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C6O4 CYCLIC
BACTERIAL MUTATION ASSAY
(*S. typhimurium* and *E. coli*)

FINAL REPORT

RTC Study no.: 76250

Sponsor:

SOLVAY SOLEXIS S.p.A.
Viale Lombardia, 20
20021 Bollate (MI)
Italy

Total number of pages: 52

Commercial Office

RTC S.p.A.
Via Tito Speri, 12/14
00040 Pomezia (Roma) - ITALY
Tel.: +39.06.91095.1 - Fax: +39.06.910.5737
www.rtc.it - e-mail: mkt@rtc.it

Head Office and Administration

RTC S.p.A.
Via Tito Speri, 12/14
00040 Pomezia (Roma) - ITALY
Tel.: +39.06.91095.1
Fax: +39.06.912.2233

RTC S.p.A.
Capitale Sociale Euro 5.164.000
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C6O4 CYCLIC
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(*S. typhimurium* and *E. coli*)

RTC Study no.: 76250

FINAL REPORT

I, the undersigned, was responsible for the preparation of this report.

[redacted]. [redacted] Date: [redacted]
(Study Director)

COMPLIANCE STATEMENT

We, the undersigned, hereby declare that the following report constitutes a true and faithful account of the procedures adopted, and of the results obtained in the performance of the study. The aspects of the study conducted by Research Toxicology Centre S.p.A. were performed in accordance with:

A. *Good laboratory practice for non clinical laboratory studies*, U.S. Food and Drug Administration, Code of Federal Regulations, 21 Part 58, 22 December 1978 and subsequent revisions.

B. Decreto Legislativo 2 March 2007 n. 50, *Attuazione delle direttive 2004/9/CE e 2004/10/CE, concernenti l'ispezione e la verifica della buona pratica di laboratorio (BPL) e il ravvicinamento delle disposizioni legislative, regolamentari ed amministrative relative all'applicazione dei principi di buona pratica di laboratorio e al controllo della loro applicazione per le prove sulle sostanze chimiche (G.U. 13 April 2007, Serie generale n. 86)* and subsequent revisions.

C. Directive 2004/10/EC of European Parliament and of the Council of 11 February 2004. *On the harmonisation of laws, regulations and administrative provisions relating to the application of the principles of good laboratory practice and the verification of their applications for tests on chemical substances.*

D. ENV/MC/CHEM(98)17 *OECD principles on Good Laboratory Practice (as revised in 1997).*

[redacted] [redacted] Date: [redacted]
(Study Director)

[redacted], Spec. Toxicology [redacted] Date: [redacted]
(Scientific Director)

QUALITY ASSURANCE STATEMENT
(Relevant to those aspects of the study conducted by RTC S.p.A.)

Study phases monitored by RTC's QAU According to current relevant Standard Operating Procedures	Quality Assurance Inspections (Day Month Year)		
	Inspection	Report to Study Director	Report to Company Management
PROTOCOL CHECK	19.03.2009	19.03.2009	19.03.2009
PROCESS-BASED INSPECTIONS RELATED TO THIS TYPE OF STUDY			
Dose preparation	11.02.2009	-	11.02.2009
Test execution	13.01.2009	-	16.01.2009

Other process-based inspections were carried out on routine activities not directly related to this type of study. The relevant documentation is kept on file although specific inspection dates are not reported here.
Associated laboratories and support functions are subject to regular facility inspections.

FINAL REPORT Review of this report by RTC's QAU found the reported methods and procedures to describe those used and the results to constitute an accurate representation of the recorded raw data.	Review completed [redacted]

[redacted]

(Head of Quality Assurance)

[redacted]

Date

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1. SUMMARY

1.1 The test item C6O4 CYCLIC was examined for the ability to induce gene mutations in tester strains of *Salmonella typhimurium* and *Escherichia coli,* as measured by reversion of auxotrophic strains to prototrophy. The five tester strains TA1535, TA1537, TA98, TA100 and WP2 *uvr*A were used. Experiments were performed both in the absence and presence of metabolic activation, using liver S9 fraction from rats pre-treated with phenobarbitone and betanaphthoflavone.

1.2 The test item was used as a solution in sterile distilled water.

1.3 The test item C6O4 CYCLIC was assayed in the toxicity test at a maximum dose level of 5000 µg/plate and at four lower concentrations spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. Concentrations are expressed in terms of active ingredient. No precipitation of the test item was observed at the end of the incubation period at any concentration. No toxicity was observed at any dose level with any tester strain.

1.4 In Main Assay I, using the plate incorporation method, the test item was assayed at the following dose levels expressed in terms of active ingredient: 5000, 2500, 1250, 625 and 313 µg/plate. No toxicity was observed at any dose level with any tester strain both in the absence and presence of S9 metabolic activation.

As no relevant increases in revertant numbers were observed at any concentration tested, a pre-incubation step was included for all treatments of Main Assay II. The test item was assayed at the same concentrations employed in Main Assay I.
Toxicity, as indicated by thinning of the background lawn and/or reduction in revertant numbers, was observed at higher dose levels with all tester strains, both in the absence and presence of S9 metabolic activation.

No precipitation of the test item was noted at the end of the incubation period at any concentration tested, in any experiment.

1.5 The test item did not induce two-fold increases in the number of revertant colonies in the plate incorporation or pre-incubation assay, at any dose level, in any tester strain, in the absence or presence of S9 metabolism.

1.6 It is concluded that the test item C6O4 CYCLIC does not induce reverse mutation in *Salmonella typhimurium* or *Escherichia coli* in the absence or presence of S9 metabolism, under the reported experimental conditions.

2. INTRODUCTION

2.1 Purpose

This report describes experiments performed to assess the mutagenic activity of the test item to *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100, and to *Escherichia coli* strain WP2 *uvr*A using the procedures developed by Ames *et al.*, 1975 and revised by Maron and Ames, 1983.

The study was designed to comply with the experimental methods indicated in:
- Test method B.13/14 'Reverse mutation test using bacteria' described in Council Regulation (EC) No. 440/2008.
- OECD Guideline for the testing of chemicals No. 471 (Adopted July 1997).
- ICH S2A Genotoxicity: Specific Aspects of Regulatory Tests, Step 5.

2.2 Principles of the method

Reverse mutation assays employ bacterial strains which are already mutant at a locus whose phenotypic effects are easily detected. The *Salmonella* tester strains have mutations causing dependence on a particular amino acid (histidine) for growth. The ability of test items to cause reverse mutations (reversions) to histidine-independence can easily be measured. The *E. coli* tester strains of the WP2 series are similarly mutant at the tryptophan locus.

Since many chemicals only demonstrate mutagenic activity after metabolism to reactive forms, in order to detect these "indirect mutagens" the test is performed in the presence and absence of a rat liver metabolising system.

2.3 Study organisation

Sponsor:

SOLVAY SOLEXIS S.p.A.
Viale Lombardia, 20
20021 Bollate (MI)
Italy

Location of Study:

Research Toxicology Centre S.p.A.
Genetic Toxicology, *in vitro* Toxicology and Immunology Department
Via Tito Speri, 12/14
00040 Pomezia (Rome)
Italy

Principal dates:

Study protocol approved by Study Director: 09-Mar-2009
Study commenced: 20-Mar-2009 (Toxicity assay treatment)
Study completed: 30-Mar-2009 (Completion of scoring Main Assay II)

Study Director:

[redacted]

Archiving:

The original data arising from this study, the original final protocol and a copy of the final report consigned will be stored in the archives of Research Toxicology Centre S.p.A. for a period of 3 years from the date of consignment of the report. At the completion of this period the Sponsor will be contacted for despatch or disposal of the material. An aliquot of the test item will be retained within the archives of the testing facility for a period of 10 years after which it will be destroyed.

3. MATERIALS AND METHODS

The methods used were in compliance with the attached Study Protocol (Addendum I).

