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8-HYDROXYISOCOUMARIN AND 3,4-DIHYDRO-8-HYDROXY-ISOCOUMARIN IN THE DEFENSIVE SECRETION OF THE TENEBRIONID BEETLE, *APSENA PUBESCENS*

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Abstract—In addition to p-benzoquinones and *o*-cresol, the tenebrionid beetle, *Apsena pubescens*, produces 8-hydroxyisocoumarin and 3,4-dihydro-8-hydroxyisocoumarin in its abdominal defensive secretion. The possible significance of these two isocoumarins in the defensive exudate of this beetle is discussed.

Key Word Index: Defensive secretion, *Apsena pubescens*, isocoumarins

INTRODUCTION

THE DEFENSIVE secretions of insects have yielded a diversity of small organic compounds, which some groups seem to produce with greater virtuosity than others. The defensive secretions of tenebrionid beetles consist most commonly of 1,4-benzoquinones with or without the admixture of 1-alkenes (TSCHINKEL, 1975a), but certain taxa within this family, such as the tribe Scaurini, are distinguished by secreting other, often unique compounds, in addition to the ordinary benzoquinones (TSCHINKEL, 1975a). Thus, TSCHINKEL (1972) reported that species of the scaurine genus *Argoporis* secrete 6-alkyl-1,4-naphthoquinones as well as benzoquinones, and indicated (1975a) that species in the related genera, *Apsena* and *Eulabis*, produced distinctive unidentified, probably phenolic, compounds. *Apsena* species produce this secretion in a pair of glands lying between the 7th and 8th sternites (TSCHINKEL, 1975b), and release it by partial eversion of the gland reservoirs (TSCHINKEL, 1975c). We now wish to report the identification of 8-hydroxyisocoumarin and 3,4-dihydro-8-hydroxyisocoumarin as major constituents in the secretion of *Apsena pubescens*.

MATERIALS AND METHODS

Collection of the secretion

The secretion was obtained by 'milking' laboratory reared beetles. The abdominal exudate was absorbed on bits of filter paper which were then extracted in carbon disulphide in 1 dram screw-cap vials.

Gas chromatography and mass spectrometry

The gas chromatographic-mass spectrometric analyses were carried out on an LKB-9000 instrument at 70 eV on either of two columns, 1% (w/w) OV-17 on 80/100 mesh Supelcoport (1.8 m × 2 mm i.d.) or 10% (w/w) SP-1000 on 60/80 mesh Supelcoport (3.7 m × 2 mm i.d.).

Preparative gas chromatography

Preparative gas chromatographic separations were conducted on a Varian Aerograph Model 1200 fitted with a column (1.5 m × 4 mm i.d.) packed with 6% (w/w) Carbowax 20 M on Chromosorb W (80/100 mesh), with nitrogen flow at 75 ml/min and at a temperature of 150°C. After elution of the benzoquinones, the temperature was programmed to 225°C at 6°C per min. Under these conditions, the two unknowns eluted at 199 and 210°C respectively, and were collected in a thermal gradient micropreparative collector (BROWNLEE and SILVERSTEIN, 1968) in glass capillary tubes. The samples were then subjected to a second pass through the GLC and sealed in these tubes until they could be used for spectroscopy. After the NMR spectra were secured, the chloroform solutions were allowed to evaporate in the NMR tubes and yielded crystalline materials. The compound of lower GC retention time melted at 123–124°C, the other compound at 56–57°C.

NMR

The NMR spectra were obtained on a Varian XL-100 spectrometer equipped with a Digilab Fourier transform system.

Reduction

Sodium borohydride reductions were run by the usual procedure with ethanol as solvent. Catalytic hydrogenation was effected with hydrogen 10% palladium on charcoal, in ethanol, at room temperature and atmospheric pressure.

Derivatizations

The acetates were prepared from crude extracts with acetic anhydride in pyridine at room temperature and the trimethylsilyl ethers with bis(trimethylsilyl) trifluoroacetamide in acetonitrile.

