Enzymatic Synthesis of M_1G-Deoxyribose

UNIT 1.2

M_1G-deoxyribose (M_1G-dR, 1,N^2-pyrimido[1,2-α]purin-10(3*H*)-one, or pyrimidopurinone; see structure in Fig. 1.2.2, below) is an endogenous exocyclic DNA adduct formed by the reaction of the dicarbonyl compound malondialdehyde (MDA) with a deoxyguanosine residue in DNA. M_1G-dR is an intermediate in the synthesis of a class of modified oligodeoxyribonucleotides that are used to study the mutagenicity and repair of M_1G.

This unit presents two methods for synthesizing M_1G-dR using enzymatic coupling. The Basic Protocol describes a procedure for coupling the nucleobase to deoxyribose, in a reaction mediated by the enzyme nucleoside 2′-deoxyribosyltransferase, followed by preparation of the modified base (see Fig. 1.2.1A). Preparation of the enzyme is described in the Support Protocol. The Alternate Protocol uses two commercially available enzymes, purine nucleoside phosphorylase and thymidine phosphorylase (see Fig. 1.2.1B). Although the enzyme preparation step is avoided, additional purification steps are required that increase the time needed to complete the synthesis and decrease the yield (see Commentary).

NOTE: Use deionized, distilled water in all recipes and protocol steps.

ENZYMATIC COUPLING USING NUCLEOSIDE 2′-DEOXYRIBOSYLTRANSFERASE

BASIC PROTOCOL

Nucleoside 2′-deoxyribosyltransferase (trans-*N*-deoxyribosylase or nucleoside:purine(pyrimidine) deoxyribosyltransferase; E.C. 2.4.2.6) catalyzes the transfer of the deoxyribosyl moiety from a deoxyribonucleoside to any other nucleoside base (see Fig. 1.2.1A). This enzyme is found exclusively in *Lactobacilli* and related microorganisms that require deoxynucleosides for growth (Carson and Wasson, 1988). The enzyme's broad specificity makes it a useful tool for synthesizing modified deoxyribonucleotides.

This protocol describes the use of this enzyme (see Support Protocol for preparation) to transfer a deoxyriboside from 2′-deoxycytidine to M_1G and produce M_1G-dR. The two synthesis steps can be carried out in a single flask, which decreases the time needed to purify M_1G-dR and significantly increases the yield of the reaction.

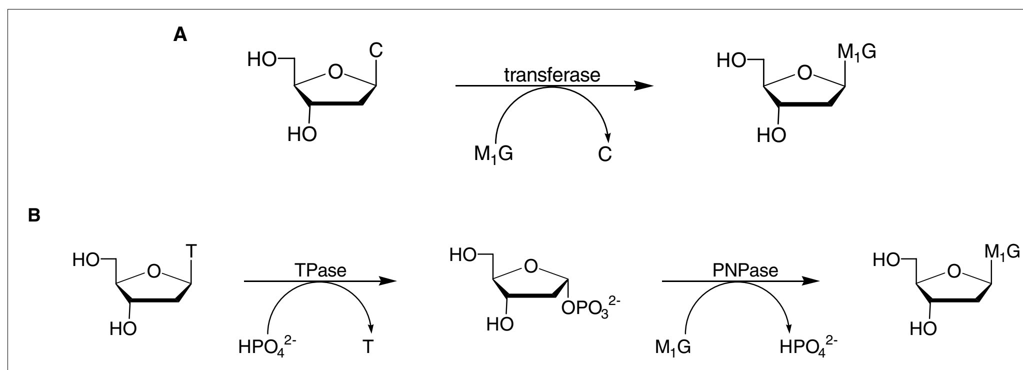

Figure 1.2.1 Enzymatic coupling reactions. (**A**) Reaction catalyzed by nucleoside 2′-deoxyribosyltransferase (Basic Protocol). (**B**) Reaction catalysed by thymidine phosphorylase and purine nucleoside phosphorylase (Alternate Protocol).

