SYNTHESIS AND HYBRIDIZATION OF OLIGONUCLEOTIDES MODIFIED AT AMP SITES WITH ADENINE PYRROLIDINE PHOSPHONATE NUCLEOTIDES

Dominik REJMAN[1,*], Petr KOČALKA[2], Radek POHL[3], Zdeněk TOČÍK[4] and Ivan ROSENBERG[5,*]

Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i., Flemingovo nám. 2, 166 10 Prague 6, Czech Republic; e-mail: [1] rejman@uochb.cas.cz, [2] kocalka@uochb.cas.cz, [3] pohl@uochb.cas.cz, [4] tocik@uochb.cas.cz, [5] ivan@uochb.cas.cz

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Dedicated to Dr. Alfred Bader on the occasion of his 85th birthday.

Three structurally diverse types of the protected pyrrolidine nucleoside phosphonates were prepared as the monomers for the introduction of pyrrolidine nucleotide units into modified oligonucleotides on the solid phase. Two different chemistries were used for incorporation of modified and natural units: the phosphotriester method for the former, i.e., monomers containing *N*-phosphonoalkyl and *N*-phosphonoacyl moieties attached to the pyrrolidine ring nitrogen atom, and phosphoramidite chemistry for the latter. Since the synthesized pyrrolidine nucleoside phosphonic acids are close mimics of the 3′-deoxynucleoside 5′-phosphates, the incorporation of one modified unit into oligonucleotides gives rise to one 2′,5′ internucleotide linkage. A series of nonamers containing two or three modified units, as well as the fully modified adenine 15-mer, were synthesized in reverse order, i.e., from the 5′ to the 3′ end of the strand. The measurement of thermal characteristics of the complexes of modified nonamers with the complementary strand revealed a destabilizing effect of the introduced modification. The modified adenine homooligonucleotide, was found to form the most stable complex with oligothymidylate of all the tested modified oligonucleotides in terms of ΔT_m per modification.

Keywords: Oligonucleotides; Pyrrolidines; Phosphonates; Nucleotides; Nucleosides.

In 1978, the use of the so-called antisense oligonucleotides (AONs) complementary to the terminal sequence of RSV (Rous sarcoma Virus) to inhibit viral replication in vitro demonstrated[1] for the first time the potential of methods of gene therapy. Two main mechanisms for antisense activity were gradually uncovered, although the problem is very complex[2,3]. The AONs bind by Watson–Crick pairing to the target RNAs and either inhibit

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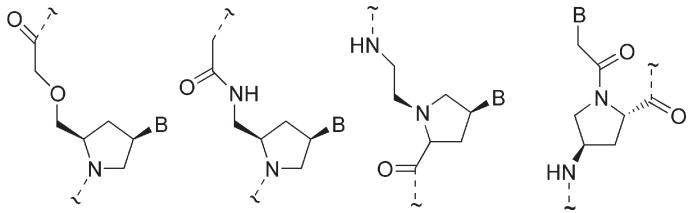
protein translation by steric hindrance by blocking ribosome processing, or activate the ubiquitous RNase H cleavage of the RNA part of the formed heteroduplex. However, chemical modifications of such oligonucleotides (ON) were soon found necessary for the development of real therapeutics and for in vivo applications, as the natural oligonucleotides are unstable toward cellular enzymes. In addition, it was found that the modified oligonucleotides must be also capable of penetrating into cells, sufficiently hybridize with their RNA target sequences, and recruit the RNase H activity while exerting no appreciable side effects[4–7]. The phosphorothioate oligonucleotides[8,9] developed as potential therapeutics of the first generation were able to meet several important demands like higher cellular uptake, enhanced metabolic stability, and the ability to elicit RNase H activity, and Vitravene, a phosporothioate oligonucleotide, was approved by FDA for treatment of HCMV (Human Cytomegalovirus)-induced retinitis[4,5]. However, non-specific interactions of phosphorothioate oligonucleotides with proteins and the presence of a large number of diastereoisomers in one phosphorothioate oligomer due to chirality of the phosphorus atom were the drawbacks that enforced the synthesis and evaluation of a series of other internucleotide linkage-modified oligonucleotides[10]. The process of assembling the chimeric (second generation) AONs composed of two or more types of modified units started, and has since reached considerable progress whereby employing, e.g., modifications chemically as different from each other as the conformationally locked nucleic acids[11], peptide nucleic acids[12], morpholino nucleic acids[13], or non-charged methylphosphonates[14], among other modifications attempting to exploit the growing knowledge of the overall stereochemistry of the nucleic acid chains[15]. Given the number of very specific biological targets to deal with, and the still incomplete information on various structural and dynamic features of nucleic acids, it is not surprising that the existing modifications of natural nucleic acid chains can only partly satisfy the needs in the ever expanding area of gene therapy methodologies[16]. Therefore, the chemical syntheses

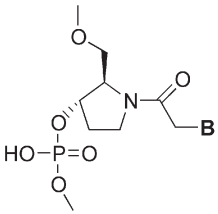


1 **2** **3** **4**

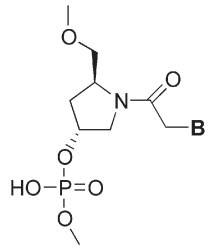
continue in order to meet the demands for more structural variants to optimize the mentioned properties of ONs and get rid of the side-effects[17].

The present work describes novel oligonucleotides combining procedures from two distinct synthetic fields: the preparation of pyrrolidine-based monomeric units and their connection by phosphonate linkages.

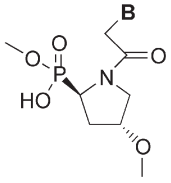
Only a few ONs containing pyrrolidine rings have been synthesized. Most of them are based on the PNA (peptide nucleic acid)-type oligonucleotides using amide moiety as the internucleotide linkage. The modified ON of type **1** was described by Altmann[18]. A homopyrimidine oligomer containing this modification exhibited a significant affinity to complementary RNA in a sequence-specific fashion, while no binding was observed to complementary DNA strand. Pentameric thymidylate ON of type **2** showed a high affinity to the complementary single-stranded RNA and DNA, whilst exhibiting kinetic preferences for RNA over DNA[19]. The modification **3** was the result of an idea of constraining flexibility and introducing positive charge in the PNA backbone via linking the α-carbon of the glycyl backbone to the β-carbon of the side chain on an (2-aminoethy)lglycyl PNA[20]. Hetero-oligomeric PNAs consisting of monomeric building blocks of type **4** showed a stronger binding to the complementary DNA than the original PNA described by Jordan[21].



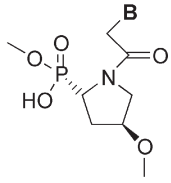
trans-3-HO-L-NAP-NA



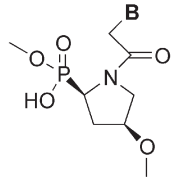
trans-4-HO-L-NAP-NA



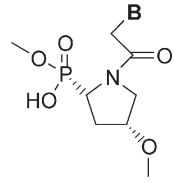
trans-L-pHypNA



trans-D-pHypNA



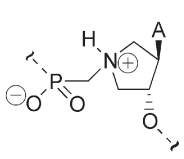
cis-L-pHypNA



cis-D-pHypNA

Modified ONs incorporating *trans*-4-hydroxy-*N*-acetyl-L-prolinol (*trans*-4-HO-L-NAP-NA), or its D analogue containing adenine and thymine as nucleobases were described by Ceulemans[22]. Efimov described synthesis of modified oligonucleotides containing 1-acetyl-4-hydroxypyrrolidine-2-phosphonic acid backbone pHypNA with various stereochemistry. These ONs exhibited high hybridization and discrimination characteristics as well as the ability to hybridize to complementary nucleic acid in low salt concentration[23].

As far as the evaluation of the role of the internucleotide backbone as a whole is concerned the replacement of phosphodiester internucleotide linkage with nonisosteric isopolar phosphonate moiety O–P–CH_2–O has been in the focus of our interest[24] because of its stability to nucleolytic enzymes. Moreover, hybridization experiments with dimers containing this kind of phosphate alteration showed that introduction of this modification was not likely to block the formation of a duplex[26,27]. A set of oligonucleotide-based HIV-I integrase inhibitors containing phosphonate modification has been also described[28].



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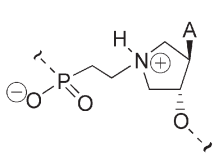

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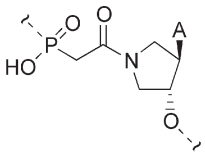

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In the present work, we deal with the compounds in which the standard pentofuranose moiety is replaced with pyrrolidine ring. Specifically, the 4′-carbon of ribose is replaced by nitrogen atom while the oxygen atom of ribose is replaced by methylene grouping. The underlying idea for this alteration was the assumption that the pyrrolidine nitrogen atom, upon protonation that was expected to occur at physiological pH, would bear positive charge which in turn should partially neutralize the internucleotide negative charge in the oligonucleotide duplex by means of a decrease in repulsion of the negatively charged chains. An increase in stability of the duplex would thus be the result. Moreover, a lower dependence on the nature of metal cation (Na^+ or Mg^{2+}) was expected. Phosphonomethyl (**5**), phosphonoethyl (**6**), and phosphonoacetyl (**7**) moieties were chosen as internucleotide linkages for the study. Modification **5** and **6** were expected to show the relation between the internucleotide linkage length and duplex stability. The idea of introduction of modification **7** containing

amide moiety consisted in the assumption that such less-flexible system could improve the entropic factor known to be important for duplex stability. As mentioned above, our modified units resemble the unnatural 2′,5′ linked system. The oligoribonucleotides containing 2′,5′-phosphodiester linkages[27] show, in contrast to DNA, a high selectivity towards complementary RNA chains. For the synthesis of desired pyrrolidine oligonucleotides with the monoester C–P–O linkage, we chose a highly efficient variant of phosphotriester method employing the (4-methoxy-1-oxido-2-pyridyl)-methyl phosphoester protecting group[29–32] which also acts as an intramolecular catalyst of the condensation reaction. The combination of the triester and standard phosphoramidite methods was used for the solid phase synthesis of the mixed-backbone ONs.

