Structure–activity relationships for chemical and glutathione S-transferase-catalysed glutathione conjugation reactions of a series of 2-substituted 1-chloro-4-nitrobenzenes

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Glutathione S-transferases (GSTs) constitute an important class of phase II (de)toxifying enzymes, catalysing the conjugation of glutathione (GSH) with electrophilic compounds. In the present study, K_m, k_{cat} and k_{cat}/K_m values for the rat GST 1-1-, 3-3-, 4-4- and 7-7-catalysed conjugation reactions between GSH and a series of 10 different 2-substituted 1-chloro-4-nitrobenzenes, and the second-order rate constants (k_s) of the corresponding base-catalysed reactions, were correlated with nine classical physicochemical parameters (electronic, steric and lipophilic) of the substituents and with 16 computer-calculated molecular parameters of the substrates and of the corresponding Meisenheimer complexes with MeS^- as a model nucleophile for GS^- (charge distributions and several energy values), giving structure–activity relationships. On the basis of an identical dependence of the base-catalysed as well as the GST 1-1- and GST 7-7-catalysed reactions on electronic parameters (among others, Hammett substituent constant σ_p and charge on *p*-nitro substituents), and the finding that the corresponding reactions catalysed by GSTs 3-3 and 4-4 depend to a significantly lesser extent on these parameters, it was concluded that the Mu-class GST isoenzymes have a rate-determining transition state in the conjugation reaction between 2-substituted 1-chloro-4-nitrobenzenes and GSH which is different from that of the other two GSTs. Several alternative rate-limiting transition states for GST 3-3 and 4-4 are discussed. Furthermore, based on the obtained structure–activity relationships, it was possible to predict the k_{cat}/K_m values of the four GST isoenzymes and the k_s of the base-catalysed GSH conjugation of 1-chloro-4-nitrobenzene.

INTRODUCTION

Cytosolic glutathione S-transferases (GSTs; EC 2.5.1.18) constitute a family of phase II isoenzymes that catalyse the conjugation of glutathione (GSH) with a variety of electrophilic compounds [1–3]. The 24–29 kDa subunits of the GSTs can form homodimers and heterodimers, and each monomer has a kinetically independent active site [4]. The enzyme active site is composed of two subsites; a G-site for binding GSH and an H-site for binding the hydrophobic electrophile. Based on, among others, amino acid sequence identities, isoelectric points, substrate selectivities and immunological reactivities, the major cytosolic GSTs are classified into five non-homologous species-independent multigene families, namely Alpha, Mu, Pi [5], Theta [6] and Sigma [7].

Many different compounds, including toxic xenobiotics and reactive products of intracellular processes such as lipid peroxidation, act as GST substrates. While the electrophilic substrates usually share the feature that they are predominantly hydrophobic molecules, they are structurally quite diverse. The electrophiles range in size from methylchloride [8] and 1,2-dibromoethane [9] to large aromatic hydrocarbons such as benzo[*a*]pyrene-7,8-diol-9,10-epoxide [10]. Numerous studies using site-directed mutagenesis [11,12], X-ray crystallography [13,14] and computer modelling [15], performed with GST isoenzymes from different classes, have contributed to our present understanding of the catalytic mechanism and the structure of GSTs. However, the direct relationship between structure and function has not often been used as an approach for characterizing GSTs.

The first time that structure–activity relationships (SARs) were described for GSTs was in 1976, when Keen et al. presented Hammett plots of the catalytic constants of rat liver GSTs 1-2 and 3-4 for the GSH conjugation of a series of 4-substituted 1-chloro-2-nitrobenzenes [16]. In a previous study we examined the ability of four rat GST isoenzymes (GSTs 1-1, 3-3, 4-4 and 7-7) from three different classes to catalyse the conjugation of GSH to eleven 2-substituted 1-chloro-4-nitrobenzenes [17]. These substrates are generally thought to be conjugated to GSH via a nucleophilic aromatic substitution (S_NAr) reaction involving formation of a delocalized carbanion (Meisenheimer complex) as the rate-determining step [18]. The enzyme kinetic parameters K_m, k_{cat} and k_{cat}/K_m were determined and compared. Subsequently we described preliminary SARs for the chemical and the rat GST 4-4-catalysed GSH conjugation reaction of the same substrates [19]. The correlations obtained were compared with the SARs for the base-catalysed GSH conjugation reaction. It appeared that the GST 4-4-catalysed reaction depends to a lesser extent on the electronic parameters of the substituents than the base-catalysed reaction, suggesting that the corresponding rate-limiting transition states differ.

Rietjens et al. [20] used classical physicochemical parameters and semi-empirical computer-calculated molecular parameters for describing SARs for the GSH conjugation of fluoronitro-

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; ΔE_{l-h}, energy difference between E_{lumo} and E_{homo}; E_{homo}, energy of the highest occupied molecular orbital; E_{lumo}, energy of the lowest unoccupied molecular orbital; GS^-, thiolate anion of GSH; GS-conjugate, glutathione conjugate; GS-**1**, GS-conjugate of **1**; GS-**7**, GS-conjugate of **7**; GST, glutathione S-transferase; MeS^-, methanethiolate anion (model nucleophile for GS^-); SAR, structure–activity relationship; S_NAr, nucleophilic aromatic substitution.

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benzenes catalysed by multi-GST-containing rat liver cytosol. A linear correlation was found between four apparent K_m values and log P, such that the apparent K_m values decrease upon increased hydrophobicity (increased log P) of the fluoronitrobenzene substrates. Linear correlations were also found between ln V_{max} and E_{lumo} (energy of the lowest unoccupied molecular orbital) and between ln V_{max} and $\Delta\Delta$HF (relative heat of formation for formation of the Meisenheimer complexes). It was concluded that, for both the base-catalysed and the GST-catalysed GSH conjugation reactions, the initial attack of GS^- (the thiolate anion of GSH) on the fluoronitrobenzenes, resulting in formation of the Meisenheimer complexes, represents the rate-limiting step in overall catalysis. This conclusion contradicts the one drawn in a previous study [19], namely that the rate-limiting transition states for the base-catalysed and the GST 4-4-catalysed GSH conjugation reactions of 2-substituted 1-chloro-4-nitrobenzenes are different. This difference might be due to the use of multi-GST-containing rat liver cytosol [20] and pure rat liver GST 4-4 [19] in these two studies.

To determine the relative contributions of different GSTs in rat liver cytosol, SARs are described in the present study for the GSH conjugation of 2-substituted 1-chloro-4-nitrobenzenes by the single GST isoenzymes 1-1, 3-3, 4-4 and 7-7, and for the same base-catalysed reaction. Nine steric, lipophilic and electronic parameters of the substituents and 16 computer-calculated molecular parameters (charge distributions and several energy values) of the substrates and Meisenheimer complexes with MeS^- (methanethiolate anion) as a model nucleophile for GS^- were used in the regression analyses. Remarkable differences were found between the base-catalysed and the GST-catalysed GSH conjugation reactions, and also between the reactions catalysed by the different GST isoenzymes. In addition, the obtained SARs were used to predict the rates at which 1-chloro-4-nitrobenzene is conjugated to GSH catalysed either by base or by the four GSTs.

MATERIALS AND METHODS

Materials

The origin, syntheses and purities of all substrates used in this study (Table 1) were described previously [17]. 1-Chloro-4-nitrobenzene was obtained from Fluka Chemie AG (Buchs, Switzerland). 1-(*S*-Glutathionyl)-2,4-dinitrobenzene (glutathione conjugate of substrate **1**; GS-**1**) and 2-(*S*-glutathionyl)-5-nitrobenzonitrile (GS-**7**) were synthesized according to Kerklaan et al. [21]. Buffers and chemical reagents were of the highest quality commercially available. GST isoenzymes were purified from rat liver (GST 1-1, 3-3 and 4-4) and kidney (GST 7-7) using affinity chromatography (*S*-hexylglutathione–Sepharose 6B), and separation of the GST isoenzymes was achieved by chromatofocusing as described previously [22]. Purity was confirmed by SDS/PAGE, isoelectric focusing and HPLC analysis as described by Vos et al. [23] and Bogaards et al. [24]. Protein was determined by the method of Lowry, using BSA as standard [25].

