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Thiol Reactivity and Its Impact on the Ciliate Toxicity of α,β-Unsaturated Aldehydes, Ketones, and Esters

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A recently introduced chemoassay has been used to determine second-order rate constants of the electrophile−nucleophile reaction of 15 α,β-unsaturated aldehydes with glutathione. The respective k_{GSH} values vary for more than 3 orders of magnitude, and are within the range determined previously for 31 α,β-unsaturated ketones and esters. Structure−reactivity analyses yield distinct relationships between k_{GSH} and structural features of the compounds. Moreover, increasing k_{GSH} increases the aldehyde toxicity toward ciliates in terms of 48 h-EC_{50} values (effective concentration yielding 50% growth inhibition of *Tetrahymena pyriformis* within 48 h). A respective log−log regression equation including both k_{GSH} and the octanol/water partition coefficient, K_{ow}, yields a squared correlation coefficient of 0.96. Comparative analysis with corresponding data for 15 ketones and 16 esters reveals systematic differences between the three compound classes with regard to the individual contributions of hydrophobicity and electrophilic reactivity to aquatic toxicity. The former is particularly pronounced for aldehydes, while the ester toxicity is largely governed by reactivity, with ketones showing an intermediate pattern that is more similar to the one of esters than of aldehydes. It follows that within the Michael acceptor domain of α,β-unsaturated carbonyls, a distinction between aldehydes and nonaldehydic derivatives appears necessary when employing electrophilic reactivity as a component for the quantitative prediction of their reactive toxicity toward aquatic organisms.

Introduction

In aquatic toxicology, the minimum or baseline toxicity of an organic compound can be well estimated applying simple quantitative structure−activity relationships (QSARs), employing the logarithmic octanol/water partition coefficient, log K_{ow}, as a surrogate for the compound's membrane affinity (*1*). Electrophilic compounds are often more toxic than iso-lipophilic baseline narcotics, thus exerting excess toxicity due to chemical reactions with nucleophilic groups of endogenous macromolecules (*2*). Here, different reaction mechanisms such as nucleophilic substitution (*3–5*), Michael-type addition (*3, 4, 6, 7*), and Schiff-base formation (*3, 4, 8*) have been identified as causes of reactive toxicity. The latter is a prominent electrophilic pathway of toxicity of aldehydes that may yield imines >C═N− upon reaction with terminal amino groups of proteins or DNA (see Scheme 1), resulting in excess toxicity toward various aquatic organisms (*9–12*), skin sensitization (*13, 14*), and mutagenicity (*15, 16*).

If the aldehydic functionality is conjugated with a double or triple bond in the α,β-position, the compound may undergo Michael-type addition (1,4-addition) as a separate electrophilic reaction pathway (see Scheme 2). Accordingly, α,β-unsaturated aldehydes contain two different electrophilic sites associated with correspondingly different toxicological pathways, the β-carbon involved in 1,4-addition (Scheme 2) and the carbonyl carbon initiating Schiff-base formation (Scheme 1).

In a previous investigation of the ciliate toxicity of 41 α,β-unsaturated carbonyls, a QSAR covering eight aldehydes, 22 esters, and 11 ketones had been derived with a squared correlation coefficient (r^2) = 0.846 and root-mean-square error (rms) = 0.35, employing reactivity toward glutathione in terms of log RC_{50} (reactive concentration 50% after 2 h exposure) as the only compound descriptor (*6*). This result suggested that for α,β-unsaturated aldehydes, the reactive toxicity is governed by their Michael-acceptor reactivity in the same manner as for corresponding esters and ketones. With regard to rat hepatocyte toxicity, however, comparative analysis of the reactivity of 11 Michael-type aldehydes toward glutathione and butylamine as models for soft protein thiol and hard amine nucleophilic sites resulted in a different conclusion (*17*). Here, only amine reactivity correlated significantly with toxicity despite overall higher reactivities toward glutathione (r^2 = 0.742 vs 0.211 for amine vs thiol reactivity), suggesting that Schiff-base formation was the dominating pathway of reactive toxicity (*17*). Possible reasons for this apparent discrepancy in the study results include the use of different toxicological end points and the relatively small number of aldehydes investigated.

In the present study, the role of Michael-type addition as one pathway of the aquatic toxicity of α,β-unsaturated aldehydes is investigated further, now using a larger set of 15 Michael-type aldehydes. To this end, our previously introduced kinetic glutathione (GSH) chemoassay (*7*) is applied to determine their second-order reaction rate constants, k_{GSH}. With these data, structure−reactivity rules for the 1,4-addition of α,β-unsaturated aldehydes to soft thiol groups are derived. Subsequent analysis of ciliate toxicity in terms of log EC_{50} (effective concentration

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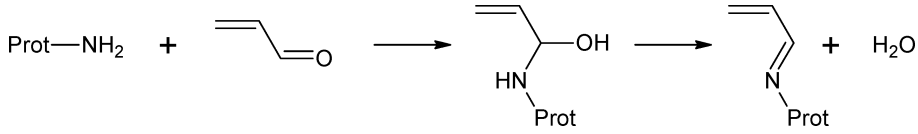
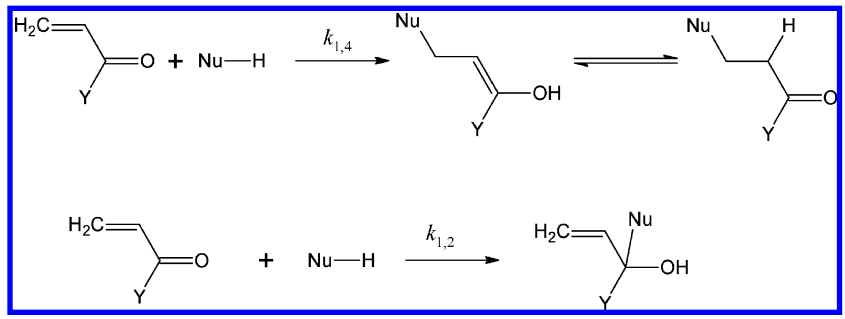
Scheme 1. Schiff-Base Formation of an α,β-Unsaturated Aldehyde with a Terminal Amino Group of an Endogenous Protein (Prot)



Scheme 2. Michael-type Addition (Conjugated 1,4-Addition, Top) and 1,2-Addition (Bottom) of a Nucleophile (Nu-H) to α,β-Unsaturated Aldehydes (Y=H), Ketones (Y=alkyl), and Esters (Y=OR)[a]



[a] The reaction with GSH as soft nucleophile is assumed to proceed predominantly via 1,4-addition, thus setting $k_{GSH} \approx k_{1,4}$ for the associated 2nd-order rate constant.

yielding 50% growth inhibition after 48 h exposure) yields a highly significant regression relationship when employing both log K_{ow} and log k_{GSH}. Comparison with 15 α,β-unsaturated ketones and 16 α,β-unsaturated esters shows further that their log k_{GSH} contribution to ciliate toxicity differs substantially from the one of the aldehydes. It follows that although all three compound classes belong to the Michael acceptor domain of α,β-unsaturated carbonyls, they differ in the relative contributions of hydrophobicity and Michael-type reactivity to aquatic toxicity. The latter suggests that for the predictive evaluation of the aquatic toxicity of Michael-type electrophiles, a separately calibrated QSAR is recommended for aldehydes in case k_{GSH} or related reactivity data are used to quantify their reactivity component of toxicity.

