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Epoxide and Thiirane Toxicity In vitro with the Ciliates *Tetrahymena pyriformis*: Structural Alerts Indicating Excess Toxicity

Franziska Schramm,[†,‡] Andrea Müller,[§] Heiko Hammer,[†,‡] Albrecht Paschke,[†] and Gerrit Schüürmann[†,‡,*]

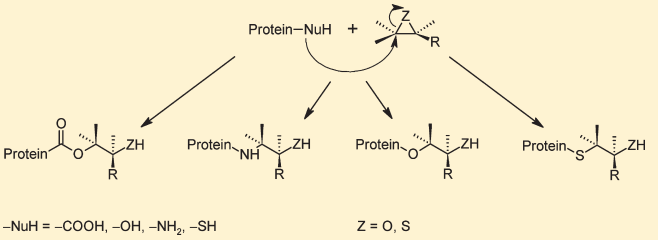
[†]UFZ Department of Ecological Chemistry, Helmholtz Centre for Environmental Research, 04318 Leipzig, Germany

[‡]Institute for Organic Chemistry, Technical University Bergakademie Freiberg, 09596 Freiberg, Germany

[§]UFZ Department of Proteomics, Helmholtz Centre for Environmental Research, 04318 Leipzig, Germany

Supporting Information

ABSTRACT: The 48 h toxicity of 18 organic narcotics, 13 epoxides, and 2 thiiranes toward the ciliates *Tetrahymena pyriformis* was determined in terms of 50% growth inhibition EC_{50}. Nominal EC_{50} was corrected for volatilization and sorption to quantify the freely dissolved compound fraction in solution. The derived baseline narcosis model served to evaluate toxicity enhancements T_e as ratios of narcosis-predicted over experimental EC_{50} values. Among the nine heterocycles with aliphatic side chains that include two thiiranes, three compounds yielded $T_e > 10$, suggesting their covalent binding at nucleophilic protein sites such as −OH, −NHR, and −SH through S_N2-type ring-opening. As a general trend of this group, T_e decreases with increasing alkyl group size. Moreover, four of the six nonaliphatic epoxides exerted substantial excess toxicities with $T_e > 10$, which could be rationalized by ring-opening activation through negative inductive effect, benzylic stabilization, and phenyl ring H-bonding. By contrast, 1,2 substituted epoxides showed narcosis-level toxicity, despite the opportunity of side-chain Schiff-base formation with protein amino groups. The resulting structural alerts enable an in silico screening of epoxides and thiiranes for their potential to exert excess toxicity. Note that observed differences in T_e sensitivity between ciliates, bacteria and fish should be taken into account when designing in vitro alternatives to fish toxicity studies.



INTRODUCTION

Oxiranes (epoxides) are three-membered heterocycles with oxygen as ring atom that occur naturally in plants, insects and microorganisms.[1] The oxirane moiety may belong to peptides, sugars, and fatty acids, and may be generated in vivo through oxidative metabolism of endogenous and xenobiotic compounds.[1,2] The negative inductive effect of oxygen makes the ring carbons electrophilic, facilitating ring-opening reactions of these strained rings.

Industrial production includes their use as synthetic intermediates for agricultural chemicals, cosmetics, and surface coatings, and as resin component. Moreover, epoxides may serve as insecticides, antibiotics, and disinfectants. Thiiranes are three-membered rings containing sulfur, and may act as herbicides, insecticides, and molluscicides.

In the absence of acidic catalysis, ring-opening proceeds through an S_N2-type mechanism with the nucleophile preferentially attacking the less substituted ring carbon (Scheme 1). This reaction pathway is the major cause for mutagenic and carcinogenic effects,[3−6] and also for protein toxicity.[7] The latter may result in skin sensitization,[8] and in excess toxicity toward aquatic species.[9−12] Electrophilic reactivity is thus toxicologically relevant, and several authors have sought to develop chemical assays, such as glutathione-based assays, to enable its quantification,[13−16] or to develop its prediction from molecular structure.[17,18]

In aquatic toxicology, reactive toxicity is often associated with an effect level above baseline narcosis.[19,20] The few respective studies available for epoxides[6,9−12] indicate that these may exert substantial excess toxicities, depending on the substitution pattern and the endpoint of interest.

In the present investigation, 48 h growth inhibition of the ciliates *Tetrahymena pyriformis* in terms of EC_{50} (effective concentration yielding 50% growth inhibition), was determined for 18 organic contaminants classified as narcotics,[21] 13 epoxides, and two thiiranes. Considering the compound loss from in vitro bioassays due to volatilization and sorption,[22−24] a quantification of these processes was achieved through targeted experiments, enabling the calculation of corrected EC_{50} values that represent the freely dissolved compound concentration.

The toxicity enhancement T_e driven by electrophilic reactivity was characterized through comparison with baseline narcosis,

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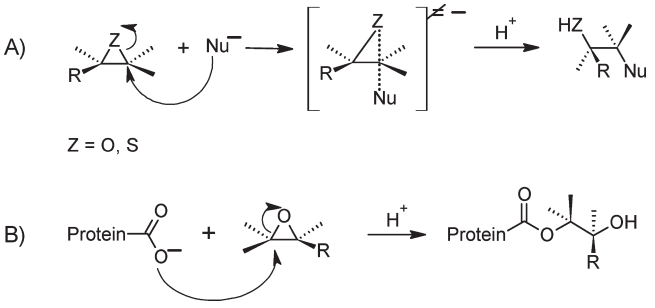
Scheme 1. (A) S_N2-Type Ring-Opening of Oxirane and Thiirane Derivatives through Their Reaction with a Nucleophile NuH under Neutral and Alkaline Conditions; The Less Substituted Ring Carbon Is the Preferred Site of Attack; (B) Protein Esterification As a Prominent Example of an S_N2-Type Reaction of Oxiranes with Endogenous Nucleophilic Sites[7]



resulting in the derivation of structural alerts that indicate the potency of epoxides and thiiranes to exert excess toxicity toward aquatic organisms.

