87. Synthesis and Characterization of the (5′ → 5″)-Ester of Adenosine 5′-Diphosphate with α-D-Ribofuranose Cyclic 1″,2″-Phosphate: a NAD Derivative Produced during tRNA Splicing

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The synthesis and spectroscopic properties of the (5′→5″)-ester of adenosine 5′-diphosphate with ribofuranose cyclic 1″,2″-phosphate **3**, a recently discovered metabolite produced during tRNA splicing, are reported.

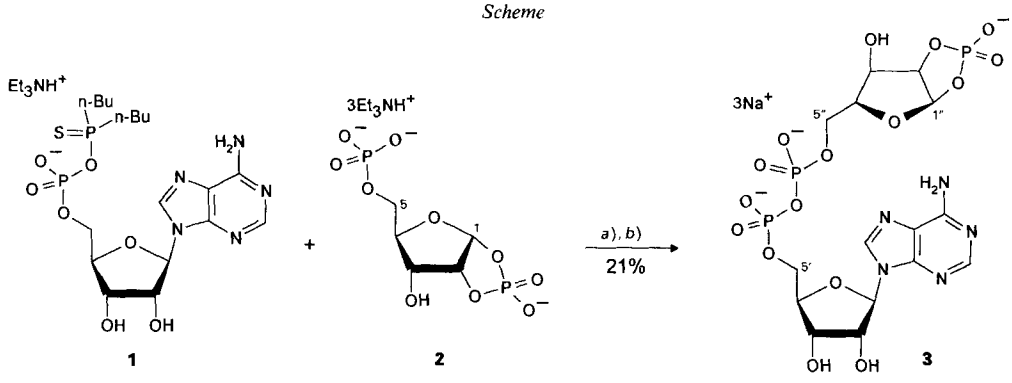
Introduction. – The (5′→5″)-ester of adenosine 5′-diphosphate with ribofuranose cyclic 1″,2″-phosphate (α-D-Rib1″,2″ > p5″pp5′A; see trisodium salt **3**) is produced during tRNA splicing in the yeast *Saccharomyces cerevisiae* and in *Xenopus* [1]. Splicing begins with excision of the intron by an endonuclease. In the second step, ligation of the two tRNA halves by a ligase generates the mature length tRNA bearing a 2′-phosphate at the splice junction. Phosphotransferase subsequently transfers this group to NAD yielding the tRNA product, nicotinamide, and α-D-Rib1″,2″ > p5″pp5′A. This pathway of processing appears to be conserved between yeast, plants, and vertebrates, and, therefore, it is likely that α-D-Rib1″,2″ > p5″pp5′A is produced in all eukaryotic cells [1–3]. The metabolic fate of this molecule was unknown until lately. However, recent studies uncovered a cellular cyclic phosophodiesterase in both yeast and wheat germ which hydrolyzes this strained five-membered phosphate regiospecifically to the ribose 1″-phosphate derivative [3]. Subsequent conversion of the anomeric phosphate to a known metabolite is then carried out, possibly by phosphatase. The yeast cyclic phosphodiesterase shows a high selectivity for α-D-Rib1″,2″ > p5″pp5′A, while plant enzymes also open nucleoside cyclic 2′,3′-phosphates to nucleoside 2′-phosphates [3] [4].

For a more detailed investigation of the aforementioned cyclic phosphodiesterases and interactions between the enzymes and their natural ligand, a supply of α-D-Rib1″,2″ > p5″pp5′A was required. Although enzymatic synthesis of this compound as the ^{32}P-labelled form has been reported [1], larger quantities were needed for more extensive studies. We report here the chemical synthesis and characterization of the trisodium salt **3** of α-D-Rib1″,2″ > p5″pp5′A.

