Enzymatic Synthesis of Nucleoside-5′-*O*-(1-thiophosphates) and (S_P)-Adenosine-5′-*O*-(1-thiotriphosphate)

by **Vladimir N. Barai***[a]), **Anatoli I. Zinchenko**[a]), **Sergei V. Kvach**[a]), **Olga I. Titovich**[a]), **Elena B. Rubinova**[b]), **Elena N. Kalinichenko**[b]), **Igor A. Mikhailopulo***[b])

[a]) Institute of Microbiology, National Academy of Sciences, 220141 Minsk, Acad. Kuprevicha 2, Belarus (e-mail: zinbar@mbio.bas-net.by)

[b]) Institute of Bioorganic Chemistry, National Academy of Sciences, 220141 Minsk, Acad. Kuprevicha 5, Belarus (phone: +358/40/085 10 55; fax: +358/17/16 24 56; e-mail: Igor.Mikhailopulo@uku.fi)

Treatment of adenosine with $PSCl_3$ in trimethyl phosphate gave, after ion-exchange chromatography, adenosine-5′-*O*-monophosphate (AMP; 28%) and adenosine-5′-*O*-monothiophosphate (AMPS; 48%). AMPS was studied as a thiophosphate residue donor in an enzymatic transphosphorylation with nucleoside phosphotransferase (NPase) of the whole cells of *Erwinia herbicola*. As exemplified by a number of natural and sugar- and base-modified nucleosides, it was demonstrated that NPase of the whole cells of *Erwinia herbicola* catalyzes the transfer of both thiophosphate and phosphate residues with a similar efficiency. An incubation of AMPS in a phosphorylating extract of *Saccharomyces cerevisiae* (K-phosphate buffer (0.3 M, pH 7.0); 3% glucose; 15 mM $MgCl_2$; 28°, 8 h), followed by ion-exchange column chromatography afforded AMP (8%), AMPS (recovered, 23%), ATP (11%), and (S_P)-adenosine-5′-*O*-(1-thiotriphosphate) ((S_P)-ATPαS); (total yield 37%; 48% based on the consumed AMPS). For comparison of physicochemical properties, adenosine was chemically transformed into ATPαS as a mixture of the (S_P) (53%) and (R_P) (44%) diastereoisomers.

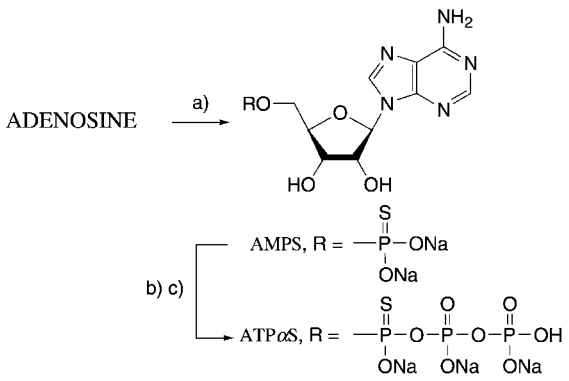
Introduction. – The individual (S_P) and (R_P) diastereoisomers of nucleoside-5′-*O*-(1-thiotriphosphates) (NTPαS) are finding diverse applications in biochemistry and molecular biology (for reviews, see [1][2]). Recently, ribonucleoside-5′-*O*-(1-thiotriphosphates) modified in the base or the pentofuranose moiety have been employed as valuable tools for elucidation of the chemical basis of RNA structure and function (Nucleoside Analog Interference Mapping (NAIM) experiment) [3]. There are a number of chemical (see, *e.g.*, [4][5]) and enzymatic [6–8] methods for the preparation of NTPαS. As might be expected, chemical methods gave rise to the formation of the (S_P) and (R_P) diastereoisomeric mixtures of NTPαS, separation of which may be achieved by HPLC [4][5]. On the contrary, the use of a coupled enzyme system comprising adenylate kinase/ATP and pyruvate kinase/phosphoenol pyruvate allowed to transform stereospecifically AMPS into (S_P)-ATPαS that was isolated as barium salt in 53% yield [8].