MATERIAL AND METHODS

Chemicals. The test set of 33 organic compounds subject to toxicity analyses comprises 18 substances classified as narcotics,[21] 13 are epoxides, and 2 thiiranes. They were purchased from Merck, Fluka, Alfa Aesar, Riedel de Haën, and Sigma Aldrich, with purities above 96% except for ethyl-3-phenylglycidate where it was ≥90% (see Supporting Information (SI)). In the following study, we expect that the response in the growth inhibition assay is due to the tested substance and not influenced by the impurities of the substance (and thus also not confounded by mixture toxicity effects).

Logarithmic octanol/water partition coefficients, log K_{ow}, and Henry's law constants H at 25 °C were obtained from EPI Suite V3.12,[25] preferring measured over calculated values whenever possible. H was converted[26] to the air−water partition coefficient, K_{aw}, at the bioassay temperature of 28 °C using the ChemProp software.[27]

50% Growth Inhibition of *Tetrahymena pyriformis*. The population growth inhibition assay with 48 h exposure was performed at 28 °C in darkness without shaking as described earlier,[28] employing 5 mL cell suspensions in 50 mL flasks closed with glass stoppers fitted with a Glindemann sealing ring (VWR, Bruchsal, Germany), and stock solutions of substances in deionized water (log K_{ow} < 1) or in DMSO (log K_{ow} > 1). It was confirmed experimentally that up to 1% (v/v) DMSO had no effect on the population growth of *T. pyriformis*. Furthermore, with this amount of DMSO there was no concentration addition effect for narcotics detectable.

Population size was measured through counting vital cells (size 20−40 μm), using the CASY Cell Counter (Innovatis AG, Reutlingen, Germany) and the Multisizer 3 Coulter-Counter (Beckmann Coulter GmbH, Krefeld, Germany). Initial range-finding with duplicates was followed by two to three independent assays with triplicates for each of the six to seven concentrations as well as for the controls. For substances dissolved in DMSO, a respective DMSO control was added. The concentration−response curve was generated using the four-parameter sigmoid function

$$y = \min + \frac{\max - \min}{1 + \left(\frac{x}{EC_{50}}\right)^{-\text{Hill slope}}} \quad (1)$$

of SigmaPlot (Systat Software, Germany). In eq 1, x denotes a toxicant concentration, y the growth inhibition expressed as percentage, min and max the minimum and maximum percentage inhibitions resulting from curve fitting (with max and min being restricted to value ranges of 90% to 110% and −5% to 5%, respectively), Hill slope the slope parameter, and EC_{50} the toxicant concentration yielding 50% growth inhibition. In addition to nominal concentrations, EC_{50} corrected for volatilization and sorption was derived as described below.

Excess Toxicity. Linear regression of log EC_{50} of the narcotics on log K_{ow} yielded baseline narcosis models (see below). Comparison of experimental with accordingly predicted narcosis-level EC_{50} provided a quantification of the toxicity enhancement T_e:[10]

$$T_e = \frac{EC_{50}(\text{narc})}{EC_{50}(\text{exp})} \quad (2)$$

A threshold of T_e = 10 (log T_e = 1) was used to discriminate between narcotic-level (T_e < 10) and excess-toxic ($T_e \geq 10$) compounds, assuming that $T_e \geq 10$ (log $T_e \geq 1$) is a strong indication that the toxicity enhancement is driven by reactive or specific toxicity. The choice of this threshold resulted from estimating general experimental uncertainty and the typical scatter around regression lines.

Exposure Concentration Corrected for Volatilization. The compound loss through volatilization was measured employing HPLC-DAD (see SI), and predicted from the volumes of the solution and headspace (air), V_w and V_a, and the air/water partition coefficient K_{aw} through equilibrium thermodynamics:

$$\Delta c_w(\text{vol}) = \frac{c_w(\text{nom}) - c_w(\text{vol})}{c_w(\text{nom})} = 1 - \left(1 - \frac{K_{aw}}{K_{aw} + \frac{V_w}{V_a}}\right) = \frac{K_{aw}}{K_{aw} + \frac{V_w}{V_a}} = f_a \quad (3)$$

In eq 3, c_w(nom) denotes the nominal compound concentration in solution, c_w(vol) the concentration in the headspace after volatilization at thermodynamic equilibrium, and Δc_w(vol) the resultant relative concentration decrease that equals the compound fraction in the headspace air compartment, f_a.

Exposure Concentration Corrected for Sorption. HPLC-DAD measurements of compound concentrations after 24 h exposure in headspace-free vials were compared with nominal concentrations (see SI). The resultant concentration decrease due to sorption, Δc_w(sorp), equals the sorbed compound fraction, f_s. Linear regression of f_s on log K_{ow} resulted in a model enabling its prediction (see below).

