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Tocopheryl acetates from the pupal exocrine secretion of the squash beetle, *Epilachna borealis* (Coccinellidae)*

A. B. Attygalle[a], S. R. Smedley[b], T. Eisner[b] and J. Meinwald[a,**]

[a]*Department of Chemistry, Cornell University, Ithaca (New York 14853-1301, USA)*
[b]*Section of Neurobiology and Behavior, Cornell University, Ithaca (New York 14853-1301, USA)*
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Abstract. The oily droplets on the pupal integumental hairs of the squash beetle *Epilachna borealis* contain a mixture of α-, β-, γ-, and δ-tocopheryl acetates as major constituents. In addition, the secretion contains a number of minor components that appear to be dehydrocongeners of the major components. This is the first report of the occurrence of acetate esters of any tocopherol in nature.
Key words. Vitamin E acetate; tocopherol; beetle; Coccinellidae; defensive secretion.

The surface of the pupa of the Mexican bean beetle, *Epilachna varivestis*, and the squash beetle, *E. borealis*, is densely covered with glandular hairs. Within hours of pupation, a tiny droplet of liquid appears at the tip of most of these hairs. Our earlier chemical investigation of *E. varivestis* showed these defensive droplets to be made up of a mixture of novel alkaloids, the azamacrolides[1]. Unexpectedly, the analogous secretion of the squash beetle was found to contain entirely different substances.

Materials and methods

Chemicals. (±)-α-Tocopheryl acetate, (±)-α-tocopherol, (+)-γ-tocopherol, and (+)-δ-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO). Samples of β-tocopherol and γ-tocotrienol were gifts of Dr Graham Burton. 5-Methyltocyl acetate, 7-methyltocyl acetate, 5,7-dimethyltocol, and α-tocotrienyl acetate were donations from Hoffmann-La Roche AG (Basel, Switzerland). Non-commercially available tocopheryl acetates were prepared from the respective tocopherols by treatment with acetic anhydride and pyridine. *N*-Methyl-*N*-bis(trifluoroacetamide) [MBTFA] was purchased from Pierce Chemical Co. (Rockford, IL).

Sample collection. Larvae of *E. borealis* were reared on a diet of zucchini squash plants (*Cucurbita pepo*, variety Milano) and allowed to pupate. Secretions from glandular hairs of one-day-(n = 50) and seven-day-(n = 50) old pupae were collected with glass capillaries, and extracted into ether or dichloromethane (50 µl). Newly shed pupal skins (n = 33) were extracted with dichloromethane (1 ml). Fresh squash leaves (2.5 g) were crushed with hexane (2 × 10 ml), and the extracts were concentrated and analyzed by gas chromatography-mass spectrometry (GC-MS). Similar GC-MS analyses were carried out after treating the evaporated extracts with MBTFA and heating the mixture for 20 min at 60 °C [2].

Analytical procedures. Samples were analyzed by gas chromatography using a Hewlett-Packard 5890 instrument equipped with a split/splitless injector and a flame ionization detector. The HP-ChemStation software program was used to acquire and integrate data. Solvent extracts were introduced by splitless injection. Low-resolution electron-ionization mass spectra were obtained using an HP 5890 gas chromatograph linked to a Finnigan ion-trap detector (ITD 800) or an HP Mass Selective Detector (MSD). Chemical-ionization mass spectra were obtained using methane as the reagent gas. Vapor phase GC/FT-IR spectra (resolution = 8 cm^{-1}) were recorded using a DB-1 coated capillary column (0.32 mm × 30 m; 250 °C for 1 min, 10 °C/min to 270 °C, 15 min) installed in a Hewlett-Packard 5890 gas chromatograph coupled to an HP 5965A IRD instrument equipped with a narrow-band (4000–750 cm^{-1}) infrared detector (mercury cadmium telluride) as described previously[3]. High-resolution GC-MS was performed on a VG 70-VSE instrument (resolution = 5000). Ultraviolet spectra were obtained using a diode-array detector (HP) linked to an HP 1090 HPLC instrument. A 100 × 2.1 mm ID ODS Hypersil (5 µm) (Hewlett-Packard) column was eluted with 10% H_2O and 90% methanol as the mobile phase.

Results

Gas chromatograms obtained from the secretion extracts, on capillary columns coated with the stationary phase DB-5, showed four major peaks representing over 98.7% of the volatile material present in the sample (fig. 1). From the mass spectra corresponding to these gas

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** Corresponding author.

