Helsinki, 16 December 2015

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

DECISION ON SUBSTANCE EVALUATION PURSUANT TO ARTICLE 46(1) OF REGULATION (EC) NO 1907/2006

For 4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline], CAS No 28768-32-3 (EC No 249-204-3)

Addressees: Registrant(s)[1] of 4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline] (Registrant(s))

This decision is addressed to all Registrants of the above substance with active registrations on the date on which the draft for the decision was first sent, with the exception of the cases listed in the following paragraph. A list of all the relevant registration numbers subject to this decision is provided as an Annex to this decision.

Registrants holding active registrations on the day the draft decision was sent are *not* addressees of this decision if they are: i) Registrant(s) who had on that day registered the above substance exclusively as an on-site isolated intermediate under strictly controlled conditions and ii) Registrant(s) who have ceased manufacture/import of the above substance in accordance with Article 50(3) of Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH Regulation) before the decision is adopted by ECHA.

Based on an evaluation by the Danish Environmental Protection Agency as the Competent Authority of Denmark (evaluating MSCA), the European Chemicals Agency (ECHA) has taken the following decision in accordance with the procedure set out in Articles 50 and 52 of Regulation (EC) No 1907/2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH Regulation).

This decision is based on the registration dossier(s) on 10 June 2014, i.e. the day until which the evaluating MSCA granted an extension for submitting dossier updates which it would take into consideration.

This decision does not imply that the information provided by the Registrant(s) in the registration(s) is in compliance with the REACH requirements. The decision neither prevents ECHA from initiating compliance checks on the dossier(s) of the Registrant(s) at a later stage, nor does it prevent a subsequent decision under the current substance evaluation or a new substance evaluation process once the present substance evaluation has been completed.

I. Procedure

Pursuant to Article 45(4) of the REACH Regulation the Competent Authority of Denmark has initiated substance evaluation for 4,4'-methylenebis[N,N-bis(2,3-epoxypropyl)aniline], CAS No 28768-32-3 (EC No 249-204-3) (TGMDA) based on registration(s) submitted by the Registrant(s) and other relevant and available information and prepared the present decision in accordance with Article 46(1) of the REACH Regulation.

[1] The term Registrant(s) is used throughout the decision, irrespective of the number of registrants addressed by the decision.

On the basis of an opinion of the ECHA Member State Committee and due to initial grounds for concern relating to mutagenicity, carcinogenicity and wide dispersive use, TGMDA was included in the Community rolling action plan (CoRAP) for substance evaluation to be evaluated in 2013. The updated CoRAP was published on the ECHA website on 20 March 2013. The Competent Authority of Denmark was appointed to carry out the evaluation.

Based on the registration dossier and other available information the evaluating MSCA considered that further information was required to clarify the abovementioned concerns. Therefore, it prepared a draft decision pursuant to Article 46(1) of the REACH Regulation to request further information. It submitted the draft decision to ECHA on 20 March 2014.

On 29 April 2014 ECHA sent the draft decision to the Registrant(s) and invited them pursuant to Article 50(1) of the REACH Regulation to provide comments within 30 days of the receipt of the draft decision.

On 2 December 2013 and 10 June 2014 the Registrant(s) submitted an updated registration dossier.

By 4 June 2014, ECHA received comments from the Registrant(s) of which it informed the evaluating MSCA without delay.

The evaluating MSCA considered the comments received from the Registrant(s) and the dossier updates. On basis of this information, Section II was amended. The Statement of Reasons (Section III) was changed accordingly.

Based on the justification provided by the Registrant(s) in the updated dossier the evaluating MSCA has accepted the read across, limited to the present substance evaluation purpose to clarify the concern identified for mutagenicity from the UVCB TGMDA substance (UVCB, substance of unknown or variable composition, complex reaction products or biological materials) (CAS 28390-91-2) to the monoconstituent TGMDA (CAS 28768-32-3). A Mammalian Erythrocyte Micronucleus Test in mice by oral gavage (OECD 474), which yielded a negative result has been performed on the UVCB substance (CAS 28390-91-2). Consequently, the requirements in the initial draft decision regarding a Mammalian Erythrocyte Micronucleus Test in mice by oral gavage (OECD 474) (and conditionally if this yielded a positive result a Mammalian Spermatogonial Chromosome Aberration Test in mice by oral gavage (OECD 483)) has been withdrawn.

