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Cyclic Disulfide C(8) Iminoporfiromycin: Nucleophilic Activation of a Porfiromycin

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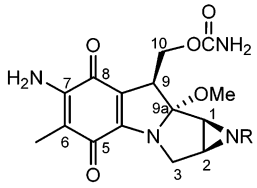
Abstract: The clinical success of mitomycin C (**1**) and its associated toxicities and resistance have led to efforts to prepare semisynthetic analogues (i.e., KW-2149 (**3**), BMS-181174 (**4**)) that have improved pharmacological profiles. In this study, we report the preparation and evaluation of the novel 7-*N*-(1′-amino-4′,5′-dithian-2′-yl)porfiromycin C(8) cyclized imine (**6**) and its reference compound, 7-*N*-(1′-aminocyclohex-2′-yl)porfiromycin C(8) cyclized imine (**13**). Porfiromycin **6** contains a disulfide unit that, upon cleavage, may provide thiol(s) that affect drug reactivity. We demonstrated that phosphines dramatically accelerated **6** activation and solvolysis in methanolic solutions ("pH 7.4") compared with **13**. Porfiromycins **6** and **13** efficiently cross-linked *Eco*RI-linearized pB*R*322 DNA upon addition of Et_3P. We found enhanced levels of interstrand cross-link (ISC) adducts for **6** and **13** compared with porfiromycin (**7**) and that **6** was more efficient than **13**. The large Et_3P-mediated rate enhancements for the solvolysis of **6** compared with **13** and a *N*(7)-substituted analogue of **1**, and the increased levels of ISC adducts for **6** compared with **13** and **7** are attributed to a nucleophile-assisted disulfide cleavage process that permits porfiromycin activation and nucleophile (MeOH, DNA) adduction. The in vitro antiproliferative activities of **6** and **13** using the A549 tumor cell line (lung adenocarcinoma) were determined under aerobic and hypoxic conditions and then compared with **7**. Both **6** and **13** were more cytotoxic than **7**, with **13** being more potent than **6**. The C(8) iminoporfiromycins **6** and **13** displayed anticancer profiles similar to **3**.

Mitomycin C (**1**) is a clinically significant antineoplastic agent.[1] Mechanisms of action have been advanced whereby drug function is initiated upon quinone ring reduction leading to aziridine ring and C(10) carbamate cleavage with DNA adduction.[2,3] In the 1960s, **1** was introduced into clinical use and today is extensively employed in combination therapies for the treatment of lung, breast, and other cancers.[1,4] The clinical success of **1** and its associated toxicities and resistance have led to an active drug development program and the preparation of over 1000 semisynthetic mitomycins.[5]

Three mitomycin analogues serve as the basis for this study. In the 1980s, Remers et al.[6] and Saito et al.[7] showed that the C(8) *imino*mitomycin **2** displayed excellent anticancer activities. Subsequently, Wang and Kohn reported that **2** underwent acid-mediated aziridine ring cleavage ~100 times faster than **1**.[8] The enhanced reactivity of **2** compared with **1** was attributed to the diminished delocalization of the indoline N(4) electrons with the adjacent $\alpha,\beta,\gamma,\delta$-unsaturated system permitting N(4)-assisted expulsion of C(9a) methoxy group and mitomycin activation. These findings suggested that **2** may react with DNA by both nonreductive and reductive-mediated pathways. In the 1980s, mitomycins **3** (KW-2149)[9] and **4** (BMS-181174)[10] were re-



1 R = H
7 R = Me

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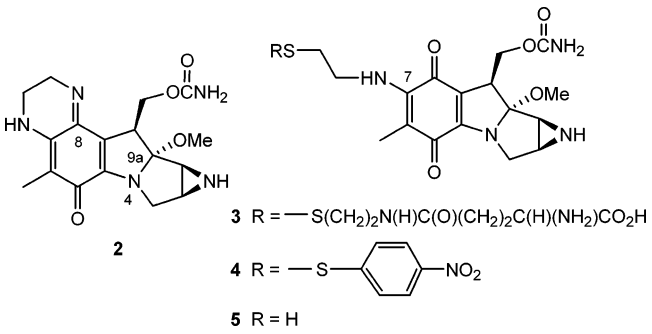
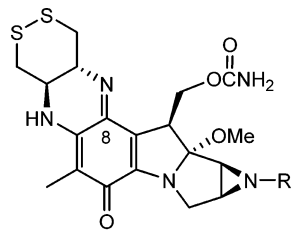
ported. Both compounds contained a C(7) aminoethylene disulfide group in place of the C(7) amino unit in **1**. Significantly, **3** was active in **1**-resistant P388 and nonhypoxic cells.[11] Both **3** and **4** were entered into clinical trials, and **3** advanced to phase II testing. Compounds **3** and **4** are members of an emerging class of multi-sulfur anticancer agents where cytotoxicity is associated, in part, with a nucleophile-assisted sulfur−sulfur cleavage transformation.[9,10,12−16] For **3** and **4**, thiol-assisted cleavage (e.g., glutathione (GSH)) of the C(7) aminodisulfide unit provides **5**, and **5** is projected to initiate drug−DNA adduction.[17−19] Thus, **2**−**4** are semisynthetic mitomycins of pharmacological interest where multiple pathways likely contribute to their bioactivity.



This study describes the synthesis and evaluation of porfiromycin **6**, which contains both a C(8) imino moiety and a strategically placed cyclic disulfide unit. The parent compound for **6**, porfiromycin[3a] (**7**), is the *N*(1a) methyl derivative of **1** and is less prone to isomerization than **1**.[20] We envisioned that **6** activation can proceed by reductive, acid, and nucleophile-mediated pathways. In this study, we document that phosphines dramatically enhanced the activation rates of **6** compared with **7**, and that C(8) iminoporfiromycins, such as **6**, effectively cross-link DNA under *nonreductive* conditions.

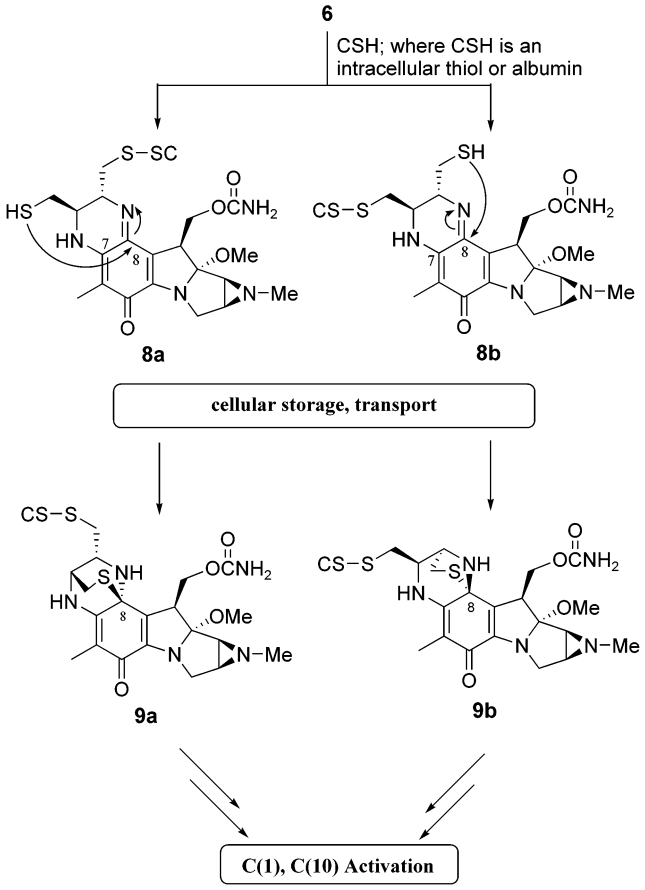
Results and Discussion

1. Substrate Design and Choice of Substrates. Porfiromycin **6** was designed to undergo nonreductive and reductive activation. We envisioned that nonreductive drug activation would



be initiated by intracellular thiol or serum albumin (CSH)-mediated disulfide cleavage to give **8a** and **8b** (Scheme 1) and

Scheme 1. Envisioned Nucleophile-Mediated Activation Pathway for **6**



that these adducts may permit either increased cellular uptake of the porfiromycin or permit drug activation to proceed by a nonreductive pathway.[17−19,21−23] Intermediates **8a** and **8b** are expected to give the C(8) modified derivatives **9a** and **9b** or the isomeric C(7) cyclic adducts (not shown), species likely to undergo ring activation and DNA adduction.[18,19] A similar pathway has been proposed for **3**.[18,19,24] Critical for generation of cyclic **9** was the placement of the disulfide unit three atoms removed from the porfiromycin C(7) site. We reported that the

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(20) For related process, see: (a) Kono, M.; Saitoh, Y.; Kasai, M.; Shirahata, K. *J. Antibiotics* **1995**, *48*, 179−181. (b) Kono, M.; Saitoh, Y.; Shirahata, K. *J. Am. Chem. Soc.* **1987**, *109*, 7224−7225.
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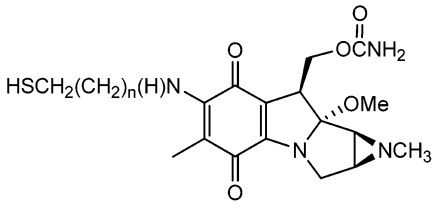
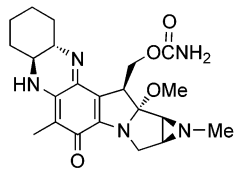
acyclic 7-*N*-(2-mercaptoethyl)porfiromycin (**10**) underwent cyclization, but the corresponding 7-*N*-(3-mercaptopropyl)porfiromycin (**11**) did not.[19] Thus, **6** was designed to be activated by reductases, acids and nucleophiles and permit cellular compartmentalization. The incorporation of the disulfide unit within the appended cyclic array distinguishes our disulfide **6** from **3** and **4**. This feature permits both thiol units to remain fixed to the porfiromycin after disulfide cleavage and, thus, be properly positioned to activate the porfiromycin and influence its pharmacological properties.



10 n = 1
11 n = 2

We initially prepared **6** and the mitomycin analogue **12**. Because compound **12** underwent slow change upon isolation[20] and storage, we focused our study on **6**. We also synthesized the corresponding cyclohexyl porfiromycin **13**[25] to serve as a reference compound for **6**.

