Bimanes. 18. (Haloalkyl)-1,5-diazabicyclo[3.3.0]octadienediones (Halo-9,10-dioxabimanes): Sulfur "Extraction" by *syn*-(1-Bromoethyl,methyl)bimane. Glutathione "Sulfide" from the Tripeptide Thiol, Glutathione

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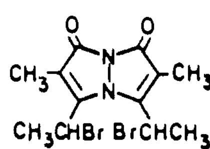
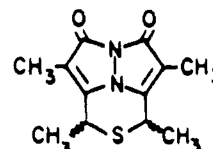
Abstract: Glutathione (GSH) and the diastereomeric dibromides [*syn*-(1-bromoethyl,methyl)bimanes] derived from *syn*-(ethyl,methyl)bimane (4,6-diethyl-3,7-dimethyl-1,5-diazabicyclo[3.3.0]octa-3,6-diene-2,8-dione) react to form a small amount of the $(GS)_2$ derivative and substantial amounts of isomeric (*cis*- and *trans*-) thia-bridged bimanes [μ-(*S*)-(methylmethylene,methyl)bimane]. The latter are accompanied by a new compound, glutathione sulfide (GSG). The process is equivalent to the "extraction" of sulfur from two thiol molecules. Formation of GSG occurs via the following steps: (1) displacement of bromide ion by the thiolate anion, GS^-, from the dibromide to yield a monoalkylthio–monobromo derivative; (2) intramolecular displacement of the remaining bromine by the neighboring alkylthio group to produce a reactive sulfonium ion; (3) disappearance of the intermediate sulfonium ion by either (a) elimination or (b) nucleophilic displacement. (3a) Base causes 1,2-elimination of H^+ and the thia-bridged bimane from the intermediate sulfonium ion, yielding "dehydro-GSH" [γ-glu-dehydroala-gly] to which GSH then adds to give two diastereomers of GSG. (3b) Nucleophilic displacement by GS^- at the cysteinyl methylene of the intermediate sulfonium ion yields GSG and the thia-bridged bimane. The dibromide diastereomers are produced by bromination of *syn*-(ethyl,methyl)bimane in roughly equal amounts, are separable by HPLC, and are stable in nonpolar solvents but interconvert readily in polar solvents. The structure of the trans isomer of μ-(*S*)-(methylmethylene,methyl)bimane has been established by X-ray crystallography. The present approach to sulfide formation from two thiol molecules in aqueous solution is the mildest yet discovered. GSG does not inhibit the reduction of GSSG by NADPH catalyzed by GSSG reductase.

The haloalkyl derivatives of *syn*-9,10-dioxabimanes (1,5-diazabicyclo[3.3.0]octadienediones)[2-5] have proven particularly useful as fluorescent labels for thiol groups in biological systems under physiological conditions.[6-24] A sensitive picomole-level analysis for thiols present in biological systems is based on the detection of the thiol derivative formed with bromobimanes.[10-13] It is therefore important to study in detail the reactions of bromobimanes with thiols. As a convenient thiol nucleophile, we chose the most important nonprotein thiol, the tripeptide glutathione [GSH, γ-L-glu-L-cys-gly]. The role of the ubiquitous glutathione in biological systems has been thoroughly reviewed elsewhere.[25] We report here the unusual products of the reaction of glutathione with certain bromobimanes. In particular, a potentially important sulfur "extraction" reaction has been discovered, the reaction involving the conversion of two molecules of thiol into a sulfide under extremely mild conditions in aqueous solution.

It was noted that the UV spectrum of the products of the reaction of glutathione and *syn*-(1-bromoethyl,methyl)bimane (**1**) had a maximum at a considerably shorter wavelength than expected. The origin of the "shifted" spectrum was traced to the presence of a thia-bridged compound, the μ-(*S*)-*syn*-$(CH(CH_3),CH_3)B$ (**2**). Isolation of the GSH-derived product yielded



1
syn-$(BrCH(CH_3),CH_3)B$
nonfluorescent



2
μ-(*S*)-*syn*-$(CH(CH_3),CH_3)B$
fluorescent

the new sulfide, GSG (**3**). The present article describes the isolation of glutathione sulfide (**3**) and the thia-bridged bimane **2** and the mechanism of their formation. Some of the possibilities inherent in this potentially useful neighboring group participation

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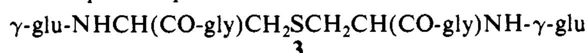
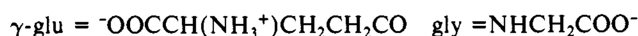
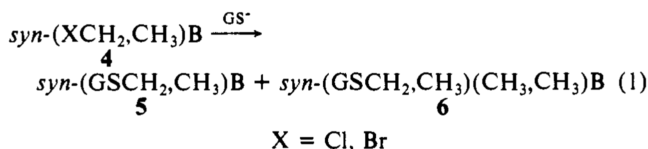
at neutral pH in aqueous solution are noted.





Results

Reaction Products. The reaction of the bis(haloalkyl) derivatives with glutathione follows a course dependent on the nature of the bimane. The bis(halomethyl)bimanes [*syn*-(halomethyl,methyl)bimane[26]] (**4**) (halo = chloro or bromo) react with glutathione to form mostly the bis(glutathione) derivative **5** (eq 1). The structure of the bis(glutathione) derivative is assigned



by using UV, NMR (GS and bimane protons are found in a 2:1 ratio), and fluorescence data. Loss of a bromine through reduction (2–8%) along with displacement yields the same GS-bimane product (**6**) as that derived from the reaction of the monohalobimanes with glutathione.

The reaction of *syn*-(1-bromoethyl,methyl)bimane (**1**) with glutathione yields a product for which the UV absorption maximum is found at a much shorter wavelength than expected (λ_{max} 365 nm rather than 392 nm). The new material (53% product) is readily extractable by organic solvents (CH_2Cl_2 or Et_2O). UV, NMR, and mass spectroscopic data lead to the identification of the compound as a mixture of two isomeric thia-bridged bimanes, *cis*- and *trans*-μ-(*S*)-*syn*-(methylmethylene,methyl)bimane (**2**). *syn*-(1-Hydroxyethyl,methyl)(ethyl,methyl)bimane (**7**) is the second major compound isolated (10% yield) from the organic-extractable material. Another four compounds, one a characteristically yellow-green fluorescent vinyl derivative, could be detected in the extracted material by TLC, but there was not enough material for purification and/or structural assignment.

The major water-soluble product is the monosulfide derived from glutathione by loss of H_2S. The monosulfide, GSG (**3**), was identified by NMR and amino acid analysis. The disulfide, GSSG (from oxidation of GSH), and minor quantities of other bimane derivatives and of partially degraded peptide products (loss of glu, or gly, etc.) are also found. Reduced glutathione, GSH, was not obtained from the column. The transformation is indicated in eq 2.

Increasing greatly the concentration of glutathione from the usual 2×10^{-4} M to ca. 05 M appeared to increase the amount of bis-substitution product at the expense of the thia-bridged derivative, as judged by the shape of the observed UV spectrum.

Diastereomeric Dibromides [*meso*- and *dl*-*syn*-(1-Bromoethyl,methyl)bimanes] (1). Thin-layer chromatography of the dibromides **1** revealed two species which migrated very close to one another. Both bright-yellow spots became fluorescent under irradiation, a response typical of bromobimanes. Separation of the isomers by HPLC was successful. The meso isomer has an NMR spectrum with a very symmetric quartet (4.749 ppm, J = 6.8 Hz) for the methine protons and singlets of equivalent chemical shift (1.617 ppm) for the two α-methyl protons. The *dl* isomer has an NMR spectrum in which there is a complicated band for the methine protons (4.659–4.904 ppm) and also singlets of different chemical shifts (1.578 and 1.630 ppm) for the α-methyl protons.

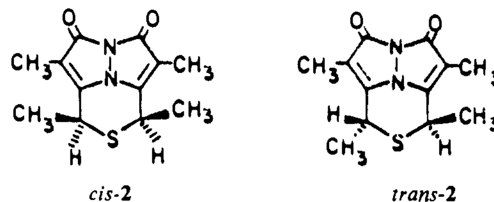
Interconversion of the dibromides occurred slowly in nonpolar solvents but quite rapidly in polar solvents, especially in those containing water, as shown by TLC or HPLC. One diastereomer in benzene did not change within 1 week but a solution in $CDCl_3$ equilibrated within 5 min (NMR spectra). The UV maxima of the isomers differed by at least 4 nm (399 vs. 403 nm). Interconversion and dehydrobromination occurred on heating; the melting points of the diastereomers were identical. Equal amounts of both isomers were produced by bromination of *syn*-(ethyl,-methyl)bimane (**8**).

Table I. Yields of Thia-Bridged Bimane [μ-(*S*)-*syn*-(Methylmethylene,methyl)bimane] (**2**) from the Reaction of Dibromobimane [*syn*-(1-Bromoethyl,methyl)bimane] (**1**) and Glutathione or Sodium Sulfide

dibromobimane[a]	reagent	reaction medium	total yield, %	% cis	% trans
meso + *dl*	GSH	CH_3CN in H_2O[b,c]	53	66	33
meso	GSH	THF in H_2O[b]	33	69	31
dl	GSH	THF in H_2O[b]	47	65	35
meso + *dl*	GSH	CH_2Cl_2[d]	0		
meso + *dl*	Na_2S	CH_3CN in H_2O	95	50	50
meso + *dl*	Na_2S	CH_2Cl_2[d]	70	25	75
meso (83%)[e]	Na_2S	CH_2Cl_2[d]		21.5	78.5
dl (77%)[e]	Na_2S	CH_2Cl_2[d]		28	72

[a] Diastereomers meso and *dl* of *syn*-(1-bromoethyl,methyl)bimane (**1**). [b] $NaHCO_3$ added. [c] In presence of 1 M KBr, cis/trans ratio 61:39. [d] Two-phase reaction; phase-transfer reagent added. [e] Approximate diastereomer purity in nonpolar solvent. Diastereomers expected to interconvert in polar solvents.

