Synthesis of Metal-Chelating Deoxycytidine-Analogue Phosphoramidites for the Automatic Synthesis of Labelled Oligonucleotides

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Abstract: The new deoxycytidine (dC) analogue phosphoramidites **1**–**3** were synthesized, which bear an ethylenediaminetetraacetic acid triethyl ester [EDTA(OEt)$_3$] attached to the exocyclic amino group through either a cyclic-C$_6$, a C$_2$, or a linear-C$_6$ tether. By this way, the chelating base dC-EDTA can be inserted, by automatic synthesis, in a site-specific manner into oligodeoxynucleotides (ODNs), in place of any dC along the sequence, and can be thus exploited, for example, for footprinting experiments. Furthermore, the analogue phosphoramidite **4**, bearing a diethylenetriaminepentaacetic acid tetraethyl ester [DTPA(OEt)$_4$] attached through the cyclic-C$_6$ tether was also prepared. The possibility of insertion of **1**–**4** into ODNs was verified.

Key words: DNA, chelates, nucleosides, oligonucleotides, footprinting

Introduction

It is well known that metal-chelating agents are very useful for the resolution of basic problems in chemistry, biology and medicine.[1–4] The simple chelator ethylenediaminecarboxylic acid and its analogues, which form stable chelates with about half of the known elements,[5,6] permit several applications in the study of biological macromolecules. In particular, the EDTA-Fe(II) chelate, in combination with hydrogen peroxide and ascorbate, produces hydroxyl radicals, which cleave the backbone of DNA with almost no sequence dependence.[7–12] Furthermore, the insertion of the chelating group into an ODN should allow its application not only in footprinting experiments but also in NMR methodology,[13] aimed at the study of interactions between nucleic acids and between nucleic acids and proteins. Therefore, we became interested in developing a convenient synthetic route allowing the insertion of the metal-chelator EDTA into an ODN. The synthesis of a DNA hybridization probe, containing an EDTA-derivatized deoxythymidine (dT-EDTA) according to the phosphoramidite chemistry, has already been reported.[14] Another method consists in the automatic synthesis of an ODN bearing a free aminoalkyl group, followed by its acylation with activated EDTA. For this purpose, a bis-anhydride, an *N*-hydroxysuccinimidic ester or a mixed anhydride of EDTA can be used. However, the latter method often leads to a mixture of different products due to cross-linking reactions, such as polymers with high molecular mass. As an approach for overcoming these problems, and with the aim of providing an EDTA-derivatized dC (dC-EDTA) alternative to the dT-EDTA,[14] we have now synthesized the dC-analogue phosphoramidites **1**–**3** (Figure 1), which have the EDTA triethyl ester attached to the exocyclic amino group through an aliphatic tether different in length and flexibility: an aminocyclohexane, an aminoethane and a linear aminohexane.

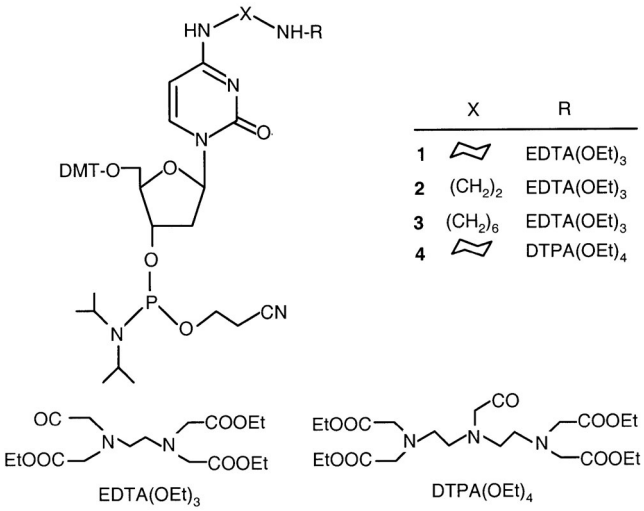

Figure 1 The new protected dC analogue phosphoramidites **1**–**4**

These products, used in combination with the above T derivative, provide the possibility of inserting a chelating agent along the sequence of nucleic acids by automatic synthesis, in a site- and type-specific manner. The three types of linkers offer the possibility of a regioselective reactivity discrimination in a rather close surrounding of the probe. To verify the possibility to extend this synthetic approach to other chelating agents, we synthesized also the phosphoramidite **4**, analogue of **1**, bearing the DTPA tetraethyl ester. The utility of all **1**–**4** for the synthesis of ODNs was tested.

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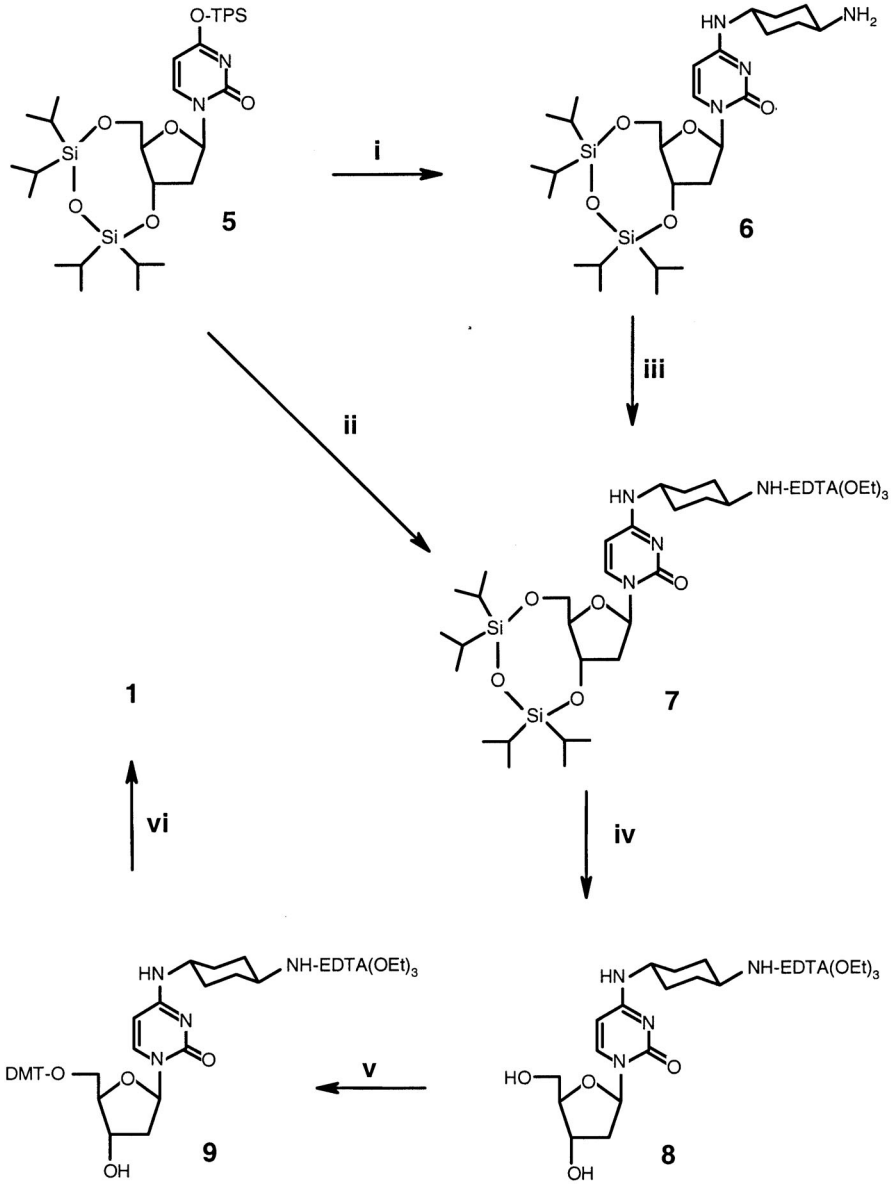
Preparation of the Phosphoramidites 1–4

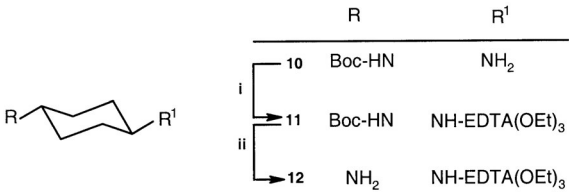
On a first attempt, we prepared the phosphoramidite **1** following a previously experienced strategy,[15] as shown in Scheme 1: the 4-*O*-[(2,4,6-triisopropylphenyl)sulfonyl]-3′,5′-*O*-(1,1,3,3,-tetraisopropyldisiloxane-1,3-diyl)-2′-deoxyuridine[15] (**5**) was reacted with *trans*-1,4-diaminocyclohexane in ethanolic solution at 60 °C for 1 hour to give **6**, which was acylated with $EDTA(OEt)_3$[16] by the mixed anhydride method [by using isobutyl chloroformate (*i*-BuCF) and *N*-methylmorpholine (NMM) for carboxy-activation], providing **7**. Alternatively, **7** was directly prepared by amination of the nucleoside **5** with **12** under the same conditions.

