Tetrahedron Letters 68 (2021) 152834

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Stereochemistry of internucleoside phosphorus atom affects sugar pucker and acid hydrolysis of N3′-P5′ thio-phosphoramidates

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ARTICLE INFO

Article history:
Received 22 November 2020
Revised 28 December 2020
Accepted 6 January 2021
Available online 28 January 2021

Keywords:
Oligonucleotide therapeutics
Nucleoside
Phosphoramidate
Stereoisomer
Diastereomer
Sugar pucker
Stereo-pure
Rate of hydrolysis

ABSTRACT

Investigation of the sugar pucker of the two diastereomers of dinucleotide N3′-P5′-thio-phosphoramidates $T_{NPS}T_{NH2}$ and $T_{NPS}T_{OH}$ shows that the S_p isomer adopts a more C3′-endo (North) sugar ring configuration than the R_p counterpart. In contrast, P-stereochemistry of oligonucleotide phosphorothioate (O3′-P5′) compounds has no effect on the nucleoside sugar puckering. This difference is also reflected in the different rate of acid hydrolysis for the two isomers. Thus, the R_p stereoisomer with less prevalent C3′-endo configuration has an acid hydrolysis rate constant ~50% higher than that of the S_p molecule. The $T_{NPO}T_{NH2}$ and $T_{NPO}T_{OH}$ dinucleotides are hydrolyzed an order of magnitude faster than $T_{NPS}T_{NH2}$ and $T_{NPS}T_{OH}$, respectively. In addition, dinucleotides with the terminal 3′-OH group are hydrolyzed two times faster than their 3′-NH_2 counterparts.

Oligonucleotide N3′-P5′-(thio)-phosphoramidate (NP/NPS) analogues in which the 3′-oxygen is replaced with nitrogen (Fig. 1), have a good stability towards nuclease digestion and form stable duplexes with complementary DNA and RNA strands. [1] These properties make them good antisense compound candidates. [2] While naturally occurring phosphodiester (OPO) bonds are hydrolytically very stable [3,4] acidic conditions lead to the hydrolysis of the backbone in phosphoramidates [5,6]. Thio-phosphoramidates (NPS) have been reported to be more resistant to hydrolysis than isosequential phosphoramidates (NPO). [5] Introduction of sulfur to the sugar-phosphate backbone leads to a chiral phosphorus center and therefore, two distinct P-stereoisomers at each chiral center, R_p and S_p, are formed. Substitution of oxygen with sulfur occurs in natural RNA and DNA and phosphorothioate is a modification present in both prokaryotes and eucaryotes [7,8].

In this work we sought to address the hypothesis, that the stereochemistry of the internucleotide thio-phosphate group may affect the sugar pucker of the adjacent nucleoside and thus results in a different acid stability of the two stereoisomers (Fig. 2). Hydrolytic stability is important because acidic conditions, particularly in cellular endosomes, will lead to partial hydrolyses of the NPS group, and thus results in a decrease in the compound's activity.

The two isomers of compounds **2** and **3** were prepared as described [9] and then separated by reversed-phase high performance liquid chromatography (RP-HPLC; Fig. 3). The fractions containing Peak1 and Peak2, (with assigned configurations S_p and R_p, respectively, details discussed below), were collected and the isolated compounds analyzed by NMR. The sugar puckering of the first (5′-) and the second (-3′) nucleoside in each isomer is reported in Table 1. A sample of the dimer **1** (Fig. 1), was also analyzed and used as a reference. The data showed that the 3′-nucleoside in the S_p isomer (Peak1) adopted a configuration that was more North (by about 10%) than the R_p isomer (Peak2). In contrast, no difference was observed in the sugar puckering for R_p vs. S_p isomers for the phosphorothioate (OPS) dimers of **1**. To investigate whether the observed difference in sugar pucker influences their hydrolytic stability, samples of the dinucleotides in citrate buffer (pH 2.5) were spiked with pyridine hydrochloride as internal standard (IS) and incubated at 40 °C. Aliquots were taken out at different time points and analyzed by LC-UV and LC-MS (Fig. 3).

