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First Biosynthetic pathway of 1-hepten-3-one in *Iporangaia pustulosa* (Opiliones)

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Arthropods produce a great variety of natural compounds, many of which have unexplored biosynthesis. Among the armored harvestmen (Arachnida: Opiliones) of the suborder Laniatores, the defensive gland exudates contain vinyl ketones and other constituents of supposed polyketide origin. We have studied the biosynthesis of 1-hepten-3-one in the Neotropical harvestman *Iporangaia pustulosa* by feeding individuals with ¹³C-labeled precursors, demonstrating its mixed acetate/propionate origin. ¹³C NMR spectroscopy showed an unusual labeling pattern suggesting different propionate sources for starting and extender units. Our analysis also indicates the presence of methylmalonyl-CoA mutase, converting acetate into propionyl-CoA via succinyl-CoA, together with other C₃ unit routes. This is the first biosynthetic study of alkyl vinyl ketones in arthropods. Our results shed light on the origin and diversification of chemical compounds in a major arthropod group.

Arthropods produce a great variety of natural compounds that are used in inter- and intra-species chemical communication. Although the identity of these info-chemicals have been intensively explored over the last 60 years¹, their biosynthesis remains vastly unexplored when compared to plants and microorganisms². Moreover, most biosynthetic studies with arthropods focus on insects, and only a few on other major arthropod groups such as arachnids, whose species may produce venoms, repellent exudates, and pheromones¹. Arachnids of the order Opiliones, for instance, have a pair of exocrine glands that release a great variety of odorous compounds³ (Fig. 1) used as alarm pheromones⁴ and defense against predators^{5–7}. However, their biosynthetic pathways have never been investigated.

Among the armored harvestmen of the suborder Laniatores, the gland exudates contain mixtures of benzoquinones, alkylated phenols, or alkyl vinyl ketones^{8,9}. Considering the phylogeny of the Neotropical family Gonyleptidae, in particular, the production of benzoquinones is the ancestral state, with at least four independent losses of these compounds¹⁰. The production of vinyl ketones, on the other hand, is derived and represents a synapomorphy of a major clade composed of five subfamilies and the production of alkyl-phenols is also derived and evolved at least five times independently¹⁰. The biosynthetic pathways of these gland exudate constituents in harvestmen may bring information on the putative origins and diversification of chemical compounds within this major arachnid group.

In a previous report, we detected 1-hepten-3-one (**1**) as the major component in the scent gland exudates of the gonyleptid harvestman *Iporangaia pustulosa*⁹. Similar vinyl ketones were also found in other gonyleptid harvestmen belonging to the clade known as K92^{8,10}. Here we describe the biosynthetic pathway of **1**, revealing the condensation of Pr + Ac + Pr units to produce a polyketide chain. This pathway depends on a methylmalonyl-CoA mutase and other enzymes comprising an ensemble of parallel propionate/methylmalonate metabolic routes. Further, we revealed that there are different sources of C₃ depending on the unit role of starter or extender. To our knowledge, this is the first biosynthetic study of an alkyl vinyl ketone in arthropods.

Results

Feeding and analysis. Four ¹³C-labeled precursors, denoted [¹³C₃]propionate, [4-¹³C]methylmalonate, [1-¹³C]acetate and [1-¹³C]glucose, were added to the diet of *Iporangaia* individuals (males and females) and their incorporation into **1** was monitored by ¹³C NMR spectroscopy. The vinyl ketone **1** produced by *Iporangaia* individuals in each feeding experiment showed specific incorporation of the precursors.

Figure 1 | Female of the harvestman *Iporangaia pustulosa* releasing two white droplets of scent gland exudate in the lateral margins of the carapace. The photo was taken by Prof. Glauco Machado.

[$^{13}\text{C}_3$]propionate incorporation. The enrichment by [$^{13}\text{C}_3$]propionate is given by the integration ratio of the satellite lines by the sum of all carbon signals^{11,12} (Fig. 2, Table 1). Positions C-1, C-2, C-5, C-6 and C-7 were labeled, and at C-1 and C-2 the enrichment was weaker. All labeled positions showed increased satellite lines.

