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Calorimetric investigations on the action of alarm pheromones in the hornet *Vespa crabro*

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Abstract

This study investigated the effects of alarm pheromone components on the heat production rates of hornets (*Vespa crabro*) by means of direct calorimetry. In a flow-through system, pheromones from hornets, honeybees (*Apis mellifera*) and yellowjackets (*Vespula vulgaris*) were sucked through a measuring vessel containing a group of hornet workers. The locomotive reaction of hornet workers was recorded as an increase of the heat production rate. Hornets exhibited a strong response to their own alarm pheromone components, mainly 2-methyl-3-butene-2-ol (MBO). They also reacted intensively upon the main alarm pheromone component of the honey bee, isopentylactetate (IPA), but less pronounced to alarm pheromone components of yellowjackets. The metabolic response of hornets to MBO was dose-dependent. The heat production rates of provoked hornet workers were similar to those of flying hornets. (z)-9-Pentacosene, a substance which is believed to be a thermoregulative brood pheromone, induced no metabolic reaction.

Keywords: Hornets; Wasps; Honeybees; Alarm pheromones; Calorimetry

1. Introduction

Pheromones serve a large variety of purposes in insects, such as finding a sexual partner, marking a territory, or production of chemical trails for orientation. In social insects, the defence of the colony is one of the most important tasks for all colony members [1,2]. A strong selection for rapid communication to recruit nestmates against predators or intruders led to the evolution of alarm pheromones. These pheromones, which can be found in honeybees, ants, wasps and termites, induce aggressive behaviour. The alarm pheromone consists typically of several components. Although wasps and hornets (Hymenoptera, Vespinae) are notorious for their aggressive behaviour when a colony is disturbed, knowledge about the nature and action of their alarm pheromones is scarce. In general, wasps (and hornets, which taxonomically belong to wasps) produce their alarm pheromone in a gland connected to the venom sac. As a consequence, their alarm pheromone is a component of the wasp venom. When wasps attack an enemy, some of the attackers spray their venom on the predator, who is then chemically marked. Other wasps will follow the scent of the pheromone, and mark the enemy themselves. The resulting snowball effect leads to a mass attack against the intruder [3].

Chemical analysis of alarm pheromones in social insects has proven to be difficult due to their high volatility and only a few components have been identified. In honeybees, isopentylacetate and 2-heptanone are the main components of the alarm pheromone. When confronted with these compounds, honeybees become extremely aggressive. Nevertheless, their alarm pheromone contains a total of 20 components. For most of them, the function and significance is unknown [4]. In the hornet *Vespa crabro*, four components have been identified so far. The main active substance seems to be 2-methyl 3-butene 2-ol [5], but quantitative data about the effectiveness of these substances are lacking.

The action of alarm pheromones is usually investigated with ethological assays, which are cumbersome and time-consuming, because they can not be performed in the laboratory but only in the field, and then only in secure areas as provoked bees, wasps or hornets may represent a hazard to humans in the vicinity of the test site. An alternative to behavioural field tests are studies on the physiological re-

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action of provoked insects. Earlier studies demonstrated the dramatic increase of honeybee heat production rates when exposed to alarm pheromones, which is mainly caused by strongly increased locomotive activities. In most cases, the chosen method was respirometry [6]. Direct calorimetry has also been tested successfully for this purpose, and descriptions of calorimeter modifications to measure metabolic responses of insects to pheromones have been described elsewhere [7]. In extension of these studies, we publish here the first comparative investigation on several substances with this method, which to our knowledge is also the first physiological study about the action of alarm pheromones on hornets.

Physiological tests, which investigate the increase in heat production rates, cannot be used as biotests in a narrow sense, because not only alarm pheromones may induce an increase of heat production, but also, e.g. brood pheromones, and the increase of heat production rates is not always necessarily an aggressive reaction. Nevertheless, such tests can be appropriate to quantify specific parameters of an alarm response when a substance has already been proven to be alarm-inducing. The compounds we investigated had different volatilities. As this investigation is a first survey, we always applied the same amount of compounds. The aspect of volatility on the efficacy of pheromones will be discussed below.