The chromatogram of compounds **1** to **3**, (Fig. 3), showed two peaks, one for each isomer. While no noticeable change was observed for **1**, it was clear that the intensity of the second peak (Peak2) decreased faster than the first peak (Peak1) in compounds **2** & **3**. Chromatograms of **4** & **5** showed only one peak (Peak1) because they do not have a chiral phosphorus atom. In addition,

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https://doi.org/10.1016/j.tetlet.2021.152834
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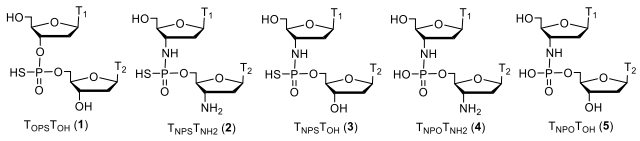

Fig. 1. Structures of dinucleotides used in the study.

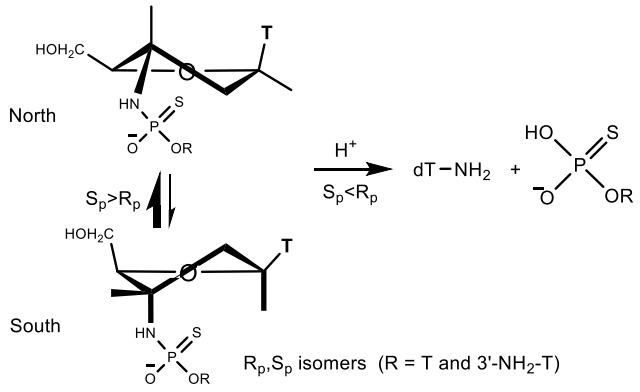

Fig. 2. Acid hydrolysis of thio-phosphoramidates. R_p and S_p isomers both adopt a C3′-endo (North) configuration. The percentage of North in the S_p isomer is more than R_p for the 5′-nucleoside in the dimer. This leads to an increased acid stability for the S_p isomer.

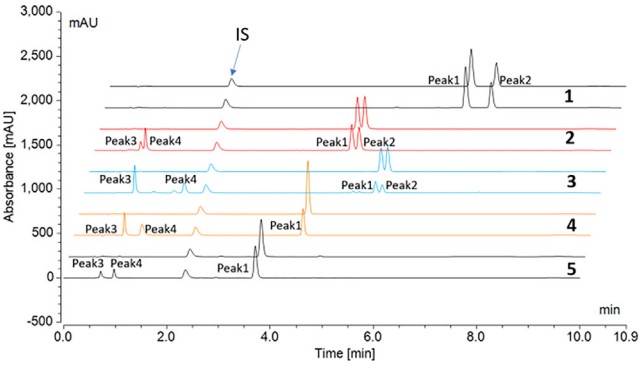

Fig. 3. Chromatograms of compounds **1** to **5** before, (top chromatogram in each pair), and after hydrolysis (bottom chromatogram) at 40 °C after incubation for 87 h for **1** to **3** and 8 h for **4** and **5**; The two stereoisomers in **1** to **3** are separated in the chromatogram (Peak1 and Peak2). The R_P stereoisomer (Peak2) is hydrolyzed faster in **2** and **3**. Compounds **4** and **5** do not have a chiral phosphorus and show only a single peak (Peak1). Acid-catalyzed hydrolysis leads to the cleavage of N3′-P5′ bond and formation of two products one of which contains a 5′-phosphate (Peak3 and Peak4). The pyridine internal standard peak is labeled as IS.

new products, corresponding to Peak3 & Peak4 were detected in the hydrolyzed samples. They are the result of 3′N—P bond cleavage, which leads to the formation of two products, one of which contains a 5′-phosphate.

