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Separation of *cis* and *trans* isomers from a mosquito repellent, CIC-4, via semi-preparative high-performance liquid chromatography and the repellent effect of each☆

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ABSTRACT

CIC-4 (1,1,4,5,6,7,8,8*a*-octahydro-3H-2-benzopyran-3-one) is a fused bicyclic lactone which acts as a non-contact insect repellent for *Aedes aegypti* at 1% concentration in 95% aqueous ethanol when applied topically to rhesus monkeys. Chromatographic and spectroscopic analyses of a synthetic sample indicated the presence of *cis*- and *trans*-fused isomers. To supply each isomer for the assessment of mosquito repellent efficacy on humans, we developed a semi-preparative high-performance liquid chromatographic separation technique for the isomer separation. A column of 5-μm silica was used for isolating milligram quantities of each isomer. By this method, each isomer was obtained in >95% gas–liquid chromatographic (GLC)-purity for biological evaluation. Supporting GLC and electron impact and chemical ionization gas chromatography–mass spectrometric data are also presented for each isomer. Biological evaluation on the human arm using *Anopheles quadrimaculatus*, *Aedes aegypti*, and *Anopheles albimanus* as the test species was effective in determining relative repellency to the standard N,N-diethyl-3-methyl-benzamide.

INTRODUCTION

In 1959, Korte *et al.* [1] reported the synthesis of DL-iridomyrmecin [2] (Fig. 1), a natural insecticide isolated from the Argentine ant, *Iridomyrmex humilis* (Mayr). He also reported methods for synthesizing a variety of related bicyclic γ- and δ-lactones, some of which exhibited insecticidal properties. It was not until 1982, however, that the insecticidal properties of iridomyrmecin and some of the other bicyclic lactones were studied in more detail [3]. At that time, it was also found that this class of com-

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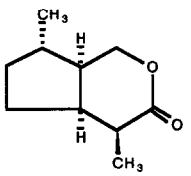
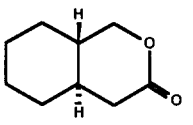
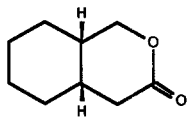


Iridomyrmecin



trans-CIC-4



cis-CIC-4

Fig. 1. Structures of iridomyrmecin, *trans*-CIC-4, and *cis*-CIC-4.

pounds demonstrated a non-contact insect repellent effect. Relatively long-lasting repellent compounds, having essentially no toxic effects on humans are in demand to prevent insect bites and disease transmission.

CIC-4 (1,1,4,5,6,7,8,8*a*-octahydro-3H-2-benzopyran-3-one) (Fig. 1) is one of these fused bicyclic δ-lactones which acts as a non-contact insect repellent. It proved effective against *Aedes aegypti* (L.) at 1% concentration in 95% aqueous ethanol when applied topically to rhesus monkeys; and was active at the same concentration against fire ants, fleas, and flies. The insect repellent properties of CIC-4 and iridomyrmecin are comparable in certain tests [3].

Since the synthesis of CIC-4 generates two geometric isomers (Fig. 1), and previous repellency data were reported for the CIC-4 mixture on monkeys [3], we felt it necessary to separate the isomers and evaluate each for efficacy on humans. We therefore report both the semi-preparative high-performance liquid chromatographic (HPLC) method for separating the geometric isomers and the repellent effects of the isomers for three species of mosquitoes: *Anopheles quadrimaculatus* Say, *Aedes aegypti*, and *Anopheles albimanus* Wiedemann.

EXPERIMENTAL

Materials and reagents

N,N-Diethyl-3-methylbenzamide (deet) was obtained from Virginia Chemicals (Portsmouth, VA, USA), and two lots of the CIC-4 mixture [containing about 1:1 isomers by gas–liquid chromatography (GLC)] were provided by Coulston (Alamagordo, NM, USA). UV-grade *n*-hexane was obtained from Baxter Burdick & Jackson (Muskegon, WI, USA), and reagent-grade anhydrous diethyl ether was obtained from Fisher Scientific (Fair Lawn, NJ, USA).

