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CHEMICAL ECOLOGY OF PHYTOPHAGOUS SCARAB BEETLES

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ABSTRACT

Sex pheromones have been characterized only for species in the subfamilies Rutelinae and Melolonthinae; aggregation pheromones have been identified for two species in the Dynastinae. Melolonthines utilize mainly amino acid derivatives and terpenoid compounds, but sex pheromones of rutelines are fatty acid derivatives. Various other species utilize japonilure-type lactones that are produced by desaturation of fatty acids, followed by hydroxylation, chain shortening, and cyclization. In marked contrast to melolonthine sex pheromone glands that are everted from the abdominal tip, ruteline sex pheromone glands consist of epithelial cells that line the inner surfaces of the pygidium and two apical sternites. Some species that are geographically and/or seasonally isolated utilize the same sex pheromone system, but chirality plays an important role in the isolation of the communication channels of two ruteline species, where one enantiomer is utilized as sex pheromone and the other is a behavioral antagonist. Olfactory receptor neurons (ORNs) are specifically tuned to these enantiomeric pheromones. It is unlikely that the specificity of these ORNs is achieved only by odorant-binding proteins. Pheromone-degrading enzymes are present in scarab beetle antennae and show considerable substrate specificity.

INTRODUCTION

The Scarabaeoidea (=Lamellicornea) form a large distinct group of highly specialized beetles (59), readily recognizable by their antennae, which are 8- to

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11-segmented (usually 10-segmented), with the last 3 to 7 segments forming a lamellate or pectinate club. The larvae are elongate, subcylindrical, and slightly to strongly curved and C-shaped; they are highly sclerotized and grub-like. The antennae of the larvae are usually 3- or 4-segmented, occasionally 2-segmented, and the penultimate segment is usually equipped with a sensorium (56). The superfamily Scarabaeoidea contains eight families: Lucanidae, Passalidae, Trogidae, Ceratocanthidae, Pleocomidae, Geotrupidae, Diphyllostomidae, and Scarabaeidae.

The family Scarabaeidae contains about 2,000 genera and 25,000 species that are usually placed in about 20 subfamilies and numerous tribes (56). The group is generally divided into Laparosticti (dung beetles) and Pleurosticti (chafers) (59). Members of the Scarabaeinae are mainly dung and carrion feeders, and adult beetles of some species provision larval burrows with balls of dung. Aphodinae are also usually dung feeders and often occur in mammalian nests (56). Adult chafers are strictly phytophagous with various feeding habits, including some species that do not feed at all in the adult stage. Dynastinae, with conspicuous horns in males, usually attack stems or roots of plants, whereas Cetoniinae prefer nectar, sap, or juice of ripening fruits and vegetables (59). Rutelinae and Melolonthinae mostly attack fresh leaves. Cetoniinae and Rutelinae are common visitors to flowers, where they feed on nectar or pollen. Larvae of Pleurosticti, such as Melolonthinae, Rutelinae, and Dynastinae are soil-dwelling white grubs that feed on living roots and may be destructive to crops. Some Cetoniinae and Dynastinae grubs feed in soil humus or litter, whereas other Cetoniinae, Trichinae, and Valginae live in decaying wood or in debris accumulated in the hollows of trees (59).

The monophyletic plant-feeding Rutelinae, Melolonthinae, Cetoniinae, and Dynastinae (18) include economically important pests in agriculture, horticulture, and forestry. Scarabs were first recorded as pests in Swiss meadows, in chronicles dating from 1478, and their populations have been monitored for more than 200 years (23).

Scarabs are widespread and endemic species (e.g. the cupreous chafer, *Anomala cuprea*, in Japan) and can be found in a wide range of environmental and economic niches. Accidental introduction of exotic species, such as the Japanese beetle, *Popillia japonica*, has also led to a steady increase in the number of problems with scarab beetles around the world (reviewed in 22).

Although a few studies have been reported on the chemical ecology of dung beetles (4, 5), most of the research in this area has been aimed at the development of environmentally sound strategies for scarab control. As a consequence, the focus has been on the economically important plant-feeding scarabs (chafers). The introduction of the Japanese beetle into the United States on nursery stock shipped to New Jersey had a tremendous influence on studies of the chemical ecology of scarab beetles. The need to survey beetle populations stimulated a

great deal of investigation aimed at the characterization of plant kairomones. The identification and synthesis of a female-released sex pheromone, japonilure (67), opened the way for further investigations on intraspecific chemical communication in scarab beetles. The identification of sex- and/or aggregation-pheromones is fundamental to promoting investigations of their feasibility for field application. In scarabs, this has been a difficult task, mainly because of the lack of uniform and consistent laboratory bioassays. This has changed dramatically with the application of alternative techniques, such as gas chromatography with an electroantennographic detector (39, 42) and gas chromatography–behavior bioassay (29, 34), which can be expeditiously used as short-cut bioassays.

This review summarizes some of the significant recent achievements in the chemical ecology of scarab beetles, with particular emphasis on chemical communication of plant-feeding scarabaeids.

CHEMICAL COMMUNICATION IN SCARAB BEETLES

Most of the emphasis of research programs on chemical communication in scarabs has been focused on the subfamilies Cetoniinae, Melolonthinae, Dynastinae, and Rutelinae because of the economic importance of these groups as agricultural and/or turf pests.

Evidence for the occurrence of sex pheromones has been documented in Cetoniinae (7), Melolonthinae (68), Dynastinae (58), and Rutelinae (33), but thus far sex pheromones have been unambiguously identified only for beetles in the most advanced rutelines and the primitive melolonthines (Figure 1). No pheromone has ever been identified in Cetoniinae, and only aggregation pheromones have been reported for a few Dynastinae.

Pre-copulatory behavior in the dung beetle *Kheper lamarcki* (Scarabaeinae) involves the production by males of a floculent secretion from the sides of the first abdominal sternites (66). Studying the chemistry of this secretion, Burger et al (4) identified hexadecenoic acid, 2,6-dimethyl-5-heptenoic acid, and (*E*)-nerolidol as its major constituents. They reported these chemicals as sex pheromone constituents for *K. larmarcki*, although traps baited with this synthetic lure did not catch any beetles (4). Later, the same group found a series of electrophysiologically active methyl and ethyl esters in the secretion of *K. larmarcki*, which were attractive to another species, *Pachylomerus femoralis* (5). In this review, I concentrate on pheromones not only that have been identified, but also for which biological activities have been demonstrated.

