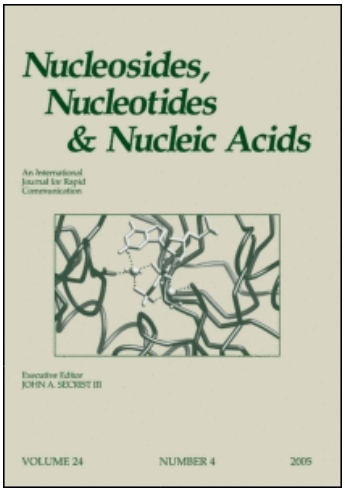

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OLIGONUCLEOTIDE ANALOGS WITH PEPTIDE INTERNUCLEOTIDE LINKAGES

Anna Varizhuk, Svetlana Kochetkova, Natalia Kolganova, Edward Timofeev, and Vladimir Florentiev
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□ *Oligonucleotide analogs containing one or a few glycine, L-, and D-alanine or L-and D-phenylalanine residues instead of phosphodiesterinternucleotide linkages were synthesized. The stability of the duplexes formed by modified oligonucleotides and their wildtype complements was studied. Oligonucleotides with D-alanine residues in internucleotide linkages form duplexes more stable than native ones (ΔT_m +0.2° C per modification), whereas other modifications destabilize the duplexes.*

Keywords Oligonucleotides; analogs; synthesis; peptide internucleotide linkages; hybridization; thermal stability

INTRODUCTION

Significant progress has been made over the last several decades in the area of oligonucleotide technology. A great number of modified oligonucleotides (ONs) meant for selective suppression of gene expression via antisense or RNA interference mechanism[1–8] have been synthesized.[9–13] However, only some of them (phosporthioate analogs, 2′-*O*-methoxyethyloligoribonucleotides, morfolino oligonucleotides, and LNA) showed sufficient activity and were brought to clinical trials,[14–17] and there is still a need to develop new ON analogs that can perform advanced functions.

We have reported a novel class of modified ONs, carrying amino acid residues in internucleoside linkages.[18] They are chirally pure, and a modified fragment can be incorporated into any predetermined position of a nucleic acid chain. The novel ON analogs represent significant interest since peptide internucleotide linkages can increase power and versatility of nucleic acid receptors, ligands, and catalysts. Moreover, the novel analogs are

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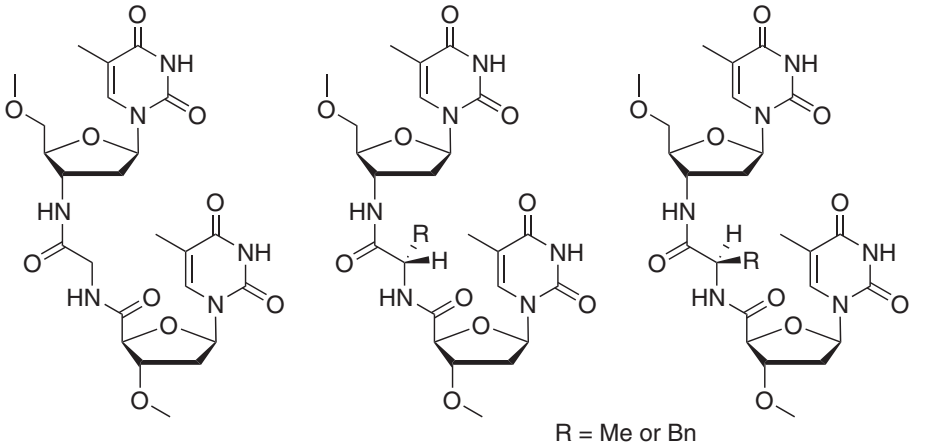
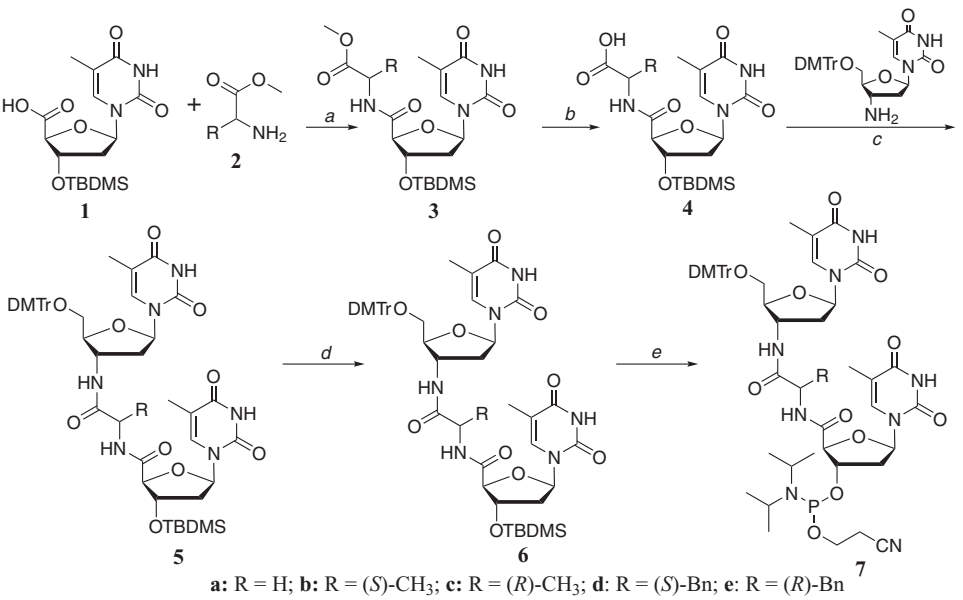
likely to demonstrate improved nuclease resistance and enhanced cellular uptake, characteristic of DNA-peptide conjugates.[19,20] The latter are extensively used to circumvent poor pharmacokinetic properties of antisense olinucleotides and si-RNA.[21, 22] In this article, we describe a full detail of the general method for facile and efficient synthesis of ONs with glycine, L- and D-alanine and L- and D-phenylalanine residues in internucleotide linkages (Scheme 1).



SCHEME 1 Fragments of ONs with glycine, L- and D-alanine and D-phenylalanine residues in internucleotide linkages.

RESULTS AND DISCUSSION

For the preparation of the modified ONs, a method for the synthesis of 3′-phosphoramidite dinucleosideblocks (**7a–7e**) was developed (Scheme 2). 3′-*O*-*t*-(Butyldimethylsilyl)thymidine 5′-carboxylc acid **1** was prepared following the modified published procedure.[23] 3′-*O*-(*t*-Butyldimethylsilyl)thymidine was oxidised by bis(acetoxy)iodobenzene in the presence of a catalytic amount of 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) in acetonitrile-water (1:1) mixture (20°C, 3 hours) to give **1** in a yield of 93%. Acid **1** was coupled with amino acids esters **2a–2e** using (benzotriazol-1-yloxy (tris-(dimethyl amino)-phosphonium hexafluorophasphate) (BOP) as a condensing agent in the presence of triethylamine (TEA) in CH_2Cl_2 (20°C, 1 hour). Esters **3a–3e** were saponified with 0.5 M KOH in an ethanol-water (3:1) mixture (20°C, 2 hour). Acid **4a** was obtained in a yield of 48%, while the yields of L- and D-alanine as well as L- and D-phenylalane derivatives **4b–4e** were over 80%. This is consistent with the published data on saponification of C-terminal glycine esters in peptides.[24] Acids **4a–4e** were coupled with 3′-amino-3′-deoxy-5′-*O*-dimethoxytritylthymidine to yield fully



SCHEME 2 Synthesis of the dinucleoside blocks containing glycine (**5a**), L- and D-alanine (**5b**, **5c**) and L- and D-phenylalanine (**5d**, **5e**) residues. Reagents and conditions: (a) BOP, TEA, CH_2Cl_2; (b) KOH, $EtOH/H_2O$; (c) BOP, TEA, CH_2Cl_2 for **4a** and DCC, NHS, CH_2Cl_2, 4°C for **4b-4e**; (d) TBAF, THF; (e) $NCCH_2CH_2OP(NPr^i_2)_2$, tetrazole, pyridine, CH_2Cl_2.

protected dinucleoside analogs **5a–5e**. The condensation conditions for **4a** (a glycine derivative) were the same as for **2a**. In the case of alanine and phenylalanine derivatives **4b–4e**, *N*,*N*-dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) were used since this enables the avoidance of epimerization at the α-carbon atom of an amino acid residue (about 15% for alanine and about 30% for phenylalanine) observed when using BOP. The optical purity of **5b–5e** was confirmed by ^{1}HNMR spectroscopy. The silyl protection was removed with 0.5 M tetrabutylammonium fluoride (TBAF) in tetrahydrofuran (THF) (20°C, 4 hours). Resulting dinucleosides **6a–6e,** bearing free 3′-hydroxyl groups, were treated with 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite in the presence of 1*H*-tetrazole and pyridine in dichloromethane to give amidites **7a–7e**. Dinucleosides **5a–e–7a–e** were characterized by MALDI-TOF mass spectrometry and the structures of **5a–5e** and **6a–6e** were confirmed by COSY NMR spectroscopy. COSY-spectra enabled assignment of proton signals to thymidine 5′-carboxylc acid and 3′-amino-3′-deoxythymidine moieties (Figure 1).

