Design, Synthesis, and Evaluation of a Novel Pyrrolobenzodiazepine DNA-Interactive Agent with Highly Efficient Cross-Linking Ability and Potent Cytotoxicity

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A novel sequence-selective pyrrolobenzodiazepine (PBD) dimer **5** (SJG-136) has been developed that comprises two C2-*exo*-methylene-substituted DC-81 (**3**) subunits tethered through their C8 positions via an inert propanedioxy linker. This symmetric molecule is a highly efficient minor groove interstrand DNA cross-linking agent (XL_{50} = 0.045 μM) that is 440-fold more potent than melphalan. Thermal denaturation studies show that, after 18 h incubation with calf thymus DNA at a 5:1 DNA/ligand ratio, it increases the T_m value by 33.6 °C, the highest value so far recorded in this assay. The analogous dimer **4** (DSB-120) that lacks substitution/unsaturation at the C2 position elevates melting by only 15.1 °C under the same conditions, illustrating the effect of introducing C2-*exo*-unsaturation which serves to flatten the C-rings and achieve a superior isohelical fit within the DNA minor groove. This behavior is supported by molecular modeling studies which indicate that (i) the PBD units are covalently bonded to guanines on opposite strands to form a cross-link, (ii) **5** has a greater binding energy compared to **4**, and (iii) **4** and **5** have equivalent binding sites that span six base pairs. Dimer **5** is significantly more cytotoxic than **4** in a number of human ovarian cancer cell lines (e.g., IC_{50} values of 0.0225 nM vs 7.2 nM, respectively, in A2780 cells). Furthermore, it retains full potency in the cisplatin-resistant cell line A2780*cis*R (0.024 nM), whereas **4** loses activity (0.21 μM) with a resistance factor of 29.2. This may be due to a lower level of inactivation of **5** by intracellular thiol-containing molecules. A dilactam analogue (**21**) of **5** that lacks the electrophilic N10−C11/N10′−C11′ imine moieties has also been synthesized and evaluated. Although unable to interact covalently with DNA, **21** still stabilizes the helix (ΔT_m = 0.78 °C) and has significant cytotoxicity in some cell lines (i.e., IC_{50} = 0.57 μM in CH1 cells), presumably exerting its effect through noncovalent interaction with DNA.

Introduction

DNA is the target for many clinically useful antitumor agents; however, few of the DNA-interactive drugs in current clinical use exhibit sequence-specificity extending beyond two to three base pairs (bp).[1] Selectivity is generally thought to favor the targeting of rapidly growing tumor cells, rather than discriminating against any fundamental differences between normal and tumor cells at the genetic level. Human genes (e.g., oncogenes) that are responsible for transforming activity in tumor cells have been identified as potential targets for new drugs. The ultimate goal is to design and synthesize agents capable of specifically inhibiting the expression of particular proteins critical for tumor cell proliferation, metastasis, or drug resistance. For complete biological specificity, such agents must be able to recognize duplex DNA in order to target individual gene sequences.[1]

The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are a family of DNA-interactive antitumor antibiotics derived from various *Streptomyces* species.[2] Well-known members include DC-81 (**1**) and tomaymycin (**2**) (Figure 1). They exert their biological activity through covalent binding via their N10−C11 imine/carbinolamine moiety to the C2-amino position of a guanine residue within the minor groove of DNA. PBD monomers span three DNA base pairs with a preference for Pu-G-Pu (where Pu = purine; G = guanine) sequences. Extensive studies have been carried out on both the solution-[3] and solid-phase[4] synthesis of PBDs, and a sound understanding of structure−activity relationships within the family has been developed.[5] The PBDs have been shown to interfere with the interaction of endonuclease enzymes with DNA[6] and to block transcription by inhibiting RNA polymerase in a sequence-specific manner,[7] processes which are thought to account for the biological activity

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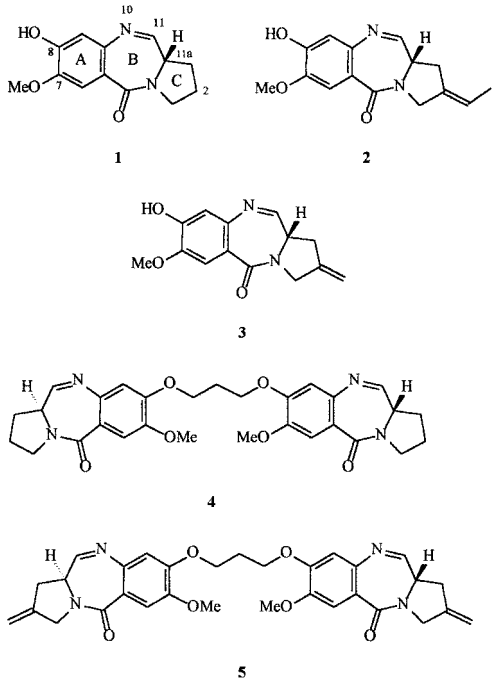


Figure 1. Structures of the PBD monomers DC-81 (**1**), tomaymycin (**2**), and C2-methylene DC-81 (**3**), and the PBD dimers **4** and **5**.

of the compounds. The PBDs have also been used as a scaffold to attach EDTA[8] and epoxide[9] moieties, leading to novel sequence-selective DNA cleaving and cross-linking agents, respectively. A homologous series of C8-diether-linked PBD dimers (e.g., **4**, Figure 1) has been synthesized, members of which exhibit potent in vitro cytotoxicity[10] and enhanced DNA-binding affinity and sequence-specificity compared to the natural product DC-81 (**1,** Figure 1).[11−13] This improved biological activity can be attributed to the ability of these compounds to cross-link DNA irreversibly, as demonstrated by a gel electrophoresis assay.[14] In addition, NMR spectroscopy[15] and molecular modeling studies[16] indicate that **4** spans six DNA base pairs, actively recognizing a 5′-GATC sequence. This represents an effective doubling of the DNA binding-site size of DC-81, which is known to be selective for Pu-G-Pu triplets.

Subsequent in vivo studies with **4** in the murine ADJ/PC6 plasmacytoma model were disappointing,[17] and the low therapeutic index observed was thought to be partly due to reaction of the molecule with cellular thiol-containing molecules prior to reaching the tumor site. PBD monomer natural products with unsaturation at the C2 position are known to be more biologically potent than their C2-saturated relatives. One explanation for this is that C2-*exo*-unsaturation can lead to lower electrophilicity at the N10−C11 position.[18,19] This may allow greater availability of the agent at the target DNA sequence due to a lower level of deactivation by cellular nucleophiles. For example, tomaymycin (**2**) is considerably more cytotoxic than DC-81 and binds more efficiently to DNA, although it is less electrophilic overall.[6,18,19] Therefore, it was of interest to synthesize and evaluate a PBD dimer with unsaturation at the C2/C2′ positions. Here, we report the synthesis and biological evaluation of an exquisitely potent bis(desmethyltomaymycin) PBD dimer, **5**.[20]

Results and Discussion

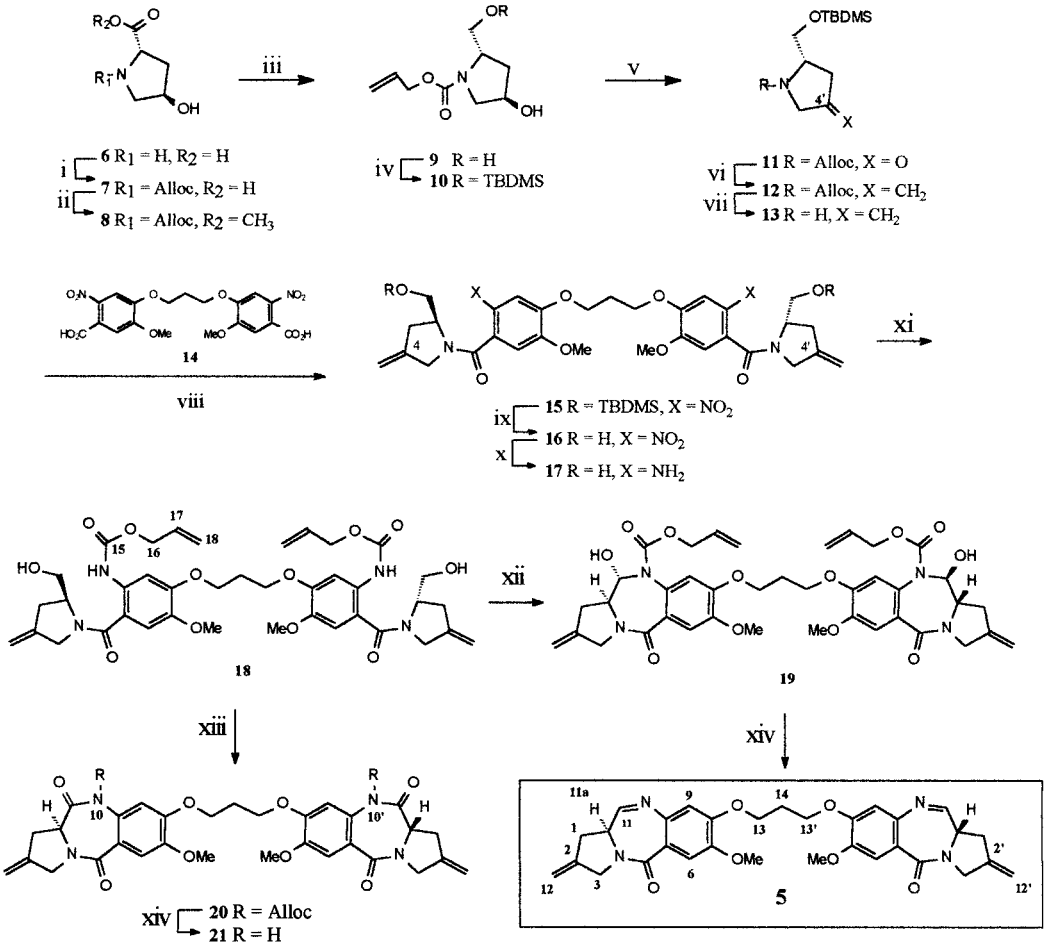
Chemistry. Synthesis of **5** was achieved by employing the B-ring cyclization strategy first reported by Fukuyama and co-workers.[21] First, commercially available *trans*-4-hydroxy-L-proline (**6**) was N-protected as the allyl carbamate **7** in 87% yield **(**Scheme 1).[22] Following esterification of **7** in modest yield (43%) using catalytic H_2SO_4 in refluxing MeOH, the methyl ester **8** was reduced to the diol **9** with $LiBH_4$ in almost quantitative yield. Differential silylation of the primary rather than secondary alcohol was achieved by employing DBU as a silyl transfer agent. Any disilylated product and unreacted diol were removed by column chromatography to provide the TBDMS ether **10** in 52% yield. Oxidation to the ketone **11** was achieved using either the Swern reaction or TPAP in the presence of NMO and 4 Å molecular sieves.[23] Both methods produced the ketone **11** in almost quantitative yield. The key C4/C4′- (i.e., pro-C2/C2′ for the final PBD structure) unsaturation was introduced by performing the Wittig reaction on ketone **11** to provide the olefin **12** in 87% yield. Initial attempts to deprotect **12** using PPh_3/Pd-$(PPh_3)_4$ in the presence of a suitable allyl scavenger (i.e., pyrrolidine,[24] dimedone,[25] or 2-ethylhexanoic acid[26]) were unsuccessful. Eventually, the Alloc group was cleaved by palladium-catalyzed hydrostannolysis with tributyltin hydride to provide amine **13** in 77% yield.[27]

