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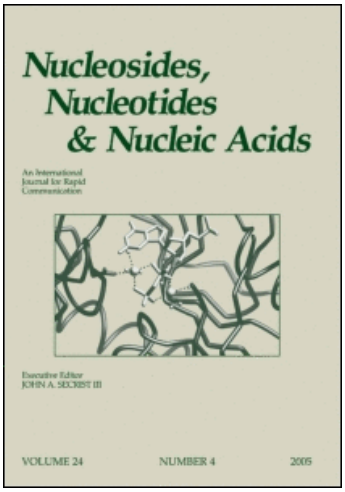

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Zoltán Bajor[a]; Gyula Sági[ab]; Zsuzsanna Tegyey[a]; Ferenc Kraicsovits[a]
[a] Chemical Research Center, Institute of Chemistry, Hungarian Academy of Sciences, Budapest, Hungary [b] Chemical Research Center, Institute of Chemistry, Hungarian Academy of Sciences, Hungary

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PNA-DNA Chimeras Containing 5-Alkynyl-pyrimidine PNA Units. Synthesis, Binding Properties, and Enzymatic Stability

Zoltán Bajor, Gyula Sági,* Zsuzsanna Tegyey,† and Ferenc Kraicsovits

Chemical Research Center, Institute of Chemistry, Hungarian Academy of Sciences, Budapest, Hungary

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ABSTRACT

Three chimeric dimer synthons (oeg_t^{NH}T, oeg_up^{NH}T and oeg_uh^{NH}T) containing thymine (t), 5-(l-propynyl)-uracil (up) and 5-(1-hexyn-1-yl)-uracil (uh) PNA units with *N*-(2-hydroxyethyl)glycine (oeg) backbone were synthesized in solution and incorporated into T_{20} oligonucleotide analogues, using standard P-amidite chemistry. Insertion of dimer blocks led to destabilization of duplexes with dA_{20} target. The smallest T_m drops were found for chimeras containing oeg_up^{NH}T dimers. Incorporation of the chimeric synthons into the 3′-end of T_{20} brought about growing resistance to 3′-exonucleolytic (SV PDE) cleavage in the order of oeg_t^{NH}T < oeg_up^{NH}T < oeg_uh^{NH}T. Due to different endonuclease activities of 3′- and 5′-exonucleases applied, placing of five consecutive dimers at the 5′-terminus resulted in a relatively smaller, but also side-chain dependent, stabilization towards the hydrolysis by 5′-exonuclease (BS PDE). Neither exonucleases (SV and BS PDE) nor an endonuclease (Nuclease P_1) could

*Correspondence: Gyula Sági, Chemical Research Center, Institute of Chemistry, Hungarian Academy of Sciences, 59–67 Pusztaszeri St., 1025 Budapest, Hungary; Fax: +36-1-438-4134; E-mail: gsagi@chemres.hu.
†Deceased.

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hydrolyse the unnatural phosphodiester bond linking the 3′-OH of thymidine to the terminal OH of *N*-(2-hydroxyethyl)glycine PNA backbone.

Key Words: 5-Alkynyl-uracils; PNA-DNA chimeras; T_{20}-analogues; Melting properties; Nuclease stability.

INTRODUCTION

Since their first synthesis by Nielsen et al.[1] peptide nucleic acids (PNA-s), containing *N*-(2-aminoethyl)glycine instead of sugar-phosphate backbone of the natural nucleic acids, have attracted great interest due to their higher duplex stability and complete resistance to both nucleases and proteases. However, the more hydrophobic pure PNA-s are prone to self-aggregation[2] in addition, they are unable to induce RNase H activity[3] which plays important role in the mechanism of action of antisense oligos. Their celluar uptake was also found to be lower[4,5] than expected earlier and they can form less stable, parallel duplexes, too.[2,6] Since these disadvantageous properties are hurdles in the application of PNA-s, as potential antisense or antigene drugs, some trials have been made to eliminate them.

According to the thorough and extended studies by Uhlmann et al.[2,6] PNA-DNA chimeras consisting of PNA and DNA blocks retain the RNase H inducing ability of natural DNA, in addition, they can form only antiparallel duplexes with the complementary DNA or RNA strands. Binding affinity of chimeras, consisting of separate homogenous PNA and DNA blocks, strongly depends on the PNA/DNA ratio.[2,7] In general, the larger is the proportion of PNA units in a chimera the higher is the T_m value of a chimera:DNA duplex relative to the native DNA:DNA counterpart. The increase of thermal stability is even higher in cases of chimera:RNA duplexes. However, beside the PNA proportion and the complementary strand, the degree of duplex stabilization depends on the type of chimera as well.[8,9] In cases of pseudo-5′-PNA-DNA-3′ chimera:DNA duplexes a strong structural perturbation occurs at the PNA-DNA junction which is likely due to the rigid amide bond between the PNA carboxy group and the 5′-amino group of modified terminal nucleotide. Therefore insertion of a single PNA unit into a DNA results in a significant drop of T_m especially when it is placed in the middle of the sequence.[10,11] However, when PNA units are positioned at the 3′- or 5′-terminus it leads to a relatively small decrease of T_m value.[11] Serum stability of PNA-DNA chimeras has also been investigated. A model 13-mer chimera with two PNA units at the 5′- and 3′-ends was found to be about 25 times more stable in human serum than the corresponding DNA counterpart or the pseudo-5′-PNA-DNA-3′ hybrids.[10] However, 5′-DNA-PNA-pseudo-3′ chimeras proved 50 times more stable in fetal calf serum relative to the DNA analogue,[12] which can also be explained by the major 3′-exonuclease activity in the serum. Nevertheless there are no data available about the stability of chimeras in the presence of pure endo- or exonucleases. The cellular uptake and water solubility of PNA-s can also be enhanced by their incorporation into PNA-DNA chimeras. Thus, the degree and

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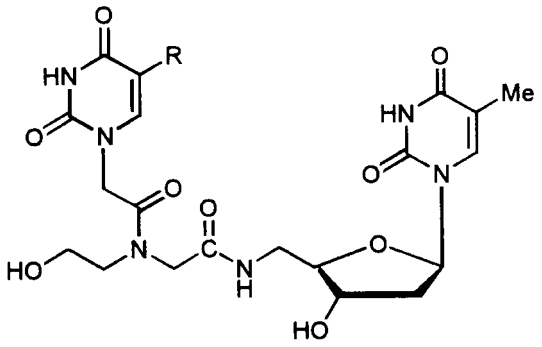

R = Me: oeg_ $t^{NH}T$; R = 1-propynyl: oeg_up^{NH}T; R = 1-hexyn-1-yl: oeg_uh^{NH}T

Figure 1. Dimer synthons incorporated into T_{20}-analogue chimeras.

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kinetics of uptake become similar to those observed for the pure DNA analogue. Another alternative way to increase the cell-penetration of PNA-s is their conjugation with Trojan peptides[13] (transportan and penetratin) which led to considerable increase of their in vitro[14] and in vivo[15] activity.

According to our and others' earlier studies on the biophysical and biochemical properties of base-modified DNA-s, the 5-alkynyl substitution of pyrimidine bases results in a significant duplex stabilization[16,17] and a higher resistance to enzymatic cleavage.[18] However, propynyl residues introduced into the 7-position of 8-aza-7-deazapurines were found to be even more stabilizing than those at the 5-position of pyrimidine bases.[19] In addition, in the case of a heptanucleotide, composed of exclusively 5-propynyl-pyrimidine deoxynucleotides, long-range cooperativity among the propynyl groups[20] and enhanced mismatch penalties[21] were observed, compared to the dT and dC containing counterparts. In correlation with the former beneficial biochemical properties, P-thio antisense oligonucleotides, containing 5-(1-propynyl)-dU and 5-(1-hexyn-1-yl)-dU units, respectively in place of thymidines, exhibited considerably higher antiviral[22] and antitumor[23] activity in vitro.

To extend these studies to PNA-DNA chimeras and to investigate the biophysical and biochemical properties of chimeras with alternating PNA and DNA units numerous T_{20} analogues, containing thymine, 5-(propyn-1-yl)-uracil or 5-(1-hexyn-1-yl)-uracil PNA monomers with *N*-(2-hydroxyethyl)glycine backbone (oeg_t, oeg_up, or oeg_uh), using 5′-amino-5′-deoxythymidine (^{NH}T) as PNA-DNA linker, have been synthesized. Application of oeg_t^{NH}T, oeg_up^{NH}T and oeg_uh^{NH}T hybrid dimer synthons (see Fig. 1) made possible the exclusive use of standard solid phase P-amidite DNA synthesis protocol for the synthesis of chimeras without employing any PNA coupling chemistry.

Hydrolysis of chimeras by exo- and endonucleases (SV PDE, BS PDE and Nuclease P_1) and thermal stability of chimera:dA_{20} duplexes have been investigated. Results are summarized in the present paper.

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RESULTS AND DISCUSSION

Synthesis

Starting from 5-iodo-uracil (**1**) first we prepared the 5-propynyl- and 5-(1-hexyn-1-yl) uracils (**2** and **3**) via Pd-catalyzed coupling of **1** with the corresponding terminal alkynes in dry DMF (see Sch. 1), by the analogy of the synthesis of 5-alkynyl-2′-deoxyuridines.[24] Carboxymethylation of **2** and **3** with bromoacetic acid in aq. NaOH solution was carried out according to the procedure reported for similar alkylation of thymine[25] resulting in the required N^1-carboxymethyl-5-substituted-uracils (**4**, **5** and **6**) with good yields. According to the comparative study of Greiner et al.[26] to attain the highest duplex stability in a 5′-DNA-PNA-pseudo-3′ chimera, the phosphodiester bridge is the most favoured linkage at the DNA-PNA junction. Therefore we coupled our base-acetic acid derivatives (**4**, **5** and **6**) to *N*-(2-hydroxyethyl)glycine *tert*-butyl ester with minor modification of the literature method.[27] Thus we isolated the required *N*-(2-hydroxyethyl)-*N*-[(5-substituted-pyrimidin-1-yl)acetyl]glycine *tert*-butyl esters (**7**, **8** and **9**) with acceptable yields. Contrary to the strategy described the coupled products were first dimethoxytritylated in dry pyridine to give the corresponding DMT-protected intermediates. Due to the weak nucleophilicity of OH group under these conditions we could not detect any unrequired lactone by-product in the reaction mixture.