3.1 Test item

Details of the test item received at RTC were as follows:

Name	:	C6O4 CYCLIC
Label name	:	cC6O4 (solution 16%)
Batch	:	150/28
Expiry date	:	31-Dec-2020
Received from	:	Solvay Solexis S.p.A.
Date received	:	05-Mar-2009
Amount received	:	337.94 ml
Description from the Sponsor	:	yellowish solution
Description at first use	:	yellowish liquid
Container	:	colourless glass bottle
Storage at RTC	:	room temperature
RTC reference number	:	11663

On 16 March 2009 and 27 March 2009 two sub-samples of 7 ml and 5ml, respectively, of the test item were transferred from the Formulation Unit to the Department of Genetic Toxicology, *in vitro* Toxicology and Immunology and stored under the same conditions. A certificate of analysis, supplied by the Sponsor, can be found in Addendum II of this report.

Solutions of the test item, as received, were prepared immediately before use in sterile distilled water. Solutions were prepared on a weight/volume basis. Concentrations were expressed in terms of active ingredient. All test item solutions were used within 2 hours and 21 minutes of the initial formulation. No assay of test item stability, nor its concentration and homogeneity in solvent were undertaken. All dose levels in this report are expressed to three significant figures.

3.2 Control items

The solvents used in this study were:
Sterile distilled water (Bieffe Medital, batch 08G2803).
Dimethylsulfoxide (DMSO) (Fluka AG, batch 1331319 30907P11).

Positive control treatments used solutions prepared as follows:

Sodium azide (Moltox, Inc., batch 6289SA) in distilled water.
9-Aminoacridine (9-AA.HCl, Moltox, Inc., batch 1004AAHC) in DMSO.
2-Nitrofluorene (Moltox, Inc., batch 2210NF) in DMSO.
2-Aminoanthracene (Moltox, Inc., batch 6306AA and Sigma, batch 58F-3462) in DMSO.
Methylmethanesulphonate (MMS) (Sigma, batch 018K3765) in distilled water.

Positive control treatments are indicated in the following table:

Tester strain	*Absence of S9*	*Presence of S9*
TA1535	sodium azide 1 μg/plate	2-aminoanthracene 1 μg/plate
TA100	sodium azide 1 μg/plate	2-aminoanthracene 1 μg/plate (2 μg/plate)
TA1537	9-amino-acridine 50 μg/plate	2-aminoanthracene 1 μg/plate
TA98	2-nitrofluorene 2 μg/plate	2-aminoanthracene 1 μg/plate (2 μg/plate)
WP2 *uvr*A	methylmethanesulphonate 500 μg/plate	2-aminoanthracene 10 μg/plate (20 μg/plate)

Concentrations refer to both treatment methods. When two values are given, the figures in brackets refer to the pre-incubation method assay.

3.3 Media

The following growth media were used:

Nutrient Broth: Oxoid Nutrient Broth No 2 was prepared at a concentration of 2.5% in distilled water and autoclaved prior to use.
This was used for the preparation of liquid cultures of the tester strains.

Nutrient Agar: Oxoid Nutrient Broth No 2 (25g) and Difco Bacto-agar (15g) were added to distilled water (1 litre) and autoclaved.
The solutions were then poured into 9 cm plastic Petri dishes and allowed to solidify and dry before use. These plates were used for the non-selective growth of the tester strains.

Minimal Agar: Minimal medium agar was prepared as 1.5% Difco Bacto-agar in Vogel-Bonner Medium E, with 2% Glucose, and poured into 9 cm plastic Petri dishes.

Top Agar: "Top Agar" (overlay agar) was prepared as 0.6% Difco Bacto-agar + 0.5% NaCl in distilled water. Prior to use 10 ml of a sterile solution of 0.5 mM Biotin + 0.5 mM Histidine (or 0.5 mM tryptophan) was added to the top agar (100 ml).

3.4 S9 tissue homogenate

Two MOLTOX rat S9 liver tissue fractions were used in this study had the following characteristics:

Lot Number	:	2273
Inducing Agents	:	Phenobarbital – 5,6-Benzoflavone
Preparation date	:	01-May-2008
Expiry date	:	01-May-2010
Received from	:	MOLTOX, Molecular Toxicology, Inc.
Date received	:	05-Dec-2008
Storage at RTC	:	Approximately -80°C
Strain	:	Sprague Dawley
Sex of donors	:	Male
Protein content	:	37.6 mg/ml

Lot Number	:	2378
Inducing Agents	:	Phenobarbital – 5,6-Benzoflavone
Preparation date	:	18-Feb-2009
Expiry date	:	18-Feb-2011
Received from	:	MOLTOX, Molecular Toxicology, Inc.
Date received	:	24-Mar-2009
Storage at RTC	:	Approximately -80°C
Strain	:	Sprague Dawley
Sex of donors	:	Male
Protein content	:	37.8 mg/ml

Production and quality control certificates can be found in Addendum III of this report

The mixture of S9 tissue fraction and cofactors (S9 mix) was prepared as follows (for each 10 ml):

S9 tissue fraction	1.0 ml
NADP (100 mM)	0.4 ml
G-6-P (100 mM)	0.5 ml
KCl (330 mM)	1.0 ml
MgCl2 (100 mM)	0.8 ml
Phosphate buffer (pH 7.4, 200 mM)	5.0 ml
Distilled Water	1.3 ml
	10.0 ml

3.5 Bacterial strains

Four strains of *Salmonella typhimurium* (TA1535, TA1537, TA98 and TA100) and a strain of *Escherichia coli* (WP2 *uvr*A) were used in this study.

TA1535 and TA100 are predominantly sensitive to base pair mutagens, TA1537 and TA98 are sensitive to frameshift mutagens. In addition to a mutation in the histidine operon, the *Salmonella* tester strains contain additional mutations which enhance their sensitivity to some mutagenic compounds. The *rfa* wall mutation results in the loss of one of the enzymes responsible for the synthesis of part of the lipopolysaccharide barrier that forms the surface of the bacterial cell wall and increases permeability to certain classes of chemicals. All strains are deficient in a DNA excision repair system (*uvr*B mutation) which enhances the sensitivity to some mutagens. TA98 and TA100 strains contain the pKM101 plasmid which activates an error prone DNA repair system.

Tester strain WP2 *uvr*A is reverted from tryptophan dependence (auxotrophy) to tryptophan independence (prototrophy) bay base substitution mutagens. In addition to the mutation in the tryptophan operon, the tester strain contains an *uvr*A DNA repair deficiency which enhance its sensitivity to some mutagenic compounds.

Permanent stocks of these strains are kept at -80°C in RTC. Overnight subcultures of these stocks were prepared for each day's work.
Bacteria were taken from vials of frozen cultures, which had been checked for the presence of the appropriate genetic markers, as follows:

Histidine requirement	:	No Growth on Minimal plates + Biotin. Growth on Minimal plates + Biotin + Histidine.
Tryptophan requirement	:	No Growth on Minimal agar plates Growth on Minimal plates + Tryptophan.
*uvr*A, *uvr*B	:	Sensitivity to UV irradiation.
rfa	:	Sensitivity to Crystal Violet.
pKM101	:	Resistance to Ampicillin.

Bacterial cultures in liquid and on agar were clearly identified with their identity.

3.6 Methods

3.6.1 Preliminary toxicity test

A preliminary toxicity test was undertaken in order to select the concentrations of the test item to be used in the main assays. In these tests a wide range of dose levels of the test item, set at half-log intervals, were used. Treatments were performed both in the absence and presence of S9 metabolism using the plate incorporation method; a single plate was used at each test point and positive controls were not included. Toxicity was assessed on the basis of a decline in the number of spontaneous revertants, a thinning of the background lawn or a microcolony formation.

3.6.2 Main experiments

Two experiments were performed including negative and positive controls in the absence and presence of an S9 metabolising system. Three replicate plates were used at each test point.

In addition, plates were prepared to check the sterility of the test item solutions and the S9 mix, and dilutions of the bacterial cultures were plated on nutrient agar plates to establish the number of bacteria in the cultures.

The first experiment was performed using a plate-incorporation method. The components of the assay (the tester strain bacteria, the test item and S9 mix or phosphate buffer) were added to molten overlay agar and vortexed. The mixture was then poured onto the surface of a minimal medium agar plate, and allowed to solidify prior to incubation.

