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Fluorescent 7-diethylaminocoumarin pyrrolobenzodiazepine conjugates: Synthesis, DNA interaction, cytotoxicity and differential cellular localization

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Abstract—The pyrrolo[2,1-*c*][1,4]benzodiazepines (PBDs) are a class of DNA minor groove binding agents that react covalently with guanine bases, preferably at Pu-G-Pu sites. A series of three fluorescent PBD–coumarin conjugates with different linker architectures has been synthesized to probe correlations between DNA binding affinity, cellular localization and cytotoxicity. The results show that the linker structure plays a critical role for all three parameters.

The design and synthesis of sequence-selective DNA-interactive agents are currently of interest for therapeutic applications, particularly in view of the recent sequencing of the human genome and the growing understanding of genetic changes associated with serious diseases such as cancer. A number of strategies have been exploited to develop ligands which bind to and disrupt DNA functioning, such as triplex-forming oligonucleotides, DNA intercalators, and major and minor groove binding agents.[1] Of the minor groove-targeted agents, the polyamides, especially those with a hairpin structure, are notable for their sequence discriminating properties and high affinity for DNA.[2] While the cell-free activity of these compounds was promising, early studies suggested that this could not be reliably translated into cellular responses.[3]

Understanding the cellular distribution of DNA-targeting molecules is particularly important as they have the potential to interact off-target which may diminish their ability to reach the genome and elicit the desired response. For these reasons, experiments with fluorophore-conjugated polyamides have been carried out to investigate their lack of cellular activity. Fluorescent BODIPY–polyamide conjugates were shown to retain affinity towards naked DNA but confocal microscopy demonstrated that they localized predominantly in the cytoplasm rather than the nucleus for >20 different cell lines (with the exception of certain T cell lines).[3,4] Further experiments showed that BODIPY conjugates accumulated in acidic cytoplasmic vesicles in rheumatoid synovial fibroblasts and HCT-116 tumor cells, whereas fluorescein coupled polyamides could enter the nuclei of HCT-116 cells.[5] A more comprehensive study of fluorophore-conjugated polyamides indicated that composition, charge, size, fluorophore type and linker structure all influenced nuclear accumulation in various different cell types.[6] Other types of DNA-interactive agents have been attached to fluorescent markers; for example, a

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Keywords: Pyrrolobenzodiazepines; PBDs; DNA-binding; DNA sequence-selective; Fluorescence conjugates; Nuclear penetration; Anticancer agents; Antitumour agents.

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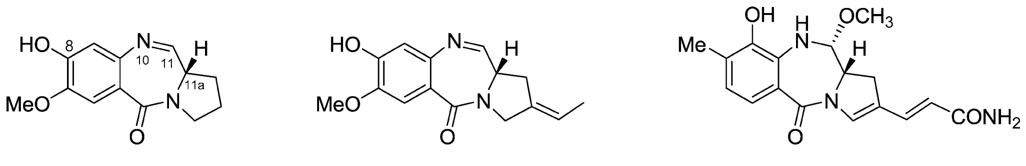
Fmoc-linked analogue of distamycin was used to show that it localizes in cytoplasmic organelles.[7]

To date there have been relatively few studies of the cellular localization of agents that interact covalently within the minor groove of DNA where off-target chemical reactions with nucleophiles such as glutathione and various proteins may be problematic. So far, only Fmoc-labeled polyamide–*seco*-cyclopropabenzindole conjugates have been studied and appear to localize in the cytoplasmic organelles of treated EMT-6 cells.[8]

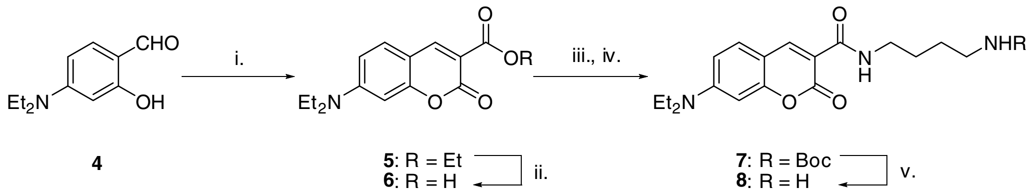
Our own recent studies have focused on sequence-selective DNA-targeting agents based on the pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) structure (see Fig. 1). These molecules have innate DNA sequence-recognition properties, binding covalently via their electrophilic N10–C11 imine moiety to embedded purine–guanine–purine sites in the minor groove. In order to evaluate the cellular localization of PBDs and investigate how this correlates with their DNA-binding and cytotoxicity, we have synthesized a number of analogues of the naturally occurring DC-81 (**1**). These are based on a fluorophore tethered to the C8-hydroxyl group of the PBD through different linkers. 7-Diethylaminocoumarin was selected as the fluorophore due to its chemical stability and favorable optical characteristics.

