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Bimanes 28: Extraction of Sulfur from Fluorescent Models for Biological Thiols

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ABSTRACT

Sulfur extraction from the tripeptide thiol, glutathione (γ-glu-cys-gly) (**1**) *via reaction with* syn-(*1-bromoethyl, methyl*)*bimane* (**2**) *yields glutathione sulfide and the thiabridged bimane,* μ(*S*)-syn-(*methylmethylene, methyl*)*bimane* (**3**) *[1]. The reaction with* **2** *has been extended to dithiols as models for important biological thiols such as reduced trypanothione. The fluorescent dithiols were derived through reductive cleavage with triphenylphosphine (tetrahydrofuan, HCl—KBr solution, pH 1.5) of the dithiatricyclic bimane esters,* $\mu(O_2C(CH_2)_nSS(CH_2)_nCO_2)$-syn-$(CH_2,CH_3)B$, n = *1, 2, 3, prepared from* syn-(*bromomethyl,methyl*)*bimane* (**4**) *and the corresponding dithiadicarboxylic acids. Sulfur extraction led to* **3** *and the cyclic sulfide derived from the dithiol in moderate yields. The dithiols, dithiothreitol and dithioerythritol, also yielded moderate amounts of* **3**. *Sterically hindered thiols (e.g., those in hemoglobin) gave* **3** *in lower yields. Treatment of human red blood cells and red cell membranes (hemoglobin-free ghosts) with* **2** *gave rise to some* **3**. *A side product in some reactions was the oxabridged bimane,* μ(*O*)-syn-(*methylmethylene,methyl*)*bimane.*

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INTRODUCTION

The disulfide bond, which is ubiquitous in the biological and biochemical world, is one of the factors contributing to the stabilization of folded proteins [2]. This bond is present in trypanothione, the oxidized form of a thiol cofactor for the parasites responsible for the diseases of trypanosomiasis and leishmaniasis in humans [3–5], occurs in cyclic vasopressin antagonists [6], and is involved in one of the most common cell reactions, the glutathione-glutathione disulfide redox system [7]. The extent of its influence on specific functions can be studied in diverse ways. Reducing the disulfide bridge to two thiol moieties results in gross changes, for example, unfolding of proteins, or little or no change, as in the binding ability of antagonists to vasopressin receptors after reduction [6]. Reoxidation of dithiols to disulfides allows the study of the restructuring of a system altered by reduction.

Complicated molecular conformational changes and loss of biological or chemical activity stem from the presence of blocking groups on the thiols derived from disulfides [8–11]. However, binding an unblocked dithiol to an antibody molecule leads to disulfide interchange, which also perturbs the conformation [12]. According to Creighton [2], a method is needed to remove the blocking group and the sulfur atom, leaving ala or ser residues, to permit

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full exploration of the disulfide trapped intermediate.

A gentler approach to obtaining information about the structural role of disulfides in molecules involves the replacement of the disulfide bond by a monosulfide bond. The effect of the transformation of cystine S—S links into lanthionine S bonds has been extensively investigated with respect to crosslinking in proteins [13, 14], the dyeing and solubility properties of wool [15–17] and hair [18], and the nutritional availability of proteins [19]. In all cases, the results suggest the occurrence of basic structural modifications. In the same vein, glutathione sulfide does not inhibit human GSSG reductase in the reduction of GSSG [1, 20].

Thus, the use of sulfur extraction seems a valid approach to study the perturbation of the structures of proteins and other compounds that have thiol groups. The replacement of two thiol groups by a monosulfide group would alter the configuration of the substrate in a way that is different from oxidation to a disulfide. However, all previously cited examples of conversion of a disulfide to a monosulfide entailed conditions, that is, high alkalinity and high temperatures, that had unwanted additional effects such as ester hydrolysis and denaturation. We have discovered a sulfur extraction procedure that obviates such complications. The reaction of the tripeptide thiol, glutathione (γ-glucys-gly) (**1**), with syn-(*1-bromoethyl,methyl*)*bimane* (*bEBBr*, **2**), at pH 7.3°C and 25°C, gave a sulfur extracted monosulfide derivative of glutathione, GSG, and a 53% yield of two thia-bridged bimanes, *cis-* and *trans-*μ(S)-*syn-*(methylmethylene,methyl)bimanes (**3**) in a 2:1 ratio. The mildness of the procedure encouraged us to extend the study of sulfur extractions to systems in which both thiol groups are present in the same molecule and to study the effect of chain length between the two moieties on the efficiency of the extraction procedure. We found bimane-containing dithiols useful for this purpose. The fluorescence of bimane derivatives makes the reaction very easy to follow. In addition, the sensitivity of bimanes to alkaline conditions is comparable to that encountered among proteins [21]. The thiol groups in human blood proteins are the targets of the fluorescent-labeling reaction of *syn-*(bromomethyl,methyl)(methyl, methyl)bimane (mBBr) [22], suggesting these proteins as substrates for sulfur extraction.

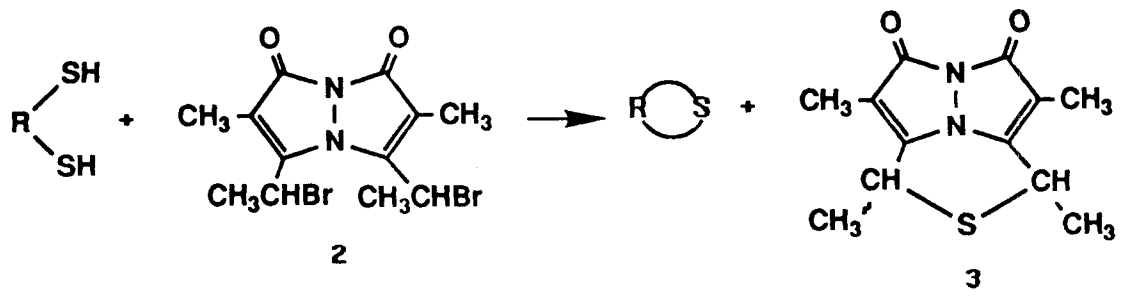
RESULTS

The reaction of various dithiols with bEBBr (**2**) in aqueous solution was studied according to the general reaction given in Equation (1).

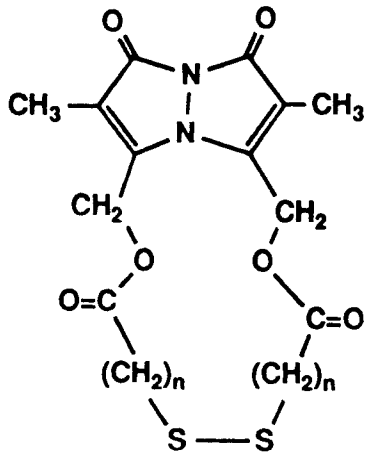
Dithiol Reactants

The thiol moieties in the dithiols were separated by bridges of between four to fifteen atoms. The simplest compounds used were dithiothreitol (DTT, **4**) and dithioerythritol (DTE, **5**). Fluorescent dithiols were prepared by reductive cleavage of the tricyclic bimane ester disulfides, μ($O_2C(CH_2)_nSS(CH_2)_nCO_2$)-*syn-*($CH_2$,$CH_3$)B (in short, BE(*n*)SS, *n* = 1,2,3). The latter were synthesized from *syn-*(bromomethyl,methyl)bimane (**6**) and the dipotassium salts of the corresponding dicarboxylic acids. The cyclic disulfide, BE(1)SS (**7**), was obtained from 2,2′-dithiodiacetic acid, BE(2)SS (**8**) from 3,3′-dithiodipropionic acid, and BE(3)SS (**9**) from 4,4′-dithiodibutyric acid. Dibenzo-18-crown-6-ether in the reaction mixture promoted the complete consumption of **6** (versus 37% recovery in the synthesis of BE(1)SS without added crown ether) and increased the product yield, for example, from 42 to 65% for BE(3)SS.

The disulfides, BE(*n*)SS, were scissioned to the bis-thiol derivatives *syn-*($HS(CH_2)_nCO_2CH_2$,CH_3)B, (in short, BE(*n*)$(SH)_2$, *n* = 1 (**10**), *n* = 2 (**11**), *n* = 3 (**12**), by triphenylphosphine in tetrahydrofuran-hydrochloric acid [23] (Equation 2). The time required for the reaction depended on the pH, being a week at pH 2.5 and 1–3 days at pH 1.5. Reactions were carried out at room temperature to minimize hydrolysis of the ester bond. The dithiol yields were not dependent on pH but did vary with structure, being highest for BE(2)$(SH)_2$ (**11**) (95–99%) and lower for **10** and **12** (ca 44%). Changing the buffer solution from HCl/NaCl (0.1 M) [24] to HCl/KBr (0.2 M) increased the yields, for example, for **11** from 44–72% (NaCl) to 66–99% (KBr), but fell (33%, **12**) in very concentrated potassium bromide (1.4 M) due to low reactant solubility. Results are summarized in Table 1.

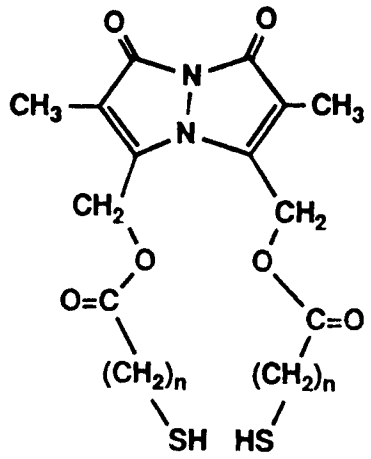


(1)



BE(n)SS

7 n=1
8 n=2
9 n=3



BE(n)(SH)$_2$

10 n=1
11 n=2
12 n=3

The solid dithiols are stable to air oxidation. The product BE(2)(SH)$_2$ (**11**) has an especially beautiful shimmering feathery appearance. In contrast to the disulfides, ^{1}H-NMR spectra show a triplet for the thiol protons and additional splitting and a decreased chemical shift for the adjacent methylene protons (Table 2). Infrared spectra show the weak SH band at 2568 cm^{-1}.

