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DESIGN, SYNTHESIS AND EVALUATION OF NOVEL BISMUSTARD CROSS-LINKED LEXITROPSINS

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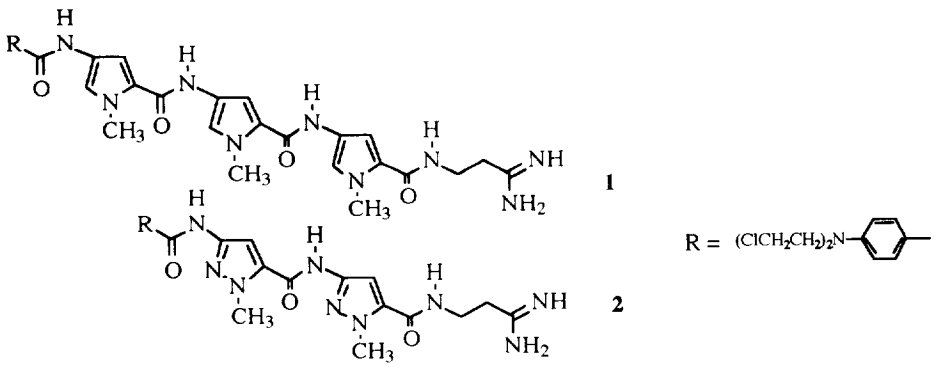
Abstract: A novel class of bis(mustard) cross-linked lexitropsins was designed and synthesized. Their interactions with a number of DNA polymers were assessed by CD and ethidium fluorometry. These data were compared with *in vitro* biological assays of cytotoxicity.

There has been considerable interest in developing DNA-targeted alkylating agents in recent years.[1] One approach is to covalently conjugate simple alkylating agents to DNA binding moieties, which in effect serve as vehicles to deliver the alkylating agents to DNA targets. Hybrid molecules fabricated as such can be expected to be more specific to DNA than simple alkylating agents and their reaction with non-DNA cellular nucleophiles should be therefore less significant, plausibly resulting in reduced side effects for a given dosage. For hybrid molecules incorporating nitrogen mustard and related haloalkyl groups as DNA reacting functional groups, the corresponding DNA binding moieties so far explored include intercalators such as acridine,[2a,b] anthracene-9,10-diones[2c] and minor groove binders such as distamycin A[3,4a,5] and its analogues,[6] 4-anilinoquinoline,[7] bisquaternary ammonium heterocycles[8] and Hoechst 33258.[9] From this research has emerged a promising compound called FCE-24517 (**1**) which is undergoing clinical trials at present[4b] and a number of other compounds such as **2**[6a] which show good *in vivo* anticancer activity. All these compounds are AT selective minor groove binding alkylators.



1 **2**

Figure 1. Minor groove targeted alkylators

Recent NMR studies on distamycin A[10] and its generalized minor groove binding analogues called lexitropsins[11a,12] elucidated a structural motif different from what was previously observed from X-ray diffraction analysis[11] and confirmed an earlier speculation from hydrodynamic studies.[13] This new motif

consists of two oligopeptide molecules, instead of one, packed in the same site of the minor groove of DNA in an antiparallel side by side orientation and covering five base pairs. The outflanking positively charged end of each peptide points in the 5' to 3' direction of the neighboring DNA strand. Generality of this structural motif was further supported from studies on imidazole-containing lexitropsins.[14] Notably, a heterodimeric motif in its matching site showed greater cooperativity than its corresponding homodimeric ones, constituting a unique pattern of strand specific information readout.[14b,d] We have recently designed covalently cross-linked dimeric lexitropsins (**3**, Figure 2) to explore this antiparallel side by side motif.[15a,b] Suitably cross-linked lexitropsins demonstrated much greater binding strength than the monomer to the alternating AT polymer where the antiparallel side by side bidentate binding is possible. The decakis(methylene) chain was identified to be the optimal cross-linker among all screened polymethylene chains, giving a binding enhancement of about 10^4 fold. In contrast, these dimers showed similar binding affinity, compared with the monomer, to the homo AT polymer, where only one oligopeptide molecule is permitted to locate in the same site of the minor groove. These interesting properties stimulated us to explore such dimeric minor groove binders as novel vehicles to deliver DNA-reacting functional groups into the minor groove of DNA. One of the functional groups we selected is the nitrogen mustard similar to that incorporated in **1** and **2**. In this communication, we present the design, synthesis and preliminary physiochemical and biological evaluation of selected nitrogen mustard-containing cross-linked lexitropsins.