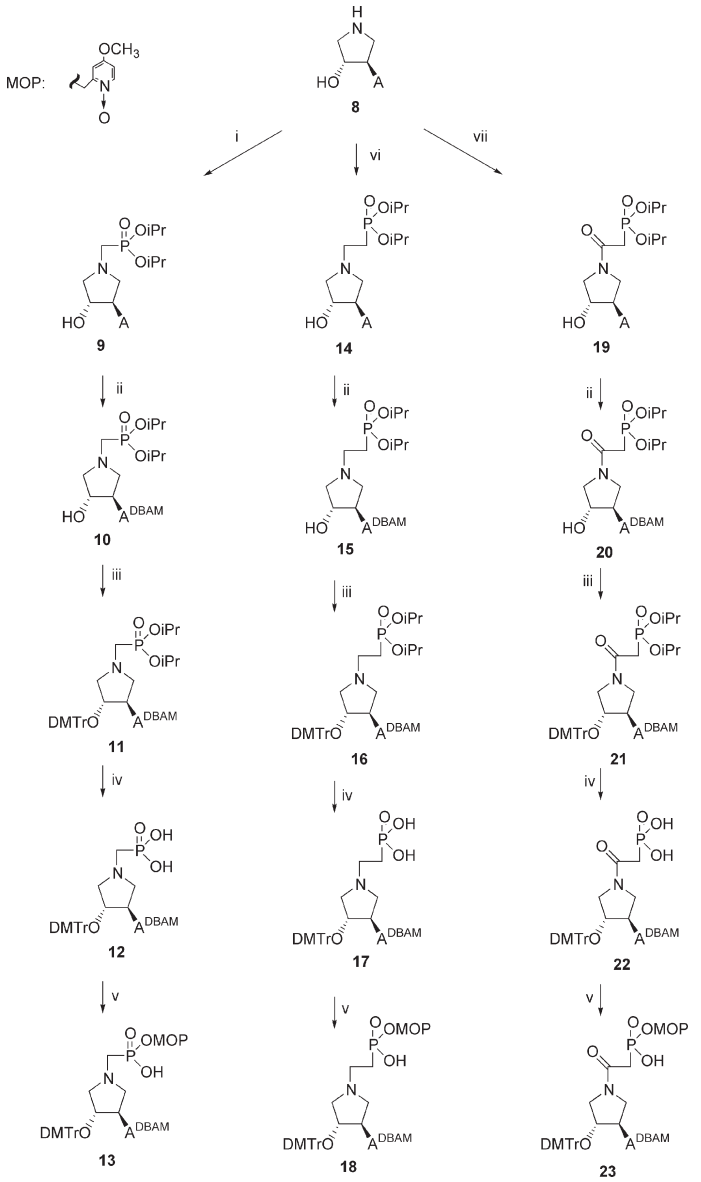
RESULTS AND DISCUSSION

Synthesis of Monomers

Pyrrolidine nucleoside intermediate **8** has been prepared according to the described procedure[33]. Phosphonylation of **8** with (i) formaldehyde and diisopropyl phosphite, (ii) diisopropyl vinylphosphonate, and (iii) *N*-hydroxysuccinimidyl diisopropylphosphonoacetate led to diisopropyl esters **9**, **14**, and **19**, respectively (Scheme 1). Adenine moieties of both phosphonates were protected with the dibutylaminomethylene (DBAM), and the free hydroxy groups were dimethoxytritylated using dimethoxytrityl chloride (DMTrCl) in pyridine. Fully protected diisopropyl phosphonates **11**, **16**, and **21** were subjected to the transesterification reaction using trimethylsilyl bromide which provided the protected phosphonic acids **12**, **17**, and **22**. The final step consisted of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) condensation with (4-methoxy-1-*N*-oxido-2-pyridyl)methanol (MOPM) followed by heating with 60% aqueous pyridine to obtain mono(4-methoxy-1-*N*-oxido-2-pyridyl)methyl (MOP) phosphonates **13**, **18**, and **23**.

Solid Phase Synthesis of Modified Oligonucleotides

Seven oligonucleotides containing either two (**ON1**, **ON2**, **ON3**), three (**ON4**, **ON5**, **ON6**), or fifteen (**ON7**) modified units were prepared. The oligomers **ON1**–**ON6** were natural oligodeoxynucleotides containing inserts of two or three modified phosphonate units. As already mentioned, a combination of standard phosphoramidite method employing common



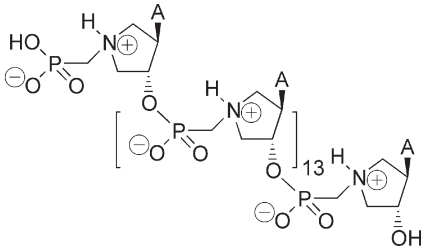
SCHEME 1

(i) H_2CO, $(iPrO)_2P(O)H$; (ii) di-*n*-butylformamide dimethyl acetal, MeOH; (iii) DMTrCl, pyridine; (iv) TMSBr, lutidine, acetonitrile; (v) 1. MOPM, TPSCl, pyridine or MOPM, DCC, pyridine; 2. 60% aq. pyridine 60 °C; (vi) diisopropyl vinylphosphonate, MeOH; (vii) *N*-hydroxysuccinimidyl diisopropyl phosphonoacetate

phosphoramidite monomers and the triester one for incorporation of the modified units was used. Certain difficulties had to be dealt with. Thus, during the phosphotriester synthesis of homooligonucleotide **ON7** containing fifteen modified units, we observed, after several condensation steps, a strongly reduced repetitive yield. We concluded that most probably, trichloroacetic acid (TCA) used in the detritylation step formed a salt with the pyrrolidine moiety (in fact, a tertiary amine) which, in turn, could decrease the condensation yield by consuming partly the activated monomer and/or TPSCl as a condensing agent (formation of a mixed anhydride of TCA with the phosphonate unit). Therefore, after detritylation step in the elongation protocol we included a "neutralization step", namely a short wash of the column with 20% TEA in acetonitrile to release TCA from its salt. This precaution was able to restore the expected repetitive yields. The solid phase synthesis of **ON7** employed the recently developed cyanoethyl-type linker suitable for the solid phase synthesis of oligodeoxynucleotides possessing terminal 3′-phosphates/phosphonates[34].

The synthesized oligonucleotides were subjected to thiophenol/triethylamine/dioxane treatment to remove MOP protecting group followed by concentrated ammonia treatment to remove the nucleobase and cyanoethyl ester protecting groups and to cleave the ON from the solid support. Crude ONs bearing 5′-*O*-dimethoxytrityl group were purified by RP HPLC and then detritylated on the same column.

In summarizing our synthetic work, the monomers **13**, **18**, and **23** for solid phase synthesis of the modified ONs were prepared and successfully used as monomers for the synthesis of two mixed-backbone 9-mers (Table I) of the sequences 5′-d(GTG **A**T**A** TGC)-3′ (**ON1**, **ON2**, and **ON3**) and 5′-d(GC**A** T**A**T C**A**C)-3′ (**ON4**, **ON5**, and **ON6**) where A represents the modified unit. A homooligonucleotide 15-mer of type **5** (**ON7**) was also synthesized using monomer **13**. The knowledge acquired with performed chemical transformations, often laborious, can be of a more general use in



Adenine fully modified pentadecamer

this less explored area of chemistry, and our elaboration of the protocol of solid-phase assembly of the mixed-type oligomers can also benefit to future syntheses combining phosphotriester and phosphoramidite strategies.

The evaluation of thermal (T_m) characteristics of the prepared oligonucleotides has revealed that, in principle, the prepared pyrrolidine oligonucleotide chains do not meet the assumption on potential stabilization of the complex with the respective oligomeric counterpart based on

TABLE I
Thermal stability of duplexes of the modified oligonucleotides with their natural counterparts (see structures **5**–**7**)

Duplex		Monomer/ scaffold	100 mM Na^+		10 mM Mg^{2+}	
			T_m °C	ΔT_m/modif °C	T_m °C	ΔT_m/modif °C
ON8/ ON9	5′-d(GTG ATA TGC)-3′ 5′-d(GCA TAT CAC)-3′	–	32.7	–	34.2	–
ON1/ ON9	5′-d(GTG **A**T**A** TGC)-3′ 5′-d(GCA TAT CAC)-3′	13/5	21.0	–5.8	23.1	–5.6
ON8/ ON4	5′-d(GTG ATA TGC)-3′ 5′-d(GC**A** T**A**T C**A**C)-3′	13/5	16.1	–5.6	17.1	–5.7
ON2/ ON9	5′-d(GTG **A**T**A** TGC)-3′ 5′-d(GCA TAT CAC)-3′	23/7	25.0	–3.8	27.1	–3.6
ON8/ ON5	5′-d(GTG ATA TGC)-3′ 5′-d(GC**A** T**A**T C**A**C)-3′	23/7	18.1	–4.9	22.9	–3.8
ON3/ ON9	5′-d(GTG **A**T**A** TGC)-3′ 5′-d(GCA TAT CAC)-3′	18/6	[a]	–	[a]	–
ON8/ ON6	5′-d(GTG ATA TGC)-3′ 5′-d(GC**A** T**A**T C**A**C)-3′	18/6	[a]	–	[a]	–
ON10/ ON11	dA_{15}/dT_{15}	–	48.0	–	–	–
ON7/ ON11	$d\mathbf{A}_{15}/dT_{15}$	13/5	34.1	–0.9	–	–
ON8/ ON12	5′-d(GTG ATA TGC)-3′ 5′-r(GCA TAT CAC)-3′	–	28.1	–	–	–
ON1/ ON12	5′-d(GTG **A**T**A** TGC)-3′ 5′-r(GCA TAT CAC)-3′	13/5	17.2	–5.4	–	–
ON2/ ON12	5′-d(GTG **A**T**A** TGC)-3′ 5′-r(GCA TAT CAC)-3′	23/7	15.2	–6.4	–	–
ON3/ ON12	5′-d(GTG **A**T**A** TGC)-3′ 5′-r(GCA TAT CAC)-3′	18/6	[a]	–	–	–

[a] No duplex formation (no sigmoidal profile was observed).

the decrease in repulsive forces due to the presence of protonated pyrrolidine ring. Instead, the strongly decreased hybridization capabilities were found, and the data show that obviously, the expected effect of the pyrrolidine ring positive charge is offset by effects of mixed 2′,5′ and 3′,5′ internucleotide linkages on oligomer stereochemistry and by effects caused by stereochemical arrangement of modified pyrrolidine units. Originally we anticipated a certain conformational adaptability of the *N*-protonated *N*-phosphonoalkyl moiety but our latest results concerning also the conformation of prolinol[35] and pyrrolidine[36] nucleoside phosphonic acids showed that these compounds existed predominantly in a *trans* arrangement of the nucleobase and *N*-phosphonoalkyl moiety (5′-phosphate mimic) and not in the expected and unambiguously advantageous *cis* orientation like in 5′-nucleotides. In addition, the fully *N*-protonated form was observed within a large range of pH values (2–10). These results suggest that just the *trans* orientation of a nucleobase and *N*-phosphonoalkyl moiety together with 2′,5′ linkage seem to be the main factors contributing to duplex destabilization.

