IDENTIFICATION OF OVIPOSITION ATTRACTANTS FOR *CULEX QUINQUEFASCIATUS* FROM FERMENTED BERMUDA GRASS INFUSIONS

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ABSTRACT. Compounds which attract and stimulate oviposition by gravid *Culex quinquefasciatus* were isolated and identified from a fermented Bermuda grass infusion. The neutral portion of the ether extract of the aqueous infusion contained the stimulatory materials. Fractionation by liquid chromatography yielded an active fraction containing phenol, 4-methylphenol, 4-ethylphenol, indole and 3-methylindole. A blend of the 5 compounds strongly stimulated oviposition, as did blends of any 4 of them. Bioassays with individual compounds showed that only 3-methylindole consistently induced oviposition, in concentrations spanning 5 orders of magnitude.

INTRODUCTION

Culex quinquefasciatus Say is an important vector of St. Louis encephalitis (SLE) and other arboviruses in the United States (Monath 1980, Reeves and Milby 1990), and in tropical regions it is a vector of Bancroftian filariasis. Adult mosquito populations have been monitored by surveys of resting sites, or with light, CO_2 or animal-baited traps (Reisen and Meyer 1990). However, these methods are inconvenient and expensive in terms of equipment and manpower, and of questionable efficacy in providing accurate mosquito population surveys. More recently, oviposition traps baited with fermented organic infusions (Reiter 1983) have gained popularity. Oviposition traps have proven to be effective for monitoring a number of species, particularly as they attract predominantly gravid females, the cohort of the population most likely to be infected and infectious. The infusions are cheap and easy to produce, and depending on the particular infusion used, the traps are also species selective, which limits processing time. Several commercial traps are now available for use with attractive infusion baits.

However, infusion-baited traps still have several disadvantages. First, servicing and maintaining the traps is still laborious, as each trap contains up to several liters of infusion, which must be changed frequently, as the attractiveness of infusions changes fairly rapidly. Second, no 2 infusions are identical, as the blend of dissolved chemical constituents will depend on factors such as the organic matter being fermented, the fermenting organism(s), temperature, age of the infusion and so forth. Thus, standardization is virtually impossible. It would be of considerable value to identify the attractive compound or blend of compounds from infusions, and to produce a synthetic attractant of known composition and concentration for baiting traps.

Gravid female mosquitoes use a combination of physical cues (e.g., reflectance, color, temperature, humidity, presence of vegetation) and chemical cues (e.g., oviposition attractants, arrestants and stimulants) to locate and recognize suitable oviposition sites (Benzon and Apperson 1988, Bentley and Day 1989). Different mosquito species apparently use different chemical cues to locate suitable sites; an odor source which is attractive to one species may be repellent to another (Kramer and Mulla 1979), reflecting the variety of habitats occupied by various species.

Chemical cues are often the products of decay of organic matter (Mulla 1979), or may be produced by immature mosquitoes and their associated bacterial fauna (Benzon and Apperson 1988). Both natural and artificial infusions of decaying organic matter have been shown to influence mosquito oviposition. Infusions have been prepared from a wide variety of materials, including hay (Hazard et al. 1967, Murphey and Burbutis 1967, Reiter 1983), grass (Gjullin et al. 1965), logs (Gjullin et al. 1965), manure (Kramer and Mulla 1979), laboratory animal chow (Kramer and Mulla 1979) and decaying fruit rinds (Lounibos 1978).

There have been few reports of systematic attempts to identify specific attractants from infusions. Gjullin (1961) determined that aqueous solutions of wood creosote stimulated oviposition by *Culex quinquefasciatus*, and some of the biologically active compounds were later identified as phenols (Ikeshoji 1975). Screening of a large number of chemicals also revealed that this species oviposits in response to furfural (Gjullin et al. 1965) and a series of alkyl carbonyl compounds (Ikeshoji and Mulla 1974).

There have been no reports of oviposition attractants or stimulants for *Culex* species being isolated from hay or grass infusions. The studies described here were undertaken to systematically isolate and characterize specific com-

pounds which influence oviposition in *Cx. quinquefasciatus* females from fermented grass infusions.