Kinetic parameters

Determination of the kinetic parameters K_m, k_{cat} and k_{cat}/K_m for the GSH conjugation of the 2-substituted 1-chloro-4-nitrobenzenes catalysed by GST isoenzymes was described previously [17]. In short, the formation of glutathione conjugates (GS-conjugates) was followed spectrophotometrically over time at 37 °C at the maximal absorbance wavelength (λ_{max}) of the GS-conjugates. The final assay medium contained 0.1 M potassium phosphate buffer (pH 6.5, 0.1 mM EDTA), GST isoenzymes (concentration depending on the substrate tested), 1 mM GSH and various concentrations of substrate. The molar absorption coefficients of the GS-conjugates were also determined previously [17]. The K_m values of GSH for the different rat GST isoenzymes when determined with various concentrations of the 2-substituted 1-chloro-4-nitrobenzenes appeared to be in the range 100–300 μM, indicating that 1 mM GSH, used to determine the kinetic constants of the substrates, saturates the GST isoenzymes.

The rate constants for the base-catalysed reaction between GSH and the 2-substituted 1-chloro-4-nitrobenzenes (k_s) were determined in a previous study [19]. This conjugation reaction was performed in a 0.1 M potassium phosphate buffer (pH 9.2) at a temperature of 50 °C. At this temperature, the k_s values for all the 2-substituted 1-chloro-4-nitrobenzenes could be determined. The final concentration of GSH in the assay medium was 10 mM, and the substrate concentration was varied from 0.05 to 4 mM. Under these experimental conditions GSH was not oxidized; this was checked using 2,2′-dinitro-5,5′-dithiodibenzoic acid. The increase in absorption at the λ_{max} of the GS-conjugates

Table 1 R groups in 2-substituted 1-chloro-4-nitrobenzenes and the corresponding reference numbers used in this study

The second-order rate constant of the base-catalysed reaction between GSH and these substrates (k_s) is shown, as well as the kinetic parameter k_{cat}/K_m for the reactions catalysed by GSTs 1-1, 3-3, 4-4 and 7-7. Kinetic constants (k_s) of the base-catalysed GSH conjugation reactions were taken from Van der Aar et al. [19]; kinetic constants (k_{cat}/K_m) of the GST-catalysed GSH conjugation reactions were taken from Van der Aar et al. [17].

		$10^2 \times k_s$ ($\mu M^{-1} \cdot min^{-1}$)	$10^2 \times k_{cat}/K_m$ ($\mu M^{-1} \cdot min^{-1}$)			
Substrate	R	Base	GST 1-1	GST 3-3	GST 4-4	GST 7-7
1	NO_2	4.2 ± 0.2	608 ± 3	716 ± 234	93 ± 19	189 ± 4
2	CHO	0.051 ± 0.001	3.3 ± 0.1	24 ± 5	1.5 ± 0.4	1.8 ± 0.1
3	COC_6H_5	0.028 ± 0.001	2.2 ± 0.2	9.1 ± 1.6	1.5 ± 0.3	–*
4	CO_2CH_3	0.043 ± 0.001	3.3 ± 0.3	8.3 ± 0.8	0.9 ± 0.1	0.9 ± 0.1
5	$CO_2(CH_2)_3CH_3$	0.039 ± 0.005	6.8 ± 0.6	18.3 ± 1.6	3.5 ± 0.1	2.8 ± 0.2
6	$CO_2C(CH_3)_3$	0.029 ± 0.002	3.1 ± 0.2	8.3 ± 0.6	–*	0.5 ± 0.1
7	CN	0.48 ± 0.01	26 ± 2	75 ± 15	8 ± 1	9.9 ± 0.1
8	CF_3	0.011 ± 0.001	2.60 ± 0.01	15 ± 2	2.00 ± 0.03	1.7 ± 0.1
9	Cl	0.0100 ± 0.0003	1.10 ± 0.05	10 ± 1	0.6 ± 0.2	0.6 ± 0.1
10	Br	0.010 ± 0.002	1.7 ± 0.3	19.3 ± 0.1	1.6 ± 0.2	0.8 ± 0.03

* No GSH conjugation was observed up to 300 μM for substrates **3** and **6**.

Table 2 Classical physicochemical parameters (σ_p, σ_p^-, $\mathscr{F}$, $\mathscr{R}$, B_1, B_5, f) of the 2-substituents in substrates 1–10 and computer-calculated molecular parameters of substrates used in the actual regression analyses

Charges were obtained from distributed multipole analysis calculations (taken from [19]).

Substrate	σ_p	σ_p^-	$\mathscr{F}$	$\mathscr{R}$	B_1	B_5	f	Charge on: *p*-Nitro substituent	Charge on: Attacked C-1 atom
1	0.78	1.27	0.67	0.16	1.70	2.44	−0.039	−0.169	0.265
2	0.42	1.02	0.31	0.13	1.60	2.36	−0.333	−0.196	0.234
3	0.43	0.87	0.30	0.16	1.92	5.98†	0.926	−0.201	0.216
4	0.45	0.76	0.33	0.15	1.64	3.36	0.181	−0.193	0.222
5	0.45	0.76	0.33	0.15	1.64	5.85	1.738	−0.193	0.219
6	0.45	–*	–*	–*	–*	–*	1.738	−0.196	0.216
7	0.66	1.01	0.55	0.19	1.60	1.60	−0.155	−0.184	0.264
8	0.54	0.71	0.38	0.19	1.99	2.61	1.223	−0.191	0.242
9	0.23	0.21	0.41	−0.15	1.80	1.80	0.933	−0.204	0.150
10	0.23	0.23	0.44	−0.17	1.95	1.95	1.134	−0.205	0.139

* This physicochemical parameter was not available for a *t*-butyl ester substituent.

† The benzene ring of the benzophenone substituent is twisted 90° out of the aromatic plane.

was recorded spectrophotometrically. The second-order rate constants of the chemical reaction (k_s) were calculated by dividing the absorption increase per min by the GSH concentration, the substrate concentration and the molar absorption coefficient of the corresponding GS-conjugate.

Inhibition experiments with GS-conjugates

GST isoenzymes (0.6–12 μg/ml) were incubated in potassium phosphate buffer (0.1 M, pH 6.5, 0.1 mM EDTA) with 0, 50 or 100 μM GS-**1** or GS-**7** for 2 min at 37 °C. Subsequently, the reaction was started by adding 1 mM GSH and 1 mM **1** or **7**. The increase in absorption at the λ_{max} of the GS-conjugates was recorded spectrophotometrically at 37 °C.

Physicochemical and computer-calculated molecular parameters

The classical physicochemical parameters used in the regression analyses were taken from the literature [26,27]. To probe the steric effects of the substituents, the multidimensional Sterimol parameters (L, B_1 and B_5) were taken [26]. The hydrophobic parameter π of Hansch and Fujita [26] and the hydrophobic fragment constant f of Rekker [27] were used to probe lipophilicity effects of the substituents. As electronic parameter, the Hammett σ constants were used [26]. Some of the substituents investigated are directly conjugated with the reaction centre, and this phenomenon may cause 'through resonance'. For this reason a different Hammett constant, named σ^- [26], was defined in order to correct for through resonance [28]. Swain and Lupton [29] separated σ into an inductive component ($\mathscr{F}$) and a resonance component ($\mathscr{R}$) [26], which were also considered in the regression analyses.

Charge and energy calculations for the substrates and the Meisenheimer intermediates with MeS^- as a model nucleophile (described in [19]) led to charge distributions over the molecules, among others at the C-1 atom attacked, at the various *ortho* substituents and at the *p*-nitro substituents, and to the energies of the highest occupied molecular orbital (E_{homo}) and of the lowest unoccupied molecular orbital (E_{lumo}) of the molecules. For the Meisenheimer complexes, the energies of formation ($\Delta E_{f,mc}$) were calculated by subtracting the energies of both the substrates and MeS^- from the energies of the corresponding Meisenheimer complexes (all computer-calculated parameters are depicted in [19]). Furthermore, the energy differences between E_{lumo} and E_{homo} (ΔE_{1-h}) were calculated and used in the regression studies. Also, the differences in charges at the attacked C-1 atom, at the *ortho* substituent and at the *p*-nitro substituent in the substrates and in the Meisenheimer intermediates were calculated and used in the correlation study. The classical physicochemical and computer-calculated parameters actually used in the regression analyses are listed in Table 2.

