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The Expanded Decision Tree: Ranking Toxic Potential

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Phase I of the External Peer Review

1. The Expanded Decision Tree (EDT)

1.1 Background

The Cramer et al. Decision Tree (CDT) (Cramer et al., 1978) prioritizes substances according to their relative toxic potential using a sequence of 33 mainly structure-based yes or no questions to which the answer either refers the user to another question or assigns the substance to one of three classes of relative toxic potential: low (Class I), intermediate (Class II), or high (Class III). While Class I contains substances predicted to have a “low order of oral toxicity,” Class III encompasses substances having structural features that suggest either “significant toxicity” or “permit no strong initial presumptions of safety” (as cited in Cramer et al., 1978). Class II substances are those with predicted intermediate toxicity. Each question in the CDT was designed based on information available at that time for chemical structure, reactivity, metabolism, toxicokinetics, biochemistry, and animal toxicology. The CDT can be applied to all chemically defined organic and organometallic substances, but not to polymers, inorganic substances, or substances with unknown structure. Since the development of the CDT in 1978, our knowledge of structure-toxicity relationships and modes of toxic action (MoA) have significantly increased along with the amount of animal toxicological data that have been acquired. Moreover, the number and types of substances in food today have expanded because we consume an increasing variety of foods, partly related to globalization of our food supply, but improvements in food production and packaging have resulted in the addition of new substances to food that improve processing, transport, and storage. Furthermore, analytical detection methodology has advanced well-beyond what was current in 1978. As lower limits of detection are developed and the complexity of the integrated global food system expands, more substances may be detected that could require a food safety review. New approach methods are needed to support evaluation of substances in the greatly expanded universe of substances in the food supply.

It is challenging and resource-intensive to prioritize, test using qualified or guideline studies, and then evaluate the safety of a wide variety of chemicals of different toxic potential, many with low-exposure scenarios. As a first step, risk assessors want a tool to screen and prioritize all substances for their relative toxic potential. While the CDT represents one of the early attempts to screen a wide variety of chemically defined substances for their relative toxicity, the Expanded Decision Tree (EDT) is a “state of the science” update and expansion of the CDT in which current mode of action (MoA), metabolism, and toxicity data are the basis for the EDT questions.

1.2 The Cramer at al. (1978) Decision Tree

While the CDT has served as a robust and useful tool for many applications, the questions in the CDT and the underpinning data have received limited attention over the past four decades. Suggested changes (Phillips et al.,1987) to the CDT have been limited to deleting question (Q) 6, re-ordering a few of the questions (Qs 18-21), expanding a few others (e.g., Qs 4, 9, and 11), adding selective reactive functional groups (e.g., nitrogen-containing functional groups), deleting an ambiguous phrase (sterically hindered) and adding a new one (readily degradable to a common component of food), and reassigning reactive moieties to a higher class (e.g., allyl-containing substances from Class II to III). New classifications suggested for selected functional groups (i.e., regrouping amines and phenols to CDT Class II) (Tluczkiewicz et al., 2011) remain unaddressed in the CDT. Analyses of No Observed Effect Level (NOEL) distributions for the three CDT classes in the Munro Database (DB) (Munro et al., 2008) and in the RepDose DB of industrial substances (Escher et al., 2010) showed considerable overlap between Classes I and III. To improve the separation among CDT classes, substances from the TTC RepDose DB (Tluczkiewicz et al., 2011) were reassigned to different CDT classes based solely on their observed NOELs, as opposed to their chemical structures. Applying this technique, groups considered for class reassignment in the CDT included primary amines and phenols.

While 31 of the 33 Qs in the CDT are structure-based, two very important Qs, 1 and 22, are non-structure based (Cramer et al., 1978). Question one tries to capture all substances that are “a normal constituent of the body or an optical isomer of such” and places them into CDT Class I. Question 22 aims to place every substance that is “a common component of food or structurally closely related to a common component of food” into Class II. As these Qs are non-structure-based and ambiguous (hence highly subjective), Cramer et al. provided definitions for ‘normal constituents of the body,’ ‘common component of food,’ and ‘structurally closely related.’ Unfortunately, the definitions themselves are ambiguous and require expert judgement and knowledge. For the definition of ‘common component of food,’ Cramer et al. states that “In something as diverse, changing and occasionally uncertain as natural occurrence, it is only possible to define a guideline, not a firm rule.” Consequently, Qs 1 and 22 may result in subjective class assignments and may depend on the user. Some compounds may end up in a higher or lower class than what would be warranted based on their true toxic potentials.

The ToxTree software (Toxtree – Toxtree - Toxic Hazard Estimation by decision tree approach (sourceforge.net)) includes the CDT and classifies compounds into their appropriate CDT classes. While, in general, a software can provide more consistent class assignments and eliminate or reduce subjectivity, implementing non-structure-based questions, such as CDT Qs 1 and 22, into a software is fraught with extreme difficulties. Patlewicz et al. (2008) discussed CDT misclassification of selected congeneric groups. In an analysis of the CDT questions and Toxtree software, Lapenna and Worth (2011) compiled recommendations for a future revision of the CDT sequence. Furthermore, Bhatia et al. (2015) described discrepancies in CDT classifications predicted by expert judgement, Toxtree software, and the QSAR Toolbox version of Toxtree commissioned by the Organisation for Economic Co-operation and Development (OECD) due to ambiguous questions in the CDT. Finally, a practical guidance was published (Roberts et al., 2015) that pointed out inconsistencies in class assignments and potential misinterpretations of the questions when applying the CDT.

1.3 Objectives of the Comprehensive Revision of the CDT

While the abovementioned researchers have made or suggested meaningful improvements to the CDT, fundamental issues remain unaddressed. Toxicological and metabolic data accumulated since 1978 have not been incorporated into the CDT. No attempts have been made to redesign the CDT such that all questions are structure-based, to eliminate or convert ill-defined terms, such as steric hindrance and terpenes into specific structure-based questions, or to design new and revise existing structure-based questions to represent "state of the science" information.

Based on much of the published work and our evaluation of the existing CDT, the primary objectives of a comprehensive revision of the CDT should include, but not be limited to, the following:

1) Questions should depend solely on chemical structure, and non-structure-based questions should be eliminated. For example, in CDT Q1, concerning whether a substance is a "normal constituent of the body," and Q22, concerning whether a substance is a "common component of food," both depend on a long and subjective list of substances that cannot be associated with specific structural features, and as such, should be removed and replaced with structure-based questions.
2) Clear language should be employed and ambiguous definitions, phrases, and questions (e.g., questions on "steric hindrance" and "terpene," often confused with terpenoid) should be eliminated. A clearer and expansive definition section for terms used in the EDT should be created. Furthermore, changes in structural class associated with steric effects should be incorporated directly into specific structure-based questions.
3) All existing CDT questions should be updated based on metabolism, toxicity, and MoA data that have become available since 1978, because the current CDT classes do not discriminate adequately among substances of different toxic potentials. For some existing questions, conditions for identifying structural features should be further refined to ensure that only substances exerting a specific type of toxicity and/or have the same MoA will result in the assignment to a specific EDT class. For some other questions, structural conditions should be broadened to capture more structures that need to be classified at that question. Yet in other instances, it may be necessary to subdivide a question into multiple questions to delineate subtle differences in toxic potential based on various features of structurally related substances (e.g., whether a functional group is present or absent on the same structural skeleton may alter detoxification).
4) New questions should be created to address a wider variety of elements, functional groups, moieties, and congeneric groups to increase the applicability of the EDT to a much broader variety of structures and to decrease the number of substances defaulting into the highest class of concern due to the lack of questions addressing the structural features they display. Designing new questions will also improve correlation between chemical structure and toxicity (i.e., No Effect Levels (NELs) (encompassing both NOELs and No Observed Adverse Effect Levels (NOAEL)), mitigating the overlap of NELs among the different CDT classes.
5) The structural classes for phenols, primary amines (Tluczkiewicz et al., 2011), and lactones (Roberts et al., 2015) should be re-evaluated based on an outlier analysis of their NEL distributions, consistent with relevant pharmacokinetic data.

6) Proper sequencing of the questions is necessary in order to avoid excessive branching and loop-backs and loop-forwards (i.e., referring the user to a previous or future question in the sequence to capture a key structural feature of the substance).

1.4 Preparation for Using the EDT

Appendix 1 helps the user understand our thinking behind each question. Appendix 2 provides the database based on which the finalized EDT TTCs were established and can also serve as examples of classifications to help the user become accustomed to and proficient in using the EDT.

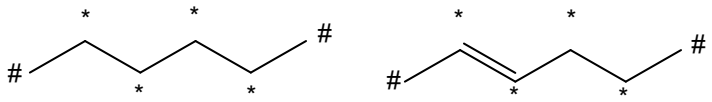
Common understanding of the scientific chemical terms is needed to reliably evaluate substances through the EDT decision tree. We attempted to provide clear definitions of terms to aid users. Some definitions are composed specifically for questions in the EDT and do not have the same meaning in the general literature. For example, the term “aliphatic” encompasses all non-aromatic compounds in the general literature. For the purposes of the EDT, aliphatic includes alkane, alkene, polyalkene, but not allenes, alkynes, polyalkynes, or alicyclic compounds. Novel definitions, such as that for aliphatic compounds, are utilized to enable simplification of many of the EDT questions. Therefore, we ask the user to review the guidelines and definitions in section 1.5 prior to and during the application of the EDT.

1.5 Applicability Domain and Definitions for Using the EDT

Although most common chemistry terms in the EDT are used as they are in scientific literature, some terms have been modified to simplify the language of the EDT questions. The following definitions were employed during the development of the EDT questions to facilitate its application and assist in resolving issues related to class assignment:

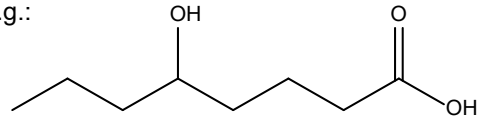
A. Applicability domain of the EDT: all compounds except unhydrolyzable polymers, proteins, elements, inorganic substances, and substances with undefined structures. Please note that ingested particles may have varying bioavailability and toxicity depending on their size. The EDT is not designed to estimate safe intake levels (i.e., TTCs) based on particle size and should only be applied to substances within its applicability domain. While there is no cutoff for molecular weight (MW) when applying the EDT, the MW range of substances in the combined EDT DB is 30.03-2285.61 Da. Some of the hydrolyzable polymers within the structural applicability domain of the EDT may have MWs that exceed this range. In case of hydrolyzable polymers, the EDT assumes complete hydrolysis to monomeric units. Additionally, please note that the EDT is designed specifically to sort compounds based on/according to their relative chronic toxic potential through oral exposure only.
B. **Skeleton/skeletal structure**: The skeletal structure of an organic compound is the series of atoms bonded together that form the essential structure of the compound. The skeleton can consist of chains, branches, and/or rings of bonded atoms. Skeleton and skeletal structure are used interchangeably throughout the EDT.
C. **Linear** means that the chain has no branching (i.e., each carbon in the chain is connected to one or two other carbon(s)). **Simply branched-chain** substances may have any number of methyl substituent(s) and/or up to two n-alkyl branches of two or more carbons at not more than two points along the main chain (these n-alkyl branches cannot be on the same carbon) with no additional branching (e.g., 3,4-diethyloctane). **Branched-chain** means that the substance contains more than two branches along the main chain that has two or more carbons. Examples:

linear:



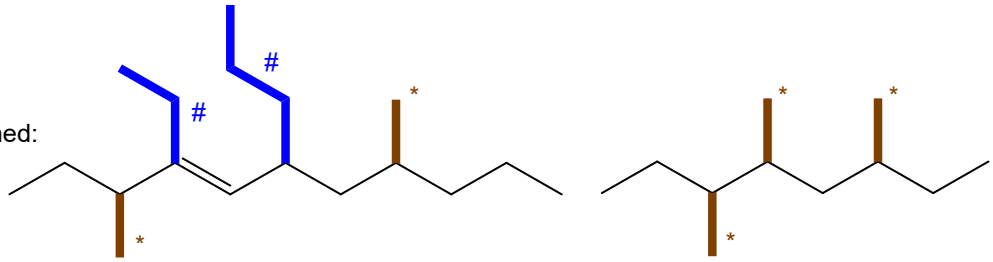
#: connected to one other carbon *: connected to two other carbons

e.g.:



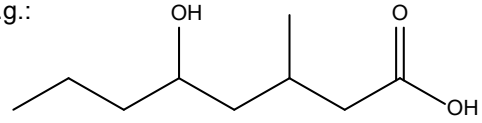
for the purposes of the EDT, the above hydroxycarboxylic acid is 'linear'

simply branched:



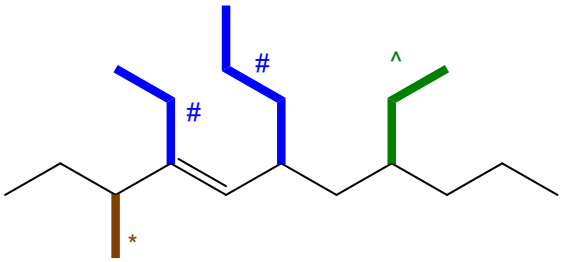
brown (also marked with *): any number of methyl substituents;
blue (also marked with #): up to 2 n-alkyl branches of 2 or more carbons at not more than 2 points along the chain

e.g.:



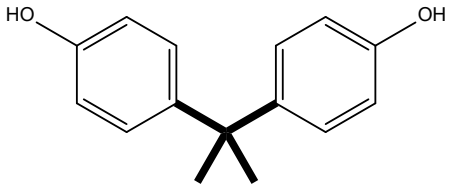
for the purposes of the EDT, the above hydroxycarboxylic acid is 'simly branched'

branched:

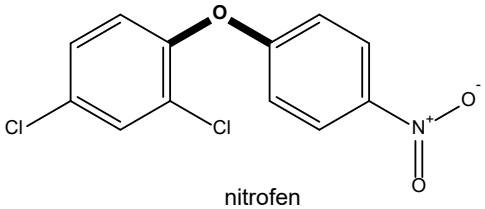


green (also marked with ^): due to 3rd branching that is other than methyl, the compound is no longer simply-branched

D. A **connector** is a structural element that links two distinct rings or fragments in a molecule through chains and/or functional groups, without fusing the rings together. Examples:

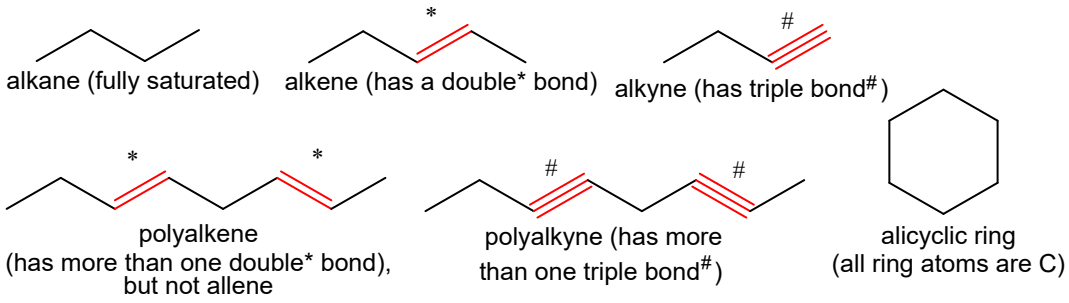


bisphenol A
(connector is bolded)

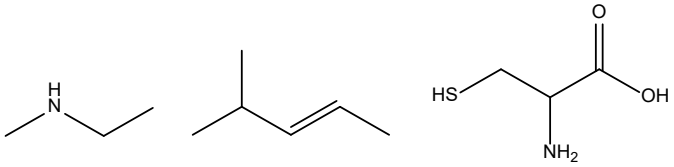


nitrofen
(connector is bolded)

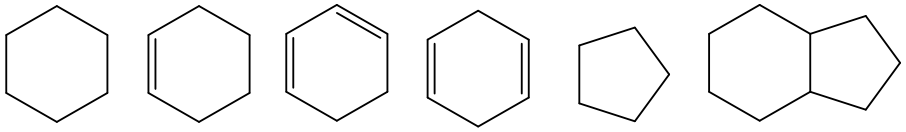
E. **Aliphatic** includes alkane, alkene, polyalkene, but not allene (C=C=C), alkyne, polyalkyne, or alicyclic compounds.



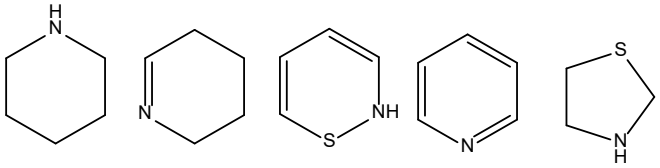
F. **Acyclic** means the absence of a ring (i.e., the molecule is open-chained).



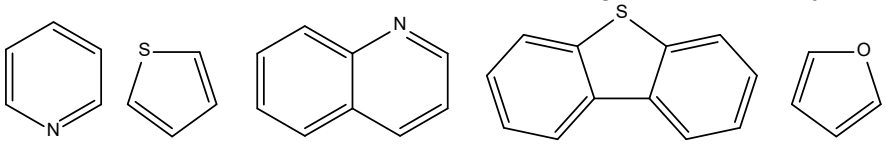
G. **Alicyclic** refers to a molecule where all rings are composed solely of carbon atoms. These rings may contain ring alkenes but do not form an aromatic ring.



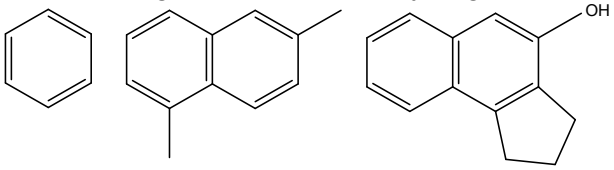
H. **Heterocyclic** refers to a molecule that contains at least one ring structure where at least one of the ring atoms is not carbon, commonly nitrogen (N), oxygen (O), or sulfur (S).



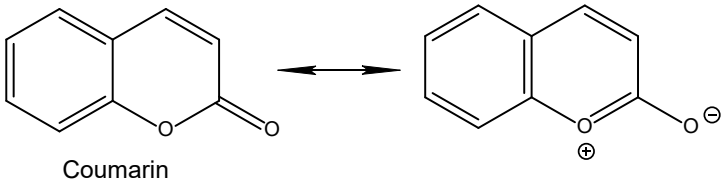
I. **Heteroaromatic** refers to a substance that contains at least one ring with at least one ring heteroatom (commonly N, O, and/or S) and a fully conjugated cyclic array of [4n+2]π electrons (e.g., furan, pyrrole, 1,3-imidazole, thiazole, and pyridine). Heteroaromatic compounds are a specific subgroup of heterocyclic compounds.



J. **Aromatic** or **aryl** (Ar) means that the substance contains at least one aromatic ring (ring with a fully conjugated cyclic array of [4n+2]π electrons) regardless of whether the aromatic ring is fused or bonded to another ring and regardless of any substitution. The aromatic ring cannot contain any ring heteroatom(s) (e.g., O, N, and S).

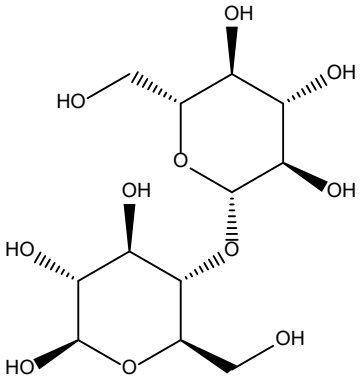


K. For the purposes of the EDT, a **pseudo-aromatic** ring means a ring that can only achieve a completed cyclic array of [4n+2]π electrons by incorporating the electron pair of a functional group into an enolic double bond, such as a lactone or lactam. Example:

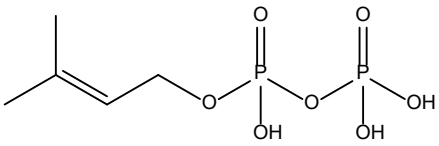


Coumarin

L. A **dimer** refers to a molecule that is formed by the combination of two identical or similar smaller molecules (monomers) through a chemical reaction. Examples:

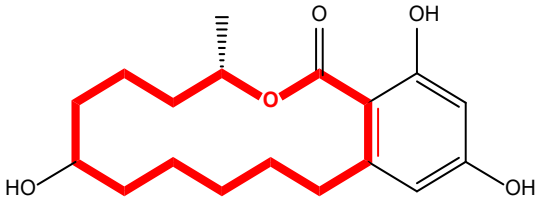


cellobiose (dimer of glucose)

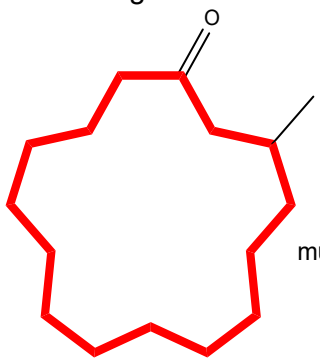


dimethylallyl diphosphate (a phosphate dimer)

M. For purposes of the EDT, a **macrocyclic** ring is a completed cyclic array of any combination of ≥11 C, O, or N atoms, with or without ring alkenes.

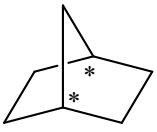


zearalanol

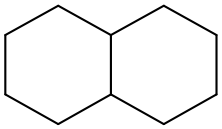


muscone

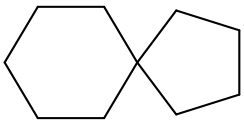
N. **Bridged** compounds have two or more rings (a ring system) that contain a bridge (i.e., a single atom or an unbranched or branched chain of atoms that connect two bridgehead atoms). Bridgehead atoms are defined as any atom that is not a hydrogen and that is part of the skeletal framework of the molecule bonded to three or more other skeletal atoms. The presence of the bridge connecting the bridgehead atoms distinguishes bridged compounds from **fused** ring compounds, which have two rings linked by two adjacent atoms, and from **spiro** compounds, which have two rings linked by a single atom. **Singly bonded** rings share a bond between one atom on each ring. Examples:



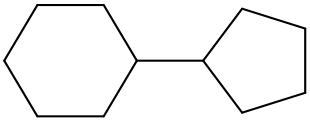
bridged
* bridgehead atoms



fused

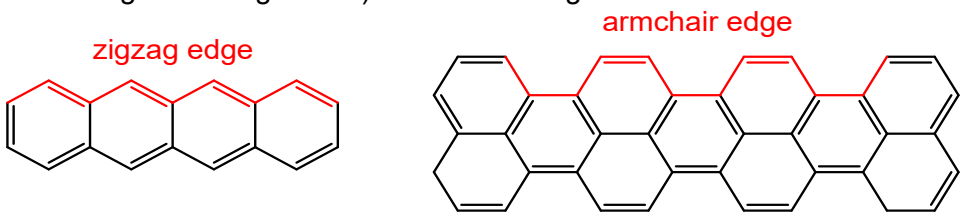


spiro

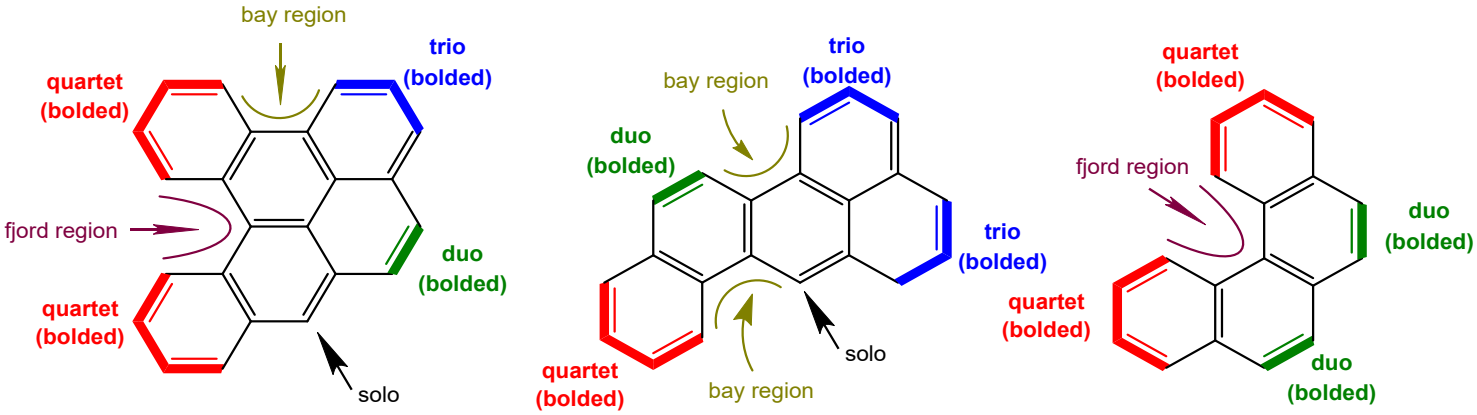


singly bonded

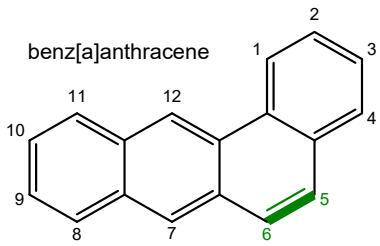
O. **Zigzag** and **armchair edge**: Zigzag edge is present when the aromatic rings fuse in a linear configuration. Armchair edges form as a result of angular fusion (i.e., angled fusion/angular configuration) of aromatic rings.

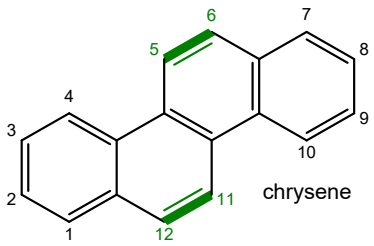


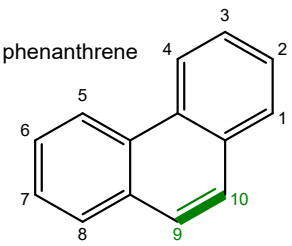
P. **Solo, duo, trio, quartet**: In polycyclic aromatic hydrocarbons (PAHs), the terms solo, duo, trio, and quartet refer to configurations where one, two, three, or four adjacent carbon atoms, respectively, are bonded to atoms other than those within the aromatic ring. For example, in the provided structures, each of the three 'trio' carbons are bonded to a hydrogen atom. That is, these trio carbons are bonded to hydrogen atoms outside the aromatic ring structure. **Bay** and **Fjord regions**: The bay region is characterized by the presence of a "bay" or "indentation" in the aromatic system. The fjord region refers to a structural feature in a molecule where there is a pronounced "fjord" or "trough" between two aromatic rings.

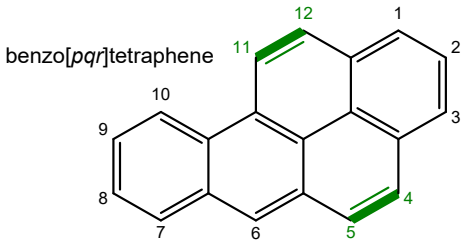


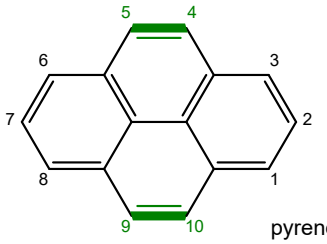
Q. **K-region**(s) is/(are) the convex armchair edge(s) of polyaromatic hydrocarbons that are joined together by angular fusion. The K-region is made of a duo (displayed in bolded green below). For examples:

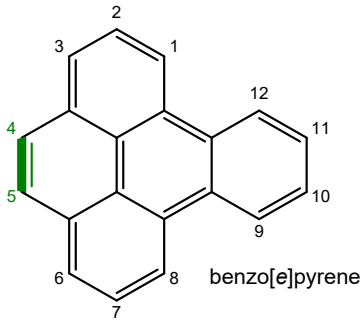


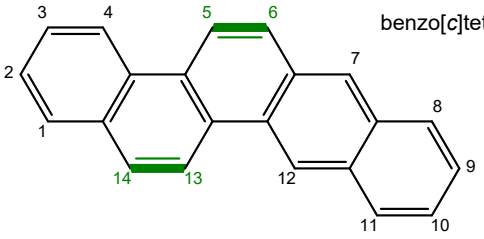




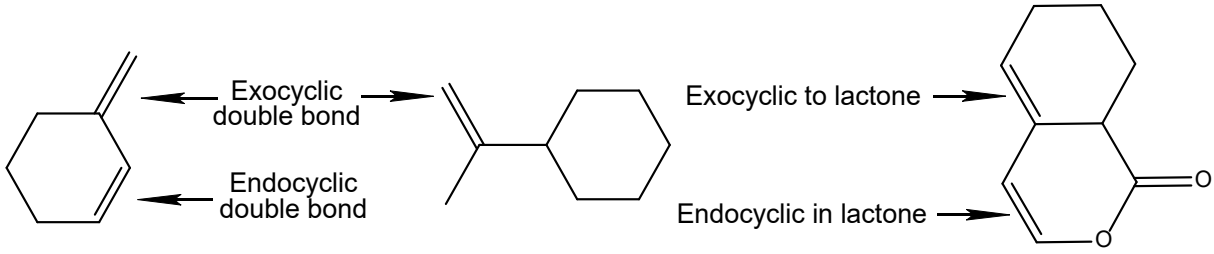




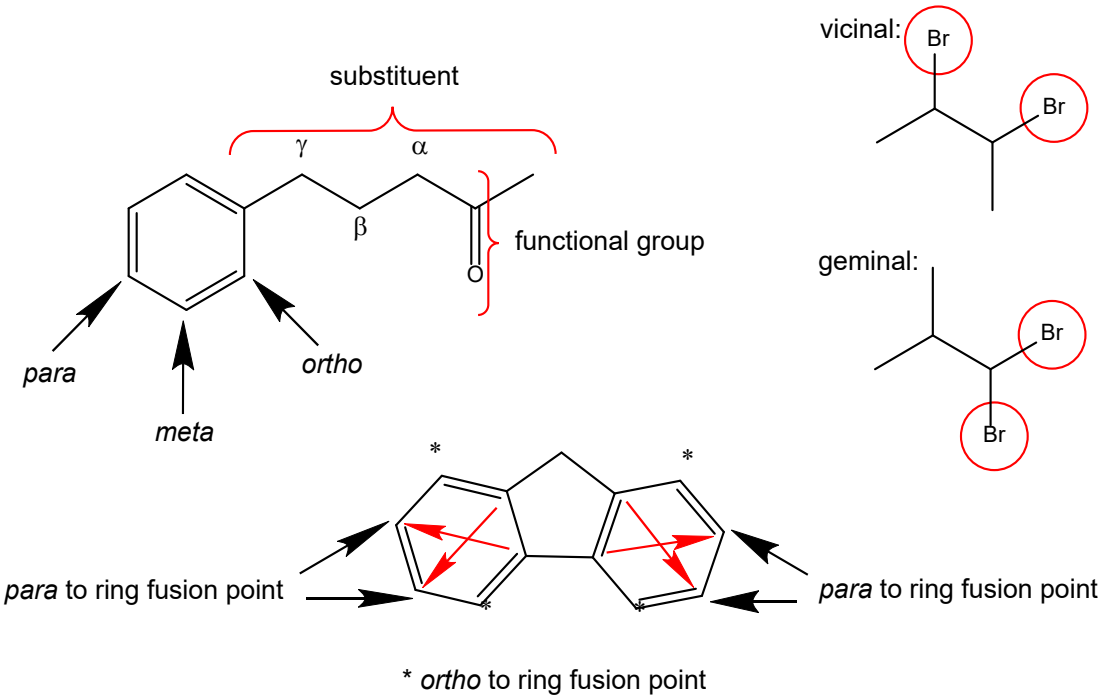




R. **Endocyclic** and **exocyclic** double bonds: If both carbon atoms connected by a double bond are members of the same ring, the double bond is said to be **endocyclic**. If at least one of the carbon atoms connected by a double bond is not a member of the same ring, the double bond is said to be **exocyclic**. Examples:

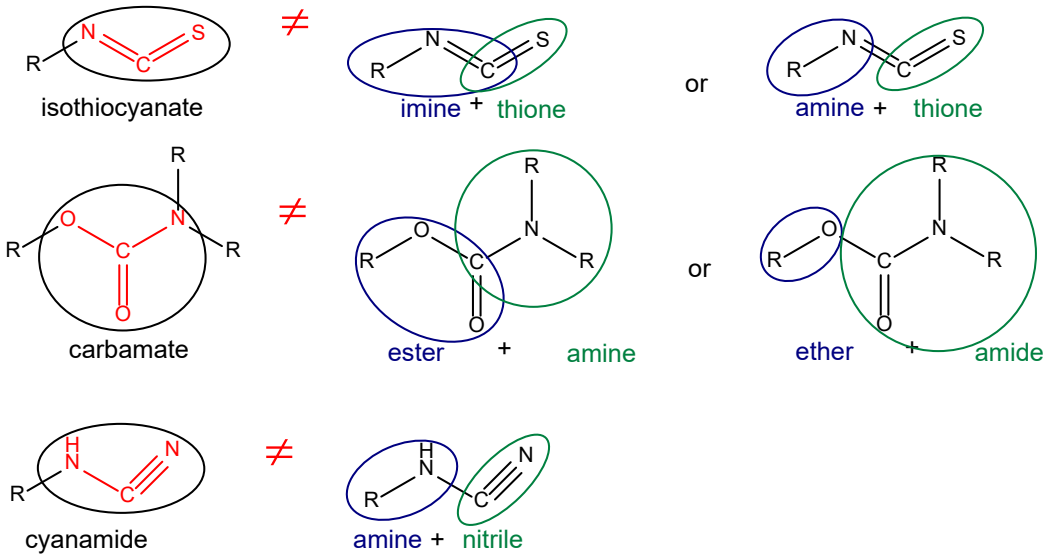


S. Positions:
   a. α, β, and γ carbon; *ortho-, meta-*, and *para*-substitution; and definitions of vicinal and geminal
   b. **α** carbon is the first carbon that attaches to a functional group, **β** is the second, **γ** is the third, and **δ** is the fourth.
   c. For six-membered aromatic or heteroaromatic rings, ***ortho***-substitution means that the two non-hydrogen substituents occupy adjacent ring atoms; ***meta***-substitution means that the substituents are separated by one ring atom; and ***para***-substitution means that the substituents are separated by two ring atoms.
   d. **Vicinal**: two functional groups or atoms attached to two adjacent atoms, and **geminal**: two functional groups or atoms attached to the same atom.



* *ortho* to ring fusion point

T. **Functional group** means a group of covalently-bound atoms of two or more elements, one of which is not hydrogen or carbon. Each functional group undergoes a characteristic set of well-known reactions independent of its individual fragments. It is important to treat the functional group as an entire molecular entity and not as a fragment (e.g., #1: R-N=C=S is an isothiocyanate and not an imine (R-N=C) and a thione (-C=S) or #2: ROC(=O)N(R_1)R_2 is a carbamate and not an ester (ROC(=O)-) and an amine (-N(R_1)R_2)). Examples:



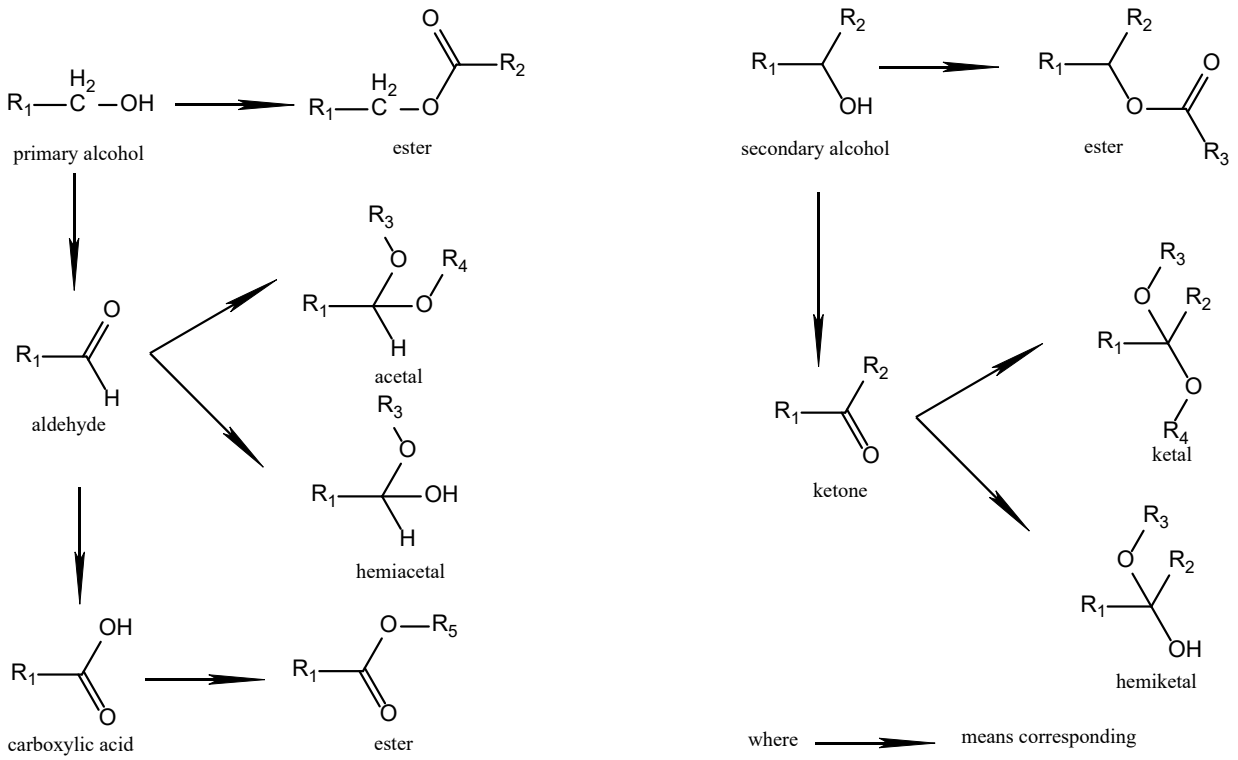
U. **Oxygenated functional group** means any of the following: alcohol (primary, secondary, or tertiary), ketone, aldehyde, carboxylic acid, ether, ester, acetal, ketal, hemiacetal, or hemiketal.

V. At Q2b only, the user is asked to identify potential **leaving groups** that are bound to phosphorus**.** Leaving groups are those atoms or groups of atoms that develop a stable negative charge following a nucleophilic substitution reaction due to inductive or resonance effects. Resonance effects operate through delocalization of π electrons present in adjacent double bonds, and inductive effects operate by polarization of electrons in sigma bonds. Both effects increase the ability of the bond to cleave and the leaving group to leave. In general, resonance effects are stronger than inductive effects and lead to more rapid bond cleavage. Resonance effects play a vital role in increasing the reactivity (and toxicity) of organophosphates in their substitution reactions with acetylcholinesterase. For the purpose of the EDT, leaving groups include, but are not limited to, those with inductive effects, such as (e.g., -CN, -SCN, and -OCN, -O-C=C, -O-C=O, $O=P(OR)_2O$-, O=P(OR)O-, $(O=)_2S(OR)O$-, or O=S(OR)O-). Because the number of possible leaving groups that can be synthesized by today's modern organic chemist is limitless, the user is encouraged to review these topics (i.e., leaving group, resonance and inductive effects) in greater depth in a standard organic chemistry text.

W. **Electron pair donors** are atoms or groups of atoms that can donate electron density. For the purpose of the EDT, these are: $-O^-$, -OR (ether), -OH (alcohol), -OC(=O)R (ester), -C(=O)OH (carboxylic acid), $-C(=O)O^-$ (carboxylate), $-NH_2$ (primary amine), -NHR (secondary amine), $-NR_2$ (tertiary amine), -NHC(=O)R (amide), -SR (thiolate), and -SH (thiol). Question regarding electron pair donors is found only in Q6d.

X. **Organyl** refers to a general class of organic fragments that contain a carbon-based structure. Specifically, it often denotes an organic group or substituent derived from an organic molecule. While organyl can apply to various types of organic groups or substituents, such as alkyl groups (e.g., methyl, ethyl), aryl groups (e.g., phenyl, tolyl), or more complex structures, the organyl group or substituent is always based on organic carbon structures.

