

QUINONES AND NON-QUINONES FROM THE DEFENSIVE SECRETION OF *UNCIGER TRANSSILVANICUS* (VERHOEFF, 1899) (DIPLOPODA, JULIDA, JULIDAE), FROM SERBIA

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Abstract – A complex mixture of compounds was identified from the secretion of specimens of *Unciger transsilvanicus*. Phenol and *p*-cresol were detected for the first time in the family Julidae, and for the second time in the order Julida. Thirteen quinones were identified, with a great relative abundance of toloquinone and 2-methoxy-3-methyl-1,4-benzoquinone. Hydroquinone was detected for the first time in the order Julida. Besides these compounds, isopentyl hexacosatetraenoate and isopentyl esters of saturated and unsaturated fatty acids with chain lengths from C₁₄ to C₂₀ were identified. The most abundant non-quinone compound was isopentyl eicosenoate. The relative abundance of quinone and non-quinone in the defensive fluid of *U. transsilvanicus* was 77% and 23%, respectively. The phylogenetic importance of the registered compounds is briefly discussed.

Key words: Diplopoda, Julidae, *Unciger transsilvanicus*, GC-MS, defense secretion, quinones, *p*-cresol, phenol, isopentyl esters of saturated and unsaturated fatty acids from C₁₄ to C₂₀, isopentyl hexacosatetraenoate

INTRODUCTION

A wide variety of arthropod species has evolved chemical defense mechanisms that have contributed to their success in terrestrial ecosystems. Generally, the arthropod defensive compounds have relatively low molecular weights and are highly volatile. They are secreted by exocrine glands and exhibit repellent, toxic or antimicrobial activities. Defensive secretions appear in millipede orders Glomerida, Julida, Spirobolida, Spirostreptida, Polydesmida, Callipodida and Polyzoniida. The defensive components in millipedes can be grouped into alkaloids, quinones, phenols and cyanogenic compounds (Eisner et al., 1978; Makarov et al., 2010, 2011, 2012).

The millipede family Julidae includes more than 600 species and represents the dominant family of Diplopoda in the western part of the Palaearctic region (Enghoff and Kime, 2011). Unfortunately, the composition of defensive fluid has been analyzed in only 19 julid species. One of the basal julid taxons is the subfamily Oncoiulinae. The relationships of this subfamily to higher julids were explained by Enghoff (1987). This subfamily is comprised of the tribes Oncoiulini and Leocogeorgiini. The small tribe Oncoiulini includes only two genera: *Unciger* Brandt, 1841 and *Chaitoiulus* Verhoeff, 1895. The genus *Unciger* includes two species: *U. foetidus* (C. L. Koch, 1838) and *U. transsilvanicus* (Verhoeff, 1899). The chemical composition of the defensive secre-

tion of Oncoiulinae has been analyzed by Röper and Heyns only (1977) who registered two benzoquinones and two hydroquinones in the ozadenes of *U. foetidus*. However, recent studies showed that in the defensive secretions within representatives of the Julidae family, as well as in other juliform millipedes, a more complex mixture of both quinones and non-quinones exists (Vujisić et al., 2011; Bodner and Raspotnig, 2012).

In order to provide further information concerning the semiochemistry of the family Julidae, we focused this study on the gland secretion of another oncoiuline species, *U. transsilvanicus*.

MATERIALS AND METHODS

Biological material

U. transsilvanicus was collected in September–November 2010 in the Drugovačka Šuma near Smederevo, Serbia. The julids were stored in plastic boxes with a layer of litter, and kept in the laboratory for a few days at 10°C in the dark. Humidity in the boxes was kept high by spraying water on the litter every day.

Chemical extraction

Ten males and 10 females were soaked in 2 ml of dichloromethane (DCM) for 3 min. To eliminate the effects of compositing–altering oxidation and degradation of compounds, a portion of the extracts was analyzed immediately by gas chromatography–mass spectrometry (GC–MS).

