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Screening and laboratory tests for stages I, II and III [1] [2]

by

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The control and eradication of vector-borne diseases of man depend, for the foreseeable future, upon the use of insecticides. DDT, lindane, and dieldrin, although they have literally worked miracles in the control or elimination of malaria, typhus, and plague, they have in many areas become ineffective due to the innate capacity of insects to develop physiological resistance to their action. The crucial importance of this acquired resistance to vector control programs may be judged by a recent summary indicating that a total of 225 species of insects and mites are resistant to one or more insecticides (BROWN, 1967), and of these, 57 species are mosquitoes (BROWN, 1968). Among the anopheline vectors of malaria, alone, there are at least 12 species resistant to DDT and 34 to dieldrin. At the very least, this resistance has caused confusion in well-established malaria eradication procedures and logistic problems in the procurement of substitute insecticides. At worst, as in Greece with *A. sacharovi,* in Central America with *A. albimanus,* and in Nigeria with *A. gambiae,* the transmission of malaria has persisted, despite prolonged residual spraying, or has recrudesced, as in Iran with *A. stephensi* and in Indonesia with *A. sundaicus.* DDT combines prolonged residual activity and maximum safety to humans, together with great economy. Its replacement with substitute materials inevitably increases the expense and complicates the procedures of malaria eradication.

Fortunately for the successful continuation of many vector control programmes — resistant strains of various insect vectors, arising as a result of selection by DDT or other insecticides, are not generally resistant to the action of all insecticides ; this is particularly true of insecticides with divergent chemical structures and modes of intoxication and detoxication. In 1957, WHO recognized that the continuing success of vector control programs, and especially malaria eradication, would be dependent upon the comprehensive evaluation of new candidate insecticides produced by the world chemical industry.

A seven-stage programme for the evaluation of new compounds was thus organized by WHO with a group of collaborating laboratories. The goal was the disco-

(1) Paper presented at the 8th International Congresses of Tropical Medicine and Malaria, Section B. 2.3, Teheran, Iran, Sept. 7-15 1968. Read by G. P. GEORGHIOU.

(2) As of July 1, 1968, the work described under stage I is being conducted at the University, of Illinois, Urbana, under the direction of R. L. METCALF. The work on insecticide resistance in mosquitoes is being continued at the University of California, Riverside, under the direction of G. P. GEORGHIOU.

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very and development of insecticides that are (*a*) fully effective against a variety of resistant species of insect vectors, (*b*) highly safe to man and animals when applied as residual sprays in human habitations, as dusts to clothing, or as larvicides, (*c*) biodegradable in animal tissues and in the environment, (*d*) highly stable and persistent when applied to a variety of building surfaces such as wood, thatch, or mud, and (*e*) of reasonable cost. This programme consists of increasingly complex and rigorous selection and has had the full cooperation of some 42 industrial firms and 4 research laboratories in 8 countries. The severity of selection may be gauged by the proportion of insecticides admitted to subsequent stages of the programme.

Stage	Insecticides admitted	
	Number	Percent
I	1290	—
II	186	14.4
III	51	27.4
IV	25	49.0
V	10	40.0
VI	6	60.0
VII	4	66.7

The work carried out in the United States involves the first three stages of the programme, i.e.,

— Stage I, « Preliminary Screening Tests » conducted at the University of California, Riverside,

— Stage II, « Advanced Laboratory Tests », and

— Stage III, « Preliminary Field Tests », pursued at both the U.S. Public Health Service Technical Development Laboratories, Savannah, Georgia, and the U.S. Department of Agriculture Laboratory, Gainesville, Florida.

Research on insecticide resistance in mosquitoes. Although this final item is not an integral part of the programme *per se,* it is being conducted with the encouragement of WHO as it fits well into the total effort for the development of new insecticides.

STAGE I

The insecticides received are precision-tested, with a minimum of delay, on two species of mosquitoes and three strains of the house fly. These tests aim at establishing dosage-mortality regression lines for each compound against both susceptible and resistant strains. The mosquito tests are performed on *Anopheles albimanus* from Panama, resistant to dieldrin, and on *Culex pipiens fatigans* (= *quinquefasciatus*) from California, naturally tolerant of DDT but susceptible to all other insecticides. Both larvae and adults are used. Adequate space is provided for the production of the several thousands of mosquitoes needed for these tests and for the propagation of the colonies.

