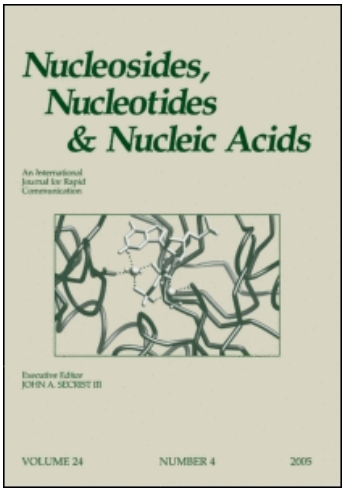

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THE SYNTHESIS OF RNA CONTAINING THE MODIFIED NUCLEOTIDES N^2-METHYLGUANOSINE AND N^6,N^6-DIMETHYLADENOSINE

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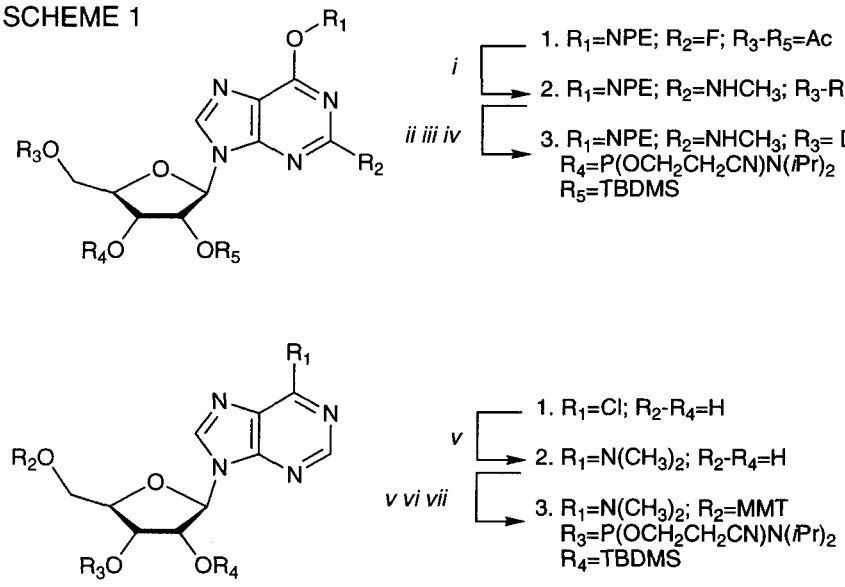
ABSTRACT: The phosphoramidites of the naturally occurring modified nucleotides N^2-methylguanosine and N^6,N^6-dimethyladenosine were synthesized and incorporated into short oligoribonucleotides. Described are the syntheses of the phosphoramidites and the procedures used to deprotect oligoribonucleotides in which the O^6 of m^2G is protected with a 2-(*p*-nitrophenyl)ethyl group.

Many RNAs contain nucleotides that are post-transcriptionally modified (for a general review of modified nucleotides see ref. [1]). These modifications increase the chemical diversity of the nucleotides found in RNAs. tRNAs are densest in modified bases, but rRNA, snRNA, and mRNA are also significantly modified. Cellular RNA are often too large to study by modern biophysical techniques, so it is necessary to synthesize shorter structural analogs. Oligo- and polyribonucleotides are synthesized *in vitro* either by transcription withT7 polymerase[2] or by solid phase phosphoramidite chemistry on automated nucleic acid synthesizers[3]. Chemical synthesis is the method of choice when modified nucleotides must be incorporated into RNA site specifically. Most modified nucleotides in the form of a protected phosphoramidite are not commercially available. Recent thermodynamic and NMR studies on the hypermethylated tetraloop of helix 45 from bacterial 16S ribosomal RNA have focused on the contributions that N^2-methylguanosine (m^2G) and N^6,N^6-dimethyladenosine (m_2^6A) make to RNA structure[4,5]. This required us to synthesize oligoribonucleotides containing both of these naturally occurring methylated nucleotides.

