Istituto Superiore di Sanità

ToxMic User Manual

ToxMic

(Structure Alerts for the *in vivo* micronucleus assay in rodents)

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User Manual

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Inroduction

The *in vivo* mutagenicity studies, shortly followed by carcinogenicity, are posing high demand for test-related recourses. Among those, the micronucleus test in rodents is the most widely used, as follow up to positive *in vitro* mutagenicity results. A recent survey of the (Q)SAR models for mutagenicity and carcinogenicity has indicated that no (Q)SAR models for *in vivo* micronucleus are available in the public domain: therefore, the development and extensive use of estimation techniques such as (Q)SARs, read-across and grouping of chemicals, might have a huge saving potential for this endpoint.

The ToxMic rulebase provides a list of structural alerts (SAs, see section "Structural Alerts") for a preliminary screening of potentially *in vivo* mutagens. These SAs are molecular functional groups or substructures that are known to be linked to the positive *in vivo* micronucleus assay.

The ToxMic rulebase is encoded as a plug-in to Toxtree application (http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=TOXTREE). This ensures that users being familiar with the Toxtree software can easily apply the features of the ToxMic plug-in to their problems.

Background

Mutagenicity testing is an important part of the regulatory hazard assessment of chemicals. It is undertaken for two main reasons: a) to detect chemicals that might cause genetic damage in germ cells, and thus increase the burden of heritable (genetic) disease in the human population; and b) to detect chemicals that might be carcinogenic (based on the assumption that mutagenesis, for example in somatic cells, is a key event in the process of carcinogenesis). Since no method is able alone to detect all possible genotoxic events, a wide array of test systems has been developed and accepted internationally in regulatory schemes.

Most often, these methods are used within a 2-tiered integrated testing approach: Tier 1 includes in vitro assays, and Tier 2 includes *in vivo* assays. As a matter of fact, mutagenicity testing was the first toxicity endpoint for which in vitro assays were accepted for regulatory testing, some 25 years ago. The latter usually comprise bacterial mutagenicity and cytogenetics tests, although gene mutation testing in cultured mammalian cells is sometimes also undertaken.

Tier 2 of the testing strategy involves the use of short-term *in vivo* studies (usually a bone-marrow cytogenetics assay) to assess whether any potential for genotoxicity detected at the Tier 1 in vitro stage is actually expressed in the whole animal. Thus, negative results in vitro are usually considered sufficient to indicate lack of mutagenicity, whereas a positive result is not considered sufficient to indicate that the chemical represents a mutagenic hazard (i.e. it could be a false positive). The above approach to genotoxicity testing has been adopted throughout the EU and has been recommended internationally as part of the strategy for predicting and quantifying mutagenic and carcinogenic hazard (see the Technical Guidance Documents at the European Chemicals Agency (EChA): http://guidance.echa.europa.eu/docs/guidance_document/information_requirements_r7a_en.pdf?vers=20_08_08).

According to an assessment carried out by the former European Chemicals Bureau (ECB), the *in vivo* mutagenicity studies, shortly followed by carcinogenicity, are posing high demand for test-related recourses (Pedersen et al. 2003;Van der Jagt et al. 2004). Among those, the micronucleus test in rodents is the most widely used, as follow up to positive in vitro mutagenicity results. A recent survey of the (Q)SAR models for mutagenicity and carcinogenicity (performed jointly by ISS and ECB) has indicated that no (Q)SAR models for *in vivo* micronucleus are available in the public domain (Benigni et al. 2007): therefore, the development and extensive use of estimation techniques such as (Q)SARs, read-across and grouping of chemicals, might have a huge saving potential for this endpoint.

Structure Alerts for the *in vivo* micronucleus assay

A Structural Alert (SA) is a molecular functional group or substructure that is known to be linked to a certain chemical property or reactivity (Benigni and Bossa 2006;Benigni and Bossa 2008).

The compilation of SAs for the *in vivo* micronucleus assay in rodents provided here, is based on both the existing knowledge on the mechanisms of toxic action and a structural analysis of the chemicals tested in the assay. The development and scope of these SAs are described in detail in the accompanying scientific document (Benigni et al. 2009). The document can be freely downloaded at http://ecb.jrc.ec.europa.eu/DOCUMENTS/QSAR/EUR_23844_EN.pdf

The result is the optimized list of alerts in Appendix 1. The SAs included in ToxMic are 35; it includes a number of the Benigni / Bossa alerts for mutagenicity and carcinogenicity (Benigni et al. 2008) together with five additional substructures identified in the course of the research on *in vivo* micronucleus. For the sake of clarity, the codes of alerts of the Benigni / Bossa rulebase in Toxtree are maintained, whereas the five additional alerts have new codes.

Classification Scheme

The processing of a query chemical by the ToxMic plug-in for Toxtree can result in two different outcomes. According to the SAs that are utilized in the ToxMic rulebase, chemicals are classified into one of the following two categories:

- Class 1 (At least one positive structural alert for the micronucleus assay)
- Class 2 (No positive alert for the micronucleus assay)

If one or more SAs for the *in vivo* micronucleus assay are found the substance is classified as positive (Class 1). If no SAs for the *in vivo* micronucleus assay are found the substance is classified as Class 2.

The Toxtree ToxMic plug-in

The ToxMic plug-in for Toxtree is Java based and utilizes the Toxtree plug-in interface to make its functionality available to the user. The following two sections describe the installation and the usage of the ToxMic plug-in for Toxtree. For a better use of this plug-in, the user should previously become familiar with Toxtree and its functionalities, as described in detail in the Toxtree installation and user manuals (http://ecb.jrc.ec.europa.eu/qsar/qsar-tools/index.php?c=TOXTREE).

Installation

The implementation of the ToxMic plug-in follows the standard plug-in mechanism of Toxtree as used in the existing decision tree methods (Verhaar scheme, Cramer rules, etc.). The functionality of the ToxMic plug-in is included in the ”ToxMic-ISS.jar” library file which has to be copied into the plug-in directory of the Toxtree installation as shown in Figure 1.

