Regulatory Toxicology and Pharmacology 168 (2026) 106079

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Metabolism-based category formation for the prioritisation of genotoxicity hazard assessment for plant protection product residues (part 5): Acetyl CoA carboxylase inhibitors

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ARTICLE INFO

Handling Editor: Dr. Martin van den Berg

Keywords:
Structural alerts
Genotoxicity
Hazard assessment
Read-across
Pesticide residues

ABSTRACT

In dietary risk assessment of plant protection products, residues of active ingredients and their metabolites need to be evaluated for their genotoxic potential. The European Food Safety Authority recommend a tiered approach focussing assessment and testing on classes of similar chemicals. A dataset of 58 inhibition of acetyl CoA carboxylase herbicides for which either Ames, chromosomal aberration or micronucleus test results were identified from publicly available regulatory submission dossiers. A set of structural space alerts were defined from this dataset, each linked to a key metabolic transformation present in the metabolic space, covering both the cyclohexadiones and aryloxphenoxypropionates classes. A hypothetical case study chemical was used to demonstrate the ability of the structural space alerts to identify metabolically related analogues with which to predict genotoxicity, including the *in vivo* micronucleus test, via read-across. In addition, the structural space alerts defined were compared to the metabolic simulators in the OECD QSAR Toolbox. The results showed the importance of expert driven methods for defining metabolic similarity for read-across for plant protection products. As with previous work in this area, the key challenge being the need for metabolism data for the development of the structural space alerts.

1. Introduction

The European Food Safety Authority (EFSA) requires an assessment of the genotoxicity potential for plant protection product residues, these being defined as any compound associated with the active ingredient (EFSA Scientific Committee, 2016). The general approach outlined in the available EFSA guidance (EFSA Scientific Committee, 2011) is that such residues should not increase the hazard to humans (and livestock). Thus, within a set of 'similar' plant protection residues, a category, a representative number need to have *in vitro* and/or *in vivo* data for gene mutation as well as structural and numerical chromosomal aberration. The availability of such data enables data-gaps within the category to be filled by read-across within this guidance, with the minimal data requirements coming from the Ames test (gene mutation) and an *in vitro* micronucleus test (structural and numerical chromosomal aberration). The availability of additional negative *in vivo* data (frequently from the micronucleus test) adds further weight of evidence to the read-across prediction (especially where exposure to the bone marrow has been demonstrated from toxicokinetic studies). If a read-across prediction of genotoxicity is negative, then no further experimental testing is required under the EFSA guidance. In contrast, a positive read-across prediction for genotoxicity requires further experimental data to be generated in a tiered approach. For example, if an initial *in vitro* micronucleus test confirms the positive read-across prediction for chromosome damage, an *in vivo* micronucleus test would be triggered.

The key step in the use of the category formation approach is the ability to confidently define 'similarity' between compounds (Enoch et al., 2010; Enoch et al., 2013; OECD, 2007). In terms of the use of

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https://doi.org/10.1016/j.yrtph.2026.106079
Received 25 September 2025; Received in revised form 26 February 2026; Accepted 1 March 2026
Available online 6 March 2026

category formation in the EFSA genotoxicity workflow noted above, defining similarity is relatively straightforward for potentially genotoxic chemicals. This is due to the key molecular initiating event for DNA-reactive genotoxicity being the formation of a covalent bond between nucleophilic centres in DNA and a compound capable of behaving as an electrophile (either directly or after metabolic activation) (Enoch and Cronin, 2010, 2012; Benigni and Bossa, 2008; Benigni et al., 2009; Mekenyan et al., 2004, 2007; Serafimova et al., 2007). The associated chemistry can be encoded easily as structural alert-based *in silico* profilers that enable compounds to be assigned to a category based on the presence of a common alert. In contrast, defining similarity between compounds that lack an alert for DNA reactivity is more challenging due to the lack of such key structural features (Schultz et al., 2018).