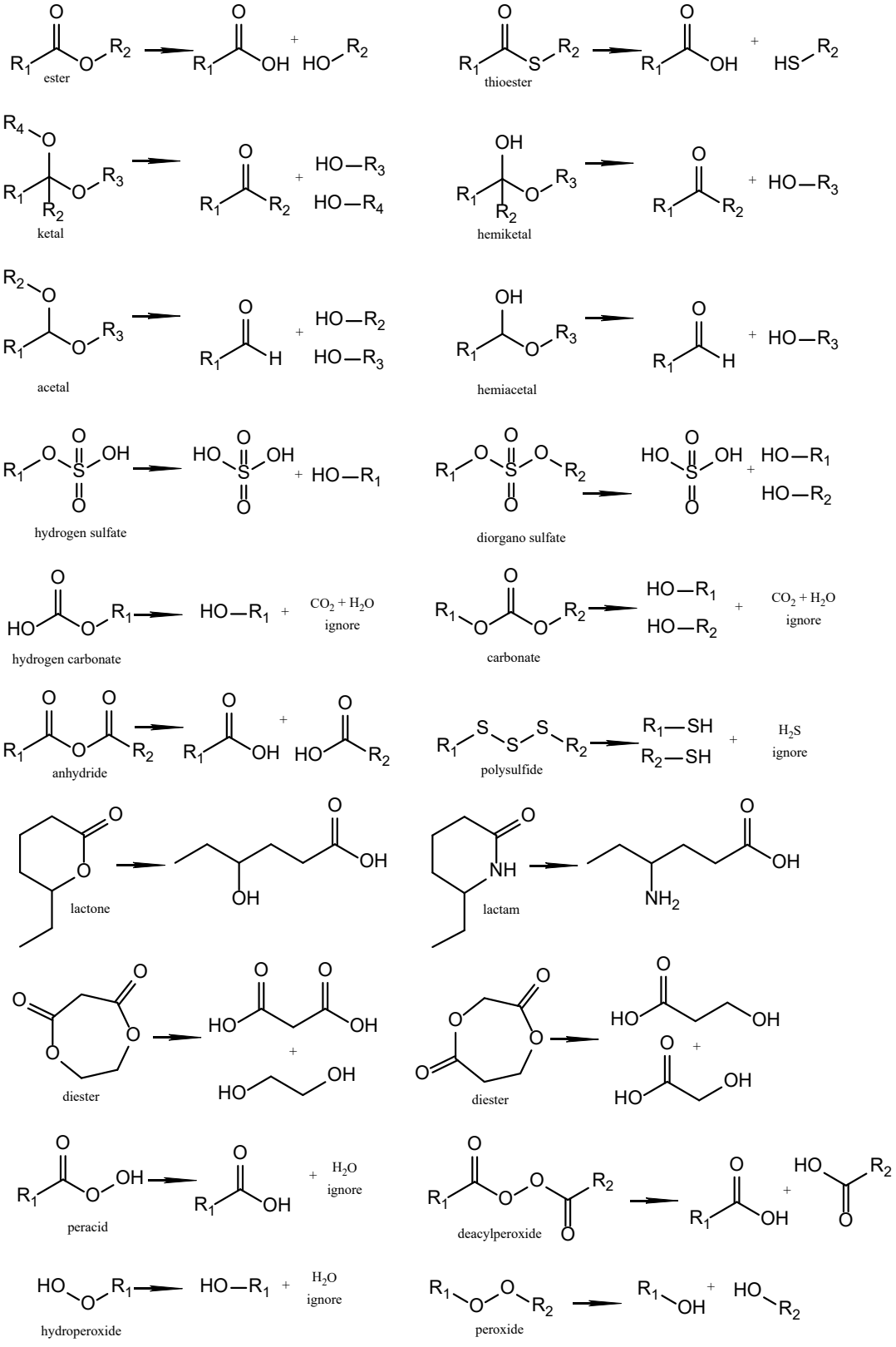
Y. The term **corresponding** refers to:
   a. A primary alcohol (e.g., 1-propanol) and its related compounds, that is, its corresponding aldehyde (i.e., propanal), carboxylic acid (i.e., propanoic acid), or an acetal, hemiacetal, or ester that hydrolyzes to yield the parent primary alcohol, or the corresponding aldehyde or carboxylic acid.
   b. A secondary alcohol (e.g., 2-butanol) and its related compounds, that is, its corresponding ketone (i.e., 2-butanone), or any ketal, hemiketal, or ester that hydrolyzes to yield the parent secondary alcohol or corresponding ketone. Examples:



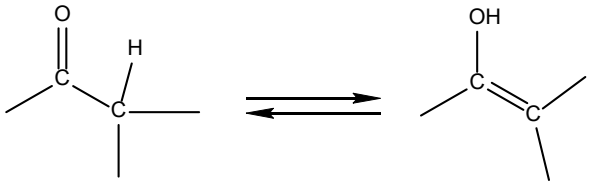
Z. The term **related** means:
   a. A member of an acyclic homologous series (e.g., 2-heptanone) different by not more than two carbons from another substance (i.e., 2-nonanone or 2-pentanone) in the series.
   b. Substances with the same functional groups (e.g., ethyl 3-ketobutanoate and propyl-3-ketopentanoate) that are expected to participate in common metabolic pathways (i.e., hydrolysis to ketoacid, β-cleavage to yield acetyl CoA and the CoA ester of the acid fragment, and complete oxidation to carbon dioxide and water).
   c. Acetal, hemiacetal, ketal, hemiketal, or ester that hydrolyzes to members of a homologous series (e.g., 2-phenylethyl acetate hydrolyzes to phenylethanol and acetic acid and 4-phenyl-1-butyl acetate hydrolyzes to 4-phenyl-1-butanol and acetic acid).

AA. Multiple questions in the EDT relate to expected **hydrolysis** or **reduction** of functional groups. Hydrolysis adds the element(s) of water to a molecule leading to either a different molecule (e.g., lactones with one cyclic ester hydrolyze to hydroxycarboxylic acids) or more than one molecule (e.g., aliphatic monoesters hydrolyzed to a carboxylic acid and an alcohol, and cyclic diesters hydrolyze to either two hydroxycarboxylic acids or to a diol and a dicarboxylic acid) (see drawing of hydrolysis reactions after this paragraph). Reduction is a chemical reaction where a species undergoes a gain of electrons or a decrease in its oxidation state. This process can involve the addition of hydrogen atoms or the removal of oxygen atoms from a molecule. Reduction is typically associated with the transfer of electrons from another substance that is being oxidized. All hydrolysis and reduction products should be evaluated using the EDT as instructed at specific questions and the structural class for the parent structure assigned based on the highest EDT class of its component molecules (e.g., if one of the hydrolysis or reduction products gets assigned to Class II and the second product to Class IV, assign the parent

compound to Class IV). See figure below for the hydrolysis and reduction reactions of common functional groups.

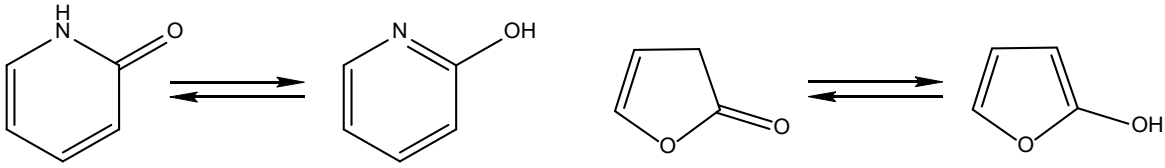


AB. **Enolization** means the interconversion (**tautomer**ism) between a keto form and an enol form. Example:

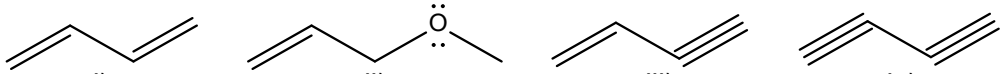


Ketone Enol

Tautomerization of heterocycles:



AC. In chemistry, **conjugated** refers to a specific arrangement of alternating single and double bonds within a molecule. This arrangement involves the overlap of p-orbitals across adjacent bonds, allowing for the delocalization of electrons across the entire system. When a double bond is adjacent to a single bond and the single bond is connected to a nitrogen or oxygen atom with lone pair electrons, these lone pairs can participate in conjugation. The lone pair on the nitrogen or oxygen can overlap with the π-system of the adjacent double bond. This interaction is often referred to as lone pair conjugation or n → π interaction and can affect the molecule's electronic structure, influencing properties such as reactivity and stability. In an alternating double bond-single bond-triple bond configuration, true conjugation does not occur because the triple bond does not participate in p-orbital overlap with the double bond in the same manner. However, there can be some electronic interaction between the double and triple bonds, though it is generally not as extensive or stabilizing as true conjugation and may affect the molecule's properties. Similarly, in a triple bond-single bond-triple bond configuration, the triple bonds do not conjugate with each other through the single bond. While there is no true conjugation here either, there may be some electronic effects or inductive interactions that can influence the molecule's stabilization and chemical properties. For the purposes of the EDT, to simplify its language, the following configurations are referred to as conjugation: i) double bond-single bond-double bond, ii) double bond-single bond-nitrogen or oxygen atom with lone pair of electrons, iii) double bond-single bond-triple bond, and iv) triple bond-single bond-triple bond.



i) ii) iii) iv)

AD. For the purposes of the EDT, we consider the moiety -CF_3 to be equivalent to one halogen. For example, if the compound has three -CF_3 moieties, we consider the compound to have a total of three halogens.

Terms in **bold letters** in the EDT questions below (section 1.7) indicate that they have been defined in section 1.5.

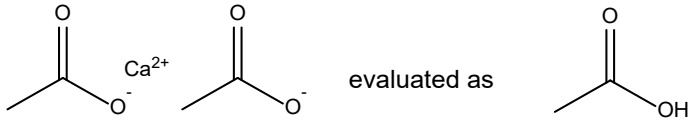
1.6 How to Use the EDT

Based on the chemical structures, definitions, and guidelines provided, the questions are answered in sequence with “yes” or “no” responses until reaching an assignment to one of the six EDT classes: Class I – VI (see section 1.8 for a detailed description of the six EDT classes). To help with classification, we provide one or more example structure(s) following each question. Moreover, in Appendix 2, we provide a large set of compounds and show how they traverse through the EDT to help the user get accustomed to using the EDT.

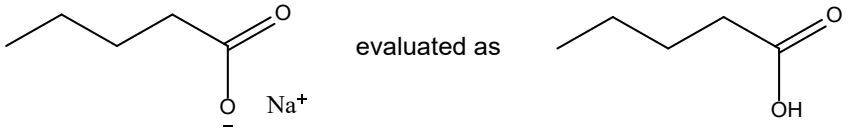
1.7 The Pre-validation EDT Questions

There are a large number of structurally diverse nontoxic substances, including substances endogenous in our bodies and common components of food. As it is impossible to formulate questions to capture all of these substances, we only attempted to devise structure-based questions for some of the most common ones. Therefore, please treat the following sub-questions in question (Q) 1 as examples rather than an exhaustive list. Combined, these sub-questions provide a basis for identifying and classifying nontoxic substances or substances with very low oral toxicity that are present in animals, in food, or are added to food along with other safe substances metabolized by high-capacity metabolic pathways.

1. Note: In Q1 only, disregard the following commonly encountered and relatively nontoxic or of low toxicity i) metal counterions: sodium, potassium, calcium, magnesium, barium, aluminum, titanium, zinc, manganese, copper, and iron; and ii) nonmetal counterions: fluoride, chloride, and bromide, and evaluate the compound of interest in its neutral form. Examples:

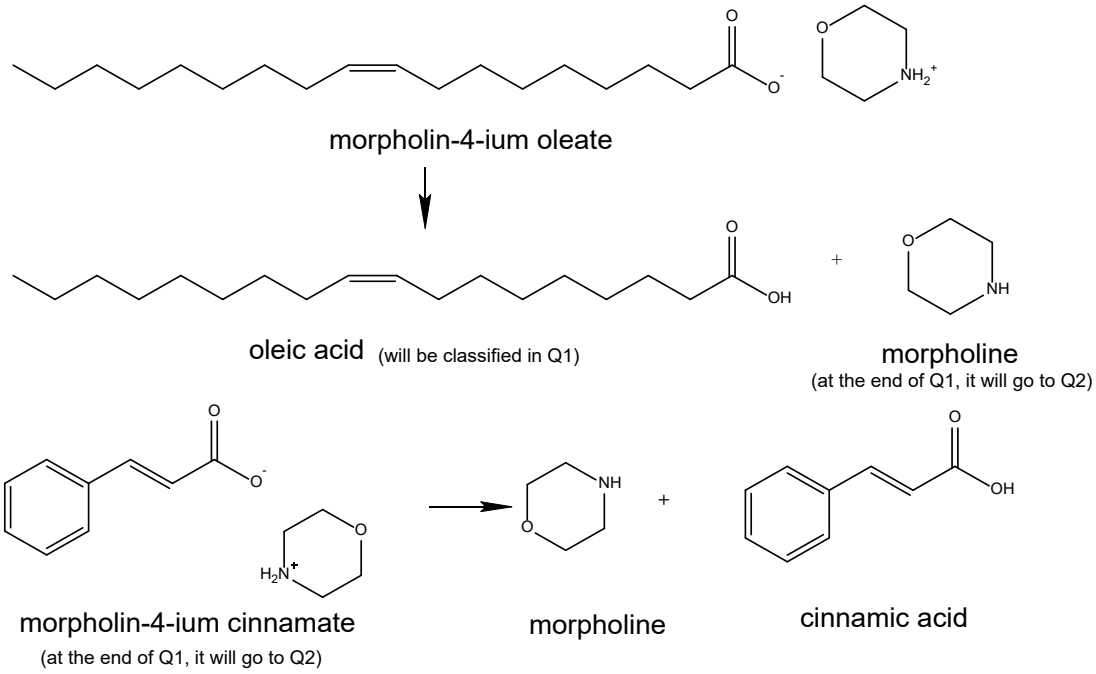


calcium acetate — acetic acid



sodium pentanoate — pentanoic acid

For compounds having other nonmetal counterions, evaluate each counterion in its neutral form in Q1 (e.g., morpholin-4-ium oleate is evaluated as morpholine and oleic acid). Disregard any counterions in subsequent questions that get classified as Class I by Q1 (in our example, oleic acid) and pass along all other counterions (morpholine) to Q2. If none of the counterions in a substance is classified in Q1 (e.g., morpholine and cinnamic acid in the case of morpholin-4-ium cinnamate), pass the substance in its original form (e.g., morpholin-4-ium cinnamate) to Q2.



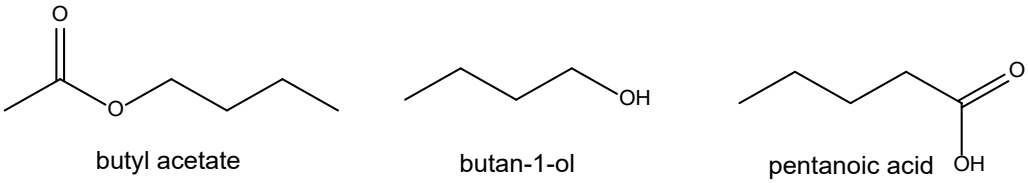
Does the substance belong to one of the structurally defined chemical categories in 1a) through 1k)?

a) A **linear aliphatic** (>1 C) primary alcohol, aldehyde, carboxylic acid, or **corresponding** hemiacetal, acetal, ester, CoA ester, carbonate, or orthoester formed from any of the above alcohols, aldehydes, and carboxylic acids <u>except</u>

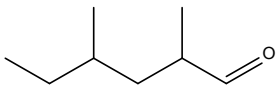
i) **linear** unsubstituted **α,β**-unsaturated aldehydes with <10 Cs or their **corresponding** acetals and hemiacetals (they will be addressed at Q28p, see example structures there, if needed),

ii) methallyl alcohol, allyl alcohol, or crotonyl alcohol and their **corresponding** acids (methacrylic acid, acrylic acid, or crotonic acid), esters, carbonates, orthoesters, acetals, hemiacetals, ketals, or hemiketals (they will be addressed at Q28i, see example structures there, if needed), <u>and</u>

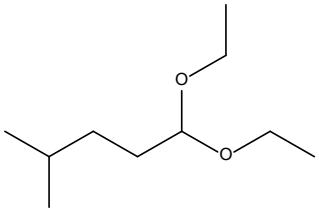
iii) compounds that fit Q1a but have ≥8 continuous **conjugated** double bonds, or



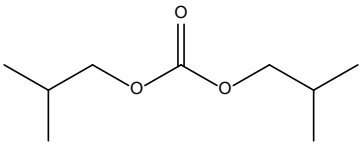
b) **Aliphatic** primary alcohol, aldehyde or carboxylic acid or the **corresponding** hemiacetal, acetal, ester, or CoA ester, carbonate, or orthoester, formed from any of the above alcohols, aldehydes, or carboxylic acids with one or more methyl substituents, <u>except</u> compounds that fit exceptions listed in Q1a ii) and iii), <u>or</u>



2,4-dimethyl-1-hexanal



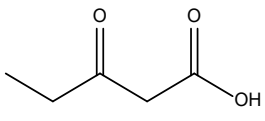
4-methylpentanal diethyl acetal



diisobutyl carbonate

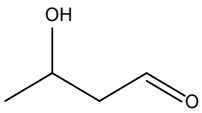
c) **Linear aliphatic** or methyl substituted (≥2 Cs)**,** except substances addressed in Q24b (see Q24b), **i)** hydroxycarboxylic acid, hydroxyester, ketoacid, ketoester, **corresponding** ketal, mono- and di-carboxylic acid, mono- and di-ester, and/or CoA ester, **ii)** substance that contains a single alcohol, ketone or **corresponding** ketal, one or more ester(s), or CoA ester in addition to the primary alcohol, aldehyde, carboxylic acid(s), or ester(s), or **iii)** a tricarboxylic acid or a triester where one of the carboxylic acids or esters is either a substituent on a **linear** carbon chain (a secondary carboxylic acid) or at the end of a side chain of a **simply-branched** compound (primary carboxylic acid), or

i:



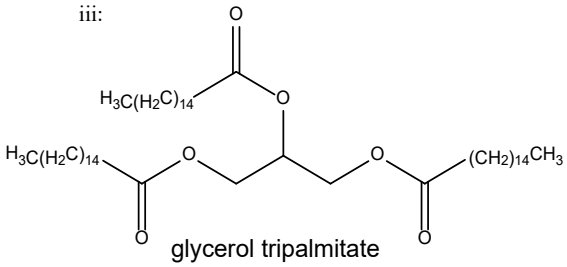
3-ketopentanoic acid

ii:



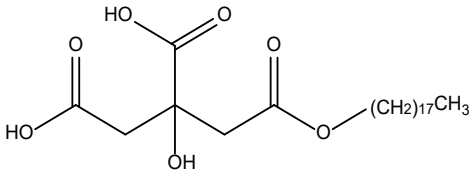
3-hydroxybutanal

iii:



glycerol tripalmitate

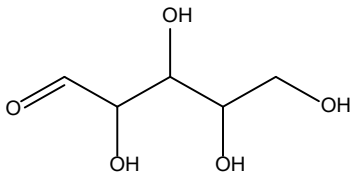
d) Substances in the fatty acid pathway, glycolysis pathway, pentose phosphate pathway, and citric acid cycle (e.g., short-chain fatty acids (C_1 to C_{10}): acetoacetate, 3-hydroxybutyrate, 2-butenoate, carnitine, glyceraldehyde, glycerol, dihydroxyacetone, lactate, malate, malonate, succinate, citrate, isocitrate, pyruvate, oxaloacetate, α-ketoglutarate, glutarate, or gluconate), and their **corresponding** esters formed from alcohols and carboxylic acids specified in 1a), 1b), or 1c), or CoA esters. (Note: To further help identify these intermediates, the reader is referred to Salway (2016).), or



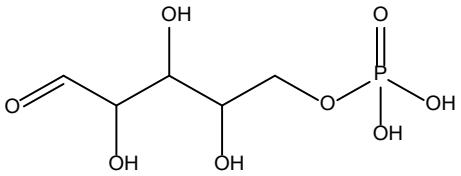
stearyl citrate

e) A monosaccharide (triose, tetrose, pentose, or hexose), a hydrolysable oligosaccharide, or a hydrolysable polysaccharide in addition to simple monosaccharide derivatives. Simple monosaccharide derivatives are **i)** phosphate esters (e.g., triose phosphate, ribose 5-phosphate, and glucose 6-phosphate), **ii)** deoxy sugars (one of the hydroxyl groups in the parent monosaccharide is replaced by an H, e.g., L-fucose (6-deoxy-L-galactose) and L-rhamonose (6-deoxy-L-mannose)), **iii)** amino sugars (one of the hydroxyl groups in the parent monosaccharide is replaced by an amino group with or

without acetylation, e.g., D-glucosamine, D-galactosamine, and D- mannosamine), and **iv)** mono- and poly-methylated, sulfated, and sulfonic acid derivatives of monosaccharides and monosaccharide derivatives (e.g., 3,4-di-O-methyl-alpha-L-rhamnose, 6-O-methyl-D-glucose, glucosamine sulfate, and 6-deoxy-6-sulfo-D-glucopyranose). These substances may also exist as the hemiacetal, acetal, hemiketal, ketal, or ester form, or as acid, or

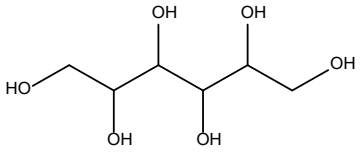


xylose

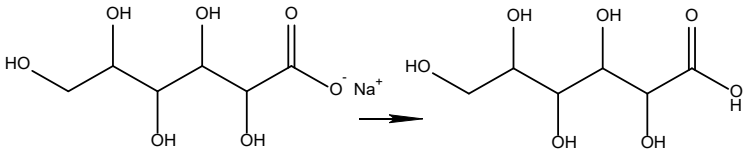


ribose-5-phosphate

f) Sugar alcohols (e.g., glycerol, erythritol, sorbitol, xylitol, galactitol, inositol, or mannitol) or sugar acids or their **corresponding** esters (i.e., monosaccharides with a carboxyl group, such as aldonic acids (e.g., gluconic acid), ulosonic acids (e.g., neuraminic acid), uronic acids (e.g., glucuronic acid), and aldaric acids (e.g., tartaric acid)), in addition to derivatives of sugar alcohols that are both alkoxylated and esterified (e.g., polysorbates), or

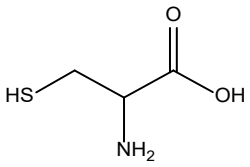


galactitol (sugar alcohol)

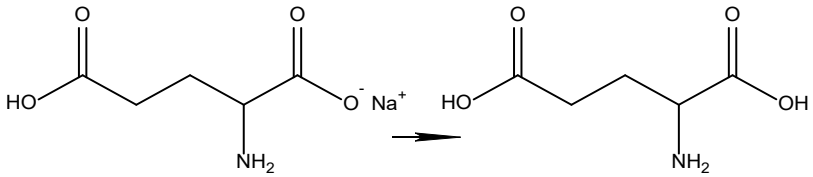


sodium gluconate evaluated as gluconic acid (sugar acid)

g) One of the twenty α-amino acids, **related** CoA esters, and esters formed from **aliphatic** alcohols; N-acetyl derivatives; di- or tri-peptides and/or simple **aliphatic** esters thereof. Intermediates and products in the synthesis of essential amino acids and in the transamination and transsulfuration pathways and degradation of essential and non-essential amino acids. For instance, intermediates in the ornithine cycle (e.g., citrulline, ornithine, and 4-hydroxyphenylpyruvate) and intermediates in the biosynthesis and degradation of non-essential amino acids (e.g., α-ketobutyrate, β-mercaptopyruvate, homocysteine, 3-thiopyruvate, α-ketoadipate, α-methylacetoacetate, 2-anthranilic acid, and α-ketoglutarate). (Note: To further help identify these intermediates, the reader is referred to Salway (2016).), or

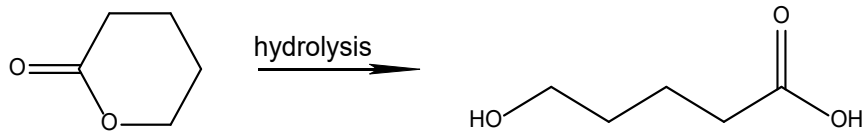


cysteine



sodium glutamate evaluated as glutamic acid

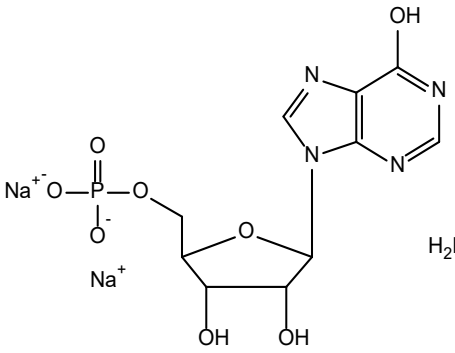
h) Lactones (i.e., monocyclic esters but not an α- or β-lactone) that undergo **hydrolysis** to form **linear aliphatic** or methyl-substituted hydroxycarboxylic acids, or



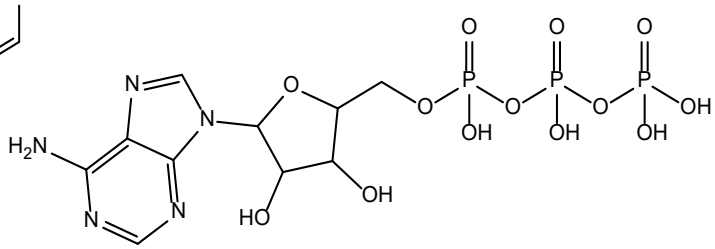
delta-valerolactone

5-hydroxypentanoic acid

i) Nucleotides, nucleosides, phospholipids, monophosphates of amino acids, or their **hydrolysis** products, or

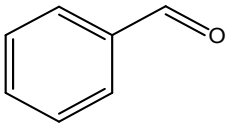


disodium inosinate
(evaluated in the neutral form)



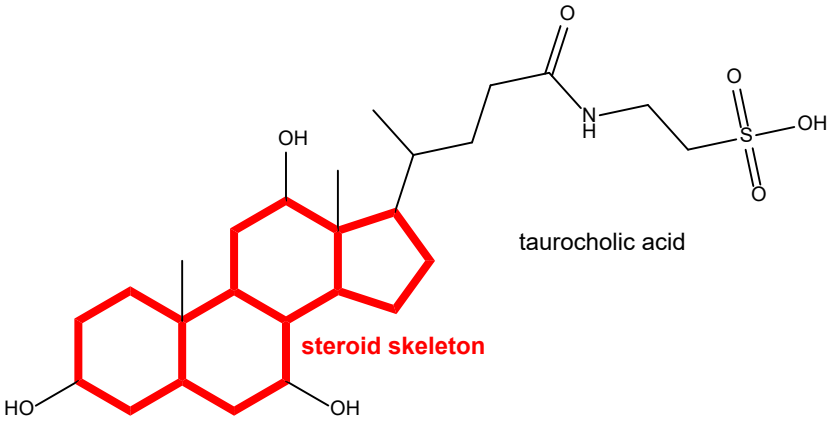
adenosine-5'-triphosphate (ATP)

j) Benzoic acid, its **related** alcohol (benzyl alcohol), aldehyde (benzaldehyde), **corresponding** alkyl acetals, hemiacetals, the CoA ester and **related** alkyl esters formed from benzyl alcohol or benzoic acid (Note: the benzene ring should not contain ring substituents other that those listed above), or



benzaldehyde

k) Bile acids, bile salts, and alkyl ester of bile acids, but no other substances containing a steroid **skeletal structure** (e.g., mineralocorticoids, such as aldosterone and progesterone), as these will be dealt with at Q6.



taurocholic acid

Please note that many structures meet the criteria in more than one sub-question of Q1. However, all structures classified at Q1 are assigned to Class I; therefore, ultimately it does not matter at which sub-question they are captured.

i) If yes to a), b), c), d), e), f), g), h), i), j), or k), assign to Class I.
ii) If no to all, proceed to Q2.

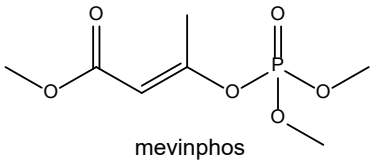
---

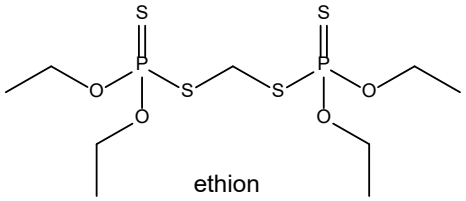
2. In Q2 only, disregard sodium, potassium, calcium, magnesium, barium, aluminum, titanium, zinc, manganese, copper, iron, ammonium, sulfate, fluoride, chloride, or bromide counterions, and evaluate the compound in its neutral form. Does the structure contain

a) covalently bound P (i.e., the P is not simply a phosphate counterion, such as in oseltamivir phosphate or in ethanol, 2,2'-iminobis-, phosphate (salt))

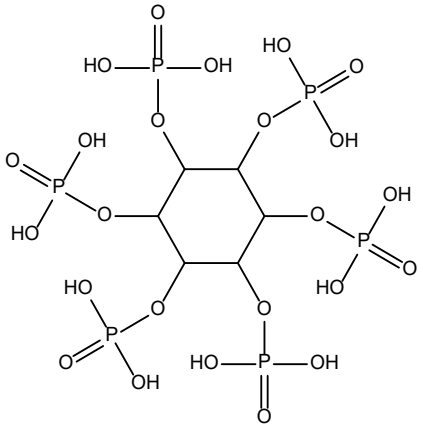
that exists as

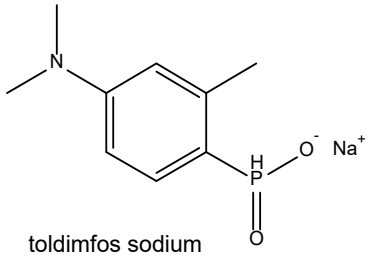
b) $O=PY(XR)_2$, $S=PY(XR)_2$, $S=P(OR)_2$-W-$(OR)_2P=S$ and $O=P(ZR)_2$-W-$(ZR)_2P=O$ where X is C, N, O, or S; W is S, N, O, or SC_nS where $n \leq 4$; Z is N or S, and Y is F-, Cl-, Br-, -S-, CN-, SCN-, OCN-, C=CO- (i.e., good **leaving groups**) and <8 C, or


mevinphos


ethion

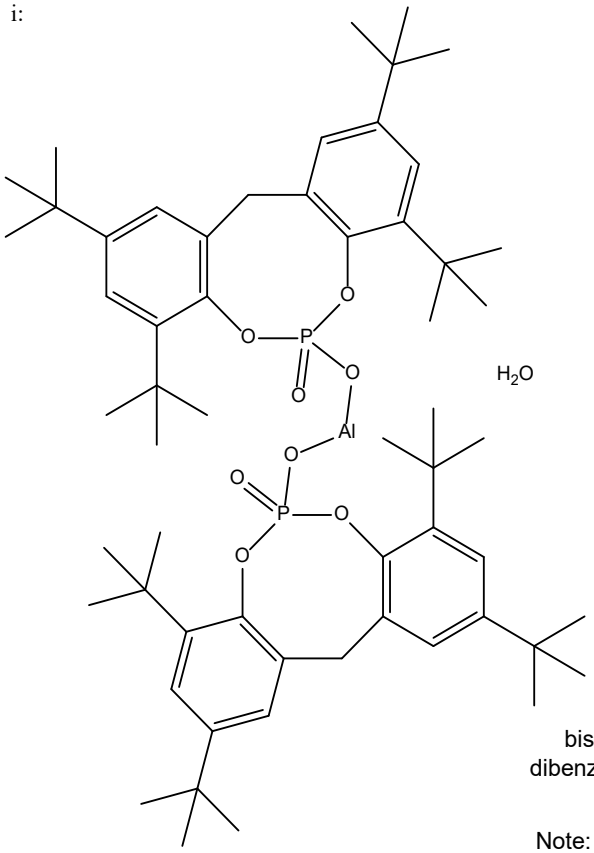
c) $O=P(OR)_3$, $P(OR)_3$, $PH_n(OR)_n$, $O=P(R)_n(OR)_n$ where (n is 1 or 2) and R is H and/or C containing at least one P-OH or their corresponding salts, or


phytic acid


toldimfos sodium

d) **i)** $O=P(OR)_3$ or **dimer** thereof ($O=P(OR)_2O(OR)_2$ P=O) or any other **dimer** that **hydrolyzes** to $O=P(OR)_2OH$ with R is H, alkyl or **aryl** and one of the R groups is ≥8Cs, or **ii)** phosphite ($P(OR)_3$ with only R is alkyl and/or **aryl** (if only alkyl groups are present, one of the alkyl groups must have ≥8Cs) with or without additional **functional groups**?

i:

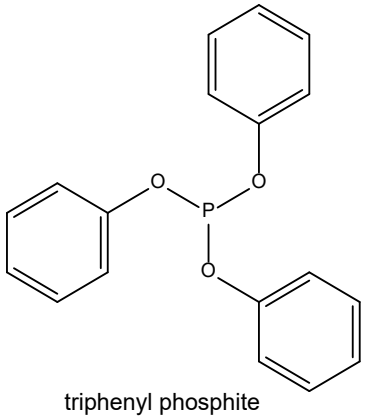


H_2O

bis((2,4,8,10-tetra-*tert*-butyl-6-oxido-12*H*-dibenzo[*d*,*g*][1,3,2]dioxaphosphocin-6-yl)oxy)-l^{2-} alumane hydrate

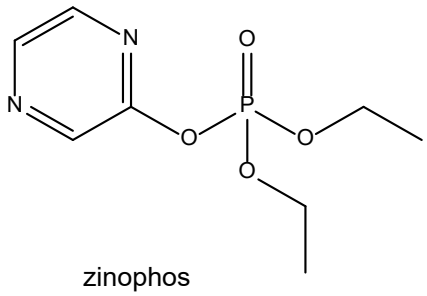
Note: hydrolysis product will satisfy requirement

ii:

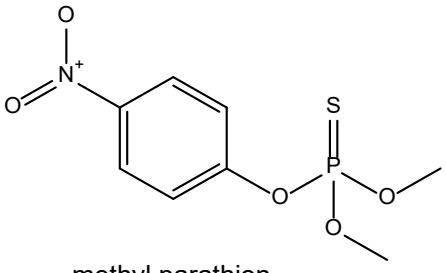


triphenyl phosphite

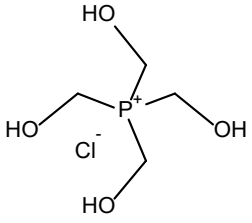
i) If no to a), proceed to Q3.
ii) If yes to a) and b), assign to Class VI.
iii) If yes to a) and either c) or d), assign to Class III, unless the substance also meets the structural criteria in Q6b), c), or d). In that case, proceed to Q6.
iv) If yes to a) but no to b), c), and d), assign to Class V.
Examples for yes to a) but no to b), c), and d):



zinophos



methyl parathion



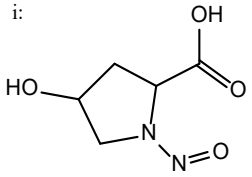
tetrakis(hydroxymethyl) phosphonium chloride

---

3. For Q3 only, disregard sodium, potassium, calcium, magnesium, barium, aluminum, titanium, zinc, manganese, copper, iron, ammonium, sulfate, fluoride, chloride, or bromide counterions, and evaluate the compound in its neutral form. Does the substance contain any of the following **functional groups** or reactive moieties?

