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Synthesis of nucleoside tetraphosphates and dinucleoside pentaphosphates from nucleoside phosphoropiperidates *via* the activation of P(V)–N bond

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ABSTRACT

A novel and efficient method for the preparation of nucleoside 5′-tetraphosphates has been developed by coupling nucleoside 5′-phosphoropiperidates with triphosphate reagent in the presence of 4, 5-dicyanoimidazole (DCI) activator. Further coupling of the nucleoside 5′-tetraphosphates with nucleoside 5′-phosphoropiperidates *via* the P(V)–N activation strategy provided a reliable synthetic method for both symmetrical and asymmetrical dinucleoside pentaphosphates.

1. Introduction

As strong agonists of the purinergic receptors, endogenous dinucleoside pentaphosphates, including Ap_5A, Ap_5G, and Gp_5G, are essential extracellular signaling molecules, and play important roles in physiological and pathological processes in cardiovascular and nervous systems [1]. Ap_5A [2] and Ap_5G [2b,3] exhibit both vasoregulatory effect and proliferative effect on vascular smooth muscle cells (VSMCs), and Gp_5G [3,4] only induces proliferation of VSMCs. In biochemical research, the Ap_5A-Zn^{2+} complex has been utilized as a probe to elucidate the configurational requirement for adenylate kinase catalysis [5]. In addition, artificial dinucleoside pentaphosphates, such as Up_5U and Ap_5T, have also been synthesized. While Up_5U showed selective agonist activity on $P2Y_2$ receptor [6], Ap_5T exhibited strong inhibitory effects on thymidine kinase, thymidylate kinase, and ribonucleotide reductase [7].

Currently, the available synthetic methods for the preparation of dinucleoside pentaphosphates are still limited compared to those for dinucleoside di-, tri-, and tetraphosphates. The most commonly employed strategy was the direct condensation of nucleoside diphosphate with another molecule of nucleoside triphosphate by using *N*,*N*′-dicyclohexylcarbodiimide (DCC) or *N*,*N*′-carbonyldiimidazole (CDI) [5a,6–8]. But this method was typically low yielding (10%–20%) and time-consuming (1–3 days). Though Jones *et al.* [9] and Taylor *et al.* [10] reported novel methods for dinucleoside pentaphosphate synthesis, the protection of the nucleosides and preparation of specific condensing reagents limited their values in practical applications. On the basis of the P(V)–N activation strategy we developed for the synthesis for nucleoside polyphosphates [11], we report in this paper a general and facile approach for the synthesis of dinucleoside pentaphosphates (Np_5N′s) *via* the 4,5-dicyanoimidazole (DCI)-promoted coupling of nucleoside 5′-phosphoropiperidates with nucleoside 5′-tetraphosphates (Np_4s). The P(V)–N activation method for the efficient synthesis of Np_4s [12] is also described.

2. Experimental

All reactions were performed in anhydrous solvents under an atmosphere of dry argon. The triethylammonium salts of nucleoside 5′-phosphoropiperidates were synthesized according to the procedure described in a previous report [11b]. Tris(tetra-*n*-butylammonium) dihydrogen triphosphate was prepared according to a known method [12a]. Ion exchange chromatography employed DEAE Sephadex A-25 exchanger. Preparative HPLC was equipped with a RP C18 column (19 mm × 250 mm, 10 μm). NMR

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spectra were obtained with a 400 MHz instrument with chemical shifts reported in parts per million (ppm, δ). IR spectra were recorded on a FT-IR spectrometer. Low-resolution mass spectra were obtained with an ion trap mass spectrometer and reported as m/z.