The crude DMT-protected esters were saponified with NaOH in aq. dioxane then carefully acidified to pH $\sim$ 5 to give the required free acids (**10**, **11** and **12**), which were isolated as triethylammonium salts in good overall yields (67–87%) after the necessary silica gel chromatographic purifications. The coupling of acids with 5′-amino-5′-deoxythymidine[28] (**13**) in DMF, in the presence of NEt_3 and TBTU, as activating agent, proceeded smoothly within 2 h in each case resulting in the 3′-free chimeric dimer synthons: oeg_t^{NH}T, oeg_up^{NH}T and oeg_uh^{NH}T (**14**, **15** and **16**) in DMT-protected form. The 3′-free dimers were then phosphitylated according to standard method to give the corresponding 3′-O-(2-cyanoethyl-N,N-diisopropyl)-phosphoramidites (**17**, **18** and **19**), as internal building blocks for the solid-phase syntheses (see Sch. 2). On the other hand, for the 3′-terminal incorporation we prepared the corresponding dimer-3′-O-succinates (**20**, **21** and **22**) which were then coupled to the solid support (LCAA-CPG, pore size 500 Å) in the presence of EEDQ, as condensing agent.

On the basis of DMT assays the loading of CPG varied from 35 to 43 μmol/g. According to the DMT-absorbance measurements coupling yields with the dimer-P-amidites were found to be 84–88% using the standard coupling programme of Synthesizer (total coupling time: 54 sec.) In order to improve the efficiency of these couplings double coupling and 4-fold waiting times were applied resulting in somewhat higher coupling yields (91–94%). The overall yields thus obtained correlated with the proportion of DMT-on final products in the crude mixtures, as determined by HPLC. As it can be seen in Table 1, the synthesized 20-mer chimeras may be arranged into three sets of trios according to the position of hybrid dimer units. In the first set (**24**, **25** and **26**) the chimeric blocks occupy a middle position, while in the second one (**27**, **28** and **29**) they are placed at the 3′-end. Chimeras of the third set (**30** ,**31** and **32**) begin with a decamer of alternate PNA and DNA units at the 5′-terminus. T_{20} (**23**), T_{11} (**33**) and the **34** chimera served as reference compounds.

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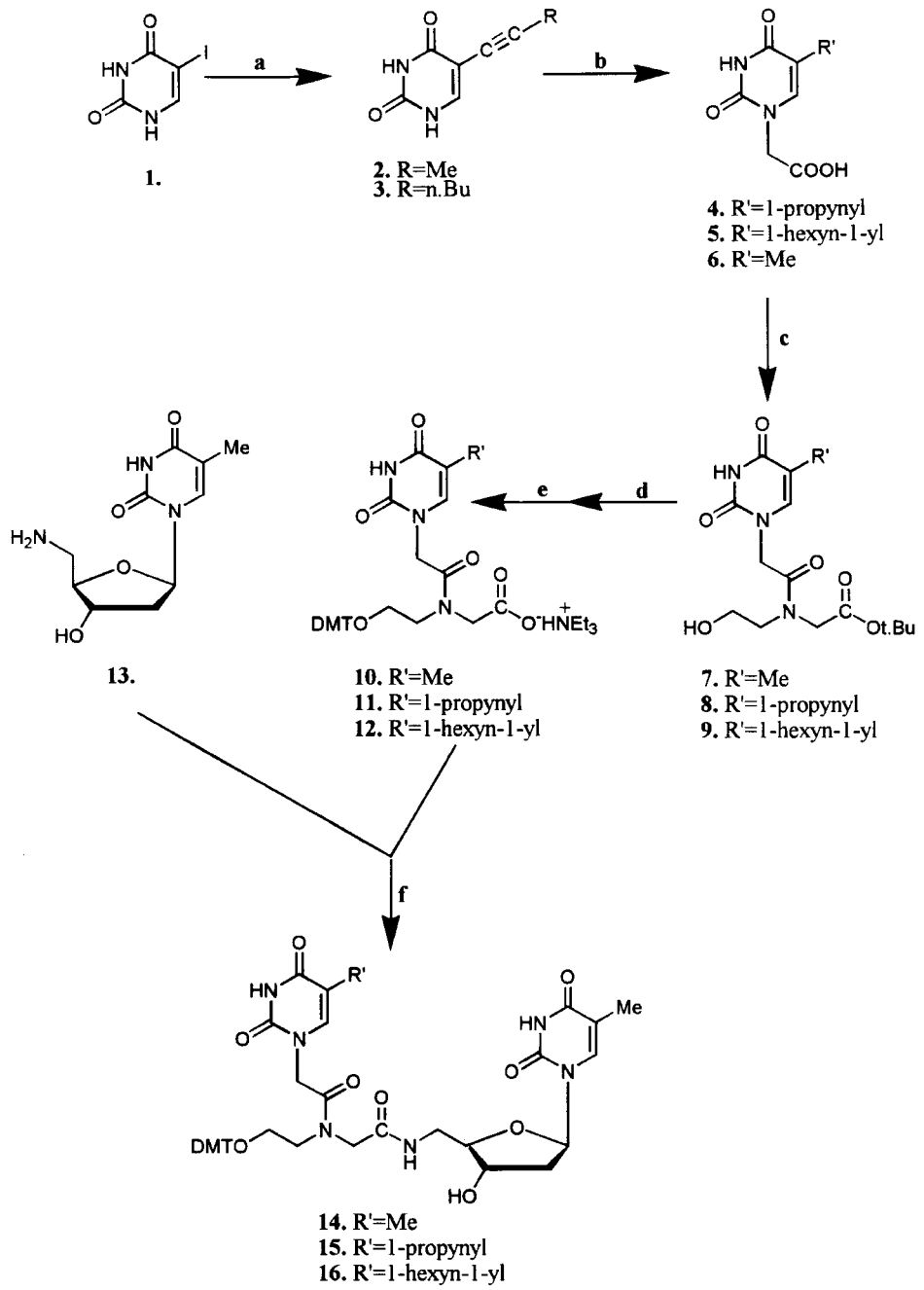

Scheme 1. (a) HC≡C–R, $(Ph_3P)_2PdCl_2$, CuI, Et_3N, DMF, rt, 18 h; (b) $BrCH_2$–COOH, 1 N aq. NaOH, cc. aq. HCl; (c) (i) HO–$(CH_2)_2$–NH–CH_2–COOt.Bu, TBTU, Et_3N, DMF, rt, 3 h; (ii) recrystallization from EtOAc; (d) (i) DMT-Cl, pyridine, rt, 18 h (ii) SiO_2 chrom; (e) (i) dioxane-water 4:1, 1 N aq. NaOH, pH = 13, rt, 3 h; (ii) 1M aq. $NaHSO_4$ to pH ~ 5.0; (iii) SiO_2 chrom; (f) TBTU, Et_3N, DMF, rt, 2 h, DCFC.

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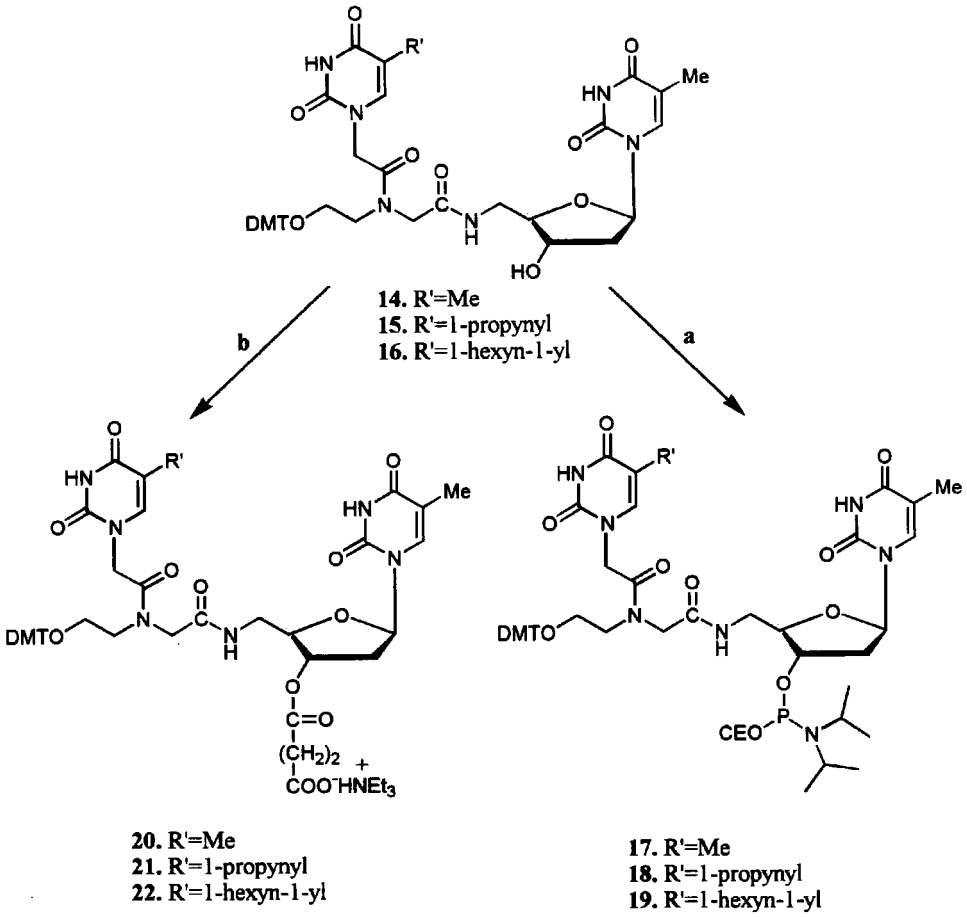

Scheme 2. (a) (i) $(i.Pr_2N)_2$–P–OCE, DIPAT, CH_2Cl_2, rt, 18 h; (ii) SiO_2 chrom; (b) (i) Succinic anhydride, DMAP, Et_3N, CH_2Cl_2, rt, 18 h; (ii) SiO_2 chrom.

