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ISOLATION, IDENTIFICATION, SYNTHESIS AND BIOLOGICAL ACTIVITY OF VOLATILE COMPOUNDS FROM THE HEADS OF *ATTA* ANTS

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Abstract—S-(+)-4-methyl-3-heptanone has been identified as the principal alarm pheromone of *Atta texana* and *Atta cephalotes*. Both enantiomers of 4-methyl-3-heptanone have been synthesized and their biological activities have been compared on both species of ants. Comparison of the geometric averages of response ratios, at threshold concentration levels on *A. texana*, showed S-(+)-4-methyl-3-heptanone to be about 100 times more active than the (−) enantiomer. A similar analysis also showed no inhibition of the activity of S-(+)-4-methyl-3-heptanone by the (−) enantiomer. A less rigorous study on *A. cephalotes* showed S-(+)-4-methyl-3-heptanone to be about 210 times more active than R-(−)-4-methyl-3-heptanone.

Both ant species produce 3-octanone, possible trace amounts of 3-octanol, and both diastereomers of 4-methyl-3-heptanol. *A. texana* also produces (+)-2-heptanol, 2-heptanone, and 3-heptanol. *A. cephalotes* contains trace amounts of 2-heptanone.

INTRODUCTION

THE CHEMICAL communication (pheromonal) systems used by ants to provide social order within a colony have been studied extensively in recent years. Alarm pheromones are conspicuous components of ant communications.

4-Methyl-3-heptanone has been identified as an alarm pheromone in species of four genera in the sub-family Myrmicinae: *Pogonomyrmex*, *Atta*, *Trachymyrmex*, and *Manica*. MCGURK *et al.* (1966) identified the compound from the mandibular glands of three species of *Pogonomyrmex* ants. MOSER *et al.* (1968) identified it, along with 2-heptanone, as the major volatile constituents of the leaf-cutting ant *Atta texana*. In *A. texana*, 4-methyl-3-heptanone was 1000 times more effective in eliciting alarm response than 2-heptanone. BLUM *et al.* (1968) found that the mandibular glands of 6 species of *Atta* contained different concentrations of 4-methyl-3-heptanone. CREWE *et al.* (1972) identified 4-methyl-3-heptanone as a major component of the mandibular gland extract of *Trachymyrmex seminole*, and FALES *et al.* (1972) identified the compound from the mandibular glands of *Manica mutica* workers.

Several studies hae been conducted to relate chemical structure and alarm activity. In an earlier work (BLUM *et al.*, 1966), and in a more recent study (AMOORE

et al., 1969), correlations of alarm activity with molecular shape were found in tests on *Iridomyrmex pruinosus*. MOSER *et al.* (1968) conducted similar tests with *A. texana* and found that any deviation in structure from that of 4-methyl-3-heptanone resulted in decreased activity.

We report here a comparative study of the volatile constituents from the mandibular glands of *A. texana* and *A. cephalotes*; we deal in particular with the relative activities of both enantiomers of 4-methyl-3-heptanone.

MATERIALS AND METHODS

A. cephalotes were obtained from the Barranca site, 60 km northwest of San Jose, Costa Rica, in July 1971. Heads from the whole bodies were obtained by vigorously shaking the frozen ants in a plastic bag. *A. texana* heads were individually separated from worker ants collected in Grand Parish Louisiana in 1968. Both ant materials were stored at $-50°C$.

Heads from *A. cephalotes* (262 g) and *A. texana* (123 g) were homogenized in distilled pentane in a Waring blender. The crude extracts were dried over anhydrous sodium sulphate and were separately concentrated, at atmospheric pressure, with a short column packed with glass beads, to volumes of 2 and 3 ml of yellow oil for *A. cephalotes* and *A. texana*, respectively.

