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Reactivity of dimethyl fumarate and methylhydrogen fumarate towards glutathione and *N*-acetyl-L-cysteine—Preparation of S-substituted thiosuccinic acid esters

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Dedicated to W. A. Schmidt, formerly Henkel KGaA, Düsseldorf, Germany, on the occasion of his 70th birthday

Abstract—Dimethyl fumarate (DMF) is used successfully to treat psoriasis. In spite of its proven clinical efficacy, the mode of metabolism and the pharmacodynamics of DMF are still not completely understood. Some previous studies have indicated that orally applied DMF for a considerable part is quickly hydrolysed to methylhydrogen fumarate (MHF) at basic pH conditions as present in the upper intestine, especially in the presence of biological fluids containing esterases. On the other hand it was shown that DMF due to its high lipophilicity rapidly penetrates into cells and may thus at least in part be absorbed after po application without being hydrolysed. On the other hand, no detectable amounts of DMF were hitherto found in plasma samples after po administration. In order to shed light on possible further routes of presystemic metabolism of DMF, studies on the reactivity towards glutathione (GSH) were carried out. GSH is present in millimolar concentrations in almost all cells. DMF due to its nature as an α,β-unsaturated carboxylic acid ester can react spontaneously with thiols via a Michael-type addition. It could be shown that DMF reacts at high rates under near-physiological conditions. Studies on the reaction kinetics at pH 7.4 show that GSH addition proceeds rapidly to yield a 1:1 mixture of both diastereomeric 2-(*S*-glutathionyl)-succinic acid dimethyl esters. MHF under identical conditions was shown to react with GSH as well leading to a mixture of four products (2 diastereomeric pairs). However, MHF reacted at a much lower rate. The structures of all thiol conjugates were confirmed unambiguously by extensive NMR measurements. GSH conjugates and the corresponding mercapturic acids on grounds of the high spontaneous reactivity observed may be expected to be major metabolites of unhydrolysed DMF which makes its way into enterocytes. On the other hand, MHF, due to its slow reaction with GSH, may have higher chances than DMF to react with more essential thiol groups in macromolecules.

1. Introduction

Fumaric acid esters (FAE) have been used for several decades in the treatment of Psoriasis vulgaris, a chronic inflammatory skin disease characterised by scaly plaques often affecting large parts of the skin.[1] Currently, a preparation containing dimethyl fumarate (DMF) along with Ca, Mg and Zn salts of the corresponding monoethylester, ethylhydrogen fumarate, is an approved drug in Germany and marketed under the name Fumaderm®. Moreover, clinical interest in fumarates and their derivatives is not limited to psoriasis but was extended to other inflammatory diseases such as multiple sclerosis, polyarthritis and others.[2–4]

The clinical efficacy and possible mechanisms of action for fumaric acid esters have been reviewed previously.[5,6] Evidence is cumulating that DMF as the main constituent of the marketed mixture is the active compound and it was suggested to evaluate it alone rather than the complex mixture of esters.[6]

However, the pharmacokinetics and metabolic fate of DMF are still not fully understood. It has been demonstrated that DMF is rapidly hydrolysed by esterases to a large part within few minutes, when exposed to various organ preparations such as, for example, pancreatic extract, intestinal perfusate and homogenate or liver

Keywords: Dimethyl fumarate; Methylhydrogen fumarate; Glutathione; Michael addition; NMR spectroscopy; Reaction kinetics.
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S9 fraction.[7] The resulting monoester, methylhydrogen fumarate (MHF), as well as other monoester derivatives showed much longer degradation half-lives when treated with, for example, liver S9 fraction.[7] Although DMF penetrated rapidly in an in vitro experiment with CACO-2 monolayers, no detectable amounts were found to penetrate across excised intestinal mucosa in an ex vivo model.[7] Consistently, MHF was detected in blood plasma after po administration of a single tablet containing DMF along with calcium monoethylfumarate to humans while neither DMF nor fumaric acid itself was found in the plasma samples in measurable amounts.[8] It thus remains an open question how DMF might exert its systemic pharmacological effects after po administration. One explanation would be that MHF is the actually active species as suggested by some authors[9] which, on the other hand, would not be in agreement with the much lower pharmacological activity of MHF in various biological assays.[10,11] Another hypothetical explanation could lie in an alternative mechanism of action or pathway of metabolism hitherto not taken into account. It must be noted that no full account on the total balance of administered DMF has been given in the mentioned absorption studies so that a fraction of the applied dose may be unaccounted for.[7,8] As already mentioned, penetration of DMF through CACO-2 cell monolayers has been demonstrated to occur very rapidly in comparison with MHF and other more polar derivatives[7] so that it can be expected that some unhydrolysed DMF is able to penetrate into cells of the intestinal mucosa. It could thus be possible that a dose of DMF in part enters the enterocytes and pervades into the bloodstream in the form of other—biologically active—metabolites which have not been detected.

Fumaric acid esters represent α,β-unsaturated carbonyl systems (enones) possessing electrophilic reactivity towards biological nucleophiles as shown previously for other enones such as, for example, acrylates[12] or the natural product helenalin.[13,14] As soft electrophiles they should react most efficiently with soft nucleophiles such as sulfhydryl groups of biological target molecules (Scheme 1).