In addition, the original draft decision included requests related to missing information in the Chemical Safety Report. This information was provided in the updated registration dossier and hence these requests were removed from the draft decision.

Commenting by other MSCAs and ECHA

In accordance with Article 52(1) of the REACH Regulation, on 23 July 2015 the evaluating MSCA notified the Competent Authorities of the other Member States and ECHA of its draft decision and invited them pursuant to Articles 52(2) and 51(2) of the REACH Regulation to submit proposals to amend the draft decision within 30 days of the receipt of the notification.

Subsequently, some Competent Authorities of the Member States and ECHA submitted proposals for amendment to the draft decision.

On 28 August 2015 ECHA notified the Registrants of the proposals for amendment to the draft decision and invited them pursuant to Articles 52(2) and 51(5) of the REACH

Regulation to provide comments on those proposals for amendment within 30 days of the receipt of the notification.

The evaluating MSCA reviewed the proposals for amendment received and amended the draft decision.

On 7 September 2015 ECHA referred the draft decision to the Member State Committee.

The Registrant did not provide any comments on the proposals for amendment, in accordance to Article 51(5). However, on 28 September 2015, the Registrants provided comments on the draft decision. The Member State Committee did not take into account the Registrant's comments on the draft decision as they were not related to the proposals for amendment made and are therefore considered outside the scope of Article 51(5).

A unanimous agreement of the Member State Committee on the draft decision was reached on 13 October 2015 in a written procedure launched on 1 October 2015.

ECHA took the decision pursuant to Article 51(6) of the REACH Regulation.

II. Information required

Pursuant to Article 46(1) of the REACH Regulation the Registrant(s) shall submit the following information using the indicated test methods/instructions (in accordance with Article 13(3) and (4) of the REACH Regulation) and the registered substance subject to the present decision (monoconstituent TGMDA, CAS 28768-32-3) or the UVCB TGMDA (CAS 28390-91-2) with the composition specified in the registration dossier:

1.A Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays in mouse or rat by oral gavage (EU B.58./OECD 488) following a 28-day exposure with a subsequent 3 day sampling period.

The following tissues shall be analysed: Glandular stomach, duodenum/jejenum, liver, and bone marrow. In accordance with paragraph 35 of the test guideline '*spermatozoa from the vas deferens/cauda epididymis and developing germ cells from the seminiferous tubules (as described in Paragraphs 32 and 33) should be collected and stored in case future analysis of germ cell mutagenicity is required.*' If the analysis of any of the somatic tissues indicates that the substance is a somatic cell mutagen, the germ cell samples shall then also be analysed.

OR

1.B *In vivo* mammalian alkaline Comet Assay in mouse or rat by oral gavage (OECD 489) as a first *in vivo* test. The following tissues should be analyzed (liver, glandular stomach and duodenum/jejenum). The optimum sampling time(s) should be determined based on kinetic data if available.

The evaluating MSCA will evaluate and interpret the result of the performed OECD 489 as well as the documentation and acceptability of the test criteria (paragraph 58-65 OECD 489). In case the evaluating MSCA finds that the result is clearly negative it can then be concluded that there is no further concern for *in vivo* gene mutagenicity.

In case the evaluating MSCA finds that the test result of the OECD 489 is positive, equivocal or that the test criteria are not acceptable the evaluating MSCA may then in a subsequent decision making process propose to request a TGR assay (Article 46(3) of the REACH

Regulation).

Test specifications for the requested OECD 489:

All critical parameters described in the OECD 489 should be carefully controlled and documented in detail in the study report (refer to section III for details). The laboratory needs to demonstrate proficiency with each individual tissue in each species they are planning to study, and will need to demonstrate that an acceptable positive response with a known mutagen (e.g. EMS) can be obtained in that tissue. For the study to be acceptable a low range of % tail DNA in controls is necessary in order to provide sufficient dynamic range to detect a potential positive effect. The evaluating MSCA must have access to the full study report including all relevant details of the study, ensuring that a clear conclusion regarding the result of the study can be drawn by the evaluating MSCA.

