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**8(e) Submission: Bicyclo 2.2.1 hept-2-ene, 5-(2-phenylethyl)-, CAS No.717099-35-9; LVE/L05-245; PMN/P06-11: Skin Sensitization: Local Lymph Node Assay in the Mouse**

Dear 8(e) Coordinator:

This letter is to inform you of the results of a Skin Sensitization Local Lymph Node Assay in the Mouse (LLNA), OECD No. 429, which is a test of Skin Sensitization.

The LLNA showed the substance to be a sensitizer under the conditions of the test. The data are being reported in accordance with EPA's 8(e) Reporting Guide (1991).

The report is attached herein. If I can be of any assistance, please contact me at 440-922-1517.

Yours very truly,

Jean Rhodes, Ph. D.  
Environmental Regulatory Expert

Product Stewardship Files/FedEx No. 798224621473



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**PENB:**

**LOCAL LYMPH NODE ASSAY IN THE MOUSE**

**PROJECT NUMBER: 1518/0073**

**AUTHOR:** A Sanders

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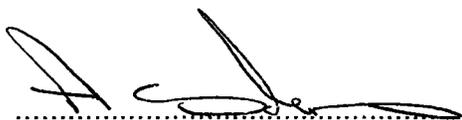
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**GLP COMPLIANCE STATEMENT**

The work described was performed in compliance with UK GLP standards (Schedule 1, Good Laboratory Practice Regulations 1999 (SI 1999/3106 as amended by SI 2004/0994)). These Regulations are in accordance with GLP standards published as OECD Principles on Good Laboratory Practice (revised 1997, ENV/MC/CHEM(98)17); and are in accordance with, and implement, the requirements of Directives 2004/9/EC and 2004/10/EC.

This report fully and accurately reflects the procedures used and data generated.



DATE: 23 NOV 2009

A Sanders  
Study Director

This report may be presented in final form as a digital (pdf) document. Such documents are prepared by scanning the paper original, and are considered of equivalent integrity and authenticity to versions produced by optical photocopy. However, in all cases the hand-signed paper original, held in secure archives, is the definitive document.

**PENB:****LOCAL LYMPH NODE ASSAY IN THE MOUSE****SUMMARY**

**Introduction.** A study was performed to assess the skin sensitisation potential of the test material in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to meet the requirements of the following:

- OECD Guideline for the Testing of Chemicals No. 429 "Skin Sensitisation: Local Lymph Node Assay" (adopted 24 April 2002)
- Method B42 Skin Sensitisation (Local Lymph Node Assay) of Commission Regulation (EC) No. 440/2008
- United States Environmental Protection Agency Health Effects Test Guidelines OPPTS 870.2600 Skin Sensitisation March 2003

**Methods.** Following a preliminary screening test in which no clinical signs of toxicity were noted at a concentration of 100%, this concentration was selected as the highest dose investigated in the main test of the Local Lymph Node Assay. Three groups, each of five animals, were treated with 50 µl (25 µl per ear) of the undiluted test material or the test material as a solution in acetone/olive oil 4:1 at concentrations of 50% or 25% v/v. A further group of five animals was treated with acetone/olive oil 4:1 alone.

One animal treated with the undiluted test material was humanely killed, pre-dose on Day 2, due to the occurrence of clinical signs of toxicity that approached the moderate severity limit set forth in the UK Home Office Project Licence.

**PENB:****LOCAL LYMPH NODE ASSAY IN THE MOUSE****1. INTRODUCTION**

A study was performed to assess the skin sensitisation potential of the test material in the CBA/Ca strain mouse following topical application to the dorsal surface of the ear. The method was designed to meet the requirements of the following:

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The assay has undergone extensive inter-laboratory validation and has been shown to reliably detect test materials that are moderate to strong sensitisers.

The strain of mouse used in these laboratories has been shown to produce satisfactory responses using known sensitisers and non-sensitisers during the in-house validation. The results of routine positive control studies are shown in Appendix 1 and Appendix 2. The results of the study are believed to be of value in predicting the sensitisation potential of the test material to man.

The study was performed between 15 October 2009 and 28 October 2009.

The animals were individually housed in suspended solid-floor polypropylene cages furnished with softwood woodflakes. Free access to mains tap water and food (2014 Teklad Global Rodent diet supplied by Harlan Teklad, Blackthorn, Bicester, Oxon, UK) was allowed throughout the study.

The temperature and relative humidity were controlled to remain within target ranges of 19 to 25°C and 30 to 70%, respectively. Any occasional deviations from these targets were considered not to have affected the purpose or integrity of the study. The rate of air exchange was approximately fifteen changes per hour and the lighting was controlled by a time switch to give twelve hours continuous light (06.00 to 18.00) and twelve hours darkness.

The animals were provided with environmental enrichment items which were considered not to contain any contaminant of a level that might have affected the purpose or integrity of the study.

