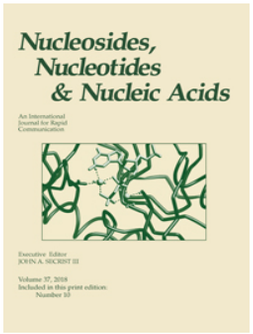

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Highly regioselective methylation of inosine nucleotide: an efficient synthesis of 7-methylinosine nucleotide

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ABSTRACT

A facile, straightforward, reliable, and an efficient chemical synthesis of inosine nucleotides such as 7-methylinosine 5′-*O*-monophosphate, 7-methylinosine 5′-*O*-diphosphate, and 7-methylinosine 5′-*O*-triphosphate, starting from the corresponding inosine nucleotide is delineated. The present methylation reaction of inosine nucleotide utilizes dimethyl sulfate as a methylating agent and water as a solvent at room temperature. It is noteworthy that the present methylation reaction proceeds smoothly under aqueous conditions that is highly regioselective to afford exclusive 7-methylinosine nucleotide in good yields with high purity (>99.5%).

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Inosine; 7-methylinosine; nucleotide; methylation; regioselective

1. Introduction

The 5′-end of cellular and eukaryotic viral RNAs that are synthesized by various RNA polymerase contain distinct cap structure $m^7G[5']ppp[5']N$, where N is any nucleotide (N = G, A, C or U).[1] The cap structure containing 7-methylguanosine residue displays a wide spectrum of molecular biology applications such as intracellular transport, splicing, translation, and protection from degradation.[2–4] Notably, the cap structure is being specifically recognized by eukaryotic initiation factor 4E (eIF4E) during the initiation of translation,[5] vaccinia virus methyltransferase VP39,[6] and the CBC80/20 nuclear cap-binding complex.[7] The presence of 7-methylguanosine moiety in the cap structure plays an important role for the recognition as it forms Watson-Crick-like hydrogen bonds with the enzymes.[5] The exploration of 7-methylguanosine nucleotide and cap analog as a potential translation inhibitor is a powerful target for anti-cancer directed therapy.[8–11] The elevated eIF4E levels in tumor cells is suppressed by the ability of these analogs to bind with eIF4E and acts as an anti-cancer agent. In addition to the synthesis of capped mRNA containing 7-methylguanosine, the synthesis of mRNA containing 5′-terminal 7-methylinosine-capped was achieved by the initiation of reovirus transcription using inosine 5′-

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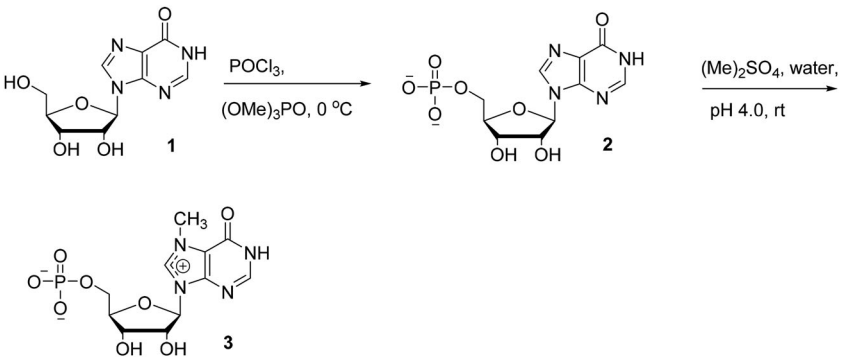
triphosphate.[12] While the use of 7-methylguanosine capped mRNA has been well documented in the literature for several molecular biology applications,[1,8] the utilization of 7-methylinosine capped mRNA is under explored.