Plots of the ratio of areas of Peak1 and Peak2 to the IS vs. time showed exponential decay curves (Fig. S1, Supporting Information) from which the rate constants were calculated. To determine the rate constants, the natural log of *y* axis in decay curves was plotted against time (Fig. 4 and Fig. S1). Graphs showed a linear relationship and slopes of the lines provided the rate constant for each sample (Table 1). Compound **1,** which was used as a reference control, did not show a noticeable degradation (Fig. S1). The obtained data confirm the previously reported increased hydrolytic stability of NPS compared to NPO linkage [5] with NPO linkage having a ten-fold higher rate of hydrolysis. In addition, the 3′-OH containing compounds **3** and **5** had rate constants two-fold higher than their 3′-NH_2 counterparts **2** and **4,** respectively. This difference can be explained by the preferential initial protonation of basic 3′-terminal nitrogen in 3′-NH_2 group, which is in spatial proximity to the internucleoside N—P linkage. Through electrostatic repulsion, this 3′-NH_3^+ proximity effect can reduce the protonation of the 3′-N of the phosphoramidate backbone, which is needed for hydrolysis [6,10]. The data also show that one of the stereoisomers of **2** and **3** (Peak2) is hydrolyzed faster with a rate constant approximately 50% higher than for the other isomer (Peak1). The conformational shift towards *C*2′-endo (South) sugar puckering renders the internucleoside phosphorus more sterically susceptible to nucleophilic attack, and thus more acid labile.

To determine which isomer, either R_p or S_p had a higher hydrolysis rate, we utilized the relative retention time of each isomer to assign R_p and S_p configurations to chromatographic peaks. It has been reported that for phosphorothioate (OPS) oligonucleotides the S_P configuration is more hydrophobic and has a better enzymatic stability than for the R_p isomer [11]. To verify the relative retention times of the two isomers under the used HPLC conditions, compound **1** was subjected to enzymatic digestion by snake venom phosphodiesterase. The two isomers were separated in the chromatogram (Fig. S2). The intensity of the first peak in the chromatogram was reduced over time, while the second peak did not show a noticeable change. The compound corresponding to the second peak was more stable enzymatically and therefore was assigned the S_p configuration as reported previously. Moreover, a comparison between stereoisomers shows that the R_p isomers of **2** and **3** have the same spatial arrangement of atoms around the phosphorus chiral center as the S_p isomer of **1** (Fig. S3) and are, therefore, the second eluted peak on the chromatogram. Based on this observation, the R_p isomer of **2** and **3** is more hydrophobic than the S_p isomer. Consequently, we conclude that the compound corresponding to the Peak2 in Fig. 3, which is hydrolyzed faster, has the R_p configuration.

In conclusion, stereochemistry of the internucleoside NPS-group phosphorus atom affects the nucleoside sugar pucker of R_p and S_p isomers. This in turn influences the rate of hydrolysis for the two stereoisomers. The R_p compound has a rate constant approximately 50% higher than that for the S_P isomer. Additionally, the NPO linkage has a 10-fold higher hydrolysis rate constant than the NPS counterpart. Moreover, the presence of basic and protonatable (at acidic and neutral pH conditions) 3′-terminal NH_2 group

Table 1
Percentage of North (C-3′ endo) configuration and acid hydrolysis rate constants of dinucleotides. The Peak1 and Peak2 correspond to S_p and R_p configurations (assigned based on the chromatographic retention times). The percentage of North configuration for NPO dimers **4** and **5** were 68/35 and 74/46 for the 5′- and 3′-nucleosides respectively. The rate constants were 7.4×10^{-6} and 2.7×10^{-5} respectively.

Compound	Sp (Peak 1)			Rp (Peak 2)		
	%North		k (s^{-1})	%North		k (s^{-1})
	5′-Nuc.	3′-Nuc.		5′-Nuc.	3′-Nuc.	
$T_{OPS}T_{OH}$ (**1**)	27 (±3)	25 (±3)	–	25 (±3)	25 (±3)	–
$T_{NPS}T_{NH2}$ (**2**)	77 (±3)	66 (±3)	8.0×10^{-7}	64 (±3)	51 (±0)	1.2×10^{-6}
$T_{NPS}T_{OH}$ (**3**)	75 (±3)	36 (±3)	2.4×10^{-6}	63 (±4)	32 (±3)	3.5×10^{-6}