METHODS AND MATERIALS

Insects: Gravid adult female *Cx. quinquefasciatus* were obtained from a colony originating from egg rafts collected from local dairy wastewater lagoons (Midhill Dairy, Norco, CA). The colony was maintained in the laboratory as previously described (Kramer and Mulla 1979). Freshly emerged females were mated and blood-fed on chicks approximately 5 days before being used in bioassays.

Grass infusion and extracts: Infusion was prepared essentially as described by Reiter (1983), by fermenting 450 g Bermuda grass cuttings, 5 g brewer's yeast, and 20 g lactoalbumen hydrolysate in 75 liters of tap water for 12 days at 25°C in a greenhouse. The brew was filtered through a 32 mesh screen and frozen until needed.

Portions of the frozen infusion were thawed and filtered through Whatman no. 1 paper. In a preliminary experiment to determine whether the biologically active materials were extractable from the aqueous infusion, a 50 ml aliquot of clear yellow, odorous filtrate was extracted with ether (3 × 25 ml), the ether extracts were combined, dried over anhydrous Na_2SO_4 and concentrated by distilling off the ether through a Vigreux column (25 cm). The concentrate was then bioassayed (vide infra).

A larger portion of the filtered infusion was fractionated as shown in Fig. 1. Filtrate (1,250 ml, pH ≈6.5) was acidified to pH 3 with 25 ml of 1 M HCl, and extracted with ether (3 × 200 ml). The ether extract (containing acidic and neutral compounds) was washed with 5% aq. $NaHCO_3$ (3 × 100 ml), leaving a colorless ether solution of the neutrals and phenolics (BG-2N; Bermuda grass, 2nd infusion, neutrals fraction). The combined $NaHCO_3$ extracts were acidified to pH 3 with 1 M HCl, and extracted with ether (3 × 100 ml), giving a yellowish, odorous organic acids fraction (BG-2A; Bermuda grass, 2nd infusion, acids).

The acidic aqueous residue from the first extraction was made basic with NaOH (2.25 g), and reextracted with ether (3 × 100 ml), giving a fraction containing organic bases (BG-2B; Bermuda grass, 2nd infusion, bases).

Ether extracts were dried over anhydrous Na_2SO_4 and concentrated to approximately 3–5 ml as described above. The concentrated extracts were transferred to glass screw-cap vials and made up to 6.25 ml with ether (1 ml of extract = 200 ml infusion).

The biologically active neutral fraction (BG-2N, 1200 ml equiv. of infusion) was concentrated under a gentle stream of nitrogen to approximately 0.1 ml, and fractionated by flash chromatography (Still et al. 1979) on silica gel (14.3 g in a 1 cm I.D. column, 230–400 mesh; Aldrich Chemical Co.), eluting with 1 column volume (ca. 70 ml) each of pentane, mixtures of ether and pentane (5, 10, 25, 50 and 100% ether), and acetone. An attempt to further purify the active materials by preparative gas chromatography was made, but the resulting subfractions had marginal activity so the method was not pursued.

Fractions were analyzed by gas chromatography, using a Hewlett-Packard 5890 GC equipped with a 20 m × 0.25 mm I.D. DB-5 column (J&W Scientific, Folsom, CA), temperature program 45°C for 2 min, 5°/min to 250°C, and injector

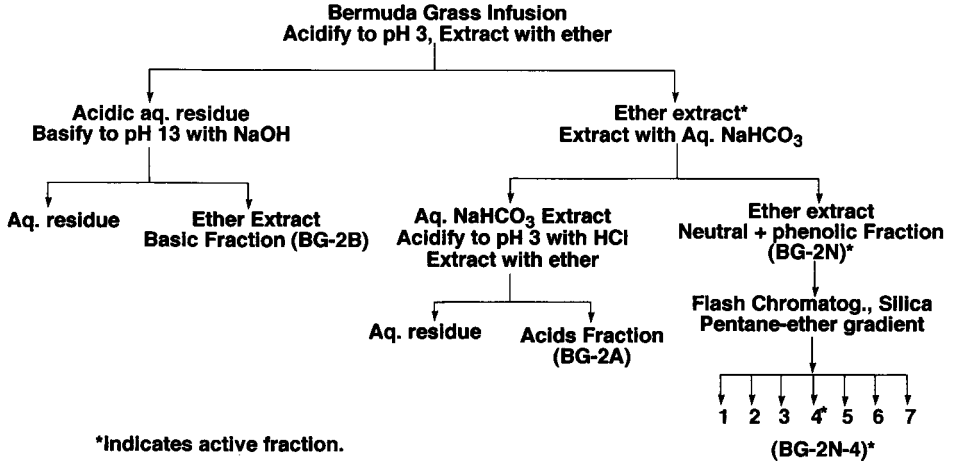

Fig. 1. Sequence of steps used to fractionate the fermented Bermuda grass infusion.