Quantitative SARs

Single- and multiple-parameter regression analyses were performed to determine correlations between the kinetic parameters of the GST isoenzyme-catalysed and base-catalysed GSH conjugation reactions on the one hand, and the physicochemical parameters (electronic, steric and lipophilic) of the substituents and the computer-calculated molecular parameters of the substrates and the Meisenheimer complexes with MeS^- as a model nucleophile for GS^- on the other. Only correlations with a Student's t test t value of $> |2|$ were considered to be significant. In case of multiple regression the intercorrelation between the two independent parameters was checked ($r < 0.5$).

RESULTS AND DISCUSSION

Comparison of GST- and base-catalysed GSH conjugation reactions

GST 3-3 showed the highest k_{cat}/K_m value for the GSH conjugation of all chloronitrobenzenes examined when compared with GSTs 1-1, 4-4 and 7-7, indicating that, for this class of compounds, the overall reaction, including binding of substrates, stabilization of reaction intermediates and release of products, is most efficiently catalysed by GST 3-3 (Table 1). The second-order rate constant of the base-catalysed GSH conjugation reaction (k_s; Table 1) was approx. 100-fold lower than the k_{cat}/K_m values of all four GSTs. Substrates **1** (1-chloro-2,4-dinitrobenzene; CDNB) and **7** showed respectively the highest and the second highest GSH conjugation rates in both the base-catalysed and the GST-catalysed reactions. Moreover, substrates **2**, **8**, **9** and **10** showed selectivity for GST 3-3.

When a regression analysis was applied between the $\log k_{cat}/K_m$ values for the four different GST isoenzymes and the log of the second-order rate constants (k_s) for the base-catalysed GSH conjugation of eight 2-substituted 1-chloro-4-nitrobenzenes, the plots in Figure 1 were obtained. GSTs 1-1 and 7-7 showed similar correlation coefficients (0.969 and 0.949 respectively) and slopes (0.92 and 0.84 respectively) (Figure 1A), while GSTs 3-3 and 4-4 showed lower values for both parameters ($r = 0.921$ and 0.914; slope = 0.62 and 0.68 respectively) (Figure 1B). Apparently, overall there are similarities between the different GST isoenzymes when compared with the base-catalysed reaction, but in detail there are major differences and an attempt will be made below to describe these differences by delineating SARs. The k_{cat}/K_m values for the four GST isoenzymes and the k_s values for the base-catalysed GSH conjugation of 2-substituted 1-chloro-4-nitrobenzenes were subjected to single- and multiple-regression analyses with nine classical physicochemical parameters and with 16 computer-calculated parameters. Only significant ($t > |2|$) correlations were considered from which the correlation coefficient was larger than 0.85.

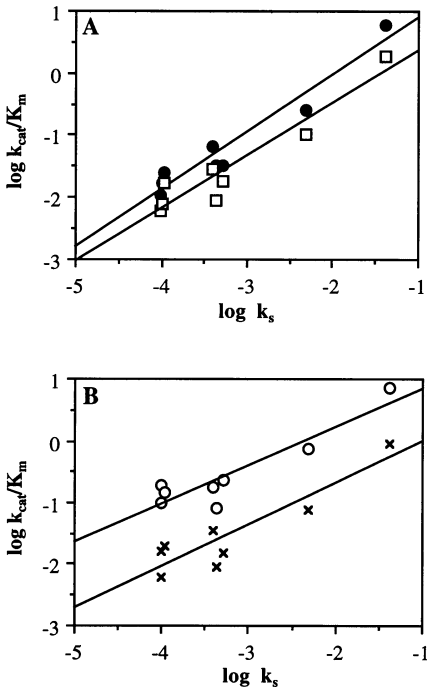


Figure 1 Plots of log k_{cat}/K_m against log k_s for the GSH conjugation of eight 2-substituted 1-chloro-4-nitrobenzenes

(**A**) GST 1-1 (●): $y = 0.92 \pm 0.10x + 1.82 \pm 0.32$, $r = 0.969$; GST 7-7 (□): $y = 0.84 \pm 0.11x + 1.18 \pm 0.38$, $r = 0.949$. (**B**) GST 3-3 (○): $y = 0.62 \pm 0.11x + 1.48 \pm 0.36$, $r = 0.921$; GST 4-4 (×): $y = 0.68 \pm 0.12x + 0.67 \pm 0.41$, $r = 0.914$.

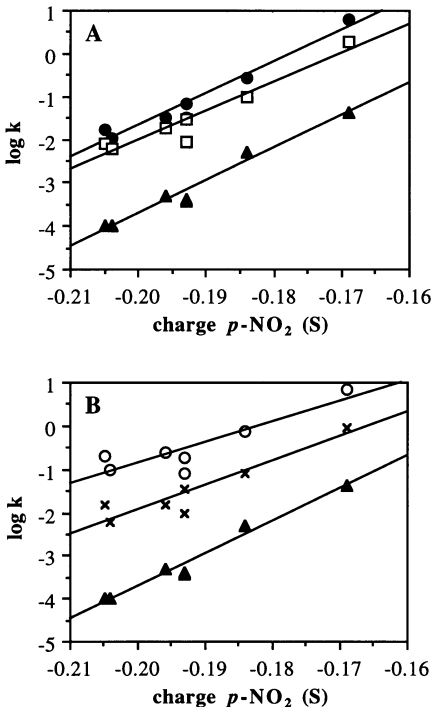


Figure 2 Correlations between the kinetic parameters for the GSH conjugation of 2-substituted 1-chloro-4-nitrobenzenes ($n = 7$) and the calculated charges on the *p*-nitro substituents of the substrates [charge *p*-NO_2 (S)]

(**A**) k_{cat}/K_m of GST 1-1 (●): $y = 74 \pm 7x + 13 \pm 1$, $r = 0.978$; k_{cat}/K_m of GST 7-7 (□): $y = 68 \pm 9x + 12 \pm 2$, $r = 0.959$; k_s (▲): $y = 76 \pm 5x + 11 \pm 1$, $r = 0.987$. (**B**) k_{cat}/K_m of GST 3-3 (○): $y = 48 \pm 11x + 9 \pm 2$, $r = 0.889$; k_{cat}/K_m of GST 4-4 (×): $y = 55 \pm 10x + 9 \pm 2$, r = 0.927; k_s (▲): $y = 76 \pm 5x + 11 \pm 1$, $r = 0.987$.

Regression analyses of K_m with molecular parameters

The K_m values of the different GST isoenzymes were not correlated significantly ($t < |2|$) with any of the classical physicochemical parameters, or had a low correlation coefficient ($r < 0.70$). Also, the computer-calculated molecular parameters of the substrates and the corresponding model Meisenheimer complexes did not show any significant correlation with the K_m values. In contrast, Rietjens et al. [20] recently reported a linear correlation ($r = -0.997$) between K_m and log P for the GSH conjugation of four fluoronitrobenzenes catalysed by rat liver cytosol. Despite the fact that the substrates used in the two studies (fluoronitrobenzenes compared with chloronitrobenzenes) are similar and are conjugated to GSH via an identical nucleophilic aromatic substitution mechanism, no such correlation was observed in the present study. This discrepancy might well be due to the fact that these authors used rat liver cytosol, thus leading to an average K_m for all GSTs present in the cytosolic mixture, whereas in the present study pure GST isoenzymes were used. Moreover, we examined 10 substrates in the regression analyses with K_m, while Rietjens et al. only used four substrates.

Single regression analyses between kinetic parameters and electronic parameters

When a regression analysis was performed between log k_s of the base-catalysed GSH conjugation reaction and σ_p, a high correlation coefficient was observed:

$$\log k_s = 4.54 \pm 0.44\sigma_p - 5.20 \pm 0.22$$
$$(r = 0.977;\ s = 0.222;\ n = 7) \quad (1)$$

This equation implies that as the 2-substituted R groups become more electron-withdrawing (increase in σ_p), the energy of the corresponding transition states is decreased (due to electron stabilization by the substituents); hence the corresponding Meisenheimer intermediates are stabilized to a greater extent and the higher k_s becomes. In eqn. (1) σ_p values are used while the substituents at the *ortho* position are varied. It is known that the empirical Hammett relation ($\log k_R/k_H = \sigma\rho$) generally fails for reactions in which substituents are varied near the reaction centre (*ortho*) due to steric effects. Sbarbati [30] determined the rate constants for the S_NAr reaction of 6-R-2-nitrochlorobenzenes with piperidine in benzene. The reactions followed the Hammett relationship if the more bulky substituents, Br and CH_3, were excluded. An approximate Van der Waals radius of 1.9 Å was proposed as the lower limit below which steric effects are negligible and differences in reaction rates are mainly determined by polar effects. From the study of Sbarbati [30] and the present study, it can therefore be concluded that σ_p parameters can be used for *ortho* substituents.

