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Synthesis of nucleoside phosphosulfates

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ABSTRACT

We describe an efficient and scalable procedure for the chemical synthesis of nucleoside 5′-phosphosulfates (NPS) from nucleoside 5′-phosphorimidazolides and sulfate bis(tributylammonium) salt. Using this method we obtained various NPS with yields ranging from 70–90%, including adenosine 5′-phosphosulfate (APS) and 2′,3′-cyclic precursor of 3′-phosphoadenosine 5′-phosphosulfate (PAPS), which are the key intermediates in the assimilation and metabolism of sulfur in all living organisms.

Adenosine 5′-phosphosulfate (APS) and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) are important intermediates involved in metabolism of sulfur in various organisms.[1] Both play a vital role in the assimilation of sulfur in plants and bacteria.[2] PAPS is the 'active sulfate' form in higher organisms, including mammals. It serves as a sulfate donor in glycosaminoglycan sulfation reactions, which are important, for instance, for blood coagulation, as well as in sulfation of peptides, proteins, lipids, hormones and other small molecules.[3,4]

Hence, both APS and PAPS have been used for studying sulfotransferases and sulforeductases in various organisms.[5] APS has also found a biotechnological use in DNA pyrosequencing.[6] Several human sulfate-transferring enzymes that utilize PAPS are related to the development of diseases such as cancer and neurodegenerative disorders,[4,7] and some have been suggested as suitable drug targets.[8] The phosphosulfate derivatives of other nucleosides have not been thoroughly investigated, nonetheless, cytidine 5′-phosphosulfate (CPS) has been identified as a putative intermediate in the biosynthesis of some sulfated steroles.[9] Moreover, nucleoside 5′-phosphosulfates (NPS) may be, in general, considered as structural analogs of nucleoside 5′-diphosphates (NDP), since they retain a similar geometry, but lack one of the phosphate's ionizable —OH group. Thus, they may be used for studying specificity of pyrophosphatases,[10] or other nucleotide-binding enzymes and proteins.

NPS are relatively unstable (easily hydrolyze to NMP and sulfate), and due to this, both their synthesis and purification are difficult. APS was chemically synthesized for the first time over 50 years ago by Baddiley et al. and Reichard & Ringertz,[11,12] but the isolated yields, if reported, were poor. PAPS and its 2′,3′-cyclic precursor have been synthesized in a similar way.[13,14] 5′-Phosphosulfates of guanosine (GPS) and uridine (UPS) have been synthesized and used to study bacterial sulforeductases, but the detailed procedures have not been provided in this report.[15,16]

Probably, due to not very promising initial results, the chemical syntheses of NPS have not been improved over the years, but instead, (chemo)enzymatic methods have been developed.[17] However, these methods are usually more costly and bear other limitations, e.g. those arising from substrate specificity of sulfotransferases. Hence, we assumed that a simple and economical chemical procedure for NPS preparation could make them more accessible and encourage their broader exploitation in biochemical and biophysical studies.

In our and others experience,[18,19] metal (II) chloride (usually $ZnCl_2$ or $MgCl_2$) mediated coupling of P-nucleophilic and P-electrophilic phosphate subunits in non-aqueous polar solvent has been a useful tool in the synthesis of nucleoside oligophosphates and their analogs modified within phosphate bridge. Hence, we decided to explore whether such approach can be used in reactions employing sulfate anion as a nucleophile. As a result, in this work we present a rapid and efficient procedure for the chemical synthesis of APS, PAPS and other NPS, based on phosphorimidazolide chemistry. We also provide, for the first time, ^{1}H and ^{31}P NMR characterization of these compounds, together with some data on their stability. Finally, we demonstrate an example of how NPS may be employed as isosteric analogs of NDP for studying nucleotide-protein interactions.

The general route for the synthesis of NPS **(2a–e)** is depicted in Scheme 1. The key step was a coupling reaction of an activated nucleoside monophosphate species, an appropriate nucleoside 5′-phosphorimidazolide (**1a–e**), with sulfate organic salt in DMF and

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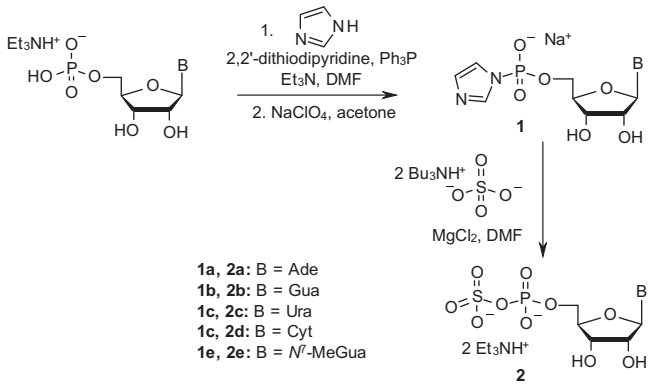