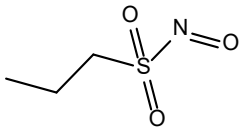
a) **i)** N-N=O (N-nitroso), N=O (nitroso), or N-OH (N-hydroxy), and the N is <u>not</u> part of a single sulfonamide function (N-SO_2-), **ii)** N-N=O (N-nitroso) or N=O (nitroso), and the N is a part of a single sulfonamide function (N-SO_2-), <u>or</u> **iii)** a C≡N (nitrile) with an amine or alcohol, **corresponding** ester or alkyl ether bonded to the alpha C, <u>or</u>

i:



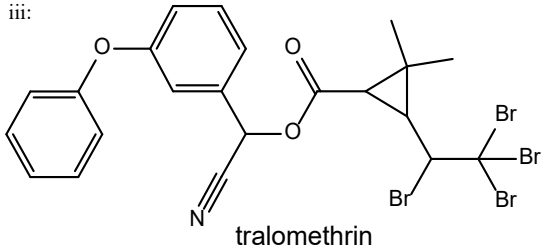
4-hydroxy-1-nitrosopyrrolidine-2-carboxylic acid
(nitrosohydroxyproline)

ii:



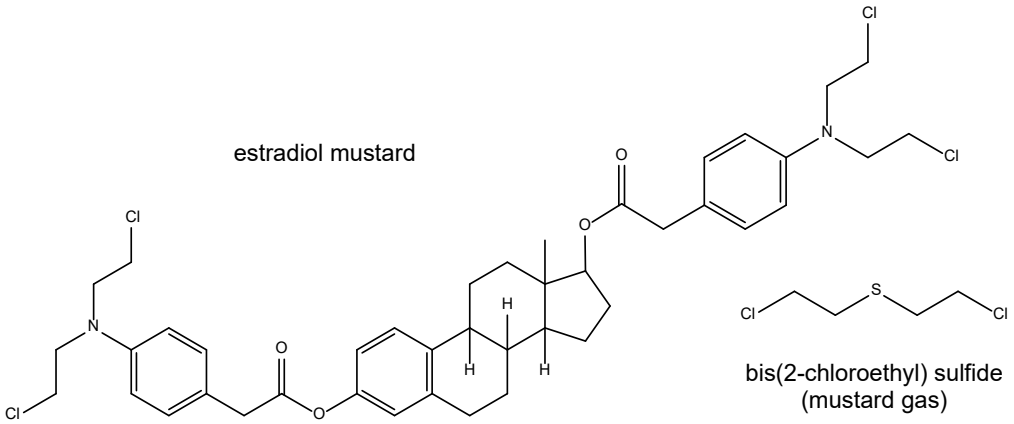
1-(nitrososulfonyl)propane

iii:



tralomethrin

b) one or more **aliphatic** chains of either $(XCC)_2Z$- or (XCC)Z- with Z is N (N not quaternary) or S and X is Cl and/or Br, <u>or</u>

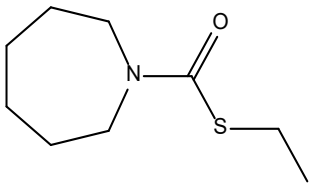


estradiol mustard

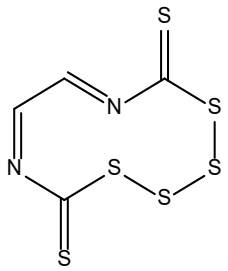
bis(2-chloroethyl) sulfide
(mustard gas)

c) **i)** thiocarbamate (both O-**organyl** ($ROC(=S)NR_2$ and $ROC(=S)N=CR_2$) and S-**organyl** thiocarbamates ($RSC(=O)NR_2$ and $RSC(=O)N=CR_2$)) or dithiocarbamates ($RSC(=S)NR_2$ and $RSC(=S)N=CR_2$) where R is H, C, N, or S, (but not part of a **heteroaromatic** ring) <u>and</u> the N is <u>not</u> part of a single sulfonamide function (N-SO_2-), <u>or</u> **ii)** thiocarbamate or dithiocarbamate where R is H, C, N, or S, (but these cannot be a part of a **heterocyclic** ring itself) <u>and</u> the N is a part of a single sulfonamide function (N-SO_2-), <u>or</u>

i:

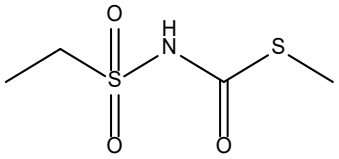


molinate



(6*Z*,8*E*)-1,2,3,4,6,9-tetrathiadiazecine-5,10-dithione

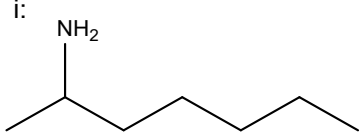
ii:



S-methyl (ethylsulfonyl)carbamothioate

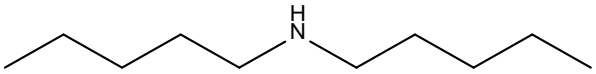
d) **i)** an **α**-methyl- or **α**-ethyl-substituted primary **linear aliphatic** amine, or its salt, or **ii)** an **aliphatic** secondary amine or its salt without any other **functional groups** except another primary or secondary amine, or

i:



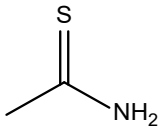
2-aminoheptane

ii:

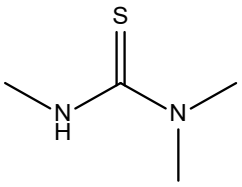


dipentylamine

e) thioamide or thiourea, or



thioacetamide



1,1,3-trimethylthiourea

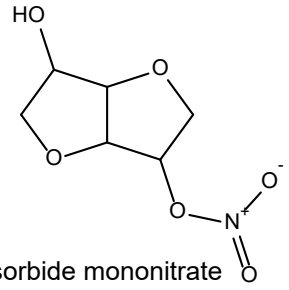
f) **i)** nitrate esters ($RONO_2$) with one or more nitrates, or

ii) a single quaternary N^+, except in any of the following forms (R is C or H):

A. iminium ion ($R_2C=N^+R_2$)
B. hydrochloride, hydrobromide, or sulfate salt of a simple **aliphatic** primary or tertiary amine
C. nitrobenzene derivatives (due to the significant toxicity data available for these substances, they are considered at Q43 and Q44)
D. choline ($(CH_3)_3\ N^+CH_2CH_2OR$) derivative
E. brominated or chlorinated compounds with Ar-N=N^+(O^-)-Ar **skeletal structure** (Ar: **aromatic** ring)
F. The positively charged N in diazo ($R_2C=N^+=N^-$ or R_2C^--$N^+\equiv N$) and azide (-N^--$N^+\equiv N$ or RN=N^+=N^-) as they are not considered quaternary, or

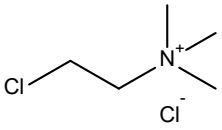
iii) at least two quaternary N^+, except in any of the above (A to F) forms, or

i:



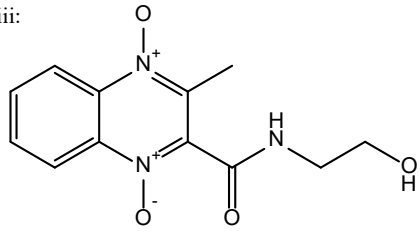
isosorbide mononitrate

ii:



(2-chloroethyl)trimethyl-ammonium chloride

iii:



olaquindox

g) **i)** a single sulfonyl carbamate ($RS(=O)_2NC(=O)OR$), sulfonyl carbohydrazide (R-$C(=O)NRNS(=O)_2R$), sulfonyl guanidine ($RS(=O)_2NC(=NR)NR_2$), or sulfonyl isocyanate ($RS(=O)_2N=C=O$), or,
ii) diazo ($R_2C=N^+=N^-$ or R_2C^--$N^+\equiv N$) (two linked nitrogen atoms (azo) at the terminal position), but not azo (RN=NR), triazeno ($RN=N-NR_2$), azide (-N^--$N^+\equiv N$ or RN=N^+=N^-),

hydrazine (R_2N-NR_2), hydrazide (-C(=O)NR-NR_2), hydrazone (R_2C=N-NR_2), guanidine (R_2NC(=NR)NR_2), amidine (R-C(=NR)NR_2) (only one amidine), oxime (R_2C=N-OH) or the **corresponding** ether (R_2C=N-OR) or the oxime as a product of the **hydrolysis** of the **corresponding** ester or lactone (R_2C=N-O-C(=O)R), carbamate (R_2NC(=O)OR) (but not oxime carbamate (R_2NC(=O)ONR_2 or R_2NC(=O)ON=R)), or isocyanate (RN=C=O) where R is C, H, N and/or S. Except for guanidine, amidine, and oxime or its precursors, none of the other **functional groups** may be part of a ring system (i.e., no atom from the **functional groups** can be a part of a ring), or

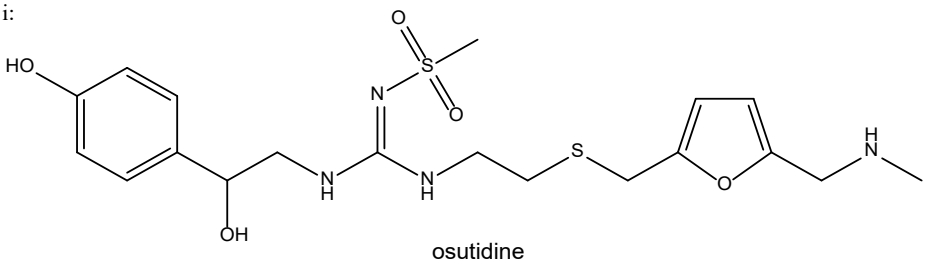
iii) a single nitrile (R-C≡N), or

iv) at least two nitriles or amidines, or

v) at least one oxime carbamate (R_2NC(=O)ONR_2 or R_2NC(=O)ON=R), or

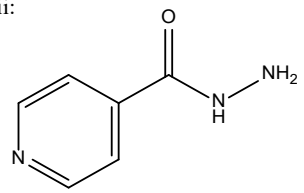
vi) one or more cyanamide(s) (R-N-C≡N), or

i:



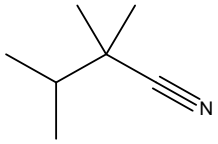
osutidine

ii:



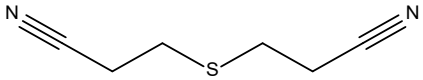
pyridine-4-carbohydrazide

iii:



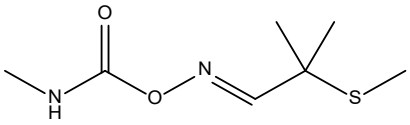
2,2,3-trimethylbutanenitrile

iv:



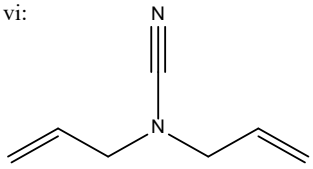
3,3'-thiobispropanenitrile

v:



aldicarb

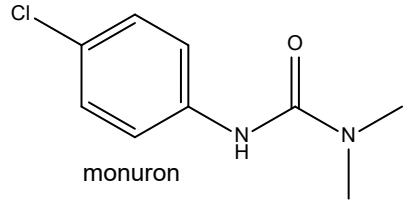
vi:



diallyl cyanamide

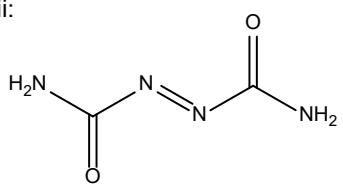
h) an isothiocyanate (S=C=NR) or ureide (RN(C=O)NR_2) where N is not bonded to an additional **oxygenated functional group** (i.e., no **oxygenated functional group** attached to the **α** carbon) and the isothiocyanate and the ureide are not part of a ring system and **i)** the substance contains one or more **aromatic** ring(s) with at least one halogen substituent or the substance contains at least one **heteroaromatic**ring or **ii)** the substance contains neither a halogen substituted **aromatic** ring nor a **heteroaromatic** ring?

i:



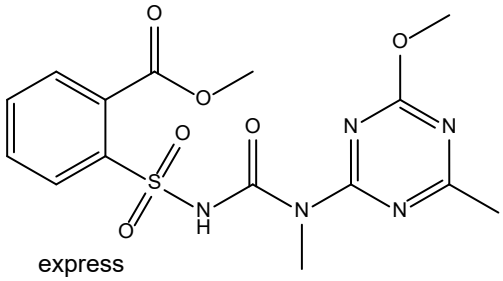
monuron

ii:



azodicarbonamide

i:



express

Note: SO_2 is not included in the definition of oxygenated functional group.

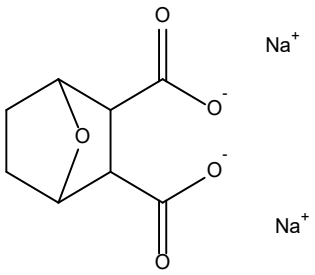
Run the substance through <u>all</u> sub-questions (a through h). Do not stop at the first yes to a sub-question. This is done to ensure that the substance gets classified based on its most reactive moiety (i.e., if the answer is yes at multiple sub-questions, assign the substance to a class at the sub-question with the highest class).

i) If yes to a(i)), a(iii)), b), c(i)), fi), f(iii)), g(iv)), or g(v)), assign to Class V.
ii) If yes to d(i)), d(ii)), e), f(ii)), g(ii)), g(iii)), g(vi), or h(i)) assign to Class IV.
iii) If yes to a(ii)), c(ii)), g(i)), or h(ii)), <u>and</u> no to all other sub-questions in Q3, assign to Class III.
iv) If no to all, proceed to Q4.

---

4. Does the structure contain elements <u>other than</u> C, H, O, N (only as trivalent N or tetravalent N^+), S (divalent (sulfide, (-S-)), tetravalent (sulfoxide (-S(=O)-)), or hexavalent S (only as sulfone (-$S(=O)_2$-), sulfamate ($ROS(=O)_2NR_2$ or $^-OS(=O)_2NR_2$), sulfonate (-$S(=O)_2O^-$ or -$S(=O)_2OR$), sulfate ($^-OS(=O)_2OR$ or $ROS(=O)_2OR$), or sulfonamide ($RS(=O)_2NR_2$)), or covalently bound F, Cl, Br, or I (Note: R is H or C)?

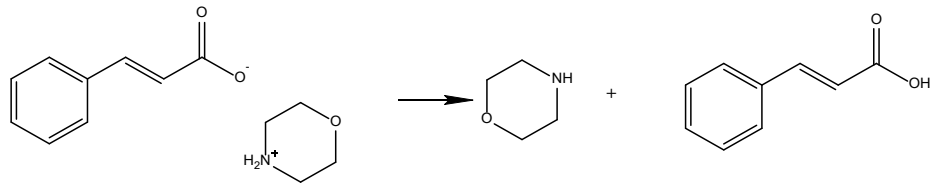
i) If yes, proceed to Q5.



disodium endothall

ii) If no, all salts that only contain C, O, H, N, or S (e.g., morpholine-4-ide, mesylate, esylate, or tosylate) should be evaluated considering neutral forms of the counterions going forward and each neutral form should be passed on to Q6 for further evaluation (e.g., the cinnamate salt of morpholine (morpholin-4-ium cinnamate) should be

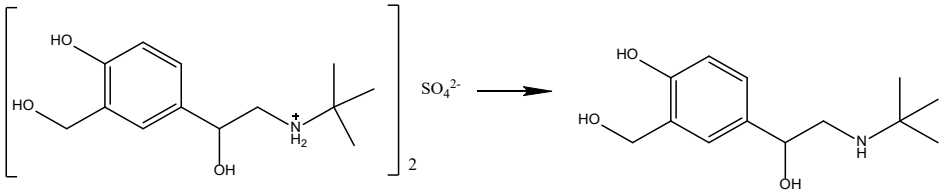
evaluated as morpholine and cinnamic acid at Q6.) As counterions, sulfate, sulfite, bisulfite, and sulfamate in their neutral forms are their corresponding acids and as such are mineral acids not intended to be evaluated by the EDT (i.e., disregard these). If the compound is already in its neutral form, pass it along to Q6.



morpholin-4-ium cinnamate

morpholine
evaluate at Q6

cinnamic acid
evaluate at Q6



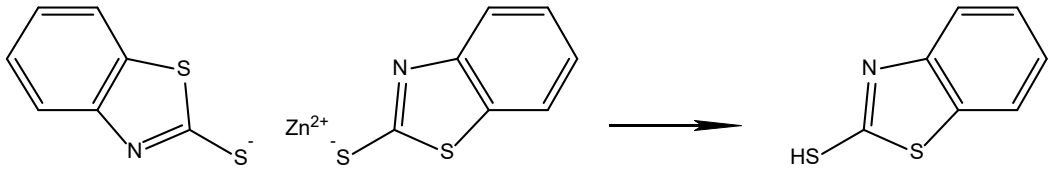
albuterol sulfate

evaluate at Q6

---

5. Do elements not listed in Q4 occur **only** as

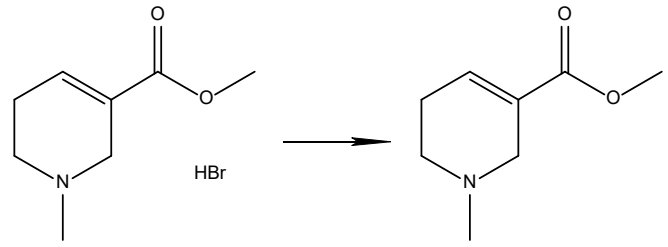
a) a sodium, potassium, calcium, magnesium, barium, aluminum, titanium, zinc, manganese, copper, or iron counterion, or



2(3H)-benzothiazolethione, zinc salt

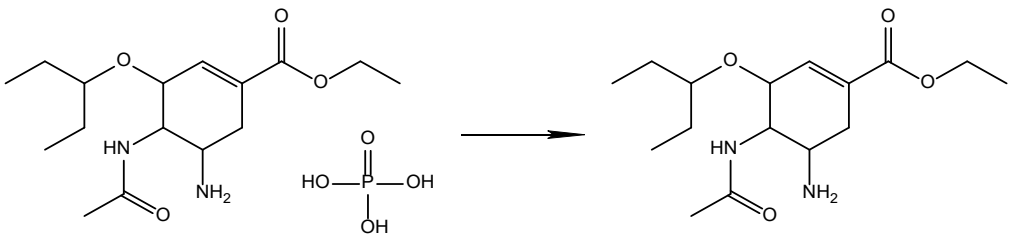
benzo[*d*]thiazole-2-thiol

b) a chloride, bromide, or fluoride counterion, or



arecoline hydrobromide

c) a phosphate counterion, or

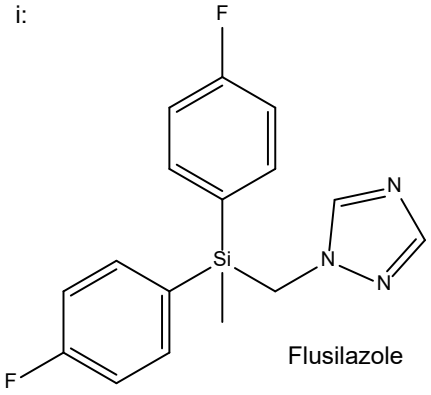


oseltamivir phosphate

d) **i)** a covalently bound silicone (Si) (more than one Si may be present) and the compound has at least one halogen and/or at least one **heterocyclic** ring, <u>or</u>

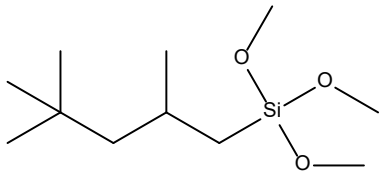
ii) a covalently bound silicone (Si) (more than one Si may be present) <u>and</u> the compound contains neither halogen(s) nor **heterocyclic** ring(s)?

i:



Flusilazole

ii:



trimethoxy(2,4,4-trimethylpentyl)silane

i) If yes to a), b), and/or c), <u>and</u> the compound has no covalently bound Si, disregard the above-listed counterions, treat the compound as the neutral substance, and proceed to Q6.

ii) If yes to a), b), and/or c), <u>and</u> the compound has at least one covalently bound Si, disregard the above-listed counterions, treat the substance as the neutral substance, and evaluate the compound at Q5d. If yes to Q5d(i)), proceed to Q6. If yes to Q5d(ii)), assign to Class II.

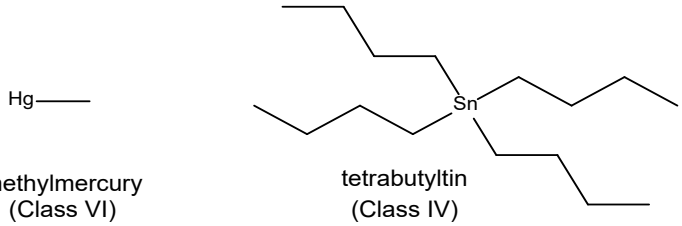
iii) If no to a), b), and c), but yes to d(i)), proceed to Q6.

iv) If no to a), b), and c), but yes to d(ii)), assign to Class II.

v) If no to a), b), c), and d), <u>and</u> the substance contains Hg, Tl, Pb, Os, Po, a Lanthanide, an Actinide, or an element in the 7th period from Group 4 to Group 18, assign to Class VI.

vi) If no to a), b), c), and d), <u>and</u> the substance contains As, Be, Cd, or Cr(VI), assign to Class V.

vii) In all other cases when the answer is no to a), b), c), and d), assign to Class IV. Examples for no:



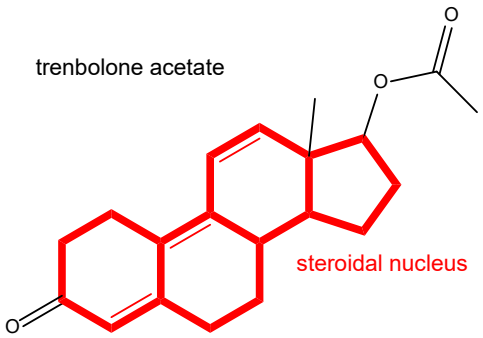
methylmercury
(Class VI)

tetrabutyltin
(Class IV)

---

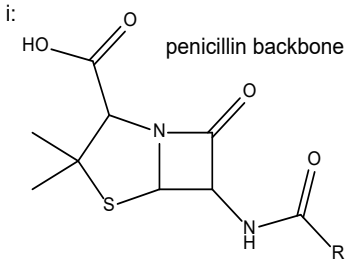
6. Does the substance contain only the elements C, H, O, N, S, P, Br, or Cl and exhibit any of the following structural features?

a) a steroidal nucleus with or without additional rings or substituents (note that bile acids are dealt with in Q1), or

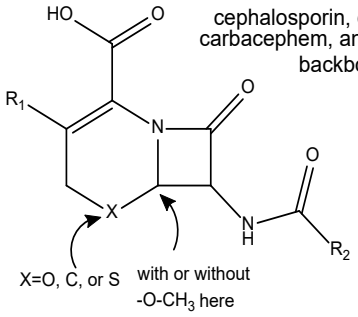


b) an amine or amide N located at the fusion point of two or more ring systems and **i)** the substance has a penicillin, cephalosporin, cephamycin, carbapenem, penem, carbacephem, or oxacephem **skeleton** or **ii)** the compound does not have any of the **skeletal structures** listed in b(i)), or

i:

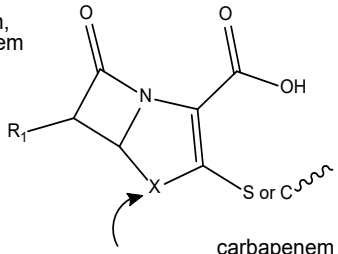


Note: R can be H, C, and/or other elements



X=O, C, or S

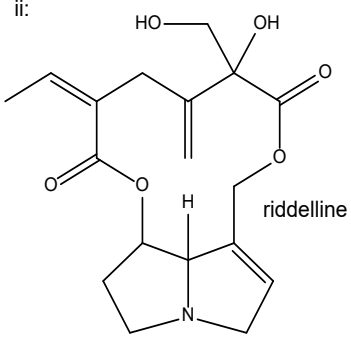
with or without $-O-CH_3$ here



X=C or S
C may be substituted

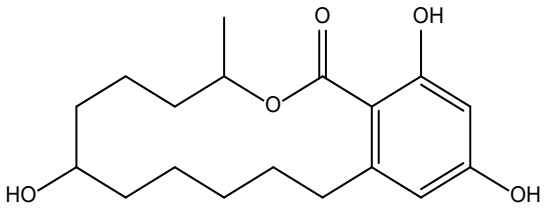
carbapenem and penem backbone

ii:



riddelline

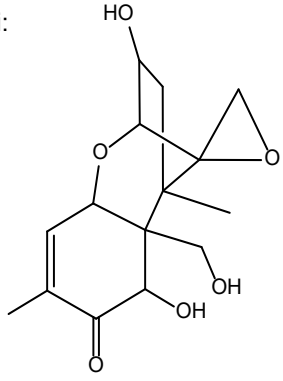
c) a **macrocyclic** ring (either **alicyclic** or **heterocyclic** (only O and/or N may be present as a heteroatom)) of ≥11 atoms, **fused**, **spiro-fused, singly bonded**, or connected by an -O- to one or more additional ring systems (additional to the above **macrocyclic** ring) with ≥2 **oxygenated functional groups** and/or one or more lactone, or



zearalanol

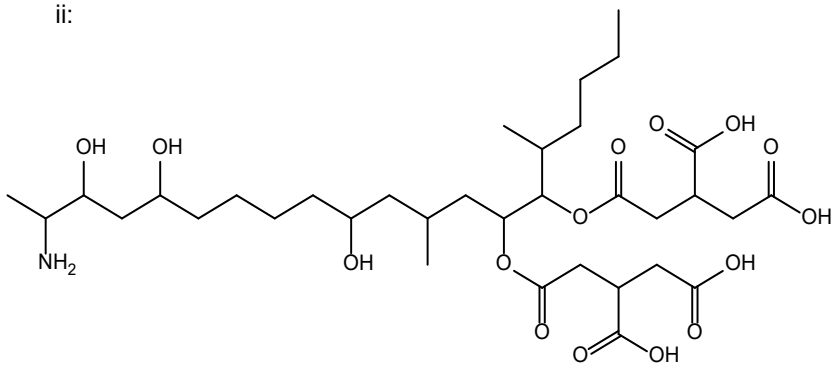
d) at least **i)** four **fused** and/or **spiro-fused** and/or **bridged alicyclic**, **heterocyclic**, **aromatic** or **heteroaromatic** rings at least one of which is an epoxide, tetrahydrofuran, dihydrofuran, furan, pyrrole, dihydropyrrole, pyrrolidine, quinone, or semiquinone or **ii)** a **linear, simply branched,** or **branched chain** of ≥20 Cs, containing at least six **electron pair donors** (except brominated triglycerides) or two lactone rings as substituents with or without additional **electron pair donors**, or

i:



deoxynivalenol

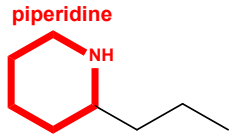
ii:



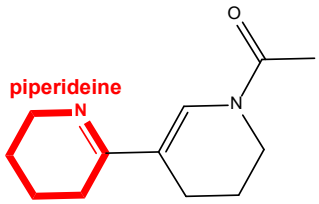
fumonisin B1

e) **i)** a piperidine or 1-piperideine ring substituted at the 2-position by a hydrocarbon chain of ≥3 Cs, a 3-pyridyl ring, or a 3-(N-acetyl-2-piperideinyl) ring or **ii)** a N-methylpyrrolidine ring substituted at the 2-position by a 3-pyridyl ring, or

i:

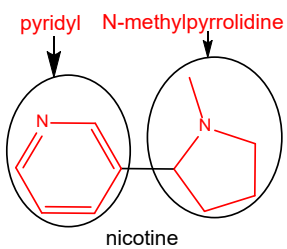


2-propylpiperidine



N-acetylhystrine

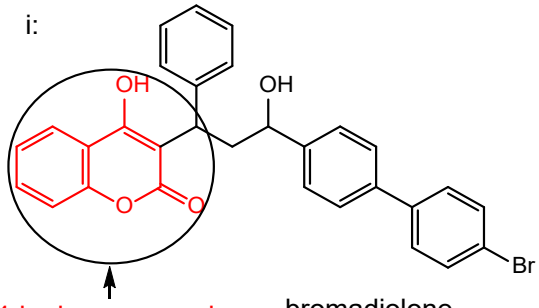
ii:



nicotine

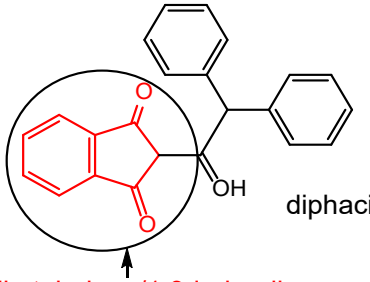
f) **i)** a 4-hydroxycoumarin ring system substituted at the 3-position either by an alkyl chain (the chain can be a part of an **alicyclic** ring) containing 1-phenyl or 1-phenyl-3-keto (or hydroxy) substituent or **ii)** a 1,3-diketoindane or 1-keto-3-hydroxyindene containing a 2-phenyl-1-keto substituent at the 2-position, or

i:



4-hydroxycoumarin bromadiolone

ii:

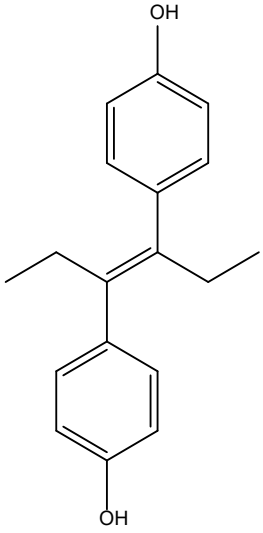


diphacinone

1,3-diketoindane/1,3-indandione

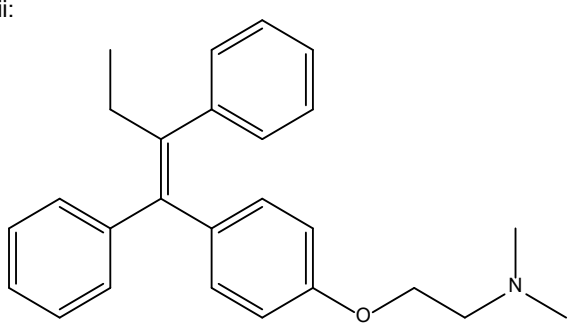
g) **i)** two benzene rings connected by a 2- or 3-carbon chain (**connector**, with or without unsaturation) and a hydroxy, **corresponding** ester, methoxy, and/or ether in the para position on each ring with or without methyl, ethyl, and/or ethylidene substitution on one or more **connector** carbons (not more than one per carbon). One or more halogen(s) are allowed anywhere on the molecule along with methyl group(s) in the meta position on the benzene ring(s) or **ii)** two benzene rings connected by a -C=C- and one **connector** carbon is substituted by a benzene ring (a total of three benzene rings) and the other **connector** carbon is either unsubstituted or substituted by a methyl or ethyl group or a halogen. Any or all of the benzene rings may be substituted by a hydroxy, **corresponding** ester, methoxy, and/or ether in the para position, but this is not required, or

i:



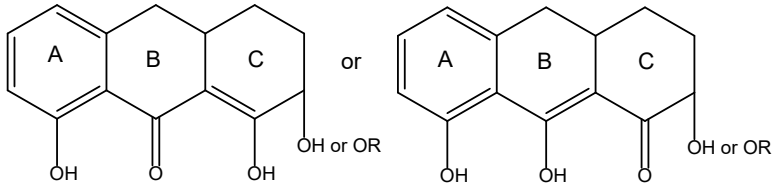
diethylstilbestrol

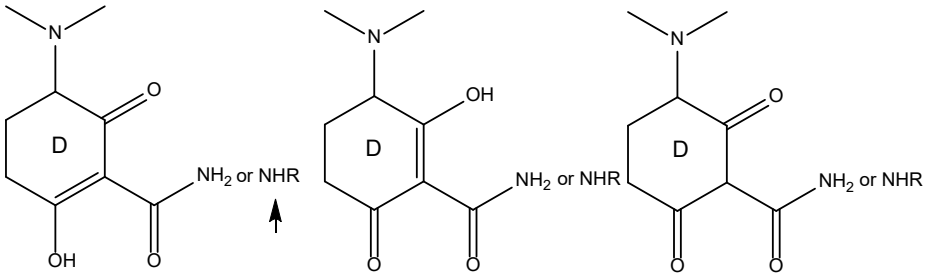
ii:



tamoxifen

h) a tetracycline **skeletal structure** consisting of four (A, B, C, and D) fused rings (D is fused to C) where rings A, B, C, and D are depicted below (note: rings A, B, and C can have additional substituents)?





i) If yes to a), b(ii)), c), d), e), or g), assign to Class V.
ii) If yes to b(i)) and the compound has a penicillin **skeletal structure**, assign to Class III. For **skeletons** other than a penicillin **skeleton**, assign to Class IV.
iii) If yes to f), assign to Class VI.
v) If yes to h), assign to Class III. vi)If no to all, proceed to Q7

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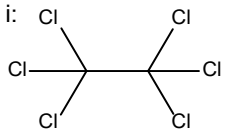
7. Is the substance

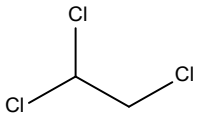
a) a compound in which carbon is covalently bonded to one or more of the following elements: Cl, Br, F, and/or I

and

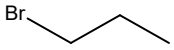
b) a saturated **acyclic** or **alicyclic** hydrocarbon with **i)** fully saturated with F, Cl, and/or Br, **ii)** a **vicinal** halide of any combination of Cl and/or Br, **iii)** ≤2 F, Cl, Br, or CF_3 (**CF_3 is 1 halogen**) in any combination except the **vicinal** position, or **iv)** ≥3 F, Cl, Br, and/or CF_3 in any combination, or

i: 

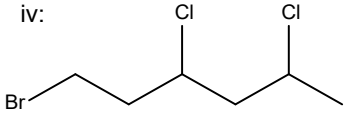
perchloroethane

ii: 

1,1,2-trichloroethane

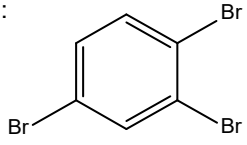
iii: 

1-bromopropane

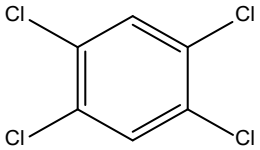
iv: 

1-bromo-3,5-dichlorohexane

c) a benzene ring substituted only by any combination of **i)** ≤3 or **ii)** ≥ 4 F, Cl, Br, and/or CF_3 (CF_3 is 1 halogen) in any arrangement without any additional substituents, or

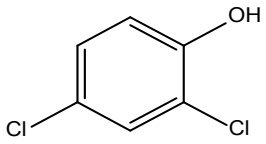
i: 

1,2,4-tribromobenzene

ii: 

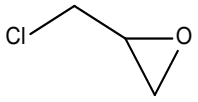
1,2,4,5-tetrachlorobenzene

d) a benzene ring substituted by ≥1 Cl and/or Br in any combination, one of which must be ***ortho*** or ***para*** to an O substituent (with O directly bonded to the benzene ring), or



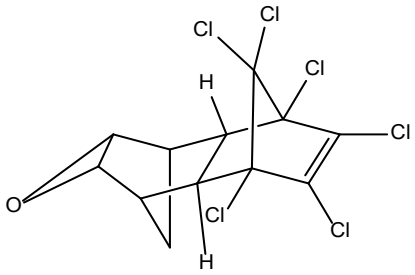
2,4-dichlorophenol

e) one or more Cl and/or Br bonded to an epoxide ring or as the only substituent(s) of an epoxide carbon side chain of ≤2 Cs, or

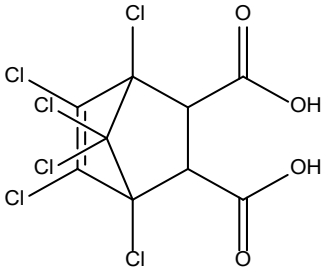


2-(chloromethyl)oxirane

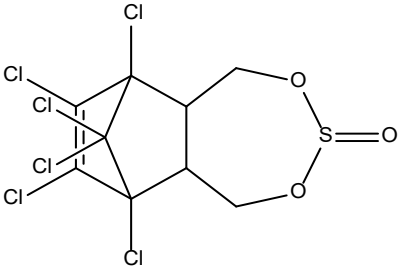
f) a mono- or poly-**alicyclic** ring system (**fused**, **spiro-fused**, or **bridged**) with ≥5 ring carbons and with ≥6 Cl and/or Br with or without additional **oxygenated functional groups** and/or a maximum of one (nonaromatic) **heterocyclic** ring (only S and/or O as ring heteroatoms may be present), or



dieldrin



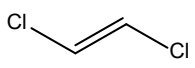
chlorendic acid



endosulfan

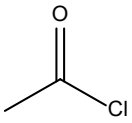
g) **i)** ≥1Cl and/or Br bonded directly to the double bonded carbon(s) of an alkene, or
ii) an **aliphatic** acyl halide (F, Cl, and/or Br), or
iii) a halogen (F, Cl, Br, and/or I) on a carbon adjacent to a carbon bearing an **aliphatic** primary or secondary alcohol oxygen or **corresponding** ether oxygen, or
iv) a halogen on a carbon bearing an ether oxygen (must be **aliphatic**), or
v) at least one halogen (F, Cl, Br, and/or I) at the alpha position of an aldehyde, ketone, carboxylic acid, ester or amide?

i:



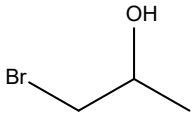
(*E*)-1,2-dichloroethene

ii:



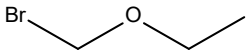
acetyl chloride

iii:



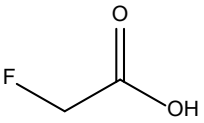
1-bromopropan-2-ol

iv:

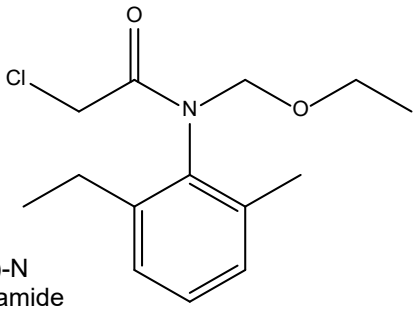


(bromomethoxy)ethane

v:



2-fluoroacetic acid



2-chloro-N-(ethoxymethyl)-N
(2-ethyl-6-methylphenyl)acetamide

Run the substance through <u>all</u> sub-questions (a through g). Do not stop at the first yes to a sub-question. This is done to ensure that the substance gets classified based on its most reactive moiety (i.e., if the answer is yes at multiple sub-questions, assign the substance to a class at the sub-question with the highest class).

i) If yes to a) <u>and</u> b(ii)), c(ii)), e), or f), assign to Class V.
ii) If yes to a) <u>and</u> b(i)), d), or g(i, ii, iii, iv, or v)), assign to Class IV.
iii) If yes to a) <u>and</u> b(iv)) or c(i)), assign to Class III.
iv) If yes to a) <u>and</u> b(iii)), assign to Class II.
v) If yes to a), <u>but no to</u> b), c), d), e), f), and g), proceed to Q8.
vi) If no to a), proceed to Q9.

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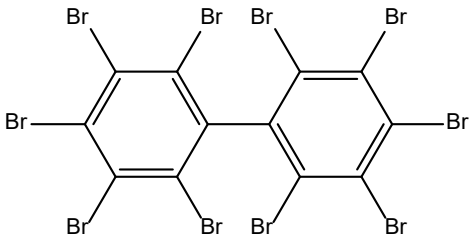
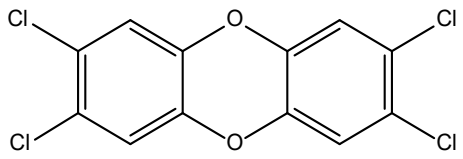
8. Is the halogenated substance

a) a dibenzodioxin, dibenzofuran, biphenyl, diphenyl ether, diphenylthio ether, or naphthalene **skeleton** fully substituted with only Cl and/or Br, <u>or</u>



perbromo-1,1'-biphenyl

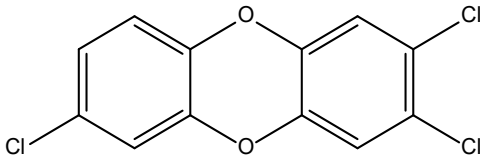
b) a dibenzodioxin, dibenzofuran, or naphthalene substituted with only Cl and/or Br in all positions that are ***para*** to the ring fusion points, and no more than 2 Cl and/or Br ***ortho*** to ring fusion points, <u>or</u>



2,3,7,8-tetrachlorodibenzo[*b*,*e*][1,4]dioxin

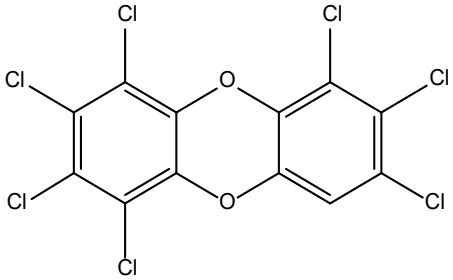
c) a dibenzodioxin, dibenzofuran, or naphthalene substituted with only Cl and/or Br **i)** at three of the four ***para*** positions or **ii)** at all (4) ***para*** positions and three ***ortho*** positions, or

i:



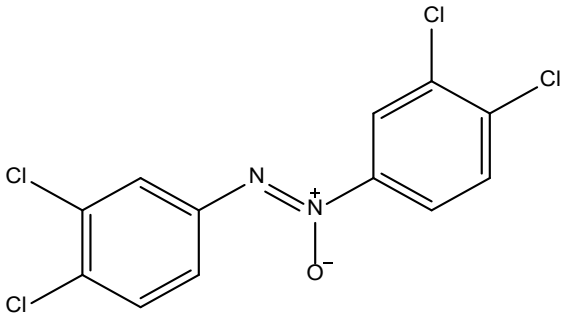
2,3,7-trichlorodibenzo[b,e][1,4]dioxin

ii:



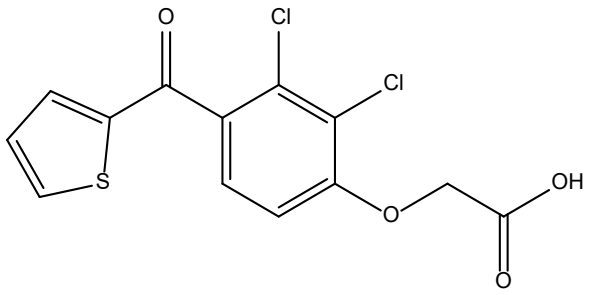
1,2,3,4,6,7,8-heptachlorodibenzo[*b*,*e*][1,4]dioxin

d) biphenyl, diphenylether, diphenylthioether, or azobenzene (Ar-N=N-Ar) and its N-oxide (Ar-N=N^{+}(O^{-})-Ar) only substituted with 3, 4, 5, or 6 Cl located only at ***meta*** or ***para*** positions or 3, 4, 5, 6, 7, or 8 Br atoms at any position or a biphenyl substituted with 4, 5, 6, or 7 Cl with at least one Cl located at the ***ortho***, ***meta***, and ***para*** positions each (does not have to be on the same ring), and each ring must be substituted by at least one Cl (i.e., no unsubstituted ring)?



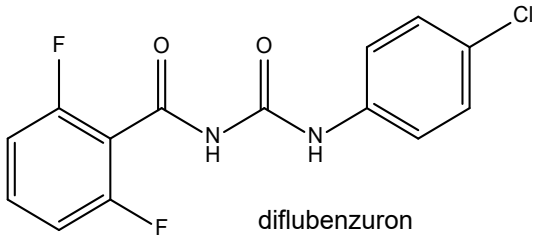
(*Z*)-1,2-bis(3,4-dichlorophenyl)diazene 1-oxide

i) If yes to a), assign to Class III.
ii) If yes to b), assign to Class VI.
iii) If yes to c) or d), assign to Class V.
iv) If no to a), b), c), and d), and the compound is **heterocyclic**, proceed to Q11.
Example:



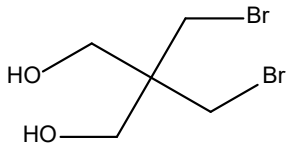
2-[2,3-dichloro-4-(thiophene-2-carbonyl)phenoxy]acetic acid

v) If no to a), b), c), and d), and the compound is **aromatic**, proceed to Q33.
Example:



diflubenzuron

vi) If no to a), b), c), and d), and the compound is neither **heterocyclic** nor **aromatic**, assign to Class IV.
Example:



dibromoneopentyl glycol

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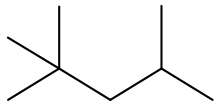
9. Is the substance a **linear** or **simply branched-chain aliphatic acyclic** hydrocarbon, except hexane and substances with a terminal double bond that is further **conjugated** with another double bond (i.e., terminal dienes)?

i) If yes, assign to Class I.



