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Functionalisation of a diene-modified hairpin mimic *via* the Diels–Alder reaction†

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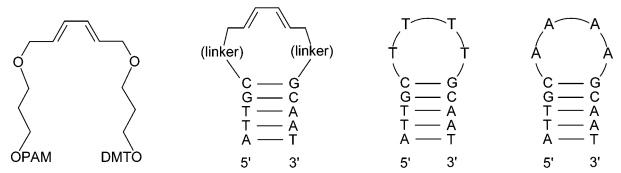
A highly stable 1,3-butadiene-derived DNA hairpin mimic and its derivatisation *via* the Diels–Alder reaction with various dienophiles are described.

The hairpin belongs to the most common secondary structural motifs found in nucleic acids.[1] In RNA, it is an essential element for the assembly of higher order structures. By enabling the proper folding, the hairpin contributes to the many functional properties of RNA. Extra stable hairpins containing four bases in the loop (tetraloops) have emerged as a distinct class of hairpins, which forms highly specific interactions with tetraloop receptor sites.[1–3] The hairpin motif is also found in DNA,[4,5] though to a much lesser extent due to the intrinsically double stranded nature of DNA. This central role of the hairpin as a structural and functional element has stimulated the design and synthesis of chemically modified hairpin analogs. Hairpin mimics can serve as tools for the investigation of structure and function of nucleic acids. In addition, they are increasingly gaining importance as building blocks for the construction of defined, nucleic acids based molecular structures.[6] Thus, the hairpin loop has been replaced with flexible oligo ethylene glycol linkers[7–9] as well as with more rigid aromatic derivatives[10–14] and metal complexes.[15,16] Furthermore, the construction of a stilbene-based hairpin mimic forming G-tetrades has been reported very recently.[17]

The Diels–Alder reaction has been used for the derivatisation[18,19] and immobilisation[20] of nucleic acids. Thus, it was shown that oligonucleotides bearing a 5′-linked diene moiety react site-specifically with dienophiles without interference of the many functional groups present in RNA[18] and DNA.[19,20] Despite its established chemoselectivity, the Diels–Alder reaction has hitherto not found widespread use for the purpose of DNA modification. Here, we show that replacement of the nucleotide loop with a 1,3-butadiene building block leads to a very stable DNA hairpin mimic, which can be further derivatised *via* Diels–Alder reaction with maleimide dienophiles carrying different pendant groups.



1 **2** **T4** **A4**

Using phosphoramidite building block **1**‡ (PAM: 2-cyanoethyl-diisopropylaminophosphinoyl; DMT: 4,4′-dimethoxytrityl), hairpin mimic **2** (linker: propane-1,3-dioxyl) was prepared *via* standard automated oligonucleotide synthesis. Oligomer **2** was purified by reverse phase HPLC and characterized by electrospray ionisation time-of-flight (ESI-TOF) mass spectrometry.§ For comparison, two analogous hairpins containing four thymidine or deoxyadenosine nucleotides in the hairpin loop, **T4** and **A4**, were synthesised.

Thermal denaturation experiments revealed that **2** forms a very stable secondary structure (Table 1). An increase in the melting temperature (Tm) of 8.9 or 9.9 °C was observed for **2** compared to the analogous hairpins containing a **T4** or **A4** loop, respectively. Tm curves (see ESI†) showed a single, cooperative transition. Furthermore, Tm values were independent of the oligomer concentration (see ESI†), which indicates a unimolecular process, *i.e.* formation and melting of the hairpin. Circular dichroism (CD) spectroscopy of **2** was consistent with a B-form DNA (see ESI†). On the basis of this information, a model of the hairpin mimic **2** was developed. According to *amber* force field calculations (*HyperChem*™),[21] both possible diene conformations (*i.e.* s-*cis* and s-*trans*) form a stable hairpin-like secondary structure. The phosphate–phosphate distance between the two strands (17.7 Å and 17.5 Å for the s-*cis* and s-*trans* conformer, respectively) corresponds well with the 17.5 Å observed in an ideal B-DNA. Since the s-*cis* isomer represents the relevant conformation for the subsequent Diels–Alder reaction, the hairpin mimic is presented in this orientation in Fig. 1. The model shows the non-nucleotidic linker on top of the helix, bridging the two nucleic acid strands. The plane of the diene is oriented parallel to the adjacent base pair, providing the proper arrangement for reaction with dienophiles.

Since the nucleotide loop of the hairpin is crucial for intra- and intermolecular interactions, introduction of different types of substituents at this site is desirable. Therefore, we next investigated the reactivity of the diene-modified hairpin mimic **2** in Diels–Alder reactions. For this purpose, the maleimide-derived dienophiles **3a–f** (Scheme 1) were allowed to react with **2** in aqueous medium (10 mM NaOAc buffer, pH 6.5, 20 °C) for 7 days.¶ After this time,

Table 1 Tm values of different hairpins[a]

hairpin	**T4**	**A4**	**2**	**4a**	**4b**	**4c**	**4d**	**4e**	**4f**
Tm [°C][b]	57.8	56.8	66.7	64.2	66.5	57.5	66.3	59.7[c]	67.1
ΔTm [°C][d]	—	−1.0	8.9	6.4	8.7	−0.3	8.5	1.9	9.3

[a] Conditions: oligomer concentration 2.5 μM, 10 mM Tris-HCl, 100 mM NaCl, pH 7.5; temp. gradient: 0.5 °C/min. [b] Melting temperatures were determined from the maximum of the first derivative of the melting curve (A_{260} against temperature); each Tm is the average of three independent experiments; exptl. error: ±0.5 °C. [c] In the presence of EDTA (10 mM). [d] Difference in Tm relative to **T4**.