We have earlier shown that the kinase system of an extract of the *Saccharomyces cerevisiae* cells effectively transforms all the purine and natural pyrimidine nucleoside-5′-monophosphates (NMP) to nucleoside-5′-triphosphates (NTP) [9]. The work presented here provides a simple synthesis of (S_P)-ATPαS from AMPS employing the kinase system of *Saccharomyces cerevisiae* as a biocatalyst. The latter was synthesized chemically from adenosine and was studied as a thiophosphate residue donor in an enzymatic transphosphorylation by using NPase of the whole cells of

Erwinia herbicola. In our previous studies, we have demonstrated that the whole cells of *E. herbicola* can be employed as a biocatalyst for the preparation of NMPs from nucleosides with 4-nitrophenyl phosphate as a donor of the phosphate residue [9].

Results and Discussion. – The starting AMPS (Na^+ salt) was prepared by the reaction of adenosine with $PSCl_3$ in trimethylphosphate in the presence of 2,6-dimethylpyridine at 0° for 3 h (*cf.*, e.g., [8]), followed by *i*) treatment with aqueous 0.5 M triethylammonium bicarbonate (TEAB) solution, *ii*) ion-exchange column chromatography, and *iii*) transformation of triethylammonium salts of the individual compounds into Na^+ salts [10]. In order of elution, AMP (28%) and adenosine-5′-*O*-monothiophosphate (AMPS; 48%) were obtained (*Scheme*).

Scheme



a) $PSCl_3$/2,6-lutidine/$PO(OMe)_3$, 0°, 3 h; DEAE-*Sephadex A-25* (HCO_3^- form; TEAB buffer); NaI/acetone (AMPS, 48%; AMP, 28%). b) AMPS/phosphorylating extract from *S. cerevisiae*, 28°, 8 h; DEAE-*Sephadex A-25* (HCO_3^- form; TEAB buffer); NaI/acetone (AMPS, recovered 23%; AMP, 8%; ATP, 11%; (S_P)-ATPαS, 37% (48% based on the consumed AMPS)). c) $PSCl_3$/2,6-lutidine/$PO(OMe)_3$, 0°, 3 h; $P_2O_7^{2-}$ ($HNBu_3^+$)$_2$/DMF, 20°, 3 h; DEAE-*Sephadex A-25* (HCO_3^- form; TEAB buffer); NaI/acetone (ATPαS, 24% (S_P)-diastereoisomer 53%; (R_P)-diastereosiomer 44%)].

As a continuation of our previous studies on enzymatic synthesis of NMPs [9], we became interested in the use of AMPS as a universal donor of the thiophosphate group. The data on substrate properties of AMPS *vs.* AMP in the transphophorylation reaction catalyzed by NPase of the whole cells of *E. herbicola* are presented in *Table 1*. The most striking finding of this enzymatic reaction consists *i*) in a high efficiency of transfer of the thiophosphate residue from a donor, AMPS, to nucleoside acceptors along with *ii*) an inability of the NPase to hydrolyze the thiophosphate group of AMPS in the absence of an acceptor (*Table 2*). In the case of the natural donor of the phosphate residue, AMP, NPase can catalyze both reactions, the transfer of the phosphate group from a donor to an acceptor and dephosphorylation of AMP to adenosine (*Table 2*). It is noteworthy that NPase displayed similar activity regarding the transfer of phosphate and thiophosphate residues from the respective donor nucleotide to a wide range of natural as well as sugar- and base-modified nucleosides (*Table 1*). This finding is in drastic contrast to NPases from plant sources, NPase

Table 1. *Activity of Nucleoside Phosphotransferase (NPase) of the Whole Cells of* E. herbicola *with AMPS* vs. *AMP as the Respective Thiophosphate or Phosphate Group Donors*