Standard compounds

A sample of 3,4-dihydro-8-methoxyisocoumarin obtained from N. S. NARASIMHAN (NARASIMHAN and BHIDE, 1971) was refluxed for 2 hr in 48% HBr/HOAc (1:1). Examination of the reaction product by GC-MS showed that it consisted of the demethylated product and 5% of a monobromo derivative. 3,4-Dihydro-8-hydroxyisocoumarin, after purification by distillation *in vacuo*, melted at 57°C.

RESULTS

Analytical GC-MS of extracts of *Apsena pubescens* secretions on OV-17 and SP-1000 indicated the

presence of four major volatile components. The major constituents eluting at lower temperature were identified as *p*-toluquinone and *p*-ethylbenzoquinone by comparison with the GC retention times and mass spectra of authentic samples. A very small amount of *o*-cresol was also detected between these two peaks.

The other two major components had strikingly similar mass spectra (Figs. 1 and 2) with molecular ions at *m/e* 162 and 164 respectively. The first compound (M^+ *m/e* 162, 93%) exhibited three consecutive losses of 28 mass units to give ions at *m/e* 134 (base peak), *m/e* 106 and *m/e* 78. Metastables were observed for all these fragmentations which could be ascribed to the loss of the elements of carbon monoxide or of ethylene. It was also noted that the mass spectrum was almost identical to that of the natural product umbelliferone (7-hydroxycoumarin) but umbelliferone had a much longer GC retention time on the same column. The other compound (M^+ *m/e* 164, 100%) displayed an abundant ion at *m/e* 134 (M-30, 98%) and fragment ions at *m/e* 106 and 78 with corresponding metastables.

After reaction with sodium borohydride and GC-MS analysis, the crude extract still contained the two unknowns suggesting that neither possessed a ketone or aldehyde group, but after catalytic hydrogenation the compound of MW 162 had disappeared and the peak corresponding to the unknown of MW 164 had increased by a corresponding amount. Therefore, the unknowns differ by only one double bond.

Treatment of the extract with bis(trimethylsilyl)-trifluoroacetamide followed by GC-MS analysis showed that both compounds had reacted with the reagent to give products exhibiting molecular ions 72 amu higher in mass, suggesting the presence of hydroxyl group in each molecule. Both trimethylsilyl derivatives displayed a very weak molecular ion and a fragment ion at M-15 (base peak) which underwent practically no further fragmentation.

Acetylation of the extract gave peaks on GC-MS corresponding to the monoacetates of the unknowns.

The NMR spectra (Table 1) were obtained on the pure substances which were separated and collected by preparative GLC on Carbowax 20 M. Both spectra in

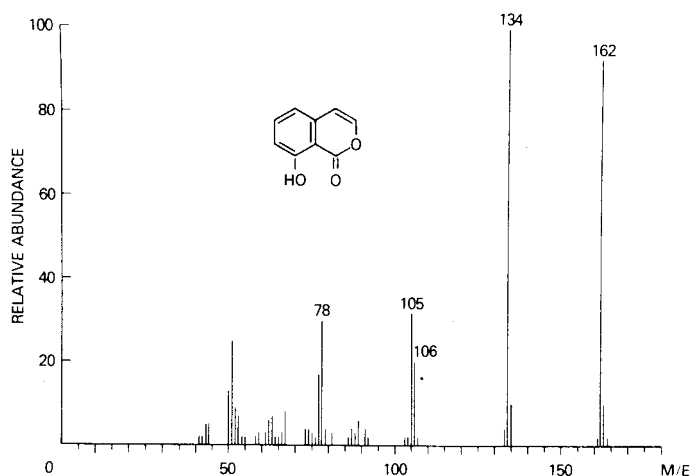


Fig. 1. Mass spectrum of 8-hydroxyisocoumarin.

