Effect of A-Ring Modifications on the DNA-Binding Behavior and Cytotoxicity of Pyrrolo[2,1-*c*][1,4]benzodiazepines†

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Received November 12, 1998

Several A-ring-modified analogues of the DNA-binding antitumor agent DC-81 (**5**) have been synthesized in order to study structure−reactivity/cytotoxicity relationships. For two molecules (**23** and **30**) the modifications required the addition of a fourth ring to give the novel dioxolo-[4,5-*h*]- and dioxano[5,6-*h*]pyrrolo[2,1-*c*][1,4]benzodiazepin-11-one (PBD) ring systems, respectively. Another three analogues (**34**, **38**, and **48**) have the native benzenoid A-ring replaced with pyridine, diazine, or pyrimidine rings to give the novel pyrrolo[2,1-*c*][1,4]pyridodiazepine, pyrrolo[2,1-*c*][1,4]diazinodiazepine, and pyrrolo[2,1-*c*][1,4]pyrimidinodiazepine systems, respectively. The other new analogues (**16a**,**b**) have extended chains at the C8-position of the DC-81 structure. During the synthesis of these compounds, a novel tin-mediated regiospecific cleavage reaction of the dioxole intermediate **18** was discovered, leading to the previously unknown *iso*-DC-81 (**20**). In addition, an unusual simultaneous nitration−oxidation reaction of 4-(3-hydroxypropoxy)-3-methoxybenzoic acid (**8**) was found to produce 3-(4-carboxy-2-methoxy-5-nitrophenoxy)propanoic acid (**9**), a key intermediate, in high yield. In general, the results of cytotoxicity and DNA-binding studies indicated that none of the changes made to the A-ring of the PBD system significantly improved either binding affinity or cytotoxicity in comparison to DC-81. This result suggests that the superior potency of natural products such as anthramycin (**1**), tomaymycin (**2**), and sibiromycin (**3**) is due entirely to differences in C-ring structure, and in particular *exo* or *endo* unsaturation at the C2-position and C2-substituents containing unsaturation. This study also provided information regarding the influence of A-ring substitution pattern on the relative stability of the interconvertible N10−C11 carbinolamine, carbinolamine methyl ether, and imine forms of PBDs.

Introduction

Currently there is interest in discovering and developing small molecules capable of binding to DNA in a highly sequence-selective manner,[1] as the ability to target and then down-regulate individual genes has potential in the therapy of genetic based diseases (e.g., cancer[2]), diagnostics, functional genomics, and target validation. The pyrrolo[2,1-*c*][1,4]benzodiazepine antitumor antibiotics (PBDs, Figure 1) are a well-known class of sequence-selective DNA-binding agents derived from *Streptomyces* species.[3] Their precise mode of interaction with DNA is unique and has been extensively studied; they bind within the minor groove of DNA, forming a covalent aminal bond between the C11-position of the central B-ring and the N2-amino group of a guanine base. A series of footprinting,[4,5] fluorescence,[6] molecular modeling,[7] and NMR[8] studies have shown that the molecules have a preferred selectivity for Pu-**G**-Pu (Pu, purine; **G**, alkylated guanine) sequences and can orientate with their A-rings pointing either toward the 3′- or 5′-end of the covalently bound strand.[3] Furthermore, the stereochemistry at the C11-position of the PBD can be in either the *R* or *S* configuration, thus giving rise to a total of four possible isomers for each DNA adduct.[3] Many members of the PBD family such as anthramycin (**1**) and tomaymycin (**2**) (Figure 1) have significant in vitro cytotoxicity, and some compounds, such as anthramycin and neothramycin (e.g., **4**), have reached various stages of clinical trials but have not progressed due to problems including cardiotoxicity or lack of efficacy, respectively.[9] However, recently there have been a number of attempts to enhance sequence selectivity and antitumor potency by synthesizing C7-[10] or C8-linked[11] PBD dimers, such as DSB-120 (**6**) which is based on the C-ring-unsubstituted PBD subunit DC-81 (**5**). DSB-120 is one of the most

† Abbreviations: EDCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; HOBT, 1-hydroxybenzotriazole; PBD, pyrrolo-[2,1-*c*][1,4]benzodiazepine.

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10.1021/jm981117p CCC: $18.00
Published on Web 05/18/1999

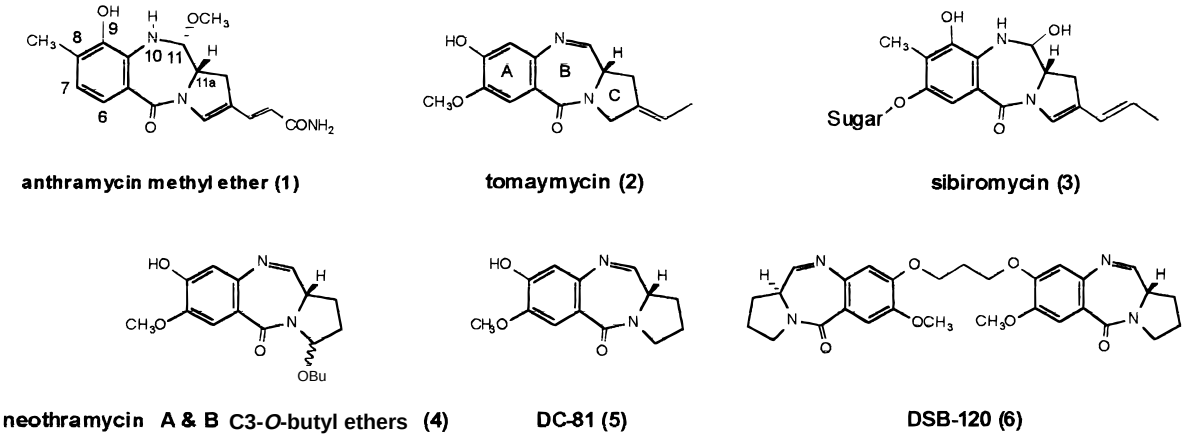


Figure 1. Structures of anthramycin (**1**), tomaymycin (**2**), sibiromycin (**3**), neothramycins A and B (**4**), DC-81 (**5**), and DSB-120 (**6**).

potent irreversible interstrand cross-linking agents known to date, capable of covalently linking two complementary DNA stands through interaction with guanine bases in the minor groove.[11,12] [**Note added in proof:** SJG-136, an analogue of DSB-120, has recently been reported to be significantly more cytotoxic than DSB-120 across a number of cell lines and to be >10-fold more potent at stabilizing DNA on a concentration basis according to thermal denaturation experiments (Gregson, S. J.; Howard, P. W.; Jenkins, T. C.; Kelland, L. R.; Thurston, D. E. Synthesis of a Novel C2/C2′-*exo* Unsaturated Pyrrolobenzodiazepine Cross-linking Agent with Remarkable DNA Binding Affinity and Cytotoxicity. *J. Chem. Soc., Chem. Commun.* **1999**, *9*, 797−798).] It is also significantly more cytotoxic than the corresponding monomer DC-81. It has been demonstrated that the pyrrolobenzodiazepines can interfere with endonuclease activity[13] and can inhibit in vitro transcription in a highly sequence-selective manner.[14]

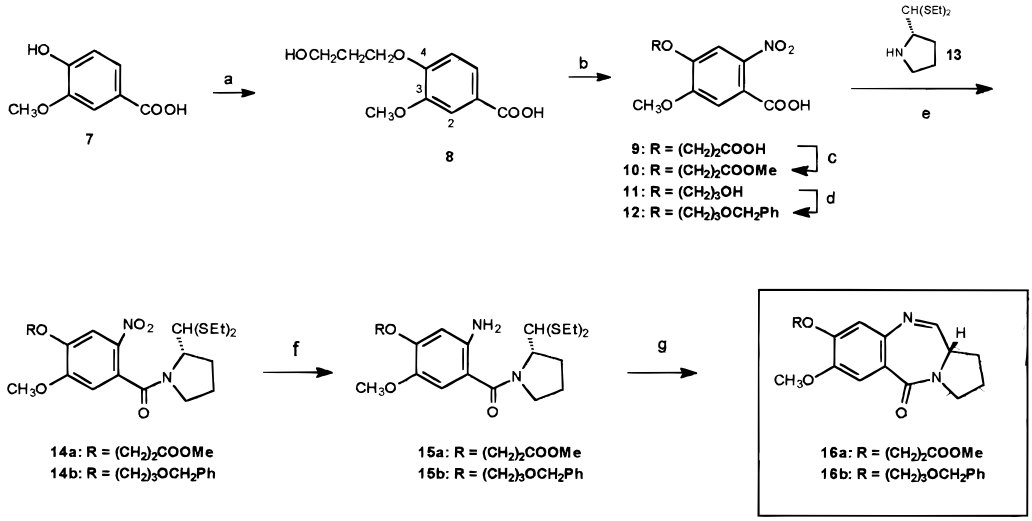
There has been a considerable effort by a number of groups to develop new synthetic routes to the PBD ring system, and these have been reviewed.[15] However, due mainly to the difficulties associated with synthesizing useful quantities of PBD analogues, little work regarding structure−activity relationships (SAR) has been published. In 1983, Hurley and Thurston tried to rationalize differences in biological activity of the known natural products using a CPK model of the DNA−PBD adduct.[16] Later, the same workers reported structure−binding and structure−cytotoxicity data for a limited series of A- and C-ring-modified synthetic analogues.[5] Doyle and co-workers have published the results of a study of bicyclic analogues of PBDs comprising only the A- and B-rings, demonstrating the importance of the presence of the C-ring for significant cytotoxicity.[17] More recently, Baraldi and co-workers have reported conversion of the aromatic A-ring of the PBD system into a pyrazole.[18] We report here the results of an investigation into the effect of modifying the A-ring complexion and substitution pattern on both DNA-binding reactivity and cytotoxic potency. Anthramycin (**1**), tomaymycin (**2**), sibiromycin (**3**), the C3-*O*-butyl neothramycins (**4**), DC-81 (**5**), the C8-benzyl (**52**) and C8-benzoyloxycarbonylamino (**53**)[19] DC-81 analogues, the A/C-ring-unsubstituted saturated analogue **50**,[19] and the pyrazine analogues[18] **49** and **51** were included in the present study for comparison.

Synthesis

C8-Substituted PBDs (16a,b) (Scheme 1). The C8-substituted DC-81 analogues **16a**,**b** (Scheme 1) were synthesized starting from vanillic acid (**7**) using the thioacetal route of Langley and Thurston.[20] Treatment of **7** with 3-bromopropanol in refluxing aqueous NaOH for 5 h afforded the alcohol **8** in >80% yield.[21] Anhydrous conditions with KOH in absolute EtOH were found to be less efficient, giving lower yields and requiring longer reaction times. Reaction of **8** with 70% aqueous HNO_3 at 0 °C[22] effected concurrent nitration at the 6-position and oxidation of the aliphatic alcohol to give the dicarboxylic acid **9** as a single product in good yield (>70%). After selective methylation (>80%) of the aliphatic carboxyl group (MeOH/*p*-TsOH) to give **10**, coupling to (2*S*)-pyrrolidine-2-carboxaldehyde diethyl thioacetal (**13**), prepared in six steps from L-proline,[20] afforded the nitro thioacetal **14a**. Subsequent reduction to **15a** with $SnCl_2 \cdot 2H_2O$ in MeOH and cyclization using $HgCl_2/CaCO_3$ afforded the PBD **16a** in the N10−C11 imine form as a stable solid after several evaporations from dry $CHCl_3$. The benzyl ether analogue **16b** was similarly prepared from the known 4-(3-hydroxypropoxy)-2-nitrovanillic acid fragment **11**. After *O*-benzylation of **11** using NaH and $PhCH_2Br$ in THF to give **12**, coupling to **13** gave the resulting amide **14b**, which was reduced (**15b**) and cyclized (**16b**) under identical conditions to that described above.

