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Stereocontrolled Synthesis of LNA Dinucleoside Phosphorothioate by the Oxathiaphospholane Approach

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Abstract—The application of the oxathiaphospholane approach for the stereocontrolled synthesis of LNA dinucleoside phosphorothioate is described. The reaction of ring opening condensation proceeds in CH_3CN solution in high yield and with over 96% stereoselectivity. One of diastereomers of LNA dinucleoside phosphorothioate (presumably R_P) was found to be readily digested by *svPDE*.

Locked nucleic acids (LNAs) (**1**, X = O, Fig. 1), defined as oligonucleotides containing one or more 2′-*O*-4′-*C*-methylene-β-D-ribofuranosyl nucleotide monomer(s), have been introduced as a new class of conformationally restricted oligonucleotide analogues.[1] LNAs were shown to form duplexes of unprecedented thermal stability with complementary DNA and RNA,[1] and were found to be stable against snake venom phosphodiesterase[1] as well as nucleases present in blood serum and cell extracts.[2] Furthermore, partly modified LNA when complexed to complementary RNA was shown to activate *Escherichia coli* RNase H, thus kindling hopes for application in antisense research/therapy.[2]

Recently, phosphorothioate LNA analogues (**1**, X = S, Fig. 1) were prepared by phosphoramidite/sulphurization methodology.[3] It was shown that the incorporation of even three LNA nucleosides into oligo(nucleoside phosphorothioate) (9-mer) greatly increased thermal stability of duplexes formed with complementary DNA or RNA (as compared to phosphorothioate modified oligonucleotides without LNA monomers).[3] However, no reference was made to the stereochemistry of internucleotide phosphorothioate moieties. In this paper, we wish to present preliminary results obtained on the application of the oxathiaphospholane approach to the stereospecific formation of internucleotide phosphorothioate bond involving LNA nucleosides. This approach was previously developed as an unique methodology allowing for the stereocontrolled synthesis of oligo(nucleoside phosphorothioate)s with a predetermined chirality at each internucleotide phosphorothioate center.[4–6]

For model stereochemical studies the 5′-*O*-protected thymine LNA nucleoside **2** was synthesized[7] and transformed into corresponding 3′-*O*-(2-thio-'spiro'-4,4-pentamethylene-1,3,2-oxathiaphospholane) derivative **3** by reaction with 'spiro'-phosphorochloridite **4**[6] followed by sulphurization with elemental sulphur (Scheme 1).[8]

Compound **3** was isolated in 90% yield by flash column chromatography on silica gel (elution with $CHCl_3$) as a mixture of diastereomers and was characterized by FAB MS and elemental analysis.[9] The diastereomers were further separated by column chromatography on Kieselgel 60H (Merck) with benzene–ethyl acetate as elut-

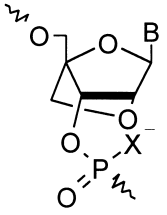

Figure 1. **1**, LNA: X = O. Phosphorothioate LNA: X = S.

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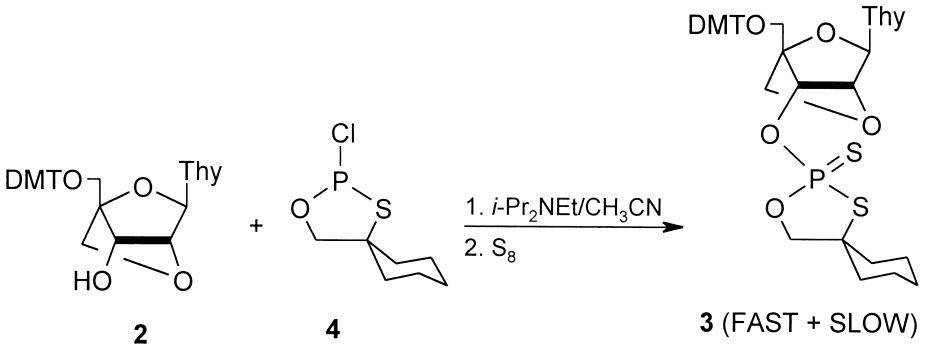


Scheme 1. The synthesis of oxathiaphospholane LNA synthon **3**.

ing system, and were designated as FAST and SLOW because of different chromatographic mobility. Selected spectroscopic data of separated diastereomers FAST-**3** and SLOW-**3** are given in Table 1.