### **3.2 Procedure**

Animals in which any adverse effects were noted that were considered to approach the moderate severity limit set forth in the UK Home Office Project Licence, were humanely killed.

#### **3.2.1 Preliminary Screening Test**

Using available information regarding the systemic toxicity/irritancy potential of the test material, a preliminary screening test was performed using one mouse. The mouse was treated by daily application of 25 µl of the undiluted test material to the dorsal surface of each ear for three consecutive days (Days 1, 2, 3). The mouse was observed twice daily on Days 1, 2 and 3 and once daily on Days 4, 5 and 6. Any signs of toxicity or excessive local irritation noted during this period were recorded. The bodyweight was recorded on Day 1 (prior to dosing) and on Day 6.

### 3.2.2.4 Terminal Procedures

**Termination:** Five hours following the administration of  $^3\text{HTdR}$  the surviving mice were killed by carbon dioxide asphyxiation followed by cervical separation. For each individual animal of each group the draining auricular lymph nodes were excised and processed. For each individual animal 1 ml of PBS was added to the lymph nodes.

**Preparation of Single Cell Suspension:** A single cell suspension of the lymph node cells for each individual animal was prepared by gentle mechanical disaggregation through a 200-mesh stainless steel gauze. The lymph node cells were rinsed through the gauze with 4 ml of PBS into a petri dish labelled with the project number and dose concentration. The lymph node cells suspension was transferred to a centrifuge tube. The petri dish was washed with an additional 5 ml of PBS to remove all remaining lymph node cells and these were added to the centrifuge tube. The lymph node cells were pelleted at 1400 rpm (approximately 190 g) for ten minutes. The pellet was resuspended in 10 ml of PBS and re-pelleted. To precipitate out the radioactive material, the pellet was resuspended in 3 ml of 5% Trichloroacetic acid (TCA).

**Determination of  $^3\text{HTdR}$  Incorporation:** After approximately eighteen hours incubation at approximately 4°C, the precipitates were recovered by centrifugation at 2100 rpm (approximately 450 g) for ten minutes, resuspended in 1 ml of TCA and transferred to 10 ml of scintillation fluid (Optiphase 'Trisafe').  $^3\text{HTdR}$  incorporation was measured by  $\beta$ -scintillation counting. The "Poly Q<sup>TM</sup>" vials containing the samples and scintillation fluid were placed in the sample changer of the scintillator and left for approximately twenty minutes. The purpose of this period of time in darkness was to reduce the risk of luminescence, which has been shown to affect the reliability of the results. After approximately twenty minutes, the vials were shaken vigorously. The number of radioactive disintegrations per minute was then measured using the Beckman LS6500 scintillation system (Beckman Instruments Inc, Fullerton, CA, USA).

### 3.3 Statistical Analysis

Data was processed to give group mean values for disintegrations per minute and standard deviations where appropriate. Individual and group mean disintegrations per minute values were assessed for dose response relationships by analysis of homogeneity of variance followed by one way analysis of variance (ANOVA). In the

## 5. RESULTS

### 5.1 Preliminary Screening Test

Clinical observations, bodyweight and mortality data are given in Table 1.

No signs of systemic toxicity were noted.

Based on this information the undiluted test material and the test material at concentrations of 50% and 25% v/v in acetone/olive oil 4:1 were selected for the main test.

### 5.2 Main Test

#### 5.2.1 Estimation of the Proliferative Response of Lymph Node Cells

The radioactive disintegrations per minute per lymph nodes for each individual animal and the stimulation index are given in Table 2.

The Stimulation Index expressed as the mean radioactive incorporation for each treatment group divided by the mean radioactive incorporation of the vehicle control group are as follows:

| Concentration (% v/v) in acetone/olive oil 4:1 | Stimulation Index | Result   |
|--|-------------------|----------|
| 25   | 5.48              | Positive |
| 50   | 8.19              | Positive |
| 100  | 13.40             | Positive |

#### 5.2.2 Clinical Observations and Mortality Data

Individual clinical observations and mortality data for test and control animals are given in Table 3.

One animal treated with the undiluted test material was humanely killed, pre-dose on Day 2, due to the occurrence of clinical signs of toxicity that approached the moderate severity limit set forth in the UK Home Office Project Licence. Signs of systemic toxicity

## PENB : LOCAL LYMPH NODE ASSAY IN THE MOUSE

**Table 1            Clinical Observations, Bodyweight and Mortality Data –  
Preliminary Screening Test**

| Concentration | Animal Number | Bodyweight (g) |       | Day      |           |          |           |          |           |   |   |   |
|---------------|---------------|----------------|-------|----------|-----------|----------|-----------|----------|-----------|---|---|---|
|               |               |                |       | 1        |           | 2        |           | 3        |           | 4 | 5 | 6 |
|               |               | Day 1          | Day 6 | Pre-Dose | Post Dose | Pre-Dose | Post Dose | Pre-Dose | Post Dose |   |   |   |
| 100           | S-1           | 18             | 19    | 0        | 0         | 0        | 0         | 0        | 0         | 0 | 0 | 0 |