Scheme 1. Synthesis of nucleoside 5′-phosphosulfates (**2a–2e**).

in the presence of divalent metal chloride. As a model reaction, we chose the synthesis of APS **(2a)**. First, adenosine 5′-monophosphate (free acid) was converted into its triethylammonium salt and activated with imidazole using triphenylphosphine/dithiodipyridine redox system to yield **1a**. We utilize this method for obtaining phosphorimidazolides, instead of the usually employed activation with CDI, since it enables very convenient isolation of bulky amounts of the desired product by simple precipitation with $NaClO_4$ acetone solution, and the product can be stored in this form for several months. As a nucleophilic reagent, we tested several organic salts of sulfate (VI): triethylammonium, pyridinium, tributylammonium and *N*,*N*-dimethyanilinium. Triethylammonium sulfate was a hygroscopic semi-solid at RT, and thus was inconvenient for storage and handling. Bis(tributylammonium) sulfate was initially obtained in a similar form, but turned into an easy-to-handle solid after drying in vacuum over P_2O_5 for several days. *N*,*N*-dimethylanilinium and pyridinium salts were solids, but produced strong HPLC signals with long retention time, which complicated the analysis of the reaction mixtures. Since all salts displayed comparable reactivity in the following coupling reaction, we selected bis(tributylammonium) sulfate as the most suitable form of organic sulfate for further studies.

Reaction of **1a** and bis(tributylammonium) sulfate (4 equiv) in DMF in the presence of anhydrous $ZnCl_2$ (10 equiv) was monitored by RP HPLC and MS(-)ES in 5 min intervals. Surprisingly, the formation of APS (**2a**) was very rapid after 20 min the expected product constituted ca. 80% of the reaction mixture (Fig. 1 A). Formation of AMP and diadenosine 5′,5′-diphosphate were observed as side reactions. However, when the reaction time was slightly elongated fairly fast conversion of **2a** into AMP was observed; after 35 min its content decreased to only 40% (Fig. 1C). Since, not only APS formation, but its decomposition was relatively rapid, we reckoned that the procedure was not fully reliable.

Interestingly, studies on the phosphosulfate bond cleavage revealed that NPS and related compounds undergo acid-catalyzed unimolecular elimination of sulfur trioxide, and that the reaction may be accelerated by divalent metal cations both in aqueous and non-aqueous media.[20] Thus, we assumed that conversion of APS into AMP in the reaction mixture was catalyzed by protons generated upon $ZnCl_2$ hydrolysis in the presence of moisture and presumably also by zinc cations. Therefore, we replaced $ZnCl_2$ with a less acidic salt, $MgCl_2$. Under these conditions the reaction was comparably fast, slightly more efficient, but most importantly the product was more stable (Fig. 1B and C). Consequently, via $MgCl_2$-activated coupling, **2a** could be synthesized and isolated after ion-exchange chromatography (DEAE-Sephadex) with 88% yield.

Employing these optimized conditions we have synthesized and isolated a set of four other nucleoside 5′-phosphosulfates (compounds **2b–e**), differing in nucleobase size and polarity (G, U, C and m^7G, Scheme 1, Table 1). In all cases the HPLC yields were almost quantitative, whereas preparative yields were above 70% (Table 1). Compounds **2a–d** did not require additional purification and after DEAE-Sephadex chromatography, were converted from triethylammonium to sodium salts by precipitation with $NaClO_4$ acetone solution. Compound **2e**, bearing a positively charged nucleoside 7-methylguanosine, likely due to its smaller negative net charge compared to other NPS, partially co-eluted with unreacted sulfate from DEAE-Sephadex, and thus, required additional purification by RP HPLC.

Structures of all final compounds have been confirmed by a combination of high resolution MS(-)ES, ^1H and ^{31}P NMR (Table 1 and Supplementary data). The NPS have nominal masses similar to those of corresponding NDP, but in contrast to them give only one signal in ^{31}P NMR. Although ^{32}S is not NMR active, NPS could also be easily distinguished from NMP by ^{31}P NMR, since their signal shifted ca. 12 ppm downfield from the signal of NMP (e.g. δ ppm + 2.16 vs −9.27 for GMP and GPS, respectively). NPS purities determined both by ^{31}P NMR and RP HPLC were all above 90%, which is a standard value for commercial APS.

