Chromophore-Modified Antitumor Anthracenediones: Synthesis, DNA Binding, and Cytotoxic Activity of 1,4-Bis[(aminoalkyl)amino]benzo[g]-phthalazine-5,10-diones†

Carmelo A. Gandolfi,‡,⊥ Gino Beggiolin,‡ Ernesto Menta,‡ Manlio Palumbo,§ Claudia Sissi,§ Silvano Spinelli,*,‡ and Francis Johnson*,∥

Research Center, Boehringer Mannheim Italia S.p.A., Viale Libertà Km 0.75, I-20052 Monza, Italy, Department of Pharmaceutical Sciences, University of Padua, Via Marzolo 5, I-35131 Padua, Italy, and Pharmacological Sciences Department, State University of New York at Stony Brook, Stony Brook, New York 11794

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As part of a program aimed at exploring the effect of the introduction of heteroatoms into the anthracene-9,10-dione chromophore, we have synthesized novel 1,4-bis[(aminoalkyl)amino]-benzo[g]phthalazine-5,10-diones (BPDs) **1** which are related to the antitumor agents ametantrone and mitoxantrone. Derivatives **1** were prepared by chromic acid oxidation of acylated benzo[g]phthalazines **5** followed by acid hydrolysis or by silylation–amination of 5,10-dihydroxybenzo[g]phthalazine-1,4-dione (**8**). The 1-[(aminoalkyl)amino]-4-amino congeners **2** were isolated in low yields as byproducts from the oxidation of **5**. Against a panel of human tumor cell lines, the benzo[g]phthalazine-5,10-diones **1** and **2** exhibited cytotoxic activity comparable or even superior to that of mitoxantrone. In compounds **1**, structure–activity relationships different than those operative in the carbocyclic series appeared to emerge. DNA-binding studies with the ametantrone-like compound **1c** and its single-armed congener **2c** indicated that the introduction of a 2,3-diaza subunit into the anthracene-9,10-dione chromophore reduces the affinity of the drug for DNA in comparison with ametantrone. On the other hand, the number of side-chain groups does not affect binding to a great extent. These findings seem to suggest mechanisms of cell death other than those induced by simple interaction of the 1,4-BPDs **1** and **2** with DNA.

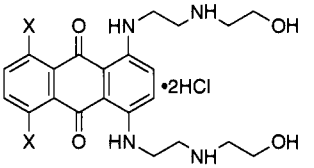
Introduction

The anticancer agents mitoxantrone and ametantrone (Chart 1) are the most representative examples of the 1,4-bis[(aminoalkyl)amino]anthracene-9,10-diones recently developed as antitumor agents.[1] Mitoxantrone in particular is currently gaining an increasingly important place in the clinical management of hematological malignancies such as chronic myelogenous leukemia, acute nonlymphoblastic leukemia, and non-Hodgkin's lymphoma, as well as in combination therapy of refractory ovarian and breast cancers.[2] Although mitoxantrone is endowed with an improved tolerability profile compared with doxorubicin and other anthracyclines, this drug is not devoid of significant toxic side effects, especially those associated with myelosuppression. Cardiac effects, especially congestive heart failure, may also be of clinical concern, particularly in patients previously treated with anthracyclines.[2]

As in the case of the anthracyclines, the mechanisms by which the antitumor anthracenediones kill cells are poorly understood and probably multimodal in their nature. DNA intercalation, nucleic acid compaction, and interference with the DNA–topoisomerase II activity resulting in protein-associated DNA strand breaks have been proposed as critical events (common also to a number of other antineoplastic agents) which lead to mitoxantrone-induced cell death.[3] Many anthraquinones, among them mitoxantrone, can undergo oxidative metabolism which results in the formation of free radicals capable of DNA alkylation and/or DNA scission, yielding nonprotein-associated DNA strand breaks.[4] Recent studies suggest that enzymes such as NAD(P)H (quinone acceptor) oxidoreductase can reduce mitoxantrone to give reactive hydroxyl radicals.[5] However, it is generally considered that quinone reduction is probably more related to the cardiotoxic side effects of mitoxantrone than to the mechanism of its antitumor activity.

The significant clinical activity of mitoxantrone makes the development of second-generation anthracenedione congeners having better therapeutic efficacy together with reduced side effects an attractive area of investigation. To date, much research has been devoted to exploring variations in the nature of the side chains and to the repositioning of the hydroxy substituents and/or the lateral side chains.[1] The introduction of a 5,8-dihydroxy substitution pattern resulted in compounds generally endowed with both increased cytotoxicity and DNA affinity.[6] However this dihydroxylation pattern

Chart 1



X = H ametantrone
X = OH mitoxantrone

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‡ Research Center, Boehringer Mannheim Italia S.p.A.

§ Department of Pharmaceutical Sciences, University of Padua.

∥ Pharmacological Sciences Department, State University of New York at Stony Brook.

⊥ Current address: Via Marcantonio Colonna 9, Milano, Italy.

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Table 1. Synthesis and Physicochemical Properties of 1,4-Bis[(aminoalkyl)amino]benzo[*g*]phthalazine-5,10-diones 1a–**g**

no.	R	method	yield (%)[a]	mp (°C dec)	molecular formula	analysis
1a	$CH_2CH_2CH_2OH$	A	34	173–176 (184–186)[b]	$C_{18}H_{20}N_4O_4$·HCl	C, H, N, Cl
1b	$CH_2CH_2N(CH_3)_2$	A	34	195–197 (257–259)[c]	$C_{20}H_{26}N_6O_2$·3HCl·1.5H_2O	C, H, N, Cl
1c	$CH_2CH_2NHCH_2CH_2OH$	A	20	122–124 (205–208)[c]	$C_{20}H_{26}N_6O_4$·3HCl	C, H, N, Cl
1d	$CH_2CH_2N(C_2H_5)_2$	B	53	173–175	$C_{24}H_{34}N_6O_2$	C, H, N
1e	$CH_2CH_2CH_2N(CH_3)_2$	B	40	122–124	$C_{22}H_{30}N_6O_2$	C, H, N
1f	CH_2CH_2-c$N(CH_2CH_2)_2CH_2$	B	67	203–204	$C_{26}H_{34}N_6O_2$	C, H, N
1g	$CH_2C_6H_5$	B	61	179–191	$C_{26}H_{20}N_4O_2$	C, H, N

[a] Overall yield of 1 from oxidation of **5** followed by deprotection of intermediate **6** (method A) or from silylation–amination of **8** (method B). [b] Monohydrochloride. [c] Trihydrochloride.

Table 2. Physicochemical Properties of 1-[(Aminoalkyl)amino]-4-aminobenzo[*g*]phthalazine-5,10-diones **2a–c**

no.	R	method	yield (%)[a]	mp (°C dec)	molecular formula	analysis
2a	$CH_2CH_2CH_2OH$	A	9	181–184 (204)[b]	$C_{15}H_{14}N_4O_3$·HCl	C, H, N, Cl
2b	$CH_2CH_2N(CH_3)_2$	A	2	181–184 (219.5–221.5)[c]	$C_{16}H_{17}N_5O_2$·2HCl	C, H, N, Cl[d]
2c	$CH_2CH_2NHCH_2CH_2OH$	A	3	155 (202–204)[c]	$C_{16}H_{17}N_5O_3$·2HCl	C, H, N, Cl

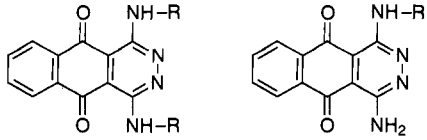
[a] Overall yield of **2** from oxidation of **5** followed by deprotection of intermediates **7** (method A). [b] Monohydrochloride. [c] Dihydrochloride. [d] A satisfactory elemental analysis could not be obtained for this compound.

seems also to be involved in the delayed lethality observed in animals treated with mitoxantrone but which is not observed with ametantrone[7] in which these hydroxy groups are lacking.

Over the past few years, the introduction of heteroatoms into the anthracenedione chromophore has been receiving increasing attention, in the expectation that such changes could potentially affect significantly the interaction of the molecules with biological targets. In particular heterocyclic analogues of anthraquinones (heteroannulated naphthoquinones) (a) should basically retain the same spatial and planar characteristics as the parent drugs for host molecular recognition such as DNA intercalation and (b) might provide additional hydrogen-bonding or basic sites, either of which could increase the affinity of the drug for DNA and/or affect the interaction with topoisomerase II.[8] In addition these heteroanalogues could be expected to possess altered redox properties.

In particular, the introduction of nitrogen atoms into different positions of the anthraquinone nucleus has been systematically investigated as an approach to the discovery of second-generation anthracenedione analogues. This effort has resulted in the preparation of several aza-[9] and diazaanthraquinones.[10]

In this paper we report the synthesis and the cytotoxic activity of novel 1,4-bis[(aminoalkyl)amino]benzo[*g*]-phthalazine-5,10-diones **1** and several related 1-[(aminoalkyl)amino]-4-amino congeners **2**[11] which are related to ametantrone and represent that part of our work which is directed toward the development of heteroanalogues of anthracene-9,10-diones. An attempt is also made to relate the observed biological activity with the DNA-binding properties of selected compounds.



1a–g **2a–c**

R = aminoalkyl, hydroxyalkyl, benzyl

Chemistry

The target benzo[*g*]phthalazine-5,10-diones (1,4-BPDs) **1** and **2** are listed in Tables 1 and 2, respectively.

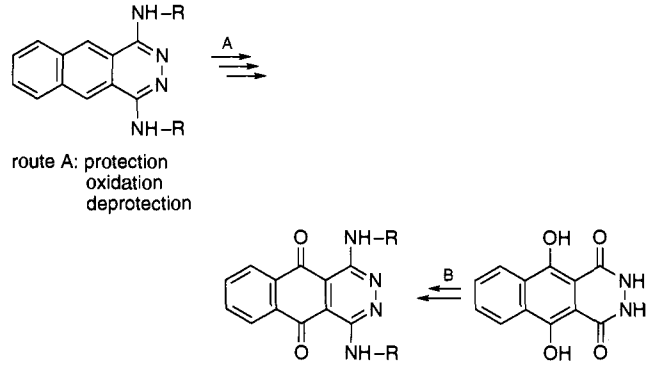


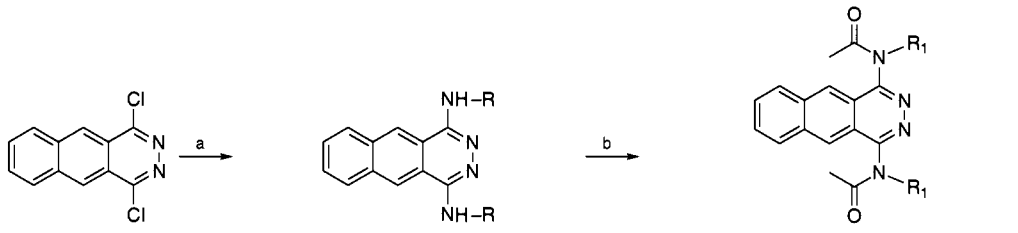
Figure 1.

