Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

12-Sep-2016

English - Or. English

ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

CASE STUDY ON THE USE OF INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT FOR IN VITRO MUTAGENICITY OF 3,3' DIMETHOXYBENZIDINE (DMOB) BASED DIRECT DYES

Series on Testing & Assessment
No. 251

JT03400414

ENV/JM/MONO(2016)49
Unclassified
English - Or. English

OECD Environment, Health and Safety Publications

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IOMC
INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS
A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2016

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FOREWORD

OECD member countries have been making efforts to expand the use of alternative methods in assessing chemicals. The OECD has been developing guidance documents and tools for the use of alternative methods such as (Q)SAR, chemical categories and Adverse Outcome Pathways (AOPs) as a part of Integrated Approaches for Testing and Assessment (IATA). There is a need for the investigation of the practical applicability of these methods/tools for different aspects of regulatory decision-making, and to build upon case studies and assessment experience across jurisdictions.

The objective of the IATA Case Studies Project is to increase experience with the use of IATA by developing case studies, which constitute examples of predictions that are fit for regulatory use. The aim is to create common understanding of using novel methodologies and the generation of considerations/guidance stemming from these case studies.

This case study was developed by Canada and the United States for illustrating practical use of IATA in a regulatory context and submitted to the 2015 review cycle of the IATA Case Studies project. This case study was reviewed by the project team and revised to consider the comments from reviewers. The document was endorsed at the 9th Task Force on Hazard Assessment meeting in June 2016.

The following three case studies were also reviewed in the project in 2015 and are published with this case study:

1. CASE STUDY ON THE USE OF INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT FOR REPEAT DOSE TOXICITY OF SUBSTITUTED DIPHENYLAMINES (SDPA), ENV/JM/MONO(2016)50, Series on Testing & Assessment No. 252.

2. CASE STUDY ON THE USE OF AN INTEGRATED APPROACH TO TESTING AND ASSESSMENT FOR HEPATOTOXICITY OF ALLYL ESTERS, ENV/JM/MONO(2016)51, Series on Testing & Assessment No. 253.

3. CASE STUDY ON THE USE OF AN INTEGRATED APPROACH FOR TESTING AND ASSESSMENT OF THE BIOACCUMULATION POTENTIAL OF DEGRADATION PRODUCTS OF 4,4'-BIS (CHLOROMETHYL)-1,1'-BIPHENYL, ENV/JM/MONO(2016)52, Series on Testing & Assessment No. 254.

In addition, a considerations document summarizing the learnings and lessons of the review experience of the case studies is published with the case studies:

> REPORT ON CONSIDERATIONS FROM CASE STUDIES ON INTEGRATED APPROACHES FOR TESTING AND ASSESSMENT (IATA) -First Review Cycle (2015): Case Studies on Grouping Methods as a Part of IATA- ENV/JM/MONO(2016)48, Series on Testing & Assessment No. 250.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology.

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INTRODUCTION

Azo colourants, which include azo dyes and azo pigments, are important industrial chemicals because of their unlimited potential to generate various colours. It was estimated in 2004 (Golka, 2004) that over 2000 azo colourants were synthesized. Since then, the number continues to increase as can be evidenced by hundreds of applications for premanufacturing/manufacturing of new azo colourants to regulatory agencies worldwide. Although azo colourants *per se* are not particularly toxic, they can be metabolized in animal bodies by enteric bacterial as well as hepatic azoreductase to mutagenic and carcinogenic constituent aromatic amines. Among the various mutagenic and carcinogenic aromatic amines that could be released from azo colourants, benzidine and some of its congeners are of utmost concern because of their carcinogenic potency and human relevance (e.g., Woo and Lai, 2012). Benzidine- and benzidine congener-derived azo colourants have been the focus of recent regulatory efforts in Canada and the United States (US).

As indicated in the Notice of Intent published in June 2010, the Government of Canada is in the process of addressing approximately 350 aromatic azo- and benzidine-based substances for potential human health and ecological risks under The Aromatic Azo- and Benzidine-Based Substances Approach (Canada 2010). There are a total of 358 aromatic azo- and benzidine based substances included in this initiative as priority substances under the Chemicals Management Plan. Azo substances are primarily used as colourants and can be found in a wide variety of products including clothing, textiles, cosmetics and personal care products. The 358 aromatic azo and benzidine based substances have been further subcategorized into smaller subgroups of related substances based on factors such as structural similarity, use and application, as well as physical and chemical properties. The subgroups allow for application of read across of certain hazard data for similar substances (analogous chemicals).

In the United States, an Action Plan was developed which addresses the use of benzidine-based dyes and benzidine congener-based dyes, both metalized and non-metalized, in products that would result in consumer exposure, such as colouring textiles. The Action Plan focused on human carcinogenicity issues. EPA intended to address risk concerns from potential exposures by adding four benzidine-based dyes to an existing TSCA section 5(a)(2) significant new use rule (SNUR) for benzidine-based substances and establishing a new SNUR for benzidine congener-based dyes, including 44 specific such dyes (U.S. EPA 2010).

In collaboration with U.S. EPA, this case study aims to illustrate a situation where the Existing Substances Risk Assessment Bureau (ESRAB) of Health Canada has applied the category and read across approach for hazard characterization. This specific case study was developed based on the work published in a recent Canadian Draft Screening Assessment Report (dSAR) for a broad group of benzidine (or congener) based dyes (Environment Canada and Health Canada 2013a). Effort was made to update predictions where necessary to use the most up to date models available. Within the screening assessment, the read across analysis was based on multiple genotoxicity assays including *in vivo* assays. The screening assessment also incorporated the potential carcinogenicity of these substances in the risk assessment. This case study focuses on assessing the *in vitro* mutagenic potential (Ames test in Salmonella *typhimurium* under reductive conditions) of a small subset of 3,3’ dimethoxybenzidine (DMOB) based azo direct dyes. The category could be expanded to other congeners in the future.

No empirical data were found for nine category members. For these substances, an integrated conclusion was derived based on read across using the general trend for category members with available empirical data. Read across is also further support by data from a supporting common metabolite and QSAR predictions across four independent models. Category members without data are expected to be

positive in the Ames assay using Salmonella *typhimurium* under reductive conditions with metabolic activation.

1. PURPOSE

1.1. Purpose of use

The purpose of this IATA approach is hazard characterization for a screening level risk assessment conducted under Canada's Chemicals Management Plan (CMP). The authority for conducting risk assessment is granted under the *Canadian Environmental Protection Act (CEPA) 1999.* Read across of *in vitro* mutagenicity potential (Ames test in Salmonella *typhimurium* under reductive conditions) supported by (Q)SAR predictions will be used for an integrated conclusion for category members with data gaps.

1.2. Category definition

A category of structurally related azo direct dyes containing the 3, 3' dimethoxybenzidine (DMOB) substructure has been established in order to aid the hazard characterization of these substances. The category was established following guidance provided by the OECD (OECD 2014). The general structure of substances covered under this category is presented in Figure 1-1. As a result of common starting materials used during their synthesis, all category members are azo substances that contain a biphenyl substructure with nitrogen and methoxy groups on the 4,4'- and 3,3'- positions, respectively. All compounds have one or more sulfonate salt substituents (Na, Li) not attached to the DMOB moiety but elsewhere on the molecule.

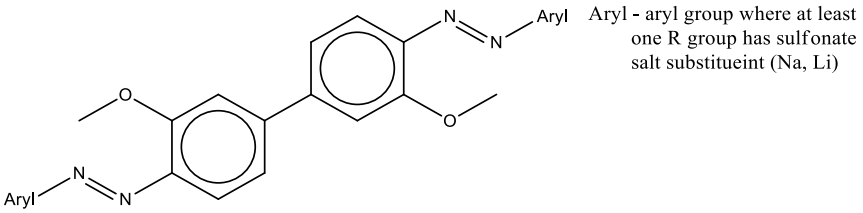


Figure 1-1 General structure common to the DMOB based azo direct dye category members

The category was developed using available data for the 13 members outlined below. However, it is anticipated that the category could apply to other azo dyes that fit the structural category definition above and are reasonably anticipated to release DMOB upon reductive metabolism of the azo bond. Although the case study is limited to analysis of dyes based on one benzidine based congener (DMOB), it is likely that the category can be expanded to include other congeners as well.

1.3. Exclusions

Not all DMOB based azo colourants are included in the category. The key deciding factor for health concern of benzidine-derived colourants is the ability of the colourant to undergo metabolic reduction to release the hazardous aromatic amine (Section 4.3.3). Based on consideration of solubility, structural differences, potential for tautomerization and metal chelation, the azo colourants may be subdivided based on their ability to release the benzidine or congener as shown in Figure 1-2. Dye colorants are generally defined as soluble substances and / or go through an application process which, at least temporarily, destroys any crystal structure by absorption, solution, and mechanical retention, or by ionic or covalent chemical bonds. Pigments colorants are generally defined as organic or inorganic solids which usually are insoluble in, and essentially physically and chemically unaffected by, the vehicle or substrate in which they are incorporated. Pigments are usually dispersed in vehicles or substrates for application. Pigments retain a crystal or particulate structure throughout the coloration process (SDC 2015).

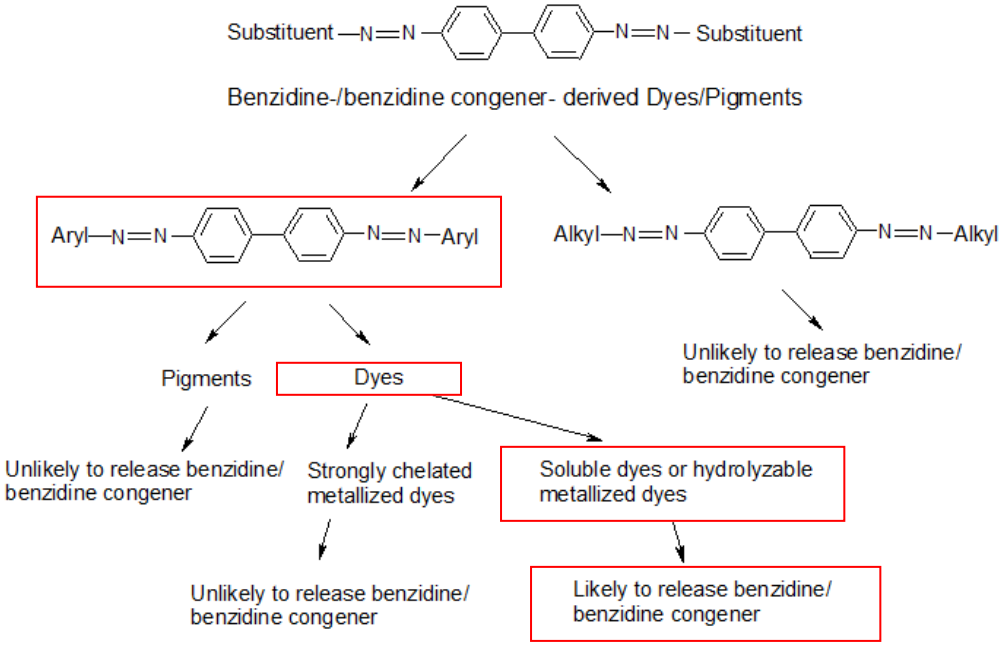


Figure 1-2 Subdivision of azo colourants based on ability to release the benzidine or congener. The focus the case study are DMOB based azo colourants that follow the metabolic pathway highlighted by red boxes.

Excluded from this category are azo colourants that are not anticipated to release DMOB such as:

A. Diarylide pigments (Figure 1-3). These compounds have very low solubility in water and as such are not substantially subject to metabolic reductive cleavage by microflora in the gut or by endogenous azo reductases in the liver or kidneys (Golka et al. 2004; Health Canada and Environment Canada 2013b). Besides low solubility, there is also possibility of inhibition of azo reduction by tautomerization of azo to hydrazone. Using the structurally related 3,3'-dichlorobenzidine coupled to diethyl malonate, DeFrance et al. (1986) showed that azo colourants based on beta-diketone coupled alkyl group exist preferentially as the tautomeric hydrazones.

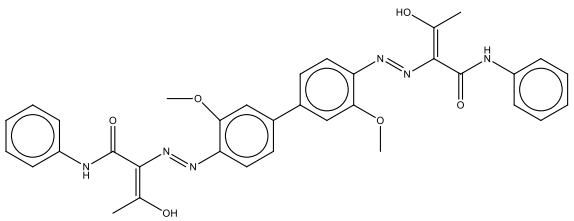


Figure 1-3 Azo diarylide pigments (substructure shown) that are based on DMOB are excluded from the category

B. Azo pigments other than diarylide pigments. Most pigments have very low solubility and as such are not substantially subject to metabolic reductive cleavage by microflora in the gut or by endogenous azo reductases in the liver or kidneys. Available evidence (e.g. NTP bioasays) of other azo pigments are mostly negative, weak or by mechanism with no human significance (e.g. spleen via hemosiderosis). As one of the regulatory actions in the US in 1993, NTP data was used to support the proposed tolerance exemptions for C.I. Pigment Blue #15 and C.I. Pigment Green

#7. The NTP decided not to perform toxicology and carcinogenesis studies of C.I. Pigment Green #7 and C.I. Pigment Blue #15 based on the lack of absorption or adverse effects in a 90-day feed study in rats and mice (NTP 1993). No DMOB based pigments that fall under this exclusion were found on the categorized Canadian DSL. However, if future substances of interest based on DMOB are examined and the compound is a pigment, it should not be considered part of this category. Furthermore, U.S.EPA (2010) dropped all azo pigments in the Benzidine dye action plan.