Table 1. Oviposition responses of gravid *Culex quinquefasciatus* to acidic, basic and neutral fractions of the ether extract of Bermuda grass infusions.

Stimulus[a]	Number of egg rafts		
	Treatment (Mean ± SD)	Control (Mean ± SD)	χ^2 [b]
Neutral extract (BG-2N)	12.3 ± 3.6	1.8 ± 1.2	46.69***
Acidic fraction (BG-2A)	8.2 ± 1.5	6.7 ± 1.6	0.91
Basic fraction (BG-2B)	6.5 ± 2.7	5.3 ± 2.6	0.69
Recomb. acids + bases (BG-2A + BG-2B)	7.0 ± 2.2	5.8 ± 2.2	0.64

[a] Bioassays replicated 6 times. Treatments = 10 ml equiv. of infusion in 100 μl ether. Control = 100 μl ether.
[b] *** = $P \geq 0.001$.

and FID detector temperatures of 250 and 275°C, respectively. Helium carrier gas was used. Fractions were further analyzed on a Hewlett-Packard 5890 GC equipped with a 20 m × 0.2 mm I.D. Ultra-2 column (Hewlett-Packard) coupled to an H.-P. 5970 mass selective detector. The temperature program was as described above. Compounds were identified by comparison of GC retention times and mass spectra with those of synthetic standards (Aldrich Chemical Co., or Eastman-Kodak for 3-methylindole. CAUTION: compounds are malodorous and/or toxic!).

Bioassays: Bioassay cages were 23 × 23 × 30.5 cm wooden frame cages covered with plastic screen, with a glass top and a muslin sleeve for access. Paper cups (4 oz.) holding 100 ml of distilled water were placed in each of the back corners, one treated with the test material, and the other with a solvent control. The positions of the cups were alternated between the different replicates. Six replicates of 20 gravid female *Cx. quinquefasciatus* (10 days old, 5 days after blood feeding) per cage were run, with cages placed side by side for each bioassay, with a 12L:10D photoperiod, and a 1 h simulated dawn and dusk period. Assays were run overnight in a well-ventilated room, at temperatures of 27 ± 2°C and a relative humidity of 40–60%. Assays were scored by counting the number of egg rafts laid in each cup. Differences between treatments and controls were determined by chi-square analyses. Chi-square values of 3.84, 6.64 and 10.83 (1 degree of freedom) corresponded to significance levels of 0.05, 0.01 and 0.001, respectively.

RESULTS

In preliminary experiments, oviposition cups treated with 1 ml of the grass infusion in 100 ml distilled water received more than 20 times as many egg rafts as the distilled water control (53:2 egg rafts, respectively). The response was concentration dependent, as 0.1 ml of infusion resulted in only a 3.8-fold increase in oviposition vs. the control. The lower concentration of bioactive material also resulted in less overall oviposition (19:5 egg rafts).

In the process of fractionation described below, some loss of volatile analytes was bound to occur during handling and concentration, especially as extracts were carried through several sequential steps. Consequently, the dose tested (in ml equivalents of crude infusion) was increased in later fractions (see tables for exact doses tested). Once active compounds were identified, these compounds were then quantified in the crude extract, and dose response curves generated.

The isolation of the bioactive compounds commenced with the extraction of an aliquot of filtered infusion with ether. The majority of the activity partitioned into the ether phase (74 vs. 27 egg rafts, $P \geq 0.001$; treatment (2 ml equivalent) vs. water control), while the residual aqueous phase was not significantly more active than the control (56 vs. 38 egg rafts, NS), indicating a fairly complete extraction of the biologically active compounds. In a direct comparison, the ether extract elicited significantly more oviposition than the aqueous residue (62 vs. 30 egg rafts, $P \geq 0.001$).