Preparation of **12** is outlined in Scheme 2: *trans*-1-amino-4-*tert*-butoxycarbonylaminocyclohexane[17] (**10**) was acylated with $EDTA(OEt)_3$ by the mixed anhydride method to give **11**. Removal of the amino-protecting group by treatment with trifluoroacetic acid afforded **12** as trifluoroacetate salt. Before using, the salt was decomposed with aqueous $NaHCO_3$ to give the free amine, which was extracted with ethyl acetate.

Quantitative removal of the 3′,5′-*O* protective group from **7** by a slight excess of tetrabutylammonium fluoride (TBAF) in tetrahydrofuran generated **8** which was 5′-*O*-dimethoxytritylated with 4,4′-dimethoxytrityl chloride (DMT-Cl) in pyridine providing **9**. This synthetic strategy was also tried for the synthesis of the $DTPA(OEt)_4$ bear-



Scheme 1 *Reagents and conditions*: i) 1,4-diaminocyclohexane, EtOH, 60 °C, 1 h, 94%; ii) **12**, EtOH, 60 °C, 1 h, 75%; iii) $EDTA(OEt)_3$, NMM, *i*-BuCF, THF, 0 °C, 18 h, 68%; iv) TBAF, THF, r.t., 18 h, 83%; v) DMT-Cl, Pyridine, r.t., 3 h, 72%; vi) *i*-Pr_2NEt, $NCCH_2CH_2OP(Cl)N(i\text{-}Pr_2)$, CH_2Cl_2, r.t., 30 min, 93%.

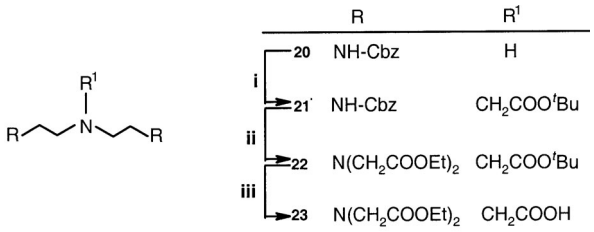


Scheme 2 *Reagents and conditions*: i) $EDTA(OEt)_3$, NMM, *i*-BuCF, THF, 0 °C, 18 h, 57%; ii) a: TFA, r.t., 30 min; b: $NaHCO_3$, EtOAc, r.t., 30 min, 81%.

ing analogue, but the 5′-*O*-dimethoxytritylation gave only traces of the desired compound, probably because of the steric hindrance of the bulky chelating group. An alternative procedure was then investigated (Scheme 3): reaction of 5′-*O*-(4,4′-dimethoxytrityl)-4-thio-2′-deoxyuridine[18] (**13**) with *trans*-1,4-diaminocyclohexane in ethanolic solution at 60 °C for 18 hours gave **14**, which was acylated with $EDTA(OEt)_3$ or $DTPA(OEt)_4$ by the mixed anhydride method giving **9** and **19**, respectively.

Any attempt to obtain **9** by reacting **13** and **12** failed, although a longer reaction time (18 h) and a higher temperature (90 °C) were employed than as for the amination conditions previously adopted. The latter strategy appeared the most convenient for the preparation of **9** and the only one useful for the preparation of **19**. We hence reacted **13** with 1,2-diaminoethane and 1,6-diaminohexane (Scheme 3) obtaining the nucleosides **15** and **16**, respectively. Acylation of these intermediates with $EDTA(OEt)_3$ according to the mixed anhydride procedure, gave **17** and **18**, respectively. Phosphitylation of **9**, **17**, **18** and **19** with 2-cyanoethyldiisopropylchlorophosphoramidite in the presence of *N*,*N*-diisopropylethylamine, followed by precipitation from hexane, afforded the corresponding phosphoramidites **1**–**4**.

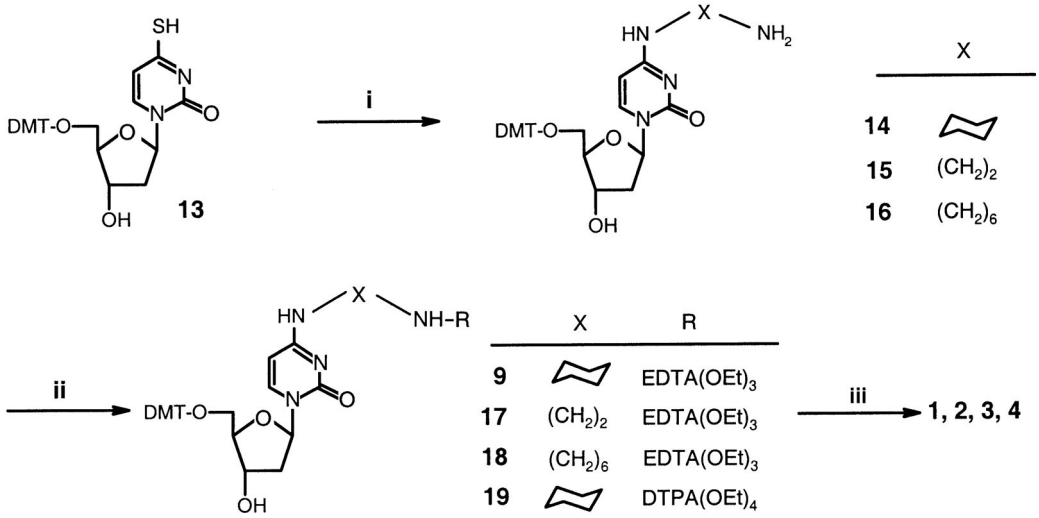
Since the preparation of the monoreactive $DTPA(OEt)_4$ (**23**) from DTPA pentaethyl ester,[7] by either enzymatic[19] or chemical[20] selective hydrolysis of one of the five ester groups, produces a mixture of symmetrical and unsymmetrical monoacids difficult to separate, we prepared **23** as follows (Scheme 4): alkylation of the unprotected secondary amine of $N^{1,5}$-(benzyloxycarbonyl)-1,5-diamino-3-azapentane[21] (**20**) with *tert*-butyl bromoacetate in acetonitrile, in the presence of *N*,*N*-diisopropylethylamine, gave **21**. After deprotection by catalytic hydrogenation on activated 10% Pd/C in formic acid (acidic condition are essential in this step to avoid the cyclization due to the internal nucleophilic displacement of *tert*-butyl ester by the newly formed primary amine), the terminal amino groups were exhaustively alkylated with ethyl bromoacetate providing **22**, by using the same conditions described for **21**. The *tert*-butyl ester was then hydrolyzed by treatment with trifluoroacetic acid giving **23**.



Scheme 4 *Reagent and conditions*: i) $BrCH_2COO$*t*-Bu, *i*-Pr_2NEt, MeCN, 0–18 °C, 18 h, 96%; ii) a: 10% Pd/C, 4% HCO_2H/MeOH, r.t., 3 h; b: $BrCH_2COOEt$, *i*-Pr_2NEt, MeCN, 0–18 °C, 18 h, 51%; iii) TFA, r.t., 3 h, 46%.

Preparation and Characterization of the ODNs

The sequence of the model dodecamers **A**–**F** (Table 1) was already used for a NMR study and comprises the consensus sequence for the specific DNA-binding sites of the glucocorticoid receptor protein.[22] The sequence of the model twentymers **G**–**M** (Table 1) was taken from the



Scheme 3 *Reagents and conditions*: i) H_2N-X-NH_2, EtOH, 60 °C, 18 h, 87–98%; ii) $EDTA(OEt)_3$ or $DTPA(OEt)_4$, NMM, *i*-BuCF, THF, 0 °C, 18 h, 60–76%; iii) *i*-Pr_2NEt, CH_2Cl_2, $NCCH_2CH_2OP(Cl)N(i\text{-}Pr_2)$, r.t., 30 min, 68–95%.

