Scientific Committee on Consumer Products

SCCP

# OPINION ON

# HC Blue n° 11

COLIPA n° B77

The SCCP adopted this opinion at its 13th plenary meeting on 2 October 2007

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 Products (SCCP), 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 Evaluation Agency (EMEA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCP
Questions concerning the safety of consumer products (non-food products intended for the consumer).
In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

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Opinion to be cited as: Opinion of the SCCP on HC Blue n° 11

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## 1. BACKGROUND

Submission I for HC Blue n° 11, with the chemical name 1-[(2'-methoxyethyl)amino]-2-nitro-4-[di-(2'-hydroxyethyl)amino]benzene, was submitted in March 1992 by COLIPA[1, 2].

The Scientific Committee on Cosmetology (SCC) adopted the opinion (SCC/1079/93) with the conclusion, *"Classification: B. Industry should provide data on skin sensitization potential from in-use data in the context of the volume used, together with any available information on the toxicological profile of the compound, e.g. from animal studies and/or, from experience in use in either the consumer on occupational context."*

Submission II for HC Blue n° 11 was submitted in December 1993 by COLIPA[2]. The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) expressed its opinion (SCCNFP/0184/99) at the plenary meeting of 23 June 1999 with the conclusion: *"Classification : 1 under the conditions in use : in oxidative hair dyes at a maximum concentration of 3.0%, in combination with hydrogen peroxide the maximum use concentration upon application is 1.5%; In hair tinting products and colouring setting lotions the concentration of Methoxyblau should not exceed 2.0%."*

The substance and its salts are currently regulated by the Cosmetics Directive (76/768/EC), Annex III, Part 2 under entry 9 on the List of provisionally allowed substances, which cosmetic products must not contain except subject to restrictions and conditions laid down.

According to the current submission III, submitted by COLIPA in July 2005, HC Blue n° 11 is used as an ingredient in direct hair dye formulations in concentrations up to 2%.

Submission III presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

## 2. TERMS OF REFERENCE

1. *Does the Scientific Committee on Consumer Products (SCCP) consider HC Blue n° 11 safe for use as an ingredient in direct hair dye formulation with an on-head concentration of maximum 2.0 % taking into account the scientific data provided?*

2. *Does the SCCP recommend any restrictions with regard to the use of HC Blue n° 11 in non-oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

[2] According to the records of COLIPA

## 3. OPINION

3.1. Chemical and Physical Specifications

### 3.1.1. Chemical identity

#### 3.1.1.1. Primary name and/or INCI name

HC Blue n° 11 (INCI)

#### 3.1.1.2. Chemical names

Ethanol, 2,2'-[[4-[(2-methoxyethyl)amino]-3-nitrophenyl]imino]bis- (CAS -9CI)
2-((2-Hydroxyethyl){4-[(2-methoxyethyl)amino]-3-nitrophenyl}amino)ethanol (IUPAC)
2,2'-[[4-[(2-methoxyethyl)amino]-3-nitrophenyl]imino]diethanol
1-((2'-Methoxyethyl)-amino)-2-nitro-4-(di(2'- hydroxyethyl)-amino)-benzene

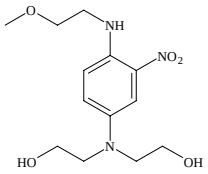
#### 3.1.1.3. Trade names and abbreviations

COS 338
Methoxyblau
COLIPA n° B 077

#### 3.1.1.4. CAS / EINECS number

CAS: 23920-15-2
ELINCS: 459-980-7 (HC Blue n° 11)

#### 3.1.1.5. Structural formula




#### 3.1.1.6. Empirical formula

Formula: $C_{13}H_{21}N_3O_5$
Available as free base and hydrochloride *(Submission 1, 1992)*

### 3.1.2. Physical form

Dark brown fine powder

### 3.1.3. Molecular weight

Molecular weight: 299.32 (free base)

### 3.1.4. Purity, composition and substance codes

Batch I-37979

Chemical characterisation by NMR and IR
NMR purity: 97% (w/w)
HPLC purity: 99.5% (peak area at 262 nm)
Water: 0.16% (w/w)
Sulfated ash: 0.07% (w/w)

Ref.: 2

**Declaration by the applicant**
The batch of COLIPA B77 used in the acute oral toxicity test is not fully analytically described. However, information is available from the laboratories that have synthesized this batch concerning the identity and purity of the material produced at that time. From this information it can be concluded that the former not fully described batch is representative and its specification is quite similar to the fully characterized batch I-37979.