C. Azo Dyes complexed with transition metals. A number of transition metals, especially chromium and cobalt, can form strong co-ordination complexes with azo dyes containing hydroxyl, carboxyl or amino groups at positions *ortho* to the azo bond (e.g, o,o'-dihydroxy azo dye). Metallized dyes fall into two classes, 1:1 metal-complexes, in which one dye molecule is complexed with one metal atom and the more modern 1:2 metal complexes, in which one metal atom is complexed with two dye molecules. Examples are shown in Figure 1-4.

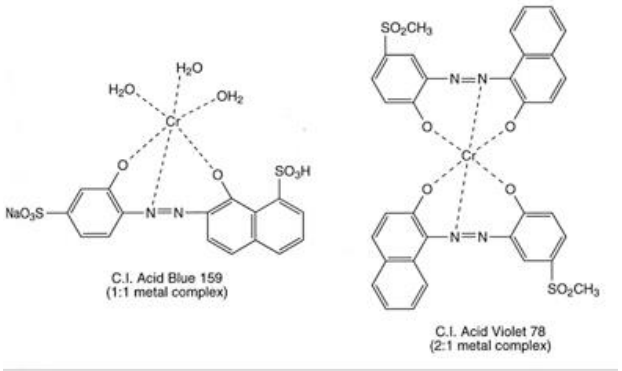


Figure 1-4 Example of azo dyes complexed with transition metals

Chromium is particularly stable in the 1:1 complex that its presence can inhibit the metabolic reduction of the azo bond. Although the dye molecule typically contains one azo bond, it is conceivable that this exclusion rationale could be applied to benzidine-derived azo dyes.

1.4. Endpoint

The category was developed to address data gaps with respect to the mutagenicity for certain azo direct dyes when exposure occurs via the oral route. The read across of data and provided justification below applies to *in vitro* mutagenicity (Ames test in *Salmonella typhimurium*) conducted under reductive metabolic conditions (Prival et al. 1984).

2. HYPOTHESIS FOR THE CATEGORY

After oral intake, the DMOB azo direct dyes are metabolically reduced through the action of microbial azoreductases in the intestine to release the aromatic amine DMOB (Figure 2-1).

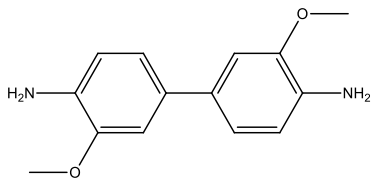


Figure 2-1: Structure of the released aromatic amine 3,3' dimethoxybenzidine (DMOB).

Released DMOB can be metabolically activated to electrophilic nitrenium and carbonium ions. The electrophilic metabolites can covalently bind to nucleophilic sites on DNA, through an SN1 substitution reaction. DNA binding is associated with DNA mutation (direct DNA acting mutagens). The DMOB based azo dyes are also structurally similar and have similar physicochemical properties. Taken together, it is hypothesised that DMOB based azo dyes behave similarly with respect to *in vitro* mutagenicity (Ames test in *Salmonella typhimurium*) conducted under reductive metabolic conditions (Prival et al. 1984) and that read across can be used to address category members that lack this test.

3. CATEGORY MEMBERS

3.1. Identification and selection of category members

The substances within this category are part of the Groupings Initiative of the Government of Canada's Chemical Management Plan (CMP). The grouping consists of 13 substances that are on Canada's Domestic Substance List (DSL) identified as priorities for action as they met the categorization criteria under section 73 of the *Canadian Environmental Protection Act* (CEPA 1999) (Environment Canada and Health Canada 2013). The DSL inventory was searched using both automated and manual methods to derive the list of substances (Table 3-1).

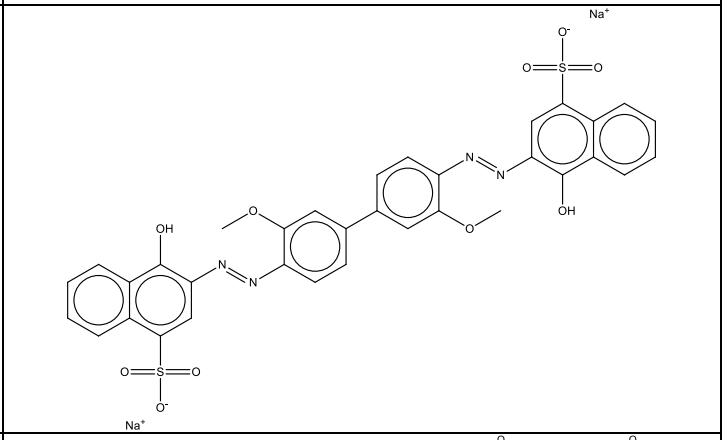
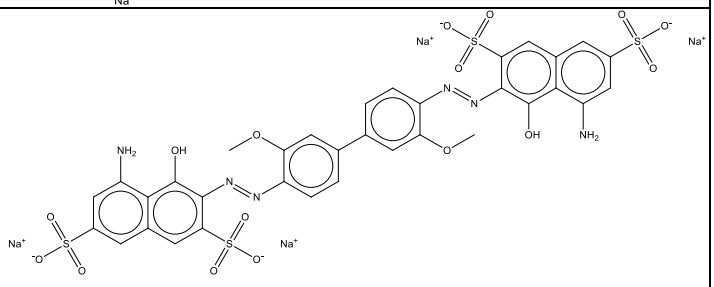
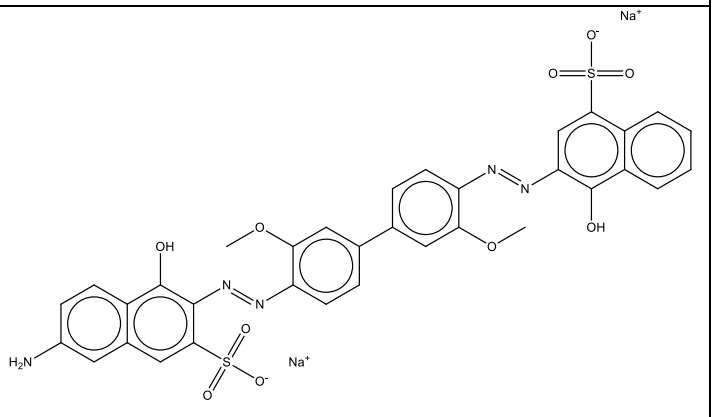
Table 3-1 Selection criteria that reflect the read-across hypothesis were applied in order to identify suitable category members

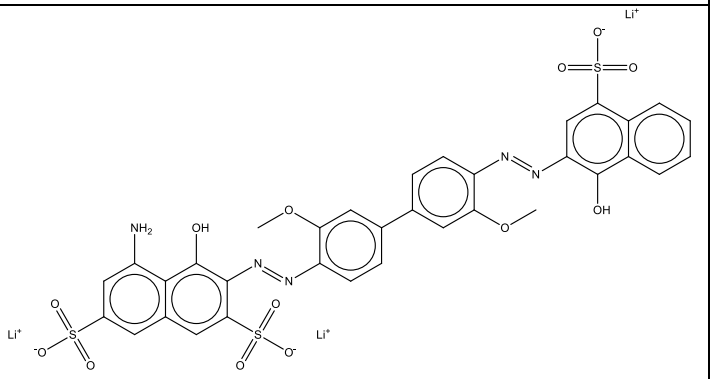
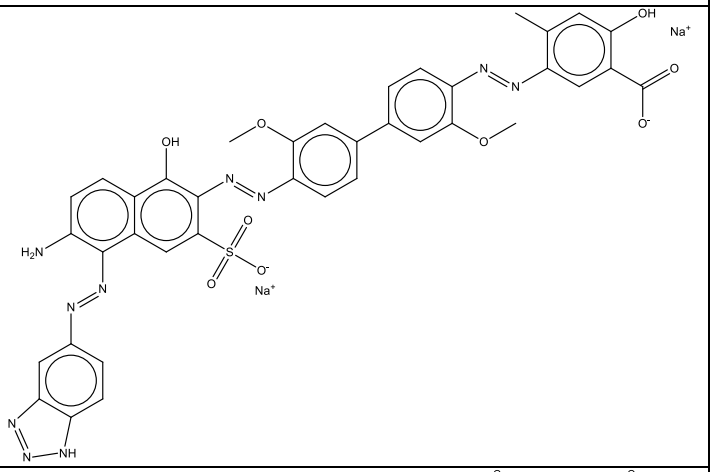
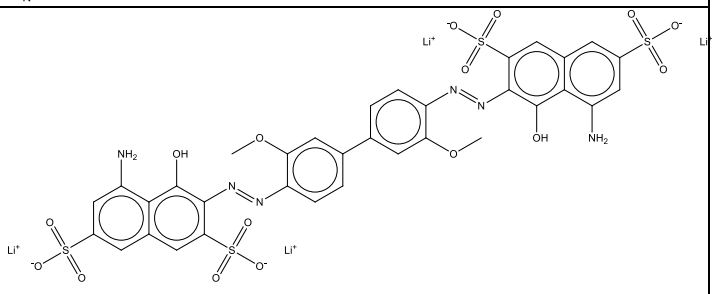
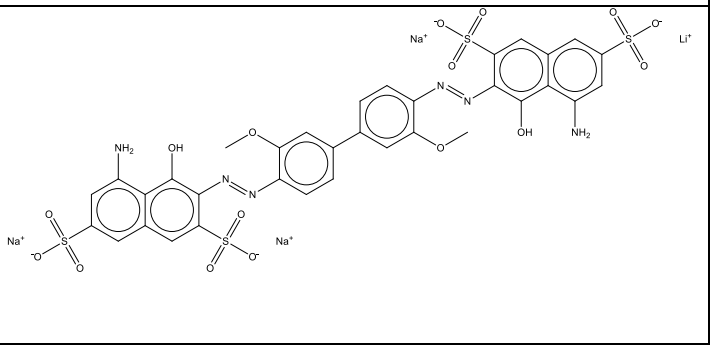
Selection criteria for analogues	Reasoning
1) Must be an azo direct dye that contains a biphenyl substructure with nitrogen and methoxy groups on the 4,4'- and 3,3'- positions, respectively (DMOB based direct dye).	Required for release of DMOB via metabolic activity of microbial azoreductases.
2) Members must have one or more sulfonate sodium or lithium salt substituent(s) not attached to the DMOB moiety but elsewhere on the molecule.	Influences key physical-chemical properties such as pKa, LogD, and water solubility. Acts to increases aqueous solubility of substances. Solubility of parent colourant is associated with the potential metabolic release of DMOB (Golka et al. 2004).