The overlay mixture was composed as follows:

(i)	Overlay agar (held at 45°C)	2	ml
(ii)	Test or control item solution	0.1	ml
(iii)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5	ml
(iv)	Bacterial suspension	0.1	ml

The second experiment was performed using a pre-incubation method.
The components were added in turn to an empty test-tube:

(i)	Bacterial suspension	0.1	ml
(ii)	Test item solution	0.1	ml
	or control item solution	0.05	ml
(iii)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5	ml

The incubate was vortexed and placed at 37°C for 30 minutes. Two ml of overlay agar was then added and the mixture vortexed again and poured onto the surface of a minimal medium agar plate and allowed to solidify.

3.6.3 Incubation and scoring

The prepared plates were inverted and incubated for approximately 72 hours at 37°C. After this period of incubation, the scoring was effected by counting the number of revertant colonies on each plate.

4. RESULTS

4.1 Sterility test

Since the test item is an aqueous sample, a preliminary sterility test was performed. An aliquot of 500 µl of the test item, as supplied, was added to 4.5 ml of nutrient broth and incubated at 37°C for about 24 hours before evaluating the outcome of the test. Based on the results obtained it was not considered necessary to filter the test item before use in any experimental procedure.

4.2 Toxicity test

The test item C6O4 CYCLIC was assayed in the toxicity test at a maximum dose level of 5000 µg/plate and at four lower concentrations spaced at approximately half-log intervals: 1580, 500, 158 and 50.0 µg/plate. Concentrations are expressed in terms of active ingredient. Results are presented in Tables 1 and 2.

No precipitation of the test item was noted at the end of the incubation period at any concentration. No toxicity was observed at any dose level with any tester strain, in the absence or presence of S9 metabolic activation. On the basis of these results a maximum concentration of 5000 µg/plate was selected for the Main Assay.

4.3 Assay for reverse mutation

Two experiments were performed; individual plate counts for these tests, and the mean and standard error of the mean for each test point, together with statistical analysis are presented in Tables 3 to 12.

In Main Assay I, using the plate incorporation method, the test item was assayed at the following dose levels expressed in terms of active ingredient: 5000, 2500, 1250, 625 and 313 µg/plate. No relevant toxicity was observed with any tester strain at any dose level.

As no relevant increases in revertant numbers were observed at any concentration tested, a pre-incubation step was included for all treatments of Main Assay II. The test item was assayed at the same dose levels as in Main Assay I. Toxicity, as indicated by thinning of the background lawn and/or reduction in revertant numbers, was observed at the higher dose levels with all tester strains, both in the absence and presence of S9 metabolic activation.

No precipitation of the test item was noted at the end of the incubation period at any concentration tested.

No increases in the number of revertant colonies were observed in the plate incorporation or pre-incubation assay, at any dose level, in any tester strain, in the absence or presence of S9 metabolism.

The sterility of the S9 mix and of the test item solutions was confirmed by the absence of colonies on additional agar plates spread separately with these solutions. Marked increases in revertant numbers were obtained in these tests following treatment with the positive control items, indicating that the assay system was functioning correctly.

5. ANALYSIS OF RESULTS

5.1 Criteria for outcome of the assays

For the test item to be considered mutagenic, two-fold (or more) increases in mean revertant numbers must be observed at two consecutive dose levels or at the highest practicable dose level only. In addition, there must be evidence of a dose-response relationship showing increasing numbers of mutant colonies with increasing dose levels.

5.2 Evaluation

Results show that mean plate counts for untreated and positive control plates fell within the normal historical range included in Appendices 1 and 2. The estimated numbers of viable bacteria/plate (titre) fell in the range of 100 - 500 million for each strain. No plates were lost through contamination or cracking. The study was accepted as valid.

The test item does not induce two-fold increases in the number of revertant colonies, at any dose level, in any tester strain, in the absence or presence of S9 metabolism. On the basis of the stated criteria it must be concluded that the test item C6O4 CYCLIC is not mutagenic to *S. typhimurium* or *E. coli* under the reported experimental conditions.

6. CONCLUSION

It is concluded that the test item C6O4 CYCLIC does not induce reverse mutation in *Salmonella typhimurium* or *Escherichia coli* in the absence or presence of S9 metabolism, under the reported experimental conditions.

7. KEY TO TABLES 1-12

7.1 Structure of Tables 3-12

These tables show, for each *Salmonella typhimurium* or *Escherichia coli* tester strain, the individual plate counts obtained for the negative and positive controls, and at each dose level of the test item. The mean number of revertant colonies and standard error of the mean are also presented. The "untreated" plates received no treatment. The titre of the bacterial cultures is given (million cells/plate).

7.2 Regression line

i) The regression analysis fits a regression line to the data by the least squares method, after square root transformation of the plate counts to satisfy normal distribution and homoscedasticity assumptions. The regression equation is expressed as:

$y = a + bx$

where y = transformed revertant numbers
a = intercept
b = slope value
x = dose level (in the units given).

ii) The regression line includes the untreated control data.

iii) Regression lines are calculated using a minimum of the three lowest dose levels, and then including the further dose levels in turn. The correlation co-efficient (r), the value of students "t" statistic, and the p-value for the regression lines are also given.

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 1 - Toxicity test without metabolic activation

STUDY NO.: 76250

SOLVENT: Sterile distilled water

EXPERIMENT: Toxicity test

Dose level (µg/plate)	TA-1535 Rev/pl.	TA-1537 Rev/pl.	TA-98 Rev/pl.	TA-100 Rev/pl.	WP2 *uvr*A Rev/pl.
Untreated	25	20	32	151	25
50.0	25	24	30	156	28
158	23	22	34	144	28
500	26	17	31	151	34
1580	27	21	36	140	28
5000	23	16	35	149	27

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 2 - Toxicity test with metabolic activation

STUDY NO.: 76250

SOLVENT: Sterile distilled water

EXPERIMENT: Toxicity test

Dose level (µg/plate)	TA-1535 Rev/pl.	TA-1537 Rev/pl.	TA-98 Rev/pl.	TA-100 Rev/pl.	WP2 *uvr*A Rev/pl.
Untreated	22	27	47	164	34
50.0	19	22	43	162	38
158	21	22	47	166	34
500	24	23	50	175	35
1580	18	25	46	173	35
5000	21	27	40	162	37

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 3 - Experiment 1 - Plate incorporation method - TA1535

STUDY NO.: 76250

SOLVENT: Sterile distilled water

Strain: TA1535 Titre: 252

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	17	12	18	16	1.9	14	21	20	18	2.2
313	18	17	18	18	0.3	19	21	19	20	0.7
625	18	21	19	19	0.9	16	25	20	20	2.6
1250	23	21	26	23	1.5	19	19	19	19	0.0
2500	19	19	24	21	1.7	21	24	26	24	1.5
5000	19	28	20	22	2.8	23	25	21	23	1.2

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	3.954	0.0007	0.64771	2.2493	0.05926
1 - 4	-	3.961	0.0007	0.83070	4.7186	0.00082
1 - 5	-	4.164	0.0002	0.54765	2.3600	0.03458
1 - 6	-	4.252	0.0001	0.48831	2.2383	0.03978
1 - 3	+	4.284	0.0004	0.27142	0.7461	0.47990
1 - 4	+	4.359	0.0001	0.08088	0.2566	0.80269
1 - 5	+	4.294	0.0002	0.52489	2.2234	0.04454
1 - 6	+	4.366	0.0001	0.53004	2.5003	0.02366

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	17	12	18	16	1.9
Sodium Azide	1 µg/pl	-	555	533	508	532	13.6
DMSO	100 µl/pl	+	17	17	19	18	0.7
2-Aminoanthracene	1 µg/pl	+	121	131	124	125	3.0

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 4 - Experiment 1 - Plate incorporation method - TA1537