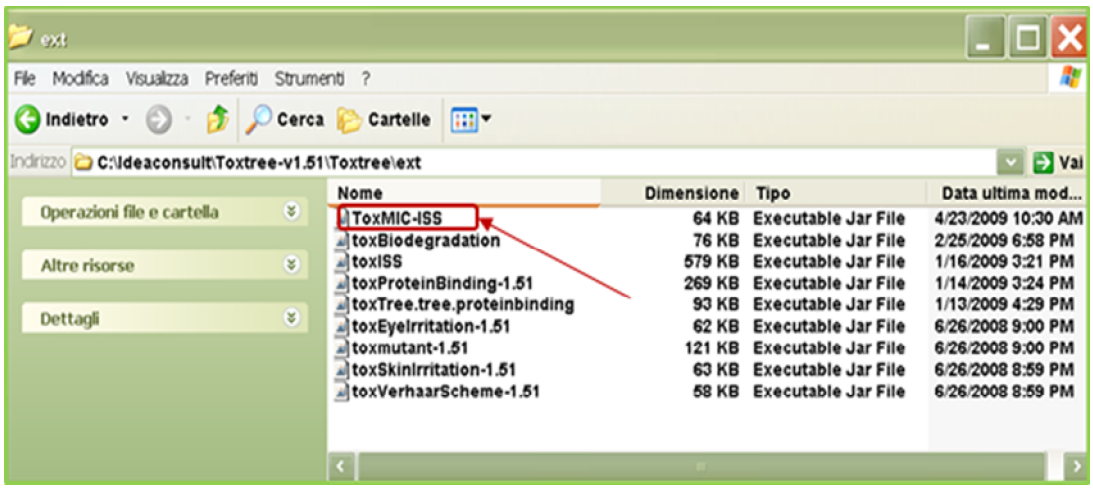


Figure 1 Installation of the ToxMic plug-in for Toxtree. The library file “ToxMic-ISS.jar” has to be copied into the “ext” plug-in directory of the Toxtree installation.

ToxMic usage

Once the ToxMic plug-in has been installed into Toxtree, it has to be activated. Within the “Select a tree” dialog window, the ToxMic plug-in can be selected from the list of available decision trees like any other Toxtree plug-in as illustrated in Figures 2 and 3.

Figure 2 The “Select a decision tree” option in the ToxMic menu bar.

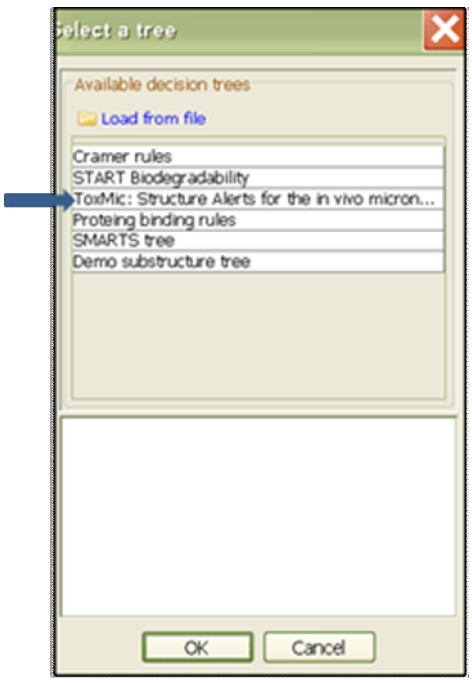


Figure 3 Decision tree selection in the “Select a tree” dialog window of Toxtree.

The Toxtree plug-in is now ready to use.

As shown in Figure 4, the SMILES of the chemical under investigation can now be entered into the Toxtree main window, and then evaluated by pressing the “Estimate” button.

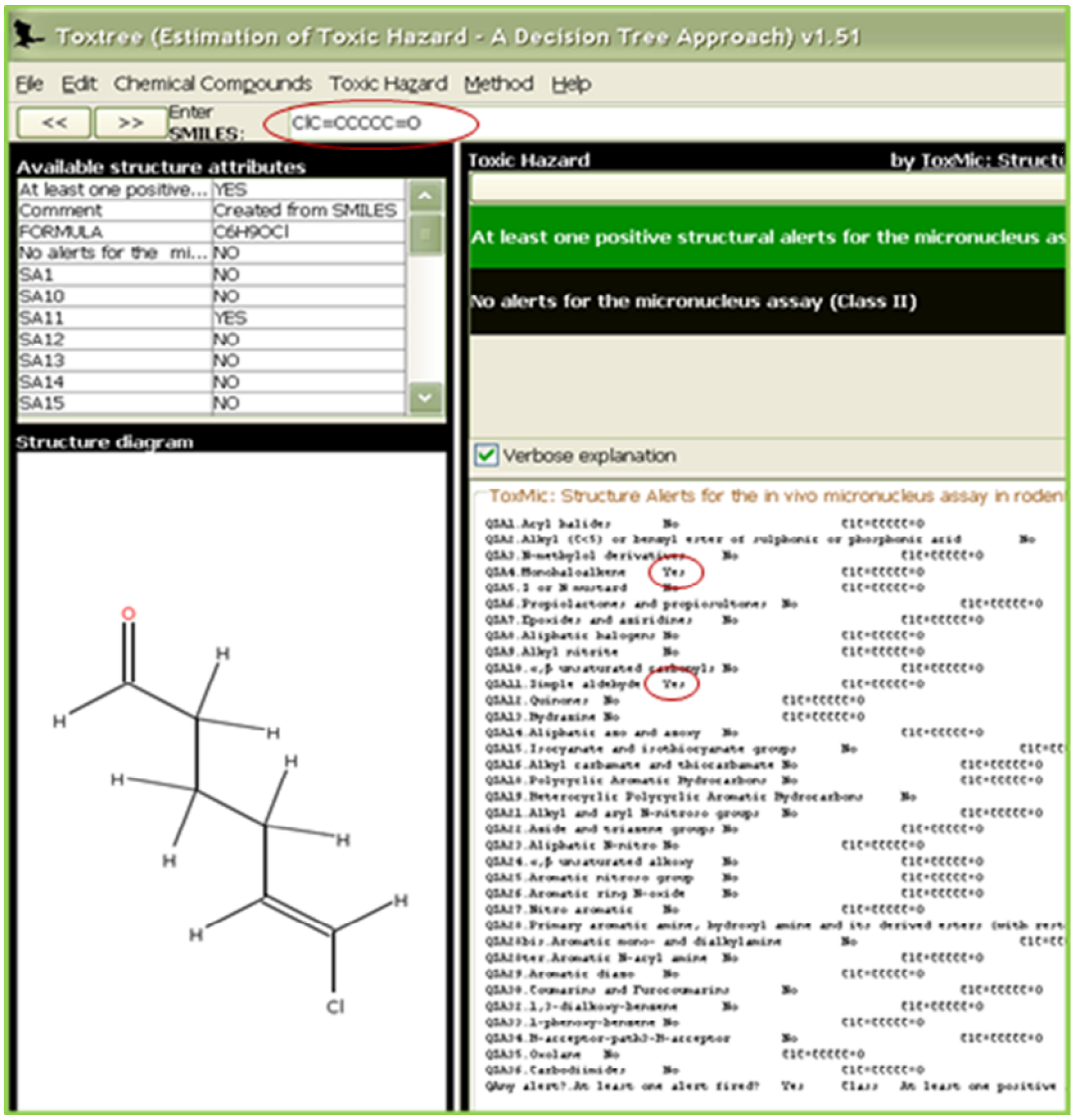


Figure 4 Example of the evaluation of sample chemical structure by ToxMic plug-in, with detailed description of the evaluation process in the main Toxtree window.