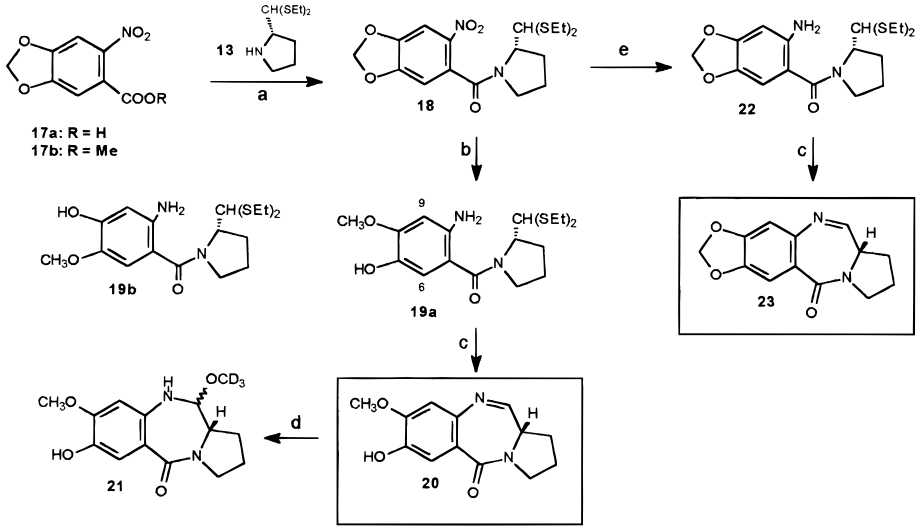
***iso*-DC-81 (20) and Dioxolo[4,5-*h*]pyrrolo[2,1-*c*]-[1,4]benzodiazepin-11-one PBD (23) (Scheme 2).** The key precursor **19a** used in the synthesis of *iso*-DC-81 (**20**) was obtained unexpectedly[23] during the synthesis of the novel dioxolo[4,5-*h*]pyrrolo[2,1-*c*][1,4]benzodiazepin-11-one PBD (**23**). The previously reported 2-nitropiperonylic acid (**17a**)[24] was coupled to (2*S*)-pyrrolidine-2-carboxaldehyde diethyl thioacetal (**13**) to afford the nitro thioacetal **18**. On the basis of past experience,[11,15,19,25,26,27] **18** was treated with $SnCl_2 \cdot 2H_2O$ in refluxing MeOH to reduce the aromatic nitro group. Surprisingly, in addition to the anticipated reduction of the nitro group, the dioxole ring opened in a completely regiospecific manner to afford **19a**.[23] The methylenedioxy proton signal at δ 6.2 in the ^{1}H NMR spectrum of **18** was replaced with a methoxy signal at δ 3.9 in the spectrum of **19a**. Comparison of the NMR data with those of the DC-81 precursor **19b**[27] indicated

Scheme 1[a]



[a] Reagents: (a) $HO(CH_2)_3Br$/aq NaOH/Δ/5 h or $HO(CH_2)_3Br$/KOH/EtOH/Δ/48 h; (b) 70% aq HNO_3/0 °C → rt/1 h; (c) MeOH/*p*-TsOH/rt; (d) NaH/$PhCH_2Br$/THF/0 °C → rt, then 10% aq NaOH (2 M)/rt/5 h; (e) $(COCl)_2$/DMF/THF/H_2O/Et_3N/**13**; (f) $SnCl_2 \cdot 2H_2O$/MeOH/Δ; (g) $HgCl_2/CaCO_3$/MeCN−H_2O (4:1).

Scheme 2[a]



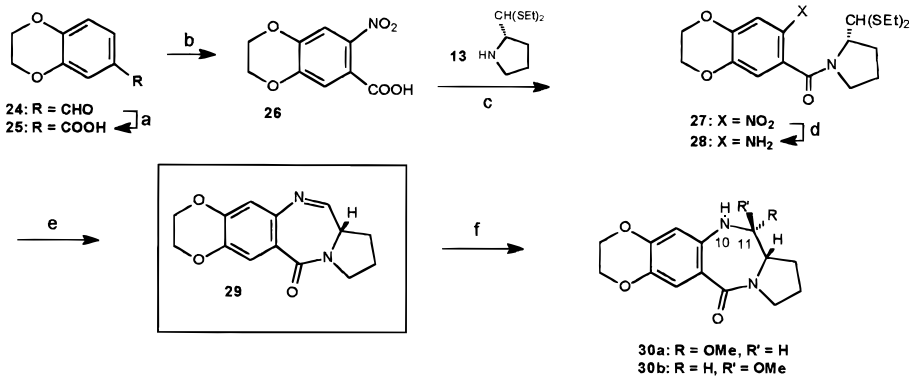
[a] Reagents: (a) $(COCl)_2$ then DMF/THF/H_2O/Et_3N/**13**; (b) $SnCl_2 \cdot 2H_2O$/MeOH/Δ/45 min; (c) $HgCl_2/CaCO_3$/MeCN−H_2O (4:1)/rt/2 h; (d) CD_3OD/rt/4.5 min; (e) MeOH/10% Pd−C/H_2/1 atm/rt/2 h.

that the two molecules were similar but not identical and suggested a difference in their A-ring substitution pattern. The H-6 and H-9 protons were still singlets (δ 6.22 and 6.85) but differed in chemical shift from the equivalent signals in the spectrum of **19b** (δ 6.27 and 6.79), suggesting a structure of type **19a**. As expected, catalytic hydrogenation (10% w/w Pd−C/CH_3OH/1 atm of H_2) of **18** selectively reduced the aromatic nitro group to give the dioxole amino thioacetal **22** in quantitative yield without cleavage of the dioxole ring. Cyclization of **19a** using $HgCl_2/CaCO_3$ afforded a product with the same molecular weight as DC-81 (**5**) (EI: M^+ 246), but ^1H NMR indicated that the aromatic protons, although still in a *para* relationship, differed in chemical shift (δ 6.81 and 7.73) from those of DC-81 (δ 6.90 and 7.51).[27] A further significant feature was the downfield shift (to δ 7.73) of one aromatic proton, most likely H-6, to a lower value than that of the H-11 signal (δ 7.67), which is usually the lowest-field nonexchangeable proton signal for a PBD.

On the basis of these factors, the structure could be unambiguously assigned as the imine form of *iso*-DC-81 (**20**). The possible role of ring strain in the $SnCl_2$-mediated cleavage of **18** was suggested by failure of the 1,4-benzodioxane analogue **27** (Scheme 3) to undergo a similar cleavage process; only reduction of the nitro group occurred under identical conditions to give the amino thioacetal **28**. Interestingly, the analogous 1,3-benzodioxole ester **17b**, prepared by esterification of **17a**, afforded the unopened amine in high yield upon treatment with $SnCl_2 \cdot 2H_2O$ in refluxing MeOH, suggesting that ring strain may not be the only factor to influence the cleavage process. Finally, cyclization of **22** afforded the novel dioxolo[4,5-*h*]pyrrolo[2,1-*c*][1,4]benzodiazepin-11-one (**23**), the first known example of a tetracyclic PBD analogue.

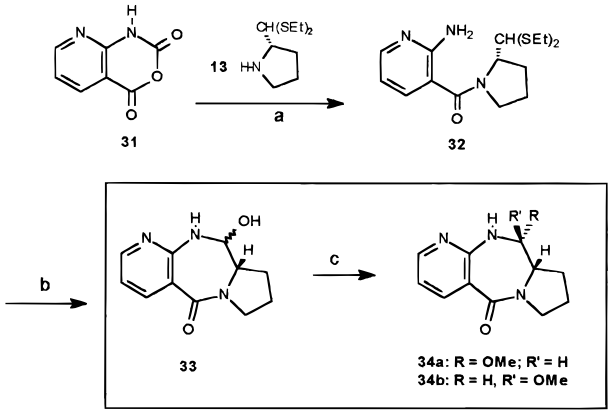
Dioxano[2,3-*h*]pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (29) (Scheme 3). Synthesis of the imine **29** started from 3,4-(ethylenedioxy)benzaldehyde (**24**) which was oxidized to the corresponding acid **25** with aqueous

Scheme 3[a]



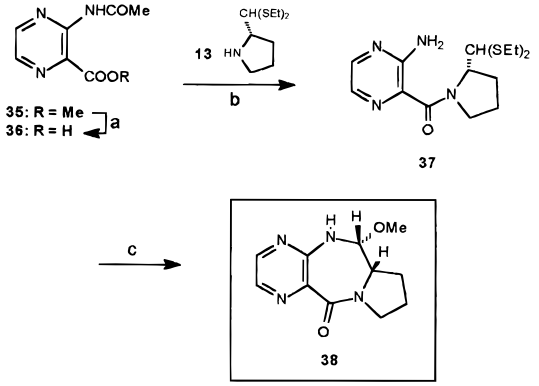
[a] Reagents: (a) aq $KMnO_4$/70−80 °C/40 min; (b) nitration;[24] (c) $(COCl)_2$/DMF/THF/H_2O/NEt_3/**13**; (d) $SnCl_2 \cdot 2H_2O$/MeOH/Δ; (e) $HgCl_2$/$CaCO_3$/MeCN−H_2O (4:1)/rt; (f) MeOH/rt/24 h.

Scheme 4[a]



[a] Reagents: (a) DMF/100 °C/6 h; (b) $HgCl_2$/$CaCO_3$/MeCN−H_2O (4:1)/rt; (c) MeOH/rt, then dry Et_2O.

Scheme 5[a]



[a] Reagents: (a) LiOH/THF−MeOH−H_2O (4:1:1)/20 min/rt; (b) HOBT/EDCI/**13**/0 °C → rt/6 h; (c) $HgCl_2$/$CaCO_3$/MeCN−H_2O (4:1)/rt, then Florisil/Et_3N/EtOAc−CH_3OH (1:1).

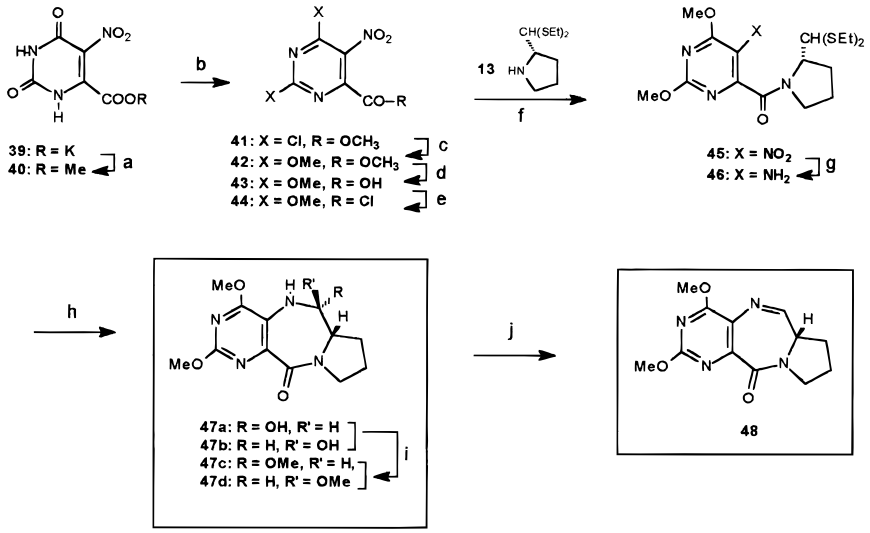
$KMnO_4$ and then nitrated to give **26**. This intermediate was coupled to **13** to afford the amide **27**, which was reduced to the amino thioacetal **28** with $SnCl_2 \cdot 2H_2O$ in MeOH. Cyclization with $HgCl_2$/$CaCO_3$ afforded the imine **29** in 70% yield. When this product was dissolved in MeOH and allowed to stand overnight at room temperature, conversion to a mixture of the methyl ethers **30a**,**b** was quantitative, as visualized by TLC and characterized by NMR. Surprisingly, treatment of this yellow oily mixture with dry Et_2O induced crystallization of the single diastereomeric (11*R*,11a*S*)-carbinolamine methyl ether **30a**. Characterization of this diastereomer was based on the chemical shift and coupling of its H-11 proton, which appeared as a doublet (δ 4.58, J = 6.2 Hz) due to coupling with the N10 proton. No coupling was observed between the H-11 and H-11a protons due to the ∼90° angle between them, and D_2O exchange caused the doublet at δ 4.58 to collapse to a singlet.

(11*R*,11a*S*)-11-Methoxy-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]pyrido[3,2-*f*]diazepin-5-one (34) (Scheme 4). The synthetic strategy developed for **34** utilized the known 3,4-dihydro-1,3-dioxo-1*H*-pyrido-[2,3-*d*][1,3]oxazine[28,29] (**31**) as starting material. Reaction with **13** in DMF at 100 °C for 6 h gave the amide **32** in 60% yield. Treatment with $HgCl_2$ and $CaCO_3$ in CH_3CN/H_2O at room temperature effected concomitant deprotection and cyclization to afford a diastereomeric mixture of the corresponding carbinolamines **33** in 70% yield. When this mixture was dissolved in MeOH and allowed to stand overnight at room temperature, conversion to a mixture of the C11-*O*-methyl ethers **34a**,**b** was quantitative. Treatment of this oily mixture with dry Et_2O induced crystallization of a single diastereomeric carbinolamine methyl ether, unambiguously assigned as the 11*R*,11a*S* diastereomer **34a**. As with compound **30a**, this assignment was made on the basis of a doublet for H-11 (δ 4.58, J = 6.2 Hz), which collapsed to a singlet upon exchange with D_2O.