Dinucleosideamidite blocks **7a–e** were used for the synthesis of modified ONs on an automated synthesizer using standard phosphoramidite protocols. Coupling time was increased to 15 minutes for modified phosphoramidites. No decrease in coupling efficiency was observed. Step-wise coupling yields were about 98–99% for both modified and unmodified

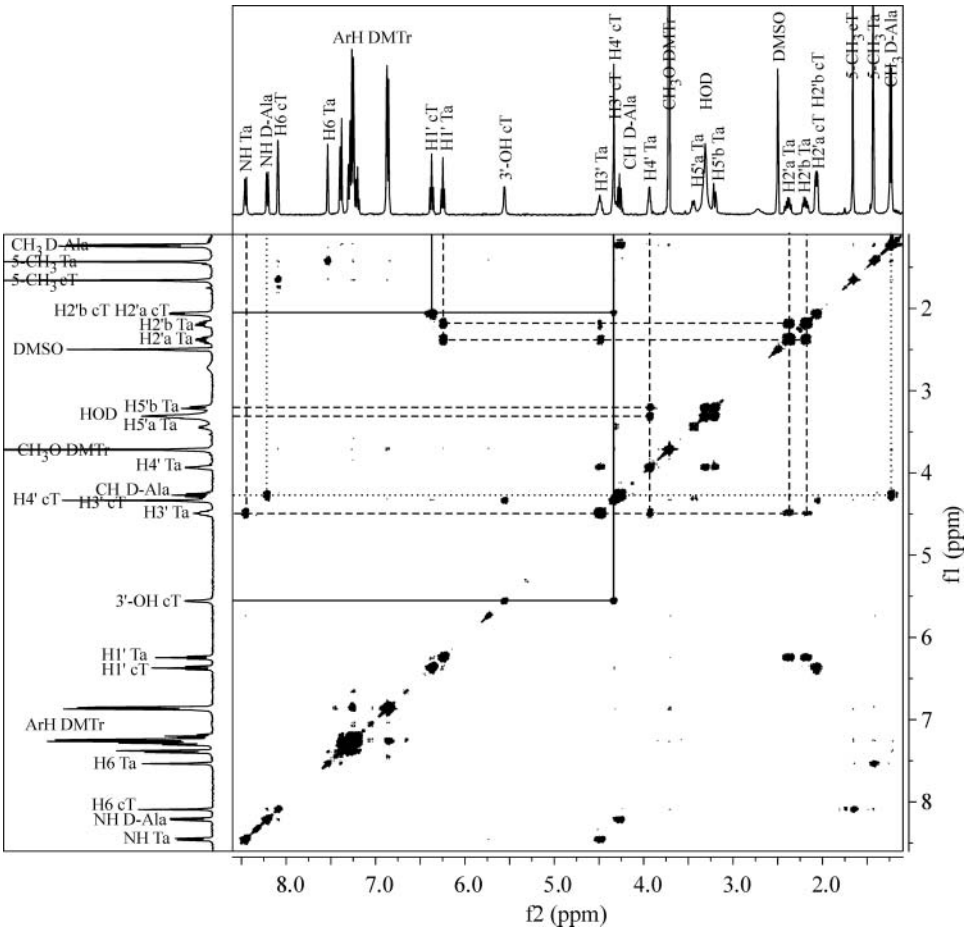


FIGURE 1 COSY NMR spectrum of compound **6c**. cT = 5′carboxylc acid moiety, Ta = 3′-amino-3′-deoxythymidine moiety.

amidites. The overall yield of the oligonucleotide synthesis was about 80%. Along with the modified ONs, isosequential and complementary wildtype ONs were synthesized. The purification of the ONs was carried out by reverse-phase HPLC. All the ONs were characterized by MALDI mass spectrometry (Table 1).

Thermal dissociation of the modified duplexes and their wildtype counterparts was measured (Figure 2).

The resulting curves enabled evaluation of the melting temperatures of the duplexes. The results are summarized in Table 2.

As evident from Table 2, modifications **D** (L-phenylalanine) and **E** (D-phenylalanine) significantly decrease duplex stability for both the random sequence ($\Delta T_m = -1.8°C$ and $-1.0°C$ per modification on an average, respectively (for each modification average ΔTm per modification was

TABLE 1 Mass spectra of the modified ONs

Sequence (5′→3′)*	*m/z*, found (calculated for $[M–H]^-$)				
	X = A	X = B	X = C	X = D	X = E
TTAACTTCACA**X**C	5061.5 (5059.39)	5074.2 (5073.43)	5074.0 (5073.43)	5147.9 (5149.51)	5150.3 (5149.51)
XAACTTCACA**X**C	5049.1 (5049.46)	5080.8 (5077.51)	5081.0 (5077.51)	5231.5 (5129.70)	5231.1 (5229.70)
G**X**TTTTTTTTTTTTG	4846.2 (4844.22)	4860.7 (4858.26)	4858.4 (4858.26)	4931.4 (4934.34)	4932.3 (4934.34)
GTTTTTT**X**TTTTTTG	4844.2 (4844.22)	4859.9 (4858.26)	4859.7 (4858.26)	4932.1 (4934.34)	4936.8 (4934.34)
GTTTTTTTTTTTT**X**G	4844.3 (4844.22)	4858.2 (4858.26)	4863.4 (4858.26)	4934.0 (4924.34)	-
G**X**TTTTTTTTTT**X**G	4834.1 (4834.29)	4864.5 (4862.35)	4862.6 (4862.35)	5014.0 (5014.54)	5017.7 (5014.54)
G**X**TTTT**X**TTTT**X**G	4826.4 (4824.36)	4867.5 (4866.44)	4866.5 (4866.44)	5095.9 (5094.73)	5095.3 (5094.73)
G**X**TT**X**TT**X**TT**X**G	4814.8 (4814.43)	4871.7 (4867.44)	4872.5 (4871.54)	5174.1 (5174.92)	5177.0 (5174.92)

Notes: *X = A, B, C, D or E-modified dinucleoside fragments containing glycine, L-alanine, D-alanine, L-phenylalanine, or D- phenylalanine residues respectively in internucleotide linkages.

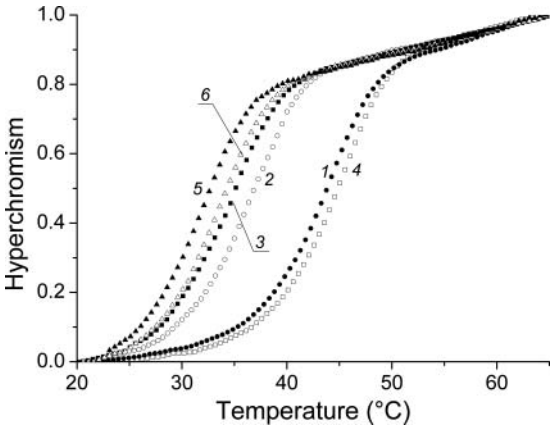


FIGURE 2 Melting curves of the modified and wildtype duplexes. Sequence (5′→3′) of a modified strand: G**X**TT**X**TT**X**TT**X**G, where X-modified dinucleoside fragments containing glycine (curve 2), L-alanine (curve 3), D-alanine (curve 4), L-phenylalanine (curve 5), or D-phenylalanine (curve 6) residues respectively in internucleotide linkages. Curve 1: unmodified duplex.

calculated as a sum of all corresponding ΔTm values reported in Table 2 divided by the total number of modifications)) and the regular sequence ($\Delta T_m = -3.4^\circ$C and -2.6°C per modification on an average, respectively). Modifications **A** (glycine) and **B** (L-alanine) cause insignificant destabilization. ($\Delta T_m = -1.1^\circ$C and -0.9°C per modification on an average, respectively, for the random sequence and $\Delta T_m = -1.9^\circ$C and -2.3°C per modification on an average, respectively, for the regular sequence). In contrast to that, modification **C** (D-alanine) has small stabilizing effect ($\Delta T_m = +0.3^\circ$C per

TABLE 2 Melting temperatures of the modified and wildtype duplexes (duplex concentration $5*10^{-6}$ M)

	T_m, °C ± 0.5, (ΔT_m, °C**)				
Sequence (5′→3′)*	X = A	X = B	X = C	X = D	X = E
TTAACTTCACATTC***		51.6			
TTAACTTCACA**X**C	50.3 (−1.3)	50.8 (−0.8)	51.6 (0.0)	49.7 (−1.9)	50.0 (−1.6)
XAACTTCACA**X**C	49.7 (−1.9)	49.9 (−1.7)	52.5 (+0.9)	58.1 (−3.5)	50.2 (−1.4)
GTTTTTTTTTTTTTTG***		44.3			
G**X**TTTTTTTTTTTTG	42.1 (−2.2)	42.0 (−2.3)	44.1 (−0.2)	39.7 (−4.6)	41.4 (−2.9)
GTTTTTT**X**TTTTTTG	42.9 (−1.4)	42.8 (−1.4)	44.6 (+0.3)	41.5 (−2.8)	42.2 (−2.1)
GTTTTTTTTTTTT**X**G	42.2 (−2.1)	42.1 (−2.2)	44.4 (+0.1)	40.9 (−3.4)	-
G**X**TTTTTTTTT**X**G	40.3 (−4.0)	39.1 (−5.2)	44.4 (+0.1)	35.9 (−8.4)	38.9 (−5.4)
G**X**TTTT**X**TTTT**X**G	38.4 (−5.9)	36.8 (−7.5)	44.8 (+0.5)	34.4 (−9.9)	36.7 (−7.6)
G**X**TT**X**TT**X**TT**X**G	37.4 (−6.9)	35.3 (−9.0)	45.3 (+1.0)	32.6 (−11.7)	34.0 (−10.3)

Notes: *X = A, B, C, D or E—modified dinucleoside residues containing glycine, L-alanine, D-alanine, L-phenylalanine, or D- phenylalanine respectively in an internucleotide linkage.

