The P-Stereocontrolled Synthesis of PO/PS-Chimeric Oligonucleotides by Incorporation of Dinucleoside Phosphorothioates Bearing an *O*-4-Nitrophenyl Phosphorothioate Protecting Group

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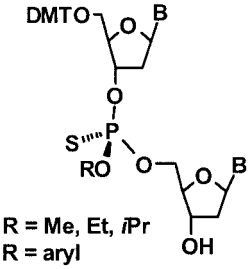
The synthesis of protected model dinucleoside (3′,5′)-*O*-aryl phosphorothioates, their separation into pure diastereomers, and their successful incorporation into oligonucleotides followed by stereospecific deprotection of the *O*-aryl phosphorothioate function with oximate ion (inversion) enables the preparation of chimeric PO/PS-oligonucleotides with a predetermined sense of *P*-chirality at each internucleotide phosphorothioate position. The absolute configuration at the phosphorus of the internucleotide *O*-aryl phosphorothioate in "dimeric building blocks" has been assigned. The 3′-terminal S_P-phosphorothioate linkages effectively protect such chimeric constructs from degradation by human plasma exonuclease

Introduction

The development of antisense strategies[1] has prompted the design of the so-called "chimeric PO/PS-oligonucleotides" containing phosphorothioates incorporated at the desired positions of a oligonucleotide chain, with the expectation that their properties as antisense therapeutics will be improved. The advantage of such chimeric PO/PS-Oligos over PS-Oligos,[2] which were successfully established as first-generation antisense drugs, involves their increased affinity towards target sequences of selected mRNA and lowered affinity towards proteins. PO/PS-Oligos are notably more resistant towards cellular exo- and endonucleases[3] compared to the unmodified PO-Oligos.[4] A satisfactory approach to the synthesis of oligonucleotides modified at the selected internucleotide linkage with phosphorothioates of predetermined sense of *P*-chirality[5] involves HPLC separation of the chimeric oligonucleotides or the synthesis of the appropriately protected dinucleotide phosphorothioates of known absolute configuration at the P-atom. Using so-called block condensation, after suitable activation at the 3′-*O*-position, these dimeric building blocks may be incorporated into a growing oligonucleotide chain by applying routine phosphoramidite chemistry.[6]

The demand for chimeric oligonucleotides increased after reports indicating that internucleotide phosphorothioates of S_P-configuration located at the 3′-end of such chimeric PO/PS-Oligos confer complete resistance towards human plasma 3′-exonucleases.[7,8] These observations kindled hopes for the evaluation of more-effective, second-generation antisense constructs and brought new challenges for the design and efficient synthesis of precursors of dinucleoside phosphorothioates of S_P-configuration, which can then be used for further incorporation into the growing oligonucleotide chain at the desired positions.



1 R = Me, Et, *i*Pr
2 R = aryl

In this laboratory, numerous dinucleoside phosphorothioate *O*-alkyl esters of general structure **1** (R = CH_3, C_2H_5, *i*Pr) have been prepared by nonstereospecific methods.[9] Since their synthesis and separation into pure diastereoisomers is difficult, we recently turned our attention to dinucleoside phosphorothioates **2**, where the phosphorothioate function carries an *O*-aryl protecting group. Early observations by Reese and Serafinowska that *O*-aryl

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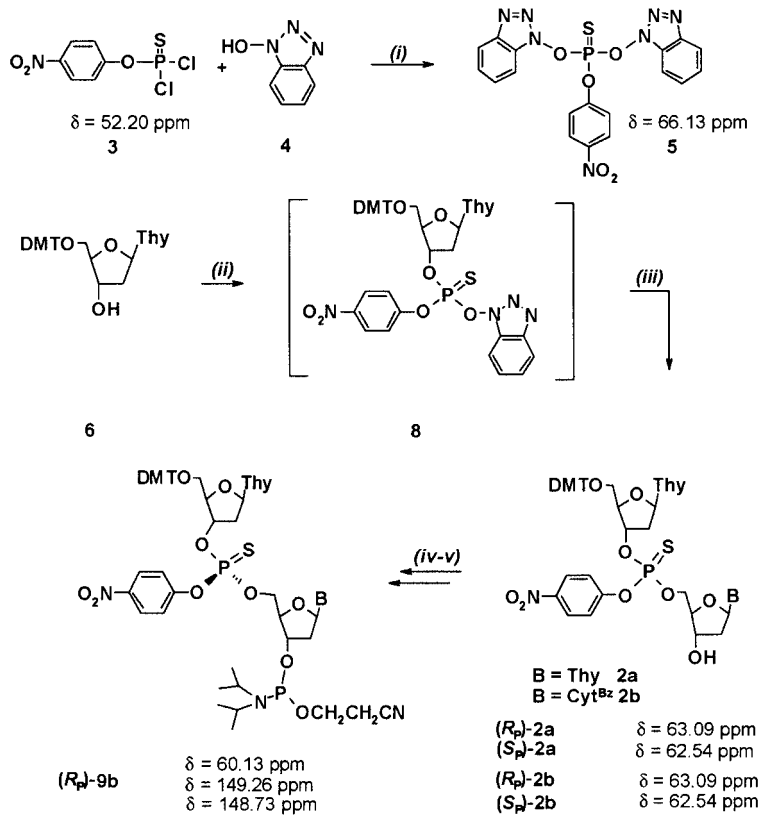


