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Synthesis, DNA binding, and cytotoxicity studies of pyrrolo[2,1-*c*][1,4]benzodiazepine-anthraquinone conjugates

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Abstract—A series of pyrrolo[2,1-*c*][1,4]benzodiazepine-anthraquinone conjugates have been prepared and evaluated for their DNA binding ability as well as anticancer activity. Some of these molecules have shown significant anticancer activity in a number of cancer cell lines.

1. Introduction

DNA is one of the main molecular targets in the design of anticancer compounds. Of late, the development of hybrid ligands, combining two modes of binding to DNA has received considerable attention. Such ligands are capable of recognizing heterogeneous DNA sequences. Anthraquinones represent an important class of anticancer agents, which generally bind to DNA by insertion and stacking between the base pairs of the DNA double helix.[1] DNA intercalation and sequential inhibition of DNA topoisomerase by these compounds is well known.[2] Mitoxantrone (**1**), as shown in Figure 1, is a lead compound in this series which is generally used in the treatment of some hematological malignancies, ovarian and breast cancers.[3] Other anthraquinone-type analogues such as bisantrene,[4] chrysophanol, and emodine[5] have exhibited significant cytotoxic activity.

The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are a class of DNA binding anticancer compounds that include the naturally occurring anthramycin and DC-81 (**2**) (Fig. 1).[6] The DNA-interactive ability and consequential biological effects are a result of the covalent bond formed between the N10/C11 carbinolamine/imine moiety in the central B-ring of the PBD moiety

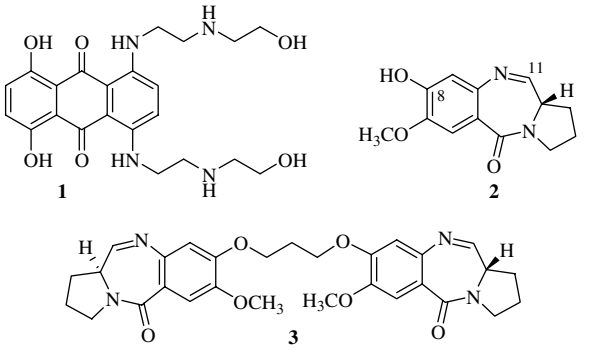

Figure 1. Chemical structures of mitoxantrone (**1**), DC-81 (**2**) and DSB-120 (**3**).

and N2 of the guanine residue in the minor groove of DNA.[7] Studies like DNA footprinting and X-ray analysis of these covalent adducts have demonstrated a high sequence specificity for GC rich regions of DNA, in particular for Pu-G-Pu triplets.[8] Rational modifications have been made to these natural products to improve and extend the sequence selectivity. One approach is the preparation of PBD dimers by coupling two PBD units through different spacers at their A-C7/A′-C7,[9] A-C8/A′-C8,[10] C-C2/C′-C2,[11] or A-C8/C-C2 positions.[12] Among these, A-C8/A′-C8 linked PBD dimers like DSB-120 (**3**) (Fig. 1) and SJG-136 have shown promising cytotoxicity and efficient DNA cross-linking properties. Alternative strategy involves the coupling

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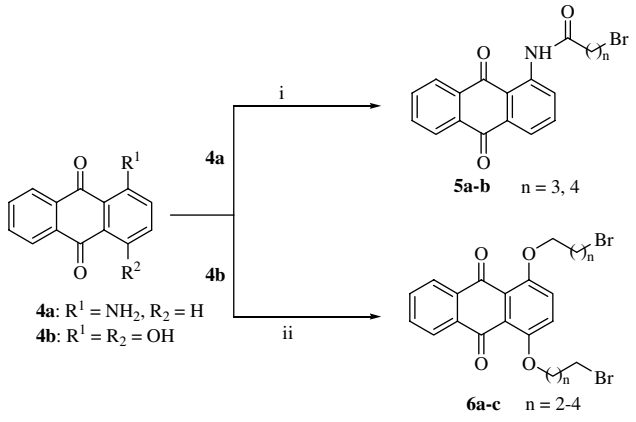
of PBDs to other DNA-interactive moieties such as cyclopropylbenzindole,[13] distamycin, and netropsin.[14] We have been interested in the development of new PBD dimers and their hybrids.[15] In this context, we have recently synthesized and studied the biological properties of C8-linked PBD-anthraquinone hybrids.[16] In continuation of these efforts, we report the full account of the design, synthesis and biological evaluation of C8-linked PBD-anthraquinone hybrids (**11a–b**) and a new class of PBD dimers linked at their C8 positions through an anthraquinone moiety (**14a–c**).