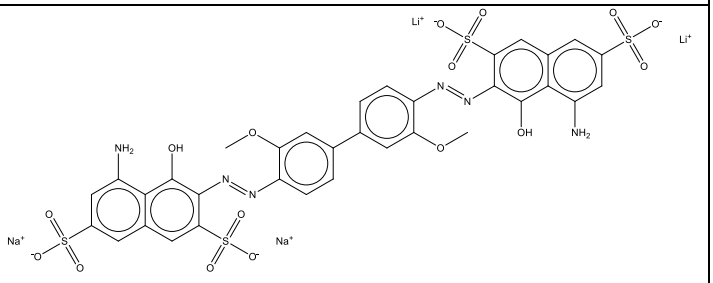
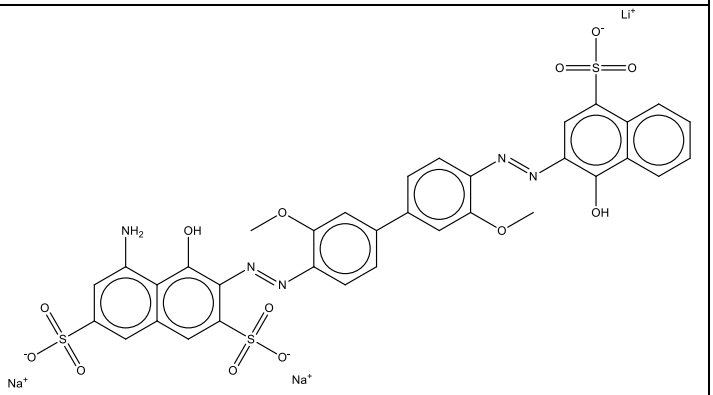
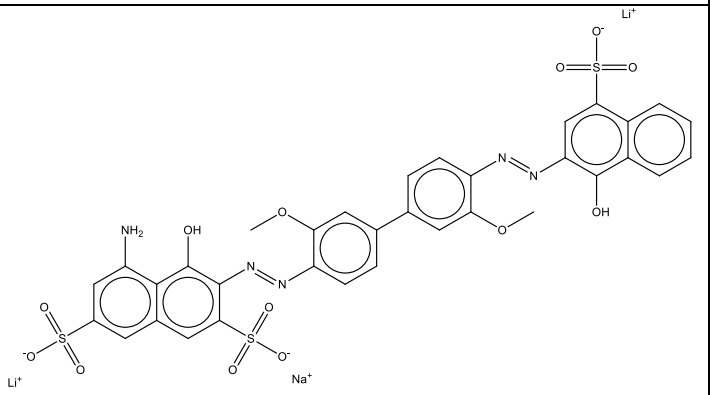
3.2. List of category members

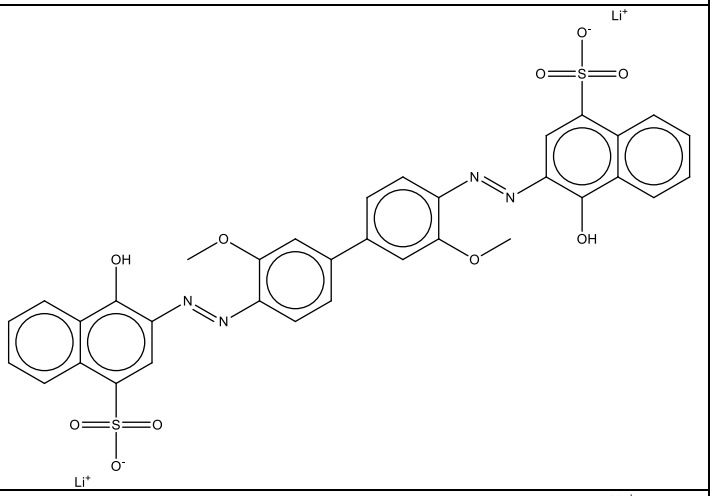
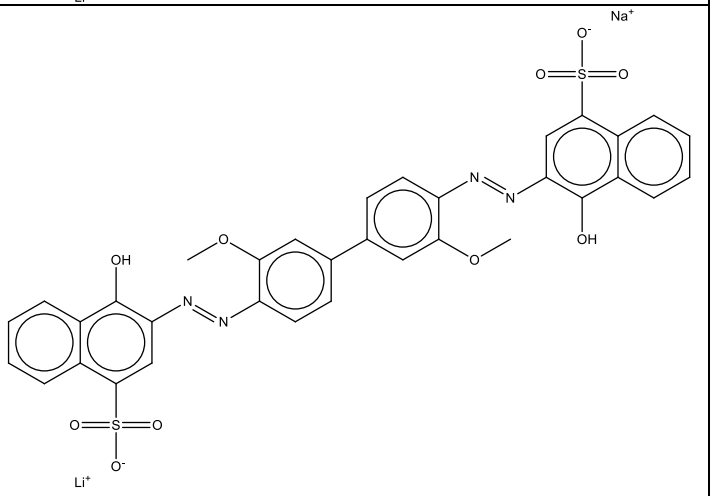
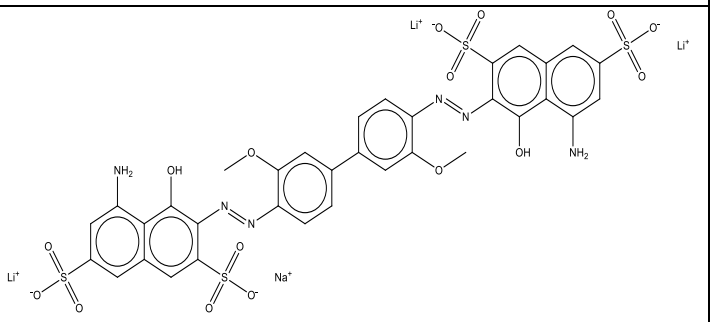
There are 13 discrete substances within the category. There is considerable overlap of the organic portion of some of the category members where only the counter ions differ. The chemical structures of the category members are presented in Table 3-2.

Table 3-2 Substance identity for discrete SDPAs and isomer mixtures

CAS RN **Common Name**	**Chemical Name**[a]	Endpoint Data[b]	**Chemical Structure**
2429-71-2 Direct Blue 8	1-Naphthalenesulfonic acid, 3,3′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[4-hydroxy-, disodium salt	Yes	
2429-74-5 Direct Blue 15	2,7-Naphthalenedisulfonic acid, 3,3′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[5-amino-4-hydroxy-, tetrasodium salt	Yes	
6449-35-0 Direct Blue 151	1-Naphthalenesulfonic acid, 3-[[4′-[(6-amino-1-hydroxy-3-sulfo-2-naphthalenyl)azo]-3,3′-dimethoxy[1,1′-biphenyl]-4-yl]azo]-4-hydroxy-, disodium salt	Yes	

CAS RN **Common Name**	**Chemical Name**[a]	Endpoint Data[b]	**Chemical Structure**
67923-89-1	2,7-Naphthalenedisulfonic acid, 5-amino4-hydroxy-3-[[4′-[(1-hydroxy-4-sulfo-2-naphthalenyl)azo]-3,3′-dimethoxy[1,1′-biphenyl]-4-yl]azo]-, trilithium salt	No	
70210-28-5 BABHS	Benzoic acid, 5-[[4′-[[6-amino-5-(1H-benzotriazol-5-ylazo)-1-hydroxy-3-sulfo-2-naphthalenyl]azo]-3,3′-dimethoxy[1,1′-biphenyl]-4-yl]azo]-2-hydroxy-4-methyl-, disodium salt	Yes	
71550-22-6	2,7-Naphthalenedisulfonic acid, 3,3′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[5-amino-4-hydroxy-, tetralithium salt	No	
75659-72-2	2,7-Naphthalenedisulfonic acid, 3,3′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[5-amino-4-hydroxy-, monolithium trisodium salt	No	

CAS RN **Common Name**	**Chemical Name**[a]	Endpoint Data[b]	**Chemical Structure**
75659-73-3	2,7-Naphthalenedisulfonic acid, 3,3′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[5-amino-4-hydroxy-, dilithium disodium salt	No	
75673-18-6	2,7-Naphthalenedisulfonic acid, 5-amino-4-hydroxy-3-[[4′-[(1-hydroxy-4-sulfo-2-naphthalenyl)azo]-3,3′-dimethoxy[1,1′-biphenyl]-4-yl]azo]-, monolithium disodium salt	No	
75673-19-7	2,7-Naphthalenedisulfonic acid, 5-amino-4-hydroxy-3-[[4′-[(1-hydroxy-4-sulfo-2-naphthalenyl)azo]-3,3′-dimethoxy[1,1′-biphenyl]-4-yl]azo]-, dilithium monosodium salt	No	

CAS RN **Common Name**	**Chemical Name**[a]	Endpoint Data[b]	**Chemical Structure**
75673-34-6	1-Naphthalenesulfonic acid, 3,3′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[4-hydroxy-, dilithium salt	No	
75673-35-7	1-Naphthalenesulfonic acid, 3,3′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[4-hydroxy-, monolithium monosodium salt	No	
75752-17-9	2,7-Naphthalenedisulfonic acid, 3,3′-[(3,3′-dimethoxy[1,1′-biphenyl]-4,4′-diyl)bis(azo)]bis[5-amino-4-hydroxy-, trilithium monosodium salt	No	

[a] Chemical name on Canada's Domestic Substance List (DSL)
[b] Ames test conducted under reductive metabolic conditions

4. JUSTIFICATION OF DATA GAP FILLING

4.1. Data gathering

4.1.1 Empirical Data

For each category member, several publically available databases were searched with a focus on Ames mutagenicity data conducted under modified conditions to facilitate the metabolic reduction of the azo bond. The modifications to the Ames assay are described in Prival et al. 1984 (the modified test system uses FMN, hamster liver S9 and a preincubation step). Data was also collected on Ames assays using other suitable modification such as pre-incubation with rat cecal bacterial as described in Reid et al. 1984.

The publically available databases or tools used to conduct the data search included:

- OECD QSAR Toolbox datasets (OECD 2013)
- Toxline (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?TOXLINE)
- ChemIDplus (http://chem.sis.nlm.nih.gov/chemidplus/)
- SciFinder (http://www.cas.org/products/scifinder)
- Literature searches using Scopus (using CAS RN, common name or chemical name)

Health Canada, under the broader azo and benzidine based substances assessment, requested specific substances tested for Ames mutagenicity using the Prival et al. 1984 modifications. Testing was performed by Integrated Laboratory Systems, Inc. This report is not published in the public domain, however results were included in this case study and the draft screening assessment for the broader risk assessment (ILS 2011; Health Canada and Environment Canada 2013a).

4.1.2 Predicted Data

The models outlined in Table 4-1 were used to generate predictions or estimates to support the IATA case study. For the majority of the models used, the neutral non-salt organic moieties were used to generate the predictions.

Table 4-1 Models used to support the DMOB direct dye case study

Model Name (version)	Description of use	Available QMRF	Reference
Advanced Chemistry Development (ACD) Percepta (v2015 – Build 2726) PhysChem module LogD module pKa Classic module LogS module	Prediction of physical-chemical properties of category members.	No*	ACD/Percepta 2015
CATABOL microbial metabolic simulator – (in OECD QSAR Toolbox v3.3)	Prediction of microbial metabolism of category members.	No	Jaworska et al. 2002

Model Name (version)	Description of use	Available QMRF	Reference
Lhasa, Derek Nexus (v3.0.1) Bacterial mutagenicity (*in vitro)*	Expert system used to profile category members for alerts related to endpoint.	Yes** Online JRC QMRF# Q13-33-36-312	Derek Nexus 2012
Toxtree (v2.5) Benigni/Bossa Rulebase for Mutagenicity and Carcinogenicity	Expert system used to profile category members for alerts related to endpoint.	Yes Online JRC QMRF# Q26-35-35-295	Toxtree 2011
Multicase Case Ultra (v1.4) Ames Salmonella *typhimurium* models with metabolic activation (S9) (A2P, A2R, AS5, A2T, A2U, A2V, A2W, A2X, A2Y, A2Z)	Hybrid QSAR (statistical and structural alerts) software for prediction of Ames mutagenicity of category members.	No	Case Ultra 2012
Leadscope Model Applier v1.8.4 (Genetic Toxicity Suite – Salmonella Mut (Genetox Statistical))	QSAR (statistical) software for prediction of Ames mutagenicity.	Yes Online	Leadscope 2012
VEGA (v1.0.8) - CAESAR mutagenicity module (v.2.1.12) – Ames test prediction	Hybrid QSAR (statistical + structural alerts) software for prediction of Ames mutagenicity of category members.	Yes Online JRC QMRF# Q35-50-46-429	VEGA 2013
OASIS TIMES (v2.27.5) Ames S9 activated (v5.05)	Hybrid QSAR (statistical + structural alerts) with metabolic simulator for prediction of Ames mutagenicity of category members.	Yes Appendix B	TIMES 2014

* Underdevelopment by model developer
** QSAR Model Reporting Format (QMRF) developed for older version of model

4.2. Data matrix

- See data matrix file for data summary (Annex)
- See Appendix A for study summaries.

4.3. Justification

Available data demonstrates that the members of this category have similar structure, physicochemical properties, metabolism, mechanistic considerations, and biological activity (predicted and empirical). Therefore, read-across is an appropriate approach to characterize the *in vitro* mutagenicity potential for members of this category where data gaps exist.

4.3.1 Structural Similarity

All category members are considered structurally similar. As a result of common starting materials used during their synthesis, all category members are azo substances that contain a biphenyl substructure with nitrogen and methoxy groups on the 4,4'- and 3,3'- positions, respectively. All substances contain

aromatic ring substructures that contain one or more sulfonated salt functional groups. The alkali metal salts are expected to dissociate in aqueous media and as a result the solubility of these compounds is increased.

4.3.2 Physicochemical Property Similarity

Identified or modelled physicochemical properties for the substances covered under the proposed category are presented in the data matrix. Alkali salts are not amenable to modelling, therefore *in silico* predictions were conducted for the substances in their neutral form. Due to similar chemical structure (sulfonated azo compounds), category members are generally similar with respect to relevant physicochemical properties. Generally, all substances covered under this category are solids (at room temperature) with low values of logD at expected pH in the small intestine. All substances are expected to be ionized at physiological pH and over the pH ranges within the GI tract. The ability for azoreductases to act on a particular azo compound is influenced by its solubility (Golka et al. 2004). Category members are considered to be sufficiently soluble in the gut for intestinal bacteria to metabolise them into their respective aromatic amines, including releasing DMOB for subsequent absorption and activation.