STUDY NO.: 76250

SOLVENT: Sterile distilled water

Strain: TA1537 Titre: 229

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	15	20	12	16	2.3	19	23	21	21	1.2
313	15	22	20	19	2.1	24	25	26	25	0.6
625	26	15	24	22	3.4	19	20	22	20	0.9
1250	23	23	19	22	1.3	28	26	23	26	1.5
2500	21	17	13	17	2.3	17	19	28	21	3.4
5000	17	22	20	20	1.5	21	24	21	22	1.0

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	3.958	0.0011	0.53730	1.6855	0.13575
1 - 4	-	4.094	0.0005	0.50989	1.8744	0.09036
1 - 5	-	4.328	0.0000	0.00666	0.0240	0.98120
1 - 6	-	4.317	0.0000	0.07307	0.2931	0.77324
1 - 3	+	4.731	-0.0001	-0.11334	0.3018	0.77156
1 - 4	+	4.636	0.0003	0.43631	1.5334	0.15619
1 - 5	+	4.765	0.0000	-0.04490	0.1621	0.87375
1 - 6	+	4.763	0.0000	-0.08031	0.3223	0.75142

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	23	17	18	19	1.9
9-Aminoacridine	50 µg/pl	-	108	116	121	115	3.8
DMSO	100 µl/pl	+	28	23	22	24	1.9
2-Aminoanthracene	1 µg/pl	+	119	105	113	112	4.1

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 5 - Experiment 1 - Plate incorporation method - WP2uvrA

STUDY NO.: 76250

SOLVENT: Sterile distilled water

Strain: WP2*uvr*A Titre: 304

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	21	28	25	25	2.0	35	34	32	34	0.9
313	20	20	28	23	2.7	31	35	36	34	1.5
625	21	27	28	25	2.2	32	27	29	29	1.5
1250	23	28	31	27	2.3	29	35	33	32	1.8
2500	30	31	20	27	3.5	33	38	29	33	2.6
5000	25	28	19	24	2.6	28	33	30	30	1.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.876	0.0001	0.07522	0.1996	0.84749
1 - 4	-	4.834	0.0003	0.34752	1.1720	0.26837
1 - 5	-	4.897	0.0001	0.29491	1.1128	0.28595
1 - 6	-	4.995	0.0000	0.01307	0.0523	0.95895
1 - 3	+	5.875	-0.0006	-0.62924	2.1420	0.06943
1 - 4	+	5.758	-0.0001	-0.26638	0.8739	0.40266
1 - 5	+	5.695	0.0000	0.00892	0.0322	0.97482
1 - 6	+	5.724	0.0000	-0.23872	0.9833	0.34010

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	21	28	25	25	2.0
MMS	500 µg/pl	-	185	169	164	173	6.3
DMSO	100 µl/pl	+	27	27	33	29	2.0
2-Aminoanthracene	10 µg/pl	+	204	193	195	197	3.4

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 6 - Experiment 1 - Plate incorporation method - TA98

STUDY NO.: 76250

SOLVENT: Sterile distilled water

Strain: TA98 Titre: 278

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	24	31	33	29	2.7	35	43	35	38	2.7
313	26	30	33	30	2.0	39	36	33	36	1.7
625	30	26	22	26	2.3	34	32	38	35	1.8
1250	33	35	31	33	1.2	40	37	42	40	1.5
2500	30	34	38	34	2.3	40	41	36	39	1.5
5000	31	34	35	33	1.2	45	46	41	44	1.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	5.468	-0.0005	-0.36065	1.0230	0.34034
1 - 4	-	5.288	0.0002	0.30198	1.0017	0.34010
1 - 5	-	5.299	0.0002	0.48961	2.0246	0.06396
1 - 6	-	5.385	0.0001	0.45801	2.0609	0.05595
1 - 3	+	6.126	-0.0004	-0.38130	1.0913	0.31129
1 - 4	+	5.994	0.0002	0.25503	0.8341	0.42372
1 - 5	+	6.017	0.0001	0.33136	1.2663	0.22763
1 - 6	+	6.000	0.0001	0.65894	3.5041	0.00294

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	100 µl/pl	-	33	30	32	32	0.9
2-Nitrofluorene	2 µg/pl	-	157	169	159	162	3.7
DMSO	100 µl/pl	+	36	43	39	39	2.0
2-Aminoanthracene	1 µg/pl	+	459	465	488	471	8.8

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 7 - Experiment 1 - Plate incorporation method - TA100

STUDY NO.: 76250

SOLVENT: Sterile distilled water

Strain: TA100 Titre: 267

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	150	168	157	158	5.2	150	171	178	166	8.4
313	161	151	154	155	3.0	181	157	167	168	7.0
625	157	169	147	158	6.4	176	165	161	167	4.5
1250	147	144	143	145	1.2	159	173	164	165	4.1
2500	149	156	160	155	3.2	170	166	161	166	2.6
5000	151	164	149	155	4.7	181	173	162	172	5.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	12.545	0.0000	-0.04005	0.1061	0.91851
1 - 4	-	12.638	-0.0004	-0.58866	2.3027	0.04405
1 - 5	-	12.485	-0.0001	-0.21556	0.7959	0.44038
1 - 6	-	12.438	0.0000	-0.07312	0.2933	0.77308
1 - 3	+	12.908	0.0001	0.04880	0.1293	0.90079
1 - 4	+	12.936	0.0000	-0.05952	0.1886	0.85422
1 - 5	+	12.928	0.0000	-0.07354	0.2659	0.79450
1 - 6	+	12.883	0.0000	0.18116	0.7368	0.47189

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	150	168	157	158	5.2
Sodium Azide	1 µg/pl	-	656	611	707	658	27.7
DMSO	100 µl/pl	+	165	173	171	170	2.4
2-Aminoanthracene	1 µg/pl	+	1352	1281	1181	1271	49.6

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 8 - Experiment 2 - Preincubation method - TA1535

STUDY NO.: 76250

SOLVENT: Sterile distilled water

Strain: TA1535 Titre: 264

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	16	23	16	18	2.3	17	20	15	17	1.5
313	16	19	20	18	1.2	23	25	27	25	1.2
625	17	25	20	21	2.3	21	21	18	20	1.0
1250	15	15	20	17	1.7	23	18	22	21	1.5
2500	13	12	16 *	14	1.2	17	14	14 *	15	1.0
5000	13	9	12 *	11	1.2	12	14	17 *	14	1.5

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.225	0.0004	0.31541	0.8794	0.40836
1 - 4	-	4.362	-0.0001	-0.18002	0.5787	0.57557
1 - 5	-	4.419	-0.0003	-0.58176	2.5789	0.02290
1 - 6	-	4.379	-0.0002	-0.75031	4.5399	0.00033
1 - 3	+	4.384	0.0005	0.32231	0.9008	0.39761
1 - 4	+	4.471	0.0001	0.18231	0.5863	0.57064
1 - 5	+	4.639	-0.0002	-0.49210	2.0382	0.06241
1 - 6	+	4.591	-0.0002	-0.63518	3.2895	0.00462

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	16	23	16	18	2.3
Sodium Azide	1 µg/pl	-	495	508	479	494	8.4
DMSO	50 µl/pl	+	19	17	20	19	0.9
2-Aminoanthracene	1 µg/pl	+	108	111	107	109	1.2

*: thinning of the background lawn

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 9 - Experiment 2 - Preincubation method - TA1537

STUDY NO.: 76250

SOLVENT: Sterile distilled water

Strain: TA1537 Titre: 233

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	15	21	18	18	1.7	17	26	25	23	2.8
313	23	23	25	24	0.7	24	25	27	25	0.9
625	20	24	17	20	2.0	24	30	28	27	1.8
1250	19	13	15	16	1.8	28	26	24	26	1.2
2500	11	8	7 *	9	1.2	17	25	23 *	22	2.4
5000	11	8	4 *	8	2.0	20	10	12 *	14	3.1

