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Biologically relevant morpholino nucleoside thio- and dithiophosphates *via* an oxathiaphospholane approach

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Appropriately protected morpholino nucleoside 6′-*O*-(2-thio)-1,3,2-oxathiaphospholanes and 6′-*O*-(2-thio)-1,3,2-dithiaphospholanes react with 3-hydroxypropionitrile in the presence of a strong base catalyst (DBU), yielding morpholino nucleoside 6′-*O*-(α-thiophosphates) and 6′-*O*-(α,α-dithiophosphates), respectively. The synthesized compounds exhibit low cytotoxicity toward human cells, indicating their favorable biocompatibility and potential for further biological and therapeutic applications.

Introduction

The nucleosides, nucleotides, and their analogs have a wide range of applications, including their use as substrates and inhibitors in enzyme research, as well as anticancer and antiviral drugs, primarily acting as replication inhibitors.[1,2] Once inside cells, these compounds are sequentially converted into nucleoside 5′-mono-, di-, and triphosphates (NMP, NDP, and NTP, respectively) through phosphorylation mediated by viral and cellular kinases.[3] These nucleos(t)ides may inhibit enzymes involved in DNA or RNA biosynthesis, including viral polymerases and kinases. Furthermore, fluorescently or radiolabeled nucleotides are valuable tools in nucleic acid research, serving as probes for various biochemical and molecular studies.[4,5]

Additionally, some of these compounds are widely used as food additives. For instance, guanosine 5′-monophosphate (GMP) and inosine 5′-monophosphates (IMP) serve as flavor potentiators.[6] Sodium uridine, cytidine, adenosine and guanosine-5′-monophosphates are used in the production of formula for infants, which has a high nucleotide content matching breast milk.[7] Scientists are actively developing and synthesizing modified nucleos(t)ides with alterations to the phosphate group or sugar moiety to expand the potential of nucleoside-based therapeutics. Among such modifications, morpholine derivatives have emerged as a flexible scaffold for drug design, owing to their ability to target a wide range of biological pathways. The versatility of morpholine derivatives is exemplified by well-known drugs like linezolid,[8] an antibiotic and gefitinib,[9] an anticancer agent, both of which incorporate morpholine into their structures, highlighting its critical role in achieving desired therapeutic outcomes.

Eckstein *et al.*[10] reported that nucleotide analogs modified by replacing an oxygen of the phosphate group with sulfur show an interesting behavior in enzymes involved in nucleic acid metabolism. Thiophosphate derivatives are powerful tools for biochemical and pharmacological studies as they are more stable than the corresponding phosphates. The Baran group[11] introduced a fundamentally new approach to the stereocontrolled synthesis of phosphorothioate nucleotides. Previously constrained by phosphorus(III) P(III)-based methodologies, they developed a phosphorus(V) P(V)-based reagent platform[12,13] that enables programmable, traceless, and diastereoselective incorporation of phosphorus-sulfur motifs. This innovative strategy allows for the efficient, cost-effective, and operationally simple synthesis of stereodefined nucleotides. As an alternative to the Baran approach, the method developed by the Stec group also offers significant advantages. Although this method was originally developed in the 1990 s and requires the synthesis of specific phosphitylating reagents, it remains highly valued and widely used today due to its operational simplicity and high efficiency. The oxathiaphospholane strategy enables rapid and scalable access to mono-, di-, and triphosphate derivatives, including nucleoside phosphorothioates[14] or phosphoramidothioates,[15] making it a versatile tool in the synthesis of nucleotide-based compounds. Its continued relevance is further supported by its adaptability to various nucleoside scaffolds[16] and compatibility with diverse nucleophilic reagents.