2.1. General procedure for the synthesis of nucleoside 5′-tetraphosphates (**5**–**8**)

To a solution of nucleoside 5′-phosphoropiperidate (0.1 mmol) in DMF (2 mL) were added tris(tri-*n*-butylammonium) dihydrogen triphosphate (0.2 mmol) and 4,5-dicyanoimidazole (DCI, 0.6 mmol). The reaction was stirred at 20 °C for 12 h and concentrated *in vacuo*. The residue was dissolved in NaOAc aqueous solution (3 mol/L, 1 mL) and EtOH (50 mL) was added. The resulting white precipitate was collected by centrifuge. The crude product was dissolved in deionized H_2O (1 mL) and loaded on a DEAE Sephadex A-25 ion exchange column (1.6 cm × 25 cm). Elution with NH_4HCO_3 buffer (linear gradient 0.3–0.6 mol/L), combination of appropriate fractions, and lyophilization afforded Np_4 in ammonium salt form. For characterization, passage of the solution of the ammonium salt in deionized H_2O through a bed of Dowex 50W-X8 ion exchange resin (Na^+ form) and lyophilization afforded nucleoside 5′-tetraphosphate as pentasodium salt. For the next step reaction, to the ammonium salt in deionized H_2O was added tetra-*n*-butylammonium hydroxide (3 equiv.), and the solution was repeatedly evaporated with deionized H_2O (1 mL × 3) to afford nucleoside 5′-tetraphosphate as more soluble tris(tetra-*n*-butylammonium) salt.

Uridine 5′-tetraphosphate, pentasodium salt (**5**): Starting from **1** (49 mg), **5** (45 mg, 67%) was obtained as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 7.99 (d, 1H, J = 8.0 Hz), 6.06–5.98 (m, 2H), 4.50–4.40 (m, 2H), 4.35–4.30 (m, 1H), 4.30–4.23 (m, 2H); ^{13}C NMR (100 MHz, D_2O): δ 166.2, 152.0, 141.7, 102.8, 88.1, 83.6 (d, $J_{P,C}$ = 9.0 Hz), 73.7, 69.8, 65.2 (d, $J_{P,C}$ = 5.1 Hz); ^{31}P NMR (D_2O, 162 MHz): δ −8.0 (d, 1P, J = 18 Hz), −11.1 (dd, 1P, J = 18 Hz), −22.3 (dd, 1P, $J_1 = J_2$ = 18 Hz), −22.5 (d, 1P, $J_1 = J_2$ = 18 Hz); IR (cm^{-1}): ν_{max} 3336, 2987, 2899, 1695, 1413, 1228, 1087, 924, 838; LRMS (ESI−): m/z calcd. for $C_9H_{15}N_2O_{18}P_4$ $[M-H]^-$ 562.9; found: 563.0.

Cytidine 5′-tetraphosphate, pentasodium salt (**6**): Starting from **2** (49 mg), **6** (41 mg, 61%) was obtained as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 7.96 (d, 1H, J = 7.2 Hz), 6.14 (d, 1H, J = 7.4 Hz), 6.01 (d, 1H, J = 4.4 Hz), 4.43–4.37 (m, 1H), 4.36–4.30 (m, 1H), 4.29–4.20 (m, 3H); ^{13}C NMR (100 MHz, D_2O): δ 166.3, 157.9, 141.7, 96.9, 89.0, 83.1 (d, $J_{P,C}$ = 8.7 Hz), 74.3, 69.6, 65.0 (d, $J_{P,C}$ = 5.6 Hz); ^{31}P NMR (D_2O, 162 MHz): δ −9.4 (d, 1P, J = 18 Hz), −14.4 (d, 1P, J = 18 Hz), −24.9 (dd, 1P, $J_1 = J_2$ = 18 Hz), −25.7 (dd, 1P, $J_1 = J_2$ = 18 Hz); IR (cm^{-1}): ν_{max} 3370, 2985, 2894, 1693, 1537, 1418, 1235, 1079, 887; LRMS (ESI−): m/z calcd. for $C_9H_{16}N_3O_{17}P_4$ $[M-H]^-$ 561.9; found: 562.0.

Adenosine 5′-tetraphosphate, pentasodium salt (**7**): Starting from **3** (52 mg), **7** (45 mg, 65%) was obtained as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 8.54 (s, 1H), 8.24 (d, 1H, J = 6.5 Hz), 6.14 (d, 1H, J = 6.1 Hz), 4.72–4.70 (m, 1H), 4.64 (dd, 1H, $J_1 = J_2$ = 4.8 Hz), 4.48–4.40 (m, 1H), 4.36–4.27 (m, 1H), 4.27–4.18 (m, 1H); ^{13}C NMR (100 MHz, D_2O): δ 155.6, 152.8, 149.2, 139.9, 118.6, 86.5, 84.3 (d, $J_{P,C}$ = 9.0 Hz), 74.2, 70.4, 65.4 (d, $J_{P,C}$ = 5.3 Hz); ^{31}P NMR (D_2O, 162 MHz): δ −11.5 (d, 1P, J = 18 Hz), −14.8 (d, 1P, J = 18 Hz), −25.8 (dd, 1P, $J_1 = J_2$ = 18 Hz), −26.2 (dd, 1P, $J_1 = J_2$ = 18 Hz); IR (cm^{-1}): ν_{max} 3354, 3049, 2795, 1714, 1492, 1453, 1260, 1087, 1044, 967, 924; LRMS (ESI−): m/z calcd. for $C_{10}H_{16}N_5O_{16}P_4$ $[M-H]^-$ 586.0; found: 586.1.