Recent research has developed the "structural space alerts" concept to address the similarity between plant protection residues that lack an alert for either covalent protein or DNA reactivity (Enoch et al., 2022a, 2022b, 2023, 2024). This concept utilises the metabolism data available in the Draft Assessment Report/Renewal Assessment Report (DAR/RAR) documents of plant protection products (available from the EFSA website) to enable category formation to be based on the presence of a set of common metabolic pathways present in the analogues within the category. It is important to note that metabolic similarity has been suggested as being a key measure of similarity when making read-across predictions within a regulatory environment (Gadaleta et al., 2020; Yordanova et al., 2021; Boyce et al., 2022). Initial development of the structural space alert concept was based on an analysis of sulphonylurea herbicides and triazole fungicides, with this work defining alerts that defined common scaffolds within the available metabolism data (Enoch et al., 2022a, 2022b). The limitation being that the resulting structural space alerts were not explicitly linked to individual metabolic transformation (as defined from *in vivo* rat metabolism). This resulted in the categories needing sub-categorisation based on expert judgement to ensure the Target (the compound with the data-gap) and the analogues all underwent the same set of metabolic transformations. More recent work using the strobilurin fungicides and the α-chloroacetamide herbicides addressed this shortcoming by explicitly linking each alert to a metabolic transformation (Enoch et al., 2023, 2024). Importantly, only metabolic transformations present in two or more compounds within a class of plant protection products were defined in this approach (i.e., metabolic transformations present in only a single compound were not covered by the structural space alerts). This approach enabled the resulting structural space alerts to be used to identify groups of compounds capable of undergoing a common set/sets of compound class specific transformations. The previous studies outlined a protocol for the development of this type of structural space alerts and examples of how they could be used to predict genotoxicity of plant protection residues via read-across (Enoch et al., 2023, 2024), without the need for expert sub-categorisation required in the earlier work (Enoch et al., 2022a, 2022b). Thus, the aim of the current study was to further extend the structural space alert concept to the inhibition of acetyl CoA carboxylase (ACCase) herbicides, using the protocol outlined for the strobilurin fungicides and α-chloroacetamide herbicides in which each structural space alert was explicitly linked to a metabolic transformation. The concept is exemplified using a case study of how the structural space alerts can be used to fill a data-gap via read-across.

2. Method

2.1. Dataset

The publicly available DAR documents were used to compile nine ACCase herbicide active ingredients, and their metabolites as identified in the rat. Of these nine DAR documents, six related to the aryloxyphenoxypropionates, with the remaining three related to the cyclohexanediones. These documents enabled a dataset of 58 compounds to be identified, of which 29 had genotoxicity data in the form of either the Ames test, *in vitro* chromosomal aberration assay or the *in vivo* micronucleus test. All compounds that were identified as being positive in the *in vitro* chromosomal aberration assay had additional data from either the *in vivo* chromosomal aberration or *in vivo* micronucleus tests showing them to be negative. This dataset expanded the chemical space for the ACCase herbicides compared to the available data in the publicly available EFSA genotoxicity dataset (available from zenodo.org/doi/10.5281/zenodo.602287). The dataset, termed the 'ACCase genotoxicity dataset' contained the following test results (*in vitro* assays with S9 fraction, Ames tests in the standard battery):

- Ames: 27 compounds (24 negative, 2 positive, 1 equivocal)
- *In vitro* chromosomal aberration: 20 compounds (16 negative, 2 positive, 2 equivocal)
- *In vivo* chromosomal aberration: 3 compounds (all negative)
- *In vitro* micronucleus: 2 compounds (both negative)
- *In vivo* micronucleus: 13 compounds (10 negative, 3 equivocal)

All chemical structures and associated genotoxicity data are available in the Supplementary Information.

2.2. Metabolic similarity profiling scheme

The development of the metabolic similarity profiling scheme utilised the same protocol as previously published (Enoch et al., 2023, 2024) and is summarised in the following three steps:

1. Definition of the metabolic maps for the ACCase herbicides: This analysis involved inspection of the available metabolism data in the DAR documents to identify common metabolic transformations with a class of compounds.
2. Scaffold identification: Key scaffolds were identified based on the parent structures and those following either cleavage or cyclisation reactions.
3. Structural space alert identification: Common sub-structures were then identified for each of the scaffolds identified in step 2 using the metabolic maps developed in step 1. These sub-structures were defined as structural space alerts that defined the atom/atoms on the scaffold upon which the metabolic transformation identified in the metabolic map occurred. These structural space alerts covered each of the structures in each pathway, expect the final structure as this structures no longer contained a metabolic transformation point (with it being the final step in the metabolism).

