Quantitative Structure-Activity Relationship Analysis of Cation-Substituted Polyaromatic Compounds as Potentiators (Amplifiers) of Bleomycin-Mediated Degradation of DNA

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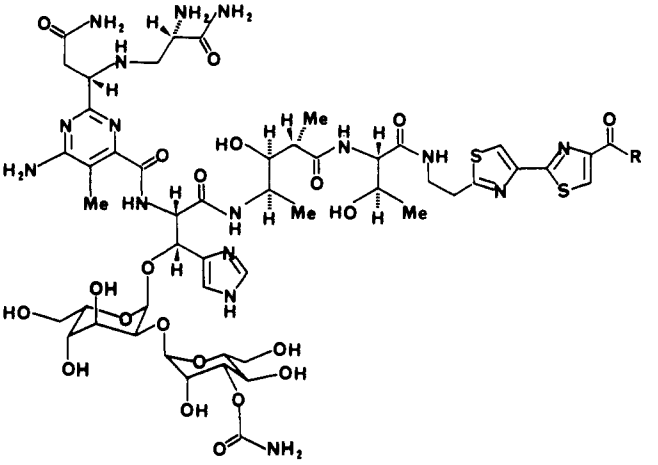
A set of 21 polyheteroaromatic compounds substituted with flexible cationic groups and of similar molecular size has been analyzed for binding with DNA and for effects on the bleomycin-mediated degradation of the DNA double helix. Increases in apparent rates of the DNA digestion were observed in all cases under the experimental conditions of noncompetitive binding of these compounds and bleomycin to DNA. Surprisingly, the quantitative structure–activity relationship analysis revealed two distinct correlations despite close structural similarities for the set of bleomycin amplifiers. These unusual results are explained in terms of the formation of two stereochemically different ternary complexes of activated bleomycin–DNA–amplifier. The relevance of this finding for the design of new bleomycin amplifiers is discussed.

Bleomycin is a generic name for a family of metal complexing glycopeptide antitumor antibiotics (Chart I). As indicated in our recent review,[1] there are apparently several modes of action by which bleomycin exerts its antitumor activity. One firmly established mechanism involves interaction with DNA in the presence of a metal ion cofactor and subsequent degradation of the double helix.[2] Although several metal complexes of bleomycin are known to bind with and cause degradation of DNA,[3] it is strongly believed that the ferrous ion induced chemistry is of the greatest importance in vivo. Many studies have shown that the chelate of bleomycin with ferrous ion reversibly forms a ternary complex with molecular oxygen, and the ternary complex is activated in solution through a one-electron reduction. The resultant activated complex binds in the minor groove of the DNA double helix, and this binding is followed by stereospecific abstraction of the deoxyribose C-4′ hydrogen with the release of small DNA fragments and inactive complex of bleomycin with ferric ion. Since the latter complex can be reduced back to the complex with ferrous ion, bleomycin acts catalytically, under proper conditions, with each turnover requiring two electrons and one molecule of dioxygen.[2]

The drug is inactivated in vivo by bleomycin hydrolase,[4] in addition to fast self-destruction of the activated complex with iron in the absence of DNA.[5] This decomposition is slower but not completely eliminated in the presence of DNA. As a result of the inactivation the drug has a relatively short lifetime in vivo.[6] Chemotherapies with increased doses of bleomycin, or even small doses applied frequently, are not acceptable because of severe, cumulative pulmonary toxicity.[6,7] This toxicity is not necessarily due to the interaction of the drug with DNA of the host organism. The current clinical use of bleomycin is limited to low doses of the drug in synergistic combinations with other anticancer agents.

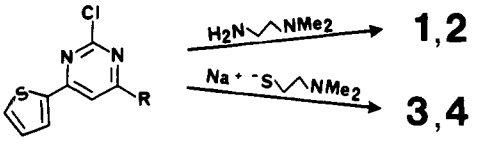
Another potential way of enhancing the activity of anticancer drugs is to find compounds that alone may have no significant activity or toxicity, but that amplify the action of the drug.[1,8] We have been interested in developing such compounds (amplifiers) that act directly at the DNA level and have focused on the bleomycin reaction.[9] Previous studies have strongly suggested that conformational changes of the DNA double helix caused by binding of amplifiers of this class are responsible for the observed bleomycin potentiation. In order to better understand the amplification phenomenon, in this paper we have analyzed

Chart I. Structures of Selected Bleomycins: BML-A2 (R = $NH(CH_2)_3{}^+SMe_2$), BLM-B2 (R = $NH(CH_2)_4NHC^+(NH_2)_2$[a]



[a] Blenoxane is a mixture consisting mainly of BLM-A2 and BLM-B2.

Scheme I



22 R = 2-furanyl

23 R = 2-thienyl

24 R = 1-methyl-2-pyrrolyl

the amplification activities of 21 DNA binding and structurally related compounds using a quantitative

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(2) For reviews, see: (a) Stubbe, J.; Kozarich, J. W. *Chem. Rev.* **1987**, *87*, 1107. (b) Hecht, S. M. *Acc. Chem. Res.* **1986**, *19*, 383.
(3) (a) Saito, I.; Morii, T.; Sugiyama, H.; Matsuura, T.; Meares, C. F.; Hecht, S. M. *J. Am. Chem. Soc.* **1989**, *111*, 2307. (b) Chang, L.-H.; Meares, C. F. *Biochemistry* **1984**, *23*, 2268. (c) Kuwahara, J.; Suzuki, T.; Sugiura, Y. *Biochem. Biophys. Res. Commun.* **1985**, *129*, 368. (d) Ehrenfeld, G. M.; Shipley, J. B.; Heimbrook, D. C.; Sugiyama, H.; Long, E. C.; van Boom, J. H.; van der Marel, G. A.; Oppenheimer, N. J.; Hecht, S. M. *Biochemistry* **1987**, *26*, 931. (e) McLean, M. J.; Dar, A.; Waring, M. J. *J. Mol. Recognit.* **1989**, *1*, 184.

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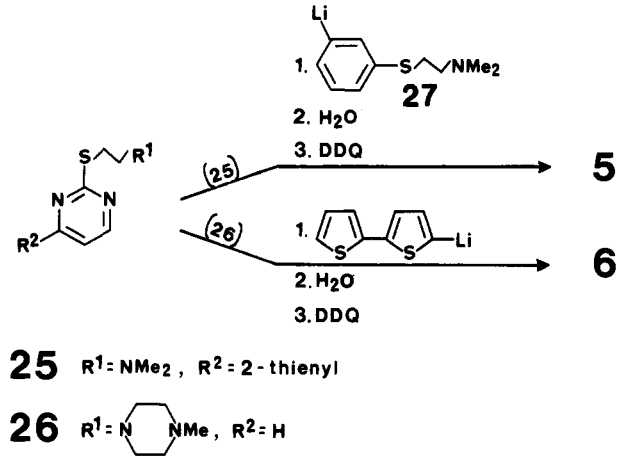
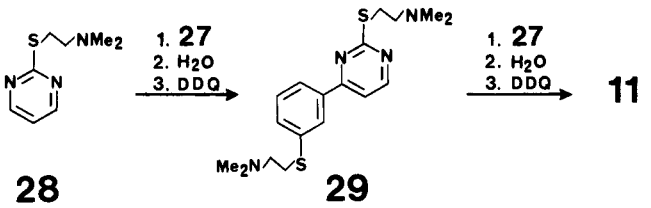
Scheme II



Scheme III



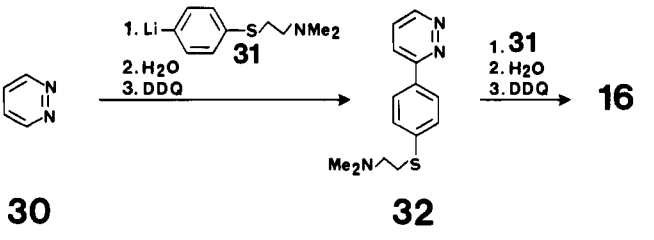
structure–activity relationship (QSAR) approach. The amplification has also been analyzed in terms of DNA binding characteristics of these compounds.

The structures of compounds **1–21** used in this study are given in Chart II. The selection of these compounds was based on their structural similarities but diverse characteristics of their interaction with DNA as discussed below.