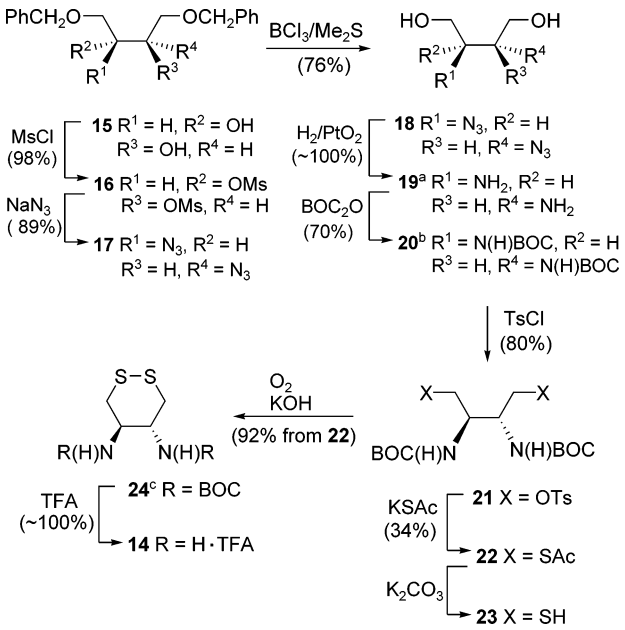


13

2. Experimental Design. C(8) Iminoporfiromycins **6** and **13** were prepared and underwent chemical and biochemical tests to determine the structural parameters that governed reactivity. First, we monitored the solvolysis rates of **6** and **13** in the absence and presence of select nucleophiles (thiols, phosphines) in buffered methanolic solutions ("pH" 7.4). Next, we determined the extent to which **6** and **13** cross-linked complementary DNA strands using linearized pB*R*322 DNA. The in vitro cytotoxicities of **6** and **13** against human lung adenocarcinoma cell line A549 were also determined. This information was not used in our assessment of the structural requirements for porfiromycin activation since neither the effective concentrations of **6** and **13** within the A549 cells nor the factors that contributed to the C(8) iminoporfiromycin inhibition of cell replication were determined.

3. Synthesis. 3.1. The Cyclic Disulfide Bridge: 4,5-Diamino-1,2-dithiane (14). Synthesis of **6** and **12** required the intermediate preparation of 4,5-diamino-1,2-dithiane (**14**).[26] Our synthesis of **14** (Scheme 2) began with commercially available (2*R*,3*R*)-1,4-dibenzyloxy-2,3-butanediol (**15**) and took advantage of the synthetic route reported by Scheurer and co-workers for (2*R*,3*R*)-2,3-diamino-1,4-butanediol (**19**).[27] Treatment of **15** with MsCl gave **16**,[27] which was then reacted with NaN_3 to afford

Scheme 2. Synthesis of Cyclic Disulfide **14**



[a] Ref 27. [b] Ref 28. [c] Ref 26.

diazide **17**.[27] NMR and TLC analyses for **17** showed only a single product suggesting that azide displacement proceeded by a S_N2-type mechanism that led to inversion. Deprotection of the benzyl group of **17** with BCl_3/Me_2S in CH_2Cl_2 gave **18**.[27] Catalytic hydrogenation of the azide groups in **18** using PtO_2 under an atmospheric pressure of H_2 afforded the key intermediate **19** ($[\alpha]_{589}^{25} = -5.0$ ($c = 0.63$, MeOH), lit.[27] $[\alpha]_{436}^{24} = -7.3$ ($c = 1.46$, MeOH)).[27] The amino groups in **19** were protected with either BOC_2O or BOC−ON to give **20**[28] and then converted to the di-tosylated derivative **21** using TsCl, Et_3N, and DMAP. The toluenesulfonyl groups were subsequently replaced by KSAc to provide **22**. Compound **22** was also obtained directly from **20** using the Mitsunobu reaction[29] (Ph_3P, DEAD with AcSH). Hydrolysis of the thioacetate groups in **22** using K_2CO_3 in aqueous MeOH gave dithiol derivative **23**, which was characterized by ^1H NMR ($CHCH_2SD$ methylene signals: δ 2.75 (br s)) when the reaction was run in a deuterated solvent (CD_3OD-D_2O). Derivative **23** was oxidized by O_2/KOH to provide **24**.[26] Compound **24** could also be obtained directly from **22** by sequential hydrolysis followed by oxidation. Several methods were attempted to remove the BOC groups in **24**. Use of either HCl/EtOAc[30] or TMSI[31] gave unsatisfactory results. With TFA,[32] we obtained diamino cyclic disulfide **14** in good yield (~100%). The overall yield of (*R*,*R*)-**14** from (*R*,*R*)-**15** was 19% (10 steps), and the reaction proceeded with stereochemical control. Compound **14** has been referenced in the patent literature,[26] but no synthetic details, spectroscopic and physical data for this diamine were provided.

3.2. Synthesis of Porfiromycin Imines 6 and 13. Using disulfide **14** we prepared imines **6** and **12** (Scheme 3).

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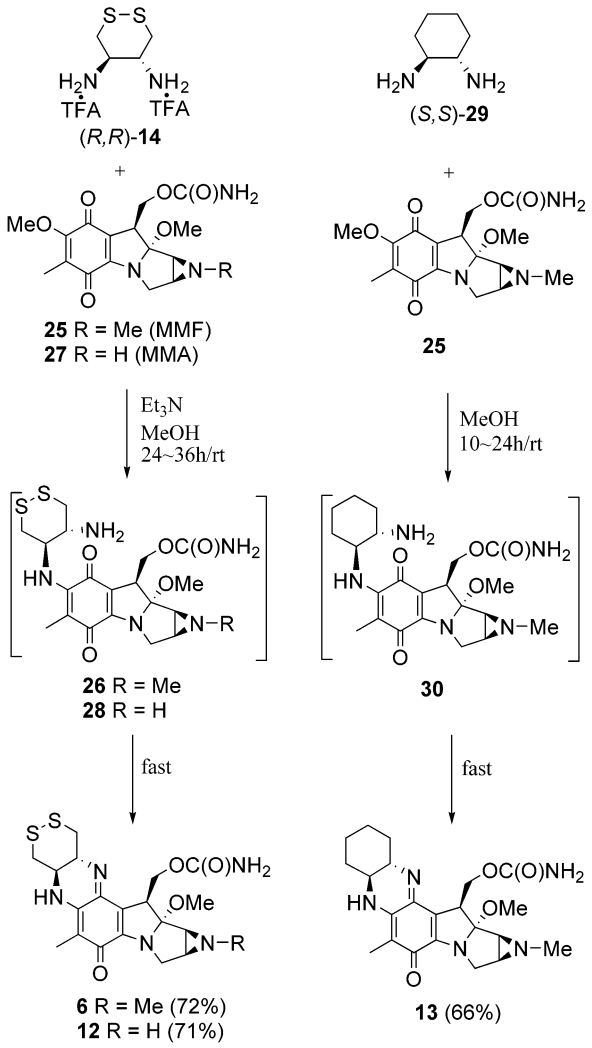
Scheme 3. Synthesis of Porfiromycin Imines **6** and **13**



Compound **14** was reacted with mitomycin F[33] (MMF, **25**) in the presence of Et_3N to give **6** (72% yield). Various reaction conditions and solvents (e.g., CH_2Cl_2, EtOH, MeOH, $CHCl_3$) were examined for this reaction, and MeOH gave the best results. The solution color changed from violet to dark red over the 36-h reaction period. The intermediate **26** was not isolated and is believed to undergo cyclization rapidly to give porfiromycin imine **6**. Using similar reaction conditions we obtained mitomycin imine **12** in 71% yield from **14** and mitomycin A[34] (MMA, **27**). Compound **12** gradually underwent change during PTLC purification (10% MeOH−$CHCl_3$) and storage. We also prepared the reference porfiromycin imine **13**[25] (66% yield) using **29** and **25**.

4. Structural Characterization of Porfiromycin (Mitomycin) Imines 6, 12, and 13. Compounds **6**, **12**, and **13** showed distinctive UV maxima at ∼224 and ∼366 nm, similar to those previously observed for other C(8) iminomitomycins.[8,25] We observed the expected upfield shift (∼23.9 ppm) for the C(8) imine carbon in the ^{13}C NMR spectra for **6**, **12**, and **13** (152.8−153.0 ppm) compared with the corresponding signal in **7** (176.8 ppm).[8,25] In addition, the C(7) and C(5a) resonances for **6**, **12**, and **13** were shifted upfield by 7.7−8.6 ppm and 10.1−10.3 ppm, respectively, while the C(8a) carbon appeared downfield (4.2−4.6 ppm) from **7**. The structures for **6**, **12**, and **13** were supported by their mass spectrum (low- and high-resolution), which showed a molecular ion peak 18 mass units (−H_2O) lower than the expected signal for the corresponding *non*-cyclized porfiromycin (mitomycin). These findings paralleled the spectral results obtained for **2**.[8]

5. Chemical Reactivity of C(8) Iminoporfiromycins: Solvolysis Studies. 5.1 Experimental Method. We first determined the rate of methanolysis of C(8) iminoporfiromycins **6** and **13** in buffered methanolic solutions ("pH" 7.4, 25 °C) in the absence and presence of nucleophiles to learn if the disulfide unit in **6** affected ring activation. The reactions were monitored by either UV−vis spectroscopy (200−600 nm) or HPLC using UV−vis detection for greater than two half-lives, when possible, and then the absorbance of the C(8) iminoporfiromycin (365 nm) was plotted against time. We used nonlinear regression analysis to fit the observed exponential decay of the iminoporfiromycin, using the SigmaPlot Program (SigmaPlot, 2001) to provide pseudo-first-order rate constants. The reactions were conducted in duplicate, and the results averaged. The products (**31** + **32**, **33** + **34**) were identified by co-injection with authentic samples using HPLC and cospotting with authentic samples using TLC.

Authentic samples of **6** and **13** solvolysis products, *cis*- and *trans*-1-methoxy-2-methylaminomitosenes **31** + **32** and **33** + **34**,[35] respectively, were prepared by maintaining the porfiromycins in acidic methanolic solutions ("pH" 5.5, 0.1 M bis-Tris·HCl, 18−21 h). In the HPLC chromatograms, we observed the expected peaks for the *cis*- and *trans*-isomers, and the two spots were also identified by TLC. The product mixtures from **6** and **13** were purified by PTLC and identified by UV−vis, mass and ^1H NMR spectroscopy. Compounds **31**−**34** displayed UV maxima at ∼253 and ∼313 nm characteristic of 2-methylaminomitosenes.[36] The mass spectra showed the expected molecular ion peaks for the 1-methoxy-2-methylaminomitosenes indicating that the imino structure remained intact during solvolysis. The ^1H NMR spectra permitted us to distinguish the *cis*- and *trans*-1-methoxy-2-methylaminomitosenes. We found that for the *cis*-isomers **31** and **33** the C(1)H−C(2)H and the C(2)H−C(3)H_β vicinal coupling constants were moderate (∼5−7 Hz) and the C(2)H−C(3)H_α coupling interaction was large (∼9 Hz). Correspondingly, for the *trans*-isomers **32** and **34** the C(1)H−C(2)H vicinal coupling was near 0 Hz, the C(2)H−C(3)H_β vicinal coupling was small (∼0−1 Hz), and the C(2)H−C(3)H_α coupling interaction was moderate (∼5 Hz). These coupling constant patterns have proven to be reliable indicators of C(1) stereochemistry.[37]

