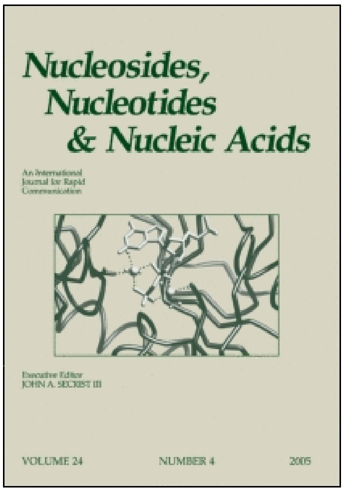

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An Efficient Process for Synthesis of 2'-*O*-methyl and 3'-*O*-methyl Guanosine from 2-aminoadenosine Using Diazomethane and the Catalyst Stannous Chloride

Anilkumar R. Kore[a]; Gaurang Parmar[a]; Srinu Reddy[a]
[a] Bioorganic Chemistry Division, Ambion, Inc., Austin, Texas, USA

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AN EFFICIENT PROCESS FOR SYNTHESIS OF 2′-*O*-METHYL AND 3′-*O*-METHYL GUANOSINE FROM 2-AMINOADENOSINE USING DIAZOMETHANE AND THE CATALYST STANNOUS CHLORIDE

Anilkumar R. Kore, Gaurang Parmar, and Srinu Reddy □ *Ambion, Inc., Bioorganic Chemistry Division, Austin, Texas, USA*

□ *An improved strategy for the selective synthesis of 2′-O-methyl and 3′-O-methyl guanosine from 2-aminoadenosine is reported by using the catalyst stannous chloride. The regioselectivity of the 2′ and 3′-O-alkylation was achieved by optimizing the addition, timing, and concentration of the catalysts and diazomethane during the methylation reaction. An efficient and selective alkylation at 2′-OH of 2-aminoadenosine was achieved by mixing a stoichiometric amount of stannous chloride at room temperature in DMF. The reaction mixture was stirred at 50°C for 1 min and immediately followed by addition of diazomethane. The resulting 2′-O-methyl 2-aminoadenosine was treated with the enzyme adenosine deaminase, which resulted in an efficient conversion to the desired 2′-O-methylguanosine (98% yield). The product was isolated by crystallization. In contrast, the methylation at 3′-OH of 2-aminoadenosine was achieved by mixing a stoichiometric amount of stannous chloride in DMF and stirring at 50°C for 15 min, followed by addition of diazomethane. The resulting mixture containing 3′-O-methyl-2-aminoadenosine in 90% yield and 2′-O-methyl-2-aminoadenosine in 10% yield was treated with the enzyme adenosine deaminase, which preferentially deaminated only 3′-O-methyl-2-aminoadenosine, resulting in the production of 3′-O-methylguanosine in 88% yield. Due to the extremely low solubility 3′-O-methylguanosine, the compound precipitated and was isolated by centrifugation. This synthetic route obviates the chromatographic purification. Selective monomethylation is achieved by using the unprotected ribonucleoside. As a result, the method described herein represents a significant improvement over the current synthetic approach by providing superior product yield and economy, a much more facile purification of 2′,3′-O-methylated isomers, and eliminating the need for protected ribonucleosides reagents.*

Keywords Nucleoside Analogues; Adenosine Deaminase; Diazomethane; Catalyst

INTRODUCTION

2′-*O*-Methylribonucleosides are widely distributed in RNA[1] and have been the focus of considerable synthetic efforts since the late 1950s. These