Thus, oligomers of type **5** and **7** containing either two (**ON1**, **ON2**) or three (**ON4**, **ON5**) modifications exhibited significantly lower melting points (T_m) than natural ones in the range from –3.5 to –6 °C per modification. Introduction of the modification of type **6** (**ON3**, **ON6**) abolished the duplex formation completely. In contrast to the type **5** (*N*-phosphonomethyl), the type **6** (*N*-phosphonoethyl), which is one methylene group longer, brings to the system one more degree of freedom. So the contributon of rotation entropy will be much higher than in the case of shorter type **5** and also in the case of isosteric but conformationally more restricted type **7** (amide bond). The entropic factor which relates to the strand pre-organization seems to be most important for the duplex stability. The results suggest the need to study the behavior of another modification combining type **5** and **7**, i.e., the P–C(O)–N nternucleotide linkage.

In contrast to studied modified nonamers, the prepared homooligomer consisting of fifteen modified units (scaffold **5**) exhibited a considerably lower decrease in the melting point of only –0.9 °C per modification which suggests that the conformation of the modified strand accommodates the conformation of the natural couterparts or vice versa. The study on pyrrolidine-based homooligomers and types of their complexes (duplex, triplex), orientation of the strands, etc. is under way.

EXPERIMENTAL

Synthesis of Monomers

Unless stated otherwise, all used solvents were anhydrous. Final products were lyophilized from dioxane or benzene, and dried over phosphorus pentoxide at 50–70 °C and 13 Pa. TLC was performed on silica gel pre-coated aluminium plates Fluka or Merck Silica gel/TLC-cards with a 254 nm fluorescent indicator. The compounds were detected by UV light (254 nm), by heating (detection of dimethoxytrityl group; orange color), and by spraying with 1% solution of 4-(4-nitrobenzyl)pyridine in ethanol followed by heating and treatment with gaseous ammonia (blue color of mono- and diesters of phosphonic acid). Preparative column chromatography was carried out on silica gel (40–60 μm; Fluka) neutralized with triethylamine (1 ml/100 g), and elution was performed at a flow rate of 40 ml/min. The following solvent systems were used for TLC and preparative chromatography: toluene–ethyl acetate 4:1 (T1), 1:1 (T2); toluene–acetone 1:1 (T3); chloroform–ethanol 9:1 (C1); ethyl acetate–acetone–ethanol–water 4:1:1:1 (H1), 12:2:2:1 (H3). The concentrations of solvent systems are given in volume percents (%, v/v). Mass spectra of compounds were recorded on a ZAB-EQ (VG Analytical) instrument using FAB (ionization with Xe, accelerating voltage 8 kV) with glycerol and thioglycerol as matrices while for the oligonucleotide molecular ion determination, MALDI-TOF measurement on a Reflex IV (Bruker Daltonics, Germany) instrument was used. NMR spectra were measured on Varian Unity 500 instrument (^{1}H at 500 MHz, ^{13}C at 125.7 MHz) in deuterated chloroform or hexadeuteriodimethyl sulfoxide (DMSO-d_6). Oligonucleotides were synthesized on automated GENSYN synthesizer (IOCB Prague) and the T_m values were measured using an UV VIS spectrophotometer Varian Cary 500.

Diisopropyl [(3*R*,4*R*)-3-(Adenin-9-yl)-4-hydroxypyrrolidin-1-yl]methylphosphonate (**9**)

14.5 M aqueous formaldehyde (1.4 ml, 20 mmol) was added to the suspension of **8** (0.9 g, 4.087 mmol) in diisopropyl phosphite (3.3 g, 20 mmol). The mixture was homogenized in ultrasonic bath and stirred at 60 °C for 5 h. Then, 0.02 M sulfuric acid (50 ml) was added and the reaction mixture was stirred at r.t. for 2 days. The solution was applied on to a column of 40 ml of Dowex 50 (H^+). The resin was washed with 50% aqueous ethanol (200 ml). The crude title product was obtained by elution with 3% ammonia in ethanol–water (1:1) mixture. Pure compound **9** was obtained by column chromatography on silica gel using linear gradient of ethanol in chloroform in a 78% yield as a colorless oil (1.27 g, 3.18 mmol). HR FAB+: calculated (M + H) 399.190967, found 399.190494. ^{1}H NMR (400 MHz, DMSO-d_6): 1.24 and 1.25 (2 × d, 12 H, J_{vic} = 6.2, (C**H**$_3$)$_2$CH); 2.56 (dd, 1 H, J_{gem} = 10.0, $J_{5b,4}$ = 4.7, H-5b-pyrr); 2.89 (dd, 1 H, J_{gem} = 15.0, $J_{H,P}$ = 10.7, CH$_a$**H**$_b$-P); 2.98 (dd, 1 H, J_{gem} = 15.0, $J_{H,P}$ = 12.4, C**H**$_a$H$_b$-P); 3.09 (d, 2 H, $J_{2,3}$ = 5.8, H-2-pyrr); 3.35 (dd, 1 H, J_{gem} = 10.0, $J_{5a,4}$ = 6.8, H-5a-pyrr); 4.40 (bm, 1 H, $J_{4,5}$ = 6.8, 4.7, $J_{4,OH}$ = 4.8, $J_{4,3}$ = 3.3, H-4-pyrr); 4.60 and 4.61 (2 × dh, 2 × 1 H, $J_{H,P}$ = 7.8, J_{vic} = 6.2, C**H**(CH$_3$)$_2$); 4.70 (td, 1 H, $J_{3,2}$ = 5.8, $J_{3,4}$ = 3.3, H-3-pyrr); 5.54 (d, 1 H, $J_{OH,4}$ = 4.8, OH); 7.23 (bs, 2 H, NH$_2$); 8.14 (s, 1 H, H-8); 8.18 (s, 1 H, H-2). ^{13}C NMR (100.6 MHz, DMSO-d_6): 24.00 and 24.08 (d, $J_{C,P}$ = 4, (**C**H$_3$)$_2$CH); 50.94 (d, $J_{C,P}$ = 163, CH$_2$P); 58.79 (d, $J_{C,P}$ = 10, CH$_2$-2-pyrr); 61.67 (CH-3-pyrr); 62.69 (d, $J_{C,P}$ = 10, CH$_2$-5-pyrr); 69.99 (d, $J_{C,P}$ = 6, **C**H(CH$_3$)$_2$); 75.06 (CH-4-pyrr); 118.87 (C-5); 139.47 (CH-8); 149.52 (C-4); 152.54 (CH-2); 156.19 (C-6). ^{31}P NMR (162 MHz, DMSO-d_6): 22.84.

Diisopropyl (3*R*,4*R*)-3-{[N^6-(Dibutylamino)methyleneadenin-9-yl]-4-hydroxypyrrolidin-1-yl}methylphosphonate (**11**)

Compound **9** (1.27 g, 3.18 mmol) was dissolved in methanol (30 ml), and dibutylformamide dimethyl acetal (0.9 ml, 3.82 mmol) was added. The reaction mixture was left aside at r.t. for 2 days and then quenched with TEAB (triethylammonium bicarbonate) (1 ml). The solvent was evaporated and the DBAM derivative **10** was obtained in 88% yield (1.51 g, 2.81 mmol) by column chromatography on silica gel using linear gradient of ethanol in chloroform as a colorless oil and used without characterization.