2.3. Chemical profiling

Chemicals were profiled using the profiling schemes within the OECD QSAR Toolbox (V4.7). A subset of the available profilers was utilised based on the results of a previous study into their suitability for read-across predictions within the plant protection chemical space (Enoch et al., 2022a, 2022b, 2023, 2024). These profilers were (CA is chromosomal aberration and MNT is the micronucleus test):

- DNA alerts for AMES, CA and MNT by OASIS
- Protein binding alerts for CA by OASIS

2.4. Metabolism prediction

Metabolism prediction was carried out using *in vivo* rat metabolism and rat liver S9 metabolism simulators in the OECD QSAR Toolbox (V4.7).

3. Results and discussion

The aim of this study was to develop a set of structural space alerts to enable the genotoxicity of the ACCase herbicides to be predicted via

read-across. A series of sub-structures linked to key metabolic transformations were defined based on an expert analysis of the metabolic information available in the DAR documents for the ACCase herbicides. The structural space alert concept enables chemical categories to be developed in which analogues undergo a common set of metabolic transformations. As discussed previously, the ability to group such compounds based on metabolic similarity increases the robustness, reliability, and repeatability of the resulting read-across predictions (Enoch et al., 2023, 2024).

3.1. Metabolic map development

The ACCase herbicides consist of two common scaffolds, these being: the cyclohexanediones (commonly referred to as the 'dims') and the arylphenoxypropionates (commonly referred to as the 'fops'). Analysis of the rat metabolism data in the publicly available DAR documents showed the cyclohexanediones to undergo four key metabolic transformations (shown in Fig. 1), whilst the arylphenoxypropionates undergo two key metabolic transformations (shown in Fig. 2). These transformations can be summarised as follows:

3.2. Cyclohexanediones

A. Thioether oxidation: the presence of a thioether moiety in the R_3 position enables oxidation reactions to occur leading to a sulphoxide, with further oxidation producing a sulphone. These reactions occur on both cyclic and acyclic thioether moieties.
B. Chain cleavage: the presence of an alkyl chain in the R_2 position (making the functional group an O-alkyl oxime) enables chain cleavage reactions to occur. These reactions remove the O-alkyl chain resulting in an imine moiety. The presence of a terminal aromatic ring system prevents this metabolism.
C. Ring opening: the cyclohexanedione scaffold can undergo a ring opening reaction to produce a dicarboxylic acid scaffold substituted by the R_3 moiety.
D. Ring cyclisation: the presence of a hydroxyl group in the *ortho*-position to oxime moiety in the cyclohexanedione scaffold enables a cyclisation reaction to occur resulting fused oxazole ring scaffold substituted by the R_1 and R_3 moieties.

3.3. Arylphenoxypropionates

A. Chain cleavage: the presence of an alkyl chain in the R_1 position leads to cleavage of the alkyl chain resulting in a carboxylic acid moiety. Further cleavage reactions remove the carboxylic acid moiety, resulting in an aromatic alcohol.
B. Ether cleavage: the presence of a nitrogen containing ring system on the 'left hand side' of the arylphenoxypropionate scaffold (as the structures are drawn in Fig. 2) results in the cleavage of the ether linkage in the arylphenoxypropionate scaffold. The nitrogen containing ring system products from this reaction can undergo tautomerism due to the presence of an alcohol moiety in the *ortho*-position to a nitrogen (Fig. 2 shows this tautomerism for pyridine/pyridine). The ether cleavage reaction does not occur when the scaffold contains a benzene ring on the 'left hand side' of the arylphenoxypropionate scaffold.

