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Synthesis of enantiomeric-pure cyclohexenyl nucleoside building blocks for oligonucleotide synthesis

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Abstract—Lipases were used for the resolution of (±) (4a*R*, 7*R*, 8a*S*)-2-phenyl-4a,7,8,8a-tetrahydro-4*H*-1,3-benzodioxine. This separation was carried out on preparative scale and used for the synthesis of eight phosphoramidites of cyclohexenyl nucleosides (D- and L-series).
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1. Introduction

Since nucleic acids with a six-membered carbohydrate moiety in the backbone are potentially more preorganized than furanose-type nucleic acids, they might have an entropic advantage during hybridization and, hence, could form more stable duplexes.[1] This is exemplified with hexitol nucleic acids (HNA)[2] and with altritol nucleic acids (ANA)[3] that demonstrate strong self-complementary hybridization and hybridization with ribonucleic acids (RNA). Their carbocyclic congeners, cyclohexane nucleic acids (CNA), also hybridize with natural nucleic acids.[4] Hexitol nucleic acids, however, have the disadvantage that they are poorly recognized by nucleic acids metabolizing enzymes which might be ascribed to their rigidity.[5] Therefore, we developed nucleosides and oligonucleotides based on a cyclohexene system. These nucleosides are more flexible than the anhydrohexitol nucleosides and are better mimics of a furanose nucleoside.[6] D-Cyclohexenyl guanine (Cycl-G) has been evaluated against a whole range of herpes viruses and its activity was comparable with those of acyclovir (ACV) and ganciclovir (GCV).[7] (±)Cyclohexenyl cytosine is a potent anti-VZV compound.[8] Cyclohexenyl adenine (Cycl-A) has been incorporated into oligonucleotides.[9] Compared to a single stranded DNA oligomer, the affinity of these cyclohexene nucleic acids (CeNAs) for complementary DNA sequences diminished slightly, while an increase was noticed for RNA complements. Moreover, a CeNA/RNA duplex could be recognized by RNase H, resulting in RNA strand cleavage in serum.[9,10]

However, the difficulty to synthesize chiral cyclohexene derivatives in high enantiomeric excess and in bulk quantities has hampered the further study of cyclohexenyl nucleosides and their oligonucleotides. It has been recognized that D- and L-nucleotides have different properties, exemplified by the findings that D-CNAs hybridize with natural nucleic acids and are RNA-selective while L-CNA hybridize either very weakly or not at all with natural nucleic acids.[4] Recently, a novel and facile method to prepare D- and L-cyclohexenyl nucleosides has been reported.[11] The resolution of these analogues via the formation of diastereomeric esters with (*R*)-(−)-methylmandelic acid is, however, a multistep and tedious work (difficult chromatographic separation), and expensive on large scale.

Therefore we developed a method for the separation of the enantiomers of a racemic intermediate of the synthetic scheme leading to cyclohexenyl nucleosides via an enzyme-catalyzed resolution strategy.[12] This method enables synthesis of the cyclohexenyl nucleosides with all four nucleobases. Further derivatization into their phosphoramidite derivatives afforded suitable building blocks for DNA synthesis as depicted in Figure 1. Here we defined the compounds or their derivatives as an 'a'-type or 'b'-type depending on the type of enantiomer that is considered. The 'a'-type has the 1′*S*, 4′*R*, 5′*S* configuration, overall resembling the D-anhydrohexitol series, while the 'b'-type has the mirror configuration with 1′*R*, 4′*S*, 5′*R* (Fig. 2).

Keywords: Cyclohexenyl nucleosides; Enzymatic resolution; Phosphoramidite; Mitsunobu reaction.
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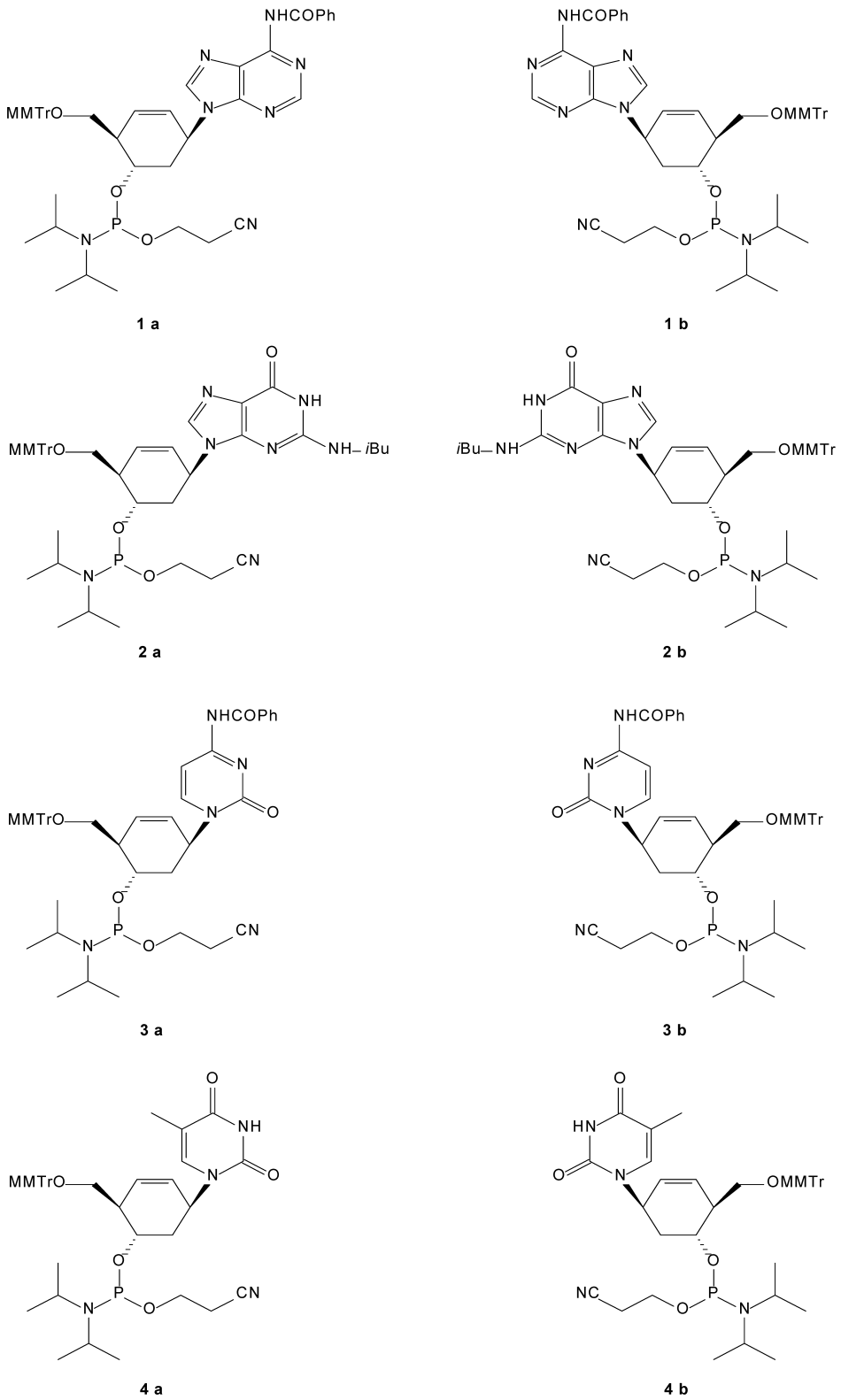


Figure 1. Cyclohexenyl nucleoside building blocks for DNA synthesis.

2. Results and discussion

2.1. Enzymatic resolution of the racemic intermediate 5

For the synthesis of cyclohexenyl nucleosides, racemic compound **5** is the key intermediate for the subsequent introduction of nucleobases. Several methods for resolving **5** could be envisaged, i.e. kinetic resolution using Sharpless epoxidation,[13] enzymatic resolution[14] or formation of diastereomeric esters.[5] The previous study on enzymatic resolution of **5** using vinyl acetate and Lipase PS (Amano) was not successful,[11] and gave only 33% of enantiomeric excess. We started our study with transesterification by screening three different lipases, Novozyme® 435, CRL

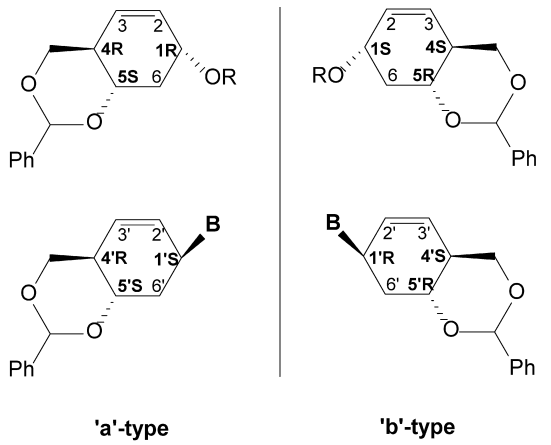


Figure 2. Nomenclature used for cyclohexenyl compounds in this manuscript.

(*Candida rugosa lipase*) and PCL (*Pseudomonas cepacia lipase*). Novozyme® 435 is a member of the lipases of *Candida antarctica B*, and is particularly useful in the synthesis of esters and amides, and has a broad substrate specificity (Fig. 3).[12]

The transesterification reactions were carried out using a test amount of *rac*-**5** (70 mg) and vinyl propionate (VP) (5 equiv.) catalysed by 14% (w/w) of enzymes at room temperature to give enantiomeric pure product *ent*-**6b** and enantiomeric pure substrate *ent*-**5a** (Table 1, Fig. 3). Vinyl propionate was used as organic solvent in individual reactions. We also examined toluene as organic solvent in each reaction (data not shown here), but found that toluene could neither improve the selectivity nor accelerate the reaction. Although none of the evaluated enzymes were synthetically useful, use of Novozyme® 435 showed the best selectivity (E=14).