Guanosine 5′-tetraphosphate, pentasodium salt (**8**): Starting from **4** (54 mg), **8** (46 mg, 64%) was obtained as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 8.14 (s, 1H), 5.93 (d, 1H, J = 6.0 Hz), 4.75–4.70 (m, 1H), 4.65–4.58 (m, 1H), 4.43–4.33 (m, 1H), 4.31–4.16 (m, 2H); ^{13}C NMR (100 MHz, D_2O): δ 159.2, 154.1, 152.0, 138.0, 116.4, 86.7, 84.4, 73.7, 70.6, 65.6; ^{31}P NMR (D_2O, 162 MHz): δ −10.4 (d, 1P, J = 19 Hz), −14.8 (d, 1P, J = 19 Hz), −25.6 (dd, 1P, $J_1 = J_2$ = 18 Hz), −26.2 (d, 1P, $J_1 = J_2$ = 18 Hz); IR (cm^{-1}): ν_{max} 3378, 2960, 2950, 1712, 1673, 1543, 1403, 1255, 1065, 914, 812; LRMS (ESI−): m/z calcd. for $C_{10}H_{16}N_5O_{17}P_4$ $[M-H]^-$ 601.9; found: 602.0.

2.2. General procedure for the synthesis of dinucleoside-5′, 5′-pentaphosphates (**9**–**11**)

To a solution of nucleoside 5′-phosphoropiperidate (0.1 mmol) in *N*-methylpyrrolidone (2 mL) were added nucleoside 5′-tetraphosphate (tetra-*n*-butylammonium salt, 0.04 mmol) and DCI (0.2 mmol). The reaction was stirred at 20 °C for 16–18 h. The white precipitation was collected by centrifuge. The crude product was dissolved in deionized H_2O (0.5 mL) and loaded on a DEAE Sephadex A-25 ion exchange column (1.6 cm × 25 cm). Elution with NH_4HCO_3 buffer (linear gradient 0.5 to 0.9 mol/L), combination of appropriate fractions, and lyophilization afforded dinucleoside pentaphosphate in ammonium salt form. To remove the small amount of contaminated polyphosphate byproducts, the ammonium salt was further purified by a preparative RP HPLC [flow rate = 20 mL/min; linear gradient of 0–10% MeOH in TEAB buffer (10 mmol/L, pH 8.0) over 15 min; UV detection at 254 nm]. Combination of appropriate fractions and lyophilization afforded dinucleoside pentaphosphate in triethylammonium salt form. Passage of the solution of the triethylammonium salt in deionized H_2O through a bed of Dowex 50W-X8 ion exchange resin (Na^+ form) and lyophilization afforded dinucleoside pentaphosphate as pentasodium salt.