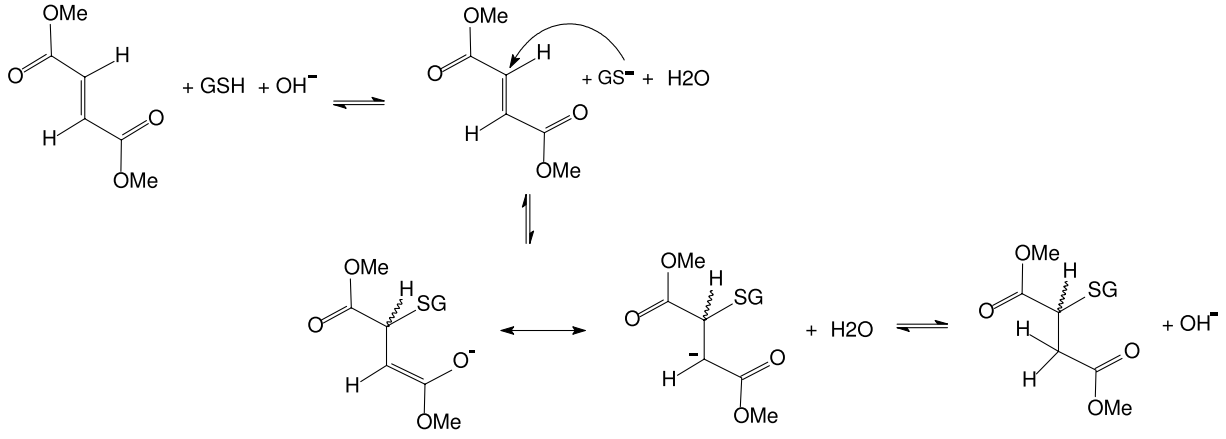
It is known that drugs can be subject to considerable phase-II metabolism in the intestinal mucosa. Enterocytes contain high concentrations of the tripeptide glutathione (gly-cys-γ-glu, GSH) which serves to detoxify electrophilic xenobiotics, either by spontaneous reaction or catalysed by glutathione-*S*-transferases.[15] Thus, it is conceivable that the fraction of unchanged DMF reaching the enterocytes is conjugated with GSH and released into the bloodstream in the form of GSH-conjugates. Furthermore, GSH adducts might be transformed into the corresponding mercapturic acids (formally conjugates with *N*-acetylcysteine).[15] Since it has been shown previously that S-alkylated GSH derivatives (i.e., drug-GSH conjugates) may still possess biological activity,[14,16–18] it is conceivable that such putative conjugates of fumarates contribute to the overall pharmacological effects. The goals of this study were therefore (a) to study in detail the reactivity of DMF and MHF towards GSH and (b) to prepare the expected conjugates in order to serve as reference compounds in analytical studies of fumarate metabolism and pharmacodynamics (Scheme 2).

2. Results and discussion

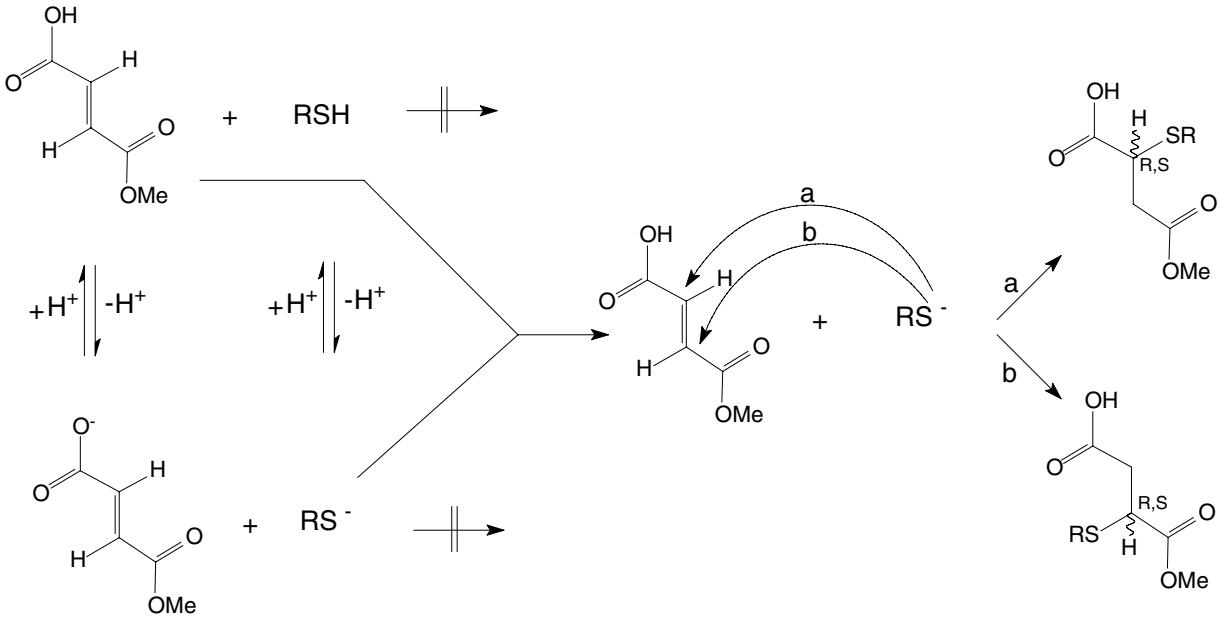
2.1. Dependence of reaction rate for DMF and GSH on pH—mechanism of reaction

Since the expected reaction (Scheme 1) must lead to abrogation of the α,β-unsaturated carbonyl system of DMF it was straightforward to conduct the studies on reaction kinetics by monitoring the decrease of the fumarates' UV absorbance in the presence of GSH in analogy to our previous report for helenanolide type sesquiterpene lactones.[14]

DMF (100 μM) and GSH (500 μM) were incubated in phosphate-buffered aqueous solution at pH 6, 7, 7.4, 8 and 9, and the reaction monitored by time-dependent UV spectroscopy at $\lambda = 226$ nm. At pH 9, the reaction under these conditions became too fast to be monitored so that the GSH concentration was lowered to 100 μM. Figure 1 shows the resulting time courses along with



Scheme 1. Reaction between DMF and thiols—the pathway via the thiolate is experimentally confirmed by the dependence of reaction rate on pH.