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Materials

Guanine hydrochloride (Sigma)
1 N HCl
Tetraethoxypropane (Aldrich)
Methanol (MeOH; Aldrich)
Potassium carbonate (Aldrich)
Nanopure water (water purified using Nanopure system from Barnstead/Thermolyne)
MES (2-[*N*-morpholino]ethanesulfonic acid; Sigma)
2′-Deoxycytidine (dC; Sigma)
1 N NaOH
Nucleoside 2′-deoxyribosyltransferase (transferase; see Support Protocol)
Dichloromethane (CH_2Cl_2; Fisher)

250-mL round-bottom flask
Oil bath, 70°C
Magnetic stir plate and stir bar
Ice bath
pH indicator strips
Büchner funnel
Whatman No. 1 filter paper
Shaking incubator, 37°C
Silica-gel thin-layer chromatography (TLC) plates
Lyophilizer
Silica gel (60 to 100 mesh; Fisher)
8 × 50–cm chromatography column

Prepare modified base

1. In a 250-mL round-bottom flask, dissolve 1 g (5.3 mmol) guanine hydrochloride in 100 mL of 1 N HCl that has been heated to 70°C using an oil bath. Stir on a magnetic stir plate until dissolved.

   The dissolution can take from 30 min to 1 hr.

2. Mix 1.3 mL (5.86 mmol) tetraethoxypropane with 1.25 mL methanol. Add this dropwise to the solution from step 1.

   Slow addition of tetraethoxypropane favors the formation of the modified base and avoids the polymerization of MDA.

3. Let the reaction mixture stir for 30 min on a magnetic stir plate, then cool to 0°C in an ice bath.

   Cooling moderates the neutralization reaction to follow.

4. Neutralize by slowly adding potassium carbonate to pH 6, verifying the pH using pH indicator strips. Take special care when approaching the desired pH.

5. Remove unreacted guanine by filtering through Whatman no. 1 filter paper on a Büchner funnel under vacuum. Wash the precipitate twice with 20 mL of Nanopure water.

   The filtrate contains the modified base. The yield of the reaction is estimated at 30%.

6. Add MES to a final concentration of 0.5 M.

   MES is used to buffer the enzymatic reaction.

7. Add 1.2 g (5.3 mmol) 2′-deoxycytidine.

8. Equilibrate the solution to pH 6.0 with 1 N HCl or 1 N NaOH.

Perform enzymatic coupling

9. Add an appropriate amount of nucleoside 2′-deoxyribosyltransferase solution and incubate overnight at 37°C with shaking.

   Each enzymatic preparation has its own concentration and activity, which must be tested empirically by assaying the preparation's ability to convert a small quantity of M_1G to M_1GdR to estimate the amount to use for the full-scale reaction.

10. Check the progress of the reaction by TLC on silica-gel plates using 9:1 (v/v) CH_2Cl_2/MeOH as the mobile phase.

    Add 50 μL methanol to 50 μL of reaction mix and spot on TLC plate using a capillary tube. If the reaction is not complete, add more enzyme and dC, but not more than one-fifth the amount used at the start of the reaction, and incubate an additional 6 to 12 hours.

11. Once the reaction is complete, lyophilize the reaction mixture to dryness.

12. Purify the crude product on a silica-gel column using 9:1 (v/v) CH_2Cl_2/MeOH for equilibration and elution. Elute by gravity and collect 50-mL fractions.

    The lyophilized reaction mixture may be added directly to the top of the packed column.

13. Confirm the purity of the product by ^1H NMR. Store the product under nitrogen at –20°C. Under these conditions, it is stable for several years.

    A typical spectrum is presented in Figure 1.2.2. The estimated yield is 10% to 15%.

