~~Confidential~~

Helsinki, 10 February 2022

Addressees
Registrant(s) of JS_7417-99-4 as listed in the last Appendix of this decision

Date of submission of the dossier subject to this decision
03/02/2021

Registered substance subject to this decision ("the Substance")
Substance name: N,N'-(methylenedi-p-phenylene)bis(aziridine-1-carboxamide)
EC number: 231-034-6
CAS number: 7417-99-4

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

DECISION ON A COMPLIANCE CHECK

Under Article 41 of Regulation (EC) No 1907/2006 (REACH), you must submit the information listed below, by the deadline of ***15 November 2023***.

Requested information must be generated using the Substance unless otherwise specified.

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

1. Short-term toxicity testing on aquatic invertebrates (Annex VII, Section 9.1.1.; test method: EU C.2./OECD TG 202)

2. Growth inhibition study aquatic plants (Annex VII, Section 9.1.2.; test method: EU C.3./OECD TG 201)

B. Information required from all the Registrants subject to Annex VIII of REACH

1. Transgenic rodent somatic and germ cell gene mutation assay (Annex VIII, Section 8.4., column 2; test method: OECD TG 488 from 2020) in transgenic mice or rats, oral route on the following tissues: liver and glandular stomach; duodenum and germ cells must be harvested and stored for up to 5 years. Duodenum must be analysed if the results of the glandular stomach and of the liver are negative or inconclusive. Germ cells must be analysed if the results of one of the somatic tissues (liver, glandular stomach or duodenum) are positive.

2. Short-term toxicity testing on fish (Annex VIII, Section 9.1.3.; test method: OECD TG 203)

3. Hydrolysis as a function of pH (Annex VIII, Section 9.2.2.1.; test method: EU C.7./OECD TG 111)

Reasons for the request(s) are explained in the following appendix/appendices:

- Appendix entitled "Reasons common to several requests";
- Appendix/Appendices entitled "Reasons to request information required" under Annexes VII to VIII of REACH respectively.

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 Annexes VII and VIII to REACH, for registration at 10-100 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". In addition, you should follow the general recommendations provided under the Appendix entitled "General recommendations 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, when adopted under Article 51 of REACH, may be appealed to the Board of Appeal of ECHA within three months of its notification to you. Please refer to http://echa.europa.eu/regulations/appeals for further information.

Failure to comply

If you do not comply with the information required by this decision by the deadline indicated above, ECHA will notify the enforcement authorities of your Member State.

Authorised[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 on Reasons common to several requests

1. **Assessment of your Quatitative structure-activity relationship Q(SAR) adaptation under Annex XI, Section 1.3**

You seek to adapt the following standard information requirements by applying (a) (Q)SAR approach(es) in accordance with Annex XI, Section 1.3:

- Short-term toxicity testing on aquatic invertebrates (Annex VII, Section 9.1.1.)
- Growth inhibition study aquatic plants (Annex VII, Section 9.1.2.)
- Short-term toxicity testing on fish (Annex VIII, Section 9.1.3.)

ECHA has considered the scientific and regulatory validity of your (Q)SAR adaptation(s) in general before assessing the specific standard information requirements in the following appendices.

Under Annex XI, Section 1.3., the following conditions must be fulfilled whenever a (Q)SAR approach is used:

1. the prediction needs to be derived from a scientifically valid model,
2. the substance must fall within the applicability domain of the model,
3. results need to be adequate for the purpose of risk assessment or classification and labelling, and
4. adequate and reliable documentation of the method must be provided.

With regard to these conditions, we have identified the following issue(s):

Lack of or inadequate documentation of the prediction (QPRF)

ECHA Guidance R.6.1.6.3 states that the information specified in or equivalent to the (Q)SAR Prediction Reporting Format document (QPRF) must be provided to have adequate and reliable documentation of the applied method. For a QPRF this includes, among others:

- the identities of close analogues, including considerations on how predicted and experimental data for analogues support the prediction.

Your registration dossier provides the following information:

- ECOSAR predictions for short-term aquatic toxicity (i.e. Invertebrate and fish) and for algae toxicity.
- QMRF and QPRF documentation is provided. However, information on the identity of the close analogues and the accuracy of their predictions is not provided.

Based on the data from the registration dossier, as you have not provided the identities of close analogues, including considerations on how predicted and experimental data for analogues support the prediction, ECHA could not establish that the prediction can be used to meet this information requirement.