### 3.1.5. Impurities / accompanying contaminants

| | | |
|---|---|---|
| N-Nitrosodiethanolamine: | 1.6 ppm | (EU CMR classification: carcinogenic category 2) |
| Pb | < 20 ppm | |
| Ni and Sb | < 10 ppm | |
| As and Cd | < 5 ppm | |
| Hg | < 1 ppm | |

Ref.: 1, 2

### 3.1.6. Solubility

Water: 3 g/l (room temperature)
Ethanol: 170 g/l (room temperature)
DMSO: 250 g/l (room temperature)

Ref.: 13

### 3.1.7. Partition coefficient (Log $P_{ow}$)

Log $P_{ow}$: 0.38 (calculated, Syracuse Vers. 1.66)

### 3.1.8. Additional physical and chemical specifications

| | |
|---|---|
| Melting point: | 61-71°C |
| Boiling point: | / |
| Flash point: | / |
| Vapour pressure: | / |
| Density: | / |
| Viscosity: | / |
| pKa: | / |
| Refractive index: | / |
| UV-VIS Spectra (200-800nm): | $\lambda_{max}$ 264 nm and 555 nm |

### 3.1.9. Homogeneity and Stability

1 mg/ml solution of HC Blue n° 11 in 0.9% HCl was stable over a period of 24 h at room temperature – a decline in the concentration by 3.8% was noted over the period.

**General Comments to physico-chemical characterisation**

- The solubility of HC Blue n° 11 in water is not determined according to the Official EC Method. Thus exact solubility in water is not known

- Log $P_{ow}$: calculated values cannot be accepted as estimates of the true physical constant without justification, indicating that the reported values are realistic.
- HC Blue n° 11 is both a secondary and tertiary amine. The nitrosamine (N-Nitrosodiethanol-amine) content in batch I-37979 is reported to be 1600 ppb. The nitrosamine content in HC Blue n° 11 should be below 50 ppb. It should not be used in combination with nitrosating agents.
- No data on the stability of HC Blue n° 11 in marketed products was provided.

## 3.2. Function and uses

HC Blue n° 11 is used as a direct dye for hair colouring products without mixing with an oxidising agent. The final concentration on head of HC Blue n° 11 can be up to 2.0%.

## 3.3. Toxicological Evaluation

### 3.3.1. Acute toxicity

#### 3.3.1.1. Acute oral toxicity

Guideline: /
Species/strain: Rat, Wistar strain
Mouse, CF1
Group size: Rat: 6 of each sex
Mouse: 10 females
Test substance: 1-(2-Methoxyethyl)-amino-2-nitro-4-[di(2-hydroxyethyl)amino]-benzene
Batch: Not specified
Purity: Not reported
Dose: Rat, 1000, 1250 or 1500 mg/kg bw
Mouse 1000, 1250, 1500 or 1750 mg/kg bw
Vehicle: deionised water
GLP: Not in compliance

In an acute oral toxicity study, the test material (purity and batch not reported) was administered by gavage to Wistar rats (6/sex/dose) at doses of 1000 – 1500 mg/kg bw and to female CF1 mice (10/dose) at doses of 1000-1750 mg/kg bw. During a two weeks observation period, mortalities and clinical-toxicological observations were recorded daily and body weight was recorded weekly. Macroscopic examination was performed in all animals found dead or after terminal sacrifice.

Results
The mortalities observed in the experiment are shown in the table below. The main symptom of toxicity was reduced activity. The test substance caused a blue colouration of extremities and urine. No changes were found in any of the organs. The surviving animals showed body weight gain over the test period.

| species and sex | dose in mg/kg bw | mortality |
|---|---|---|
| **female mice, CF1** | 1750 | 10 / 10 |
| | 1500 | 8 / 10 |
| | 1250 | 4 / 10 |
| | 1000 | 2 / 10 |

| species and sex | dose in mg/kg bw | mortality |
|---|---|---|
| **female rat, Wistar** | 1500 | 6 / 6 |
| | 1250 | 1 / 6 |
| | 1000 | 2 / 6 |
| **male rat, Wistar** | 1500 | 6 / 6 |
| | 1250 | 3 / 6 |
| | 1000 | 0 / 6 |

The $LD_{50}$ was calculated to be 1250 mg/kg bw in male and female rats and 1275 mg/kg bw in female mice.

Ref.: 3

Comment
The study design resembles that of OECD Guideline 401. No information on the purity or batch of the test material is provided. In the submission it is stated that, on the basis of information from the laboratories that have synthesized the test material at that time, it can be concluded that the used batch is representative and its specification is quite similar to the fully characterized batch I-37979. The purity of I-37979 is reported to be 99.5 area % (HPLC).

Although the study is not performed according to modern standards, it is useful for evaluation.

#### 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
Species: New Zealand White rabbit
Group: 3 males
Substances: B077 (SAT040260)
Batch: I-37979
Purity: 99.5% (HPLC)
Dose: 0.5g under semi-occlusion
GLP: in compliance

Three animals were used in the test. Each animal served as its own control. Approximately 24 hours prior to the treatment, the dorsal fur was shaved, to expose an area of about 150 $cm^2$. Each animal was treated by dermal application of 0.5 grams of the test substance. The test substance was moistened and applied to the skin of one flank, using a semi-occlusive patch of 2x3 cm. The patch was mounted on tape which was wrapped around the abdomen and secured with an elastic bandage.
Animals were examined for signs of erythema, eschar and oedema formation at approximately 1 hour, 24, 48 and 72 hours after termination of the exposure.