Finally, we employed a slightly modified approach to attempt the synthesis of PAPS starting from commercially available 3′-adenylic acid (3′-AMP) by a route presented in Scheme 2, path A. The crucial intermediate, 3′-phosphoadenosine 5′-phosphorimidazolide, was attempted in a one pot three-step reaction. First, 3′-AMP was 5′-phosphorylated in Yoshikawa conditions,[21] then reacted with excess imidazole and finally hydrolyzed to monoimidazolide in mildly basic pH. However, during the first step, 3′-AMP and its 5′-posphorylated derivative converted into 2′,3′-cyclic forms. Thus, 2′,3′-cyclic phosphoadenosine 5′-phosphorimidazolide **(3)** was obtained as the actual product of the reaction sequence. The product was isolated by DEAE Sephadex with only 50% yield, since the initial phosphorylation step was moderately efficient (about 70% by

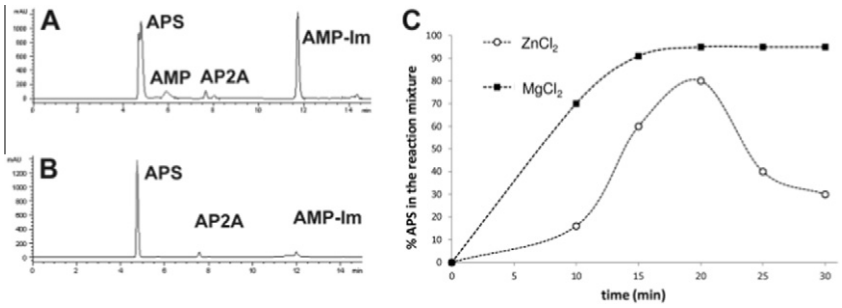
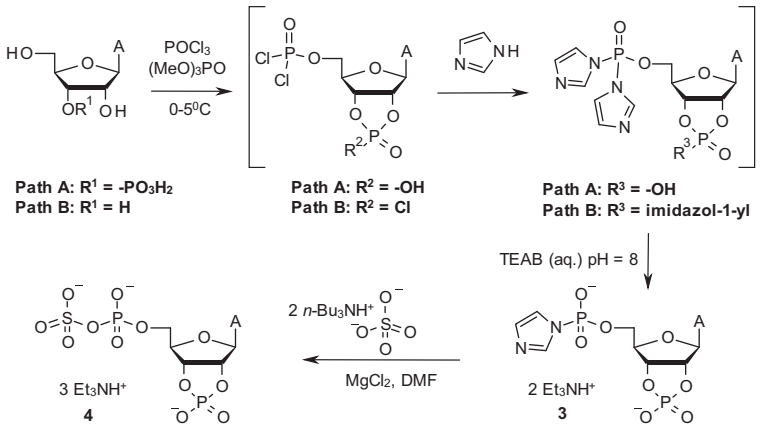


Figure 1. Example RP HPLC profiles from reactions of **1a** (AMP-Im) with bis(tributylammonium) sulfate (4 equiv) in DMF at RT in the presence of either (A) 10 equiv $ZnCl_2$ or (B) 10 equiv $MgCl_2$; (C) APS content in the same reaction mixtures as a function of time.

Table 1
Synthesized nucleoside 5′-phosphosulfates

Entry	Compound		Reaction time (min)	HPLC Conversion (%)[a]	Isolated yield (%)[b]	HPLC R_t (min)[c]	HRMS(-)ES *m/z* found (Calculated)	^{31}P NMR δ (ppm)[d]	^{31}P NMR purity (%)[d]
	No	Abbreviation							
1	**2a**	APS	20	92	88	3.3	426.0137 (426.0126)	−9.74	95
2	**2b**	GPS	20	91	84	3.0	442.0083 (442.0075)	−9.75	96
3	**2c**	UPS	30	96	86	2.3	402.0019 (402.0014)	−9.73	92
4	**2d**	CPS	18	100	84	2.3	402.9872 (402.9854)	−9.88	>99
5	**2e**	m^{7}GPS	20	85	72	4.9	456.0241 (456.0232)	−10.36	93
6	**4**	2′,3′-cPAPS	15	88	70	3.3	487.9693 (487.9684)	20.59; −9.93	>99

[a] Determined by RP HPLC analysis of reaction mixtures before DEAE-Sephadex purification.
[b] Determined by optical density unit measurements of the starting phosphorimidazolides (**1a–e**, **3**) and isolated final compounds.
[c] For final product.
[d] Determined in D_2O, at 25 °C and 162 MHz.



