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New approach to the synthesis of oligodeoxyribonucleotides modified with phosphorothioates of predetermined sense of *P*-chirality

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Abstract—Appropriately protected, diastereomerically pure dinucleoside phosphorothioates, obtained by the stereocontrolled oxathiaphospholane method, were *S*-alkylated with 2-nitrobenzyl bromide and then converted into their 3′-*O*-phosphoramidites. The corresponding *S*-protected dinucleotide building blocks were successfully used for the synthesis of oligonucleotides containing *P*-stereodefined phosphorothioate bonds at preselected positions.

Oligonucleotides containing, at selected position(s), internucleotide phosphorothioate linkages of a predetermined sense of *P*-chirality (*PS*-oligos) are attractive as probes in mechanistic studies of various enzymes, particularly endo- and exonucleases.[1] They are accessible via the phosphoramidite approach[2] where at selected steps the routine oxidation of intermediary phosphites with iodine–water is replaced by sulfurization.[3] However, the resulting *PS*-oligos are a mixture of diastereoisomers that have to be separated, preferably by RP-HPLC.[4] In numerous cases such an attempted separation is not effective.[5] Pure diastereoisomers of *PS*-oligos modified with a single phosphorothioate moiety are accessible via preparation of dinucleoside phosphorothioates containing an *O*-protected phosphorothioate function, their separation at the dinucleotide stage, 3′-*O*-activation with phosphoramidite and subsequent incorporation into the oligonucleotide chain at a selected position.[6] Appropriate protection of the phosphorothioate function is necessary since unprotected phosphorothioate diesters are converted effectively into phosphates under treatment with iodine–water, which is unavoidable during chain elongation. Attention has to be paid to proper selection of the phosphorothioate-protecting group which, after completion of the synthesis of the desired oligonucleotide, has to be removed without loss of sulfur and with known stereochemistry.[6,7]

Recently, we demonstrated that dinucleoside-(3′,5′)-*O*-*p*-nitrophenyl phosphorothioates can be used as building blocks due to adventitious separation of the diastereomers (this is not always the case for dinucleoside (3′,5′)-phosphorothioate *O*-alkyl triesters) and clean stereoinvertive removal of the *p*-nitrophenyl protection by means of *syn*-4-nitrobenzaldoxime/1,1,3,3-tetramethylguanidine.[8]

Here, we present an alternative approach based upon the stereocontrolled synthesis of dinucleoside phosphorothioates using the oxathiaphospholane method (OTP) developed in our laboratory.[9] Suitably protected 3′-*O*-(2-thio-1,3,2-oxathiaphospholane) nucleosides **1** were afforded from the reaction of the 3′-OH group of 5′-DMT-*O*-protected nucleosides with *N*,*N*-diisopropylamino-1,3,2-oxathiaphospholane, followed by sulfurization and chromatographic separation into pure *P*-diastereoisomers.[10] The yields from the DBU-promoted ring opening condensations with 5′-OH-nucleosides were excellent and the stereospecificity was above 99.5%. Therefore, the corresponding *P*-chiral dinucleoside phosphorothioates **3** of high diastereoisomeric purity are now available. Diastereomerically pure

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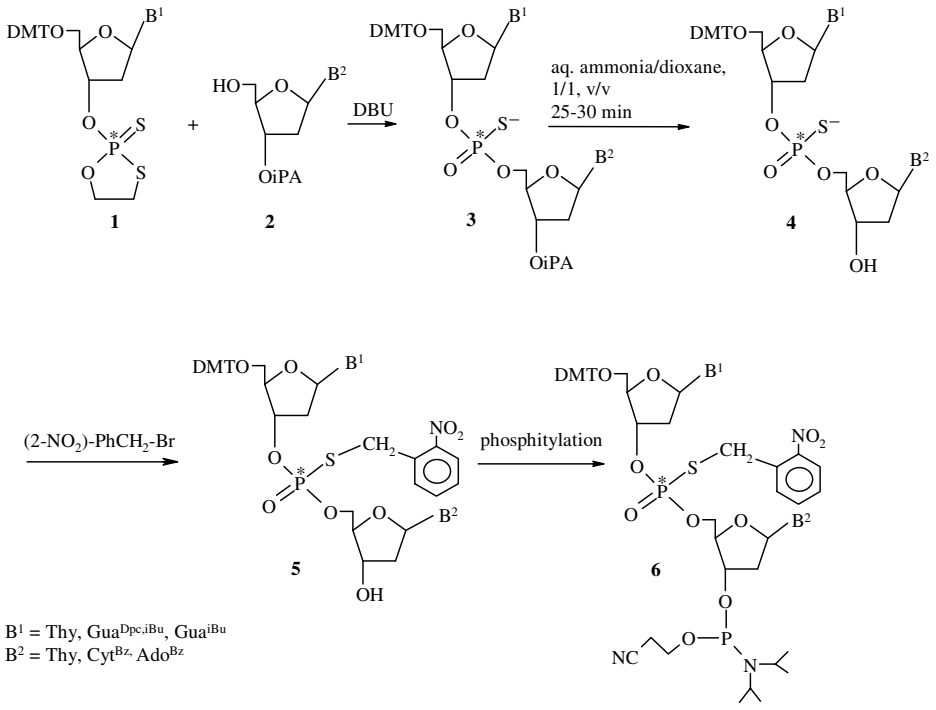