(11*R*,11a*S*)-11-Methoxy-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]pyrazino[2,3-*f*]diazepin-5-one (38) (Scheme 5). The pyrazine analogue **38** was synthesized from the known methyl 3-(acetylamino)-2-pyrazinecarboxylate (**35**)[30,31] which was initially hydrolyzed to the corresponding acid **36**. Treatment with EDCI/HOBT followed by **13** effected simultaneous coupling and *N*-deacetylation to afford the amino thioacetal **37**. After cyclization with $HgCl_2$/$CaCO_3$, purification of the reaction mixture by flash chromatography using TEA-treated Florisil and EtOAc/MeOH as solvent afforded a single carbinolamine methyl ether unambiguously characterized as the 11*R*,11a*S* diastereomer **38**. As with compounds **30a** and **34a**, this structural assignment was made on the basis of a doublet for H-11 (δ 4.57, J = 6 Hz) which collapsed to a singlet upon exchange with D_2O.

(11a*S*)-7,9-Dimethoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]pyrimido[4,5-*f*]diazepin-5-one (48) (Scheme 6). Synthesis of the pyrimidine analogue **48** started from the readily available 5-nitroorotic acid salt

Scheme 6[a]



[a] Reagents: (a) MeOH/H^+; (b) $POCl_3/\Delta$; (c) CH_3ONa; (d) aq NaOH/rt; (e) $(COCl)_2$/rt; (f) **13**/Et_3N/rt; $SnCl_2 \cdot 2H_2O$/MeOH/2 h/Δ; (h) $HgCl_2/CaCO_3$/MeCN−H_2O (4:1)/rt; (i) MeOH/rt/24 h; (j) 0.01 Torr/6 h/rt.

(**39**) which was converted to the corresponding pyrimidinic acid derivative **43**[32] in 50% overall yield through a four-step sequence involving esterification (**40**), chlorination with phosphoryl chloride (**41**), exchange with NaOMe (**42**), and finally hydrolysis at room temperature. After conversion to the acid chloride (**44**), treatment with **13** in THF in the presence of Et_3N afforded the nitro amide **45** which was reduced smoothly by refluxing with $SnCl_2 \cdot 2H_2O$ in MeOH for 2 h to give the amino thioacetal **46** in nearly quantitative yield. Simultaneous deprotection and cyclization of the thioacetal with $HgCl_2/CaCO_3$ gave a mixture of carbinolamines **47a**,**b** which was converted into a diastereomeric mixture of the corresponding carbinolamine methyl ethers **47c**,**d** by allowing to stand in MeOH at room temperature for 24 h. Interestingly, this diastereomeric mixture could be converted to the single imine species **48** by vacuum treatment (0.01 Torr) for 6 h at room temperature.

Results and Discussion

Thermal Denaturation Studies. The PBD compounds were assessed as DNA-modifying agents by determining the thermal helix → coil or melting stabilization (ΔT_m) afforded to double-stranded calf thymus DNA (CT-DNA) following incubation at 37 °C. This 'pseudorandom' DNA duplex was selected to eliminate possible sequence-dependent binding effects. All of the compounds examined effected thermal stabilization of the CT-DNA duplex (Table 1) with a greater effect upon the G/C-rich or high-temperature (i.e., $T > T_m$) portion of the DNA melting curve compared to the A/T-rich or low-temperature (i.e., $T < T_m$) region. Negligible effect (<0.3 °C) was separately determined for binding to poly-[d(A-T)]$_2$, even following prolonged DNA−ligand contact times (data not shown). Such differential behavior is consistent with exclusive binding to guanine bases, as previously reported for this class of DNA-reactive compounds (e.g., refs 3, 11, 13, 33).

Table 1 shows that PBDs with a saturated pyrrolidine C-ring (i.e., **4**, **5**, **16a**,**b**, **20**, **23**, **29**, **34a**,**b**, **38**, and **48**−**53**) provide the smallest ΔT_m shifts, with significant

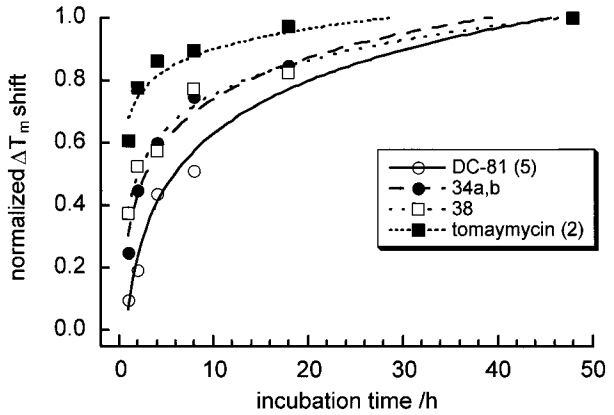
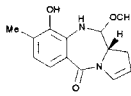
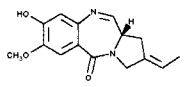
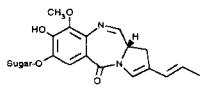
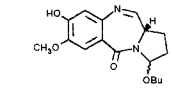
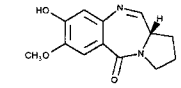
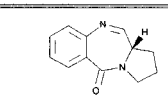
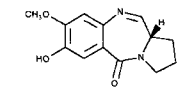
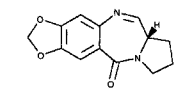
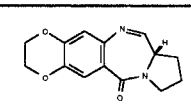
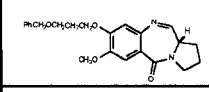
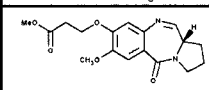
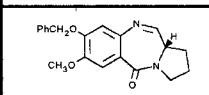
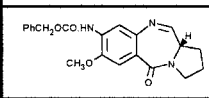
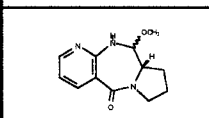
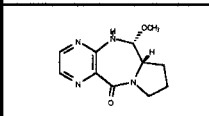
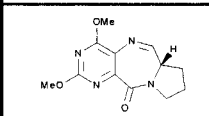
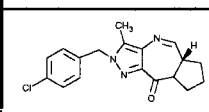
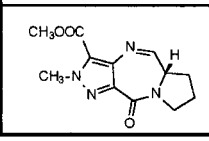


Figure 2. Time course for the secondary, time-dependent ligand-induced thermal stabilization of CT-DNA following incubation at 37 °C with tomaymycin (**2**), DC-81 (**5**), **34a**,**b**, and **38** at a fixed 5:1 [DNAp]:[ligand] molar ratio (see Experimental Section). Normalized ΔT_m shifts represent $[T_m(t) - T_m(0)]/[T_m(\infty) - T_m(0)]$ for each compound at time t, where the maximal value was taken after 48 h. The final induced T_m shifts were 0.72 ± 0.12, 0.53 ± 0.10, 0.74 ± 0.09, and 2.60 ± 0.13 °C for **5**, **34a**,**b**, **38**, and **2**, respectively. $T_m(0)$ values determined for each PBD without incubation were taken from Table 1.

modifications of the aromatic A-ring having only a modest influence on the overall induced stabilization. In contrast, a C2-exocyclic (i.e., **2**) or C2/C3-endocyclic (e.g., **1** or **3**) double bond in the C-ring results in a marked increase in binding affinity, with a clear rank order of **3** > **1** > **2**. A 3-*O*-butyl substituent in the C-ring (i.e., **4**) also exerts a very weak activating effect.

The ΔT_m shifts determined after incubation for 0, 4, and 18 h indicate that covalent adduct formation is time-dependent and generally slow, except for compounds **1**−**3** where 30−95% of the maximal effect is achieved without any preincubation. Incubation at 20 °C instead of 37 °C resulted in significantly reduced reactivities, with a typical ~4−5-fold increase in ligand−DNA contact time required to achieve comparable helix stabilization (data not shown). Figure 2 shows the time course for the slower, secondary component of binding to CT-DNA (100 μM) for a fixed concentration (20 μM)

Table 1

No.	Structure	Cytotoxicity (IC_{50} μM)[a] L1210	ADJ/PC6	CH1	ΔT_m (°C)[b] after incubation for: 0 h	4 h	18 h
1		0.022	0.0028	0.32	9.4	11.2	13.0
2		0.0037	0.0018	0.00013	1.0	2.4	2.6
3		0.0029	0.000017	0.04	15.7	15.9	16.3
4		2.7	1.3	0.43	0.5	0.6	0.8
5[c]		0.38	0.33	0.10	0.3	0.50	0.70
50[c]		>100	38	42	0.3	0.4	0.6
20		2.70	2.70	0.20	0.3	0.5	0.6
23		1.5	1.9	0.26	0.1	0.2	0.4
29		2.70	3.40	0.46	0.1	0.3	0.5
16b		2.40	2.30	0.29	0.2	0.4	0.6
16a		-	-	4.4	0.3	0.5	0.6
52[c]		3.0	0.37	1.0	0.2	0.4	0.4
53[c]		4.5	3.2	1.8	0.1	0.2	0.4
34a/b		13	> 50	43	0.3	0.5	0.5
38		-	-	17	0.3	0.6	0.7
48		42	-	-	0.1	0.2	0.3
49[c]		0.5	13.5	11.5	0.2	0.3	0.4
51[c]		35	> 50	> 100	0.2	0.3	0.5

[a] Dose of PBD required to inhibit cell growth by 50% compared to PBD-free controls. Compounds were dissolved in DMSO and then diluted to provide a final concentration of 0.05% v/v DMSO. Incubation times (37 °C) were L1210, 3 days; PC6, 4 days. [b] Thermal denaturation studies with CT-DNA (ΔT_m for a 5:1 molar ratio of DNAp:PBD, following incubation at 37 °C for the times shown). T_m of the DNA alone = 67.83 ± 0.06 °C; values shown are ±(0.06−0.16) °C. [c] See Experimental Section for source of compounds **1**−**4** and also compounds **5** and **49**−**53** which were synthesized according to literature procedures.

of tomaymycin (**2**), DC-81 (**5**), **34a**,**b**, and **38** during 0−48 h incubation at 37 °C. For each compound, the induced DNA stabilization follows a first-order kinetic dependence, with an overall rank order for reactivity of **2** > **34a**,**b** ∼ **38** > **5**. The increased kinetic reactivity of the pyridine (**34a**,**b**) and pyrazine (**38**) derivatives compared to the simpler benzenoid PBD structure (e.g., **5**, **23**, **29**, or **50**) suggests that polar effects and/or protonation factors may contribute to the DNA-binding mechanism. Similarly, the introduction of a bulky alkyl group onto the concave imine face of the molecule (e.g., **51**) appears to weaken interaction with the DNA host, suggesting that steric and/or orientation effects may contribute to the alkylation process. Penetration of the DNA minor groove is essential for N2-alkylation of a guanine base and will be favored by ligands that adopt a complementary isohelical shape but impaired by deleterious steric effects that cannot be accommodated by structural rearrangement of either the ligand or host DNA molecule. In summary, it is notable that the naturally occurring C-ring-modified PBDs examined (i.e., **1**−**3**) appear to provide both greater differential thermal stabilization of the DNA duplex and significantly enhanced kinetic reactivity during covalent adduct formation.

Electrophilicity. The electrophilic properties of *iso*-DC-81 (**20**) were directly compared to DC-81 (**5**) by dissolving a sample of each in CD_3OD and monitoring changes by ^1H NMR over a 22-h period. Both isomers initially reacted at the same rate, with the N10−C11 imine signals completely disappearing after 4.5 min in each case, and both compounds showing a similar initial predominance of the (C11*S*)-methyl ether form (e.g., **21**: C11 *R*:*S* ratio = approximately 1:1.6). However, at equilibrium (22 h), although there was still a predominance of the C11*S* isomer in the case of *iso*-DC-81 (*R*:*S* ratio = 1:1.2), there was an excess of the C11*R* isomer (*R*:*S* ratio = 1.3:1) with DC-81.[23,27] The preference for C11*S* stereochemistry in **21** at equilibrium is unusual for PBD compounds which, with few exceptions, prefer the C11*R* configuration for C11-substituents. A steric explanation for the difference in behavior between *iso*-DC-81 (**20**) and DC-81 (**5**) upon reaction with CD_3OD is unlikely, but electronic factors in *iso*-DC-81 associated

with π-conjugation between the C7-hydroxyl group situated in the *para* position to the electrophilic N10−C11 imine functionality may play a role.