HPLC

An ALC-100 liquid chromatograph (equipped with two Model 6000A pumps and a U6K injector) was used for analytical HPLC; and a Delta Prep 3000 liquid chromatograph, both from Waters (Milford, MA, USA), was used for semi-preparative HPLC. Both instruments were controlled by an 840 data and chromatography control station (a Digital Professional 380 computer connected via two Systems Interface Modules). A Waters Model 440 absorbance detector with an extended wavelength module and a Waters Lambda-Max Model 481 LC Spectrophotometer, were used at 214 nm for UV absorbance detection for analytical and semi-preparative HPLC runs, respectively; fractions were collected manually. A 5-μm Hypersil, Shandon Southern Products (Chesshire, UK), stainless-steel column (23 × 0.43 cm I.D.) and a 5-μm silica, YMC (Morris Plains, NJ, USA), stainless-steel column (25 × 2.0 cm I.D.) were used for analytical and semi-preparative separations, respectively.

Samples for analytical HPLC were 5.0 mg of neat CIC-4, and samples for semi-preparative HPLC were 50 mg of CIC-4 in sufficient diethyl ether–*n*-hexane (15:85) to make 1.0 ml of solution.

Analytical HPLC required a flow-rate of 2.0 ml/min, and semi-preparative HPLC required a flow-rate of 30.0 ml/min; the eluent was diethyl ether–*n*-hexane (15:85, v/v).

GLC

Hewlett-Packard (Baltimore, MD, USA) 5880A and 5830A, and a Shimadzu GC-9A (Columbia, MD, USA) gas chromatograph, each equipped with a capillary injector system and a flame ionization detector, were used for GLC analyses. A Hewlett-Packard 5730A gas chromatograph, equipped with a flame ionization detector, was also used for GLC analyses. A Hewlett-Packard dimethyl silicone fused-silica capillary column (12 m × 0.2 mm I.D.)

was used on the 5880A; an Analabs (Norwalk, CT, USA) 3% butanediol adipate on Gas Chrom Q (100–120 mesh) packed column (12 ft. × 1/8 in. O.D.) was used on the 5730A; a Restek Corp. (Bellefonte, PA, USA) Stabilwax (15 m × 0.53 mm I.D.) was used on the 5830A; and Supelco, (Bellefonte, PA, USA) Supelcowax 10 (60 m × 0.25 mm I.D., 0.25 mm film thickness) and Supelco SPB 1701 (30 m × 0.25 mm I.D.) fused-silica capillary columns were used on the GC-9A gas chromatograph.

GC-mass spectrometry (MS)

Electron impact (EI)-MS were recorded on a Finnigan (San Jose, CA, USA) GC–MS INCOS 50 quadrupole mass spectrometer. The GC component was equipped with a J&W Scientific (Deerfield, IL, USA) DB-1 fused-silica capillary column (60 m × 0.25 mm I.D.). The DB-1 column was held at 100°C for 1 min, then programmed at 20°C/min to 280°C, and held at 280°C until peak elution. In the EI-mode, 70 eV was used.

Chemical ionization (CI)-MS were recorded on a Finnigan Model 4510 mass spectrometer equipped with an INCOS data system. The GC component was equipped with a J&W Scientific DB-1 fused-silica capillary column (30 m × 0.32 mm I.D., 0.25 μm film thickness). The DB-1 column was held at 70°C for 2 min, then programmed at 10°C/min to 260°C, and held at 260°C until peak elution (helium carrier pressure, 13 p.s.i.). An ammonia pressure of 0.6 torr was used with the source at 60°C.

IR analyses

A Perkin-Elmer (Norwalk, CT, USA) IR Spectrophotometer Model 882 was used for IR analyses.

Mosquito repellent bioassay on humans

Anopheles quadrimaculatus, Aedes aegypti, and *Anopheles albimanus* mosquitoes were each used in the repellent bioassay on human volunteers.

Each test substance was dissolved in acetone, and the resulting solution was used to impregnate a cotton muslin bandage cloth (5 × 10 cm) at the rate of 1.0 mg/cm^2. The treated cloth was stapled over a 4 × 9 cm rectangular opening which had been cut in a 12.7 × 20.3 cm file card. The cloth was allowed to dry for 15 min before testing.