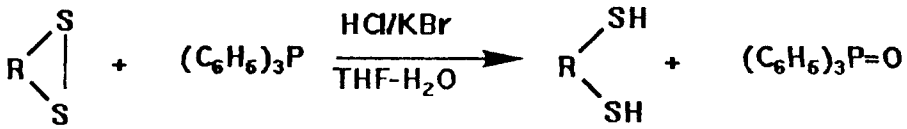
Other reagents were ineffective for reduction of the disulfide bond: low concentrations of sodium borohydride at pH 7–8 (high concentrations destroyed bimane); sodium sulfide and sodium dithionite at neutral pH with or without a phase transfer reagent (bicarbonate and more basic solutions hydrolyzed bimanes); tin and acetic acid (tin and hydrochloric acid and zinc and acetic acid destroyed bimanes); hydrogenation over palladium on charcoal; 2-mercapto-ethanol and thioglycerol in saturated bicarbonate solution gave many fluorescent products but not dithiol.

The oxidation of BE(2)(SH)$_2$ (**11**) with diamide (diazenedicarboxylic acid bis-*N*,*N*-dimethylamide) [25] gave the disulfide BE(2)SS (**8**) (31%), a fluroescent oligomeric substance (38%), and fluorescent material that could not be removed easily from the chromatography column.

Dithiol Reactions with bEBBr (**2**)

The thiabridged μ(S)-*syn*-(methylmethylene,methyl)bimane (**3**) was the main identifiable product from the reaction of **2** with DTT (**4**) and DTE (**5**) (21% yield). Reaction of **2** with dithiothreitol (**4**) gave 56% *cis*-**3**; reaction of **2** and **5** gave 44% *cis*-**3**. The products from **4** were studied in detail. The cyclic bis substitution product of **2** by **4**, the 4-carbon bridged μ(SCH$_2$CH(OH)CH(OH)CH$_2$S)-*syn*-(CH(CH$_3$),CH$_3$)bimane (**13**) was isolated in 15% yield. Small amounts of *syn*-(ethyl,methyl)bimane (**14**) (1%), *syn*-(1-hydroxyethyl,methyl)(ethyl, methyl)bimane (**15**) (2.6%), and unreacted **2** (7.3%) and **4** (4%) were obtained. Altogether, 46% of the **2** and 19% of the **4** were accounted for. The sulfur extracted product of **4**, 3,4-dihydroxytetrahydrothiophene (HTS), was not found. The last and largest chromatographic fraction (38% of total product weight), contained several unknown, probably oligomeric, compounds, some fluorescent and others only iodine positive. ^{1}H-NMR spectra showed protons related to those of both **2** and **4**.

The reaction of the BE(*n*)(SH)$_2$ compounds and **2** gave the products summarized in Table 3. The major products were μ(S)-*syn*-(methylmethyl-



(2)

TABLE 1 Reaction Conditions and Yields for the Reduction of BE(*n*)SS to BE(*n*)$(SH)_2$

BE(n)*SS*	*mmole*	*$Ø_3P$ mmole*	*THF mL*	*Buffer sol (mL)*	*t(days)*	*BE*(n)*(SH)*$_2$	*Yield mmole (%)*
7, n = 1	0.201	0.51	8	HCl/0.2 M KBr pH 2.5 (2.5)	7	**10**, n = 1	0.086 (42.8)
7, n = 1	0.225	0.576	9	HCl/0.2 M KBr pH 2.5 (2.5)	5	**10**, n = 1	0.104 (46.2)
7, n = 1	0.120	0.411	6	HCl/0.2 M KBr pH 1.5 (6)	2	**10**, n = 1	0.055 (46.8)
8, n = 2	0.051	0.128	2	HCl/0.2 M NaCl pH 2.5 (0.5)	7	**11**, n = 2	0.023 (44)
8, n = 2	0.081	0.147	3	HCl/0.2 M NaCl pH 2.5 (0.75)	6	**11**, n = 2	0.058 (72)
8, n = 2	0.105	0.255	4	HCl/0.1 M KBr pH 2.5 (1.0)	3		
8, n = 2	0.246	0.870	9	HCl/0.4 M KBr pH 2.5 (0.5)	1	**11**, n = 2	0.335 (95)
8, n = 2	0.188	0.362	6.5	HCl/0.2 M KBr pH 2.5 (1.5)	6	**11**, n = 2	0.125 (66)
8, n = 2	0.512	1.28	30	HCl/0.2 M KBr pH 1.5 (30)	3	**11**, n = 2	0.506 (99)
9, n = 3	0.156	0.375	8.8	HCl/1.1 M KBr pH 2.5 (1.0)	10	**12**, n = 3	0.052 (33)
9, n = 3	0.187	0.450	8	HCl/0.2 M KBr pH 1.5 (8)	2	**12**, n = 3	0.080 (42.6)
9, n = 3	0.555	1.405	33	HCl/0.2 M KBr pH 1.5 (33)	5	**12**, n = 3	0.258 (46.5)

ene,methyl)bimane (**3**) and the sulfur extracted products, the tricyclic monothia derivatives $\mu(O_2C(CH_2)_nS(CH_2)_nCO_2)$-*syn*-$(CH_2,CH_3)$B (in short, BE($n$)S, n = 1 (**16**), n = 2 (**17**), n = 3 (**18**). Yields of both **3** and BE(n)S decrease as n increases; for **3**, from 33.9% to 11.3% and for BE(n)S, from 19.5% **16** (n = 1) to little or no **18** (n = 3). The ratio of the yields of BE(n)S to **3** was the same in two cases, 0.68 for n = 1 and 0.60 for n = 2; the *cis*-**3**/*trans*-**3** ratio was 1.5. Authentic samples of **16** and **17** were prepared by reaction of *syn*-(bromomethyl,methyl) bimane (**6**) with the dipotassium salts of 2,2′-thiodiacetate and 3,3′-thiodipropionate, respectively. The identification of the small amount of **18** present in the product **15** was based on comparison of NMR spectra of BE(n)SS and Be(n)S compounds (Table 4). The signals of the methylene protons between the carboxylate and the thia groups of the BE(n)S series, n = 1 and 2, are increased in complexity and are shifted to higher fields than those of the Be(n)SS derivatives. Signals attributable to BE(3)S (**18**) were observed but attempts to isolate the material by preparative TLC failed.

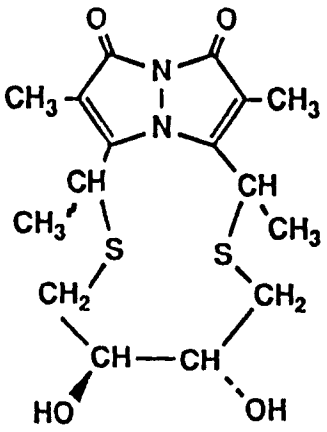
Unreacted dithiols, BE(n)$(SH)_2$, were recovered in all three cases (n = 1, 21%, n = 2, 23%, n = 3, 6%); unreacted **2** was found mainly in the reaction with **10**, n = 1 (15% entrained in precipitated polymer). Additional products included partially reduced or hydrolyzed derivatives of **2** such as *syn*-(1-bromoethyl,methyl)(ethyl,methyl)bimane (**19**) and *syn*-(1-hydroxyethyl,methyl)(ethyl,methyl)-bimane (**15**). Excluding polymer, one could account for 60% of the n = 2 dithiol, 40% of the n = 1 dithiol, and 20% of the n = 3 dithiol. The reactions of **2** and BE(n)$(SH)_2$ were carried out at pH 6 rather than pH 7.3 on the basis of higher yields of **3** found in preliminary studies.

BE(2)$(SH)_2$ (**11**) was reacted with *syn*-(bromomethyl,methyl)(methyl,methyl)bimane (**21**), pH 7.3, to give a 73% yield of the product containing three bimane rings [4-(methylene,methyl)(methyl, methyl)bimane)-4-thiabutanoyl,methyl]bimane (**22**). The absorption coefficient (15,400) of this compound is about three times that of a simple bimane.

TABLE 2 ^{1}H-NMR Spectrum Differences in the Series BE(n)SS and BE(n)$(SH)_2$[a]

	n = 1	n = 2	n = 3
(. . . .CH_2—S—)$_2$[b]	3.650(s)	2.984(t)	2.796(t)
. . . .CH_2—SH[b]	3.351(d)	2.838–2.776(m)	2.589(q)
. . . .CH_2—SH[c]	2.071(t)	1.652(t)	1.354(t)

[a] δ-Values given in ppm.
[b] For methylene protons.
[c] For thiol proton.



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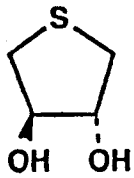
μ(SCH$_2$CH(OH)CH(OH)CH$_2$S)-*syn*-(CH(CH$_3$),CH$_3$)B



HTS

Hemoglobin Thiols

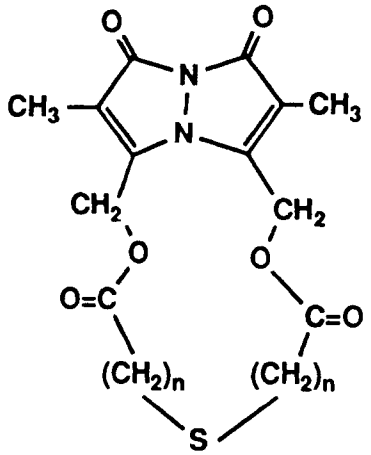
Human red blood cells were examined as a thiol source. *Syn*-(1-bromoethyl,methyl)bimane (**2**) was reacted for an average of 18 hrs at 37°C under sterile conditions with erythrocytes (Rbc) prepared from freshly drawn heparinized blood, erythrocyte ghosts (G), and hemoglobin (Hb) solution prepared by dialysis from hemolyzed erythrocytes. Human hemoglobin has two reactive thiol groups, β-cys 93, at pH 7.3, and erythrocytes also contain 0.5 eq GSH per hemoglobin. Precautions were taken to avoid lysis of the cells. In one experiment, hemoglobin (Hb III) was reacted for two weeks at high dilution. Only material extractable by organic solvents was analyzed; the product yield was calculated from UV absorption maxima. The results are summarized in Table 5.

The thiabridged μ(S)-*syn*-(methylmethylene,methyl)bimane (**3**) was found in all three sets of experiments in yields between 20% (exp. Rbc I) and 2.5% (exp. Hb III). In one case (exp. Rbc III), erythrocytes yielded 62% *trans*-**3** and 38% *cis*-**3**. Traces of *syn*-(ethyl,methyl)bimane (**14**), *syn*-(1-bromoethyl,methyl)(ethyl,methyl)bimane (**19**) and *syn*-(1-hydroxyethyl,methyl)(ethyl,methyl) bimane (**15**) were found. Up to 23% unreacted **2** was recovered in some runs.