We further investigated the Novozyme® 435-catalyzed transesterification of *rac*-**5** with formation of *ent*-**7b** and *ent*-**5a** with different acetyl donors (Table 1). Vinyl acetate (VA) and isopropenyl acetate (IPA) were respectively used both as acetyl donor and as organic solvent. A moderate selectivity as expressed in an E value of 22 was obtained with isopropenyl acetate. Under these circumstances, the reaction reached a point of conversion of 49% within 20 h.

Having obtained improved results by using Novozyme® 435 and isopropenyl acetate, we tried to increase the enantioselectivity by further changing the reaction conditions (Table 1). We did not examine a temperature effect on selectivity since testing-scale reactions would not generate much heat which otherwise might have significant influence on temperature. Toluene, octane, chloroform and

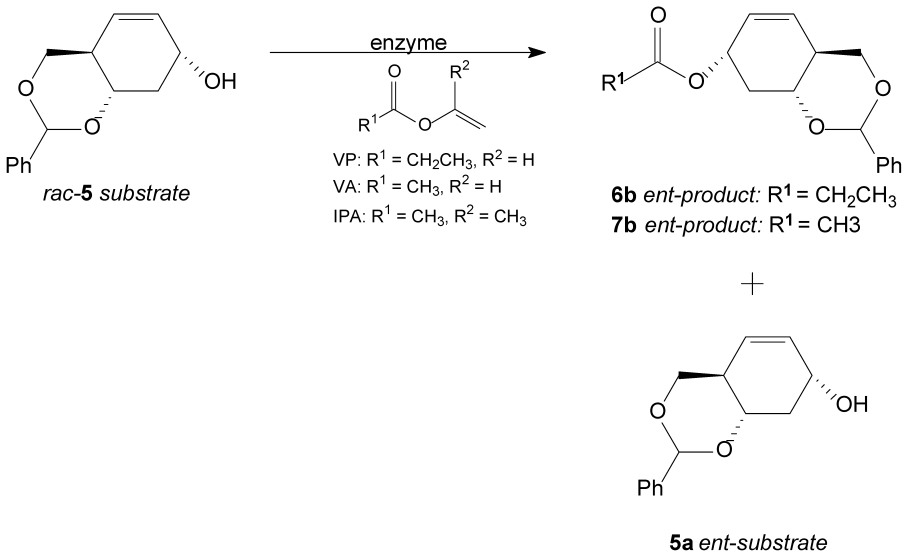


Figure 3. Transesterification of *rac*-**5** with different enzymes.

Table 1. Transesterification of *rac*-**5** with vinyl propionate in the presence of different enzymes, with different acetyl donors and under various conditions using Novozyme® 435 and isopropenyl acetate

Reagents	Enzymes	Conditions solvent /additive	Conversion (%)	Time (h)	e.e.$_s$ (%)	e.e.$_p$ (%)	E
Vinyl propionate	Novozyme® 435	–/–	30	3	37	82	14
Vinyl propionate	CRL	–/–	49	27	51	35	3
Vinyl propionate	PCL	–/–	45	27	40	46	4
Vinyl acetate	Novozyme® 435	–/–	66	43	79	39	5
Isopropenyl acetate	Novozyme® 435	–/–	49	20	93	74	22
Isopropenyl acetate	Novozyme® 435	*Toluene*/–	64	43	65	47	7
Isopropenyl acetate	Novozyme® 435	*Octane*/–	59	20	62	29	3
Isopropenyl acetate	Novozyme® 435	*$CHCl_3$*/–	41	24	69	86	28
Isopropenyl acetate	Novozyme® 435	*CH_2Cl_2*/–	49	20	95	84	50
Isopropenyl acetate	Novozyme® 435	*CH_2Cl_2/Et_3N*	71	20	>99	49	19
Isopropenyl acetate	Novozyme® 435	–/*Et_3N*	60	20	95	70	20

dichloromethane were examined as organic solvent for the transesterification reactions. Both the reactions using chloroform and dichloromethane as organic solvent exhibited good (E=28) to excellent (E=50) selectivity. Attempts were tried to add some additives such as triethylamine, which might change the pH environment and thus the enzymatic activity, which could lead to further improvement of selectivity. The results shown in Table 1, however, revealed that triethylamine enhanced the reaction rate but had negative effect on the selectivity. A test reaction with vinyl butyrate (data not shown here) using the same conditions afforded the highest selectivity (E=127) but with a relatively slow reaction rate (52% conversion, 39 h), which is not favored for economical and practical reasons.

We also investigated the enzyme-catalyzed (PLE, CRL, CAL-B) hydrolysis of *rac*-**7**, which gave the product *ent*-**5a** and the substrate *ent*-**7b** (Fig. 4, Table 2).

None of the enzyme-catalyzed hydrolysis reactions showed useful selectivity as well as good enzymatic activity. The PLE-catalyzed hydrolysis of the racemic ester **7** at room temperature in a 0.1 M phosphate buffer (pH 8.0) in the presence of 10% (v/v) acetone proceeded somewhat faster than in the presence of 10% (v/v) *t*-BuOH, but did not improve the selectivity. CRL-catalyzed hydrolysis exceptionally gave *ent*-**5b** as the product and *ent*-**7a** as the residual substrate, which indicates that the enzyme may favor the 'b-type' configuration of the racemic substrate. This phenomenon was also observed for the hydrolysis of *rac*-**6** and *rac*-**8**. We then turned our attention to the hydrolysis of different racemic esters *rac*-**6**, *rac*-**8** (Fig. 4, Table 2). As seen from the table, the hydrolysis reaction was best conducted by PLE-catalysed hydrolysis of *rac*-**8** in a 0.1 M phosphate buffer solution at pH 8.0 in the presence of 10% (v/v) *t*-BuOH affording *ent*-**5a** and *ent*-**8b** with a selectivity of (E=45). The resolution might be further optimized by changing cosolvent, i.e. acetone or *t*-BuOMe. However, all enzyme-catalyzed hydrolysis reactions had a much lower reaction rate than transesterifications. This could be explained as the former reaction was carried out in heterogeneous phases.

In view of the obtained results, enzyme-catalyzed transesterification was finally adopted for the separation of the racemic intermediate **5** considering both economical and practical aspects.

2.2. Separation and purification of enantiomers of rac-5

The overall procedure for separation and purification of racemic **5** is shown in Figure 5. The transesterification was carried out with 14% (w/w) of Novozyme® 435 catalyzed acylation of racemic substrate *rac*-**5** using 5 equiv. of isopropenyl acetate as the acetyl donor in 50 volumes of dichloromethane at room temperature. Chiral high performance liquid chromatography (HPLC)[11] was used to follow the reaction to determine the end point. The reaction was

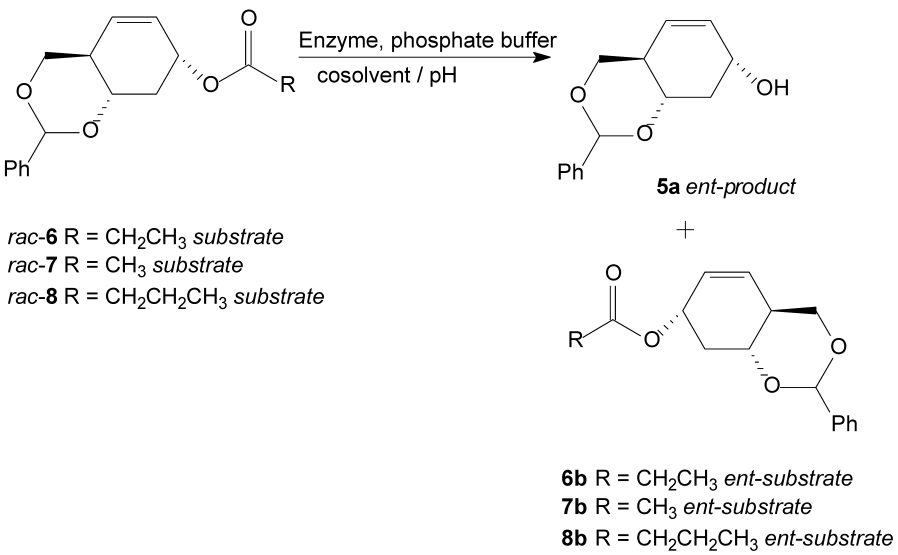


Figure 4. Hydrolysis of different racemic esters *rac*-**6** *rac*-**7** and *rac*-**8**.

