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A simple and rapid synthesis of nucleotide analogues containing a phosphorothioate moiety at the terminal position of the phosphate chain

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Abstract—A straightforward method for the synthesis of nucleotide analogues bearing a phosphorothioate moiety at the terminal position of the polyphosphate chain is described. Several nucleoside 5′-(2-thiodiphosphates) and 5′-(3-thiotriphosphates) were synthesized by treatment of the appropriate nucleotide imidazolide derivative with a ca. 4-fold excess of thiophosphate triethylammonium salt in DMF in the presence of zinc chloride. The HPLC reaction yields varied from 80% to 100%, in the majority of cases exceeding 90%. Separation was accomplished by Sephadex ion-exchange chromatography or reverse-phase HPLC with preparative yields of about 70%.

1. Introduction

Nucleotide analogues in which one of the non-bridging oxygens is replaced by sulfur have received much attention as molecular tools for the investigation of fundamental processes concerning nucleic acid metabolism. Nucleoside phosphorothioates are isosteric and pseudoisoelectronic with natural nucleotides and retain a similar negative net charge under physiological conditions. However, this minimal alteration of the phosphate moiety often imposes a dramatic change in biological activity. 'Terminal phosphorothioates', modified at the terminal position of the polyphosphate chain, possess several unique properties that make them especially attractive as nucleotide analogues. It is well known that a terminal phosphorothioate moiety significantly improves resistance to phosphatases, but usually does not affect the substrate properties towards kinases.[1] Therefore, terminal phosphorothioates serve as non-hydrolysable nucleotide analogues to investigate the role of phosphate transfer in versatile biological systems.[1–4] Moreover, ATPγS is commonly used as a thiophosphoryl group donor to obtain 5′-thiophosphorylated oligonucleotides or thiophosphorylated proteins resistant to protein phosphatases.[5–9] The resistance of terminal phosphorothioates to hydrolases results in the ability to form stable enzyme–substrate complexes which are useful in numerous functional and structural studies on proteins.[10–12] Furthermore, it was shown that non-hydrolyzable terminal purine and pyrimidine phosphorothioates are activators of extracellular G-protein-coupled P2 receptors.[13] Apart from these briefly described applications in biochemical and biological studies, the terminal phosphorothioate group also offers the possibility of irreversible (via S-alkylation) or reversible (via disulfide bond) functionalization with versatile ligands.[14,15]

One of the difficulties encountered in exploiting terminal phosphorothioates is that only a few of them are commercially available or easy to synthesize. Although many investigations have been carried out with the aim of chemical or enzymatic synthesis of ribo- and

Keywords: Phosphorothioates; Nucleotide analogues; Phosphorimidazolides; Chemical synthesis.
Abbreviations: ADPβS, adenosine 5′-(2-thiodiphosphate); GDPβS, guanosine 5′-(2-thiodiphosphate); IDPβS, inosine 5′-(2-thiodiphosphate); UDPβS, uridine 5′-(2-thiodiphosphate); dCDPβS, 2′-deoxycytidine 5′-(2-thiodiphosphate); m^7GDPβS, 7-methylguanosine 5′-(2-thiodiphosphate); ATPγS, adenosine 5′-(3-thiotriphosphate); GTPγS, guanosine 5′-(3-thiotriphosphate); m^7GTPγS, 7-methylguanosine 5′-(3-thiotriphosphate); ImpG, guanosine 5′-phosphorimidazolide; ImpA, adenosine 5′-diphosphate imidazolide; 2,2′-DTDP, 2,2′-dithiodipyridine; Ade, adenin-9-yl; Gua, guanin-9-yl; Hyp, hypoxanthin-9-yl; Ura, uracil-1-yl; Cyt, cytosin-1-yl; m^7Gua, 7-methylguanin-9-yl.
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deoxyribonucleotide terminal phosphorothioates,[16–21] a single, all-purpose and efficient method is conspicuously lacking. Enzymatic thiophosphorylation seems to be efficient and uncomplicated, however, restrictions arising from enzyme specificity, problems with up-scaling and high cost are major disadvantages. Two main synthetic routes have been employed for the preparation of terminal phosphorothioates. In the first, adenosine 5′-(3-thiotriphosphate) and 5′-(2-thiodiphosphate) could be prepared by condensation of ADP or AMP, respectively, with β-cyanoethyl phosphorothioate under conditions originally developed by Michelson,[22] with subsequent removal of the β-cyanoethyl group via alkaline elimination.[18] In the second route, the key reactants were AMPS or ADPαS, respectively, and 2′,3′-methoxymethylidene AMP which were also coupled together by the Michelson procedure.[19–21] The unprotected ribose ring was then cleaved with periodate and subjected to alkaline conditions resulting in β-elimination. Finally, the methoxymethylidene protecting group was removed under acidic conditions yielding the desired terminal phosphorothioate. A similar approach was applied for the synthesis of GDP analogues.

