Pergamon

Tetrahedron, Vol. 52, No. 30, pp. 10011-10024, 1996

0040-4020/96 $15.00 + 0.00

PII: S0040-4020(96)00532-7

Synthesis and Duplex Stability of Oligodeoxynucleotides Containing Stereoregular or Stereorandom Octylphosphonate Linkages

Matthias Mag, Kerstin Jahn, Gerhard Kretzschmar, Anusch Peyman and Eugen Uhlmann*

Hoechst Aktiengesellschaft, G 838, D-65926 Frankfurt am Main, Germany

Abstract: The synthesis of oligodeoxynucleotide pentadecamers containing two octylphosphonate linkages [3'-O-P(=O)(*n*-C_8H_{17})-O-5'] with stereoregular or stereorandom chirality is described. The introduction of random octylphosphonate linkages was performed using a monomeric nucleoside octylphosphonamidite as synthon whereas the introduction of stereoregular linkages could be accomplished by the use of stereoregular dimers containing a preformed octylphosphonate linkage. The novel oligodeoxynucleotides were characterized by electrospray ionization mass spectrometry and the influence of chirality of the modified linkages on the duplex stability was studied. Furthermore, end-capped oligodeoxynucleotides having two octylphosphonate linkages at either end which were directed against HSV-1 mRNA have been synthesized for investigation as antisense drugs.

The synthesis of chemically modified oligodeoxynucleotides has attracted considerable attention in recent years owing to their applicability for various biochemical purposes, including their potential use as antisense agents.[1-4] Among the many types of oligodeoxynucleotide analogs described in the literature, phosphate-modified oligomers such as phosphorothioates and methylphosphonates have been investigated most thoroughly.[5-7] While phosphorothioates retain the anionic charge of the phosphodiester linkage, methylphosphonate moieties are uncharged. The reasons for the great attention given to these two classes of compounds are: (i) they can be prepared by means of automated solid-phase synthesis using commercially available monomers; (ii) they possess increased resistance against nucleases as compared to unmodified oligodeoxynucleotides, (iii) they form reasonably stable duplexes with complementary nucleic acids. A disadvantage is the chirality of their internucleotide linkage in that the phosphate-modified oligodeoxynucleotides are obtained from standard solid-phase synthesis as diastereomeric mixtures.

In an evaluation program for oligodeoxynucleotides containing non-ionic internucleotide linkages, such as alkyl- or phenylphosphonates, we considered alkylphosphonates with longer alkyl chains to be of particular interest. From a chemical point of view octylphosphonate oligodeoxynucleotides may be more stable to the alkaline conditions used for deprotection than methylphosphonate analogs. With respect to their use as antisense agents, octylphosphonates may have improved stability against nucleases and enhanced cellular uptake as compared to unmodified oligodeoxynucleotides. So far, octylphosphonate oligodeoxynucleotides have not been

studied in detail most likely due to the unavailability of appropriate synthetic procedures. To our knowledge there is only one report concerning the preparation of this class of compounds.[8] However, no binding studies with mixed base sequences were reported .

As with the methylphosphonate internucleoside residue, the octylphosphonate group is chiral, each linkage existing either in Rp or Sp configuration. Since the synthetic procedure using monomeric methyl- or octylphosphonamidites is not stereospecific, the corresponding oligodeoxynucleotide product results in a mixture of 2^n diastereomers, where n is the number of modified linkages within the oligodeoxynucleotide. The importance of stereochemistry in the hybridization of uncharged derivatives has been demonstrated by Lesnikowski *et al.*[9] They reported the synthesis of homo-thymidine octamer oligodeoxynucleotides with all-Rp or all-Sp methylphosphonate linkages, except for the central one, which was racemic. The melting temperature (Tm) of the Rp-enriched form was 38 °C under conditions where normal Tg and the stereorandom oligomer gave a Tm of only 13 °C. The Sp-enriched form showed a Tm of less than 2 °C.

Here we report a method for automated solid-phase synthesis of oligodeoxynucleotide pentadecamers containing two stereoregular or random octylphosphonodiester linkages. The influence of the absolute configuration at phosphorus on duplex stability of these compounds is described. Furthermore, the synthesis of three end-capped oligodeoxynucleotides containing octylphosphonate linkages paired with phosphorothioate linkages is described.

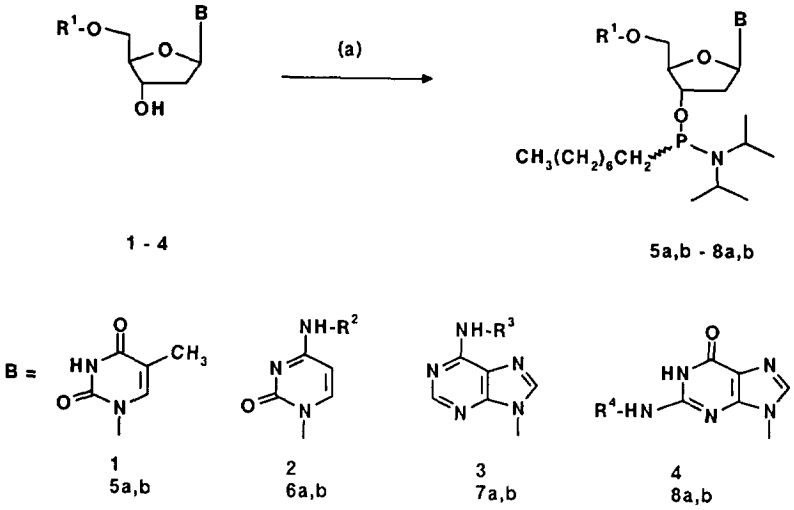
RESULTS AND DISCUSSION

Synthesis of Monomeric Building Blocks

For the introduction of stereoregular internucleoside linkages into defined positions of an oligodeoxynucleotide we first prepared octylphosphonate dimers of defined stereochemistry. The monomeric deoxynucleoside-3'-octylphosphonamidite synthons **5a,b-8a,b** (Scheme 1) which are needed for dimer synthesis were prepared by reaction of the appropriately protected nucleosides **1-4** with *ent*-chloro-*N,N*-diisopropylamino-octylphosphine (1.5 equivalents) in the presence of *N,N*-diisopropylethylamine (3.0 equivalents) at room temperature (2 hours). After flash chromatography the desired synthons were obtained as colourless solids as a 1:1 mixture of diastereomers. The building blocks were then characterized by ^{1}H NMR and ^{31}P NMR spectroscopy.