2. Chemistry

Initial attempts to couple 1-amino anthraquinone **4a** with (2*S*)-*N*-[4-[(*n*-carboxyalkyl)oxy]-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal[11] in the presence of EDCI and HOBt in different solvents gave the intermediates **9a–b** in poor yields, probably due to the low solubility of 1-amino anthraquinone in the reaction solvent. Similarly, the coupling of 1, 4-dihydroxyanthraquinone **4b** with (2*S*)-*N*-[4-(*n*-bromoalkoxy)-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal[15a] gave the desired intermediates **12a–c** in 15–20% yields. On the contrary, the synthesis of the final molecules via **5a–b** and **6a–c** intermediates made the coupling reactions more efficient (Scheme 1). Reaction of **4a** in the presence of pyridine in toluene with bromo alkyl acid chloride gave compounds **5a–b** in 80–82% yield. Reaction of **4b** with dibromo alkanes in the presence of K_2CO_3 in acetone gave the intermediates **6a–c** in high yields. The other precursor (2*S*)-*N*-[4-hydroxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal **8** was prepared from compound **7**, (2*S*)-*N*-[4-benzyloxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal, which was synthesized starting from vanillin according to the literature method.[17] The coupling of intermediates **5a–b** and **6a–c** with **8** provided the desired intermediates **9a–b** and **12a–c**, respectively, in 70–92% yield. Reductions of the nitro group using $SnCl_2{\cdot}2H_2O$ in methanol gave amines **10a–b** and **13a–c**, respectively.



Scheme 1. Reagents and conditions: (i) bromo alkyl acid chloride, pyridine, toluene, 60 °C, 4 h; (ii) dibromoalkane, K_2CO_3, acetone, reflux, 24 h.

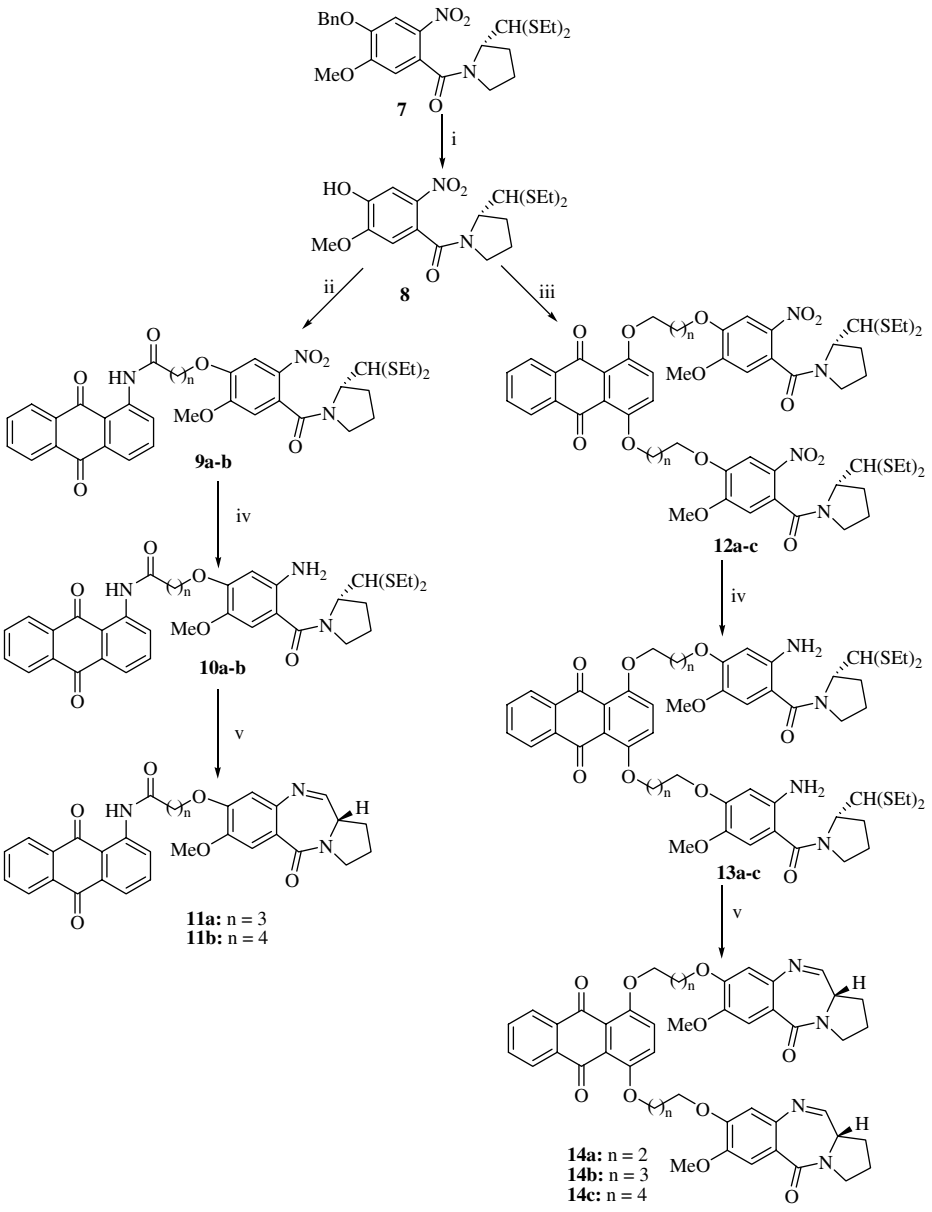
The deprotection of the thioacetal groups of **10a–b** and **13a–c** with $HgCl_2/CaCO_3$ resulted in the formation of the target compounds **11a–b** and **14a–c**[18] (Scheme 2).