Two converging strategies to 1,4-BPDs **1** have been developed (Figure 1).

(A) Oxidation of Protected 1,4-Bis(alkylamino)-benzo[*g*]phthalazines. Anthracene-9,10-diones can be obtained by the oxidation of anthracenes.[12a] The same oxidative procedure applied to 1,4-bis(alkylamino)-benzo[*g*]phthalazines **3**[13] was expected to give the target 5,10-quinones **1**, provided that the 1,4-diamino subunit of **3** and the amino and/or hydroxy groups present in the side chains were appropriately protected.[12b] Accordingly, 1,4-bis(alkylamino)benzo[*g*]phthalazines **3a–c** were prepared from 1,4-dichlorobenzo[*g*]phthalazine (**4**) by adaptation of a published procedure.[13] Attempted oxidation of these unprotected derivatives resulted in extensive decomposition and therefore the fully protected derivatives **5** were prepared by exhaustive acetylation of **3** (Scheme 1).

The introduction of the 5,10-quinone moiety into the protected derivatives **5** required some effort in order to minimize oxidative degradation of the side chains. After some investigation, effective conditions for the oxidation of the protected benzo[*g*]phthalazines **5** were finally found by the use of chromic acid in warm acetic acid. This procedure provided us with reasonable yields (35–47%) of the acylated 1,4-bis(alkylamino-substituted)-quinones **6**. However concomitant formation of varying amounts of 1-(*N*-acetylalkylamino)-4-aminobenzo[*g*]-phthalazine-5,10-diones **7** could not be completely suppressed (Scheme 2). The 4-amino diones **7** probably originate by oxidative degradation of one of the side

Scheme 1[a]

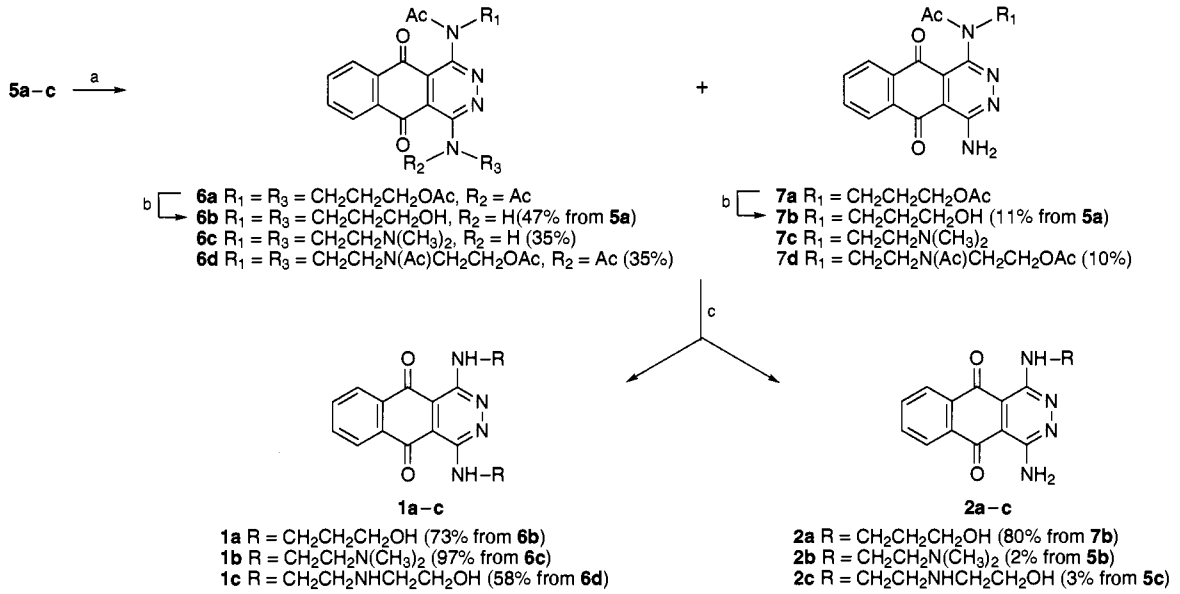


3a R = $CH_2CH_2CH_2OH$ (98%)
3b R = $CH_2CH_2N(CH_3)_2$ (90%)
3c R = $CH_2CH_2NHCH_2CH_2OH$ (59%)

5a R = $CH_2CH_2CH_2OAc$ (67%)
5b R = $CH_2CH_2N(CH_3)_2$ (36%)
5c R = $CH_2CH_2N(Ac)CH_2CH_2OAc$ (80%)

[a] (a) NH_2R, 105–125 °C, 8 h; (b) Ac_2O, pyridine.

Scheme 2[a]



6a $R_1 = R_3 = CH_2CH_2CH_2OAc$, R_2 = Ac
6b $R_1 = R_3 = CH_2CH_2CH_2OH$, R_2 = H (47% from **5a**)
6c $R_1 = R_3 = CH_2CH_2N(CH_3)_2$, R_2 = H (35%)
6d $R_1 = R_3 = CH_2CH_2N(Ac)CH_2CH_2OAc$, R_2 = Ac (35%)

7a $R_1 = CH_2CH_2CH_2OAc$
7b $R_1 = CH_2CH_2CH_2OH$ (11% from **5a**)
7c $R_1 = CH_2CH_2N(CH_3)_2$
7d $R_1 = CH_2CH_2N(Ac)CH_2CH_2OAc$ (10%)

1a–c

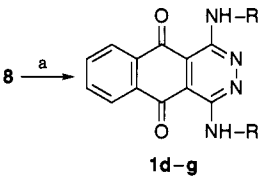
1a R = $CH_2CH_2CH_2OH$ (73% from **6b**)
1b R = $CH_2CH_2N(CH_3)_2$ (97% from **6c**)
1c R = $CH_2CH_2NHCH_2CH_2OH$ (58% from **6d**)

2a–c

2a R = $CH_2CH_2CH_2OH$ (80% from **7b**)
2b R = $CH_2CH_2N(CH_3)_2$ (2% from **5b**)
2c R = $CH_2CH_2NHCH_2CH_2OH$ (3% from **5c**)

[a] (a)CrO_3, $AcOH/H_2O$, 60 °C; (b) 1 N NaOH, 25 °C; (c) aq HCl, 55–90 °C.

Scheme 3[a]



1d–g

[a] (a) HMDS (9 equiv), RNH_2 (11 equiv), PTSA or $(NH_4)_2SO_4$ (cat.), 170 °C, 24–43 h, 40–67% yield.

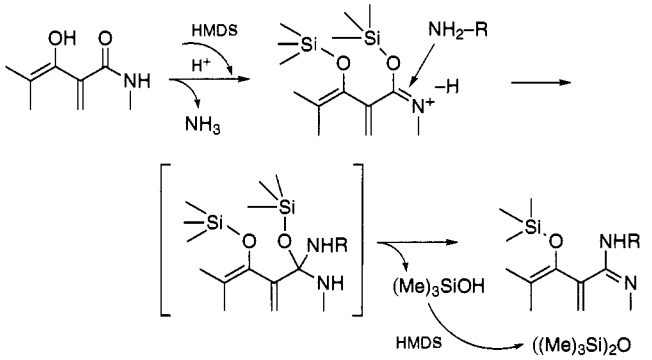


Figure 2.

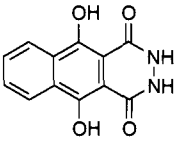
chains, possibly triggered by partial deacetylation of the amidino moiety.

The mixture of the red acetylated quinones **6** and **7** could be separated either by silica gel column chromatography, as in the case of the mixtures of **6c/7c** and **6d/7d**, or on analytical TLC silica gel plates, with **6a/7a**. However basic hydrolysis of the crude mixture of **6a** and **7a** yielded the monoacetyl derivatives **6b** and **7b** which were easily separated by silica gel column chromatography. In general the acetylated quinones **6** and **7** showed quite complex ^{1}H-NMR patterns, very often with broad signals and splitting of the resonances of the *N*-acetyl group, probably due to the occurrence either of slowly exchanging rotamers or of amide geometrical isomerism.

Only **7a,b** could be obtained in pure enough form to permit their structural elucidation by ^{1}H-NMR and electron impact or FAB mass spectrometry. In the case of compounds **7c,d**, structures were deduced on the basis of their chromatographic behavior, from ^{1}H-NMR analysis of the crude chromatographic fractions containing the compounds and from the structure of the final compounds **2b,c** obtained by acid hydrolysis (see below). Acid hydrolysis was also used to convert compounds **6b–d** to **1a–c**. However overall yields from **5** were only moderate (20–34%). Similar acid hydrolysis of **7b–d** afforded the 4-amino derivatives **2** in 2–9% overall yields from **5**.

(B) Functionalization of the Hydroquinone 8 (5,10-Dihydroxy-1,4-dioxo-1,2,3,4-tetrahydrobenzo[*g*]phthalazine). The moderate yields in which 1,4-BPDs **1** were produced by the above oxidative route prompted us to investigate an alternative process starting from the hydroquinone **8**, which is an inexpensive

and easily available material.[14] It was expected that



8

functionalization of the pyridazine ring of **8** with amines followed by a facile hydroquinone–to–quinone oxidation would represent a convenient route to the target compounds **1**. Despite numerous attempts, the conversion of the pyridazine ring of the hydroquinone **8** to the corresponding 1,4-dichloro derivative failed under a variety of standard chlorination conditions, including the use of $POCl_3$, PCl_5, or $SOCl_2$.

