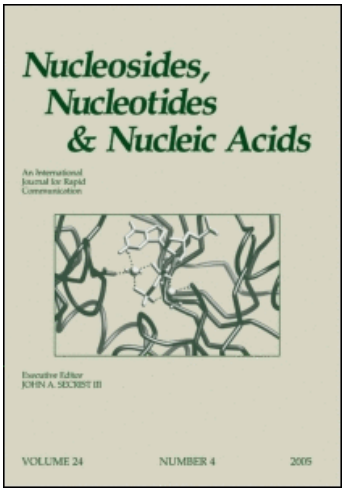

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Understanding High Diastereomeric Discrimination in Formation of Oligoribonucleotide Phosphorothioate Linkages: The First Study of pKa-Dependent Activation in Solid-Supported Coupling of 2′-*O*-Substituted Ribonucleoside Phosphoramidites

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ABSTRACT

Activation of 2′-*O*-substituted ribonucleoside phosphoramidites with various activators during solid-supported synthesis of phosphorothioate oligonucleotides was studied. The Rp:Sp diastereomeric composition of resulting phosphorothioate linkage dependent on pKa of activator utilized for coupling.

Key Words: Coupling; Stereochemistry; Activators; Oligomerization; Solid support; pKa; Phosphoramidite; Phosphorothioates.

In the past few years 2′-*O*-alkyl (especially 2′-*O*-methoxyethyl and 2′-*O*-methyl) oligoribonucleotides have emerged as promising modifications, showing promise as therapeutic agents for treatment of multiple diseases through an antisense mechanism of action. Synthesis of oligonucleotides is currently a well-established procedure that is carried out automatically on solid-phase using phosphoramidite

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chemistry. This reaction usually requires an activator with a desired pka to undergo quantitative, fast and clean reaction. A wide variety of promotors including 5-(ethylthio)-1*H*-tetrazole (ETT), imidazolium triflate Im Tf), pyridinium trifluoroacetate (PTFA) 4,5-dicyanoimidazole (DCI) have been reported to date. Efficient sulfurization is then performed using phenylacetyl disulfide (PADS) or 3*H*-1,2-Benzodithiol-3-one 1,1-dioxide (Beaucage reagent).

An unappreciated problem concerning the use of phosphorothioate oligonucleotides in antisense-based therapy is their polydiastereoisomerism. Extensive study using phosphorothioate oligonucleotides with defined P-stereochemistry is warranted to gain insights on the impact of diastereoisomerism on antisense activity, both in in vitro, and in in vivo systems. For this purpose, a practical synthetic approach is required that would provide access to stereospecific/stereoselective Rp or Sp phosphorothioate oligonucleotides. Stec's oxathiaphospholane approach is the most advanced method to date, even though other methods have also been reported in literature. However, this approach has not been applied for large-scale synthesis of antisense drugs. We describe here a practical solid-phase approach towards stereoselective synthesis of 2′-*O*-alkyl substituted PS oligonucleotides using pKa of activator as a handle to control the diastereoselectivity. To the best of our knowledge, control of pKa of activator to modulate the diastereoselectivity has not been described in the literature.

EXPERIMENTAL DESIGN

The experimental approach chosen was to synthesize oligonucleotides about 5-mer in length in which a single phosphorus center was replaced by a phosphorothioate linkage. Oligonucleotide syntheses were performed on a Pharmacia Oligo-Pilot II DNA/RNA synthesizer by the phosphoramidite method (Tables 1 and 2). Phosphate diester linkages were incorporated via oxidation of phosphite triesters using a 15% (v/v) solution of *tert*-butyl hydroperoxide in CH_3CN for 15 min. Phosphorothioate linkages were introduced by sulfurization with a 0.2 M solution of phenylacetyl disulfide in CH_3CN/3-picoline (1:1 v/v) for a contact time of 2 min. Final detritylation at the end of synthesis was performed on column before deprotection and cleavage. After standard ammonia cleavage the products were filtered and the filtrate evaporated under reduced pressure. The residue was dissolved in D_2O (0.5 mL) and transferred to a 5 mm NMR tube for analysis. Good separation of two phosphorothioate diastereomer signals was observed and a minimum signal-to-noise ratio of 200 was obtained for all samples analyzed.