We expected to find a correlation between the k_s values and the Hammett electronic parameter corrected for through-resonance (σ_p^-), because of the ability for through-resonance of the 2-substituents of substrates **1**–**7**. When a regression analysis was applied between log k_s and σ_p^- using the same data set as in eqn. (1), the following equation was produced:

$$\log k_s = 2.08 \pm 0.56\sigma_p^- - 4.77 \pm 0.46$$
$$(r = 0.837;\ s = 0.549;\ n = 7) \quad (2)$$

Although the correlation is significant ($t > |2|$), the correlation coefficient is considerably lower ($r = 0.837$) than that of eqn. (1) ($r = 0.977$). Moreover, the dependence of k_s on σ_p (4.54) is more than twice as great as that on σ_p^- (2.08), also indicative of the low importance of through-resonance in this series of substrates. Also, when a regression was applied between the k_s values of all substrates able to exhibit through-resonance (substrates **1**–**7**) and σ_p^-, a relatively low correlation coefficient was observed ($r = 0.874$). Previously, Chen et al. [31] reported a high linear correlation between $\log k_s$ and σ_p^- for five 4-substituted 1-chloro-2-nitrobenzenes. The present results suggest that through-resonance via substituents at the *ortho* position is not very efficient, possibly because of steric hindrance in the Meisenheimer intermediates between the substituents at C-2 and the Cl atom at C-1. In the ground state C-1 is sp^2-hybridized, and as such occupies less space when compared with an sp^3-hybridized C-1 in the Meisenheimer intermediate.

In determining eqn. (1), the CF_3-substituted substrate (**8**) was omitted because it was an outlier according to statistical rules. It appeared that substrate **8** was an outlier in regression analyses between k_s and all other electronic parameters considered. Most likely, this can be explained by the negatively charged electrostatic field around the *ortho* substituent, caused by the electronegative F atoms [32], leading to repulsion of GS^- when attacking the substrate. Furthermore, substrates **3** and **6** were excluded from the regression analyses because no accurate enzyme kinetic data were obtained for the GST 7-7- and 4-4-catalysed conjugations, respectively, due to the fact that no GSH conjugation was observed up to 300 μM substrate. For the comparison between the base-catalysed and GST-catalysed GSH conjugations, identical data sets were included into the SARs. When the complete available data set was used in the regression analyses, however, none of the omitted substrates (**3**, **6** and **8**) appeared to be an outlier and no significant differences in coefficients were observed.

When a similar regression analysis was applied between the $\log k_{cat}/K_m$ values for the four different GSTs and σ_p, the following Hammett equations were determined:

$$\log k_{cat}/K_m \text{ 1-1} = 4.30 \pm 0.72\sigma_p - 3.07 \pm 0.36 \quad (r = 0.936;\ s = 0.361;\ n = 7) \quad (3)$$

$$\log k_{cat}/K_m \text{ 3-3} = 2.74 \pm 0.80\sigma_p - 1.75 \pm 0.40 \quad (r = 0.838;\ s = 0.399;\ n = 7) \quad (4)$$

$$\log k_{cat}/K_m \text{ 4-4} = 3.21 \pm 0.77\sigma_p - 2.97 \pm 0.38 \quad (r = 0.882;\ s = 0.383;\ n = 7) \quad (5)$$

$$\log k_{cat}/K_m \text{ 7-7} = 3.96 \pm 0.77\sigma_p - 3.30 \pm 0.38 \quad (r = 0.916;\ s = 0.386;\ n = 7) \quad (6)$$

The slopes in these Hammett equations (ρ values) give an indication of the sensitivity of the GSH conjugation reactions to the electronic effects of the substituents. Obviously, the base-catalysed reaction depends most on the electronic character of the substituents ($\rho = 4.54$; eqn. 1). The ρ values of the GST 3-3- and 4-4-catalysed reactions (2.74 and 3.21 respectively) differ slightly but significantly from that of the base-catalysed reaction (4.54). In contrast, the ρ values of the GST 1-1- and 7-7-catalysed GSH conjugation reactions (4.30 and 3.96 respectively) do not differ significantly from that of the base-catalysed reaction, indicating that the latter three catalysts depend in a similar way on the electronic properties of the *ortho* substituents. Also, from the relatively high correlation coefficients of the Hammett equations in the case of the base-, GST 1-1- and GST 7-7-catalysed reactions (eqns. 1, 3 and 6), when compared with the GST 3-3- and 4-4-catalysed reactions (eqns. 4 and 5), it may be concluded that the latter two reactions depend to a lesser extent on the electronic properties of the *ortho* substituents, probably due to the influence of the GST 3-3 and 4-4 proteins. These results suggest that there are significant differences in the transition states of the GST 3-3- and 4-4-catalysed conjugation reactions of 2-substituted 1-chloro-4-nitrobenzenes on the one hand and the GST 1-1-, GST 7-7- and base-catalysed conjugation reactions on the other.

Similar results were obtained when regression analyses were performed with computer-calculated molecular parameters (Table 2 and Figure 2). The log of the kinetic parameters (k_{cat}/K_m for the four different GST isoenzymes, and k_s) was plotted against the charge on the *p*-nitro substituent in seven substrates [charge *p*-NO_2 (S)]. This parameter indirectly represents the charge on the *ortho* substituent: when the electron-withdrawing capacity of the *ortho* substituent is increased (for example by the substitution Cl to NO_2), the net charge on the *p*-nitro substituent is decreased (-0.204 to -0.169, substrates **9** and **1**, Table 2). Figure 2(A) shows the k_{cat}/K_m values for GSTs 1-1 and 7-7 and the second-order rate constant (k_s) for the base-catalysed GSH conjugation against the charge on the *p*-NO_2 substituent. The slopes of the lines in this plot do not differ significantly (GST 1-1, 74 ± 7; GST 7-7, 68 ± 9; base, 75 ± 5), suggesting that these three catalysts depend in a similar way on the electronic effects of the *ortho* substituent. Figure 2(B) shows analogous regression analyses with GSTs 3-3 and 4-4 as catalysts. The slopes of the GST 3-3- (48 ± 11) and 4-4- (55 ± 10) catalysed reactions differ significantly from that of the base-catalysed reaction (75 ± 5). These results again suggest that GSTs 3-3 and 4-4 depend less on the electronic character of the *ortho* substituents than do GSTs 1-1 and 7-7.

Alternative rate-limiting transition states

The base-catalysed addition of GSH to CDNB (**1**, the most widely used model substrate for determination of GST activities) proceeds via a nucleophilic aromatic substitution reaction and is thought to occur via a rate-limiting σ-bond formation in the Meisenheimer intermediate [18]. Because of the identical dependence of the reactions catalysed by base, GST 1-1 and GST 7-7 on the electronic properties of the 2-substituents, these two GST isoenzymes probably catalyse the GSH conjugation of 2-substituted 1-chloro-4-nitrobenzenes via a similar transition state as in the base-catalysed reaction. For GSTs 3-3 and 4-4, however, it is possible that other steps besides the formation of a Meisenheimer complex are partially rate-limiting.