In your comments on the draft decision, you agreed with ECHA that the dossier information on the identity of the close analogues was not included in the QPRF. But with your comments you have provided new information including the information on the identities of the analogues substances and new experimental data which gives more robust and reliable prediction.

Further, you indicate the following: *"in order to comply with the ECHA requirement, the registration dossier will be updated by including this new information and filling in the corresponding sections of the QPRF"*

We have assessed all this information and identified the following issue:

The substance is outside the applicability domain of the model

Under ECHA Guidance R.6.1.5.3., a prediction is within the applicability domain of the model, when, among others, the substance falls within descriptor, structural, mechanistic and metabolic domain.

In your comments to the draft decision, you have provided documentation about the prediction (QPRF) and the model (QMRF), including information on the substances in the training sets of the ECOSAR models for substituted ureas.

The Substance is outside the applicability domain of the model because it falls outside the fragment domain of the model. Among others, the substance includes two aziridyl fragments. These fragments are not represented in the training set of ECOSAR models for the substituted urea class.

As a result of the common deficiency described above, your adaptations do no meet the requirements of Annex XI, Section 1.3. Further we have identified additional deficiencies specific to the information requirements you intended to adapt, which also add to the overall conclusion. Those are addressed under the corresponding endpoint(s) in the following Appendices.

For all these reasons, your adaptations are rejected.

Regarding your comments on a possible update of the registration dossier, please note that this decision does not consider updates of the registration dossiers after the date on which you were notified of the draft decision according to Article 50(1) of REACH (see section 5.4. of ECHA's Practical Guide "How to act in Dossier Evaluation). You remain responsible for complying with this decision by the set deadline.

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

1. Short-term toxicity testing on aquatic invertebrates

Short-term toxicity testing on aquatic invertebrates is an information requirement under Annex VII to REACH (Section 9.1.1.).

In your dossier, you have adapted this information requirement by using Annex XI, Section 1.3 by providing the following information:

- Q(SAR) prediction using the ECOSAR model.

In your comments on the draft decision you have provided an updated document including the information on the substances in the training sets of the ECOSAR models. We have assessed the information provided in your dossier and in your comments and as explained in the Appendix on Reasons common to several requests, your adaptation is rejected.

Therefore, the information requirement is not fulfilled

Study design

The Substance is difficult to test due to the rapid hydrolysis (half-life of <10 hours at 20°C and pH 7).

OECD TG 202 specifies that, for difficult to test substances, you must consider the approach described in OECD GD 23 or other approaches, if more appropriate for your substance. In all cases, the approach selected must be justified and documented. Considering that the Substance is rapidly hydrolysable it is important to take into account the relative toxicities of the parent test chemical and hydrolysis products to determine the appropriate test design and test media preparation methods for the Substance.

Taking the rapid hydrolysis of the parent substance into account, it may be difficult to achieve and maintain the desired exposure concentrations of the Substance or its hydrolysis products. Therefore, you must monitor the test concentration(s) of the Substance, or its hydrolysis products, throughout the exposure duration and report the results.

2. Growth inhibition study aquatic plants

You have adapted this information requirement by using Annex XI, Section 1.3 by providing the following information:

- Q(SAR) prediction using the ECOSAR model.

In your comments on the draft decision you have provided an updated document including the information on the substances in the training sets of the ECOSAR models.

In your comments you also indicate that the training set includes more analogues with more experimental data which gives a more reliable and robust prediction, i.e. the training set includes 4 substances and not only 1 as it was provided initially in your dossier.

We have assessed this information and identified the following issues:

A. Your adaptation is rejected for the issues identified in the Appendix on Reasons common to several requests.
B. An additional reason for the rejection of your adaptation is the following:

Under Annex XI, Section 1.3., one of the condition that must be fulfilled whenever a (Q)SAR approach is used is that the prediction needs to be derived from a scientifically valid model.

Under ECHA Guidance R.6.1.3., a (Q)SAR model must fulfil the principles described in the OECD Guidance document on the validation of (Q)SAR models (ENV/JM/MONO(2007)2) to be considered scientifically valid. For that purpose, the fourth OECD principle requires that appropriate measures of the internal performance (i.e. goodness-of-fit and robustness using the learning data set) and predictivity (using a test data set) of the model are available.