μ-(*S*)-*syn*-(Methylmethylene,methyl)bimane (2). As noted above, the reaction of *syn*-(1-bromoethyl,methyl)bimanes (**1**) with glutathione in aqueous solution gave CH_2Cl_2-extractable material in reasonably high yield. The major extractable products were



cis-**2** *cis*-μ-(*S*)-*syn*-(CH(CH_3),CH_3)B *trans*-**2** *trans*-μ-(*S*)-*syn*-(CH(CH_3),CH_3)B

a mixture of the two isomers (cis and trans) of the thia-bridged μ-(*S*)-*syn*-(methylmethylene,methyl)bimane (**2**) as shown by NMR, UV, mass spectra, and X-ray crystallography. Reaction of **1** with sodium sulfide gave the same two isomeric products.

The cis and trans isomers were separable only by HPLC. X-ray crystallography identified the lower melting fraction as the trans isomer. The NMR spectra showed a difference between the isomers: the methine proton appeared at lower field and the β-methyl protons at higher field for the trans isomer in comparison with the cis isomer. In the trans isomer, the α-methyl proton signals are not completely equivalent. In solution the isomers were stable and not interconvertible. The trans isomer absorbed at a longer wavelength (λ_{max} 353 nm) than the cis isomer (λ_{max} 349 nm). Material recovered from thin films produced by sublimation of the trans isomer in high vacuum onto a sapphire window[27] revealed 20% trans-to-cis isomerization, while the cis isomer remained unchanged under similar conditions. Pure trans isomer absorbs at a shorter wavelength than the cis isomer (λ_{max} 350 and 365 nm, respectively).

The yield of μ-(*S*)-*syn*-(methylmethylene,methyl)bimane (**2**) was between 33% and 95%; the cis/trans ratio in the isomer mixture varied from 2.2 to 0.3 in various reactions (Table I). Equal amounts of cis- and trans-bridged compounds were produced with aqueous sodium sulfide, while reaction with glutathione gave more cis isomer (cis/trans 1.5). The diastereomeric composition of the starting material had no effect on the isomer ratios. Reaction with sodium sulfide using a phase-transfer agent and a two-phase reaction mixture gave much less cis isomer (cis/trans ca 0.3). Reaction of **1** with glutathione could not be effected under these conditions.

X-ray Structure of the Trans Isomer of μ-(*S*)-*syn*-(Methylmethylene,methyl)bimane (*trans*-2). The crystal structure was

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Table II. Amino Acid Contents of Chromatographic Fractions[a]

fraction[b]	glu[c,d]	gly[c,e]	lan[c]	(a, b)[f]	cys[c]	ser[c]	ala[c]	sample,[g] mg
78.5-2	3.40	3.40	0.63	(48.9, 51.1)	1.05		0.091	1.156
78.5-3	3.38	3.49	0.69	(48.9, 51.1)	0.97	0.038	0.078	1.144
77.5-1	3.62	3.67	1.05	(47.8, 52.2)	0.67	0.023	0.057	1.19
77-1	2.88	3.40	0.63	(48.9, 51.1)	0.32	0.019	0.0926	0.967
77-2	3.77	3.95	1.69	(47.5, 52.5)	n.m.	0.015	0.066	1.21
77-3	2.62	2.74	1.23	(47.1, 52.9)	0.18	0.029	0.020	0.888
77-4	3.42	3.60	1.67	(46.9, 53.1)	0.18	0.020	0.077	1.17
76-1	3.15	3.19	1.53	(46.3, 53.7)	0.11	0.024	0.091	1.06
76-2	2.68	2.84	1.32	(46.5, 53.5)	0.15	0.047	0.266	0.946
76-3	3.42	3.69	1.68	(46.5, 53.5)	0.22	0.063	0.149	1.20
76-4	2.39	2.53	1.12	(46.4, 53.6)	0.11	0.078	0.164	0.822
76-5	3.42	3.75	1.65	(46.6, 53.4)	0.13	0.036	0.048	1.17
75.5-1	3.17	3.56	1.45	(46.0, 54.0)	0.16	0.033	0.041	1.081
75.5-2	2.98	3.21	1.17	(44.8, 55.2)	n.m.	0.053	0.072	0.926
75.5-3	0.87	1.08	0.19	(47.0, 53.0)	0.13	0.017	0.018	0.284
75	1.68	2.06	0.52	(45.6, 54.1)	0.21	0.031	0.013	0.562

[a] The fractions were obtained by chromatography on cellulose as described in the text of the water-soluble products of the rxn of *syn*-(1-bromoethyl,methyl)B and glutathione. [b] The fractions are labeled according to the percent of 2-propanol in the 2-propanol/water solvent used to eluate the given fraction, followed by a number designating the subfraction from which the sample was taken. [c] The amino acids found are as follows: glu, γ-glutamic; gly, glycine; lan, lanthionine; cys, cystine; ser, serine; ala, alanine. The areas obtained for light absorption by ninhydrin reaction product were converted to millimoles by measured calibration factors. [d] Average glu found was 96.7% (σ_{n-1} 4.7) of 1/2 of the sum of lan + cys. [e] Average gly found was 101.4% (σ_{n-1} 4.7) of 1/2 of the sum of lan + cys. [f] Percents of lan isomers a and b. [g] Sum of the weights of all amino acids found in a sample.

solved by a combination of direct methods and Fourier techniques (MULTAN78 and MULTAN80). The refinements were carried out by full-matrix least squares, including the positional and anisotropic thermal parameters of all the non-hydrogen atoms. All hydrogens were located in electron density difference maps at an intermediate stage of the refinement and were assigned isotropic temperature factors. Their atomic parameters were not refined, except for a partial adjustment of the coordinates with low-order data below $\sin \theta/\lambda = 0.50$ Å^{-1}. The least-squares calculations were based on the experimental weights [$w = 1/\sigma^2(F_o)$], the quantity minimized being $w/(\Delta F)^2$.

trans-μ-(*S*)-*syn*-(Methylmethylene,methyl)bimane (*trans*-**2**) crystallizes with two molecules of the bridged dioxabimane in the asymmetric unit. Their configurations are the same (with one axial and one equatorial methyl group in the bridge), but their conformations are different. The most pronounced variation is in the fold of each one of the dioxabimanes about the central N–N bonds, with 144.5° in one molecule and 152.1° in the other. The folding in the former is comparable to that observed in μ-(*S*)-(methylene,methyl)-9,10-dioxabimane (141.8°).[28] The distribution of bond lengths around the N atoms is clearly associated with changes in the overall conformation, in excellent agreement with previous results showing consistent correlations between the conformation adopted by the dioxabimane moiety, hybridization on nitrogen, and the detailed geometry of the molecule.[29] The crystal packing is stabilized by van der Waals forces including several C–H···O interactions. The molecular structure is shown in Figure 1.

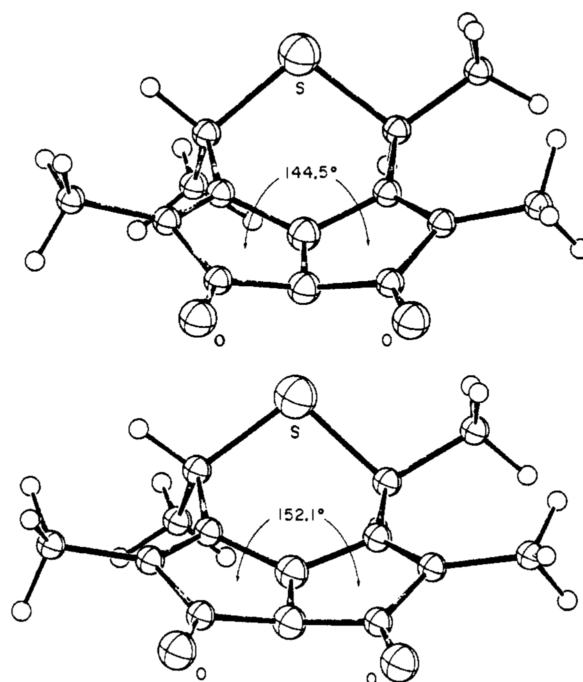

Figure 1. Crystal structure of *trans*-μ-(*S*)-*syn*-(methylmethylene,methyl)bimane (*trans*-**2**) showing the two slightly different forms present in the asymmetric unit. The bond lengths and bond angles for each of the two forms are listed in Table V.

Separation and Analysis of Water-Soluble Products. The water-soluble products formed in the reaction of **1** with glutathione are separated by partition chromatography on cellulose using eluants varying from 100% to 65% 2-propanol–water. Complete purification on plates is limited by the loss of product due to incomplete extraction from the cellulose. The fluorescent compounds are visualized under a UV lamp and α-amino acids and peptides by reaction with ninhydrin. NMR analysis indicated that some fractions contain partially hydrolyzed peptides, which show lower glycine or γ-glutamyl proton intensities than expected. *syn*-(1-Glutathionylethyl,methyl)(ethyl,methyl)bimane (**9**) was in evidence (1:1 ratio of bimane and glutathione NMR peaks) but not in sufficient quantity for isolation.

The yield of the bis(glutathionyl) derivative, *syn*-(1-glutathionylethyl,methyl)bimane (**10**), was very low (ca. 2%) and separated by chromatography from glutathione disulfide with difficulty. The NMR spectrum showed a 1:2 ratio of bimane and glutathionyl protons and indicated that two diastereomers, in a ratio of 3:2, were present.

Glutathione Sulfide (3). Most of the water-soluble products are eluted with solvents containing between 79% and 75% 2-propanol. The first compound eluted is glutathione disulfide. As the polarity of the eluant increases, succeeding ninhydrin-positive, nonfluorescent fractions exhibit NMR spectra which change gradually from that of glutathione disulfide to that of glutathione sulfide (**3**). The change is most notable in the region of the cysteine proton signals (Figure 2).

Two other analytical methods, (1) amino acid analysis and (2) NADPH disappearance via GSSG reductase, were used on a representative number of column fractions.

