Bimanes. 17. (Haloalkyl)-1,5-diazabicyclo[3.3.0]octadienediones (Halo-9,10-dioxabimanes): Reactivity toward the Tripeptide Thiol, Glutathione

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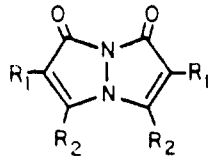
Abstract: Rate constants for the reaction of (haloalkyl)-1,5-diazabicyclo[3.3.0]octadienediones (halo-9,10-dioxabimanes) with the tripeptide thiol, glutathione (GSH), are reported. Certain bromobimanes are fluorescent labeling agents for thiols. Monohalobimanes and glutathione react by either displacement or reduction (replacement of alkylhalogen by hydrogen). Dihalobimanes react with glutathione in two stages: (1) displacement of a halogen by GS^- yields a monobromo–monoalkylthio intermediate, which reacts further either by (2a) an intermolecular displacement pathway, forming a bis-substituted product, or by (2b) an intramolecular displacement (neighboring group) pathway, the resulting sulfonium ion reacting further as described elsewhere.[13] Reduction is also observed for dihalobimanes. *syn*-($BrCH_2$,CH_3)(CH_3,CH_3)B is slightly more reactive than the corresponding anti derivative and 10 times as reactive as benzyl bromide. Either α-methyl substitution or replacement of bromine by chlorine seriously diminishes the rate constants for reaction with nucleophiles. The *syn*-(bromoalkyl,chloro)bimanes are more than 10 times as reactive as the *syn*-(bromoalkyl,methyl)bimanes but are also more susceptible to reduction by glutathione.

The haloalkyl derivatives of 1,5-diazabicyclo[3.3.0]octadienediones (9,10-dioxabimanes or in short, "bimanes") (syn-, **1**, and anti-, **2**) are important in the syntheses of bimane derivatives.[2-4]



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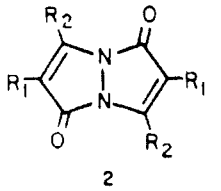

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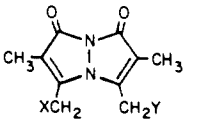
syn-Bromobimanes are useful as fluorescent labeling agents for thiol groups in biological systems under physiological conditions[5-8] and in a highly sensitive analysis for biological thiols at the picomolar level.[9-11] Rate constants for halobimane reactions with thiols in water are of interest for two reasons. First, it is important to quantitate the reactivity of bimane derivatives in reactions known for other alkyl halides. Second, a sound kinetic basis is needed for the design and choice of labeling agents. The tripeptide thiol, glutathione (GSH) [γ-glu-cys-gly],[12] is the major nonprotein thiol in many biological systems and a readily available water-soluble thiol. We report here the rate constants for the reaction of glutathione with a number of halobimanes, with special attention to the effect of substitutions at both the R_1 and R_2 positions of the bimane ring on reactivity. A particularly interesting outgrowth of the present study is the "sulfur extraction" reaction described in the following article.[13]

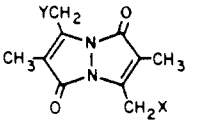
Results

Halobimanes. The kinetics of the reaction of both monohaloalkyl and bis(haloalkyl) derivatives of *syn*- and *anti*-bimanes with glutathione have been studied, including derivatives of *syn*-(CH_3,CH_3)B (**3a-f**), *anti*-(CH_3,CH_3)B (**4a,b**), *syn*-(CH_3CH_2,CH_3)(CH_3,CH_3)B (**5a-c**), *syn*-(CH_3CH_2,CH_3)B (**6a,b**), and *syn*-(CH_3,Cl)B (**7a,b**). The short-form nomenclature has been described previously;[2] abbreviations used for the labeling agents are indicated under the formulas. Most of the kinetic studies were carried out under "physiological" conditions, with [GSH] of 2×10^{-3} M at 25.0 °C and pH 7.3. The *syn*-(bromomethyl,chloro)bimanes (**7a,b**) were studied at lower pH (6.35, 6.01) because of their high reactivity toward glutathione.



3

a, X = Br, Y = H (mBBr)
b, X = Cl, Y = H
c, X = Y = Br (bBBr)
d, X = Y = Cl
e, X = Br, Y = OH
f, X = Br, Y = $N(CH_3)_3^+$ (qBBR)



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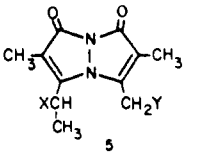
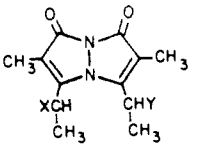
a, X = Br, Y = H
b, X = Y = Br



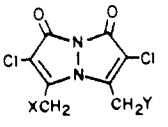
5

a, X = Br, Y = H
b, X = H, Y = Br
c, X = Y = Br



6

a, X = Br, Y = H (mEBBr)
b, X = Y = Br (bEBBr)
c, X = OH, Y = H



7

a, X = Br, Y = H
b, X = Y = Br
c, X = Y = H

Reaction Kinetics. The reaction product from (halolakyl)-bimanes and glutathione (GSH) has an absorption maximum at

