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Diplodomica I. Chemical Composition of repugnatorial secretions of Cuban endemic millipede gen. *Rhinocricus* sp. A case of Applied Chemical Ecology †

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Abstract:
The composition of the repugnatorial secretion of some populations of the Cuban endemic millipede *Rhinocricus* inhabiting in the western region of the Cuban archipelago has been developed. Several quinonoids metabolites were identified. From some individuals (males), collected in forest and karst regions, 850 μg of a deep brown-red secretion were obtained, and directly introduced into analytical instrumental (GC-MS and FTIR). The analysis of FTIR and GC-MS spectra shows that major components of secretions are substituted hydroquinones, 1,4-benzoquinones and aldehydes. The biological effects of these defensive secretions were evaluated on pathogenic microorganisms, showing interesting antimicrobial action. The ecological and evolutionary significance of chemical differentiation in millipedes populations are under study.

Keywords: millipede; defensive secretion; chemical composition, quinonoids; antimicrobial; chemical ecology.

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1. Introduction

Advances in the chemistry of natural products isolated from terrestrial invertebrate and their chemical ecology, via metabolomics and chemogenomics, offer new opportunities for bioprospecting of biodiversity in searching for new molecular entities with potential bioactivity and industrial applications, starting from minimum quantities of biological material including defensive secretions against predators or any ecological disturbance. The biodiversity of the (edaphic) fauna of terrestrial invertebrates of the Cuban archipelago has not been properly chemo-prospected; the efforts, presented here, are oriented to the isolation, structural elucidation, molecular mapping and strategic development of leading molecular systems based on secondary metabolites that would constitute a new potential pharmacological, agrochemical and structural entities (NPE + NAE +

NSE). There are no reports detailing chemical ecology, biological activity and structural-compositional aspects of defensive secretions of Millipedes inhabiting in different eco-geographical regions of Cuban tropical archipelago [1-3]. The reported results constitute the first chemical analysis of repugnatorial ejections isolated and collected from individuals of some populations of the Cuban endemic millipede gen. *Rhinocricus* sp., inhabiting in the wester zone of the Cuban neotropical archipelago. Their microbiocidal action *vs.* microbial pathogens is reported.

2. Materials and Methods

All reagents used were supplied by MERCK and were used without prior purification.

2.1. IR spectroscopy

The infrared spectra were recorded on a PHILIPS ANALYTICAL FTIR PU-9600 spectrophotometer; the samples were prepared in potassium bromide (KBr) tablets at 25°C and silicon discs. Alternatively, the spectra were recorded in a JASCO-Canvas 4600, Japan system in CsBr tablets at 25 °C

2.2. NMR spectroscopy

NMR spectra were recorded on a BRUKER AC-250 instrument at 25 ° C. The protonic chemical (δ) shifts are given in ppm, using tetramethylsilane as internal reference (TMS, δ = 0.0) and as a solvent $CDCl_3$. The chemical shifts (δ) for 13C refer to the central peak of the $CDCl_3$ solvent at 77.03 ppm.

2.3. Gas chromatography coupled to mass spectrometry (GC-MS)

A Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA, USA) with 5973 quadrupole detection system (GC-MS) was used. The separations were carried out through a capillary column of Ultra 2 type (J & W Scientific, USA), 12 m long and 0.22 mm of internal diameter. As carrier gas was used He at a flow of 1 mL/min. Temperature ramp: 60 ^{0}C with increments of 10 ^{0}C/min up to 300 ^{0}C (isothermal 5 min.). Run time 30 min. Injection volume 2 μL at a temperature of 280 ^{0}C, in split mode (1:10 ratio). The ionization source was IE at 70 eV operating at 230 ^{0}C. Acquisition mode: Full Scan. Range of m / e 40-700.