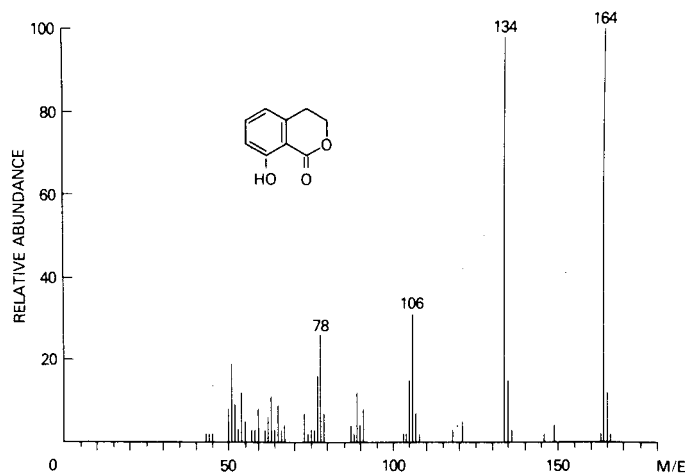


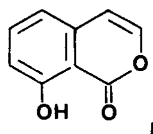
Fig. 2. Mass spectrum of 3,4-dihydro-8-hydroxyisocoumarin.

Table 1. Nuclear magnetic resonance data

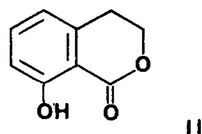
Proton	Absorption (ppm) Compound I MW 162	Compound II MW 164
H-3	1H 7.23 (d, J = 5.6 Hz)	2H 4.57 (t, J = 6 Hz)
H-4	1H 6.51 (d, J = 5.6 Hz)	2H 3.05 (t, J = 6 Hz)
H-5	1H 7.00 (broad d spaced 8 Hz)	1H 6.93 (broad d spaced 8 Hz)
H-6	1H 7.61 (broad t spaced 8 Hz)	1H 7.42 (broad t spaced 8 Hz)
H-7	1H 6.89 (broad d spaced 7 Hz)	1H 6.75 (broad t spaced 8 Hz)
Hydroxyl H	10.99 (s)	10.96 (s)
	disappears upon CD_3OD addition	

$CDCl_3$ displayed signals for three adjacent aromatic protons and a signal at low field for a hydrogen bonded phenolic proton which disappeared upon addition of CD_3OD. In addition, the spectrum of the first compound exhibited a pair of doublets at δ 6.51 and 7.23 (1H each, J = 5.6 Hz) and that of the other compound showed two triplets (2H each, J = 6 Hz) at δ 3.05 and 4.57. Integration indicated a total of six protons for compound I and eight protons for compound II.

All the above data are consistent with only one structure for each of the unknowns, namely 8-hydroxyisocoumarin (I) and 3,4-dihydro-8-hydroxy-isocoumarin (II).



I

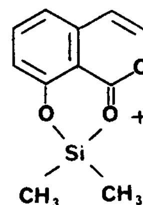


II

Neither of the two compounds is described in the literature. The dihydroisocoumarin is mentioned in a German patent (Chemical Abstract, 1975) but details of its preparation or physical properties are not given. However, 8-methoxyisocoumarin and 3,4-dihydro-8-methoxyisocoumarin have been synthesized recently for the first time (NARASIMHAN and BHIDE, 1970, 1971) and the NMR data reported for these compounds are in excellent accord with the NMRs of the corresponding hydroxyisocoumarins I and II. Treatment of 3,4-dihydro-8-methoxyisocoumarin in refluxing HBr HOAc yielded a product identical to compound II by comparison of the GC retention times, GC-MS spectra and melting points of the two compounds.

These structures also helped to clarify the interpretation of the mass spectra data. It is obvious that the loss of 30 amu from the molecular ion of compound II results from the expulsion of the elements of formaldehyde by a retro Diels–Alder reaction. The same behavior has been observed (BRAND *et al.*, 1973) in the spectrum of 3,4-dihydro-8-hydroxy-3-methylisocoumarin which loses the corresponding elements of acetaldehyde (44 amu) from the molecular ion.

