Isocycloseram/Emamectin Benzoate

Isocycloseram/Emamectin Benzoate SC (A23220A) – Skin Sensitisation Local Lymph Node Assay

Final Report

TEST GUIDELINE(S): OECD 429 (2010)
EC 440/2008 B.42 (2012)

AUTHOR(S): Dr. Eva Dony

COMPLETION DATE: 26 August 2020

PERFORMING LABORATORY: ICCR-Roßdorf GmbH
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SPONSOR(S): Syngenta Ltd
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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study performed in the test facility of ICCR-Roßdorf GmbH, In den Leppsteinswiesen 19, 64380 Rossdorf, Germany was conducted in compliance with Good Laboratory Practice Regulations:

"Chemikaliengesetz" (Chemicals Act) of the Federal Republic of Germany, "Anhang 1" (Annex 1), in its currently valid version

"OECD Principles of Good Laboratory Practice", as revised in 1997 [C(97)186/Final]

EC Commission Directive 2004/10/EC

These procedures are compatible with Good Laboratory Practice regulations specified by regulatory authorities throughout the European Community, the United States (EPA and FDA), and Japan (MHW, MAFF, and METI).

There were no circumstances that may have affected the quality or integrity of the study.

E.D

26 August 2020

Dr. Eva Dony
Study Director
Toxicology *in vivo*

Date

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To be completed for USA EPA submission only:

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FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

ICCR Study Number: 2108100
Test Substance: Isocycloseram/Emamectin Benzoate SC (A23220A)
Study Director: Dr. Eva Dony
Study Title: Isocycloseram/Emamectin Benzoate SC (A23220A) – Skin Sensitisation Local Lymph Node Assay

Study based activities at the Test Facility ICCR-Roßdorf GmbH were audited and inspected. The details of these audits and inspections are given below.

Type of Inspection	Date(s) of Inspection	Date Reporting to Study Director, Test Facility Management
Study Plan Verification	30 April 2020	30 April 2020
Process – based Test System Preparation & Application Test Item Preparation	 08 May 2020 02 June 2020	 08 May 2020 02 June 2020
Report Audit	16 July 2020	16 July 2020

General facilities and activities where this study was conducted were inspected on an annual basis and results are reported to the relevant responsible person and Management.

This statement is to confirm, that this report reflects the raw data.

Quality Assurance

H. Pilawa

26 August 2020
Date

Quality Assurance Auditor
ICCR-Roßdorf GmbH

GENERAL INFORMATION

Contributors
The following contributed to this report in the capacities indicated:

Name	Title
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Study Dates

Study initiation date:	04 May 2020
Experimental start date:	06 May 2020
Experimental completion date:	23 June 2020

Deviations from the Guidelines
None

Retention of Samples
Raw data.

Performing Laboratory Test Substance Reference Number
[S 2092211]

Other
Records and documentation relating to this study will be maintained in the archives of ICCR-Roßdorf GmbH for a period of 4 years from the date on which the Study Director signs the final report. This will include but may not be limited to the Study Plan, raw data, amendments (if any), and the Report generated during the course of this study.

At termination of the aforementioned period, the records and documentation will be transferred to the GLP compliant Rhenus Archiv Services GmbH, Frankfurt am Main for further archiving up to a total archiving period of 15 years.

A sample of the test substance will not be archived.

ICCR-Roßdorf GmbH will retain in its archive a copy of the study plan and final report, and any amendments indefinitely.

Deviations from the study plan

The relative humidity in the animal room was between 18-65% instead of 45-65% for several days due to a defective air humidifier.

This deviation to study plan, however, does not affect the validity of the study.

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

In the study the test substance Isocycloseram/Emamectin Benzoate SC (A23220A) formulated in 1% aqueous Pluronic® was assessed for its possible skin sensitising potential.

For this purpose a local lymph node assay was performed using test substance concentrations of 5, 10, and 25% (w/w). The highest concentration tested was the highest concentration that could be achieved whilst avoiding systemic toxicity and excessive local skin irritation as confirmed by two pre-experiments.

1.2 Results

The animals did not show any signs of systemic toxicity during the course of the study and no cases of mortality were observed. The animals showed a very slight erythema of the ear skin on test day 3 only (Score 1).

In this study Stimulation Indices (S.I.) of 0.8, 1.3, and 1.6 were determined with the test substance at concentrations of 5, 10, and 25% in 1% aqueous Pluronic®, respectively.

1.3 Conclusion

The test substance Isocycloseram/Emamectin Benzoate SC (A23220A) was **not a skin sensitiser** under the test conditions of this study.

2.0 INTRODUCTION

2.1 Purpose

The test is designed to assess the skin sensitisation potential (delayed type hypersensitivity) of the test substance in the mouse following topical application to the dorsal surface of the ear.