Results
Under the conditions of the study, the undiluted test substance produced no visible reactions to the skin at any time point. Purple staining of the treated skin by the test

substance was observed throughout the observation period but this did not hamper the scoring of the skin reactions.

Conclusion
Under the conditions of the study, B077 was not irritant when applied to the intact rabbit skin.

Ref.: 4

### 3.3.2.2. Mucous membrane irritation

| | |
|---|---|
| Guideline: | OECD 405 |
| Species: | New Zealand White rabbit |
| Group: | 3 males |
| Substances: | B077 (SAT040260) |
| Batch: | I-37979 |
| Purity: | 99.5% (HPLC) |
| Dose: | 62 mg (volume approximately 0.1ml) |
| GLP: | in compliance |

The equivalent of 0.1 ml of the test substance was instilled into the conjunctival sac of one eye of the test animals. The substance remained in permanent contact with the eyes until rinsing with warm tap water, 24 hours after instillation. The other eyes served as controls.
The eye irritation reactions were scored approximately 1 hour, 24, 48 and 72 hours and 7 days after instillation of the test solution.

Results
Instillation of the test substance into one eye of each of three rabbits resulted in irritation of the conjunctivae, which consisted of redness and chemosis. The irritation had completely resolved in one animal within 48 hours and in two animals within 72 hours. No iridial irritation or corneal opacity were observed, and treatment of the eyes with 2% fluorescein, 24 hours after test substance instillation revealed no corneal epithelial damage.

Conclusion
Under the conditions of the study, the undiluted test substance was irritating to the rabbit eye.

Ref.: 5

## 3.3.3. Skin sensitisation

**Local Lymph Node Assay (LLNA)**

| | |
|---|---|
| Guideline: | OECD 429 |
| Species: | Mice; strain CBA/CaOlaHsd |
| Group: | Three dose groups and one control group; 4 females in each |
| Substances: | B077 (SAT040260) |
| Batch: | I-37979 |
| Purity: | 99.5% (HPLC) |
| Dose: | 5, 10 and 25% solutions in ethanol:water (7:3 v/v) |
| GLP: | in compliance |

The dermal sensitization properties of B077 were investigated in healthy mice of the CBA/CaOlaHsd strain. Three dose groups and a control group (receiving the vehicle only) of four female mice each, were chosen. The test item was topically applied to the dorsal surface of the ears to analyse the sensitization activity by measuring the proliferative response of lymph node cells.
A homogenous dilution of the test item in a mixture of ethanol:water (7:3 v/v) was made shortly before each dosing.

Ethanol:water (7:3 v/v) was used because the test material shows a higher solubility in this vehicle than in the normally used acetone:olive oil (4:1 v/v). In addition, the used vehicle does not chemically react with B077 as suspected for acetone or other organic solvents.
The highest technically applicable, non-irritating test item concentration was found in a pre-test with four mice. Based on these test results 5%, 10% and 25% solutions were chosen for the main study.
Each test group of mice was treated by topical (epidermal) application to the dorsal surface of each ear lobe (left and right) with the different test item concentrations. The application volume, 25 µl, was spread over the entire dorsal surface of each ear lobe once daily for three consecutive days. The control group was treated with the vehicle exclusively. Five days after the first topical application, all mice were administered with radio-labelled thymidine ($^3$HTdR) by intravenous injection via the tail vein.
Approximately five hours after $^3$HTdR application all mice were euthanized. The draining lymph nodes were excised and pooled for each experimental group. After preparation of the lymph nodes, disaggregation and overnight precipitation of macromolecules, these precipitations were re-suspended and transferred to scintillation vials.
The level of $^3$HTdR incorporation was then measured by scintillation counting. The proliferative response of lymph node cells is expressed as the ratio of $^3$HTdR incorporation into lymph node cells of treated animals relative to that recorded in control mice (stimulation index).
An appropriate reference (α-hexylcinnamaldehyde) was used as positive control to demonstrate the sensitivity of the test system.
The proliferative capacity of pooled lymph node cells was determined by quantifying the incorporation of $^3$H-methyl thymidine.

Results
No symptoms of local toxicity at the ears of the animals and no systemic toxicity was observed during the study up to the highest tested concentration of 25% (w/v).

| ***Test Item Concentration*** | ***S.I.*** |
|---|---|
| **5% (w/v)** | 1.1 |
| **10% (w/v)** | * |
| **25% (w/v)** | 1.2 |

* The data is not available due to partial spillage of the lymph node suspension labelled with $^3$HTdR during preparation of cell suspensions.

The Stimulation Index (S.I.) was below 3 in all dose groups. No dose response relation was noted. Calculation of the EC3 value was not performed as none of the test concentrations produced a stimulation index of 3 or above.