4.3.3 Metabolic Similarity

As described, the potential for metabolic reduction of the azo bond to yield aromatic amines is typically the determining factor in the genotoxic mode of action for azo type substances (Brown and De Vito 1993). The similarity hypothesis of the category is based on the consideration that after oral intake, the DMOB azo direct dyes are metabolically reduced through the action of azoreductase of microflora in the intestine to release the aromatic amine DMOB. The ability of the azo bond to be reduced for a particular substance is influenced by its solubility (Golka et al. 2004). Therefore, the potential for the category members to undergo metabolic azo reductions to aromatic amine metabolites was determined based on an analysis of *in vivo* and *in vitro* metabolic assays.

In vivo metabolism studies provide the most direct evidence for azo bond reduction, and this type of study was found for two category members, Direct Blue 8 and 15. In both instances, the aromatic amine DMOB was identified in the urine and feces of one or more mammalian species that were orally exposed to the dye. The amounts of the aromatic amines present in the urine and feces were greater than those present as impurities in the testing material, indicating *in vivo* metabolic reduction of the azo bond (Lynn et al. 1980; Bowman et al. 1982; Nony et al. 1983; Bowman et al. 1983).

In vitro metabolism studies were found for one category member, Direct Blue 15. The aromatic amines generated from the reductive cleavage of the azo bond were identified following incubation of the dye with either intestinal contents from humans, monkeys or rats. In the study, the potential for reductive cleavage to aromatic amines was demonstrated. A DMOB metabolite was detected through GC/MS (Cerniglia et al. 1982a, 1982b).

Another line of evidence that was considered in the assessment of metabolic azo bond reduction for category members is the results obtained from the Ames assay under reductive metabolic conditions. These reductive conditions include incubation with intestinal contents or incorporation of the Prival modifications (Prival et al. 1984). If the Ames test yielded positive results only after such conditions were employed, then the potential for the substance to reduce to aromatic amine metabolites *in vivo* was inferred (Environment Canada and Health Canada 2013a). Three category members (Direct Blue 15, Direct Blue 8 and BABHS) were evaluated in this type of assay, and comparing the results with studies where reductive conditions were not used support for activation only after azo bond reduction (Prival et a. 1984; Reid et al. 1984; Krishna et al. 1986; EI DuPont de Nemours and Co. Inc. 1978; Gregory et al. 1981; Brown and Dietrich 1983; ILS 2011).

In the absence of empirical data, metabolic azo bond reduction was predicted using a metabolic simulator within the OECD QSAR Toolbox software package (OECD 2013). Guidance on how to use the OECD Toolbox is available online (OECD 2015). Microbial metabolic transformations were simulated using the Oasis CATABOL simulator (Jaworska et al. 2002). The simulator is thought to be an approximate model for the microbial transformations found in the intestine. All category members were predicted to release DMOB through reductive metabolism of the azo bond.

Where data exists for category members, there is consensus across *in vivo, in vitro* and *in silico* methods that these azo direct dyes can undergo reductive metabolism of the azo bond to form respective aromatic amines including DMOB. Untested category members are predicted using *in silico* tool to also undergo reductive metabolism at the azo bond.

4.3.4 Mechanistic Similarity

The DMOB Azo Direct Dyes contain substructures that alert for potential DNA binding according to molecular profiling using two expert systems, Derek Nexus (v3.0.1) and Toxtree (v2.5) (Derek Nexus 2012 and Toxtree 2011) through the formation of a reactive metabolite.

Research on the association of azo compounds with mutagenicity has been well documented. The molecular initiating event has been well established by several research groups and has been reviewed in the literature (Brown and DeVito 1993). Certain azo dyes are mutagenic after reductive cleavage of the azo linkage to their aromatic amine metabolites. The azo linkage is the most labile portion of an azo molecule and the potential for azo compounds to become mutagens is often determined by their ability to undergo enzymatic breakdown in mammalian organisms or micro-organisms (Figure 2-2) (Brown and DeVito 1993). In mammalian organisms azo-reductases are present in various organs such as the liver, kidney, lung, heart, brain and muscle tissues (Fouts et al. 1957). Azo-reductases are also present in the microflora of the gut and skin (Rafii et al. 1990; Stingley et al. 2010). Endogenous azo-reductases of the various tissues of the body as well as azoreductase activity of gut and skin microflora can contribute to metabolic activation of azo compounds leading to mutagenic potential. The majority of mutagenic azo compounds require metabolic activation (reduction and cleavage of the azo linkage to the component aromatic amines) in order to show mutagenicity for *in vitro* test systems (Brown and DeVito 1993). After aromatic azo bond reduction, the produced aromatic amines also require further metabolic activation to bind DNA (Figure 2-3). The first step involves N-hydroxylation and N-acetylation, and the second step involves O-acylation yielding acyloxy amines. These compounds can degrade to form highly reactive nitrenium and carbonium ions. These electrophilic reactants may readily bind covalently cellular DNA and RNA (Brown and DeVito, 1993).

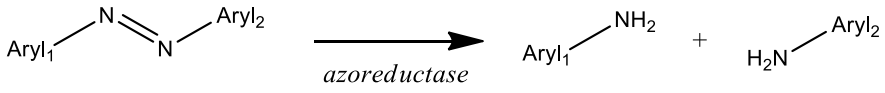


Figure 2-2: Cleavage of aromatic azo bond can yield aromatic amine metabolites that can potentially bind to DNA leading to gene mutations

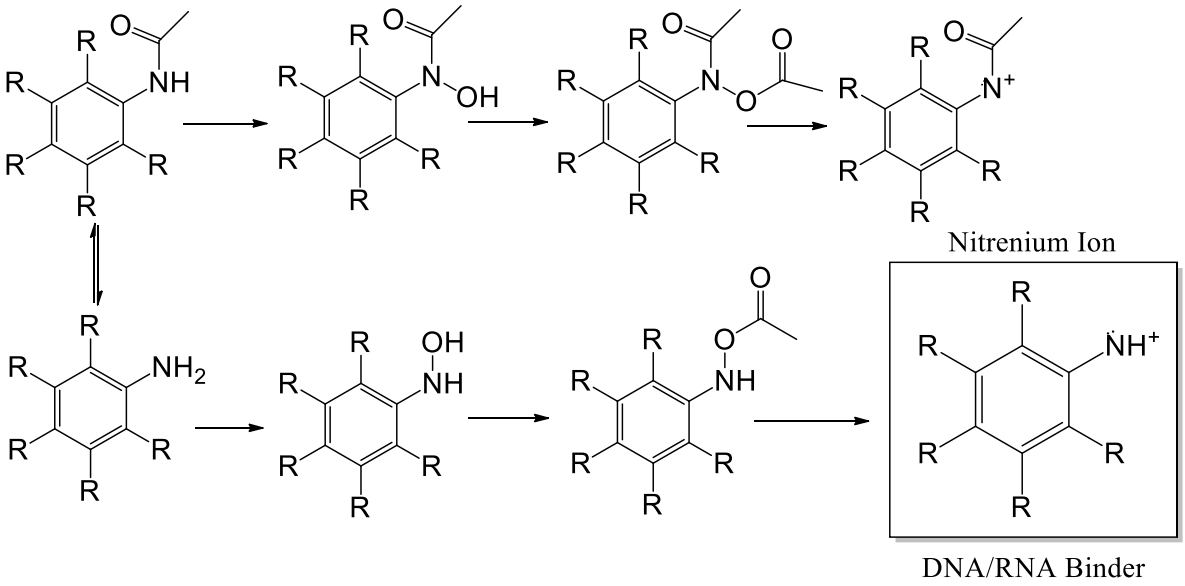


Figure 2-3: Metabolically activated aromatic amine to electrophilic substance that can interact with DNA or RNA (Adapted from Brown & DeVito, 1993)

It is important to note that other mechanism may apply to other specific azo dyes for mutagenicity (e.g. quinone-imine formation of certain aromatic amine metabolites). However, for the DMOB based direct dyes in this category, the above described mechanism is believed to be the major mechanism responsible for mutagenicity of category members.

4.3.5 Trend in Empirical Toxicological Data

Available data for category members and the application of read across is presented in the data matrix. Study summaries are presenting in Appendix A.

In vitro genotoxicity studies were found for Direct Blue 8 (CAS RN 2429-71-2), Direct Blue 15 (CAS RN 2429-74-5), Direct Blue 151 (CAS RN 6449-35-0) and BABHS (CAS RN 70210-28-5). All four dyes were mutagenic in the presence of metabolic activation and reductive conditions in bacterial mutagenicity assays (Prival et al. 1984; Reid et al. 1984; Zhou et al. 1987; ILS 2011). While Direct Blue 15 and BABHS also showed some equivocal or positive results under metabolic activation only, incorporation of reductive conditions resulted in an increase in mutagenic potency for Direct Blue 15 (Prival et al. 1984; Reid et al. 1984). The positive results in the absence of reductive conditions may be attributed to the use of commercial samples of dyes in the testing protocols that may have mutagenic impurities (e.g. DMOB impurity).

Data was also collected for the common category metabolite DMOB. DMOB was generally positive in Salmonella *typhimurium* strains TA98, TA100, and TA1538 with metabolic activation, but negative without metabolic activation (Probst et al. 1981; Chung et al. 2000).

Where data is available for the Ames mutagenicity test in Salmonella *typhimurium* under reductive conditions with metabolic activation, the category members are consistently positive (see Table 3-1). The common aromatic amine metabolite for the category members, DMOB, is also positive in the Ames assay with metabolic activation for most tested strains.

4.3.6 Trend in Predicted Toxicological Data using QSAR Models

Predictions for Ames mutagenicity across four independent QSAR models were made for all category members to build support for the category and read across due to the paucity of data for many members. Predictions across the substances were mixed. For CAS RNs 2429-74-5, 67923-89-1, 71550-22-6, 75659-72-2, 75659-73-3, 75673-18-6, 75673-19-7, 75752-17-9 three out of four predictions are positive with negative predictions coming only from CaseUltraT. CAS RN 2429-71-2, CAS 6449-35-0, 70210-28-5, 756-34-6, and 75673-35-7 have mixed predictions with equal number of positive and negative/indeterminate predictions across the four models.

For models base on statistical algorithms alone, it is not clear if the azo compounds in the respective training sets were tested under the appropriate reductive modifications. This would have a significant influence on the models. The defined endpoint for the QSAR models is not the Ames test under reductive conditions, rather Ames tests with metabolic activation only. With the exception of the OASIS TIMES model which simulates metabolism prior to screening for alerts (including reductive metabolism of the azo bond), the model results could be confounded by this distinction.

CaseUltraT (version 1.4) (CaseUltraT 2012)

All category members are predicted to be negative in the Ames Salmonella *typhimurium* models with metabolic activation (S9) (A2P, A2R, AS5, A2T, A2U, A2V, A2W, A2X, A2Y, A2Z). In the majority of the models, the substances are flagged for deactivating alerts based on the sulfonated aromatic azo moiety. However, the dyes have an aromatic component that is not sulfonated (the DMOB moiety). While the other released aromatic amines may be deactivated due to sulfonation (described in the alert in Derek Nexus), DMOB is also released and is not sulfonated. Therefore, the deactivating alert generated by Case Ultra does not apply to the whole compound.

VEGA / CAESAR (version 2.1.12) (VEGA 2013)

All compounds are predicted to be mutagens in the CAESAR mutagenicity model. However, in certain cases the compounds fall out of the applicability domain or produce a warning (e.g. some atom-centered fragments of the compound have not been found in the compounds of the training set, or are rare fragments, etc). The model is considered to accurate when comparing category members with *in vitro* data and their prediction result. The model also highlights an alert for the disazo substances for mutagenicity which provided mechanistic support for the prediction (alert from the Benigni/Bossa rulebase for mutagenicity.

Leadscope Model Applier (version 1.6) (Leadscope Model Applier 2012)

Results are mixed across the substances. All compounds were within the model domain as defined by the software developers. The azo structural feature is considered a "positive" contributor to mutagenicity while the sulfonate groups are "negative" contributors. For dyes that contain 3,5-diamino-4-hydroxynaphthalene-2,7-disulfonic acid in the structure, these are predicted to be positive (e.g. Direct Blue 8). When examining the features contributing to the prediction, the aromatic amine in this substructure (not associated with the azo bond in the parent structure) appears to be adding significant weight towards a positive prediction (198 out of 295 substances in the training set with a primary aromatic amine feature are positive in the Ames test). Direct Blue 8 is part of the training set compounds of the model. The substance is listed as "negative" in the training set. It is not clear if the azo compounds in the respective training sets were tested under the appropriate reductive modifications. This would have a significant influence on this model and the outcome. Confidence in the predictions for the case study is considered low.