5.2. Methanolysis in the Absence of Nucleophiles. At "pH" 7.4, the rate of **6** solvolysis was similar to that of **13** (Table 1A, k_{obs} (d^{-1}): **6**, 0.062; **13**, 0.069). HPLC and TLC analyses indicated that the products were the expected *cis*- and *trans*-1-methoxy-2-methylaminomitosenes (**6**: **31** + **32**; **13**: **33** + **34**). Correspondingly, mitomycins such as **1** and **7** did not undergo appreciable change in buffered methanolic solutions at "pH" 7.4 (10 d), demonstrating that conversion of the C(8) quinone unit to the imine facilitated mitosene production (data not

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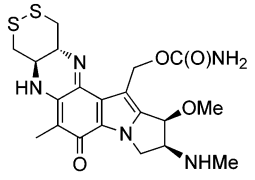
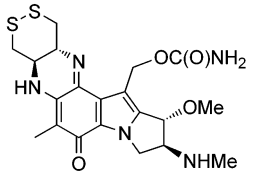
Table 1. Methanolysis Rates for **6** and **13** Using Nucleophiles at "pH" 7.4[a]

reagents		6		13	
	equiv	k_{obs} (d^{-1})	$t_{1/2}$ (d)	k_{obs} (d^{-1})	$t_{1/2}$ (d)
A. no nucH		0.062	11	0.069	10
B. L-DTT	2	0.072	9.6	0.073	9.5
	5	0.120	5.9	0.077	9.0
	10	0.140	5.0	0.079	8.8
	20[b,c]	0.240	2.9	0.390	1.8
	50[b,c]	0.390	1.8	0.410	1.7
	100[b,c]	0.870	0.8	0.430	1.6
C. GSH	2	0.083	8.3	–	–
	5	0.085	8.2	–	–
D. Et_3P	0.5[c]	0.080	8.7	0.144	4.8
	2[c]	0.130	5.5	0.151	4.6
	5	16.6	0.042	0.128	5.4
	10	33.3	0.021	0.147	4.7
	20	83.2	0.0083	0.141	4.9
	50	166	0.0042	–	–
	100	415	0.0017	–	–
E. TCEP (HCl)	2	0.120	5.8	–	–
	5	5.50	0.13	–	–
	10	8.30	0.083	–	–
F. Ph_3P	2	0.220	3.1	–	–
	5	0.260	2.7	–	–
	10	0.460	1.5	–	–

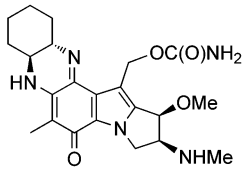
[a] Reactions were run in buffered methanolic solution (0.1 M Tris·HCl, "pH" 7.4) at 25 °C. The reactions were run in duplicate and the values averaged. The data were obtained using a Cary 3Bio Varian UV−visible spectrophotometer and the reactions monitored at 365 ± 3 nm unless indicated. The concentration of the porfiromycin was 0.03 mM unless indicated. [b] The concentration of the porfiromycin was 0.06 mM. [c] The data were obtained using HPLC.



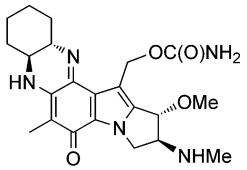
31
cis



32
trans



33
cis



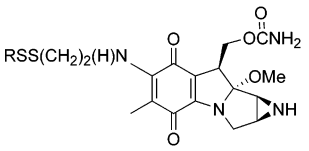
34
trans

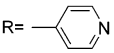
shown). We also monitored the solvolysis of **6** and **13** at "pH" 5.5 (k_{obs} (d^{-1}): **6**, 6.30; **13**, 12.8) and compared these values with the "pH" 7.4 results. The rate of methanolysis increased by 102-fold for **6** and 186-fold for **13**. These results are in agreement with previous studies and are consistent with the acid-catalyzed loss of methanol at the C(9) and C(9a) pathways previously proposed for both mitomycins[38] and C(8) iminomitomycins.[8] On the basis of these observations we concluded that the disulfide group exerted little effect on porfiromycin activation in the absence of nucleophiles between "pH" 5.5 and 7.4.

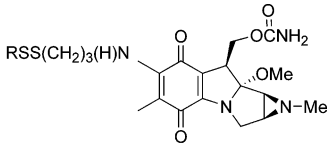
(38) (a) Stevens, C. L.; Taylor, K. G.; Munk, M. E.; Marshall, W. S.; Noll, K.; Shah, G. D.; Shah, L. G.; Uzu, K. *J. Med. Chem.* **1964**, *8*, 1−10. (b) Tomasz, M.; Lipman, R. *J. Am. Chem. Soc.* **1979**, *101*, 6063−6067. (c) Iyengar, B. S.; Remers, W. A. *J. Med. Chem.* **1985**, *28*, 963−967. (d) Hornemann, U.; Keller, P. J.; Takeda, K. *J. Med. Chem.* **1985**, *28*, 31−36. (e) Rebek, J.; Shaber, S. H.; Shue, Y.-K.; Gehret, J.-C.; Zimmerman, S. *J. Org. Chem.* **1984**, *49*, 5164−5174. (f) McClelland, R. A.; Lam, K. *J. Am. Chem. Soc.* **1985**, *107*, 5182−5186. (g) Boruah, R. C.; Skibo, E. B. *J. Org. Chem.* **1995**, *60*, 2232−2243.

5.3. Solvolysis in the Presence of Nucleophiles. We measured the rate of solvolysis of C(8) iminoporfiromycins **6** and **13** at "pH" 7.4 in the presence of select nucleophiles (Table 1B−F). We chose two thiols (L-DTT, GSH) and three phosphines (Et_3P, tris(2-carboxyethyl)phosphine hydrochloride[39] (TCEP·HCl), Ph_3P). Ph_3P was selected because it was found to trigger the leinamycin activation cascade.[40]

5.3.1. Thiols. First, we examined the effect of L-DTT on activation of **6** and **13**. L-DTT has previously been used to cleave the unsymmetrical disulfide units in the KW-2149 (**3**) and BMS-181174 (**4**) analogues **35** and **36**.[18,24]

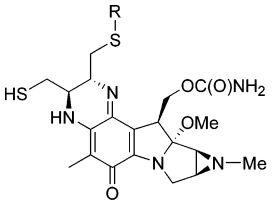


35 R= 

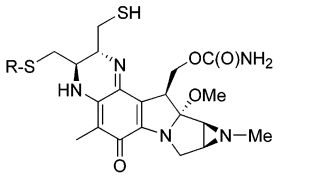


36 R= 

We observed that the **6** and **13** activation rates gradually increased (6.3−14-fold) as the number of equivalents of L-DTT increased from 0 to 100 (Table 1B). Product analyses (HPLC, TLC) indicated the formation of *cis*- and *trans*-1-methoxy-2-methylaminomitosenes (**6**: **31** + **32**; **13**: **33** + **34**). Only small increases in the activation rate were observed over the first 10 equiv of L-DTT, with **6** undergoing a 2.3-fold increase in activation and **13** a 1.1-fold rate increase. These findings indicated that if L-DTT-mediated disulfide cleavage had occurred in **6** then either the newly generated thiol **37** provided only a slight rate boost or the thiol **37** rapidly regenerated the cyclic disulfide **6**.



37a R = $SCH_2CH(OH)CH(OH)CH_2SH$
38a R = P^+Et_3



37b R = $SCH_2CH(OH)CH(OH)CH_2SH$
38b R = P^+Et_3

Use of higher levels of L-DTT (10−100 equiv) led to further increases in the methanolysis rates for **6** and **13**. Since both compounds were similarly affected, the high L-DTT levels likely promoted ring activation by routes not central to the disulfide unit in **6**. These pathways can include L-DTT-mediated reduction of the C(8) iminoquinone ring, L-DTT addition to the C(8) imine unit, and L-DTT-initiated removal of the C(9) proton leading to the loss of methanol at C(9) and C(9a).

When GSH was used as the nucleophile with **6** we obtained results similar to those with L-DTT (Table 1C). The activation rate of **6** was not measurably affected by GSH (2−5 equiv) addition, and the products were the *cis*- and *trans*-1-methoxy-2-methylaminomitosenes **31** + **32** (HPLC, TLC analyses). These results mirrored those observed for L-DTT, a finding that suggests that reclosure of the intermediate GSH-cleaved disul-

(39) Burns, J. A.; Butler, J. C.; Moran, J.; Whitesides, G. A. *J. Org. Chem.* **1991**, *56*, 2648−2650.
(40) Zang, H.; Breydo, L.; Mitra, K.; Dannaldson, J.; Gates, K. S. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1511−1515.

fide to **6** competes with porfiromycin activation. This notion is also supported by the relative rates of GSH activation of **6** and **3**. The half-live for GSH (5 equiv) mediated solvolysis of **6** was ~8 d, while acyclic disulfide **3** underwent efficient activation at near neutral pH within 2 h when treated with excess GSH.[17] Compound **3** is less likely to regenerate starting material upon GSH cleavage than **6**. Results similar to **3** were observed for **35** upon treatment with DTT.[18]

5.3.2. Phosphines. Next, we determined the effect of alkyl and aryl phosphines on **6** and **13** activation (Table 1D−F). The nonbonding electron pair present on phosphorus permit phosphines to be classified as both organic bases and nucleophiles.[41,42] Phosphine nucleophilicity depends on the reaction type (S_N1, S_N2), the phosphorus substituents, the electrophile (hard, soft), and the displaced leaving group. Nevertheless, phosphines are often more nucleophilic than their sulfur and nitrogen counterparts[42a] and have been used in desulfurization reactions[43] and to cleave cyclic and protein disulfide bonds.[44−46] In addition, phosphines are known to react with carbonyl groups and aziridines.[47]

Use of 5 equiv of Et_3P with **6** led to a 270-fold increase in 2-methylaminomitosene product formation, and use of 10 equiv of Et_3P enhanced the rate 540-fold (Table 1D). Only modest increases (~2-fold) were observed for the reference compound **13** when Et_3P (10−20 equiv) was added. Far greater rate differences were observed when we compared **6** with a *N*(7)-substituted analogue of **1** where no appreciable mitosene production occurred after 8 d with Et_3P (50 equiv).[48] The Et_3P-mediated activation rate of **6** was linear with phosphine concentration (5−100 equiv). Interestingly, the rapid rate increases for **6** were observed only after addition of 5 or more Et_3P equiv. We suspect that this represents the threshold level of Et_3P needed for porfiromycin nucleophilic activation and likely reflects the consumption of Et_3P by adventitious amounts of O_2 present in reaction medium and that were not purged by Ar. This is the first example of a phosphine activation of a porfiromycin (mitomycin).

Our finding that Et_3P affected only **6** activation and not **13** indicated that the disulfide group in **6** was involved in the drug activation process. Accordingly, we suggest that Et_3P reacted with the disulfide unit to generate thiol **38**, which in turn activated the quinone ring permitting 2-methylaminomitosene formation. The 2-fold rate increase in solvolysis observed for **13** upon Et_3P (0.5−20 equiv) addition was similar to the result obtained with L-DTT and may reflect additional roles for Et_3P (e.g., C(8) iminoquinone reduction, imine addition, base-catalyzed removal of methanol at C(9) and C(9a)) in the overall drug activation process.