Cytotoxicity. As with previous studies on pyrrolobenzodiazepines, a broad correlation between cytotoxicity and DNA-binding affinity was observed.[3,5,11,13,14,18] The C-ring-unsaturated compounds **1**−**3** had the highest DNA-binding affinity as judged by thermal denaturation studies and were also the most cytotoxic across the three cell lines examined (i.e., range of IC_{50} values = 0.000017−0.32 μM). Sibiromycin (**3**) was the most cytotoxic compound examined in the L1210 and ADJ/PC6 cell lines and also gave the largest ΔT_m value of 16.3 °C after incubation for 18 h (see Table 1). Although tomaymycin (**2**) was more cytotoxic than anthramycin in the cell lines examined, the order of DNA-binding affinity was reversed with anthramycin showing a higher binding affinity as judged by the thermal denaturation data.

Interesting SAR data were obtained with respect to substitution of the pyrrolobenzodiazepine A-ring. The fact that some substitution of the A-ring is required for significant cytotoxicity is clear from comparing the unsubstituted PBD **50** (IC_{50} = 38 to >100 μM) with DC-81 (**5**) (IC_{50} = 0.10−0.38 μM). However, alkyl loading of the C8-hydroxyl group of DC-81, as with CH_3-$OOCCH_2CH_2$− (**16a**) or $PhCH_2OCH_2CH_2CH_2$− (**16b**), reduces the cytotoxicity (IC_{50} = 0.29−4.4 μM). Similarly, a C8-*O*-benzyl functionality reduces the cytotoxicity (**52**: IC_{50} = 0.37−3.0 μM) compared to DC-81, as does a C8-carbamate grouping (**53**: IC_{50} = 1.8−4.5 μM). Intriguingly, *iso*-DC-81 (**20**), with the C7- and C8-substituents reversed in comparison to DC-81, has similar DNA-binding affinity to DC-81 but is 7−8-fold less cytotoxic in the L1210 and ADJ/PC6 cell lines. Similarly, neothramycin C3-*O*-butyl ether (**4**) is interesting in that the C3-substituent enhances DNA-binding affinity compared to DC-81 but cytotoxicity is reduced in all cell lines examined. Inconsistencies of this type may reflect differences in membrane transport or metabolism.

Finally, adding the dioxazole (**23**) or dioxazine (**29**) rings to the existing A-ring of DC-81 significantly reduces DNA binding affinity (ΔT_m = 0.1−0.5 °C) and cytotoxicity (IC_{50} = 0.26−3.4 μM) compared to DC-81 (0.3−0.7 °C, 0.1−0.38 μM). Changing the benzenoid A-ring to a pyridine (**34a**,**b**), pyrazine (**38**), pyrimidine (**48**), or pyrazole (**49** and **51**) also appears to reduce cytotoxicity (with the exception of **49** in L1210 leukemia) based on the limited data available. However, the pyridine (**34a**,**b**) and pyrazine (**38**) analogues had an increased kinetic reactivity toward DNA compared to DC-81 (see Figure 2 and discussion above).

Effect of A-Ring Substitution Pattern on the Stability of the N10−C11 Functionality. The N10−C11 position of the PBD structure can exist in either the imine (e.g., DC-81, **5**), carbinolamine (e.g., **47a**,**b**, Scheme 6), or carbinolamine methyl ether (e.g., **47c**,**d**, Scheme 6) form depending upon the precise structure of the molecule, the method of synthesis, and/or the chemical environment in which it is formed or stored.[3] PBDs are thought to interconvert between these different forms via a highly electrophilic N10−C11 iminium species.[3] In particular, the A-ring substitution pattern can significantly influence the relative stability of individual N10−C11 forms. For example, it is known that substituents donating electrons by resonance such as C7-OCH_3 and C8-OH groups can promote stability of the N10−C11 imine form, whereas mesomeric electron-withdrawing groups such as a C7-nitro can stabilize the carbinolamine form.[34] Thus, for the novel compounds reported here, those with C7/C8-oxygen substituents, whether free (i.e., **16a**,**b** and **20**) or part of a ring structure (i.e., **23** and **29**), were all formed and isolated as N10−C11 imine species. Compounds **52**, **53**, DC-81 (**5**), the neothramycins (**4**), and tomaymycin (**2**) with similarly substituted A-rings also seem to prefer to exist in the imine form, as does the A-ring unsubstituted PBD (**50**) and the pyrazole analogues **49** and **51**. In the latter case, this might be explained by the relatively electron-rich nature of the five-membered nitrogen-containing A-ring heterocycles.

Interestingly, the three compounds with six-membered heterocyclic A-rings (**34a/b**, **38**, and **48**) were all initially formed as either carbinolamines (**33** and **47a**,**b**) or carbinolamine methyl ethers (**38**). This may be due to the relatively electron-deficient nature of six-membered rings with aza substitution. Carbinolamine mixtures **33** and **47a**,**b** could be readily converted to the methyl ether forms **34a**,**b** and **47c**,**d** by allowing them to stand overnight in MeOH. Anthramycin (**1**) also prefers to exist in the methyl ether form possibly due to the C9-hydroxyl substituent which may withdraw charge inductively.

Of further interest was crystallization of the single (C11*R*)-methyl ether forms of **29**, **33**, and **38** by treating a solution of the mixtures of methyl ethers (i.e., **30a/b**, **34a/b**, and **38**, respectively) with Et_2O. This behavior is similar to that of anthramycin, which crystallizes from methanol in the pure (C11*R*)-methyl ether form.

Finally, the imine **48** was unusual in being formed from a mixture of the carbinolamine methyl ethers **47c**,**d** by standing in a vacuum for 6 h at room temperature. The procedure normally used to convert N10−C11 carbinolamines or carbinolamine methyl ethers into the corresponding imine form involves several cycles of evaporation from $CHCl_3$.[3,15]

Conclusions

In summary, the studies reported here, which include the synthesis of five novel ring systems (**23**, **29**, **34a**,**b**, **38**, and **48**), demonstrate the importance of the structure of the C-ring of the PBD system for both DNA-binding affinity and cytotoxicity. Despite the number of compounds studied with a diversity of A-ring modifications, not one with a saturated and unsubstituted C-ring had cytotoxicity (IC_{50} = 0.10 to >100 μM) or ΔT_m values (0.1−0.8 °C over 18 h) approaching the level of the three naturally occurring C-ring-unsaturated/substituted compounds (**1**−**3**) (IC_{50} = 0.000017−0.32 μM, ΔT_m = 1.0−16.3 °C over 18 h). Preliminary molecular modeling studies suggest that C2-*endo* or C2-*exo* unsaturation causes a flattening of the C-ring, thus leading to a better fit in the minor groove, and this will be reported in detail elsewhere.

Although A-ring electron-donating substituents do not appear to be a prerequisite for DNA binding (e.g., **50**), they can influence binding affinity and cytotoxicity to

some extent. For example, DC-81 has a significantly higher cytotoxicity (0.1−0.38 μM) and slightly improved DNA binding (ΔT_m = 0.3−0.7 °C) compared to *iso*-DC-81 (corresponding values of 0.2−2.7 μM and 0.3−0.6 °C, respectively), even though the molecules are isomeric with only the C7- and C8-substituents transposed. Similarly, DC-81 (**5**) has a significantly enhanced DNA-binding affinity and cytotoxicity compared to the unsubstituted analogue **50** (ΔT_m = 0.3−0.6 °C, IC_{50} = 42−100 μM). Extending the length of the substituent at the C8-position of DC-81 (e.g., **16a**,**b**) reduces both DNA-binding and cytotoxicity by a small but significant degree, whereas incorporating the C7- and C8-oxygen atoms into a fused five- or six-membered ring (i.e., **23** and **29**) reduces the DNA-binding affinity while causing a more substantial reduction in cytotoxicity (IC_{50} = 0.26−3.4 μM) compared to DC-81 (IC_{50} = 0.1−0.38 μM). The loss of correlation between in vitro DNA-binding affinity and cytotoxicity for some of the compounds compared to DC-81 (**5**) (e.g., **4** and **20**) may reflect differences in membrane transport or metabolism in the tumor cells.

Preliminary modeling studies of both the dioxazole (**23**) and dioxazine (**29**) analogues suggest that the extra rigidity introduced by the new rings may give rise to some steric interactions with functional groups disposed on opposite walls of the host minor groove. The C7-methoxy/C8-hydroxy pattern of substituents in DC-81 is more flexible and seems to allow the molecule to pivot so as to achieve an optimal groove fit. Finally, neither aza substitution of the A-ring, as in the pyridine (**34a**,**b**), diazine (**38**), or pyrimidine (**48**) analogues, nor conversion of the A-ring to a five-membered pyrazine, as in **49** or **51**, improve either the cytotoxicity or the DNA-binding affinity.

Of further interest is the effect of the A-ring substitution pattern on the relative stability of the N10−C11 form of the PBD structure, with the six-membered heterocyclic A-ring compounds (**33**, **38**, and **47**) appearing to behave as A-ring electron-deficient PBDs and the five-membered pyrazole analogues (**49** and **51**) and the novel dioxolo[4,5-*h*] (**23**) and dioxano[5,6-*h*] (**29**) ring systems as A-ring electron-rich PBDs. However, from a biological standpoint, the relevance of the interconversion of the N10−C11 forms is uncertain as once dissolved in an aqueous medium all PBDs are likely to exist predominantly in their carbinolamine forms.[3]

In summary, these SAR data indicate that improvements to the DNA binding and/or cytotoxicity of pyrrolobenzodiazepine monomers might be better achieved by modification of the C-ring.

Experimental Section

General. Unless otherwise stated, all solvents and reagents were obtained from Aldrich Chemical Co. Ltd. and used as supplied. Anhydrous solvents were prepared by distillation under a dry N_2 atmosphere in the presence of the appropriate drying agent and stored over 4-Å molecular sieves or sodium wire. Petroleum ether refers to the fraction boiling at 60−80 °C. Thin-layer chromatography (TLC) was performed using Bakerflex (Silica Gel IB2-F) or GF254 silica gel plates with fluorescent indicator. Column chromatography was performed using Merck Kieselgel 60 (60−200 mesh) or 200−400 mesh for flash chromatography with the indicated eluting solvents. Melting points (mp) were determined on either a Gallenkamp P1384 digital apparatus or a Buchi capillary melting point apparatus and are uncorrected. Infrared (IR) spectra were recorded in Nujol or as indicated using a Perkin-Elmer 298 instrument. ^{1}H and ^{13}C NMR were recorded for solutions in $CDCl_3$ or Me_2SO-d_6 using either a Bruker AC200 (200 MHz) or a JEOL GSX (270 MHz) FT-NMR spectrometer operating at 293 ± 1 K; ^{13}C NMR peak assignments were confirmed by DEPT experiments. Chemical shifts (δ, ppm) are reported downfield from internal Me_4Si. Spin multiplicities are described as: s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), or m (multiplet). Mass spectra (MS) were recorded using a JEOL JMS-DX 303 GC mass spectrometer (EI mode, 70 eV, 390−420 K source; CI mode, isobutane); accurate molecular masses (HRMS) were determined by peak matching using perfluorokerosene (PFK) as an internal mass marker. Optical rotations at the Na-D line were obtained at 20 °C using a Perkin-Elmer 141 polarimeter. All new compounds reported gave IR, ^{1}H NMR, and ^{13}C NMR spectra in agreement with their assigned structures. HRMS was used for all novel PBD compounds and many intermediates reported in this paper, as combustion analysis was hampered by problems with hydration and/or slow decomposition under vacuum or thermal drying conditions due to the interconvertibility of the N10−C11 functionality. Furthermore, most of the aromatic amines (i.e., **15a**,**b**, **19a**, **22**, **28**, **32**, **37**, and **46**) were prone to oxidation and so were not fully characterized but used directly in the next step as soon as possible after synthesis. Anthramycin (**1**) and tomaymycin methyl ethers (**2**), neothramycin (as a mixture of isomeric 3-*O*-butyl ethers) (**4**), and sibiromycin (**3**) were gifts from Hoffmann-La Roche Corp. (New Jersey), Fujisawa Corp. (Ibaraki, Japan), Institute of Microbial Chemistry (Tokyo, Japan), and Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan), respectively. DC-81 was synthesized in this laboratory.[23,27] Compounds **5**,[25,27] **49**,[18] **50**,[19] **51**,[18] **52**,[25] and **53**[19] shown for comparative purposes in Table 1 were synthesized according to literature procedures.

4-(3-Hydroxypropoxy)-3-methoxybenzoic Acid (8). Method A. 3-Bromo-1-propanol (130 g, 93.5 mol) was added to a vigorously stirred solution of vanillic acid (**7**; 146.8 g, 0.87 mol) in aqueous NaOH (70 g, 1.74 mol in 440 mL). After reflux for 5 h, the mixture was cooled and treated with 2.25 M HCl (500 mL) and the resulting precipitate collected by filtration and dried. Recrystallization from 2-butanone gave **8** as a colorless solid (190 g, 96%): mp 160−161 °C.