The basic principle underlying the Local Lymph Node Assay (LLNA) is that sensitisers induce a primary proliferation of lymphocytes in the lymph node draining the application site. Primary lymphocyte proliferation is assessed during the sensitising (induction) phase of the response. This proliferation is proportional to the dose applied (and to the potency of the allergen) and provides a simple means of obtaining an objective, quantitative measurement of sensitisation. The LLNA assesses this proliferation as a dose response in which the proliferation in test groups is compared to that in vehicle treated controls.

Lymphocyte proliferation is quantified by measuring the incorporation of radiolabelled thymidine into lymph node cells by β-Scintillation Counting.

2.2 Justification

This study should provide a rational basis for risk assessment to the sensitising potential of the test substance in man.

2.3 Animal Welfare

The in-life experimental procedures undertaken during the course of this study were subject to the provisions of Germany's 'Tierschutzgesetz' (TierSchG, Animal Act) in its currently valid version, as well as with Germany's 'Verordnung zum Schutz von zu Versuchszwecken oder zu anderen wissenschaftlichen Zwecken verwendeten Tieren (Tierschutz-Versuchstierverordnung, TierSchVersV)'[1] in its currently valid version.

This study design is regularly inspected and approved by the veterinary authorities (Regierungspräsidium Hessen). The study was also conducted in accordance with the OECD guidance document on recognition, assessment and use of clinical signs as humane endpoints for experimental animals used in safety evaluation.

The number of animals used were the minimum that is consistent with scientific integrity and regulatory acceptability, consideration having been given to the welfare of individual animals in terms of the number and extent of procedures to be carried out on each animal.

2.4 Regulatory Testing Guidelines

The study was performed in compliance with the following regulations or guidelines:

- OECD Guidelines for Testing of Chemicals, Updated Guideline 429: Skin Sensitisation: Local Lymph Node Assay (adopted 22 July 2010).
- Commission Regulation (EU) 2017/735 amending, for the purpose of its adaptation to technical progress, the Annex to Regulation (EC) No. 440/2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH): Chapter B.42: 'Skin Sensitisation: Local Lymph Node Assay', updated 06 July 2012.

[1] Regulation for the protection of animals used for experimental and other scientific purposes (Regulation on Experimental Animal Protection, TierSchVersV)

3.0 MATERIALS AND METHODS

3.1 Test Substance

The test substance and the information concerning the test substance were provided by the Sponsor.

Identification:	Isocycloseram/Emamectin Benzoate SC (A23220A)
Batch:	TSC002-041-001
Content of isocycloseram:	17.5% w/w corresponding to 201 g/L
Content of emamectin benzoate:	4.18% w/w corresponding to 48.1 g/L
Physical state / Appearance:	Brown liquid
Recertification Date:	31 January 2023
Storage Conditions:	At room temperature
Stability in Solvent:	Not indicated by the Sponsor

Correction for content of active ingredients was not made.

3.2 Chemicals

^{3}H-Methyl thymidine:	Perkin Elmer (aqueous solution), specific activity: 74 GBq/mmol (2 Ci/mmol), concentration: 37 MBq/mL (1 mCi/mL)
Trichloroacetic acid:	Purity: min. 99%
Phosphate buffered saline:	1 tablet solved in 200 mL deionized water

3.3 Vehicle

1% aqueous Pluronic®

3.4 Test Substance Preparation

3.4.1 Vehicle and dose selection

A solubility experiment was performed according to the recommendations given by OECD 429. The highest test item concentration, which could be technically used was 100% of the undiluted test item. Test item suspension at different concentrations was prepared using 1% aqueous Pluronic®. Vortexing was used to formulate the test substance.

To determine the highest non-irritant test concentration that at the same time did not induce signs of systemic toxicity, two pre-test were performed in two animals each and stated in raw data and report.

In the first pre-test, two mice were treated by (epidermal) topical application to the dorsal surface of each ear with test substance concentrations of 50 and 100% once daily each on three consecutive days. In the second pre-test, two mice were treated with 10 and 25%.

Prior to the first application of the test substance and before sacrifice the body weight was determined. Clinical signs were recorded at least once daily. Eventual signs of local irritation were documented and a score was used to grade a possible erythema of the ear skin. Furthermore, prior to the first application of the test substance (day 1), on day 3 and before sacrifice (day 6) the ear thickness was determined using a micrometer. Additionally, for both animals, the ears were punched after sacrifice (day 6) at the apical area using a biopsy punch (Ø 8 mm corresponding to 0.5 cm^2) and were immediately pooled per animal and weighed using an analytical balance.

Eventual ear irritation was considered to be excessive if an erythema of the ear skin of a score value ≥3 was observed at any observation time and/or if an increase in ear thickness of ≥25% was recorded on day 3 or day 6.

