~~Confidential~~

Helsinki, 10 March 2022

Addressees
Registrants of JS_Acid_Green_111 listed in the last Appendix of this decision

Date of submission of the dossier subject of a decision
28/08/2019

Registered substance subject to this decision, hereafter 'the Substance'
Substance name: Trisodium 4-amino-5-hydroxy-3-[[4-[[2-oxo-1-[(phenylamino)carbonyl]propyl]azo]phenyl]azo]-6-[(4-sulphonato-1-naphthyl)azo]naphthalene-2,7-disulphonate
EC number: 279-093-7

Decision number: Please refer to the REACH-IT message which delivered this communication (in format TPE-D-XXXXXXXXXX-XX-XX/F)

DECISION ON TESTING PROPOSAL(S)

Based on Article 40 of Regulation (EC) No 1907/2006 (REACH), you must submit the information listed below by ***17 June 2024***.

The requested information must be generated using the Substance unless otherwise specified.

A. Information required from the Registrants subject to Annex VII of REACH

1. *In vitro* cytogenicity study in mammalian cells (test method: OECD TG 473) or *In vitro* micronucleus study (test method: OECD TG 487); and subsequently

2. *In vivo* genetic toxicity study to be selected according to the following specifications:

    a. If the results of the *in vitro* test requested under A.1 are **negative**:

    In vivo mammalian alkaline comet assay (test method: OECD TG 489) in rats, oral route, on the following tissues: liver, glandular stomach and duodenum.

    b. If the results of the *in vitro* test requested under A.1 are **positive**:

    In vivo mammalian alkaline comet assay (test method: OECD TG 489) combined with *in vivo* mammalian erythrocyte micronucleus test (test method: OECD TG 474) in rats, oral route. For the comet assay the following tissues shall be analysed: liver, glandular stomach and duodenum.

Reasons for the request(s) are explained in the following appendix entitled "Reasons to request information required under Annexes VII of REACH".

Information required depends on your tonnage band

You must provide the information listed above for all REACH Annexes applicable to you, and in accordance with Articles 10(a) and 12(1) of REACH, the information specified in Annex VII to REACH, for registration at 1-10 tonnes per year (tpa), or as a transported isolated intermediate in quantity above 1000 tpa.

You are only required to share the costs of information that you must submit to fulfil your information requirements.

How to comply with your information requirements

To comply with your information requirements you must submit the information requested by this decision in an updated registration dossier by the deadline indicated above. You must also update the chemical safety report, where relevant, including any changes to classification and labelling, based on the newly generated information.

You must follow the general testing and reporting requirements provided under the Appendix entitled "Requirements to fulfil when conducting and reporting new tests for REACH purposes". For references used in this decision, please consult the Appendix entitled "List of references".

Appeal

This decision can be appealed to the Board of Appeal of ECHA within three months of its notification. An appeal, together with the grounds thereof, has to be submitted to ECHA in writing. An appeal has suspensive effect and is subject to a fee. Further details are described under: http://echa.europa.eu/regulations/appeals.

Approved[1] under the authority of Mike Rasenberg, Director of Hazard Assessment

[1] As this is an electronic document, it is not physically signed. This communication has been approved according to ECHA's internal decision-approval process.

Appendix A: Reasons to request information required under Annex VII of REACH

This decision is based on the examination of the testing proposals you submitted.

1. In vitro cytogenicity study in mammalian cells or In vitro micronucleus study

Under Annex VII, Section 8.4, column 2 of REACH, further mutagenicity studies must be considered in case of a positive result in an *in vitro* gene mutation study in bacteria. ECHA guidance R.7a, section R.7.7.6.3 (p.570), further specifies that "*REACH Annex VII substances for which only a bacterial gene mutation test has been conducted and for which the result is positive should be studied further, according to the requirements of Annex VIII*." It is necessary to request an *in vitro* cytogenicity test as an additional test to further investigate the mutagenicity of the substance in accordance with the REACH integrated testing strategy. The obtained *in vitro* data will inform on the genotoxic concern(s) associated with the substance and help identify the most adequate follow-up *in vivo* study.

Your dossier contains positive results for the *in vitro* gene mutation study in bacteria (OECD TG 471; [redacted], 2017), which raise the concern for gene mutation.

