SCCS/1579/16
Final

Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Orange No. 6 (B125)

The SCCS adopted this Opinion by written procedure
on 7 November 2016

About the Scientific Committees

Two 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) and the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) and are made up of scientists appointed in their personal capacity.

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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All Declarations of Working Group members are available on the following webpage:
http://ec.europa.eu/health/scientific_committees/experts/declarations/sccs_en.htm

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This opinion has been subject to a commenting period of 8 weeks (from 15 November 2016 to 10 January 2017) after its initial publication.
There were no comments received and the final version of the opinion remained unchanged compared to the preliminary one.

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1. BACKGROUND

Submission I on the hair dye HC Orange No. 6 (B125), with the chemical name Di-[2-[(E)-2-[4-[bis(2-hydroxyethyl)aminophenyl]vinyl]pyridin-1-ium]-ethyl]disulphide dimethanesulfonate (CAS 1449653-83-1), was transmitted by Cosmetics Europe in December 2015.

The new ingredient HC Orange No. 6 (B125) is intended to be used in non-oxidative hair colouring products at a maximum concentration of 0.5%.

2. TERMS OF REFERENCE

(1) In light of the data provided, does the SCCS consider HC Orange No. 6 (B125) safe when used in non-oxidative hair colouring products at a maximum concentration of 0.5%?

(2) Does the SCCS have any further scientific concerns with regard to the use of HC Orange No. 6 (B125) in cosmetic products?

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

HC Orange No. 6

3.1.1.2 Chemical names

Di-[2-[(E)-2-[4-[bis(2-hydroxyethyl)aminophenyl]vinyl]pyridin-1-ium]-ethyl]disulphide dimethanesulfonate
CAS NAME: Pyridinium, 1,1'-(dithiodi-2,1-ethanediyl) bis[2-[(1E)-2-[4- [bis(2-hydroxyethyl) amino] phenyl] ethenyl]-, methanesulfonate (1:2)

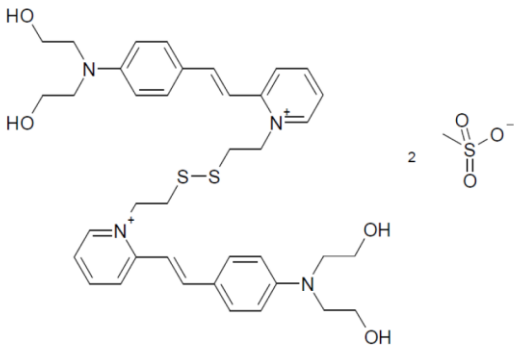
3.1.1.3 Trade names and abbreviations

Vibracolor Copper Orange

3.1.1.4 CAS / EC number

CAS: 1449653-83-1

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

$C_{38}H_{48}N_4O_4S_2$, 2 CH_3O_3S

3.1.2 Physical form

Red powder

3.1.3 Molecular weight

Molecular weight: 879.2 (688.9 + 190.2)

3.1.4 Purity, composition and substance codes

Chemical identification of HC Orange No. 6 was performed by IR, ^{1}H-NMR, ^{13}C-NMR, fluorescence spectroscopy and mass spectrometry using electrospray ionization (ESI) in the alternated positive and negative ion modes, where the expected doubly-charged ion of the colorant and the methylsulfonate anion were mainly observed as the base peaks of the corresponding spectra at m/z 344.4 and m/z 95.1

Table Summarising Composition of Vibracolor Copper Orange for the batches 014L001, 015D001 and Op. 07/13:

	014 L 001	015 D 001	Op. 07/13	
HPLC determination	97.4% w/w	91.8% w/w (1)	93.0% w/w (1)	
R0062327A (Aldehyde) (area% or % w/w)	0.01 (area%)	0.036 (2)	0.0146 (2)	
R0070527B (area% or % w/w)	0.40 (area%)	0.73 (3)	0.45 (3)	
Sum of other impurities > 0.1% (area% or % w/w)	0.7 (area%)	1.7 (1)	265nm 2.8 (area%)	450nm 2.5 (area%)
Sum of other impurities < 0.1% (area%)	0.45	0.41	Not performed	
Water content (% w/w)	0.9 (estimated value El. Ana.)	4.3 (K.F. Method)	0.8 (mean value) (K.F. Method)	
Methanesulfonate ion (Theoretical value: 21.6 %w/w) (% w/w)	23.0	19.5	21.0	
Acetate ion (% w/w)	-	0.05	< 0.1	
Chloride ion (% w/w)	-	0.11	Not Performed	
Elemental analysis	Conform			
Nitrosamines (µg/kg) Total N-Nitroso compounds (expressed as NO)	<50	<50	<50	
Residual solvents (µg/g) (4)				
Ethanol	<1000 (Detected)	1400	4500	
Isopropanol	4800	- (5)	<1000 (Detected)	
Heavy metals	conform	conform	conform	
Ashes (% w/w)	<0.1	-	-	
Total (% w/w)	100.3	99.1	97.5	

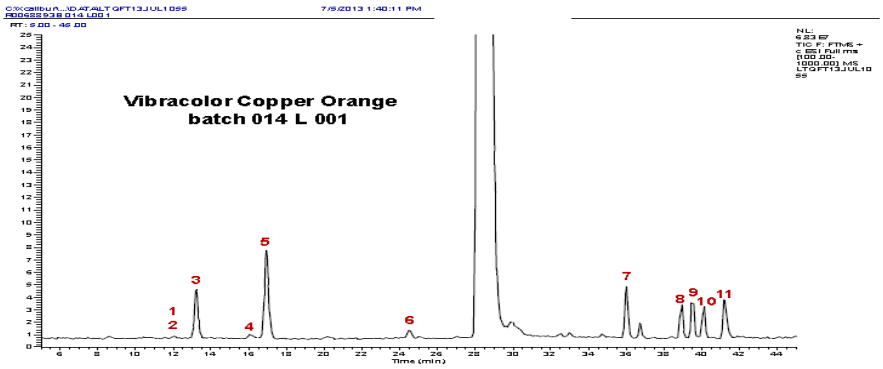
(1) Determined against Vibracolor Copper Orange (R0068893B) 014 L 001 (97.4% pure)
(2) Against batch R0062327A 001P 001 reference standard (99.4% pure)
(3) Against batch R0070527B 002 L 001 reference standard (96.7% pure)
(4) Residual solvents content: All solvents used in the process were checked. Only ethanol and isopropanol were detected with content above 0.1% (w/w).
(5) Solvent not used in this batch

SCCS comment

The applicant should provide analytical data on the method used to quantify methanesulfonate ion.