The obtained compound was co-evaporated with pyridine (3 × 40 ml). Then, 4,4′-dimethoxytrityl chloride (1.7 g, 5 mmol) and silver triflate (1.28 g, 5 mmol) were added to the solution of **10** (1.51 g, 2.81 mmol) in pyridine (30 ml). The reaction mixture was stirred at r.t. overnight, quenched with methanol (3 ml), diluted with ethyl acetate (200 ml), and washed with saturated solution of sodium hydrogencarbonate. Organic layer was dried over sodium sulfate, concentrated, and applied on to a column of silica gel. The title product was obtained using linear gradient of ethanol in chloroform in 77% yield (1.81 g, 2.16 mmol) as a yellowish oil. HR-MS: for $C_{46}H_{63}N_7O_6P$ $(M + H)^+$ calculated 840.45774, found 840.45815. ^{1}H NMR (400 MHz, $CDCl_3$): 0.95 and 0.96 (2 × t, 2 × 3 H, J_{vic} = 7.3, C**H**$_3$CH$_2$CH$_2$CH$_2$N); 1.25, 1.27, 1.28 and 1.29 (4 × d, 4 × 3 H, J_{vic} = 6.2, (C**H**$_3$)$_2$CH); 1.32–1.45 (m, 4 H, CH$_3$C**H**$_2$CH$_2$CH$_2$N); 1.60–1.71 (m, 4 H, CH$_3$CH$_2$C**H**$_2$CH$_2$N); 2.24 (dd, 1 H, J_{gem} = 10.0, $J_{5b,4}$ = 5.7, H-5b-pyrr); 2.73 (dd, 1 H, J_{gem} = 15.0, $J_{H,P}$ = 11.7, CH$_a$**H**$_b$-P); 2.80 (dd, 1 H, J_{gem} = 15.0, $J_{H,P}$ = 12.2, C**H**$_a$H$_b$-P); 2.84 (dd, 1 H, J_{gem} = 10.0, $J_{5a,4}$ = 6.8, H-5a-pyrr); 3.04 (d, 2 H, $J_{2,3}$ = 4.6, H-2-pyrr); 3.40 (t, 2 H, J_{vic} = 7.3, CH$_3$CH$_2$CH$_2$C**H**$_2$N); 3.67 and 3.70 (2 × s, 2 × 3 H, CH$_3$O); 3.74 (t, 2 H, J_{vic} = 7.3, CH$_3$CH$_2$CH$_2$C**H**$_2$N); 4.37 (ddd, 1 H, $J_{4,5}$ = 6.8, 6.7, $J_{4,3}$ = 2.5, H-4-pyrr); 4.67 (dh, 2 H, $J_{H,P}$ = 7.8, J_{vic} = 6.2, C**H**(CH$_3$)$_2$); 5.11 (td, 1 H, $J_{3,2}$ = 4.6, $J_{3,4}$ = 2.5, H-3-pyrr); 6.58 and 6.65 (2 × m, 2 × 2 H, H-*m*-C$_6$H$_4$-DMTr); 7.07–7.15 (m, 3 H, H-*m*,*p*-C$_6$H$_5$-DMTr); 7.22 and 7.23 (2 × m, 2 × 2 H, H-*o*-C$_6$H$_4$-DMTr); 7.37 (m, 2 H, H-*o*-C$_6$H$_5$-DMTr); 7.93 (s, 1 H, H-8); 8.56 (s, 1 H, H-2); 9.00 (s, 1 H, CH=N). ^{13}C NMR (100.6 MHz, $CDCl_3$): 13.68 and 13.92 (**C**H$_3$CH$_2$CH$_2$CH$_2$N); 19.75 and 20.18 (CH$_3$**C**H$_2$CH$_2$CH$_2$N); 24.04, 24.08 and 24.11 (d, $J_{C,P}$ = 4, (**C**H$_3$)$_2$CH); 29.25 and 31.00 (CH$_3$CH$_2$**C**H$_2$CH$_2$N); 45.08 (CH$_3$CH$_2$CH$_2$**C**H$_2$N); 51.26 (d, $J_{C,P}$ = 165, CH$_2$P); 51.75 (CH$_3$CH$_2$CH$_2$**C**H$_2$N); 55.05 and 55.08 (CH$_3$O); 60.26 (d, $J_{C,P}$ = 9, CH$_2$-2-pyrr); 60.63 (CH-3-pyrr); 62.44 (d, $J_{C,P}$ = 11, CH$_2$-5-pyrr); 70.59 and 70.62 (d, $J_{C,P}$ = 7, **C**H(CH$_3$)$_2$); 79.15 (CH-4-pyrr); 87.35 (C-DMTr); 112.98 and 113.05 (CH-*m*-C$_6$H$_4$-DMTr); 125.38 (C-5); 126.81 (CH-*p*-C$_6$H$_5$-DMTr); 127.76 (CH-*m*-C$_6$H$_5$-DMTr); 127.94 (CH-*o*-C$_6$H$_5$-DMTr); 129.94 and 130.05 (CH-*o*-C$_6$H$_4$-DMTr); 135.98 and 136.00 (C-*i*-C$_6$H$_4$-DMTr); 140.42 (CH-8); 145.03 (C-*i*-C$_6$H$_5$-DMTr); 151.25 (C-4); 152.26 (CH-2); 158.18 (CH=N); 158.44 and 158.80 (C-*p*-C$_6$H$_4$-DMTr); 159.90 (C-6). ^{31}P NMR (162 MHz, $CDCl_3$): 26.79.

(4-Methoxy-1-oxido-2-pyridyl)methyl (3*R*,4*R*)-3-{[N^6-(Dibutylamino)-methyleneadenin-9-yl]-4-(4,4′-dimethoxytrityl)oxypyrrolidin-1-yl}methylphosphonate (**13**)

Compound **11** was co-evaporated with acetonitrile (2 × 30 ml) and dissolved in the same solvent (20 ml). Lutidine (3 ml, 26 mmol) and bromotrimethylsilane (1.7 ml, 13 mmol) were added under argon. The reaction mixture was left at r.t. under argon overnight. Acetonitrile was removed in vacuo, the residue was dissolved in the mixture of TEAB and methanol, and the volatiles were evaporated. Phosphonic acid **12** was obtained by column chromatography on silica gel using a linear gradient of H1 in ethyl acetate in 68% yield

(1.4 g, 1.46 mmol). NMR signals were too broad to be analyzed so this intermediate was used without further characterization.

A mixture of compound **12** (0.8 g, 0.83 mmol) and (4-methoxy-1-*N*-oxido-2-pyridyl)methanol (0.4 g, 2.5 mmol) was co-evaporated with pyridine (2 × 10 ml) and dissolved in the same solvent (10 ml). TPSCl (1.52 g, 5 mmol) was added, and the reaction mixture was stirred at r.t. overnight. Pyridine was removed in vacuo, and the crude reaction mixture was purified on silica gel column using a linear gradient of H3 in ethyl acetate followed by linear gradient of H1 in H3. The obtained material was heated at 60 °C in 60% aqueous pyridine (30 ml) for 5 days. The solvents were removed, and the obtained residue was partitioned between chloroform and TEAB. Organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. The crude material was co-evaporated with methanol (2 × 20 ml) and dissolved in the same solvent (30 ml). Dibutylformamide dimethyl acetal (0.5 ml, 2.12 mmol) was added, and the mixture was left for 2 days at r.t. 2 M aqueous TEAB (1 ml) was added and the reaction mixture was concentrated in vacuo, dissolved in chloroform, and washed with TEAB. The product was obtained in a 29% yield (0.24 g, 0.24 mmol) by reverse phase chromatography using linear gradient of methanol in water as a white amorphous fluffy solid after lyofilization from water. HR FAB+: calculated (M + H) 893.411525, found 893.408434. ^{1}H NMR (500 MHz, $CDCl_3$): 0.95 and 0.96 (2 × t, 2 × 3 H, J_{vic} = 7.4, C**H**$_3$CH$_2$CH$_2$CH$_2$N); 1.22 (t, 3 H, J_{vic} = 7.3, C**H**$_3$CH$_2$N); 1.39 and 1.65 (2 × m, 2 × 4 H, CH$_3$C**H**$_2$C**H**$_2$CH$_2$N); 2.52 and 2.88 (2 × bm, 2 H, H-5-pyrr); 2.93–3.07 (m, 6 H, H-2-pyr, CH$_2$P and CH$_3$C**H**$_2$N); 3.40 (t, 2 H, J_{vic} = 7.3, CH$_3$CH$_2$CH$_2$C**H**$_2$N); 3.66 and 3.69 (2 × s, 2 × 3 H, CH$_3$O-DMtr); 3.70 (t, 2 H, J_{vic} = 7.3, CH$_3$CH$_2$CH$_2$C**H**$_2$N); 3.76 (s, 3 H, CH$_3$O-MOP); 4.57 (bm, 1 H, H-4-pyrr); 5.12 (bm, 1 H, H-3-pyrr); 5.14 (bd, 2 H, $J_{H,P}$ = 6.8, CH$_2$-MOP); 6.57 and 6.63 (2 × m, 2 × 2 H, H-*m*-C$_6$H$_4$-DMTr); 6.70 (dd, 1 H, $J_{5,6}$ = 7.2, $J_{5,3}$ = 3.5, H-5-MOP); 7.05–7.14 (m, 3 H, H-*m*,*p*-C$_6$H$_5$-DMTr); 7.15–7.22 (m, 5 H, H-3-MOP and H-*o*-C$_6$H$_4$-DMTr); 7.32 (m, 2 H, H-*o*-C$_6$H$_5$-DMTr); 7.94 (s, 1 H, H-8); 8.06 (d, 1 H, $J_{6,5}$ = 7.2, H-6-MOP); 8.46 (s, 1 H, H-2); 9.02 (s, 1 H, CH=N). ^{13}C NMR (125.7 MHz, $CDCl_3$): 8.46 (C**H**$_3$CH$_2$N); 13.70 and 13.92 (C**H**$_3$CH$_2$CH$_2$CH$_2$N); 19.77 and 20.18 (CH$_3$C**H**$_2$CH$_2$CH$_2$N); 29.24 and 31.02 (CH$_3$CH$_2$C**H**$_2$CH$_2$N); 45.07 (CH$_3$CH$_2$CH$_2$C**H**$_2$N); 45.37 (CH$_3$C**H**$_2$N); 51.84 (CH$_3$CH$_2$CH$_2$C**H**$_2$N); 52.06 (d, $J_{C,P}$ = 150, CH$_2$P); 55.06 and 55.10 (CH$_3$O-DMTr); 56.15 (CH$_3$O-MOP); 60.82 (CH-3-pyrr); 61.25 (CH$_2$-MOP); 61.80 (CH$_2$-2-pyrr); 62.59 (CH$_2$-5-pyrr); 75.40 (CH-4-pyrr); 87.33 (C-DMTr); 108.83 (CH-3-MOP); 110.65 (CH-5-MOP); 113.00 and 113.10 (CH-*m*-C$_6$H$_4$-DMTr); 125.43 (C-5); 126.81 (CH-*p*-C$_6$H$_5$-DMTr); 127.77 and 127.9 (CH-*o*,*m*-C$_6$H$_5$-DMTr); 129.87 and 130.02 (CH-*o*-C$_6$H$_4$-DMTr); 135.87 and 135.90 (C-*i*-C$_6$H$_4$-DMTr); 139.66 (CH-6-MOP); 141.03 (CH-8); 144.93 (C-*i*-C$_6$H$_5$-DMTr); 150.85 (C-2-MOP); 151.18 (C-4); 152.09 (CH-2); 158.42, 158.50 and 158.58 (CH=N and C-*p*-C$_6$H$_4$-DMTr); 158.78 (C-4-MOP and C-6).

