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7-*N*-(Mercaptoalkyl)mitomycins: Implications of Cyclization for Drug Function

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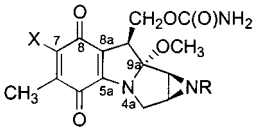
Abstract: The Kyowa Hakko Kogyo and Bristol-Myers Squibb companies reported that select mitomycin C(7) aminoethylene disulfides displayed improved pharmacological profiles compared with mitomycin C (**1**). Mechanisms have been advanced for these mitomycins that differ from **1**. Central to many of these hypotheses is the intermediate generation of 7-*N*-(2-mercaptoethyl)mitomycin C (**5**). Thiol **5** has been neither isolated nor characterized. Two efficient methods were developed for mitomycin (porfiromycin) C(7)-substituted thiols. In the first method, the thiol was produced by a thiol-mediated disulfide exchange process using an activated mixed mitomycin disulfide. In the second route, the thiol was generated by base-mediated cleavage of a porfiromycin C(7)-substituted thiol ester. We selected four thiols, 7-*N*-(2-mercaptoethyl)-mitomycin C (**5**), 7-*N*-(2-mercaptoethyl)porfiromycin (**12**), 7-*N*-(2-mercapto-2-methylpropyl)mitomycin C (**13**), and 7-*N*-(3-mercaptopropyl)porfiromycin (**14**), for study. Thiols **5** and **12**−**14** differed in the composition of the alkyl linker that bridged the thiol with the mitomycin (porfiromycin) C(7) amino substituent. Thiol generation was documented by HPLC and spectroscopic studies and by thiol-trapping experiments. The linker affected the structure of the thiol species and the stability of the thiol. We observed that thiols **5** and **12** existed largely as their cyclic isomers. Evidence is presented that cyclization predominantly occurred at the mitomycin C(7) position. Correspondingly, alkyl linker substitution (**13**) or extension of the linker to three carbons (**14**) led to enhanced thiol stability and the predominant formation of the free thiol species. The dominant reaction of thiols **5** and **12**−**14** or their isomers was dimerization, and we found no evidence that thiol formation led to mitosene production and aziridine ring-opening. These findings indicated that thiol generation was not sufficient for mitomycin ring activation. The potential pharmacological advantages of mitomycin C(7) aminoethylene disulfides compared with **1** is discussed in light of the observed thiol cyclization pathway.

The discovery of mitomycin C (**1**) and mitomycin A (**2**) in the late 1950s heralded a new era in cancer chemotherapy.[1,2] Findings documenting that the mitomycins[3] targeted oxygen-deficient cells provided new opportunities for cancer treatment through the use of bioreductive alkylating agents.[4] The clinical success of **1** led to intense efforts by medicinal chemists to prepare new mitomycins with improved pharmacokinetic and therapeutic properties. Over 1000 compounds have been synthesized and evaluated.[5] In the mid-1980s, the Kyowa Hakko Kogyo and the Bristol-Myers Squibb companies reported that mitomycin C(7) aminoethylene disulfides **3**[6] and **4**[7] displayed



1 X = NH_2, R = H

2 X = OCH_3, R = H

3 X = $N(H)(CH_2)_2SS(CH_2)_2N(H)C(O)(CH_2)_2CH(NH_2)CO_2H$, R = H (KW-2149)

4 X = $N(H)(CH_2)_2SS$–C$_6$H$_4$–NO_2, R = H (BMS-181174)

5 X = $N(H)(CH_2)_2SH$, R = H

12 X = $N(H)(CH_2)_2SH$, R = CH_3

13 X = $N(H)CH_2C(CH_3)_2SH$, R = H

14 X = $N(H)(CH_2)_3SH$, R = CH_3

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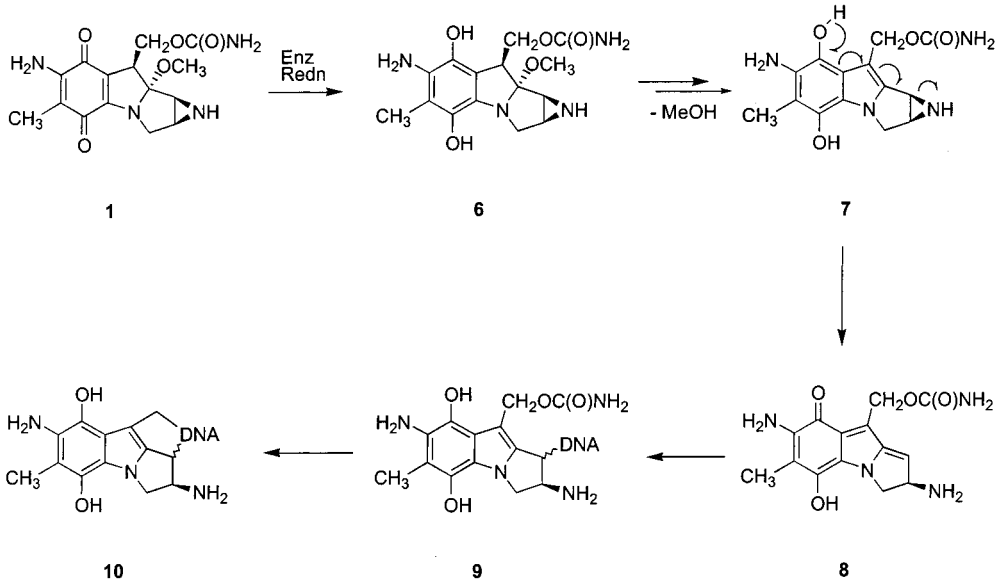
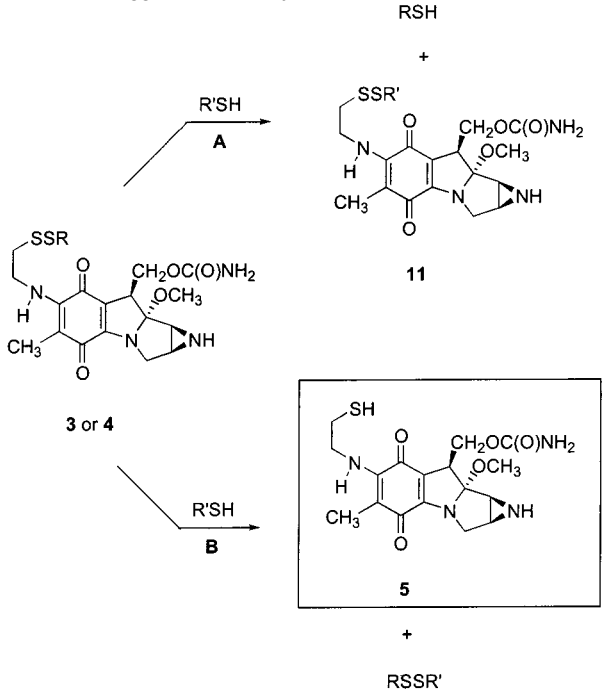
Scheme 1. Proposed Mechanism for the Mode of Action of Mitomycin C



improved pharmacological profiles compared with **1**.[8,9] Significantly, **3** was active in both **1**-resistant P388 and *non*-hypoxic cells.[10] Both **3** and **4** were entered into clinical trials, and **3** advanced to phase II testing.

Suggestions were made that the therapeutic advantage of **3** and **4** stemmed from the mitomycin C(7) disulfide unit, the disulfide-cleaved product **5**, or both[11−16] and that the mode of action of **3** and **4** differed from the accepted enzymatic reductive activation mechanism for **1**[3b,c,17,18] (Scheme 1). Studies showed that administering **3** to tumor-bearing mice led to the serum albumin conjugate **11** (R′S = serum albumin) (Scheme 2, route A) and that **11** resided for longer periods in the plasma than **3**.[14] In addition, mechanistic investigations suggested that glutathione (GSH)-induced cleavage of **3** and **4** led to **5** (Scheme 2, route B), and that **5** can *initiate* drug−DNA adduction processes.[15,16]

Scheme 2. Suggested Pathways for **3** and **4** Function



Central to many theories concerning **3** and **4** function is thiol **5**. 7-*N*-(2-Mercaptoethyl)mitomycin C (**5**) has been neither isolated nor characterized.[13] In this paper, we provide details on the formation of **5** and related thiols **12**−**14** and document the reactivity of mitomycin C(7)-substituted thiols. Our findings permit us to speculate on the importance of the C(7) aminoethylene disulfide unit in mitomycins **3** and **4** for drug function.