We compared a total of nine substances and one substance mixture. Three of these substances have already been described and tested as alarm pheromones of the hornet *V. crabro*. We included also five substances which are alarm pheromones in other wasp or bee species (*Vespula vulgaris* and *Apis mellifera*, respectively) in order to investigate interspecific reactions of hornets to pheromones of other species they may encounter under natural conditions. In addition, we reinvestigated the effect of (z)-9-pentacosene on the thermoregulative behaviour of hornets, as this substance has been described as a brood pheromone, i.e. a substance that induces an increase in heat production of adult hornets in order to warm their brood.

2. Experimental

2.1. Hornets

A total of seven hornet (*V. crabro*) nests have been relocated from their original nest sites to the garden of the Institute for Biology of the Free University of Berlin. The nests were placed in wooden nest boxes. For the calorimetric experiments, hornets were caught at the entrance of the nest box with tweezers or a net and carried immediately to the laboratory, where they were provided with food (“bee bread”, a mixture of bee honey and pollen). After a short sedation period, a group of five hornets was transferred to the calorimeter vessel for each experiment. All tests were performed between July and October 2001.

2.2. Calorimetry

Heat production rates of groups of hornet workers with and without influence of pheromones were measured by means of an isoperibolic, heat conduction calorimeter with two twin units (Type Calvet, Setaram MS 70, Lyon, France) with a continuous flow of air at a rate of 1.3 l/min. The calorimeter temperature was regulated to 20 °C. The calorimeter was calibrated under experimental conditions (including air flow through the vessel) by means of an electrical resistor (ATE RB25, 47 Ω, Conrad Elektronik, Hirschau, Germany). The resistor was placed in each measuring vessel and connected to a power supply (Triple power supply EA-PS2316-050, Elektro-Automatik, Viersen, Germany) with small wires (Ø = 1 mm without isolation) which were led through the air supply tubes. The calorimeter calibration of both twin units rendered a sensivity of 51.2 μV/mW (twin unit 1) and 54.5 μV/mW (twin unit 2), respectively. For application of pheromone to the hornets, air was sucked through a washing flask, which contained a piece of filter paper soaked with pheromone, and subsequently through the measuring vessel (Fig. 1). An identical air stream was led through the reference vessel. The addition of pheromone to the air and the time of exposure were regulated by means of a three-way valve. The measuring and reference vessel had a volume of 100 ml each.

At the beginning of each experiment, the heat production rate of undisturbed hornets was recorded for 15 min. After that, the three-way valve was switched and the insects were exposed to the pheromone for 60 s. The valve was closed then and the heat production rate in the calming-down period was monitored for 30 min. Before and after each experiment, the base line was recorded without the insects but with air flow through the calorimeter vessels. For calculation of the specific heat production rate, body mass of the hornets was determined by means of a fine mechanical balance (Typ 414/13, Sauter, Ebingen, Germany) to the nearest 0.1 mg.

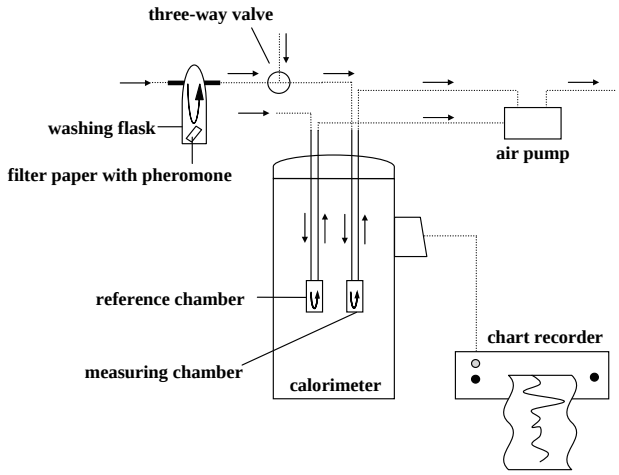

Fig. 1. Scheme of calorimetric set-up. Not to scale. Arrows indicate air flow. The air flow amounted to 1.3 l/min.