3. DNA interactions: Thermal denaturation studies

The DNA binding affinity of the PBD-anthraquinone hybrids (**11a–b**) and anthraquinone-PBD dimers (**14a–c**) was investigated by thermal denaturation studies using calf thymus (CT) DNA. The studies have been carried out at DNA/PBD molar ratios of 5:1. The increase in melting temperature of DNA (ΔT_m) for each compound was examined at 0 h and 18 h of incubation at 37 °C (Table 1). The compounds **11a** and **11b** elevated the helix melting temperature of CT-DNA by 9.1 °C and 5.2 °C, respectively, after incubation at 37 °C for 18 h. In case of anthraquinone-PBD dimers, compound **14a** has been found to be an efficient stabilizing agent of double stranded CT-DNA. This compound elevates the helix melting temperature of CT-DNA by 17.9 °C, whereas compounds **14b** and **14c** have shown a ΔT_m of 1.6 °C and 6.0 °C, respectively, after incubation at 37 °C for 18 h. It is interesting to observe that as the spacer length increases from three to four carbons, there is a decrease in the DNA binding ability, on further increase from four to five carbons, the DNA binding ability is enhanced. These results indicate the importance of spacer chain length in such PBD hybrids. The naturally occurring DC-81 shows a ΔT_m of 0.7 °C, whereas DSB-120, the synthetic dimer of DC-81, provides a ΔT_m of 15.1 °C under similar conditions. These results clearly demonstrate that compound **14a** has a significant DNA binding ability. It is also interesting to note that PBD-anthraquinone hybrid **11a** exhibited a higher ΔT_m than the anthraquinone-PBD dimer **14b** having same spacer length.

4. Cytotoxicity

Compounds **11a–b** and **14a–c** have been evaluated for their in vitro cytotoxicity in selected human cancer cell lines like Hop62 (lung), SiHa (cervix), MCF7 and ZR-75-1 (breast), Colo205 (colon), PC3 (prostate), and A2780 (ovarian), by employing the sulforhodamine B (SRB) assay. The results described in Table 2. show that all the new compounds are significantly cytotoxic, with the concentration of the drug that produced 50% inhibition of cell growth (IC_{50}) ranging from 0.5 to 10 μM. Among all the compounds synthesized, compound **14a** displayed higher cytotoxicity with an IC_{50} value of <1 μM against all the cell lines examined. Similarly, compound **14c** has IC_{50} values of <1 μM against SiHa, Colo205 and A2780 cell lines. Compound **14b**, comprising of two PBD units and four-carbon spacer exhibited mild cytotoxicity against all the cell lines tested. Compound **11a**, which has one PBD unit tethered to anthraquinone through a four-carbon spacer has shown higher cytotoxicity against ZR-75-1, A2780, HOP62, and PC3 cell lines with IC_{50} values ranging from 0.5 to 0.8 μM. Compound



Scheme 2. Reagents and conditions: (i) CH_2Cl_2, BF_3-OEt/EtSH, 16 h; (ii) **5a–b**, K_2CO_3, acetonitrile, reflux, 15 h; (iii) **6a–c**, K_2CO_3, acetonitrile, reflux, 24 h; (iv) $SnCl_2$-$2H_2O$, MeOH, reflux, 4 h; (v) $HgCl_2$, $CaCO_3$, CH_3CN/H_2O, rt, 12 h.

Table 1. Thermal denaturation data for PBD-anthraquinones (**11a–b**, **14a–c**) with calf thymus (CT) DNA

Compound	[PBD]:[DNA] molar ratio[b]	Induced ΔT_m[a] after incubation at 37 °C (°C)	
		0 h	18 h
11a	1:5	8.9	9.1
11b	1:5	5.1	5.2
14a	1:5	15.8	17.9
14b	1:5	1.2	1.6
14c	1:5	5.7	6.0

[a] For CT-DNA alone at pH 7.00 ± 0.01, T_m = 69.4 °C ± 0.01 (mean value from 10 separate determinations), all ΔT_m values are ± 0.1–0.2 °C.

[b] For a 1:5 molar ratio of [PBD]/[DNA], where CT-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].

11b has also shown similar cytotoxicity with IC_{50} values ranging from 0.5 to 0.8 μM against ZR-75-1, A2780, MCF7, and PC3 cell lines.

5. Conclusion

In conclusion, we have described the synthesis, DNA binding ability, and anticancer activity of PBD-anthraquinone conjugates. Among all the compounds synthesized, compound **14a**, which has two PBD units tethered to anthraquinone ring system through a three-carbon spacer, exhibits higher DNA binding ability. In addition, these compounds have also displayed promising anticancer activity in various cell lines and have provided an insight for future direction in the development of such conjugates.

Table 2. In vitro anticancer activity data for PBD-anthraquinone conjugates (**11a–b**, **14a–c**)

Compound	IC_{50}[a] (μM)						
	Hop62[b]	SiHa[c]	MCF7[d]	ZR-75-1[d]	PC3[e]	Colo205[f]	A2780[g]
11a	0.8	1.8	1.4	0.5	0.8	1.8	0.5
11b	1.0	1.2	0.6	0.5	0.8	<1.0	0.5
14a	<1.0	0.8	0.6	0.5	0.6	<1.0	0.6
14b	—[h]	9.0	9.0	—[h]	7.0	10.0	10.0
14c	1.4	0.8	1.2	1.5	1.0	<1.0	0.5
ADR	1	1.5	0.5	0.6	0.5	2.0	0.5

[a] Dose of the compound required to inhibit cell growth by 50% compared to untreated cell controls, values are derived from IC_{50} graphs. All experiments were done in triplicate wells and each experiment was repeated thrice.
[b] Lung cancer.
[c] Cervix cancer.
[d] Breast cancer
[e] Prostate cancer.
[f] Colon cancer.
[g] Ovarian cancer.
[h] IC_{50} value not attained at the concentrations used in the assay; ADR, adriamycin.