Acceptor	NPase activity [unit · mg^{-1}]		Acceptor	NPase activity [unit · mg^{-1}]	
	Donor			Donor	
	AMPS	AMP		AMPS	AMP
2′-dAdo	6.9	4.6	Ara-Gua	3.0	5.7
3′-dAdo	4.9	3.0	Ara-Cyt	1.5	3.3
2′-dGuo	8.6	12.8	Ara-Thy	7.8	4.5
2′-dUrd	5.0	8.0	Ara-Ura	3.3	2.6
2′-dCyd	7.8	5.8	Thd$^{3'NH_2}$	10.0	15.6
Cyd	8.2	8.7	AZT	5.7	4.5
Urd	10.0	3.5	5Br-2′-dUrd	6.0	6.1
Thd	8.0	7.0	5I-2′-dUrd	5.0	4.5
			BVDU	2.2	0.8

Table 2. *Phosphotransferase and Phosphatase Activities of NPase of the Whole Cells of* E. herbicola *towards AMPS and AMP*

Substrate	Enzymatic activity			
	Transphosphorylation[a])		Dephosphorylation[b])	
	unit · mg^{-1}	rel. [%]	unit · mg^{-1}	rel. [%]
AMPS	8.6	67.2	0.08	1.2
AMP	12.8	100	6.8	100

[a]) 2′-dGuo was used as an acceptor in these experiments. [b]) Without an acceptor nucleoside.

activity of which was found to be much lower in relation to the thiophosphate group than in relation to the phosphate group [2].

An incubation of AMPS in a phosphorylating extract prepared from baker's yeast [9] (see *Exper. Part*) at 28° for 8 h, followed by ion-exchange column chromatography, afforded AMP (8%), AMPS (recovered, 23%), ATP (11%), and (S_P)-ATPαS (37%; 48% based on the consumed AMPS). The yield of (S_P)-ATPαS was not optimized. All individual compounds were obtained as amorphous Na^+ salts [10] and characterized by UV spectroscopy, HPLC comparisons with authentic samples, and by ^{1}H- and ^{31}P-NMR spectroscopy in the case of (S_P)-ATPαS as well. The ^{31}P-NMR spectrum of (S_P)-ATPαS is presented in the *Figure*. Moreover, for comparison, adenosine was chemically transformed into ATPαS as a mixture of the (S_P)- and (R_P)-diastereoisomers (53 and 44%, resp.) in a 'one-pot' procedure [5].

The HPLC and ^{31}P-NMR data are evidenced in favor of the formation of the (S_P)-diastereoisomer of ATPαS in an enzymatic phosphorylation of AMPS by a phosphorylating extract prepared from commercial baker's yeast (*cf.* the HPLC and ^{31}P-NMR data for the (S_P) and (R_P) diastereoisomers of ATPαS in [4]).

In conclusion, the approach described here represents a simple synthesis of (S_P)-ATPαS, which shows certain advantages over both chemical [4][5] and enzymatic [8] methods. Despite the rather moderate yield of the desired (S_P)-ATPαS, this method is rapid and does not require protection of any functional groups of nucleoside.

