Syntheses of Specifically ^{15}N-Labeled Adenosine and Guanosine

This unit describes the specific incorporation of ^{15}N into the N7 and amino positions of adenosine (Basic Protocol 1), and conversion of the adenosine to guanosine labeled at the N1, N7, and amino positions (Basic Protocol 2). Two variations of the procedures are also presented that include either ^{12}C or ^{13}C at the C8 position of adenosine, and ^{13}C at either the C8 or C2 position of guanosine. These ^{13}C tags permit the incorporation of two ^{15}N-labeled nucleosides into an RNA strand while ensuring that their nuclear magnetic resonance (NMR) signals can be distinguished from each other by the presence or absence of C-N coupling. While the major application of these specifically ^{15}N-labeled nucleosides is NMR, the additional mass makes them useful in mass spectrometry (MS) as well. The procedures can also be adapted to synthesize the labeled deoxynucleosides. The Support Protocol describes the synthesis of 7-methylguanosine.

CAUTION: The procedures in this unit use a number of highly toxic and dangerous reagents. Raney nickel is pyrophoric when dry and may burst into flames if not kept wet. Phosphorous oxychloride ($POCl_3$) is very reactive and hydrolyzes to hydrochloric and phosphoric acids, which are both highly corrosive to skin and tissue. Cyanogen bromide is very dangerous; improper use of this reagent has led to deaths in the laboratory. All reactions must be done with great care in an appropriate chemical fume hood.

SYNTHESES OF [7,NH_2-$^{15}N_2$]- AND [8-^{13}C-7,NH_2-$^{15}N_2$]ADENOSINE

BASIC PROTOCOL 1

As shown in Figure 1.6.1, the procedures described here (Pagano et al., 1995; Zhao, 1997; Shallop and Jones, 2000) start with the inexpensive pyrimidine 4-amino-6-hydroxy-2-mercaptopyrimidine and introduce the first ^{15}N label by a direct nitrosation in high yield. Reduction of the nitroso group to an amino group is followed by ring closure using either diethoxymethylacetate in dimethylformamide (DMF) to give ^{12}C at the C8 position, or [^{13}C]sodium ethyl xanthate to give ^{13}C at the C8 position. Removing the thiol group(s) with Raney nickel forms hypoxanthine, which can readily be converted to 6-chloropurine, a good substrate for enzymatic transglycosylation. The second ^{15}N label is then introduced into the nucleoside by displacement of the chloride by $^{15}NH_3$, which is generated in situ.

Materials

4-Amino-6-hydroxy-2-mercaptopyrimidine monohydrate, also called 6-amino-2-thioxo-1,2-dihydro-4(3*H*)-pyrimidinone (Aldrich)
1 N HCl
[^{15}N]Sodium nitrite ([^{15}N] $NaNO_2$; Isotec or Cambridge Isotope Laboratories)
2:98 to 40:60 (v/v) gradient of acetonitrile/0.1 M triethylammonium acetate (TEAA), pH 6.8
95% (v/v) ethanol, 4°C
Acetone, 4°C
Phosphorous pentoxide (P_2O_5)
Saturated aqueous $NaHCO_3$
Sodium hydrosulfite ($Na_2S_2O_4$)
Glacial acetic acid
96% (v/v) formic acid
Nitrogen gas source
Dimethylformamide (DMF), anhydrous
Diethoxymethyl acetate (DEMA), for ^{12}C synthesis only
Acetonitrile, room temperature and 4°C

Contributed by Barbara L. Gaffney and Roger A. Jones
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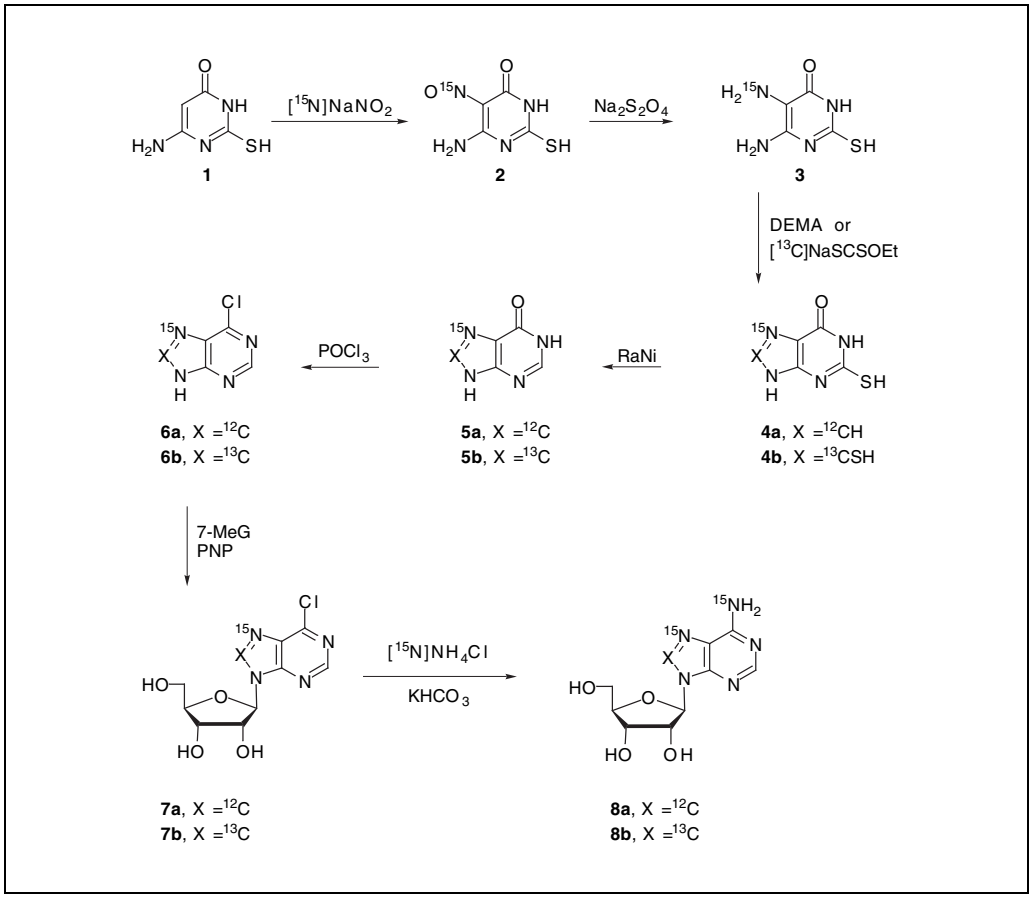