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(5) (a) Kosower, N. S.; Kosower, E. M.; Newton, G. L.; Ranney, H. M., *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 3382-3386. (b) Kosower, N. S.; Kosower, E. M. *Methods Enzymol.*, in press.
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0002-7863/86/1508-4527$01.50/0

Table I. Rate Constants for Reaction of Halogen Derivatives of *syn*- and *anti*-9,10-Dioxabimanes with Glutathione at 25 °C in Water at pH 7.3[a]

	bimane	k_2,[b] M^{-1} s^{-1}	λ_{measd}, nm	runs	k_{rel}
3a	*syn*-($BrCH_2$,CH_3)(CH_3,CH_3)	5.54	440	4	1.0
	+ 0.1 M TMA^+Br^-[c]	6.83	440	2	1.20
	+ 1.0 M TMA^+Br^-[c]	7.75	440	3	1.39
3c	*syn*-($BrCH_2$,CH_3)	5.60	440	4	1.0
		7.62	460	4	1.36
	+ 0.1 M TMA^+Br^-[c]	5.32[d]	440	3	0.95
	+ 1.0 M TMA^+Br^-[c]	8.82	440	3	1.58
3b	*syn*-($ClCH_2$,CH_3)(CH_3,CH_3)	0.130	440	1	0.023
3d	*syn*-($ClCH_2$,CH_3)	0.138	440	1	0.025
5a + 5b	*syn*-($BrCH_2$,CH_3)(CH_3CH_2,CH_3)	3.6	440	1	0.64
	+ *syn*-(CH_3,CH_3)($BrCH(CH_3)$,CH_3)	0.026[e]			0.0047
5c	*syn*-($BrCH_2$,CH_3)($BrCH(CH_3)$,CH_3)	1.95	440	1	0.35
		0.24[f]			0.043
		1.14	430	1	0.20
		0.24[f]			0.043
6a	*syn*-($BrCH(CH_3)$,CH_3)(CH_3CH_2,CH_3)	0.045	440	1	0.0081
		0.016	440	1	0.0029
		0.022[d]	430	3	0.0040
6b	*syn*-($BrCH(CH_3)$,CH_3)	0.215	440	1	0.039
		0.074[d]	420	2	0.014
3e	*syn*-($BrCH_2$,CH_3)($HOCH_2$,CH_3)	3.47	440	3	0.63
3f	*syn*-($BrCH_2$,CH_3)($(CH_3)_3N^+CH_2$,CH_3)Br^-	34.3[g]	440	3	>6.2
4a	*anti*-($BrCH_2$,CH_3)(CH_3,CH_3)	3.64	320	2	0.65
		3.22	360	6	0.58
4b	*anti*-($BrCH_2$,CH_3)	2.90	340	2	0.52
		3.08[c]	360	3	0.55
7a	*syn*-($BrCH_2$,Cl)(CH_3,Cl)	61[c]	440	4	11
		7.40		3	7.8[h]
7b	*syn*-($BrCH_2$,Cl)	ca. 40[i]			
		7.2[j]	440	3	7.8[h]

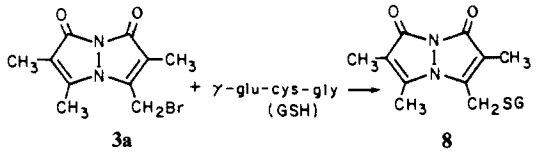
[a] Reactions were carried out under pseudo-first-order conditions, using ca. 2×10^{-3} M glutathione and 1×10^{-4} M bimane. [b] Values are ±1–3%, except as noted. [c] Tetramethylammonium bromide. [d] ±6–8%. [e] After 500-s reaction time. [f] After 300-s time. [g] Reaction solution: 0.4×10^{-3} M glutathione, 0.27×10^{-4} M **3f**. [h] pH 6.35, k_{rel} calculated by using $k_2 = 0.95$ for **3a** at pH 6.4. [i] Estimated by TLC from ratio of **7b** and **7a** at pH 6.01 (see text). [j] After 25-s reaction time (pH 6.35).

somewhat shorter wavelengths than the (haloalkyl)bimanes. After rapid combination of halobimane and glutathione solutions, the rate of loss of the halobimane was followed spectroscopically at a wavelength chosen to optimize the overall change in optical density. Excess glutathione is used, so that the absorbance decreases were first order in the halobimane. At lower concentrations of glutathione, the rate decreased in accordance with a reaction first order in glutathione. The pseudo-first-order rate constants (from plots of log $[D(t) - D_\infty]$ vs. time) were converted to second-order constants by dividing by the glutathione concentration. The rate constants for the reaction of various halobimane derivatives with glutathione are listed in Table I.

Reasonably linear first-order plots were obtained for the reaction of most of the halo derivatives, although reaction with several dihalides showed two-phase behavior. The plots decreased in slope after 30–50% reaction (factors: **3c**, 1.3; **5c**, 8], indicating a rate of replacement for the second halide not very different from that of the first.