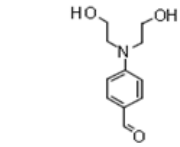
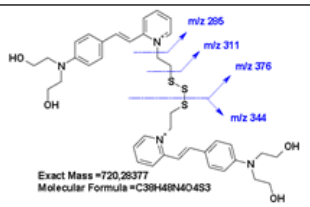
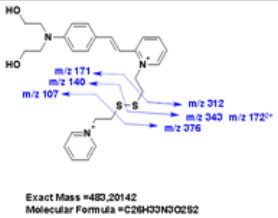
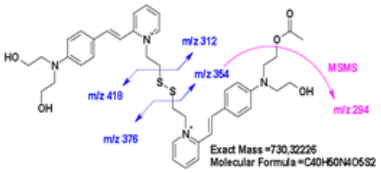
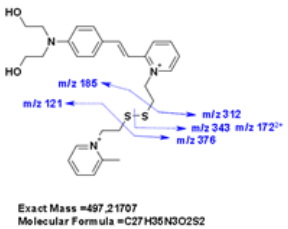
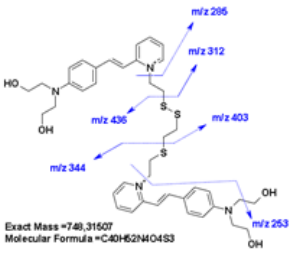
3.1.5 Impurities / accompanying contaminants

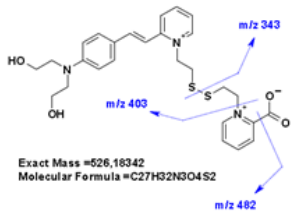
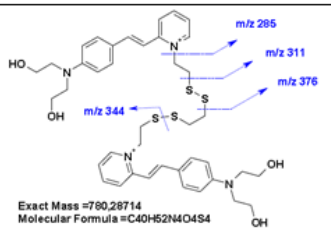
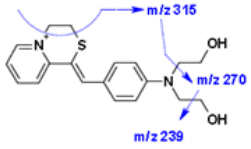
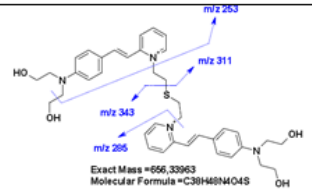
Several impurities were detected in the three batches. Two of them were identified as R0062327A (Rt = 12.0min) and R0070527B (Rt = 17.4min). A quantification of the impurities with a level greater than 0.1% (area%) were performed against the primary reference standard Vibracolor Copper Orange batch 014 L 001.
Potential impurities for Vibracolor Copper Orange can be starting material, synthesis intermediaries or by-products. Sum of the impurities above 0.1% (area %) content: About 1 to 3 %.



Total Ion Current profile of the impurities present in the batch #5 of the Vibracolor Copper Orange colorant using the Total Ion Current detection (FT-ICR)

Characterisation of the structure of the impurities present in the two batches 014 L 001 and 015 D 001 of the Vibracolor Copper Orange colorant at a level greater than 0.1%, as determined from the UV profile performed by HPLC/ESI/MS and HPLC/ESI/MS^n, the results of which are presented in the table below.

R0062327A (Aldehyde) impurity #1, tR = 12 min	 Exact Mass =209 Molecular formula =C11H15NO3	impurity #7 tR=35.2 min	 Exact Mass =720,28377 Molecular Formula =C38H48N4O4S3
impurity #2, tR = 12 min	 Exact Mass =483,20142 Molecular Formula =C26H33N3O2S2	impurity #8 tR=38.3 min	 Exact Mass =730,32226 Molecular Formula =C40H50N4O5S2
impurity #3 tR=14.0 min	 Exact Mass =497,21707 Molecular Formula =C27H35N3O2S2	impurity #9 tR=38.7 min	 Exact Mass =748,31507 Molecular Formula =C40H52N4O4S3

impurity #4 tR=16.0 min		impurity #10 tR=39.2 min	
R0070527B impurity #5 tR=17.3 min		impurity #11 tR=40.3 min	
impurity #6 tR=24.8 min			

Sum of other impurities content in:
- Vibracolor Copper Orange batch 014 L 001: 0.7% (Area%)
- Vibracolor Copper Orange batch 015 D 001: 1.7% w/w
Total N-Nitroso compounds content (expressed as NO): < 50 µg/kg

SCCS comment
Information regarding hazard classification for the impurities R0062327A (Aldehyde) and R0070527B should be provided. Any impurity belonging to CMR (carcinogenic, mutagenic and reproduction toxic) classes must be quantified.
Since Vibracolor Copper Orange is in the form of a methanesulfonate salt, any potential methane sulfonate impurities such as ethyl methanesulfonate should be reported and quantified.

3.1.6 Solubility

The solubility of HC Orange No. 6 was evaluated at 23°C according to the EP 5.07/5.11 method.

Water: between 100 and 500g/L (freely soluble), pH = 6.8.
Absolute Ethanol: less than 0.1g/L (practically insoluble)
DMSO: between 0.1 and 1g/L (very slightly soluble)
Corn Oil: less than 0.1g/L (practically insoluble)

3.1.7 Partition coefficient (Log P_{ow})

Log P_{OW}: < -2 (batch 014 L001)
Theoretical value: -2.7 (ClogP SYBIL, v.5.2) - error code 10, valid estimate for difficult structure.

3.1.8 Additional physical and chemical specifications

Melting point: batch 014 L 001:106°C (ΔH = 4J/g) and 182°C (ΔH = 63J/g)
batch 015 D 001: 104°C (ΔH = 28J/g) and 151°C (ΔH = 41J/g)
Boiling point: /
Flash point: /
Vapour pressure: /
Density: /
Viscosity: /
pKa: 1.8 to 3.5 (25°C and ionic strength 0.15M)- experimental(GLpKa Sirius)
Refractive index: /
pH at 23±2°C: 7.9 ± 0.2
Half-wave oxidation potential at pH 9.9 (voltametry): 595mV/ECS
Surface tension in water (air/water interface): Measured at 1% weight on Vibracolor Copper Orange batch 015 D 001 at 23±2°C and after an equilibrium time equal to 15 min:
The batch solution has no effect on water surface tension (γ= 72.8±0.3 mN/m).
Critical Micellar Concentration is not detected.
UV-Vis spectrum (200nm-800 nm): λmax at 442 and 277 nm in water/acetonitrile 95:5(v/v)

3.1.9 Homogeneity and Stability

According to the analytical certificate, the compound is considered to be stable when stored at room temperature, away from light and shielded from humidity.

SCCS comment
Stability of HC Orange No 6 at concentration of 0.5% and under use conditions should be provided.

3.2 Function and uses

The ingredient HC Orange No. 6 is intended to be used in non-oxidative hair colouring products at a maximum concentration of 0.5%.

3.3 Toxicological evaluation

3.1.1 Acute toxicity

3.3.1.1 Acute oral toxicity

No acute oral toxicity study for HC Orange No. 6 was submitted.

In the 14-day dose-range finding study in rats, HC Orange No 6 did not induce deaths at dose levels up to 1000 mg/kg/day. Based on these results, it can be concluded that oral exposure to HC Orange No. 6 does not induce acute toxicity by the oral pathway.

3.3.1.2 Acute dermal toxicity

No data submitted.

3.3.1.3 Acute inhalation toxicity

No data submitted.

3.3.1.4 Acute intraperitoneal toxicity

No data submitted.