**Tm difference between modified and natural duplexes.

***Unmodified oligonucleotide. Corresponding duplex was used as a reference when calculating ΔT_m.

modification on an average for the random sequence and $\Delta T_m = +0.2°C$ per modification on an average for the regular sequence). Apparently, D-amino acid derivatives tend to form generally more stable duplexes than L-analogs.

CONCLUSION

In summary, we have described a novel promising class of oligonucleotide analogs and a general strategy for their synthesis. Analogs with glycine, L-, and D-alanine and L-and D-phenylalanine residues in internucleotide linkages have been synthesized and their ability to form stable duplexes with natural complements has been studied. ONs with D-alanine residues satisfy the stability criterion set for modified ONs. D-amino acid derivatives seem generally more promising with respect to their hybridization properties. This may be of great importance for in vivo applications of the novel ON analogs. Nonnatural D-amino acids can be used to avoid enzymatic degradation of peptide internucleotide linkages by carboxypeptidases.The herein presented general approach to DNA modification potentially offers a wide range of functional group incorporation. The synthetic strategy worked out for hydrophobic amino acids can be optimized to design ON analogs with additional functionalities, e.g. lysine-containing analogs. This study is currently underway. Amino acid side chain functionalities can be used for attaching various reporter groups.

EXPERIMENTAL

All reagents were commercially available (Fluka, Switzerland) and used without further purification. 3′-Deoxy-3′-azidothymidine was provided by Association AZT (Russia). All solvents were purchased from Khimmid (Russia). Dichloromethane was dried by distillation from phosphorus pentaoxide, pyridine was distilled from calcium hydride, and THF was distilled from lithium alumohydride prior to use. Flesh column chromatography (CC) was performed on silica gel Kieselgel 60 (0.040–0.063 mm, Merck, Germany). TLC was performed on silica gel Kieselgel 60 F_{254} precoated plates (Merck) with detection by UV using the following solvent systems (compositions expressed as *v*/*v*): ethanol-methylene chloride 1:65 (A),1:32 (B), 1: 19 (C), 1:9 (D), 1:9 + 0.1% TEA.^{1}HNMR spectra were recorded on a Bruker AMXIII-400 NMR spectrometer (Germany) on solutions in deuterated dimethyl sulfoxide (DMSO-d6). Chemical shifts are given in parts per million (ppm). The coupling constants (*J*) are given in Hz. Abbreviations used: cT (thymidine 5′-carboxylc acid moiety), Ta(3′-amino-3′-deoxythymidine moiety). The signals were assigned using COSY experiments. The ^{1}HNMR data were processed usingMestReNova version 6.1.1 (Mestrelab Research SL, Spain). MALDI TOF

mass spectra were acquired on a compact IV mass spectrometer (Kratos, UK) using linear flight path.

ONs were synthesized on an ABI 3400 DNA synthesizer (Applied Biosystems, USA) using standard phosphoramidite protocols and purified using preparative scale reverse-phase HPLC on a 250 mm × 4.0 mm^2 Hypersil C18 column with detection at 260 nm. Chromatography of dimethoxytrytil-protected ONs was performed using 10–50% gradient of CH_3CN in 0.05 M TEAA (triethylammonium acetate). Detritylated oligonucleotides were further purified in 0–25% gradient of CH_3CN in TEAA buffer. Melting curves of the duplexes were recorded on a UV 160-A spectrometer (Shimadzu, Japan), equipped with a thermostatic system, in 20 mM sodium phosphate buffer, 100 mM NaCl, 01 mM ethylenediaminetetraacetic acid (EDTA). pH 7.0, concentration of each duplex being $5{\cdot}10^{-6}$M. Samples were denatured at 95°C for 5 minutes and slowly cooled to 20°C prior to measurements. A_{260} (duplex absorbance) was measured as a function of temperature. It was registered every 0.5°C from 20 to 70°C. Thermodynamic parameters of duplex formation were obtained by performing nonlinear regression analysis using Data Fit version 9.0.059 (Oakdale Engineering, USA). The calculation method taking into account temperature dependence of UV absorbance of duplexes and single strands was applied.

3′-O-(tert-Butyldimethylsilyl)thymidine-5′-carboxylic acid (1)

3′-*O*-(*tert*-Butyldimethylsilyl)uridine (4.12 g, 11.6 mmol, prepared following the published procedure[25]) was dissolved in acetonitrile (25 mL). Bis(acetoxy)iodo benzene (BAIB) (9.02 g, 28 mmol), TEMPO (0.5 g, 3.2 mmol) and water (25 ml) were added. The mixture was stirred for 2 hours at 20°C and gradually poured into 0.5 N KOH (190 mL). After stirring for 10 minutes at 20°C the mixture was extracted with CH_2Cl_2 (3 × 50 mL). Combined organic layers were washed with water (50 mL). Combined water layers were acidified carefully under cooling to pH 2 with 2 N HCl. The lade-down sediment was filtered, washed with water at filter and dried to give compound **1** as white crystals. Yield 4.22 g, 98.2%. ^{1}HNMR: 13.265 (1 H, s, COOH), 11.307 (1 H, s, H3), 8.004 (1 H, d, ^4J 1.1, H6), 6.305 (1 H, dd, $J_{1',2'a}$ 8.5, $J_{1',2'b}$ 5.9, H1′), 4.680–4.614 (1 H, m, H3′), 4.293 (1 H, d, $J_{3',4'}$ 1.1, H4′), 2.162–2.034 (2 H, m, H2′a, H2′b), 1.786 (3 H, d, ^4J 1.1, 5-CH_3), 0.892 (9 H, s, *t*-BuSi), 0.125 (3 H, s, CH_3Si), 0.121 (3 H, s, CH_3Si).

General Procedures

General Procedure for Preparation of Compounds 3a–3e (Amide Formation with BOP as a Condensing Reagent)

To a stirred solution of amino acid hydrochloride (1 mmol) in dry CH_2Cl_2 (10 mL), TEA (0.47 mL, 3.4 mmol), acid **1** (0.41 g, 1.1 mmol) and BOP (0.53 g, 1.2 mol) were added. The mixture was stirred for 1 hour at 20°C, diluted with water (50 mL) and extracted with CH_2Cl_2 (3 × 30 mL).

Combined organic layers were washed with saturated $NaHCO_3$(aqueous) (2 × 30 mL) and water (30 mL). The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (CC) using the solvent systems specified below to give **3a–3e** as colorless foam.

Methyl (3′-O-(tert-butyldimethylsilyl)thymidine-5′-carboxamido)acetate (3a)

CC solvent system: 3% of ethanol in CH_2Cl_2, yield 67.9%, R_f = 0.56 (Solvent system C).^{1}HNMR: 11.315 (1 H, s, H3 Ta), 8.603 (1 H, t, J 5.9, NH Gly), 7.861 (1 H, d, ^4J 1.2, H6 Ta), 6.328 (1 H, dd, $J_{1',2'a}$ 9.1, $J_{1',2'b}$ 5.5, H1′ Ta), 4.584–4.527 (1 H, m, H3′ Ta), 4.278 (1 H, d, $J_{3',4'}$ 1.4, H4′ Ta), 3.911 (2 H, dd, J 5.9, 1.2, CH_2Gly), 3.654 (3 H, s, CH_3O Gly), 2.229 (1 H, ddd, $J_{1',2'a}$ 9.1, $J_{3',2'a}$ 5.1, $^2J_{2'a,2'b}$ 13.9, H2′a Ta), 2.078 (1 H, ddd, $J_{1',2'b}$ 5.5, $J_{3',2'b}$ 1.6, $^2J_{2'a,2'b}$ 13.9, H2′b Ta), 1.765 (3 H, d, ^4J 1.0, 5-CH_3 Ta), 0.890 (9 H, s, t-BuSi), 0.119 (3 H, s, CH_3Si), 0.107 (3 H, s, J 3.1, CH_3Si).