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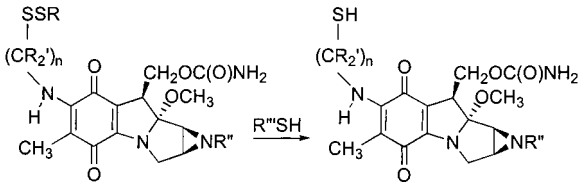
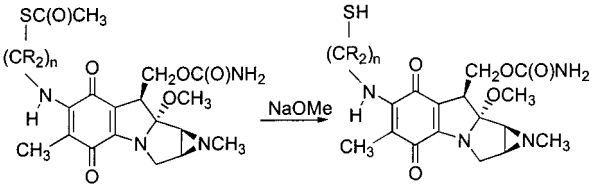
Scheme 3. Synthetic Approaches to Mitomycin (Porfiromycin) Thiols

Route A: Thiol-Mediated Disulfide Cleavage Reaction

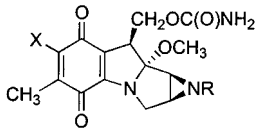


Route B: Base-Mediated Thiol Ester Cleavage Reaction



Results

1. Experimental Design and Choice of Substrates. We designed mitomycins that upon chemical activation gave mitomycin C(7)-substituted thiols. Two general pathways were developed (Scheme 3, routes A and B). In one route, mitomycins (porfiromycins) **15** containing an activated C(7) disulfide unit were prepared and treated with an external thiol (R‴SH) to initiate disulfide exchange[19] to provide thiol **16** (Scheme 3, route A). This pathway is similar to the drug activation steps[11−13] proposed for KW-2149 (**3**) and BMS-181174 (**4**) (see Scheme 2, route B). D,L-Dithiothreitol (D,L-DTT) was the external thiol of choice because of its reducing power and because most D,L-DTT reactions proceed to completion.[20] We also employed L-DTT, GSH, and *N*,*N*′-dimethyl-*N*,*N*′-bis(mercaptoacetyl)-hydrazine[21] (DMH) (data not shown) in the disulfide cleavage experiments. In the second pathway, porfiromycin **17** containing a thiol ester moiety within the C(7) unit were prepared and then the thiol ester bond cleaved with base[22] (NaOMe) to yield the porfiromycin C(7)-substituted thiol **18** (Scheme 3, route B). Porfiromycin[3b] (**19**) is the *N*(1a)-methyl derivative of **1**. We introduced a methyl group at N(1a) in **17** to prevent base-mediated rearrangement to the isomeric albomitomycin analogue.[23] The thiol-generating step (**15** → **16**, **17** → **18**) for the two protocols distinguished and defined their utility and

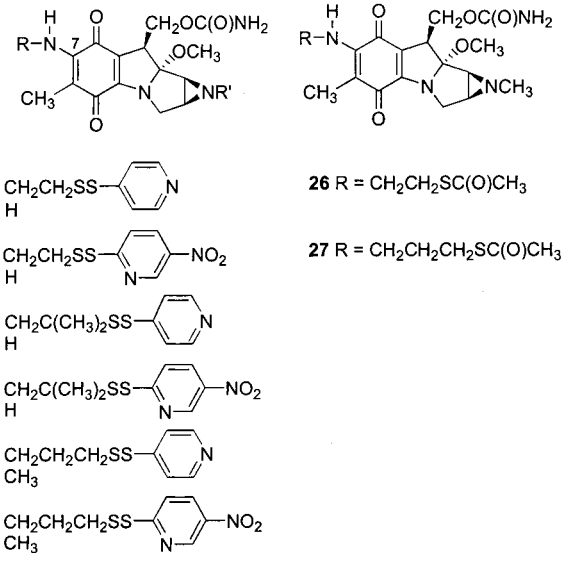


19 X = NH_2, R = CH_3

47 X = OCH_3, R = CH_3

limitations. The DTT-mediated cleavage of **15** to give thiol **16** proceeded at near-neutral "pH" values in MeOH with the concomitant release of a substituted thiopyridone readily detected by HPLC. In these reactions, however, complex product profiles were sometimes observed due to the presence of thiols (DTT, substituted thiopyridines) that reacted with the generated mitomycin thiol **16**. Correspondingly, NaOMe cleavage of thiol ester **17** gave **18** at low temperatures without production of competing thiols, but these reactions proceeded only under basic conditions.[24]

Mitomycins (porfiromycins) **20**−**27** were prepared to provide information concerning the structure and reactivity of mitomycin (porfiromycin) C(7)-substituted thiols. These eight mitomycins



(porfiromycins) can be classified by the C(7) terminal substituent (disulfide, thiol ester) and the structural composition of the linker that bridged the C(7) terminal group with the mitomycin (porfiromycin) C(7) amino substituent. Compounds **20**−**25** each contained a terminal disulfide unit while **26** and **27** each possessed a terminal thiol ester moiety. In **20**, **21**, and **26**, we maintained the ethylene bridge found for **3** and **4**, and in **22**−**25** and **27**, we either modified the ethylene bridge by methyl substitution (**22**, **23**) or by extension of the ethylene linker to a propylene group (**24**, **25**, **27**). Alterations in the ethylene linker were made to enhance the stability of the generated free thiol.

2. Syntheses. Compounds **20**−**23** were prepared from **2** using a modified procedure reported by Vyas and co-workers[25] for mitomycin C(7)-substituted disulfides. This route (Scheme 4) benefited from the rapid synthesis of the intermediate mixed disulfides and proceeded in two steps to a 42−90% overall yield.

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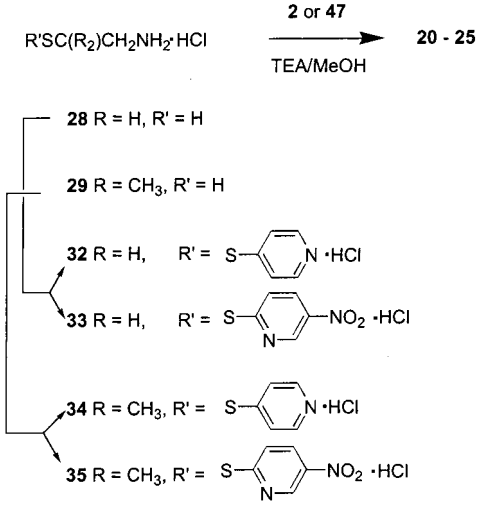
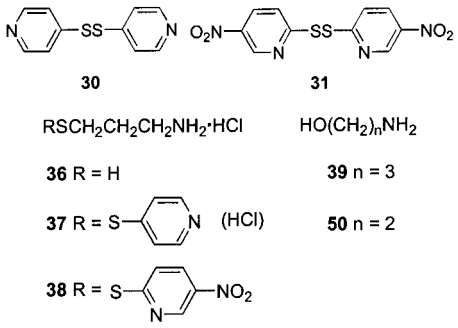
(24) Use of lower levels of NaOMe (e.g., "pH" 9) led to either no or low levels of reaction. Similarly, use of NH_2NH_2 or NH_2OH in place of NaOMe was unsuccessful.

Scheme 4. Preparative Pathways for Mitomycin Disulfides **20**−**25**



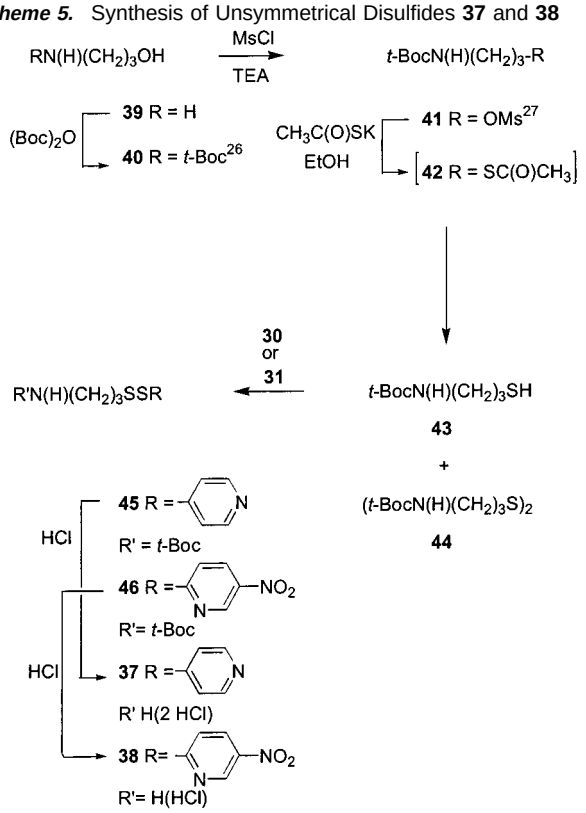
Disulfides **32**−**35** were prepared by treating the aminoalkanethiol hydrochlorides **28** and **29** with either 4,4′-dithiodipyridine (**30**) or 2,2′-dithiobis(5-nitropyridine) (**31**). Use of excess



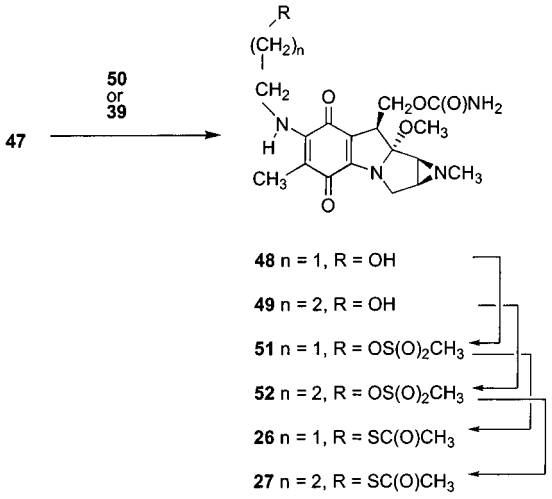
30 and **31** and the incremental addition of the aminoalkanethiol hydrochloride (**28**, **29**) to a dilute alcoholic solution of the symmetrical disulfide (**30**, **31**) under Ar ensured the quantitative formation of the mixed disulfides (**32**−**35**) and the prevention of the disulfide from the aminoalkanethiol hydrochloride. The reaction mixture was not purified but was immediately treated with either **2** or **47**. Since 3-aminopropanethiol hydrochloride (**36**) was not commercially available, we prepared mixed disulfides **37** and **38** by an alternative route beginning with 3-aminopropanol (**39**) (Scheme 5). Treatment of mitomycin F[28] (**47**) with **37** and **38** gave porfiromycin C(7)-substituted disulfides **24** and **25**, respectively.