Method B. Solid KOH (3.2 g, 57 mmol) was added to a stirred solution of vanillic acid (**7**; 1.0 g, 5.95 mmol) in boiling EtOH (35 mL), and the mixture refluxed under N_2 for 30 min. A solution of 3-bromo-1-propanol (5.2 g, 37.4 mmol) in EtOH (15 mL) was added dropwise over 30 min while the reaction mixture stirred at 0 °C. After warming to room temperature, the mixture was refluxed for 48 h until TLC ($MeOH-CHCl_3$, 1:3 v/v) indicated complete loss of starting material. After cooling and removal of the EtOH by evaporation in vacuo, the aqueous residue was acidified to pH 1 (1 M HCl) and the resultant precipitate collected by filtration and dried to afford **8** as a light-brown solid (1.02 g, 76%): mp 158−160 °C; IR (KBr) ν 3700−2120, 1670, 1595, 1515, 1460, 1425, 1350, 1280, 1235, 1185, 1145, 1110, 1050, 1035, 940, 925, 870, 820, 765 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 1.88−1.93 (m, 2H), 3.58 (t, 2H, J = 6.2 Hz), 3.82 (s, 3H), 4.11 (t, 2H, J = 6.3 Hz), 4.66 (br s, 1H), 7.05 (d, 1H, J = 8.3 Hz), 7.46 (s, 1H), 7.57 (d, 1H, J = 8.3 Hz), 12.68 (br s, 1H); ^{13}C NMR (Me_2SO-d_6) δ 31.9, 55.4, 57.2, 65.3, 111.7, 112.0, 122.7, 123.2, 148.3, 152.0, 167.1; MS (EI) *m*/*z* (relative intensity) 226 ($[M]^{\bullet+}$, 35), 168 (100), 153 (65), 125 (10), 111 (5), 97 (15), 79 (10), 65 (10), 51 (15); HRMS calcd 226.0705 ($C_{11}H_{14}O_5$), found 226.0528.

3-(4-Carboxy-2-methoxy-5-nitrophenoxy)propanoic Acid (9). The alcohol **8** (3 g, 13.3 mmol) was added in portions over 10 min to aqueous HNO_3 (70% w/w, 15 mL) at 0 °C. The mixture was stirred at 0 °C for 15 min and then allowed to warm to room temperature over 45 min. The mixture was added to ice−water (200 mL), and the resultant yellow suspension extracted with EtOAc (3 × 30 mL). The combined organic phase was dried (Na_2SO_4) and evaporated in vacuo to afford a yellow solid which was recrystallized from EtOAc−

petroleum ether to give **9** (2.0 g, 53%): mp 178−180 °C; IR (Nujol) ν 3200−2500, 1700−1670, 1590, 1570, 1510, 1450, 1360, 1260, 1210, 1040 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.87 (t, 2H, J = 6.6 Hz), 3.94 (s, 3H), 4.36 (t, 2H, J = 6.5 Hz), 7.14 (s, 1H), 7.45 (s, 1H), 10.68 (br s, 2H); ^{13}C NMR ($CDCl_3$) δ 33.0, 56.5, 65.2, 108.4, 111.2, 122.7, 141.1, 149.2, 152.5, 167.3, 172.5; MS (EI) *m/z* (relative intensity) 286 ($[M + H]^{\cdot+}$, 20), 241 (10), 213 (100), 169 (20), 152 (5), 111 (20), 96 (5), 79 (5), 73 (15), 55 (10); HRMS calcd 285.0511 ($C_{11}H_{14}NO_8$), found 285.0538.

Methyl 3-(4-Carboxy-2-methoxy-5-nitrophenoxy)propanoate (10). A solution of **9** (2.0 g, 7.0 mmol) and *p*-TsOH (157 mg, 0.83 mmol) in MeOH (30 mL) was stirred at room temperature for 12 h followed by removal of the MeOH in vacuo and crystallization of the residue from water. The resultant solid was collected and recrystallized from EtOAc−petroleum ether to give **10** as a colorless solid (1.5 g, 72%): mp 176−177 °C; IR (KBr) ν 3070−2700, 1710, 1650, 1550, 1470, 1380, 1330, 1250, 1170, 1010, 830, 800, 760, 730 cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 2.86 (t, 2H, J = 5.8 Hz), 3.64 (s, 3H), 3.90 (s, 3H), 4.33 (t, 2H, J = 5.8 Hz), 7.29 (s, 1H), 7.62 (s, 1H); ^{13}C NMR (Me_2SO-d_6) δ 33.4, 51.5, 56.3, 64.9, 108.1, 111.3, 121.4, 141.2, 148.9, 151.6, 165.9, 170.9; MS (EI) *m/z* (relative intensity) 299 ($[M]^{\cdot+}$, 55), 240 (10), 213 (5), 188 (30), 160 (10), 87 (100), 58 (30); HRMS calcd 299.0633 ($C_{12}H_{13}NO_8$), found 299.0625.

General Procedure for Preparation of the 2-Nitrobenzoylpyrrolidine-2-carboxaldehyde Diethyl Thioacetals (14a,b). DMF (2 drops) was added to a stirred suspension of the nitrobenzoic acid (**10** or **12**) (1.48 mmol) and oxalyl chloride (0.29 g, 2.28 mmol) in dry THF (15 mL), and stirring continued for 4 h. After evaporation of the solvent in vacuo, the resulting yellow solid was dissolved in dry THF (10 mL) and the solution added dropwise over 25 min to a vigorously stirred suspension of (2*S*)-pyrrolidine-2-carboxaldehyde diethyl thioacetal[20] (**13**; 0.4 g, 1.95 mmol), Et_3N (0.34 g, 3.36 mmol), and ice−water (0.6 mL) cooled in an ice bath. After addition was complete, the mixture was warmed to room temperature and stirred for a further 1.5 h. After removal of the THF by evaporation in vacuo, the residue was diluted with water (100 mL) and extracted with EtOAc (3 × 25 mL). The aqueous phase was adjusted to pH 3 with 12 M HCl and extracted with EtOAc (2 × 50 mL). The combined organic phase was washed with water (3 × 25 mL) and brine (3 × 25 mL) and then dried ($MgSO_4$). Removal of the solvent in vacuo gave the crude product (**14a** or **14b**) which could be purified by flash chromatography (EtOAc−hexane, 1:1 v/v; TLC: EtOAc−hexane, 3:2 v/v) to afford the pure amide as a light-yellow syrup.

***N*-[5-Methoxy-4-(2′-methoxycarbonylethoxy)-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (14a).** In a typical reaction, 0.5 g (1.67 mmol) of **10** gave an oily crude product which was subjected to flash chromatography (EtOAc−hexane, 55:45 v/v) to afford pure **14a** as a yellow oil (0.6 g, 74%). An analytical sample was crystallized from EtOAc−hexane: mp 28 °C; IR (Nujol) ν 1790, 1720, 1560, 1450, 1360, 1270; ^1H NMR ($CDCl_3$) δ 1.33−1.38 (m, 6H), 1.82−2.32 (m, 4H), 2.75−2.80 (m, 4H), 2.88 (t, 2H, J = 6.9 Hz), 3.25 (m, 2H), 3.75 (s, 3H), 3.93 (s, 3H), 4.38 (t, 2H, J = 6.9 Hz), 4.72 (m, 1H), 4.88 (d, 1H, J = 3.8 Hz), 6.83 (s, 1H), 7.73 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 15.0, 24.6, 26.3, 26.6, 27.2, 29.5, 33.9, 50.2, 52.16, 52.8, 56.50, 61.1, 65.0, 108.9, 109.4, 128.6, 137.2, 147.2, 154.5, 166.6, 170.9; MS (EI) *m/z* 486 ($[M]^{\cdot+}$, 4), 352 (30), 351 (17), 283 (14), 282 (100), 251 (5), 208 (4), 107 (5) 93 (4), 87 (34), 59 (22); HRMS calcd 486.1484 ($C_{21}H_{30}N_2O_7S_2$), found 486.1494; $[\alpha]^{21}_D$ −111.9° (*c* = 0.1, $CHCl_3$).

***N*-(4-Benzyloxypropoxy-5-methoxy-2-nitrobenzoyl)-pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (14b).** In a typical reaction, 0.5 g (1.67 mmol) of **12** gave 0.58 g (77%) of pure **14b** as a yellow oil: IR (neat) ν 3000−2810, 1630, 1570, 1500, 1410, 1325, 1270, 1210, 1180, 1090, 1045 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.31−1.39 (m, 6H), 1.73−2.38 (m, 6H), 2.70−2.85 (m, 4H), 3.22−3.35 (m, 2H), 3.68 (t, 2H, J = 5.9 Hz), 3.93 (s, 3H), 4.22 (t, 2H, J = 6.4 Hz), 4.53 (s, 2H), 4.69−4.76 (m, 1H), 4.88 (d, 1H, J = 3.9 Hz), 6.81 (s, 1H), 7.26−7.34 (m, 5H), 7.70 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 15.0, 15.1, 24.6, 26.3, 26.6, 27.2, 29.3, 50.2, 52.8, 56.5, 61.1, 66.3, 66.6, 73.1, 108.3, 109.2, 127.6, 128.1, 128.4, 137.3, 138.2, 148.5, 154.4, 166.7; MS (EI) *m/z* (relative intensity) 548 ($[M]^{\cdot+}$, 5), 519 (2), 413 (27), 345 (27), 344 (100), 286 (7), 254 (2), 222 (3), 135 (20), 91 (50); HRMS calcd 548.2015 ($C_{27}H_{36}O_6N_2S_2$), found 548.2015.

General Procedure for Reduction of the *N*-(2-Nitrobenzoyl)pyrrolidine-2-carboxaldehyde Diethyl Thioacetals (14a,b). A solution of the nitro thioacetal (0.64 mmol) and $SnCl_2 \cdot 2H_2O$ (0.58 g, 2.58 mmol) in MeOH (25 mL) was refluxed for 40 min until TLC (EtOAc−hexane, 4:1 v/v) indicated that reaction was complete. The solvent was removed by evaporation in vacuo, and the residue was cooled to 0 °C and treated with saturated aqueous $NaHCO_3$. The resulting solid was triturated with EtOAc (2 × 50 mL) and the mixture allowed to stir at room temperature for 1 h. The suspension was filtered through a short bed of Celite which was rinsed with EtOAc (2 × 50 mL). The combined filtrate was evaporated in vacuo to afford the corresponding amino diethyl thioacetal as a pale-yellow foam. Further purification by flash chromatography (EtOH−hexane, 3:7 v/v) afforded the corresponding amine as a yellow syrup which was used directly in the next step due to potential stability problems.

***N*-[2-Amino-5-methoxy-4-(2′-methoxycarbonylethoxy)-benzoyl]pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (15a).** In a typical reaction, 0.35 g of **14a** (0.72 mmol) gave crude **15a** as a yellow oil (0.3 g, 90%) which was used directly in the next step without further purification: ^1H NMR ($CDCl_3$) δ 1.24 (m, 6H), 1.70−2.04 (m, 4H), 2.23 (m, 1H), 2.72 (m, 4H), 2.87 (t, 2H, J = 6.9 Hz), 3.68 (m, 2H), 3.72 (s, 3H), 3.75 (s, 3H), 4.29 (t, 2H, J = 6.9 Hz), 4.68−4.71 (m, 3H), 6.26 (s, 1H), 6.82 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 24.2, 29.6, 34.0, 46.7, 51.9, 53.7, 56.17, 64.3, 111.0, 111.8, 120.7, 140.6, 147.8, 150.3, 162.5, 164.6, 171.1.

***N*-[2-Amino-4-(3′-benzyloxypropoxy)-5-methoxybenzoyl]-pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (15b).** In a typical reaction, 0.35 g (0.64 mmol) of **14b** gave 0.25 g (76%) of the amine **15b**. Due to the relative instability of this compound it was used directly in the next step without extensive characterization.

General Procedure for $HgCl_2$/$CaCO_3$-Mediated B-Ring Cyclization. A suspension of the amino thioacetal (0.38 mmol), $HgCl_2$ (264 mg, 0.97 mmol), and $CaCO_3$ (96 mg, 0.96 mmol) in MeCN−H_2O (4:1 v/v, 15 mL) was stirred slowly at room temperature for 2−6 h until TLC (EtOAc−hexane, 4:1 v/v) indicated complete loss of starting material. After removal of the volatile solvent in vacuo, the residue was triturated with EtOAc (2 × 50 mL) which was washed with aqueous $NaHCO_3$ (2 × 50 mL) and brine (2 × 25 mL), and the combined aqueous layer was back-extracted with EtOAc (1 × 25 mL). The combined organic phase was evaporated and the residue purified by flash chromatography (MeOH/$CHCl_3$, 1:4 v/v) to provide the pure PBD as a yellow oil. In some cases, the oil could be diluted with dry Et_2O and the organic phase evaporated in vacuo to afford a yellow solid.