Table 1
List of all compounds investigated in this study

Compound	Abbreviation	Species and biological function	Physical data	Source of chemical
2-Methyl-3-buten-2-ol	MBO	Alarm pheromone of *V. crabro* (hornet)	bp: 96–98.5 °C, MW: 86.13	1
3-Methyl-3-buten-1-ol	MBO 331	Alarm pheromone of *V. crabro* (hornet)	VP: 0.266 kPa, bp: 130–132 °C, MW: 86.13	1
3-Methyl-2-buten-1-ol	MBO 321	Alarm pheromone of *V. crabro* (hornet)	bp: 139–140 °C, MW: 86.13	1
4-Penten-1-ol	Pentenol	Alarm pheromone of *V. crabro* (hornet)	bp: 135 °C, MW: 86.13	1
Isopentyl acetate	IPA	Alarm pheromone of *A. mellifera* (honey bee)	VP: 0.53 kPa, bp: 143 °C, MW: 130.19	1
Isovaleraldehyde	IVS	Alarm pheromone of *V. vulgaris* (yellowjacket)	VP: 40 000 kPa, bp: 89–91 °C, MW: 86.13	1
Isovaleric acid	IVAL	Alarm pheromone of *V. vulgaris* (yellowjacket)	VP: 500 kPa, bp: 176 °C, MW: 102.13	1
Isobutyric acid	IBS	Alarm pheromone of *V. vulgaris* (yellowjacket)	VP: 0.13 kPa at 14.7 °C, bp: 153–154 °C, MW: 88.11	1
N-(3-methylbutyl)acetamide	NAmd	Alarm pheromone of *V. vulgaris* (yellowjacket)	MW: 129.20	2
(z)-9-Pentacosene	Pentacosene	Brood pheromone of *V. crabro* (hornet)	MW: 186.21	3
Hexane	Hexane	Solvent for (z)-9-pentacosene	VP: 160 000 kPa, bp: 69 °C, MW: 86.18	1
Compound mixture				
MBO + MBO 331 + MBO 321, 1:1:1	MBO-mixture	Alarm pheromone of *V. crabro* (hornet)	MW: 86.13	1; mixture made by authors

VP: vapour pressure at 20 °C (if left out, information about VP was not available); bp: boiling point at 101.3 kPa (1 atm); MW: molecular weight; source of chemical—1: Aldrich Chemie GmbH, Taufkirchen, Germany; 2: Frinton Laboratories, New Jersey, USA; 3: synthesis by Prof. W. Franke, Institute for Organic Chemistry, University of Hamburg, Germany.

The calorimeter signal was recorded by means of a chart recorder (Type L2005, Linseis, Selb) at a sensivity of 50 mV and a paper speed of 20 cm/h. The mean heat production rates of the hornets were evaluated by electronic integration (Digikon, Kontron, Munich) of the power–time (*P–t*) curves.

2.3. Pheromones

Table 1 gives an overview of all chemicals tested in this study. If not mentioned otherwise, in each experiment 200 μl of pheromone was placed on the filter paper.

2.4. Evaluation of curve parameters and statistics

To evaluate the metabolic response of hornets to the pheromones, the following parameters in the *P–t* curves were investigated (Fig. 2): (i) p_{max}, maximum specific heat production rate after pheromone application; (ii) $t_{p_{max}}$, time from application of pheromone to p_{max}; (iii) a_p, rate of increase of p to p_{max}, calculated as p_{max} divided by $t_{p_{max}}$; (iv) p_A, the increase of p above that before substance application.

Values given are the median and first and third quartile. The increase of p after pheromone application to p_{max} was tested for statistical significance with a Wilcoxon test (exact, two-tailed). In order to investigate whether all values belonged to the same statistical population, we conducted a Kruskal–Wallis test for variance (two-tailed Monte Carlo-significance, confidence interval 99% at 10 000 samples). When significantly different, we conducted a post hoc test for all parameters evaluated in this study [8]. Significance level for all tests was $\alpha = 0.05$.