Materials and Methods

Materials. The 46 test chemicals used in this study were purchased from Merck (Darmstadt, Germany), Alfa Aesar (Karlsruhe, Germany), Sigma (Missouri), Acros Organics (Geel, Belgium), and Fluka (Buchs, Switzerland). Purity of all compounds was ≥95%. Reduced glutathione (GSH), dimethyl sulfoxide (DMSO), 2,2′-dinitro-5,5′-dithiodibenzoic acid (DTNB), disodium hydrogen phosphate (anhydrous), and potassium dihydrogen phosphate (anhydrous) were obtained from Merck (Darmstadt, Germany) at *p.a.* grade. For the *Tetrahymena pyriformis* bioassay, proteose-peptone and tryptone, both from Difco (Detroit), were used. Doubly distilled water was obtained from a "GFL 2102" distillation apparatus (GFLmbH, Germany).

Kinetic GSH Chemoassay. The electrophilic reactivity of 46 α,β-unsaturated aldehydes, ketones, and esters was quantified in terms of second-order reaction rate constants, k_{GSH}, employing glutathione as a nucleophile. The k_{GSH} values were determined using our spectrometric kinetic GSH chemoassay (*7*). For the subset of 31 ketones and esters, the respective rate constants were taken from previous publications (*7, 18*). In short, we used well plates (96 wells, total volume = 2 mL each) as reaction batches. In these, mixtures of DMSO and phosphate buffer (Na_2HPO_4/KH_2PO_4) containing 0.14 × 10^{-3} mol/L GSH and the electrophilic contaminant were incubated. After different reaction times ($0 \le t$ [min] ≤ 420), the remaining GSH was quantified by adding 40 μL of DTNB into each batch. Subsequently, the absorbance was determined at 412 nm (SpectraMax 384 Plus, Molecular Devices Corporation). The GSH concentration (c_{GSH}) was then determined using an external calibration.

Determination of Reaction Rate Constants. 2nd-order rate constants for the reaction of less reactive electrophiles with GSH (k_{GSH}) were determined employing *pseudo*-1st-order kinetics according to

$$\ln\left(\frac{c_{GSH}}{c_{GSH}^0}\right) = -k_{GSH}^{pseudo} t \quad (1)$$

Here, c_{GSH}^0 and c_{GSH} quantify the GSH concentrations at the beginning ($t = 0$) and after a defined reaction time t, respectively. Linear regression according to eq 1 yields pseudo-1st-order rate constants k_{GSH}^{pseudo}, which are converted to second-order rate constants k_{GSH} through application of $k_{GSH}^{pseudo} = k_{GSH} c^0$, where c^0 denotes the electrophile concentration at $t = 0$. Pseudo-1st-order conditions were met if the change in electrophile concentration, $c(t) - c^0$, was ≤5% during the observed reaction time. For more reactive compounds, second-order kinetics were applied. Regression of ln c_{GSH} − ln c on reaction time t yielded slope k'. Hence, the second-order rate constant (k_{GSH}) was derived from slope k' according to

$$k_{GSH} = k'/(c_{GSH}^0 - c^0) \quad (2)$$

GSSG Formation (Oxidative GSH Loss). The oxidative GSH loss through DMSO and solution-phase oxygen has been quantified by the pseudo-1st-order rate constant $k_{GSSG}^{pseudo} = 0.115 \times 10^{-2}$ min^{-1} and has been taken into account for the calculation of k_{GSH} as described previously (*7*).

***Tetrahymena pyriformis* Bioassay.** For most compounds, EC_{50} values quantifying the effective concentration that yields 50% growth inhibition of the ciliates *Tetrahymena pyriformis* after 48 h exposure were available from the literature (*9, 19, 20*). However, for some electrophiles these data were missing and generated employing our in-house *Tetrahymena pyriformis* bioassay protocol based upon the approach of Schultz (*21*). First, the ciliates were cultured without shaking at 28 °C in proteose-peptone-trypton-salts medium (pH = 7.2) containing 5 g/L proteose-peptone, 5 g/L tryptone, and 0.2 g/L K_2HPO_4. As inoculum in the *Tetrahymena pyriformis* population growth inhibition assay, a 24 h culture from the exponential phase with a cell concentration of 0.2 × 10^5 cell/mL was used.

Stock solutions of compounds were either prepared in doubly distilled water (log $K_{ow} < 1$) or in DMSO (log $K_{ow} > 1$) with the latter resulting in a final DMSO−water ratio of ≤1% (v/v). The bioassay accomplished at 28 °C without shaking in darkness was conducted in a concentration−response scenario using the protocol

Table 1. Data Set of 46 α,β-Unsaturated Carbonyls[a]