HIV-1 retro-transcribed proviral DNA and comprises the so-called polypurine tract.[23] Solutions ca. 0.06 M of normal, commercial phosphoramidites and of the phosphoramidites **1**–**4** in anhydrous MeCN were used for the preparation of the normal and mono-labelled ODNs. The solid-phase coupling reaction of the phosphoramidites of the labelled bases resulted in lower yields in comparison to those (96–98%) of the normal commercial phosphoramidites and we attributed this to the bulkiness of the molecule. In fact, the coupling yield of **1**–**3** in **C**–**E** and in **I**–**M** was about 50–60%, and the coupling yield of **4** in **F** was only about 10%. Furthermore, the coupling yields of **1**–**4** were not raised by the extension of the coupling time from 25 seconds to 12 minutes. However, in the subsequent couplings in **C**–**E** and **I**–**M**, the average yields were again in the range 96–98%, so that the synthesis of the ODNs labelled with **1**–**3** could be completed, allowing an acceptable overall yield and the recovery of material sufficient for any purpose. In the case of the coupling of **4** in **D**, the yield dropped so drastically that its use proved only possible either at the 5′ end, or at positions very close to it. The potential use of phosphoramidite **4** was not further investigated. All ODNs were characterized by HPLC and PAGE, and also by ESI-MS (Table 1).

Furthermore, since the aim of the research was to prepare ODNs carrying chelating groups, as molecular probes acting upon hybridization to a complementary single strand (ss) ODN, first we formed double strand (ds) hybrids made of the dodecameric **B** with **C**–**E**, and of **A** with **B** as reference; additionally we prepared the twentymeric ds hybrids made of **H** with **I**–**M** and of **G** with **H**. The formation of the ds hybrids was first confirmed by nondenaturing PAGE. Then we tried to measure their thermal melting point (Tm), by heating and monitoring the variation of absorbance at 260 nm, in order to assess the influence of the introduction of the chelating group on their formation and

Table 1 Synthetic Oligo-5′-deoxyribonucleotides-3′ **A**–**M**

ODN	Sequence[a]	Average Mass	Ion Species[b]
A	CCAGAACAGTGG	3658.18 3679.46	$[M - H]^{-4}$ (m/z = 913.58) $[M - H]^{-3}$ (m/z = 1218.44), $[(M + Na) - H]^{-4}$ (m/z = 919.03), $[(M + Na) - H]^{-3}$ (m/z = 1225.72), $[(M + 2\ Na) - H]^{-4}$ (m/z = 924.50), $[(M + 2\ Na) - H]^{-3}$ (m/z = 1233.00)
B	CCAGAACTGTTCTGG	3613.54 3612.40	$[M - H]^{-4}$ (m/z = 902.09), $[M - (H_2O) - H]^{-4}$ (m/z = 897.8)
C	CCAGAAC[1]AGTGG	4050.02	$[M - H]^{-5}$ (m/z = 808.92), $[M - H]^{-4}$ (m/z = 1011.49)
D	CCAGAAC[2]AGTGG	3977.53 3996.72	$[M - H]^{-5}$ (m/z = 794.50) $[(M - C_6H_{11}NO_4) - H]^{-5}$ (m/z = 762.14), $[(M - C_6H_{11}NO_4) - H]^{-4}$ (m/z = 952.92), $[(M - C_5H_9NO_4) - H]^{-5}$ (m/z = 764.75), $[(M - C_5H_9NO_4) - H]^{-4}$ (m/z = 956.90)
E	CCAGAAC[3]AGTGG	4052.04	$[M - H]^{-5}$ (m/z = 809.39), $[M - H]^{-4}$ (m/z = 1012.01)
F	[4]CCAGAACAGTGG	4151.21	nd[c]
G	AGTCCCCCCTTTTCTTTTAA	5966.00 5968.94	$[M - H]^{-6}$ (m/z = 993.32)
H	TTAAAAGAAAAGGGGGGACT	5933.96 6263.18	$[M - H]^{-5}$ (m/z = 1185.78)
I	AGTCCCCCC[1]TTTTCTTTTAA	6337.84 6340.29	$[(M - C_6H_{11}NO_4) - H]^{-7}$ (m/z = 881.49)
L	AGTCCCCCC[2]TTTTCTTTTAA	5978.08 6016.20	$[(M - C_{10}H_{15}N_2O_7) - H]^{-7}$ (m/z = 813.64), $[(M - C_8H_{14}N_2O_6) - H]^{-7}$ (m/z = 819.09)
M	AGTCCCCCC[3]TTTTCTTTTAA	6339.18 6342.31	$[(M - C_6H_{11}NO_4) - H]^{-7}$ (m/z = 881.64)

[a] $C^{1,2,3,4}$ indicate the type of modified nucleotide, with reference to the modified phosphoramidite (1, 2, 3, 4) used for the automatic synthesis at the labelled position.
[b] ESI-MS characterization of the ODNs as negative ions.
[c] nd = not determined.

stability. In fact, the Tm value was found to be 44 °C for the unmodified ds dodecameric **A**/**B** hybrid, while in all **B**/**C**–**E** hybrids one could observe an increasing hyperchromic effect in the thermal denaturation profile, but apparently no well-defined Tm. On the contrary, the Tm of the ds twentymeric hybrids proved measurable in all cases and was found to be 54 °C for the unmodified **G**/**H**, 45 °C for the modified **H**/**L** and 41 °C for both modified **H**/**I** and **H**/**M**.

Conclusion

In conclusion, we have developed a versatile method for the preparation of DNA models containing a dC nucleotide functionalized with a chelating group. By this method, in particular the EDTA chelator can be inserted along the sequence of an ODN by automatic synthesis, despite the lowering of the coupling yield occurring in coincidence with the insertion of the labelled nucleotide. The present derivatives should, in principle, be able to give Watson–Crick pairing, forming a ds helix with limited structural perturbations, depending on the hindrance of the modification, since the tethered chelator can protrude into the major groove. In practice, however, the formation of the ds hybrid necessary for the exploitation of such probes upon molecular recognition, seems to require that the probe be inserted into an ODN of adequate length and composition, so that a sufficient number of inter-strand interactions can occur and stabilize the whole system, compensating for local perturbations.

Melting points were determined on a Büchi B-540 apparatus and are uncorrected. UV and IR absorption spectra were performed with a Shimadzu UV-2101PC and a Perkin-Elmer 521 recording spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer. Optical rotations were determined with a Schmidt-Haensch 1604 polarimeter. Silica gel for column chromatography and TLC silica gel plates were from Merck AG (Darmstadt, Germany). Elemental analyses (where necessary a sample was further purified by preparative TLC) were performed by Servizio Microanalisi of CNR, Area della Ricerca di Roma, and were within ±0.4% of the theoretical values. Solvents were purified and dried according to standard laboratory procedures.[24] ESI-MS experiments were performed on an Applied Biosystems Sciex QSTAR Pulsar hybrid quadrupole time-of-flight mass spectrometer (Sciex, Canada), equipped with an ion spray source. The thermal denaturation profiles were evaluated on a Cary 3 UV/Vis spectrophotometer equipped with a Peltier device for temperature control. The samples were placed in 1 cm path length quartz cells and the absorbance increase was monitored from 5 to 95 °C.

N^4-(*trans*-4-Aminocyclohexyl)-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2′-deoxycytidine (6)

The protected nucleoside **5**[15] (818 mg, 1.11 mmol) and *trans*-1,4-diaminocyclohexane (634 mg, 5.55 mmol) in absolute EtOH (5.0 mL) were allowed to react for 1 h in a tightly stopped flask at 60 °C. After removal of the solvent, the residue was taken up in $CHCl_3$ (30 mL), washed with aq sat. $NaHCO_3$ (3 × 30 mL), then with H_2O until neutral. The organic layer was dried (Na_2SO_4) and evaporated to give **6** (586 mg, 94%) as a white foam, which was used without further purification; R_f = 0.32 (NH_3 sat. EtOAc–MeOH, 70:30); $[\alpha]_D^{20}$ +51 (*c* = 1, EtOH).

IR ($CHCl_3$): 3392, 2949, 2869, 2253, 1643, 1495, 1145 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 0.72–1.45 [m, 28 H, 4 × (C*H*$_3$)$_2$CH and 2 × $CH_{2cyclohexyl}$], 1.53–1.69 (m, 4 H, 2 × $CH_{2cyclohexyl}$), 1.80–2.80 [m, 8 H, 4 × (CH$_3$)$_2$C*H*, 2 × $CH_{cyclohexyl}$, H-2′ and H-2″], 3.60–3.81 (m, 2 H, H-5′ and H-5″), 3.94–4.11 (m, 1 H, H-4′), 4.18–4.55 (m, 1 H, H-3′), 5.55 (d, 1 H, *J* = 8.0 Hz, H-5), 6.04 (t, 1 H, *J* = 6.0 Hz, H-1′), 7.70 (d, 1 H, *J* = 8.0 Hz, H-6).

UV (EtOH): λ_{max} = 273 (10000), 240 nm (7300).

Anal. Calcd for $C_{27}H_{50}N_4O_5Si_2 \cdot H_2O$: C, 55.44; H, 8.96; N, 9.58. Found: C, 55.41; H, 9.01; N, 9.72.