To have appropriate robustness, a model must be built from a training set which includes a sufficient number of substances. The minimum number of substances depends on the number of variables or descriptors included in the model. The ratio between the number of substances and the number of variables or descriptors must be at least 5.

You have provided ECOSAR predictions according to the « Urea substituted » chemical class model.

According to the documentation provided in your dossier, it is reported that the Urea substituted model includes only 1 substance in its training set and the r^2 is "Not available". Due to these statistics, the model has not been considered scientifically valid.

Based in the information submitted in your comments on the draft decision, the training set of your model is based on 1 descriptor and 4 substances. This means that the ratio between the number of substances and the number of variables or descriptors is less than five. So you have not established the robustness, and thereby the scientific validity, of the model.

We also note the very low value for the r^2 (0.17) in the updated documentation provided in your comments for the Urea substituted model.

Based on the above, the adaptation is rejected.

Therefore, the information requirement is not fulfilled.

Study design

OECD TG 201 specifies that for difficult to test substances OECD GD 23 must be followed. As already explained above, the Substance is difficult to test. Therefore, you must fulfil the requirements described in 'Study design' under Appendix A.1.

Appendix B: Reasons to request information required under Annex VIII of REACH

1. Transgenic rodent somatic and germ cell gene mutation assay

Under Annex VIII, Section 8.4, column 2 of REACH, the performance of an appropriate *in vivo* somatic cell genotoxicity study must be considered if there is a positive result in any of the *in vitro* genotoxicity studies in Annex VII or VIII.

The ECHA Guidance R.*7a*[2] states that following a positive result in an *in vitro* test, "*adequately conducted somatic cell in vivo testing is required to ascertain if this potential can be expressed in vivo. In cases where it can be sufficiently deduced that a positive in vitro finding is not relevant for in vivo situations (e.g. due to the effect of the test substances on pH or cell viability, in vitro-specific metabolism: see also Section R.7.7.4.1), or where a clear threshold mechanism coming into play only at high concentrations that will not be reached in vivo has been identified (e.g. damage to non-DNA targets at high concentrations), in vivo testing will not be necessary*.".

Your dossier contains positive results for the *in vitro* gene mutation study in bacteria which raise the concerns for gene mutation. It is noted that the substance induced a particularly strong mutagenic effect in strains TA100 and TA1535 with and without metabolic activation, at a level similar or higher than the concurrent positive controls. ECHA concludes that the Substance is a very potent *in vitro* mutagen in bacteria.

Therefore, the concern should be followed by *in vivo* testing.

However, no data from an *in vivo* somatic cell genotoxicity study is available in the dossier. Moreover, you did not provide any considerations explaining that the genotoxic potential of the substance cannot be expressed *in vivo*, based e.g. on lack of relevance for *in vivo* situations or the existence of threshold mechanism.

Instead, you have provided the following reasoning for not performing the study: "*It is possible to make a conclusive hazard assessment in accordance with Annex I of REACH without additional testing on the basis of structure-activity relationship with a known mutagen".*

ECHA understands that you sought to adapt this information requirement according to the general rules for adaptation of section 1.5. of Annex XI of REACH Regulation.

ECHA has evaluated the provided information accordingly and identified the following issues:

Grouping of substances and read-across approach

Annex XI, Section 1.5. specifies two conditions which must be fulfilled whenever a read-across approach is used. Firstly, there needs to be structural similarity between substances which results in a likelihood that the substances have similar physicochemical, toxicological and ecotoxicological properties so that the substances may be considered as a group or category (addressed under 'Scope of the grouping'). Secondly, it is required that the relevant properties of a substance within the group may be predicted from data for reference substance(s) within the group (addressed under 'Assessment of prediction(s)').

Additional information on what is necessary when justifying a read-across approach can be found in the ECHA Guidance R.6 and related documents.

[2] ECHA Guidance R.7a, section R.7.7.6.3, p.570.

A. Predictions for toxicological properties

You have provided a read-across justification document in IUCLID under the endpoint "genotoxicity".

You read-across between the structurally similar substance 1-Aziridinepropanoic acid, 1,1'-[2-[[3-(1-aziridinyl)-1-oxopropoxy]methyl]-2-ethyl-1,3-propanediyl] ester (EC:257-765-0) as source substance and the Substance as target substance.