Scheme 1. Reagents and conditions: (i) **4** (2.1 equiv.), pyridine, room temp, 0.5 h; (ii) **6** in pyridine/dioxane, added dropwise, room temp, 1 h; (iii) nucleoside (*N*-protected) **7** (1.1 equiv.), Me-Imi (4 equiv.), room temp, 1.5 h; (iv) silica-gel column chromatography and separation of diastereomers; (v) phosphitylation: $(i\mathrm{Pr}_2\mathrm{N})_2\mathrm{POCH}_2\mathrm{CH}_2\mathrm{CN}$ (2 equiv.), 1*H*-tetrazole (4 equiv.), MeCN, 1 h; silica-gel column chromatography purification.

protecting groups at internucleotide phosphorothioates can be selectively removed stereospecifically by treatment with oximate ion were encouraging in this respect.[10] We also expected that separation of the diastereoisomers of **2** would be more effective. To prove these hypotheses we prepared two pairs of the protected dinucleoside *O*-aryl phosphorothioates **2** as model compounds, namely $^{DMT}T_{PS(O\text{-}Aryl)}T$ (**2a**) and $^{DMT}T_{PS(O\text{-}Aryl)}C^{Bz}$ (**2b**) using *O*,*O*-di(hydroxybenzotriazoyl) *O*-4-nitrophenyl phosphorothioate (**5**) as a coupling reagent (Scheme 1).[11]

Results and Discussion

A one-pot reaction, as depicted in Scheme 1, yielded dinucleotides **2a** and **2b** as a diastereomeric mixture in an approximate 1:1 ratio, as estimated by ^{31}P NMR spectroscopy, with an average yield of 65–70% (calculating from starting 5′-*O*-DMT thymidine). These dinucleotides were purified and separated into their diastereomers FAST-**2** and SLOW-**2** by silica-gel column chromatography.

The assignment of the absolute configuration of isomeric FAST- and SLOW-**2**[12] was performed by employing 2D NMR techniques, with use of the Pulse Field Gradient (PFG) system to reduce the measurement time and to improve the quality of the spectra (e.g. by reduction of T_1 noise).[13] From the analysis of the interactions between the 5′,5″ protons and the aromatic protons of the aryl group of the internucleotide bond (ROESY spectra), we concluded that the FAST eluting isomer **2a** has the S_P-configuration, whereas the SLOW eluting counterpart **2a** has the R_P-configuration. It is worth mentioning that for dinucleotides with an aryloxy group like 2-chlorophenoxy (data not presented here) or 4-nitrophenoxy linked to the internucleotide phosphorothioate, the simplified but reliable assignment of absolute configuration is feasible. Magnetic shielding (ring-current) effects carry useful structural information about the site-specific proton shifts, and have been observed for a large number of proteins and nucleic acids.[14] For a partially protected diastereomeric **2**, the shielding effect of the aromatic protecting group strongly influences the chemical shifts of H1′ (δ = 6.38 ppm for SLOW-**2a** and δ = 6.46 ppm for FAST-**2a**), H3′ (δ = 5.37 ppm for SLOW-**2a** and δ = 5.54 ppm for FAST-**2a**), and H2′ of the thymidine.

An analogous analysis was performed for isomeric protected phosphotriesters **2b**, and established the R_P-configuration for SLOW-**2b**, and the S_P-configuration for FAST-**2b** (see Figures 1 and 2).

Semi-empirical PM3 and molecular mechanics calculations (using the MM2 force field)[15] revealed that for (R_P)-**2** the distances between the aryl group and H1′ and H3′ exceed 5.0 Å, and beyond this distance the shielding effect of the ring (if any) is very small,[14] which supports the

