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Thermally Induced Actinidine Production in Biological Samples

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ABSTRACT: Actinidine, a methylcyclopentane monoterpenoid pyridine alkaloid, has been found in many iridoid-rich plants and insect species. In a recent research on a well-known actinidine- and iridoid-producing ant species, *Tapinoma melanocephalum* (Fabricius) (Hymenoptera: Formicidae), no actinidine was detected in its hexane extracts by gas chromatography−mass spectrometry analysis using a common sample injection method, but a significant amount of actinidine was detected when a solid injection technique with a thermal separation probe was used. This result led us to hypothesize that heat can induce the production of actinidine in iridoid-rich organisms. To test our hypothesis, the occurrence of actinidine was investigated in four iridoid-rich organisms under different sample preparation temperatures, including two ant species, *T. melanocephalum* and *Iridomyrmex anceps* Roger (Hymenoptera: Formicidae), and two plant species, *Actinidia polygama* Maxim (Ericales: Actinidiaceae) and *Nepeta cataria* L. (Lamiales: Lamiaceae). Within a temperature range of 50, 100, 150, 200, and 250 °C, no actinidine was detected at 50 °C, but it appeared at temperatures above 100 °C for all four species. A positive relationship was observed between the heating temperature and actinidine production. The results indicate that actinidine could be generated at high temperatures. We also found that the presence of methylcyclopentane monoterpenoid iridoids (iridodials and nepetalactone) was needed for thermally induced actinidine production in all tested samples. These results suggest that the presence of actinidine in iridoid-rich plants and ants might be a consequence of using high temperatures during sample preparation.

KEYWORDS: *iridoids, high temperature, Tapinoma melanocephalum, Iridomyrmex anceps, Actinidia polygama, Nepeta cataria*

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INTRODUCTION

Actinidine, a methylcyclopentane pyridine monoterpenoid alkaloid, was first reported in the silver vine, *Actinidia polygama* Maxim (Ericales: Actinidiaceae), in 1959 and was named after this plant.[1] For the past six decades, actinidine has been studied in many plants in the families Actinidiaceae, Bignoniaceae, Caprifoliaceae, Loganiaceae, Pandanaceae, and Valerianaceae,[1−5] more likely because it is a feline attractant.[4−6] In addition, it has also been found in many insect species (i.e., ants, sawflies, rove beetles, and stick insects) and presumably functions as a defensive chemical.[7−12] Recently, it was reported as a biosynthetic compound in two *Tapinoma* ant species and used in chemotaxonomy.[13]

Actinidine and methylcyclopentane monoterpenoid iridoids often co-occur in biological samples. For example, silver vine plants are rich not only in actinidine but also in iridodiols and matatabilactones, such as neomatatabiol, iridomyrmecin, isoiridomyrmecin, neonepetalactone, dihydronepetalactone, and isodihydronepetalactone.[1,4−6,14] Iridodial and its stereoisomers are key intermediates in the biosynthesis of many iridoids in plants.[15−18] Actinidine, iridodials, and some other iridoid lactones such as iridomyrmecin, isoiridomyrmecin, nepetalactone, dihydronepetalactone, and isodihydronepetalactone also co-occur in the pygidial glands of ants in the subfamily Dolichoderinae.[11−13,19−21] Traces of actinidine, five iridoid dialdehydes, and three iridoid lactones coexist in the larvae of several species of Chrysomelid beetles.[22] Iridodials have been reported as defensive chemicals and pheromones in ants in the subfamily Dolichoderinae and other insects.[11−13,19] Although the fact that actinidine is not a biosynthetic compound in insects has been suspected,[21,23] it has never been experimentally demonstrated.

In contrast to a previous report on the existence of actinidine in *Tapinoma melanocephalum* (Fabricius) (Hymenoptera: Formicidae),[11] in our recent investigation, no actinidine was detected in the hexane extracts of *T. melanocephalum* using a normal sample injection method in gas chromatography−mass spectrometry (GC−MS) analysis.[24] However, in a preliminary study, actinidine was detected when a solid injection technique with a thermal separation probe (TSP) was used. These results indicated a possibility that the heating sample in the injector may induce/enhance the production of actinidine. Solid injection techniques were often used in the discovery of actinidine in a number of insects.[7,8,25−27] In these studies, glands, secretions, and even whole insect bodies were injected directly into the GC−MS system using the solid injection technique, and the sample was pyrolyzed at 200−300 °C.[28] Although thermally induced production of actinidine in insects has been suspected,[29] it has never been proven experimentally.

