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PAPER

Synthesis of novel deoxynucleoside *S*-methylphosphonic acids using *S*-(diisopropylphosphonomethyl)isothiouronium tosylate, a new equivalent of mercaptomethylphosphonate†

Ivana Kóšiová, Miloš Buděšínský, Natalya Panova and Ivan Rosenberg*

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The synthesis of the novel nucleotide analogues 5′-deoxynucleoside-5′-*S*-methylphosphonates, starting from 5′-deoxy-5′-haloribonucleosides, 5′-*O*-tosylribonucleosides, and 2′-*O*-triflylnucleosides, is described. The phosphonothiolation of these compounds was achieved using *S*-(diisopropylphosphonomethyl)isothiouronium tosylate, a new, odourless, and efficient equivalent of mercaptomethylphosphonate. The thiolate anion of mercaptomethylphosphonate was generated *in situ* from the isothiouronium salt in both protic and aprotic solvents using two equivalents of sodium *iso*-propoxide. The prepared nucleoside 5′-*S*-methylphosphonates were deprotected, and the free phosphonic acids were transformed into diphosphoryl derivatives (the NTP analogues). Both mononucleotides and NTP analogues were studied as substrates/inhibitors of several enzymes that are involved in the nucleoside/nucleotide metabolism.

Introduction

In various areas where relatively subtle chemical modifications are needed to study biologically important systems and their functions *in vivo*, the replacement of oxygen atoms with sulfur, a change frequently employed in such investigations, is known to bring distinct new properties into the organic molecule. In contrast to the oxygen atom, the sulfur atom is bulkier, more lipophilic, and considerably more nucleophilic. In the field of nucleic acid chemistry, the well-known oligonucleotide phosphorothioates[1] may serve as an example to document dramatic changes that are due to the oxygen–sulfur replacement. These changes include increased lipophilicity and thus increased cellular uptake, increased nuclease stability, and a strong, non-specific binding to proteins.

In our laboratory, we have been continuously interested in the synthesis of novel isopolar phosphonate-based analogues of mono- and oligonucleotides and their biophysical and biological evaluation.[2–7] Modified oligodeoxynucleotides containing phosphodiester and phosphonate C3′–O–P–CH_2–O–C5″ internucleotide linkages in an alternating mode are capable of eliciting RNase H activity[8] and were found to hybridise with an RNA target similar to the natural ones.[9] In addition, several modified 2′,5′-linked tetrameric oligoadenylates containing regioisomeric phosphonate C2′–O–CH_2–P–O–C5″ internucleotide linkages were recognised as potent agonists and antagonists of RNase L.[10] The fact that all these oligomers contain an ether–ester type phosphonate linkage prompted us to extend the scope of this research by elaborating a suitable method for the synthesis of nucleotides containing the thioether–ester C5′(2′)–S–CH_2–P(O)$(OH)_2$ moiety instead of the C5′(2′)–O–CH_2–P(O)$(OH)_2$ one, as reported earlier.[10–13] We describe here the synthesis of *S*-methylphosphonates and some of their diphosphoryl derivatives (NTP analogues).

To accomplish the key thiophosphonylations (see below), we considered the use of mercaptomethylphosphonates first. The synthesis of mercaptomethylphosphonates using various precursors has been reported in the literature several times. The preparation involves their *in situ* generation by the alkaline hydrolysis of *S*-acetylthiomethyl phosphonate,[14,15] the reduction of phosphonodithioformate with sodium borohydride in acetonitrile,[16] and the reaction of lithium methylphosphonate carbanion with elemental sulfur.[17] Mercaptomethylphosphonates are useful intermediates for the synthesis of many organic compounds,[18–24] among them are certain biologically active derivatives.[15,25]

A literature search revealed that the respective dialkylphosphonomethylisothiouronium salts, a possible equivalent of mercaptomethylphosphonate, have not been synthesised so far. It was, therefore, worth trying to prepare *S*-diisopropylphosphonomethylisothiouronium tosylate, and prove its usefulness for the task.

Results and discussion

The new compound for the intended transformations, *S*-(diisopropylphosphonomethyl)isothio-uronium tosylate (**2a**), was

Institute of Organic Chemistry and Biochemistry, Academy of Sciences v. v. i., Flemingovo 2, 166 10 Prague 6, Czech Republic. E-mail: ivan@uochb.cas.cz

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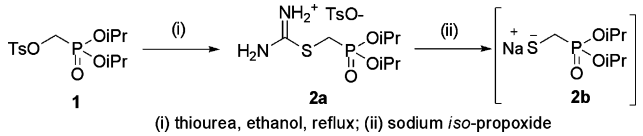
smoothly prepared from diisopropyl tosyloxymethylphosphonate[26] (**1**, Scheme 1) and thiourea by refluxing in ethanol.