The functionalization of the pyridazine ring of **8** with amines followed by a smooth hydroquinone–to–quinone oxidation, to give the target compounds **1**, was finally accomplished in a single step. This involved a modification of the one step/one pot silylation amination technique developed by Vorbrueggen[15] for the conversion of lactam moieties in heterocyclic systems to the corresponding amidino groups. The silylation–amination of **8** was carried out at reflux temperature (170 °C) in a large excess of both HMDS (9 equiv) and the chosen amine (11 equiv) in the presence of an acid catalyst such as *p*-toluenesulfonic acid or ammonium sulfate (Scheme 3). The quinones **1d–g** were directly isolated at the end of the reaction after methanolic workup in the presence of air, often as crystalline materials. The final hydroquinone–quinone oxidation occurred extremely easily, not requiring any oxidant other than atmospheric oxygen.

In the silylation–amination conversion of the pyridazinedione to a bis(alkylamino)pyridazine, silylation probably occurs initially at all four oxygen atoms. However the intermediate bis(silyloxy)pyridazine ring is much more reactive to amine substitution than the silylated hydroquinone moiety (Figure 2) because of both steric and electronic reasons. Thus one obtains excellent selectivity in contrast to our previous attempts to effect this conversion with a combination of chlorination and amination reactions. The silylation–amination of **8** is only poorly effected with relatively low-boiling amines such as *N,N*-dimethylethylenediamine or with amines such as 2-[(2-aminoethyl)amino]ethanol, which are unstable at the high reaction temperatures (not shown). In these cases the preparation of the corresponding quinones **1b,c** was carried out according to route **A**.

Biological Results

In Vitro Cytotoxic Activity. Table 3 shows the in vitro cytotoxic activity of 1,4-BPDs **1** against the human colon adenocarcinoma cell line LoVo and its subline which is resistant to doxorubicin (LoVo/Dx). This cell line has the P-glycoprotein-mediated mechanism of multidrug resistance (MDR) and is resistant to a number of intercalating agents such as various anthracyclines, mitoxantrone, and ametantrone.[16] The cytotoxic activity of the known benzo[*g*]phthalazine **3a**[13] and of the new congener **3c** is also reported for comparison.

With the exception of compounds **1c,g**, in general the novel 1,4-BPDs **1** are 2–12 times more cytotoxic than

Table 3. Cytotoxic Activity of Benzo[*g*]phthalazine-5,10-diones **1a–g** and Benzo[*g*]phthalazines **3a,c** against Human Colon Adenocarcinoma Cell Lines LoVo and LoVo/Dx

compound	IC_{50}(M)[a] LoVo	IC_{50}(M)[a] LoVo/Dx	RI[b]
1a[c]	7.6×10^{-8}	$<2.0 \times 10^{-6}$	<26
1b[d]	7.3×10^{-8}	4.4×10^{-7}	6.0
1c[d]	2.7×10^{-6}	3.9×10^{-5}	14.4
1d	1.8×10^{-7}	1.7×10^{-6}	9.4
1e	4.9×10^{-8}	1.0×10^{-6}	20.4
1f	2.6×10^{-7}	1.2×10^{-6}	4.6
1g	1.4×10^{-6}	1.3×10^{-6}	0.9
3a	$>9.6 \times 10^{-6}$	3.6×10^{-5}	>3.7
3c	$>8.1 \times 10^{-6}$	$>2.6 \times 10^{-4}$	–
ametantrone	5.8×10^{-7}	9.2×10^{-5}	158.6
mitoxantrone	9.7×10^{-9}	5.3×10^{-7}	54.6
doxorubicin	4.3×10^{-8}	6.0×10^{-6}	139.5

[a] IC_{50} = concentration of compound required to inhibit the cellular growth by 50% after 144 h of drug exposure, as determined by the MTT assay.[18] Each experiment was run at least three times, and the results are presented as an average value. [b] Resistance index = (IC_{50} LoVo/Dx)/(IC_{50} LoVo). [c] Hydrochloride. [d] Trihydrochloride.

Table 4. Cytotoxic Activity of Benzo[*g*]phthalazine-5,10-diones **1b,c** and **2b,c** against a Panel of Human Tumor Cell Lines

compound	IC_{50} (M)[a] of cell lines[b] HT-1080	HT-29	MCF-7	A-375
1b[c]	4.6×10^{-8}	9.4×10^{-9}	9.5×10^{-10}	3.3×10^{-8}
1c[c]	1.3×10^{-6}	4.1×10^{-7}	7.2×10^{-7}	nt[d]
2b[e]	3.4×10^{-8}	6.5×10^{-9}	5.0×10^{-9}	nt[d]
2c[e]	8.3×10^{-9}	5.2×10^{-9}	4.7×10^{-8}	4.7×10^{-10}
mitoxantrone	6.6×10^{-8}	1.7×10^{-8}	2.0×10^{-8}	2.6×10^{-8}

[a] IC_{50} = concentration of compound (mol/L) required to inhibit the cellular growth by 50% after 144 h of drug exposure, as determined by the MTT assay. [b] Human cell lines: HT-1080 fibrosarcoma; HT-29 colon adenocarcinoma; MCF-7 breast adenocarcinoma; A-375 melanoma. [c] Trihydrochloride. [d] nt = not tested. [e] Dihydrochloride.

the structurally related ametantrone, the most active compounds being the dimethylamino derivatives **1b,e** and the (3-hydroxypropyl)amino derivative **1a**. The low potency of the 2,3-diazaametantrone congener **1c** appears to be remarkable. The cytotoxic activity of 1,4-BPDs **1** does not depend so much on chain length (compare **1b** with **1e**) as it does on steric variations in the substituent on the distal nitrogen atom (compare **1b,d,f**). The presence of a distal basic nitrogen atom is not a prerequisite to cytotoxic activity, the (3-hydroxypropyl)amino derivative **1a** being one of the most cytotoxic compounds. The 1,4-BPDs **1a,c** are also much more cytotoxic than the non-quinonoid congeners **3a,c**. This evidence underlines the importance of the quinone subunit for cytotoxic activity in the benzo[*g*]phthalazine series.

The 1,4-BPDs are definitely less cytotoxic than mitoxantrone in the LoVo cell line. However it is interesting to note that the resistance index of the 1,4-BPDs is remarkably reduced in comparison to both mitoxantrone and ametantrone and that the bis(benzylamino) derivative **1g** is not cross-resistant at all. These data suggest that 1,4-BPDs **1** are capable of overcoming at least in part the MDR mechanism operating in the LoVo cell line induced by doxorubicin.

The cytotoxic activity of the [2-(dimethylamino)ethyl]amino (**1b**) and 2,3-diazaametantrone (**1c**) congeners was also examined against a panel of human tumor cell lines. The results are reported in Table 4 which also lists the activities of the related 1-alkylamino 4-amino

Table 5. Spectrophotometric and Thermodynamic Parameters for the Binding of **1c**, **2c**, and Ametantrone to Calf Thymus DNA in ETN (0.15 M), pH 7.0, $T = 25$ °C[a]

	free		bound			$K_i \times 10^{-4}$	
	λ_{max}	ϵ_{max}	λ_{max}	ϵ_{max}	λ_{iso}	(M^{-1})	n (bp)
1c	640	4660	677	3880	680	4.7 ± 0.1	3.0 ± 0.1
2c	610	4530	655	4130	646	4.5 ± 0.1	3.0 ± 0.1
ametantrone	626	16100	642	13600	637	34 ± 1	2.7 ± 0.1

[a] See Experimental Section.

Table 6. Linear and Circular Dichroism Data for the Visible Band in the Complex of **1c**, **2c**, and Ametantrone to Calf Thymus DNA in ETN (0.15 M), pH 7.0, $T = 25$ °C[a]

compound	LD[b]	α_L (deg)	$\Delta\epsilon$
1c	−0.13	~90	0.7
2c	−0.13	~90	0
ametantrone	−0.13	~90	−4.5

[a] See Experimental Section. [b] LD_r for calf thymus DNA = −0.14.

derivatives **2b,c**. Compound **1b** is definitely more potent than the ametantrone-like congener **1c**. Moreover, both the 4-amino-substituted compounds **2b,c** show very high cytotoxic activity, comparable or even superior to that of mitoxantrone.

DNA-Binding Studies. Unlike their congeners **1b** and **2b**, compounds **1c** and **2c** exhibit remarkably different cytotoxic activities. In this connection we tried to correlate their biological response with their DNA-binding properties. The thermodynamic and spectroscopic data of complexes formed between **1c** or **2c** and calf thymus DNA are summarized in Table 5.

The data relative to ametantrone are reported for comparison in the same table. They are in fairly good agreement with the binding parameters reported in the literature for this compound under similar experimental conditions.[3d,e] The affinity for DNA of ametantrone is about 8 times higher than that of compounds **1c** or **2c**. Previous measurements (not shown) confirmed that aggregation phenomena are not observed for the tested drugs. Clearly both diaza derivatives show very close thermodynamic DNA-binding parameters under physiological conditions. Hence differences in affinities for the nucleic acid cannot be responsible for the different biological activities.

In addition to affinity, the mode and complex geometry of interaction with DNA could play a relevant role. To assess possible modifications in these parameters, linear and circular dichroism measurements were performed. The results are reported in Table 6. Both drugs intercalate into the double-helical structure of DNA, as shown by the reduced linear dichroism of the bound ligands, very close to the values obtained for ametantrone and free DNA. However, the circular dichroism spectra in the ligand absorption region differ considerably among the examined drugs, being positive for **1c**, almost zero for **2c**, and large and negative for ametantrone (Table 6). These results suggest that the average orientation/location of the test drug−molecules within the base pair pocket of DNA is altered. Indeed, this fact might play an important role in eliciting different cytotoxic responses by the above compounds.

Conclusions

The benzo[*g*]phthalazine-5,10-diones **1** and **2** represent a novel class of cytotoxic chromophore-modified anthracene-9,10-diones structurally related to ametantrone. Members of this family are endowed with broad cytotoxic activity against a panel of human tumor cell lines, comparable or even superior to that of mitoxantrone. Clearly, the introduction of the 2,3-diaza subunit into the anthraquinone chromophore exerts a profound effect on the biological properties of the molecules. This is reflected by the relative inactivity of the ametantrone-like derivative **1c** in different experimental models and by the pronounced cytotoxicity of the (3-hydroxypropyl)amino derivative **1a**, which lacks any basic site in the side chains, suggesting that in the 1,4-BPD series different structure−activity relationships are operative than in the anthracenedione series. The potent cytotoxic activity of the monoalkylamino congeners **2b,c** is also worthy of note. DNA-binding studies on the ametantrone-like compounds **1c** and **2c** indicate that the introduction of a 2,3-diaza subunit into the anthracene-9,10-dione chromophore reduces the affinity of the drug for DNA in comparison to ametantrone. On the other hand, the number of side-chain groups does not affect binding to any great extent, whereas it modulates the average orientation of the drug with respect to DNA. This would in turn affect recognition of the drug−DNA complex by a third agent (e.g., topoisomerases or other DNA-processing enzymes) and lead to a modified pharmacological response. Alternatively, the above findings could be explained in terms of mechanisms of cell death other than those requiring binding to DNA as a key step.