In this study, the hypothesis that heat can induce the production of actinidine in iridoids containing biological samples was tested by reinvestigating the occurrence of

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actinidine in four organisms under different sample preparation temperatures, including two ant species, *T. melanocephalum* and *Iridmyrmex anceps* Roger (Hymenoptera: Formicidae), and two plant species, *A. polygama* Maxim (Ericales: Actinidiaceae) and *Nepeta cataria* L. (Lamiales: Lamiaceae). These ants and plants are well known as producers of actinidine and iridoids (e.g., iridodials, nepelatactone, and iridodiols). First, we analyzed both iridoids and actinidine in the hexane extractions of these four organisms that had been heated at different temperatures. Second, in order to determine whether actinidine can be produced from the iridoids in biological samples, the occurrence of actinidine in actinidine-free ants was investigated after they were mixed with authentic iridoids and heated at high temperatures. Finally, the correlation between the quantity of detected actinidine and temperature was established.

MATERIALS AND METHODS

Biological Samples—Ant and Plant Materials. Three colonies of *T. melanocephalum* were collected in March 2016 from Tianhe District (113.35658964N, 23.1458646672E) in Guangzhou City, Guangdong Province, China. Four colonies of *I. anceps* were collected in July 2017 from Bole County (114.31185838665N, 23.265124186825E) in Guangdong Province, China. Ants were separated from mound soil using a method described by Shi et al[24] Three colonies of *Solenopsis invicta* Buen (Hymenoptera: Formicidae) were collected in October 2016 from Fogang County (N23.753396°N, E113.485293°E) in Guangdong Province, China. Fire ant colonies were extracted from soil by a water drip method.[30] Each colony was reared in a plastic container (45 × 38 × 15 cm), with the inner wall coated with Fluon F4-1 (Guangzhou Xingshengjie Sci. and Tech. Co., Ltd.), to prevent the ants from escaping. The yellow mealworm, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae), a cotton ball saturated with a 20% honey water solution, and another cotton ball with distilled water were separately placed in a Petri dish (7 × 1.5 cm). All colonies were maintained at 26 ± 2 °C and a photoperiod of 12:12 (L:D) h. The plants of silver vine, *A. polygama*, and catnip, *N. cataria*, were purchased from Bikazi Forest Inc. (Yichang, Hubei, China) and from Qiangxian Inc. (Weifang, Shandong, China), respectively. All plants were kept in a greenhouse.

Effects of Heat Treatment on the Production of Actinidine in Ants and Plants. *Heat Treatment.* An oil bath heater (HH−S1, Changzhou Aohua Instrument Co., Ltd., China) with dimethyl silicon oil (Tianjin Kemiou Chemical Reagent Co., Ltd., China) was used for heating samples. Each sample was placed into a 25 mL HD round bottom pressure tube vessel bottle (borosilicate glass 3.3, Shanghai Di Glass Instrument Co., Ltd., China). Air in the bottle was replaced by high-purity N_2. The sealed bottle was placed in dimethyl silicon oil and then heated at different temperatures for 10 min.

Actinidine Content in Heated Biological Samples. Two experiments were conducted. In experiment 1, the ant (five workers) and plant (0.1 g leaves) samples were first heated at 250 °C for 10 min, extracted with 1 mL of hexane for 10 min, and analyzed using GC−MS. The hexane extracts of unheated samples (live ants and fresh plants) were used as a control. The objective was to determine whether heat treatment affected the production of actinidine. In experiment 2, a pair of pygidial glands and the whole body of *T. melanocephalum* were analyzed using the TSP technique coupled with GC−MS. The objective was to determine whether this solid sample injection method induced actinidine production in *T. melanocephalum*.