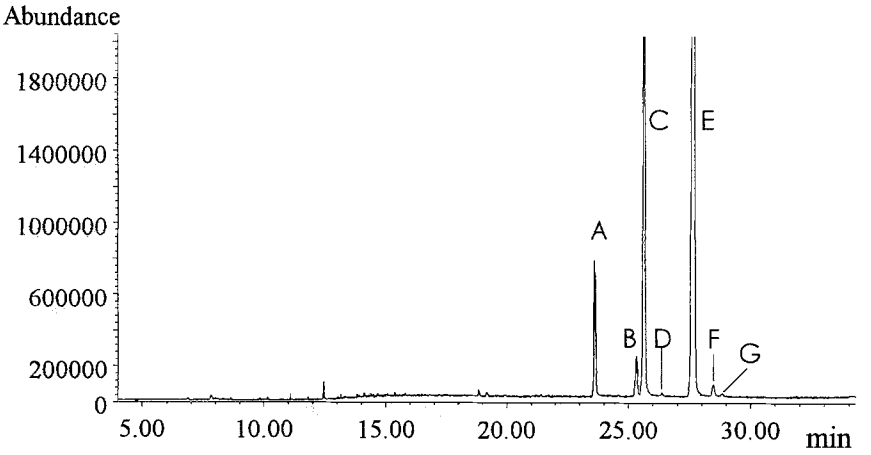


Figure 1. Reconstructed gas chromatogram obtained from dichloromethane extract of *E. borealis* pupal secretion. Refer to table 1 for peak identifications. (Mass Selective Detector; 25 m × 0.2 mm DB-5 coated fused-silica capillary; oven temperature was held at 60 °C for 4 min and programmed at 20 °C/min to 280 °C).

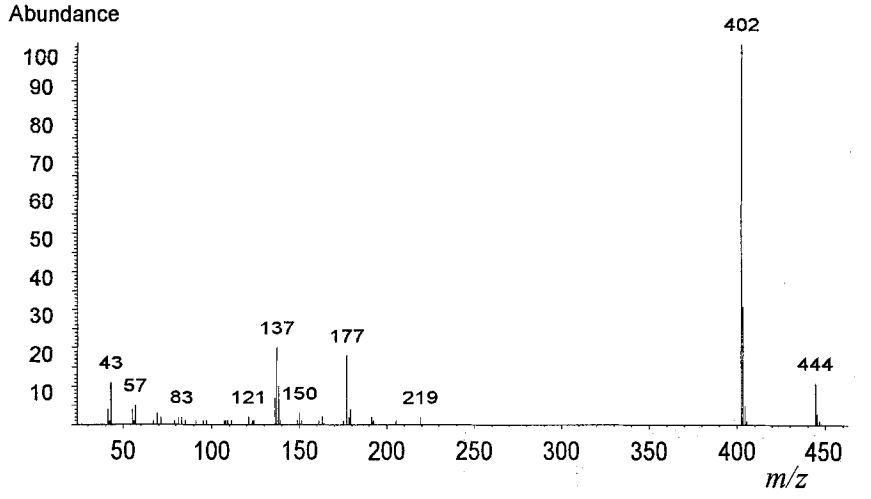


Figure 2. Mass spectrum corresponding to Peak A of the chromatogram shown in figure 1 (70 eV Electron-impact ionization; Mass Selective Detector).

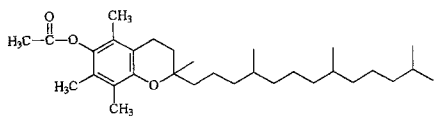
chromatographic peaks, obtained by GC-MS, it was evident that all four of these components are structurally related. The base peak in all the spectra results from the facile loss of 42 mass units from the molecular ion. The mass spectrum corresponding to gas chromatographic peak **A** (fig. 1) is depicted in figure 2 as a typical example, and the other spectra are presented in Table 1.

