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Synthesis and Binding Properties of Oligodeoxynucleotides Containing Phenylphosphon(othio)ate Linkages

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Abstract—A method for the synthesis of chimeric oligodeoxynucleotides comprised of phosphodiester and phenylphosphonate [3′-O-P(=O)(C_6H_5)-O-5′] or phenylphosphono-thioate [3′-O-P(=S)(C_6H_5)-O-5′] linkages has been developed. Synthesis was performed using suitably protected nucleoside phenylphosphonamidites as building blocks following an adjusted solid-phase phosphoramidite synthesis protocol. The new oligodeoxy-nucleotide analogues were characterized by electrospray ionization- and matrix-assisted laser desorption mass spectrometry, as well as by ^{31}P NMR spectroscopy. Additionally, their binding properties to complementary oligodeoxynucleotides has been studied. © 1997 Elsevier Science Ltd.

Introduction

In recent years, the synthesis of chemically modified oligo(deoxy)nucleotides has attracted considerable attention due to their potential use as antisense and antigene agents.[1–3] From the plethora of different oligodeoxynucleotide analogues described in the literature, phosphate modified oligomers such as methylphosphonates and phosphorothioates have been investigated most carefully. In contrast to phosphorothioates, which retain the anionic charge of the phosphodiester linkage, methylphosphonate moieties are uncharged. In an evaluation program for oligodeoxynucleotides containing non-ionic internucleotide linkages,[4] such as methylphosphonothioates, octylphosphonates and benzylphosphonates, were studied.[5,6] An antisense oligonucleotide containing seven benzylphosphonate linkages directed against the pre-S-region of the duck hepatitis B virus resulted in a 40% inhibition of viral replication.[6] Thus we considered phenylphosphonate oligodeoxynucleotides to be of particular interest, since the planar nature of the phenyl group could be expected to cause only weak destabilization of the resulting duplex. Furthermore, phenylphosphon(othio)ate modified oligodeoxynucleotides may have improved stability against nucleases combined with enhanced cellular uptake, compared to unmodified oligodeoxynucleotides.

Until now, the properties of phenylphosphonate oligodeoxynucleotides have not been studied, most likely due to the unavailability of appropriate synthetic procedures. Only the synthesis of dinucleoside phenylphosphonates has been described in the literature.[7–9] In a first report by Agarwal and Riftina,[7] the phenylphosphonate analogue of TpT was prepared in 60% yield using phenylphosphonoditriazolide and appropriately protected nucleosides. Solution synthesis of the same modified dinucleotide, in 65% yield, has also been accomplished by Löschner by means of dichlorophenylphosphine.[8] Recently, Hashmi et al.[9] described a third method for the synthesis of dinucleoside phenylphosphonates using 5′-*O*-dimethoxytritylthymidine-3′-*O*-phenylphosphonate triethylammonium salts and 6-nitrobenzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (NBOP) as coupling reagent. Using this procedure, reminescent to phosphotriester chemistry, the fully protected TpT analogue was isolated in 95% yield. Here we report the preparation of appropriately protected monomeric phenylphosphonamidite building blocks, and their use for automated solid phase synthesis of novel oligodeoxynucleotide analogues containing phenylphosphonate or phenylphosphonothioate linkages. Furthermore, the binding properties of the phenylphosphon(othio)ate oligodeoxynucleotides to their complementary nucleic acids is described.

Key words: oligonucleotide, phenylphosphon(othio)ate, hybridization, solid-phase synthesis, antisense.

Results and Discussion

Synthesis of the monomeric building blocks

For the synthesis of the monomeric deoxynucleoside-3′-phenylphosphonamidite building blocks **5–8**, the corresponding 5′-*O*-dimethoxytrityl-*N*-acylated nucleoside derivatives **1–4** were phosphitylated with *rac*-chloro-*N,N*-diisopropylphenylphosphine in the presence of *N,N*-diisopropylaminoethyl amine (Scheme 1). The