(1) Amino Acid Analysis (AA). The amino acid composition of individual fractions was evaluated by using an experimentally determined calibration factor for each amino acid to convert the

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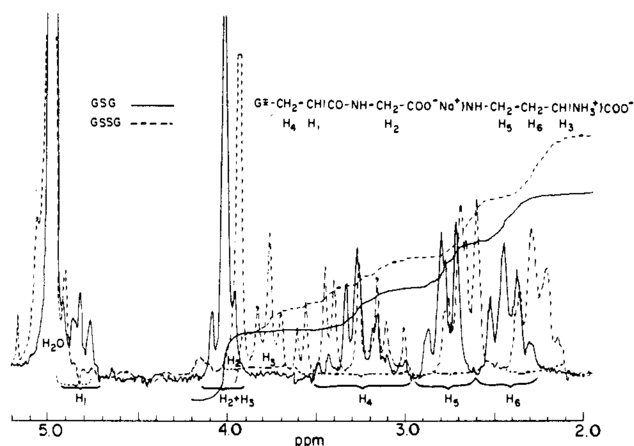


Figure 2. NMR spectra of glutathione sulfide (GSG, 3) and glutathione disulfide (GSSG) in D_2O.

integrated areas into millimoles (Table II). Beginning with the eluant, 79% 2-propanol, successive fractions contained less and less cystine, derived from GSSG, and more and more of the two diastereomers of lanthionine ("cysScys"), derived from GSG. The diastereomer ratio in lanthionine from an independent source is 40.7:59.3 (isomer 1:isomer 2, in order of isomer appearance in amino acid analysis). The ratio of the isomers in the lanthionine derived from glutathione sulfide (3) varied with fraction from 49:51 to 45:55. The amounts of γ-glutamic and glycine remained relatively constant and equivalent in each fraction. On the average, 1.93 glutamic and 2.04 glycine were found for 1.00 (lanthionine + cystine). For each 1.00 glutamic acid or glycine, serine (0.005–0.01) and alanine (0.02–0.03) were also detected, with as much as 0.025 serine and 0.09 alanine in the fraction eluted by 76% 2-propanol. The total weight of each sample analyzed was calculated as the sum of the weights of the component amino acids.

(2) Kinetics of NADPH Disappearance via GSSG Reductase (EK). The kinetics of NADPH loss catalyzed by GSSG reductase reflects the glutathione disulfide content of a sample. No sample caused the oxidation of NADPH in the absence of GSSG reductase. The amount of NADPH oxidized was consistent with the amount of glutathione found; that is, two thiol groups appeared for every NADPH which was consumed. No thiol was found in samples for which there was no NADPH consumption and the rate of NADPH lost was related to the thiol content. Glutathione sulfide did not promote the loss of NADPH and did not inhibit the reduction of GSSG by GSSG reductase. The GSSG content of successive fractions decreased, beginning with the 79% 2-propanol eluate, with results that correlate well with the glutathione disulfide contents based on amino acid analysis in Table III. However, high polarity and low polarity eluants gave material with more cystine by AA analysis than that detectable by GSSG reductase.

The enzymatic analysis probably gives a better account of the amount of glutathione disulfide, since amino acid analysis (and NMR) detects cystine derived from partially hydrolyzed glutathione disulfide (loss of gly or glu). The yield of glutathione sulfide is derived from the amino acid analysis. It was assumed that there is no appreciable hydrolysis of glutathione sulfide, i.e., that each lanthionine represents one glutathione sulfide. The weight of each column fraction is known. From amino acid analyses, the total weight of each sample could be estimated and the amount of glutathione sulfide and glutathione disulfide in each fraction calculated. In the conversion of millimole contents derived from the analyses to weights, it was assumed that glutathione disulfide and glutathione sulfide are present as the disodium salts. (The samples are derived from reactions carried out at pH 7.5.) The glutathione disulfide and glutathione sulfide contents estimated in this way are reported in Table IV.

The glutathione sulfide and glutathione disulfide contents of fractions which were not analyzed were estimated by interpolation. The overall analysis shows that the yield of glutathione sulfide

Table III. Comparison of Glutathione Disulfide Content Measured Enzymatically or by Amino Acid Analysis

fraction[b]	mg of GSSG by EK[c,d]	mg of GSSG by AA[c,e]
90–3	<0.033	0.015
88.5	<0.03	0.041
87	<0.05	0.140
86.5	0	0.111
78.5–2	0.587	0.690
78.5–3	0.586	0.636
77.5–1	0.409	0.439
77–1	0.206	0.209
77–2	0.135	n.m.
77–3	0.103	0.119
77–4	0.075	0.120
76–1	0.058	0.070
76–2	0.046	0.099
76–3	0.047	0.146
76–4	0.032	0.069
76–5	0.029	0.086
75.5–1	0.024	0.103
75.5–2	0.067	n.m.
75.5–3	0.033	0.088
75	0.033	0.134

[a,b] As in Table II. [c] Glutathione disulfide content (in milligrams). [d] Based on the quantity of glutathione found after reduction of glutathione disulfide with NADPH catalyzed by GSSG reductase. [e] Based on the assumption that all cystine measured is derived from glutathione disulfide in the sample, i.e., that 1 cys = 1 GSSG.

Table IV. Yields of Glutathione Sulfide (GSG) and Glutathione Disulfide (GSSG)[a]

fraction[b]	wt, mg	GSG(Na_2),[c] %	GSSG(Na_2),[d] %
78.5–2	43.48	34.5	56.1
78.5–3	44.24	37.7	55.1
77.5–1	33.04	55.0	36.9
77–1	23.64	74.9	22.1
77–2	31.85	87.7	12.0
77.3	27.17	86.5	12.5
77–4	25.87	89.2	6.8
76–1	31.09	90.7	5.5
76–2	25.76	87.3	4.9
76–3	14.46	87.5	4.2
76–4	18.91	85.7	4.2
76–5	8.42	88.6	2.6
75.5–1	20.82	84.2	2.4
75.5–2	17.12	79.1	7.8
75.5–3	9.02	11	3.2
75	10.87	32	2.8

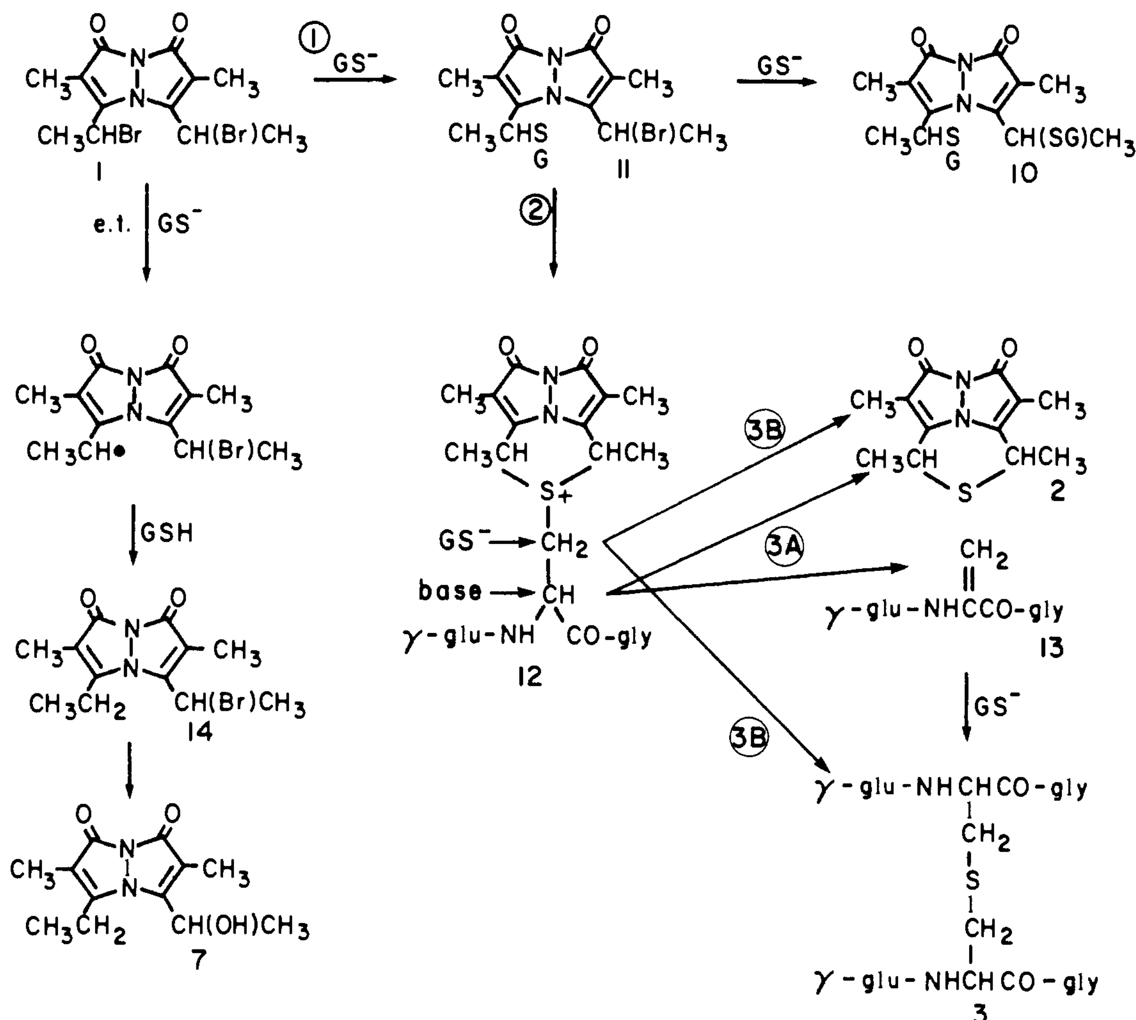
[a,b] As in Table II. [c] Calculated from amino acid analyses, assuming that 1 langhionine (lan) = 1 glutathione sulfide (GSG). [d] Based on the quantity of glutathione found after reduction of glutathione disulfide with NADPH catalyzed by GSSG reductase.

(3) (0.665 mmol, 56%) is approximately equal to the yield of the ring compound, μ-(*S*)-*syn*-(methylmethylene,methyl)bimane (2) (0.63 mmol, 53%). The yield of the bis(glutathionyl)bimane derivative **10** (2%) is negligible. About 0.59 mmol glutathione disulfide was obtained. In summary, 67% of the initial bimane and 95% of initial glutathione are accounted for.