Temperature and pH Variation. The rate of reaction of the bromobimane **3a** (mBBr) has been studied over the temperature range 15–35 °C (activation energy, 16 ± 1.5 kcal/mol) and over the pH range 1.2–9.0. The rate constants are linear in $[OH^-]$ (Table II).

Reaction Products. Mono(haloalkyl)bimanes react with glutathione (GSH) to yield a substitution product, a monoalkylated glutathione (RSG), as illustrated for mBBr (**3a**) in eq 1.



(1)

3a **8**

syn-($BrCH_2$,CH_3)(CH_3,CH_3)B nonfluorescent — *syn*-($GSCH_2$,CH_3)(CH_3,CH_3)B fluorescent

The glutathione derivatives of *syn*-bimanes are strongly fluorescent, while the anti counterparts are phosphorescent on a TLC plate, as expected.[14] The fluorescent alkylated glutathione derivatives (RSG) are water soluble. Unreacted bromobimanes and other products can be extracted from the reaction solution with organic solvents. In the case of *syn*-($BrCH_2$,Cl)(CH_3,Cl)B (**7a**), the reduction product, *syn*-(CH_3,Cl)B (**7c**), is obtained in 57% yield (eq 2).

$$\underset{\textbf{7a}}{\textit{syn}\text{-}(BrCH_2,Cl)(CH_3,Cl)B} + GS^- \rightarrow \underset{\textbf{9}}{\textit{syn}\text{-}(GSCH_2,Cl)(CH_3,Cl)B} + \underset{\textbf{7c}}{\textit{syn}\text{-}(CH_3,Cl)B} \quad (2)$$

The spectroscopic changes observed during the reactions of halobimanes **7** are recorded in Table III, and the product composition is shown in Table IV.

The glutathione derivatives (RSG) are purified by chromatography on cellulose plates using 72% aqueous 2-propanol as the

Table II. Temperature and pH Effects on the Rate Constants for Reaction of Glutathione in Water with syn-(Bromomethyl,methyl)(methyl,methyl)bimane[a]

temp, °C	pH	k_2, M^{-1} s^{-1} [b]	runs
15	9.0	168[c]	4
	7.8	7.43	3
	7.3	2.25	2
25	7.3	5.54	4
	6.4	0.95	3
	5.6	0.14	2
	5.0	0.038	1
	4.2	0.0073[d]	2
36	7.3	15.8	5
40	4.2	0.031	1
	3.0	0.001	1
	2.1	0.0012	1
	1.2	0.0006	1

[a] As in Table I. [b] ±3%, except as noted. [c] ±10%. [d] Half-lives [GSH]: 6.8 h, 3.79×10^{-3} M; 13.3 h, 2.04×10^{-3} M.

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Table III. Absorption Maxima of Solutions of Bimanes [*syn*-(R_2,Cl)(R_2,Cl)B] Reacted with Glutathione at pH 7.31 or 6.01

compd	R_2	R_2	pH	*t*,[a] min	maxima, nm CH_3CN[b]	maxima, nm water[b]
7c	CH_3	CH_3			375	385
7a	CH_3	CH_2Br	7.31, 6.0	0	386	402
			7.31	3[c]	374[d]	391[e]
			6.01	10	380[d]	394[f]
			6.01	60[c]	374[d]	392[f]
7b	CH_2Br	CH_2Br		0	391	407
			7.31	3[c]	374[d]	391[e]
			6.01	10	378[d]	391[e]
			6.01	60[b]	374[d]	394[e]

[a] Time allowed for reaction after mixing of bimane and glutathione at pH 6.01; *t* = 0, no glutathione. Reaction was stopped by lowering pH to 2.5. [b] Solvent used for spectroscopic measurements. [c] Reaction complete. [d] CH_2Cl_2 extract was evaporated to obtain sample. [e] Aqueous portion. [f] Reaction solution not extracted with methylene chloride.

Table IV. Products of Reaction of Bimanes [*syn*-(R_2,Cl)(R_2,Cl)B] with Glutathione at pH 7.31 or 6.01

compd	R_2	R_2	pH	*t*,[a] min	nonpolar[c] (% yield)	polar[d] (% yield)
7a	CH_3	CH_2Br	7.31	3[b]	**7c** (56)	**9** (25)
			6.01	10	**7a** + **7c** (60)	
			6.01	60[b]	**7c** (57)	
7b	CH_2Br	CH_2Br	7.31	3[b]	**7c** (43)	**9** (44)
			6.01	10	**7a** + **7c** (41)	**9** (32)
			6.01	60[b]	**7c** (39)	**9** (44)

[a,b] As in Table III. [c] Products extracted with methylene chloride; analysis by TLC and UV; **7c**, $R_2 = R_2 = CH_3$. [d] Aqueous residue; TLC identical for all; **9**, $R_2 = CH_3$, $R_2 = GSCH_2$, by NMR.