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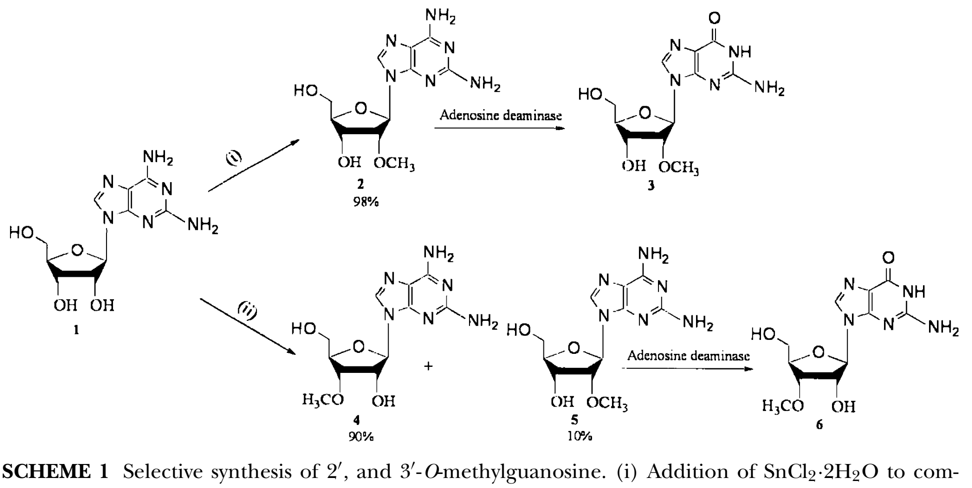
Address correspondence to Anilkumar Kore, Ambion, Inc., Bioorganic Chemistry Division, 2130 Woodward St., Austin, TX, 78744. E-mail: akore@ambion.com

synthetic efforts were further stimulated by recent applications of 2′-*O*-methyloligoribonucleotides in studying pre-mRNA splicing[2] and the structure of splicesomes[3] as well as in the preparation of nuclease resistant hammerhead ribozymes.[4] 2′-*O*-Alkyl substituted oligonucleotides have also emerged as a second-generation antisense construct with improved hybridization properties and favorable nuclease resistance and pharmacological profiles.[5] Recently, 2′-*O*-methylguanosine has been used to synthesize anti-reverse cap analogs with superior translational properties.[6] 3′-*O*-Methylguanosine was used to synthesize a chain-terminating dinucleotide mRNA cap analog[7] and was also reported as an anti-reverse cap analog[8] that has shown better translation efficiency over unmodified cap analogs.

A survey of the literature revealed a variety of problems encountered in the preparation and isolation of 2′ and 3′-*O*-methylribonucleosides.[9–13] Yields were often very low and the presence of the 3′-*O*-methyl-isomer complicated the purification, usually achieved by anion-exchange chromatography on Dowex. Robins et al.[14] markedly improved synthesis by demonstrating the utility of using one equivalent of diazomethane and the catalyst stannous chloride to mono-methylation the 2′,3′-diol moiety. Yields were relatively high except in case of guanosine. However, the mixture of the 2′-*O*-methyl and 3′-*O*-methyl isomers synthesized through this approach necessitates laborious ion-exchange chromatography to separate the isomers. In particular, the recent advances in protecting group methodology using the 3′, 5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)[15] and methylene-bis (diisopropylsilylchloride) MDPSCl$_2$[16] protecting group have now eliminated the isomer problem. In a subsequent publication by Robins et al.[17] a 51% yield of 2′-*O*-methyl-2-aminoadenosine starting from 2,6-diaminopurine riboside and a 47% yield of the 3′-*O*-methyl-2-aminoadenosine were obtained. Conversion to the 2′ and 3′-*O*-methylguanosine was accomplished with adenosine deaminase. In light of the growing interest in oligonucleotide-based therapeutics, and a key synthon in synthesis of anti-reverse cap analogs, it is increasingly important to develop a cost effective synthesis of 2′-*O*-methyl and 3′-*O*-methylguanosine. This guanosine derivatives are essential reagents for the production of modified RNA that is indispensable for many biological assays. Indeed, the alkylations of the 2′ and 3′-OH groups offer several advantages including an improved binding affinity to the target RNA, enhanced nuclease resistance and chemical stability against depurination, improved pharmacokinetics, and decreased toxicity. In this communication, we report an efficient synthesis of 2′, and 3′-*O*-methylguanosine from 2-aminoadenosine, which eliminates the problematic purification and the need for protection of the 3′, 5′-OH groups of the nucleosides.