Discussion

The formation of "glutathione sulfide" (GSG) (3) [equivalent to a "sulfur extraction" from two thiols] in the reaction of *syn*-(1-bromoethyl,methyl)bimane (1) occurs in steps: (1) displacement of bromide ion by the thiolate anion, GS^-, from the dibromide **1** to yield a monoalkylthio-monobromo derivative, GSBr (**11**); (2) intramolecular displacement of the bromine in **11** by the neighboring alkylthio group to produce a reactive sulfonium ion, GSB^+ (**12**); (3) disappearance of the intermediate sulfonium ion by either (a) elimination or (b) nucleophilic displacement. (3a) Base causes 1,2-elimination of H^+ and the thia-bridged bimane (2) from the intermediate sulfonium ion (**12**), yielding "dehydroglutathione" [γ-glu-dehydroala-gly] (**13**) to which GSH then adds to give either of the two diastereomers of GSG (3). (3b) Nucleophilic displacement by GS^- at the cysteinyl

Scheme I. Interconversion of *dl* and Meso Isomers of *syn*-(1-Bromoethyl,methyl)bimane (**1**) by the Grob-Winstein Mechanism



[a] Steps on the primary reaction route are marked with circled numbers. Glutathione anion (GS^-) reacts with **1** either by nucleophilic displacement (step 1) to give GSBBr (**11**) or by electron transfer to yield a radical which extracts a hydrogen from GSH to yield a monobromobimane (**14**). The latter hydrolyzes to form the alcohol **7**. The intermediate **11** can react with a second GS^- to produce the bis(glutathionyl) derivative (**10**) or by intramolecular displacement of bromide (step 2) to yield the sulfonium ion, GSB^+ (**12**). The GSB^+ (**12**) can react with base by elimination to give the thia-bridged bimane (**2**) and dehydroglutathione (**13**) (step 3A) or by displacement at the cysteinyl methylene to yield **2** and glutathione sulfide, GSG (**3**) (step 3B). The dehydroglutathione reacts with GS^- to form **3**.

methylene of the intermediate sulfonium ion (**12**) yields GSG (**3**) and the thia-bridged bimane (**2**). The reactions leading to the formation of the thia-bridged bimane and glutathione sulfide are summarized in Scheme I.

Thia-Bridged Bimane (2) Diastereomers. The first step is a nucleophilic displacement reaction like those which lead to bimane-labeled thiols, including glutathione, in biological systems.[6-15] The rate constant for the reaction of **1** with glutathione is no more than twice as great as that for the monobromo compound, *syn*-(1-bromoethyl,methyl)(ethyl,methyl)bimane (**14**), as reported in the preceding paper.[26] These rate constants are much smaller (factor of 100–200) than those for the reaction of the monobromobimane **15** and the corresponding bis-(bromo) derivative, *syn*-(1-bromomethyl,methyl)bimane (**4**, X = Br), with glutathione.

The second step, a classical neighboring group reaction, must be considered in light of the difference between the reactivity of a thioether and a thiolate ion, which is ca. 10^5.[25] The concentration of the external thiolate, GS^-, is ca. 10^{-5} M (for 10^{-3} M GSH at pH 7.3) and differs from that of the intramolecular alkylthio group (>10 M) by a factor of at least 10^6. All other things being equal, the internal alkylthio group will react faster than the external thiolate. Raising the concentration of glutathione by 100 did lead to more bis(glutathionyl) product. However, ring closure in the present case has some geometric and entropic constraints which will disfavor the internal nucleophile in comparison with the external anion. In particular, the direction of approach of the external nucleophile will engage somewhat more overlap with the conjugated system than the direction open to the internal nucleophile.[30]

In the case of *syn*-(1-bromomethyl,methyl)bimane (**4**), bis substitution predominates without sign of the intervention of an intermediate sulfonium ion. (Spectra taken during kinetic measurements[26] show no sign of unusual intermediates; no thia-bridged bimane can be detected nor is there bimane ring opening.) The rate constant for reaction of the 1-bromoethyl derivative **1** with external nucleophile is at least 100 times smaller than that for the bromomethyl derivative. Since the alkylthio group nucleophilicity is lowered somewhat by the geometry of the approach but less affected than an external nucleophile by steric effects from the adjacent methyl group, intramolecular reaction will be favored and ring closure should predominate.

A related neighboring group reaction is found in the reaction of bis(1,2-bromomethyl)benzene with 2-hydroxyethanethiol to form the sulfonium ion, 2-(2-hydroxyethyl)-1,3-dihydroisothianaphthenium ion.[31] The points made here should be useful for the design of other dihalides suitable for the "sulfur extraction" reaction. Comments on intramolecular sulfur participation have been made by Eliel.[32]

(30) Eliel, E.; Knox, D. E. *J. Am. Chem. Soc.* **1985**, *107*, 2946–2952.
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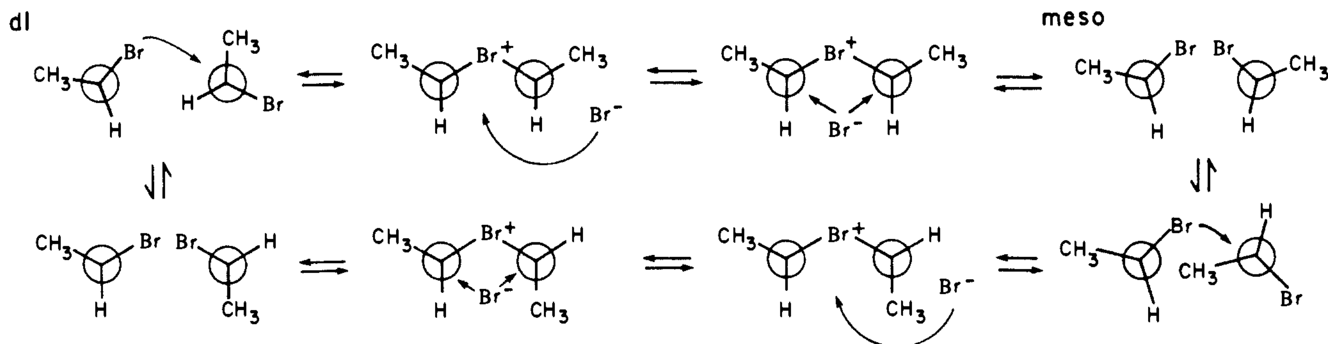
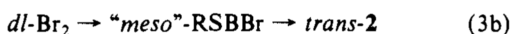
Scheme II. Interconversion of *dl* and Meso Isomers of *syn*-(1-Bromoethyl,methyl)bimane (**1**) by the Grob–Winstein Mechanism



The proportion of *cis*- and *trans*-thia-bridged bimane (**2**) formed from the dibromides and glutathione (ca. 2:1) is different from that derived from sulfide ion (1:1 in aqueous buffer, 1:3 with a phase-transfer agent). The starting dibromide is a mixture (1:1) since interconversion between *meso*- and *dl*-dibromides must be at least as rapid in water as it is in chloroform or acetonitrile. In the latter solvents, equilibration is apparently complete in less than 5 min. A mechanism for interconversion, analogous to that proposed by Grob and Winstein[33] for the interconversion of diastereomeric dibromocholestanes, is shown in Scheme II.

The significant differences in the cis/trans ratios obtained with different reagents can be rationalized in terms of the following points after consideration of the transition-state geometries. 1. The *meso*-dibromide **1** reacts with GS^- to yield *dl*-RSBBr about twice as fast as *dl*-dibromide **1** reacts to yield *meso*-RSBBr. However, each diastereomer reacts with the smaller SH^- ion at about the same rate. Electrostatic repulsion between the attacking GS^- and the carbon–bromine should be somewhat greater in the transition state from the *dl*-**1** than that from *meso*-**1**. In the phase-transfer reaction with hydrosulfide, SH^-, the cation favors reaction with *dl*-**1** over that with *meso*-**1** as a result of electrostatic interaction. 2. Bromide ion as an external nucleophile competes very poorly with the thioether as an intramolecular nucleophile. This is shown by the slightly changed cis/trans ratio (61:39 vs. 66:34) for a reaction carried out in the presence of 1 M KBr. Bromide ion should not affect the products derived from SH^- at all. The reactions are summarized in eq 3a and 3b.

$$meso\text{-}Br_2 \rightarrow \text{“}dl\text{”-RSBBr} \rightarrow cis\text{-}\mathbf{2} \quad (3a)$$



$$dl\text{-}Br_2 \rightarrow \text{“}meso\text{”-RSBBr} \rightarrow trans\text{-}\mathbf{2} \quad (3b)$$

The sulfonium ion is not likely to be subject to diastereomeric equilibration by bromide ion because the reactivity of bromide ion will not be high (low orbital overlap in the transition state, steric hindrance, and somewhat lower leaving ability for sulfonium vs. bromide ion). However, the proton on the peptide carbon β to the S^+ may be labile. (See following discussion.)

Glutathione Sulfide (3) Diastereomers. Whereas the steric conformation of the thia-bridged bimane is established in the initial displacement and ring closure steps, the implications of the finding that GSG is a 1:1 mixture of the two diastereomers (based on the *meso*- and *dl*-lanthionine contents in amino acid analysis) must be considered with respect to the reactions of the sulfonium ion (**12**).

Elimination. The formation of thia-bridged bimane by elimination, yielding dehydroglutathione followed by addition of GS^-, should give both diastereomers of GSG. Elimination from 2-(carboxyethyl)dimethylsulfonium ion yields dimethyl sulfide.[34] The reactivity of α-(acylamino)-α,β-unsaturated amino acid amides toward thiols is well-known.[35,36] Addition of glutathione to dehydroglutathione, with L-cysteine as the central amino acid, should yield equal amounts of two forms of glutathione sulfide (**3**) (LL-S-LL and/or LL-S-LD).

Addition of water to dehydroglutathione would yield the serine analogue of glutathione, γ-glu-ser-gly (GOH), a compound which has been recently synthesized by standard techniques.[37] In fact, small yields of serine are detected in some of the GSG fractions.

Asymmetric induction has been reported to occur in the formation of adducts from dehydropeptides.[35] Asymmetric induction by the asymmetric center on L-glu in the γ-glu-dehydroala-gly in the proposed reaction with glutathione (L-glu-L-cys-gly) cannot be significant, since roughly equal quantities of *dl*- and *meso*-lanthionine are found. The slightly enhanced content of one of the lanthionine isomers in some fractions (see description of amino acid analysis in Results section) might reflect (a) a small degree of asymmetric induction, (b) separation of the **3** diastereomers during partition chromatography, or (c) a small degree of preferential loss of one of the lanthionine isomers during storage before analysis.

Nucleophilic Displacement. The second plausible mechanism (step 3b) for the formation of GSG is a nucleophilic displacement by GS^- on a CH_2 of the sulfonium ion (**12**). L-Lanthionine has been synthesized by desulfurization of the disulfide, *N,N'*-bis(trifluoroacetyl)-L-cystine dimethyl ester by tris(diethylamino)phosphine, followed by treatment with base.[38] Displacement by a thiolate on the methylene next to a thiophosphonium group is thought to be the mechanism of the reaction by which L-lanthionine is synthesized.[39] The four stereoisomers of β-methyllanthionine have been synthesized by a displacement reaction.[40] The displacement mechanism requires an explanation of why both diastereomers of GSG were obtained and why the cysteinyl carbon was attacked in preference to the carbons α to the bimane ring.