Chemical analyses and identification

GC and GC–MS analyses were performed on an Agilent 7890A GC system equipped with a 5975C inert XL EI/CI MSD and a FID detector connected by a capillary flow technology 2-way splitter with make-up gas. An HP-5MSI capillary column (Agilent Technologies, 0.25 mm i.d., 30 m length, 0.25 µm film thickness) was used. The samples were injected in splitless mode and the injection volume was 1 µl. The carrier gas (He) flow rate was 1.6 ml/

min at 40°C (constant pressure mode). The column temperature was programmed linearly in a range of 40–300°C at a rate of 10°C/min with an initial 1-min and a final 8-min hold. Mass spectra were acquired in electron ionization mode (EI) with ion energy of 70 eV, and chemical ionization (CI) mode with ion energy of 150 eV. CI mass spectra were obtained in positive mode with isobutane as the reagent gas. The scan range was m/z 40–550 in EI mode and m/z 60–550 in CI mode.

A library search and mass spectral deconvolution and extraction were performed using NISTAMDIS (Automated Mass Spectral Deconvolution and Identification System) software, ver. 2.70. Our library containing more than 5 000 mass spectra (more than 100 mass spectra of defensive compounds from arthropods), and the commercially available NIST11 and Wiley07 libraries were searched. Identification of the secretory compounds was confirmed by CI–MS spectra.

The relative percentages of the identified compounds were computed from the corresponding GC–FID peak areas.

Analysis of the whole-body DCM extract showed the presence of three groups of compounds: (a) 13 quinones (I, III, V–XV), (b) phenol (II), and *p*-cresol (IV) (Table 1, Fig. 1), and (c) isopentyl hexacosatetraenoate (isopentyl ester of C₂₆:4 fatty acid), and isopentyl esters of saturated and unsaturated fatty acids with chain lengths from C₁₄ to C₂₀ (Fig. 1). We identified the following quinones: 1,4-benzoquinone (I), 2-methyl-1,4-benzoquinone (toloquinone) (III), 2-ethyl-1,4-benzoquinone (V), 2-hydroxy-3-methyl-1,4-benzoquinone (VI), 2-methoxy-3-methyl-1,4-benzoquinone (VII), 2-methoxy-1,4-benzoquinone (VIII), hydroquinone (IX), 2,3-dimethoxy-1,4-benzoquinone (X), 2-methyl-hydroquinone (XI), 2,3-dimethoxy-hydroquinone (XII), 2-methyl-3,4-methylenedioxyphenol (XIII), 2,3-dimethoxy-5-methyl-1,4-benzoquinone (XIV), and 2,3-dimethoxy-5-methylhydroquinone (XV). The relative abundances of quinones and non-quinones in the defensive fluids of *U. transsilvanicus* were 77% and 23%, respectively.

The most abundant non-quinone compound was isopentyl eicosenoate (isopentyl ester of C₂₀:1 acid), and the identification of non-quinone components was tentative.

RESULTS AND DISCUSSION

U. transsilvanicus has pairs of ozadenes laterally on nearly all pleurotergites. The structure of the ozadene is similar to that of other juliform millipedes. It consists of a more or less spherical sac, efferent duct and ozopore. Muscles attached to the duct bring about emission of the defensive secretion from the sac through the ozopore and outside the organism.

The defensive fluids of *U. transsilvanicus* contain quinone, cresol and ester fractions. The three main components in the defensive secretion of *U. transsilvanicus* are the quinones 2-methyl-1,4-benzoquinone (**III**) (56.2% of the secretion), 2-methoxy-3-methyl-1,4-benzoquinone (**VII**) (26.1% of the secretion) and 2-methyl-3,4-methylenedioxyphenol (**XIII**) (9.0% of the secretion). These three allomones have relative abundance of 91.3% in the quinone fraction (Table 1). Compounds **III** and **VIII** have also been detected as the main allomones in *Cylindroiulus boleti* (C. L. Koch, 1847), *Leptoiulus trilineatus* (C. L. Koch, 1847) and *Megaphyllum bosniense* (Verhoeff, 1897) (Vujisić et al., 2011). However, in this three species compound **VII** has the great relative abundance, while in *U. transsilvanicus* compound **III** is dominant. All other benzoquinones and hydroquinones (**I**, **V-VI**, **XVIII-XII**, and **XIV-XV**) represent minor components in the defensive fluids of the analyzed species. Similar quinone profiles were also observed in other julids analyzed by Huth (2000) and Vujisić et al. (2011). Apart from hydroquinone, all compounds of *U. transsilvanicus* represent common allomones in genera *Cylindroiulus*, *Leptoiulus* or *Megaphyllum*. Hydroquinone has been known only in the defensive fluids of Spirobolida and Spirostreptida (Wu et al., 2007; Deml and Huth, 2000). This finding represents the first record of this compound in the whole Julida order. Its presence in *U. transsilvanicus* confirms that within juliform millipedes *sensu lato* great quinone consistency is

present, supporting morphological and molecular data on their close relationships.

