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Polyhydroxylated Azepanes as New Motifs for DNA Minor Groove Binding Agents

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Abstract—The synthesis of 1,3-bis-[3,4,5,6-tetrahydroxyazepane-*N*-*p*-phenoxy] and 1,3-bis-[3,4,5,6-tetrahydroxyazepane-*N*-*p*-benzyloxy] propanes is reported. These compounds have been prepared to investigate the potential of incorporating iminosugars as useful recognition elements in DNA minor groove binding agents. The compounds were shown to have very moderate binding affinities for DNA in thermal denaturation and ethidium bromide displacement assays when compared with propamidine. They were also found to possess some in vitro anticancer activity that did not correlate with their DNA binding affinity.

The aromatic diamidines 1,5-bis-[4-amidinophenoxy) pentane (pentamidine) **1** and 1,3-bis-[4-amidinophenoxy) propane (propamidine) **2** have been shown to bind in the minor groove of DNA (Fig. 1).[1] Pentamidine is currently being used for the treatment and prophylaxis of *Pneumocystis carinii* pneumonia in patients with AIDS.[2] However, the clinical use of pentamidine and other aromatic diamidines has been restricted by their significant toxicity, leading to adverse effects including hypotension and hypoglycemia, and their limited oral availability.[3] Pentamidine and related drugs have also been shown to have a high affinity for the imidazoline I2 receptor found on rat liver membranes suggesting that the amidine group may be responsible for the toxicity.[4]

A survey of established DNA-binding antibiotics reveals that a significant number have minor groove binding elements composed of oligosaccharides. One well studied example is the calicheamicin γ_1^I aryl tetrasaccharide that has been shown to bind specifically to homopurine-homopyridine tracts such the TCCT sequence.[5] Kahne and coworkers have demonstrated that methyl [(1-4)-(3-*N*-((2-guanidino)acetamido))-2,3-dideoxy-α-L-fuco-pyranoside]$_5$, a rationally designed oligosaccharide, functions as a DNA minor groove binding ligand (MGBL) with a dissociation constant of $\sim 10^{-6}$ M.[6] Inspired by these observations, we have designed and synthesised ligands to evaluate the tetrahydroxyazepane group, an iminosugar, as an alternative cationic ligand terminator to the amidine group in DNA binding ligands.

Polyhydroxyazepanes have several properties that make them potentially useful as MGBL's. The flexibility of the seven-membered ring (compared with five- or six-membered rings) would allow the hydroxyl groups to adopt a variety of positions increasing the probability of them forming hydrogen bonds with the N-3 of the purine, the urea carbonyl of the pyrimidine bases (H-bond acceptors), or the 2-amino of guanine (H-bond donor) which point into the minor groove. The primary advantage of the level of hydroxylation in polyhydroxyazepanes is their high water solubility, allowing them to circumvent the problem of poor bioavailability seen with many other MGBL's. The chirality of the polyhydroxyazepanes can also be controlled, allowing access to a range of diastereomers, and possibly leading to improved sequence selectivity.

In this preliminary study, bis-azepanes that share the dicationic nature and core structure of propamidine

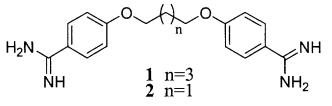

Figure 1.

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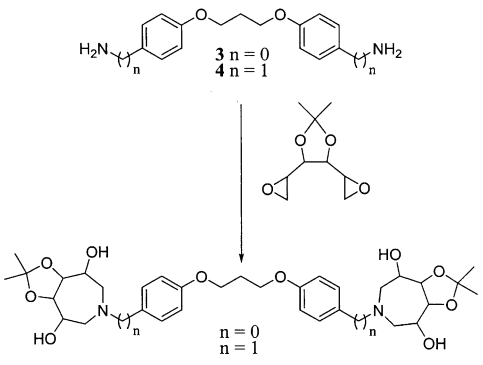
have been prepared, allowing a direct comparison of their DNA binding abilities and biological activity.