Thermally Induced Production of Actinidine from Iridoids and Biological Tissue or Materials. In experiment 3, the hexane extracts of the ant (five workers) and plant (0.1 g leaves) samples, without ant or plant material, were heated at 250 °C for 10 min and then re-extracted using 1 mL of hexane for 10 min and analyzed using GC−MS. The unheated sample was used as a control. The objective was to determine whether the materials associated with the ant or plant samples were necessary for actinidine production in the heating process. In experiment 4, three types of samples including five *S. invicta* workers, 1 μg synthetic iridodials, and a mixture of *S. invicta* workers and synthetic iridodial were heated at 250 °C for 10 min and then extracted using 1 mL of hexane for 10 min and analyzed using GC−MS. The objective was to determine if synthetic iridodials could be converted to actinidine after being heated with *S. invicta* workers (which do not produce actinidine). In experiment 5, the procedure was identical to experiment 4 except that the following three types of samples were used: five *S. invicta* workers, 1 μg nepetalactone, and a mixture of *S. invicta* workers and nepetalactone. Nepetalactone was isolated from *N. cataria* leaves (see the following section for the isolation method). The objective was to determine if nepetalactone could be converted to actinidine after being heated with *S. invicta* workers.

Temperature-Dependent Production of Actinidine. In experiment 6, the relationship between the actinidine production in ants *T. melanocephalum* and temperature of heat treatment was investigated. Five workers were first heated at 50, 100, 150, 200, or 250 °C for 10 min, extracted with 1 mL of hexane for 10 min, and then analyzed using GC−MS. The actinidine content was quantified (see the following section for the quantification method of actinidine). In experiment 7, five *I. anceps* workers were first heated at 50, 100, 150, 200, or 250 °C for 10 min, extracted with 1 mL of hexane for 10 min, and then analyzed using GC−MS. The actinidine content was quantified. In experiment 8, actinidine was quantified for the mixture of five *S. invicta* workers and 1 μg synthetic iridodials, after the mixture was heated at different temperatures of 50, 100, 150, 200, or 250 °C for 10 min. The heated mixture was then extracted with 1 mL of hexane for 10 min and analyzed using GC−MS. The actinidine content was quantified. In experiment 9, actinidine was quantified for the mixture of five *S. invicta* workers and 10 μg nepetalactone, after the mixture was heated at different temperatures of 50, 100, 150, 200, or 250 °C for 10 min. The heated mixture was then extracted with 1 mL of hexane for 10 min and analyzed using GC−MS. The actinidine content was quantified.

Water in all hexane extractions was removed by anhydrous sodium sulfate (Chinasun Specialty Products Co., Ltd., China). Hexane was evaporated under N_2 to dryness and then readjusted to 1 mL. Two microliters of concentrated extracts were injected into a GC−MS system . There were five replicates for each treatment.

Separation of Actinidine and Nepetalactone in Ants and Plants Using Column Chromatography. *T. melanocephalum* workers (~1 g or 5000 workers) were anesthetized using carbon dioxide and were evenly divided in five bottles (25 mL HD round bottom pressure tube vessel bottles). The sealed bottles were submerged into dimethyl silicon oil and then heated at 250 °C for 10 min. After the bottle was cooled down to room temperature, each ant sample was extracted in 15 mL of hexane for 10 min. All extractions were then pooled, and water was removed by adding 3 g anhydrous sodium sulfate. At last, the pooled hexane extraction was evaporated under N_2 to dryness, and the dry residue was readjusted to 5 mL of hexane. The extraction was subjected to 10 g silica gel (SiO_2; 200−300 mesh; Qingdao Marine Chemical Factory, China) column chromatography (CC) (Φ2.5 × 300 cm) and eluted with the gradient mixtures of hexane and acetone (10:1 to 7:1). Sixty fractions (each fraction was ca. 20 mL) were collected and respectively analyzed by GC−MS. The fractions in which actinidine accounted for over 95% peak areas were combined and evaporated under N_2 to dryness. The samples (1−2 mg, actinidine purity ≥95%) were dissolved in 1 mL $CDCl_3$ and then used for ^1H and ^{13}C NMR analyses.

N. cataria leaves (10 g) were extracted in 100 mL of hexane for 24 h. Water in the extraction was removed by adding 3 g anhydrous sodium sulfate and then evaporated under N_2 to dryness, and the dry residue was readjusted to 5 mL of hexane. The concentrated extract was subjected to 10 g silica gel (SiO_2; 200−300 mesh; Qingdao Marine Chemical Factory, China) CC (Φ2.5 × 300 cm) and eluted with the gradient mixtures of hexane and acetone (20:1 to 9:1). Sixty fractions (each fraction was ca. 20 mL) were collected and respectively analyzed by GC−MS. The fractions in which

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nepetalactone accounted for over 95% peak areas were pooled and evaporated under N_2 to dryness and readjusted to 1 mL of hexane.