The positive control (α-hexylcinnamaldehyde) induced an increase of the stimulation index of 1.5, 2.3 and 8.4 at concentrations of 5, 10 and 25% (w/v), respectively, in acetone:olive oil, 4:l (v/v). An EC3 value of 11.7% (w/v) was derived.

Conclusion
Under the conditions of the study, B077 was found to be a non-sensitizer when tested up to the highest achievable concentration of 25% (w/v) in ethanol:water (7:3 v/v).

Ref.: 6

### 3.3.4. Dermal / percutaneous absorption

**Dermal absorption / percutaneous penetration out of a basic hair dyeing formulation *in vitro* (excised pig skin)**

| | |
|---|---|
| Guideline: | OECD 428 |
| Species: | Pig |
| Group: | 1 male, 1 female; dermatomed skin of 0.75mm |
| Substances: | B077 (SAT040260) |
| Batch: | I-37979 |
| Purity: | 99.5% (HPLC) |
| Dose: | 0.4 mg/cm² skin within a i) direct dye formulation and ii) in a water:ethanol (3 :1) solution; 8 replicates per experiment. |
| GLP: | in compliance |

The dermal absorption/percutaneous penetration of B077 out of a standard direct dye cream and a water:ethanol (3:1) solution was studied on the clipped excised skin of two young pigs (one male, one female).
The skin integrity of the frozen (–20°C) skin discs was checked by measuring the trans-dermal electrical resistance. The intact, clipped excised pig skin of the flanks area was exposed for 30 minutes to the test substance in the standard direct hair dyeing cream and the water:ethanol (3:1) solution without occlusion. The content of B077 in both application formulations was 2%.
The dermal absorption/percutaneous penetration of the test substance was investigated in about 20 mg direct dye formulation per cm² pig skin. Therefore the resulted dose of the test substance was approx. 0.4 mg/cm² skin. Skin discs of 1.0 cm² were exposed to the formulations for 30 minutes, terminated by gently rinsing with a 0.01% Tween 80 solution and water.
Each the formulation and the solution were analysed in two experiments with eight replicates per experiment for adsorbed, absorbed and penetrated amount of the test substance. The receptor fluid used was Dulbecco´s phosphate buffered saline (pH 7.35). In the static system, samples of the receptor fluid were drawn before the application of the test substance formulation and 0.5, 1, 2, 4, 6, 24, 29 and 48 hours after application. The removed volume was replaced by fresh receptor fluid.

The composition of the direct dye cream is shown in the table below.

| Ingredient | Conc. in % |
|---|---|
| COLIPA n° B 077 | 2.00 |
| Hydrenol D | 6.00 |
| Lorol techn. | 6.00 |
| Eumulgin B1 | 3.00 |
| Eumulgin B2 | 3.00 |
| PHB-methyl ester | 0.30 |
| PHB-propyl ester | 0.20 |
| Phenoxy ethanol | 1.00 |
| Polidiol 400 | 5.00 |
| Eumulgin RH 40 | 1.00 |
| Natrosol 250 HR | 1.00 |
| NaOH | for pH adjustment |
| Tartaric acid (saturated) | for pH adjustment |
| Water | ad 100 |
| | pH 9.0 |

Results
The quantities that had penetrated during the 30 minute exposure to B77 containing formulations and within 48 hours after application, are shown in the following table. Both the amounts absorbed and penetrated were taken as systemically available.

| Analysed Sample | **Direct dye cream** | | **Water : ethanol (3:1)** | |
|---|---|---|---|---|
| | **[% of dose]** | **[µg/cm²]** | **[% of dose]** | **[µg/cm²]** |
| Skin rinsings | 91.1 | - | 92.4 | - |
| Adsorption (stratum corneum) | 0.13 | 0.54 | 0.61 | 2.31 |
| Not Bio-available | 91.2 | - | 93.0 | - |
| Absorption (epidermis/dermis) | 0.30 | 1.22 | 0.53 | 2.02 |
| Penetration (receptor fluid) | 0.26 | 1.06 | 0.04 | 0.17 |
| Bio-available | 0.56 | 2.27 | 0.58 | 2.19 |
| Total recovery / mass balance | 91.3 | - | 93.7 | - |

In Direct Dye formulation: 0.558 ± 0.273 %; 2.27 ± 1.12 µg/cm². $A_{max}$ 1.0183%; 3.993 µg/cm².

In ethanol:water solution: 0.579 ± 0.159 %; 2.19 ± 0.59 µg/cm². $A_{max}$ 0.877%; 3.367 µg/cm².

Conclusion
In these *in vitro* dermal penetration studies the amount of B077 systemically available from a standard direct hair dye formulation was found to be 2.27 ± 1.12 µg/cm² with an $A_{max}$ of 3.993 µg/cm², which should be used for calculating the MOS.

Ref.: 13

Comment
Although the study was not performed according to the SCCP Notes of Guidance (skin from only 2 donors was used), the study was well performed and accepted for evaluation.