[4- ^{13}C]methylmalonate, [1- ^{13}C]acetate and [1- ^{13}C]glucose incorporation. For each carbon signal of **1** in the ^{13}C NMR spectrum, the ratio of the signal height was calculated using one non-enriched position (C-4 for [1- ^{13}C]acetate and [4- ^{13}C]methylmalonate; C-5 for [1- ^{13}C]glucose), furnishing the R value. The $r = R_{\text{labeled group}}/R_{\text{control group}}$ was calculated for all the positions of **1**. The enrichment at each site was obtained by multiplying r by 1.1, which is the natural abundance of ^{13}C (Table 2)^{11,12}. Experiment with [4- ^{13}C]methylmalonate produced **1** enriched at C-1 (1.3%), C-6 (1.4%) and C-7 (1.5%) (Supplementary Data S3). Individuals fed on [1- ^{13}C]acetate produced **1** showing increased intensities at C-1 (2.5%), C-3 (3.0%), C-5 (1.6%) and C-6 (1.3%) (Supplementary Data S4). [1- ^{13}C]glucose incorporation yielded **1** with isotopic enhancement

Table 1 | Enrichment of 1-hepten-3-one (**1**) from the harvestman *Iporangaia pustulosa* after [$^{13}\text{C}_3$]propionate incorporation

C	% ^{13}C - $^{13}\text{C}^a$	J c-c	
		(Hz)	Position
1	15.25	66.85	2
2	16.17	66.87	1
3		Not enriched	
4		Not enriched	
5	39.18	27.51	6
6	48.63	34.73	7
		32.39	5
7	45.06	34.55	6

at C-2, C-4, C-6 and C-7 of 1.3% (Supplementary Data S5), with a lower global incorporation and ^{13}C NMR signal enhancement than observed in [1- ^{13}C]acetate feeding experiment. The sample was too diluted and the carbonyl signal was not observed.

Discussion

Given that the biosynthesis of secondary metabolites of harvestmen had never been studied before, we used Morgan's suggestion² of an aceto-propionin origin for aliphatic ketones structurally similar to **1** as a first approach. To test this hypothesis, we selected ^{13}C NMR spectroscopy and ^{13}C -labeled precursors (acetate and propionate), which allowed the exact position of the unit incorporation into the intact molecule of **1**. *Iporangaia pustulosa* has a defense secretion with **1** as major constituent, allowing ^{13}C NMR analysis of the crude exudate, with the additional advantage of surviving feeding experiments in the laboratory.

Feeding *Iporangaia* individuals on [$^{13}\text{C}_3$]propionate yielded **1** labeled at C-1, C-2, C-5, C-6 and C-7 (Table 1). The ^{13}C enrichment increased the intensities of the satellites and reduced the central signal of the singly labeled isotopomers. The doublets at C-7 and C-5 and double doublet at C-6 have characteristic ^{13}C - ^{13}C coupling constants, revealing the intact incorporation of the [$^{13}\text{C}_3$]propionate precursor at these positions (Fig. 2, Table 2). Satellite signals (doublets) were also enhanced at C-1 and C-2 indicating the incorporation of a second [$^{13}\text{C}_3$]propionate unit and loss of one labeled carbon. The ^{13}C enrichment at C-1 and C-2 (~15%) was less effective than at C-5, C-6 and C-7 (~44%), suggesting alternative C_3 units for initiating (propionate) and extending (methylmalonate) the polyketide chain

Figure 2 | Results of the experiment in which *Iporangaia pustulosa* individuals were fed [$^{13}\text{C}_3$]propionate. (a) ^{13}C NMR spectrum (125 MHz, CDCl_3) of the exudate containing **1** as major compound. (b) Extended signals of ^{13}C enriched positions showing ^{13}C - ^{13}C coupling patterns.