Diisopropyl 2-[(3*R*,4*R*)-3-(Adenin-9-yl)-4-hydroxypyrrolidin-1-yl]ethylphosphonate (**14**)

Diisopropyl vinylphosphonate (1.4 g, 7 mmol) was added to a suspension of compound **8** (0.7 g, 3.18 mmol) in methanol (30 ml). The mixture was refluxed for 5 days, and the solution was applied on to a column of Dowex 50 (40 ml). The resin was washed with 50% aqueous ethanol (200 ml). The crude product was obtained by elution with 3% ammonia in ethanol–water (1:1) mixture. Titled compound was obtained in a 69% yield (0.9 g, 2.19 mmol) by column chromatography on silica gel using linear gradient of ethanol in chloroform as a colorless oil. HR FAB+: calculated (M + H) 413.206617, found 413.208356.

^{1}H NMR (400 MHz, DMSO-d_6): 1.234, 1.240, 12.42 and 1.243 (4 × d, 4 × 3 H, J_{vic} = 6.2, (C**H**$_3$)$_2$CH); 1.92 (m, 2 H, CH$_2$P); 2.43 (dd, 1 H, J_{gem} = 9.8, $J_{5b,4}$ = 4.6, H-5b-pyrr); 2.66 (m, 2 H, CH$_2$N); 2.90 (dd, 1 H, J_{gem} = 9.6, $J_{2b,3}$ = 5.1, H-2b-pyrr); 3.00 (dd, 1 H, J_{gem} = 9.6, $J_{2a,3}$ = 7.1, H-2a-pyrr); 3.18 (dd, 1 H, J_{gem} = 9.8, $J_{5a,4}$ = 7.0, H-5a-pyrr); 4.41 (bm, 1 H, $J_{4,5}$ = 7.0, 4.6, $J_{4,OH}$ = 5.0, $J_{4,3}$ = 3.7, H-4-pyrr); 4.57 and 4.58 (2 × dh, 2 × 1 H, $J_{H,P}$ = 7.8, J_{vic} = 6.2, C**H**(CH$_3$)$_2$); 4.72 (td, 1 H, $J_{3,2}$ = 7.1, 5.1, $J_{3,4}$ = 3.7, H-3-pyrr); 5.52 (d, 1 H, $J_{OH,4}$ = 5.0, OH); 7.21 (bs, 2 H, NH$_2$); 8.13 (s, 1 H, H-8); 8.24 (s, 1 H, H-2). ^{13}C NMR (100.6 MHz, DMSO-d_6): 23.97 and 23.99 (d, $J_{C,P}$ = 4, (C**H**$_3$)$_2$CH); 25.60 (d, $J_{C,P}$ = 139, CH$_2$P); 48.95 (CH$_2$N); 57.17 (CH$_2$-2-pyrr); 60.58 (CH$_2$-5-pyrr); 61.60 (CH-3-pyrr); 69.41 (d, $J_{C,P}$ = 6, C**H**(CH$_3$)$_2$); 75.26 (CH-4-pyrr); 118.88 (C-5); 139.77 (CH-8); 149.56 (C-4); 152.48 (CH-2); 156.18 (C-6). ^{31}P NMR (162 MHz, DMSO-d_6): 28.46.

Diisopropyl 2-{(3*R*,4*R*)-3-[N^6-(Dibutylamino)methyleneadenin-9-yl]-4-(4,4′-dimethoxytrityl)oxypyrrolidin-1-yl}ethylphosphonate (**16**)

Compound **14** (0.9 g, 2.19 mmol) was dissolved in methanol (20 ml), and dibutylformamide dimethyl acetal (0.72 ml, 3 mmol) was added. The reaction mixture was left aside at r.t. for 2 days and the reaction was quenched with TEAB (1 ml). On evaporation of the solvent, the desired product **15** was obtained in a 95% yield (1.15 g, 2.08 mmol) by column chromatography on silica gel using a linear gradient of ethanol in chloroform as a yellowish oil, and was used without characterization.

Compound **15** was co-evaporated with pyridine (3 × 40 ml) and dissolved in the same solvent (20 ml). Dimethoxytrityl chloride (1.4 g, 4 mmol) and silver triflate (1 g, 4 mmol) were added to the solution, and the reaction mixture was stirred at r.t. overnight. The reaction was quenched with methanol (3 ml), diluted with ethyl acetate (200 ml), and washed with saturated solution of sodium hydrogencarbonate. Organic layer was dried over sodium sulfate, concentrated, and the residue applied on a column of silica gel. The product was obtained in a 79% yield (1.4 g, 1.64 mmol) using linear gradient of ethanol in chloroform as a yellowish oil. HR FAB+: calculated (M + H) 854.473397, found 854.476457. ^{1}H NMR (500 MHz, CDCl$_3$): 0.95 and 0.96 (2 × t, 2 × 3 H, J_{vic} = 7.3, C**H**$_3$CH$_2$CH$_2$CH$_2$N); 1.27, 1.28 and 1.29 (3 × d, 12 H, J_{vic} = 6.2, (C**H**$_3$)$_2$CH); 1.35–1.49 (m, 4 H, CH$_3$C**H**$_2$CH$_2$CH$_2$N); 1.61–1.71 (m, 4 H, CH$_3$CH$_2$C**H**$_2$CH$_2$N); 1.78 (m, 2 H, CH$_2$P); 2.07 (dd, 1 H, J_{gem} = 10.0, $J_{5b,4}$ = 5.8, H-5b-pyrr); 2.55–2.72 (m, 3 H, H-5a-pyrr and CH$_2$N); 2.79 (dd, 1 H, J_{gem} = 9.9, $J_{2b,3}$ = 2.9, H-2b-pyrr); 2.85 (dd, 1 H, J_{gem} = 9.9, $J_{2b,3}$ = 6.6, H-2a-pyrr); 3.40 (t, 2 H, J_{vic} = 7.3, CH$_3$CH$_2$CH$_2$C**H**$_2$N); 3.67 and 3.70 (2 × s, 2 × 3 H, CH$_3$O); 3.72 (t, 2 H, J_{vic} = 7.3, CH$_3$CH$_2$CH$_2$C**H**$_2$N); 4.38 (ddd, 1 H, $J_{4,5}$ = 7.1, 5.8, $J_{4,3}$ = 2.9, H-4-pyrr); 4.66 (dh, 2 H, $J_{H,P}$ = 8.0, J_{vic} = 6.2, C**H**(CH$_3$)$_2$); 5.12 (dt, 1 H, $J_{3,2}$ = 6.6, 2.9, $J_{3,4}$ = 2.9, H-3-pyrr); 6.58 and 6.65 (2 × m, 2 × 2 H, H-*m*-C$_6$H$_4$-DMTr); 7.07–7.15 (m, 3 H, H-*m*,*p*-C$_6$H$_5$-DMTr); 7.21 and 7.23 (2 × m, 2 × 2 H, H-*o*-C$_6$H$_4$-DMTr); 7.36 (m, 2 H, H-*o*-C$_6$H$_5$-DMTr); 7.93 (s, 1 H, H-8); 8.56 (s, 1 H, H-2); 9.02 (s, 1 H, CH=N). ^{13}C NMR (125.7 MHz, CDCl$_3$): 13.66 and 13.89 (CH$_3$CH$_2$CH$_2$CH$_2$N); 19.78 and 20.21 (CH$_3$CH$_2$CH$_2$CH$_2$N); 24.00, 24.05 and 24.06 (d, $J_{C,P}$ = 4, (CH$_3$)$_2$CH); 26.32 (d, $J_{C,P}$ = 141, CH$_2$P); 29.29 and 31.08 (CH$_3$CH$_2$CH$_2$CH$_2$N); 45.17 (CH$_3$CH$_2$CH$_2$CH$_2$N); 48.60 (CH$_2$N); 51.80 (CH$_3$CH$_2$CH$_2$CH$_2$N); 55.07 and 55.11 (CH$_3$O); 58.40 (CH$_2$-2-pyrr); 60.26 (CH$_2$-5-pyrr); 60.53 (CH-3-pyrr); 70.06 and 70.10 (d, $J_{C,P}$ = 6, CH(CH$_3$)$_2$); 79.08 (CH-4-pyrr); 87.38 (C-DMTr); 113.04 and 113.11 (CH-*m*-C$_6$H$_4$-DMTr); 125.48 (C-5); 126.82 (CH-*p*-C$_6$H$_5$-DMTr); 127.75 (CH-*m*-C$_6$H$_5$-DMTr); 128.02 (CH-*o*-C$_6$H$_5$-DMTr); 129.92 and 130.02 (CH-*o*-C$_6$H$_4$-DMTr); 136.05 and 136.07 (C-*i*-C$_6$H$_4$-DMTr); 140.52 (CH-8);

145.05 (C-*i*-C_6H_5-DMTr); 151.38 (C-4); 152.38 (CH-2); 158.25 (CH=N); 158.52 and 158.60 (C-*p*-C_6H_4-DMTr); 159.97 (C-6). ^{31}P NMR (202.3 MHz, $CDCl_3$): 27.50.