The optimum sampling time(s) may be substance- or route-specific, sampling times should be determined where available from kinetic data (e.g., the time (Tmax) at which the peak plasma or tissue concentration (Cmax) is achieved, or at the steady state for multiple administrations). In order to maximize the ability to detect short-lived lesions animals should be euthanized and tissues collected at or soon after Tmax is reached. In the absence of kinetic data a suitable compromise for the measurement of genotoxicity is to sample at 2-6 h after the last treatment for two or more treatments, or at both 2-6 and 16-26 h after a single administration, although care should be taken to necropsy all animals at the same time after the last (or only) dose. Information on the appearance of toxic effects in target organs (if available) may also be used to select appropriate sampling times.

Further testing may later be relevant in accordance with Article 46 of the REACH Regulation, depending on the results obtained in the studies requested above.

Deadline for submitting the required information

Pursuant to Article 46(2) of the REACH Regulation, the Registrant(s) shall submit to ECHA an update of the registration(s) containing the information required by this decision[2], including robust study summaries, an update of the Chemical Safety Report, and where relevant, full study reports, so that the evaluating MSCA has access to all relevant details of the studies, ensuring that a clear conclusion regarding the results of the studies can be drawn. This information shall be submitted by:

- **23 June 2017** if a Transgenic Rodent Assay is performed
  or
- **02 January 2017** if a Comet Assay is performed.

III. Statement of reasons

Based on the evaluation of all relevant information submitted on TGMDA and other relevant and available information, ECHA concludes that further information is required in order to enable the evaluating MSCA to complete the evaluation of whether the substance constitutes a risk to human health.

Testing for mutagenicity

TGMDA has been shown to cause gene mutations *in vitro* in a bacterial reverse mutation assay (Ames test prior to the adoption of the guideline) by ([redacted]) (Klimisch 2, reliable

[2] The deadline set by the decision already takes into account the time that registrants may require to agree on who is to perform any required tests and the time that ECHA would require to designate a registrant to carry out the test(s) in the absence of the aforementioned agreement by the registrants (Article 53(1) of the REACH Regulation).

with restrictions), which was performed according to the Japanese guidelines for screening mutagenicity testing of chemicals. TGMDA also caused gene mutations in a supporting study in the bacterial reverse mutation test (Ames test prior to the adoption of the guideline, [redacted] Klimisch 3, not reliable). TGMDA was positive only with metabolic activation in both studies. No data are available concerning gene mutagenicity *in vivo* and there is therefore concern as to whether TGMDA and/or its reactive metabolites can cause gene mutagenicity in somatic and/or germ cells *in vivo*.

Comments from the Registrant(s):

The Registrant(s) agrees that due to concern for gene mutagenicity *in vivo*, testing on the potential of the substance to cause gene mutation is necessary. However, rather than a Transgenic Rodent Somatic and Germ cell Gene Mutation Assay in mouse or rat by oral gavage (EU B.58./OECD 488), the Registrant(s)suggested that the *in vivo* alkaline Comet Assay would be suitable to detect somatic and germ cell mutation.

The evaluating MSCA has carefully considered the proposal by the Registrant to choose the considerably cheaper Comet Assay instead of a TGR Assay as follow-up. The OECD test guideline 489 for the *In Vivo* Mammalian Alkaline Comet Assay was recently adopted (26 September 2014).

Considerations on the endpoint:
The Comet Assay is a genotoxicity test. It does not measure mutations, but short-lived strand breaks that may be repaired, i.e. the DNA damage effect may or may not be persistent, it may be lethal to the cell and hence not result in a permanent mutation, or it may not be successfully repaired and then be fixed into a permanent mutation.