OASIS TIMES (version 2.27.5) (TIMES 2014)

OASIS TIMES is a unique predictive tool as it employs a metabolic simulator and predicts activity for parent compounds as well as metabolites. All substances are predicted to be mutagenic, where the activity is due to metabolic reduction of the azo bond to release DMOB. DMOB is further N-hydroxylated and this metabolite is flagged for a mutagenic alert with the software. However, all members are out of the model structure domain. If a prediction is out of the applicability domain this does not necessarily mean that the prediction is not valid, or is incorrect. It indicates that the uncertainty about the reliability of the model is increased, as the performance statistics from the training and/or validation datasets might not be applicable to this specific substance. The models used in this case study set the applicability domain thresholds differently by default and some are stricter than others. Given the conservative applicability domain categorization that is applied in the TIMES model and the fact that the model correctly predicts the mutagenic properties of substances with empirical data, the model is deemed reliable despite domain of applicability warnings. This model also has the benefit of supporting the prediction with a mechanistic interpretation.

5. STRATEGY FOR INTEGRATED CONCLUSION OF DATA GAP FILLING

5.1. Integrated conclusion

No empirical data were found for nine category members. For these substances, an integrated conclusion was derived based on read across using the general trend for category members with available empirical data. Read across is also further support by data from a supporting common metabolite. QSAR predictions from OASIS Times and the CAESAR software support the conclusion. The predictions with these programs are supported by defined structural alerts related to mechanism described in this case study. QSAR models that are based on statistical algorithms alone, produced conflicting results when comparing with empirical data. Category members without data are expected to be positive in the Ames assay using Salmonella *typhimurium* under reductive conditions with metabolic activation.

5.2. Uncertainty

The potential for the chemical grouping and read across approach to introduce uncertainty into the hazard assessment of chemicals is well acknowledged by regulatory agencies including Health Canada and Environment Canada. There are various areas that contribute to the overall uncertainty when applying the approach. There is uncertainty associated with the data, assumptions, and predictions used to justify similarity and analogue suitability between the group members. There is also toxicological uncertainty with the prediction of hazard that can be derived when using read-across (evaluated based on the number and suitability of analogues contributing data, source study quality, likelihood of effect and potency concordance between target and source chemical).

Presented here is a preliminary effort to document the various areas of uncertainty associated with the data and methods used for the similarity justification (Section 5.2.1). Some consideration of the uncertainty with prediction of hazard when applying read across for repeat dose toxicity is also conducted in a separate step (Section 5.2.2). Limited guidance is available in the open literature for assessing uncertainty with respect to the read across approach. Two recent and related published frameworks were consulted when assessing the read across uncertainty within our case study (Wu et al. 2010, Blackburn and Stuard 2014).

5.2.1 Analogue Suitability Rating for Each Case of Read Across

The process used in this case study to justify similarity within subgroups of DMOB based direct dyes follows closely the framework proposed by Wu et al. 2010. The overall flow chart from this publication was used to assign a 'suitability rating'. For the use of read-across, Table 5-1 outlines the applicable questions from this flow chart and the associated areas of uncertainty based on the data, assumptions, and predictions used for the determination. The 'suitability rating' is then used in a subsequent step to assign an uncertainty category to the overall read across.

Table 5-1 Analogue suitability rating for read-across within the DMOB based direct dye category

Chemical ID		
Target Chemical(s) CAS RN: 67923-89-1; 71550-22-6; 75659-72-2; 75659-73-3; 75673-18-6; 75673-19-7; 75673-34-6; 75673-35-7; 75752-17-9		**Source Chemical(s) for Read Across** CAS RN: 2429-71-2; 2429-74-5; 6449-35-0; 70210-28-5
Similarity Evaluation		
Evaluation Criteria	**Decision tree question(s) in Wu et al. 2010**	**Uncertainty**
Structural Similarity	Do the target and analogue share a major substructure feature or functional group: **YES**	All category members are discrete substances with well-defined structures curated from reliable databases. As a result of common starting materials used during their synthesis, all category members are azo substances that contain a biphenyl substructure with nitrogen and methoxy groups on the 4,4'- and 3,3'- positions, respectively. All substances contain aromatic ring substructures that contain one or more sulfonated salt functional groups. Critical functional groups are consistent across category members.
Metabolic Similarity	Could the target and analogue metabolize to each other or converge to a common stable metabolite or reactive metabolite with the same mode of action? **YES**	Based on metabolic *in vivo* and *in vitro* data for multiple category members (3 of 13), there is evidence that the azo bond can be metabolically reduced and aromatic amines, including DMOB, are released. However, there is uncertainty with respect to metabolism for the category members without empirical data. Although a predictive model was used to simulate metabolism for these data poor category members, there is high uncertainty associated with the results due to the model domain issues.
Mechanistic Similarity		SAR models were used to screen category members for mechanistic alerts associated with mutagenicity. For all category members, the expert systems describe the mechanism as metabolism to aromatic amines that are subsequently metabolised to electrophilic compounds that covalently bind to DNA. *No modulating structural features were noted for mutagenicity for any category member.* Although specific mechanistic studies for the category members could not be located, the mechanism of aromatic azo compound mutagenicity in general is well studied (Brown and DeVito 1993).
Phys/Chem Properties	Could any other part of the molecule have the potential	Category members were considered appropriately similar with respect to physicochemical properties although there is

	to change the toxicity of the analogue relative to the target?	moderate uncertainty associated with the analysis as the majority of values were predicted with *in silico* tools using neutral non-salt forms of the substances. *No modulating structural features were noted for mutagenicity for any category member when screening with expert systems.*
Overall "suitability rating" based on Wu et al. 2010 decision tree: **Suitable with preconditions** The precondition is that the target and analogues undergo similar metabolism when administered via the oral route to converge on a common metabolites DMOB. Uncertainty could be reduced with more empirical data on the metabolism of the target chemicals.		

[a] Uncertainty associated with underlying data used for analysis
[b] Consistency within the data

5.2.2 Assigning an Uncertainty Category for Read-Across

A systematic framework to describe potential areas of additional uncertainty that may arise in read across (evaluated based on the number and suitability of analogues contributing data, and effects/ potency concordance) has been recently published (Blackburn and Stuard 2014). The framework was developed for consistent application through the use of a questionnaire for evaluating and documenting consideration of these potential additional sources of uncertainty by risk assessors. The framework and questionnaire was developed for cases of quantitative read across (e.g. read across of effect level for repeat dose toxicity). The questionnaire was not completed for this case study. However, factors outlined in the paper (number of analogues, robustness of analogue data set, and concordance of effects were considered (Table 5-2) to derive an overall uncertainty category for the read across.

Table 5-2 Uncertainty associated with the prediction of hazard using read across

Analogue Data Set Characteristics	**Comment**
Number of analogues contributing data	Four (4) suitable category members with data used for read across. Data from supporting metabolite also considered.
Robustness of analogue data set	The available studies for the category members with data were judged to be reliable and conducted under the appropriate conditions. However, many studies were missing information regarding purity of the test substances which has confounded the analysis to some extent.
Concordance of effect(s)	Available empirical data for the Ames assay using Salmonella *typhimurium* under reductive metabolic conditions were positive where data exists for category members (3 of 13). A common metabolite DMOB was also positive in the Ames test. All the category members were predicted to be positive with a QSAR consensus model approach with the exception of CAS RN 70210-28-5 which was equivocal.
Overall uncertainty of read across prediction: Low Read across data (of sufficient quality for risk assessment) is contributed by at least 1 'suitable' analogue for the target. Highly concordant toxicity effects in data set across category.	

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Office of Pollution, Prevention and Toxics, Washington, DC, USA.

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APPENDIX A – STUDY SUMMARIES

Health Canada has formatted the study summaries using IUCLID 5.4 which utilizes the OECD Harmonized Templates for endpoint study records. The .i5z electronic files are available from Health Canada upon request. Presented here is the output derived from the IUCLID CSR plug-in (Consumer Safety Report) modified to only show the sections relating to toxicokinetics and *in vitro* mutagenicity for the category members with empirical data.

Toxicokinetics (metabolism)

Non-human information

Table A-1. *In vivo* studies on metabolism for category members

CAS RN	Method	Results	Reference
2429-71-2	rat (Fischer 344) oral: gavage Exposure regime: single acute exposure Doses/conc.: 0 and 1 mL aqueous solution equivalent to 2 mg of 100% dye	Main ADME results: metabolism: metabolites detected and identified in urine. excretion: peak concentrations of metabolites observed between 0 and 12 hours post-treatment in rat urine. Metabolites identified: yes Details on metabolites: 3,3'-dimethoxybenzidine, mono- and di-acetyldimethoxybenzidine and alkaline hydrolysable conjugates in urine.	Bowman, M.C. et al. (1983)
2429-74-5	dog (mongrel) female oral: feed Exposure regime: single acute exposure followed by 3 days urine collection Doses/conc.: 100 mg/kg	Main ADME results: metabolism: metabolite was observed and identified in the urine of treated dogs. excretion: concentration of metabolite in dog urine was 0.03% of administered dose. Metabolites identified: yes Details on metabolites: 3,3'-dimethoxybenzidine was detected in urine and confirmed by GC/MS.	Lynn et al. (1980)
2429-74-5	rat (Sprague-Dawley) male oral: gavage Exposure regime: daily for 10 days Doses/conc.: 100 mg/kg	Main ADME results: metabolism: metabolites were found in the urine of treated rats. excretion: concentration of metabolite in rat urine was 0.17 +/- 0.18% of administered dose; excretion cannot be attributed to the level of impurity. Metabolites identified: yes Details on metabolites: 3,3'-dimethoxybenzidine	Lynn et al. (1980)

CAS RN	Method	Results	Reference
		was found in rat urine and confirmed by GC/MS.	
2429-74-5	rat (Fischer 344) male oral: gavage Exposure regime: single acute exposure; urine and feces were collected at various intervals for 192 hours. Doses/conc.: 0 and 12 mg/kg b.w. (radiolabelled)	Main ADME results: metabolism: metabolites were detected and identified in the urine. excretion: 18.8% of dose is in urine and 74.4% of dose is in feces, of which, 12% of dose is present as intact dye. Peak excretion occured during the 8-16 hour interval, with no metabolites detected after 25 hours by gas chromatography. Metabolites identified: yes Details on metabolites: Metabolites detected in the urine: 3,3'-dimethoxybenzidine (0.22% of dose in the free amine fraction and 0.48% in the alkaline hydrolyzable conjugate fraction) and monoacetyldimethoxybenzidine (0.27% of dose) and diacetyldimethoxybenzidine (0.22% of dose) in the free amine fraction. Unextractable metabolites accounted for 17.6% of the dose (indication of high polarity). Metabolites were detected using gas chromatography and radiochemical assays.	Bowman et al. (1982)

Table A-2. *In vitro* studies on metabolism for category members

CAS RN	Method	Results	Reference
2429-71-2	anaerobic bacterial suspensions from CD rat intestinal contents Exposure regime: 48 hours Doses/conc.: 0 and 15 nM	Main ADME results: metabolism: Total reduction of the dye occurred within 4 hours of incubation with anaerobic bacterial suspensions isolated from rat intestinal contents; 82% of the dye was converted to the benzidine congener. Metabolites identified: yes Details on metabolites: 3,3'-dimethoxybenzidine identified by GC/MS.	Cerniglia et al. (1982a)
2429-71-2	anaerobic bacterial suspensions from CD rat, rhesus monkey intestinal contents and human feces Exposure regime: 48 hours Doses/conc.: 0 and 3.13 nmol/ml (estimate based on 188 nmol dye added to 60 ml of	Main ADME results: metabolism: 88 to 100% of the dye was reduced within 6 hours by anaerobic bacteria from rat and monkey intestinal contents and human feces. Metabolites identified: yes Details on metabolites: 3,3'-dimethoxybenzidine was identified by GC/MS.	Cerniglia et al. (1982b)

CAS RN	Method	Results	Reference
	media; does not account for volume of water vehicle)		

Mutagenicity

In vitro data

Table A-2. *In vitro* genotoxicity studies

CAS RN	Method	Results	Reference
119-90-4 DMOB Supporting Common Metabolite	bacterial reverse mutation assay (e.g. Ames test) (gene mutation) S. typhimurium, other: G46, TA1535, TA100, C3076, TA1537, D3052, TA1538, TA98 (met. act.: with) E. coli WP2 (met. act.: with) E. coli WP2 uvr A (met. act.: with) Test concentrations: 0, 0.3nmol/ml Positive control substance(s): 2-acetylaminofluorene Positive control substance(s): N-ethyl-N-nitro-N-nitrosoguanidine	Test results: negative for S. typhimurium, other: G46 ; met. act.: with negative for S. typhimurium TA 1535 ; met. act.: with positive for S. typhimurium TA 100 ; met. act.: with negative for S. typhimurium, other: C3076 ; met. act.: with negative for S. typhimurium TA 1537 ; met. act.: with negative for S. typhimurium, other: D3052 ; met. act.: with positive for S. typhimurium TA 1538 ; met. act.: with positive for S. typhimurium TA 98 ; met. act.: with negative for E. coli WP2 ; met. act.: with negative for E. coli WP2 uvr A ; met. act.: with negative for S. typhimurium, other: G46 ; met. act.: without negative for S. typhimurium TA 1535 ; met. act.: without negative for S. typhimurium TA 100 ; met. act.: without negative for S. typhimurium, other: C3076 ; met. act.: without negative for S. typhimurium TA 1537 ; met. act.: without negative for S. typhimurium, other: D3052 ; met. act.: without negative for S. typhimurium TA 1538 ; met. act.: without negative for S. typhimurium TA 98 ; met. act.:	Probst et (al. 1981)