The compound under investigation is searched for the presence of all structural alerts for the *in vivo* micronucleus assay and the number of found alerts is kept.

If at least one alert -notifying a chemical potentially positive for the micronucleus assay- is found, the compound under investigation is declared as Class 1 (“At least one positive structural alert for the micronucleus assay”).

If no alerts are found at all, the compound is declared as Class 2 (“No positive alert for the micronucleus assay”)

The detailed summary of all alerts found in the compound under investigation is shown in the main Toxtree window (Figure 4).

Decision tree editing

The “Method”-Edit decision tree” menu of Toxtree can be used in order to edit an existing decision tree, for example ToxMic (Figure 5). The “Load from file” submenu can be used for loading any tree in the “Decision tree editor”.

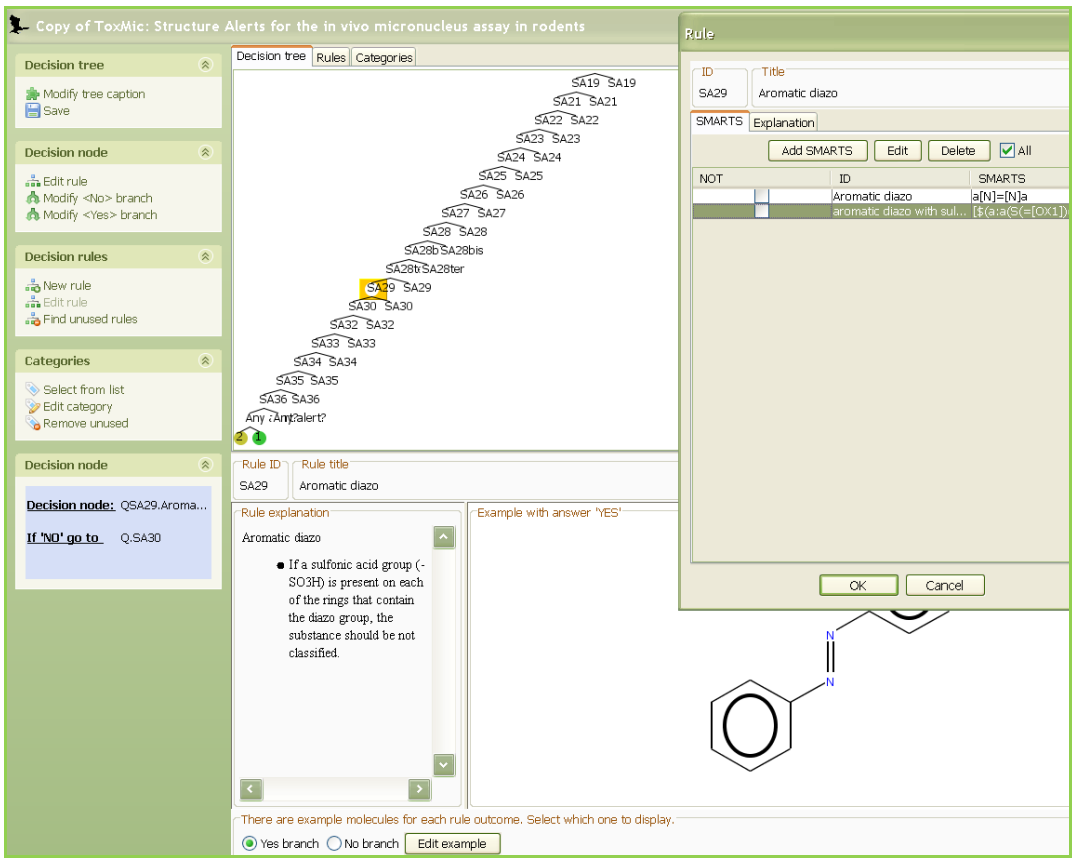


Figure 5. Decision tree editor on a copy of ToxMic rules

Before exiting from the Decision tree editor, users should save the edited decision tree by using the “File”-“Save” menu on the upper left corner of the main Decision tree editor window. A reminder is displayed if the user tries to exit the Decision tree editor without having saved his work.

References

Benigni R, Bossa C. 2006. Structural alerts of mutagens and carcinogens. Curr Comput -Aid Drug Des 2:169-176.

Benigni R, Bossa C. 2008. Structure Alerts for carcinogenicity, and the Salmonella assay system: a novel insight through the chemical relational databases technology. Mutat Res Revs 659:248-261.

Benigni, R., Bossa, C., Jeliazkova, N. G., Netzeva, T. I., and Worth, A. P. The Benigni / Bossa rulebase for mutagenicity and carcinogenicity - a module of Toxtree. EUR 23241 EN. 2008. Luxenbourg, Office for the Official Publications of the European Communities. EUR - Scientific and Technical Report Series.

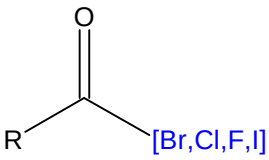
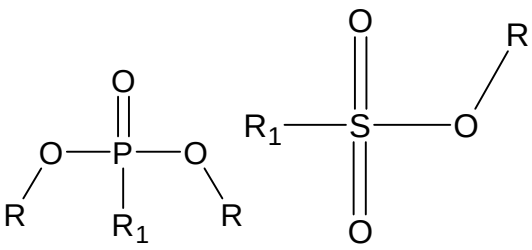
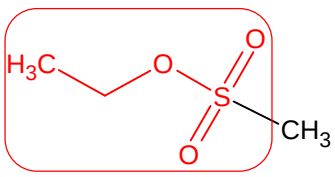
Benigni, R., Bossa, C., Netzeva, T. I., and Worth, A. P. Collection and evaluation of (Q)SAR models for mutagenicity and carcinogenicity. EUR 22772 EN. 2007. Luxembourg, Office for the Official Publications of the European Communities. EUR - Scientific and Technical Research Series.