Scarab Sex Pheromones

RUTELINES Indications that a sex pheromone was produced by female Japanese beetles were noted as early as 1926 (27). However, when Fleming confined

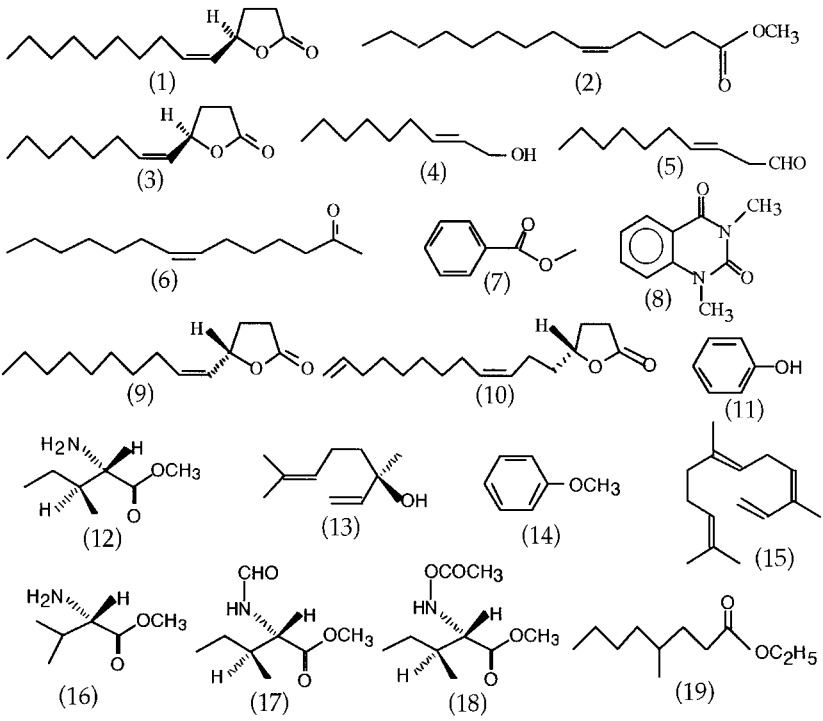


Figure 1 Semiochemicals involved in chemical communication of scarab beetles. (*1*) (*R,Z*)-5-(—)-(1-decenyl)oxacyclopentan-2-one; (*2*) methyl 5-(*Z*)-tetradecenoate; (*3*) (*R,Z*)-5-(—)-(1-octenyl)oxacyclopentan-2-one; (*4*) 2-(*E*)-nonenol; (*5*) 2-(*E*)-nonenal; (*6*) 7-(*Z*)-tetradecen-2-one; (*7*) methyl benzoate; (*8*) 1,3-dimethyl-2,4-(1*H*,3*H*)-quinazolinedione; (*9*) (*S,Z*)-5-(+)-(1-decenyl)oxacyclopentan-2-one; (*10*) (*R,Z*)-7,15-hexadecadien-4-olide; (*11*) phenol; (*12*) L-isoleucine methyl ester; (*13*) (*R*)-(—)-linalool; (*14*) anisole; (*15*) (*Z,E*)-α-farnesene; (*16*) L-valine methyl ester; (*17*) N-formyl L-isoleucine methyl ester; (*18*) N-acetyl L-isoleucine methyl ester; (*19*) ethyl 4-methyloctanoate.

virgin and field-collected females and beetles of both sexes in traps, he found that traps baited with the living insects captured no more beetles than the few caught in unbaited traps (11). Ladd conducted field studies that confirmed the presence of a pheromone and demonstrated that it was produced in the abdomen; however, solvent extracts of the abdomen were not attractive (25).

Tumlinson & Klein undertook a series of studies aimed at the full characterization of the sex pheromone. The semiochemical was collected by rinsing the glass vessels used to hold virgin females. The extracts were purified in a series of chromatographic steps, each step being monitored by a bioassay (27).

Attempts to develop a laboratory bioassay in a number of olfactometers and wind tunnels did not consistently yield any quantifiable orientation behavior (8). The bioassay was conducted by placing 50 to 100 female-equivalents per day in a Petri dish, exposing the dish on the ground (where male beetles were actively searching for females), and determining the number of males responding to the dish, compared with the number responding to three virgin female beetles held in a cage in the same area during the same period (27). The pheromone was tentatively identified as (*Z*)-5-(1-decenyl)oxacyclopentan-2-one. This racemic lactone failed to attract male beetles in the field and even inhibited the response of males to virgin females (67). The addition of 50 to 5000 ng of the racemic lactone to a petri dish containing a cage of three virgin females reduced the response of males to females by 80 to 100%. Although the role of chirality in insect chemical communication was almost terra incognita at that time, Tumlinson et al (67) speculated that the lack of activity of the racemic lactone was due to enantiomeric inhibition. Preparation of the two enantiomeric forms of this lactone and field tests demonstrated that the (*R*)-enantiomer (japonilure) (Figure 1, structure *1*) was active, whereas admixtures of as little as 1% of the (*S*)-enantiomer significantly reduced male attraction (67). Enantiomeric composition of the pheromone could not be determined analytically because the amount of natural pheromone was too small for the techniques available at that time. However, based on the inhibitory effect of the (*S*)-stereoisomer, the sex pheromone was characterized as (*R*)-japonilure.

Japonilure has been commonly used with food-type lures (see below) that act synergistically with the synthetic sex pheromone. The combined lure increased catches of males and females over the combined captures by the two lures separately, although the performance of the attractants varies throughout the flight season (24).

Based on the observation that either sex of the Japanese beetle tethered on grape leaves or an extract of either sex attracted males, Iwabuchi & Takahashi suggested that chemical communication in the Japanese beetle also involves a male-released aggregation pheromone (21). On the other hand, Loughrin et al (53) studied host location in the Japanese beetle and proposed a different scenario. Beetles that emerge early in the season respond to odors from a wide variety of plants, and, when a beetle locates an acceptable host, feeding is stimulated and in turn induces volatile release from the damaged leaves. Later-emerging beetles then can exploit the induced volatile blends as a more efficient means of host location. This sequence of events may help to explain the aggregative behavior of the Japanese beetle without a beetle-produced aggregation pheromone (53).