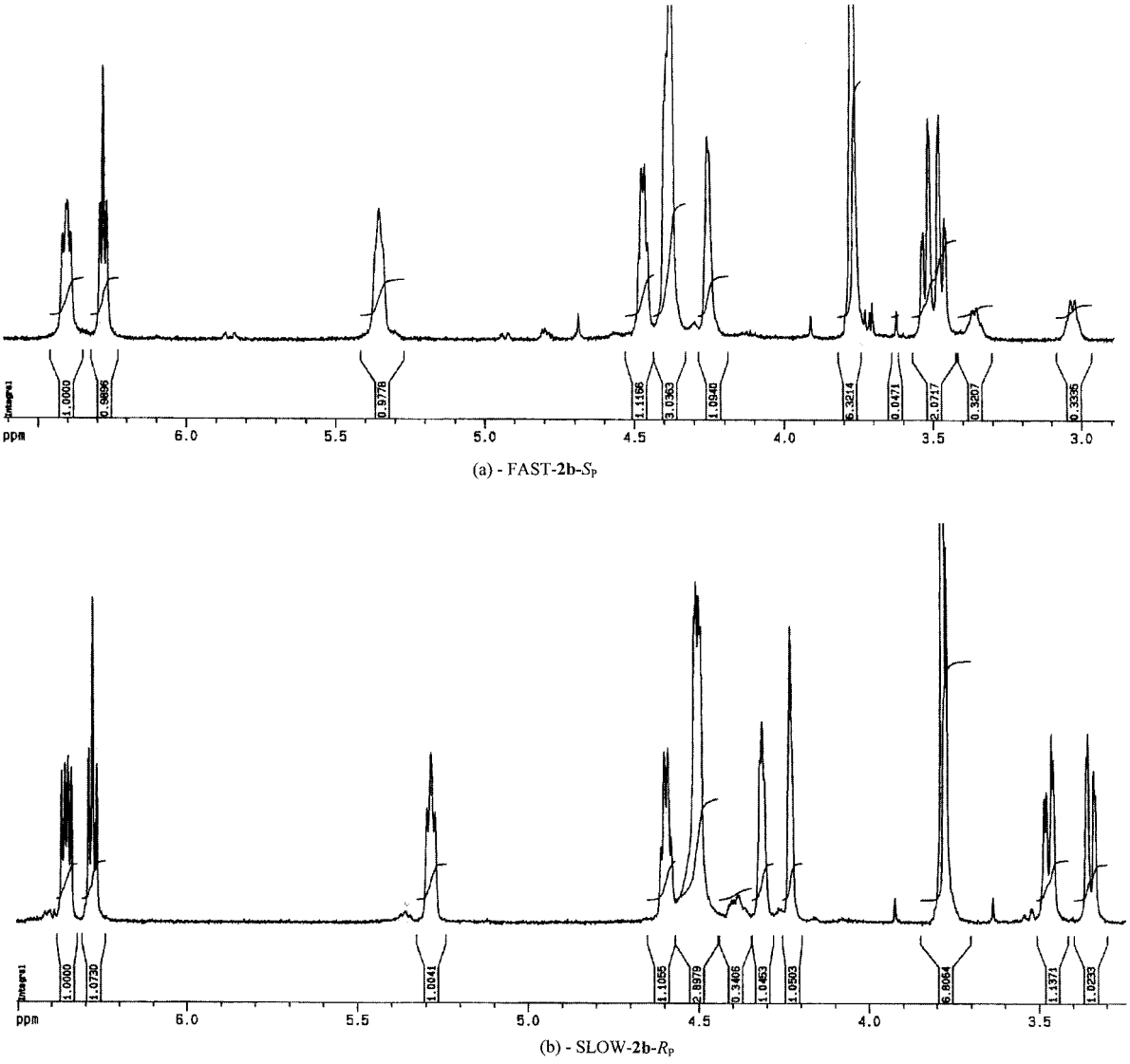


Figure 1. ^{1}H NMR spectra (500 MHz, in $CDCl_3$) of (a) FAST-**2b** and (b) SLOW-**2b**.

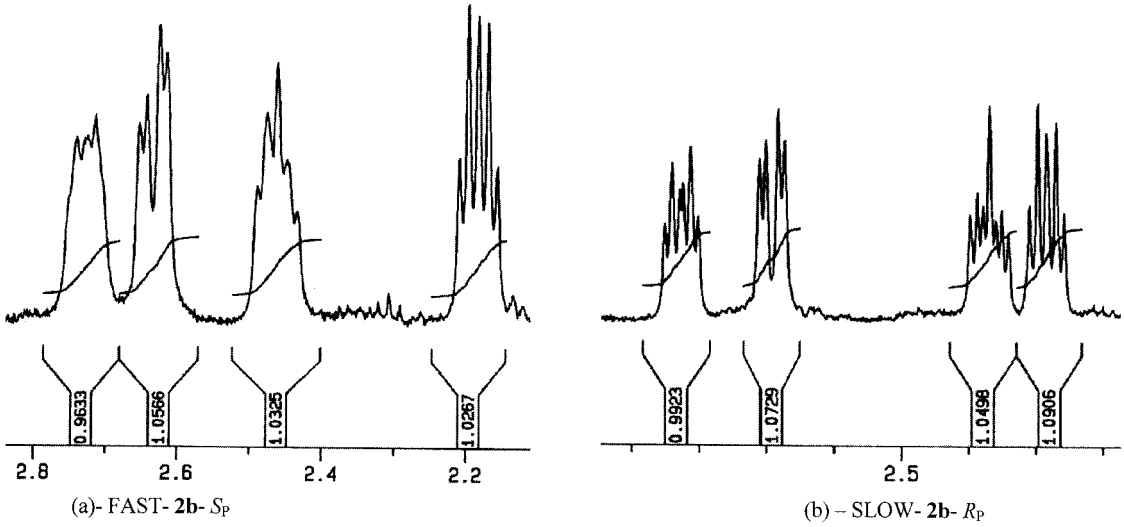


Figure 2. Fragment of ^{1}H NMR spectra (500 MHz, in $CDCl_3$) showing the H2′ region of (a) FAST-**2b** and (b) SLOW-**2b**.

above assignment. Based upon these findings we assigned the absolute configuration of the precursors of dimeric building blocks FAST-**2** as S_P and SLOW-**2** as R_P.