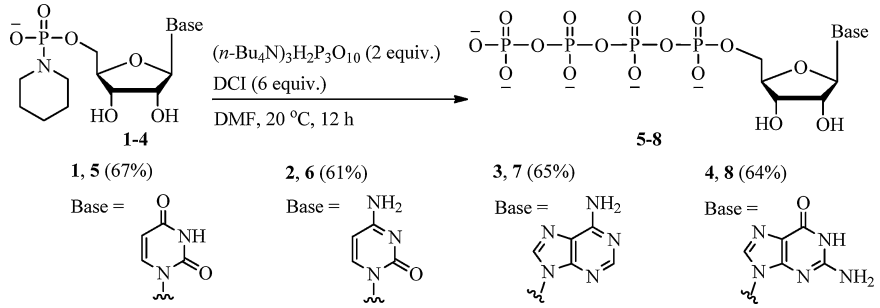
P^1,P^5-Diadenosine-5′,5′-pentaphosphate, pentasodium salt (**9**): Starting from **7** (52 mg), **9** (14 mg, 35%) was obtained as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 8.38 (s, 2H), 8.11 (d, 2H, J = 6.0 Hz), 6.06–5.98 (m, 2H), 4.75–4.68 (m, 2H), 4.60–4.51 (m, 2H), 4.39–4.32 (m, 2H), 4.31–4.15 (m, 4H); ^{13}C NMR (100 MHz, D_2O): δ 154.4, 151.8, 148.1, 138.9, 117.3, 85.7, 73.7, 69.4, 67.6, 64.3; ^{31}P NMR (D_2O, 162 MHz): δ −11.5 (m, 2P), −22.9 (m, 3P); IR (cm^{-1}): ν_{max} 3324, 3149, 2975, 1714, 1492, 1404, 1260, 1087, 1044, 967, 924; LRMS (ESI−): m/z calcd. for $C_{20}H_{28}N_{10}O_{55}P_5$ $[M-H]^-$ 915.0; found: 915.1.

P^1,P^5-Diguanosine-5′,5′-pentaphosphate, pentasodium salt (**10**): Starting from **8** (52 mg), **10** (14 mg, 34%) was obtained as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 8.00 (s, 2H), 5.80 (s, 2H), 4.60–4.44 (m, 4H), 4.35–4.15 (m, 6H); ^{13}C NMR (100 MHz, D_2O): δ 158.6, 153.5, 151.3, 137.4, 115.8, 86.4, 83.6, 73.1, 70.0, 65.0; ^{31}P NMR (D_2O, 162 MHz): δ −11.0 (m, 2P), −22.4 (m, 3P); IR (cm^{-1}): ν_{max} 3388, 2964, 2922, 1673, 1543, 1413, 1250, 1065, 914, 816; LRMS (ESI−): m/z calcd. for $C_{20}H_{28}N_{10}O_{24}P_5$ $[M-H]^-$ 947.0; found: 947.1.

P^1-Adenosine-5′-P^5-guanosine-5′-pentaphosphate, pentasodium salt (**11**): Starting from **7** (52 mg), **11** (13 mg, 31%) was obtained as a white solid. ^{1}H NMR (400 MHz, D_2O): δ 8.33 (s, 1H), 8.04 (s, 1H), 7.91 (s, 1H), 5.95 (d, 1H, J = 5.8 Hz), 5.70 (d, 1H, J = 6.3 Hz), 4.63 (m, 2H), 4.45 (m, 2H), 4.25–4.20 (m, 2H), 4.13–4.10 (m, 4H); ^{13}C NMR (100 MHz, D_2O): δ 159.0, 155.6, 154.0, 153.0, 151.8, 149.2, 140.1, 137.9, 118.6, 116.3, 86.8 (×2), 84.2 (×2), 74.4, 73.5, 70.5 (×2), 65.5 (×2); ^{31}P NMR (162 MHz, D_2O): δ −11.0 (m, 2P), −22.8 (m, 3P); IR (cm^{-1}): ν_{max} 3783, 3455, 2950, 2673, 2480, 1722, 1706, 1618, 1442, 1380, 1243, 1122, 1060, 948, 800, 732; LRMS (ESI−): m/z calcd. for $C_{20}H_{28}N_{10}O_{23}P_5$ $[M-H]^-$ 931.0; found: 931.1.

3. Results and discussion

As shown in Scheme 1, nucleoside 5′-tetraphosphates (**5**–**8**) were efficiently synthesized by treating nucleoside 5′-phosphoropiperidates (**1**–**4**) with 2.0 equiv. of tris(tetra-*n*-butylammonium)



Scheme 1. The P(V)–N activation method for the synthesis of nucleoside 5′-tetraphosphates (**5**–**8**).