In an attempt to identify the sex pheromone of the soybean beetle, *Anomala rufocuprea*, Tamaki (63) developed an indoor bioassay for walking behavior

in a glass tube and collected the semiochemical with Tenax GC® and Porapak Q®. Purification of the crude extract by various chromatographic steps and monitoring the activity with the walking bioassay led to the isolation of methyl 5-(*Z*)-tetradecenoate (Figure 1, structure *2*). The synthetic sex pheromone was very active both in the indoor bioassays (64) and in field tests (55). Traps using either pheromones, virgin females, or black lights yielded the maximum number of captures at 30 to 60 min after sunset. The fact that the pheromone traps captured beetles for a longer period of time during a day compared with the virgin female traps (55) suggested that the copulatory activity was regulated by female release of the pheromone in a narrow time window.

The response-guided strategy for the isolation of sex pheromones, i.e. the repeated fractionation of a crude extract and testing of its biological activity, did not work for some scarab species because of behavioral complications and/or the fact that most of the time the natural products were tiny peaks buried in a highly contaminated mixture. These complications could be alleviated by the utilization of gas chromatography with an electroantennographic detector (GC-EAD). The stage for holding the beetle antennae was modified in order to allow the lamellae to be open and to face the air flow during GC-EAD measurements (42). Utilizing this technique, the major constituent of the sex pheromone for the cupreous chafer, *A. cuprea*, was identified as (*Z*)-5-(—)-(1-octenyl)oxacyclopentan-2-one, buibuilactone (Figure 1, structure *3*) (28). Utilizing the state-of-the-art capillary chiral chromatography and an amplification strategy, the absolute configuration of buibuilactone was determined to be (*R*). Further investigations by GC-EAD revealed that *A. cuprea* also utilizes japonilure (1) as a second component (45). It has been analytically determined that japonilure from both *A. cuprea* sex pheromone system and the Japanese beetle has the same (*R*)-stereochemistry (43; WS Leal, unpublished data).

Another technique that has been successfully applied in the identification of scarab sex pheromones is the gas chromatography–behavior bioassay. With this technique, we have demonstrated that the small cherry beetle, *Anomala schonfeldti*, utilizes 2-(*E*)-nonenol (Figure 1, structure *4*) as a sex pheromone (30, 34), and the compound is very active in the field (15). However, GC-EAD measurements showed that 2-(*E*)-nonenal (Figure 1, structure *5*), an unavoidable contaminant of the synthetic sex pheromone, is also electrophysiologically active (WS Leal, unpublished data). Highly purified pheromone samples have been prepared to test the role of the aldehyde in pheromonal communication. Although no traces of 2-(*E*)-nonenal could be detected by conventional analytical techniques, such as GC or GC-MS, GC-EAD showed the occurrence of electrophysiologically detectable amounts of the aldehyde. This obstacle has prevented the assessment of the role of the aldehyde in the

chemical communication of *A. schonfeldti* (WS Leal, unpublished). This can be done only by testing male responses to 2-(*E*)-nonenol (Figure 1, structure *4*) that is completely devoid of 2-(*E*)-nonenal (Figure 1, structure *5*) versus a binary mixture.

The sex pheromone of the Oriental beetle, *Blitopertha orientalis*, has been identified by GC-behavior bioassay as a 7:1 mixture of (*Z*)- and (*E*)-tetradec-7-en-2-one (Figure 1, structure *6*) (29). The same compound has been identified in the American population of the Oriental beetle, *Anomala orientalis* (75). Baraud (2) revised the genus and suggested that *Blitophertha* and *Anomala* should be named *Exomala*. Therefore, it is not entirely surprising that the American and Japanese populations of the Oriental beetle utilize the same sex pheromone. Although the ketone compound (Figure 1, structure *6*) alone is very active in the field (10, 35), there is a second component in the pheromone system of the Oriental beetle (35). The scanty amounts of the semiochemical collected from female beetles have prevented its chemical characterization.

Even with pheromone blends that consist of just a few semiochemicals, closely related scarab species have attained isolated chemical communication channels. *Anomala daimiana*, for example, co-occurs in the field with *A. schonfeldti* and *A. cuprea* and utilizes a pheromone system of 2-(*E*)-nonenol (Figure 1, structure *4*) and buibuilactone (Figure 1, structure *3*), compounds that are common to the other two species (46). Perhaps the role of a second component in the pheromonal activity prevents cross-attraction among these three species.

More interestingly, species that are geographically and/or seasonally isolated utilize the same sex pheromone system. In the genus *Anomala*, *A. cuprea*, *A. octiescostata*, and *A. albopilosa sakishimana* produce a pheromone blend composed of buibuilactone (Figure 1, structure *3*) and japonilure (Figure 1, structure *1*) (28, 37, 38, 45). Both *A. cuprea* and *A. octiescostata* occur in the mainland of the Japanese archipelago (Honshu), but the flight activity of the former occurs in summer, whereas the latter is active in early spring. On the other hand, *A. albopilosa sakishimana* is native to Miyako Island in the south of Japan. Thus, there was probably limited selective pressure for the evolution of sex pheromone systems with different constituents (32). In this connection, it was interesting to investigate the pheromone system for *A. albopilosa albopilosa*, which shares the same habitat and has a common flight season with *A. cuprea*, although this species has not attained the status of agricultural pest. *A. albopilosa albopilosa* utilizes a more complex pheromone blend, i.e. buibuilactone, 2-(*E*)-nonenol, 2-(*E*)-nonenal, and methyl benzoate (Figure 1, structures *3*, *4*, *5*, and *7*, respectively); only the major component is a common constituent of the sex pheromones of both species (36). The chemical signature of the different pheromone blends provides species-specific chemical signals for the two

species. Temporal differences in mating activities also play a role in their isolation. While the diel rhythm of pheromone release and mating activity in *A. albopilosa albopilosa* showed a peak at the beginning of the scotophase (36), pheromone release in *A. cuprea* reached a peak late in the photophase (WS Leal, unpublished data). Species specificity of the chemical communication channels of the northern and southern masked chafers, *Cyclocephala borealis* and *Cyclocephala lurida*, is also attained by temporal rather than chemical differences in the pheromone systems. Normally, these two species are active at different times at night, with *C. lurida* mating before 11 PM and *C. borealis* after midnight. However, when *C. lurida* females were confined in traps above ground, *C. borealis* males were attracted and captured. Similarly, female *C. borealis* in traps attracted male *C. lurida* (58).