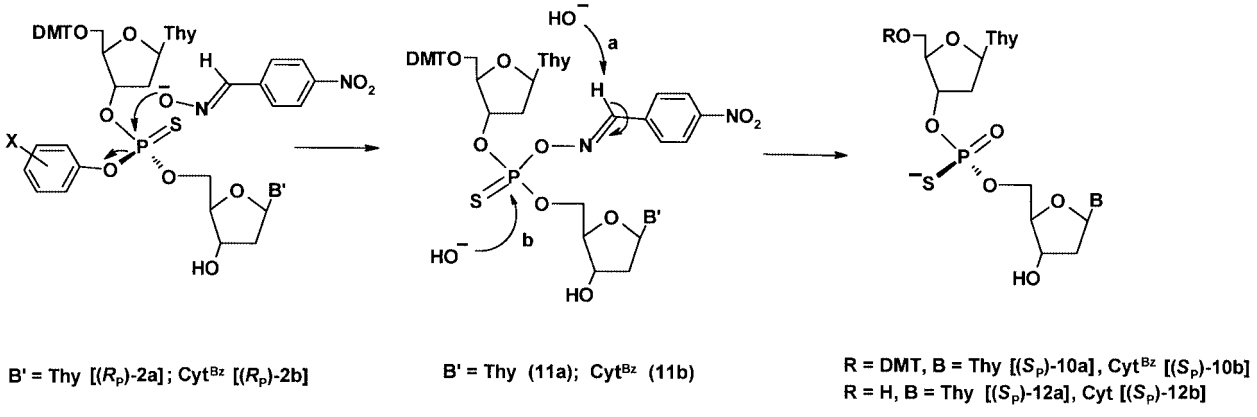
Stereochemistry of Deprotection of the Phosphorothioate Triesters 2

Deprotection procedures for the aryl protecting groups of the internucleotide phosphotriester linkages in phosphates were developed in the 1970s and 1980s with the discovery of the phosphotriester approach for RNA synthesis.[16] The application of the conjugated bases of oximes, in particular *syn*-4-nitrobenzaldoximate and pyridine-carboxaldoximate, as effective deprotecting reagents for model 2-chlorophenyl diethylphosphates and 4-nitrophenyl diphenylphosphates, was studied by Reese et al.[17] It was also confirmed that the removal of a 2,5-dichlorophenyl protecting group from the phosphorothioate internucleotide bond is stereospecific.[10,18] However, since the absolute configuration of the starting phosphotriester was not known, it was not possible to assign the stereochemical pathway of the deprotection process.

In our experiments, the deprotection of the diastereomeric mixture enriched in FAST ($T_{PS}C^{Bz}$) isomer (S_P)-**2b** [$dr > 98\%$; $\delta = 62.54$ (major), 62.12 ppm (minor)] was performed in dioxane/water solution in the presence of an excess of *syn*-4-nitrobenzaldoxime and 1,1,3,3-tetramethylguanidine (TMG). This reaction was followed by ^{31}P NMR spectroscopy and was complete within two hours.[19] Similar stereospecific deprotection was performed with the SLOW isomer (R_P)-**2b** ($dr = 94\%$). In the process of these deprotections no other signals were observed in the NMR spectrum: only substrates **2b** and the deprotected phosphorothioate diesters (R_P)-**10** ($\delta = 57.37$ ppm) and (S_P)-**10** ($\delta = 57.5$ ppm), respectively, were detected.

It was postulated earlier that in the rate-determining step the conjugate base of the oxime attacks the phosphorus atom of the corresponding triester phosphate to form the intermediate oxime ester **11**.[20] The intermediate **11** rapidly fragments to form the substituted benzonitriles and the corresponding phosphodiester **10** (Scheme 2, path a).[21] The question arose, however, whether substitution of the phosphoryl oxygen by sulfur could modify this proposed deprotection mechanism, and whether a different mechanism may operate. This different mechanism could involve attack of the hydroxide at the phosphorus center of the oxime triester (Scheme 2, path b), analogous to the mechanism demonstrated by Bunton and Ihara for 4-nitrophenyl diphenylphosphate.[22] To distinguish between the two possible mechanisms, the deprotection of the triester **2** was performed in a solution containing [^{18}O] water, such that the product of hydrolysis of the intermediary oxime ester **11** (path b) should be labeled with ^{18}O. It was found that after deprotection in dioxane/$H_2{}^{18}O$ (99.8% atom $H_2{}^{18}O$, 4:1 v/v), no incorporation of ^{18}O into phosphorothiate **10** was detected by FAB-MS. The assignment of the absolute configuration of the completely deprotected dinucleotide (3′,5′)-phosphorothioates **12** d($T_{PS}C$) was performed by enzymatic digestion followed by HPLC analysis of the products.[23,24]

Fully deprotected FAST-**12b**,[25] obtained from the phosphotriester FAST-**2b**, was completely degraded by the R_P-specific *Crotalus adamanteus* snake venom phosphodiesterase (Capd svPDE) within 18 hours, and was not degraded by the S_P-specific nuclease P1 (nP1). Accordingly, the phosphorothioate SLOW-**12b** was not degraded by nuclease Capd, but was hydrolyzed by nP1.[26] The above results confirm that the SLOW isomer (R_P)-**2b**, as assigned by NMR analysis, was deprotected with oxime/TMG to give the corresponding diester phosphorothioate (S_P)-**12b**, while the FAST isomer (S_P)-**2b** is the precursor of the isomer (R_P)-**12b**. The inversion of configuration supports a mechanism involving attack of the oximate anion at the triester phosphorothioate center, followed by β-elimination of benzonitrile (path a). Since the oximate ion is a classical α-nucleophile bearing nonbonding pairs of electrons adjacent to the nucleophilic center,[27] with a reactivity greater than what would be expected from the pK_a value of 9.95,[20] it attacks the phosphorus atom with inversion of configuration. ^{31}P NMR studies of the deprotection reaction did not indicate formation of any intermediate, only formation of the deprotected phosphodiesters. This observation is consistent with