N^4-[*trans*-4-(2-{[2-Bis(ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}acetylamino)cyclohexyl]-3′,5′-*O*-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)-2′-deoxycytidine (7)

Method A: Prepared from **5** (480 mg, 0.65 mmol) and **12** (307 mg, 0.65 mmol) in abs EtOH (3.0 mL) according to the procedure used for **6**.

Method B: To a solution of EDTA(OEt)$_3$ (203 mg, 0.54 mmol) and NMM (54 mg, 0.54 mmol) in anhyd THF (5.0 mL), cooled to –15 °C, was added *i*-BuCF (74 mg, 0.54 mmol) with stirring. After 20 min, a solution of **6** (317 mg, 0.54 mmol) in anhyd THF (3.0 mL) was added and the mixture was kept overnight at 4 °C. After removal of the solvent at reduced pressure, the residue was diluted with EtOAc (20 mL), washed with aq sat. $NaHCO_3$ (2 × 20 mL) and brine (15 mL), dried (Na_2SO_4) and evaporated.

Both methods, after flash chromatography (CH_2Cl_2–MeOH, 98:2), provided **7** as a white foam; Method A: 450 mg (75%); Method B: 343 mg (68%); R_f = 0.34 ($CHCl_3$–MeOH, 95:5); $[\alpha]_D^{20}$ +43 (*c* = 1, EtOH).

IR ($CHCl_3$): 3313, 2940, 2867, 1737, 1642, 1500, 1275, 1115 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 0.82–1.50 [m, 37 H, 4 × (C*H*$_3$)$_2$CH, 3 × OCH$_2$C*H*$_3$ and 2 × $CH_{2cyclohexyl}$], 1.60–1.75 (m, 4 H, 2 × $CH_{2cyclohexyl}$), 1.80–2.20 [m, 6 H, 4 × (CH$_3$)$_2$C*H*, H-2′ and H-2″], 2.22–2.52 (m, 2 H, 2 × $CH_{cyclohexyl}$), 2.70–2.81 (m, 4 H, NCH_2CH_2N), 3.26 (s, 2 H, NCH_2CON), 3.39 (s, 2 H, C*H*$_2$CO$_2$Et), 3.50 (s, 4 H, 2 × C*H*$_2$CO$_2$Et), 3.62–3.82 (m, 2 H, H-5′ and H-5″), 3.92–4.50 (m, 8 H, H-4′, 3 × OC*H*$_2$CH$_3$ and H-3′), 5.65 (d, 1 H, *J* = 8.0 Hz, H-5), 6.05 (br s, 1 H, H-1′), 7.70 (d, 1 H, *J* = 8.0 Hz, H-6).

UV (EtOH): λ_{max} 273 (14600), 235 nm (11100).

Anal. Calcd for $C_{43}H_{76}N_6O_{12}Si_2$: C, 55.82; H, 8.28; N, 9.08. Found: C, 55.96; H, 8.22; N, 9.32.

N^4-[*trans*-4-(2-{[2-Bis(ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino]}acetylamino)cyclohexyl]-2′-deoxycytidine (8)

To a solution of **7** (284 mg, 0.30 mmol) in THF (2.0 mL) was added TBAF (157 mg, 0.60 mmol) and the mixture was kept overnight at r.t. After removal of the solvent at reduced pressure, the residue was dissolved in H_2O (20 mL) and washed with Et_2O (2 × 15 mL). The aqueous phases were back extracted with $CHCl_3$ (3 × 20 mL) and the pooled organic layers were dried (Na_2SO_4), filtered and evaporated under reduced pressure. Product **8** was obtained as a white foam homogeneous on TLC (170 mg, 83%); R_f = 0.25 (EtOAc–MeOH, 80:20); $[\alpha]_D^{20}$ +23 (*c* = 1, EtOH).

IR ($CHCl_3$): 3315, 2934, 1737, 1643, 1501, 1276, 1113 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 0.81–1.15 (m, 13 H, 3 × OCH$_2$C*H*$_3$ and 2 × $CH_{2cyclohexyl}$), 1.68–2.34 (m, 8 H, 2 × $CH_{2cyclohexyl}$, 2 × $CH_{cyclohexyl}$, H-2′ and H-2″), 2.72–2.83 (m, 4 H, NCH_2CH_2N), 3.24 (s, 2 H, NCH_2CON), 3.40 (s, 2 H, C*H*$_2$CO$_2$Et), 3.48 (s, 4 H, 2 × C*H*$_2$CO$_2$Et), 3.75–3.92 (m, 3 H, H-4′, H-5′ and H-5″), 4.12 (q, 6 H, *J* = 7.5 Hz,

3 × OCH_2CH$_3$), 4.48 (br s, 1 H, H-3′), 5.75 (d, 1 H, *J* = 8.0 Hz, H-5), 6.15 (br s, 1 H, H-1′), 7.82 (d, 1 H, *J* = 8.0 Hz, H-6).

UV (EtOH): λ_{max} = 273 nm (12800).

Anal. Calcd for $C_{31}H_{50}N_6O_{11}$: C, 54.53; H, 7.38; N, 12.31. Found: C, 54.61; H, 7.42; N, 12.58.

trans-1-_tert_-Butoxycarbonylamino-4-(2-{[2-bis(ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}acetylamino)cyclohexane (11)

Prepared from **10**[17] (2.45 g, 2.65 mmol) and EDTA(OEt)$_3$ (997 mg, 2.65 mmol) according to the procedure used for **7** (Method B). After washings [in this preparation an additional washing with 1 M $KHSO_4$ (2 × 15 mL) was done] and evaporation of the solvent, **11** was crystallized from hexane and obtained as a white solid by filtration (870 mg, 57%); mp 105–108 °C; R_f = 0.46 ($CHCl_3$–MeOH, 95:5).

IR ($CHCl_3$): 3434, 3324, 2983, 1736, 1705, 1659, 1503, 1236, 1169 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 1.31 (t, 9 H, *J* = 7.5 Hz, 3 × OCH$_2$CH_3), 1.42 [s, 9 H, C(CH$_3$)$_3$], 1.80–2.17 (m, 10 H, 4 × CH$_{2cyclohexyl}$ and 2 × CH$_{cyclohexyl}$), 2.75–2.86 (m, 4 H, NCH$_2$CH$_2$N), 3.30 (s, 2 H, NCH$_2$CON), 3.38 (s, 2 H, CH_2CO$_2$Et), 3.42 (s, 4 H, 2 × CH_2CO$_2$Et), 4.16 (q, 6 H, *J* = 7.5 Hz, 3 × OCH_2CH$_3$).

Anal. Calcd for $C_{27}H_{48}N_4O_9$: C, 56.63; H, 8.45; N, 9.78. Found: C, 56.72; H, 8.34; N, 9.67.

trans-1-Amino-4-(2-{[2-bis(ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}acetylamino)cyclohexane (12)

To an ice-cooled solution of **11** (1.10 g, 1.9 mmol) in CH_2Cl_2 (8.0 mL) was added TFA (4.0 mL) and the mixture was allowed to warm to r.t. After the reaction was complete (TLC), the solvent was evaporated under reduced pressure. The residue was coevaporated with anhyd Et_2O (4 ×) to remove the excess of TFA, then triturated with Et_2O. The crude trifluoroacetate salt was obtained as a white solid (1.02 g, 91%). Decomposition of the salt with aq sat. $NaHCO_3$ (20 mL), followed by extraction with EtOAc (50 mL), afforded **12** as the free amine (colorless oil) which was immediately used in the next step (735 mg, 81%); mp 92–94 °C (TFA salt); R_f = 0.30 (*n*-BuOH–AcOH–H_2O, 60:20:20).

IR (TFA salt, KBr): 3448, 2989, 1734, 1658, 1420, 1204, 1134 cm^{-1}.

^{1}H NMR (free amine) ($CDCl_3$): δ = 1.21 (t, 9 H, *J* = 7.5 Hz, 3 × OCH$_2$CH_3), 1.67 (s, 2 H, NH$_2$), 1.85 (br s, 10 H, 4 × CH$_{2cyclohexyl}$ and 2 × CH$_{cyclohexyl}$), 2.72–2.83 (m, 4 H, NCH$_2$CH$_2$N), 3.21 (s, 2 H, NCH$_2$CON), 3.35 (s, 2 H, CH_2CO$_2$Et), 3.46 (s, 4 H, 2 × CH_2CO$_2$Et), 4.14 (q, 6 H, *J* = 7.5 Hz, 3 × OCH_2CH$_3$).

Anal. Calcd for $C_{24}H_{41}F_3N_4O_9 \cdot H_2O$: C, 47.68; H, 7.17; N, 9.27. Found: C, 47.56; H, 7.21; N, 9.33.