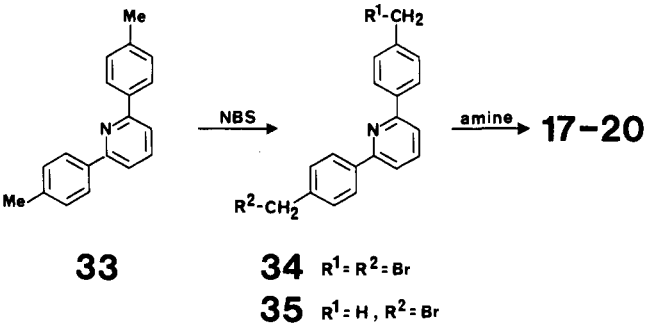
Chemistry

A nucleophilic displacement of chloride in readily available 2-chloropyrimidines[10] **22–24** by treatment with 2-(dimethylamino)ethylamine or sodium 2-(dimethylamino)ethanethiolate gave the corresponding amplifiers

Scheme IV



Scheme V



1–4 (Scheme I). A similar chemistry was used in the synthesis of substituted pyrimidines **25** and **26**, the precursors to **5** and **6**, respectively. Thus, treatment of **25** with 3-[[2-(dimethylamino)ethyl]thio]phenyllithium (**27**) followed by aromatization of the resultant dihydropyrimidine derivative in the reaction with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) furnished tricyclic derivative **5** (Scheme II). The addition reaction of a 2,2′-bithien-5-yllithium reagent with **26** followed by a similar treatment with DDQ gave **6**. This addition/aromatization method has been used previously[9f,g,11,12] to synthesize **7–10**, **12**, **13**, and **15** from the appropriate pyrimidines and lithium reagents. Its synthetic value is further demonstrated in this work for similar preparations of **14**, substituted pyrimidine **11** (Scheme III), and substituted pyridazine **16** (Scheme IV).

The synthesis of a series of disubstituted pyridines **17–20** is given in Scheme V. Bromination of 2,6-di-*p*-tolylpyridine (**33**) with *N*-bromosuccinimide (NBS) gave, depending on conditions, a bis(bromomethyl)[13] (**34**) or a bromomethyl derivative (**35**) as the major product. These compounds were treated with amines to give **17–20**.

DNA Binding

Binding Strength. The aromatic ring systems of **1–20** consist of three unfused rings while propidium (**21**) has a fused, three-ring system which is also cationic. None of the tricyclic compounds **1–20** is protonated at a ring nitrogen[14] under the conditions of this work (pH 7). The protonation of their basic amino substituents depends on the separation of the adjacent amino groups.[15] Thus,

(4) Sebti, S. M.; DeLeon, J. C.; Ma, L.-T.; Hecht, S. M.; Lazo, J. S. *Biochem. Pharmacol.* **1989**, *38*, 141.
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(7) Catravas, J. D. *Dev. Oncol.* **1988**, *53*, 118.
(8) Brown, D. J.; Grigg, G. W. *Med. Res. Rev.* **1982**, *2*, 193. (b) Brown, D. J. *Chem. Aust.* **1987**, 116.
(9) (a) Wilson, W. D.; Tanious, F. A.; Barton, H. J.; Wydra, R. L.; Jones, R. L.; Boykin, D. W.; Strekowski, L. *Anti-Cancer Drug Des.* **1990**, *5*, 31. (b) Strekowski, L.; Harden, D. B.; Wydra, R. L.; Stewart, K. D.; Wilson, W. D. *J. Mol. Recognit.* **1989**, *2*, 158. (c) Strekowski, L.; Mokrosz, J. L.; Tanious, F. A.; Watson, R. A.; Harden, D.; Mokrosz, M.; Edwards, W. D.; Wilson, W. D. *J. Med. Chem.* **1988**, *31*, 1231. (d) Strekowski, L.; Mokrosz, M.; Mokrosz, J. L.; Strekowska, A.; Allison, S. A.; Wilson, W. D. *Anti-Cancer Drug Des.* **1988**, *3*, 79. (e) Strekowski, L.; Wilson, W. D.; Mokrosz, J. L.; Strekowska, A.; Koziol, A. E.; Palenik, G. J. *Anti-Cancer Drug Des.* **1988**, *2*, 387. (f) Wilson, W. D.; Strekowski, L.; Tanious, F. A.; Watson, R. A.; Mokrosz, J. L.; Strekowska, A.; Webster, G. D.; Neidle, S. *J. Am. Chem. Soc.* **1988**, *110*, 8292. (g) Strekowski, L.; Strekowska, A.; Watson, R. A.; Tanious, F. A.; Nguyen, L. T.; Wilson, W. D. *J. Med. Chem.* **1987**, *30*, 1415.
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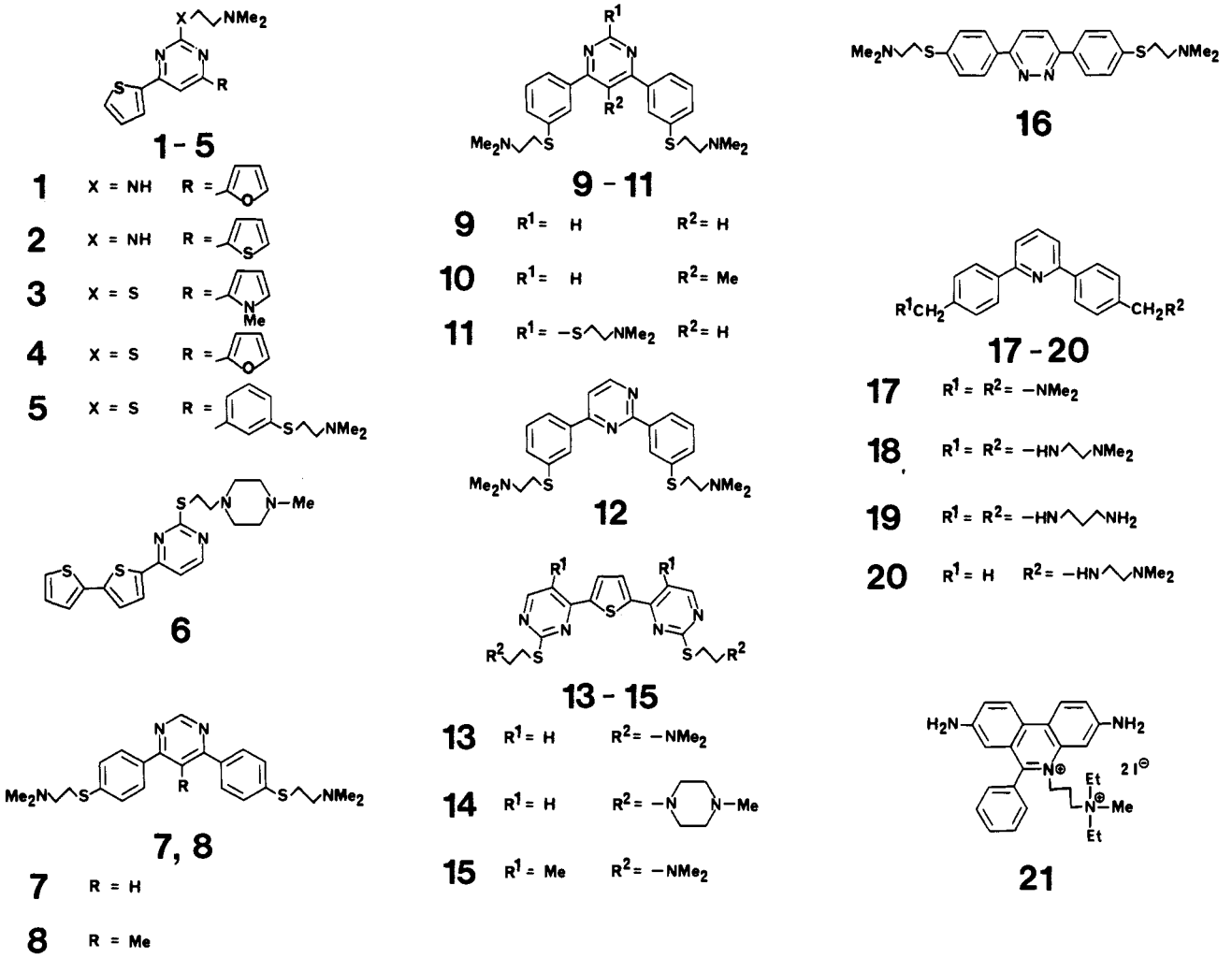
(15) Protonation of both amino functions in an aliphatic diamine at pH 7 requires the separation of the nitrogen atoms by at least three methylene groups: Perrin, D. D.; Dempsey, B.; Serjeant, E. P. *pK_a Prediction for Organic Acids and Bases*; Chapman & Hall: London, 1981. See also ref 9d.