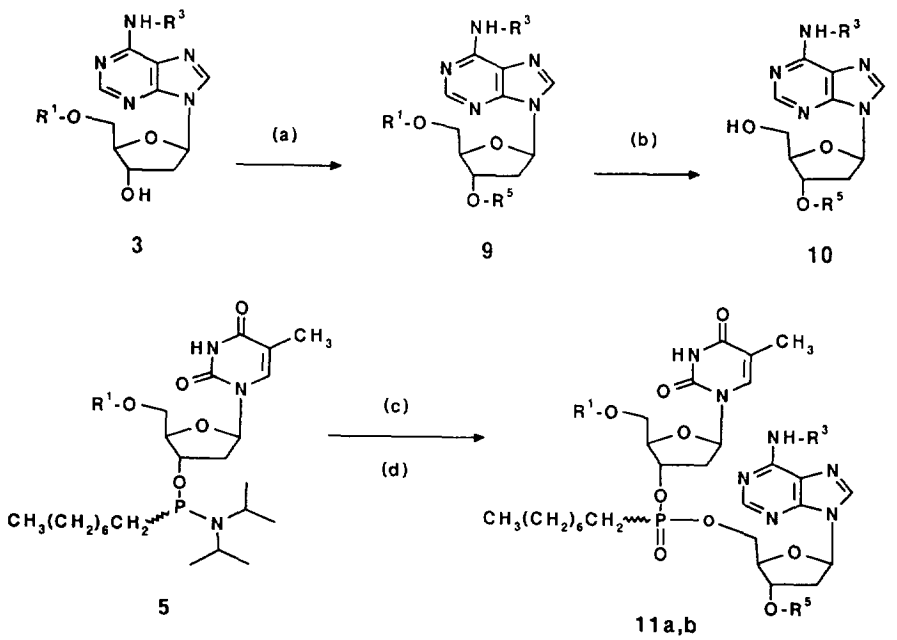
Synthesis of Dimeric Building Blocks

For the synthesis of fully protected octylphosphonate dimers **11a,b** (Scheme 2) N^6-benzoyl-3'-O-levulinoyl-2'-deoxyadenosine (**10**) was first prepared starting from 5'-O-dimethoxytrityl-N^6-benzoyl-2'-deoxyadenosine (**3**). The 3'-O-levulinoyl group was introduced as previously described[10] yielding the 3',5'-bis-protected nucleoside **9** in 82% yield. Removal of the 5'-O-dimethoxytrityl transient protecting group with 80% acetic acid at room temperature gave **10** in 86% yield.



Reagents and Conditions: (a) $CH_3(CH_2)_6CH_2$-P(Cl)N(i-C_3H_7)$_2$, Et-N(i-C_3H_7)$_2$, methylene chloride, 2 hours, room temperature (R^1: dimethoxytrityl, R^2: phenoxyacetyl, R^3: benzoyl, R^4: *iso*-butanoyl).

Scheme 1: Synthesis of Monomeric Octylphosphonamidites **5a,b - 8a,b**.



Reagents and Conditions: (a) [$CH_3C(O)(CH_2)_2C(O)]_2O$, *N,N*-dimethylamino pyridine, pyridine; (b) 80 % CH_3COOH; (c) 10, tetrazole, methylene chloride; (d) iodine/water/pyridine (R^5: levulinoyl).

Scheme 2. Synthesis of the partially protected octylphosphonate dimers **11a,b.**

The internucleoside octylphosphonate linkage 3'-O-P(=O)(n-C_8H_{17})-O-5' in dimers **11a,b** was formed by the reaction of 1.5 equivalents of the octylphosphonamidite **5a,b** in methylene chloride with one equivalent of **10** in the presence of 3.0 equivalents of tetrazole. After the reaction was allowed to proceed for 2 hours at ambient temperature, the initial P(III) linkage was oxidized *in situ* to the corresponding octylphosphonate **11a,b** by iodine/water. Flash chromatography of the crude product on silica gel resulted in a 1:1 mixture of diastereomers of **11a,b** as a colourless amorphous solid in 49% yield. The purity of this diastereomeric mixture was checked by ^{1}H NMR and ^{31}P NMR spectroscopy (singlets at 34.5 and 34.9 ppm).

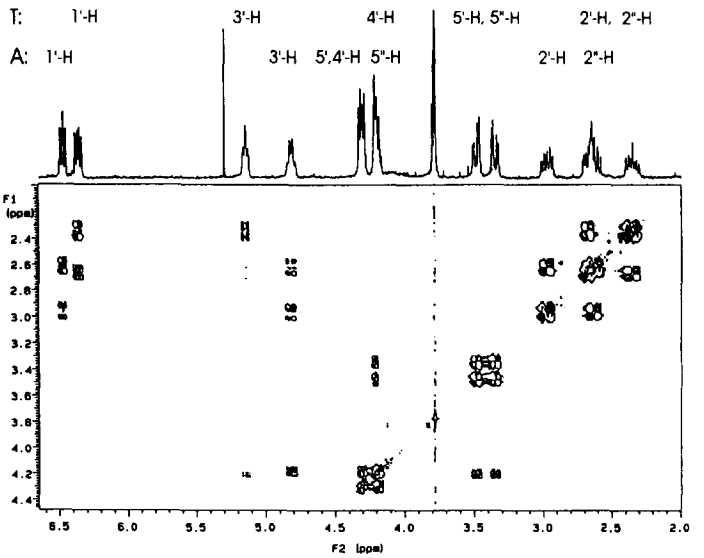


Figure 1: Relevant part of the 300 MHz-^{1}H,^{1}H COSY NMR spectrum of **12a**. (T, A: Assignment of signals for the protons of the deoxyfuranosyl part of thymidine or deoxyadenosine, respectively).