Freely Dissolved Compound Concentration. Correction of the nominal compound concentration for both volatilization and sorption yielded the freely dissolved concentration in aqueous

Table 1. Set of 18 Organic Narcotics with Their Logarithmic Octanol/Water Partition Coefficients (log K_{ow}), Neutral Hydrolysis Half-Lives ($t_{1/2}$), Dimensionless Henry's Law Constants (K_{aw}), Compound Fractions in Air (f_a), Sorbed (f_s) and Freely Dissolved (f_w), Experimental 48 h Toxicity Towards the Ciliates *Tetrahymena pyriformis* (log EC_{50}), and Hill Slopes of the Associated Concentration-Response Curves.[a]

substance	CAS	log K_{ow}	$t_{1/2}$ [d]	K_{aw} (28 °C)	f_a [%]	f_s [%]	f_w [%]	log EC_{50} [M]	Hill slope
ethylene glycol	107−21−1	−1.20	n.a.	3.39×10^{-6}	0.01	0.00	99.99	-0.90 ± 0.03	2.5 ± 0.4
methanol	67−56−1	−0.77	n.a.	2.24×10^{-4}	0.47	0.00	99.53	-0.32 ± 0.01[b]	5.9 ± 7.8[b]
ethanol	64−17−5	−0.31	n.a.	2.49×10^{-4}	0.52	0.00	99.48	-0.55 ± 0.01	7.4 ± 1.8
acetone	67−64−1	−0.24	n.a.	1.91×10^{-3}	3.84	0.00	96.16	-0.86 ± 0.02	4.1 ± 0.8
2-butanon	78−93−3	0.29	n.a.	2.78×10^{-3}	3.48	0.00	96.52	-1.21 ± 0.02	3.6 ± 0.4
butanol	71−36−3	0.88	n.a.	4.49×10^{-4}	0.93	0.49	98.58	-1.62 ± 0.06	2.9 ± 0.8
methyl tertiary butyl ether	1634−04−4	0.94	n.a.	2.90×10^{-2}	58.58	0.95	41.03	-1.88 ± 0.04	2.0 ± 0.3
dichloromethan	75−09−2	1.25	2.54×10^{5}	1.45×10^{-1}	70.61	3.37	28.40	-2.66 ± 0.04	7.5 ± 5.5
trichloromethan	67−66−3	1.97	1.12×10^{6}	1.65×10^{-1}	77.53	8.98	20.45	-2.84 ± 0.05[b]	2.5 ± 0.5[b]
1-hexanol	111−27−3	2.03	n.a.	8.90×10^{-4}	1.82	9.44	88.91	-2.59 ± 0.03	3.6 ± 0.7
1-heptanol	111−70−6	2.31	n.a.	9.89×10^{-4}	2.02	11.62	86.59	-3.14 ± 0.04	2.8 ± 0.6
1-octanol	111−87−5	3.00	n.a.	1.30×10^{-3}	2.65	17.00	80.80	-3.73 ± 0.02	4.6 ± 0.8
1,2,4-trichlorobenzene	120−82−1	4.02	n.a.	6.63×10^{-2}	46.30	24.95	40.30	-4.68 ± 0.05	3.7 ± 1.1
diphenyl ether	101−84−8	4.21	n.a.	1.30×10^{-2}	14.48	26.43	62.92	-4.91 ± 0.03	2.6 ± 0.6
1,2,3,4-tetrachlorobenzene	634−66−2	4.60	n.a.	3.64×10^{-2}	32.09	29.46	47.90	-5.35 ± 0.05	1.4 ± 0.2
4-bromodiphenyl ether	101−55−3	4.94	n.a.	2.25×10^{-3}	2.84	32.11	65.96	-5.24 ± 0.04[b]	2.8 ± 0.8[b]
1-dodecanol	112−53−8	5.13	n.a.	1.23×10^{-3}	7.12	33.59	61.68	-5.16 ± 0.01	17.7 ± 1.9
pentachlorobenzene	608−93−5	5.17	n.a.	3.41×10^{-2}	30.69	33.90	45.81	-5.14 ± 0.11	0.9 ± 0.1

[a] Logarithmic octanol/water partition coefficient, log K_{ow}, was predicted using EPISuite,[25] and dimensionless Henry's law constant K_{aw} at 28 °C was calculated using our model[26] as implemented in the ChemProp software,[27] starting from experimental or predicted K_{aw} at 25 °C.[25] Hydrolysis half-lives at pH 7 were predicted using the ChemProp software,[27] n.a. means not available (not predictable), indicating that hydrolysis is very unlikely to play a role. The compound fractions in air (f_a), sorbed (f_s), and freely dissolved (f_w) are predicted using eqs 3−5. EC_{50} [mol/L] denotes the effective compound concentration yielding 50% growth inhibition of the ciliates *Tetrahymena pyriformis* after 48 h exposure, which was corrected using eqs 3−5. The actual log EC_{50} error is slightly unsymmetric (but symmetric for EC_{50}), with the larger errors being used for the symmetric intervals given. Hill slope refers to the respective parameter in eq 1, characterizing the steepness of the concentration−response curve. [b] Taken from a previous publication.[29]

solution, c_w(free):

$$c_w(\text{free}) = c_w(\text{nom}) \cdot (1 - f_a) \cdot (1 - f_s) = c_w(\text{nom}) \cdot f_w \quad (4)$$

Statistics. Model performance was characterized through the following parameters: r^2, squared correlation coefficient; q^2_{cv}, predictive squared correlation coefficient from leave-1-out cross validation; rms, root-mean-square error of calibration; rms_{cv}, cross-validated root-mean-square error of prediction; $F_{i,n-(i+1)}$, *F*-test value (with i = number of variables, and n = number of compounds).