Chart II. Structures of Bleomycin Amplifiers 1-21



compounds 1–4, 6, and 20 are monocationic, 5, 7–10, and 12–18 are dicationic, 11 is tricationic, and 19 is tetracationic at pH 7. The electrostatic interaction of these cationic groups with the anionic DNA backbone enhances groove binding or intercalation of the aromatic portion of the molecule by increasing the overall stability of the molecule–DNA complex.[16] Previous studies have shown that compounds binding with DNA by this dual mode also show increased bleomycin amplification in comparison to the activity of both noncationic aromatic derivatives and nonaromatic polycationic amines.[9]

Addition of DNA to 1–21 caused hypochromic changes and shifts to longer wavelength in absorption spectra for all compounds. Compounds 1, 3–6, 11, 14, and 16–20 were titrated into DNA at a range of concentrations low enough that isosbestic points were obtained, and the free and bound extinction coefficients were used to construct binding isotherms.[17] These isotherms in the form of Scatchard plots were analyzed with the site exclusion model of McGhee and von Hippel[18] (eq 1), where v is the

$$v/C = K(1 - nv)[(1 - nv)/(1 - (n - 1)v]^{n-1} \quad (1)$$

moles of compound bound per DNA base pair, C is the free compound molarity, K is the observed binding equilibrium constant, and n is the number of base pairs per binding site.

Binding constants for these compounds are given in Table I. Binding constants for 2, 7–10, 12, 13, 15, and 21, obtained by the same method and reported previously,[9f,g,12,17] are also included in Table I.

Binding Specificity. Scatchard binding isotherms were also determined for the binding of 18 and 20 to synthetic DNA polymers, poly[d(A-T)]·poly[d(A-T)] and poly[d(G-C)]·poly[d(G-C)]. Both compounds show a preference for binding at A-T base pairs with the ratios $K_{A\text{-}T}/K_{G\text{-}C}$ of 6.7 for 18 and 3.6 for 20. Previously we reported the base pair binding specificity for 7, 8, and 13 as 12.0, 4.2, and 0.27, respectively.[9f,g] Thus, compound 13 prefers to bind adjacent to G-C base pairs while 7, 8, 18, and 20 show opposite base pair specificity.

Mode of Binding: NMR. NMR spectroscopy is a powerful tool for evaluating binding modes in the small molecule–DNA complex.[9f,19] Thus, the outside binding, due entirely to electrostatic attraction, causes little, if any, shifts in the proton signals of the molecule, imino proton signals of DNA base pairs, and DNA phosphorus signals. Groove binding induces shifts in the proton spectrum of the bound molecule, which are usually smaller than 0.3 ppm and can be either negative or positive, but it does not

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(18) McGhee, J. D.; von Hippel, P. H. *J. Mol. Biol.* **1974**, *86*, 469.
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Table I. DNA Binding, Connectivity Indexes, and Potentiation of BLM-A2-Mediated Digestion of DNA for Amplifiers 1-21

	DNA binding			bleomycin amplification[a]		
no.	$K \cdot 10^{-3}$, M^{-1}	n	connectivity index $^1X^V$	k_{amp}, min^{-1}	k_{amp}/k_s	log (k_{amp}/k_s)
1	65	2.3	7.063	0.0296	1.51	0.179
2	158[b]	3.3	7.063	0.0298	1.52	0.182
3	70	2.3	7.455	0.0341	1.74	0.240
4	165	2.5	6.966	0.0316	1.61	0.207
5	>2000	~3	9.595	0.0348	1.77	0.249
6	188	2.4	8.786	0.0395	2.01	0.304
7	727[c]	2.9	10.091	0.0292	1.49	0.173
8	28[c]	3.5	10.513	0.0218	1.11	0.046
9	419[d]	2.3	10.091	0.0254	1.29	0.112
10	38[d]	~4	10.513	0.0206	1.05	0.022
11	>5000	2.7	12.225	0.0421	2.15	0.332
12	427[d]	2.4	10.101	0.0248	1.26	0.102
13	>5000[b]	3.3	9.328	0.0280	1.43	0.155
14	>2000	2.5	12.963	0.0287	1.47	0.166
15	>5000[b]	3.8	10.161	0.0261	1.33	0.124
16	>1000	~3	10.108	0.0416	2.12	0.326
17	200	~3	8.962	0.0239	1.22	0.086
18	>1000	3.5	11.377	0.0465	2.37	0.375
19	>1000	3.5	10.772	0.0493	2.52	0.401
20	26	3.0	9.105	0.0286	1.46	0.164
21	>5000[e]	3.0	11.288	0.0458	2.34	0.360

[a] The apparent rate constants for the BLM-mediated digestion of DNA in the presence of an amplifier are denoted as k_{amp} and k_s, respectively. The constant $k_s = 0.0196 \pm 0.0003$ min^{-1} was obtained with BLM-A2. Blenoxane gave slightly lower k_s but the amplification ratios k_{amp}/k_s for BLM-A2 and blenoxane were essentially identical. [b] Data from ref 11. [c] Data from ref 9f. [d] Data from ref 12. [e] Data for ref 17.

usually have any significant effect on the DNA imino and phosphorus spectra. Evidence for intercalation is based on (i) upfield shifts for the signals of the aromatic protons of the molecule, normally larger than 0.4 ppm, (ii) upfield shifts for the signals of the DNA imino base pair protons, and (iii) downfield shifts in DNA phosphorus spectra. Propidium (**21**) is a classical intercalator.[17]

The proton NMR assignments for **1–20** were determined by analysis of chemical shifts, coupling constants, and NOE results. The assignments for compounds not reported previously are given in the Experimental Section. Upon addition of DNA all compounds **1–20** gave upfield shifts for the aromatic protons. The following lowest and largest shifts (in ppm) were obtained at the molar ratio of one molecule per three DNA base pairs (see names in the Experimental Section for the proton numbering): **1**, 0.38 (H4′), 0.70 (H5); **2**,[11] 0.60 (H4′, H5′), 0.84 (H5); **3**, ~0.5 (H4′), ~0.8 (H5); **4**, 0.37 (H4′), 0.71 (H5″); **5**, 0.38 (H2″), 0.86 (H3′); **6**, 0.48 (H4″), 0.80 (H4′); **7**,[9f] 0.41 (H2′, H3′, H5′, H6′), 0.51 (H2); **8**,[9f] <0.05 (H2′, H3′, H5′, H6′), 0.05 (H2); **9**,[12] 0.45 (H2′), 0.70 (H2); **10**,[12] 0.05 (H4′), 0.07 (H2); **11**, 0.47 (H4′), 0.61 (H6′); **12**,[12] 0.51 (H4′), 0.86 (H5); **13**,[11] 0.47 (H6′), 0.76 (H3, H4); **14**, 0.45 (H6′), 0.74 (H3, H4); **15**,[11] 0.40 (H6′), 0.54 (H3, H4); **16**, >0.5 (H2′), >0.7 (H4, H5); **17**, 0.29 (H3′, H5′), 0.66 (H4); **18**, 0.34 (H3′, H5′), 0.58 (H4); **19**, 0.34 (H3′, H5′), 0.58 (H4); **20**, >0.2 (H3″, H5″), >0.6 (H4). All side-chain protons for all compounds **1–20** shifted 0.13 ppm or less over the same titration range. Compounds **1–7**, **9**, and **11–20** all induced a 0.2–0.5 ppm upfield shift in the DNA imino proton signal and caused an approximately 0.2–0.4 ppm downfield shift in the DNA ^{31}P NMR signal. By contrast, addition of **8** or **10** to DNA did not result in a significant shift for the DNA imino proton or ^{31}P signals under the same conditions.[9f,12]

We conclude, therefore, that aromatic portions of **1–7**, **9**, and **11–20** at least partially intercalate with DNA base pairs while **8** and **10** are not DNA intercalators. The observed changes in ^1H NMR spectra of **8** and **10** upon addition of DNA are consistent with the groove binding mode for these compounds.

