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PREPARATION OF (2′-5′)-OLIGONUCLEOTIDES BASED ON 6-*N*-BENZYLAMINOPURINERIBOSIDE USING THE *Spicaria violacea* FUNGAL ENZYME COMPLEX

T. I. Kulak,[1*] O. V. Tkachenko,[1] O. Yu. Grishan,[1] T. A. Kukharskaya,[2] L. A. Eroshevskaya,[2] E. N. Kalinichenko,[1] and A. I. Zinchenko[2]

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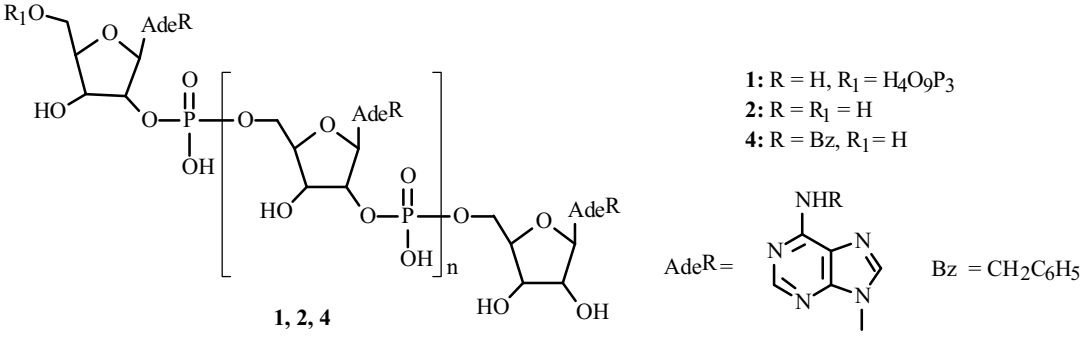
A method for chemico-enzymatic synthesis of (2′-5′)-oligonucleotides with 6-N-benzylaminopurineriboside as the nucleoside units was proposed. The method consisted of enzymatic hydrolysis of the oligonucleotides with mixed (2′-5′)-(3′-5′)-phosphodiester bonds that were prepared by polymerization of 6-N-benzyladenosine-2′(3′)-monophosphate by using (3′-5′)-specific nuclease and phosphatase contained in the filtrate of culture medium of the mycelial fungus Spicaria violacea.

Key words: (2′-5′)-oligoadenylates, 6-*N*-benzylaminopurineriboside, *Spicaria violacea*, nuclease, enzymatic hydrolysis.

(2′-5′)-Oligoadenylates are oligonucleotides incorporating several adenosine molecules bonded through a (2′-5′)-phosphodiester bond [ppp5′A2′p(5′A2′p)$_n$5′A, **1**] and are one of the principal mediators of interferon antiviral action in mammal cells [1-4]. (2′-5′)-Oligoadenylates are active toward animal and plant viruses [5, 6] and affect on cell growth and development [7, 8]. The principal types of biological activity are typical of compounds containing an oligonucleotide chain of at least three nucleoside units. Dephosphorylated oligomers **2** and their synthetic analogs, in particular, those containing 6-*N*-benzyladenosine (**3**) in various positions of the oligonucleotide chain exhibit interesting biological properties [6, 9]. This nucleoside is a riboside of the natural phytohormone 6-*N*-benzylaminopurine (BAP) and possesses cytokine activity [10]. Analogs of the A(2′p5′A)$_2$ trimer that contain BAP-riboside moieties in various positions of the chain exhibit plant growth-stimulating properties, possess antiviral activity toward plant viruses [6], activate ribonuclease L, and inhibit growth of syncytium induced by the HIV-1 virus [9].



1) Institute of Bioorganic Chemistry, National Academy of Sciences, Belarus, 220141, Minsk, ul. Akad. V. Kuprevicha, 5/2, e-mail: kulak@iboch.bas-net.by; 2) Institute of Microbiology, National Academy of Sciences of Belarus, 220141, Minsk, ul. Akad. V. Kuprevicha, 2. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 64-68, January-February, 2009. Original article submitted July 1, 2008.