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.399	0.0004	0.29922	0.8297	0.43410
1 - 4	-	4.596	-0.0004	-0.41083	1.4250	0.18462
1 - 5	-	4.719	-0.0007	-0.82886	5.3418	0.00013
1 - 6	-	4.529	-0.0004	-0.82454	5.8293	0.00003
1 - 3	+	4.757	0.0008	0.55031	1.7438	0.12472
1 - 4	+	4.882	0.0003	0.36824	1.2525	0.23889
1 - 5	+	5.037	-0.0001	-0.23365	0.8664	0.40198
1 - 6	+	5.159	-0.0003	-0.72443	4.2035	0.00067

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	50 µl/pl	-	20	21	22	21	0.6
9-Aminoacridine	50 µg/pl	-	101	162	152	138	18.9
DMSO	50 µl/pl	+	27	27	25	26	0.7
2-Aminoanthracene	1 µg/pl	+	107	105	92	101	4.7

*: thinning of the background lawn

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 10 - Experiment 2 - Preincubation method - WP2uvrA

STUDY NO.: 76250

SOLVENT: Sterile distilled water

Strain: WP2*uvr*A Titre: 313

Dose-level [µg/pl]	Without metabolic activation: Plate counts			Mean	S. E.	With metabolic activation: Plate counts			Mean	S. E.
Untreated	18	21	25	21	2.0	31	31	32	31	0.3
313	30	32	30	31	0.7	27	27	26	27	0.3
625	27	24	27	26	1.0	33	35	27	32	2.4
1250	31	33	28	31	1.5	27	34	35	32	2.5
2500	22	24	23 *	23	0.6	24	30	22 *	25	2.4
5000	25	32	29 *	29	2.0	22	20	20 *	21	0.7

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	4.836	0.0008	0.46480	1.3889	0.20744
1 - 4	-	4.892	0.0006	0.59215	2.3237	0.04250
1 - 5	-	5.151	0.0000	-0.08198	0.2966	0.77148
1 - 6	-	5.095	0.0000	0.15178	0.6142	0.54770
1 - 3	+	5.450	0.0000	0.03096	0.0819	0.93699
1 - 4	+	5.418	0.0002	0.24987	0.8161	0.43348
1 - 5	+	5.559	-0.0002	-0.38595	1.5084	0.15536
1 - 6	+	5.590	-0.0002	-0.74254	4.4344	0.00042

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	18	21	25	21	2.0
MMS	500 µg/pl	-	162	153	139	151	6.7
DMSO	50 µl/pl	+	23	24	22	23	0.6
2-Aminoanthracene	20 µg/pl	+	184	184	173	180	3.7

*: thinning of the background lawn

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 11 - Experiment 2 - Preincubation method - TA98

STUDY NO.: 76250

SOLVENT: Sterile distilled water

Strain: TA98 Titre: 278

Dose-level [µg/pl]	Without metabolic activation Plate counts			Mean	S. E.	With metabolic activation Plate counts			Mean	S. E.
Untreated	36	36	40	37	1.3	39	39	37	38	0.7
313	33	33	38	35	1.7	36	36	41	38	1.7
625	29	34	37	33	2.3	42	37	36	38	1.9
1250	36	35	28	33	2.5	38	48	38	41	3.3
2500	31	38	36 *	35	2.1	35	30	38 *	34	2.3
5000	30	24	28 *	27	1.8	24	25	24 *	24	0.3

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	6.091	-0.0005	-0.53265	1.6651	0.13983
1 - 4	-	6.025	-0.0003	-0.44374	1.5659	0.14845
1 - 5	-	5.924	0.0000	-0.14337	0.5223	0.61023
1 - 6	-	5.992	-0.0001	-0.63457	3.2842	0.00467
1 - 3	+	6.173	0.0000	-0.00749	0.0198	0.98474
1 - 4	+	6.122	0.0002	0.36516	1.2404	0.24315
1 - 5	+	6.258	-0.0001	-0.31406	1.1927	0.25431
1 - 6	+	6.370	-0.0003	-0.83048	5.9634	0.00002

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
DMSO	50 µl/pl	-	34	28	33	32	1.9
2-Nitrofluorene	2 µg/pl	-	171	193	161	175	9.5
DMSO	50 µl/pl	+	40	45	39	41	1.9
2-Aminoanthracene	2 µg/pl	+	654	604	647	635	15.6

*: thinning of the background lawn

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

TABLE 12 - Experiment 2 - Preincubation method - TA100

STUDY NO.: 76250

SOLVENT: Sterile distilled water

Strain: TA100 Titre: 264

Dose-level [µg/pl]	Without metabolic activation Plate counts				Mean	S. E.	With metabolic activation Plate counts				Mean	S. E.
Untreated	166	151	167		161	5.2	171	171	162		168	3.0
313	165	166	159		163	2.2	171	162	176		170	4.1
625	157	162	168		162	3.2	167	178	160		168	5.2
1250	119	95	93	*	102	8.4	94	114	108	*	105	5.9
2500	60	78	62	*	67	5.7	75	76	61	*	71	4.8
5000	76	60	71	*	69	4.7	75	68	55	*	66	5.9

Regression analysis:

Points	S9	Intercept	Slope	Corr. coeff.	t	P-value
1 - 3	-	12.719	0.0001	0.08026	0.2130	0.83738
1 - 4	-	13.259	-0.0022	-0.83593	4.8165	0.00071
1 - 5	-	13.210	-0.0020	-0.94478	10.3947	0.00000
1 - 6	-	12.452	-0.0010	-0.84726	6.3802	0.00001
1 - 3	+	12.980	0.0000	0.01914	0.0506	0.96102
1 - 4	+	13.529	-0.0022	-0.85193	5.1448	0.00043
1 - 5	+	13.446	-0.0021	-0.94855	10.8010	0.00000
1 - 6	+	12.742	-0.0011	-0.87601	7.2655	0.00000

Positive and negative controls

Treatment		S9	Plate counts			Mean	S. E.
Untreated		-	166	151	167	161	5.2
Sodium Azide	1 µg/pl	-	649	625	607	627	12.2
DMSO	50 µl/pl	+	119	121	134	125	4.7
2-Aminoanthracene	2 µg/pl	+	945	1021	975	980	22.1

*: thinning of the background lawn

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

APPENDIX 1 - Historical control data without metabolic activation (2000-2008)

STUDY NO.: 76250

	Untreated Plate incorporation	Untreated Pre-incubation	Positive control Plate incorporation	Positive control Pre-incubation
TA1535				
Mean value	18	18	565	567
SD	2.6	2.4	76.4	73.5
n	185	190	185	190
TA1537				
Mean value	17	17	169	164
SD	1.9	1.8	33.2	41.1
n	184	187	184	187
TA98				
Mean value	31	32	183	180
SD	3.2	3.0	24.3	23.4
n	187	179	187	179
TA100				
Mean value	143	142	782	791
SD	15.4	15.2	123.5	121.9
n	187	186	187	186
WP2uvrA				
Mean value	28	27	180	210
SD	3.1	2.9	17.9	42.5
n	127	122	127	122

SD : standard deviation
n : number of experiments

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*

APPENDIX 2 - Historical control data with metabolic activation (2000-2008)

STUDY NO.: 76250

	Untreated Plate incorporation	Untreated Pre-incubation	Positive control Plate incorporation	Positive control Pre-incubation
TA1535				
Mean value	16	16	142	96
SD	1.8	1.8	28.3	12.7
n	185	189	185	189
TA1537				
Mean value	21	20	120	97
SD	2.4	2.1	19.9	11.5
n	184	188	184	188
TA98				
Mean value	39	39	611	661
SD	3.7	2.9	137.7	179.7
n	187	169	187	169
TA100				
Mean value	152	150	1261	1135
SD	15.7	14.2	196.5	186.9
n	188	184	188	184
WP2uvrA				
Mean value	35	34	205	191
SD	3.4	3.1	35.2	32.6
n	126	117	126	117

SD : standard deviation
n : number of experiments

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*

ADDENDUM I - Study Protocol

STUDY NO.: 76250

RESEARCH TOXICOLOGY CENTRE - ROMA

C604 CYCLIC BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli*)