That geographically or seasonally isolated species of the same genus utilize the same sex pheromone systems prompted us to screen previously identified sex pheromones. 2-(*E*)-Nonenol was a male attractant to *Anomala dubia* and *Anomala vitis* (65) in Hungary, but none of the tested pheromones were attractive to *Anomala marginata* in Florida (6).

Generally, rutelines utilize sex pheromone constituents that are fatty-acid derivatives (see below), but the sex pheromone for *Phyllopertha diversa* is a very unusual alkaloid with medicinal properties (Figure 1, structure *8*) (52). Interestingly, this alkaloid is very attractive to males in the field, despite its low volatility, as compared with other scarab pheromones. It is conceivable that owing to the swarming behavior of males in the field, chemical communication in *P. diversa* is achieved with a short-range semiochemical that plays a pivotal role for pinpoint localization of females.

MELOLONTHINES The first sex pheromone of a scarab beetle, which was identified from a grass grub native to New Zealand, *Costelytra zealandica* (9), was phenol (Figure 1, structure *11*). Two years before the identification of the pheromone, a commercial adhesive Pliobond® was found to be highly attractive to male beetles (reviewed in 32), of which the active material is the phenol/formaldehyde resin Durez 12687®, an ingredient used in the manufacture of Pliobond.

Not until 1991 was the second pheromone discovered from another melolonthine. Females of the large black chafer *Holotrichia parallela* display an interesting calling behavior by everting an abdominal gland. Fractionation of ether extracts of this gland, followed by behavioral bioassays led to the identification of L-isoleucine methyl ester (Figure 1, structure *12*) as the major sex pheromone constituent (40). Further investigations showed that (*R*)-linalool (Figure 1, structure *13*) was a key component of the *H. parallela* pheromone system (47). Traces of L-valine methyl ester (Figure 1, structure *16*) were also

found in gland extracts, but addition of this compound to the binary system did not significantly increase trap catches. Behavioral patterns of this species do not follow a circadian rhythm but rather an unusual periodicity. Adults remain in the soil during the day time, coming to the surface every other evening after sunset. The rhythm of appearance was documented for four years in the field and also observed in the laboratory (71). Examination of the pheromone titer showed that the amount of L-isoleucine methyl ester in the glands decreased dramatically in the night following calling. In addition, the ability of males to respond behaviorally to the sex pheromone showed peaks every other day (47). This circabidian rhythm is unusual not only in pheromone production, but also in the biology of insects.

Melolonthines are unique not only in the circabidian periodicity of pheromone production but also in their mating behavior. Examination of the chemistry of the pheromone gland of female *Holotrichia consanguinea* led to the identification of anisole (Figure 1, structure *14*), which was very attractive in the field to males and females (50). Females of some scarab species can often detect and be attracted to their own pheromones in the field, but the number of captured females, though significantly higher than in control traps, is much smaller than the number of captured males (WS Leal, unpublished data). In order to determine the sex ratio of beetles attracted during the calling period of *H. consanguinea*, capture data were analyzed by sex at 5- to 10-min intervals. Throughout the period of mating activity, the average sex ratio of beetles caught by three field traps separated by 10 m was very close to 1:1, which is the same sex ratio as in beetles present in the field. This result could prompt one to call anisole an aggregation pheromone. However, both sexes that were attracted to this semiochemical also displayed sexual behavior, which strongly suggests that this is a sex pheromone. Males attracted to anisole tried to copulate with any beetle in the vicinity. They also fluttered their wings when sitting on the trap lips or the strings used to hang the traps. On the other hand, females were equally attracted to the same semiochemical, and those that were not trapped displayed calling behavior on the traps (50). The adaptive basis of response to the pheromone by both sexes is not clear. It might not have been selected to attract females because it increases competition for mates. However, if the aggregation has any advantage other than mate finding, natural selection is expected to favor the use of a pheromone that is not sex specific (50).

The pheromone chemistry of the cranberry white grub, *Phyllophaga anxia*, is more complex. Five compounds from female effluvium gave reproducible EAD activity, with L-valine and L-isoleucine methyl esters (Figure 1, structure *16*) giving the strongest electrophysiological responses (76). A synthetic 3:1 blend of L-valine and L-isoleucine methyl esters on a rubber septum was attractive in the field (76).

The occurrence of amino acid–derived sex pheromones in melolonthines is unique and remarkably different from the fatty acid derivative sex pheromones of rutelines. Related compounds have also been identified as sex pheromones for two *Phyllophaga* species native to Costa Rica. The sex pheromone systems for *P. elemans* and *P. viciana* are composed of N-formyl L-isolucine methyl ester (Figure 1, structure *17*), N-acetyl L-isoleucine methyl ester (Figure 1, structure *18*), and L-isoleucine methyl ester (Figure 1, structure *12*). In field experiments, only males have been captured in traps baited with synthetic lures and no captures were observed in the control traps (WS Leal, P Shannon, E Hidalgo, M Ono, PHG Zarbin & AC Oehschlager, unpublished data).

Yardin & Shani (68) reported evidence for the occurrence of a female-released sex pheromone in *Maladera matrida*, an exotic species in Israel. They suggested a two-stage mechanism in the chemical communication of this melolonthine. First, males cause damage to their host plants, and both sexes are then attracted to host plant volatiles. Second, females emit sex pheromone while feeding on the host plants or shortly after. GC-EAD analysis showed the occurrence of an electrophysiologically active component in the airborne extracts from females. This EAD-active component was identified as (*Z,E*)-α-farnesene (Figure 1, structure *15*) (69), but proof of pheromonal activity is yet to be reported.

Most of the melolonthine pheromones identified so far are either amino acid derivatives or terpenoid compounds, whereas ruteline sex pheromones are fatty acid derivatives. Intriguingly, we have identified that the major constituent of the sex pheromone for the yellowish elongate chafer, *Heptophylla picea*, is (*R,Z*)-7,15-hexadecadien-4-olide (Figure 1, structure *10*) (39). Although we have not yet studied the biosynthesis of this pheromone, it is likely to be derived from oleic acid.