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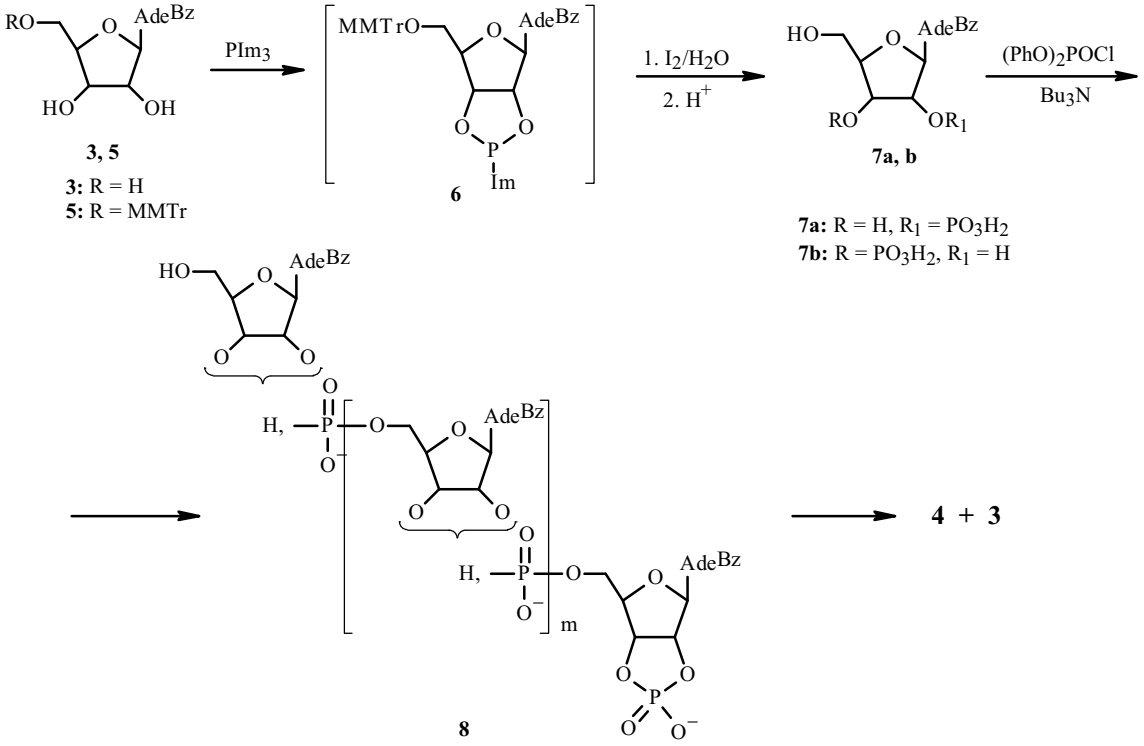
Chemical methods for synthesizing (2′-5′)-oligoadenylates are labor-intensive and expensive. Thus, the literature method for preparing 6-*N*-benzyladenylyl(2′-5′)-6-*N*-benzyladenylyl(2′-5′)-6-*N*-benzyladenosine (**4**, n = 1) from 6-*N*-benzyl-adeonsine (**3**) is based on the triester method of synthesizing oligonucleotides that includes 11 synthetic steps and requires performing 9 column chromatographies [6].

We have previously shown that short dephosphorylated (2′-5′)-oligoadenylates (**2**) can be prepared by enzymatic hydrolysis of long-chain oligonucleotides containing mixed (2′-5′)-(3′-5′)-internucleotide bonds by using (3′-5′)-specific nuclease and phosphatase in the filtrate of culture medium (FCM) of the mycelial fungus *Spicaria violacea* [11]. The goal of the present work was to develop a chemico-enzymatic method for synthesizing (2′-5′)-oligonucleotides containing 6-*N*-benzyladenosine (**3**) moieties as the nucleoside units.