6. Experimental

6.1. 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 vapour unless otherwise stated. Chromatography was performed using Acme silica gel (100–200 mesh). Most of the reaction solvents were purified by distillation under nitrogen from the indicated drying agent and used fresh dichloromethane (calcium hydride), acetone (potassium permanganate), and acetonitrile (phosphorous pentoxide). ^{1}H NMR spectra were recorded on a Varian Gemini 200 MHz spectrometer using tetramethyl silane (TMS) as an internal standard. Chemical shifts are reported in parts per million (ppm) downfield from tetramethyl silane. Spin multiplicities are described as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Coupling constants are reported in Hertz (Hz). Low resolution mass spectra were recorded on a VG-7070H Micromass mass spectrometer at 200 °C, 70 eV with trap current of 200 μA, and 4 kV acceleration voltage. FABMS spectra were recorded on LSIMS-VG-AUTOSPEC-Micromass. Melting points were recorded on Electrothermal 9100 and are uncorrected.

6.2. *N*-(9,10-Dihydro-9,10-dioxo-1-anthracenyl)-1-bromobutanamide (5a)

1-Aminoanthraquinone (**4a**, 223 mg, 1 mmol) was dissolved in 100 mL of toluene containing a catalytic amount of pyridine. The mixture was warmed to 60 °C and 4-bromobutanoyl chloride (371 mg, 2 mmol) was added dropwise. After the reaction mixture was stirred for 4 h, the solvent was removed under reduced pressure to get the crude product. The crude product was purified by column chromatography (20% EtOAc–hexane) to afford compound **5a** as a yellow solid (305 mg, 82%). ^{1}H NMR ($CDCl_3$): δ 2.22–2.36 (m, 2H), 2.62–2.78 (m, 2H), 3.48–3.60 (m, 2H), 7.65–7.80 (m, 3H), 7.96 (d, 1H, J = 6.70 Hz), 8.18–8.24 (m, 2H), 9.04 (d, 1H, J = 8.90 Hz), 12.34 (s, 1H); MS (EI): m/z 371 $[M]^+$.

6.3. *N*-(9,10-Dihydro-9,10-dioxo-1-anthracenyl)-1-bromopentanamide (5b)

The compound **5b** was prepared following the method described for the compound **5a** employing 1-aminoanthraquinone (**4a**, 223 mg, 1 mmol) and 5-bromo pentanoyl chloride (399 mg, 2 mmol) and the crude product was purified by column chromatography to afford the compound **5b** as a yellow solid (313 mg, 81%). ^{1}H NMR ($CDCl_3$): δ 1.88–2.12 (m, 4H), 2.51–2.63 (m, 2H), 3.40–3.56 (m, 2H), 7.65–7.86 (m, 3H), 8.02 (d, 1H, J = 6.90 Hz), 8.20–8.32 (m, 2H), 9.12 (d, 1H, J = 9.20 Hz), 12.36 (s, 1H); MS (EI): m/z 386 $[M+1]^+$.

6.4. 1,4-Bis-(3-bromopropyloxy)anthracene-9,10-dione (6a)

To a solution of 1,4-dihydroxyanthraquinone (**4b**, 480 mg, 2 mmol) in acetone (30 mL) were added anhydrous potassium carbonate (1.1 g, 8 mmol) and 1,3-dibromopropane (1.21 g, 6 mmol) and the mixture was refluxed for 24 h. After completion of the reaction, potassium carbonate was removed by filtration and the solvent was evaporated under reduced pressure to get the crude product. This was further purified by column chromatography (30% EtOAc–hexane) to afford the compound **6a** as a yellow solid (791 mg, 82%); mp 117–119 °C; ^{1}H NMR ($CDCl_3$): δ 2.40–2.52 (m, 4H), 3.88 (t, 4H, J = 6.51 Hz), 4.26 (t, 4H, J = 5.53 Hz), 7.34 (s, 2H), 7.71–7.77 (m, 2H), 8.15–8.18 (m, 2H); MS (FAB): 480 $[M]^+$.

6.5. 1,4-Bis-(4-bromobutyloxy)anthracene-9,10-dione (6b)

The compound **6b** was prepared following the method described for the compound **6a**, employing 1,4-dihydroxyanthraquinone (**4b**, 480 mg, 2 mmol) and 1,4-dibromobutane (1.29 g, 6 mmol), and the crude product was purified by column chromatography (30% EtOAc–hexane) to afford the compound **6b** as a yellow

solid (826 mg, 81%); mp 108–110 °C; ^{1}H NMR ($CDCl_3$): δ 2.0–2.32 (m, 8H), 3.58 (t, 4H, J = 6.45 Hz), 4.10 (t, 4H, J = 5.40 Hz), 7.25 (s, 2H), 7.62–7.77 (m, 2H), 8.12–8.18 (m, 2H); MS (FAB): 508 $[M]^+$.