RESULTS AND DISCUSSION

Volatilization Correction of Exposure. Compound loss through volatilization to the vial headspace reduces the concentration in solution, and thus the actual exposure of the test organism. The suitability of the mass balance model of eq 3 was confirmed with analytical HPLC-DAD measurements in bioassay vials at 24 h exposure time for the following three substances: glycidyl methacrylate (log K_{aw}(28 °C): −4.82), isobutyl acrylate (−1.43), and toluene (−0.51). The difference between experimental and calculated volatilization losses, Δc_w(vol) = f_a (compound fraction in the headspace) was less than 4%, with f_a ranging from essentially 0% to about 85% (see SI).

The results thus confirm that eq 3 describes the volatilization loss properly, and that the used vials with glass stoppers are sufficiently airtight for volatile chemicals. Accordingly, eq 3 has been used to correct experimental EC_{50} for volatilization.

Sorption Correction of Exposure. Neutral organic compounds can sorb to medium components, cell debris and the glass wall. To quantify this effect, the freely dissolved concentration has been measured in headspace-free samples containing cell suspensions of nonvital cells and cell debris.

To ensure that the sorption process alone was responsible for the loss of aqueous concentration, hydrolysis half-lives were estimated using the ChemProp software[27] (Tables 1 and 2). For most of the tested substances—except styrene-7,8-oxide—the hydrolysis half-life is larger ($t_{1/2}$ > 6.12 days) than the 48 h exposure time. The loss of the nominal concentration was determined after 24 h exposure. Consequently, the observed compound loss is due to sorption to medium components in the aqueous medium.

The results achieved for nine compounds with log K_{ow} from 0.29 to 5.17 show losses due to sorption, Δc_w(sorp) = f_s (sorbed compound fraction) from essentially 0% to 38% (see SI). The resultant regression equation

$$f_s = 0.078(\pm 0.013) \cdot \log K_{ow} - 0.066(\pm 0.047) \quad (5)$$

$$n = 9, r^2 = 0.84, q^2_{cv} = 0.80, \text{rms} = 0.056, \text{rms}_{cv} = 0.076, F_{1,7} = 37.9$$

was used for correcting experimental EC_{50} for sorption.

Baseline Narcosis. The narcotic mode of action is generally understood as reversible impairment of cellular membrane functions. For waterborne narcotics, toxicity is usually expected

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Table 2. Set of Nine Three-Ring Heterocycles with Aliphatic Substituents and Six Epoxides with Non-Aliphatic Substituents Together with Their Log K_{ow}, Neutral Hydrolysis Half-Lives ($t_{1/2}$), K_{aw}, Compound Fractions in Air (f_a), Sorbed (f_s), and Freely Dissolved (f_w), and Experimental Data Characterizing Their 48 h Toxicity Towards the Ciliates *Tetrahymena pyriformis*.[a]

name	no.	CAS	log K_{ow}	$t_{1/2}$ [d]	K_{aw} (28 °C)	f_a [%]	f_s [%]	f_w [%]	log EC_{50} [M]	log T_e	Hill slope
Aliphatic Epoxides and Thiiranes											
1,2-propylene oxide	A1	75−56−9	0.37	12.62	3.16×10^{-3}	6.2	0.00	93.80	-2.36 ± 0.01	1.05	6.9 ± 1.5
thiirane	A2	420−12−2	0.81	n.a.	1.48×10^{-2}	23.6	0.00	76.40	-3.23 ± 0.10	1.53	0.9 ± 0.2
2-methyl-1,2-epoxypropane	A3	558−30−5	0.83	6.36	9.76×10^{-3}	16.9	0.10	83.02	-2.52 ± 0.05	0.80	1.7 ± 0.3
1-butene oxide	B1	106−88−7	0.86	12.62	8.28×10^{-3}	14.8	0.33	84.92	-2.43 ± 0.07	0.69	1.7 ± 0.3
1,2-propylene sulphide	B2	1072−43−1	1.22	n.a.	1.99×10^{-2}	29.4	3.13	68.39	-3.18 ± 0.11	1.12	0.8 ± 0.2
1,2-epoxy-3-methylbutane	B3	1438−14−8	1.28	10.98	1.30×10^{-2}	21.5	3.60	75.67	-2.51 ± 0.09	0.40	0.8 ± 0.1
1,2-epoxyhexane	C1	1436−34−6	1.85	12.62	1.74×10^{-2}	26.7	8.04	67.41	-2.91 ± 0.04	0.30	1.2 ± 0.1
octene-1,2-oxide	C2	2984−50−1	2.83	12.62	3.16×10^{-2}	39.8	15.68	50.76	-3.83 ± 0.07	0.37	1.9 ± 0.4
1,2-epoxydodecane	C3	2855−19−8	4.79	12.62	1.02×10^{-1}	68.2	30.94	21.96	-5.01 ± 0.05	−0.16	2.0 ± 0.4
Nonaliphatic Epoxides											
isopropyl glycidyl ether	D1	4016−14−2	0.52	12.71	1.68×10^{-4}	0.5	0.00	99.50	-2.53 ± 0.04	1.08	1.5 ± 0.2
glycidyl methacrylate	D2	106−91−2	0.81	1045.50	1.53×10^{-5}	0.0	0.00	100.00	-3.21 ± 0.03	1.51	2.0 ± 0.1
styrene-7,8-oxide	D3	96−09−3	1.59	1.24	7.60×10^{-4}	1.6	6.02	92.48	-4.01 ± 0.05	1.63	5.7 ± 2.5
phenyl glycidyl ether	E1	122−60−1	1.61	12.71	4.02×10^{-5}	0.1	6.17	93.74	-3.58 ± 0.03	1.18	1.5 ± 0.1
ethyl 3-phenylglycidate	E2	121−39−1	2.55	293.30	1.13×10^{-5}	0.0	13.49	86.51	-3.60 ± 0.05	0.38	1.9 ± 0.4
chalcone α,β-epoxide	E3	5411−12−1	3.36	9.36	4.97×10^{-7}	0.0	19.80	80.20	-3.63 ± 0.08	−0.30	1.4 ± 0.3