The α-proton on the cys moiety would be rendered considerably more acidic by the positive charge on the sulfonium ion β to this position, a circumstance which would lead to racemization in one portion of the GSG. The position for attack by GS^- on the sulfonium ion is understood on the basis of steric and orbital overlap factors. The crystal structure of **2** (Figure 1) serves as a useful model for the sulfonium ion. Approach of a nucleophile to a carbon adjacent to the ring from the side opposite the leaving group is hindered by the methyl on that carbon and the methyl at the R_1 position on the bimane ring. The importance of both the leaving sulfur and the entering nucleophilic atom in determining the structure of the transition state has been shown by isotope effect studies in sulfonium ion displacements.[41] In addition, the π-orbitals of the bimane ring would not stabilize the

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transition state for displacement because the orbital connecting the entering and leaving groups is far from parallel to those of the bimane ring, an important factor in displacements on cyclic sulfonium ions. Dibenzylethylsulfonium cation reacts with thiocyanate anion to give exclusively benzyl thiocyanate, whereas cyclic benzylsulfonium ions are attacked to a smaller extent at the benzylic position.[30]

The reaction of GSH with the unsymmetrical dibromo compound, *syn*-(bromomethyl,methyl)(1-bromoethyl,methyl)bimane (**17**), gives mostly the thia-bridged bimane.[26] Initial attack on the bromomethyl group is followed by formation of the sulfonium through a neighboring group reaction. Although attack at the CH_2 next to the bimane ring would be less hindered sterically, the orbital overlap factor is unfavorable, and reaction at the cysteinyl CH_2 is still preferred. It may be inferred that formation of bis(glutathionyl) derivatives occurs by way of two displacement reactions on the dibromide and not via the sulfonium ion, one of the possibilities indicated in eq 5, ref 26.

The formation of glutathione sulfide (**3**) from glutathione and the dibromide **1** is an example of a remarkably mild method for the generation of a sulfide from two thiol molecules. The mechanism is quite straightforward. Neighboring group participation of the alkylthio group[42] in the first-formed GSBBr (**11**) intermediate leads to a cyclic sulfonium ion. The thia-bridged bimane is either eliminated from the sulfonium ion (to form "dehydroglutathione") or displaced by GS^-. The synthesis of glutathione sulfide (even the mixture of isomers: LL-S-LL or LL-S-LD) is simple and potentially useful, but the generality of the synthesis remains to be tested. Sulfides of various types are of great importance in biological systems.

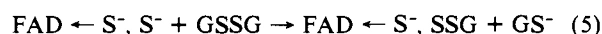
Other methods for generating dehydroalanine[35,36,43-45] utilize concentrated bases, methods which may be less suitable for stereochemically defined peptides. For example, exposure of *S*-(2,4-dinitrophenyl)glutathione[46] to 0.5 M $NaOD/D_2O-(CD_3)_2SO$ leads to a solution which has ^1H NMR signals for the vinyl hydrogens of a dehydroalanine group, presumably within dehydroglutathione.[47] Dehydroglutathione forms by elimination of glutathione persulfide (GSS^-) on treatment of the glutathione disulfide, with sodium hydroxide, pH 13, exhibiting both ^1H NMR and ^{13}C NMR signals for the dehydroalanine.[48] Glutathione persulfide decomposes on exposure to cyanide ion, apparently forming thiocyanate.[49] Treatment of wool with sodium cyanide (0.3 M) for 8 h at 20 °C leads to ca. 12% conversion of the cystine to lanthionine, probably via elimination of RSS^-, decomposition of RSS^- by CN^-, and addition of RS^- to a dehydroalanine residue.[50] Whether the mechanism involves elimination or substitution, the present procedure is gentle and offers the possibility of producing sulfides in neutral aqueous solution. Formation of dehydro derivatives via concentrated base treatments of cysteine or cystine derivatives could not be used for biological material and would not be generally useful for many peptide derivatives (cf. the useful discussion on the presence of dehydroalanine in histidine ammonialyase[51]).

There is a possibility that the dibromo compound might participate in a reaction which replaces two thiol centers in polypeptides or proteins with a sulfide link. For such a conversion to be successful, the most reactive thiol group reacts according to steps 1 and 2 of the reaction sequence for the formation of glutathione sulfide, to give the sulfonium ion. The latter decomposes as in steps 3a or 3b, reacting with the most reactive (and/or accessible) nucleophiles (cys or lys). It would be most interesting to compare such altered proteins with those obtained by the equilibrium transfer alkyation method of Mitra and Lawton.[52] One might also imagine the transformation of reducible disulfide links via dithiols into nonreducible sulfide bonds. Reducible disulfide bonds are critical for the in vivo activity of diphtheria toxin;[53] conversion of the disulfide bond to a sulfide might create a nontoxic but antigenic protein.

It should be noted that bis(3,4-dibromomethyl)benzoic acid was said to react with cysteine peptides to yield a sulfonium salt via intermolecular and then intramolecular displacement of bromide ion.[54] However, no full report of the results has appeared, and subsequent articles report using the dibromo compound for synthesis of thiophenes[55] or for the inhibition of fumarase,[56] histidine being the amino acid moiety attacked at neutral pH.

Glutathione Disulfide (GSSG) Reductase and Glutathione Sulfide (3). The structure of human glutathione disulfide (GSSG) reductase has been refined to 2 Å.[57] The flavin adenine dinucleotide (FAD) binding site has been elucidated in great detail by a combination of amino acid sequence determinations[58] and structural analysis.[59,60] However, the binding site for glutathione disulfide has not yet been identified. It is of considerable interest that glutathione sulfide (GSG) is not an inhibitor of GSSG reductase. A mechanism for the reduction of glutathione disulfide by NADPH with yeast GSSG reductase has been developed.[61] A simplified version of the mechanism is given in eq 4–6. (The

$$\text{NADPH} + \text{FADS-S} \rightarrow \text{NADP}^+ + \text{FAD} \leftarrow \text{S}^-, \text{S}^- \quad (4)$$



$$\text{FAD} \leftarrow \text{S}^-, \text{S}^- + \text{GSSG} \rightarrow \text{FAD} \leftarrow \text{S}^-, \text{SSG} + \text{GS}^- \quad (5)$$

$$\text{FAD} \leftarrow \text{S}^-, \text{SSG} \rightarrow \text{FADS-S} + \text{GS}^- \quad (6)$$

arrow between the thiolate and the FAD represents a charge-transfer complex, an idea proposed by Kosower in 1966[62] and now confirmed by crystal structure.) It is clear that **3** should not undergo the reduction reaction, but one or both of the isomers of **3** (LL-S-LL or LL-S-LD) might have (a) inhibited the enzyme or (b) reacted to form glutathione and dehydroglutathione. The high specificity of the reductase requires a specific molecular environment around the disulfide bond. The glutathione derivative, *S*-(2,4-dinitrophenyl)glutathione, is an inhibitor of human GSSG reductase, but X-ray crystallographic comparisons indicate that the conjugate does not bind in the same way as glutathione disulfide.[63] A potential inhibitor for GSSG reductase is homoglutathione sulfide (L-glu-L-homocys-*S*-(G)-gly, G = L-glu-"L-cys"-gly), which might be made by "sulfur extraction" from a mixture of homoglutathione and glutathione, followed by a difficult fractionation.

Thermal Stability of Thia-Bridged Bimane (2). An interesting new reaction of bimanes is evident in the partial conversion of the trans-thia-bridged bimane (*trans*-**2**) to the cis-isomer during

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Table V. Bond Distances (Å) and Bond Angles (deg) of *trans*-μ-(*S*)-*syn*-(Methylmethylene,methyl)bimane (*trans*-2) [Columns a and b Refer to the Two Crystallographically Independent Molecules of the Asymmetric Unit]

atoms	distance, Å a	distance, Å b	atoms	distance, Å a	distance, Å b
N1–N5	1.390 (3)	1.394 (3)	C6–C7	1.354 (4)	1.350 (4)
N1–C2	1.429 (4)	1.418 (4)	C6–C15	1.502 (4)	1.486 (4)
N1–C8	1.422 (4)	1.418 (4)	C7–C8	1.467 (4)	1.462 (4)
C2–C3	1.463 (4)	1.463 (4)	C7–C12	1.490 (4)	1.496 (4)
C2–O9	1.208 (4)	1.210 (4)	C8–O10	1.214 (4)	1.212 (4)
C3–C4	1.350 (4)	1.357 (4)	C13–C14	1.516 (4)	1.510 (4)
C3–C11	1.483 (4)	1.499 (4)	C13–S17	1.826 (4)	1.843 (3)
C4–N5	1.383 (3)	1.398 (3)	C15–C16	1.518 (4)	1.533 (4)
C4–C13	1.497 (4)	1.497 (4)	C15–S17	1.838 (4)	1.831 (3)
N5–C6	1.384 (3)	1.397 (4)			

atoms	angles, deg a	angles, deg b	atoms	angles, deg a	angles, deg b
C2–N1–C8	134.8 (3)	129.1 (3)	N5–C6–C7	109.1 (3)	109.9 (3)
C2–N1–N5	108.2 (3)	108.4 (3)	C7–C6–C15	136.6 (3)	133.1 (3)
N5–N1–C8	107.4 (3)	107.8 (3)	C6–C7–C12	131.6 (3)	129.5 (3)
N1–C2–O9	124.6 (3)	124.4 (3)	C6–C7–C8	108.1 (3)	108.2 (3)
N1–C2–C3	104.7 (3)	105.4 (3)	C8–C7–C12	120.2 (3)	122.3 (3)
C3–C2–O9	130.7 (3)	130.2 (4)	N1–C8–C7	105.5 (3)	105.4 (3)
C2–C3–C11	123.0 (3)	119.9 (3)	C7–C8–O10	130.7 (3)	131.0 (3)
C2–C3–C4	108.2 (3)	108.2 (3)	N1–C8–O10	123.8 (3)	123.6 (3)
C4–C3–C11	128.8 (3)	131.8 (3)	C4–C13–S17	108.8 (2)	109.7 (2)
C3–C4–C13	132.2 (3)	135.3 (3)	C4–C13–C14	112.3 (3)	114.4 (3)
C3–C4–N5	109.9 (3)	109.3 (3)	C14–C13–S17	113.4 (2)	106.4 (2)
N5–C4–C13	117.8 (3)	115.3 (3)	C6–C15–S17	108.5 (2)	107.8 (2)
N1–N5–C4	108.6 (2)	108.5 (3)	C6–C15–C16	115.5 (3)	112.1 (3)
C4–N5–C6	129.8 (3)	126.8 (3)	C16–C15–S17	106.6 (2)	113.7 (2)
N1–N5–C6	109.8 (3)	108.6 (3)	C13–C17–C15	100.8 (2)	101.8 (2)
N5–C6–C15	114.2 (3)	116.9 (3)			

distillation of the compound onto a sapphire window for spectroscopic measurements. The cis isomer under the same conditions does not isomerize to the trans isomer, implying that the cis isomer is somewhat more stable. A reasonable pathway for the isomerization involves C–S bond cleavage, rotation of the carbon radical, and re-formation of the C–S bond.