The use of methyl iodide has been the most commonly used methylating agent for the synthesis of 7-methylguanosine nucleotide, starting from the corresponding guanosine nucleotide.[3,13–15] However, the reaction requires longer time to complete and often results in poor yields due to the poor solubility of guanosine nucleotide in the presence of organic solvent. Another method involves the use of methyl methanesulfonate as a methylating agent under aqueous conditions for the methylation of guanosine moiety but a wide variety of generality has not been achieved using this reagent.[16] We have developed an efficient synthesis of various substituted 7-methylguanosine nucleotides in good yields involving the use of dimethyl sulfate as a methylating agent under aqueous conditions.[17–23] It has been reported in the literature that 7-methylinosine was achieved by the reaction of inosine using methyl iodide as a methylating agent.[24] While the synthesis of 7-methylinosine and several types of substituted 7-methylguanosine nucleotide have been reported,[17–24] to the best of our knowledge, no synthesis of 7-methylinosine nucleotide has been reported in the literature. Given the presence of delocalized positive charge to the nucleobase for both 7-methylgunaosine and 7-methylinosine, we envisaged that the properties of 7-methylinosine nucleotide will be similar to the properties of 7-methylguanosine nucleotide and these analogs will be used in the area of mRNA cap analogs and cancer immunotherapy. Therefore, the development of new chemical method for the synthesis of 7-methylinosine nucleotide is demanded in order to explore the potential applications of these analogs. Our continuous interest in the area of nucleic acid chemistry,[18–23,25–28] prompted us to develop a reliable chemical method for the synthesis of inosine nucleotides. Herein, we report the first example of an efficient chemical synthesis of 7-methylinosine nucleotides such as 7-methylinosine 5′-*O*-monophosphate (m^7IMP), 7-methylinosine 5′-*O*-diphosphate (m^7IDP), and 7-methylinosine 5′-*O*-triphosphate (m^7ITP) in good yields with high purity (>99.5%).

2. Results and discussion

The reaction pathway leading to the formation of 7-methylinosine 5′-*O*-monophosphate (**3**) is depicted in Scheme 1. The required starting material (i.e.,) inosine 5′-*O*-monophosphate (**2**) was obtained by the monophosphorylation of inosine (**1**) using $POCl_3$ as a phosphorylating agent and trimethyl phosphate as a solvent.[29] The methylation reaction of **2** with dimethyl



Scheme 1. Synthesis of 7-methylinosine 5′-*O*-monophosphate (**3**).

sulfate as a methylating agent using water as a solvent at pH 4.0 afforded the corresponding 7-methylinosine 5′-*O*-monophosphate (**3**) in 80% yield. It is noteworthy that the methylation reaction is highly regioselective. No other regioisomers was formed as evidenced by HPLC of the crude reaction mixture. The regiochemistry of **3** was confirmed ^{1}H NMR based on the disappearance of H-8 proton in **3**. The ^{1}H NMR spectrum of **2** showed a characteristic singlet peak for H-8 proton at δ 8.10,[29] whereas, in the case of **3**, no characteristic peak was seen for the H-8 proton probably due to the presence of delocalized positive charge to the nucleobase. This observation is in agreement with our previously reported 7-methylguanosine analogs in which there was no characteristic peak for H-8 proton in the ^{1}H NMR spectrum.[18–23] The regiochemistry of **3** was further corroborated based on 2D-NOESY spectrum. The purity of final isolated product **3** was checked by HPLC (Figure 1). The structure of **3** was confirmed by ^{1}H and ^{13}C NMR and mass data.

In a similar manner, the synthetic strategy for the formation of 7-methylinosine 5′-*O*-diphosphate (**6**) is shown in Scheme 2. The activation of inosine monophosphate **2** with imidazole in the presence of triphenylphosphine and 2,2′-dipyridyl disulfide, followed by the phosphorylation of resulting imidazolide **4** with $(Bu_3NH)_3PO_4$ in the presence of zinc chloride as a catalyst afforded the corresponding inosine-5′-diphosphate (**5**) in 75% yield.[29] Next, treatment of inosine diphosphate **5** with dimethyl sulfate under optimized aqueous conditions afforded the highly regioselective 7-methylinosine 5′-*O*-diphosphate (**6**) in 75% yield.