In your comments on the draft decision you indicate that (1) the tested sample used in the available OECD TG 471 study ([redacted], 2017; study sponsor: [redacted]) has been re-evaluated and '*some doubts on the characterisation and the potentiality for uncontrolled impurities have been arisen, since during the revision of all available data, the positive result in bacteria, […] seemed questionable*'. (2) You also refer to other Ames studies available with a number of azo-dye substances that gave negative results.

ECHA notes the following:

(1) The available Ames study ([redacted], 2017) is GLP compliant and has a reliability score of 1 with no deviations from the OECD TG 471 reported in your dossier. Moreover, according to the information under the test material section, the composition of the Substance tested is '*as per the legal entity composition in section 1.2*' of your IUCLID dossier.

In your comments you only speculate that there are '*some doubts*' concerning the substance that has been tested in the Ames study however, you have not provided any supporting information on the test material used, to substantiate your claim.

In the absence of such information concerning the adequacy and reliability of the study, ECHA considers the available OECD TG 471 study ([redacted], 2017) as a valid study, based on the information reported in the dossier.

Therefore, considering the positive results obtained in the study with the Substance (positive with a reductive metabolic activation system in *S. typhimurium* strains: TA 1537 and TA 100, with and without metabolic activation and TA 98 with metabolic activation), further mutagenicity studies must be considered, as explained above.

(2) In your comments you also refer to the Ames data of other azo-dyes. More specifically, '*Ames studies available on butiramidate-co-complexes sharing a substructure with the target substance*'.

However, you have not provided any justification or documentation to explain how and why this information can be used to predict the outcome on mutagenicity for the Substance.

In the absence of such justification and/or relevant documentation to substantiate your claim, ECHA cannot assess the relevance of such comments in respect to the mutagenic properties of the Substance.

Finally, ECHA notes that in your comments you also provide a QSAR toolbox prediction for the main constituent of the Substance, indicating '*no alert found*'. However, as already explained above, your dossier contains positive results (OECD TG 471) with the Substance which raise a concern for gene mutation. The predicted information from the QSAR toolbox does not remove the concern raised from an adequate and reliable study with the Substance.

1.1. Information provided to fulfil the information requirement

You have submitted a testing proposal for an *in vivo* mammalian alkaline comet assay to be performed with the Substance to further investigate the mutagenicity of the substance.

However, no information from an *in vitro* cytogenicity study or an *in vitro* micronucleus study on the Substance in mammalian cells is available in the dossier.

ECHA therefore considers that an appropriate *in vitro* cytogenicity or micronucleus study is necessary to further investigate the mutagenicity of the Substance and to help identify the most adequate follow-up *in vivo* study.

For the following reasons ECHA further considers that the data provided in your dossier does not meet the conditions for adaptation of the information on an *in vitro* cytogenicity study in mammalian cells or an *in vitro* micronucleus study:

In the justification provided in your dossier you indicate that the Substance, being an azo-dye, will "*not be expected to give positive results in mammalian cells, where reductive metabolic conditions are not applied*". Moreover you indicate that that the specific metabolic pathway explaining the positive Ames findings "*cannot be simulated adequately using mammalian cells in vitro*".

Under Section 8.4.2., Column 2, Annex VIII to REACH, the study may be omitted 1) if adequate data from an *in vivo* cytogenicity test are available or 2) if the Substance is known to be carcinogenic category 1A or 1B or germ cell mutagenic category 1A, 1B or 2.

We note the following:
1) You have not provided an *in vivo* chromosomal aberration test; and
2) The Substance is not known to be Carc. 1A/1B or germ cell muta 1A, 1B, or 2.

Therefore, the requirements of Section 8.4.2., Column 2, Annex VIII to REACH for an adaptation of the information on an *in vitro* cytogenicity study in mammalian cells or an *in vitro* micronucleus study are not met.

1.2 Test design

Either the *in vitro* cytogenicity study in mammalian cells (test method OECD TG 473) or the *in vitro* micronucleus study (test method OECD TG 487) are considered suitable.

1.3 Outcome

Under Article 40(3)(c) of REACH, you are requested to carry out the additional test, as indicated above.