Scheme 1

The precipitation with diethyl ether gave **2a** as a white solid in excellent yield. The product is odourless (an advantage over mercaptomethylphosphonate and its *S*-acetyl derivative) and stable at 4 °C.

The generation of the thiolate anion **2b** *in situ* was achieved by the alkaline hydrolysis of **2a** with two equivalents of sodium *iso*-propoxide in *iso*-propanol or DMF. The use of *iso*-propoxide to hydrolyse **2a** was chosen for its compatibility with the phosphonate ester group (to prevent a possible base-catalysed transesterification). The subsequent reaction with 5′-bromo-5′-deoxyadenosine **3a**[27] (prepared by a modified reaction of adenosine with triphenyl phosphine and carbon tetrabromide in pyridine in the presence of *N*-methylimidazole, see Experimental), 5′-deoxy-5′-iodoguanosine[28] **3b**, 5′-deoxy-5′-iodo-2′,3′-*O*-isopropylideneuridine[29] **3c** (Scheme 2), and 5′-*O*-tosylribonucleosides[30,31] **6**, **7** (Scheme 3) afforded the desired 5′-deoxyribonucleoside 5′-*S*-methylphosphonates **5**.

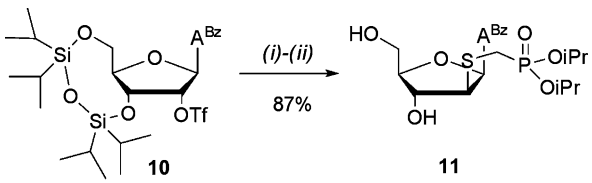
In contrast to 5′-haloribonucleosides, the use of fully protected 5′-*O*-tosylribonucleosides provided better yields (Table 1) due to the easier purification of the fully protected nucleoside phosphonates.

To prepare 2′-deoxy-2′-*S*-methylphosphonate **11**, we checked the reactivity of the protected 2′-*O*-triflyladenosine[32] **10** with the thiolate anion **2b** generated from **2a** (Scheme 4). Also, in this case, the nucleophilic displacement of the triflyloxy group for the thioate **2b** proceeded smoothly and provided an excellent yield of

Table 1

Nucleoside	Base	Solvent	Time/h	Product	Yield (%)
3a	A	iPrOH	12	**4a**	62
3b	G		18	**4b**	48
3c	U		16	**4c**	64[a]
6	A^{Bz}	DMF	1	**8 (4a)**	79 (72)[b]
7	C^{Bz}		1	**9 (4d)**	85 (76)[b]
10	A^{Bz}	DMF	2	**11**	87

[a] After removal of 2,3′-*O*-isopropylidene group. [b] After removal of 2,3′-*O*-methoxymethylidene and *N*-benzoyl groups.



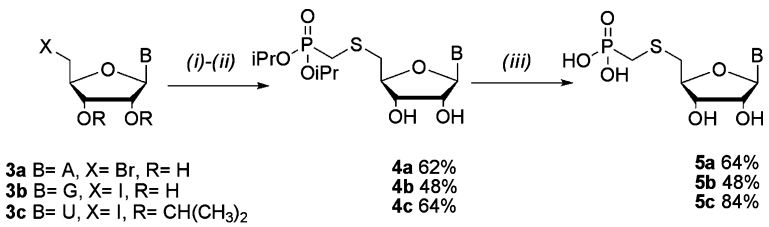
Scheme 4

the desired arabino *S*-methylphosphonate **11** after removal of the disiloxanyl group (Table 1).

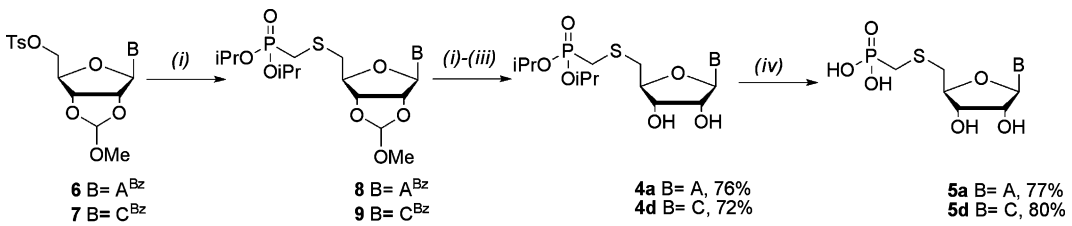
This compound was prepared as a building unit for the synthesis of the modified 2′-5′ oligoadenylates. In our recent study[10] on the RNase L agonists/antagonists, the tetramer containing an *arabino*-configured 2′-*O*-methylphosphonate unit (similar to **11** but with the 2′-oxygen instead of 2′-sulfur atom) was recognised as a potent nanomolar antagonist.