Partially separated diastereomers of **3** were reacted with N^4-benzoyl-3′-*O*-acetyldeoxycytidine **5** in acetonitrile solution under conditions of oxathiaphospholane synthesis[4] in the presence of 1,4-diazabicyclo[5.4.0]undec-7-ene (DBU) as activator. Thus, **3** (100 mg, 12.8 mmol) and **5** (48 mg, 12.8 mmol) were combined, dried overnight at high vacuum and dissolved under argon in 700 μL of dry CH_3CN (water content 10.1 ppm as determined by coulometric titration, 654 KF Coulometer, Metrohm). DBU (20 μL, 12.9 mmol) was added and the resulting solution was stirred for 10 min at rt. After evaporation of the solvent, the residue was dissolved in 50% aq acetic acid (4 mL), kept for 2 h at rt, and evaporated. The residue was dissolved in 25% aq ammonia (7 mL), and kept overnight at 55 °C in a tightly closed vessel. After evaporation of ammonia the product of reaction was analyzed by ^{31}P NMR. The major product of the reaction was chimeric LNA dinucleoside phosphorothioate **6** (94%; isomer **6a**, δ 56.00 ppm, and isomer **6b**, δ 56.77 ppm) accompanied by small amounts of the corresponding LNA 3′-monophosphorothioate **7**[10] (6%, δ 47.52 ppm) (Scheme 2).

Starting from the LNA substrate **3** of isomers ratio FAST/SLOW = 92/8, the product **6** with isomer ratio **6a**/**6b** = 90.5/9.5 was obtained. Similarly, LNA oxathiaphospholane **3** FAST/SLOW = 15/85 gave LNA dinucleotide **6** with isomer composition **6a**/**6b** = 18/82. The products were further isolated by preparative RP HPLC.[11] The physicochemical data of purified diastereomers of **6** are listed in Table 2.

From the aforementioned results it may be concluded that the formation of internucleotide phosphorothioate linkage involving LNA nucleoside via oxathiaphospholane route proceeds highly efficiently, in a stereocontrolled way, with the stereoselectivity 96.5–98.5%. In order to assign absolute configuration at phosphorus in diastereomeric LNA dinucleotides **6** some enzymatic

Table 1. Physicochemical characteristics of FAST/SLOW isomers of **3**

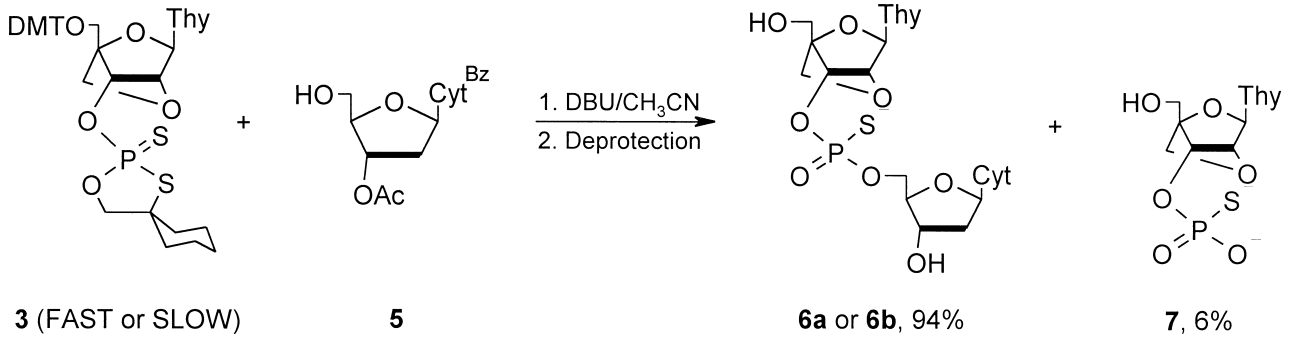
Isomer	TLC[a] (R_f)	^{31}P NMR[b] δ (ppm)	^{1}H NMR[c] δ (ppm)		
			2′H	3′H	1′H
FAST-**3**	0.77	106.01	4.76 (s)	5.16 (d)	5.71 (s)
SLOW-**3**	0.70	107.27	4.61 (s)	5.29 (d)	5.73 (s)

[a]Benzene-ethyl acetate (1:1, v/v) as developing system.
[b]In CD_3CN.
[c]In $CDCl_3$.