6.6. 1,4-Bis-(5-bromopentyloxy)anthracene-9,10-dione (6c)

The compound **6c** was prepared following the method described for the compound **6a**, employing 1,4-dihydroxyanthraquinone **4b** (480 mg, 2 mmol) and 1, 5-dibromopentane (1.38 mg, 6 mmol) and the crude product was purified by column chromatography (30% EtOAc–hexane) to afford the compound **6c** as a yellow solid (904 mg, 84%); mp 75–77 °C; ^{1}H NMR ($CDCl_3$): δ 1.75–2.07 (m, 12H), 3.49 (t, 4H, J = 6.59 Hz), 4.08 (t, 4H, J = 6.13 Hz), 7.25 (s, 2H), 7.69–7.75 (m, 2H), 8.13–8.20 (m, 2H); MS (FAB): 536 $[M]^+$.

6.7. (2*S*)-*N*-[4-Hydroxy-5-methoxy-2-nitrobenzoyl]pyrrolidine-2-carboxaldehyde diethyl thioacetal (8)

To a stirred solution of EtSH (1.20 g, 19 mmol) and $BF_3.OEt_2$ (1.419 g, 10 mmol) was added dropwise compound **7** (491 mg, 1 mmol) in dichloromethane (20 mL) at room temperature. Stirring was continued until the reaction was completed and then the solvent was evaporated under reduced pressure. The reaction mixture was quenched with 5% $NaHCO_3$ solution (20 mL), extracted with dichloromethane (2× 20 mL) and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure and purified by column chromatography (50% EtOAc–hexane) to afford compound **8** as pale yellow solid (344 mg, 86%). ^{1}H NMR ($CDCl_3$): δ 1.28–1.41 (m, 6H), 1.78–2.36 (m, 4H), 2.67–2.87 (m, 4H), 3.20–3.31 (m, 2H), 3.96 (s, 3H), 4.62–4.70 (m, 1H), 4.82 (d, 1H, J = 3.64 Hz), 6.77 (s, 1H), 7.67 (s, 1H); MS (FAB): 400 $[M]^+$.

6.8. (2*S*)-*N*-{4-[*N*-(9,10-Dihydro-9,10-dioxo-1-anthracenyl)-propane-3-carboxami-de]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (9a)

To a solution of **8** (401 mg, 1 mmol) in dry acetonitrile (30 mL) were added anhydrous K_2CO_3 (553 mg, 4 mmol) and **5a** (409 mg, 1.1 mmol). The reaction mixture was refluxed for 15 h. After completion of reaction, K_2CO_3 was removed by filtration and the solvent was evaporated under reduced pressure, the crude product was purified by column chromatography (50% EtOAc–hexane) to afford compound **9a** (623 mg, 90%). ^{1}H NMR ($CDCl_3$): δ 1.21–1.38 (m, 6H), 1.53–2.42 (m, 6H), 2.62–2.81 (m, 6H), 3.10–3.28 (m, 2H), 3.91 (s, 3H), 4.20–4.30 (m, 2H), 4.60–4.70 (m, 1H), 4.80 (d, 1H, J = 3.62 Hz), 6.74 (s, 1H), 7.68 (s, 1H), 7.71–7.85 (m, 3H), 8.02 (d, 1H, J = 6.80 Hz), 8.20–8.30 (m, 2H), 9.15 (d, 1H, J = 8.70 Hz), 12.38 (br s, 1H); MS (FAB): 692 $[M+1]^+$.

6.9. (2*S*)-*N*{4-[*N*-(9,10-Dihydro-9,10-dioxo-1-anthracenyl)-butane-4-carboxamide]-oxy-5-methoxy-2-nitrobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (9b)

The compound **9b** was prepared following the method described for the compound **9a**, employing **8** (401 mg, 1 mmol) and **5b** (425 mg, 1.1 mmol) and the crude product was purified by column chromatography (50% EtOAc–hexane) to afford the compound **9b** (649 mg, 92%). ^{1}H NMR ($CDCl_3$): δ 1.21–1.42 (m, 6H), 1.60–2.40 (m, 8H), 2.62–2.85 (m, 6H), 3.15–3.30 (m, 2H), 3.95 (s, 3H), 4.10–4.25 (m, 2H), 4.65 (m, 1H), 4.84 (d, 1H, J = 3.64 Hz), 6.78 (s, 1H), 7.68 (s, 1H), 7.75–7.90 (m, 3H), 8.05 (d, 1H, J = 6.70 Hz), 8.20–8.35 (m, 2H), 9.15 (d, 1H, J = 8.90 Hz), 12.38 (br s, 1H); MS (FAB): 706 $[M+1]^+$.

6.10. (2*S*)-*N*-{4-[*N*-(9,10-Dihydro-9,10-dioxo-1-anthracenyl)-propane-3-carboxami-de]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (10a)

The compound **9a** (692 mg, 1 mmol) was dissolved in methanol (20 mL) and added $SnCl_2{\cdot}2H_2O$ (1128 mg, 5 mmol) was refluxed for 4 h. The reaction mixture was cooled and the methanol was evaporated under reduced pressure. The residue was carefully adjusted to pH 8 with saturated $NaHCO_3$ solution and then extracted with ethyl acetate (2× 30 mL). The combined organic phase was washed with brine (15 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to afford the amino diethyl thioacetal **10a** as yellow oil (503 mg, 76%). The compound was directly used in the next step due to potential stability problem.