## 3.3.5. Repeated dose toxicity

### 3.3.5.1. Repeated Dose (28 days) oral / dermal / inhalation toxicity

No data submitted

### 3.3.5.2. Sub-chronic (90 days) oral / dermal / inhalation toxicity

| | |
|---|---|
| Guideline: | OECD 408 (1998) |
| Species/strain: | Rat, Wistar Hannover strain HsdBrlHan:Wist |
| Group size: | 10 animals per sex and dose, 5 per sex in recovery groups |
| Test Substance: | B 077 |
| Batch: | I-37979 |
| Purity: | 99.5% (HPLC) |
| Dose levels: | 0, 80, 160, 320 mg/kg bw/day (in an aqueous solution containing 0.5% carboxymethylcellulose) |
| Route: | Oral, gavage |
| Exposure period: | 13 weeks, 4 weeks recovery |
| GLP: | in compliance |

Wistar rats (10/sex) were treated with B 077 for 91 consecutive days by oral gavage at doses of 0, 80, 160 or 320 mg/kg bw/day. Additionally, 10 rats (5 per sex) in both a control and a high dose group were assessed for recovery of treatment-related effects, four weeks after the last administration. The animals were checked daily for mortality and signs of intoxication. A detailed clinical assessment (including neurotoxicity parameters), body weight and food consumption were recorded weekly. Motor activity and sensory reactivity was assessed during week 12 and during week 4 of the recovery period. Haematology, clinical chemistry and urinalysis were performed in week 13. Ophthalmoscopy was performed in week 13 on all control and high-dose animals. At termination the animals were killed, macroscopied and selected organs were weighed. An extensive range of organs and tissues was histologically examined.

Results
One female of the control group died due to mis-dosing. No toxicologically relevant effects on clinical signs, neurotoxicity parameters, motor activity, ophthalmoscopy, body weight gain and food consumption were observed. An increase in white blood cell count was observed in the high dose females at the end of treatment and recovery periods. Increases, generally statistically significant, in bilirubin, total cholesterol, triglycerides and urea were observed in the high dose animals at the end of the treatment period. A very slight, but statistically significantly decrease in sodium level was also seen in high dose males. These animals recovered from these changes except for bilirubin in the females. An increase in specific gravity of the urine in all animals receiving the test material was probably due to excretion of the test substance or its metabolite via urine and was considered not toxicologically significant.

Absolute and relative kidney weights were statistically significantly increased (up to 24%) in all females treated with the test item at the end of the treatment period and the change was still present at the end of recovery period. Histological examination of the kidneys revealed statistically significant increases in tubular cell vacuolation, mainly localised in the medullary region and sometimes extending to the inner cortex, and yellow-brown, intra-cytoplasmic pigmentation in the mid-and high-dose groups.

Absolute and relative liver weights were statistically significantly increased (up to 26%) in a dose-related manner in all treated males and in high dose females at the end of the treatment period. Histological examination of the liver revealed statistically significant centrilobular hepatocytic hypertrophy in animals of the high-dose group.

A generalised violet coloration on the skin of the animals from all treated groups, and of the stomach and content of the urinary bladder of some high dose animals were not regarded as adverse.

Conclusion
The study authors considered the NOAEL to be 160 mg/kg bw/day.

Ref.: 2

Comment
It is not clear on which basis the study authors dismiss the statistically significant changes in kidney histology and kidney weight observed in the mid-dose animals.
In the females of the lowest dose group, also histological changes in the kidneys are observed (tubular cell vacuolisation), and kidney weight is slightly increased, but not statistically significant. This should be taken into consideration when calculating the Margin of Safety.

On the basis of the histological changes in the kidneys, in the mid and high dose animals, accompanied by increases in absolute and relative kidney weights, the SCCP sets the NOAEL at 80 mg/kg bw/day.

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 gene mutation assay**

| | |
|---|---|
| Guideline: | OECD 471 |
| Species/strain: | *Salmonella typhimurium*, TA1535, TA1537, TA98, TA100 and TA102 |
| Replicates: | Three plates per concentration in two independent experiments |
| Assay conditions: | Plate incorporation (experiment I) and pre-incubation (experiment (II) method, both in the presence and absence of phenobarbital and beta-naphthoflavone induced rat liver S9-mix. |
| Test substance: | B 077 |
| Batch: | I-37979 |
| Purity: | 99.5% (HPLC) |
| Concentrations: | 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate |
| Solvent: | DMSO |
| GLP: | In compliance |

B 077 was tested in a preliminary toxicity test in TA98 and TA100 at eight concentrations from 3 to 5000 µg/plate. Based on this toxicity test the highest concentration tested in the two main tests was 5000 µg/plate both in the absence and presence of metabolic activation. Negative (solvent) and positive controls were included in all experiments in accordance with OECD guidelines.