Thermal Melting Studies

As shown in Table 1, placing of one oeg_t^{NH}T or oeg_uh^{NH}T dimer unit in the middle of T_{20} (**24** and **26**) causes about −9°C lowering of T_m value relative to that of the reference T_{20}:dA_{20} duplex. However, in the case of T_9-oeg_up^{NH}T-T_9 (**25**) the T_m-drop is only −7.6°C. The same substitutions at the 3′-end (**27**, **28** and **29**) have less pronounced effect. The smallest drop of the T_m value (−1.8°C) was found again for the propynyl-substituted analogue (**28**). However, chimera:dA_{20} duplexes having a decamer block of alternate PNA and DNA units at the 5′-terminus (**30**, **31** and **32**) showed significant drops (approx. 16–20°C) in T_m values. Thermal stabilities of these duplexes were close to that of T_{11}:dA_{11} suggesting that chimeric parts of these analogues are hardly involved in cooperative binding to the complementary strand. According to Bergmann et al.[10] a phosphodiester → phosphamide exchange

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Table 1. Melting points of the reference and chimera:dA_{20} duplexes.[a]

Compd.	Sequence	T_m (°C)	ΔT_m (°C)[b]	ΔT_m (°C)[c]
23	T_{20}	57.4	0	
24	T_9-oeg_t^{NH}T-T_9	48.3	−9.1	0
25	T_9-oeg_up^{NH}T-T_9	49.8	−7.6	+1.5
26	T_9-oeg_uh^{NH}T-T_9	48.5	−8.9	+0.2
27	T_{18}-oeg_t^{NH}T	54.7	−2.7	0
28	T_{18}-oeg_up^{NH}T	55.6	−1.8	+0.9
29	T_{18}-oeg_uh^{NH}T	55.0	−2.4	+0.3
30	(oeg_t^{NH}T)$_5$-T_{10}	36.7	−20.7	0
31	(oeg_up^{NH}T)$_5$-T_{10}	41.6	−15.8	+4.9
32	(oeg_uh^{NH}T)$_5$-T_{10}	37.9	−19.5	+1.2
33	T_{11}	38.6	−18.8	
34	(T_2-oeg_t^{NH}T)$_5$	11.3	−46.1	

[a]Measured in 0.5 M NaCl, 10 mM $MgCl_2$, 20 mM Tris (pH 7.4) buffer.
[b]For these ΔT_m values the T_m of T_{20}:dA_{20} duplex (57.4 °C) is the reference.
[c]For these ΔT_m values T_m − s of the corresponding oeg_t^{NH}T containing chimera:dA_{20} duplexes are the references.

in the middle of a DNA sequence results in T_m drops of 1.0–2.4°C, depending on the modified nucleoside. The lower T_m value of our (oeg_t^{NH}T)$_5$-T_{10}:dA_{20} duplex (36.7°C) relative to that of T_{11}:dA_{11} (38.6°C) might also be explained by this small destabilizing effect. (However, if the 5 oeg_t^{NH}T blocks are alternating with TT in the full length of sequence (see compd. **34**) it results in dramatic decrease of the T_m ($\Delta T_m = -46.1$°C), consequently this arrangement may cause full destruction of the duplex structure.) Similarly to the first and second set, the smallest T_m-drop (−15.8°C) was found again for the chimera having five oeg_up^{NH}T dimers (**31**). Compared to **30**, it means that, in spite of the alternating mixed backbone structure, the propynyl group still has a duplex stabilization effect of about $\Delta T_m = +1.0$°C/dimer unit, relative to the methyl group. In the case of **25** and **28** similar T_m rises ($\Delta T_m = +1.5$ and +0.9°C, respectively) were observed which can be attributed to the known increased π–π stacking interaction between the neighbouring bases, caused by the conjugated triple bond.[17] A similar, but much smaller effect ($\Delta T_m = +0.2$–0.3°C/dimer unit in all the three sets) was observed for the hexynyl group (compounds **26**, **29**, and **32**). In these cases the duplex stabilizing effect of triple bond is compensated by the hydrophobic destabilizing effect of long alkyl chain, which protrudes into the major groove and probably disrupts the water structure around the phosphate moiety.[29,30]

Enzymatic Hydrolyses

The 3′-modified analogues containing one dimer block at the 3′-end (**27**, **28** and **29**) were hydrolysed by snake venom phosphodiesterase (SV PDE) and were found to be 33–53 times more resistant than T_{20}, as shown by the corresponding 1/R values (see Table 2). In accordance with our earlier results[18] the alkynyl analogues (**28** and

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Table 2. SV PDE hydrolysis half-times of chimeras modified at the 3′-end.[a]

Compd.	Sequence	$t_{1/2}$ (min)	Relative rate (R)	1/R
23	T_{20}	1.3	1	1
27	T_{18}-oeg_t^{NH}T	43	3.02×10^{-2}	33.1
28	T_{18}-oeg_up^{NH}T	58	2.24×10^{-2}	44.6
29	T_{18}-oeg_uh^{NH}T	69	1.88×10^{-2}	53.1

[a]Hydrolysis mixtures were incubated at 37°C, in 5 mM $MgCl_2$, 50 mM Tris (pH 8.8) buffer. The enzyme (phosphodiesterase I. from Crotalus adamanteus venom, SIGMA) concentrations were 4×10^{-3} and 4×10^{-4} unit/mL for chimeras and T_{20}, respectively. The substrate concentration was 4 A_{260} unit/mL in each experiment. Determination of $t_{1/2}$ values is described in the experimental.

29) proved more stable compard to the oeg_t^{NH}T-containing counterpart (**27**). It can be seen that the longer is the 5-side chain of pyrimidine base in the PNA moiety the more resistant is the chimera to the 3′-exonucleolytic cleavage. The $t_{1/2}$ values of propynyl and hexynyl analogues (**28** and **29**) were 1.3 and 1.6 times higher, respectively compared to that of **27**.

The resistance to SV PDE hydrolysis can also be enhanced significantly by incorporation of more consecutive dimer blocks. According to our recent findings, reported in a preliminary form,[31] positioning of 1, 2 or 3 oeg_t^{NH}T blocks at the 3′-end of T_{12} resulted in 50-, 96- and 145-fold increase of hydrolysis half-time, respectively relative to the $t_{1/2}$ of T_{12}. In addition, RP HPLC profiles of the final hydrolysis mixtures, after 1 day digestion, displayed different retention times (t_R) for the peaks of chimeric hydrolysis products. These t_R values increased with increasing number of dimer blocks incorporated, indicating longer and longer chimeric fragments. It proves that even the double and triple chimeric blocks remained intact, i.e., the enzyme could not hydrolyse the unnatural phosphodiester bond between the 3′-OH of thymidine and the terminal OH of *N*-(2-hydroxyethyl)glycine PNA backbone. It is in good agreement with earlier results[32,33] connected with the enzymatic stability of oligonucleotides containing different acyclic nucleosides.

The 5′-modified analogues (**30**, **31** and **32**) were hydrolysed by bovine spleen phosphodiesterase (BS PDE). Compared to the SV PDE hydrolyses, in these cases 250 times higher enzyme concentrations (expressed in unit/mL) were necessary to attain measurable hydrolysis rates. These chimeras, which contain five chimeric dimer blocks at the 5′-terminus, in spite of the much longer chimeric strands, were found to be only 6–8 times more resistant than the reference T_{20} (see Table 3). This resistance also increased with increasing length of 5-alkynyl substituent of the PNA-uracil moiety. The propynyl analogue (**31**) and the hexynyl one (**32**) were 1.3 and 1.4 times more stable, respectively than the oeg_t^{NH}T containing counterpart (**30**).

Analogous incorporation of 1, 2 or 3 oeg_t^{NH}T units into the 5′-terminus of T_{12} also led to increasing resistance to the hydrolysis by BS PDE. As indicated by the HPLC profiles, similarly to those of the previous digestions by SV PDE, the longer chimeric blocks also remained intact which refers to the complete resistance of this phosphodiester bond to the 5′-exonucleolytic cleavage, too.

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Table 3. BS PDE hydrolysis half-times of chimeras modified at the 5′-end.[a]

Compd.	Sequence	$t_{1/2}$ (min)	Relative rate (R)	1/R
23	T_{20}	7.7	1	1
30	(oeg_t^{NH}T)$_5$-T$_{10}$	45	0.17	5.8
31	(oeg_up^{NH}T)$_5$-T$_{10}$	59	0.13	7.7
32	(oeg_uh^{NH}T)$_5$-T$_{10}$	64	0.12	8.3

[a]Hydrolysis mixtures were incubated at 37°C, in 5 mM $MgCl_2$, 50 mM Tris (pH 6.5) buffer. The enzyme (phosphodiesterase II. from bovine spleen, SIGMA) concentrations were 1.0 and 0.1 unit/mL for chimeras and T_{20}, respectively. The substrate concentration was 4 A_{260} unit/mL in each case. Determination of $t_{1/2}$ values is described in the experimental.

