EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

2,6-Dihydroxyethylaminotoluene

COLIPA n° A138

Scientific Committees
on consumer safety
on emerging and newly identified health risks
on health and environmental risks

The SCCS adopted this opinion at its 12th plenary meeting

of 20 September 2011

About the Scientific Committees

Three independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.
They are: the Scientific Committee on Consumer Safety (SCCS), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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This opinion has been subject to a commenting period of four weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged.

Revised opinions carry the date of revision.

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1. BACKGROUND

Submission I for 2,6-Dihydroxyethylaminotoluene was submitted in May 1995 by COLIPA[1].

The Scientific Committee on Consumer Products and Non Food Products intended for Consumers (SCCNFP) adopted its opinion SCCNFP/0697/03 at the 25th plenary meeting of 20 October 2003 with the conclusion, that "The data submitted were insufficient for a final evaluation. The SCCNFP is of the opinion that 2,6-Dihydroxyethyl aminotoluene should fulfil the demands of the SCCNFP "Strategy Paper" (doc. n° SCCNFP/0720/03 of 24 June 2003) as to mutagenicity of possible reaction products. Also it has to be shown that under in use conditions no nitrosation can occur. Moreover, an appropriate sensitization study is required. The compound as such can be regarded as safe."

The substance is currently regulated by the Cosmetics Directive (76/768/EC), Annex III, Part 1 under entry 9 on the List of substances, which cosmetic products must not contain except subject to restrictions and conditions laid down.

According to current submission II, 2,6-dihydroxyethylaminotoluene is used as a precursor in hair colouring products. The final concentration on-head is proposed up to 1.0% (calculated for the free base).

2. TERMS OF REFERENCE

Does the Scientific Committee on Consumer Safety (SCCS) consider 2,6-dihydroxyethylaminotoluene safe for the consumers, when used as a precursor in oxidative hair dye formulations with a concentration on-head of maximum 1.0% taking into account the scientific data provided?

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

2,6-Dihydroxyethylaminotoluene (INCI)

3.1.1.2. Chemical names

1-Methyl-2,6-bis-(2-hydroxyethylamino)-benzene
Ethanol, 2,2'-[(2-methyl-1,3-phenylene)diimino]bis-
2,6-Di-(2-hydroxyethylamino)toluene;
2,6-bis-(β-hydroxyethylamino)toluene;
2,6-bis[(2-hydroxyethyl)amino]toluene
2,6-di(2-hydroxyethylamino)-toluol

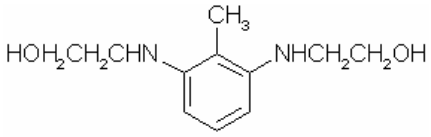
3.1.1.3. Trade names and abbreviations

HC Violet AS, HC Purple BS, WS I-111
COLIPA n° A138

3.1.1.4. CAS / EC number

CAS: 149330-25-6
EC: 443-210-1

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{11}H_{18}N_2O_2$

3.1.2. Physical form

Odourless, light brown-greyish powder

3.1.3. Molecular weight

Molecular weight: 210.28 g/mol

3.1.4. Purity, composition and substance codes

Batch PVS 11/02 = SAT 040796

Identity:	verified by NMR-spectroscopy, IR- and UV-spectrometry
Purity by NMR assay:	101% (w/w)
Purity by HPLC assay:	99.7% (area)
Sulphated ash	1.1% (w/w)
Impurities:	
2,6-Diaminotoluene	< 50 ppm (detection limit)
Sulphate ion	0.1% (w/w)
Solvent content (water):	0.4% (w/w)
Heavy Metal Content:	
Pb	< 20 ppm
Sb and Ni	< 10 ppm
As and Cd	< 5 ppm
Hg	< 1 ppm

Batch Pt. 1/91 and Batch 2

Identity:	verified by HPLC chromatography, UV-/VIS-spectroscopy and thin layer chromatography
Purity by HPLC:	> 99.8 area%
Impurities:	unspecified 0.07area% (HPLC)

Comment
Batches No. 2 and 1/91 seem to have been used in toxicological testing and the HPLC purity of both batches is similar to that of batch PVS 11/02 (HPLC peak area ≥ 99.7%).

3.1.5. Impurities / accompanying contaminants

See 3.1.4
NDELA content in HC violet AS; Syntharo, Lot #2010101: <25 ppb

3.1.6. Solubility

Water:	39.9 g/L	at 20°C, pH 8.2, measured by EC Method A.6
Ethanol:	10 – 100 g/l	at room temperature
DMSO:	> 100 g/l	at room temperature

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 0.037 (pH 7, 23°C) measured by EC Method A.8

3.1.8. Additional physical and chemical specifications

Melting point:	115 – 121 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/

UV_Vis spectrum (200-800 nm): λmax at 221nm and 293 nm

3.1.9. Homogeneity and Stability

The homogeneity and stability of 2,6-dihydroxyethylaminotoluene in test suspensions is described as follows:

0.1%, 1% and 10% suspensions of 2,6-dihydroxyethylaminotoluene were prepared in 0.5% aqueous CMC and stored at room temperature under access of daylight. Analyses were performed immediately after and after 2h, 4h and 24 h storage. Deviations of 5% of the initially determined concentration were tolerated.

2,6-Dihydroxyethylaminotoluene was assumed to be homogeneously distributed in the preparation, when values of the 3 samples were within ± 10% of the mean.

Ref.: 14, 16

General Comments to physico-chemical characterisation

- 2,6-Dihydroxyethylaminotoluene is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content in 2,6-dihydroxyethylaminotoluene is not reported. The NDELA content is reported for one batch of 2,6-Dihydroxyethylaminotoluene. However, NDELA content cannot be related to possible nitrosamine that can be formed by 2,6-Dihydroxyethylaminotoluene
- Stability of 2,6-dihydroxyethylaminotoluene in typical hair dye formulations is not reported.