Scheme 1. Synthesis of *S*-protected *P*-stereodefined dinucleoside units.

phosphorothioate dimers are *S*-alkylated and converted into building blocks via 3′-*O*-deprotection followed by activation with phosphoramidite. The obvious advantage of the present method results from the fact that only four oxathiaphospholane monomers **1** have to be separated into their R_P and S_P diastereoisomers in order to access all 16 diastereomerically pure common dinucleotides **3**. As depicted in Scheme 1, diastereomerically pure compounds **1** were condensed with appropriately protected nucleosides **2** providing the dinucleoside phosphorothioates **3**. As established earlier, the slow-migrating diastereoisomers of **1** furnished the dinucleotides **3** of R_P configuration (Scheme 1).[11]

Removal of the *iso*-propoxyacetyl protecting group[12] from the 3′-OH position of **3** was performed using 28% aqueous ammonia/dioxane mixture (1/1, v/v) at room temperature in a time-controlled reaction, monitored by TLC. Typically, the reaction was terminated after 25–30 min by removal of ammonia under slightly reduced pressure. Dimers **4** were obtained in preparative yields >80%. Extended reaction times (up to 120 min) led to removal of the diphenylcarbamoyl (Dpc) protecting group from *O*-6 of the guanine nucleobase. Before the conversion of dimers **4** into building blocks **6**, the sulfur atom of the internucleotide phosphorothioate function was alkylated with 2-nitrobenzyl bromide,[13] which was selected among several tested chloro- and nitrobenzyl halides, as the most suitable *S*-protecting agent. The 2-nitrobenzyl protecting group was found to be stable during oligonucleotide synthesis and could be efficiently and stereospecifically removed by means of thiophenolate anion[7] before aqueous ammonia-assisted cleavage of the oligonucleotide from the solid support. The alkylation reaction was optimized for the diastereomeric mixture of dithymidine phosphorothioate **4** ($B^1 = B^2 =$ Thy). Physical data and yields of **5** are given in Table 1. Routine phosphitylation of **5** with 2-cyanoethyl bis-(*N*,*N*-diisopropyl)phosphoramidite/1-*H*-tetrazole in acetonitrile,[2] followed by silica gel chromatographic purification, afforded the desired products **6**, 80–90% yields, which were ready to use as building blocks for standard phosphoramidite synthesis of oligonucleotides.

Table 1. Spectral and chromatographic characteristics of *S*-protected dinucleoside phosphorothioates **5** (Scheme 1)

No.	*P*-conf.	B^1	B^2	MALDI-TOF	^{31}P NMR (δ, ppm)	Yield **4** → **6** (%)	R_f ($CHCl_3$/MeOH, 9/1)[a]
5	Mix	Thy	Thy	999.4[b] $[M+H]^+$	29.00; 29.18	91	0.31
5a	R_P	$Gua^{dpc,\ i\text{-}Bu}$	Cyt^{Bz}	1380.3 (1379)	26.59	66	0.76
5b	S_P	$Gua^{dpc,\ i\text{-}Bu}$	Cyt^{Bz}	1380.3 (1379)	26.96	57	0.71
5c	R_P	Thy	Ado^{Bz}	1111.9 (1111)	27.83	84	0.30
5d	S_P	Thy	Ado^{Bz}	1111.7 (1111)	27.96	82	0.33
5a′	R_P	$Gua^{i\text{-}Bu}$	Cyt^{Bz}	1182.8 (1182)	27.29	75	0.52
5b′	S_P	$Gua^{i\text{-}Bu}$	Cyt^{Bz}	1182.8 (1182)	27.65	79	0.45

[a] Thin layer chromatography, silica gel 60 F_{254} (Merck).
[b] FAB MS.

Table 2. Sequences and characteristics of oligonucleotide phosphorothioates **7**–**15**

PS-ODN No.	Sequence 5′ → 3′	*P*-conf.	HPLC[a] DMT-ON t_R (min)[b]	MALDI-TOF DMT-OFF		HPLC[a] DMT-OFF t_R (min)[b]	Yield (A_{260})
				Calcd	Exp.		
7	GACTTPSTCGAT	Mix	20.42	3032	3031	19.61	15.0
8	AGGGTTGAGGCTAGpsCTACAACGATCATCTGT	R_P	19.40	9586	9591	21.14	11.3
9	AGGGTTGAGGCTAGpsCTACAACGATCATCTGT	S_P	21.01	9586	9592	20.97	27.0
10	AGGGTTGAGGpsCTAGCTACAACGATCATCTGT	R_P	19.51	9586	9583	20.85	14.3
11	AGGGTTGAGGpsCTAGCTACAACGATCATCTGT	S_P	21.62	9586	9580	20.70	12.0
12	AGGGTTGAGGCTpsAGCTACAACGATCATCTGT	R_P	19.91	9586	9591	16.21	7.2
13	AGGGTTGAGGCTpsAGCTACAACGATCATCTGT	S_P	21.13	9586	9589	14.79	10.0
14	AGGGTTGAGGCTAGCTpsACAACGATCATCTGT	R_P	19.54	9586	9577	14.83	7.0
15	AGGGTTGAGGCTAGCTpsACAACGATCATCTGT	S_P	19.39	9586	9585	14.79	15.3