The ether extract was partitioned into acidic (BG-2A), basic (BG-2B), and neutral + phenolic (BG-2N) fractions. Only the neutral + phenolic fraction (BG-2N) was significantly active in bioassays; the acids and bases fractions, alone or recombined, were not significantly different from the distilled water control (Table 1). It was expected that the acidic fraction might show some deterrency, as Hwang et al. (1982) demonstrated that short chain carboxylic acids deterred *Cx. quinquefasciatus* oviposition at levels of 0.06% acid in water. The lack of a repellent effect in our bioassays may be due to the concentration of test compounds used, estimated to be at least 10-fold less than that used in the previous study.

The neutral fraction was further fractionated by flash chromatography on silica gel, taking 7

fractions. The first 2 (nonpolar) fractions were significantly repellent when tested vs. distilled water, resulting in decreased oviposition compared to the control (Table 2). Only the fourth fraction (BG-2N-4) resulted in increased oviposition (Table 2). The total recombination of all the fractions also resulted in large numbers of egg rafts (Table 2), suggesting that little or no biological activity had been lost or destroyed in the fractionation process. The fact that the total recombination produced a 10-fold difference in the number of egg rafts laid versus the control, whereas fraction BG-2N-4 produced only a 3-fold difference, suggests that some of the other fractions may synergise the activity in BG-2N-4.

All possible combinations of 6 of the 7 fractions, and a total recombination of all 7 fractions (Fig. 2) were bioassayed (Fig. 2). All recombinations were significantly more stimulatory than the distilled water controls, but the recombination which did not contain fraction BG-2N-4 was least active, corroborating the results of testing the individual BG-2N-X (X = 1–7) fractions (Table 2), where only BG-2N-4 was significantly active alone.

In a further series of experiments, the subtractive recombinations of 6 of the 7 fractions (see Fig. 2 for combinations) were tested versus the total recombination of all 7 fractions. In this case, there were no significant differences between any of the subtractive recombinations and the total recombinations (data not shown).

The fraction which was significantly active alone (BG-2N-4) was fractionated further by preparative gas chromatography on an OV-101 column. Bioassays demonstrated that the resulting subfractions had marginal activity, suggesting that biological activity was due to synergistic interactions between compounds in different subfractions. Consequently, this fractionation method was not pursued.

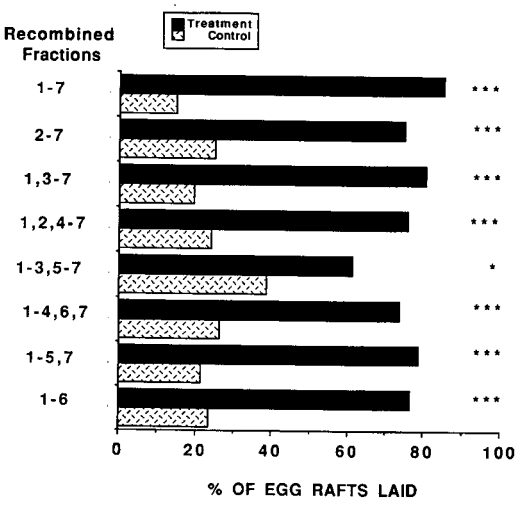

Fig. 2. Ovipositional responses of gravid female *Culex quinquefasciatus* to recombinations of fractions BG-2N-(1–7). Six replicates/treatment. * = significant at $P = 0.05$ level; *** = significant at $P = 0.001$ level.

Compounds in fraction BG-2N-4 were identified by analysis with coupled capillary gas chromatography-mass spectrometry. Five compounds, namely phenol, 4-methylphenol, 4-ethylphenol, indole and 3-methylindole (ratio 19:100:8:1:6) were conclusively identified by comparison of mass spectra and retention times with those of standards. In particular, the substitution patterns of the 4-alkylphenols were conclusively determined by comparison of retention times to those of the alkylphenol standards with methyl and ethyl groups in the 2, 3 or 4 positions. Only the retention times of the 4-alkylphenols matched those of the compounds in fraction BG-2N-4.

Table 2. Oviposition responses of gravid *Culex quinquefasciatus* to flash chromatography subfractions of the neutral fraction BG-2N.