Porfiromycins **26** and **27** were prepared using the four-step procedure outlined in Scheme 6. Mitomycin F[28] (**47**) was converted to the porfiromycin derivatives **48**[29] and **49**[30] with ethanolamine (**50**) and 3-aminopropanol (**39**), respectively. Treatment of **48** and **49** in THF with methanesulfonyl chloride and triethylamine yielded mesylates **51** and **52**, respectively. Com-

Scheme 5. Synthesis of Unsymmetrical Disulfides **37** and **38**



Scheme 6. Preparative Pathway for Porfiromycin Thiol Esters **26** and **27**



pounds **51** and **52** proved too unstable to permit chromatographic purification. Accordingly, crude **51** and **52** were treated with thiolacetic acid and triethylamine to give **26**[29] and **27**. The overall yield of **26** and **27** from **2** (the precursor of **47**) was 40−57%.

3. Mitomycin (Porfiromycin) C(7)−Substituted Thiols That Cyclize. 3.1. Thiol-Mediated Disulfide Cleavage of 20 and 21: Attempts To Prepare Thiol 5. 3.1.1. The HPLC Profile. We first prepared thiol **5**. Accordingly, **20** and **21** were treated with D,L-DTT. We varied the temperature (0 to −78

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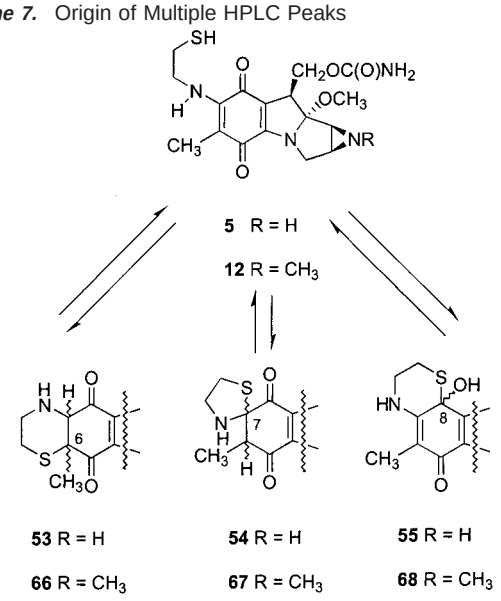
Scheme 7. Origin of Multiple HPLC Peaks



5 R = H

12 R = CH_3

53 R = H **66** R = CH_3

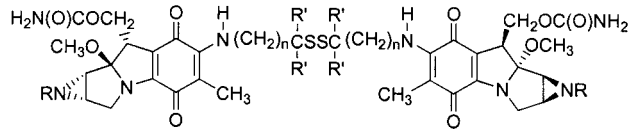
54 R = H **67** R = CH_3

55 R = H **68** R = CH_3

°C), the ratio of D,L-DTT to mitomycins **20** and **21** (1−11:1), the sequence of addition of mitomycin C(7)-substituted disulfide (**20**, **21**) and D,L-DTT, the solvent (methanol, buffered methanol, acetone), the "pH" of the methanolic solution (5.5, 6.5), and the concentration of the mitomycin in solution (0.1−100 mM). The experiments were monitored by HPLC (200−400 nm, photodiode array detection), and select products were verified by comparison with authentic samples. Treatment of a deaerated (Ar) methanolic (−78 °C) solution containing **21** and D,L-DTT (10 equiv) led to the complete consumption of **21** (t_R 32.1 min) and the appearance of three peaks in the HPLC profile (365 nm) between t_R 22.1−23.9 min (designated **5** and **53**−**55**, Scheme 7; see figure in Supporting Information and ref 13). These peaks accounted for 40−80% of the mitomycin profile, and the reaction profile remained essentially unchanged at −78 °C for ∼3 days. The same peaks between t_R 22.1−23.9 min were observed when we used DMH in place of DTT, acetone for methanol, and **20** in place of **21** (data not shown). All reactions gave detectable amounts of the mitomycin C(7) symmetrical disulfide **56**[31] (t_R 28.4 min). In the D,L-DTT-mediated reactions, additional peaks were observed at t_R 26.1 (**57**), 26.6 (**58**), 28.0,[32a] and 30.3[32b] min. The relative amounts of these adducts in the product profile depended on the reaction conditions. Use of more concentrated solutions and high molar equivalents of D,L-DTT led to increased yields of the t_R 26.1 and 26.6 min compounds, which approached 30% of the total mitomycin HPLC profile. Interestingly, the relative amounts of the t_R 26.1 min (**57**) peak was consistently higher than the 26.6 min (**58**) peak, an observation that may reflect the relative stabilities of these two products.

3.1.2. Analysis of the HPLC Profile: The t_R 22.1−23.9, 26.1, and 26.6 min Peaks. The identities of the t_R 22.1−23.9, 26.1, and 26.6 min HPLC peaks were puzzling. We expected a single peak for thiol **5**. We tentatively assigned the t_R 22.1−23.9 min HPLC peaks to thiol **5** and/or the corresponding C(6) (**53**), C(7) (**54**), and C(8) (**55**) cyclic isomeric adducts (Scheme 7).

We sought additional evidence that the t_R 22.1−23.9 min HPLC peaks were associated with isomeric forms of **5**. Attempts to isolate these peaks gave **56**. Verification of **56** was accomplished by reacting **2** with cysteamine hydrochloride (**28**) or cystamine dihydrochloride (**59**).[31] We characterized thiol **5**

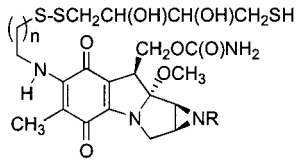


56 n = 1, R = H, R' = H

69 n = 1, R = CH_3, R' = H

73 n = 1, R = H, R' = CH_3

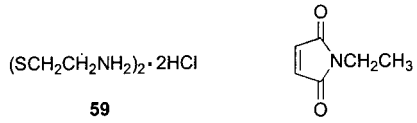
74 n = 2, R = CH_3, R' = H



57 L-DTT, n = 1, R = H

58 D-DTT, n = 1, R = H

77 L-DTT, n = 2, R = CH_3



59 **60**

and/or cyclized adducts **53**−**55** using the derivatization reagents **30**, **31**, and *N*-ethylmaleimide (NEM) (**60**).

3.1.3. Thiol 5 Derivatization Studies Using 30, 31, and 60. Addition of either **30** or **31** to the D,L-DTT-treated **20** and **21** reactions at room temperature and below led to the loss of the t_R 22.1−23.9 (**5**, **53**−**55**), 26.1 (**57**), and 26.6 (**58**) (when observed) min HPLC peaks and the generation of either **20** or **21** as the major mitomycin product (HPLC analyses; see figure in Supporting Information and ref 13). These findings indicated that the t_R 22.1−23.9, 26.1, and 26.6 min HPLC peaks can be converted to thiol **5** and then trapped as thiol adducts **20** and **21** under the derivatization conditions. Since the t_R 26.1 and 26.6 min peaks were not observed when we used DMH in place of D,L-DTT these compounds are likely to be D,L-DTT adducts[33] that undergo further change prior to derivatization.

We attempted without success to isolate the t_R 26.1 (**57**) and 26.6 (**58**) min HPLC compounds by treating either **20** or **21** with D,L-DTT on a semipreparative scale. Additional information concerning the structure of these compounds was obtained by carrying out a parallel experiment with L-DTT in place of D,L-

(31) (a) Kono, M.; Saito, Y.; Goto, J.; Shirahata, K.; Morimoto, M.; Ashizawa, T. Mitomycin Analogs (Kyowa Hakko Kogyo Co., Ltd.) Eur. Pat. Appl. EPO 116,208A1, August 22, 1984; *Chem. Abstr.* **1985**, *102*, 6059. (b) Senter, P. D.; Langley, D. R.; Manger, W. E.; Vyas, D. M. *J. Antibiot.* **1988**, *41*, 199−201.

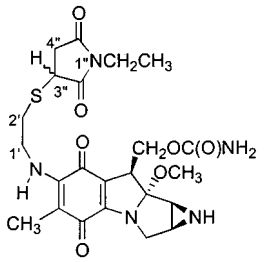
(32) (a) We have tentative information concerning the structure of the 28.0 min HPLC peak. Repetition of this reaction under semipreparative conditions led to the isolation of two compounds corresponding to this signal whose HPLC retention times (instrument B) corresponding to 27.6 and 27.7 min. Mass spectroscopic studies indicated that these adducts were the diastereomeric DTT-bridged mitomycin disulfides. For the 27.6 min HPLC adduct, MS (+ES) *m/e* 939.0 $[M + H]^+$ (23%), 470.1 $[M + 2H]^{2+}$ (100%); for the 27.7 min HPLC adduct, MS (+ES) *m/e* 938.8 $[M + H^+]^+$ (8%), 469.9 $[M + 2H]^{2+}$ (100%). (b) We have not identified this adduct.

(33) For structurally related adducts, see: Li, Y.-J.; Rothwarf, D. M.; Scheraga, H. A. *J. Am. Chem. Soc.* **1998**, *120*, 2668−2669.

DTT. L-DTT gave an HPLC profile nearly identical to that of D,L-DTT except that only the t_R 26.1 min peak (**57**) was detected. Co-injection of the D,L-DTT and L-DTT reactions suggested that the t_R 26.1 min adduct **57** was derived from L-DTT. These cumulative results indicate that the t_R 26.1 and 26.6 min peaks are the L- and D-diastereomeric mixed disulfides **57** and **58**, respectively, and that both compounds can be converted to thiol **5** and then trapped with disulfides **30** and **31**.