6.11. (2*S*)-*N*-{4-[*N*-(9,10-Dihydro-9,10-dioxo-1-anthracenyl)-butane-4-carboxamide]-oxy-5-methoxy-2-aminobenzoyl}pyrrolidine-2-carboxaldehyde diethyl thioacetal (10b)

The compound **10b** was prepared following the method described for the compound **10a**, employing the compound **9b** (706 mg, 1 mmol) to afford the amino diethyl thioacetal **10b** (500 mg, 74%).

6.12. 7-Methoxy-8-[*N*-(9,10-dihydro-9,10-dioxo-1-anthracenyl)-propane-3-carboxamide]-oxy-(11a*S*)-1,2,3,11a tetrahydro-5H-pyrrolo [2,1-*c*][1,4]benzodiazepin-5-one (11a)

A solution of amino thioacetal **10a** (662 mg, 1 mmol), $HgCl_2$ (597 mg, 2.2 mmol) and $CaCO_3$ (240 mg, 2.4 mmol) in 15 mL of acetonitrile/water (4:1) was stirred slowly at room temperature overnight. The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through celite. The clear yellow organic supernatant was extracted with ethyl acetate (2× 20 mL). The organic layer was washed with saturated $NaHCO_3$ (20 mL), brine (20 mL), and the combined organic phase was dried over anhydrous Na_2SO_4. The organic layer was evaporated under reduced pressure and the crude product was purified by column chromatography (90% EtOAc–hexane) to afford the compound **11a** as a pale yellow solid (301 mg, 56%). mp 75–76 °C; $[\alpha]_D^{26}$ + 349.5 (c 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$): δ 1.95–2.10 (m, 2H), 2.20-2.40 (m, 4H), 2.78–2.86 (m, 2H), 3.50–3.81 (m, 3H), 3.91 (s, 3H), 4.15–4.26 (m, 2H), 6.76 (s, 1H), 7.42 (s, 1H), 7.55 (d, 1H, J = 4.25 Hz), 7.70–7.82 (m, 3H), 8.0 (d, 1H, J = 6.90 Hz), 8.20–8.30 (m, 2H), 9.15 (d, 1H, J = 8.70 Hz), 12.38 (br s, 1H); MS (FAB): 538

$[M+1]^+$. Anal. Calcd for $C_{31}H_{27}N_3O_6$: C, 69.26; H, 5.06; N, 7.82. Found: C, 69.29; H, 5.02; N, 17.90.

6.13. 7-Methoxy-8-[*N*-(9,10-Dihydro-9,10-dioxo-1-anthracenyl)-butane-4-carboxamide]-oxy-(11a*S*)-1,2,3,11a tetrahydro-5H-pyrrolo [2,1-*c*][1,4]benzodiazepin-5-one (11b)

The compound **11b** was prepared following the method described for the compound **11a** employing **10b** (676 mg, 1 mmol) to afford the compound **11b** as a pale yellow solid (287 mg, 52%). mp 79–81 °C; $[\alpha]_D^{26} + 277$ (*c* 0.5, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 1.85–2.40 (m, 8H), 2.60–2.78 (m, 2H), 3.51–3.80 (m, 3H), 3.93 (s, 3H), 4.15–4.20 (m, 2H), 6.78 (s, 1H), 7.42 (s, 1H), 7.60 (d, 1H, $J = 4.34$ Hz), 7.65–7.83 (m, 3H), 8.0 (d, 1H, $J = 6.90$ Hz), 8.20–8.25 (m, 2H), 9.15 (d, 1H, $J = 8.80$ Hz), 12.38 (br s, 1H); MS (FAB): 552 $[M+1]^+$. Anal. Calcd for $C_{32}H_{29}N_3O_6$: C, 69.68; H, 5.30; N, 7.62. Found: C, 69.64; H, 5.33; N, 7.59.

6.14. 1,4-Bis-{3-[(2*S*)-*N*-(4-oxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal]propyloxy}anthracene-9,10-dione (12a)

To a solution of **6a** (482 mg, 1 mmol) in dry acetonitrile (30 mL) were added anhydrous K_2CO_3 (829 mg, 6 mmol) and **8** (801 mg, 2 mmol). The reaction mixture was refluxed for 24 h. After completion of reaction, K_2CO_3 was removed by filtration and the solvent was evaporated under reduced pressure, the crude product was purified by column chromatography (80% EtOAc–hexane) to afford compound **12a** (907 mg, 81%). ^1H NMR ($CDCl_3$): δ 1.24–1.40 (m, 12 H), 1.92–2.15 (m, 12H), 2.62–2.82 (m, 8H), 3.12–3.25 (m, 4H), 3.92 (s, 6H), 4.05–4.32 (m, 8H), 4.60–4.72 (m, 2H), 4.82 (d, 2H, $J = 3.66$ Hz), 6.75 (s, 2H), 7.24 (s, 2H), 7.61–7.74 (m, 4H), 8.02–8.18 (m, 2H); MS (FAB): 1121 $[M+1]^+$.