2,2,4-trimethylpentane

ii) If no, proceed to Q10.

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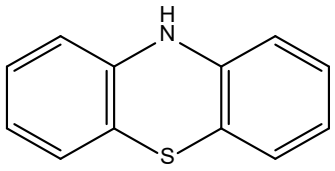
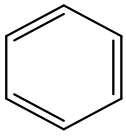
10. Is the substance **heterocyclic**?

i) If yes, proceed to Q11.



phenothiazine

ii) If no, proceed to Q23.



benzene

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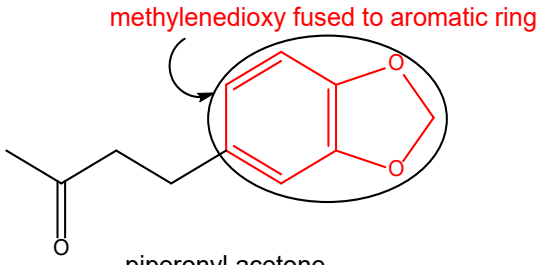
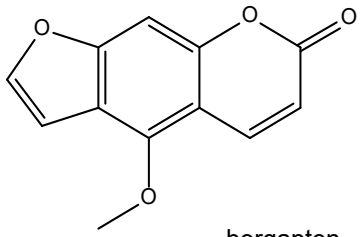
11. Does the substance contain one or more of the following: ester (but not cyclic diester and lactone; these **functional groups** together with lactams are dealt with in Q12), thioester, hemiacetal, acetal (other than cyclic methylenedioxy **fused** to an **aromatic** ring), hemiketal, ketal, sulfate, mono- or poly-glycoside (i.e., glycone), carbonate, anhydride and/or polysulfide?

i) If no to Q11 and the compound is a cyclic methylenedioxy **fused** to an **aromatic** ring, proceed to Q33. For all other compounds, if no to Q11, proceed to Q12.

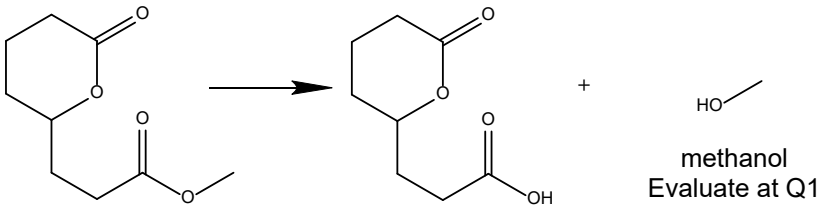


piperonyl acetone
Proceed to Q33



bergapten
Proceed to Q12

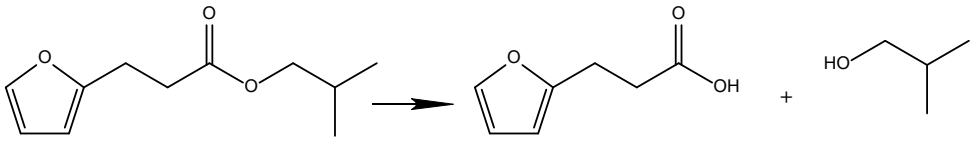
ii) If yes to Q11, and the compound is a lactone, lactam, or cyclic diester, **hydrolyze** the **functional groups** listed in the question, but do not **hydrolyze** the lactone, lactam, and cyclic diester moieties. After **hydrolysis**, send the lactone, lactam, and cyclic diester to Q12 and all other **hydrolysis** products to Q1. If yes to Q11 and the compound is not a lactone, lactam, or cyclic diester, assume the **heterocyclic** substance is **hydrolyzed** or **reduced** (exclusively for sulfide linkages), and evaluate any **heterocyclic** products at Q13 and all other product(s) at Q1.



methyl 3-(6-oxotetrahydro-2*H*-pyran-2-yl)propanoate

3-(6-oxotetrahydro-2*H*-pyran-2-yl)propanoic acid
Evaluate lactone at Q12

methanol
Evaluate at Q1



isobutyl furylpropionate

3-(furan-2-yl)propanoic acid
Evaluate at Q13

2-methylpropan-1-ol
Evaluate at Q1

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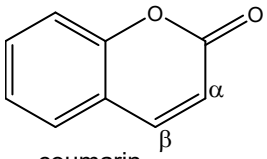
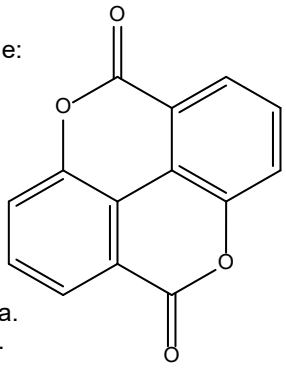
12. Is the **heterocyclic** substance

a) an **α,β**-unsaturated lactone **fused** to an **alicyclic**, **aromatic,** or **heteroaromatic** ring such that the lactone ring can attain a completed cyclic array of 4n+2 π electrons assuming **enolization** of the lactone carbonyl (aka **pseudoaromaticity**) (exception: compounds with an ellagic acid **skeletal structure**. If ellagic acid **skeleton** is present, proceed to Q12e)), or



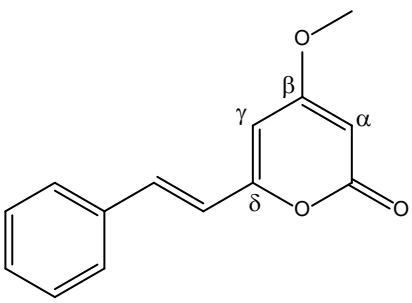
coumarin

Exception: ellagic acid backbone:

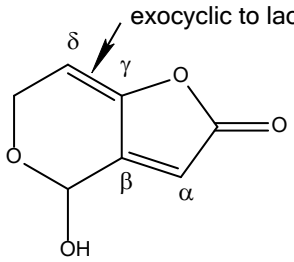


Note: regardless of the substitution pattern on the ellagic acid backbone, if the lactones are present as a part of the ellagic acid backbone, respond no at Q12a. These compounds are evaluated at Q12e. (See example at Q12e.)

b) an **α,β**- and **γ,δ-conjugated** δ-lactone or an **α,β**-unsaturated-γ-lactone containing an **exocyclic** (to the lactone) alkene at the **γ**-position (the γ-lactone cannot be fused to a benzene ring), or

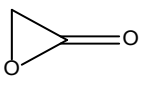


5,6-dehydrokawain
(δ–lactone)

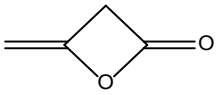


4-hydroxy-4,6-dihydrofuro[3,2-c]pyran-2-one
(γ-lactone)

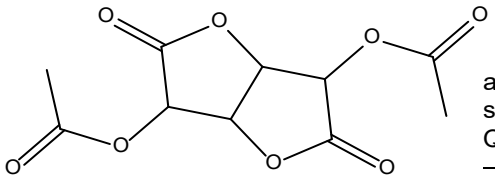
c) an α- or β-lactone or substance containing two or more lactone rings, or



oxiran-2-one
(α-lactone)

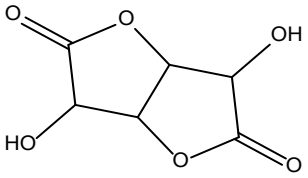


diketene
(β-lactone)



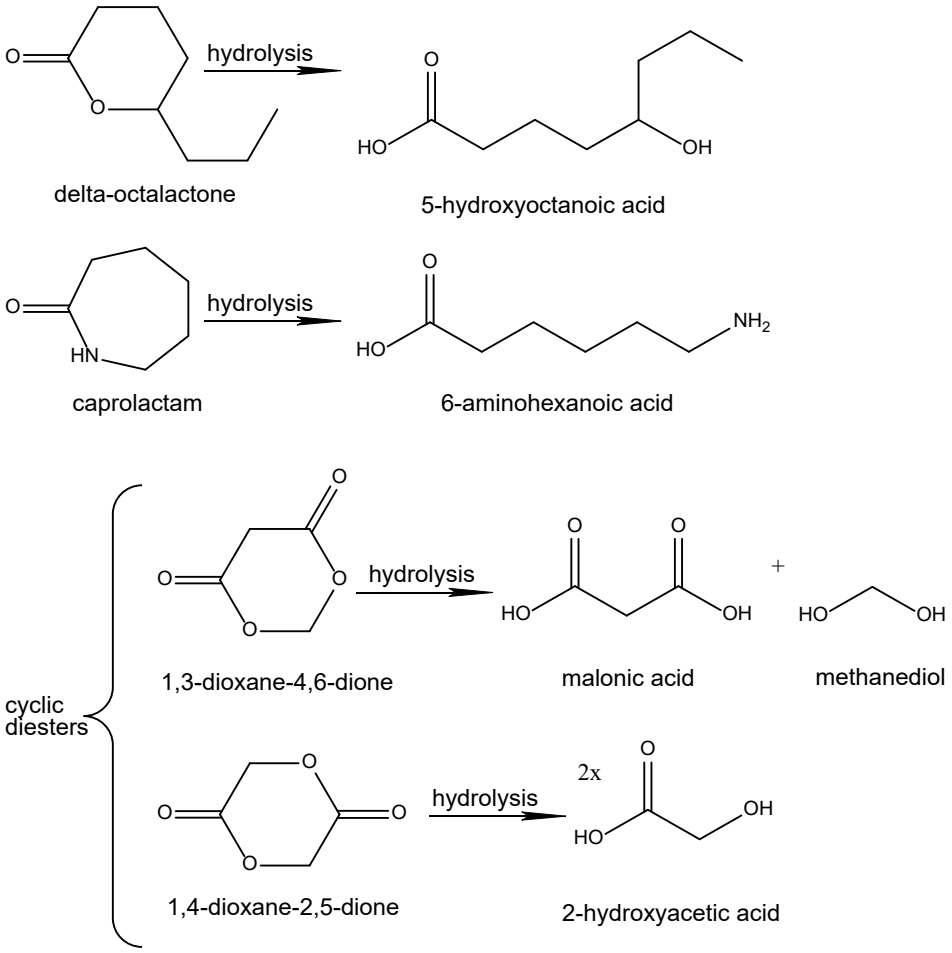
glucaron

after hydrolysis of side chain esters at Q11, evaluated as

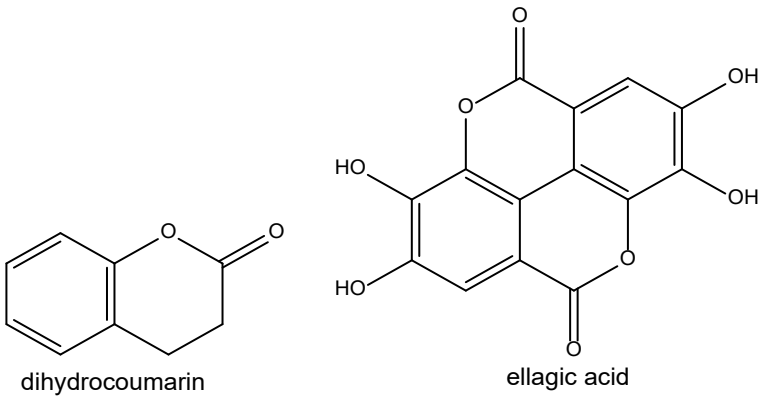


3,6-dihydroxytetrahydrofuro[3,2-*b*]furan-2,5-dione

d) a cyclic diester or lactone that **hydrolyzes** to a **linear aliphatic** or simply **branched-chain** hydroxycarboxylic acid, dicarboxylic acid, and/or diol or a simple secondary lactam (γ,δ, ε, …) that **hydrolyzes** to a **linear or simply branched aliphatic** aminocarboxylic acid not bonded to any other ring system, or

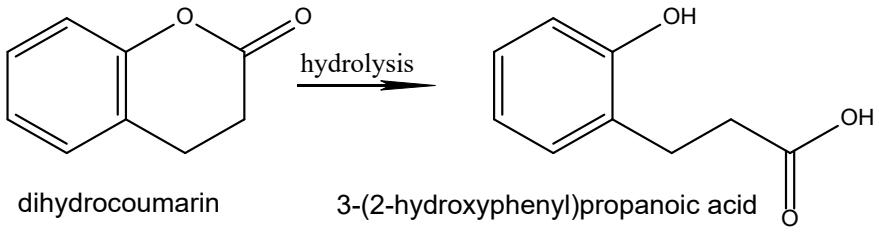


e) a lactone (γ, δ, ε, …) **fused**, **singly bonded**, or connected by a carbon chain of ≤4 Cs to an **alicyclic**, **aromatic**, or **heterocyclic** ring(s) <u>without</u> containing a continuous cyclic array of 4n+2 π electrons within the lactone<u>?</u>



i) If yes to a), b), or c), assign to Class IV.
ii) If yes to d), assume **hydrolysis** and proceed to Q1 to evaluate all **hydrolysis products**. See examples for lactone, lactam, and cyclic diester **hydrolysis** provided after sub-question d).

iii) If yes to e), consider that the lactone is **hydrolyzed** to an **alicyclic**-, **aromatic**-, or **heterocyclic**-ring substituted hydroxycarboxylic acid derivative. Proceed to Q30, Q33, or Q10 to evaluate the **alicyclic**, **aromatic**, or **heterocyclic hydrolysis** products, respectively. Note: if the compound contains a mixed ring system (such as a combination of **alicyclic** and **heterocyclic** rings), go to Q10.
Example:



dihydrocoumarin 3-(2-hydroxyphenyl)propanoic acid

iv) If no to a), b), c), d), and e), proceed to Q13.

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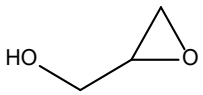
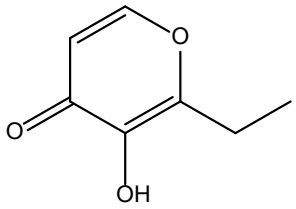
13. Does the substance contain one or more three-membered **heterocyclic** rings containing either a single N, O, or S?

i) If yes, proceed to Q14.



glycidol

ii) If no, proceed to Q15.



ethyl maltol

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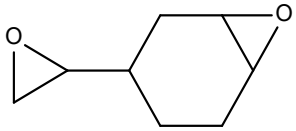
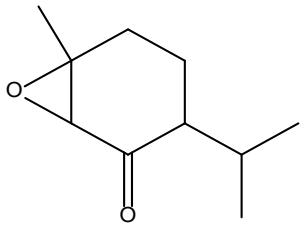
14. Is the substance

a) a polyepoxide (≥2 epoxide rings) or



4-vinylcyclohexene diepoxide

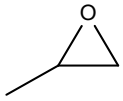
b) a monoepoxide containing a total number of ≥6 Cs?



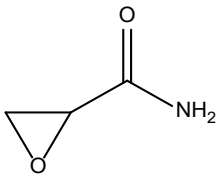
piperitone oxide

i) If yes to 14a), assign to Class V.
ii) If yes to 14b), and the epoxide is substituted by or **fused** to a poly**aromatic** ring system, proceed to Q33. In all other cases, assign to Class III.
iii) If no, assign to Class IV.
Examples for no reply:

Class IV:

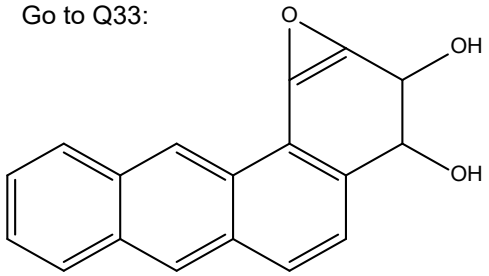


propylene oxide



glycidamide

Go to Q33:



2,3-dihydrotetrapheno[1,2-*b*]oxirene-2,3-diol

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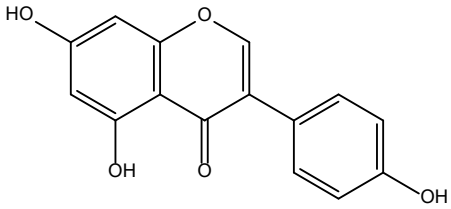
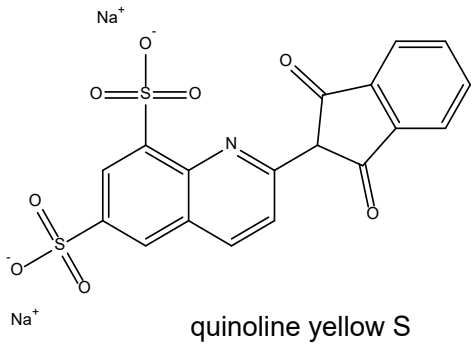
15. Is the **heterocycle** a six-membered ring containing only a single ring O with or without a ketone or alcohol ring substituent at the 4 position (no other substitutions are allowed at this position) and the **heterocyclic** ring is [2.3]-**fused** to one benzene ring and connected at the 5 or 6 position by a single bond (i.e., **singly bonded**) to a second benzene ring (i.e., commonly recognized as the flavonoid carbon **skeleton**)? The benzene rings should be substituted by more than 2 phenolic hydroxy and/or methoxy substituents with each benzene ring having at least one phenolic hydroxy or methoxy substituent.

i) If yes, proceed to Q28.



genistein

ii) If no, proceed to Q16.



quinoline yellow S

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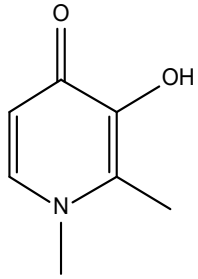
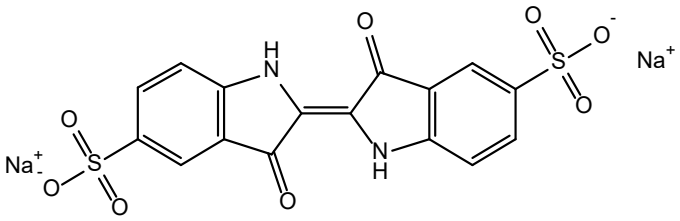
16. Does the **heterocyclic** ring contain an **α**-ketoenol moiety (C=C(OH)C=O) in which the enolic double bond is further **conjugated** with a heteroatom (O or N) possessing a non-bonding electron pair or another double bond?

i) If yes, assign to Class III.



deferiprone

ii) If no, proceed to Q17.



FD & C Blue No. 2

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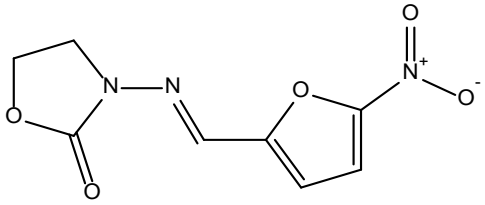
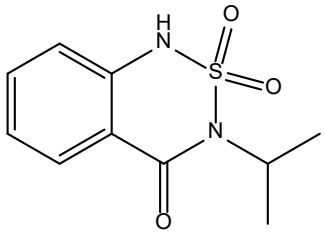
17. Does the substance contain one or more **heteroaromatic** rings?

i) If yes, proceed to Q19.



furazolidone

ii) If no, proceed to Q18.



bentazon

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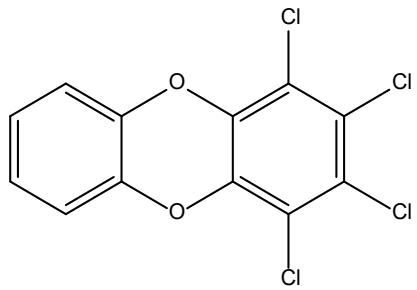
18. Does the **heterocyclic** ring(s) contain

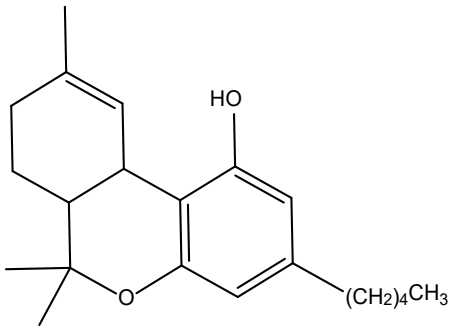
a) **i)** a dibenzo-p-dioxin **skeletal structure** or **ii)** at least three rings that are **fused**, **bridged**, **spiro-fused**, and/or **singly bonded**, or

i:



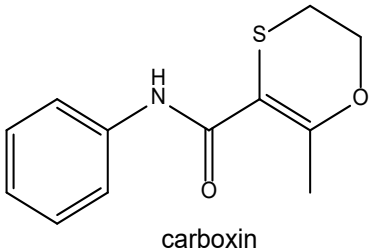
1,2,3,4-tetrachlorodibenzo-p-dioxin

ii:



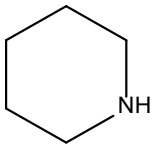
delta-9-tetrahydrocannabinol

b) substituents (note: the heteroatoms contained within the ring are not considered substituents) *other than* **linear** or **simply branched aliphatic** chains of ≤6 Cs, **alicyclic** ring, **bridged** chain (≤6 Cs), only one **aromatic** ring (**fused**, **singly bonded**, or connected by an **aliphatic** carbon chain of ≤4 Cs or connected by an -O-), with or without primary alcohols, secondary alcohols, aldehydes, ketones, carboxylic acids, lactone or lactam, primary amines (cannot be bonded to a ring nitrogen), thiols, thioesters, polysulfides, sulfides, sulfoxides, single sulfonate, sulfonamide, or sulfone as a substituent or part of the ring or a single ring sulfamate, methoxy, ethoxy, or polyoxyethylene ($-OCH_2-CH_2-)_x$ with x is 2, 3, or 4, or



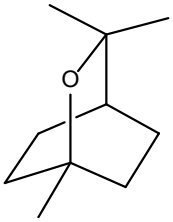
carboxin

c) no substituents on the ring (i.e., it is an unsubstituted ring)?

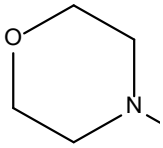


piperidine

i) If yes to a(i)), assign to class V.
ii) If yes to a(ii)) or b) or for ≥2 sulfur moieties in b), proceed to Q47.
iii) If yes to c), assign to Class III.
iv) If no to a), b), and c), proceed to Q28.
Examples for no:



eucalyptol



ethylmorpholine

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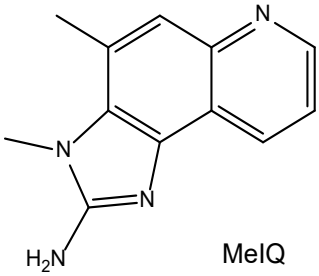
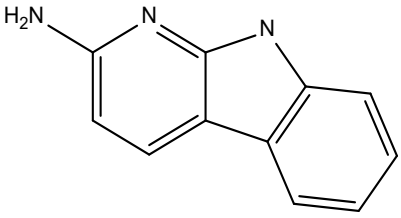
19. Does the **heteroaromatic** substance contain

a) ≥3 **fused** and/or **singly bonded aromatic** or **heteroaromatic** rings in which one of the rings is a 2-aminoimidazolyl or 2-aminopyridyl ring, or

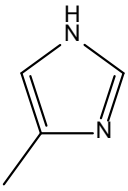


MeIQ



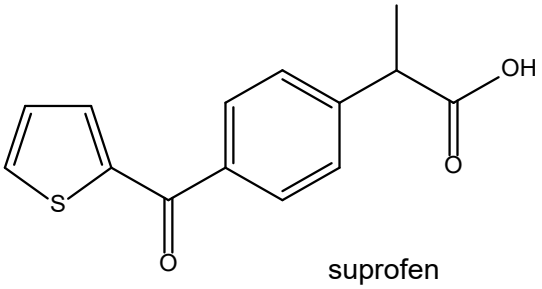
AαC

b) a 5- or 4- methyl- or ethyl- imidazole ring, or



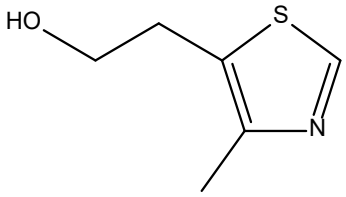
4-methylimidazole

c) a thiophene ring, or



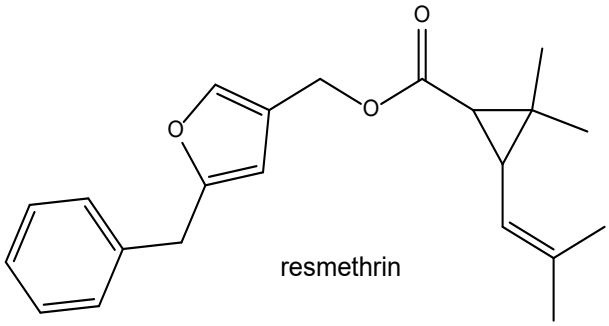
suprofen

d) a thiazole ring substituted at the 2, 4 and/or 5-position(s) by alkyl or **aryl** substituents (the **aryl** ring cannot be **fused** to the thiazole ring) with or without **oxygenated functional group**s (the ester should be an alkyl ester)?

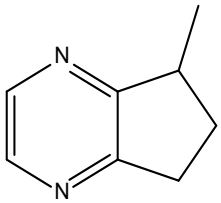


4-methyl-5-thiazoleethanol

i) If yes to a), assign to Class V.
i) If yes b) or c), assign to Class IV.
ii) If yes to d), assign to Class III.
iii) If no to a), b), c), and d), proceed to Q20.
Examples for no:



resmethrin



5H-5-methyl-6,7-dihydrocyclopentapyrazine

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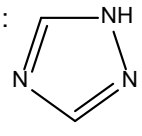
20. Does the **heteroaromatic** compound contain

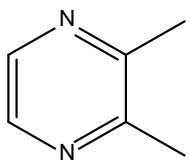
a) only one **heteroaromatic** ring that is **i)** unsubstituted, **ii)** substituted, but not by a ring(s), or **iii)** substituted by one or more cyclopropylamine ring, <u>or</u>

i:



1,2,4-triazole

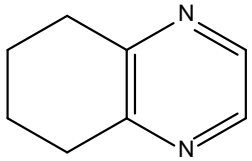
ii:



2,3-dimethylpyrazine

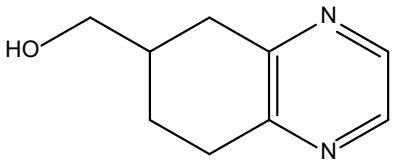
b) only one **heteroaromatic** ring **fused** or **singly bonded** to an **alicyclic** ring and the rings are **i)** unsubstituted or **ii)** substituted, or

i:



5,6,7,8-tetrahydroquinoxaline

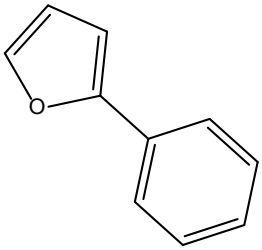
ii:



(5,6,7,8-tetrahydroquinoxalin-6-yl)methanol

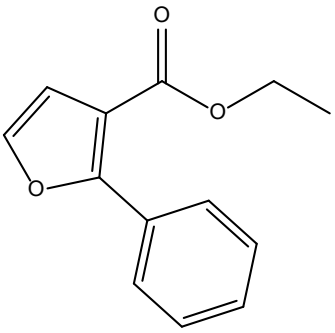
c) only one **heteroaromatic** ring **singly bonded** to an **aryl** ring and the rings are **i)** unsubstituted or **ii)** substituted, or

i:



2-phenylfuran

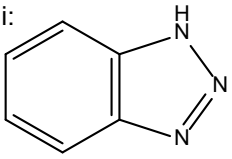
ii:



2-phenyl-3-carbethoxyfuran

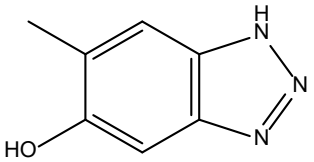
d) only one **heteroaromatic** ring **fused** to an **aromatic** ring and the rings are **i)** unsubstituted or **ii)** substituted, or

i:



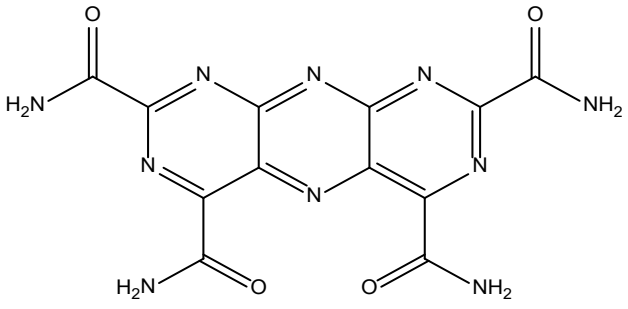
1,2,3-benzotriazole

ii:



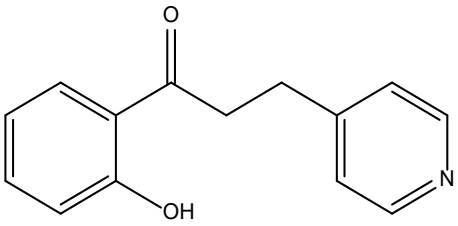
6-methyl-1*H*-benzo[*d*][1,2,3]triazol-5-ol

e) substituted or unsubstituted ring system composed of any combination of at least three **aromatic** and **heteroaromatic** rings or at least 3 **heteroaromatic** rings if no **aromatic** rings are present, or



pyrimido[5,4-*g*]pteridine-2,4,6,8-tetracarboxamide

f) **heteroaromatic** ring or ring systems other than in a), b), c), d), and e)?



1-(2-hydroxyphenyl)-3-(4-pyridyl)propan-1-one

i) If yes to a(iii)), assign to Class V.
ii) If yes to a(i)) or d(i)), assign to Class IV.
iii) If yes to e) or f), assign to Class IV unless one or more sulfonate or sulfamate substituents are present, in which case proceed to Q47.
iv) If yes to b(i)) or c(i)), assign to Class III.
v) If yes to a(ii)), b(ii)), c(ii)), or d(ii)), proceed to Q21.
Note: If no to a) through e), f) must be yes.

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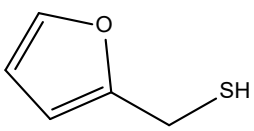
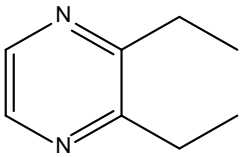
21. Does the **heteroaromatic** substance contain any of the reactive moieties listed in Q28 c), e), g), m), n), q), or r)?

i) If yes, proceed to Q28 and assign to Class III, IV, or V as appropriate.

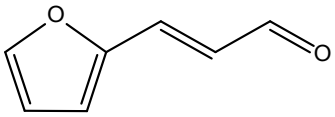


furfuryl mercaptan

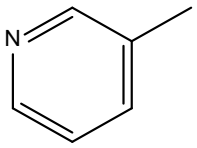


2,3-diethylpyrazine

ii) If no, proceed to Q22.



furylacrolein



beta-picoline

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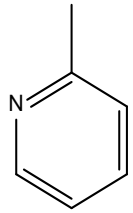
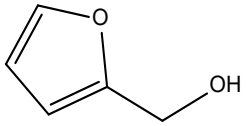
22. Is/Are the ring(s) substituted only by one or more **aliphatic** chains with or without one or more ring hydroxy, methoxy, ethoxy and/or one or more side chain primary alcohol, secondary alcohol, aldehyde, ketone, carboxylic acid, primary amide, methoxy or ethoxy, monosulfide, or sulfoxide?

i) If yes, assign to Class III.

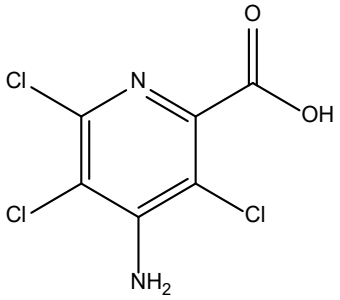


2-methylpyridine



furfuryl alcohol

ii) If no, proceed to Q47.



picloram

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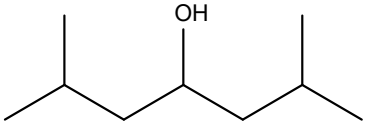
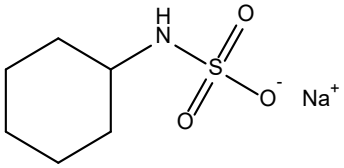
23. Is the structure **acyclic**?

i) If yes, proceed to Q24.



2,6-dimethylheptan-4-ol

ii) If no, proceed to Q29.



sodium cyclamate

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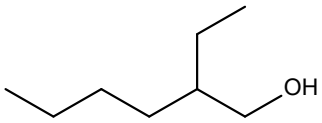
24. Is the **acyclic** substance

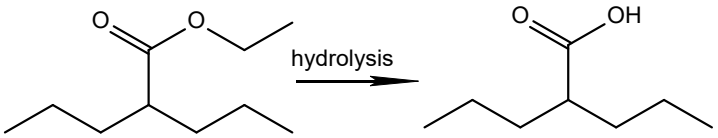
a) **i)** a primary alcohol, the primary alcohol's **corresponding** aldehyde or carboxylic acid, with no other **functional group**s, and a chain length of 5-8 Cs containing only one 2-alkyl substituent (2-4 Cs) or **ii)** an ester, acetal, or hemi-acetal for which at least one of the **hydrolysis** products satisfies the structural requirements in i), or

i:



2-ethyl-1-hexanol

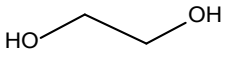
ii:



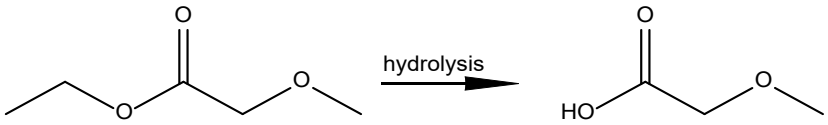
ethyl 2-propylpentanoate

2-propyl-pentanoic acid
(hydrolysis product satisfies requirements in i)

b) an **α**-hydroxy- or **α**-alkoxy-ethanoic acid, its **corresponding** alcohol or aldehyde, or an ester, acetal, or hemi-acetal that **hydrolyses** to an **α**-hydroxy- or **α**-alkoxy-ethanoic acid, its **corresponding** alcohol or aldehyde where the alkoxy substituent adjacent to the above **oxygenated functional groups** has ≤4 Cs?



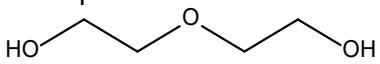
ethylene glycol



ethyl 2-methoxyacetate

2-methoxyacetic acid

i) If yes to a) or b), assign to Class III.
ii) If no to a) and b), proceed to Q25.
Example for no:



diethylene glycol

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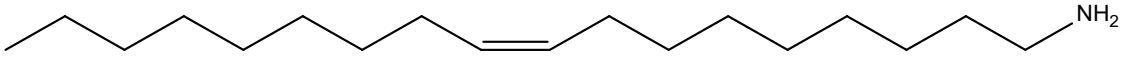
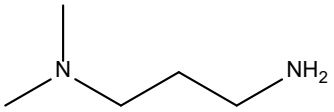
25. Is the substance a **a)** primary and/or tertiary **aliphatic** amine (if tertiary, only one tertiary amine may be present) or **b)** primary, secondary, and tertiary amide and both a) and b) of a chain length ≥12 Cs or a combination of carbons, oxygens, and nitrogens (for tertiary amines N is counted as part of the chain) with or without **oxygenated functional groups** but no other **functional groups**?

i) If yes to a) or b), assign to Class III.



oleylamine

ii) If no to a) and b), <u>and</u> more than one tertiary amine substituents are present, go to Q47.
In all other cases, if no to Q25, proceed to Q26.



N',N'-dimethylpropane-1,3-diamine

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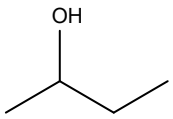
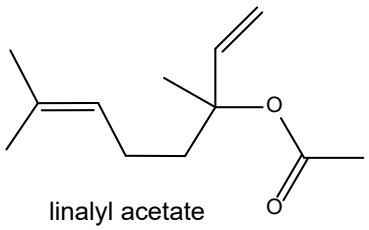
26. Is the structure a **linear** or **simply branched aliphatic** substance (methylene branching is allowed as well) or a **linear** or simply branched alkyne, either unsubstituted or containing any one or a combination of only the following **functional groups**:

a) any combination of six or less of primary alcohols, secondary alcohols, aldehydes, carboxylic acids, acetal, hemiacetal, esters, carbonates, sulfate esters, or alkynes. In addition to or instead of the above **functional groups**, four or less ethers may also be present, and/or



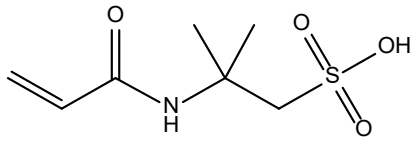
2-butanol

b) one each of one or more of the following: hemiketal, ketal, tertiary amine, sulfoxide, thiol, dithiol, monosulfide, polysulfide, thioester, tertiary alcohol or **corresponding** ester, primary or N-alkyl secondary amide, polyoxyethylene $(-OCH_2-CH_2-)_x$ or polyoxypropylene $(-OCH_2-CH_2-CH_2-)_x$ with $x>1$ but ≤ 4, a trimethyl ammonium moiety, a secondary amine but only when monosulfide, polysulfide, sulfoxide, sulfone or primary alcohol, aldehyde, carboxylic acid or ester **functional group** is also present, and/or a maximum of two primary amines and up to two ketones, and/or



linalyl acetate

c) one sulfone, sulfonate, sulfonamide, sulfamate, or thionosulfate group?

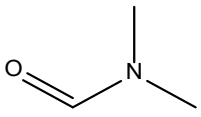


2-acrylamido-2-methyl-1-propane sulfonic acid

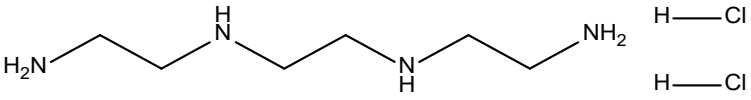
i) If the substance is a **linear** or **simply branched aliphatic** substance (methylene branching is allowed as well) or **linear** or **simply branched** alkyne that is unsubstituted or if yes to a), b), and/or c), proceed to Q27.

ii) If no to a), b), and c), proceed to Q47.

Examples for no:



dimethylformamide



triethylenetetramine dihydrochloride

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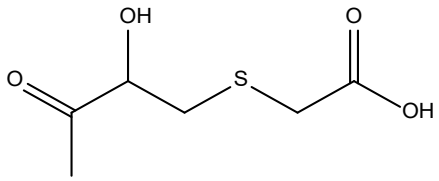
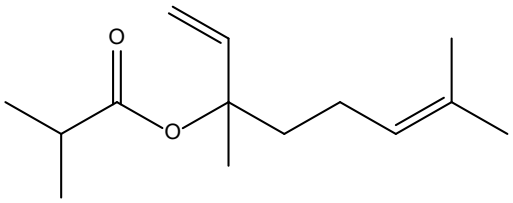
27. Does the structure contain more than three different **functional groups**? The following metabolically **related functional groups** count as one: i) ester, orthoester, and carboxylic acid; ii) hemiketal, ketal, and ketone; iii) hemiacetal, acetal, and aldehyde; iv) primary alcohol and methoxy; and v) thioester and thiol.

i) If yes, assign to Class IV.



2-((2-hydroxy-3-oxobutyl)thio)acetic acid

ii) If no, proceed to Q28.