Scheme 2. Oximate-mediated removal of aryl protecting groups; path a: base-catalyzed β-elimination of 4-nitrobenzonitrile from the oxime ester **11** (retention of configuration); path b: hydrolysis of the oxime ester **11** (inversion of configuration).

a rate-determining stereoinversion step of the transesterification reaction, followed by fast β-elimination of the corresponding benzonitrile and formation of the internucleotide phosphorothioate.

Synthesis of Chimeric Oligonucleotides with Incorporated Phosphorothioate Funcionalities of Predetermined Absolute P-Configuration

To check the applicability of the diastereomerically pure dimeric building blocks with *O*-aryl protection of the internucleotide phosphorothioates for synthesis of chimeric PO/PS oligonucleotides we prepared representative chimeric constructs (Table 1). The partially protected phosphotriesters **2b** were synthesized, separated into FAST- and SLOW-eluting diastereoisomers (vide supra, Scheme 1), phosphitylated at the 3′-OH position, and purified by silica-gel column chromatography. The corresponding 3′-*O*-(β-cyanoethyl-*N*,*N*-diisopropyl phosphoramidites) (**9b**) were used for the coupling reactions with the CPG-bound oligonucleotides prepared by phosphoramidite chemistry. All oligomers were prepared on a 1-μmol scale with an ABI 394 Synthesizer (Applied Biosystems Inc., Foster City, USA). Incorporation of **9b** was achieved by prolonging the coupling time to 10 min, without further modifications of the standard protocol.

Table 1. Chimeric oligonucleotides obtained by the phosphoramidite method with incorporated *dimeric building blocks*.

	Chimeric oligonucleotides with the specified modifications	Absolute conf.	MALDI TOF[b]
13	5′-T_{PS}CTTTT-3′[a]	R_P	(1763) 1762
13	5′-T_{PS}CTT TT-3′	S_P	(1763) 1762
14	5′-TCTTTTT_{PS}C-3′	S_P	(2373) 2373
15	5′-T_{PS}CTTTTT_{PS}C-3′	S_P	(2373) 2373
16	5′-T_{PS}CTCCCAGCGTGCGCCAT-3′	R_P,S_P[c]	(5424) 5425
17	5′-T_{PS}CTCCCAGCGTGCGCCAT-3′	S_P	(5424) 5424
18	5′- TGACGT_{PS}CATTTTTGACGTCA-3′	S_P	(6414) 6413[d]
19	5′- TGACGT_{PS}CATTTTTGACGTCA-3′	R_P,S_P	(6111) 6111
20	5′-T_{PS}CTCCCAGCGTGCGCCAT_{PS}C-3′	S_P	(5729) 5725

[a] T_{PS}C-dinucleoside (3′,5′)-phosphorothioate. [b] Theoretical molecular masses are given in parentheses. [c] Obtained according to the standard phosphoramidite procedure with a single sulfurization step. [d] Contains a DMT group.

Since incorporation of the diastereomerically pure phosphorothioate at the terminal 3′-position of the chimeric oligonucleotide is of special interest due to effective protection against exonuclease degradation,[7,8] we prepared a CPG support functionalized with diastereomerically pure (>96%) dinucleoside (3′,5′)-*O*-arylphosphorothioate **2b** linked to the support through a standard succinyl linker.[4] The use of (R_P)-**2b** and (S_P)-**2b** allowed the preparation of supports with a loading capacity of 28.6 and 20.1 μmol g^{-1}, respectively. These supports were used for synthesis of the corresponding chimeric oligonucleotides (**14**, **15**, and **20**; Table 1).

The enhanced stability of the chimeric oligonucleotides **14** and **15** towards exonucleases was confirmed in experiments with snake venom phosphodiesterase (svPD) and 50% human serum.[8] We found that oligonucleotides **14** and **15** protected by the 3′-terminal S_P-phosphorothioate function remained almost intact (96%) after two hours incubation with svPD, and were stable in more than 60% after eight hours of incubation in 50% human serum at 37 °C. In control experiments under the same conditions unmodified octanucleotide d(TCTTTTTC) was completely degraded within one hour of incubation. Comprehensive analysis of such protection is given elsewhere.[28]

Conclusions

The data presented here reveal that the partially protected diastereomerically pure dinucleoside (3′,5′)-phosphorothioates **2** with 4-nitrophenyl groups protecting the internucleotide phosphorothioate centers are convenient substrates for the "dimeric building blocks" strategy. They can be efficiently used for incorporation of diastereomerically pure phosphorothioate linkages into the chimeric oligonucleotides using standard phosphoramidite chemistry. The use of the aryl protecting groups, and the 4-nitrophenyl group in particular, allows for separation of the dinucleoside (3′,5′)-*O*-aryl-phosphorothioates into diastereomerically pure isomers by silica-gel column chromatography. These protecting groups can be removed from dinucleotides **2** and from chimeric oligonucleotides **13**–**20** in a stereospecific manner, with inversion of configuration at the internucleotide phosphorothioate center, by treatment of the LCAA CPG-bound oligonucleotides with 4-nitrobenzyloximate ions, before standard deprotection procedures. The partially protected phosphotriesters SLOW-**2** are precursors for the S_P-phosphorothioate diesters, whereas FAST-**2** serve as precursors of R_P internucleotide phosphorothioate diesters.