(11a*S*)-7-Methoxy-8-(2′-methoxycarbonylethoxy)-1,2,3,-11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (16a). In a typical reaction, 0.32 g of **15a** (0.70 mmol) was completely cyclized after 6 h (TLC: EtOAc−MeOH, 9:1 v/v) to give a yellow oil which was diluted with dry Et_2O, and the organic phase evaporated in vacuo (4 × 20 mL) to afford **16a** as a yellow solid (0.15 g, 64%): ^1H NMR ($CDCl_3$) δ 2.02−2.08 (m, 4H), 2.29−2.34 (m, 2H), 2.91 (t, 2H, J = 6.6 Hz,), 3.49 (m, 1H), 3.73 (s, 3H), 3.92 (s, 3H), 4.35 (t, 2H, J = 6.6 Hz,), 6.85 (s, 1H), 7.51(s, 1H), 7.67 (d, J = 4.4 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 24.2, 29.6, 34.0, 46.7, 51.9, 53.7, 56.2, 64.3, 111.0, 111.8, 120.7, 140.6, 147.8, 150., 162.5, 164.6, 171.1; MS (EI) *m/z* (relative intensity) 332 ($[M]^{\cdot+}$, 100), 301 (6), 217 (10), 203 (4), 201 (2), 177 (3), 166 (3), 87 (25), 70 (10); HRMS calcd 332.1402 ($C_{17}H_{20}N_2O_5$), found 332.1372.

(11a*S*)-8-(3′-Benzyloxypropoxy)-7-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (16b). In a typical reaction, 0.25 g of **15b** (0.48 mmol) gave 0.15 g (79%) of **16b** as a yellow oil: ^1H NMR ($CDCl_3$) δ 2.02−2.38 (m, 6H), 3.51−3.88 (m, 5H), 3.92 (s, 3H), 4.18−4.24 (m,

2H), 4.52 (s, 2H), 6.84 (s, 1H), 7.42−7.49 (m, 5H), 7.53 (s, 1H), 7.66 (d, 1H, J = 4.4 Hz); ^{13}C NMR ($CDCl_3$) δ 24.2, 29.4, 29.6, 46.6, 53.7, 56.1, 66.1, 66.7, 73.0, 110.6, 111.5, 120.2, 127.5, 127.6, 127.7, 127.8, 128.4, 138.3, 140.6, 147.8, 150.8, 162.4, 164.5; MS (EI) m/z (relative intensity) 394 ([M]$^{\cdot+}$, 68%), 393 (100), 303 (25), 287 (15), 259 (44), 258 (92), 257 (60), 254 (19), 245 (35), 231 (16), 229 (19), 217 (18), 149 (13), 99 (65), 91 (96); HRMS calcd 394.1892 ($C_{23}H_{26}O_4N_2$), found 394.1886; $[\alpha]^D_{23}$ +295.5° (c = 0.56, $CHCl_3$).

(2*S*)-*N*-[4,5-(Methylenedioxy)-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (18). DMF (2 drops) was added to a stirred suspension of 2-nitropiperonylic acid (**17a**) (0.5 g, 2.56 mmol) and oxalyl chloride (0.45 g, 4.46 mmol) in dry THF (15 mL), and stirring continued for 4 h. Evaporation of the solvent in vacuo gave a yellow residue which was dissolved in dry THF (10 mL) and added dropwise over 20 min to a vigorously stirred suspension of (2*S*)-pyrrolidine-2-carboxaldehyde diethyl thioacetal (**13**; 0.58 g, 2.84 mmol), Et_3N (0.5 g, 5.0 mmol), and ice−water (0.6 mL) cooled in an ice bath. The mixture was warmed to room temperature and stirred for 1.5 h, and the THF was evaporated in vacuo. The residue was diluted with water (2 × 20 mL), and this solution was extracted with EtOAc (3 × 20 mL). The aqueous phase was then adjusted to pH 3 (12 M HCl) and extracted with EtOAc (2 × 25 mL). The combined organic phase was washed with water (2 × 25 mL) and brine (2 × 20 mL), dried (Na_2SO_4), and then concentrated in vacuo to afford a dark-red oil which was purified by flash chromatography (EtOAc−hexane, 1:1 v/v; TLC: EtOAc−hexane, 3:2 v/v) to afford the amide **18** (0.67 g, 66%) as a pale-yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.22−1.37 (m, 6H), 1.75−2.33 (m, 4H), 2.64−2.86 (m, 4H), 3.23−3.36 (m, 2H), 4.64−4.71 (m, 1H), 4.84 (d, 1H, J = 4.1 Hz), 6.17 (s, 2H), 6.81 (s, 1H), 7.61 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.9, 15.0, 24.7, 26.2, 26.6, 27.3, 50.3, 52.7, 61.1, 103.5, 105.2, 106.9, 130.6, 139.2, 148.3, 152.7, 166.1; IR (KBr) ν 3000−2820, 2300, 1640, 1530, 1490, 1440, 1390, 1330, 1250, 1150, 1130, 1040, 940, 870, 810, 750 cm^{-1}; MS (EI) m/z (relative intensity) 398 ([M]$^{\cdot+}$, 17), 369 (4), 263 (60), 230 (4), 194 (100), 178 (7), 135 (61), 120 (24), 107 (5), 70 (6), 55 (14), 45 (4); HRMS calcd 398.0965 ($C_{17}H_{22}O_5N_2S_2$), found 398.0970.

(2*S*)-*N*-(2-Amino-5-hydroxy-4-methoxybenzoyl)pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (19a). A solution of the nitro thioacetal **18** (440 mg, 1.1 mmol) and $SnCl_2$•$2H_2O$ (1.12 g, 5 mmol) in MeOH (30 mL) was refluxed for 45 min, after which time TLC (EtOAc−hexane, 3:1 v/v) indicated complete loss of starting material. The solvent was evaporated in vacuo and the residue cooled to 0 °C and then quenched with water (2 × 30 mL). The resulting viscous yellow liquid was triturated with EtOAc (2 × 50 mL) and the mixture allowed to stir at room temperature for 1 h. The resultant suspension was filtered through a short bed of Celite which was rinsed with EtOAc (2 × 25 mL). The combined organic phase was washed with water (2 × 30 mL) and brine (2 × 25 mL), then dried (Na_2SO_4), and concentrated in vacuo. The resulting yellow foam was purified by flash chromatography (EtOAc−hexane, 4:1 v/v) to give **19a** (200 mg, 49%) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.21−1.32 (m, 6H), 1.66−2.24 (m, 4H), 2.60−2.73 (m, 4H), 3.57−3.63 (m, 2H), 3.81 (s, 3H), 4.67−4.71 (m, 4H), 6.22 (s, 1H), 6.85 (s, 1H), 6.91 (br s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.9, 15.1, 25.2, 26.4, 27.2, 29.7, 51.7, 53.1, 55.7, 60.9, 99.9, 111.9, 114.4, 137.1, 140.8, 149.3, 169.8; IR (KBr) ν 3600−2800, 1615, 1520, 1440, 1400, 1340, 1270, 1210, 1100, 1030, 970, 875, 840, 760 cm^{-1}; MS (EI) m/z (relative intensity) 370 ([M]$^{\cdot+}$, 13), 246 (4), 235 (17), 167 (11), 166 (100), 149 (5), 138 (13), 75 (6), 55 (4), 43 (5); HRMS calcd 370.1385 ($C_{17}H_{26}O_3N_2S_2$), found 370.1424.

(11a*S*)-7-Hydroxy-8-methoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (20, *iso*-DC-81). A suspension of the amino thioacetal **19a** (150 mg, 0.4 mmol), $HgCl_2$ (275 mg, 1.0 mmol), and $CaCO_3$ (0.1 g, 1.0 mmol) in MeCN−H_2O (4:1 v/v, 15 mL) was stirred slowly at room temperature for 2 h until TLC (EtOAc−hexane, 4:1 v/v) indicated complete loss of starting material. After evaporation of the solvent in vacuo, the residue was dissolved in EtOAc (2 × 50 mL) and washed with saturated aqueous $NaHCO_3$ (2 × 30 mL) and brine (2 × 20 mL), and the combined aqueous layer was back-extracted with EtOAc (25 mL). The combined organic phase was concentrated in vacuo to afford a residue, which was purified by flash chromatography (MeOH−$CHCl_3$,1:4 v/v) to give **20** (55 mg, 55%) as a yellow oil: IR (KBr) ν 3600−3050, 3000−2720, 1610, 1515, 1450, 1275, 1215, 760 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 2.02−2.07 (m, 2H, H-1), 2.28−2.36 (m, 2H, H-2), 3.52−3.88 (m, 3H, H-3, H-11a), 3.92 (s, 3H, OCH_3), 6.82 (s, 1H, H-6), 7.28 (br s, 1H, 8-OH), 7.66 (d, 1H, J = 4.6 Hz, H-11) and 7.73 (s, 1H, H-9); ^{13}C NMR ($CDCl_3$) δ 24.2, 29.6, 46.7, 53.7, 56.1, 109.3, 115.6, 120.7, 139.9, 144.8, 149.8, 162.2, 164.7; MS (EI) m/z (relative intensity) 246 ([M]$^{\cdot+}$, 100), 217 (22), 177 (8), 150 (13), 122 (15), 107 (5), 89 (9), 70 (30), 45 (9); HRMS calcd 246.1004 ($C_{13}H_{14}N_2O_3$), found 246.1041; $[\alpha]^D_{23}$ +64° (c = 1.28, $CHCl_3$).

(2*S*)-*N*-(2-Amino-4,5-methylenedioxybenzoyl)pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (22). The nitro thioacetal **18** (0.5 g, 1.25 mmol) was dissolved in MeOH (20 mL), and Pd−C (10% w/w, 0.15 g) was added. The mixture was hydrogenated at room temperature and atmospheric pressure until no further uptake of H_2 was observed (ca. 2 h). The mixture was filtered through Celite and the filtrate concentrated in vacuo to afford **22** as a pale-yellow oil (0.39 g, 85%), which was used directly in the next step: IR (neat) ν 3540−3370, 3360−3240, 3080−2770, 1630, 1585, 1540, 1485, 1445, 1400, 1380, 1340, 1260, 1235, 1215, 1150, 1035, 930, 855, 830, 700 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.21−1.40 (m, 6H), 1.62−2.29 (m, 4H), 2.61−2.79 (m, 4H), 3.55−3.63 (m, 2H), 4.49−4.60 (br s, 2H), 4.62−4.74 (m, 2H), 5.87 (s, 2H), 6.24 (s, 1H), 6.73 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 14.9, 15.1, 25.2, 26.4, 26.5, 27.2, 51.7, 53.2, 60.9, 98.0, 101.0, 107.8, 111.3, 139.1, 143.1, 150.0, 170.0.

(11a*S*)-7,8-(Methylenedioxy)-1,2,3,11a-tetrahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (23). The method used to synthesize *iso*-DC-81 (**20**) from the amino thioacetal **19a** was used (see above). In a typical reaction, 0.38 g (1.03 mmol) of **22** gave **23** (0.18 g, 72%) as a thick yellow syrup: ^{1}H NMR ($CDCl_3$) δ 1.97−2.18 (m, 2H), 2.07−2.39 (m, 2H), 3.42−3.85 (m, 3H), 6.04 (s, 2H), 6.75 (s, 1H), 7.43 (s, 1H), 7.65 (d, 1H, J = 4.6 Hz); ^{13}C NMR ($CDCl_3$) δ 24.1, 29.4, 46.6, 53.5, 102.0, 106.6, 108.5, 121.8, 142.2, 146.3, 150.2, 162.6, 164.2; MS (EI) m/z (relative intensity) 244 ([M]$^{\cdot+}$, 100%), 243 (83), 215 (14), 175 (20), 148 (22), 120 (18); HRMS calcd 244.0847 ($C_{13}H_{12}O_3N_2$), found 244.0911; $[\alpha]^D_{23}$ +795° (c = 0.54, $CHCl_3$).