Larval tests are conducted by means of a variation of the standard WHO method wherein groups of 20, fourth-instar larvae are transferred to waxed paper cups containing 100 ml of water and are then treated with varying concentrations of the toxicant in 1 ml of acetone per cup. At least 5 concentrations are used within the range producing 5 to 95 per cent mortality. The tests are repeated on 3 or more days, and the results are averaged and plotted on standard log-probit graph paper. A compound must have an LC_{95} of less than 1 ppm to be recommended for further evaluation at advanced stages.

In the adult tests (GEORGHIOU and METCALF, 1961), 3-day old sugar-fed mosquitoes are anaesthetized with CO_2, counted in groups of 20, and transferred by vacuum to shell vials lined with filter paper. These papers are treated, one hour in advance, with various concentrations of the test compound. After a 60-minute exposure period the mosquitoes are transferred to paper cups fitted with transparent plastic lids, for observation of mortality 24 hours later. A compound must have an LC_{50} of less than 16 µg/cm² to be recommended for further evaluation.

About 1300 compounds have been tested in Stage I since the programme was initiated in 1960. Of these, approximately 15 % have met the minimum toxicity standards for admission to stage II. The relative severity of these standards may be visualized in the following table by examination of the toxicity of a number of « promising » compounds which were discussed in a recent WHO publication (1967). DDT has also been included for further comparison.

TABLE 1

Stage I. — Relative toxicity of various insecticides to *Anopheles albimanus*

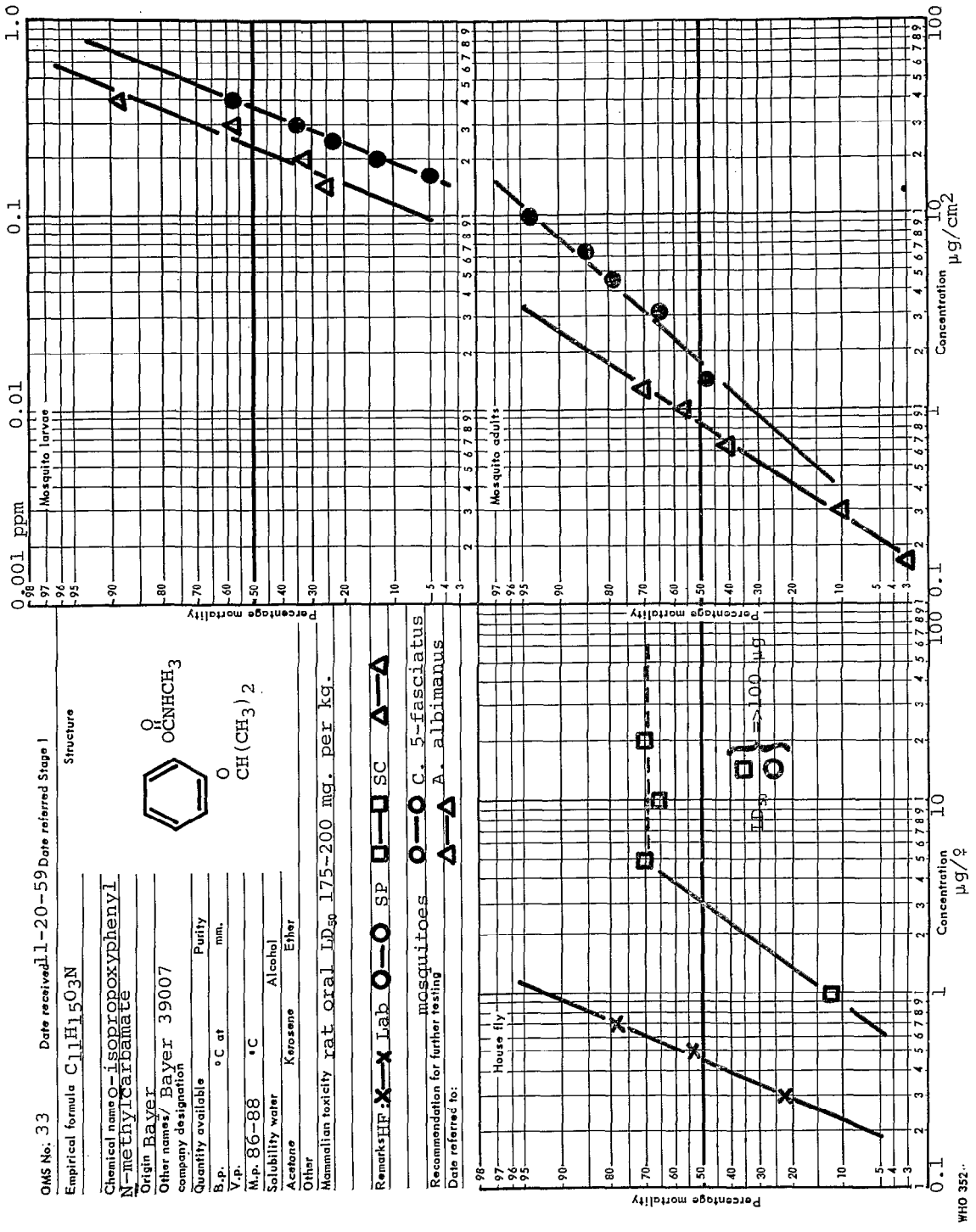
Compound	Larval LC_{50} (ppm)	Compound	Adult LC_{50} (γ/cm²)
Dursban	0.006	Dursban	0.18
Abate	0.011	D.D.T.	0.23
Fenitrothion	0.012	Carbamult	0.55
D.D.T.	0.015	Arprocarb	0.82
Fenthion	0.016	Bromophos	0.95
Bromophos	0.034	Fenthion	1.20
Dicapthon	0.092	Fenitrothion	2.80
Malathion	0.100	Malathion	4.30
Arprocarb	0.230	Dicapthon	6.90
Carbamult	0.300	Abate	> 16.00
Standard	1.0	Standard	16,00