Table 2. Physicochemical characteristics of isomeric **6**

Isomer	HPLC[11] R_t (min)	^{31}P NMR[a] δ (ppm)	MALDI TOF MS *m*/*z*
6a	14.93	56.00	575.2[b]
6b	16.77	56.79	574.8[b]

[a]In CD_3CN.
[b]Calcd MW 574.48.



Scheme 2. Oxathiaphospholane method of synthesis of LNA dinucleotide **6**.

experiments were performed involving snake venom phosphodiesterase (*svPDE*) and nuclease P1 (*nP1*). *SvPDE* is known to be an R_P-specific nuclease whereas *nP1* preferentially hydrolyzes internucleotide phosphorothioate linkages of S_P configuration.[12,13] It was found that under standard conditions[14] none of diastereomers of **6** was hydrolyzed by *nP1* to a measurable extent. The application of *svPDE* was more conclusive. It was shown that, although **6b** isomer was still intact after 24 h incubation with the enzyme,[15] the **6a** isomer was readily hydrolyzed to yield deoxycytidine 5′-*O*-phosphorothioate and thymine LNA nucleoside. On this basis it was possible to assign the absolute configuration *R* at phosphorus atom for the diastereomer **6a**. Furthermore, since the stereochemical course of the oxathiaphospholane ring opening condensation was earlier established,[6] we could also assign the R_P configuration for the diastereomer FAST-**3**. The successful digestion of **6a** by *svPDE* is of special interest in the light of reported stability of 14-mer oligothymidylate LNA (non-phosphorothioate) against this nuclease.[16]

In conclusion, the results presented in this paper provide the evidence that oxathiaphospholane methodology can be successfully applied for the stereocontrolled synthesis of LNA dinucleoside phosphorothioates in solution. Further studies on the adaptation of this approach to the requirements of solid-phase oligonucleotide synthesis are underway.

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7. LNA nucleoside **2** was prepared from α-D-allofuranose by the procedure described in detail earlier (Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607) followed by 4,4′-dimethoxytritylation of the 5′-hydroxyl function.
8. Phosphorochloridite **4** (170 mmol) was added at rt into a solution of **2** (170 mmol) and diisopropylethylamine (210 mmol) in dry CH_3CN (4 mL). After 10 min elemental sulphur (400 mmol) was added and the mixture was stirred overnight at rt. ^{31}P NMR of the crude mixture showed two signals; δ 106.07 ppm (51.6%), 107.34 ppm (48.4%).
9. White powder; elemental analysis for $C_{39}H_{43}N_2O_9PS_2$: calcd/found C 60.14/59.28, H 5.56/5.57, N 3.59/3.42, P 3.98/3.47, S 8.23/8.51; MS (+FAB) *m/z* 778.1 (calcd MW 778.87).
10. As proved in a separate experiment the by-product **7** was formed as a result of hydrolysis of **3** with residual water present in a reaction mixture. When acetonitrile containing only 1.1 ppm of water was used, no by-product **7** could be detected by ^{31}P NMR.
11. 2.1×220 mm column (Brownlee) filled with PTH C-18, 5 μ; linear gradient of acetonitrile (0.73%/min) in 0.1 TEAB, pH 7.0.
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14. **6a** or **6b** (0.2 nmol) was incubated for 24 h at 37 °C with 25 μg of *nP1* (EC 3.1.30.1, Boehringer Mannheim) in a buffer containing 100 mM Tris–HCl (pH 7.2) and 1 mM $ZnCl_2$. After denaturing of the enzyme (2 min at 95 °C) the products were analyzed by RP HPLC.[11] These conditions were proved successful to hydrolyze the non-LNA dinucleoside phosphorothioate $d(T_{PS}C)$ (S_P isomer).
15. **6a** or **6b** (0.2 nmol) was incubated for 24 h at 22 °C with 10 μg of *svPDE* (EC 3.1.15.1, Boehringer Mannheim) in a buffer containing 100 mM Tris–HCl (pH 8.5) and 15 mM $MgCl_2$. After denaturing of the enzyme (2 min at 95 °C) the products were analyzed by RP HPLC[11] and identified by co-elution with an authentic sample. Under these conditions, the non-LNA dinucleoside phosphorothioate $d(T_{PS}C)$ (R_P isomer) was readily hydrolyzed.
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