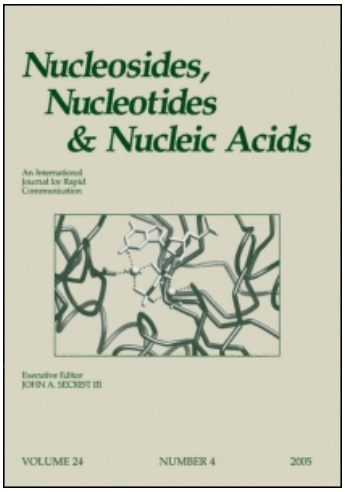

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Synthesis of [*bis*(Inosine-5')]-tetraphosphate and [*bis*(Inosine-5')]-pentaphosphate Analogues Bearing the Residues of Methylenediphosphonic Acid

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SYNTHESIS OF [BIS(INOSINE-5')]-TETRAPHOSPHATE AND [BIS(INOSINE-5')]-PENTAPHOSPHATE ANALOGUES BEARING THE RESIDUES OF METHYLENEDIPHOSPHONIC ACID

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Abstract: Various methods of synthesis of metabolically stable phosphonate analogues of bisnucleoside oligophosphates containing two residues of methylenediphosphonic acid in the oligophosphate chain are studied. Phosphonate analogues of Ip_4I and Ip_5I are prepared.

Natural bisnucleoside oligophosphates Np_nN can function in cells as metabolic signals, inhibitors of some enzymes, or as initiators of processes catalyzed by enzymes [1, 2].

Recently, a new type of purinergic brain receptors was discovered, for which agonists are bisadenosine oligophosphates Ap_nA (n = 4, 5) and antagonists are bisinosine oligophosphates Ip_nI (n = 4, 5) [3]. Some phosphonate analogues of these bisnucleoside oligophosphates also revealed agonist or antagonist properties toward these receptors [4].

Np_nN have some specific features if compared with nucleoside 5'-triphosphates: they are less polar, exhibit considerable stacking interactions, and can rather effectively form complexes with bivalent metal ions [1]. All this results in intricate interpretations of the Np_nN effect on biological processes. To study the mechanism of action of these polyphosphate nucleotides, their phosphonate analogues may be useful. Some approaches for

the preparation of Np_4N analogues as well as their biological application were developed by Blackburn [5]. Syntheses of Ap_4A analogues bearing methylenediphosphonic acid residues were published in [6, 7].