ASSIGNMENT OF STEREOCHEMISTRY

The Rp and Sp configurations of 2′-*O*-methoxyethyl modified phosphorothioate linkages were tentatively assigned based on an analogous molecule viz. 2′-*O*-methyl oligoribonucleotide phosphorothioates. Thus, the upfield shift in ^{31}P NMR signal of 2′-*O*-methoxyethyl modified phosphorothioate diester linkage was assigned the Sp configuration and the downfield shift signal was assigned the Rp configuration. Work on assigning the absolute configuration is under progress.

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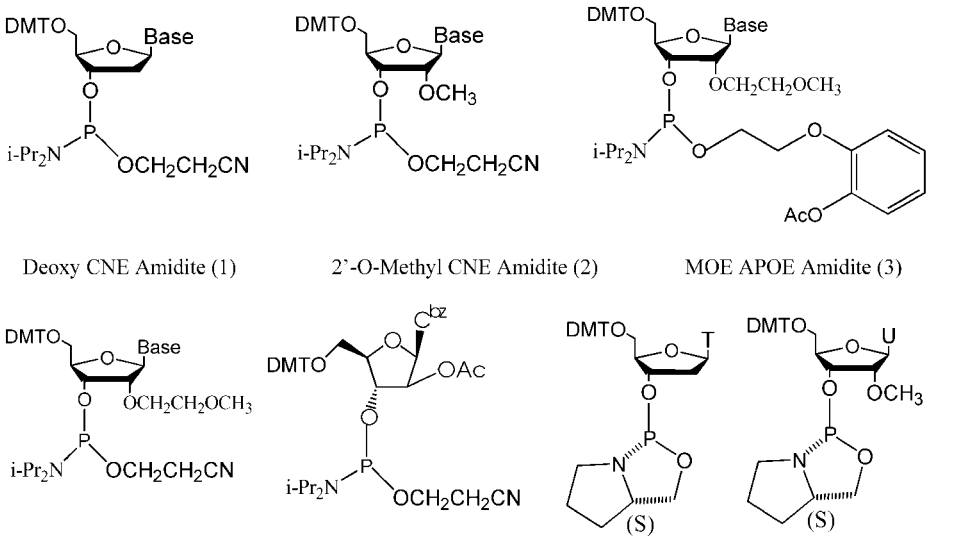

Figure 1. Structures of various amidites used during the investigation.

SEPARATION OF PHOSPHORAMIDITE DIASTEREOMERS

The two diastereoisomers of commercially available 5′-*O*-DMT-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropyl phosphoramidite of 5-methyl-2′-*O*-methoxyethyluridine were separated cleanly using flash silica gel chromatography on 15 g scale and used for investigation. Coupling of each diastereomer under above coupling conditions with different activators (viz. ETT, 1H-tetrazole, PTFA, DCI and ImTf) did not show any significant difference compared to racemic mixture (data shown in table above; oligo #1) even though they also showed similar pka-based diastereomeric ratio trend thereby indicating that individual isomers do not play any role in the above observed phenomenon.

Table 1. Synthesis of various oligonucleotides for stereochemical evaluation.

Oligo No.	Oligonucleotide synthesized	Amidite used for coupling
1	5′-MOE U^{me} ps TTTT	4
2	5′-MOE U^{me} ps MOE U^{me} TTT	4
3	5′-MOE U^{me} ps TTTT	3
4	5′-dC ps MOE U^{me} TTT	1
5	5′-(2′-*O*-Me) U^{me} ps TTTT	2
6	5′-*ara*-C ps TTTT	5
7	5′-T ps TTTT	6
8	5′-(2′-*O*-Me) U^{m} ps TTTT	7

Table 2. Coupling of different amidites with various activators.