As discussed elsewhere (32), the pheromones of melolonthines may have evolved from a primary defensive role. The reasoning is that the semiochemicals are ubiquitous compounds that are utilized per se or as immediate precursors; they are produced in huge amounts; enantiomeric purity is not critical; and most of them have antimicrobial activities. Additional strong evidence for the secondary function hypothesis for the origin of sex pheromones in scarab beetles is the serendipitous discovery of attraction of male *Cyclocephala lurida* (Dynastinae) to grubs (16). GC-EAD analysis of the airborne volatiles and whole-body extracts from female beetles and whole-body extracts from grubs showed the occurrence of a common component in the grub and adult female extracts that was electrophysiologically active on male antennae (WS Leal, KF Haynes & J Meinwald, unpublished data). GC- behavior bioassay experiments showed that this EAD-active compound was involved in attraction of male *C. lurida*, as males gathered at the GC outlet soon after the peak was eluted from the column, and some males displayed pre-copulatory behavior (WS Leal,

KF Haynes & J Meinwald, unpublished data). The production of the adult female sex pheromone by immature stages of the same species remains enigmatic. Potentially, this compound may play some alternative role in the immature stage. Haynes & Potter (17) speculated that the ontogeny of the sex pheromone signal in *C. lurida*, in which a sex pheromone may be derived from volatile chemicals that are lost in adult males but remain in females, represents another, previously undescribed route for evolution of chemical communication.

I have tested the occurrence of adult sex pheromone in a few scarab species utilizing adult male antennae as sensing elements for GC-EAD. No traces of the semiochemicals could be detected in larval extracts from *A. cuprea*, *Exomala orientalis*, and *H. parallela* (WS Leal, unpublished data). It may be that this phenomenon is restricted only to Dynastinae or even to *Cyclocephala* species.

Aggregation Pheromones

Examination of volatile extracts obtained from aerations of either *Oryctes monoceros* (Dynastinae) males, females, or both sexes combined showed two male-specific compounds. One of them was EAD-active and was identified as ethyl 4-methyloctanoate (Figure 1, structure *19*) (13). Field experiments showed small, but significant, catches of males and females in traps baited with synthetic lures. Although 4-methyloctanoic acid was also male specific, its activity was not tested, probably because it was not EAD-active. However, EAD responses could be easily missed, because of the low signal-to-noise responses of this beetle's antennae in EAD measurements. The same ethyl ester has been identified as an aggregation pheromone for *Oryctes rhinoceros* (14). Airborne volatile collections showed the occurrence of two other male-specific components in this species. In addition to 4-methyloctanoic acid, an EAD-active ethyl 4-methylheptanoate was detected. Field trapping experiments supported that only ethyl 4-methyloctanoate was attractive, as significantly more males and females were captured in the pheromone-baited traps. Based on the fact that in field tests racemic ethyl 4-methyloctanoate and the (*S*)-stereoisomer were equally attractive, whereas traps baited with the (*R*)-enantiomer caught significantly fewer beetles, the pheromone was fully characterized as ethyl (*S*)-4-methyloctanoate (14). Thus, the same aggregation pheromone is utilized by these two geographically isolated species: *O. monoceros*, which is one of the most destructive pests of commercial coconut, oil, and date palms in Africa, and *O. rhinoceros*, which is one of the most important pests of coconut and oil palms in South and Southeast Asia.

Laboratory bioassays suggested that in addition to this aggregation pheromone, chemical communication in *O. rhinoceros* involves a female-produced sex pheromone (14), but the identification of the latter has not been pursued.

AGONIST-ANTAGONIST ACTIVITIES OF ENANTIOMERIC PHEROMONES

Stereochemical discrimination may be considered the ultimate refinement of chemical communication. Its importance with respect to (*E*)- and (*Z*)-isomerism was recognized with the characterization of bombykol. The existence of chiral pheromones has been recognized in the pioneering works of Silverstein (61) and in a few studies of enantiomeric effects on insect behavior (reviewed in 60), but the most clear-cut case for the significance of (*R*)/(*S*) configurations in insect semiochemicals was demonstrated in the Japanese beetle (67). Although the stereochemistry of the sex pheromone was analytically determined only recently (43), Tumlinson et al showed that female beetles appear to release predominantly the (*R*)-enantiomer, but the presence of the (*S*)-stereoisomer dramatically reduces the response of responding males. Recent studies on chemical communication of scarab beetles showed the occurrence of various chiral pheromonal systems, but in most cases the non-natural enantiomer had no effect on the insect's behavior. However, the identification of the sex pheromone for the Osaka beetle, *Anomala osakana*, disclosed a very interesting mechanism of mutual behavioral agonist-antagonist activities of enantiomeric pheromones (Figure 2). The Osaka beetle produces and responds to (*S*)-japonilure (Figure 1, structure *9*), the activity of which is completely inhibited by the presence of the Japanese beetle sex pheromone. Chiral gas chromatography with an electroantennographic detector (48) demonstrated that both the Japanese and Osaka beetles had olfactory neurons tuned to each enantiomeric form of the lactone (31).

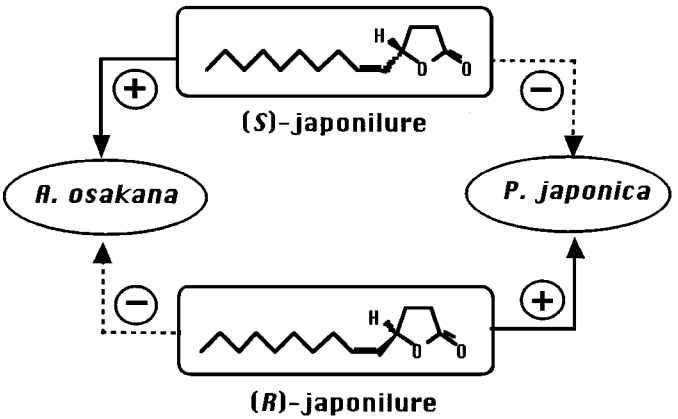


Figure 2 Schematic view of the mechanism of reciprocal behavioral agonist-antagonist activities of chiral pheromones.

Other *Anomala* species, however, for which the non-natural enantiomer has no behavioral roles, appear to lack olfactory receptor neurons (ORNs) specific to the antipode of the pheromone (WS Leal, unpublished data). The existence of olfactory neurons specific to each enantiomer, the behavioral antagonist effect of the pheromone antipode, and the fact that these two species share a common habitat in Japan strongly support the existence of a reciprocal agonist-antagonist mechanism that provides species-specific chemical signals for the two species with the use of the two enantiomeric forms of a chiral pheromone.