Despite the potent in vitro cytotoxic activity, the benzo[*g*]phthalazine-5,10-diones **1** and **2** are devoid of significant in vivo antileukemic activity in mice (data not shown). The reasons for this in vivo inactivity are not presently understood and might involve inappropriate distribution, metabolic inactivation, and/or nonselective mechanisms of cell killing. Further studies aimed at elucidating these mechanisms are underway and will be reported later.

Experimental Section

Melting points were determined on a Thomas-Hoover or Buchi-535 apparatus and are uncorrected. Proton and carbon NMR spectra were taken on a Brucker AC 200 or General Electric QE-300 pulsed Fourier transform spectrometer using TMS as internal standard. A Beckman Uvikon 860 spectrophotometer was used for UV spectral determinations. IR spectra were recorded on a Perkin Elmer 1710 infrared Fourier transform spectrometer in Nujol films, unless otherwise stated. Molecular weights were determined by electron impact (EI), FAB, or positive LSIMS mass spectrometry on a KRATOS MS 80 spectrometer or VG AutoSpec mass spectrometer. Precoated silica gel plates (silica gel 60 F_{254}; Merck) with fluorescent indicator or reversed phase plates (RP-18 F_{254}s; Merck) were used for thin layer chromatography (TLC). SiO_2 (70−230 mesh) was used for all column chromatography separations. All mixtures of solvents were by volume. Microanalyses were performed by Redox s.n.c. (Cologno Monzese, Milan, Italy).

Ametantrone dihydrochloride and mitoxantrone dihydrochloride were synthesized at the Chemical Department of Boehringer Mannheim Italia. Doxorubicin hydrochloride (adriblastina) was obtained from Farmitalia Carlo Erba S.p.A. Calf thymus DNA was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Its extinction coefficient was 6600 M^{-1} cm^{-1} at 260 nm.

1,4-Dichlorobenzo[*g*]phthalazine (4). (a) A stirred suspension of naphthalene-2,3-dicarboxylic acid (13.90 g, 64 mmol) in acetic anhydride (95 mL) was heated to reflux until complete

solution was obtained (about 20 min). After cooling to room temperature, the cream-colored crystals which separated were filtered and washed with diethyl ether to give naphthalene-2,3-dicarboxylic acid anhydride (12.06 g, 95% yield): mp 248.2–250 °C; ^{1}H-NMR (DMSO-d_6) δ 7.88 (m, 2H), 8.33 (m, 2H), 8.78 (s, 2H); IR (Nujol, major peaks, cm^{-1}) 1836, 1804, 1762, 929, 917, 890, 735, 722.

(b) Hydrazine hydrate (80% solution, 17.5 mL) was added in one portion to a mechanically stirred suspension of the naphthalene-2,3-dicarboxylic acid anhydride (11.92 g, 60 mmol) in glacial acetic acid (300 mL): an exothermic reaction ensued with copious evolution of fumes, and most of the anhydride dissolved. The mixture was heated rapidly to reflux to give an almost clear solution from which a slightly yellow solid began to separate after a few minutes. The mixture was refluxed for 6 h; then it was left overnight at room temperature. The precipitated solid was filtered, washed with water (500 mL), and dried to give 1,2,3,4-tetrahydro-1,4-dioxobenzo-[*g*]phthalazine as a cream-colored powder (11.92 g, 93% yield): mp >360 °C (from DMF) (lit.[17] mp 345 °C); ^{1}H-NMR (DMSO-d_6) δ 7.75 (m, 2H), 8.29 (m, 2H), 8.77 (s, 2H), 11.52 (br s, 2H).

(c) Dry pyridine (6.0 mL) was slowly added to a suspension of 1,2,3,4-tetrahydro-1,4 dioxobenzo[*g*]phthalazine (10.00 g, 47 mmol) in phosphorus oxychloride (100 mL), and the mixture was heated for 2.5 h at 100 °C. The yellow solution obtained was allowed to cool to 40 °C (below this temperature a yellow solid separates), and the volatiles were removed by distillation at reduced pressure, the external bath temperature being 60–65 °C. The yellow solid obtained was triturated with diethyl ether (120 mL), filtered, washed with diethyl ether (120 mL), and partitioned under vigorous stirring between ice-cold water (200 mL) and ethyl acetate (200 mL) for 10 min. The solid which separated was filtered, washed first with water (50 mL) and then with diethyl ester (50 mL), and finally dried over P_2O_5 to give **4** as a yellow powder (10.19 g, 87% yield): mp 224–228 °C (lit.[13] mp 217–219 °C); ^{1}H-NMR (DMSO-d_6) δ 7.90 (m, 2H), 8.50 (m, 2H), 9.10 (s, 2H).

1,4-Bis[*N*-(3-hydroxypropyl)amino]benzo[*g*]phthalazine (3a). A solution of **4** (4.84 g, 19 mmol) in 1-amino-3-propanol (dried over 3 Å molecular sieves) was heated at 125 °C for 5 h. After cooling to room temperature, the orange solution obtained was poured into a stirred solution of potassium carbonate (96 g) in water (240 mL). The yellow solid which separated was filtered, washed first with a few milliliters of water and then with absolute ethanol (20 mL), and finally dried under vacuum at 50 °C to give **3a** (6.095 g, 98% yield): mp 225–228 °C (from ethanol/water) (lit.[13] mp 231–232 °C); ^{1}H-NMR (DMSO-d_6) 1.86 (m, 4H), 3.57 (m, 8H), 4.87 (br s, 2H, exchangeable with D_2O), 6.76 (br s, 2H, exchangeable with D_2O), 7.68 (m, 2H), 8.09 (m, 2H), 8.81 (s, 2H); ^{13}C-NMR (DMSO-d_6) δ 32.47, 38.42, 58.82, 118.19, 122.15, 127.71, 128.57, 133.20, 148.95; UV (EtOH) λ_{max} (nm) (log ϵ) 234 (4.76), 254 (4.61), 361 (3.60), 412 (3.66).

1,4-bis[*N*-[2-(dimethylamino)ethyl]amino]benzo[*g*]phthalazine (3b). A solution of **4** (3.82 g, 15.3 mmol) in *N,N*-dimethylethylenediamine (38 mL) was heated to reflux for 8 h. After cooling to room temperature, the solution was poured into a saturated sodium chloride solution (200 mL) and the white precipitate which separated was redissolved by addition of water (20 mL). The solution was extracted with ethyl acetate (4 × 80 mL), and the combined organic layers were dried over anhydrous sodium sulfate and rotoevaporated. The orange solid obtained was triturated with diethyl ether (20 mL) to give crude **3b** (4.87 g, 90% yield) which was used for the next step without further purification: mp 150–154 °C; ^{1}H-NMR ($CDCl_3$) δ 2.30 (s, 12H), 2.70 (t, 4H), 3.70 (q, 4H), 5.55 (br t, 2H), 7.62 (m, 2H), 8.05 (m, 2H), 8.35 (s, 2H).

1,4-Bis[*N*-[2-[(2-hydroxyethyl)amino]ethyl]amino]benzo[*g*]phthalazine (3c). Under a nitrogen atmosphere compound **4** (3.164 g, 12.7 mmol) was added to dry (over 3 Å molecular sieves) 2-[(2-aminoethyl)amino]ethanol (30 mL). The mixture was heated at 120 °C for 4 h, and the red solution that resulted was then cooled to room temperature and poured into 20% aqueous ammonium sulfate (100 mL). After removal of some less polar impurities by extraction with ethyl acetate (2 × 25 mL), the aqueous phase was repeatedly extracted with THF (10 × 50 mL). The THF extracts were combined and thoroughly dried over sodium sulfate. After removal of the solvent at reduced pressure, the residue was dried by azeotropic distillation with absolute ethanol (150 mL, three times). Finally a solution of the obtained residue in absolute ethanol (6 mL) was cooled to −20 °C for 12 hours, and then it was diluted with ethyl acetate (70 mL). The yellow precipitate obtained was filtered to give **3c** (2.03 g, 41% yield). A further crop of 0.92 g (18% yield) of **3c** could be obtained by exhaustive extraction of the ammonium sulfate mother liquor with THF (700 mL total) followed by the same procedure as described above: mp 129–131 °C; ^{1}H-NMR (DMSO-$_{d6}$) δ 2.65 (t, 4H), 2.85 (t, 4H), 3.50 (m, 8H), 4.40 (br), 7.70 (m, 2H), 8.10 (m, 2H), 8.80 (s, 2H); ^{13}C-NMR (DMSO-d_6) δ 35.06, 48.12, 51.23, 60.00, 118.04, 122.07, 127.59, 128.47, 133.09, 148.74; UV (H_2O) λ_{max} (nm) (log ϵ) 255 (3.81), 292 (3.85), 381 (3.65). Anal. ($C_{20}H_{28}N_6O_6$) Calcd: C, 62.48; H, 7.34; N, 21.86. Found: C, 60.33; H, 7.47; N, 21.18.