N^2-methylguanosine and N^6,N^6-dimethyladenosine were prepared as phosphoramidites (**SCHEME 1**) and incorporated by solid phase synthesis. N^6,N^6-dimethyladenosine was

SCHEME 1



i. 40%(aq.) CH_3NH_2, RT for 10 min.
ii. DMTCl, RT for 18 hrs. in pyridine.
iii. $AgNO_3$, TBDMSCl, RT for 3 hrs. in THF.
iv. (Cl)P(OCH_2CH_2CN)N(*i*Pr)$_2$, (*i*Pr)$_2$NEt, 0°C for 5 min., RT for 90 min. in CH_2Cl_2.
v. $HN(CH_3)_2$•HCl, $N(Et)_3$, RT for 1 hr. in DMF.
vi. MMTCl, $N(Et)_3$, RT for 1 hr.
vii. $AgNO_3$, TBDMSCl, RT for 3 hrs. in THF.
viii. (Cl)P(OCH_2CH_2CN)N(*i*Pr)$_2$, (*i*Pr)$_2$NEt, RT for 90 min. in CH_2Cl_2.

prepared by treating 6-chloropurine riboside with dimethylammonium chloride using trimethylamine as catalyst[6]. The synthon of N^2-methylguanosine was prepared by converting O^6-(4-NPE)-2',3',5'-tris-*O*-acetyl-guanosine to the 2-fluoro derivative using 30% HF/ 70% pyridine[7,8]. This was converted to O^6- NPE-N^2-methylguanosine by treatment with 40% aqueous methylamine. The 5'-hydroxyls of both nucleosides were protected with a trityl group, and the 2'-hydroxyls were protected with *tert*-butyldimethylsilyl chloride (TBDMSCl). The final synthons were prepared by phosphitylation of the 3'-hydroxyl with β-cyanoethyl-*N*,*N*-diisopropyl chlorophosphoramidite and incorporated into several oligoribonucleotides ranging in length from 6-14 nucleotides using standard phosphoramidite coupling procedures[9].

RNAs were cleaved from the solid support and the base protecting groups removed in a 3:1 mixture of ammonia (conc.) and ethanol (55°C overnight). Further deprotection was

carried out in two steps. First, the 2'-TBDMS protecting groups were removed by treatment with neat triethylamine-trihydrofluoride (overnight at room temp.). Second, NPE groups were removed from RNAs containing m^2G by treatment with 1.0 M tributylammonium fluoride (TBAF) in THF (24 hr at room temp.). In principle a single TBAF treatment could remove the 2'-TBDMS and the NPE groups, but TEA·3HF is superior to TBAF in removing the 2'-TBDMS groups[10]. However, NPE is stable to TEA·3HF, requiring a separate deprotection step with TBAF. The yields and quality of synthesis of oligoribonucleotides that included the modified nucleotides were indistinguishable, by polyacrylamide gel electrophoresis, from oligoribonucleotides composed of the four standard bases. The oligoribonucleotides were purified and desalted as previously described[11]. Base composition analysis was performed on an RNA containing both substitutions to confirm that m^2G and m_2^6A were properly incorporated into the RNA.

Allerson et al. recently reported a different strategy for incorporating alkylated bases into RNA[12]. They prepared the phosphoramidite of the 2-fluoroinosine and post-synthetically modified it to m^2G using methylamine deprotection of the oligoribonucleotide. Their strategy is a versatile method that allows for the incorporation of a wide variety of alkyl amines, but does not permit more than one type of alkyl amine to be incorporated per oligoribonucleotide, which is a requirement for the study of the helix 45 tetraloop[5]. In this report we show that the phosphoramidites of m^2G and m_2^6A can be used to incorporate these methylated bases into oligoribonucleotides, increasing the diversity of RNA that can be studied. These reagents will make it possible to determine how such modifications affect RNA structure and stability.

EXPERIMENTAL METHODS

Preparation of 5'-O-Monomethoxytrityl-N^6,N^6-dimethyladenosine (1). In 7.5 ml DMF 6-chloropurine (1.0 g, 3.5 mmol) was dissolved. To this solution dimethylamine•HCl (0.57 g, 7 mmol) and triethylamine (2.5 ml, 17.5 mmol) were added. Immediately, the chloride salt of triethylammonium began to precipitate. After 1 hr at room temperature, silica gel TLC (15% MeOH in $CHCl_3$ (R_f 0.33)) showed the reaction to be complete. The reaction mixture was filtered to remove the precipitate, and the precipitate was washed with acetone. The filtrate was evaporated to an oil, and N^6,N^6-dimethyladenosine recrystalized by addition of 10 ml EtOH. The nucleoside was filtered to remove the mother liquor and the remaining solid was dried overnight under high vacuum. N^6,N^6-dimethyladenosine was