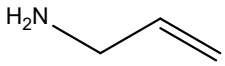


linalyl isobutyrate

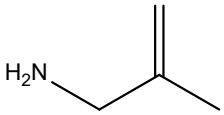
---

28. Does the substance contain any one or more of the following moieties or is the substance an

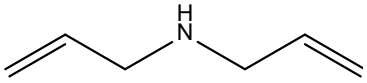
a) allyl amine, β-methylallylamine, or their corresponding secondary amide or the corresponding tertiary amide of diallylamine, and di(β-methyl-allyl)amine, or



allyl amine

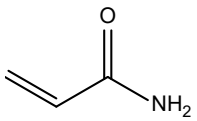


beta-methylallylamine



diallylamine

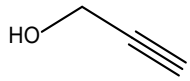
b) acrylamide or N-alkyl or **aryl**-substituted acrylamide without any other **functional groups**, or



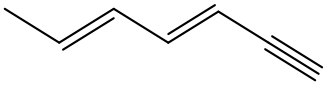
acrylamide

c) alkyne **i) conjugated** with one or more alkyne, alkene, carbonyl group/s or adjacent to the **corresponding** alcohols (e.g., 2-butyn-1-ol) or a terminal alkyne regardless of **conjugation** or **ii)** unconjugated and not a terminal alkyne, or

i:

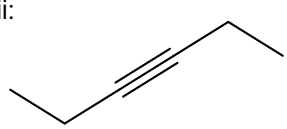


propargyl alcohol



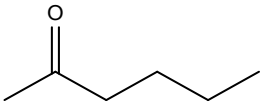
(3*E*,5*E*)-hepta-3,5-dien-1-yne

ii:



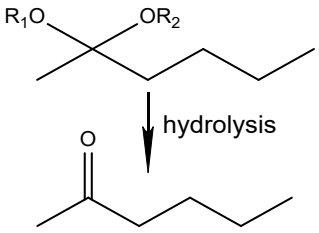
hex-3-yne

d) **i)** hexane; or 2-hexanone, 3-heptanone, or 5-nonanone or their **corresponding** hemiketals or ketals or **ii)** 2,5-hexadione, 2,5-heptadione, or 2,5-nonadione with or without methyl or methoxy substituents between the ketone functions, or their **corresponding** hemiketals or ketals, or

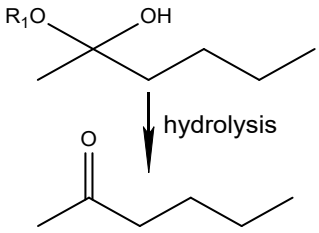


2-hexanone
(monoketone)

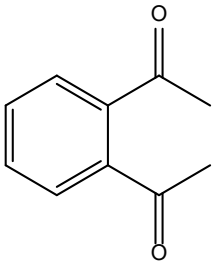
ketal that hydrolyzes to corresponding monoketone:



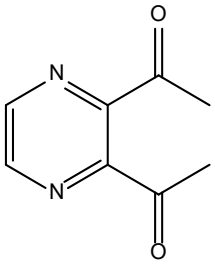
hemiketal that hydrolyzes to corresponding monoketone:



e) **aromatic** or **heteroaromatic** substance with *o*-diacetyl substituents (e.g., 1,2-diacetylbenzene (**aromatic**) and 2,3-diacetylpyrazine (**heteroaromatic**)), its **corresponding** alcohols, hemiketals, ketals, or diethyl precursor (e.g., 1,2-diethylbenzene and 2,3-diethylpyrazine, respectively), or

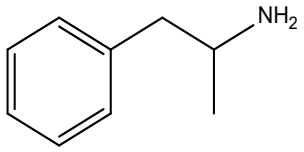


1,2-diacetylbenzene



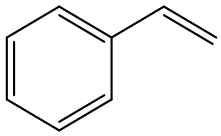
2,3-diacetylpyrazine

f) β-phenylethylamine (primary or secondary but not tertiary amine) moiety with or without additional alkyl, hydroxy, methoxy, or ethoxy substitution, or



amphetamine

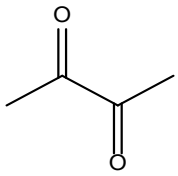
g) **aromatic**, **heteroaromatic**, or mono**heterocyclic** substance with one or more terminal vinyl (i.e., $RHC=CH_2$) group(s) as the only ring substituent(s), or



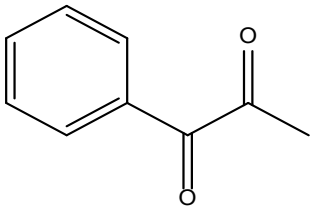
styrene

h) **i) acyclic** or **aromatic** ring substituted **α**-diketone or its **corresponding** hemiketal or ketal or **ii)** an **aliphatic** dialdehyde without α,β-unsaturation as the only **functional group**s, or

i:

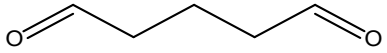


diacetyl



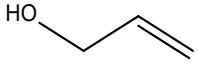
1-phenyl-1,2-propanedione

ii:

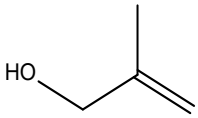


pentane dialdehyde

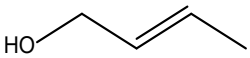
i) allyl alcohol, methallyl alcohol, methacrolein, crotonyl alcohol, crotonaldehyde, or **corresponding** ester (e.g., allyl hexanoate or crotonyl acetate), carbonate, orthoester, acetal, hemiacetal, ketal, or hemiketal or



allyl alcohol

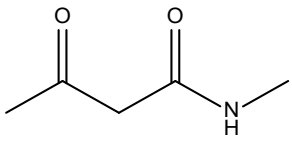


methallyl alcohol

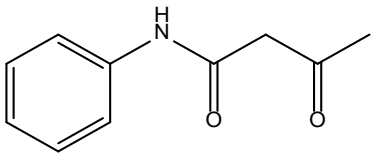


crotonyl alcohol

j) **aliphatic β**-diketone or **β**-ketoamide moiety (may be a substituent on a ring), or

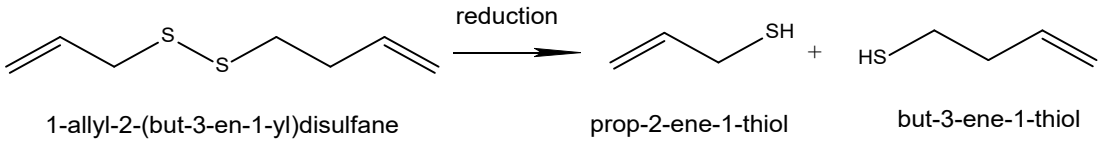


N-methylacetoacetamide

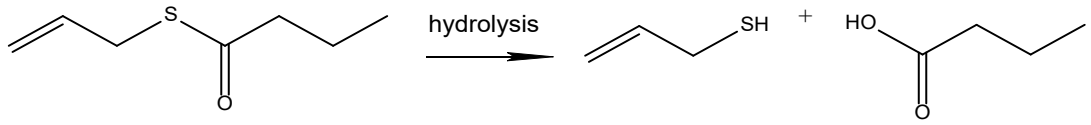


acetoacetanilide

k) allyl thiol, mono- or di-allyl disulfide that is **reduced** to allyl thiol, or allyl thioester that is **hydrolyzed** to allyl thiol, or

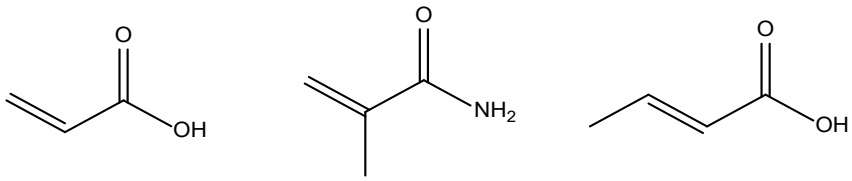


1-allyl-2-(but-3-en-1-yl)disulfane | prop-2-ene-1-thiol | but-3-ene-1-thiol



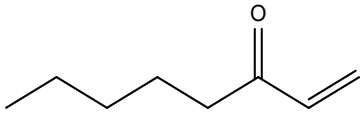
S-allyl butanethioate | prop-2-ene-1-thiol | butyric acid

l) acrylic acid, methylacrylic acid, methacrylamide, or crotonic acid and **corresponding** esters (e.g., ethyl acrylate), or



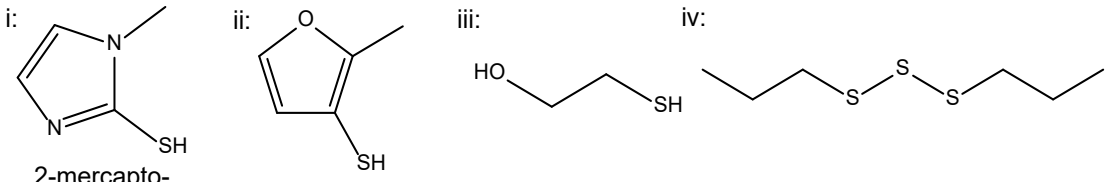
acrylic acid | methacrylamide | crotonic acid

m) ketone, a ketal, a hemiketal, or secondary alcohol (or **corresponding** ester) directly bonded to a terminal alkene, or



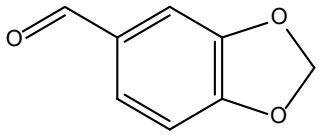
vinyl amyl ketone

n) a mono- or di-thiol, thioester, thiocarbonate, or disulfide **i)** in which S is connected by a single bond to the 2-position of an imidazole or pyrimidine ring, **ii)** in which S is connected by a single bond to a **heteroaromatic** ring, **iii)** as a substituent of an **alicyclic** ring (may be part of the **alicyclic** ring, not only a substituent), **aromatic** or **heterocyclic** ring, or as a substituent of a **linear** or **branched aliphatic** chain (either an **acyclic** compound or a substituent on an **alicyclic**, **aromatic**, **heterocyclic**, or **heteroaromatic** ring, or **iv)** a polysulfide with S_n where n≥3, or



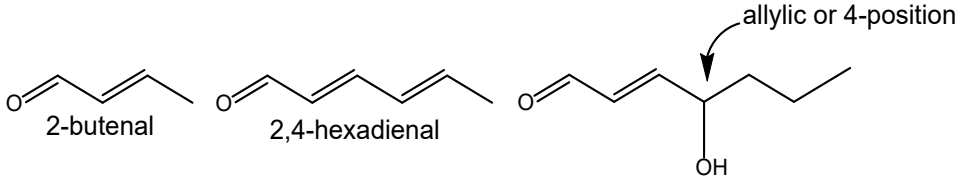
2-mercapto-1-methylimidazole | 2-methyl-3-furanthiol | 2-mercaptoethanol | dipropyl trisulfide

o) a methylenedioxy ring **fused** to an **aromatic** ring, or



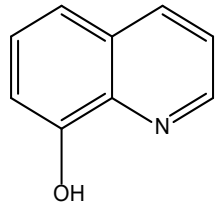
piperonal

p) **linear aliphatic α,β**-unsaturated aldehyde or dialdehydes of <10 Cs, or their **corresponding** acetals or hemiacetal, or their corresponding continuously **conjugated** di- or tri-enal with or without a hydroxy or hydroperoxy substituent(s) at the allylic (e.g., 4-) position of mono **α,β**-unsaturated compounds, or



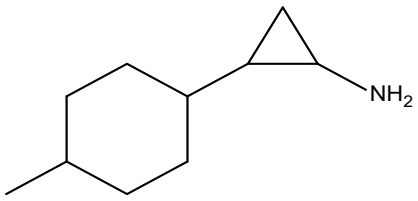
2-butenal 2,4-hexadienal (*E*)-4-hydroxyhept-2-enal

q) **aromatic** or **heteroaromatic** substance containing a substituent hydroxyl, ether, aldehyde, or ketone that is separated from a ring or substituent N by two ring carbons (note: the ring carbons can be on the same or on different rings), ($HOC{=}CNH_2$ or NC-C=O) (e.g., *o*-aminophenol, 8-hydroxyquinoline, or 2-acetylpyrrole), or



8-hydroxyquinoline

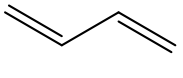
r) aminocyclopropyl moiety, or



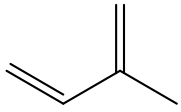
2-(4-methylcyclohexyl)cyclopropan-1-amine

s) **linear** or **simply branched-chain aliphatic acyclic** hydrocarbon with or without one or more $=CH_2$ branches that has a terminal diene and **i)** ≤ 6 Cs or **ii)** > 6 Cs?

i:

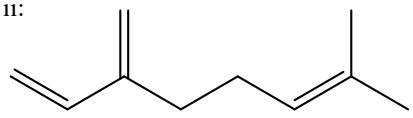


1,3-butadiene



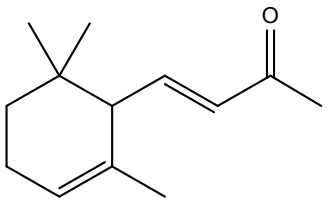
isoprene

ii:

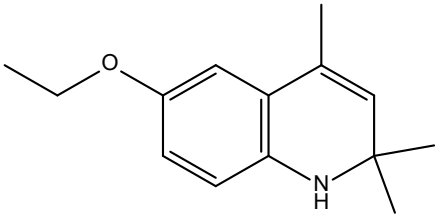


beta-myrcene

i) If yes to 28 c(i)), n(i)), or r), assign to class V.
ii) If yes to 28 a), b), c(ii)), d), e), f), n(ii)), n(iv)), p, or s(i)) assign to Class IV.
iii) If yes to 28 g), h(i)), h(ii)), i), j), k), l), m), n(iii)), o), q), or s(ii)) assign to Class III.
iv) If no to a) through s) and the compound is not **heterocyclic**, assign to Class II.
v) If no to a) through s) and the compound has at least one **heterocyclic** ring, assign to Class II only if at least one of the **heterocyclic** rings contains either a cyclic anhydride, one or more cyclic ester, one or more cyclic amide (N can be connected to another N or S), an imidazolidinone, and/or a 5- or 6-membered ring with 1, 2, or 3 ring oxygen atoms with or without a single ring double bond with or without additional ring N and/or S atoms. Note: **heterocyclic** rings may be substituted. In the case of all other **heterocycles**, if no to a) through s), assign to class III.
Examples for no to a) through s):



alpha-Ionone



ethoxyquin

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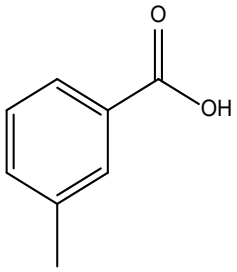
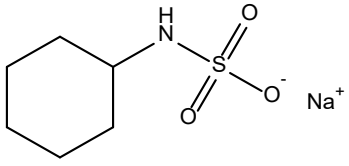
29. Does the substance contain one or more **aromatic** rings?

i) If yes, proceed to Q33.



3-methylbenzoic acid

ii) If no, proceed to Q30.



sodium cyclamate

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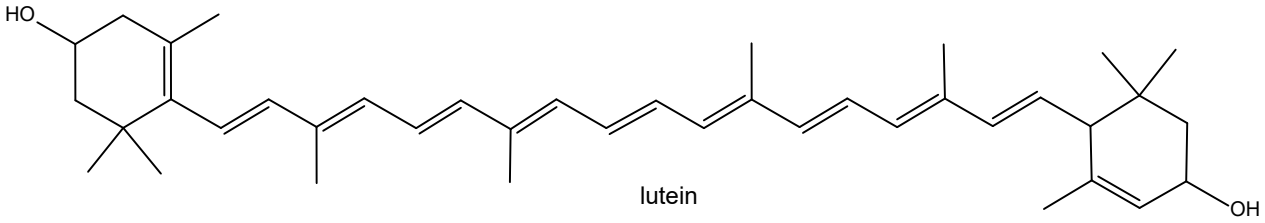
30. Does the **alicyclic** substance contain one, two, or three rings with

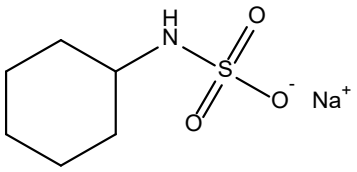
a) **alicyclic** ring structures containing ≤30 ring Cs, unsubstituted or substituted with or without **linear** or **simply branched aliphatic chains** each of ≤12 Cs per ring* with or

without one or more of the following **functional groups**: alcohol, aldehyde (except for **vicinal** dialdehydes), acetal, carboxylic acid, ester, ketone (including ring ketone), ketal, thiol, sulfide, sulfoxide, primary or tertiary amine, or primary or secondary amide (*If one long chain connects two rings, the chain can contain up to 24Cs.), <u>or</u>



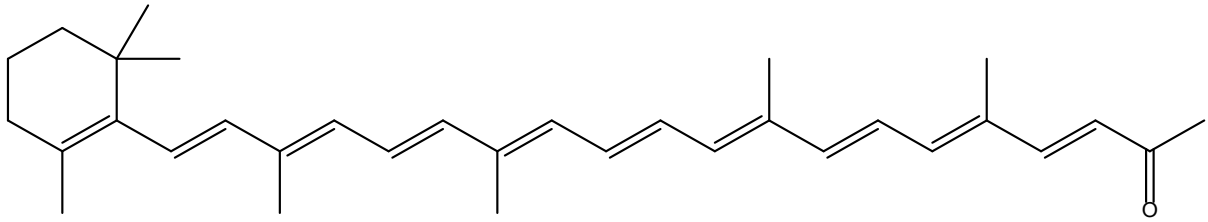
lutein

b) a sulfonate, sulfonamide, or sulfamate?



sodium cyclamate

i) If yes to a), proceed to Q31.
ii) If yes to b), assign to Class I.
iii) If no to a) and b), proceed to Q47.
Example for no:



5,9,14,18-tetramethyl-20-(2,6,6-trimethylcyclohexen-1-yl)icosa-3,5,7,9,11,13,15,17,19-nonaen-2-one
(Citranaxanthin)

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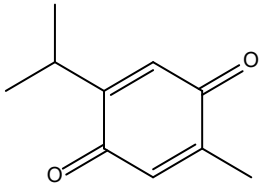
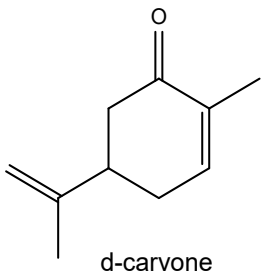
31. Is the **alicyclic** substance an *o*- or *p*-quinone with or without substitution by one or more alkyl substituent of ≤6 Cs with no additional **functional groups**?

i) If yes, assign to Class III.



thymoquinone

ii) If no, proceed to Q32.



d-carvone

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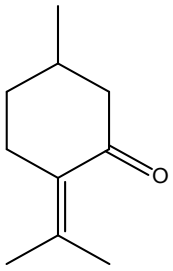
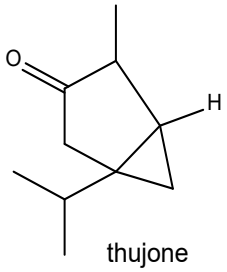
32. Is the substance a mono or bicyclic ring that contains

a) a cyclohexane or cyclohexene ring with i) ketone or ketal and ii) an isopropylidene or isobutylidene side chain adjacent to the ketone function, or



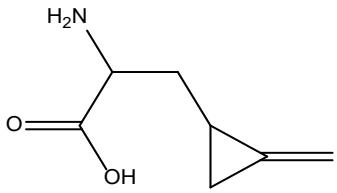
pulegone

b) a ring ketone or ketal with a 4-methyl-1-isopropyl bicyclo[3.1.0]-2- or 3-cyclohexanone carbon **skeleton**, or



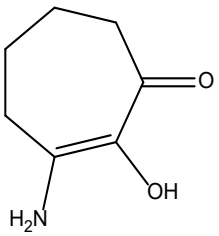
thujone

c) a cyclopropyl ring with an **exocyclic** or **endocyclic** alkene, or

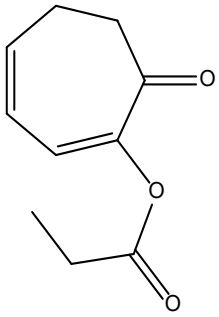


hypoglycin A

d) Does the **alicyclic** ring contain an **α**-ketoenol moiety (C=C(OH)C=O), or the **corresponding α**-ketoester (C=C(OC(=O)R)C=O) in which the enolic double bond is further **conjugated** with an O or N atom possessing a non-bonding electron pair or another double bond?

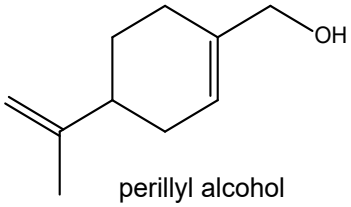


3-amino-2-hydroxycyclohept-2-en-1-one



7-oxocyclohepta-1,3-dien-1-yl propionate

i) If yes to a) or d), assign to Class III.
ii) If yes to b) or c), assign to Class IV.
iii) If no to a), b), c), and d), proceed to Q28.
Example for no:



perillyl alcohol

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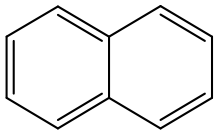
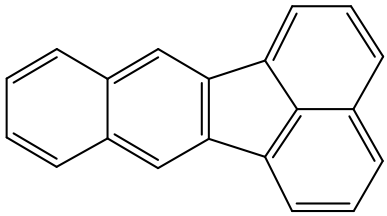
33. Is the substance

a) an unsubstituted benzene ring (i.e., benzene) or composed of 2 or 3 unsubstituted **fused aromatic** rings, or



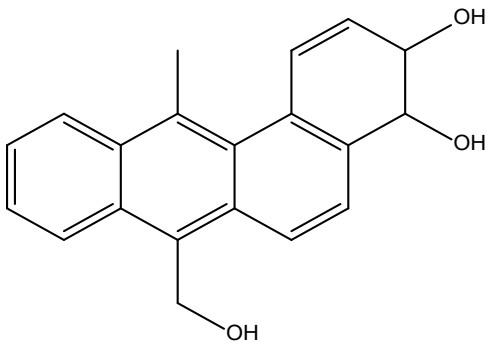
naphthalene

b) unsubstituted and composed of >3 **fused aromatic** rings, or



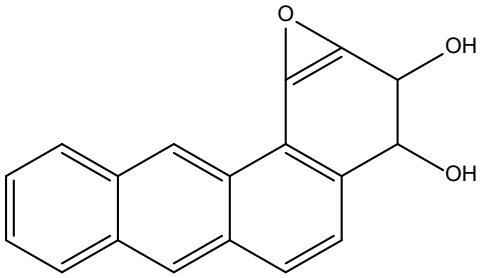
benzo[k]fluoranthene

c) a poly**aromatic** ring system of three or more **fused** rings containing either one or more -CH_3, -CH_2CH_3, -CH_2F, -CH_2Cl, -CH_2Br, -CH_2I, -C(=O)H, -CH_2OH, -CH_2OCH_3, -CH_2-O-$S(=O)_2OH$, -CH_2-O-$S(=O)_2CH_3$, -CH_2-O-$S(=O)_2CF_3$, -CH_2-O-C(=O)R, -CH_2-O-CH_2-Ar (Ar is benzene), and/or -CH_2-O-gluc substituents, or

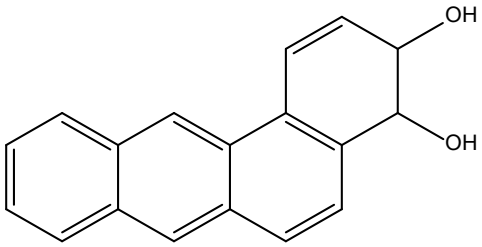


7-(hydroxymethyl)-12-methyl-3,4-dihydrotetraphene-3,4-diol

d) a poly**aromatic** ring system of three or more **fused** rings substituted by any combination of diol(s) and/or epoxide(s) in the **K-region** and/or on **bay or fjord region trio**(s) **and/or quartet**(s)?

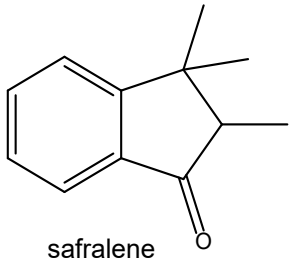


2,3-dihydrotetrapheno[1,2-*b*]oxirene-2,3-diol



3,4-dihydrotetraphene-3,4-diol

i) If yes to a), assign Class IV.
ii) If yes to b), c), or d), assign Class V.
iii) If no to a), b), c), and d), proceed to Q34.
Example for no:



safralene

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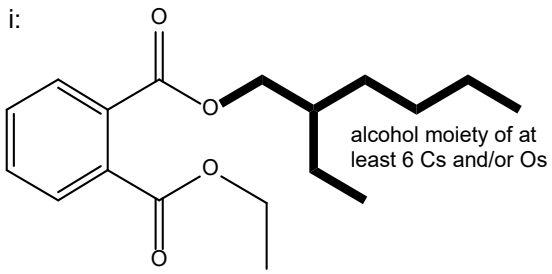
34. Is the substance

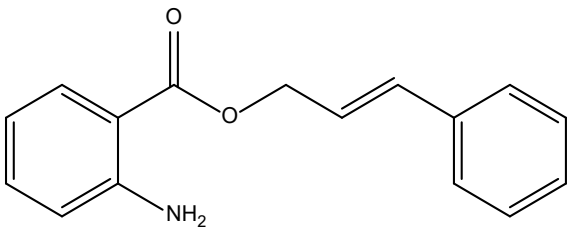
a) **i)** an o-phthalate diester that contains at least one alcohol moiety of ≥6 atoms (Cs and/or Os) or **ii)** a benzoic acid ester substituted at the *o*-position by a moiety bearing a non-bonding pair of electrons (e.g., -OR, -OH, $-NH_2$, CO_2H) or

i:



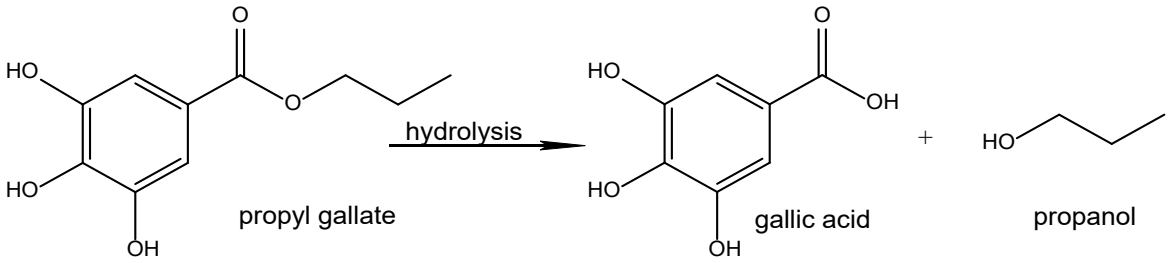
ethyl-(2-ethylhexyl) phthalate

ii:



cinnamyl anthranilate

b) an ester, orthoester, thioester, acetal, ketal, hemiacetal, hemiketal, sulfate ester, or anhydride that would be anticipated to be completely **hydrolyzed**?



propyl gallate

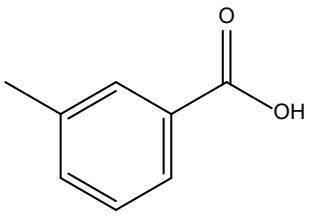
gallic acid

propanol

proceed to Q1

proceed to Q1

i) If yes to a(i)) or a(ii)), assign to Class III.
ii) If yes to b), assume **hydrolysis** and start the evaluation of the **hydrolysis** products at Q1.
iii) If no to a(i)), a(ii)), and b), proceed to Q35.
Example for no:



3-methylbenzoic acid

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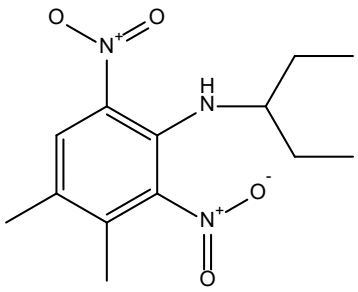
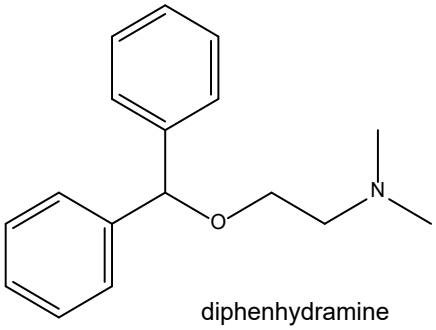
35. Does the substance contain

a) only one **aromatic** ring (additional ring(s) that are not **aromatic** are allowed) or



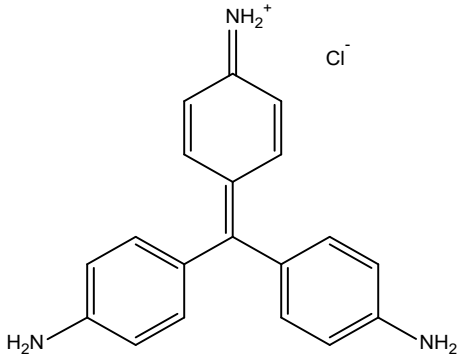
pendimethalin

b) only two **aromatic** rings (additional ring(s) that are not **aromatic** are allowed)?



diphenhydramine

i) If yes to a), proceed to Q38.
ii) If yes to b), proceed to Q36.
iii) If no to a) and b), proceed to Q47.
Example for no:



C.I. Basic Red 9

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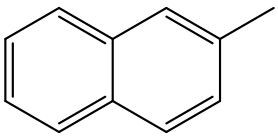
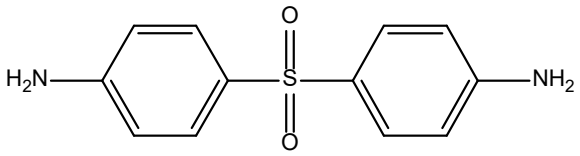
36. Is the binuclear substance

a) **fused** (e.g., naphthalene or azulene), <u>or</u>



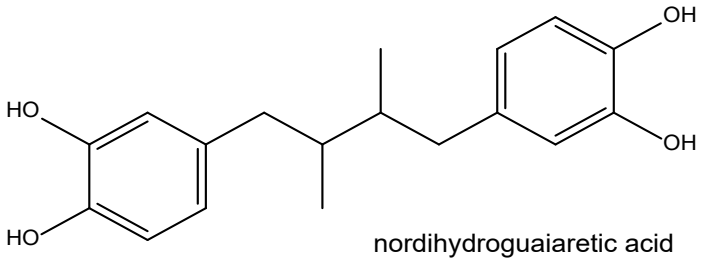
2-methylnaphthalene

b) unfused with benzene rings either **singly bonded** or connected by an -O- or one or more -S- (divalent (-S-), tetravalent (-S(=O)-) or hexavalent (-S(=O)$_2$-)), or -N- (e.g., -N=N-), <u>or</u>



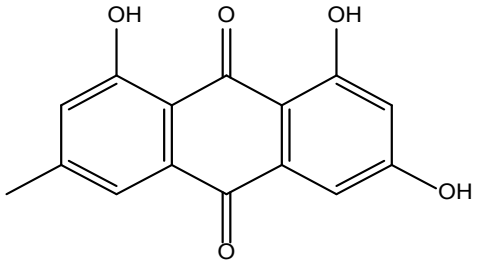
dapsone

c) unfused but linked by either one **linear aliphatic** chain of ≤6 Cs or a **simply branched aliphatic chain** with ≤2 branches of ≤2 Cs each and a total of ≤8 Cs. The connecting chain may contain -O-, and one or more -S- (divalent, tetravalent, or hexavalent), -N- (e.g., -N=N-), or not more than 3 amino acids, <u>or</u>



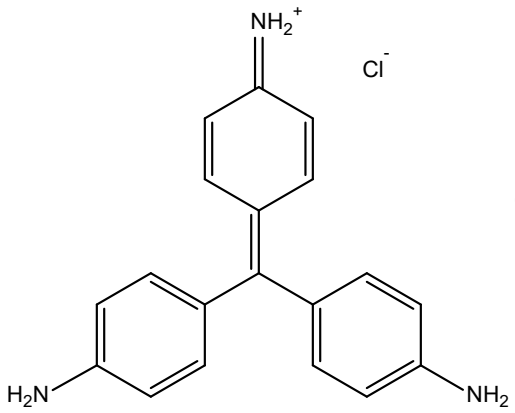
nordihydroguaiaretic acid

d) unfused linked at ***ortho*** positions by a single bond (i.e., **singly bonded**) and a **linear** or **simply branched** chain of ≤4 Cs <u>or</u> two **linear** or **simply branched aliphatic chains** of ≤4 Cs each?



emodin

i) If yes to a), proceed to Q37.
ii) If yes to b), c), or d), proceed to Q41.
iii) If no to a), b), c), and d), proceed to Q47.
Example for no:



C.I. Basic Red 9

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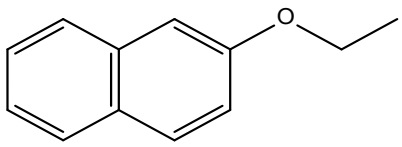
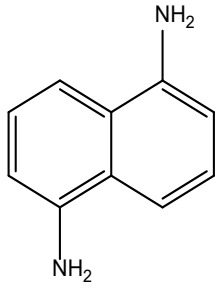
37. Does the **fused** ring system contain only the following substituent(s)

a) one or more alkyl substituent(s) each of ≤4 Cs and/or at least one hydroxy, methoxy, ethoxy, primary or secondary alcohol, aldehyde, ether, ketone, carboxylic acid, or ester, or



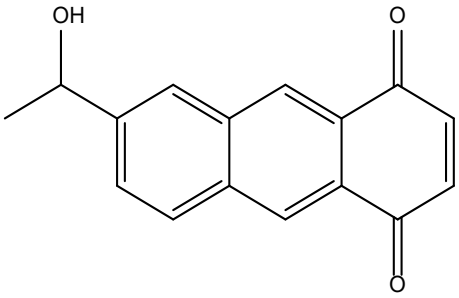
2-ethoxynaphthalene

b) either one primary amine (or its N-acetyl amide) and/or one nitro group or two primary amines (or their N-acetyl amide) or two nitro groups at any position with or without one alkyl substituent of ≤2 Cs and no other **functional groups**, or



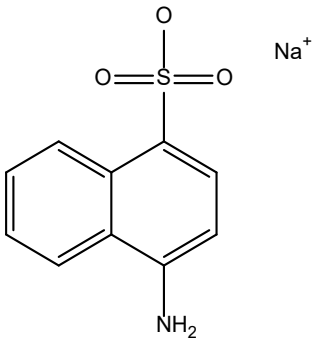
1,5-naphthalenediamine

c) an *o*- or *p*-quinone with or without additional alkyl chains of ≤4 Cs and/or the following **oxygenated functional groups**: hydroxy, methoxy, ethoxy, primary or secondary alcohol, aldehyde, carboxylic acid, or ester?



6-(1-hydroxyethyl)anthracene-1,4-dione

i) If yes to a) or c), assign to Class III.
ii) If yes to b), assign to Class V.
iii) If no to a), b), and c), proceed to Q47.
Example for no:



sodium naphthionate

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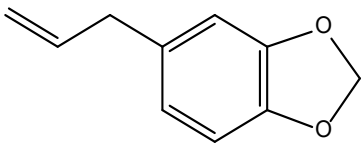
38. Is the substance a single benzene ring that consists only of

a) 2'-alkene or a 1'-hydroxy or 1'-ester of the 2'-alkene and

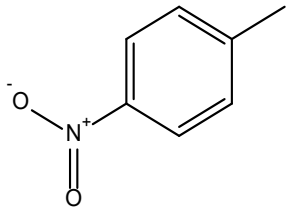
b) one or more alkoxy groups, one of which must be ***para*** to the hydrocarbon chain? (Note: The *p*-alkoxy includes the alkoxy of a 3,4-methylenedioxy substituent.)

i) If yes to a) and b), assign to Class IV.



safrol

ii) If no to a) and/or b), proceed to Q39.



4-nitrotoluene

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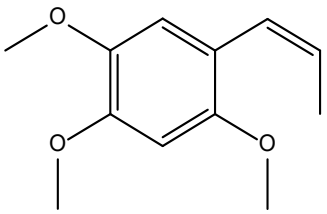
39. Is the substance a single benzene ring substituted only by

a) a hydrocarbon chain of 2 or 3 Cs containing a 1'-alkene with or without a terminal **oxygenated functional group** (i.e., hydroxy, aldehyde, carboxylic acid, or **corresponding** hemiacetals, acetal, or alkyl ester) and

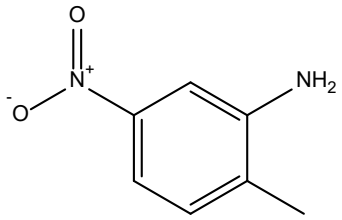
b) one *o*-hydroxy or one or more methoxy groups one of which is *o*- to the hydrocarbon chain?

i) If yes to a) and b), assign to Class III.



beta-asarone

ii) If no to a) and/or b), proceed to Q40.



2-methyl-5-nitroaniline

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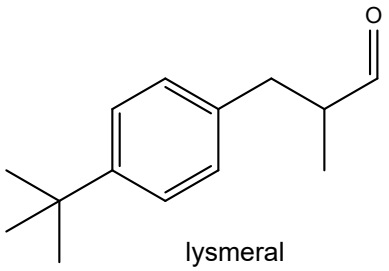
40. Is the substance

a) **i)** benzoic acid or its precursors (i.e., toluene, benzyl alcohol, or benzaldehyde) or **ii)** 3-phenylpropanoic or 3-phenylpropenoic acid or their **corresponding** alcohol or aldehyde with or without side chain alkyl substituents of ≤6 Cs, **and** i) or ii) is substituted at the ***para*** position by a tertiary butyl, isopropyl, or isobutyl group with no other ring substituents?, or

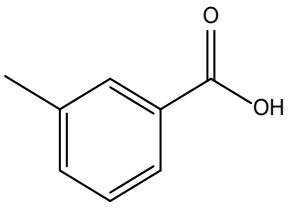


lysmeral

b) a benzoic acid, benzaldehyde, or benzyl alcohol that is ring substituted by

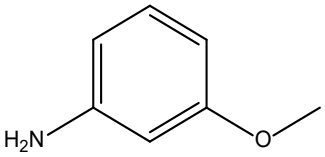
i) any combination of one or more hydroxy (except for *o*-hydroxybenzoic acid derivatives) or ether of ≤4 Cs and/or

ii) a single **linear** alkyl substituent of ≤4 Cs with or without hydroxy or ether present?



3-methylbenzoic acid

i) If yes to a(i)) or a(ii)), assign to Class III.
ii) If yes to b), assign to Class I.
iii) If no to a) and b), proceed to Q41.
Example for no:



m-anisidine

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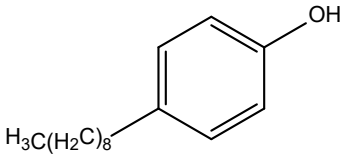
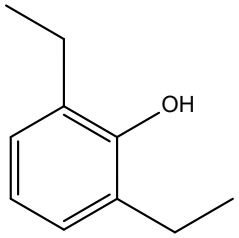
41. Does the substance have only one or a maximum of two **aromatic** ring(s) and is substituted by not more than one phenolic -OH per **aromatic** ring and

a) one or more *o*- or *p*- (to the phenolic -OH) alkyl substituents of ≥ 4 Cs, or



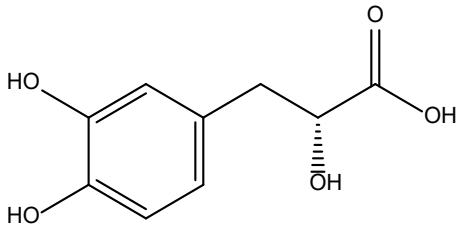
4-n-nonylphenol

b) two *o*-alkyl substituents of ≥1 C but ≤8 Cs?