Experimental Section

General Remarks: THF was distilled from $LiAlH_4$ directly into the reaction vessel. NEt_3 was distilled from CaH_2, and stored under argon over CaH_2 pellets. Dioxane was refluxed with sodium wire for 3–5 h. 1-Hydroxybenzotriazole was purchased from Fluka (98%, 13% water content). The protected nucleosides were purchased from JBL, Scientific Inc., or Promega, or were prepared using standard procedures. HPTLC plates (Silica gel 60F_{254}, precoated for nano-TLC) were purchased from Merck. Glass, gas-tight syringes (Hamilton), reaction vessels, and magnetic stirrers were dried overnight under high vacuum.

Synthesis of 4-Nitrophenyl Phosphorothiodichloridate (3): This compound was prepared as described previously.[26] Yield: 62%. ^{1}H NMR ($CDCl_3$): δ = 7.51 (dd, $J_{H,H}$ = 7.3, $J_{H,H}$ = 2.3 Hz) 8.32 (dd, $J_{H,H}$ = 9.3, $J_{H,H}$ = 1.0 Hz) ppm. ^{31}P NMR ($CDCl_3$): δ = 52.25 ppm. EI-MS: *m*/*z* = 271.3, 273.1. M.p. 51–52 °C (ref.[25] 52 °C).

Synthesis of 4-Nitrophenyl *O*,*O*-Bis(1-hydroxybenzotriazolyl)phosphorothioate (5): 1-Hydroxybenzotriazole hydrate (98% purity, 13% water content, Fluka) was dried by co-evaporation with pyridine (three times), followed by high vacuum drying over P_2O_5 (16 hours). Anhydrous 1-hydroxybenzotriazole (1.2 mmol) was dis-

solved in pyridine (8 mL) and kept overnight with activated 4 Å molecular sieves (beads 8–12 mesh, Aldrich 4 Å, activated at 220 °C for 6 h). A solution of 4-nitrophenyl phosphorothiodichloridate (0.6 mmol) in dioxane (2 mL) was then added dropwise to this solution and the reaction mixture was stirred for 1 h at room temperature. The phosphorylating agent **5** (^{31}P NMR: δ = 66.13 ppm; 95% purity) was used directly for condensation reactions.

Synthesis of Dinucleoside (3′,5′)-Phosphorothioates (2): Nucleosides were dried by co-evaporation with pyridine (twice) and were kept under vacuum overnight. 5′-*O*-DMT-thymidine (**6**; 0.272 g, 0.5 mmol) was dissolved in 2 mL of dioxane and added to the phosphorylating agent (prepared as described above). The reaction mixture was stirred at room temperature for 1 h, and reaction progress was monitored by HP TLC [$CHCl_3$/MeOH, 9:1 (v/v)]. The reaction mixture containing ester **8** was added to thymidine (**2a**) or *N*-benzoyl deoxycytidine (**2b**) dissolved in 2 mL of pyridine (**7**, 0.55 mmol) with *N*-methylimidazole (0.4 g, 180 µL, 5 equiv.). The resulting mixture was stirred at room temp. for 1.5 hours, then quenched with NEt_3 (0.2 mL), concentrated to 1/4 volume, diluted with CH_2Cl_2, and washed with brine (twice) and water. The aqueous layers were additionally extracted with chloroform. Combined organic fractions were dried with $MgSO_4$ and concentrated. Preliminary purification involved a silica-gel chromatography (Kieselgel 60, 230–400 mesh, eluent: chloroform/methanol). Subsequent column chromatography resulted in diastereomerically pure products. First: silica gel 60-230-400 mesh; eluent: chloroform/ethanol 0→5%. Second: silica gel 60 H; eluent: ethyl acetate/methanol (0→10% MeOH).

FAST-2a: HP-TLC [chloroform/ethanol, 9:1(v/v)] R_f = 0.36. ^{1}H NMR ($CDCl_3$): δ = 1.82 (C5-CH_3b), 2.18 (H2′b), 2.37 (H2′′b), 2.46 (H2′a), 2.65 (H2′′a), 2.93 (3′-O*H*), 3.47 (H5′a), 4.13 (H4′b), 4.38 (H4′a), 4.40, 4.42 (H5′,H5′′b), 4.52 (H3′b), 5.53 (H3′a), 6.33 (H1′b), 6.45 (H1′a), 6.82 (arom. C2-*H*), 6.84 (arom., C3-*H*), 7.31 (C6-*H*b), 7.56 (C6-Ha) ppm. ^{31}P NMR ($CDCl_3$): δ = 63.43 ppm. Yield: 23%.