dried further by repeated evaporations with anhydrous pyridine. N^6,N^6-dimethyladenosine and *p*-anisylchlorodiphenylmethane (1.4 g, 4.55 mmol) were dissolved in 5 ml pyridine, and triethylamine (0.74 ml, 5.25 mmol) was added. The reaction appeared to be ca. 70 % complete after 2 hrs at room temperature (silica gel TLC). Additional amounts of *p*-anisylchlorodiphenylmethane (0.40 g, 1.3 mmol) and triethylamine (0.20 ml, 1.42 mmol) were added and the reaction was stirred an additional 1.5 hrs, at which point the reaction was essentially complete. The reaction was quenched with the addition of 1 ml MeOH, combined with 25 ml $CHCl_3$ and extracted twice with 25 mls of 1 M $NaHCO_3$. The organic layer was dried over Na_2SO_4 and reduced to an oil by rotary evaporation. Compound **1** (2.01 g, 98% yield) was obtained as a faint yellow solid by purification on a silica gel column with a step gradient of MeOH (0% to 10%) in $CHCl_3$. TLC (2% MeOH in $CHCl_3$), R_f 0.57. ^{1}H NMR ($CDCl_3$) δ, ^{1}H NMR ($CDCl_3$) δ 8.19 (s, 1H, H8), 7.95 (s, 1H, H2), 7.41 (d, 4H, ArH), 7.39 (d, 2H, ArH), 7.25-7.15 (m, 6H, ArH), 6.75 (t, 2H, ArH),5.99 (d, 1H, H1'), 5.12 (m, 1H, H2'), 4.51 (m, 1H, H3'), 4.44 (m, 1H, H4'), 3.75 (s, 3H, -OCH_3), 3.53 (br, 6H, -$N(CH_3)_2$), 3.44 (dd, 1H, H5'), 3.28 (dd, 1H, H5'').

Preparation of 5'-O-Monomethoxytrityl-2'-O-t-butyldimethylsilyl- N^6,N^6*-adenosine (2).* Nucleoside **2** (1.9 g, 3.4 mmol) was dissolved in 34 ml THF, and pyridine (1.12 ml, 13.6 mmol), $AgNO_3$ (0.81 g, 4.4 mmol), and TBDMS-Cl (0.72 g, 4.4 mmol) were added to the reaction. A cloudy white precipitate immediately formed. The reaction was stirred at room temperature for 3 hrs. Additional $AgNO_3$ (0.12g, 1.0 mmol) and TBDMS-Cl (0.24 g, 1 mmol) were added. After an additional 1.7 hrs, silica TLC showed that >90% of **1** had reacted. The precipitate was removed by filtration, the reaction combined with 60 ml of 5% $NaHCO_3$, and the solution was twice extracted with 100 ml of CH_2Cl_2. The combined organic layers were dried over $NaSO_4$ and evaporated to an oil. The oil was redissolved in benzene, evaporated, redissolved in CH_2Cl_2 (ca. 30 ml), and the silylated products were precipitated with hexanes (ca. 150 ml). The ratio the 2'-O-silylated nucleoside (R_f 0.75) to the 3'-O-silylated nucleoside (R_f 0.62) was approximately 2:1 by TLC (1:3; ether/CH_2Cl_2). Compound **2** was purified from a silica gel column with a step gradient of ether (12% to 24%) in CH_2Cl_2. Two columns were necessary to obtain pure compound **2** as a white solid (0.960 g, 42% yield): ^{1}H NMR ($CDCl_3$) δ 8.25 (s, 1H, H8), 7.93 (s, 1H, H2), 7.45 (d, 4H, ArH), 7.33 (d, 2H, ArH), 7.29-7.16 (m, 6H, ArH), 6.72 (t, 2H, ArH), 6.02 (d, 1H, H1'), 4.97 (m, 1H, H2'), 4.30 (m, 1H, H3'), 4.22 (m, 1H, H4'), 3.78 (s, 3H, -OCH_3), 3.53 (br, 6H, -$N(CH_3)_2$), 3.53 (dd, 1H, H5'), 3.35 (dd, 1H, H5''), 0.84 (s, 9H, *tert*-butyl), -0.01 (s, 3H, -$SiCH_3$), -0.12 (s, 3H, -$SiCH_3$).