Final Protocol
prepared for

SOLVAY SOLEXIS S.p.A.
Viale Lombardia, 20
20021 Bollate (MI)
Italy

by

RESEARCH TOXICOLOGY CENTRE S.p.A.
Via Tito Speri, 12/14
00040 Pomezia (Roma)
Italy

RTC Enquiry Number: 75839
RTC Study Number: 76250

March 2009

Commercial Office

RTC S.p.A.
Via Tito Speri, 12
00040 Pomezia (Roma) - ITALY
Tel.: + 39.06.91095.1
Fax: + 39.06.910.5737
e-mail: mkt@rtc.it
www.rtc.it

Head Office and Administration

RTC S.p.A.
Via Tito Speri, 12
00040 Pomezia (Roma) - ITALY
Tel.: + 39.06.91095.1
Fax: + 39.06.912.2233
P.O. Box 15301-00143 - Roma Eur Laurentino

RTC S.p.A.
Capitale sociale Euro 5.164.000
C.C.I.A.A. n° 375376
Reg. Soc. Trib. di Roma n° 2828/72
Cod. Fisc.: 00653120584
Partita IVA: 00920611001

BACTERIAL MUTATION ASSAY
(*S. typhimurium* and *E. coli*)

MANAGEMENT OF STUDY

Scientific Director : [redacted]

Head of Genetic Toxicology,
in vitro Toxicology and Immunology : [redacted]

Study Director : [redacted]

Sponsor : SOLVAY SOLEXIS S.p.A.
Viale Lombardia, 20
20021 Bollate (MI)
Italy

Monitor : [redacted]

QUALITY ASSURANCE

Quality Assurance Manager : [redacted].

LOCATION OF STUDY

The study will be performed at : Research Toxicology Centre S.p.A.
Via Tito Speri, 12/14
00040 Pomezia (Roma)
Italy

The laboratory facilities, archives and administration are located at this site.

TIME SCHEDULE OF STUDY

The Study will be conducted with a time schedule agreed between the Sponsor and RTC.

TEST ITEM IDENTITY

The test item will be: C604 CYCLIC

BACTERIAL MUTATION ASSAY
(*S. typhimurium* and *E. coli*)

1. INTRODUCTION

1.1 Objective

To assay the test item for the ability to induce gene mutations in *Salmonella_typhimurium* and *Escherichia coli*, as measured by reversion of auxotrophic strains to prototrophy.

1.2 Regulatory requirements

This study will be conducted in compliance with the GLP regulations of:

- US FDA [21 CFR part 58, 22 December 1978] and subsequent revisions;
- Directive 2004/10/EC of the European Parliament and of the Council of 11 February 2004;
- ENV/MC/CHEM(98)17 "OECD principles on Good Laboratory Practice – as revised in 1997";
- Decreto Legislativo no. 50 of 2 March 2007 and subsequent revisions.

In addition, the study is designed to comply with the experimental methods indicated in the guidelines of:

- EEC Council Directive 2000/32, Annex 4D.
- OECD Guidelines for the testing of chemicals No. 471 (Adopted July 1997).
- ICH S2A Genotoxicity: Specific Aspects of Regulatory Tests, Step 5.

1.3 Principles of the method

Reverse mutation assays employ bacterial strains which are already mutant at a locus whose phenotypic effects are easily detected. The *Salmonella* tester strains have mutations causing dependence on a particular amino acid (histidine) for growth. The ability of test items to cause reverse mutations (reversions) to histidine-independence can easily be measured. The *E. coli* tester strains of the WP2 series are similarly mutant at the tryptophan locus.

Since many chemicals only demonstrate mutagenic activity after metabolism to reactive forms, in order to detect these "indirect mutagens" the test is performed in the presence and absence of a rat liver metabolising system.

2. TEST ITEM

2.1 It is the responsibility of the Sponsor to supply the test item, accompanied by analytical data confirming the identity, purity, stability, strength and composition of the substance, the solubility and stability in the proposed vehicle and details of any known hazards to laboratory staff. The test item should be accompanied by a certificate of analysis.

2.2 Approximately one year after the submission of the final report, remaining amounts of the test item will be destroyed by incineration. An aliquot of the substance will be retained within the archives of the testing facility for a period of ten years after which it will be destroyed.

2.3 The test item identity is indicated on previous pages of this protocol.

2.4 Unless otherwise indicated by the Sponsor the storage conditions for the test item will be room temperature.

2.5 The precautions necessary when handling either the test item or prepared formulations of the test item are based on information supplied by the Sponsor. The minimum safety precautions necessary are detailed under the RTC Hazard Classification System, according to RTC standard procedures.

2.6 The amount of test item received and used will be recorded according to standard procedures.

2.7 Fresh dilutions of the test item as supplied will be prepared for each day's work at appropriate concentrations. One sterility test, using nutrient broth, will be performed before commencing all experimental treatments. Concentrations of solutions will be expressed in terms of active ingredient. Preferred solvent will be sterile distilled water.

2.8 No assay of test item stability, nor its concentration and homogeneity in the solvent/vehicle will be undertaken, nor samples of formulated test item consigned to the Sponsor, without express instructions from the Sponsor. No determination of the absorption of the test item in the test system will be made without express instructions from the Sponsor.

3. MATERIALS

3.1 Bacterial strains

Stocks of *Salmonella* tester strains (TA 1535, TA 1537, TA 1538, TA 98, TA 100, TA 97 and TA 102 and some other related strains) were obtained from Dr. B.N.Ames, University of California. Stocks of *E. coli* tester strains (WP2, WP2 *uvr*A and WP2 *uvr*A pKM101) were obtained from Life Science Research, Occold, Suffolk, UK. Permanent stocks are kept at -80°C, and overnight subcultures of these stocks are prepared for each day's work. The cultures used in each experiment contain a high titre of viable bacteria (1-5 x 10^9 cells per ml). The titre of each tester strain culture will be demonstrated in each experiment through the determination of viable cell numbers using nutrient agar plates.

The presence of the appropriate genetic markers in these strains is checked on a monthly basis for those in regular use, and as necessary for other strains, as follows:

Histidine requirement	:	No Growth on Minimal plates + Biotin. Growth on Minimal plates + Biotin + Histidine.
Tryptophan requirement	:	No Growth on Minimal agar plates Growth on Minimal plates + Tryptophan.
*uvr*A, *uvr*B	:	Sensitivity to UV irradiation.
rfa	:	Sensitivity to Crystal Violet.
pKM101	:	Resistance to Ampicillin.

Strain identity is also confirmed by reference to the spontaneous reversion levels and responses to mutagens during use. Bacterial cultures in liquid and on agar are clearly identified with their identity.

Detailed information about the genetic constitution of the tester strains may be found in the cited publications of Dr. B.N.Ames and Drs. M.H.L. Green and W.J. Muriel.

3.2 Media

The following growth media will be used:

Nutrient Broth: Oxoid Nutrient Broth No 2 will be prepared at a concentration of 2.5% in distilled water and autoclaved prior to use.
This will be used for the preparation of liquid cultures of the tester strains.

Nutrient Agar: Oxoid Nutrient Broth No 2 (25g) and Difco Bacto-agar (15g) will be added to one litre of distilled water and autoclaved.
The solution will then be poured into plastic Petri dishes and allowed to solidify and dry before use. These plates will be used for the non-selective growth of the tester strains. Incubations on Nutrient Agar will be for approximately 48 or 72 hours.

Minimal Agar: Minimal medium agar will be prepared as 1.5% Difco Bacto-agar in Vogel-Bonner Medium E, with 2% Glucose, and poured into plastic Petri dishes.

Top Agar: "Top Agar" (overlay agar) will be prepared as 0.6% Difco Bacto-agar + 0.5% NaCl in distilled water. This solution will be autoclaved, and stored. Prior to use 10 ml of a sterile solution of 0.5 mM Biotin + 0.5 mM Histidine (or 0.5 mM tryptophan) will be added to 100 ml of the top agar.

All incubations will be at 37°C.

