Effect of Linker Length on DNA-binding Affinity, Cross-linking Efficiency and Cytotoxicity of C8-linked Pyrrolobenzodiazepine Dimers

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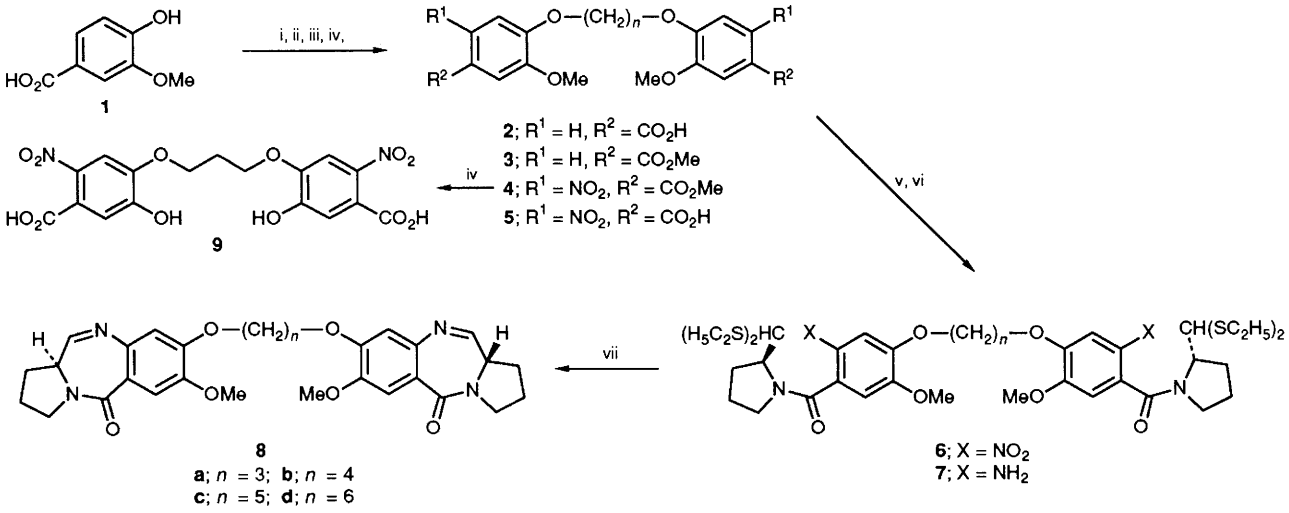
An efficient synthesis of a homologous series of C8-linked pyrrolobenzodiazepine dimers is reported; compounds with an odd number of methylenes ($n = 3$ or 5) in the linker show a higher affinity for DNA, enhanced cross-linking efficiency, and are more cytotoxic compared with compounds with either $n = 4$ or 6.

It is generally accepted that a DNA interstrand cross-link is a highly cytotoxic lesion,[1a] and many bifunctional alkylating agents such as the nitrogen mustards and cisplatin are used clinically as antitumour agents.[1b] Although the basis for tumour-cell selectivity remains unknown, it is possible that cross-linked DNA adducts prove challenging to cellular repair mechanisms,[2] or that the agents target genes associated with the control of cell growth.[3] We recently reported[4] the design and synthesis of a novel irreversible guanine-specific DNA interstrand cross-linking agent, DSB-120 (**8a**; $n = 3$), based on the naturally occurring pyrrolo[2,1-*c*][1,4]benzodiazepine (PBD) antitumour antibiotic DC-81 (**10**),[5] which bonds in the minor groove of DNA *via* covalent interaction between the C11-position and N2 of guanine. PBD monomers are known to be sequence selective with a preference for PuGPu triplets.[6] NMR spectroscopy and modelling studies indicate that DSB-120 spans six DNA base-pairs (twice the binding-site size of DC-81) and actively recognises a 5′-GATC sequence.[4] We report here the synthesis of a homologous series of C8-linked bifunctional DNA alkylating agents in order to probe the effect of linker length on binding affinity, DNA cross-linking efficiency and cytotoxicity.

The overall synthetic strategy is shown in Scheme 1. A versatile approach has been developed to join two units of vanillic acid **1** with α,ω-dihaloalkanes of varying length to provide the dimer acids **2a–d**. Dibromo- and dichloro-alkanes gave inferior yields compared to the diiodo-compounds, and varying the chain length from $n = 3$ to 6 did not affect the efficiency of the reaction. By using this approach, the formation of mixtures of mono- and bis-alkylated products is avoided. The procedure involved refluxing **1** with the di-iodoalkanes in the presence of aq. NaOH for 48 h to afford **2a–d** in 33–65% yields. However, all attempts to obtain the nitro acids of type **5** by direct nitration of **2a–d** failed using a variety of reaction conditions, including $NaNO_2$–H_2SO_4, $SnCl_4$–HNO_3 and H_2SO_4–HNO_3, owing to the insoluble nature of the dimer acids. Following conversion into the corresponding methyl esters **3a–d**, nitration with $SnCl_4$–HNO_3 proceeded smoothly to afford the nitro esters **4a–d** in high yield. An initial attempt to hydrolyse the methyl ester **4a**