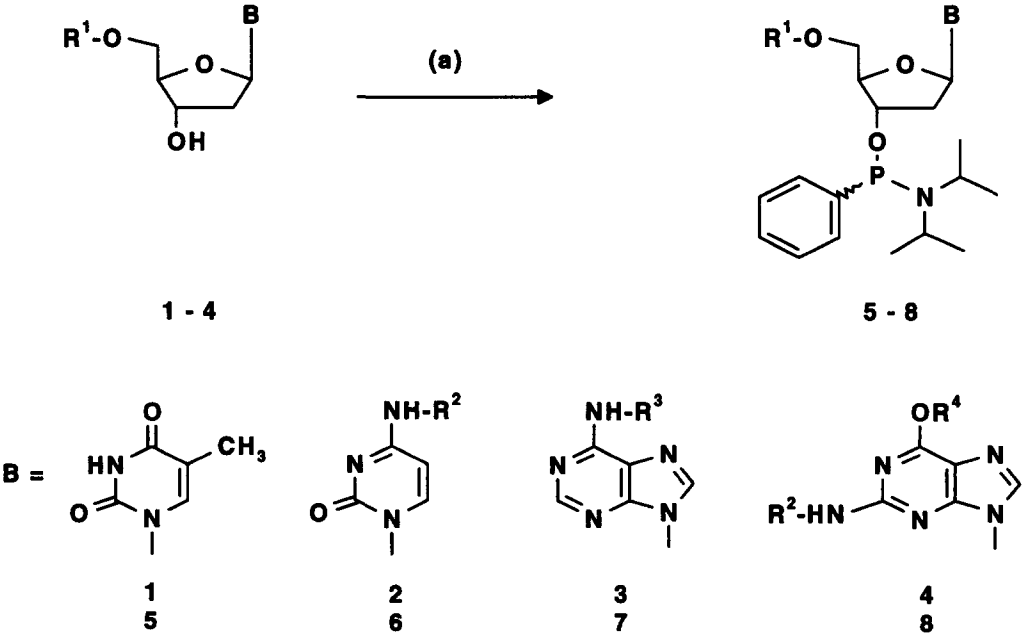
phosphitylation reagent was prepared by reaction of dichlorophenylphosphane with an excess of diisopropylamine in diethylether. Vacuum distillation of the crude product afforded the desired *rac*-chloro-*N,N*-diisopropylaminophenylphosphine in 57% yield (^{31}P NMR: 133.5 ppm). It should be noted that attempts to prepare the corresponding bis-*N,N*-diisopropylaminophenylphosphine by this route were unsuccessful. Surprisingly, the reactivity of *rac*-chloro-*N,N*-diisopropylaminophenylphosphine is only modest, requiring relatively long reaction times to obtain the deoxynucleoside-3′-phenylphosphonamidites **5–8** as mixtures of diastereoisomers. The best yields of **5–8** (62–76%) were obtained by reaction of partially protected nucleosides **1–4** with 2.5 equiv of the phosphitylating reagent for three days at room temperature. The new building blocks **5–8** were characterized by FABMS, ^{1}H NMR and ^{31}P NMR (two singlets at 115–118 ppm).

The reaction of O^6-unprotected 5′-*O*-(4,4′-dimethoxytrityl)-N^2-isobutyryl-2′-deoxyguanosine[10] with *rac*-chloro-*N,N*-diisopropylaminophenylphosphine under these conditions resulted in phosphitylation at the O^6-positon of guanine in addition to the desired phosphitylation of the 3′-hydroxyl group. The bis-phosphitylated deoxyguanosine monomer was found to be much more lipophilic than the other monomers **5–7**, and two sets of resonance signals could be observed in the ^{31}P NMR spectrum at 115.6:117.7 ppm (3′-*O*-P) and 123.5:123.6 ppm (O^6-P), respectively, in a ratio of 1:1.

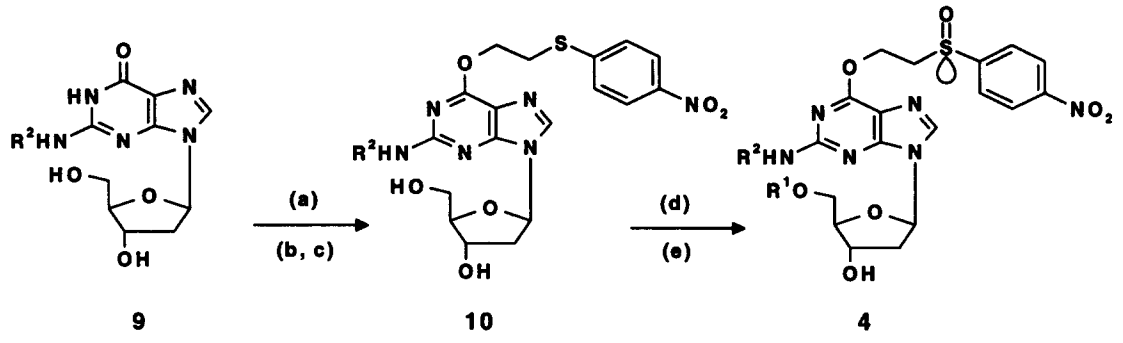
To overcome this side reaction, we introduced the 4-nitrophenylsulfoxyethyl (NPSOE) group[11,12] at the amide function of the guanine moiety. The O^6-NPSOE-protecting group can be cleaved by β-elimination with concentrated ammonia (2 h, 55 °C), ethanolamine (3 h, room temperature) or ethylenediamine (3 h, room temperature). Synthesis of the NPSOE-protected 2′-deoxyguanosine building block **4** was performed using the transient protection method of Jones[13] (Scheme 2) starting from N^2-isobutanoyl-2′-deoxyguanosine **9**, which was reacted first with *N*-(trimethylsilyl)-imidazole in dioxane to give a 3′,5′-*O*-disilyl protected intermediate. This was further reacted in a Mitsunobu reaction[14,15] with 2 equiv of each triphenylphosphine, 4-nitrophenyl-2-hydroxyethylsulfide and diethyl azodicarboxylate and desilylated with fluoride resulting in the O^6-protected N^2-isobutanoyl-2′-deoxyguanosine **10** in 74% yield.