[a] The compound numbering refers to Scheme 2. Logarithmic octanol/water partition coefficient, log K_{ow}, was predicted using EPISuite,[25] and dimensionless Henry's law constant K_{aw} at 28 °C was calculated using our model[26] as implemented in the ChemProp software,[27] starting from experimental or predicted K_{aw} at 25 °C.[25] Hydrolysis half-lives at pH 7 were predicted using the ChemProp software,[27] n.a. means not available (not predictable), suggesting that hydrolysis is very unlikely to play a role. The compound fractions in air (f_a), sorbed (f_s) and freely dissolved (f_w) are predicted using eqs 3-5. EC_{50} [mol/L] denotes the effective compound concentration yielding 50% growth inhibition of the ciliates *Tetrahymena pyriformis* after 48 h exposure, corrected using eq 3-5. The actual log EC_{50} error is slightly unsymmetric (but symmetric for EC_{50}), with the larger errors being used for the symmetric intervals given. T_e denotes the toxicity enhancement (eq 2) relative to the narcosis level (eq 6), and Hill slope refers to the respective parameter in eq 1.

to correlate with hydrophobicity in terms of log K_{ow}. In Table 1, the 48-h growth inhibition of *Tetrahymena pyriformis* is summarized for 18 organics classified previously as narcotics.[21] Besides the corrected log EC_{50}, the compound fractions in air (f_a), sorbed (f_s) and freely dissolved (f_w) (eqs 3−5) are listed. The Hill slopes are given as well, characterizing the steepness of the respective concentration−response curves (eq 1).

As can be seen from Table 1, the log K_{ow} range of 6.4 (from −1.20 to 5.17) is larger than the log EC_{50} range of ca. 4.5. Interestingly, the Hill slope varies by a factor of almost 20 (from 0.9 to 17.7).

The plot of log EC_{50} vs log K_{ow} (not shown) reveals ethylene glycol and dichloromethane as excess-toxic outliers probably due to biotransformation to electrophilic metabolites or direct alkylation of endogenous nucleophilic sites (dichloromethane). Their omission yields the following regression equation:

$$\text{Log } EC_{50}[\text{M}] = -0.87(\pm 0.02)\cdot \log K_{ow} - 0.99(\pm 0.08) \quad (6)$$

$$n = 16, r^2 = 0.99, \text{rms} = 0.19, q^2_{cv} = 0.99, \text{rms}_{cv} = 0.23, F_{1,15} = 1213$$

In the present study, eq 6 was used for predicting narcosis-level EC_{50} to assess T_e.

Figure 1 shows the regression line of eq 6 and a previously published narcosis baseline[30] with uncorrected nominal concentration data. Slope (−0.80) and intercept (−0.83 mol/L) of the latter come close to the ones (−0.80, −0.92) of a regression equation employing nominal log EC_{50} values of the present 16 compounds. Their comparison with eq 6 reveals that with increasing log K_{ow}, nominal EC_{50} tends to increasingly underestimate the compound toxicity.

Aliphatic Epoxide and Thiirane Toxicity Toward *Tetrahymena pyriformis*. Among the 15 three-ring heterocycles, seven epoxides and the two thiiranes can be classified as aliphatic derivatives because of respective substituents (compounds A1 to C3 in Scheme 2 and Table 2). Their physicochemical and toxicological data are summarized in Table 2. Here, log K_{ow} ranges from 0.37 to 4.79, while EC_{50} varies by only 2.5 log units.

This reduced toxicity variation is due to the fact that for the lower-hydrophobic derivatives, the observed toxicity is larger than expected from baseline narcosis. It indicates a substantial contribution of reactive mechanisms to EC_{50} due to electrophilic attack at nucleophilic sites of proteins (see Scheme 1).

The largest toxicity enhancements T_e as compared to baseline narcosis (eqs 2 and 6) are observed for thiirane (A2; log T_e = 1.53), 1,2-propylene sulfide (B2; log T_e = 1.12), and 1,2-propylene oxide (A1; log T_e = 1.05). Comparison of the latter two suggests that thiiranes are similarly reactive toward a S_N2-type attack of nucleophiles as epoxides. A possible reason is that their smaller ring strain (83 vs 114 kJ/mol)[31] and their smaller ring-carbon charge (O is more electronegative than S), which would suggest lower reactivity, is compensated by the better leaving group ability of RS^- as compared to RO^-.