3.4.2 Test Substance Preparation

The test substance was placed into an appropriate container on a tared balance, and 1% aqueous Pluronic® was added (weight per weight).

The different test substance concentrations were prepared individually. Homogeneity of the test substance in vehicle was maintained during treatment using a magnetic stirrer.

The preparations were made freshly before each dosing occasion.

3.5 Test System and Supporting Information

Test system:	Mice, CBA/CaOlaHsd
Rationale:	Recognised as the recommended test system.
Source:	Envigo RMS B.V., Inc Postbus 6174 5960 AD Horst / The Netherlands
Number of animals for the pre-tests:	4 (2 females for each pre-test)
Number of animals for the main study:	20 females
Number of animals per group:	5 females (nulliparous and non-pregnant)
Number of test groups:	3
Number of control (vehicle) groups:	1
Age (beginning of treatment):	8 - 12 weeks
Body weight:	see Appendix 1 and 2
Identification:	The animals were distributed into the test groups at random. All animals belonging to the same experimental group were kept in one cage. In the main experiment, the animals were individually

	marked. In the pre-experiment, animals were identified by cage number.
Acclimation:	At least 5 days prior to the start of dosing under test conditions after health examination. Only animals without any visible signs of illness were used for the study.

3.5.1 Husbandry

The animals were kept conventionally. The experiment was conducted under standard laboratory conditions.

Housing:	group
Cage Type:	Makrolon Type II (pre-test) / III (main study), with wire mesh top
Bedding:	granulated soft wood bedding
Feed:	2018C Teklad Global 18% protein rodent diet (certified), *ad libitum*
Water:	tap water, *ad libitum*
Environment:	temperature 22 $\pm$ 2°C relative humidity approx. 45-65% (except for deviation) artificial light 6.00 a.m. - 6.00 p.m. ventilation at least eight air changes per hour

3.6 Experimental Design and Study Conduct

3.6.1 Test substance administration

Each test group of mice was treated by (epidermal) topical application to the dorsal surface of each ear with a corresponding test substance concentrations of 5, 10, or 25% in 1% aqueous Pluronic®. The application volume, 25 µL/ear/day, was spread over the entire dorsal surface (∅ ~ 8 mm) of each ear once daily for three consecutive days. A further group of mice (control animals) was treated with an equivalent volume of the relevant vehicle alone (control animals).

3.6.2 Administration of ^{3}H-methyl-thymidine

Five days after the first topical application (day 6) 250 µL of phosphate-buffered saline containing 20.8 µCi of ^{3}H-methyl thymidine (equivalent to 83.1 µCi/mL ^{3}HTdR) were injected into each test and control mouse via the tail vein.

3.6.3 Terminal procedure

Approximately five hours after treatment with ^{3}HTdR all mice were euthanized by using CO_2, which was, after harvesting of the lymph nodes, followed by cervical dislocation to ensure death. For each individual animal of each group, the draining auricular lymph nodes were excised and processed.

3.6.4 Preparation of single cell suspensions

The draining lymph nodes were rapidly excised and pooled per animal (2 nodes per animal). Single cell suspensions (in phosphate buffered saline) of pooled lymph node cells were prepared by gentle mechanical disaggregation through stainless steel gauze (200 µm mesh size). After washing two times with phosphate buffered saline (approx. 10 mL) the lymph node cells were resuspended in 5 % trichloroacetic acid (approx. 3 mL) and incubated at approximately +4 °C for approximately 18 hours for precipitation of macromolecules.

3.6.5 Determination of cellular proliferation (incorporation of ^{3}HTdR)

The precipitates were then resuspended in 5 % trichloroacetic acid (1 mL) and transferred to scintillation vials with 10 mL of scintillation liquid and thoroughly mixed. The level of ^{3}HTdR incorporation was then measured in a β-scintillation counter. Similarly, background ^{3}HTdR levels were also measured in two 1 mL-aliquots of 5 % trichloroacetic acid. The β-scintillation counter expresses ^{3}HTdR incorporation as the number of radioactive disintegrations per minute.

3.6.6 Observations

Clinical Observations

All animals were observed on a daily basis, including pre- and post-dose observations on days 1, 2 and 3. Any clinical signs of systemic toxicity, local skin irritation or signs of ill health during the study were recorded.

Determination of Ear Thickness

In the pre-test, the ear thickness was determined prior to the first application of the test substance (day 1), on day 3, and on day 6 prior to sacrifice using a micrometer.

Determination of Ear Weights

In the pre-test, after the lymph nodes have been excised, both ears of mice were punched at the apical area using a biopsy punch (Ø 8 mm corresponding to 0.5 cm^2). For each animal both punches were immediately weighed per animal using an analytical balance. The values obtained were taken down manually. The results are described in the report.