3.4. Structural space alert development

Analysis of metabolism data for the cyclohexanediones class of ACCase herbicides identified three key metabolic scaffolds in the dataset (Fig. 1). These scaffolds being the cyclohexanedione ring, the fused oxazole ring and the dicarboxylic acid. These scaffolds were used to identify a set of four structural space alerts for the cyclohexanedione class of compounds (Table 1). Two of these alerts covered the multiple structures capable of undergoing the metabolic transformations related to pathways A and B in Fig. 1 (ACCase-dim-1 and ACCase-dim-2 in Table 1). The remaining two alerts relate products of scaffold transformation due to either cyclisation or ring opening reactions (ACCase-dim-3 and ACCase-dim-4 in Table 1). A further three alerts were defined for the aryloxyphenoxypropionates class of ACCase herbicides, these being based on the identification of a set of three common scaffolds (metabolism data in Fig. 2, structural space alerts in Table 2). Two of these alerts cover the multiple structures related to pathway A and B in Fig. 2 (ACCase-fop-1 and ACCase-fop-2 in Table 2), with the third alert

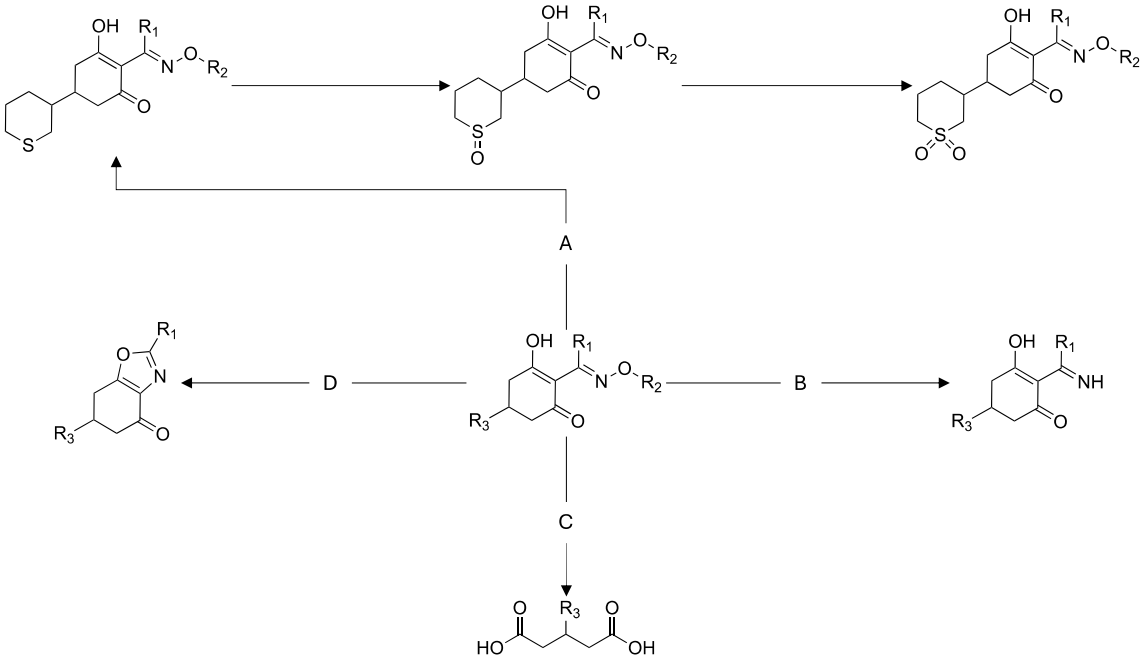


Fig. 1. Summary of the common metabolic pathways in the rat for the cyclohexanedione class of ACCase herbicides (R_1 = alkyl carbon, R_2 = alkyl carbon, R_3 = cyclic/acyclic thioether, benzene).