Scheme 2. Synthesis of 2′,3′-cPAPS (**4**).

HPLC). A reaction of **3** with bis(tributylammonium) sulfate in the presence of $MgCl_2$ under conditions analogous for compounds **2a–e** resulted in the formation of 2′,3′-cPAPS (**4**) as a major product (88% HPLC conversion) together with minute amounts of PAP. After DEAE-Sephadex and RP HPLC purification, **4** was isolated in 70% yield. It has been reported previously that **4** can be converted into PAPS by ribonuclease-T_2 with ca. 70% yield.[14] Since the 2′,3′-cyclicli-sation of 3′-AMP during phosphorylation was unavoidable, we simplified the synthesis of **3** by replacing the first step with exhaustive phosphorylation of adenosine (Scheme 2, path B).

Finally, we searched for optimal conditions for NPS storage both as solid samples and in aqueous solutions. In general agreement with previous studies,[20] we found that the 1 mM NPS solutions containing EDTA were stable for longer than 24 h at pH 9 and 7. At pH 2, hydrolysis to corresponding NMP took place with an approximated half-time of 12–18 h. Moreover, we have found that aqueous samples alkalized with sodium bicarbonate could be stored for several weeks at 4 °C without decomposition. Addition of EDTA and Na_2CO_3 also stabilized solid samples, which under such conditions remained intact after over 1 year of storage, whereas samples without any additives underwent partial decomposition ranging from 10% to even 40%.

NPS may be considered as analogs of NDP, and hence, used to determine specificity of some nucleotide binding proteins. To demonstrate this we treated compound **2e** as an isoster of naturally occurring molecule m^{7}GDP and tested its interaction with eukaryotic initiation translation factor 4E (eIF4E) by means of fluorescence quenching titration. eIF4E is a protein responsible for the recognition of eukaryotic m^{7}G mRNA cap during translation initiation.[22] From biophysical and crystallographic studies,[23] it is known that hydrogen bonding and salt bridges between the negatively charged phosphate chain and positively charged aminoacids in the eIF4E's cap binding cavity are important for complex stabilization.

Table 2
Binding affinity of m^{7}GPS (**2e**) for eIF4E protein

Compound	$K_{AS\ (eIF4E)}$ (μM^{-1})[a]	$\Delta G°$ (kcal/mol)
m^{7}GDP[b]	28.9 ± 1.5	−10.00 ± 0.03
m^{7}GPS (**2e**)	1.74 ± 0.44	−8.37 ± 0.15
m^{7}GMP[b]	1.5 ± 0.1	−8.28 ± 0.04

[a] Determined in 50 mM Hepes/KOH (pH 7.2), 100 mM KCl, 0.5 mM EDTA, 2 mM DTT at 20 ± 0.2 °C.
[b] Data from Ref.[24].

The terminal sulfate in m^{7}GPS bears only one ionizable hydroxyl group, in contrast to two groups in the terminal phosphate of m^{7}GDP, and thus has a smaller net negative charge under physiological pH. Consequently, the equilibrium binding affinity constant (K_{AS}) for m^{7}GPS-eIF4E complex determined by fluorescence quenching titration was ~10-fold lower than the corresponding value for the m^{7}GDP-eIF4E complex (Table 2). The $\Delta\Delta G^0$ value of −1.6 kcal/mol may correspond to the stabilizing interaction provided by the terminal phosphate's second negative charge in m^{7}GDP. Notably, the K_{AS} value for eIF4E-m^{7}GPS is similar to that of eIF4E-m^{7}GMP complex, which corresponds well with the fact that both nucleotides have similar net charge under experimental conditions.

In summary, we proposed a general method for the synthesis of nucleoside 5′-phosphosulfates. The synthesis is straightforward, very efficient and can be performed starting from commercially available nucleotides or nucleosides without the need of

employing protecting groups or harsh reaction conditions. The method can also be useful for the synthesis of nucleoside phosphosulfates and related compounds that are unavailable via enzymatic methods (e.g. those modified within nucleobase or sugar moiety). We synthesized, isolated with good yields (70–90%) and characterized APS and four other NPS. Using a slightly modified approach we also chemically synthesized 2′,3′-cPAPS. Beside the biotechnological utility of APS in pyrosequencing, these nucleoside phosphosulfates may serve as tools for more detailed studies on the mechanisms and substrate specificity of sulfotransferases and sulforeductases as well as, if reckoned as structural analogs of NDP, for studying other proteins and enzymes.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.04.039.

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