It may be argued that the standards used are not severe enough, i.e., the cutoff points for either larval or adult toxicity are too high. We should point out, however, that about 85 % of the compounds received in stage I failed to move to stage II because they did not meet these standards. It is true that the more successful compounds used in the past, such as DDT and dieldrin, have much lower LC_{50} values than our present cutoff points. But it must be remembered that although high toxicity to mosquitoes is a very desirable characteristic, other considerations such as mammalian toxicity, residual activity, and cost of production are also important. Since these properties are usually not known to us at the time of testing in stage I, we consider it advisable not to exclude some seemingly marginal compounds from further consideration in the eventuality that other desirable properties might offset the disadvantage of low toxicity.

In addition it should be pointed out that the complexities of a vector control programme, including the presence of resistant races of vectors, differing environmental conditions, and the logistics of supply, are such that it is highly advisable to have a large number of suitable insecticidal compounds available for use as needed.

This table also illustrates the well-known fact that a good larvicide is not necessarily a good adulticide and vice-versa. For instance, Abate, one of the most toxic larvicides, is of marginal toxicity to adults. Conversely, carbamult and arprocarb, two good adulticides, are rather poor larvicides. This fact is taken into consideration in the screening programme by assuring that each compound is tested against both of the life stages of *Anopheles* and *Culex*. A compound may be recommended for further evaluation on the basis of its performance on larvae only, on adults only, or on both.

The results of all screening tests with each compound, including data on house flies, are recorded on final WHO data sheets. A sample of the form with data on arprocarb (Baygon®, OMS 33) is shown in fig. 1. These data sheets provide for instant visual comparison of the level of toxicity of each compound, of cross-resistance (data on the



house fly), and of the degree of heterogeneity of response of each strain to the insecticide (as manifested by the slopes of the 1d-p lines). Copies of the data sheets are sent to all the participants in the programme and constitute a valuable permanent record of the initial performance of each compound.

STAGE II

Once they have cleared stage I, the successful compounds are moved to stage II for « advanced laboratory testing » at Savannah, Georgia and at Gainesville, Florida. In both laboratories the insecticides are tested on larvae and adults of additional species of mosquitoes.

At the Savannah laboratory, the insecticides are tested on adults for :

1. **Contact action,** as space sprays (30-second exposure) against :

Anopheles albimanus,
Aedes aegypti,
Aedes taeniorhynchus, and
Culex pipiens fatigans (= *quinquefasciatus*).