The extract from *A. texana* was subjected to fractionation by gas chromatography, whereas the *A. cephalotes* extract was cooled overnight at 0°C, and centrifuged to remove precipitated solids before gas chromatographic fractionation. The following columns were used (stainless steel unless otherwise noted): column A, 5% Hi Eff diethyleneglycol succinate (DEGS) on Chromosorb G 60/80 mesh, 3 m × 6·3 mm (o.d.) fitted with precolumn packed with Chromosorb G 60/80 mesh, 30 cm^3/min He flow rate, 95 and 90°C isothermal for *A. cephalotes* and *A. texana*, respectively; column B, 4% Carbowax 20M on Chromosorb G 60/80 mesh, 7·3 m × 3 mm, 30 cm^3/min He flow rate, 95 and 103°C; column C, 4% 1,2,3 Tris-(2-cyanoethoxy)-propane (TCEP) on Chromosorb G 60/80 mesh, 7·3 m × 3 mm, 20 cm^3/min He flow rate, 90 and 97°C isothermal; column D, Carbowax 20M capillary column, 30·5 m × 0·5 mm, 3 cm^3/min He flow rate, 80°C isothermal; column E, DEGS capillary column, 30·5 m × 0·5 mm, 3 cm^3/min He flow rate, 70°C isothermal; column F, Apiezon L capillary column, 15·3 m × 0·5 mm, 3 cm^3/min He flow rate, 87°C isothermal; and column G, 4% Carbowax 20M on Chromosorb G 60/80 mesh, 6·1 m × 6·3 mm, glass column, 60 cm^3/min He flow rate, 130°C isothermal.

A thermal gradient collector (BROWNLEE and SILVERSTEIN, 1968) was used to collect fractions of column effluent in 30·5 cm glass capillary tubes.

Spectra of both the synthetic and natural compounds were obtained as follows: Mass spectra were obtained on a Hitachi RMU-6 mass spectrometer equipped with a micro inlet system, which allowed direct introduction of 10 to 20 μg samples collected in the capillary tubes. Infrared spectra were obtained on a Perkin Elmer Model 621 grating i.r. spectrophotometer; a Barnes Engineering 4 μl cavity cell was used with a variable path length cell in the reference beam to balance out the

carbon tetrachloride solvent. A sample size of ~25 μg was used routinely. NMR spectra were obtained on a Varian HA-100 spectrometer; Wilmad microtubes of ~36 μl capacity were filled with CCl_4 solutions containing 1% TMS and about 500 μg of sample. Optical rotations were obtained on a Durren Jasco Model ORD/UV/CD-5 recording spectropolarimeter. Samples ranging in concentration from 0·25 to 10 mg/ml in hexane were introduced into a Durren Jasco cylindrical metal cell with a 10 mm fixed path length and a 0·5 ml volume. Gas–liquid chromatography was done on a Varian Model 1740 gas chromatograph fitted with flame ionization detectors and a splitter with a split ratio of 100 : 1.

Bioassays (MOSER *et al.*, 1968) of compounds isolated from *A. cephalotes* were performed by one of us (R. G. R.) on a colony of *A. cephalotes*. The biological responses of *A. texana* to racemic 4-methyl-3-heptanone and 2-heptanone have previously been reported (MOSER *et al.*, 1968). Bioassays on *A. texana* of the enantiomers of 4-methyl-3-heptanone were performed by one of us (J. C. M.) on a laboratory colony of *A. texana*.

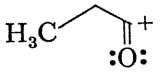
Samples of 2-heptanone, 4-methyl-3-heptanone, 3-octanone, and 3-heptanone were obtained from Aldrich Chemical Co. Sodium borohydride reduction of each ketone in isopropyl alcohol produced the corresponding alcohols, in 72, 50, 89, and 64 per cent yields, respectively, for bioassay, gas chromatographic retention time studies, and spectral comparisons.

RESULTS

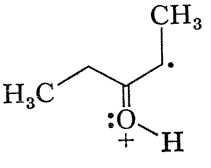
Isolation and identification

A. cephalotes. The yellow oil from *A. cephalotes* (2 ml) was chromatographed on column A at 95°C, and three fractions were collected: WHX-1 (6·2–7·5 min), WHX-2 (9·5–10·5 min), and WHX-3 (11·8–13·4 min). All three fractions were further purified on Column B. Fractions WHX-1A (10·0–11·2 min) and WHX-2A (15·8–17·8 min) were collected at 95°C and fraction WHX-3A (16·3–20·0 min) was collected at 103°C.

The mass spectrum of WHX-1A showed a molecular weight of 128, and the i.r. spectrum showed a ketone carbonyl absorption band at 1710 cm^{-1}. The mass spectrum showed a base peak at 57



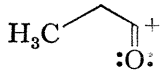
and a significant fragment at 86



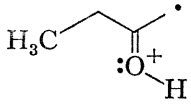
(SILVERSTEIN and BASSLER, 1967) indicating a methyl branch at carbon four.

These data suggested 4-methyl-3-heptanone as a likely structure for WHX-1A. These spectra and those of an authentic sample of 4-methyl-3-heptanone were congruent. The natural compound had an optical rotation of $[\alpha]_D^{27} = +22{\cdot}0 \pm 0{\cdot}4°$ (C, 1, hexane).