Quantification of Actinidine. Ant workers, plant fresh leaves, and the mixture of five *S. invicta* workers and authentic iridoids were analyzed after being heated under different temperatures (50, 100, 150, 200, or 250 °C). The actinidine content was quantified for the following samples: (1) five workers of *S. invicta*, (2) five fire ant workers with 1 μg (1*R*, 2*S*, 5*R*, 8*R*)-iridodial, (3) 0.1 g fresh leaf of *A. polygama*, (4) 0.1 g fresh leaf of *N. cataria*, and (5) five fire ant workers with 1 μg nepetalactone. The internal response factor (IRF) of purified actinidine (separated from the heated product of *T. melanocephalum* workers using CC) and *n*-octadecane was used to calculate the amount of actinidine.[24]

There were five replicates for each treatment. The sample heated by oil bath was extracted in 5 mL of hexane for 10 min. The combined hexane extract was evaporated under N_2 to dryness and then readjusted to 1 mL of hexane (with 10 ng/μL *n*-octadecane as an internal standard). Two microliters of extract were injected into a GC−MS system.

GC−MS Analysis. An Agilent 7890A gas chromatograph, coupled with an Agilent 5975C mass selective detector (GC−MS), was used for qualitative and quantitative analyses. An HP-5 fused silica capillary column (30 m × 0.25 mm ID), with a film thickness of 0.25 μm (Agilent Technologies, United States), was used. The temperature was programmed from 50 °C (held for 1 min) to 280 °C at 10 °C/min and held at 280 °C for 5 min. The solvent delay was 3 min. Helium was used as the carrier gas. Electron ionization mass spectra were recorded from *m*/*z* 50 to 330 at 70 eV, with the ion source temperature of 230 °C.

Chemical identifications were confirmed by the comparison of mass spectra of unknown compounds with authentic standards. Synthetic iridodials [(1*R*, 2*S*, 5*R*, 8*R*)-iridodial as the major isomer] were provided by Dr. Kamal Chauhan, Invasive Insect Biocontrol & Behavior Laboratory, USDA-ARS, Beltsville, MD. When standards were not available, identification would be based on comparison with the mass spectra in the literature[12,13] and the computer library (NIST 12 library).

NMR Analysis. The alkaloid, a major component of heated product from *T. melanocephalum* workers, was identified by nuclear magnetic resonance (NMR) spectra. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 400 spectrometer with an operating frequency of 400.13 MHz for proton and 151 MHz for carbon at the Key Laboratory of Nature Pesticide and Chemical Biology, Ministry of Education, South China Agricultural University, Guangzhou, China. Tetramethylsilane (TMS) was adopted as the internal standard for ^{1}H NMR spectra and the solvent peaks for ^{13}C NMR spectra. Chemical shifts (δ_H and δ_C) are quoted as downfield from TMS. The identification would be based on comparison with the NMR spectra in the literature.[13,31]

Statistical Analysis. The mean values and standard errors of the mean were calculated for all tests. One-way analysis of variance test (SPSS 19.0; SPSS Inc., Chicago, IL, USA) was used by least-significant difference test to assess and compare the influence of different temperatures on the amount of actinidine production. Values of $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

Actinidine Content in Heated Biological Samples. The total ion chromatograms of the hexane extracts of two ant species were compared between the unheated and heated treatments (Figure 1A). An alkaloid was detected in all heated samples but not in unheated ones. The chemical structure of the alkaloid, which was purified from the heated product of *T. melanocephalum* workers, was clarified by GC−MS and ^{1}H and ^{13}C NMR analyses (Figure S1−S3). It was identical to the mass spectrum[12] and NMR spectra of actinidine found in the literature.[13,31] For unheated samples, the dominant components were monoterpenes including the six isomers of

