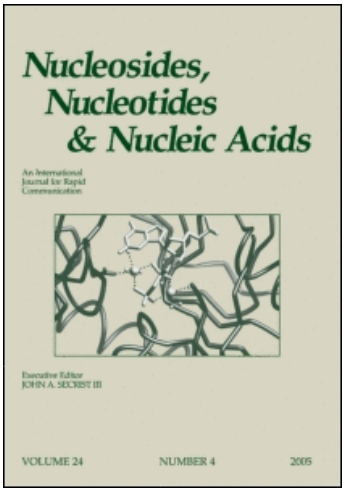

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ENZYMATIC RESOLUTION AND BASE PAIRING PROPERTIES OF *D*- AND *L*-CYCLOHEXENYL NUCLEIC ACIDS (CeNA)

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▫ *An enzymatic transesterification reaction afforded large scale resolution of the cyclohexenol precursor needed for preparation of both series of CeNA building blocks. CeNA oligos of "D-like" chirality display a strong and selective interaction with RNA, while preserving RNase H activity, and therefore have potential as antisense constructs. CeNAs of opposite chirality form a self-pairing system on their own.*

INTRODUCTION

Although studied for over a decade, antisense oligonucleotides remain an important tool for possible therapeutic intervention. Among the many modifications of oligonucleotides to date, only a few demonstrate an increase in duplex stability and few of them allow RNase H to cleave the target RNA. In 1988, we started a program with the intent to substitute a 6-membered ring for the 5-membered (deoxy)ribofuranose ring.[1] These 6-membered rings should confer increased rigidity to antisense constructs and therefore have less entropy loss upon

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formation of a double stranded complex. As a result these constructs should be endowed with higher affinity for a partner provided the correct orientation of the base moieties is present. This endeavour afforded us the 1,5-anhydrohexitol nucleic acids (HNA), with a strong and selective recognition of RNA, yielding the desired A-type double helix.[2–4] At the same time, however, the HNA stiffnes prohibits RNase H recognition. It was reasoned that introduction of a double bond should increase the flexibility allowing adaptation and enzymatic recognition, while hopefully preserving the increased affinity for complementary targets. A project on cyclohexenyl oligonucleotides (CeNA) was thus started.

Synthesis of the desired monomers as required for incorporation into oligonucleotides (see Figure 1A) is not straightforward, however, with a lengthy chiral synthesis hampering further development of this new antisense backbone.[5] A neat strategy for preparation of the racemic mixture of the nucleosides was reported, and these can be separated by tedious chromatography of the diastereomeric esters with (*R*)-methyl-mandelic acid.[6] We now report an enzyme-catalyzed resolution of the enantiomers of the intermediate cyclohexenol derivatives. This strategy affords sufficient quantities to allow preparation of all four building blocks of the cyclohexenyl analogues, for both the *D*- and *L*-like series.[7]

It was described already that CeNA may adopt two half-chair conformations ($^3H_2$and $^2H_3$). The former one with axially oriented base moieties is energetically favoured. Being a good mimic of a furanose ring in its C3′-endo conformation, this analogue is preferred for hybridization with RNA.[5,8] As of the presence of the double bond, these CeNAs can undergo conformational changes similar to those of natural nucleic acids[8,9] (Figure 1B). The constraint of the six-membered ring, endows these nucleoside analogues a ribonucleoside-like conformation with strong affinity for RNA. The presence of the double bond on the other hand allows sufficient flexibility within a CeNA-RNA double helix, to be recognized by RNase H. However, these preliminary results were obtained with only a single CeNA-unit