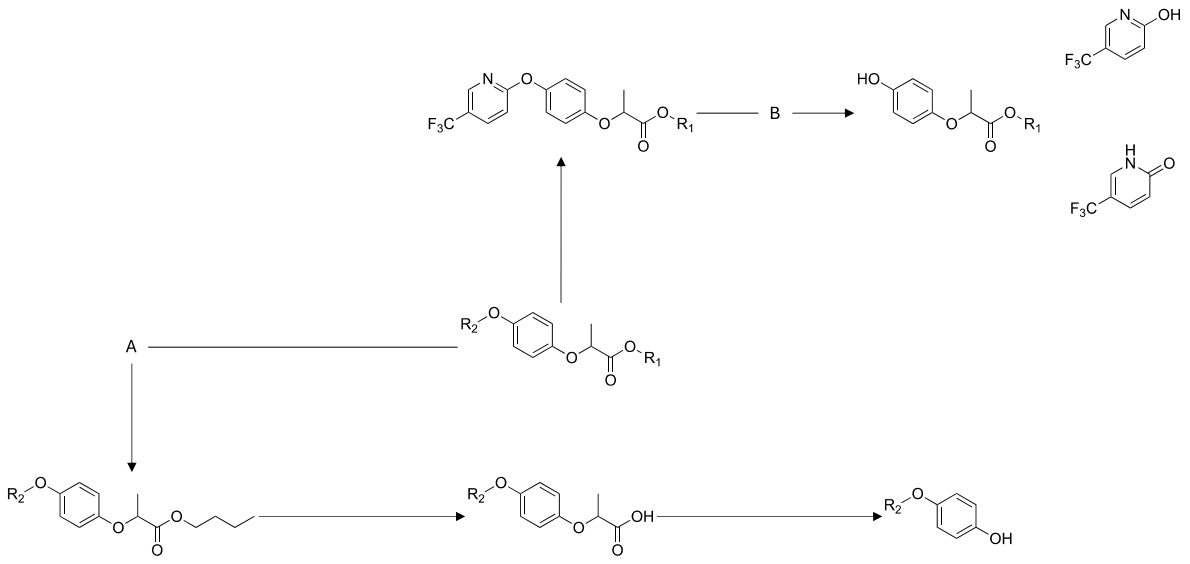
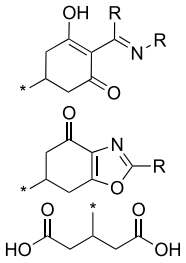
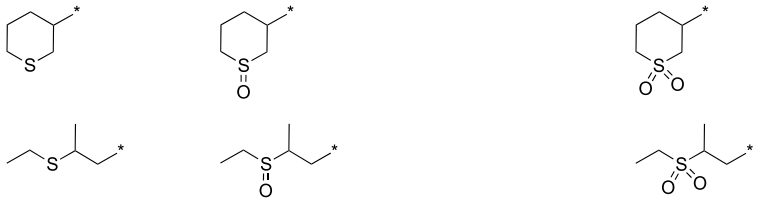
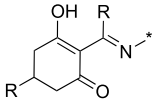
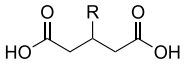
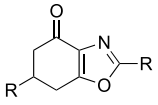


Fig. 2. Summary of the common metabolic pathways in the rat for the aryloxyphenoxypropionates class of ACCase herbicides (R_1 = alkyl, R_2 = aromatic carbon).

Table 1
Structural space alerts for the cyclohexanedione class ('dims') of ACCase herbicides (* = attachment position between scaffold and metabolic substituent, R = common scaffold attachment position - any atom).

Alert (Pathway – see Fig. 1)	Scaffold	Metabolic substituent
ACCase-dim-1 (Dim pathway A)		
ACCase-dim-2 (Dim pathway B)		
ACCase-dim-3 (Dim pathway C)		Product of scaffold metabolism
ACCase-dim-4 (Dim pathway D)		Product of scaffold metabolism

relating to the products of the metabolism of the arylphenoxypropionate scaffold (ACCase-fop-3 in Table 2).

3.5. Read-across case study: 'Zuzanoxydim'

A hypothetical, cyclohexanedione, compound was created to demonstrate the utility of the structural space alerts in predicting genotoxicity via read-across (this compound was generated by mixing Cycloxydim and Tepraloxydim). In addition to the parent compound, a series of metabolites were generated by applying the metabolic information outlined in Fig. 1 (metabolite structures as shown in Table 3). The structural space alerts shown in Table 1 were used to profile these compounds and to identify metabolically related analogues from the ACCase genotoxicity dataset. This analysis resulted in three chemical categories based around pathways A and B, pathways A and C, and pathway D (as outlined in Fig. 1). The results of these categories are outlined below (all category data are available in the Supplementary Information):