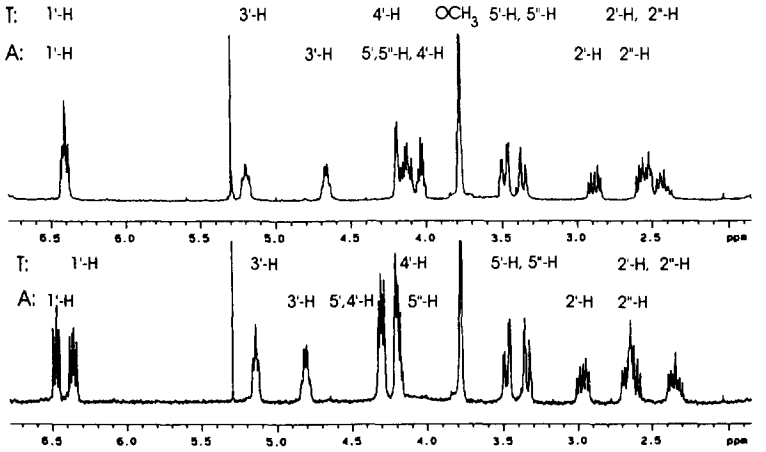
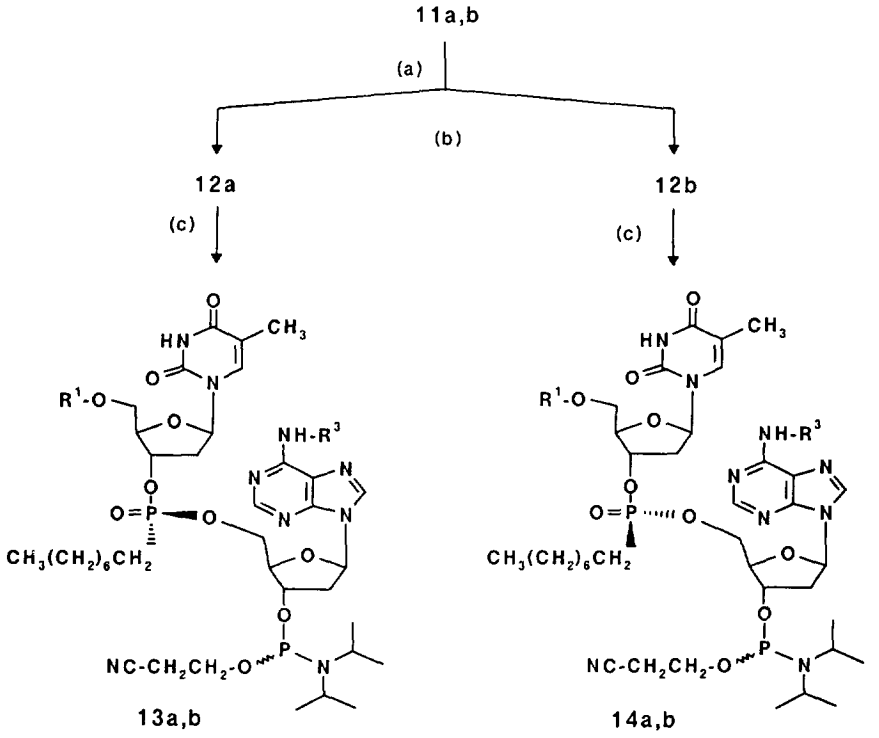


Figure 2: Relevant part of the 300 MHz-^{1}H NMR spectra of **12a** (lower) and **12b** (upper; T, A: see Figure 1).

Next the 3'-hydroxyl protecting group had to be be removed to allow for phosphitylation. The levulinic ester could be selectively cleaved by a buffered hydrazine solution (0.5 M in pyridine/acetic acid, 3:2, v:v)[10] without harming any of the other protecting groups yielding **12a,b** (Scheme 3). At this stage of the synthesis the two diastereomers of **12a,b** were separated by flash chromatography on silica gel (dichloromethane/methanol, 99:1, v:v). Pure fractions were combined and evaporated and the mixed fractions were rechromatographed. The individual diastereomers were then characterized by ^{1}H NMR spectroscopy (Figures 1 and 2) and the purity was checked by ^{31}P NMR experiments (**12a**: 35.1 ppm and **12b**: 35.6 ppm). Both diastereomers were > 99% pure. The absolute configuration of both octylphosphonate dimers has not been experimentally assigned so far.

In the next step, the 3'-hydroxyl groups of dimers **12a** and **12b** were phosphitylated with *rac*-2-cyanoethoxy-*N,N*-diisopropylaminochlorophosphine (1.2 equivalents) in the presence of *N,N*-diisopropyethylamine (2.4 equivalents). After stirring for 30 minutes at room temperature, work-up and purification by flash chromatography the dimeric 3'-phosphoramidite building blocks **13a,b** and **14a,b** could be isolated in 92% and 89% yield, respectively.



Reagents and Conditions: (a) N_2H_4 / CH_3COOH / pyridine; (b) separation of diastereoisomers by flash chromatography; (c) $NCCH_2CH_2O$-P(Cl)N(i-C_3H_7)$_2$, Et-N(i-C_3H_7)$_2$, methylene chloride.

Scheme 3: Preparation of dimer building blocks **13a,b** and **14a,b**. (Note that the absolute stereochemistry is shown as expected from analogy to methylphosphonates due to the higher melting temperature of RpMe analogs).

Solid-Phase Synthesis of Octylphosphonate oligodeoxynucleotides

The new dimeric phosphoramidite building blocks **13a,b** and **14a,b** were employed for the synthesis of the pentadecamers **15** and **16**, each having two octylphosphonate internucleoside linkages within their sequences as indicated in *Table 1*. Synthesis of the oligomers was performed using 0.1M solutions of **13a,b** or **14a,b** in acetonitrile (ten-fold excess over the start nucleoside) at 1 μmol scale under standard conditions, but with an extended coupling time of 600 s (Cycle II, Table 2).