Stimulus[a]	Number of egg rafts: Treatment (Mean ± SD)	Number of egg rafts: Control (Mean ± SD)	χ^2 [b]
BG-2N-1	4.3 ± 1.2	7.5 ± 2.4	(5.08)*
BG-2N-2	4.5 ± 2.4	7.8 ± 3.3	(5.41)*
BG-2N-3	9.3 ± 2.0	9.5 ± 1.6	0.01 NS
BG-2N-4	13.3 ± 2.7	4.5 ± 2.4	26.25***
BG-2N-5	8.0 ± 1.4	8.2 ± 2.1	0.01 NS
BG-2N-6	6.3 ± 2.4	7.0 ± 1.8	0.20 NS
BG-2N-7	6.8 ± 2.1	8.3 ± 1.6	0.89 NS
Recombination, BG-2N-1 to 7	16.7 ± 1.5	1.7 ± 1.0	73.64***

[a] Treatments = 10 ml equivalent of infusion in 100 µl ether; control = 100 µl ether, except for BG-2N-7, where 100 µl acetone was used, as this was the acetone-eluted fraction.

[b] * = $P \geq 0.05$; *** = $P \geq 0.001$. χ^2 values in brackets indicate that the test stimulus was repellent.

A mixture of the 5 compounds identified from fraction BG-2N-4 (phenol, 4-methylphenol, 4-ethylphenol, indole, 3-methylindole, in the ratio 19:100:8:1:6, mimicking the ratio in fraction BG-2N-4) was prepared for testing. Bioassays of this mixture at two concentrations (10 and 100 μg of 4-methylphenol, the major component, per liter water, minor components in lesser amounts in ratio above; 10 and 100 parts per billion) showed the test mixture to be biologically active, resulting in 54 vs. 12 ($P \geq 0.001$) and 78 vs. 12 ($P \geq 0.001$) egg rafts, respectively, versus the water control.

Subtractive recombinations of the 5 identified compounds were bioassayed in an effort to identify one or more compounds which must be present for the mixture to be ovipositionally active. However, all combinations of 4 of the 5 components were significantly more active than the distilled water control, suggesting that there may be a considerable degree of flexibility tolerated in the active mixture, and that the components may substitute for each other, or that activity may be the result of additive or synergistic effects. This may reflect both the complexity of the blend of chemicals which would normally be associated with oviposition sites, and that a variety of oviposition sites with variable chemical profiles may be acceptable.

Dose response trials spanning 5 orders of magnitude were conducted for each of the 5 compounds (Table 3). Consistently significant and reproducible responses versus distilled water controls were obtained with all doses of 3-methylindole, and with the 2 highest doses of indole. However, careful analysis of the indole standard by GC-MS revealed that it contained trace amounts (0.1%) of 3-methylindole, suggesting that the biological activity seen at the higher doses of indole was actually due to the trace amounts of 3-methylindole present.

In further tests to determine the threshold concentration needed to elicit biological activity, the response to 3-methylindole at a concentration of 1 ng/liter (1 part/trillion) was not significantly different than the response to the distilled water control. Because significant responses were obtained with 3-methylindole at a concentration of 10 ng/liter (Table 3), the threshold concentration must be between 1 and 10 ng/liter (1–10 parts per trillion).

Sporadic and inconsistent results were obtained with 4-ethylphenol; significant levels of oviposition were obtained in 4 of 15 bioassays, at several different dosages.

To place the dose response data in a context relevant to the crude grass infusion, a second aliquot of the infusion was extracted exactly as described previously, and the 5 identified components were quantified in the extract (BG-2N) by gas chromatography-mass spectrometry. The compounds were present in the crude extract of the infusion in the following amounts: phenol, 0.27 mg/liter; 4-methylphenol, 1.99 mg/liter; 4-ethylphenol, 0.073 mg/liter; indole, trace ,<0.01 mg/liter; 3-methylindole, 0.25 mg/liter. In preliminary bioassays (*vide supra*), the crude infusion had been diluted by a factor of 100 or 1,000 for bioassay. Thus, the concentrations of the various components in the dilutions were as follows: phenol, 2.7 and 0.27 μg/liter; 4-methylphenol, 19.9 and 1.99 μg/liter; 4-ethylphenol, 0.73 and 0.073 μg/liter; indole,~0.1 and 0.01 μg/liter; 3-methylindole, 0.25 and 0.025 μg/liter. These concentrations all fall within the range of concentrations at which the synthetic chemicals were tested (Table 3).