Methyl (2S)-2-(3′-O-(tert-butyldimethylsilyl)thymidine-5′-carboxamido)propanoate (3b)

CC solvent system: 1,5% of ethanol in CH_2Cl_2, yield 85.6%,R_f = 0.40 (Solvent systemA). ^{1}HNMR: 11.301 (1 H, s, H3 Ta), 8.612 (1 H, d, J 7.3, NH L-Ala), 7.928 (1 H, d, ^4J 0.9, H6 Ta), 6.299 (1 H, dd, $J_{1',2'a}$ 8.9, $J_{1',2'b}$ 5.6, H1′ Ta), 4.525–4.473 (1 H, m, H3′ Ta), 4.381 (1 H, quintet, J 7.32, CH L-Ala), 4.268 (1 H, d, $J_{3',4'}$ 1.0, H4′ Ta), 3.636 (3 H, s, CH_3O L-Ala), 2.307 (1 H, ddd, $J_{1',2'a}$ 8.9, $J_{3',2'a}$ 5.1, $^2J_{2'a,2'b}$ 13.7, H2′a Ta), 2.070 (1 H, ddd, $J_{1',2'b}$ 5.6, $J_{3',2'b}$ 1.5, $^2J_{2'a,2'b}$ 13.7, H2′b Ta), 1.761 (3 H, d, ^4J 0.9, 5-CH_3 Ta), 1.329 (3 H, d, J 7.3, CH_3 L-Ala), 0.897 (9 H, s, *t*-BuSi), 0.128 (3 H, s, CH_3Si), 0.117 (3 H, s, CH_3Si).

Methyl (2R)-2-(3′-O-(tert-butyldimethylsilyl)thymidine-5′-carboxamido)propanoate (3c)

CC solvent system: 1,5% of ethanol in CH_2Cl_2, yield 86.3%,R_f = 0.40 (Solvent system A). ^{1}HNMR: 11.314 (1 H, s, H3 Ta), 8.612 (1 H, d, J 7.2, NH D-Ala), 7.872 (1 H, d, ^4J 0.9, H6 Ta), 6.302 (1 H, dd, $J_{1',2'a}$ 8.8, $J_{1',2'b}$ 5.6, H1′ Ta), 4.567–4.503 (1 H, m, H3′ Ta), 4.341 (1 H, quintet, J 7.2, CH D-Ala), 4.263 (1 H, d, $J_{3',4'}$ 1.5, H4′ Ta), 3.655 (3 H, s, CH_3O D-Ala), 2.339 (1 H, ddd, $J_{1',2'a}$ 8.8, $J_{3',2'a}$ 5.1, $^2J_{2'a,2'b}$ 13.6, H2′a Ta), 2.088 (1 H, ddd, $J_{1',2'b}$ 5.6, $J_{3',2'b}$ 2.0, $^2J_{2'a,2'b}$ 13.6, H2′b Ta), 1.768 (3 H, d, ^4J 0.9, 5-CH_3 Ta), 1.329 (3 H, d, J 7.3, CH_3 D-Ala), 0.897 (9 H, s, *t*-BuSi), 0.128 (3 H, s, CH_3Si), 0.117 (3 H, s, CH_3Si).

Methyl (2S)-2-(-3′-O-(tert-butyldimethylsilyl)thymidine-5′-carboxamido)-3-phenylpropanoate (3d)

CC solvent system: 3% of ethanol in CH_2Cl_2, yield 85.2%, R_f = 0.51 (Solvent system A). ^{1}HNMR (DMSO): 11.326 (1 H, s, H3 Ta), 8.593 (1 H, d,

J 7.7, NH L-Phe), 7.804 (1 H, d, ^4J 1.1, H6 Ta), 7.314–7.149 (5 H, m, ArH), 6.218 (1 H, dd, $J_{1',2'a}$ 9.1, $J_{1',2'b}$ 5.4, H1′ Ta), 4.564(1 H, ddd, $J_{NH,\alpha\text{-H}}$7.7, $J_{\alpha\text{-H},\beta\text{ Ha}}$6.0, $J_{\alpha\text{-H},\beta\text{-Hb}}$8.8, α-H L-Phe), 4.363–4.282 (1 H, m, H3′ Ta), 4.230 (1 H, d, $J_{3',4'}$ 1.3, H4′ Ta), 3.655 (3 H, s, CH_3O L-Phe), 3.106 (1 H, dd,$J_{\alpha\text{-H, Ha}}$ 6.0, $^2J_{Ha,Hb}$ 13.9,β-Ha L-Phe), 3.010 (1 H, dd, $J_{\alpha\text{-H, Hb}}$ 8.8, $^2J_{Ha,Hb}$ 13.9, β-Hb L-Phe), 2.266–2.162 (1 H, m, H2′a Ta), 2.035 (1 H, ddd, $J_{1',2'b}$ 5.4, $J_{3',2'b}$ 1.5, $^2J_{2'a,2'b}$13.3, H2′b Ta), 1.745 (3 H, d, ^4J 1.1, 5-CH_3 Ta), 0.876 (9 H, s, *t*-BuSi), 0.085 (3 H, s, CH_3Si), 0.075 (3 H, s, CH_3Si).

Methyl (2R)-2-(3′-O-(tert-butyldimethylsilyl)thymidine-5′-carboxamido)-3-phenylpropanoate (3e)

CC solvent system: 3% of ethanol in CH_2Cl_2, yield 84.7%, R_f = 0.57 (Solvent system A). ^{1}HNMR (DMSO): 11.311 (1 H, s, H3 Ta), 8.672 (1 H, d, J 8.1, NH D-Phe), 7.897 (1 H, d, ^4J 1.1, H6 Ta), 7.310–7.174 (5 H, m, ArH), 6.268 (1 H, dd, $J_{1',2'a}$ 9.3,$J_{1',2'b}$ 5.4, H1′ Ta), 4.641 (1 H, ddd, $J_{NH,\alpha\text{-H}}$ 8.1, $J_{\alpha\text{-H},\beta\text{ Ha}}$ 5.0, $J_{\alpha\text{-H},\beta\text{-Hb}}$9.7, α-H D-Phe), 4.244 (1 H, br. s, H4′ Ta), 4.227–4.193 (1 H, m, H3′ Ta), 3.660 (3 H, s, CH_3O D-Phe), 3.121 (1 H, dd,$J_{\alpha\text{-H, Ha}}$ 5.0,$^2J_{Ha,Hb}$13.9, β-Ha D-Phe), 2.947 (1 H, dd, $J_{\alpha\text{-H, Hb}}$ 9.7, $^2J_{Ha,Hb}$13.9, β-Hb D-Phe), 2.145–2.054 (1 H, m, H2′a Ta), 1.989 (1 H, ddd, $J_{1',2'b}$ 5.4, $J_{3',2'b}$ 1.1, $^2J_{2'a,2'b}$12.7, H2′b Ta), 1.753 (3 H, d, ^4J1.1, 5-CH_3), 0.861 (9 H, s, *t*-BuSi), 0.058 (3 H, s, CH_3Si), 0.041 (3 H, s, CH_3Si).

Hydrolysis of Esters 3a–3e (General Procedure for Preparation of Acids 4a–4e)

To a stirred solution of the methyl ester (2 mmol) in ethanol (6 mL), 2 N KOH (2 mL) was added. The mixture was stirred for 2 hours at 20°C, diluted with water (12 mL) and pH was adjusted to neutral with 2 N HCl. The solution was evaporated to half its original volume and acidified to pH 2 with 2 N HCl. The lade-down sediment was filtered, washed with cold water at filter and dried to give **3a–3c** as white powder or **3d**, **3e** as colorless oil.

(3′-O-(tert-Butyldimethylsilyl)thymidine-5′-carboxamido)acetic acid (4a)

Yield 48%.^{1}HNMR: 12.688 (1 H, s, COOH Gly), 11.313 (1 H, s, H3 Ta), 8.447 (1 H, t, J 5.7, NH Gly), 7.893 (1 H, d, ^4J 0.7, H6 Ta), 6.338 (1 H, dd, $J_{1',2'a}$ 9.1, $J_{1',2'b}$ 5.5, H1′ Ta), 4.602–4.542 (1 H, m, H3′ Ta), 4.274 (1 H, d, $J_{3',4'}$ 0.8, H4′ Ta), 3.852 (1 H, dd, $J_{Ha,NH}$6.1, $^2J_{Ha,Hb}$ 17.5, Ha Gly), 3.767 (1 H, dd, $J_{Hb,NH}$5.7, $^2J_{Ha,Hb}$ 17.5, HbGly), 2.259–2.165 (1 H, m, H2′a Ta), 2.069 (1 H, ddd, $J_{1',2'b}$ 4.4, $J_{3',2'b}$ 1.7, $^2J_{2'a,2'b}$ 13.1, H2′b Ta), 1.768 (3 H, d, ^4J 0.7, 5-CH_3 Ta), 0.891 (9 H, s, *t*-BuSi), 0.120 (3 H, s, CH_3Si), 0.111 (3 H, s, CH_3Si).