The known PBD dimer core[13] **14** was converted to the corresponding acid chloride by treatment with oxalyl chloride followed by a catalytic amount of DMF. Subsequent coupling to **13** provided the bis-nitro-amide **15** in 74% yield. The TBDMS protecting groups were removed rapidly under mild conditions using TBAF in THF to afford the bis-nitro-alcohol **16** in 94% yield. Reduction of the nitro groups was achieved by employing $SnCl_2 \cdot 2H_2O$ in refluxing MeOH.[28] The resulting bis-aniline **17** was obtained in 61% yield with the C4/C4′-unsaturation intact. Following Alloc protection of the anilino moieties in 50% yield, **18** was subjected to Swern oxidation in an attempt to synthesize the protected carbinolamine **19**. Unfortunately, **18** was prone to over-oxidation to the tetralactam **20** under these conditions. Instead, the spontaneous oxidation/ring closure was achieved by treating **18** with TPAP/NMO/4 Å molecular sieves. Although the yield was modest (32%), the formation of tetralactam **20** was not observed using TPAP. Finally, treatment of **19** with $Pd(PPh_3)_4$/PPh_3/pyrrolidine[24] afforded the novel PBD dimer[20] **5** in 77% yield. Cleavage of the N10/N10′-Alloc protecting groups from **20** under identical conditions afforded **21**, the first example of a PBD tetralactam dimer, in 43% yield.

Biophysical Studies. (a) DNA Binding Studies. The DNA binding affinity of the novel C2-functionalized PBD dimers **5** and **21** was examined by thermal denaturation using calf thymus (CT) DNA.[6] Studies on **5** were carried out at [DNA]/[ligand] molar ratios of 100:1, 50:1, and 5:1. The increase in helix melting temperature (ΔT_m) for each ratio was examined after 0, 4, and 18 h incubation at 37 °C (Table 1). Data for **21**, **4**, and tomaymycin (**2**) are included in Table 1 for comparison.

The data clearly show that **5** is a highly efficient stabilizing agent for double-stranded calf thymus DNA. For a [ligand]/[DNA] molar ratio of 1:5, **5** elevates the helix melting temperature of CT DNA by an unprec-

Scheme 1[a]



[a] Reagents and conditions: (i) Alloc-Cl, aq. NaOH, THF, 0 °C, 87%; (ii) MeOH, H_2SO_4, Δ, 43%; (iii) $LiBH_4$, THF, 0 °C, 99%; (iv) TBDMS-Cl, TEA, DBU, CH_2Cl_2, 52%; (v) TPAP, NMO, 4 Å sieves, CH_2Cl_2−CH_3CN, 92%; or $(COCl)_2$, DMSO, TEA, CH_2Cl_2, −70 °C, 95%; (vi) Ph_3PCH_3Br, KO^{t}Bu, THF, 0 °C, 87%; (vii) Bu_3SnH, $Pd(PPh_3)_2Cl_2$, H_2O, CH_2Cl_2, 77%; (viii) $(COCl)_2$, DMF, THF then **13**, TEA, H_2O, 0 °C, 74%; (ix) TBAF, THF, 0 °C, 94%; (x) $SnCl_2 \cdot 2H_2O$, MeOH, Δ, 61%; (xi) Alloc-Cl, pyridine, CH_2Cl_2, 0 °C, 50%; (xii) TPAP, NMO, 4 Å sieves, CH_2Cl_2, CH_3CN, 32%; (xiii) $(COCl)_2$, DMSO, TEA, CH_2Cl_2, −45 °C 51%; (xiv) $Pd(PPh_3)_4$, PPh_3, pyrrolidine, CH_2Cl_2, CH_3CN, 0 °C, 77% for **5** and 43% for **21**.

Table 1. Thermal Denaturation Data for Tomaymycin (**2**), **4**, **5**, and **21** with Calf Thymus DNA

compound	[PBD]:[DNA] molar ratio[b]	induced ΔT_m (°C)[a] after incubation at 37 °C for 0 h	4 h	18 h
5	1:100	7.1	8.0	9.1
5	1:50	11.3	12.3	15.0
5[c]	1:5	25.7	31.9	33.6
21	1:5	0.78	–	–
4	1:5	10.2	13.1	15.1
tomaymycin (**2**)	1:5	0.97	2.38	2.56

[a] For CT-DNA alone at pH 7.00 ± 0.01, ΔT_m = 67.83 ± 0.06 °C (mean value from 30 separate determinations). All ΔT_m values are ±0.1−0.2 °C. [b] For a 1:5 molar ratio of [ligand]/[DNA], calf thymus DNA concentration = 100 μM and ligand concentration = 20 μM in aqueous sodium phosphate buffer [10 mM sodium phosphate + 1 mM EDTA, pH 7.00 ± 0.01]. [c] The ΔT_m for **5** at a [ligand]:[DNAp] molar ratio of 1:5 increased to a value of 34.4 °C after 72 h incubation.

edented 33.6 °C after incubation at 37 °C for 18 h. In the same experiment, the DC-81-based dimer **4** gives a ΔT_m of 15.1 °C, illustrating the significant effect of introducing C2/C2′-unsaturation. As usually observed for the PBD dimers, **5** exerts most of its effect upon the GC-rich or high-temperature regions of the DNA melting curves. In a fashion similar to **4**, **5** provides some 60−80% of its stabilizing effect without prior incubation, suggesting a kinetic effect in the PBD reactivity profile. However, the comparative ΔT_m curves (not shown) indicate that, on a concentration basis alone, **5** is ≥10-fold more efficient than **4**. Even at a [DNA]/[ligand] molar ratio of 100:1, **5** exhibits significantly greater DNA-binding affinity than that for the corresponding monomer tomaymycin at a lower 5:1 molar ratio. Furthermore, after 72 h incubation at a [DNA]/[ligand] ratio of 5:1, the ΔT_m for **5** increases to a value of 34.4 °C, suggesting a plateau saturation level for covalent DNA interaction. Although the tetralactam **21** is significantly less effective at stabilizing DNA than **5**, the results confirm that it does provide some helix stabilization, elevating the DNA melting temperature by 0.78 °C. This result clearly demonstrates that replacement of the electrophilic N10−C11/N10′−C11′ bis-imine moieties present in **5** with bis-lactam functionalities abolishes covalent DNA-interaction, presumably leaving only the noncovalent component of interaction. This phenomenon has been previously observed with PBD monomers.[29]

(b) DNA Cross-Linking Studies. The DNA cross-linking efficiency of **5** was investigated using an assay involving linear double-stranded DNA derived from the

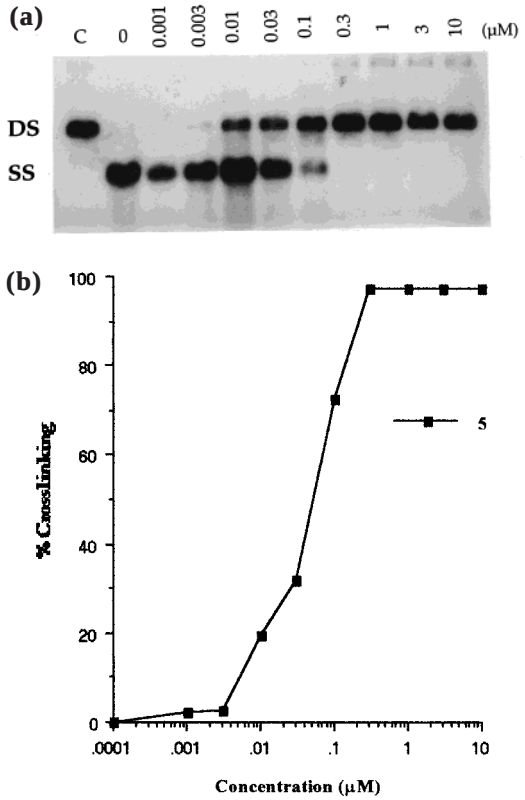


Figure 2. (a) Autoradiograph of a neutral agarose gel showing DNA interstrand cross-linking by **5** in linear ^{32}P-end-labeled pBR322 DNA following a 2 h incubation at 37 °C. The lanes are as follows: C, double-stranded DNA control; 0, single-stranded DNA control; and 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, 10.0 μM **5**. DS and SS are double- and single-stranded DNA, respectively. (b) Concentration dependence of DNA cross-linking for **5** in linear ^{32}P-end-labeled pBR322 DNA from gel in Figure 2a.

Table 2. DNA Cross-Linking Data for **4**, **5**, and Melphalan with pBR322 DNA

compound	XL_{50}[a] (μM)
4	0.055
5	0.045
melphalan	20.0

[a] Concentration of agent required for 50% cross-linking of pBR322 DNA.

plasmid pBR322 (4632 bp, linearized with *Hind* III and then ^{32}P-end-labeled).[14] Following denaturation conditions which completely separate the DNA strands, the presence of an interstrand cross-link results in renaturation to double-stranded DNA during electrophoresis in a neutral agarose gel.

The highly efficient DNA cross-linking ability of **5** is illustrated in the autoradiograph in Figure 2a which is quantitated by laser densitometry in Figure 2b. After 2 h incubation at 37 °C, cross-linking is measurable above background at 0.001 μM, with 100% cross-linking at doses of >0.3 μM. The concentration required to produce 50% double-stranded (i.e., interstrand cross-linked) DNA was calculated from Figure 2b and is shown in Table 2. Compound **5** is a more efficient cross-linking agent than **4** (XL_{50} values: 0.045 μM versus 0.055 μM) and is 440-fold more efficient than the major groove cross-linking agent melphalan.

Molecular Modeling of Interstrand Cross-Link Formation. Studies on the energetics of binding were carried out using an established protocol previously used to examine the interactions of DNA with both reversible and irreversible groove-binding agents.[9,16,30−32] Helical analysis shows that upon binding to **5** the DNA maintains its B-like conformation with the helical rise for the adduct between DNA and **5** being 3.55 Å and the mean helical rotation, 37.0° (9.7 bp/turn). This suggests that there is little or no disruption of DNA secondary structure upon adduct formation and cross-linking and that base-pairing or induced distortion effects are absent (see Figure 3). This behavior has been reported previously for the d(CIC***G****ATC*ICG)$_2$−**4** complex.[16] Both **4** and **5** are well accommodated within the minor groove, such that very little of either molecule remains exposed beyond the periphery of the host DNA duplex (Figure 3). This feature may be responsible for the observed resistance to repair enzymes that are reliant upon tracing distortion or helical perturbation in DNA induced by most alkylating agents.