The cards and attached cloths were taped over a nylon stocking-covered forearm so that only the treated cloth allowed the mosquitoes access to the skin. The hand and wrist were protected with a rubber glove and tape. The arm was exposed for 1 min in a stock cage (37 × 38 × 46 cm), which contained approximately 1500 5- and 7-day-old female mosquitoes. Failure of the repellent candidate was indicated by >3 bites through the treated cloth.

The minimum effective dose (MED) was determined on chemicals that were effective at the 1.0 mg/cm^2 dosage. The method of testing was the same, except that the treatment dose was reduced by half until >3 bites/min were received at the lowest dose. The MEDs were determined on the fresh treatments (air-dried for 15 min) and after 24 h.

The repellent, deet, was used as a standard of comparison for mosquito repellency in the bioassay.

RESULTS AND DISCUSSION

The elution order of the CIC-4 isomers for GLC and HPLC (Fig. 2) columns was *trans* followed by *cis*. (The actual assignment of isomer configuration was accomplished via NMR analyses [4].) The HPLC peaks preceeding the *trans* and *cis* isomers were strong UV absorbers since the weight of the HPLC-recovered CIC-4 isomers was equal to that of the CIC-4 mixture being injected. In this study, milligram quantities approaching 1.0 g of each isomer of CIC-4 were obtained semi-preparatively on 5-μm silica for mosquito repellency tests. The retention times of the two isomers were 33 min (*trans*-CIC-4) and 41 min (*cis*-CIC-4) (Fig. 2).

Preliminary GLC data revealed that the CIC-4 mixture contained two substances in a 1:1 ratio. The isomers were analyzed by GLC on a dimethyl silicone capillary column (Table I) as they were separated and then analyzed on 3% butanediol adipate, Stabilwax, Supelcowax 10, and Supelco SPB 1701 columns for confirmation. The best GLC separation of the isomers was afforded by Supelcowax 10 with *trans* at 26.5 and *cis* at 28.5 min. The GLC purity of the separated geometric isomers was >95%.

IR analysis of the CIC-4 mixture showed an ester carbonyl absorption at 1735 cm^{-1}; the range for an acyclic δ-saturated lactone is 1735–1750 cm^{-1} [5]. Lack of a hydroxy absorption in both the alcohol and carboxylic acid areas of the spectrum indicated

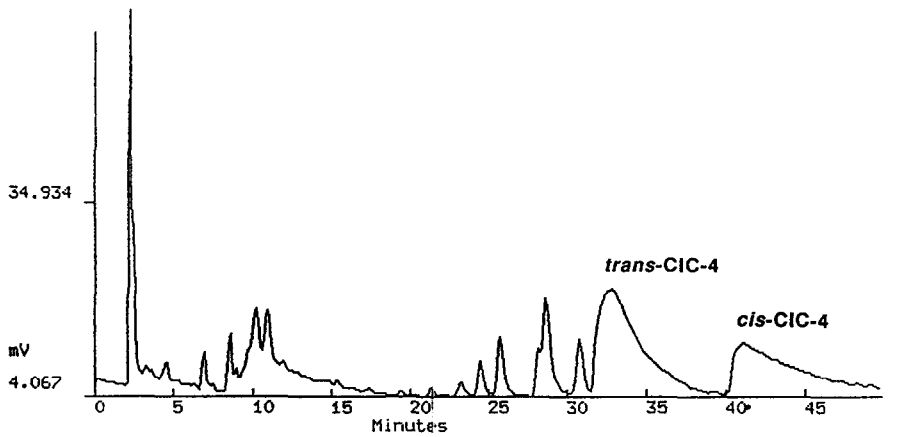


Fig. 2. Separation of *trans*-CIC-4 and *cis*-CIC-4 on 5-μm silica (25 × 2.0 cm I.D.). Eluent, diethyl ether–*n*-hexane (15:85, v/v), flow-rate, 30 ml/min; detector, 214 nm.

that the lactone ring was intact and that no hydrolytic cleavage had occurred.