We also tested whether **20** or **21** produced in the thiol derivatization experiments originated from **56** present in the product mixture. Treatment of **56** with D,L-DTT (1−2 equiv) followed by either **30** (1.4 equiv) or **31** (3 equiv) at room temperature yielded **20** and **21**, respectively. However, the rate of conversion of **56** to either **20** or **21** was measurably slower (room temperature, 1−3 days) than the corresponding rate of **20** and **21** production after either **30** or **31** was added to the D,L-DTT-treated **20** and **21** reactions (room temperature, <2 min). This demonstrated that **56** was not the precursor to these adducts.

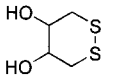
We obtained similar findings when we used **60** in place of either **30** or **31** in the thiol derivatization experiments. Addition of **60** to D,L-DTT-treated methanolic solutions of **20** and **21** led to the disappearance of the t_R 22.1−23.9 (**5, 53**−**55**), 26.1, (**57**), and 26.6 (**58**) min HPLC peaks and the concomitant generation of two HPLC peaks at t_R 25.4 and 26.0 min corresponding to NEM-substituted adducts **61** and **62,** respectively.



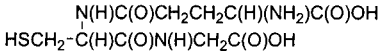
61 3"*S* or 3"*R*

62 3"*R* or 3"*S*

3.2. GSH-Mediated Disulfide Cleavage of 20 and 21. Compounds **57** and **58** are the expected initial products for D,L-DTT-mediated cleavage of **20** and **21** along with the released thiopyridinone. Few DTT-based disulfide adducts have been reported[33] since subsequent intramolecular cyclization and disulfide cleavage to give oxidized DTT (**63**) is energetically favor-



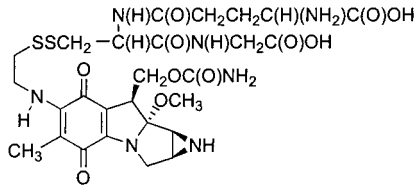
63



64

able. Our evidence that the t_R 26.1 and 26.6 min HPLC peaks are **57** and **58** is circumstantial and is based on the apparent conversion of these products to **20**, **21**, **61**, and **62** upon addition of the thiol derivatization agent and the detection of a single DTT-bound adduct **57** with L-DTT. Accordingly, we obtained additional support for mixed disulfide **57** and **58** formation. Mitomycin C(7) aminoethylene disulfides **20** and **21** were treated with a thiol that would generate a mixed disulfide product capable of isolation and characterization. We chose GSH (**64**). This cellular thiol has special significance in light of the mechanistic proposals, suggesting GSH initiates **3** and **4** function (Scheme 2, route B).[15,16]

Use of GSH (10 equiv) for mitomycin disulfide **20** and **21** cleavage in MeOH (22 °C) led to the formation of a new HPLC product at t_R 18.0 min (**65**) (>85%). It was significant that we

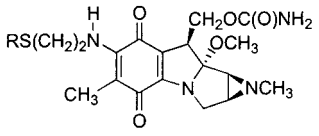


65

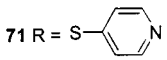
observed only low amounts of dimer **56** (~8%). By repeating the reaction on the preparative scale using **20** followed by chromatographic purification, we isolated and characterized the t_R 18.0 min compound as the mitomycin-GSH mixed disulfide **65**.

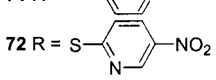
3.3. NaOMe-Mediated Cleavage of 26: Attempts to Prepare Thiol 12. Our assignment of the t_R 22.1−23.9 min HPLC peaks in the DTT-mediated cleavage reactions of **20** and **21** to thiol **5** and isomeric cyclized adducts **53**−**55** required further support. We sought an independent route to a comparable species and determined whether base cleavage of thiol ester **26** provided **12**. This method would generate **12** in the absence of competing thiols, such as DTT and thiopyridone byproducts.

Cleavage of the thiol ester bond in **26** was accomplished using NaOMe at −78 °C under dilute conditions. The HPLC product profile (see figure in Supporting Information and ref 13) was cleaner than those recorded for the D,L-DTT-cleavage reactions of **20** and **21**. We observed three peaks between t_R 23.9 and 26.6 min; these HPLC signals have been assigned to thiol **12** and/or the corresponding isomeric forms **66**−**68** (Scheme 7). We attributed the longer retention times (2−3 min) of **12** and **66**−**68** compared with **5** and **53**−**55** to the increased hydrophobicity of the porfiromycin caused by *N*(1a)-methylation.[34] Low levels (~10%) of the dimeric porfiromycin disulfide **69** (t_R 31.2 min) were observed along with an unidentified peak (t_R 29.1 min, 7−18%).[35] Noticeably absent from the HPLC profile for the base-mediated reactions were the corresponding later retention time peaks (t_R >26 min) assigned to thiol- D,L-DTT adducts (**57**, **58**). We monitored (HPLC) the base cleavage of **26** against time. The reaction occurred rapidly under these conditions (<1 h), and the product profile remained largely unaltered for 3 days. We verified **69** in the HPLC profile by preparing an authentic sample by treating **47** with cystamine dihydrochloride (**59**).[31a] The major product isolated was **70**.



70 R = $S(CH_2)_2NH_2$

71 R = 

72 R = 

(34) A similar difference in HPLC retention times was observed for mitomycin C (16.7 min) and porfiromycin (18.1 min).

(35) The 29.1 min HPLC peak has not been identified and may correspond to a cyclic adduct. Addition of trapping reagents led to the disappearance of this adduct.

Evidence supporting that the t_R 23.9−26.6 min HPLC peaks corresponded to thiol **12** and/or **66**−**68** was provided by thiol−disulfide exchange reactions. Successive treatment of a methanolic solution of **26** with NaOMe followed by **30** led to the loss of the three peaks between t_R 23.9−26.6 min and the t_R 29.1 min signal and gave **71** (t_R 30.2 min) as the sole porfiromycin product (HPLC analysis, see figure in Supporting Information). Similarly, **26** furnished **72** (t_R 34.4 min) after treatment with base and **31**. The identities of **71** and **72** were confirmed by co-injection with authentic samples prepared by treatment of **47**[28] with mixed disulfides **32** and **33**, respectively.

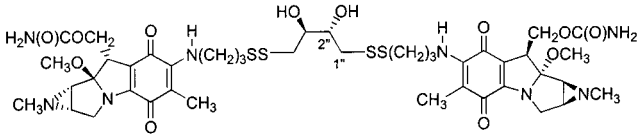
3.4. Correlation of the DTT- and NaOMe-Mediated Routes to Mitomycin (Porfiromycin) Thiols 5 and 12. Multiple HPLC peaks associated with thiols **5** (t_R 22.1−23.9 min) and **12** (t_R 23.9−26.6 min) were observed after DTT and NaOMe treatment of mitomycins **20** and **21** and porfiromycin **26**, respectively. We confirmed that these HPLC peaks were comparable by treating methanolic solutions of porfiromycin mixed disulfides **71** and **72** with D,L-DTT (−78 °C). Under these conditions, we observed the same peaks (t_R 23.9−26.6 min) obtained with thiol ester **26** and NaOMe. Furthermore, addition of either **30** or **31** to these D,L-DTT cleaved products led to the disappearance of the multiple HPLC peaks (t_R 23.9−26.6 min) and the production of **71** and **72**, respectively.

4. Mitomycin (Porfiromycin) C(7)-Substituted Thiols That Do Not Cyclize. 4.1. Introduction. The DTT- and NaOMe-generated HPLC products for mitomycin (porfiromycin) C(7) aminoethanethiols **5** and **12** were assigned as a mixture of free thiol and cyclic adducts. Attempts to isolate **5** and **12** gave only symmetrical disulfides **56** and **69**, respectively. We sought to prepare thiols that do not cyclize and so targeted **13** and **14**. In **13**, we placed a *gem*-dimethyl unit adjacent to the thiol moiety, and in **14**, we incorporated a propylene bridge between the thiol unit and the porfiromycin C(7) amino substituent. We reasoned in **13** that the *gem*-dimethyl unit would sterically impede quinone cyclization processes (C(6)−C(8)) and diminish the rate of free thiol dimerization to symmetrical disulfide **73**, while in **14** the propyl side chain would entropically diminish intramolecular cyclization processes.

4.2. Mitomycin Thiol 13: Effect of the *Gem*-Dimethyl Unit. We generated **13** in acetone at room temperature using **22** and D,L-DTT. Mixed disulfide cleavage proceeded at a rate considerably slower than that observed for **20** and **21**. Moreover, the **22** HPLC product profile for this transformation (see figure in Supporting Information) was considerably cleaner than previously observed for mitomycin disulfides **20** and **21**. A single major mitomycin peak was observed at t_R 25.1 min along with two minor peaks at t_R 23.2 and 27.0 min. We have tentatively assigned the t_R 25.1 min peak to thiol **13**. No mitomycin dimer **73** was produced, and little differences in the HPLC profile were observed after 1 day of storage at room temperature. Attempts to isolate thiol **13** by preparative TLC were unsuccessful. We found evidence that the three HPLC peaks at t_R 23.2, 25.1 and 27.0 min were associated with free thiol **13** when we sequentially treated **22** with D,L-DTT (acetone, room temperature) and then with excess **31** to give quantitatively **23** (HPLC analysis). We were unable to cleave the mixed disulfide bond in **23** with D,L-DTT (acetone, room temperature) (HPLC and TLC analyses).

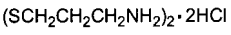
4.3. Porfiromycin Thiol 14: Effect of the Propylene Unit. We sought to prepare free thiol **14** using the same strategies implemented for **5** and **12**.