TABLE 3 Product Yields from the Reaction of *syn*-(2-Bromoethyl,methyl)bimane **2** with the Dithiols BE(*n*)(SH)$_2$ at pH 6

	10 (*n* = 1)	**11** (*n* = 2)	**12** (*n* = 3)
2 recovered[a]	15	3.6	0
BE(*n*)(SH)$_2$ recovered[a]	21	22.7	5.7
μ(S)-B[b] (**3**)	34 (55% cis)	25.3 (60% cis)	11.3 (59% cis)
mEBBr[b] (**19**)	0	0	2.5
mEBOH[b] (**15**)	0	17.6	8.5
BE(*n*)S	22.1 (**16**)	15.4 (**17**)	6.9 (**18**)[c]
BE(*n*)SS	0	25.2 (**8**)	6.0 (**9**)
BE(*n*)S/3[d]	0.65	0.59	0.61
2 accounted for[e]	43.6	52	22
BE(*n*)(SH)$_2$ accounted for[f]	40.2	62	18

[a] % of starting material recovered at end of reaction. The yields are based on the amount of **2** actually consumed.
[b] μ(S) = μ(S)-*syn*-(CH(CH$_3$),CH$_3$)B; mEBBr = *syn*-(BrCH(CH$_3$),CH$_3$)(CH$_2$CH$_3$,CH$_3$)B; mEBOH = *syn*-(HOCH(CH$_3$),CH$_3$)(CH$_2$CH$_3$,CH$_3$)B.
[c] The identification of **18** is not definitive.
[d] Ratio of yields of BE(*n*)S and of **3**.
[e] Sum of all **2**-related materials.
[f] Sum of all BE(*n*)(SH)$_2$-related materials.



BE(n)S

16 n=1
17 n=2
18 n=3

Hidden glutathione was not detected in Hb through reaction with mBBr, showing that the thia-bridged product must arise from HbSH [26].

Oxabridged Bimane

A new product, μ(O)-*syn*-(methylmethylene,methyl) bimane (**23**), was isolated by chromatography from the dichloromethane-extractable products of the reaction of Rbc and Hb with **2** in yields similar to those of the thiabridged derivative **3**. Formation of **23** from **2** did not depend on the presence of Hb or Rbc since the reaction of **2** and water or NaCl-phosphate buffer at room temperature over several days gave **23** in 2–2.3% yields with 88–85% recovery of unreacted **2**. Reactions under more drastic conditions (2 weeks at 60°C) consumed **2** and gave a 25% yield of **23**. The compound **23** was also found as a minor side product of the synthesis of *syn*-(hydroxyethyl,methyl)bimane through trifluoracetolysis of **2** [27]. The *trans*-**23**/*cis*-**23** ratio was **2** in the rbc experiment. The isomers of **23** could be resolved by TLC whereas the isomers of **3** were separable only by HPLC. Insufficient material was available for crystallization or determination of the crystal structure. The analysis of the cis and trans mixture was based on the NMR spectra through a comparison with the chemical shifts of *cis*- and *trans*-**3** in NMR spectra.

DISCUSSION

The discovery and elucidation of the sulfur extraction reaction [1, 20] led us to the exciting thought that the reaction might be quite general. This view has now been shown to be correct albeit within considerable limitations imposed by the variety of possible reactions that can occur.

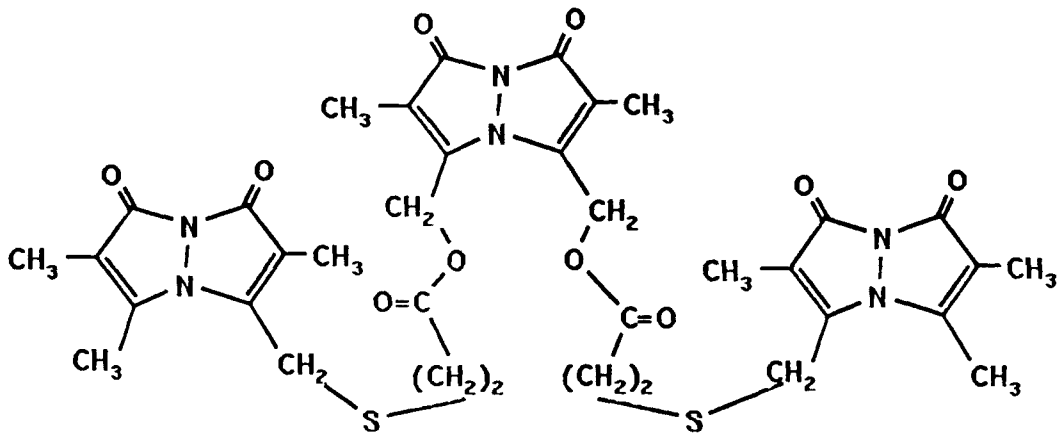
Reactions of Dithiols

The sulfur extraction reaction was originally discovered for the conversion of glutathione (GSH, **1**) to the monosulfide, GSG. The reaction has now been extended to the intramolecular domain with a series of dithiols derived from bimane esters, using bEBBr (**2**) as the agent of sulfur extraction. The course of the reaction is shown in Scheme 1. The first step (1) involves displacement of bromide by thiolate, forming a monobromothiol. Three possible reactions can then occur: (2) displacement of the remaining bromide by the remaining thiol, (3) displacement of the remaining bromide by the sulfide to form a sulfonium ion, or (4) displacement of a bromide in a second dibromide molecule by the second thiol. Reaction (2) yields a cyclic bis-sulfide as found in one case, **13**, formed in 15% yield. The sulfonium ion formed by reaction (3) can disappear in two ways, via the intramolecular route (5) to give the desired *sulfide* along with the thiabridged bimanes, *cis*- and *trans*-μ(S)-*syn*-(methylmethylene,methyl)bimanes (**3**) or by an intermolecular route (6) to yield oligomer A. A different oligomer (B) is formed by reaction (4). The amount of sulfur extraction is measured by the yield of **3**, which should equal the sum of the yields of monosulfide (step 5) and oligomer A (step 6).

TABLE 4 ^{1}H-NMR Chemical Shifts for the Disulfides, BE(*n*)SS, and the monosulfides, BE(*n*)S[a]

	α-CH_3	β-CH_2	$CH_2(1)$	$CH_2(2)$	$CH_2(3)$
BE(1)SS **7**	1.986	5.155	3.650(s)		
BE(1)S **16**	1.977	5.105	3.409(s)		
BE(2)SS **8**	1.958	5.125	2.845(t)	2.984(t)	
BE(2)S **17**	1.954	5.083	2.787(ct)	2.904(ct)	
BE(3)SS **9**	1.973	5.159	2.602(t)	2.079(qt)	2.796(t)
BE(3)S **18**	1.962	5.165	2.545(ct)	1.955(broad)	2.722(ct)

[a] δ-values given in ppm.



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Reaction Conditions

The experimental conditions for the reaction of **1** with **2**, under which sulfur extraction was maximized (53%), were used in the present investigation. The highest yield (34%) of **3** was obtained for the reaction of BE(1)$(SH)_2$ (**10**) with **2**. The lower yields obtained in this study may reflect the circumstance that the reaction parameters might not have been optimal.

There are several factors to be considered. First is the reactivity of the thiol itself, which is related to its pK_a. The electronic environment of SH in **10** is closest to that of GSH since the ester linkage is also two carbons removed from the thiol group. As the number of methylene units increases in the series BE(n)$(SH)_2$, one expects the acidity of the SH groups to decrease and thus the undissociated thiol concentration to increase. However, as the stability of thiolate versus thiol decreases, the nucleophilicity of the thiolate increases. Thus, the reactivity of the series in substitution reactions may not change much with increasing chain length. In DTT (**4**) and DTE (**5**) the hydroxyls adjacent to the thiols have approximately the same effect on the thiol acidity as does the further removed carbonyl moiety in the glutathione molecule. This is shown by the similarity of the pK_a values for the thiols [28, 29] **4**, 8.3 and 9.5, **5**, 9.0 and 9.9, and GSH, 9.2.

The product desired from a dithiol is a mono-

TABLE 5 Product Analysis of the Reaction of *syn*-(2-Bromoethyl,methyl)bimane (**2**) and Human Blood[a]

Blood[b] *exp.*	*SH/2*[c] *μmol used*	**2** *μmol used*	**2** *% recovered*	*μ(S)EB*[d] **3**	*μ(O)EB*[e] **23**	*mEBOH*[d] **15**	*mEBBr*[d] **19**	*(Et,Me)*[d] **14**
Rbc I[f]	1.0	1.25		20	6.8			
Rbc II[g]	50	59.5	35	5.8	1.1	0.8		0.80
Rbc III[f]	40	44.5	0.1	12.5[h]	4.9[i]		trace	2
G	0.5	0.65		6				
Hb I[j]	2.5	3.12		17.6	8.2			
Hb II[j]	32	36.9	47	12.8	7.4			
Hb III[j]	40	52.9		2.5	1.0			

[a] Refers only to products extractable by organic solvents.

[b] Rbc refers to human erythrocytes, G to erythrocyte ghosts, and Hb to hemoglobin. Average incubation period in each experiment was 18 h at 37°C except for Hb III where reaction time was two weeks.

[c] Hb has 2 reactive SH moieties. Rbc suspensions contain 1 eq Hb (2 eq SH) and 0.5 eq GSH.

[d] % yield based on μmol SH/2 available.

[e] % yield based on μmol **2** consumed.