Synthesis

Two families of ligands have been prepared based on 1,3-bis-[alkyl-*N*-*p*-phenoxy] and 1,3-bis-[alkyl-*N*-*p*-benzyloxy]propanes. These would be expected to differ significantly in the pK_a of their azepane nitrogens (pK_a 5.5–6.0 for a *N,N*-dialkylphenylamine; 7.3–7.8 for a *N,N*-dialkyl-*p*-methoxybenzylamine; compared to 11.4 for *p*-methoxybenzamidine) and the linear flexibility of the ligands. The synthetic route initially investigated (Scheme 1) was a highly convergent one in which the central 1,3-bis-[*p*-aminophenoxy] or 1,3-bis-[4-aminomethylphenoxy] propane core **3** or **4** was reacted with the appropriate bis-epoxide derived from D-mannitol or L-iditol to produce the required bis-azepane.

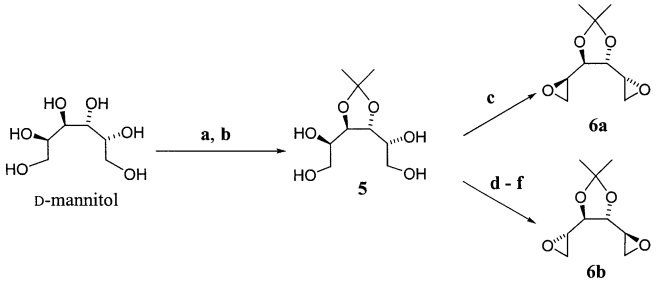
The formation of tetrahydroxyazepanes from bis-epoxides by a nucleophilic opening/aminocyclisation has previously been described by Depezay,[7] Lohray[8] and Wong.[9,10] To investigate the effects of different stereochemistry on DNA binding two different C_2-symmetric bis-epoxides were selected. The common intermediate, 3,4-*O*-isopropylidene-D-mannitol **5**, was prepared by converting D-mannitol to its triacetonide using dimethoxypropane and a catalytic amount of toluene sulfonic acid, followed by removal of the terminal acetonides using acidic aqueous methanol.[10] We found that 1,2:5,6-dianhydro-3,4-*O*-isopropylidene-D-mannitol **6a** required for the formation of the bis-azepanes **10a** and **13a** was most efficiently produced using a route devised by Lohray et al. (Scheme 2).[11] 3,4-*O*-Isopropylidene-D-mannitol **5** was treated with trimethylorthoacetate in the presence of pyridinium *para*-toluene sulphonate. The orthoesters produced were then converted to α-bromoacetates by treatment with acetylbromide and triethylamine in dichloromethane. The acetyl esters were then hydrolyzed with potassium carbonate, causing spontaneous cyclisation to give the bis-epoxide **6a**. Overall this gave 1,2:5,6-dianhydro-3,4-*O*-isopropylidene-D-mannitol **6a** in 72% yield from D-mannitol. The alternative C_2-symmetric bis-epoxide, 1,2:5,6-dianhydro-3,4-*O*-isoproptylidene-L-iditol **6b** was prepared from D-mannitol following the route described by Depezay et al. (Scheme 2).[12] Conversion of the primary hydroxyls of **5** to their benzoyl esters and the secondary alcohols to their tosylates was followed by ester hydrolysis and cyclisation with inversion at C-2 and C-4 to give **6b** in an overall yield of 47% from D-mannitol.



Scheme 1. Proposed route to bis-azepanes.

The bis-epoxides **6a** and **6b** were then reacted with either 1,3-bis-(4-aminophenoxy)propane **3** or 1,3-bis-(4-aminomethyl phenoxy)propane **4**. However, in neither case was the required bis-azepane isolated. Rather than undergoing the second epoxide opening through a 7-*endo*-tet-type aminocyclisation to generate the azepane, the second epoxide appeared to have reacted with another 1,3-bis-(4-aminoaryloxy)propane molecule leading to polymer formation. Changes in the reaction conditions and the ratio of the bis-epoxide to bis-amine did not give any of the required product. An alternative strategy as shown in Scheme 3 was therefore investigated. 3,4,5,6-Tetrahydroxyazepanes **7a** and **7b** were synthesized from 4-acetoxyphenylamine and 1,2:5,6-dianhydro-3,4-*O*-isopropylidene-D-mannitol **6a** or D-iditol **6b**, respectively, by heating in water. The protected azepanes were then treated with 2 M NaOH in methanol to remove the acetyl group. The bis-azepanes were then obtained by treatment of the phenol **8a** or **8b** with potassium carbonate in dry ethanol followed by addition of 1,3-dibromopropane. The resulting mixture was heated under reflux for 4 days and the required di-isopropylidene bis-azepane **9a** or **9b** was obtained by column chromatography in 40 or 27% yield, respectively. The isopropylidene groups were removed by treatment with 0.5 M hydrochloric acid giving 1,3-bis-[(3*R*,4*R*,5*R*,6*R*)-3,4,5,6 - tetrahydroxy - azepane - *N* - *p* - phenoxy]propane dihydrochloride **10a,** and the (3*S*,4*R*,5*R*,6*S*)-diastereoisomer **10b** as beige solids.