Determination of Body Weights

The body weights were recorded on day 1 (prior to dosing) and prior to sacrifice (pre-test) or prior to treatment with ^{3}HTdR (main experiment).

3.7 Data Evaluation

3.7.1 Interpretation of raw data

The proliferative response of the lymph node cells is expressed as the number of radioactive disintegrations per minute per lymph nodes of each animal (DPM/animal) and as the ratio of ^{3}HTdR incorporated into lymph node cells of test animals relative to that recorded for lymph nodes of control animals (Stimulation Index; S.I.).

Before DPM/animal values were determined, mean scintillation-background DPM was subtracted from test and control raw data.

A test substance is regarded as a sensitiser in the LLNA if the following criteria are fulfilled:

- First, that exposure to at least one concentration of the test substance resulted in an incorporation of ^{3}HTdR at least 3-fold or greater than that recorded in control mice, as indicated by the Stimulation Index.
- Second, that the data are compatible with a conventional dose response, although allowance must be made (especially at high topical concentrations) for either local toxicity or immunological suppression.

3.7.2 General calculations

The mean values and standard deviations were calculated in the body weight tables.

Where appropriate, the EC3 value were calculated according to the equation

$$EC3 = (a-c)\,[(3-d)/(b-d)] + c$$

where EC3 is the estimated concentration of the test substance required to produce a 3-fold increase in draining lymph node cell proliferative activity; (a, b) and (c, d) are respectively the co-ordinates of the two pair of data lying immediately above and below the S.I. value of 3 on the local lymph node assay dose response plot.

All calculations conducted on the DPM values were performed with a validated test script of "*R*", a language and environment for statistical computing and graphics.

Within the program the Dean-Dixon-Test and Grubb's Test were used for identification of possible outliers. An outlier (DPM value determined for animal 8) was identified in both statistical outlier tests, but was not excluded from any calculations since exclusion of the outlier would not change the overall test result.

Both biological and statistical significance were considered together.

3.8 Positive Control Data

The sensitivity and reliability of the experimental technique employed was assessed by use of α-hexyl cinnamaldehyde dissolved in acetone/olive oil (4+1 v/v) (compound listed in OECD 429 Guideline) which is known to have skin sensitisation properties in mice. The periodic positive control experiment was performed using CBA/CaOlaHsd mice in April 2020, see Appendix 1 and 2.

4.0 RESULTS AND DISCUSSION

4.1 Pre-Experiment

In the first pre-experiment two mice were treated by (epidermal) topical application to the dorsal surface of each ear with test substance concentrations of 50 and 100% once daily each on three consecutive days.

At the tested concentrations the animals showed an erythema of the ear skin (score 1 to 2). Signs of systemic toxicity included increased activity, piloerection, fur loss, hunched posture, partially closed eyes, decreased activity, elevated tail, tremor, and fasciculations. The animal treated with 100% test item concentration was euthanised on day 4 due to deterioration of clinical symptoms (tremor, tippy toe walk, moribund appearance, substantial body weight loss).

Therefore, a second pre-experiment was performed using concentrations of 10 and 25%. At the tested concentrations the animals did not show any signs of systemic toxicity. At the tested concentration of 25% the animal showed a very slight erythema of the ear skin (score 1). The animal treated with 10% test item concentration did not show any signs of local irritation.
See Table 2 for more details.

On the basis of these data the test substance in the main study was assayed at 5, 10, and 25%.

4.2 Results in the Main Experiment

Stimulation Indices
In this study Stimulation Indices of 0.8, 1.3, and 1.6 were determined with the test substance at concentrations of 5, 10, and 25% in 1% aqueous Pluronic®. The EC3 value could not be calculated, since none of the tested concentrations induced a S.I. greater than the threshold value of 3.

Calculations of group mean DPM values, standard deviations and and individual results of the data can be found in Table 3 and 4.

Viability / Mortality
No deaths occurred during the study period.

Clinical Signs
No signs of systemic toxicity were observed during the study period. The animals showed a very slight erythema of the ear skin (Score 1).
The individual data are included in Table 5.

Body Weights
The body weight of the animals, recorded prior to the first application and prior to treatment with ^{3}HTdR, was within the range commonly recorded for animals of this strain and age.

The individual body weight values are included in Table 6.