To investigate the stability of the longest chimeric blocks in the presence of an endonuclease too, 5′-T$_6$-(oeg_t^{NH}T)$_3$-3′ and 5′-(oeg_t^{NH}T)$_3$-T$_6$-3′ were incubated with Nuclease P_1. After 1 day digestion the peaks of HPLC profiles in both cases were found to be identical with those obtained by the same analyses of the corresponding SV PDE or BS PDE hydrolysis mixtures. It proves that the unnatural phosphodiester bond is resistant not only to exonucleases but to endonuclease as well.

The fact that the 5′-modified analogues were found to be much less resistant to the hydrolysis by 5′-exonuclease (BS PDE) than the 3′-modified derivatives to the 3′-exonuclease (SV PDE) digestion, may be explained by the significant difference between the endonuclease activity of the two enzymes.

CONCLUSIONS

In the present work a facile and simple synthetic route for the preparation of 3 chimeric PNA-DNA dimer synthons has been described. Longer coupling cycle and larger reagent excess were necessary to attain acceptable (>90%) coupling yields when dimer P-amidites were used for the solid-phase synthesis. Due to perturbation of the duplex structure caused by the inhomogenous mixed backbone, incorporation of chimeric blocks led to considerable drop of T_m values relative to that of the reference T$_{20}$:dA$_{20}$ duplex. The degree of T_m drops strongly depended on the number and position of dimer units incorporated. The chimeric part of (oeg_t^{NH}T)$_5$-T$_{10}$ did not form duplex at all, while the methyl → propynyl exchange in the uracil PNA moiety resulted in noticeable duplex stabilization, even if it was not enough to compensate the destabilizing effect of the mixed backbone. For chimeras containing 5-hexynyl-uracil PNA units, the duplex stabilizing effect of triple bond and destabilizing effect of the long alkyl side-chain still gave a little positive ΔT_m value (+0.2°C/mod). Incorporation of dimer blocks increased the stability against exonucleases in each case. While the unmodified T$_{20}$ was rapidly digested by a 3′-exonuclease (SV PDE) PNA-DNA chimeras, having one chimeric dimer block at the 3′-terminus (**27**, **28** and **29**), proved especially stable towards this exonucleolytic cleavage. We have found that BS PDE had higher endonuclease activity compared to that of SV PDE since, in spite of the incorporation of 5 dimer blocks, the $t_{1/2}$ values of 5′

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modified analogues (**30**, **31** and **32**) increased only by 6–8 times relative to that of T_{20}. In cases of both enzymes the stability of chimeras depended on the length of 5-substituent of PNA base moiety. Within one set the hydrolysis rates decreased in the order of oeg_t^{NH}T > oeg_up^{NH}T > oeg_uh^{NH}T, which can be attributed to the increased inhibition of the substrate binding to the active site by the longer alkynyl side-chains. Neither exonucleases (SV PDE and BS PDE) nor an endonuclease (Nuclease P_1) could hydrolyse the unnatural phosphodiester bond between the secondary OH of thymidine and the terminal OH of *N*-(2-hydroxyethyl)glycine PNA moiety.

Our results call the attention to PNA-DNA chimeras having limited number of PNA residues with 5-propynyl- or 5-hexynyl-uracil base at the 3′- or 5′-terminus, as possible antisense candidates with suitable binding affinity to the complementary targets and high resistance towards nucleolytic degradations.

EXPERIMENTAL SECTION

Materials and Methods

5-Iodo-uracil was purchased from Aldrich, 1,2-dibromo-propane, 1-hexyne, CuI, EEDQ, TBTU and $PdCl_2$ from Fluka. DMF and NEt_3 were distilled from CaH_2 and stored over molecular sieves (4 Å). Precoated SiO_2 plates (Kieselgel 60 HF_{254}, 0.2 mm) were used for TLC and Kieselgel 60 (0.04–0.063 mm and 0.063–0.2 mm) (Merck) for column chromatographic separations. Solvent systems used were the following: A: $CHCl_3$–MeOH 9:1, B: $CHCl_3$–MeOH 2:1, C: $CHCl_3$–MeOH 9:1 + 1% TEA, D: $CHCl_3$–MeOH 4:1 E: EtOAc-MeOH 9:1 + 2% TEA. HPLC analyses and purification of the crude DMT-on oligonucleotides were performed with a Jasco HPLC system using Hypersi ODS column (8 × 250 mm). The flow rate was 3 mL/min, the gradient was 5 → 50% acetonitrile in 0.1M aq. NH_4OAc solution. Infrared spectra were recorded in KBr pellets with a Nicolet Magna 750 FT–IR spectrophotometer. ^1H and ^{31}P NMR spectra were recorded with a Varian XL-400 multinuclear instrument at 400 MHz. Chemical shifts are given in ppm relative to Me_4Si and H_3PO_4, as internal standards, respectively. ESI mass spectra were obtained with a Perkin Elmer SCIEX, API 2000 tandem mass spectrometer equipped with electrospray ion source both in positive and negative ion mode. Samples were directly injected into aq. acetonitrile. The flow rate was 0.2 mL/min. Elemental analyses were carried out by the Environmental and Analytical Chemistry Department of the Chemical Research Center. Automated solid-phase oligonucleotide syntheses were run on a MilliGen/Biosearch 8700 DNA Synthesizer using T- and dimer-loaded LCAA-CPG-s (pore size: 500 Å), as solid supports. T_m points of duplexes with dA_{20} target were measured with a Hewlett Packard 8452A UV-VIS spectrophotometer (detection at 260 nm). Linear variations of temperature as a function of time were regulated by a compatible microcomputer using Absorbe 3.0 software. In cases of endonuclease (Nuclease P_1 from Penicillium citrinum, SIGMA) digestions the enzyme (c = 0.02 unit/mL) and substrates (c = 4 A_{260} unit/mL in each case) were incubated at 37°C, in 5 mM $MgCl_2$, 50 mM Tris (pH 5.3) buffer. Half times of enzymatic hydrolyses ($t_{1/2}$ values) were determined by IE HPLC analysis. Samples were

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taken from the hydrolysis mixtures after given intervals (0, 5, 15, 30, 60 and 120 min.), freezed then applied to an analytical ion-exchange HPLC column. A_{260} absorbance values of the starting 20-mer were plotted against the time to give the $t_{1/2}$ value, belonging to the half-A_{260} value of the starting T_{20} analogue. The HPLC analyses mentioned were performed with a Merck HPLC system (LaChrom L-7100 pump, Bischoff Lambda 1010 UV-VIS detector, detection at 260 nm), using MN Nuleogen-DEAE 60-7 column (4 × 125 mm) equipped with a MN Nucleogen DEAE 60-7 guard column (4 × 30 mm). Elution was carried out using linear 0 → 1.0 M KCl gradient in aq. 20 mM NaOAc containing 20% acetonitrile programmed over a 200 min. period with a flow rate of 2 mL/min.

5-(1-Propynyl)-uracil (2). 5-Iodo-uracil (**1**) (9.52 g = 40 mmol) was dissolved in dry DMF (100 mL) and dry TEA (11 mL = 80 mmol). The solution was purged with argon then $(Ph_3P)_2$ $PdCl_2$ (2.80 g = 4 mmol) and CuI (1.52 g = 8 mmol) were added. After 5 min propyne (17 mL = 0.3 mol), that was previously generated from 1,2-dibromopropane,[34] was introduced with strirring at ambient temperature for 1 h. The mixture was left to stand overnight then the solvent was evaporated in vacuo to give yellow amorphous residue. It was suspended in 0.5 N aq. NaOH (150 mL), the insoluble Pd salts were filtered and washed with further 0.5N aq. NaOH solution (50 mL) and water (20 mL). The filtrate was treated with charcoal, filtered then acidified with cc. aq. HCl (7.0 mL) to pH ~ 5. The precipitate was filtered and washed with 0.5 M aq. HCl. Since according to the TLC, it still contained Cu-salts and a more polar impurity it was stirred with cc. aq. NH_3 solution for 15 min then filtered. Thus we isolated solid product that was pure by TLC. It was dried over P_2O_5 in vacuo overnight to give 2.80 g = 18.6 mmol of 5-(1-propynyl)-uracil (**2**), as a pale grey powder. Yield: ~47%. R_f(A): 0.33; IR ν [cm^{-1}] = 3214 m (NH), 2261 vw (C≡C), 1715 s (as C=O), 1684 s (s C=O), 1628 m (C=C), 1434 m, 1234 m, 855 m; ^{1}H NMR (DMSO-d_6): δ = 2.00 (3H, s, CH_3), 7.70 (1H, s, H6), 11.18 and 11.35 (2H, 2bs, 2NH); ESI MS m/z (%): 151.0 (42) $[M+H]^+$, 157.0 (100) $[M+Li]^+$, $C_7H_6N_2O_2$ requires 150.13. Anal. calcd for $C_7H_6N_2O_2$; C, 56.00; H, 4.03; N, 18.66. Found: C, 55.78; H, 4.19; N, 18.47.