When TCEP (2−10 equiv) was used in place of Et_3P, we again observed increased rate enhancements for **6** activation (Table 1E). Significant increases in the solvolysis rates were only observed with 5 or more equiv of the phosphine. With 5 equiv, the rate enhancement was 89-fold, and with 10 equiv, it was 134-fold.

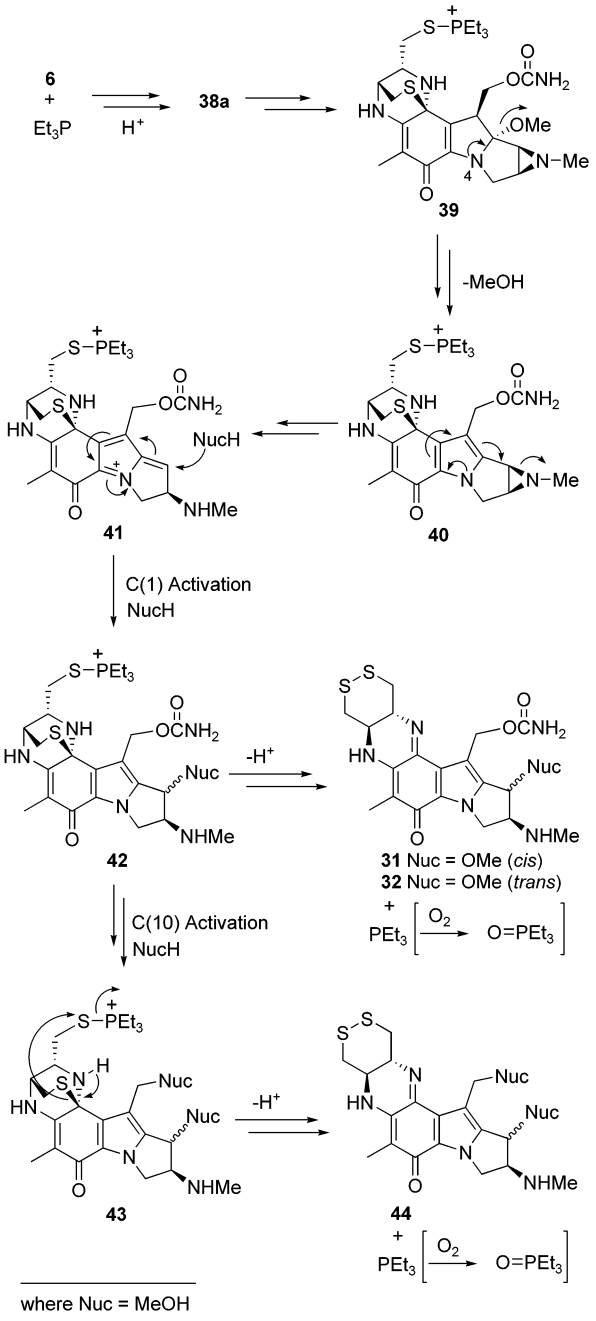
The final phosphine employed was Ph_3P. The rate enhancements observed for **6** activation were less than those with Et_3P and TCEP (Table 1F). We detected a 4.2-fold activation with 5 equiv of Ph_3P and a 7.4-fold activation with 10 equiv. We have attributed the diminished level of activation for these transformations to the decreased nucleophilicity of aromatic (Ph_3P) versus aliphatic (Et_3P, TCEP) phosphines.[41,49]

The reaction rates for most entries in Table 1 were determined by UV−vis spectroscopy on which we observed a decrease in the absorption for the starting C(8) iminoporfiromycin (~365 nm) and a concomitant increase in absorption for the C(8) imino-2-methylaminomitosene products (~313 nm). When the 0.5 and 2 equiv Et_3P reactions were monitored by HPLC, the loss of starting porfiromycin was seen to correlate with the UV−vis spectroscopic data. In these cases, we observed the HPLC peaks corresponding to *cis*- (**31**, t_R 31.8 min) and *trans*- (**32**, t_R 29.7 min) 1-methoxy-2-methylaminomitosenes. However, when HPLC was used to monitor the methanolysis of **6** with ≥ 10 equiv of Et_3P (data not shown) the progressive loss of **6** was observed, but we could not monitor the concomitant increase in **31** and **32**. For the 10 equiv Et_3P reaction, we detected at the outset (0−1 h) **31** and **32**, but these products diminished with time and no new adducts were recorded by HPLC. When 20 equiv of Et_3P were employed we were only able to follow the loss of **6** and did not observe **31** and **32**. These results differed from those observed for **13**. Treatment of **13** with Et_3P (10 and 20 equiv) led to the production of **33** and **34** (data not shown). Correspondingly, with Ph_3P (2−10 equiv) we were able to follow **31** and **32** production on HPLC as **6** was consumed (data not shown). Thus, we suspect that the excess Et_3P present in the reaction medium reacted with the 1-methoxy-2-methylaminomitosene products (**31** and **32**) leading to adducts that were either unstable or unable to elute from the HPLC column under the experimental conditions. Several unsuccessful attempts were made to identify the products generated under these high Et_3P conditions (data not shown). First, we passed O_2 through the solution (2 d) but observed no change in the HPLC and TLC profiles. Second, we reduced the temperature for the **6** plus Et_3P (10 equiv) reaction from 25 °C to −50 °C. The HPLC product profiles were similar to those observed at room temperature. Finally, we treated the *cis*-(**31**) and *trans*-(**32**) 1-methoxy-2-methylaminomitosenes with Et_3P (10 equiv, buffered methanol, "pH" 7.4, 25 °C). After 1 h, we were unable to detect **31** and **32** (HPLC, TLC analyses). We concluded that the excess Et_3P (≥10 equiv) in the **6** methanolysis reactions consumed the initially generated **31** and **32** products.

5.4. Reflections on the Mechanism for Phosphine-Mediated 6 Solvolysis. Addition of phosphines to methanolic solutions of **6** led to rapid porfiromycin consumption and 2-methylaminomitosene production (Table 1D−F). We observed that C(8) iminoporfiromycin activation depended on the strength and concentration of the phosphine. Analysis of the corresponding data for **13** showed that the large rate enhancements were attributable to a phosphine-mediated disulfide cleavage process. We propose that the rate-limiting step is the phosphine rupture

(41) (a) Overman, L. E.; Matzinger, D.; O'Connor, E. M.; Overman, J. D. *J. Am. Chem. Soc.* **1974**, *96*, 6081−6089. (b) Overman, L. E.; Petty, S. T. *J. Org. Chem.* **1975**, *40*, 2779−2782. (c) Overman, L. E.; O'Connor, E. M. *J. Am. Chem. Soc.* **1976**, *98*, 771−775.
(42) (a) Bartlett, P. D.; Lohaus, G.; Weis, C. D. *J. Am. Chem. Soc.* **1958**, *80*, 5064−5069. (b) Hudson, F. *Organic Chemistry: Structure and Mechanism in Organo-Phosphorous Chemistry*, Vol 6; Blomquist, A. T., Ed.; Academic Press: London and New York, 1965; p 16.
(43) Moore, C. G.; Trego, B. R. *Tetrahedron* **1962**, *18*, 205−218.
(44) Sweetman, B. J.; Maclaren, J. A. *Aust. J. Chem.* **1966**, *19*, 2347−2354.
(45) Maclaren, J. A.; Sweetman, B. J. *Aust. J. Chem.* **1966**, *19*, 2355−2360.
(46) Lee, S. H.; Kohn, H. *Heterocycles* **2003**, *60*, 47−56.
(47) Fan, R.-H.; Hou, X.-L. *J. Org. Chem.* **2003**, *68*, 726−730.
(48) Lee, S. H. Ph.D. Dissertation, 2003, University of North Carolina, Chapel Hill.
(49) Parker, A. J.; Kharasch, N. *J. Am. Chem. Soc.* **1960**, *82*, 3071−3075.

Scheme 4. Proposed Phosphine-Mediated Activation Pathway for **6** in MeOH



of the disulfide bond in **6**. In Scheme 4, we postulate that disulfide cleavage occurred at the sulfur atom closest to the C(8) imine bond leading to thiol **38a**. Alternatively, disulfide cleavage may have occurred at the other sulfur site to give **38b** (pathway not shown). There is precedent for the proposed phosphine-mediated disulfide transformation (**6** → **38a**). Alkyl phosphines have been utilized to cleave disulfide units in peptides,[44,45] and recently we showed that Et_3P and TCEP efficiently cleaved cyclic disulfides (e.g., DTT^{ox}) under neutral to basic conditions.[46] A similar transformation has also been reported for leinamycin and Ph_3P.[40]

We project that thiol **38a** undergoes intramolecular cyclization at the C(8) imine carbon to give **39**. Disruption of the N(4)−C(5a)−C(8a)−C(8)−N conjugated system in **39** unleashes the N(4) electrons leading to 1-methoxy-2-methylaminomitosene

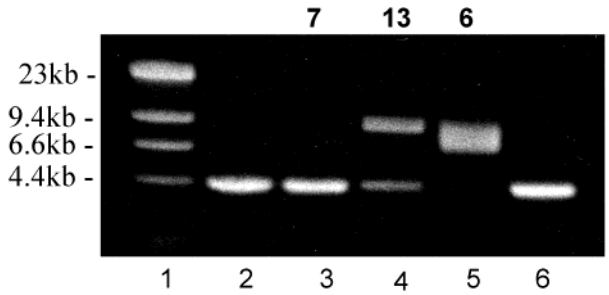


Figure 1. Denaturing 1.2% Alkaline Agarose Gel for **6**, **7**, and **13** (0.05 mM) using Et_3P (5 equiv**)**. DNA cross-linking experiments for **6**, **7**, and **13** at 0.05 mM concentration using *Eco*RI-linearized pB*R*322 plasmid DNA and Et_3P (5 equiv). All reactions were incubated at room temperature (2 h). Lane 1: λ Hind III DNA molecular weight marker. Lane 2: control (only linearized pB*R*322). Lane 3: **7** + Et_3P (5 equiv). Lane 4: **13** + Et_3P (5 equiv). Lane 5: **6** + Et_3P (5 equiv). Lane 6: only Et_3P (5 equiv).

40. Formation of **40** is expected to lead to rapid aziridine ring opening[8] to give **41** followed by attack of the nucleophile (NucH) to give **42**. An alternative activation pathway is conceivable where thiol **38a** gives the C(7) cyclized species, which then undergoes further nucleophilic attack to give the leucoporfiromycin. We have previously suggested a comparable route for **5** upon thiol activation of **3** and **4**.[19] Both pathways are supported by the kinetic studies in which the solvolysis products in methanol were **31** and **32** (HPLC, TLC analyses) and the reaction rates depended on the nucleophile and its concentration.