You have provided the following reasoning for the prediction of toxicological properties:

You state that according to ECHA Guidance R.7a, page 566 "*since the substance shares structural characteristics with known mutagens (harmonised CLP classification of analogue CAS 52234-82-9: Mutagen Category 2) it is possible to make a conclusive hazard assessment in accordance with Annex I of REACH without additional testing on the basis of structure-activity relationship alone.*"

You also state that the two substances share "*the same functional group (aziridine)"* and "*they also have comparable values for the relevant molecular properties. Therefore, the results obtained with the substance CAS No. 52234-82-9 can be used for the read-across approach".* You have self-classified the Substance as Muta 2 (H341), based on the harmonized classification of the structurally similar substance 1-Aziridinepropanoic acid, 1,1'-[2-[[3-(1-aziridinyl)-1-oxopropoxy]methyl]-2-ethyl-1,3-propanediyl] ester (EC:257-765-0). You have supported your reasoning, providing mechanistic information, using QSAR Toolbox (v.4.1.) profilers.

ECHA understands that you predict the properties of the Substance using a read-across hypothesis which assumes that different compounds have the same type of effects. The properties of your Substance are predicted to be quantitatively equal to those of the source substance.

ECHA identified the following deficiencies with regards to prediction of toxicological properties.

Missing supporting information to compare properties of the category members

Annex XI, Section 1.5 of the REACH Regulation states that "*physicochemical properties, human health effects and environmental effects or environmental fate may be predicted from data for reference substance(s)*". For this purpose "*it is important to provide supporting information to strengthen the rationale for the read-across*"[3]. The set of supporting information should allow to verify the crucial aspects of the read-across hypothesis and establish that the properties of the Substance can be predicted from the data on other category members.

As indicated above, your read-across hypothesis is based on the assumption that the structurally similar substances cause the same type of effect(s). In this context, relevant, reliable and adequate information allowing to compare the properties of the Substance and of the source substance(s) is necessary to confirm that both substance cause the same type of effects. Such information can be obtained, for example, from bridging studies of comparable design and duration for the Substance and of the source substance(s).

To support your hypothesis that the Substance and the source substance will have same genotoxicity properties, you have provided mechanistic information, using QSAR Toolbox

[3] Guidance on information requirements and chemical safety assessment Chapter R.6: QSARs and grouping of Chemicals, Section R.6.2.2.1.f

(v.4.1.) profilers. You concluded that the Substance and the source substance have DNA binding properties, based on the presence of the aziridine functional group. No *in vitro* or *in vivo* experimental genotoxicity data with the source substance is included in your technical dossier.

ECHA notes that apart from the common functional group (aziridine) that you identified, the Substance and source substance are largely structurally different. The Substance has an aromatic carboxamide core structure, while the source substance has e.g. alkyl (methyl, ethyl, propyl) groups. You have not established if the differences in the chemical structures of the substances would have an impact on their genotoxic properties.

The information obtained from the QSAR Toolbox structural alerts is based on the structural similarities, however the structural differences were not covered. According to the data included in your dossier, the Substance caused positive responses in a bacterial reverse mutation assay and was negative in an *in vitro* micronucleus study in mammalian cells. No information on the actual genotoxic properties of the source substance has been included in your dossier other than a reference to its harmonised classification.

In the comments to the draft decision you disagree with ECHA's assessment. You did not provide any new information but in principle reiterate that "*since the known mutagen (source substance) shares the same only group with an alert with the target substance, we consider that it is possible to deduce that the target substance could be also considered as a mutagen* in accordance with the statement of ECHA Guidance R.7a, page 566". You claim that "*the QSAR ToolBox is used to identify structural alerts for relevant endpoints (DNA binding, protein binding, Ames test, micronucleus, etc.) in a substance, that is, in any part or group of the substance*" and that "*the results showed that aziridine was the only group identified with an alert in both substances".* You further claim that "*[…] parts structurally different between the substances were also evaluated but no other alerts were identified in any other part or group of either substance.*"

Regarding your repeated reference to ECHA Guidance R.7a, page 566, ECHA clarifies that the Guidance text explicitly explains that "*the registrant still has to provide sufficient information to meet the requirements of Annexes VII to X*" or fulfil "*the general rules of Annex XI for adaptation of the standard testing regime*".

For your adaptation according to Annex XI, Section 1.5 you did not provide any new information to substantiate your read-across hypothesis in your comments on the draft decision, but soley refer again to the information obtained from the OECD QSAR Toolbox.