For this reason a positive result in the *in vivo* Comet assay in somatic cells is only indicative of possible somatic mutagenicity and it is currently uncertain whether the presence of such results on their own are clear enough to indicate somatic mutagenicity, i.e. whether such results alone are sufficient to fulfill the CLP criteria for a Muta Cat. 2 classification. Furthermore, the OECD 489 has not yet been validated for detecting germ cell mutagenicity. Positive results in gonadal cells, however, indicates that the test substance or its metabolites have caused DNA damage in the mixed population of somatic and germ cells in the gonads. Therefore, if such a result is obtained by a laboratory which has shown proficiency for detecting such effects in gonadal cells, this result will be indicative of germ cell mutagenicity. This means that if there is sufficient evidence of *in vivo* mutagenicity in somatic cells this result in germ cells would be sufficient to conclude that the substance is also a germ cell mutagen (i.e. should be classified Muta Cat. 1b according to the CLP Regulation).

According to the ECHA guidance on Mutagenicity (Endpoint Specific guidance R.7a, version 3.0, August 2014) "for substances that appear preferentially to induce gene mutations, the TGR assays are the most appropriate and usually preferred tests to follow-up an *in vitro* gene mutation positive result and detect, *in vivo*, substances that induce gene mutations."

3R considerations:
Both the Comet Assay and the TGR Assay utilize a total of ≥ 25 animals per test. This means that if a TGR Assay is chosen as the standalone follow-up only 25 animals will be utilized in total because the concern for somatic and germ cell mutagenicity can be resolved in the same animals in accordance with two of the 3R principles of reduction and refinement.

If the option of conducting a Comet Assay as a first *in vivo* test is chosen ≥ 25 animals will

be utilized for this test. A positive result in the Comet Assay will then (c.f. the above considerations) most likely have to be followed-up by conducting a TGR Assay for both somatic and germ cell mutagenicity utilizing an additional ≥25 animals, resulting in ≥50 animals in total.

Further considerations on the concern for germ cell mutagenicity:
Currently, the Comet Assay (OECD 489) is as mentioned above not fully validated to measure DNA strand breaks in mature germ cells (paragraph 10, OECD 489: "*protocol modifications together with improved standardization and validation studies are deemed necessary before the comet assay on mature germ cells (e.g. sperm) can be included in the test guideline. [...] Genotoxic effects as measured by the comet assay in testicular cells at different stages of differentiation have been described in the literature (34) (35). However, it should be noted that gonads contain a mixture of somatic and germ cells. For this reason, positive results in whole gonad (testis) are not necessarily reflective of germ cell damage; nevertheless, they indicate that tested chemical(s) and/or its metabolites have reached the gonad.*"

Consequently, the Comet Assay is not equivalent to the TGR assay for the identification of germ cell mutagens and in particular it is not appropriate to use negative test results to conclude that a substance does not induce gene mutations in germ cells.

By using the TGR Assay TG 488 it is possible to sample both somatic cells as well as germ cells from the same animals in the study. It is then possible to analyze for germ cell mutagenicity without conducting a new animal study in case mutagenic effects are identified in any of the somatic tissues, "*sampling cells from seminiferous tubules in addition to spermatozoa from the vas deferens/cauda epididymis following only a 28 + 3 day sampling regimen would provide some coverage of cells exposed across the majority of phases of germ cell development*" (paragraph 33 OECD 488).

Current limitations of the Comet Assay:
As reflected throughout the current version of the OECD 489 (April 2014) several critical parameters pertaining to the transient nature of strand breaks, application of negative and positive controls, and technical parameters of gel electrophoresis affect the results of the Comet Assay and must be carefully controlled and documented (please see also 'Acceptability Criteria' (paragraph 58-65) and Annex 3: Current Limitations of the Assay' in the OECD 489).

Focus points of both the OECD 489 and Annex 3 include, but are not limited to the following: "*The laboratory needs to demonstrate proficiency with each individual tissue in each species they are planning to study, and will need to demonstrate that an acceptable positive response with a known mutagen (e.g. EMS) can be obtained in that tissue.*" (paragraph 17). "*Vehicle/negative control data should be collected so as to demonstrate reproducibility of negative data responses, and to ensure that the technical aspects of the assay were properly controlled or to suggest the need to re-establish historical control ranges (see paragraph 22).*" (paragraph 18).