SLOW-2a: HP-TLC [chloroform/ethanol, 9:1 (v/v)] R_f = 0.30. ^{1}H NMR ($CDCl_3$): δ = 1.49 (d, C5-CH_3a), 1.80 (d, C5-CH_3b), 2.25 (m, H2′b), 2.39 (m, H2′′a), 2.45 (m, H2′′b), 2.72 (dd, H2′′a), 3.35 (dd, H5′b), 3.46 (dd, H5′a), 3.53 (s, 3′-O*H*) 4.28 (m, H4′b), 4.33 (m, H4′a), 4.45 (dd, H5′,H5′′b,a), 4.60 (m, H3′b), 5.38 (dd, H3′a), 6.30 (t, H1′b), 6.38 (dd, H1′a), 6.82 (m, arom. C2-*H*), 6.85 (m, arom., C3-*H*) ppm. ^{31}P NMR ($CDCl_3$): δ = 63.65 ppm. Yield: 21%.

FAST-2b: HP-TLC [chloroform/ethanol, 9:1 (v/v)] R_f = 0.27 [EtOAc/MeOH, 95:5 (v/v)]. ^{1}H NMR ($CDCl_3$): δ = 1.49 (C5-CH_3b), 2.19 (H2′b), 2.72 (H2′′b), 2.447 (H2′a), 2.64 (H2′′a), 2.93 (3′-O*H*), 3.50 (H5′a), 4.13 (H4′b), 4.24 (H4′), 4.39 (H5′,H5′′b), 4.48 (H3′b), 5.35 (H3′a), 6.28 (H1′b), 6.40 (H1′a), 6.82 (arom., C2-*H*), 6.84 (arom., C3-*H*), 7.31 (C6-*H*b), 7.56 (C6-Ha) ppm. ^{31}P NMR ($CDCl_3$): δ = 60.98 ppm. MS FAB: *m*/*z* = 1073.8 [M – H], 1075.9 [M + H] (calcd.: 1074.3). Yield: 27%.

SLOW-2b: HP-TLC [chloroform/ethanol, 9:1 (v/v)] R_f = 0.20. ^{1}H NMR ($CDCl_3$): δ = 1.52 (d, C5-CH_3a), 2.28 (m, H2′b), 2.36 (m, H2′′a), 2.69 (dd, H2′′b), 2.82 (m, H2′′b), 3.33 (m, H5′a), 3.47(dd, H5′a), 4.22 (m, H4′a), 4.31 (m, H4′b), 4.51 (m, H5′, H5′′b), 4.59 (m, H3′b), 5.28 (m, H3′a), 6.28 (m, H1′b), 6.35 (m, H1′a). ^{31}P NMR ($CDCl_3$): δ = 61.70 ppm. Yield: 25%.

Stereochemistry of Removal of the 4-Nitrophenyl Protecting Group: Each diastereomer of dinucleotide **2a** (0.066 M, 0.033 mmol) was dissolved in dioxane/water (1:2 v/v; 0.5 mL), and a solution of 1,1,3,3-tetramethylguanidinium oximate (0.6 M, 0.3 mmol, 0.05 mL) was added. The resulting homogeneous solution was stirred at room temperature for 12–22 h, and then evaporated under reduced pressure. The reaction progress was monitored by HP TLC (20% MeOH in $CHCl_3$) and ^{31}P NMR spectroscopy. The reaction was quenched by dilution of the reaction mixture with 500 µL of a 1:1 (v/v) solution of CH_3CN/0.1 M TEAB buffer (pH 7). ^{31}P NMR yield > 98%. Crude products **10a** were analyzed by MALDI MS: *m*/*z* = 863 [M + H$^+$] (calcd: 863). RP HPLC (LC Talk, LDC Analytical) purification of **10a** was carried out: column ECONOSIL C18 5µ, Buffer A: 0.1 M TEAB, pH 7; Buffer B: 40% MeCN in buffer A; flow: 1 mL min^{-1}, linear gradient: 85% A (0.1 M TEAB) to 100% B over 20 min. (FAST-**10a**: R_t = 19.13 min; SLOW-**10a**: R_t = 19.33 min). Pure fractions of phosphodiesters **10a** (DMT-on) were analyzed by MALDI TOF *m*/*z* = 863 [M + H$^+$] (for experiments carried out in H_2O and [^{18}O]H_2O) ppm. ^{31}P NMR (of reaction mixture): FAST-**10a**: δ = 57.5 ppm; SLOW-**10a**: δ = 57.21 ppm.

Enzymatic Digestion: Each diastereomer **10** (**a** and **b**) (1 mg) was detritylated, and the fully deprotected dinucleotide **12** (**a** and **b**) was purified by HPLC (LC Talk, LDC Analytical); column ECONOSIL C18 5µ, Buffer A: 0.1 M TEAB, pH 7; Buffer B: 40% MeCN in buffer A; flow: 1 mL min^{-1}, linear gradient: 100% A (0.1 M TEAB) to 50% B over 20 min. FAST-**12a**: R_t = 15.33 min; SLOW-**12a**: R_t = 15.21 min (yield 82%). FAST-**12b**: R_t = 17.03 min; SLOW-**12b**: R_t = 17.69 min (yield 84%).