4.3.1. L-DTT-Mediated Disulfide Cleavage Reactions: Preparation of Thiol 14. When we added a methanolic L-DTT (2 equiv) solution to a deaerated (Ar) methanolic solution of **25**, we saw a simple HPLC profile consisting of three principal porfiromycin products: one corresponding to **14** (t_R 27.2 min), one to the porfiromycin dimer **74** (t_R 33.1 min), and a third compound whose HPLC retention time was t_R 31.7 min (**75**)



75

(see figure in Supporting Information) along with an unidentified minor adduct. We confirmed formation of **74** by co-injection (HPLC) and cospot (TLC) of the reaction mixture with an authentic sample. Repetition of the reaction with **24** in place of **25** provided similar results. We prepared an authentic sample of symmetrical disulfide **74** by treating **47** with 3-amino-1-propyl disulfide·2HCl (**76**) in methanolic TEA solution that was

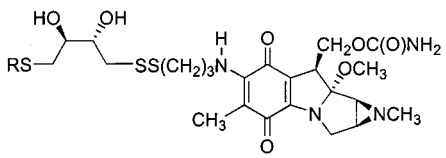


76

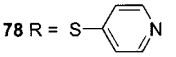
not deaerated. Compound **76** was prepared from **44** by *t*-Boc deprotection with HCl.

Repeating the reaction of **25** with L-DTT on a semipreparative scale followed by chromatographic purification of the reaction mixture permitted isolation of the t_R 31.7 min compound **75**. We have assigned this compound as the L-DTT-bridged bis-disulfide **75** on the basis of ^{1}H NMR, ^{13}C NMR, UV−visible, and mass spectral data. This compound is a structural analogue of the suspected DTT-bridged adducts[32] (t_R 28.0 min) observed in DTT-mediated cleavage reactions beginning with either **20** or **21**.

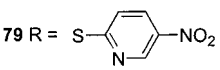
We determined the effect of solution "pH" (buffered methanolic solutions) on the L-DTT-mediated cleavage process for **25**. The reaction proceeded faster at "pH" 7.4 than at "pH" 5.5. Furthermore, we observed that at "pH" 5.5 (70 min), the relative yield of the L-DTT-bridged bisporfiromycin disulfide **75** (61%) increased and the yield of porfiromycin thiol **14** (19%) decreased, compared with the "pH" 7.4 transformation (5 min) (**75**, 35%; **14**, 61%). These results are consistent with the notion that L-DTT cleavage of **25** first generated L-DTT-porfiromycin disulfide **77**, which is similar to **57**. We suspect that **77** is more



77 R = H



78 R =



79 R =

stable at "pH" 5.5 than at "pH" 7.4, leading to higher levels of the coupled product **75**. Correspondingly, at "pH" value 7.4,

77 undergoes accelerated intramolecular cyclization to give **14** and oxidized DTT (**63**).

4.3.2. Thiol 14 Derivatization Studies Using 30 and 31. Chemical derivatization experiments provided support for the assignment of the t_R 27.2 min HPLC peak as free thiol **14**. HPLC analysis of a methanolic solution (0 °C) of **25** sequentially treated with L-DTT and then **30** showed two major peaks at t_R 30.5 and 32.2 min. The latter peak has been assigned as **24** and was confirmed by co-injection (HPLC) of the reaction solution with an authentic sample. We identified the t_R 30.5 min adduct by repeating the reaction on a semipreparative scale. Spectroscopic analysis of the isolated product was consistent with the mixed bisdisulfide **78**.

These findings indicated that **77** generated after L-DTT cleavage of **25** is, in part, converted to **14** and then trapped by **30** to give **24** and, in part, reacts with **30** to afford **78**. We repeated the thiol disulfide exchange experiment with **24** in place of **25** and **31** in place of **30**. We observed similar results and the appearance of a new peak (t_R 33.9 min) that has been tentatively assigned as **79**, on the basis of its retention time. This peak eluted ~3.4 min later than the **78** peak (t_R 30.5 min). The HPLC retention time difference between **78** and **79** is nearly the same found for **24** (t_R 32.2 min) and **25** (t_R 35.2 min). We did not further characterize the t_R 33.9 min HPLC peak.

4.3.3. NaOMe-Mediated Cleavage of 27: Preparation of Thiol 14. Porfiromycin thiol **14** was generated from methanolic solutions of **27** with NaOMe at −78 °C. The HPLC product profile for this reaction (see figure in Supporting Information) differed from the corresponding reaction of **26**. For **26**, we observed multiple peaks while **27** gave two major peaks at t_R 28.1 and 33.8 min in an approximate 7:3 ratio, respectively, along with a minor signal at t_R 35.6 min. We assigned the t_R 28.1 min peak to thiol **14** and the t_R 33.8 min signal to the symmetrical disulfide **74**. Thiol ester bond cleavage in **27** occurred rapidly at −78 °C, and the product profile remained largely unchanged after 1 day. Elevation of the reaction temperature from −78 °C to room temperature converted thiol **14** to disulfide **74**.

4.3.4. Thiol Derivatization Studies Using 30 and 31. Thiol derivatization reactions provided support that the t_R 28.1 min peak corresponded to free thiol **14**. First, treatment of methanolic solution of **27** with NaOMe followed by **30** at −78 °C gave **24** and **74**. The identities of **24** and **74** were confirmed by co-injection of authentic samples with the reaction mixture. HPLC analysis of the reaction both before and after **30** was added indicated that the relative amount of dimer **74** was not affected by **30** addition. An analogous reaction was conducted using **31** in place of **30** to provide **25** and **74** (see HPLC profile (figure) in Supporting Information).

4.3.5. Structural Characterization of Thiol 14. The simplicity of the HPLC product profile for the NaOMe-mediated cleavage of **27** permitted us to characterize thiol **14** by UV−visible and ^{1}H NMR spectroscopy. The UV−visible spectrum obtained by HPLC photodiode array detection showed two major absorptions at ~220 and ~370 nm in a near 1:1 ratio. This spectrum is similar to that observed for porfiromycin (**19**).

We modified our experimental procedure to permit characterization of **14** by ^{1}H NMR spectroscopy. A $NaOCD_3$−CD_3OD solution was added to a concentrated CD_3OD solution containing **27** at −78 °C and then the solution temperature raised to −4 °C and monitored by ^{1}H NMR spectroscopy. The initial ^{1}H NMR spectrum after 0.5 h showed the consumption of 7-*N*-(3-acetylthiopropyl)porfiromycin (**27**) and the production of **14** (Figure 1a). In particular, we observed the disappearance of the characteristic thioacetyl signal (δ 2.28) in **27** and the appearance of the C(2′) and the C(3′) methylene protons at δ 1.85 and 2.56 as multiplet and triplet signals, respectively. These values (δ 1.85, 2.56) are upfield from comparable resonances for the symmetrical porfiromycin disulfide **74** (δ 1.99, 2.75). This difference in chemical shift values is diagnostic of thiol and disulfide structures.[36] Maintenance of the basic NMR solution for 9 h led to the gradual loss of the signals associated with **14** and the quantitative production of disulfide **74** (Figure 1b). The conversion of **14** to **74** prevented us from acquiring the ^{13}C NMR spectrum of **14**.

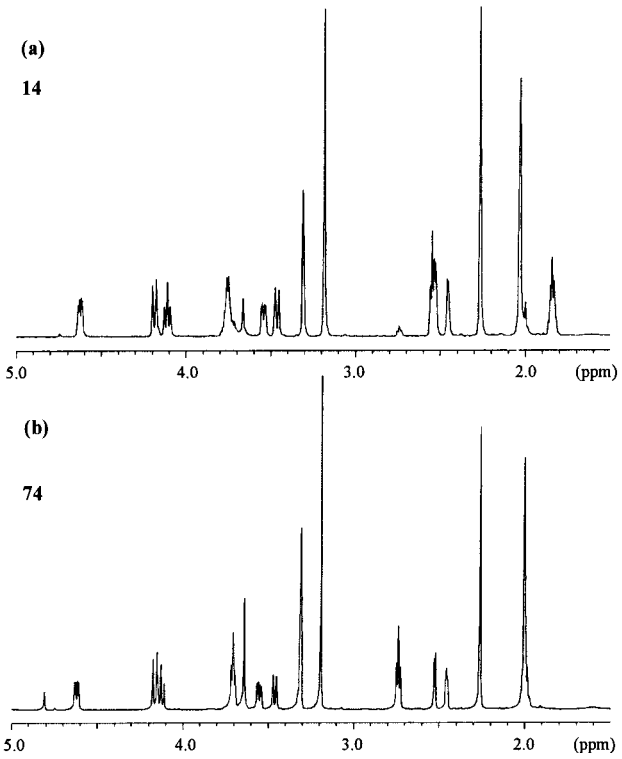


Figure 1. (a) ^{1}H NMR spectrum of 7-*N*-(3′-mercaptopropyl)porfiromycin (**14**) in CD_3OD. (b) ^{1}H NMR spectrum of 7-*N*,7′-N-dithiobis(3,1-propanediyl)bisporfiromycin (**74**) in CD_3OD.

Discussion

Synthesis of Mitomycin (Porfiromycin) C(7)-Substituted Thiols. We utilized two methods that enabled us to generate mitomycin (porfiromycin) C(7)-substituted thiols, isomers, or both. The first method was a thiol-mediated disulfide exchange process[19] using an activated mixed mitomycin (porfiromycin) disulfide (Scheme 3, route A). Incorporation of either the 4-thiopyridine (**20**, **22**, **24**, **71**) or the 5-nitro-2-thiopyridine (**21**, **23**, **25**, **72**) unit in the mitomycin (porfiromycin) disulfide permitted selective and rapid disulfide cleavage with external thiols (e.g., DTT). The second route was the base-mediated cleavage of thiol esters[22] (Scheme 3, route B). Accordingly, we incorporated a thiol acetyl unit at the C(7) terminal end in **26** and **27**, which permitted efficient NaOMe cleavage.