You acknowledge that there are structural differences between the source substance and the Substance. However, you have not provided an adequate justification why the structural differences do not impact the read-across prediction.

ECHA points out that while the similarity in presence or absence of structural alerts may indicate that the structural differences do not influence the reactivity of the substance e.g. on DNA, this information does not confirm, on its own, that the Substance and the source substance have similar toxicological properties such as *in vivo* genotoxicity. As already explained in the initial draft decision, you have not provided any experimental genotoxicity studies with the source substance to support your hypothesis that the Substance and the source substance would have the same mutagenic potential.

Therefore, ECHA reiterates that in the absence of experimental data with the source substance, it is not possible to compare the genotoxicity profile between the Substance and

the source substance and to ascertain that their genotoxicity properties are likely to be similar despite the identified DNA binding properties based on the common aziridine functional group.

Conclusions on the read-across approach

As explained above, you have not established that relevant properties of the Substance can be predicted from data on the analogue substance. Therefore, your adaptation does not comply with the general rules of adaptation as set out in Annex XI, Section 1.5. and your grouping and read-across approach is rejected.

ECHA considers that an appropriate *in vivo* follow up mutagenicity study is necessary to address the concern identified *in vitro*.

i. Test selection

According to the ECHA Guidance R.7a, Section R.7.7.6.3, the transgenic rodent somatic and germ cell gene mutation assay ("TGR assay", OECD TG 488) and the *in vivo* mammalian alkaline comet assay ("comet assay", OECD TG 489) are suitable to follow up a positive *in vitro* result on gene mutation.

However, as indicated in a proposal for amendment (PfA) submitted by one of the Member State Competent Authorities, the TGR assay is the gold standard test to investigate gene mutations *in vivo*, as it can detect permanent gene mutations (whereas the comet assay is an indicator test detecting only putative DNA lesions). Moreover, in case of positive results in somatic cells, the TGR assay is the most appropriate test to enable the classification of a substance as germ cell mutagen category 2 because it is a mutagenicity test, whereas the comet assay is only a genotoxicity test.

Furthermore, as described in the OECD TG 488, the TGR allows the detection of gene mutations both in somatic cells and in germ cells (according to the current OECD TG 489, para.10, the comet assay is not appropriate to detect DNA damage in germ cells). If positive results are obtained in the TGR assay in both somatic and germ cells, a classification as germ cell mutagen category 1B is warranted. Since the Substance demonstrated a very potent mutagenic effect in the Ames test, an indicative test such as the comet assay is not appropriate and may result in unnecessary additional animal testing if investigations in germ cells would be necessary. Therefore, a test detecting permanent mutations, the TGR assay, is required to 1) follow up the clear gene mutation concern for the Substance and 2) generate data that is the most appropriate for classification and labelling of the Substance.

In your comments to the PfA, you made the following arguments (the text between square brackets was added by ECHA to facilitate the reading/summary of your arguments) to conclude that both methods TGR and comet assay should be valid in order to assess the potential for mutagenicity of the Substance:

1) According to ECHA Guidance Chapter R.7a, Section R.7.7.6.3, both the TGR assay and the comet assay are suitable to follow up a positive *in vitro* result on gene mutation.
2) Kirkland D. *et al*. (2019) concluded that comet and TGR generally identify the same compounds (mainly potent mutagens) as genotoxic in liver, stomach and colon.
3) According to ECHA guidance [on 'Three recently approved *in vivo* genotoxicity test Guidelines'[4]], the comet assay may be performed on gonadal cells; so both TGR assay (OECD TG 488) and comet assay (OECD TG 489) can examine target tissues (including germ cells) and site-of-contact tissues (i.e. skin, epithelium of the respiratory or gastro-

[4] ECHA document entitled 'Three recently approved in vivo genotoxicity test Guidelines' https://echa.europa.eu/documents/10162/1128894/oecd_test_guidelines_genotoxicity_en.pdf/56ab5788-0103-4716-8903-59ab0c942efe?t=1520932890820

intestinal tract).

4) ECHA considers that [the comet assay on gonadal cells] may be relevant for the overall assessment of possible germ cell mutagenicity including classification and labelling according to the CLP Regulation. Based on the wording of the [second bullet point] of the criteria of CLP Regulation (EC) No. 1272/2008 for Muta 1B, it is possible to deduce that not only a germ cell mutagenicity test is necessary to be able to classify a substance with category 1B but also a genotoxicity test performed in germ cell *in vivo* such as the comet assay can be considered as sufficient.