0 = No signs of systemic toxicity

## PENB : LOCAL LYMPH NODE ASSAY IN THE MOUSE

Table 3 Individual Clinical Observations and Mortality Data

| Concentration<br>(% v/v) in<br>acetone/olive oil<br>4:1 | Animal<br>Number | Day 1        |              | Day 2                     |              | Day 3        |              | Day<br>4 | Day<br>5 | Day<br>6 |
|---|------------------|--------------|--------------|---------------------------|--------------|--------------|--------------|----------|----------|----------|
|   |                  | Pre-<br>Dose | Post<br>Dose | Pre-<br>Dose              | Post<br>Dose | Pre-<br>Dose | Post<br>Dose |          |          |          |
| Vehicle   | 1-1              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 1-2              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 1-3              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 1-4              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 1-5              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
| 25  | 2-1              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 2-2              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 2-3              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 2-4              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 2-5              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
| 50  | 3-1              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 3-2              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 3-3              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 3-4              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 3-5              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
| 100   | 4-1              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 4-2              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 4-3              | 0            | 0            | HLPt<br>PRl<br>HoDh<br>∇X |              |              |              |          |          |          |
|   | 4-4              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |
|   | 4-5              | 0            | 0            | 0                         | 0            | 0            | 0            | 0        | 0        | 0        |

0 = No signs of systemic toxicity      H = Hunched posture      L = Lethargy      P = Pilo-erection  
 Ho = Hypothermia      Pt = Ptosis      Dh = Dehydration      RI = Laboured respiration  
 ∇ = Bodyweight loss noted (3 g) animal weighed 13 g  
 X = Animal humanely killed due to the occurrence of clinical signs of toxicity that approached the moderate severity limit set forth in the UK Home Office Project Licence

## PENB : LOCAL LYMPH NODE ASSAY IN THE MOUSE

**Appendix 1 Current Positive Control Study for the Local Lymph Node Assay**

**Introduction.** A study was performed to assess the sensitivity of the strain of mouse used at these laboratories to a known sensitiser. The methodology for the LLNA is detailed in the OECD Guideline for the Testing of Chemicals, No. 429, and Method B.42 of Commission Regulation (EC) No. 440/2008. The study described in this document is based on these test methods but has been refined in order to reduce the number of animals required. The reduced LLNA (rLLNA) has been endorsed by the non-Commission members of the European Centre for the Validation of Alternative Method (ECVAM) Scientific Advisory Committee (ESAC) at its 26<sup>th</sup> meeting held on 26 – 27 April 2007 at ECVAM, Ispra, Italy.

Test Material:  $\alpha$ -Hexylcinnamaldehyde, tech., 85%  
Project number: 0039/1107  
Study dates: 11 September 2009 to 17 September 2009

**Methods.** A group of five animals was treated with 50  $\mu$ l (25  $\mu$ l per ear) of  $\alpha$ -Hexylcinnamaldehyde, tech., 85% as a solution in acetone/olive oil 4:1 at a concentration of 15% v/v. A further control group of five animals was treated with acetone/olive oil 4:1 alone. The control group was shared with Harlan project number 0673/0012.

**Results.** The Stimulation Index expressed as the mean radioactive incorporation for the treatment group divided by the mean radioactive incorporation of the vehicle control group is as follows:

| Concentration (% v/v) in acetone/olive oil 4:1 | Stimulation Index | Result   |
|--|-------------------|----------|
| 15   | 3.70              | Positive |

**Conclusion.**  $\alpha$ -Hexylcinnamaldehyde, tech., 85% was considered to be a sensitiser under the conditions of the test.

## PENB : LOCAL LYMPH NODE ASSAY IN THE MOUSE

## Appendix 3 Vehicle Determination Record

| Vehicle                    | Concentration                                   | Method of Preparation | Description of Formulation | Suitability*        |
|----------------------------|---|-----------------------|----------------------------|---------------------|
| acetone/olive oil<br>(4:1) | 50%<br>0.5 ml test material<br>+ 0.5 ml vehicle | shaken                | solution                   | suitable for dosing |

\* = Suitable for dosing if formulation is a solution or fine homogenous suspension which can be administered via a micropipette

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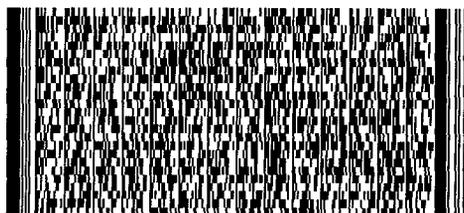
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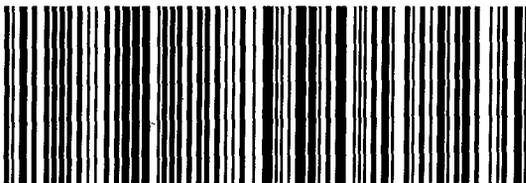


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