5-(1-Hexyn-1-yl)-uracil (3). Starting from the same amount (40.0 mmol) of **1** the alkynylation was carried out on similar way but in this case only 3 equiv = 0.12 mol = 13.5 mL of 1-hexyne was added. Due to the lower water solubility of the product after removal of DMF the residue was stirred with 0.5 M aq. KOH (88 mL = 44 mmol) at 40°C, for 1 h then filtered. Since the solid precipitate still contained some main product it was further stirred with 150 mL of warm 1M aq. KOH and the insoluble Pd and Cu salts were filtered. The filtrate was treated with cc. aq. HCl (23.0 mL) which resulted in the precipitation of a pale yellow solid. After 1 h standing in the refrigerator the precipitate was filtered, washed with water (15 mL) then dried in vacuo, over P_2O_5. Thus we obtained 4.15 g = 21.6 mmol of 5-(1-hexyn-1-yl)-uracil (**3**). Yield: 57%, R_f(A): 0.40; IR ν [cm^{-1}] = 3200 m (NH), 2958 m (as CH_2), 2933 m (s CH_2), 2240 vw (C≡C), 1730 s (as C=O), 1686 s (s C=O), 1632 m (C=C), 1429 m, 1215 m; ^{1}H NMR (DMSO-d_6): δ = 0.88(3H, t, CH_3), 1.30–1.65 (4H, m, $(CH_2)_2$), 2.38 (2H, t, C≡C–CH_2), 7.70 (1H, s, H6), 11.10 and 11.30 (2H, 2 bs, 2 NH); ESI MS m/z (%): 193.0 (90) $[M+H]^+$, 199.0 (100)

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$[M + Li]^+$, 215.2 (31) $[M + Na]^+$, $C_{10}H_{12}N_2O_2$ requires 192.21. Anal. calcd for $C_{10}H_{12}N_2O_2$:C, 62.48; H, 6.29; N, 14.57. Found: C, 62.31; H, 6.47; N, 14.40.

5-(1-Propynyl)-uracil-1-yl-acetic acid (4). Crude **2** (2.60 g = 17.3 mmol) was dissolved in 1 N aq. NaOH with stirring at 40°C, cooled to room temperature then the solution of bromoacetic acid (3.61 g = 26 mmol) in water (20 mL) was added dropwise. After 4 h stirring the mixture was carefully acidified with 1 N aq. HCl to pH ~ 5. Some unreacted **2** was precipitated then removed by filtration. The pH of the filtrate was then adjusted to ~1.0 with cc. aq. HCl, which resulted in precipitation of the required pure 5-(propyn-1-yl)-uracil-1-yl acetic acid (**4**). It was dried in vacuo, over P_2O_5 for 2 days to give 2.11 g = 10.14 mmol of **4**, as a pale yellow powder. Yield: 58.6%, R_f(B): 0.43–0.58 diffuse; IR ν [cm^{-1}] = 3170 m (NH), 3200–2800 m (OH carboxylic), 2246 vw (C≡C), 1738 s (as C=O uracil), 1696 s (C=O carboxylic), 1680 s (s C=O uracil), 1632 m (C=C), 1477 m, 1424 m, 1376 m, 1245 m, 1218 m; ^{1}H NMR (DMSO-d_6): δ = 2.00 (3H, s, CH_3), 4.40 (2H, s, CH_2), 7.95 (1H, s, H6), 11.60 (1H, s, NH), 13.30 (1H, bs, COOH); ESI MS m/z (%): 209.2 (57) $[M + H]^+$, 215.2 (100) $[M + Li]^+$, 207.1 (100) $[M - H]^-$, 415.3 (11) $[2M - H]^-$,$C_9H_8N_2O_4$ requires 208.16. Anal. calcd for $C_9H_8N_2O_4$: C, 51.93; H, 3.87; N, 13.45. Found: C, 51.74; H, 4.04; N, 13.31.

5-(1-Hexyn-1-yl)-uracil-1-yl-acetic acid (5). The procedure described for the propynyl analogue had to be slightly modified due to the lower water solubility of **3**. The crude **3** (1.92 g = 10.0 mmol) was stirred with 0.5N aq. NaOH (70 mL) at 50°C but it did not become quite homogeneous even after 1 h. The insoluble part was filtered off then a solution of bromoacetic acid (2.08 g = 15 mmol) in water (10 mL) was added dropwise. After 24 h stirring the pH was set to ~6.0 with 1 N aq. HCl (10.0 mL) which led to the precipitation of some unreacted **3** along with a little (<10%) main product. Further acidification of the filtrate with cc. aq. HCl (1.7 mL) resulted in the precipitation of the required main product 5-(1-hexyn-1-yl)-uracil-1-yl-acetic acid (**5**) that was filtered, washed with some cold water and dried over P_2O_5, in vacuo. Thus we isolated 1.85 g = 7.39 mmol of **5**, as while solid. Yield: 74%, R_f(A): 0.14; IR ν [cm^{-1}] = 3170 m (NH), 3200–2800 m (OH carboxylic), 2960 m (as CH_2), 2925 m (s CH_2), 2238 vw (C≡C), 1736 s (as C=O uracil), 1703 s (C=O carboxylic), 1680 s (s C=O uracil), 1630 m (C=C), 1467 m, 1201 m; ^{1}H NMR (DMSO-d_6): δ = 0.90 (3H, t, CH_3), 1.40 (4H, m, $(CH_2)_2$), 2.30 (2H, t, C≡C–CH_2–), 4.42 (2H, s, CH_2), 8.00 (1H, s, H6), 11.70 (1H, s, NH), 13.20 (1H, bs, COOH); ESI MS m/z (%): 249.1 (100) $[M - H]^-$, 499.3 (29) $[2M - H]^-$, 749.2 (6) $[3M - H]^-$, $C_{12}H_{14}N_2O_4$ requires 250.24. Anal. calcd for $C_{12}H_{14}N_2O_4$: C, 57.59; H, 5.64; N, 11.19. Found: C, 57.47; H, 5.78; N, 11.08.

Thymin-1-yl-acetic acid (6). This compound has been described in the literature[25] but only R_f and ^{1}H NMR data were reported. IR ν [cm^{-1}] = 3185 m (NH), 3200–2800 m (OH carboxylic), 1740 s (as C=O uracil), 1707 s (C=O carboxylic), 1663 s (s C=O uracil), 1633 s (C=C); ESI MS m/z (%) 183.1 (100) $[M - H]^-$, 367.0 (41) $[2M - H]^-$, $C_7H_8N_2O_4$ requires 184.14. Anal. calcd for $C_7H_8N_2O_4$: C, 45.65; H, 4.38; N, 15.21. Found: C, 45.48; H, 4.55; N, 15.07.

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General Procedure for the Coupling of N^1-Carboxymethyl-5-substituted-uracils with *N*-(2-Hydroxyethyl)glycine *tert*-butyl ester. *N*-(2-hydroxyethyl)glycine *tert*-butyl ester[27] (0.96 g = 5.5 mmol) was dissolved in DMF (10 mL) then any of N^1-carboxymethyl-5-substituted-uracils (**4, 5** or **6**) (5.0 mmol), TBTU (1.76 g = 5.5 mmol) and TEA (1.54 mL = 11 mmol) were added and the mixture was left to stir at ambient temperature for 3 h. The solvent was removed in vacuo, the residue was dissolved in CH_2Cl_2, evaporated to dryness again and the solid foam obtained was recrystalized from EtOAc (25 mL). After 16 h standing at 4°C the solid product was filtered, washed with EtOAc and dried in vacuo to give the required coupled products, as white solids.

***N*-(2-Hydroxyethyl)-*N*-(thymin-1-yl-acetyl)glycine *tert*-butyl ester (7).** This compound has been described and characterized in the literature.[27] We give only some own, additional data: Yield: 76%, R_f(A): 0.39; mp.: 169–174°C; IR ν [cm^{-1}] = 3464 m (OH), 3163 w (NH), 2962 w (as CH_2), 2933 w (s CH_2), 1733 s (C=O ester), 1697 s (as C=O uracil), 1670 s (s C=O uracil), 1473 m, 1215 m, 1163 m; ESI MS m/z (%) 342.1 (78) $[M+H]^+$, 348.1 (47) $[M+Li]^+$, 359.2 (97) $[M+NH_4]^+$, 364.3 (20) $[M+Na]^+$, 380.2 (100) $[M+K]^+$, $C_{15}H_{23}N_3O_6$ requires 341.44. Anal. calcd for $C_{15}H_{23}N_3O_6$: C, 52.76; H, 6.79; N, 12.31. Found: C, 52.68; H, 6.87; N, 12.22.

***N*-(2-Hydroxyethyl)-*N*-[(5-propynyl-uracil-1-yl)acetyl]glycine *tert*-butyl ester (8).** Yield: 65%, R_f(A): 0.43; mp.: 189–192°C; IR ν [cm^{-1}] = 3471 w (OH), 3170 w (NH), 2968 m (as CH_2), 2935 m (s CH_2), 1736 s (C=O ester), 1700 s (as C=O uracil), 1677 s (s C=O uracil), 1661 s (>N–C=O), 1468 m, 1370 m, 1161 m; ^{1}H NMR (DMSO-d_6) δ = 1.45–1.52 (9H, 3s, $OCMe_3$), 2.03 (3H, s, CH_3), 3.40–3.75 (5H, m, CH_2–CH_2–OH), 4.05 and 4.28 (2H, 2s, glycine CH_2, rotamers), 4.58 and 4.80 (2H, 2s, B–CH_2, rotamers), 7.82 and 7.85 (1H, 2s, H6, rotamers), 11.62 (1H, s, NH); ESI MS m/z (%) 366.1 (54) $[M+H]^+$, 372.3 (100) $[M+Li]^+$, 383.2 (63) $[M+NH_4]^+$, 388.3 (25) $[M+Na]^+$, 404.2 (13) $[M+K]^+$, $C_{17}H_{23}N_3O_6$ requires 365.46. Anal. calcd for $C_{17}H_{23}N_3O_6$: C, 55.87; H, 6.34; N, 11.50. Found: C, 55.72; H, 6.42; N, 11.39.