2. **Residual action** by application of suspensions at several dosages to plywood panels, and making biweekly then monthly evaluations, until mortality drops below 70 %. The test species are resistant strains of :

Anopheles albimanus,
Anopheles quadrimaculatus,
Aedes aegypti,
Culex p. fatigans (= *quinquefasciatus*).

TABLE 2

Stage II. Residual action
Weeks of 70 percent or greater effectiveness of experimental insecticide residues on plywood panels against *A. quadrimaculatus, A. albimanus,* and *C. quinquefasciatus.* Savannah, Georgia 1968 (1)

Compound (2)	*A. quadrimaculatus* (g/m²)			*A. albimanus* (g/m²)			*C. quinquefasciatus* (g/m²)		
	0.5	1	2	0.5	1	2	0.5	1	2
O.M.S.-852 ...	2	2	2	—	—	—	2	2	2
O.M.S.-888 ...	2	2	10	14+	14+	14+	2	6	10
O.M.S.-1011 ..	2	10	26+	6	14	26+	2	14	26+
O.M.S.-1102 ..	2	2	2	—	—	26+	2	2	26+
O.M.S.-1103 ..	2	25+	25+	5	25+	25+	2	25+	25+
O.M.S.-1206 ..	2	17	26+	6	17	26+	2	17	22
O.M.S.-1236 ...	2	6	26	22	26	26	10	26	26+
O.M.S.-1244 ..	10	14	26+	14	18	26+	14	18	26+
O.M.S.-1246 ..	10	22	26+	10	22	26+	6	14	26+
O.M.S.-1247 ..	2	6	14	6	6	14	2	6	14
O.M.S.-1249 ..	6	18	26	14	18	26	10	26	26
O.M.S.-1271 ..	2	2	2	—	—	—	2	2	2

+ Test concluded.
(1) TAYLOR (R. T.) and SCHOOF (H.-F.). — *Promising residual insecticides for control of adult mosquitoes* (In manuscript.)
(2) Chemical names of compounds are given in table 6.

The same properties are also tested against larvae, i.e.,

1. **Contact action,** by determination of LC_{95} values by the standard WHO method on the following species :

Anopheles quadrimaculatus,
Aedes aegypti,
Aedes triseriatus,
Culex p. fatigans (= *quinquefasciatus*).

2. **Residual action,** by application of ethanol solutions of the insecticides to water, at various concentrations, and determination of residual toxicity at weekly intervals thereafter, against larvae of :

Aedes aegypti, and
Culex p. fatigans (=*quinquefasciatus*).

At the Gainesville laboratory, the tests on adult mosquitoes are aimed at determining (1) the contact and (2) the residual action of the compounds by different methods than those employed at Savannah.

1. **Contact action** is assayed against *Aedes taeniorhynchus* by sprays in a wind tunnel, in volumes of 1/4 ml of insecticide solution injected in an air stream moving at 4 m.p.h Compounds with LC_{50} values of 0.05 % or less are accepted for further testing.

2. **Residual action** is determined by application of the compounds at the rate of 1 g/m^2 to plywood panels. The panels are tested 1 week after treatment, again after 4 weeks, and every 4 weeks thereafter for a period of 24 weeks, or until they become ineffective, whichever occurs first. Panels are considered ineffective in tests with mosquitoes when they fail to produce at least 70 % mortality. In each test female *Anopheles quadrimaculatus* Say are exposed under half sections of petri dishes on the treated panels for 60 minutes. Compounds that cause a minimum of 70 % mortality during at least 8 weeks of aging are recommended for further evaluation.

Larval tests at Gainesville are performed against *Anopheles quadrimaculatus* by the standard WHO method, but with acetone substituted as the solvent. Compounds that cause at least 95 % mortality at 0.1 ppm are recommended for advancement to stage III.

It is readily apparent that the tests in stage II provide further essential information not only on the intrinsic toxicity of the compounds against additional species of mosquitoes but also on the persistence of residues in aquatic environments and on wood. Approximately 27 % of the compounds tested in stage II have been recommended for advancement to stage III.