Different tissues and different species, as well as different vehicles and routes of administrations, may give different negative control % tail DNA values. "*No inter-laboratory studies have been conducted in tissues other than liver and stomach, therefore no recommendation has been established for how to achieve a sensitive and reproducible response in tissues other than liver, such as expected positive and negative control ranges. For the liver, agreement on setting a lower limit to the negative control value also could not be reached.*" (OECD 489, Annex 3 Current limitations of the Assay, paragraph 3).

"*Laboratories should use quality control methods, such as control charts (e.g. C-charts or X-bar charts (48)), to identify how variable their data are, and to show that the methodology is 'under control' in their laboratory. Selection of appropriate positive control substances, dose ranges and experimental conditions (e.g. electrophoresis conditions) may need also to be optimised for the detection of weak effects (see paragraph 17).*" (OECD 489 paragraph 21). "*Any changes to the experimental protocol should be considered in terms of their consistency with the laboratory's existing historical control databases. Any major inconsistencies should result in the establishment of a new historical control database*" (OECD 489 paragraph 22)."

Due to the transient nature of strand breaks the optimum sampling time is critical. "*Some types of DNA damage may be short-lived, i.e. may be repaired too quickly to be observed 24 hours or more after the last dose. There is no identifiable list of the types of short-lived damages, nor of the substances which are likely to cause this type of damage, nor is it known over what time period this type of damage can be detected. The optimum sampling time(s) may also be substance- or route-specific and sampling times should be determined from kinetic data (for example the time, Tmax, at which the peak plasma or tissue concentration is achieved), when such data are available.*" (OECD 489, Annex 3 Current limitations of the Assay, paragraph 1).

Also, the length of time from euthanasia to removal of tissues may be critical for the detection of strand breaks (OECD 489 paragraph 19). In order to maximize the ability to detect short-lived lesions animals should be euthanized and tissues collected at or soon after Tmax is reached. The laboratory needs to be proficient in harvesting multiple tissues from a single animal. Historical and contemporary positive and vehicle control data should be included in the study report. The scoring of cells must be done quantitatively using an automated or semi-automated image-analysis system.

The Comet Assay Working Group (Speit et al. 2015) does not currently recommend the use of frozen samples in the *in vivo* Comet Assay, which means that samples from multiple tissues must be processed right away. "*Currently there is no agreement on how to best freeze and thaw tissues, and how to assess whether a potentially altered response may affect the sensitivity of the test.*" (OECD 489, Annex 3 Current limitations of the Assay, paragraph 5).

<u>Critical variables of the TGR Assay OECD 488:</u>
"*The sampling time is a critical variable because it is determined by the period needed for mutations to be fixed. This period is tissue-specific and appears to be related to the turnover time of the cell population, with bone marrow and intestine being rapid responders and the liver being much slower. A suitable compromise for the measurement of mutant frequencies in both rapidly and slowly proliferating tissues is 28 consecutive daily treatments (as indicated in Paragraph 26) and sampling three days after the final treatment; although the maximum mutant frequency may not manifest itself in slowly proliferating tissues under these conditions.*" (paragraph 30, OECD 488).

<u>Conclusion:</u>
ECHA maintains the conclusion to request a Transgenic Rodent Assay OECD 488 to be performed for 28 days in liver, glandular stomach , duodenum/jejenum, bone marrow and germ cells. The Comet Assay proposed by the Registrant is an indicator test measuring short-lived DNA damage (strand breaks) and not mutagenicity. Moreover, the OECD 489 is not considered appropriate to measure DNA strand breaks in mature germ cells (paragraph 10).
In order to obtain data, which is adequate for the purpose of classification and labelling and/or risk assessment to make a robust and final conclusion on this substance evaluation a

TGR study is required.