The coumarin fragment was prepared by Knoevenagel condensation of benzaldehyde **4** with diethyl malonate to give ester **5**, hydrolysis of which produced the acid **6**. Conversion to the acid chloride followed by reaction with mono-Boc-protected 1,4-diaminobutane gave the *N*-Boc-butylamino derivative **7**. Deprotection with 95% TFA provided the free amine **8** (Scheme 1). The simplest conjugate **11**, where the PBD is joined to the coumarin fragment via a C_3-linker, was synthesized by reaction of the previously reported *N*-10-Alloc-protected 8-(3-aminopropyl)-PBD (**9**)[10] with the coumarin acid (**6**), to give the coupled intermediate **10**. Alloc deprotection using $Pd[PPh_3]_4$ gave the desired conjugate **11**[9] (Scheme 2). The equivalent C_8-linked homologue **17** was synthesized in a similar manner (Scheme 3). The protected PBD **12**[11] was *O*–debenzylated and the result-

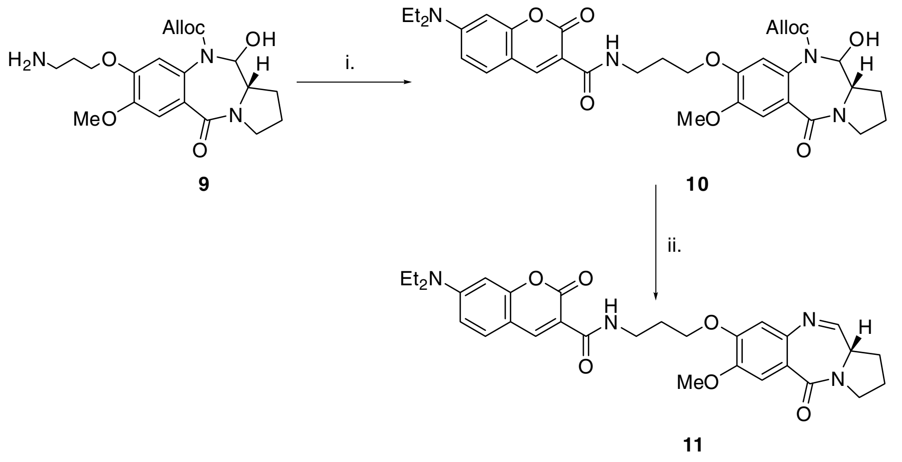


1: DC-81 **2**: Tomaymycin **3**: Anthramycin Methyl Ether

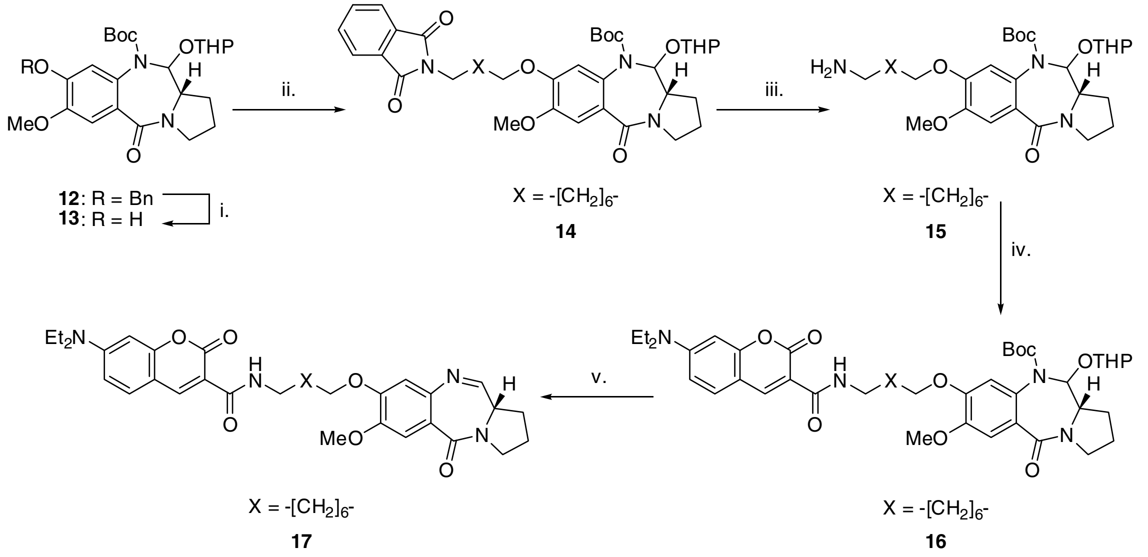
Figure 1. Examples of naturally occurring PBD molecules.