2. In vivo genetic toxicity study

Under Annex VII Section 8.4., column 2 of REACH, further mutagenicity studies must be considered in case of a positive result in an *in vitro* gene mutation study in bacteria.

Your dossier contains positive results for the *in vitro* gene mutation study in bacteria (OECD TG 471; [redacted], 2017), which raise the concern for gene mutations.

2.1 Information provided to fulfil the information requirement

You have submitted a testing proposal for an *in vivo* mammalian alkaline comet assay to be performed with the Substance.

ECHA requested your considerations for alternative methods to fulfil the information requirement for Genetic toxicity *in vivo*. You provided your considerations concluding that there were no alternative methods which could be used to adapt the information requirement(s) for which testing is proposed. ECHA has taken these considerations into account.

ECHA agrees that an appropriate *in vivo* follow up genotoxicity study is necessary to address the concerns identified *in vitro.*

2.2 Test selection

ECHA notes that the proposed *in vivo* mammalian alkaline comet assay ("comet assay", OECD TG 489) is suitable to follow up a positive *in vitro* result on gene mutation.

However, as explained above, under section 1, the adaptation provided to waive the *in vitro* cytogenicity study or an *in vitro* micronucleus study does not meet the requirements of Section 8.4.2., Column 2, Annex VIII to REACH. Therefore, by this decision, ECHA also requests an *in vitro* cytogenicity study or an *in vitro* micronucleus study (for the reasons see above Appendix A.1.), which may raise a concern for chromosomal aberration in case of positive results.

In case there is also a concern for chromosomal aberration, you must combine the comet assay and the *in vivo* mammalian erythrocyte micronucleus test ("MN test", OECD TG 474) into a single study (see OECD TG 489 para. 33; OECD TG 474 para. 37c; ECHA Guidance R.7a, Section R.7.7.6.3). While the comet assay can detect primary DNA damage that may lead to gene mutations and/or structural chromosomal aberrations, the MN test can detect both structural chromosomal aberrations (clastogenicity) and numerical chromosomal aberrations (aneuploidy). A combined study will thus address both concerns for chromosomal aberration as well as gene mutation.

The combined study, together with the results of the *in vitro* mutagenicity studies, can be used to make definitive conclusions about the mechanism(s) inducing *in vivo* mutagenicity and lack thereof. Furthermore, the combined study can help reduce the number of tests performed and the number of animals used while addressing (structural and numerical) chromosomal aberrations as well as gene mutations.

Therefore, you must wait for the results of the *in vitro* test requested under A.1. and, depending on these results, to conduct either a) Comet assay if the test results of request A.1 are negative; or b) Comet assay combined with MN test if the test results of request A.1 are positive. The deadline set in this decision allows for sequential testing.

In your comments on the draft decision you suggest to first perform a '*confirmatory Ames study*' and in case of a positive result in the new Ames study you agree with the testing strategy indicated above.

However, as already explained under Section 1, you have not provided, either in the dossier or in your comments on the draft decision, any substantiated justification why the Ames study ([redacted], 2017) that is available in your dossier cannot be considered adequate and reliable.

Therefore, based on the absence of this information and considering the positive results obtained in the Ames study ([redacted], 2017) you must perform the studies requested in this decision.

2.3 Specification of the study design

*a) Comet assay (if the test results of request A.1 are **negative**)*

You did not specify the species to be used for testing. According to the test method OECD TG 489, rats are routinely used in this test. Therefore you must perform the study in rats.

You proposed testing by the oral route. Having considered the anticipated routes of human exposure and adequate exposure of the target tissue(s) performance of the test by the oral route is appropriate.

In your testing proposal you only refer to "*an in vivo assay to evaluate genotoxic activity in tissues/organs, including the gastrointestinal tract*". ECHA notes that in line with the test method OECD TG 489, the test must be performed by analysing tissues from liver as primary site of xenobiotic metabolism, glandular stomach and duodenum as sites of contact. There are several expected or possible variables between the glandular stomach and the duodenum (different tissue structure and function, different pH conditions, variable physico-chemical properties and fate of the Substance, and probable different local absorption rates of the Substance and its possible breakdown product(s)). In light of these expected or possible variables, it is necessary to analyse both tissues to ensure a sufficient evaluation of the potential for genotoxicity at the site of contact in the gastro-intestinal tract.