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

Compound	Synthesized Sequence of the Oligodeoxynucleotides	Building Block	Cycle Used	Overall Yield (%)
15	d(GAC $Tp_{Oc}A$ CGATG $Tp_{Oc}A$ CTG)	**13a,b**	I, II	78%
16	d(GAC $Tp_{Oc}A$ CGATG $Tp_{Oc}A$ CTG)	**14a,b**	I, II	79%
17a-d	d(GAC $Tp_{Oc}A$ CGATG $Tp_{Oc}A$ CTG)	**5a,b**	I, III	84%
18	d(GAC TAC GAT GTA CTG)	standard	I	91%
19a-d	d(GAC $Tp_{Me}A$ CGATG $Tp_{Me}A$ CTG)	standard	I	86%
20	d(CAG TAC ATC GTA GTC)	standard	I	88%

Yields were determined by trityl colour quantitation at 498 nm. p_{Oc}: octylphosphonate linkage, p_{Me}: methylphosphonate linkage.

Table 2: Synthesis cycles used for automated oligodeoxynucleotide synthesis.

Reagent	Function	Cycle I	Cycle II	Cycle III
3% trichloroacetic acid/CH_2Cl_2	to column	24 s	24 s	24 s
	Detritylation	20 s	20 s	20 s
amidite + tetrazole	to column	5 s	5 s	5 s
	Condensation	30 s	600 s	300 s
Ac_2O/*N*-methyl-imidazole/pyridine	to column	10 s	10 s	10 s
	Capping	5 s	5 s	5 s
I_2/H_2O/pyridine	to column	8 s	8 s	8 s
	Oxidation	22 s	22 s	22 s

Cycle I: standard cycle; II: cycle for the introduction of a modified dimer building block; III: cycle for the coupling of the octylphosphonamidites **5a,b-8a,b**

The coupling efficency of the internucleotide bond formation with the modified dimer building block **13a,b** or **14a,b** was about 97% as estimated by dimethoxytrityl colour quantitation at 498 nm. Treatment with concentrated ammonia for 1 hour at room temperature cleaved the protected oligodeoxynucleotides from the solid support. After evaporation to dryness in vacuo the protecting groups were removed with a mixture of ethylene diamine/ethanol/acetonitrile/water (50.0:23.5:23.5:3.0, v:v:v:v) at room temperature during 6 hours.

Unfortunately, ethylene diamine readily transaminates the N^4-amide of the commonly used N^4-benzoylated deoxycytidine. In order to minimize the extent of transamination we replaced the benzoyl protecting group by the more labile phenoxyacetyl group.[11] The pre-treatment with ammonia, as described above, removed most of the N^4-phenoxyacetyl groups prior to treatment with the ethylene diamine mixture necessary to cleave the other protecting groups. Additionally, N^4-phenoxyacetyl-dC is less susceptible against transamination.[12]

All oligodeoxynucleotides were purified by C18 RP-HPLC with a gradient of acetonitrile in triethylammonium acetate on a preparative Nucleosil column. The purification by RP-HPLC is straightforward because the two octylphosphonate residues function as a purification handle, similar to the trityl group in the trityl-on chromatography of oligodeoxynucleotides. The modified oligomers, being more hydrophobic, were retained stronger on the reversed phase column than the less lipophilic truncated sequences.

For the synthesis of **17a-d** containing two octylphosphonate linkages with random chirality we used the monomer octylphosphonamidite building block **5a,b** (Table 1). Synthesis was performed with a ten-fold excess of **5a,b** in acetonitrile. The modified monomer was employed in the coupling reaction of the fourth and eleventh reaction cycle using cycle III. Interestingly, the coupling yield using this modified thymidine building block was as high as with a standard phosphoramidite. **19a-d** containing two random methylphosphonate linkages was synthesized to allow for the comparison of its duplex stability with that of **17a-d**.

To test the usefulness of the nucleoside octylphosphonamidites **7a,b** and **8a,b** in automated solid-phase oligodeoxynucleotide synthesis we prepared three end-capped oligodeoxynucleotides, which are outlined in *Table 3*. Compounds with this "minimal" modification should on one the hand be reasonably stable against nucleolytic degradation, since the major nucleolytic activity in serum is a 3'-exonuclease. On the other hand, the enhanced lipophilicity of the octylphosphonate oligodeoxynucleotides may promote their cellular uptake. Oligodeoxynucleotide **21a-p** is protected against 3'-exonucleases with two phosphorothioate linkages at the 3'-end, while the 5'-terminus is end-capped with two octylphosphonate linkages. Oligodeoxynucleotide **22a-p** is modified in the opposite way, having the octylphosphonates at the 3'-end and the phosphorothioates at the 5'-end. In oligodeoxynucleotide **23a-p** two octylphosphonate linkages were introduced at each terminus rendering this oligodeoxynucleotide very lipophilic.

Table 3: Synthesis of end-capped oligodeoxynucleotides.

Compound	Synthesized Sequence	Building Block	Cycle Used	Overall Yield (%)
21a-p	d($Gp_{Oc}Ap_{Oc}CGTTCCTCCTCCTGCGGGAp_SAp_SG$)	**7a,b / 8a,b**	I, III	84%
22a-p	d($Gp_SAp_SCGTTCCTCCTCCTGCGGGAp_{Oc}Ap_{Oc}G$)	**7a,b**	I, III	91%
23a-p	d($Gp_{Oc}Ap_{Oc}CGTTCCTCCTCCTGCGGGAp_{Oc}Ap_{Oc}G$)	**7a,b / 8a,b**	I, III	86%

Yields were determined by trityl colour quantitation at 498 nm. p_{Oc}: octylphosphonate linkage, p_S: phosphorothioate linkage.

The introduction of the modified building blocks was performed using a longer coupling time (300 s for cycle III instead of 30 s for standard cycle I). Coupling yields for the introduction of octylphosphonate linkages were > 98%. Sulfurization reaction was performed with 0.5 M tetraethylthiuram disulfide solution in acetonitrile

for 20 minutes to generate phosphorothioate internucleoside linkages.[13] Purification of **21a-p - 23a-p** was performed as described for **15** and **16**.

The novel backbone modified oligodeoxynucleotides were characterized by means of electrospray ionization mass spectrometry to show that internucleoside octylphosphonate linkages were incorporated. For the molecular weight of the diastereomers **15-17a-d** we computed 4784.55 g/Mol ($C_{163}H_{217}N_{57}O_{86}P_{14}$). All three spectra showed the correct molecular weight (**15**: 4784.14 ± 0.32, **16**: 4784.25 ± 0.61 and **17a-d**: 4784.05 ± 0.66).