Scheme 1. Reagents and conditions: (i) diethyl malonate, EtOH, reflux, 48%; (ii) NaOH, H_2O, MeOH, 97%; (iii) $(COCl)_2$, DMF, CH_2Cl_2; (iv) $H_2N(CH_2)_4NHBoc$, Et_3N, CH_2Cl_2, 70% (2 steps); (v) 95% aq TFA, 92%.



Scheme 2. Reagents: (i) **6**, EDCI, DMAP, DMF, 81%; (ii) $Pd[PPh_3]_4$, pyrrolidine, CH_2Cl_2, 89%.

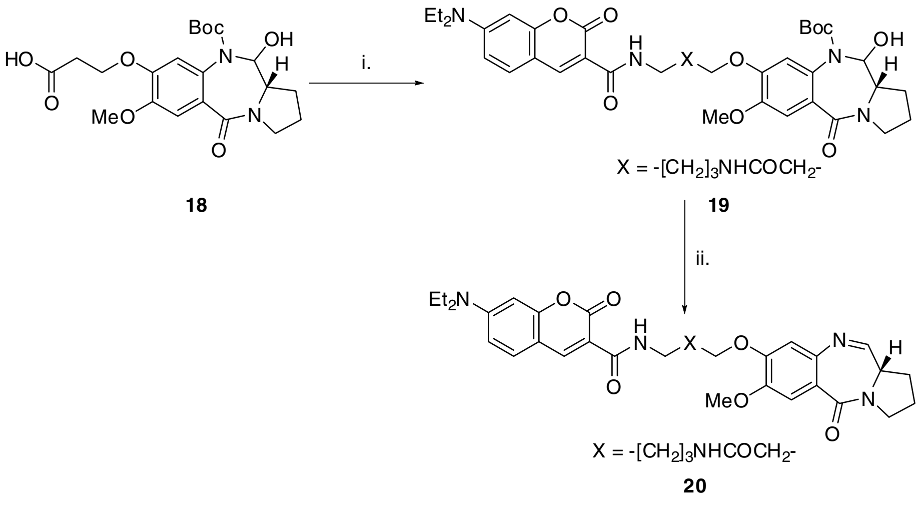


Scheme 3. Reagents and conditions: (i) H_2, 10% Pd/C, EtOH, 100%; (ii) *N*-8-bromooctylphthalimide, K_2CO_3, DMF, 100%; (iii) N_2H_4, MeOH; (iv) **6**, EDCI, DMAP, DMF, 50% (2 steps); (v) 95% aq TFA, 95%.

ing phenol (**13**) treated with *N*-8-bromooctylphthalimide to give **14**. Hydrazinolytic cleavage gave the PBD-octylamine **15**, which was coupled to the coumarin acid (**6**) to provide conjugate **16**. Treatment with 95% TFA effected removal of both the Boc and THP protecting groups to provide the C^8-linked PBD–coumarin conjugate **17**.[12] Finally, for the PBD–coumarin conjugate with an amide linker (**20**), the PBD acid **18** was coupled to the coumarin fragment **8** to give conjugate **19**. Removal of the Boc group using 95% TFA afforded the PBD–coumarin conjugate **20**[13] (Scheme 4).

The conjugates had similar fluorescence wavelengths to the free coumarin **5** (i.e., λ_{max} absorption ~410–420 nm, emission 455–460 nm). However, fluorescence was significantly more intense for the conjugated agents, which was attributed to the presence of an amide rather than an ester linkage at the coumarin 3-position. While the PBD molecules alone showed some innate fluorescence (e.g., absorption 320 nm, emission 420 nm for the C-8 methyl ether of **1**), their direct visualization in microscopy applications is less practicable due to the requirement for excitation by a UV laser rather than light at visible wavelengths.