3.2. Function and uses

2,6-Dihydroxyethylaminotoluene is used as a precursor for hair colours. It reacts with primary intermediates to form the final dye-stuff. The reaction can be accelerated by addition of an oxidising agent (e.g. hydrogen peroxide), but it can also be achieved by air oxidation. The final concentration on head of COLIPA A138 can be up to 1.0%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from SCCNFP/0697/03

Guideline:	OECD n° 401 / ECB 1 / Limit test
Species/strain:	Sprague Dawley rats
Group size:	5 males and 5 females
Test substance:	A 138 (in water)
Batch:	#2
Dose:	2000 mg/kg bw
Observ. Period:	14 days
GLP:	in compliance

Five male (140-152 g) and five female (129-140 g) Sprague-Dawley rats were used for the test. The method used followed OECD Guideline n° 401 (1981), referenced as Method B1 in Commission Directive 84/449/EEC.
The rats were given a single oral dose of test material as a suspension in distilled water at a dose level of 2000 mg/kg bw. They were observed for 14 days after the day of dosing and were then killed for gross pathological examination.

Results
There were no mortalities and all animals showed the expected gain in body weight during the study. Hunched posture and lethargy were noted in all animals on the day of dosing. No abnormalities were noted at necropsy. The acute median lethal dose (LD50) of the test compound was found to be greater than 2000 mg/kg bw in rats.

Ref.: 4

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline:	OECD 404 (1992)
Species/strain:	rabbit, white New Zealand (SPF Crl:NZW)
Group size:	3 males
Test substance:	2,6-di(2-hydroxyethylamino)-toluol
Batch:	Pt. 1/91
Purity:	99.8 area%
Vehicle:	water
Dose level:	single dose of 500 mg
GLP:	in compliance
Study period:	14 – 28 March 1995

The acute dermal irritation/corrosion of 2,6-di(2-hydroxyethylamino)-toluol was tested in three albino rabbits. The substance was applied in a single dose of 500 mg to a shaved dorsal area of trunk and covered with a gauze patch and aluminium foil which holds in contact with the skin by an occlusive dressing. Exposure duration was 4 hours. Thereafter residual substance was removed with water. Animals were examined for mortality, clinical signs and signs of irritation response 60 minutes, 24, 48 and 72 hours after patch removal.

Results
The treated skin area of all animals showed no signs of erythema or oedema formation.

Conclusion
2,6-di(2-hydroxyethylamino)-toluol was not irritant to rabbit skin under the conditions of the experiment.

Ref.: 5

3.3.2.2. Mucous membrane irritation

Guideline: OECD n° 405
Species/strain: New Zealand White Rabbits
Group size: 3
Test substance: 1 % (w/v) a.i. in water
Batch: #2
Purity: 99.5%
Dose: 0.1 ml
GLP: in compliance

Three New Zealand white rabbits were used. Their body weights ranged from 2.73 to 3.06 kg. The test was performed according to OECD Guideline n° 405 (1987), referenced as Method B5 in Commission Directive 84/449/EEC.
The test material was prepared as a 1 % (w/v) dilution in distilled water. An amount of 0.1 ml of the test material preparation was instilled into the conjunctival sac of the right eye, the left eye remained untreated and was used for control purposes. Assessment of ocular damage/irritation was made at about 1, 24, 48 and 72 h following treatment according to the scale of Draize.

Results
The test substance was not irritating at 1% in water.

Ref.: 3 (subm I)

Guideline: OECD 405 (1987)
Species/strain: rabbit, white New Zealand (SPF Crl:NZW)
Group size: 3 males
Test substance: 2,6-di(2-hydroxyethylamino)-toluol
Batch: Pt. 1/91
Purity: 99.8 area%
Vehicle: /
Dose level: 100 mg (neat)
GLP: in compliance
Study period: March 1995

The acute eye irritation/corrosion of 2,6-di(2-hydroxyethylamino)-toluol was tested in three albino rabbits. The substance was applied in a single dose of 100 mg to one of the eyes of each animal. The untreated eye was used as control. The eyes were washed out 24 hours after instillation of the test substance.

The animals were examined for clinical signs and the eyes were examined for lesions of conjunctivae, Cornea and iris 60 minutes, 24, 48, 72 and 96 hours after application of the test substance.

Results
One hour after instillation a slight redness and a swelling of the conjunctivae with lids about half closed were observed. 24 hours after application a diffuse, crimson colour but only light swelling were seen. All animals were free of irritation signs 96 hours after application.
Ref.: 6

Comment
Under the conditions of the test, 2,6-di(2-hydroxyethylamino)-toluol was irritant to the rabbit eye.

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)
Species/strain:	mouse, CBA strain, inbred, SPF-quality
Group size:	20 females (4 groups of 5 animals), nulliparous and non-pregnant
Test substance:	2,6-Di-(2-hydroxyethylamino)toluene
Batch:	PVS 11/02
Purity:	99.7 area% (HPLC)
Vehicle:	ethanol:water (7:3 v/v)
Concentration:	0, 10, 25 and 50%
Positive control:	alpha-hexylcinnamic aldehyde (in acetone:olive oil 4:1)
GLP:	in compliance
Study period:	7 – 21 June 2005

Three groups of five female mice each were treated with 2,6-di-(2-hydroxyethylamino)toluene at concentrations of 10, 25 and 50 % in ethanol:water by topical application to the dorsum of each ear lobe (25 µL) on three consecutive days. A control group of four mice was treated with the vehicle (ethanol:water (7:3 v/v)) only. Five days after the first topical application the mice were injected intravenously into a tail vein with ^{3}H-methyl thymidine.
Approximately five hours after intravenous injection, the mice were sacrificed, the draining auricular lymph nodes excised and pooled per group. The proliferative capacity of pooled lymph node cells was determined by the incorporation of ^{3}H-methyl thymidine measured in a ß-scintillation counter.