3,4-(Ethylenedioxy)benzoic Acid (25). An aqueous solution of $KMnO_4$ (13.84 g, 0.084 mol in 300 mL) was added over 40 min to a stirred solution of 3,4-(ethylenedioxy)benzaldehyde (**24**; 10 g, 61 mmol) in water (250 mL) at 70−80 °C. After 40 min the reaction mixture was basified (10% aqueous KOH) and the resulting precipitate collected by filtration and rinsed with water (3 × 50 mL). The combined filtrate was acidified (12 M HCl) and the resulting solid separated and dried to afford **25** as a colorless powder (8.4 g, 76%): mp 136 °C; IR (KBr) ν 3300−2300, 1680, 1610, 1590, 1500, 1430, 1290, 1260, 1240, 1190, 1120, 1070 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 4.30 (s, 4H), 6.95 (d, 1H, J = 8.8 Hz), 7.44 (m, 2H), 12.80 (s, 1H).

4,5-(Ethylenedioxy)-2-nitrobenzoic Acid (26). The nitro acid **26** was prepared from **25** in 68% yield using the method of Rault and co-workers:[24] mp 210 °C; IR (KBr) ν 3300−2600, 1685, 1520, 1580, 1455, 1420, 1345, 1305, 1275, 1130, 1060, 990, 890 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 7.55 (s, 1H), 7.25 (s, 1H), 4.35 (s, 4H).

***N*-[2-Amino-4,5-(ethylenedioxy)benzoyl]pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (28).** The nitro thioacetal **27** (0.62 g, 1.5 mmol) was converted into the amino thioacetal (0.38 g, 66%) using the same $SnCl_2$•H_2O method as described above for **14a**,**b**. This material was used directly in the next step: IR (neat) ν 3660−3100, 3000−2780, 1625, 1580, 1550, 1495, 1390, 1300, 1240, 1160, 1050, 885 cm^{-1}.

Dioxano[2,3-*h*]pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (29). A suspension of **28** (0.15 g, 0.46 mmol), $HgCl_2$ (0.3 g, 2.2 equiv), and $CaCO_3$ (0.12 g, 2.5 equiv) in MeCN−H_2O (4:1 v/v, 5 mL) was stirred at room temperature for 24 h. The

reaction mixture was diluted with EtOAc (15 mL) and filtered through a short Celite bed. The filtrate was extracted with saturated $NaHCO_3$ (2 × 10 mL) and brine (2 × 10 mL), and the combined aqueous phase was back-extracted with EtOAc (2 × 10 mL). The combined organic phase was then dried (Na_2SO_4) and evaporated in vacuo and the residual oil purified by flash chromatography on silica gel (acetone−hexane, 1:1 v/v) to afford the imine **29** as a yellow oil (70 mg, 69%): IR (neat) ν 3410, 3030, 2990, 1630, 1270 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.7−2.1 (m, 4H), 3.5−3.8 (m, 3H), 4.47 (m, 1H), 5.7 (br s, 1H), 6.61 (dd, 1H, J = 6, 8 Hz), 7.15 (br s, 1H), 7.47 (dd, 1H, J = 2, 6 Hz), 8.17 (dd, 1H, J = 2, 4 Hz).

(11*R*,11a*S*)-11-Methoxydioxano[5,6-*h*]-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one (30a,b). A solution of **29** (60 mg, 0.27 mmol) in MeOH (10 mL) was stirred at room temperature for 24 h. After removal of the solvent in vacuo, the resulting solid residue of carbinolamine methyl ethers **30a**,**b** was crystallized from dry Et_2O to afford **30a** as a white solid (30 mg, 48%): mp 187 °C; IR (Nujol) ν 3310, 1600, 1530, 1080 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.7−2.3 (m, 4H), 3.32 (s, 3H), 3.76−3.87 (m, 3H), 4.58 (d, 1H, J = 6.2 Hz), 6.79 (dd, 1H, J = 6, 8 Hz), 7.4 (br s, 1H), 8.2 (dd, 1H, J = 2, 6 Hz), 8.3 (dd, 1H, J = 2, 4 Hz); ^{13}C NMR ($CDCl_3$) δ 22.6, 29.3, 30.9, 49.1, 54.5, 57.8, 58.9, 87.2, 114.4, 142.5, 151.2, 151.3, 153.7, 164.7; $[\alpha]^D_{23}$ = +630.0 (c = 0.66, $CHCl_3$).

(2*S*)-*N*-(2-Amino-3-pyridinylcarbonyl)pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (32). A small round-bottom flask containing the isatoic anhydride **31** (0.3 g, 1.82 mmol) and DMF (3 mL) was immersed in an oil bath at 100 °C. A solution of **13** (315 mg, 1.54 mmol) in DMF (2 mL) was added dropwise over 5 min, and the mixture was cooled, diluted with water (10 mL), and extracted with EtOAc (4 × 20 mL). The combined organic phase was dried (Na_2SO_4) and evaporated under reduced pressure to afford crude **32** as a yellow oil. Column chromatography (8.5:1:0.5 v/v CH_2Cl_2−MeOH−toluene) afforded pure **32** as a pale-yellow oil (180 mg, 30%): IR (Nujol) ν 3320, 1670, 1580, 1500 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.64 (t, 6H, J = 7.0 Hz), 1.77 (m, 1H), 1.86−1.94 (m, 2H), 2.22 (m, 1H), 2.56−3.26 (m, 2H), 3.54 (q, 4H, J = 7.0 Hz), 4.61−4.69 (m, 2H), 5.54 (br s, 2H), 6.58 (dd, 1H, J_1 = 4 Hz, J_2 = 6 Hz), 7.46 (dd, 1H, J_1 = 2 Hz, J_2 = 6 Hz), 8.03 (dd, 1H, J_1 = 2 Hz, J_2 = 4 Hz); ^{13}C NMR ($CDCl_3$) δ 14.9, 15.0, 25.2, 26.3, 26.5, 27.3, 51.2; 52.9, 61.1, 112.7, 156.6, 168.4. This material was used directly in the next step.

Epimeric Mixture of (11*R*,11a*S*)- and (11*S*,11a*S*)-11-Hydroxy-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]-pyrido[3,2-*f*]diazepin-5-one (33). A solution of **32** (0.15 g, 0.46 mmol), $HgCl_2$ (0.3 g, 2.2 equiv), and $CaCO_3$ (0.12 g, 2.5 equiv) in MeCN−water (4:1, 5 mL) was stirred at room temperature for 24 h. The reaction mixture was then diluted with EtOAc (15 mL) and filtered through a short pad of Celite. The filtrate was extracted with saturated $NaHCO_3$ (2 × 10 mL) and brine (2 × 10 mL), and the combined aqueous phase was back-extracted with EtOAc (2 × 10 mL). The combined organic phase was dried over Na_2SO_4 and evaporated. The resulting oily residue was subjected to flash chromatography on silica gel (acetone−hexane, 50%) to afford **33** as a yellow oil (70 mg, 69%): IR (neat) 3410, 3030, 2990, 1630, 1270 cm^{-1}; ^1H NMR ($CDCl_3$) 1.7−2.1 (m, 4H), 3.5−3.8 (m, 3H), 4.47 (m, 1H), 5.7 (bs, 1H), 6.61 (dd, 1H, J = 6.0 Hz, J = 8.0 Hz), 7.15 (bs, 1H), 7.47 (dd, 1H, J_1 = 2 Hz, J_2 = 8 Hz), 8.17 (dd, 1H, J_1 = 4.0 Hz, J_2 = 6.0 Hz).

(11*R*,11a*S*)-11-Methoxy-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]pyrido[3,2-*f*]diazepin-5-one (34a). Carbinolamine **33** (60 mg, 0.26 mmol) was dissolved in MeOH (10 mL), and the solution stirred at room temperature for 24 h. The solvent was evaporated under reduced pressure to give a mixture of the carbinolamine methyl ethers **34a**,**b** as a solid residue. Crystallization from dry ether afforded the single diastereomer **34a** as a white solid (30 mg, 47%): mp 187 °C; IR (Nujol) 3310, 1600, 1530, 1080 cm^{-1}; ^1H NMR ($CDCl_3$) 1.7−2.3 (m, 4H), 3.32 (s, 3H), 3.76−3.87 (m, 3H), 4.58 (d, 1H, J = 6 Hz), 6.79 (dd, 1H, J_1 = 6 Hz, J_2 = 8 Hz), 7.4 (bs, 1H), 8.2 (dd, 1H, J_1 = 2 Hz, J_2 = 6 Hz), 8.3 (dd, 1H, J_1 = 2 Hz, J_2 = 8 Hz); ^{13}C NMR ($CDCl_3$) 22.8, 30.9, 49.1, 54.5, 58.9, 87.2, 114.4, 142.5, 151.2, 151.4, 153.8, 164.7. Anal. ($C_{12}H_{15}N_3O_2$) C, H, N.

3-Acetamido-2-pyrazinecarboxylic Acid (36). Lithium hydroxide monohydrate (680 mg, 3 equiv) was added to a solution of **35** (1.05 g, 5.33 mmol) in THF−H_2O−MeOH (4:1:1 v/v, 20 mL), and the mixture stirred at room temperature for 20 min. The resulting suspension was acidified with aqueous 1 M HCl and the white precipitate collected by filtration and recrystallized from EtOH to give pure **36** (0.92 g, 95%): mp >300 °C; IR (KBr) ν 3480, 3340, 1720, 1610 cm^{-1}; ^1H NMR ($CDCl_3$) δ 3.5 (s, 3H), 7.41 (br s, 2H, O*H* & N*H*), 7.87 (d, 1H, J = 2.2 Hz), 8.23 (d, 1H, J = 2.2 Hz).

***N*-(3-Amino-2-pyrazinylcarbonyl)pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (37).** A solution of **36** (318 mg, 1.76 mmol), hydroxybenzotriazole (HOBT; 261 mg, 1.1 equiv), and EDCI (371 mg, 1.1 equiv) in dry DMF (50 mL) was cooled to 0 °C and treated dropwise with a solution of **13** (312 mg, 1 equiv) in DMF (25 mL). The reaction mixture was allowed to warm to room temperature and stirred for 6 h. The solvent was then removed in vacuo and the oily residue dissolved in EtOAc (50 mL) and washed with aqueous 0.5 M HCl (2 × 20 mL), saturated aqueous $NaHCO_3$ (2 × 20 mL), and brine (20 mL). The organic phase was dried (Na_2SO_4) and evaporated to afford crude **37**. Flash chromatography (7:3 v/v EtOAc−petroleum ether) afforded pure **37** as a pale-yellow oil (380 mg, 66%): IR (neat) ν 3450, 3320, 1600, 1440, 1420 cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.1−1.38 (dt, 6H, J = 8 Hz), 1.6−2.3 (m, 5H), 2.62−2.83 (q, 4H, J = 8.0 Hz), 3.66−3.88 (m, 2H), 4.70−4.80 (m, 1H), 6.12 (br s, 2H, NH_2), 7.88 (d, 1H, J = 2.4 Hz), 8.03 (d, 1H, J = 2.4 Hz); $[\alpha]^D_{20}$ −66.7° (c = 0.574, MeOH). This material was used directly in the next step.

(11*R*,11a*S*)-11-Methoxy-1,2,3,10,11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]pyrazino[2,3-*f*]diazepin-5-one (38). The amide **32** (200 mg, 0.61 mmol) was dissolved in MeCN−H_2O (4:1 v/v, 60 mL), and $HgCl_2$ (364 mg, 2.2 equiv) and $CaCO_3$ (153 mg, 2.5 equiv) were added. The suspension was stirred at room temperature for 48 h and then diluted with EtOAc (15 mL), and the salts were removed by filtration. The solvent was removed in vacuo and the residue purified by flash chromatography (TEA−treated Florisil, 1:1 v/v, EtOAc−MeOH). Crystallization from Et_2O gave pure **38** as a colorless solid (89.6 mg, 73%): mp 167 °C dec; IR (KBr) ν 3450, 1630 cm^{-1}; ^1H NMR (Me_2SO-d_6) δ 8.45 (d, 1H, J = 6 Hz), 8.25 (d, 1H, J = 2 Hz), 8.07 (d, 1H, J = 2 Hz), 4.57 (d, 1H, J = 6 Hz), 3.87 (m, 1H), 3.7−3.4 (m, 2H), 3.28 (s, 3H), 2.3−1.5 (m, 4H); ^{13}C NMR (Me_2SO-d_6) δ 162.2, 150.7, 144.9, 134.1, 130.2, 86.4, 57.6, 54.4, 48.5, 30.1, 22.1; $[\alpha]^D_{20}$ +224.2° (c = 0.19, MeOH). Anal. ($C_{11}H_{14}N_4O_2$) C,H,N: calcd 56.4, 6.04, 23.92; found 56.6, 6.00, 23.88.