CAS RN	Method	Results	Reference
		without negative for E. coli WP2 ; met. act.: without negative for E. coli WP2 uvr A ; met. act.: without	
119-90-4 DMOB Supporting Common Metabolite	bacterial reverse mutation assay (e.g. Ames test) (gene mutation) S. typhimurium, other: TA 98 and TA 100 (met. act.: with and without) Test concentrations: 0, 3, 10, 30, 100, 300, 1000 µg/plate Positive control substance(s): 2-nitrofluorene (TA 98 without S9) Positive control substance(s): 2-aminofluorene (both strains with S9) Positive control substance(s): N-methyl-N-nitro-N'-nitrosoguanidine (TA 100 without S9)	Test results: positive for S. typhimurium TA 98 ; met. act.: with ; negative controls valid: yes; positive controls valid: yes negative for S. typhimurium TA 98 ; met. act.: without ; negative controls valid: yes; positive controls valid: yes positive for S. typhimurium TA 100 ; met. act.: with ; negative controls valid: yes; positive controls valid: yes negative for S. typhimurium TA 100 ; met. act.: without ; negative controls valid: yes; positive controls valid: yes	Chung et al (2000)
2429-71-2 Direct Blue 8	bacterial reverse mutation assay (e.g. Ames test) (gene mutation) S. typhimurium TA 1538 (met. act.: with) Test concentrations: 0, 0.25, 0.5 and 1.0 µMole/assay Positive control substance(s): Dimethoxybenzidine rat cecal flora and FMN modifications	Test results: negative for S. typhimurium TA 1538(rat S9 activation) ; met. act.: with positive for S. typhimurium TA 1538(rat S9 activation and rat cecal bacteria) ; met. act.: with positive for S. typhimurium TA 1538(hamster S9 activation and FMN) ; met. act.: with	Reid et al. (1984)
2429-74-5 Direct Blue 15	bacterial reverse mutation assay (e.g. Ames test) (gene mutation) S. typhimurium TA 98 (met. act.: with) Test concentrations: 0, 0.1, 0.3	Test results: positive (more potent than without modification) for S. typhimurium TA 98(with modifications (including FMN)) ; met. act.: with ; positive controls valid: yes positive for S. typhimurium TA 98(without modification) ; met. act.: with ; positive	Prival et al. (1984)

CAS RN	Method	Results	Reference
	and 1.0 µmol/plate Positive control substance(s): 3,3'-Dichlorobenzidin Positive control substance(s): Direct Blue 6 Positive control substance(s): 2-Fluorenyl-acetamide Positive control substance(s): 4-Nitro-o-phenylenediamine Positive control substance(s): Nitrofurantoin Prival modification	controls valid: yes	
2429-74-5 Direct Blue 15	bacterial reverse mutation assay (e.g. Ames test) (gene mutation) S. typhimurium TA 1538 (met. act.: with) Test concentrations: 0, 0.25, 0.5, and 1.0 µMole/assay Positive control substance(s): Dimethoxybenzidine rat cecal flora and FMN modifications	Test results: positive (rat S9 activation) for S. typhimurium TA 1538 ; met. act.: with positive (more potent than without reductive conditions) for S. typhimurium TA 1538(rat S9 activation and rat cecal bacteria) ; met. act.: with positive (more potent than without reductive conditions) for S. typhimurium TA 1538(hamster S9 activation and FMN) ; met. act.: with ; cytotoxicity: yes (at highest dose)	Reid et al. (1984)
2429-74-5 Direct Blue 15	bacterial reverse mutation assay (e.g. Ames test) (gene mutation) S. typhimurium TA 98 (met. act.: with) Test concentrations: 0, 0.10, 0.30, 1.00 and 3.00 mg/plate Positive control substance(s): 2-aminoanthracene Prival modification	Test results: positive for S. typhimurium TA 98(with modifications (including FMN and hamster S9)) ; met. act.: with ; vehicle controls valid: yes; positive controls valid: yes positive for S. typhimurium TA 98(with modifications (including FMN, but in the presence of rat S9)) ; met. act.: with ; cytotoxicity: yes (at higher doses) ; vehicle controls valid: yes; positive controls valid: yes	Krishna et al. (1986)
2429-74-5 Direct Blue 15	bacterial forward mutation assay (gene mutation) S. typhimurium, other: SV50	Test results: positive for S. typhimurium, other: SV50(with modifications (including FMN and hamster S9)) ; met. act.: with ; vehicle controls valid:	Krishna et al. (1986)

CAS RN	Method	Results	Reference
	(met. act.: with) Test concentrations: 0, 0.10, 0.30, 1.00 and 3.00 mg/plate Positive control substance(s): 2-aminoanthracene arabinose resistant assay with Prival modification	yes; positive controls valid: yes positive for S. typhimurium, other: SV50(with modifications (including FMN, but in the presence of rat S9)) ; met. act.: with ; vehicle controls valid: yes; positive controls valid: yes	
2429-74-5 Direct Blue 15	bacterial reverse mutation assay (e.g. Ames test) (gene mutation) S. typhimurium, other: TA 98, TA 100, TA 1535, TA 1537 and TA 1538 (met. act.: with and without) Test concentrations: 0, 500, 1000, 2500, 5000 and 1000 µg/plate Positive control substance(s): 2-acetylaminofluorene plate incorporation method	Test results: negative for S. typhimurium TA 98 ; met. act.: with negative for S. typhimurium TA 98 ; met. act.: without negative for S. typhimurium TA 100 ; met. act.: with negative for S. typhimurium TA 100 ; met. act.: without negative for S. typhimurium TA 1535 ; met. act.: with negative for S. typhimurium TA 1535 ; met. act.: without negative for S. typhimurium TA 1537 ; met. act.: with negative for S. typhimurium TA 1537 ; met. act.: without negative for S. typhimurium TA 1538 ; met. act.: with negative for S. typhimurium TA 1538 ; met. act.: without	EI Dupont De Nemours and Co Inc.; (1978)
2429-74-5 Direct Blue 15	bacterial reverse mutation assay (e.g. Ames test) (gene mutation) S. typhimurium, other: TA 98 and TA 100 (met. act.: with) Test concentrations: 0, 0.1, 0.3 and 1.0 µmole/plate FMN, riboflavin and rat cecal bacteria modifications	Test results: positive for S. typhimurium TA 98(with riboflavin) ; met. act.: with ; vehicle controls valid: yes positive for S. typhimurium TA 98(without riboflavin) ; met. act.: with ; vehicle controls valid: yes positive for S. typhimurium TA 98(under anaerobic conditions and with FMN) ; met. act.: with positive for S. typhimurium TA 98(under anaerobic conditions and with rat cecal bacteria) ; met. act.: with ; vehicle controls valid: yes positive for S. typhimurium TA 100(with riboflavin) ; met. act.: with ; vehicle controls valid: yes	Brown and Dietrich (1983)

CAS RN	Method	Results	Reference
		positive for S. typhimurium TA 100(without riboflavin) ; met. act.: with ; vehicle controls valid: yes positive for S. typhimurium TA 100(under anaerobic conditions and with FMN) ; met. act.: with ; vehicle controls valid: yes positive for S. typhimurium TA 100(under anaerobic conditions and with rat cecal bacteria) ; met. act.: with ; vehicle controls valid: yes	
6449-35-0 Direct Blue 151	bacterial reverse mutation assay (e.g. Ames test) (gene mutation) S. typhimurium TA 98 (met. act.: with) Test concentrations: 0, 0.25 and 0.50 µmoles/plate Prival modification	Test results: positive for S. typhimurium TA 98(with modifications) ; met. act.: with	Zhou et al. (1987)
70210-28-5 BABHS	bacterial reverse mutation assay (e.g. Ames test) (gene mutation) S. typhimurium, other: TA 98 and TA 100 (met. act.: with) Test concentrations: 0, 8.64, 25.9, 77.8, 233.3, 700 and 2100 µg/plate Positive control substance(s): benzidine Prival modification	Test results: positive for S. typhimurium TA 98(with modifications (including FMN)) ; met. act.: with ; vehicle controls valid: yes; positive controls valid: yes not determined (technical problem) for S. typhimurium TA 98(without modification) ; met. act.: with ; vehicle controls valid: yes; positive controls valid: yes positive for S. typhimurium TA 100(with modifications (including FMN)) ; met. act.: with ; vehicle controls valid: yes; positive controls valid: yes ambiguous for S. typhimurium TA 100(without modification) ; met. act.: with ; vehicle controls valid: yes; positive controls valid: yes	[ILS] Integrated Laboratories Systems, Inc (2011)

APPENDIX B – QMRF DOCUMENTS

QSAR Model Reporting Format (QMRF) Documents that are not available online were compiled for this case study. These QMRF documents were created by the model developers and not Health Canada.

(Q)SAR Model Reporting Format (QMRF) #1 – OASIS TIMES

Model version: Ames mutagenicity v.10.10
Platform version: OASIS TIMES 2.27.16
Name: Ames mutagenicity
Author: LMC, University "Prof. As. Zlatarov", Bourgas, Bulgaria
Date: 20 November, 2014
E-mail: omekenya@btu.bg
www: http://www.oasis-lmc.org/

Section 1. QSAR identifier

1.1. QSAR identifier (title)

In vitro Ames Mutagenicity with S9 metabolic activation

1.2. Other related models

POPs v2.60.3 [Mutagenicity + S9]; CANADIAN POPs v1.2.4 [Mutagenicity + S9].

1.3. Software coding the model

Model version: Ames mutagenicity v.10.10
Platform version: OASIS TIMES 2.27.16
Name: In vitro Ames Mutagenicity with S9 metabolic activation
Developer: LMC, University "Prof. As. Zlatarov", Bourgas, Bulgaria
Coding language: DELPHI XE3

Section 2. Date of QMRF

2.1. Date of QMRF

20 November 2014

2.2. QMRF author(s) and contact details

Name: Laboratory of Mathematical Chemistry
Affiliation: Laboratory of Mathematical Chemistry, University "Prof. As. Zlatarov", "Yakimov" St. #1, 8010 Bourgas, BULGARIA
URL: http://www.oasis-lmc.org
E-mail: omekenya@btu.bg

2.3. Date of QMRF update(s)

N/A

2.4. QMRF update(s)

N/A

2.5. Model developer(s) and contact details

Name: R. Serafimova, M. Todorov, T. Pavlov, S. Kotov, E. Jacob, A. Aptula, O. Mekenyan
Affiliation: Laboratory of Mathematical Chemistry, University "Prof. As. Zlatarov", "Yakimov" St. #1, 8010 Bourgas, BULGARIA
URL: http://www.oasis-lmc.org
E-mail: omekenya@btu.bg

2.6. Date of model development and/or publication

2006 November

2.7. Reference(s) to the main scientific and/or software package

R. Serafimova, M. Todorov, T. Pavlov, S. Kotov, E. Jacob, A. Aptula, O. Mekenyan. Identification of the structural requirement for mutagencity by incorporating molecular flexibility and metabolic activation of chemicals. II. General Ames mutagenicity model. Chem. Res. Toxicol., 662-676, (**2007**).

2.8. Availability of information about the model

http://oasis-lmc.org/products/models/human-health-endpoints/mutagenicity-(ames).aspx

2.9. Availability of another QMRF for exactly the same model

Not available.

Section 3. Defining the endpoint – OECD Principle 1

3.1. Species

Salmonella typhimurium
For more details see: OECD GUIDELINE FOR TESTING OF CHEMICALS
http://www.oecd-ilibrary.org/environment/test-no-471-bacterial-reverse-mutation-test_9789264071247-en

3.2. Endpoint

In vitro: Ames mutagenicity with metabolic activation
According to JRC pre-classification list of endpoints:
No. 207 QMRF Human Health Effects, QMRF 4.10 Mutagenicity.