Results
No symptoms of local toxicity at the ears of treated mice and no systemic findings were observed during the study period. The Stimulation Index (S.I.) was below 3 in all dose groups. No dose response relation was noted.

Compound	Concentration %	Stimulation Index
2,6-Di-(2-hydroxyethylamino)toluene	10	1.2
	25	1.0
	50	0.9
alpha-hexylcinnamic aldehyde	5	1.5
	10	2.9
	25	6.1

Conclusion
2,6-Di-(2-hydroxyethylamino)toluene is not a sensitizer.

Ref.: 7

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428
Species/strain:	pigs both sexes
Group size:	8 dermatomed skin preparations from two young pigs
Skin thickness:	680-720 µm
Skin integrity:	TER >7kΩ
Test substance:	Experiment A: [^{14}C]-labelled "A 138" included in a hair dye formulation TM 0038-1a was mixed with a developer, not containing hydrogen peroxide. Experiment B: [^{14}C]-labelled "A 138" included in a hair dye formulation TM 0038-1a was mixed with another developer, containing hydrogen peroxide.
Batch:	PVS 11/02
Purity:	Purity non radiolabeled: 99.7% HPLC Purity radiolabeled: 97.7% HPLC
Receptor fluid:	Dulbecco's phosphate buffered saline
Test item:	A 138
Dose:	20 mg/cm² (corresponding to 0.21 mg/cm²)
Application area:	1 cm²
Method of Analysis:	Liquid scintillation
GLP:	in compliance
Study period:	2005

The test substance was studied as an ingredient of representative formulations:

Experiment A: A 138 included in a hair dye formulation TM 0038-1a was mixed with a developer, not containing hydrogen peroxide.
Experiment B: A 138 included in a hair dye formulation TM 0038-1a was mixed with a developer, containing hydrogen peroxide.
Eight integrity checked dermatomed skin preparations of two young pigs of both sexes were used in each experiment. Skins were inserted in static penetration cells (Franz-cells) with an application area of 1.0 cm². The non-occlusive exposure under temperature controlled conditions lasted 30 minutes before rinsing.
The test substance formulation was applied topically to the horny layer of the skin in nominal quantities of 20 mg/cm², which corresponded to nominally 0.21 mg of the test substance per cm^2 for the experiments A and B.
48 hours after the application, the *stratum corneum* was removed by repeated stripping with adhesive tapes to obtain the adsorbed test substance. The remaining skin was taken to determine the absorbed test substance. The penetration was calculated from the mass of the test substance in the receptor fluid, consisting of phosphate buffered saline. The overall amount of bioavailable test substance is defined as the sum of absorbed and penetrated quantities.

Results
The mean values of bioavailability were 1.97 ± 0.57 µg/cm² and 2.33 ± 0.70 µg/cm² for the formulation without and with hydrogen peroxide respectively.

Ref.: 17

Comment
The number of donors was only two. The mean + 2SD (3.73 µg/cm²) should be used for the MoS calculation under oxidative conditions.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral toxicity

No data submitted

3.3.5.2. Sub-chronic (90 days) toxicity (oral, dermal)

Dose Range Finding in Rats

The test substance was administered orally per gavage to 5 groups of 5 male and 5 female Wistar rats each once a day for 14 consecutive days. An equally sized negative control group was treated with distilled water. The test substance was administered freshly dissolved in distilled water (doses of 100 mg/kg and less) respectively suspended in a 0.5% aqueous CMC (carboxymethylcellulose; doses of 316 mg/kg bw and more) solution at a dose volume of 10 ml per kg body weight. Doses of 0 (control), 10, 32, 100, 316 and 1000 mg test substance per kg body weight and day were used.

Investigations performed: Observations in life, body weight, feed consumption and terminal necropsy.

Results
Mortality
All animals survived until the scheduled termination of the study.

Body weight
There were no significant differences of any dosed group to the control group.

Feed consumption
There were no significant differences of any dosed group to the control group.

Observations in life
Bedding material of all animals dosed with 1000 mg/kg bw was stained bright brown, resembling the colour of the test substance. This is most likely due to urinary excretion of the test substance and/or its metabolites. These stains are not regarded as toxic changes.
Some of the animals of the highest dosed group (1000 mg/kg bw) suffered from transient apathy, starting about 30 min after test substance administration and lasting for about another 30 min. This is regarded to be a toxic change.

Necropsy findings
A few single findings are altogether interpreted as incidental without test substance relationship.

Dose suggestions for a 90-Day Study:
The dose of 316 mg/kg bw represents the No-adverse-effect-level for both sexes, where no significant differences to the control group were found. Test compound related alterations in the highest dosed group (1000 mg/kg bw) were of only low severity. Therefore, a dose of 1000 mg per kg bw/d may be used as the high dose in the main study whereas 100 and ca. 300 mg per kg bw/d may serve as low respectively mid dose.

Ref.: 13

Taken from SCCNFP/0697/03

Guideline: OECD n° 408

Species/strain:	Wistar rats
Group size:	15/sex + 20/sex for control and high dose group
Test substance:	A 138 in 0.5% aqueous Na-carboxymethylcellulose solution
Batch:	Pt 1/91
Purity:	>99.8 area% (HPLC)
Dose:	0, 100, 316 and 1000 mg/kg bw/d
Exposure period:	90 days
Route:	oral, gavage
GLP:	in compliance

80 male and 80 female Wistar rats, Crl:(WI) BR, SPF, were used. The age at first administration was approximately 7 - 10 weeks and the mean body weight of the animals at the beginning of the study ranged between 197 - 219 g for males and 159 - 168 g for females.
The study was conducted according to OECD-Guideline 408. The test substance (purity >99.5%) was suspended in a 0.5% aqueous Na-carboxymethylcellulose solution and preparations were made freshly every day immediately before use. Doses of 100, 316 and 1000 mg test substance/kg bw/d were applied to groups of 15 male and 15 female rats for 90 or 91 consecutive days in males and 91 or 92 consecutive days in females. An equally sized control group received the vehicle only. In all groups the dose volume was 10 ml/kg bw. Recovery was investigated in two groups of 10 males and 10 females each; one high-dose recovery group and one control recovery group, that were treated as their corresponding groups for 92 days and then maintained without treatment for an additional 28 days. Observations in life, ophthalmoscopy, body weight, feed consumption, haematology and clinical chemistry, urinalysis, gross pathology, organ weight determination and histopathology were performed.