Mixed (2′-5′)-(3′-5′)-oligonucleotides are substrates for subsequent enzymatic hydrolysis by the enzyme complex from FCM of *Spicaria violacea* and were synthesized by polymerization of 6-*N*-benzyladenosine-2′(3′)-monophosphate (**7**) obtained from 6-*N*-benzyladenosine (**3**). 6-*N*-Benzyl-5′-*O*-monomethoxytrityladenosine (**5**) [6] was prepared by reaction of **3** with monomethoxytritylchloride (MMTrCl) in pyridine in 85% yield. Then it was treated successively with solutions of tri(imidazolyl)phosphite (PIm_3) in THF:pyridine, iodine in THF:pyridine:water, and aqueous HCl. The reaction of **5** with PIm_3 should lead to formation of only cyclic phosphite **6** because the primary 5′-hydroxyl of **5** is blocked by the MMTr group [12, 13]. Treatment of the reaction mixture obtained from oxidation of **6** by iodine in the presence of water and pyridine [14] with HCl (0.2 M) gave first rapid removal of the acid-labile MMTr group and then hydrolysis of the cyclophosphate group that is also unstable in acidic media [15, 16]. The resulting mixture of 6-*N*-benzyladenosine 2′(3′)-monophosphates (**7a** and **7b**) was isolated in 66.7% yield by chromatography over DEAE-Sephadex A-25 in the HCO_3^- -form.



The formation from **7a** and **7b** of **3** via enzymatic hydrolysis using alkaline phosphatase confirmed that they contained a phosphate group. The location of the phosphate on C(2′) or C(3′) of **7a** and **7b**, respectively, was confirmed by the spin—spin coupling constants of the corresponding carbohydrate protons with the P atom in the PMR spectrum ($^3J_{2',P}$ = 7.4 Hz for 2′-monophosphate **7a** and $^3J_{3',P}$ = 7.0 Hz for 3′-isomer **7b**). Two resonances at –1.10 and –0.57 ppm for 2′- and 3′-monophosphates **7a** and **7b**, respectively, were observed in the ^{31}P NMR spectrum of the resulting mixture of nucleotides (H^+-form).

Phosphates **7** were polymerized by treatment with diphenylchlorophosphate and tri-*n*-butylamine in dioxane [16, 17]. The resulting 6-*N*-benzyl derivatives of the oligoadenylates with mixed (2′-5′)-(3′-5′)-phosphodiester bond (**8**) by nuclease and phosphatase in FCM of *S. violacea* were hydrolyzed at 55°C in sodium acetate buffer (pH 6.0) containing $MgCl_2$ (10 mM). Aliquots of the reaction mixture were analyzed by TLC during the course of the reaction.

Incubation of the (2′-5′)-(3′-5′)-oligoadenylates **8** with FCM of *S. violacea* led to their hydrolysis and the formation of **3** and an array of short-chain oligonucleotides with high chromatographic mobility in polar media compared with the values for the components of the starting mixture of **8**.

Enzymatic hydrolysis of 6-*N*-benzylated oligoadenylates **8** by the enzyme complex from FCM of *S. violacea* occurred much slower than for the analogous unmodified adenosine derivatives (the time for completion of the hydrolysis increased by more than four times even if the amount of FCM used increased by 2.5 times). This was apparently explained by differences in the three-dimensional structure of the oligoadenylates with free NH_2 groups and their 6-*N*-benzyl derivatives **8** [6].