For the sake of clarity, in the oxathiaphospholane (OTP) method, P-chiral nucleoside 3′-*O*-(2-thio-1,3,2-oxathiaphospholane)

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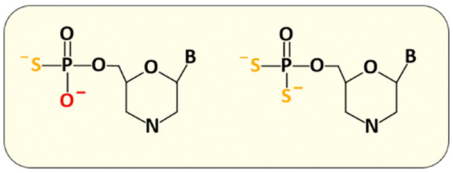


Fig. 1 Schematic representation of morpholino nucleoside thiophosphates (left) and dithiophosphates (right).

monomers are obtained as mixtures of P-diastereomers, which can be separated by silica gel column chromatography. These isolated diastereomers are employed in the synthesis of P-stereodefined phosphorothioate analogs. However, in this study, we do not pursue stereochemically defined synthesis. Instead, the OTP strategy is used as a convenient and efficient approach for the rapid preparation of nucleoside thiophosphate or dithiophosphate analogs.

In this study, we present an expansion of the oxathiaphospholane approach, a well-established and versatile synthetic strategy previously utilized for the preparation of P-stereodefined phosphorothioate derivatives. This extended methodology enables the efficient synthesis of a new class of compounds, morpholino nucleoside α-thiophosphates and α,α-dithiophosphates (Fig. 1).

Nucleoside phosphorothioates (PS) are nucleotide analogs in which a single non-bridging oxygen atom in the phosphate group is replaced by sulfur. In dithiophosphates ($P(S)_2$), however, both non-bridging oxygen atoms are substituted with sulfur. Notably, dithiophosphates possess a symmetrical structure similar to that of natural phosphates, in contrast to the asymmetrical structure of phosphorothioates.

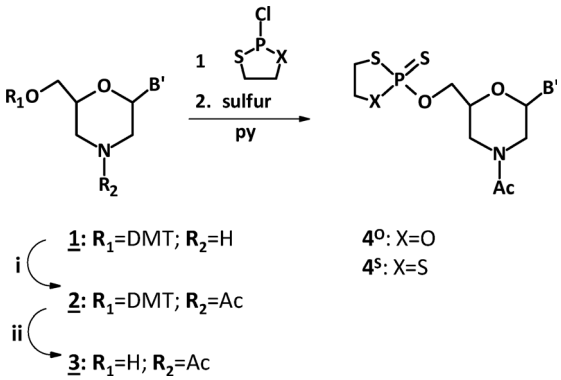
The oxathiaphospholane-based synthesis described herein provides a robust and scalable route to these novel nucleotide analogs, laying the groundwork for further exploration of their biological properties, including their potential incorporation into cyclic dinucleotides (CDNs), or triphosphate forms for enzymatic or therapeutic applications.

Results and discussion

Preparation of oxathiaphospholane (6′-OTPs, 4^O) and dithiaphospholane (6′-DTPs, 4^S) derivatives of morpholino nucleosides

Morpholino nucleosides (**1**) were synthesized according to published literature protocols.[17] Briefly, 5′-*O*-dimethoxytrityl ribonucleoside was oxidatively converted in the acyclic dialdehyde derivative, followed by reductive amination. The target morpholino nucleosides were obtained in good yields. Subsequent acetylation afforded *N*-acetyl morpholino nucleosides (**2**), which were purified by flash chromatography (Fig. S5, SI), yielding the products in quantitative amounts (above 90%, Fig. S2–S4, SI). The acetylation was carried out under standard conditions using acetic anhydride in pyridine, a well-established protocol for introducing *N*-acetyl protection (Scheme 1).

The resulting acetylated nucleosides (**2**) were detritylated using 1.5% DCA in methylene chloride.[21] The crude products



Scheme 1 The synthesis of oxathiaphospholane (6′-OTPs) and dithiaphospholane (6′-DTPs) monomers; Conditions: (i). 1.2 eq Ac_2O, in anhydrous pyridine; (ii). 1.5% DCA in methylene chloride.