All these synthetic routes share several disadvantages. They are chiefly related to purine nucleotides and are either lengthy, low-yielding or otherwise preparatively complicated (e.g., require transient nucleoside protection or maintenance of strictly anhydrous conditions). Finally, in each case, one of the steps involves treatment under alkaline conditions. Therefore, we assumed that neither of these approaches is suitable for the synthesis of compounds bearing a nucleobase that is unstable under alkaline conditions, such as 7-methylguanine. These 7-methylguanosine 5′-polyphosphates are mononucleotide analogues of the eukaryotic 5′ mRNA cap and act as potent translational inhibitors.[23] The synthesis of m^7GTPγS was of great interest to us, as this compound could act as a strong translational inhibitor with improved resistance to enzymatic degradation and, therefore, elongated half-life under physiological conditions.

Hence, the work reported herein was aimed at developing a simpler and more general method for the synthesis of nucleoside 5′-(2-thiodiphosphates) and 5′-(3-thiotriphosphates). As a result, a novel, convenient and high-yielding approach via efficient coupling between appropriate nucleotide imidazolide derivatives and excess phosphorothioate in DMF in the presence of zinc chloride is described.

The synthesis of a pyrophosphate bond via coupling between methyl phosphorimidazolidate with GDP in DMF in the presence of a metal chloride was initially reported by Kadokura et al.[24] The reaction rate was substantially accelerated when carried out in the presence of an 8-fold excess of $ZnCl_2$, which acts as a Lewis acid catalyst. Later, Stepinski et al. reported coupling reactions under analogous conditions involving guanosine 5′-phosphorimidazolide and an appropriate nucleoside 5′-diphosphate.[25] A similar approach has been successively exploited for the synthesis of mono- and dinucleoside 5′-di-, tri-, tetra- and even pentaphosphates and their analogues.[26–29] In comparison to the previously applied coupling reactions performed in aqueous solutions,[30] coupling in an organic solvent leads to shorter reaction times, reduces the hydrolysis of phosphorimidazolides, and thus formation of unwanted by-products. In this work, further evaluation of this approach utilising the phosphorothioate anion as a novel efficiently coupling nucleophile is presented.

The first attempt at a synthesis of nucleoside 5′-(2-thiodiphosphates) involved the coupling reaction between guanosine 5′-phosphorimidazolide (ImpG) and thiophosphoric acid sodium salt in DMF in the presence of zinc chloride. However, due to the poor solubility of Na_3PSO_3 in DMF the reaction did not proceed in the expected direction. To overcome this problem, thiophosphate was converted into a more lipophilic triethylammonium salt. In this case, the coupling reaction proceeded smoothly yielding the desired guanosine 5′-(2-thiodiphosphate) **1b** in excellent yield (over 90% as indicated by analytical reverse-phase HPLC). Unexpectedly, the reaction rate with phosphorothioate anions was noticeably greater than that of the analogous reaction with phosphate anions. HPLC analysis indicated complete substrate disappearance within 15–20 min. In contrast, the synthesis of GDP via coupling of ImpG with the phosphate TEA salt under analogous conditions required about two hours for completion. It seemed that under the applied conditions, the phosphorothioate anion is a much better nucleophile than the phosphate anion; therefore, it was reasonable to investigate whether a metal chloride catalyst was necessary for efficient product formation. No formation of the phosphorothioate–phosphate mixed anhydride occurred in the absence of metal chloride, even after extended reaction times.

To demonstrate the utility of the synthetic approach, a series of reactions were performed, resulting in nucleoside 5′-(2-thiodiphosphates) **1a**–**f** and 5′-(3-thiotriphosphates) **2a**–**c**; these reactions are summarized in Table 1. In all cases the reaction times did not exceed 20 min.

The general synthetic route is depicted in Figure 1. Conversion of the nucleotides into the appropriate imidazo-

Table 1.

	B	R	HPLC retention time[a] (min)	HPLC yield (%)
Nucleoside 5′-(2-thiodiphosphate)				
ADPβS (**1a**)	Ade	–OH	4.1	94
GDPβS (**1b**)	Gua	–OH	3.5	98
IDPβS (**1c**)	Hyp	–OH	4.5	95
UDPβS (**1d**)	Ura	–OH	2.5	82
dCDPβS (**1e**)	Cyt	–H	3.0	77
m^7GDPβS (**1f**)	m^7Gua	–OH	4.2	93
Nucleoside 5′-(3-thiotriphosphate)				
ATPγS (**2a**)	Ade	–OH	2.7	98
GTPγS (**2b**)	Gua	–OH	2.5	97
m^7GTPγS (**2c**)	m^7Gua	–OH	3.5	98

[a] Conditions: flow 1.3 mL/min, linear gradient 0–25% methanol in 0.05 M ammonium acetate pH 5.9 within 15 min.