EI-GC–MS fragmentation patterns were similar for the two isomers (1:1 ratio) (Table II) except that *trans*-CIC-4 had a base peak of *m/e* 87 and *cis*-CIC-4 had a base peak of *m/e* 67. Also, the *cis* isomer showed an appreciable amount of *m/e* 136 $(M-18)^+$ while the *trans* isomer showed only a trace of this ion. Although this is an uncommon fragmentation for esters, the *cis* isomer seems to be more susceptible than the *trans* to form this fragment. An MS library search identified isoiridomyrmecin and isomyrmecin as compounds with similar fragmentation patterns.

CI-GC–MS analysis with NH_3 (Table II) of CIC-4 (1:1 isomer ratio) established the molecular weight of each resolved isomer as 154. Analysis of each isomer with N^2H_3 revealed identical data that confirmed the absence of exchangeable protons, thus supporting the cyclic lactone structure.

In the bioassay against *Anopheles quadrimaculatus* (Table III), CIC-4 mixture (lots 1 and 2), the isomers, and deet were equally effective for 5 days at 1.0 mg/cm^2. The 24-hr post-treatment MED test revealed that all the samples and deet were equally repellent at 0.125 mg/cm^2, while the 15-min test revealed that CIC-4 mixture (lots 1 and 2) and the isomers were effective at 0.008 mg/cm^2 (deet was effective at 0.004 mg/cm^2).

TABLE I

GAS–LIQUID CHROMATOGRAPHIC ANALYSES OF CIC-4

Column	Parameters	Sample	Retention (min)		% Purity (isomer ratio)
3% Butanediol adipate	90°C for 2 min, 4°C/min to 210°C, 30 ml He/min	CIC-4	21.2,	22.2	(50:50)
Stabilwax	60–220°C at 10°C/min, 7 ml He/min	CIC-4	13.3,	13.8	99.1[a]
Supelcowax 10	100°C for 1 min, 10°C/min to 220°C, 1 ml He/min	CIC-4	26.5,	28.5	99.0[a] (51.2:48.8)
Dimethylsilicone	50°C for 5 min, 20°C/min to 105°C, held 7.25 min,	CIC-4	9.7,	10.2	(50.8:49.0)
	10°C/min to 125°C, held 13 min, 2.0 ml He/min	*trans*-CIC-4	9.7		95.7
		cis-CIC-4		10.2	100.0
Supelco SPB 1701	70–200°C at 15°C/min, held 30 min, He at 2.0 kg/cm^2	CIC-4	27.6,	28.7	(50.9:48.0)
		trans-CIC-4	27.6		94.2
		cis-CIC-4		28.7	98.8

[a] Total isomer (*trans* and *cis*) content in sample.

TABLE II

GAS–LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC ANALYSES OF CIC-4

MS mode	*trans*-CIC-4 (*m/e*)	*cis*-CIC-4 (*m/e*)
Electron impact	41, 57, 67, 87 (100), 95, 124, trace 136 $[M-18]^+$	41, 57,67 (100), 87, 95, 124, 136 (28) $[M-18]^+$
Chemical ionization (NH_3)	172 $[M+NH_4]^+$ 189 $[M+(NH_3)_2H]^+$ 206 $[M+(NH_3)_3H]^+$ 326 $[2M+NH_4]^+$	172 $[M+NH_4]^+$ 189 $[M+(NH_3)_2H]^+$ 206 $[M+(NH_3)_3H]^+$ 326 $[2M+NH_4]^+$
Chemical ionization (N^2H_3)	176 $[M+N^2H_4]^+$ 196 $[M+(N^2H_3)_2{}^2H]^+$ 216 $[M+(N^2H_3)_3{}^2H]^+$ 330 $[2M+N^2H_4]^+$	176 $[M+N^2H_4]^+$ 196 $[M+(N^2H_3)_2]^+$ 216 $[M+(N^2H_3)_3{}^2H]^+$ 330 $[2M+N^2H_4]^+$

