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A substituted triaza crown ether as a binding site in DNA conjugates

Stefan Vogel,[a] Katja Rohr,[a] Otto Dahl[b] and Jesper Wengel*[a]

[a] *Nucleic Acid Center†, Department of Chemistry, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark. E-mail: jwe@chem.sdu.dk*

[b] *Department of Chemistry, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen, Denmark*

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Synthesis of an asymmetrically substituted triaza crown ether, its incorporation into the 3′-end and 5′-end of nine-mer oligonucleotides, and the influence of various alkane-diamine ligands on duplex thermostabilities are reported.

Functionalized crown ethers are of special interest in terms of host–guest chemistry, and their selectivity for complexation of metal cations of different sizes and for organic molecules such as amines and amino acids is a starting point for promising applications.[1] So far, only one example of incorporation of a crown ether moiety, a monoaza crown ether, into an oligonucleotide (ON) has been reported but no complexation studies were performed.[2]

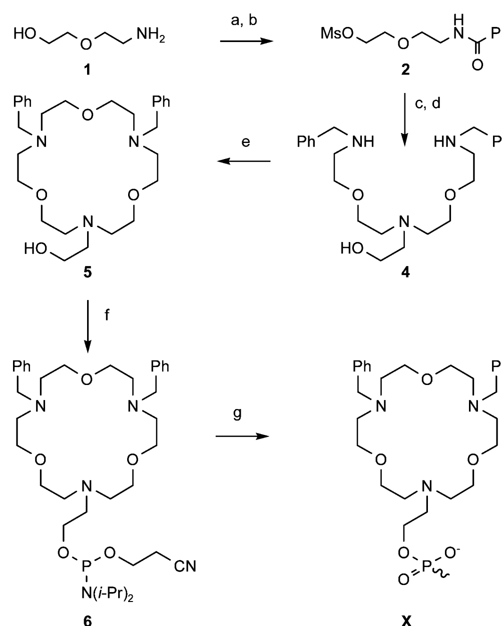
In searching for a model receptor acting as amine or amino acid binding site in ONs we have focused on crown ethers. Although 18-crown-6 and similar crown ethers are capable of binding primary ammonium cations,[3,4] binding to alkali metal cations is appreciably stronger.[5] A remarkable exception are triaza crown ethers which show about ten times stronger complexation of primary ammonium cations than sodium or potassium cations.[6] A protonated amine ligand is complexed by a network of alternating O···H–N and N···H–N hydrogen bonds with the arrangement of the three nitrogens in a triaza crown ether matching the symmetry axis of a primary ammonium cation enabling the formation of three strong N···H–N hydrogen bonds. We therefore decided to focus on conjugating a triaza crown ether to ONs, and the phosphoramidite **6** designed for automated end-conjugation of ONs was synthesized (Scheme 1).

Commercially available amine **1** was first monobenzoylated and then mesylated to give building block **2** which after treatment with ethanolamine afforded a diamide. Subsequent reduction led to triamine **4** as a suitable intermediate for a macrocyclization reaction.[7] Thus, treatment of triamine **4** with diethyleneglycol ditosylate furnished the macrocycle **5** which was eventually converted into the phosphoramidite **6**‡ (Scheme 1).

The triaza crown ether unit **X** (Scheme 1 and Fig. 1) was incorporated at the 5′-end and the 3′-end of 9-mer ONs (**ON7** and **ON8**, see Fig. 1). In order to avoid low temperature denaturations in these first studies, **ON7** was synthesized with three affinity-enhancing LNA§ monomers (Fig. 1).[8–10] Synthesis of **ON7–ON9** was performed in 0.2 μmol scale on an automated DNA synthesizer using the phosphoramidite approach.[11] Standard procedures were used with modifications as described previously[12] for amidite **6** (including 10 min coupling time using 1*H*-tetrazole as activator). Initially, coupling of amidite **6** was not successful. Careful NMR monitoring of the coupling reaction revealed water bound to the hygroscopic crown as the major problem. Removal of residual water was finally achieved by stirring amidite **6** in acetonitrile with 4 Å molecular sieves for 24 h. The anhydrous solution obtained was immediately used for coupling on the DNA synthesizer. **ON8** was synthesized using commercial so-called "inverted amidites" (3′-*O*-DMT amidites, > 98% coupling yields using 2 min coupling time), amidite **6** (92% coupling yield) and a universal support. **ON7** and **ON9** were synthesized using standard DNA amidites and LNA amidites (> 98% coupling yields using 2 and 10 min coupling time, respectively), amidite **6** (for **ON7**, 98% coupling yield) and standard supports. The coupling yields for amidite **6** were estimated by trityl monitoring after having performed an additional coupling reaction with a commercial DNA phosphoramidite without a preceding capping step on a sample of the support after coupling with amidite **6**. Satisfactory purities (> 80%) of **ON7–ON9** were verified by capillary gel electrophoresis. Peaks, although only minor ones, corresponding to the calculated masses of **ON7** and **ON8** were obtained by MALDI-MS analysis supporting the composition of these molecules.¶ Association of the crown ether moieties of **ON7** and **ON8** with the matrix is one possible explanation for the problems experienced during MALDI-MS analysis. However, the composition of **ON8** was furthermore verified by ESI-MS analysis ($[M - 3H]^{3-}$ *m/z* 1099; calcd. 1099; major peak).