3.3.2 Irritation and corrosivity

3.3.2.1 Skin irritation

Guideline:	*In vitro* Episkin^{SM} Skin Irritation Test, ECVAM validated protocol (ESAC statement 2007)
Test system:	Reconstructed Human Epidermis Model Episkin^{SM} (small model, 0,38cm^2)
Replicates:	3 different tissue batches
Test substance:	R0068893B (HC Orange No. 6)
Test batch:	R0068893B 015 D001
Purity:	91.8%
Test item:	neat substance
Dose level:	10 ± 2mg
Treatment period:	15 minutes
Post-treatment incubation time:	42 hours
Positive control:	10µl of 50mg/ml aqueous solution of Sodium Dodecyl Sulfate
Negative control:	10µl of PBS+
Direct interaction with MTT:	negative
Colouring of tissue:	positive
GLP:	in compliance
Study period:	October- November 2013

The test item, positive and negative controls were tested in triplicate. One additional negative control which followed the same treatment as the negative control except the MTT incubation period was added. Three additional tissues were used and followed the same treatment with the test item as the other tissues, except for the MTT incubation period. These tissues were used as additional specific controls in order to quantify the non-specific colour due to the colouring interactions of HC Orange No. 6 with the tissue. 10 ± 2 mg of the test substance and 10 µl of the different controls were applied onto the epidermis. After a 15-minute treatment period at room temperature, tissues were rinsed with PBS+ and the epidermis was transferred in 2 ml/well of fresh maintenance medium for 42 ± 1 hours at 37°C, 5% CO_2 and 95% humidity. Maintenance culture media were kept frozen at -20°C for further IL-1α measurements. After the 42-hour incubation period, each epidermis unit was transferred to another 12-well plate containing 2 ml/well of dye solution (0.30 mg/ml MTT in assay medium), except for the negative control and the test item-treated epidermis without MTT, which were transferred in another 12-well plate containing 2 ml/well fresh assay medium. After a 3-hour ± 15-minute incubation period at 37.0°C, 5.0% CO_2 and 95% humidity, a biopsy of the entire epidermis was taken. For all tissues with the test item, the superficial epidermis layer (containing most of the remaining colour) was removed and discarded. The epidermis was separated from the collagen matrix and both were transferred into a tube containing 500 µl acidified isopropanol. Formazan crystals were extracted and after homogenisation the optical density was measured at 570 nm versus acidified isopropanol as blank and the % cell viability was calculated.
IL-1α released in the culture medium was determined by a classic quantitative sandwich immunoassay technique. Monoclonal specific IL-1α antibodies were pre-coated onto

microplates. 200 µl of standards or samples were added in the wells enabling IL-1α to bind to immobilised antibody. After washing, an enzyme-linked polyclonal antibody specific to Il-1α was added to the wells. A substrate solution was added and the intensity of the colour developed was measured at 450 nm.

Results
The mean viability value for undiluted HC Orange No. 6 was 89.2 ± 3.6 % and the mean IL-1α release was 10.3 ± 3.1 pg/mL.

Conclusion
Under the conditions of this study, undiluted HC Orange No. 6 is predicted to be non-irritant to the skin.

Ref.: 3

3.3.2.2 Mucous membrane irritation / Eye irritation

Guideline:	OECD 437 (September 2009)
Test material:	Bovine cornea
Replicates:	6 corneas per condition
Test item:	R0068893B (HC Orange No. 6)
Test batch:	R0068893B 015 D001
Purity:	91.8 %
Test item:	20% (w/w) in 0.9% (w/v) NaCl
Treatment period:	4 hours ± 10 minutes
Positive control:	20% (w/v) Imidazole in 0.9% (w/v) NaCl
Negative control:	0.9% (w/v) NaCl
GLP:	in compliance
Study period:	May 2013

Bovine eyes (from cattle aged less than 12 months) were collected at slaughterhouses and prepared within 4 hours of collection. Too big eyes or defective corneas were rejected. 750 ± 8 µl of test item diluted at 20% (w/w) in 0.9% (w/v) NaCl was applied onto the cornea (category: solid non surfactant). The test item remained in contact with the isolated cornea for 4 hours ± 10 minutes. Six corneas per condition were used. At the end of the contact period, the corneas were rinsed and prepared for measurement of opacity (changes in light transmission). Three of these corneas were further used to measure the permeability (evaluation of transfer of 5 mg/ml fluorescein through the cornea by measuring the optical density at 490 nm of the media in the ocular posterior compartment). The remaining three corneas were kept for histological analysis. The IVIS score, which is the combination of opacity and permeability, was then calculated. Negative and positive control substances were tested according to the same experimental conditions.

Results
The IVIS score obtained for HC Orange No. 6 diluted at 20% (w/w) in NaCl 0.9% after 4-hour contact was 10.3 ± 1.5; IVIS for the positive control was calculated to be 184.8 ± 4.4. Corneas stained orange upon application of the test item. Histological examination revealed changes in the epithelium characterised by minimal to slight erosion/ulceration, minimal to slight cell dissociation and minimal to mild cell alteration. There were no changes in the corneal stroma or endothelium.

Conclusion
Under the conditions of this study, HC Orange No. 6 diluted at 20% (w/w) in physiological saline was concluded to be neither corrosive nor severely irritant to eyes. Additionally, histological evaluation of the cornea suggested only minimal to slight eye irritation potential for HC Orange No. 6 at the concentration tested.

Ref.: 11

SCCS comment
Because colouring of the cornea has occurred, interference with both the opacity and the permeability measurement endpoints of the BCOP assay are to be expected, which may lead to false estimates of the eye irritation potential. Under the conditions of this study, an eye irritation potential of HC Orange No. 6 at 20% (w/w) cannot be excluded. Considering that the maximum intended concentration of HC Orange No. 6 in a hair dye product is 0.5% (w/w), it can be assumed that eye irritation will be of limited concern.

3.3.3 Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429
Species/strain:	Female CBA/J mice
Group size:	4 mice per group (main study); 2 mice per group (pre-test)
Test substance:	R0068893B
Batch:	014 L 001
Purity:	> 95% (HPLC, UV detection)
Vehicle:	Dimethyl sulfoxide (DMSO)
Concentration:	0.5, 1, 5, 10 or 25% (w/v)
Positive control:	alpha-hexylcinnamaldehyde (HCA) at 25% (v/v) in Acetone/Olive Oil (4:1; AOO).
GLP:	In compliance
Study period:	May – June 2010

In a pre-test it was shown that the highest technically achievable concentration in DMSO (25%) did not induce any clinical signs of toxicity and no increase in ear thickness. In the main LLNA study, mice were topically treated with HC Orange No. 6 at 0.5, 1, 5, 10 or 25% (w/v) in DMSO. The negative control group received the vehicle alone.
The test item or vehicle was applied to the ears on three consecutive days. On day five, mice were sacrificed and auricular lymph nodes were sampled, pooled per group, and the proliferation of lymphocytes was evaluated by measuring the incorporation of [^{3}H]-methylthymidine.

Results
There were no lymphoproliferative responses, with SI values of 0.43, 0.67, 1.48, 2.13 and 2.41 obtained at HC Orange No. 6 concentrations of 0.5%, 1%, 5%, 10% and 25%, respectively.

Conclusion
Under the conditions of this study, HC Orange No. 6 did not induce delayed contact hypersensitivity and was therefore considered to be devoid of sensitising potential.

SCCS comment
HC Orange red No. 6 induced a dose-dependent increase of lymphocyte proliferation. For all tested concentrations, the SI values were below 3. Under the conditions of this study, HC Orange red No. 6 is not considered a skin sensitiser.

