BRINE SHRIMP (*ARTEMIA SALINA*) AS A CONVENIENT BIOASSAY FOR AVERMECTIN ANALOGS

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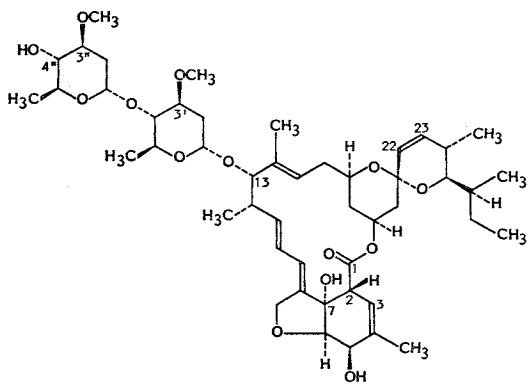
(Received for publication December 24, 1988)

The avermectins are a family of naturally occurring macrocyclic lactones which are potent antiparasitic agents.[1] For several years we have been involved in a program of chemical modification of naturally occurring avermectins (*e.g.*, avermectin B_{1a} (**1**) and avermectin B_{2a} (**2**)) in an attempt to discover new compounds with improved antiparasitic activity. An important problem in the avermectin project has been the lack of a rapid and convenient method for determining the activity of new avermectin analogs. Results from most of our bioassays were not available for several weeks after a compound was submitted. We have therefore developed and report herein a simple and convenient bioassay (based on brine shrimp) that provides the necessary information quickly and can be carried out by chemists with no biological facilities or training.

Examination of the literature suggested two possible systems which could serve as useful bioassays for avermectin analogs. First, the use of *Caenorhabditis elegans* in a rapid screening assay for anthelmintic compounds has been described.[2] In addition, *C. elegans* has also been used in a simple bioassay of avermectin analogs.[3,4] However, this assay requires a considerable amount of effort to grow and maintain the requisite cultures and is not well suited for use in a chemistry laboratory. An alternative possibility involved the use of brine shrimp (*Artemia salina*) larvae. Brine shrimp larvae have frequently been used in the past as a bioassay for general toxicity of organic compounds.[5~8] In addition, this system has also been used for determination of the toxicity of a few avermectin analogs but the method as described was not suitable for our purpose.[9] Although none of the methods described in the literature met all of the criteria that we had established for our assay (quick results, minimal effort, reproducible, applicable to a broad range of analogs) the fact that brine shrimp larvae are extremely easy to grow and maintain prompted us to explore this system further.

The methods described in the literature generally use low concentrations ($\leq 2\%$) of an organic solvent in salt water for the assay. Due to the very low water solubility of the avermectins we felt that a much higher (*ca.* 10%) solvent concentration would be necessary. We therefore decided to examine a number of organic solvents to see if we could find one which would have no effect on brine shrimp larvae at this level. The solvents we chose to study were acetonitrile, DMF, DMSO, dioxane, ethanol, 2-propanol, methanol, and THF. We placed 0.025 ml of each solvent in separate wells of a plastic 96-well tissue culture plate (Costar)



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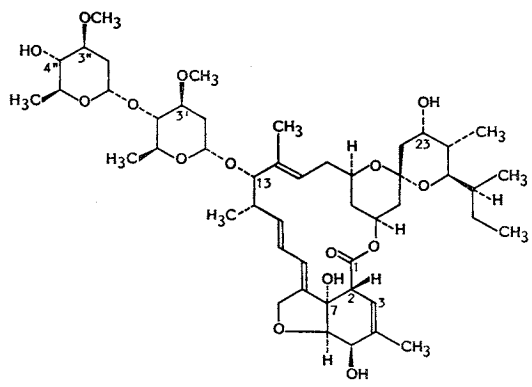

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and added 0.200 ml of a suspension of brine shrimp (*ca.* 30~40 larvae) in 3% (w/v) salt water. The brine shrimp were thus exposed to a 11% aqueous solution of the solvent tested. The plate was allowed to stand uncovered at room temperature for 1 hour. Examination of the plate under a microscope showed that the brine shrimp exposed to 11% acetonitrile or 11% dioxane were completely immobilized. The shrimp exposed to the other solvents all showed significantly impaired movement but were not completely paralyzed. The plate was re-examined after an additional 3 hours at room temperature. It was found that while most of the brine shrimp looked the same as they had earlier the ones exposed to acetonitrile showed substantial recovery from their earlier paralysis. By the time an additional 2 hours had elapsed there was essentially no difference between brine shrimp exposed to 11% acetonitrile and a control group exposed only to salt water. On average there were 2~5 dead brine shrimp in the salt water control wells (out of 30~40 larvae) and 3~6 dead brine shrimp in the acetonitrile containing wells (out of 30~40 larvae). The live shrimp in the two sets of wells appeared to be identical. We believe that this reversal of the acetonitrile-induced anesthesia is due to evaporation of the acetonitrile from the open tray during the experiment. NMR experiments clearly showed the disappearance of the acetonitrile during the assay. On the basis of this experiment we selected 11% acetonitrile as the solvent for our bioassay.