The benzyl bis-azepane homologues, 1,3-bis-[(3*R*,4*R*,5*R*,6*R*) - 3,4,5,6 - tetrahydroxyazepane - *N* - *p* - benzyloxy]-



Scheme 2. (a) 2,2-DMP, pTsOH, 95%; (b) 1:10 MeOH/H_2O, pTsOH, 75%; (c) (i) $MeC(OMe)_3$, PPTS, DCM; (ii) Et_3N, AcBr, DCM; (iii) K_2CO_3, MeOH, 95%. (d) BzCl, pyr, DCM, 72%; (e) TsCl, pyr, 100%; (f) K_2CO_3, MeOH, DCM, 85%.

propane dihydrochloride **13a** and the (3*S*,4*R*,5*R*,6*S*)-diastereoisomer **13b** were produced by a similar route except that 4-allyloxybenzylamine was used in place of 4-acetoxyphenylamine because the reactions with the bis-epoxides were cleaner and the allyl groups could be removed easily prior to coupling with 1,3-dibromopropane as shown in Scheme 4. All intermediates and final products were fully characterized by NMR (^{1}H, ^{13}C, DEPT), HRMS and elemental analysis.

Biological Activity

3,4,5,6-Tetrahydroxyazepanes have been shown to be good competitive inhibitors of glycosidase enzymes from plant origin,[13] their 2,5-dialkyl derivatives to have some activity as competitive inhibitors of HIV and FIV proteases,[14] malto-oligosaccharides containing tetrahydroxyazepanes have been shown to inhibit human α-amylase.[15] Lohray et al. have shown that (3*S*,4*R*,5*R*,6*S*)-tetrahydroxyazepane and *N*-benzyl (3*R*,4*R*,5*S*,6*R*)-tetrahydroxyazepane have modest activities against several cancer cell lines (GI_{90} 20−90 μM) with the highest activity being observed against the SNB-75 CNS cancer cell line.[16] The site of action in this case has not been not determined.

We have tested propamidine **2** together with the bis-azepanes and their acetonide precursors against a number of cell lines in in vitro biological tests. Literature GI_{50} data are also given on doxorubicin (intercalator which induces topoisomerase II mediated cleavage) and chlorambucil (a DNA alkylator) as benchmark agents.[17] Data for the compounds found to be active against HCT116 and HT29 human colon cancer, and MCF7 human breast cancer cell lines are given in Table 1. MCF7/ADR is a mutant form of the breast cancer cell line that is resistant to doxorubicin.

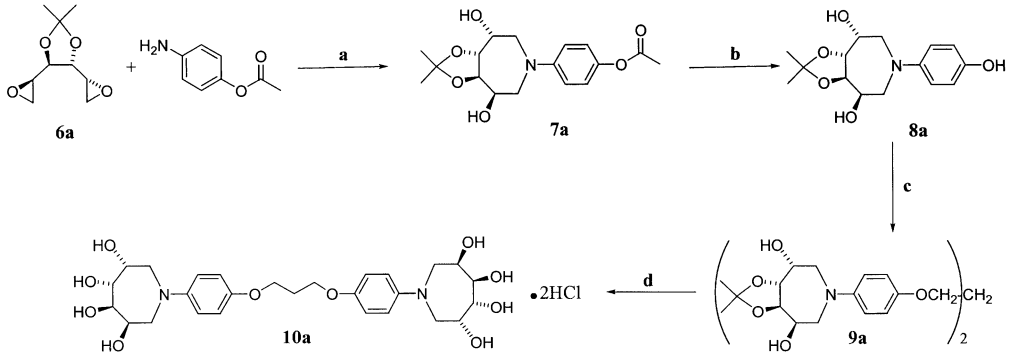
The results shown in Table 1 demonstrate that three of the bis-azepane derivatives are more effective than chlorambucil, of similar activity to propamidine and much less effective than doxorubicin in inhibiting the growth of the colon cancer cell lines. One of the acetonide protected bis-azepanes **12a** is a better growth inhibitor than any of the drugs in the doxorubicin resistant breast cancer cell line, although all compounds tested only had modest activity in this case. As the bis-azepane with the best DNA binding affinity **13a** has the poorest inhibitory activity of those tested, it is unlikely that this biological activity is due to DNA binding, unless the acetonide groups on **12a** aid transport across the cell membrane and are then hydrolyzed within the cell to give compound **13a.** In comparison, compound **13a** may have very poor cellular uptake due to its increased polarity.