Table 2 | Labeling and ^{13}C enrichment of 1-hepten-3-one (1) from feeding of *Iporangaia pustulosa* individuals with labeled precursors

		[1- ^{13}C] acetate	[4- ^{13}C] methyl-malonate	[1- ^{13}C] glucose
C	δ (ppm)	% ^{13}C	% ^{13}C	% ^{13}C
1	127.9	2.5	1.3	Not measured ^a
2	136.6	0.9	0.4	1.3
3	201.2	3.0	0.7	Not measured ^b
4	39.4	1.1	1.1	1.3
5	26.1	1.6	1.1	1.1
6	22.8	1.3	1.4	1.3
7	13.9	0.9	1.5	1.3

Signals in bold type represent significant incorporation in groups 1, 2 and 3.

^aC-1 signal was overlapped by the solvent signal.

^bC-3 signal not visualized due to sample dilution and it was considered not enriched.

of 1. In the same way, [4- ^{13}C]methylmalonate incorporation yielded labelling at C-1 and C-7, in agreement with two C_3 units incorporated into the polyketide chain of 1. C-3 and C-4 showed no ^{13}C incorporation from C_3 labeled precursors, suggesting that these two carbons arise from an acetate unit. In fact, when [1- ^{13}C]acetate was incorporated into the diet, the C-3 signal increased 3% (Table 1) and [1- ^{13}C]glucose incorporation enriched C-4 in 1.3% (Table 1). This last result was expected as [1- ^{13}C]glucose is first metabolized producing two acetate units, one unlabelled and one labelled at C-2 after a TCA cycle yielding a weaker enrichment.

This labeling pattern indicates that biosynthesis of 1 occurs via polyketide synthases (PKS) notwithstanding the lack of evidence of their presence in arthropods (see review in ref. 13). On the basis of the PKS mode of action¹⁴ we propose assembling to initiate with a propionyl-CoA (C_3), which is extended by a malonyl-CoA (C_2) and a methylmalonyl-CoA (C_3) (Fig. 3). Hydrolysis of the thioester and decarboxylation yields the saturated ketone precursor of 1 (Fig. 3c). The introduction of the terminal double bond of 1 can be assigned to an unusual desaturase (Fig. 3d), but this mechanism was not assessed in our experiments. Known desaturases in insects do not insert double bonds at the terminal nor at α , β -carbonyl positions². A saturated analog of 1 (4-methyl-3-hexanone) was isolated from the ant *Harpegnathos saltator* and also revealed a mixed acetate-propionate origin by heavy isotope labeling¹⁵. Among arachnids, the storage mite *Chortoglyphus arcuatus* was shown to add one acetate and four propionate units to produce the aggregation pheromone (4R,6R,8R)-4,6,8-trimethyldecan-2-one (chortolure)¹⁶.

A closer examination of the ^{13}C NMR spectra from labeling experiments with [4- ^{13}C]methylmalonate, [1- ^{13}C]acetate and [1- ^{13}C]glucose revealed intriguing labeling patterns relative to the C_3 units (Fig. 4). The unexpected labeling of 1 at C-6, by adding [4- ^{13}C]methylmalonate to the diet of *Iporangaia* individuals suggests the existence of a methylmalonyl-CoA mutase. The isotopic enhancement at positions C-6 (1.5%) and C-7 (2.3%) complies with the methylmalonyl-CoA-succinyl-CoA interconversion, leading to [2- ^{13}C] and [3- ^{13}C]propionyl-CoA biosynthesis as consequence of the equilibrium succinyl-CoA-succinate¹⁷ (Fig. 5a1). Stronger enrichment at C-7 indicates the incorporation of [3- ^{13}C]propionyl-CoA and the enrichment at

Figure 4 | Labeling pattern of 1 by feeding *Iporangaia pustulosa* individuals with ^{13}C labeled precursors. Colored ball indicate ^{13}C labeled positions. Green signals means expected and magenta means unexpected labeling. The ball sizes correspond to ^{13}C enrichment (%).