3.3. Comment on endpoint

Efforts in (Q)SAR modeling of Mutagenicity and Carcinogenicity are much more pronounced that for any of the other human health endpoints. Major difficulties in modeling the carcinogenicity endpoint are principal due to the diversity of carcinogenicity pathways and the nonavailability or subcritical number of reliable data sets, which have hampered the applicability of the (Q)SAR approach. Because of this, efforts have been focused on the modeling of in vitro mutagenicity, which is better defined by underlying molecular interaction mechanisms. Generally, genotoxic chemicals cause a broader spectrum of adverse effects including interactions with DNA, altering its structure and information content; segregation of DNA in chromosome structure; damaging normal replication processes, etc. The mutagenic chemicals are more restricted in their mode of action, increasing the occurrence of mutation in populations of cell and/or organisms only on account of interaction with DNA. To detect and measure this potency, a short-term, simple, and inexpensive in vitro assay was designed, namely, the Ames test, which identifies genetic damage caused by chemicals on bacterial cells. The derived in vitro Ames mutagenicity model S9 is combined with metabolic simulator used for predicting metabolic activation of chemicals with the S9 mix. The explicit generation of metabolites allowed the DNA reactivity model to be applied not only to parent chemicals but also their stable metabolites.

3.4. Endpoint units

Qualitative – positive/ negative

3.5. Dependent variable

Observed Mutagenicity with S9

3.6. Experimental protocol

Bacterial Reverse Mutation Assay (e.g. Ames test)

3.7. Endpoint data quality and variability

High quality, chemicals provided by National Toxicology Program (NTP) and Badische Anilin und Soba-Fabrik AG (BASF AG)

Section 4. Defining the algorithm – OECD Principle 2

4.1. Type of model

Structural alerts based model

4.2. Explicit algorithm

Prediction of Bacterial mutagenicity.
Mutagenicity of chemicals, we have combined the alerting group approach with a pattern recognition type of model to delineate reactivity of chemicals toward DNA within a given interaction mechanism. The explicit generation of metabolites allowed the DNA reactivity model to be applied not only to parent chemicals but also their stable metabolites.
More details about the algorithm could be found in the reference in 2.7.

4.3. Descriptors in the model

Name: Molecular weight (MW)
Description of parameters:
Molecular weight - relative molecular mass, Da

4.4. Descriptor section

Not applicable (see 4.3)

4.5. Algorithm and descriptor generation

Not applicable (see 4.3)

4.6. Software name and version for descriptor generation

Not applicable (see 4.3)

4.7. Chemicals/Descriptors ratio

Not applicable (see 4.3)

Section 5. Defining the applicability domain of the model – OECD Principle 3

5.1. Description of the applicability domain of the model

The domain consists of the following sub-domain layers:
1. General parametric requirements.
The variations of molecular parameters that may affect the quality of the measured endpoint significantly are included here (such as molecular weight, etc.). The domain of general parametric includes the range of variation of hydrophobicity (*log K*OW) and Molecular weight (*MW*) of chemicals in training set.
2. Structural domain.
The structural component of the model is based on the structural similarity between chemicals in the training set which were correctly predicted by the model. The structural neighborhood of atom-centered fragments (accounting for the first neighbours) extracted from correctly and incorrectly predicted parent structures from the training set is used to determine this similarity.
The target chemical could contain the following types of ACF:

- Fragments present in correctly predicted training chemicals only (i.e. correct fragments),
- Fragments found both in correctly and non-correctly predicted training chemicals (i.e. fuzzy fragments). These fragments are treated as correct fragments,
- Fragments present in non-correctly predicted training chemicals only (i.e. incorrect fragments),
- Fragments not present in the training chemicals (i.e. unknown fragments).

A chemical belongs to the structural domain of the model if it could be partitioned only on correct fragments. The user is able to analyse how important are unknown and incorrect fragments (if present in the target) and to make a decision about their effect on the quality of prediction. The distribution of

structural characteristics of the target chemical and accepted thresholds is used as a criterion to determine how well the target is represented in the structural space of correctly predicted chemicals. The accepted domain thresholds for Mutagenicity are as follows:

- Correct = 100%
- Incorrect = 0%

A chemical is considered In Domain if it is classified to belong to all sub-domain levels. The information implemented in the applicability domain is extracted from the correctly predicted training chemicals used to build the model and in this respect the applicability domain determines practically the interpolation space of the model.

5.2. Method used to assess the applicability domain

The approach use to determine and assess the domain is described in:
Dimitrov S, Dimitrova G., Pavlov T., Dimitrova D., Patlewicz G., Niemela J., Mekenyan O., A stepwise approach for defining the applicability domain of SAR and QSAR models, *J. Chem. Inf. Model.*, 45, 839-849 (2005).

5.3. Software name and version for the applicability domain assessment

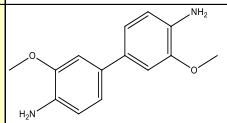
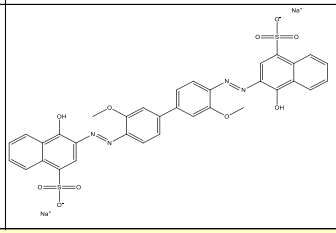
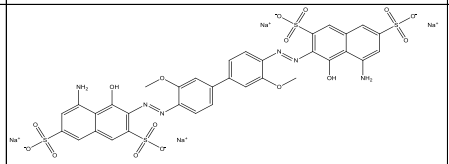
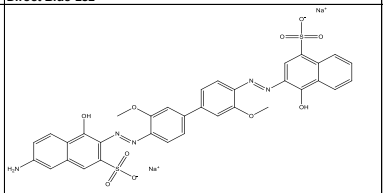
The LMC software OASIS Domain Manager v.1.09 (which is embedded in OASIS platform) is used to determine the applicability domain.
http://oasis-lmc.org/products/software/domain-manager.aspx

5.4. Limits of applicability

 General properties requirements:

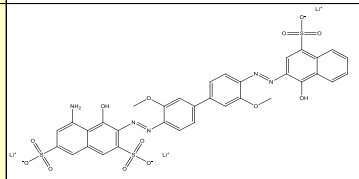
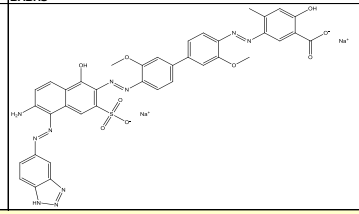
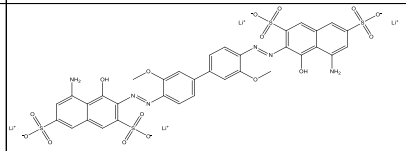
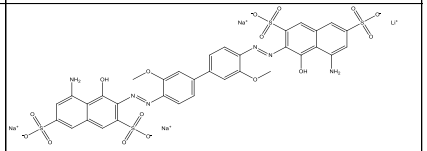
Property	Domain	Target chemical
log KOW	[-13.155; 21.646]	2.06
MW, Da	[18.014; 1560.198]	155

ANNEX - DATA MATRIX

Chemical ID					
		Supporting Common Metabolite	**Member 1**	**Member 2**	**Member 3**
CAS		119-90-4	2429-71-2	2429-74-5	6449-35-0
Name		3,3'-Dimethoxybenzidine (DMOB)	1-Naphthalenesulfonic acid, 3,3'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-hydroxy-, disodium salt **Direct Blue 8**	2,7-Naphthalenedisulfonic acid, 3,3'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-, tetrasodium salt **Direct Blue 15**	1-Naphthalenesulfonic acid, 3-[[4'-[(6-amino-1-hydroxy-3-sulfo-2-naphthalenyl)azo]-3,3'-dimethoxy[1,1'-biphenyl]-4-yl]azo]-4-hydroxy-, disodium salt **Direct Blue 151**
Structure					
Summary of data gap filling					
Target endpoint1 (modified Ames mutagenicity)	Experimental result (GLP)				
	Experimental result (non-GLP)	Positive S. typhimurium (TA 98; TA 100; TA 1538) with S9 (rat) (Probst et al. 1981) Positive S. typhimurium (TA 98; TA 100) with S9 (rat) (Chung et al. 2000)	Positive S. typhimurium (TA 1538) with S9 (hamster) + FMN (Reid et al. 1984) Positive S. typhimurium (TA 1538) with S9 (rat) + rat cecal bacteria (Reid et al. 1984)	Positive S. typhimurium (TA 98) with S9 (hamster) + FMN modified according to Prival et al. (Prival et al. 1984) Positive S. typhimurium (TA 1538) with S9 (hamster) + FMN (Reid et al. 1984) Positive S. typhimurium (TA 1538) with S9 (rat) + rat cecal bacteria (Reid et al. 1984) Positive S.typhimurium (TA 98) with S9 (hamster or rat) + FMN (Krishna et al. 1986) Positive S.typhimurium (SV50) with S9 (hamster or rat) + FMN (Krishna et al. 1986) Positive S. typhimurium (TA 98 / TA 100) with S9 + FMN/riboflavin or rat cecal bateria (Brown and Dietrich 1983)	Positive S. typhimurium (TA 98) with S9 (hamster) + FMN (Zhou et al. 1987)
	Integrated conclusion (eg. read-across)				
Molecular profiling related to the category hypothesis					
Expert system 1 (Benigni/Bossa Rulebase for Mutagenicity and Carcinogencity in Toxtree v2.5))****			Positive Genotoxic Alert: Aromatic Diazo	Positive Genotoxic Alert: Aromatic Diazo	Positive Genotoxic Alert: Aromatic Diazo
Expert system 2 (Derek Nexus, v3.0.1)			Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound	Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound	Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound

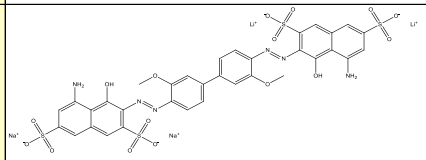
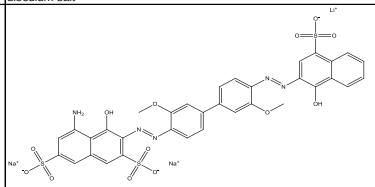
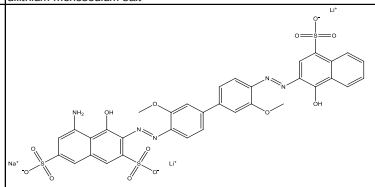
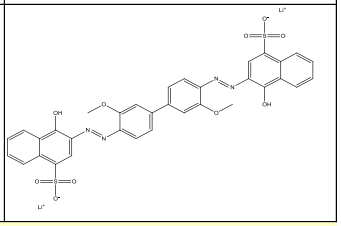
Chemical ID					
		Supporting Common Metabolite	**Member 1**	**Member 2**	**Member 3**
CAS		119-90-4	2429-71-2	2429-74-5	6449-35-0
Physical-chemical data					
Physical form			Powder (ChemicalBook 2008)	Powder (ChemicalBook 2008)	
Density - modelled (g/cm^3)**** ACD Percepta v2015 (2726)- PhysChem module			1.53	1.82	1.57
LogD - modelled**** ACD Percepta v2015 (2726)- LogD module			-1.42 @ pH 6.5	-9.73 @ pH 6.5	-2.61 @ pH 6.5
pKa_1 - modelled**** ACD Percepta v2015 (2726) - pKa Classic module			-0.2 ± 0.4	-1.9 ± 0.4	-1.2 ± 0.4
Water solubility - measured (mg/L)				3.0×10^4 (Brown 1992)	
Water solubility - modelled (mg/L)**** ACD Percepta v2015 (2726) - LogS module			11.3 @ pH 6.5	2.5×10^4 @ pH 6.5	76.5 @ pH 6.5
Metabolism					
Support for Reduction of Azo Bond (*in vivo*)			Yes	Yes	
Support for Reduction of Azo Bond (*in vitro*)				Yes	
Support for Reduction of Azo Bond (*in silico*) CATABOL microbial metabolic simulator - in OECD QSAR			Yes	Yes	Yes
Ames assay positive only or enhanced with reductive metabolic conditions			Yes	Yes (positive in certain assays without modifications, however more potent with; impurities maybe confouding results)	
Supporting data related to the target endpoint(s)					
*In silico (QSAR)*****	Multicase Case UltraT v1.4 (Ames mutagenicity)		Negative Inside AD	Negative Inside AD	Negative Inside AD
	Leadscope Model Applier v1.6 (Ames mutagenicity)***		Negative Inside AD P=0.38	Positive Inside AD P=0.71	Indeterminate Inside AD P=0.58
	VEGA/ CAESAR, v.2.1.12 (Ames mutagenicity)		Mutagen Outside AD	Mutagen Warning AD	Mutagen Inside AD
	OASIS TIMES (Ames mutagenicity)		Mutagen Out of AD Active is: Metabolite	Mutagen Out of AD Active is: Metabolite	Mutagen Out of AD Active is: Metabolite