compound	CAS number	k_{GSH} [L mol^{-1} min^{-1}]	(±)Δk_{GSH} [L mol^{-1} min^{-1}]	log k_{GSH} [L mol^{-1} min^{-1}]	log K_{ow}[b]	log EC_{50}[mol/L]
15 α,β-Unsaturated Aldehydes						
2-octynal	1846-68-0	487	26	2.69	2.07	−4.72[c]
phenyl propiolaldehyde	2579-22-8	446	28	2.65	1.32	−4.34[c]
methyl acrolein	78-85-3	203	6	2.31	0.74	−3.67[c]
2-ethyl acrolein	922-63-4	59.4	0.9	1.77	1.23	−3.91[d]
trans-2-pentenal	1576-87-0	28.3	1.7	1.45	1.09	−3.66[d]
trans-2, *cis*-6 nonadienal	557-48-2	22.8	2.2	1.36	2.84	−4.34[d]
trans-2-octenal	2548-87-0	18.0	0.1	1.26	2.57	−4.20[d]
4-methyl-2-pentenal	5362-56-1	10.6	0.5	1.03	1.51	−3.82[d]
trans-2-decenal	3913-81-3	10.1	0.1	1.00	3.55	−4.85[d]
trans,*trans*-2,4-hexadienal	142-83-6	6.74	0.34	0.83	1.37	−3.75[d]
trans,*trans*-2,4-heptadienal	4313-03-5	5.65	0.21	0.75	1.86	−3.86[d]
trans,*trans*-2,4-nonadienal	5910-87-2	3.49	0.43	0.54	2.84	−4.23[d]
3-methyl-2-butenal	107-86-8	1.71	0.07	0.23	1.15	−3.09[d]
trans-2-methyl-2-butenal[e]	497-03-0	0.474	0.022	−0.32	1.15	−2.86[d]
trans-2-methyl-2-pentenal[e]	623-36-9	0.350	0.021	−0.56	1.64	−2.98[d]
15 α,β-Unsaturated Ketones[f]						
1-pentene-3-one	1629-58-9	1261	63	3.10	0.90	−4.52[c]
1-hexene-3-one	1629-60-3	1173	30	3.07	1.39	−4.66[d]
1-octene-3-one	4312-99-6	1074	13	3.03	2.37	−4.92[d]
3-hexyne-2-one	1679-36-3	80.0	1.3	1.90	0.52	−4.32[d]
3-pentene-2-one	625-33-2	26.7	0.9	1.43	0.82	−3.54[d]
2-octene-4-one	4643-27-0	26.1	0.5	1.42	2.29	−4.01[d]
2-cyclopentene-1-one	930-30-3	25.6	0.1	1.41	0.71	−3.64[d]
4-hexene-3-one	2497-21-4	24.2	0.2	1.38	1.31	−3.93[d]
3-heptene-2-one	1119-44-4	12.5	0.3	1.10	1.80	−3.70[d]
3-octene-2-one	1669-44-9	11.4	0.2	1.06	2.29	−3.74[d]
3-nonene-2-one	14309-57-0	10.8	0.2	1.03	2.79	−3.98[d]
3-methyl-3-pentene-2-one[e]	565-62-8	0.779	0.018	−0.11	1.37	−2.66[d]
4-methyl-3-pentene-2-one[e]	141-79-7	0.208	0.007	−0.68	1.37	−2.36[d]
2-methyl-2-cyclopentene-1-one[e]	1120-73-6	0.200	0.001	−0.70	1.26	−2.17[d]
3-methyl-2-cyclopentene-1-one[e]	2758-18-1	0.074	0.002	−1.13	1.26	−1.68[d]
16 α,β-Unsaturated Esters[f]						
methyl propiolate	922-67-8	117	8	2.07	0.09	−4.77[d]
ethyl propiolate	623-47-2	105	5	2.02	0.58	−4.70[d]
propargyl acrylate	10477-47-1	51.4	2.9	1.71	0.94	−4.06[d]
allyl acrylate	999-55-3	19.6	1.1	1.29	1.57	−3.68[d]
methyl acrylate	96-33-3	11.4	0.3	1.06	0.73	−3.55[d]
ethyl acrylate	140-88-5	10.6	0.1	1.03	1.22	−3.52[d]
n-propyl acrylate	925-60-0	10.2	0.3	1.01	1.71	−3.53[d]
n-butyl acrylate	141-32-2	8.54	0.20	0.93	2.20	−3.52[d]
tert-butyl acrylate	1663-39-4	2.50	0.09	0.40	2.09	−3.27[d]
methyl-*trans*-2-octenoate[e]	7367-81-9	0.785	0.030	−0.11	3.10	−3.76[d]
propargyl methacrylate[e]	13861-22-8	0.220	0.020	−0.66	1.49	−2.63[d]
methyl crotonate[e]	623-43-8	0.164	0.005	−0.79	1.44	−2.08[d]
ethyl crotonate[e]	623-70-1	0.161	0.002	−0.79	1.63	−2.24[d]
methyl methacrylate[e]	80-62-6	0.072	0.004	−1.14	1.28	−1.78[d]
ethyl methacrylate[e]	97-63-2	0.058	0.005	−1.24	1.77	−2.07[d]
methyl tiglate[e]	6622-76-0	0.007	0.001	−2.15	1.69	−2.38[d]

[a] Experimental data for their reactivity towards glutathione in terms of 2nd-order rate constants, k_{GSH}, calculated logarithmic octanol/water partition coefficients, log K_{ow}, and experimental values for their 48 h ciliate toxicity, log EC_{50} (effective concentration yielding 50% growth inhibition of *Tetrahymena pyriformis*). [b] log K_{ow} calculated using KOWWIN v 1.67 (*23*). [c] In-house *Tetrahymena pyriformis* bioassay. [d] Taken from literature and converted to mol/L (*9, 19, 20*). [e] Reactivity corrected by pseudo-1st-order rate constant of oxidative GSH loss through GSSG formation by DMSO and solution-phase oxygen, $k_{GSSG}^{pseudo} = 0.115 \times 10^{-2}$ min^{-1} (*7*). [f] Reactivity data for 31 ketones and esters published previously (*7, 18*).