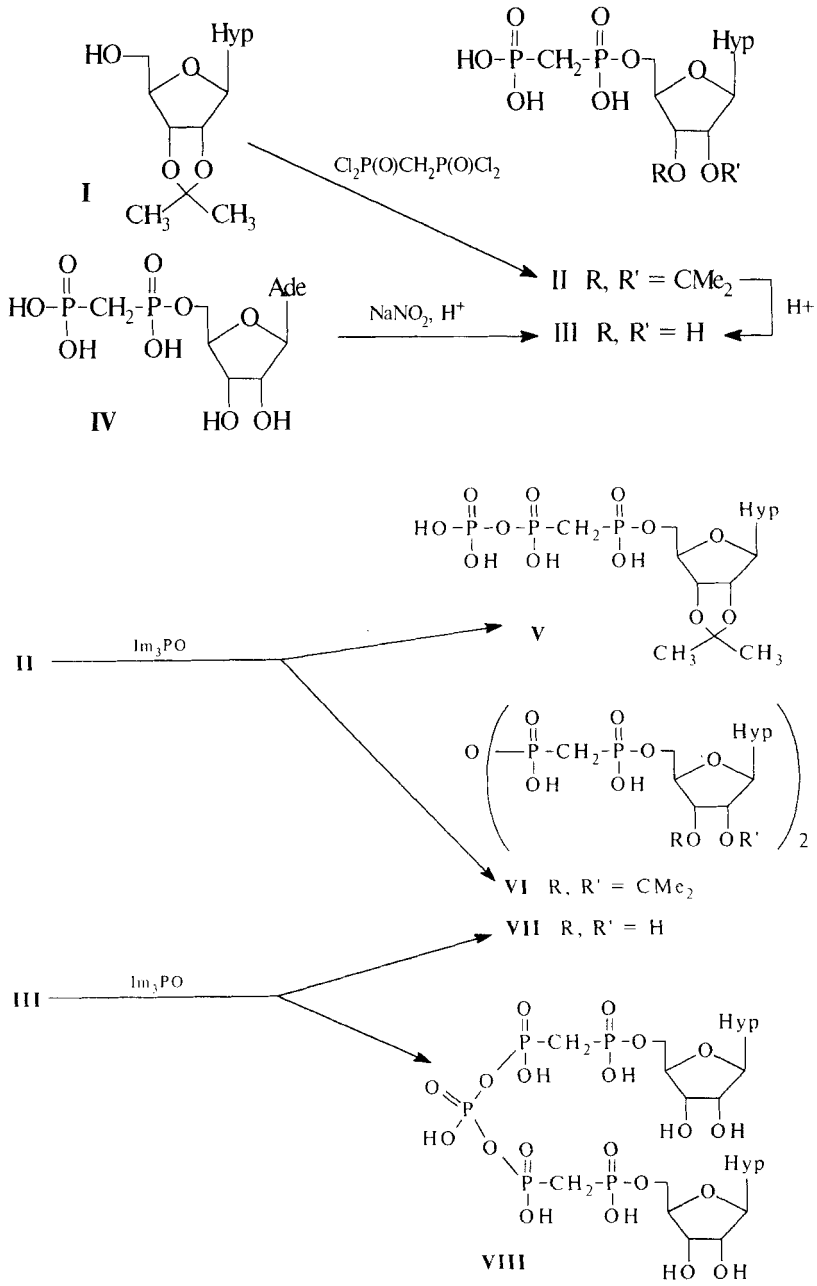
Phosphonate Np_nN analogues differ from natural compounds by their stability toward hydrolyzing enzymes or by their inhibitory properties [8, 9], by the properties of metal complexes [10], and conformational flexibility of the oligophosphate chain.

The aim of this work was to develop simple procedures for the synthesis of Ip_nI (n = 4, 5) phosphonate analogues containing in the phosphate chain either two residues of methylenediphosphonic acid (MDP) (compound VII) or two MDP fragments attached to a phosphate residue (compound VIII).

The starting compound, inosine 5'-methylenediphosphonate (III), can be obtained in different ways (Scheme 1). We tried several methods to synthesize compound (III) [11, 12] or to find a convenient one-step way to its dimer (VI). But in the case of inosine derivatives we did not obtain satisfactory results. The reaction of 2',3'-O-isopropylideneinosine (I) with methylenebis(phosphonic dichloride) in triethylphosphate in the presence of a base yielded derivative (II), which was deprotected in 60% HCOOH to give (III). This compound was also obtained through oxidative deamination of adenosine 5'-methylenediphosphonate (IV) synthesized as in [6].

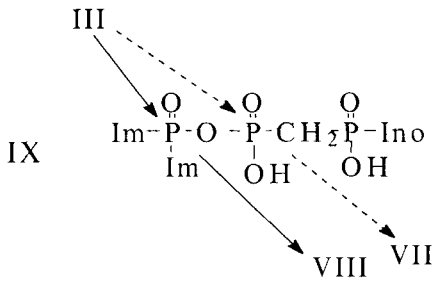
To introduce a residue of phosphoric acid between phosphonate fragments, we treated diphosphonate (II) with tris(imidazolido)phosphate in absolute DMF. However, the compound bearing five phosphorus atoms was not found. The products isolated were the analogue of triphosphate (V) and tetraphosphonate derivative (VI). We failed to deprotect the latter to the corresponding (VII), since under acid conditions of deblocking the hydrolysis of the P-O-P bond prevailed over hydrolysis of the isopropylidene group. Nevertheless, starting from nucleotide (III) and Im_3PO, we isolated both compound (VII), which is an Ip_4I analogue, and compound (VIII), an Ip_5I analogue, in satisfactory yields (44 and 11%, respectively).