4.3 Discussion

In order to study the possible skin sensitising potential of Isocycloseram/Emamectin Benzoate SC (A23220A), three groups each of five female mice were treated once daily with the test substance at concentrations of 5, 10, and 25% in 1% aqueous Pluronic® by topical application to the dorsum of each ear for three consecutive days. The maximum concentration tested was the highest concentration that could be achieved whilst avoiding systemic toxicity and excessive local skin irritation as confirmed by two pre-experiments. A control group of five mice was treated with the vehicle (1% aqueous Pluronic®) only. Five days after the first topical application the mice were injected intravenously into a tail vein with radio-labelled thymidine (^{3}H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed, the draining auricular lymph nodes excised and pooled per animal. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes, which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of ^{3}H-methyl thymidine measured in a β-scintillation counter.

All treated animals survived the scheduled study period and no signs of systemic toxicity were observed. The animals showed a very slight erythema of the ear skin (Score 1).

A test substance is regarded as a sensitiser in the LLNA if the exposure to one or more test concentration resulted in a 3-fold or greater increase in incorporation of ^{3}HTdR compared with concurrent controls, as indicated by the Stimulation Index (S.I.). The estimated concentration of test substance required to produce a S.I. of 3 is referred to as the EC3 value.

In this study Stimulation Indices of 0.8, 1.3, and 1.6 were determined with the test substance at concentrations of 5, 10, and 25% in 1% aqueous Pluronic®. The EC3 value could not be calculated, since none of the tested concentrations induced a S.I. greater than the threshold value of 3.

5.0 CONCLUSIONS

The test substance, Isocycloseram/Emamectin Benzoate SC (A23220A) was **not a skin sensitiser** under the test conditions of this study.

6.0 REFERENCES

1. OECD Guidelines for Testing of Chemicals, Updated Guideline 429: Skin Sensitisation: Local Lymph Node Assay (adopted 22 July 2010).

2. Kimber I., Hilton J. and Weisenberger C. (1989). The murine local lymph node assay for identification of contact allergens: a preliminary evaluation of in situ measurement of lymphocyte proliferation. Contact Dermatitis, 21, 215-220.

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8. W. J. Dixon (1950): Analysis of extreme values. The Annals of Mathematical Statistics, 21, 488-506.

TABLES SECTION

TABLE 1 Identification of the Animals by their Individual Markings

The animals were distributed to the different test groups as follows:

Group	Concentration[a] %	Number of Animals per Group	Animal Numbers (Group Housing)
1 Control Group*	—	5	1 - 5
2 Low Dose	5	5	6 - 10
3 Mid Dose	10	5	11 - 15
4 High Dose	25	5	16 - 20

*vehicle group = 1% aqueous Pluronic®

a) concentrations as determined in a pre-experiment.

TABLE 2 Results of the Pre-Tests

Pre-Test 1
Body Weights

Animal No.	Concentration %	Body Weight (g) (Sacrifice: Day 6 for Animal 1, Day 4 for Animal 2)			
		prior 1st Application	prior to Sacrifice	Difference Day 1 to Day of Sacrifice	Difference %
1	50	20.9	21.7	0.8	3.8
2	100	19.2	16.2	3.0	-15.6

Ear Thickness

Animal No.	Conc. %	Ear Thickness								
		prior to 1st Application (µm)			prior to 3rd Application (µm)			prior to Necropsy (µm)		
		Right Ear	Left Ear	Mean	Right Ear	Left Ear	Mean	Right Ear	Left Ear	Mean
1	50	245	250	247.5	250	255	252.5	235	240	237.5
2	100	245	240	242.5	255	265	260.0	animal euthanised on day 4		

Animal No.	Difference Day 1 to Day 3 (µm)	Ear Swelling Day 3 (%)	Difference Day 1 to Day 6 (µm)	Ear Swelling Day 6 (%)
1	5.0	2.0	-10.0	-4.0
2	17.5	7.2	animal euthanised on day 4	

Ear Weights

Animal No.	Concentration %	Ear Weights after Necropsy (mg per animal)	% Increase Compared to Vehicle Values
1	50	25.22	6.4
2	100	animal euthanised on day 4	

Mean of historical controls (1% aqueous Pluronic®): 23.7 mg/ animal

Ear Erythema

Animal No.	Score								
	Pre Dose Day 1	Post Dose Day 1	Pre Dose Day 2	Post Dose Day 2	Pre Dose Day 3	Post Dose Day 3	Day 4	Day 5	Day 6
1	0	0 SR	1	1 SR	1 SR H P PC UA ET T FA OA	1 SR H P PC UA	1 SR H P PC UA T	1 H P PC T UA	0 OA
2	0	0 SR	1	1 SR	1 SR H P PC UA ET T FA OA FL	2 SR H P PC UA FL*	2 SR H P PC UA T FL EU	animal euthanised on day 4	

SR=Substance residuals H=Hunched posture P=Piloerection PC=Partially closed eyes
UA=Decreased activity ET=Elevated tail T=Tremor FA=Fasciculations
OA=Increased activity FL=Fur loss EU=Euthanasia (deterioration of clinical symptoms: tremor, tippy toe walk, moribund appearance, weight loss)* burrows itself in the bedding, scratches itself