1,4-bis[*N*-(3-acetoxypropyl)-*N*-acetylamino]benzo[*g*]phthalazine (5a). A stirred solution of 1,4-bis[*N*-(3-hydroxypropyl)amino]benzo[*g*]phthalazine (**3a**; 1.82 g, 5.57 mmol) in dry pyridine (6.5 mL) and acetic anhydride (6.5 mL) was heated at 110 °C for 4 h 30 min. Excess pyridine and acetic anhydride were removed by distillation at reduced pressure, and the yellow solid obtained was taken up in methylene chloride (100 mL). The organic solution was washed with water (2 × 50 mL), saturated sodium bicarbonate solution (2 × 50 mL), and brine (1 × 50 mL) and finally dried over anhydrous sodium sulfate. Removal of the solvent under reduced pressure left an oily residue, still containing traces of pyridine, which was treated with diethyl ether (30 mL) under stirring for 30 min. The yellow solid obtained was filtered and recrystallized from absolute ethanol (10 mL) to give 1.86 g (67% yield) of a pale-yellow crystalline material: mp 157–158 °C; the ^{1}H-NMR showed very broad resonances, ^{1}H-NMR ($CDCl_3$, TMS) δ 1.97 (s, 12H), 2.20 (m, 4H), 3.67 (br, 2H), 4.23 (t, 2H), 4.54 (br, 2H), 7.84 (m, 2H), 8.24 (m, 2H), 8.63 (m, 2H); ^{13}C-NMR ($CDCl_3$) δ 20.69, 22.80, 27.69, 61.74, 122.58, 124.91, 129.13, 129.80, 135.49, 156.65, 170.213, 170.256, 170.686; IR (Nujol, major peaks, cm^{-1}) 1727, 1668, 1250, 1200, 1130, 1095, 895, 770; positive ion FAB (thioglycerin) 495.0651 (M^+).

1,4-Bis[*N*-[2-(dimethylamino)ethyl]-*N*-acetylamino]benzo[*g*]phthalazine (5b). A solution of **3b** (1.21 g, 3.4 mmol) in pyridine (3 mL) and acetic anhydride (2 mL) was stirred at room temperature overnight. After removal of the volatiles at reduced pressure, the residue was taken up in chloroform (50 mL) and the solution was washed with saturated brine and brought to pH 8 by the addition of 1 N sodium hydroxide solution. Drying and removal of the solvent left an oily residue which was purified by silica gel column chromatography, eluant: ethyl acetate/methanol/triethylamine, 16/2/1. Compound **5b** was obtained as a yellow oil (0.54 g, 36% yield): ^{1}H-NMR ($CDCl_3$) δ 1.88 (s, 6H), 2.12 (s, 12H), 2.61 (t, 4H), 4.29 (br, 2H), 4.13 (q, 2H), 7.75 (m, 2H), 8.20 (m, 2H), 8.75 (br s, 2H).

1,4-Bis[*N*-[2-[*N*-acetyl-*N*-(2-acetoxyethyl)amino]ethyl]-*N*-acetylamino]benzo[*g*]phthalazine (5c). Acetic anhydride (13 mL) was added to a stirred suspension of **3c** (3.03 g, 7.86 mmol) in dry pyridine (15 mL), and the solution obtained was heated at 85 °C for 3.5 h while protected from light. After removal at reduced pressure of the excess of pyridine and acetic anhydride, the residue was taken up in methylene chloride (100 mL) and the organic solution was washed with 5% sodium bicarbonate soltuion (4 × 60 mL). The combined aqueous layers were re-extracted with methylene chloride (50 mL), the pooled organic phases were washed with brine and dried, and the solvent was removed by rotary evaporation. The residue was purified by silica gel column chromatography eluting with methylene chloride/methanol, 20/1, to give 4.02 g (80% yield) of pure compound **5c** as a pale-yellow foam: ^{1}H-NMR ($CDCl_3$) δ 1.92 and 2.01 and 2.08 and 2.21 (4 s, 18H), 3.35–4.05 (br m, 10H), 4.15 and 4.29 and 4.50–4.75 (m, 6H), 7.81 (n, 2H), 8.22 (m, 2H), 8.60 (m, 2H); IR (KBr, major peaks, cm^{-1}) 1741, 1673, 1643.

1-[*N*-(3-Hydroxypropyl)-*N*-acetylamino]-4-[*N*-(3-hydroxypropyl)amino]benzo[*g*]phthalazine-5,10-dione (6b) and 4-Amino-1-[*N*-(3-hydroxypropyl)-*N*-acetylamino]benzo[*g*]phthalazine-5,10-dione (7b). A solution of chromium trioxide (5.44 g, 95 mmol) in water (3.5 mL) and acetic acid (31 mL) was added to a well-stirred solution of **5a** (5.40 g, 10.9 mmol) in acetic acid (45 mL) at such a rate that the temperature did not rise over 35 °C. The addition required about 1 h. The mixture was then heated at 55 °C for 1 h. The excess of chromium trioxide was destroyed by the addition of 2-propanol (8 mL) at 10 °C, and after stirring for 1 h at room temperature, the reaction mixture was poured into water (500 mL) and extracted with chloroform (3 × 100 mL). The combined organic solutions were washed first with water (4 × 200 mL), then with saturated sodium bicarbonate solution (2 × 100 mL), and finally with brine (2 × 50 mL). Drying and removal of the solvent led to a mixture of **6a** and **7a** (3.99 g) which was dissolved in methanol (30 mL) and treated with 1 N NaOH (16 mL) at room temperature for 45 min. After dilution with a saturated solution of NaH_2PO_4 (100 mL) and water (200 mL), the wine-red solution was extracted with methylene chloride (3 × 100 mL). Drying of the combined organic solutions and removal of the solvent left a red residue (2.67 g) which was applied to the top of a silica gel chromatographic column. By elution with ethyl acetate/*n*-hexane/methanol, 20/5/1, 4-amino-1-[*N*-(3-hydroxypropyl)-*N*-acetylamino]benzo[*g*]phthalazine-5,10-dione (**7b**; 0.42 g, 11% yield from **5a**) was obtained as a brown powder: mp 201–203 °C (from 2-propanol) ^{1}H-NMR (DMSO-d_6) (mixture of two rotamers) δ 1.60 (m, 2H), 1.68 and 2.23 (2 s, 3H), 3.12 (m, 2H), 4.09 (m, 2H), 4.32 and 4.52 (2 t, 1H), 7.92 and 7.72 (2 m, 4H), 8.29 and 8.45 (2 br s, 2H); ^{13}C-NMR (DMSO-d_6) δ 21.79 and 22.48, 30.89 and 31.75, 44.79 and 47.17, 58.03 and 58.69, 109.76 and 110.15, 125.82 and 125.95, 126.31, 132.29, 134.87, 145.50, 157.44 and 157.88, 169.39 and 171.17, 182.79, 185.84 and 186.13; IR (Nujol, major peaks, cm^{-1}) 3405, 1679, 1634, 1588; EI *m/z* (rel intensity) 42.9 (84.3), 210.9 (64.0), 252.9 (100), 298.0 (58.2), 340.0 (M^+, 14.0), 341.0 (4.4), 342.0 (0.8); UV (MeOH) λ_{max} (nm) 252, 452.

By further elution with ethyl acetate/methanol, 92/8, 1-[*N*-(3-hydroxypropyl)-*N*-acetylamino]-4-[*N*-(3-hydroxypropyl)amino]benzo[*g*]phthalazine-5,10-dione (**6b**; 2.04 g, 47% yield from **5a**) was obtained as an amorphous purple powder: mp 67–70 °C (from diethyl ether); ^{1}H-NMR (DMSO-d_6) (mixture of two rotamers) δ 1.61 and 1.88 (2 m, 4H), 1.63 and 2.21 (2 s, 3H), 3.10 and 3.35 (2 m, 4H), 3.55 (q, 2H), 3.81 (m, 2H), 3.35 and 4.09 (2 m, 1H), 4.35 (t, 1H), 4.51 and 4.70 (2 t, 1H), 8.02 (br m, 4H), 9.05 and 9.20 (br m, 1H); EI *m/z* (rel intensity) 43.0 (82.5), 210.9 (100), 355.0 (51.6), 356.0 (55.3), 399.1 (2.8, M^+); IR ($CHCl_3$, major peaks, cm^{-1}) 1681, 1651.

1,4-Bis[*N*-(3-acetoxypropyl)-*N*-acetylamino]benzo[*g*]-phthalazine-5,10-dione (6a) and 4-Amino-1-[*N*-(3-acetoxypropyl)-*N*-acetylamino]benzo[*g*]phthalazine-5,10-dione (7a). The reaction mixture (28 mg) obtained from a small scale oxidation of compound **5a** (48 mg, 0.097 mmol) with chromium trioxide (48.6 mg added in four portions) in aqueous acetic acid as described above was divided into two portions which were applied to two analytical TLC plates (silica gel, 5 × 20 cm). The plates were eluted twice with ethyl acetate/*n*-hexane/methanol, 20/5/1. From the orange faster moving band, pure compound **6a** (13 mg, 25% yield) was recovered as a glass red solid: ^{1}H-NMR ($CDCl_3$) (mixture of rotamers with broad resonances) δ 2.06, 2.47, 4.22, 7.85, 8.13; positive ion FAB (thioglycerin) 527 $(M + 3H)^+$; EI *m/z* (rel intensity) 43.2 (80.7), 57.2 (28.7), 101.2 (100), 149.1 (39.7), 524.3 (1.1, M^+), 525.2 (0.4), 526.3 (0.2). From the red slower moving band, compound **7a** (contaminated by traces of **6a**) was obtained (4 mg, 12%): positive ion FAB (thioglycerin) 385 $(M + 3H)^+$; EI *m/z* (rel intensity) 43.2 (100), 101.1 (64.1), 253.2 (54.8), 279.2 (52.8), 382.3 (M^+).

1-[*N*-[2-(Dimethylamino)ethyl]-*N*-acetylamino]-4-[*N*-[2-(dimethylamino)ethyl]amino]benzo[*g*]phthalazine-5,10-dione (6c). A solution of chromium trioxide (0.44 g, 4.2 mmol) in water (0.4 mL) and acetic acid (3.5 mL) was dropped at room temperature into a well-stirred solution of **5b** (0.32 g, 0.73 mmol) in acetic acid (3 mL). The separation of a dark material was observed. After stirring for 1 h, the reaction mixture was heated to 60 °C (external oil bath) for 1.66 h. Then a further portion of chromium trioxide (0.16 g, 1.5 mmol) dissolved in water (0.2 mL) and acetic acid (2 mL) was added at 60 °C. After further heating at 60 °C for 30 min, the reaction mixture was cooled to room temperature and treated with 2-propanol (1.5 mL). After 10 min, brine (8 mL) was added and the pH was adjusted to 9 with 35% NaOH solution while cooling. Chloroform was added, and the biphasic mixture was filtered to remove some precipitated material. The aqueous phase was repeatedly extracted with chloroform and then discarded. The precipitate was dissolved in water, and the aqueous solution was re-extracted with chloroform. Drying and removal of the solvent from the combined organic solutions left a red waxy material (0.14 g). The pure compound **6c** (0.11 g, 35% yield) was obtained as a red-brick powder by silica gel column chromatography, eluting with ethyl acetate/methanol/triethylamine, 9/1/0.1, followed by crystallization from diethyl ether: mp 119–122 °C; ^{1}H-NMR (DMSO-d_6) (mixture of two rotamers) δ 1.7 (s, 6H), 1.80 and 2.05 (2 s, 3H), 2.15 and 2.48 (2 m, 2H), 2.30 (s, 6H), 2.70 (t, 2H), 3.30 and 3.60 (2 m, 2H), 3.82 (m, 2H), 7.85, (m, 2H), 8.10 and 8.20 (2 m, 2H), 9.20 and 9.30 (2 br t, 1H); IR (Nujol, major peaks, cm^{-1}) 3348, 1672, 1650, 1592, 1540, 737; UV (H_2O) λ_{max} (nm) 300, 477.