(**3**) were subsequently purified by flash silica gel column chromatography and characterized by HRMS, affording isolated yields of 65–78% (Fig. S6–S9, SI). Suitably protected nucleosides (**3a–d**), thoroughly dried overnight to eliminate residual moisture, were phosphitylated using 2-chloro-1,3,2-oxathiaphospholane[18] or 2-chloro-1,3,2-dithiaphospholane,[19] followed by sulfurization with elemental sulfur, yielding the corresponding compounds 4^O**a–d** and 4^S**a–d**. Although these two the phosphitylating reagents are not commercially available, their preparation is well established, with reliable synthetic protocols described in the literature.[19,20] Crude (4^O**a–d** and 4^S**a–d**) products were purified by flash silica gel column chromatography and then isolated with 65–81% yield in the form of amorphous powders after drying at high vacuum. They were characterized by HR MS and NMR (Table 1, Fig. S10–S30, SI). A lower yield was observed for the 6′-OTPs (and 6′-DTPs) monomers compared to the *N*-OTP analogs described in the previous work.[20]

Preparation of morpholino nucleoside 6′-*O*-(α-thiophosphates) and 6′-*O*-(α,α-dithiophosphates)

The conversion reaction of oxa- and dithiaphospholanes (4^O and 4^S) to thio- (5^O) and dithiophosphates (5^S) was performed in anhydrous acetonitrile solution, at room temperature, using

Table 1 Chromatographic and spectroscopic characteristics of oxathiaphospholane (6′-OTPs) and dithiaphospholane (6′-DTPs) derivatives of morpholino nucleosides

	Yield (%)[a]	MM calc. (Da)	HR MS (*m*/*z*)[b]	δ ^{31}P NMR (ppm)[c]
OTP-mU (4^O**a**)	81	407	406.0308	106.13, 105.43
OTP-mC (4^O**b**)	76	510	509.0717	106.36, 105.56
OTP-mA (4^O**c**)	73	534	533.0833	105.82, 105.34
OTP-mG (4^O**d**)	65	516	n.d.	105.15, 104.67
DTP-mU (4^S**a**)	77	423	422.0077	123.65
DTP-mC (4^S**b**)	74	526	525.0489	124.28
DTP-mA (4^S**c**)	70	550	549.0605	124.41
DTP-mG (4^S**d**)	68	532	531.0709	124.48

[a] Yield of the isolated product (for OTP: as mixture of P-diastereomers). [b] Recorded with a SYNAPT G2-Si high definition mass spectrometer. [c] In anhydrous CD_3CN.

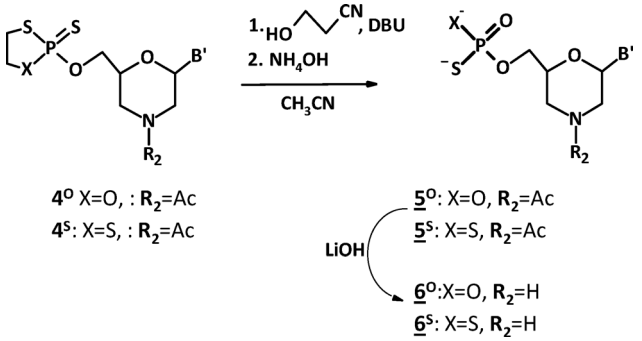
5 molar equivalents of 3-hydroxypropionitrile and 1 molar equivalent of 1,8 diazabicyclo[5.4.0]undec-7-ene (DBU) (Scheme 2).

After 20 minutes of reaction, quantitative formation of product was observed by ^{31}P NMR analysis of the crude reaction mixture. After evaporation of the solvent, the residue was treated with 30% aqueous ammonia at 55 °C for 10 hours to afford the desired thiophosphates ($\mathbf{5^O}$) and dithiophosphates ($\mathbf{5^S}$). The crude products were purified by semi-preparative RP-HPLC (Fig. S32, SI) to give pure compounds in 51–60% isolated yields (Table S1, SI) and characterized by HRMS (Fig. S33–S40, SI). Treatment of the obtained materials with aqueous 1 M LiOH effected N-deacetylation, affording samples $\mathbf{6^O}$ and $\mathbf{6^S}$, which were desalted by RP-HPLC and analyzed by analytical RP-HPLC and NMR (Fig. S41–S72, SI). Following this verification step, the compounds were subjected to cytotoxicity evaluation in human cells.