X-ray Structure of *trans*-2. The bond distances and bond angles in the structure of *trans*-2 are given in Table V. There are two different molecules in the unit cell. An interesting difference between the two is the carbon–sulfur distance, which is appreciably longer in the molecule with the smaller dihedral angle.

Experimental Section

General. Instruments used are as follows. ^{1}H NMR spectra: Chemical shifts are given in δ values (references: HDO, 5.000 (D_2O solvent)[64] or $(CH_3)_4Si$, 0.00 (organic solvents)), Bruker WH-90 and AM-360 spectrometers. Ultraviolet and visible spectra: Cary Model 17 spectrophotometer. Fluorescence spectra: Hitachi-Perkin-Elmer MPF-4 fluorescence spectrometer. Mass spectra: Du Pont 21-491B mass spectrometer. IR spectra: Perkin-Elmer Model 177. HPLC: Waters Assoc. Model 6. X-ray structural determination: measured at room temperature on an Enraf-Nonius CAD-4 diffractometer equipped with a graphite monochromator, employing Mo Kα radiation (λ = 0.7107 Å) and an ω–2θ scan technique. The scan rate varied according to the detected intensity between 1.0 and 4.0 deg min^{-1}. Intensity data were not corrected for absorption or extinction effects.

***trans*-μ-(*S*)-*syn*-(Methylmethylene,methyl)bimane (*trans*-2).** Crystal data: $C_{12}H_{14}N_2O_2S$, M_r = 250.3, triclinic, a = 8.814 (2) Å, b = 8.592 (4) Å, c = 16.2272 (8) Å, α = 89.33 (44)°, β = 102.35 (3)°, γ = 93.58 (3)°, V = 1198.0 $Å^3$, Z = 4, d_c = 1.388 g cm^{-3}, $F(000)$ = 5280, μ(Mo Kα) = 2.49 cm^{-1}, space group $P\bar{1}$. Data collection: 2θ limits 0–50°, scan range 0.80 + 0.30 tan θ, number of unique observations above 0, 3785. Refinement: 307 parameters refined using 2992 reflections with $I > 3\sigma_I$. Final R = 0.040, R_w = 0.055, goodness of fit (GOF) = 1.75 e.

Chromatography. Water-soluble material was chromatographed on cellulose (microcrystalline, Merck) columns (8 × 1 cm). The eluant was a mixture of 2-propanol and water and was varied as follows: (% 2-PrOH) 95, 90–86 (1% steps), 85–75 (0.5% steps), and 65. Solvents were made up by volume, and the composition was adjusted to give constant refractive index increments (LOC Refractomonitor). Nitrogen pressure (1.5 bar) was used to maintain the flow at 15 mL/h. Columns were run over a 5–6-day period without interruption, using an LKB automatic fraction collector (10–15-mL fractions). All connections were made with Teflon except for the Viton section at the flow stopper. Subsequent treatment of the fractions was carried out with a minimum of heating to avoid hydrolysis of peptides. 2-Propanol was removed under vacuum at room temperature, and water was removed by lyophilization (Virtis Freezemobile).

Compounds soluble in organic solvents were chromatographed on silica gel 60 or 60H (Merck). TLC separations were done with silica gel on plastic sheets (Merck or Kodak) or glass plates (Merck, 250 μm, preparative scale), using ethyl acetate as the developer. Water-soluble compounds were chromatographed on cellulose (plastic sheets, 20 μm, or glass plates, 100 μm), using 68–72% 2-propanol. Compounds were visualized either with UV light (fluorescence) or iodine (silica plates only) or through reaction with ninhydrin (α-amino acid groups react, solution of 0.3 g of ninhydrin/100 mL of 1-butanol + 3 mL acetic acid sprayed on chromatogram which is then heated with hot air).[65] HPLC separations were carried out by using SI 100 or SI 60 columns.

Solvents and Materials. Dichloromethane (Anal), acetonitrile, and 2-propanol (Merck) were used without further purification. Ethyl acetate (Anal, Merck) and cyclohexane (Merck) were distilled. Deionized, distilled water was used. Glutathione (GSH) and glutathione disulfide (GSSG) (Sigma) were converted to sodium salts with $NaHCO_3$ in water (2.2 equiv/mol of GSH, 4.4 equiv/mol of GSSG) and isolated by lyophilization. Lanthionine (Sigma) was used.

Sodium γ-L-Glutamyl-L-cysteinylglycinate (GSH, Na Salt). ^{1}H NMR (D_2O): 2.203–2.460 (m, 2 H, CH_2 glu), 2.675–2.851 (m, 2 H, CH_2 glu), 3.073–3.161 (d, 2 H, J = 6.7 Hz, CH_2 cys), 3.921 (t, 1 H, J = 6.2 Hz, CH cys), 3.986 (s, 2 H, CH_2 gly), 4.656 (t, 1 H, J = 6.3 Hz, CH glu).

Disodium Bis(γ-L-glutamyl)-L-cysteinyldiglycinate (GSSG, Na Salt). ^{1}H NMR: 2.188–2.437 (m, 4 H, CH_2 glu), 2.649–2.827 (m, 4 H, CH_2 glu), 3.031–3.647 (m, 4 H, CH_2 cys), 3.878 (t, 2 H, J = 6.2 Hz, CH cys), 3.989 (s, 4 H, CH_2 gly), 4.899 and under (H_2O, 1 H, CH glu). The chemical shifts (δ) (intensity ratios) of the cystine methylene protons are 3.031 (0.47), 3.3135 (0.49), 3.190 (0.88), 3.295 (0.98), 3.438 (0.85), 3.490 (1.00), 3.598 (0.49), 3.647 (0.46).

9,10-Dioxa-*syn*-(1-bromoethyl,methyl)bimane [*syn*-($CHBr(CH_3)$,-CH_3)B] (1). Bromine (8.0 g, 2.8 mL, 50 mmol) was added to *syn*-(ethyl,methyl)bimane [*syn*-(CH_3CH_2,CH_3)B] **(8)**[66] (5.006 g, 22.7 mmol) and sodium bicarbonate (4.005 g, 47.7 mmol) in dichloromethane (150 mL). The solution, protected from light, was stirred for 1 h and filtered, the solvent evaporated, and the residue recrystallized from hot ethyl acetate to give a yellow crystalline product. Chromatography of the residue from the mother liquor on silica H (eluant, CH_2Cl_2) gave additional product. Yield, 7.1 g (18.8 mmol) (86%). The dibromo compound is not fluorescent in any form but is transformed into fluorescent materials upon irradiation on a TLC plate, properties characteristic of bromobimanes.

Separation and Properties of Diastereomeric Dibromides. The two diastereomers [meso:racemic (*dl*)] of *syn*-(1-bromoethyl,methyl)bimane (**1**) are formed in a 1:1 ratio and can be observed with TLC as two adjacent spots. The isomer mixtures (injected as a CH_2Cl_2 solution) are separated by HPLC (SI-60 column) [eluant: cyclohexane–ethyl acetate (60:40)]. In nonpolar or aprotic solvents (tetrahydrofuran, dichloromethane, benzene), the diastereomers are stable. In chloroform or acetonitrile, equilibration of the isomers is rapid, leading to a mixture containing 43% meso. The stability of **1** (mixed isomers) in an aqueous solution of tetrahydrofuran and sodium bicarbonate was determined by TLC.

Meso isomer: mp 180–181.5 °C. IR (KBr): 2990, 2925, 1730, 1655, 1585, 1425, 1305, 1245, 1210, 1040, 980, 740, 660 cm^{-1}. ^{1}H NMR (C_6D_6): 1.373 (d, 6 H, J = 7 Hz), 1.617 (s, 6 H, α-CH_3), 4.749 (q, 2 H, J = 6.8 Hz). UV (λ_{max}, 3% CH_3CN/H_2O, pH 7.29): (65% meso) 403 nm (ε 4500), 258 (13 500). Racemic (*dl*) isomer: mp 179.5–182 °C. IR (KBr): 2990, 2930, 1740, 1660, 1590, 1425, 1310, 1245, 1205, 1040, 980, 740, 655 cm^{-1}. ^{1}H NMR (C_6D_6): 1.472 (d, 6 H, J = 7.4 Hz), 1.578 (s, 3 H, α_1-CH_3), 1.630 (s, 3 H, α_2-CH_3), 4.659–4.904 (m, 2 H). UV (λ_{max}, 3% CH_3CN/H_2O, pH 7.29): (75% racemic) 399 nm (ε 4500), 257

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

(65) Stahl, E. *Thin-Layer Chromatography*; Academic: New York, 1965; p 496.

(66) Kosower, E. M.; Zbaida, D.; Baud'huin, M.; Goldberg, I., unpublished results.

(67) Carried out by the group of Prof. N. S. Kosower, Department of Human Genetics, Sackler School of Medicine, Tel-Aviv University.

(13 200). Mixed diastereomers: mp 175–177 °C. After melting, the isomers are equilibrated and vinyl derivatives are present (TLC).