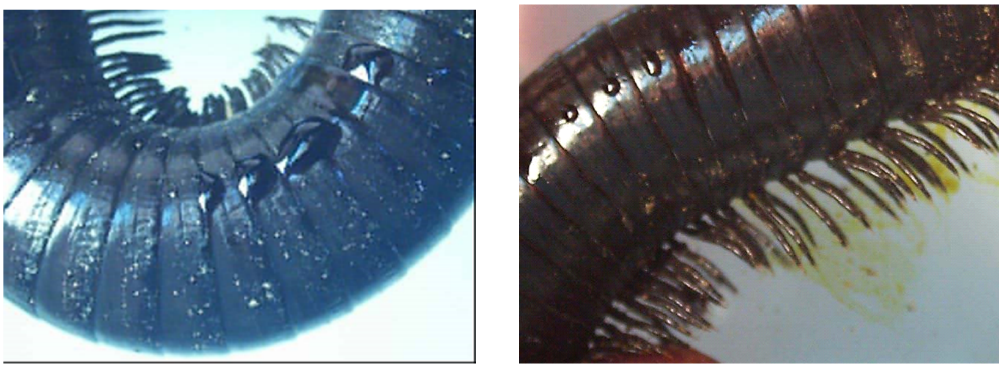
The following databases were used for structural characterization: Nist98 (National Institute of Standards and Technology, USA), PMW_TOX2 (Wiley Library and Pfleger Maurer Weber (PMW), National Metrology Institute of Japan (NMIJ); and National Institute of Advanced Industrial Science and Technology (AIST). Also, the databases where the chemical composition of the invertebrate defensive secretions (www.pherobase.com), and their GC-MS (Libraries for the rapid identification of metabolites in complex biological samples, Max- Planck Institute of Plant Molecular Physiology, Germany) are reported were considered. Using the reported sources and m/e data from the GC-MS, the most likely structures and their fragmentation mechanisms are postulated.

2.4. Biological material

Adult individuals (males) of the millipede species gen. *Rhinocricus* sp. (Diplopoda, order Spirobolida, fam. Rhinocricidae) were collected in the following sites (6, forest and karst regions): Yumurí Valley (Matanzas), Banao (Sancti Spíritus), La Coca (Habana, Florido Field: 82008'14 ″O; 230 09 '22″ N), Isla de La Juventud (820 49' 14 ″O; 210 52 '18″ N), La Palma (Pinar del Río: 830 33' 15 ″O; 220 45 '24″ N), Cuevas de Alamar (Habana) during the months of May-July 2015, 2016, 2017 and 2018. The specimens were kept in the Edaphic Fauna Laboratory of the Faculty of Biology, University of La Havana, Cuba and in the Organic Chemistry Laboratory of the Faculty of Science & Technology at Technical University of Esmeraldas, Ecuador (voucher 001-003).

2.5. Extraction and Isolation

To obtain the defensive secretions (ejected by the ozopores located in the dorso-lateral part of the millipede´s body) of intense reddish-brown coloration, the male individuals are stimulated by gently pressure. The secretion (650-800 µg / individual) is absorbed on Whatman 40 filter paper and cooled to -10 ^{0}C. The filter paper is extracted with diethyl ether (5 x 2 mL) and the ether extracts are concentrated with N_2 flow (g) to dryness and stored at -10 ° C in 1 mL Eppendorf vials, and sealed with parafilm for further analysis. To each vial, with the dry extract, DCM (1mL) is added, the mixture is stirred, filtered through 0.45 µm frit, and injected into the GC-MS (2-20 µl). The collection of the secretion is depicted in Figure 1 (a,b).



(**a**) (**b**)

Figure 1. (**a**). droplets of secretion onto dorso-lateral surface of millipede´s body ready for collecting *via* glass capillary; (**b**) collecting millipede defensive secretion *via* filter paper

2.6. Bioassay

For the detection of antimicrobial activity, the defensive solution (0.5-1000 µg / mL) was placed in Petri dishes containing nutrient agar, aseptically adding the inoculum of the species of pathogenic microorganism to be analyzed in filter paper discs (4 mm diameter). The plates were incubated (72 h) at room temperature and the minimum inhibitory concentrations (MIC) were calculated.
The general protocol is depicted in the Figure 2.