RESULTS AND DISCUSSION

The biological value of 2′ and 3′-*O*-alkylated nucleosides has sparked a considerable effort directed toward the development of an efficient synthetic approach. Due to its insolubility in most organic solvents, the direct methylation of unprotected guanosine is not convenient. Therefore, we looked for an alternative strategy for the selective alkylations of the 2′ and 3′-OH group in unprotected 2-aminoadenosine by using diazomethane as an alkylating agent and stannous chloride as a catalyst and subsequent enzymatic deamination of the products by the enzyme adenosine deaminase to the 2′ and 3′-*O*-methylguanosines. The same strategy has been reported by Robins et al.[17] Following their synthetic strategy, we discovered that parameters such as temperature, the concentration of catalyst and addition of the alkylating agent to the reaction mixture significantly impacts the reaction selectivity and yield. This observation encouraged us to re-investigate the reaction with 2-aminoadenosine. Under our experimental conditions, an efficient and selective alkylation at 2′-OH of 2-aminoadenosine was achieved by mixing a stoichiometric amount of stannous chloride at room temperature in DMF. After stirring the reaction mixture at 50°C for 1 min, diazomethane was added all at once. In contrast, in the reported method,[17] the catalyst in hot DMF and diazomethane was added drop wise over 3–4 h. The methylation at 3′-OH of 2-aminoadenosine was achieved by mixing a stoichiometric amount of stannous chloride in DMF and the reaction mixture was stirred at 50°C for 15 min, followed by at once addition of diazomethane. The resulting mixture of 3′-*O*-methyl nucleoside (90%) and 2′-*O*-methyl nucleoside (10%) was treated with the enzyme adenosine deaminase, which under experimental conditions result in preferential deamination of the 3′ moiety within 36 h (Scheme 1). Deamination of the 2′ group occurs only after extremely prolonged incubations with a vast excess of adenosine deaminase. Although it is difficult to speculate its exact mechanism in the reaction, we strongly believe that the high yield and specificity are attributed to the acidity and



SCHEME 1 Selective synthesis of 2′, and 3′-*O*-methylguanosine. (i) Addition of $SnCl_2 \cdot 2H_2O$ to compound (**1**) in DMF at room temperature, followed by immediate addition of diazomethane at 50°C and stirring overnight. (ii) Addition of $SnCl_2 \cdot 2H_2O$ to compound (**1**) at 50°C in DMF stirring for at least 15 min, followed by addition of diazomethane and stirring overnight.

pKa value of 2′ and 3′ hydroxyl group of nucleoside in the presence of catalyst and diazomethane. It is also known that nucleosides that contain an acidic proton in the base can be monomethylated on the sugar by raising the concentration of catalyst and addition of dilute solution of diazomethane.[14]

Preparation of Diazomethane Stock Solution

0.2 M Diazomethane was prepared by a reported procedure[14] with a slight change in protocol. To an ice-cold mixture of 100 mL of 40% aqueous KOH and 150 mL of 1,2-dimethoxyethane (glyme) was slowly added (7.5 g; 72.75 mmol) of N-nitroso-N-methylurea with vigorous stirring. Stirring was continued for an additional 40 min at 0°C and the phases were allowed to separate. The upper organic layer was decanted and dried over KOH pellets for at least 15 min. KOH pellets were changed while drying. The dried solution was filtered before use. Standardization of the diazomethane solution using 0.2 *N* benzoic acid in glyme and back titration with 0.1 *N* NaOH gave an average value of 0.2 *N* CH_2N_2 in glyme.