Figure 1 shows the data distribution of log EC_{50} vs log K_{ow} together with the narcosis line (eq 6) and a parallel line representing log T_e = 1. It is seen that most of the aliphatic epoxides (filled squares) belong to the (operationally defined)

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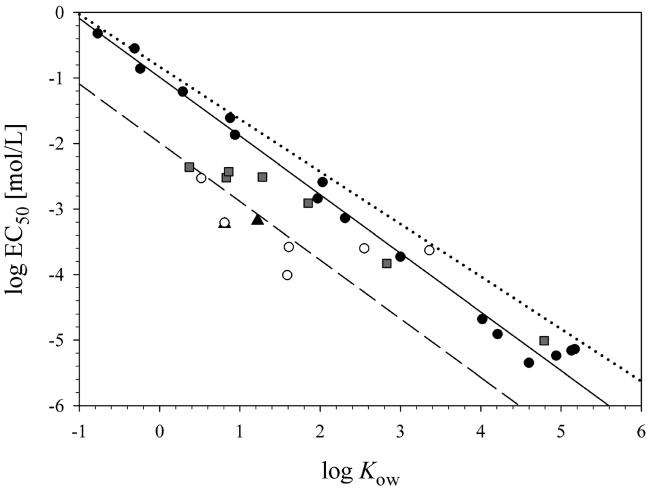
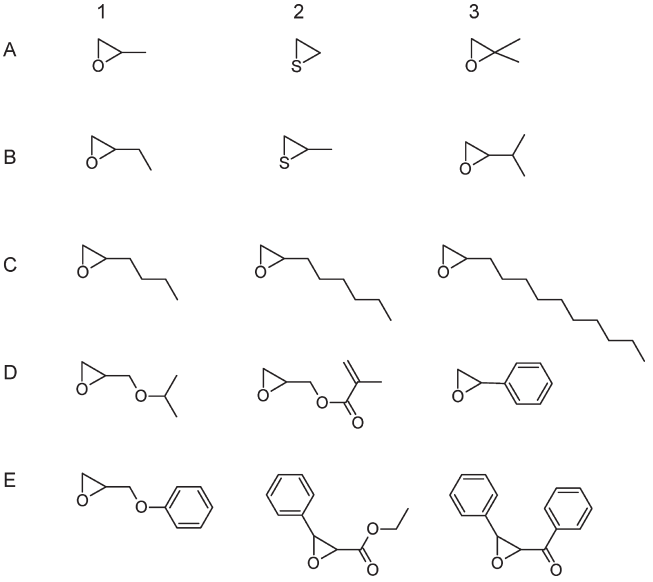


Figure 1. Log EC_{50} vs log K_{ow} for 16 baseline narcotics (see text and Table 1) and for 15 three-ring heterocycles covering 13 epoxides and two thiiranes (see text and Table 2). EC_{50} denotes the effective concentration yielding 50% growth inhibition of the ciliates *Tetrahymena pyriformis* after 48 h exposure, corrected for volatilization and sorption according to eqs 3−5. Filled circles: narcotics; filled squares: aliphatic epoxides; filled triangles: thiiranes (the left one at log K_{ow} = 0.81 is almost hidden by an unfilled circle); unfilled circles: nonaliphatic epoxides. The respective linear regression (eq 6) is shown as solid line, while the dashed line parallel to baseline narcosis represents $T_e = 10$. The dotted line represents a literature baseline narcosis model based on nominal EC_{50}.[30]

Scheme 2. Chemical Structures of the 15 Three-Ring Heterocycles Listed in Table 2[a]



[a] Aliphatic epoxides and thiiranes: 1,2-Propylene oxide (A1), thiirane (A2), 2-methyl-1,2-epoxypropane (A3), 1-butene oxide (B1), 1,2-propylene sulfide (B2), 1,2-epoxy-3-methylbutane (B3), 1,2-epoxyhexane (C1), octene-1,2-oxide (C2), and 1,2-epoxydodecane (C3). Nonaliphatic epoxides: Isopropyl glycidyl ether (D1), glycidyl methacrylate (D2), styrene-7,8-oxide (D3), phenyl glycidyl ether (E1), ethyl 3-phenylglycidate (E2), and chalcone α,β-epoxide (E3).

narcosis-level range of log $T_e < 1$ (where the observed T_e can still be explained through statistical scatter, measurement errors and a possible laboratory bias).

Increasing length of the aliphatic side chain increases toxicity (and thus reduces EC_{50}) because of increasing K_{ow}, but reduces T_e, as is illustrated when comparing 1,2-propylene oxide (A1), 1-butene oxide (B1), 1,2-epoxyhexane (C1), 1,2-epoxyoctane (C2), and 1,2-epoxydodecane (C3). It confirms an early observation[9] that with increasing hydrophobicity, the aquatic toxicity of reactive chemicals tends to approach the narcosis level. Moreover, the structural isomers 2-methyl-1,2-epoxypropane (A3) and 1,2-epoxybutane (B1) have similar K_{ow} values as well as similar EC_{50} and T_e values.