The calculated binding energies (Table 3) show that PBDs with unsaturation in the C-ring (i.e., **5** and the hypothetical *exo*-CH_2 monomer, **3**, Figure 1) form more-favorable adducts than analogues with fully saturated C-rings (i.e., **4** and DC-81, respectively). Introduction of the exocyclic methylene group at the C2 position, where the $sp^3 \rightarrow sp^2$ hybridization change stiffens the C-ring, leads to a more planar arrangement that enables this portion of the molecule to fit more snugly in the minor groove. Importantly, atomic clash with the groove walls is ameliorated by the superior isohelical matching of the ligand with the groove contours.

Covalent interaction between the DNA and each dimer is approximately twice as favorable as that for the corresponding monomers, reflecting their difunctional character and the tolerance afforded to the inert 1,3-propanedioxy linker present between the PBD A-rings. Both halves of the dimer behave in an essentially identical fashion without disruption of the host DNA integrity. The flexible linker between the A-rings of each subunit makes favorable non-bonding contacts with the floor and walls of the minor groove which enhances overall binding.

Both DC-81 (**1**) and the C2-*exo*-unsaturated monomer (**3**) generally prefer to bind with their aromatic A-rings oriented toward the 3′-end of the alkylated DNA strand (i.e., 3*S*), although this is sensitive to sequence context. In contrast, the spatially separated PBD units in each dimer prefer to adopt the alternative 5*S* orientation. This difference largely reflects the superior hydrogen bonding capability in the 5*S*-5*S* or "reverse" cross-link configuration. Interestingly, this 5*S*-5*S*/3*S*-3*S* preference is very marked (13 kcal mol^{-1}) for the more discriminating **4**, although the differential is absent for the more reactive **5** where formation of forward (5′-AGATCT) and reverse (5′-TCTAGA) cross-links are essentially isoenergetic. The different behaviors predicted for cross-linking suggest that the DNA reactivity profiles are particularly sensitive to the isohelical characteristics of the dimer molecules.

In Vitro Cytotoxicity in Cisplatin-Resistant Cell Lines. The cytotoxicity of **5** in a panel of human ovarian cell lines (SKOV-3, A2780/A2780*cis*R, and CH1/CH1*cis*R) was determined using the 96 h continuous exposure sulforhodamine B (SRB) growth delay assay.[33] The cell

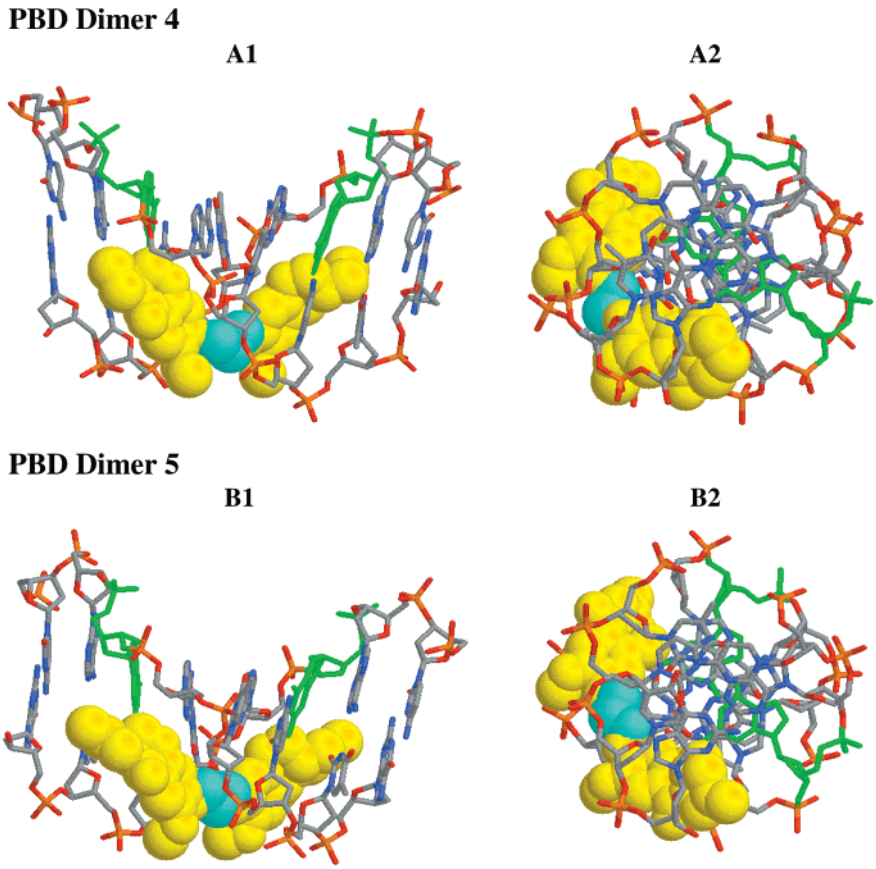


Figure 3. Energy-minimized models for the minor groove cross-linked adducts formed with d(CGCA***G****ATC*TGCG)$_2$ and either **4** (A1, A2) or the more extended dimer **5** (B1, B2). Only the central eight base pairs are shown for each structure with all hydrogen atoms removed for clarity. The bound molecules are highlighted in space fill; color scheme: PBD subunits (yellow), inert ligand C8−C8′ diether linkage (cyan), and alkylated guanine bases (green). Panels A2 and B2 represent views looking down the long dyad or *z*-axis of the DNA duplex, showing the effective burial of the dimer molecules within the minor groove conduit. The only exposed molecular fragments are the 7-methoxy substituents in each PBD A-ring.

Table 3. Interaction Energies for DNA Cross-Linking by the PBD Dimers **4** and **5** and for Monoalkylation by the Corresponding PBD Monomers **1** and **3** Using Defined Oligonucleotide Duplex Targets

DNA sequence[a]	PBD cmpd	ΔE_{vdW}[b] kcal mol^{-1}	ΔE_{elec}[b] kcal mol^{-1}	ΔE_{HB}[b] kcal mol^{-1}	E_{bind}[b] kcal mol^{-1}
5′-CGCA***G****ATC*TGCG	**4**	−53.0	−38.1	−4.6	−88.5
	5	−58.5	−43.7	−5.7	−104.1
	1[c]	−29.1/−25.7	−20.6/−14.0	−0.9/−2.8	−48.0/−40.5
	3[c]	−30.0/−24.2	−22.6/−24.1	−1.3/−2.8	−51.5/−48.7
5′-CGCT*CTA****G***AGCG	**4**	−62.5	−39.7	−3.4	−101.4
	5	−60.1	−46.5	−2.7	−104.3
	1[c]	−27.5/−28.5	−17.2/−21.6	−3.0/−2.5	−45.6/−50.2
	3[c]	−25.1/−29.1	−22.7/−23.6	−2.8/−2.0	−48.8/−52.0
5′-CGCA***G****A*GCG	**1**[c]	−17.5/−26.3	−13.0/−6.3	−2.2/−2.0	−31.7/−34.8
	3[c]	−27.1/−23.0	−33.1/−23.2	−2.1/−2.5	−61.2/−48.0

[a] Sequence for one strand of the modeled host DNA duplexes, showing the alkylated guanine and the spanned cross-linking site for the PBD dimers. The embedded 5′-A***G****ATC*T and 5′-T*CTA****G***A tracts present twin optimal 5′-*AGA* sites for the PBD monomers for "forward" (3*S*-3*S*) and "reverse" (5*S*-5*S*) cross-linking, respectively (see text).Where equivalent sites are present in the sequence, only one site was examined for the PBD monomers. [b] ΔE_{vdW}, ΔE_{elec}, and ΔE_{HB} are the calculated van der Waals, electrostatic, and H-bonded energy contributions to the overall binding interaction energy (E_{bind}) as defined by $E_{bind} = E_{(adduct)} - [E_{(free\ DNA)} + E_{(free\ compound)}]$ where each term refers to the energy-minimized covalent adduct or native reactant. [c] Energy values refer to the alternative 3*S* or 5*S* alignments of the PBD monomer with respect to the covalently modified guanine.

lines were chosen on the basis of their inherent sensitivity to the DNA cross-linking agent cisplatin and the availability of sub-lines with acquired resistance to cisplatin. IC_{50} values for human leukemic K_{562} were obtained using the microculture tetrazolium (MTT) assay.[34]

The IC_{50} values obtained for **5** (Table 4) show that it is exquisitely cytotoxic, exhibiting the lowest IC_{50} values so far obtained for any synthetic PBD monomer or dimer. In each cell line, **5** is significantly more potent than either **4** or the clinically used antitumor agent cisplatin. In the CH1 cell line, **4** is approximately 6-fold more active than cisplatin, whereas **5** is 1500-fold more potent. It is generally assumed that an interstrand cross-link, if not repaired, will interfere with the process of DNA replication and lead to cell death. The cisplatin-resistant sub-line CH1*cis*R owes its resistance to the enhanced repair of platinum-DNA adducts. Whereas **4**

Table 4. In Vitro Cytotoxicity Data for the PBD Dimers **4** and **5**, the Tetralactam **21**, and Cisplatin in Cisplatin-Resistant Cell Lines

	IC_{50} (μM)[a]			
cell line	**5**	**21**	**4**	cisplatin
SKOV-3	0.0091	28	0.74	10.5
A2780	0.0000225	2.5	0.0072	0.265
A2780*cis*R	0.000024	11	0.21	8.4
RF[b]	1.1	4.4	29.2	32
CH1	0.00012	0.57	0.033	0.18
CH1*cis*R	0.0006	2.9	0.022	1.10
RF[b]	5	3.5	0.7	6.1
K_{562}[c]	0.0425	ND	0.25	ND

[a] Dose of PBD required to inhibit cell growth by 50% compared to PBD-free controls. The cells were incubated with the compounds for 96 h at 37 °C. [b] RF is the resistance factor (IC_{50} resistant/parent). [c] K_{562} is a human leukemia cell line in which IC_{50} values were measured using an MTT assay following a 1 h exposure to drug; ND = not determined.

showed no cross-resistance in the CH1*cis*R line, **5** and cisplatin gave resistance factors of 5 and 6.1, respectively. Conversely, the potency of **5** is maintained in the human chronic myelogenous cell line K_{562}, whereas **4** is approximately 6-fold less effective. The most significant results were obtained with the human ovarian carcinoma cell lines SKOV-3 and A2780/A2780*cis*R. The IC_{50} values for **5** in the A2780/A2780*cis*R pair of cell lines are extremely low (~0.00002 μM) and very little cross-resistance is observed (RF = 1.1). In contrast, **4** shows potent cytotoxicity in the parent A2780 line but, like cisplatin, experiences almost 30-fold resistance in the A2780*cis*R cells (RF = 29.2). The A2780*cis*R subline is known to possess elevated GSH levels, an increased level of repair of DNA-cisplatin adducts, and a decreased ability to uptake cisplatin.[33]

Similar results were obtained with the intrinsically cisplatin-resistant SKOV-3 cell line, which possesses levels of glutathione 4-fold higher than cisplatin-sensitive ovarian carcinoma cells.[35] It is thought that **4** failed in initial in vivo studies due to its interaction with biological nucleophiles, such as glutathione or thiol-containing proteins in the blood and tissue.[17] Unlike **4**, the behavior of **5** in SKOV-3 and A2780 *cis* R suggests that the potency of the drug is unaffected by high cellular GSH levels as predicted during the design of the molecule. In summary, these results highlight the effect that C2-unsaturation has on in vitro cytotoxicity and suggest that **5** should perform significantly better than **4** in in vivo studies.[17]

Surprisingly, the tetralactam **21** exhibits limited cytotoxicity in most of the ovarian carcinoma cell lines. The IC_{50} of 0.57 μM in the CH1 cell line is particularly striking. This activity is in accord with the thermal denaturation data; presumably the cytotoxic effects are due to noncovalent interaction with DNA, a phenomenon previously observed with the PBD monomers.[29] Furthermore, comparison of the IC_{50} data for **5** and **21** clearly demonstrates that, for maximum cytotoxicity, an electrophilic imine or carbinolamine moiety is essential at the N10−C11 position of the PBD units.