The mass spectrum of WHX-2A indicated a molecular weight of 128. The i.r. showed a carbonyl group (1718 cm^{-1}), and the NMR revealed a four proton multiplet at $\delta 2{\cdot}65$ suggesting two methylene groups adjacent to a carbonyl group. A distorted triplet at $\delta 0{\cdot}8$ indicated a shielded methyl group adjacent to a methylene group. Further inspection of the mass spectrum revealed major peaks at 57

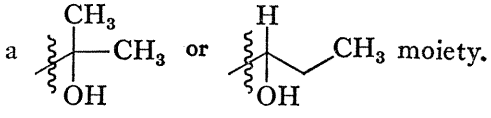


and at 72

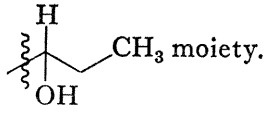


indicating an ethyl ketone not branched α to the carbonyl group (SILVERSTEIN and BASSLER, 1967). These data led us to 3-octanone as the likely structure for WHX-2A. The i.r. and mass spectrum of the natural compound were identical with those of synthetic 3-octanone.

WHX-3A had a molecular weight of 130 with a base peak in the mass spectrum at 59, which, together with the i.r. band at 3620 cm^{-1}, suggested



(SILVERSTEIN and BASSLER, 1967). The one proton multiplet in the NMR at $\delta 3{\cdot}27$ supported the



The i.r. and mass spectra of WHX-3A with those of an authentic sample of 4-methyl-3-heptanol were congruent. The optical rotation of natural 4-methyl-3-heptanol from *A. cephalotes* was $[\alpha]_D^{25} \sim +0{\cdot}8°$ (C, 1, hexane).

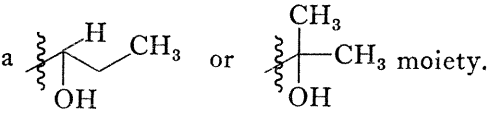
Each of the isolated compounds from *A. cephalotes*, except 4-methyl-3-heptanol, gave a single peak on columns, D, E, and F; no peak distortion was noted on co-injection with authentic samples. Isolated 4-methyl-3-heptanol gave a single peak on column F, but two equal-size peaks on columns D, E, and G, corresponding to a 1 : 1 ratio of the diastereomers.

A. texana. The yellow oil from *A. texana* (3 ml) was chromatographed on column A at 90°C, and five fractions were collected; WHY-1 (7·5–8·8 min), WHY-2 (8·8–10·2 min), WHY-3 (10·2–13·4 min), WHY-4 (13·4–14·9 min), and WHY-5 (14·9–17·6 min). All five fractions were rechromatographed on column C. Fractions WHY-1A (13·6–16·0 min), WHY-2A (17·1–19·0 min), WHY-3A (16·9–19·7 min), and WHY-3B (21·4–24·0 min) were collected at 90°C. WHY-4A (14.7–18·6 min) and WHY-5A (15·2–18·4 min) were collected at 97°C.

WHY-1A was identified as 4-methyl-3-heptanone from mass and i.r. spectra and comparison of these spectra with those of an authentic sample of 4-methyl-3-heptanone. The optical rotation of the natural compound was $[\alpha]_D^{25} = +22{\cdot}1 \pm 0{\cdot}4°$ (C, 1, hexane). As previously described for *A. cephalotes*, WHY-3B was identified as 3-octanone and WHY-5A was identified as 4-methyl-3-heptanol. The optical rotation of 4-methyl-3-heptanol from *A. texana* was $[\alpha]_D^{25} = -3{\cdot}2°$ (C, 1, hexane). When WHY-5A was rechromatographed on column G, two peaks were present in a ratio of about 2 : 1 corresponding to the two diastereomeric forms of 4-methyl-3-heptanol.

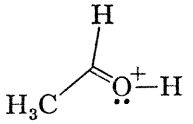
WHY-2A was identified as 2-heptanone from the congruency of its mass spectrum with that of synthetic 2-heptanone. The compound has previously been identified from the mandibular glands of *A. texana* (MOSER *et al.*, 1968).

WHY-3A was present in such a small quantity that only a mass spectrum could be obtained. The base peak at 59 suggested that we were again dealing with an alcohol containing



The largest molecular weight fragments in the spectrum were at *m/e* 87 and *m/e* 98. If the molecular weight of the alcohol was 116, then these fragments would correspond to M-29 and M-18, loss of an ethyl group and water molecule, respectively. The M-29 fragment corresponds to the secondary alcohol moiety previously described. The compound was tentatively identified as 3-heptanol; the published mass spectral data of 3-heptanol (CORNU and MASSOT, 1966) as well as the mass spectrum of an authentic sample of 3-heptanol were congruent with that of the isolated compound.