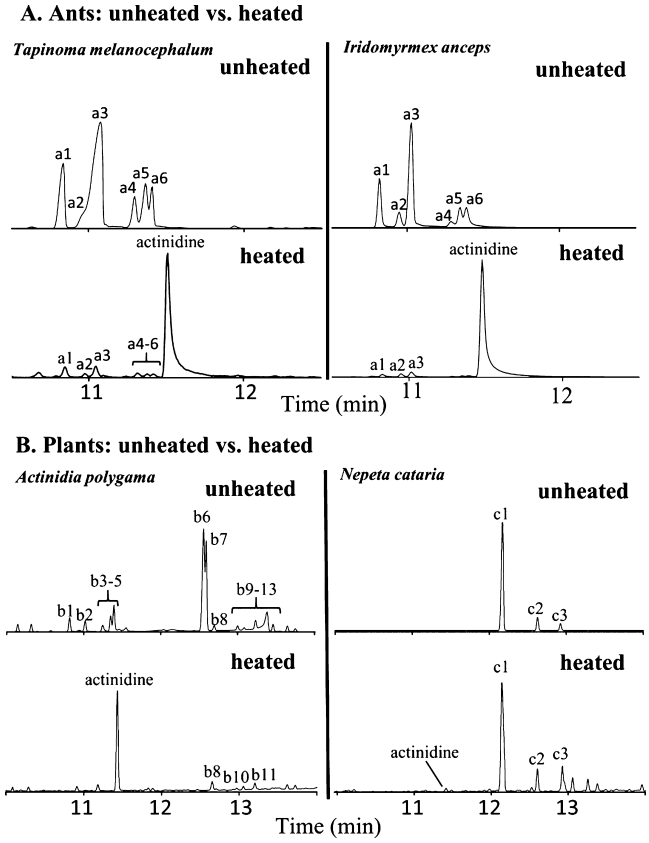

Figure 1. Total ion chromatograms of the hexane extracts of the two ants, *Tapinoma melanocephalum* and *Iridomyrmex anceps*, at an ambient temperature(unheated) and at 250 °C (heated) for 10 min (A) and the hexane extracts of fresh leaves from the two plants, *Actinidia polygama* and *Nepeta cataria*, at an ambient temperature (unheated) and at 250 °C (heated) for 10 min (B). Peak assignment: a1−a6: six isomers of iridodial; b1−b13: methylcyclopentane monoterpenoid iridoids, b6 and b7: iridodiols; c1 and c2: nepetalactones; c3: dihydronepetalactone. All components were identified by MS.

iridodials and 6-methyl-5-hepten-2-one in *T. melanocephalum*[24] and *I. anceps* workers (Figure S4). Actinidine was the major component in all heated samples. Although much less abundant, some of these monoterpenes in the unheated samples were still detectable in the heated samples.

The total ion chromatograms of the hexane extracts of two plant species in the unheated and heated treatments were compared in Figure 1B. The extracts of silver vine fresh leaves contained 13 components (b1−b13), tentatively identified as methylcyclopentane monoterpenoid iridoids (Figure S5), and the extracts of catmint plants contained three components (c1−c3), which were tentatively identified as two isomers of nepetalactone and dihydronepetalactone (Figure S6) in the unheated samples of the two plants (Figure 1B). Nepetalactone and dihydronepetalactone are the characteristic compounds in the genus *Nepeta*.[4,6,15,16,32] A significant actinidine peak was shown in silver vine samples, and a trace of actinidine was detected in catmint, in the heated samples (Figure 1B). As for catmint, the total ion chromatograms of the heated and unheated samples were similar, except for a slight difference in abundance. Actinidine has been reported in the essential oils of *Nepeta leucophylla* and *Nepeta clarkei* from steam distillation.[32]

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The total ion chromatograms of *T. melanocephalum* samples using TSP are shown in Figure 2. Actinidine was detected in the pygidial glands and whole bodies of *T. melanocephalum* workers using the solid injection technique but not in their hexane extracts.