Results
One male animal (low-dose group) died accidentally during blood sampling on day 29. All other animals survived until the scheduled sacrifice. A dose-dependent light to dark staining of skin, fur, urine and bedding material was observed.
In all high-dosed animals, transient apathy was reported within the first two weeks of treatment within about half an hour after gavage. Salivation was noted for a short time after application of the test substance in one animal of the mid-dose group and practically in all high-dosed animals.
In a few animals, abnormal head posture and stereotype was observed occasionally. Serum bilirubin was significantly elevated in males of the high-dose group. Serum creatinine was significantly lower in mid- and high-dosed females.
In the urine of mid- and high-dosed groups, bilirubin and urobilinogen were found in abnormally high concentrations. High-dosed recovery group males showed a significantly increased relative liver weight. The urine pH was significantly lowered in male rats of the high-dose group. In male animals of the high-dose group, renal tubular epithelial basophilia was observed. A trend towards a dose-related increase in relative kidney weight was seen in males, but comparison of the relative kidney weights in the low-dose group and the corresponding control group showed no differences (% of bw control : 0.77 - 0.91%; low-dose group: 0.79 - 0.91%). An increased absolute and relative kidney weight was observed in high-dosed recovery group males only.
High-dosed females had a significantly increased relative weight of kidneys and high-dosed recovery females had an increased absolute adrenal weight.
Study results have been described by means of descriptive statistics. Due to multiple testing, some probably unspecific effects have been reported as "significantly different from actual corresponding controls" (i.e., elevated mean cell volume of females on day 84, caused by an unusual low mean of the control group).

Target organs of the substance toxicity were the liver (based on serum bilirubin, urine urobilinogen and bilirubin, organ weight changes) and the kidney (based on serum

creatinine, organ weights and histopathological changes, i.e., tubular epithelial basophilia). The NOAEL was considered to be 100 mg/kg bw/d.

Ref.: 14

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Assay

Guideline:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium* TA1535, TA1537, TA98, TA100, TA102
Replicates:	triplicate. One study with each strain
Treatment:	plate incorporation assay
Test substance:	A138
Batch:	PVS 11/02
Purity:	99.7 area%
Vehicle:	DMSO
Concentration:	pre-experiment: 3, 10, 33, 100, 333, 1000, 2500, 5000 µg/plate (TA98 and TA100) Experiment 1: 33, 100, 333, 1000, 2500, 5000 µg/plate (TA1535, TA1537 and TA102)
Positive control:	without S9-mix Sodium azide in deionised water (TA1535, TA100) 4-nitro-o-phenylenediamine in DMSO (TA1537, TA98) Methyl methane sulfonate in deionised water (TA102) With S9-mix 2-aminoanthracene in DMSO (TA1535, TA1537, TA98, TA100, TA102)
GLP:	in compliance
Study period:	23 November – 20 December 2004

A138 was tested for the induction of gene mutation in the above mentioned *Salmonella* strains with and without Phenobarbital/β-naphthoflavone induced rat liver S9-mix. Eight concentrations were used in the pre-test for toxicity with TA98 and TA100. This data are reported as part on the main test (experiment 1).

Results
No toxicity was observed up to the highest tested concentration (5000 µg/ml) evident as a reduction in the number in revertants or reduction in background growth, in any of the tested strains either with or without metabolic activation. There was a substantial and concentration related increase in revertant colonies in strain TA98 with metabolic activation, but not without metabolic activation. No mutagenic effect was observed in the other tester strains.

Conclusion
Under the test conditions of this study A138 did induce frameshift mutations in bacteria with S9-mix.

Ref.: 8

***In vitro* Mammalian Cell Gene Mutation Test**

Guideline:	OECD 476 (1997)
Species/strain:	The mouse lymphoma cell line L5178Y
Replicates:	Two independent experiments with two parallel cultures
Test substance:	A138
Batch:	PVS 11/02
Purity:	99.9 area%
Vehicle:	DMSO
Concentrations:	Experiment I without S9-mix: 131.3, 262.5, 525, 1050, and 2100 µg/ml with S9-mix: 131.3, 262.5, 525, 1050, and 2100 µg/ml Experiment II without S9-mix, 131.3, 262.5, 525, 1050, and 2100 µg/ml
Treatment:	Exp I: 4 h treatment with and without S9-mix, 72 h expression period
Control:	Exp II: 24 h treatment without S9-mix, 48 h expression period
GLP:	in compliance
Study period:	9 November 2004 – 3 January 2005

A138 was tested in the mouse lymphoma assay both with and without phenobarbital/β-naphthoflavone induced rat liver S9-mix for the induction of gene mutations (and chromosomal aberrations). The substance was tested in a pre-test for toxicity up to the maximum recommended concentration (10 mM). The concentrations in the main test were based on toxicity measured in the pre-test.