N^4-(_trans_-4-Aminocyclohexyl)-5′-_O_-(4,4′-dimethoxytrityl)-2′-deoxycytidine (14)

A solution of **13** (300 mg, 0.55 mmol) and 1,4-diaminocyclohexane (628 mg, 5.55 mmol) in abs EtOH (5.0 mL) was allowed to react for 18 h in a tightly stoppered flask at 60 °C. After the usual work up (see preparation of **6**), **14** was recovered as a white foam homogeneous on TLC and was used without further purification (302 mg, 87%); R_f = 0.22 (NH_3 sat. CH_2Cl_2–MeOH, 85:15); $[\alpha]_D^{20}$ +42 (*c* = 1, MeCN).

IR ($CHCl_3$): 3431, 2855, 1641, 1504, 1462, 1174 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 0.90–1.42 (m, 8 H, 4 × CH$_{2cyclohexyl}$), 1.60–2.19 (m, 2 H, H-2′ and H-2″), 2.31–2.72 (m, 2 H, 2 × CH$_{cyclohexyl}$), 3.25–3.49 (m, 2 H, H-5′ and H-5″), 3.72 (s, 6 H, 2 × PhOCH_3), 3.90–4.10 (m, 1 H, H-4′), 4.29–4.54 (m, 1 H, H-3′), 5.35 (br s, 1 H, H-5), 6.25 (t, 1 H, *J* = 6.0 Hz, H-1′), 6.61–6.88 and 7.02–7.51 (2 m, 13 H, Ar), 7.73 (d, 1 H, *J* = 8.0 Hz, H-6).

UV (EtOH): λ_{max} = 275 (10400), 234 nm (23800).

Anal. Calcd for $C_{36}H_{42}N_4O_6 \cdot 0.5H_2O$: C, 68.07; H, 6.74; N, 8.82. Found: C, 68.00; H, 6.83; N, 8.90.

N^4-(2-Aminoethyl)- 5′-_O_-(4,4′-dimethoxytrityl)-2′-deoxycytidine (15)

Prepared from **13**[18] (154 mg, 0.28 mmol) and 1,2-diaminoethane (85 mg, 1.41 mmol) according to the procedure used for **14**. The product was recovered as a white foam homogeneous on TLC and was used without further purification (157 mg, 98%); R_f = 0.25 (NH_3 sat. CH_2Cl_2–MeOH, 85:15); $[\alpha]_D^{20}$ +69 (*c* = 2, EtOH).

IR ($CHCl_3$): 3378, 2961, 1646, 1509, 1234, 1178 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 1.81–2.29 (m, 2 H, H-2′ and H-2″), 2.60–2.95 (m, 4 H, CH$_2$CH$_2$), 3.20–3.45 (m, 2 H, H-5′ and H-5″), 3.70 (s, 6 H, 2 × PhOCH_3), 3.91–4.08 (m, 1 H, H-4′), 4.30–4.52 (m, 1 H, H-3′), 5.40 (br s, 1 H, H-5), 6.25 (br s, 1 H, H-1′), 6.56–6.86 and 7.00–7.47 (2 m, 13 H, Ar), 7.73 (br s, 1 H, H-6).

UV (EtOH): λ_{max} = 275 (9800), 231 nm (19600).

Anal. Calcd for $C_{32}H_{36}N_4O_6$: C, 67.12; H, 6.34; N, 9.78. Found: C, 67.42; H, 6.26; N, 9.88.

N^4-(6-Aminohexyl)-5′-_O_-(4,4′-dimethoxytrityl)-2′-deoxycytidine (16)

Prepared from **13** (295 mg, 0.54 mmol) and 1,6-diaminohexane (308 mg, 2.70 mmol) according to the procedure used for **14**. The product (310 mg, 96%) was recovered as a white foam homogeneous on TLC (NH_3 sat. CH_2Cl_2–MeOH, 85:15) and was used without further purification. Compound **16** is described in the literature.[18]

5′-_O_-(4,4′-Dimethoxytrityl)-N^4-[_trans_-4-(2-{[2-bis(ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}acetylamino)cyclohexyl]-2′-deoxycytidine (9)

Method A: Nucleoside **8** (155 mg, 0.23 mmol), previously coevaporated with anhyd pyridine and dried overnight under high vacuum, was dissolved in anhyd pyridine (4 mL), then DMT-Cl (142 mg, 0.42 mmol) was added at r.t. After 3 h, the reaction was quenched with MeOH (0.1 mL) and the mixture was concentrated to a small volume. The residue was diluted with CH_2Cl_2 (25 mL) and washed with aq sat. $NaHCO_3$ (2 × 20 mL) and brine (20 mL). The organic extract was dried (Na_2SO_4), filtered, evaporated and finally coevaporated with toluene to remove the excess of pyridine.

Method B: Prepared from **14** (250 mg, 0.40 mmol) and EDTA(OEt)$_3$ (150 mg, 0.4 mmol) according to the procedure used for **7** *(Method B)*.

Both methods, after flash chromatography (EtOAc–MeOH–TEA, 90:9.5:0.5), provided **9** as a pale yellow foam (*Method A*: 163 mg, 72%; *Method B*: 279 mg, 71%); R_f = 0.51 (EtOAc–MeOH, 80:20); $[\alpha]_D^{20}$ +21 (*c* = 1, EtOH).

IR ($CHCl_3$): 3302, 2951, 1736, 1642, 1502, 1284, 1177 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 1.05–1.45 (m, 13 H, 3 × OCH$_2$CH_3 and 2 × CH$_{2cyclohexyl}$), 1.70–2.30 (m, 6 H, 2 × CH$_{2cyclohexyl}$, H-2′ and H-2″), 2.50 (br s, 1 H, 2 × CH$_{cyclohexyl}$), 2.70–2.81 (m, 4 H, NCH$_2$CH$_2$N), 3.22 (s, 2 H, NCH$_2$CON), 3.46 (s, 2 H, CH_2CO$_2$Et), 3.50 (s, 4 H, 2 × CH_2CO$_2$Et), 3.75 (s, 6 H, 2 × PhOCH_3), 4.12 [q, 9 H, *J* = 7.5 Hz, 3 × OCH_2CH$_3$, (obscured H-4′, H-5′ and H-5″)], 4.50 (m, 1 H, H-3′), 5.40 (d, 1 H, *J* = 8.0 Hz, H-5), 6.25 (t, 1 H, *J* = 6.0 Hz, H-1′), 6.64–6.90 and 7.10–7.48 (2 m, 13 H, Ar), 7.72 (d, 1 H, *J* = 8.0 Hz, H-6).

UV (EtOH): λ_{max} = 274 (14000), 234 (24700) nm.

Anal. Calcd for $C_{52}H_{68}N_6O_{13}\cdot0.5H_2O$: C, 62.82; H, 7.00; N, 8.45. Found: C, 62.96; H, 6.94; N, 8.68.

5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{4}-[2-(2-{[2-bis(ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}acetylamino)ethyl]-2′-deoxycytidine (17)

Prepared from **15** (300 mg, 0.52 mmol) and EDTA(OEt)$_3$ (197 mg, 0.52 mmol) according to the procedure used for **7** (*Method B*). After purification of the crude material on silica gel (CH_2Cl_2–MeOH–TEA, 98:1.5:0.5) the product was recovered as a pale yellow foam (290 mg, 60%); R_f = 0.28 (CH_2Cl_2–MeOH–TEA, 98:1.5:0.5); $[\alpha]_D^{20}$ +37.5 (*c* = 1, EtOH).

IR ($CHCl_3$): 3295, 3014, 1736, 1648, 1509, 1234, 1201 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 1.36 (t, 9 H, *J* = 7.5 Hz, 3 × OCH$_2$C*H*$_3$), 1.68–2.30 (m, 6 H, CH_2CH_2, H-2′ and H-2″), 2.75 (s, 4 H, $NCH_2CH_2N_{EDTA}$), 3.26 (s, 2 H, NCH_2CON), 3.36 (s, 2 H, C*H*$_2$CO$_2$Et), 3.48 (s, 4 H, 2 × C*H*$_2$CO$_2$Et), 3.76 (s, 6 H, 2 × PhOC*H*$_3$), 4.09 [q, 9 H, *J* = 7.5 Hz, 3 × OC*H*$_2$CH$_3$, (obscured H-4′, H-5′ and H-5″)], 4.60–4.88 (m, 1 H, H-3′), 5.46 (d, 1 H, *J* = 7.5 Hz, H-5), 6.30 (t, 1 H, *J* = 6.0 Hz, H-1′), 6.75–6.92 and 7.10–7.50 (2 m, 13 H, Ar), 7.70 (d, 1 H, *J* = 7.5 Hz, H-6).