WHY-4A had a molecular weight of 116 and a base peak at 45



indicative of a methyl branched secondary alcohol (SILVERSTEIN and BASSLER, 1967). The i.r. showed an O—H stretching band at 3625 cm^{-1} and the NMR had a one proton multiplet at δ3·65 indicative of a methine proton on a carbon atom containing oxygen. A three proton doublet at δ1·12 suggested a methyl group

adjacent to a carbon atom containing oxygen. From these data, the compound was tentatively identified as 2-heptanol. The mass and i.r. spectra were congruent with those of a synthetic sample of 2-heptanol. The optical rotation of the natural compound was $[\alpha]_D^{25} \sim +1{\cdot}1°C$ (C, 1, hexane).

Each of the isolated compounds from *A. texana*, except 4-methyl-3-heptanol gave a single peak on columns D, E, and F; no peak distortion was noted on co-injection with authentic samples.

Trace amounts of 3-octanol seem to be present in both ant species. However, its identification is based only on matching retention times with those of an authentic sample on Columns A and B.

The amounts of each isolated compound from both ant species were determined (see Table 1) by comparison of GLC peak areas of standards with those of the natural compounds.

TABLE 1—CONCENTRATIONS OF VOLATILE HEAD COMPOUNDS

Compound (fraction)	*A. Texana* (amount μg/average worker)	*A. Cephalotes* (amount μg/average worker)
4-Methyl-3-heptanone (WHY-1A), (WHX-1A)	0·59*	18·8
4-Methyl-3-heptanol (WHY-5A), (WHX-3A)	0·21	0·90
2-Heptanone (WHY-2A)	0·16*	Trace <1%†
2-Heptanol (WHY-4A)	0·13	Absent
3-Octanone (WHY-3B), (WHX-2A)	0·03	0·70
3-Octanol (tentative identification)	Trace <1%‡	Trace ≪1%†
3-Heptanol (WHY-3A)	0·01	Absent

* Compounds previously identified (MOSER *et al.*, 1968).
† Relative to amount of 4-Methyl-3-heptanone in *A. cephalotes*.
‡ Relative to amount of 4-methyl-3-heptanone in *A. texana*.

Both enantiomers of 4-methyl-3-heptanone were synthesized in high optical purity, and their absolute configurations were established from the known absolute configurations of precursor acids (RILEY and SILVERSTEIN, submitted to *Tetrahedron*; RILEY *et al.*, 1974). The (+) enantiomer isolated from both ant species was thus shown to have the S configuration. The synthetic ketones had the following optical rotations: S(+)-4-methyl-3-heptanone $[\alpha]_D^{27} = +21{\cdot}0 \pm 0{\cdot}4°$ (C, 1, hexane) and R-(−)-4-methyl-3-heptanone $[\alpha]_D^{25} = -21{\cdot}5 \pm 0{\cdot}4°$ (C, 1, hexane).

Since both ant species produced the same enantiomer with identical rotations, and since this rotation was higher than those obtained for the synthetic enantiomers, it seems a reasonable assumption that the isolated enantiomers are optically pure.

Optical purities were determined for both synthetic enantiomers based on the maximum rotation of +22·1°C observed for the naturally occurring ketone from *A. texana*. On this basis, the optical purity of the (+) enantiomer was 97·5 per cent, and of the (−) enantiomer, 98·7 per cent.

Bioassay

The threshold response level of *A. texana* workers to each of the synthetic enantiomers was determined by means of the bioassay previously described (MOSER *et al.*, 1968). The lowest concentration of each enantiomer at which 50 per cent of the ants present (20–100 workers of mixed sizes) raised their antennae was designated the threshold response level. The threshold response level of *A. texana* to each enantiomer was determined ten times by the above procedure, and their threshold response levels were compared with a corresponding threshold response level to 2-heptanone, which was determined following each assay of an enantiomer of 4-methyl-3-heptanone. A measure of response is thus reported as a ratio of the threshold response levels of each enantiomer to that of 2-heptanone. Comparison of the geometric averages of response ratios for both enantiomers showed S-(+)-4-methyl-3-heptanone to be about 100 times more active than the (−) enantiomer.