Nucleoside phosphonates **4a–d** were deprotected by treatment with bromotrimethylsilane and 2,6-lutidine in acetonitrile to afford phosphonic acids **5a–d** in good yields (Table 2).

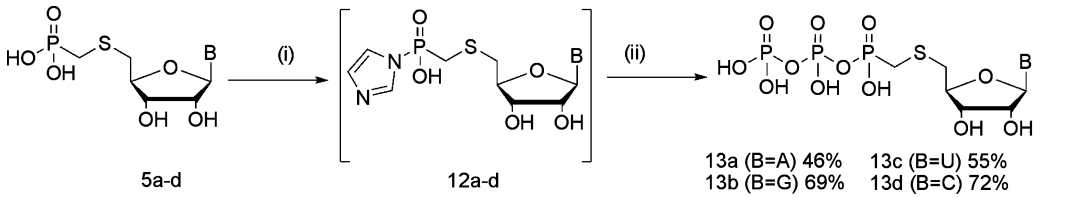
The free phosphonic acids **5a–d** were transformed into diphosphoryl derivatives **13a–d** (the NTP analogues) by Lohrmann's procedure[33] *via* phosphonoimidazolides **12a–d** as intermediates.



Scheme 2



Scheme 3



Scheme 5

Table 2

Phosphonate	Base	Phosphonic acid	Yield (%)	Diphosphoryl derivative	Yield (%)
4a	A	**5a**	64	**13a**	46
4b	G	**5b**	48	**13b**	69
4c	U	**5c**	84	**13c**	55
4d	C	**5d**	80	**13d**	72

The activation of **5** with triphenylphosphine and 2,2-dipyridyl disulfide in the presence of imidazole afforded the corresponding phosphonoimidazolides which were treated, after precipitation, with a 0.5 M solution of bis(tributylammonium)pyrophosphate in DMSO (Scheme 5). The NTP analogues **13a–d** were obtained in very good yields (Table 2) after purification on a POROS 50 HQ anion exchanger column in ammonium hydrogen carbonate.

The prepared triphosphate analogues **13a–d** exhibited very high IC_{50} values (100–250 μM) in an HCV RNA-dependent RNA polymerase competition assay.[34,35] With regard to the study with 5′-nucleotidases and purine nucleoside phosphorylase, no inhibition by the free phosphonic acids **5a–d** was observed.

Conformational analysis of compounds 4a–d and 5a–d

The vicinal proton coupling constants and the program PSEUROT,[36] based on the concept of pseudorotation,[37] were used to obtain the information regarding the conformation of the furanose ring in compounds **4a–d** and **5a–d**. The NMR conformational studies show that with nucleotides in solution, mostly two preferred conformers of the furanose ring are present in a fast equilibrium and can be fully described by five parameters (phase angles for both conformers (P(N), P(S)), puckering amplitudes (ϕ_m(N), ϕ_m(S)) and the molar concentration of one conformer (X(N) or X(S)).

Because there are only three ^3J(H,H) in the furanose ring of **4a–d** and **5a–d**, we started our PSEUROT calculation with the assumption that ϕ_m(N) = ϕ_m(S) and changed their values in 2° steps over the range of 30° to 46°, calculating P(N), P(S) and X(N) to obtain the best fit between the calculated and observed ^3J(H,H) values. The actual set of J values and a periodicity of the Karplus relation gives two solutions, A and B, for each ϕ_m (two different combinations of P(N) and P(S)). Table 3 shows the observed ^3J(H,H) values and two ranges of calculated combinations of P(N), P(S) and X(N) that give an excellent agreement between the calculated and observed ^3J(H,H) (rms ~ 0).

It can be seen from Table 3 that the calculated data are very similar, except in populations X(N) and X(S). In compounds **4a–c** and **5a–c**, the S-type conformer somewhat prevails, while in nucleosides **4d** and **5d** with the cytosine base, the N-type conformer is more populated.

To find a "physically correct" combination of the N- and S-type conformers from the alternative ones obtained from the PSEUROT calculations, we decided to explore the conformational behaviour of the studied compounds by molecular modeling. The uracil derivative **4c** (without any substituents on the sulfur atom) was chosen as the model compound. Twenty conformers, defined by phase angle P in 18° steps and ϕ_{max} = 38°, were constructed and their geometries were energetically optimised with only restrained endocyclic torsion angles using the AMBER version of the HYPERCHEM 8.0 program package.