Table 2. Hydrolysis of *rac*-**6**, *rac*-**7** and *rac*-**8** with different enzymes

Substrates (R=)	Enzymes	Conditions cosolvent/pH	Conversion (%)	Time (h)	e.e.$_s$ (%)	e.e.$_p$ (%)	E
$-CH_3$	PLE	*t*-BuOH/8.0	11	22	<1	30	2
$-CH_3$	PLE	Acetone/8.0	44	24	27	46	4
$-CH_2CH_3$	PLE	*t*-BuOH/8.0	35	17	40	68	8
$-CH_2CH_2CH_3$	PLE	*t*-BuOH/8.0	13	10	9	95	45
$-CH_3$	CRL*	*t*-BuOH/7.5	17	24	26	65	6
$-CH_2CH_3$	CRL*	*t*-BuOH/7.5	85	24	30	2	1
$-CH_2CH_2CH_3$	CRL*	*t*-BuOH/7.5	27	4	24	11	2
$-CH_3$	CAL-B	*t*-BuOH/7.5	21	25	10	16	1
$-CH_2CH_2CH_3$	CAL-B	*t*-BuOH/7.5	21	30	15	63	4

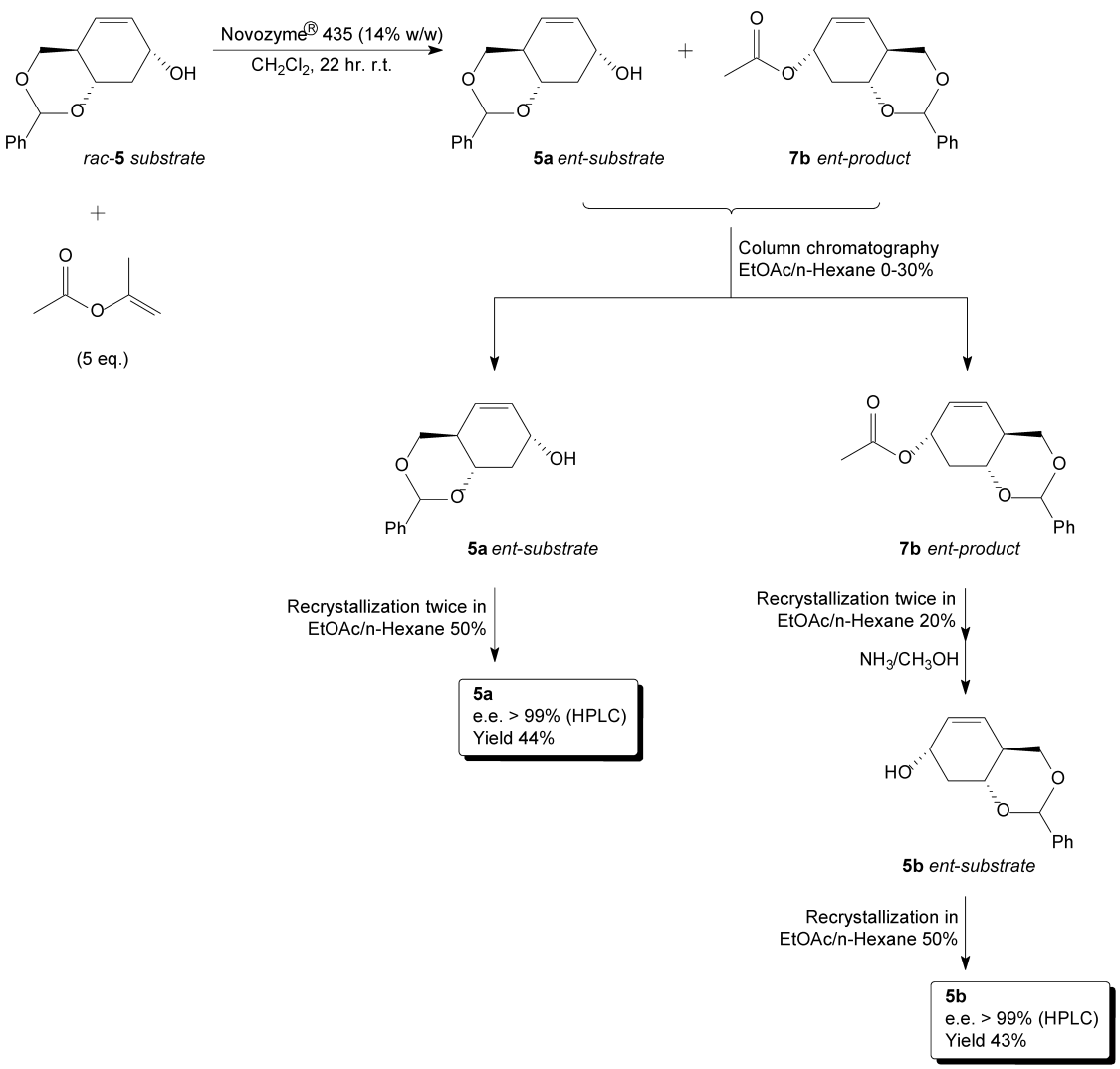


Figure 5. Procedure for separation and purification of enantiomers of **5**.

stopped after 22 h (52% conversion) and optically pure substrate **5a** (e.e.$_s$>97%) and product **7b** (e.e.$_p$>85%) were obtained, which were subsequently separated easily by column chromatography.

Following two recrystallizations from 50% of ethyl acetate in *n*-hexane, a colorless needle crystal of the enantiomeric pure **5a** with an enantiomeric excess value (e.e.) of over 99% was obtained in an overall yield of 44% (calculation based on *rac*-**5**). The absolute configuration was established via comparison of its guanine nucleoside derivative with an authentic sample which was described in the literature.[11] High optical purity (e.e.>97%) for the isolated product of *ent*-**7b** was achieved by recrystallizing twice from 20% ethyl acetate in *n*-hexane. Subsequently *ent*-**7b** was further treated with saturated ammonia/methanol solution to afford *ent*-**5b**. Likewise, recrystallized from 50% of ethyl acetate in *n*-hexane, **5b** was afforded as a colorless needle with e.e. value of over 99% in an overall yield of 43%.

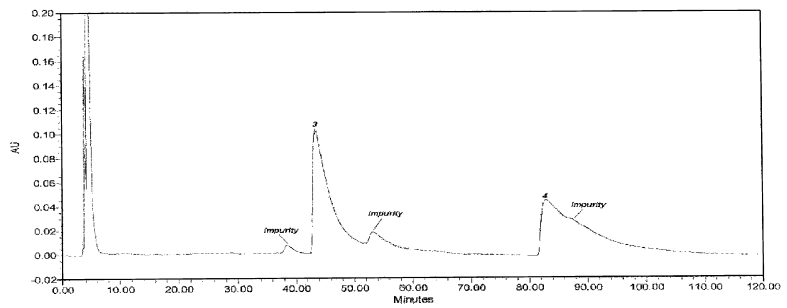
Figure 6 shows the enzymatic separation of the racemic mixture of **5** by chiral chromatography. A 1:1 mixture of **5a** and **5b** is shown in (A), while (B) and (C) show the results for enzyme-catalyzed hydrolysis and transesterification with a selectivity of (E=2) and (E=50), respectively. The identification of the obtained optically pure **5a** and **5b** are shown in (D) and (E), respectively.

In general, compared with enzyme-catalyzed hydrolysis and other described chemical methods, Novozyme® 435-catalyzed transesterification is the most facile approach to achieve the highest selectivity with economical and practical advantages.

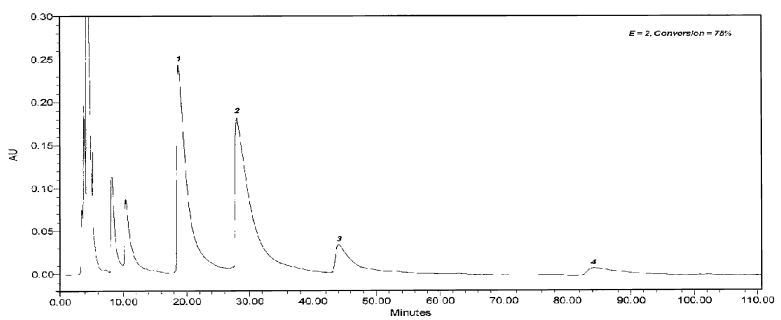
2.3. Synthesis of cyclohexenyl nucleoside building blocks

For investigation of the properties of cyclohexenyl containing nucleic acids, the enantiomeric pure protected phosphoramidite nucleosides of the four natural nucleobases were synthesized independently. The synthesis started with the previously separated enantiomerically pure (e.e.>99%) cyclohexene precursors **5a** and **5b**. The purine base moieties (adenine or 2-amino-6-chloropurine) were introduced via a direct nucleophilic displacement strategy using the Mitsunobu condensation reaction,[15] to give nucleosides **9a**,**b** (yield of 45%) and **10a**,**b** (yield of 37%)[7,11] (Fig. 7). The adenine base of **9a**,**b** was thereafter protected by the benzoyl protecting group, followed by removal of the