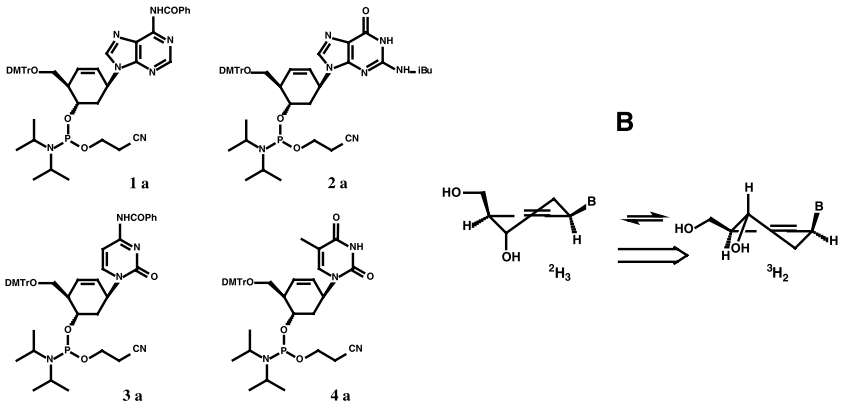

FIGURE 1 Cyclohexenyl nucleoside building blocks for DNA synthesis of the a-type or the *D*-like series. Pannel B: conformational equilibrium within the CeNA series.

incorporated in an otherwise regular DNA helix, and awaited confirmation. In addition, the influence of chirality of nucleic acids on hybridization is studied here in evaluating homochiral *D*-CeNA and *L*-CeNA for their hybridization capacity with DNA and RNA, respectively, and with itself.

Racemic compound **5** is the key intermediate for the subsequent introduction of nucleobases, and is obtained via a Diels-Alder reaction. Several methods for resolving **5** could be envisaged, i.e., kinetic resolution using sharpless epoxidation, enzymatic resolution, or formation of diastereomeric esters. At first, several conditions for enzyme-catalyzed hydrolysis of benzylidene-protected cyclohexenyl esters **6–8** were studied, but none showed the desired selectivity and activity profiles. Best results were obtained with PLE-catalyzed hydrolysis of the butyryl ester with a selectivity of $E = 45$ (Figure 2).

However, all hydrolysis reactions had a much lower rate then the following transesterifications. It was found already that transesterification of the cyclohexenol ***rac-5*** with Lipase PS afforded only 33% of enantiomeric excess.[7] We looked for improvement using different lipases, co-solvents and reagents, which afforded a transesterification methodology with Novozyme® 435 and isopropenyl acetate. Followed by chromatographic separation and a double crystallization, this methodology afforded a clean separation in good yield. Vinyl buyrate afforded the highest selectivity ($E = 127$), but with slow reaction rate (52% conversion in 39 h) and is economically more expensive. (Figure 3). The final methodology uses 14% (w/w) of Novozyme® 435 with 5 equiv. of isopropenyl acetate in 50 volumes of CH2Cl2 at RT (about 52% conversion after 22 h, $E = 50$). The method developed allows isolation of both optically pure cyclohexenol enantiomers on a preparative scale. These were used for introducing the base moieties via a Mitsunobu protocol.

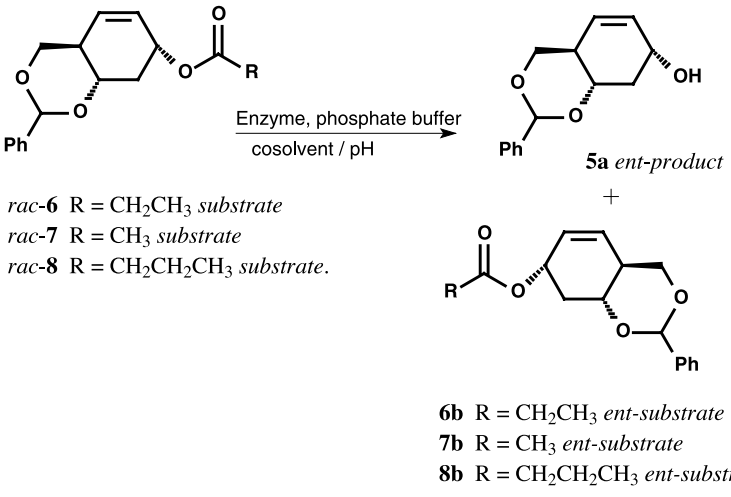

FIGURE 2 Hydrolysis of different racemic esters *rac*-**6**, *rac*-**7**, *rac*-**8** with different lipases and esterases.