***N*-(2-Hydroxyethyl)-*N*-[(5-hexynyl-uracil-1-yl)acetyl]glycine *tert*-butyl ester (9).** Yield 58%, R_f(A): 0.50; mp.: 230–235°C, IR ν [cm^{-1}] = 3480 w (OH), 3178 w (NH), 2970 m (as CH_2), 2935 m (s CH_2), 1738 s (C=O ester), 1702 s (as C=O uracil), 1680 s (s C=O uracil), 1664 s (>N–C=O), 1466 m, 1375 m, 1160 m; ^{1}H NMR ($CDCl_3$) δ = 0.90 (3H, t, CH_3), 1.35–1.60 (13H, m, $OCMe_3$ and –$(CH_2)_2$), 2.40 (2H, t, C≡C–CH_2), 3.43–3.80 (5H, m, CH_2–CH_2–OH), 4.00 and 4.20 (2H, 2s, glycine, CH_2, rotamers), 4.48 and 4.75 (2H, 2s, B–CH_2, rotamers), 7.30 and 7.38 (1H, 2s, H6, rotamers), 9.20 (1H, bs, NH); ESI MS m/z (%) 408.1 (50) $[M+H]^+$, 414.4 (100) $[M+Li]^+$, 425.2 (52) $[M+NH_4]^+$, 430.3 (27) $[M+Na]^+$, 446.2 (17) $[M+K]^+$ $C_{20}H_{29}N_3O_6$ requires 407.54. Anal. calcd for $C_{20}H_{29}N_3O_6$: C, 58.94; H, 7.17; N, 10.31. Found: C, 58.75; H, 7.30; N, 10.20.

***N*-(Thymin-1-yl-acetyl)-*N*-(2-dimethoxytrityloxy-ethyl)glycine triethylammonium salt (10).** (The reported synthesis[27] of this compound followed a different strategy) Solution of **7** (1.70 g = 5.0 mmol) in dry pyridine (10 mL) was cooled to 10°C in water

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bath then DMT-Cl (1.86 g = 5.5 mmol) was added in three portions within 1 h. The mixture was stirred at ambient temperature for 3 h then it was poured into ice-water (~150 mL). After separation of the supernatant aqueous phase the yellow syrup was dissolved in CH_2Cl_2 (100 mL) and washed with water (2 × 20 mL). The organic phase was dried with Na_2SO_4, filtered and evaporated to dryness. This crude product was redissolved in CH_2Cl_2 (15 mL) and added dropwise to cold n.hexane (300 mL). The off-white precipitate was filtered and dried in vacuo, over P_2O_5 to give 3.14 g = 4.88 mmol of tritylated ester, as a white powder. Yield: 98%, R_f(A): 0.61. This crude product (1.92 g = 3.0 mmol) was dissolved in a mixture of dioxane (13 mL) and water (3 mL) and the pH of solution was set to ~13 with 1M aq. NaOH. The mixture was stirred at 20°C for 3h then cooled in ice-water bath and carefully acidified to pH ~ 5.0 with 2N aq. $NaHSO_4$. Then it was diluted with water (30 mL) and extracted with CH_2Cl_2 (2 × 50 mL). The CH_2Cl_2 solution was evaporated, the residue was dissolved in $CHCl_3$–TEA (1%) (8.0 mL) and was applied to a silica gel column (70 g of Kieselgel 60, 0.063–0.2 mm) that was eluted with $CHCl_3$–MeOH 4:1 + 2% TEA. The appropriate fractions were combined and evaporated to give 1.39 g = 2.02 mmol of **10**, as triethylammonium salt. Yield: 67,3%. R_f(C): 0.26; IR ν [cm^{-1}] = 3210 w (NH), 3200–2800 w (OH carboxylic), 2939 m (as CH_3 OMe), 2840 w (s CH_3 OMe), 1716 s (as C=O uracil), 1680 s (C=O carboxylic), 1674 s (s C=O uracil), 1650 s (>N–C=O), 1632 m (C=C), 1608 m and 1509 m (aromatic), 1250 m, 117 m, 1033 m; ^{1}H NMR (DMSO-d_6):δ = 1.30 (9H, t, 3 Me of TEA), 1.75 and 1.90 (3H, CH_3, rotamers), 3.00 (6H, q, 3CH_2 of TEA), 3.25 and 3.38 (2H, 2t, CH_2–N, rotamers), 3.52–3.65 (2H, m, DMT–O–CH_2), 3.80 (6H, s, 2 OMe of DMT), 3.88 and 4.05 (2H, 2s, glycine CH_2, rotamers), 4.58 and 4.85 (2H, 2s, B–CH_2, rotamers), 6.75–7.45 (14H, m, aromatic and H6), 11.30 (1H, bs, NH); ESI MS m/z (%) 594.4 (100) $[M + Li]^+$, 605.2 (31) $[M + NH_4]^+$, 586.3 $[M - H]^-$, $C_{32}H_{33}N_3O_8$ requires 587.68. Anal. calcd for $C_{32}H_{33}N_3O_8$: C, 65.40; H, 5.66; N, 7.15. Found: C, 65.51; H, 5.78; N, 7.21.

The alkynyl derivatives were prepared and purified on similar way resulting in,the corresponding 5-propynyl- and 5-hexynyl-substituted PNA monomers (**11** and **12**).

***N*-[(5-Propynyl-uracil-1-yl)acetyl]-*N*-(2-dimethoxytrityloxy-ethyl)glycine triethylammonium salt (11).** Yield: 81%, R_f(C): 0.27; IR ν [cm^{-1}] = 3220 w (NH), 3200–2800 w (OH carboxylic), 2940 w (as CH_3 OMe), 2852 w (s CH_3 OMe), 1733 s (as C=O uracil), 1699 s (C=O carboxylic), 1682 s (s C=O uracil), 1651 s (>N–C=O), 1608 m and 1509 m (aromatic), 1464 m, 1251 s, 1177 m, 1033 m; ^{1}H NMR ($CDCl_3$): δ = 1.25 (9H, t, 3Me of TEA), 1.90 (3H, s, C≡C–CH_3), 3.03 (6H, q, 3 CH_2 of TEA), 3.27 and 3.40 (2H, 2s, H_2C–N, rotamers), 3.54–3.68 (2H, m, DMT–O–CH_2), 3.82 (6H, s, 2 OMe of DMT), 3.90 and 4.07 (2H, 2s, glycine CH_2, rotamers), 4.60 and 4.90 (2H, 2s, B–CH_2, rotamers), 6.60–7.42 (14H, m, aromatic and H6), 11.38 (1H, bs, NH); ESI MS m/z 610.3 $[M - H]^-$, $C_{34}H_{33}N_3O_8$ requires 611.70. Anal. calcd for $C_{34}H_{33}N_3O_8$: C, 66.75; H, 5.44; N, 6.87. Found: C, 66.80; H, 5.62; N, 6.95.

***N*-[(5-Hexynyl-uracil-1-yl)acetyl]-*N*-(2-dimethoxytrityloxy-ethyl)glycine triethylammonium salt (12).** Yield: 87%, R_f(C): 0.30; IR ν [cm^{-1}] = 3200–2800 w

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(OH carboxylic), 3180 w (NH), 2956 w (as CH_3 OMe), 2933 w (s CH_3 OMe), 1714 s (as C=O uracil), 1700 s (C=O carboxylic), 1685 s (s C=O uracil), 1654 s (>N–C=O), 1608 s and 1510 s (aromatic), 1251 s, 1177 m, 1034 m; ^{1}H NMR ($CDCl_3$): $\delta = 0.85$ (3H, t, CH_3 of hexynyl), 1.10–1.65 (13H, m, 3 Me of TEA and 2 CH_2 of hexynyl), 2.30 (2H, t, C≡C–CH_2), 3.04 (6H, q, 3, CH_2 of TEA), 3.26 and 3.40 (2H, 2s, H_2C–N, rotamers), 3.55 and 3.68 (2H, 2s, DMT–O–CH_2), 3.80 (6H, s, 2 OMe of DMT), 3.92 and 4.10 (2H, 2s, glycine CH_2, rotamers), 4.61 and 4.92 (2H, 2s, B–CH_2, rotamers), 6.50–7.50 (14H, m, aromatic and H6), 11.41 (1H, bs, NH); ESI MS m/z 652.3 $[M-H]^-$, $C_{37}H_{39}N_3O_8$ requires 653.78. Anal. calcd for $C_{37}H_{39}N_3O_8$: C, 67.97; H, 6.01; N, 6.43. Found: C, 68.05; H, 6.12; N, 6.48.