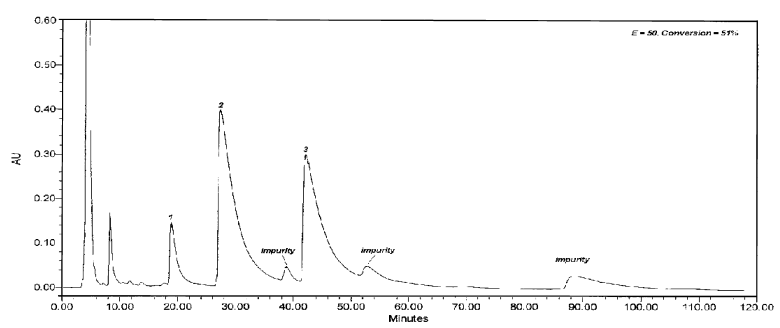
Panel A



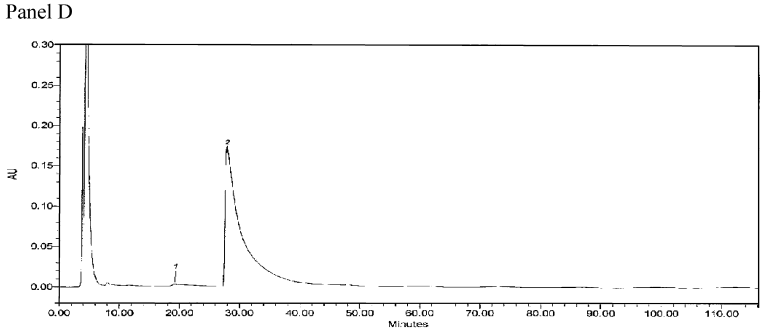
Panel B



Panel C



Panel D



Panel E

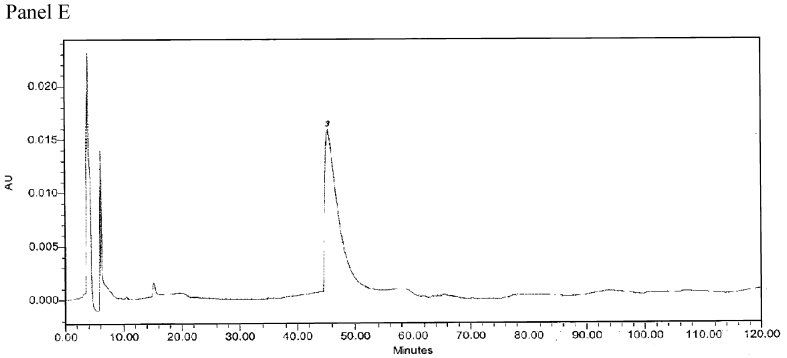


Figure 6 (*legend opposite*)

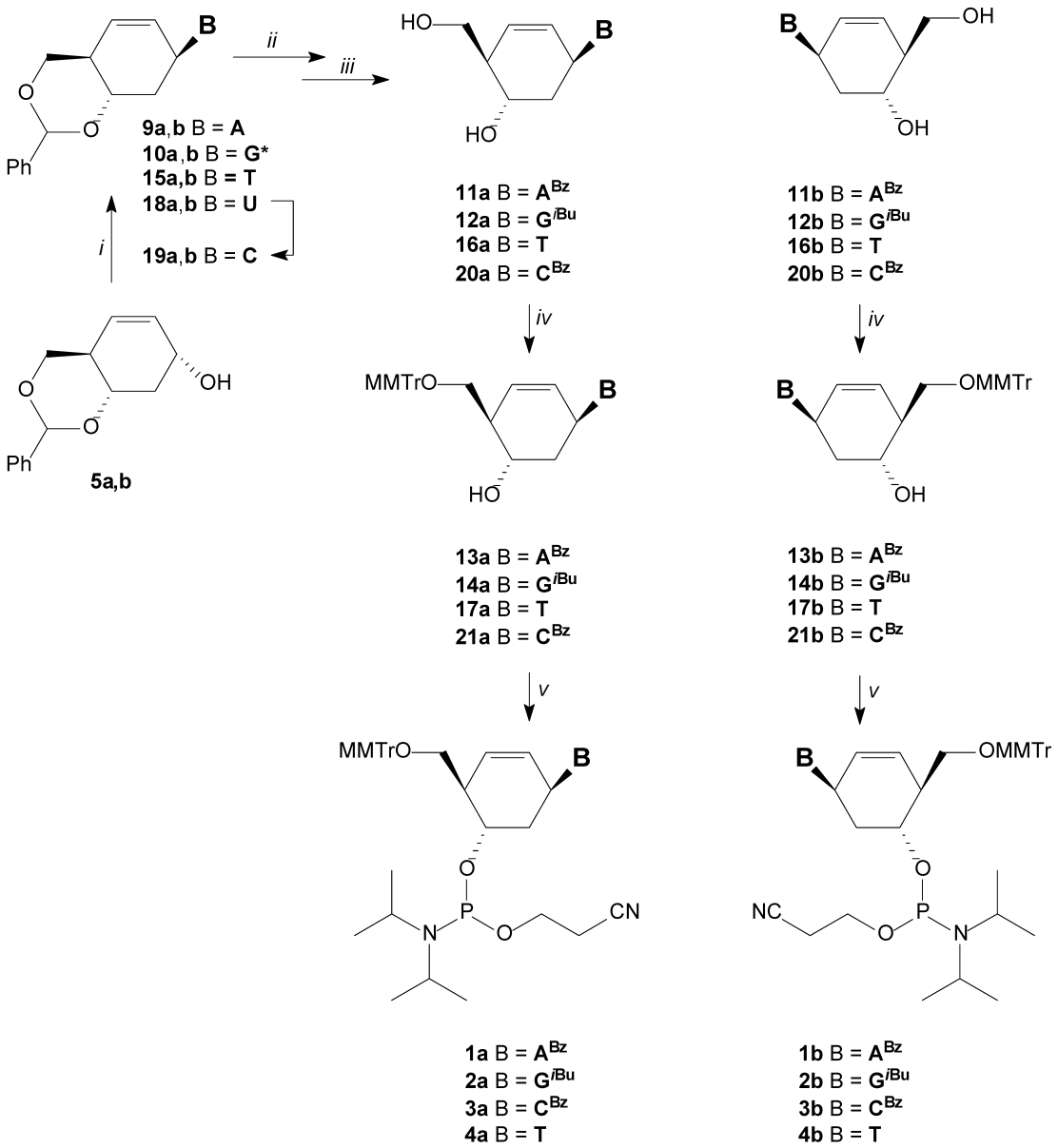


Figure 7. Synthesis for phosphoramidite building blocks **1**–**4** (a- and b-series). (i) Mitsunobu reaction; (ii) BzCl, pyridine for A and C; for G: first TFA/H_2O (80%), then TMS-Cl, isobutyric anhydride, pyridine, 0 °C rt for G; (iii) TFA/H_2O (80%), 40 h; (iv) MMTrCl, pyridine, rt; (v) $(iPr)_2N(CE)PCl$, $(iPr)_2NEt$, CH_2Cl_2.

benzylidene protecting group using 80% TFA/H_2O solution at room temperature for 2 days to give **11a**,**b** in 48% yield. The congeners **10a**,**b** on the other hand were first treated with 80% TFA/H_2O solution for 40 h, followed by protection of the 2-NH_2 position with the isobutyryl group via a transient protection approach to afford **12a**,**b** in 32% yield.[16] The monomethoxytritylation of **11a**,**b** and **12a**,**b** at the primary hydroxyl groups (4′-CH_2OH) was less straightforward. The bis-tritylated product as well as the mono-tritylated product at the secondary alcohol (5′-OH) group were likewise obtained in a total yield of 20–30%. These side reactions could be avoided by starting the reaction at 0 °C and gradually raising the temperature to room temperature with careful control of the reaction time by monitoring with TLC. The secondary hydroxyl groups (5′-OH) of **13a**,**b** and **14a**,**b** were further reacted with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite to yield enantiomeric pure **1a**,**b** and **2a**,**b** (yield of 86–92% and 66–74%, respectively), as the nucleotide building blocks.

Figure 6. Enzymatic resolution of the racemic mixture of **5**. (A) Chromatogram for the racemic mixture of **5**. Chromatographic conditions: Chiralpak AD (250×4.6 mm) Mobile phase: *n*-hexane/EtOH 98:2, Detection: UV_{220} nm; Flow rate: 1.0 mL/min; Injection volume: 10 μL. Peak 3=**5a**, peak 4=**5b**. (B) Analysis of the PLE-catalyzed hydrolysis reaction of *rac*-**8**. Chromatographic conditions: see (A). Peak 1=**8a**, peak 2=**8b**, peak 3=**5a**, peak 4=**5b**. (C) Analysis of the Novozyme®-catalyzed transesterification of *rac*-**5**.Chromatographic conditions: see (A). Peak 1=**6a**, peak 2=**6b**, peak 3=**5a**. (D) Analysis of **6b** obtained following column chromatography of the Novozyme®-catalyzed transesterification reaction of *rac*-**5**. Chromatographic conditions: see (A). Peak 1=**6a**, peak 2=**6b**. (E) Analysis of **5a** obtained following column chromatography of the Novozyme®-catalyzed transesterification reaction of *rac*-**5**. Chromatographic conditions: see (A). Peak 3=**5a**.

Alkylation of pyrimidine bases (cytosine and thymine) is more problematic than that of purines. The thymine base could be directly coupled to the sugar part by the Mitsunobu procedure. When the benzylidene protecting groups were removed the unprotected thymine nucleoside analogues **16a** and **16b** (overall yield of 16%) were obtained which were monotritylated at the primary hydroxyl group to afford **17a** and **17b** (yield of 59%). The secondary hydroxyl groups (5′-OH) were then phosphitylated to obtain the phosphoramidites, **4a** and **4b** (81–85% yield). The cytosine nucleoside **19a**,**b** (overall yield 41%) could be obtained starting from the uracil congener **18a**,**b**.[17] Following protection of the cytosine base with a benzoyl group, the benzylidene group was removed in acidic medium, to obtain the cyclohexenyl cytosine nucleosides **20a**,**b** (yield of 16–21%). Finally, following the tritylation at 4′-OH, compounds **21a**,**b** was converted to the protected phosphoramidite building block **3a**,**b** (yield of 77–87%).