In principle, it is possible that the release of the respective GS-conjugates from the active site of GSTs 3-3 and 4-4 is rate-limiting. This phenomenon was indeed found for the release of 1-(*S*-glutathionyl)-2,4-dinitrobenzene (GS-**1**) from the active site of GST 3-3 [11]. A hydrogen bond between Ser^{209} and Tyr^{115} in GST 3-3 was proposed to be formed, so that elimination of GS-**1** from the active site would be restricted. In GST 4-4, however, Ser^{209} is replaced by an alanine [33], and formation of a similar specific hydrogen bond is not likely [15]. Also, for GSTs 1-1 and 7-7, the rate-limiting release of GS-conjugates is not likely. The amino acid residue corresponding to Tyr^{115} in GST 1-1 is a valine or an isoleucine (depending on the alignment used [34]), both of which are incapable of forming hydrogen bonds. GST 7-7 is composed of 209 amino acid residues [35], and upon alignment with GST 3-3 it has been concluded that there is no residue corresponding to Ser^{209}. In conclusion, the physical process of formation of a hydrogen bond blocking the active site of GST isoenzymes and leading to rate-determining product release is not very likely to be the reason for the different dependence of

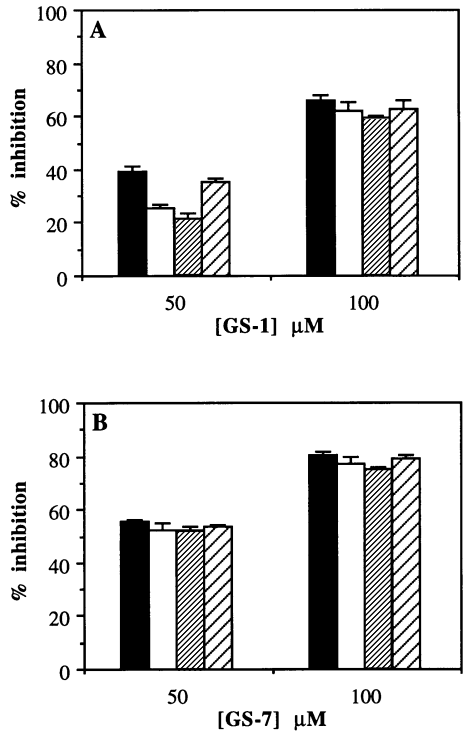


Figure 3 Inhibition of GSTs 1-1 (■), 3-3 (□), 4-4 (▨) and 7-7 (▨) after a 2 min incubation with (A) GS-1 and (B) GS-7

(**A**) GS-**1** was present at 50 or 100 μM; and residual activity towards 1 mM GSH and 1 mM CDNB (**1**) was measured. (**B**) GS-**7** was present at 50 or 100 μM; residual activity towards 1 mM GSH and 1 mM **7** was measured.

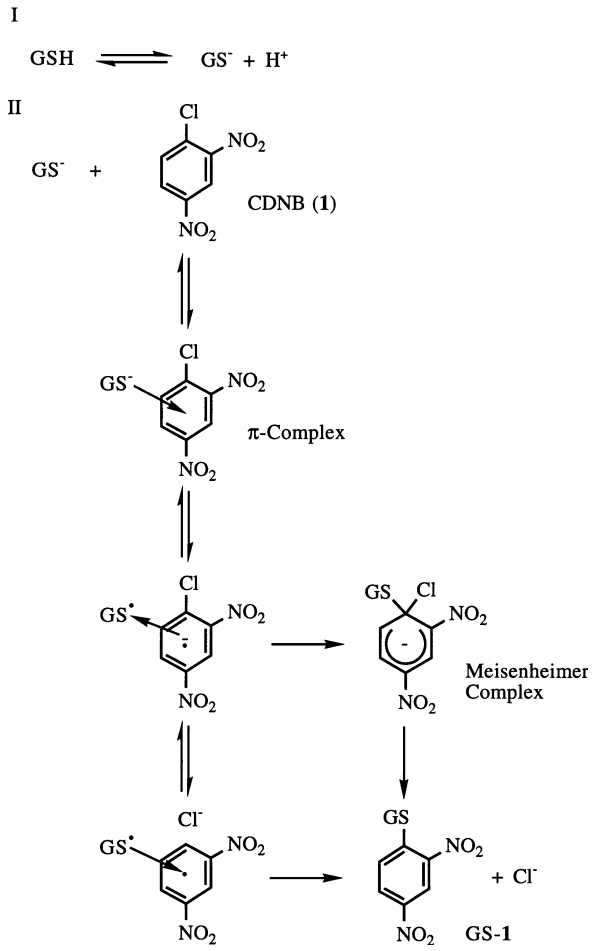
GSTs 3-3 and 4-4 on one hand, and GSTs 1-1 and 7-7 on the other, on the electronic properties of the *ortho* substituents.

However, rate limiting product release could also be influenced by the high affinity of GS-conjugates for the active site. In principle, it is possible that the GS-conjugates of substrates **1**–**10** have a higher affinity for the active sites of GSTs 3-3 and 4-4 than for the active sites of GSTs 1-1 and 7-7, leading to (partially) rate-limiting product release in the case of the Mu-class GSTs. In the present study, inhibition experiments were performed to study the effects of the GS-conjugates of substrates **1** and **7** (GS-**1** and GS-**7** respectively) on the GST-catalysed GSH (1 mM) conjugation of **1** and **7** (1 mM). In Figure 3, the percentage inhibition of the four GST isoenzymes is plotted against the concentration of GS-conjugate. A concentration of 50 μM GS-**1** inhibited the GSH conjugation of **1** by approx. 23 % for GSTs 3-3 and 4-4 and by approx. 37 % for GSTs 1-1 and 7-7, while 50 μM GS-**7** inhibited the GSH conjugation of **7** by approx. 50 % for all four GSTs. A concentration of 100 μM GS-**1** inhibited the GSH conjugation of **1** by about 60 % for all GSTs, and 100 μM GS-**7** inhibited the GSH conjugation of **7** by about 77 % for all GSTs. If GSTs 3-3 and 4-4 have a significantly higher affinity for GS-conjugates, greater inhibition should have been found than with GSTs 1-1 and 7-7. This was not observed, and it is therefore unlikely that rate-limiting product release is responsible for the lower dependence of GSTs 3-3 and 4-4 on the electronic properties of the *ortho* substituents.

Rate-limiting release of Meisenheimer intermediates from the active site as the initial product instead of the final GS-conjugates could, in theory, also be a possible reason for the observed discrepancy between GSTs 1-1/7-7 and GSTs 3-3/4-4. The stability of the enzyme–intermediate complexes and the transition states for release are probably influenced by the electron density and distribution in the Meisenheimer complexes as well, and thus a relationship with σ_p might be expected. The correlation coefficients of the regression between the k_{cat}/K_m of GSTs 3-3 and 4-4 and σ_p observed in the present study, however, were not very high ($r = 0.838$ and 0.882 respectively) when compared with those of GSTs 1-1 and 7-7 ($r = 0.936$ and 0.916 respectively). The release of the Meisenheimer complexes out of the active site of GSTs 3-3 and 4-4 is therefore unlikely to be rate-determining.

The decreased dependence of GSTs 3-3 and 4-4 on the electronic properties of the *ortho* substituents when compared with GSTs 1-1 and 7-7 could also reflect an earlier, more reactant-like, transition state for the Mu-class enzymes. GSH is efficiently deprotonated by GSTs via a stabilizing hydrogen bond with a Tyr residue in the G-site, leading to a decrease in the pK_a of GSH from about 9 in aqueous solution to 5.7–7.0 when bound to GSTs [31]. Except for Theta-class GSTs, all GSTs possess such a Tyr residue in the G-site (GST 1-1, Tyr^8; GST 3-3, Tyr^6; GST 4-4, Tyr^6; GST 7-7, Tyr^7) [35]. The efficiency of this deprotonation reaction, however, depends on the electrostatic environment of this Tyr residue. Liu et al. [36] proposed an on-face hydrogen bond between the side-chain hydroxy group of Thr^{13} and the π-system of the aromatic benzene ring of Tyr^6 in GST 3-3. This interaction enhances the effect of Tyr^6 on the pK_a of bound GSH and results in a further lowering of the acid dissociation constant of GSH by 0.7 unit. In GST 4-4, residue 13 is an Ala, which is unable to form this type of hydrogen bond [15,33], and the pK_a of GSH bound to GST 4-4 is significantly greater (6.6) than that when bound to GST 3-3 (5.7). GSTs 1-1 and 7-7 both have a Tyr residue in the position corresponding to Tyr^6 in GSTs 3-3 and 4-4, and also have an S-containing amino acid in the position corresponding to position 13 (Met and Cys respectively) [34,37]. In principle, it is possible to form a specific hydrogen bond between the π-system of Tyr and the free electrons of the S atom in Met or Cys in GSTs 1-1 and 7-7, although this bond is expected to be weaker than with an O atom as in Thr. Moreover, for this kind of interaction it is important that the orientation of the amino acids involved is optimal. GSTs 3-3 and 4-4 have a positively charged His residue at position 14 in common. In contrast, this position is occupied by a negatively charged Glu residue in GSTs 1-1 and 7-7. Apart from these studies [15,31,36], detailed research on the electrostatic environment of GSH in the active site of different GST isoenzymes, and the possible effects on the GSH conjugation mechanism, has not been performed as yet. The final pK_a of GSH in GSTs and the rate of formation of GS^-, which depend on the G-site and its environmental amino acids, may possibly influence the formation of an earlier, more reactant-like, transition state for GSTs 3-3 and 4-4, as suggested in the present study.