2,6-diethylphenol

i) If yes a) or b), assign to Class III.
ii) If no to a) and b), proceed to Q42.
Example for no:



(2R)-3-(3,4-dihydroxyphenyl)-2- hydroxypropanoic acid

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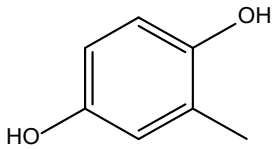
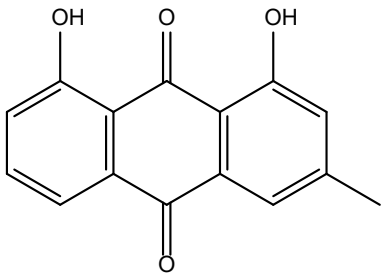
42. Is the substance an

a) *o*- or *p*-hydroquinone, or its methoxy or ethoxy derivative with no additional **oxygenated functional group**, or



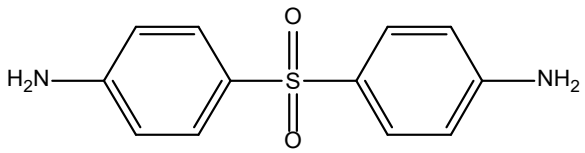
2-methylhydroquinone

b) *o*- or *p*-quinone with **fused aryl** ring(s) (e.g., naphthoquinone), with or without additional alkyl chains (≤4 Cs), and/or one **alicyclic** or an additional **heterocyclic** ring and/or containing the following **oxygenated functional groups**: hydroxy, methoxy, ethoxy, primary or secondary alcohol, aldehyde, ketone, and/or carboxylic acid?



chrysophanol

i) If yes to a), assign to Class II.
ii) If yes to b), assign to Class III.
iii) If no to a) and b), proceed to Q43.
Example for no:



dapsone

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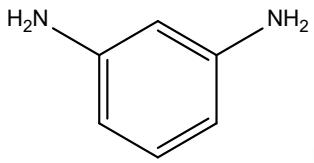
43. Is the substance

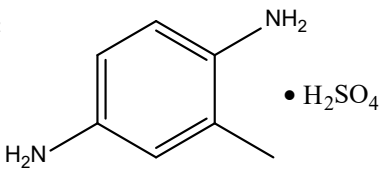
a) a diaminobenzene (or its **related** N-acetyl or N-propionyl derivative), nitroaniline (or its **related** N-acetyl or N-propionyl derivative (i.e., of amine)), or dinitrobenzene either **i)** unsubstituted or substituted with one or more halogens and/or -CF_3 moieties directly bonded to the benzene ring (note: other substituents may also be present in addition to the listed substituents) or **ii)** substituted with one or more ring alkyl substituents of ≤4 Cs only, or

i:



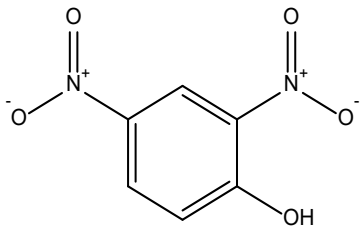
m-phenylenediamine

ii:



2,5-toluenediamine sulfate

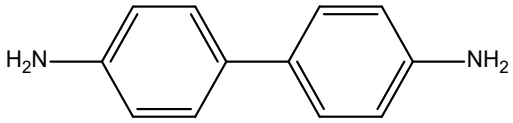
b) a diaminobenzene (or its **related** N-acetyl or N-propionyl substituent), nitroaniline (or its **related** N-acetyl or N-propionyl substituent), or dinitrobenzene with a ring hydroxy, N-(2-hydroxyethyl)-, carboxy, methoxy, ethoxy, with or without additional ring alkyl substituents of ≤4 Cs except those adjacent (*o*-position) to the **oxygenated functional group**. Alkyl substituents may be unsubstituted or substituted by primary or secondary alcohol, aldehyde, ketone, carboxylic acid, ether, or ester substituents, or



2,4-dinitrophenol

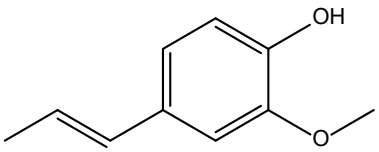
c) a biphenyl, methylenebis(phenyl) and homologues with linkages of ≤4 Cs (a maximum of 4 Cs linking the two phenyl rings), diphenyl ether, diphenylthioether, or diphenylsulfuryl containing **i)** a single primary amine, N-acetyl derivative, or nitro group at the 4-position

(i.e., ***para*** to the **connector**) or **ii)** diamine (or N-acetyl derivative), nitroamine (or N-acetyl derivative), or dinitro groups at the 4,4'- positions and both i) and ii) with or without additional alkyl, methoxy, halogen substituents but not substituted by any other **functional group**?



benzidine

i) If yes to a(i)), a(ii)), c(i)), or c(ii)), assign to Class V.
ii) If yes to b), assign to Class III.
iii) If no to a), b), and c), proceed to Q44.
Example for no:



isoeugenol

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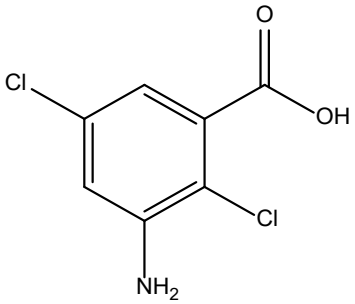
44. Is the substance aniline (or its **related** N-acetyl or N-propionyl derivative) or nitrobenzene with only the following substituents

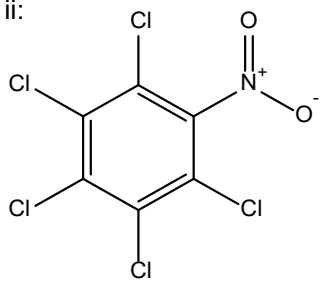
a) **i)** one or more Cl and/or Br substituents with at least one halogen at the *o*- or *p*-position with one or more alkyl substituents of ≤ 4 Cs, hydroxy, carboxy, methoxy, or ethoxy in any of the remaining positions or **ii)** one or more Cl and/or Br substituents with at least one halogen at the *o*- or *p*-position, or

i:



3-amino-2,5-dichlorobenzoic acid

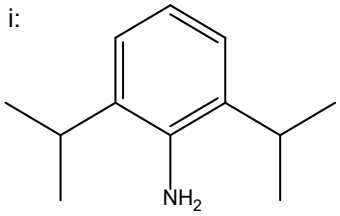
ii:



pentachloronitrobenzene

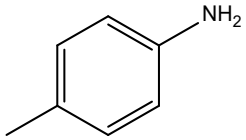
b) **i)** with two alkyl substituents of ≤5 Cs at the *o*-positions or **ii)** alkyl group(s) of ≤4 Cs at any other position (other than at the two *o*-alkyl position) and the compounds described in i) and ii) of this sub-question cannot have any additional **functional groups**, or

i:



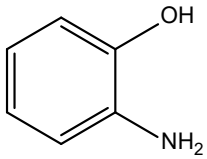
2,6-diisopropyl-aniline

ii:



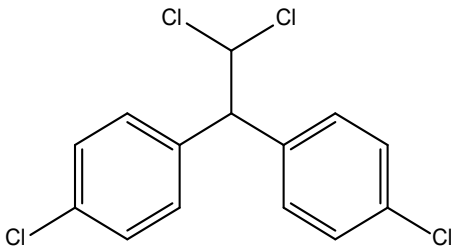
p-toluidine

c) one or more hydroxy, N-(2-hydroxyethyl), carboxy, methoxy, or ethoxy with or without additional alkyl substituents of ≤4 Cs with or without the **oxygenated functional groups**?



o-aminophenol

i) If yes to a(ii)), assign to Class V.
ii) If yes to b(ii)), assign to Class IV.
iii) If yes to a(i)), b(i)), or c), assign to Class III.
iv) If no to a), b), and c), proceed to Q45.
Example for no:



4,4'-(2,2-dichloroethane-1,1-diyl)bis(chlorobenzene)

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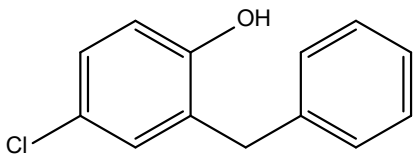
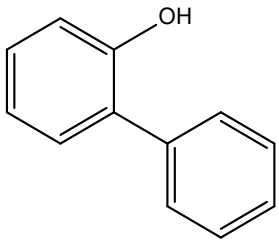
45. Disregarding any combination of **aromatic** ring hydroxy, methoxy, ethoxy, or carboxylic acid, does the mono- or binuclear system contain substituents *other than* **linear**, **simply branched aliphatic chain(s)**, and/or **alicyclic** ring(s) each of ≤8 Cs total, an alkyne (alkynes are evaluated at Q28), a β-ethylamine, a methylenedioxy group **fused** to a benzene ring, together with or without one or more side chain substituent alcohol, methoxy, ethoxy, ketone, aldehyde, carboxylic acid, a mercaptan, thioester, polysulfide, or monosulfide (or it's S-oxide), primary amide, β-ketoamide, secondary amides (but only for simple peptides connecting ≤5 amino acids or their N-acyl derivative), or esters (or sulfate ester), ketals, or acetals that can be **hydrolyzed** to ring substituents of ≤8 Cs?

i) If yes, proceed to Q46.



chlorophene

ii) If no, proceed to Q28.



2-phenylphenol

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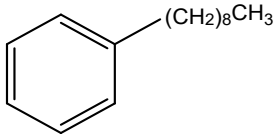
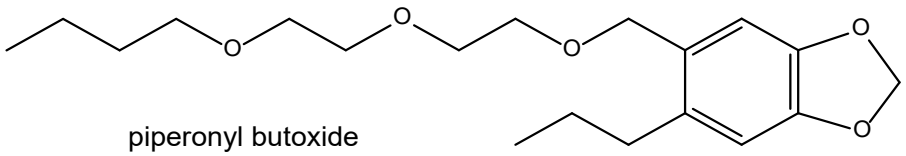
46. Does the substance contain the following moieties with or without those identified in Q45 but no other **functional groups** or moieties

a) **aliphatic** hydrocarbon chains of 9 or 10 Cs <u>or</u>



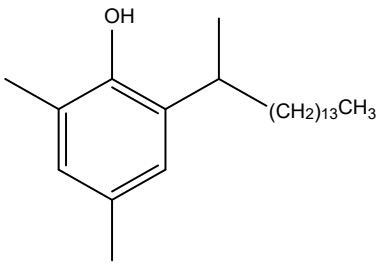
nonylbenzene

b) one or more polyoxyethylene or polyoxypropylene chain(s) ((-O-CH_2-CH_2-)$_n$ and/or (-O-CH_2(CH_3)CH_2-)$_n$) with n≤4 (in total) bonded either to the **aromatic** ring or **aliphatic** side chain?



piperonyl butoxide

i) If yes to a), assign to Class III.
ii) If yes to b), assign to Class II.
iii) If no to a) and b), proceed to Q47.
Example for no:



2-(hexadecan-2-yl)-4,6-dimethylphenol

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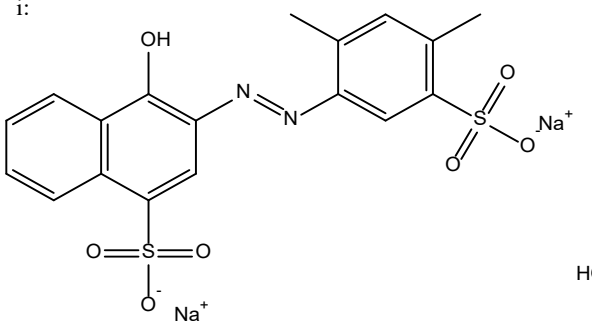
47. Does the substance contain

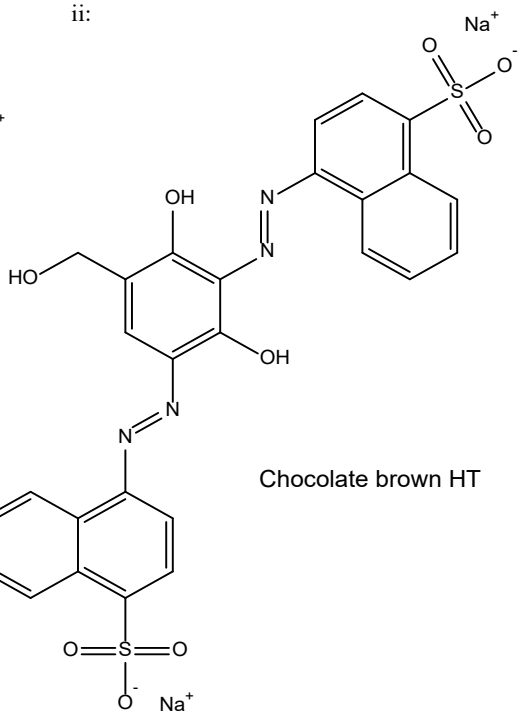
a) **i)** one or more azo (-N=N-) or N-N=C-C=O ↔ -N=N-C=C-O **functional groups** in which each N is bonded to a structural fragment bearing at least one sulfonate, sulfamate, or carboxylate <u>per each fragment</u> and ≤20 Cs per structural fragment without any primary amines except those adjacent to a sulfonate, sulfamate, or carboxylate substituent, or **ii)** one or more azo groups and one or more sulfonate, sulfamate, or carboxylate, but not on each fragment, <u>or</u>

i:



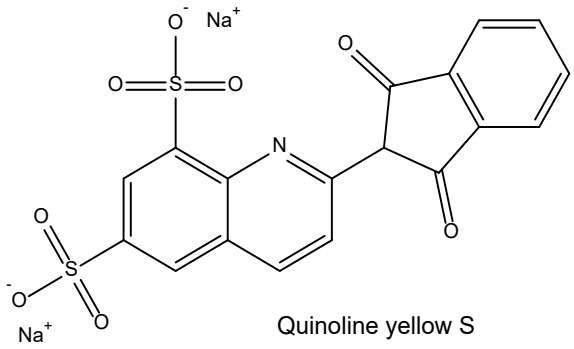
FD&C Red No. 4

ii:



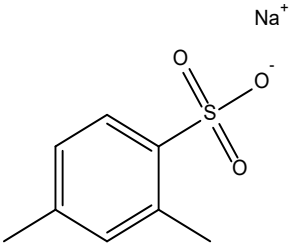
Chocolate brown HT

b) ≥2 sulfonate or sulfamate substituents where there is at least one sulfonate or sulfamate for every ≤10 Cs (note: but no azo functionality), <u>or</u>



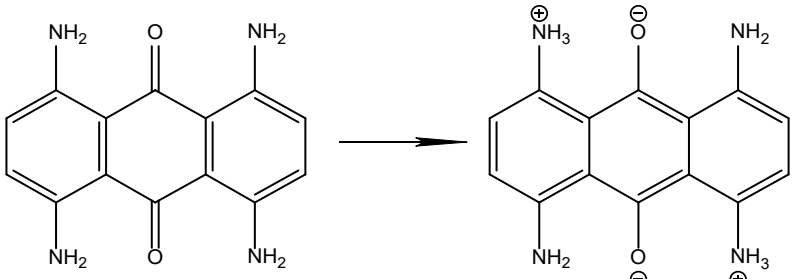
Quinoline yellow S

c) one sulfonate or sulfamate for every ≤20 Cs (but no azo functionality), or



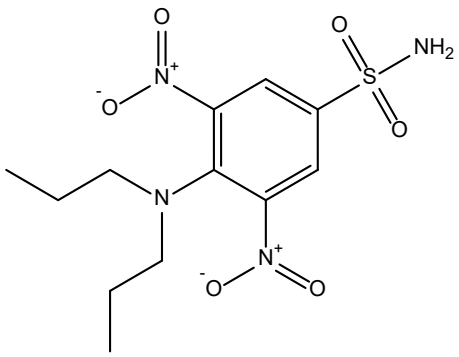
sodium 2,4-dimethylbenzenesulfonate

d) three or more **fused aromatic** and/or **heteroaromatic** rings that can extend conjugation through ring substituents (N or C=O) with the formation of a zwitterion (e.g., N^+ and O^-), or



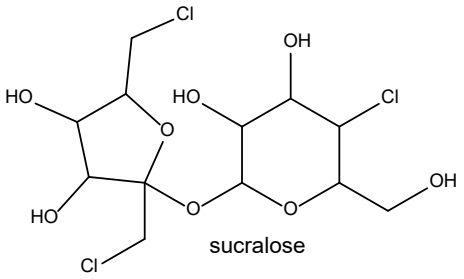
C.I. Disperse Blue zwitterion

e) a mono-**aromatic** benzenesulfonamide containing ≤15 total Cs, or

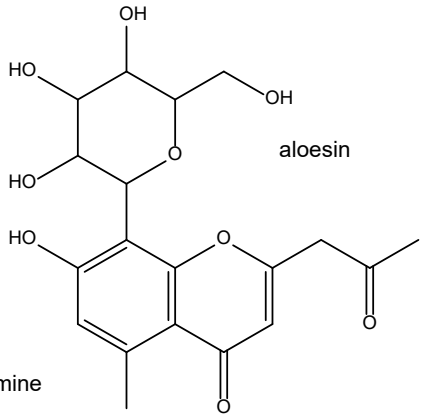


oryzalin

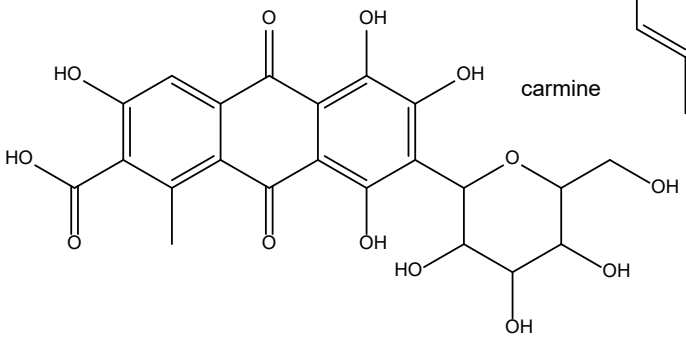
f) at least two or more **alicyclic** ring(s), nonaromatic **heterocyclic** ring(s) (only O is allowed as ring heteroatom), and/or **aromatic** ring(s) and at least one of the rings is a tetrahydropyran (oxane) ring and the tetrahydropyran ring is either **singly bonded** or connected to the rest of the molecule by an -O- in the position next to the tetrahydropyran O and all other (i.e., four) tetrahydropyran ring carbons are substituted by any combination of -OH (minimum 2 must be present), -CH_2-OH, -COOH, and/or Cl (a maximum of 1 Cl). Additionally, except for the fully substituted tetrahydropyran ring, every other ring should have at least two substitutions. The allowed substitutions are any combination of -OH, -COOH, =O, and alkyl (with or without sidechain substitutions), or



sucralose

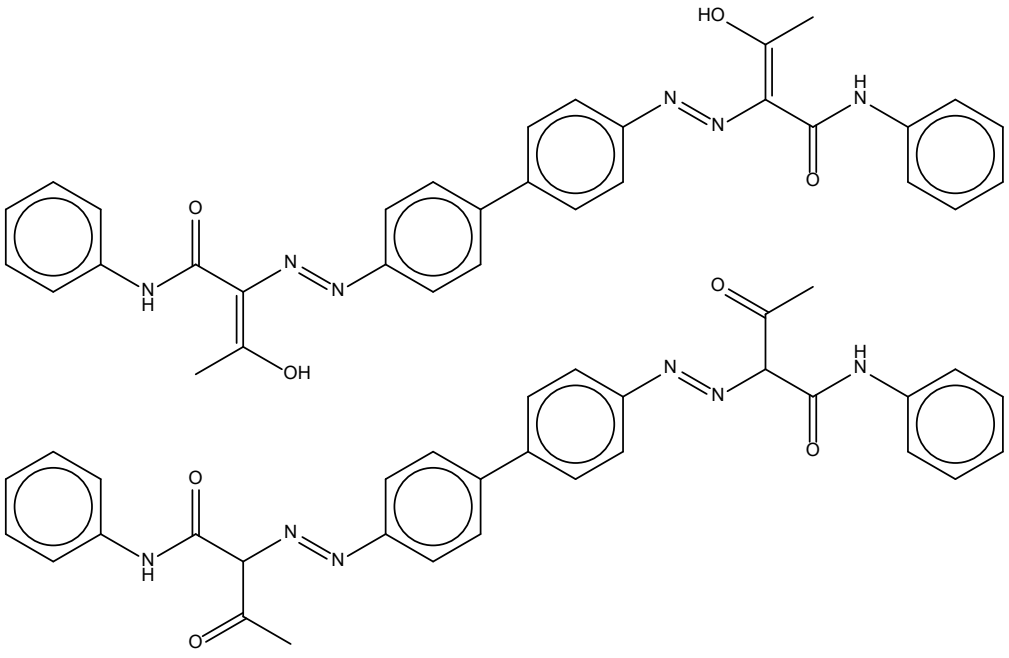


aloesin

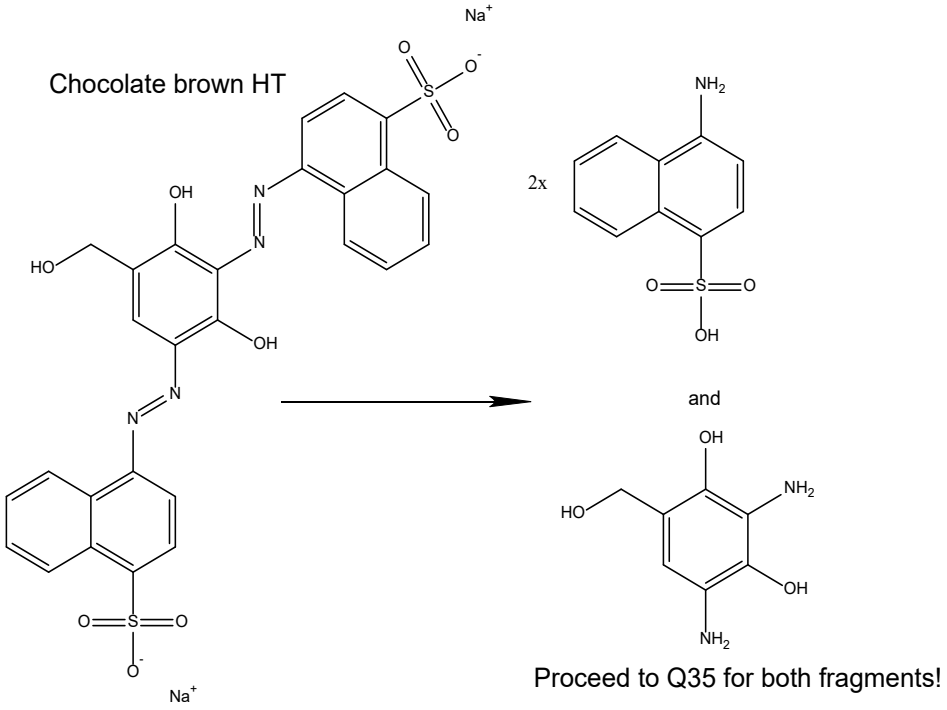


carmine

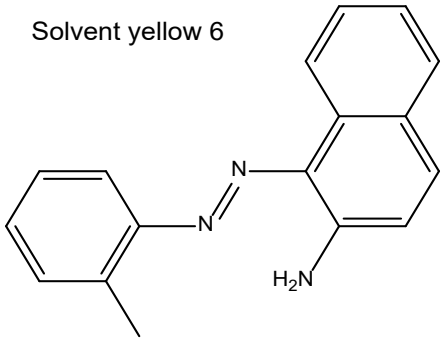
g) any of the **skeletons** below (with or without any **aromatic** ring substitution)?



i) If yes to a(i)) or b), assign to Class I.
ii) If yes to a(ii)), assume the reductive cleavage of the azo function(s). For all **heteroaromatic** fragment(s) produced after **reduction**, proceed to Q19. For all **heterocyclic** (nonaromatic) fragment(s), proceed to Q11. For all **aromatic** fragment(s), proceed to Q35. For all **alicyclic** fragments containing amine substituent, assign the fragment to Class IV. (Note: each azo function may undergo **reduction** in the intestinal lumen resulting in the formation of the corresponding amine fragment. Please note that N-N=C-C=O ↔ -N=N-C=C-O group can participate in azo type **reduction**.)
Example:



iii) If yes to c), f), or g) assign to Class II.
iv) If yes to d) or e), assign to Class III.
v) If no to a), b), c), d), and e), assign to Class IV.
Example for no:



1.8 Description of the Pre-validation EDT Classes

The EDT assigns substances to one of six levels of toxic potentials; from Class I that captures compounds with the lowest toxic potential to Class VI that aims to capture compounds with the highest toxic potential. A description of the types of substances in the original (pre-validation) EDT Chemistry, Toxicity, and Metabolism Database (EDT DB) for each of the classes follows:

- **Class I:** Class I includes two general categories of structures for which there is no predicted safety concern even at relatively high levels of exposure. One type contains those substances and their downstream catabolites that are substrates for ubiquitous high-capacity pathways utilized by carbohydrates, fats, proteins, and nucleotides used for growth and maintenance of animals. The catabolic end products include carbon dioxide, urea, and water. These pathways operate in all animals. Hence, species differences are not a significant concern, and most types of laboratory animals are relevant models for human health assessments. The second type contains those structures with selected functional groups that restrict reactivity or absorption from the gastrointestinal tract. These substances mainly are excreted from the body unchanged.
- **Class II:** In general, substances that fall into Class II display structural features that allow for ready metabolic detoxication *via* Phase I and/or Phase II pathways. At increasing levels of exposure, these pathways may become saturated (e.g., saturation of a Phase I CYP isozyme or Phase II glycine conjugation), amplifying less important intoxication pathways.
- **Class III:** For many congeneric groups in Class III, the metabolism or MoA of the substance is sufficiently different between the animal model and humans (i.e., usually the animal model is more sensitive) to warrant not placing these substances in the next highest structural class (Class IV). This group also contains substances exerting biological effects in an animal model that are not relevant to humans (e.g., α-2m-globulin nephropathy).
- **Class IV**: Class IV contains those substances with structural features expected to react with biomolecules, leading to toxicity in both the animal model and humans. This class also contains substances for which no EDT question could be designed (i.e., it is the major EDT default class).
- **Class V**: Similar to Class IV, Class V contains substances with structural features expected to react with biomolecules, leading to toxicity in both the animal model and humans, but possess even higher toxic potential than Class IV substances. Because of their expected biological activity, many of the Class V substances in the EDT DB are intended for use as herbicides, rodenticides, and other pesticides, while others are natural toxins.
- **Class VI**: Class VI substances in the EDT DB are very toxic at extremely low levels over a short time period. They are mostly chemical warfare agents, certain organophosphate insecticides, selected polychlorinated dibenzodioxins, and other halogenated compounds.

2. The Original (Pre-validation) Expanded Decision Tree Chemistry, Toxicity, and Metabolism Database

2.1 Creation of the Original (Pre-validation) EDT Chemistry, Toxicity, and Metabolism Database (EDT DB)

The EDT DB was created to serve as one of the main bases for the development of new EDT questions and updates of the old CDT questions. We aimed to collect toxicity studies, metabolism, and chemical data for a large number of substances. We identified NELs for a diverse set of chemical structures present in food, whether ordinarily present as nutrients, substances intentionally added to food, or used in food packaging, or unavoidably present due to the food source, preparation, processing, or contamination. In addition, we included data in the EDT DB on substances other than those found in food, such as, but not limited to, those present in cosmetics and pharmaceutical preparations and known environmental toxins.

The substances found in the Munro DB were the starting point for the EDT DB. In addition, we added a large number of studies for new substances harvested from online DBs and reports from authoritative bodies such as Joint WHO/FAO Expert Committee on Food Additives (JECFA), Joint FAO/WHO Meeting on Pesticide Residues (JMPR), US Environmental Protection Agency Integrated Risk Information System (EPA IRIS), US EPA High Production Volume Information System (EPA HPVIS), US EPA Pesticides: Reregistration, California EPA (CalEPA), European Chemicals Agency (ECHA), and European Food Safety Authority (EFSA). Moreover, we added substances to the DB for which animal safety studies were available in the published literature, substances found in FDA's Office of Food Additive Safety (OFAS) administrative records on food and color additives, Generally Recognized As Safe (GRAS) ingredients, food contact substances, and substances with study reports by the National Toxicology Program (NTP). We conducted searches using Google, Google Scholar, and PubMed. The search terms included phrases such as "safety of [chemical name]," "toxicity of [chemical name]," "mode of action of [chemical name]," "carcinogenicity of [chemical name]," with [chemical name] representing both common and IUPAC names for all chemicals.

For many substances, authoritative bodies do not agree as to the NEL and LEL. We reconciled any differences in NEL and LEL values for a substance by our independent evaluation. In general, we adopted the lowest NEL and LEL from these evaluations, except when a NEL was assigned based on an effect that was either not relevant to humans (e.g., α-2μ-globulin nephropathy) or was not adverse (e.g., minor reversible liver effects due to increased metabolic load). To determine whether an effect is adverse, non-adverse, adaptive, or an artifact, FDA consulted various sources, including Pandiri et al. (2017). When we could not determine whether an effect was adverse or not, to err on the side of caution, we assumed that it was adverse. We note that we did not reevaluate the statistical significance of all observations as it was beyond the scope of the project's resources.

We tried to limit the number of substances belonging to a specific congeneric group within each EDT Class to avoid over-weighting the database with too many substances of low toxicity (i.e., high NELs) or high toxicity (i.e., low NELs) from a single closely related congeneric group. In addition to NELs and LELs, study details are included (i.e., species, strain, sex, route, duration, dose levels, endpoints, and summary of results), as well as basic data on each substance's toxicokinetics and metabolic fate in the appropriate animal model and humans; or if not available, metabolism of a close structural analog or predictions from

available commercial software were used, along with results obtained from in vitro metabolism studies. The goal of collecting these data was to help evaluate the influence (or the lack) of absorption and metabolism on the toxicity of the compound rather than to gather comprehensive ADME data for our database. Metabolism data was used to understand how the metabolism of a compound may shift depending on the dose level tested and how this metabolic shift may affect its toxicity (i.e., a compound may be detoxicated to safe metabolites that are easily eliminated at low dose levels, but the metabolism may shift to the production of toxic metabolite(s) at higher dose levels once the detoxication pathways are saturated). We included references for toxicity, toxicokinetics, and metabolism data in the EDT DB. Additionally, for each substance in the EDT DB, a range of descriptors and physiochemical properties (i.e., name, synonyms, CAS number, SMILES code, chemical formula, molecular weight, and water-octanol partition coefficient) are included.

Certain compounds in the literature may have numerous CAS numbers. To help minimize duplicate entries in the EDT DB for the same substance under different CAS numbers, in addition to checking for duplicate CAS numbers, we also scanned the DB for duplicate names and SMILES codes.

Consistent with recommendations from various publications, we represent toxic potency using study duration adjusted NELs expressed in mmol/kg body weight (bw)/day[1] (Escher et al., 2010; Tluczkiewicz et al., 2011). This approach allows for comparisons of NELs between substances based on the number of molecules rather than molecular mass. For example, 0.1 mmol or 7 mg of acetone (molecular weight (MW)=70 mg/mmol) contains the same number of molecules as 0.1 mmol or 111 mg of ciguatoxin (MW=1111 mg/mmol). When comparing the toxic potency of different substances, a weight-to-weight comparison must consider the differences in their molecular weights. Therefore, mole-based NEL adjustments provide a scientifically robust approach for developing structural classes of *relative* toxicity. Moreover, mole-based NELs can improve sensitivity in detecting potential toxicity, particularly for substances with very low mass but high biological activity, ensuring that even low concentrations of highly potent substances are adequately evaluated.

2.2 Criteria for Data Collection and Derivation of Duration, Purity, and Dosing Schedule Adjusted NELs

2.2.1 Criteria for Data Collection

Guidelines on the inclusion of toxicity studies and determination of study NELs were developed and applied to address studies with different duration, type, and route of exposure, species and sex differences, and relevance of toxic endpoints and MoA to humans.

We aimed at collecting studies with a broad toxicological focus for inclusion in the EDT DB. That is, studies where an extensive battery of testing was performed, and the study did not focus on one specific endpoint (such as testicular toxicity or hepatotoxicity). Endpoint specific studies were only included if the study focused on the most sensitive toxic endpoint of the compound as shown by other supporting studies and yielded the lowest

[1] In addition to expressing NELs using units of mmol/kg bw/day, NELs with units of mg/kg bw/day are also listed in the EDT DB.

NEL. Studies conducted using an adequate number of animals were included in the EDT DB to ensure that we could determine whether an effect observed is statistically significant or not compared to controls. We also aimed at collecting studies where the reporting was adequate to determine whether an effect reported was adverse or not.

To support the assessment of data poor congeneric groups, we included studies that did not have an adequate number of animals or had limited reporting[2] to help us formulate EDT questions for the congeneric group in question and to help us determine the appropriate class assignment. To ensure the EDT is sufficiently conservative, in cases where sufficient information was not provided, adversity and significance of the findings were assumed in cases where a more appropriate study was not available.

While our intent was to include studies with at least two dose levels in addition to the controls, some of the substances were tested only in single-dose studies. Therefore, single-dose oral studies (e.g., propyl disulfide) were included if the NEL was within an order of magnitude as that of other members of the congeneric group in multiple dose level subchronic or chronic studies (e.g., dimethyl disulfide).

As the EDT was designed to sort compounds based on/according to their relative chronic *oral* toxic potential, we aimed at collecting oral studies for the EDT DB. Exactly 95% of the studies in the original (pre-validation) EDT DB were performed via the oral route of administration.[3] No oral studies existed for 5% of the substances in the EDT DB. The breakdown of the type of studies by the route of administration are presented in Table 1 below.

Table 1. Distribution of studies in the original (pre-validation) EDT DB based on the route of exposure

Exposure route	Number of studies	Percent of all studies (%)
Dermal	1	0.05
Osmotic minipump	4	0.2
Subcutaneous	5	0.3
Intravenous	13	0.7
Intraperitoneal	24	1.3
Inhalational	48	2.5
Oral	1805	95

As shown in Table 1, for 48 substances in the EDT DB, the chosen representative study involved inhalational exposure. For almost all of these substances, no oral study was available. Inhalation studies in which systemic adverse effects were observed at the LEL were included in the EDT DB but not those with only localized adverse effects (e.g., upper respiratory tract nasal hyperplasia and irritation) and no systemic toxicity. We calculated systemic doses from inhalational exposures. In the final analysis, none of the inhalational

[2] . “Limited reporting” refers to the amount of data and information provided regarding study observations. In some cases, only a brief summary of the study results was available, lacking detailed information on the findings.

[3] For the purposes of the EDT, oral studies are any studies where the test material was delivered into the stomach (i.e., feed, gavage, drinking water (or juice), capsule administration, and nasogastric intubation).

NELs fell within the low 5th percentile NELs used for TTC calculations. The only non-oral study that fell within the low 5th percentile NEL was an intravenous study for a Class V substance.

Our intent was to select NELs from chronic studies; however, for some substances either no chronic study exists, the chronic study did not yield a NEL, or the NEL for the shorter duration study is lower than that for the chronic study. Therefore, we also included NELs in our DB from studies that are not chronic in duration.

2.2.2 Derivation of Duration Adjusted NELs

To account for the non-chronic duration of subacute and sub-chronic studies (other than reproductive and/or developmental studies), the EDT uses duration adjustment factors to estimate the chronic NEL based on these non-chronic studies. A duration factor of 1 is used for studies lasting >98 days, 3 for studies lasting 84-98 days, and 10, the most conservative factor used by any regulatory agency, for studies lasting <84 days.

A review of the scientific literature, regulatory guidance, and technical documents indicated that different adjustment factors are employed to convert subacute and subchronic NELs to chronic NELs. For instance, both FDA's Q3D Elemental Impurities Guidance for Industry (FDA, 2015) and ICH Q3C(R6) Maintenance of the Guideline for Residual Solvents (ICH, 2016) use the following variable factors to account for toxicity studies of short-term exposure: 1 for studies that last at least one half lifetime (1 year for rodents and rabbits, 7 years for dogs and monkeys) and for reproductive studies in which the whole period of organogenesis is covered; 2 for a 6-month study in rodents or 3.5-year study in non-rodents; 5 for a 3-month study in rodents or a 2-year study in non-rodents; and finally, 10 for studies of a shorter duration. On the other hand, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Technical Report No. 110 titled Guidance on Assessment Factors to Derive a DNEL (derived no-effect-level) does not use any adjustments for exposure duration for local effects, and uses an adjustment factor of 2 for subchronic studies (90-day studies) and a factor of 6 for subacute studies (28-day studies) for systemic effects (ECETOC, 2010). These numbers are virtually identical to those proposed by the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) Technical Guidance Document (ECHA, 2012). The values by ECETOC and REACH are based on central estimates (50th percentile) of the distributions for the relationships among subacute/subchronic/chronic NELs (Malkiewicz et al., 2009). Our subchronic to chronic adjustment factor of 3 is in between the factor of 5 (for rodents) found in FDA's Q3D Guidance and ICH's Q3C(R6) Guideline and the factor of 2 recommended by ECETOC and REACH. The value we used therefore represents a middle ground between various recommendations. Moreover, this is the same conversion factor that Munro et al. (1996) used to group subchronic NELs with NELs obtained from chronic studies to derive the cumulative distribution of NELs when they proposed the establishment of TTC levels for the three CDT classes.

In reproductive and/or developmental studies, systemic parental NO(A)EL, reproductive NO(A)EL, and developmental NO(A)EL values are normally provided (or LO(A)EL if no NO(A)EL can be established). For these studies, we assign the lowest NO(A)EL as the overall study NO(A)EL. If the systemic parental or reproductive NO(A)ELs are chosen as the overall study NO(A)EL, we apply duration adjustment factors of either 3 or 10 to generate chronic NO(A)ELs, with the specific factor selected based on the study length. However, if the developmental NO(A)EL is lower than that for either or both parents

and the reproductive NO(A)EL, we select the developmental NO(A)EL without adjusting for study duration. The reason for not applying a duration adjustment factor to developmental NO(A)ELs is that adverse developmental effects arise from in utero exposure within a predefined and relatively short time frame; they are not the result of chronic exposure to the test article by the fetus. This approach aligns with the ICH Harmonised Guideline (ICH, 2016), which specifies a duration adjustment factor (AF) of 1 for "reproductive studies in which the whole period of organogenesis is covered." Additionally, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) Technical Report No. 110 (ECETOC, 2010) indicates that for developmental toxicity, "an AF for exposure duration is not necessary provided that the experimental exposure includes the entire period of gestation, parturition, and the first four days of postnatal life." Consequently, an adjustment factor for exposure duration is generally not required, resulting in an "informed" AF of 1.

2.2.3 Adjustment of NELs Based on Dosing Schedule and Test Article Purity

In some studies, the test material was not administered every day. For these studies, we adjusted for dosing schedule. For example, for a gavage study with a NEL of 50 mg/kg bw given 5 days per week, we calculated the daily dose by multiplying the NEL the number of times the substance was given each week divided by 7. For the above example: (50 mg/kg bw x 5 day/week)/7 days/week=35.7 mg/kg bw/day.

When purity of the test substance was available, for substances with less than 95% purity, the NEL was adjusted for purity. For example, for a study with a NEL of 50 mg/kg bw/day and a purity of 80%, the study NEL was changed to 40 mg/kg bw/day ((50/80)x100).

2.2.4 Consideration of Sex- and Species-specific Effects and Metabolism When Establishing NELs

Regarding sex-specific effects, if the NEL for one sex of a species has been established as irrelevant to humans (e.g., α_{2u}-globulin mediated nephrotoxicity in male rats), we selected the NEL for the other sex of the same species. For instance, we included toxicological data (i.e., NEL and LEL values) for female rats only in the EDT DB for aliphatic, alicyclic, or aromatic ketones or hydrocarbons that possess sufficient molecular weight and lipophilicity, which cause α_{2u}-globulin-type nephropathy—a non-relevant endpoint to humans that is observed exclusively in male rats.

For species-specific effects, if the NEL for both sexes of the same species had been shown to be irrelevant in humans (e.g., chronic progressive nephropathy, a common spontaneous kidney disease of aging rats, including F344 rats (McInnes, 2017)), we chose a NEL from a more relevant species, if available.

We also evaluated toxic effects in the context of enzyme catalyzed and uncatalyzed metabolism[4], metabolic options available, saturation of these options under conditions of the study, reactivity of intermediates, and disposition of the metabolites formed in humans and

[4] Uncatalyzed metabolism refers to biochemical reactions that occur without the assistance of enzymes. These reactions typically include hydrolysis (the breakdown of compounds by the addition of water, which can occur without enzyme involvement) and non-enzymatic conjugation (reactions where small molecules (like glutathione) may react with electrophiles without specific enzyme catalysis).

animal models. These factors, together with the NEL range for the congeneric group, also were considered in the assignment of structural class.