The analysis of the reaction course allows us to suggest that at the initial stage β-phosphorus of (III) attacks Im_3PO to give intermediate (IX).



SCHEME 1

At this point additional (III), acting as a nucleophile, attacks either the γ-phosphorus of (IX) yielding (VIII) after hydrolysis of the remaining imidazole group, or (III) attacks the β-phosphorus of (IX) (Im_2PO_2- is a leaving group) giving (VII) (Scheme 2).



SCHEME 2

The structures of the compounds synthesized were confirmed by UV and NMR spectra. The ^{31}P NMR spectrum of compound (VII) was in coincidence with that of the corresponding bisadenosine derivative [6]. It is worth noting the difference in the ^{31}P NMR spectra of inosine derivatives bearing MDP residues. The 2',3'-isopropylidene-protected compounds (II, V, and VI) had clear P-α, P-β doublets with J = 8 Hz, whereas for unprotected III and VII the signals corresponding to P-α and P-β atoms were observed as singlets. Different modes of signal splitting for similar phosphorus atoms of nucleoside phosphonates was earlier described in [13, 14].

Phosphonates (VII) and (VIII) evidently differed: compound (VII) had two singlets, whereas compound (VIII) had a singlet corresponding to P-α, a doublet of P-β, and a triplet corresponding to P-γ atoms with the coupling constant 19.5 Hz.

EXPERIMENTAL

UV spectra were recorded on a Specord M40 (UV-VIS) and Shimadzu UV-1201 spectrophotometers. The yields were determined according to UV absorption of products, using molar extinction coefficients as in [1, 6]. ^1H NMR spectra (200 MHz) with *t*-BuOH as an internal standard and ^{31}P NMR

spectra (81 MHz) (proton decoupled) with phosphoric acid as an external standard were registered on a Bruker WP-200 SY in D_2O. Mass spectra were registered on a COMPACT MALDI-4 (Kratos Analytical, United States) on 2-amino-5-nitropyridine matrix. Column chromatography was performed on cellulose DE-32 (Whatman, England), LiChroprep RP-18 (25-40 μ), Dowex 50W x 8 (Merck, Germany), and Spheron DEAE-100 (Lachema, Czech Republic). For TLC, Kieselgel 60 F254 plates and PEI cellulose plates (Merck) in 0.25 M phosphate buffer were used. 2',3'-Isopropylideneinosine was from Sigma (USA); methylenebis(phosphonic dichloride) from Aldrich; tris(imidazolido)phosphate was prepared according to [15].

2',3'-O-Isopropylideneinosine 5'-methylenediphosphonate, bisammonium salt (II). To a solution of nucleoside (I) (150 mg, 0.487 mmol) in triethyl phosphate (3 ml), Bu_3N (0.24 ml, 1 mmol) and methylenebis(phosphonic dichloride) (150 mg, 0.6 mmol) were added, and the mixture was stirred. After 24 h at 20°C, Bu_3N (0.5 ml, 2 mmol) and water (5 μl, 0.3 mmol) were added, and the solution was stirred for 6 h. Then, 1 M NH_4HCO_3 (200 μl) and after 0.5 h water (200 ml) and 25% aqueous ammonia (100 μl) were added. The mixture was chromatographed on a DEAE Spheron column (10 x 80 mm) in a linear gradient of NH_4HCO_3 (0→0.5 M, 500 ml). The fraction eluted at 0.1-0.2 M was concentrated and repurified on a LiChroprep RP-18 column (20 x 150 mm) in a linear gradient of MeOH (0→0.25%) in 0.05 M NH_4HCO_3 (500 ml). The solution of compound (II) was evaporated and freeze-dried to give 54 mg (22%). ^1H NMR (δ, ppm; *J*, Hz): 8.25s and 8.04s (2H, H-2 and H-8), 6.12d (1H, *J* 2, H-1'), 5.29m and 5.09m (2H, H-2' and H-3'), 4.52m (1H, H-4'), 3.98m (2H, H-5'), 1.97t (2H, *J* 20, PCH_2P), 1.52s and 1.31s (6H, two CH_3). ^{31}P NMR (δ, ppm; *J*, Hz): 19.15d (*J* 8, P-α), 15.84d (*J* 8, P-β).