Binding Studies

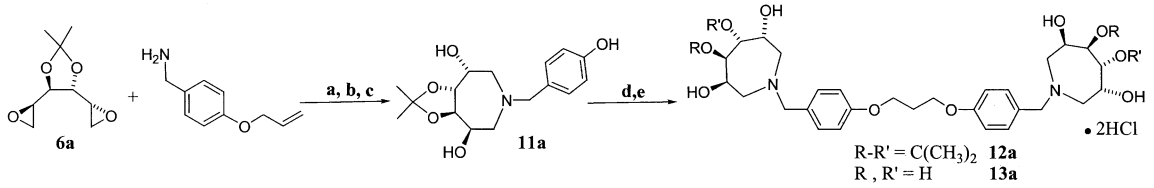
To determine the effectiveness of bis-azepanes as DNA binding agents, both thermal denaturation[18] and ethidium bromide displacement studies[19] have been conducted.

Thermal denaturation

A comparison of thermal denaturation profiles of DNA in the presence and absence of ligand provides the simplest means of detecting binding, and also ascertaining relative binding strength. Melting curves were measured at 260 nm following a modification of the method described by Cory for the investigation of pentamidine analogues.[18] Whilst amidine groups would be protonated



Scheme 3. (a) H_2O, 95 °C, 40%; (b) 2 M NaOH, MeOH, 75%; (c) $Br(CH_2)_3Br$, K_2CO_3, EtOH, reflux, 4 days, 40%; (d) 0.5 M HCl, 95%.



Scheme 4. (a) H_2O, 60 °C, 69%; (b) *t*-BuOK, DMSO 100 °C, 81%; (c) HgO, $HgCl_2$ in 10:1 acetone/water, 87%; (d) K_2CO_3, $Br(CH_2)_3Br$, EtOH, reflux for 4 days, 31%; (e) 0.5 M HCl, 100%.

Table 1. Activities against colon and breast cancer cell lines[a]

Compd	HCT116 GI_{50} (μM)[b]	HT29 GI_{50} (μM)[b]	MCF7 GI_{50} (μM)[b]	MCF7/ADR GI_{50} (μM)[b]
Propamidine **2**	11 (±2.8)	>50	9 (±3.6)	33 (±2.1)
Doxorubicin[c]	0.023	0.056	0.013	16.7
Chlorambucil[c]	105	129	30	56
9a	32 (±5.0)	40 (±1.0)	>50	>50
12a	6 (±0.6)	23 (±2.5)	24 (±9.0)	10 (±1.5)
13a	>50	24 (±0.6)	>50	>50

[a]Values are means of three experiments, standard deviation is given in parentheses (50 μM was maximum concn tested).
[b]In vitro GI_{50} (concn required to inhibit growth by 50%).
[c]Values from ref 17.