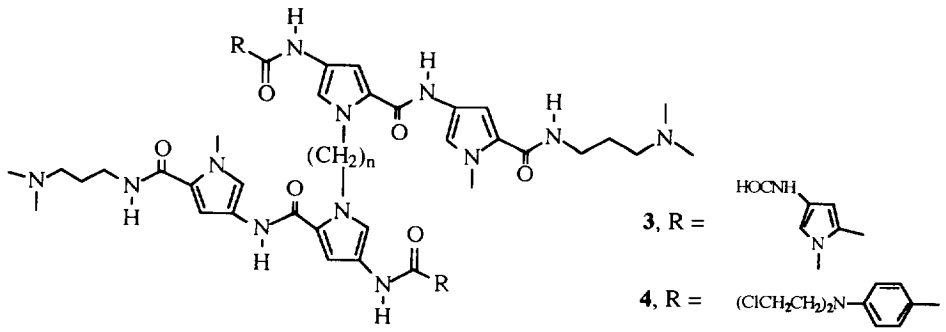
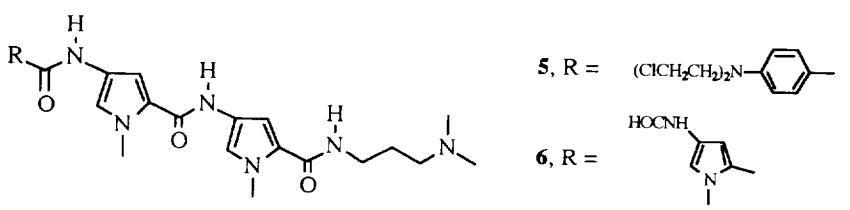
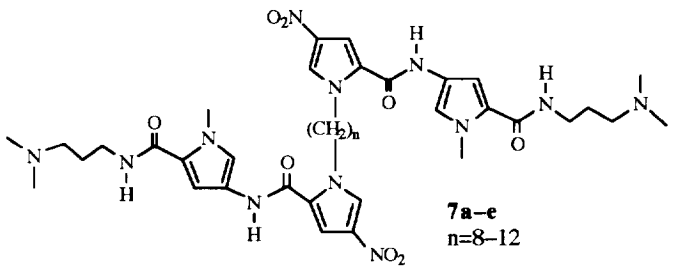


Figure 2. Design of bismustard cross-linked lexitropsins

The first generation of nitrogen mustard cross-linked lexitropsins possess the general structure **4** (Figure 2), which has the 4-N,N-di(2-chloroethyl)aminobenzoyl moiety replacing the 4-formylamino-1-methylpyrrole-2-carbonyl moiety at the amino end of the oligopeptide strand of **3**. CD titration and ethidium displacement studies with homo AT and alternating AT polymers (see below) revealed that the mustard-containing monomer **5** behaves similarly to the corresponding non-functionalized monomer **6** and the side by side binding mode remains effective for the mustard-containing monomer **5**, supporting the validity of design. We might anticipate that the bismustard dimers would not only function similarly to the monomer **5**, when only one oligopeptide strand is allowed to reside in the groove, but also to demonstrate a novel way of interaction with DNA when the side by side antiparallel binding is permitted. The latter interaction might enhance the possibilities of intra- and inter- strand alkylation reactions, and increasing the cytotoxic potency. In fact, a minor groove targeted "split mustard" with the chloroethyl group situated at both ends of the molecule, thus providing similar intra- and inter- strand alkylation possibilities, was shown to be highly potent.[8]



In order to synthesize these molecules, intermediates **7a–e** were prepared from pyrrole and the corresponding 1,n-dibromoalkane (n=8–12) following a previously established procedure.[15a] Catalytic hydrogenation of **7** with platinum oxide in methanol led to unstable diaminotetrapyrrole intermediates which were applied to the next step without purification. The coupling reaction with 4-N,N-di(2-chloroethyl)aminobenzoic acid[16] was performed at 55 °C in DMF solution in the presence of DCC and HOBt. Compounds **4a–e** were isolated in ~50% yield.[17]



7a–e
n=8–12

Binding of these bismustard dimers to DNA was first evaluated with the ethidium displacement assay.[18,19a] With the excitation light at 525 nm, monomer **5** and dimers **4a-e** (n=8-12) do not show any detectable fluorescence emission at 600 nm, in the absence or presence of the four different DNA polymers shown in Table 1. Furthermore, these ligands do not show any appreciable quenching effect when an ethidium fluorescence quenching assay was used.[19c,15a] Thus, the decrease of fluorescence intensity upon addition of ligands to solutions of ethidium bromide and each DNA polymer can be exclusively attributed to the ethidium displacement by ligands. The total ligand concentration required to reduce the ethidium fluorescence to 50% of the initial value, the so-called C_{50},was obtained for each ligand and DNA pair.