Probably the most intriguing chemical findings in the defensive fluids of *U. transsilvanicus* are phenol and *p*-cresol (Table 1). In Julida, *p*-cresol and phenol have been previously known only from the basal parajulid *Oriulus venustus* (Wood, 1864) (Kluge and Eisner, 1971). There are no findings of these compounds in members of the orders Spirobolida and Spirostreptida. It is well known that *p*-cresol and phenol represent main allomones in the defensive fluids of Callipodida (Makarov et al., 2011). Within the order Polydesmida both compounds have been detected in seven species belonging to different families (Shear et al., 2007; Kuwahara et al., 2011). We believe that the finding of *p*-cresol and phenol in the defensive fluids of another basal julid, *U. transsilvanicus*, may contribute to further phylogenetic analyses. According to the distribution in Julida, Polydesmida and Callipodida, *p*-cresol and phenol are likely to represent plesiomorphic gland components of three eugnathan clades. The relationship of the eugnathan clades Juliformia (Julida, Spirobolida, Spirostreptida), Merocheta (Polydesmida) and Nematophora (Chordeumatida, Callipodida, Stemmiulida) remains unresolved (Shear et al., 2007) and this semiochemical "puzzle" may be of interest in future analyses. In other words, it is probably that millipede phylogeny is somehow reflected in the specific chemistry of extant taxa, as noted for some other arthropods (e.g. Rasputnig, 2012).

The second interesting feature of the defense secretion of *U. transsilvanicus* is the considerably high abundance of non-quinones (23%). Different non-quinones, such as long chain acetates, fatty acid esters, alcohol and alkenals (Huth, 2000; Vujisić et al., 2011; Bodner and Rasputnig, 2012; Shimizu et al., 2012) were detected in 18 of the 26 chemically scanned members of the order Julida. This is the first report of isopentyl hexacosatetraenoate and isopentyl esters of saturated and unsaturated fatty acids with chain lengths from C₁₄ to C₂₀ in the millipede in general. The most abundant isopentyl ester was isopentyl eicosenoate. It is interesting that isopentyl acetate

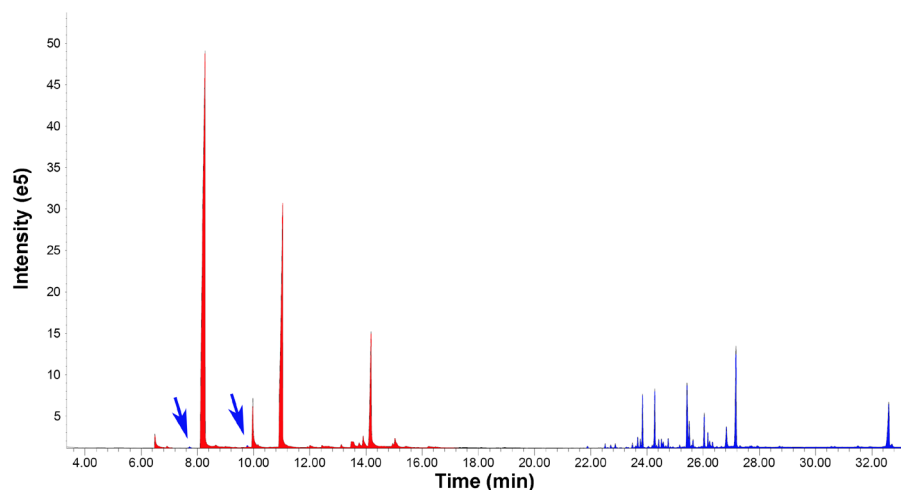


Fig. 1. GC-FID profile of DCM extracts of julid millipede species *Unciger transsilvanicus* (Verhoeff, 1899), (a) red color = quinones, (b) blue color = non-quinones (arrows indicate phenol and *p*-cresol).