When the above chromic acid oxidation of **5b** was run on a multigram scale, the formation of very small amounts of a yellow faster moving compound (probably **7c**) was detected by TLC (SiO_2; AcOEt/MeOH/TEA, 9/1/0.1). From the oxidation of 4.507 g (10.31 mmol) of **5b** followed by chromatographic purification as noted above, 1.07 g of **6c** contaminated by trace amounts of **7c** was obtained, along with 0.55 g of pure **6c** (approximately 36% overall yield).

1,4-Bis[*N*-[2-[*N*-acetyl-*N*-(2-acetoxyethyl)amino]ethyl]-*N*-acetylamino]benzo[*g*]phthalazine-5,10-dione (6d). A solution of chromium trioxide (3.31 g, 33.1 mmol) in water (2.8 mL) and acetic acid (22 mL) was added during 45 min to a stirred solution of **5c** (3.83 g, 6 mmol) in acetic acid (33 mL). After 3 h at 60 °C, the dark solution was cooled, treated with 2-propanol (8 mL), poured into 5% $NaHCO_3$ solution (600 mL), and extracted with chloroform (3 × 200 mL). The combined organic layers were washed with water (2 × 100 mL), ice-cold 5% $NaHCO_3$ solution (3 × 100 mL), and finally brine (100 mL). After drying over Na_2SO_4 and removal of the solvent at reduced pressure, a red residue was obtained which by TLC analysis (SiO_2; ethyl acetate/methanol/triethylamine, 16/4/0.1) was shown to consist essentially of the red slower moving spot of the hexaacetyl derivative **6d** together with an orange faster moving spot (probably the triacetyl derivative **7d**). This residue was purified by silica gel column chromatography. Elution with ethyl acetate followed by ethyl acetate/methanol/triethylamine, 16/2/0.2, removed the faster moving orange spot of **7d** (0.28 g, approximately 10% yield) which was obtained contaminated by some less polar material and used as such for the next step (see below).

Further elution with ethyl acetate/methanol/triethylamine, 16/2/0.2, gave compound **6d** (1.34 g, 35% yield) as an orange foam: ^{1}H-NMR ($CDCl_3$) (mixture of rotamers with very broad resonances) δ 1.80, 1.95, 2.05, 2.16, 2.19, and 2.51 (br s, 18H), 3.62, 4.20 and 4.58 (br m, 16H), 7.86 (m, 2H), 8.13 (m, 2H).

1,4-Bis[*N*-(3-hydroxypropyl)amino]benzo[*g*]phthalazine-5,10-dione (1a). A solution of **6b** (1.969 g, 4.94 mmol) in ethanol (25 mL) was added to 10 N HCl (6.5 mL) and heated at 65 °C for 1.5 h. After cooling to room temperature, the blue solution was diluted with water (100 mL) and extracted with chloroform (50 mL). The organic phase was discarded. The aqueous solution was neutralized with solid $NaHCO_3$ and extracted with chloroform (100 mL). From the chloroform solution a dark-blue solid separated which was filtered. The chloform solution was dried and the solvent removed to give pure **1a** as a blue powder (0.52 g, 29% yield). TLC analysis of the dark-blue solid precipitated from the chloroform layer showed this solid to be compound **1a** contaminated by a yellow very polar material. After two washings with water (2 × 50 mL), this solid was redissolved in ethanol (20 mL) containing 10 N HCl (1 mL) and reprecipitated by the addition of 5% $NaHCO_3$ solution (100 mL). The collected material was finally extracted into boiling chloroform (150 mL), and the solvent

was removed to give a further crop of pure **1a** (0.76 g, 73% overall yield): mp 173–176 °C (from MeOH; after melting the compound becomes again solid at 180–190 °C and remelts at 225 °C); ^{1}H-NMR ($CDCl_3$) δ 1.96 (quint, 4H), 2.80 (br, 2H), 3.75 (m, 4H), 3.93 (q, 4H), 7.84 (m, 2H), 8.19 (m, 2H), 8.75 (br t, 2H); UV (MeOH) λ_{max} (nm) (log ϵ) 251 (4.46), 340 (3.61), 703 (3.77); EI *m/z* (rel intensity) 105.1 (43.8), 127.1 (34.2), 211.1 (100.0), 356.2 (39.2, M^+); TLC (SiO_2 GF, 250 μm, eluant AcOEt/*n*-hexane/MeOH, 20/5/1) R_f 0.10.

O,O-Diacetyl Derivative. The *O,O*-diacetyl derivative of **1a** was prepared by acetylation of **1a** (0.029 g, 0.81 mmol) in 1/1 pyridine/acetic anhydride (0.8 mL) for 1 h at room temperature. The dark-blue solid which separated was filtered, washed with diethyl ether, and recrystallized from a large volume of MeOH, yielding blue needles of the *O,O*-diacetyl derivative (0.019 g, 53% yield): mp 142–145 °C; ^{1}H-NMR ($CDCl_3$) δ 2.12 (s and quint, 6H and 4H), 3.91 (q, 4H), 4.27 (t, 4H), 7.82 (m, 2H), 8.20 (m, 2H), 8.77 (br t, 2H); ^{13}C-NMR ($CDCl_3$) δ 20.97, 28.59, 38.74, 62.59, 106.78, 126.19, 132.08, 134.34, 150.36, 171.22, 186.98; IR (Nujol, major peaks, cm^{-1}) 3348 (sharp), 1737, 1644.

Monohydrochloride Salt. The monohydrochloride salt of **1a** was prepared by bubbling anhydrous hydrogen chloride into a cooled (0 °C) solution of **1a** (0.27 g, 0.76 mmol) in methanol/chloroform, 1/1 (26 mL). After 15 min the suspension was diluted with diethyl ether (80 mL) and the precipitate filtered under a nitrogen atmosphere to give the monohydrochloride salt of compound **1a** (**1a**·HCl) (0.23 g, 77% yield): mp 184–186 °C; ^{1}H-NMR (DMSO-d_6) δ 1.85 (m, 4H), 3.55 (t, 4H), 3.70 (t, 4H), 7.95 (m, 2H), 8.20 (m, 2H), 8.80 (br, 2H); UV MeOH) λ_{max} (nm) (log ϵ) 249 (4.44), 307 (3.73), 697 (3.65); positive LSIMS $[M + H]^+$ *m/z* 357.1. Anal. ($C_{18}H_{21}ClN_4O_4$) Calcd: C, 55.03; H, 5.39; N, 14.26; Cl, 9.02. Found: C, 54.93; H, 5.47; N, 14.13; Cl, 10.07.

4-Amino-1-[*N*-(3-hydroxypropyl)amino]benzo[*g*]phthalazine-5,10-dione (2a). A solution of **7b** (0.40 g, 1.18 mmol) in ethanol (8 mL) and 37% HCl (0.8 mL) was heated at 55 °C for 1 h. After cooling to room temperature, the solution was poured in $NaHCO_3$ saturated solution (100 mL). The blue solid which separated was filtered, washed with water, and dried under vacuum at 50 °C to give **2a** (0.28 g, 80% yield): mp 181–184 °C; ^{1}H-NMR (DMSO-d_6/$CDCl_3$) δ 1.96 (m, 2H), 3.72 (m, 2H), 3.91 (m, 2H), 4.30 (t, 1H, exchangeable with D_2O), 7.17 (br s, 2H, exchangeable with D_2O), 7.86 (m, 2H), 8.22 (m, 2H), 8.90 (br t, 1H, exchangeable with D_2O): UV (MeOH) λ_{max} (nm) (log ϵ) 244 (4.41), 334 (3.62), 656 (3.77); ^{13}C-NMR (DMSO-d_6) δ 32.15, 38.46, 58.68, 106.10, 106.14, 125.72, 125.80, 131.90, 132.07, 134.48, 134.55, 150.68, 151.67, 186.38, 186.53; EI *m/z* (rel intensity) 44.1 (100), 253.1 (55.6), 254.1 (80.9), 298.1 (66.6, M^+); TLC (SiO_2 GF, 250 μm, eluant AcOEt/*n*-hexane/MeOH, 20/5/1) R_f 0.35.

Monohydrochloride Salt. Anhydrous HCl was bubbled during 10 min into a stirred, cooled (0 °C) suspension of **2a** (0.12 g, 0.40 mmol) in MeOH/$CHCl_3$, 1/1 (30 mL). Diethyl ether (100 mL) was then slowly added at 0 °C to the almost complete solution, and after 1 h the blue precipitate was collected by suction under a nitrogen stream to give the monohydrochloride salt of **2a** (**2a**·HCl) (0.1 g, 75% yield): mp 204–205 °C; ^{1}H-NMR (CD_3OD) 1.97 (m, 2H), 3.61 (t, 2H), 3.76 (t, 2H), 8.0 (m, 2H), 8.20 (m, 2H); UV (MeOH) λ_{max} (nm)(log ϵ) 298 (3.74), 644 (3.59); EI $[M]^+$ *m/z* 298.0. Anal. ($C_{15}H_{15}ClN_4O_3$) Calcd: C, 53.93; H, 4.52; N, 16.77; Cl, 10.61. Found: C, 53.12; H, 4.49; N, 16.36; Cl, 10.60.