Results
Toxicity, measured as reduction in the revertant colonies (from 60-80%) compared to control, was observed at the highest concentration tested (5000 µg/plate) in both assays both with and without metabolic activation in TA98 and TA102 and from 1000 and 2500 µg/plate in TA1537 and TA1535, respectively, in the pre-incubation test with S9-mix. There was a slight increase in revertant colony numbers (1.8 fold) in the preincubation assay with TA100 and TA1537 without S9-mix at the highest concentration tested. However, this increase was minor, not reproducible and there was no tendency of higher mutation rates with increasing concentrations at the lower tested concentrations and therefore not considered biological relevant. No increase in revertant colonies was observed in the other tester strains following treatment with B 077 at any dose level, either in the presence or absence of metabolic activation. All positive controls used gave a distinct increase of induced revertant colonies.

Conclusion
B 077 was not considered to be mutagenic in the *in vitro* bacterial mutagenicity test in the presence or absence of S9-mix.

Ref.: 7

***In vitro* Mammalian Cell Gene Mutation Test (*tk*-locus)**

| | |
|---|---|
| Guideline: | OECD 476 |
| Species/strain: | Mouse lymphoma cell line L5178Y (*tk*-locus) |
| Replicates: | Duplicate cultures in two independent experiments |
| Metabolic act.: | Phenobarbital and beta-naphthoflavone induced rat liver S9-mix |

| | | |
|---|---|---|
| Test substance: | B077 | |
| Batch: | I-37979 | |
| Purity: | 99.5% (HPLC) | |
| Concentrations: | Experiment I: | 187.5, 375, 750, 1500 and 2250 µg/ml without S9-mix, and 93.8, 187.5, 375, 750, 1500 and 2250 µg/ml with S9-mix |
| | Experiment II: | 18.8, 37.5, 75, 150, 300 and 3000 µg/ml without S9-mix |
| Treatment: | Experiment I: | 4 hours treatment both in the absence and presence of S9-mix and 72 hours expression period |
| | Experiment II: | 24 hours treatment in the absence of S9-mix and 48 hours expression period |
| Solvent: | DMSO | |
| GLP: | In compliance | |

B 077 was evaluated for its genotoxic activity at the *tk*-locus in the mouse lymphoma cell line L5178Y. A preliminary toxicity test was performed with concentrations up to 10 mM (3000 µg/ml). Two independent main experiments using duplicate cultures each were performed. The first experiment was performed with a treatment period of 4 hours where 6 and 5 concentrations were tested with and without S9-mix, respectively. The concentration range chosen for evaluation were based on toxicity of the test compound.
Dimethylsulphoxide (DMSO) was used as solvent control, while methylmethanesulphonate (MMS µg/ml) without and cyclophosphamide (CPA) with metabolic activation system were used as positive controls. Mutant frequency, colony size and cell survival (measured as relative total growth and cloning efficiency) were determined.

Results
In experiment I, severe toxic effects (relative suspension growth (RSG) less than < 10%) were detected in both parallel cultures at 2250 µg/ml both with and without S9-mix. At 1500 µg/ml RSG was 36.8 and 14.4 without S9-mix and 6.4 and 9.2 with S9-mix in the two parallel cultures respectively. In experiment II, RSG was 6% and 4% in the duplicate cultures respectively at the highest tested concentration (3000 µg/ml), and 16.4 and 29 at the second highest concentration (300 µg/ml). No reproducible dose-dependent increase in mutant colony numbers was observed in either of the two main experiments (not even concentrations with severe toxic effect), except an isolated moderate increase at 1500 µg/ml in one culture in experiment I with S9-mix. However, this isolated effect is not considered to be of biological relevance.
The concurrent positive controls induced a distinct increase in mutation frequency.

Conclusion
Under the test conditions used in this study, B077 was not considered to be mutagenic and/or clastogenic in mammalian cells, either in the absence or in the presence of metabolic activation.

Ref.: 8

### *In vitro* mammalian micronucleus assay

| | | |
|---|---|---|
| Guideline: | / | |
| Species/strain: | Chinese hamster V79 cell line | |
| Replicates: | Duplicate cultures in two independent experiments. | |
| Metabolic act.: | Phenobarbital and beta-naphthoflavone induced rat liver S9-mix | |
| Test substance: | B 077 | |
| Batch: | I-37979 | |
| Purity: | 99.5% (HPLC) | |
| Concentrations: | Experiment I: | 500, 750, 1000 and 2000 µg/ml (with and without S9-mix) |
| | Experiment II: | 1500, 1750, 2000 and 2250 µg/ml (with S9-mix) |

Solvent: DMSO
GLP: In compliance

Time of exposure and dose selection were performed according to OECD 473.
Chinese hamster V79 cell line was used to examine the induction of micronuclei by the test substance. The concentrations of the test compound chosen for evaluation were based on a preliminary toxicity test (eight concentrations in the range 23.4-3000 µg/ml). In the main study, two independent experiments using duplicate cultures were performed. In both experiments cells were treated for 4 hours followed by a 20-hour recovery period. In experiment I four concentrations were tested with and without S9-mix. A confirmatory experiment (experiment II) using more narrow dilution steps was performed to verify the positive result observed in experiment I in the presence of S9-mix. At least 1000 cells in each culture were scored for micronuclei.
The test substance was dissolved in DMSO. Colcemid and cyclophosphamide were used as positive control in the absence and the presence of S9-mix, respectively. Culture medium was used as a negative control.