Table V. Absorption Maxima for Bimanes [*syn*-(R_2,CH_3)(R_2,CH_3)B] in Water, pH 7.3

compd	R_2	R_2	maxima, nm
3a	CH_3	$BrCH_2$	395
3b	CH_3	$ClCH_2$	392
8	CH_3	$GSCH_2$	390.5[a]
3e	$HOCH_2$	$BrCH_2$	400
3e	$HOCH_2$	$GSCH_2$	393
3c	$BrCH_2$	$BrCH_2$	404
3d	$ClCH_2$	$ClCH_2$	399
12	$GSCH_2$	$GSCH_2$	393[b]
5a + **5b**	$BrCH_2$ + CH_3	CH_3CH_2 + $BrCH(CH_3)$	396
5a + **5b**	$BrCH_2$ + CH_3	CH_3CH_2 + $BrCH(CH_3)$	392[c]
5c	$BrCH_2$	$BrCH(CH_3)$	402.5
5c	$BrCH_2$	$BrCH(CH_3)$	364[c]
6a	CH_3CH_2	$BrCH(CH_3)$	395
15	CH_3CH_2	$GSCH(CH_3)$	391
6b	$BrCH(CH_3)$	$BrCH(CH_3)$	402
6b	$BrCH(CH_3)$	$BrCH(CH_3)$	365[c]

[a] Product of reaction of glutathione with both **3a** and **3b**. [b] Product of reaction of glutathione with both **3c** and **3d**. [c] Maximum observed for the solution after reaction with GSH.

developer. Structures are assigned by using (a) UV spectra (λ_{max} 390 nm, typical for monosubstituted bimanes in water), (b) ^1H NMR spectroscopy (characteristic chemical shifts and splittings of substituted bimanes along with those of glutathione in a 1:1 ratio), and (c) fluorescence (λ_{max} 480 nm, typical for *syn*-bimane derivatives in water). The UV absorption maxima in aqueous solution are summarized in Table V. The NMR shifts for bimane derivatives are given in Tables VI and VII.

The reaction of the dibromobimanes with glutathione follows a course dependent on the structure of the bimane. The dibromo derivatives of *syn*- and *anti*-(CH_3,CH_3)B, **3c** and **4b**, form mostly the bis-substituted glutathione derivatives, along with some monosubstituted GS-bimane resulting from reduction (eq 3).

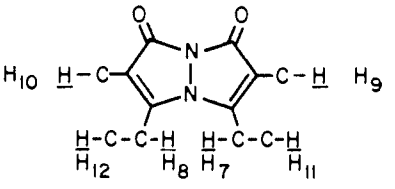
$$(BrCH_2,CH_3)B + 2GS^- \rightarrow (GSCH_2,CH_3)B + (GSCH_2,CH_3)(CH_3,CH_3)B \quad (3)$$

Table VI. ^1H NMR Chemical Shifts for Bimanes [*syn*-(R_2,R_1)(R_2',R_1')B]

bimane	solvent	R_2	R_2'	R_1	R_1'
$R_2 = CH_3$, $R_2' = CH_2OH$ $R_1 = CH_3$, $R_1' = CH_3$	$CDCl_3$	2.42	4.61	1.81	1.85
$R_2 = CH_3$, $R_2' = CH_2Br$ $R_1 = CH_3$, $R_1' = CH_3$	$CDCl_3$	2.46	4.35	1.82	1.87
$R_2 = CH_2OH$, $R_2' = CH_2Br$ $R_1 = CH_3$, $R_1' = CH_3$	CD_3CN	4.63	4.69	1.84	2.22
$R_2 = CH_2OH$, $R_2' = CH_2SG$[a] $R_1 = CH_3$, $R_1' = CH_3$	D_2O	4.27	4.01	2.14	2.14
$R_2 = CH_2Br$, $R_2' = CH_2Br$ $R_1 = CH_3$, $R_1' = CH_3$	$CDCl_3$	4.62	4.62	2.31	2.31
$R_2 = CH_2CH_3$, $R_2' = CHBr(CH_3)$ $R_1 = CH_3$, $R_1' = CH_3$	$CDCl_3$	CH_2 1.90 (q) CH_3 0.64 (t)	CH 4.22 (q) CH_3 1.41 (d)	1.43	1.74

[a] G = glutathione – SH. The NMR signals for the G protons are not included in this table.

Table VII. ^1H NMR Data for Glutathione and Glutathione Derivatives of Bimanes



$^-OOCCH(NH_2)CH_2CH_2CONHCH(CH_2SH)CONHCH_2COO^-$
H_3 H_6 H_5 H_1 H_4 H_2

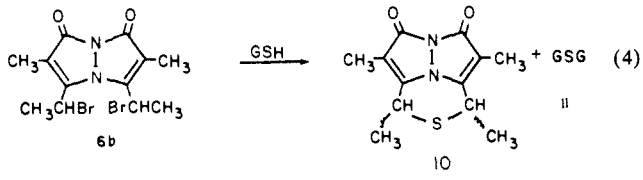
H	GSH[a]	GSB[b]	$(GS)_2B$[c]	GSEB[d]
H_1	4.501 (t, *J* = 6.3 Hz)	4.883–4.742	under H_2O	ca. 4.817
H_2	4.214 (s)	4.133	4.286	3.999
H_3	4.064 (t, *J* = 6.2 Hz)	3.940–3.898	hidden	3.846
H_4	3.213–3.145 (d)	3.585–3.138	3.589–3.259	3.357–3.259
H_5	2.894–2.718 (m)	ca. 2.548	2.763	2.705–2.682
H_6	2.519–2.287 (m)	ca. 2.323	2.388	2.356
H_7		3.986 (s)	4.015 (s)	3.963
H_8		2.659 (s)		3.089
H_9		2.092 (s)	2.147 (s)	2.203 (s)
H_{10}		2.004 (s)	2.147 (s)	2.043 (s)
H_{11}				1.952–1.873 (d)
H_{12}				1.505 (t, *J* = 7.8 Hz)