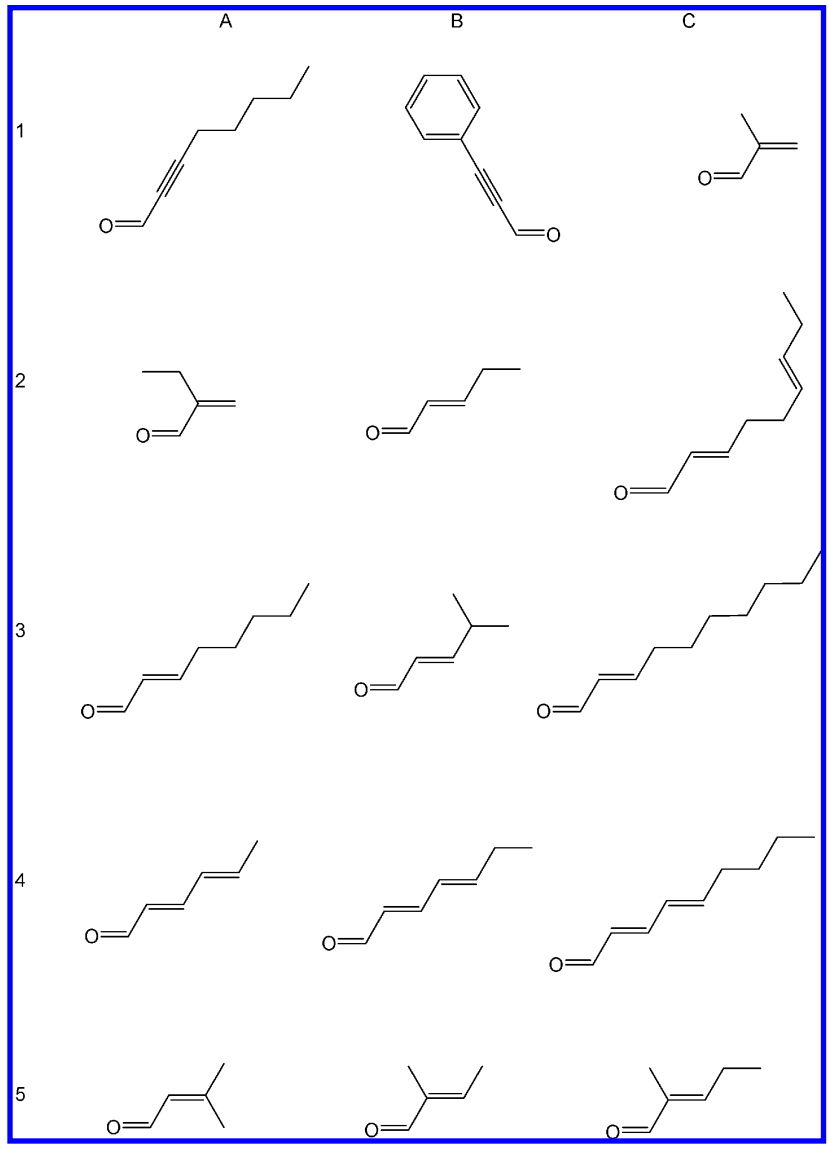
described by Müller and Herbarth (*22*). Cultures of *Tetrahymena pyriformis* were exposed to the chemicals for 48 h. For cell counting, a CASY Cell Counter (Innovates AG Bielefeld, Germany) was used. The toxicity of the substances was quantified by determining their average effective concentration yielding 50% growth inhibition (EC_{50}) using the four-parametric logistic equation of SigmaPlot 11 software (Systat Software GmbH Erkrath, Germany) with the *Y*-axis as cell counts/milliliter normalized as a percentage of the control and solvent-control, and the *X*-axis as the toxicant concentration in millimoles/liter.

Statistical Parameters. To evaluate the statistical performance of linear regression equations employing log k_{GSH}, the following parameters have been employed: squared correlation coefficient, r^2; predictive squared correlation coefficient evaluated through leave-1-out cross validation, q_{cv}^2; root-mean-square error of calibration, rms; cross-validated root-mean-square error of prediction, rms_{cv}; *F*-test value, $F_{i,n-(i+1)}$ (with i = number of variables, and n = number of compounds).

Results and Discussion

Aldehyde Reactivity toward Glutathione. In the upper part of Table 1, the second-order rate constants, k_{GSH}, of the reaction with glutathione (GSH) are listed for all 15 newly investigated α,β-unsaturated aldehydes (see Scheme 3 for their chemical structures). The values span over more than 3 orders of magnitude, ranging from 0.35 L mol^{-1} min^{-1} (log $k_{GSH} = -0.56$) for *trans*-2-methyl-2-pentenal to 487 L mol^{-1} min^{-1} (log $k_{GSH} = 2.69$) for 2-octynal. Comparison with the previously studied 31 α,β-unsaturated ketones and esters (*7, 18*) (lower part of Table 1) reveals that the latter cover a still broader range of reactivity, with the low and high end being represented by methyl tiglate ($k_{GSH} = 0.007$ L mol^{-1} min^{-1}, log $k_{GSH} = -2.15$) and 1-pentene-3-one ($k_{GSH} = 1261$ L mol^{-1} min^{-1}, log $k_{GSH} = 3.10$).

Scheme 3. Chemical Structures of the 15 α,β-Unsaturated Aldehydes Listed in Table 1[a]



[a] 2-octynal (A1), phenyl propiolaldehyde (B1), 2-methyl acrolein (C1), 2-ethyl acrolein (A2), *trans*-2-pentenal (B2), *trans*-2,*cis*-6-nonadienal (C2), *trans*-2-octenal (A3), 4-methyl-2-pentenal (B3), *trans*-2-decenal (C3), *trans*,*trans*-2,4-hexadienal (A4), *trans*,*trans*-2,4-heptadienal (B4), *trans*,*trans*-2,4-nonadienal (C4), 3-methyl-2-butenal (A5), *trans*-2-methyl-2-butenal (B5), and *trans*-2-methyl-2-pentenal (C5).

With regard to structure−reactivity relationships, the following trends become visible. First, β-carbon substitution reduces the aldehyde Michael-acceptor reactivity as compared to α-carbon substitution. An example of Table 1 is given by 2-ethyl acrolein as compared to *trans*-2-pentenal (compounds A2 and B2 of Scheme 3), the latter of which is less reactive by a factor of 2 as compared to the former (k_{GSH} 59.4 vs 28.3 L mol^{-1} min^{-1}). Actually, this is in line with expectation for two reasons: On the one hand, the steric accessibility of the β-carbon is more affected by β-substitution than by α-substitution. On the other hand, alkyl groups have a positive inductive effect that reduces the electrophilic character of the β-carbon if attached to this site.

Second, alkyl substitution at both the α- and β-carbon reduces the Michael-acceptor reactivity of aldehydes substantially. This is seen through comparison of 2-ethyl acrolein with its isomer *trans*-2-methyl-2-butenal (B5 in Scheme 3), with a factor of 125 between their k_{GSH} values (59.4 vs 0.474 L mol^{-1} min^{-1}). Comparative analysis with single substitution at either the α- or β-carbon (2-ethyl acrolein and *trans*-2-pentenal as discussed above) shows further that the combined effect is highly overadditive. A possible reason could be that in this latter case, the slower 1,2-addition involving the carbonyl carbon (see Scheme 2) becomes preferred through transition-state stabilization. Note further that double alkyl substitution at the β-carbon as represented by 3-methyl-2-butenal (A5 in Scheme 3) yields a still ∼4-fold higher reactivity (k_{GSH} 1.71 L mol^{-1} min^{-1}) than α,β-alkyl substitution (*trans*-2-methyl-2-butenal; k_{GSH} 0.474 L mol^{-1} min^{-1}). A clarification of this surprisingly large α,β-alkylation effect, however, requires further investigation, either through experimental analysis of the products actually generated or through computational analysis of the site-specific reactivities.