Scheme 2. Reaction between MHF and thiols. From the observation that reaction products at C-2 and C-3 are formed in approximately equal amounts (via routes a and b, respectively), it can be deduced that MHF reacts exclusively in the protonated form, which must react with the deprotonated thiolate, so that the low rate of reaction is explained by the small concentration of both reacting species at physiological pH.

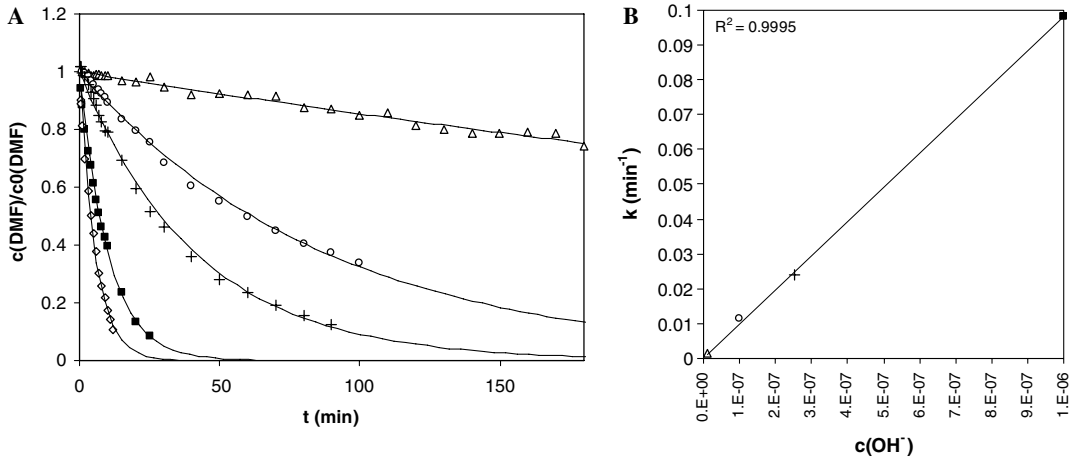


Figure 1. Reaction time courses for 100 μM DMF and GSH at different pH values (cGSH = 500 μM at pH 6–8, 100 μM at pH 9). (A) Time course diagrams for pH 6.0 (open triangles), 7.0 (open circles), 7.4 (plus signs), 8.0 (squares) and 9.0 (rhombus). Numerical values see Table 1. (B) (Pseudo)first-order rate constants determined from A plotted vs. c(OH^-) demonstrating the linear dependence of reaction rate on basicity of the medium.

their exponential trendlines. From the excellent fit of the exponential functions it could be deduced that the reaction under these conditions follows first-order kinetics. The resulting (pseudo)first-order rate constants are reported in Table 1. A clear dependence of reaction rates on pH is evident (see Fig. 1), and a linear relationship exists between the observed rate constants and the basicity of the medium ($r^2 = 0.999$). This clearly proves that the reaction—as commonly observed in thiol additions[19]—proceeds via the thiolate anion as reactive species. The fast progress of the reaction in the physiological pH range indicates that spontaneous reaction of DMF with GSH (i.e., not catalysed by GSH-*S*-transferase) may be an important route of metabolism in living cells.

2.2. Kinetic studies at pH 7.4

In order to further assess the physiological relevance of GSH addition to DMF, the reaction was carried out at varying GSH and constant DMF concentration (100 μM) and monitored as described above. Figure 2 shows the resulting curves for equimolar ratio as well as 2-, 5-, 7.5-, 10- and 15-fold excess GSH. The reaction was found to follow a first-order rate law (r^2 for all exponential functions fitted to these data >0.99). The first-order rate constants thus obtained are reported in Table 2.

It was found by plotting the resulting rate constants vs. the ratio of reactants (Fig. 2B) that these data are con-

Table 1. Dependence of reaction rate between DMF and GSH on pH value

pH	k [min^{-1}]	r^2
6.0	0.0016	0.982
7.0	0.0133	0.996
7.4	0.0240	0.997
8.0	0.0983	0.998
9.0[a]	0.1758	0.993

Reaction time-courses at c(DMF) = 100 μM, for pH 6–8 at c(GSH) = 500 μM, for pH 9 at c(GSH) = 100 μM since the reaction at 500 μM was too fast to be monitored (see Fig. 1A). The rate constants represent the exponents of the regression functions shown as trend lines in Figure 1A, and r^2 represents the squared correlation coefficients between these functions and the experimental data.
[a] At c(GSH) = 100 μM.

nected very well by a linear relationship which allowed extrapolation to an even higher excess of GSH. Thus, for 100 μM DMF and 10 mM GSH, a rate constant of 0.54 corresponding to a reaction half-life of 1.3 min was predicted.

From these data it becomes clear that DMF under physiological conditions (high intracellular excess GSH between 0.5 and 10 mM[15]) will spontaneously form covalent adducts with GSH within minutes. It can thus be expected that a considerable part of the DMF that penetrates from the intestinal lumen into enterocytes will be conjugated with GSH. This finding may explain the lack of detectable amounts of unchanged DMF in the acceptor fluid in the ex vivo experiment[7] and in blood plasma after po administration.[8]

2.3. Reactivity of MHF and GSH

Analogous experiments with methyl hydrogen fumarate (MHF) were carried out. Figure 2C shows the progress of the reaction of 100 μM MHF with 1.5 mM GSH incubated at pH 7.4, in comparison with the corresponding curve for DMF. The resulting first-order rate constant for MHF is lower than for DMF by a factor of approximately 30 corresponding to a reaction half-life of about 4 h (vs. 9 min for DMF). Thus, MHF can be expected to remain largely unaffected by spontaneous GSH conjugation which is in agreement with its occurrence in the acceptor fluids in ex vivo and in vivo experiments.[7,8] At the same time, this low reactivity of MHF indicates that it may be available in concentrations high enough for other biological effects since it will be less susceptible to deactivation by GSH addition.