Results and Discussion. – The successful synthesis involves a coupling of the two known compounds **1** and **2** (*Scheme*). Adenosine derivative **1** is commonly used for the preparation of unnatural NAD derivatives [5–7] and was synthesized according to the literature protocol [8]. In contrast to the reported method, however, it was purified by

reversed-phase HPLC to furnish the triethylammonium salt. α-D-Ribofuranose cyclic 1,2-phosphate (**2**) was prepared by the method of *Fathi* and *Jordan* [9]. The coupling of **1** with **2** in pyridine in the presence of silver(I) acetate worked acceptably on a milligram scale, but yielded only trace amounts of the desired product with larger quantities of starting materials. Critical improvements to the procedure were a switch to the solvent DMF/pyridine to counter the poor pyridine solubility of **2**, and the addition of a limited quantity (*ca.* 25 equiv.) of H_2S at the end of the reaction to precipitate silver salts. The desired compound then represented the major product and was finally isolated in 21% yield as the trisodium salt **3**. Further synthetic optimizations were not carried out.

Scheme



a) AgOAc, DMF, pyridine, 4 h, r.t., then H_2S. *b*) Reversed-phase HPLC, then cation exchange.

Table. *Selected Chemical Shifts* [ppm] *and Coupling Constants* J [Hz] *of Various Ribose Cyclic 1,2-Phosphate Derivatives*

	Ribose cyclic 1,2-phosphate[a])	**2**	**3**[b])
H–C(1)	5.91 (J = 4.1, 17.0[c]))	5.87 (J = 4.0, 17.0[c]))	5.77 (J = 4.2, 17.1[c]))
H–C(2)	4.85	4.83 (J = 8, 4)	4.78
H–C(3)	3.94	4.15	4.12
H–C(4)	3.72	4.12	4.12
2 H–C(5)	4.09	3.90 (J = 12.0, 6.1, 3.7), 4.06 (J = 12.0, 4.4, 1.7)	4.04, 4.20
C(1)	101.0 (J = 4.1[d]))	101.9 (J = 4.1[d]))	102.5 (J = 3.7[d]))
C(2)	78.4	79.4	80.0
C(3)	69.7 (J = 6.3[d]))	70.4 (J = 6[d]))	70.8 (J = 6[d]))
C(4)	80.3	80.2 (J = 8.5[d]))	80.4 ($J \approx 8$[d]))
C(5)	59.7	63	66.8
P(1)	17.4	18.8[e])	18.6
P(5)	–	2.7[f])	–11.0 (J = 21[g])), –11.0 (J = 21[g]))

[a]) Data taken from [10]; chemical shifts in ^{31}P-NMR spectra relative to internal HPO_4^{2-}. [b]) For convenience, the ribose and phosphate moieties of **3** are numbered as in **2**. [c]) $J(H–C(1),P(1))$. [d]) J(C,P). [e]) 17.0 ppm in [9]. [f]) 1.3 ppm in [9]. [g]) J(P,P).

Characterization of compound **3** was performed with a combination of spectroscopic techniques and simple enzyme reactions to assure the integrity of the (5′→5″)-diphosphate and cyclic 1″,2″-phosphate linkages. Proof that the latter moiety is intact comes from comparison of the ^{1}H-, ^{13}C-, and ^{31}P-NMR spectra of **3** with those of **2** and ribose cyclic 1,2-phosphate [10] (see *Table*). ^{1}H-NMR Chemical shifts of these three derivatives are very similar, and the coupling constants $J(H-C(1),P(1))$ and $J(H-C(1),H-C(2))$ are nearly identical. A 2D-COSY spectrum of **3** (*Fig.*) shows the expected connectivity. ^{13}C-NMR Chemical shifts and J(C,P)'s of the three compounds also match very closely with the largest variation for C(5), as expected. Each of the resonances in the ^{31}P-NMR spectrum of **3** is strongly supportive of the anticipated structure; P(1) is coupled to no