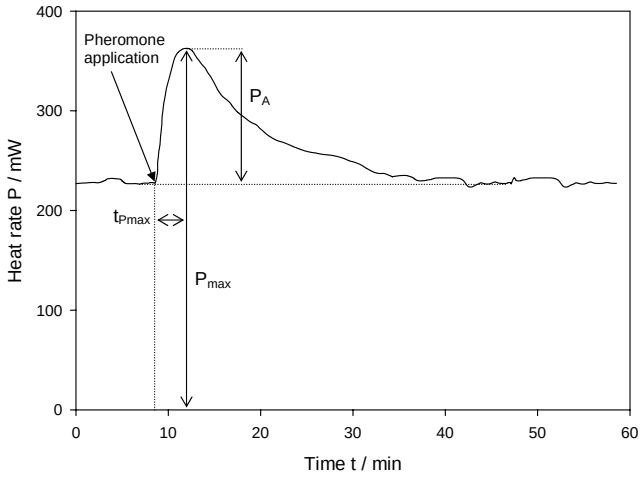

Fig. 2. Heat production rate of five hornet workers after application of 200 μl 3-methyl-2-butene-1-ol as an example for *P–t* curves before and after pheromone application. All parameters for *P–t* curve evaluation are shown. P_A = increase of specific heat production rate above the level before pheromone application; P_{max} = maximum specific heat production rate; $t_{p_{max}}$ = time from pheromone application until P_{max}.

3. Results

In all cases, the heat production rate of hornets increased after pheromone application (Fig. 3). The sharp increase stood in clear contrast to the smooth curve before application. After the maximum heat production rate (P_{max})[1] was reached, the curve decreased, either to the same level as before substance application or below that level. As the curve progression following P_{max} was not uniform in the experiments, we only analysed the action of pheromone compounds up to the point when the maximum heat production rate (P_{max}) was reached. No deleterious effects on hornets were observed in those cases when the heat production rates fell below the pre-application level during the recovery phase.

All intraspecific substances (MBO, MBO 331, MBO 321, Pentenol) provoked a significant increase of the heat production rate (p_A and p_{max}), but no significant statistical differences in the strength of the reaction between the substances was observed. All interspecific substances (IPA, IVS, IVAL, IBS, NAmd) caused a significant increase of p_A and p_{max} as well. The strongest interspecific alarm response was observed with IPA, the main alarm phermone component of the honeybee. The weakest response was caused by NAmd, one of the several pheromone components of the yellowjacket. IPA and NAmd differed significantly in the relative increase p_A and in the maximum rate p_{max}. The mixture of all MBO-compounds (MBO, MBO 331 and MBO 321 in a ratio of 1:1:1) showed no synergistic effects. The response of hornets to this mixture was statistically indistinguishable from the response to any of the single MBO components.

The reaction times $t_{p_{max}}$ of the hornets against pheromones were not significantly different, neither for the intraspecific nor the interspecific ones, with the exception of MBO 331 against IVS. The rate of increase of the heat production rate after substance application a_p was statistically different between MBO 331 and MBO. Slight differences between the increase rate could be observed in interspecific pheromones, but only the difference between IPA and NAmd was statistically significant.

The metabolic response of hornets to MBO was dose-dependent (Fig. 4). Significant differences were observed in p_A between 1 μl MBO and all other amounts (25, 50 and 200 μl) and in p_{max} between 1 μl MBO and 50 μl as well as 200 μl MBO.

Although (z)-9 pentacosene has been previously described as a brood pheromone, it provoked no thermogenic reaction in the hornets (Fig. 3). The increase in p_A and p_{max} did not differ from the increase caused by hexane (control ex-

[1] The reader should be aware that *P* denotes the heat production rate of the experimental hornet group, whereas *p* is the specific heat production rate, defined as *P* divided by body mass. As the body mass of hornets was not significantly different in our experiments, and for a better comparability of our results, we only present the values for *p* in this paper.