Benigni, R., Bossa, C., Tcheremenskaia, O., and Worth, A. P. Development of Structure Alerts for the *in vivo* micronucleus assay in rodents. EUR 23844 EN. 2009. Luxembourg, Office for the Official Publications of the European Communities. EUR - Scientific and Technical Research Series.

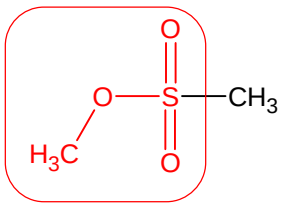
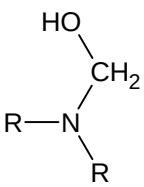
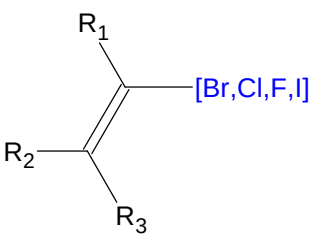
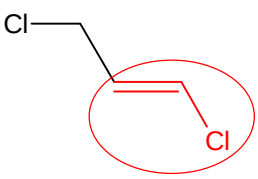
Pedersen, F., de Brujin, J., Munn, S. J., and Van Leeuwen, K. Assessment of additional testing needs under REACH. Effects of (Q)SARs, risk based testing and voluntary industry initiatives. JRC report EUR 20863 EN. 2003. Ispra, EUR.

Van der Jagt, K., Munn, S. J., Torslov, J., and de Brujin, J. Alternative approaches can reduce the use of test animals under REACH. Addendum to the Report "Assessment of addtional testing needs under REACH. Effects of (Q)SARs, risk based testing and voluntary industry initiatives". JRC Report EUR 21405 EN. 2004. Ispra, European Commission Joint Research Centre.

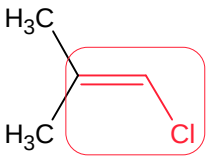
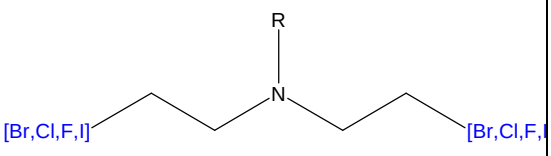
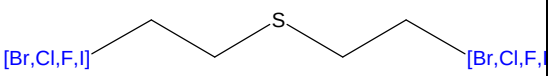
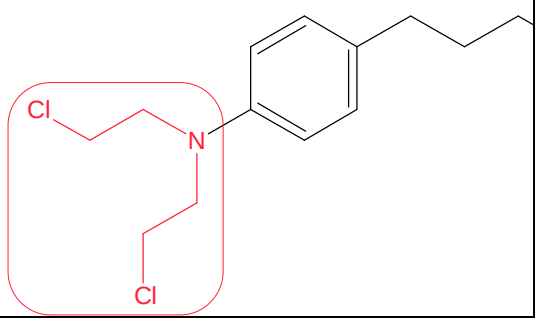
APPENDIX 1. Structural Alerts included in the ToxMic plug-in.

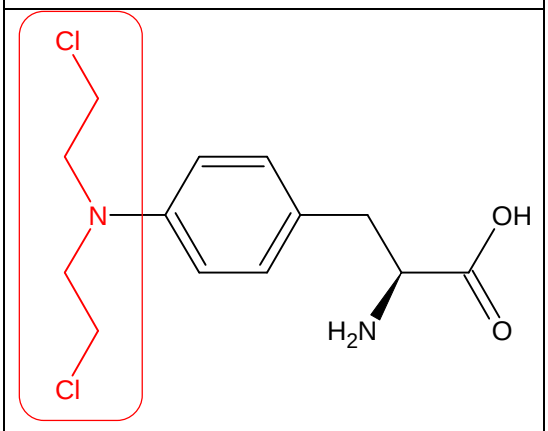
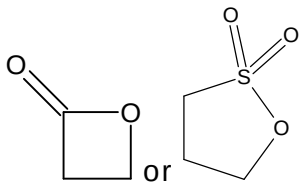
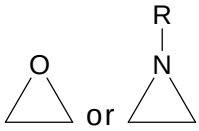
STRUCTURAL ALERT	DETAILS AND EXAMPLES
SA_1: Acyl halides 	R = any atom/group, except OH, SH No representatives
SA_2: alkyl (C<5) or benzyl ester of sulphonic or phosphonic acid 	R= Alkyl with C<5 (potentially substituted by halogens), or benzyl R1= any atom/group except OH, SH, O-, S^-  Name: Ethyl Methanesulfonate CAS: 62-50-0 In vivo Micronucleus (Rodent): Positive Reference: NTP[1]

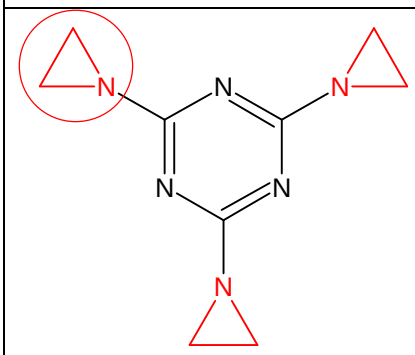
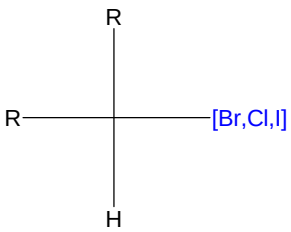
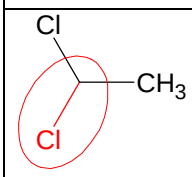
[1] National Toxicology Program, http://ntp.niehs.nih.gov/