*** P = probability that a chemical is positive
**** Modelled neutral form of compound (non-salt)

Chemical ID					
		Member 4	**Member 5**	**Member 6**	**Member 7**
CAS		67923-89-1	70210-28-5	71550-22-6	75659-72-2
Name		2,7-Naphthalenedisulfonic acid, 5-amino-4-hydroxy-3-[[4'-[(1-hydroxy-4-sulfo-2-naphthalenyl)azo]-3,3'-dimethoxy[1,1'-biphenyl]-4-yl]azo]-, trilithium salt	Benzoic acid, 5-[[4'-[[6-amino-5-(1H-benzotriazol-5-ylazo)-1-hydroxy-3-sulfo-2-naphthalenyl]azo]-3,3'-dimethoxy[1,1'-biphenyl]-4-yl]azo]-2-hydroxy-4-methyl-, disodium salt **BABHS**	2,7-Naphthalenedisulfonic acid, 3,3'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-, tetralithium salt	2,7-Naphthalenedisulfonic acid, 3,3'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-, monolithium trisodium salt
Structure					
Summary of data gap filling					
Target endpoint1 (modified Ames mutagenicity)	Experimental result (GLP)				
	Experimental result (non-GLP)		Positive S. typhimurium (TA 98 / TA 100) with S9 (hamster) + FMN (ILS 2011) - unpublished report for Health Canada		
	Integrated conclusion (eg. read-across)	**Read Across** **overall trend of category** Positive S.typhimurium (TA 98, 100, 1538) with reductive modifications and metabolic activation		**Read Across** **overall trend of category** Positive S.typhimurium (TA 98, 100, 1538) with reductive modifications and metabolic activation	**Read Across** **overall trend of category** Positive S.typhimurium (TA 98, 100, 1538) with reductive modifications and metabolic activation
Molecular profiling related to the category hypothesis					
Expert system 1 (Benigni/Bossa Rulebase for Mutagenicity and Carcinogencity in Toxtree v2.5))****		Positive Genotoxic Alert: Aromatic Diazo	Positive Genotoxic Alert: Aromatic Diazo	Positive Genotoxic Alert: Aromatic Diazo	Positive Genotoxic Alert: Aromatic Diazo
Expert system 2 (Derek Nexus, v3.0.1)		Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound	Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound	Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound	Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound

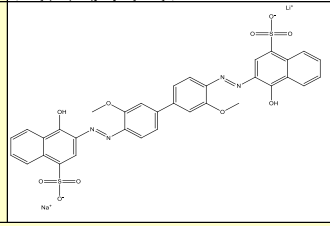
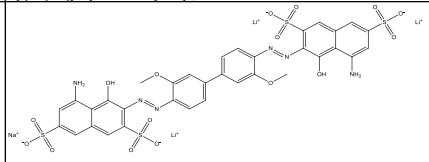
Chemical ID					
		Member 4	**Member 5**	**Member 6**	**Member 7**
CAS		67923-89-1	70210-28-5	71550-22-6	75659-72-2
Physical-chemical data					
Physical form					
Density - modelled (g/cm^3)**** ACD Percepta v2015 (2726)- PhysChem module		1.68	1.61	1.82	1.82
LogD - modelled**** ACD Percepta v2015 (2726)- LogD module		-5.42 @ pH 6.5	0.51 @ pH 6.5	-9.73 @ pH 6.5	-9.73 @ pH 6.5
pKa_1 - modelled**** ACD Percepta v2015 (2726) - pKa Classic module		-1.5 ± 0.4	1.0 ± 0.4	-1.9 ± 0.4	-1.9 ± 0.4
Water solubility - measured (mg/L)					
Water solubility - modelled (mg/L)**** ACD Percepta v2015 (2726) - LogS module		706 @ pH 6.5	12.7 @ pH 6.5	2.5 x 10^4 @ pH 6.5	2.5 x 10^4 @ pH 6.5
Metabolism					
Support for Reduction of Azo Bond (*in vivo*)					
Support for Reduction of Azo Bond (*in vitro*)					
Support for Reduction of Azo Bond (*in silico*) CATABOL microbial metabolic simulator - in OECD QSAR		Yes	Yes	Yes	Yes
Ames assay positive only or enhanced with reductive			Yes (ambiguous results without modifications)		
Supporting data related to the target endpoint(s)					
*In silico (QSAR)***	Multicase Case UltraT v1.4 (Ames mutagenicity)	Negative Inside AD	Negative Inside AD	Negative Inside AD	Negative Inside AD
	Leadscope Model Applier v1.6 (Ames mutagenicity)***	Positive Inside AD P=0.70	Indeterminate Inside AD P=0.48	Positive Inside AD P=0.71	Positive Inside AD P=0.71
	VEGA/ CAESAR, v.2.1.12 (Ames mutagenicity)	Mutagen Warning AD	Mutagen Inside AD	Mutagen Warning AD	Mutagen Warning AD
	OASIS TIMES (Ames mutagenicity)	Mutagen Out of AD Active is: Metabolite	Mutagen Out of AD Active is: Metabolite	Mutagen Out of AD Active is: Metabolite	Mutagen Out of AD Active is: Metabolite

*** P = probability that a chemical is positive
**** Modelled neutral form of compound (non-salt)

Chemical ID					
		Member 8	**Member 9**	**Member 10**	**Member 11**
CAS		75659-73-3	75673-18-6	75673-19-7	75673-34-6
Name		2,7-Naphthalenedisulfonic acid, 3,3'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-, dilithium disodium salt	2,7-Naphthalenedisulfonic acid, 5-amino-4-hydroxy-3-[[4'-[(1-hydroxy-4-sulfo-2-naphthalenyl)azo]-3,3'-dimethoxy[1,1'-biphenyl]-4-yl]azo]-, monolithium disodium salt	2,7-Naphthalenedisulfonic acid, 5-amino-4-hydroxy-3-[[4'-[(1-hydroxy-4-sulfo-2-naphthalenyl)azo]-3,3'-dimethoxy[1,1'-biphenyl]-4-yl]azo]-, dilithium monosodium salt	1-Naphthalenesulfonic acid, 3,3'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-hydroxy-, dilithium salt
Structure					
Summary of data gap filling					
Target endpoint1 (modified Ames mutagenicity)	Experimental result (GLP)				
	Experimental result (non-GLP)				
	Integrated conclusion (eg. read-across)	**Read Across** **overall trend of category** Positive S.typhimurium (TA 98, 100, 1538) with reductive modifications and metabolic activation	**Read Across** **overall trend of category** Positive S.typhimurium (TA 98, 100, 1538) with reductive modifications and metabolic activation	**Read Across** **overall trend of category** Positive S.typhimurium (TA 98, 100, 1538) with reductive modifications and metabolic activation	**Read Across** Positive S.typhimurium (TA 98, 100, 1538) with reductive modifications and metabolic activation
Molecular profiling related to the category hypothesis					
Expert system 1 (Benigni/Bossa Rulebase for Mutagenicity and Carcinogencity in Toxtree v2.5))****		Positive Genotoxic Alert: Aromatic Diazo	Positive Genotoxic Alert: Aromatic Diazo	Positive Genotoxic Alert: Aromatic Diazo	Positive Genotoxic Alert: Aromatic Diazo
Expert system 2 (Derek Nexus, v3.0.1)		Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound	Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound	Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound	Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound

Chemical ID					
		Member 8	**Member 9**	**Member 10**	**Member 11**
CAS		75659-73-3	75673-18-6	75673-19-7	75673-34-6
Name		2,7-Naphthalenedisulfonic acid, 3,3'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-, dilithium disodium salt	2,7-Naphthalenedisulfonic acid, 5-amino-4-hydroxy-3-[[4'-[(1-hydroxy-4-sulfo-2-naphthalenyl)azo]-3,3'-dimethoxy[1,1'-biphenyl]-4-yl]azo]-, monolithium disodium salt	2,7-Naphthalenedisulfonic acid, 5-amino-4-hydroxy-3-[[4'-[(1-hydroxy-4-sulfo-2-naphthalenyl)azo]-3,3'-dimethoxy[1,1'-biphenyl]-4-yl]azo]-, dilithium monosodium salt	1-Naphthalenesulfonic acid, 3,3'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-hydroxy-, dilithium salt
Physical-chemical data					
Physical form					
Density - modelled (g/cm^3)**** ACD Percepta v2015 (2726)- PhysChem module		1.82	1.68	1.68	1.53
LogD - modelled**** ACD Percepta v2015 (2726)- LogD module		-9.73 @ pH 6.5	-5.42 @ pH 6.5	-5.42 @ pH 6.5	-1.42 @ pH 6.5
pKa_1 - modelled**** ACD Percepta v2015 (2726) - pKa Classic module		-1.9 ± 0.4	-1.5 ± 0.4	-1.5 ± 0.4	-0.2 ± 0.4
Water solubility - measured (mg/L)					
Water solubility - modelled (mg/L)**** ACD Percepta v2015 (2726) - LogS module		2.5×10^4 @ pH 6.5	706 @ pH 6.5	706 @ pH 6.5	11.3 @ pH 6.5
Metabolism					
Support for Reduction of Azo Bond (*in vivo*)					
Support for Reduction of Azo Bond (*in vitro*)					
Support for Reduction of Azo Bond (*in silico*) CATABOL microbial metabolic simulator - in OECD QSAR		Yes	Yes	Yes	Yes
Ames assay positive only or enhanced with reductive					
Supporting data related to the target endpoint(s)					
*In silico (QSAR)****	Multicase Case UltraT v1.4 (Ames mutagenicity)	Negative Inside AD	Negative Inside AD	Negative Inside AD	Negative Inside AD
	Leadscope Model Applier v1.6 (Ames mutagenicity)***	Positive Inside AD P=0.71	Positive Inside AD P=0.70	Positive Inside AD P=0.70	Negative Inside AD P=0.38
	VEGA/ CAESAR, v.2.1.12 (Ames mutagenicity)	Mutagen Warning AD	Mutagen Warning AD	Mutagen Warning AD	Mutagen Out of AD
	OASIS TIMES (Ames mutagenicity)	Mutagen Out of AD Active is: Metabolite	Mutagen Out of AD Active is: Metabolite	Mutagen Out of AD Active is: Metabolite	Mutagen Out of AD Active is: Metabolite

*** P = probability that a chemical is positive

Chemical ID			
		Member 12	**Member 13**
CAS		75673-35-7	75752-17-9
Name		1-Naphthalenesulfonic acid, 3,3'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-hydroxy-, monolithium monosodium salt	2,7-Naphthalenedisulfonic acid, 3,3'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[5-amino-4-hydroxy-, trilithium monosodium salt
Structure			
Summary of data gap filling			
Target endpoint1 (modified Ames mutagenicity)	Experimental result (GLP)		
	Experimental result (non-GLP)		
	Integrated conclusion (eg. read-across)	**Read Across** Positive S.typhimurium (TA 98, 100, 1538) with reductive modifications and metabolic activation	**Read Across** **overall trend of category** Positive S.typhimurium (TA 98, 100, 1538) with reductive modifications and metabolic activation
Molecular profiling related to the category hypothesis			
Expert system 1 (Benigni/Bossa Rulebase for Mutagenicity and Carcinogencity in Toxtree v2.5))****		Positive Genotoxic Alert: Aromatic Diazo	Positive Genotoxic Alert: Aromatic Diazo
Expert system 2 (Derek Nexus, v3.0.1)		Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound	Plausible Bacterium mutagenicity (*in vitro*) Alert: Aromatic Azo Compound

Chemical ID			
		Member 12	**Member 13**
CAS		75673-35-7	75752-17-9
Physical-chemical data			
Physical form			
Density - modelled (g/cm^3)**** ACD Percepta v2015 (2726)- PhysChem module		1.53	1.82
LogD - modelled**** ACD Percepta v2015 (2726)- LogD module		-1.42 @ pH 6.5	-9.73 @ pH 6.5
pKa_1 - modelled**** ACD Percepta v2015 (2726) - pKa Classic module		-0.2 ± 0.4	-1.9 ± 0.4
Water solubility - measured (mg/L)			
Water solubility - modelled (mg/L)**** ACD Percepta v2015 (2726) - LogS module		11.3 @ pH 6.5	2.5 x 10^4 @ pH 6.5
Metabolism			
Support for Reduction of Azo Bond (*in vivo*)			
Support for Reduction of Azo Bond (*in vitro*)			
Support for Reduction of Azo Bond (*in silico*) CATABOL microbial metabolic simulator - in OECD QSAR		Yes	Yes
Ames assay positive only or enhanced with reductive			
Supporting data related to the target endpoint(s)			
*In silico (QSAR)****	Multicase Case UltraT v1.4 (Ames mutagenicity)	Negative Inside AD	Negative Inside AD
	Leadscope Model Applier v1.6 (Ames mutagenicity)***	Negative Inside AD P=0.38	Positive Inside AD P=0.71
	VEGA/ CAESAR, v.2.1.12 (Ames mutagenicity)	Mutagen Out of AD	Mutagen Warning AD
	OASIS TIMES (Ames mutagenicity)	Mutagen Out of AD Active is: Metabolite	Mutagen Out of AD Active is: Metabolite

*** P = probability that a chemical is positive
**** Modelled neutral form of compound (non-salt)