The identification of the protected cyclohexenyl nucleosides and their enantiomers are exemplified with compounds **11a** and **11b**. Their structures were deduced from ^{1}H NMR analysis. Table 3 shows the chemical shifts and coupling constants for all protons. The β-conformation was previously confirmed by ^{1}H NMR analysis of its racemic unprotected congener.[6] Here the small value of the coupling constant between H-2′ and H-1′ ($J_{2'\text{-}1'}$=2.4 Hz) might also indicate that the base moiety occupies the axial position. The two enantiomers of the compound **11** give similar spectroscopic data.

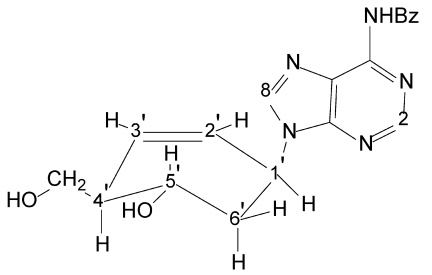
3. Conclusion

In summary, enzymatic resolution of the racemic **5** by employing a transesterification reaction with Novozyme® 435 allows the isolation of optically pure enantiomers in preparative scale. In comparison with other chemical methods,[11] the developed method is highly efficient, easy to perform and economical for multigram preparations. The different enantiomeric series of nucleoside phosphoramidite derivatives with the four natural nucleobases were synthesized as building blocks and they will be used for the synthesis of the corresponding nucleic acids analogues (CeNA).[9]

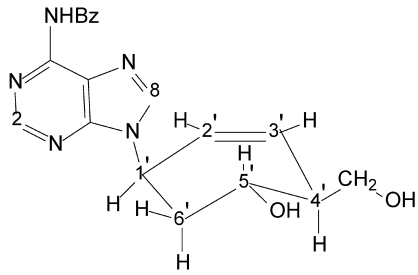
4. Experimental

All solvents used for reactions are analytical grade or freshly distilled. 1,4-Dioxane was refluxed on sodium/benzophenone and distilled. Anhydrous pyridine was refluxed on potassium hydroxide and distilled. PLE (40 KU/306 mg, suspension in 3.2 M $(NH_4)_2SO_4$) solution was purchased from Roche Diagnostics. CRL (26 U/mg), PCL (40 U/mg) were purchased from Fluka. CAL-B

Table 3. ^{1}H NMR chemical shifts (δ) and coupling constants (*J*) of **11a** and **11b**



11a



11b

Proton	Coupled to proton	**11a**		**11b**	
		δ (ppm)	*J* (Hz)	δ (ppm)	*J* (Hz)
1′(m)		5.47		5.45	
2′(ddd)	4′	5.93	1.7	5.94	1.5
	1′		2.4		2.4
	3′		10.0		9.9
3′(dd)	4′	6.13	2.5	6.07	2.7
	2′		10.0		9.9
4′(m)		2.50		2.50	
5′(m)	6′eq	3.81	2.9	3.80	2.9
	4′		5.7		5.7
	6′ax		9.6		9.6
5′-OH (d)	5′	4.74	4.4	4.75	–
6′(m)		2.03–2.37		2.01–2.41	
$HOCH_{2a}$-(m)	4′	3.53	5.1	3.54	5.1
	$HOCH_{2b}$–		10.3		10.3
$HOCH_{2b}$-(m)	4′	3.61	5.4	3.60	5.3
	$HOCH_{2a}$–		10.6		10.6
–OH (t)	$HOCH_2$–	4.68	5.4	4.68	–
2 (s)		8.21		8.24	
8 (s)		7.97		7.91	
Bz		7.26–7.60		7.25–7.57	

(Chirazyme® L-2, lyo, 120 U/mg) was purchased from Boehringer Mannheim. Novozyme® 435 (10 U/mg) was donated by Novo-Nordisk A/S. Enzymatic reactions were run at room temperature. Enantiomer compositions were determined by chiral HPLC analysis with a Chiralpak AD column (250×4.6 mm) on a Waters 6000 controller liquid chromatograph equipped with a Waters 2487 UV detector. ^{1}H NMR was determined with a 200 MHz Varian Gemini spectrometer with tetramethylsilane (TMS) as internal standard for ^{1}H NMR spectra and the same apparatus was used for ^{13}C NMR determination with DMSO-d_6 (39.6 ppm) or $CDCl_3$ (76.9 ppm) as internal standard for the ^{13}C NMR spectra (s=singlet, d=doublet, dd=double doublet, t=triplet, br s=broad singlet, br d=broad doublet, m=multiplet). ^{31}P NMR spectra was obtained as 85% H_3PO_4 as external standard. Exact mass measurements were performed on a quadrupole time-of-flight mass spectrometer (Q-Tof-2, Micromass, Manchester, UK) equipped with a standard electrospray-ionization (ESI) interface; samples were infused in *i*-PrOH/H_2O 1:1 at 3 μL/min column chromatography was performed on ICN silica gel 63–200 μm, 60 Å. Precoated aluminium sheets (Fluka Silica gel/TLC-cards, 254 nm) were used for TLC; the spots were examined with UV.

Chiral HPLC analysis for determination of enantiomer composition: Mobile phase: *n*-hexane/EtOH 98:2, flow rate: 1 mL/min, UV wavelength: 220 nm, t_R (1)=18.7, t_R (2)=28.1, t_R (3)=43.8, t_R (4)=83.5 min.

4.1. Transesterification reactions of *rac*-5

The selectivity of the reaction is expressed as the enantiomeric ratio (*E*), which mathematically links to the conversion (*c*) of the reaction,[18] and the optical purities of substrate (e.e.$_s$) and product (e.e.$_p$). The dependence of the selectivity and the conversion of the reaction is:

for the product

$$E = \frac{\ln[1 - c(1 + \text{e.e.}_p)]}{\ln[1 - c(1 - \text{e.e.}_p)]}$$

for the substrate

$$E = \frac{\ln[(1 - c)(1 - \text{e.e.}_s)]}{\ln[(1 - c)(1 + \text{e.e.}_s)]}$$

where *c*, conversion; e.e., enantiomeric excess of substrate (S) or product (P); *E*, enantiomeric ratio.

The following equation is recommended instead because only values for the optical purities of substrate and the product need to be measured.[19]

$$E = \ln\left(\frac{1 - \text{e.e.}_s}{1 + \frac{\text{e.e.}_s}{\text{e.e.}_p}}\right) / \ln\left(\frac{1 + \text{e.e.}_s}{1 + \frac{\text{e.e.}_s}{\text{e.e.}_p}}\right)$$

With Novozyme® 435. *rac*-**5**[11] (70 mg, 0.30 mmol) and vinyl propionate (5 mL, 46.8 mmol) were mixed. Subsequently, Novozyme® 435 (10 mg, 100U) was added and the reaction mixture was stirred at room temperature. The reaction was stopped at 3 h (30% conversion) by filtration. Chiral HPLC analysis of a sample from the filtrate showed the presence of **6b** *ent*-product with 82% e.e. and of **5a** *ent*-substrate with 37% e.e.

With CRL. The reaction was carried out with *rac*-**5** (70 mg, 0.30 mmol) and vinyl propionate (5 mL, 46.8 mmol). CRL (10 mg, 260U) was added under the above conditions. The reaction was stopped at 27 h (49% conversion) by filtration. Chiral HPLC analysis of a sample from the filtrate showed the presence of **6b** *ent*-product with 35% e.e. and of **5a** *ent*-substrate with 51% e.e.

With PCL. The reaction was carried out with *rac*-**5** (70 mg, 0.30 mmol) and vinyl propionate (5 mL, 46.8 mmol). PCL (10 mg, 400U) was added under the above conditions. The reaction was stopped at 27 h (45% conversion) by filtration. Chiral HPLC analysis of a sample from the filtrate showed the presence of **6b** *ent*-product with 46% e.e. and of **5a** *ent*-substrate with 40% e.e.

With vinyl acetate. *rac*-**5** (70 mg, 0.30 mmol) was dissolved in vinyl acetate (5 mL, 54 mmol) and Novozyme® 435 (10 mg, 100U) was subsequently added at room temperature. The reaction was stopped at 43 h (66% conversion) by filtration. Chiral HPLC analysis of a sample from the mixture showed the presence of **7b** *ent*-product with 39% e.e. and of **5a** *ent*-substrate with 79% e.e.

With isopropenyl acetate. *rac*-**5** (70 mg, 0.30 mmol) with isopropenyl acetate (145 mg, 1.5 mmol) and Novozyme® 435 (10 mg, 100U) was added under the above conditions. The reaction was stopped at 20 h (49% conversion) by gel filtration. HPLC analysis of a sample from the filtration showed the presence of **7b** *ent*-product with 74% e.e. and **5a** *ent*-substrate with 93% e.e.

The procedures for transesterification of *rac*-**5** with various solvents and additives are given here exemplified by using CH_2Cl_2 and CH_2Cl_2/Et_3N.

With CH_2Cl_2. *rac*-**5** (70 mg, 0.30 mmol) and isopropenyl acetate (145 mg, 1.5 mmol) were dissolved in CH_2Cl_2 (5 mL). Subsequently, Novozyme® 435 (10 mg, 100U) was added and the reaction mixture was stirred at room temperature. The reaction was stopped at 20 h (49% conversion) by filtration. Chiral HPLC analysis of a sample from the mixture showed the presence of **7b** *ent*-product with 84% e.e. and of **5a** *ent*-substrate with 95% e.e.