UV (EtOH): λ_{max} = 275 (16500), 234 nm (35600).

Anal. Calcd for $C_{48}H_{62}N_6O_{13}\cdot0.5H_2O$: C, 61.33; H, 6.75; N, 8.94. Found: C, 61.58; H, 6.66; N, 9.05.

5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{4}-[6-(2-{[2-bis(ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}acetylamino)hexyl]-2′-deoxycytidine (18)

Prepared from **16**[18] (212 mg, 0.37 mmol) and EDTA(OEt)$_3$ (139 mg, 0.37 mmol) according to the procedure used for **7** (*Method B*). After purification of the crude material on silica gel (CH_2Cl_2–MeOH–TEA, 99:0.5:0.5) the product was obtained as a pale yellow foam (280 mg, 76%); R_f = 0.33 (CH_2Cl_2–MeOH, 90:10); $[\alpha]_D^{20}$ +27.5 (*c* = 1, EtOH).

IR ($CHCl_3$): 3324, 3011, 1736, 1646, 1509, 1250, cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 1.12–1.66 (m, 17 H, 3 × OCH$_2$C*H*$_3$ and 2–5 CH$_{2hexyl}$), 1.85–2.30 (m, 2 H, H-2′ and H-2″), 2.75 (s, 4 H, NCH_2CH_2N), 3.15–3.55 (m, 15 H, NCH_2CON, 3 × CH$_2$CO$_2$Et, H-4′, H-5′, H-5″ and 1,6 CH$_{2hexyl}$), 3.78 (s, 6 H, 2 × PhOC*H*$_3$), 4.12 (q, 6 H, *J* = 7.5 Hz, 3 × OC*H*$_2$CH$_3$), 4.40 (br s, 1 H, H-3′), 5.45 (d, 1 H, *J* = 8.0 Hz, H-5), 6.25 (br s, 1 H, H-1′), 6.68–6.86 and 7.12–7.40 (2 m, 13 H, Ar), 7.80 (br s, 1 H, H-6).

UV (EtOH): λ_{max} 274 (13600), 234 nm (28000).

Anal. Calcd for $C_{52}H_{70}N_6O_{13}\cdot0.5H_2O$: C, 62.70; H, 7.18; N, 8.44. Found: C, 62.63; H, 7.22; N, 8.52.

5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{4}-[*trans*-4-(*N*,*N*-bis{2-[bis(2-ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}-acetylamino)cyclohexyl]-2′-deoxycytidine (19)

Prepared from **14** (125 mg, 0.20 mmol) and **23** (101 mg, 0.20 mmol) according to the procedure used for **7** (*Method B*). After purification of the crude material on silica gel (CH_2Cl_2–MeOH–TEA, 98:1.5:0.5) the product was recovered as a pale yellow foam (147 mg, 66%); R_f = 0.55 (EtOAc–MeOH, 80:20); $[\alpha]_D^{20}$ +39.2 (*c* = 1, EtOH).

IR ($CHCl_3$): 2936, 1743, 1642, 1499, 1177 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 0.95 (d, 4 H, *J* = 6.5 Hz, 2 × CH$_{2cyclohexyl}$), 1.06–1.25 (m, 12 H, 4 × OCH$_2$C*H*$_3$), 1.40 (d, 4 H, *J* = 6.5 Hz, 2 × CH$_{2cyclohexyl}$), 1.75–2.28 (2 m, 4 H, H-2′, H-2″ and 2 × CH$_{cyclohexyl}$), 2.48 and 2.90 (2 m, 8 H, 2 × NCH_2CH_2N), 3.09 (s, 2 H, NCH_2CON), 3.50 [s, 10 H, 4 × C*H*$_2$CO$_2$Et (obscured 5′ and 5″)], 3.75 (s, 6 H, 2 × PhOC*H*$_3$), 4.15 [q, 9 H, *J* = 7.5 Hz, 4 × OC*H*$_2$CH$_3$ (obscured H-4′)], 4.53 (m, 1 H, H-3′), 5.38 (br s, 1 H, H-5), 6.29 (t, 1 H, *J* = 6.0 Hz, H-1′), 6.58–6.85 and 7.15–7.42 (2 m, 13 H, Ar), 7.80 (br s, 1 H, H-6).

UV (EtOH): λ_{max} = 275 (13300), 235 nm (28300).

Anal. Calcd for $C_{58}H_{79}N_7O_{15}$: C, 62.52; H, 7.15; N, 8.80. Found: C, 62.78; H, 7.23; N, 9.14.

5′-O-(4,4′-Dimethoxytrityl)-*N*^{4}-[*trans*-4-(2-{[2-bis(ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}acetylamino)cyclohexyl]-2′-deoxycytidine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (1)

To a stirred solution of **9** (137 mg, 0.14 mmol) (previously coevaporated with anhyd pyridine and dried overnight under high vacuum) and *i*-Pr$_2$NEt (72 mg, 0.56 mmol) in anhyd CH_2Cl_2 (3.0 mL) was added 2-cyanoethyldiisopropylchlorophosphoramidite (66 mg, 0.28 mmol) by a syringe over 2 min under N_2. The mixture was left for 30 min at r. t., diluted with CH_2Cl_2 (30 mL) and washed with ice-cold brine (2 × 20 mL). The organic phase was dried (Na_2SO_4), filtered, concentrated to small volume at low pressure and added dropwise to stirred *n*-hexane cooled to –78 °C. The product, recovered as oil, was redissolved in benzene and lyophilized (155 mg, 93%, mixture of diastereomers); R_f = 0.67 and 0.64 (EtOAc–MeOH, 85:15); $[\alpha]_D^{20}$ +15 (*c* = 1, EtOH).

IR ($CHCl_3$): 3274, 1736, 1642, 1503, 1380, 1113 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 1.04 (d, 4 H, *J* = 6.6 Hz, 2 × CH$_{2cyclohexyl}$), 1.16–1.30 [m, 21 H, 3 × OCH$_2$C*H*$_3$ and 2 × (C*H*$_3$)$_2$CHN], 1.43 (d, 4 H, *J* = 6.6 Hz, 2 × CH$_{2cyclohexyl}$),1.85–2.01 (m, 2 H, H-2′ and H-2″), 2.03–2.18 [m, 2 H, 2 × (CH$_3$)$_2$C*H*N], 2.20–2.33 (m, 2 H, 2 × CH$_{cyclohexyl}$), 2.61 (t, 2 H, *J* = 6.6 Hz, CH_2CN), 2.70–2.86 (m, 4 H, NCH_2CH_2N), 3.27 (s, 2 H, NCH_2CON), 3.54 (s, 2 H, C*H*$_2$CO$_2$Et), 3.48–3.67 (m, 3 H, H-4′, H-5′ and H-5″), 3.97 (s, 4 H, 2 × C*H*$_2$CO$_2$Et), 3.79 (s, 6 H, 2 × PhOC*H*$_3$), 4.50 (br s, 1 H, H-3′), 4.16 (q, 6 H, *J* = 7.2 Hz, 3 × OC*H*$_2$CH$_3$), 4.54–4.68 (m, 2 H, CH_2OP), 5.29 (d, 1 H, *J* = 8.0 Hz, H-5), 6.37 (br s, 1 H, H-1′), 6.79–6.84 and 7.20–7.33 (2 m, 13 H, Ar), 7.95 (d, 1 H, *J* = 8.0 Hz, H-6).

^{13}C NMR ($CDCl_3$): δ = 171.41, 171.08, 170.45 (CO_{EDTA}), 162.98 (C-4), 160.19 (*C*OMe), 158.66 (C-2), 144.51, 135.55, 130.20, 128.35, 127.93, 127.04, 113.23 (Ar and C-6), 117.55 (CN), 105.23 (C-5), 76.63–77.48 (C-1′ and C-4′, obscured by $CDCl_3$), 60.70 (C-5′), 60.64 (C-3′), 58.71, 58.44 and 58.20 (O*C*H$_2$CH$_3$), 55.95 (PhO*C*H$_3$), 55.28 (CH_2OP), 55.25, 53.39, 53.12 and 52.15 (N*C*H$_2$CO), 51.71 (NCH_2CH_2N), 47.38 and 47.05 ($CHN_{cyclohexyl}$), 43.40 and 43.17 [N*C*H(CH$_3$)$_3$], 31.82 and 31.29 (CH$_{2cyclohexyl}$), 24.70 and 24.50 [NCH(*C*H$_3$)$_3$], 20.41 (*C*H$_2$CN), 14.29 (OCH$_2$*C*H$_3$).

UV (EtOH): λ_{max} = 274 (12300), 235 nm (26800).