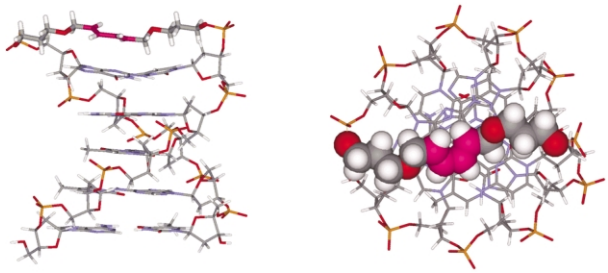


Fig. 1 Molecular model of 1,3-butadiene-derived DNA hairpin mimic **2** (*HyperChem*™ 7.0, *amber* force field); view perpendicular (left) and along the helical axis. The synthetic linker is displayed in a space filling representation (right) and the butadiene moiety (highlighted in red) is shown in the s-*cis* conformation.

† Electronic supplementary information (ESI) available: synthesis of phosphoramidite **1**; reverse-phase HPLC trace of conjugate **4f**; thermal denaturation experiments and circular dichroism (CD) spectra of hairpin analogs **2** and . See http://www.rsc.org/suppdata/cc/b4/b405223d/

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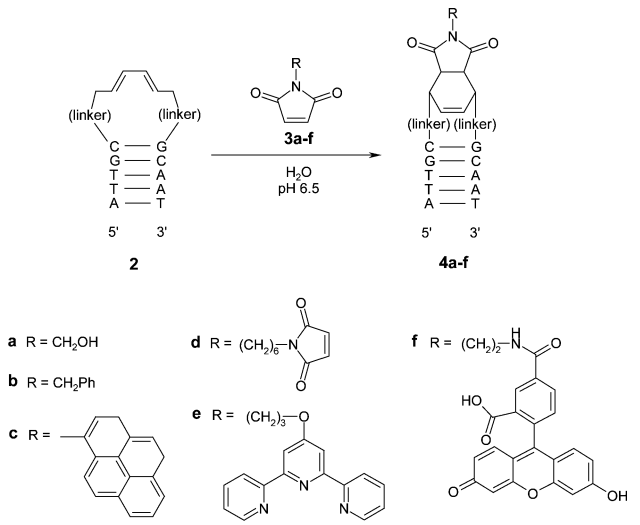
analytical HPLC indicated completeness for all reactions. After removal of excess maleimide reagent by extraction with CH_2Cl_2, the reaction mixtures were concentrated and the conjugates were purified by reverse phase HPLC. The identities of products **4a–f** were confirmed by mass spectrometry.§

The obtained pure conjugates were further analysed regarding their structural properties. As with the parent hairpin mimic **2**, cooperative melting processes were observed for **4a–f** (see ESI†). All conjugates formed stable secondary structures with Tm values between 58 and 67 °C (see Table 1). Even bulky substituents (*e.g.* **4e** or **f**) were very well tolerated. With the exception of **4c**,‖ all conjugates showed Tm values which are 2 to 9 °C higher than the ones of the natural hairpins **T4** and **A4**. Again, as with **2**, Tm values were independent of the oligomer concentration, confirming a hairpin structure. Furthermore, CD spectra of all conjugates showed the typical pattern for B-form DNA (see ESI†).

These results demonstrate that the diene-modified hairpin mimic **2** is amenable to derivatisation though Diels–Alder reaction with suitable dienophiles. A variety of different groups can be attached to the hairpin loop, including aromatic residues, fluorescent dyes, metal coordinating ligands, as well as chemically reactive groups (*e.g.* **4d**), which can serve as a site for attachment of further substituents. In addition, the use of bifunctional maleimides, such as **3d**, should allow the construction of larger structures by linking different hairpin mimics.[19] We are currently studying the assembly of higher order structures using this approach.

In conclusion, a variety of hairpin analogs has been synthesised *via* Diels–Alder reaction of different maleimides with a diene-modified DNA hairpin mimic. The method allows the introduction of a variety of different functionalities into the loop region of DNA hairpins. Attachment of a range of different groups to the hairpin linker does not impede the formation of stable, hairpin-like structures as verified by thermal denaturation experiments and CD spectroscopy.



Scheme 1 Diels–Alder reaction of 1,3-butadiene-based hairpin mimic **2** with substituted maleimides.

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Notes and references

‡ Amidite **1**: ^{31}P NMR data: δ ($CDCl_3$) 147.7 ppm; ESI-TOF MS $[M+H]^+$ *m*/*z* 733.3971; calcd. 733.3981.

§ ESI-MS: *m*/*z* exp./calcd. for $[M-H]^-$ for **2**: 3319/3319; **4a**: 3346/3346; **4b**: 3507/3506; **4c**: 3615/3616; **4d**: 3595/3595; **4e**: 3817/38178; **4f**: 3705/3705.

¶ All Diels–Alder reactions were carried out at 20 °C to ensure the hairpin structure of **2**. Reactions were carried out in the presence of an excess (10 equiv.) of the dienophiles (**3a–f**).

‖ The somewhat lower stability of conjugate **4c** may be a result of the absence of a linker between the pyrene and the imide, thus leading to unfavourable steric interactions between the pyrene and the neighbouring nucleotides.

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