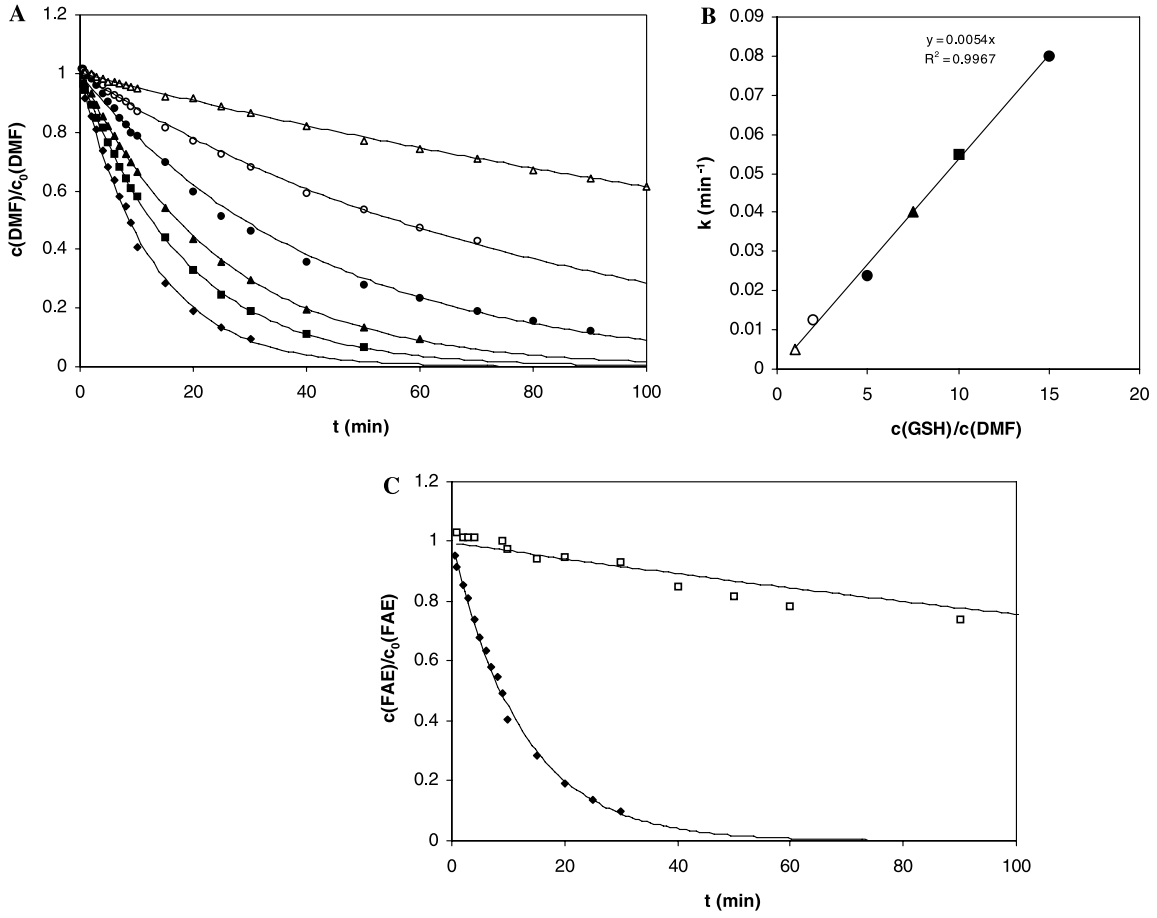


Figure 2. Reaction time courses for 100 μM DMF and various concentrations of GSH at pH 7.4. (A) Time course diagrams for c(GSH) = 100 μM (open triangles), 200 μM (open circles), 500 μM (filled circles), 750 μM (filled triangles), 1000 μM (filled squares) and 1500 μM (filled rhombi). Numerical data see Table 2. (B) Rate constants determined from A plotted vs. concentration ratio of GSH/DMF. (C) Comparison of the reactivity of DMF (rhombi) and MHF (squares) under identical conditions: pH 7.4, c(fumarate) = 100 μM, c(GSH) = 1500 μM.

Table 2. Dependence of reaction rates between DMF and GSH on GSH concentration

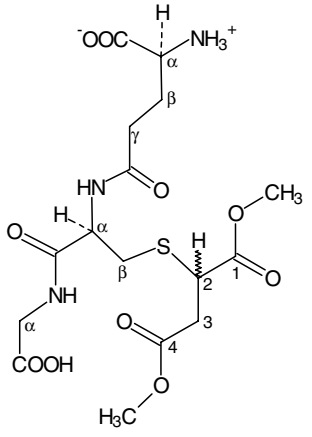
c(GSH) [μM]	k [min^{-1}]	r^2
100	0.0049	0.998
200	0.0125	0.997
500	0.0240	0.997
750	0.0403	0.999
1000	0.0550	0.999
1500	0.0801 (0.0027)	0.997 (0.942)

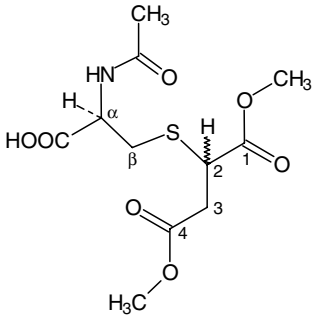
Reaction time-courses were recorded at pH 7.4 and c(DMF) = 100 μM for various c(GSH) (see Fig. 2A). The rate constants represent the exponents of the regression functions shown as trend lines in Figure 2A, and r^2 represents the squared correlation coefficients between these functions and the experimental data. Values in brackets: corresponding data for 100 μM MHF at 1500 μM GSH.