With CH_2Cl_2/Et_3N. *rac*-**5** (70 mg, 0.30 mmol), isopropenyl acetate (145 mg, 1.5 mmol) and Et_3N (10.6 μL, 75 μmol) were dissolved in CH_2Cl_2 (5 mL). Subsequently, Novozyme® 435 (10 mg, 100U) was added and the reaction mixture was stirred at room temperature. The reaction was stopped at 20 h (71% conversion) by filtration. Chiral HPLC analysis of a sample from the mixture showed the presence of **7b** *ent*-product with 49% e.e. and of **5a** *ent*-substrate with >99% e.e.

4.1.1. (±)-(4a*R*,7*R*,8a*S*)-2-Phenyl-4a,7,8,8a-tetrahydro-4*H*-1,3-benzodioxin-7-yl acetate (*rac*-7). To a mixture of *rac*-**5** (0.72 g, 3.1 mmol) in pyridine (10 mL), acetyl chloride (0.35 mL, 4.9 mmol) was added at 0 °C and the mixture was stirred at room temperature for 6 h. The reaction was poured into saturated aqueous $NaHCO_3$ and stirred for half-an-hour. The mixture was extracted with CH_2Cl_2 and the organic layer was separated, dried over

anhydrous Na_2SO_4 and co-evaporated with toluene in vacuo. The crude was purified by column chromatography (EtOAc/*n*-hexane, 0–30%) to afford 0.78 g of *rac*-**7** (yield 94%).

^{1}H NMR ($CDCl_3$) δ 1.88–1.99 (m, 1H, H-8), 2.09 (s, 3H, $-CH_3$), 2.48–2.69 (m, 2H, H-8, H-4a), 3.59–3.80 (m, 2H, 4-CH_2O-), 4.28 (dd, 1H, J=10.8, 4.6 Hz, H-7), 5.50–5.72 (m, 3H, H-8a, H-5, H-6), 5.62 (s, 1H, 2-CHPh), 7.27–7.51 (m, 5H, aromatic-H) ppm; ^{13}C NMR ($CDCl_3$) δ 20.8 ($-CH_3$), 33.9 (C-8), 39.5 (C-4a), 69.5 (C-7), 70.1 (4-CH_2O-), 76.1 (C-8a), 101.8 (2-CHPh), 125.9, 126.7, 128.1, 128.4, 128.7, 137.8 (aromatic-C) 170.3 ($-OCO-$) ppm. LISMS (CH_3OH/H_2O) 275.1 $(M+H)^+$.

4.1.2. (±)-(4a*R*,7*R*,8a*S*)-2-Phenyl-4a,7,8,8a-tetrahydro-4*H*-1,3-benzodioxin-7-yl propionate (rac-6). *rac*-**5** (0.72 g, 3.1 mmol) in pyridine (10 mL) was reacted with propionyl chloride (0.39 mL, 4.5 mmol) using the previous procedure to afford 0.78 g (yield 90%) of *rac*-**6** as a colorless oil.

^{1}H NMR ($CDCl_3$) δ 1.11 (t, 3H, $-CH_2CH_3$), 1.82–1.94 (m, 1H, H-8), 2.28 (q, 2H, $-CH_2CH_3$), 2.21–2.47 (m, 2H, H-8, H-4a), 3.59–3.92 (m, 2H, 4-CH_2O-), 4.54 (dd, 1H, J=10.7, 4.2 Hz, H-7), 5.53–5.61 (m, 3H, H-8a, H-5, H-6), 5.59 (s, 1H, 2-CHPh), 7.39–7.51 (m, 5H, aromatic-H) ppm; ^{13}C NMR ($CDCl_3$) δ 9.04 ($-CH_2CH_3$), 27.6 ($-CH_2CH_3$), 32.4 (C-8), 39.8 (C-4a), 68.0 (C-7), 70.1 (4-CH_2O-), 76.1 (C-8a), 102.1 (2-CHPh), 125.9, 126.7, 128.1, 128.4, 128.7, 138.3 (aromatic-C), 170.4 ($-OCO-$) ppm. LISMS (CH_3OH/H_2O) 289.1 $(M+H)^+$.

4.1.3. (±)-(4a*R*,7*R*,8a*S*)-2-Phenyl-4a,7,8,8a-tetrahydro-4*H*-1,3-benzodioxin-7-yl-butyrate (rac-8). *rac*-**5** (0.70 g, 3.0 mmol) and 30 mg of DMAP in pyridine (10 mL) reacted with butyric anhydride (0.74 mL, 4.5 mmol) using above procedure to afford 0.83 g (yield 91%) of *rac*-**8** as a colorless oil.

^{1}H NMR ($CDCl_3$) δ 0.93 (t, 3H, $-CH_2CH_2CH_3$), 1.61 (m, 2H, $-CH_2CH_2CH_3$), 1.80–1.95 (m, 1H, H-8), 2.22–2.47 (m, 4H, H-8, $-CH_2CH_2CH_3$, H-4a), 3.80–3.95 (m, 2H, 4-CH_2O-), 4.54 (m, 1H, H-7), 5.53–5.61 (m, 3H, H-8a, H-5, H-6), 5.59 (s, 1H, 2-CHPh), 7.39–7.53 (m, 5H, aromatic-H) ppm; ^{13}C NMR ($CDCl_3$) δ 10.5 ($-CH_2CH_2CH_3$), 18.4 ($-CH_2CH_2CH_3$), 32.4 (C-8), 36.1 ($-CH_2CH_2CH_3$), 41.4 (C-4a), 67.9 (C-7), 69.1 (4-CH_2O-), 75.8 (C-8a), 102.7 (2-CHPh), 125.8, 126.7, 127.6, 128.6, 131.7, 138.8 (aromatic-C) 172.4 ($-OCO-$) ppm. LISMS (CH_3OH/H_2O) 302.2 $(M+H)^+$.

4.2. Resolution of *rac*-7

With PLE. The ester *rac*-**7** (200 mg, 0.73 mmol) dissolved in (a) *t*-BuOH (4 mL) or (b) acetone (4 mL) was added to the phosphate buffer solution (36 mL, pH 8.0, adjusted by 1 N HCl). Subsequently, PLE (0.1 mL, 130U) was added and the mixture was efficiently stirred at room temperature while the pH value was held constant at 8.0 by the addition of 1 M NaOH solution with a pH-stat autotitrator. The reaction was stopped after 22 h (11% conversion) and 24 h (44% conversion), respectively, and the mixture was extracted continuously with CH_2Cl_2 (50 mL) for 17 h (Soxlet apparatus). The organic phase was collected, dried over anhydrous Na_2SO_4 and concentrated in vacuo. Chiral HPLC analysis of a sample of the residue showed the presence of **5a** *ent*-product with 30% e.e. and of **7b** *ent*-substrate with <1% e.e. for (a); and of **5a** *ent*-product with 46% e.e. and of **7b** *ent*-substrate with 27% e.e. for (b).

With CRL. The hydrolysis was carried out with *rac*-**7** (200 mg, 0.73 mmol), *t*-BuOH (4 mL) and CRL (5 mg, 130U) in aqueous phosphate buffer solution (36 mL, pH 8.0, adjusted by 1 N HCl). Work-up was done as described above after 24 h (17% conversion). Chiral HPLC analysis of a sample of the residue showed the presence of **5b** *ent*-product with 65% e.e. and of **7a** *ent*-substrate with 26% e.e.

With CAL-B. The hydrolysis was carried out with *rac*-**7** (200 mg, 0.73 mmol), *t*-BuOH (4 mL) and CAL-B (20 mg, 2.4 KU) in phosphate buffer solution (36 mL, pH 7.5, adjusted by 1 N HCl). Work-up as described above after 25 h (21% conversion). Chiral HPLC analysis of a sample of the residue showed the presence of **5a** *ent*-product with 16% e.e. and of **7b** *ent*-substrate with 10% e.e.

4.3. Resolution of *rac*-6

With PLE. The hydrolysis was carried out with *rac*-**6** (200 mg, 0.69 mmol), *t*-BuOH (4 mL) and PLE (0.1 mL, 130U) in phosphate buffer solution (36 mL, pH 7.5, adjusted by 1 N HCl). Work-up after 17 h (35% conversion). Chiral HPLC analysis of a sample of the residue showed the presence of **5a** *ent*-product with 68% e.e. and of **6b** *ent*-substrate with 40% e.e.

With CRL. The hydrolysis was carried out with *rac*-**6** (200 mg, 0.69 mmol), *t*-BuOH (4 mL) and CRL (5 mg, 130U) in aqueous phosphate buffer solution (36 mL, pH 7.5, adjusted by 1 N HCl). Work-up after 24 h (85% conversion). Chiral HPLC analysis of a sample of the residue showed the presence of **5b** *ent*-product with 2% e.e. and of **6a** *ent*-substrate with 30% e.e.

4.4. Resolution of *rac*-8

With PLE. The hydrolysis was carried out with *rac*-**8** (200 mg, 0.66 mmol), *t*-BuOH (4 mL) and PLE (0.1 mL, 130U) in aqueous phosphate buffer solution (36 mL, pH 7.5, adjusted by 1 N HCl). Work-up after 10 h (13% conversion). Chiral HPLC analysis of a sample of the residue showed the presence of **5a** *ent*-product with 95% e.e. and of **8b** *ent*-substrate with 9% e.e.

With CRL. The hydrolysis was carried out with *rac*-**8** (200 mg, 0.66 mmol), *t*-BuOH (4 mL) and CRL (5 mg, 130U) in aqueous phosphate buffer solution (36 mL, pH 7.5, adjusted by 1 N HCl). Work-up after 4 h (27% conversion). Chiral HPLC analysis of a sample of the residue showed the presence of **5b** *ent*-product with 11% e.e. and of **8a** *ent*-substrate with 24% e.e.