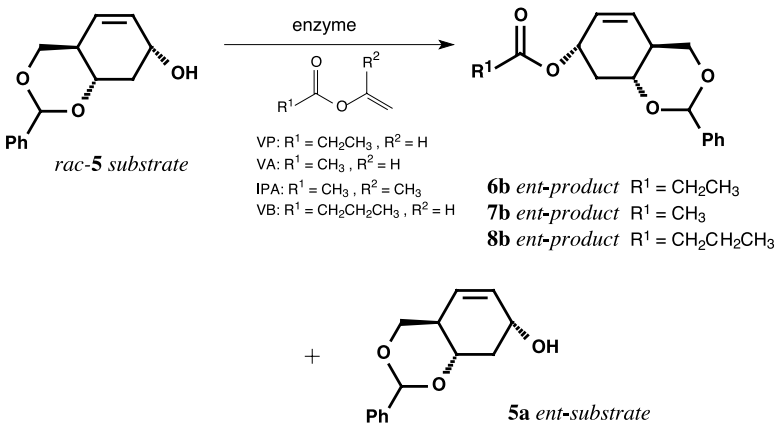

FIGURE 3 Transesterification of *rac*-**5** with different enzymes and substrates.

Further elaboration into phosphoramidites (Figure 1) allowed straightforward assembly into oligonucleotides (Figure 4) in acceptable yield.

D-CeNAs resembling the natural configuration, upon incorporation into DNA strands only slihtly decrease the affinity for DNA strands, while with RNA complements there is a slight and sometimes even large increase in affinity. This can be seen in the following Table 1, with examples for incorporation of A and T CeNA building blocks (A_1, T_1), but several new examples have confirmed these findings. With fully modified sequences, however, the picture is clear, with CeNA displaying an increased affinity for RNA targets (ΔT_m/mod = 0.6 to 1.2°C). This increase per basepair is 1°C less in comparison with the HNA skeleton (entries 5, 6,

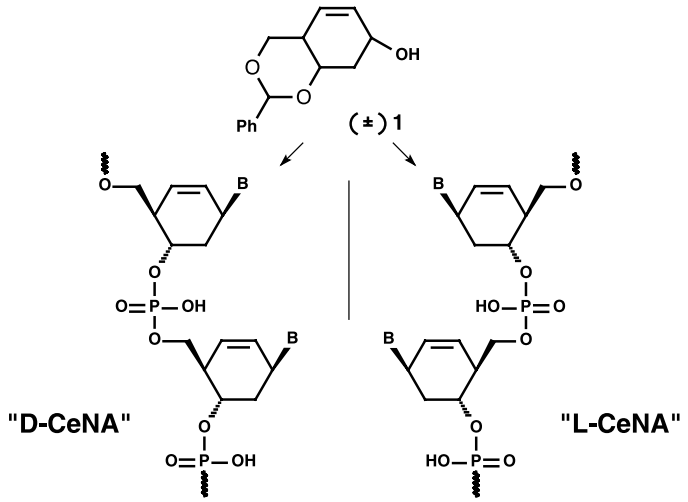

FIGURE 4 Elaboration of the racemic cyclohexenol into, respectively, *D*-like and *L*-like cyclohexenyl oligonucleotides.