To allow cleavage of the NPSOE-group under mild alkaline conditions at a later step of the synthesis, the sulfide intermediate **10** was oxidized with $NaIO_4$ to the sulfoxide, which after dimethoxytritylation resulted in the fully protected deoxyguanosine derivative **4** as a mixture of two diastereomers. The 4-nitrophenyl-2-hydroxyethylsulfide **11** was prepared from 2-chloroethanol via nucleophilic aliphatic substitution of the chloro atom by 4-nitrothiophenol using the procedure of Bennett and Berry.[16]

In an attempt to synthesize **4** in a more straightforward way, we reacted 2-(4-nitrophenylsulfonyl)-ethanol directly with **9** under Mitsunobu conditions. However, no desired alkylated product could be isolated, probably because the proposed substituted ethoxyphosphorane intermediate of the Mitsunobu reaction was subject to a rapid β-elimination reaction[14,17] as a consequence of the



Scheme 1. Synthesis of monomeric phenylphosphonamidites **5–8** [*R/S*]. *Reagents and Conditions*: (a) *rac*-C_6H_5-P(Cl)N(*i*-C_3H_7)$_2$, Et-N(*i*-C_3H_7)$_2$, methylene chloride, 3 days, rt (R^1: dimethoxytrityl, R^2: *iso*-butanoyl, R^3: benzoyl, R^4: 4-nitrophenylsulfoxyethyl). Compounds **5–7** are mixtures of 2-diastereomers and compound **8** is a mixture of 4-diastereomers, respectively.



Scheme 2. Synthesis of O^6-protected 2-deoxyguanosine derivative 4. *Reagents and Conditions*: (a) *N*-(trimethylsilyl)-imidazole, dioxane, 30 min; (b) *p*-NO_2-C_6H_4-SCH_2 CH_2 OH (11), triphenyl phosphine, diethylazodicarboxylate, 2 h; (c) hydrogen fluoride, pyridine, 2 h, 0 °C; (d) $NaIO_4$, methanol, H_2O, 48 h; (e) dimethoxytrityl chloride, triethylamine, 4 h (all reactions were carried out at room temperature, unless otherwise indicated).

electron withdrawing nature of the nitrophenyl sulfonyl group.

Finally, phosphitylation of the fully protected deoxyguanosine derivative **4** with *rac*-chloro-*N,N*-diisopropylphenylphosphine afforded the desired phenylphosphonamidite building block **8** as a mixture of four diastereomers. After purification of the crude product by flash chromatography, **8** was isolated in 72% yield and characterized by means of FABMS, ^{1}H- and ^{31}P NMR spectroscopy (pseudo singlets at 115.6 and 117.7 ppm).

Synthesis of oligodeoxynucleotides containing phenylphosphon(othio)ate linkages

To test the usefulness of the nucleoside phenylphosphonamidites **5–8** in automated solid phase oligodeoxynucleotide synthesis, we prepared several modified oligodeoxynucleotides whose sequences and backbone modification patterns are outlined in Table 1. Introduction of the phenylphosphonate linkage was achieved using cycle II (Table 2) with a tenfold excess of the corresponding phosphonamidite **5–8** in acetonitrile. Cycle II differs from standard cycle I only in the prolonged coupling time (300 s instead of 25 s). All oligodeoxynucleotides were synthesized in the 'trityl-on' mode to facilitate reversed phase HPLC purification.

Although synthesis of oligodeoxynucleotide methylphosphonothioates has been reported before,[18,19] arylphosphonothioates are completely unknown in the literature. Oligonucleotides containing phenylphosphonothioate linkages were prepared using the nucleoside phenylphosphonamidites **5–8** in combination with cycle III (Table 2). The sulfurization reaction was performed with 0.5 M tetraethylthiuramdisulfide solution in acetonitrile[20] for 20 min, to generate the phenylphosphonothioate internucleoside linkages. Synthesis of this new type of compound was exemplified by preparation of two oligodeoxynucleotides containing four (**16**) and 11 (**19**) phenylphosponothioate linkages. Yields per cycle for the introduction of phenylphosphonothioate and phenyphosphonate linkages were in the range of 98%.

Table 1. Sequences of synthesized oligodeoxynucleotides and overall coupling yields of the syntheses

Compound	Sequence	Synthesized sequence of the oligodeoxynucleotide[a]	Cycle	Yield[b]
12	1	d(AAGGAGATAAGCCCGCTAAA)	I	84%
13	1	d($A_\phi A_\phi$GGAGATAAGCCCGCTA$_\phi A_\phi$A)	I, II	78%
14a	2	d(GACGTTCCTCCTGCGGGAAG)	I	81%
14b	2	d($G_S A_S$CGTTCCTCCTGCGGGA$_S A_S$G)	I	n.d.
15	2	d($G_\phi A_\phi$CGTTCCTCCTGCGGGA$_\phi A_\phi$G)	I, II	72%
16	2	d($G_\psi A_\psi$CGTTCCTCCTGCGGGA$_\psi A_\psi$G)	I, III	75%
17	2	d($G_\phi A_\phi$CGT$_\phi$TCC$_\phi$TC$_\phi$CT$_\phi$GCG$_\phi$GGA$_\phi A_\phi$G)	I, II	72%
18	2	d($G_\phi A_\phi$CG$_\phi$TT$_\phi$CC$_\phi$TC$_\phi$CT$_\phi$GC$_\phi$GG$_\phi$GA$_\phi A_\phi$G)	I, II	68%
19	2	d($G_\psi A_\psi$CG$_\psi$TT$_\psi$CC$_\psi$TC$_\psi$CT$_\psi$GC$_\psi$GG$_\psi$GA$_\psi A_\psi$G)	I, III	68%
20	3	d(ACACCCAATTCTGAAAATGG)	I	86%
21	3	d($A_\phi C_\phi$ACCCAATTCTGAAAATGG)	I, II	81%
22	4	d(ACACCCAATTCTGAAAATGGATA)	I	83%
23	4	d(ACACCCAATTCTGAAAATGGA$_\phi$T$_\phi$A)	I, II	80%
24	4	d($A_\phi C_\phi$ACCCAATTCTGAAAATGGA$_\phi$T$_\phi$A)	I, II	71%

[a] ϕ indicates a phenylphosphonate, S phosphorothioate and ψ a phenylphosphonothioate linkage.
[b] Overall yields were determined by trityl colour quantitation at 498 nm (n.d.: not determined).