Ref: 12

3.3.4 Dermal / percutaneous absorption

***In vitro* percutaneous absorption under non-oxidative conditions**

Guideline:	OECD TG 428 (2004)
Test system:	Frozen human dermatomed skin (400 µm)
Membrane integrity:	Checked by electrical resistance, at least 10 kΩ
Replicates:	12 intact skin samples (4 donors)
Test substance:	[vinyl–2,2'-14C]-R0068893B
Batch:	CFQ41583
Purity:	>97%
Test item:	Hair dye formulation (P1104361, batch number LG 03/11/12) containing 0.5% w/w R0068893B
Dose applied:	20 mg/cm^2 of the test formulation (100 µg/cm^2 R0068893B)
Exposure area:	2.54 cm^2
Exposure period:	20 minutes
Sampling period:	24 hours
Receptor fluid:	Degassed phosphate buffered saline (PBS)
Solubility in receptor fluid:	Ultra-pure water: between 100 and 500 g/L
Absolute ethanol:	less than 100 g/L
Mass balance analysis:	Provided
Tape stripping:	Yes (20)
Method of Analysis:	Liquid scintillation counting (LSC)
GLP:	In compliance
Study period:	18 March - 16 April 2013

Human skin samples were obtained from a tissue bank. Each membrane was stored frozen at approximately -20°C, on aluminium foil until required for use. Skin membranes were cut from the samples at a thickness setting of 400 µm using an electric dermatome.
Prior to dosing, the membrane integrity was checked by measuring electrical resistance. The doses were applied to the surface of 12 intact skin membranes (from 4 human donors) at a rate of 20 mg/cm^2, corresponding to a nominal concentration of 100 µg/cm^2 of R0068893B. At the end of the 20-minute exposure period, the skin surface was washed with water (10 x 1270 µL), and then by 2% sodium dodecyl sulphate (SDS) in water (1 x 1270 µL). Between each set of washes, the washing fluid was aspired three times. The skin surface was washed further with water (10 x 1270 µL), after which the skin surface was dried with cotton wool swabs. Following decontamination, the cells were returned to the water bath for the remainder of the 24-hour run time. Following this, a 24-hour mass balance procedure was performed. The skin surface was washed with sponges soaked in 3% Teepol L® in water and further sponges pre-wetted with water. The *stratum corneum* was removed by a tape-stripping process, removing up to a maximum of 20 strips from each skin membrane. The flange skin was cut away from the dermis and the epidermis on the remaining skin disc and the epidermis was separated from the dermis using a heat separation technique.
The penetration process was monitored using [^{14}C]-radiolabelled R0068893B, which was incorporated into the formulation, prior to application. The 'Hair dye formulation' was analysed by HPLC to assess radiochemical purity and concentration. The distribution of R0068893B within the test system was measured and a 24-hour penetration profile was determined by collecting receptor fluid samples 20 minutes, 1, 2, 4, 8, 12, and 24 hours following application. The samples were analysed by liquid scintillation counting (LSC).

Results
The HPLC method used was found to be suitably sensitive to confirm the radiochemical purity of [^{14}C]-R0068893B when formulated in the 'Hair dye formulation'. HPLC analysis confirmed the stability of the test materials for a period exceeding the length of the study.
Three of the 12 dosed cells either had a mass balance outside the range of 85-115% (SCCS, 2010) or a penetration profile that indicated that the membrane had become damaged over

the course of the 24-hour run and were therefore rejected and not included in the mean ± SD.
Mean recovery of the applied test material was good at 91.6%, with individual cell values ranging from 86.0% to 100% (n=9).
Table 1 shows the distribution of R0068893B in the test system.

Table 1: Summary of R00668893B distribution in the test system

Test compartment n = 9	**µg R0068893B per cm²**		**% of applied dose**	
	Mean	**SD**	**Mean**	**SD**
Donor chamber	0.062	0.046	0.054	0.040
Skin wash at 20 minutes	106	6.00	91.3	5.18
Skin wash at 24 hours	0.190	0.152	0.164	0.131
Stratum corneum	0.022	0.013	0.019	0.011
Living epidermis	0.049	0.080	0.042	0.069
Dermis	0.001	0.001	0.001	0.001
Flange	0.006	0.004	0.005	0.004
Receptor fluid	0.001	0.0004	0.0009	0.0004
Total non-absorbed	106	6.03	91.6	5.20
Systemically available	**0.045**	**0.078**	**0.039**	**0.067**
Total recovered	106	6.08	91.6	5.25

Total non-absorbed = Sum of donor chamber, skin wash, flange and stratum corneum
Systemically available = Sum of remaining skin, dermis and receptor fluid
Stratum corneum = Amount in tape strips
Remaining skin = Tissue remaining after tape stripping

Conclusion
The results obtained in this study indicate that R0068893B present at 0.5% in a hair dye formulation penetrated through human dermatomed skin at an extremely slow rate. The extent of R0068893B penetrating through human skin to the receptor fluid amounted to only 0.0009% (0.001 ± 0.0004 µg/cm^2) of the applied dose, after 24 hours.
The mean total systemically available dose of R0068893B was 0.039% of the applied dose (corresponding to 0.045 ± 0.078 µg/cm^2).

SCCS comment
SCCS considers that the mean + 2SD i.e. 0.201 µg/cm^2 should be used for the MoS calculation because of the short exposure time.

Ref.: 4,5

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose (14 days) oral toxicity: dose finding for 90-day study

Guideline: /
Species/strain: Rat / Wistar Hannover
Group size: 5 animals/sex/group
Test substance: R0068893B
Batch: R0068893B 015 D001
Purity: 91.8 ± 1%
Vehicle: deionised water
Dose levels: 0, 100, 300 and 1000 mg/kg bw/day

Dose volume: 4 ml/kg
Route: oral
Administration: by gavage
GLP: in compliance
Study period: September 2011 – May 2013

The study was performed to obtain preliminary information on the major toxic effects of the test item in order to select appropriate dose levels for further sub-chronic toxicity studies.
Test substance was administered by oral gavage to male and female rats (5/sex/group) for 14 days at doses of 0, 100, 300 and 1000 mg/kg bw/day. The doses were based on a previous study where 1000 mg/kg bw/day did not produce excessive toxic effects.

Results
The test item did not cause any mortality or clinical signs of toxicity during the study.
No statistically significant effects on body weight were observed in either sex. However, the body weight gains over the 14-day treatment period were lower in both males (-19.1%) and females (-34.4%) at 1000 mg/kg bw/day (statistically significant in females only).

In females at 1000 mg/kg bw/day, there were statistically higher values of food consumption, but no other changes (e.g. clinical signs) were found. The higher consumption values for this dose group were punctual and not dose-related.

With regard to clinical pathology, in females dose-related higher cholesterol levels at 100, 300 and 1000 mg/kg bw/day compared to the control group were observed. No additional alterations were observed except for some isolated, small or not dose-related differences.

Statistically significantly higher adrenal gland weights (absolute and relative) were observed at 1000 mg/kg bw/day in males. However, the adrenal glands were not examined microscopically and the significance of this finding could not be determined. Nothing was observed during macroscopic and microscopic examinations, except for stomach erosion in one male at 1000 mg/kg bw/day.

Conclusion
Only small effects were observed at 1000 mg/kg bw/day in both male and female rats. Thus, the doses recommended for the 13-week repeated dose oral toxicity study were 100, 300 and 1000 mg/kg bw/day.