The mass spectrum of the major component (Peak **E**, fig. 1) showed a molecular ion at *m/z* 472 and a base peak at *m/z* 430 (corresponding to the loss of C_3H_6 or CH_2CO from the parent ion). The molecular ion at *m/z* 472 was confirmed by chemical-ionization mass spectrometry, which showed a very significant (90%) peak at *m/z* 473. The high-resolution electron-ionization mass spectrum of this compound afforded an accurate mass for the molecular ion of 472.3962 (calculated 472.3916 for $C_{31}H_{52}O_3$). Similarly, the formula of the ion at *m/z* 430 was determined to be $C_{29}H_{50}O_2$ (observed mass = 430.3864; calculated mass for $C_{29}H_{50}O_2$ = 430.3811). Thus the loss of 42 mass units from the parent ion is due to a loss of a CH_2CO group. Since the mass spectra of acetylphenols usually show a facile ketene loss (42 mass units), and since the spectrum of component **E** also showed a peak at *m/z* 43, it was evident that **E** is a phenolic acetate. In our library of mass spectra[4], there are sixteen entries for acetates of molecular weight 472. Of these, the spectrum of α-tocopheryl acetate is indistinguishable from that of **E**. This identification was confirmed by comparing the mass spectrum of **E** with that from the literature[5] and from an authentic sample. Finally, the gas chromatographic retention time and the vapor phase infrared spectrum of **E** were identical to those obtained from synthetic α-tocopheryl (vitamin E) acetate.

Table 1. Volatile components in *E. borealis* pupal secretion.

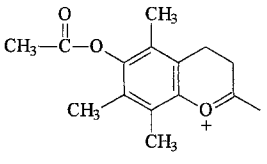
Component	Relative %	Mass spectrum, m/z (%)
A	6.90	445 (M^++1, 3), 444 (M^+, 11), 404 (5), 403 (31), 402 (100), 219 (2), 192 (1), 191 (2), 179 (4), 178 (2), 177 (17), 163 (2), 150 (3), 139 (1), 138 (10), 137 (20), 136 (6), 109 (1), 108 (1), 107 (1), 83 (2), 71 (2), 69 (3), 67 (1), 57 (5), 55 (4), 43 (11), 41 (4).
B	2.50	459 (M^++1, 3), 458 (M^+, 8), 418(5), 417(28), 416(100), 233(2), 193(7), 192(7), 191(12), 163(2),152(3), 151(16), 150(17), 71(2), 69(2), 57(4), 55(2), 43(9), 41(3)
C	30.70	459 (M^++1, 4) 458 (M^+, 11), 418 (5), 417 (30), 416 (100), 233 (2), 193 (6), 191 (9), 164 (2), 152 (6), 151 (31), 150 (8), 71 (2), 69 (2), 57 (4), 55 (3), 43 (10), 41 (3).
D	0.20	457 (M^++1, 5), 456 (M^+, 13), 431 (6), 430 (19), 417 (3), 416 (10),415 (26), 414 (100), 358 (4), 343 (2), 233 (2), 205 (2), 203 (2), 193 (6), 192 (3), 191 (14), 189 (3), 177 (2), 175 (3), 165 (7), 164 (8), 163 (3), 152 (9), 151 (53), 150 (13), 139 (2), 123 (2), 111 (2), 107 (2), 97 (2), 95 (2), 83 (2), 81 (3), 77 (2), 70 (2), 69 (5), 67 (4), 56 (2), 55 (6), 45 (2), 43 (10), 41 (8).
E	58.60	473 (M^++1, 3), 472 (M^+, 9), 432 (5), 431 (31), 430 (100), 247 (2), 207 (8), 205 (4), 166 (4), 165 (30), 164 (13), 71 (2), 69 (2), 57 (4), 55 (3), 43 (10), 41 (3).
F	0.80	471 (M^++1, 4), 470 (M^+, 11), 431 (2), 430 (9), 429 (34), 428 (100), 372 (4), 247 (2), 207 (10), 205 (6), 203 (2), 189 (2), 166 (6), 165 (50), 164 (22), 136 (2), 121 (3), 91 (2), 83 (2), 81 (2), 69 (5), 67 (2), 57 (2), 56 (3), 55 (7), 43 (6), 41 (4).
G	0.30	471(M^++1, 4), 470(M^+, 8), 452(3), 431(2), 430(3), 429(31), 428(100), 411(2), 410 (5), 281 (2), 247 (2), 245 (2), 231 (2), 209 (3), 207 (8), 206 (2), 205 (6), 203 (2), 193 (2), 191 (4), 190 (3), 189 (3), 177 (2), 176 (3), 175 (2), 166 (4), 165 (36), 164 (18), 163 (2), 159 (2), 152 (2), 151 (6), 137 (2), 136 (2), 135 (3), 125 (2), 121 (3), 95 (2), 93 (2), 83 (2), 81 (2), 79 (2) 71 (2), 70 (2), 69 (12), 67 (2), 55 (5), 53 (2), 44 (3), 43 (7), 42 (3), 41 (5).