Score: 0 = No visible erythema 1 = Very slight erythema 2 = Well defined erythema 3 = Moderate to severe erythema 4 = Severe erythema to formation of eschar which prevents grading of erythema

Pre-Test 2

Body Weights

Animal No.	Concentration %	Body Weight (g)			
		prior 1st Application	prior to Sacrifice (Day 6)	Difference Day 1 to Day 6	Difference %
1	10	21.9	22.5	0.6	2.7
2	25	21.1	19.8	-1.3	-6.2

Ear Thickness

Animal No.	Conc. %	Ear Thickness								
		prior to 1st Application (µm)			prior to 3rd Application (µm)			prior to Necropsy (µm)		
		Right Ear	Left Ear	Mean	Right Ear	Left Ear	Mean	Right Ear	Left Ear	Mean
1	10	230	230	230.0	235	245	240.0	240	240	240.0
2	25	235	220	227.5	235	235	235.0	235	235	235.0

Animal No.	Difference Day 1 to Day 3 (µm)	Ear Swelling Day 3 (%)	Difference Day 1 to Day 6 (µm)	Ear Swelling Day 6 (%)
1	10.0	4.3	10.0	4.3
2	7.5	3.3	7.5	3.3

Ear Weights

Animal No.	Concentration %	Ear Weights after Necropsy (mg per animal)	% Increase Compared to Vehicle Values
1	10	25.47	7.5
2	25	24.80	4.6

Mean of historical controls (1% aqueous Pluronic®): 23.7 mg/ animal

Ear Erythema

Animal No.	Score								
	Pre Dose Day 1	Post Dose Day 1	Pre Dose Day 2	Post Dose Day 2	Pre Dose Day 3	Post Dose Day 3	Day 4.	Day 5	Day 6
1	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	1	0	0	0

Score: 0 = No visible erythema
1 = Very slight erythema
2 = Well defined erythema
3 = Moderate to severe erythema
4 = Severe erythema to formation of eschar which prevents grading of erythema

TABLE 3 Calculation and Results of Individual Data

Vehicle: 1% aqueous Pluronic®

Test substance concentration			DPM values measured	DPM−BG per animal (2 lymph nodes) [a)]	S.I.[b)]
%	Group no.	Animal no.			
---	---	BG I	19	---	---
---	---	BG II	16	---	---
0	1	1	809	791.5	---
0	1	2	1252	1234.5	---
0	1	3	2113	2095.5	---
0	1	4	4492	4474.5	---
0	1	5	2475	2457.5	---
5	2	6	983	965.5	0.4
5	2	7	1381	1363.5	0.6
5	2	8	3414	3396.5	1.5
5	2	9	1618	1600.5	0.7
5	2	10	1681	1663.5	0.8
10	3	11	2437	2419.5	1.1
10	3	12	2424	2406.5	1.1
10	3	13	2928	2910.5	1.3
10	3	14	4013	3995.5	1.8
10	3	15	2603	2585.5	1.2
25	4	16	1451	1433.5	0.6
25	4	17	2962	2944.5	1.3
25	4	18	5900	5882.5	2.7
25	4	19	3789	3771.5	1.7
25	4	20	3894	3876.5	1.8

BG = Background (1 ml 5% trichloroacetic acid) in duplicate
1 = Control Group for the test substance
2-4 = Test Groups
S.I. = Stimulation Index
a) = values corrected for mean background value (BGI and BGII).
b) = Stimulation Indices relative to the mean of the control group (Group 1)

TABLE 4 Calculation of Stimulation Indices per Dose Group

Test substance concentration	Group Calculation		
	Mean DPM per animal (2 lymph nodes) [a)]	SD	**S.I.**
Vehicle Control Group (1% aqueous Pluronic®)	2210.7	1428.8	**1.0**
5% Isocycloseram/Emamectin Benzoate SC (A23220A)	1797.9	934.6	**0.8**
10% Isocycloseram/Emamectin Benzoate SC (A23220A)	2863.5	664.6	**1.3**
25% Isocycloseram/Emamectin Benzoate SC (A23220A)	3581.7	1615.3	**1.6**

a) Mean DPM/animal was determined by dividing the sum of the measured values from lymph nodes of all animals within a group by the number of animals in that group (5 animals)