9,10-Dioxa-μ-(S)-syn-(methylmethylene,methyl)bimane [μ-(S)-syn-(CH(CH_3),CH_3)B] (2) (SH^- Reaction). Method 1.[4] A mixture of meso and racemic *syn*-(1-bromoethyl,methyl)bimanes (**1**) (100 mg, 0.265 mmol) in dichloromethane (14 mL) was stirred for 7 h with sodium sulfide (168 mg, 1 mmol) in water (4 mL) containing hexadecyltrimethylammonium bromide (15.2 mg). After exhaustive extraction with CH_2Cl_2, the organic layers were combined, dried ($MgSO_4$), and evaporated and the residue (60 mg) chromatographed on a thick silica plate (ethyl acetate) to yield 46.6 mg (0.186 mmol, 70%) of product **2**: mp 133–134 °C. ^1H NMR ($CDCl_3$) (two isomers, ratio 3:1) [isomer a]: 1.751 (d, 6 H, J = 7.0 Hz), 1.904 (s, 3 H, α_1-CH_3), 1.911 (s, 3 H, α_2-CH_3), 4.185 (q, 2 H, J = 7.1 Hz), [isomer b] 1.831 (d, 6 H, J = 7.3 Hz), 1.885 (s, 6 H, α-CH_3's), 4.064 (q, 2 H, J = 7.3 Hz). UV (λ_{max}, 3% chloroform/dioxane): 350 nm (ϵ 5700), 215 (36 000). UV (3% CH_3CN/H_2O, pH 7.3): 366.5 nm, 255 sh, 234. Mass spectrum: m/e 250 (M^+).

Method 2:[4] A mixture of meso and racemic *syn*-(1-bromoethyl,-methyl)bimanes (**1**) (100 mg, 0.265 mmol) in CH_3CN (20 mL) was added over 1.5 h to sodium sulfide (0.0875 g, 0.5 mmol) in water (90 mL, phosphate buffer pH 7.3) and CH_3CN (10 mL) and stirred overnight. The organic extract (CH_2Cl_2 and diethyl ether) was chromatographed on silica (eluant CH_2Cl_2) to yield 63.4 mg of product (0.25 mmol) (95%) containing cis and trans isomers in a 1:1 ratio (HPLC (SI-100 column) eluant: ethyl acetate–cyclohexane, 40:60).

Reaction of syn-(1-Bromoethyl,methyl)bimane (1) with Glutathione. The reaction conditions and reactant ratios were selected to maximize the yield of the ring compound [μ-(S)-(CH(CH_3),CH_3)B] (**2**). Small scale reactions were used to avoid precipitation of **1** and involved running reactions in triplicate. Three three-necked flasks, each equipped with a Teflon-coated stirrer, and two addition funnels were used. All parts of the apparatus were covered with aluminum foil to avoid exposure of **1** to light. Flasks were loaded with CH_3CN (10 mL), water (60 mL), and sodium bicarbonate (67 mg, 0.80 mmol). One addition funnel contained dibromobimane [*syn*-(BrCH(CH_3),CH_3)B] (**1**) (150 mg, 0.40 mmol) in CH_3CN (30 mL); one funnel contained glutathione (268.6 mg, 0.87 mmol) and sodium bicarbonate (147 mg, 1.75 mmol) in water (75 mL). At ambient temperature, aliquots (6 mL all at once) of the bimane solution were added alternately with aliquots (15 mL over 30 min) of the glutathione solution with the last aliquot of the glutathione solution being added slowly over 1 h. The reaction solutions were stirred overnight, each extracted with CH_2Cl_2 (4 × 50 mL) and then with diethyl ether (1 × 25 mL). The extracts were dried ($MgSO_4$) and evaporated and the residues weighed (63–68 mg per reaction flask, total 194 mg). Organic solvents were removed under reduced pressure at room temperature from the aqueous solutions which were lyophilized to give 490–505 mg of residue from each reaction, the total being 1.46 g.

The residue from the organic solvent extracts was chromatographed on silica gel. The products were, in order of elution, (a) (eluant: CH_2Cl_2 with 2% ethyl acetate) the thia-bridged bimane **2**, μ-(S)-*syn*-(CH-(CH_3),CH_3)B, 157 mg (0.63 mmol, 53%) as a mixture of two isomers (2:3, HPLC), (b) (eluant: ethyl acetate) the alcohol *syn*-(1-hydroxyethyl,methyl)(ethyl,methyl)bimane (**7**) [*syn*-(HOCH(CH_3),CH_3)-(CH_3CH_2, CH_3)B], 10% yield.

The thia-bridged bimane isomers were separated by HPLC (SI-100 column) in the same way as the diastereomers of **1**. Slow evaporation of a solution of one isomer [a] in CH_3CN gave a crystal suitable for X-ray crystallography, whereas 2-propanol, ethyl acetate, or ethanol gave disordered yellow crystals. Structure determination revealed that isomer a had the trans configuration.

***trans*-μ-(S)-syn-(CH(CH_3),CH_3)B (*trans*-2)**: mp 148.5–149.5 °C (from CH_3CN), 146.5–147.5 °C (from 2-propanol). IR (KBr): 2995, 2940, 1740, 1670, 1600, 1445, 1385, 1180, 1155, 1035, 740 cm^{-1}. ^1H NMR ($CDCl_3$): 1.754 (d, 6 H, J = 7 Hz), 1.904 (s, 3 H, α_1-CH_3), 1.911 (s, 3 H, α_2-CH_3), 4.185 (q, 2 H, J = 6.8 Hz). UV (λ_{max}, CH_3CN): 353 nm (ϵ 5300), 233.5 (14 900); UV (λ_{max}, 3% CH_3CN/dioxane): 351 nm (ϵ 5500), 232 (13 700); UV (λ_{max}, 3% CH_3CN/H_2O): 367.5 nm (ϵ 4400), 255 sh (6000), 234 (15 900); UV (λ_{max}, thin film (0.1 μm) on sapphire window,[27] 80% trans + 20% cis) 350 ± 2 nm (0 °C), 347 ± 2 (−180 °C). Fluorescence (λ_{max}, (ϕ_F) CH_3CN): 444 nm, 465 sh (0.77). UV (3% CH_3CN/H_2O, pH 7.31): 470 nm, 470 sh, 482 (0.64). Mass spectrum: m/e 250 (M^+).

***cis*-μ-(S)-syn-(CH(CH_3),CH_3)B (*cis*-2)**: mp 195–196 °C (from 2-propanol. IR (KBr): 2980, 2940, 1735, 1675, 1610, 1415, 1180, 1110, 1030, 985, 745 cm^{-1}. ^1H NMR ($CDCl_3$): 1.834 (d, 6 H, J = 7.3 Hz), 1.888 (s, 6 H, α-CH_3), 4.074 (q, 2 H, J = 7.3 Hz). UV (λ_{max}, CH_3CN): 349 nm (ϵ 5000), 232 (16 500). UV (λ_{max}, 3% CH_3CN/dioxane): 347.5 nm (ϵ 5400), 232 (15 200). UV (λ_{max}, 3% CH_3CN/H_2O): 365 nm (ϵ 4200), 255 sh (5900), 234 (17 900). UV (λ_{max}, thin film (0.1 μm) on sapphire window[27]): 365 ± 2 nm (0 °C), 362 ± 2 (−180 °C). Fluorescence (λ_{max}, (ϕ_F) CH_3CN) 444 nm, 465 sh (0.74). UV (3% CH_3CN/H_2O, pH 7.3): 470 nm, 470 sh, 483 (0.66). Mass spectrum: m/e 250 (M^+).

***syn*-(1-Hydroxyethyl,methyl)(ethyl,methyl)bimane [*syn*-(HOCH-(CH_3),CH_3)(CH_3CH_2,CH_3)B] (7).** The alcohol (eluant: ethyl acetate) was obtained in 10% yield: mp 174 °C. IR (KBr): 3420, 2990, 2940, 1770, 1645, 1580, 1425, 1240, 1075, 1030, 740 cm^{-1}. ^1H NMR ($CDCl_3$): 1.272 (t, 3 H, J = 7.3 Hz, CH_3 of CH_3CH_2), 1.562 (d, 3 H, J = 7.0 Hz, CH_3 of CH(CH_3)), 1.777 (s, 3 H, α-CH_3 adjacent to CH_3CH_2), 1.849 (s, 3 H α-CH_3 adjacent to HOCH(CH_3)), 2.845 (q, 2 H, J = 7.3 Hz, CH_2), 4.473 (s, 1 H, OH), 5.075 (q, 1 H, J = 6.8 Hz, CH]. UV (λ_{max}, CH_3CN): 375 nm (ϵ 5600), 250 sh (7200), 232.5 (15 000). UV (λ_{max}, 3% CH_3CN/H_2O): 392 nm (ϵ 4500), 258 (2500), 231 (13 500). Fluorescence (λ_{max}, (ϕ_F) CH_3CN): 439 nm, 462 sh (0.76). Mass spectrum: m/e 236 (M^+).

The water-soluble material (1.38 g, 92% of the total obtained in the set of reactions described above) was chromatographed on cellulose. In order of elution, the fractions were (a) (eluant: 95–89.5% 2-PrOH, 409 mL) (0.20 g) sodium bromide (silver nitrate test for bromide ion); (b) (eluant: 89–82% 2-PrOH, 1082 mL) (265 mg) ninhydrin-positive compounds, including *syn*-(GSCH(CH_3),CH_3)(CH_3CH_2,CH_3)B (**9**), ring-opened glutathionylbimane derivatives (nonfluorescent, only peptide and low field proton signals), and partially hydrolyzed ring-opened glutathionylbimanes. No reduced glutathione (GSH) was present; (c) (eluant: 81–80% 2-PrOH, 173 mL) (40 mg) mixtures of *syn*-((GSCH(CH_3),-(CH_3)B (**10**) and glutathione disulfide (GSSG); (d) (eluant: 79–75% 2-PrOH, 1810 mL) (796 mg) two compounds, glutathione disulfide (GSSG) and glutathione sulfide (GSG) (**3**) present. The fractions were analyzed by TLC, the rate of GSSG reduction by NADPH (GSSG reductase) amino acid analysis, and NMR.

9,10-Dioxa-syn-(1-glutathionylethyl,methyl)bimane [syn-(CHSG-(CH_3),CH_3)B] (10). A mixture of two diastereomers (meso and racemic (*dl*)) is present. Isomers a and b are assigned but not identified: ^1H NMR D_2O: 1.942 (d, 6 H, J = 7.0 Hz, CH_3 of CH_3CH, isomer a), 2.147–2.457 (m, 16 H, CH_3 of CH_3CH, isomer b, α-CH_3, CH_2 glu), 2.662–2.829 (m, 4 H, CH_2 glu), 3.100–3.655 (m, 4 H, CH_2 cys), 3.851–4.054 (m, 8 H, CH of CH_3CH, CH cys, CH_2 gly (3.976)), 4.630–4.927 (m, 2 H, CH glu). Isomers a and b are in a ratio of 3:2.