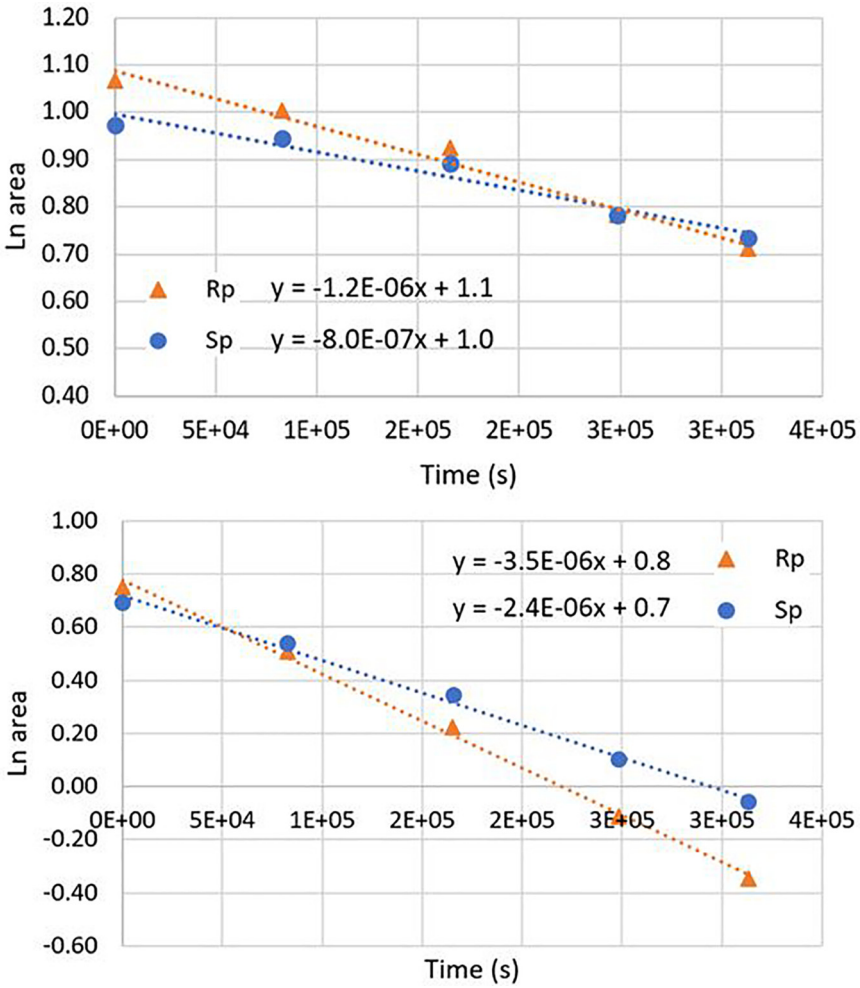

Fig. 4. Acid-catalyzed hydrolysis rate assessments for the NPS group. Plots of natural log of the ratio of areas of Peak1 and Peak2 to the IS vs. time for $T_{NPS}T_{NH2}$ (top) and $T_{NPS}T_{OH}$ (bottom). The hydrolysis rate constants were determined from the slope of the lines. R^2 values were 0.96 (R_p), 0.98 (S_p) in the top graph and 0.99 (R_p & S_p) in the bottom graph.

results in 2-fold reduction of NP and NPS hydrolysis rates, in comparison with neutral 3′-OH counterparts. The findings may be important for designing more advanced and efficacious nucleic acid based therapeutic agents.

Author contributions

The manuscript was written, and the work was done through contributions of both authors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We would like to thank our colleagues at the Janssen Pharmaceutical Companies especially Saúl Martínez-Montero for useful discussions.

Appendix A. Supplementary data

Materials and Methods, Synthesis and acid hydrolysis, Enzymatic digestion, LC-UV and LC-MS analysis, Analysis of sugar pucker by NMR, Figures S1–S3. Supplementary data to this article can be found online at https://doi.org/10.1016/j.tetlet.2021.152834.

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