Mode of Binding: Viscometric Titrations. The above conclusion is strongly supported by the observed increases in viscosity of sonicated calf thymus DNA upon addition of compounds **1–7**, **9**, and **11–20**. The maximum reduced specific viscosity ratio η/η_0 was in the range 1.8 ± 0.3, typically seen for intercalators.[16] Furthermore, selected compounds from this group caused unwinding of supercoiled DNA as expected for an intercalation binding mode. The unwinding angles,[9f,11,12] determined by the Vinograd method,[20] are 19°, 8°, 11°, 14°, 17°, and 10° for **2**, **7**, **9**, **12**, **13**, and **15**, respectively.

Also in agreement with the classification of **8** and **10** as groove-binding molecules,[21] they caused a negligible increase in viscosity of linear DNA ($\eta/\eta_0 < 1.05$) and did not unwind supercoiled DNA.

Groove-Binding Specificity. Recently we have conducted molecular modeling studies[9f] on the intercalation of **7** at the G-C site of d(TACGTA). The minor groove binding model was not feasible due to a large number of close contacts including prohibitive steric interactions of the sulfur atoms of **7** with the DNA backbone. Two other models, complex A, with the molecule threaded through DNA and the side chains located in both grooves, and complex B, with the molecule intercalated to DNA from the major groove and both side chains located in this groove, gave low-energy interactions. On the basis of stopped-flow kinetics experiments, which indicate that the bound-state lifetime is very short, it was concluded that intercalation of **7** from the major groove (complex B) is the more feasible interaction model.

The bleomycin amplification experiments with **7** conducted as described below but with increasing concentrations of **7** strongly support this model. In these experiments the maximum enhancement of the bleomycin-mediated degradation of DNA was reached at the molar ratio 1:2.5 of **7** per DNA base pair and it did not change even at the ratio 2:1, when not only all DNA binding were saturated with **7** but also when a large excess of **7** was free in solution. Essentially the same result was obtained by

(20) Revet, B.; Schmir, M.; Vinograd, J. *Nature, New Biol.* **1971**, *229*, 10.
(21) Zimmer, C.; Wahnert, U. *Prog. Biophys. Mol. Biol.* **1986**, *47*, 31.

quantitative analysis of the DNA degradation products using HPLC. Since bleomycin binds in the minor groove with the binding constant[22] several times smaller than the constant for **7**, it must be concluded that **7** does not compete with bleomycin for binding in the DNA minor groove.[9b]

This analysis has a direct implication on groove-binding specificity of **17–20**, which must be the same as that with **7**. Thus, compounds **7** and **17–20** all contain isosteric aromatic portions with the same substitution pattern. The unfavorable steric clash in the minor groove, as already discussed, is expected to be even more severe with the methylene moieties of **17–20** than with the sulfur atoms of **7** because of the relative size of these groups.[23,24]

Bleomycin Amplification

In order to develop better bleomycin amplifiers, it is extremely important to understand their structure–activity relationship and the molecular basis for bleomycin amplification. Such work requires the availability of simple, but sensitive and reliable, assays to monitor the kinetics of bleomycin-mediated degradation of DNA in the presence and absence of potential amplifiers. These must be in vitro tests so that the effects of amplifiers at DNA can be distinguished from secondary effects of transport and metabolism.

Of three such methods developed recently, the viscometric assay is characterized by the highest accuracy and reproducibility.[9c] It mimics the biological conditions of low concentration of iron and high molecular ratio of DNA nucleotides to bleomycin, where the drug acts as a catalyst with many turnovers. The viscometric assay was used in this work for correlation analysis.

The DNA degradation experiments were conducted directly in a viscometer at 37 ± 0.01 °C in buffered solutions (pH = 7.00, $[Na^+]$ = 0.015 M) preequilibrated with air to ensure the same concentration of molecular oxygen in all experiments. The concentration of calf thymus DNA was 1.7×10^{-4} M base pairs with the following molar ratios of other components to DNA base pairs: BLM-A2, 1:100; Fe(II), 1:50; amplifier, 1:5.35. Under these conditions and in the presence of dithiothreitol as an iron-reducing agent, the DNA viscosity changes in time are described by the biphasic eq 2,

$$(\eta/\eta_0)_t = ae^{-k_f t} + be^{-k_s t} \quad (2)$$

where η_0 is the initial reduced specific viscosity for DNA before the addition of bleomycin, η is the reduced specific viscosity for DNA at the digestion time t, k_f is the apparent rate constant for the first (fast) process, k_s is the apparent rate constant for the second (slow) process, and a and b are the amplitudes for the fast and slow phases, respectively. The fast degradation is due to the fast activation of the BLM–Fe(II) complex and the subsequent fast reaction of the activated complex with DNA.[25] This reaction is important at the initial degradation stage, after addition of a ferrous salt to the mixture. The rate-determining step for the slow process is reactivation of the BLM–Fe(III) complex. This second slow process can be monitored with high accuracy and reproducibility to compare activities of different amplifiers.

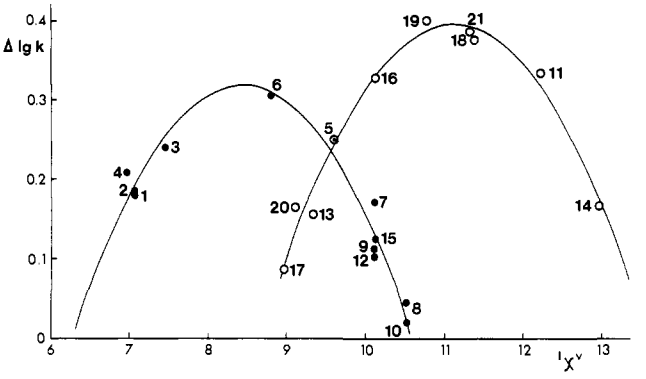

Figure 1. QSAR analysis of compounds **1–21** as amplifiers of BLM-A2-mediated degradation of DNA. The left and right parabolas correspond to the eqs 3 and 4, respectively.

The apparent rate constant k_s = 0.0196 ± 0.0003 min^{-1} was obtained with BLM-A2 and in the absence of an amplifier. The corresponding rate constants k_{amp} in the amplified DNA degradations and the amplification activities k_{amp}/k_s are given in Table I. These amplification values were obtained for the same ratio of 1:5.35 of bound molecules **1–21** to DNA base pairs. The analysis of binding of the amplifiers with DNA using eq 1 revealed that a fraction of molecules close to unity is complexed under the conditions of this work for compounds with $K > 100\,000$ M^{-1}. For relatively weakly binding molecules **1**, **3**, **8**, **10**, and **20**, their concentrations were increased to arrive at the same DNA complexation.

Synthetic DNA Polymers. The amplification work was also conducted with poly[d(A-T)]·poly[d(A-T)] and poly[d(G-C)]·poly[d(G-C)] and amplifier **7** under the same concentration conditions as above, but a different test system. The reactions were monitored by HPLC quantitation of DNA bases released from the polymers, as described previously.[9b] Due to the relatively low molecular weights of these synthetic polymers, the viscometric assay could not be used.

In the absence of amplifier the apparent initial degradation rate for poly[d(A-T)]·poly[d(A-T)] was faster than that for poly[d(G-C)]·poly[d(G-C)] by a factor of 1.11 ± 0.05. In the presence of **7** these degradation rates increased by a factor of 1.55 ± 0.05 in both cases. Thus, the same enhancement effect is observed with **7** bound strongly to A-T base pairs and weakly to G-C base pairs. With high molecular weight calf thymus DNA the amplification activity measured by the HPLC method was 1.8 ± 0.1 under the same conditions.

QSAR

Recently we found an excellent Hansch-type parabolic correlation[9d] among the experimental amplification results for aliphatic cationic polyamines, the bleomycin amplifiers, and calculated third-order connectivity indices $^3X_p^v$. The $^3X_p^v$ indices have been chosen for the correlation analysis because they characterize well the extended conformations of these chain molecules complexed with DNA. In this work a similar correlation analysis for compounds **1–21** was performed with the valence molecular connectivity indices $^1X^v$, which efficiently encode the additive and constitutive nature of the complex molecules including their basic electronic and stereochemical properties.[26] The calculated

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indices $^1X^v$ and the log (k_{amp}/k_s) values used in the analysis are also given in Table I.