All dimeric lexitropsins bind to the alternating AT polymer more strongly than monomer **5**, a characteristic expected from the bidentate side by side antiparallel binding.[15] However, the enhancement is below what is expected from the previous studies on the corresponding polypyrrolecarboxamide dimers. Monomer **5** binds much more strongly than all dimers to the homo AT polymer, which is in contrast to the parallel polypyrrolecarboxamide series. These data seem to suggest that the aggregation of dimers is playing a role in dictating their binding strength even at the concentration near 1 μM. In the case of binding to the alternating AT polymer, dimers still bind to the DNA better than the monomer **5**, because there is a compensating effect from the bidentate binding. However, when the compensating effect is absent, as in the case of binding to the homo AT polymer, dimers become significantly worse binders. Apparently, the 4-N,N-di(2-chloroethyl)aminobenzoyl moiety is significantly more hydrophobic than the 4-formylamino-1-methyl-pyrrole-2-carbonyl moiety, which augments the aggregation tendency of bismustard-containing dimers. In fact, all bismustard-containing dimers are poorly soluble in aqueous solution even when prepared as their dihydrochloride salts, in sharp contrast to the corresponding polypyrrolecarboxamide dimers **3**.[15a,b,19b] While monomer **5** shows a poor affinity to the poly(dG-dC) polymer, all dimers seem much more tolerant of the GC polymer. Overall, the monomer **5** shows a strong preference for the homo AT polymer, binding somewhat less to the alternating AT polymer and poorly to the alternating GC polymer. Its side by side antiparallel binding is less cooperative (the cooperativity factor ~0.32)[19c] than that of monomer **6** (the cooperativity factor ~5.2).[15a] All dimers bind to the alternating AT polymer strongly and significantly less to the homo AT polymer, a result best accommodated by assuming the bidentate binding mechanism to the former DNA. Dimer **4a** (n=8) turns out to be a somewhat better binder than other dimers to each DNA polymer. The averaged binding affinity is further assessed by using calf thymus DNA. All dimers bind better than monomer **5** and the dimer **4a** is the best among them. Apparently, the strong discriminating binding of the monomer makes it bind poorly to a random sequence. In other words, its preference for AT-rich sequences is more than offset by the poor binding to GC-rich sequences.

Table 1.[20]

	poly(dA-dT)		poly(dA)-poly(dT)		poly(dG-dC)		CT DNA		%	Cytotoxicity
	C_{50}	K_b	C_{50}	K_b	C_{50}	K_b	C_{50}	K_b	Alkylation	TD_{50} (μM)
5	2.85	3.41	0.199	6.06	11.0	1.81	5.10	1.07	11	5.0
4a	0.802	121	0.535	1.79	1.64	12.5	1.02	5.60	7.1	>79.2
4b	1.30	72.8	1.31	0.683	1.97	10.4	1.74	3.20	6.7	>79.2
4c	1.78	52.6	1.10	0.820	1.96	10.4	2.02	2.74	7.3	44.2
4d	1.10	86.7	1.20	0.749	2.46	8.25	1.63	3.42	12	35.8
4e	1.06	90.1	1.31	0.683	2.11	9.66	3.17	1.73	--	36.3

We further studied the interaction of these nitrogen mustard lexitropsins with DNA by CD titration.[21] The dimer **4c** was selected as the representative ligand in the series. The induction peak at 330 nm was monitored against the added ligand per nucleotide (γ'). Nearly identical initial slopes (γ' 0–0.05) of the two titration curves suggest that the monomer **5** binds to the homo AT DNA in a manner similar to the tripyrrolecarboxamide monomer **6** (Figure 3). However, **5** achieves saturation of the DNA matrix faster than **6**, consistent with greater binding strength of **5**. It is evident that the final saturation δε of **5** is smaller than **6**, which can be accounted for by the larger binding site size it possesses. The surprising observation is that the dimer **4c** generated a rather small induction in comparison with both monomers, in contrast to dimers **3**.[15a,b] One possible explanation is that dimers aggregate in aqueous solution to such an extent that the effective binding of individual molecules to DNA is significantly weakened, consistent with the binding strength measurements.