Preparative separation of the products from enzymatic hydrolysis was carried out by column chromatography over DEAE-Sephadex A-25. Precipitation by NaI in acetone produced the sodium salts of compounds that were assigned the following structures based on results from enzymatic hydrolysis and NMR spectroscopy: 6-*N*-benzyladenylyl(2′-5′)-6-*N*-benzyladenosine (**4**, n = 0), 6-*N*-benzyladenylyl-(2′-5′)-6-*N*-benzyladenylyl(2′-5′)-6-*N*-benzyladenosine (**4**, n = 1), and 6-*N*-benzyladenylyl(2′-5′)-6-*N*-benzyladenylyl-(2′-5′)-6-*N*-benzyladenylyl-(2′-5′)-6-*N*-benzyladenosine (**4**, n = 2) in yields calculated for **7** of 21.4, 10.1, and 2.4%, respectively.

Treatment of compounds **4** with snake venom phosphodiesterase, which cleaves both (3′-5′)- and (2′-5′)-internucleotide bonds, formed 6-*N*-benzyladenosine (**3**) and its 5′-monophosphate (**9**) in the following ratios: 1:1 for **4** (n = 0), 1:2 for **4** (n = 1), and 1:3 for **4** (n = 2). Oligonucleotides **4** were stable to the action of nuclease S_1 of *Aspergillus oryzae*, which can hydrolyze only (3′-5′)-internucleotide bonds. This indicates that these compounds have (2′-5′) phosphodiester bonds. The stability to the action of bacterial alkaline phosphatase of the resulting oligomers **4** confirmed that they lacked terminal phosphates. The results indicate that derivatives **4** are a dinucleosidemonophosphate (n = 0), trinucleosidediphosphate (n = 1), and tetranucleosidetriphosphate (n = 2), the 6-*N*-benzyladenosine in which are bonded by (2′-5′)-phosphodiester bonds.

The structures of compounds **4** were confirmed by NMR spectroscopy. Thus, the PMR spectrum of **4** (n = 0) contained resonances for protons of two nucleosides; of **4** (n = 1), three nucleosides. The PMR spectrum of **4** (n = 0) had spin—spin coupling constant for proton H-2′ of one of the nucleosides with the P atom ($J_{2',P}$ = 9.1 Hz). The PMR spectrum of **4** (n = 1) agreed with that for 6-*N*-benzyladenylyl(2′-5′)-6-*N*-benzyladenylyl-(2′-5′)-6-*N*-benzyladenosine that was prepared by the triester method [6]. The ^{31}P NMR spectrum of **4** (n = 0) had one singlet corresponding to the internucleotide phosphodiester group whereas that of trimer **4** (n = 1) had two such resonances (–0.97 and –1.39 ppm).

Thus, nuclease from FCM of *S. violacea* performed selective hydrolysis of (3′-5′)-phosphodiester bonds in mixed (2′-5′)-(3′-5′)-oligomers **8** that contain nucleoside units of 6-*N*-benzyladenosine analogous to that which was observed for compounds based on adenosine [11]. The lack of reaction products with terminal 2′,3′-cyclophosphates indicates that treatment of oligomers **8** with the enzyme complex from FCM of *S. violacea* also caused hydrolysis of terminal 2′,3′-cyclophosphates in the oligomers. The action of phosphatase in FCM caused dephosphorylation of the resulting mono- and oligonucleotides, which produced nucleoside **3** and short-chain (2′-5′)-oligomers **4**.

The described method for synthesizing (2′-5′)-oligonuclotides based on BAP-riboside that uses the enzyme complex of *S. violacea* is rather simple and convenient to implement. It does not require isolation of highly pure enzymes and can produce the target compounds in satisfactory yields.

EXPERIMENTAL

6-*N*-Benzyladenosine (**3**) was prepared by microbiological transribosylation of 6-*N*-benzyladenine using uridine as the carbohydrate donor [18].

6-*N*-Benzyladenosine-5′-*O*-monophosphate (**9**) that was used as a reference for hydrolysis of oligomers **4** by snake venom phosphodiesterase was prepared by reaction of **3** with $POCl_3$ in trimethylphosphate [19].