(2S)-2-(3′-O-(tert-Butyldimethylsilyl)thymidine-5′-carboxamido)propanoic acid (4b)

Yield 81.5%. ^{1}HNMR: 12.659 (1 H, s, COOH L-Ala), 11.299 (1 H, s, H3 Ta), 8.434 (1 H, d, J 7.4, NH L-Ala), 7.958 (1 H, d, ^4J 1.0, H6 Ta), 6.295 (1 H, dd, $J_{1',2'a}$ 8.9, $J_{1',2'b}$ 5.6, H1′ Ta), 4.615–4.470 (1 H, m, H3′ Ta), 4.277 (1 H, qintet, J 6.8, CH L-Ala), 4.272 (1 H, d, $J_{3',4'}$ 1.1, H4′ Ta), 2.306 (1 H, ddd, $J_{1',2'a}$ 9.0, $J_{3',2'a}$ 5.1, $^2J_{2'a,2'b}$ 13.3, H2′a Ta), 2.069 (1 H, ddd, $J_{1',2'b}$ 5.2, $J_{3',2'b}$ 1.2, $^2J_{2'a,2'b}$ 13.3, H2′b Ta), 1.766 (3 H, d, ^4J 1.0, 5-CH_3 Ta), 1.318 (3 H, d, J 7.3, CH_3 L-Ala), 0.893 (9 H, s, *t*-BuSi), 0.123 (3 H, s, CH_3Si), 0.112 (3 H, s, CH_3Si).

(2R)-2-(3′-O-(tert-Butyldimethylsilyl)thymidine-5′-carboxamido)propanoic acid (4c)

Yield 87.6%. ^{1}HNMR: 12.737 (1 H, s, COOH D-Ala), 11.308 (1 H, s, H3 Ta), 8.472 (1 H, d, J 7.2, NH D-Ala), 7.911 (1 H, br. s, H6 Ta), 6.316 (1 H, dd, $J_{1',2'a}$ 8.4, $J_{1',2'b}$ 5.7, H1′ Ta), 4.615–4.470 (1 H, m, H3′ Ta), 4.369–4.141 (2 H, m, H4′; CH D-Ala), 2.298–2.152 (1 H, m, H2′a Ta), 2.088 (1 H, ddd, $J_{1',2'b}$ 5.2, $J_{3',2'b}$ 1.2, $^2J_{2'a,2'b}$ 12.8, H2′b Ta), 1.768 (3 H, br. s, 5-CH_3 Ta), 1.329 (3 H, d, J 7.3, CH_3 D-Ala), 0.897 (9 H, s, *t*-BuSi), 0.128 (3 H, s, CH_3Si), 0.117 (3 H, s, CH_3Si).

(2S)-2-(3′-O-(tert-Butyldimethylsilyl)thymidine-5′-carboxamido)propanoic acid (4d)

A modified procedure was used. After stirring for 2 hours at 20°C, the mixture was diluted with water (100 mL) and pH was adjusted to 3 with 2 N HCl. The mixture was extracted with $CHCl_3$ (3 × 30 mL). Combined organic layers were washed with water (30 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated. Yield 94.7%.^{1}HNMR: 12.831 (1 H, s, COOH L-Phe), 11.334 (1 H, s, H3 Ta), 8.372 (1 H, d, J 8.0, NH L-Phe), 7.739 (1 H, d, ^4J 1.2, H6 Ta), 7.310–7.157 (5 H, m, ArHL-Phe), 6.194 (1 H, dd, $J_{1',2'a}$9.1, $J_{1',2'b}$5.5, H1′ Ta), 4.541 (1 H, ddd, $J_{NH,\alpha\text{-}H}$8.0, $J_{\alpha\text{-}H,\beta\ Ha}$ 5.1, $J_{\alpha\text{-}H,\beta\text{-}Hb}$9.1, α-H L-Phe),4.340–4.275 (1H, m, H3′ Ta), 4.210 (1 H, d, $J_{3',4'}$ 1.4, H4′ Ta), 3.147 (1 H, dd, $J_{\alpha\text{-}H,Ha}$ 5.1, $^2J_{Ha,Hb}$ 13.9, β-Ha L-Phe), 3.010 (1 H, dd, $J_{\alpha\text{-}H,Hb}$9.1, $^2J_{Ha,Hb}$ 13.9, β-Hb L-Phe), 2.193 (1 H, ddd, $J_{1',2'a}$9.1, $J_{3',2'a}$5.4, $^2J_{2'a,2'b}$ 13.8, H2′a Ta), 2.098 (1 H, ddd, $J_{1',2'b}$ 5.5, $J_{3',2'b}$ 1.5, $^2J_{2'a,2'b}$ 13.8, H2′b Ta), 1.728 (3 H, d, ^4J 1.2, 5-CH_3 Ta), 0.889 (9 H, s, *t*-BuSi), 0.079 (3 H, s, CH_3Si), 0.071 (3 H, s, CH_3Si).

(2R)-2-(3′-O-(tert-Butyldimethylsilyl)thymidine-5′-carboxamido)propanoic acid (4e)

The compund was prepared by the same procedure as **4d**. Yield 87.6%.^{1}HNMR: 12.903 (1 H, s, COOH D-Phe), 11.299 (1 H, s, H3 Ta), 8.492 (1 H, d, J 8.5, NH D-Phe), 7.928 (1 H, d, ^4J 1.0, H6 Ta), 7.336–7.116

(5 H, m, ArH D-Phe), 6.278 (1 H, dd, $J_{1',2'a}$ 9.4, $J_{1',2'b}$ 5.3, H1′ Ta), 4.571 (1 H, ddd, $J_{NH,\alpha\text{-H}}$8.5, $J_{\alpha\text{-H},\beta\text{ Ha}}$ 5.1, $J_{\alpha\text{-H},\beta\text{-Hb}}$9.7, α-H L-Phe), 4.251 (1H, br. s, H4′ Ta), 4.228–4.184 (1 H, m, H3′ Ta), 3.142 (1 H, dd, $J_{\alpha\text{-H, Ha}}$5.1, $^2J_{\text{Ha, Hb}}$ 13.9, β-Ha D-Phe), 2.917 (1 H, dd, $J_{\alpha\text{-H, Hb}}$ 9.7, $^2J_{\text{Ha, Hb}}$13.9, β-HbD-Phe), 2.138–2.031 (1 H, m, H2′a Ta), 2.019- 1.922 (1 H, m, H2′b Ta), 1.754 (3 H, d, ^4J 1.0, 5-CH_3 Ta), 0.863 (9 H, s, *t*-BuSi), 0.058 (3 H, s, CH_3Si), 0.039 (3 H, s, CH_3Si).

N-[2-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-2-oxoethyl]-3′-O-(tert-butyldimethylsilyl)thymidine-5′-carboxamide (5a)

The compound was prepared following the general procedure described for **3a-3e**(amide formation with BOP as a condensing reagent) from 3′-amino-3′-deoxy-5′-*O*-dimethoxytritylthymidine (1 mmol) and acid **4a** (1.1 mmol) using 1.2 mmol of BOP and 2.4 mmol of TEA. CC solvent system: 5% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 86.3%, R_f = 0.38 (Solvent system C).^{1}HNMR: 11.309 (2 H, s, H3 Ta, H3 cT), 8.376 (1 H, d, *J* 7.4, 3′-NH Ta), 8.293 (1 H, t, *J* 5.9, NH Gly), 7.937 (1 H, d, ^4J 1.0, H6 cT), 7.544 (1 H, d, ^4J 1.0, H6 Ta), 7.423–6.843 (13 H, m, ArHDMTr), 6.343 (1 H, dd, $J_{1',2'a}$ 9.1, $J_{1',2'b}$ 5.5, H1′ cT), 6.235 (1 H, t, $J_{1',2'a,b}$ 6.6, H1′ Ta), 4.612–4.567 (1 H, m, H3′ cT), 4.547–4.462 (1 H, m, H3′, Ta), 4.292 (1 H, d, $J_{3',4'}$1.1, H4′ cT), 3.955–3.896 (1 H, m, H4′ Ta), 3.834 (1 H, dd, $J_{\text{Ha, NH}}$5.9, $^2J_{\text{Ha, Hb}}$ 16.5, Ha Gly), 3.729 (6 H, s, CH_3O DMTr), 3.692 (1 H, dd, $J_{\text{Hb, NH}}$5.9, $^2J_{\text{Ha, Hb}}$ 16.5, HbGly), 3.317 (1 H, dd, $J_{4',5'a}$ 4.7, $^2J_{5'a,5'b}$ 10.4, H5′a Ta), 3.210 (1 H, dd, $J_{4',5'b}$ 2.4, $^2J_{5'a,5'b}$ 10.4, H5′b Ta), 2.430–2.331 (1 H, m, H2′a Ta), 2.272–2.141 (2 H, m, H2′b Ta, H2′a cT), 2.048 (1 H, ddd, $J_{1',2'b}$ 5.5, $J_{3',2'b}$ 1.6, $^2J_{2'a,2'b}$ 12.0, H2′b cT), 1.722 (3 H, d, ^4J 1.0, 5-CH_3cT), 1.470 (3 H, d, ^4J 1.0, 5-CH_3 Ta), 0.883 (9 H, s, *t*-BuSi), 0.108 (3 H, s, CH_3Si), 0.098 (3 H, s, CH_3Si). MS: *m/z* 952.9. Calculated (calc.) 952.12 $[M–H]^-$ ($C_{49}H_{59}N_6O_{12}Si$).