Results
Precipitation of the test substance was observed at 750 µg/ml with and without S9-mix. Cytotoxicity in experiment I, indicated by reduced cell numbers, was in the range 94.2-28.8% and 74.7-6.3% (at 500-2000 µg/ml) with and without S9-mix, respectively, compared to solvent control.

In experiment I in the absence of S9-mix, a dose-related increase (0.35, 0.6, 0.7 and 1.7%) in micronuclei was observed at concentrations from 500 to 2000 µg/ml. Only the increase at 2000 µg/ml was significant ($p<0.05$). However, the value (1.7%) was within the laboratory's historical control data (0-2%). In the presence of S9-mix a statistically significant increase (5.1%) of micronuclei was observed at 2000 µg/ml. This positive result was confirmed in experiment II where statistically significant increases in micronuclei of 2.85, 5.7, 11.3 and 7% were observed at 1500, 1750, 2000 and 2250 µg/ml, respectively.

Conclusion
Under the test conditions used in this study B 077 was clastogenic and/or aneugenic in Chinese hamster V79 cells *in vitro* in the presence of metabolic activation.

Ref.: 9

### 3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

**Mouse bone marrow micronucleus test**

Guideline: OECD 474
Species/strain: Mouse, strain NMRI
Group size: 5 males, 5 females per dose group and per sacrifice time
Test substance: B 077
Batch: I-37979
Purity: 99.5% (HPLC)
Dose level: 0, 125, 250 and 500 mg/kg bw administered as single doses
Route: Intraperitoneal
Vehicle: 20% DMSO, 80% corn oil
Sacrifice times: 24 hours after dosing and 48 hours (high dose only)
GLP: In compliance

The potential of B077 to induce micronuclei in polychromatic erythrocytes (PCE) was investigated in bone marrow from mice. The animals were dosed once intraperitoneally. For the high dose, two groups were treated to allow sampling after 24 and 48 hours.

A pre-toxicity test was performed to find the highest applicable dose without significant effects on the survival rates but with clear signs of toxicity and this dose was used as maximum dose in the main study.
Bone marrow cells were sampled from sacrificed mice 24 hours after dosing in all dose groups and positive control animals and additionally after 48 hours in the high dose group. 2000 PCEs per animal were scored for the incidence of micronuclei. Toxicity on the bone marrow was reported for each animal as the number of PCE per 2000 erythrocytes.
Positive (cyclophosphamide (CPA)) and negative (vehicle) controls were included.

Results
B 077 showed no cytotoxic effect in the bone marrow as the number of PCE per 2000 erythrocytes did not substantially change after treatment with the test item; however, the urine of the treated animals was blue indicating the systemic distribution of the test substance.
There was no statistically significant or biologically relevant increase in the number of micronuclei in mice of any B 077 treated group compared to the respective vehicle control group.
The positive control (CPA) induced a distinct and significant increase in micronucleated PCEs.

Conclusion
Under the test conditions used in this study B 077 was not clastogenic and/or aneugenic in the *in vivo* micronucleus test in mice.

Ref.: 10

### 3.3.7. Carcinogenicity

No data submitted

### 3.3.8. Reproductive toxicity

#### 3.3.8.1. Two generation reproduction toxicity

No data submitted

#### 3.3.8.2. Teratogenicity

**Prenatal development toxicity study**

| | |
|---|---|
| Guideline: | OECD 414 |
| Species/strain: | Rat, Wistar Hannover strain HsdBrlHan:Wist |
| Group size: | 25 pregnant females per dose group |
| Test Substance: | B 077 |
| Batch: | I-37979 |
| Purity: | 99.5% (HPLC) |
| Dose levels: | 0, 100, 200, 400 mg/kg bw/day (aqueous solution containing 0.5% carboxymethylcellulose) |
| Treatment period: | Days 5 - 19 of gestation, oral gavage |
| GLP: | In compliance |

B 077 was administered pregnant rats orally by gavage once daily from day 5 to day 19 post coitum at doses of 0, 100, 200 or 400 mg/kg bw/day. Each group consisted of 25 rats. Control animals were dosed with the vehicle alone. Females were sacrificed on day 20 post coitum and the foetuses were removed by Caesarean section. The examination of the dams and foetuses was performed in accordance with international recommendations.

Results
In the maternal animals, no signs were noted at pre- and post-dose observations.

Food consumption was slightly but statistical significantly decreased in the high dose group on day 9. On day 9 slight reductions in body weight gain were noted in the mid- and high-dose groups when compared to controls, reaching statistically significance in the high dose group. No treatment related effects were seen on uterus and corrected body weight. No relevant macroscopic changes were detected in treated females that could be considered treatment-related.