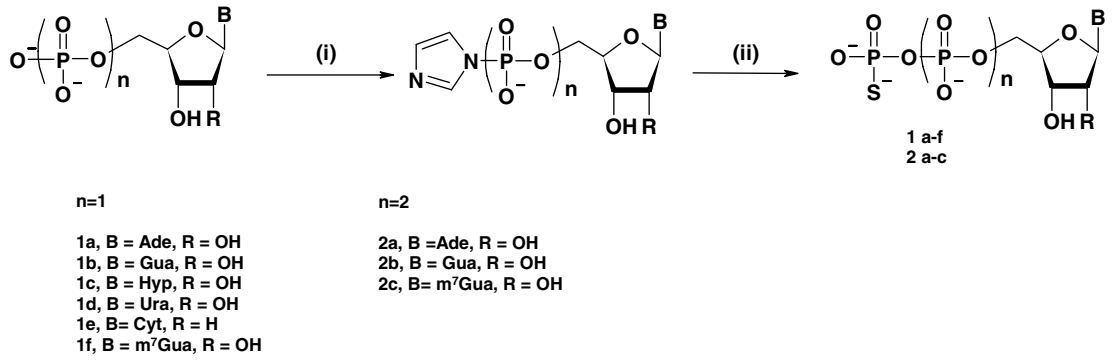

Figure 1. General scheme for the synthesis of nucleoside 5′-(2-thiodiphosphates) and 5′-(3-thiotriphosphates). Reagents: (i) imidazole, 2,2′-DTDP/Ph_3P, TEA, DMF; (ii) PSO_3^{3-}, $ZnCl_2$, DMF.

lide derivatives was easily achieved using imidazole and triphenylphosphine/2,2′-dithiodipyridine as the condensing agent.[31,32] The activated compounds were reacted with a 4-fold excess of the triethylammonium salt of thiophosphoric acid in DMF to give the respective nucleoside 5′-(2-thiodiphosphates) or 5′-(3-thiotriphosphates). Monitoring by analytical reverse-phase of HPLC revealed that in all cases, only one major product was formed in very good yield. An example of HPLC reaction profile is depicted in Figure 2A. Purification could be achieved by standard methods: Sephadex ion-exchange chromatography was used for large-scale reactions, whereas for separation of small amounts of compounds, HPLC purification was employed. However, probably due to slow, but observable decomposition of products in aqueous solutions, preparative yields were about 20% lower than HPLC yields. The structures of all newly synthesized compounds were confirmed by MS ESI-spectrometry, ^{1}H NMR and ^{31}P NMR (Table 2). An example of ^{31}P NMR spectrum is depicted in Figure 2B.

In conclusion, a novel method for the synthesis of biologically important nucleoside 5′-(2-thiodiphosphates) and 5′-(3-thiotriphosphates) is described. The new synthetic approach is facile, efficient and can be applied to obtain nucleotides containing 7-methylguanosine, which would be difficult to prepare using other methods.

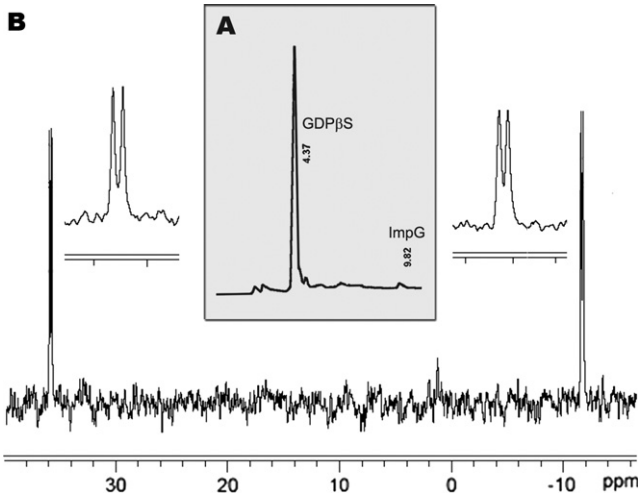

Figure 2. (A) HPLC profile of the coupling reaction between ImpG and thiophosphate triethylammonium salt in DMF in the presence of $ZnCl_2$. (B) ^{31}P NMR spectrum of GDPβS recorded in D_2O, at 161.9 MHz.