Ref: 6

3.3.5.2 Sub-chronic (90 days) toxicity (oral)

Guideline: OECD TG 408 (1998)
Species/strain: Rats Wistar Hannover
Group size: 10 animals/sex/group
Test substance: R0068893B
Batch: R0068893B 015 D 001 (provided by Sponsor)
Purity: 91.8±1%
Vehicle: deionized water
Dose levels: 0, 100, 300, 1000 mg/kg bw/day
Dose volume: 4 ml/kg bw/day
Route: oral
Administration: gavage
GLP: in compliance
Study period: 21 December 2011 – 4 April 2014
The test substance was administered by oral gavage to male and female rats (10/sex/group) at dose levels of 0, 100, 300, 1000 mg/kg bw/day once daily for 90 days, in

a constant dosage volume of 4 ml/kg. The dose levels were based on the findings in the 14-day dose-range finding study described above.

Results
The stability of the substance was confirmed with the certificate of analysis provided by the sponsor. The stability results for the test substance in the lowest and highest dosing formulations were satisfactory for 7 days. Thus, test solutions were prepared weekly and stored at 2-8 °C prior to use under inert gas and protected from light. Test solutions were stirred continuously during administration to maintain homogeneity.

No deaths occurred during the study. No treatment-related clinical findings were observed, including during the ophthalmological examination.

There was a treatment-related decrease in body weight and body weight gain at 1000 mg/kg bw/day when compared to controls (body weight gain: -18% in males and -19 % in females). There was also a decrease of lower magnitude in males only at 100 mg/kg bw/day. This finding was considered to be a chance event due to the absence of a dose-response relationship and because it was observed in males only.

There were statistically significant changes in food consumption in both males and females in treated rats compared to control groups at some time points. However, these changes were small in magnitude, not dose proportional, and did not statistically affect overall food consumption, except in males at 100 mg/kg bw/day. These findings were therefore considered unrelated to the administration of the test item.

With regard to the functional observational battery, a statistically significantly lower number of times passing through the centre and higher freezing times were noted in 1000 mg/kg/day males during the Open Field Evaluation. Furthermore, lower mean hindlimb foot splay measurements at heights of 40 cm and 30 cm, were observed in males at 300 and 1000 mg/kg bw/day, respectively. In females at 1000 mg/kg/day, the mean righting reflex time at 30 cm height was minimally higher when compared with the control group. These changes were isolated, of minimal magnitude, lacked consistency within a sex and/or were observed in a single sex. Finally, all values remained within the historical control range, and were then considered as normal biological variations unrelated to the administration of the test item.

No prominent alterations were found in the haematological parameters in treated rats compared to the controls, except for lower Activated Partial Thromboplastin Time observed in male rats at all dose levels. These effects on Activated Partial Thromboplastin Time were statistically significant at 1000 mg/kg bw/day. However, these differences from controls were principally due to incidentally high values in control males. Since values observed in test item-treated groups remained within the historical control data range for rats of this strain and age, they were considered to be the result of normal biological variation.

With regard to clinical chemistry parameters, few statistically significant or otherwise notable differences between control and test item-dosed groups were observed. Most changes were consistent with normal biological variation for this strain of rats at this age, were of minor magnitude, inconsistent between sexes, and unrelated to dose. Thus, they were considered to be incidental and unrelated to administration of test item.

For the urinalysis parameters there were no changes of toxicological significance in treated groups compared to the controls. At 1000 mg/kg bw/day, urine was orange-coloured at 1000 mg/kg/day, which was considered to be related to the colour of the test item and/or its metabolites. Therefore, these changes were considered to be without toxicological significance.
Isolated, statistically significant changes in organ weights occurred in the treated rats. However, these changes were considered unrelated to treatment because most of the

differences were of small magnitude and not dose related, limited to a single sex or not observed in relative weights (to brain weight). The increases in relative (to body weight) brain and testis weights observed in males at 1000 mg/kg bw/day compared to controls, were considered to be secondary to the body weight changes. Furthermore, the absolute and relative (to organ weight) heart weights were slightly to moderately higher in both sexes at 1000 mg/kg/day. However, in the absence of associated macroscopic or microscopic changes and of similar changes in relative (to brain weight) weights, these changes were considered to be of no toxicological significance. At 1000 mg/kg bw/day, the kidneys weights in females displayed consistent changes, but they were of small magnitude and were considered to be of no toxicological significance.

A few macroscopic lesions were observed, involving both the control and the 300 mg/kg bw/day group that were considered unrelated to treatment.
Scattered microscopic lesions were seen in control and treated rats at very low incidence, but none of the findings was considered related to the administration of the test item.

Conclusion
According to the applicant, the NOAEL was considered to be 300 mg/kg bw/day for both males and females.

SCCS comment
The SCCS agrees with the NOAEL of 300 mg/kg bw/day.

Ref.: 7

3.3.5.3 Chronic (> 12 months) toxicity

3.3.6 Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline: OECD TG 471 (1997)
Species/Strain: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA 102
Replicates: triplicates plates in two separate experiments
Test substance: R0068893B (hair-dye B125 - HC Orange No 6)
Batch: R0068893B 014 L 001 (red powder)
Purity: 98.4% (UV detector)
Solvent: water (at concentrations up to at least 62.50 mg/ml)
Positive controls: yes
Concentrations: experiment I: 0, 5, 16, 50, 160, 500, 1600 and 5000 µg/plate without and with S9-mix. experiment II: 0, 160, 300, 625, 1250, 2500 and 5000 µg/plate with and without with S9-mix
Treatment: direct plate incorporation incubated for 3 days protected from light with and without S9-mix
GLP: in compliance
Study period: 21 November 2013 - 12 February 2014

Results

Experiment 1 treatments of all the tester strains were performed in the absence and presence of S-9, using final concentrations of R0068893B at 5, 16, 50, 160, 500, 1600 and 5000 µg/plate, plus vehicle and positive controls. Following these treatments, evidence of toxicity in the form of a slight thinning of the background bacterial lawn was observed at

5000 µg/plate in strains TA98, TA1537 and TA102 in the presence of S-9. A reduction in the number of revertant colonies was also observed at 5000 µg/plate in strain TA100 in the absence of S-9.

Experiment 2 treatments of narrowed concentration intervals were employed covering the range 160-5000 µg/plate, in order to examine more closely those concentrations of R0068893B approaching the maximum test concentration. In addition, all treatments in the presence of S-9 were further modified by the inclusion of a pre-incubation step, in order to increase the range of mutagenic chemicals that could be detected using this assay system. Following these treatments, evidence of toxicity was observed at 5000 µg/plate in all strains in the absence of S-9 and at 2500 and/or 5000 µg/plate in all strains in the presence of S-9. In addition, a reduction in revertant numbers was observed at 300 µg/plate in strain TA1537 in the absence of S-9, but this was only observed at a single concentration at the lower end of the range. Negative and positive controls were in accordance with the OECD guideline.

Conclusion
R0068893B did not induce gene mutants in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of Salmonella typhimurium when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate in the absence and in the presence of a rat liver metabolic activation system (S-9).