A slight but statistically significant reduction in mean foetal weight (5%) was noted in the high dose group. External examination of foetuses did not show any treatment related effects. No relevant changes that could be considered treatment-related were noted at visceral examination of foetuses between control and treated groups. A general retardation of the ossification of the skull, 5th and 6th sternebrae, metacarpal, thoracic centrum and sacral arches was observed in the mid- and/or high dose groups at skeletal examination.

In conclusion, a slight, but statistically significant reduction in body weight gain and food consumption was noted in the high dose group when compared to controls on Day 9. A statistically significant reduction in mean foetal weight was also noted in this group.

At skeletal examination, a general retardation in the ossification of several bones was observed in the mid- and/or high dose groups. These changes were considered to be a consequence of the presence of foetuses with a lower foetal weight and due to the signs of marginal maternal toxicity observed in the high dose groups.

Based on the effect on body weight gain the maternal NOAEL is 200 mg/kg bw/day. The NOAEL for foetal toxicity was 100 mg/kg bw/day, on the basis of the retarded ossification. In this study the test chemical is not teratogenic.

Ref.: 3

### 3.3.9. Toxicokinetics

No data submitted

### 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**

**(HC Blue n°11)**
**(Direct / semi-permanent)**

| | | | |
|---|---|---|---|
| **Maximum absorption through the skin** | **A (µg/cm²)** | **=** | **3.993 µg/cm²** |
| **Skin Area surface** | **SAS (cm²)** | **=** | **700 cm²** |
| **Dermal absorption per treatment** | **SAS x A x 0.001** | **=** | **2.795 mg** |
| **Typical body weight of human** | | **=** | **60 kg** |
| **Systemic exposure dose (SED)** | **SAS x A x 0.001/60** | **=** | **0.046 mg/kg bw** |
| **No observed adverse effect level (90day, rat, oral gavage)** | **NOAEL** | **=** | **80 mg/kg bw** |

| | | | |
|---|---|---|---|
| **Margin of Safety** | **NOAEL / SED** | **=** | **1739** |

### 3.3.14. Discussion

This risk assessment relates to the use of HC Blue n° 11 in non-oxidative hair dye formulations only.

*Physico-chemical properties*
HC Blue n° 11 is used as an ingredient in direct hair dye formulations in concentrations up to 2%. The solubility of HC Blue n° 11 in water is not determined according to the Official EC Method, and thus exact solubility in water is not known. The calculated value of Log $P_{ow}$ cannot be accepted as an estimate of the true physical constant without justification, indicating that the reported value is realistic.
HC Blue n° 11 is both a secondary and a tertiary amine. Its nitrosamine (N-Nitrosodiethanol-amine) content in batch I-37979 is reported to be 1600 ppb.
No data on the stability of HC Blue n° 11 in marketed products was provided.

*General toxicity*
The $LD_{50}$ was calculated to be 1250 mg/kg bw in male and female rats and 1275 mg/kg bw in female mice.
A No Observed Adverse Effect Level (NOAEL) for HC Blue n° 11 of 80 mg/kg bw/day was established in a subchronic oral toxicity study in rats, based on histological changes in the kidneys and increased kidney weight.
In a teratogenicity study in rats the NOAEL for embryo/foetotoxicity was 100 mg/kg bw. The NOAEL of maternal toxicity was 200 mg/kg bw.

*Irritation / sensitisation*
Under the conditions of the study, HC Blue n° 11 was not irritant when applied to the intact rabbit skin. Undiluted, it was irritating to the rabbit eye.
It was found to be a non-sensitizer when tested up to the highest achievable concentration of 25% (w/v) in ethanol:water (7:3 v/v) in a LLNA study.

*Dermal absorption*
In these *in vitro* dermal penetration studies the amount of B077 systemically available from a standard direct hair dye formulation was found to be 2.27 ± 1.12 µg/cm² with an $A_{max}$ of 3.993 µg/cm² which should be used for calculating the MOS.

*Mutagenicity / genotoxicity*
Three types of mutation (gene mutation, structural chromosome mutation and aneuploidy) were investigated. HC Blue n° 11 did not induce mutations in bacteria nor in mammalian cells at the *tk-locus*. HC Blue n° 11 did induce micronuclei in mammalian cells *in vitro*. However, this genotoxic effect could not be confirmed in an *in vivo* micronucleus assay. Therefore, HC Blue n° 11 can be considered to have no *in vivo* genotoxic potential and additional tests are not necessary.

*Carcinogenicity*
No data submitted

## 4. CONCLUSION

This risk assessment relates to the use of HC Blue n° 11 in non-oxidative hair dye formulations only.

The nitrosamine content in the test substance used in submission III was 1600 ppb. HC Blue n° 11 is both a secondary and a tertiary amine. It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

Provided that the nitrosamine content is < 50 ppb, the SCCP is of the opinion that the use of HC Blue n°11 as a non-oxidative hair dye at a maximum concentration of 2.0% on the head does not pose a risk to the health of the consumer.

## 5. MINORITY OPINION

Not applicable

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