Duplex Stability

To elucidate the influence of chirality of the modified oligodeoxynucleotides **15-17a-d** on duplex stability melting temperature experiments were performed. All analogs were found to form duplexes with cooperative melting transitions at physiological salt concentration (140 mM NaCl). The Tm values measured are displayed in *Table 4*. It can be seen that substitution of octylphosphonate linkage for a phosphodiester internucleoside linkage leads to a reduction in Tm upon hybridization to DNA. The difference in Tm between the unmodified oligonucleotide **18** and the oligomer **17a-d** having stereorandom octylphosphonate linkages is -6.7 °C. In contrast, the Tm of stereorandom methylphosphonate analog **19a-d** differs from the unmodified oligonucleotide by only -1.5 °C. Consequently, the stereochemically uniform oligodeoxynucleotides **15** and **16** also showed reduced binding affinity when hybridized to **20** (ΔTm = -4.5 and -8.0 °C, respectively). In analogy to results found with methylphosphonates,[9] where introduction of a Rp methylphosphonate results in a significantly higher Tm as compared to the introduction of a Sp methylphosphonate, we suggest that the absolute configuration of the octylphosphonate linkage of **15** is likely to be Rp whereas that of **16** is likely to be Sp.

Table 4: Tm values of the oligodeoxynucleotides **15-19**.

Compound	T_m Value (°C)	Reduction in T_m relative to **18** + **20** (°C)	Configuration of modified linkages	Modification of Oligonucleotide
15 + **20**	51.2	-4.5	possibly Rp	p_{Oc}
16 + **20**	47.7	-8.0	possibly Sp	p_{Oc}
17a-d + **20**	49.0	-6.7	random	p_{Oc}
18 + **20**	55.7	--	unmodified	--
19a-d + **20**	54.2	-1.5	random	p_{Me}

Measured in 10 mM HEPES buffer (pH 7.5) at 140 mM NaCl (p_{Oc}: Octylphosphonate, p_{Me}: Methylphosphonate).

The effect of alkylphosphonodiester linkages on the hybrid stability results from four factors: (1) the modified linkages are uncharged (no repulsive Coulombic interactions), (2) steric effects of the substituents, (3) electronic effects, and (4) changes in hydration of the modified duplex as compared to the unmodified one (differences in the water activity).

From modelling studies it is obvious that in the Sp (pseudoaxial) configuration the phosphonate octyl group hinders base stacking due to its inward orientation towards the deoxyribose rings and bases. For linkages

with Rp configuration (pseudoequatorial) it is reasonable to assume that the octyl residue is oriented away from the major groove and towards the solvent. Thus, no sterical destabilization of the duplex occurs, but there is an unfavorable contact between the solvent water and the hydrophobic octyl residue, which could lower the binding affinity. It may be possible for the Rp octyl group to rotate into the minor groove, which would reduce these detrimental solvent interactions.

SUMMARY AND CONCLUSION

The present work describes methods for the synthesis of nucleoside octylphosphonamidite monomers and nucleoside dimers containing diasteriomerically pure octylphosphonate linkages. The application of these building blocks for efficient solid-phase synthesis of oligonucleotides containing stereorandom or stereochemically pure octylphosphonate linkages has been demonstrated, and the influence of the chirality of the modified linkages on duplex stability has been determined.

To study the influence of the chirality of the modified linkages on the duplex stability we have synthesized several oligodeoxynucleotides. The synthesis of three oligodeoxynucleotide pentadecamers containing two octylphosphonate linkages with stereoregular (Rp / Rp or Sp / Sp) or stereorandom (Rp, Sp / Rp, Sp $\Rightarrow 2^2 = 4$ diastereomers) chirality has been accomplished. The above modified oligodeoxynucleotides are stable under the standard synthesis conditions. Deprotection of oligodeoxynucleotides was performed using a two-step protocol using concentrated ammonia followed by ethylene diamine. After C18 RP-HPLC purification the oligodeoxynucleotides were characterized by electrospray ionization mass spectrometry.

Due to the introduction of octylphosphonate linkages and the lack of negative charges the modified oligodeoxynucleotides are much more lipophilic as compared to their natural congeners. The effect of replacement of phospodiester by octylphosphonodiester linkages on hybrid stability with complementary DNA was investigated. All modified oligodeoxynucleotides **15-17a-d** form duplexes with their complementary sequence which have cooperative melting transitions at physiological salt conditions (140 mM NaCl) and all duplexes are destabilized relative to the unmodified duplex. The difference in Tm was -3.4 °C per modification for the random (**14a-d**) and about -2.3 and -4.0 °C per modification for the stereoregular configured oligodeoxynucleotides **15** and **16** as compared to the unmodified oligodeoxynucleotide **18**. In analogy to results found with methylphosphonates we suggest that the absolute configuration of the octylphosphonate linkages of **15** is most likely to be Rp and **16** is most likely to be Sp.

The aim of this work was to investigate the synthesis and duplex stability of oligodeoxynucleotides containing octylphosphonate linkages. Chimeric octylphosphonodiester/phosphodiester molecules offer several advantages over normal phosphodiester oligodeoxynucleotides. These advantages include enhanced resistance to exonucleases which are ubiquitous in serum and the cytoplasm and the higher lipophilicity which may promote the cell uptake of this class of oligodeoxynucleotides. Regarding the biological activity, the observed reduced duplex stabilities of oligodeoxynucleotides containing octylphosphonate linkages must not necessarily be a disadvantage. Thus, Giles and Tidd found a 4-fold enhancement of RNase H activity using a chimeric methylphosphonodiester/phosphodiester antisense effector with a Tm 18.8 °C lower than the all-phosphodiester

oligodeoxynucleotide.[14] This result suggests that the general assumption of increasing activity with increased duplex stability may not be always appropriate if RNase H is involved in antisense effects.

The introduction of the octylphosphonodiester linkage is compatible with the synthesis of chimeric methylphosphonodiester/phosphodiester or phosphorothioate/phosphodiester oligodeoxynucleotides. Furthermore, oligodeoxynucleotides containing octylphosphonothioate linkages can also be synthesized very easily by using tetraethylthiuram disulfide in the oxidation step instead of iodine/water. Several chimeric octylphosphonodiester/phosphodiester oligodeoxynucleotides including the end-capped sequences **21a-p - 23a-p** against HSV 1 were synthesized. These antisense compounds are now under investigation with respect to their potency to selectively block virus replication.