Third, increasing the alkyl chain length decreases the thiol reactivity moderately. A corresponding example of Table 1 are the three compounds *trans*-2-pentenal, *trans*-2-octenal, and *trans*-2-decenal (B2, A3 and C3 in Scheme 3), showing a decrease in k_{GSH} by factors of 1.6 (k_{GSH} 28.3 vs 18.0 L mol^{-1} min^{-1}) and 1.8 (18.0 vs 10.1), respectively. The compounds *trans*-2-methyl-2-butenal and *trans*-2-methyl-2-pentenal (B5 and C5) form a second example, differing in their k_{GSH} values by a factor of 1.4 (0.474 vs 0.350). A third example is the series *trans*,*trans*-2,4-hexadienal, *trans*,*trans*-2,4-heptadienal, and

Table 2. Linear Regression of the Ciliate Toxicity (48 h Growth Inhibition of *Tetrahymena pyriformis*), log EC_{50} [mol/L], against the Logarithmic Rate Constant of Reaction with Glutathione, log k_{GSH} [L mol^{-1} min^{-1}], for α,β-Unsaturated Carbonyl Compounds[a]

compound class	*n*	*b*	*c*	r^2	rms	q_{cv}^2	rms_{cv}	$F_{1,n-2}$
			log $EC_{50} = b \log k_{GSH} + c$					
aldehydes	15	−0.405 (±0.127)	−3.43 (±0.19)	0.44	0.43	0.28	0.50	10.2
ketones	15	−0.687 (±0.053)	−2.80 (±0.09)	0.93	0.25	0.90	0.30	169
esters	16	−0.656 (±0.080)	−3.04 (±0.10)	0.83	0.37	0.75	0.47	67.6
ketones and esters	31	−0.645 (±0.046)	−2.95 (±0.07)	0.87	0.34	0.84	0.38	194
all	46	−0.613 (±0.048)	−3.05 (±0.07)	0.78	0.40	0.76	0.42	160

[a] Regression equations and associated statistics are given for 15 aldehydes, 15 ketones, and 16 esters as well as for the combined set of 46 compounds. Slope and intercept of the regression equation are denoted by *b* and *c*, respectively; *n* = number of compounds; r^2 = squared correlation coefficient; rms = root-mean-square error; q_{cv}^2 = squared leave-1-out cross-validation correlation coefficient; rms_{cv} = cross-validation root-mean-square error; $F_{1,n-2}$ = *F*-test value; log k_{GSH} and log EC_{50} values are listed in Table 1.

trans,*trans*-2,4-nonadienal (A4, B4, and C4), showing a decrease in k_{GSH} by factors of 1.2 and 1.6, respectively (6.74 vs 5.65 vs 3.49). Interestingly, 2-methyl acrolein and 2-ethyl acrolein (C1 and A2) as α-alkylsubstituted aldehydes show a much more pronounced reactivity difference by a factor of 3.4 (k_{GSH} 203 vs 59.4).

Fourth, aldehydes with a conjugated triple bond are more reactive toward thiol than double-bonded counterparts. Indeed, the two top-reactive aldehydes in Table 1 have a conjugated triple bond (A1 and B1 in Scheme 2), and 2-octynal is more reactive than its double-bonded counterpart *trans*-2-octenal (A3) by a factor of ∼27. Note further that among the α,β-unsaturated esters, the two most reactive compounds, methyl propiolate and ethyl propiolate (k_{GSH} 117 and 105 L mol^{-1} min^{-1}, see the lower part of Table 1) also have a conjugated triple bond. Nevertheless, triple-bonded Michael acceptors are electronically harder than their double-bonded counterparts (*18*), showing that there is no simple relationship between hardness (or softness) and the electrophilic reactivity toward the soft thiol group of glutathione.

Fifth, fully conjugated dienals are less reactive as Michael acceptors than dienals with an isolated double bond. This is seen through comparison of the isomers *trans*,*trans*-2,4-nonadienal and *trans*-2,*cis*-6-nonadienal (C4 and C2 in Scheme 3; k_{GSH} 3.49 vs 22.8 L mol^{-1} min^{-1}). A possible explanation is that in the extended conjugated system, both the β- and δ-carbon share the partial positive charge, which apparently results in a less pronounced overall electrophilicity as compared to the standard Michael-acceptor situation with a larger electron deficiency at the β-carbon (see Scheme 2).

Sixth, the Michael-acceptor reactivity of α,β-unsaturated aldehydes is in the same range as compared to the ones of their isomeric ketone counterparts. This is illustrated through the isomers *trans*-2-pentenal and 3-pentene-2-one with k_{GSH} values of 28.3 and 26.7 L mol^{-1} min^{-1}, respectively. A further example is given by the isomers 2-methyl-2-pentenal and 3-methyl-3-pentene-2-one that have k_{GSH} values of 0.350 and 0.779 L mol^{-1} min^{-1}, respectively.

Seventh, replacement of the carbonyl H of α,β-unsaturated aldehydes by a methyl ester function is accompanied by a substantial reduction of the Michael-acceptor reactivity. Indeed, the thiol reactivity of *trans*-2-methyl-2-butenal is larger by a factor of 68 than the one of methyl tiglate, keeping in mind that both compounds have quite low k_{GSH} values (0.474 vs 0.007 L mol^{-1} min^{-1}), with methyl tiglate being at the low end of sensitivity of our kinetic chemoassay (*7*). Comparing *trans*-2-pentenal (ethyl group at β-C) with methyl crotonate that however has a methyl group at β-C provides another example of this qualitative trend, with k_{GSH} values of 28.3 vs 0.164 L mol^{-1} min^{-1} corresponding to a more than 100-fold difference in thiol reactivity (see Table 1).

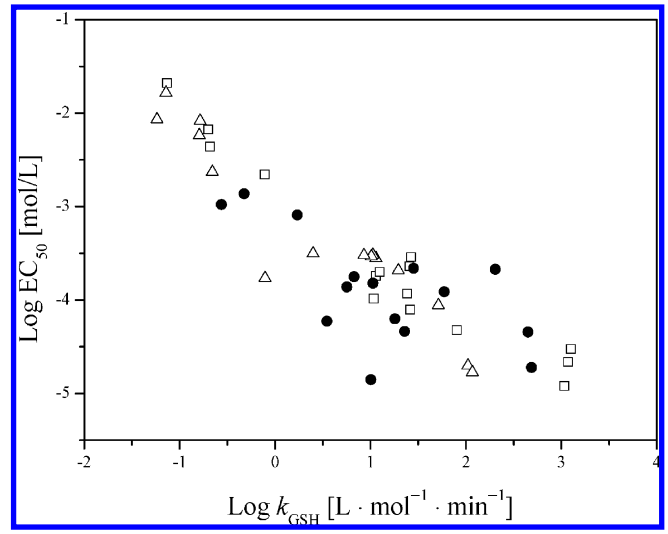


Figure 1. Ciliate toxicity in terms of log EC_{50} (48 h growth inhibition 50% for *Tetrahymena pyriformis*, mol/L) vs electrophilic reactivity toward glutathione in terms of the logarithmic 2nd-order rate constant, log k_{GSH} [L mol^{-1} min^{-1}], for 46 α,β-unsaturated carbonyls: 15 aldehydes (●), 15 ketones (□), and 16 esters (Δ) listed in Table 1.