General Procedure for Preparation of Dinucleosides 5b–5e (Condensation with DCC and NHS)

3′-Amino-3′-deoxy-5′-*O*-dimethoxytritylthymidine (0.41 g, 0.72 mmol) was dissolved in dry CH_2Cl_2, acid (0.79 mmol) and NHS (0.09 g, 0.72 mmol) were added. The mixture was cooled to 0°C in an ice-water bath with stirring, and DCC (0.16 g, 0.79 mmol) was added. The mixture was kept overnight at 4°C, then filtered. The filtrate was diluted with water (30 mL) and extracted with ethyl acetate (3 × 30 mL). Combined organic layers were washed with saturated $NaHCO_3$(aqueous) (30 mL) and water (30 mL). The organic layer was dried over Na_2SO_4, filtered and evaporated. The residue was purified by CC using the solvent systems specified below to give **5d–5e** as hard foam.

N-[(2S)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxopropane-2-yl]-3′-O-(tert-butyldimethylsilyl)thymidine-5′-carboxamide (5b)

CC solvent system: 3% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 83.3%, $R_f = 0.48$ (Solvent system B). (H5′a Ta signal is hidden under the signal of residual HOD. Identification by COSY-spectrum.) ^{1}HNMR: 11.298 (2 H, s, H3 Ta, H3 cT), 8.331 (1 H, d, *J* 7.8, 3′-NH Ta), 8.256 (1 H, d, *J* 7.6, NH L-Ala), 7.952 (1 H, d, ^4J 1.0, H6 cT), 7.557 (1 H, d, ^4J 1.0, H6 Ta), 7.427–6.837 (13 H, m, ArHDMTr), 6.320 (1 H, dd, $J_{1',2'a}$ 8.7, $J_{1',2'b}$ 5.7, H1′ cT), 6.225 (1 H, t, $J_{1',2'a,b}$ 6.4, H1′ Ta), 4.612–4.567 (2 H, m, H3′ cT, H3′ Ta), 4.294 (1 H, d, $J_{3',4'}$ 1.5, H4′ cT), 4.293 (1 H, quintet, *J* 7.4, CH L-Ala), 3.921–3.862 (1 H, m, H4′ Ta), 3.732 (6 H, s, CH_3O DMTr), 3.250–3.190 (2 H, m, H5′a Ta, H5′b Ta), 2.430–2.331 (2 H, m, H2′a cT, H2′a Ta), 2.223–2.131 (1 H, m, H2′b Ta), 2.063 (1 H, ddd, $J_{1',2'b}$ 5.7, $J_{3',2'b}$ 2.1, $^2J_{2'a,2'b}$ 13.4, H2′b cT), 1.756 (3 H, d, ^4J 1.0, 5-CH_3cT), 1.464 (3 H, d, ^4J 1.0, 5-CH_3 Ta), 1.184 (1 H, d, *J* 7.3, CH_3 D-Ala), 0.876 (9 H, s, *t*-BuSi), 0.103 (3 H, s, CH_3Si), 0.093 (3 H, s, CH_3Si). MS: *m/z* 966.8. Calc. 966.15 $[M–H]^-$ ($C_{50}H_{61}N_6O_{12}Si$).

N-[(2R)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxopropane-2-yl]-3′-O-(tert-butyldimethylsilyl)thymidine-5′-carboxamide (5c)

CC solvent system: 2% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 87.5%, $R_f = 0.6$ (Solvent system B).^{1}HNMR: 11.296 (2 H, s, H3 Ta, H3 cT), 8.476 (1 H, d, *J* 7.3, 3′-NH Ta), 8.293 (1 H, d, *J* 7.5, NH L-Ala), 7.983 (1 H, d, ^4J 0.9, H6 cT), 7.537 (1 H, d, ^4J 0.9, H6 Ta), 7.418–6.804 (13 H, m, ArHDMTr), 6.320 (1 H, dd, $J_{1',2'a}$ 8.8, $J_{1',2'b}$ 5.6, H1′ cT), 6.248 (1 H, t, $J_{1',2'a,b}$ 6.7, H1′ Ta), 4.537–4.448 (2 H, m, H3′ cT, H3′ Ta), 4.311 (1 H, d, $J_{3',4'}$ 1.4, H4′ cT), 4.273 (1 H, quintet, *J* 7.1, CH L-Ala), 3.961–3.922 (1 H, m, H4′ Ta), 3.717 (3 H, s, CH_3O DMTr), 3.712 (3 H, s, CH_3O DMTr), 3.204 (1 H, dd, $J_{4',5'b}$ 2.2, $^2J_{5'a,5'b}$ 10.3, H5′b Ta), 2.420–2.351 (1 H, m, H2′a Ta), 2.224–2.151 (2 H, m, H2′a cT, H2′b Ta), 2.038 (1 H, ddd, $J_{1',2'b}$ 5.7, $J_{3',2'b}$ 1.8, $^2J_{2'a,2'b}$ 13.3, H2′b cT), 1.656 (3 H, d, ^4J 0.9, 5-CH_3cT), 1.430 (3 H, d, ^4J 0.9, 5-CH_3 Ta), 1.237 (1 H, d, *J* 7.3, CH_3 D-Ala), 0.876 (9 H, s, *t*-BuSi), 0.099 (3 H, s, CH_3Si), 0.086 (3 H, s, CH_3Si). MS: *m/z* 990.4. Calc. 990.14 $[M + Na]^+$ ($C_{50}H_{62}N_6O_{12}SiNa$).

N-[(2S)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxo-3-phenylpropane-2-yl]-3′-O-(tert-butyldimethylsilyl)thymidine-5′-carboxamide (5d)

CC solvent system: 2% of ethanol in CH_2Cl_2+ 0.1% TEA, yield 89%, R_f = 0.42 (Solvent system B). ^{1}HNMR:11.332 (1 H, s, H3 cT), 11.299 (1 H, s, H3 Ta), 8.451 (1 H, d, *J* 7.6, 3′-NH Ta), 8.319 (1 H, d, *J* 8.3, NH L-Phe), 7.740 (1 H, d, ^4J 1.0, H6 cT), 7.537 (1 H, br. s, H6 Ta), 7.455–6.816 (18 H, m, ArHDMTr, ArHL-Phe), 6.271–6.125 (2 H, m, H1′ cT, H1′ Ta), 4.620–4.509 (1H, m, α-H L-Phe), 4.498–4.383 (1 H, m, H3′ Ta), 4.348–4.282 (1 H, m, H3′

cT), 4.231 (1 H, d, $J_{3',4'}$1.7, H4′ cT), 3.719 (7 H, br. s, CH_3O DMTr, H4′ Ta), 3.234–3.154 (2 H, m, H5′a Ta, H5′b Ta), 2.962–2.756 (2 H, m, β-Ha, β-Hb L-Phe), 2.414–2.278 (1H, m, H2′a Ta), 2.268–2.096 (2H, m, H2′a cT, H2′b Ta),2.033 (1 H, ddd, $J_{1',2'b}$5.4, $J_{3',2'b}$1.7, $^2J_{2'a,2'b}$12.9, H2′b cT), 1.719 (3 H, d, ^4J 1.0, 5-CH_3cT), 1.463 (3 H, d, ^4J0.7, 5-CH_3 Ta), 0.861 (9 H, s, *t*-BuSi), 0.069 (3 H, s, CH_3Si), 0.059 (3 H, s, CH_3Si). MS: *m/z*1068.1. Calculated 1066.23 $[M + Na]^+$ ($C_{56}H_{66}N_6O_{12}SiNa^+$).