- Category 1 - pathways A and B: Four Target chemicals, Zuzanoxydim and Z-metabolites 1 – 3, were identified as being able to undergo the metabolism defined in pathways A and B. Eight analogues that were able to undergo these two pathways were identified. Of these analogues, seven were negative in the Ames test (one being equivocal), three were negative in the *in vitro* chromosomal aberration test (two being equivocal), one was negative in the *in vitro* micronucleus test, two were negative in the *in vivo* chromosomal aberration test, one was negative in the *in vivo* micronucleus test (one being equivocal). Interestingly, the equivocal test data were related to the same analogue – further data from the *in vivo* unscheduled DNA synthesis assay for this chemical showed it to be negative for genotoxicity (chemical ID ACCase-5 in the Supplementary Information). Overall,

Table 2
Structural space alerts for the arylphenoxypropionate class ('fops') of ACCase herbicides (* = attachment position between scaffold and metabolic substituent, R = common scaffold attachment position - any atom).

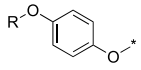
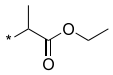
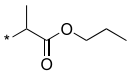
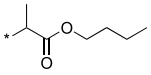
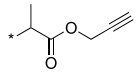
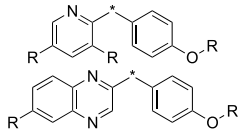
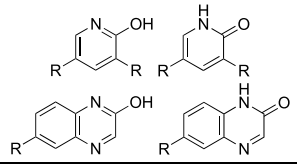
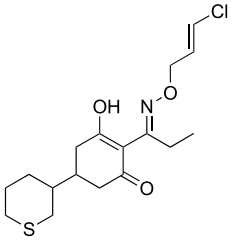
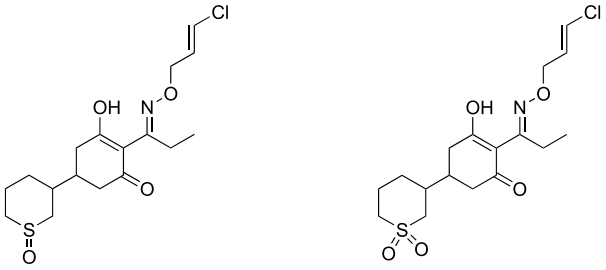
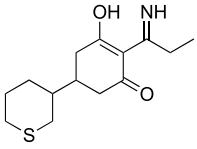
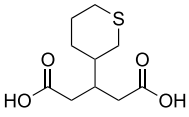
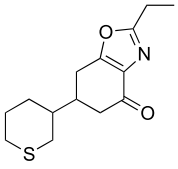
Alert (Pathway – see Fig. 2)	Scaffold	Metabolic substituent
ACCase-fop-1 (Fop pathway A)		*–H      
ACCase-fop-2 (Fop pathway B)		* is oxygen Scaffold metabolism
ACCase-fop-3 (Fop pathway B)		Product of scaffold metabolism

Table 3
Chemical structure of 'Zuzanoxydim' and its potential metabolites generated by the application of the metabolic information in Fig. 1.

ID	Structure	Applied metabolic pathway
Zuzanoxydim		Parent
Z-metabolite 1 (left structure) Z-metabolite 2 (right structure)		A
Z-metabolite 3		B
Z-metabolite 4		C
Z-metabolite 5		D

these data allow a weight of evidence to be developed that chemicals metabolised along pathways A and B are not genotoxic.

- Category 2 - pathways A and C: A single Target chemical, Z-metabolite 4, was identified by the being able to undergo the metabolism defined in pathways A and C. Three analogues that were able to undergo these two pathways were identified. All three of the analogues were negative in the Ames test, with one of them also being negative in the *in vitro* chromosomal aberration test. For this category the lack of *in vivo* test data related to the combination of pathways prevents the prediction of genotoxicity from these data alone. However, the weight of evidence for pathway A (considering the data outlined above for pathways A and B) suggests that this pathway is not likely to be genotoxic. Further inspection of the profiling results of the ACCase genotoxicity dataset identifies an additional analogue capable of undergoing pathway C, with this analogue also being negative in the Ames, and *in vitro* chromosomal aberration tests. Despite the availability of this additional chemical related to pathway C further testing is required to rule out genotoxicity associated with this pathway – within the EFSA guidance (EFSA Scientific Committee, 2011) this testing would be an *in vitro* micronucleus test, which if negative would be sufficient to rule out any further *in vivo* testing to support the premise that pathway C is not associated with genotoxicity.
- Category 3 - pathways A and D: A single Target chemical, Z-metabolite 5, was identified as being able to undergo the metabolism defined in pathways A and D. Two analogues that were able to undergo these two pathways were identified, with both being negative in the Ames test. One of the analogues tested positive in the *in vitro* chromosomal aberration, but subsequently tested negative in the *in vivo* micronucleus test. The second chemical has a single additional test carried out in the *in vitro* micronucleus test, this being negative. These data suggest that neither pathway A nor D lead to genotoxicity.