Tang and Chang [38] recently proposed an alternative chemical mechanism for the base-catalysed nucleophilic aromatic substitution of GSH in CDNB in reverse micelles. This mechanism involves ionization of GSH to give the GS^- thiolate anion and the subsequent attack of GS^- on C-1 to form the corresponding GS-conjugate through a π-complex and Meisenheimer complex intermediates and radical–radical anion electron-transfer complexes (Scheme 1). This mechanism is based on the identification of both π-complex and radical anion–radical pair charge-transfer complexes for the reaction of OH^- and CDNB in aprotic solvents by hydrogen exchange and NMR spectroscopic analysis [39]. The reaction mechanism shown in Scheme 1 might also apply to GSTs, since the active sites of these enzymes possibly resemble the aprotic environment in micelles [38]. The base-catalysed GSH conjugation of substrates **1**–**10** is performed in protic



Scheme 1 Proposed chemical mechanism for the base-catalysed nucleophilic aromatic substitution of GSH and CDNB (1) in reverse micelles

Step I, ionization of GSH to give the GS^- thiolate anion; step II, attack of GS^- on C-1 to form GS-**1** through π-complex and Meisenheimer complex intermediates and radical–radical anion electron-transfer complexes.

potassium phosphate buffer, and is therefore expected to proceed directly via the corresponding Meisenheimer intermediates and not via the radical intermediates. It is interesting to speculate that the fact that the reactions catalysed by GSTs 1-1 and 7-7 resemble base-catalysed GSH conjugation as far as their dependence on the electronic properties of the *ortho* substituents is concerned might imply that GSTs 1-1 and 7-7 share a relatively protic active site and, by inference, that substrates **1**–**10** are conjugated directly via a Meisenheimer complex. On the other hand, the GST 3-3- and GST 4-4- catalysed GSH conjugation of substrates **1**–**10** might proceed via the radical intermediates, possibly due to the relatively aprotic active sites of these isoenzymes.

Multiple regression analyses between kinetic and electronic parameters

The Hammett σ constant can be separated into a field ($\mathscr{F}$) and a resonance ($\mathscr{R}$) component [29]. Field effects are due to the intrinsic electronegativity of atoms and to the dipoles of functional groups. They operate by donating or withdrawing electrons either through σ-bonds or through space. Resonance effects

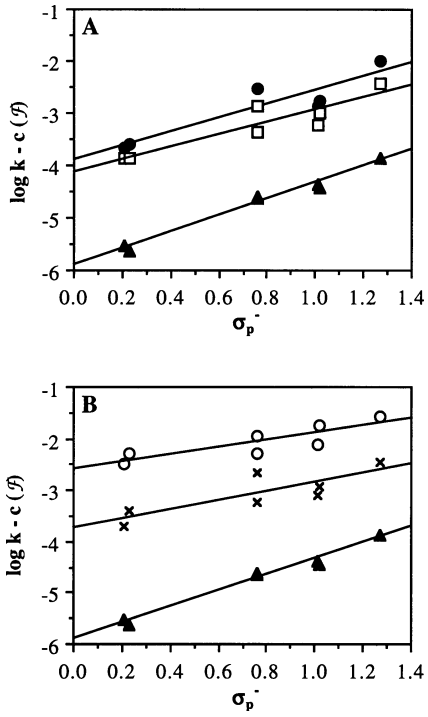


Figure 4 Effects of $\mathscr{F}$ and σ_p^- on the GSH conjugation of 2-substituted 1-chloro-4- nitrobenzenes

Reactions were catalysed by (**A**) GST 1-1 (●), GST 7-7 (□) and base (▲); (**B**) GST 3-3 (○), GST 4-4 (×) and base (▲). Eqns. (7)–(11) were transformed to $\log k - c_1(\mathscr{F}) = c_2(\sigma_p^-) + c_3$.

operate by donating or withdrawing electrons by p-orbital overlap with the aromatic ring pi electrons. When a multiple regression was performed between the experimental kinetic constants (k_s and k_{cat}/K_m) and $\mathscr{F}$ and σ_p^- (i.e. σ_p corrected for through-resonance), much higher correlation coefficients were obtained than with the individual physicochemical parameters ($r = 0.70$–0.88):

$$\log k_s = 3.72 \pm 0.38\mathscr{F} + 1.57 \pm 0.13\sigma_p^- - 5.91 \pm 0.16 \quad (r = 0.995;\ s = 0.114;\ n = 7) \quad (7)$$

$$\log k_{cat}/K_m \text{ 1-1} = 4.17 \pm 0.94\mathscr{F} + 1.32 \pm 0.31\sigma_p^- - 3.90 \pm 0.40 \quad (r = 0.969;\ s = 0.282;\ n = 7) \quad (8)$$

$$\log k_{cat}/K_m \text{ 3-3} = 3.60 \pm 0.68\mathscr{F} + 0.70 \pm 0.22\sigma_p^- - 2.58 \pm 0.29 \quad (r = 0.969;\ s = 0.203;\ n = 7) \quad (9)$$

$$\log k_{cat}/K_m \text{ 4-4} = 3.62 \pm 0.96\mathscr{F} + 0.88 \pm 0.32\sigma_p^- - 3.73 \pm 0.41 \quad (r = 0.948;\ s = 0.289;\ n = 7) \quad (10)$$

$$\log k_{cat}/K_m \text{ 7-7} = 4.03 \pm 0.87\mathscr{F} + 1.20 \pm 0.29\sigma_p^- - 4.14 \pm 0.37 \quad (r = 0.970;\ s = 0.260;\ n = 7) \quad (11)$$

Despite the fact that the contribution of $\mathscr{F}$ to all eqns. (7)–(11) is about the same for all catalysts, it is obvious that the addition of an extra field effect to σ_p^- increases the correlation coefficients. The identical dependence of the catalysts on $\mathscr{F}$ could possibly be explained by the fact that there is no significant interaction of the *ortho* substituents with amino acids in the active sites of the GST isoenzymes which are differentially influencing the intrinsic electronegativity or the dipoles of the substituents. Figure 4,

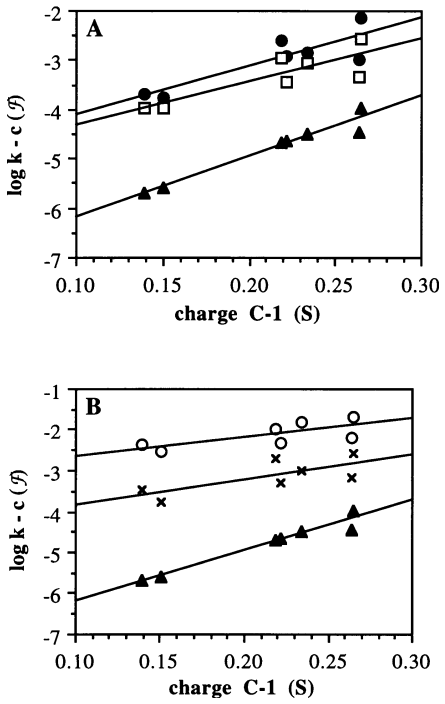


Figure 5 Effects of $\mathscr{F}$ and the charge on C-1 in the substrates on the base-catalysed (k_s) and GST-catalysed (k_{cat}/K_m) GSH conjugation of 2-substituted 1-chloro-4-nitrobenzenes

The original equations were transformed to $\log k - c_1(\mathscr{F}) = c_2(\text{charge C-1}) - c_3$. The c_1 coefficient is identical for all equations (approx. 4.0), while c_2 and c_3 differ. (**A**) GST 1-1 (●): $c_2 = 9.79 \pm 3.05$, $c_3 = 5.08$, $r = 0.952$; GST 7-7 (□): $c_2 = 8.73 \pm 2.96$, $c_3 = 5.18$, $r = 0.949$; k_s (▲): $c_2 = 12.19 \pm 1.49$, $c_3 = 7.39$, $r = 0.989$; (**B**) GST 3-3 (○): $c_2 = 4.59 \pm 2.35$, $c_3 = 3.11$, $r = 0.944$; GST 4-4 (×): $c_2 = 6.26 \pm 2.95$, $c_3 = 4.48$, $r = 0.928$; k_s (▲): as in (**A**).

in which eqns. (7)–(11) are transformed to $\log k - c_1(\mathscr{F}) = c_2(\sigma_p^-) + c_3$, shows that the GST 1-1- and GST 7-7-catalysed reactions depend in a similar way on the σ_p^- values of the *ortho* substituents as does the base-catalysed GSH conjugation reaction (Figure 4A), while the GSTs 3-3- and GST 4-4-catalysed reactions depend much less on the σ_p^- values (Figure 4B). This again supports the suggestion that GST 3-3 and 4-4 catalyse the GSH conjugation of 2-substituted 1-chloro-4-nitrobenzenes via a different rate-determining transition state than in the base-, GST 1-1- and GST 7-7-catalysed reactions.