3.3 S9 mix

The S9 liver tissue fraction will be prepared according to RTC standard procedures or will be obtained from an appropriate supplier (MOLTOX, Molecular Toxicology, Inc., USA). Induction of drug metabolising enzyme-levels is routinely performed using phenobarbitone and betanaphthoflavone (Mixed Induction); induction with Aroclor 1254 will be performed if specifically requested by the Sponsor. Records pertaining to the preparation of the S9 fraction are kept on file at RTC. The mixture of S9 tissue fraction and cofactors (S9 mix) will be prepared as follows (for each 10 ml):

S9 tissue fraction	1.0 ml
NADP (100 mM)	0.4 ml
G-6-P (100 mM)	0.5 ml
KCl (330 mM)	1.0 ml
MgCl2 (100 mM)	0.8 ml
Phosphate buffer (pH 7.4, 200 mM)	5.0 ml
Distilled Water	1.3 ml
	10.0 ml

3.4 Control items

Positive control treatments will be used in each experiment. The positive control agents are obtained commercially and characterised by their labelling, and their stability determined from the scientific literature. Sodium azide and methylmethanesulphonate will usually be dissolved in distilled water; 9-aminoacridine, 2-nitrofluorene and 2-aminoanthracene will usually be dissolved in DMSO. Determination of the stability and concentration of solutions of these agents will not be undertaken since it is sufficient to provide evidence for the correct expected response of the test system to them.

4. PRELIMINARY TOXICITY TEST

4.1 Experimental design

In order to establish the concentrations of test item to be used in the main assay, a preliminary toxicity test will be performed.
This test follows the method described in section 6.1, using only one plate per dose level, a single S9 mix concentration (10%) and covering a wide range of concentrations of the test item.

The highest dose-level for this preliminary test, unless limited by the solubility of the test item, will be 5 mg/plate, and the lower dose-levels will be spaced at approximately half-log intervals.

4.2 Selection of dose-levels

The toxicity will be assessed on the basis of a decline in the number of spontaneous revertants or a thinning of the background lawn. The highest dose-level for the mutation assays will be selected as a concentration which elicits moderate toxicity. If there is no evidence of toxicity following treatment with the test item, then the highest dose-level will be 5 mg/plate.

5. EXPERIMENTAL DESIGN

Each experiment will include solvent/vehicle and positive controls, and at least five doses of the test item, tested in the absence and presence of an S9 metabolising system. Three replicate plates will be used at each test point. If a first experiment produces a clear positive response, no further experiments will be undertaken.
If negative or equivocal results are obtained in the first experiment a confirmatory experiment will be performed using the pre-incubation method. A further experiment may be undertaken if inconsistent results are obtained. The five bacterial strains *S. typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* WP2 *uvr*A will be used in this study.

Solvent/vehicle controls: untreated and solvent vehicle controls will be prepared for each experiment; when the solvent is distilled water, these will be considered to be equivalent and only one set of controls will be performed.

Positive controls: treatments are indicated in the following table:

Tester strain	***Absence of S9***	***Presence of S9***
TA1535	sodium azide 1 µg/plate	2-aminoanthracene 1 µg/plate
TA100	sodium azide 1 µg/plate	2-aminoanthracene 1 µg/plate (2 µg/plate)
TA1537	9-amino-acridine 50 µg/plate	2-aminoanthracene 1 µg/plate
TA98	2-nitrofluorene 2 µg/plate	2-aminoanthracene 1 µg/plate (2 µg/plate)
WP2 *uvr*A	methylmethanesulphonate 500 µg/plate	2-aminoanthracene 10 µg/plate (20 µg/plate)

Concentrations refer to both treatment methods. When two values are given, the figures in brackets refer to the pre-incubation method assay.

Test item: the highest dose-level of the test item to be used will be selected as described above. Further dose levels will be selected at intervals of a factor of two.

Where it seems advisable, further test points or controls may be included in experiments.

In addition, plates will be prepared to check the sterility of the test item solutions and the S9 mix, and dilutions of the bacterial cultures will be plated on nutrient agar plates to establish the number of bacteria in the cultures.

6. ASSAY PROCEDURE

6.1 Plate-incorporation

The components of the assay (the tester strain bacteria, the test item and S9 mix or phosphate buffer) will be added to molten overlay agar and vortexed. The mixture will then be poured on the surface of a minimal medium agar plate, and allowed to solidify prior to incubation.

The overlay mixture will be composed as follows:

(i)	Overlay agar (held at 45°C)	2 ml
(ii)	Test or control item solution	0.1 ml
(iii)	S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml
(iv)	Bacterial suspension	0.1 ml

The volume of test item solution, as indicated, will usually be 0.1 ml; in the event that it is necessary to alter this volume, the quantities used will be carefully recorded.

6.2 Pre-incubation

The components will be added in turn to an empty test-tube:

(i) Bacterial suspension	0.1 ml
(ii) Test or control item solution	0.05 ml
(iii) S9 mix or phosphate buffer (pH 7.4, 0.1 M)	0.5 ml

The volume of test item solution, as indicated, will usually be 0.05 ml. Where control or test items are dissolved in aqueous solvents, the volume used may be 0.1 ml. In the event that it is necessary to alter this volume, the quantities used will be carefully recorded.

The treatment mixture will be vortexed and placed at 37°C for 30 minutes. Two ml of overlay agar will then be added and the mixture vortexed again and poured onto the surface of a minimal medium agar plate and allowed to solidify.

6.3 Incubation and scoring

The prepared plates will be inverted and incubated for approximately 72 hours at 37°C. When the test item is a liquid at ambient temperature, the plates will be incubated in separate closed containers for each dose-level. After this period of incubation, the plates may be held at 4°C prior to scoring. Scoring is effected by counting the number of revertant colonies on each plate, either manually, or using a Cardinal - Automatic colony counting system (Perceptive Instruments). Contaminated plates will be considered on a case-by-case basis.

7. REPORTING

7.1 Presentation of data

The data will be presented in tabular form. The individual plate counts for each experiment will be given, together with the means and standard errors of the means, and regression analyses.

7.2 Evaluation of data

For the test item to be considered mutagenic, two-fold (or more) increases in mean revertant numbers must be observed at two consecutive dose-levels or at the highest practicable dose-level only. In addition there must be evidence of a dose- response relationship showing increasing numbers of mutant colonies with increasing dose-levels.
Evaluation of Ames test data based on a 'doubling rate' has been shown to be as effective as statistical techniques in allowing the correct interpretation of test results (Chu et al. 1981).

7.3 Reporting procedure

A Draft Report will be supplied, and a Final Report issued subsequently to include any agreed changes or amendments. If comments have not been received within 6 months of despatch of the audited draft report, the final report will be issued.
If any corrections or additions are required to the Final Report, these will be in the form of an amendment by the Study Director. The amendment will clearly identify that part of the Final report that is being added to or corrected, and the reasons for the changes, and will be signed and dated by the person responsible.

7.4 Final report

The following information and data will be included in the final report:

- name and address of the facility performing the study and the dates on which the study was initiated and completed;
- objective and procedures stated in the approved protocol, including approved changes to the original protocol;
- the test article, identified by name, chemical name or chemical number;
- method used;
- any unforeseen circumstances that may have affected the quality or integrity of the study;
- data generated while conducting the study;
- statistical methods employed for analysing the data;
- a summary of the data, an analysis of the data and a statement of the conclusions drawn from the analysis;
- historical untreated and positive control data;
- the name and signature of the Study Director;
- the location where all raw data, specimens and final report are to be stored;
- Quality Assurance statement.

One original unbound, one copy bound and a PDF version will be supplied.

7.5 Records kept

Full records will be maintained of all aspects of study conduct, along with the results of all measurements and observations. Prior to final archiving of the study data a full list will be prepared of all records associated with the study.

7.6 Archiving

All raw data, records and documentation arising from this study, including the original final protocol and a copy of the final report consigned generated during the course of this study will be retained at RTC. Archiving will be provided for a period of 3 years after which the Sponsor will be contacted for instructions regarding despatch or disposal of the material. The original final report will be despatched to and archived by the Sponsor.