Anal. Calcd for $C_{61}H_{85}N_8O_{14}P$: C, 61.81; H, 7.23; N, 9.45. Found: C, 62.93; H, 6.95; N, 9.33.

5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{4}-[2-(2-{[2-bis(ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}acetylamino)ethyl]-2′-deoxycytidine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (2)

Prepared from **17** (207 mg, 0.22 mmol) according to the procedure used for **1** (230 mg, 92%, mixture of diastereomers); R_f = 0.58 and 0.50 (CH_2Cl_2–MeOH, 90:10); $[\alpha]_D^{20}$ +16 (*c* = 1, MeCN).

IR ($CHCl_3$): 3293, 2988, 1735, 1651, 1509, 1234, 1057 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 1.00–1.40 [m, 21 H, 3 × OCH$_2$C*H*$_3$ and 2 × (C*H*$_3$)$_2$CHN], 1.70–2.26 [m, 8 H, NHC*H*$_2$C*H*$_2$NH , H-2′, H-2″ and 2 × (CH$_3$)$_2$C*H*N], 2.53 (br s, 2 H, CH_2CN), 2.70–2.88 (m, 4 H, NCH_2CH_2N), 3.25 (s, 2 H, NCH_2CON), 3.37 (s, 2 H, C*H*$_2$CO$_2$Et), 3.45–3.68 (m, 3 H, H-4′, H-5′ and H-5″), 3.50 (s, 4 H, 2 × C*H*$_2$CO$_2$Et), 3.76 (s, 6 H, 2 × PhOC*H*$_3$), 4.15 (q, 6 H, *J* = 7.2 Hz, 3 × OC*H*$_2$CH$_3$), 4.45 (br s, 1 H, H-3′), 4.50–4.90 (m, 2 H, CH_2OP), 5.40 (d, 1 H, *J* = 8.0 Hz, H-5), 6.53 (br s, 1 H, H-1′), 6.65–6.90 and 7.15–7.45 (2 m, 13 H, Ar), 7.90 (d, 1 H, *J* = 8.0 Hz, H-6).

^{13}C NMR ($CDCl_3$): δ = 171.32, 171.12, 171.03 (CO_{EDTA}), 163.20 (C-4), 160.28 (*C*OMe), 158.65 (C-2), 144.49, 135.57, 130.12, 128.25, 126.97, 113.20 (Ar and C-6), 116.80 (CN), 104.89 (C-5), 77.63–76.36 (C-1′ and C-4′, obscured by $CDCl_3$), 60.73 (C-5′), 58.49 (C-3′), 58.21–57.20 (OCH_2CH_3), 55.22 ($PhOCH_3$), 54.80 (CH_2OP), 53.92–52.51 (NCH_2CO), 52.33 ($NCH_2CH_2N_{EDTA}$), 45.39 and 45.27 (NCH_2CH_2N), 43.34 and 43.12 [$NCH(CH_3)_3$], 22.98–22.48 [$NCH(CH_3)_3$], 20.14 (CH_2CN), 14.17 (OCH_2CH_3).

UV (EtOH): λ_{max} = 274 (13000), 235 nm (28100).

Anal. Calcd for $C_{57}H_{79}N_8O_{14}P$: C, 60.52; H, 7.04; N, 9.91. Found: C, 60.42; H, 7.12; N, 9.89.

5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{4}-[6-(2-{[2-bis(ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}acetylamino)hexyl]-2′-deoxycytidine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (3)

Prepared from **18** (126 mg, 0.13 mmol) according to the procedure used for **1** (148 mg, 96%, mixture of diastereomers); R_f = 0.46 and 0.40 (CH_2Cl_2–MeOH, 90:10); $[\alpha]_D^{20}$ +15.2 (*c* = 1, MeCN).

IR ($CHCl_3$): 3324, 2971, 1736, 1649, 1509, 1250, 1180, 1034 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 0.90–1.50 [m, 29 H, 3 × OCH_2CH_3, 2 × $(CH_3)_2CHN$ and 2–5 CH_{2hexyl}], 1.61–1.90 (m, 2 H, H-2′ and H-2″), 1.98–2.45 [m, 4 H, 2 × $(CH_3)_2CHN$ and CH_2CN], 2.62–2.83 (m, 4 H, NCH_2CH_2N), 3.15–3.56 (m, 15 H, NCH_2CON, 3 × CH_2CO_2Et, H-4′, H-5′, H-5″ and 1,6 CH_{2hexyl}), 3.75 (s, 6 H, 2 × $PhOCH_3$), 4.15 (q, 6 H, *J* = 7.5 Hz, 3 × OCH_2CH_3), 4.30 (br s, 1 H, H-3′), 4.40–4.70 (m, 2 H, CH_2OP), 5.30 (d, 1 H, *J* = 8.0 Hz, H-5), 6.35 (br s, 1 H, H-1′), 6.65–6.87 and 7.11–7.35 (2 m, 13 H, Ar), 7.85 (br s, 1 H, H-6).

^{13}C NMR ($CDCl_3$): δ = 171.32, 170.78, 170.51 (CO_{EDTA}), 163.12 (C-4), 160.21 (*C*OMe), 158.13 (C-2), 143.96, 135.07, 129.65, 127.80, 126.46, 112.68 (Ar and C-6), 117.23 (CN), 103.91 (C-5), 77.13–75.86 (C-1′ and C-4′, obscured by $CDCl_3$), 60.20 (C-5′), 60.06 (C-3′), 58.27, 57.97 and 57.60 (OCH_2CH_3), 55.54 ($PhOCH_3$), 54.72 (CH_2OP), 54.41 and 53.20 (NCH_2CO), 52.55 and 51.82 (NCH_2CH_2N), 44.89 and 44.75 [$NCH(CH_3)_3$], 42.91 and 42.59 (1,6 CH_{2hexyl}), 29.15–28.35 (2-5 CH_{2hexyl}), 24.07–23.92 [$NCH(CH_3)_3$], 19.50 (CH_2CN), 13.70 (OCH_2CH_3).

UV (EtOH): λ_{max} = 275 (11700), 236 nm (27200).

Anal. Calcd for $C_{61}H_{87}N_8O_{14}P$: C, 61.70; H, 7.39; N, 9.44. Found: C, 61.82; H, 7.43; N, 9.38.

5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{4}-[*trans*-4-(*N*,*N*-bis{2-[bis(2-ethoxycarbonylmethylamino)ethyl]ethoxycarbonylmethylamino}acetylamino)cyclohexyl]-2′-deoxycytidine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite (4)

Prepared from **19** (100 mg, 0.09 mmol) according to the procedure used for **1** (80 mg, 68%, mixture of diastereomers); R_f = 0.37 and 0.44 ($CHCl_3$–MeOH, 96:4); $[\alpha]_D^{20}$ +25 (*c* = 1, MeCN).

IR ($CHCl_3$): 3274, 1736, 1642, 1503, 1380, 1113 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 1.05 (d, 4 H, *J* = 6.5 Hz, 2 × $CH_{2cyclohexyl}$), 1.14–1.31 [m, 24 H, 4 × OCH_2CH_3 and 2 × $(CH_3)_2CHN$], 1.38 (d, 4 H, *J* = 6.5 Hz, 2 × $CH_{2cyclohexyl}$), 1.84–2.31 [2 m, 6 H, H-2′, H-2″, 2 × $(CH_3)_2CHN$ and 2 × $CH_{cyclohexyl}$], 2.61–2.64 and 2.67–2.85 (2 m, 10 H, CH_2CN and 2 × NCH_2CH_2N), 3.13 (s, 2 H, NCH_2CON), 3.53 [s, 10 H, 4 × CH_2CO_2Et (obscured 5′ and 5″)], 3.79 (s, 6 H, 2 × $PhOCH_3$), 4.16 [q, 9 H, *J* = 7.5 Hz, 4 × OCH_2CH_3 (obscured H-4)], 4.55 (m, 1 H, H-3′), 4.53–4.76 (m, 2 H, CH_2OP), 5.30 (d, 1 H, *J* = 8.0 Hz, H-5), 6.31 (br s, 1 H, H-1′), 6.81–6.86 and 7.27–7.39 (2 m, 13 H, Ar), 7.78 (br s, 1 H, H-6).

^{13}C NMR ($CDCl_3$): δ = 171.38, 170.89, 170.71 (CO_{DTPA}), 162.59 (C-4), 160.45 (*C*OMe), 158.35 (C-2), 147.37, 135.42, 129.75, 129.01, 127.89, 127.12, 113.86 (Ar and C-6), 116.85 (CN), 77.65–76.80 (C-1′ and C-4′, obscured by $CDCl_3$), 60.80 (C-5′), 60.51 (C-3′), 58.85, 58.37, 58.02 (OCH_2CH_3), 55.70 ($PhOCH_3$), 55.13 (CH_2OP), 55.01–52.28 (NCH_2CO), 51.71 (NCH_2CH_2N), 47.15 and 47.05 ($CHN_{cyclohexyl}$), 43.38 and 43.12 [$NCH(CH_3)_3$], 31.98–30.95 ($CH_{2cyclohexyl}$), 24.89–24.12 [$NCH(CH_3)_3$], 20.40 (CH_2CN), 14.57 (OCH_2CH_3).