TABLE 5 Observations in the Main Experiment

Concentration (%) in 1% aqueous Pluronic®	Animal Number	Day 1		Day 2		Day 3		Day 4	Day 5	Day 6
		Pre Dose	Post Dose	Pre Dose	Post Dose	Pre Dose	Post Dose			
Vehicle (1% aqueous Pluronic®)	1	0	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0	0
	4	0	0	0	0	0	0	0	0	0
	5	0	0	0	0	0	0	0	0	0
5%	6	0	0	0	0	0	1	0	0	0
	7	0	0	0	0	0	1	0	0	0
	8	0	0	0	0	0	1	0	0	0
	9	0	0	0	0	0	1	0	0	0
	10	0	0	0	0	0	1	0	0	0
10%	11	0	0	0	0	1	1	0	0	0
	12	0	0	0	0	1	1	0	0	0
	13	0	0	0	0	1	1	0	0	0
	14	0	0	0	0	1	1	0	0	0
	15	0	0	0	0	1	1	0	0	0
25%	16	0	0	0	0	1	1	0	0	0
	17	0	0	0	0	1	1	0	0	0
	18	0	0	0	0	1	1	0	0	0
	19	0	0	0	0	1	1	0	0	0
	20	0	0	0	0	1	1	0	0	0

Score: 0 = No visible erythema
1 = Very slight erythema
2 = Well defined erythema
3 = Moderate to severe erythema
4 = Severe erythema to formation of eschar which prevents grading of erythema

TABLE 6 Body Weights in the Main Experiment

Animal No.	Group No.	Concentration %	Initial Weight (g)		Weight prior to treatment with ^{3}HTdR (g)	
			Individual	Mean ± SD*	Individual	Mean ± SD
1	1	Control Group (1% aqueous Pluronic®)	19.3	19.8±0.9	20.0	20.2±0.9
2	1		21.0		21.4	
3	1		20.4		20.6	
4	1		19.6		18.9	
5	1		18.8		20.0	
6	2	5% Isocycloseram/ Emamectin Benzoate SC (A23220A)	23.7	20.8±1.8	22.4	21.2±1.1
7	2		20.3		21.5	
8	2		21.1		22.0	
9	2		19.7		19.5	
10	2		19.1		20.8	
11	3	10% Isocycloseram/ Emamectin Benzoate SC (A23220A)	21.4	21.0±0.5	21.1	21.7±0.7
12	3		20.9		21.6	
13	3		20.6		22.8	
14	3		20.5		21.7	
15	3		21.7		21.3	
16	4	25% Isocycloseram/ Emamectin Benzoate SC (A23220A)	20.5	19.3±1.0	21.4	20.3±1.0
17	4		19.9		21.1	
18	4		18.0		20.5	
19	4		19.0		19.0	
20	4		18.9		19.7	

*SD= Standard Deviation

APPENDICES SECTION

APPENDIX 1 Results of the GLP Positive Control

Experiment performed in April 2020 (study number 1992300). Positive control substance: α-Hexylcinnamaldehyde

Vehicle: acetone:olive oil (4:1 v/v))

Test item concentration %	Group	Measurement DPM	Calculation			Result
			DPM-BG[a)]	number of lymph nodes	DPM per lymph node[b)]	**S.I.**
---	BG I	15	---	---	---	---
---	BG II	20	---	---	---	---
0	1	10165	10147.5	8	1268.4	**1.00**
5	2	20257	20239.5	8	2529.9	**1.99**
10	3	25046	25028.5	8	3128.6	**2.47**
25	4	56701	56683.5	8	7085.4	**5.59**

1 = Control Group
2-4 = Test Group
[a)] = The mean value was taken from the figures BG I and BG II
[b)] = Since the lymph nodes of the animals of a dose group were pooled, DPM/node was determined by dividing the measured value by the number of lymph nodes pooled

Calculation of the EC3 value:

	Test item concentration %	S.I.
Test Group 3	10 (a)	2.47 (b)
Test Group 4	25 (c)	5.59 (d)
EC3 = (a-c) [(3-d)/(b-d)] + c = 12.5% (w/v)		

a,b,c,d = Co-ordinates of the two pairs of data lying immediately above and below the S.I. value of 3 on the LLNA dose response plot.

APPENDIX 2 Historical Positive Control Data

These values represent historical control data of the last 10 periodic positive control experiments.

Positive Control Substance	Date	Concentration / Vehicle	S.I. values
alpha- Hexyl-cinnamaldehyde	April 2020	25% in acetone:olive oil (4+1 v/v)	5.59
	November 2019*		8.19
	April 2019		8.10
	October 2018		10.33
	April 2018		9.85
	October 2017		5.7
	April 2017		10.1
	October 2016		11.8
	April 2016		7.8
	October 2015		17.6

*the experiment was performed approximately 2 weeks in delay of the usual schedule, due to difficulties concerning the supply of ^{3}HTdR

APPENDIX 3 Copy of GLP Certificate

HESSEN

Gute Laborpraxis/Good Laboratory Practice

GLP-Bescheinigung/Statement of GLP Compliance
(gemäß/according to § 19b Abs. 1 Chemikaliengesetz)