Table 1. Chemical composition of the cresol and quinone components of defensive secretions in *Unciger transsilvanicus* (Verhoeff, 1899) analyzed by GC-FID and GC-MS.

t_R (min) ^a		Compounds	Relative abundance (%) ^b
			<i>Unciger transsilvanicus</i>
6.5	I	1,4-benzoquinone	1.3
7.7	II	phenol	0.1
8.2	III	2-methyl-1,4-benzoquinone	56.2
9.3	IV	<i>p</i> -cresol	T ^c
9.8	V	2-ethyl-1,4-benzoquinone	0.1
10.0	VI	2-hydroxy-3-methyl-1,4-benzoquinone	3.2
11.0	VII	2-methoxy-3-methyl-1,4-benzoquinone	26.1
12.0	VIII	2-methoxy-1,4-benzoquinone	0.2
12.4	IX	hydroquinone	0.4
13.1	X	2,3-dimethoxy-1,4-benzoquinone	0.2
13.4	XI	2-methylhydroquinone	1.0
13.9	XII	2,3-dimethoxyhydroquinone	0.9
14.1	XIII	2-methyl-3,4-methylenedioxyphenol	9.0
14.5	XIV	2,3-dimethoxy-5-methyl-1,4-benzoquinone	0.1
15.0	XV	2,3-dimethoxy-5-methylhydroquinone	1.2

^aObtained from GC-MS data

^bCalculated from GC-FID peak areas

^cTrace (less than 0.1%)

is known as attractant, pheromone, allomone, and even kairomone, in one Nematoda and a couple of Diptera, Coleoptera and Hymenoptera species (Terrill and Dusenbery, 1996; Brockmann et al., 2006).

Identifying new non-quinones in the defensive secretion of *U. transsilvanicus* raises two questions

connected with millipede semiochemistry, at least in julids. Firstly, does the millipede defensive mixture in addition to its repellent function also have a protective role? It is known that quinones function as repellents or toxicants for numerous potential predators. However, non-quinonic components registered in different millipedes are known as important mol-

ecules in other arthropods' intraspecific communications. It is possible that the same molecules in Diplopoda have similar functions to those in other soil animals. Secondly, do non-quinones represent chemotaxonomic markers? A recent report showed diversity in julid non-quinones (saturated fatty acid esters with dominant *n*-Hexyl laurate in *Anaulaciulus* sp.; aliphatic alkenals, heptenal, octenal, nonenal, decanal, with dominant (*E*)-2-octenal in *Allajulus dicentrus* (Latzel, 1884); long chain acetates in *Blaniulus guttatus* (Fabricius, 1798); hexyl ester of alkanolic acid and derivatives of unsaturated fatty acids in some representatives of genera *Cylindroiulus*, *Enantiulus*, or *Julus*; or hexyl esters of saturated and unsaturated C₁₄-C₂₀ fatty acids in some members of the genera *Cylindroiulus*, *Leptoiulus*, or *Megaphyllum*). Such variety of non-quinonic profiles in different julid taxa may represent important chemotaxonomic potential.

Intraspecific comparison within the genus *Unciger* is limited. Röper and Heyns (1977) detected four compounds in the defensive fluids of *U. foetidus*: 1,4-benzoquinone, 2-methoxy-3-methyl-1,4-benzoquinone, 2-methyl-1,4-hydroquinone and 2-methoxy-3-methyl-hydroquinone. This is a considerably smaller number of compounds than in *U. transsilvanicus*, which is probably the result of the high sensitivity of modern equipment used for characterization of the chemical composition of defensive secretion in *U. transsilvanicus*. It is interesting that we did not identify 2-methoxy-3-methyl-hydroquinone in the analyzed species. This compound has recently been detected in a few millipedes from the genera *Cylindroiulus*, *Leptoiulus*, and *Megaphyllum* (Huth, 2000; Vujisić et al., 2011). For the time being, with the limited number of analyzed species it is difficult to evaluate the importance of the absence of 2-methoxy-3-methyl-hydroquinone in the analyzed species.

To summarize, herein we demonstrate the presence of a complex mixture of defensive fluids in the analyzed species. Three groups of compounds were identified: quinones, cresol-related compounds and non-quinones. Quinones have a repellent function and their diversity in *U. transsilvanicus* generally is

common in other analyzed julid species. The first finding of phenol and *p*-cresol, as well as esters, in a member of the family Julidae has phylogenetic importance. Finally, this study shows that the detected compounds in *U. transsilvanicus* are not only protective chemicals, but may have additional functions, at least in intraspecies communication.

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