In addition to the synthesis of 7-methylinosine monophosphate **3** and diphosphate **6**, the present reaction strategy was successfully extended to the synthesis of 7-methylinosine 5′-*O*-triphosphate (**8**) as depicted in Scheme 3. The inosine triphosphate **7** was achieved using “one pot, three-step” Ludwig synthetic strategy.[29] The monophosphorylation reaction of inosine (**1**) with phosphorus oxychloride using trimethyl phosphate as a solvent, followed by the triphosphorylation reaction with tributylammonium pyrophosphate using

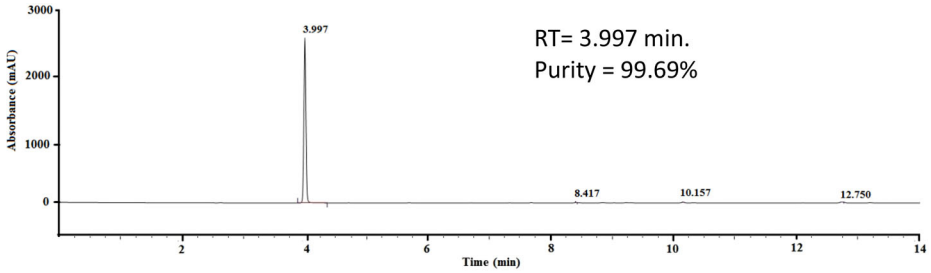
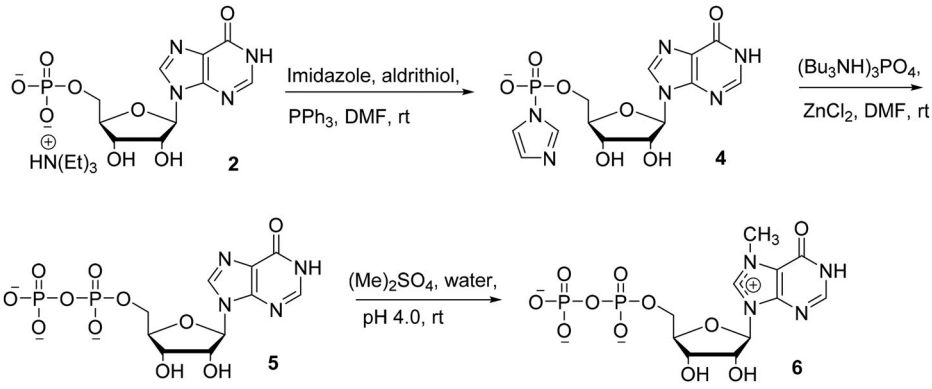


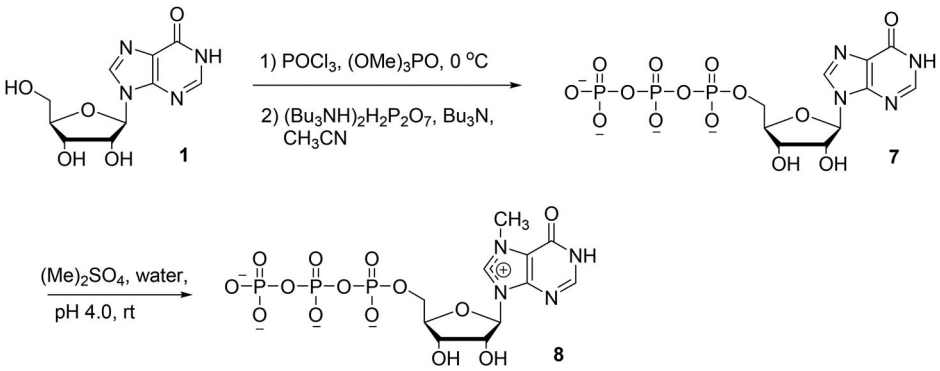
Figure 1. HPLC spectrum of 7-methylinosine 5′-*O*-monophosphate (**3**).



Scheme 2. Synthesis of 7-methylinosine 5′-*O*-diphosphate (**6**).

acetonitrile as a solvent and tributylamine as a base afforded inosine-5′-triphosphate (**7**) in 62% yield. The methylation reaction of **7** with dimethyl sulfate under optimized aqueous conditions furnished the highly regioselective 7-methylinosine 5′-*O*-triphosphate (**8**) in 74% yield.