2.3 Inclusion of Carcinogens in the Original (Pre-validation) EDT Chemistry, Toxicology, & Metabolism Database

In some cancer risk assessment paradigms, non-genotoxic carcinogens are assumed to produce nonlinear dose-response curves. Therefore, nongenotoxic carcinogens may have a threshold of exposure below which tumor development is not anticipated. In contrast, the dose response to genotoxic carcinogens is, historically, assumed to be linear, that is, a straight line approaching zero (Nohmi, 2018). This means any exposure to a genotoxic carcinogen is assumed to have a risk, and the higher the exposure, the greater the risk.

The absence of a threshold in the action of genotoxic carcinogens was postulated decades ago but has been challenged continually for scientific and practical reasons. More recent science has shown that thresholds) exist for several genotoxic carcinogens, (Fukushima et al., 2014; Kakehashi et al., 2014; Hengstler et al., 2003; Zito, 2001; Müller and Kasper, 2000; Lutz and Kopp-Schneider, 1999; Nohmi and Tsuzuki, 2016).

Diverse protective mechanisms may contribute to no-effect thresholds for genotoxic carcinogens. Key mechanisms contributing to threshold doses are carcinogen detoxication (metabolic inactivation), lack of activation, scavenging mechanisms, and DNA repair or error-free translesion DNA synthesis (Kaina et al., 2015; Nohmi, 2018). Elimination of cells harboring premutagenic DNA lesions by apoptosis and other cell death pathways and reduced proliferation rates within tissues may minimize the effects of mutation and may therefore contribute to threshold dose effects. Consequently, carcinogens that show a threshold effect in carcinogenesis studies may have a threshold of exposure below which tumor development is not anticipated, regardless of whether they are genotoxic or non-genotoxic.

Therefore, as long as toxicological studies yielded an overall NO(A)EL for which neither carcinogenic nor noncarcinogenic effects were observed for a carcinogenic substance, we included the substance in the DB and used its NO(A)EL to calculate its class TTC, regardless of whether it was a non-genotoxic or genotoxic carcinogen. We note that there are non-threshold mode of actions and other factors, such as short-term study durations (less than 1 or 2 years), that might have contributed to the fact that a NO(A)EL could be derived for some carcinogens.

FDA would like to emphasize that while we included these compounds in our database and designed EDT questions to capture them, it is ultimately up to each user, including regulatory agencies, to determine whether to apply the EDT for nongenotoxic and/or genotoxic carcinogens based on specific regulatory frameworks and program areas. The EDT is a scientific tool designed to inform safety and risk assessment by providing a prediction for oral chronic potency and establish conservative threshold exposures. It is not intended to replace assessment of potential genotoxicity or carcinogenic risk assessment when such an evaluation is warranted, nor does it represent an approach to satisfy a regulatory requirement stipulated in existing rules or regulations.

3. The Pre-validation Threshold of Toxicological Concern Levels (TTCs)

3.1 The Threshold of Toxicological Concern

Munro et al. (1996) compiled a DB of 2,941 NOELs from chronic and sub-chronic oral toxicity studies for 613 organic substances likely in commerce. To approximate chronic NOELs, subchronic NOELs were divided by a factor of three. Each chemical was assigned to one of the three CDT classes. For each class, the authors created cumulative distributions of the NOELs and determined the lowest 5th percentile NOEL value for each of the three classes. A safety factor of 100 was applied to each of the 5th percentile NOEL values, and the resulting values were converted from NOEL values in mg/kg bw/day to mg/person (p)/day by multiplying by a factor of 60 (60 kg, treated as the default body weight of an adult person by Munro et al.) to obtain a TTC value for each CDT class. The TTC values are considered conservative threshold values because 1) the 5th percentile NOELs were used during calculations providing 95% confidence that the NOEL of another substance in the same class would not have a NOEL less than the class 5th percentile NOEL and 2) a conservative safety factor of 100 was applied to the 5th percentile NOEL.

Munro et al. (1996) presented three TTC values: 1.8 mg/p/day for Class I, 0.54 mg/p/day for Class II, and 0.09 mg/p/day for Class III. The analysis suggested that an experimentally derived ADI for any structurally defined substance would likely be higher than the CDT estimated threshold, suggesting that the majority of substances could be considered safe up to the CDT threshold values. Substances with intakes greater than their class TTC values and having little or no existing safety-related data would require additional evaluation and potentially the provision of additional toxicity, metabolism, or intake data for their safety evaluations.

Based on additional analysis of NOELs, a congeneric group specific TTC value of 18 µg/p/day was proposed for organophosphates, and later for the combined group of organophosphates and carbamates, along with a TTC level of 90 µg/p/day for organohalogens (Kroes, et al., 2004; Leeman et al., 2014). An even lower endpoint specific TTC of 0.15 µg/p/day was suggested for substances with a structural alert for genotoxicity (Kroes et al., 2004; Müller et al., 2006). When organophosphates were removed from the Munro et al. (1996) DB, the structural Class III TTC value increased from 90 to 180 µg/p/day, and when organophosphates and organohalogens were removed, this value further increased to 600 µg/p/day (Munro et al., 2008). Exclusion of organophosphates and carbamates from the Munro et al. (2008) dataset raises the Class III TTC to 132 µg/p/day, exclusion of organohalogens raises the Class III TTC to 108 µg/p/day, and elimination of organophosphates, carbamates, and organohalogens increases this value to 240 µg/p/day (Leeman et al., 2014).

3.2 The Adaptation of the CDT-TTC Approach for Safety Assessment

While created in 1978, the CDT remained in relative obscurity until 1995, when JECFA recommended that a safety evaluation procedure for flavoring agents that incorporated the CDT and the TTC levels of the CDT classes into the evaluation process “should be applied to the evaluation of a number of flavoring agents belonging to different chemical classes in order to assess its utility in practice” at a future meeting (as cited in WHO, 1995). In 1996, JECFA used the procedure to evaluate three groups of flavoring agents and found the procedure to provide “a sound basis” for their safety evaluation (WHO, 1997). Additionally, JECFA recommended that the procedure should be used at future meetings for the safety

evaluation of groups of flavoring agents and discussed a safety evaluation procedure that included the application of the TTC approach (WHO, 1995; Munro et al., 1996) and the use of the CDT to assign chemical substances to one of three classes of toxic potential. The procedure was tested and adopted by JECFA the following year in a pilot program evaluating three chemical categories containing 46 flavoring agents (JECFA, 1996). Each year since the procedure was adopted, JECFA received data for different chemical categories (later recognized as congeneric groups) for its safety evaluation. According to the initial procedure, each substance in a congeneric group is first screened by passing it through the CDT, resulting in its assignment to one of the three CDT classes. The substance then is evaluated for its metabolic fate. If the substance is considered adequately detoxified under reported conditions of intake and if the intake is less than the TTC for the respective CDT structural class, it is considered safe under current conditions of intake. However, if the intake is greater than the TTC threshold and/or the substance is not readily detoxified, additional data are needed to evaluate the safety of the substance. Those data were available for some of the other members of the congeneric group being reviewed, so relatively few additional studies were needed. The JECFA evaluation procedure has been successfully used for almost three decades for the safety assessment of flavoring agents. More recently, the procedure has been modified to address genotoxicity concerns. The JECFA approach to flavoring agents has become a model for the safety evaluation of other substances with low-exposure scenarios. The scientific data underpinning JECFA's conclusions for different chemical categories, encompassing more than 2,800 flavoring substances, are available (https://www.who.int/groups/joint-fao-who-expert-committee-on-food-additives-(jecfa)/publications/toxicological-monographs) and many appear in peer-reviewed publications (e.g., Adams et al., 1996, 2002, 2007; Newberne et al., 1999; Smith et al., 2002).

Other non-food ingredient regulatory bodies have applied the TTC approach, including assessment of mutagenic impurities in pharmaceutical preparations by the European Medicines Agency (EMA) and FDA (EMA, 2018; FDA, 2018) and the evaluation of genotoxic constituents in herbal substances and preparations by EMA (EMA, 2007). Based on numerous applications and validation of the TTC concept as described above, and the EFSA and WHO review of the TTC approach (EFSA and WHO, 2016), the TTC concept now is widely recognized as useful in the screening, prioritization, and safety evaluation of substances with low exposure scenarios.

3.3 Derivation of the Pre-validation EDT TTC Levels

The EDT DB contains 1,900 substances, 1,628 of which have established NELs. Given the available data, only LELs could be established for the remaining 272 substances. Because the EDT DB of 1,628 NELs is sufficiently robust for its purpose, we chose not to generate additional NELs from LELs.

The NELs (mmol/kg bw/day) within the EDT DB span a range of 11 orders of magnitude ($1.25x10^{2}$ to $3.11x10^{-9}$) while the NELs (mg/kg bw/day) within the Munro DB span only 6 orders of magnitude. This indicates that the substances in the EDT DB have NO(A)ELs that cover nearly double the range of those in the Munro DB. Consequently, FDA concluded that it was essential to double the number of classes used by Munro to adequately account for the expanded range observed in the EDT DB compared to the Munro DB.

When deriving TTCs for EDT Classes I through V, we used only NELs from studies with a minimum duration of 84 days. Class VI substances are those that are very toxic, even at

very low intake levels over a short period of time. Consequently, we only have 52 substances in Class VI, and 46 of these have a NEL. Thirty-five of these yielded a NEL in a study of 84 days or longer. Therefore, to calculate the Class VI TTC, we decided to also use the 11 NELs from studies with a duration of less than 84 days (but no one-day studies). We used a factor of 10 to adjust for the short duration to calculate chronic DNELs, as described earlier. As noted earlier, to derive a chronic NEL from a study with a duration that is less than subchronic, 6 or 10 is normally used. We used the most conservative factor of 10 to ensure that the Class VI TTC is protective for the most toxic substances that exist.

For each EDT Class, we determined the lowest 5th percentile NEL, simply taken from the data, in units of mmol/kg bw/day using Excel's percentile function. (Please note that only NELs were used for the calculation of the TTCs and no ADIs or RfDs.) We calculated the TTCs using a conservative safety factor of 100 (a factor of 10 for interspecies and 10 for intraspecies variation), in units of µmol/kg bw/day. Additionally, using the median MW of each structural class, we calculated the six EDT TTC values in units of µg/kg bw/day to make it easier to compare the EDT TTCs to the Cramer TTCs. We note that we have decided not to calculate TTC values in units of µg/person (p)/day as different regulatory agencies use various values (60, 70, or 80 kg) for the average adult body weight in their safety evaluations and risk assessments (US EPA, 2011; EFSA, 2012; AUS-DHHS, 2012; Portier et al., 2007). We leave it up to each individual regulatory agency and the various regulatory programs within an agency to determine what they find the appropriate adult body weight to be and calculate TTCs in µg/p/day; and whether they want to calculate separate TTCs for the various life stages of children (each with different average body weights). Additionally, as neonates and infants have different metabolic capabilities than older children (Fernandez et al., 2011), special considerations should be made for neonates and infants.

3.4 The Pre-validation EDT TTC Values

Table 2 provides the pre-validation EDT TTCs calculated based on the data contained in the EDT DB according to the method described in section 3.3.

Table 2. The pre-validation EDT TTCs

EDT Class	**I**	**II**	**III**	**IV**	**V**	**VI**
EDT TTC (µmol/kg bw/d)	2.34	3.07×10^{-1}	8.80×10^{-2}	1.24×10^{-2}	1.09×10^{-4}	9.40×10^{-7}
Median MW	172.26	166.13	164.25	237.45	286.30	319.65
EDT TTC (µg/kg bw/d)	403	51	14	2.9	0.031	0.00030
Total # of substances	223	352	321	606	346	52
# of substances used for TTC calculation*	180	264	229	405	188	46

* The discrepancy between the total number of substances in each class and those used for TTC calculations arises because some studies provided only LOAELs and no NOAELs, which prevented their inclusion in the calculations. Additionally, NO(A)ELs from studies shorter than 84 days were excluded from TTC calculations for Classes I-V.

Formula:

TTC (μg/kg bw/d)=[(5^{th} percentile NEL (mmol/kg bw/d) x 1000 (μmol/mmol) x med. MW]/100

where 100 is the factor used for inter- (10) and intraspecies (10) variation and med stands for median.

To show that using the median MWs to calculate the TTCs is a valid method, the TTCs were also calculated directly from the duration adjusted NELs with units of mg/kg bw/day without the use of the median MW. A comparison of the data showed that the TTCs obtained using the median MW of each class are comparable with the TTCs calculated without the use of the median MW (see Table 3).

Table 3. Comparison of the pre-validation EDT TTCs calculated various ways

EDT Class	**I**	**II**	**III**	**IV**	**V**	**VI**
EDT TTC (μg/kg bw/d)*	403	51	14	2.9	0.031	0.00030
EDT TTC (μg/kg bw/d)**	401	53	16	2.4	0.032	0.00046
* Calculated from the duration adjusted NELs (mmol/kg bw/d) using the class median MWs (these values are considered to be the pre-validation EDT TTCs) **Calculated directly from the duration adjusted study NELs (mg/kg bw/d) without using median MWs						

Percentage of substances assigned to Classes I through VI by the EDT are (%, (class)): 11.7% (I), 18.5% (II), 16.9% (III), 31.9% (IV), 18.2% (V), and 2.73% (VI). The percentage of substances assigned to CDT default Class III in the Munro (73%) or Tluczkiewicz (77%) (Munro et al., 1996; Tluczkiewicz et al., 2011) DBs was significantly reduced in default Class IV in the EDT DB (<32%). Additionally, a large number of substances in EDT Class IV were placed into this class based on their toxic potentials (i.e., EDT questions probing their structural features and toxic potentials exist), and they are not just simply defaulted into Class IV.

The 5^{th} and 95^{th} percentile NELs of each EDT class are within approximately two orders of magnitude in Classes I through IV. Different congeneric groups with a similar NEL range constitute a structural class. Hence, a congeneric group of 14 organophosphites with bulky substituents that inhibit the oxidation of phosphite to phosphate (captured at Q2) and exhibit a NEL range from 0.041 to 2.64 mmol/kg bw/day are assigned to Class III, as is the congeneric group of 10 heterocycles containing an α-ketoenol moiety in which the enolic double bond is further conjugated (captured at Q16) spanning a NEL range of 0.018 to 1.56 mmol/kg bw/day.

Based on the relatively narrow range of NELs of EDT structural classes I through IV and the very low NELs for Classes V and VI, overlap among EDT classes is significantly less than the overlap of NELs for the three classes presented in the Munro et al., (1996) DB or the RepDose DB (Escher et al., 2010; Tluczkiewicz et al., 2011) (Figure 1). However, this comparison should be taken in the context that considerable overlap is unavoidable, given

that the substances included in these other DBs are sorted into only three structural classes because of the limited number of questions in the CDT, which does not allow for sufficient differentiation of such a diverse set of structures.

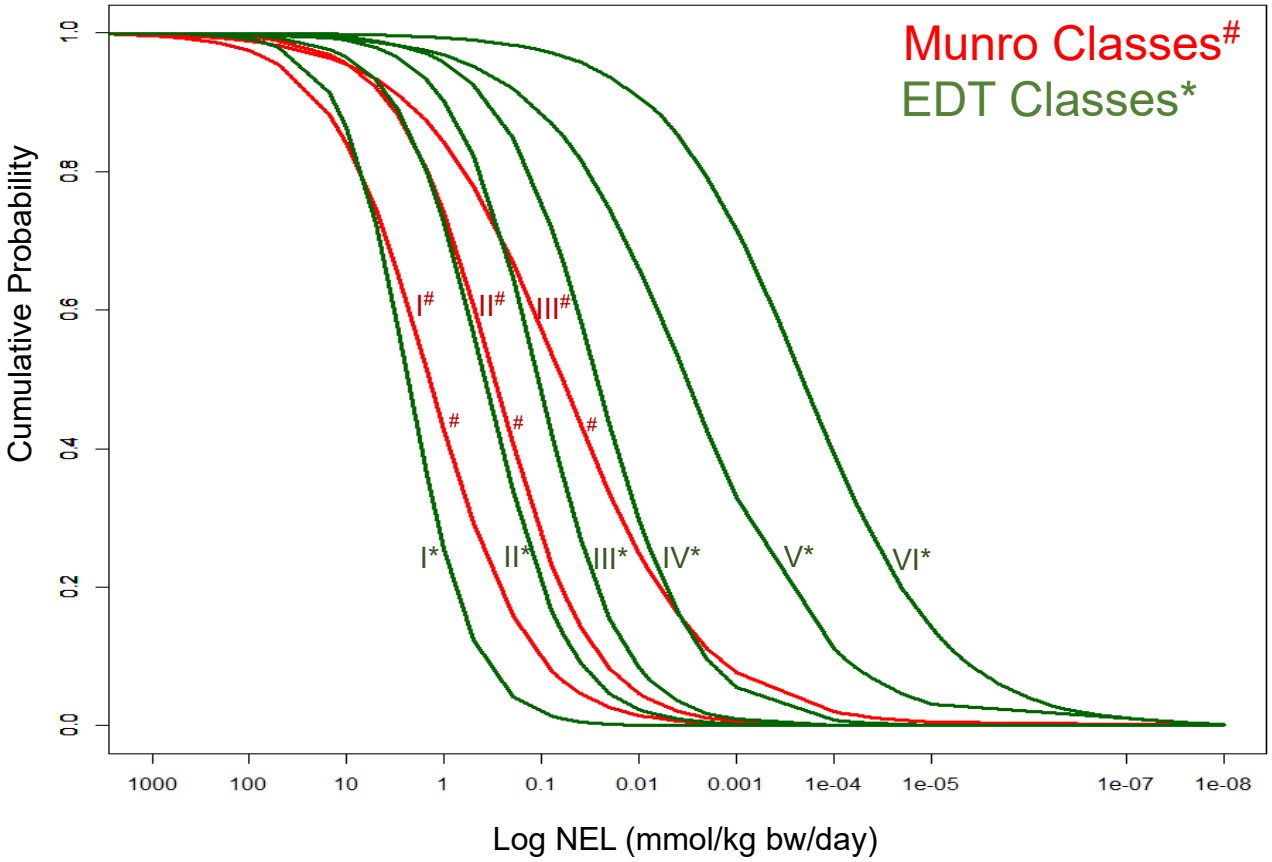


Figure 1. Overlap of NELs: CDT vs EDT

4. The Validation of the Expanded Decision Tree

4.1 The Purpose of the Validation

The validation of the EDT was carried out to show that i) the preliminary EDT TTCs are protective when applied to a large set of naïve compounds and ii) the EDT is fit for its purpose and can accurately sort compounds with a broad range of structural variation based on/according to their relative chronic oral toxic potential.

4.2 The Creation of the External Validation Database

As validation is very important in demonstrating that the EDT is fit for its purpose, we needed to create a new DB containing compounds other than the 1,900 found in the EDT DB. The data for the external validation DB were harvested from the ToxVal DB (https://comptox.epa.gov/dashboard/chemical-lists/TOXVAL_V5) that the US Environmental Protection Agency (EPA) has been building for years. The harvested data were based on all data that was available to EPA as of February 8, 2021, and some of the data that FDA received were not publicly available on EPA's ToxVal DB site at that time.

The data were filtered down by EPA to a subset containing compounds with defined structures and which were tested in subchronic and chronic oral toxicological studies that produced NELs. For each compound in the external validation DB, EPA provided the following information: preferred name, CAS number, SMILES code, average mass, ToxVal ID, type of data (i.e., NOAEL or NOEL), numeric qualifier (whether the NEL was equal to the value given or was equal to or larger than the value given (in cases where the NEL was the top dose level tested, and as such the true NEL might be higher)), the numeric value of the NEL, the unit for the NEL, whether the dose corresponding to the NEL was nominal or the actual dose received, risk assessment class and study type (i.e., whether the study is a chronic, subchronic, or reproductive toxicity study), study duration, species, strain, sex, exposure type (only oral studies were kept), exposure method (diet (or feed), gavage, capsule, drinking water, or unspecified), critical effect, reference, and URL for the toxicological data.

4.3 Processing and Verification of the Data in the External Validation Database

4.3.1 Elimination of Duplicate Substances from the External Validation Database

In the first step, the substances in the external validation DB were cross-referenced with those found in the EDT DB, and all substances were deleted from the external validation DB that were found in both DBs (i.e., the duplicate substances). We note that some compounds can exist in various forms. For example, acetic acid may exist as the free acid or may form a salt with sodium, potassium, calcium, magnesium, and numerous other ions. Once ingested, they dissociate to acetate and the inorganic counterion. Some common counterions (e.g., calcium) are either nontoxic or have very low oral toxicity (i.e., safe even at relatively high intake levels, intake levels that are higher than the EDT Class I TTC). We consider these salts (e.g., sodium and calcium acetate) and the neutral form of the substance (in the above example acetic acid) toxicologically equivalent. In these cases, usually the organic part of the compound drives the compound's toxic potential. Therefore, we only allowed one form to be present in the external validation DB, and if one form was already present in the EDT DB, we deleted these substances from the external validation DB.

That stated, not all inorganic counterions are created equal. For example, cadmium ion is more toxic than calcium ion. While in the case of calcium acetate the toxicity of calcium would not drive the toxicity of calcium acetate, in the case of cadmium acetate, the driving force of toxicity is clearly the cadmium ion, a toxic inorganic element. As in this case the toxicity is mostly due to the presence of the inorganic counterion, we would not consider calcium and cadmium acetate as toxicologically equivalent. Additionally, these substances would not be classified at the same EDT question and would not be placed into the same EDT class. Hence, if a toxic salt form of a compound was present in the external validation DB along with a salt form possessing a 'non-toxic' or of 'low toxicity' counterion, and as a result these substances were classified at different EDT questions, we kept both entries in the external validation DB. Alternately, if one form (e.g., the nontoxic counterion) was present in the EDT DB, we included the other form (in this case the toxic counterion) in the external validation DB.

Certain compounds may have numerous CAS numbers. For example, according to PubChem, Lindane has the following CAS numbers: 319-84-6, 319-85-7, 319-86-8, 608-73-1, 58-89-9, 6108-10-7, 6108-11-8, 6108-12-9, and 6108-13-0 along with five deleted (no

longer used) CAS numbers (NCBI, 2021). We had Lindane in our EDT DB under the CAS number 58-89-9 and in the external validation DB with the CAS number 6108-10-7. We also found that within the external validation DB, a few compounds were entered more than once with different CAS numbers. For example, beta-ionone was found in the external validation DB under the names beta-Ionone (CAS 79-77-6) and 4-(2,6,6-Trimethyl-cyclohex-1-enyl)-but-3-en-2-one (CAS 14901-07-6), seemingly two different substances. We deleted all duplicates. To help minimize duplicates, in addition to checking for duplicate CAS numbers and names, we also scanned each DB for duplicate SMILES codes and for partial matches. Moreover, once classified, compounds were grouped by question, sub-question, and sub-sub-question. As non-toxic or low-toxic potential counterions are disregarded by the EDT, various salt forms and the neutral form of the same compound are normally classified under the same question, sub-question, or sub-sub-question. For salts, the presence of other salt forms or the neutral form was manually examined within the same question, sub-question, and sub-sub-question. The form with the best representative study was chosen to represent the substance and its various salt and neutral forms.

4.3.2 Elimination of Substances Outside the Applicability Domain of the EDT from the External Validation Database

In the next step, we eliminated all compounds not in the applicability domain of the EDT (i.e., unhydrolyzable polymers, proteins, elements, inorganic substances, and substances with undefined structures in addition to most mixtures).[5] While some mixtures were easy to identify based on the "preferred name" in the external validation DB, for others it was not obvious that they were mixtures. For example, we had one row of entry for 4-methyl-2,6-dimethoxyphenol (CAS 6638-05-7) in the external validation DB; a single structurally defined substance. When we reviewed the associated toxicological data provided in the external validation DB, it became apparent that the test article in the 90-day oral study in rats was Scansmoke SEF7525, a complex mixture of numerous components (EFSA, 2012b). 4-Methyl-2,6-dimethoxyphenol constituted only 6.2 to 9.2% of this mixture. Hence, we deemed this study to be inappropriate to represent 4-methyl-2,6-dimethoxyphenol for the purpose of this validation.

In cases where the true structure of the test substance was unclear and could not be elucidated, these substances were deleted from the external validation DB. For example, "copper napthenate,"[6] CAS number of 1338-02-9, in the validation DB obtained from EPA was not associated with a SIMLES code. Searching for structural information in PubChem, ChemSpider, and ChemIDplus using the provided name and/or CAS number resulted in various potential structures. Also, a search of Regulation.gov for documents on the registration review and/or human health effects data and/or risk assessment of this compound yielded various structures represented by the same name and CAS number. As

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[5] Some mixtures with one major component that made up a large percentage of a composition may have been kept in the external validation DB. In these cases, the study was listed under the major component and the dose levels and the NEL were adjusted based on the percent composition of the main ingredient.
[6] Original spelling from the external validation DB. On certain websites the spelling of the compound was copper naphthenate (same CAS).

such, this substance and those exhibiting similar issues were removed from the external validation DB.

4.3.3 Dealing with Read-across Data

A cursory review of the toxicological data in the external validation DB from the EPA indicated that some of the NELs for a specific substance were actually not for that substance but for a read-across substance (especially those for which ECHA was listed as the reference). For the read-across studies, we changed the name of the substance in the external validation DB to the name of the true test article used in the toxicological study as long as that true test substance was present neither in the EDT DB nor in the external validation DB. All other read-across data were deleted.

4.3.4 Verification and Selection of NEL Values for Each Study in the External Validation Database

In addition to confirming that each study is listed under the test item employed in that study, we verified that the correct NEL value was listed for each study. To achieve this, we located and reviewed the original study report, if available, along with any other documents containing the study details. In addition, we searched opinions and risk and safety assessment reports from a large number of authoritative bodies such as FDA, EPA (Human Health Risk Assessment documents, IRIS, RED, and HPVIS), CalEPA, ECHA, EFSA, EMA, JECFA, JMPR, and others. We found that in many cases the authors and/or the above bodies did not agree on NOAELs for specific studies. In these cases, we used our own judgement to settle on the most appropriate NOAEL value for each study in the external validation DB using the same NEL selection criteria as used for the original (pre-validation) EDT DB. As an example, the relatively simple case of Chlorpyrifos-methyl is presented below.

Chlorpyrifos-methyl (CAS 5598-13-0) is an organophosphate pesticide used to control insects in stored grain and other food products. It is a methyl ester derivative of chlorpyrifos, which is another widely known organophosphate insecticide. Chlorpyrifos-methyl has a similar mode of action to chlorpyrifos, targeting the nervous systems of pests by inhibiting the enzyme acetylcholinesterase, which is crucial for nerve function.

Chlorpyrifos-methyl was tested in chronic dietary toxicity/oncogenicity study in rats at 0, 0.05, 0.1, 1, or 50 mg/kg bw/day (Barna-Lloyd et al., 1991). The original study report by Barna-Lloyd et al. (1991) is not publicly available. A literature search for this substance yielded that JMPR (JMPR, 2009), EPA (EPA, 2015), and EFSA (EFSA, 2019) evaluated the safety of this substance and considered this study in their evaluations.

According to JMPR, “A NOAEL of 1 mg/kg bw per day can be determined for this study, based on decreased brain cholinesterase activity, increased adrenal weights and associated histopathology at 50 mg/kg bw per day. Animals were fasted prior to termination; it is therefore possible that terminal cholinesterase inhibition was underestimated in this study. However, reassurance is gained from cholinesterase results in a previous 2-year rat study (Barna-Lloyd, Szabo & Davis, 1991).” JMPR goes on stating that “A histopathology review panel performed a “blind” reading of the adrenal slides from the study of Barna-Lloyd, Szabo & Davis (1991). The review included a scoring for severity of vacuolation that was absent from the original study. The review panel concluded that the findings of adrenal vacuolation at 1 mg/kg bw per day and below were consistent with background findings and

that the only dose producing clear effects was the top dose of 50 mg/kg bw per day (Table 19) (Bruner & Gopinath, 2000)."

According to EPA, "In the rat combined chronic toxicity/ carcinogenicity study (MRID 42269001), the NOAEL and LOAEL for RBC ChEI were established at 1.0 and 50.0 mg/kg/day, respectively, but there were no indications of clinical signs. At 50 mg/kg/day in the rat, body weight decreases, alterations in the adrenals (increased weight, slight to moderate vacuolation with lipid accumulation in the zona fasciculata) were observed."

And finally, according to EFSA, "The main effect following short- to long-term repeated oral administration of chlorpyrifos-methyl was the inhibition of acetylcholinesterase (AChE) activity, which, at high-dose levels, was leading to endogenous cholinergic overstimulation resulting in typical cholinergic symptoms. Erythrocyte (red blood cell (RBC)) AChE inhibition was the critical effect in all studies conducted with rats, mice and dogs. Additionally, the adrenals (increased weight, hypertrophy and vacuolation of cells of the zona fasciculata) were identified as target organ of chlorpyrifos-methyl in rats. The relevant no observed adverse effect level (NOAEL) for short-term toxicity was 0.65 mg/kg body weight (bw) per day from the 28-day toxicity study in mice and 0.1 mg/kg bw per day for long-term exposure from the 2-year study in rats[7] based on significant decrease of RBC AChE activity in both studies and adrenal toxicity upon long-term exposure in rats only."

FDA assigned a NOAEL of 1 mg/kg bw/day to the chronic dietary toxicity/oncogenicity study of chlorpyrifos-methyl based on the available evidence and a review of international evaluations. While EFSA identified effects on erythrocyte acetylcholinesterase (RBC AChE) activity and adrenal glands at lower doses (0.1 and 1 mg/kg bw/day), both JMPR and EPA determined that the effects on RBC AChE activity were inconsistent over time and lacked a clear dose-response relationship. Importantly, a histopathology review panel concluded that adrenal vacuolation observed at these doses was consistent with background findings, reaffirming that the adverse effects occurred at the top dose of 50 mg/kg bw/day. Therefore, FDA aligns with JMPR and EPA in considering the NOAEL for this study to be 1 mg/kg bw/day, as it reflects the highest dose without consistent adverse effects and provides a scientifically robust basis for regulatory decisions.

4.3.5 Selection of the Best Representative Study for Each Substance in the External Validation Database

For most of the compounds that remained after the elimination process described in the prior sections (4.3.1-4.3.3), multiple studies were listed in the external validation DB. Therefore, after verifying the identity of the test article and the correct NEL, species, and duration for each study, we set out to identify the single best representative study for each substance in the external validation DB.

Choosing the best representative study with the most appropriate NEL for each substance was fraught with difficulties. To help us determine the most appropriate study, we turned to safety and risk assessments performed by authoritative bodies such as EPA (IRIS, RED, and HPVIS), CalEPA, FDA, EMA, EFSA, JECFA, JMPR, and others. As with differences in agreement on the NOAEL for a specific study, assessments from these groups do not always agree on what is the best representative study for the evaluation of

[7] Barna-Lloyd et al., 1991

chronic oral toxicity to derive an Acceptable Daily Intake (ADI) level or a Reference Dose (RfD).

For example, in the case of Tolclofos-methyl (CAS 57018-04-9), EFSA (2018) chose the NOAEL from a 9-month study in mice as the "relevant short-term oral NOAEL" and the NOAEL from a 2-year study in mice as "the relevant long-term NOAEL" and used these values to calculate an ADI. In comparison, the EPA (2012) deemed the 2-year study in mice "unacceptable"[8] and chose to combine a 26-week study in dogs with a 90-day study in rats as "co-critical" studies for their risk assessment. In cases like this (i.e., where authoritative bodies did not agree on the best representative study for a substance), we used our best judgement to choose the most appropriate representative study based on our review of all the data and information available for the substance using the same study selection criteria as that for the original (pre-validation) EDT DB.

We note that during our review, we noticed that studies existed for certain compounds that were more appropriate to establish a NEL for a compound than those listed in the external validation DB. For example, lenacil (CAS 2164-08-1) had two 13-week studies listed in dogs and rats with NELs of 44 and 40.6 mg/kg bw/day, respectively, with EFSA (2009) listed as the reference. During our review of the EFSA paper, a 2-year carcinogenicity study with an overall NEL of 12 mg/kg bw/day came to our attention. As we deemed this 2-year carcinogenicity study to be more appropriate to represent the chronic toxicity of the compound, we entered this study into the external validation DB and chose it as the representative study for lenacil.

4.3.6 Adjusting NELs for Dosing Schedule and Purity

In some studies, the test material is not administered every day. For these studies, we adjusted for dosing schedule just as we did for the original (pre-validation) EDT DB. For example, for a gavage study with a NEL of 50 mg/kg bw given 5 days per week, we calculated the daily dose by multiplying the NEL the number of times the substance was given each week divided by 7. For the above example: (50 mg/kg bw x 5 day/week)/7 days/week=35.7 mg/kg bw/day.

When purity of the test substance was available, for substances with less than 95% purity, the NEL was adjusted for purity. For example, for a study with a NEL of 50 mg/kg bw/day and a purity of 80%, the study NEL was changed to 40 mg/kg bw/day (50x0.80).

[8] EPA (2012) listed the following reasons for the "unacceptable" rating of the 2-year study in mice: 1) a maximum tolerated dose not achieved; 2) stability and homogeneity analyses of the test diet were not reported; 3) data from the pilot study were not reported; 4) the study authors reported that the first two analyses of the test diet concentration revealed that the control diet was contaminated with 1.8 to 3.0 µg/g of S3349 (Tolclofos-methyl) (the study authors indicated that these results may not be accurate); 5) a large amount of the individual data was handwritten and illegible (therefore, validation of many parameters (body weights, hematology, clinical chemistry, urinalyses, and organ weights) was not possible); and finally 6) individual data were not reported for clinical observations or palpable mass observation.

4.3.7 Additional Processing of the Data in the External Validation Database

In the unprocessed external validation DB, NELs were provided using various units, such as mg/kg bw/day, ppm, or percent in the diet. All of these values were replaced with the corresponding or equivalent values expressed in mg/kg bw/day. For some the unit of the NEL was listed as 'other.' For these, we corrected the unit based on the information obtained from the references and then calculated the corresponding values in mg/kg bw/day.

We note that we did not reevaluate the statistical significance of all observations as it was beyond the scope of the project's resources.

Once we finalized the compounds in the external validation DB and chose the most appropriate NEL for each, we calculated the NEL for each substance in the DB in units of mmol/kg bw/day. We then adjusted the NELs based on study duration employing the same adjustment factors as those used to calculate duration adjusted NELs in the EDT DB (section 2.2). Next, each compound was classified according to its EDT class, and the EDT question at which they were classified was recorded.

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Appendix 1: Short explanations for the Pre-Validation Expanded Decision Tree Questions

Q1: There are a large number of structurally diverse substances that are metabolized by high-capacity pathways, including substances endogenous in our bodies and common components of food. As it is impossible to formulate questions to capture all of these substances, we only attempted to devise structure-based questions for some of the most common ones. Therefore, please treat the sub-questions in Q1 as examples rather than

an exhaustive list and apply your own judgement to capture additional substances found in food that are metabolized by high-capacity pathways. Combined, these provide the user with a basis for identifying and classifying nontoxic substances or substances with very low oral toxicity that are present in animals or in food or are added to food along with other substances metabolized by high-capacity pathways.

Q2: Organophosphorus substances possess a wide range of toxic potential. At Q2, we proposed three structure-based sub-questions that assign organophosphorous substances to structural Classes III, V, or VI, in addition to Q1e) and i) that assign some nontoxic organophosphorus compounds to Class I. We note that certain toxins containing phosphorus are assigned to Class V at Q6.

Question 2 assesses the relative reactivity of organophosphorous substances and its relationship with neurotoxicity. Neurotoxicity arises when a serine residue in acetylcholinesterase (AChE) replaces the leaving group on an organophosphate or thiophosphate, inactivating AChE, an enzyme that breaks down the neurotransmitter acetylcholine into choline and acetate (Fukuto, 1971, 1990; Klaassen et al., 2013). While the primary targets of organophosphorus compounds are the central and peripheral nervous systems, immunotoxicity, hepatotoxicity, and other target organ toxicities have been reported along with carcinogenicity (Ahmadian et al., 2018; IARC, 2017; Klaassen et al., 2013).

Sub-question 2b) identifies organophosphorus compounds with improved leaving group ability and increased electrophilicity of P, which increases the rates of reaction with the serine residue in AChE, leading to significant toxicity (Worek et al., 2004). Part 2c) assesses the competition between the rate of phosphate ester hydrolysis and the reaction with AChE. Partial hydrolysis increases the rate of further hydrolysis of phosphate, which favorably competes with the reaction with AChE (Worek et al., 2004). Part 2di) identifies those phosphates with reduced rates of S_N2 reactions with AChE due to the presence of bulky alkyl and aryl substituents (i.e., steric effects) that slow the rate of S_N2 reaction of phosphates with AChE.

The relative rate of oxidation of P in phosphites to biologically active phosphates is addressed in sub-questions 2c) and 2d(ii)). Answering yes to these two sub-questions indicates that the oxidation of phosphite to phosphate is so slow that these compounds display low biological activity. Finally, if P is present (i.e., yes to 2a) but the answer is no to all other structure-based sub-questions in Q2, the phosphorous-containing substance defaults to Class V (e.g., tributyl phosphate).

Q3: Question 3 identifies congeneric groups of substances that have reactive nitrogen- and/or sulfur-containing functional groups or moieties that are most frequently associated with enhanced toxicity (Kalgutkar et al., 2005; Mirvish, 1995). In some cases, elevated toxicity is associated with the presence of more than one functional group of the same type (Q3f(iii)) (quaternary N^+) and Q3g(iv)) (nitriles and amidines)) (U.S. EPA, 1996; U.S. EPA NCEA, 1987a, 1987b). Due to the relative toxicity of these groups, they are assigned to Classes III, IV, and V.

The most toxic substances identified by Q3 are captured by sub-sub-questions a(i)), a(iii)), b), c(i)), f(i)), f(iii)), or g(iv)). For example, nitroso derivatives (Q3a(i)) have multiple target organs as carcinogens with the kidney and liver as the two major target organs along with other targets such as the bladder, esophagus, and lung (Magee & Barnes,

1967). *N*-nitroso compounds (Q3a(i)) are among the most potent carcinogens known (Lijinsky, 1987; SCCS, 2012). Tumors can be produced in a wide variety of tissues, such as, but not limited to, esophagus, stomach, duodenum, colon, lung, liver, kidney, and urinary bladder (Bruning-Fann & Kaneene, 1993; Lijinsky, 1987; Mirvish, 1995). N-nitroso compounds require metabolic activation to yield α-hydroxynitrosamines, which decompose to yield monoalkylnitrosamines, alkyldiazohydroxides, and nitrogen separated ion pairs (Mirvish, 1995).

Of lower toxicity than Class V substances, but still potent toxins, are certain primary amines (Q3d(I)) and aliphatic secondary amines (Q3d(ii)), thioamides and thioureas (Q3e), specified quaternary N compounds (Q3f(ii)), and a group of N-containing reactive moieties (Q3g(ii) and g(iii)) (Class IV). Isothiocyanates and ureides (Q3h), along with nitroso- and N-nitroso compounds (3a(ii)), thiocarbamates (3c(ii)), sulfonyl carbamate ($RS(=O)_2NC(=O)OR$), sulfonyl carbohydrazide ($R\text{-}C(=O)NRNS(=O)_2R$, sulfonyl guanidine ($RS(=O)_2NC(=NR)NR_2$), or sulfonyl isocyanate ($RS(=O)_2N=C=O$), (Q3g(I)) exhibit the lowest toxic potential identified by Q3, and are assigned to Class III (Komae et al., 1998; NCI, 1978).

Q4: Question 4 sends all compounds containing elements other than C, O, H, N, and/or S and compounds containing noncovalently bound P, F, Cl, Br, and I to the next question to either sort them into various classes of relative toxicity (e.g., methylmercury) or for further instructions as to which counterions are disregarded from Q6 and onward (e.g., K^+, Cl^-, or Na^+).

Q5: Sub-questions 5a) through 5c) assess substances for the presence of common biological cations (e.g., Na^+ and Ca^{2+}) and anions (e.g., Cl^- and Br^-) exhibiting no significant toxic potential. Due to dynamic changes in acidity in biological systems, the salt and neutral forms interconvert and can be concluded to be physiologically equivalent. Sub-question 5d) aims at addressing organosilicon compounds. Those having halogen(s) and/or heterocyclic ring(s) are passed along for further evaluation, while those with relatively low toxic potential are classified here.