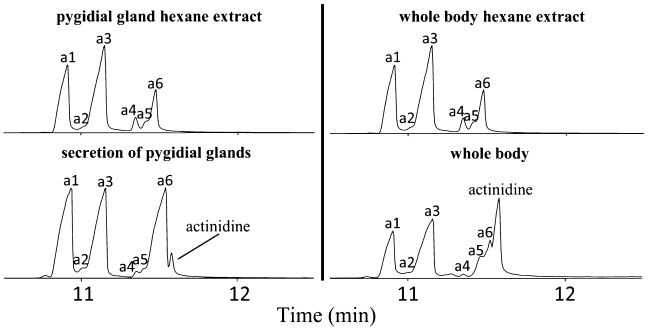

Figure 2. Total ion chromatograms of pygidial glands or whole bodies and the hexane extracts of *Tapinoma melanocephalum* workers using the TSP technique (pyrolyzing at 250 °C).

For all the four biological samples, actinidine was detected only when the samples were heated before solvent extraction or solid sample injection technique was used, indicating that actinidine may not naturally occur in all these species. Actinidine was previously reported in the secretions of *T. melanocephalum* workers collected from Florida, USA.[11] However, in our earlier study, the compound was not detected in *T. melanocephalum* workers collected from either Guangdong, China or Florida, USA.[24] This study demonstrated that actinidine can be thermally induced not only in *T. melanocephalum* but also in *I. anceps*.

In this study, for both plant species, actinidine was detected only in the heated leaf samples but not in unheated leaf samples. It was reported that actinidine was present in the dried samples of four plants[2−4] and in the samples of iridoid-rich biological materials when high temperature is involved during sample preparation.[7−9,25−27] Heating is commonly used for drying plant materials. Although heat can increase the drying rate of plant materials and minimize the hydrolysis of some compounds, artifacts can be generated during the drying process.[33] The heat involved in the drying process may contribute to the production of actinidine in plant materials.

It is possible that other factors, instead of heat, may also contribute to the production of actinidine, such as chemical profiles and fresh degree of the samples and the use of ammonia in the sample extraction/purification process. More research is needed to identify other potential factors that could induce actinidine production.

The solid injection technique coupled with gas chromatographic analysis was often used to identify the volatile chemicals of insects.[7−10,25,26,29,34,35] For example, in the chemical analysis of the defensive secretions of rove beetles (Coleoptera: Staphylinidae), the beetle's abdomen was pinched with a pair of forceps, and the defensive glands with adjacent tissues were removed and directly introduced into the injection port of the GC−MS system for chemical analysis.[8] During the procedure, the samples were exposed to pyrolyzing temperatures. Both actinidine and iridodials were often detected in these studies. In this study, when TSP was used, actinidine was detected in the whole body or tissue samples of *T. melanocephalum* workers, indicating that high temperatures might induce actinidine production in the samples.

Iridodials and Nepetalactone, and Biological Tissue/Materials, are Required for the Thermally Induced Production of Actinidine. In contrast to the whole body or tissue samples, actinidine was not detected in any hexane extracts using TSP analysis (Figure 2). Furthermore, heating hexane extracts at 250 °C did not produce any actinidine (Figure 3). This result suggests that iridoids and biological tissue/materials are the prerequisites for thermally induced actinidine production.

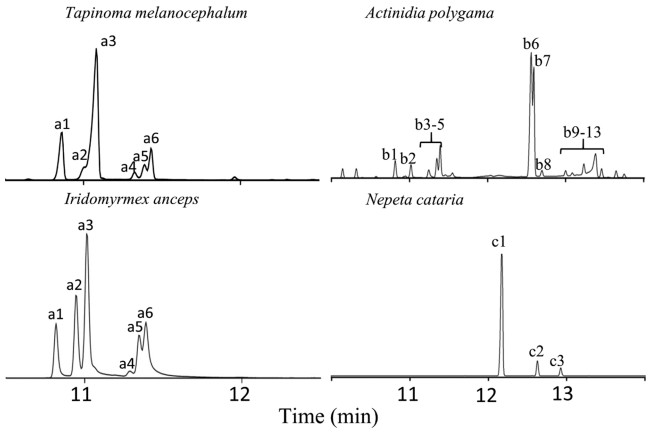

Figure 3. Total ion chromatograms of the hexane extracts of ants and plants heated at 250 °C for 10 min (C).

The total ion chromatograms of five *S. invicta* workers (actinidine- and iridoid-free ants) (Figure S7), synthetic (1*R*, 2*S*, 5*R*, 8*R*)-iridodial or nepetalactone isolated from *N. cataria* leaves, and the *S. invicta* workers treated with these two compounds are shown in Figure 4. Actinidine was produced only in *S. invicta* workers that were pretreated with synthetic iridodials or isolated nepetalactone.