Enzymatic hydrolysis of oligonucleotides was carried out in culture medium of fungus strain *S. violacea* BM-105D that was preserved in the Belarusian Collection of Nonpathogenic Microorganisms of the Microbiology Institute of the National Academy of Sciences of Belarus as number BIM F-329. The cultivation conditions for mycelial fungus *S. violacea* and preparation of the filtrate of culture medium containing phosphatase and nuclease have been previously described [20].

We used bacterial alkaline phosphatase (Fluka), snake venom phosphodiesterase (Boehringer), and nuclease S_1 of *Aspergillus oryzae* (NPO Vector).

Enzymatic dephosphorylation of nucleotides **7a** and **7b** by alkaline phosphatase was carried out in Tris-HCl buffer (0.1 M, pH 9.0). Enzymatic hydrolysis of compounds **4** by snake venom phosphodiesterase was performed in Tris-HCl buffer (0.1 M, pH 8.7) containing $MgCl_2$ (10 mM). The action of nuclease S_1 on the resulting oligonucleotides was studied using solutions of them in sodium-acetate buffer (0.04 M, pH 4.7) containing $ZnCl_2$ (0.1 mM) [17]. The composition of the reaction mixtures from enzymatic hydrolysis was analyzed by TLC. Spots of reaction products were eluted by ethanol:water (1:1). Optical density of the resulting solutions was measured at 270 nm. The amounts of compounds were calculated taking into account their molar absorption coefficients.

TLC was performed on Kieselgel 60 F254 plates (Merck) using solvent systems $CHCl_3$:CH_3OH (4:1, A) and 2-propanol:aqueous ammonia (25%):water (7:1:2, B). Column chromatography used DEAE-Sephadex A-25 anion-exchanger (Pharmacia).

UV spectra were recorded on a Specord M-400 spectrophotometer. PMR spectra in DMSO-d_6 (TMS internal standard) and D_2O (*t*-BuOH internal standard), chemical shifts of protons are given relative to TMS taking the chemical shift of *t*-BuOH vs. TMS as 1.27 ppm) were recorded on an Avance-500 spectrometer (Bruker). Resonances of protons were assigned using 2D correlation spectroscopy (COSY). ^{31}P NMR spectra were recorded on the same instrument using H_3PO_4 (85%) as a standard with full decoupling of protons.

Elemental analyses of the products agreed with those calculated.

6-*N*-Benzyladenosine-2′(3′)-*O*-monophosphate (7a, 7b). A solution of 6-*N*-benzyl-5′-*O*-monomethoxytrityladenosine (**5**, 1.26 g, 2 mmol) [6] in THF:pyridine (4:1, 54 mL) was cooled to 0°C; stirred; treated under an Ar atmosphere with a solution of tri(imidazol-1-yl)phosphine, prepared by addition of PCl_3 (0.53 mL, 6 mmol) to a solution of imidazole (2.45 g, 36 mmol) in THF (48 mL) at 0°C with subsequent filtration of the precipitated imidazole hydrochloride; stirred for 15 min at 0°C; treated with a solution of iodine (1.52 g, 6 mmol) in THF:H_2O:pyridine (4:4:1, 45 mL), stored for 30 min, treated dropwise with $Na_2S_2O_3$ solution until colorless, and evaporated to dryness. The solid was evaporated with toluene (2 × 15 mL) in order to remove traces of pyridine.