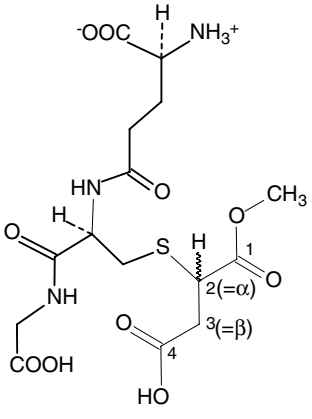
2.4. Preparation of thiol conjugates of DMF and MHF and spectroscopic characterisation

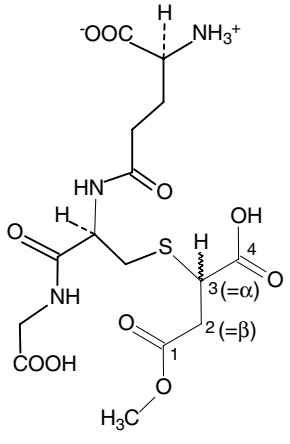
It has previously been shown that addition of GSH to α,β-unsaturated carbonyl structures of complex natural products is highly stereoselective.[13,14,18]

Since the covalent addition of GSH to DMF introduces a chiral centre at C-2 of the resulting thiosuccinic acid ester, the question arose whether one of the resulting diastereomers would be favoured. In order to prove unambiguously the structure of the reaction product(s) and to observe possible stereoselectivity during the reaction, equimolar amounts of DMF and GSH were incubated at pH 7.4 for 18 h and the resulting products investigated by one- and two-dimensional NMR spectroscopy.









Structures of the GSH conjugates of DMF (**GS-DMS**) and MHF (**2-GS-MHS**, **3-GS-MHS**) and of the N-acetylcysteine conjugates of DMF (**NAC-DMS**); Diastereomers A and B (see tables 3, 4 and 5) differ in the stereochemistry at C-2 (C-3 in 3-GS-MHS). Assignment of the absolute stereochemistry remained unsolved.

It could be shown that both possible diastereomers of dimethyl-2-(*S*-glutathionyl)- succinate (**GS-DMS**) were formed in a molar ratio of 1:1, that is, no stereoselectivity was observed. However, in spite of the high degree of similarity between the two diastereomers (which were found to be inseparable by TLC and HPLC), it was possible to clearly distinguish most of their NMR signals in the spectra of the 1:1 mixture (NMR data see Table 3).

Due to the inseparability of the two compounds, it is presently not possible to assign the distinct sets of resonances to the *R*,*R*- or the *R*,*S*-configured diastereomer.

GSH conjugates of electrophilic xenobiotics under normal cellular conditions are commonly subject to further metabolism catalysed by γ-glutamyltransferase, dipepdidase and cysteine-acetyltransferase leading to mercapturic acids (formally conjugates of *N*-acetylcysteine).[15] Thus, in future studies on the metabolic fate of DMF, such mercapturic acids will have to be taken into account. Therefore, DMF and *N*-acetylcysteine (NAC) were incubated under essentially the same conditions as described above for GSH and the resulting products analysed by NMR spectroscopy (data see Table 4). It was found that complete reaction to two diastereomeric products (dimethyl-2-[*S*-(*N*-acetyl)-L-cysteinyl]-succinate, **NAC-DMS**), analogous to those obtained with GSH, had occurred. The NMR spectra in a very similar manner as observed with the GSH adducts displayed characteristic shift differences between the two diastereomers once more allowing for assignment of all relevant signals to two distinct sets. As with the GSH conjugates, chromatographic separation was not possible up to present so that the NMR resonances could not be assigned to the *R*,*R*- and *R*,*S*-configured diastereomers.

Since MHF had shown some reactivity with GSH in the kinetic experiments, it was of interest to study the resulting products' structure. To this end, GSH was incubated with a twofold molar excess of MHF in phosphate buffer at pH 7.4. After 48 h, the reaction mixture was freeze-dried and analysed by ^{1}H NMR spectroscopy which revealed the presence of four products, which were identified by extensive NMR measurements as two diastereomeric pairs of methyl-2-(*S*-glutathionyl)-hydrogen succinate (**2-GS-MHS**) and methyl-3-(*S*-glutathionyl)-hydrogen succinate (**3-GS-MHS**), respectively (NMR data see Table 5) in a ratio of approximately 1:1. To remove excess MHF, the mixture was submitted to gel chromatography on Sephadex LH-20/water, yielding a mixture of **2-GS-MHS** and **3-GS-MHS** in a ratio of approximately 3:7.

2.5. Implications for the mechanism of reaction between MHF and GSH

The formation of both, the 2- and 3-*S*-glutathionyl conjugates of MHF, **2-GS-MHS** and **3-GS-MHS**, in similar amounts reveals some interesting details of the reaction mechanism between MHF and GSH. Regarding the potency of the carboxylic acid/carboxylate and the ester

Table 3. NMR data of diastereomeric GSH-conjugates of DMF, **GS-DMS**, (125 and 500 MHz for ^{13}C and ^{1}H, respectively, D_2O)

Position	Diastereomer A					Diastereomer B				
	δ_C	mult.	δ_H	mult.	*J* (Hz)	δ_C	mult.	δ_H	mult.	*J* (Hz)
DMS-										
1	174.32	s				174.32	s			
2	42.72	d	3.807	dd	6.0; 9.1	41.80	d	3.789	dd	6.0; 9.1
3	36.12	t	2.935	dd	9.1; 17.2	36.45	t	2.942	dd	9.1; 17.2
			2.777	dd	6.0; 17.2			2.817	dd	6.0; 17.2
4	173.59[a]	s				173.60[a]	s			
1-OCH_3	53.69[a]	q	3.709	s	(3H)	53.72[a]	q	3.709	s	(3H)
4-OCH_3	53.00	q	3.624[a]	s	(3H)	53.00	q	3.622[a]	s	(3H)
cys-										
α	53.55	d	4.542	dd	4.7; 9.3	53.04	d	4.537	dd	5.0; 8.6
β	33.52	t	3.174	dd	4.7; 14.2	32.96	t	3.140	dd	5.0; 14.2
			2.867	dd	9.3; 14.2			2.954	dd	8.6; 14.2
CO	171.86[a]	s				171.84[a]	s			
gly-										
α	43.73	t	3.70[b]			43.73	t	3.70[b]		
COO	176.58[a]	s				176.56[a]	s			
γ-glu										
α	54.47	d	3.68[b]			54.47	d	3.68[b]		
β	26.61[a]	t	2.069	m	(2H)	26.57[a]	t	2.069	m	(2H)
γ	31.80[a]	t	2.445	m	(2H)	31.78[a]	t	2.445	m	(2H)
γ-CO	175.31[a]	s				175.22[a]	s			
COO	174.64[a]	s				174.52[a]	s			