Inosine 5'-methylenediphosphonate, bisammonium salt (III). Method A. Compound (II) (85 mg, 0.17 mmol) was dissolved in 60% HCOOH (2 ml), after 2 h at 20°C the solution was evaporated, coevaporated with water (3 x 5 ml), and the residue was purified on a LiChroprep RP-18 column (10 x 150 mm) eluting with water. The fractions containing compound (III) were

concentrated in a vacuum to the volume of 3 ml, neutralized with 25% aqueous ammonia, and evaporated to dryness. The yield 56 mg (72%). UV (water): λ_{max} 249 nm (10200). ^{1}H NMR ppm, δ, Hz): 8.41s and 8.08s (2H, H-2 and H-8), 5.99d (1H, *J* 5.4, H-1'), 4.66m (1H, H-2'), 4.44m (1H, H-3'), 4.23m (1H, H-4'), 4.04m (2H, H-5'), 2.05t (2H, *J* 19.5, PCH_2P). ^{31}P NMR: 18.46s (P-α), 14.94s (P-β).

Method B. To a solution of bisammonium salt of (IV) (50 mg, 0.11 mmol) in a 2 : 1 AcOH-H_2O mixture (1 ml), sodium nitrite (20 mg, 0.28 mmol) was added, the reaction mixture was stirred for 12 h at 20°C, evaporated, coevaporated with water (5 x 5 ml), and dried in a vacuum. The residue was dissolved in water (1 ml), loaded on a Dowex 50W x 8 column (20 x 100 mm), and eluted with water. The fractions corresponding to the first peak were concentrated to 0.5 ml, adjusted to pH 7 with 25% aqueous ammonia, and purified on a LiChroprep RP-18 column (20 x 200 mm) eluting with 0.02 M NH_4HCO_3, and evaporated to give (III) (32 mg, 64%). The second peak contained starting (IV) (15 mg).

2',3'-O-Isopropylideneinosine-5'-methylenediphosphonatephosphate, tetraammonium salt (V) and P-α, P-α'-bis[2',3'-O-isopropylideneinosine-5'-methylenediphosphonate], tetraammonium salt (VI). Compound (II) (23 mg, 0.046 mmol) and Bu_3N (26 μl, 0.11 mmol) were dissolved in DMF (1 ml), then the solution of Im_3PO in MeCN (120 μl, 0.03 mmol) was added, and, after stirring at 20°C for 66 h, 1 M NH_4HCO_3 (1 ml) was added. The mixture was stirred for 1 h, 25% aqueous ammonia (300 μl) was added, the mixture was diluted with water to 150 ml, and the solution was loaded on a DEAE Spheron column (10 x 80 mm). The products were eluted in a linear gradient of NH_4HCO_3 (0→0.5 M, 500 ml). The target fractions were concentrated and repurified on a LiChroprep RP-18 column (20 x 150 mm) in a linear gradient of MeOH in 0.05 M NH_4HCO_3, (0→25%, 500 ml) to give (V) (5 mg, 18%) and (VI) (4 mg, 18%). Compound (V): UV (water): λ_{max} 249 nm (18400). ^{1}H NMR: 8.18s and 8.02s (2H, H-2 and H-8), 6.08d (1H, *J* 2, H-1'), 5.28m and 5.07m (2H, H-2' and H-3'), 4.51m (1H, H-4'), 3.94m (2H, H-5'), 1.91t (2H, *J* 20, PCH_2P), 1.51s and 1.28s (6H, two CH_3). ^{31}P NMR: 18.49d (*J* 8, P-α),