Conclusions

Introduction of C2-*exo*-unsaturation into the C-rings of PBD dimer **4** to give **5** leads to a significant increase in both DNA cross-linking efficiency and the ability to stabilize double-stranded DNA as evaluated by thermal denaturation studies. Modeling studies suggest that this is due to a flattening of the C-rings by the C2/C2′-methylene groups, leading to a more snug fit in the minor groove and a lower-energy adduct. This appears to be reflected in the exquisite cytotoxicity of **5**, particularly toward some cisplatin-resistant cell lines, where it is possible that the low-energy non-distortive adducts formed remain invisible to the DNA repair surveillance systems. Furthermore, C2-*exo*-unsaturation is known to reduce the electrophilicity of PBD molecules, thus decreasing the likelihood of collateral interactions with thiol-containing proteins and other nucleophilic molecules such as glutathione.[18,19] This suggests that **5** may have greater bioavailablity than **4**.

In addition to the significant in vitro cytotoxicity in cisplatin-resistant ovarian cell lines (e.g., IC_{50} = 0.024 nM in A2780*cis*R), selective cytotoxicity has been observed for **5** in the NCI's 60 cell line screen. In addition, significant antitumor activity has been achieved in both the Hollow Fiber assay and a number of human tumor xenografts, and these data will be reported elsewhere. Compound **5** is currently in preclinical development[36] and has potential in a number of human cancers including cisplatin-resistant ovarian disease.

Experimental Section

Synthetic Chemistry. Reaction progress was monitored by thin-layer chromatography (TLC) using GF_{254} silica gel, with fluorescent indicator on glass plates. Visualization was achieved with UV light and iodine vapor unless otherwise stated. Flash chromatography was performed using 14 cm of J.T. Baker 30−60 μm silica gel. The majority of reaction solvents were purified by distillation under nitrogen from the indicated drying agent and used fresh: dichloromethane (calcium hydride), tetrahydrofuran (sodium benzophenone ketyl), methanol (magnesium methoxide), acetonitrile (calcium hydride), and toluene (sodium benzophenone ketyl). Extraction and general chromatography solvents were bought and used without further purification from J.T. Baker. All organic chemicals were purchased from Aldrich Chemical Co. Drying agents and inorganic reagents were purchased from BDH.

IR spectra were recorded with a Perkin-Elmer FT/IR-Paragon 1000 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a JEOL GSX 270 MHz (67.8 MHz for ^{13}C NMR spectra) FT-NMR operating at 20 °C ± 1 °C. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Spin multiplicities are described as s (singlet), br s (broad singlet), d (doublet), br d (broad doublet), t (triplet), q (quartet), quint (quintet), or m (multiplet). NMR interpretations are quoted for the pro-PBD numbering system (see Figure 1). Mass spectra were recorded on a JEOL JMS-DX 303 GC-mass spectrometer. Electron impact (EI) mass spectra were measured at 70 eV, chemical ionization (CI) spectra were obtained using isobutane as reagent gas, and fast atom bombardment (FAB) spectra were recorded using thioglycerol as a matrix. Accurate molecular masses were determined by peak matching using perfluorokerosene (PFK) as an internal standard. Optical rotations were measured at ambient temperature using a Bellingham and Stanley ADP 220 polarimeter.

(2*S*,4*R*)-*N*-(Allyloxycarbonyl)-4-hydroxypyrrolidine-2-carboxylic Acid (7). A solution of allyl chloroformate (29.2 mL, 33.2 g, 275 mmol) in THF (30 mL) was added dropwise to a suspension of *trans*-4-hydroxy-L-proline (**6**; 30 g, 229 mmol) in a mixture of THF (150 mL) and H_2O (150 mL) at 0 °C (ice/acetone), while maintaining the pH at 9 with 4 M NaOH. After being stirred at 0 °C for 1 h, the mixture was saturated with solid NaCl and extracted with EtOAc (100 mL). The aqueous phase was separated, washed with further EtOAc (100 mL),

and then adjusted to pH 2 with 12 M HCl. The resulting milky emulsion was extracted with EtOAc (2 × 100 mL), washed with brine (200 mL), dried ($MgSO_4$), filtered, and evaporated in vacuo to give the allyl carbamate **7** as a viscous oil (42.6 g, 87%): $[\alpha]^{20}_D = -62.1°$ ($c = 0.69$, MeOH); ^{1}H NMR ($CDCl_3$ + DMSO-d_6) (rotamers) δ 5.98−5.81 (m, 1H), 5.40−5.14 (m, 2H), 4.64−4.42 (m, 4H, Alloc, H2 and H11a), 3.82−3.51 (m, 2H, H3), 2.34−2.08 (m, 2H, H1); ^{13}C NMR ($CDCl_3$ + DMSO-d_6) (rotamers) δ 175.0/174.5 (C11), 155.1/154.6 (C5), 132.9/132.8, 117.6/116.7, 69.5/68.8 (C2), 65.9/65.8, 58.0/57.7 (C11a), 55.0/54.5 (C3), 39.3/38.3 (C1); MS (EI) *m/z* (rel intensity) 215 ($[M]^{+\bullet}$, 10) 197 (12), 170 (100), 152 (24), 130 (97), 126 (34), 112 (50), 108 (58), 86 (11), 68 (86), 56 (19); IR (neat) 3500−2100 (br), 1745, 1687, 1435, 1415, 1346, 1262, 1207, 1174, 1133, 1082 cm^{-1}; HRMS $[M]^{+\bullet}$ Calcd for $C_9H_{13}NO_5$ *m/z* 215.0794, Obsd *m/z* 215.0791.

Methyl (2*S*,4*R*)-*N*-(Allyloxycarbonyl)-4-hydroxypyrrolidine-2-carboxylate (8). A solution of **7** (43 g, 200 mmol) in MeOH (300 mL) was treated with concentrated H_2SO_4 (4.5 mL) and heated at reflux for 2 h. After cooling to room temperature, the reaction mixture was treated with TEA (43 mL) and concentrated in vacuo. The residue was dissolved in EtOAc (300 mL), washed with brine (200 mL), dried ($MgSO_4$), filtered, and evaporated under reduced pressure to give a viscous oil. Purification by flash chromatography (40% EtOAc/petroleum ether 40°−60°) gave the pure ester **8** as a yellow oil (19.6 g, 43%): $[\alpha]^{23}_D = -79.0°$ ($c = 0.35$, $CHCl_3$); ^{1}H NMR ($CDCl_3$) (rotamers) δ 5.98−5.78 (m, 1H), 5.35−5.16 (m, 2H), 4.65−4.45 (m, 4H, Alloc, H2 and H11a), 3.75/3.72 (s, 3H, CO_2CH_3), 3.70−3.54 (m, 2H, H3), 3.13/3.01 (br s, 1H, O*H*), 2.39−2.03 (m, 2H, H1); ^{13}C NMR ($CDCl_3$) (rotamers) δ 173.4/173.2 (C11), 155.0/154.6, 132.6/132.4, 117.6/117.3, 70.0/69.2 (C2), 66.2, 57.9/57.7 (C11a), 55.2/54.6 (C3), 52.4 (CO_2CH_3), 39.1/38.4 (C1); MS (EI) *m/z* (rel intensity) 229 ($[M]^{+\bullet}$, 7), 170 (100), 144 (12), 126 (26), 108 (20), 68 (7), 56 (8); IR (neat) 3438 (br), 2954, 1750, 1694, 1435, 1413, 1345, 1278, 1206, 1130, 1086 cm^{-1}; HRMS $[M]^{+\bullet}$ Calcd for $C_{10}H_{15}NO_5$ *m/z* 229.0950, Obsd *m/z* 229.0940.

(2*S*,4*R*)-*N*-(Allyloxycarbonyl)-4-hydroxy-2-(hydroxymethyl)pyrrolidine (9). A solution of ester **8** (19.5 g, 85 mmol) in THF (326 mL) was cooled to 0 °C and treated portion-wise with $LiBH_4$ (2.78 g, 128 mmol). The stirred reaction mixture was allowed to warm to room temperature over 2.5 h under a N_2 atmosphere, after which TLC (50% EtOAc/petroleum ether 40°−60°) revealed the complete consumption of starting material. The mixture was cooled to 0 °C and carefully treated with water (108 mL) and then 2 M HCl (54 mL). After concentration in vacuo, the mixture was adjusted to pH 7 with 10 M NaOH, saturated with solid NaCl, and then extracted with EtOAc (5 × 100 mL). The combined organic phase was washed with brine (200 mL), dried ($MgSO_4$), filtered, and evaporated in vacuo to furnish the pure diol **9** as a colorless oil (16.97 g, 99%): $[\alpha]^{24}_D = -57.0°$ ($c = 0.61$, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 6.01−5.87 (m, 1H), 5.36−5.20 (m, 2H), 4.84 (br s, 1H, H11), 4.60 (d, 2H, $J = 5.31$ Hz), 4.39 (br s, 1H, H11), 4.18−4.08 (m, 1H, H2), 3.90−3.35 (m, 4H, H3, H11a, and O*H*), 3.04 (br s, 1H, O*H*), 2.11−2.03 (m, 1H, H1), 1.78−1.69 (m, 1H, H1); ^{13}C NMR ($CDCl_3$) δ 157.1, 132.6, 117.7, 69.2 (C2), 66.4/66.2 (Alloc and C11), 59.2 (C11a), 55.5 (C3), 37.3 (C1); MS (EI) *m/z* (rel intensity) 201 ($[M]^{+\bullet}$, 2), 170 (100), 144 (6), 126 (26), 108 (20), 68 (9); IR (neat) 3394 (br, OH), 2946, 2870, 1679, 1413, 1339, 1194, 1126, 1054 cm^{-1}; HRMS $[M]^{+\bullet}$ Calcd for $C_9H_{15}NO_4$ *m/z* 201.1001, Obsd *m/z* 201.1028.