Assignments were confirmed by COSY, HMQC and HMBC experiments.
[a] Assignment interchangeable between A and B.
[b] Multiplicity not determined, shift value determined from cross peaks in 2D spectra.

Table 4. NMR data of diastereomeric NAC adducts of DMF, **NAC-DMS** (125 and 500 MHz for ^{13}C and ^{1}H, respectively, D_2O)

Position	Diastereomer A					Diastereomer B				
	δ_C	mult.	δ_H	mult.	J (Hz)	δ_C	mult.	δ_H	mult.	J (Hz)
1	174.77[a]	s				174.68[a]	s			
2	41.77	d	3.768	dd	5.9; 9.3	42.60	d	3.768	dd	5.9; 9.3
3	36.51	t	2.900	dd	9.3; 17.2	36.16	t	2.912	dd	9.3; 17.2
			2.752	dd	5.9; 17.2			2.788	dd	5.9; 17.2
4	173.65	s				173.65	s			
1-OCH_3	53.62[a]	q	3.684[a]	s	(3H)	53.58[a]	q	3.675[a]	s	(3H)
4-OCH_3	52.94	q	3.603	s	(3H)	52.94	q	3.603	s	(3H)
cys-										
α	54.51	d	4.286	dd	4.3; 8.5	54.87	d	4.277	dd	4.5; 8.4
β	33.64	t	3.098	dd	4.1; 13.9	34.27	t	3.069	dd	4.4; 13.7
			2.839	dd	8.4; 13.8			2.923	dd	8.0; 13.6
COO	176.71	s				176.71	s			
ac-										
CH_3	22.26	q	1.944[a]	s	(3H)	22.26	q	1.941[a]	s	(3H)
CO	174.02[a]	s				173.95[a]	s			

Assignments were confirmed by COSY, HMQC and HMBC experiments.
[a] Assignment interchangeable between A and B.

Table 5. NMR data of isomeric GSH conjugates of MHF (100 and 400 MHz for ^{13}C and ^{1}H, respectively, D_2O)

Position	**2-GS-MHS**								**3-GS-MHS**							
	δ_C		mult.	δ_H		mult.	J (Hz)		δ_C		mult.	δ_H		mult.	J (Hz)	
	A	B		A	B		A	B	A	B		A	B		A	B
MHS-																
COOH	176.16	176.13	s						177.55	177.52	s					
α	43.05	43.03	d	3.640	3.631	dd	6.4; 9.4	5.1;8.8	45.21	44.51	d	3.513	3.494	dd	6.0; 9.0	6.0; 9.1
β	37.58	37.12	t	2.735	2.711	dd	9.2; 16.7	9.4; 16.8	37.27	37.01	t	2.743	2.723	dd	9.0; 16.8	9.0; 17.1
				2.568	2.546	dd	6.1; 16.5	6.1; 16.9				2.609	2.583	dd	6.2; 16.4	5.7; 16.7
CO	174.67		s						173.88	173.84	s					
OCH_3	53.12	53.28	q	3.607	3.593	s	(3H)		52.55		q	3.522	3.520	s	(3H)	
cys-																
α	53.03	52.76	d	4.453	4.442	dd	4.6; 9.3	4.6; 9.2	53.52	53.31	d	4.439	4.427	dd	4.6; 8.9	4.5; 8.9
β	33.17	32.66	t	3.064	3.005	dd	4.6; 14.4	4.8; 13.8	32.52		t	3.039	2.959	dd	4.7; 14.0	5.0; 14.0
				2.773	2.773	dd	9.2; 14.1	9.2; 14.1				2.858	2.847	dd	8.9; 14.0	8.6; 14.0
CO	172.07	172.03	s						172.27		s					
gly-																
α	42.57		t	3.697	3.686	s			42.51		t	3.692	3.690	s		
COO	175.0–174.9[a]		s						175.0–174.9[a]		s					
γ-glu																
α	54.12		d	3.628		dd[t]	6.2		54.12		d	3.606		dd[t]	6.4	
β	26.20		t	1.985		m			26.25		t	1.985		m		
γ	31.34		t	2.354		m			31.37		t	2.354		m		
γ-CO	175.0–174.9[a]		s						175.0–174.9[a]		s					
COO	174.00		s						174.00		s					

Assignments were confirmed by COSY, HMQC and HMBC and DEPT experiments.
[a] Signal overlap, no exact determination possible.

carbonyl group to withdraw electrons from C-3 and C-2, respectively, it would be expected that the C-3 regioisomer should be formed exclusively. Under the chosen near-physiological conditions in buffered aqueous solution at pH 7.4, the carboxyl group is deprotonated to a major extent and will thus possess no activating effect on C-2. At the same time, C-3 would be expected to receive some activation by the ester carbonyl group and hence be the only possible site of reaction. At the same time, however, C-3 would be deactivated by the negative partial charge of the neighbouring deprotonated carboxy group which will repel an attacking thiolate anion. Thus, if the deprotonated form were the reacting species, it would be expected that the C-3 regioisomer would result as the only product. On the other hand, the low rate of reaction might be due to the non-deprotonated neutral MHF molecule being the reactive species. Neutral MHF with a pK_a about 3.3 represents only a minor fraction of about 0.01% at pH 7.4, which could cause the slow reaction rate.