EXPERIMENTAL

General

Thin layer chromatography was performed on precoated Merck TLC glass plates (silica gel 60). Flash chromatography was carried out on Merck silica gel 60. HPLC was carried out on Waters 600 for analytical runs, Waters Delta Prep 3000 for preparative runs, using Nucleosil C18 reversed phase columns (4 x 250 mm or 20 x 250 mm, 5 μm, acetonitrile in triethylammonium acetate pH 7 (5 to 45% in 30 min., flow: 1.0 ml/min for analytical runs, 15 ml/min for preparative separations). Melting curves were recorded on a Varian Cary 1 spectrophotometer equipped with a temperature programmer. Oligodeoxynucleotides (0.5 A_{260}-unit) were mixed with complementary DNA (0.5 A_{260}-units). The duplex was heated to 80 °C and then cooled down. The solutions containing duplex DNA were then heated from 25 to 80 °C at a rate of 0.5 °C per minute and A_{260} was recorded against temperature. NMR measurements: One- and two-dimensional ^1H NMR spectra were recorded on a Varian VXR 300 S spectrometer using standard pulse sequences. Tetramethylsilane was used as internal reference and the cited chemical shifts are given in ppm downfield to this standard. ^{31}P NMR experiments were performed on the same spectrometer. The spectra were recorded at 121.5 MHz using proton noise decoupling. The spectrometer was referenced with a $CDCl_3$ sample containing a 85% H_3PO_4 filled glass capillary.

[Rp/Sp]-5'-O-(4,4'-Dimethoxytrityl)-thymidine-3'-O-(*N,N*-diisopropylamino, *n*-octyl)-phosphine (5a,b)

The 5'-O-protected nucleoside **1** (2.7 g, 5.0 mmol) was dried overnight under high vacuum and dissolved under argon in 40 ml CH_2Cl_2. To this solution, *N,N*-diisopropylethylamine (3.9 g, 30.0 mmol) and monochloro-*N,N*-diisopropylamino-octylphosphine (2.1 g, 7.5 mmol) were added. The reaction was monitored by tlc. On completion of the reaction (2 h, r. t.) the mixture was diluted with ethyl acetate (200 ml). The ethyl acetate solution was washed with 2.5% $NaHCO_3$ soln. (50 ml) followed by saturated brine (100 ml). After drying over anhydrous Na_2SO_4 the solution was evaporated in vacuo and the residue purified by flash chromatography on silica gel using a step gradient of ethyl acetate in hexane (40 - 70%) containing triethylamine (0.5%). Yield: 3.5g

(88%), R_f: 0.39/0.46 (ethyl acetate/hexane, 1:1, v:v), 300 MHz-^{1}H NMR ($CDCl_3$): 0.82-1.64 (m, 32H, T-CH_3, 4xCH_3, octyl-H); 3.40 (m, 4H, 5',5"-H, 2xCH); 3.79 (2xs, 6H, 2xOCH_3); 4.11 (m, 1H, 4'-H); 4.55 (m, 1H, 3'-H); 6.40 (m, 1H, 1'-H); 6.81 (m, 4H, ortho to OCH_3); 7.20-7.44 (m, 9H, aromat.-H); 7.64 (2xψd, 1H, 6-H); 8.52 (2xbs, 1H, NH). ^{31}P NMR: 127.5 and 127.8 ppm.

[Rp/Sp]-5'-O-(4,4'-Dimethoxytrityl)-*N*^{4}-phenoxyacetyl-2'-deoxycytidine-3'-O-(*N,N*-diisopropylamino, *n*-octyl)-phosphine (6a,b)

The 2'-deoxycytidine analog **6a,b** was prepared as described for **5a,b** using 5.0 mmol 5'-O-(4,4'-dimethoxytrityl)-*N*^{4}-phenoxyacetyl- 2'-deoxycytidine. Yield: 3.6g (78%), R_f: 0.48/0.57 (ethyl acetate/hexane, 1:1, v:v). ^{31}P NMR: 128,6 and 128,9ppm.

[Rp/Sp]-5'-O-(4,4'-Dimethoxytrityl)-*N*^{6}-benzoyl-2'-deoxyadenosine-3'-O-(*N,N*-diisopropylamino, *n*-octyl)- phosphine (7a,b)

The 2'-deoxyadenosine analog **7a,b** was prepared as described for **5a,b** using 5.0 mmol 5'-O-(4,4'-dimethoxytrityl)- *N*^{6}-benzoyl-2'-deoxyadenosine. Yield: 3.2g (71%), R_f: 0.39/0.46 (ethyl acetate/hexane, 1:1, v:v). ^{31}P NMR: 127.0 and 128.1 ppm.

[Rp/Sp]-5'-O-(4,4'-Dimethoxytrityl)-N^2-isobutanoyl-2'-deoxyguanosine-3'-O-(*N,N*-diisopropylamino, *n*-octyl)-phosphine (8a,b)

The 2'-deoxyguanosine anaolg **8a,b** was prepared as described for **5a,b** using 5.0 mmol 5'-O-(4,4'-dimethoxytrityl)-*N*^{2}-isobutanoyl-2'-deoxy-guanosine. Yield: 3.3g (75%), R_f: 0.24 (ethyl acetate/hexane, 1:1, v:v). 300 MHz-^{1}H NMR ($CDCl_3$): 0.78-1.60 (m, 35 H, 7xCH_3, 7xCH_2); 1.97 (m, 1H, 2'). ^{31}P NMR: 126.4 and 127.2 ppm.