Ref.: 8

In vitro Mammalian Cell Gene Mutation Test (Hprt-locus)

Guideline:	OECD TG 476 (1997)
Cells:	L5178Y tk+/- (3.7.2C) mouse lymphoma cells
Replicates:	duplicate cultures in two independent experiments
Test substance:	R0068893B (hair-dye B125 - HC Orange No 6)
Batch:	R0068893B 014 L 001 (red powder)
Purity:	>95% (UV detector)
Solvent:	DMSO/Water
Positive controls:	yes
Concentrations:	experiment I: 0, 660. 1200, 1400, 1600, 1800 and 2000 µg/mL without S9-mix; 0, 100, 200, 300, 400, 500, 600, 800 and 1000 µg/mL with S9-mix experiment II 0, 500. 750, 1000, 1250, 1500, 1750 and 2000 µg/mL without S9-mix; 0, 200, 400, 500, and 700 µg/mL with S9-mix experiment III: 0, 500. 1000, 2000, 3000, 4000, 4500 and 5000 µg/mL without S9-mix experiment IV: 0, 750. 1500, 2250, 3000, 4000, 4500 and 5000 µg/mL without S9-mix
Treatment:	experiment I: 3 h treatment both with and without S9-mix; expression period 7 days experiment II: 3 h treatment both with and without S9-mix; expression period 7 days experiment III: 3 h treatment without S9-mix; expression period 7 days experiment IV: 3 h treatment without S9-mix; expression period 7 days
GLP:	in compliance
Study period:	5 November 2010 – 30 May 2011

Results
In experiment 1, six concentrations, ranging from 660 to 2000 µg/mL in the absence of a rat liver metabolic activation system S-9 and eight from 100 to 1000 µg/mL in the presence of S-9, were tested. Post-treatment precipitate was observed at 1000 µg/mL in the presence of S-9, but there was again no post-treatment precipitate in the absence of S-9. Seven days after treatment the highest concentrations analysed to determine viability and 6-thioguanine (6TG) resistance were 2000 µg/mL in the absence of S-9 and 1000 µg/mL in the presence of S-9, which gave 74% and 90% of relative survival (RS), respectively.
In experiment 2, seven concentrations, ranging from 500 to 2000 µg/mL in the absence of S-9 and four concentrations, ranging from 200 to 700 µg/mL in the presence of S-9, were tested. Post-treatment precipitate was observed at 700 µg/mL and above in the presence of S-9 but there was again no post-treatment precipitate in the absence of S-9. Seven days after treatment the highest concentrations analysed to determine viability and 6TG resistance were 2000 µg/mL in the absence of S-9 and 700 µg/mL in the presence of S-9, which gave 82% and 89% RS, respectively.
In experiment 3, seven concentrations, ranging from 500 to 5000 µg/mL, were tested in the absence of S-9. Seven days after treatment the highest concentration analysed to determine viability and 6TG resistance was 5000 µg/mL in the absence of S-9, which gave 25% RS.
In experiment 4, seven concentrations, ranging from 750 to 5000 µg/mL, were tested in the absence of S-9. Seven days after treatment the highest concentration selected to determine viability and 6TG resistance was 5000 µg/mL in the absence of S-9, which gave 58% RS.

In experiments 1 and 2, no statistically significant increases in mutant frequency (MF) were observed following treatment with R0068893B at any concentration tested in the absence and presence of S-9 and there were no significant linear trends. In experiment 3, a significant increase in MF was observed at 4500 µg/mL and a statistically significant linear trend was also observed. However, the MF value was less than three times the historical control and no such increase in MF was observed in higher concentration (5000 µg/mL) and the effect was not reproduced in experiment 4. This isolated observation was therefore not considered biologically relevant.

Conclusion
R0068893B (hair-dye B125 - HC Orange No 6) did not induce gene mutations at the *Hprt* locus in L5178Y mouse lymphoma cells when tested under the conditions employed in this study. These conditions included treatments up to the limit of solubility in culture medium (up to 5000 µg/mL - the maximum recommended concentration according to the current regulatory guidelines) in the absence of S-9.

Ref.: 9

SCCS comment:
Only 3 h exposure was used.

Micronucleus Test in Human Lymphocytes

Guideline:	OECD TG 487 (2010)
Cells:	human lymphocytes
Replicates:	duplicate cultures in 2 independent experiments
Test substance:	R0068893B
Batch:	R0068893B 014 L 001 (red powder)
Purity:	>95% (UV detector)
Solvent:	DMSO/Water
Positive controls:	yes
Concentrations and treatment:	
	experiment I: vehicle DMSO (limited solubility)

	3 h treatment + 21 recovery 0, 200, 400, 600, 800, 1000, 1200, 1400, 1500, 1750 and 2000 µg/ml without S9-mix 3 h treatment + 21 recovery 0, 200, 400, 600, 800, 1000, 1200, 1400, 1500 and 1750 with S9-mix 24 h treatment + 0 recovery 0, 200, 400, 600, 800, 1000, 1200, 1400, 1500 and 1750 without S9-mix experiment II: vehicle water (enabling higher concentrations to be tested) 3 h treatment + 21 recovery 0, 250, 500, 1000, 2000, 3000, 4000 and 5000 µg/ml without S9-mix 24 h treatment + 0 recovery 0, 250, 500, 1000, 2000, 3000, 4000 and 5000 µg/ml without S9-mix Treatment: experiment I and II: 3 + 21 hour treatment both with and without S9-mix; test article added at 48 h following culture initiation (stimulation by PHA). Cultures were sampled 3 or 24 h after the beginning of the treatment. (i.e. 72 h after culture initiation)
GLP:	in compliance
Study period:	27 January 2011 – 24 March 2014

Results

R0068893B was tested in an in vitro micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two male donors in two sets of experiments. Treatments covering a broad range of concentrations were performed both in the absence and presence of metabolic activation (S-9) from Aroclor 1254-induced animals. The dimethyl sulphoxide (DMSO) was used as the vehicle. The highest concentrations used in Experiments 1 and 2 were either 5000 µg/mL or were limited by solubility in culture medium and were determined following Range-Finder Experiments 1 and 2. The test article concentrations for micronucleus analysis were selected by evaluating the effect of R0068893B on the replication index (RI). In the Micronucleus Experiments, micronuclei were analysed at three or four concentrations, limited by the observation of post-treatment precipitate under each treatment condition.

Treatment of cells with R0068893B (hair-dye B125 - HC Orange No 6) in the absence and presence of S-9 in Experiment 1 in DMSO and in the absence of S-9 in Experiment 2 in water resulted in frequencies of binucleated cells with micronuclei (MNBN) that were similar to (and not significantly different from) those observed in concurrent vehicle controls at all concentrations analysed. The MNBN cell frequencies of all R0068893B treated cultures under all treatment conditions in Experiments 1 and 2 fell within the normal ranges.

Conclusion

R0068893B (hair-dye B125 - HC Orange No 6) did not induce micronuclei in cultured human peripheral blood lymphocytes in the absence and presence of S-9. The maximum concentration analysed under all treatment conditions was either 5000 µg/mL (the maximum concentration recommended by the test guidelines) or was limited by the solubility of the test article in culture medium.

Ref.: 10

SCCS overall comment on mutagenicity

Under the experimental conditions used, HC Orange No 6 did not show gene mutations and did not induce an increase in micronucleated cells and, consequently, is not considered to be mutagenic, clastogenic and/or aneugenic in human lymphocytes.