The solid after evaporation was dissolved in THF:dioxane:H_2O (1:1:1, 78 mL), treated with conc. HCl (2.4 mL), stored for 16 h, neutralized with aqueous ammonia (25%) until the pH was 7, and evaporated to dryness. The solid after evaporation was dissolved in water (400 mL) and extracted with $CHCl_3$ (2 × 200 mL). The aqueous layer was diluted to 800 mL, placed on a column with DEAE-Saphadex A-25 in the HCO_3^- -form (350 cm^3), and eluted with water (500 mL) and a gradient of TEAB from 0 to 0.5 M (total volume 3.0 L). Fractions containing a mixture of di(triethylammonium) salts of 2′- and 3′-monophosphates of 6-*N*-benzyladenosine (**7a** and **7b**) were combined and evaporated. The solid was evaporated with ethanol to remove traces of TEAB. The solid after evaporation was dissolved in the minimal volume of water:ethanol (1:1), placed on a column with cation-exchanger Dowex 50W×4 in the H^+-form (80 cm^3), and eluted with water (200 mL) and water:ethanol (1:1). Fractions containing phosphates **7a** and **7b** in the H^+ -form were combined and evaporated to afford a mixture of nucleotides **7a** and **7b** (0.58 g, 66.7%) as a white powder, R_f 0.37 (B), UV spectrum (MeOH, λ_{max}, nm): 270 (log ε 4.28).

6-*N*-Benzyladenosine-2′-*O*-monophosphate (7a). PMR spectrum (DMSO-d_6, δ, ppm, J/Hz): 8.52 (1H, m, NH), 8.33 (1H, s, H-2), 8.18 (1H, s, H-8), 7.31-7.16 (5H, m, C_6H_5), 6.06 (1H, d, H-1′, $J_{1',2'}$ = 6.53), 5.17 (1H, m, H-2′, $J_{2',1'}$ = 6.53, $J_{2',3'}$ = 4.77, $J_{2',P}$ = 7.4), 4.68 (2H, m, C$\underline{H}_2$Ph), 4.36 (1H, dd, H-3′, $J_{3',2'}$ = 4.77, $J_{3',4'}$ = 2.5), 3.98 (1H, m, H-4′), 3.64 (1H, dd, H-5′, $J_{5',4'}$ = 3.1, $J_{5',5''}$ = 12.0), 3.52 (1H, dd, H-5″, $J_{5'',4'}$ = 3.0, $J_{5'',5'}$ = 12.0). ^{31}P NMR spectrum (DMSO-d_6, δ, ppm): –1.10 s.

6-*N*-Benzyladenosine-3′-*O*-monophosphate (7b). PMR spectrum (DMSO-d_6, δ, ppm, J/Hz): 8.52 (1H, m, NH), 8.37 (1H, s, H-2), 8.18 (1H, s, H-8), 7.31-7.16 (5H, m, C_6H_5), 5.89 (1H, d, H-1′, $J_{1',2'}$ = 7.11), 4.77 (1H, m, H-2′, $J_{2',1'}$ = 7.11, $J_{2',3'}$ = 5.3, $^4J_{2',P}$ < 1.0), 4.68 (2H, m, C$\underline{H}_2$Ph), 4.63 (1H, m, H-3′, $J_{3',2'}$ = 5.3, $J_{3',P}$ = 7.0), 4.22 (1H, m, H-4′), 3.67 (1H, dd, H-5′, $J_{5',4'}$ = 3.0, $J_{5',5''}$ = 12.3), 3.55 (1H, dd, H-5″, $J_{5'',4'}$ = 3.1, $J_{5'',5'}$ = 12.3). ^{31}P NMR spectrum (DMSO-d_6, δ, ppm): –0.57 s.

Polymerization of 6-*N*-Benzyladenosine-2′(3′)-*O*-monophosphate (7a, 7b). Phosphates (**7a**, **7b**, 0.57 g, 1.3 mmol) were treated with methanol:ethanol (1:1, 26 mL) and tri-*n*-octylamine (0.46 g, 1.3 mmol), refluxed for 15 min until the starting powder dissolved, cooled, and evaporated to dryness. The solid was evaporated with benzene (3 × 10 mL), dissolved in dioxane (3.2 mL), stirred, treated with diphenylchlorophosphate (0.39 mL, 1.9 mmol) and tri-*n*-butylamine (0.91 mL, 3.8 mmol), stirred for 2 h, treated with diphenylchlorophosphate (0.39 mL, 1.9 mmol) and tri-*n*-butylamine (0.91 mL, 3.8 mmol), stirred for 20 h, evaporated to half the volume, and poured into ether (150 mL). The powdery white precipitate was filtered off, washed with ether (2 × 20 mL), and dried in air to afford alkylammonium salts of (2′-5′)-(3′-5′)-oligonucleotides **8** (0.68 g).