Special Precautions for Diazomethane

Diazomethane is an explosive, insidious poison and a strong irritant, which may cause delayed hypersensitivity and cancer.[18] Concentrated solutions may explode violently, especially if impurities are present. Rough surfaces, such as ground glass, may also cause explosions.[19] Therefore, it should be prepared by experienced chemists with proper glassware. A Diazald kit with clear-seal joints from Sigma-Aldrich is quite useful, and it is specially designed for the safe preparation of diazomethane.[20]

Cautions: Because of the highly toxic and explosive nature of diazomethane, all reactions involving its preparation and use should be carried out in an efficient chemical fume hood and behind a safety shield. Avoid use of PVC tubing or other plastic tubings with Diazald kits.[20]

Synthesis of 2,6-Diamino-9-(2-*O*-methyl-β-D-ribofuranosyl) purine (2). In a typical reaction, 2-aminoadenosine (**1**) (2.5 g; 8.84 mmol) and $SnCl_2 \cdot 2H_2O$ (0.115 g; 0.51 mmol) in 170 mL of N,N-dimethylformamide were stirred at room temperature for 10 min. The mixture was then heated to 50°C and 130 mL of 0.2 M diazomethane solution was then added all at once. The resulting yellow solution became cloudy and stirring at 50°C was continued overnight. Complete disappearance of (**1**) with formation of a single product was observed by tlc and analytical HPLC. The bright yellow color of the reaction mixture became faintly yellow after overnight stirring. This was a good indication of the selective 2′-*O*-methylation; as for the 3′-*O*-methylation (described below), the bright yellow color became cloudy and colorless solution. The mixture was evaporated and the residue dissolved in water and evaporated four times with 150 mL water to get 2,6-Diamino-9-(2-*O*-methyl-*β*-D-ribofuranosy) purine (**2**) as pure white powder. The resulting solid was recrystallized from water and then from MeOH to give (2.4 g; 8.1 mmol) in (96%) yield, mp 121–122°C, UV max at 280, 256, and 216 nm. The product was identical to an authentic sample by HPLC, UV, and ^{1}H NMR.[14] This product (**2**) was used for enzymatic deamination.

2′-*O*-Methylguanosine 3. A solution of adenosine deaminase (calf spleen, Worthington Biochemical Corporation, Lakewood, NJ, USA.) (0.0064 g; 111 U) in 0.1 M Tris, pH 7.5 (15 mL), 0.1 M sodium phosphate, pH 7.4 (0.75 mL), and DMSO (3.63 mL) was added to a solution of compound (**2**) (2.4 g; 8.1 mmol) in 0.1 M Tris, pH 7.5 (22 mL), 0.1 M sodium phosphate, pH 7.4 (1.5 mL), and DMSO (5.54 mL). The cloudy white mixture was stirred at room temperature for 36 h. During the deamination time, compound (**3**) was precipitated, which was centrifuged for 30 min at 5000 rpm. The product was analysed by HPLC. (Figures 1a and b) It was recrystallized from water and then from MeOH to give (2.3 g; 7.73 mmol) (92%) of compound (**3**), mp 233–235°C. (Lit. mp, 234–236°C).[14] The compound (**3**) was identical to an authentic sample by HPLC, UV, and ^{1}H NMR.[14]

Synthesis of 2,6-Diamino-9-(3-*O*-methyl-*β*-D-ribofuranosyl) Purine 4. In a typical reaction, compound (**1**) (2.5 g; 8.84 mmol) and $SnCl_2 \cdot 2H_2O$ (0.115 g; 0.51 mmol) in 170 mL of N,N-dimethylformamide were stirred at 50°C for 15 min and 130 mL of 0.2 M diazomethane solution was added all at once. The reaction mixture was stirred overnight at 50°C. Complete disappearance of compound (**1**) with formation of two products was observed by tlc and analytical HPLC. The starting bright yellow color of the reaction mixture became a cloudy and colorless solution. HPLC analysis of the crude reaction mixture showed that 90% of the 3′ derivative and 10% of the 2′ derivative had been formed. The mixture was evaporated and the residue dissolved in water and evaporated four times with 150 mL water to get the mixture of 3′ (**4**) and 2′ isomer (**5**) as a white powder. The resulting solid was recrystallized from water and then from MeOH to give (2.4 g; 8.1 mmol) in 96% yield. The mixture of compounds (**4**) and (**5**) were directly used for the enzymatic deamination by adenosine deamination.

