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Oligonucleotides with 1,5-anhydrohexitol nucleoside building blocks: crystallization and preliminary X-ray studies of h(GTGTACAC)

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Hexitol nucleic acids are oligonucleotides built up from natural nucleobases and a phosphorylated 1,5-anhydrohexitol backbone. The anhydrohexitol oligonucleotide h(GTGTACAC) was synthesized using phosphoramidite chemistry and standard protecting groups. Crystals of h(GTGTACAC) were obtained at either 279 or 289 K by the hanging-drop vapour-diffusion technique using a 24-matrix screen for nucleic acid fragments. The crystals diffract beyond 2.0 Å resolution and belong to the hexagonal space group $P6_222$ (or $P6_422$) with unit-cell parameters a = 36.42 and c = 63.33 Å.

1. Introduction

Antisense oligonucleotides can be considered as a new class of potential therapeutic agents. However, modifications of the classical DNA backbone are necessary in order to overcome the problems of low stability and cellular uptake. One such recent modification (Van Aerschot *et al.*, 1995) is the use of 1,5-anhydrohexitol building blocks (Fig. 1) instead of the normal β-D-2′-deoxyribose. The insertion of an extra methylene group between C1′ and O4′ has an enormous influence on the stability of complexes between these modified hexitol nucleic acids (HNA) and DNA or RNA. HNA duplexes are even more stable: the melting temperature of $(hT)_{13}.(hA)_{13}$ (349 K) is considerably higher than that of its natural analogue (307 K). This hexitol modification is one of the strongest hybridizing antisense compounds presently known.

The crystal structures of the hexitol guanine (hG), cytosine (hC), thymine (hT), inosine (hI) and iodouracil [h(IdU)] building blocks have shown the chair conformation for the hexitol ring, with the base oriented in the axial position and *anti* conformation (Verheggen *et al.*, 1995; Declercq *et al.*, 1996). As a consequence, the incorporation of 1,5-anhydrohexitol sugars will greatly influence the three-dimensional structure of the HNA duplex. From circular dichroism measurements one can conclude that the HNA double helix formed is of a new type (Hendrix *et al.*, 1997). A complete structure determination will elucidate the geometry and hydration of these new HNA duplexes and may explain their extra stability.

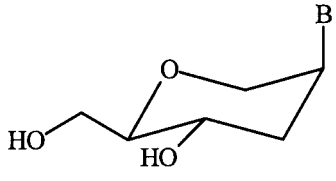

Figure 1
1,5-Anhydrohexitol building block.

2. Methods and results

2.1. Synthesis of h(GTGTACAC)

The modified oligonucleotide was assembled on an LCAA-CPG support functionalized with the 1,5-anhydrohexitol deoxycytidine analogue (hC) (Verheggen *et al.*, 1993; Van Aerschot *et al.*, 1995). Synthesis was performed on a 10 µmol scale on an ABI-392 synthesizer using the monomethoxytritylated nucleoside phosphoramidite analogues at 0.12 *M* concentration. The standard protocol was slightly adapted with extension of the coupling time to 10 min and a 5 min detritylation procedure. Standard deprotection (33% aqueous NH_3, 16 h at 328 K) was followed by anion-exchange purification at pH 12 on a Mono-Q column HR 10/10 (Pharmacia) with a NaCl gradient (five runs). Gel filtration on a Biogel P2 column (280 × 25 mm, exclusion limit 1800 Da) afforded the purified material (yield 240 OD) as its sodium salt.

2.2. Crystallization

Crystallization conditions were screened using a 24-matrix screen for nucleic acid fragments (Berger *et al.*, 1996). Crystals were grown at 289 K by the hanging-drop vapour-diffusion technique using Linbro multiwell tissue-culture plates. Well shaped crystals appeared in the droplets after 1 d using the conditions 10%(*v*/*v*) MPD, 12 m*M* spermine chloride, 80 m*M* Na^+ or K^+, 20 m*M* Ba^{2+} and 40 m*M* cacodylate buffered at either pH 6.0 or 7.0. Two distinctive crystal forms were observed: long needles with average dimensions 0.05 × 0.05 × 0.3 mm and, under the K^+-containing conditions, diamond-like crystals with average dimensions 0.25 × 0.25 ×

Table 1
Data-collection statisitics.