6.15. 1,4-Bis-{4-[(2*S*)-*N*-(4-oxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal]butyloxy}anthracene-9,10-dione (12b)

The compound **12b** was prepared following the method described for the compound **12a**, employing **6b** (510 mg, 1 mmol) and **7** (810 mg, 2 mmol) and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **12b** (872 mg, 76%); H NMR ($CDCl_3$): δ 1.25–1.40 (m, 12H), 2.0–2.32 (m, 16H), 2.62–2.80 (m, 8H), 3.12–3.25 (m, 4H), 3.92 (s, 6H), 4.08–4.40 (m, 8H), 4.60–4.71 (m, 2H), 4.82 (d, 2H, $J = 3.62$ Hz), 6.77 (s, 2H), 7.23 (s, 2H), 7.62–7.78 (m, 4H), 8.02–8.18 (m, 2H); MS (FAB): 1149 $[M+1]^+$.

6.16. 1,4-Bis-{5-[(2*S*)-*N*-(4-oxy-5-methoxy-2-nitrobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal]pentyloxy}anthracene-9,10-dione (12c)

The compound **12c** was prepared following the method described for the compound **12a**, employing **6c** (538 mg, 1 mmol) and **8** (801 mg, 2 mmol) and the crude product was purified by column chromatography (80% EtOAc–hexane) to afford the compound **12c** (824 mg, 70%); ^1H NMR ($CDCl_3$): δ 1.23–1.42 (m, 12H), 1.83–2.18 (m, 20H), 2.65–2.85 (m, 8H), 3.12–3.28 (m, 4H), 3.93 (s, 6H), 4.05–4.20 (m, 8H), 4.60–4.71 (m, 2H), 4.83 (d, 2H, $J = 3.66$ Hz), 6.77 (s, 2H), 7.24 (s, 2H), 7.60–7.73 (m, 4H), 8.02–8.18 (m, 2H); MS (FAB): 1177 $[M+1]^+$.

6.17. 1,4-Bis-{3-[(2*S*)-*N*-(4-oxy-5-methoxy-2-aminobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal]propyloxy}anthracene-9,10-dione (13a)

The compound **12a** (1.12 g, 1 mmol) was dissolved in methanol (40 mL) and added $SnCl_2{\cdot}2H_2O$ (2.256 g, 10 mmol) was refluxed for 4 h. The reaction mixture was cooled and the methanol was evaporated under vacuum. The residue was carefully adjusted to pH 8 with saturated $NaHCO_3$ solution and then extracted with ethyl acetate (2× 30 mL). The combined organic phase was washed with brine (15 mL), dried over anhydrous Na_2SO_4, and evaporated under vacuum to afford the amino diethyl thioacetal **13a** as yellow oil (895 mg, 82%) and due to potential stability problems directly used in the next step.

6.18. 1,4-Bis-{4-[(2*S*)-*N*-(4-oxy-5-methoxy-2-aminobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal]butyloxy}anthracene-9,10-dione (13b)

The compound **13b** was prepared following the method described for the compound **13a**, employing the compound **12b** (1.15 g, 1 mmol) to afford the amino diethyl thioacetal **13b** (895 mg, 80%).

6.19. 1,4-Bis-{5-[(2*S*)-*N*-(4-oxy-5-methoxy-2-aminobenzoyl)pyrrolidine-2-carboxaldehyde diethyl thioacetal]pentyloxy}anthracene-9,10-dione (13c)

The compound **13c** was prepared following the method described for the compound **13a**, employing the compound **12c** (1.18 g, 1 mmol) to afford the amino diethyl thioacetal **13c** (963 mg, 84%).

6.20. 1,4-Bis-{3-[7-Methoxy-8-oxy-(11a*S*)-1,2,3,11a tetrahydro-5H-pyrrolo [2,1-*c*][1,4]benzodiazepin-5-one]propyloxy}anthracene-9,10-dione (14a)

A solution of amino thioacetal **13a** (1.19 g, 1 mmol), $HgCl_2$ (1.19 mg, 4.4 mmol), and $CaCO_3$ (480 mg, 4.8 mmol) in acetonitrile–water (4:1) was stirred slowly at room temperature overnight. The reaction mixture was diluted with ethyl acetate (30 mL) and filtered through celite. The clear yellow organic supernatant was extracted with ethyl acetate (2× 20 mL). The organic layer was washed with saturated $NaHCO_3$ (20 mL), brine (20 mL) and the combined organic phase was dried over anhydrous Na_2SO_4. The organic layer was evaporated under vacuum and the crude product was purified by column chromatography (15% MeOH–EtOAc) to afford the compound **14a** as a yellow solid (414 mg, 51%); mp 125–126 °C; $[\alpha]_D^{26} + 304.5$ (*c* 1, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 1.96–2.05 (m, 4H), 2.20–2.50 (m, 8H), 3.55–3.85 (m, 6H), 3.92 (s, 6H), 4.20–4.32 (m, 4H), 4.42–4.52 (m, 4H), 6.85 (s, 2H), 7.22 (s, 2H), 7.40 (s, 2H), 7.52 (d, 2H, $J = 4.39$ Hz), 7.65–7.71 (m, 2H), 8.02–8.15 (m, 2H); MS (FAB): 813

$[M+1]^+$. Anal. Calcd for $C_{46}H_{44}N_4O_{10}$: C, 67.97; H, 5.46; N, 6.89. Found: C, 68.01; H, 5.35; N, 6.86.