In addition to heat, the results reveal that the presence of *S. invicta* worker was also required to convert (1*R*, 2*S*, 5*R*, 8*R*)-iridodial or nepetalactone to actinidine, indicating that the presence of biological tissues may be a requirement for such

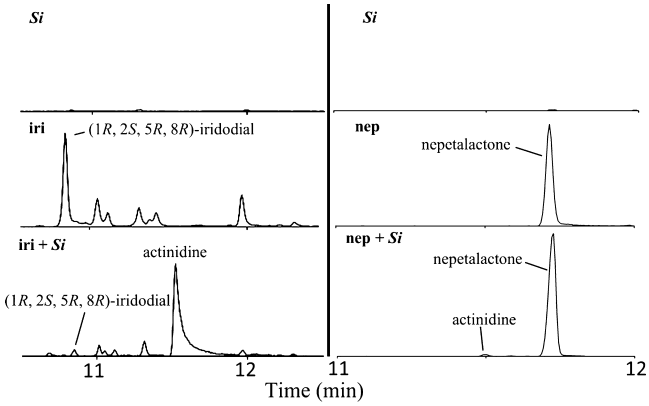

Figure 4. Total ion chromatograms of heated samples (250 °C for 10 min), including five *Solenopsis invicta* worker (*Si*) (actinidine-/iridoid-free ants) hexane extraction, synthetic (1*R*, 2*S*, 5*R*, 8*R*)-iridodial (iri) or nepetalactone (nep) isolated from *Nepeta cataria* leaves, and five *S. invicta* workers plus 1 μg synthetic (1*R*, 2*S*, 5*R*, 8*R*)-iridodial (iri + *Si*) or nepetalactone (nep + *Si*).

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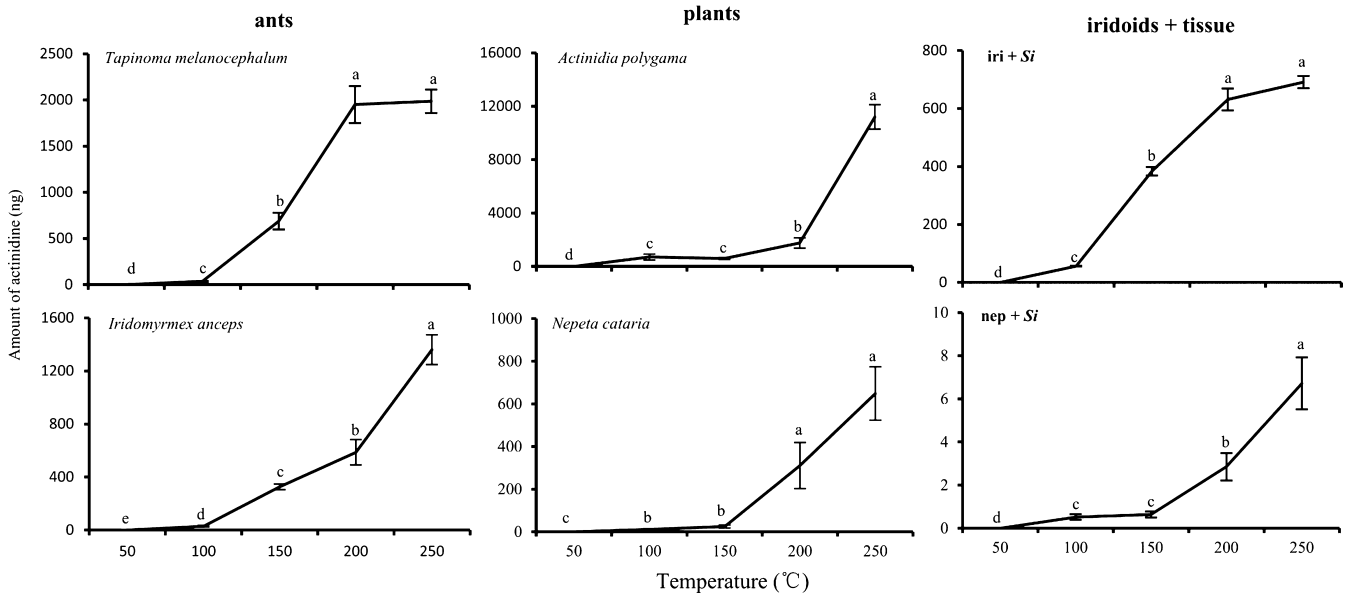