Depicted in Scheme 4 is a proposed pathway for C(10) activation (**42** → **43** → **44**). Although we did not detect C(1), C(10)-disubstituted products (i.e., **44**), the disruption of the N(4)−C(5a)−C(8a)−C(8)−N conjugated system in **42** is expected to activate the C(10) site toward nucleophilic substitution (see Sections 6.2 and 6.5).

6. C(8) Iminoporfiromycins−DNA Bonding Profiles. Mitomycin−DNA modifications include both monofunctional and bifunctional alkylation processes (intrastrand and interstrand cross-links (ISC)).[3] The efficiency of DNA cross-linking depends on the structure of the mitomycin and the activation conditions. Compound **6** was designed to undergo activation under nonreductive (e.g., nucleophilic) and reductive conditions leading to DNA adduction.

6.1. Experimental Method. The ability of C(8) iminoporfiromycins **6** and **13** to cross-link complementary *Eco*RI-linearized pB*R*322 DNA was determined using denaturing alkaline agarose gel electrophoresis as reported by Cech.[50a] The size of the DNA product(s) was estimated using λ DNA digested with *Hin*dIII as a molecular weight marker. We adopted the term DNA ISC for interstrand cross-linked (ISC) DNA adducts. The extent of DNA ISC formation for **6** and **13** was determined under nonreductive (Et_3P) and reductive ($Na_2S_2O_4$) conditions.

6.2. Effect of Et_3P on DNA ISC. Preliminary experiments led us to choose 5 equiv of Et_3P and 50 μM porfiromycins **6**, **7**, and **13**. The reactions were run at room temperature (2 h). We found that both **6** and **13** formed DNA ISC (**6**: 96%, **13**: 73%) while **7** exhibited low levels of DNA ISC (5%) (Figure 1). Interestingly, the band for the **6** DNA ISC was more diffuse and encompassed DNA products with enhanced gel mobilities, compared with **13**. Although the origin of this phenomenon has

(50) (a) Cech, T. R. *Biochemistry* **1981**, *20*, 1431−1437. (b) Tepe, J. J.; Williams, R. M. *J. Am. Chem. Soc.* **1999**, *121*, 2951−2955.

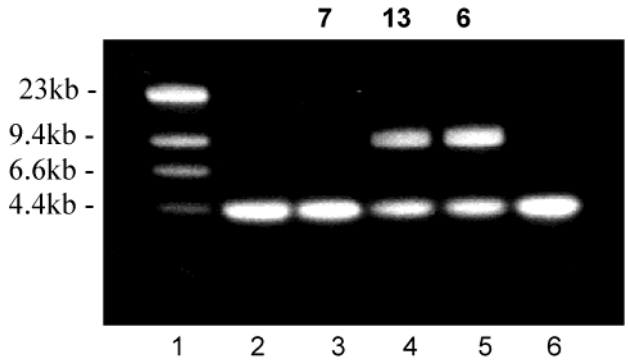


Figure 2. Denaturing 1.2% Alkaline Agarose Gel for **6**, **7**, and **13** (0.01 mM) using L-DTT (5 equiv). DNA cross-linking experiments for **6**, **7**, and **13** at 0.01 mM concentration using *Eco*RI-linearized p*BR*322 plasmid DNA and L-DTT (5 equiv). All reactions were incubated at room temperature (2 h). Lane 1: λ Hind III DNA molecular weight marker. Lane 2: control (only linearized p*BR*322). Lane 3: **7** + L-DTT (5 equiv). Lane 4: **13** + L-DTT (5 equiv). Lane 5: **6** + L-DTT (5 equiv). Lane 6: only L-DTT (5 equiv).

not been determined, we suspect that the diffuse band may result from extensive DNA ISC adduction,[51a] multiple ISC adducts within the linearized DNA fragment, and DNA ISC isomers that differed in their site of DNA ISC formation.[51b,c] We expect that adducts with altered DNA structures will display different mobilities under denatured agarose gel conditions.[51b]

We attempted to distinguish the Et_3P-mediated activation of **6** and **13** further by using lower porfiromycin concentrations (10 μM) (Supporting Figure 1). Under these conditions, **6** efficiently generated DNA ISC (89%), but the extent of DNA ISC for **13** was 35% and for **7** was ~2%. Again, we observed that the band for **6** DNA ISC adducts was more diffuse than that for **13**.

The enhanced DNA ISC efficiency of **6** compared with **13** upon Et_3P addition is attributed to the role of the disulfide unit in the drug activation steps. Moreover, the observation of DNA ISC adducts documents that Et_3P activation of **6** leads to modification of both the C(1) and C(10) sites within the porfiromycin (Scheme 4). The DNA studies were unable to differentiate the extent of DNA ISC within any given DNA fragment. The increased efficiency of **6** compared with **13** paralleled the trend observed in the kinetic studies (Table 1D), but the large difference (5 equiv Et_3P: 130-fold) found in the kinetic studies were not observed in the DNA experiments. We believe that only qualitative comparisons of these data sets can be made and that direct comparisons are not warranted unless the number of DNA ISC sites are known.

6.3. Effects of Thiols. We next determined the effects of L-DTT and GSH on porfiromycin **6** and **13** DNA ISC transformations and compared the results with **7**. Using the conditions established for Et_3P, we treated aqueous buffered solutions containing DNA and **6**, **7**, or **13** (10 μM) with L-DTT (5 equiv) at room temperature (2 h). Gel analysis (Figure 2) showed that L-DTT (5 equiv) led to appreciable levels of DNA ISC for **6** (61%) and **13** (48%) and a low level (5%) for **7**. Only minor differences existed in the ISC bands for **6** and **13**. A similar result was obtained for GSH using the same conditions (ISC: **6**, 48%; **13**, 47%; **7**, 3%; see Supporting Figure 2).

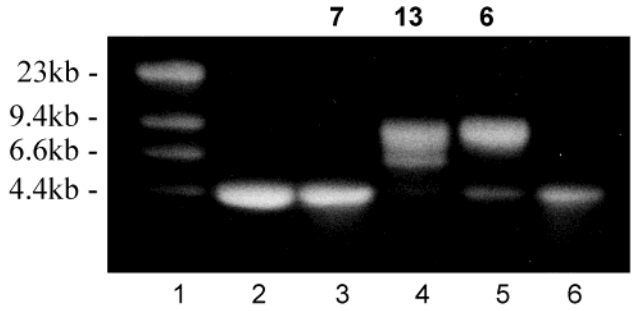


Figure 3. Denaturing 1.2% Alkaline Agarose Gel for **6**, **7**, and **13** (0.2 mM) using $Na_2S_2O_4$ (1 equiv). DNA cross-linking experiments for **6**, **7**, and **13** at 0.2 mM concentration using *Eco*RI-linearized p*BR*322 plasmid DNA and $Na_2S_2O_4$ (1 equiv). All reactions were incubated at 0 °C (1 h). Lane 1: λ Hind III DNA molecular weight marker. Lane 2: control (only linearized p*BR*322). Lane 3: **7** + $Na_2S_2O_4$ (1 equiv). Lane 4: **13** + $Na_2S_2O_4$ (1 equiv). Lane 5: **6** + $Na_2S_2O_4$ (1 equiv). Lane 6: only $Na_2S_2O_4$ (1 equiv).

The modest increase in DNA ISC with L-DTT (5 equiv) for **6** compared with **13** paralleled the differences observed in the solvolysis rates for these compounds (Table 1B: **6**, 0.12 d^{-1}; **13**, 0.077 d^{-1}). Surprising to us, however, was the amount of DNA ISC produced for each compound with L-DTT, which neared 50%, despite the finding that both compounds underwent slow solvolysis. We have attributed the high percentage of DNA ISC to the number of potential adduction sites within the 4361-bp *Eco*RI-linearized p*BR*322 DNA. Mitomycin C ISC processes have been shown to proceed at 5′CG•5′CG sites.[52] Thus, ISC adduction at any one of these many sites would lead to a slower migrating band in the denaturing 1.2% alkaline agarose gel, despite the fact that only low levels of either **6** or **13** were activated. In light of this analysis we are uncertain to attribute the modest difference in the extent of DNA ISC for **6** and **13** with L-DTT (Figure 2) to L-DTT-mediated activation of the **6** disulfide unit.

6.4. Effect of the Chemical Reductant Sodium Dithionite ($Na_2S_2O_4$). Mitomycins, such as **1** and **7**, are activated in vitro by chemical reductants (e.g., $Na_2S_2O_4$[52,53]) and in vivo by reductases.[1,3,54] Thus, previous studies have examined the extent of DNA ISC for mitomycins (porfiromycins) under reductive conditions.[52] In most studies, chemical reductants were employed.[52,53] We chose to use $Na_2S_2O_4$ and previously employed conditions that led only to efficient mitomycin C(1) activation.[55] Porfiromycins **6**, **7**, and **13** served as our test compounds, and the reactions were conducted at 0 °C (1 h) using 0.2 mM drug concentration and 1 equiv of $Na_2S_2O_4$. We found that under these conditions C(8) iminoporfiromycins **6** and **13** provided high levels of DNA ISC (**6**: 94%, **13**: 96%) while **7** showed the expected low amounts (5%) (Figure 3). Therefore, we concluded that under reductive conditions, imines **6** and **13** were more effective in forming DNA ISC than was **7**. These results may reflect the ease with which iminoquinones undergo reduction[56] or the ability of $Na_2S_2O_4$ or its byproduct, SO_3^{2-}[53b] to assist porfiromycin activation by either C(8) imine addition

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or C(9) proton abstraction. Interestingly, in these experiments the **13** DNA ISC products (Figure 3, lane 4) were more diffuse than the corresponding **6** adducts and appeared as two, closely spaced bands. The gel findings provided no evidence that the disulfide unit in **6** played a role in porfiromycin activation under reductive conditions, and thus we suspect iminoquinone reduction superseded thiol-activation pathways (e.g., Scheme 1).

6.5. Reflections Concerning the DNA Bonding Profiles for 6 and 13 and the Differences in the Kinetic and DNA Adduction Findings. Significant levels of DNA ISC were obtained for **6** and **13** when phosphine (Et_3P) and thiols (L-DTT, GSH) were added. Careful adjustment of the reaction condition permitted us to differentiate **6** from **13** with Et_3P (Figure 1 and Supporting Figure 1). With Et_3P, we observed the appearance of a diffuse DNA band corresponding to the DNA ISC products. We have partially attributed this band to extensively cross-linked DNAs. If this is the case, then the calculated percentage cross-linked efficiencies of **6** and **13** underestimated the degree of ISC within the DNA and the role of the disulfide unit in **6** in promoting these transformations.