Relationship between Thiol Reactivity and Ciliate Toxicity. As outlined in the Introduction, the electrophilic attack at nucleophilic sites of protein side chains is likely to result in distinct toxicity of the correspondingly reactive compound. Moreover, earlier investigations have shown significant correlations between 48 h ciliate toxicity in terms of log EC_{50} (effective concentration yielding 50% growth inhibition of *Tetrahymena pyriformis*) and thiol reactivity for α,β-unsaturated ketones, esters, and aldehydes as well as halogenated carbonyls (*5*–*7*).

For the present set of 15 Michael-type aldehydes with newly determined k_{GSH} values, linear regression of log EC_{50} (*9, 19, 20*) against log k_{GSH} yields $r^2 = 0.44$, which is much lower than respective correlations for 15 ketones ($r^2 = 0.93$) and 16 esters ($r^2 = 0.83$) taken from our previous studies (*7, 18*) (see Table 2). Inspection of the regression equations reveals further that the slope (regression coefficient *b* in Table 2) is significantly smaller for aldehydes than for both ketones and esters (0.4 vs 0.7), reflecting a correspondingly lower sensitivity of toxicity on the compound electrophilicity for aldehydes as compared to the other carbonyls. In Figure 1, log EC_{50} [mol/L] is plotted against log k_{GSH} [L mol^{-1} min^{-1}] for all three compound classes, showing an overall curvilinear shape.

Among the eight aldehydes analyzed previously with regard to RC_{50} toward GSH (*6*), acrolein (RC_{50} = 0.086 mM), 2-butenal (RC_{50} = 0.22 mM), and 2-hexenal (RC_{50} = 0.76 mM)

Table 3. Linear Regression of the Ciliate Toxicity (48 h Growth Inhibition of *Tetrahymena pyriformis*), log EC_{50} [mol/L], against the Logarithmic Rate Constant of Reaction with Glutathione, log k_{GSH} [L mol^{-1} min^{-1}], for α,β-Unsaturated Carbonyl Compounds[a,b]

compound class	*N*	*a*	*b*	*c*	r^2	rms	q_{cv}^2	rms_{cv}	$F_{2,n-3}$
			$\log EC_{50} = a \log K_{ow} + b \log k_{GSH} + c$						
aldehydes	15	−0.526 (±0.042)	−0.433 (±0.036)	−2.45 (±0.09)	0.96	0.11	0.94	0.15	142
ketones	15	−0.242 (±0.085)	−0.683 (±0.043)	−2.44 (±0.15)	0.96	0.19	0.93	0.26	134
esters	16	−0.148 (±0.166)	−0.693 (±0.090)	−2.82 (±0.28)	0.84	0.36	0.66	0.54	33.7
esters	15[b]	−0.227 (±0.129)	−0.830 (±0.081)	−2.59 (±0.22)	0.91	0.27	0.80	0.42	58.9
ketones and esters	30[b]	−0.181 (±0.079)	−0.715 (±0.043)	−2.60 (±0.14)	0.91	0.27	0.88	0.33	141
all	45[b]	−0.333 (±0.065)	−0.671 (±0.041)	−2.45 (±0.12)	0.87	0.31	0.84	0.34	142

[a] Regression equations and associated statistics are given for 15 aldehydes, 15 ketones, and 16 esters as well as for the combined sets of 30 ketones and esters and 45 ketones, esters, and aldehydes. The statistical parameters are *n* = number of compounds, r^2 = squared correlation coefficient, rms = root-mean-square error, q_{cv}^2 = squared leave-1-out cross-validation correlation coefficient, rms_{cv} = cross-validation root-mean-square error, $F_{2,n-3}$ = *F*-test value. Log k_{GSH} and log EC_{50} values are listed in Table 1. [b] Methyl tiglate excluded.

are not part of the present compound set. For the latter two compounds, RC_{50} can be converted to k_{GSH} through

$$\log k_{GSH}[M^{-1}\,min^{-1}] = -0.998(\pm 0.023) \log RC_{50}[mM] + 0.732(\pm 0.019) \quad (3)$$

Eq 3 had been derived for the intermediate reactivity range 0.21 mM ≤ RC_{50} ≤ 34.8 mM (*7*), and thus is not applicable to acrolein. Inclusion of the accordingly calculated log k_{GSH} values for 2-butenal (1.39) and 2-hexenal (0.85) and their log EC_{50} [mol/L] values (−4.06, −3.77) (*6*) yields very similar log EC_{50} vs log k_{GSH} regression results for the augmented set of 17 Michael-type aldehydes (slope = −0.406 (±0.117), intercept = −3.43 (±0.17), $r^2 = 0.44$, rms = 0.40, $q_{cv}^2 = 0.29$, rms_{cv} = 0.47, and $F_{1,15} = 12.0$).

A possible explanation for the somewhat lower impact of Michael-acceptor reactivity on the toxicity of α,β-unsaturated aldehydes could be their parallel ability to act as Schiff-base formers when attacking amino groups (Scheme 1). Because k_{GSH} quantifies only the Michael-type reactivity as outlined in Scheme 2, it does not address the additional Schiff-base formation reactivity and thus accounts only for a part of the aldehydic reactivity profile. As mentioned above, Schiff-base formation had indeed been advocated as a major reactive component of toxicity of α,β-unsaturated aldehydes, besides an additional influence through the rate of metabolic detoxification to carboxylic acids mediated by aldehyde dehydrogenases (*17*). However, a detailed investigation of the lysine adduct formation of acrolein and methyl vinyl ketone suggests that also for α,β-unsaturated aldehydes, initial Michael addition rather than Schiff-base formation is governing the toxicological process (*24*).

In Table 2, Michael-type ketones and esters yield similar slopes (−0.69 vs −0.66) and intercepts (−2.8 vs −3.0) despite differences in the regression statistics (r^2 0.93 vs 0.83). Consequently, the combined model results in again similar regression coefficients (slope −0.65, intercept −3.0) with intermediate statistics ($r^2 = 0.87$, rms = 0.34) and can be used for predicting log EC_{50} of both compound classes.