The correlation analysis gave two distinct functions, eqs 3 and 4, additionally illustrated in Figure 1.

$$\log (k_{amp}/k_s) = -4.485 + 1.139^1X^v - 0.068(^1X^v)^2 \quad (3)$$

n = 12 (compounds **1–10**, **12**, **15**), r = 0.933, s = 0.025, confidence level = 99.9%

$$\log (k_{amp}/k_s) = -7.678 + 1.453^1X^v - 0.065(^1X^v)^2 \quad (4)$$

n = 10 (compounds **5**, **11**, **13**, **14**, **16–21**), r = 0.980, s =0.022, confidence level = 99.9%

As can be seen from Figure 1, the activity value for **5** fits either correlation well.

Biological Results

Our studies in broth cultures have shown that many yeasts are extremely resistance to bleomycin. In particular the drug is highly ineffective against *Candida lipolytica*. It was of interest, therefore, to determine whether or not combinations of bleomycin with amplifier would be effective against this yeast.

Qualitative experiments were conducted with *C. lipolytica* spread on agar plates. Initial tests demonstrated that 50 μL of blenoxane (3.2×10^{-4} M) or 50 μL of selected amplifiers (3.2×10^{-3} M) did not produce inhibition when applied individually. However, the inhibition zones, 1.7 cm in diameter, were observed for the combinations composed of 25 μL of blenoxane and 25 μL of amplifier **6** or **12**. In the same way amplifiers **11**, **15**, and **18** together with the drug produced 1.5-cm inhibition zones. The combinations with other selected compounds **1**, **3**, **5**, **9**, and **13** were not effective under the same conditions. These results clearly demonstrate the importance of other factors, in addition to DNA binding, for a successful bleomycin amplification in a biological system.

Discussion

As can be seen from Table I, all compounds **1–21** bind with DNA with a wide range of binding constants but with the number n of base pairs in the complex not larger than 4. The bleomycin–iron chelate also requires only a few base pairs for DNA binding because this chelate is rather compact.[2] Thus, under the conditions with the ratio 1:5.35 of amplifier to DNA base pair and the catalytic amount of bleomycin, the amplifier and bleomycin molecules do not compete for binding with DNA. Even in the presence of amplifiers that show some base pair binding specificity, there always will be favorable binding sites for bleomycin. This conclusion is based on the well-established facts that (i) binding of the activated bleomycin chelate with DNA is a necessary condition for DNA cleavage, and this cleavage occurs predominantly at 5′-pyrimidine-G-T-purine-3′ and 5′-pyrimidine-G-C-purine-3′ sequences, but (ii) other alternating pyrimidine–purine sequences are cleaved with only slightly reduced efficiency.[2] Our findings that poly[d(A-T)]·poly[d(A-T)] and poly[d(G-C)]·poly[d(G-C)] are cleaved with similar initial rates and that these degradations are similarly enhanced in the presence of the A-T specific amplifier **7** are consistent with the above data. These results also show that natural heterogeneous DNA can be used for quantitative analysis of bleomycin amplification by compounds that exhibit diverse features in their interaction with the DNA.

The most surprising result of this study is that the amplification results fall into two distinct groups (Figure 1) which are quite well fitted by two distinct QSAR correlations. In order to understand this rather unusual dual mode of amplification, we have attempted to correlate the DNA binding characteristics of amplifiers **1–21** and their effects on the DNA structure in the amplifier–DNA complex with the two functions. An analysis of the data collected in Table I clearly shows that there is no correlation between the DNA binding strengths for **1–21** and their amplification activity. Both weakly and strongly binding compounds can follow either function and the binding strengths for compounds within the same function do not parallel the corresponding amplification activities. The two functions are also not due to different base pair binding specificities. For example, the amplification activities for G-C-specific **13** and A-T-specific **18** and **20** follow the same equation (4). In a similar way the groove-binding specificity can be ruled out as a basis for classification of the amplifiers because the activities for the major groove intercalators **7** and **17–20** fit eqs 3 and 4, respectively. In addition, propidium (**21**) can apparently bind from either groove.[16,17] The analysis of DNA unwinding upon binding of the amplifiers with DNA also fails to explain the dual bleomycin amplification. For example, compounds **2** and **13** unwind DNA to a similar extent and show similar amplification activities which follow different functions. It should be noted, however, that compounds **8** and **10** do not unwind DNA within the experimental error and are weak bleomycin amplifiers. Thus, at least qualitatively, these results are consistent with the amplifier-induced conformational changes of the DNA double helix as a condition for the bleomycin potentiation. The above analysis also suggests that fine features of the DNA distortion, which are necessary to facilitate interaction of the activated bleomycin complex with the double helix, may provide an explanation for the amplification, including the dual amplification pattern.

We strongly suggest that the two functions (3 and 4) are due to different DNA binding modes for the corresponding amplifiers which, in turn, result in different, amplifier-induced stereochemical changes in the helix. More specifically, it appears that DNA groove binding compounds and nonclassical intercalators, the interaction of which also includes a substantial groove-binding mode, fit eq 3 while classical DNA intercalators, with a negligible groove interaction for the intercalating aromatic system, follow eq 4.

The major stereochemical difference between the DNA complexes of these two groups of compounds is in the DNA base pair propeller twist. This twist may be slightly enhanced upon groove binding and, normally, it is substantially enhanced in the nonclassical intercalation complex of an unfused polyaromatic compound with DNA.[9e,f] The available evidence strongly suggests that intercalation and groove-binding modes of such unfused compounds should be viewed as two variable depth potential wells on a continuous energy surface.[11] By contrast, the classical intercalation of planar fused-ring systems and the unfused systems that can easily attain a coplanar conformation in the intercalation complex, is characterized by one deep energy minimum. The stereochemical consequence of the classical intercalation is a decrease of the base pair propeller twist in the complex.[16,17] In summary, the base pairs are significantly twisted in the groove binding and nonclassical intercalation modes while they are virtually coplanar as part of the intercalation complex in a classical sense.

The available evidence strongly suggests that local conformational effects in the DNA–amplifier complex result in a better stereochemical fit for binding of the activated bleomycin.[9a,b] This favorable interaction of the

activated bleomycin with DNA may occur even a few base pairs away from the amplifier binding site because the conformational changes are known to be transmitted along the DNA double helix.[16] It can be expected that these amplifier-induced bleomycin binding sites are stereochemically different for the two classes of amplifiers which produce different distortions of the helix. Moreover, the subsequent binding of the activated bleomycin with DNA must result in two different structures of the activated bleomycin–DNA complexes. This analysis is not only consistent with the observed dual amplification pattern but it agrees also with other studies which have shown that bleomycin-A2 forms two different complexes with DNA in the presence of ferrous ion.[27a] This dual binding has been interpreted in terms of two different interaction types of the bithiazole portion of bleomycin with DNA. One suggested interaction model involves partial intercalation of the bithiazole while the second model apparently does not involve the intercalation. Additional studies on the interaction of a variety of bithiazole derivatives with synthetic DNA polymers have strongly supported this hypothesis.[27b,c] It is important to note that the dual bleomycin binding should result in a dual amplification pattern, as observed.

As can be seen from Figure 1, the highest enhancements of bleomycin chemistry under conditions of this work are produced by propidium (**21**) and unfused compounds **18** and **19**, all apparently interacting with DNA through a classical intercalation mode. The bleomycin potentiation can be increased further by increasing concentration of an amplifier, provided solubility of the DNA–amplifier complex is not a problem. For example, the bleomycin-mediated degradation of DNA has been potentiated 11-fold in the presence of a rather modest amplifier **13** taken in the molar ratio of 1:2 per DNA base pair, under otherwise similar conditions.[9g] Analogus increases in concentrations of amplifiers that fit eq 3 produce relatively smaller increases in bleomycin potentiation. The best bleomycin amplifiers, thus, are either fused or unfused polyaromatic molecules which fit eq 4. We strongly suggest that all such amplifiers are capable of forming classical intercalation complexes with DNA.