Result
No precipitation of the test item was observed in the main experiments up to the maximum tested concentration. No relevant toxic effects occurred in the first experiment with and without metabolic activation up to the maximum concentration of 2100 µg/ml corresponding to approximately 10 mM. In the second experiment the test item induced minor toxic effects in culture 2 as indicated by a relative total growth of just below 50% of survival.
No relevant increase of the mutant frequency was observed in both main experiments. The threshold of twice the mutant frequency of the corresponding solvent control was not reached at any concentration and the mutant frequency remained well within the historical range of negative and solvent controls.
The positive control mutagens showed a distinct increase in induced total mutant colonies and an increase of the relative quantity of small versus large colonies.

Conclusion
Under the experimental conditions reported, A138 is considered to be not genotoxic in the mouse lymphoma assay.

Ref.: 9

In vitro Micronucleus Test

Guideline:	OECD 487 (draft 2004)
Species/strain:	Chinese Hamster V79 cells
Replicates:	Three independent experiments with duplicate cultures
Test item:	A138
Batch:	PVS 11/02
Purity:	99.9 area%
Vehicle:	DMSO
Concentrations:	Exp. I: without S9-mix: 525, 1050, 2100 µg/ml with S9-mix: 525, 1050, 2100 µg/ml Exp. IIA: Without S9-mix: 525, 1050, 2100 µg/ml with S9-mix: 525, 1050, 2100 µg/ml

	Exp. IIB: Without S9-mix: 1050, 1400, 2100 µg/ml
Performance:	Exp I: without S9-mix: 4 h treatment, recovery period 20 h with S9-mix: 4 h treatment, recovery period 20 h Exp IIA: without S9-mix: 20 h treatment, recovery period 4 h with S9-mix: 4 h treatment, recovery period 44 h Exp IIB: without S9-mix: 20 h treatment, recovery period 4 h
Positive controls:	Colcemid in the absence of S9-mix, cyclophosphamide in the presence of S9-mix
GLP:	In compliance
Study date:	February 2005 – June 2005

The test agent was investigated for its clastogenic and aneugenic potential in the *in vitro* micronucleus assay. In a preliminary toxicity test the highest concentration used (2100 µg/ml) was the highest recommended concentration (10mM). Due to low toxicity this concentration was also used as the highest concentration in the main study.

Results
No test item precipitation was observed in culture medium 4 hrs after start of treatment either in the absence and the presence of S9-mix in the main tests
In the absence and the presence of S9-mix, no cytotoxicity indicated by reduced cell numbers and/or XTT activity of below 40% of control was observed up to the highest applied test item concentration. In the confirmatory Experiment IIB after 20 hrs treatment slight toxic effects indicated by cell numbers of 54.1% of control were observed at the highest applied concentration (2100 µg/ml).

After 4 hrs treatment in Experiment I and IIA, neither in the absence nor in the presence of S9-mix, a biologically relevant increase in the percentage of micronucleated cells was observed up to the highest applied concentration. The rates of micronucleated cells after treatment with the test item were close to the range of the solvent control values and clearly within the range of the historical control data.

In Experiment IIA at 48 hrs preparation interval in the presence of S9-mix, a single statistically significant increase was observed after treatment with 2100 µg/ml. Although this value was statistically significant compared to the low response in the solvent control data, the response is within the historical control data range. Therefore, the statistical significance has to be regarded as biologically irrelevant.
However, in Experiment IIA, in the absence of S9-mix a dose-related increase slightly exceeding the upper border of the historical control data range was observed after 20 hrs treatment with 2100 µg/ml. In this experimental part, the response of the positive control groups was lower than expected. To corroborate this observation Experiment IIB was performed with narrower dilution steps. In this confirmatory experiment after 20 hrs treatment with 2100 µg/ml the rate of micronucleated cells slightly but statistically significantly exceeded the historical control data range.

Finally, due to the confirmation of a slight genotoxic effect after 20 hrs continuous treatment in the absence of S9-mix, the test item has to be regarded as genotoxic.
The positive control substances produced a distinct increase in the number of micronucleated cells, thus demonstrating the sensitivity of the test system used for the endpoints investigated in this study. In Experiment IIA in the absence of S9-mix the responses after treatment with 50 and 25 ng/ml Colcemid were lower than expected. However, due to a distinct increase in the percentage of micronucleated cells in one of the test groups exposed with the test item the validity of the experiment was confirmed.

Therefore, this observation has no detrimental impact on the validity of this experimental part.

Conclusion,
It can be stated that in the study described and under the experimental conditions reported, A138 induced micronuclei in Chinese hamster V79 cells in the absence of metabolic activation, and A138 is therefore considered to be an *in vitro* clastogen and/or aneugen
Ref.:10

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

***In vivo* Mammalian Erythrocytes Micronucleus Test (1)**

Guideline:	OECD 474
Species/strain:	Male and female mice of the Crl:NMRI strain
Group size:	Five mice per dose and sex
Test substance:	A138
Batch:	PVS 11/2
Purity:	99.7 area% (HPLC)
Vehicle:	30% DMSO / 70% CMC (2%)
Dose level:	0, 312.5, 625 and 1250 mg/kg bw (10 ml/kg)
Route:	Intraperitoneal
Sacrifice time:	24 and 48 (only for the high dose level)
Control:	Cyclophosphamide monohydrate (40 mg/kg bw) and the vehicle, respectively served as positive and negative controls.
GLP:	in compliance
Study period:	September 2005-January 2006

The highest applicable dose, 1250 mg A138 per kg bw, was estimated by pre-experiments. This dose was without significant effects on the survival rates, but with clear signs of toxicity. At a higher dose (1500 mg/kg bw) two treated animals died. Bone marrow of femurs was prepared 24 and 48 (only for the high dose level) hours after application of the test substance. For each animal at least 2,000 polychromatic erythrocytes (PCE) obtained from femoral bone marrow were examined. The frequency of micronuclei was calculated for each animal and dose group.