Component designations in column 1 correspond to gas chromatographic peaks in fig. 1. 70 eV Electron ionization spectra presented in column 3 were recorded using a Mass Selective Detector.



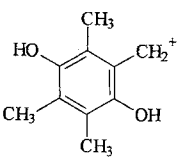
α-tocopheryl acetate (5,7,8-trimethyltocopheryl acetate)

With the identity of component **E** established, it became clear that components **A**, **B**, and **C** are lower homologues of α-tocopheryl acetate. The mass spectrum of α-tocopheryl acetate shows a weak but significant peak at *m/z* 247 that arises from the loss of the sixteen-carbon ($C_{16}H_{33}$, 225-dalton) isoprenoid side chain by an α-cleavage.

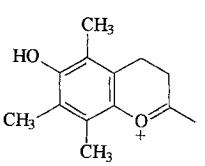


m/z 247

The spectra of **B** and **C** show a corresponding aromatic fragment at *m/z* 233, and that of **A** at *m/z* 219 (fig. 1; all resulting from loss of 225 daltons), indicating that this side chain is common to all four components. Furthermore, the spectrum of α-tocopheryl acetate shows prominent fragment ions at *m/z* 165 and 205, which can be represented as shown below.



m/z 165

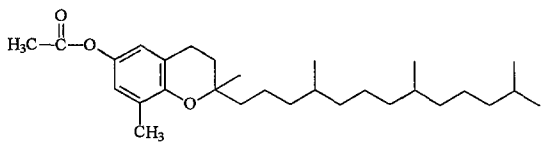


m/z 205

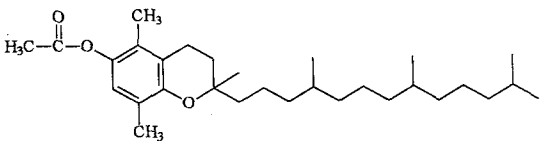
The corresponding peaks in the spectra of **A**, **B**, and **C** occur at *m/z* 137 and 177; 151 and 191; and 151 and 191, respectively. Therefore, it was clear that **A** has two aromatic methyl substituents less, and **B** and **C** one

methyl group less, than α-tocopheryl acetate. Thus, it appeared likely that **A**, **B**, and **C** are the acetyl derviatives of the other naturally occurring tocopherols.

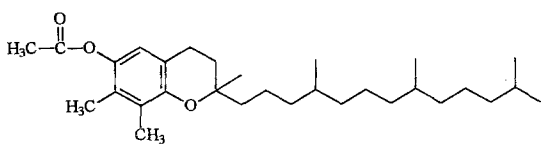
To identify **A**, we obtained the mass spectra, under identical conditions, of the three possible isomeric compounds bearing a single aromatic methyl substituent at the 5, 7, and 8 positions. These data, however, were not helpful since the mass spectra of all three isomers were virtually identical. Gas chromatography on DB-5 stationary phase showed that the retention time of 5-methyltocyl acetate is shorter than that of **A**; however, no distinction between 7- and 8-methyltocyl acetates could be made on this basis since these two isomers coelute under the conditions used. On the other hand, the vapor phase infrared spectra of the three isomers (Attygalle and Meinwald, unpublished) are significantly different from each other, and the spectrum of 8-methyltocyl acetate (δ-tocopheryl acetate) was indistinguishable from that of component **A**. Similarly, for the identification of components **B** and **C**, GC, MS, and IR data of 5,7-, 5,8- and 7,8-dimethyltochopheryl acetates were recorded from authentic samples. For these isomers, the mass spectra were once more very similar. The retention time of the 5,7-isomer was much shorter than those of **B** and **C**, while those of 5,8- and 7,8-dimethyltocopheryl acetates were identical to the retention times of component **B** and **C** respectively. Finally, the vapor phase IR spectrum of 7,8-dimethyltocopheryl acetate (γ-tocopheryl acetate) was observed to be indistiguishable from that of **C**. These data serve to identify major components **A**, **B**, **C**, and **E** definitively.