There are several interesting features that merit comments for the present methylation reaction. First, in all cases, the methylation reaction is high regioselective affording the corresponding single regioisomer, 7-methylinosine nucleotide in good yields. The other possible regioisomer, 1-methylinosine nucleotide was not formed under our optimized reaction conditions. It is to be noted that the synthesis of 1-methylinosine was achieved by the methylation reaction of inosine using trimethyloxosulfonium hydroxide as a methylating agent.[30] Second, the present methylation reaction proceeds smoothly under aqueous conditions. This is in striking contrast with the literature method that utilizes organic solvent for the methylation of purine nucleotides.[13–15] Finally, the purification procedure for the synthesis of inosine nucleotide is simple, straightforward, and furnishes a final pure product with extremely high purity (>99.5%) as evidenced by HPLC. It is to be noted that the successful molecular biology applications depend on the high purity of nucleotide.



Scheme 3. Synthesis of 7-methylinosine 5′-*O*-triphosphate (**8**).

In conclusion, we have developed an efficient and reliable chemical method for the synthesis of 7-methylinosine nucleotides such as 7-methylinosine 5′-*O*-monophosphate, 7-methylinosine 5′-*O*-diphosphate, and 7-methylinosine 5′-*O*-triphosphate in good yields. It is noteworthy that the present developed methylation reaction is highly regioselective and the final product is isolated with extremely high purity (>99.5%) as evidenced by HPLC. Further work is in progress to study the potential biological applications of these analogs and will be reported in due course.

2.1. Experimental

2.1.1. General

All of the commercial reagents and solvents are used as such without further purification. Inosine was purchased from Sigma-Aldrich. Compounds such as inosine-5′-monophosphate (**2**), inosine-5′-diphosphate (**5**), and inosine-5′-triphosphate (**7**) were prepared according to a reported method.[29] ^{1}H NMR spectra were recorded in D_2O on a Bruker 400 MHz and ^{31}P NMR were recorded on a Bruker 162 MHz. Chemical shifts are reported in ppm, and signals are described as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). ESI mass was recorded on an Applied Biosystems/Sciex MDX API 150 model. HPLC was run on a Thermo Scientific DIONEX Ultimate 3000 using a Hypersil SAX column. FPLC (fast protein liquid chromatography) was performed with an ÄKTA purifier (GE Healthcare) using a DEAE Sepharose column.

2.2. Synthesis of 7-methylinosine 5′-O-monophosphate (3)

To a stirred solution of inosine-5′-monophosphate (**2**) (1.0 g, 2.70 mmol) in 20.0 mL of water, acetic acid was added slowly to adjust the pH of the solution to 4.0. To this mixture, dimethyl sulfate (3.8 mL) was added drop wise

over a period of 30 min. and the reaction mixture was allowed to stir at rt for 4 h. As the methylation proceeds, the pH drops down to around 2.0 and the pH was readjusted back to 4.0 using 1M NaOH solution. Then, the reaction mixture was extracted with ethyl acetate (2 × 25 mL) to remove unreacted excess dimethyl sulfate. The collected aqueous solution was adjusted to pH 6.5 and loaded on a DEAE Sepharose column. The desired product was eluted using a linear gradient of 0-1M TEAB and the fractions containing the product were pooled, evaporated, and co-evaporated with water (3 × 100 mL). The TEA salt of 7-methylinosine 5′-*O*-monophosphate thus obtained was subjected to ion-exchange with sodium perchlorate (5.0 g) in acetone (100.0 mL) for two times to afford the sodium salt of 7-methylinosine 5′-*O*-monophosphate **3** as a fine white powder (Yield 0.83 g, 80%). ^{1}H NMR (D_2O, 400 MHz) δ 8.36 (s, 1H, H-2), 6.26 (d, $J = 3.2$ Hz, 1H, H-1′), 4.70 (m, 1H), 4.41 (m, 2H), 4.23 (m, 1H), 4.20 (s, 3H, CH_3), 4.10 (m, 1H); ^{31}P NMR (D_2O, 162 MHz) δ 0.38 (s, 1P); MS (m/z): 362 $[M\text{-}H]^-$.