The fact that a chiral sex pheromone of one scarab species elicits a negative behavioral response in allospecific receivers has also been demonstrated (31), but reciprocal behavioral antagonism was not observed.

SEX PHEROMONE GLANDS

Sex pheromone glands of rutelines have been located by extracting various parts of the body, by analyzing the extracts by GC-MS, and by histological and morphological studies. Female-specific epithelial cells line the inner surfaces of the anal plate and the two apical sternites, and the volatile compounds of these cells are probably released through pores that appear in the cuticular layer adjacent to these glandular cells only in females (62). On the other hand, scarab beetles in the genera *Holotrichia* and *Phyllophaga* (Melolonthinae) display a typical calling behavior by extruding the abdominal tip, which exposes a ball-shaped sac (32, 47, 50). Sex pheromones of a few melolonthines have been identified from whole-extracts of these abdominal tissues, but the fine structures of the glands remained unknown. Analysis of extracts from the abdominal tips, as well as from the anal plate and the two apical sternites of *H. parallela*, showed that pheromone was present only in the abdominal sacs (62). Conversely, the pheromone of *H. picea* (Melolonthinae) was detected in the anal plate and the two apical sternites, no trace of which was detected in abdominal tips of females (62).

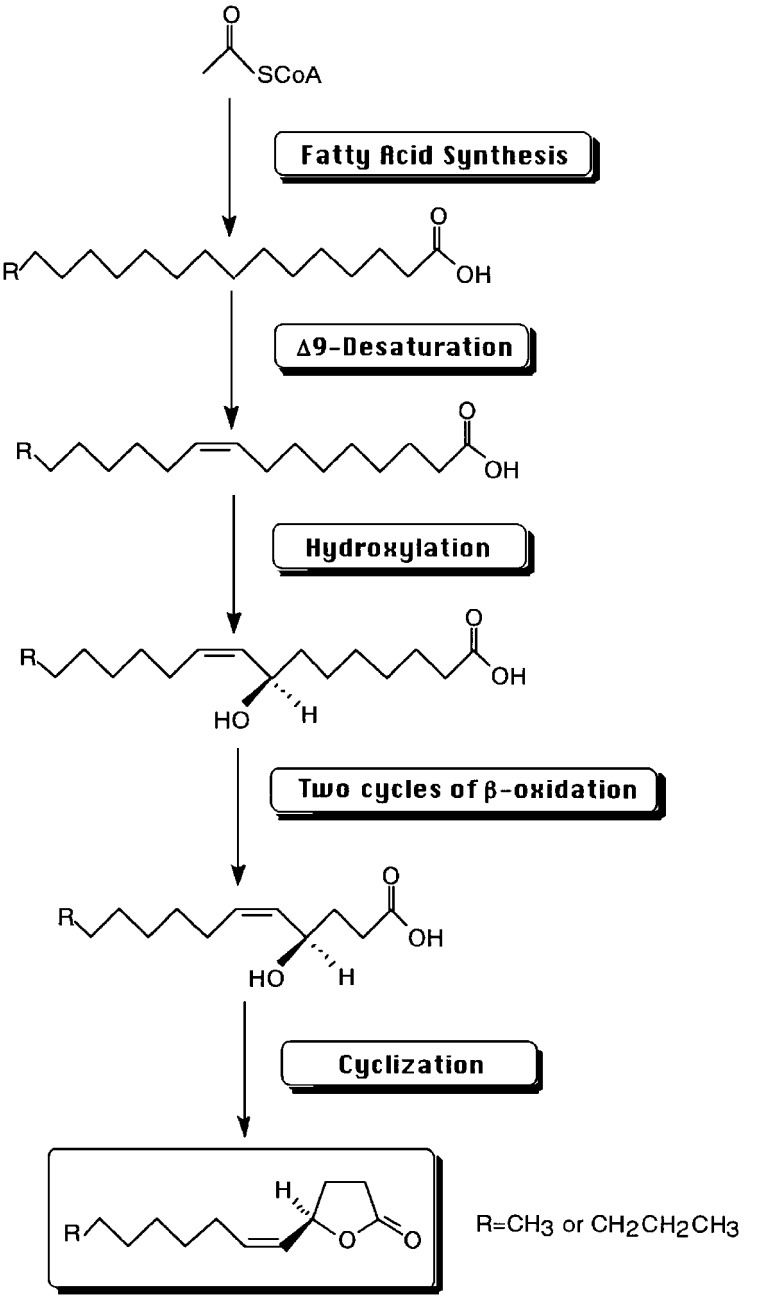
The structure involved in sex pheromone attraction of female *Rizotrogus aequinoctialis* (Melolonthinae) has been described as a gland of milky color and round in form, located at the end of the abdomen. During calling, females have been observed to display a big bulge of intersegmental membrane containing two dark bodies that appear at the end of the abdomen. In order to identify the parts of the body that emit the pheromone, male attraction was studied in the field towards caged females, freshly excised membranous structures, and females without these structures. Zamatailov (74) concluded that "males were most strongly attracted to isolated intersegmental membranous structures." Anatomical studies have shown that below the membrane, there are two round bodies, one on each side of the vagina, structures that are located inside the

bulging membrane during attraction of males. A smaller part of these organs looks like an orange grain, and a bigger part looks like a reservoir of dark-gray color and ends in a canal, which is not connected to reproductive organs. There is no evidence in this concise report that the organs described are really involved in pheromone production. It seems that Zamatailov found that males were attracted to abdominal tissues, and because of their location in the abdomen, these structures were assigned as pheromone glands. A similar structure was also observed in *H. parallela*, consisting of a couple of small mushroom-like organs situated laterally on the mid part of the sac that is everted during calling behavior. Although the mushroom-like organs contained many secretory cells, no trace of the pheromone was found in their extracts, but only in the remaining part of the whole abdominal tip (WS Leal, unpublished data). On the other hand, Hoyt & Osborne (19) demonstrated that in another melolonthine, *C. zealandica*, sex pheromone was produced by symbiotic bacteria in colleterial glands that lie beneath the seventh sternite, ventral to the vagina.