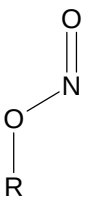
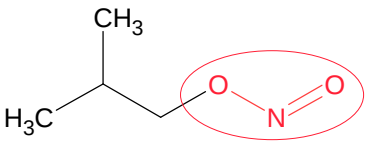
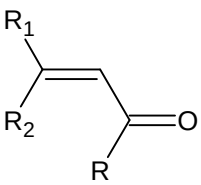
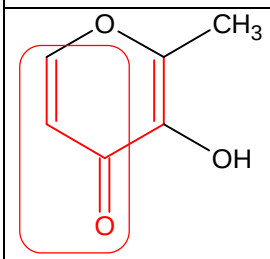
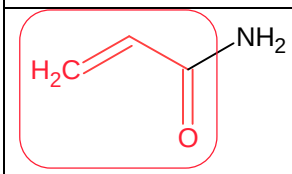
	 Name: Methyl Methanesulfonate CAS: 66-27-3 In vivo Micronucleus (Rodent): Positive Reference: CCRIS[2]
SA_3: N-methylol derivatives 	R = any atom/group No positive representative
SA_4: Monohaloalkene 	R_1, R_2 (or R_3) = H or Alkyl R_3 (or R_2) = any atom/group except halogens  Name: 1,3-dichloropropene CAS: 542-75-6 In vivo Micronucleus (Rodent): Positive Reference: NTP

[2] Chemical Carcinogenesis Research Information System, http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CCRIS

	 Name: Dimethylvinyl Chloride CAS: 513-37-1 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_5: S or N mustard  or  	R = any atom/group
	 Name: Chloroambucil

	Reference: NTP
	 Name: Melphalan CAS: 148-82-3 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_6 Propiolactones or propiosultones 	Any substance with the displayed substructures No representatives
SA_7:Epoxides and aziridines 	R = any atom/group
	 Name: Ethylene Oxide CAS: 75-21-8

	(Rodent): Positive Reference: CCRIS
	 Name: Triethylenemelamine CAS: 51-18-3 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_8: Aliphatic halogens 	R = any atom/group
	 Name: 1,2-dibromoethane CAS: 106-93-4 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: 1,1-dichloroethane CAS: 75-34-3 In vivo Micronucleus

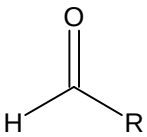
	(Rodent): Positive Reference: CCRIS
SA_9: Alkyl nitrite 	R= any alkyl group
	 Name: Isobutyl Nitrite CAS: 542-56-3 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_10: Unsaturated carbonyls 	R1 and R2 = any atom/group, except alkyl chains with C>5 or aromatic rings. R= any atom/group, except OH, O^-
	 Name: Maltol CAS: 118-71-8 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
	

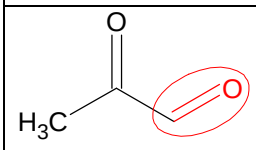
<table>
<tr><td></td><td>Name: Acrylamide

CAS: 79-06-1

In vivo Micronucleus (Rodent): Positive

Reference: CCRIS</td></tr>
<tr><td rowspan="3">SA_11: Simple aldehyde

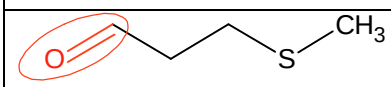
 
</td><td>R= aliphatic or aromatic carbon

unsaturated aldehydes are excluded</td></tr>
<tr><td> 

Name: Pyruvaldehyde

CAS: 78-98-8

In vivo Micronucleus (Rodent): Positive

Reference: Leadscope[3]</td></tr>
<tr><td> 

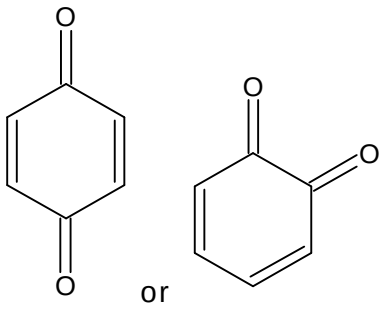
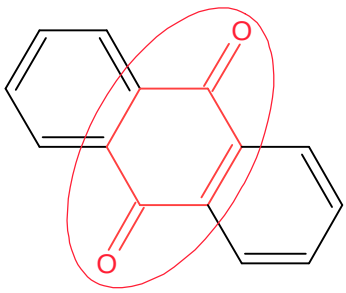
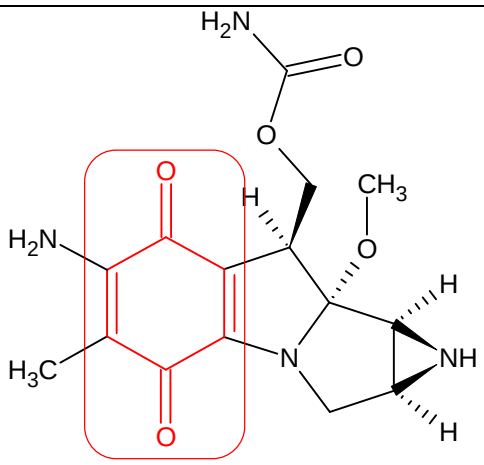
Name: 3-(methylthio)propionaldehyde

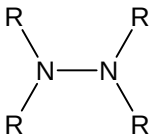
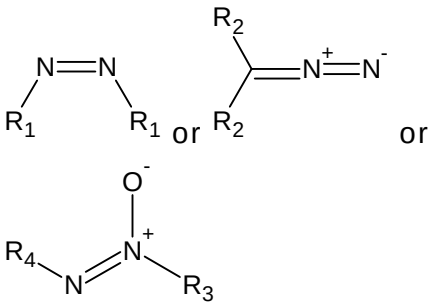
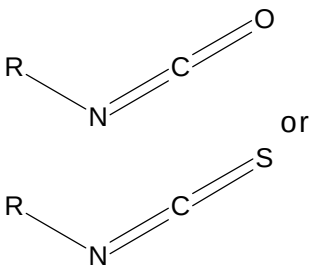
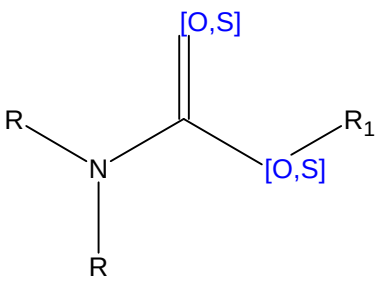
CAS: 3268-49-3

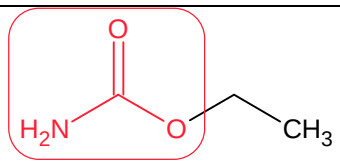
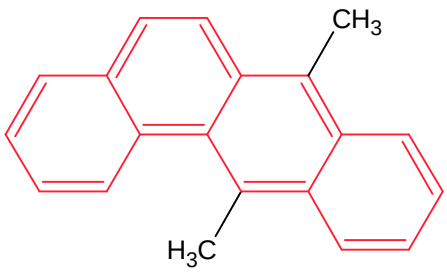
In vivo Micronucleus (Rodent): Positive