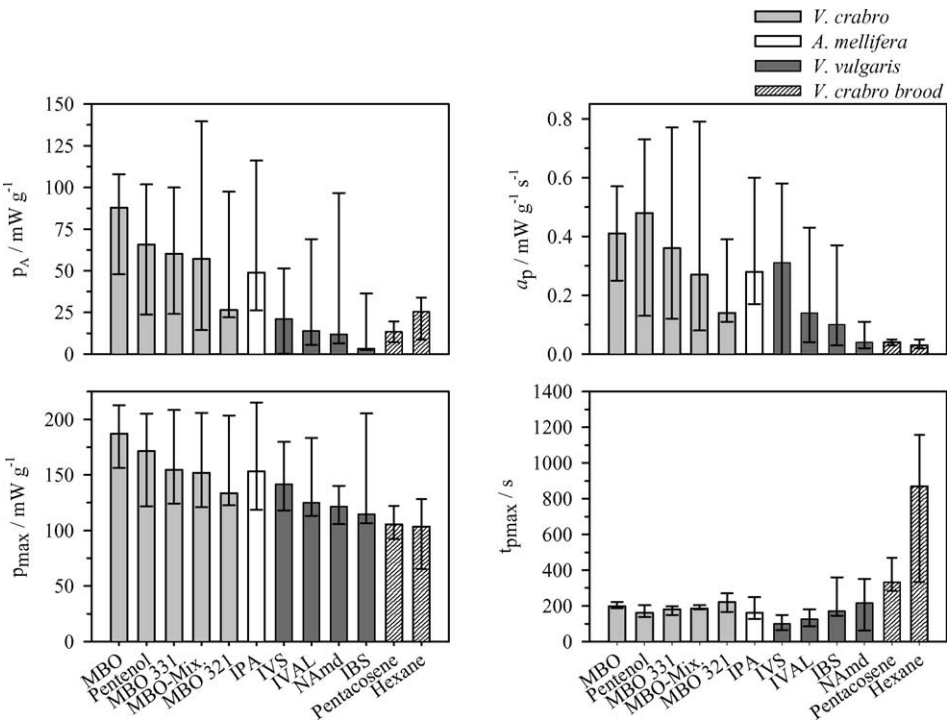

Fig. 3. Medians of different curve parameters of the heat production rate after application of 200 μl alarm pheromone components, the supposed thermoregulative pheromone z-9-pentacosene and hexane (solvent for pentacosene; control experiment). Bars indicate first and third quartiles. Parameter abbreviations—p_{max}: maximum specific heat production rate after pheromone application; p_A = increase of specific heat production rate above the level before pheromone application; $t_{p_{max}}$: time from pheromone application to p_{max}; a_p: rate of increase of p to p_{max}, calculated as p_{max} divided by $t_{p_{max}}$. Number of experiments: $n = 10$ for MBO; $n = 8$ for pentenol; $n = 9$ for MBO 331; $n = 8$ for MBO-mixture; $n = 8$ for MBO 321; $n = 9$ for IPA; $n = 8$ for IVS; $n = 9$ for IVAL; $n = 7$ for IBS; $n = 8$ for NAmd; $n = 6$ for pentacosene; $n = 6$ for hexane. For abbreviations of chemicals see Table 1.

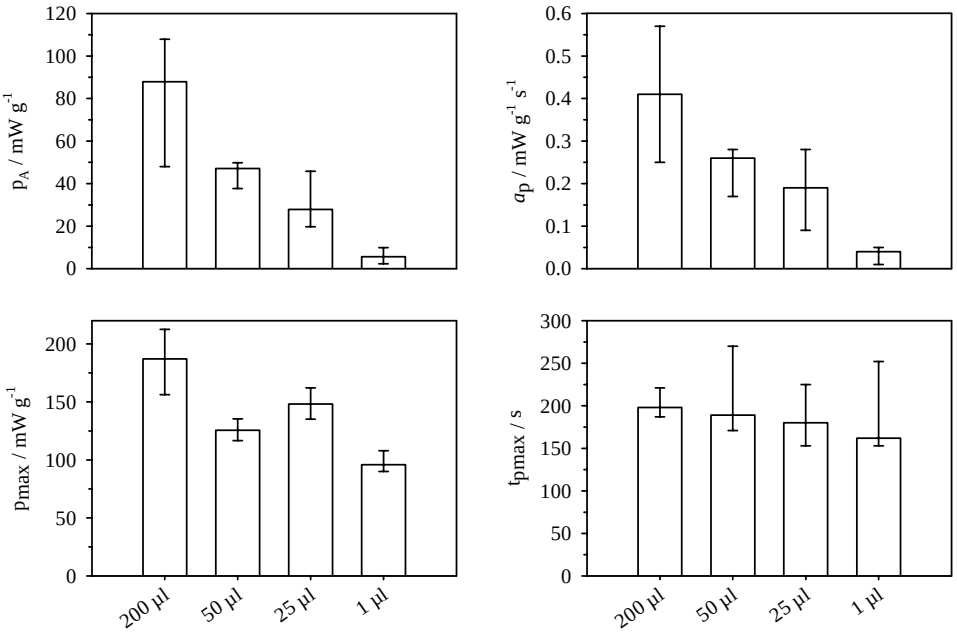

Fig. 4. Medians of different curve parameters of the heat production rate after application of different amounts (1, 25, 50 and 200 μl) of 2-methyl-3-buten-2-ol (MBO). Bars indicate first and third quartiles. Parameter abbreviations as in Fig. 3. Number of experiments: $n = 10$ for 200 μl MBO; $n = 7$ for 50 μl MBO; $n = 7$ for 25 μl MBO; $n = 7$ for 1 μl MBO.