(36) Pretsch, E.; Clerc, T.; Seibl, J.; Simon, W. *Tables of Spectral Data for Structure Determination of Organic Compounds*, 2nd ed. Springer-Verlag Berlin, 1989.

In total, four different thiols were selected for preparation and evaluation. The first was 7-*N*-(2-mercaptoethyl)mitomycin C (**5**), which corresponded to the putative intermediate generated in the KW-2149 (**3**) and BMS-181174 (**4**) activation processes[11−13] (Scheme 2, path B). For this thiol, we used a thiol-mediated disulfide exchange reaction using either **20** or **21**. The second was the porfiromycin analogue of **5**, 7-*N*-(2-mercaptoethyl)porfiromycin (**12**). To produce this thiol, we employed both a thiol-mediated disulfide exchange reaction using **71** and **72** and the base-mediated thiol ester cleavage route using **26**. The final two compounds were the *gem*-dimethyl analogue of **5**, 7-*N*-(2-mercapto-2-methylpropyl)mitomycin C (**13**) and the **12** homologue 7-*N*-(3-mercaptopropyl)porfiromycin (**14**). Synthesis of **13** was accomplished using a thiol-mediated disulfide exchange process from **22** while both synthetic strategies (thiol-mediated disulfide cleavage of **24** and **25**, base-mediated thiol ester cleavage of **27**) were employed to generate **14**.

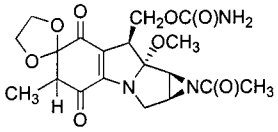
Each thiol preparative method had advantages and disadvantages. The thiol-mediated disulfide exchange process (Scheme 3, route A) proceeded rapidly in methanolic and acetone solutions at low temperatures (≤0 °C) for **20**, **21**, **24**, **25**, **71**, and **72**. Elevated temperatures (22 °C) were needed for *gem*-dimethyl derivative **22**. Moreover, when we replaced the 4-thiopyridone-leaving group in **22** with the 5-nitro-2-thiopyridone to give **23**, we observed no reaction upon DTT addition at room temperature. DTT-mediated thiol cleavage occurred at "pH" 5.5, 6.5, and 7.4 in MeOH. These reaction parameters allowed us to monitor mitomycin (porfiromycin) thiol generation and subsequent processes under mild conditions. Important limitations of this protocol, however, were the need to use excess external thiol (3−11 equiv) at low temperatures to generate the mitomycin (porfiromycin) thiol and the introduction into the reaction solution of multiple external thiols (e.g., DTT, 4-thiopyridine, 5-nitro-2-thiopyridine) that could react with the newly generated mitomycin (porfiromycin) thiol. The NaOMe cleavage route of porfiromycin thiol esters **26** and **27** (Scheme 3, route B) eliminated the need for external thiols. This reaction proceeded rapidly at both room temperature and low temperatures (0 to −78 °C). Despite this advantage, the method required our using basic solutions ("pH" 10−11).[24]

HPLC and Spectroscopic Characterization of the DTT- and NaOMe-Generated Mitomycin (Porfiromycin) Products. DTT activation of methanolic and acetone solutions containing either **20** or **21** generated distinctive chromatograms containing three peaks (t_R 22.1−23.9 min) that correlated with mitomycin C(7)-substituted thiol **5** formation. We expected a single peak. The same set of peaks was observed regardless of the starting material (**20**, **21**), method of activation (D,L-DTT, L-DTT, DMH), solvent (acetone, methanol), "pH" of the methanol solution (5.5, 6.5), or temperature (room temperature to −78 °C). A comparable pattern (t_R 23.9−26.6 min) was observed upon base cleavage of porfiromycin thiol ester **26**. The same profile was obtained using D,L-DTT and either disulfide **71** or **72**. This finding demonstrated that both thiol synthetic strategies (Scheme 3) generated the same intermediates. Addition of thiol-trapping reagents (**30**, **31**, **60**) led to the rapid disappearance of these three peaks and the concomitant appearance of peaks for the mitomycin (porfiromycin) C(7)-substituted thiol-modified products.

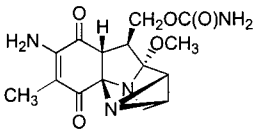
We have assigned the multiple HPLC peaks obtained from mitomycins **20** and **21** to the mitomycin free thiol **5** and/or isomers **53**−**55**. A similar assignment has been given to the products (**12**, **66**−**68**) from porfiromycins **26**, **71**, and **72**. The observation of multiple products for both **5** and **12** was surprising. The corresponding alcohols, (2-hydroxyethyl)mitomycin C (t_R 18.4 min, data not shown) and porfiromycin alcohol **48** (t_R 19.8 min) showed only a single peak in the HPLC.

A qualitatively different HPLC profile was observed for reactions that generated thiols **13** and **14**. For **13**, we observed a major peak (t_R 25.1 min) flanked by two minor peaks (t_R 23.2, 27.0 min) while for **14** we detected only a single major peak (t_R 28.2 min). We have assigned the t_R 25.1 min and the t_R 28.2 min peaks to free thiols **13** and **14**, respectively. Addition of thiol-trapping reagents (**30**, **31**, **60**) led to the disappearance of these major and minor HPLC peaks and the production of a new signal associated with the thiol-modified derivative.

The UV−visible absorption spectra (photodiode array detection) for the multiple peaks assigned to **5** and **12** and/or isomers were of interest. All of the adducts exhibited absorptions at 225−245 and 365−376 nm in an approximate 1:1 ratio. This pattern was comparable with **1**, **19**, and **48** (UV−vis$_{max}$ λ 222−225 (∼1), 360−366 nm (∼1)). We asked whether disruption of the quinone unit by C(6)−C(8) cyclization would alter the UV−visible absorption spectrum associated with mitomycin C and porfiromycin derivatives. Only a few reports have appeared on the UV−visible spectrum of quinone-modified mitomycin (porfiromycin) analogues. Kanda and Kasai showed that the C(6), C(7)-modified mitomycin **80** exhibited major absorptions



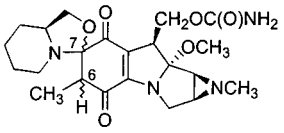
80



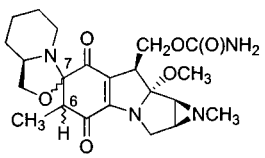
81

at 237 and 359 nm in a l:1 ratio.[37] Kono and co-workers reported that albomitomycin C (**81**), a C(4a), C(8a)-modified isomer of **1**, displayed absorptions at 242 and 344 nm in a 1:1 ratio.[23]

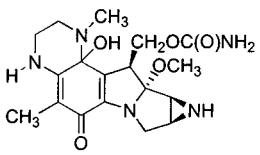
Recently, we determined the UV−visible spectra of porfiromycins **82** and **83**[38] and mitomycin **84**.[39] The spectra of **82** and



82



83



84

83 consisted of major absorptions at 235−238 and 353−362 nm in a 1.2:1 ratio while **84** exhibited absorptions at 240 and 410 nm in a 0.9:1 ratio.

(37) Kanda, Y.; Kasai, M. *J. Org. Chem.* **1990**, *55*, 2515−2518.
(38) Na, Y.; Kohn, H. *Heterocycles* **2001**, *55*, 1347−1363.
(39) Wang, S.; Kohn, H. *J. Org. Chem.* **1996**, *61*, 9202−9206.

The UV−visible absorption maximums observed for the multiple peaks associated with thiols **5** and **12** indicate that these adducts are likely to be C(7) or C(6) cyclized adducts. Since no C(6) cyclized adducts have been reported, we suggest these are the free thiols **5** and **12** and the corresponding isomeric C(7) adducts **54** and **67**, respectively. In principle, C(7) cyclization can produce four isomers that differ in their C(7) and C(6) stereochemistry.

The UV−visible spectra (photodiode array detection) for **13** and **14** were comparable with **1** and **19**. For **13**, we observed major absorptions between 240−243 and 364−377 nm in an approximate 1:1 ratio for both the major (25.1 min) and the minor (23.2, 27.0 min) HPLC peaks. Correspondingly, porfiromycin thiol **14** exhibited absorptions at 220 and 370 nm in an approximate 1:1 ratio.

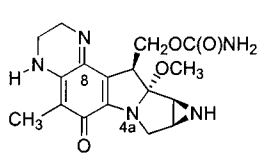
Efforts to obtain structural information concerning thiols **5** and **12**−**14** (or isomers) by NMR spectroscopy were only partially successful. Use of a concentrated sample of **20** and D,L-DTT led to a mixture of disulfides **56**−**58** along with suspected thiol **5**. Similarly, when we attempted to prepare a concentrated sample ($NaOCD_3$−CD_3OD) of **12** from thiol ester **26** suitable for NMR spectroscopy, HPLC analysis indicated that the predominant species in solution was **69** (data not shown). We found that disulfide **22** was not a suitable candidate to generate a NMR sample of the free thiol **13** since D,L-DTT-mediated disulfide cleavage occurred slowly and required our using a large excess of D,L-DTT. We were successful in obtaining a ^{1}H NMR spectrum for thiol **14**. Preparation of a concentrated solution of thiol ester **27** in $NaOCD_3$−CD_3OD provided a spectrum (Figure 1a) consistent with free thiol **14**. Key to our assignment was the detection of a multiplet at δ 1.85 and a triplet at δ 2.56 for the C(2′) and C(3′) methylene protons, respectively. It is significant that we observed no signals associated with C(6)−C(8) cyclic adducts. Attempts to obtain the corresponding ^{13}C NMR spectrum were unsuccessful since **14** converted to disulfide **74** during the NMR acquisition time (NMR and HPLC analysis).