Special care was taken in the deprotection of the oligodeoxynucleotides containing phenylphosphon-(othio)ate linkages by treating the oligonucleotides with a mixture of ethylenediamine or ethanolamine and ethanol:acetonitrile:water (50.0:23.5:23.5:3.0; v:v:v:v),[21] since concentrated ammonia commonly used to deblock phosphodiester oligodeoxynucleotides rapidly degrades the phenylphosphon(othioate)ate linkage at elevated temperature (55 °C). However, both ethylendiamine and ethanolamine readily transaminate the N^4 amine of N^4-benzoyl-2′-deoxycytidine. Therefore, the *iso*-butanoyl group was used for the protection of the exocyclic amino function of 2′-deoxycytidine which can be deblocked by a brief pre-treatment with concentrated ammonia at room temperature without significant transamination.[21]

Purification of the 5′-*O*-dimethoxytritylated oligonucleotides was achieved by C18 reversed-phase HPLC. Figure 1 shows the analytical C18 reversed phase HPLC of the crude synthesis product of **15**. Peak group A represents the mixture of the sixteen diastereomers of **15**, while peak group B is caused by failure sequences. The oligodeoxynucleotides were characterized by electrospray ionization (ESI), or matrix-assisted laser desorption (MALDI) mass spectrometry (MS) as well as by ^{31}P NMR spectroscopy to prove the incorporation of the phenylphosphonate linkages. As an example, the ^{31}P NMR spectrum of the oligodeoxynucleotide **23** is illustrated in Figure 2 showing two resonances located at 0.4 and 23.0 ppm which correspond to the phosphodiester (A) and phenylphosphonate (B) linkages, respectively. The integrated areas of the NMR peaks A and B gave the correct PO/Pϕ ratio of 20:2.

Binding properties of phenylphosphon(othio)ate modified oligonucleotides

To study the influence of the phenylphosphon-(othioate)ate linkage on the duplex stability of the modified oligodeoxynucleotides, melting temperature experiments were performed. All investigated oligonucleotide analogues form duplexes having cooperative melting transitions at physiological salt concentrations. As can be seen from Table 3, substitution of a phosphodiester linkage by a phenylphosphon(othio)ate internucleoside linkage leads to a reduction of the T_m values of the corresponding duplexes, except for oligodeoxynucleotides **21** and **23** which, surprisingly, have a slightly higher T_m value than their natural congeners. Introduction of a phenylphosphonothioate linkage results in a higher destabilization of the duplex than introduction of a phenylphosphonate linkage. Furthermore the destabilization (ΔT_m of 1.0–1.3 K per modification) is more than adding the phenyl and thioate increments (0.6–0.8 K).

Summary and Conclusion

We have synthesized new monomeric phenylphosphonamidite building blocks **5–8** of suitably protected nucleosides that serve as synthons for the introduction of phenylphosphon(othio)ate linkages into oligodeoxynucleotides. In case of the deoxyguanosine monomer, protection of the O^6-position with the NPSOE group, in deviation from the standard protection scheme, was necessary to avoid phosphitylation of the amide function of guanine. Furthermore, use of the labile *iso*-butanoyl group for protection of the exocyclic amino function of deoxycytidine proved to be useful to minimize transamination[21] during deprotection of the phenylphospon(othio)ate oligodeoxynucleotides, using a short pre-treatment with ammonia followed by treatment with ethylene diamine.

The new building blocks **5–8** were successfully used for the synthesis of several chimeric oligodeoxynucleotides containing phosphodiester, phenylphosphonate and phenylphosphonothioate linkages at desired positions within their sequences. All new oligonucleotide analogues were characterized by ESI- or MALDI-MS, and some were also shown by ^{31}P NMR spectroscopy to contain the expected ratio of phosphodiester and phenylphosphonate linkages.

Additionally, the effect of replacement of phosphodiester by phenylphosphono(thioate) linkages on hybrid stability with complementary DNA was investigated. Duplex stability against the complementary RNA showed no measurable difference (Table 3) in T_m. Thus for this sequence DNA was taken as reference. All modified oligodeoxynucleotides formed stable duplexes with their complementary sequences, showing cooperative melting transitions at physiological salt conditions. Most of the duplexes were found to be destabilized relative to the unmodified duplexes (ΔT_m between −0.3

Table 2. Synthesis cycles used for automated oligodeoxynucleotide synthesis

Reagent	Function	Cycle I[a]	Cycle II[b]	Cycle III[c]
3% Trichloroacetic acid/CH_2Cl_2	Detritylation	30 s	30 s	30 s
Amidite + tetrazole	Condensation	25 s	300 s	300 s
Ac_2O/*N*-methylimidazole/pyridine	Capping	10 s	10 s	10 s
Iodine/water/pyridine	Oxidation	30 s	30 s	—
0.5 M Tetraethylthiuram-disulfide in CH_3CN	Sulfurization (before capping)	—	—	1200 s

[a]Cycle I: standard cycle for phosphodiester linkages
[b]Cycle II: for phenylphosphonate linkages and RNA
[c]Cycle III: for phenylphosphonothioate linkages.