Nucleotides: AMP, GMP, IMP, UMP, dCMP, ADP and GDP were purchased as sodium salts or acidic forms; commercially non-available nucleotides: m^7GMP and m^7GDP were prepared as reported.[33] Trisodium thiophosphate dodecahydrate ($Na_3PSO_3 \cdot 12H_2O$) is also commercially available or can be synthesized by alkaline hydrolysis of thiophosphoryltrihalide. Analytical HPLC was performed on a Spectra-Physics SP8800 apparatus, using a Supelcosil LC-18-T reverse-phase column (4.6 × 250 mm, flow rate 1.3 mL/min) with a linear gradient 0–25% of methanol in 0.05 M ammonium acetate buffer at pH 5.9 within 15 min. Ion-exchange chromatography was performed on a DEAD-Sephadex A-25 column using a TEAB buffer gradient in deionized water. Preparative HPLC was performed on a Waters 600E Multisolvent Delivery System apparatus, using a Waters HR-C-18 reverse-phase column (19 × 300 mm, flow rate 5.0 mL/min) with isocratic 0.05 M ammonium acetate buffer (pH 5.9) as the mobile phase.

2. Chemical syntheses

Representative syntheses are presented below.[34,35]

2.1. Conversion of trisodium thiophosphate into the TEA salt

Trisodium thiophosphate dodecahydrate Na_3PSO_3 (2 g) was passed through a Dowex 50 WX 8 column (TEA form) followed by evaporation of the eluate to an oily residue and re-evaporation twice with anhydrous (99.8%) ethanol. In this form, the compound is far less stable and at room temperature undergoes fast decomposition with loss of sulfur. However, it can be stored at −20 °C for up to three weeks, or even longer if stored as a frozen water/ethanol solution. The TEA salt obtained was slightly contaminated with PO_4^{3-}, similar to commercially available Na_3PSO_3 (5–10% as indicated by ^{31}P NMR).

Table 2.

	ADPβS **1a**	GDPβS **1b**	IDPβS **1c**	UDPβS **1d**	dCDPβS **1e**	m^7GDPβS **1f**	ATPγS **2a**	GTPγS **2b**	m^7GTPγS **2c**
H1′	6.11	5.83	6.13	6.01	6.33	6.05	6.01	5.93	6.07
H2′	4.79	4.64	4.81	4.51	2.41; 2.35[a]	4.73	4.70	4.79	4.75
H3′	4.62	4.52	4.67	4.43	4.69	4.59	4.50	4.54	4.66
H4′	4.39	4.24	4.40	4.29	4.26	4.41	4.26	4.35	4.40
H5′, H5″	4.25	4.12	4.27	4.26	4.19	4.35	4.14	4.20	4.34
H2	8.55	—	8.53	—	—	—	8.43	—	—
H5	—	—	—	8.03	8.03	—	—	—	—
H6	—	—	—	6.00	6.15	—	—	—	—
H8	8.23	8.05	8.21	—	—	9.23	8.13	8.12	—[b]
CH_3	—	—	—	—	—	4.15	—	—	4.14
Pα	−11.63	−11.17	−11.59	−11.71	−11.54	−11.66	−11.03	−10.50	−10.89
Pβ	37.02	33.50	34.69	35.58	34.04	35.79	−23.67	−23.34	−22.97
Pγ	—	—	—	—	—	—	38.97	37.97	34.64
MS ESI	441.97	457.99	442.93	418.95	401.96	471.99	522.00	537.95	552.03
Calcd. mass	442.00	458.00	442.98	418.97	401.99	472.01	521.96	537.96	551.98

^{1}H NMR and ^{31}P NMR spectra were recorded in D_2O at 25 °C at 399.94 MHz and 161.90 MHz, respectively. ^{1}H NMR chemical shifts are referenced to sodium 3-trimethylsilyl-[2,2,3,3-d_4]-propionate (TSP) and ^{31}P NMR chemical shifts are referenced to 20% phosphoric acid in D_2O as an external standard. Mass spectra were recorded using negative electrospray ionization.

[a] Signals of the H2′ and H2″ of the 2′-deoxyribose moiety.

[b] Exchangeable proton.