Preparation of 5'-O-Monomethoxytrityl-2'-O-t-butyldimethylsilyl-N^6,N^6-adenosine-3'-O-(2-Cyanoethyl) N,N-*diisopropylphosphoramidite (3).* Compound **2** (0.5 g, 0.70 mmol) and dimethylaminopyridine (ca. 10 mg) were dissolved in 10 ml dry THF and kept under N_2. Triethylamine (0.5 ml, 2.86 mmol) and 2-cyanoethoxy *N,N*-diisopropylaminochlorophosphine (0.19 ml, 0.85 mmol) were added dropwise to the mixture. The reaction was complete after 3 hrs at room temperature. The reaction mixture was diluted with CH_2Cl_2 (100 ml) and washed with brine (50 ml). The organic extract was dried over $MgSO_4$ and concentrated *in vacuo*. Flash chromatography (hexane/acetone/Et_3N (50:44:6)) afforded 0.56 g of a white powder (83 %, 0.63mmol). ^{31}P NMR ($CDCl_3$) δ: 150.0, 147.5.

*Preparation of 2',3',5' tris-*O*-acetyl-2-fluoro-O^6-[2-(4-nitrophenyl)ethyl]-inosine (4).* The nucleoside, 2',3',5' tris-*O*-acetyl-O^6-[2-(4-nitrophenyl)ethyl]-guanosine **(5)**, was prepared as previously described[8]. An admixture of triphenyl phosphine and **5** (ca. 8.90 g, ca. 9.6 mmol) was dissolved in 50 ml of 30% HF/70% pyridine at -30°C. The reaction was stirred for four min. and poured onto 100 g of ice. The aqueous phase was extracted with CH_2Cl_2 (2x100ml). The organic layer was back-extracted with 5% $NaHCO_3$ (2x100 ml) and H_2O (2x100 ml), dried over Na_2SO_4 and evaporated to a yellow foam *in vacuo*. The estimated yield of crude **4** (ca. 3.5g, ca. 6.2 mmol) was 65%. An analytical sample was purified and characterized by TLC and ^1H NMR. TLC (10 MeOH in CH_2Cl_2): R_f 0.74. ^1H NMR ($CDCl_3$) δ 8.17 (d, 2H, ArH *o* to -NO_2), 8.06 (s, 1H, H8), 7.67 (d, 2H, ArH *m* to -NO_2), 6.13 (d, 1H, H1'), 5.84 (m, 1H, H2'), 5.59 (m, 1H, H3'), 4.85 (t, 2H, -$CH_2\underline{CH_2}O$), 4.45-4.37 (m, 3H, H4',H5', H5''), 3.31 (t, 2H, -$\underline{CH_2}CH_2O$), 2.15 (s, 3H, $CH_3C(O)$-), 2.14 (s, 3H, $CH_3C(O)$-), 2.07 (s, 3H, $CH_3C(O)$-).

Preparation of O^6-[2-(4-nitrophenyl)ethyl]-N^2-methylguanosine (6). Nucleoside **4** (ca. 2.4g, 4.3 mmol) was dissolved in 100 ml of 48% aqueous methylamine, and the reaction was stirred at room temperature for 1 hr. The product was extracted with 100 ml of CH_2Cl_2. The organic layer was back-extracted with 5% $NaHCO_3$ (2x100ml), H_2O (2x100ml), dried over Na_2SO_4, and concentrated *in vacuo*. The crude product was purified by flash chromatography (1-8% MeOH/CH_2Cl_2). Compound **6** was obtained as a yellow solid (1.23g, 60% yield). TLC (10% MeOH in CH_2Cl_2): $R_f < 0.1$. ^1H NMR (DMSO-d_6) δ 8.17 (d, 2H, ArH *o* to -NO_2), 8.05 (s, 1H, H8), 7.62 (d, 2H, ArH *m* to -NO_2), 5.79 (d, 1H, H1'), 5.33 (br, 1H, -OH(C2')), 5.11 (br, 1H, -OH(C3')), -4.93 (br, 1H, -OH(C5')), 4.70 (t, 2H, -$CH_2C\underline{H_2}O$), 4.57 (m, 1H, H2'), 4.14 (m, 1H, H3'), 3.89 (m, 1H, H4'), 3.64 (d, 1H, H5'), 3.53 (d, 1H, H5''), 3.26 (t, 2H, -$\underline{CH_2}CH_2O$), 2.81 (s, 3H, -$NHCH_3$).