SEX PHEROMONE BIOSYNTHESIS

Most of the progress in our understanding of pheromone biosynthesis is due to the extensive efforts that have centered on Lepidoptera, apart from a few scattered reports on other economically important pests. Since the identification of japonilure, it has been suggested that the pheromone originated from fatty acids, but this remained a speculation because the pheromone-producing glands were not known. The discovery of the pheromone glands in scarabs paved the way for a careful investigation of biosynthesis of pheromones in rutelines (51). Using deuterated precursors, we elucidated the whole biosynthetic pathway for buibuilactone and japonilure in *A. cuprea* (51). The reaction pathway starts from saturated fatty acids and involves their desaturation, followed by hydroxylation, chain shortening, and cyclization (Figure 3). Perdeuterated palmitoleic acid was applied to investigate the mechanism of hydroxylation. Retention of all deuterium atoms in the lactones implied that this reaction was a direct process. The perdeuterated pheromone elicited electrophysiological response in a male antenna similar to that evoked by the natural pheromone, but surprisingly, the perdeuterated pheromone eluted earlier than the unlabeled buibuilactone. The product obtained from racemic (9,10-d_2)-8-hydroxypalmitoleic (and oleic)

Figure 3 Route for the biosynthesis of buibuilactone ($R = CH_3$) and japonilure ($R = CH_2CH_2CH_3$) in scarab beetles. The stereochemistry of these semiochemicals is determined in an earlier step of hydroxylation.


O
SCoA
Fatty Acid Synthesis
R
O
OH
Δ9-Desaturation
R
O
OH
Hydroxylation
R
O
OH
HO
H
Two cycles of β-oxidation
R
O
OH
HO
H
Cyclization
H
R
O
O
R=CH3 or CH2CH2CH3

acids was also racemic. Thus, the hydroxylation of fatty acids seems to be the only stereospecific reaction in the pathway, and subsequent steps do not discriminate between enantiomers.

PLANT KAIROMONES

When the Japanese beetle was found in southern New Jersey in mid-August, 1916, little was known about its biology, probably because it was a pest of minor importance in its native environment, though nowadays it is an economically important pest in Japan as well. In New Jersey, the beetle found a generally favorable climate, large areas of permanent turf for developing the immature stages, almost 300 species of plants to satisfy its voracious appetite, and at that time no important natural enemies (12). Because the beetle population annually increased to more destructive levels and spread into new areas, efforts were made to identify botanical attractants that could be used to survey its spread and that might have a direct role in its control (27).

The initial stage of this chemical prospecting for plant kairomones was ecologically oriented, but later it turned into a trial-and-error testing of many substances, alone and in combinations. The observation that the Japanese beetle is particularly attracted to certain plant species has led to examination of the attractancy of essential oils of these plants and some of the constituents of these oils. Field tests showed that the beetles were attracted to geraniol and eugenol. A 10:1 mixture of these two chemicals was used as a standard attractant for the survey conducted by the USDA until 1940 (11). This standard lure was replaced by a mixture of anethole plus eugenol (9:1) that was used until 1965. A mixture of phenethyl proprionate and eugenol (3:7) was subsequently adopted as the standard survey lure by state and federal agencies. Finally, Ladd and collaborators (26) found that a three-component lure, phenethyl proprionate plus eugenol plus geraniol (3:7:3) was more effective, and this is currently the most widely used standard food-type lure for surveys of the Japanese beetle populations.

The success of the Japanese beetle program motivated worldwide trial-and-error tests with attractants for other scarab species. In Japan, most of the emphasis was on geraniol, eugenol, anethole, phenethyl proprionate, and phenethyl butyrate, pure or in combinations (70). Although lures for some species were identified, a seven-year program failed to find any attractant for the major target pests, the soybean beetle, *A. rufocuprea*, and the cupreous chafer, *A. cuprea*. On some occasions, the screening of plant metabolites has led to the successful identification of highly effective plant kairomones, such as methyl antranilate. Traps baited with this lure caught 5 times as many *A. rufocuprea* as the synthetic sex pheromone (20). Even the catches of males were higher (2.5 times)

with methyl antranilate than with the sex pheromone. Interestingly, this lure showed no attractiveness for any other species that were observed in the field, including *A. cuprea*, *A. puncticollis*, *P. japonica*, *E. orientalis*, *H. parallela*, and *Oxycetonia jucunda*.

Ecologically oriented approaches do not necessarily lead to a better lure than a trial-and-error program. This was the case with the plant kairomone attractant for *A. octiescostata* (44). The observation that adult beetles feed voraciously on dandelion, *Taraxacum officinale*, led to the characterization of the kairomones from this plant as *cis*-3-hexenyl acetate, benzaldehyde, phenylacetaldehyde, benzyl alcohol, phenethyl alcohol, phenylacetonitrile, and benzyl benzoate in the ratio 4:8:14:3:5:19:11. Although this lure increased trap catches, it was not better than anethol plus geraniol plus phenethyl proprionate (9:0.5:0.5), obtained by trial and error (44).

In marked contrast to pheromone research, most of the investigation in food-type lures has been aimed only at the development of attractants for population surveys and/or scarab control, to which studies of insect-plant interaction have taken a back seat in these programs. Interestingly, most of the kairomones identified thus far are flower scents. Since scarabs feed on, rather than pollinate, flowers, these semiochemicals are unlikely to be beneficial to the flowers as far as scarab-plant interactions are concerned.

Leaf volatiles have also been identified as scarab attractants (WS Leal, unpublished data), but they are not widely known as lures because their high volatility makes their application difficult. It has been suggested that the application of the plant growth regulator ethephon on edible ginger, *Zingiber officinale*, increases the formation of unidentified plant volatile(s) that lead to the attraction and feeding of the Chinese rose beetle, *Adoretus sinicus* (1).

Sometimes the lead for new lures is quite unexpected, such as the observation that the beetle *A. octiescostata* is attracted to a cigarette brand, Mild Seven®. Field experiments showed that beetles were attracted to cigarette stubs as well as to people smoking this cigarette brand. Although the attractant could be extracted with organic solvent (hexane), it is unclear whether this attraction is due to chemical(s) from tobacco leaves or from the flavor compounds used in the cigarette preparation (WS Leal, unpublished data).