Results
After treatment with the test item the number of polychromatic erythrocytes (PCEs) was not substantially decreased as compared to the mean value of PCEs of the vehicle control thus indicating that the test substance did not exert any cytotoxic effects in the bone marrow. However, systemic distribution of the test item was demonstrated by systemic toxicity and a distinct yellow/orange colour of the excreted urine of treated animals.

The analysis of 2000 PCE´s per animal of the test item treated animals showed a dose dependent increase in the frequencies of micronucleated PCE´s, which did not exceed the historical control range. This increase could be confirmed by increasing the number of evaluated PCE´s to 6000 per animal. The increase was statistically significant for the value obtained for the high dose test group at the 24 h preparation interval (0.145%) as compared to the vehicle control group (0.085%). The increase in micronuclei (0.140) was not statistically significant different from control when exposed for 48 h with the highest dose. However, all values are within the historical control range (0.01-0.15%), leaving the possibility that the observed dose dependence at 24 h exposure is not biologically relevant. The positive control (CPA) showed a substantial increase of induced micronucleus frequency.

Conclusion

The study author concluded that, under the experimental conditions reported, the results were inconclusive regarding the mutagenic potential of A138. Therefore a second study was performed (see ref 12 below).

Ref.: 11

***In vivo* Mammalian Erythrocytes Micronucleus Test (2)**

Guideline:	OECD 474
Species/strain:	Male and female mice of the Crl:NMRI strain
Group size:	Five mice per dose and sex
Test substance:	A138
Batch:	PVS 11/2
Purity:	99.7 area% (HPLC)
Vehicle:	30% DMSO / 70% CMC (2%)
Dose level:	0, 312.5, 625 and 1250 mg/kg bw (10 ml/kg bw)
Route:	Intraperitoneal
Sacrifice time:	24 and 48 (only for the high dose level)
Control:	Cyclophosphamide monohydrate (40 mg/kg bw) and the vehicle, respectively served as positive and negative controls.
GLP:	in compliance
Study period:	December 2005-January 2006

The study was performed in the same way as above and with the same doses.

Result
As in the first experiment A138 did not exert any cytotoxic effects in the bone marrow measured as the ratio of PCE to total number of erythrocytes. The bioavailability of the test item was, however confirmed as the excreted urine of treated animals had a distinct yellow/orange colour, and by the observed general toxicity of the test item.

In this study there was no biologically relevant enhancement in the frequency of the detected micronuclei compared to corresponding negative controls at any preparation interval after intraperitoneal administration of the test item and with any dose level used.
All obtained values were within the historical control range. The comparison of the test group dosed with 1250 mg/kg bw at 48 h preparation interval with the vehicle control showed that the observed increase in the frequency of the micronucleated cells (0.140%) was statistically significant from control (0.065%). This increase (0.140%) was also observed in the 48 h preparation interval of the first study. But the increase in the first study was not statistically significant due to a slightly higher negative control value (0.068%). The study authors state that the relevance of this observed effect is questionable, since there are no indications that a delayed mutagenic effect is to be expected. Such a delay could be due to either a mitotic delay caused by high bone marrow cytotoxicity or to kinetics of the test item leading to bioavailability of the test item in the target organ only at a later time point. In this study test item was already detected in the urine of treated animals at the 6 h post treatment observation interval. This shows that the test item was at the latest bioavailable at this time point, therefore the increase observed in the micronucleus frequency in the pertinent test group could not be attributed to a delayed distribution of the test item. The analysis of the PCE to total erythrocytes ratio did not give any indications of induced bone marrow cytotoxicity. Thus, a mitotic delay is also not induced. Given the fact that the individual and group values of the micronucleus rates of this test group are within the historical control range, the study authors considered the statistical significance of the observed increases as biologically irrelevant. In addition the increase in micronucleated cells was not statistically significant in the first study.

Conclusion

From the results obtained in these two *in vivo* studies, it was concluded that A138 has no mutagenic potential (clastogenicity and aneugenicity) in this *in vivo* micronucleus test in bone marrow cells when administered intraperitoneally to mice.

Ref.: 12

Remark
The SCCS agrees to the conclusion because 1) the observed effects were not reproducible in two similar studies and 2) the increase in PCEs with micronuclei were within the historical control range at all tested doses and time points.

3.3.7. Carcinogenicity

Taken from SCCNFP/0697/03

Malignant transformation of C31-1-mouse M2-fibroblasts *in vitro*

The test substance was dissolved in dimethylsulfoxide (DMSO) and tested in a concentration range of 50-4000 µg/ml (without and with addition of an external metabolising system; S9, Aroclor-1254 induced). C3H-mouse M2-fibroblasts were used as indicator cells. The solvent served as negative control. Positive control substances were N-methyl-N'-nitro-N-nitrosoguanidine (0.5 µg/ml), methylcholanthrene (10 µg/ml) and 2-acetylaminofluorene (10 µg/ml).

Results
The test substance was tested up to concentrations inducing significant cytotoxicity; the compound was detoxified by microsomal metabolism. 2,6-Dihydroxyethylaminotoluene was inactive at inducing malignant transformation in vitro. The positive controls yielded the expected results indicating the proper functioning of the indicator cells and the external metabolising system. The cell transformation test used has not been validated.

Ref.: 13 (subm I)

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Dose range finding study in rats

At least six mated female rats were used per dose group. The test substance was suspended in 0.5 % aqueous Na-carboxymethylcellulose and administered by oral gavage at doses of 10, 32, 100, 316, and 1000 mg/kg bw/d from day 6 to 15 of gestation, the control group received water. Signs of toxicity, body weight and food consumption were recorded. After dissection from the uterus the foetuses were weighed and externally examined.

Results
At 100 mg/kg bw/d statistically significant body weight reduction was noted. No changes in the foetal parameters were noted. For the main study the doses 40, 200 and 1000 mg/kg bw/d were chosen.