ECHA's response to your arguments are the following:

1) ECHA acknowledges that, according to ECHA guidance, both the TGR assay and the comet assay are suitable to follow up (*in vivo,* in somatic cells) a positive *in vitro* result on gene mutation.
2) We are aware of the article of Kirkland *et al*. 2019 and of its conclusion. We however do not believe that the TGR assay and the comet assay are equivalent or have similar weight in the mutagenicity assessment, and in particular for the classification and labelling (see point 4 below).
3) In the ECHA document of 2018 'Three recently approved *in vivo* genotoxicity test Guidelines', there is a footnote indicating that "Comet assay may be performed on gonadal cells (which contain a mixture of somatic and germ cells)". We however note that the OECD TG 489 for the comet assay does not provide any specific recommendation for the investigation of gonadal cells or germ cells. The OECD TG 489 even states (para.10): "*the OECD/OCDE 489 standard alkaline comet assay as described in this guideline is not considered appropriate to measure DNA strand breaks in mature germ cells*".
4) ECHA maintains that comet assay data obtained on gonadal cells 'may be relevant for the overall assessment of possible germ cell mutagenicity including classification and labelling according to the CLP Regulation'. However, we consider that comet data alone cannot be sufficient to warrant a classification as Muta 1B. Indeed ECHA does not agree with your interpretation of the CLP text:
    a. When the CLP text states "- *positive result(s) from in vivo somatic cell mutagenicity tests in mammals, in combination with some evidence that the substance has potential to cause mutations to germ cells*", it is textually referring to positive results in 'mutagenicity tests' (such as the TGR assay), in combination with supporting evidence showing the ability to cause mutations in germ cells. This understanding is confirmed in section 3.5.2.4., page 366, of the Guidance on the Application of the CLP Criteria (version 5.0, from July 2017)[5].
    b. When the CLP text continues with "*It is possible to derive this supporting evidence from mutagenicity/genotoxicity tests in germ cells in vivo, or by demonstrating the ability of the substance or its metabolite(s) to interact with the genetic material of germ cells*", it is actually explaining what this 'supporting evidence' can be derived from: so the supporting evidence showing the ability to cause mutations in germ cells can be derived from *in vivo* mutagenicity/genotoxicity tests in germ cells or from the demonstration of the ability to interact with the genetic material of germ cells. In other words, a positive *in vivo* genotoxicity test (such as the comet assay) performed in germ cell can be considered relevant as a <u>supporting evidence</u> of the ability of the substance to cause mutations in germ cells. On the other hand, positive data from a comet assay, on somatic or germ cells, cannot be considered equivalent to a positive '*in vivo* somatic cell mutagenicity tests in mammals', because the comet assay is a genotoxicity test and not a mutagenicity test.

[5] https://echa.europa.eu/documents/10162/2324906/clp_en.pdf/58b5dc6d-ac2a-4910-9702-e9e1f5051cc5

In conclusion, the TGR assay is the only OECD test that allows the investigation of both somatic cells and germ cells and has the capacity to generate data that could warrant a classification as Muta 1B. We thus consider the TGR assay as the only adequate *in vivo* test to investigate the mutagenicity of this extremely potent *in vitro* mutagen.

ii. Test design

According to the test method OECD TG 488, the test must be performed in transgenic mice or rats and the test substance is usually administered orally.

Based on the recent update[6] of OECD TG 488, you are requested to follow the new 28+28d regimen, as it permits the testing of mutations in somatic tissues and as well as in tubule germ cells from the same animals.

According to the test method OECD TG 488, the test must be performed by analysing tissues from liver as slowly proliferating tissue and primary site of xenobiotic metabolism and from glandular stomach and duodenum as rapidly proliferating tissue and site of direct 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 mutagenicity at the site of contact in the gastro-intestinal tract. However, duodenum must be stored (at or below −70 ºC) until the analysis of liver and glandular stomach is completed; the duodenum must then be analysed only if the results obtained for the glandular stomach and for the liver are negative or inconclusive.

iii. Germ cells

You are also requested to collect the male germ cells (from the seminiferous tubules) at the same time as the other tissues, in order to limit additional animal testing. According to the OECD TG 488, the tissues (or tissue homogenates) can be stored under specific conditions and used for DNA isolation for up to 5 years (at or below −70 ºC). This duration is sufficient to allow you or ECHA, to decide on the need for assessment of mutation frequency in the collected germ 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 in case of positive results in somatic cells, and is proportionate considering that it is a standard and simple approach.