3.3.6.2 Mutagenicity / Genotoxicity in vivo

3.3.7 Carcinogenicity

3.3.8 Reproductive toxicity

3.3.8.1 Two generation reproduction toxicity

3.3.8.2 Other data on fertility and reproduction toxicity

3.3.8.3 Developmental Toxicity

Prenatal Developmental Toxicity Study in Rats: dose-range finding study

Guideline:	OECD TG 414 (2001)
Species/strain:	Rat, Wistar Hannover
Group size:	10 females/group
Test substance:	R0068893B
Batch:	R0068893B 015 D 001
Purity:	91.8±1 %
Vehicle:	deionized water
Dose levels:	0, 100, 300 and 1000 mg/kg bw/day
Dose volume:	4 mL/kg bw
Route:	Oral
Administration:	Gavage
GLP:	In compliance
Study period:	5 October 2011- 16 October 2013

The test solutions (at 100, 300 and 1000 mg/kg bw/day) or deionized water were administered to the mated females rats daily (control group- 8 animals, group 100 mg/kg/day9 animals, group 300 mg/kg/day-9 animals, group 1000 mg/kg/day-6 animals), by gavage, from implantation until one day prior to the scheduled caesarean section (i.e. from day 6 to day 19 of gestation). A constant dose-volume of 4 mL/kg was used.

Results

No chemical analysis of the test item solutions was carried out. The stability, homogeneity and concentration of the test solutions were not determined for this study.

No mortality or clinical symptoms were observed in any of the groups.

No biologically or statistically significant differences in body weight, body weight gain or food consumption were observed between the exposed and the control group.

A small but not statistically and biologically significant decrease in corrected maternal body weight was observed in all treated dams.

No biologically relevant changes were observed in reproduction parameters between the control and the exposed dams. There were no changes observed at necropsy in either treated or control dams.

Foetal evaluation revealed no changes in body weights and placental weights between the exposed and control groups. Similarly no external alterations were noted in foetuses from the exposed or control dams.

Conclusion
Based on the results the authors of the report recommended the following doses of the test item for the prenatal developmental oral toxicity study in rats: 100, 300 and 1000 mg/kg/day.

Ref: 1

Prenatal Developmental Toxicity Study in Rats

Guideline:	OECD TG 414 (2001)
Species/strain:	Rat, Wistar Hannover
Group size:	25 females/group
Test substance:	R0068893B
Batch:	R00688938 015 0 001
Purity:	91.8±1 %
Vehicle:	deionized water
Dose levels:	0, 100, 300 and 1000 mg/kg bw/day
Dose volume:	4 mL/kg bw
Route:	Oral
Administration:	Gavage
GLP:	In compliance
Study period:	24 November 2011- 2 February 2015

The dose levels were based on the findings in the 14-day dose-range finding study.
The test solutions (at 100, 300 and 1000 mg/kg bw/day) or deionized water were administered to the mated females rats daily (25/group), by gavage, from implantation until one day prior to the scheduled caesarean section (i.e. from day 6 to day 19 of gestation). A constant dose-volume of 4 mL/kg was used. Animals that were not pregnant were excluded from calculations regarding mean maternal bodyweights, food consumption and gestational parameter (n= 19, 19, 20 and 23, for 0, 100, 300 and 1000 mg/kg bw/day, respectively). ln addition, one rat from the control group with total resorptions was excluded from the calculation of mean body weight, body weight gain, food consumption, corrected maternal body weight and uterine weight. Furthermore, the proportion of live foetuses was calculated excluding this dam with total resorptions.

Results
The stability of the substance was confirmed with the certificate of analysis provided by the sponsor. Test solutions were prepared at the lowest and highest concentrations. Duplicate samples were taken on the day of preparation and after 4 and 7 days storage at +2°C to +8°C. The concentration of the test item in control and test solutions was determined at the beginning and at the end of the treatment period. The acceptance criterion with regard to both stability and concentration at each time-point was that mean concentration was ±10% of the nominal value. All values were within the acceptable range. The test solutions were prepared daily and administered within 2 hours after preparation.

No mortality was observed in any group during the study.

Body weight and body weight gain measurements showed a decrease in body weight gain in dams given 1000 mg/kg/day between days 6 and 12 (23%), with subsequent recovery. This resulted in a slight decrease in overall body weight gain (-7.1% and -18.8% without and with correction for uterus weight, respectively). Although the changes did not reach statistical significance, according to the authors of the study report they were likely to be treatment related and an indication of slight maternal toxicity.

Corrected body weight gain measurements revealed a higher mean unopened uterine weight in the dams exposed to 100 and 300 mg/kg/day compared to the control group (+16.4% and +17.6%, respectively). These differences were not statistically significant and

were considered by the authors of the report to be related to the higher number of foetuses in both groups, therefore being not related to the treatment. Corrected maternal body weight gain was lower in dams exposed to 300 and 1000 mg/kg/day (-20.9% mid and -18.8% high dose, when compared to the controls). According to the authors of the report, the changes were likely to be treatment related and were considered to be of toxicological significance, despite the fact that they did not achieve statistical significance or show any dose-related trend.

Food consumption measurements did not reveal any biologically or statistically significant changes between the groups.

No significant effects were observed in the gestational parameters or maternal necropsy findings in any treated group when compared to the control group.

No changes in mean placenta weight were observed when compared to control values. Although mean foetal body weight was slightly and not significantly lower at 1000 mg/kg/day in comparison to the control values, the authors of the report considered the effect to be attributed to dosing with R0068893B.
External examinations of the foetuses, except one foetus excluded from the analysis due to spontaneous, non-test-item related malformations, did not reveal any variations or malformations in control or exposed dams.
No soft tissue malformations were observed in treated or control foetuses.
The total number of soft tissue variations was significantly higher in foetuses from dams exposed to 1000 mg/kg/day compared to the control group. This was primarily due to the higher incidence of bilateral dilatation of the renal pelvis, which was significantly different from controls on a foetal (+9.7%) and litter (+36.4%) basis. Since these changes occurred in the highest dose group and because the incidence values were outside of the historical control range, they were considered by the authors of the report to be related to the administration of R00688938 and to be secondary to the maternal toxicity observed at 1000 mg/kg/day.
The few skeletal malformations observed in treated and control foetuses were of the type and incidence commonly observed in rats of this strain and were evenly distributed among groups. Therefore they were considered by the authors of the report to be unrelated to exposure to R00688938.

Applicant's summary of foetal skeletal variations and retardations observed in the study is presented below:

Foetal skeletal variations:

- The litter incidence of supernumerary ribs was significantly higher in all treated groups, while the foetal incidence was significantly higher only at 1000 mg/kg/day. The foetal incidence remained within historical control data range, although for litters it was outside this range. Despite the differences observed in litter incidence, these changes did not show any dose-relationship and the associated changes in foetal incidence were of low magnitude;
- Foetal and/or litter incidences of some other skeletal variations were lower than for controls in some or all groups given R006688938 (e.g. bipartite sternebrae, enlarged fontanel). According to the authors of the report, these changes were considered to be unrelated to exposure to R00688938 and of no biological/toxicological significance;
- A higher incidence of dumbbell-shaped sternebrae was observed in foetuses from dams exposed to 300 and 1000 mg/kg/day. Similar changes in incidences of dumbbell-shaped thoracic vertebrae were observed in foetuses at 1000 mg/kg/day. These incidences at 1000 mg/kg/day were significantly increased over control values, except for the foetal incidence of dumbbell-shaped sternebrae;
- The incidence values of dumbbell-shaped thoracic vertebrae were within historical control range and these findings were thus considered to be unrelated to the

administration of R00688938. In contrast, the increased incidences of dumbbell-shaped sternebrae at 300 and 1000 mg/kg/day were considered treatment related since the incidence values observed are outside the historical control range.