Cytotoxicity screening of potential therapeutic (di)thiophosphates

Cytotoxicity assessment serves as a fundamental step in the early-phase of characterization[21] of novel compounds, providing crucial information on their safety and potential applicability in therapeutics or diagnostic. Evaluating the safety profile of such molecules, especially in terms of their impact on human cells, is essential before considering any further biological application. In this context, the serves as a widely accepted *in vitro* model for assessing cytotoxic effects on skin-related tissues. The present study aimed to examine the influence of studied (di)thiophosphates ($\mathbf{6^O}$ and $\mathbf{6^S}$) on the viability of human HaCaT keratinocytes, providing preliminary insights into their biocompatibility.

The series of nucleotide derivatives was tested for their impact on the viability of human keratinocytes HaCaT. The results showed limited cytotoxicity of all tested compounds towards human HaCaT cells. After a 24-hour incubation, the effect of the compounds on the survival rate of the cells did not exceed 10%. The cytotoxic effect was not observed even after extending the incubation time to 72 hours (Fig. 2). The maximum reduction in cell viability did not exceed 20% after treatment with the tested derivatives at a concentration of



Scheme 2 The synthesis of morpholine nucleoside 6′-*O*-(α-thiophosphates) and 6′-*O*-(α,α-dithiophosphates) by the oxathiaphospholane approach.

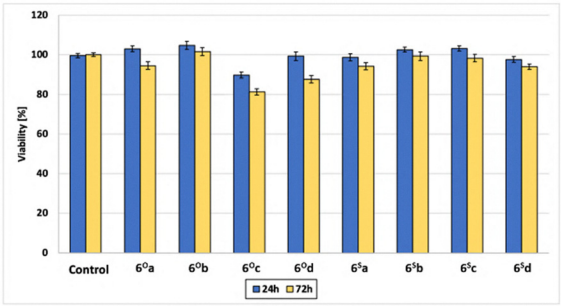


Fig. 2 The viability of HaCaT cells after 24 and 72 hours of incubation with 100 μM modified nucleotides. Data are presented as mean values of at least three independent experiments ± SE.

100 μM after 24 and 72 hours. Thus, obtained data indicate the non-toxic nature of this class of modified nucleotides, which makes them good candidates as tools for further biological applications.

Conclusions

In conclusion, an efficient synthetic protocol was developed for the preparation of morpholino nucleoside (di)thiophosphates. The results demonstrate that the oxathiaphospholane methodology can be successfully applied to obtain phosphorothioate and phosphorodithioate derivatives of morpholino analogs.

Preliminary cytotoxicity assays using HaCaT human keratinocyte cell lines revealed that these newly synthesized analogs exhibit negligible toxicity, suggesting a favorable biocompatibility profile. This is an encouraging result, as it indicates that the compounds may be well tolerated in biological systems, a critical requirement for any therapeutic or diagnostic application.

Given their chemical novelty and biological promise, these compounds are poised for further exploration. Ongoing and future studies will focus on the synthesis of the corresponding nucleoside 5′-triphosphate derivatives and/or cyclic dinucleotides (CDNs), which are known to play pivotal roles in immune signaling and antiviral defense. This work lays a strong foundation for the continued development of morpholino thiophosphate and dithiophosphate analogs as next-generation nucleotide-based agents in chemical biology and medicinal chemistry.

Author contributions

K. J. supervised the project. K. J., R. P., A. C. prepared and edited the manuscript. K. J., J. J., A. S. and W. S. performed all the chemical synthesis and data analysis. R. P. and A. C. designed and performed the biological evaluation of compounds activity and data analysis. All authors approved the final version of the manuscript.

Conflicts of interest

The authors declare no competing financial interest.

Data availability

The data supporting this article have been included as part of the SI. Supplementary information: Detailed experimental procedures and associated data. See DOI: **https://doi.org/10.1039/d5nj02865e**.

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