UV (EtOH): λ_{max} = 275 (12700), 234 nm (25200).

Anal. Calcd for $C_{67}H_{96}N_9O_{16}P$: C, 61.20; H, 7.36; N, 9.59. Found: C, 61.38; H, 7.45; N, 9.37.

[Bis[2-(benzyloxycarbonylamino)ethyl]amino]acetic Acid *tert*-Butyl Ester (21)

To an ice-cooled solution of *N*^{1,5}-(benzyloxycarbonyl)-1,5-diamino-3-azapentane[21] (**20**; 2.2 g, 6.0 mmol) in anhyd MeCN (30 mL) was added *i*-Pr_2NEt (1.29 g, 10.0 mmol), followed by *tert*-butyl bromoacetate (1.56 g, 8.0 mmol), maintaining the reaction temperature below 0 °C. After the mixture was stirred overnight at r.t., the solution was concentrated at reduced pressure. The residue was taken up in EtOAc (50 mL) and washed with aq sat. $NaHCO_3$ (2 × 25 mL) and brine (25 mL). The organic phase was dried (Na_2SO_4), filtered and evaporated. The product (colorless oil) was used without further purification (2.8 g, 96%). This compound is described in the literature.[25]

[Bis[2-bis(ethoxycarbonylmethylamino)ethyl]amino]acetic Acid *tert*-Butyl Ester (22)

A solution of **21** (2.53 g, 5.20 mmol) in a mixture of formic acid 4% in MeOH (50 mL) was added dropwise to a stirred suspension of 10% Pd/C (550 mg) in 4% formic acid in MeOH (50 mL). The stirring was continued at r.t. until deblocking was complete (about 2 h), then was filtered and evaporated under reduced pressure. The residue was coevaporated twice with MeOH and triturated with Et_2O, filtered and dried under vacuum. To an ice-cooled solution of the deblocked product (1.5 g, 5.0 mmol) and ethyl bromoacetate (3.9 g, 25 mmol) in anhyd MeCN (30 mL) was added *i*-Pr_2NEt (1.29 g, 10.0 mmol), maintaining the reaction temperature below 0 °C. After the mixture was stirred overnight at r.t., the solution was concentrated at reduced pressure. The residue was taken up in EtOAc (50 mL) and washed with aq sat. $NaHCO_3$ (2 × 25 mL) and brine (25 mL). The organic phase was dried (Na_2SO_4), filtered and evaporated. The product, after silica gel flash chromatography (CH_2Cl_2–MeOH, 98:2), was recovered as a colorless oil (1.50 g, 51%).

IR ($CHCl_3$): 1734, 1372, 1205 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 1.24 (t, 12 H, *J* = 7.5 Hz, 4 × OCH_2CH_3), 1.42 [s, 9 H, $(CH_3)_3C$], 2.85 (s, 8 H, 2 × NCH_2CH_2N), 3.30 (s, 2 H, CH_2CO_2Bu-*t*), 3.50 (s, 8 H, 4 × CH_2CO_2Et), 4.16 (q, 8 H, *J* = 7.5 Hz, OCH_2CH_3).

Anal. Calcd for $C_{26}H_{47}N_3O_{10}$: C, 55.60; H, 8.43; N, 7.48. Found: C, 55.82; H, 8.59; N, 7.76.

[Bis[2-bis(ethoxycarbonylmethylamino)ethyl]amino]acetic Acid (23)

A solution of **22** (1.46 g, 2.60 mmol) in TFA (2.0 mL) was allowed for 2 h at r.t. After evaporation of TFA at reduced pressure, the residue was repeatedly coevaporated with anhyd Et_2O and then purified on silica gel ($CHCl_3$–*i*-PrOH, 80:20). The product was recovered as colorless oil (600 mg, 46%). This compound is described in the literature as mixture of symmetrical and unsymmetrical isomers.[19]

IR ($CHCl_3$): 2958, 1737, 1380, 1211 cm^{-1}.

^{1}H NMR ($CDCl_3$): δ = 1.21 (t, 12 H, *J* = 7.5 Hz, 4 × OCH_2CH_3), 3.10 (s, 8 H, 2 × NCH_2CH_2N), 3.55 (s, 8 H, 4 × CH_2CO_2Et), 3.62 (s, 2 H, CH_2CO_2H), 4.12 (q, 8 H, *J* = 7.5 Hz, OCH_2CH_3), 11.20 (s, 1 H, CO_2H).

Anal. Calcd for $C_{22}H_{39}N_3O_{10}$: C, 52.57; H, 7.78; N, 8.31. Found: C, 52.34; H, 7.36; N, 8.38.

Automatic Synthesis and Characterization of ODNs

The automatic synthesis of the ODNs was performed at a 1 μmole scale, on an Perseptive Biosystems Expedite 8909 synthesizer, leaving the terminal 5′-*O*-DMT-group on. The phosphoramidites **1**–**4** (0.06 M) in anhyd MeCN, were used for the synthesis of the ODNs **C**–**F** and **I**–**M** (Table 1) without having to change any parameter in the standard DNA synthesis protocol of the machine. Having used Ac-protected dC (Beckmann) and usual standard chemicals (Proligo Biochemie) for the synthesis, the nonlabelled ODNs **A**, **B**, **G**, **H** were cleaved from the solid support by a mixture of ammonia and methylamine (AMA, Beckmann) in 10 min at r.t., then the solution was heated for 10 min at 55 °C in order to deprotect the exocyclic amino groups. In order to avoid ammonolysis of the EDTA ethyl esters to amides, the labelled ODNs **C**–**F**, **I**–**M** instead, were cleaved form the solid support and deprotected by a solution of NaOH 0.4 M in MeOH–H_2O (4:1), overnight at r.t., followed by quenching with a solution of triethylammonium acetate (TEAA) buffer 2 M, pH 7.0. After SpeedVac removal of AMA, or directly after quenching with TEAA 2 M, the samples were loaded onto an HPLC column Vydac C18, 300 Å, 5μ, 22 × 50 mm eluted at a flow rate of 6 mL/min. The failure sequences were eluted first with MeCN 10% in TEAA 0.1 M, pH 7.0, for 10 min. Afterwards the terminal DMT was removed on column from the full-length ODN by 0.5% TFA. Then the DMTr-free full-length ODN was eluted by a linear gradient 0–45% MeCN in TEAA 0.1 M, pH 7.0, in 20 min. A further purification by HPLC was accomplished with a SAX Dionex® Nucleopack100 column, 9 × 250 mm, eluting at a flow-rate of 3 mL/min, with a linear gradient 10–30% of $NaClO_4$ 0.5 M in Tris-HCl 25 mM buffer, pH 8, in 15 min.

The collected fractions were liophylized and freed from Tris-HCl and $NaClO_4$ by elution on a Phenomenex C18 column, 9 × 250 mm, at a flow-rate of 3 mL/min, with a linear gradient 3–40% of MeCN in TEAA 0.1 M, pH 7.0, in 20 min, and then liophylized again.

The ODNs were analyzed by HPLC both on a Vydac C18, 300 Å, 5μ, 250 × 4.6 mm column and on a SAX Dionex® Nucleopack100, 250 × 4 mm column, eluting at a flow-rate of 1 and 1.5 mL/min respectively, each one with the same eluents as above. The ss ODNs were also analyzed by PAGE on 15% polyacrylamide, 7 M urea gels (1 mm) containing 50 mM Tris-borate pH 8.0, 0.1 mM EDTA buffer (Sigma) staining with ethidium bromide. ODNs **L**-**M** rich in dC proved not to be stained in this way. The ODNs were also hybridized to form ds, by heating the appropriate mixtures in NaH_2PO_4 (10 mmol), pH 7.2, NaCl (100 mmol), EDTA 0.1 (mmol), at 95 °C for 2 min and allowing them to reach slowly r.t. The ds ODNs were analyzed both by HPLC and PAGE under analogous conditions as above, but omitting urea in PAGE. ESI Mass Spectra were obtained by direct infusion (10 μL/min) of a solution containing 15 ppm/μL of the analytes dissolved in MeOH–H_2O (1:1) solution containing 2% NH_4OAc at the optimum ion spray voltage of 4.200 eV. The Tms were evaluated by heating the ds ODNs in the hybridization buffer.

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