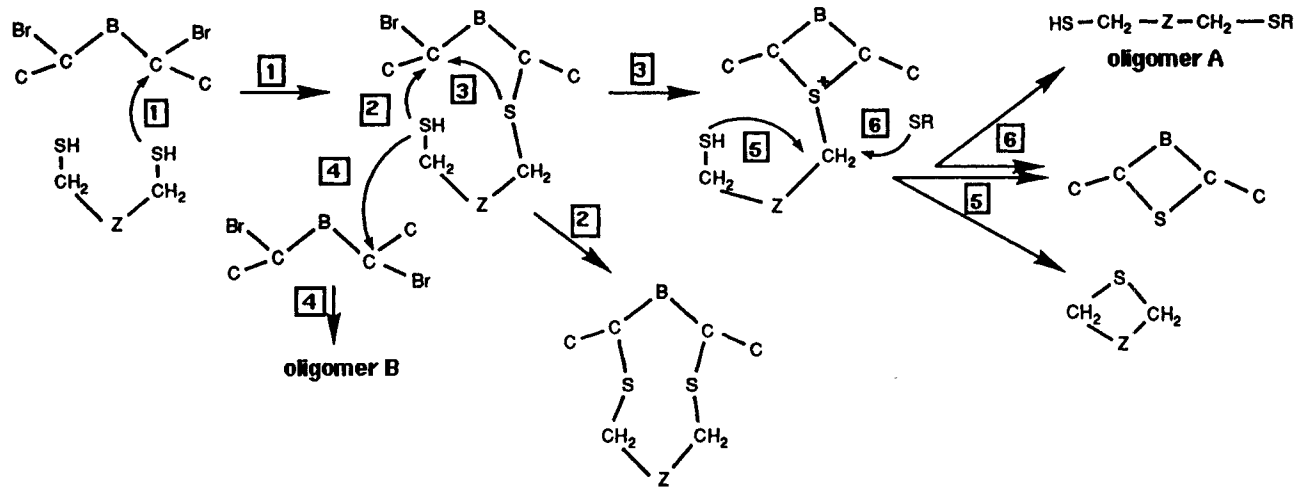
[f] 8% Rbc/mL equivalent to 0.4 μmol Hb and 0.2 μmol GSH. Cells in Rbc III did not lyse during a *period equal* to two half lives for the reaction.

[g] 10% Rbc/mL; cells lysed so that the reaction took place in the solution and not in the cells.

[h] 62% trans- and 38% cis-**3**.

[i] 65% trans- and 35% cis-**23**.

[j] Hb contents/mL is 0.5 μmol for I, 1.6 μmol for II, and 0.4 μmol for III.



SCHEME 1 Reaction paths in the Interaction between *syn*-(1-Bromoethyl,methyl)bimane (**2**) and the Dithiols **4**, **5**, **10**, **11**, and **12**.

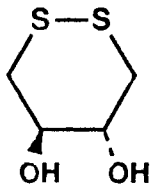
mer but sulfur extraction (yield of **3**) can also give rise to oligomer. The product yields are useful in evaluating structural effects on the reaction pathways. In the reaction of **2** with the dithiol **4**, a cyclic disulfide was formed (15% yield) indicating that this particular intramolecular thiol reacts at about the same rate as the intramolecular sulfide nucleophile (20% yield of **3**).

A thiolate ion is ca 10^5 more nucleophilic than a thioether group. However, the second thiol of DTT (**4**) is only about 10% dissociated under the reaction conditions. Thus the essentially equivalent contributions of the thiol and sulfide to the product suggests that a factor equivalent to 10^4 in rate favors the thioether. Intramolecuular reaction of the thioether involves an energetically favorable 6-membered ring transition state, whereas reaction of the thiolate anion must be via a less favorable 11-membered ring transition state. If half the strain energy (taken as similar to cyclododecane) is acquired at the transition state, the rate would be diminished by an amount equivalent to 0.5 × 43 kJ (cyclododecane − cyclohexane)/4.165 = 5.25 kcal/mol [30] or almost 10^4. A similar competition should be evident for less strained larger rings, but it was not practical to carry out the reaction at higher dilution.

For the dithiols BE(n)$(SH)_2$, the ring size in the transition state for forming compounds analogous to **13** is between 18 and 22 atoms. Entropy rather than strain energy should influence the competition of the thiolate with the sulfide. However, no monomeric bis-substituted product equivalent to **13** was isolated. The yield of sulfur extraction as determined by the yield of **3** decreases from 34% for n = 1 to 11% for n = 3. Trimeric and higher oligomers were obtained. Intermolecular reaction is more important than intramolecular reaction. The decrease in the formation of **3** with increasing n from Be(n)$(SH)_2$ is curious and may reflect an increase in the accessibility of other molecules, that is, that aggregation of the hydrophobic molecules in the aqueous medium favors intermolecular reaction. The pH of the reaction for this series was lowered from 7.3 to 6 in order to diminish the concentration of thiolate anion (factor of 20) and make the thioether more competitive in nucleophilic attack on the second bromine. There was indeed a qualitative improvement in the yield of **3**.

Sulfur Extraction Results

The fact that the yield of the monomeric monosulfide product is not uniform shows that the sulfur extraction process depends on structure. Reaction with either DTT (**4**) or DTE (**5**) requires an intramolecular sulfur abstraction (reaction 5, Scheme 1), which entails the formation of the strained five-membered ring compound (HTS) [31]. (Strain energy in the transition state might be ca 6 kcal/mole, taking into account strain in the 5-membered ring, the fact that the sulfur atom is larger, and that the **3** leaving group might be restricted with respect to the five-membered ring). In any event, the intermolecular path (reaction 6, Scheme 1) is favored



HTSS

and only oligomeric derivatives of **4** were isolated. In contrast, in the reduction of GSSG by **4**, an unstrained six-membered ring disulfide, HTSS is formed in a reaction [31] for which the K_{eq} (1.3×10^4 at pH 7) reflects the high stability of the product. The monosulfide derivative of **4**, HTS, is not readily converted under our reaction conditions [33–35] to thiophene, a low boiling compound that would have been difficult to trap and identify.

As the distance increases between the thiol groups in the BE(*n*)SH series, flexibility is introduced into the system and an intramolecular thiolate attack (reaction 5, Scheme 1) for sulfur extraction becomes sterically and energetically more feasible. The transition state for the product BE(*n*)S, with a ring size ranging from 12 to 16, is strained by a maximum of 5 kcal (see above discussion). This is apparently a low enough barrier to permit about two thirds of the total sulfur extraction (measured by the yield of **3**) to proceed by the intramolecular process to give the monomeric product. The intermolecular attack (reaction 6, Scheme 1) is thus of secondary importance.

Formation of Dithiols

The breaking and forming of protein disulfide bonds by thiol-disulfide interchange reagents, such as DTT, 2-mercaptoethanol, and GSH [36–38], are considered rapid, specific, and have predictable rate constants (Equation 3).

$$2RS^- + R'SSR' \rightleftharpoons 2R'S^- + RSSR \quad (3)$$

However, our attempts at reduction of BE(*n*)SS derivatives with DTT, thioglycerol, or 2-mercaptoethanol were not successful. Reduction of the disulfides, BE(*n*)SS, to the dithiols was finally achieved by a modification of the Overman method, using triphenylphosphine and potassium bromide in acidic medium [23, 24]. The steps in the reduction are shown in Equation (4)

$$RSSR' + (C_6H_5)_3P \longrightarrow RS^- + (C_6H_5)_3P^+SR' \xrightarrow{H_2O} RSH + R'SH + (C_6H_5)_3P{=}O \quad (4)$$

The highest dithiol yields were obtained (up to 99%) for **8**, $n = 2$, but were lower (~40%) for most of the dithiols.

Higher molecular weight fluorescent products were not isolated. The isolated dithiols proved to be quite stable to handling so that it was unlikely that the products were reoxidized during isolation. The low yields may stem from the reversibility of the first reaction since, except for BE(1)SS, the yield increased by a factor of ca 1.4 when the pH was lowered from 2.5 to 1.5.

Cyclic Disulfide Esters BE(n)*SS*

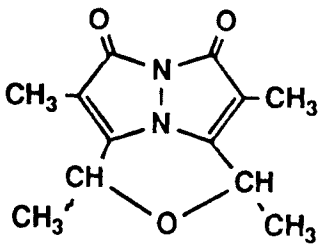
The formation of the thirteen to seventeen-membered rings by the simple displacement reaction between bBBr (**6**) and an alkane dicarboxylate is somewhat unexpected. Ring size in the synthesis of the tricyclic bimanes BE(*n*)SS **7** to **9** did not seem to have much effect on the yields (45–55%). Changing the disulfide to a monosulfide linkage as in BE(*n*)S **16** and **17** had no effect on yield. The addition of crown ether improved conversion as it facilitated dissolution of the potassium salts and thereby allowed more consumption of starting material. The conclusion is that the length and folding ability of the carboxylic acid chain were of relatively little importance in the reaction.

Hemoglobin and Human Erythrocytes

Monobromobimane labels the two reactive thiol groups in human hemoglobin (Hb) as well as the thiol group of glutathione (0.25 eq SH/eq SH Hb) on reaction with intact erythrocytes (Rbc). The bis bromoethyl derivative, bEBBr (**2**), reacts with pure GSH to give as much as 53% μ(S)-*syn*-(methylmethylene,methyl)bimane (**3**). From reaction with intact rbc, one should expect ~0.12 eq **3**/eq HbSH to arise from GSH, were HbSH as reactive as GSH toward **2**. Reaction of **2** with rbc ghosts, in which there is neither HbSH nor GSH, will yield **3** only as a result of reaction with membrane protein SH groups. In fact, a 6% yield of **3** was obtained, based on the quantity of **2** used. Rbc yielded up to 20% of **3**, allowing the possibility that at least some fraction of **3** is a product of HbSH and **2**. In reaction of **2** with pure hemoglobin (Hb) (dialyzed), in which no hidden glutathione could be detected, the yield of **3** (up to 17.6%) can be attributed only to sulfur abstraction from HbSH. One can conclude that sulfur extraction can take place in a protein system of reasonable complexity.

Since not all of the bEBBr (**2**) was recovered or could be accounted for by formation of thiol products or hydroxybimanes, other reactions must have consumed **2**. At least some polymers were formed, both from BE(*n*)$(SH)_2$ and from hemoglobin, judging from the insolubility and texture of some of the products.

The isolation of the oxygen-bridged analog of **3**, μ(O)-*syn*-(methylmethylene,methyl)bimane (**23**), was an unexpected bonus of the research, since it had not been found in other reactions carried out with **2**. Reaction of **2** with water at higher temperatures readily gave detectable amounts of **23**. The circumstance that rbc experiments were done at 37°C while earlier experiments had been done at ambient temperatures may account for the formation of **23**. The ultraviolet absorption maxima of the isomers of **23** at 333 nm or 335 nm in acetonitrile suggest a fairly bent bimane with a dihe-



23

dral angle estimated as 139° or 140°, whereas the corresponding μ(S)-*syn*-(methylmethylene, methyl)bimane has absorption maxima at 345 nm and a dihedral angle of 142° [39].