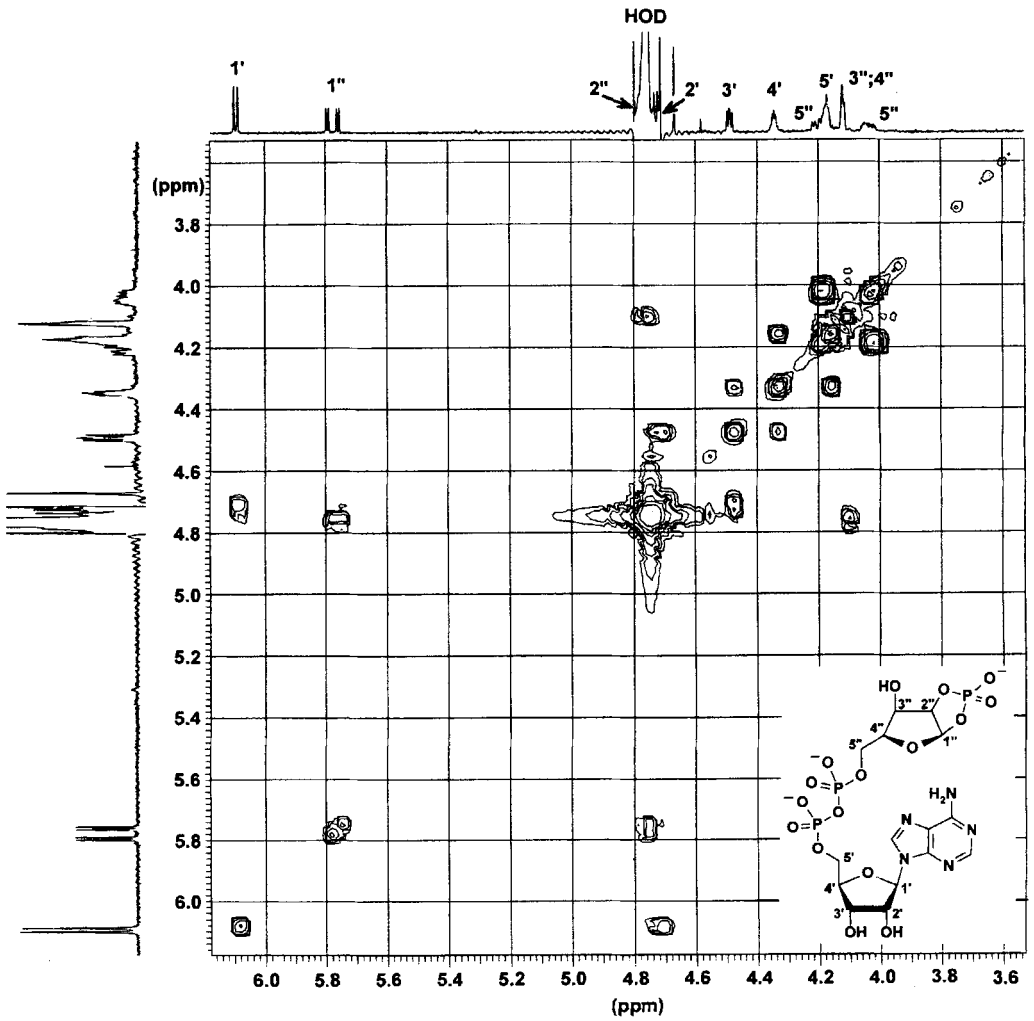


Figure. *Part of 2D-COSY spectrum* (500 MHz) *of* **3** *in* D_2O

other P-nucleus, and its chemical shift is characteristic of a ribose cyclic 1,2-phosphate [10]. The remaining upfield *AB* system is expected for the (5′→5″)-diphosphate linkage, and the chemical shift of −11 ppm is in agreement with internal phosphate moieties.

Additional evidence for the nature of the phosphate groups was available from several enzyme reactions which were monitored by TLC. Compound **3** was unaffected by calf intestinal phosphatase, which specifically hydrolyzes phosphomonoesters. Exposure of **3** to RNase T2 on the other hand gave ADP-ribose 2″-phosphate (α-D-Rib2″p5″pp5′A), whereas ADP-ribose 1″-phosphate (α-D-Rib1″p5″pp5′A), was produced from the reaction with the aforementioned wheat-germ cyclic phosphodiesterase. Products of hydrolysis with RNase T2 and cyclic phosphodiesterase were converted to ADP-ribose (α-D-Rib5″pp5′A) upon further incubation with calf intestinal phosphatase. Specific cleavage of the diphosphate linkage of **3** using pyrophosphatase yielded AMP. Compound **3** migrated on TLC, in two solvent systems, with the same R_f values as those determined previously [1] [3] for an enzymatically prepared sample of α-D-Rib1″,2″p5″pp5′A providing further strong evidence of the structure.