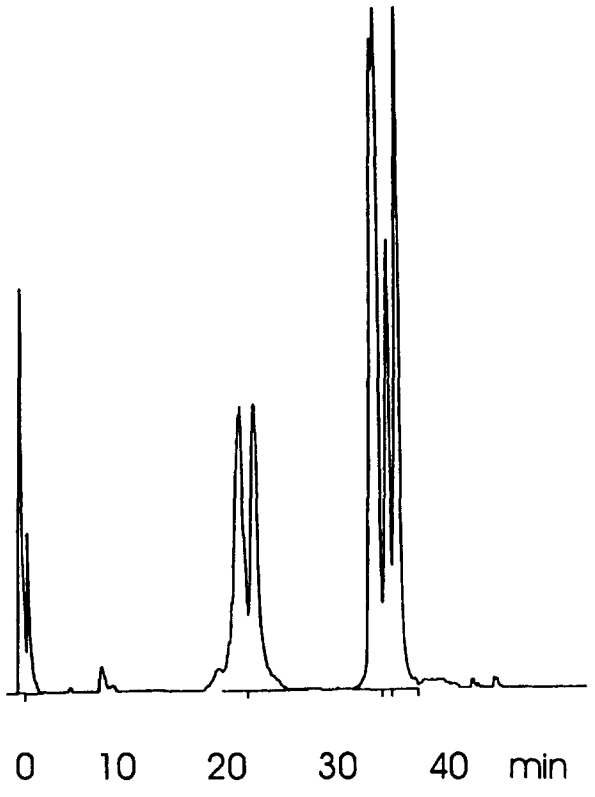


Figure 1. Analytical C18 reversed-phase HPLC of crude compound **15**. Peak group (A) 35 min, peak group (B) 20 min.

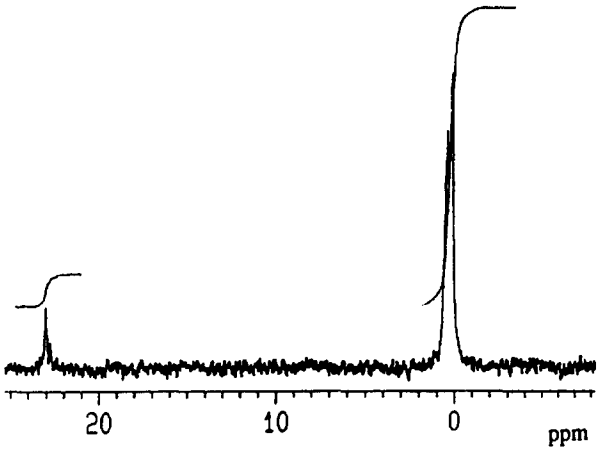


Figure 2. ^{31}P NMR spectrum of oligodeoxynucleotide **23** measured in D_2O at 298 K.

and −1.3 °C per modification). Surprisingly, two of the phenylphosphonate modified oligodeoxynucleotides (**21** and **23**) exhibited slightly higher T_m values than their natural congeners (ΔT_m = 0.2–0.5 °C per modification). At present, it is unclear why only those two modified oligodeoxynucleotides showed improved binding affinity. It could either be due to the specific sequence of these two oligonucleotides, or to the fact that these two were the only oligonucleotides in our series which were modified at just one terminus.

We have shown previously[5] that the introduction of a lipophilic octylphosphonate linkage into oligodeoxynucleotides resulted in a decrease of the T_m value by −3.4 °C per modification. Interestingly, introduction of a lipophilic, planar phenyl group in this study decreased the T_m value only by −0.3 to −1.0 °C per modification. This destabilizing effect is similar to the one observed for the introduction of the commonly used phosphorothioate and methylphosphonate linkages.

Chimeric phenylphosphon(othio)ate/phosphodiester molecules offer several advantages over normal phosphodiester oligodeoxynucleotides, including enhanced resistance to exonucleases which are ubiquitous in serum and the cytoplasm. Moreover, using our synthetic procedure the phenylphosphon(othio)ate linkages can be introduced into oligodeoxynucleotides according to

Table 3. T_m values of duplexes of modified oligonucleotides with their complementary oligodeoxnucleotides

Compound	Sequence	Modification	T_m Value (°C)[a] against DNA/RNA	ΔT_m per modification (°C) against DNA/RNA
12	1	—	66.8	—
13	1	4 ϕ	63.6	−0.8
14a	2	—	66.6/65.8	—
14b	2	4 S	65.6/64.8	−0.3/−0.3
15	2	4 ϕ	64.6/64.6	−0.5/−0.3
16	2	4 ψ	61.5/61.8	−1.3/−1.0
17	2	9 ϕ	57.2	−1.0
18	2	11 ϕ	56.5	−0.9
19	2	11 ψ	55.5	−1.0
20	3	—	60.0	—
21	3	2 ϕ	61.0	+0.5
22	4	—	65.5	—
23	4	2 ϕ	65.9	+0.2
24	4	4 ϕ	63.8	−0.4