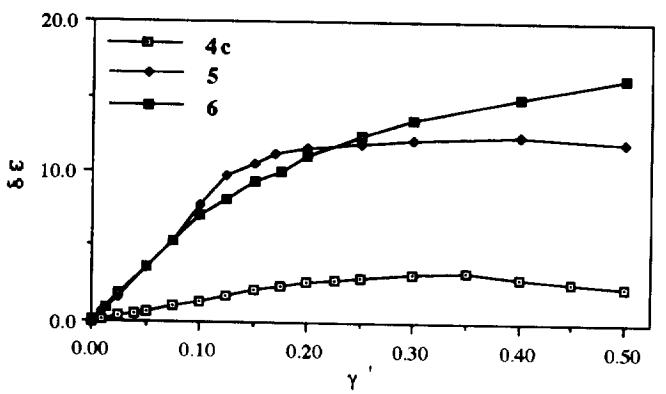


Figure 3. CD titration with poly(dA)-poly(dT): δε is the specific differential extinction coefficient ($mdegM^{-1}cm^{-1}$); γ' is the number of ligand pernucleotide; total [DNA] = 80 μM; buffer: 10 mM NaCl, 10 mM Tris-HCl, 0.25 mM EDTA; temperature, 23 °C.

CD titrations with the alternating AT polymer are shown in Figure 4. Monomers **5** and **6** are similar to each other up to γ' = 0.15. The significant departure after this point suggests that monomer **5** has a less cooperative side by side binding and its approach to the final saturation is slow. The poorer cooperativity was verified by the binding constant measurements discussed above. The relatively poor cooperativity could result from a somewhat different side by side antiparallel binding structure, which might also lead to a quite different extinction coefficient. Perhaps the steric hindrance between the bis(chloroethyl)amino side chain and the dimethylaminopropyl side chain is playing some role here. Again, it is observed that the dimer **4c** has a rather low CD induction. In addition to the ligand aggregation factor mentioned previously, the cross-linker could serve to enforce a certain orientation which is not optimal for the side by side binding of mustard-containing lexitropsins.

The ability of these compounds to alkylate DNA from the minor groove was evaluated using the method developed by Arcamone et al.[4a] Incubation of 160 μM calf thymus DNA with 16 μM of ligands at 37 °C for 24 h was followed by dissolution of 20 mg SDS into the reaction mixture. Comparison of CD at ~330 nm before and after the addition of SDS gave a measure of how much ligand was irreversibly bound to the DNA matrix

from the minor groove.[22] Monomer **5** and **4d** (n=11) appear to be more efficient alkylators. The alkylation ability of dimers **4a-c** is poorer than that of monomer **5**, despite their greater average binding strength. This could be due to the slower alkylation, possibly resulting from the imperfect positioning of the mustard moiety in the side by side motif and the weaker effective binding to the homo AT sequence, and the faster competing deactivation of the dimers by processes such as hydrolysis and hydrochloride elimination of the mustard moiety.

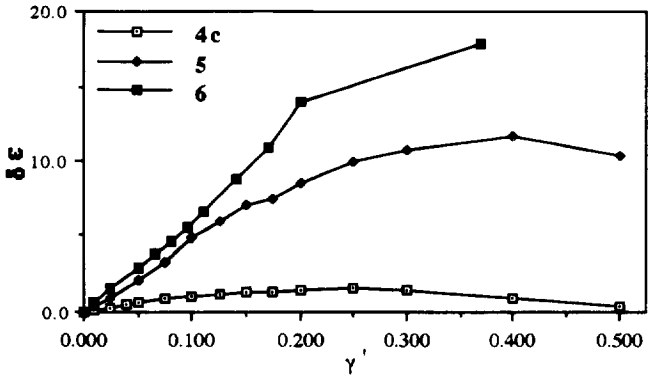


Figure 4 CD titration with poly(dA-dT): $\delta\varepsilon$ is the specific differential extinction coefficient ($mdegM^{-1}cm^{-1}$); γ' is the number of ligand pernucleotide; total [DNA] = 80 μM; buffer: 10 mM NaCl, 10 mM Tris-HCl, 0.25 mM EDTA; temperature, 23 °C.

The cytotoxicities of mustard-containing lexitropsins were measured against KB human cancer cells.[23] As the corresponding lexitropsin controls **6** and **3** are significantly less potent,[24] the cytotoxicity enhancement could be mainly attributed to the alkylation effect. Monomer **5** is significantly more cytotoxic than dimers **4**, which correlates with its strong preference to the homo AT sequence and better alkylation ability from the minor groove. The greater alkylation ability of **4d** may account for its greatest cytotoxic potency among the five dimers. It is noted that other factors such as cellular distribution and metabolic deactivation may also influence the cytotoxicity result but are not assessed in the present study.