EXPERIMENTAL

General

Instruments used are as follows: ^{1}H-NMR spectra: Bruker WH-90 and AM-360 spectrometers. Chemical shifts are given in δ-values (ppm) downfield from tetramethylsilane as 0.000 with the following abbreviations to designate the multiplicity of individual signals: ct = complex triplet, q = quartet, qt = quintet. Ultraviolet and visible spectra: Cary Model 17 spectrophotometer. Fluorescence spectra: Hitachi-Perkin-Elmer MPF-4 fluorescence spectrophotometer. Mass spectra: Du Pont 21-491B mass spectrometer. IR spectra: Perkin-Elmer Model 177 spectrophotometer and Nicolet 5DX FTIR spectrometer. Certain samples were measured as deposits on a silver halide fiber using infrared fiberoptic spectroscopy with a specially designed cell [40–42].

Chromatography

Flash chromatography on Merck Kieselgel 60 (230–400 mesh) used solvent elution at the rate of 2.5 cm/30–40 sec, and eluants: dichloromethane/cyclohexane (95:5) to acetonitrile/methanol (4:1). TLC separations were done with silica gel (Merck Kieselgel 60 F_{254}) on plastic sheets (0.2 mm) or glass plates (0.25 mm and 2 mm, prep. scale), the latter being cleaned by elution with distilled solvent before use.

Solvents and Materials

Ethyl acetate and cyclohexane were distilled. *Deionized distilled* water was used. Other solvents were used without further purification. Buffers, pH 6 and 7, contained KH_2PO_4 and Na_2HPO_4. Bromobimanes and other bimanes were synthesized according to published procedures [43–45] or were made available by others within our group. *Syn*-(1-bromoethyl,methyl)bimane (bEBBr, **2**) was a mixture of the two diastereomers, dd/ll and meso. The isomers, *cis*- and *trans*-μ(S)-*syn*-(methylmethylene,methyl)bimanes (μ(S)-*syn*-(CH(CH$_3$),CH$_3$)B, **3**) have been reported [20]. Dithiothreitol (**4**) and dithioerythritol (**5**) were obtained from Sigma. Blood was drawn from human volunteers.

Dipotassium Salts of Dicarboxylic Acids

Equivalent amounts of potassium bicarbonate and dicarboxylic acid were heated at reflux in acetonitrile (2 h). The insoluble salt was filtered off and dried in an oven (110°C, 3–4 h). Dipotassium 2,2′-dithiodiacetate, 2,2′-thiodiacetate, 3,3′-dithiodipropionate, 3,3′-thiodipropionate, and 4,4′-dithiodibutyrate were used as prepared.

μ(*1,6-(3,4-Dithia)-hexanedioyloxy*)-syn-(*methylene,methyl*)*bimane* [μ(*O$_2$CCH$_2$SSCH$_2$CO$_2$*)-syn-(*CH$_2$,CH$_3$*)*B, BE(1)SS*] (**7**)

A suspension of dipotassium 2,2′-dithiodiacetate (0.269 g, 1.04 mmol) and *syn*-(bromomethyl, methyl)bimane (bBBr, **6**) (0.331 g, 0.95 mmol) in acetonitrile (50 mL) was stirred rapidly and heated (60–70°C) overnight. The solvent was evaporated and the residue flash chromatographed (eluant, dichloromethane with 10–15% ethyl acetate) to give unreacted **6**, 0.122 g (37% recovery), and diester **7**, 0.101 g (45.5% yield). Addition of dibenzo-18-crown-6 ether (0.5 eq) increased the yield to 50% with complete consumption of **6**.

μ(*O$_2$CCH$_2$SSCH$_2$CO$_2$*)-syn-(*CH$_2$,CH$_3$*)*B, BE(1)SS* (**7**). Sublimes, 190–200°C. IR(neat): 1740, 1670, 1600, 1430, 1280, 1230, 1130, 1000, 730 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.982 (s, 3H, α-CH_3), 3.649 (s, 2H, CH_2—S), 5.157 (s, 2H, β-CH_2). UV: λ_{max} (ϵ_{max}) (CH_3CN): 380 nm (4000), 250 sh (6000), 235 (10100). Fluroescence: λ_{max} (ϕ_F) (CH_3CN): 440 nm, 460 sh (0.4). Mass spectrum: *m/e* 370 (49%) [M$^+$].

μ(*1,8-(4,5-Dithia)-octanedioyloxy*)-syn-(*methylene,methyl*)*bimane* [μ(*O$_2$CCH$_2$CH$_2$SSCH$_2$CH$_2$CO$_2$*)-syn-(*CH$_2$,CH$_3$*)*B, BE(2)SS*] (**8**)

Dipotassium 3,3′-dithiodipropionate and **6** were reacted in the manner described for the synthesis of **7**. The product was obtained in 59% yield after flash chromatography using elution with dichloromethane containing 20–30% ethyl acetate.

μ(*O$_2$CCH$_2$CH$_2$SSCH$_2$CH$_2$CO$_2$*)-syn-(*CH$_2$,CH$_3$*)*B, BE(2)SS* (**8**). mp 199–201°C. IR(neat): 1735, 1668, 1609, 1431, 1386, 1361, 1210, 1127, 1028, 1007, 740 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.948 (s, 3H, α-CH_3), 2.846 (t, 2H, J = 7 Hz, CH_2CO), 2.983 (t, 2H, J = 7 Hz,

CH_2S), 5.137 (s, 2H, β-CH_2). UV: λ_{max} (ϵ_{max}) (CH_3CN): 378 nm (6900), 250 sh (6800), 230 (15000). Fluorescence: λ_{max} (ϕ_F) (CH_3CN): 440 nm, 460 sh (0.43). Mass spectrum: *m/e* 398 (2%) [M^+].

μ(1,10-(5,6-Dithia)-decanedioyloxy)-syn-(methylene,methyl)bimane [μ($O_2CCH_2CH_2CH_2SSCH_2CH_2CH_2CO_2$)-syn-($CH_2$,$CH_3$)B, BE(2)SS] (**9**)

Dipotassium-dithiodibutyrate and **6** were reacted in the manner described for the synthesis of **7**. The product was obtained in 42% yield after flash chromatography as noted for the synthesis of **8**. Addition of dibenzo-18-crown-6 ether increased the yield to 65%.

μ($O_2CCH_2CH_2CH_2SSCH_2CH_2CH_2CO_2$) - syn-($CH_2$,$CH_3$)B, BE(2)SS (**9**). mp 185°C. IR(KBr): 1730, 1670, 1600, 1440, 1380, 1360, 1310, 1215, 1110, 1020, 1010, 910, 775, 730 cm^{-1}; ^{1}H-NMR ($CDCl_3$): 1.968 (s, 3H, α-CH_3), 2.070 (qt, 2H, J = 7 Hz, —CH_2—), 2.592 (t, 2H, J = 7 Hz, CH_2CO), 2.788 (t, 2H, J = 7 Hz, CH_2S), 5.148 (s, 2H, β-CH_2); UV: λ_{max} (ϵ_{max}) (CH_3CN): 375 nm (6500), 250 sh (6500), 230 (15300). Fluorescence: λ_{max} (ϕ_F) (CH_3CN): 440 nm, 460 sh (0.4). Mass spectrum: *m/e* 426 (42%) [M^+].

μ(1,5-(3-Thia)-pentanedioyloxy)-syn-(methylene,methyl)bimane [μ($O_2CCH_2SCH_2CO_2$)-syn-(CH_2,CH_3)B, BE(1)S] (**16**)

Dipotassium 2,2′-thiodiacetate and **6** were reacted in the manner described for the synthesis of **7**. The product was obtained in 48% yield after flash chromatography as noted for the synthesis of **8**.

μ($O_2CCH_2SCH_2CO_2$)-syn-(CH_2,CH_3)B, BE(1)S (**16**). mp 227°C. IR(KBr): 1740, 1675, 1620, 1410, 1370, 1280, 1230, 1190, 1140, 1040, 1000, 950, 750, 730, 659 cm^{-1}; ^{1}H-NMR ($CDCl_3$): 1.977 (s, 3H, α-CH_3), 3.409 (s, 2H, CH_2S), 5.105 (s, 2H, β-CH_2). UV: λ_{max} (ϵ_{max}) (CH_3CN): 378 nm (6400), 250 sh (4800), 230 (10400). Fluorescence: λ_{max} (ϕ_F), CH_3CN): 440 nm, 460 sh (0.39). Mass spectrum: *m/e* 338 (100%) [M^+].

μ(1,7-(4-Thia)-heptanedioyloxy)-syn-(methylene,methyl)bimane [μ($O_2CCH_2CH_2SCH_2CH_2CO_2$)-syn-($CH_2$,$CH_3$)B, BE(2)S] (**17**)

Dipotassium 3,3′-thiodipropionate and **6** were reacted in the manner described for the synthesis of **7**. The product was obtained in 54% yield after flash chromatography as noted for the synthesis of **8**.

μ($O_2CCH_2CH_2SCH_2CH_2CO_2$) - syn - ($CH_2$,$CH_3$)B, BE(2)S (**17**). mp 234°C. IR (neat): 1739, 1678, 1613, 1438, 1415, 1370, 1339, 1237, 1218, 1197, 1154, 1097, 993, 720 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.954 (s, 3H, α-CH_3), 2.787 (m, 2H, CH_2CO), 2.904 (m, 2H, CH_2S), 5.084 (s, 2H, β-CH_2). UV: λ_{max} (ϵ_{max}) (CH_3CN): 379 nm (3600), 250 sh (3000), 230 (9500). Fluorescence: λ_{max} (ϕ_F) (CH_3CN): 440 nm, 460 sh (0.4). Mass spectrum: *m/e* 366 (100%) [M^+].