DNA profiling: Two of the available profiling schemes in the OECD QSAR Toolbox, DNA alerts for AMES, CA and MNT by OASIS, and Protein binding alerts for CA by OASIS profilers have been shown to be useful for adding to the weight of evidence for read-across predictions using structural space alerts (Enoch et al., 2022a, 2022b, 2023, 2024). Profiling the chemicals in categories 1-3 showed none of them to trigger any alerts related to DNA or protein binding relevant to genotoxicity. These profiling results support the negative predictions for genotoxicity made for chemical categories 1-3.

Metabolism profiling: It has been suggested that metabolic simulators can be used to add to the weight of evidence that chemicals within a category are not diss-similar (EFSA Scientific Committee et al., 2025). In supporting a prediction of the absence of genotoxicity, as is the case for the categories in this study, such profiling would need to show that none of the category members are metabolised into electrophiles. Profiling of chemical categories 1-3 (described above) using the *in vivo* rat metabolism simulator, rat liver S9 metabolism simulator, DNA alerts for AMES, CA and MNT by OASIS profiler, and Protein binding alerts for CA by OASIS profiler in the OECD QSAR Toolbox (V4.7) showed this to be the case (all data available in the Supplementary Information). Despite this, closer inspection of the *in vivo* rat metabolism simulator (chosen as the metabolic map in Fig. 1 was derived from rat data) highlights the low predictivity of these profiling schemes. As an example, the parent structure, Zuzanoxydim, the only pathway that is predicted in-line with the experimental data (summarised in Fig. 1) is pathway A, relating to the oxidation of the thioether moiety to a sulphoxide and then sulphone (predicted pathway A in Fig. 3). The other pathway that the *in vivo* rat metabolism simulator predicts being hydroxylation of the ethyl chain in combination with the oxidation of the thioether moiety (predicted pathway B in Fig. 3). This poor predictivity of simulated metabolism for plant protection products is in keeping with other, more detailed, studies that also showed similar results (Scholz et al., 2023; Clark, 2018). These results also demonstrate the key advantage of the structural space alert concept in which metabolic similarity is embedded in the approach on a class-by-class basis (Enoch et al., 2022a, 2022b, 2023, 2024). However, it is important to note that the relatively small dataset, in which only 29 compounds out of the 58 available have genotoxicity data, is a limiting factor in the applicability domain of the ACCase structural space alerts.

Uncertainty: There are a number of areas of uncertainty in the application of the ACCase structural space alerts when making read-across predictions for genotoxicity. Primarily these are related to the limited genotoxicity data for the compounds in the dataset, with only 29 (out of 58) having been tested in either the Ames, chromosomal aberration, or micronucleus assays. This leads to the developed structural space alerts have a relatively narrow applicability domain. The applicability domain is further limited by the fact that only 13 of the compounds with Ames data have been tested in the *in vivo* micronucleus assay. Finally, the need for expert judgement in the development of the