ODOR PERCEPTION IN SCARAB BEETLES

Scarab beetles are characterized by lamellate antennae, formed by leaf-like extensions of the terminal segments, on which the olfactory sensilla are located. According to Meinecke (54), most of the many variants of the scarab olfactory sensilla can be regarded as modifications of three basic types: recessed pore plates, pore plates on sockets, and hair-like sensilla. Comparison of 50 beetle

species has shown that a cladogram based solely on morphological characters of the olfactory sensilla matches remarkably well those based on entirely different characters. Comparative morphology of the presumably primitive types of each group indicates that the ancestral type might be the sensillum coeloconicum, one of the most common types of olfactory sensillum in insects. Using single sensillum recordings, we (41) demonstrated that pheromone-sensitive receptor neurons are located in sensilla placodea without pits, which encompass a large area of the posterior parts of the lamellae.

In *A. cuprea*, these receptor neurons are specific to the natural buibuilactone, whereas its antipode remains undetected. The mutual behavioral agonist-antagonist activities of enantiomeric pheromones are mirrored in the ORNs of the Osaka and Japanese beetles (BS Hansson & WS Leal, manuscript in preparation). In these two species, two ORNs, each detecting one enantiomer of the pheromone, have been found in most sensilla. In the Osaka beetle, the pheromone (*S*)-japonilure was detected by a receptor neuron characterized by a large action potential amplitude, whereas a small spiking receptor neuron was tuned to the behavioral antagonist, (*R*)-japonilure. On the other hand, in the Japanese beetle, the large spiking neuron is tuned to (*R*)-japonilure, and the small spiking receptor neuron detected the antipode. It is worth mentioning that these ORNs are located in the same olfactory plates. In the Japanese beetle, all sensilla containing pheromone-detecting receptor neurons corresponded to this pattern, whereas in the Osaka beetle some sensilla contained only a receptor neuron tuned to one of the enantiomers, while the antipode elicited no response in the second receptor neuron.

MOLECULAR BIOLOGY OF SIGNAL TRANSDUCTION

At least three classes of proteins are involved in recognition of chemical signals in insects: (*a*) odorant binding proteins (OBPs), (*b*) odorant receptors placed on the membrane of olfactory neurons, and (*c*) degrading enzymes present in the sensillar lymph surrounding these neuronal cells. It is believed that at least in Lepidoptera there is a specific protein in each of these three classes involved in pheromone recognition; thus proteins involved in recognition of pheromones have been set apart from the proteins involved in recognition of general odorants. It has been suggested that pheromone binding proteins (PBPs) are not merely passive transporters of lipophilic ligands but rather selective for certain ligands (reviewed in 3). Thus, PBPs may act as a selective filter that binds and consequently translocate only distinct, physiologically relevant ligands. Pelosi (57) derives this model for the role of PBPs from the chemoreception mechanisms in bacteria. Under this hypothesis, the odorant molecules would never reach the neuronal membrane, but the olfactory message could be conveyed by

the PBPs, in turn activated by the odorants. This model looks appealing because the receptors would thus be protected from foreign, potentially harzadous molecules carried by the air stream. However, this model poses some problems when it has to fit with the intracellular mechanism of olfactory transduction. The role of G-proteins in olfactory transduction has been established, as well as the identity of the olfactory proteins belonging to the family of seven transmembrane domain receptors. The ligands for receptors of this class, however, are not proteins but small organic molecules. Therefore, an interaction of PBPs with the known olfactory receptors seems unlikely.

The discovery of the reciprocal agonist-antagonist activities of enantiomeric pheromones in the Osaka and Japanese beetles stimulated us to investigate whether PBPs of these two species are able to discriminate the two enantiomeric forms of the pheromone. We have prepared both enantiomers of japonilure in tritiated form by stereoselective synthesis of precursors and catalytic reduction of the corresponding alkyne and have characterized antennal specific proteins from various scarab species. As far as the N-terminal amino acid sequences for the putative PBPs from Osaka and Japanese beetles are concerned, these proteins are identical or highly homologous. In addition, the proteins bind weakly radiolabeled pheromones. These experiments suggest that the ligand specificity of PBPs may not be as high as had been postulated. Unexpectedly, these ligands were bound by a PBP from a moth, *Samia cynthia ricini*, which utilizes (*Z,Z*)-6,11-hexadecadienal as a pheromone. Another line of evidence that points to the lack of specificity of PBP/OBP is the occurrence of *A. cuprea* PBP/OBP not only in olfactory receptors related to pheromone perception, but also in the general odorant sensilla (49). In rutelines, pheromone and general odorant receptors are clearly separated on lamellae of the beetles. Analysis of the protein extracts from each part of the lamellae showed the same putative PBPs present in both types of olfactory plates (49). It is worth noting that species that utilize unrelated pheromone structures have different PBPs. For example, antenna-specific proteins of *H. parallela* and *P. diversa* differ remarkably from the same proteins from *A. cuprea* and *P. japonica* (H Wojtasek & WS Leal, unpublished data).

We observed that soluble antennal proteins from the extracts of several species can degrade buibuilactone and japonilure, even those from beetles that do not use this group of compounds as their pheromones. Activity staining of native PAGE showed that species possessing high levels of antennae-specific esterases have a significant preference for the (*R*)-stereoisomer. However, this specificity seems one-sided because species that utilize the (*S*)-enantiomer do not degrade it more quickly (72, 73). Interestingly, the recently identified alkaloid pheromone from *P. diversa* is readily degraded by antennal enzymes of this species. On the other hand, beetles that utilize lactones as their pheromones

possess little or no ability to metabolize the alkaloid. Because of its unusual structure, it is not surprising that only species that utilize the alkaloid in chemical communication have developed the ability to degrade it. Lactones and esters, on the other hand, are common odorants, and thus the presence of appropriate enzymes in a large number of species is expected (73). These data suggest that antennae-specific pheromone-degrading enzymes in scarab beetles have evolved to participate in deactivation of pheromone or other odorants.

FUTURE PROSPECTS

Utilizing the newly established techniques in chemical ecology, pheromones and other semiochemicals should be identified in many other scarab species in the near future. Their chemical structures will give a better perspective of the evolution of chemical communication in scarab beetles. The area of pheromone production may be explored with respect not only to biosynthesis but also to the role of PBAN-like peptides and other physiological factors.

Single sensillum recordings have demonstrated that two colocalized olfactory receptor neurons in antennae of scarab beetles handle pheromone information and "stop signal" with selectivity higher than that of any other biological systems. Electrophysiological approaches and molecular biology of signal transduction may be explored within the foreseeable future to get a better understanding of chiral discrimination in nature using scarabs as a model.

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