General Method for the Coupling of PNA Monomers (10, 11 or 12) with 5′-Amino-5′-deoxythymidine (13). The mixture of a PNA monomer (2.5 mmol), **13** (0.60 g = 2.5 mmol) and TBTU (0.80 g = 2.5 mmol) was dissolved in DMF (25 mL) and TEA (0.70 mL = 5.0 mmol) then stirred at room temperature for 2 h. The solvent was removed then the residue was taken up with CH_2Cl_2 (50 mL) and washed with brine (2 × 10 mL). The organic phase was dried with Na_2SO_4 and evaporated to dryness. The residue was purified by dry column flash chromatography[35] (70 g of Kieselgel 60 HF_{254}, eluant: $CHCl_3$–MeOH 1–10% + 1% TEA). The pure fractions were combined, evaporated and dried over P_2O_5 to give the following coupled products:

***N*-[(Thymin-1-yl)acetyl]-*N*-(2-dimethoxytrityloxy-ethyl)glycine-(5′-deoxythymidin-5′-yl)amide (14).** Yield: 59%, R_f(D): 0.68; IR ν [cm^{-1}] = 3180 w (NH), 1716 s (as C=O uracil), 1685 s (–NH–C=O), 1672 s (s C=O uracil), 1650 s (>N–C=O), 1608 m and 1510 m (aromatic), 1252 m, 1178 w, 1033 w; ^{1}H NMR ($CDCl_3$): $\delta = 1.70$–1.95 (6H, 3s, 2 Me of T-s, rotamers), 2.55–2.72 (2H, m, H2′ab), 3.15–3.60 (6H, m, H5′ab and DMT–O–$(CH_2)_2$), 3.80 (6H, s, 2 OMe of DMT), 4.20–4.42 (3H, m, H4′ and glycine CH_2), 4.70–5.05 (3H, m, H3′ and B–CH_2), 5.33 (1H, d, 3′-OH), 6.05 (1H, t, 5′-NH), 6.22 (1H, dd, Hi′), 6.75–7.50 (15H, 2m, aromatic + 2 H6), 11.28–11.35 (2H, bs, 2 NH); ESI MS m/z 817.3 $[M+Li]^+$, $C_{42}H_{46}N_6O_{11}$ requires 810.91. Anan. calcd for $C_{42}H_{46}N_6O_{11}$: C, 62.20; H, 5.72; N, 10.36. Found: C, 62.07; H, 5.85; N, 10.25.

***N*-[(5-Propynyl-uracil-1-yl)acetyl]-*N*-(2-dimethoxytrityloxy-ethyl)glycine-(5′-deoxythymidin-5′-yl)amide (15).** Yield: 82%, R_f(D): 0.70; IR ν [cm^{-1}] = 3200 w (NH), 1713 s (as C=O uracil), 1686 s (–NH–C=O), 1670 s (s C=O uracil), 1656 s (>N–C=O), 1607 w and 1509 m (aromatic), 1465 m, 1250 m, 1178 w, 1033 w; ^{1}H NMR ($CDCl_3$): $\delta = 1.85$ (3H, s, 5-CH_3 of T), 2.03 (3H, s, C≡CCH_3), 2.48–2.70 (2H, m, H2′ab), 3.20–3.63 (6H, m, H5′ab and DMT–O–$(CH_2)_2$), 3.82 (6H, s, 2 OMe of DMT), 4.25–4.40 (3H, m, H4′ and glycine CH_2), 4.70–4.98 (3H, m, H3′ and B–CH_2), 5.50–5.80 (2H, m, 3′OH and 5′-NH), 6.10 (1H, dd, H1′), 6.80–7.50 (15H, 2 m, aromatic and 2 H6), 11.30–11.38 (2H, bs, 2 NH); ESI MS m/z 841.3 $[M+Li]^+$, $C_{44}H_{46}N_6O_{11}$ requires 834.93. Anal. calcd for $C_{44}H_{46}N_6O_{11}$: C, 63.29; H, 5.55; N, 10.07. Found: C, 63.14; H, 5.71; N, 9.98.

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***N*-[(5-Hexnyl-uracil-1-yl)acetyl]-*N*-(2-dimethoxytrityloxy-ethyl)glycine-(5′-deoxythymidin-5′-yl)amide (16).** Yield: 77%, R_f(D): 0.77; IR ν [cm^{-1}] = 3180 w (NH), 1716 s (as C=O uracil), 1687 s (–NH–C=O), 1670 s (s C=O uracil), 1653 s (>N–C=O), 1608 w and 1510 m (aromatic), 1465 m, 1252 m, 1178 w, 1034 w; ^{1}H NMR ($CDCl_3$): δ = 0.90 (3H, t, CH_3 of hexynyl), 1.35–1.70 (4H, m, $(CH_2)_2$ of hexynyl), 1.88 (3H, s, 5-CH_3 of T), 2.39 (2H, t, C≡C–CH_2), 2.72–2.90 (2H, m, H2′ab), 3.20–3.65 (6H, m, H5′ab and DMT–O–$(CH_2)_2$), 3.80 (6H, s, 2 OMe of DMT), 4.20–4.50 (3H, m, H4′ and glycine CH_2), 4.70–4.90 (3H, m, H3′ and B–CH_2), 5.32 (1H, d, 3′-OH), 5.75 (1H, t, 5′-NH), 6.15 (1H, dd, H1′), 6.80–7.50 (15H, 2 m, aromatic and 2 H6), 11.32–11.41 (2H, bs, 2 NH); ESI MS m/z 883.3 $[M + Li]^+$, $C_{47}H_{52}N_6O_{11}$ requires 877.01. Anal. calcd for $C_{47}H_{52}N_6O_{11}$: C, 64.36; H, 5.98; N, 9.58. Found: C, 64.21; H, 6.11; N, 9.47.

General Procedure for the Phosphitylation of Chimeric Dimers. The chimeric dimer (**14**, **15** or **16**) was dissolved in dry CH_2Cl_2 (20 mL) then diisopropylammonium tetrazolide (DIPAT, 1.5 mmol) and bis-diisopropylamino-2-cyanoethyl-phosphite (0.87 mL = 3.0 mmol) were added with stirring in Ar atmosphere. The mixture was left to stir at ambient temperature overnight then it was diluted with CH_2Cl_2 (30 mL) and washed with cold, 2% (w/v) aq. Na_2CO_3 (2 × 20 mL) then with brine (20 mL). The CH_2Cl_2 solution was dried with Na_2SO_4, filtered and evaporated to dryness. The residue was dissolved in EtOAc-TEA 19:1 (5 mL) and applied to a silica gel column made of Kieselgel 60 (0.04–0.06 mm, 70 g) which was eluted with linear gradient of EtOAc-TEA (5%)→ EtOAc-MeOH (5%)–TEA (5%) (300–300 mL). The appropriate pure fractions were combined, evaporated and dried in vacuo, over P_2O_5 to give the required 3′-O-(β-cyanoethyl-N,N-diisopropyl)-phosphoramidites (**17**, **18** and **19**), as white solid foams.

***N*-(Thymin-1-yl-acetyl)-*N*-(2-dimethoxytrityloxy-ethyl)glycine-(5′-deoxythymidin-5′-yl)amide-3′-O-(N,N-diisopropylamino-2-cyanoethyl)phosphite (17).** Yield: 84%, R_f(E): 0.50; ^{1}H NMR ($CDCl_3$): δ = 1.05–1.20 (12H, 4s, 4Me), 1.81 (3H, s, 5-Me of T), 1.87 (3H, s, 5-Me of t), 2.20–2.52 (2H, m, H2′ab), 2.60 (2H, t, CH_2–O), 3.17–3.30 (2H, m, $HCMe_2$), 3.35–3.45 (2H, m, H5′ab), 3.52–3.73 (6H, m, DMT–O–$(CH_2)_2$ and NC–CH_2), 3.78 (6H, s, OMe), 4.07 and 4.18 (2H, 2s, glycine CH_2), 4.44 (1H, m, H4′), 4.77 and 4.90 (2H, 2s, B–CH_2), 5.12 (1H, m, H3′), 5.85 (1H, t, 5′-NH), 6.26 (1H, dd, H1′), 6.75–7.48 (15H, m, aromatic and 2 H6), 11.20–11.35 (2H, bs, 2NH) ^{31}P NMR ($CDCl_3$): δ = 148.7 and 149.0 ppm. Anal. calcd for $C_{51}H_{63}N_8O_{12}P$: C, 60.58; H, 6.28; N, 11.08; P, 3.06. Found: C, 60.34; H, 6.46; N, 11.02; P, 2.98.

***N*-[(5-Propynyl-uracil-1-yl)-acetyl]-*N*-(2-dimethoxytrityloxy-ethyl)glycine-(5′-deoxythymidin-5′-yl)amide-3′-O-(N,N-diisopropylamino-2-cyanoethyl)phosphite (18).** Yield: 60%, R_f(E): 0.55; ^{1}H NMR ($CDCl_3$): δ = 1.02–1.16 (12H, 4s, 4Me), 1.79 (3H, s, 5-Me of T), 1.98 (3H, s, C≡C–Me), 2.21–2.54 (2H, m, H2′ab), 2.62 (2H, t, CH_2–O), 3.16–3.28 (2H, m, $HCMe_2$), 3.30–3.48 (2H, m, H5′ab), 3.53–3.73 (6H, m, DMT–O–$(CH_2)_2$ and NC–CH_2), 3.80 (6H, s, OMe), 4.10 and 4.21 (2H, 2s, glycine CH_2), 4.47 (1H, m, H4′), 4.79 and 4.93 (2H, 2s, B–CH_2), 5.15 (1H, m, H3′), 5.90 (1H, t, 5′-NH), 6.28

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(1H, dd, H1′), 6.72–7.53 (15H, m, aromatic and 2 H6), 11.20–11.33 (2H, bs, 2NH) ^{31}P NMR ($CDCl_3$): $\delta = 149.4$ ppm. Anal. calcd for $C_{53}H_{63}N_8O_{12}P$: C, 61.49; H, 6.13; N, 10.83; P, 2.99. Found: C, 61.37; H, 6.32; N, 10.70; P, 2.87.