1,4-Bis[*N*-[2-(dimethylamino)ethyl]amino]benzo[*g*]phthalazine-5,10-dione (1b). A sample of compound **6c** (1.11 g, approximately 2.60 mmol) containing trace amounts of compound **7c** (see above) was dissolved in H_2O/37% HCl, 1/2 (6 mL), and heated at 90 °C for 6 h. After cooling to room temperature, the solution was diluted with water (5 mL) and brought to pH 9 with 20% NaOH while cooling. The blue solid which separated was filtered, dried, and then purified by silica gel column chromatography. Elution with CH_2Cl_2/MeOH, 16.5/3.5, removed compound **2b**. Further elution with CH_2Cl_2/MeOH/TEA, 18/2/0.2, gave compound **1b** (0.968 g, 97% yield) as blue crystals: mp 195–197 °C (from methylene chloride/methanol); ^{1}H-NMR ($CDCl_3$) δ 2.38 (s, 12H), 2.70 (t, 4H), 3.90 (q, 4H), 7.80 (m, 2H), 8.22 (m, 2H), 8.82 (br t, 2H).

Trihydrochloride Salt. Compound **1b** (0.59 g, 1.54 mmol) was dissolved in chloroform (20 mL), and the solution was filtered. The insoluble portion was washed with chloroform (15 mL). The combined filtrates were cooled to 0 °C and treated under a nitrogen atmosphere with 5.8 N HCl in 2-propanol (1.59 mL) diluted with chloroform (5.0 mL). After stirring for 3 h at room temperature, the precipitate formed was filtered under a nitrogen atmosphere and washed with diethyl ether to give the trihydrochloride salt of **1b** (**1b**·3HCl) as a hygroscopic violet powder (0.467 g, 62% yield): mp 257–259 °C; ^{1}H-NMR (DMSO-d_6) δ 2.80 (s, 12H), 3.41 (m, 4H), 4.10 (m, 4H), 7.95 (m, 2H), 8.18 (m, 2H), 8.60 (br s, 2H, exchangeable with D_2O), 10.40 (br s, 2H, exchangeable with D_2O): UV (MeOH) λ_{max} (nm) 243, 332, 628. Anal. ($C_{20}H_{29}Cl_3N_6O_2$·1.5H_2O) Calcd: C, 46.30; H, 6.17; N, 16.20; Cl, 20.50. Found: C, 46.04; H, 5.94; N, 16.03; Cl, 20.64.

4-Amino-1-[*N*-[2-(dimethylamino)ethyl]amino]benzo[*g*]phthalazine-5,10-dione (2b). This compound was isolated by silica gel column chromatography from the crude mixture obtained by the above-described hydrolysis of **6c** (contaminated by **7c**). Compound **2b** (0.060 g, 1.9% overall yield from **5b**) was obtained by elution with CH_2Cl_2/MeOH, 16.5/3.5: mp 181–184 °C; ^{1}H-NMR ($CDCl_3$) 2.40 (s, 6H), 2.75 (t, 2H), 3.95 (m, 2H), 6.80 (br s, 2H), 7.70 (m, 2H), 8.25 (m, 2H), 8.88 (br, m, 1H).

Dihydrochloride Salt. The dihydrochloride salt of **2b** was prepared at 0 °C by treatment of a solution of **2b** (0.045 g, 0.14 mmol) in chloroform (15 mL) and methanol (2 mL) with a 6.8 N solution of HCl in 2-propanol (0.1 mL) diluted with chloroform (3 mL). After 15 min, the mixture was diluted with diethyl ether (10 mL) and filtered to give the dihydrochloride salt of **2b** (**2b**·2HCl) as a violet powder (0.047 g, 85% yield): mp 219.5–221.5 °C; ^{1}H-NMR (DMSO-d_6) δ 2.80 (d, 6H), 3.35 (m, 2H), 4.00 (m, 2H), 7.90 (m, 2H), 8.15 (m, 2H), 8.60 (br, exchangeable with D_2O), 10.05 (br, 1H, exchangeable with D_2O); UV (MeOH) λ_{max} (nm) 240, 336, 625; EI $[M]^+$ *m/z* 311.1. Although this compound appeared to be homogeneus by TLC analysis (SiO_2, eluant CH_2Cl_2/MeOH/concentrated NH_4OH, 18.5/1.5/0.2, R_f 0.28; RP-18, eluant 0.2 N HCl/CH_3CN, 80/20, R_f 0.44), a satisfactory elemental analysis could not be obtained due to contamination by inorganic material. Anal. Calcd for $C_{16}H_{19}Cl_2N_5O_2$: C, 50.01; H, 4.98; N, 18.23; Cl, 18.45. Found: C, 42.03; H, 4.43; N, 15.03; Cl, 16.83.

1,4-Bis[*N*-[2-[2-hydroxyethyl)amino]ethyl]amino]benzo[*g*]phthalazine-5,10-dione (1c). A solution of **6d** (0.80 g, 1.2 mmol) in 37% HCl (8 mL) was heated at 90 °C for 6 h. After cooling to 0 °C, the solution was made alkaline with 20% NaOH and extracted with chloroform (4 × 70 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated to about 20 mL, whereafter compound **1c** crystallized as a blue-black powder (0.29 g, 58% yield): mp 122–124 °C; ^{1}H-NMR ($CDCl_3$/DMSO-d_6) δ 2.88 (t, 4H), 3.05 (t, 4H), 3.70 (t, 4H), 3.90 (br, 4H), 7.80 (br, 2H), 8.80 (br, 2H), 8.90 (br, 2H); TLC (RP-18, eluant methanol/acetonitrile/concentrated NH_4OH, 9/1/1) R_f 0.28.

Trihydrochloride Salt. The trihydrochloride salt of **1c** was prepared at 0 °C under a nitrogen atmosphere by dropwise treatment of a solution of **1c** (0.26 g, 0.68 mmol) in $CHCl_3$/MeOH, 10/1 (16.5 mL), with a 6.3 N solution of HCl in 2-propanol (0.9 mL) diluted with $CHCl_3$ (4 mL). After 2 h at 0 °C, the precipitate was filtered under nitrogen and washed with chloform and then diethyl ether to give the trihydrochloride salt of **1c** (**1c**·3HCl) as a blue-black powder (0.27 g, 76% yield): mp 205–208 °C; ^{1}H-NMR (DMSO-d_6) 3.05 (m, 4H), 3.25 (m, 4H), 3.67 (m, 4H), 4.00 (m, 4H), 8.00 (m, 2H), 8.18 (m, 2H), 8.60 (br), 8.90 (br); UV (HCl, 0.1 N) λ_{max} (nm) (log ϵ) 294 (4.98), 356 (4.46), 583 (4.37); positive LSIMS $[M + H]^+$ *m/z* 415.2. Anal. ($C_{20}H_{29}Cl_3N_6O_4$) Calcd: C, 45.86; H, 5.58; N, 16.04; Cl, 20.30. Found: C, 44.52; H, 5.57; N, 15.53; Cl, 19.74.

4-Amino-1-[*N*-[2-[(2-hydroxyethyl)amino]ethyl]amino]benzo[*g*]phthalazine-5,10-dione (2c). A solution of the crude orange faster moving compound **7d** isolated during the chromatographic purification of **6d** (0.27 g) in 37% HCl (4 mL)

was heated at 90 °C for 9 h. After cooling to 0 °C, the solution was made alkaline with 20% NaOH and extracted three times with chloroform. The combined organic solutions were washed with brine and dried over Na_2SO_4, and the solvent was removed at reduced pressure. The residue was recrystallized from chloroform to give **2c** as a blue crystalline material (60 mg, 3% overall yield from **5c**): mp 155 °C; ^{1}H-NMR ($CDCl_3$) δ 2.88 (t, 2H), 3.05 (t, 2H), 3.65 (t, 2H), 3.90 (q, 2H), 6.75 (br s, 2H), 7.80 (br m, 2H), 8.20 (br m, 2H), 9.00 (br t, 1H); UV (H_2O) λ_{max} (nm) (log ϵ) 614 (3.66).

Dihydrochloride Salt. The dihydrochloride salt of **2c** was prepared at 0 °C by treatment of a solution of **2c** (45 mg, 0.14 mmol) in $CHCl_3/CH_3OH$, 5/1 (3 mL), with a 6.3 N solution of HCl in 2-propanol (0.25 mL) diluted with $CHCl_3$ (0.5 mL). After 2 h at 0 °C, the mixture was diluted with diethyl ether (4 mL) and filtered under a nitrogen atmosphere to give the dihydrochloride salt of **2c** (**2c**·2HCl) (51 mg, 80% yield): mp 202–204 °C; ^{1}H-NMR in DMSO-d_6 gave very broad resonances; UV (HCl, 0.1 N) λ_{max} (nm) (log ϵ) 614 (3.68); EI $[M]^+$ *m/z* 327.1. Anal. ($C_{16}H_{17}N_5O_3$·2HCl·H_2O) Calcd: C, 45.94; H, 5.06; N, 16.74. Found: C, 45.27; H, 4.82; N, 16.21.

General Procedure for the Preparation of 1,4-Bis[*N*-(aminoalkyl)amino]benzo[*g*]phthalazine-5,10-diones by Silylation–Amination of 5,10-Dihydroxy-1,4-dioxo-1,2,3,4-tetrahydrobenzo[*g*]phthalazine (8): 1,4-Bis[*N*-[2-(1-piperidino)ethyl]amino]benzo[*g*]phthalazine-5,10-dione (1f). In a single-necked, round-bottom flask equipped with a short-path distillation apparatus and a magnetic stirring bar were placed 5,10-dihydroxy-1,4-dioxo-1,2,3,4-tetrahydrobenzo[*g*]-phthalazine (**8**; 0.70 g, 2.87 mmol), *p*-toluenesulfonic acid monohydrate (0.11 g, 0.57 mmol), hexamethyldisilazane (5.45 mL, 25.8 mmol), and *N*-(2-aminoethyl)piperidine (4.50 mL, 31.6 mmol). The mixture was gradually heated with stirring at 170 °C (external oil bath), and after the copious evolution of ammonia subsided, a nitrogen atmosphere was applied. The solution was intermittently heated at 170 °C for about 27 h, while two additional portions of *p*-toluenesulfonic acid were added after 8 h (127 mg, 0.66 mmol) and 18 h (125 mg, 0.66 mmol); the progress of the reaction was monitored by TLC analysis or by monitoring the increase in the volume of the distillate. The nitrogen flow was removed, and methanol (7 mL) was added to the dark reaction mixture which was then refluxed for 15 min. After cooling to room temperature, the dark precipitate was collected and washed first with ethanol and finally with diethyl ether to give **1f** as a blue powder (0.56 g, 42% yield): mp 203 °C dec.