Reference: Leadscope</td></tr>
</table>

[3] Leadscop Database, http://www.leadscope.com/

SA_12: Quinones 	Any substance with the displayed substructures  Name: 9,10-Anthraquinone CAS: 84-65-1 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: Mitomycin C CAS: 50-07-7 In vivo Micronucleus (Rodent): Positive Reference: CCRIS & NTP

SA_13: Hydrazine 	R= any atom/group
	No positive representative
SA_14: Aliphatic azo and azoxy 	R1= Aliphatic carbon or hydrogen R2, R3 = Any atom/group R4 = Aliphatic carbon
	No representatives
SA_15: isocyanate and isothiocyanate groups 	R= any atom/group
	No positive representative
SA_16: alkyl carbamate and thiocarbamate 	R = Aliphatic carbon or hydrogen R1 = Aliphatic carbon

	 Name: Urethane CAS: 51-79-6 In vivo Micronucleus (Rodent): Positive Reference: CCRIS & NTP
SA_18: Polycyclic Aromatic Hydrocarbons	Three or more fused rings, not heteroaromatic
	 Name: 7,12-Dimethylbenz(a)anthracene CAS: 57-97-6 In vivo Micronucleus (Rodent): Positive Reference: CCRIS & NTP
SA_19: Heterocyclic Polycyclic Aromatic Hydrocarbons	Three or more fused rings, heteroaromatic
	No positive representative
SA_21: alkyl and aryl N-nitroso groups	R1= Aliphatic or aromatic carbon, R2= Any atom/group

<table>
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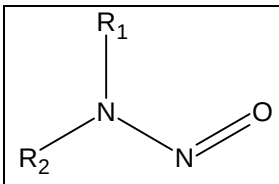
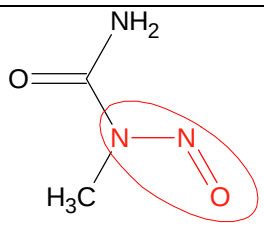
<td>  </td>
<td> 
Name: N-methyl-N-nitrosourea
CAS: 684-93-5
In vivo Micronucleus (Rodent): Positive
Reference: NTP</td>
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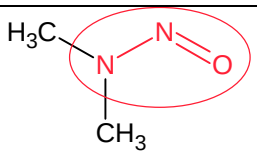
<td> 
Name: N-nitrosodimethylamine
CAS: 62-75-9
In vivo Micronucleus (Rodent): Positive
Reference: CCRIS</td>
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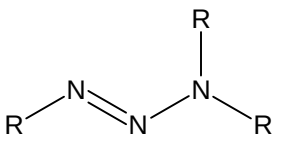
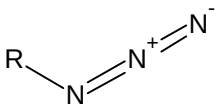
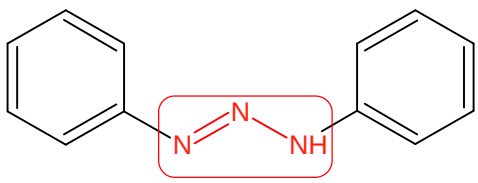
<td>SA_22: azide and triazene groups
  or
  </td>
<td>R= Any atom/group
 
Name: Diazoaminobenzene
CAS: 136-35-6
In vivo Micronucleus (Rodent): Positive
Reference: CCRIS & NTP</td>
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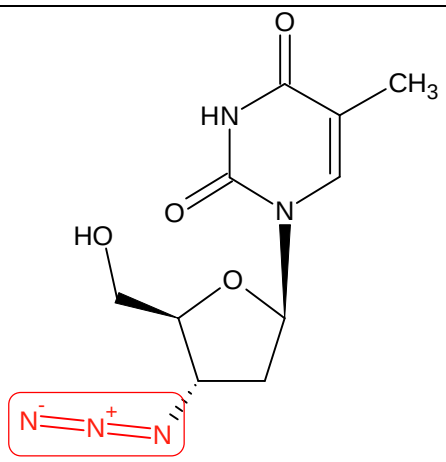
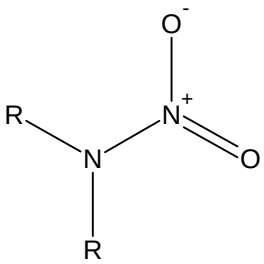
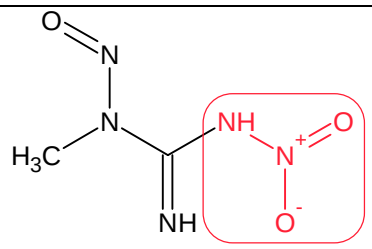
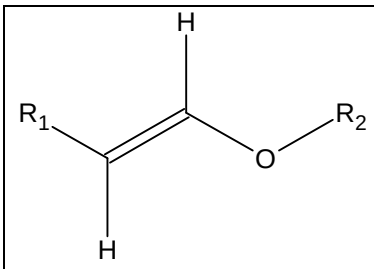
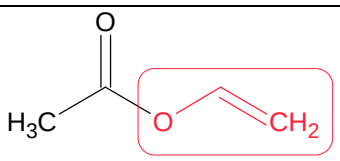
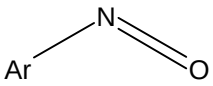
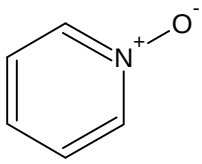
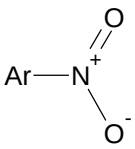
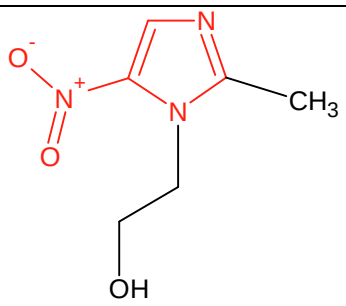
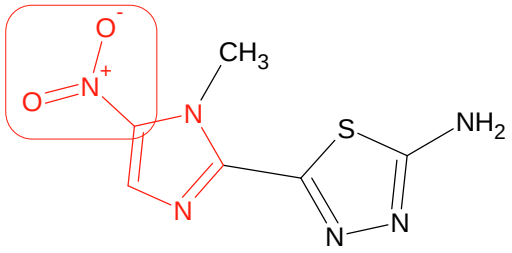
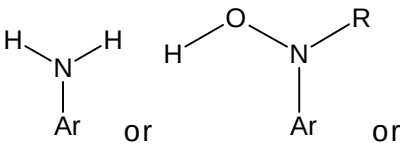
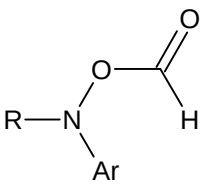
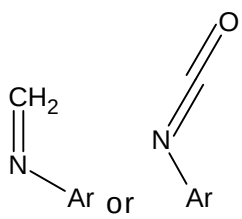
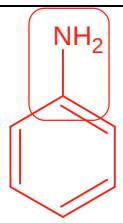
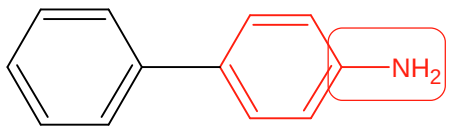
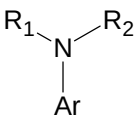
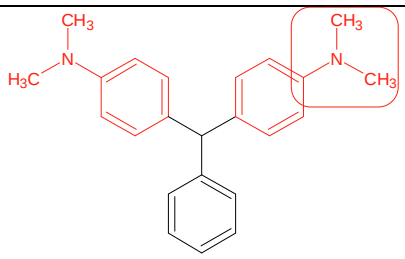
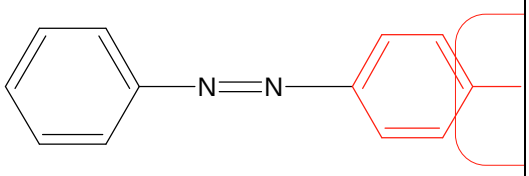
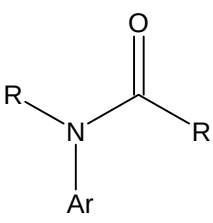
	 Name: Zidovudine CAS: 30516_87-1 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_23: aliphatic N-nitro group  	R= Aliphatic carbon or hydrogen  Name: N-methyl-N'-nitro-N-nitrosoguanidine CAS: 70-25-7 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_24: Unsaturated aliphatic alkoxy group	R1= Any aliphatic Carbon R2 = Aliphatic or aromatic carbon