3′-*O*-Methylguanosine 6. A crude reaction mixture of compounds (**4**) and (**5**) of (2.4 g; 8.1 mmol) was treated identically to the above-described condition to obtain (2.25 g; 7.56 mmol) 94% of recrystallized (**6**), mp 263°C. (Lit. mp, 258°C).[14] Under experimental conditions, the 3′ is preferentially deaminated within 36 h and that only after longer incubations with more

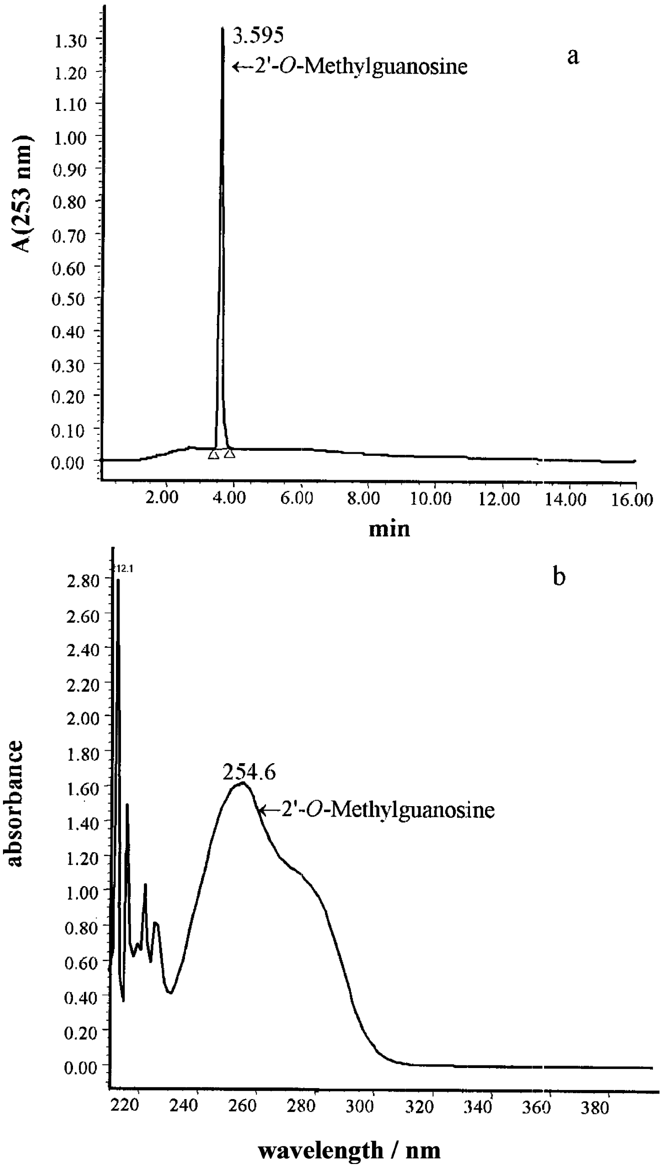


FIGURE 1 (a) Anion-exchange HPLC analysis of crude reaction mixture of (**3**). HPLC Column used: Hypersil SAX, 5 μm, 250 × 4.6 mm. Using a gradient system from 0 to 100% of buffer B over a period of 16 min. Buffer A was 5 mM $(NH_4)H_2PO_4$, pH 2.8, and buffer B was 750 mM $(NH_4)H_2PO_4$, pH 3.7. (b) After enzymatic deamination UV spectra of (**3**) in water.