Our assay is based on immobilization of brine shrimp (*A. salina*) larvae by avermectin derivatives. The assay is carried out as follows: Brine shrimp (*A. salina*) eggs obtained from a local pet store are added to 3% (w/v) aqueous NaCl solution in a crystallizing dish to hatch (*ca.* 250 mg of eggs in 400 ml of salt water). After 24 hours under a fluorescent light at *ca.* 28°C (temperature maintained by placing the dish on top of an oven) the live larvae are harvested by using a pipette to siphon out larvae attracted to a light source (penlight flashlight). This procedure affords a concentrated suspension of live larvae in salt water. Each test compound (0.025 ml of a solution containing 1 mg/ml of the compound in acetonitrile) is placed separately in a well of the first column of a 96-well (8 rows ×12 columns) culture plate. An additional 0.025 ml of acetonitrile is then added to each well in the first column and (after mixing) half of the volume of each well is transferred to the corresponding well of the next column. An additional 0.025 ml of acetonitrile is then added to each well in the second column and (after mixing) half of the volume of each well is transferred to the corresponding well of the next (third) column. The process is then repeated until each well in the row contains 0.025 ml of solution. Each row of the plate thus contains a decreasing concentration of a different test compound (2-fold serial dilution from left to right). The suspension of brine shrimp larvae is then added to the wells such that each well contains 0.025 ml of acetonitrile solution and 0.200 ml of brine shrimp suspension (final avermectin concentrations range from 55,500 ng/ml to 27 ng/ml from left to right). The dilutions and transfers are carried out using a multipipette (8 tips) which allows rapid setup of the assay. The tray is then allowed to stand (uncovered) at room temperature for 6 hours. In each assay several wells containing acetonitrile plus brine shrimp suspension (but no compound) and others containing brine shrimp suspension only (no organics) are set aside as negative controls. Avermectin B_1 is run as a positive control in every assay. The brine shrimp in all wells containing acetonitrile are rapidly paralyzed but those in wells without an avermectin analog recover completely after about 4 hours. When the assay is evaluated after 6 hours the control wells containing acetonitrile (but no compound) are essentially identical to those containing only salt water. The assay is evaluated by examining (with a microscope or a powerful magnifying glass ($\geq 16\times$)) each row of the plate from left to right (decreasing concentration) and determining the minimum concentration of compound which causes 100% of the brine shrimp to lose mobility (immobilized but not necessarily completely paralyzed). This concentration is defined as the 100% immobilization concentration (IC_{100}) for the compound. Duplicate assays are run for each compound and the average value of the two assays is reported. Most of the time the duplicate assays give identical results. The IC_{100} for the two assays has never differed by more than a factor of two (one well difference). Occasionally the solution in the well containing the highest concentration of a com-

Table 1. Bioactivity of representative avermectin analogs.

Compound	*Artemia salina*[a] IC_{100} (ng/ml)	*Tetranychus urticae*[b] EC_{90} (μg/ml)
Avermectin B_2	165	>0.05[f]
13-β-Fluoroivermectin aglycone	220	0.01
Avermectin B_1	277[c]	0.04
13-α-Fluoroivermectin aglycone	325	0.05
22,23-Dihydroavermectin B_1 (ivermectin)	430[d]	0.05
13-Deoxyivermectin aglycone	430	0.05
4''-*epi*-Acetylamino-4''-deoxyavermectin B_1	540[e]	0.20
Ivermectin monosaccharide	870	0.10
Ivermectin aglycone	870	>0.10
4''-*epi*-Amino-4''-deoxyavermectin B_1	1,730	0.25
13-β-Chloroivermectin aglycone	1,730	0.25
4''-*epi*-Methylamino-4''-deoxyavermectin B_1	1,730	0.30
9-Phenylthio-8-hydroxy-8,9-H_2-avermectin B_1	>55,500	$\gg$0.25
7-*O*-Trimethylsilylavermectin B_1	>55,500	$\gg$0.25
2-*epi*-Avermectin B_1	>55,500	4.00

[a] Brine shrimp data obtained as described above, average of 2 assays except as noted.
[b] Two-spotted spider mite (on bean plants) data obtained as described in refs 10 and 11.
[c] Average of 63 assays. [d] Average of 3 assays. [e] Average of 4 assays.
[f] Note that with a few exceptions there is a rough correlation between the two assays.
EC_{90}: Effective concentration 90.

pound (55,500 ng/ml) is cloudy suggesting that the compound may be precipitating as the acetonitrile evaporates. However, this is only observed at the highest concentration and the reproducibility of the assay implies that the compounds remain in solution long enough to interact with the brine shrimp even at this concentration.

To date we have tested over 175 compounds in this assay. A good dose-response curve has been observed for all compounds and the results have been consistent and reproducible. Under the conditions of the test the results appear to be reproducible to within a factor of two (thus an observed value of 220 ng/ml implies that the actual value is somewhere between 110 and 440 ng/ml). The test is therefore accurate enough to determine the approximate activity of the compound (IC_{100} values for compounds tested to date have ranged from 110 to >55,500 ng/ml; see Table 1 for IC_{100} values for representative avermectins). Finally, there appears to be a rough correlation between this assay and our other antiparasitic/insecticidal bioassays (although there are exceptions; see Table 1 for a comparison of brine shrimp data with two-spotted spider mite (*Tetranychus urticae*) data[10,11]).

In conclusion, we have developed a method for the use of brine shrimp larvae for the rapid determination of the approximate activity of avermectin analogs. The method requires no special equipment and can be carried out by research personnel with no formal biological training. It is simple, inexpensive, and provides quick results with minimal effort. The data thus obtained are reproducible and are useful for predicting the antiparasitic and/or insecticidal activity of these important compounds.

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