Why did we see appreciable levels of DNA ISC with **13** but not with **7** upon addition of nucleophiles (Et_3P, L-DTT, GSH)? The imino group in **13** (**6**) is more easily reduced than the quinone unit in **7**,[56] and phosphine and thiols can both serve as reducing agents.[44,45] Furthermore, the imino unit in **13** (**6**) is expected to undergo nucleophilic addition.[57] Generating a C(8) tetrahedral intermediate deconjugates the N(4)−C(5a)−C(8a)−C(8)−N unsaturated system and activates the C(8) iminoporfiromycin toward 2-methylaminomitosene formation. The finding that C(8) iminoporfiromycins undergo extensive DNA ISC compared with **7** has not been reported.

The HPLC data for the **6** kinetic methanolysis experiments with L-DTT and Et_3P showed no measurable amounts of C(1), C(10)-dimethoxy adducts **44** (Nuc = OMe) yet we obtained clear evidence of **6** C(1), C(10) modification in the presence of DNA in aqueous buffered solutions. A similar result was obtained for **13**. How do we reconcile these results? Previous studies have shown that the C(1) site is 10−100 times more reactive than C(10) under reductive conditions.[58] If this is a generalizable finding, we would expect to see few C(1), C(10)-dimethoxy products[59] in our kinetic studies. This difference in C(1) and C(10) site reactivity is more difficult to discern with the *Eco*RI DNA (4361 bp) where even a single ISC would lead to the appearance of a slower migrating band in the denaturing 1.2% alkaline agarose gel.

7. Cytotoxicities. 7.1. Experimental Approach. We tested whether C(8) iminoporfiromycins **6** and **13** inhibited tumor cell line replication using an in vitro antiproliferative activity assay[60] at the Kyowa Hakko Pharmaceutical Company (Shizuoka, Japan). The tests were conducted using the human tumor cell line A549 (lung adenocarcinoma) because of its sensitivity to KW-2149 (**3**). The antiproliferative activities of these compounds were determined under aerobic and hypoxic conditions and then compared with **3**. A distinguishing feature previously found for **3**, compared with other mitomycins (e.g., **1**), was the low ratios determined from the IC_{50} values under hypoxic and aerobic conditions (IC_{50} (hypoxic)/IC_{50} (aerobic)).[61] For **3**, this ratio was 3.4 and for **1** and **7** it was 8.2 and >7.6, respectively. Thus, we were interested to see if either **6** or **13** had ratios of activities comparable with either **3** or **1** (**7**). The cells were precultured at 37 °C (24 h) under either aerobic or hypoxic conditions and then treated with the drug candidates (72 h).

Table 2. Antiproliferative Activities for C(8) Iminoporfiromycins[a]

	IC_{50} (μmol/L)		IC_{50} (hypoxic)
compd	aerobic	hypoxic	IC_{50} (aerobic)
6	0.56	2.3	4.1
13	0.11	0.47	4.1
1	2.4	20	8.2
7	13	>100	>7.6
3	0.12	0.41	3.4

[a] A549 cell line was used. The IC_{50} (μmol/L) value is the concentration that inhibits cell replication by 50% under the assay condition.

7.2. Antiproliferative Activities. The in vitro antiproliferative activity test data for **1**, **3**, **6**, **7**, and **13** using the A549 tumor cell line are listed in Table 2. In general, the cell proliferation rate under hypoxic conditions was slower than under aerobic conditions. We observed that **6** was 25-fold more potent than **7** under aerobic and >50-fold more cytotoxic under hypoxic conditions. The ratio of activity (hypoxic/aerobic) for **6** was 4.1, for **7** was >7.6, and for **3** was 3.4. When we compared **6** with its cyclohexyl equivalent **13**, we found it was ∼5-fold less active than **13** under aerobic and hypoxic conditions, and the activity ratio (hypoxic/aerobic) remained constant (4.1). KW-2149 (**3**) showed potent activity under both aerobic and hypoxic conditions and was 4.7−5.6-fold more potent than **6**. The porfiromycin **6** and **13** in vitro antiproliferative profiles with the A549 cell line showed no apparent advantages by incorporating a disulfide unit in the appended cyclohexyl ring. Thus, under these conditions we saw no evidence that the nucleophile-mediated activation pathway (Scheme 1) contributed to cell growth inhibition.

Conclusions

We report the synthesis and evaluation of the novel C(8) iminoporfiromycin **6**. This compound contained two key structural elements that were expected to enhance drug activation and DNA adduction—the C(8) imino group and the disulfide unit. Earlier studies documented that when the C(8) carbonyl group in **1** was replaced with an imino unit to give **2** there was an approximate 100-fold increase in the rate of ring activation under acidic conditions.[8] Recent findings have documented that the disulfide unit likely contributes to the enhanced activity of **3** and may be necessary for its efficient activation and usage under aerobic conditions. Accordingly, we envisioned that **6** would be activated by reductases, acids, and nucleophiles. Distinguishing **6** from **3** and **4** was accomplished by incorporating the disulfide unit in a fused six-membered ring system. This feature permitted both thiol units that were generated upon disulfide cleavage to remain appended to the porfiromycin and positioned to activate the porfiromycin and influence its pharmacological properties.

We found that moderate amounts of Et_3P dramatically enhanced **6** activation compared with **13**. With 10 equiv of Et_3P

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the difference in reactivity was 230-fold. Far greater rate differences have been observed when **6** was compared with a *N*(7)-substituted analogue of **1**,[48] which underwent no appreciable change after 8 d with Et_3P (50 equiv). These findings are consistent with a Et_3P-mediated activation pathway (Scheme 4) that involves phosphine attack of the disulfide unit in **6**. Consistent with this pathway, the rates of **6** consumption increased with increasing phosphine concentrations and with increasing nucleophilicity of the phosphine. This is the first example in which phosphines were used to activate mitomycins (porfiromycins). The efficiency of **6** to generate DNA ISC was assessed. We found significantly enhanced levels of DNA ISC for **6** and **13** compared with **7** and that **6** was found more efficient than **13**. These results substantiate the nucleophile-mediated pathway for mitomycin (porfiromycin) activation and documents its efficiency for DNA ISC. This route complements the previously reported acid-catalyzed and reductive pathways for mitomycin (porfiromycin) activation.

Experimental Section

(General experimental details can be found in the Supporting Information.)

(2*R*,3*R*)-2,3-Bis(*tert*-butyloxycarbonylamino)-1,4-bis(acetylthio)-butane (22).

Method A. Ph_3P (125 mg, 0.48 mmol) was dissolved in THF (2 mL) and DEAD (74 μL, 0.47 mmol) was added at room temperature. The solution was cooled to 0 °C and stirring was continued (20 min). Compound **20** (64 mg, 0.20 mmol) in THF (0.5 mL) and AcSH (34 μL, 0.48 mmol) were added with stirring. The reaction was allowed to stir at 0 °C (1 h) and then at room temperature (16 h). The mixture was concentrated in vacuo and H_2O (20 mL) was added to the residue. The mixture was extracted with EtOAc (2 × 20 mL) and the combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. Purification by PTLC (1:2 EtOAc/hexanes) afforded **22** as a white solid: yield, 38 mg (44%); mp 156−166 °C; $[\alpha]_{589}^{25} = -31$ ($c = 0.26$, $CHCl_3$); R_f 0.40 (1:2 EtOAc/hexanes); IR (KBr) 3363, 2985, 2360, 1689, 1527, 1173, 625 cm^{-1}; ^{1}H NMR (acetone-d_6) δ 1.41 (s, 18 H, OC(CH$_3$)$_3$), 2.31 (s, 6 H, C(O)CH$_3$), 2.93−3.17 (m, 4 H, SCH$_2$), 3.85−3.96 (m, 2 H, C*H*NH), 5.91 (app d, $J = 9$ Hz, 2 H, NHCO), the ^{1}H NMR data were in agreement with the HETCOR spectrum; ^{13}C NMR (acetone-d_6) δ 28.7 (OC(*C*H$_3$)$_3$), 30.6 (C(O)*C*H$_3$), 32.5 (SCH$_2$), 54.6 (CHNH), 79.3 (O*C*(CH$_3$)$_3$), 156.9 (NHCO), 195.5 (*C*OCH$_3$), the ^{13}C NMR data were in agreement with the HETCOR spectrum; MS (+CI) *m*/*z* 437 [M+1]$^+$; M_r (+CI) 437.177 73 [M+1]$^+$ (calcd for $C_{18}H_{33}N_2O_6S_2$ 437.178 01).

Method B. To a cooled (0 °C) solution of **20** (64 mg, 0.20 mmol), Et_3N (86 μL, 0.60 mmol) and DMAP (58 mg, 0.48 mmol) in CH_2Cl_2 (6 mL) was slowly added a solution of TsCl (116 mg, 0.6 mmol) in CH_2Cl_2 (1 mL) for 1 h. The reaction was allowed to stir at 0 °C (30 min) and then at room temperature (24 h). Dichloromethane (20 mL) was added and then the reaction mixture was successively washed with aqueous 0.1 N HCl (30 mL) and aqueous 10% $NaHCO_3$ (30 mL) solutions. The organic layer was dried ($MgSO_4$) and concentrated in vacuo to afford **21** as a yellow solid: yield, 100 mg (80%); R_f 0.30 (1:2 EtOAc/hexanes); ^{1}H NMR ($CDCl_3$) δ 1.39 (s, 18 H, OC(CH$_3$)$_3$), 2.45 (s, 6 H, PhC*H*$_3$), 3.87−4.04 (m, 6 H, TsOC*H*$_2$, C*H*NH), 5.11 (br s, 2 H, NHCO), 7.36 (d, $J = 8.4$ Hz, 4 H, Ph (ortho)), 7.76 (d, $J = 8.4$ Hz, 4 H, Ph (meta)); ^{13}C NMR ($CDCl_3$) δ 21.6 (Ph*C*H$_3$), 28.2 (OC(*C*H$_3$)$_3$), 50.4 (CHNH), 68.5 (TsO*C*H$_2$), 80.2 (O*C*(CH$_3$)$_3$), 128.0 (Ph, meta), 130.1 (Ph, ortho), 132.3 (Ph, para), 145.3 (Ph, ipso), the signal for the NHCO resonance was not detected.

To a stirred solution DMF (7 mL) of **21** (100 mg, 0.16 mmol) was added KSAc (46 mg, 0.40 mmol). After warming to 60 °C, stirring was continued (3 h) and then the solvent was removed in vacuo. H_2O (40 mL) was added to the residue, and the mixture was extracted with EtOAc (2 × 40 mL). The combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. Purification by PTLC (1:2 EtOAc/hexanes) afforded **22** as a white solid: yield, 24 mg (34%); mp 154−163 °C; ^{1}H NMR ($CDCl_3$) δ 1.43 (s, 18 H, OC(CH$_3$)$_3$), 2.35 (s, 6 H, C(O)CH$_3$), 3.05−3.12 (m, 4 H, SCH$_2$), 3.75−3.86 (m, 2 H, C*H*NH), 4.86 (br s, 2 H, NHCO); ^{13}C NMR ($CDCl_3$) δ 28.3 (OC(*C*H$_3$)$_3$), 30.5 (C(O)*C*H$_3$), 31.7 (SCH$_2$), 54.0 (CHNH), 79.8 (O*C*(CH$_3$)$_3$), 156.2 (NHCO), 195.7 (*C*OCH$_3$).