The location of the hydroxyl group in the 8 position on the aromatic ring, already evident from the existence of a lowfield hydrogen bonded proton in the NMR spectra, could also have been deduced from the mass spectra of the trimethylsilyl ethers of the two compounds. The lack of further fragmentation of the M-15 ions, base peaks in the spectra of the two trimethylsilyl derivatives, can be explained by the formation of an ion such as III. Loss of a methyl group from the molecular ion and stabilization with the adjacent carbonyl give rise to the very stable cyclic ion III (BRAND *et al.*, 1973).



III

DISCUSSION

The identification of substituted isocoumarins in the abdominal glandular exudate of *A. pubescens* further underscores the natural product idiosyncracies of scaurine tenebrionids. Species in at least five genera in this tribe produce compounds other than benzoquinones in these secretions (TSCHINKEL, 1975a). The identification of 8-hydroxyisocoumarin and 3,4-dihydro-8-hydroxyisocoumarin in this *Apsena* species and 6-alkyl-1,4-naphthoquinones in *Argoporis* species (TSCHINKEL, 1972) provide strong grounds for concluding that species in different scaurine taxa produce a variety of unrelated compounds. This conclusion is also consistent with the demonstration that the defensive secretion of a species of *Scaurus* contains about fifty compounds in its abdominal secretion (TSCHINKEL, 1975a).

Although substituted isocoumarins and 3,4-dihydroisocoumarins have been widely found in plants, molds, lichens and bacteria (BARRY, 1964), these compounds have rarely been encountered as animal natural products. Indeed, mellein (3,4-dihydro-8-hydroxy-3-methylisocoumarin) is the only substituted isocoumarin which has been identified as an animal glandular product. BRAND *et al.* (1973) identified this compound in the mandibular gland secretions of three species of male carpenter ants

(*Camponotus* spp.). Mellein appears to be utilized as a sex pheromone by these ants but it is not unlikely that this compound also functions as a part of a defensive secretion when these male ants are molested.

o-Cresol also constitutes a novel product of the abdominal defensive glands of tenebrionids. The only cresol previously identified as an exocrine product of tenebrionids is *m*-cresol, the major constituent in the exudate from the prothoracic glands of *Zophobas rugipes* (TSCHINKEL, 1969). Although the presence of even a small amount of *o*-cresol in the secretion of *A. pubescens* probably increases the deterrent efficiency of this exudate, the real significance of this compound must be regarded as obscure.

The pharmacological and antibiotic activities of substituted 8-hydroxyisocoumarins range from antifungal activity (NAKAJIMA *et al.*, 1976a,b; BALLIO *et al.*, 1966), contraction of tracheal muscles (OHASHI *et al.*, 1963), effects on blood pressure (UMEZAWA *et al.*, 1972), growth stimulation and inhibition in plants (KAMEDA *et al.*, 1973; TANAKA *et al.*, 1974), antibacterial activity (BENDZ, 1959) and induction of sweet taste (YAMAMOTO and SATO, 1974). Although it is not yet possible to judge the importance of these observations to the evolution of *Apsena* defensive secretion, it is clear that the 8-hydroxyisocoumarins are broadly pharmacoactive and antibiotic substances. Perhaps the most peculiar coincidence is that the fungi *Marismius ramealis* and *M. graminum* produce the compounds 8-hydroxy-3-methylisocoumarin and 6-methyl-1,4-naphthoquinone, respectively (BENDZ, 1959), while the closely related tenebrionids *Apsena* and *Argoporis* produce 8-hydroxyisocoumarin and 6-methyl-1,4-napthoquinone, respectively. Considering the rarity of these compounds as animal natural products, it seems unlikely that this coincidence is based on chance alone. Some common factor must predispose both fungi and beetles to produce these compounds. Perhaps the biosynthetic pathways leading to isocoumarins and naphthoquinones are related, but it is not possible to interpret the presence of these compounds in *Apsena* without knowing the selective pressures which brought about their evolution. Predation is usually suggested as the primary natural selective factor for the evolution of 'defensive secretions'. Beyond some suggestive evidence that *Apsena grossa* is preyed upon by Channel Islands Fox at a lower rate than expected from its abundance (DOYEN, 1974), very little information on the predators of *Apsena* exists. The possibility that selective factors other than predation might be important cannot be ruled out.

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