a) 0.1 OD of each diastereomer **12** (**a** and **b**) was treated with 1 µg of nuclease P1 (from *Penicillium citricum*, Boehringer GmbH, no. 10432620-12) in 1 µL of buffer 10X (100 mM tris-HCl, pH 7.2; 1.0 mM $ZnCl_2$) and 8 µL of H_2O at room temperature. Aliquots (2 µL) were taken after 1 h, 2 h, and 4 h and analyzed by HPLC with the same gradient as described below.

b) 0.1 OD of each diastereomer **12** (**a** and **b**) was treated with 0.5 µg of phosphodiesterase Capd (*Crotalus adamanteus* venom, Amersham Life Science, no. 108044) in 1 µL of buffer 10X (25 mM Tris-HCl pH 8.5; 5 mM $MgCl_2$) and 8.5 µL of H_2O at 37 °C. The reaction times were 1 h, 2 h, 4 h, and 12 h. Products were analyzed by HPLC.

Derivatization of Long-Chain Alkylamine CPG Support with Dinucleoside Phosphorothioate: Succinylated LCAA-CPG (200 mg, CPG Product no. CPG 00500A), partially protected dinucleoside (3′,5′)-phosphorothioate (55 mg, SLOW-**2**), and DMAP (12 mg) were dried overnight at high vacuum, followed by addition of 1-(3-dimethylaminopropyl) ethylcarbodiimide (DEC, 92 mg, 0.05 mM), pyridine (2 mL), and triethylamine (60 µL). This reaction mixture was shaken at 28 °C for 24 h. After the reaction was complete (trityl assay), the CPG was filtered off, washed with small portions of MeOH, CH_2Cl_2 and MeCN, and dried under vacuum. The functionalized support was suspended in acetic anhydride capping reagent, stirred for 2 h at room temperature, filtered off, and washed with CH_2Cl_2. The support was dried at high vacuum and stored at –20 °C. The nucleotide loading (28.6 µmol g^{-1}) was determined by trityl analysis. Yield: 195 mg (95%).

Synthesis of Chimeric Oligonucleotides. a) Synthesis of Chimeric Oligonucleotides with 3′-End Modification: Solid support derivatized with **2b** as described above was placed in a 1-µmol column and attached to the DNA synthesizer. Subsequent couplings were routinely performed with commercially available nucleoside 3′-phosphoramidites according to a standard protocol for the phosphoramidite method of oligonucleotide synthesis. Standard RP HPLC isolation provided pure **14**.

b) Synthesis of Chimeric Oligonucleotides with 3′- and 5′-Internucleotide Phosphorothioate Modification: Solid support derivatized with **2b** as described above was placed in a 1-µmol column and attached

to the DNA synthesizer. For the synthesis of compounds **15** and **20** the subsequent couplings were routinely performed with commercially available nucleoside 3′-phosphoramidites according to a standard protocol for the phosphoramidite method of oligonucleotide synthesis. 5′-*O*-(4,4′-Dimethoxytrityl)-2′-thymidyl (3′,5′)-3′-*O*-[(4-nitrophenyl)(diisopropylamino)phosphoramidityl]-2′-N^4-benzoyl-2′-deoxycytidine) phosphorothioate (**9b,** vide infra) was dried overnight, dissolved in MeCN (0.08 M), and used as a reagent for coupling at the synthesizer. The coupling time was 10 min; no other steps were modified or changed. Average yield of coupling (calculated from the trityl assay): 98%.

c) Synthesis of Chimeric Oligonucleotides with "Internal" or 5′-Terminal Modification: Diastereomerically enriched isomer SLOW-**2b** (R_P/S_P: 95:5; 20 mg) was co-evaporated with dry MeCN (three times), dried overnight under high vacuum, and dissolved in dry MeCN (300 μL). 2-Cyanoethyl tetraisopropylphosphordiamidite (6.5 μL) was added to this solution, followed by a 0.5 M solution of 5-ethylthiotetrazol (25 μL). The reaction mixture was stirred for 15 min and used without further purification [^{31}P NMR: δ = 149.26, 148.73, 60.13 ppm for (R_P)-**9b**] for the coupling reaction on a solid support. A 1-μmol column was used for preparation of compounds **16**–**19**. Partially synthesized oligonucleotides **18** and **10** were detached from the DNA synthesizer after completion of the unmodified sequences by the phosphoramidite method, and manual coupling with **9b** was performed; reaction time: 10 min. The consecutive coupling with five nucleosides in the case of compounds **16**–**19** was performed automatically by phosphoramidite chemistry after attachment of the column to the DNA synthesizer. After the synthesis of compounds **16**–**19** was accomplished, the DMT group was removed, and the column was detached from the DNA synthesizer. Average yield of coupling (calculated from the trityl assay): 98%.

d) Deprotection: The solid support-bound oligonucleotides were treated with a 0.4 M solution of *syn*-4-nitrobenzaldoxime and 1,1,3,3-tetramethylguanidine in dioxane/water (1 mL) for 1 h, followed by treatment with NH_4OH at 55 °C for 16 h. Purification of the oligonucleotides was performed by HPLC (RP-C18 SUPELCOSIL, 1 mL min^{-1}, gradient 2–20% MeCN, 0.1 M TEAB buffer, pH 7.5). Their purity was additionally confirmed by gel electrophoresis. Results of MS analysis are included in Table 1.

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