(4*R*,5*R*)-*trans*-4,5-Bis(*tert*-butyloxycarbonylamino)-1,2-dithiane (24). To a stirred solution of **22** (31 mg, 0.07 mmol) in MeOH−H_2O (5:1, 2 mL) was added K_2CO_3 (58 mg, 0.42 mmol). After stirring at room temperature (30 min), KOH (8 mg, 0.15 mmol) was added and O_2 was bubbled through the solution (5 h). The solvent was removed in vacuo and H_2O (20 mL) was added to the residue. The mixture was extracted with EtOAc (2 × 20 mL) and the combined organic layers were dried ($MgSO_4$) and concentrated in vacuo. Purification by PTLC (1:2 EtOAc/hexanes) afforded **24** as a sticky solid: yield, 23 mg (92%); $[\alpha]_{589}^{25} = +46$ ($c = 0.19$, $CHCl_3$); R_f 0.51 (1:2 EtOAc/hexanes); IR (KBr) 3363, 2978, 2360, 1682, 1520, 1057 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 18 H, OC(CH$_3$)$_3$), 2.83 (br s, 2 H, SC*H*H′), 3.15 (br s, 2 H, SCH*H*′), 3.71 (br s, 2 H, C*H*NH), 5.06 (br s, 2 H, NHCO); ^{13}C NMR ($CDCl_3$) δ 28.4 (OC(*C*H$_3$)$_3$), 40.5 (SCH$_2$), 55.4 (CHNH), 80.1 (O*C*(CH$_3$)$_3$), 155.7 (NHCO); MS (+CI) *m*/*z* 351 [M+1]$^+$; M_r (+CI) 351.140 62 [M+1]$^+$ (calcd for $C_{14}H_{27}N_2O_4S_2$ 351.141 23).

(4*R*,5*R*)-*trans*-4,5-Diamino-1,2-dithiane·2TFA (14). Compound **24** (21 mg, 0.06 mmol) was dissolved in TFA (2.0 mL) and stirring was continued at room temperature (30 min). The reaction was concentrated in vacuo to afford **14** as a viscous oil: yield, 23 mg (~100%); $[\alpha]_{589}^{25} = +22$ ($c = 0.37$, MeOH); IR (neat) 3433, 2900, 1689, 1203 cm^{-1}; ^{1}H NMR (CD_3OD) δ 3.00 (app dd, $J = 15.0$, 6.0 Hz, 2 H, SC*H*H′), 3.50−3.63 (m, 4 H, SCH*H*′, C*H*NH$_2$); ^{13}C NMR (CD_3OD) δ 34.1 (SCH$_2$), one signal was not detected and is believed to overlap with one of the solvent peaks; MS (+CI) *m*/*z* 151 [M-2TFA+1]$^+$; M_r (+CI) 151.035 94 [M-2TFA+1]$^+$ (calcd for $C_4H_{11}N_2S_2$ 151.036 37).

7-*N*-(1′-Amino-4′,5′-dithian-2′-yl)porfiromycin C(8) Cyclized Imine (6) ([(4a*R*,9a*S*,10a*S*,10b*R*,11*S*,12a*R*)-10b-Methoxy-6,10-dimethyl-7-oxo-1,4,4a,5,7,9,9a,10,10a,10b,11,12a-dodecahydroazireno[2′,3′:6,7]pyrrolizino[2,3-*f*][1,2]dithiino[4,5-*b*]quinoxalin-11-yl]methyl Carbamate). To an anhydrous methanolic solution (1 mL) of **14** (5.4 mg, 0.014 mmol) and Et_3N (8 μL, 0.06 mmol) was added a methanolic solution (1 mL) of **25** (4.3 mg, 0.012 mmol). The reaction solution was stirred at room temperature (1.5 d) and then the solvent was removed in vacuo. Purification by PTLC (10% MeOH−$CHCl_3$) afforded **6**: yield, 4.0 mg (72%); HPLC t_R 29.2 min; R_f 0.57 (10% MeOH−$CHCl_3$); UV−vis (CH_3CN-H_2O) λ_{max} 224, 276 (sh), 366 nm; ^{1}H NMR (pyridine-d_5, 300 MHz) δ 1.86 (s, C(6)CH$_3$), 2.12 (dd, $J =$ 4.5, 1.5 Hz, C(2)H), 2.29 (s, N(1a)CH$_3$), 2.59 (d, $J = 4.5$ Hz, C(1)H), 3.13−3.26 (C(2′)H, C(3′)*H*H′, C(6′)*H*H′), 3.28 (s, C(9a)OCH$_3$), 3.43−3.58 (C(1′)H, C(3′)H*H*′, C(6′)H*H*′), 3.62 (br d, $J = 12.0$ Hz, C(3)*H*H′), 4.01 (dd, $J = 11.7$, 4.5 Hz, C(9)H), 4.38 (d, $J = 12.0$ Hz, C(3)H*H*′), 5.00 (dd, $J = 11.7$, 10.2 Hz, C(10)*H*H′), 5.71 (dd, $J = 10.2$, 4.5 Hz, C(10)H*H*′), 6.73 (br s, C(7)NH), the signal for the C(10)OC(O)NH$_2$ protons was not detected and is believed to be beneath the solvent peak, the ^{1}H NMR assignments were consistent with the COSY spectrum; ^{13}C NMR (pyridine-d_5, 75 MHz) δ 8.2 (C(6)*C*H$_3$), 38.7 (C(3′)), 40.0 (C(6′)), 43.1 (N(1a)*C*H$_3$), 43.5 (C(2)), 47.2 (C(9)), 47.7 (C(1)), 49.2 (C(9a)O*C*H$_3$), 51.1 (C(3)), 57.0 (C(2′)), 63.2 (C(1′)), 63.3 (C(10)), 106.5 (C(6)), 107.1 (C(9a)), 115.1 (C(8a)), 140.8 (C(7)), 148.0 (C(5a)), 152.8 (C(8)), 158.3 (C(10a)), 179.3 (C(5)); MS (+FAB) *m*/*z* 464 [M+1]$^+$; M_r (+FAB) 464.142 35 [M+1]$^+$ (calcd for $C_{20}H_{26}N_5O_4S_2$, 464.142 62).

7-*N*-(1′-Amino-4′,5′-dithian-2′-yl)mitomycin C C(8) Cyclized Imine (12) ([(4a*R*,9a*S*,10a*S*,10b*R*)-10b-Methoxy-6-methyl-7-oxo-1,4,-4a,5,7,9,9a,10,10a,10b,11,12a-dodecahydroazireno[2′,3′:6,7]pyrrolizino-[2,3-*f*][1,2]dithiino[4,5-*b*]quinoxalin-11-yl]methyl Carbamate). To an

anhydrous methanolic solution (1 mL) of **14** (8.7 mg, 0.023 mmol) and triethylamine (11 μL, 0.08 mmol) was added a methanolic solution (1 mL) of **27** (5.4 mg, 0.015 mmol). The reaction solution was stirred at room temperature (2 d) and then the solvent was removed in vacuo. Purification by PTLC (10% MeOH−$CHCl_3$) afforded **12**: yield, 4.8 mg (71%); HPLC t_R 27.6 min; R_f 0.45 (10% MeOH−$CHCl_3$); UV−vis (CH_3CN-H_2O) λ_{max} 225, 366 nm; ^{1}H NMR (pyridine-d_5, 300 MHz) δ 1.85 (s, C(6)CH_3), 2.71 (dd, J = 3.9, 1.8 Hz, C(2)H), 3.08−3.25 (m, C(1)H, C(2′)H, C(3′)*H*H′, C(6′)*H*H′), 3.30 (s, C(9a)OCH_3), 3.42−3.61 (m, C(1′)H, C(3′)H*H*′, C(6′)H*H*′), 3.64 (br d, J = 11.9 Hz, C(3)*H*H′), 4.05 (dd, J = 11.4, 4.2 Hz, C(9)H), 4.46 (d, J = 11.9 Hz, C(3)H*H*′), 5.26 (dd, J = 11.4, 10.2 Hz, C(10)*H*H′), 5.77 (dd, J = 10.2, 4.2 Hz, C(10)H*H*′), 6.70 (br s, C(7)NH), the signals for the N(1a)H and C(10)$OC(O)NH_2$ protons were not detected and are believed to be beneath the solvent peaks; ^{13}C NMR (pyridine-d_5, 75 MHz) δ 8.3 (C(6)*C*H_3), 34.0 (C(2)), 38.1 (C(1)), 38.8 (C(3′) or C(6′)), 47.7 (C(9)), 49.4 (C(9a)-O*C*H_3), 56.6 (C(2′)), 63.4 (C(10)), 63.5 (C(1′)), 106.9 (C(6)), 107.5 (C(9a)), 114.9 (C(8a)), 140.9 (C(7)), 148.9 (C(5a)), 153.1 (C(8)), 158.6 (C(10a)), 178.8 (C(5)), the signals for C(3) and either C(6′) or C(3′) were not detected and are believed to overlap with the observed peaks; MS (+ESI) m/z 450 $[M+1]^+$.

Methanolysis of C(8) Iminoporfiromycin 6 To Give *cis*-(31) and *trans*-(32) C(1) 1-Methoxy (8) Imino-2-methylaminomitosenes. Compound **6** (4.8 mg, 0.01 mmol) was dissolved in a buffered methanolic solution (0.1 M bis-Tris·HCl, "pH" 5.5, 2 mL) and then stirred at room temperature (21 h). The solvent was removed under reduced pressure and the residue was purified using PTLC (10% MeOH−$CHCl_3$) to provide the desired compounds.

Compound 31: yield, 1.7 mg (37%); HPLC t_R 31.8 min; R_f 0.26 (10% MeOH−$CHCl_3$); UV−vis (CH_3CN-H_2O) λ_{max} 255, 319 nm; ^{1}H NMR (pyridine-d_5, 300 MHz) δ 2.00 (s, C(6)CH_3), 2.48 (s, NHC*H*$_3$), 3.16−3.34 (m, C(2′)H, C(3′)*H*H′, C(6′)*H*H′), 3.51 (s, C(1)OCH_3), 3.52−3.64 (m, C(1′)H, C(3′)H*H*′, C(6′)H*H*′), 3.73−3.82 (m, C(2)H), 4.09 (dd, J = 11.7, 9.3 Hz, C(3)*H*H′), 4.92 (dd, J = 11.7, 7.5 Hz, C(3)-H*H*′), 5.01 (d, J = 5.1 Hz, C(1)H), 5.76 (1/2ABq, J = 12.9 Hz, C(10)-*H*H′), 5.94 (1/2ABq, J = 12.9 Hz, C(10)H*H*′), 6.61 (br s, C(7)NH), the signals for the C(2)NH and C(10)$OC(O)NH_2$ protons were not detected and are believed to be beneath the solvent peaks; MS (+FAB) m/z 464 $[M+1]^+$; M_r (+FAB) 464.141 49 $[M+1]^+$ (calcd for $C_{20}H_{26}N_5O_4S_2$, 464.142 62).