Scheme 1 *Reagents and conditions*: i, I-$(CH_2)_n$-I, aq. NaOH, THF, reflux, 48 h; ii, dimethyl sulfate, K_2CO_3, acetone, reflux, 30 min; iii, $SnCl_4$, HNO_3, CH_2Cl_2, −20 °C, 15 min; iv, aq. NaOH, THF; v, oxalyl chloride, THF, Et_3N, H_2O, (2*S*)-pyrrolidine-2-carbaldehyde diethyl thioacetal,[8] 4 h; vi, $SnCl_2{\cdot}2H_2O$, MeOH, reflux, 40 min; vii, $HgCl_2$, $CaCO_3$, MeCN, H_2O, 2.5 h

Table 1 Thermal denaturation (ΔT_m values), DNA cross-linking efficiency, and *in vitro* cytotoxicity data for the C8-linked PBD dimers and DC-81

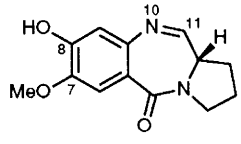
Compound	Linker *n* =	ΔT_m/°C[a]	$C_{50\%}$/μmol dm^{-3}[b]	IC_{50}/μmol dm^{-3} L1210[c]	ADJ/PC6[c]	CH1[c]	K_{562}[d]
8a	3	15.1	0.055	0.01	0.0005	0.003	0.2
8b	4	4.1	1.00	1.2	0.35	0.05	2.5
8c	5	8.1	0.070	0.0045	0.0004	0.00032	0.5
8d	6	7.0	0.750	0.34	0.002	0.002	1.0
DC-81 (**10**)	—	0.7	—	0.38	0.33	0.1	NA
Melphalan	—	—	20.0	3.0	0.02	2.0	35

[a] Thermal denaturation studies with calf thymus DNA (see text). [b] Concentration of drug required for 50% cross-linking of pBR322 DNA (see text). [c] IC_{50} is the drug dose that inhibits cell growth by 50% compared with solvent controls. Compounds were dissolved in DMSO to provide a final concentration of 0.05% DMSO. Incubation times (37 °C) were: L1210, 3 days; ADJ/PC6, 4 days; CH1, 9 days; [d] K_{562} is a human leukaemia cell line in which IC_{50} values were measured using an MTT assay following a 1 h exposure to drug. NA = Result not available.

by refluxing in aq. NaOH gave a high-melting solid identified as the diacid hydrolysis product **9** in which demethylation of the aromatic ether had occurred. The electron-withdrawing *p*-nitro groups may be responsible for this phenomenon.[7] However, mild hydrolysis of the ester with aq. NaOH at room temperature for 6 h afforded **5a** in quantitative yield. Coupling of nitro acids **5a–d** with (2*S*)-pyrrolidine-2-carbaldehyde diethyl thioacetal[8] afforded the bis(amides) **6a–d** in approximately 65% yield, which were subsequently reduced to the amino thioacetals **7a–d**. Cyclization[8] with $HgCl_2$–$CaCO_3$ afforded the target C8-dimers **8a–d** in good yields.†

The general DNA-binding affinity of PBD dimers **8a–d** was examined by thermal denaturation studies.[9] For a 5:1 mol ratio of DNA-ligand (calf thymus DNA concentration = 100 μmol dm^{-3}, 10 mmol dm^{-3} phosphate buffer, pH 7.0) an increase in helix melting temperature (ΔT_m) is observed for each dimer compared with untreated control DNA following incubation at 37 °C for 18 h (Table 1). In the same experiment, the monomer DC-81 (**10**) gives only a small increase in T_m, consistent with the notion that the PBD dimers **8a–d** stabilise DNA through the formation of interstrand cross-links.

The DNA cross-linking efficiency of dimers **8a–d** was investigated using an assay involving linear double-stranded



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DNA derived from the plasmid pBR322 (4362 bp, linearised with *Hind* III and then ^{32}P-end-labelled).[10] Following complete denaturation to the single-stranded form, the presence of an interstrand cross-link results in renaturation to double-stranded DNA during electrophoresis in a neutral agarose gel (Fig. 1). Quantitation of the autoradiograph using laser densitometry allowed calculation of the concentration of each agent required to effect 50% cross-linking (Table 1).

Compounds **8a** (*n* = 3) and **8c** (*n* = 5) are broadly similar in cross-linking efficiency, whereas dimers **8b** (*n* = 4) and **8d** (*n* = 6) approximately 18- and 14-fold less efficient, respectively. This data is consistent with the determined ΔT_m values, which reflect the differences in ability of the compounds to stabilise DNA helix-coil transitions. Compound **8a** binds to double-stranded DNA more tightly than **8c** even though they have similar cross-linking efficiencies. This may reflect differences in their non-covalent binding, since they differ in isohelicity with respect to the DNA minor groove. It is also possible that mono adducts or intrastrand cross-links can be formed that would influence the thermal denaturation data but not the cross-linking assay.