Analysis of compounds which fit the corresponding eqs 3 and 4 reveals that both structural and stereoelectronic features in the molecules greatly affect their DNA interaction mode and, therefore, their amplification activity. None of the unfused derivatives containing a methyl group ortho to the torsional bond joining aromatic rings is capable of forming an intercalation complex in a classical sense. This observation is nicely illustrated by the pair **13** and its methyl derivative **15** which fit eqs 4 and 3, respectivley. It is also consistent with other studies which have showed that the methyl group is too bulky to be part of a planar intercalation complex.[28] On the other hand, **13** with a protonated dimethylamino cationic substituent and its piperazino-substituted analogue **14** exhibit the same activity and fit the same equation (4). These results are consistent with the same interaction model for **13** and **14** in which the tricyclic aromatic system intercalates with DNA base pairs and the two cationic substituents, although structurally different, provide a similar electrostatic interaction with the anionic DNA backbone.[11] As a result, a similar DNA distortion is produced and a similar bleomycin potentiation is observed in both cases.

We have shown recently that intercalation of polyaromatic systems with DNA base pairs is strongly affected by π-electron polarization within these systems.[9c,12] The phenomenon is especially important for molecules with a short cationic chain because the favorable positioning of the cation in close proximity to DNA phosphate dictates the stereochemistry of intercalation. Only the molecules that have the polyaromatic system polarized extensively in the direction of the side cationic chain, so that the intercalating site constitutes a positive part of the dipole, show strong intercalative binding with DNA. These stereoelectronic effects are clearly operative in the interactions of a nonclassical intercalator **7**, which fits eq 3, and the suggested classical intercalators **17–20**, which fit eq 4. With **7** the two electronegative nitrogens of the pyrimidine cause polarization which is unfavorable for a strong interaction of the molecule with π-electron systems of DNA base pairs. As a result, the interaction of **7** with DNA involves a nonclassical intercalation accompanied by a substantial groove-binding mode. By contrast, the nitrogen atom of the central pyridine ring in **17–20** decreases the electron density at the opposite site of the pyridine, the primary interaction site of these molecules with DNA base pairs. The favorable stereoelectronic effect in **17–20** induces a much better overlap of these molecules with the DNA base pairs in comparison to the interaction of the isosteric aromatic system of **7**. The observed different DNA binding strengths for the particular compounds **17–20** are due to different cationic side chains which affect the overall complex stability.

Conclusions

The above analysis is important to the development of guidelines for the design of improved bleomycin amplifiers. The best amplifiers interact with DNA through a classical intercalation mode. However, our preliminary biological results with a simple eukaryotic model indicate that transport of the cationic amplifiers into cells will have to receive careful attention for a successful amplification in vivo. The design of such improved amplifiers must include increased lipophilicity. This problem may be attacked by structural modification of the cationic side chain which does not intercalate with DNA. We have shown that such a structural change has little effect on the DNA interaction strength and amplification activity of the amplifier molecule.

Experimental Section

Melting points (Pyrex capillary) are uncorrected. Unless stated otherwise, ^{1}H NMR spectra were obtained on a Varian VXR-400 (400 MHz) spectrometer at 65 °C in a phosphate buffer (pH 7) with TSP as an internal standard, for a direct comparison with the spectra obtained in the presence of DNA. Elemental analyses were within 0.3%, 0.1%, and 0.2% for C, H, and N, respectively. All reactions with organolithium reagents were conducted in Et_2O distilled from sodium benzophenone ketyl immediately before use and under static pressure of nitrogen. The glassware was dried at 140 °C, assembled hot, and cooled in a stream of nitrogen. The liquids were transferred with syringes.

3-[[2′-(Dimethylamino)ethyl]thio]phenyllithium (**27**), 4-[[2′-(dimethylamino)ethyl]thio]phenyllithium (**31**), and 2,2′-bithiophen-5-yllithium in Et_2O were generated in bromine–lithium exchange reactions using a general procedure.[9f,10,12]

The following compounds were synthesized by published procedures: 4,6-bis[4′-[[2″-(dimethylamino)ethyl]thio]phenyl]-pyrimidine[9f] (**7**), 4,6-bis[4′-[[2″-(dimethylamino)ethyl]thio]-phenyl]-5-methylpyrimidine[9f] (**8**), 4,6-bis[3′-[[2″-(dimethyl-

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amino)ethyl]thio]phenyl]pyrimidine[12] (9), 4,6-bis[3′-[[2″-(dimethylamino)ethyl]thio]phenyl]-5-methylpyrimidine[12] (10), 2,4-bis[3′[[2″-(dimethylamino)ethyl]thio]phenyl]pyrimidine[12] (12), 2,5-bis[2′[[2″-(dimethylamino)ethyl]thio]pyrimidin-4′-yl]-thiophene[9g] (13), and 2,5-bis[2′-[[2″-(dimethylamino)ethyl]-thio]-5′-methylpyrimidin-4′-yl]thiophene[11] (15). Propidium iodide (21) was obtained from Calbiochem.

N-[2‴-(Dimethylamino)ethyl]-4-furan-2′-yl-6-thien-2″-ylpyrimidin-2-amine (1). A mixture of 2-chloro-4-furan-2′-yl-6-thien-2″-ylpyrimidine[10] (22, 0.52 g, 2 mmol), *N,N*-dimethylethylenediamine (0.5 mL, 4.5 mmol), and EtOH (5 mL) was heated under reflux for 20 min. Silica gel chromatography with hexanes/Et_3N/EtOH (80:17:3) afforded 0.53 g (85%) of 1 as an oil: ^1H NMR δ 3.00 (s, NMe_2), 3.50 (m, CH_2), 3.90 (m, CH_2), 6.74 (dd, J = 1.6 and 3.4 Hz, H4′), 7.29 (dd, J = 3.6 and 4.8 Hz, H4″), 7.34 (d, J = 3.4 Hz, H3′), 7.47 (s, H5), 7.74 (d, J = 4.8 Hz, H5″), 7.82 (d, J = 1.6 Hz, H5′), 7.93 (d, J = 3.6 Hz, H3″). Anal. ($C_{16}H_{18}N_4OS$) C, H, N.

N-[2″-(Dimethylamino)ethyl]-4,6-dithien-2′-ylpyrimidin-2-amine Dihydrobromide (2·2HBr). The reaction of 2-chloro-4,6-dithien-2′-ylpyrimidine[10] (23) with *N,N*-dimethylethylenediamine was conducted and worked up as described above to give 2 (yield 90%) as an oil. Product 2 was transformed into a dihydrobromide and crystallized as described previously: mp 256–270 °C (lit.[11] mp 255–270 °C).

2-[[2‴-(Dimethylamino)ethyl]thio]-4-(1′-methylpyrrol-2′-yl)-6-thien-2″-ylpyrimidine Hydrobromide (3·HBr). Metallic sodium (0.12 g, 5.2 mmol) was reacted with anhydrous ethanol (10 mL) and the resultant solution of sodium ethoxide was treated with 2-(dimethylamino)ethanethiol hydrochloride (0.37 g, 2.6 mmol). The mixture was stirred briefly, then treated with 2-chloro-4-(1′-methylpyrrol-2′-yl)-6-thien-2″-ylpyrimidine[10] (24; 0.55 g, 2 mmol), and stirred for 10 h at 70 °C. Silica gel chromatography with hexanes/Et_3N/EtOH (80:15:5) afforded 0.69 g (75%) of 3 as an oil. The hydrobromide of 3 was obtained by using a general procedure[29] and crystallized from EtOH: mp 204–206 °C; ^1H NMR δ 2.97 (s, NMe_2), 3.59 (s, CH_2CH_2), 3.95 (s, N1′-Me), 6.33 (m, H4′), 6.99 (m, H3′), 7.05 (m, H5′), 7.27 (dd, J = 3.8 and 4.8 Hz, H4″), 7.64 (s, H5), 7.73 (d, J = 4.8 Hz, H5″), 7.91 (d, J = 3.8 Hz, H3″). Anal. ($C_{17}H_{20}N_4S_2$·HBr) C, H, N.

2-[[2‴-(Dimethylamino)ethyl]thio]-4-furan-2′-yl-6-thien-2″-ylpyrimidine Hydrobromide (4·HBr). The product was obtained from 2-chloro-4-furan-2′-yl-6-thien-2″-ylpyrimidine[10] (22) by using the procedure described above and crystallized from MeOH: yield 78%; mp 111–112 °C; ^1H NMR δ 2.95 (s, NMe_2), 3.30 (t, J = 7 Hz, CH_2), 3.45 (t, J = 7 Hz, CH_2), 6.71 (dd, J = 1.8 and 3.6 Hz, H4′), 7.23 (dd, J = 4.0 and 5.2 Hz, H4″), 7.26 (d, J = 3.6 Hz, H3′), 7.55 (s, H5), 7.71 (d, J = 5.2 Hz, H5″), 7.77 (d, J = 1.8 Hz, H5′), 7.88 (d, J = 4.0 Hz, H3″). Anal. ($C_{16}H_{17}N_3OS_2$·HBr) C, H, N.