DNA thermal denaturation[15,16] results for the compounds are presented in Table 1. The data show that the simple coumarin **5** is a poor stabilizing ligand for calf thymus (CT) DNA and there is no time-dependent increase in ΔT_{m} during the 72 h exposure. In contrast, the hybrid agents **11** and **17** are effective at stabilizing CT-DNA. Their ΔT_{m} values increase with exposure time, confirming the occurrence of time-dependent covalent binding typical for PBD agents.[17] Interestingly, the short-chain homologue **11** (i.e., 3 methylenes in linker) is more potent and reacts faster than its longer-chain counterpart **17** (i.e., 8 methylenes). Compound **20** is considerably less effective than the related conjugate **17**



Scheme 4. Reagents: (i) **8**, EDCI, DMAP, DMF, 98%; (ii) 95% aq TFA, 89%.

Table 1. Cytotoxicity and DNA thermal denaturation data for **5** and PBD conjugates **11**, **17**, and **20**

Compound	Cell line IC_{50}[a] (μM)		Induced ΔT_m (°C)[b,c] after incubation at 37 °C for			
	OVCAR-5[d]	LOX IMVI[d]	0 h[e]	4 h[e]	18 h[e]	72 h[e]
5	>10	>10	0.1	0.2	0.2	0.2
11	3.2 ± 1.04	1.4 ± 0.24	2.9	3.4	3.6	3.9
17	7.8 ± 0.33	4.5 ± 0.22	1.1	1.4	2.0	2.9
20	>10	>10	0.3	0.7	0.9	1.0

[a] Determined by Alamar blue assay (96 h drug exposure).[14] Each value represents the mean ± standard deviation of three independent experiments.

[b] For calf thymus (CT) DNA alone, $\Delta T_m = 67.82 \pm 0.07$ °C (mean from >110 experiments).[15,16] All ΔT_m values are ±0.08–0.12 °C.

[c] For a fixed 1:5 molar ratio of [ligand]/[DNA], DNA concentration = 50 μM (bp) and ligand concentration = 20 μM, in aqueous sodium phosphate buffer [10 mM sodium phosphate + 1 mM EDTA, pH 7.00 ± 0.01]. Solutions were incubated at 37 ± 0.1 °C for the time shown.

[d] 8-Methyl DC-81 was inactive at 1 μM or below in this assay.

[e] Values of 0.3, 0.5, 0.7, and 0.8 °C were determined for DC-81 (**1**) at the same 1:5 ligand/DNA molar ratio after incubation for 0, 4, 18, and 72 h, respectively.

despite the similar linker length. It behaves in a comparable manner to the simple PBD monomer DC-81 (**1**).

The responses of OVCAR-5 and LOX IMVI tumor cells to **5** and the conjugates are also presented in Table 1. These cell lines were selected on the basis of intrinsic sensitivity (LOX IMVI melanoma) and known resistance (OVCAR-5 ovarian carcinoma) to PBD molecules in xenograft studies.[18] The free coumarin **5** was inactive in both cell lines at the highest concentration tested. In contrast, the PBD–coumarin conjugates had effects that were dependent on their linker structure. The C_3-linked compound **11** was the most active agent tested, with IC_{50} values in the two cell lines of 3.2 μM and 1.4 μM after incubation for 96 h, with cytotoxic effects evident in the more sensitive melanoma cell line after 24 h (IC_{50} 1.5 μM). The C_8-linked compound **17** was less active, with IC_{50} values of 7.8 μM and 4.5 μM after 96 h. However, incorporation of an amide unit within the C_8 linker (i.e., **20**) resulted in reduced activity.

Cellular uptake and localization of the fluorescent PBD conjugates within the OVCAR-5 and LOX IMVI cells are shown in Figure 2. The detection limit for all compounds was >0.1 μM. The free coumarin **5** was localized and concentrated in the cytoplasm of both cell lines (from 1 h at 1 μM concentration; data not shown). Compound **20** showed a very weak cytoplasmic localization, similar to the fluorophore **5** alone, at a concentration of 10 μM suggesting poor cellular penetration. In contrast, conjugate **11** could be observed to enter cells and accumulate in the nucleus of both cell lines after 1 h exposure at concentrations above 1 μM. Compound **17** could be observed in the cytoplasm of both cell lines (1–10 μM) and appeared to be present in the nucleus after 24 h (10 μM).