TABLE 1 Influence of Incorporation of Single and Multiple CeNA Monomers in DNA Strands on the Affinity for DNA and RNA Complementary Sequences

Entry	Sequence	DNA complement	RNA complement
1	5′-CCAGTGATATGC-3′	49.8	44.0
2	5′-CCAGTG A_1TATGC-3′	49.4	45.1
3	5′-CCAGTG A_1T A_1TGC-3′	48.5	45.6
4	5′-CC A_1GTG A_1T A_1TGC-3′	48.1	49.2
5	5′-CCAGTGhATATGC-3′	49.5	46.9
6	5′-CCAGTGhAThATGC-3′	49.3	48.7
7	5′-CChAGTGhAThATGC-3′	47.5	53.1
8	5′-GGTCACTATACG-3′	46.1	46.1
9	5′-GGTCACT$_1$ATACG-3′	44.8	49.1
10	5′-GGTC$_1$A$_1$C$_1$T$_1$A$_1$T$_1$ACG-3′	37.7	53.5
11	5′-GCGTAGCG-3′	–	21.0
12	5′-r(GCGTAGCG)-3′	–	47.6
13	5′-G$_1$C$_1$G$_1$T$_1$A$_1$G$_1$C$_1$G$_1$-3′	–	50.8
14	5′-h(GCGTAGCG)-3′	–	54.4

7, and 14), but the CeNA modification preserves RNase H cleavage activity for the target sequence. This cleavage however takes place at much higher enzyme concentrations, resulting in a 600-fold lower Kcat in comparison with a DNA antisense oligo (not shown). Where the affinity for DNA targets is reduced (ΔT_m/mod = 1 to 2°C), the selectivity of interaction remains unaffected, analogous to the selectivity for RNA complements.

Table 2 gives an overview of the strength of different homopolymer interactions within an A-T or A–U context, a duplex, however, with different overall shape already within the natural sequences. One clearly notices the effect of constraint comparing the interactions of A_1 or hA with RNA in comparison with the DNA-RNA duplex (lines 3 and 6 versus line 1, with a rise from 15 to 34°C). Especially enlightning therefore is the strong HNA-HNA interaction with a T_m of 78°C versus

TABLE 2 T_m (°C) Determination of the Complexes Formed Between Homo-Modified Oligonucleotides and Their DNA, RNA, HNA, and CeNA Complement, Respectively

Entry	Sequence	DNA	RNA	HNA	CeNA
1	$(dA)_{13}$	34.0	15.2	45/58[d]	37–43[b]
2	$(dT)_{13}$	34.0	32.0	21	33.5
3	$(A_1^*)_{13}$	33.5	34.2	72.5	ND[c]
4	$(A_2^*)_{13}$	–[a]	26	48.5/43[e]	67
5	$(T_2^*)_{13}$	37–43[b]	37–43[b]	37–43[b]	67
6	$(hA)_{13}$	21	33	78	37–43[b]
7	$(hT)_{13}$	45/58[d]	50	78	72.5

[a]No melting transition detected.
[b]Shallow melting curve without pronounced inflection point (T_2 containing duplex).
[c]Not determined (ND).
[d]Double transition, respectively for triplex and duplex.
[e]Dissociation and reassociation values, respectively, when determined at 0.2°C/min.

72.5°C for the CeNA-HNA and 67°C for the CeNa-CeNA counterparts. The latter duplex was studied with oligomers of opposite chirality (the *L*-series) in view of insufficient supply of the required T_1 monomer. Herewith, however, we uncovered that the CeNAs of opposite chirality constitute a strong self-pairing system on their own, resembling *L*-RNA sequences, but not pairing with natural DNA or RNA. This is actually the first example of a "nonnatural-like" spiegelmer. The unknown thymine-thymine interaction as seen before within the HNA series (entry 4, T_m 48.5°C), was noticed here in both CeNA series as well, and is responsible for the shallow melting curves with inflection point at 37–43°C. Finally, an unexpected interaction occus between the A_2 homopolymer with polyuridine (T_m of 26°C), which cannot be explained either.

In view of the strong and selective interaction of CeNAs with RNA complements, and the preservation of RNaseH recognition of a CeNA-RNA duplex, the CeNAs have a strong potential for antisense strategies.

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