2,3″-Bis[[2‴-(dimethylamino)ethyl]thio]-4-thien-2′-yl-6-phenylpyrimidine Dihydrobromide (5·2HBr). The addition reaction of lithium reagent[12] 27 (2.9 mmol) with 2-[[2″-(dimethylamino)ethyl]thio]-4-thien-2′-ylpyrimidine[29] (25; 0.74 g, 2.8 mmol) in Et_2O (25 mL), the subsequent dehydrogenation of the resultant dihydropyrimidine adduct by treatment with DDQ (0.70 g, 3.1 mmol), and then workup were conducted by using a general procedure for the arylation of the pyrimidine ring developed by us recently.[10] Silica gel chromatography with hexanes/Et_3N/EtOH (80:15:5) afforded 0.84 g (73%) of 5 as an oil. The dihydrobromide of 5 was obtained by using a general procedure[29] and crystallized from EtOH/Et_2O: mp 238–239 °C; ^1H NMR δ 2.95 (s, NMe_2), 3.01 (s, NMe_2), 3.41 (m, 2 CH_2), 3.56 (m, 2 CH_2), 7.30 (dd, J = 3.6 and 4.8 Hz, H4′), 7.58 (t, J = 8 Hz, H5″), 7.67 (d, J = 8 Hz, H4″), 7.70 (s, H5), 7.80 (d, J = 4.8 Hz, H5′), 7.87 (d, J = 8 Hz, H6″), 7.92 (d, J = 3.6 Hz, H3′), 7.97 (s, H2″). Anal. ($C_{22}H_{28}N_4S_3$·2HBr) C, H, N.

4-(2′,2″-Bithiophene-5′-yl)-2-[[2‴-(N-methylpiperazino)-ethyl]thio]pyrimidine Dihydrobromide (6·2HBr). A general procedure[10] was used for the reaction of 2,2′-bithiophene-5-yllithium with pyrimidine 26; subsequent aromatization of the resultant dihydropyrimidine and then chromatographic purification of the product gave 6 in a 70% yield: mp 85–86 °C. Amine 6 was transformed into a dihydrobromide 6·2HBr: mp 265–269 °C; ^1H NMR δ 2.78 (s, Me), 2.96 (m, 2 CH_2), 3.16 (m, 3 CH_2), 3.39 (m, CH_2), 7.21 (dd, J = 3.6 and 5.2 Hz, H4″), 7.40 (d, J = 4.0 Hz, H4′), 7.44 (d, J = 3.6 Hz, H3″), 7.51 (d, J = 5.2 Hz, H5″), 7.53 (d, J = 5.2 Hz, H5), 7.84 (d, J = 4.0 Hz, H3′), 8.47 (d, J = 5.2 Hz, H6). Anal. ($C_{19}H_{22}N_4S_3$·2HBr) C, H, N.

2-[[2″-(Dimethylamino)ethyl]thio]-4,6-bis[3′-[[2‴-(dimethylamino)ethyl]thio]phenyl]pyrimidine Trihydrobromide (11·3HBr·$^1/_2H_2O$). A standard addition reaction[10] of lithium reagent[12] 27 with 2-[[2′-(dimethylamino)ethyl]thio]pyrimidine[9g] (28) followed by treatment with DDQ and then chromatography (hexanes/Et_3N/EtOH 7:3:1) furnished 2,3′-bis-[[2″-(dimethylamino)ethyl]thio]-4-phenylpyrimidine (29) as an oil (yield 56%). Treatment of 29 with hydrobromic acid followed by crystallization of the resultant salt from EtOH/Et_2O gave 29·2HBr: mp 253–254 °C; ^1H NMR δ 2.92 (s, NMe_2), 3.00 (s, NMe_2) 3.40 (m, 2 CH_2), 3.66 (m, 2 CH_2), 7.65 (dd, J = 8 Hz, H4′), 7.74 (m, H5 and H5′), 8.02 (d, J = 8 Hz, H6′), 8.15 (s, H2′), 8.70 (d, J = 5.2 Hz, H6). Anal. ($C_{18}H_{26}N_4S_2$·2HBr) C, H, N.

Substitution of 29 for 28 in the reaction with lithium reagent 27 followed by the same procedure furnished 11 as an oil (yield 30%), which was characterized as a hydrobromide salt, 11·3HBr·1/2H_2O: mp 196–198 °C; ^1H NMR δ 2.86 (s, 4 Me), 2.90 (s, 2 Me), 3.31 (m, 2 CH_2), 3.40 (m, 2 CH_2), 3.50 (m, CH_2), 3.65 (m, CH_2), 7.63 (t, J = 8 Hz, H5′), 7.70 (d, J = 8 Hz, H4′), 7.91 (s, H5), 7.99 (d, J = 8 Hz, H6′), 8.10 (s, H2′). Anal. ($C_{28}H_{39}N_5S_3$·3HBr·$^1/_2H_2O$) C, H, N.

2,5-Bis[2′-[[2″-(N-methylpiperazino)ethyl]thio]pyrimidin-4′-yl]thiophene Tetrahydrobromide (14·4HBr·2H_2O). The published procedure[9g] for the preparation of 13 was used without modification for the reaction of 2,5-dilithiothiophene with 2-[[2′-(*N*-methylpiperazino)ethyl]thio]pyrimidine (26) and subsequent workup. Hydrobromide 14·4HBr·2H_2O was obtained in a 14% yield after crystallization from EtOH: mp 250–252 °C; ^1H NMR δ 2.78 (s, 2 Me), 2.91 (m, 6 CH_2), 3.20 (m, 6 CH_2), 7.39 (d, J = 5.2 Hz, H5′), 7.76 (s, H3 and H4), 8.45 (d, J = 5.2 Hz, H6′). Anal. ($C_{26}H_{36}N_8S_3$·4HBr·2H_2O) C, H, N.

3,6-Bis[4′-[[2″-(dimethylamino)ethyl]thio]phenyl]-pyridazine Dihydrobromide (16·2HBr). The reaction of 4-[[2′-(dimethylamino)ethyl]thio]phenyllithium[9f] (31, 7.4 mmol) with pyridazine (30, 0.60 g, 7.4 mmol) in Et_2O (40 mL) was conducted under the general conditions.[9f,10] The subsequent oxidation with DDQ and then chromatography (hexanes/Et_3N/EtOH, 7:2:1) was followed by crystallization from toluene/hexanes to furnish 0.76 g (40%) of 3-[4′-[[2″-(dimethylamino)ethyl]thio]phenyl]pyridazine (32): mp 86–88 °C; ^1H NMR (60 MHz, $CDCl_3$/TMS) δ 2.26 (s, 6 H), 2.55 (t, J = 7 Hz, 2 H), 3.07 (t, J = 7 Hz, 2 H), 7.2–8.0 (m, 6 H), 9.00 (m, 1 H). Anal. ($C_{14}H_{17}N_3S$) C, H, N.

The subsequent reaction of 32 (0.49 g, 1.9 mmol) with lithium reagent 31 (1.9 mmol) under the same conditions followed by the same workup furnished 0.29 g (36%) of 16: mp 180–182 °C. Compound 16 was treated with excess HBr in EtOH and the resultant precipitate was crystallized from EtOH to give 16·2HBr: mp 288–289 °C; ^1H NMR δ 2.95 (s, 4 Me), 3.45 (m, 4 CH_2), 7.69 (d, J = 8.0 Hz, H3′ and H5′), 8.06 (d, J = 8.0 Hz, H2′ and H6′), 8.22 (s, H4 and H5). Anal. ($C_{24}H_{30}N_4S_2$·2HBr) C, H, N.