The straightforward synthetic access to this compound has enabled further studies of its properties and its hydrolysis by various enzymes. These will be reported elsewhere.

Experimental Part

General. Pyridine (*Fluka*) and DMF (*Fluka*) were purified by distillation from KOH and CaH_2, resp., onto 4 Å molecular sieves. *N,N′*-Dicyclohexylmorpholino-4-carboxyamidine (MDCA) was obtained from *Aldrich Chemical Co.* The 5-*O*-phospho-α-D-ribofuranosyl 1-pyrophosphate (= α-D-ribofuranose 5-(dihydrogen phosphate) 1-(trihydrogen diphosphate); *ca.* 80% pure) and adenosine 5′-monophosphate (= disodium 5′-adenylate) were purchased from *Sigma Chemical Co.* and were used without further purification. Other reagents were obtained from *Fluka* and used without purification. Calf intestinal phosphatase and RNase T2 were obtained from *Boehringer* and *Calbiochem*, resp. Pyrophosphatase from *Crotalus Adenantius* venom was purchased from *Sigma Chemical Co.* Cyclic phosphodiesterase from wheat germ was isolated and purified as reported previously [4]. Prep. reversed-phase HPLC: *Waters LC4000* apparatus fitted with a *Merck-Lichrosphor*™*-300* column (*C18*, 12 μm particle size, 250 × 50 mm). Anal. reversed-phase HPLC: *Waters 600* apparatus fitted with a *Nucleosil C18* column (5 μm particle size, 250 × 46 mm). Optical rotations: *Perkin-Elmer-241* polarimeter. UV Spectra (λ max (ε), in nm): *Shimadzu-UV-160A* spectrometer. NMR Spectra: *Bruker-AC-250*, *Bruker-DPX-400*, *Varian-XL-300*, and *Varian-Unity-500* spectrometers: δ in ppm and coupling constants *J* in Hz; external ref. for ^{13}C-NMR in H_2O (dioxane) and ^{31}P-NMR (H_3PO_4), internal solvent peaks as ref. in all other cases; ^{1}H assignments of **1–3** were confirmed by 2D-COSY. Matrix-assisted laser-desorption ionization time-of-flight (MALDI-TOF) mass spectra: *Linear-Scientific-LDI-1700* spectrometer; negative mode; samples were dissolved in a matrix solution containing ammonium citrate and 2,4,6-trihydroxyacetophenone (THA). High-resolution mass spectra (HR-MS): *VG-70SE* spectrometer; positive mode.

5′-Adenylic Dibutylphosphinothioic Anhydride Triethylammonium Salt (**1**). Disodium 5′-adenylate (725 mg, 1.79 mmol) and MDCA (525 mg, 1.79 mmol) were dried separately by co-evaporation with pyridine (2×) and were then dissolved in pyridine (17 ml). Dibutylphosphinothioic bromide (prepared according to [8]; 920 mg, 3.58 mmol) was added and the soln. stirred at r.t. for 3 h. The solvent was evaporated, the crude product taken up in H_2O, the mixture extracted once with Et_2O, and the aq. phase freeze-dried to give a white solid (1.66 g). Samples of the crude product (1.4 g total) were dissolved in aq. MeOH and purified by prep. reversed-phase HPLC (50 mM $(Et_3NH)OAc$ (pH 7) for 1 min, then with increasing MeOH at 1.7%/min, flow rate 60 ml/min): t_R (product) 33.2 min. Solvents were evaporated to give, after extensive drying under high vacuum, **1** (395 mg, 35%). White amorphous solid. UV (MeOH): 260 (15000). ^{1}H-NMR (250 MHz, CD_3OD): 0.79 (*t*, *J* = 7.5, Me); 0.8 (*t*, *J* = 7.5, Me); 1.17 (*t*, *J* = 7.5, 3 Me); 1.28 (*m*, 2 MeCH_2); 1.52 (*m*, 2 CH_2CH$_2$P); 1.90–2.12 (*m*, 2 CH$_2$CH_2P); 3.06 (*q*, *J* = 7.5, 3 CH$_2$); 3.19 ((D)MeOH); 4.06–4.14 (*m*, H–C(4′), 2 H–C(5′)); 4.31 (*dd*, *J* = 5.5, 2.5 H–C(3′)); 4.59 (*dd*, *J* = 6.0, 5.5, H–C(2′)); 5.97 (*d*, *J* = 6.0, H–C(1′)); 8.09 (*s*, H–C(2) Ade); 8.40 (*s*, H–C(8) Ade). ^{13}C-NMR (62.5 MHz, CD_3OD): 9.2 (3 Me); 14.0 (*m*, 2 Me); 24.7 (*d*, *J* = 18, 2 CH_2CH$_2$CH$_2$P); 26.0 (*m*, 2 CH$_2$CH_2CH$_2$P);