C-1 (1.9%) is provided by direct incorporation of [4- ^{13}C]methylmalonyl-CoA as the second extender unit (Fig. 5c). Moreover, labeling of 1 at propionate units with [1- ^{13}C]acetate incorporation (Fig. 4) suggests the conversion of acetate into propionate/methylmalonate. The presence of methylmalonyl-CoA would yield [1- ^{13}C]methylmalonyl-CoA (Fig. 5c), undetectable due to the loss of labeled carbon, as depicted in Figure 3c. Methylmalonyl-CoA mutase was previously reported in polyketide biosynthesis of insects^{18–20} and other arthropods²¹, suggesting that 1 from *I. pustulosa* possesses similar biosynthetic origin.

Enrichment at C-5 (1.6%) and C-6 (1.3%) are similar with the [1- ^{13}C]acetate feeding, suggesting that both labelings arise from the same pool of starting units, either [1- ^{13}C] or [2- ^{13}C]propionyl-CoA. The biosynthesis of the C_3 unit from C_2 via α -oxidation and decarboxylation of butyrate has been reported for pheromone biosynthesis of *Carpophilus* beetles (Nitidulidae)²², however this mechanism is not in accordance with the observed labeling pattern. Chan et al.²³ reviewed the sources of propionate in several microorganisms and, in the crotonyl-CoA carboxylase/reductase pathway, the acetate is converted into propionate after condensation of two acetyl-CoA units yielding a butyrate²⁴. The presence of a crotonyl-CoA mutase pathway in *I. pustulosa* would explain [1- ^{13}C]acetate labeling of 1 at C-5 and C-6, which corresponds to [1- ^{13}C] and [3- ^{13}C]propionate incorporation (Fig. 6a). Enrichment at C-1 of 1 by feeding on [1- ^{13}C]acetate corresponds to the incorporation of a [3- ^{13}C]methylmalonyl-CoA extender unit, whose origin can be linked to methionine and/or threonine catabolism via acetate (Fig. 6c).

[4- ^{13}C]methylmalonate incorporation enriches positions C-6 and C-7 of 1, corresponding to a C_3 starter unit while the C_3 extender unit labels only C-1 and not C-2, (Fig. 5). This labeling pattern indicates the existence of parallel propionate routes in *I. pustulosa* for the C_3 units as starter and extender to be loaded during the polyketide chain assembly. Analyzing [1- ^{13}C]acetate incorporation, the labeling at C-6 and non-labeling at C-2 (Fig. 6) are further evidences of these parallel

Figure 3 | Proposed biosynthetic pathway for hepten-3-one (1) from the harvestman *Iporangaia pustulosa*. (a) Condensation of propionyl-CoA with malonate. (b) Condensation with methylmalonyl-CoA. (c) Reduction, dehydration, reduction, thioester hydrolysis and decarboxylation. (d) Dehydrogenation of the saturated ketone.

Figure 5 | Proposed biosynthesis of **1** from the harvestman *Iporangaia pustulosa* based on [4-¹³C]methylmalonate feeding experiment and observed labelling pattern. Isotopomers of **1** and precursor are not indicated separately.

route. Additionally, the [¹³C₃]propionate ¹³C enrichment at C-1 and C-2 (15.25 and 16.27%, respectively) corresponding to the extender unit is weaker than at C-5, C-6 and C-7 (39.18%, 48.63% and 45.06%, respectively), the starter unit, which reinforces the hypothesis of parallel propionate route (Table 2).

Feeding on [4-¹³C]methylmalonate yields no labeling at C-3 nor at C-4 of **1**, corresponding to the acetate portion of **1** polyketide chain. Consequently, we can say that harvestman propionate catabolism occurs via succinate, contrary to the alternative biosynthetic pathway via 3-hydroxypropionate to acetate for insects^{25,26}. Our results also indicate that propionate in harvestmen may arise from three alternative biosynthetic pathways (succinyl-CoA, crotonyl-CoA and methionine and/or threonine catabolism) (Fig. 7). Bacterial symbionts are known to participate in the biosynthesis of arthropod secondary metabolites^{27,28} and may be an alternative source of C₃ units for the biosynthesis of harvestman defense molecules like **1**.