Sample Preparation for Analysis. Each fraction to be analyzed was lyophilized, weighed, and then redissolved in D_2O (10 μL/mg of sample). Aliquots (10 μL) were transferred to three vials (ca. 1 mg each), one for TLC, one for kinetics of an enzyme-catalyzed reaction (EK), and one for amino acid analysis (AA). The actual weight of the sample was determined by AA as described below. The remainder of the sample was lyophilized and set aside for examination by NMR. All samples were stored at −10 °C until used.

Thin-Layer Chromatography. TLC of mixtures of glutathione sulfide and glutathione disulfide with 68% 2-propanol as the developer did not separate the two compounds. However, as the proportion of glutathione disulfide diminished in the fraction, the R_f of the spot decreased. The strength of the color produced by ninhydrin reflected the relative contents of ninhydrin-positive compounds in the sample.

Glutathione Disulfide Analysis with GSSG Reductase.[60] The samples were dissolved in 1.0 mL of water. Aliquots were taken for reaction with NADPH in the presence of GSSG reductase. Both the rate of oxidation of NADPH (λ_{max} 340 nm) and the total NADPH consumed were measured, the rate being proportional to the concentration of glutathione disulfide. The SH content of the reaction mixture was determined using Ellman's reagent and reported as milligrams of glutathione disulfide per weight sample.

Amino Acid Analysis. Glutamic acid, glycine, cystine, and lanthionine contents of representative samples were determined by using an amino acid analyzer.

Calibration of the Instrument Response. Three weighed samples each of glutathione disulfide (GSSG) were dissolved in water (1 mL each) and of lanthionine (lan) in 2.5% aqueous ammonium hydroxide (1 mL each). Different proportions of the two sample sets were randomly mixed (eight samples in all). The samples were hydrolyzed and brought to 1.5-mL volume with water and 20-μL aliquots taken for analysis. Two sets of analyses were required for each sample, one for glu, gly, and lan and the other for gly and cys, because under the optimum conditions for lan, cys and gly overlap. Lan consists of two isomers which were assumed to give the same instrumental response. The amount of each amino acid is reported as an area factor, and since the millimole content of each sample is known, the calibration factor, after compensating for dilutions, is defined as

$$f_{cal} = \text{area total}/\text{millimole total}$$

The following values were obtained: for glu, $f_{glu} = 0.4992 \times 10^5 \equiv 0.50$

$\times 10^5$, σ_{n-1} 0.0257 $\times 10^5$; for gly, f_{gly} = 0.50145 $\times 10^5 \equiv 0.50 \times 10^5$, σ_{n-1} 0.0267 $\times 10^5$; for lan, f_{lan} = 0.6032 $\times 10^5 \equiv 0.60 \times 10^5$, σ_{n-1} 0.0187 $\times 10^5$. In the analysis for cys, the area of gly reported is not the same as the area in the glu-gly-lan study. Therefore, the ratio of f_{cys} to f_{gly} for these analyses was calculated, 1.1549, σ_{n-1}: 0.0556, and applied to the above value of gly to give f_{cys} = 0.58 $\times 10^5$.

The amount of glutathione disulfide and glutathione sulfide (3) was calculated using two glu and two gly for each cys or lan. The millimole values for serine and alanine were used without calibration. The weight of each fraction analyzed was calculated as the sum of the weights of all the amino acids in the fraction and was found to range between 1.2 and 0.8 mg (Table II).

NMR Analysis. The fractions containing glutathione sulfide and glutathione disulfide showed no NMR signals attributable to the bimane moiety. Changes in the NMR spectra were observed in the region of the cysteine proton signals (Figure 2).

Glutathione Sulfide (3) (GSG). ^{1}H NMR (D_2O): 2.274–2.503 (m, 4 H, CH_2 glu), 2.698–2.874 (m, 4 H, CH_2 glu), 2.987–3.488 (m, 4 H, CH_2 cys), 3.974–4.081 (2 H, J = 6.0 Hz, CH cys), 4.012 (s, 4 H, CH_2 gly), 4.772–4.925 (m, 2 H, CH glu}.

The specific chemical shifts δ (intensity ratios) for the lanthionine methylene protons are 2.987 (0.10), 3.015 (0.05), 3.089 (0.15), 3.145 (0.33), 3.171 (0.23), 3.262 (1.00), 3.321 (0.54), 3.425 (0.18), and 3.448 (0.10). The appearance and increasing strength of the signal at δ 3.262 as well as the downfield shift of the lan methyne protons were used as a measure of glutathione sulfide in the sample. The yields of glutathione disulfide and glutathione sulfide in fractions not analyzed were estimated by interpolation using the results from the GSSG reductase experiments used for the GSSG values and of the amino acid analysis for the GSG. The total yield of glutathione disulfide was estimated as 0.388 g (0.59 mmol) and of glutathione sulfide as 0.416 g (0.665 mmol).

Acknowledgment. We are grateful to the European Research Office (U.S. Army), the United States–Israel Binational Science Foundation, and the donors of the Petroleum Research Fund, administered by the American Chemical Society, for support. We appreciate the assistance of Prof. N. S. Kosower, Department of Human Genetics, Sackler School of Medicine, Tel-Aviv University, in carrying out the enzymatic analyses of GSSG content and the Amino Acid Analysis Laboratory of the Weizmann Institute of Science for carrying out the amino acid analyses and for their efforts in improving the accuracy of the analysis for lanthionine. We thank Dr. Joshua Hermolin for carrying out the experiments involving thin films of the thia-bridged bimanes. We are grateful to a referee for some very useful comments.

Registry No. *dl*-**1**, 102420-25-7; *meso*-**1**, 102420-26-8; *cis*-**2**, 102420-27-9; *trans*-**2**, 102420-28-0; **3**, 102420-29-1; **7**, 102420-30-4; **8**, 99240-32-1; **9**, 102284-84-4; **10** (isomer 1), 102518-90-1; **10** (isomer 2), 102434-57-1; **10** (isomer 3), 102517-60-2; GSH, 70-18-8; GSSG, 27025-41-8.

Nucleophilic Addition to Olefins. 16.[1] Unusual Substituent Effects in the Reaction of Amines with β-Nitrostyrenes. Solvent Effect on Intrinsic Rate Constants

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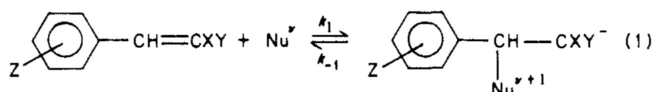
Abstract: A kinetic study of the addition of piperidine and morpholine to β-nitrostyrene in water and in 70% Me_2SO–30% water at 20 °C and of piperidine to 4-NO_2-, 4-CN-, 3-Cl-, 4-Br-, 4-OMe-, and 4-NMe_2-substituted β-nitrostyrenes in water at 20 °C is reported. The reaction leads to a zwitterionic adduct, $ArCH(R_2NH)^+CHNO_2^-$ ($T^{\pm}$), which is in rapid equilibrium with its conjugate base, $ArCH(R_2N)CHNO_2^-$ (T^-). Rate constants for nucleophilic attack (k_1) and its reverse (k_{-1}) both increase slightly along the series H, 4-Br, 3-Cl, 4-CN, and 4-NO_2 with Hammett $\rho(k_1) = 0.27 \pm 0.02$ and $\rho(k_{-1}) = 0.33 \pm 0.05$, while for the equilibrium constant, $K_1 = k_1/k_{-1}$, $\rho_{eq}(K_1) \approx 0$. The small size of the substituent effects is attributed to the near cancellation of two opposing factors: the combined effect of negative charge development and rehybridization of the α-carbon and the effect of the positive charge of the amine nitrogen. The fact that $\rho(k_1)$ and $\rho(k_{-1})$ are larger than $\rho(K_1)$ indicates a transition-state imbalance which arises from a lag in the resonance stabilization and solvation of the negatively charged $CHNO_2$ moiety. K_1 for the 4-OMe and 4-NMe_2 derivatives deviates negatively while k_1 and k_{-1} show positive deviations from the Hammett plots defined by the other substituents. The negative deviation of K_1 is attributed to resonance stabilization of the olefin which is a common phenomenon. The positive deviations for k_1 and k_{-1} are unusual because they suggest an even greater resonance stabilization in the transition state. These observations are attributed to the energetic advantage of having the negative charge delocalized into the nitro group at the transition state. This avoids the problem of the late development of resonance stabilization of the negative $CHNO_2$ moiety which is the main reason for the high intrinsic barriers in nitronate ion forming reactions. A two-point Brønsted plot for the piperidine and morpholine reactions with β-nitrostyrene yields β_{nuc} = 0.26 (β_{nuc}^n = 0.25) and an intrinsic rate constant, log k_0 = 2.10 ($k_0 = k_1 = k_{-1}$ at K_1), in water. In 70% Me_2SO β_{nuc} = 0.32 (β_{nuc}^n = 0.37) and log k_0 = 2.90. The increase in log k_0 upon addition of Me_2SO is consistent with late solvation of the negative charge being a factor contributing to the high intrinsic barrier of nitronate ion forming reactions. When in the kinetic experiments the equilibrium is approached from the T^- side, protonation of $T^{\pm}$ on carbon becomes significant at high buffer concentrations, to yield $ArCH(R_2NH)^+CH_2NO_2$ (T^+) as a transient. This allowed evaluation of various kinetic and thermodynamic parameters of T^+.

The study of substituent effects on the reaction of nucleophiles with activated olefins of the type



where X and Y are electron-withdrawing groups, has yielded interesting information about the transition states of these reactions. For example with XY = $(COO)_2C(CH_3)_2$ (benzylidene Meldrum's acids) it was observed that the normalized Hammett ρ-values, $\rho_n(k_1) = \rho(k_1)/\rho(K_1)$, are larger than the normalized β_{nuc}-values, $\beta_{nuc}^n = \beta_{nuc}/\beta_{eq}$, for the addition of amines[2] or aryloxide ions.[3] This disparity in the substituent effects or "imbalance" was attributed to a lag in the charge delocalization into the

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0002-7863/86/1508-4541$01.50/0 © 1986 American Chemical Society