[a] Glutathione. [b] *syn*-(Glutathionylmethyl,methyl)(methyl,methyl)-bimane (**8**). [c] *syn*-(Glutathionylmethyl,methyl)bimane (**12**). [d] *syn*-(1-Glutathionylethyl,methyl)(ethyl,methyl)bimane (**15**).

The dibromobimane, *syn*-($BrCH_2$,Cl)B (**7b**), is rapidly reduced to the monobromobimane (**7a**) after mixing with glutathione. About 39% yield of *syn*-(methyl,chloro)bimane (**7c**) is obtained through further reduction. The water-soluble product of this reaction is the same (by TLC) as that obtained from the monobromobimane (**7a**) reaction with glutathione. Details are summarized in Tables III and IV. It should be noted that the UV maximum of the *syn*-($BrCH_2$,Cl)B (**7b**) is at a longer wavelength (407 nm) than those of other bromo derivatives.

The reaction of the *syn*-(1-bromoethyl,methyl)bimane (**6b**) with glutathione yielded small amounts of the expected bis(glutathionyl), along with monoglutathionyl, derivatives. The major products were an organic-solvent-soluble thia-bridged bimane (**10**),

formed from the bridged sulfonium ion, and a *sulfide*, GSG (**11**). These results are fully described in the following paper[13] (eq 4).



(4)

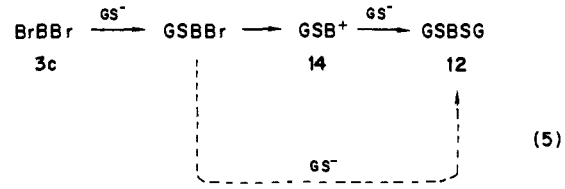
Discussion

The reaction of glutathione and halobimanes raises the following questions: (1) What precisely are the reactants? (2) What is the reactivity of a halobimane in comparison with a better known system like a benzyl halide? (3) What are the effects of variation in the structure of the bimane?

The rate of the reaction between glutathione and the monobromobimane, **3a** (mBBr), increases linearly with [OH^-] (a plot of log k vs. pH has a slope of about 1), showing that the major reactive species at pH 7.3 is the anion GS^-. Reaction rates could be measured even at pH 1, since bimanes are very weak bases,[2] but the constants decreased no further between pH 1 and 3. The values at low pH suggest that the undissociated thiol reacts about 3×10^{-5} times as fast as the thiolate anion. Temperature has a modest effect on the reaction rate. The activation energy is ca. 16 ± 1.5 kcal/mol for both the thiolate anion and the thiol.

In the case of the reaction of GS^- with dihalobimanes, one must consider whether the first substitution product (GSBX) reacts with a second GS^- or forms a sulfonium ion by a neighboring group reaction. From the relative reactivities (10^{-5}) of glutathione (thiol as a model for thioether in GSBX) and GS^- (cf. Table II) and their relative concentrations [ca. 10^6],[13] intramolecular reaction should be favored by a factor of ca. 10. In fact, this is the case for both **5c** and **6b**, as shown by the results in the following paper. An inverse ratio is found for a different pair of reactants. The rate constant for the reaction of benzyl bromide with glutathione at pH 7.3 is ca. 0.42 M^{-1} s^{-1} [15] and with methionine (thioether as the nucleophile) is 0.08 M^{-1} s^{-1}.[15] These rate constants yield a factor of 0.02 for the intramolecular vs. intermolecular reaction but do not take into account possible strain and entropy effects. A more extensive analysis of intramolecular vs. intermolecular reactivities (especially with respect to enzymes) is found in the book by Fersht.[16]

The product of the reaction of the dibromobimane **3c** with glutathione is the bis(glutathione) derivative **12** with no trace of the thia-bridged bimane (μ-(S)-bimane, **13**) being found. Were there a sulfonium ion intermediate (**14**), its reaction with GS^- at the position next to the bimane ring, in the intramolecular pathway, would give a product identical with that formed by a completely intermolecular pathway. The two pathways are shown schematically in eq 5.