(2*S*,4*R*)-*N*-(Allyloxycarbonyl)-2-(*tert*-butyldimethylsilyloxymethyl)-4-hydroxypyrrolidine (10). A solution of diol **9** (16.97 g, 84 mmol) and TEA (11.7 mL, 8.5 g, 84 mmol) in CH_2Cl_2 (235 mL) was allowed to stir for 15 min at room temperature and then treated with *tert*-butyldimethylsilyl chloride (TBDMSCl, 9.72 g, 64 mmol) and DBU (16.8 mmol, 2.51 mL, 2.56 g). The reaction mixture was allowed to stir for a further 16 h under a N_2 atmosphere. After dilution with EtOAc (500 mL), the organic phase was washed with saturated aqueous NH_4Cl (160 mL) and brine (160 mL), then dried ($MgSO_4$), filtered, and evaporated in vacuo to give an oil. TLC (50% EtOAc/ petroleum ether 40°−60°) revealed the presence of the major product, unreacted diol, and the presumed disilylated derivative. Flash chromatography (20−100% EtOAc/ petroleum ether 40°−60°) gave the monosilylated compound **10** as a pale yellow oil (13.85 g, 52%): $[\alpha]^{21}_D = -58.6°$ ($c = 1.14$, $CHCl_3$); ^{1}H NMR ($CDCl_3$) (rotamers) δ 6.01−5.86 (m, 1H), 5.34−5.18 (m, 2H), 4.59−4.49 (m, 3H, Alloc and H11a), 4.06−3.50 (m, 5H, H3, H2 and H11), 2.20−2.01 (m, 2H, H1), 0.87 (s, 9H, SiC(CH_3)$_3$), 0.00 (s, 6H, Si(CH_3)$_2$); ^{13}C NMR ($CDCl_3$) (rotamers) δ 155.0, 133.1, 117.6/117.1, 70.3/69.7 (C2), 65.9/65.6, 63.9/62.8 (C11), 57.8/57.4 (C11a), 55.7/55.2 (C3), 37.3/36.6 (C1), 25.9 (SiC(CH_3)$_3$), 18.2 (SiC(CH_3)$_3$), −5.5 (Si(CH_3)$_2$); MS (EI) *m/z* (rel intensity) 316 ($[M + H]^{+\bullet}$, 29), 315 ($[M]^+$, 4), 300 (26), 284 (4), 261 (8), 260 (50), 259 (100), 258 (100), 218 (13), 215 (10), 214 (52), 200 (12), 170 (100), 156 (40), 126 (58), 115 (33), 108 (41), 75 (35); IR (neat) 3422 (br), 2954, 2858, 1682, 1467, 1434, 1412, 1358, 1330, 1255, 1196, 1180, 1120, 1054 cm^{-1}; HRMS $[M]^{+\bullet}$ Calcd for $C_{15}H_{29}NO_4Si$ *m/z* 315.1866, Obsd *m/z* 315.1946.

(2*S*)-*N*-(Allyloxycarbonyl)-2-(*tert*-butyldimethylsilyloxymethyl)-4-oxopyrrolidine (11). Method A. A solution of DMSO (12.9 mL, 14.3 g, 183 mmol) in CH_2Cl_2 (90 mL) was added dropwise to a solution of oxalyl chloride (45.1 mL of a 2.0 M solution in CH_2Cl_2, 90.2 mmol) at −60 °C (dry ice/ acetone) under a N_2 atmosphere. After the mixture was stirred at −70 °C for 30 min, a solution of the alcohol **10** (25.8 g, 81.9 mmol) in CH_2Cl_2 (215 mL) was added dropwise at −60 °C. The reaction mixture was allowed to stir for 1.5 h at −70 °C and was then treated dropwise with TEA (57.2 mL, 41.5 g, 410 mmol) and allowed to warm to 10 °C. The reaction mixture was then treated with brine (150 mL) and acidified to pH 3 with 12 M HCl. The organic phase was separated and washed with brine (200 mL), then dried ($MgSO_4$), filtered, and concentrated in vacuo. Purification of the resulting orange oil by flash chromatography (40% EtOAc/petroleum ether 40°−60°) furnished the ketone **11** as a pale yellow oil (24.24 g, 95%).

Method B. A solution of **10** (4.5 g, 14.3 mmol) in CH_2Cl_2 (67.5 mL) was treated with CH_3CN (7.5 mL), powdered molecular sieves (4 Å, 3.54 g), and NMO (2.4 g, 20.5 mmol). After the mixture was stirred for 15 min at room temperature, TPAP (0.24 g, 0.7 mmol) was added to the reaction mixture, and a green to black color change was observed. The reaction mixture was stirred for a further 2.5 h until TLC (50% EtOAc/ petroleum ether 40°−60°) indicated complete consumption of starting material. Concentration of the reaction mixture in vacuo followed by flash chromatography (50% EtOAc/petroleum ether) gave the pure ketone **11** as a yellow oil (4.1 g, 92%): $[\alpha]^{22}_D = +1.25°$ ($c = 10.0$, $CHCl_3$); ^{1}H NMR ($CDCl_3$) (rotamers) δ 6.00−5.90 (m, 1H), 5.35−5.22 (m, 2H), 4.65−4.63 (m, 2H), 4.48−4.40 (m, 1H, H11a), 4.14−3.56 (m, 4H, H3 and H11), 2.74−2.64 (m, 1H, H1trans), 2.46 (d, 1H, $J = 18.7$ Hz, H1cis), 0.85 (s, 9H, SiC(CH_3)$_3$), 0.00 (s, 6H, Si(CH_3)$_2$); ^{13}C NMR ($CDCl_3$) (rotamers) δ 210.1 (C2), 154.1, 132.7, 118.0/117.7, 66.0/65.8, 65.0 (C11), 55.7 (C11a), 53.6 (C3), 40.8/40.1 (C1), 25.7 (SiC(CH_3)$_3$), 18.1 (SiC(CH_3)$_3$), −5.7/-5.8 (Si(CH_3)$_2$); MS (CI) *m/z* (rel intensity) 314 ($[M + H]^{+\bullet}$, 100), 256 (65); IR (neat) 2930, 2858, 1767, 1709, 1409, 1362, 1316, 1259, 1198, 1169, 1103, 1016 cm^{-1}; HRMS $[M]^{+\bullet}$ Calcd for $C_{15}H_{27}NO_4Si$ *m/z* 313.1710, Obsd *m/z* 313.1714.

(2*S*)-*N*-(Allyloxycarbonyl)-2-(*tert*-butyldimethylsilyloxymethyl)-4-methylidenepyrrolidine (12). Potassium *tert*-butoxide (41.0 mL of a 0.5 M solution in THF, 20.5 mmol) was added dropwise to a suspension of methyltriphenylphosphonium bromide (7.29 g, 20.4 mmol) in THF (20 mL) at 0 °C under a N_2 atmosphere. After the mixture was stirred for 2 h at 0 °C, a solution of ketone **11** (3.20 g, 10.2 mmol) in THF (10 mL) was added dropwise, and the reaction mixture was allowed to warm to room temperature. After being stirred for 30 min, the reaction mixture was diluted with EtOAc (150 mL) and water (150 mL), and the organic layer was separated, washed with brine, dried ($MgSO_4$), filtered, and evaporated in vacuo to give a yellow oil. Purification by flash chromatography (5% EtOAc/petroleum ether 40°−60°) gave the pure olefin **12** as a colorless oil (2.76 g, 87%): $[\alpha]^{21}_D = -22.2°$ ($c =$

0.25, $CHCl_3$); ^1H NMR ($CDCl_3$) (rotamers) δ 6.02−5.87 (m, 1H), 5.31 (ddd, 1H, J = 1.7, 3.1, 17.2 Hz), 5.21 (dd, 1H, J = 1.5, 10.4 Hz), 4.99−4.61 (m, 2H, H12), 4.60 (d, 2H, J = 4.9 Hz), 4.19−3.98 (m, 2H, H11), 3.93−3.87 (m, 1H, H11a), 3.66−3.42 (m, 2H, H3), 2.80−2.56 (m, 2H, H1), 0.87 (s, 9H, SiC(CH_3)$_3$), 0.03−0.02 (m, 6H, Si(CH_3)$_2$); ^{13}C NMR ($CDCl_3$) (rotamers) δ 154.4, 145.1/144.1 (C2), 133.1 (C7), 117.5/117.2, 107.5/106.9 (C12), 65.8/65.6, 63.7/63.1 (C11), 58.7/58.3 (C11a), 51.1 (C3), 34.9/34.2 (C1), 25.8 (SiC(CH_3)$_3$), 18.2 (SiC(CH_3)$_3$), −5.5 (Si(CH_3)$_2$); MS (CI) m/z (rel intensity) 312 ([M + H]$^{+\bullet}$, 82), 296 (9), 279 (5), 255 (20), 254 (100), 168 (8), 122 (14); IR (neat) 3083, 2954, 2847, 1709, 1533, 1467, 1404, 1360, 1310, 1252, 1207, 1174, 1103, 1076, 1006 cm^{-1}; HRMS [M + H]$^{+\bullet}$ Calcd for $C_{16}H_{30}NO_3Si$ m/z 312.1995, Obsd (FAB) m/z 312.1976.

(2*S*)-2-(*tert*-Butyldimethylsilyloxymethyl)-4-methylidenepyrrolidine (13). A catalytic amount of $PdCl_2(PPh_3)_2$ (92 mg, 0.131 mmol) was added to a mixture of the allyl carbamate **12** (1.0 g, 3.22 mmol) and H_2O (0.34 mL, 18.9 mmol) in CH_2Cl_2 (30 mL). After the mixture was stirred for 5 min at room temperature, Bu_3SnH (0.96 mL, 1.04 g, 3.57 mmol) was added rapidly in one portion. A mildly exothermic reaction occurred immediately with vigorous gas evolution. The mixture was then stirred for 16 h at room temperature under N_2 when TLC (50% EtOAc/petroleum ether 40°−60°) revealed formation of the free amine. After dilution with CH_2Cl_2 (30 mL), the solution was dried ($MgSO_4$), filtered, and evaporated in vacuo to give an orange oil. Purification by flash chromatography (50−100% EtOAc/petroleum ether 40°−60°) afforded the pyrrolidine **13** as a pale orange oil (0.56 g, 77%): $[\alpha]^{21}_D = -3.90°$ (c = 5.0, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 4.93/4.90 (2 × t, each 1H, J = 2.0 Hz, H12), 3.68−3.46 (m, 4H, H11 and H3), 3.30−3.21 (m, 1H, H11a), 2.53−2.41 (m, 2H, H1 and NH), 2.26−2.17 (m, 1H, H1), 0.90 (s, 9H, SiC(CH_3)$_3$), 0.06 (s, 6H, Si(CH_3)$_2$); ^{13}C NMR ($CDCl_3$) δ 150.0 (C2), 104.7 (C12), 64.7 (C11), 60.5 (C11a), 51.3 (C3), 34.9 (C1), 25.9 (SiC(CH_3)$_3$), 18.3 (SiC(CH_3)$_3$), −5.4 (Si(CH_3)$_2$); MS (EI) m/z (rel intensity) 227 ([M]$^{+\bullet}$, 8), 212 (6), 170 (36), 96 (8), 82 (100), 75 (11); IR (neat) 3550−3100, 3074, 2929, 2857, 1664, 1472, 1424, 1391, 1380, 1361, 1255, 1190, 1101, 1006, cm^{-1}; HRMS [M + H]$^{+\bullet}$ Calcd for $C_{12}H_{26}NOSi$ m/z 228.1784, Obsd (FAB) m/z 228.1777.