Interestingly, *in vitro* cytotoxicity data in human K_{562} and rodent ADJ/PC6 cell lines (Table 1) correlate with both the

† All compounds gave satisfactory spectral data: selected data for **8a**: ^{1}H NMR ($CDCl_3$, 270 MHz): δ 2.01–2.17 (m, 2H), 2.28–2.45 (m, 8H), 3.50–3.87 (m, 6H), 3.92 (s, 6H), 4.22–4.33 (m, 4H), 6.85 (s, 2H), 7.51 (s, 2H), 7.66 (d, 2H, *J* 4.4 Hz). ^{13}C NMR ($CDCl_3$): δ 24.2, 28.8, 29.6, 46.7, 53.7, 56.1, 65.4, 110.7, 111.6, 120.3, 140.6, 147.8, 150.6, 162.4, 164.6.

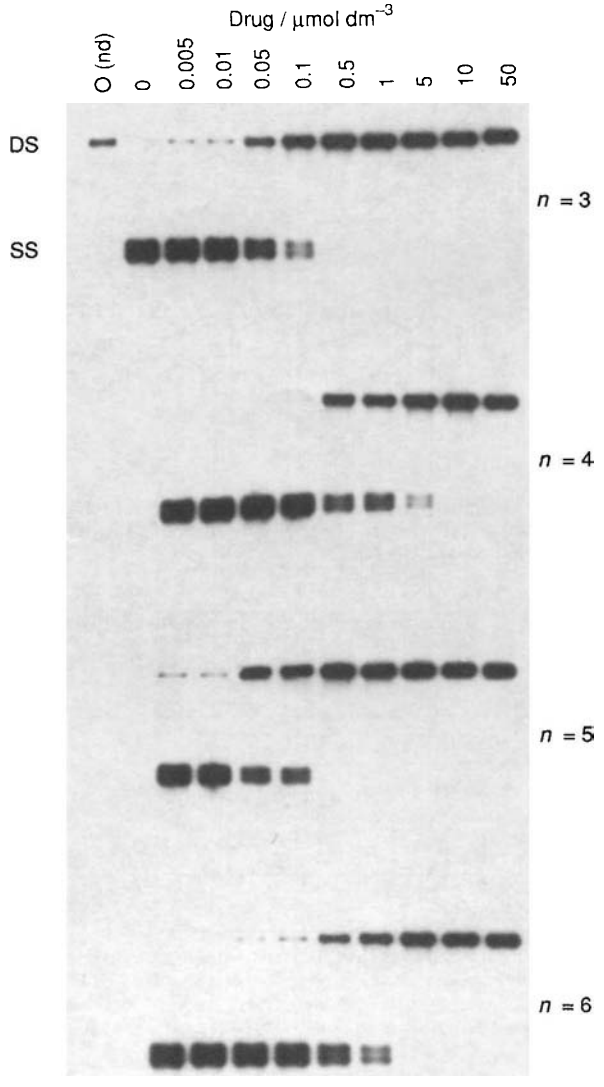

Fig. 1 Autoradiograph of a neutral agarose gel showing DNA interstrand cross-linking by **8a–d** in linear ^{32}P-end-labelled pBR322 DNA. Drug reactions (2 h at 37 °C) were in 25 mmol dm^{-3} triethanolamine/1mmol dm^{-3} EDTA pH 7.2 buffer with 10 ng DNA in a final volume of 50 μl. Reaction was terminated by addition of an equal volume of 0.6 mol dm^{-3} sodium acetate, 20 mmol dm^{-3} EDTA and 100 μg cm^{-3} tRNA, and the DNA precipitated with ethanol. Dried pellets were taken up in strand separation buffer (30% DMSO in 1 mmol dm^{-3} EDTA). Denaturation for 2 min at 90 °C was followed by immediate chilling in an ice-water bath. Electrophoresis was carried out on 0.8% submerged horizontal agarose gels at 40 V for 16 h with tris(acetate) running buffer. Double- (DS) and single-stranded (SS) DNA were quantitated by laser densitometry. O (nd) is the non-denatured double-stranded DNA control.

thermal denaturation data and the cross-linking efficiencies. There is a similar correlation for the rodent L1210 and human CH1 cell lines, except that the positions of **8a** and **8c** in the overall rank-order are transposed. As shown in Table 1, **8a** is approximately 300-fold more efficient at cross-linking DNA than the clinically-used major-groove cross-linking agent melphalan, and is significantly more cytotoxic across the four cell lines studied. Considering the lower activity of the monomer DC-81, this data suggests that interstrand cross-links probably represent the cytotoxic lesions leading to cell death.

In summary, an efficient synthesis of C8-linked pyrrolobenzodiazepine dimers of varying linker length has been achieved. We find a significant correlation between DNA-binding affinity, cross-linking efficiency and cytotoxicity, with the $n = 3$ and $n = 5$ homologues **8a** and **8c** displaying optimal effects compared to the dimers with an even number of methylenes in the linker.

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