In summary, we have designed and synthesized a novel class of bismustard cross-linked lexitropsins. Preliminary binding, alkylation and cytotoxicity analysis reveal some properties of the cross-linked lexitropsins as carriers to deliver DNA reactive functional groups to their targets. We are currently undertaking a more extensive and detailed evaluation of this group of molecules by other biophysical and biochemical means as well as designing a new generation of bismustard cross-linked lexitropsins.

Acknowledgments

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17. All spectroscopic and analytical data of intermediates and final compounds **4a-e** are consistent with the structures shown. For example, **4c** has mp 135-137 °C; IR (KBr): 3297, 2927, 1637, 1515, 1275; ^{1}H-NMR (DMSO-d6): 10.02 (s, 2H, 2 x NH), 9.91 (s, 2H, 2 x NH), 8.08 (t, J = 5.0 Hz, 2H, 2 x $NHCH_2$), 7.84 (d, J = 8.5 Hz, 4H, 4 x PhH), 7.32 (d, J = 1.0 Hz, 2H, 2 x PyH), 7.17 (d, J = 1.0 Hz, 2H, 2 x PyH), 7.00 (d, J = 1.0 Hz, 2H, 2 x PyH), 6.83 (d, J = 8.5 Hz, 4H, 4 x PhH), 6.81 (d, J = 1.0 Hz, 2H, 2 x PyH), 4.28 (t, J = 6.0 Hz, 4H, 2 x $PyNCH_2$), 3.79 (m, 22H, 2 x $N(CH_2CH_2Cl)_2$ + 2 x $PyNCH_3$), 3.17 (m, 4H, 2 x $NHCH_2CH_2$), 2.23 (t, J = 6.0 Hz, 4H, 2 x $CH_2N(CH_3)_2$), 2.12 (s, 12H, 2 x $N(CH_3)_2$), 1.60 (m, 4H, 2 x $NHCH_2CH_2$), 1.20 (m , 16H, $(CH_2)_8$). HRFAB (*m/z*): $C_{64}H_{88}N_{14}O_6{}^{35}Cl_3{}^{37}ClH$ (M + H), calc., 1291.5814; found, 1291.5871.
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19. (a) Because the fluorometry displacement and CD experimental conditions are much milder than those of the alkylation experiment and the greatest alkylation percentage under the latter condition is only 12% (Table 1), the effect of alkylation on the reversible binding under the fluorometry and CD conditions is neglected.[4a,6b,6c] (b) The concentration of the ligand stock solution previously used for CD titration and fluorometry displacement experiments is 1.6 mM. A 10-time dilution was usually necessary to ensure that a homogeneous solution could be obtained for the bismustard-containing dimers. However, this should not make the solubility a problem for the cytotoxicity measurements. (c) The cooperativity factor = $k_{21} / k_{11} \approx (3.41^2 / 6.46) / 6.46 \approx 0.32$.
20. (a) C_{50} is in μM and K_b in $10^6 M^{-1}$. Both ethidium displacement and quenching experiments follow conditions used previously.[15a] (b) Binding constants to homo AT and alternating AT DNA's are calculated using the same way as in the previous studies. The effective binding constant of monomer **5** to poly(dA-dT) is an average of two stepwise binding constants (1:1 and 2:1 modes), i.e., the square root of the product of two binding constants. Binding constants of dimers are those of the 1:1 mode. (c) Binding constants to poly(dG-dC) and CT DNA are calculated as follows: K_b=19.8 / (C_{50}-0.0599) and K_b = 5.38 / (C_{50}-0.0587), assuming the ligand binding site size equal to four. (d) % Alkylation of dimer **4e** cannot be easily obtained because the CD induction is weak. (d) Standard deviations in terms of percentage of mean values: C50, 6.0 [p(dA-dT)], 7.1 [p(dA)-p(dT); 20% for **5**], 3.1 [p(dG-dC)], 6.7 [CT DNA]; Kb, 4.7 [p(dA-dT)], 7.8 [p(dA)-p(dT); 29% for **5**], 3.1 [p(dG-dC)], 6.9 [CT DNA]; % alkylation, 10.
21. Experimental conditions for CD titration are the same as previously employed.[15a]
22. The % alkylation calculation method is based on the assumption that the covalent alkylation does not cause significant degree of CD change, which was confirmed by comparing CD before and after incubation.
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