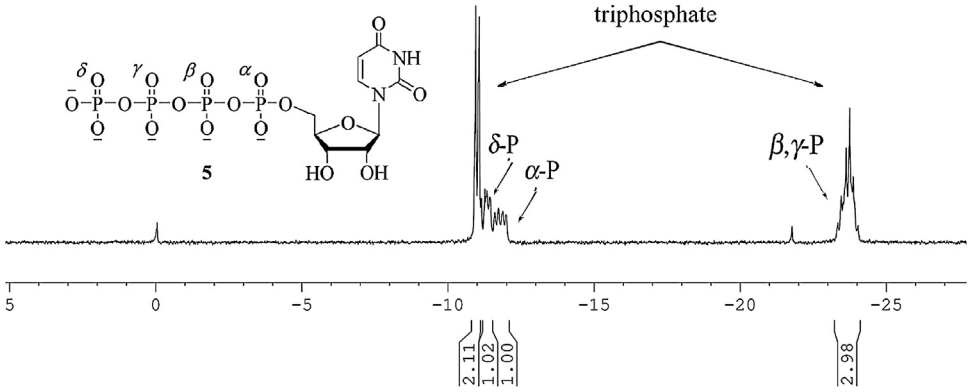
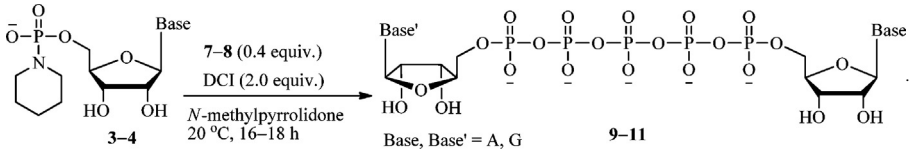


Fig. 1. The ^{31}P NMR spectrum of crude reaction mixture of Up_4 (**5**).

Table 1
The P(V)–N activation method for the synthesis of dinucleoside pentaphosphates (**9**–**11**).



Entry	Base	Base′	Reaction time (h)	Product	Isolated yield (%)
1	A (**3**)	A (**7**)	16	Ap_5A (**9**)	35
2	G (**4**)	G (**8**)	18	Gp_5G (**10**)	34
3	G (**4**)	A (**7**)	18	Ap_5G (**11**)	31

triphosphate and 6.0 equiv. of DCI in anhydrous DMF at 20 °C for 12 h. The precipitated crude products were separated by filtration. Ethanol precipitation of the sodium salts followed by ion exchange chromatography afforded **5**–**8** in good isolated yields ranging from 61% to 67%. The ^{31}P NMR spectrum of crude reaction mixture of **5** (Fig. 1) showed that the conversion of **1**–**5** was smooth and clean. The calculated ^{31}P NMR yield of **5** was close to 90%. Compared to nucleoside 5′-triphosphate synthesis [11a], longer reaction time was required, indicating that the nucleophilicity of triphosphate reagent was lower than that of pyrophosphate. However, due to the highly negatively charged nature, the isolated yields of **5**–**8** were slightly lower than those of nucleoside di- and tri-phosphates. It was also noticed that Np_4s were less stable than their NTP counterparts. When the aqueous solution of **7** was stored at −20 °C, about 10% of **7** decomposed after 6 months.

In the following research, adenosine 5′-phosphoropiperidate (**3**) was coupled with Ap_4 (**7**) in 1:0.5 molar ratio [11d] in *N*-methylpyrrolidone at 20 °C with or without DCI. ^{31}P NMR tracing experiments showed that there was almost no reaction between the reactants after 24 h without DCI. This result was in accordance with the observed low reactivity of phosphoropiperidate toward phosphate nucleophiles in our previous research. In contrast, addition of acidic DCI (2.0 equiv.) promoted the reaction to completion in 16 h. But when **3** disappeared, there was still significant amount of **7** (~20%) unreacted due to its low nucleophilicity and the self-condensation of **3**. Decrement of the equivalent of **7** to 0.4 effectively promoted its consumption, and the ^{31}P NMR yield of Ap_5A (**9**) was improved to around 50%. But we found that ion exchange chromatography alone was difficult to completely separate the desired Np_5N' products from other closely related long chain polyphosphate impurities. Therefore, RP-HPLC was employed afterward to ensure the purity of these highly negatively charged compounds. As shown in Table 1, three naturally occurring Np_5N's (**9**–**11**) were synthesized and isolated in 31%–35% yields following this P(V)–N activation method.

4. Conclusion

In summary, we developed efficient P(V)–N activation methods for the synthesis of both nucleoside 5′-tetraphosphates and dinucleoside pentaphosphates. Compared to the known methods, these new approaches feature easily accessible starting materials, mild reaction conditions, and moderate to good isolated yields, and provide facile and reliable access to Np_4s and Np_5N's.

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