N^6-Benzoyl-3'-O-levulinoyl-2'-deoxyadenosine (10)

5'-O-(4,4'-Dimethoxytrityl)-N^6-benzoyl-3'-O-levulinoyl-2'-deoxyadenosine (**9**) was prepared in analogy to the 2'-deoxyguanosine compound as described by Jones.[10] Crude **9** (7.5 g, 10 mmol) was dissolved in 80 % acetic acid (75 ml) and stirred for 30 min at room temperature. The resultant orange solution was evaporated in vacuo and the residue was partitioned between ethyl acetate (500 ml) and 5% $NaHCO_3$ (150 ml). The organic phase was extracted with water (50 ml) followed by sat. brine (100 ml) and dried with Na_2SO_4. After evaporation to dryness the crude product was purified by flash chromatography on silica gel eluting with a step gradient of methanol in ethyl acetate (2-5%). Yield: 3.6 g (79%), R_f: 0.57 (CH_2Cl_2/CH_3OH, 9:1, v:v), 300 MHz-^{1}H NMR ($CDCl_3$): 2.21 (s, 3H, CH_3); 2.50 (m, 1H, 2'-H); 2.61 (m, 2H, CH_2); 2.81 (m, 2H, CH_2); 3.12 (m, 1H, 2"-H); 3.92 (m, 2H, 5', 5"-H); 4.24 (m, 1H, 4'-H); 5.54 (m, 1H, 3'-H); 5.86 (dd, 1H, 5'-OH); 6.36 (dd, 1H, 1'-H); 7.46-7.63 (m, 3H, aromat., meta and para to C=O); 8.00 (m, 2H, aromat., ortho to C=O); 8.12 (s, 1H, 8-H); 8.73 (s, 1H, 2-H); 9.28 (s, 1H, NH).

[Rp/Sp]-5'-O-(4,4'-Dimethoxytrityl)-P-deoxy-P-octyl-thymdylyl-(3'→5')-N^6-benzoyl-3'-O-levulinoyl-2'-deoxyadenosine (11a,b)

Compound **5a,b** (4.08 g, 5.25 mmol) and **10** (1.58 g, 3.50 mmol) were dissolved in dry CH_2Cl_2 (30 ml). Tetrazole (0.74 g, 10.50 mmol) was added to the solution and the mixture was stirred at r. t. for 3 h. After oxidation of the resultant P(III) linkage with I_2/H_2O (10 min, DNA synthesizer standard solution) the reaction mixture was diluted with ethyl acetate (200 ml). The solution was washed with 5% $NaHCO_3$, 5% Na_2SO_3 and saturated NaCl solution (50 ml each) and dried over Na_2SO_4. Evaporation and flash chromatography of the residue on silica gel (4% CH_3OH in ethyl acetate containing 1% triethylamine) afforded the desired dimer **11a,b** as a 1:1 mixture of diastereomers. Yield: 2.0 g (49%), R_f: 0.64/0.68 (CH_2Cl_2/CH_3OH, 9:1, v:v), 300 MHz-^{1}H NMR (DMSO-d6): 1.80 (m, 3H, CH3); 1.00-1.38 (m, 12H, 6xCH_2); 1.45 (m, 3H, T-CH_3); 2.70 (m, 2H, P-CH_2); 2.08 (m, 1H, 2'-H[2]); 2.12 (2xs, 3H, CH_3C=O); 2.36 (m, 1H, 2'-H[2]); 2.55 (m, 2H, CH_2-C=O); 2.78 (m, 2H, O-C=O-CH_2); 3.04 (m, 1H, 2'-H [1]); 3.22 (m, 2H, 5',5"-H [2]); 3.72 (2xs, 6H, 2xOCH_3); 4.00-4.35 (m, 4H, 4'-H [1], 4'-H [2], 5',5"-H [1]); 5.04 (m, 1H, 3'-H [2]); 5.40 (m, 1H, 3'-H [1]); 6.18 (m, 1H, 1'-H [2]); 6.49 (m, 1H, 1'-H [1]); 6.87 (m, 4H, ortho to OCH_3); 7.14-8.07 (m, 15H, 6-H, aromat.-H); 8.72 (m, 2H, 2-H, 8-H); 11.18 (2xs, 1H, NH); 11.35 (bs, 1H, NH). ^{31}P NMR (DMSO-d6): s at 34.5 and 34.8 ppm (ratio 1:1).

[Rp]-5'-O-(4,4'-Dimethoxytrityl)-P-deoxy-P-octyl-thymdylyl-(3'→5')-N^6-benzoyl-2'-deoxyadenosine (12a)
[Sp]-5'-O-(4,4'-Dimethoxytrityl)-P-deoxy-P-octyl-thymdylyl-(3'→5')-N^6-benzoyl-2'-deoxyadenosine (12b)

The fully protected octylphosphonate dimer **11a,b** (2.0 g, 1.7 mmol) was treated with 5 ml of a solution of pyridine/acetic acid (3:2, v:v) containing 0.5 M hydrazine hydrate at 4 °C. The reaction was quenched with ice after 15 min. The solution was diluted with ethyl acetate (150 ml), washed with 5% $NaHCO_3$ soln. (30 ml) followed by sat. brine (75 ml), dried over Na_2SO_4 and evaporated. The residual oil was flash chromatographed on silica gel eluting with 2% CH_3OH in CH_2Cl_2. Diastereomerically pure fractions were combined and evaporated. Mixed fractions were also evaporated and the residue was again separated by flash chromatography. **12a:** Yield: 0.68 g (38%), R_f: 0.44 (CH_2Cl_2/CH_3OH, 9:1, v:v), 300 MHz-^{1}H NMR ($CDCl_3$): 0.79-1.9 (m, 20 H, T-CH_3, octyl-H); 2.37 (m, 1H, 2"-H [1]); 2.62 (m, 2H, 2'-H [1], 2"-H [2]); 2.98 (m, 1H, 2'-H [2]); 3.40 (m, 2H, 5',5"-H [1]); 3.79 (m, 6H, 2xOCH_3); 4.19 (m, 2H, 4'-H [1], 5"-H [2]); 4.30 (m, 2H, 4'-H [2], 5'-H [2]); 4.80 (m, 1H, 3'-H [2]); 5.12 (m, 1H, 3'-H [1]); 6.37 (dd, 1H, [1]); 6.48 (dd, 1H, 1'-H [2]); 6.81 (d, 4H, ortho to OCH_3); 7.18-8.01 (m, 15H, 6-H, aromat.-H); 8.26 (s, 1H, 2-H); 8.78 (s, 1H, 8-H); 9.27 (bs, 1H, NH); 9.46 (bs, 1H, NH). ^{31}P NMR (CDCl3): s at 35.1ppm. **12b:** Yield: 0.61 g (33%), R_f: 0.50 (CH_2Cl_2/CH_3OH, 9:1, v:v), 300 MHz-^{1}H NMR ($CDCl_3$): 1.78-1.82 (m, 20H, T-CH_3, octyl-H); 2.41 (m, 1H, 2"-H [1]); 2.56 (m, 2H, 2'-H [1], 2"-H [2]); 2.88 (m, 1H, 2'-H [2]); 3.41 (m, 2H, 5',5"-H [1]); 3.78 (m, 6H, 2xOCH_3); 4.02 (m, 1H, 4'-H [2]; 4.10 (m, 2H, 5',5"-H [2]); 4.18 (m, 1H, 4'-H [1]); 4.66 (m, 1H, 3'-H [2]); 5.19 (m, 1H, 3'-H [1]); 6.39 (m, 2H, 1'-H [1], 1'-H [2]); 6.80 (m, 4H, ortho to OCH_3); 7.17-8.02 (m, 15H, 6-H, aromat.-H); 8.14 (s, 1H, 2-H); 8.77 (2xs, 2H, 8-H, NH); 9.29 (bs, 1H, NH). ^{31}P NMR ($CDCl_3$): s at 35.6 ppm.