2.2. Guanosine 5′-monophosphate imidazolide, ImpG

Guanosine 5′-monophosphate sodium salt was converted into the triethylammonium salt using Dowex ion-exchange resin. Guanosine 5′-monophosphate triethylammonium salt (300 mg, 0.66 mmol), imidazole (360 mg, 5.3 mmol) and 2,2′-dithiodipyridine (440 mg, 2.0 mmol) were suspended in anhydrous DMF (10 mL). Subsequently, triethylamine (95 μL) and triphenylphosphine (524 mg, 2.0 mmol) were added and the mixture was stirred at room temperature for 6 h. The resulting clear solution was poured into a flask containing 150 mg of anhydrous sodium perchlorate dissolved in 30 mL of acetone. After cooling at 4 °C for 3 h, the resulting precipitate was filtered, washed with dry acetone and dried overnight in a vacuum desiccator over P_4O_{10} to give 260 mg of guanosine 5′-monophosphate imidazolide sodium salt. Yield 90%.

2.3. Guanosine 5′-(2-thiodiphosphate), GDPβS

Guanosine 5′-monophosphate imidazolide sodium salt (115 mg, 0.28 mmol) was mixed with phosphorothioate triethylammonium salt (320 mg, ca. 1.2 mmol) and the resultant mixture was suspended in 3.5 mL of DMF. Subsequently, anhydrous zinc chloride (305 mg, 2.3 mmol) was added and rapid dissolution of the reagents was observed. After ca. 15–20 min reverse-phase HPLC revealed complete substrate disappearance ($t_R = 9.8$ min) and formation of one major product ($t_R = 3.5$ min). The reaction was quenched by addition of EDTA solution (920 mg, 2.5 mmol in 35 mL of water) and brought to pH ~7 with sodium bicarbonate. Chromatographic isolation was performed on a DEAE-Sephadex A25 column with a linear gradient of triethylammonium bicarbonate (from 0 to 0.9 M TEAB in deionized water). Collected fractions eluted at a buffer concentration of 0.60–0.65 M gave 1900 absorbance units (260 nm) of GDPβS. Evaporation with addition of ethanol to decompose TEAB buffer and drying of the resulting solid residue over P_4O_{10} overnight yielded 120 mg of guanosine 5′-(2-thiodiphosphate) triethylammonium salt. Yield 70%.

2.4. Adenosine 5′-diphosphate imidazolide, ImppA

Adenosine 5′-diphosphate triethylammonium salt (350 mg, 0.55 mmol), imidazole (375 mmol, 5.5 mmol) and 2,2′-dithiodipyridine (485 mg, 2.2 mmol) were suspended in DMF (10 mL). Subsequently, triethylamine (170 μL) and triphenylphosphine (577 mg, 2.2 mmol) were added, and the mixture was stirred at room temperature. After ca. 5 h the reaction mixture was poured into a flask containing anhydrous sodium perchlorate (300 mg) dissolved in 30 mL of dry acetone. After cooling at 4 °C for 3 h, the resulting precipitate was filtered, washed with dry acetone and dried overnight in a vacuum desiccator over P_4O_{10} to afford 310 mg of adenosine 5′-diphosphate imidazolide sodium salt. Yield 95%.

2.5. Adenosine 5′-(3-thiotriphosphate), ATPγS

Adenosine 5′-diphosphate imidazolide sodium salt (125 mg, 0.24 mmol) was mixed with 300 mg of thiophosphate triethylammonium salt and the resultant mixture was suspended in 5 mL of DMF. Anhydrous zinc chloride (260 mg, 1.9 mmol) was added and rapid dissolution of the reagents was observed. After 20 min, the reaction was quenched by addition of EDTA solution (670 mg, 2.0 mmol in 30 mL of water) and brought to pH ~7 with sodium bicarbonate. HPLC analysis revealed formation of a product with $t_R = 2.7$ min and a yield exceeding 90%. Chromatography performed on a DEAE-Sephadex A25 column with a linear gradient of triethylammonium bicarbonate (from 0 to 1.2 M TEAB in deionized water) gave ca. 1900 absorbance units (260 nm) of ATPγS. After evaporation with addition of ethanol, the residue was redissolved in water

and freeze-dried twice to afford 135 mg of adenosine 5′-(3-thiotriphosphate) triethylammonium salt. Yield 73%.

Acknowledgements

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34. Compounds **1a**–**d** and **2a** were prepared in large-scale reactions (starting from 100 to 150 mg of the respective imidazolide derivative). These compounds were isolated as triethylammonium salts after Sephadex ion-exchange chromatography. Compounds **1e**, **1f**, **2b** and **2c** were prepared in small scale reactions (starting from ca. 20 mg of the respective imidazolide). These compounds were isolated as ammonium salts after semi-preparative RP HPLC separation.
35. Due to poorer solubility in DMF, nucleotides: m^7GMP, UMP and dCMP required about a 2-fold larger excess of imidazole and other reagents than described for GMP to achieve efficient conversion into phosphorimidazolides.