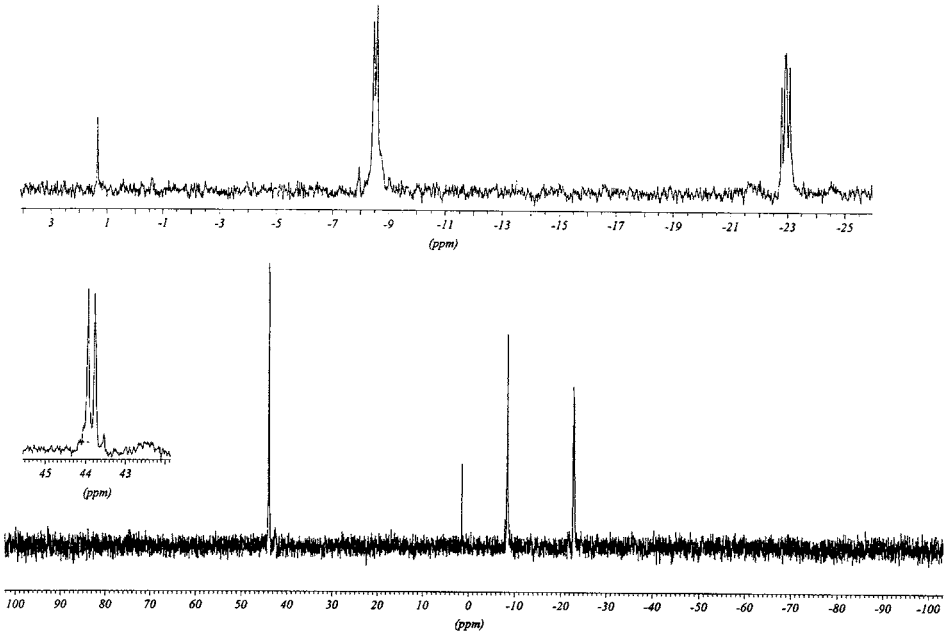


Figure. *The ^{31}P-NMR spectrum of (S_P)-ATPαS in D_2O prepared by an enzymatic phosphorylation of AMPS*

Experimental Part

1. *General.* TLC: Standard silica gel *$60F_{254}$* (*Merck*, Germany); solvent system i-PrOH/25% aq. NH_3/H_2O, 11:7:2. HPLC: *Waters* apparatus (column *Nova-Pac C-18* (3.9 × 300 mm, *Waters*); *Waters* pump control module, *Waters 515* HPLC pump; *Waters 996* photodiode array detector operating at 254 nm); isocratic elution with the following buffer: 7% MeCN in 0.1M KH_2PO_4, (*v*/*v*) at a flow rate 0.7 ml/min (time of analysis 15 min). UV Spectra: in EtOH on a *Specord M-400* spectrometer (*Carl Zeiss*, Germany). ^{1}H- and ^{31}P-NMR Spectra: at 400.13 and 161.98 MHz, at 23° on an *Bruker AM-400WB* spectrometer in D_2O with Me_4Si and H_3PO_4 as external standards, respectively; assignments of δ(H) by selective homonuclear decoupling experiments; δ(TMS) and *J* values in ppm and Hz, resp.

Natural nucleosides and their 5′-monophosphates (for TLC and HPLC comparisons) as well as 3′-azido- (AZT) and 3′-amino- ($Thd^{3'NH_2}$) -3′-deoxythymidines, 1-(β-D-arabinofuranosyl)thymine (ara-Thy) and -uracil (ara-Ura), and 2′-deoxyuridine (2′d-Urd) used in the present study are commercially available products. The following compounds have been prepared as described previously: 1-(β-D-arabinofuranosyl)cytosine (ara-C) and its 5′-monophosphate (ara-CMP) by chemical methods [11]; 9-(β-D-arabinofuranosyl)guanine (ara-G) by enzymatic transglycosylation [12], and its 5′-monophosphate by a chemical method [13]; 5-bromo- (5Br-Urd), 5-iodo- (5I-Urd), and (*E*)-5-bromovinyl- (BVDU) -2′-deoxyuridine by an enzymatic transglycosylation [14]. Purities of all compounds were checked by TLC and HPLC, and the structures were established by ^{1}H-NMR spectroscopy. Bis(tributylammonium) pyrophosphate was prepared according to *Moffatt* [10].

2. *Biocatalysts.* The biomass of *E. herbicola* 47/3 cells possessing high NPase (E.C. 2.7.1.77) activity was grown in 250-ml *Erlenmeyer* flasks containing 50 ml of the following medium ($g \cdot l^{-1}$): Na_2HPO_4 (6.0), KH_2PO_4 (3.0), NaCl (0.5), NH_4Cl (1.0), $MgSO_4$ (0.12), bactopeptone (*Sigma*, USA) (3.0), and yeast extract (*Serva*, Heidelberg, Germany) (5.0). The initial pH of the culture medium was adjusted to 7.0. Cultivation was carried out on a circular shaker (180–200 rpm) at 28° for 24 h. Biomass was harvested by centrifugation (4500 × g, 10 min), suspended in two volumes of 0.85% NaCl, and separated by centrifugation (4500 × g, 10 min). This procedure was repeated to furnish the cells as a paste. To standardize the quantity of the cells taken into a reaction, dry weight of the cells in a paste was measured after drying an aliquot at 105° for 24 h.