TABLE III

REPELLENT EFFECTIVENESS ON CLOTH OF CIC-4 MIXTURE, *trans*-CIC-4, AND *cis*-CIC-4 ON THREE SPECIES OF MOSQUITOES

Chemical	No. of days effective[a] on cloth at 1.0 mg/cm²	MED[b] (mg/cm²) on cloth at post-treatment time	
		15 min	24 hr
Anopheles quadrimaculatus			
CIC-4, lot 1	6	0.008	0.125
CIC-4, lot 2	6	0.008	0.125
trans-CIC-4	5	0.008	0.125
cis-CIC-4	6	0.008	0.125
Deet standard	6	0.004	0.125
Aedes aegypti			
CIC-4, lot 1	1	0.125	1.0
CIC-4, lot 2	1	0.125	1.0
trans-CIC-4	1	0.125	1.0
cis-CIC-4	1	0.125	1.0
Deet standard	5	0.032	0.5
Anopheles albimanus			
CIC-4, lot 1	1	0.5	1.0
CIC-4, lot 2	1	0.5	1.0
trans-CIC-4	1	0.125	1.0
cis-CIC-4	1	0.5	1.0
Deet standard	5	0.5	0.5

[a] All treatments tested until >3 bites were received during a 1-min exposure.
[b] Minimum effective dose.

In tests of CIC-4 (lots 1 and 2), *trans*-CIC-4, and *cis*-CIC-4 at the 1.0 mg/cm² against *Aedes aegypti*, each substance was as effective as deet for 1 day; deet repelled for 5 days. The MED 15-min post-treatment test revealed that CIC-4 mixture (lots 1 and 2) and the isomers were effective at 0.125 mg/cm², whereas deet was effective at 0.032 mg/cm². The 24-h MED for CIC-4 mixture (lots 1 and 2) and the isomers was 1.0 mg/cm²; the MED of deet was 0.5 mg/cm².

At 1.0 mg/cm² against *Anopheles albimanus*, CIC-4 mixture (lots 1 and 2) and its separated isomers were as effective as deet for 1 day, but deet repelled for 5 days. The 15-min post-treatment MED tests revealed that CIC-4 (lots 1 and 2), *cis*-CIC-4, and deet were equal in repellency at 0.5 mg/cm²; whereas, *trans*-CIC-4 was effective at 0.125 mg/cm². After 24 h, CIC-4 mixture (lots 1 and 2) and the isomers were effective at 1.0 mg/cm²; deet was effective at 0.5 mg/cm².

Thus the MED on cloth against *Anopheles albimanus* revealed a difference in the repellency of the *trans*- and *cis*-CIC-4 isomers, but no differences were found with the other mosquitoes.

CONCLUSIONS

Milligram quantities approaching 1.0 g of *cis* and *trans* isomers of CIC-4 (1,1,4,5,6,7,8,8*a*-octahydro-3H-2-benzopyran-3-one) were obtained from a 1:1 mixture by a semi-preparative HPLC separation technique. By this method, each isomer was >95% GLC-pure and each was evaluated for its efficacy in mosquito repellency tests. GLC, EI- and CI-GC–MS data supported the fact that the CIC-4 mixture contained configurational isomers and that these were present in approximately a 1:1 ratio.

The cloth bioassay on the human arm using *Anopheles quadrimaculatus*, *Aedes aegypti*, and *Anopheles albimanus* as the test species indicated that there were no differences in repellency between lots 1 and 2 of CIC-4, and there were no appreciable differences in rellency between *trans*-CIC-4, *cis*-CIC-4, and the CIC-4 mixture. Moreover, the deet standard was more effective than CIC-4 or its separated isomers in both MED and duration of effectiveness, except with *Anopheles quadrimaculatus* where there was equivalent duration of effectiveness for CIC-4 (lots 1 and 2), the separated isomers, and deet for 5 days.

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The authors are also appreciative of the personal communication (1990) provided by Dr. F. Coulston claiming that new collaborative research with Dr. F. Korte (Technical University of Munich, Germany) produced simular results; *i.e.*, in mosquito tests (*Aedes aegypti* and *Anopheles quadrimaculatus*), the CIC-4 isomers showed no difference in repellent effects and that the isomers were as effective as the CIC-4 mixture.

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