The general trend of decreasing epoxide T_e with increasing length of the aliphatic side chain has been reported earlier for both fish toxicity (14 d LC_{50} guppy;[9] 24 h LC_{50} goldfish[10]) and bacteria toxicity (30 min bioluminescence and 24 h growth inhibition of *Vibrio fischeri*),[12] and holds more generally for reactive organics. Interspecies comparison reveals that *Tetrahymena pyriformis* is more sensitive toward reactive toxicity mechanisms of aliphatic epoxides than *Vibrio fischeri*, where even 1,2-propylene oxide did not exert excess toxicity. However, with the guppy *Poecilia reticulata* log T_e values of 1.0−1.9 had been observed for 1,2-epoxyhexane, 1,2-epoxybutane and 1,2-epoxypropane,[9] contrasting with respective ciliate log T_e values of 0.40−1.05.

Coming back to Table 2, the larger toxicity of 1,2-propylene sulfide (B2) as compared to its oxygen analogue 1,2-propylene oxide (A1) by 0.8 log units is driven by a corresponding log K_{ow} difference. By contrast, its larger toxicity as compared to the essentially isohydrophobic 1,2-epoxy-3-methylbutane (B3) is driven by a larger log T_e (1.12 vs 0.40), reflecting a correspondingly larger electrophilic reactivity. The latter difference in toxicity and reactivity is thus probably due to a respective difference in steric shielding, suggesting a substantial respective sensitivity of the heterocycle S_N2-type reactivity under physiological conditions.

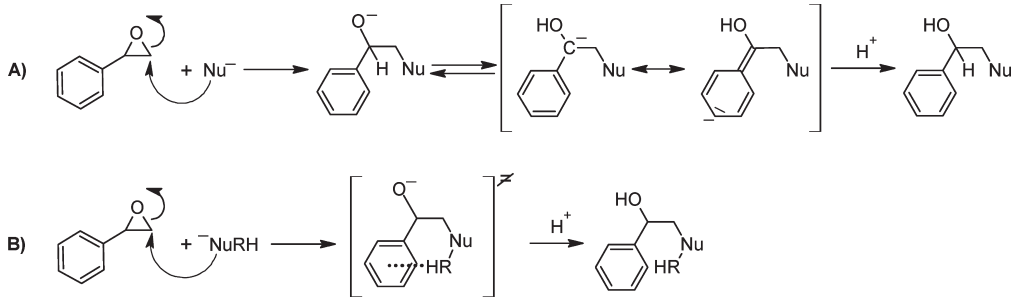
As noted above, the heterocycle ring-opening is regioselective, preferring a nucleophilic attack at the less-substituted ring carbon because of smaller steric shielding. Interestingly, the difference in log T_e − taken as rough surrogate for reactivity − is ca. 0.4 between 1,2-propylene oxide (A1) and 1-butene oxide (B1) as well as between thiirane (B2) and 1,2-propylene sulfide (B2), in both cases differing in just one methyl group. This appears to confirm the sensitivity of the reactive toxicity of three-ring heterocycles to steric shielding.

The Hill slopes of the concentration−response curves observed for the aliphatic epoxides and thiiranes vary by a factor of 9 (from 0.8 to 6.9; Table 2). They are in the range observed for the narcotics except for 1-dodecanol (17.7, Table 1). Note further that among the aliphatic epoxides, 1,2-propylene oxide with the largest Hill slope has the smallest toxicity but a significant log T_e (1.05), and that the apparently similarly reactive sulfur analogue 1,2-propylene sulfide has a Hill slope at the low end of the values observed. It shows that the steepness of the concentration−response curve appears to be not informative with regard to discriminating narcosis-level from excess toxicity.

Nonaliphatic Epoxide Toxicity Toward *Tetrahymena pyriformis*. For the six nonaliphatic epoxides (D1 to E3), K_{ow} and K_{aw} vary by 2.9 and 3.3 orders of magnitude, respectively, and EC_{50} by 1.5 log units (Table 2). T_e goes up to a factor of 43 with four values being above 10 (log $T_e > 1$).

Styrene epoxide (D3) shows the largest toxicity enhancement over baseline narcosis with a log T_e of 1.63, suggesting a reactive mode of action. Scheme 3 offers two explanations for

Scheme 3. Possible Reaction Mechanisms Explaining the Enhanced Toxicity of Styrene-7,8-oxide; (A) The S_N2-Type Ring-Opening through Reaction with an Endogenous Nucleophile Is Facilitated through a Tautomeric Equilibrium Coupled with a Mesomeric Stabilization; (B) The Transition-State Energy of the S_N2-Type Reaction Is Lowered through H-Bonding between Respective Donor Groups of Protein Side Chains and the Aromatic Styrene Ring As H-Bond Acceptor



this finding: On the one hand, the nucleophilic attack at the epoxide carbon could be facilitated through a tautomeric equilibrium coupled with a mesomeric stabilization. On the other hand, the aromatic ring could serve as H-bond acceptor, thus reducing the transition-state energy. Aliphatic epoxides lack both opportunities, making the substantial T_e difference reasonable. Note further that the estimated hydrolysis half-life is 1.24 days, indicating that hydrolysis may have contributed to the compound loss. Thus, the freely dissolved compound concentration may have been still smaller than predicted with eqs 3−5, resulting in a still larger toxicity enhancement

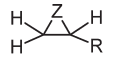
Both glycidyl ethers (D1 and E1) have similar log T_e values around 1.1−1.2. This contrasts with respective *Vibrio fischeri* results where their log T_e differed by ca. 1.[12] At present, we have no explanation for this difference in species sensitivity. Nevertheless, S_N2-type epoxide ring-opening may contribute to the observed excess toxicity, facilitated through a negative inductive effect of the $-CH_2-OR$ substituent. For phenyl glycidyl ether (E1), an additional pathway would be an S_N2 reaction at the methylene carbon, cleaving the phenolate anion as good leaving group.[12]

The second largest T_e of 32 among the nonaliphatic epoxides was observed for glycidyl methacrylate (D2). Its larger T_e as compared to both glycidyl ethers suggests the contribution of a Michael-type reaction at the acrylate β-carbon.[12] Accordingly, both S_N2-type epoxide ring-opening and side-chain Michael-type addition may take place.