N-[(2R)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxo-3-phenylpropane-2-yl]-3′-O-(tert-butyldimethylsilyl)thymidine-5′-carboxamide (5e)

CC solvent system:% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 92%, R_f = 0.51 (Solvent system B). ^{1}HNMR:11.291 (2 H, br. s, H3 cT, Ta), 8.549 (1 H, d, *J*7.3, 3′-NH Ta), 8.462 (1 H, d, *J* 8.5, NH D-Phe), 8.009 (1 H, d, ^4J 1.0, H6 cT), 7.563 (1 H, d, ^4J 0.6, H6 Ta), 7.425–6.812 (18 H, m, ArHDMTr, ArH D-Phe), 6.279 (1H, dd, $J_{1',2'a}$9.2, $J_{1',2'b}$5.4, H1′ cT), 6.209 (1 H, t, $J_{1',2'a,b}$ 6.6, H1′ Ta), 4.610 (1H, dt, $J_{\alpha\text{-H,NH}}$, $J_{\alpha\text{-H},\beta\text{-Hb}}$ 8.5, $J_{\alpha\text{-H,Ha}}$ 5.2, α-H D-Phe), 4.541–4.440 (1 H, d, H3′ Ta), 4.303 (1 H, br. s, H4′ cT), 4.149 (1 H, d,$J_{3',2'a}$3.9, H3′ cT), 3.948–3.885 (1H, m, H4′ Ta), 3.716 (6 H, s, CH_3O DMTr), 3.404–3.330 (1H, m, H5′a Ta), 3.192(1 H, dd, $J_{4',5'b}$ 2.4, $^2J_{5'a,5'b}$ 10.5, H5′b Ta), 3.013 (1H, dd, $J_{\alpha\text{-H,Ha}}$5.2, $^2J_{Ha,Hb}$ 13.8, β-Ha D-Phe), 2.830 (1 H, dd, $J_{\alpha\text{-H,Hb}}$8.5, $^2J_{Ha,Hb}$ 13.8, β-Hb D-Phe), 2.341 (1H, dd, $J_{1',2'a}$6.6, $^2J_{2'a,2'b}$ 12.5, H2′a Ta), 2.102 (1H, dd, $J_{1',2'b}$ 6.6, $^2J_{2'a,2'b}$ 12.3, H2′b Ta), 2.018 (1H, dd, $J_{1',2'a}$9.2, $J_{3',2'a}$3.9,$^2J_{2'a,2'b}$ 12.5, H2′a cT),1.954 (1H, dd, $J_{1',2'b}$ 5.4, $^2J_{2'a,2'b}$ 12.5, H2′b cT),1.643 (3 H, d, ^4J 1.0, 5-CH_3cT), 1.441 (3 H, d, ^4J 0.6, 5-CH_3 Ta), 0.861 (9 H, s, *t*-BuSi), 0.061 (3 H, s, CH_3Si), 0.031 (3 H, s, CH_3Si). MS: *m/z*1067.1. Calculated 1066.23 $[M + Na]^+$ ($C_{56}H_{66}N_6O_{12}SiNa^+$).

General Procedure for Cleavage of 3′-*O-tert*-butyldimethylsilyl Protection (Preparation of Dinucleosides 6a–6e)

To a stirred solution of a dinucleoside (0.73 mmol) in dry THF (1.5 mL), 1 M solution of tetrabutylammonium fluoride in dry THF (1.5 mL) was added. The mixture was stirred for 4 hours at 20°C, then TEA (0.2 mL) was added, and the mixture was partitioned between water (30 mL) and $CHCl_3$ (40 mL). The water layer was extracted with $CHCl_3$ (2 × 40 mL). Combined organic layers were washed with water, dried over Na_2SO_4, filtered, and concentrated. The residue was purified by silica gel column chromatography (CC) using the solvent systems specified below.

N-[2-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-2-oxoethyl]-thymidine-5′-carboxamide (6a)

CC solvent system: 15% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 86.1%, R_f = 0.4 (Solvent system D). ^{1}HNMR: 11.231 (1 H, s, H3 cT), 11.208 (1 H, s,

H3 Ta), 8.301 (1 H, d, J 7.4, 3′-NH Ta), 8.215 (1 H, t, J 5.9, NH Gly), 7.956 (1 H, d, ^4J 0.9, H6 cT), 7.464 (1 H, d, ^4J 0.9, H6 Ta), 7.364–6.747 (13 H, m, ArHDMTr), 6.305 (1, dd, $J_{1',2'a}$ 8.6, $J_{1',2'b}$ 6.1, H1′ cT), 6.155 (1 H, t, $J_{1',2'a,b}$ 6.6, H1′ Ta), 5.579 (1 H, d, J 4.2, 3′-OH cT), 4.479–4.377 (1 H, m, H3′ Ta), 4.368–4.307 (1 H, m, H3′ cT), 4.240 (1 H, d, $J_{3',4'}$0.8, H4′ cT), 3.884–3.816 (1 H, m, H4′ Ta), 3.725 (1 H, dd, $J_{Ha,NH}$ 6.1, $^2J_{Ha,Hb}$ 16.5, Ha Gly), 3.653 (6 H, s, CH_3O DMTr), 3.635 (1 H, dd, $J_{Hb,NH}$ 3.1, $^2J_{Ha,Hb}$ 16.5, Hb Gly), 3.232 (1 H, dd, $J_{4',5'a}$ 4.6, $^2J_{5'a,5'b}$ 10.3, H5′a Ta), 3.133 (1 H, dd, $J_{4',5'b}$ 2.2, $^2J_{5'a,5'b}$ 10.3, H5′b Ta), 2.352–2.257 (1 H, m, H2′a Ta), 2.152–2.064 (1 H, m, H2′b Ta), 2.053–1.957 (2 H, m, H2′a cT, H2′b cT), 1.647 (3 H, d, ^4J 0.9, 5-CH_3 cT), 1.393 (3 H, d, ^4J 0.9, 5-CH_3 Ta). MS: m/z 838.8. Calc. 837.86 $[M–H]^-$ ($C_{43}H_{45}N_6O_{12}$).

N-[(2S)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxopropane-2-yl]-thymidine-5′-carboxamide (6b)

CC solvent system: 10% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 92%, $R_f = 0.47$ (Solvent system C).^{1}HNMR: 11.294 (1 H, s, H3 cT), 11.276 (1 H, s, H3 Ta), 8.340 (1 H, d, J 7.9, 3′-NH Ta), 8.304 (1 H, d, J 7.5, NH L-Ala), 8.078 (1 H, d, ^4J 1.1, H6 cT), 7.546 (1 H, d, ^4J 0.9, H6 Ta), 7.427–6.833 (13 H, m, ArHDMTr), 6.338 (1 H, dd, $J_{1',2'a}$ 8.9, $J_{1',2'b}$ 5.7, H1′ cT), 6.217 (1 H, t, $J_{1',2'a,b}$ 6.4, H1′ Ta), 5.550 (1 H, d, J 4.2, 3′-OH cT), 4.548–4.452 (1 H, m, H3′ Ta), 4.358–4.221 (3 H, m, H3′ cT, H4′ cT, CH L-Ala), 3.904–3.844 (1 H, m, H4′ Ta), 3.734 (6 H, s, CH_3O DMTr), 3.242–3.184 (2, Hm, H5′a Ta, H5′b Ta), 2.410–2.309 (1 H, m, H2′a Ta), 2.222–2.113 (2 H, m, H2′a cT, H2′b Ta), 2.077 (1 H, ddd, $J_{1',2'b}$ 5.7, $J_{3',2'b}$ 1.8, $^2J_{2'a,2'b}$ 12.5, H2′b cT), 1.748 (3 H, d, ^4J 1.1, 5-CH_3 cT), 1.462 (3 H, d, ^4J 0.9, 5-CH_3 Ta), 1.175 (1 H, d, J 7.1, CH_3 L-Ala). MS: m/z 852.7. Calc. 851.88 $[M–H]^-$ ($C_{44}H_{47}N_6O_{12}$).

N-[(2R)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxopropane-2-yl]thymidine-5′-carboxamide (6c)

CC solvent system: 10% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 94.5%, $R_f = 0.44$ (Solvent system C).^{1}HNMR: 11.266 (2 H, s, H3 cT, H3 Ta), 8.454 (1 H, d, J 7.4, 3′-NH Ta), 8.211 (1 H, d, J 7.4, NH D-Ala), 8.092 (1 H, d, ^4J 0.9, H6 cT), 7.534 (1 H, d, ^4J 0.9, H6 Ta), 7.422–6.822 (13 H, m, ArHDMTr), 6.373 (1 H, t, $J_{1',2'a,b}$ 7.4, H1′ cT), 6.246 (1 H, t, $J_{1',2'a,b}$ 6.7, H1′ Ta), 5.557 (1 H, d, J 3.4, 3′-OH cT), 4.540–4.446 (1 H, m, H3′ Ta), 4.363–4.221 (2 H, m, H3′ cT, H4′ cT), 4.271 (1 H, quintet, J 7.19, CH D-Ala), 3.966–3.910 (1 H, m, H4′ Ta), 3.720 (3 H, s, CH_3O DMTr), 3.716 (3 H, s, CH_3O DMTr), 3.320 (1 H,dd, $J_{4',5'a}$ 4.2, $^2J_{5'a,5'b}$ 10.4, H5′a Ta), 3.206 (1 H, dd, $J_{4',5'b}$ 2.4, $^2J_{5'a,5'b}$ 10.4, H5′b Ta), 2.431–2.342 (1 H, m, H2′a Ta), 2.229–2.148 (2 H, m, H2′b Ta), 2.095–2.040 (2 H, m, H2′a cT, H2′b cT), 1.661 (3 H, d, ^4J 0.9, 5-CH_3 cT), 1.433 (3 H, d, ^4J 0.9, 5-CH_3 Ta), 1.236 (1 H, d, J 7.1, CH_3 D-Ala). MS: m/z 876.2. Calc. 875.87 $[M + Na]^+$ ($C_{44}H_{48}N_6O_{12}SiNa$).