(4-Methoxy-1-oxido-2-pyridyl)methyl 2-{(3*R*,4*R*)-3-[N^6-(Dibutylamino)-methyleneadenin-9-yl]-4-(4,4′-dimethoxytrityl)oxypyrrolidin-1-yl}ethylphosphonate (**18**)

Compound **16** (1.4 g, 1.64 mmol) was co-evaporated with acetonitrile (2 × 30 ml) and dissolved in the same solvent (15 ml). Lutidine (2.3 ml, 20 mmol) and bromotrimethylsilane (1.3 ml, 10 mmol) were added under argon. The reaction mixture was left overnight at r.t. under argon, acetonitrile was removed in vacuo, the residue was dissolved in a mixture of aqueous TEAB and methanol and the solvents evaporated again. Phosphonic acid **17** was obtained in a 73% yield (1.17 g, 1.2 mmol) by column chromatography on silica gel using a linear gradient of H1 in ethyl acetate as a yellowish oil. NMR signals were too broad to be analyzed so this intermediate was used without further characterization.

A mixture of compound **17** (0.58 g, 0.6 mmol) and (4-methoxy-1-oxido-2-pyridyl)methanol (0.3 g, 1.78 mmol) was co-evaporated with pyridine (2 × 10 ml) and dissolved in the same solvent (10 ml). TPSCl (1.1 g, 3.6 mmol) was added. The reaction mixture was stirred at r.t. overnight. Pyridine was removed in vacuo, and the crude reaction mixture was purified on a silica gel column using linear gradient of H3 in ethyl acetate followed by linear gradient of H1 in H3. The obtained material was heated at 60 °C in 60% aqueous pyridine (30 ml) for 5 days, and the solvents were removed. The obtained residue was partitioned between chloroform and TEAB. Organic layer was dried over anhydrous sodium sulfate and concentrated in vacuo. Crude material was co-evaporated with methanol (2 × 20 ml) and dissolved in the same solvent (30 ml). Dibutylformamide dimethyl acetal (0.5 ml, 2.1 mmol) was added, and the mixture was left at r.t. for 2 days. Then 2 M aqueous TEAB (1 ml) was added and the reaction mixture was concentrated in vacuo, dissolved in chloroform, and washed with TEAB. The product was obtained in a 25% yield (0.15 g, 0.15 mmol) by reverse phase chromatography using a linear gradient of methanol in water as a white amorphous fluffy solid after lyofilization from dioxane. HR FAB+: calculated (M + H) 907.427175, found 907.424525. ^{1}H NMR (400 MHz, $CDCl_3$): 0.94 and 0.96 (2 × t, 2 × 3 H, J_{vic} = 7.4, C**H**$_3$CH$_2$CH$_2$CH$_2$N); 1.27 (t, 3 H, J_{vic} = 7.3, C**H**$_3$CH$_2$N); 1.38 and 1.65 (2 × m, 2 × 4 H, CH$_3$C**H**$_2$C**H**$_2$CH$_2$N); 1.92 (bdt, 2 H, $J_{H,P}$ = 17.7, J_{vic} = 8.0, CH$_2$P); 2.41 (bm, 1 H, H-5b-pyrr); 2.80 (bdd, 1 H, J_{gem} = 10.8, $J_{5a,4}$ = 7.1, H-5a-pyrr); 2.94 (bt, 2 H, J_{vic} = 8.0, CH$_2$N); 3.03 (q, 2 H, J_{vic} = 7.3, CH$_3$C**H**$_2$N); 3.11 and 3.23 (2 × m, 2 H, H-2-pyrr); 3.40 (t, 2 H, J_{vic} = 7.3, CH$_3$CH$_2$CH$_2$C**H**$_2$N); 3.67 and 3.70 (2 × s, 2 × 3 H, CH$_3$O-DMtr); 3.71 (t, 2 H, J_{vic} = 7.3, CH$_3$CH$_2$CH$_2$C**H**$_2$N); 3.81 (s, 3 H, CH$_3$O-MOP); 4.57 (bm, 1 H, H-4-pyrr); 5.15 (bm, 1 H, H-3-pyrr); 5.15 (bd, 2 H, $J_{H,P}$ = 7.3, CH$_2$-MOP); 6.59 and 6.66 (2 × m, 2 × 2 H, H-*m*-C_6H_4-DMTr); 6.71 (dd, 1 H, $J_{5,6}$ = 7.3, $J_{5,3}$ = 3.5, H-5-MOP); 7.08–7.15 (m, 3 H, H-*m*,*p*-C_6H_5-DMTr); 7.16–7.22 (m, 5 H, H-3-MOP and H-*o*-C_6H_4-DMTr); 7.32 (m, 2 H, H-*o*-C_6H_5-DMTr); 7.92 (s, 1 H, H-8); 8.07 (d, 1 H, $J_{6,5}$ = 7.3, H-6-MOP); 8.47 (s, 1 H, H-2); 9.02 (s, 1 H, CH=N). ^{13}C NMR (100.6 MHz, $CDCl_3$): 8.46 (**C**H$_3$CH$_2$N); 13.67 and 13.89 (**C**H$_3$CH$_2$CH$_2$CH$_2$N); 19.73 and 20.15 (CH$_3$**C**H$_2$CH$_2$CH$_2$N); 25.49 (d, $J_{C,P}$ = 128, CH$_2$P); 29.22 and 30.98 (CH$_3$CH$_2$**C**H$_2$CH$_2$N); 45.12 (CH$_3$CH$_2$CH$_2$**C**H$_2$N); 45.20 (CH$_3$**C**H$_2$N); 50.82 (CH$_2$N); 51.84 (CH$_3$CH$_2$CH$_2$**C**H$_2$N); 55.06 and 55.10 (CH$_3$O-DMTr); 56.20 (CH$_3$O-MOP); 56.52 (CH$_2$-2-pyrr); 59.46 (CH$_2$-5-pyrr); 60.54 (CH-3-pyrr); 60.76 (d, $J_{C,P}$ = 4, CH$_2$-MOP); 77.44 (CH-4-pyrr); 87.48 (C-DMTr); 108.69 (CH-3-MOP); 110.81 (CH-5-MOP); 113.07 and 113.15 (CH-*m*-C_6H_4-DMTr); 125.62 (C-5); 126.92 (CH-*p*-C_6H_5-DMTr); 127.82 and 127.94

(CH-*o*,*m*-C_6H_5-DMTr); 129.82 and 129.99 (CH-*o*-C_6H_4-DMTr); 135.64 (C-*i*-C_6H_4-DMTr); 139.73 (CH-6-MOP); 141.14 (CH-8); 144.68 (C-*i*-C_6H_5-DMTr); 150.77 (d, $J_{C,P}$ = 7, C-2-MOP); 151.09 (C-4); 152.12 (CH-2); 158.34 (CH=N); 158.48 and 158.57 (C-*p*-C_6H_4-DMTr); 158.74 (C-4-MOP); 159.80 (C-6). ^{31}P NMR (162 MHz, $CDCl_3$): 22.49.

Diisopropyl 2-[(3*R*,4*R*)-3-(Adenin-9-yl)-4-hydroxypyrrolidin-1-yl]-2-oxoethylphosphonate (**19**)

A solution of *N*-hydroxysuccinimidyl(diisopropoxyphosphoryl)acetate, prepared in situ from (diisopropoxyphosphoryl)acetatic acid (0.7, 3.1 mmol), *N*-hydroxysuccinimide (0.36 g, 3.1 mmol) and DCC (1.28 g, 6.2 mmol) in acetonitrile (12 ml), was added to (3*R*,4*S*)-3-(adenin-9-yl)-4-hydroxypyrrolidine (**8**) (0.24 g, 1.1 mmol). The suspension was concentrated in vacuo and ethyldiisopropylamine (0.38 ml, 2.2 mmol) and ethanol (4 ml) were added to the residue. The suspension was stirred at r.t. for 2 days. Then 25% aqueous ammonia (100 ml) was added and, after 30 min, the mixture was concentrated and the residue co-evaporated with ethanol (3 × 50 ml). The titled product was obtained in 36% yield (170 mg) by column chromatography on silica gel using linear gradient of methanol in chloroform as a yellowish oil. HR-MS: for $C_{11}H_{16}N_1O_2$ $(M + H)^+$ calculated 427.185882, found 427.183416. NMR confirmed a 1:1 mixture of amide rotamers. ^{1}H NMR (400 MHz, DMSO-d_6): 1.23–1.28 (8 × d, 8 × 3 H, J_{vic} = 6.1, (C**H**$_3$)$_2$CH); 3.00 (dd, 1 H, J_{gem} = 14.6, $J_{H,P}$ = 11.1, CH$_a$**H**$_b$P); 3.04 (dd, 1 H, J_{gem} = 14.6, $J_{H,P}$ = 10.9, C**H**$_a$H$_b$P); 3.10 (t, 1 H, J_{gem} = $J_{H,P}$ = 14.7, CH$_a$**H**$_b$P); 3.13 (t, 1 H, J_{gem} = $J_{H,P}$ = 14.7, C**H**$_a$H$_b$P); 3.28 (bdd, 1 H, J_{gem} = 12.7, $J_{H,P}$ = 1.6, H-5b-pyrr); 3.53 (dd, 1 H, J_{gem} = 10.8, $J_{5b,4}$ = 5.7, H-5b-pyrr); 3.58 (ddd, 1 H, J_{gem} = 12.7, $J_{5a,4}$ = 6.0, $J_{H,P}$ = 1.6, H-5a-pyrr); 3.84 (ddd, 1 H, J_{gem} = 12.5, $J_{2b,3}$ = 7.5, $J_{H,P}$ = 1.6, H-2b-pyrr); 3.90 (ddd, 1 H, J_{gem} = 12.5, $J_{2b,3}$ = 6.5, $J_{H,P}$ = 1.6, H-2a-pyrr); 4.07 (dd, 1 H, J_{gem} = 12.7, $J_{5,4}$ = 6.3, H-5a-pyrr); 4.16 (dd, 1 H, J_{gem} = 11.3, $J_{2b,3}$ = 5.4, H-2b-pyrr); 4.25 (dd, 1 H, J_{gem} = 11.3, $J_{2b,3}$ = 7.0, H-2a-pyrr); 4.61 (m, 1 H, H-4-pyrr); 4.63 (m, 4 H, C**H**$(CH_3)_2$); 4.69 (m, 1 H, H-4-pyrr); 4.80 (ddd, 1 H, $J_{3,2}$ = 7.5, 6.5, $J_{3,4}$ = 6.3, H-3-pyrr); 4.85 (dt, 1 H, $J_{3,2}$ = 7.0, 5.4, $J_{3,4}$ = 5.4, H-3-pyrr); 5.80 (d, 1 H, $J_{OH,4}$ = 4.4, OH); 5.86 (d, 1 H, $J_{OH,4}$ = 4.9, OH); 7.27 (bs, 4 H, NH_2); 8.09 (s, 1 H, H-8); 8.14 and 8.15 (2 × s, 2 × 1 H, H-2); 8.20 (s, 1 H, H-8). ^{13}C NMR (100.6 MHz, DMSO-d_6): 23.72–24.02 (d, $J_{C,P}$ = 4, $(CH_3)_2$CH); 34.94 (d, $J_{C,P}$ = 133, CH_2P); 47.62 and 48.84 (CH_2-2-pyr); 51.27 and 52.20 (CH_2-5-pyr); 59.10 and 60.21 (CH-3-pyr); 70.51–70.70 (d, $J_{C,P}$ = 6, C**H**$(CH_3)_2$); 70.90 and 71.91 (CH-4-pyrr); 119.23 (C-5); 139.13 and 139.53 (CH-8); 149.65 and 149.70 (C-4); 152.53 and 152.60 (CH-2); 156.27 (C-6); 163.59 and 163.75 (d, *J* = 6, C=O). ^{31}P NMR (162 MHz, DMSO-d_6): 20.60 and 20.65.