A phosphorylating extract was prepared from commercial baker's yeast with slight modification of the procedure described in [9]. The yeast cells were dried at 28° for 24 h and then treated with 0.3M K-phosphate buffer (pH 7.0; 10 ml/g of dry yeast) containing 15 mM $MgCl_2$ and 3% glucose. The suspension was kept for 3 h at 28° and then centrifuged (8000 × g, 5 min). The supernatant was used as the source of phosphorylating enzymes.

3. *Assay of NPase Activity.* The progress of the NMPS's and NMP's synthesis with the use of the whole *E. herbicola* cells was monitored by HPLC and TLC [9]. The reaction mixture (1 ml) containing donor (10 mM) of the thiophosphate group, AMPS, or phosphate group, AMP, nucleoside acceptor (50 mM), sodium acetate buffer (0.2M, pH 4.5), and wet paste of intact *E. herbicola* cells (6.0 mg, calculated as abs. dry weight) was incubated at 50° with gentle stirring. Aliquots of the reaction medium were analyzed by the TLC on *Silufol UV-254* plates (*Serva*, Germany; i-PrOH/25% aq. NH_3/H_2O 7:2:1 (*v*/*v*)) and/or by HPLC with a molar extinction coefficients for long-wave maximum for nucleoside under investigation. The amount of the enzyme producing 1 nmol of NMPS per min was taken to be one unit.

4. *Adenosine-5′-*O*-(1-thiophosphate)*. A soln. of adenosine (0.257 g, 0.96 mmol) in trimethyl phosphate (2.5 ml) was heated on the glycerin bath from 20° to 100° during 20 min and then rapidly cooled to 0°. To this soln., 2,6-lutidine (0.4 ml, 3.5 mmol) and then $PSCl_3$ (0.3 ml, 3 mmol) were added, and the mixture was stirred for 3 h at 0°. The mixture was poured into 0.5M TEAB (20 ml), stirred at 20° for 3 h, and evaporated. The residue was chromatographed on a DEAE-*Sephadex A-25* (HCO_3^--form, 2.5 × 30 cm) column with a linear gradient (0.001 → 0.6M, pH 7.6; 2 × 500 ml) of TEAB buffer. The product-containing fractions were collected, evaporated to dryness, and transformed into Na^+ salts [10] to give, in order of elution, adenosine-5′-*O*-monophosphate (AMP; 106 mg, 28%) and AMPS (188 mg, 48%) as amorphous powders; TLC: R_f 0.51 and 0.56, resp. HPLC of AMPS: t_R 7.58 min (λ_{max} 258 nm; 96.7%); this product contained AMP according to the HPLC, t_R 5.31 min (λ_{max} 258 nm; 2.6%). ^{1}H-NMR ((D$_6$)DMSO): 8.50 (*s*, H–C(8)); 8.16 (*s*, H–C(2)); 7.32 (br. *s*, C(6)–NH_2)); 5.92 (*d*, *J*(1′,2′) = 6.5, H–C(1′)); 4.68 (*t*, *J*(2′,3′) = 6.5, H–C(2′)); 4.24 (br. *s*, H–C(3′)); 4.06 (br. *s*, H–C(4′)); 3.84 (br. *s*, H–C(5′)).

5. *Synthesis of Adenosine-5′-*O*-(1-thiotriphosphate)*. a) *Enzymatic Synthesis.* A mixture containing AMPS (Na^+ salt; 56.7 mg, 0.14 mmol) and the phosphorylating extract (6 ml) was incubated at 28° for 8 h with gentle stirring. The reaction was stopped by boiling for 3 min with subsequent clarification of the mixture by centrifugation (5000 × g, 10 min). The supernatant was chromatographed on a DEAE *Sephadex A-25* (HCO_3^- form, 1.8 × 27 cm) column with a linear gradient (0.001 → 1.0M, pH 7.6; 2 × 400 ml) of TEAB buffer. In order of elution, the following products were isolated and transformed to their Na^+ salts: AMP (4 mg, 8%), AMPS (13 mg, 23%), ATP (9 mg, 11%; HPLC: t_R 3.67 min (λ_{max} 258 nm)), and (S_p)-ATPαS (30.5 mg, HPLC: t_R 4.21 min (λ_{max} 258 nm); 37%; 48% based on the consumed AMPS).