With CAL-B. The hydrolysis was carried out with *rac*-**8** (200 mg, 0.66 mmol), *t*-BuOH (4 mL) and CAL-B (20 mg, 2.4 KU) in aqueous phosphate buffer solution (36 mL, pH

7.5, adjusted by 1 N HCl). Work-up after 30 h (21% conversion). Chiral HPLC analysis of a sample of the residue showed the presence of **5a** *ent*-product with 63% e.e. and of **8b** *ent*-substrate with 15% e.e.

4.5. Isolation of enantiomerically pure cyclohexene precursor

The precursor *rac*-**5** (14.0 g, 60.2 mmol) and isopropenyl acetate (32.8 mL, 301 mmol) were dissolved in dichloromethane (700 mL). Subsequently, Novozyme® 435 (2 g, 20 KU) was added and the reaction mixture was stirred at room temperature. The reaction was stopped at 22 h (52% conversion) by filtration. The filtrate was concentrated in vacuo and submitted to column chromatography (EtOAc/*n*-hexane, 0–30%). The first portion of eluent was concentrated to afford a white solid which was twice recrystallized from 20% EtOAc in *n*-hexane giving a white needle crystal of **6b** *ent*-product (8.07 g, 98% e.e.). Following treatment with 50 mL of a saturated ammonia/methanol solution at room temperature for 14 h, the reaction mixture was concentrated and co-evaporated with methanol to afford a pale-yellow oil which was purified by column chromatography (EtOAc/*n*-hexane, 50:50, R_f=0.5). A white solid was obtained and recrystallized twice from 50% EtOAc in *n*-hexane twice affording a white needle crystal of **5b** (6.1 g, yield 89%) with enantiomeric excess (e.e.) >99% (overall yield 43% starting from *rac*-**5**). The second fraction from the first chromatographic purification was concentrated and thereafter crystallized twice from 50% EtOAc in *n*-hexane to afford a white needle crystal of **5a** (6.2 g, yield 44%) with enantiomeric excess (e.e.) >99%.

4.6. Synthesis of cyclohexenyl nucleoside building blocks

For all reactions, analytical grade solvents were used. All moisture-sensitive reactions were carried out in oven-dried glassware (100 °C) under a nitrogen atmosphere.

Compound **9a**,**b** were synthesized using the procedure for preparation of the racemic isomer as described in literature.[7] Compound **10a**,**b** were prepared using the synthetic method for racemic Cycl-G.[11] The other isomers **18a**,**b**, **19a**,**b** and **15a**,**b** were obtained analogously to the preparation of their racemic isomers.[20]

4.6.1. N^6-Benzoyl-9-[(1′*S*,4′*R*,5′*S*)-5′-hydroxy-4′-(monomethoxytrityl)oxymethyl-2′-cyclo-hexenyl]adenine (13a). To a solution of **9a** (590 mg, 1.71 mmol) in pyridine (10 mL) at 0 °C was added benzoyl chloride (0.52 mL, 5.13 mmol) and kept at room temperature overnight. The reaction mixture was cooled to 0 °C, and saturated aqueous $NaHCO_3$ (5 mL) was added and the mixture was extracted with CH_2Cl_2 (3×50 mL). The combined organic layer was washed with H_2O (20 mL), concentrated, and co-evaporation with toluene. The residue was treated with saturated ammonia/methanol solution (25 mL) for 5 min. Following evaporation of the solvent and co-evaporation with methanol, the residue was further treated with 80% CF_3COOH in water for 40 h. The reaction mixture was concentrated and co-evaporated with toluene and methanol three times. The crude was purified by silica gel column chromatography (CH_3OH/CH_2Cl_2, 0–10%, R_f=0.3) to give **11a** (430 mg, 1.18 mmol, yield 70%). To a solution of the obtained **11a** (co-evaporated three times with freshly dried pyridine) in dry pyridine (5 mL) at 0 °C under nitrogen was added monomethoxytrityl chloride (436 mg, 1.41 mmol) in portions. After the mixture was stirred at 0 °C for 1 h, the temperature was raised to room temperature. The reaction mixture was treated with CH_3OH (5 mL) at 0 °C after 22 h reaction. After the mixture was stirred at room temperature for 0.5 h, the resulting mixture was concentrated. The residue was co-evaporated with toluene and methanol, and chromatographed on silica gel (CH_3OH/CH_2Cl_2, 0–2%, Et_3N 1%) to give **13a** (470 mg, 0.74 mmol, 63% yield, overall yield of 43%) as a white foam.

^{1}H NMR ($CDCl_3$) δ 2.03–2.40 (m, 2H, H-6′, 6″), 2.55 (m, 1H, H-4′), 3.04 (br s, 1H, 5′-OH), 3.26 (t, 1H, *J*=8.6 Hz, $-OCH_2-$), 3.57 (dd, 1H, *J*=4.4, 9.2 Hz, $-OCH_2-$), 3.81 (br s, 4H, $-OCH_3$, H-5′), 5.47 (m, 1H, H-1′), 5.93 (br s, 2H, H-2′, H-3′), 6.87 (d, 2H, *J*=9.2 Hz, aromatic H), 7.26–7.61 (m, 15H, aromatic H), 7.91 (s, 1H, H-8), 8.05 (d, 2H, aromatic H), 8.81 (s, 1H, H-2), 9.12 (br s, 1H, 6-N*H*) ppm; ^{13}C NMR ($CDCl_3$) δ 35.7 (C-6′), 44.4 (C-4′), 49.6 (C-1′), 55.2 ($-OCH_3$), 65.9 ($-OCH_2-$), 66.7 (C-5′), 87.4 ($-OC^{TrMM}$), 123.9 (C-2′), 113.3, 127.3, 128.0, 128.1, 128.3, 128.9, 130.3, 132.7, 135.0, 143.9, 158.9, (aromatic C), 133.9 (C-3′), 141.9 (C-8), 149.6 (C-2), 151.7 (C-6), 152.6 (C-4), 164.8 ($-NHC{=}O$) ppm. HRMS calcd for $C_{39}H_{36}N_5O_4$ $(M+H)^+$: 638.2767, found 638.2770.

4.6.2. N^6-Benzoyl-9-[(1′*R*,4′*S*,5′*R*)-5′-hydroxy-4′-(monomethoxytrityl)oxymethyl-2′-cyclohexenyl]adenine (13b). Starting from 770 mg (2.2 mmol) of **9b**, an amount of 618 mg (0.96 mmol, overall yield of 44%) of **13b** was obtained. Spectroscopic data are the same as for **13a**.

4.6.3. N^2-Isobutyryl-9-[(1′*S*,4′*R*,5′*S*)-5′-hydroxy-4′-(monomethoxytrityl)oxymethyl-2′-cyclohexenyl]guanine (14a). To a solution of **10a** (460 mg, 1.66 mmol) in dry pyridine (12 mL) at 0 °C under nitrogen was added dropwise trimethylsilyl chloride (1.06 mL, 8.29 mmol). After the mixture was stirred for 1 h, isobutyric anhydride (0.83 mL, 4.98 mmol) was added slowly. The mixture was stirred at 0 °C for 1 h, warmed to room temperature and kept stirring for an additional 14 h. The reaction mixture was then cooled in an ice-water bath and quenched with water (12 mL). The resulting mixture was stirred at room temperature for 15 min and concentrated. The residue was purified by column chromatography (CH_3OH/CH_2Cl_2, 5–20%, R_f=0.4) to afford **12a** (730 mg, 2.10 mmol, yield 63%). Following the monomethoxytritylation procedure as for preparation of **13a**, using 972 mg (3.15 mmol) of monomethoxytrityl chloride in 15 mL pyridine, **14a** was obtained (380 mg, 0.61 mmol, 29% yield, overall yield of 37%) as a pale yellow foam.

^{1}H NMR ($CDCl_3$) δ 1.20 (dd, 6H, *J*=4.9, 6.8 Hz, $-CH(CH_3)_2$), 2.01–2.25 (m, 2H, H-6′, 6″), 2.50 (m, 2H, H-4′, 2-N*H*CO–), 3.21 (m, 2H, $-OCH_2-$, 5′-O*H*), 3.50 (dd, 1H, *J*=4.6, 9.3 Hz, $-OCH_2-$), 3.80 (s, 3H, $-OCH_3$), 3.90 (m, 1H, H-5′), 5.07 (m, 1H, H-1′), 5.85 (br s, 2H, H-2′, H-3′), 6.85 (d, 2H, *J*=8.8 Hz, aromatic H), 7.23–7.46 (m, 12H, aromatic H), 7.51 (s, 1H, H-8), 8.47 (br s, 1H, 1-N*H*) ppm; ^{13}C NMR ($CDCl_3$) δ 18.8 ($-CH(CH_3)_2$), 35.7 (C-6′), 36.3