Diisopropyl 2-{(3*R*4,*R*)-3-[N^6-(Dibutylamino)methyleneadenin-9-yl]-4-(4, 4′-dimethoxytrityl)oxypyrrolidin-1-yl}-2-oxoethylphosphonate (**21**)

Compound **19** (0.17 g, 0.4 mmol) was dissolved in methanol (2 ml) and dibutylformamide dimethyl acetal (0.28 ml, 1.2 mmol) was added. The reaction mixture was left aside at r.t. for 5 h and then quenched with 2 M TEAB (0.8 ml). The mixture was concentrated and co-evaporated with ethanol (2 × 10 ml) and then with dry pyridine (2 × 10 ml). To the residue in pyridine (10 ml), DMTrCl (0.34 g, 1 mmol) and silver triflate (0.26 g, 1 mmol) were added. The mixture was stirred at r.t. for 5 days. The reaction was quenched with dry methanol (1 ml). The suspension was filtered through a pad of Celite, the cake was washed several times with DCM, and the organic layer was extracted with aqueous sodium bicarbonate. The organic layer was concentrated and the title compound was obtained in 50% yield

(173 mg) by chromatography on silica gel using linear gradient of methanol in chloroform as a yellowish foam. HR-MS: for $C_{11}H_{16}N_1O_2$ $(M + H)^+$ calculated 868.455814, found 868.452661. NMR spectra confirmed a 1:1 mixture of amide rotamers. ^{1}H NMR (400 MHz, DMSO-d_6): 0.93 and 0.94 (2 × t, 2 × 6 H, J_{vic} = 7.2, C**H**$_3$CH$_2$CH$_2$CH$_2$N); 1.17–1.26 (8 × d, 24 H, J_{vic} = 6.2, (C**H**$_3$)$_2$CH); 1.32 and 1.61 (2 × m, 2 × 8 H, CH$_3$C**H**$_2$C**H**$_2$CH$_2$N); 2.58–2.76 and 2.82–2.98 (2 × m, 2 × 2 H, CH$_2$P); 3.28–3.50 (m, 6 H, H-5-pyrr and CH$_3$CH$_2$CH$_2$C**H**$_2$N); 3.58–3.75 (m, 6 H, H-5-pyrr and CH$_3$CH$_2$CH$_2$C**H**$_2$N); 3.65 and 3.70 (2 × s, 12 H, CH$_3$O); 3.87 (m, 2 H, H-2-pyrr); 4.06 (dd, 1 H, J_{gem} = 10.6, $J_{2b,3}$ = 9.1, H-2b-pyrr); 4.23 (dd, 1 H, J_{gem} = 10.6, $J_{2a,3}$ = 8.3, H-2a-pyrr); 4.50–4.62 (m, 4 H, C**H**(CH$_3$)$_2$); 4.69 and 4.72 (2 × dt, 2 × 1 H, $J_{4,3}$ = 7.5, $J_{4,5}$ = 6.6, H-4-pyrr); 5.25 and 5.32 (2 × ddd, 2 × 1 H, $J_{3,2}$ = 9.1, 8.3, $J_{3,4}$ = 7.5, H-3-pyrr); 6.61 and 6.75 (2 × m, 2 × 4 H, H-*m*-C$_6$H$_4$-DMTr); 6.97 and 6.98 (2 × m, 4 H, H-*o*-C$_6$H$_4$-DMTr); 7.05–7.25 (m, 14 H, H-*o*,*m*,*p*-C$_6$H$_5$-DMTr and H-*o*-C$_6$H$_4$-DMTr); 8.32, 8.34, 8.36 and 8.38 (4 × s, 4 × 1 H, H-2 and H-8); 8.99 (s, 2 H, CH=N). ^{13}C NMR (100.6 MHz, DMSO-d_6): 13.67 and 13.85 (**C**H$_3$CH$_2$CH$_2$CH$_2$N); 19.27 and 19.75 (CH$_3$**C**H$_2$CH$_2$CH$_2$N); 23.64–23.86 (d, $J_{C,P}$ = 4, (**C**H$_3$)$_2$CH); 28.85 and 30.63 (CH$_3$CH$_2$**C**H$_2$CH$_2$N); 34.27 and 34.60 (d, $J_{C,P}$ = 133, CH$_2$P); 44.58 and 51.08 (CH$_3$CH$_2$CH$_2$**C**H$_2$N); 45.49 and 47.47 (CH$_2$-2-pyrr); 49.63 and 50.83 (CH$_2$-5-pyrr); 54.99 and 55.11 (CH$_3$O); 58.44 and 59.21 (CH-3-pyrr); 70.37–70.47 (d, $J_{C,P}$ = 7, **C**H(CH$_3$)$_2$); 73.28 and 73.96 (CH-4-pyrr); 86.31 and 86.37 (C-DMTr); 113.15 and 113.31 (CH-*m*-C$_6$H$_4$-DMTr); 126.12 and 126.21 (C-5); 126.83 126.88, 127.49, 127.60 and 127.84 (CH-*o*,*m*,*p*-C$_6$H$_5$-DMTr); 129.45 and 129.92 (CH-*o*-C$_6$H$_4$-DMTr); 135.36, 135.42, 135.44 and 135.61 (C-*i*-C$_6$H$_4$-DMTr); 142.15 and 142.41 (CH-8); 145.08 and 145.11 (C-*i*-C$_6$H$_5$-DMTr); 151.65 and 151.71 (C-4); 151.77 (CH-2); 158.01 (CH=N); 158.17, 158.24, 158.41 and 158.49 (C-*p*-C$_6$H$_4$-DMTr); 159.60 and 159.63 (C-6); 163.11 and 163.18 (d, $J_{C,P}$ = 6, CO).

(4-Methoxy-1-oxido-2-pyridyl)methyl 2-{(3*R*,4*R*)-3-[N^6-(Dibutylamino)methyleneadenin-9-yl]-4-(4,4′-dimethoxytrityl)oxypyrrolidin-1-yl}-2-oxoethylphosphonate (**23**)

Compound **21** was co-evaporated with acetonitrile (2 × 10 ml) and dissolved in the same solvent (3 ml). Under argon atmosphere, lutidine (0.23 ml, 2 mmol) and bromotrimethylsilane (0.13 ml, 1 mmol) were added. The reaction mixture was left at r.t. under argon atmosphere overnight and then concentrated in vacuo. To the residue, 0.1 M TEAB (6 ml) and chloroform (20 ml) were added. The organic layer was separated, dried over anhydrous sodium sulfate, filtered and on evaporation of solvents, the compound **22** was obtained in 80% yield (0.13 g) by column chromatography on silica gel using a linear gradient of H1 in ethyl acetate as a yellowish foam. NMR signals were too broad to be analyzed so this intermediate was used without further characterization.

A mixture of compounds **22** (0.13 g, 0.16 mmol) and (4-methoxy-1-oxido-4-pyridyl)methanol (0.47 g, 0.3 mmol) was co-evaporated with pyridine (2 × 10 ml) and dissolved in the same solvent (3 ml). Then DCC (0.1 g, 0.5 mmol) was added. The mixture was left to stand aside at r.t. After 4 days, water (2 ml) was added and the mixture was heated at 60 °C overnight. Then concentrated, partitioned between chloroform and 0.1 M aqueous TEAB, dried over anhydrous sodium sulfate. The titled compound was obtained in 39% yield (63 mg) by column chromatography on silica gel using linear gradient of H1 in H3 folowed by reverse phase chromatography using linear gradient of methanol in water as a yellowish amorphous fluffy solid (after lyofilization from dioxane). HR-MS: for $C_{11}H_{16}N_1O_2$ $(M + H - Et_3N)^+$ calculated 921.406440, found 921.414407. NMR spectra confirmed a 1:1 mixture of amide