In case the TGR analysis would show a positive result in at least one of the somatic tissues (liver, stomach or duodenum), ECHA requests you to analyse the germ cells because this is necessary to generate the appropriate information for possible classification and labelling of the substance as germ cell mutagen category 1B.

2. Hydrolysis as a function of pH

Hydrolysis as a function of pH is an information requirement under Annex VIII to REACH (Section 9.2.2.1).

You have provided the following information

i. OECD TG 111, key study, [redacted] (2018)

[6] The updated OECD TG 488, adopted on 26 June 2020, is available on OECD website at https://www.oecd-ilibrary.org/docserver/9789264203907-en.pdf?expires=1596539942&id=id&accname=guest&checksum=D552783C4CB0FC8045D04C88EFFBFA66.

We have assessed this information and identified the following issue:

To fulfil the information requirement, a study must comply with OECD TG 111. The guideline describes a tiered approach whereby each tier is triggered by the results of the previous tier. If major hydrolysis products (i.e. corresponding to ≥ 10% of the applied dose) are formed in a second test (tier 2), these must be identified (third tier), quantified and the results interpreted.

Your registration dossier provides an OECD TG 111 including the following:

- A second test (tier 2) showing that the hydrolysis percentage of the test substance reached 90 % at the end of the test, and the formation of hydrolysis products.
- A third test (tier 3), in which you report that the main hydrolysis product that was identified by the end of the test is Tributyl acetylcitrate. However you have not provided any further information (i.e. the relative abundance and interpretation of the results) on the hydrolysis products obtained.

Based on the information that you have provided in the dossier regarding the identity of hydrolysis products, ECHA notes that the reported main hydrolysis product (Tributyl acetylcitrate) does not relate in any way to the chemical structure of the Substance n,n'-(methylenedi-p-phenylene)bis(aziridine-1-carboxamide). You have not provided any interpretation of the results obtained that could explain this discrepancy.

In the absence of adequate justification on the identity of the hydrolysis product(s) and its percentage, this study does not meet the requirement to identify the relevant hydrolysis products as specified in OECD TG 111.

On this basis, the information requirement is not fulfilled.

3. Short-term toxicity testing on fish

You have adapted this information requirement by using Annex XI, Section 1.3 and provided the following information:

- Q(SAR) prediction using the ECOSAR model.

In your comments on the draft decision you have provided an updated document including the information on the substances in the training sets of the ECOSAR models.

We have assessed the information provided in your dossier and in your comments and as explained in the Appendix on Reasons common to several requests, your adaptation is rejected.

Study design

OECD TG 203 specifies that for difficult to test substances OECD GD 23 must be followed. As already explained above, the Substance is difficult to test. Therefore, you must fulfil the requirements described in 'Study design' under Appendix A.1.

Appendix C: 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[7].

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[8].

[7] https://echa.europa.eu/practical-guides
[8] https://echa.europa.eu/manuals

Appendix D: Procedure

This decision does not prevent ECHA from initiating further compliance checks at a later stage on the registrations present.

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

The compliance check was initiated on 11 December 2020.

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

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

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

ECHA received proposal(s) for amendment and modified the draft decision.

ECHA invited you to comment on the proposed amendment(s) and referred the modified draft decision to the Member State Commitee.

Your comments on the proposed amendment were taken into account by the Member State Committee.

The Member State Committee reached a unanimous agreement on the draft decision in its MSC-77 written procedure and ECHA took the decision according to Article 51(6) of the REACH Regulation.

Appendix E: List of references - ECHA Guidance[9] 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)[10]

RAAF - considerations on multiconstituent substances and UVCBs (RAAF UVCB, March 2017)[11]

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[12]

[9] https://echa.europa.eu/guidance-documents/guidance-on-information-requirements-and-chemical-safety-assessment
[10] https://echa.europa.eu/support/registration/how-to-avoid-unnecessary-testing-on-animals/grouping-of-substances-and-read-across
[11] https://echa.europa.eu/documents/10162/13630/raaf_uvcb_report_en.pdf/3f79684d-07a5-e439-16c3-d2c8da96a316
[12] 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 F: Addressees of this decision and their corresponding information requirements

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.