Ref.: 15

Taken from SCCNFP/0697/03

Guideline: OECD 414

Species/strain:	Wistar rats
Group size:	24 females
Test substance:	A138 gavage in CMC
Batch:	Pt 1/91
Purity:	> 99.8 area% (HPLC)
Dose levels:	0, 40, 200 and 1000 mg/kg bw
Treatment period:	day 6 to day 15
GLP:	in compliance

Virgin female and male Wistar rats (Crl: (WI) BR) were used. The mean body weight of females at day 0 of gestation ranged from 275 to 279 g. The test was performed according to OECD 414.
The test substance (purity >99.5%) was suspended in an aqueous 0.5% Na-carboxymethylcellulose solution.
Beginning on day 6 of gestation (day 0 = day of detecting vaginal plug or sperms), the test substance was applied by gavage once daily until day 15 to groups of 24 mated female rats at doses of 40, 200 or 1000 mg/kg bw (groups A, B and C). The control group K received the vehicle only. Signs of toxicity, body weights and food consumptions of the dams were recorded.
On day 20, the dams were necropsied and examined for the number of corpora lutea, implantations, viable foetuses, early and late deaths. The viable foetuses were weighed, sexed and examined for gross malformations. Approximately one half of the foetuses was examined for skeletal anomalies and the other half for internal anomalies.

Results
In the control group, significantly more litters with foetuses with incompletely ossified or unossified hyoid were observed in comparison with all dosed groups. In one control foetus an abnormal curvature of the spine and fused sternebrae were noted.
According to the authors, no test-substance related effects on dams or foetuses were diagnosed in groups A (40 mg/kg bw) and B (200 mg/kg bw). In all dosed groups the observed effects on foetuses were not considered as test-substance related since they were infrequent and not dose-dependent.
A slight maternal toxicity caused by the test substance in the highest dose group C (1000 mg/kg bw) was assumed due to a "statistically significant" decreased body weight at the beginning of the dosing period. This finding is limited to one recorded measurement period only and inconsistent with the overall weight development. Thus, the relevance of this result is limited, no clear-cut dose for maternal toxicity was observed. A slight but dose-dependent increase in post implantation losses was noted (mean values: group K, 5.8; group A, 6.4; group B, 7.8; group C, 10.9) but not interpreted as abnormal.

Conclusion
The overall NOAEL is 200 mg/kg bw.

Ref.: 16

3.3.9. Toxicokinetics

Guideline:	/
Species/Strain:	Rats, male, female, Sprague-Dawley, SPF-quality
Test substance:	^{14}C-labelled test substance incorporated in a hair dye formulation (**A, D**) and in an aqueous solution (**B, C, E**)
Batch:	2(WSI-111) (Purity: 99%)
Study groups:	**A:** 0.5 h dermal exposure (formulation), sacrifice after 72 h **B:** 0.5 h dermal exposure (solution), sacrifice after 72 h **C:** 72 h oral exposure (solution) **D:** 0.5 h dermal exposure (formulation), sacrifice after 24 h **E:** 24 h oral exposure (solution)
Dose level:	**A** 0.66 mg/cm² of the test substance (56.9 mg/cm² of formulation)

	B 0.56 mg/cm² of the test substance (33.6 mg/cm² of solution)
	D 0.65 mg/cm² of the test substance (56.6 mg/cm² of formulation)
	C 26.3 mg/kg bw
	E 26.9 mg/kg bw
Exposure time:	30 min dermal exposure and 24 h or 72 h follow up
GLP:	not in compliance

5 male and 5 female Sprague-Dawley rats (Him: OFA, SPF) were used in each of the 5 experiments. The body weights were approximately 200 g. The ^{14}C-labelled test substance was integrated in a hair dyeing formulation (0.18% 14C-"WSI-111", 0.98% "WSI-111", 1% p-Phenylenediamine hydrochloride, 97.94% basic formulation) or used as a solution in water (**B**: 16.7 mg/ml, **C** and **E**: 5 mg/ml). The stability of the test substance (in solution and in formulation) was checked and considered satisfactory. The hair dyeing formulation was mixed with oxigenta lotion (containing 6% hydrogen peroxide) before application. The test substance was applied to the clipped dorsal skin of rats (3 cm x 3 cm) for 30 min and was then washed off. The concentration on skin was 0.58% (formulation) and 0.5% (solution). Radioactivity of rinsings, application site, urine, faeces, blood, organs and carcass was estimated by liquid scintillation counting. Groups **C** and **E** received the test solution (about 26 mg/kg bw) orally by stomach tube. Group **C** was sacrificed after 72 h and urine, faeces, organs, and carcass without gastrointestinal tract were examined for radioactivity. Group **E** was killed after 24 h and radioactivity was determined in the blood.

Results, dermal application
Under the experimental conditions, total recoveries of the test substance of 97.7% (formulation) and 99.3% (aqueous solution) were obtained. The majority of the applied ^{14}C-labelled test substance was removed from the skin with the washing procedure (95.5% for the formulation and 96.1% for the aqueous solution). The amount of ^{14}C penetrated was calculated by adding the amounts eliminated from the body (i.e. urine 0-72 h plus faeces 0-72 h) and the amounts of ^{14}C still being present in the carcass.

When the formulation was used (group **A**), the application site contained a mean ^{14}C-activity of 2.1% of the dosed ^{14}C and 3% when the test substance solution was applied. In the animals of group **D**, the blood level of radioactivity was highest at 35 min p.a. and declined with a half-life of approximately 50 min. In groups **A** and **B**, the observed detection limit ranged from ca. 0.0005% dose/g for thyroids to 0.00002% dose/g for large organs (i.e., ca. 0.03-0.001 µg equivalents of the test substance/g). Mean radioactivity concentrations in blood and the 14 analysed organs were all below or at the detection limit in groups **A** and **B** at 72 h after dosing. The radioactivity was excreted mainly via urine (82 - 89% of eliminated ^{14}C) and to a lesser extent via faeces (11 - 18% of eliminated ^{14}C). The excretion was fast: A mean of 99% of the totally eliminated ^{14}C was excreted within the first 24 h in groups **A** and **B**. Relatively highest concentrations of radioactivity were determined in group **A** in thyroids, adrenals and femur and in group **B** in thyroids, carcass and skin.