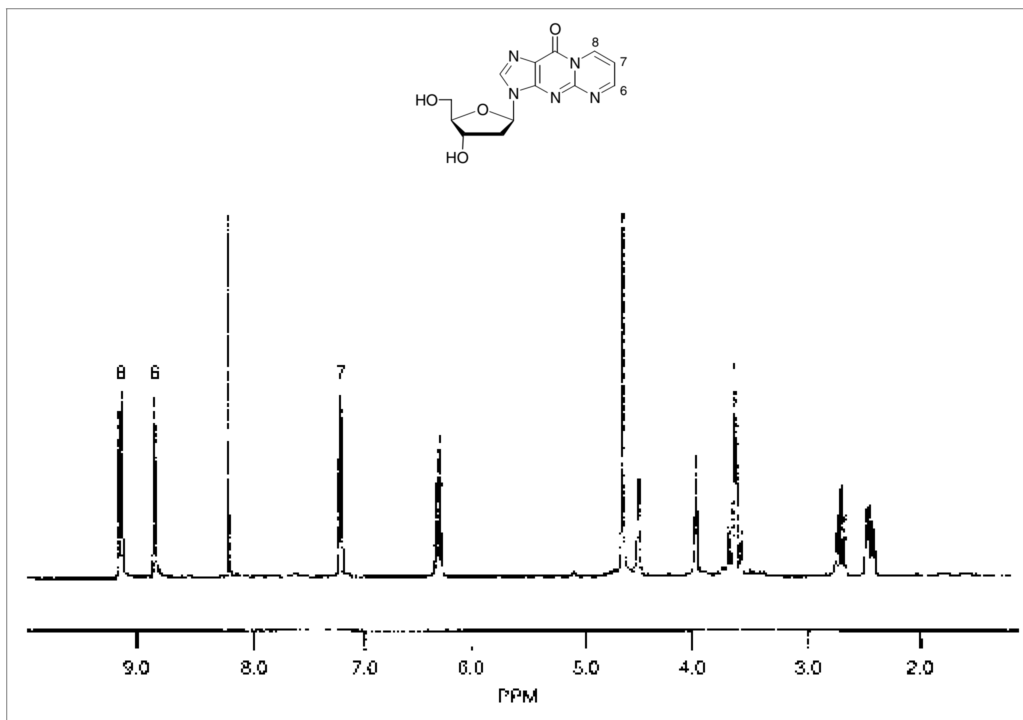

Figure 1.2.2 ^1H NMR spectrum of M_1-GdR in D_2O.

SUPPORT PROTOCOL

PREPARATION OF NUCLEOSIDE 2′-DEOXYRIBOSYLTRANSFERASE

Nucleoside 2′-deoxyribosyltransferase was first isolated by McNutt (1952). Partial purification of the enzyme from *Lactobacillus leichmannii* has been described by Beck and Levin (1963), and its complete purification and crystallization by Uerkvitz (1971). This protocol is based upon the latter method. The partial purification described here is sufficient for obtaining enzyme to be used in the Basic Protocol.

Materials

Lactobacillus broth AOAC (see recipe)
Lactobacillus helveticus culture
0.15 M NaCl (4°C)
50 mM potassium phosphate buffers, pH 6.0 and 6.9 (see *APPENDIX 2A*; dilute with Nanopure water to desired molarity)
50 mM potassium phosphate, pH 5.1, containing 10 g/L NaCl

250-mL Erlenmeyer flask
Centrifuge and rotors (e.g., Sorvall GS-3 and SS-34)
Microtip sonicator (Virsonic 100)
BCA Protein Assay (Pierce; optional) or equivalent

Purify the enzyme

1. Using aseptic technique, place 25 mL of *Lactobacillus* broth in an Erlenmeyer flask. Add a loopful of *Lactobacillus helveticus* commercial stock and incubate overnight at 37°C without shaking.
2. Transfer the 25-mL culture into 1000 mL of fresh *Lactobacillus* broth. Grow 18 hr at 37°C without shaking.
3. Cool to 4°C and divide into centrifuge tubes. Centrifuge the tubes 10 min at 7000 × *g* (6500 rpm in a GS-3 rotor), 4°C.
4. Resuspend pellets in 100 mL cold 0.15 M NaCl and centrifuge again as in step 3. Repeat.
5. Suspend cell pellets in a total of 20 mL of 50 mM potassium phosphate, pH 6.0.
6. Sonicate 10 times for 1 min each time with a Virsonic 100 microtip sonicator at a setting of 4 to 5.
7. Centrifuge 30 min at 28,000 × *g* (15,000 rpm in an SS-34 rotor), 4°C. Save the supernatant.
8. Wash the pellets twice by resuspending in a minimal volume of 0.15 M NaCl and centrifuging as in step 7. Save the supernatants.
9. Combine the supernatants from the previous two steps, and dialyze overnight against 4 L of 50 mM potassium phosphate, pH 5.1, containing 10 g/L NaCl.
10. Heat 10 min at 55°C, then immediately place on ice.

    The heating denatures all heat-sensitive proteins that are present in the extract, thereby enriching for transferase, which is not heat sensitive.

11. Centrifuge again as in step 7.
12. Collect the supernatant and dialyze overnight in 50 mM potassium phosphate, pH 6.9.