Our finding that thiols **5** and **12** existed predominantly as cyclized adducts while **14** existed as the uncyclized, free thiol species can be rationalized on the basis of the linker size. Cyclization of **5** (**12**) at C(7) led to a five-membered-ring adduct while the corresponding cyclization for **14** gave a six-membered-ring adduct. A comparable difference in ring size exists for **5** (**12**) and **14** if cyclization proceeds at either C(6) or C(8). This difference provides an entropic advantage for cyclization for thiols **5** and **12** compared with **14**. Similar entropic factors likely contributed to the facile formation of **82** and **83**.[38]

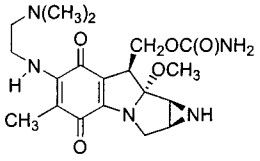
Generation and Stability of Thiols: Insight into Structure. Our inventory of mitomycins (porfiromycins) consisted of 10 compounds (**20**−**27**, **71**, **72**) the substitution patterns (CH_2CH_2, $CH_2C(CH_3)_2$) and linker sizes (C2, C3) of which varied. Together these compounds permit us to determine whether the linker affects the chemical reactivity of mixed mitomycin (porfiromycin) disulfides and porfiromycin thiol esters and the stability of the generated thiol. In KW-2149 (**3**) and BMS-181174 (**4**), an unsubstituted C2 (CH_2CH_2) bridge separates the terminal disulfide unit and the mitomycin C(7) amino substituent. We found that the ease with which the mixed mitomycin disulfides and porfiromycin thiol esters underwent thiol formation was independent of the length of the linker. Treatment of **20**, **21**, **24**, **25**, **71**, and **72** with DTT led to the selective cleavage of the disulfide bond at low temperatures (≤0 °C). Similarly, treatment of **26** and **27** with NaOMe in MeOH at −78 °C led to rapid cleavage of the thiol ester bond. Correspondingly, we observed that incorporation of a *gem*-dimethyl unit within the linker noticeably reduced the ease with which these mitomycins (**22**, **23**) underwent disulfide cleavage with D,L-DTT in comparison with **20** and **21**. For example, for **22**, D,L-DTT (DMH)-mediated cleavage only occurred at room temperature (acetone), and no reaction was observed for **23** under these conditions (HPLC, TLC analyses). We suspect that the bulky *gem*-dimethyl group adjacent to the disulfide unit hampered bond breakage.

Different methods (disulfide exchange, thiol ester cleavage) and conditions (solvents, temperatures, concentrations) were used to generate thiols or isomers **5** and **12**−**14** and/or isomers. This variation does not permit us to gain definitive conclusions concerning the relative solution stabilities of the generated thiol intermediates. However, several observations appear valid. The dominant reaction product for **5**, **12**−**14**, and/or isomers in either basic or near-neutral methanolic solutions was dimerization. *No* evidence was obtained for mitomycin ring activation. We observed neither mito*sene* production (loss of MeOH at C(9) and C(9a)) nor ring-opening of the aziridine unit. The ease with which thiols **5**, **12**−**14**, and/or cyclized isomers underwent dimerization depended on the size and the substitution pattern of the C(7) amino substituent. We found that when the linker was C2, dimerization to the symmetrical disulfides (**5** → **56**, **12** → **69**) proceeded more rapidly at 0 °C than when the bridge unit was C3 (**14** → **74**). For example, under basic conditions (NaOMe−MeOH), the approximate half-life for thiol (thiolate) **14** was 4.5 h, but for **12**, dimerization occurred within minutes at 0 °C. We also observed that the *gem*-dimethyl unit in **13** substantially decreased the ease in which this thiol dimerized to the symmetrical disulfide **73**.

The absence of mitosene adducts in the reaction profiles was striking. A similar finding was observed by Kono and co-workers.[40] Mitomycin (porfiromycin) reduction to the hydroquinone perturbs the delocalization of the N(4a) electrons with the C(5a)−C(8a)−C(8)−O(8) α,β-unsaturated carbonyl bond system and promotes expulsion of the C(9a) methoxy group and mitosene production leading to aziridine ring-opened adducts. This reaction proceeds rapidly at neutral "pH" in water[41] and alcoholic solvents at 0 °C.[42] Similarly, we have demonstrated that conversion of the C(8) quinone ring in C(7) amino-substituted mitomycins to the corresponding C(8) (i.e., **85**) imine accelerates mitosene formation and aziridine ring-



85



86

opening processes presumably by diminishing N(4a) delocalization with the adjacent α,β-unsaturated imine system.[12,43] For

(40) Kono, M.; Saitoh, Y.; Kasai, M.; Shirahata, K.; Morimoto, M.; Ashizawa, T. *J. Antibiot.* **1993**, *46*, 1428−1438.
(41) Boruah, R. C.; Skibo, E. B. *J. Org. Chem.* **1995**, *60*, 2232−2243.
(42) (a) Kohn, H.; Li, V.-S.; Tang, M.-s. *J. Am. Chem. Soc.* **1992**, *114*, 5501−5509. (b) Li, V.-S.; Choi, D.; Tang, M.-s.; Kohn, H. *Biochemistry* **1995**, *34*, 7120−7126.
(43) Wang, S.; Kohn, H. *J. Org. Chem.* **1997**, *62*, 5404−5412.

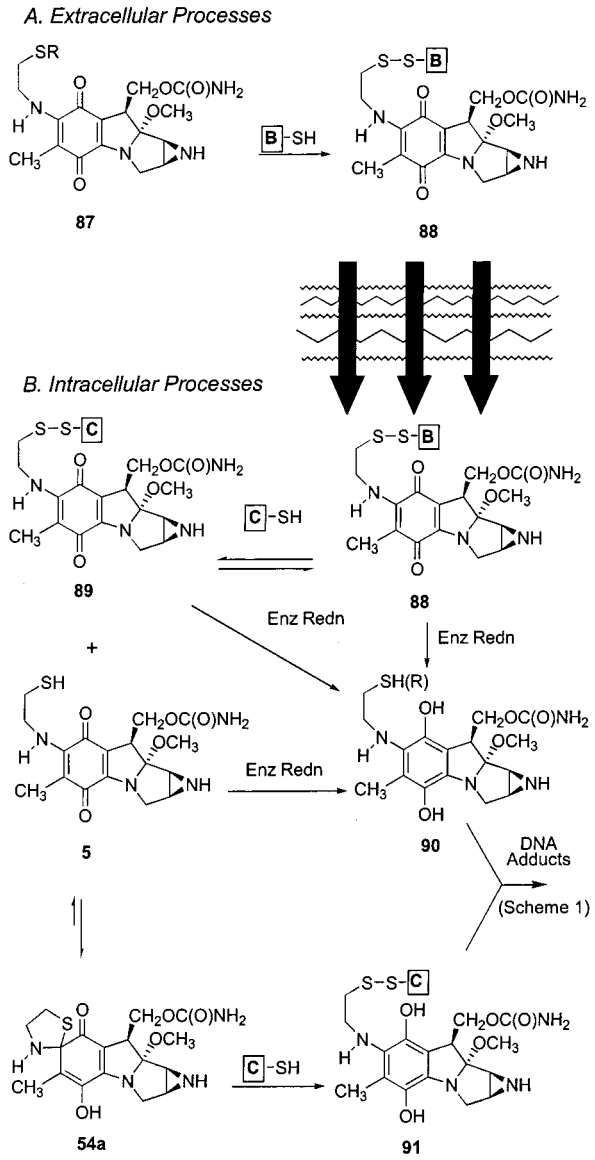
example, the half-life for conversion of **85** to the ring-opened C(1)-substituted methoxymitosene adducts in buffered methanolic solutions (Tris-HCl, "pH" 7.4, 25 °C) was 40 h while no reaction was observed for **86** after 10 days.[12] These findings indicate that the structure of the *cyclized* mitomycin (porfiromycin) thiol (**53**−**55**, **66**−**68**) has a significant impact on the ease with which the mitomycin (porfiromycin) is converted to the mito*sene*. For **55** (**68**), C(8) cyclization leads to a hybridization change at C(8) from sp^2 to sp^3 and the loss of delocalization of the N(4a) electron pair with the adjacent α,β-unsaturated carbonyl system in the starting material. For **53** (**66**) and **54** (**67**), cyclization does not significantly perturb N(4a) delocalization. These observations predict that **55** (**68**) would rapidly be converted to mito*sene* adducts at neutral "pH" values under our reaction conditions, but **53** (**66**) and **54** (**67**) would not. To date, only C(7)[37,38] and C(8)[39] cyclized mitomycin (porfiromycin) intermediates have been characterized. No C(6) adducts have been reported, a fact that may reflect the effect of the C(6) methyl group on substitution reactions at this site. We concluded, therefore, that the HPLC peaks observed for thiols **5** and **12** likely corresponded to **54** and **67** and the free thiol.