[a] Hypersil BDS C-18 column, 4 × 250 mm, eluting buffers—A: 1 M triethylammonium bicarbonate, pH 7.5; B: 40% acetonitrile/60% A; DMT-ON gradient—B: 15% → 100% in 0 → 20 min; B: 100% in 20 → 35 min; DMT-OFF gradient—B: 0% → 50% in 0 → 20 min; 50% → 100% in 20 → 24 min; 100% in 24 → 32 min; 100% → 0% in 32–36 min.

[b] Purity of oligomers was >98%.

S-Protected *P*-chiral phosphorothioate dinucleosides were successfully introduced into the DNA chain of the model octamer **7** and eight 31-mers (**8**–**15**). These later constructs containing the 15-nt consensus sequence of a catalytic domain of deoxyribozyme 10–23[14] and substrate recognition arms complementary to the selected region (from 1801 to 1817) of the human aspartyl protease Asp2 (BACE) mRNA[15] were designed for mechanistic studies on the mode of action of the deoxyribozyme 10–23.

The syntheses of *PS*-modified DNAzymes were performed on 1 μmol scale according to the routine protocol with the coupling time for the modified units extended to 120 s. The coupling yields for the incorporation of the building blocks **6** were in the range of 90–99% (DMT cation assay), depending on the incorporated dimer. After the syntheses were complete, the oligonucleotides were treated with 1 M piperidine/acetonitrile solution to remove the 2-cyanoethyl protecting groups from the phosphate functions. This deprotection protocol was designed to avoid *P*-S^- center modification with the acrylonitrile released during phosphate deprotection.[16,17] Subsequently, the polymer-bound oligonucleotides were treated for a short time with a mixture of dioxane/triethylamine/thiophenol (1/2/2, v/v/v). HPLC analysis confirming that treatment of the oligomers with thiophenolate for 5 min resulted in effective removal of the *S*-protecting group from the phosphorothioate function.

It should be pointed out that in the case of Gua^{Dpc} derivatives, this procedure did not result in substitution of the *O*-diphenylcarbamoyl moiety with a *S*-Ph residue, though such a transformation was observed when the guanosine protected with Dpc was exposed to thiophenol for 2–3 h.[18]

The oligomers with deprotected internucleotide bonds were further treated with 28% aqueous ammonia, followed by RP-HPLC (DMT-ON) purification, acidic removal of the DMT group, and a final RP-HPLC purification (DMT-OFF).[4] The purity of the oligomers, determined by reverse phase HPLC and polyacrylamide gel electrophoresis (20% polyacrylamide/7 M urea) (data not shown) was >98%. The structure of the oligomers was confirmed by MALDI-TOF mass spectrometry (Table 2). The absolute configuration at the phosphorus atoms in the phosphorothioate internucleotide bonds of oligonucleotides **8**–**15** was confirmed enzymatically using snake venom phosphodiesterase (PDE I) and 3′-endonuclease P1 (nP1) (see Supplementary data).[19,20]

The methodology presented in this letters demonstrates that *S*-protected diastereomerically pure dinucleoside phosphorothioates **5** can be prepared effectively from nucleoside oxathiaphospholanes **1**. These, after *S*-protection and 3′-OH activation, are introduced into the DNA chain by solid-phase phosphoramidite methodology. This approach permits the preparation of *PS*-deoxyribozymes with a predetermined sense of chirality at the phosphorus atom of the internucleotide phosphorothioate function at each selected internucleotide position of the catalytic core. Since some of the catalytic nucleic acids facilitate magnesium-assisted hydrolysis of phosphodiester bonds in complementary RNA, their activity is expected to be modulated by thio-substitutions in selected positions of the catalytic core. In subsequent studies, the stereochemical aspects of reactions catalyzed by chimeric 10–23 DNAzymes containing a single *PS* linkage of R_P- and S_P-configuration will be evaluated. In addition, future studies will be targeted at the preparation of diastereomerically pure di-*ribo* nucleoside phosphorothioates and their incorporation into the RNA chain. Various sources report that such *P*-chiral oligoribonucleotides containing phosphorothioate functions are indispensable tools for evaluation of the stereochemical outcome of RNA-binding[21] and RNA-cleaving proteins.[19]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2005.07.158. The following Supplementary data is available in electronic version: detailed synthetic procedures for **1**–**6**; spectral data of oxathiaphospholanes **1** and dimers **3** and **4**, chromatographic profiles and MALDI-TOF mass spectra of oligomer **14** and HPLC analysis of the products of enzymatic digestion of **12** and **13**.

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