(2*S*)-1,1′-[[(Propane-1,3-diyl)dioxy]-bis[(2-nitro-5-methoxy-1,4-phenylene)carbonyl]]-bis[2-(*tert*-butyldimethylsilyloxymethyl)-4-methylidenepyrrolidine] (15). A catalytic amount of DMF (2 drops) was added to a solution of dimer acid[13] **14** (0.66 g, 1.42 mmol) and oxalyl chloride (0.31 mL, 0.45 g, 3.55 mmol) in THF (12 mL), and the mixture was stirred for 16 h under a N_2 atmosphere. Following the removal of volatiles in vacuo, the resulting bis-acid chloride was redissolved in THF (10 mL) and added dropwise to a stirred mixture of amine **13** (0.65 g, 2.86 mmol), H_2O (0.84 mL), and TEA (0.83 mL, 0.60 g, 5.93 mmol) in THF (2 mL) maintained at 0 °C under N_2. The reaction mixture was allowed to warm to room temperature and then stirred for a further 2 h, at which time TLC (EtOAc) revealed the complete consumption of starting material. After removal of the THF by evaporation in vacuo, the residue was partitioned between H_2O (100 mL) and EtOAc (100 mL), and the separated aqueous layer washed with EtOAc (3 × 50 mL). The combined organic phase was then washed with saturated aqueous NH_4Cl (100 mL) and brine (100 mL), and then dried ($MgSO_4$), filtered, and concentrated in vacuo to afford a dark orange oil. Purification by flash chromatography (50% EtOAc/petroleum ether 40°−60°) afforded the pure bis-amide **15** as a pale yellow glass (0.93 g, 74%): $[\alpha]^{21}_D$ = −51.1° (c = 0.08, $CHCl_3$); ^1H NMR ($CDCl_3$) (rotamers) δ 7.77/7.74 (2 × s, 2H, H6/H6′), 6.81/6.76 (2 × s, 2H, H9/H9′), 5.09−4.83 (m, 4H, H12/H12′), 4.60 (m, 2H, H11a/H11a′), 4.35−4.31 (m, 4H, H13/H13′), 4.08−3.74 (m, 14H, H11/H11′, H3/H3′ and 2 × OCH_3 at C7/C7′), 2.72−2.45 (m, 6H, H1/H1′ and H14), 0.91/0.79 (2 × s, 18H, SiC(CH_3)$_3$), 0.09/−0.09/−0.12 (s × 3, 12H, Si(CH_3)$_2$); ^{13}C NMR ($CDCl_3$) (rotamers) δ 166.2 (C5/C5′), 154.7/154.5 (C_{quat}), 148.4/148.2 (C_{quat}), 144.1/143.2 (C_{quat}), 137.2 (C_{quat}), 128.2/127.4 (C_{quat}), 110.1/108.6 (C6/C6′), 109.1/108.3 (C9/C9′), 107.5 (C12/C12′), 65.7/65.5 (C13/13′), 63.9/62.6 (C11/C11′), 60.2 (C11a/C11a′), 58.1/56.6 (OCH_3 at C7/C7′), 52.8/50.5 (C3/C3′), 35.0/33.9 (C1/C1′), 30.8/28.6 (C14), 25.8/25.7 (SiC(CH_3)$_3$), 18.2 (SiC(CH_3)$_3$), −5.5/-5.6 (Si(CH_3)$_2$); MS (EI) m/z (rel intensity) 885 ([M]$^{+\bullet}$, 7), 828 (100), 740 (20), 603 (3), 479 (26), 391 (27), 385 (25), 301 (7), 365 (10), 310 (14), 226 (8), 222 (13), 170 (21), 168 (61), 82 (39), 75 (92); IR (Nujol) 2923, 2853, 2360, 1647, 1587, 1523, 1461, 1429, 1371, 1336, 1277, 1217, 1114, 1061, 1021 cm^{-1}; HRMS [M + H]$^{+\bullet}$ Calcd for $C_{43}H_{65}N_4O_{12}Si_2$ m/z 885.4138, Obsd (FAB) m/z 885.4165.

(2*S*)-1,1′-[[(Propane-1,3-diyl)dioxy]bis[(2-nitro-5-methoxy-1,4-phenylene)carbonyl]]bis[2-(hydroxymethyl)-4-methylidenepyrrolidine] (16). A solution of TBAF (3.98 mL of a 1 M solution in THF, 3.98 mmol) was added to the bissilyl ether **15** (1.41 g, 1.59 mmol) in THF (35 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature, and saturated aqueous NH_4Cl (120 mL) was added after 30 min. The reaction mixture was extracted with EtOAc (3 × 80 mL), and the combined extracts were washed with brine (80 mL), then dried ($MgSO_4$), and evaporated in vacuo to give a dark oil. Flash chromatography (97% $CHCl_3$/MeOH) provided the diol **16** as a pale orange solid (0.98 g, 94%): $[\alpha]^{19}_D = -31.9°$ (c = 0.09, $CHCl_3$); ^1H NMR ($CDCl_3$) (rotamers) δ 7.75/7.71 (2 × s, 2H, H6/H6′), 6.96/6.84 (2 × s, 2H, H9/H9′), 5.08, 5.02/4.88 (3 × br s, 4H, H12/H12′), 4.61−4.50 (m, 2H, H11a/H11a′), 4.35−4.33 (m, 4H, H13/H13′), 4.02−3.65 (m, 14H, H11/H11′, H3/H3′ and 2 × OCH_3 at C7/C7′), 2.88−2.43 (m, 6H, H1/H1′ and H14); ^{13}C NMR ($CDCl_3$) (rotamers) δ 167.9/166.9 (C5/C5′), 154.9/154.3 (C_{quat}), 148.4/148.2 (C_{quat}), 143.3/142.6 (C_{quat}), 137.2/137.0 (C_{quat}), 127.6/127.3 (C_{quat}), 109.1 (C6/C6′), 108.4 (C12/C12′), 108.2 (C9/C9′), 65.6/65.4 (C13/C13′), 64.5/63.3 (C11/C11′), 60.5/60.0 (C11a/C11a′), 56.8/56.7 (OCH_3 at C7/C7′), 52.9 (C3/C3′), 35.0/34.3 (C1/C1′), 29.6/28.6 (C14); MS (FAB) (rel intensity) 657 ([M + H]$^{+\bullet}$, 10), 639 (2), 612 (1), 544 (4), 539 (1), 449 (16), 433 (9), 404 (8), 236 (32), 166 (65), 151 (81), 112 (82), 82 (100); IR (Nujol) 3600−3200 (br), 2923, 2853, 2360, 1618, 1582, 1522, 1459, 1408, 1375, 1335, 1278, 1218, 1061 cm^{-1}; HRMS [M + H]$^{+\bullet}$ Calcd for $C_{31}H_{37}N_4O_{12}$ m/z 657.2408, Obsd (FAB) m/z 657.2388.

(2*S*)-1,1′-[[(Propane-1,3-diyl)dioxy]bis[(2-amino-5-methoxy-1,4-phenylene)carbonyl]]-bis[2-(hydroxymethyl)-4-methylidenepyrrolidine] (17). A mixture of **16** (0.98 g, 1.49 mmol) and $SnCl_2{\cdot}2H_2O$ (3.36 g, 14.9 mmol) in MeOH (35 mL) was heated at reflux, and the progress of the reaction was monitored by TLC (90% $CHCl_3$/MeOH). After 45 min, the solvent was evaporated in vacuo and the resulting residue cooled in ice, treated carefully with saturated aqueous $NaHCO_3$ (120 mL), and then diluted with EtOAc (120 mL). After the mixture was stirred for 16 h at room temperature, the inorganic precipitate was removed by filtration through Celite. The organic phase was separated, washed with brine (100 mL), then dried ($MgSO_4$), filtered, and evaporated in vacuo to give a brown solid. Flash chromatography (95% $CHCl_3$/MeOH) afforded the bis-amine **17** as an orange solid (0.54 g, 61%): $[\alpha]^{19}_D = -31.8°$ (c = 0.30, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 6.74 (s, 2H, H6/H6′), 6.32 (s, 2H, H9/H9′), 5.00 and 4.93 (2 × br s, 4H, H12/H12′), 4.54 (br s, 2H, H11a/H11a′), 4.24−4.14 (m, 4H, H13/H13′), 3.98−3.50 (m, 14H, H11/H11′, H3/H3′ and 2 × OCH_3 at C7/C7′), 2.76 (dd, 2H, J = 8.6, 15.9 Hz, H1/H1′trans), 2.46−2.41 (m, 2H, H1/H1′cis), 2.33−2.28 (m, 2H, H14); ^{13}C NMR ($CDCl_3$) δ 171.0 (C5/C5′), 151.0 (C_{quat}), 143.5 (C_{quat}), 141.3 (C_{quat}), 140.6 (C_{quat}), 112.4 (C6/C6′), 111.9 (C_{quat}), 107.8 (C12/C12′), 102.4 (C9/C9′), 65.2 (C13/C13′), 65.0 (C11/C11′), 59.8 (C11a/C11a′), 57.1 (2 × OCH_3 at C7/C7′), 53.3 (C3/C3′), 34.4 (C1/C1′), 29.0 (C14); MS (FAB) (rel intensity) 597 ([M]$^{+\bullet}$, 1.5), 596 ([M]$^{+\bullet}$, 13), 484 (14), 389 (10), 371 (29), 345 (5), 224 (8), 206 (44), 166 (100), 149 (24), 112 (39), 96 (34), 81 (28); IR (Nujol) 3600−3000 (br), 3349, 2922, 2852, 2363, 1615, 1591, 1514, 1464, 1401, 1359, 1263, 1216, 1187, 1169, 1114, 1043 cm^{-1}; HRMS [M + H]$^{+\bullet}$ Calcd for $C_{31}H_{41}N_4O_8$ m/z 597.2924, Obsd (FAB) m/z 597.2896.