2.3. Synthesis of 7-methylinosine 5′-O-diphosphate (6)

To a stirred solution of inosine-5′-diphosphate (**5**) (1.0 g, 2.12 mmol) in 20.0 mL of water, acetic acid was added to adjust the pH of the solution to 4.0. To this mixture, dimethyl sulfate (3.0 mL) was added drop wise over a period of 30 min. and the reaction mixture was allowed to stir at rt for 5 h. As the methylation proceeds, the pH drops down to around 2.0 and the pH was readjusted back to 4.0 using 1M NaOH solution. After completion of the reaction, it was extracted with ethyl acetate (2 × 25 mL) to remove unreacted excess dimethyl sulfate. The collected aqueous solution was adjusted to pH 6.5 and loaded on a DEAE Sepharose column. The desired product was eluted using a linear gradient of 0-1M TEAB and the fractions containing the product were pooled, evaporated and co-evaporated with water (3 × 100 mL). The TEA salt of 7-methylinosine 5′-*O*-diphosphate thus obtained was subjected to ion-exchange with sodium perchlorate (5.0 g) in acetone (100.0 mL) for two times to afford the sodium salt of 7-methylinosine 5′-*O*-diphosphate **6** as a fine white powder (Yield 0.77 g, 75%). ^{1}H NMR (D_2O, 400 MHz) δ 8.28 (s, 1H, H-2), 6.19 (d, $J = 3.2$ Hz, 1H, H-1′), 4.63 (m, 1H), 4.34 (m, 2H), 4.24 (m, 1H), 4.13 (s, 3H, CH_3), 4.07 (m, 1H); ^{31}P NMR (D_2O, 162 MHz) δ −9.58 (d, $J = 21.0$ Hz, 1P), −11.03 (d, $J = 21.0$ Hz, 1P); MS (m/z): 442 $[M\text{-}H]^-$.

2.4. Synthesis of 7-methylinosine 5′-O-triphosphate (8)

To a stirred solution of inosine-5′-triphosphate (**7**) (1.0 g, 1.74 mmol) in 20.0 mL of water, acetic acid was added to adjust the pH of the solution to

4.0. To this mixture, dimethyl sulfate (2.5 mL) was added drop wise over a period of 30 min. and the reaction mixture was allowed to stir at rt for 5 h. As the methylation proceeds, the pH drops down to around 2.0 and the pH was readjusted back to 4.0 using 1 M NaOH solution. After completion of the reaction, the mixture was extracted with ethyl acetate (2 × 25 mL) to remove unreacted excess dimethyl sulfate. The collected aqueous solution was adjusted to pH 6.5 and loaded on a DEAE Sepharose column. The desired product was eluted using a linear gradient of 0-1M TEAB and the fractions containing the product were pooled, evaporated and co-evaporated with water (3 × 100 mL). The TEA salt of 7-methylinosine 5′-*O*-triphosphate thus obtained was subjected to ion-exchange with sodium perchlorate (5.0 g) in acetone (100.0 mL) for two times to afford the sodium salt of 7-methylinosine 5′-*O*-triphosphate **8** as a fine white powder (Yield 0.76 g, 74%). ^{1}H NMR (D_2O, 400 MHz) δ 8.29 (s, 1H, H-2), 6.17 (d, $J = 3.2$ Hz, 1H, H-1′), 4.62 (m, 1H), 4.38 (m, 2H), 4.27 (m, 1H), 4.15 (s, 3H, CH_3), 4.11 (m, 1H); ^{31}P NMR (D_2O, 162 MHz) δ −8.78 (d, $J = 17.9$ Hz, 1P), −11.15 (d, $J = 19.6$ Hz, 1P), −22.16 (t, $J = 19.6$ Hz, 1P); MS (m/z): 522 $[M\text{-}H]^-$.

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