Methyl 5-Nitroorotate (40). The potassium salt of 5-nitroorotic acid (5 g, 2 mmol) was dissolved in MeOH (15 mL), then concentrated H_2SO_4 (4.2 mL) was added, and the mixture was refluxed for 8 h. The resulting precipitate was collected by filtration, washed with water, and crystallized from water to afford **40** (4.5 g, 78%): mp 199−200 °C.[32]

Methyl 2,6-Dichloro-5-nitro-4-pyrimidincarboxylate (41). Compound **31** (1 g, 4.65 mmol) was dissolved in $POCl_3$ (4 mL) and diethylaniline (1.33 mL). The solution was stirred at room temperature for 30 min and then refluxed for a further 20 min. The solvent was removed under vacuum, and the oily residue was poured onto ice and water and extracted with Et_2O (3 × 20 mL). The ethereal layer washed successively with 0.5 M HCl (10 mL), aqueous 1 M $NaHCO_3$ (10 mL), and brine (10 mL). The organic layer was dried (Na_2SO_4) and evaporated under reduced pressure to afford a solid that was crystallized from cyclohexane (1.07 g, 92%): mp 84−85 °C.

Methyl 2,6-Dimethoxy-5-nitro-4-pyrimidinecarboxylate (42). Dichloropyrimidine **35** (300 mg, 1.19 mmol) was added to a methanolic solution of NaOMe (freshly prepared from sodium metal (0.06 g, 2.62 mmol) and MeOH (15 mL)) at 0 °C. After reflux for 4 h, the solvent was removed under reduced pressure and the residue triturated with water (10 mL) and extracted with EtOAc (3 × 10 mL). The combined

organic phase was dried and evaporated in vacuo to give a crude product which was subjected to flash chromatography (7:3 v/v EtOAc−petroleum ether) to afford **42** as a yellow oil (239 mg, 82%): ^{1}H NMR ($CDCl_3$) δ 3.97 (s, 3H), 4.11 (s, 3H), 4.16 (s, 3H).

2,6-Dimethoxy-5-nitro-4-pyrimidinecarboxylic Acid (43). The ester **42** (1.56 g, 6.42 mmol) was dissolved in MeOH (10 mL) and treated with aqueous 1 M NaOH (10 mL). The solution was stirred at room temperature for 18 h and the volatile solvent evaporated in vacuo. The aqueous layer was acidified with aqueous 0.5 M HCl to pH 4 and extracted with EtOAc (3 × 20 mL). The combined organic phase was dried (Na_2SO_4) and evaporated in vacuo to afford a residue which was crystallized from EtOAc−petroleum ether to afford **43** as a yellow solid (0.8 g, 55%): mp 79−80 °C; IR (KBr) ν 3500, 1720, 1570, 1350, 1250, 1200 cm^{-1}; ^{1}H NMR (Me_2SO-d_6) δ 7.65 (br s, 1H), 4.14 (s, 3H), 4.08 (s, 3H).

2,6-Dimethoxy-5-nitro-4-pyrimidinecarboxylic Acid Chloride (44). The acid **43** (0.5 g, 2.18 mmol) was dissolved in dry C_6H_6 (50 mL) and DMF (2 drops), and oxalyl chloride (0.2 mL, 2.18 mmol) was added dropwise. The solution was stirred at room temperature for 3 h and the solvent then removed by evaporation in vacuo. The residue was dissolved in dry C_6H_6 (20 mL) and the solvent evaporated in vacuo, with this cycle being repeated once. The crude acid chloride **44** was used directly in the next step without further purification.

***(2S)*-*N*-(2,6-Dimethoxy-5-nitropyrimidin-4-ylcarbonyl)-pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (45).** A solution of crude **44** in dry THF (10 mL) was added dropwise to a solution of (2*S*)-pyrrolidinecarboxaldehyde diethyl thioacetal (0.5 g, 2.2 mmol) in dry THF (30 mL) and TEA (0.8 mL) at 0 °C. The mixture was allowed to warm to room temperature and stirred for 24 h. The solvent was removed in vacuo, the residue was dissolved in EtOAc (20 mL), and this solution was washed with aqueous 0.5 M HCl (2 × 20 mL), saturated $NaHCO_3$ (2 × 20 mL), and brine (2 × 20 mL). The organic phase was dried (Na_2SO_4) and evaporated in vacuo to afford **45** as a pale-yellow oil (0.7 g, 77%): ^{1}H NMR ($CDCl_3$) δ 1.30 (t, 3H, J = 7 Hz), 1.34 (t, 3H, J = 7.0 Hz), 2.17−2.27 (m, 3H), 2.78 (q, 2H, J = 7.0 Hz), 2.82 (q, 2H, J = 7.0 Hz), 3.52−3.57 (m, 2H), 4.12 (s, 3H), 4.15 (m, 1H), 4.20 (s, 3H), 4.68 (m, 1H), 4.77 (d, 1H, J = 3.4 Hz).

***(2S)*-*N*-(5-Amino-2,6-dimethoxypyrimidin-4-ylcarbonyl)pyrrolidine-2-carboxaldehyde Diethyl Thioacetal (46).** The nitro amide **45** (0.65 g, 1.56 mmol) was dissolved in MeOH (20 mL) and $SnCl_2 \cdot 2H_2O$ (1.6 g, 5 equiv) added. The mixture was refluxed for 3 h, then cooled, adjusted to pH 8 with saturated $NaHCO_3$ solution, and extracted with EtOAc (2 × 30 mL). The combined organic phase was dried (Na_2SO_4) and then evaporated in vacuo to afford **46** as a yellow oil (0.5 g, 83%): IR (Nujol) ν 3480, 3350, 2980, 1730, 1370, 1260 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.20 (t, 3H, J = 7 Hz), 1.34 (t, 3H, J = 7 Hz), 2.0−2.3 (m, 3H), 2.6−2.9 (m, 3H), 3.88 (s, 3H), 3.94 (m, 4H), 4.05 (s, 3H), 4.6−4.75 (m, 1H), 4.76 (d, 1H, J = 3.4 Hz), 4.95 (br s, 2H).

(11*R*,11a*S*)- and (11*S*,11a*S*)-7,9,11-Trimethoxy-1,2,3,10,-11,11a-hexahydro-5*H*-pyrrolo[2,1-*c*][1,4]pyrimido[4,5-*f*]-diazepin-5-one (47a,b). The amino thioacetal **46** (0.5 g, 1.29 mmol) was dissolved in MeCN−H_2O (4:1 v/v, 20 mL), and $HgCl_2$ (0.77 g, 2.2 equiv) and $CaCO_3$ (0.32 g, 2.5 equiv) were added. The suspension was stirred at room temperature for 48 h, diluted with EtOAc (15 mL), and centrifuged (7000 rpm) for 10 min. The supernatant was collected and washed with saturated $NaHCO_3$ (2 × 15 mL) and brine (2 × 15 mL). The combined aqueous phase was back-extracted with EtOAc (3 × 30 mL), and the combined organic phase was dried (Na_2SO_4), filtered through Celite, and evaporated in vacuo to afford a mixture of the (C11*R*)- and (C11*S*)-carbinolamines **47a**,**b** as an oily residue. This was dissolved in MeOH and the solution stirred at room temperature for 24 h. After evaporation of the solvent in vacuo, the methyl ethers **47d**,**e** were obtained. Purification by HPLC (2:8 v/v H_2O−MeOH) afforded pure **47d/e** as a yellow oil (0.227 g, 60%): ^{1}H NMR ($CDCl_3$) δ 1.5−2.3 (m, 4H), 2.7−2.8 (m, 2H), 3.55−3.95 (m, 3H), 4.02 (s, 3H), 4.06 (s, 3H), 4.32 (m, 1H), 4.45 (d, 1H, J = 4 Hz), 4.55 (br s, 1H).

(11a*S*)-7,9-Dimethoxy-1,2,3,11a-tetrahydro-5*H*-pyrrolo-[2,1-*c*][1,4]pyrimido[4,5-*f*]diazepin-5-one (48). The methyl ethers **47a**,**b** (0.1 g, 0.39 mmol) were subjected to reduced pressure (0.01 Torr) for 6 h at room temperature to afford **48** as a yellow oil (78 mg, 88%): ^{1}H NMR ($CDCl_3$) δ 1.9−2.4 (m, 4H), 3.64−3.95 (m, 3H), 4.1 (s, 3H), 4.12 (s, 3H), 7.75 (d, 1H, J = 3.8 Hz). Anal. ($C_{12}H_{14}N_4O_3$) C,H,N.

Thermal Denaturation Studies. The compounds were subjected to DNA thermal denaturation (melting) studies[33] using calf thymus DNA (CT-DNA, type-I, highly polymerized sodium salt, 42% G+C (Sigma)) at a fixed concentration of 100 μM (DNAp), determined using an extinction coefficient of 6600 (M phosphate)$^{-1}$ cm^{-1} at 260 nm.[35] Aqueous solutions were prepared in pH 7.00 ± 0.01 buffer containing 10 mM NaH_2PO_4/Na_2HPO_4 and 1 mM EDTA. Working solutions containing CT-DNA and the test compound (20 μM) were incubated at 37.0 ± 0.1 °C for 0−18 h (or 48 h) using an external water bath. Samples were monitored at 260 nm using a Varian-Cary 1E spectrophotometer fitted with a Peltier heating accessory. Heating was applied at a rate of 1 °C/min in the 45−98 °C temperature range, with optical and temperature data sampling at 200-ms intervals. A separate experiment was carried out using buffer alone, and this baseline was subtracted from each DNA melting curve. Optical data were imported into the Origin 3.5 computer package (MicroCal Inc., Northampton, MA) for analysis. DNA helix → coil transition temperatures (T_m) were determined at the midpoint of the normalized melting profiles using a published procedure.[13,33] Results are given as the mean ± standard deviation for at least three determinations. Ligand-induced alterations in DNA melting behavior (ΔT_m) are given by: $\Delta T_m = T_m$(DNA + ligand) − T_m-(DNA), where the T_m value for the free CT-DNA is 67.83 ± 0.06 °C (averaged from 50 runs). All compounds were dissolved in HPLC-grade MeOH to give working solutions containing (less than or equal to) 0.6% v/v MeOH; T_m results were corrected for the effects of MeOH cosolvent using a linear correction term. Other DNAp-to-ligand molar ratios (i.e., 10:1 and 2:1) were also examined in the case of selected PBDs (**2**, **5**, **23**, and **29**) to ensure that the 5:1 [DNAp]:[ligand] ratio used in this assay did not result in saturation of the host DNA duplex.

In Vitro Cytotoxicity Studies. Compounds were screened in the 10^{-4}−10^{-11} M range at decreasing 10-fold concentrations. Each compound was dissolved in DMSO to give <0.05% v/v DMSO in contact with the cells, which was nontoxic. Compounds were evaluated in the L1210 mouse leukemia (antimetabolite-sensitive), the ADJ/PC6 mouse plasmacytoma (alkylating agent-sensitive), and the CH1 human ovarian cell line (platinum-sensitive). L1210 and ADJ/PC6 cells were grown as suspension cultures, whereas CH1 was grown as a monolayer. Suspension cultures were counted using a Coulter counter, and the CH1 colonies were counted with a colony counter. The chemotherapeutic index of activity is expressed as the IC_{50}, or compound dose found to inhibit cell growth by 50% as compared to the solvent controls.

L1210 Leukemia. Day 0 cells were set up at 10^4 cells/mL in RPMI 1640 medium containing 10% horse serum. Day 1 cells were counted to ensure that they were in the logarithmic growth phase and were then exposed to the compound which was left in contact with the cells for 48 h at 37 °C. Day 3 cells were counted, and the percentage inhibition of growth was calculated by comparison with controls, which were treated with solvent alone.

ADJ/PC6 Plasmacytoma. Day 0 cells were set up at 3−5 × 10^4 cells/mL in Dulbecco's medium containing 20% horse serum. Day 1 cells were counted to ensure they were in the logarithmic phase of growth and were then exposed to the compound which was left in contact with the cells for 72 h at 37 °C. Day 4 cells were counted, and the percentage inhibition of growth was calculated by comparison with controls, which were treated with solvent alone.

CH1 Human Ovarian. Day 0 cells were set up in T_{25} flasks (100−200 cells/flask) in Dulbecco's medium containing 10% fetal calf serum and left overnight to allow the cells to attach. On Day 1 the compound was added and left in contact with the cells at 37 °C for the duration of the experiment. Day 9 colonies were usually visible and were fixed in EtOH (100%), stained with methylene blue, and counted.

Acknowledgment. This work was supported in part by the Cancer Research Campaign U.K. (Grant SP1938/0301 to D.E.T., T.C.J., and J.A.H.). Jane Rimington is thanked for her help in the preparation of this manuscript.

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JM981117P