Germ cells
You may consider to collect the male gonadal cells from the seminiferous tubules in addition to the other aforementioned tissues in the comet assay, as it would optimise the use of animals. You can prepare the slides for male gonadal cells and store them for up to 2 months, at room temperature, in dry conditions and protected from light. Following the generation and analysis of data on somatic cells in the comet assay, you should consider analysing the slides prepared with gonadal cells.

This type of evidence may be relevant for the overall assessment of possible germ cell mutagenicity including classification and labelling according to the CLP Regulation.

*b) Comet assay combined with MN test (if the test results of request A.1 are **positive**)*

According to the test method OECD TG 489, rats are routinely used in this test. Therefore, the combined test (OECD TG 489 and OECD TG 474) must be performed in rats. Having considered the anticipated routes of human exposure and the need for adequate exposure of the target tissue(s) performance of the test by the oral route is appropriate.

In line with the test method OECD TG 489, the test must be performed by analysing tissues from liver as primary site of xenobiotic metabolism, glandular stomach and duodenum as

sites of contact. There are several expected or possible variables between the glandular stomach and the duodenum (different tissue structure and function, different pH conditions, variable physico-chemical properties and fate of the Substance, and probable different local absorption rates of the Substance and its possible breakdown product(s)). In light of these expected or possible variables, it is necessary to analyse both tissues to ensure a sufficient evaluation of the potential for genotoxicity at the site of contact in the gastro-intestinal tract.

The combination of OECD TGs 489 and 474 should not impair the validity of and the results from each individual study. Careful consideration should be given to the dosing, and tissue sampling for the comet analysis alongside the requirements of tissue sampling for the mammalian erythrocyte micronucleus test (see OECD TG 489, e.g. Bowen *et al*. 2011 [1]).

Germ cells
You may consider to collect the male gonadal cells from the seminiferous tubules in addition to the other aforementioned tissues in the comet assay, as it would optimise the use of animals. You can prepare the slides for male gonadal cells and store them for up to 2 months, at room temperature, in dry conditions and protected from light. Following the generation and analysis of data on somatic cells in the comet assay, you should consider analysing the slides prepared with gonadal cells.

Reference:
[1] Bowen DE et al. (2011) Evaluation of a multi-endpoint assay in rats, combining the bone-marrow micronucleus test, the comet assay and the flow-cytometric peripheral blood micronucleus test. *Muta Res*;722:7–19.

2.4 Outcome

Under Article 40(3)(b) your testing proposal is accepted under modified conditions and you are requested to conduct the test with the Substance, as specified above.

Appendix B: Requirements to fulfil when conducting and reporting new tests for REACH purposes

A. Test methods, GLP requirements and reporting

1. Under Article 13(3) of REACH, all new data generated as a result of this decision must be conducted according to the test methods laid down in a European Commission Regulation or to international test methods recognised by the Commission or ECHA as being appropriate.

2. Under Article 13(4) of REACH, ecotoxicological and toxicological tests and analyses must be carried out according to the GLP principles (Directive 2004/10/EC) or other international standards recognised by the Commission or ECHA.

3. Under Article 10(a)(vi) and (vii) of REACH, all new data generated as a result of this decision must be reported as study summaries, or as robust study summaries, if required under Annex I of REACH. See ECHA Practical Guide on How to report robust study summaries[2].

B. Test material

Before generating new data, you must agree within the joint submission on the chemical composition of the material to be tested (Test material) which must be relevant for all the registrants of the Substance.

1. Selection of the Test material(s)

   The Test material used to generate the new data must be selected taking into account the following:
   - the variation in compositions reported by all members of the joint submission,
   - the boundary composition(s) of the Substance,
   - the impact of each constituent/ impurity on the test results for the endpoint to be assessed. For example, if a constituent/ impurity of the Substance is known to have an impact on (eco)toxicity, the selected Test material must contain that constituent/ impurity.

2. Information on the Test material needed in the updated dossier
   - You must report the composition of the Test material selected for each study, under the "Test material information" section, for each respective endpoint study record in IUCLID.
   - The reported composition must include all constituents of each Test material and their concentration values and other parameters relevant for the property to be tested.