The mean percutaneous penetration of the test substance was 0.078% of the administered ^{14}C for the formulation (0.515 µg/cm²) and 0.128% (0.838 µg/cm²) for the aqueous solution

Results, oral dosing
After application of 26.3 mg test substance/kg bw, 83.7% were recovered in urine and 0.068% in the carcass at 72 h p.a. (group **C**). The radioactivity was excreted mainly via urine (88% of eliminated ^{14}C) and to a lesser extent via faeces (12% of eliminated ^{14}C). The excretion was fast: 99% of the total eliminated radioactivity was excreted within the first 24 h. In group **C**, the detection limit ranged from approximately 0.002% dose/g for thyroids to 0.00006% dose/g for large organs (i.e., ca. 0.01 and 0.003 µg equivalents of test substance/g). As far as can be judged from the very low ^{14}C concentrations, the distribution into the organs was not too different from that observed after dermal application.

In the blood and in the 14 analysed organs, the ^{14}C-concentrations were below or near the detection limit. At 72 h p.a., relatively highest concentrations of radioactivity were found in thyroids, skin and kidneys, lowest in testes, brain and muscle. In the animals of group **E**, the blood levels of radioactivity were highest at 35 min. p.a. and declined with an initial half-life of approximately 40 min.
An oral absorption of at least 83.8% (i.e., 22 mg test substance/kg bw) was calculated.

Conclusion
Experiments carried out with radio-labelled test formulations in toxicokinetic investigations including cutaneous and oral (gavage) application showed a low dermal penetration rate – between 0.08 and 0.13% of the applied radioactivity – compared with an absorption rate of about 84% after oral application; in both cases, more than 99% of the radioactivity was excreted during the first 24 hours after application, about 88% via urine.

Ref.: 15 (subm I)

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

Not applicable

3.3.14. Discussion

Physico-chemical properties
2,6-Dihydroxyethylaminotoluene is used as a precursor for hair colours. It reacts with primary intermediates to form the final dye-stuff. The reaction can be accelerated by addition of an oxidising agent (e.g. hydrogen peroxide), but it can also be achieved by air oxidation. The final concentration on head of 2,6-Dihydroxyethylaminotoluene can be up to 1.0%.
2,6-Dihydroxyethylaminotoluene is a secondary amine, and thus, it is prone to nitrosation. Nitrosamine content in 2,6-dihydroxyethylaminotoluene is not reported. It should not be used together with nitrosating agents. Nitrosamine content should be <50 ppb. The NDELA content is reported for one batch of 2,6-Dihydroxyethylaminotoluene. However, NDELA content cannot be related to possible nitrosamine that can be formed by 2,6-Dihydroxyethylaminotoluene

Stability of 2,6-Dihydroxyethylaminotoluene in typical hair dye formulations is not reported.

Irritation, sensitisation
HC Violet AS is not irritant to rabbit skin. The neat substance is irritant to the rabbit eye. A 1% solution was not irritant. 2,6-Dihydroxyethylaminotoluene is not a sensitizer.

Dermal absorption
The mean value of dermal absorption of HC Violet AS was 2.33 ± 0.70 µg/cm^{2}under oxidative conditions. As only two donors were uses, the mean + 2SD (3.73 µg/cm²) could be used for the MoS calculation.

Toxicokinetics
Experiments carried out with radio-labelled test formulations in toxicokinetic investigations including cutaneous and oral (gavage) application showed a dermal penetration rate between 0.08 and 0.13% of the applied radioactivity – compared with an absorption rate of about 84% after oral application; in both cases more than 99% of the radioactivity was excreted via the kidneys/urine.

General toxicity
2,6-Dihydroxyethylaminotoluene is of low acute toxicity; > 2 000 mg/kg bw in rats. The NOAEL, derived from a 90 day study in rats, is 100 mg/kg bw/d, target organs were the liver and the kidneys. The results of a teratogenicity study showed an overall NOAEL of 200 mg/kg bw/d. No teratological abnormalities were recorded. No reproductive toxicity study was provided.

Mutagenicity
2,6-Dihydroxyethylaminotoluene was tested for the 3 genetic endpoints: gene mutations, structural and chromosomal aberrations. The test item induced gene mutations in bacteria with metabolic activation, but it did not induce gene mutations (or chromosomal aberrations) in a mouse lymphoma assay. SCCS does not agree with the applicant that a negative mammalian cell gene mutation test can overrule a positive bacteria gene mutation test.

It was clastogenic and or aneugenic in an *in vitro* micronucleus assay. The clastogenic/aneugenic effect was not confirmed *in vivo*. No *in vivo* study has been performed to investigate gene mutations. Therefore it cannot be ruled out that the substance is genotoxic.

Carcinogenicity
2,6-Dihydroxyethylaminotoluene did not induce transformation in a non-validated in vitro transformation test.

4. CONCLUSION

The SCCS is of the opinion that the positive results found in the *in vitro* gene mutation assay in bacteria were not confirmed nor ruled out in an appropriate *in vivo* test on the same genetic endpoint. Consequently, a final conclusion on the genotoxic potential of 2,6-dihydroxyethylaminotoluene cannot be drawn.

2,6-Dihydroxyethylaminotoluene is a secondary amine, and thus, it is prone to nitrosation. It should not be used together with nitrosating agents. Nitrosamine content should be <50 ppb.

5. MINORITY OPINION

Not applicable

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