Figure 5. Actinidine production of hexane extracts of five workers of *Tapinoma melanocephalum*, *Iridomyrmex anceps*, or 0.1 g fresh plant leaves of *Actinidia polygama* or *Nepeta cataria*, or five *Solenopsis invicta* workers (*Si*) with 1 μg synthetic (1*R*, 2*S*, 5*R*, 8*R*)-iridodial (iri) or with 1 μg nepetalactone (nep) before the hexane extraction. All samples were heated at 50, 100, 150, 200, and 250 °C for 10 min and then extracted with hexane. Values are the means [standard error (n = 5)]. Values with different letters are significantly different (P < 0.05).

heat-induced actinidine synthesis. It was reported that iridodials can react with ammonia to produce actinidine.[36,37] Biological tissues at high temperature, *S. invicta* workers in this case, may serve as a source of ammonia. As ammonia production was common for biological tissues at elevated temperatures,[38,39] the presence of iridoids becomes a determining factor for heat-induced actinidine production in all organisms. Actinidine was detected in some plants when ammonia was used in the extraction of the organism or during the process of purification.[40−42] Iridoids are found in a wide variety of plants and some animals.[4−6,9−14,35,43] If heating is involved during sample preparation, actinidine may be produced from iridoids in these iridoid-rich biological samples.

Some previous works showed that pyridine monoterpene alkaloids have been synthesized from the iridoid glycosides, whereas ammonia was used in extraction.[42,44,45] Hence, in addition to methylcyclopentane monoterpenoid iridoids, other iridoids may be induced to produce pyridine monoterpene alkaloids when heating is involved or ammonia is used during sample preparation.

Temperature-Dependent Production of Actinidine. Actinidine production in ants and plants at different temperatures is shown in Figure 5. No actinidine was detected at 50 °C for all four species, but it appeared when the temperature was over 100 °C, and its concentration increased gradually as the temperature increased. A similar pattern was observed for *S. invicta* workers pretreated with synthetic iridodials or isolated nepetalactone. In addition, actinidine production increased over time as the mixture of fire ants and nepetalactone was heated at 250 °C (Figure S8).

Heating was often used during sample pretreatment in the analytical process. During the heating process, the reaction of iridoids with ammonia, generated from the nitrogen-containing compounds in biological tissue/material,[38,39] can produce actinidine. The temperature of pyrolysis and ammonia production is different for different types of nitrogen-containing compounds. TSP may not be a good method for analyzing biological samples with iridoids as it is hard to use an injection port temperature less than 100 °C. Also, for non-TSP chemical analysis, it is necessary to avoid temperatures above 100 °C during sample preparation.

In conclusion, the study showed that actinidine may not be a product of biosynthesis in all the four tested species. It could be a product of reaction(s) of iridoids with tissues/other biological materials at high temperatures (over 100 °C). Although this study cannot exclude the possibility that actinidine is biosynthesized in other organisms and even in these four tested species from different geographical origins, it should be enough to alert scientists to avoid misinterpreting actinidine found in any biological samples when heating is involved during sample preparation. This research also provided robust methods for analyzing iridoid-rich biological samples.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c02540.

Mass spectrum, ^{1}H and ^{13}C NMR spectra of an alkaloid from the extracts of *T. melanocephalum* workers heated, total ion chromatogram of the extracts of whole bodies of *I. anceps* and *S. invicta* workers, total ion chromatogram of the extracts of *A. polygama* and *N. cataria*, total ion chromatograms of the hexane extracts of ants and plants with heat treatment, total ion chromatograms of the hexane extracts of *S. invicta* workers, and amount of actinidine in *S. invicta* workers heated with 10 μg nepetalactone at 250 °C for 5, 10, 15, 20, or 25 min (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GC−MS, gas chromatography−mass spectrometry; TSP, thermal separation probe; NMR, nuclear magnetic resonance; SPME, solid-phase microextraction.

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