High correlation coefficients were also found when a multiple regression analysis was performed between k_s or k_{cat}/K_m, the charges on the attacked C-1 atom in the substrates and the $\mathscr{F}$ values. When the original equations are transformed to $\log k - c_1(\mathscr{F}) = c_2(\text{charge C-1}) - c_3$, the plots in Figure 5 are obtained. All examined catalysts depend in the same way on $\mathscr{F}$ (c_1 = approx. 4.0), while the dependence on the charge on the attacked C-1 atom in the substrates varies depending on the catalyst. The coefficients for the charge on the attacked C-1 atom in GSTs 1-1 and 7-7 (9.79 ± 3.05 and 8.73 ± 2.96 respectively) do not differ significantly from that of the base-catalysed reaction (12.19 ± 1.49) (Figure 5A) while, in contrast, those for GSTs 3-3 and 4-4 (4.59 ± 2.35 and 6.26 ± 2.95 respectively) do (Figure 5B). The fact that the GST 3-3- and GST 4-4-catalysed reactions apparently depend less on the charge on C−1 can be explained by a more efficient deprotonation of GSH by these enzymes, suggested above to be caused by the different environment of GSH and the hydrogen-bonding Tyr residue in GSTs 3-3 and 4-4 when compared with GSTs 1-1 and 7-7. It is also possible that hydrogen-bond formation between certain atoms in the substrates and amino acids in the active sites of GSTs 3-3 and 4-4 could change the charge distributions in the substrates, particularly at C-1. In line with this suggestion, De Groot et al. described a specific hydrogen-bond interaction between Arg^{42} of GST 4-4 and the *o*-nitro group of CDNB [15].

In a previous study, the field effect $\mathscr{F}$ and the resonance component $\mathscr{R}$ (the two constituents of σ_p) were simultaneously correlated with k_s and with k_{cat}/K_m of GST 4-4 [19]. It appeared that the correlation coefficient increased substantially when compared with analysis using σ_p alone and that, for both catalysts, an identical dependence on $\mathscr{F}$ was found. Similar results were found when using k_s and k_{cat}/K_m of three out of the four GST isoenzymes examined:

$$\log k_{cat}/K_m \text{ 1-1} = 3.06 \pm 0.86\mathscr{R} + 5.32 \pm 1.00\mathscr{F} - 3.61 \pm 0.45 \quad (r = 0.959;\ s = 0.324;\ n = 7) \quad (12)$$

$$\log k_{cat}/K_m \text{ 4-4} = 1.98 \pm 0.86\mathscr{R} + 4.40 \pm 1.00\mathscr{F} - 3.53 \pm 0.45 \quad (r = 0.935;\ s = 0.323;\ n = 7) \quad (13)$$

$$\log k_{cat}/K_m \text{ 7-7} = 2.66 \pm 0.90\mathscr{R} + 5.10 \pm 1.04\mathscr{F} - 3.87 \pm 0.47 \quad (r = 0.950;\ s = 0.338;\ n = 7) \quad (14)$$

$$\log k_s = 3.67 \pm 0.52\mathscr{R} + 5.08 \pm 0.61\mathscr{F} - 5.56 \pm 0.27 \quad (r = 0.985;\ s = 0.198;\ n = 7) \quad (15)$$

Eqns. (12)–(15) show that all catalysts have similar coefficients for $\mathscr{F}$ (approx. 5), again implying that there are no significant interactions of the *ortho* substituents with amino acids in the active site of GSTs 1-1, 4-4 and 7-7 that are differentially influencing the intrinsic electronegativity or the dipoles of the substituents. The coefficients for $\mathscr{R}$ of GSTs 1-1 and 7-7 do not differ significantly from that of the base-catalysed reaction, while the corresponding coefficient for GST 4-4 is lower. GST 3-3 showed no statistically significant correlation with $\mathscr{F}$ and $\mathscr{R}$, because the *t*-value in the Student's *t* test was 1.7 for the $\mathscr{R}$ parameter. This observation, together with the low dependence of GST 4-4 on $\mathscr{R}$ indicates that in these Mu-class GSTs the p-orbital overlap of the substituent with the aromatic ring pi electrons is not very efficient.

Multiple regression analyses with GST 3-3

In the present study all kinetic parameters, both base-catalysed and GST-catalysed, were subjected to single and multiple regression analyses using the classical physicochemical parameters and the computer-calculated molecular parameters of the substrates and the model Meisenheimer complexes. Only for GST 3-3 was a high correlation coefficient observed when $\log k_{cat}$ was correlated with the Hammett σ_p constant and the Sterimol B_5 factor:

$$\log k_{cat} \text{ 3-3} = 2.18 \pm 0.35\sigma_p - 0.29 \pm 0.04B_5 + 1.47 \pm 0.22 \quad (r = 0.976;\ s = 0.177;\ n = 8) \quad (16)$$

In the Sterimol system, L is defined as the length of the substituent along the axis of the bond between the first atom of the substituent and the parent molecule, and B_5 is the maximum width [40]. In forming eqn. (16), substrate **6** was not included because no B_5 value is available for a *t*-butyl ester substituent. Furthermore, the CHO-substituted substrate (**2**) appeared to be an outlier. A regression analysis between $\log k_{cat}$ of GST 3-3 and the individual parameters gave correlation coefficients of only

0.636 and 0.770 for σ_p and B_5 respectively. The fact that the combined correlation of σ_p and B_5 was only found to be significant for GST 3-3 indicates that there is probably a unique steric restriction in the conjugation of 2-substituted 1-chloro-4-nitrobenzenes by GST 3-3.

Base- versus GST-catalysed reactions

Figure 1 shows the correlation between the enzyme kinetic parameter k_{cat}/K_m of the GSTs and the second-order rate constant of the base-catalysed reaction (k_s). We checked whether the correlation coefficients of these equations could be increased by addition of steric, lipophilic and electronic parameters. Inclusion of the lipophilicity fragment constant f of Rekker [27] gave the following equations:

$$\log k_{cat}/K_m \text{ 1-1} = 1.07 \pm 0.08 \log k_s + 0.29 \pm 0.10 f + 2.13 \pm 0.23 \quad (r = 0.989;\ s = 0.152;\ n = 8) \quad (17)$$

$$\log k_{cat}/K_m \text{ 4-4} = 0.86 \pm 0.11 \log k_s + 0.36 \pm 0.14 f + 1.04 \pm 0.33 \quad (r = 0.964;\ s = 0.217;\ n = 8) \quad (18)$$

$$\log k_{cat}/K_m \text{ 7-7} = 0.99 \pm 0.12 \log k_s + 0.29 \pm 0.14 f + 1.49 \pm 0.34 \quad (r = 0.973;\ s = 0.225;\ n = 8) \quad (19)$$

Addition of f does not result in a substantial increase in the correlation coefficients of the equations for GSTs 1-1 and 7-7 (0.969 to 0.989 and 0.949 to 0.973 respectively), while the correlation coefficient of the equation for GST 4-4 does increase significantly (0.914 to 0.964) (for equation, see legend to Figure 1). In the case of GST 4-4 an extra 10% of the data points are explained by including f, indicating that the active site of GST 4-4 is relatively lipophilic. On including f in the regression between $\log k_{cat}/K_m$ of GST 3-3 and $\log k_s$, no significant relationship was found [$t(f) = 0.8$], suggesting that lipophilicity is of less importance for GST 3-3. When the amino acid residues of GSTs 3-3 and 4-4 that are located within 5 Å of the centre of the H-site are compared, there are only five differences [15]. In GST 3-3 positions 9 and 111 are occupied by Val and Ile respectively, while GST 4-4 contains Ile and Ala respectively at these positions. These differences do not have a significant influence on the lipophilicity of the active site of the corresponding GSTs. Positions 13, 108 and 209 in GST 3-3 are occupied by Thr, Met and Ser respectively, all of which bear electronegative atoms and are capable of forming hydrogen bonds. In GST 4-4, however, these positions are occupied by Ala, Leu and Ala respectively; these are all relatively lipophilic amino acid residues and are incapable of forming hydrogen bonds.