Compound 32: yield, 1.7 mg (37%); HPLC t_R 29.7 min; R_f 0.27 (10% MeOH−$CHCl_3$); UV−vis (CH_3CN-H_2O) λ_{max} 255, 316 nm; ^{1}H NMR (pyridine-d_5, 300 MHz) δ 1.99 (s, C(6)CH_3), 2.47 (s, NHC*H*$_3$), 3.17−3.38 (m, C(2′)H, C(3′)*H*H′, C(6′)*H*H′), 3.54 (s, C(1)OCH_3), 3.55−3.78 (m, C(1′)H, C(3′)H*H*′, C(6′)H*H*′), 3.92 (d, J = 5.1 Hz, C(2)H), 4.53 (d, J = 12.6 Hz, C(3)*H*H′), 4.64 (dd, J = 12.6, 5.1 Hz, C(3)H*H*′), 5.11 (s, C(1)H), 5.75 (1/2ABq, J = 12.9 Hz, C(10)*H*H′), 5.88 (1/2ABq, J = 12.9 Hz, C(10)H*H*′), 6.51 (br s, C(7)NH), the signals for the C(2)-NH and C(10)$OC(O)NH_2$ protons were not detected and are believed to be beneath the solvent peaks; MS (+FAB) m/z 464 $[M+1]^+$; M_r (+FAB) 464.142 47 $[M+1]^+$ (calcd for $C_{20}H_{26}N_5O_4S_2$, 464.142 62).

Methanolysis of C(8) Iminoporfiromycin 13 To Give *cis*-(33) and *trans*-(34) 1-Methoxy C(8) Imino-2-methylaminomitosenes.[35] Compound **13** (2.0 mg, 0.004 mmol) was dissolved in a buffered methanolic solution (0.1 M bis-Tris·HCl, "pH" 5.5, 1 mL) and then stirred at room temperature (18 h). The solvent was removed under reduced pressure and the residue was purified using PTLC (10% MeOH−$CHCl_3$) to provide the desired compounds.

Compound 33:[35] yield, 0.6 mg (30%); HPLC t_R 29.0 min; R_f 0.26 (10% MeOH−$CHCl_3$); UV−vis (CH_3CN-H_2O) λ_{max} 251, 315 nm; ^{1}H NMR (pyridine-d_5, 300 MHz) δ 1.19−1.32 (m, C(4′)*H*H′, C(5′)*H*H′), 1.40−1.48 (m, C(3′)*H*H′, C(6′)*H*H′), 1.54−1.58 (m, C(4′)H*H*′), 1.64−1.68 (m, C(5′)H*H*′), 1.96−2.02 (m, C(3′)H*H*′), 2.12 (s, C(6)CH_3), 2.38 (s, NHC*H*$_3$), 2.45−2.50 (m, C(6′)H*H*′), 2.93 (ddd, J = 11.7, 11.7, 2.7 Hz, C(2′)H), 3.28 (ddd, J = 11.7, 11.7, 2.7 Hz, C(1′)H), 3.51 (s, C(1)-OCH_3), 3.58−3.62 (m, C(2)H), 4.01 (dd, J = 11.5, 9.0 Hz, C(3)*H*H′), 4.88 (dd, J = 11.5, 7.5 Hz, C(3)H*H*′), 5.84 (1/2ABq, J = 13.2 Hz, C(10)*H*H′), 6.11 (1/2ABq, J = 13.2 Hz, C(10)H*H*′), 6.57 (br s, C(7)-NH), the signals for the C(1)H, C(2)NH and C(10)$OC(O)NH_2$ protons were not detected and are believed to be beneath the solvent peaks; MS (+FAB) m/z 428 $[M+1]^+$; M_r (+FAB) 428.230 34 $[M+1]^+$ (calcd for $C_{22}H_{30}N_5O_4$, 428.229 78).

Compound 34:[35] yield, 0.9 mg (45%); HPLC t_R 27.9 min; R_f 0.29 (10% MeOH−$CHCl_3$); UV−vis (CH_3CN-H_2O) λ_{max} 251, 315 nm; ^{1}H NMR (pyridine-d_5, 300 MHz) δ 1.17−1.48 (m, C(4′)*H*H′, C(5′)*H*H′, C(3′)*H*H′, C(6′)*H*H′), 1.52−1.56 (m, C(4′)H*H*′), 1.62−1.66 (m, C(5′)-H*H*′), 1.95−1.99 (m, C(3′)H*H*′), 2.10 (s, C(6)CH_3), 2.42 (s, NHC*H*$_3$), 2.42−2.45 (m, C(6′)H*H*′), 2.93 (ddd, J = 12.3, 12.3, 3.0 Hz, C(2′)H), 3.23−3.30 (m, C(1′)H), 3.55 (s, C(1)OCH_3), 3.86 (d, J = 5.1 Hz, C(2)H), 4.51 (d, J = 12.9 Hz, C(3)*H*H′), 4.63 (dd, J = 12.9, 5.1 Hz, C(3)H*H*′), 5.05 (s, C(1)H), 5.90 (1/2ABq, J = 12.6 Hz, C(10)*H*H′), 5.98 (1/2ABq, J = 12.6 Hz, C(10)H*H*′), 6.45 (br s, C(7)NH), the signals for the C(2)NH and C(10)$OC(O)NH_2$ protons were not detected and are believed to be beneath the solvent peaks; MS (+FAB) m/z 428 $[M+1]^+$; M_r (+FAB) 428.229 56 $[M+1]^+$ (calcd for $C_{22}H_{30}N_5O_4$, 428.229 78).

General Procedure for the Solvolysis of Porfiromycins (Kinetic Studies). To a buffered methanolic solution (0.1 M Tris·HCl, "pH" 7.4; 0.1 M bis-Tris·HCl, "pH" 5.5) (final volume 1.5 mL) maintained at 25 °C containing the porfiromycin (10−60 μL of 4 mM methanolic solution, final concentration 0.03−0.17 mM) was added a methanolic solution (5−50 μL) of the nucleophile of choice (stock solution: 4−20 mM, final nucleophile concentration 0.015−3.0 mM). The reaction was monitored by UV−visible spectroscopy (200−600 nm), and typically followed for greater than two half-lives. The "pH" of the solution was determined at the conclusion of the reaction and found to be within ± 0.1 pH units of the original solution. The reaction products (**31** + **32**, **33** + **34**) were identified by co-injection with authentic samples using HPLC and cospotting with authentic samples using TLC. The λ_{max} of porfiromycin (~365 nm) was plotted versus time and found to decrease in a first-order decay (exponential decay) process. The nonlinear regression analysis to fit the observed exponential decay by SigmaPlot Program (SigmaPlot, 2001) yielded pseudo-first-order rate constants (k_{obs}) and half-lives ($t_{1/2}$). The reactions were done in duplicate and the results averaged.

General Procedure for Alkaline Agarose Gel Electrophoresis.[50] The agarose gels were prepared by adding 1.2 g of agarose to 100 mL of an aqueous 100 mM NaCl and 2 mM EDTA solution (pH 8.0). The suspension was heated in a microwave oven until all of the agarose was dissolved (1 min). The gel was poured and was allowed to cool and solidify at room temperature (1 h). The gel was soaked in an aqueous alkaline running buffer solution (50 mL) containing 40 mM NaOH and 1 mM EDTA (1 h) and then the comb was removed. The buffer solution was refreshed prior to electrophoresis.

To an aqueous solution of ~85 μL of H_2O (sterile) and 2.5 μL of 1 M Tris·HCl (pH 7.4) was added a solution of linearized pB*R*322 (5 μL, 5 μg) in 10 mM Tris solution containing 1 mM EDTA (pH 8.0). After deaeration with Ar (15 min), porfiromycin (1−5 μL of 1−4 mM DMSO solution, final concentration 0.01−0.2 mM) and a nucleophile of choice (1−5 μL of 1−20 mM DMSO solution, final concentration 0.01−1.0 mM) were added and the resulting solution (final volume 100 μL) was incubated at room temperature (2 h). The solution was washed with 1:1 PhOH/$CHCl_3$ (100 μL) and $CHCl_3$ (2 × 100 μL), and precipitated (12.1 μL of 3 M NaOAc and 250 μL of EtOH, −70 °C (10 min)). The mixture was centrifuged at 0 °C (15 min), and the EtOH was decanted off and evaporated in vacuo. The remaining DNA was dissolved in 25 μL of 10 mM Tris solution containing 1 mM EDTA (pH 8.0). Agarose loading dye (5 μL) was added to the sample (5 μL) and the samples were loaded onto the wells. The gel was run at 75 mA/25 V (30 min) and then at 145 mA/38 V (3−4 h). The gel was then neutralized for 45 min in an aqueous 100 mM Tris pH 7.0 buffer

solution containing 150 mM NaCl, which was refreshed every 15 min. The gel was stained with an aqueous 100 mM Tris pH 7.5 buffer solution (100 mL) containing ethidium bromide (20 μL of an aqueous ethidium bromide stock solution (10 mg/10 mL)) and 150 mM NaCl for 20 min. The background staining was then removed by soaking the gel in an aqueous 50 mM NH_4OAc and 10 mM β-mercaptoethanol solution (3 h). The gel was then analyzed by two methods. In one method, the gel was visualized by UV and photographed using Polaroid film 667. In the second method, the gel was analyzed with a Storm 860 phosphorimager operating in the blue fluorescence mode and ImageQuant 5.0 software (Molecular Dynamics, Sunnyvale, CA).

General Procedure for Antiproliferative Activity Test.[60] In vitro antiproliferative tests were conducted using human tumor cell line A549 (lung adenocarcinoma) by Dr. Hitoshi Arai (Kyowa Hakko Kogyo Co., Shizuoka, Japan). The cells (2×10^3 cells/well) were precultured at 37 °C (24 h) in 96-well microtiterplates containing the culture medium (RPMI-1640 supplemented with 10% (v/v) fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin) under either aerobic (5% of CO_2 and 95% of air) or hypoxic (5% of CO_2 and $<2\%$ of O_2) conditions. The cells were then treated with the drug candidates (1 h), washed twice with the medium and further incubated (71 h) in the drug-free medium. The antiproliferative activity of drugs against tumor cells was measured by MTT assay. Cell growth (%) was calculated by the equation, $\{[A - A_o]/[A_c - A_o]\} \times 100$ (A: absorbance, A_o: blank absorbance, A_c: control absorbance), and the activity was expressed by IC_{50} values (concentration required for 50% inhibition).

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Supporting Information Available: General methods and synthetic procedures for the preparation of **20**, supporting figure for DNA ISC experiments for **6**, **7**, and **13** with Et_3P and GSH (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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