[a]Measured in 10 mM HEPES buffer (pH 7.5) at 140 mM NaCl (ϕ: phenylphosphonate, S: phosphorothioate, ψ: phenylphosphonothioate); sequence 1: d(AAGGAGATAAGCCCGCTAAA), 2: d(GACGTTCCTCCTGCGGGAAG); 3: d(ACACCCAATTCTGAAAATGG), 4: d(ACACCCAATTCTGAAAATGGATA).

the 'minimal' protection strategy,[22] which is a combination of end-capping and protection at internal pyrimidine residues which are the major cleavage sites of endonuclease degradation. Since this strategy reduces the number of modifications needed to make an oligodeoxynucleotide stable against degradation, the use of uniformly phenylphosphon(othio)ate modified oligodeoxynucleotides, which have poor solubility in aqueous medium, can be avoided.

Several chimeric phenylphosphon(othio)ate/phosphodiester oligodeoxynucleotides against HSV-1 and HIV-1 were synthesized which are now under investigation as antisense drugs to selectively block virus replication. Further studies will show, whether the higher lipophilicity of this class of oligodeoxynucleotides can promote their cellular uptake and consequently their biological activity.

Experimental

General

Thin-layer chromatography, HPLC, as well as measurements of NMR, ESI-MS and T_m values were performed as described previously.[5]

[R_P/S_P]-chloro-*N,N*-diisopropylamino-phenylphosphine. To a solution of *N,N*-diisopropylamine (20.9 g, 500 mmol) in diethylether (250 mL) was added, under an argon atmosphere within 1 h at 0 °C, *P,P*-dichlorophenylphosphine (8.9 g, 50 mmol). The reaction was stirred for additonal 16 h at ambient temperature. The precipitated hydrochloride was filtered off under argon and washed twice with ether (2 × 100 mL). The combined organic layers were evaporated in vacuo and the residual oil was purified by distillation over a 15 cm Vigreux column under vacuum yielding a colourless oil that slowly crystallized upon standing. Yield: 7.0 g (57%), bp: 105 °C (0.3 Torr), 300 MH, ^{1}H NMR ($CDCl_3$) 1.03 (m, 12H, 4 × CH_3), 3.39 (m, 2H, CH), 7.43 (m, 3H, phenyl), 7.70 (m, 2H, phenyl); ^{31}P NMR ($CDCl_3$) 133.5 ppm.

5′-*O*-(4,4′-dimethoxytrityl)-N^2-isobutanoyl-O^6-[*R/S*]-(4-nitrophenylsulfoxyethyl)-2′-deoxyguanosine (4). Nucleoside **10** (5.2 g, 10 mmol; synthesis described below) was dissolved in methanol (100 mL) and a solution of sodiummetaperiodate (3.7 g, 17 mmol) in water (50 mL) was added. After being stirred for 48 h the suspension was concentrated to one third of its original volume and extracted with ethyl acetate (5 × 150 mL). The combined organic layers were dried with Na_2SO_4 and evaporated to a pale yellow foam. The resulting N^2-isobutanoyl-O^6-[*R/S*]-(4-nitrophenylsulfoxyethyl)-2′-deoxyguanosine was characterized as an intermediate. Yield: 5.0 g (94%), R_f: 0.26 ($CHCl_3$:CH_3OH, 9:1, v:v), 300 MH, ^{1}H NMR ($CDCl_3$) 1.26 (d, 6H, 2 × CH_3), 2.42 (m, 1H, 2″-H), 2.84 (m, 2H, 2′-H, CH-Ib), 3.52 (t, 2H, S-CH_2), 3.86 (2 × dd, 2H, 5′,5″-H), 4.16 (m, 1H, 4′-H), 5.02 (t, 2H, OCH_2), 4.84 (m, 1H, 3′-H), 6.30 (dd, 1H, 1′-H), 7.43 (dt, 2H, aromat.-H, AA′BB′), 8.02 (s, 1H, 8-H), 8.06 (dt, 2H, aromat.-H, AA′BB′), 8.20 (bs, 1H, NH). FABMS (matrix: 3-nitrobenzylalcohol): M^- = 534; calculated: M = 534.

The N^2-isobutanoyl-O^6-[*R/S*]-(4-nitrophenylsulfoxyethyl)-2′-deoxyguanosine (10 mmol scale) was then subjected to a Mitsunobu reaction as described previously.[11] Yield: 5.9 g (71%), R_f 0.50 ($CHCl_3$:CH_3OH, 9:1, v:v), 300 MH, ^{1}H NMR ($CDCl_3$) 1.16 (m, 6H, 2 × CH_3), 2.59 (m, 1H, 2″-H), 2.72 (m, 2H, 2′-H, N^2-CH), 3.33–3.59 (m, 4H, 5′,5″-H, S-CH_2), 3.75 (s, 6H, 2 × OCH_3), 4,21 (m, 1H, 4′-H), 4.79–5.11 (m, 2H, OCH_2), 6.52 (m, 1H, 1′-H), 6.76 (m, 4H, aromat.-H ortho to OCH_3), 7.13–7.43 (m, 9H, aromat.-H), 7.84 (m, 2H, AA′BB′), 7.97 (s, 1H, 8-H), 8.13 (2 × s, 1H, NH), 8.25 (m, 2H, AA′BB′). FABMS (matrix: 3-nitrobenzylalcohol + KCl): MH^+ = 837; estimated: M^+ = 836.