Eine GLP-Inspektion zur Überwachung der Einhaltung der GLP-Grundsätze gemäß Chemikaliengesetz bzw. Richtlinie 2004/9/EG wurde durchgeführt in

Assessment of conformity with GLP according to Chemikaliengesetz and Directive 2004/9/EEC at:

☒ Prüfeinrichtung/Test facility ☐ Prüfstandort/Test site

ICCR-Roßdorf GmbH
Institute for Competent Contract Research
In den Leppsteinswiesen 19
64380 Roßdorf

(Unverwechselbare Bezeichnung und Adresse/Unequivocal name and adress)

Prüfungen nach Kategorien/Areas of Expertise
(gemäß/according ChemVwV-GLP Nr. 5.3/OECD guidance)

2 Prüfungen zur Bestimmung der toxikologischen Eigenschaften	**2** Toxicity studies
3 Prüfungen zur Bestimmung der erbgutverändernden Eigenschaften (in vitro und in vivo)	**3** Mutagenicity studies
8 Analytische Prüfungen an biologischen Materialien	**8** Analytical and clinical chemistry testing

22.11.2018, 21.02.2019, 12. bis 14.03.2019
Datum der Inspektion/Date of Inspection
(Tag Monat Jahr/day month year)

Die genannte Prüfeinrichtung befindet sich im nationalen GLP-Überwachungsverfahren und wird regelmäßig auf Einhaltung der GLP-Grundsätze überwacht.

The above mentioned test facility is included in the national GLP Compliance Programme and is inspected on a regular basis.

Auf der Grundlage des Inspektionsberichtes wird hiermit bestätigt, dass in dieser Prüfeinrichtung die oben genannten Prüfungen unter Einhaltung der GLP- Grundsätze durchgeführt werden können.

Based on the inspection report it can be confirmed, that this test facility is able to conduct the aforementioned studies in compliance with the Principles of GLP.

Im Auftrag

Brandt

Dr. Astrid Brandt, Referentin, Wiesbaden, den **23. Oktober 2019**
(Name und Funktion der verantwortlichen Person/
Name and function of responsible person)

HESSISCHES MINISTERIUM FÜR UMWELT, KLIMASCHUTZ, LANDWIRTSCHAFT UND VERBRAUCHERSCHUTZ

Hessisches Ministerium für Umwelt, Klimaschutz, Landwirtschaft und Verbraucherschutz,
Mainzer Straße 80, D 65189 Wiesbaden
(Name und Adresse der GLP-Überwachungsbehörde/Name and address of the GLP Monitoring Authority)

English name and address of the GLP Monitoring Authority: Hessian Ministry of Environment, Climate Prodection, Agriculture and Consumer Protection; Department II 10; P.O. Box 31 09; 65189 Wiesbaden
Translation of stamp inscription:
Hessian Ministry for Environment, Area for Agricultural Use and Consumer Protection

APPENDIX 4 Certificate of Analysis

syngenta

Syngenta Crop Protection AG
GLP Testing Facility WMU
Analytical Development & Product Chemistry
Breitenloh 5
4333 Münchwilen, Switzerland

Certificate of Analysis

A23220A

isocycloseram/emamectin benzoate SC (200/050)

TSC002-041-001

Batch Identification	TSC002-041-001
Other Batch ID	1122866
Product Code	**A23220A**
Other Product Code(s)	isocycloseram/emamectin benzoate SC (200/050)
Chemical Analysis (Active Ingredient content)	
- **Identity of the Active Ingredient(s)***	**confirmed**
- **Content of isocycloseram***	**17.5 % w/w corresponding to 201 g/l**
- **Content of emamectin benzoate***	**4.18 % w/w corresponding to 48.1 g/l**
	The Active Ingredient(s) content is within the FAO limits.
Methodology used for Characterization / Recertification	LC, chiral LC, oscillating density meter
Physical Analysis	
- **Appearance**	**brown liquid**
- **Density***	**1150 kg/m³**
Stability:	
- **Storage Temperature**	**< 30°C**
- **Recertification Date**	**End of January 2023**

If stored under the conditions given above, this test substance can be considered stable until the recertification date is reached.
This Certificate of Analysis summarizes data which originates either from a single study or from several individual studies. Tests marked with an asterisk (*) have been conducted in compliance with GLP.
Raw data, documentation, study plans, any amendments to study plans and reports pertaining to this/these study/studies are stored under the study number(s) referenced below within the archives of the GLP Testing Facility WMU at Syngenta Crop Protection AG, Switzerland

Study number of batch characterization: CHMU200180
Study number(s) of batch recertification:

Authorization: 19-Feb-2020

Dr. Karine Heintz
Analytical Development & Product Chemistry

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