Foetal skeletal retardations:

- The incidences of incomplete ossification of interparietal bone in foetuses from dams exposed to 300 and 1000 mg/kg/day were significantly higher than in the control group. According to the authors of the report, these high incidences were considered to be unrelated to treatment since they remained within historical control data range;
- A higher foetal incidence of unossified metacarpals was noted at 300 and 1000 mg/kg/day, statistically significant at 1000 mg/kg/day. This finding was considered by the authors of the report to be treatment related at 1000 mg/kg/day since the incidence value observed was outside historical control range;
- At 300 mg/kg/day, higher incidences of unossified caudal vertebrae, incomplete parietal bone ossification and incomplete ossification of sacral vertebrae were noted, whereas the incidence of incomplete supraoccipital bone ossification was higher at 100 and 300 mg/kg/day, compared to the control group. These changes reached statistical significance but were considered by the authors of the report to be unrelated to treatment in the absence of dose-related relationship, specifically in the absence of similar changes at the highest dose level of 1000 mg/kg/day.

Conclusion

Based on the above results, the authors of the study report established the No Observed Adverse Effect Level (NOAEL) for maternal toxicity of R0068893B at 100 mg/kg/day and the NOAEL for foetal developmental toxicity at 300 mg/kg/day.

Ref.: 2

SCCS comment

The study showed that the corrected maternal body weight gain was lower in dams exposed to 300 and 1000 mg/kg/day (-20.9% mid and -18.8% high dose, when compared to the controls). SCCS agrees that the changes could be treatment related and should be considered to be of toxicological significance, despite the fact they did not achieve statistical significance or show any dose-related trend. Therefore the NOAEL for maternal toxicity can be established at 100 mg/kg.

The SCCS agrees that the test item R00688938 did not show any teratogenic potential.

Most foetal skeletal variations were observed at 1000 mg/kg/day and their incidences were outside of historical control range. Moreover, they were associated with other foetus developmental disturbances (e.g. decreased body weight). Some foetal skeletal variations which were observed at 100 and 300 mg/kg were most likely caused by retarded ossification due to slight maternal toxicity at these doses. Therefore, they should not be treated as toxicologically relevant, even if they were statistically significant. The SCCS agrees that the NOAEL value for developmental toxicity can be established at 300 mg/kg/day.

3.3.9 Toxicokinetics

No data

3.3.9.1 Toxicokinetics in laboratory animals

3.3.9.2 Toxicokinetics in humans

3.3.10 Photo-induced toxicity

3.3.10.1 Phototoxicity / photo-irritation and photosensitisation

/

3.3.10.2 Photomutagenicity / photoclastogenicity

/

3.3.11 Human data

/

3.3.12 Special investigations

/

3.3.13 Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

(non-oxidative conditions)
(on-head concentration 0,5 %)

Absorption through the skin	**A**	**=**	**0.201 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.12 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**1.94x10^{-3} mg/kg bw**
No observed adverse effect level (prenatal, developmental, oral, rat)	**NOAEL**	**=**	**100 mg/kg bw/d**
Bioavailability 50%*		**=**	**50 mg/kg bw/d**
Margin of Safety	**adjusted NOAEL/SED**	**=**	**25773**

* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.14 Discussion

Physicochemical properties

The SCCS notes the presence of ethanol in the batches used for the chemical analysis. Since Vibracolor Copper Orange is in the form of a methanesulfonate salt, any potential methane sulfonate impurities such as ethyl methanesulfonate (which may be formed in the presence of ethanol) should be reported and quantified.

General toxicity

No acute oral toxicity study with R0068893B was submitted but repeated dose studies did not show the potential for acute toxicity.

Daily administration of 100, 300 and 1000 mg/kg bw/day R0068893B by oral gavage to male and female Wistar Hannover rats for 90 days resulted in a treatment-related decrease in body weight and body weight gain at 1000 mg/kg bw/day when compared to controls (body weight gain: -18% in males and -19 % in females). Therefore the NOAEL for this study can be established at 300 mg/kg.

Developmental toxicity

Some foetal skeletal variations which were observed at 100 and 300 mg/kg/day were most likely caused by retarded ossification due to slight maternal toxicity at these doses. The SCCS concluded that the NOAEL value for developmental toxicity can be established at 300 mg/kg/day.

The study showed that the corrected maternal body weight gain was lower in dams exposed to 300 and 1000 mg/kg/day (-20.9% mid and -18.8% high dose, when compared to the controls). SSCS agree that the changes could be treatment related and should be considered to be of toxicological significance, despite the fact they did not achieve statistical significance or show any dose-related trend. Therefore the NOAEL for maternal toxicity can be established at 100 mg/kg.

Irritation/sensitisation

The *in vitro* tests did not indicate skin irritancy. While an eye irritation potential at 20% cannot be excluded, it can be assumed that this will be of no concern in view of the 0.5% intended use concentration.

The *in vivo* tests did not indicate a skin-sensitising potential.

Dermal absorption

The submitted documents indicate a very low rate of dermal penetration. Because of the relatively short exposure time in the test system, the mean + 2 SD is used for the calculation of the MoS.

Mutagenicity

The genotoxicity of HC Orange No. 6 was investigated in the three endpoints of genotoxicity: gene mutations, structural chromosome aberrations and aneuploidy. Di-[2-[(E)-2-[4-[bis(2-hydroxyethyl)aminophenyl]vinyl]pyridin-1-ium]-ethyl]disulfide dimethanesulfonate did not induce gene mutants in five strains of Salmonella typhimurium (TA98, TA100, TA1535, TA1537 and TA102) up to concentration 5000 µg/plate in the absence and in the presence of a rat liver metabolic activation system (S-9 mix), it did not induce gene mutants at the *Hprt* locus in L5178Y mouse lymphoma cells in the absence or presence of S-9 mix up to 5000 µg/mL and did not inducemicronuclei in cultured human peripheral blood lymphocytes either in the absence or the presence of S-9 mix.

Thus, Di-[2-[(E)-2-[4-[bis(2-hydroxyethyl)aminophenyl]vinyl]pyridin-1-ium]-ethyl] disulfide dimethanesulfonate can be considered to have no genotoxic potential and additional tests are unnecessary.

4. CONCLUSION

(1) In light of the data provided, does the SCCS consider HC Orange No. 6 (B125) safe when used in non-oxidative hair colouring products at a maximum concentration of 0.5%?

The SCCS considers HC Orange No. 6 (B125) safe when used in non-oxidative hair colouring products at a maximum concentration of 0.5%.

(2) Does the SCCS have any further scientific concerns with regard to the use of HC Orange No. 6 (B125) in cosmetic products?

The SCCS has concerns regarding a potential presence of methanesulfonates impurities, in particular ethyl methanesulfonate. Information on these impurities has not been provided.

5. MINORITY OPINION

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6. REFERENCES

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