These assumptions were confirmed by quantum mechanical calculations which were carried out at various levels of theory (results of AM1 semi-empirical, ab initio RHF/6-31G(d) and density functional theory RB3LYP/6-311+G(2d,p) calculations are summarized in Table 6) using the program package Gaussian.[20] Under the assumptions of frontier molecular orbital theory (FMO[21]), that the lowest unoccupied molecular orbital (LUMO) of the electrophile accepts electrons from the highest occupied molecular orbital (HOMO) of the nucleophile, the potential reactivity of an electrophile is an inverse function of its LUMO energy (i.e., the lower the LUMO energy, the higher the tendency to form a covalent bond with the nucleophile). Thus, it is possible to estimate the reactivity of different electrophiles towards a given nucleophile by comparing their LUMO energies. The LUMO energy calculated for the neutral form of MHF by all chosen methods is very similar to that of DMF so that it would be expected to have a similar reactivity. The LUMO energy for deprotonated MF^- is calculated to be higher by 0.17 eV (corresponding to 16.4 kJ/mol) indicating that this species would be far less reactive towards a thiolate. The dominance of MF^- in solution thus explains the low overall reactivity observed.

Moreover, from the calculated contributions of the reactive centres' atomic orbitals (AOs) to the LUMO of a molecule it is possible to estimate the preferred site of reaction. In the case of MHF, the sum of contributions of the p_z AOs of C-2 and C-3 to the LUMO were calculated as 0.846 and 0.840, respectively. These very similar values, which are very close to the value calculated for both carbons in DMF (0.838), indicate that reaction of MHF in the neutral form with a nucleophile will not show a very pronounced preference for either C-2 or C-3. In case of deprotonated MF^-, values of 0.675 and 1.087 were calculated for C-2 and C-3, respectively, clearly showing that C-3, as expected, would be strongly preferred over C-2 as the site of nucleophilic attack if MF^- were the reactive species.

The thermodynamic stability of the two resulting MHF conjugates was finally compared by calculating their free energy at the RB3LYP/6-311+G(2d,p) level. Since calculations at this level of accuracy with a full GSH or cysteine molecule would be extremely time consuming, methanethiol (MeSH) was chosen as model nucleophile.

The free energies calculated for the C-2 and the C-3 conjugate with MeSH (2-MeS-MHS and 3-MeS-MHS, respectively) differ by only 0.045 kJ/mol corresponding to an equilibrium constant of 1.02, that is, both derivatives are predicted to be essentially equal in energy and will hence be equally populated at equilibrium which is in very good agreement with our experimental data.

It may hence be concluded that MHF can react only from the neutral state which, however, is present only in very low concentration at this pH. Lowering the pH would increase the fraction of protonated MHF but at the same time decrease the concentration of thiolate available so that no increase in reaction rate can be expected by lowering the pH. Consistently, the reaction of MHF with GSH at 100/1500 μM at pH 6.0 was too slow to determine a rate constant while increasing the pH from 7.4 to 8.0 led to a slight increase in the rate constant from 0.003 to 0.004 due to higher thiolate concentration.

3. Conclusions

In conclusion, the present results corroborate our hypothesis that a likely reason for the lack of detectable amounts of DMF in blood plasma after po administration may be the rapid conjugation with GSH. DMF reacts quickly with GSH at concentrations which may be reached under physiological conditions. MHF, in contrast, is much less reactive towards GSH which is in agreement with its occurrence in blood after po administration. The low reactivity and long reaction half-life indicate that MHF will remain largely unaffected by

Table 6. Results of quantum mechanical calculations for DMF and MHF

	DMF		MHF		MF^-	
LUMO energies (eV) obtained at different levels of theory						
AM1	−0.035		−0.039		0.130	
RHF/6-31G(d)	0.071		0.066		0.245	
RB3LYP/6-311+G(2d,p)	−0.095		−0.102		0.067	
	DMF		MHF		MF^-	
	C-2	C-3	C-2	C-3	C-2	C-3
Contributions of p_z AOs to LUMO (RB3LYP/6-311+G(2d,p)						
$2p_z$	−0.1386	0.1386	0.1414	−0.1361	−0.0910	0.1656
$3p_z$	−0.2091	0.2091	0.2132	−0.2052	−0.1366	0.2508
$4p_z$	−0.2806	0.2806	0.2853	−0.2717	−0.1850	0.3233
$5p_z$	−0.2095	0.2095	0.2063	−0.2273	−0.2629	0.3472
$\|\Sigma\|$	0.8378	0.8378	0.8462	0.8403	0.6755	1.0869
Free energy of MHF- MeSH conjugates, 298 K, RB3LYP/6-311+G(2d,p)						
G^{298} 2-MeS-MHS (a.u.)	−933.86188810					
G^{298} 3-MeS-MHS (a.u.)	−933.86187116					
ΔG^{298} (a.u.)	0.00001694					
ΔG^{298} (kJ/mol)	0.0445					

GSH conjugation inside cells. MHF may thus account for some of the pharmacodynamic effects of orally administered fumaric acid ester preparations since it will have higher chances than DMF to interact with more essential thiol groups in macromolecules.

The fumaric acid ester conjugates prepared and characterised here for the first time[23] are currently being used as reference samples in studies aimed at the elucidation of the complete metabolic balance of DMF. Furthermore, investigations on possible contributions of these conjugates to the pharmacological effects of fumaric acid esters have been initiated.