(2*S*)-1,1′-[[(Propane-1,3-diyl)dioxy]bis[(2-allyloxycarbonylamino-5-methoxy-1,4-phenylene)carbonyl]]bis[2-(hydroxymethyl)-4-methylidenepyrrolidine] (18). Pyridine (0.47 mL, 0.46 g, 5.82 mmol) was added to a stirred

solution of the bis-amine **17** (0.857 g, 1.44 mmol) in CH_2Cl_2 (30 mL) at 0 °C. This mixture was treated dropwise with a solution of allyl chloroformate (0.33 mL, 0.38 g, 3.15 mmol) in CH_2Cl_2 (10 mL). After being stirred for 2.5 h at room temperature, the reaction mixture was diluted with CH_2Cl_2 (60 mL), washed with 1 M HCl (2 × 50 mL), H_2O (80 mL), and brine (80 mL), then dried ($MgSO_4$), filtered, and evaporated in vacuo. The residue was purified by flash chromatography (70−100% EtOAc/petroleum ether 40°−60°) to afford the bis-carbamate **18** as a pale orange glass (0.548 g, 50%): ^{1}H NMR ($CDCl_3$) δ 8.58 (br s, 2H, N*H*), 7.56 (s, 2H, H6/H6′), 6.78 (s, 2H, H9/H9′), 6.03−5.88 (m, 2H, H17/H17′), 5.39−5.21 (m, 4H, H18/H18′), 5.00 and 4.93 (2 × br s, 4H, H12/H12′), 4.70−4.57 (m, 4H, H16/H16′), 4.30−4.25 (m, 4H, H13/H13′), 4.17−3.90 (m, 8H, H11/H11′and H3/H3′), 3.81−3.54 (m, 8H, H11a/H11a′ and 2 × OC*H*$_3$ at C7/C7′), 2.76 (dd, 2H, J = 8.5, 15.9 Hz, H1/H1′trans), 2.49−2.44 (m, 2H, H1/H1′cis), 2.36−2.28 (m, 2H, H14); ^{13}C NMR ($CDCl_3$) δ 170.3 (C5/C5′), 153.8 (C15/C15′), 150.5 (C_{quat}), 144.8 (C_{quat}), 143.1 (C_{quat}), 132.5 (C17/C17′), 130.7 (C_{quat}), 118.1 (C18/C18′), 116.8 (C_{quat}), 110.9 (C6/C6′), 108.1 (C12/C12′) 106.9 (C9/C9′), 65.7 (C16/C16′), 65.4 (C13/C13′), 65.1 (C11/C11′), 59.8 (C11a/C11a′), 56.5 (2 × OCH_3 at C7/C7′), 53.9 (C3/C3′), 34.2 (C1/C1′), 29.7/29.2 (C14); MS (FAB) (rel intensity) 765 ([M + H]$^{+\bullet}$, 10), 652 (32), 594 (4), 539 (2), 481 (51), 441 (31), 290 (3), 249 (13), 232 (38), 192 (83), 166 (49), 149 (32), 114 (100).

1,1′-[[(Propane-1,3-diyl)dioxy]bis[(11*S*,11a*S*)-10-(allyloxycarbonyl)-11-hydroxy-7-methoxy-2-methylidene-1,2,3,10,11,11a-hexahydro-*5H*-pyrrolo[2,1-*c*][1,4]benzodiazepin-5-one] (19). A stirred solution of the bis-carbamate **18** (150 mg, 0.196 mmol) in CH_2Cl_2/CH_3CN (12 mL, 3:1) was treated with powdered molecular sieves (4 Å, 0.2 g) and NMO (70 mg, 0.598 mmol) at room temperature. After the mixture was stirred at room temperature for 15 min, TPAP (7 mg, 19.9 μmol) was added and stirring continued for a further 2 h, after which time TLC (95% $CHCl_3$/MeOH) indicated formation of the cyclized product and the presence of some unreacted starting material. The mixture was treated with a further portion of NMO (35 mg, 0.299 mmol) and TPAP (3.5 mg, 9.96 μmol), and it was allowed to stir for 30 min, when TLC showed complete reaction. Solvent removal in vacuo afforded a black residue which was purified by flash chromatography (98% $CHCl_3$/MeOH) to give the pure bis-carbinolamine derivative **19** as a white solid (47 mg, 32%): ^{1}H NMR ($CDCl_3$) δ 7.23 (s, 2H, H6/H6′), 6.74 (s, 2H, H9/H9′), 5.90−5.65 (m, 2H, H17/H17′), 5.60 (d, 2H, J = 8.2 Hz, H11/H11′), 5.26−5.07 (m, 8H, H12/H12′ and H18/H18′), 4.67−4.10 (m, 14H, H16/H16′, H3/H3′, H13/H13′ and 2 × OH at C11/C11′), 3.89 (s, 6H, 2 × OCH_3 at C7/C7′), 3.63 (m, 2H, H11a/H11a′), 2.91 (dd, 2H, J = 8.8, 15.8 Hz, H1/H1′trans), 2.68 (d, 2H, J = 16.1 Hz, H1/H1′cis), 2.42−2.24 (m, 2H, H14); ^{13}C NMR ($CDCl_3$) δ 166.7 (C5/C5′), 150.1 (C_{quat}), 149.0 (C_{quat}), 141.7 (C_{quat}), 131.7 (C17/C17′), 130.6 (C_{quat}), 128.9 (C_{quat}), 128.8 (C_{quat}), 118.3 (C18/C18′), 114.7 (C6/C6′), 110.7 (C9/C9′), 109.8 (C12/C12′), 85.9 (C11/C11′), 66.9 (C16/C16′), 66.0 (C13/C13′), 59.7 (C11a/C11a′), 56.1 (2 × OCH_3 at C7/C7′), 50.7 (C3/C3′), 35.0 (C1/C1′), 29.7/29.1 (**C14**); MS (FAB) (rel intensity) no molecular ion, 743 (16), 725 (17), 632 (13), 574 (8), 548 (13), 490 (10), 481 (9), 441 (7), 425 (6), 257 (12), 232 (20), 192 (46), 166 (52), 149 (100), 91 (59); IR (Nujol) 3234 (br), 2923, 2853, 2361, 1707, 1604, 1515, 1464, 1410, 1377, 1302, 1267, 1205, 1163, 1120, 1045 cm^{-1}.

1,1′-[[(Propane-1,3-diyl)dioxy]bis[(11a*S*)-7-methoxy-2-methylidene-1,2,3,11a-tetrahydro-*5H*-pyrrolo[2,1-*c*][1,4]-benzodiazepin-5-one] (5). A catalytic amount of tetrakis-(triphenylphosphine)palladium (11 mg, 9.52 μmol) was added to a stirred solution of bis-carbamate **19** (139 mg, 0.183 mmol), Ph_3P (4.8 mg, 18.3 μmol), and pyrrolidine (27 mg, 0.380 mmol) in CH_2Cl_2/CH_3CN (13 mL, 10:3 v/v) at 0 °C under a N_2 atmosphere. The reaction mixture was allowed to warm to room temperature, and progress of the reaction was monitored by TLC (95% $CHCl_3$/MeOH). After 135 min, TLC revealed the complete consumption of starting material and formation of a highly fluorescent product. The solvent was evaporated in vacuo, and the resulting residue was purified by flash chromatography (98% $CHCl_3$/MeOH) to give the target PBD dimer **5** as a pale orange glass (78 mg, 77%). This material was repeatedly evaporated from $CHCl_3$ in vacuo to generate the bis-imine form: $[\alpha]^{21}_D$ = +357.7° (c = 0.07, $CHCl_3$); reverse-phase HPLC (C_4 stationary phase, 65% MeOH/H_2O mobile phase, 254 nm) R_t = 6.27 min, % peak area = 97.5%; ^{1}H NMR ($CDCl_3$) (imine form) δ 7.68 (d, 2H, J = 4.4 Hz, H11/H11′), 7.49 (s, 2H, H6/H6′), 6.85 (s, 2H, H9/H9′), 5.20/5.17 (2 × br s, 4H, H12/H12′), 4.46−4.19 (m, 8H, H13/H13′ and H3/H3′), 3.93 (s, 6H, 2 × OCH_3 at C7/C7′), 3.89−3.80 (m, 2H, H11a/H11a′), 3.12 (dd, 2H, J = 8.6, 16.2 Hz, H1/H1′trans), 2.94 (d, 2H, J = 16.3 Hz, H1/H1′cis), 2.45−2.38 (m, 2H, H14); ^{13}C NMR ($CDCl_3$) (imine form) δ 164.7 (C5/C5′), 162.6 (C11/C11′), 150.7 (C_{quat}), 147.9 (C_{quat}), 141.5 (C_{quat}), 140.6 (C_{quat}), 119.8 (C_{quat}), 111.5 (C6/C6′), 110.7 (C9/C9′), 109.4 (C12/C12′), 65.4 (C13/C13′), 56.1 (2 × OCH_3 at C7/C7′), 53.8 (C11a/C11a′), 51.4 (C3/C3′), 35.4 (C1/C1′), 28.8 (C14); MS (FAB) (rel intensity) (imine form) 773 ([M + H + (2 × thioglycerol adduct)]$^{+\bullet}$, 3), 665 ([M + H + thioglycerol adduct]$^{+\bullet}$, 7), 557 ([M + H]$^{+\bullet}$, 9), 464 (3), 279 (12), 257 (5), 201 (5), 185 (43), 166 (6), 149 (12), 93 (100); IR (Nujol) 3600−3100 (br), 2923, 2849, 1599, 1511, 1458, 1435, 1391, 1277, 1228, 1054, 1011 cm^{-1}; HRMS [M + H]$^{+\bullet}$ Calcd for $C_{31}H_{33}N_4O_6$ m/z 557.2400, Obsd (FAB) m/z 557.2394; Calcd for $C_{31}H_{32}N_4O_6$: C 65.57, H 5.85, N 9.60. Found: C 65.50, H 5.86, N 9.56.