To quantify the amount of total protein purified, the BCA Protein Assay (Pierce) can be used.

For the purpose of this experiment, this level of purification is sufficient. Store the protein in aliquots at –20°C or proceed to coupling experiments (see Basic Protocol).

Assay the enzyme

13. Prepare five 5-mL aliquots of filtrate containing M_1G (see Basic Protocol, step 8).

14. Add to the filtrate 50, 100, 150, 200, and 250 µL of supernatant containing the enzyme (from step 12).

15. Incubate overnight at 37°C with shaking.

16. Spot 10 µL of each reaction mixture three times on a TLC plate using 9:1 (v/v) CH_2Cl_2/MeOH as the mobile phase. Determine presence of M_1G-dR.

ENZYMATIC COUPLING USING PURINE NUCLEOSIDE PHOSPHORYLASE AND THYMIDINE PHOSPHORYLASE

ALTERNATE PROTOCOL

Purine nucleoside phosphorylase (PNPase; E.C. 2.4.2.1) catalyzes the displacement of phosphate from deoxyribose-1-phosphate on purines and purine analogs. The stereochemistry of purine attachment produces the naturally occurring β-isomers. Although ribose-1-phosphate is commercially available, it may be more conveniently and cost-effectively generated in situ by thymidine phosphorylase (TPase; E.C. 2.4.2.4)–catalyzed phosphorolysis of thymidine (see Fig. 1.2.1).

This Alternate Protocol can be used to avoid the transferase preparation required for the Basic Protocol. First the modified base is synthesized and purified, then the enzymatic coupling is initiated, and finally the product is separated on a medium-performance liquid chromatography (MPLC) column. Due to the instability of M_1G, only 2% to 5% of product is recovered, regardless of the yields achieved in the transribosylation step.

Additional Materials *(also see Basic Protocol)*

Thymidine (e.g., Sigma)
Purine nucleoside phosphorylase (PNPase; Sigma)
Thymidine phosphorylase (TPase; Sigma)
20 mM potassium phosphate, pH 7.3 (*APPENDIX 2A*)
MPLC buffer: 20% methanol in water

UV lamp (254 and 365 nm)
mPLC column (Baker C_{18}-40 µm, 30 × 500 mm)

Prepare modified base

1. Prepare modified base (see Basic Protocol, steps 1 to 5).

2. Concentrate the filtrate under vacuum.

3. Prepare a slurry of the residue with ~10 g silica gel.

4. Purify by chromatography on a silica-gel column using 9:1 (v/v) CH_2Cl_2/MeOH for equilibration and elution.

5. Collect and combine the fractions containing M_1G as determined by UV fluorescence.

   M_1G base is fluorescent under the long-wavelength (365 nm) of the UV lamp.

6. Evaporate under vacuum and store under nitrogen at –20°C.

7. Verify the purity of M_1G base by ^1H NMR.

   The estimated yield is 2% to 5%.

Perform enzymatic coupling

8. Dissolve 37 mg (0.2 mmol) M_1G base and 73 mg (0.6 mmol) thymidine in 200 mL of 20 mM potassium phosphate buffer. Adjust the pH to 7.3 with 1 N HCl or 1 N NaOH.

9. Add 20 U TPase and 30 U PNPase.

10. Incubate the solution 18 hr at 37° to 39°C with shaking.

    Verify the completion of the enzymatic coupling by thin-layer chromatography (see Basic Protocol, step 10). If the reaction is not complete, continue the purification without further incubation.

11. Concentrate the reaction under vacuum.

    This and all subsequent vacuum steps may be performed using a side-arm flask with water aspiration.

12. Prepare a slurry of the residue with ~5 g silica gel.

13. Purify by chromatography on a silica-gel column using 9:1 (v/v) CH_2Cl_2/MeOH for equilibration and elution. Elute by gravity and collect 50-mL fractions.

14. Collect the fractions containing M_1G-dR as determined by yellow fluorescence at 365 nm.

    M_1G and M_1G-dR have different elution times.

15. Evaporate to dryness under vacuum. Redissolve two aliquots in 2 mL MPLC buffer each.

16. Purify by MPLC on a Baker C_{18} 40 µm, 30 × 500–mm column, eluting with 20% methanol in water (isocratic) at a flow rate of 3 mL/min.

    This second column is necessary to separate thymidine from M_1G-dR.