Table 4. Analytical data for the phosphoramidites

	mmol of starting material	Yield (%)	R_f	HRMS $(M+H)^+$		^{31}P NMR
			n-Hexane/acetone/TEA (49:49:2)	Calcd	Found	
1a	0.72	86	0.46	838.3846 For $C_{48}H_{53}N_7O_5P$	838.3859	148.357, 148.393
1b	0.90	92	0.46	838.3846 For $C_{48}H_{53}N_7O_5P$	838.3840	148.308, 148.345
2a	0.57	66	0.38	820.3951 For $C_{45}H_{55}N_7O_6P$	820.3944	146.115, 147.142
2b	0.62	74	0.38	820.3951 For $C_{45}H_{55}N_7O_6P$	820.3953	146.085, 147.148
3a	0.40	87	0.51	814.3733 For $C_{47}H_{53}N_5O_6P$	814.3759	148.314, 148.465
3b	0.73	77	0.51	814.3733 For $C_{47}H_{53}N_5O_6P$	814.3732	148.314, 148.459
4a	0.77	85	0.51	725.3467 For $C_{41}H_{50}N_4O_6P$	725.3472	147.632, 148.127
4b	1.01	81	0.51	725.3467 For $C_{41}H_{50}N_4O_6P$	725.3455	147.644, 148.163

(−*C*H(CH$_3$)$_2$), 44.3 (C-4′), 49.4 (C-1′), 55.2 (−O*C*H$_3$), 65.6 (−O*C*H$_2$−), 66.4 (C-5′), 87.2 (−O*C*^{TrMM}), 121.6 (C-5), 124.3 (C-2′), 113.3, 127.2, 128.1, 128.3, 130.3, 135.1, 144.1, 158.9 (aromatic C), 133.4 (C-3′), 138.0 (C-8), 147.2 (C-2), 147.8 (C-4), 155.8 (C-6), 178.6 (−NH*C*=O) ppm. HRMS calcd for $C_{36}H_{38}N_5O_5$ $(M+H)^+$: 620.2872, found 620.2869.

4.6.4. N^2-Isobutyryl-9-[(1′*R*,4′*S*,5′*R*)-5′-hydroxy-4′-(monomethoxytrityl)oxymethyl-2′-cyclohexenyl]guanine (14b). Starting from 600 mg (2.16 mmol) of **10b**, an amount of 420 mg (0.68 mmol, overall yield of 31%) of **14b** was obtained. Spectroscopic data are the same as for **14a**.

4.6.5. 1-[(1′*S*,4′*R*,5′*S*)-5′-Hydroxy-4′-(monomethoxytrityl)oxymethyl-2′-cyclohexenyl]-thymine (17a). 15a (750 mg, 2.21 mmol) was treated with 80% CF_3COOH in H_2O for 40 h. After evaporation and co-evaporation with toluene and methanol, the residue was purified by column chromatography (CH_3OH/CH_2Cl_2, 0–10%, R_f=0.25) to give **16a** (334 mg, 1.32 mmol, yield 60%). Following the procedure used for preparation of **13a**, using 778 mg (2.52 mmol) of monomethoxytrityl chloride in 15 mL pyridine, **17a** was obtained (410 mg, 0.78 mmol, 59% yield, overall yield of 35%) as a white foam.

^{1}H NMR ($CDCl_3$) δ 1.75 (s, 3H, −C*H*$_3$), 1.95–2.00 (m, 2H, H-6′, 6″), 2.43 (m, 1H, H-4′), 2.73 (d, 1H, 5′-OH), 3.19 (m, 1H, −OC*H*$_2$−), 3.51 (dd, 1H, *J*=4.4, 9.5 Hz, −OC*H*$_2$−), 3.80 (br s, 4H, −OC*H*$_3$, H-5′), 5.26 (m, 1H, H-1′), 5.62 (m, 1H, H-2′), 5.91 (m, 1H, H-3′), 6.86 (d, 2H, *J*=8.8 Hz, aromatic H), 6.97 (s, 1H, H-6), 7.22–7.46 (m, 12H, aromatic H), 8.41 (br s, 1H, 3-N*H*) ppm; ^{13}C NMR ($CDCl_3$) δ 12.3 (−*C*H$_3$), 35.1 (C-6′), 44.0 (C-4′), 50.5 (C-1′), 55.2 (−O*C*H$_3$), 65.4 (−O*C*H$_2$−), 66.3 (C-5′), 87.3 (−O*C*^{TrMM}), 110.1 (C-5), 124.9 (C-2′), 113.3, 127.3, 128.1, 128.3, 130.3, 135.1, 144.1, 158.8 (aromatic C), 134.4 (C-3′), 137.4 (C-6), 150.7 (C-2), 163.6 (C-4) ppm. HRMS calcd for $C_{32}H_{32}N_2O_5Na$ $(M+Na)^+$: 547.2208, found 547.2212.

4.6.6. 1-[(1′*R*,4′*S*,5′*R*)-5′-Hydroxy-4′-(monomethoxytrityl)oxymethyl-2′-cyclohexenyl]-thymine (17b). Starting from 875 mg (2.57 mmol) of **15b**, an amount of 540 mg (1.03 mmol, overall yield 40%) of **17b** was obtained. Spectroscopic data are the same as for **17a**.

4.6.7. N^4-Benzoyl-1-[(1′*S*,4′*R*,5′*S*)-5′-hydroxy-4′-(monomethoxytrityl)oxymethyl-2′-cyclohexenyl]cytosine (21a). To a solution of **19a** (660 g, 2.04 mmol) in pyridine (10 mL) at 0 °C was added benzoyl chloride (0.72 mL, 6.12 mmol) and the mixture was kept at room temperature overnight. The reaction mixture was cooled to 0 °C, and saturated aqueous $NaHCO_3$ (5 mL) was added and extracted with CH_2Cl_2 (3×50 mL). The combined organic layer was washed with H_2O (15 mL), concentrated, and co-evaporated with toluene. The residue was treated with saturated ammonia/methanol solution (25 mL) for 5 min. Following evaporation of the solvent and co-evaporation with methanol, the residue was further treated with 80% CF_3COOH in H_2O for 40 h. The reaction mixture was concentrated and co-evaporated with toluene and methanol three times. The crude was purified by silica gel column chromatography (CH_3OH/CH_2Cl_2, 0–10%, R_f=0.45) to give **20a** (560 mg, 1.64 mmol, yield 76%). Tritylation according to the preparation of **13a**, using 608 mg (6.30 mmol) of monomethoxytrityl chloride in 15 mL pyridine, afforded **21a** (320 mg, 0.52 mmol, 32% yield, overall yield of 26%) as a white foam.

^{1}H NMR ($CDCl_3$) δ 1.98–2.11 (m, 2H, H-6′, 6″), 2.29 (m, 1H, H-4′), 2.36 (br, 1H, 5′-OH), 3.10 (m, 1H, −OCH_2−), 3.73 (m, 1H, −OCH_2−), 3.79 (s, 3H, −OCH_3), 4.14 (m, 1H, H-5′), 5.41 (m, 1H, H-1′), 5.68 (m, 1H, H-2′), 6.05 (m, 1H, H-3′), 6.83 (d, 2H, *J*=8.8 Hz, aromatic H), 7.20–7.52 (m, 18H, aromatic H, H-5), 7.98 (d, 1H, H-6), 8.65 (br s, 1H, 4-NH) ppm; ^{13}C NMR ($CDCl_3$) δ 35.5 (C-6′), 44.8 (C-4′), 53.3 (C-1′), 55.2 (−O*C*H$_3$), 63.3 (−O*C*H$_2$−), 64.0 (C-5′), 87.0 (−O*C*^{TrMM}), 95.8 (C-5), 123.6 (C-2′), 113.3, 127.2, 128.1, 128.8, 130.2, 135.9, 138.0, 144.1, 158.7 (aromatic C), 132.8 (C-3′), 147.1 (C-6), 156.0 (C-2), 162.1 (C-4), 167.5 (−NH*C*=O) ppm. HRMS calcd for $C_{38}H_{36}N_3O_5$ $(M+H)^+$: 614.2654, found 614.2647.

4.6.8. N^4-Benzoyl-1-[(1′*R*,4′*S*,5′*R*)-5′-hydroxy-4′-(monomethoxytrityl)oxymethyl-2′-cyclohexenyl]cytosine (21b). Starting from 920 mg (2.84 mmol) of **19b**, an amount of

540 mg (1.58 mmol, overall yield of 31%) of **21b** was obtained. Spectroscopic data are the same as for **21a**.

4.7. Synthesis of the amidite building blocks (1a, 1b, 2a, 2b, 3a, 3b, 4a and 4b)

4.7.1. General procedure for phosphoramidite synthesis. The phosphitylation reaction was carried out on 0.4–1 mmol of the monomethoxytritylated derivative (**13a**,**b**, **14a**,**b**, **17a**,**b** and **21a**,**b**, respectively) in 5–10 mL dichloromethane using freshly distilled diisopropylethylamine (3 equiv.) and 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (1.5 equiv.) under argon. The reaction mixture was stirred at room temperature for 60 min when TLC indicated complete reaction. Water (3 mL) was added, the solution was stirred for 10 min and partitioned between CH_2Cl_2 (50 mL) and aqueous $NaHCO_3$ (30 mL). The organic phase was washed with aqueous NaCl (3×30 mL) and the aqueous phases were back extracted with CH_2Cl_2 (30 mL). Evaporation of the organics left an oil which was flash purified on 45 g of silica gel (hexane/acetone/TEA, 49:49:2) to afford the product as a foam after co-evaporation with dichloromethane. Dissolution in 3 mL of dichloromethane and double precipitation in 160 mL cold (−60 °C) hexane afforded the desired product as a white powder. The obtained material was dried in vacuo and stored under nitrogen at −20 °C until use for oligonucleotide synthesis.

Yields, starting quantity, R_f values, mass analysis and ^{31}P NMR data are given in Table 4.

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