Reductive Cleavage of the Disulfide Bond to Dithiols

The disulfide, BE(*n*)SS (1 eq) and triphenylphosphine (2.25 eq) were added together to a mixture of THF (4 mL/0.1 mmol BE(*n*)SS) and acid aqueous KBr (Type 1 or 2). 1: 0.1 M HCl was added to adjust the pH of aq KBr or NaCl (0.2 M to 1.1 M) to pH 2.5. Addition of THF raised the pH; additional 0.1 M HCl restored pH 2.5. The dithiol yield was raised with greater amounts of solution (Table 1). 2: Equal volumes of a 0.2 M KBr/0.1 M HCl solution and THF were mixed (final pH 1.5–2.0. All aqueous solutions were flushed with N_2 before use. The reaction mixture was stirred (1–7d) in darkness and under N_2. After dilution with water, the reaction solution was extracted with dichloromethane (under N_2), the organic layer dried ($MgSO_4$) and evaporated, and the residue flash chromatographed. Streaking of the product, which absorbs iodine more strongly than the disulfide, is evident in both 1- and 2-dimensional TLC.

syn-*(2-Thioeacetyloxymethylene,methyl)bimane* [syn-($HSCH_2CO_2CH_2$,CH_3)B, BE(1)($SH)_2$] (**10**). Dichloromethane:ethyl acetate [9–19:1] was used as eluant to yield 46% **10**, which could be kept for two days in a pH 6 solution without decomposition (TLC). ^{1}H-NMR ($CDCl_3$): 1.983 (s, 3H, α-CH_3), 2.071 (t, 1H, J = 8.5 Hz, SH), 3.551 (d, 2H, J = 8.5 Hz, CH_2—S), 5.246 (s, 2H, β-CH_2).

syn-*(3-Thiolpropionyloxymethylene,methyl)-bimane* [syn-($HSCH_2CH_2CO_2CH_2$,CH_3)B, BE(2)($SH)_2$] (**11**). Dichloromethane:ethyl acetate [9–25:1] was used as eluant to yield 44–99% **11** (Table 1), mp 95°C. IR (neat): 2568 (w, SH), 1740, 1668, 1655, 1594, 1449, 1439, 1417, 1381, 1290, 1213, 1182, 1146, 1030, 995, 732 cm^{-1}. ^{1}H-NMR ($CDCl_3$): 1.652 (t, 1H, J = 8.2 Hz, SH), 1.982 (s, 3H, α-CH_3), 2.741 (t, 2H, J = 6.05 Hz, CH_2CO), 2.838–2.776 (m, 2H, CH_2—S), 5.185 (s, 2H, β-CH_2). UV: λ_{max} (ϵ_{max}) (CH_3CN): 378 nm (4200), 228 (12,300). Fluorescence: λ_{max} (ϕ_F) (CH_3CN): 438 nm, 464 sh (0.26). Mass spectrum: *m/e* 400(100%) [M^+].

Reaction of **11** *with diamide.* BE(2)$(SH)_2$ (**11**) (16.1 mg, 0.040 mmol) and diamide (diazenedicarboxylic bis-*N*,*N*′-dimethylamide) (6.0 mg, 0.04224 mmol) in aqueous solution at pH 7.3 (12 mL) were stirred overnight under N_2. The product was extracted with dichloromethane, dried ($MgSO_4$),

evaporated, and flash chromatographed to yield the dithiol **11**, 3.0 mg (0.0075 mmol), the disulfide **8**, 4.0 mg (0.0101 mmol) (30.8% yield), and fluorescent polymeric material, 5.0 mg. Other fluorescent materials remained on the column.

syn - (*4 - Thiolbutyroyloxymethylene, methyl*)-*bimane* [syn-($HS(CH_2)_3CO_2CH_2$,CH_3)*B*, *BE*(3)(SH)$_2$] (**12**). Dichloromethane:ethyl acetate [9:1] was used as eluant to yield 33–46.5% **12** (Table 1). Two phases appeared in the reaction with 1.1 M KBr (pH 2.5); the yield of **12** was 33%. ^{1}H-NMR ($CDCl_3$): 1.354 (t, 1H, J = 8.1 Hz, SH), 1.958 (qt, 2H, J = 7.1 Hz, —CH_2—), 1.973 (s, 3H, α-CH_3), 2.561 (t, 2H, J = 7.3 Hz, CH_2CO), 2.589 (q, 2H, J = 8.1 Hz, CH_2S), 5.157 (s, 2H, β-CH_2).

Unsuccessful Attempts to Effect Disulfide Reduction to Dithiols

$NaBH_4$. BE(2)SS (**8**) (2.1 mg) in acetonitrile (1 mL) was reacted with $NaBH_4$ (2.1 or 9.2 mg) and $NaHCO_3$ (67.1 mg). No change in TLC was detected before loss of bimane (loss of fluorescence).

Na_2S. (1) **8** (4.8 mg) in ethanol and Na_2S in water, pH 8 (1 mL) were reacted for 4 h. The pH was adjusted with HCl to pH 5–6. No bimanes could be extracted with dichloromethane. (2) **8** (2.7 mg) in dichloromethane (1.4 mL), Na_2S (2.5 mg) in water (0.5 mL) and hexadecyl trimethylammonium bromide (1.1 mg) were reacted under nitrogen for 24 h. No reaction was observed (TLC).

$Na_2S_2O_4$. No change was found for **8** (1.7 mg) in diethyl ether (5 mL) in the presence of sodium dithionite (9.7 mg) in water (10 mL).

Tin. (1) No reaction was noted for **8** (2.0 mg) in dichloromethane (1.5 mL) and Sn (4.3 mg) in glacial acetic acid (1.5 mL) over 24 h but (2) **8** (2 mg) in dichloromethane (1.5 mL) and Sn (4 mg) and HCl (1.5 mL) led to loss of bimane.

Zinc. **8** (2 mg) in ether (4 mL) and Zn (6 mg) in acetic acid (6 mL) reacted within 4 h to destroy the bimane.

$HOCH_2CH_2SH$. **8** (2.2 mg) in acetonitrile (1 mL) and 2-mercaptoethanol (0.48 mg) in aqueous $NaHCO_3$ solution (1 mL) reacted to yield several fluorescent products other than dithiol (TLC). Dithreitol (**4**) was also ineffective under the same conditions.

Palladium on Charcoal. **8** (20.5 mg) and hydrogen (1 atm) were reacted in presence of Pd/C (20.7 mg) in ethanol (2 mL) for three days. Chromatography of the reaction mixture yielded only unreacted **8**.

Reaction of Dithiols with Bromobimanes. Method A: Dithiothreitol (**4**) *with* syn-(*1-Bromoethyl,methyl*)*bimane* (**2**)

A three-necked flask equipped with a teflon-coated stirrer and two addition funnels, all protected from light and kept under N_2, was loaded with water (200 mL) and CH_3CN (55 mL). At ambient temperature, aliquots (10 mL) of bEBBr (**2**) (0.567 g, 1.5 mmol) in CH_3CN (90 mL) were added from one funnel every 15 min. Aliquots (20 mL) of DTT (**4**) (0.232 g, 1.5 mmol) and $NaHCO_3$ (0.360 g, 4.3 mmol) in water (180 mL) were added dropwise from the second funnel over a period of 15 min. The reaction solution was stirred overnight, then extracted with dichloromethane (4 × 40 mL), then overnight with a liquid–liquid extractor. The extracts were combined, dried ($MgSO_4$) and evaporated, the residue (0.441 g) was flash chromatographed to afford (a) (eluant: CH_2Cl_2) **2**, 57.4 mg (0.152 mmol) (10% yield), (b) (eluant: CH_2Cl_2:ethyl acetate (9:1)) μ(S)-*syn*-($CH(CH_3)$,CH_3)B (**3**) (56% cis, 44% trans), 69.3 mg (0.277 mmol) (20% yield), (c) (eluant: CH_2Cl_2:ethyl acetate (1:1)) **4**, 9.0 mg (0.058 mmol) and *syn*-(CH_2CH_3,CH_3)B (**14**), 3.2 mg (0.015 mmol) (1% yield), (d) (eluant: CH_2Cl_2:CH_3CN (56:44)) *syn*-($HOCH(CH_3)$,CH_3)(CH_2CH_3,CH_3)B (mEBOH, **15**), 12.7 mg (0.0536 mmol) (3.6% yield) and μ($SCH_2CH(OH)CH(OH)CH_2S$)-*syn*-($CH(CH_3)$,CH_3)B (**13**) (43% cis, 57% trans) 81.3 mg (0.215 mmol) (15% yield), (e) (eluants: CH_3CN to CH_3CN: CH_3OH(1:1)), 0.12 g oligomers with many components with ^{1}H-NMR peaks related to **4** and **2**.

μ(*1,4-Dithia-2,3-dihydroxybutyl*)-syn-(*methylmethylene,methyl*)*bimane*, μ($SCH_2CH(OH)CH(OH)CH_2S$)-syn-[$CH(CH_3)$,CH_3]*B* (**13**). Compound **13** was purified by preparative TLC: mp 215–218°C. NMR spectra show two isomers, assigned as cis and trans, in analogy with **3**. ^{1}H-NMR ($CDCl_3$): 1.586 (d, 6H, J = 6.8 Hz, *trans*-β-CH_3) and 1.629 (d, 6H, J = 7.0 Hz, *cis*-β-CH_3), 2.037 (s, 6H, *cis*-α-CH_3) and 2.066 (s, 6H, *trans*-α-CH_3), 2.975–2.425 (m, 6H, μ-group), 3.150 (d, 2H, J = 11.3 Hz, *cis*-OH) and 3.734 (d, 2H, J = 7.8 Hz, *trans*-OH), 4.446 (qt, 2H, *cis*- and *trans*-H). UV: λ_{max} (ϵ_{max}) (CH_3CN): 382 nm (4600), 236 (11500). Fluorescence: λ_{max} (ϕ_F) (CH_3CN): 438 nm, 465 (0.26). Mass spectrum: *m/e* 370 (44%) [M^+].

Reaction of Dithioerythritol (**5**) *with* **2**

Method A was used. CH_3CN (15 mL) and water (50 mL) were placed in the reaction vessel, **2** (0.151 g, 0.4 mmol) in CH_3CN (25 mL) in one addition funnel, and **5** (0.069 g, 0.45 mmol) and $NaHCO_3$ (0.067 g, 0.8 mmol) in water (50 mL) in the second addition funnel. Aliquot sizes were reduced to 5 and 10 mL, respectively. At the end of the reaction, more $NaHCO_3$ was added to raise the pH from 6 to ca 7.5. Flash chromatography of the residue after extraction

yielded unreacted **2**, 6.0 mg (0.016 mmol) and **3** (44% cis, 56% trans), 21.6 mg (0.086 mmol) (22.4% yield). Other products were not studied.