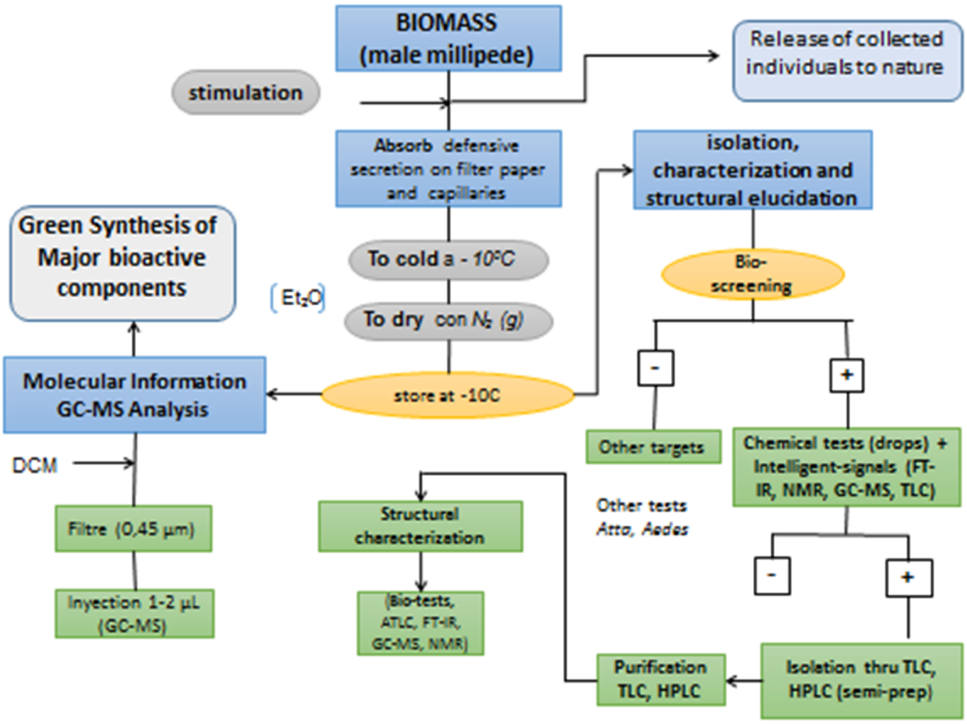


Figure 2. General protocol for studying defensive secretions isolated from Cuban endemic millipede gen. *Rhinocricus* sp., inhabiting in the western zone of the Cuban archipelago

3. Results

The analysis of the chemical composition of defensive secretions of terrestrial invertebrates facilitates the generation of a structural data of great significance for modeling and design studies not only of metabolic pathways, but also optimizes the synthesis of NSE and NPE with potential application in various areas of human activity, increasing the added value (intellectual, heritage and technology) of biodiversity as an eco-sustainable source of secondary metabolites, the result of applying the chemo-bioprospecting to biodiversity (Cuba and, at present, Ecuador), facilitate the understanding of the need for its systemic conservation.

Due to the absence of molecular information and structural knowledge about the chemical composition of the repugnatorial secretions of the Cuban endemic millipedes (gen. *Rhinocricus* sp), which constitute the fundamental component of the Cuban edaphic fauna biomass, and their possibility of application in obtaining broad-spectrum micro-biocidal agents, it was decided to study, preliminary, these secretions collected from different population which inhabiting in the wester eco-geographical zone of the Cuban tropical archipelago.

The analysis, by thin layer chromatography (chromogenic reactions on SiO_2 plates doped with oxalic acid and silver nitrate), of the repugnatorial secretions, collected *in situ*, of the populations under study of millipedes of the gen. *Rhinocricus* sp revealed that all defensive secretions are poly-component mixtures. The uses of specific reagents suggest the presence of quinonoid metabolites (phenols and benzoquinones) with a certain degree of substitution. (Figure 3).