8. STUDY CONDUCT

8.1 Language

English language and Italian language versions of the study protocol, Standard Operating Procedures and other study documents may be used interchangeably. Similarly, English and Italian renderings of chemical names, including that of the test material will be considered to be equivalent.

8.2 Scientific decisions

The procedures described in this protocol may not comprehensively cover all the circumstances that can arise in the assay of test items. When the study director considers it advisable to modify the procedures described for the selection of a solvent, selection of dose levels, interpretation of the outcome of the study or other aspects of the study conduct, he/she will record carefully the decision he has reached and the reasoning which led to it.

8.3 Quality assurance

According to the RTC quality assurance programme, defined in the RTC QA SOPs, this study will be subjected to the following procedures:

- the protocol will be inspected,
- study/process based inspections of procedures/facilities will be carried out at intervals adequate to assure the integrity of the study,
- the report will be inspected to assure that it accurately describes the methods and Standard Operating Procedures and that the results accurately reflect the raw data.

Periodic reports on these activities will be made to management and the Study Director.
All raw data pertaining to the study will be available for inspection by the Sponsor's representative and regulatory authorities (following authorisation from the Sponsor).

9. DEPARTURES FROM REGULATORY REQUIREMENTS

Items which are the responsibility of the Sponsor are indicated in sections 2.1, 2.4, 2.8 and 3.3 of this protocol. Since full compliance with regulatory requirements may depend on the performance of some of these items, the Sponsor should ensure that appropriate actions are initiated or undertaken.

10. REFERENCES

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Mutation Research 189, 83-91.

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Recommendations for the performance of bacterial mutation assays.
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U.K.E.M.S., Swansea, 1983.

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S. Venitt and J.M. Parry (eds.)
IRL Press, Oxford, 1984.

PROTOCOL APPROVAL PAGE

for

R.T.C. S.p.A.

APPROVED BY [redacted] Date

Study Director

RELEASED BY [redacted] Date

Scientific Director

RTC Enquiry Number: 75839
RTC Study Number: 76250

March 2009

PROTOCOL APPROVAL PAGE

for

SOLVAY SOLEXIS S.p.A.

AUTHORISED BY [redacted] [redacted]
Date

Name and Title* [redacted]

* Please print or type your name and company status below your signature.

Date of Protocol Approval: [redacted]

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli)*

ADDENDUM II - Certificate of analysis

STUDY NO.: 76250

SOLVAY SOLEXIS

R,D&T Analytical & Structural
Characterization Department
Viale Lombardia, 20 – 20021 Bollate (MI) - Italy

Sample Description:
C_6O_4 Ammonium Salt, sample 150/28

Analysis Requests:
Ammonium chloride and fluoride determination
Salt title quantification
Organic impurity determination

Method (Equipment):
Ionic Chromatography
Potentiometry
NMR Spectroscopy

Performance:
MDL: na
MQL: na

Results:
The analyzed sample shows the following weight/volume of:
NH4F =28 mg/l
NH4Cl = 601 mg/l
C6O4 (NH4 titration) = 160 g/l

The organic impurities are dioxolen based and represent 0.8% by mol on total mol of organic phase of sample.

[redacted]

Analytical & Structural Characterization Department

C6O4 CYCLIC: BACTERIAL MUTATION ASSAY (*S. typhimurium* and *E. coli)*

ADDENDUM III - S9 production and quality control certificates

STUDY NO.: 76250

[redacted] POST MITOCHONDRIAL SUPERNATANT (S-9)
PRODUCTION & QUALITY CONTROL CERTIFICATE

LOT NO.: 2273 **SPECIES:** Rat **PREPARATION DATE:** May 1, 2008
PART NO.: 11-105 **STRAIN:** Sprague Dawley **EXPIRATION DATE:** May 1, 2010
VOLUME: 1 & 2 ml **SEX:** Male **BUFFER:** 0.154 M KCl
TISSUE: Liver **INDUCING AGENT(s):** Phenobarbital - 5,6-Benzoflavone

REFERENCE: Matsushima, et. al., In: In Vitro Metabolic Activation in Mutagenesis Testing (F.J. de Serres, Ed.), Elsevier, 1976. p. 85.
STORAGE: At or below 70°C

BIOCHEMISTRY:

- PROTEIN
37.6 mg/ml — Assayed according to the method of Lowry et al., JBC **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	55.5
PROD	2B1	19.7
BROD	2B1	19.9
MROD	1A2	11.2

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 31.0, 14.2, 57.4, 10.4 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS
Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 24 - 48 h incubation at 35°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. *his*+ Revertants EtBr/ TA98	CPA/ TA1535
342.8	1528

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* **129**:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames (*Mutat. Res.***113**:173, 1983).

	µl S9 per plate/number *his*+ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 µg)	181	297	387	650	855	1065
2-AA (2.5 µg)	184	365	1075	1727	2026	1283

[redacted]

[redacted] **POST MITOCHONDRIAL SUPERNATANT (S-9)**
PRODUCTION & QUALITY CONTROL CERTIFICATE

LOT NO.: 2378 **SPECIES:** Rat **PREPARATION DATE:** February 18, 2009
PART NO.: 11-105 **STRAIN:** Sprague Dawley **EXPIRATION DATE:** February 18, 2011
VOLUME: 1 & 2 ml **SEX:** Male **BUFFER:** 0.154 M KCl
TISSUE: Liver **INDUCING AGENT(s):** Phenobarbital - 5,6-Benzoflavone

REFERENCE: Matsushima, et. al., In: In Vitro Metabolic Activation in Mutagenesis Testing (F.J. de Serres, Ed.), Elsevier, 1976. p. 85.
STORAGE: At or below - 70°C

BIOCHEMISTRY:

- PROTEIN

37.8 mg/ml — Assayed according to the method of Lowry et al., JBC **193**:265, 1951 using bovine serum albumin as the standard.

- ALKOXYRESORUFIN-0-DEALKYLASE ACTIVITIES

Activity	P450	Fold - Induction
EROD	IA1, IA2	60.7
PROD	2B1	25.9
BROD	2B1	23.5
MROD	1A2	34.8

Assays for ethoxyresorufin-0-deethylase (EROD), pentoxy-, benzyl- and methoxyresorufin-0-dealkylases (PROD, BROD, & MROD) were conducted using a modification of the methods of Burke, et al., *Biochem Pharm* **34**:3337, 1985. Fold-inductions were calculated as the ratio of the sample vs. uninduced specific activities (SA's). Control SA's (pmoles/min/mg protein) were 14.0, 7.5, 22.6, 2.6 for EROD, PROD, BROD and MROD, respectively.

BIOASSAY:

- TEST FOR THE PRESENCE OF ADVENTITIOUS AGENTS

Samples of S-9 were assayed for the presence of contaminating microflora by plating 1.0 ml volumes on Nutrient Agar and Minimal Glucose (Vogel-Bonner E, supplemented with 0.05 mM L-histidine and D-biotin) media. Triplicate plates were read after 24 - 48 h incubation at 35°C. The tested samples met acceptance criteria.

- PROMUTAGEN ACTIVATION

No. *his*+ Revertants EtBr/ CPA/ TA98	TA1535
276.8	868

The ability of the sample to activate ethidium bromide (EtBr) and cyclophosphamide (CPA) to intermediates mutagenic to TA98 and TA1535, respectively, was determined according to Lesca, et al., *Mutation Res* **129**:299, 1984. Data were expressed as revertants per µg EtBr or per mg CPA.

Dilutions of the sample S9, ranging from 0.2 - 10% in S9 mix, were tested for their ability to activate benzo(a)pyrene (BP) and 2-aminoanthracene (2-AA) to intermediates mutagenic to TA100. Assays were conducted using duplicate plates as described by Maron & Ames (*Mutat. Res.***113**:173, 1983).

	µl S9 per plate/number *his*$^+$ revertants per plate					
Promutagen	0	1	5	10	20	50
BP (5 µg)	113	186	281	431	801	1105
2-AA (2.5 µg)	114	196	486	1249	1433	1497

[redacted]

Approved: [redacted] 2/23/09