By contrast, additional inclusion of the 15 aldehydes leads to a regression model of significantly reduced quality ($r^2 = 0.78$, $q_{cv}^2 = 0.76$, rms = 0.40; see Table 2), reflecting the substantial difference in the impact of Michael-acceptor electrophilicity on toxicity between aldehydes on the one side and ketones and esters on the other side. Because of the latter, merging α,β-aldehydes with corresponding ketones and esters into a combined QSAR is not recommended for predictive applications.

Contributions of log K_{ow} and log k_{GSH} to Ciliate Toxicity. Inclusion of hydrophobicity in terms of the logarithmic octanol/water partition coefficient, log K_{ow}, yields the regression equations for the ciliate toxicity in terms of log EC_{50} (48 h growth inhibition) as summarized in Table 3. For the 15 α,β-unsaturated aldehydes, r^2 increases to 0.96, and rms decreases from 0.43 (Table 2) to 0.11 (Table 3), indicating that the combined consideration of log K_{ow} and log k_{GSH} can well explain most of the variation in their aquatic toxicity. Moreover, the log k_{GSH} regression coefficient is very similar for the models without and with log K_{ow} (0.405 vs 0.433 for *b*, see Tables 2 and 3). The latter reflects the fact that hydrophobicity and Michael-type electrophilicity vary essentially independently across the 15 aldehydes under investigation (indeed, the respective squared intercorrelation coefficient is $r^2 = 0.01$). The associated ranges of variation are 2.8 log K_{ow} units, 3.3 log k_{GSH} units, and 2.0 log EC_{50} units, respectively (see Table 1).

Inclusion of 2-butenal (log K_{ow} = 0.60) and 2-hexenal (log K_{ow} = 1.58) with log k_{GSH} derived from experimental RC_{50} values (*6*) according to eq 3 (*7*) now yields slightly different regression parameters (a = −0.452 (±0.063), b = −0.439 (±0.057), c = −2.62 (±0.14)) with significantly inferior statistics ($n = 17$, $r^2 = 0.88$, rms = 0.19, $q_{cv}^2 = 0.81$, rms_{cv} = 0.24, $F_{2,14} = 51.6$). Inspection of the data distribution reveals 2-butenal as the outlier with the EC_{50} overestimated (and thus toxicity underestimated) by 0.56 log units. Indeed, according to the structure−reactivity relationships presented above, 2-butenal would be expected to be slightly more reactive than *trans*-2-pentenal, which contrasts with an even slightly smaller log k_{GSH} value for the former (1.39 vs 1.45). With the removal of 2-butenal from the regression of log EC_{50} on both log K_{ow} and log k_{GSH}, the results are essentially identical ($n = 16$, a = −0.523 (±0.042), b = −0.431 (±0.035), c = −2.46 (±0.09), $r^2 = 0.96$, rms = 0.11, $q_{cv}^2 = 0.94$, $rms_{cv} = 0.15$, $F_{2,13} = 146$) to the ones achieved for the 15 aldehydes with directly determined log k_{GSH} values. In Figure 2, predicted vs experimental log EC_{50} is plotted for all three compound classes, employing the class-specific regression models of Table 3.

The above-mentioned study of the hepatocyte toxicity (fresh hepatocytes isolated from male rats) of 11 Michael-type aldehydes reported also associated log K_{ow} values (*17*). Here, inclusion of log K_{ow} does not improve the correlation with toxicity: For modeling hepatocyte log LC_{50} (lethal concentration 50% quantifying 2 h-MTT cytotoxicity (*17*)), log K_{ow} combined with either log k_{GSH} (resulting in $r^2 = 0.22$) or log k_{NH2} (r^2 = 0.76) is statistically not significant; the associated intercorrelations between log K_{ow} and reactivity are $r^2 = 0.66$ (log k_{GSH}) and r^2 = 0.05 (log k_{NH2}), respectively. At first sight, these findings confirm the dominant impact of amine reactivity on the hepatocyte toxicity of Michael-type aldehydes as reported earlier (*17*). Note, however, that their LC_{50} range was only ~0.8

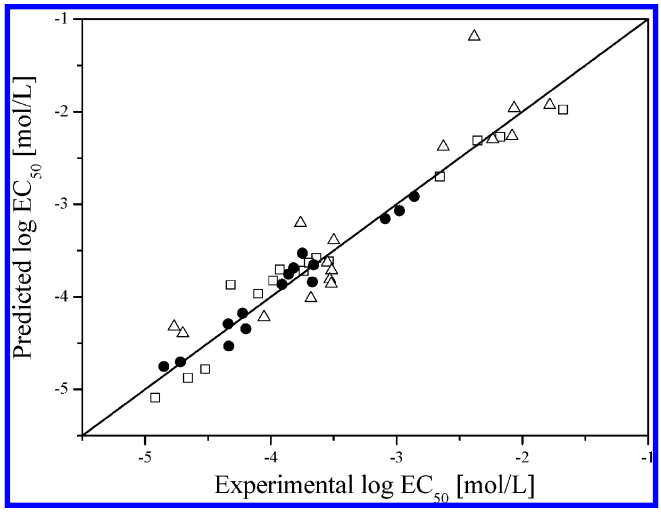


Figure 2. Predicted vs experimental ciliate toxicity in terms of log EC_{50} (48 h growth inhibition 50%) for 46 α,β-unsaturated carbonyls: 15 aldehydes (●), 15 ketones (□), and 16 esters (Δ) (see Table 1 and Scheme 3), employing the regression equations based on log K_{ow} and log k_{GSH} for aldehydes ($r^2 = 0.96$, rms = 0.11), ketones ($r^2 = 0.96$, rms = 0.19), and esters ($r^2 = 0.91$, rms = 0.27, calibrated for $n = 15$ without methyl tiglate; see Table 3). The largest outlier is the ester methyl tiglate (log EC_{50} [mol/L] predicted = −1.19 vs experimental = −2.38).

log units and reduces to ∼0.4 log units (−4.00 ≤ log LC_{50} [M] ≤ −3.62) when removing acrolein (log LC_{50} [M] = −4.40). By contrast, log EC_{50} quantifying ciliate toxicity varies by ∼2 orders of magnitude for our present set of 15 α,β-unsaturated aldehydes (−2.86 ≤ log EC_{50} [M] ≤ −4.72) and by 3.2 log units when taking into account also the 15 ketones and 16 esters (−1.68 ≤ log EC_{50} [M] ≤ −4.92). Accordingly, it appears that a larger variation in hepatocyte toxicity is needed for assessing its actual dependence on hydrophobicity and reactivity.