2,6-Bis[4′-(bromomethyl)phenyl]pyridine (34). The following method gave much purer 34 and in greater yield than that reported.[13] A mixture of 2,6-di-*p*-tolylpyridine (33; 2.6 g, 10 mmol), *N*-bromosuccinimide (NBS, 3.3 g, 18.5 mmol), benzoyl peroxide (20 mg), and CCl_4 (50 mL) was refluxed for 18 h, then cooled, and the precipitated product 34 was filtered. The filtrate was treated with NBS (1.2 g, 6.7 mmol) and a catalytic amount of benzoyl peroxide, and the reflux was continued for an additional 18 h. The combined precipitates were crystallized from CCl_4 to give 2.6 g (62%) of 34: mp 178–180 °C (lit.[13] mp 150–153 °C). The ^1H NMR spectrum of 34 was identical with that reported.[13]

2,6-Bis[4′-[(dimethylamino)methyl]phenyl]pyridine Dihydrobromide (17·2HBr). A mixture of 34 (0.17 g, 0.4 mmol), THF (10 mL), and a 40% aqueous solution of dimethylamine (1 mL, 8 mmol) was refluxed for 1 h in a flask equipped with a dry ice condenser. Then the mixture was concentrated under reduced pressure, made alkaline with NaOH, and extracted with Et_2O. Removal of Et_2O was followed by chromatography on silica gel (hexanes/Et_3N/EtOH 80:15:5) to give 0.11 g (78%) of 17. The

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product dissolved in EtOH was treated with excess of HBr, and the precipitated salt was crystallized from EtOH to give a dihydrobromide, **17**·2HBr: mp 263–265 °C; ^{1}H NMR δ 2.90 (s, NMe_2), 4.36 (s, C4′-CH_2), 7.68 (d, J = 8 Hz, H3′ and H5′), 7.92 (d, J = 8 Hz, H3 and H5), 8.10 (t, J = 8 Hz, H4), 8.18 (d, J = 8 Hz, H2′ and H6′). Anal. ($C_{23}H_{27}N_3$·2HBr), C, H, N.

2,6-Bis[4′-[[[2″-(dimethylamino)ethyl]amino]methyl]phenyl]pyridine Tetrahydrobromide (18·4HBr·2H$_2$O). A solution of *N,N*-dimethylethylenediamine (20 mL) in THF (50 mL) was stirred at 5 °C and treated dropwise with a solution of **34** (3.1 g, 7.5 mmol) in THF (25 mL). Then the mixture was stirred at 23 °C for 10 h. Workup as described above furnished 3.0 g (50%) of **18**·4HBr·2H_2O: mp 239–241 °C; ^{1}H NMR δ 2.80 (s, 4 Me), 3.06 (t, J = 6.8 Hz, CH_2), 3.19 (t, J = 6.8 Hz, CH_2), 4.00 (s, C4′-CH_2), 7.59 (d, J = 8 Hz, H3′ and H5′), 7.87 (d, J = 8 Hz, H3 and H5), 8.06 (t, J = 8 Hz, H4), 8.10 (d, J = 8 Hz, H2′ and H6′). Anal. ($C_{27}H_{37}N_5$·4HBr·2H_2O) C, H, N.

2,6-Bis[4′-[[(3″-aminopropyl)amino]methyl]phenyl]pyridine Tetrahydrobromide (19·4HBr). Treatment of a large excess of 1,3-diaminopropane with **34** in the manner described above and then followed by the same workup furnished **19**·4HBr in a 45% yield: mp 273–274 °C; ^{1}H NMR 1.70 (m, CCH_2C), 3.00 (m, CH_2), 3.10 (m, CH_2), 3.95 (s, C4′-CH_2), 7.58 (d, J = 8 Hz, H3′ and H5′), 7.85 (d, J = 8 Hz, H3 and H5), 8.02 (t, J = 8 Hz, H4), 8.10 (d, J = 8 Hz, H2′ and H6′). Anal. ($C_{25}H_{33}N_5$·4HBr) C, H, N.

2-[4′-[[[2‴-(Dimethylamino)ethyl]amino]methyl]phenyl]-6-(4″-methylphenyl)pyridine Dihydrobromide (20·2HBr·H$_2$O). A mixture of 2,6-di-*p*-tolylpyridine (1.9 g, 7.5 mmol), NBS (0.45 g, 3 mmol), benzoyl peroxide (20 mg), and CCl_4 was heated under reflux for 16 h and then cooled. The precipitated dibromo derivative **34** was filtered off, and then CCl_4 was removed under reduced pressure from the solution containing mainly a monobromo derivative, **35**. The residue was dissolved in THF (20 mL) and allowed to react with *N,N*-dimethylethylenediamine (10 mL) dissolved in THF (20 mL). Workup as described above was followed by silica gel chromatography (hexanes/Et_3N/EtOH 8:1:1) to give 0.70 g (20%) of **20** as an oil. A hydrobromide salt was prepared and crystallized from EtOH to give **20**·2HBr·H_2O: mp 234–235 °C; ^{1}H NMR δ 2.44 (s, C4″-Me), 2.79 (s, NMe_2), 3.06 (t, J = 6.8 Hz, CH_2), 3.18 (t, J = 6.8 Hz, CH_2), 4.00 (s, C4′-CH_2), 7.44 (d, J = 8 Hz, H3″ and H5″), 7.58 (d, J = 8 Hz, H3′ and H5′), 7.82 (d, J = 8 Hz, H3 and H5), 7.98 (d, J = 8 Hz, H2″ and H6″), 8.02 (t, J = 8 Hz, H4), 8.08 (d, J = 8 Hz, H2′ and H6′). Anal. ($C_{23}H_{27}N_3$·2HBr·H_2O) C, H, N.

Bleomycin and DNA Samples. Bleomycin-A2 and blenoxane were obtained from the Bristol-Myers Co. through the courtesy of Dr. William T. Bradner. Calf thymus DNA (Worthington Biochemical) was phenol extracted, dialyzed in pipes buffer (see below), and characterized as previously described.[17] High molecular weight DNA was used in the bleomycin amplification experiments and sonicated samples were used in the viscometric titrations and binding studies (800 ± 200 base pairs), and the NMR experiments (200 ± 50 base pairs). Commercial samples of synthetic DNA polymers (Pharmacia) were dialyzed in the pipes buffer before use. All stocks solutions were stored at −30 °C.

Methods. Procedures for viscometric titrations,[30] spectrophotometric binding,[17] NMR measurements,[31] and monitoring the bleomycin amplification by the viscometric method[9c,d] and HPLC[9b] have been recently presented. All bleomycin amplification experiments were conducted in pipes buffer (pH 7.00, [Na^+] 0.015 M) at 37 ± 0.1 °C.

Biological Assay. Biological effects of selected amplifiers were evaluated by using the following modification of the antibiotic/agar diffusion test.[32] Broth cultures of *Candida lipolytica* were surface inoculated onto 6-cm petri dishes containing 5 mL of 8% glucose, 0.67% yeast nitrogen base (Difco), and 2% agar (Difco). In control experiments 1.1-cm cellulose disks were treated with 50 μL of blenoxane (3.2×10^{-4} M stock solution) or 50 μL of a stock solution of an amplifier (3.2×10^{-3} M) and placed in the center of the inoculated plates. The plates were incubated in the dark at 22 °C for 48 h, after which time no inhibition zone was observed in each case. Then the experiments were conducted with the cellulose disks each containing 25 μL of the blenoxane solution and 25 μL of the amplifier solution. The inhibition zones were measured after 48 h of incubation at 22 °C in the dark.

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Chemically Stable, Lipophilic Prodrugs of Phosphoramide Mustard as Potential Anticancer Agents

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Benzyl phosphoramide mustard (**3**), 2,4-difluorobenzyl phosphoramide mustard (**4**), and methyl phosphoramide mustard (**5**) were examined as lipophilic, chemically stable prodrugs of phosphoramide mustard (**2**). These phosphorodiamidic esters are designed to undergo biotransformation by hepatic microsomal enzymes to produce **2**. The rate of formation of alkylating species, viz., **2**, from these prodrugs and their in vitro cytotoxicity toward mouse embryo Balb/c 3T3 cells were comparable to or better than that of cyclophosphamide (**1**). Preliminary antitumor screening against L1210 leukemia in mice, however, suggests that these prodrugs are devoid of any significant antitumor activity in vivo.

Cyclophosphamide (CP, **1**) is a widely used anticancer and immunosuppressive agent. Its chemistry and pharmacology have been reviewed by several investigators.[1-7] Cyclophosphamide is itself a prodrug that is activated by hepatic microsomal mixed-function oxidase (MFO) catalyzed C_4-hydroxylation. The resulting 4-hydroxycyclophosphamide (4-OH-CP) undergoes ring opening to aldophosphamide (Aldo), followed by generation of cytotoxic

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