6.21. 1,4-Bis-{4-[7-Methoxy-8-oxy-(11a*S*)-1,2,3,11a tetrahydro-5H-pyrrolo [2,1-*c*][1,4]benzodiazepin-5-one]butyloxy}anthracene-9,10-dione (14b)

The compound **14b** was prepared following the method described for the compound **14a** employing **13b** (1.12 mg, 1 mmol) to afford the compound **14b** as a yellow solid (386 mg, 46%); mp 120–122 °C; $[\alpha]_D^{26} + 143.5$ (*c* 0.75, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 1.96–2.35 (m, 16H), 3.50–3.84 (m, 6H), 3.92 (s, 6H), 4.12–4.38 (m, 8H), 6.82 (s, 2H), 7.23 (s, 2H), 7.42 (s, 2H), 7.56 (d, 2H, $J = 4.39$ Hz), 7.62–7.72 (m, 2H), 8.02–8.18 (m, 2H); MS (FAB): 841 $[M+1]^+$. Anal. Calcd for $C_{48}H_{48}N_4O_{10}$: C, 68.56; H, 5.75; N, 6.66. Found: C, 68.59; H, 5.77; N, 6.58.

6.22. 1,4-Bis-{5-[7-Methoxy-8-oxy-(11a*S*)-1,2,3,11a tetrahydro-5H-pyrrolo [2,1-*c*][1,4]benzodiazepin-5-one]pentyloxy}anthracene-9,10-dione (14c)

The compound **14c** was prepared following the method described for the compound **14a** employing **13c** (1.15 mg, 1 mmol), to afford the compound **14c** as a yellow solid (417 mg, 48%); mp 92–94 °C; $[\alpha]_D^{26} + 424$ (*c* 0.5, $CHCl_3$); ^1H NMR ($CDCl_3$): δ 1.95–2.32 (m, 20H), 3.53–3.82 (m, 6H), 3.92 (s, 6H), 4.07–4.13 (m, 8H), 6.76 (s, 2H), 7.23 (s, 2H), 7.44 (s, 2H), 7.58 (d, 2H, $J = 4.40$ Hz), 7.65–7.69 (m, 2H), 8.02–8.16 (m, 2H); MS (FAB): 869 $[M+1]^+$. Anal. Calcd for $C_{50}H_{52}N_4O_{10}$: C, 69.11; H, 6.03; N, 6.45. Found: C, 69.07; H, 5.59; N, 6.51.

6.23. Thermal denaturation studies

The DNA binding affinity of the novel anthraquinone-PBD conjugates (**11a**–**b** and **14a**–**c**) has been evaluated through thermal denaturation studies with duplex-form calf thymus DNA (CT-DNA) using modified reported procedure.[19,20] Working solutions in aqueous buffer (10 mM NaH_2PO_4/Na_2HPO_4, 1 mM Na_2EDTA, pH 7.00 ± 0.01) counting CT-DNA (100 μM in phosphate) and the PBD (20 μM) were prepared by addition of concentrated PBD solutions in DMSO to obtain a fixed [PBD]/[DNA] molar ratio of 1:5. The DNA–PBD solutions are incubated at 37 °C for 0, 18, and 36 h prior to analysis. Samples are monitored at 260 nm using a Beckman DU-7400 spectrophotometer fitted with high performance temperature controller and were heated at 1 °C/min in the range of 40–95 °C. DNA helix coil transition temperatures (T_m) were obtained from the maxima in the d(A260)/dT derivative plots. Drug-induced alterations in DNA melting temperatures are given by: $\Delta T_m = T_m$ (DNA+PBD)-Tm (DNA alone), where the T_m value for the PBD-free CT-DNA is 69.4 °C ± 0.01.

6.24. In vitro evaluation of cytotoxic activity

The compounds **11a**–**b** and **14a**–**c** were evaluated for in vitro activity against selected human tumor cell lines, derived from six cancer types (lung cancer, cervix cancer, breast cancer, prostate cancer, colon cancer, ovarian cancer). For each compound, dose–response curves against each cell line were measured. Sulforhodamine B (SRB) protein assay[21,22] has been used to estimate cell viability or growth.

The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine and were inoculated into 96-well microtiter plates in 90 μL at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37 °C, 5% CO_2, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs. Aliquots of 10 μL of the drug dilutions were added to the appropriate microtiter wells already containing 90 μL of cells, resulting in the required final drug concentrations.

Plates were incubated further for 48 h and assay was terminated by the addition of 50 μL of cold trichloro acetic acid (TCA) (final concentration, 10% TCA) and incubated for 60 min at 4 °C. The plates were washed five times with tap water and air-dried. Sulforhodamine B (SRB) solution (50 μL) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. The residual dye was removed by washing five times with 1% acetic acid. The plates were air-dried. Bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on an ELISA plate reader at a wavelength of 540 nm with 690 nm reference wavelength. Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percentage growth was expressed as the (ratio of average absorbance of the test well to the average absorbance of the control wells) × 100.

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