enzyme also deaminated the 2′ isomer. (Figures 2a and b) Compounds (**2**) and (**4**) have a strong UV max at 280 and 256 nm, while after enzymatic deamination compounds (**3**) and (**6**) have a typical guanosine UV absorption at 253 nm and a sholder at 270 nm (Figure 2b). The product (**6**) was identical to an authentic sample by HPLC, UV, and ^{1}H NMR.[14]

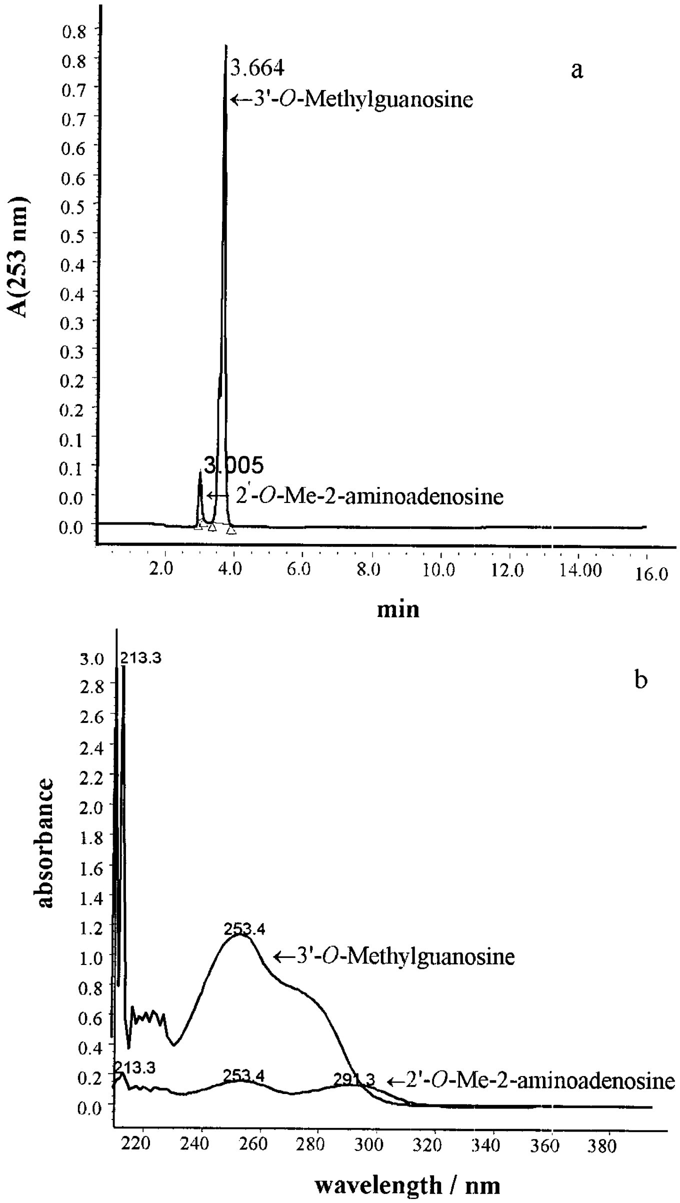


FIGURE 2 (a) Anion-exchange HPLC analysis of crude reaction mixture of (**4**) and (**5**). HPLC conditions were same as in Figure 1a. (b) After enzymatic deamination UV spectra of (**6**) and undigested (**5**) in water.

CONCLUSION

In conclusion, we have developed a versatile and efficient synthetic route enabling the selective preparation of 2′-*O*-methylguanosine and 3′-*O*-methylguanosine, which have a considerable value in biological assays. This synthetic route obviates the chromatographic purification necessary for previously published methods and requires no protection of the starting nucleoside unprotected ribonucleoside, resulting in improved product yields and a simpler and more cost-effective procedure.

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