	 Name: Vinyl Acetate CAS: 108-05-4 In vivo Micronucleus (Rodent): Positive Reference: NTP

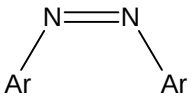
SA_25: aromatic nitroso group 	Ar = Any aromatic/heteroaromatic ring
	No positive representative
SA_26: aromatic ring N-oxide 	Any aromatic or heteroaromatic ring
	No positive representative
SA_27: Nitro-aromatic 	Ar = Any aromatic/heteroaromatic ring: -chemicals with ortho-disubstitution, or with an ortho carboxylic acid substituent are excluded; -chemicals with a sulfonic acid group (-SO3H) on the same ring of the nitro group are excluded.
	 Name: Metronidazole CAS: 443-48-1 In vivo Micronucleus (Rodent): Positive Reference: CCRIS

	 Name: CL 64855 CAS: 19622-55-0 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_28: primary aromatic amine, hydroxyl amine and its derived esters   or amine generating group:  	Ar = Any aromatic/heteroaromatic ring R= Any atom/group -Chemicals with ortho-disubstitution, or with an ortho carboxylic acid substituent are excluded. -Chemicals with a sulfonic acid group (-SO3H) on the same ring of the amino group are excluded.

	 Name: Aniline CAS: 62-53-3 In vivo Micronucleus (Rodent): Positive Reference: CCRIS & NTP
	 Name: 4-Biphenylamine CAS: 92-67-1 In vivo Micronucleus (Rodent): Positive Reference: NTP
SA_28bis: Aromatic mono- and dialkylamine 	Ar = Any aromatic/heteroaromatic ring R1 = Hydrogen, methyl, ethyl R2 = Methyl, ethyl -Chemicals with ortho-disubstitution, or with an ortho carboxylic acid substituent are excluded. -Chemicals with a sulfonic acid group (-SO3H) on the same ring of the nitro group are excluded.

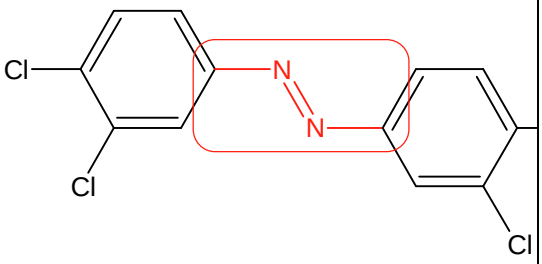
	 Name: Leucomalachite Green CAS: 129-73-7 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: 4-Dimethylaminoazobenzene CAS: 60-11-7 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
SA_28tris: aromatic N-acyl amine  	Ar = Any aromatic/heteroaromatic ring R = Hydrogen, methyl • Chemicals with ortho-disubstitution, or with an ortho carboxylic acid substituent are excluded. • Chemicals with a sulfonic acid group (-SO3H) on the same ring of the nitro group are excluded. No positive representative

SA_29: Aromatic diazo



Ar = Any aromatic/heteroaromatic ring

- Chemicals with a sulfonic acid group (-SO3H) on both rings that contain linked to the diazo group are excluded.

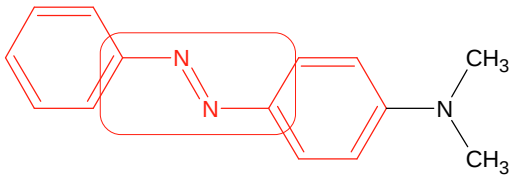


Name: 3,3',4,4'-Tetrachloroazobenzene

CAS: 14047-09-7

In vivo Micronucleus (Rodent): Positive

Reference: NTP

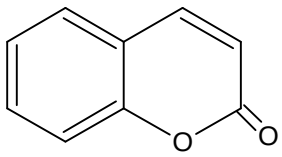
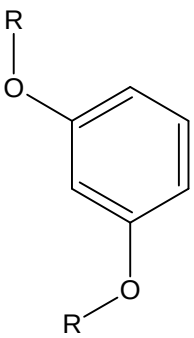
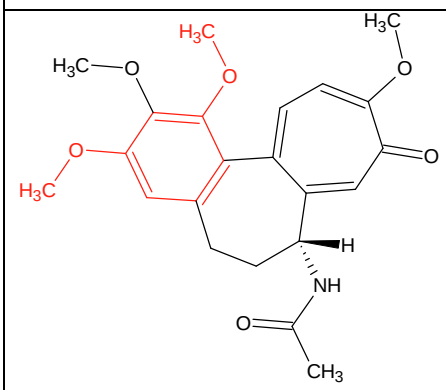
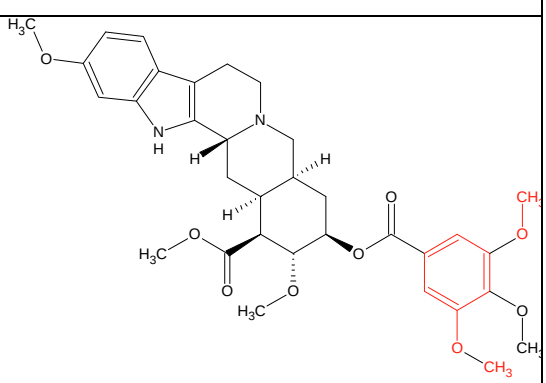