The combined mother liquors were poured into water (100 mL) and extracted with ethyl acetate (3 × 80 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and concentrated to dryness. The residue was recrystallized from absolute ethanol (15 mL) to give a second crop of pure **1f** (0.33 g, 25% yield): mp 203–204 °C dec; ^{1}H-NMR ($CDCl_3$) δ 1.40–1.58 (m, 4H), 1.60–1.75 (m, 8H), 2.50 (t, 8H), 2.75 (t, 4H), 3.90 (q, 4H), 7.78 (m, 2H), 8.22 (m, 2H), 8.88 (br t, 2H); ^{13}C-NMR ($CDCl_3$) δ 24.48, 26.02, 38.87, 54.49, 57.64, 126.23, 134.08; UV (EtOH) λ_{max} (nm) (log ϵ) 252 (4.54), 342 (3.67), 709 (3.83). Anal. ($C_{26}H_{34}N_6O_2$) Calcd: C, 67.51; H, 7.41; N, 18.17. Found: C, 67.37; H, 7.40; N, 18.09.

1,4-Bis[*N*-[2-(diethylamino)ethyl]amino]benzo[*g*]-phthalazine-5,10-dione (1d). This compound was obtained according to the above general procedure of silylation–amination from the reaction of **8** (0.60 g, 2.45 mmol), *N,N*-diethylethylenediamine (3.83 mL, 26.9 mmol), hexamethyldisilazane (4.65 mL, 22.05 mmol), and *p*-toluenesulfonic acid monohydrate (0.49 g, 2.62 mmol; added in four portions over the 43 h reaction time). The desired product **1d** (0.57 g, 53% yield) was obtained on workup: mp 173–175 °C dec; ^{1}H-NMR ($CDCl_3$) δ 1.10 (t, 12H), 2.63 (q, 8H), 2.83 (t, 4H), 3.88 (q, 4H), 7.79 (m, 2H), 8.21 (m, 2H), 8.87 (t, 2H, exchangeable with D_2O); ^{13}C-NMR ($CDCl_3$) δ 12.04, 39.63, 47.11, 51.80, 108.0, 126.24, 132.31, 134.05, 150.0, 186.9; UV (EtOH) λ_{max} (nm) (log ϵ) 252 (4.54), 342 (3.68), 709 (3.83). Anal. ($C_{24}H_{34}N_6O_2$) Calcd: C, 65.73; H, 7.81; N, 19.16. Found: C, 65.90; H, 7.85; N, 19.13.

1,4-Bis[*N*-[3-(dimethylamino)propyl]amino]benzo[*g*]-phthalazine-5,10-dione (1e). This compound was obtained according to the above general procedure of silylation–amination by reaction of **8** (0.40 g, 1.63 mmol) with 3-(dimethylamino)propylamine, hexamethyldisilazane, and *p*-toluenesulfonic acid monohydrate for 43 h. The crude compound was purified by silica gel column chromatography (eluant: ethyl acetate/methanol/concentrated ammonium hydroxide, 16/4/0.5) to give 0.273 g (40% yield) of **1e**: mp 122–124 °C dec (from ligroine); ^{1}H-NMR ($CDCl_3$) δ 1.93 (q, 4H), 2.26 (s, 12H), 2.45 (t, 4H), 3.88 (q, 4H), 7.79 (m, 2H), 8.20 (m, 2H), 8.80 (br t, 2H, exchangeable with D_2O); ^{13}C-NMR ($CDCl_3/D_2O$) δ 27.62, 39.60, 45.52, 57.43, 126.18, 134.20; UV (EtOH) λ_{max} (nm) (log ϵ) 252 (4.52), 342 (3.66), 709 (3.81); positive LSIMS $[M + H]^+$ *m/z* 411. Anal. ($C_{22}H_{30}N_6O_2$) Calcd: C, 64.37; H, 7.37; N, 20.47. Found: C, 63.48; H, 7.30; N, 19.71.

1,4-Bis(*N*-benzylamino)benzo[*g*]phthalazine-5,10-dione (1g). This compound was obtained according to the above general procedure of silylation–amination from the reaction of **8** (0.10 g, 0.41 mmol), benzylamine (0.49 mL, 4.5 mmol), hexamethyldisilazane (0.78 mL, 3.69 mmol), and ammonium sulfate (33 mg, 0.25 mmol; added in three portions over the 24 h reaction time). By the usual workup procedure, 0.10 g (61% yield) of **1g** was obtained. **1g**: mp 179–191 °C dec (from CH_2Cl_2/MeOH); ^{1}H-NMR ($CDCl_3$) δ 5.05 (d, 4H), 7.30–7.50 (m, 10H), 7.81 (m, 2H), 8.20 (m, 2H), 8.98 (br t, 2H, exchangeable with D_2O); UV (EtOH) λ_{max} (nm) (log ϵ) 252 (4.58), 342 (3.73, 694 (3.83). Anal. ($C_{26}H_{20}N_4O_2$) Calcd: C, 74.27; H, 4.79; N, 13.32. Found: C, 74.30; H, 4.73; N, 13.35.

Biological Studies. In Vitro Cytotoxic Activity. Human Colon Adenocarcinoma LoVo and LoVo/Dx. LoVo and LoVo/Dx were cultured in Ham's F12 medium (Gibco) supplemented with 10% fetal calf serum, 1% L-glutamine (200 mM), 1% BME vitamins solution (100X), 2% hepes buffer solution (1 M in 0.85% NaCl), and 1% penicillin (5000 UI/mL)–streptomycin (5000 μg/mL) solution. Cells were split twice a week and maintained at 37 °C in an atmosphere of CO_2. The cell lines were periodically tested for Mycoplasma contamination by the DAPI (4',6-diamidine-2-phenylindole dihydrochloride) test (Boehringer Mannheim). For the cytotoxicity evaluation of the compounds, the MTT colorimetric assay[18] was used. Briefly, the MTT assay is based on mitochondrial reduction of a tetrazolium salt by living cells. The viable cell number is proportional to the production of formazan salt which can be read spectrophotometrically at 570 nm. Cell lines (2.5×10^4 cells/mL) were plated in 96-microwell plates (Nunclon Delta, Nunc, Roskilde, Denmark) and preincubated for 24 h. After this time, the tumor cell lines were exposed for 144 h to drugs dissolved in an appropriate solvent. The drug concentration inhibiting 50% of cellular growth (IC_{50}, μg/mL) and the resistance index (RI: IC_{50} of resistant cell line/IC_{50} of sensitive cell line) were calculated.

HT-1080 Fibrosarcoma, HT-29 Colon Adenocarcinoma, MCF-7 Breast Adenocarcinoma, and A-375 Melanoma. Cell cultures were adapted to growth in completed RPMI 1640 medium; 2.5×10^4 cells/mL for each cell line were plated in 96-microwell plates and preincubated for 24 h. After this time, the tumor cell lines were exposed to drugs dissolved in an appropriate solvent for 144 h. After solubilization of the formazan salts by DMSO, the absorbance of a single well was determined by a microplate spectrophotometer and the drug concentration inhibiting 50% of cellular growth (IC_{50}, M) was calculated.

DNA-Binding Studies. Spectrophotometric Experiments. Spectrophotometric experiments were performed using a Perkin Elmer Lambda 5 instrument. All measurements were carried out in TRIS (10 mM) with EDTA (2 mM) and sodium chloride to adjust the ionic strength (IS) to 0.15 M (ETN), at pH 7.0 and 25 °C. Experiments were performed by addition of a known amount of DNA to solutions containing a given concentration of the ligand. As isosbestic points were observed throughout the titrations, the amount of bound and free ligand (C_b and m, respectively) were determined from the absorption readings at a fixed wavelength (usually corresponding to the maximum changes in absorption) according to the equations:

$$C_b = (A_f - A/A_f - A_b)C_0$$

$$m = C_0 - C_b$$

where A_f is the absorption of the free compound, A_b is the absorption of the bound compound, A is the absorption of a mixture of the free and bound compounds, and C_0 is the total concentration of the compound. The data so collected were analyzed using the neighbor exclusion model:[19]

$$r/m = K_i(1-nr)[(1 - nr)/[1 - (n - 1)r]]^{n-1}$$

where r is the number of ligand molecules bound per DNA phosphate, K_i is the intrinsic binding constant, and n is the number of consecutive lattice covered by a ligand molecule. A best-fitting least-squares procedure was used.

Circular Dichroism. Circular dichroism (CD) measurements were performed on a Jasco J 500 A spectrophotometer equipped with an IBM PC and a Jasco J interface. Spectra were recorded in ETN (IS = 0.1), pH 7.0, and $T = 25$ °C. Data are reported as $\Delta\epsilon$.

$$\Delta\epsilon = [(100 \times \Theta)/(c \times d)]/3300 \text{ mol}^{-1} \text{ cm}^{-1}$$

where Θ is the measured ellipticity (deg), d is the cell path length (cm), and c is the molar concentration.

Linear Dichroism. Linear dichroism (LD) measurements were performed on an adapted J 500 A spectrophotometer equipped with an IBM PC and a Jasco J interface. DNA was oriented in a rotating quartz cell (600–800 rpm). Each spectrum was accumulated four times. Experiments were carried out in ETN (IS = 0.1), pH 7.0, and $T = 25$ °C. The reduced dichroism was measured as follows:

$$LD_f = LD/A_{iso}$$

where LD is the linear dichroism define as $A_{\parallel} - A_{-}$ and A_{iso} is the absorbance of the isotropic solution. For DNA:

$$LD_r = S \times {}^3/_2(3\cos^2 \alpha - 1)$$

where S ($0 < S < 1$) is an orientation function and α is the angle between the chromophore transition moment and the helix axis. Assuming a value of $\alpha = 90°$ for the nucleotide bases in DNA, for a ligand bound to it, it follows[20] that:

$$\alpha_L = \arccos[{}^1/_3 - [(LD_r)_L/3(LD_r)_{DNA}]]^{1/2}$$

where $(LD_r)_L$ is the reduced linear dichroism for the ligand, $(LD_r)_{DNA}$ is the reduced LD for DNA, and α defines the ligand–DNA relative orientation. For intercalated systems, $\alpha_L \sim 90°$ and $(LD_r)_L \sim (LD_r)_{DNA}$.

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