This information is needed to assess whether the Test material is relevant for the Substance and whether it is suitable for use by all members of the joint submission.

Technical instructions on how to report the above is available in the manual on How to prepare registration and PPORD dossiers[3].

[2] https://echa.europa.eu/practical-guides
[3] https://echa.europa.eu/manuals

Appendix C: Procedure

ECHA started the testing proposal evaluation in accordance with Article 40(1) on 27 November 2020.

ECHA held a third party consultation for the testing proposal(s) from 21 January 2021 until 8 March 2021. ECHA did not receive information from third parties.

ECHA followed the procedure detailed in Articles 50 and 51 of REACH.

ECHA notified you of the draft decision and invited you to provide comments.

ECHA took into account your comments and did not amend the requests.

ECHA notified the draft decision to the competent authorities of the Member States for proposals for amendment.

As no amendments were proposed, ECHA adopted the decision under Article 51(3) of REACH.

Appendix D: List of references - ECHA Guidance[4] and other supporting documents

Evaluation of available information
Guidance on information requirements and chemical safety assessment, Chapter R.4 (version 1.1., December 2011), referred to as ECHA Guidance R.4 where relevant.

QSARs, read-across and grouping
Guidance on information requirements and chemical safety assessment, Chapter R.6 (version 1.0, May 2008), referred to as ECHA Guidance R.6 where relevant.

Read-across assessment framework (RAAF, March 2017)[5]

RAAF - considerations on multi-constituent substances and UVCBs (RAAF UVCB, March 2017)[6]

Physical-chemical properties
Guidance on information requirements and chemical safety assessment, Chapter R.7a (version 6.0, July 2017), referred to as ECHA Guidance R.7a in this decision.

Toxicology
Guidance on information requirements and chemical safety assessment, Chapter R.7a (version 6.0, July 2017), referred to as ECHA Guidance R.7a in this decision.

Guidance on information requirements and chemical safety assessment, Chapter R.7c (version 3.0, June 2017), referred to as ECHA Guidance R.7c in this decision.

Environmental toxicology and fate
Guidance on information requirements and chemical safety assessment, Chapter R.7a (version 6.0, July 2017), referred to as ECHA Guidance R.7a in this decision.

Guidance on information requirements and chemical safety assessment, Chapter R.7b (version 4.0, June 2017), referred to as ECHA Guidance R.7b in this decision.

Guidance on information requirements and chemical safety assessment, Chapter R.7c (version 3.0, June 2017), referred to as ECHA Guidance R.7c in this decision.

PBT assessment
Guidance on information requirements and chemical safety assessment, Chapter R.11 (version 3.0, June 2017), referred to as ECHA Guidance R.11 in this decision.

Guidance on information requirements and chemical safety assessment, Chapter R.16 (version 3.0, February 2016), referred to as ECHA Guidance R.16 in this decision.

Data sharing
Guidance on data-sharing (version 3.1, January 2017), referred to as ECHA Guidance on data sharing in this decision.

OECD Guidance documents[7]

[4] https://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment
[5] https://echa.europa.eu/support/registration/how-to-avoid-unnecessary-testing-on-animals/grouping-of-substances-and-read-across
[6] https://echa.europa.eu/documents/10162/13630/raaf_uvcb_report_en.pdf/3f79684d-07a5-e439-16c3-d2c8da96a316
[7] http://www.oecd.org/chemicalsafety/testing/series-testing-assessment-publications-number.htm

Guidance Document on aqueous–phase aquatic toxicity testing of difficult test chemicals – No 23, referred to as OECD GD 23.

Guidance document on transformation/dissolution of metals and metal compounds in aqueous media – No 29, referred to as OECD GD 29.

Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption – No 150, referred to as OECD GD 150.

Guidance Document supporting OECD test guideline 443 on the extended one-generation reproductive toxicity test – No 151, referred to as OECD GD 151.

Appendix E: Addressees of this decision and the corresponding information requirements applicable to them

You must provide the information requested in this decision for all REACH Annexes applicable to you.

Registrant Name	Registration number	Highest REACH Annex applicable to you
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

Where applicable, the name of a third party representative (TPR) may be displayed in the list of recipients whereas ECHA will send the decision to the actual registrant.