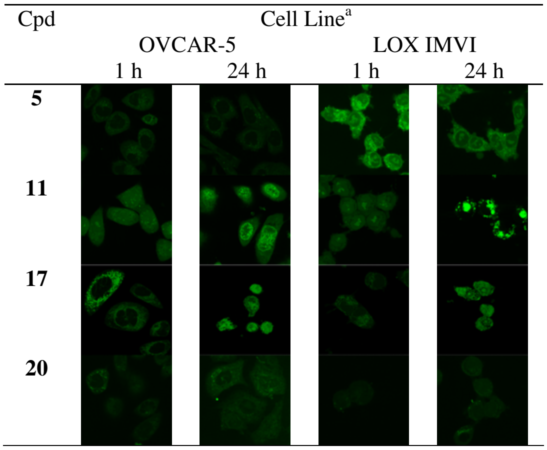


Figure 2. Fluorescence microscopy of cells treated with **5** and PBD conjugates **11**, **17**, and **20**. [a]Cells were treated for 48 h with 1 μM or 10 μM of agent. Data are shown for 1 h and 24 h time points for a 10 μM concentration.

These results demonstrate the critical effect of the linker structure in governing the behavior of these conjugates. The simple coumarin **5** has no intrinsic DNA-binding ability, little tendency to accumulate in the nucleus of cells, and negligible cytotoxic and cell membrane-damaging effects (data not shown). However, greater fluorescence intensity is seen in the cytoplasm of treated LOX IMVI melanoma cells relative to OVCAR-5. The conjugates **11**, **17**, and **20** differ significantly in their cellular distribution properties, DNA interaction behavior and cytotoxic potencies. Compound **11**, with the short C_3 linker exhibits the greatest stabilizing effect on DNA melting and the fastest DNA binding kinetics, presumably through additional hydrogen bonding and van der Waals interactions with groups on the floor and walls of the DNA minor groove. Localization in the nuclei of both treated cell lines is similarly rapid and occurs during the first 1–4 h of treatment. Nuclear condensation (confocal microscopy) and cell killing (trypan blue exclusion assays, data not shown) evident at longer exposure times are consistent with the cytotoxicity of the agent as determined using the Alamar blue assay. Lengthening the linker between the PBD and the coumarin from 3 to 8 methylene units (i.e., **11** → **17**) has a detrimental effect on the interaction of the conjugate with DNA; the kinetics of association are slower and the ultimate effect on thermal denaturation is smaller. Cellular distribution of this compound was also different. It clearly localized within the cytoplasm of both cell lines after 1 h, with a more uniform distribution, including the nuclear compartment, after 24 h. Cytotoxicity was also diminished relative to compound **11**. Interestingly, the introduction of an amide unit into the linker (i.e., **20**) abolished most of the interaction with DNA. The rigidity introduced into the chain by the amide functionality may prevent the molecule from adopting a favorable DNA-binding conformation. The potential for the amide to generate additional H-bonding interactions with the floor of the host minor groove did not appear to help in this case, although other placements in the linker might be more effective. As anticipated, based on its poor DNA binding, **20** is less cytotoxic and shows poor cellular penetration, with minor accumulation in the cytoplasmic compartment in both cell lines.

In summary, as demonstrated by Dervan and co-workers for the hairpin polyamide family of DNA-interactive agents,[6] these data reveal the important influence of the linker within these PBD conjugates on the extent and time course of DNA binding, in vitro cytotoxic potency and cellular distribution. This information provides a useful starting point for the design of new PBD conjugates with optimized linkers and fluorophores that may be of interest for biophysical optical assays and for studying various mechanisms of inherent and acquired resistance (e.g., drug efflux, DNA repair, glutathione conjugation) or sub-cellular distribution in intact cells.

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13. Analytical data for compound **20**. ^{1}H NMR (CDCl$_3$, 400 MHz) 8.82 (1H, br s), 8.68 (1H, s), 7.65 (1H, d, *J* = 4.3 Hz), 7.50 (1H, s), 7.42 (1H, d, *J* = 9.0 Hz), 6.84 (1H, s), 6.64 (1H, dd, *J* = 9.0, 2.5 Hz), 6.58 (1H, br s), 6.49 (1H, d, *J* = 2.3 Hz), 4.33 (2H, m), 3.92 (3H, s), 3.83–3.69 (2H, m), 3.48–3.31 (9H, m), 2.32–2.28 (2H, m), 2.07–2.03 (2H, m), 1.68–1.59 (4H, m), 1.24 (6H, t, *J* = 7.1 Hz); HRMS ([M+H]$^{\cdot+}$) $C_{34}H_{42}N_5O_7$ calc. 632.3079 found 632.3055.
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