17. Collect the fluorescent fractions and evaporate to dryness under vacuum.

18. Verify the purity of the product by ^1H NMR.

    A typical spectrum is presented in Figure 1.2.2 (see Basic Protocol). The estimated yield is 50% to 60%.

REAGENTS AND SOLUTIONS

Use Nanopure water (water purified using Nanopure system from Barnstead/Thermolyne) where indicated, and deionized, distilled water in all other recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A;*** *for suppliers, see* ***SUPPLIERS APPENDIX.***

Lactobacillus broth AOAC

Mix 38 g of *Lactobacillus* broth AOAC (Difco) into 1000 mL Nanopure water. Heat to boiling for 2 min. Autoclave 30 min and allow to cool to room temperature. Prepare fresh for each run.

COMMENTARY

Background Information

Adducts formed between electrophiles and nucleic acid bases are believed to play a key role in chemically induced mutations and cancer (Singer and Grunnenberger, 1983). Chemical synthesis of deoxynucleoside adducts provides not only authentic standards for comparison to biologically derived materials but also reagents for the synthesis of adducted nucleotides (Basu and Essigmann, 1988). The preparation of certain classes of deoxynucleoside adducts is problematic because of the instability of the intermediates under the conditions used for synthetic manipulations (e.g., the acid lability of purine deoxyribosides). Synthetic approaches to preparing sensitive deoxynucleosides include coupling of adducted bases to activated deoxyribose derivatives (Srivasta et al., 1988) and attachment of deoxyribose moieties to modified bases (Garner and Ramakanth, 1988). However, there are inherent difficulties in controlling both the regiochemistry (e.g., to achieve attack at the N7 versus N9 atom of a purine) and the stereoselectivity (e.g., an S_N2 attack on a sugar isomer is needed to generate the desired linkage). Chemical synthesis increases the difficulty of obtaining the correct regioselectivity, whereas enzymatic coupling on nonmodified bases generates only one regioselective isomer.

Enzymatic coupling of purine or pyrimidine analogs to deoxyribose has been used to synthesize a number of compounds, including isotopically substituted deoxynucleosides, antitumor agents, and biological active molecules (Holy and Votruba, 1987; Krenitsky et al., 1981, 1986; Muller et al., 1996). When PNPase and TPase are used on M_1G (see Alternate Protocol), N9 linkage is shown to be preferred over N7. With the use of transferase (see Basic Protocol), complete regioselectivity (for the N9 isomer only) is obtained.

Critical Parameters

Since the pyrimidopurinone M_1G-dR synthesized in these procedures is base labile, the pH must stay below 7.5 for all protocols. M_1G base is even less stable than M_1G-dR.

The Basic Protocol does not require purification of the modified base; the transferase can be added to the crude mixture. The desired nucleoside can be purified by simple column chromatography. This strategy not only shortens the time required for the synthesis, but also significantly improves the yield. In comparison the Alternate Protocol, which uses a combination of two commercially available enzymes, is more demanding in terms of time and purification. M_1G base must be purified; moreover, when the crude mixture is reacted with the combination of PNPase and TPase, transribosylation is inefficient. Also, due to the close polarity of thymidine and M_1G-dR (in a variety of solvents system used), a combination reversed-phase and straight-phase column must be employed to obtain pure material.

The transferase used in the Basic Protocol and prepared in the Support Protocol has a higher selectivity and produces only the N9 isomer. The two-enzyme method of the Alternate Protocol produces a mixture of N7 and N9, increasing the difficulty of purification. Once a stock solution of transferase is prepared, the Basic Protocol is much easier to perform, less time-consuming, and gives better yields.

Anticipated Results

The two protocols described in this unit allow preparation of the desired modified nucleoside in good yields. The Basic Protocol should give yields between 10% and 15% and the Alternate Protocol gives between 2% and 5%. Figure 1.2.2 shows the ^{1}H NMR spectrum of M_1G-dR.

Time Considerations

Preparation of transferase (see Support Protocol) may take 3 to 4 days. The one-step condensation procedure using transferase (see Basic Protocol), from the synthesis of the modified base to the purification of the nucleoside, should take 24 hr with lyophylization. The alternative method using commercial phosphorylases (see Alternate Protocol) should take 2 or 3 days.

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Key Reference

Uerkvitz, 1971. See above.

Decribes the purification and crystallization of the transferase.

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