To test for inhibition on the part of the (−) enantiomer, twice as much racemic 4-methyl-3-heptanone, relative to (+)-4-methyl-3-heptanone, was bioassayed by the same procedure.* A comparison of these data with those for the (+) enantiomer showed that the racemic mixture and the (+) enantiomer were of comparable activity, and thus that the (−) enantiomer was not inhibiting the activity of the (+) enantiomer.

Although at that time their presence in *A. texana* had not been confirmed, the biological activity of the compounds, 4-methyl-3-heptanol, 2-heptanol, and 3-octanone had previously been determined (MOSER *et al.*, 1968). These values are included in Table 2, which summarizes the response ratios for all compounds that were bioassayed.

The response of *A. cephalotes* to each enantiomer of 4-methyl-3-heptanone was determined in a similar manner, albeit less rigorously. On the basis of 3 replicates that were run for each enantiomer, it was estimated that the (+) enantiomer was about 210 times more active than the (−) enantiomer. 3-Octanone was as active as R-(−)-4-methyl-3-heptanone, but 4-methyl-3-heptanol elicited no detectable response from *A. cephalotes*.

* Since there is only half as much of the (+) enantiomer in the racemic form of 4-methyl-3-heptanone as compared with the pure (+) form of the ketone, the concentration of racemic ketone in the first dilution from the saturated vapour was doubled. If the geometric average of response ratios observed for the racemic mixture was not significantly lower than that observed for the (+) enantiomer, then little or no inhibition by the (−) enantiomer could be taking place.

TABLE 2—THRESHOLD RESPONSE RATIOS: COMPOUND/2-HEPTANONE

Compound	*A. texana*	*A. cephalotes*
S-(+)-4-Methyl-3-heptanone	$1{\cdot}3 \times 10^{-3}$	$4{\cdot}7 \times 10^{-4}$
R-(−)-4-Methyl-3-heptanone	$1{\cdot}3 \times 10^{-1}$	$1{\cdot}0 \times 10^{-1}$
(±)-4-Methyl-3-heptanone	$(1{\cdot}0 \times 10^{-2}$;* $4{\cdot}0 \times 10^{-3}$	†
4-Methyl-3-heptanol	$(1{\cdot}0 \times 10^{+1})$*	‡
2-Heptanone	$(1{\cdot}0 \times 10^{0})$;* $1{\cdot}0 \times 10^{0}$	$1{\cdot}0 \times 10^{0}$
2-Heptanol	$(1{\cdot}0 \times 10^{+1})$*	†
3-Octanone	$(1{\cdot}0 \times 10^{-1})$*	$1{\cdot}0 \times 10^{-1}$
3-Octanol	†	†
3-Heptanol	†	†

* Ratios in parentheses obtained from previously reported response values (MOSER *et al.*, 1968).

† Compound not tested on this species.

‡ No response was observed for 4-methyl-3-heptanol.

DISCUSSION

It is obvious that no simple statement can encompass the complexity and diversity of interactions at receptor sites.

At one extreme, we here observe receptors that can sense the subtle differences between enantiomers. At the other extreme, we note that butyl acetate (an artifact) and 2-heptanone (the pheromone) elicit the same response from *Iridomyrmex pruinosus* (AMOORE *et al.*, 1969). These two compounds are grossly similar in size and shape, but grossly different in chemical and other physical properties. Between these extremes, are examples of discrimination among structural isomers (e.g. BLUM *et al.*, 1966, 1971; MOSER *et al.*, 1968; AMOORE *et al.*, 1969).

The earliest documentation of synergism in a pheromone system was the demonstration that the bark beetle, *Ips paraconfusus* Lanier (= *confusus*), responded in the field to a mixture of three terpene alcohols, each of which was individually inactive (SILVERSTEIN *et al.*, 1966). In fact, as techniques are refined, it appears that multicomponent pheromones are 'more the rule than the exception' (SILVERSTEIN, 1971), and indeed the advantages of several components has been pointed out by WRIGHT (1964). Synergism involving two geometric isomers in definite proportions for optimum response has recently been demonstrated (HUMMEL *et al.*, 1973; KLUN *et al.*, 1973).

In the two species of leaf-cutting ants, *A. texana* and *A. cephalotes*, almost all of the biological activity contained in the mandibular glands is associated with the enantiomer, S-(+)-4-methyl-3-heptanone, which is more active than the artifact, the (−) enantiomer. Enantiomers have identical chemical and physical properties in an achiral medium, but are distinguishable in their interactions with plane polarized light or with chiral substances such as enzymes. Thus both ant species are

required to have sites that preferentially accept the spatial disposition of S-(+)-4-methyl-3-heptanone.

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