(5)

It is difficult to distinguish between these two routes. The reactivity to extramolecular nucleophiles is diminished by steric hindrance, as shown by the rate constant for the bis(1-bromoethyl) derivative, **6b**, in comparison to **3c**. The intramolecular pathway is found to take precedence over intermolecular reaction. The nature of the product changes to that resulting from elimination of a thia-bridged bimane from the sulfonium ion.[13]

Reactivity of Halobimanes. Monobromobimane is 13 times as reactive as benzyl bromide.[15] (Bromoalkyl)bimanes are thus fairly reactive alkyl halides. The reactions with thiols are nucleophilic displacement reactions on the basis of the effect of α-methyl substitution. In addition, qualitative experiments show that silver ion does not promote ionization of bromide from mBBr. The alkylation of glutathione has been reviewed by Friedman.[17]

Bromobimanes are 43 times as reactive as chlorobimanes toward glutathione. Both dichlorides and dibromides react at almost the same rate as the monohalides, shown impressively in the case of the bromoalkyl derivatives of the "mixed" bimane, *syn*-(CH_3CH_2,CH_3)(CH_3,CH_3)B. There are three possible bromo derivatives, a $BrCH_2$ compound (**5b**), a $BrCH(CH_3)$ compound (**5a**), and a dibromide compound (**5c**) in which there are both $BrCH_2$ and $BrCH(CH_3)$ groups. The monobromo $BrCH_2$ derivative reacts at almost the same rate as the corresponding compound derived from *syn*-(CH_3,CH_3)B (**3a**, mBBr). The monobromo $BrCH(CH_3)$ compound reacts at about the same rate as the monobromo compound **6a** (mEBBr) derived from *syn*-(CH_3CH_2,CH_3)B. The dibromo derivative having both $BrCH_2$ and $BrCH(CH_3)$ groups (**5c**) exhibits two rates of reaction with glutathione, one similar to that of the $BrCH_2$ compound **5b** and a second similar to that of the other monobromo compound **5a**. However, in the dibromo compound **6b** derived from *syn*-(CH_3CH_2,CH_3)B, the second bromoalkyl substituent increases the rate constant by a factor of about 5.

A trimethylammonio cation attached to the R_2'-alkyl group (**3f**) raises the rate constant by more than a factor of 6, apparently an "electrostatic" catalysis like that produced by added tetramethylammonium bromide (rate factor 1.4 at 1.0 M salt). The local cation concentration introduced by the neighboring group is equivalent to 10 M cation, for which a rate increases by a factor of 14 might be expected. The high reactivity of **3f** (qBBr) means that solutions for labeling thiol groups should be freshly prepared. Uncharged dipolar groups (e.g., the OH in **3e**) slightly decrease the reactivity of bromobimanes.

Substitution of a methyl group for a hydrogen in the methylene of **3a** (mBBr) lowers the rate by a factor of at least 100. Both the monobromo derivative, *syn*-($BrCH(CH_3)$,CH_3)-(CH_3CH_2,CH_3)B (**6a**), and the dibromo compound, *syn*-($BrCH(CH_3)$,CH_3)B (**6b**), react much more slowly with glutathione than **3a** and **3c**, respectively. A similar decrease is found for displacement reactions on benzyl halides and must be a steric effect.

Replacement of the CH_3 at R_1 with Cl increases reactivity toward glutathione by a factor of 10 and favors reduction over substitution. The R_1 = Cl derivatives are thus not particularly suitable for biological thiol labeling procedures[5b] in spite of their higher reactivity. The rate of reduction of the dibromo derivative **7b** is very high, $k_2 > 40$ M^{-1} s^{-1}. Electron-transfer is the likely mechanism for reduction. Reduction by electron transfer has been observed for ($BrCH_2$,CH_3)(CH_3,CH_3)B in preparations of thioethers[2] or in reactions with photosynthetic reaction centers.[18]

Experimental Section

General. Instruments used were as follows. UV and visible spectra: Cary Model 17 spectrophotometer equipped with a thermostated cell holder having a built-in variable-speed magnetic stirring apparatus. Fluorescence spectra: Hitachi–Perkin-Elmer MPF-4 fluorescence spectrophotometer. ^1H NMR spectra: Bruker WH-90 (chemical shifts are given in δ values (reference: HDO 5.000 (D_2O solvent))).[19] pH measurement: Orion Model 801 digital pH meter.

Solvents and Materials. Dichloromethane (analytical grade), acetonitrile (spectroscopic), and 2-propanol were used without further purification. Distilled deionized water was used. Bromobimanes were available within our group or were prepared according to established procedures.[2-4]

Chromatography. Water-soluble compounds were chromatographed on cellulose-coated glass plates (thickness 100 μm, Merck) using 68–72%

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(19) Although HDO as a reference signal (5.000) in ^1H NMR is not as reproducible as an added solute (DSS), we have found very little variation (±0.03 ppm) in the position of particular protons. In addition, we wished to recover the samples in pure form.

aqueous 2-propanol for development. Compounds were located through fluorescence or through the color produced with ninhydrin. Organic-soluble compounds were chromatographed on silica gel plastic plates (Merck) using ethyl acetate as the developer and UV to check the location of the material.