For ethyl 3-phenylglycidate (E2) and chalcone α,β-epoxide (E3), the T_e values indicate narcosis-level toxicity. These compounds are the only epoxides of the present test set with substituents at both ring carbons, suggesting a correspondingly reduced ring-carbon reactivity due to steric hindrance. However, both contain a reactive side chain, offering the possibility of Schiff-base formation between an endogenous amine group and the carbonyl carbon. It seems that this does not play a role with *Tetrahymena pyriformis*, while with *Vibrio fischeri* log T_e values up to 1.6 had been observed.[12] Note further that Figure 1 includes also the six nonaliphatic epoxides.

Coming back to Figure 1, the observed decrease of T_e with increasing log K_{ow} would suggest a cutoff around log K_{ow} 2 above which epoxides and thiiranes appear to exert narcosis-level toxicity toward *Tetrahymena pyriformis*. In view of the limited number of respective data available, however, we consider this finding preliminary, apart from the fact that other species may show other cut-offs.

Scheme 4. Structural Alerts for Three-Ring Heterocycles Covering Epoxides (Z = O) and Thiiranes (Z = S), Indicating the Potential for Exerting Excess Toxicity Towards the Ciliates *Tetrahymena pyriformis* with Toxicity Enhancements T_e > 10 (see eq 2)[a]



Z = O, S

R = H, CH_3, C_6H_5, Y, CH_2-Y

[a] R stands for hydrogen, methyl, phenyl, electron-withdrawing substituents Y, and reactive side chains CH_2-Y (e.g. Y = halogen or Michael acceptor). Specification of the latter is based on extrapolation from literature data with fish.[9,10].

Structural Alerts. From the present set of 15 oxiranes and thiiranes, the following rules can be derived. First, epoxides and thiiranes with at least one unsubstituted ring carbon have the potential for T_e > 10, provided the substituent is not a larger alkyl group. Here, the T_e sensitivity toward the alkyl group size differs significantly for different species. While T_e > 10 is confined to the methyl substituent with *Tetrahymena pyriformis*, respective toxicity enhancements were observed with the guppy up to 1,2-epoxyhexane (C_4 alkyl group).[9] By contrast, *Vibrio fischeri* yielded narcosis-level toxicity even for 1,2-epoxypropane.[12]

Second, substituents Y containing heteroatoms close or conjugated to the ring activate the heterocycle for S_N2-type ring-opening, provided one of the two ring carbons is unsubstituted. In the present test set, Y was confined to —CH_2—O—R with R being isopropyl or phenyl, but it is likely that other groups—if not too bulky—will also yield substantial toxicity enhancements. Note that with fish, T_e values of 692 and 6275 were observed for glycidol (—CH_2—OH) and 1,3-butadienediepoxide.[9]

Third, substantial T_e values may be caused by reactive mechanisms facilitated through mesomeric stabilization of the intermediate (benzylic stabilization) or, alternatively, by H-bond interaction with phenyl substituents, both of which is possible for styrene epoxide. Interestingly, for this compound the observed T_e of 85 with *Tetrahymena pyriformis* is close to the one reported for the guppy (72).[9]

Fourth, reactive side chains offer alternative toxicity pathways, alone or in addition to heterocycle ring-opening. Among the

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presently investigated compounds, phenyl glycidyl ether may be subject to an S_N2 reaction at the methylene carbon that is activated through both a negative inductive effect and the presence of a good leaving group. However, the narcosis-level toxicities of both ethyl-3-phenylglycidate and chalcone α,β-epoxide—contrasting with respective *Vibrio fischeri* results[12]—suggests that *Tetrahymena pyriformis* is less sensitive to Schiff-base formation.

The above-mentioned chemical reactions are conditioned by substructural features of the epoxides and thiiranes. In Scheme 4, the corresponding structural alerts likely associated with excess toxicity are summarized.

As compared to *Vibrio fischeri*,[12] *Tetrahymena pyriformis* are more T_e sensitive for heterocycles with small alkyl substituents, but less T_e sensitive concerning 1,2 substitution and with regard to Schiff-base formation as side-chain reaction. Moreover, literature data[9] reveal a larger T_e sensitivity of the fish species guppy (*Poecilia reticulata*) toward aliphatic epoxides as discussed above. These findings suggest that in the context of in vitro alternatives to fish toxicity studies, a judicious selection of nonanimal assays will be needed to compensate for individual differences in species sensitivity as observed here for electrophilic compounds and their potential for exerting excess toxicity.

■ ASSOCIATED CONTENT

Supporting Information. Further details of the experimental procedures, and analytical measurement results for the compound loss under bioassay conditions due to volatilization and sorption, respectively. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +49-341-235-1262; fax +49-341-235-1785; e-mail gerrit.schuurmann@ufz.de.

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