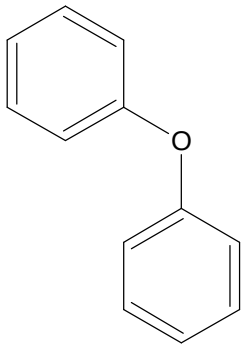
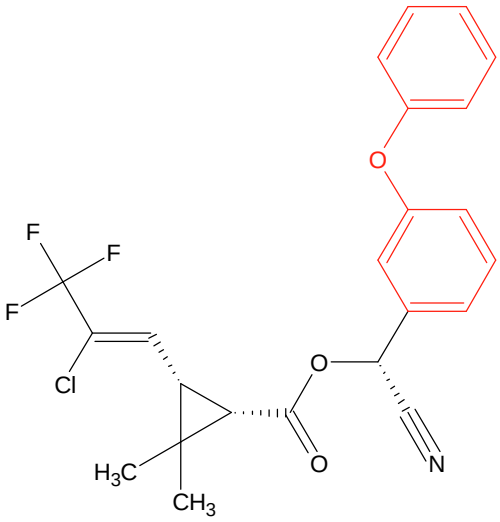
Name: 4-Dimethylaminoazobenzene

CAS: 60-11-7

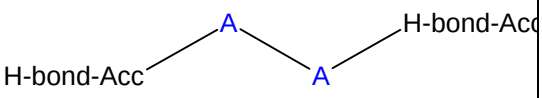
In vivo Micronucleus (Rodent): Positive

Reference: CCRIS

SA_30: Coumarins and Furocoumarins 	Any substance with the displayed substructure
	No positive representative
SA_32: 1,3-dialkoxy-benzene 	R= any alkyl group
	 Name: Colchicine CAS: 64-86-8 In vivo Micronucleus (Rodent): Positive Reference: CCRIS
	 Name: Reserpine CAS: 50-55-5 In vivo Micronucleus (Rodent): Positive Reference: NTP

SA_33: 1-phenoxy-benzene	Any substance with the displayed substructure.
	 Name: Lambda-cyhalothryn CAS: 91465-08-6 In vivo Micronucleus (Rodent): Positive Reference: CCRIS

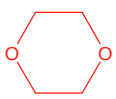
SA_34: hacceptor-path3-hacceptor



A= Any atom, except Hydrogen

H-bond-Acc= Any atom that is a potential Hydrogen bond acceptor:

a) any doubly bonded oxygen;
b) any singly bonded oxygen, such as anion A-O-or hydroxyl A-OH;
c) Uncharged imine, nitrile, or aromatic N. Examples of imines include C=NH, or C=N-Ak; aromatic N includes ARO -N-ARO, where both ARO-N bonds are cyclic, aromatic;
d) an ether oxygen in the form C-O-C, where: neither C is substituted by a doubly-bonded N, S, or O; neither C is part of a carbonyl; at most one C is aromatic; the O is acyclic;
e) C-O-C is cyclic, both C are sp3 hybridized;
f) Any doubly bonded sulfur in thioxomethyl C=S, where S has no other attachments.

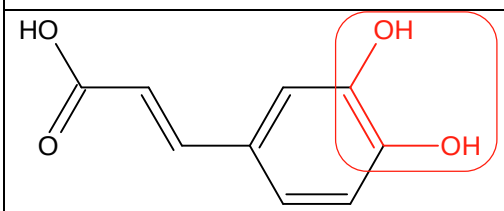


Name: p-Dioxane

CAS: 123-91-1

In vivo Micronucleus (Rodent): Positive

Reference: CCRIS & NTP



Name: 3,4-Dihydroxycinnamic acid

CAS: 331-39-5

In vivo Micronucleus (Rodent): Positive

Reference: NTP

<table>
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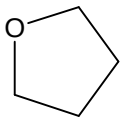
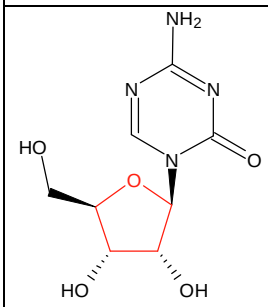
  </td><td>Any substance with the displayed substructure.

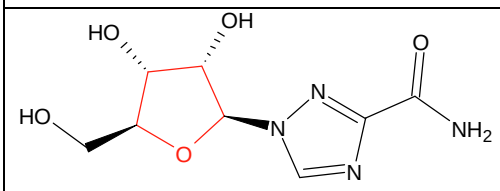
 

Name: 5-Azacytidine

CAS: 320-67-2

In vivo Micronucleus (Rodent): Positive

Reference: NTP

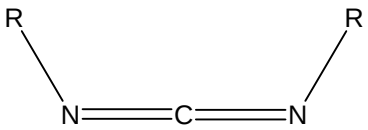
 

Name: Ribavirin

CAS: 36791-04-5

In vivo Micronucleus (Rodent): Positive

Reference: NTP</td></tr>
<tr><td>SA_36: Carbodiimides

  </td><td>R= any alkyl group</td></tr>
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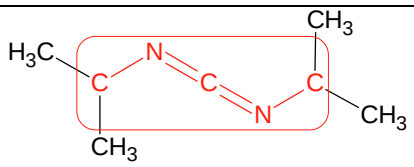
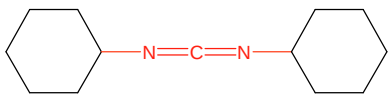
	 Name: Diisopropylcarbodiimide CAS: 693-13-0 In vivo Micronucleus (Rodent): Positive Reference: NTP
	 Name: Dicyclohexylcarbodiimide CAS: 538-75-0 In vivo Micronucleus (Rodent): Positive Reference: NTP