35.6 (*d*, J = 68, 2 $CH_2CH_2CH_2P$); 47.7 (3 CH_2); 6.70 (*m*, C(5′)); 72.4 (C(3′)); 76.0 (C(2′)); 85.7 (*d*, J = 13, C(4′)); 88.9 (C(1′)); 120.2 (C(5) Ade); 141.1 (C(8) Ade); 151.0 (C(4) Ade); 153.9 (C(2) Ade); 157.3 (C(6) Ade). ^{31}P-NMR (101 MHz, CD_3OD): 98.5 (*d*, J = 34.5, P=S); −10.1 (*d*, J = 34.5, P=O). MALDI-TOF-MS: 522.5 ($C_{18}H_{30}N_5O_7P_2S^-$; calc. 522.5). Anal. calc. for $C_{18}H_{31}N_5O_7P_2S \cdot 1\ Et_3N \cdot 2.5\ H_2O$ after equilibration in air: C 43.04, H 7.68, N 12.55, S 4.79; found: C 43.15, H 7.41, N 12.37, S. 5.57.

α-D-*Ribofuranose 5-[Bis(triethylammonium) Phosphate] Cyclic 1,2-[(Triethylammonium) Phosphate]* **2**. As described in [9], from α-D-ribofuranose 5-(dihydrogen phosphate) 1-(trihydrogen diphosphate). ^{1}H-NMR (500 MHz, D_2O): 1.22 (*t*, J = 7.3, 9 *Me*CH_2); 3.14 (*q*, J = 7.3, 9 Me*C*H_2); 3.90 (*ddd*, J = 12.0, 6.1, 3.7, 1 H–C(5)); 4.06 (*ddd*, J = 12.9, 4.4, 1.7, H–C(5)); 4.15 (*m*, H–C(3), H–C(4)); 4.76 (H_2O); 4.83 (*dd*, J = 4,4, 8.8, H–C(2)); 5.87 (*dd*, J = 17.1, 4.0, H–C(1)). ^{13}C-NMR (125 MHz, D_2O): 9.1 (*Me*CH_2); 47.5 (*Me*CH_2); 63.0 (*d*, J = 4.2, C(5)); 70.4 (*d*, J = 6, C(3)); 79.4 (C(2)); 80.2 (*d*, J = 8.5, C(4)); 101.9 (*d*, J = 4.1, C(1)). ^{31}P-NMR (101 MHz, D_2O): 18.8 (P(1)); 2.7 (P(5)).