In the Sterimol system, L is defined as the length of the substituent along the axis of the bond between the first atom of the substituent and the parent molecule, and B_1 is the minimum width [40]. When B_1 is included in the regression between the k_{cat}/K_m values and k_s, the following equations were obtained:

$$\log k_{cat}/K_m \text{ 1-1} = 1.04 \pm 0.09 \log k_s + 1.22 \pm 0.51 B_1 + 0.05 \pm 0.77 \quad (r = 0.986;\ s = 0.175;\ n = 8) \quad (20)$$

$$\log k_{cat}/K_m \text{ 3-3} = 0.76 \pm 0.08 \log k_s + 1.51 \pm 0.49 B_1 - 0.70 \pm 0.75 \quad (r = 0.973;\ s = 0.171;\ n = 8) \quad (21)$$

$$\log k_{cat}/K_m \text{ 4-4} = 0.83 \pm 0.10 \log k_s + 1.65 \pm 0.61 B_1 - 1.71 \pm 0.93 \quad (r = 0.966;\ s = 0.213;\ n = 8) \quad (22)$$

$$\log k_{cat}/K_m \text{ 7-7} = 0.98 \pm 0.10 \log k_s + 1.49 \pm 0.58 B_1 - 0.97 \pm 0.89 \quad (r = 0.978;\ s = 0.203;\ n = 8) \quad (23)$$

Inclusion of B_1 does not significantly increase the correlation coefficients of GSTs 1-1 and 7-7 (from 0.969 to 0.986 and from 0.949 to 0.978 respectively). For GSTs 3-3 and 4-4, however, the addition of B_1 does cause an increase in the correlation coefficients (from 0.921 to 0.973 and from 0.914 to 0.966 respectively), leading to an additional 10% of the data points which can be explained. This suggests that there is a steric factor involved in the GSH conjugation of 2-substituted 1-chloro-4-nitrobenzenes when catalysed by GSTs 3-3 and 4-4, but not by GSTs 1-1 and 7-7.

Table 3 Classical physicochemical parameters and computer-calculated molecular parameters of 1-chloro-4-nitrobenzene (S) and of the corresponding model Meisenheimer complex (MC) used in the prediction of GST activity based on the obtained SARs

Charges were obtained from distributed multipole analysis calculations. Energy values (kJ/mol) were obtained from SV6-31G calculations.

Parameter	Value
σ_p	0
σ_p^-	0
$\mathscr{F}$	0
$\mathscr{R}$	0
B_5	0
Charge C-1 (S)	0.176
Charge *ortho* (S)	0.113
Charge *p*-NO_2 (S)	−0.216
E_{lumo} (S)	28.4
E_{homo} (S)	−1015.0
Charge C-1 (MC)	0.117
Charge *ortho* (MC)	0.060
Charge *p*-NO_2 (MC)	−0.467
E_{lumo} (MC)	464.4
E_{homo} (MC)	−344.1
$\Delta E_{f,mc}$	−18.1

Table 4 Calculated kinetic parameters for the base-catalysed (k_s) and the GST-catalysed (k_{cat}/K_m) GSH conjugation of 1-chloro-4-nitrobenzene based on the obtained SAR equations

SAR equations	$10^4 \times k_s$ ($\mu M^{-1} \cdot min^{-1}$)	$10^2 \times k_{cat}/K_m$ ($\mu M^{-1} \cdot min^{-1}$)			
		GST 1-1	GST 3-3	GST 4-4	GST 7-7
σ_p	0.06	0.09	1.80	0.11	0.05
Charge *p*-NO_2	0.09	0.13	2.10	0.15	0.08
$\mathscr{F} + \sigma_p^-$	0.02	0.02	0.26	0.02	0.01
$\mathscr{F}$ + charge C-1	0.06	0.04	0.50	0.04	0.02
$\mathscr{F} + \mathscr{R}$	0.03	0.03	0.38	0.03	0.02

Predictions with 1-chloro-4-nitrobenzene

Based on the SAR equations described in the present study, it is possible to predict at what rate 2-substituted 1-chloro-4-nitrobenzenes will be conjugated to GSH (spontaneously or GST-catalysed). Thus for 1-chloro-4-nitrobenzene, in which the 2-substituent R = H (**11**), the classical physicochemical parameters of the substituent were taken from literature and the molecular parameters of this possible substrate and the corresponding Meisenheimer complex with MeS^- as a model nucleophile for GS^- were calculated (for methods, see [19]) (Table 3). Using the different molecular parameters of **11** (Table 3) in all the above-mentioned SAR equations for the different GST isoenzymes and the base-catalysed reaction, it was calculated that the only measurable GSH conjugation of substrate **11** would be expected with GST 3-3 (Table 4). The average calculated k_{cat}/K_m for GST 3-3 is $(1.0 \pm 0.8) \times 10^{-2}\ \mu M^{-1} \cdot min^{-1}$. The S.D. is high because of

the use of different SAR equations and their corresponding errors, which are shown in the *s*-values and the S.D. of the coefficients. The other GST isoenzymes are predicted to have such a low GSH conjugation rate that it will not be measurable (approx. $0.05 \times 10^{-2}\ \mu M^{-1} \cdot min^{-1}$).

GST- and base-catalysed GSH conjugation of compound **11** was checked experimentally. The λ_{max} of compound **11** (282 nm) and the corresponding GS-conjugate (340 nm) and its molar absorption coefficient ϵ ($2.1 \pm 0.5\ mM^{-1} \cdot cm^{-1}$) were determined. As predicted, only GST 3-3 showed measurable GS-conjugate formation with compound **11**. The apparent K_m was $1295 \pm 80\ \mu M$, the k_{cat} was $10.6 \pm 0.2\ min^{-1}$ and the k_{cat}/K_m was $0.82 \pm 0.04\ \mu M^{-1} \cdot min^{-1}$. Compared with other 2-substituted 1-chloro-4-nitrobenzenes, characterized kinetically in a previous study [17], substrate **11** has a relatively high K_m ($1295\ \mu M$), suggestive of low apparent affinity. 2-Chloro-5-nitropyridine also showed a high K_m ($1074\ \mu M$) with GST 3-3 [17], indicating that, when the *ortho* position is not occupied by a substituent, the apparent affinity towards GST 3-3 decreases. Most likely, there are important interactions between this kind of substrate and the complementary parts of the active site of GST 3-3. This observation agrees with the above conclusion that there is a steric restriction in the conjugation of 2-substituted 1-chloro-4-nitrobenzenes by GST 3-3. The k_{cat}/K_m value for **11** [$(0.82 \pm 0.04) \times 10^{-2}\ \mu M^{-1} \cdot min^{-1}$] with GST 3-3 was the lowest found within this series of substrates (Table 1), possibly because of the relatively high K_m value and thus low apparent affinity.

Conclusions

Experimentally determined K_m, k_{cat} and k_{cat}/K_m values for the GST 1-1-, 3-3-, 4-4- and 7-7-catalysed conjugation reactions between GSH and a series of ten 2-substituted 1-chloro-4-nitrobenzenes, and the second-order rate constant (k_s) of the corresponding base-catalysed reaction, were correlated with nine classical physicochemical parameters of the substituents and 16 computer-calculated molecular parameters of the substrates and of the corresponding Meisenheimer complexes with MeS^- as a model nucleophile for GS^-. The SARs obtained for the reactions catalysed by GSTs 1-1 and 7-7 were always similar to those for the base-catalysed reaction, strongly suggesting that these catalysts depend in the same way on the electronic properties of the 2-substituents. GSTs 3-3 and 4-4, however, showed SARs with significantly lower coefficients, indicating that these enzymes depend less on the electronic properties of the 2-substituents. These results lead us to propose that GSTs 3-3 and 4-4 have a different rate-determining transition state in the GSH conjugation reaction with 2-substituted 1-chloro-4-nitrobenzenes. Several alternative rate-limiting transition states are discussed for the GST 3-3- and GST 4-4-catalysed nucleophilic aromatic substitution of 2-substituted 1-chloro-4-nitrobenzenes. Based on the obtained SARs, it appears to be possible to predict the k_{cat}/K_m values of the GST isoenzymes and the k_s of the base-catalysed GSH conjugation of 1-chloro-4-nitrobenzene.

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