To study complexation of alkanediamine ligands with **ON7** and **ON8** and the importance of ligand chain length, a thermal denaturation study was performed and the melting temperatures (T_m values) were determined (Table 1). **ON7** was hybridized with its complementary strands **ON8** or **ON9** to give duplexes bearing either two triaza crown ethers (**ON7**:**ON8**; Series A) or a single triaza crown ether (**ON7**:**ON9**; Series B). In order to distinguish effects of ligand complexation by the crown ether unit **X** from nonspecific interactions of the ligand with the duplex, we also studied duplex **ON10**:**ON11**‖ (Series C).

With no ligand present, duplex **ON7**:**ON8** (Series A) displayed a slightly higher thermal stability (T_m = 42.5 °C) than



Scheme 1 *Reagents and conditions* a) $(PhCO)_2O$, EtOH, reflux, 90%, b) MsCl, pyridine, 80%, c) $HO(CH_2)_2NH_2$, NEt_3, MeCN, 70%, d) $LiAlH_4$, THF, 90%, e) $TsO(CH_2)_2O(CH_2)_2OTs$, Na_2CO_3, MeCN, 50%, f) $NC(CH_2)_2OP(Cl)N(i\text{-}Pr)_2$, $(i\text{-}Pr)_2NEt$, CH_2Cl_2, 50%, g) DNA synthesizer.

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duplex **ON7**:**ON9** (Series B) bearing only one triaza crown ether unit **X** (T_m = 41.0 °C). Different trends with respect to the dependence of the thermal stabilities on ligand chain length and ligand concentration for Series A, B and C were observed as depicted in Table 1 (ΔT_m values are calculated relative to the T_m value recorded for each series in the absence of a ligand).

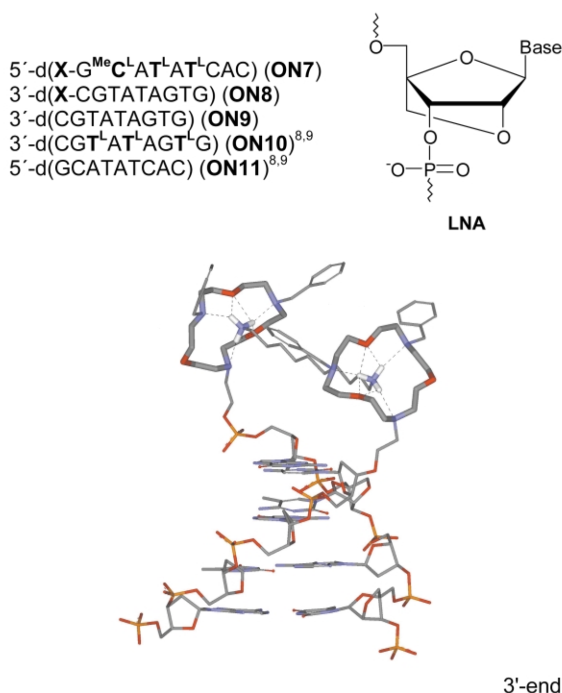

Fig. 1 Sequences synthesized, the structure of LNA nucleotide monomers (**T^L** and **^MeC^L** are LNA thymin-1-yl and LNA 5-methylcytosin-1-yl monomers, respectively) and a representation of the putative mode of complexation of a alkanediamine ligand by the two triaza crown ether units in the oligonucleotide duplex **ON7**:**ON8**; only four base pairs are shown in the structural model in which the dotted lines indicate probable hydrogen bonds between the triaza crown ether units and ligand **14**. Alkanediamine ligands used: $H_2N(CH_2)_nNH_2$; n = 6 (**12**), n = 8 (**13**), n = 10 (**14**) and n = 12 (**15**).