Number of reflections used	10972
Number of unique reflections	1134
Resolution range (Å)	14–2.5
Outermost resolution shell (Å)	2.54–2.50
Overall $I/\sigma(I) > 3$ (%)	93.2
Outermost shell $I/\sigma(I) > 3$ (%)	86.4
Overall completeness	99.6
Overall R_{merge} (%)	6.0
Outermost R_{merge} (%)	12.2
Overall multiplicity	9.6

0.25 mm (Fig. 2). After some days, the morphology of the crystals changed. The needles showed splitting, while the edges of the diamond-like crystals became rounded.

The quality of fresh crystals was checked on a Xentronics area-detector system equipped with a rotating-anode source and showed the necessity of using synchrotron radiation. Finally, crystals were grown near the synchrotron site at both 279 and 289 K, and the diamond-like crystals with dimensions 0.2 × 0.2 × 0.2 mm proved to diffract well beyond 2.0 Å.

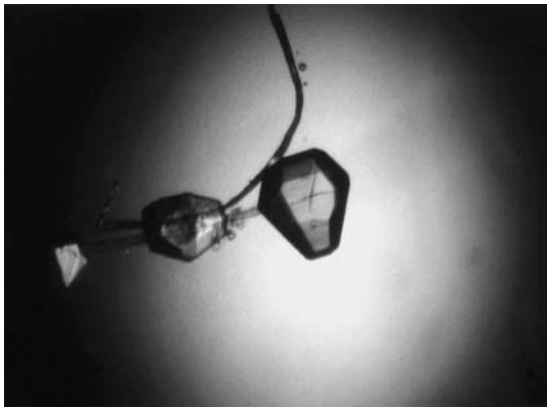

Figure 2
A typical diamond-like crystal of the hexitol-containing octamer h(GTGTACAC) obtained by hanging-drop vapour diffusion. The dimensions of the central crystal are 0.3 × 0.3 × 0.3 mm.

Figure 3
A 2° oscillation image of the h(GTGTACAC) crystal taken using an MAR Research image plate on beamline 5.2R (Elettra, Trieste). The resolution at the edge of the image is 2.5 Å.

3. Data collection and processing

Intensity data were collected at 100 K on an MAR imaging-plate detector at beamline 5.2R of the synchrotron ELETTRA at Trieste (λ = 1.000 Å) using cryo-cooling techniques. Prior to flash freezing of the crystal, 2.5 µl of a 50%(*v*/*v*) aqueous MPD solution was added to the crystal-containing drop. For scaling purposes, two data sets were collected at high (1.8 Å) and low (2.6 Å) resolution over a 90° φ range and increments of 1 and 2° with crystal-to-detector distances of 140 and 220 mm, respectively. The crystal did not show any sign of decay during the data collection. The data sets were processed with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1996). The space group was determined to be either $P6_222$ or $P6_422$ with unit-cell dimensions a = 36.42, c = 69.33 Å, which allows for one strand in the asymmetric unit and a volume per base pair of 1659 Å^3. Structure determination by molecular replacement is currently being carried out using a model built by molecular-dynamics calculations (De Winter *et al.*, 1998).

An experimental low-resolution diffraction pattern is shown in Fig. 3. Although the high-resolution diffraction pattern showed diffraction to 1.9 Å, careful analysis showed that the data between 1.9 and 2.5 Å were of much lower quality than the rest; therefore, a cut-off was chosen at 2.5 Å. Some data-collection statistics are given in Table 1.

The natural analogue of h(GTGTACAC) and the potentially Z-forming sequence d(GTGTACAC) has been crystallized as A-DNA in two different crystal forms with tetragonal (Jain & Sundaralingam, 1989) and hexagonal (Jain *et al.*, 1991; Thota *et al.*, 1993) unit cells. The hexagonal crystal structure belongs to space group $P6_122$ with cell parameters a = 32.12 Å and c = 78.51 Å, resulting in a smaller volume per base pair (1467 Å^3) than found for the hexitol octamer. However, the volume per base pair has been found to be very variable in A-DNA (∼1300 to ∼1800 Å^3; Heinemann, 1991). So far, no well diffracting needle-shaped crystals, which may possibly be the tetragonal variant of h(GTGTACAC), have been obtained. Further optimization of the crystallization conditions is in progress.

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