rotamers: ^{1}H NMR (400 MHz, $CDCl_3$): 0.92–1.20 (m, 12 H, C**H**$_3$CH$_2$CH$_2$CH$_2$N); 1.24 (t, 18 H, C**H**$_3$CH$_2$N); 1.27–1.47, 1.55–1.74 and 1.92 (3 × m, 16 H, CH$_3$C**H**$_2$C**H**$_2$CH$_2$N); 2.95–3.22 (m, 4 H, CH$_2$P); 3.25–3.54 (m, 22 H, H-5-pyrr, CH$_3$CH$_2$CH$_2$C**H**$_2$N and CH$_3$C**H**$_2$N); 3.60–3.70 (m, 2 H, H-5-pyrr); 3.71, 3.72, 3.73 and 3.74 (4 × s, 12 H, CH$_3$O-DMTr); 3.856 and 3.860 (2 × s, 6 H, CH$_3$O-MOP); 3.95 (dd, 1 H, J_{gem} = 12.2, $J_{2b,3}$ = 5.2, H-2b-pyrr); 4.03 (dd, 1 H, J_{gem} = 12.2, $J_{2a,3}$ = 5.2, H-2a-pyrr); 4.34 (dd, 1 H, J_{gem} = 10.7, $J_{2b,3}$ = 7.6, H-2b-pyrr); 4.50 (dd, 1 H, J_{gem} = 10.6, $J_{2a,3}$ = 8.2, H-2a-pyrr); 4.63 and 4.73 (2 × q, 2 × 1 H, $J_{4,3}$ = $J_{4,5}$ = 6.4, H-4-pyrr); 5.05 (ddd, 1 H, $J_{3,2}$ = 7.2, 5.2, $J_{3,4}$ = 6.4, H-3-pyrr); 5.16 (ddd, 1 H, $J_{3,2}$ = 8.2, 7.6, $J_{3,4}$ = 6.4, H-3-pyrr); 5.21 (d, 4 H, $J_{H,P}$ = 7.4, CH$_2$P-MOP); 5.57–6.75 (m, 10 H, H-5-MOP and H-*m*-C$_6$H$_4$-DMTr); 7.08–7.32 (m, 18 H, H-*o*-C$_6$H$_4$-DMTr and H-C$_6$H$_5$-DMTr); 7.39 and 7.41 (2 × d, 2 × 1 H, $J_{3,5}$ = 3.5, H-3-MOP); 7.97 and 8.10 (2 × s, 2 × 1 H, CH-8); 8.02 and 8.04 (2 × d, 2 × 1 H, $J_{6,5}$ = 7.2, H-6-MOP); 8.39 and 8.44 (2 × s, 2 × 1 H, CH-2); 9.01 and 9.02 (2 × s, 2 × 1 H, CH=N), ^{31}P NMR (162 MHz, $CDCl_3$): 14.45 and 14.60.

Synthesis of Oligonucleotides

All used solvents were anhydrous. 1-H-tetrazole was resublimed at reduced pressure. The starting phosphonate monomers were lyofilized from dioxane as TEA salts. Before using in the synthesizer, they were dissolved in pyridine and dried over molecular sieves for 8 h. The HPLC analysis of oligonucleotides was carried out on a Nucleosil 100-5 C18 HD (4.6 × 150 mm) column with liquid chromatograph LC5000 (Ingos, Czech Republic) using gradient of acetonitrile in 0.1 M TEAA. Preparative HPLC of oligonucleotides was performed using the same system with different columns, as follows: Dynamax C18 (Rainin, USA) column was used for purifications of oligonucleotides bearing terminal dimethoxytrityl group. Free oligonucleotides were purified on Phenomenex C18 (Phenomenex, USA) (10 × 250 mm) column. The detritylation took place on Dynamax PureDNA (Rainin, USA) (10 × 50 mm) column. All oligonucleotide solutions were evaporated in a centrifuge evaporator (Labconco, USA). Thermal characteristic of the prepared oligonucleotides were measured on Cary 100 (Varian, Australia) UV/VIS spectrophotometer.

The solid phase synthesis of oligonucleotides was carried out on GENESYN (IOCB R&D) synthesizer using the "trityl on" method, on the scale of 0.5 μmol (~20 mg of solid support was used for each synthesis). Table II shows the synthesis protocols for oligonucleotides **ON1**–**ON6**. Oligonucleotide **ON7** was synthesized by phosphotriester method. The average yield of the condensation step was in the range of 97–98% while the overall oligonucleotide yield reached 66–74%.

The (4-methoxy-1-oxido-2-pyridyl)methyl protecting group of phosphonate moieties of the oligonucleotides bound on solid support was removed by treatment with thiophenol–triethylamine–dioxane (1:1:2 v/v/v) at r.t. for 48 h. The CPG (controlled pore glass) support containing the oligonucleotide was washed with acetonitrile and dried in the stream of argon. The oligonucleotide-bearing support placed in 37% aqueous ammonia (2 ml) was then kept at 55 °C for 16 h to remove remaining protecting groups and to cleave the oligonucleotide from the support. The solution of free oligonucleotide bearing terminal dimethoxytrityl group was evaporated after addition of 2 M aqueous TEAB (200 μl) to avoid cleavage of DMTr group. The oligonucleotide was dissolved in 0.4 M TEAA (1 ml) and applied on Dynamax C18; 300 A; 10 × 250 mm column. The chromatography was carried out using linear gradient from 0.1 M TEAA in 10% acetonitrile to 0.1 M TEAA in 25% acetonitrile during 20 min, and then to 0.1 M TEAA in 50% acetonitrile over additional 30 min. The pu-

TABLE II
Protocol for solid phase synthesis of phosphonate analogues of oligonucleotides **ON1**–**ON6**

Step	Process	Solvent/Reagent	Volume, ml	Time, s
1	Washing	CH_3CN	3	30
2	Capping	(i) Ac_2O–collidine–CH_3CN (2:3:6) (ii) 6% DMAP in CH_3CN	0.2 0.2	60
3	Washing	CH_3CN	3	30
		Start of phosphotriester elongation cycle		
4	Washing	DCE	3	20
5	Detritylation	3% TCA in DCE	6	70
6	Washing	pyridine	3	60
7	Condensation[b]	(j) 0.05 M P-component (jj) 0.1 M TPSCl	0.2	300–1800
8	Washing	pyridine CH_3CN	1 1	20 10
9	Capping	as step 2		
10	Washing	CH_3CN	3	30
		End of phosphotriester elongation cycle		
		Start of phosphoramidite elongation cycle		
11	Washing	DCE	3	20
12	Detritylation	3% TCA in DCE	6	70
13	Washing	CH_3CN	3	30
14	Condensation[c]	0.1 M amidite 0.5 M tetrazole	0.075 0.090	140
15	Washing	CH_3CN	1	10
16	Oxidation	0.01 M iodine in CH_3CN–collidine–water (65:6:30)	0.6	40
17	Washing	CH_3CN	1	10
18	Capping	As step 2		
19	Washing	CH_3CN	3	30
		End of phosphoramidite elongation cycle		
		Postsynthetic procedures		
23	Drying with argon			
24	Removal of MOP group	thiophenol–Et_3N–dioxane (1:1:2)	1	48 h
25	Washing	CH_3CN	30	
26	Cleavage from support, and final deprotection	37% aqueous NH_3, 55 °C	2	16 h

[a] Alternating pulses of 20-μl segments of (i) and (ii). [b] Manual injection of freshly prepared solution of the appropriate phosphonate and TPSCl in pyridine. [c] Alternating pulses of 15-μl segments of (j) and (jj) reagents (tetrazole solution first).

rified oligonucleotide was evaporated, dissolved in 0.4 M TEAA (1 ml) and applied on Dynamax C18; 300 A; 10 × 50 mm column where detritylation took place according to the protocol shown in Table III.

TABLE III
Protocol for detritylation on Dynamax C18 PureDNA column

Step	Process	Flow rate ml min^{-1}	Time min
1	(i) Column washing with methanol (ii) Equilibration 0.1 M TEAA	9.4	5
2	Sample injection (1 ml, 0.94–4.7 OD_{260})		–
3	Washing 0.1 M TEAA		2
4	Detritylation 0.5% TFA		2
5	Washing 0.1 M TEAA		4
6	Elution with linear gradient 0→100% B in A A = 0.1 M TEAA B = 0.1 M TEAA in 50% acetonitrile	3	15

The oligonucleotide was dissolved in 0.4 M TEAA (1 ml) and applied on Dynamax C18; 300 A; 10 × 250 mm column. The chromatography was carried out using a linear gradient from 0.1 M TEAA in 10% acetonitrile to 0.1 M TEAA in 25% acetonitrile at a flow rate of 3 ml min^{-1}. The exact conditions depended on temperature. Removal of salts on NAP-10 Pharmacia column filled with Sephadex G-25 in 1% aqueous ammonia was the last step. The pure oligonucleotide was then lyophilized and stored at –20 °C. Structures of the prepared ONs were proved by MALDI-TOF mass spectrometry (Table IV).

Measurements of T_m Values

T_m values of duplexes of the prepared ONs with complementary oligodeoxyribonucleotide were measured in 50 mM Tris-HCl (pH 7.2); 10 mM Mg^{2+} (or 100 mM Na^+), and 1 mM EDTA at overall oligonucleotide concentration of 4 μM.

An ON solution was heated to 60 °C, cooled to room temperature, and transferred to the measuring cuvette placed in thermostated cell. The cuvette with the sample was heated at a temperature gradient of 5 °C min^{-1} to 60 °C in the spectrophotometer, and it was continued at this temperature for 5 min. The temperature was then left to decrease to 10 °C at a temperature gradient of 1 °C min^{-1} and the absorbance was measured. The absorbance was also measured at the reverse direction of temperature from 10 to 60 °C (1 °C min^{-1}).

TABLE IV
MALDI-TOF determination of **ON1**–**ON7** molecule size

Oligonucleotide	Sequence	Calcd	Found (MALDI-TOF)	Monomer/ **A** scaffold
ON1	5′-d(GTG **A**T**A** TGC)-3′	2718.544	2719.995	**13**/5
ON2	5′-d(GTG **A**T**A** TGC)-3′	2774.533	2775.791	**23**/7
ON3	5′-d(GTG **A**T**A** TGC)-3′	2746.575	2747.651	**18**/6
ON4	5′-d(GC**A** T**A**T C**A**C)-3′	2630.564	2631.737	**13**/5
ON5	5′-d(GC**A** T**A**T C**A**C)-3′	2714.549	2715.448	**23**/7
ON6	5′-d(GC**A** T**A**T C**A**C)-3′	2672.611	2673.806	**18**/6
ON7	d$\mathbf{A}_{15}$	4459.191	4460.07 (4461.05)	**13**/5

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