STAGE III

In this stage (Savannah) the promising materials from stage II are evaluated under simulated field conditions. Residual treatments for adult control are applied by hand equipment to various surfaces such as thatch, clay, whitewashed clay, cement, plaster, etc., and evaluated at biweekly intervals with mosquitoes confined under a plastic cone. In addition, treatments are made to experimental huts which have clay-lined walls and an exterior screened porch. Free-flying adults are used in the hut studies. In other adulticide tests, insecticidal fogs are evaluated against caged mosquitoes at sites 150 feet and 300 feet from the line of discharge. In larvicide tests, various formulations of the candidate compounds are applied to small field plots and the effectiveness assessed at periodic intervals.

In stage III tests the residues are exposed to the normal weathering encountered on operational programmes. The chief difference between these tests and full field evaluation is that laboratory-reared resistant strains of *Anopheles quadrimaculatus, Anopheles albimanus,* and *Culex quinquefasciatus* are used.

TABLE 3

Stage III. Residual adulticide evaluation
Number of weeks indicated compounds gave 90 percent or greater mortality of dieldrin-resistant *Anopheles quadrimaculatus* released in treated experimental huts for 2, 4, or 8 hours. Savannah, Georgia, 1966 (1)

Compound (2)	Dosage g/m²	Exposure 2	Time	Hours
O.M.S.-33	2	> 20	—	—
O.M.S.-597	2	12	> 18	—
O.M.S.-658	2	> 20	—	—
O.M.S.-708	2	6	17	—
O.M.S.-712	1	*	5	17
O.M.S.-716	1	9	> 20	—
O.M.S.-971	2	> 18	—	—

* Mortality below 90 percent when first tested on week 4.
(1) MATHIS, WILLIS and SCHOOF (H. F.). — Residual activity of several new insecticides to adult *Anopheles quadrimaculatus* in adobelined experimental huts. *Mosquito News.* In press.
(2) Chemical names of the compounds are given in table 6.

Finally a few words concerning our studies on resistance. Since the tremendous effort and expense involved in this programme are the consequence of the innate capacity to develop resistance, it would be remiss if this potential in mosquitoes was not determined prior to the introduction of new compounds in large scale field trials. Similarly, since many field populations are already resistant to one or more insecticides,

TABLE 4

Stage III. Space spray evaluation
Percent kill of adult female mosquitoes 150 and 300 feet from the point of discharge of thermal fogs. Savannah, Georgia, 1967 (1)

Insecticides (2)	No. Runs	*Ae. aegypti*		*A. albimanus*		C. *quinque.*		*Ae. taenior.*	
		150'	300'	150'	300'	150'	300'	150'	300'
O.M.S.-1 (6 oz.) *	12	94	84	96	93	61	38	90	52
O.M.S.-595 (4 oz.)	4	98	87	71	51	30	7	80	25
O.M.S.-658 (6 oz.)	12	85	70	66	61	62	45	44	40
O.M.S.-786 (6 oz.)	4	46	9	15	9	35	1	7	1
O.M.S.-1170 (2 oz.)	4	66	45	93	76	24	14	58	41
O.M.S.-1197 (2 oz.)	4	98	88	98	94	95	82	78	67

* Oz. of technical grade per gallon of fuel oil
(1) TAYLOR (R. T.) and SCHOOF (H. F.). — Field effectiveness of five organophosphorus compounds as thermal fogs. *Mosquito News.* (In manuscript).
(2) Chemical names of the compounds are given in table 6.

TABLE 5

Stage III. Residual larvicide test
Weeks of 90 percent kill of *Anopheles albimanus* when introduced into flooded soil samples treated with granular insecticides. Savannah, Georgia. 1967 (1)

Insecticide (2)	Lb./ Acre	3-day flooded soil samples		10-day flooded soil samples	
		Wet *	Dry **	Wet	Dry
O.M.S.-971	1	> 12	> 12	> 12	> 12
O.M.S.-33	2	6	< 2	> 12	< 2
O.M.S.-29	4	4	< 2	> 12	< 2
O.M.S.-708	4	4	6	> 12	> 12
O.M.S.-43	2	4	< 2	8	< 2

* 3.75 inches rainfall per week.
** 0.25 inch rainfall per week.
(1) SCHOOF (H. F.) and TAYLOR (R. T.). — Experimental field treatments with larvicides for control of *Anopheles* and *Aedes* mosquitoes. (In manuscript).
(2) Chemical names of the compounds are given in table 6.

new compounds must also be evaluated for their performance against all existing types of resistance. Our objective is to obtain by selection pressure, and to maintain in the laboratory, reference strains of mosquitoes that are resistant to insecticides representative of the major groups of compounds in current use or in the process of development. New compounds may then be tested routinely against these resistant strains as a means of avoiding costly and unpleasant surprises at a later, crucial stage of the programme.