(5′→5″)-Ester of Adenosine 5′-(Hydrogen Disodium Diphosphate) with α-D-*Ribofuranose Cyclic 1″,2″-(Sodium Phosphate)* (**3**). The salts **1** (26 mg, 42.5 μmol) and **2** (50 mg, 85.0 μmol) were dried separately by co-evaporation of their suspensions in pyridine. To dry **1** (2 co-evaporations) in pyridine (2 ml), dry **2** in DMF (0.5 ml) and pyridine (1.0 ml) was added under Ar, followed by a soln. of AgOAc (35 mg, 0.21 mmol) in pyridine (2 ml; prepared under Ar). The mixture was stirred for 4 h at r.t. under Ar in the dark (precipitation after 0.5 h). Then *ca.* 0.15M H_2S in pyridine/H_2O 1:1 (prepared by bubbling H_2S into pyridine/H_2O 1:1; 14 ml, *ca.* 2 mmol) was added under vigorous stirring (immediate formation of a black precipitate). After 5 min, Ar was bubbled through the soln. to drive off excess H_2S, and the mixture was filtered through *Celite*®. The yellow soln. was evaporated at r.t., the residue redissolved in H_2O, and the soln. filtered through cotton and evaporated: brown oily solid (47 mg). This solid was purified by prep. reversed-phase HPLC (50 mM $(Et_3NH)OAc$ (pH 7) for 1 min, then with increasing MeCN at 0.2%/min, flow rate 60 ml/min): t_R (product) 22 min. Solvents were evaporated to give, after extensive drying under high vacuum, a hygroscopic white solid (19 mg). ^{1}H-NMR: product associated with a mixture of triethylammonium and MCDA cations. This mixture was combined with a similar mixture obtained from 25 mg of **2** in a second batch. The combined mixture (27 mg) was dissolved in H_2O and passed slowly down a cation-exchange column (*Dowex 50* × *8*, Na^+ form). The UV-absorbing fraction was lyophilized: **3** (9 mg, 21%). Hygroscopic amorphous solid. Anal. HPLC: 98% pure. $[\alpha]_D^{23} = -10$ (c = 0.0033, H_2O). UV (H_2O): 259 (15500). ^{1}H-NMR (500 MHz, D_2O): 4.04 (*m*, 1 H–C(5″)); 4.12 (*m*, H–C(3″), H–C(4″)); 4.17 (*m*, 2 H–C(5′)); 4.20 (*m*, 1 H–C(5″)); 4.34 (*m*, H–C(4′)); 4.49 (*dd*, J = 3.4, 5.1, H–C(3′)); 4.72 (*m*, H–C(2′)); 4.76 (H_2O); 4.78 (*m*, H–C(2″)); 5.77 (*dd*, J = 17.1, 4.2, H–C(1″)); 6.09 (*d*, J = 5.9, H–C(1′)); 8.21 (*s*, H–C(2)); 8.46 (*s*, H–C(8)). ^{13}C-NMR (75 MHz, D_2O): 65.0 (C(5′)); 66.8 (C(5″)); 70.8 (*J*(C,P) = 6, C(3″)); 71.9 (C(3′)); 75.8 (C(2′)); 80.0 (C(2″)); 80.4 (*J*(C,P) ≈ 8, C(4″)); 85.6 (*J*(C,P) = 8,4, C(4′)); 88.4 (C(1′)); 102.5 (*J*(C,P) = 3.7, C(1″)); 141.8 (C(8)); 154.5 (C(2)). ^{31}P-NMR (101 MHz, D_2O, pDapp 6): −11.01 (*d*, J = 21.3, P(5′)); −10.95 (*d*, J = 21.3, P(5″)); 18.8 (P(1″)). MALDI-MS: 620 ($[M - H]^-$). HR-MS: 665.9966 ($C_{15}H_{21}N_5O_{16}P_3Na_2^+$; calc. 665.9992).

Enzyme Assays. Samples of **3** (20 μg) were treated with four enzymes. Incubation with calf intestinal phosphatase (1 U) in 100 mM *Tris* · HCl, 320 mM $(NH_4)_2SO_4$, 0.1 mM $MgCl_2$, and 0.01 mM $ZnCl_2$ at pH 8.0 and 37° was carried out for 30 min. Incubation with RNase T2 (0.5 U) in 10 mM NaOAc and 1 mM Na_2EDTA at pH 4.5 and 37° was carried out for 2 h. Reaction with purified wheat-germ cyclic nucleotide phosphodiesterase (30 ng [3] [4]) was performed at 30° for 2 h in 50 mM *Tris* · HCl and 0.01% *Triton X-100*. Incubation with pyrophosphatase from *Crotalus Adenantius* venom (1 U) was carried out at 37° in 100 mM *Tris* · HCl and 140 mM $MgCl_2$ at pH 7.5 for 2–3 h.

Products from these reactions were analysed by TLC on both PEI (polyethyleneimine) cellulose in 0.75M LiCl and cellulose in sat. $(NH_4)_2SO_4$ soln. 1M NaOAc (pH 5.2)/i-PrOH 80:18:2 and visualized under UV light (254 nm).

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