Thus, the relation between conformer geometry and calculated energy was obtained over the entire pseudorotation pathway (see Fig. 1). The calculated ϕ_{max} in the entire range of P adopted realistic values between 34.5° and 39.5. There are two low-energy minima with P ~ 36° and ~180°, differing by approximately 0.22 kcal mol^{-1}, and the third energy minimum with approximately 1 kcal higher energy content and therefore much less probability of population. Therefore, only set A of the conformation parameters from PSEUROT is in acceptable agreement with the calculated energy in the pseudorotation pathway.

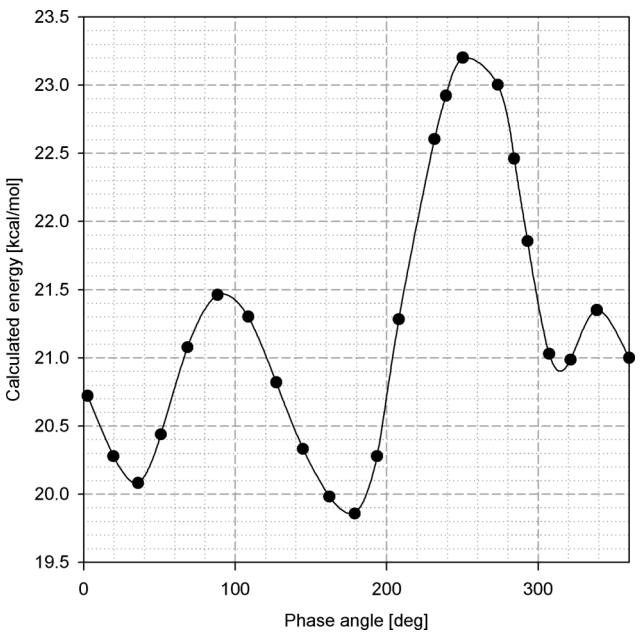


Fig. 1 Conformation analysis of the furanose ring in the uracil derivative **4c** (without any substituents on the sulfur atom) by molecular mechanics (AMBER in HYPERCHEM 8.0).

Table 3 Pseudorotation analysis of compounds **4a–d** and **5a–d** (The $\phi_{max}(N) = \phi_{max}(S)$ approximation was used and its value changed with 2° steps in the range of 30°–46°. Phase angles $P(N)$, $P(S)$ and populations $X(N)$ and $X(S)$ were optimised. Ranges of calculated parameters for set A and B are given for rms ≤ 0.001. The given $P(N)$ and $P(S)$ values were rounded to the closest unit)

Comp.	Solvent	$J(1',2')$	$J(2',3')$	$J(3',4')$	Set	$\Phi_m(N) = \Phi_m(S)$	$P(N)$	$X(N)$	$P(S)$	$X(S)$
4a	DMSO	5.58	5.17	4.00	A	34 to 44	42 to 68	0.40 to 0.35	182 to 213	0.60 to 0.65
					B	34 to 40	−11 to −34	0.40 to 0.46	178 to 135	0.60 to 0.54
4b	DMSO	6.00	4.80	3.60	A	36 to 44	29 to 62	0.34 to 0.30	175 to 206	0.66 to 0.70
					B	36 to 44	−4 to −40	0.36 to 0.44	159 to 140	0.64 to 0.56
4c	DMSO	5.46	5.46	4.44	A	36 to 42	60 to 70	0.46 to 0.42	199 to 214.	0.54 to 0.58
					B	34 to 44	−18 to −43	0.41 to 0.49	134 to 121	0.59 to 0.51
4d	DMSO	4.44	5.47	5.40	A	36 to 40	52 to 58	0.56 to 0.53	204 to 214	0.44 to 0.47
					B	38 to 42	−29 to −37	0.55 to 0.58	119 to 113	0.46 to 0.42
5a	D_2O	5.70	5.46	4.20	A	34 to 42	58 to 74	0.44 to 0.40	189 to 214	0.56 to 0.60
					B	34 to 42	−19 to −42	0.38 to 0.45	136 to 125	0.62 to 0.55
5b	D_2O	5.89	5.40	3.96	A	34 to 44	57 to 79	0.41 to 0.37	187 to 216	0.59 to 0.63
					B	34 to 42	−18 to −42	0.36 to 0.43	139 to 128	0.64 to 0.57
5c	D_2O	4.93	5.16	4.50	A	34 to 40	37 to 53	0.46 to 0.42	186 to 208	0.54 to 0.58
					B	34 to 40	−11 to −32	0.48 to 0.53	146 to 132	0.52 to 0.47
5d	D_2O	4.28	5.34	5.88	A	36 to 38	47 to 50	0.61 to 0.60	196 to 204	0.39 to 0.40
					B	36 to 42	−14 to −32	0.56 to 0.60	123 to 111	0.44 to 0.40