With a return to the ciliate toxicity of α,β-unsaturated esters and ketones, inclusion of log K_{ow} yields only minor increases in the explained log EC_{50} variation (r^2 0.96 vs 0.93 and 0.84 vs 0.83, respectively; see Tables 2 and 3). In addition, the ester log K_{ow} regression coefficient a is statistically not significant; here, the standard error of a is even larger than a. It follows that for the 16 α,β-unsaturated esters, their EC_{50} variation by ∼3 orders of magnitude is mainly driven by their Michael-type reactivity as quantified through k_{GSH}.

The largest ester outlier is methyl tiglate, with a second-order rate constant at the low end of sensitivity of our kinetic GSH chemoassay (log $k_{GSH} = -2.15$ (*7*)), a moderate hydrophobicity (log K_{ow} = 1.69), and a log EC_{50} overestimation (toxicity underestimation) by 0.81. Although methyl tiglate is formally an α,β-unsaturated carbonyl, its very low reactivity suggests its ciliate toxicity to be driven solely by hydrophobicity. Indeed, application of a K_{ow}-based regression model for aliphatic (not α,β-unsaturated) esters (log EC_{50} [M] = −0.79 log K_{ow} − 1.07 (*25*)) yields a predicted log EC_{50} (−2.41) for methyl tiglate that is close to its experimental value (−2.38). Removal of methyl tiglate results in significantly improved regression statistics for the remaining 15 esters (r^2 0.91 vs 0.84, rms = 0.27 vs 0.36; see Table 3), and the associated regression equation is the currently recommended model for α,β-unsaturated esters.

While the two-variable regression statistics are similar for aldehydes and ketones, the contributions of hydrophobicity and Michael-acceptor electrophilicity to log EC_{50} differ significantly. As can be seen from Table 3, the log K_{ow} regression coefficient is more than twice as large for aldehydes as for ketones (−0.53 vs −0.24), and with regard to log k_{GSH} there is a more than 1.5-fold difference in the opposite direction (−0.43 vs −0.68). Moreover, most of the ketone EC_{50} variation of ∼3 orders of magnitude can be traced back to electrophilic reactivity, while for aldehydes the log K_{ow} contribution to aquatic toxicity is much more pronounced (see above and Table 2). It follows that although both compound groups belong to the Michael-acceptor domain, their small but systematic difference in chemical structure translates into a systematic difference in the dependence of their aquatic toxicity on hydrophobicity and reactivity. Note further that with regard to log k_{GSH}, the reactivity contribution to toxicity is largest for esters (except for methyl tiglate; regression coefficient $b = -0.83$ for $n = 15$ in Table 3), followed by ketones (−0.68) and aldehydes (−0.43).

Although log K_{ow} appears to be not important for the ciliate toxicity of the presently selected esters (despite variations of three log units for both K_{ow} and EC_{50}; see Table 1), its linear combination with log k_{GSH} yields a statistically significant and robust regression model for the combined subset of 30 esters (without methyl tiglate, see above) and ketones ($r^2 = 0.91$, $q_{cv}^2 = 0.88$; see Table 3 and Figure 2). This model is expected to provide also reasonable log EC_{50} estimates for Michael-type acceptors containing both α,β-unsaturated ketone and ester groups, keeping in mind that this would represent a moderate extension of the chemical domain addressed so far.

Finally, inclusion of all α,β-unsaturated aldehydes, ketones and esters except methyl tiglate into one regression model results in only moderate statistics ($r^2 = 0.87$) due to the significantly different behavior of the aldehydes as discussed above. Consequently, this combined model is not recommended for application except for screening the toxicity of Michael acceptors that contain both α,β-unsaturated aldehyde and ketone or ester groups, again keeping in mind that such more complex Michael acceptors are not present in the chemical domain of Table 1. Apart from that, its statistics are superior to the correspondingly combined model employing only log k_{GSH} (r^2 0.87 vs 0.78, rms 0.31 vs 0.40; see Tables 2 and 3) reflecting the substantial impact of log K_{ow} on the ciliate toxicity of aldehydes.

As outlined above, the regression contribution of log K_{ow} to log EC_{50} is much more pronounced for aldehydes than for ketones, and only marginal for esters, despite a larger log K_{ow} variation for esters (3.0) than for aldehydes (2.8) and ketones (2.1). Conversely, the reactivity contribution to toxicity in terms of log k_{GSH} is largest for esters, followed by ketones and aldehydes. At present, we have no mechanistic explanation for these findings. A possible hypothesis would be that the observed differences in the toxicological roles of log K_{ow} and log k_{GSH} reflect differences in the preferred sites of covalent attack at endogenous macromolecules. So far, however, we are not aware of respective experimental information. For the time being, the results indicate that for the predictive evaluation of the aquatic toxicity of α,β-unsaturated carbonyls, attention should be paid to respective differences between individual compound classes of the Michael-acceptor domain.

The presently derived regression models for mechanistically interpreting and predicting ciliate toxicity are based on experimentally determined log k_{GSH} values. As shown recently, however, there are currently being developed computational chemistry methods to predict log k_{GSH} from molecular structure for α,β-unsaturated carbonyls (*18*). Accordingly, the newly determined log k_{GSH} data for aldehydes may also serve as reference values for calibrating respective calculation tools, which in turn could be used to predict, directly from molecular structure, the aquatic toxicity of Michael-acceptor electrophiles. The latter appears particularly attractive as an in silico com-

ponent of integrated testing strategies for REACH (*26*), thus enabling a predictive evaluation of the aquatic toxicity of α,β-unsaturated carbonyls.

Conclusions

The kinetic chemoassay employing glutathione as a model nucleophile is a sensitive tool for determining the Michael-acceptor reactivity of α,β-unsaturated aldehydes in terms of second-order rate constants k_{GSH}. The results show that alkyl substitution at the β-carbon reduces k_{GSH} more than at the α-carbon, and respective substitution at both the α and β positions yields a still substantially increased reduction in electrophilic reactivity. Moreover, k_{GSH} is significantly larger for aldehydes with an α,β triple bond than for their double-bonded counterparts. Within the chemical domain of α,β-unsaturated carbonyls, the thiol reactivity of aldehydes is comparable to the one of isomeric ketones but much higher than that of respective methyl esters. However, the impact of thiol reactivity on aquatic toxicity is smaller by a factor of 1.5 on the log scale for aldehydes than for both ketones and esters. The latter indicates that across the Michael acceptor domain, the contributions of log K_{ow} and log k_{GSH} to aquatic toxicity vary significantly. The high statistical quality of the respective regression relationship for α,β-unsaturated aldehydes suggests that for this compound class, reactive toxicity is governed by the Michael-acceptor pathway rather than by Schiff-base formation.

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