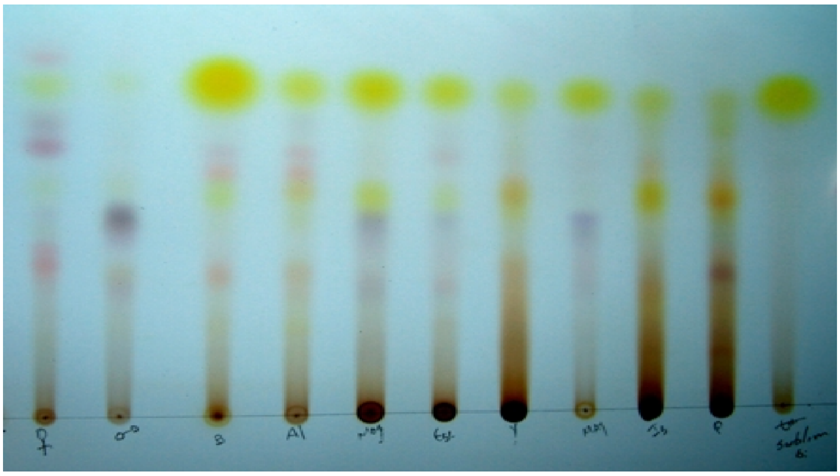
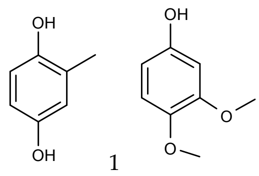
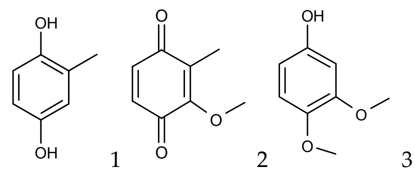
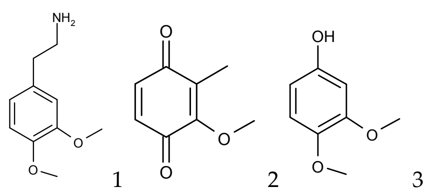


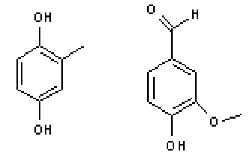
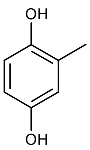
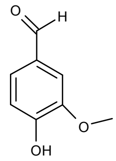
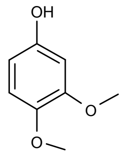
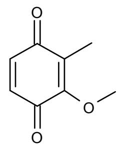
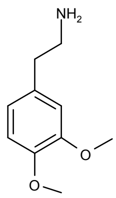
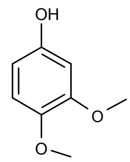
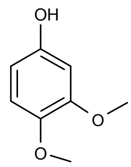
Figure 3. Thin Layer Chromatography (TLC) of the defensive secretions of millipede gen. *Rhinocricus* sp, collected at the 6 collection sites of the study

The compositional-structural analysis, by GC-MS and FTIR, of the millipede defensive secretions of the gen. *Rhinocricus* sp. that inhabits the western eco-geographical zone of the Cuban archipelago, collected in the 6 described sites (*vide supra*) revealed a great heterogeneity both in composition and in structural variations and molecular patterns.

The results of the GC-MS analysis are reported in Table 1.

Table 1. Secondary Metabolites detected in millipede defensive secretions from 6 populations under study of gen. *Rhinocricus sp.* inhabiting the Cuban archipelago

Collection site	**Components**	**Retention time (Rt, min.) and % in the defensive secretion**
Isla de la Juventud		1. 2-methylhydroquinone **Rt** = 2,622 min. (38 %) 2. 3, 4-dimethoxyphenol **Rt** = 3,226 min. (61, 8%)
Valle de Yumurí		1. 2-methylhydroquinone **Rt** = 2,622 min. (33, 6 %) 2. 1-methyl-2-methoxy-1,4-benzoquinon /(traces only) 3. 3, 4-dimethoxyphenol **Rt** =3,226 min. (66 %)
Alamar		1. Phenyl-3,4-di-methoxy-ethyl-1-amine **Rt**= 5,352 min. (25 %) 2. 1-methyl-2-methoxy-1,4-benzoquinon **Rt**=5,464 (26%) 3. 3, 4-dimethoxyphenol **Rt** =3,226 min. (47 %)

	 4 & 5	4. 2-methylhydroquinone /(traces only) 5. 4-hydroxy-3-methoxy-benzaldehyde/(traces only)
Banao	 1  2  3  4  5	1. 2-methylhydroquinone **Rt**=2,622 min. (3,2%) 2. 4-hydroxy-3-methoxy- benzal-dehyde **Rt** =2,909 min. (5,3%) 3. 3, 4-dimethoxyphenol **Rt**=3,240 min. (10,6%) 4. 1-methyl-2-methoxy-1,4-benzoquinon **Rt**=5,374 min. (26,6%) 5. Phenyl-3,4-di-methoxy-ethyl-1-amine **Rt**= 5,330 min. (51%)
La Palma	 1	1. 3, 4-dimethoxyphenol **Rt**=3,246 min. (100%)
La Coca	 1	1. 3, 4-dimethoxyphenol Rt= 3,388 min. (0.9 %) The main components are hydrocarbons C_{11}-C_{30}