Buffer Solutions. All buffer solutions were standardized against Fisher Standard pH buffer: pH 1–2, H_2SO_4; pH 4–5.6, $I = 0.1$, acetic acid–sodium acetate; pH 6–8, $I = 0.1$, KH_2PO_4 or $NaHPO_4 \cdot 2H_2O$ and Na_2HPO_4; pH 9, $I = 0.1$, bicarbonate–carbonate.[20]

Kinetic Procedure. Bromobimane solutions in acetonitrile (ca. 3×10^{-3} M) were stable if stored at 0–5 °C and protected from light. Glutathione (GSH) solutions (ca. 2×10^{-3} M) were prepared daily in the appropriate buffers. The actual concentration of glutathione was determined several times a day with DTNB,[21] 0.4×10^{-3} M in 1% sodium citrate solution. [DTNB (1 mL) and glutathione (0.1 mL) were diluted to 5 mL with buffer and the thiolate anion determined at λ 412 nm (ϵ 1.35×10^4).] A solution of glutathione (2.9 mL) was thermostated in a quartz cell (1 cm) containing a small magnetic stirring bar for ca. 10 min. The bimane solution (0.1 mL) was injected rapidly with a 200-μL micropipet and the change in absorbance with time at an appropriate wavelength recorded. The wavelength was chosen to give the maximum difference in absorbance between starting material and product.

In the reaction of *syn*-(bromomethyl,chloro)bimane, **7b**, with glutathione, the initial rate of reduction to the monobromo derivatives, **7a**, is too rapid to be measured with the instrumentation used in the present work. The rate is estimated as follows: An aliquot (0.9 mL) of a glutathione solution (2×10^{-3} M at pH 6.01) is introduced into a vial. A solution of **7b** (0.1 mL, 1×10^{-3} M) is injected rapidly. At a designated time, the reaction is quenched with sufficient 0.1 N hydrochloric acid (0.1 mL) to give pH ca. 2.5. TLC of a sample (0.01 mL) on a silica plate allows a comparison of the light-induced fluorescence of *syn*-(bromomethyl,chloro)bimane (**7b**) and of *syn*-(bromomethyl,chloro)(methyl,-chloro)bimane (**7a**). Reaction times as short as 3 s could be achieved. The time, 10 s, at which the two spots were equal was taken as the half-life of the reaction.

Isolation and Identification of Reaction Products. 9,10-Dioxa-*syn*-(glutathionylmethyl,methyl)(methyl,methyl)bimane [*syn*-(GSCH$_2$,CH$_3$)(CH$_3$,CH$_3$)B] (8). *syn*-(BrCH$_2$,CH$_3$)(CH$_3$,CH$_3$) (**3a**) (21.8 mg, 0.080 mmol) in CH_3CN (1.5 mL) was added to a solution of glutathione (24.4 mg, 0.079 mmol) and $NaHCO_3$ (20.3 mg, 0.242 mmol) in water (6 mL) and the mixture stirred for 1 h. After extraction with CH_2Cl_2, the aqueous layer was lyophilized and the residue (55.5 mg) chromatographed on a cellulose plate (75% aqueous 2-propanol). ^{1}H NMR (D_2O): 2.004 (s, 3 H, α_1-CH_3), 2.092 (s, 3 H, α_2-CH_3), 2.323 (m, 2 H, CH_2 glu), 2.548–2.810 (m, 2 H, CH_2 glu), 2.659 (s, 3 H, β-CH_3), 3.030–3.585 (m, 2 H, CH_2 cys), 3.940 (t, 1 H, CH cys), 3.986 (s, 2 H, β-CH_2), 4.133 (s, 2 H, CH_2 gly), 4.742–4.883 (m, 1 H, CH glu). UV (λ_{max} (nm) (ϵ), 3% CH_3CN/H_2O, pH 7.3): 390.5 (3300), 257 sh (7300), 234 (12000). Fluorescence (λ_{max} (nm) (ϕ_F), 3% CH_3CN/H_2O, pH 7.3): 469 sh, 486, 500 sh (0.28).

9,10-Dioxa-*syn*-(glutathionylmethyl,methyl)bimane [*syn*-(GSCH$_2$,CH$_3$)B] (12). *syn*-(BrCH$_2$,CH$_3$)B (**3c**) (55.2 mg, 0.158 mmol) in CH_3CN (3.8 mL) was reacted with glutathione (97.4 mg, 0.317 mmol) and sodium bicarbonate (53.4 mg, 0.636 mmol) in water (15 mL) as described for **3a**. Some **3c** was recovered by extraction with CH_2Cl_2 (1.5 mg). UV (λ_{max} (nm), 3% CH_3CN/H_2O): 396, 255, 240 sh. After lyophilization, the residue (163.3 mg) was chromatographed on a cellulose plate (75% aqueous 2-propanol). ^{1}H NMR (D_2O): 2.147 (s, 6 H, α-CH_3), 2.285-2.529 (m, 4 H, CH_2 glu), 2.789 (t, 4 H, $J = 7.2$ Hz, CH_2 glu), 3.081–3.669 (m, 4 H, CH_2 cys), 4.081 (t, 2 H, $J = 6.3$ Hz, CH cys), 4.214 (s, 4 H, CH_2 gly), 4.292 (d, 4 H, β-CH_2), 4.751–4.921 (m, 2 H, CH glu). UV (λ_{max} (nm) (ϵ), 3% CH_3CN/H_2O, pH 7.3): 393 (4500), 234.