δ-tocopheryl acetate (8-methyltocopheryl acetate)

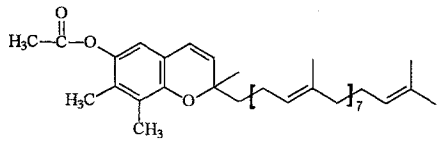


β-tocopheryl acetate (5,8-dimethyltocopheryl acetate)



γ-tocopheryl acetate (7,8-dimethyltocopheryl acetate)

From the mass spectra of minor components **D**, **F**, and **G** (Table 1), it was evident that they are novel compounds structurally related to the well-known tocopherol acetates. Components **F** and **G** are isomers with a composition ($C_{31}H_{50}O_3$) corresponding to dehydroderivatives of α-tocopherol acetate. Since the mass spectra of these two compounds do not show prominent signals at m/z 203 and 245, which would have been expected from an α-cleavage of a long saturated side chain, an additional double bond does not appear to be in the dihydropyran ring. (By contrast, the spectrum of O-acetylplastochromenol-8 shows very prominent α-cleavage peaks at 189 and 231[6]). Presumably, these minor compounds bear an extra double bond in the long side chain. Component **D**, which is similarly related to β- or γ-tocopheryl acetate, also appears to bear a carbon-carbon double bond in the side chain.



O-acetylplastochromenol-8

Additional evidence bearing on the nature of these trace components, and reinforcing our conclusions about the major compounds, was provided by ultraviolet absorption data obtained during HPLC analyses of the pupal secretion. The liquid chromatogram (with UV detection at 280 nm) showed five peaks. The retention times of the three major peaks observed at 4.68, 5.59, and 6.73 min were in agreement with those obtained from synthetic samples of δ-, γ- and α-tocopherol acetate, respectively. (Under the conditions used, β- and γ-tocopherol acetates coelute). The UV spectra corresponding to those three peaks were very similar, with a major absorption maximum at 202 nm bearing a shoulder at 224 nm, followed by a secondary absorption at 285–286 nm, indicating the presence of an aromatic system with oxygenated substituents. The spectrum corresponding to the peak at 6.73 min was identical to that obtained from an authentic sample of α-tocopheryl acetate, and similar to that reported in the literature[7]. In addition to the major three peaks, the liquid chromatogram of the pupal secretion showed two minor peaks which eluted before the major components. These two peaks, at 2.78 min and 3.46 min, may represent the aforementioned dehydrotocopheryl acetates detected by GC-MS analysis. However, the long-wavelength UV absorption maxima corresponding to these minor HPLC peaks occur at 296 nm, suggesting some significant difference in the chromophore. This difference cannot be attributed to the presence of a double bond in the pyran ring, since the introduction of a double bond causes a much larger bathochromic shift (the UV spectrum of O-acetylplastochromenol-8 shows a λmax at 314 nm). The 296 nm absorption is most simply rationalized as a result of hydrolysis of the phenolic acetate to liberate the free phenol.

These tocopheryl acetates do not appear to be reabsorbed by the insects before adult emergence, since they were found in significant amounts in dichloromethane extracts of shed pupal skins (in fact, the vapor phase infrared spectra were obtained from pupal skin extracts). Finally, hexane extracts of squash leaves were analyzed by GC-MS in order to determine whether tocopherols or their acetates are present in significant quantities in the beetles' diet. While we were unable to detect tocopheryl acetates in squash leaf extracts, low levels of free tocopherols were found by derivatization of the extract to give the corresponding trifluoroacetates. The mass spectra of these esters are highly characteristic, showing strong fragment ions at *m/z* 233 (25), 273 (51) and 498 (67); 247 (17), 287 (10) and 512 (25); and 261 (100), 301 (40) and 526 (93); for monomethyl, dimethyl, and trimethyltocyl derivatives, respectively. The presence of tocopherols in squash leaves suggests that the beetles sequester and esterify the free phenols obtained from their diet.

Discussion

We have evidence indicating that the defensive action of the exocrine secretion of *E. borealis* is attributable to components other than the tocopheryl acetates themselves. α-Tocopheryl acetate, both in feeding tests with ants (*Crematogaster ashmeadi*) and in irritancy tests with cockroaches[8], proved essentially inert. The secretion itself, however, in assays with ants, showed contact deterrence. We are currently attempting to isolate whatever component(s) might be responsible for this action. We presume the tocopheryl acetates to serve primarily as carrier molecules.

While tocopherols are widely distributed in the plant kingdom, and occur also in some animals[9], to our knowledge this is the first report of acetylated tocopherols occurring in nature. This finding is of some interest, since α-tocopheryl acetate has been considered to originate only from industrial sources. In fact, the amount of tocopheryl acetate in municipal waste has been taken as an indicator of the domestic use of α-tocopheryl-acetate-containing products[10].

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