N-[(2S)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxo-3-phenylpropane-2-yl]-thymidine-5′-carboxamide (6d)

CC solvent system: 7% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 89.1%, $R_f = 0.59$ (Solvent system D). ^{1}HNMR: CC solvent system:% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 92%, $R_f = 0.51$ (Solvent system B). ^{1}HNMR: 11.343 (1 H, s, H3 cT), 11.320 (1 H, s, H3 Ta), 8.510 (1 H, d, J 7.8, 3′-NH Ta), 8.443 (1 H, d, J 8.2, NH L-Phe), 7.948 (1 H, d, ^4J0.9, H6 cT), 7.583 (1 H, br. s, H6 Ta), 7.480–6.835 (18 H, m, ArHDMTr, ArHL-Phe), 6.302 (1H, t, $J_{1',2'a,b}$7.8, H1′ cT), 6.241 (1 H, t, $J_{1',2'a,b}$ 6.5, H1′ Ta), 5.456 (1H, d, J 4.3, 3′-OH cT), 4.609–4.452 (2H, m, α-H D-Phe, H3′ Ta), 4.322 (1 H, br. s, H4′ cT), 4.259 (1H, br. s, H3′ cT), 3.826–3.701 (7H, m, H4′ Ta, CH_3O DMTr), 3.240 (2H, br. s, H5′CH_2 Ta), 2.931–2.775 (2H, m, β-Ha, β-Hb D-Phe), 2.393 (1H, ddd,$J_{1',2'a}$, 6.5,$J_{3',2'a}$, 7.0,$^2J_{2'a,2'b}$12.9, H2′a Ta), 2.204 (1H, dt, $J_{1',2'b}$, $J_{3',2'b}$ 6.5, $^2J_{2'a,2'b}$12.9, H2′b Ta), 2.139–2.056(2H, m, H2′a, bcT), 1.753 (3 H, br. s, 5-CH_3cT), 1.510 (3 H, br.s, 5-CH_3 Ta). MS: m/z 952.3. Calculated 952.8 $[M + Na]^+$ ($C_{50}H_{52}N_6O_{12}Na^+$).

N-[(2R)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxo-3-phenylpropane-2-yl]thymidine-5′-carboxamide (6e)

CC solvent system: 7% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 96.3%, $R_f = 0.57$ (Solvent system D). ^{1}HNMR:11.314 (1 H, s, H3 cT), 11.259 (1 H, s, H3 Ta), 8.499 (1 H, d, J 7.4, 3′-NH Ta), 8.281 (1 H, d, J 8.4, NH D-Phe), 8.039 (1 H, d, ^4J 1.0, H6 cT), 7.503 (1 H, d, ^4J 0.7, H6 Ta), 7.428–6.764 (18 H, m, ArHDMTr, ArH D-Phe), 6.312 (1H, dd, $J_{1',2'a}$ 5.4, $J_{1',2'b}$ 9.4, H1′ cT), 6.201 (1 H, t, $J_{1',2'a,b}$ 6.6, H1′ Ta), 5.456 (1H, d, J 4.1, 3′-OH cT), 4.573 (1H, dt, $J_{\alpha\text{-H,NH}}$, $J_{\alpha\text{-H},\beta\text{-Hb}}$8.5, $J_{\alpha\text{-H,Ha}}$ 5.7, α-H D-Phe), 4.502–4.405 (1 H, m, H3′ Ta), 4.310 (1 H, br. s, H4′ cT), 4.029 (1H, t, $J_{3',3'\text{-OH}}$, $J_{3',2'b}$ 4.1, H3′ cT), 3.956–3.869 (1H, m, H4′ Ta), 3.712 (6 H, s, CH_3O DMTr), 3.371–3.295 (1H, m, H5′a Ta), 3.201 (1 H, dd, $J_{4',5'b}$2.3, $J_{5'a,5'b}$ 10.5, H5′b Ta), 3.003 (1H, dd, $J_{\alpha\text{-H,Ha}}$ 5.7, $J_{Ha,Hb}$ 13.7, β-Ha D-Phe), 2.830 (1 H, dd, $J_{\alpha\text{-H,Hb}}$8.5, $J_{Ha,Hb}$ 13.7, β-HbD-Phe), 2.321 (1H, dt$J_{1',2'a}$, $J_{3',2'a}$ 6.6, $^2J_{2'a,2'b}$13.3, H2′a Ta), 2.080 (1H,ddd,$J_{1',2'b}$6.6, $J_{3',2'b}$ 6.3, $^2J_{2'a,2'b}$13.3,H2′b Ta), 1.980 (1H, dd, $J_{1',2'a}$ 5.4, $^2J_{2'a,2'b}$ 12.9, H2′a cT), 1.880 (1H, ddd, $J_{1',2'b}$9.4, $J_{3',2'b}$4.1, $^2J_{2'a,2'b}$12.9, H2′bcT), 1.663 (3 H, d, ^4J 1.0, 5-CH_3cT), 1.461 (3 H, d, ^4J 0.7, 5-CH_3 Ta). MS: m/z 952.3. Calculated 951.97 $[M + Na]^+$ ($C_{50}H_{52}N_6O_{12}Na^+$).

General Procedure for Preparation of Amidites 7a–7e

To a stirred solution of a dinucleoside (0.8 mmol) in dry CH_2Cl_2 (3 mL), pyridine (0.19 mL, 2.4 mmol) and tetrazole (0.11 g, 1.6 mmol) were added, and the reaction mixture was stirred for 30 minutes at 20°C. Then 2-cyanoethyl-*N,N,N′,N′*-tetraisopropylphosphoramidite in CH_2Cl_2 (2 mL) was added, and the mixture was stirred for 2 hours at 20°C. TEA (0.1 mL) was

added, the mixture was partitioned between water (20 mL) and CH_2Cl_2 (30 mL). The water layer was extracted with CH_2Cl_2 (2 mL). Combined organic layers were washed with water (30 mL), saturated NaCl (aqueous) (30 mL). The organic layer was dried over Na_2SO_4, filtered and concentrated. The residue was purified by CC using the solvent systems specified below.

N-[2-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-2-oxoethyl]-3′-deoxy-3′-[(2-cyanoethoxy)(diisopropylamino)phosphinooxy]thymidine-5′-carboxamide (7a)

CC solvent system: 5% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 76%, R_f = 0.49 (Solvent system D).^{31}P NMR: 150.79, 150.24.MS: *m/z* 1039.0. Calculated 1038.08 $[M–H]^-$ ($C_{52}H_{62}N_8O_{13}P$).

N-[(2S)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxopropane-2-yl]- 3′-deoxy-3′-[(2-cyanoethoxy)(diisopropylamino) phosphinooxy]thymidine-5′-carboxamide (7b)

CC solvent system: 4% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 73%, R_f = 0.54 (Solvent system C). ^{31}P NMR: 150.53, 150.20.MS: *m/z* 1053.2. Calculated 1052.10 $[M–H]^-$ ($C_{53}H_{64}N_8O_{13}P$).

N-[(2R)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxopropane-2-yl]-3′-deoxy-3′-[(2-cyanoethoxy)(diisopropylamino) phosphinooxy]thymidine-5′-carboxamide (7c)

CC solvent system: 4% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 76%, R_f = 0.56 (Solvent system C). ^{31}P NMR: 150.51, 150.26. MS: *m/z* 1052.7. Calculated 1052.10 $[M–H]^-$ ($C_{53}H_{64}N_8O_{13}P$).

N-[(2S)-1-{(5′-O-(4,4′-Dimethoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxo-3-phenylpropane-2-yl]-3′-deoxy-3′-[(2-cyanoethoxy)(diisopropylamino) phosphinooxy]thymidine-5′-carboxamide (7d)

CC solvent system: 2% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 76%, R_f = 0.79 (Solvent system B).^{31}P NMR: 150.47, 150.25. MS: *m/z* 1152.7. Calculated 1152.19 $[M–Na]^+$ ($C_{59}H_{69}N_8O_{13}PNa^+$).

N-[(2R)-1-{(5′-O-(4,4′-Dimetoxytrityl)-3′-deoxythymidine-3′-yl)amino}-1-oxo-3-phenylpropane-2-yl]-3′-deoxy -3′-[(3′-deoxy-3′-[(2-cyanoethoxy) (diisopropylamino)phosphinooxy]thymidine-5′-carboxamide (7e)

CC solvent system: 2% of ethanol in CH_2Cl_2 + 0.1% TEA, yield 77.2%, R_f = 0.78 (Solvent system B).^{31}P NMR: 151.21, 150.56. Mass spectrum: *m/z* 1128.7. Calculated 1128.20 $[M+ H]^-$ ($C_{59}H_{69}N_8O_{13}P$).

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