Preparation of 5'-O-(4,4'-Dimethoxytrityl)-O^6-[2-(4-nitrophenyl)ethyl]-N^2-methylguanosine (7). Nucleoside **6** (1.23g, 2.76 mmol) was dried by co-evaporation with pyridine and suspended in 30 ml of dry pyridine. To this mixture 4,4'-dimethoxytrityl chloride (DMT-Cl) (1.03 g, 3.04 mmol) was added in three equal portions over one hour. The reaction was stirred under argon for 18 hours at room temperature, quenched with MeOH (15 ml), and concentrated to an oil *in vacuo*. This oil was redissolved in CH_2Cl_2, and the organic phase was washed with saturated $NaHCO_3$ (2x50 ml), and H_2O (2x50 ml). The organic layer was dried over Na_2SO_4, and reconcentrated to an oil. Purification by flash chromatography (1-8% MeOH/CH_2Cl_2) afforded **8** (0.88g, 42% yield) as a yellow foam. TLC (5% MeOH in CH_2Cl_2): R_f 0.42. ^{1}H NMR ($CDCl_3$) δ 8.15 (d, 2H, ArH *o* to -NO_2), 7,85 (s, 1H, H8), 7.48 (d, 2H, ArH *m* to -NO_2), 7.28-7.19 (m, 9H, ArH), 6.75 (t, 4H, ArH), 5.83 (d, 1H, H1'), 4.99 (br), 4.75 (t, 2H, -$CH_2C\underline{H}_2O$), 4.72 (m, 1H, H2'), 4.43 (m, 1H, H3'), 4.39 (m, 1H, H4'), 3.76 (s, 6H, -OCH_3), 3.41 (d, 1H, H5'), 3.28 (t, 2H, -$C\underline{H}_2CH_2O$), 3.23 (d, 1H, H5''), 2.97 (s, 3H, -$NHCH_3$).

*Preparation of 5'-O-(4,4'-Dimethoxytrityl)-2'-O-(*tert*-butyldimethylsilyl)-O^6-[2-(4-nitrophenyl)ethyl]-N^2-methylguanosine (8).* Nucleoside **7** (0.88g, 1.17 mmol) was dissolved in 40 ml THF. Dry pyridine (1 ml, 12.9 mmol) and $AgNO_3$ (238 mg, 1.4 mmol) were added, and the mixture was sonicated extensively to dissolve the $AgNO_3$. TBDMS-Cl (228 mg, 1.5 mmol) was added and the solution became instantly turbid. After 3 hrs at room temperature the reaction was still incomplete (30 % unreacted **7**), so additional portions of pyridine (0.25 ml, 3.2 mmol), TBDMS-Cl (53 mg, 0.35 mmol), and $AgNO_3$ (60 mg, 0.35 mmol) were added. After three additional hrs. the reaction was more than 90% complete with two major products. The 2'-regioisomer (R_f 0.88) had the faster mobility by TLC (5% MeOH in CH_2Cl_2) and was produced in a 2:1 ratio over the 3'-regioisomer (R_f 0.80). The precipitate was removed by passing the mixture over celite into saturated $NaHCO_3$ (50 ml) The mixture was extracted with CH_2Cl_2, and the organic phase was extracted with saturated $NaHCO_3$ (2x30ml), H_2O (2x30ml), dried over Na_2SO_4, and concentrated *in vacuo*. Purification by flash chromatography (1% triethylamine/1-3% MeOH/CH_2Cl_2) afforded **8** (0.412g, 36% yield) as a yellow foam. ^{1}H NMR ($CDCl_3$) δ 8.16 (d, 2H, ArH *o* to -NO_2), 7.73 (s, 1H, H8), 7.48 (d, 2H, ArH *m* to -NO_2), 7.43-20 (m, 9H, ArH), 6.80 (t, 4H, ArH), 5.88 (d, 1H, H1'), 5.00 (br, 1H, -OH(C3')), 4.65 (t, 2H, -$CH_2C\underline{H}_2O$), 4.37 (m, 1H, H2'), 4.20 (m, 1H, H3'), 3.79 (s, 6H, -OCH_3), 3.64 (m, 1H, H4'), 3.49 (d, 1H, H5'), 3.37 (d, 1H, H5''), 3.28 (t, 2H, -$C\underline{H}_2CH_2O$), 2.84 (s, 3H, -$NHCH_3$), 0.86 (s, 9H, *tert*-butyl), -0.10 (s, 3H, -$SiCH_3$), -0.12 (s, 3H, -$SiCH_3$).