DISCUSSION

The bioassays described above using synthetic chemical attractants indicate that mosquitoes were acutely sensitive to chemical stimuli, with significant amounts of oviposition occurring in response to as little as 10 ng/liter of 3-methylindole in water (10 parts/trillion). This is several

Table 3. Numbers of *Culex quinquefasciatus* egg rafts laid in response to various concentrations of synthetic test compounds.[a]

	Concentration of stimulus[b,c]														
	0.01 μg/liter			0.1 μg/liter			1.0 μg/liter			10 μg/liter			100 μg/liter		
Compound	T	C	χ^2	T	C	χ^2	T	C	χ^2	T	C	χ^2	T	C	χ^2
Phenol	23	22	0.02	19	27	1.39	27	27	0	32	20	2.77	26	18	1.45
4-methylphenol	17	22	0.64	13	23	2.78	26	34	1.07	31	26	0.44	19	21	0.10
4-ethylphenol	47	41	0.41	40	43	0.11	54	37	3.18	48	31	3.66	47	34	2.09
Indole	32	33	0.02	37	34	0.13	39	37	0.05	51	21	12.50	64	21	21.75
3-methylindole	26	9	8.26	28	8	11.11	42	12	16.67	43	19	9.29	55	17	20.06

[a] Results are the means from 2 sets of 6 replicates, 20 females/replicate.
[b] Stimulus added to 100 ml water, in 100 μl ether; control cup treated with 100 μl ether.
[c] χ^2 for 95% confidence level = 3.84; for 99% confidence level = 6.63; for 99.9% confidence level = 10.83.

orders of magnitude lower in dose than has been used previously in testing other potential mosquito oviposition stimulants (Gjullin 1961, Ikeshoji and Mulla 1974, Bentley et al. 1981). In addition, the range of acceptable concentrations appears to be broad, with significant levels of oviposition occurring in response to doses of 3-methylindole spanning 5 orders of magnitude.

Indole and 3-methylindole (skatole) are ubiquitous natural products, being found in sources as diverse as floral odors, animal excreta, animal scent-marking or defensive secretions, and as products of fermentation of proteinaceous organic material by microorganisms. However, to our knowledge, neither indole nor skatole have been previously implicated as semiochemicals used by mosquitoes.

Phenol and alkylated phenols are also widely distributed natural products, being found in a wide variety of plants and in the excreta and secretions of many animals. Phenolics are also produced during the degradation of organic matter, particularly plant material rich in lignins and other polyphenolics.

Phenols have been demonstrated by several research groups to influence mosquito oviposition. Ikeshoji (1975) isolated phenol and a series of mono-, di- and trimethyl phenols from extracts of wood creosote, and several of the compounds were found to induce oviposition by several *Aedes*, *Armigeres* and *Culex* species.

In more detailed studies, Bentley et al. (1979) isolated 4-methylphenol from infusions of decaying paper birch, and in laboratory bioassays this compound was found to be an attractant for both male and female *Ae. triseriatus* (Say). In a later study, Bentley et al. (1981) demonstrated that several compounds involved in oviposition site selection and oviposition by *Ae. triseriatus* only exerted their effects when perceived by contact chemoreceptors, even though the compounds were easily volatile enough to be perceived by olfaction.

The work reported here is a preliminary investigation with a crude but effective bioassay. This type of bioassay was used because it is simple and reproducible. However, this bioassay does not provide any indication as to the role played by each chemical stimulus in the steps of the ovipositional sequence, such as long-range attraction, arrestment and stimulation to oviposit. These types of subtleties would not be obvious with the bioassay that we used, although similar mechanisms may operate with *Culex* species. More detailed bioassays aimed at defining the roles played by various chemicals in the entire sequence of behaviors leading to oviposition need to be developed, now that at least one compound which results in increased oviposition, by whatever mechanism, has been identified.

In summary, 3-methylindole has been identified as a semiochemical that strongly influences oviposition by *Cx. quinquefasciatus*. Other compounds such as alkylated phenols further enhance ovipositional activity. The isolation and identification of these compounds from fermented grass infusions mimicking natural oviposition water represents the first step in the development of synthetic attractant blends for *Culex* mosquito monitoring and control programs from cheap and readily available compounds.

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