5′-*O*-(4,4′-dimethoxytrityl)-thymidine-3′-*O*-[R_P/S_P]-(*N,N*-diisopropylamino, phenyl)-phosphine (5). The protected nucleoside **1** (5.4 g, 10 mmol)[10] was dried and dissolved in CH_2Cl_2 (50 mL). To this solution, *N,N*-diisopropylethylamine (13 mL, 75 mmol) and *rac*-chloro-*N,N*-diisopropylamino-phenylphosphine (6.1 g, 25 mmol) were added. After 72 h at room temperature the reaction mixture was cooled (0 °C) and quenched with water (2 mL). After 20 min the solution was diluted with ethyl acetate (300 mL) and washed with 5% $NaHCO_3$ soln. (2 × 75 mL) followed by saturated brine (2 × 75 mL). After drying over Na_2SO_4 the solution was evaporated in vacuo and the residue was purified by flash chromatography on silica gel using ethyl acetate containing triethylamine (0.5%). Yield: 5.7 g (76%), R_f 0.75 ($CHCl_3$:CH_3OH, 9:1, v:v), 300 MH, ^{1}H NMR ($CDCl_3$) 0.98–1.25 (m, 12H, 4 × CH_3), 1.43 (2 × d, 3H, 5-CH_3), 2.35 (m, 1H, 2′-H), 2.58 (m, 1H, 2′-H), 3.20–3.54 (m, 4H, 5′,5″-H, 2 × N-CH), 3.78 (m, 6H, 2 × OCH_3), 4.25 (m, 1H, 4′-H), 4.80 (m, 1H, 3′-H), 6.49 (m, 1H, 1′-H), 6.78 (m, 4H, aromatic-H ortho to OCH_3), 7.20–7.96 (m, 15H, aromatic-H, 6-H), 8.30 (bs, 1H, NH); ^{31}P NMR ($CDCl_3$): s at 117.27 and 117. 31 ppm.

5′-*O*-(4,4′-dimethoxytrityl)-N^4-isobutanoyl-2′-deoxycytidine-3′-*O*-[R_P/S_P]-(*N,N*-diisopropylamino, phenyl)-phosphine (6). The phosphonamidite **6** was prepared from nucleoside **2** (6.0 g, 10 mmol)[21] and purified as described for the preparation of **5**. Yield: 5.2 g (65%), R_f 0.73 ($CHCl_3$:CH_3OH, 9:1, v:v), 300 MH, ^{1}H NMR ($CDCl_3$) 0.98–1.26 (m, 18H, 6 × CH_3), 2.29 (m, 1H, 2′-H), 2.54 (m, 1H, CO-CH), 2.85 (m, 1H, 2′-H), 3.21–3.56 (m, 5′,5″-H, 2 × N-CH), 3.78 (m, 6H, 2 × OCH_3), 4.29 and 4.38 (2 × m, 1H, 4′-H), 4.70 and 4.81 (2 × m, 1H, 3′-H), 6.32 and 6.37 (2 × t, 1H, 1′-H), 6.74–6.88 (m, 4H, aromat.-H ortho to OCH_3), 7.04–7.60 (m, 16H, aromat.-H, 5-H, 6-H), 8,18 (bs, 1H, NH); ^{31}P NMR ($CDCl_3$):s at 118.02 and 118.60 ppm.

5′-*O*-(4,4′-dimethoxytrityl)-N^6-benzoyl-2′-deoxyadenosine-3′-[R_P/S_P]-*O*-(*N,N*-diisopropylamino, phe-

nyl)-phosphine (7). The phosphonamidite **7** was prepared from nucleoside **3** (6.0 g, 10 mmol)[10] and purified as described for the preparation of **5**. Yield: 5.4 g (62%), R_f 0.82 ($CHCl_3$:CH_3OH, 9:1, v:v), 300 MH, ^1H NMR ($CDCl_3$) 0.98–1.27 (m, 12H, 4 × CH_3), 2.78 (m, 1H, 2′-H), 2.98 (m, 1H, 2′-H), 3.24–3.54 (m, 4H, 5′,5″-H, 2 × N-CH), 3.75 (m, 6H, 2 × OCH_3), 4.43 (m, 1H, 4′-H), 4.92 (m, 1H, 3′-H), 6.60 (m, 1H, 1′-H), 6.70–6.83 (m, 4H, aromat.-H ortho to OCH_3), 7.12–8.05 (m, 19H, aromat.-H), 8.21 (2 × s, 1H, 2-H), 8.72 (2 × s, 1H, 8-H), 9.08 (2 × s, 1H, NH); ^{31}P NMR ($CDCl_3$): s at 115.73 and 118.23 ppm.