Pathway of Thiol-Mediated Cleavage Reactions of Mitomycin (Porfiromycin) C(7)-Substituted Disulfides. A major preparative route employed in our studies for mitomycin (porfiromycin) C(7)-substituted thiols was thiol (e.g., DTT)-mediated cleavage of unsymmetrical mitomycin (porfiromycin) disulfides **20**−**25**, **71**, and **72**. Our studies documented that these reactions proceeded along a thiol−disulfide exchange pathway.[19] First, we detected (HPLC) intermediates **57** and **58** when either **20** or **21** was treated with D,L-DTT. Second, substitution of GSH for D,L-DTT gave **65** in high yields (>85%). Compound **65**, unlike **57** and **58**, cannot undergo *intra*molecular disulfide exchange to give **5**, thus explaining the stability of this adduct and the yield of this transformation. Third, HPLC analysis of the D,L-DTT- and GSH-mediated cleavage reactions of **20** and **21** showed that **57**, **58**, and **65** appeared rapidly with time (data not shown), suggesting that these compounds represented initial reaction products. Finally, we isolated the DTT-bound adducts **75** and **78** after treatment of **25** with L-DTT followed by **30**. Compounds **75** and **78** likely originated from L-DTT adduct **77**.

Reflections on the Mechanism of Action of KW-2149 and BMS-181174. Our results allowed conclusions concerning the proposed thiol **5** generation step for KW-2149 (**3**) and BMS-181174 (**4**) (Scheme 2, route B) and the reactivity of thiol **5**. We observed that thiol generation readily occurred for **20**−**22**, **24**, **25**, **71**, and **72** under mild conditions, indicating that this is a plausible event for **3** and **4**. We further showed that thiol (thiol intermediates) stability increased with alkyl substitution of the mitomycin C(7) thiol linker (**13** vs **5**) and with the length of the linker (**14** vs **5** and **12**). When the linker was held to an ethylene (C2) unit (**5**, **12**), isomeric intermediates predominated and spectroscopic analysis suggested the formation of C(7) spiro-adducts (**54**, **67**). Finally, thiol **5** and **12**−**14** generation in MeOH did not lead to mitomycin ring activation and mito*sene* production, indicating that simple thiol formation, in and of itself, is not sufficient to initiate either **3** or **4** activation.[11−13]

What are the causative factors for the improved preclinical pharmacological properties of KW-2149 and BMS-181174 over conventional mitomycins, and why is maximal activity achieved

Scheme 8. Proposed Pathway for KW-2149 (**3**) and BMS-181174 (**4**) Function



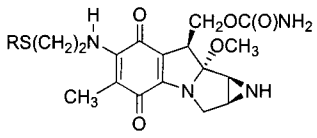
for unsymmetrical mitomycin C(7) disulfides,[44] symmetrical mitomycin C(7) disulfides,[40,44] and mitomycin C(7) thiol esters[29] containing C2 bridging elements?

The absence of mitomycin ring activation upon thiol generation **5** asks whether other factors are responsible for the different pharmacological properties observed for **3** and **4** compared with **1**. Recall that both **3** and **4** exhibit significant activity in **1**-resistant tumor cell lines.[8−10] Furthermore, cells that are resistant to **1** and express low levels of the reductase DT-diaphorase[45] are not cross-resistant to **3**.[8b,e] Despite these pharmacological differences, DNA adduction studies indicate that the sequence specificity for DT-diaphorase-activated **1** and glutathione-treated **3** are nearly identical,[16] suggesting that both transformations proceeded by comparable molecular mechanisms. How can these findings be explained? Several possibilities exist. We have diagrammed one in Scheme 8, which focuses on the unique chemi-

(44) Shirahata, K.; Kasai, M.; Kono, M.; Morimoto, M.; Ashizawa, T.; Saito, Y. Disulfide Compounds and Antineoplastic Agents. Japan Pat. Appl. JP86/0085, February 21, 1986.
(45) Prakash, A. S.; Beall, H.; Ross, D.; Gibson, N. W. *Biochemistry* **1993**, *32*, 5518−5525.

cal properties of the disulfide unit in KW-2149 and BMS-181174 and the properties and reactivities of the C(7) spiro-adduct **54**.

We divide our discussion into the extracellular and intracellular processes that may occur for **3** and **4**, which are represented by **87** in Scheme 8. In the first stage (Scheme 8A), the mitomycin C(7) amino-substituted disulfide **87** undergoes thiol−disulfide exchange with biological thiol B-SH (e.g., serum albumin, glutathione) in the plasma to generate **88**.[14−16] Support for this step comes from the metabolic studies of Kobayashi and co-workers, who showed that iv administration of **3** to mice led to the rapid production of **56**, **92**, and **93** in the plasma and



92 R = CH_3

93 R = S-albumin

that the plasma half-lives for **56**, **92**, and **93** were 12, 13, and 40 min, respectively.[14] Further evidence for this process comes from Masters, McAdam, and Hartley, who showed that **3** is activated extracellularly by serum.[15,16] They further suggested that an unidentified species that passively enters the cells more rapidly than **3** and can efficiently modify DNA is produced upon activation. The recent investigation by Yasuzawa and Tomer documented that a human serum albumin (HSA)−KW-2149 adduct (**93**)[46] is produced upon incubating HSA with **3**. Finally, we observed that **20**, **21**, **24**, **25**, **71**, and **72** all underwent rapid thiol-mediated disulfide exchange under mild conditions. Formation of small mixed disulfides **88** by extracellular processes then permits passage across the cell membrane.

In the second stage of our hypothesis (Scheme 8B), passage of **88** into the cell permits drug activation by one of two pathways. The first is enzymatic reduction. We envision that **88** reacts, in part, with intracellular thiols C−SH (e.g., GSH) by a thiol−disulfide exchange process to give **89** and **5**. All three mitomycins (**5**, **88**, **89**) are capable of undergoing quinone reduction and ring activation by a route comparable to that proposed for **1** (Scheme 1). The second activation pathway is unique for mitomycin C(7)-substituted thiols with C2 (ethylene) linkers. We have proposed that **5** largely exists as the C(7) spiro isomer **54a** (drawn as the enol tautomer). Reaction of **54a** with an intracellular thiol (C−SH) at sulfur leads to C(7)−S heterolytic cleavage and aromatization of the quinone ring, providing reduced mitomycin **91**.[47] Leucomitomycin **91** is in the same reduction state as **90** and **6** (Scheme 1) and is expected to undergo ring activation. This pathway (**54a** + C−SH → **91**) is an unconventional mitomycin activation pathway and provides a possible explanation why cell lines deficient in DT-diaphorase may be susceptible to **3**.

The proclivity of mitomycin C(7) disulfides with a C2 bridge to undergo cyclization may provide another potential pharmacological advantage for **3** and **4**. Formation of **54** upon drug activation may prevent unwanted consumption and destruction of the drug by reductases bound to cellular membranes and present in the cytosol, thereby preventing the drug from reaching its nuclear DNA target.

There is support for key features in Scheme 8B. First, generation of **5** (**12**) under a variety of conditions provided HPLC profiles consistent with the formation of isomeric adducts. Second, both the UV−visible properties of the cyclic adducts and the absence of aziridine ring-opened mitosene production suggested that **5** (**12**) cyclization occurred predominantly at C(7). Third, we found that **5** (**12**) and/or isomers underwent dimerization more rapidly than either **13** or **14**. This finding suggested that dimerization may have incurred, in part, by the heterolytic pathway outlined in Scheme 8B (**54a** → **91**) where C−SH is **5**. Fourth, Tomasz and co-workers demonstrated addition of large excesses of GSH to both **3** and **4** led to mitosene production (UV analysis) and DNA interstrand cross-link formation.[11] Fifth, a heterolytic *intramolecular* thiol-initiated cleavage pathway comparable to **54a** → **91** has been postulated for the facile reduction of mitomcyin A (**2**) by D,L-DTT.[48−50]

Conclusions

A series of mitomycin C(7)-substituted thiols were prepared that examine the effect of structural composition of the unit that bridges the thiol with the mitomycin core on thiol reactivity. We learned that the linker affects thiol structure and thiol stability. Special emphasis was given to mitomycins where the linker is two carbons in light of the pharmacological properties reported for KW-2149 (**3**) and BMS-181174 (**4**). Our studies indicated that the thiol **5** generated from these drugs exist as the C(7) cyclic species. C(7)-Spiromitomycins, such as **54**, may provide special advantages for drug function that include alternative pathways for ring activation and the generation of intermediates not prone to unwanted activation by reductases. Finally, previous investigations indicate that the pharmacological properties of **5**−conjugates (e.g., **88**) contribute to drug efficacy.

Acknowledgment. This paper is dedicated to Professors Roy A. Olofson (The Pennsylvania State University) and Ronald Breslow (Columbia University), mentors and friends, and the two Universities they have continuously served. This study was supported by grants from the National Institutes of Health (CA29756) and the Robert A. Welch Foundation (E-607). We thank Drs. Masaji Kasai and Hitoshi Arai (Kyowa Hakko Kogyo Co., Ltd., Osaka, Japan) for generously supplying mitomycin A.

Supporting Information Available: Full experimental details for the synthesis and characterization of **20**−**27**, **32**−**35**, **37**, **38**, **40**−**46**, **51**, **52**, **56**, **61**, **62**, **65**, **69**−**76**, and **78**). Full experimental details and procedures for the generation and trapping of thiol intermediates **5,** and **12**−**14**. HPLC chromatograms of mitomycin (porfiromycin) thiol and/or isomers and their trapped products (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0124313

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(47) Intracellular thiols include glutathione, cysteine-containing proteins, and **5**. Reaction of **54a** with **5** provides a dimeric mitomycin adduct. We have recently shown that dimeric mitomycins can cross-link DNA: Na, Y.; Li, V.-S.; Nakanishi, Y.; Bastow, K. F.; Kohn, H. *J. Med. Chem.* **2001**, *44*, 3453−3462.
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(50) A *homolytic* route has been proposed for the corresponding *bi*molecular reaction where the rate-limiting step is an intramolecular redox reaction involving the (homolytic) dissociation of the sulfur-bound quinone species to give a reduced mitomycin A species.[49]