Oligo No.	Synthesis scale (μmole)	Activator used	Diastereomeric ratio (Rp/Sp)
1	172	ETT	69.61/30.39
1	167	1*H*-Tetrazole	57.68/42.32
1	162	PTFA	51.19/48.81
1	171	DCI	45.16/54.84
1	159	Im Tf	27.51/72.49
2	176	ETT	69.00/31.00
2	166	1*H*-Tetrazole	56.08/43.92
2	168	PTFA	49.07/50.93
2	169	DCI	45.44/54.56
2	168	Im Tf	25.82/74.18
3	155	ETT	73.09/26.91
3	165	1*H*-Tetrazole	63.13/36.87
3	92	PTFA	54.83/45.17
3	163	DCI	53.14/46.86
3	157	Im Tf	35.49/64.51
4	180	ETT	54.33/45.67
4	178	1*H*-Tetrazole	55.28/44.72
4	102	PTFA	55.63/44.37
4	166	DCI	54.51/45.49
4	84	Im Tf	54.87/45.13
5	155	ETT	68.42/31.58
5	165	1*H*-Tetrazole	57.65/42.35
5	163	PTFA	53.64/46.36
5	163	DCI	44.12/55.88
5	157	Im Tf	29.57/70.43
6	105	ETT	57.82/42.18
6	106	1*H*-Tetrazole	57.57/42.43
6	101	PTFA	57.41/42.59
6	94	DCI	57.84/42.16
6	105	Im Tf	57.98/42.02
7	118	ETT	26.29/73.71
7	120	1*H*-Tetrazole	26.17/73.83
7	130	PTFA	26.76/73.24
7	132	DCI	25.55/74.45
7	122	Im Tf	25.86/74.14
8	118	ETT	11.38/88.62
8	152	1*H*-Tetrazole	11.48/88.52
8	201	PTFA	11.56/88.44
8	131	DCI	11.52/88.48
8	116	Im Tf	12.32/87.68

CONCLUSION

Activation of 2′-*O*-substituted ribonucleoside phosphoramidites with various activators during solid-supported synthesis of phosphorothioate oligonucleotides

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was studied. The Rp : Sp diastereomeric composition of resulting phosphorothioate linkages depended on pKa of activator utilized for coupling. Relatively more acidic activators such as 5-(ethylthio)-1*H*-tetrazole biased the linkage towards Rp-diastereomer whereas less acidic activators like imidazolium triflate afforded more of Sp-diastereomer. Variations in 2′-*O*-substitution on ribose sugar, and nature of nucleosides (pyrimidines, purines, deoxyribose, 2′-*O*-alkylribose) on the 5′-end of oligonucleotide attached to solid support undergoing coupling had no measurable impact on diastereoselectivity of phosphorothioate linkages. Change of *O*-protecting group on phosphorous had minor impact on the outcome. In case of salts of imidazoles, change of cation had little impact whereas change of anion had measurable impact on the ratio of Rp:Sp diastereomers. In the case of deoxyribose amidite synthons, coupling to 5′-hydroxyl of deoxyribonucleoside or 2′-*O*-(2-methoxyethyl)ribonucleoside on solid support was not influenced by activator pKa and afforded nearly 1:1 diastereomeric mixtures. Interestingly, coupling of 2′-*ara*-cytidine, which contains 2′-*O*-substitution but in the *ara*-configuration was not influenced by activator pKa demonstrating that 2′-*ribo* configuration is crucial for the observed phenomenon. Finally, coupling of conformationally restricted bicyclic oxazaphospholidine amidite of either thymidine or 2′-*O*-methyluridine that has been designed to slow down the rate of p-epimerization by increasing the energy barrier to pseudo rotation did not exhibit any variation in diastereoselectivity with respect to activators, affording stereoselectively the Sp diastereomer in all cases.

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