[Rp]-5'-O-(4,4'-Dimethoxytrityl)-P-deoxy-P-octyl-thymdylyl-(3'→5')-N^6-benzoyl-2'-deoxy-adenosine-3'-O-[Rp/Sp]-(2-cyanoethyl, *N,N*-diisopropylamino)phosphite (13a,b)

The dry octylphosphonate dimer **12a** (0.53 g, 0.5 mmol) was dissolved under argon in CH_2Cl_2 (5 ml). To this solution, *N,N*-diisopropylethylamine (0.16 g, 1.2 mmol) and chloro-2-cyanoethoxy-*N,N*-diisopropylamino-phosphine (0.14 g, 0.6 mmol) were added. After stirring for 30 min at r. t. the reaction mixture was diluted with ethyl acetate (100 ml). This solution was extracted with sat. brine (50 ml) and dried over Na_2SO_4 (30 min). After evaporation the residue was purified by flash chromatography on silica gel using CH_2Cl_2 containing triethylamine (1%). Yield: 0.58 g (92%), R_f: 0.75 (CH_2Cl_2/CH_3OH, 9:1, v:v), ^{31}P NMR ($CDCl_3$): ψs at 34.5 and 150.0 ppm.

[Sp]-5'-O-(4,4'-Dimethoxytrityl)-P-deoxy-P-methyl-thymdylyl-(3'→5')-N^6-benzoyl-2'-deoxy-adenosine-3'-O-[Rp/Sp]-(2-cyanoethyl, *N,N*-diisopropylamino)phosphite (14a,b)

This compound was prepared as described for **13a,b**. Yield: 0.56 g (89%), R_f: 0.79 (CH_2Cl_2/CH_3OH, 9:1, v:v), ^{31}P NMR ($CDCl_3$): ψs at 35.1 and 150.1 ppm

Oligodeoxynucleotide Synthesis

Oligodeoxynucleotides were synthesized on an Applied Biosystems DNA synthesizer model 394 using the synthesis cycles described in *Table 2* (1 μM scale). Oligodeoxynucleotides were automatically cleaved from the support with conc. ammonia (1 hour, r. t.). The ammonia solution was evaporated to dryness and the residue was then treated with 1 ml of ethylene diamine/C_2H_5OH/CH_3CN/H_2O (50.0:23.5:23.5:3.0, v:v:v:v).[12] After 6 hours at room temperature, the solution was diluted to a volume of 15 ml with water and neutralized (pH 7.5) with acetic acid.

Acknowledgements

We would like to thank Dr. H. J. Kleiner for providing the chloro-*N,N*-diisopropylamino-octylphosphine. Furthermore we have to thank Dr. G. Becker for his help in recording the NMR spectra and Dr. H.-W. Fehlhaber for measurement of electrospray ionization mass spectra.

REFERENCES

1. Uhlmann, E.; Peyman, A. *Chem. Rev.* **1990**, 90, 543-584.
2. Milligan, J.F.; Matteucci, M.D.; Martin, J.C. *J. Med. Chem.* **1993**, 36, 1923-1937.
3. Crooke, S.T. *Ann. Rev. Pharmacol. Toxicol.* **1992**, 32, 329-376.
4. Thuong, N. T.; Hélène, C. *Angew. Chem.* **1993**, 105, 697-723; *Angew. Chem. Int. Ed. Engl.* **1993**, 32, 666-690.
5. Stein, C.A.; Cheng, Y.-C. *Science* **1993**, 261, 1004-1012.
6. Ts'o, P.O.P.; Aurelian, L.; Chang, E.; Miller, P. S. In *Antisense Strategies*; Annals New York Academy of Sciences; Baserga, R.; Denhardt, D. T. Eds.; The New York Academy of Sciences.; New York **1992**, Vol. 660, pp. 159-177.
7. Cook, P. D. *Anticancer Drug. Des.* **1991**, 6, 585-607.
8. Roelen, H.C.P.F.; Van den Elst, H.; Van der Marel, G.A.; Van Boom, J.H. *Tetrahedron Lett.* **1992**, 33, 2357-2360.
9. Lesnikowski, Z.J.; Jaworska, M.; Stec, W.J. *Nucleic Acids Res.* **1990**, 18, 2109-2115.
10. Jones, R. In *Oligonucleotide Synthesis*; Gait, M. Ed.; IRL Press; Oxford **1984**, pp. 23-34.
11. Singh, R. K.; Misra, K. *Ind. J. Chem.* **1988**, 27B, 409-417.
12. Hogrefe, R.I.; Vaghefi, M.M.; Reynolds, M.A.; Young, K.M.; Arnold Jr., L.J. *Nucleic Acids Res.* **1993**, 21, 2031-2038.
13. Vu, H.; Hirschbein, B. L., *Tetrahedron Lett.* **1991**, 32, 3005-3008.
14. Giles, R.V.; Tidd, D.M. *Anti-Cancer Drug Design* **1992**, 7, 37-48.

(*Received in Germany* 14 *March* 1996; *accepted* 31 *May* 1996)