Method B: Reaction of BE(n)(*SH*)$_2$ *with* **2**

N_2-purged phosphate buffer (pH 6.0) (3.2 mL/0.01 mmol BE(*n*)$(SH)_2$) was added to BE(*n*)$(SH)_2$ (1 eq) in CH_3CN (1 mL). **2** (1 eq) in CH_3CN (1 mL/0.01 mmol **2**) was added rapidly and the reaction mixture (protected from light) stirred in the dark, under N_2, until the **2** had been consumed (TLC). The reaction mixture was extracted with dichloromethane until the aqueous layer was no longer fluorescent. The organic extract was dried ($MgSO_4$), evaporated, and the residue flash chromatographed. The products were **2** (eluant: CH_2Cl_2:cyclohexane (19:1)), **3** (eluant: CH_2Cl_2:ethyl acetate (50:1)), BE(*n*)$(SH)_2$ (eluant: CH_2Cl_2:ethyl acetate (9:1)) followed by products of increasing polarity (eluants: polarity up to CH_3CN:CH_3OH (9:1)). The product yields are based on the amount of **2** consumed.

syn-(*2-Thiolacetyloxymethylene,methyl*)*bimane* (**10**) *with* **2**

In triplicate experiments, a total of BE(1)$(SH)_2$ (**10**) (85.6 mg, 0.230 mmol) and **2** (85.8 mg, 0.227 mmol) were reacted over 2 days. An insoluble yellow precipitate, presumably polymer, was filtered off (35.4 mg). Products identified were (a) **2**, 12.6 mg (0.033 mmol), (b) **3** (55% cis), 16.5 mg (0.066 mmol) (34.2% yield), (c) **10**, 18.4 mg (0.050 mmol), (d) (eluant: CH_2Cl_2:ethyl acetate (1:1)) BE(1)S (**16**), 14.5 mg (0.043 mmol) (22.1% yield), (e) (eluant: CH_3CN to CH_3CN:CH_3OH (9:1)) 49.7 mg compounds with ^{1}H-NMR peaks related to those of **2** and **10**.

syn-(*3-Thiolpropionyloxymethylene, methyl*)*bimane* (**11**) *with* **2**

In triplicate experiments, a total of BE(2)$(SH)_2$ (**11**) (0.173 g, 0.432 mmol) and **2** (0.164 g, 0.434 mmol) were reacted over 2 days. No precipitate formed. Products identified were (a) **2**, 5.9 mg (0.016 mmol), compound X, 12.0 mg (0.031 mmol), (b) **3** (60% cis), 26.6 mg (0.106 mmol) (25.3% yield), (c) **11**, 39.2 mg (0.098 mmol), (d) (eluant: CH_2Cl_2:ethyl acetate (4–1:1)) BE(2)S (**17**), 23.0 mg (0.063 mmol) (15.0% yield), BE(2)SS (**8**), 42.0 mg (0.106 mmol) (25.2% yield), (e) (eluant: ethyl acetate) **15** 17.4 mg (0.074 mmol) (17.6% yield), (f) (eluant: (CH_3CN to CH_3CN:CH_3OH (4:1)), 0.1489 g compounds with ^{1}H-NMR peaks related to those of **2** and **17**.

syn-(*4-Thiolbutanoyloxymethylene, methyl*)*bimane* (**12**) *with* **2**

In five identical experiments, a total of BE(3)$(SH)_2$ (**12**) (0.153 g, 0.356 mmol) and **2** (0.132 g, 0.350 mmol) were reacted over 1 day. No precipitate formed. Products identified were (a) **3** (59% cis), 9.6 mg (0.038 mmol) (11.0% yield), (b) (eluant: CH_2Cl_2: ethyl acetate (12:1)) *syn*-[BrCH(CH_3), CH_3](CH_2CH_3,CH_3)B (mEBBr) (**19**), 2.6 mg (0.009 mmol) (2.5% yield), (c) **12**, 8.5 mg (0.020 mmol), (d) (eluant: CH_2Cl_2: ethyl acetate (4–1:1)) BE(3)SS (**9**), 9.0 mg (0.021 mmol) (6.0% yield), (e) (eluant: ethyl acetate) BE(3)S (**18**), 9.5 mg (0.024 mmol) (6.9% yield), and **15** 7.0 mg (0.030 mmol) (8.5% yield) (f) (eluant: CH_3CN to CH_3CN:CH_3OH (4:1)) 0.1944 g compounds with ^{1}H-NMR peaks related to those of **2** and **12**.

μ(1,9-(5-Thia)-nonanedioyloxy-syn-(*methylene, methyl*)*bimane* [*μ*($O_2C(CH_2)_3S(CH_2)_3CO_2$)-syn-($CH_2$,$CH_3$)*B, BE(3)S*] (**18**). The structure of **18** is inferred from ^{1}H-NMR of a column fraction: 1.962 (s, 6H, *α*-CH_3), 2.049–1.9955 (m, 4H, —CH_2—), 2.545 (ct, 4H, CH_2CO), 2.722 (ct, 4H, CH_2S), 5.165 (s, 4H, *β*-CH_2). The material contained some **15** with peaks that overlapped somewhat with those of **18**. Thus, the peak integrations were not exact but were in agreement with the structural assignment. Separation of **18** from **15** by preparative TLC, although successful for **15**, did not give pure material.

syn-(*3-Thiolpropionyloxymethylene, methyl*)*bimane* (**11**) *with* syn-(*Bromomethyl,methyl*)(*methyl, methyl*)*bimane* (**21**)

BE(2)(SH_2) (**11**) (13.8 mg, 0.035 mmol) and mBBr (**21**) (20.0 mg, 0.075 mmol) in a mixture of CH_3CN (4 mL) and phosphate buffer pH 7.3 (12 mL) were stirred overnight. After the usual workup, the residue was flash chromatographed to yield (a) **21**, 2.1 mg (0.008 mmol), (b) **11**, 4.1 mg (0.010 mmol), (c) (eluant: ethyl acetate:CH_3CN(1:1)) **22**, a trisbimane, 19.6 mg (0.025 mmol) (73% yield), and (d) 8.1 mg unidentified material.

(*4*-syn-[syn-(*Methylene, methyl*)(*methyl, methyl*)*bimane*]-*4-thiabutanoyl,methyl*)*bimane* (syn-[(syn-(CH_2,CH_3)(CH_3,CH_3)*B-SCH*$_2$*CH*$_2$*CO*$_2$*CH*$_2$,CH_3]*B*) (**22**). ^{1}H-NMR ($CDCl_3$): 1.810 (s, 6H, *α*-CH_3 of **21**), 1.860 (s, 6H, *α*′-CH_3 of **21**), 1.932 (s, 6H, *α*-CH_3 of **11**), 2.395 (s, 6H, *β*-CH_3 of **21**), 2.752 (t, 4H, J = 6.7 Hz, CH_2CO), 2.8933 (m, 4H, —CH_2S), 3.739 (s, 4H, *β*-CH_2 of **21**), 5.171 (s, 4H, *β*-CH_2 of **11**). UV: λ_{max} (ϵ_{max}) (CH_3CN): 375 nm (15400), 229 (26400). Fluorescence: λ_{max} (ϕ_F) (CH_3CN): 439.7 nm, 463.6 sh, 480 sh (0.07).

Reactions of Human Blood with syn-(*1-Bromoethyl,methyl*)*bimane* (**2**)

Erythrocytes. Human erythrocytes from freshly drawn heparinized blood were washed twice with NaCl (0.15 M) and suspended in 0.01 M phosphate

buffer—0.135 M NaCl (PBS) (pH 7.4). The resultant 8% red blood cell suspension (rbc), contained 0.4 μmol hemoglobin (Hb) and 0.2 μmol GSH (Ellman's reagent) per mL. At the reaction pH, each Hb had two reactive SH groups. A solution of **2** in acetonitrile (50 mM) was mixed with the rbc suspension to give 1 to 1.5 eqs bromo groups per SH, and the mixture incubated at 37°C for 18 h under sterile conditions. The dichloromethane extract of the reaction mixture was analysed. Experiments: (1) Rbc I: 8% rbc (2 mL) and **2** (1.25 μmol); (2) Rbc II: 10% rbc (80 mL) and **2** (59.5 μmol, 20.8 μmol recovered), cell lysis occurred; (3) Rbc III: 8% rbc (80 mL, divided into 10 flasks of 8 mL each) and **2** (5 μmol per flask), no cell lysis for at least two half lives of the reaction of **2**.

Erythrocyte Ghosts. (Experiment G). Ghosts were prepared from washed erythrocytes by mixing with hypotonic phosphate buffer (5 mM, pH 8.0). The suspension was centrifuged and the pellet was washed repeatedly with the phosphate buffer until the supernatant solution was colorless. A final wash with NaCl (10 mM) gave hemoglobin-free white ghosts. A ghost suspension in PBS (5 mL, 1 μmol SH) was added to **2** (0.5 μmol). The mixture was incubated and extracted with dichloromethane as above.

Hemoglobin. Human erythrocytes were washed twice with NaCl (0.15 M). Erythrocytes (80%) were hemolyzed by diluting with phosphate buffer (5 mM, pH 8.0) (3 vols/vol rbc suspension) or by freezing and thawing of a 50% cell suspension and then centrifuged. The hemoglobin solution was dialyzed in the cold against several changes of water for 18 hr. After dialysis, no GSH was found in MPA extract of the Hb solution (Ellman's reagent). The reaction of the hemoglobin solution, Hb, with **2** was carried out as described above under sterile conditions: (1) Hb I: Hb 0.5 μmol/mL (5 mL) and **2** (3.12 μmol in 0.3 mL CH_3CN); (2) Hb II: Hb 1.6 μmol/mL (20 mL) and **2** (36.9 μmol, 17.3 μmol recovered), dichloromethane extraction was complicated by polymeric Hb at the interface; (3) Hb III: Hb 0.4 μmol/mL (100 mL) and **2** (52.9 μmol), 2 week reaction time, the dichloromethane extraction being easier in this experiment.

Product Analysis

The residue from the extracts were separated by preparative TLC (Developer: Ethyl acetate or CH_2Cl_2 and ethyl acetate (7:3)) and extracted (Soxhlet) from the silica gel (CH_2Cl_2 or ethyl acetate). Quantitation was based on UV absorption (known ϵ values). The maximum amounts could not exceed half of the total amount of available thiol; the yields are calculated on this basis. The products were identified by TLC (R_f values) and UV (λ_{max}) and by ^{1}H-NMR and mass spectrometry where there was sufficient material. Compounds identified were: Unreacted **2** (λ_{max} 392 nm (ϵ_{max}) 5400); μ(S)-*syn*-(CH(CH$_3$),CH$_3$)B (**3**) 350 nm (5200), μ(O)-*syn*-[CH(CH$_3$),CH$_3$]B (**23**) 333 nm (5200); mEBOH (**15**) 375 nm (5600); and *syn*-(C_2H_5,CH_3)B (**14**) 369 nm. Details are given in Table 5.