and, therefore, positively charged at pH 7.50, this would not be true of the phenylamine derivatives **10a/b** and possibly not of the benzylamine derivatives **13a/b** used in our study. We therefore conducted the thermal denaturation studies at both pH 7.5 and pH 5.0. Calf thymus DNA (50 μM) (Sigma D-1501) was taken up in a buffer solution composed of 5 mM TRIS (pH 7.5) or 10 mM sodium citrate/sodium phosphate (pH 5.0), 50 μM EDTA, 10 mM sodium chloride and 0.25% DMSO. This gave melting temperatures of the DNA of 69.5 °C at pH 7.5 and 67.2 °C at pH 5.0. Table 2 summarizes the changes observed on adding either propamidine or one of the ligands under investigation (5 μM). It can be seen that propamidine stabilises the DNA duplex to give an increase in the melting temperature of +8.2 °C at pH 7.5, falling to +6.3 °C at pH 5.0, whilst none of the bis-azepanes gave any apparent stabilisation at pH 7.5 within experimental error, and only one, **13a** gave a slight increase at pH 5.0. Even at this lower pH, it is possible that the majority of nitrogens of the phenylamine ligands are still unprotonated. The studies were repeated leaving the ligands to equilibrate for 12 h with the DNA prior to determining the denaturation temperature. In this case, several of the ligand–DNA mixture melting temperatures were elevated indicating that some stabilisation of the DNA duplex structure was occurring, but again, not to the extent of that seen with propamidine. The polyhydroxylated bis-azepanes are significantly broader than propamidine, and they may exhibit slower DNA binding kinetics (milliseconds rather than microseconds). This would not fully account for the increases in melting temperature observed. Most minor groove binding ligands including propamidine and pentamidine have a preference for binding AT rich DNA sequences.[1] It was therefore decided to repeat the thermal denaturation studies using both poly(dA).poly(dT) and poly(dG–dC)$_2$ at pH 5.0. Whilst poly(dG–dC)$_2$ did not melt below 95 °C in our buffer system, poly(dA).poly(dT) had a melting temperature of 53.3 °C. The increase in melting temperature on addition of ligand were found to be +25.7 °C for propamidine; +0.8 °C for **13a**; and +0.5 °C for **13b**; other compounds did not affect the melting temperature. In this case, preincubation of the ligand with the DNA for 12 h did not effect the DNA melting temperature.

Overall, the thermal denaturation studies indicated that one of the five bis-azepane ligands, **13a** had a stabilizing affect on the DNA, and this was very weak compared with that of propamidine **2**.

Table 2. Change in melting temperature of calf thymus DNA

Ligand	pH 5.0[a,b,c] (°C)	pH 5.0[a,b,d] (after 12 h) (°C)	pH 7.5[a,b,c] (°C)
Propamidine **2**	+6.3	+6.8	+ 8.2C
9a	+0.1	+0.4	- 0.1
12a	+0.1	+0.5	+ 0.1
10a	0.0	+0.7	+ 0.1
13a	+0.3	+1.2	+ 0.1
13b	+0.2	—	—

[a]Values are means of three experiments.
[b]50 μM DNA, 5 μM ligand, 10 mM NaCl, 50 μM EDTA, 0.25% DMSO.
[c]5 mM Tris.
[d]10 mM sodium phosphate–sodium citrate.

Ethidium bromide displacement

Using a procedure developed by Cain et al.[19] it is possible to measure the relative binding affinity of a ligand for DNA by determining the C_{50} value of the ligand. This is the concentration of the ligand required to displace 50% of the ethidium from a preformed, intercalated complex. It is also possible to calculate the apparent binding constant for a ligand given that the binding constant for ethidium [$K_e = 9.5\times10^{-6}$ M(bp)$^{-1}$] is known under the conditions used. Table 3 summarizes the results for those ligands that did affect the fluorescence due to the intercalated ethidium at pH 7.0. One of the acetonide-protected bis-*N*-phenylazepanes **9a** had a low affinity for the DNA, whilst the two unprotected *N*-benzylazepanes **13a** and **13b** displayed apparent binding affinities of the same order of magnitude as that of propamidine. None of the other compounds tested, **9b**, **10a/b** or **12a/b,** caused a change in fluorescence.

Table 3. Ethidium bromide displacement assays

Ligand	C_{50} (μM)[a,c]	K_{app} (M^{-1})[b]
Propamidine **2**	25.6 (±1.5)	4.7×10^5
9a	703 (±161)	1.7×10^4
13a	61.6 (±5.3)	1.9×10^5
13b	97.0 (±7.9)	1.2×10^5
Distamycin[d]	1.8	6.7×10^6

[a]Values are means of three experiments, standard deviation is given in parentheses.
[b]$K_{app} = (1.26\times K_e)/C_{50}$: ethidium, $K_e = 9.5\times10^{-6}$ M(bp)$^{-1}$.
[c]1 μM DNA, 1.26 μM ethidium, 2 mM HEPES, 9.4 mM NaCl, 10 μM EDTA.
[d]From ref 20.

Conclusions

This preliminary study has demonstrated that bis-tetrahydroxyazepanes do have modest growth inhibitory activity in cancer cell lines and are capable of binding to DNA at low pH, although not with the affinity of ligands such as propamidine. Future research will involve conducting DNA footprinting studies on compound **13a**, and the preparation of bis-azepanes with a reduced number of hydroxyl groups, or with the hydroxyls modified as their methyl ethers, which may exhibit better minor groove binding abilities.

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