9,10-Dioxa-*syn*-(1-glutathionylethyl,methyl)(ethyl,methyl)bimane [*syn*-(CHGS(CH$_3$),CH$_3$)(CH$_3$CH$_2$,CH$_3$)B] (15). *syn*-(BrCH(CH$_3$),-CH$_3$)(CH$_3$CH$_2$,CH$_3$)B (**6a**) (31 mg, 0.11 mmol) in CH_3CN (4.5 mL) was reacted with glutathione (31.1 mg, 0.11 mmol) and sodium bicarbonate (25 mg, 0.3 mmol) in water (15 mL) as described for **3a**. After extraction and lyophilization, the residue (60 mg) was chromatographed on a cellulose plate. ^{1}H NMR (D_2O): 1.524 (t, 3 H, $J = 7.2$ Hz, CH_3 of CH_3CH_2), 1.932 (d, 3 H, $J = 7.0$ Hz, CH_3 of CH_3CH), 2.062 (s, 3 H, α-CH_3 adjacent to CH_3CH_2), 2.219 (s, 3 H, α-CH_3 adjacent to CH_3CH), 2.323–2.476 (m, 2 H, CH_2 glu), 2.621–2.849 (m, 2 H), (CH_2 glu), 3.050–3.510 (m, 4 H, CH_2 cys and CH_2 of CH_3CH_2), 3.893–4.208 (m, 2 H, CH cys and CH of CH_3CH), 4.162 (s, 2 H, CH_2 gly), 4.602–4.847 (m, CH glu). UV (λ_{max} (nm) (ϵ), 3% CH_3CN/H_2O, pH 7.3): 391 (4100), 254 sh (12700), 200–210 (18200). Fluorescence (λ_{max} (nm) (ϕ_F), 3% CH_3CN/H_2O, pH 7.3): 470 sh, 484, 497 sh (0.35).

Reaction of *syn*-(Bromomethyl,chloro)(methyl,chloro)bimane [*syn*-(BrCH$_2$,Cl)(CH$_3$,Cl)B] (7a) and of *syn*-(Bromomethyl,chloro)bimane [*syn*-(BrCH$_2$,Cl)B] (7b) with Glutathione. A bimane solution (3×10^{-3} M in CH_3CN, 200 μL) was added to 5.8 mL of glutathione solution (2.1×10^{-3} M, pH 7.3) in a separatory funnel covered with aluminum foil. After 2 min, the solution was extracted with CH_2Cl_2 (2×5 mL); the extracts were evaporated, redissolved in CH_3CN (3 mL), and analyzed by UV and TLC (silica plate, EtOAc). The aqueous layer was lyophilized and redissolved in water (3 mL) for UV and TLC analysis (cellulose plate, 70% aqueous 2-propanol, ninhydrin spray).

For reactions at pH 6.0, glutathione and bimane were mixed to give 5 mL of reaction solution containing 0.47×10^{-3} M glutathione with 0.22×10^{-3} M **7a** and 0.94×10^{-3} M glutathione with 0.20×10^{-3} M **7b**. After an appropriate time ~ (10 or 60 min), 0.1 N HCl (0.8 mL) was added to reduce the pH to 2.5. The samples were then treated as described above for pH 7.3.

After 5 min of reaction, a mixture of glutathione (0.244 mmol), **7b** (0.051 mmol), and sodium bicarbonate (0.346 mmol) in water (100 mL) and CH_3CN (10 mL) was brought to pH 3 with 0.1 N H_2SO_4. After extraction with CH_2Cl_2, the aqueous residue was lyophilized and chromatographed on a cellulose column, using 2-propanol–water mixtures (95–82% 2-propanol; see following paper[13]). Elution with 89–87% 2-propanol yielded a fluorescent, ninhydrin-positive product, containing some unreacted glutathione. ^{1}H NMR (D_2O) indicated that the product is *syn*-(GSCH$_2$,Cl)(CH$_3$,Cl)B (**9**, with bimane methyl at δ 2.713, as part of the methylene signal for γ-glu of glutathione). The bimane methylene proton signals are found with the signal for the methylene protons of gly and the methine proton of cys (4.239–4.167). Spectrum integration shows a ratio of 3:2 protons for the bimane CH_3:CH_2 protons. The UV absorption maximum is at 393 nm in H_2O.

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Registry No. 3a, 71418-44-5; **3b**, 76421-73-3; **3c**, 68654-25-1; **3d**, 76421-74-4; **3e**, 102284-79-7; **3f**, 71418-45-6; **4a**, 74235-78-2; **4b**, 74235-77-1; **5a**, 102306-05-8; **5b**, 102284-80-0; **5c**, 102284-81-1; **6a**, 88848-54-8; **6b**, 88848-55-9; **7a**, 74235-79-3; **7b**, 102284-82-2; **8**, 88859-65-8; **9**, 102306-06-9; **12**, 102284-83-3; **15**, 102284-84-4; GSH, 70-18-8.

(20) *Biochemistry Handbook*; Long, C., Ed.; Wiley: New York, 1971; pp 30–36.

(21) 5,5′-Dithiobis(2-nitrobenzoic acid) (Ellman's reagent).