Table 1 Thermal denaturation experiments in the presence of alkanediamine ligands **12–15**[a]

Duplex series	Eq.	T_m/°C	ΔT_m/°C
Series A (**ON7**:**ON8**)		42.5	
Ligand **12**	1/10	42.0/42.0	−0.5/−0.5
Ligand **13**	1/10	45.0/44.0	+2.5/+1.5
Ligand **14**	1/10	46.0/45.0	+3.5/+2.5
Ligand **15**	1/10	44.5/45.0	+2.0/+2.5
Series B (**ON7**:**ON9**)		41.0	
Ligand **12**	1/10	40.0/44.5	−1.0/+3.5
Ligand **13**	1/10	43.5/43.5	+2.5/+2.5
Ligand **14**	1/10	43.5/42.0	+2.5/+1.0
Ligand **15**	1/10	44.5/43.5	+3.5/+2.5
Series C (**ON10**:**ON11**)		43.5	
Ligand **12**	1/10	42.5/42.5	−1.0/−1.0
Ligand **13**	1/10	42.0/42.5	−1.5/−1.0
Ligand **14**	1/10	42.5/42.0	−1.0/−1.5
Ligand **15**	1/10	41.5/41.5	−2.0/−2.0

[a] Melting temperatures [T_m values (ΔT_m values are calculated relative to the T_m value recorded in the absence of a ligand)] measured as the maximum of the first derivative of the melting curve (A_{260} *vs.* temperature; 10 °C to 80 °C with an increase of 1 °C min^{-1}) recorded in medium salt buffer (10 mM sodium phosphate, 100 mM sodium chloride, 0.02 mM EDTA, pH 7.0) using 1 µM concentrations of the two complementary strands. Hyperchromicity values and transition intervals were similar for all melting curves. Each T_m value was determined in two independent experiments and ΔT_m values were within ±0.5 °C consistent for the two experiments. "Eq." denotes the molar equivalent(s) of ligand used relative to duplex. See caption of Fig. 1 for structures of alkanediamine ligands **12–15**.

For duplex Series A involving duplexes with two juxtapositioned crown ether units **X**, optimal complexation of an alkanediamine ligand should be for one equivalent of ligand of the appropriate length allowing complexation-driven reversible end-joining of the two strands of the duplex with concomitant increased thermal stabilities. For duplex Series B, complexation of the alkanediamine ligands was expected not to strongly influence the thermal stability and no difference between the different ligands and ligand concentrations was expected. In Series A, all ligands induced an increase in T_m by 1.5–3.5 °C except for the shortest ligand **12**, and, in general, similar results were obtained for Series B. Overall, no strong effect of the equivalents of ligand present on the obtained ΔT_m values was observed

The results obtained in Series C are in strong contrast to those obtained in Series A and B and therefore support specific complexation of the alkanediamine ligands with the crown ether unit(s) in Series A and B. Interstrand cross complexation involving either the two juxtapositioned crown ether units in Series A (*e.g.*, with 1 eq. of ligands **13** and **14**) or one crown ether unit and the phosphate backbone (in Series A and B) would explain the affinity enhancing effects observed. The comparable results obtained with one and ten equivalents of ligands render any conclusion regarding the actual mode of complexation impossible at this stage.

Oligonucleotide crown ether conjugates have been introduced herein as a novel class of potentially very useful molecular building blocks for construction of self-assembling molecular receptors. We are currently studying conformationally restricted variants of the systems introduced herein and synthesizing ^{15}N-labelled crowns and ligands for NMR structural studies.

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Notes and references

‡ Amidite **6**: ^{31}P NMR data: δ (CH_3CN) 148.5 ppm; MALDI-MS ([M + Na]$^+$ *m/z* 708.4224; calcd. 708.4229.

§ We have defined LNA as an oligonucleotide containing one or more conformationally locked 2′-*O*,4′-*C*-methylene-β-D-ribofuranosyl nucleotide monomer(s) ("LNA monomer(s)").

¶ MALDI-MS *m/z* for **ON7**: [M − H]$^-$ 3327; calcd. 3325; [M − 2H + Na]$^-$ 3349; calcd. 3347; MALDI-MS *m/z* for **ON8**: [M − H]$^-$ 3299; calcd. 3298; [M − 2H + Na]$^-$ 3322; calcd. 3320.

‖ This duplex was available and chosen as reference because of close resemblance to the **ON7**:**ON8** and **ON7**:**ON9** duplexes with respect to sequence, composition and structure (see ref. 13 and ref. 14 for relevant NMR structural work).

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