Table 4 Pseudorotation analysis of compounds **4a–5d** (The pseudorotation parameters $\Phi_m(N)$, $\Phi_m(S)$, $P(N)$ and $P(S)$ are fixed to calculated values from the energy minimisation in Fig. 1, and only $X(N)$ and $X(S)$ were optimised)

Compound	$X(N)$	$\Phi_m(N)$	$P(N)$	$X(S)$	$\Phi_m(S)$	$P(S)$	rms	$\Delta J(1',2')$	$\Delta J(2',3')$	$\Delta J(3',4')$
4a	0.39			0.61			0.245	−0.12	0.39	−0.11
4b	0.34			0.66			0.051	−0.06	0.05	−0.05
4c	0.44			0.56			0.382	0.03	0.66	−0.02
4d	0.55	38	36 ($_4T^3$)	0.45	37	179 ($_3T^2$)	0.369	−0.15	0.60	−0.15
5a	0.40			0.60			0.392	0.06	0.68	0.01
5b	0.37			0.63			0.366	0.06	0.63	0.01
5c	0.46			0.54			0.292	−0.28	0.34	−0.24
5d	0.59			0.41			0.262	−0.02	0.45	−0.04

To check the consistency between the results of geometry optimisation (calculated by energy minimisation; Fig. 1) and those obtainable from NMR data, we applied PSEUROT using fixed values of $P(N)$, $P(S)$, $\phi_{max}(N)$ and $\phi_{max}(S)$ (corresponding to the lowest energy conformers in Fig. 1) and only the ratio of $X(N):X(S)$ variable in the whole series of compounds **4a–d** and **5a–d**. The results are shown in Table 4. Although the rms values are somewhat worse than in Table 3, we believe that they can give a realistic picture of the conformational behaviour of the studied compounds. It is not surprising that the structural similarity of **4a–d** and **5a–d** can result in the same type of the preferred N- and S-conformers with a higher population of S-type $_3T^2$ in **4a–c** and **5a–c** (in accordance with its calculated lower energy; Fig. 1). The reason for a preference for the N-type conformer $_4T^3$ in the cytidine derivatives **4d** and **5d** is not clear. The calculated N- and S-type conformers are shown for **4c** in Fig. 2.

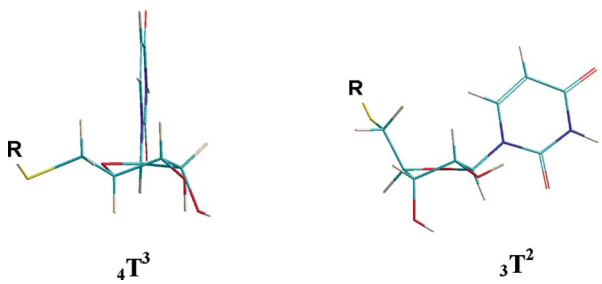


Fig. 2 The calculated N- and S-type conformers ($_4T^3$ and $_3T^2$) shown on the model of the uracil derivative **4c**.

In summary, the novel compounds 5′-deoxy-5′-phosphonomethylthioribonucleosides were synthesised by the reaction of 5′-deoxy-5′-haloribonucleoside derivatives with diisopropyl mercaptomethylphosphonate generated *in situ* from its new synthetic equivalent, the *S*-(diisopropylphosphonomethyl)isothiouronium tosylate. The NMR conformational study of both protected and unprotected nucleotides revealed a preference for the N-type conformer in the case of the only cytidine derivatives (the reasons are unclear). Novel modified 5′-ribonucleotides and their diphosphoryl derivatives (the NTP analogues) were tested as potential inhibitors of cytosolic and mitochondrial 5′-nucleotidases, and purine nucleoside phosphorylase and the NTP compounds were tested as the substrates/inhibitors of the HCV RNA-dependent RNA polymerase. Although none of the prepared compounds were recognised as inhibitors or substrates by the selected enzymes, the phosphonomethylthio motif can be further exploited in regard to its incorporation into the modified oligonucleotides, in which the internucleotide linkages C3′(2′)–O–P–**CH_2–O**–C5″ and C3′(2′)–**O–CH_2**–P–O–C5″ that have already been evaluated will be substituted with C3′(2′)–O–P–**CH_2–S**–C5″ or C3′(2′)–**S–CH_2**–P–O–C5. Further work on this topic is underway and will be reported elsewhere.

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