5′-*O*-(4,4′-dimethoxytrityl)-N^2-isobutanoyl-O^6-[*R*/*S*]-(4-nitrophenylsulfoxyethyl)-2′-deoxyguanosine-3′-*O*-[R_P/S_P]-(*N*,*N*-diisopropylamino, phenyl)-phosphine (8). The phosphonamidite **8** was prepared from nucleoside **4** (6.0 g, 10 mmol) and purified as described for the preparation of **5**. Yield: 3.8 g (72%), R_f 0.70 ($CHCl_3$:CH_3OH, 9:1, v:v), 300 MH, ^1H NMR ($CDCl_3$) 1.00–1.34 (m, 18H, 6 × CH_3), 2.60–2.99 (m, 3H, 2′,2″-H, N^2-CH), 3.20–3.71 (m, 6H, 5′,5″-H, S-CH_2, 2 × P-N-CH), 3.75 (m, 6H, 2 × OCH_3), 4.38 (m, 1H, 4′-H), 4.78–5.06 (m, 3H, 3′-H, OCH_2), 6.44 (m, 1H, 1′-H), 6.74 (m, 4H, aromat.-H ortho to OCH_3), 7.24–8.30 (m, 19H, aromat.-H, 8-H); ^{31}P NMR ($CDCl_3$) s at 115.56, 115.58, and φs at 117.71 ppm. FABMS (matrix: 3-nitrobenzylalcohol + KCl): MH^+ = 1044; calcd: M = 1043.

N^2-isobutanoyl-O^6-(4-nitrophenylthioethyl)-2′-deoxyguanosine (10). To a stirred suspension of dry N^2-isobutyryl-2′-deoxyguanosine (**9**) (3.4 g, 10 mmol)[10] in 50 mL dioxane was added *N*-(trimethylsilyl)-imidazole (3.7 mL, 25 mmol). The mixture was stirred for 30 min at room temperature and triphenylphosphine (5.2 g, 20 mmol), 4-nitrophenyl-2-hydroxyethylsulfide (4.0 g, 20 mmol) and diethylazodicarboxylate (3.2 mL, 20 mmol) were added successively. After 3 h of stirring at ambient temperature a 1 M HF/pyridine solution (50 mL) was added and stirring was continued for additional 10 min. The reaction mixture was diluted with ethyl acetate (300 mL) and the resultant solution was extracted with 5% $NaHCO_3$ solution (2 × 75 mL) followed by saturated brine (100 mL). After drying over anhydrous Na_2SO_4 the solution was evaporated in vacuo and the residue was purified by flash chromatography on silica gel. As eluent dichlormethane:methanol (95:5, v:v) was used. Fractions containing homogeneous product were combined and concentrated to a yellow foam. Yield: 3.9 g (74%), R_f 0.34 ($CHCl_3$:CH_3OH, 9:1, v:v), 300 MH, ^1H NMR ($CDCl_3$) 1.26 (d, 6H, 2 × CH_3), 2.42 (m, 1H, 2″-H), 2.84 (m, 2H, 2′-H, CH-Ib), 3.52 (t, 2H, S-CH_2), 3.86 (2 × dd, 2H, 5′,5″-H), 4.16 (m, 1H, 4′-H), 4.77 (t, 2H, OCH_2), 4.84 (m, 1H, 3′-H), 6.30 (dd, 1H, 1′-H), 7.43 (dt, 2H, aromat.-H, AA′BB′), 8.02 (s, 1H, 8-H), 8.06 (dt, 2H, aromat.-H, AA′BB′); 8.20 (bs, 1H, NH).

4-Nitrophenyl-2-hydroxyethylsulfide (11). 4-Nitrothiophenol (15.5 g, 100 mmol) was suspended in ethanol (30 mL). A solution of KOH (6.0 g) in water (40 mL) and 2-chloroethanol (15 mL, 225 mmol) was added. The reaction mixture was refluxed for 3 h and then cooled down to room temperature. After standing overnight at 4 °C the product had crystallized. The sulfide was filtered off, washed thoroughly with water (500 mL) and dried over P_4O_{10} in vacuo. The crude product was purified by flash chromatography using methylene chloride:methanol (97:3, v:v). Yield: 12.5 g (63%), mp: 57 °C, R_f 0.53 ($CHCl_3$:CH_3OH, 9:1, v:v), UV (CH_3OH): 336 nm (ε = 16600); 268 nm (ε= 2200), 300 MH, ^1H NMR ($CDCl_3$) 2.25 (bs, 1H, OH), 3.25 (t, 2H, SO_2CH_2), 3.88 (φt, 2H, OCH_2), 7.37 (d, 2H, aromat.-H, AA′BB′), 8.09 (d, 2H, AA′BB′).

Oligodeoxynucleotide synthesis

All oligonucleotides were synthesized at one micromolar scale on an Applied Biosystems DNA synthesizer model 394 using the synthesis cycles described in Table 2. Removal of the *iso*-butanoyl groups and cleavage of the oligonucleotide from the solid support was accomplished by an one hour treatment with concentrated ammonia at the DNA synthesizer (standard end-procedure). After evaporation to dryness the residue was treated with 1 mL of ethylenediamine: C_2H_5OH:CH_3CN:H_2O (50.0:23.5:23.5:3.0, v:v:v:v) to remove the other protecting groups. After 6 h at room temperature, the solution was diluted to a volume of 15 mL with water and neutralized (pH 7.5) with acetic acid. This solution was directly soaked onto a C18 reversed-phase HPLC column and eluted using acetonitrile in 50 mM triethylammonium acetate. From each fraction one A_{260}-unit was removed, detritylated and analysed on a 16% polyacrylamide/7 M urea gel. Fractions containing homogeneous product were pooled and lyophilized to a colourless powder. The isolated yields were in the range of 25–45 A_{260}-units per μmol synthesis.

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