*9,10-Dioxa-μ(O)*syn-(*methylmethylene, methyl)bimane*[*μ(O)*-syn(*CH(CH$_3$),CH$_3$)B*] (**23**)

Compound **23** was first isolated as a by-product in 11.7% yield from the synthesis of *syn*-[HOCH(CH$_3$),CH$_3$]B [27] through reaction of **2** (0.6131 g, 1.62 mmol) with CF_3COONa (0.577 g, 4.1 mmol) by heating in acetonitrile at reflux. NMR spectra indicated cis and trans isomers, as found for the sulfur homologue, **3**. ^{1}H-NMR ($CDCl_3$): 1.604 (d, 3H, J = 6.8 Hz, *trans*-β-CH_3), 1.655 (d, 3H, J = 6.8 Hz, *cis*-β-CH_3), 1.860 (s, 3H, *trans*-α-CH_3), 1.881 (S, 3H, *cis*-α-CH_3), 4.701 (q, 1H, J = 6.8 Hz, *cis*-CH), 5.020 (q, 1H, J = 6.8 Hz, *trans*-CH). UV: λ_{max} (ϵ_{max}) (CH_3CN): 335 nm (5200), 233. Fluorescence: λ_{max} (ϕ_F) (CH_3CN): 440 nm, 464 sh, 478 sh (0.68). Mass spectrum: *m/e* 234 (72%) [M^+]. One product of the Rbc III experiment had λ_{max} 333 nm and an NMR corresponding to the cis-isomer.

In separate experiments, **2** (7.5 mg, 0.020 mmol) in CH_3CN (0.5 mL) was reacted with water (5 mL) or PBS (5 mL) for 18 h at 37°C (similar to Rbc III). Precipitated **2** was filtered off and weighed. Residual **2** on the walls of the flask was dissolved in acetonitrile (5 mL) and analyzed by UV. The residue obtained by evaporation of the dichloromethane extract of the aqueous filtrate was separated by preparative TLC. Three bands were obtained for each experiment and analyzed by UV (CH_3CN), indicating the presence of **2** (λ_{max} 396 nm), **23** (333 nm), and an unknown (370 nm). Reaction of **2** in water gave recovered **2**, 6.66 mg (0.018 mmol) (88% recovery), (**23**), 4.5×10^{-5} mmol (2% yield) and an unknown, (assuming ϵ = 5500) 8.4×10^{-5} mmol (3.8% yield). Reaction of **2** in PBS gave recovered **2**, 6.42 mg (0.017 mmol) (85% yield), **23**, 6.6×10^{-5} mmol (2.3% yield) and an unknown, 1.2×10^{-4} mmol (4.1% yield).

In another experiment, **2** (0.6608 g, 1.77 mmol) and $KHCO_3$ (0.368 g, 3.6 mmol) in water (600 mL) were heated (60°C) for 2 weeks. The residue obtained from a dichloromethane extract was flash chromatographed. No unreacted **2** was recovered. The yield of **23** was 0.1017 g (0.44 mmol) (24.6% yield).

Hidden GSH in Hemoglobin

In parallel experiments, mBBr, *syn*-(BrCH$_2$,CH$_3$) (CH$_3$,CH$_3$)B (**21**) was reacted with (1) PBS, pH 7.4, and with (2) Hb in PBS. An equivalent volume of 2% metaphosphoric acid, MPA, was then added to

each, the mixtures centrifuged and the supernatants extracted with dichloromethane. As a reference, *syn*-(CH_2SG,CH_3)(CH_3CH_3)B (**24**) was prepared by reacting **21** (0.030 mmol) in acetonitrile (0.5 mL) with GSH (0.028 mmol) in PBS, pH 7.4, (5 mL) for 30 min, then adding an equivalent volume of 2% MPA, and extracting the solution with dichloromethane. The dichloromethane extracts were examined by TLC (developer: ethyl acetate) and were the same for all experiments, the main spot being unreacted mBBr (**21**) and two minor fluorescent spots. The aqueous layers were examined by TLC on cellulose (developer: *i*-PrOH:water (76:24)) and visualized with ninhydrin. There was no evidence for the formation of **24** in either of the two experiments. GSH was also not detected in the Hb solution by Ellman's reagent.

REFERENCES

[1] A. E. Radkowsky, E. M. Kosower, D. Eisenberg, I. J. Goldberg, *J. Am. Chem. Soc., 108,* 1986, 4532.
[2] T. E. J. Creighton, *Mol. Biol. 129,* 1979, 411.
[3] A. H. Fairlamb, P. Blackburn, P. Ulrich, B. T. Chait, A. Cerami, *Science, 227,* 1985, 1485.
[4] S. L. Shames, A. H. Fairlamb, A. Cerami, C. T. Walsh, *Biochem., 25,* 1986, 3519.
[5] A. H. Fairlamb, *Parasitology, 99S,* 1989, S93.
[6] M. Manning, J. P. Przybylski, A. Olma, W. A. Klis, M. Kruszynski, N. C. Wo, G. H. Pelton, W. H. Sawyer, *Nature, 329,* 1987, 839.
[7] N. S. Kosower, E. M. Kosower, *Int. Rev. Cytol., 54,* 1978, 109.
[8] H. P. J. Misra, *Biol. Chem., 249,* 1974, 2151.
[9] M. Friedman, J. C. Zahnley, J. R. Wagner, *Anal. Bioch., 106,* 1980, 27.
[10] Y. Goto, K. J. Hamaguchi, *Biochem., 86,* 1979, 1433.
[11] L. G. Chavez, H. A. Scheraga, *Biochemistry, 19,* 1980, 1005.
[12] T. E. Creighton, *Method. Enzymol., 131,* 1986, 83.
[13] R. S. Asquith, M. S. Otterburn, *Adv. Exp. Med. Biol., 86B,* 1977, 93.
[14] M. Friedman, J. W. Finley, L. Yeh, *Adv. Exp. Med. Biol., 86B,* 1977, 213.
[15] M. Friedman, *Adv. Exp. Med. Biol., 105,* 1978, 613.
[16] M. S. Ellison, H. P. Lundgren, *Text. Res. I., 48,* 1978, 697.
[17] I. Strenken, H. Zahn, *Proc. Int. Wool Text. Res. Conf. 7th, 5,* 1985, 49.
[18] E. Tolgyesi, F. Fang, *Hair Res., [Proc. Int. Congr.] 1st,* 1979 (pub. 1981), 116.
[19] R. F. Hurrell, K. J. Carpenter, W. J. Sinclair, M. S. Otterburn, R. S. Asquith, *Br. J. Nutr., 35,* 1976, 383.
[20] A. E. Radkowsky, E. M. Kosower, *J. Am. Chem. Soc., 108,* 1986, 4527.
[21] H. Kanety, E. M. Kosower, *J. Org. Chem., 47,* 1982, 4222.
[22] N. S. Kosower, E. M. Kosower, *Methods Enzymol., 143,* 1987, 76.
[23] L. E. Overman, D. Matzinger, E. M. O'Connor, J. D. Overman, *J. Am. Chem. Soc., 96,* 1974, 6081.
[24] L. E. Overman, E. M. O'Connor, *J. Am. Chem. Soc., 98,* 1976, 771.
[25] N. S. Kosower, E. M. Kosower, *Method. Enzymol., 143,* 1987, 264.
[26] N. S. Kosower, E. M. Kosower: Functional Aspects of Glutathione Disulfide and Hidden Forms of Glutathione, in I. M. Arias, W. A. Jakoby (eds), *Glutathione: Physiological Aspects,* Raven Press, New York, pp. 159–174 (1976).
[27] A. E. Radkowsky, unpublished results.
[28] R. M. C. Dawson, D. C. Elliot, W. H. Elliot, K. M. Jones (eds), *Data for Biochemical Research,* 3rd Ed., Clarendon, Oxford, pp. 380–381 (1986).
[29] E. M. Kosower: Structure and Reactions of Thiols with Special Emphasis on Glutathione, in D. Dolphin, R. Poulson, O. Avramovic (eds), *Coenzymes and Cofactors, Volume III, Part A: Glutathione: Chemical, Biochemical and Medical Aspects,* Wiley, New York, pp. 103–146 (1989).
[30] U. Burkert, N. L. Allinger, *Molecular Mechanics,* ACS Monograph 177, Am. Chem. Soc., Washington, D. C., 1982.
[31] W. W. Cleland, *Biochemistry, 3,* 1964, 480.
[32] S. Capasso, Z. Zagari, *Acta Crystallogr., B37,* 1981, 1437.
[33] G. W. Kilmer, M. D. Armstrong, G. R. Brown, V. du Vigneaud, *J. Biol. Chem., 145,* 1942, 495.
[34] A. I. Kosak, R. L. Holbrook, *Science, 117,* 1953, 231.
[35] T. P. Doumani, U. S. 2,532,612 (1951), *Chem. Abs. 45,* (1951), 3868.
[36] T. E. Creighton, *J. Mol. Biol., 96,* 1975, 767.
[37] Z. Shaked, R. P. Szajewski, G. M. Whitesides, *Biochem., 19,* 1980, 4156.
[38] R. P. Szajewski, G. M. Whitesides, *J. Am. Chem. Soc., 102,* 1980, 2011.
[39] D. Marciano, M. Baud'huin, B. Zinger, I. Goldberg, E. M. Kosower, *J. Am. Chem. Soc., 112,* 1990, 7320.
[40] S. Simhony, E. M. Kosower, A. Katzir, *Applied Phys. Lett., 49,* 1986, 253.
[41] S. Simhony, E. M. Kosower, A. Katzir, *Biochem. and Biophys. Res. Comm., 142,* 1987, 1059.
[42] S. Simhony, A. Katzir, E. M. Kosower, *Anal. Chem., 60,* 1988, 1908.
[43] E. M. Kosower, B. Pazhenchevsky, *J. Am. Chem. Soc., 102,* 1980, 4983.
[44] E. M. Kosower, B. Pazhenchevsky, H. Dodiuk, H. Kanety, D. Faust, *J. Org. Chem., 46,* 1981, 1666.
[45] E. M. Kosower, B. Pazhenchevsky, H. Dodiuk, M. Ben-Shoshan, H. Kanety, *J. Org. Chem., 46,* 1981, 1673.