As an alternative option, which may require more animals but potentially less testing costs, a Comet Assay OECD 489 is requested in somatic tissue (liver, glandular stomach, duodenum/jejenum) as a first *in vivo* test. The test must be conducted according to the specifications in the decision. The evaluating MSCA will evaluate and interpret the results of the performed OECD 489 as well as the documentation and acceptability of the test criteria (paragraph 58-65 OECD 489). In case the evaluating MSCA finds that the result is clearly negative it can then be concluded that there is no further concern for *in vivo* gene mutagenicity.

In case the evaluating MSCA finds that the test result of the OECD 489 is positive, equivocal or that the test criteria are not acceptable the evaluating MSCA may then in a new decision making process propose to request a TGR test conducted according to OECD 488 in glandular stomach, duodenum/jejenum, liver, bone marrow and germ cells to be able to conclude on the concerns for somatic and germ cell gene mutagenicity.

Overall conclusion concerning mutagenicity

In conclusion, the concern of whether TGMDA and/or its metabolites causes gene mutations *in vivo* in germ cells and/or somatic cells remains. There is no alternative to obtain this information other than to conduct experimental studies, in particular there is no *in vitro* method available.

Therefore, pursuant to Article 46(1) of the REACH Regulation, the Registrant(s) are required to carry out one of the following studies using the registered substance subject to the present decision (monoconstituent TGMDA, CAS 28768-32-3) or the UVCB TGMDA (CAS 28390-91-2):

1.A Transgenic Rodent Somatic and Germ Cell Gene Mutation Assays in mouse or rat by oral gavage (EU B.58./OECD 488) following a 28-day exposure with a subsequent 3 day sampling period

or

1.B *In vivo* mammalian alkaline Comet Assay in mouse or rat by oral gavage (OECD 489) as a first *in vivo* test. The following tissues should be analyzed (liver,glandular stomach and duodenum/jejenum). The optimum sampling time(s) should be determined based on kinetic data if available.

It is up to the Registrant(s) whether the study should be conducted with the UVCB or the monoconstituent TGMDA. The overlap between the two substances is 82,0%. The impurities (or the reactions intermediated substances) related to the UVCB are higher in the UVCB and two impurities are not present in the monoconstituent. However, for the monoconstituent, only 97,0% of all components are surveyed, where 99.0% are surveyed for the UVCB. The two impurities could therefore be present in the monoconstituent under the detection limit.

IV. Avoidance of unnecessary testing by data- and cost-sharing

In relation to the experimental stud(y/ies) the legal text foresees the sharing of information and costs between Registrant(s) (Article 53 of the REACH Regulation). Registrant(s) are therefore required to make every effort to reach an agreement regarding each experimental study for every endpoint as to who is to carry out the study on behalf of the other Registrant(s) and to inform ECHA accordingly within 90 days from the date of this decision

under Article 53(1) of the REACH Regulation. This information should be submitted to ECHA using the following form stating the decision number above at: https://comments.echa.europa.eu/comments_cms/SEDraftDecisionComments.aspx

Further advice can be found at http://echa.europa.eu/regulations/reach/registration/data-sharing.

If ECHA is not informed of such agreement within 90 days, it will designate one of the Registrant(s) to perform the stud(y/ies) on behalf of all of them.

V. Information on right to appeal

An appeal may be brought against this decision to the Board of Appeal of ECHA under Articles 52(2) and 51(8) of the REACH Regulation. Such an appeal shall be lodged within three months of receiving notification of this decision. Further information on the appeal procedure can be found on the ECHA's internet page at http://www.echa.europa.eu/regulations/appeals. The notice of appeal will be deemed to be filed only when the appeal fee has been paid.

Authorised[3] by Leena Ylä-Mononen, Director of Evaluation

Annex I: List of registration numbers for the addressees of this decision. This annex is confidential and not included in the public version of this decision.

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

References:

[redacted]

Speit G, Kojima H, Burlinson B, Collins et al. (2015). 'Critical issues with the in vivo comet assay: A report of the comet assay working group in the 6th International Workshop on Genotoxicity Testing (IWGT)'.Mutat Res Genet Toxicol Environ Mutagen.783:6-12.