Preparation of 5'-O-(4,4'-dimethoxytrityl)-2'-O-(tert-butyldimethylsilyl)-O^6-[2-(4-nitrophenyl)ethyl]-N^2-methylguanosine-3'-O-(2-Cyanoethyl N,N-*diisopropylphosphoramidite (9).* Nucleoside **8** (0.412 g, 0.424 mmol) was dissolved in dry CH_2Cl_2 (10 ml) and cooled to 0°C. A solution containing 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (0.145 ml, 0.636 mmol), diisopropylamine (0.145 ml, 0.848 mmol), and *N*-methylimidazole (17.4 µl, 0.212 mmol) in CH_2Cl_2 (10 ml) was cooled to 0°C and added dropwise to the solution containing **8**. The solution was allowed to warm to room temperature. After 90 min. NMR of the reaction mixture in the H1' proton region showed nearly complete conversion to the phosphoramidite **9**. The reaction was concentrated *in vacuo* and purified by flash chromatography (1% triethylamine/1-2% MeOH/CH_2Cl_2) yielding a mixture of diastereomers (0.365mg, 71% yield). ^{31}P NMR (CH_3CN) δ 151.2 and 149.9.

Oligoribonucleotide synthesis and purification. The N^6,N^6-dimethyladenosine and the N^2-methylguanosine phosphoramidites were incorporated into RNA molecules using standard solid phase synthetic techniques[3]. RNAs were cleaved from the solid support and the base protecting groups were removed in a 3:1 mixture of ammonia (conc.) and ethanol (55°C overnight). To each 1 µmol of synthetic RNA, 1.5 ml of neat triethylamine trihydrofluoride was added and aggitated for 12-24 hrs. This mixture was diluted with 300 µl H_2O and precipitated with 10 ml *n*-butanol (-20°C for four hrs). After centrifugation the supernatant was decanted and the RNA pellet dried under vacuum. For oligoribonucleotides containing NPE-O^6 protected N^2-methylguanosine the NPE group was removed by dissolving the residue in 1.8 ml of 1.0 M TBAF in THF and agitating for 24 hrs. The RNA was precipitated by adding 600 µl of 1 M NaCl, 1.8 ml H_2O, and 7.2 ml EtOH, and storing at -20°C for four hours. After centrifugation the supernatant was decanted and the RNA pellet dried under vacuum. Fully deprotected RNAs were purified by denaturing (5 M urea) polyacrylamide (20%) gel electrophoresis. RNA was eluted from gel slices by repeated incubation (3 x 25 ml) in 1 M NH_4OAc at 55°C for three hours. The RNAs were desalted using C_{18} Sep-pak cartridges (Millipore) following the manufacture's instructions.

Base Composition Analysis. Oligoribonucleotide base composition was confirmed by digesting 1 OD of the oligoribonucleotide 5'-GGA CCm^{2}G Gm$_2^6$Am$_2^6$A GGU CC-3', which contains both m^{2}G and m$_2^6$A, with nuclease P1 (ca. 2 units) overnight at 50°C and dephosphorylating with calf intestine alkaline phosphatase (2 units) at 37° for three hours. The nucleoside products were resolved by reverse-phase HPLC using a Vydac (C_{18}) 4.6 x

250 mm column with 50 mM ammonium phosphate (pH 5.2) and methanol as buffers. In addition to the peaks for the four standard bases (A, C, G, and U), two additional peaks were observed. These were confirmed to be m^2G and m_2^6A by coinjection with authentic samples. Retention times: C(9.67 min.), U(10.03 min.), G(10.52 min.), m^2G(11.52 min.), A(11.52 min.), and m_2^6A(12.70 min.).

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