(S$_p$)-ATPαS: ^{1}H-NMR (D$_2$O): 8.62 (*s*, H–C(8)); 8.21 (*s*, H–C(2)); 6.10 (*d*, *J*(1′,2′) = 5.92, H–C(1′)); H–C(2′) and H–C(3′) resonances are overlapped by the intense HOD signal; 4.38 (*m*, H–C(4′)); 4.30 (*ddd*, *J*(5′,4′) = 2.49, *J*(5′,P) = 7.79, *J*(5′,5″) = 11.83, H–C(5′)); 4.23 (*ddd*, *J*(5′,4′) = 3.27, *J*(5′,P) = 5.61, H–C(5′)). ^{31}P-NMR (H$_2$O): 43.85 (*d*, *J*(Pα,Pβ) = 27.46, Pα); −8.52 (*d*, *J*(Pγ,Pβ) = 19.33, Pγ); −22.93 (*dd*, Pβ).

b) *Chemical Synthesis.* A soln. of adenosine (0.267 g, 1.0 mmol) in trimethyl phosphate (2.6 ml) was heated on a glycerin bath from 20° to 100° during 20 min and then rapidly cooled to 0°. 2,6-Lutidine (0.46 ml, 4.0 mmol) and then $PSCl_3$ (0.2 ml, 2.0 mmol) were added to the soln., and the mixture was stirred at 0° for 5 h. A mixture of 0.5M bis(tributylammonium) pyrophosphate in anh. DMF (3.5 ml) and Bu_3N (0.5 ml) was then added, and stirring was continued at r.t. for 30 min. The mixture was poured into 0.5M TEAB (10 ml), stirred at 20° for 3 h, and evaporated. The residue was chromatographed on a DEAE-*Sephadex A-25* (HCO_3^- form, 2.5 × 30 cm) column with a linear gradient (0.001 → 1.0M, pH 7.6; 2 × 700 ml) of TEAB buffer. The product-containing fractions were collected, evaporated, dissolved in MeOH, and treated with a NaI soln. in acetone [10]. The precipitate was centrifuged (4000 × g, 5 min), washed twice with acetone and centrifuged, dried *in vacuo* over $CaCl_2$ at r.t. overnight to give 0.143 g (24%) of ATPαS as Na^+ salt. This product showed one spot on TLC with R_f 0.35. HPLC Analysis displayed two main peaks with the following t_R values: 4.21 min (λ_{max} 258 nm; 53%) and 4.94 min (λ_{max} 258 nm; 44%), which correspond to the (S_p) and (R_p) diastereoisomers, respectively.

(S$_p$)-ATPαS: ^{1}H-NMR (D$_2$O): 8.60 (*s*, H–C(8)); *ca.* 8.16 (*s*, H–C(2)); *ca.* 6.10 (*d*, *J*(1′,2′) ≅ 5.80, H–C(1′)); H–C(2′) and H–C(3′) resonances are overlapped by the intense HOD signal; 4.25–4.40 (*m*, H–C(4′), 2 H–C(5′)). ^{31}P-NMR (H$_2$O): 43.81 (*d*, *J*(Pα,Pβ) = 27.46, Pα); −7.99 (*d*, *J*(Pγ,Pβ) = 19.65, Pγ); −22.73 (*dd*, Pβ).

(R$_p$)-ATPαS: ^{1}H-NMR (D$_2$O): 8.53 (*s*, H–C(8)); 8.17 (*s*, H–C(2)); 6.07 (*d*, *J*(1′,2′) = 5.92, H–C(1′)); H–C(2′) and H–C(3′) resonances are overlapped by the intense HOD signal; 4.25–4-40 (*m*, H–C(4′),

2 H–C(5′)). ^{31}P-NMR (H_2O): 43.58 (*d*, *J*(Pα,Pβ) = 28.49, Pα); −7.92 (*d*, *J*(Pγ,Pβ) = 19.33, Pγ); −22.73 (*dd*, Pβ).

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