4. Experimental

4.1. Instrumentation

NMR spectra were recorded on Bruker DRX 500 and Varian Mercury 400BB spectrometers (500.13/400.33 MHz for ^{1}H, 125.13/100. 66 MHz for ^{13}C experiments) at 298/296 K, respectively, in D_2O. Spectra are referenced to external TMS. ESI-Mass spectra were recorded on Finnigan LCQ Deca XP (DMF-conjugates) and on a Waters-Micromass Quattro-LCZ (MHF conjugates) spectrometers in the direct inlet mode.

UV spectroscopic measurements were performed on a Beckman DB-G double beam photometer at room temperature (22 ± 3 °C).

Computational chemistry: all mentioned calculations were carried out using Gaussian 03 W, Rev. B.05 ond Pentium IV/Windows XP PC system. Force field optimized start geometries for each molecule were energy minimized using subsequently the semi-empirical AM1 Hamiltonian, ab initio RHF/6-31G(d), density functional theory RB3LYP/6-31G(d) and RB3LYP/6-311+G(2d,p). All parameters were used in their default settings as implemented in Gaussian. Free energies for the MHF-MeSH model conjugates were derived from frequency calculations of the final 6-311+G(2d,p) geometries.

4.2. Chemicals

DMF and MHF were obtained from Fumapharm AG, Lucerne, Switzerland. Their purity was confirmed by ^{1}H NMR analysis. GSH and CysNAc were purchased from Sigma Chemical Co, St. Louis, MO, USA. Buffers between pH 6.0 and 8.0 used were prepared using bidistilled water and 0.1 M KH_2PO_4/0.1 M NaOH, for pH 9.0 a glycine/NaOH buffer was used. Buffers were prepared according to Ref. 22.

4.3. DMF conjugates

4.3.1. Dimethyl-2-(*S*-glutathionyl)- succinate (GS-DMS). To a solution of 50 mg GSH (0.16 mmol) in 5 mL phosphate buffer, pH 7.4, 23 mg DMF (0.16 mmol), dissolved in 2 mL acetone was slowly added under stirring. After stirring for 1 h, the reaction mixture was left overnight. After evaporating the acetone under reduced pressure, the aqueous solution was freeze-dried. NMR spectroscopy of the residual in D_2O confirmed completeness of the reaction and formation of two diastereomeric products in equimolar ratio. NMR data see Table 3.

ESI-MS: pos. ion mode: 490 $[M+K]^+$ (80); 474 $[M+Na]^+$ (100), 452 $[M+H]^+$ (78); neg. ion mode: 901 $[2M-H]^-$ (5); 450 $[M-H]^-$ (100); 306 $[M-(DMF+H)]^-$ (20).

4.3.2. Dimethyl-2-[*S*-(*N*-acetyl)-L-cysteinyl]-succinate (NAC-DMS). To a solution of 28 mg *N*-acetyl-L-cysteine (0.17 mmol) in 5 mL phosphate buffer pH 7.4, 25 mg DMF (0.17 mmol), dissolved in 2 mL acetone was slowly added under stirring. After stirring for 1 h, the reaction mixture was left overnight. After evaporating the acetone under reduced pressure, the aqueous solution was freeze-dried. NMR spectroscopy of the residual in D_2O confirmed completeness of the reaction and formation of two diastereomeric products in equimolar ratio. NMR data see Table 4.

ESI-MS: pos. ion mode: 346 $[M+K]^+$ (48); 330 $[M+Na]^+$ (100), 308 $[M+H]^+$ (53); neg. ion mode: 613 $[2M-H]^-$ (100); 306$[M-H]^-$ (50); 162 $[M-(DMF+H)]^-$ (26).

4.4. MHF conjugates

Methyl-2-(*S*-glutathionyl)-hydrogen succinate and methyl-3-(*S*-glutathionyl)-hydrogen succinate (**2-GS-MHS** and **3-GS-MHS**, respectively):

To a solution of 59 mg GSH (0.19 mmol) in 5 mL phosphate buffer, pH7.4, 50 mg MHF (0.38 mmol), dissolved in 2 mL acetone was slowly added under stirring. After stirring for 1 h, the reaction mixture was left for two days. After evaporating the acetone under reduced pressure, the aqueous solution was freeze-dried. The completeness of reaction was confirmed by NMR spectroscopy of the residual in D_2O. The excess MHF was removed by gel chromatography on Sephadex LH-20/water. In the eluates, MHF was detected on TLC plates by UV absorbance at 254 nm, the GS-MHS conjugates by spraying with ninhydrin (0.25%) in acetone. UV-negative and ninhydrin positive eluates were combined and freeze-dried yielding the mixture of 2-GS-MHS and 3-GS-MHS. NMR data see Table 5.

ESI-MS: pos. ion mode: 476 $[M+K]^+$ (100); 460 $[M+Na]^+$ (54), 438 $[M+H]^+$ (27).

4.5. Reaction kinetics

For the UV spectroscopic measurements of reaction kinetics, a stock solution of GSH in degassed H_2O was freshly prepared in such concentration that a volume of 10–20 μL could be added to the buffer (all buffers were degassed by flushing with N_2) in the measuring and reference cuvette, in order to obtain the final specified concentration. A stock solution of DMF or MHF was prepared in acetone in such concentration that a volume of 10 μL could be added to the GSH solution to obtain the final specified concentration. Ten microlitres of ace-

tone was added to the reference cuvette instead of the fumarate solution. The final volume in both, the measuring and reference cuvette was 2.00 mL. The UV absorbance at 226 nm was monitored over time starting at $t = 0.5$ min. The resulting reaction time-courses were normalized to the theoretical absorbance at $t = 0$ (obtained by extrapolation of exponential trend lines fitted to the experimental curves to $t = 0$ min; each time course represents the mean of three measurements). Observed (pseudo)first-order rate constants (k [min^{-1}]) were deduced by least-squares fitting of exponential functions to the average of three normalized time-courses (obtained from three independent measurements). In these exponential functions the exponent is equal to $-k$. Data analysis was carried out using Microsoft Excel.

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