Given that the production of vinyl ketones is a synapomorphy of the clade composed of the subfamilies Gonyleptinae, Hernandariinae, Sodreaninae, Progonyleptoidellinae, and Caelopyginae¹⁰, the biosynthetic pathway described here is probably similar in other harvestman species belonging to this major group of gonyleptids. Moreover, given that the production of vinyl ketones is derived from an ancient state of the benzoquinone production in gonyleptid harvestmen¹⁰, our next challenge is to investigate whether distantly

Figure 6 | Proposed biosynthesis of **1** from the harvestman *Iporangaia pustulosa* based on [1-¹³C]acetate feeding experiment and observed labelling pattern. Isotopomers of **1** and precursors are not indicated separately.

related species partially share the biosynthetic pathways of these two major classes of metabolites. Previous studies with tenebrionid beetles have shown that two 1,4-benzoquinones also reported in the scent gland secretion of several gonyleptid species (2-methyl-1,4-benzoquinone and 2-ethyl-1,4-benzoquinone) are derived from a pathway using acetate and propionate as precursors, suggesting a

Figure 7 | Propionate metabolism in the harvestman *Iporangaia pustulosa*.

polyketide origin^{29,30}. Therefore, similar precursors for both benzoquinones and vinyl ketones could help to explain evolutionary transitions among gonyleptid species.

Methods

Harvestmen. Individuals of *Iporangaia pustulosa* were collected from a large fragment of Atlantic forest in the state of São Paulo, southeastern Brazil. The collection of the animals in the field and the experiments in the laboratory complied with the current laws of the Brazilian government. Before beginning the experiment, a dorso-ventral pressure was applied on all individuals to empty the gland sacs. The pool of harvestmen was divided into four groups and fed on: 1) canned sardines with 5% w/w [¹³C]sodium acetate (Cambridge Isotope Laboratories, CIL) (n = 14 individuals); 2) canned sardines with 5% w/w [4-¹³C]sodium methylmalonate (Supplementary Method S7) (n = 11 individuals); 3) canned sardines with 1% w/w [1-¹³C]glucose (CIL) (n = 10 individuals) and 4) [¹³C₃]propionate (Aldrich) (n = 28 individuals). The control group was the exudate extracted before initiating each experiment (n = 30 individuals). The experiment was set up over a period of 60 days (for groups 1 to 3) and 30 days for group 4, with feeding renewal every 48 h. The number of individuals analyzed in each experimental group is the total that survived for the whole period of feeding (minimum of 10 individuals). The gland exudates were collected with dewaxed cotton wool and extracted from the cotton wool with deuteriochloroform or deuterobenzene.

NMR analyses of 1. ¹³C NMR signal assignment of 1 has been described in the literature³¹. Previous assignments were refined with 2D-NMR spectra (¹H, ¹³C HSQC) (Supplementary Data S9) of the synthetic standard⁹. NMR spectra of 1 were acquired with either a Bruker Avance III 11 tesla (for [1-¹³C]acetate, [4-¹³C]methylmalonate and [1-¹³C]propionate groups) or a Varian Inova spectrometer 11 tesla ([1-¹³C]glucose group), both operating at 125.75 MHz, 25°C, acquisition time 0.55 s, and collecting about 40,000 scans, taking care to have equal scan numbers for samples belonging to the same experiment (sample and control).

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Author contributions

D.F.O.R., F.C.W. and A.J.M. are the chemists responsible for the experimental design and data interpretation. G.M. is the biologist responsible for the identification and collection of the individuals and furnished technical support to raise *Iporangaia pustulosa* individuals in the laboratory.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

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