To err on the side of caution, most radioactive elements were placed in Class VI. Even though organometallic and inorganic substances containing metal ions are widely used, the toxicological data for these chemicals are lacking. To complicate the situation, even for the same element (e.g., Cr), the toxicities of the different oxidation states of the same element (e.g., Cr(II), Cr(III), or Cr(VI)) can be very different. Even within the same oxidation state (e.g., Cr(III)), toxicity can vary greatly depending on the identity of the counterion or ligand present in the substance. For example, the median lethal doses (LD_{50}) of different Cr(III) compounds are 440 mg/kg for Cr(III) chloride, 3360 mg/kg for Cr(III) acetate, and >15,000 mg/kg for Cr(III) oxide in rats; while the LD_{50} of Cr(VI) oxide is only 52 mg/kg and the LD_{50} of Cr(II) chloride is 1870 mg/kg (Egorova & Ananikov, 2017). Due to the complex nature of metal and organometal toxicity, fully addressing these is beyond the scope of the EDT at this time. Nonetheless, we invite the public to propose refinements for sorting them into the appropriate EDT classes.

Q6: Question 6 attempts to identify structural features associated with many toxins. Question 6a) identifies compounds with a steroidal skeletal structure, such as the sex hormone estradiol, the corticosteroid dexamethasone, and certain natural toxicants such as the steroidal alkaloids solanidine and chaconine. Substances with nitrogen(s) at the ring fusion

point(s) can exhibit increased toxicity. Question 6b(ii)) classifies pyrrolizidine alkaloids (e.g., riddelliine and lasiocarpine) found in plants and certain toxic secondary metabolites produced by organisms of the fungus kingdom (mycotoxins) (e.g., cyclochlorotine and verrucarin), in addition to other toxic substances. On the other hand, Q6b(i)) aims at capturing some commonly used antibiotics with nitrogen at the ring fusion point that are not as toxic as substances captured by Q6b(ii)). Q6c) identifies mycoestrogens (xenoestrogens produced by fungi) or related synthetic derivatives (e.g., zearalenone, zearalenol, and zearalanol), antihelmintic and insecticidal avermectines and their derivatives (e.g., ivermectin, doramectin, and abamectin), along with other groups of natural toxins and their derivatives. Question 6d(i)) identifies the most common structural features of a wide variety of toxins, such as certain mycotoxins (aflatoxins (e.g., B1, B2, G1, and M1) and trichothecenes (e.g., nivalenol, vomitoxin, and fusarenon-X)), naturally-occurring ergoline alkaloids and their synthetic derivatives (e.g., ergine, ergometrine, and LSD), phycotoxins (e.g., azaspiracid and ciguatoxin 1), additional steroidal alkaloids (e.g., jervine), and opiates (alkaloids) such as codeine and morphine, along with other toxic substances. Question 6d(ii)) is designed to identify additional natural toxins, such as fumonisin mycotoxins (e.g., fumonisin B1, B2, B3, and B4). Additional toxic alkaloids, such as nicotine, coniine, and anabasine, are identified at Q6e). Question 6f) aims to identify a variety of anticoagulants, such as warfarin, acenocoumarol, bromadiolone, and diphenadione. Question 6g) aims to capture nonsteroidal estrogens, namely stilbestrols (Q6gi) and triphenylethylenes (Q6g(ii)). As their name suggest, they are selective estrogen receptor modulators. Finally, Q6h) captures additional substances commonly used as antibiotics to prevent them from defaulting into one of the classes of high concern at later questions.

We acknowledge that many benign substances that meet structure-based criteria in Q6a) through 6g) will be assigned to toxic Class V at this step. However, one of the primary goals of the EDT is to be comprehensive in classifying substances found in food, both when intentionally added and unavoidably present. Therefore, structure-based questions were designed to address chemical categories of known naturally occurring toxins and other compounds exhibiting elevated toxicity. In some cases, based on their complex molecular scaffolding, natural toxins that do not meet the criteria in 6a) through 6g) will be assigned to other structural classes (i.e., IV, V, or VI) via other questions in the EDT. For instance, the natural toxin tetrodotoxin, a guanidine derivative, and the mushroom toxin gyromitin, a hydrazide, both are assigned to Class IV at Q3e), while ochratoxins A and C are assigned to Class IV at Q47. The authors realize the limitations inherent in selecting a limited number of toxicant classes.

Q7: Questions 7 and 8 address compounds containing halogens. Halogenated alkanes, cycloalkanes, aromatic, and heteroaromatic compounds exhibit a wide range of toxicity depending on the number, position, and type of halogen in the compound, carbon-carbon bond saturation, the presence of functional groups other than halogens, available hydrogens, lipophilicity, and the species, sex, and conditions of the toxicity study.

These structural differences result in metabolic differences among halogenated compounds leading to a wide range of toxicities. For example, structures with two vicinal halogens (Q7b(ii)) (e.g., 1,2-dibromo-3-chloropropane (DBCP)) are prone to toxicity due to the formation of reactive intermediates (e.g., 2-bromoacrolein in the case of DBCP) via glutathione (GSH) and CYP450-mediated mechanisms (Anders, 2004; Weber et al., 1995)

and exhibit high toxicity (e.g., DBCP) (Rao et al., 1983). Vicinal halides, therefore, are assigned to Class V. Nephrotoxic haloalkenes (7g(i)) are bioactivated via the GSH-dependent multistep β-lyase pathway (Anders, 2004), and are placed into Class IV. On the other hand, GSH conjugation of monohalides (7b(iii)) is a detoxication reaction (Guengerich, 2005) and, in general, monohalides exhibit relatively lower toxicities compared to other halogenated compounds (e.g., chloroethane (NTP, 1989a), bromoethane (NTP, 1989b), 1-bromopropane (NTP, 2011), and 1-chlorobutane (NTP, 1986)), and are placed in Class II.

Q8: Question 8 deals with halogenated polyaromatic ring systems that, depending on the position(s) and the number of halogen substitution(s) and the planarity of the ring system, can be extremely toxic. For example, 2,3,7,8-tetrachlorodibenzo-p-dioxin and 2,3,7,8-tetrabromodibenzo-p-dioxin are two of the most toxic substances tested in animal models (Birnbaum et al., 1991; Hooth et al., 2012).

Q9: Question 9 assigns simple aliphatic hydrocarbons to Class I, none of which have shown any significant evidence of toxicity except those listed as exceptions in the question (Carreón & Herrick, 2012; TCEQ, 2015). All compounds that are noted as exceptions will be dealt with in Q28.

Q10: Question 10 is a simple sorting question that separates heterocyclic substances from all others.

Q11: Question 11 looks for substances that undergo gastrointestinal hydrolysis or reduction, depending on the functional groups present, to yield, in many cases, multiple products. From this point forward in the EDT, each substance produced by hydrolysis is evaluated individually at the appropriate step (e.g., isobutyl furylpropionate is hydrolyzed to 3-(furan-2-yl)propanoic acid and 2-methylpropan-1-ol and evaluated at Q13 and Q1, respectively). The highest structural class for the hydrolysis products is considered to be the structural class for the initial substrate (i.e., as 3-(furan-2-yl)propanoic acid is a Class III substance and 2-methylpropan-1-ol is a Class I substance, the overall class for isobutyl furylpropionate is Class III); please see this example in the supplementary data section.

We note that certain functional groups integral to the heterocycle do not hydrolyze. Therefore, heterocyclic 1,3-dithiolanes (Cashman & Williams, 1990) and 1,3-oxathiolanes (Cashman et al., 1990) do not hydrolyze and are treated as such at Q13 and onward. Additionally, a cyclic methylenedioxy group fused to an aromatic ring does not hydrolyze. It is oxidized *via* a well characterized cytochrome (CYP) pathway (Delaforge et al., 1999). Heterocyclic acetals, hemiacetals, ketals, hemiketals (Bissig & Muecke, 1988), and thioesters hydrolyze; and as such, their hydrolysis products are generated and proceed to further evaluation.

Q12: Question 12 separates lactones or lactams (Q12d) that hydrolyze to open chain aliphatic compounds, which in turn are completely catabolized; or those (Q12e) that hydrolyze to a hydroxy- and carboxy-substituted alicyclic, heterocyclic, or aromatic derivative that is readily excreted as such or in the conjugated form. Compounds addressed at Q12a) through 12c), either can attain aromaticity through enolization of the lactone carbonyl (Q12a & b) (Billecke et al., 2000), or react directly with GSH due to increased ring strain

(Q12c) (Dijkstra, 1975). Substances identified in Q12a) through 12c show increased toxicity in animal models (Becci et al., 1981; Fredricks et al., 1981; Simon et al., 2002); therefore, they are assigned to Class IV.

Q13: Question 13 is a sorting question and sends three-membered heterocyclic rings for further evaluation at Q14.

Q14: Question 14 concern three-membered heterocyclic rings (i.e., epoxides, aziridines, and thiiranes). Substances with two or more epoxide rings (Q14a) show evidence of increased toxicity, likely due to increased protein crosslinking, while monoepoxides with more molecular complexity (Q14b) and added functional groups provide additional detoxication options (Dunnington et al., 1981; Sauer et al., 1997).

Q15: Question 15 screens for plant flavonoids such as isoflavonoids, flavones, flavonols, flavanones, flavans, and anthocyanidins. Flavonoids are ubiquitous in fruits and vegetables and, as such, in the human diet. Moderate consumption of flavonoids from dietary sources is considered to be safe and most flavonoids end up in Class II at Q28.

Flavonoids usually have low bioavailability or are not orally bioavailable (Ma et al., 2014; Ueno et al., 1983). Glycoside conjugates of polyphenols are readily hydrolyzed on the brush border of intestinal epithelial cells. Metabolism occurs both in the gastrointestinal tract and after absorption. Absorbed polyphenols are metabolized through hydrolysis, sulfation, glucuronidation, and/or O-demethylation. Urinary excretion of parent substances or metabolites is proportional to the extent of ring hydroxylation. Biliary excretion also occurs. Metabolites not absorbed in the small intestine may undergo further metabolism in the large intestine. Both glycosylated and aglycone metabolites may be excreted in the feces. Intestinal microflora may also cleave conjugated moieties, with the resultant aglycones undergoing ring fission, leading to phenolic acid and cinnamic acid derivatives. These metabolites may be absorbed and ultimately excreted in the urine.

Q16: Question 16 screens substances for the α-ketoenol moiety in the presence of adjoining electron-donating substituents. Reminiscent of the biological activity of vitamin C, substances that possess an α-ketoenol moiety are oxidized in the presence of metal ions (Fe^{3+}) to yield a carbon-centered radical (Hiramoto et al., 1996a; Hiramoto et al., 1996b; Li et al., 1998; Yamashita et al., 1998). The radical can further react with molecular oxygen to form a peroxy radical ($ROO^{\cdot}$) capable of reacting with cellular constituents, resulting in toxicity. Oxidative stress has been reported *in vitro* (Hiramoto et al., 1996a) at high concentrations and *in vivo* (Shelby et al., 1993) at high dose levels. However, long-term studies show no evidence of carcinogenicity in rats (Kelly & Bolte, n.d.; Munday & Kirkby, 1973).

Q17: Question 17 is a simple sorting question that separates substances containing a heteroaromatic ring from other heterocyclic substances. Heterocyclic substances are treated in Q18a) through 18c), and those substances containing a heteroaromatic ring are addressed in Q19-22.

Q18: Question 18a(i)) assigns dibenzo-p-dioxins not addressed at previous questions to Class V due to their high relative toxic potential.

The toxicity and metabolism of other heterocyclic compounds with three or more fused, spiro-fused, bridged, or singly bonded rings, with or without substituents, have been, in general, less extensively studied than alicyclic, aromatic or heterocyclic, or heteroaromatic substances with one or two rings. Therefore, some of the polyheterocyclic systems addressed in Q18a(ii)) are assigned to default Class IV at Q47. Also, those heterocyclics that contain substituents other than simple common substituents listed in Q18b) eventually are assigned to an EDT class at Q47. If a substance contains one or more of the substituents listed in Q18b), it bears structural features that either make the compound not readily absorbed or are known or expected to participate in metabolic detoxication reactions and subsequent rapid excretion. Heterocyclics containing common substituents that support increased metabolic disposition are evaluated at Q28 for other structural moieties associated with increased toxicity before a final classification of Class II can be made. Sufficient data is available to classify unsubstituted heterocycles (Q18c)). A “no” response forces compounds to be evaluated further and finally classified at Q28.

Q19: Question 19 evaluates subgroups of heteroaromatic substances that have been more thoroughly investigated and for which a mode of action has been proposed. Question 19a) concerns a group of polyheteroaromatic amines (PHAA) that show carcinogenicity in animal models (Chen et al., 2017; Ohgaki et al., 1986). PHAA in food is thought to be produced from chemical substances (e.g., creatinine, amino acids) found in meat during its processing, preservation, and cooking (Gallus & Bosetti, 2016). Among these reactants, creatinine is a molecular moiety common to the more potent carcinogenic and mutagenic PHAAs (Chen et al., 2017).

Questions 19b) and Q19c) deal with biologically reactive imidazole and thiophene derivatives. Relatively few alkyl-substituted imidazole derivatives have been investigated for their potential toxicity. Therefore, our knowledge of the effect of the substituents and their positions on the toxicity of a substituted imidazole is limited, hence the limited scope of Q19b. Due to the increased toxicity of the thiophene ring compared to other heteroaromatic ring systems, all thiophene derivatives are assigned to a more toxic class (Class IV). Metabolic activation of thiophene occurs via facile oxidation of the ring S to yield the more reactive sulfoxide and epoxidation of the thiophene double bond (Dansette et al., 1991; Dansette et al., 1998; Dansette et al., 1992; Gramec et al., 2014; Mansuy et al., 1991).

Question 19d) deals with selected thiazole derivatives that show high-dose hepatotoxicity and nephrotoxicity in animal models and in humans. At relatively high dose levels, metabolism to the 4,5-epoxide of the thiazole ring, followed by ring opening and thiazole ring cleavage, yield α-diketone and toxic thioamide or thiourea metabolites that have been related to the nephrotoxicity and hepatotoxicity endpoints for some thiazole derivatives (EFSA, 2008; Mizutani et al., 1994; Obach et al., 2008).

Q20: Question 20 further sorts heteroaromatic substances based on whether they are unsubstituted or substituted by acyclic substituents or rings, the type of substituent ring and its connection to the heteroaromatic ring, and the number of heteroaromatic rings. Cyclopropyl amine-substituted heteroaromatic substances are placed in Class V, a class of very high toxicity. The cyclopropylamine group is an inactivator of CYP 450 and other enzymes and can covalently bind to macromolecules (Kalgutkar et al., 2005). Heteroaromatic compounds in which the heteroaromatic ring is fused or singly bonded to

an alicyclic ring or fused to an aromatic ring with no other substitution are assigned to Class III, while others are placed in Class IV, depending on substitution. The rest are sent for further classification at either Q21 for certain highly reactive moieties or Q47 for functional groups that decrease their toxicities.

Q21: If “yes” is answered for Q21, the user is sent to Q28 to determine if selected reactive moieties are present (e.g., a terminal alkene, a thiol, or an o-diacetyl) that would indicate an increase in potential for toxicity. A “yes” answer here results in assignment to Class III, IV, or V.

Q22: Question 22 evaluates heteroaromatic substances for structural features and functional groups that decrease toxicity mostly by providing additional venues for detoxication. Classification of heterocycles and heteroaromatics concludes with Q22.

Q23: Question 23 is a sorting question that directs open-chain substances to Q24 and directs cyclic (i.e., alicyclic and aromatic) substances to Q29. This separation is primarily, but not solely, due to significant differences in these substances’ metabolic disposition.

Q24: Question 24 is a two-part question that deals with the reproductive and developmental effects reported predominantly in rats exposed to aliphatic acids and their precursors. In our review of the literature, no reproductive or developmental effects have been reported in humans following low, reasonably expected occupational or environmental exposures to substances meeting the structural requirements of this question. At high dose levels, 2-propylpentanoic acid (valproic acid), 2-ethylhexanoic acid, and their alcohol, ester, as well as aldehyde precursors (Jauniaux et al., 1994; Nau & Scott, 1987, 1986) consistently show reproductive and developmental effects.

Q25: In multiple instances, oral repeated-dose toxicity studies conducted in rats and dogs with tallow-derived analogs or C13-C15-alkyl, ethoxylated amines showed local effects on the gastrointestinal tract (ECHA, 2011a; SCC, 1993). Also, in several of the oral studies, histiocytosis (the presence of foamy macrophages) was noted in the small intestines and mesenteric lymph nodes (ECHA, 2011a; Sheppard, 1982). The prevailing scientific opinion is that, without additional evidence of concurrent toxicity, the presence of foamy macrophages in the intestine should not be considered an adverse effect (CIR, 2015; Boyer et al., 2018). However, until longer-term studies are performed, an intermediate conservative assignment of Class III is applied for purposes of the EDT. Additionally, based on the limited data available, compounds belonging to fatty amides possess relatively low or intermediate toxicities; therefore, they were placed in Class III (Health Canada, 2018; U.S. EPA, 2010).

Q26: Substances that contain only the listed functional groups or any of the allowed combinations of those functional groups identified in Q26a) through 26c) may be regarded as simple organic structures. These simple organic structures should be metabolized through known metabolic detoxication pathways or readily excreted without adverse biochemical, physiological, or pharmacological effects. Other structural features that are known or are expected to be exceptions to this general statement are classified further at Q28. The limitations on the number of occurrences of different functional groups within a

structure were prompted in part by the types of structures from which toxicity and metabolism data were available.

Q27: The CDT limited functional groups to fewer than three. However, data are now available to differentiate between three different functional groups. Compounds with three or fewer functional groups are classified into Classes II to V based on the presence or absence of the functional groups identified in Q28. Predicting toxicity and metabolism of compounds with four or more unrelated functional groups is difficult due to their complexity. As a result, these compounds are placed in default Class IV, indicating no initial presumption of safety.

Q28: This question is a terminal question that addresses a number of biologically reactive moieties that exhibit increased potential for toxicity in animals and, therefore, are assigned to Classes III, IV, or V depending on the relative toxic potential of the moiety. If the answer is “no” to all sub-questions, the substance defaults to Class II.

Substances assigned to Class V include conjugated alkynes (Q28c(I)) that form intermediate reactive oxirenes, ketenes, or allenes (Zhao et al., 2018), terminal alkynes regardless of conjugation that can generate reactive ketene (Q28c(I)) (Kalgutkar et al., 2005), long-lived reactive enolic thiols (Q28n(I)) (*Enzymatic Basis of Detoxification*, 1980; *Metabolic Basis of Detoxication: Metabolism of Functional Groups*, 1982), and the aminocyclopropyl moiety, a well-known inhibitor of CYP450 and other human enzymes (Q28r) (Guengerich, 2001; Kalgutkar et al., 2005).

Class IV includes allylamine derivatives (Q28a) that exhibit cardiovascular toxicity (Conklin & Boor, 1998); acrylamide and its derivatives (Q28b) that are associated with neurotoxicity and carcinogenicity at high concentrations in rodents (Burek et al., 1980; M. J. Miller et al., 1982); certain internal alkynes (Q28c(ii)) that can react with nucleophiles formed by oxidation of internal alkyne carbon followed by rearrangement to the oxirene (Kalgutkar et al., 2005); aliphatic γ-diketones (Q28d) associated with neurotoxicity recognized as “giant axonal swelling” (Sayre et al., 1986), also reported for aromatic *o*-diacetyl derivatives (Q28e) (Gagnaire et al., 1991); neuroactive β-phenethylamine derivatives (Q28f) (Zanda & Fattore, 2017); a subgroup of heteroaromatic thiols (Q28n(ii)) and polysulfides with S_n n≥3 (Q28n(iv)) that form reactive perthiol intermediates producing cellular oxidative stress (Munday et al., 2003); biologically active aldehydes and dialdehydes with α,β-unsaturation (Q28p) (Anke & Sterner, 1991; Morales et al., 1992); and certain carcinogenic terminal dienes (Q28s(I)) (NTP, 1993, 1999).

The compounds addressed in Q28i) and Q28l) are placed in Class III. In Q28i), α,β-unsaturated aldehydes conjugate with GSH directly or undergo allylic hydroxylation *via* lipid peroxidase to yield 4-hydroxyalkenals (Esterbauer et al., 1982) that also conjugate with GSH (Esterbauer et al., 1975; Winter et al., 1987). The GSH redox cycle maintains adequate levels of GSH in animal cells (Nelson & Cox, 2005) and is a major intracellular mechanism involved in the detoxication of α,β-unsaturated aldehydes (Janzowski et al., 2003; Witz, 1989). The addition of GSH across the electrophilic carbon-carbon double bond is catalyzed by the enzyme glutathione S-transferase but can also occur at a lower rate in a non-enzymatic reaction (Eisenbrand et al., 1995; Grootveld et al., 1998). The cellular formation and fate of α,β-unsaturated aldehydes have been directly linked to the depletion of cellular GSH and increased lipid peroxidation that are part of a phenomenon known as oxidative stress. Oxidative stress results when free radicals react with proteins, polypeptides, RNA and DNA bases, and particularly polyunsaturated fatty acid chains of

phospholipids in cell membranes. In Q28l), organ toxicity of these small molecule α,β-unsaturated acids and their corresponding esters involve irritation of the rodent forestomach (Greim et al., 1995). Prolonged exposure to high concentrations of these irritating substances is associated with necrosis of the forestomach (Ghanayem et al., 1985a, 1985b).

Questions 28h(I)) and Q28j) identify substances that contain aliphatic α- and β-diketones, respectively. These substances are classified into Class III. Volatile α-diketones may exhibit respiratory toxicity during repeated exposures at high in vivo concentrations (Anders, 2017; Morgan et al., 2016), while the β-ketoamides (Q28j) tend to complex metal ions (e.g., Fe) leading to the presence of Heinz bodies and exhibit effects on the spleen, erythron, and liver (OECD, 1998). An aromatic or heteroaromatic ring directly bonded to a terminal vinyl group is a conjugated diene (Q28g) that is sterically and electronically available for CYP-induced epoxidation and Michael-type reaction with GSH, leading to intermediary metabolites that may react with protein and DNA nucleophiles (Carlson, 2010; Laffon et al., 2003). Question 28k) addresses allyl thiol and compounds that can be reduced or hydrolyzed to allyl thiol. These compounds can react with GSH and affect cellular redox status in addition to reacting directly with proteins involved in various physiological processes and, consequently, exert toxicity (Miron et al., 2010). α,β-Unsaturated ketones (Q28m), an important group of flavoring and fragrance substances, can react with GSH enzymatically or non-enzymatically via nucleophilic addition to the β-carbon due to the resonance interaction with the carbonyl group that renders it electrophilic (Portoghese et al., 1989). Regardless, they are relatively unreactive electrophiles, and even when they are sufficiently electrophilic to react with GSH, the rates of reaction with GSH are much greater than with the guanine component of nucleotides. In addition, aliphatic dialdehydes without α,β-unsaturation (Q28h(ii)), certain sulfur-containing compounds (Q28n(iii)), compounds containing a methylenedioxy ring fused to an aromatic ring (Q28o), certain aldehydes (Q28p(I) and p(iv)), some longer-chain terminal dienes (Q28s(ii)), and other compounds (Q28q) are placed in Class III.

Please note that question 28 does not include all reactive moieties and was created based on the available data.

Q29: This question separates alicyclic skeletal structures (Q30 through Q32) from aromatic structures (Q33 through Q47).

Q30: This question identifies alicyclic substances that have substituents (Q30a) that undergo ready detoxication and rapid excretion, and those that are poorly absorbed and undergo elimination (Q30b). A “yes” answer at Q30a) sends the user to Q31 and Q32 to check for unique structural features that potentially increase toxicity. A “yes” at Q30b) identifies substances that contain certain moieties that are ionic under physiological conditions, and as such, are not readily absorbed.

Q31: This question identifies and classifies unsubstituted and alkyl substituted alicyclic *o*- or *p*-quinones. In general, quinones, being α,β-unsaturated ketones, react with GSH in biological systems. At elevated levels of exposure, the loss of GSH facilitates cellular oxidative stress and liver toxicity (Monks & Jones, 2002). Their electrophilic properties depend on the presence of substituents.

Q32: Question 32 identifies groups of naturally occurring substances that exhibit toxicity both in animals and humans. Present in a variety of mint plant families (e.g., *Mentha pulegium* (pennyroyal), *Mentha piperita* (peppermint) and *Mentha arvensis* (corn mint)), pulegone and structurally-related substances (e.g., piperitenone) and some of their metabolites (e.g., 5-hydroxypulegone) possess an α,β-unsaturated ketone that oxidizes and then cyclizes to form a reactive menthofuran (proximate hepatotoxic agent) that oxidizes and ring opens to yield the ultimate hepatotoxic agent γ-ketoenal (World Health Organization (WHO), 2001). Many of these compounds are addressed in Q32a).

Thujone, a major component of wormwood oil (*Artemisia absinthium L.*), and umbellulone, present in California bay laurel (*Umbellularia californica*, aka headache tree), are structurally related alicyclic terpene ketones that cause a variety of neurological symptoms in humans. In animals, thujone is a potent neurotoxin that affects the *gamma*-amino butyric acid system (Tripathi & Mishra, 2016), while umbellulone acts *via* its selective TRPA1-agonism as a trigeminovascular stimulator, which provides a possible explanation for headache (Nassini et al., 2012). These compounds are addressed in Q32b).

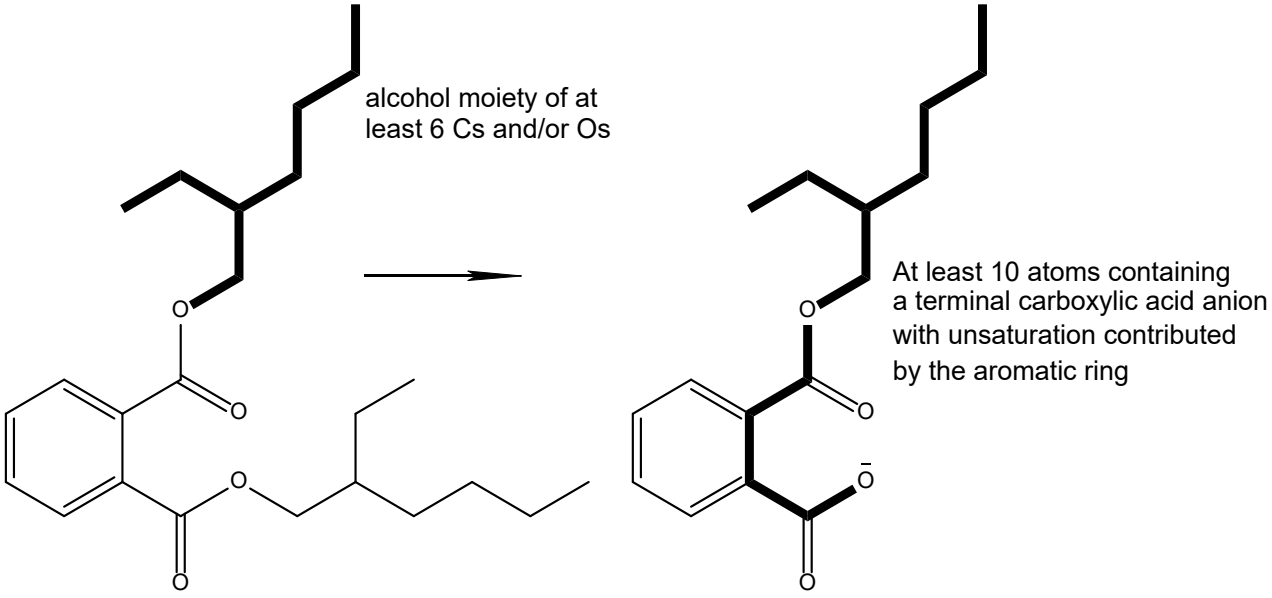
Acute exposure to hypoglycin (from the consumption of unripe fruit of the ackee tree) causes Jamaican vomiting sickness, also known as toxic hypoglycemic syndrome, *via* the inhibition of β-oxidation of fatty acids (Gordon, 2015; Wenz et al., 1981) while chronic exposures lead to toxicity of the liver, kidney, and spleen (Blake et al., 2006; Gordon, 2015). Hypoglycin and other related compounds containing a cyclopropyl ring with an exocyclic or endocyclic alkene are dealt with in Q32c).

The alicyclic analog of the group of heterocyclic α-ketoenols in Q15 (e.g., β-thujaplicin (hinokitiol), sotolone, maltol, and furaneol) show similar biological properties (i.e., cellular oxidant) and metabolic fates (glucuronic acid conjugation and excretion) as their heterocyclic analogues and are addressed in Q32d) (Roscher et al., 1997; Williams & Schlatter, 2006).

If at the end of Q31 and Q32, the answers are “no,” the substance is sent to Q28 to evaluate for the presence of reactive moieties at sub-questions a) through s). The absence of the reactive moieties described in Q28 results in assignment of the alicyclic substance to Class II.

Q33: The structures of PAHs determine whether they are carcinogenic or not and the type of cancer they cause. In most cases, the initial step in the activation of PAHs is CYP450 oxidation to reactive electrophilic species that can interact with nucleic acids and proteins (Androutsopoulos et al., 2009; Flesher & Lehner, 2016; Henkler et al., 2012; Xue & Warshawsky, 2005). In non-methylated PAHs (parental unsubstituted PAHs are addressed in Q33b), methylation at meso positions at the most reactive center is an important step in carcinogenesis (methylated PAHs are addressed in Q33c) (Flesher & Lehner, 2016). Substitution of meso-methyl groups with functional groups and moieties listed in Q33c) imparts carcinogenesis. These functional groups are capable of generating a long-lived but reactive electrophilic arylmethyl carbocation that can react with cellular nucleophiles, leading to cancer. CYP450-mediated monooxygenation of PAHs to reactive epoxides and follow-up products (dihydrodiols (diols) and diol-epoxides) can result in electrophilic products capable of binding to macromolecules and are carcinogenic (Henkler et al., 2012). These diols, epoxides, and diol epoxides are addressed in Q33d.

Q34: Question 34a(i)) evaluates the extent of hydrolysis of aromatic diesters. For *o*-phthalates, partial hydrolysis of the diester yields a monoester with an *o*-carboxylate anion. If the resulting monoester contains an alcohol fragment that has a chain length ≥6 Cs and/or Os, then the resulting monoester will contain a chain (carbons and oxygen) length of at least 10 atoms containing a terminal carboxylic acid anion with unsaturation contributed by the aromatic ring.



bis-(2-ethylhexyl) phthalate

This part (in bold) of the molecule resembles a (Z)-2-α,β-unsaturated fatty acid salt that may serve as a ligand for the activation of peroxisome proliferator-activated receptor alpha (PPAR-α), a ligand-activated transcriptional factor that belongs to the family of nuclear receptors. PPAR-α regulates the expression of genes involved in fatty acid β-oxidation and is a major regulator of energy homeostasis in animal models. The activation of PPAR-α is associated with reported reproductive effects in laboratory animals (Schoonjans et al., 1996).

Question 34 a(ii)) identifies aromatic benzoic acid esters with an o-substituent bearing an atom with a free electron pair (e.g., -OH, -OR, -NH2, -COOH) on an atom bonded directly to the aromatic ring. The o-substituent is known to inhibit enzymes of the carboxyesterase and dehydrogenase families. Question 32b) identifies other aromatic esters that will undergo hydrolysis.

Q35: Question 35 is a simple sorting question that separates mononuclear (Q35a), from binuclear (Q35b) and polynuclear ("no" to Q35a and b) ring systems. Note that polynuclear ring systems that participate in ligand activation of the aryl hydrocarbon receptor are considered in Q33.

Q36: Question 36 is also a sorting question that separates two fused aromatic rings (Q36a) from unfused benzene rings connected by a single bond or O, N, or S (Q36b) or unfused rings connected by one (Q36c) or two (Q36d) carbon chains, the latter yielding a third ring (alicyclic). The fused rings are considered at Q37, while the non-fused ring systems are

dealt with at Q41. These non-fused rings are screened for functional groups at Q42 and onward in the EDT.

Q37: In Q37a) and Q37c), epoxidation of alkyl-substituted naphthalene, a toxication pathway, competes with side-chain hydroxylation, a detoxication pathway, improving excretion and leading to reduced toxicity. In guinea pigs, rats, and mice, ring epoxidation accounts for a variable percentage of the metabolism of the monomethylated naphthalenes, leading to innocuous diol and mercapturic acid urinary metabolites under conditions where these detoxication pathways are not overwhelmed by toxication pathways. Also, side-chain hydroxylation followed by oxidation of the methyl substituent to yield a carboxylic acid conjugate (a detoxication pathway) competes favorably with epoxidation (Griffin et al., 1982; Grimes & Young, 1956; Melancon et al., 1982; Teshima et al., 1983). Other ring alkyl substituents, such as isopropyl or diisopropyl, undergo side chain oxidation, and virtually no epoxide or dihydrodiol metabolites are detected (Kojima et al., 1984; Kojima et al., 1985; Kojima et al., 1982; Kojima et al., 1978; Kojima et al., 1979).

Question 37b) identifies naphthalene substituted with one or two $-NO_2$, $-NH_2$, or its N-acetyl amide substituents. Reduction of nitro or hydrolysis of N-acetyl produce the corresponding amine substituent. Oxidative metabolism of the amino substituent yields a highly-reactive electrophile, the nitrenium ion, that has been shown to form covalent adducts with proteins and nucleic acids (and may also cross-link them) that can eventually produce carcinogenic effects (Cheung et al., 1997; Johnson & Cornish, 1978; Josephy & Novak, 2013).

Q38: Question 38 identifies aromatic compounds that contain only one or more alkoxy substituents, one of which is located in the *para* position to an allyl substituent or their corresponding 1'-hydroxy or 1'-hydroxyester. The 1'-position, being both an allylic and benzylic position, is subject to metabolic activation (toxication), hydroxylation and subsequent sulfation (Delaforge et al., 1980b; Wislocki et al., 1976), and incipient formation of an electrophilic carbocation that has been associated with hepatotoxicity, and protein (Gardner et al., 1996) and DNA adduct formation at higher concentrations (Borchert et al., 1973; Chan & Caldwell, 1992; Delaforge et al., 1980a; Delaforge et al., 1980b; E. C. Miller et al., 1983; J. A. Miller & Miller, 1977).

Q39: Question 39 assesses the effect of an *o*-hydroxy or *o*-methoxy substituent on the metabolism of an adjacent alkyl or alkenyl chain of two or three carbons on a benzene ring. *o*-Hydroxy- or *o*-methoxy- derivatives of styrene or 1-propenylbenzene derivatives (such as asarone) (Wiseman et al., 1987) are expected to possess a different metabolic fate than congeners without such an *o*-substituent (Solheim & Scheline, 1976). The presence of a substituent with a negative charge or a non-bonding electron pair *ortho* to phenethyl acetaldehyde inhibits the rate of oxidation to the corresponding phenylacetic acid. This long-lived *o*-hydroxyphenylacetaldehyde has been shown to be a potent proximate hepatotoxin (Born et al., 2000).

Q40: Oxidation of the alcohol analog or β-oxidative cleavage of the cinnamyl derivatives yields a *p*-substituted benzoic acid. These metabolites (e.g., 4-*tert*-butylbenzoic acid, 4-isopropylbenzoic acid (cuminic acid), 4-isopropylbenzyl alcohol, and 4-isopropylbenzaldehyde) are reproductive toxins in rodents (BASF SE, 2004; Laue et al., 2020; Laue et al., 2017). Upon repeated exposures, these long-lived organic acids are

associated with decreased ovary weight and interfere with implantation of the embryo (Bernauer et al., 2017; ECHA, 2011b; Furuhashi et al, 2007). In male rats, *p*-substituted benzoyl-CoA conjugates collect in testicular cells and impair male reproduction by adversely affecting CoA-dependent processes required for spermatogenesis (Laue et al., 2020; Laue et al., 2017).

Q41: Mononuclear phenols from Q34 and unfused binuclear phenols from Q36 are evaluated for steric effects by Q41. The basis for this question is that detoxication via conjugation versus toxication via *o*- or *p*-hydroxylation leading to reactive quinone is affected by the size and position of alkyl substituents. Bulky *o*-substituents hinder conjugation and excretion (i.e., detoxication) allowing *p*-hydroxylation and quinone formation, thereby increasing toxicity. Alkyl substitution at the *p*-position and/or less steric hindrance at the *o*-position favor conjugation, leading to decreased toxicity (detoxication).

Q42: Question 42 classifies aromatic hydroquinones (Q42a) and anthro- and naphthoquinones (Q42b) with different alkyl substituents and/or oxygenated functional groups. Hydroquinones are phenol derivatives that can be readily conjugated and excreted primarily in the urine (Class II), while quinones (Class III) are biologically reactive due to their oxidative and electrophilic properties that are modulated by the presence of substituents (Monks & Jones, 2002; Nordlund et al., 2006).

Q43: Question 43a(I)) evaluates the effects of ring halogen and alkyl substituents on the relative toxicity of diaminobenzene, nitroaniline, and dinitrobenzene and their corresponding N-acyl derivatives. In the second part of the question (Q43a(ii)), the effect of alkyl substitution on these compounds is evaluated. In question (Q43b), the EDT assesses the effect of oxygenated substituents on the relative toxicity of these same substances. Oxygenated functional groups provide a detoxication pathway involving conjugation and excretion, decreasing the toxicity of these substances. In the third part (Q43c), the effect of the number and position of amino- and nitro-substituents on biphenyl are evaluated for relative toxicity.

Q44: This question deals with data-rich derivatives of aniline and nitrobenzene that have a wide variety of ring substituents. The nitro group of nitrobenzene is mainly metabolized to aniline, which may be further metabolized to N-hydroxylamine, a hemolytic agent in animals (U.S. EPA, 2009; NCI, 1978). Halogens increase the rate of oxidation of aniline to form hydroxylamine, thereby increasing toxicity (Q44a); oxygenated substituents decrease the extent of oxidation to the *N*-hydroxylamine (Cnubben et al., 1994) by providing competing detoxication pathways (Q44c) (e.g., conjugation and excretion), thereby decreasing toxicity. Also, *o*-alkyl substituents provide steric hindrance that slows the rate of oxidation of aniline (Q44b), thereby decreasing toxicity relative to aniline and chloroaniline derivatives.

Q45: The functional groups listed in Q45 provide metabolic handles that mainly are oxidized to yield more polar functional groups, which allow for efficient excretion, thus reducing the toxicity of substituted benzenes. These compounds are passed along to Q28, where they are placed in Classes II to IV based on the functional groups present in the molecule. All other aromatic substances are sent to Q46 for further sorting or classification.

Q46: Compounds with the listed structural features exhibit low toxicity (e.g., phenoxyethanol and piperonyl butoxide) (ECHA, 2003; NCI, 1979). All other compounds are sent to Q47 for final classification, with most defaulting to Class IV.

Q47: Question 47 is a terminal question. In Q47a(i)), appropriate sulfonation is associated with rapid excretion and low toxicity (Guyton & Reno, 1975; Guyton & Stanovick, 1975). If the sulfonate or sulfamate is not on every structural fragment that would result from intermediate metabolism (e.g., reduction of an azo function in an azobenzene to yield an aniline derivative), the compound would display enhanced toxicity compared to compounds bearing at least one sulfonate or sulfamate per each fragment. In Q47b) and Q47c), where no intermediate metabolism is expected, the relative number of carbons to sulfonic acid groups determines the relative amount of the sulfonamide secreted and relative toxicity. In Q47d), the formation of a zwitterion through extended conjugation throughout the molecule will result in a lack of absorption and ready excretion. In Q47e), the relative number of carbons to sulfonamides in large part determines the excretion of the sulfonamide. Finally, Q47f) and Q47g) try to prevent certain compounds of low order of oral toxicity from defaulting into Class IV.

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