7), and in cuticular wax it is palmitic acid (8). Dipping young fresh tobacco leaves into chloroform for 30 sec removed very small quantities of linolenic acid, and even a chloroform dip of 2 hr removed only 3.5 μg of linolenic acid per g of fresh tissue even though tobacco leaves contain 2 to 5 mg of linolenic acid per g fresh weight (7). A 30-sec dip released 97.5% of the total duvatrienediol. In view of these data we suggest that duvatrienediol is a component of the tobacco leaf surface waxes.

Duvatrienediol has a carbon skeleton identical to that of neophytadiene except for the ring structure. A large amount of neophytadiene has been isolated from wax fractions of cured tobacco leaves, but these fractions did not consist of surface waxes only (9–11). The present attempt to extract a significant quantity of neophytadiene from the surface wax fraction was not successful.

All of the duvatrienediol appeared to be in its free diol form, since alkaline hydrolysis with 15% KOH in methanol resulted in no quantitative increase of duvatrienediol in the duvatrienediol fraction, nor an appearance of duvatrienediol in the alkane fraction. Duvatrienediol was very stable in alkaline condition; however, it was extremely labile in acidic condition and several degradation products were observed with 0.5% H_2SO_4 in ethanol. Duvatrienediol slowly decomposed under laboratory conditions and a complex mixture of products resulted. The decomposition of duvatrienediol was also observed by Roberts and Rowland (1). Attempts to form acetyl or trifluoroacetyl derivatives in basic conditions with pyridine as a solvent were not successful. Trimethylsilyl derivatives, however, were formed readily.

Young (upper) leaves had higher duvatrienediol concentrations than older (lower) leaves and almost half of the surface wax of young leaves was duvatrienediol (Table 1). The duvatrienediol concentration of young leaves was about three times as high as the alkane concentration, but in older tobacco leaves the duvatrienediol to alkane ratio was 1:1. Both the alkane and duvatrienediol concentrations decreased with leaf age; however, the duvatrienediol concentration decreased at a faster rate.

It is interesting to note that while three different varieties of *N. tabacum* (Burley 21, Burley 37, and Kentucky 16) contained similar concentrations of duvatrienediol, *N. glutinosa* did not contain any detectable duvatrienediol. Young *N. tabacum* leaves had a high duvatrienediol content, but 6-day-old tobacco seedlings, which did not have secondary leaves, contained no duvatrienediol. It is quite likely that duvatrienediol is not a major component of all leaf waxes.

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REFERENCES

1. Roberts, D. L., and R. L. Rowland. 1962. Macrocylic diterpenes. α- and β-4,8,13-duvatriene-1,3-diols from tobacco. *J. Org. Chem.* **27**: 3989–3995.
2. Nicholas, H. J. 1973. Terpenes. *In* Phytochemistry Vol. II. L. P. Miller, ed. Van Nostrand Reinhold Co. N. Y. 254–309.
3. Kolattukudy, P. E. 1973. Analysis of surface lipids. *Analabs, Inc. Research Notes.* **13**: 1–5.
4. Lemieux, R. U., and E. Rudloff. 1955. Periodate–permanganate oxidations. *Can. J. Chem.* **33**: 1701–1713.
5. Kolattukudy, P. E. 1970. Biosynthesis of cuticular lipids. *Ann. Rev. Plant Physiol.* **21**: 163–192.
6. Martin, J. T., and B. E. Juniper. 1970. Cuticles of plants. St. Martins Press Inc. N.Y. 121–156 and 222–277.
7. Tso, T. C., and H. Chu. 1970. Effect of growth, senescence, and curing on fatty acid composition of tobacco. *Agron. J.* **62**: 512–514.
8. Carruthers, W., and R. A. W. Johnstone. 1959. Composition of a paraffin wax fraction from tobacco leaf and tobacco smoke. *Nature* **184**: 1131–1132.
9. Caldicott, A. B., and G. Eglinton. 1973. Surface waxes. *In* Phytochemistry. Vol. III. L. P. Miller, ed. Van Nostrand Reinhold Co. N.Y. 162–194.
10. Rowland, R. L. 1957. Flue-cured tobacco. II. Neophytadiene. *J. Amer. Chem. Soc.* **79**: 5007–5010.
11. Wright, H. E., Jr., W. W. Burton, and R. C. Berry, Jr. 1959. Carotenoids and related colorless polyenes of aged Burley tobacco. *Arch. Biochem. Biophys.* **82**: 107–116.