We have made substantial progress in the development of strains of *Culex p. fatigans* fully resistant to a chlorinated hydrocarbon (DDT), a cyclodiene (dieldrin), and a carbamate (arprocarb) ; strains with low resistance to the organophosphate fenthion and with slight tolerance to fenitrothion have also been developed. Progress has been slower with *Anopheles albimanus*, but we now have strains fully resistant to DDT and dieldrin, and strains with slight tolerance toward arprocarb and fenitrothion.

Since the question of resistance will be discussed in detail in Section B. 2. 4 it is not necessary for us to go into further details now. Suffice it to say that this vital problem is being investigated as one of the many facets of the WHO insecticide development programme.

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TABLE 6

Chemical names of compounds cited in this paper

O.M.S.-1	diethyl mercaptosuccinate *S*-ester with *O,O*-dimethyl phosphorodithioate (malathion)
O.M.S.-29	α-naphthyl methylcarbamate (carbaryl)
O.M.S.-33	*o*-isopropoxyphenyl methylcarbamate (carbaryl)
O.M.S.-43	*O,O*-dimethyl *O*-(4-nitro-*m*-tolyl) phosphorothioate (fenitrothion)
O.M.S.-595	dimethyl 2,4,5-trichloro-α-(chloromethylene) benzyl phosphate
O.M.S.-597	3,4,5-trimethylphenyl methylcarbamate
O.M.S.-658	*O*-(4-bromo-2,5-dichlorophenyl) *O,O*-dimethyl phosphorothioate (bromophos)
O.M.S.-708	4-benzothienyl methylcarbamate (mobam)
O.M.S.-712	2-chloro-1-(2,4-dichlorophenyl) vinyl dimethyl phosphate.
O.M.S.-716	3-methyl-5-isopropylphenyl methylcarbamate.
O.M.S.-786	*O,O,O',O'*-tetramethyl *O,O'*-thiodi-*p*-phenylene phosphorothioate (Abate).
O.M.S.-852	hexyl propargyl sulfite.
O.M.S.-888	1,1-dichloro-2,2-bis (*p*-chlorophenyl)-2-fluoroethane.
O.M.S.-971	*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate (Dursban).
O.M.S.-1011	3,4,5,6-tetrahydrophthalimidomethyl *dl*-cis-transchrysanthemate (phthalthrin).
O.M.S.-1102	*o*-(1,3-dioxolan-2-yl) phenyl methylcarbamate.
O.M.S.-1103	*o*-(4,5-dimethyl-1,3-dioxolan-2-yl) phenyl methylcarbamate.
O.M.S.-1170	phenylglyoxylonitrile oxime *O*-ester with *O,O*-diethyl phosphorothioate.
O.M.S.-1197	*o*-chlorophenylglyoxylonitrile oxime *O*-ester with *O,O*-diethyl phosphorothioate.
O.M.S.-1206	2-benzyl-4-furfuryl *dl*-cis-trans-chrysanthemate.
O.M.S.-1244	4-hydroxy-2-mercaptovaleric acid gamma-lactone *S*-ester with *O*-butyl methylphosphonodithioate.
O.M.S.-1246	4-hydroxy-2-mercaptobutyric acid gamma-lactone *S*-ester with *O*-isobutyl ethylphosphonodithioate.
O.M.S.-1247	4-hydroxy-2-mercaptobutyric acid gamma-lactone *S*-ester with *O*-isobutyl methylphosphonodithioate.
O.M.S.-1249	4-hydroxy-2-mercaptobutyric acid gamma-lactone *S*-ester with *O*-butyl ethylphosphonodithioate.
O.M.S.-1271	*o*-(1,3-dithiolan-2-yl) phenyl methylcarbamate.