Secondary Metabolites detected in millipede defensive secretions from 6 populations under study of gen. *Rhinocricus sp.* inhabiting the Cuban archipelago

The results of the *in vitro* bioassays to evaluate the microbiocidal action *vs.* pathogenic microorganisms of the defensive secretions are reported in Table 2

Table 2.

Microorganism	Toxicological doses MIC
Fonsecae pedrosi	MIC = 6,0 µg /mL
Candida albicans	MIC = 30,0 µg /mL
Microsporum gypeseum	MIC = 78,0 µg /mL
Microsporus canis	MIC = 56,3 µg /mL
Trycophyton mentagraphytes	MIC = 27,0 µg /mL
Epidermophyton flocossum	MIC = 68,0 µg /mL
Trycophyton rubrum	MIC = 44,0 µg /mL

Microbiocidal evaluation of defensive repugnatorial secretions from *Rhinocricus* sp. vs. pathogenic microorganisms

4. Discussion

The taxonomic description of millipede species (endemic and regional) that inhabit the Cuban archipelago reveals the existence of aprox. 75 species for central and eastern geographical areas of Cuba. In Cuba there are very few reports on secondary metabolites, the general chemical composition (majority or minority) or their concentration ratios for different species, sub-species of millipedes, and their populations. In a preliminary approach, trying to apply, roughly, the concept of integrating, and structurally populating, the chemical space [4] of defensive secretions of endemic millipedes of the Rhinocricidae family and populations of endemic genus *Rhinocricus* sp, the possibility of studying metabolic profiles (chemical-compositional) of the defensive secretions of various populations of millipedes that inhabit the western part of Cuba was evaluated.

The theoretical basis on which we based our decision was:

- Endemic character of the described species (gen. *Rhinocricus*) and geographically differentiated populations (70-450 Km).
- Paleo-geological and phytogeographic patterns (native populations and their variations in the last 25-30 x 10^6 years) that frame high endemism between botanical species and terrestrial invertebrates and considerations on structural singularity (chemistry) derived from the origins of invertebrate Cuban biota (continental connections).
- Possibility of correlating morphology-metabolism / secondary metabolite-eco-geographic patterns, in the search for specific chemo-types or biomarkers that facilitate the systematic classification of the species or sub-species of millipedes (including gen. *Rhinocricus* sp).
- Applicability of secondary metabolites isolated and extracted from defensive secretions from millipedes gen. *Rhinocricus* such as NSE-NPE as molecular resource for advanced applied chemistry and its systemic integration to conservation strategies

Based on the results described, a molecular distribution pattern of quinonoid molecular systems (substituted phenols and 1,4-benzoquinones) can be inferred. This is represented, as *molecular mapping*, in the Figure 4