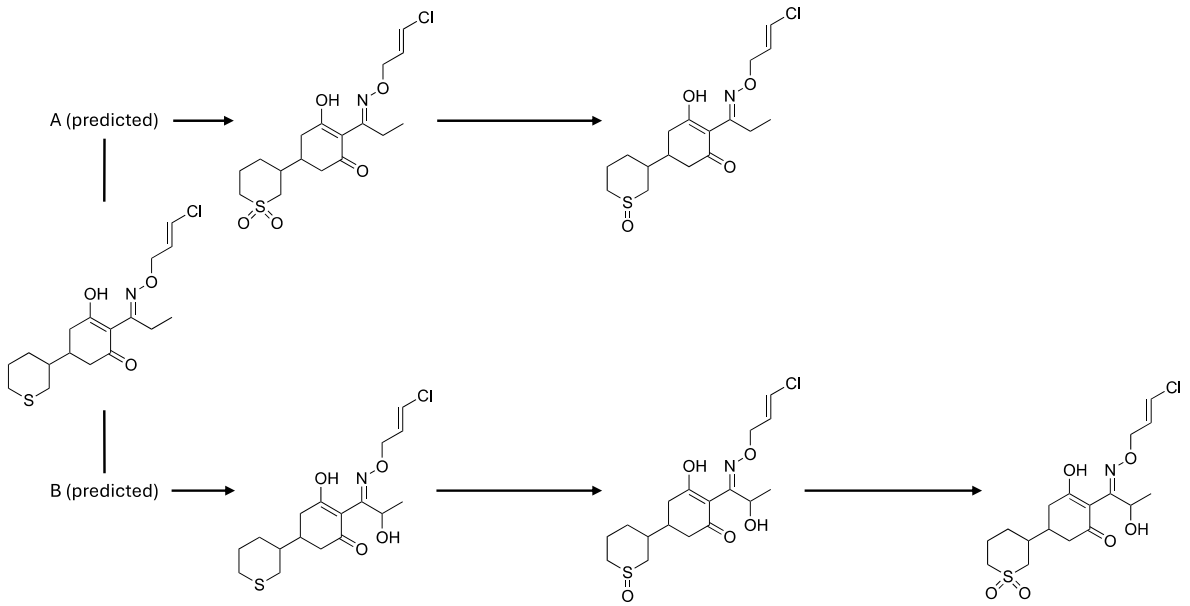


Fig. 3. Summary of metabolism predicted by the *in vivo* rat metabolism simulator in the OECD QSAR Toolbox (V.47) for the hypothetical compound, 'Zuzanoxydim'.

structural space alerts does involve a level of subjectivity which can lead to further uncertainty in the resulting read-across predictions. The need for expert judgement in the development of structural space alerts is also a limitation in their applicability to other plant protection product classes – something that the alternative matched molecular pairs approach is not limited by (Enoch et al., 2025).

4. Conclusions

This study has expanded the structural space alert concept extending it to cover both structural classes of compounds that make up the ACCase herbicides. To this end, expert judgment was used to identify four key metabolic pathways for the cyclohexanediones and two for the aryloxyphenoxypropionates ACCase herbicides. This metabolic information was used to develop a set of seven structural space alerts covering these transformations and their products. As with previous work in this area, a case study outlined how these structural space alerts could be used to fill genotoxicity data-gaps via read-across, including for the *in vivo* micronucleus test. In addition, this research also showed the significant benefit of the structural space alert concept for defining metabolic similarity as compared to the metabolism profiling schemes in the OECD QSAR Toolbox. Finally, the work in this manuscript, in conjunction with the others in this series, demonstrates the importance of expert judgement approaches, such as the structural space alert concept, for defining metabolic similarity for read-across. Such research is key for the regulatory acceptance of read-across predictions for plant protection products.

CRediT authorship contribution statement

S.J. Enoch: Writing – review & editing, Writing – original draft, Funding acquisition, Formal analysis, Conceptualization. **Z. Hasarova:** Data curation. **M.T.D. Cronin:** Writing – review & editing. **K. Bridgwood:** Writing – review & editing, Project administration, Data curation. **S. Rao:** Writing – review & editing. **A. Hueser:** Writing – review & editing. **F.M. Kluxen:** Writing – review & editing. **M. Frericks:** Writing – review & editing, Project administration, Data curation, Conceptualization.

Funding body

Crop Life Europe are gratefully acknowledged for funding this research.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Zuzana Hasarova reports financial support was provided by Crop Life Europe.

Acknowledgements

This research received funding from CropLife Europe.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2026.106079.

Data availability

All data have been made available in the SI

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