Enzymatic Hydrolysis of Mixed (2′-5′)-(3′-5′)-Oligonucleotides 8 by Enzyme Complex Nuclease/Phosphatase of Fungus *S. violacea*. A mixture of oligomers **8** (0.2 g) was treated with water (17.7 mL), sodium-acetate buffer (6.7 mL, 1 M, pH 6.0), $MgCl_2$ solution (0.67 mL, 0.5 M), and FCM of *S. violacea* (10.4 mL) [20]; stored in a thermostat at 55°C for 6 h; treated with FCM (10.4 mL); stored for another 12 h; heated on a boiling water bath for 3 min; and centrifuged for 10 min at 2,600 rpm. The supernatant was diluted with water to 400 mL, placed on a column with DEAE-Sephadex A-25 in the HCO_3^--form (120 cm^3), and eluted with water until absorption in UV light disappeared and then with a gradient of TEAB from 0 to 0.5 M (total volume 2 L). Fractions containing pure oligoadenylates **4** (n = 0-2) were combined and evaporated. The solids were evaporated with ethanol. Precipitation from methanol solutions by NaI solution (1 M) in acetone produced sodium salts of the following compounds (in order of elution from the column):

6-*N*-Benzyladenylyl(2′-5′)-6-*N*-benzyladenosine (**4**, n = 0, 32.8 mg, 21.4% calculated for **7a** and **7b**), R_f 0.80 (B), UV spectrum (MeOH, λ_{max}, nm): 270 (4.52). PMR spectrum (D_2O, δ, ppm, J/Hz): 8.19 (1H, s), 8.15 (s, 1H), 7.87 (1H, s), 7.82 (1H, s, 2H-2, 2H-8), 7.41-7.22 (10H, m, $2C_6H_5$), 6.17 (1H, d, H-1′, $J_{1',2'} = 5.22$), 5.83 (1H, d, H-1′, $J_{1',2'} = 3.63$), 5.23 (1H, m, H-2′, $J_{2',P} = 9.1$);

6-*N*-Benzyladenylyl(2′-5′)-6-*N*-benzyladenylyl(2′-5′)-6-*N*-benzyladenosine (**4**, n = 1, 16.0 mg, 10.1%), R_f 0.78 (B), UV spectrum (MeOH, λ_{max}, nm): 270 (4.71). PMR spectrum (D_2O, δ, ppm, J/Hz): 8.13 (1H, s), 8.08 (1H, s), 8.04 (1H, s), 7.98 (1H, s), 7.81 (1H, s), 7.75 (1H, s, 3H-2, 3H-8), 7.37-7.18 (15H, m, $3C_6H_5$), 6.08 (1H, d, H-1′, $J_{1',2'} = 5.19$), 6.01 (1H, d, H-1′, $J_{1',2'} = 4.44$), 5.86 (1H, d, H-1′, $J_{1',2'} = 3.00$), 5.07 (1H, m, H-2′, $J_{2',P} = 8.4$). ^{31}P NMR spectrum (D_2O, δ, ppm): −1.39 s, −0.97 s;

6-*N*-Benzyladenylyl(2′-5′)-6-*N*-benzyladenylyl(2′-5′)-6-*N*-benzyladenylyl-(2′-5′)-6-*N*-benzyladenosine (**4**, n = 2, 3.8 mg, 2.4%), R_f 0.75 (B), UV spectrum (MeOH, λ_{max}, nm): 270 (4.74).

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