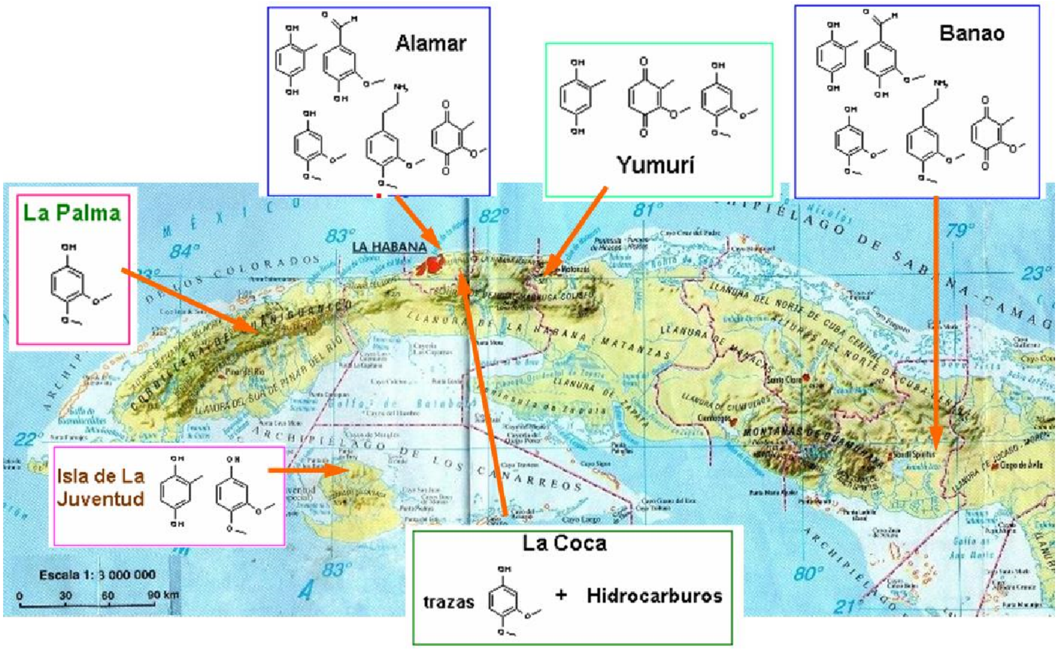


Figure 4. *Molecular mapping* of compositions of defensive secretions collected from millipedes gen. *Rhinocricus* sp., inhabiting in the wester zone of Cuban archipelago

In our study, a great variability and heterogeneity have been observed in the substances that constitute the defensive secretions of the millipedes gen. *Rhinocricus* sp in the populations under study (intra-specific variation). According to [5-7], there may be variations, both inter-specific and intra-specific, depending on sex, age, stage of biological development, climatic season, population or the evolution of the secretion mechanism, in addition to the diet of individuals, which adds even greater variability to the chemistry of secondary metabolites of repugnatorial secretions [8]. Also, within the same family or class if there is any degree of geographic isolation, variations in the chemical composition of their secretions are favored with respect to the major components because the predators (invertebrates and vertebrates) are not the same, nor their ecological pressure, in two geographically distant areas [9].

The insular conditions of Cuba favor evolutionary processes of rapid differentiation at the molecular level (concentrational variations of major components and oxidative enzymatic systems at the mitochondrial level) that take place in short periods of time in small eco-geographical spaces reaching great molecular heterogeneity. The study of the geographic distribution patterns and variations of the compositions in the repugnatorial secretions of diplópodos (gen. *Rhinocricus* sp) reveals tendencies of the intra-species chemical micro-evolution and its dependencies *vs*. ecological, biotic and edafo-climatic parameters.

The mapping (molecular spacial distribution) of the obtained results reveals a significant geographical heterogeneity of the chemotypes observed in the defensive secretions of millipedes in the western area of the Cuban archipelago and the major components identified and quantified for each population of the *Rhinocricus* species.

5. Conclusions

It was evidenced the existence of eco-geographical variations and potential chemotypes in 6 populations of millipede gen. *Rhinocricus* sp. that inhabit in Cuban tropical archipelago (West eco-geographical region) The five chemotypes determined were: Yumurí Valley-Isla de la Juventud (two components, 31/33 and 61.8 / 66% respectively); Alamar-Banao collection sites (5-9 components with remarkable differences in secondary metabolite concentrations); La Palma (1 component, 100%, phenolic nature); Coca (mixture of aliphatic hydrocarbons with traces of substituted phenols); and Yumurí Valley (quinona >90%). Two new molecular entities for defensive secretion of endemic Spirobolida gen. *Rhinocricus* sp. in Cuban tropical archipelago (Banao and Alamar collection area) were determined: 2-(3,4-dimethoxy-phenyl)-ethylene-1-amine and 4-hydroxy-5-methoxy-benzaldehyde (vanillin). The *molecular mapping* of compositions of the defensive secretions isolated from *Rhinocricus* sp. populations revealed the existence of a singular compositional heterogeneity (aromatic, benzoquinone and hydrocarbon nature components with specific substitution patterns) in the western area of the Cuban archipelago. Repugnatorial secretions showed interesting microbiocidal activity *vs*. pathogenic micro-organisms [10, 11]. Studies on the evolutionary and chemical significance of these poly-component mixtures are currently under development.

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Conflicts of Interest: The authors declare no conflict of interest.

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