5.75dd (*J* 8, 21.5, P-β), -9.34d (*J* 21.5, P-γ). Compound (VI): UV (water): λ_{max} 249 nm (10300). ^{1}H NMR: 8.21s and 8.00s (2H, H-2 and H-8), 6.06d (1H, *J* 2, H-1'), 5.23m and 5.06m (2H, H-2' and H-3'), 4.45m (1H, H-4'), 3.94m (2H, H-5'), 2.14t (2H, *J* 20, PCH_2P), 1.52s and 1.31s (6H, two CH_3). ^{31}P NMR: 18.34d (*J* 8, P-α, P-α'), 8.19d (*J* 8, P-β, P-β').

P-α, P-α'-Bis[inosine-5'-methylenediphosphonate], tetraammonium salt (VII) and [P-α, P-α'-bis(inosine-5'-methylenediphosphonate)]phosphate, pentaammonium salt (VIII). DMF (2 ml) and Bu_3N (66 μl, 0.28 mmol) were added to a solution of (III) (65 mg, 0.14 mmol) in water (2 ml), the solution was evaporated to a volume of 2 ml, and coevaporated with DMF (3 ml x 3). A solution of 0.25 M Im_3PO in DMF (420 μl, 0.11 mmol) was added under vigorous stirring. After stirring at 22°C during 72 h, the reaction mixture was quenched with 25% aqueous ammonia (2 ml) and water (5 ml) and extracted with chloroform (3 x 5 ml). The aqueous layer was concentrated, loaded on a LiChroprep RP-18 column (20 x 250 mm, 25 x 40 μ), and then eluted with 0.01 M NH_4HCO_3. The fractions corresponding to the first peak were concentrated and chromatographed on a DE-32 cellulose (HCO_3^- form) column (25 x 150 mm) in a linear gradient of NH_4HCO_3 in 15% MeOH (400 ml, 0.2→0.6 M). Compounds (VII) and (VIII) were eluted at 0.36-0.38 M and 0.40-0.43 M, respectively, and repurified on a LiChroprep RP-18 column (25 x 160 mm) eluting with 0.05 M NH_4HCO_3. The second peak eluted from the DE-32 cellulose column contained compound (VII), which was repurified on a LiChroprep RP-18 column as above. The total yield of (VII) was 28 mg (44%). UV (water): λ_{max} 249 nm (18500). ^{1}H NMR: 8.21s and 7.99s (2H, H-2 and H-8), 5.88d (1H, *J* 5.8, H-1'), 4.40-4.55m (2H, H-2', H-3'), 4.22m (1H, H-4'), 3.99m (2H, H-5'), 2.22t (2H, *J* 19.5, PCH_2P). ^{31}P NMR: 17.68s (P-α, P-α'), 7.52s (P-β, P-β'). MS, m/e: 833.9 $[M]^+$. Calc. $C_{22}H_{30}N_8O_{19}P_4$: 840.

Compound (VIII). The yield 8 mg (11%). λ_{max} 249 nm (18000). ^{1}H NMR: 8.47s and 8.15s (2H, H-2 and H-8), 6.06d (1H, *J* 5.6 Hz, H-1'), 4.53m (1H, H-2'), 4.32m (1H, H-3'), 4.14m (1H, H-4'), 4.04m (2H, H-5'),

2.39t (2H, *J* 19.5, PCH_2P). ^{31}P NMR: 17.18s (P-α, P-α'), 8.95d (*J* 19.5, P-β, P-β'), -21.88t (*J* 19.5, P-γ). MS, m/e: 916.0 $[M+1]^+$, 938.5 $[M + Na]^+$. Calc. $C_{22}H_{31}N_8O_{22}P_5$: 914.5.

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