periment), which served as solvent for pentacosene in our experiments.

4. Discussion

All substances tested in our study provoked an increase of the heat production rate of hornets. Among substances which had so far been identified as alarm pheromones in the hornet *V. crabro*, MBO induced the strongest reaction. This is in good accord with other studies [5].

The vapour pressures of alarm pheromone compounds seemingly had no effect on the heat production rates or reaction time of hornets. The vapour pressure of IVS amounts to 40 000 kPa compared to MBO 331 with 0.266 kPa (see Table 1), but all parameters we evaluated in our experiments were not significantly different. Nevertheless, more detailed information on vapour pressure of pheromone compounds would surely increase our understanding why alarm pheromones consist of several compounds, probably with different volatilities. One could speculate that the compounds act subsequently, or that some compounds serve for rapid alarming, and others for a more enduring marking of a predator.

Nevertheless, the reaction to MBO was dose-dependent. In this case, p_A was the most robust parameter for the identification of differences between varying concentrations of the pheromone, because in some cases the heat production rate of the hornets was not precisely on the same level before substance application, and p_A presents the increase of p relative to the heat production rate before substance application. The specific heat production rate of provoked hornets increased up to values which have been previously described for flying hornets [9]. As the calorimeter chambers with a volume of 100 ml provide sufficient space for locomotive activities, it is likely that the hornets tried to fly when responding to their alarm pheromone. In some cases, we observed hornets still beating their wings after the vessel was removed from the calorimeter.

In our experiments, we found no statistical differences in the action of MBO, MBO 321 and MBO 331. All three substances were equally active. This is contrary to other findings, which describe that MBO 321 and MBO 331 provoke much smaller effects compared to MBO [6]. Nevertheless, the rate of increase of the heat production rate a_p is statistically different between MBO and MBO 331 in our study. This may be a hint that the MBO compounds (MBO, MBO 321 and MBO 331) exhibit a slightly shifted temporal pattern of activity, which may prolong the total action of the alarm pheromone and thus lead to smaller p_A and p_{max}. Further biotests will be needed to clarify this. No synergistic effects of the different MBOs where observed by the application of an MBO mixture. In honeybees, such effects have been described previously in a study using the same method and experimental set-up as the present one [7].

The hornets could be provoked not only by their own, but also by several alarm pheromones of other insect species. The strongest reaction in our study was against IPA, the main alarm pheromone component of the honeybee *A. mellifera*. As hornets are natural predators of the honeybee, it is not unlikely that the reaction against their alarm pheromone is adaptive and occurs under natural circumstances. Hornets reacted less sensitively to alarm pheromones of yellowjackets, with the weakest reaction on NAmd, a main alarm pheromone component of the wasp *V. vulgaris*. Hornets are frequently reported to hunt for prey in front and even inside honeybee colonies, whereas they are seldom found in the close vicinity of wasp nests [10]. This may explain the difference of their reactions to honeybee and yellowjacket pheromones.

Hornets as well as other social wasps heat up and thermoregulate their nests [11,12]. In earlier studies, (z)-9-pentacosene has been described as a brood pheromone produced by older pupae. In the presence of (z)-9-pentacosene, hornet workers are reported to warm their brood [13]. In our study, (z)-9-pentacosene had no significant effect on the heat production rates of hornets compared to the solvent hexane in the control experiment. It therefore seems doubtful to us that this substance acts as a brood or thermoregulative pheromone. It occurs in nearly all nest structures and even on the cuticle of adult hornets and is thus a rather unspecific substance in the colony [14]. If active at all, pentacosene certainly affects the thermoregulative behaviour of hornets only when together with other, up to now undetermined factors.

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