1,1′-[[(Propane-1,3-diyl)dioxy]bis[(11a*S*)-7-methoxy-2-methylidene-1,2,3,10,11,11a-hexahydro-*5H*-pyrrolo[2,1-*c*]-[1,4]benzodiazepin-5,11-dione] (21). A solution of DMSO (0.17 mL, 0.19 g, 2.43 mmol) in CH_2Cl_2 (10 mL) was added dropwise to a solution of oxalyl chloride (0.60 mL of a 2.0 M solution in CH_2Cl_2, 1.20 mmol) at −45 °C under a N_2 atmosphere. After the mixture was stirred at −45 °C for 1 h, a solution of **18** (0.41 g, 0.54 mmol) in CH_2Cl_2 (10 mL) was added dropwise at −45 °C. The reaction mixture was allowed to stir for 2 h at −45 °C before treating dropwise with TEA (0.76 mL, 0.55 g, 5.40 mmol) in CH_2Cl_2 (10 mL) and allowing to warm to 10 °C. TLC (95% $CHCl_3$/MeOH) indicated complete consumption of starting material. The mixture was then treated with brine (100 mL) and acidified to pH 3 with 12 M HCl. The organic phase was separated and washed with brine (100 mL), then dried ($MgSO_4$), filtered, and concentrated in vacuo to afford **20** as an orange oil (0.21 g). Without further purification, the intermediate **20** was dissolved in CH_2Cl_2 (20 mL), cooled to 0 °C, and treated sequentially with PPh_3 (7.6 mg, 29.0 μmol), pyrrolidine (41 mg, 0.576 mmol), and a catalytic amount of tetrakis(triphenylphosphine)palladium (16.6 mg, 14.4 μmol). The mixture was allowed to warm to room temperature and was then stirred for 2 h, at which point TLC (95% $CHCl_3$/MeOH) revealed that the reaction was complete. The solvent was evaporated in vacuo, and the residue was purified by flash chromatography (97% $CHCl_3$/MeOH) to afford the tetralactam **21** as a yellow glass (70 mg, 43%): $[\alpha]^{22}_D$ = +274.1° (c = 0.06, $CHCl_3$); ^{1}H NMR ($CDCl_3$) δ 8.95 (br s, 2H, D_2O exchangeable, N*H*), 7.40 (s, 2H, H6/H6′), 6.62 (s, 2H, H9/H9′), 5.14/5.08 (2 × br s, 4H, H12/H12′), 4.38 (br d, 2H, J = 16.3 Hz, 1 × H3/H3′), 4.20−4.10 (m, 8H, 1 × H3/H3′, H11a/H11a′ and H13/H13′), 3.85 (s, 6H, 2 × OCH_3 at C7/C7′), 3.43 (br d, 2H, J = 15.9 Hz, 1 × H1/H1′), 2.84−2.75 (m, 2H, 1 × H1/H1′), 2.34−2.30 (m, 2H, H14); ^{13}C NMR ($CDCl_3$) δ 170.1 (C11/C11′), 165.2 (C5/C5′), 151.5 (C_{quat}), 146.8 (C_{quat}), 141.4 (C_{quat}), 129.8 (C_{quat}), 119.0 (C_{quat}), 112.4 (C6/C6′), 109.0 (C12/C12′), 105.3 (C9/C9′), 65.4 (C13/C13′), 56.8/56.3 (C11a/C11a′ and 2 × OCH_3 at C7/C7′), 51.5 (C3/C3′), 31.8 (C1/C1′), 28.5 (C14); MS (EI) (rel intensity) 588 ([M]$^{+\bullet}$, 39), 570 (100), 554 (5), 461 (13), 314 (27), 300 (17), 274 (24), 257 (15), 236 (19), 166 (12), 82 (19); IR (Nujol) 3700−3200, 2923, 2854, 1697, 1607, 1518, 1491, 1463, 1435, 1377, 1267, 1227, 1183, 1119, 1020 cm^{-1}; HRMS [M + H]$^{+\bullet}$ Calcd for $C_{31}H_{33}N_4O_8$ m/z 589.2298, Obsd (FAB) m/z 589.2282.

Thermal Denaturation Studies. The compounds were subjected to DNA thermal denaturation (melting) studies[29] 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.[37] 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 400 Bio 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 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.[29] 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 ≤0.6% v/v MeOH; T_m results were corrected for the effects of MeOH co-solvent by using a linear correction term. Other [DNAp]/[ligand] molar ratios (i.e., 50:1 and 100:1) were examined in the case of **5** to ensure that the fixed 5:1 ratio used in this assay did not result in saturation of the host DNA duplex.

DNA Cross-Linking Assay. The agarose gel electrophoresis assay was carried out with **5** using pBR322 DNA and T4 polynucleotide kinase (PNK) purchased from Northumbria Biological Ltd. and used as supplied. Bacterial alkaline phosphatase (BAP) and *Hind* III enzymes were purchased from BRL and used as supplied.

Linearization of pBR322 DNA. A mixture of circular pBR322 DNA (10 μg in 20 μL), React 2 aqueous buffer (3 μL, [500 mM Tris-HCl, pH 8, 100 mM $MgCl_2$, 500 mM NaCl]), *Hind* III (3 μL, 30 units), and water (4 μL) was incubated at 37 °C for 1 h. Aqueous 4 M NaOAc was added to give a 0.3 M solution, then EtOH (95% v/v, 3 volumes) was added, and the mixture was chilled. After centrifugation and removal of supernatant, the pellet was lyophilized.

Dephosphorylation of Linearized DNA. A mixture of linearized DNA in water (15 μL), BAP buffer (4 μL, [50 mM Tris-HCl, pH 8, 600 mM NaCl]), and BAP (1 μL, 100 units) was incubated at 65 °C for 1 h. After extraction with Tris/EDTA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA)/saturated phenol (1:1 v/v), the aqueous layer was collected, and the phenol layer back-extracted with buffer. The combined aqueous phase was extracted twice with $CHCl_3$/isoamyl alcohol (24:1 v/v), then aqueous 4 M NaOAc was added to give a 0.3 M solution, followed by EtOH (3 volumes). After chilling and centrifugation, the supernatant was removed and the pellet lyophilized.

5′-End Labeling. The resultant dephosphorylated DNA was 5′-labeled with [γ-^{32}P]-ATP (5000 Ci/mmol, Amersham plc). A mixture of the DNA (10 μg in 30 μL water), forward reaction buffer (9 μL, [300 mM Tris-HCl, pH 7.8, 75 mM 2-thioethanol, 50 mM $MgCl_2$, 1.65 μM ATP]), [γ-^{32}P]-ATP (3 μL, 30 μCi), and PNK (4 μL, 24 units) was incubated at 37 °C for 30 min. Aqueous NH_4OAc (7.5 M, 0.5 volume) and EtOH (2 volumes) were added, and the mixture was chilled and centrifuged (13000 rpm/10 min). The supernatant was removed and the pellet resuspended in 20 μL Tris/EDTA buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.3 M NaOAc). After reprecipitation with EtOH (60 μL) and centrifugation, the supernatant was removed and the pellet washed with EtOH (20 μL) and lyophilized. The DNA was then resuspended in sterile double-distilled water at 1 μg/μL. Approximately 10 ng of labeled DNA was used for each experiment.

Reaction Protocols. Reactions were performed with **4**, **5**, and melphalan at concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1.0, 3.0, and 10 μM in 25 mM triethanolamine/1 mM EDTA at pH 7.2 and 37 °C. Reactions were terminated after 2 h by the addition of an equal volume of stop solution (0.6 M NaOAc/20 mM EDTA/100 μg/mL tRNA). After precipitation of the DNA by addition of EtOH (3 volumes) followed by centrifugation and the removal of supernatant, the pellet was dried by lyophilization.

Electrophoresis. Samples were dissolved in strand separation buffer (10 μL of 30% DMSO/1 mM EDTA/0.04% bromophenol blue/0.04% xylene cylanol), heated at 90 °C for 2 min, and then chilled immediately in an ice−water bath prior to loading. Control non-denaturated samples were dissolved in 6% sucrose/0.04% bromophenol blue (10 μL) and loaded directly. Electrophoresis was performed on a 20 cm 0.8% submerged horizontal agarose gel at 40 V for 16 h using a 40 mM Tris/20 mM HOAc/2 mM EDTA gel running buffer.

Autoradiography. Gels were dried at 80 °C on one layer of Whatman 3 mm and one layer of DE81 filter papers, with a vacuum-connected Bio-Rad Model 583 gel drier. Autoradiography was performed with Hyperfilm MP (Amersham plc) for 4 h at −70 °C using a DuPont-Cronex Lighting-Plus intensifying screen, and the percentage double-stranded cross-linked DNA was calculated.

Molecular Modeling Studies. The strategy and protocol used for the modeling studies were adapted from our recent studies of covalent DNA-PBD adduct formation.[16,32] Two host DNA 12-mer duplexes of sequence 5′-CGCA***G****ATC*TGCG and 5′-CGCT*CTA****G***AGCG (where the spanned and G−G cross-linked sequences and the alkylated guanine bases are highlighted) were selected for energy calculations as each of the embedded tracts offer optimal 5′-A**G**A sites for alkylation by a PBD unit. The symmetric 5′A***G****ATC*T duplex core tract has been shown previously to be a favored sequence for cross-linking by the dimer **4**.[16] We have recently shown that **4** and **5** can form interstrand DNA cross-links at sites with 5′-**G**ATC and 5′-CTA**G** duplex sequences (unpublished results). By analogy, the "reverse" cross-link 5′-T*CTA****G***A core sequence retains the preferred 5′-A**G**A sequence at each site, but the PBD orientations are reversed with respect to the modified guanines. Each strand was terminated with an alternating G/C base stretch of sufficient length to confer duplex stability during extended dynamics simulations, particularly to prevent strand separation or "fraying".

The interaction of dimers **4** and **5** together with their corresponding monomeric subunits were examined with each host DNA duplex sequence. The PBD monomers were also modeled using the 5′-CGC*A****G****A*GCG duplex to enable comparison with a single optimized and embedded 5′-A**G**A alkylation site and to remove the influence of any asymmetric flanking base sequence. The C11(*S*) geometry was adopted for all PBD molecules studied, as this stereochemistry is known to lead to energetically favored adducts with the exocyclic C2-NH_2 group of an embedded guanine in the context of a B-DNA duplex.[32] Thus, the forward and reverse cross-links examined can be described as 3*S*-3*S* and 5*S*-5*S* structures due to the enforced head-to-head tethering through the distal A-rings of the PBD subunits. Here, the numbering used refers to the orientation of the aromatic A-ring with respect to the alkylated strand. However, as both 3*S* and 5*S* alignments can be achieved for a PBD monomer, this allows an estimation of the energy penalty resulting from their symmetric tethering in a dimer ligand.

In Vitro Cytotoxicity Studies. In vitro cytotoxicity was evaluated using the human ovarian carcinoma cell lines SKOV-3, A2780, and CH1, together with the cisplatin-resistant counterparts of the A2780 and CH1 lines (A2780*cis*^{R} and CH1*cis*^{R}, respectively). Viable cells were seeded in growth medium (160 μL) into 96-well microtiter plates and allowed to attach overnight. The PBDs **4**, **5**, and **21**, and cisplatin, were dissolved in DMSO (to give a 20 mM concentration in each case) immediately prior to adding to the cells in quadruplicate wells. Final drug concentrations in the wells ranged from 100 μM to 2.5 nM as follows: 100, 25, 10, 2.5, 1 μM, and 250, 100, 25, 10, 2.5 nM. This was achieved by diluting the drugs in growth medium and then adding 40 μL to the existing well volume of 160 μL to give the final concentrations stated above. After 4 days (96 h), the medium was removed and the

remaining cells were fixed using 10% trichloroacetic acid on ice for 30 min. Wells were then washed 3−4 times with tap water and air-dried overnight, and 100 μL of 0.4% sulforhodamine B (dissolved in 1% glacial HOAc) was added to each well. Staining was allowed to continue for 10−15 min; the wells were then washed 3−4 times with 1% acetic acid and air-dried, and Tris base (100 μL of 10 mM) was added to each one. The plates were then shaken and absorbance readings taken at 540 nm using a plate reader. From plots of concentration versus percentage absorbance (compared to 8 untreated wells), IC_{50} values were calculated using the Quattro-Pro software package.

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Supporting Information Available: Purity table for target molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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