***N*-[(5-Hexynyl-uracil-1-yl)-acetyl]-*N*-(2-dimethoxytrityloxy-ethyl)glycine-(5′-deoxythymidin-5′-yl)amide-3′-O-(N,N-diisopropylamino-2-cyanoethyl)phosphite (19).** Yield: 59%, R_f(E): 0.59; ^{1}H NMR ($CDCl_3$): $\delta = 0.70$ (3H, t, Me of hexynyl), 1.05–1.36 (16H, m, 4Me and 2CH_2 of hexynyl), 1.81 (3H, s, 5-Me of T), 2.21–2.32 (1H, m, H2′a), 2.40 (2H, t, C≡C–CH_2), 2.43–2.53 (1H, m, H2′b), 2.62 (2H, t, CH_2–O), 3.14–3.30 (2H, m, $HCMe_2$), 3.33–3.48 (2H, m, H5′ab), 3.52–3.73 (6H, m, DMT–O–$(CH_2)_2$ and NC–CH_2), 3.80 (6H, s, OMe), 4.12 and 4.21 (2H, 2s, glycine CH_2), 4.40 (1H, m, H4′), 4.80 and 4.94 (2H, 2s, B–CH_2), 5.15 (1H, m, H3′), 5.94 (1H, t, 5′-NH), 6.28 (1H, dd, H1′), 6.70–7.55 (15H, m, aromatic and 2 H6), 11.22–11.36 (2H, bs, 2NH) ^{31}P NMR ($CDCl_3$): $\delta = 148.9$ and 148.6 ppm. Anal. calcd for $C_{56}H_{69}N_8O_{12}P$: C, 62.43; H, 6.46; N, 10.40; P, 2.87. Found: C, 62.30; H, 6.58; N, 10.27; P, 2.71.

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General Procedure for the Succinylation of Chimeric Dimers. A 3′-free dimer (**14**, **15** or **16**) (0.15 mmol) was dissolved in dry CH_2Cl_2 (4.0 mL) then TEA (0.30 mmol = 40 μL), 4-dimethylamino-pyridine (28 mg = 0.23 mmol) and succinic anhydride (45 mg = 0.45 mmol) were added. The mixture was stirred at room temperature overnight, then it was diluted with CH_2Cl_2 (30 mL), transferred to a separatory funnel, washed with 10% (w/v) aq. NaH_2PO_4 (2 × 15 mL) and water (15 mL). The organic phase was dried with Na_2SO_4, filtered and evaporated to dryness. The crude product was purified by vacuum flash chromatography on a Kieselgel 60 HF_{254}(30 g) column using $CHCl_3$–MeOH (1–10%)-TEA (1%), as eluant. The pure fractions were combined and evaporated then dried in vacuo, over P_2O_5. Thus we isolated the corresponding dimer-3′-O-succinate triethylammonium salts (**20**, **21** and **22**), as white solids.

***N*-(Thymin-1-yl-acetyl)-*N*-(2-dimethoxytrityloxy-ethyl)glycine-(5′-deoxythymidin-5′-yl)amide-3′-O-succinate triethylammonium salt (20).** Yield: 95%, R_f(D): 0.40; IR ν [cm^{-1}] = 3180 w (NH), 3150–2800 w (OH carboxylic), 1737 s (C=O ester), 1717 s (as C=O uracil), 1695 s (–NH–C=O), 1675 s (C=O carboxylic), 1656 s (>N–C=O), 1608 w and 1510 m (aromatic), 1464 m, 1250 m, 117 w; ^{1}H NMR ($CDCl_3$): $\delta = 1.20$ (9H, t, 3 Me of TEA), 1.78 (3H, s, 5-Me of T.), 1.92 (3H, s, 5-Me of t), 2.25–2.40 (2H, m, H2′ab), 2.48–2.70 (4H, m, 2 CH_2 of Su), 2.90 (6H, q, 3 CH_2 of TEA), 3.38–3.47 (2H, m, H5′ab), 3.55–3.72 (4H, m, DMT–O–$(CH_2)_2$), 3.80 (6H, s, 2 OMe), 4.10 and 4.19 (2H, 2s, glycine CH_2), 4.50 (1H, m, H4′), 4.80 and 4.90 (2H, 2s, B–CH_2), 5.15 (1H, m, H3′), 5.85 (1H, t, 5′-NH), 6.05 (1H, t, H1′), 6.80–7.40 (15H, 2m, 13 aromatic and 2 H6); ESI MS m/z 917.1 $[M + Li]^+$, $C_{46}H_{50}N_6O_{14}$ requires 910.98. Anal. calcd for $C_{46}H_{50}N_6O_{14}$: C, 60.64; H, 5.53; N, 9.23. Found: C, 60.71; H, 5.72; N, 9.30.

***N*-[(5-Propynyl-uracil-1-yl)-acetyl]-*N*-(2-dimethoxytrityloxy-ethyl)glycine-(5′-deoxythymidin-5′-yl)amide-3′-O-succinate triethylammonium salt (21).** Yield: 77%,

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R_f(D): 0.44; IR ν [cm^{-1}] = 3150–2800 w (OH carboxylic), 3180 w (NH), 1739 s (C=O ester), 1715 s (as C=O uracil), 1695 s (–NH–C=O), 1670 s (C=O carboxylic), 1660 s (>N–C=O), 1608 w and 1510 m (aromatic), 1463 m, 1251 m, 117 w; ^{1}H NMR ($CDCl_3$): δ = 1.25 (9H, t, 3 Me of TEA), 1.82 (3H, s, 5-CH_3 of T), 2.03 (3H, s, C≡C–CH_3), 2.22–2.42 (2H, m, H2′ab), 2.46–2.72 (4H, m, $(CH_2)_2$ of Su), 2.97 (6H, q, 3 CH_2 of TEA), 3.35–3.45 (2H, m, H5′ab), 3.50–3.75 (4H, m, DMT–O–$(CH_2)_2$), 3.79 (6H, s, 2OMe of DMT), 4.13 and 4.24 (2H, 2s, glycine CH_2), 4.53 (1H, m, H4′), 4.81 and 4.92 (2H, 2s, B–CH_2), 5.19 (1H, m, H3′), 5.90 (1H, t, 5′-NH), 6.12 (1H, t, H1′), 6.75–7.44 (15H, 2m, aromatic + 2 H6); ESI MS m/z 941.2 $[M + Li]^+$, $C_{48}H_{50}N_6O_{14}$ requires 935.00. Anal. calcd for $C_{48}H_{50}N_6O_{14}$: C, 61.66; H, 5.39; N, 8.99. Found: C, 61.78; H, 5.52; N, 9.10.

***N*-[(5-Hexynyl-uracil-1-yl)-acetyl]-*N*-(2-dimethoxytrityloxy-ethyl)glycine-(5′-deoxythymidin-5′-yl)-amide-3′-O-succinate triethylammonium salt (22).** Yield: 86%, R_f(D): 0.50; IR ν [cm^{-1}] = 3180 w (NH), 1741 s (C=O ester), 1715 s (as C=O) uracil), 1695 s (–NH–C=O), 1680 s (C=O carboxylic), 1667 s (s C=O uracil), 1658 s (>N–C=O), 1607 w and 1510 m (aromatic), 1462 m, 1251 m, 1177 w; ^{1}H NMR ($CDCl_3$): δ = 0.83 (3H, t, CH_3 of hexynyl), 1.28–1.62 (13H, m, 3 Me of TEA + $CH_2)_2$ of hexynyl), 1.83 (3H, s, 5–CH_3 of T), 2.20–2.45 (4H, m, H2′ab and C≡C–CH_2), 2.48–2.75 (4H, m, $(CH_2)_2$ of Su), 3.00 (6H, q, 3 CH_2 of TEA), 3.32–3.44 (2H, m, H5′ab), 3.48–3.74 (4H, m, DMTO–$(CH_2)_2$), 3.80 (6H, s, 2 OMe), 4.15 and 4.25 (2H, 2s, glycine CH_2), 4.50 (1H, m, H4′), 4.78 and 4.87 (2H, 2s, B–CH_2), 5.17 (1H, m, H3′), 5.95 (1H, t, 5′–NH), 6.15 (1H, t, H1′), 6.73–7.50 (15H, 2m, aromatic + 2 H6); ESI MS m/z 983.2 $[M + Li]^+$, $C_{51}H_{56}N_6O_{14}$ requires 977.08. Anal. calcd for $C_{51}H_{56}N_6O_{14}$: C, 62.69; H, 5.78; N, 8.60. Found: C, 62.77; H, 5.92; N, 8.72.

General Method for the Binding of 3′-O-Succinates to LCAA-CPG. The mixture of a dimer-3′-succinate (**20**, **21** or **22**) (0.50 mmol), LCAA-CPG (pore size: 500 Å) (0.25 g ~ 10 μmol) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (124 mg = 0.50 mmol) were suspended in dry CH_3Cl_2 (8.0 mL) and rotated slowly for 2 days. The solid support was filtered, washed with CH_2Cl_2 (2 × 5 mL) and CH_3CN (5 mL), then it was resuspended in the mixture of CAP A (5 mL) and CAP B (5 mL) reagents and rotated for additional 4 h in order to completely acetylate the unreacted NH_2 groups. The loaded CPG was filtered, washed with dry THF (2 × 5 mL) and CH_2Cl_2 (5 mL) finally dried in dessicator over P_2O_5 and paraffin shavings. The loadings, by DMT assays, were found to be 43, 38 and 35 μmol/g for **20**, **21** and **22**, respectively.

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ABBREVIATIONS

SV PDE	snake venom phosphodiesterase
BS PDE	bovine spleen phosphodiesterase
TBTU	O-(benzotriazol-1-yl)-N,N,N^1,N^1-tetramethyluronium tetrafluoroborate
DMT	4,4′-dimethoxytriphenylmethyl
LCAA-CPG	long-chain alkylamino controlled pore glass
EEDQ	2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline
DEAE	diethylaminoethyl
DCFC	dry column flash chromatography
DIPAT	diisopropylammonium tetrazolide
DMAP	4-dimethylamino-pyridine

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