Figure 1.6.1 Steps for synthesis of [7,NH_2-$^{15}N_2$]- and [8-^{13}C-7,NH_2-$^{15}N_2$]adenosine using Basic Protocol 1. DEMA, diethoxymethyl acetate; 7-MeG, 7-methylguanosine; [^{13}C]NaSCSOEt, [^{13}C]sodium ethyl xanthate; PNP, purine nucleoside phosphorylase; RaNi, Raney nickel.

[^{13}C]NaSCSOEt (see recipe), for ^{13}C synthesis only
NaOH
50% aqueous Raney 2800 nickel (RaNi) slurry (Aldrich)
Dipotassium salt of EDTA
Boiling water
Phosphorous oxychloride ($POCl_3$)
N,*N*-Dimethylaniline
5% (v/v) NH_3, (diluted with water from 30% concentrated aqueous ammonia)
Ethyl acetate
Ethyl ether
1 M HCl
7-Methylguanosine (see Support Protocol)
0.02 M K_2HPO_4
3 M NaOH
Purine nucleoside phosphorylase (Sigma)
[^{15}N]Ammonium chloride ([^{15}N]NH_4Cl; Isotec or Cambridge Isotope Laboratories)
Dimethylsulfoxide (DMSO), anhydrous
$KHCO_3$, anhydrous

100- and 250-mL round-bottom flasks
Small glass vials
1-, 3-, 10-, and 20-ml syringes
Vacuum desiccator
Rubber septa fitted with large-bore vent needles
Condenser
Rotary evaporator, connected to water aspirator and a vacuum pump, the latter with a dry-ice trap
Oil bath (silicone oil), 130°C
Separatory funnel
Continuous extraction apparatus for solvents lighter than water (Aldrich)
30°C oven with shaker
50-mL bomb with Teflon liner (Parr Instrument)
80°C oven

Additional reagents and equipment for analytical and preparative reversed-phase high-performance liquid chromatography (HPLC; *UNIT 10.5*)

Synthesize [5-^{15}N]-6-amino-5-nitroso-2-thioxo-1,2-dihydro-4(3H)-pyrimidinone (S.2)

1. Weigh 0.805 g (5.0 mmol) of 4-amino-6-hydroxy-2-mercaptopyrimidine monohydrate into a 100-mL round-bottom flask with a stir bar.

2. Add 25 mL of 1 N HCl and chill the suspension 10 min in an ice bath.

3. Weigh 0.385 g (5.5 mmol, 1.1 eq) [^{15}N]$NaNO_2$ into a small glass vial, dissolve it in ~1 mL water, and draw the solution into a 3-mL syringe.

4. Slowly add the sodium nitrite to the reaction mixture over ~5 min.

   If the addition is too fast, the reaction may bubble over. Before long, the mixture will change from yellow to red.

5. Stir the mixture for ~7 hr in the ice bath, while monitoring the reaction for completeness by reversed-phase HPLC with a gradient of 2:98 to 40:60 acetonitrile/0.1 M TEAA over 5 min.

   The peak representing the starting material should diminish to <3%, and a new peak representing the product should appear.

   Any reversed-phase column can be used. The sample should be injected immediately after mixing for comparison.

6. Collect **S.2** by vacuum filtration and wash it with 5 mL cold water, then 5 mL cold 95% ethanol, and finally 5 mL cold acetone.

7. Without removing it from the funnel, dry **S.2** over P_2O_5 in a vacuum desiccator overnight.

   The yield is usually >95%. See Table 1.6.1 for various data on this and other intermediates and products.

Synthesize [5-^{15}N]-5,6-diamino-2-thioxo-1,2-dihydro-4(3H)-pyrimidinone (S.3)

8. Scrape out most of **S.2** from the funnel into a 100-mL round-bottom flask. Rinse the funnel with portions of saturated aqueous $NaHCO_3$ and add them to the flask to give a final volume of 40 mL.

9. Add a stir bar and place the mixture in an ice bath for 10 min. Stir gently.

10. Quickly weigh 2.61 g (15 mmol, 3 eq) $Na_2S_2O_4$ into a small beaker. Using a spatula, gradually add it in portions to the reaction over 20 min.

    The sodium hydrosulfite smells bad, so it should be kept in the back of the hood. If the addition is too fast, the reaction will bubble over.

11. Insert a rubber septum containing a large-bore vent needle.

12. Stir the mixture for ~7 hr in the ice bath, while monitoring the reaction for completeness by HPLC as described (step 5).

    The mixture will change from red to yellow.

13. Slowly add 1.6 mL glacial acetic acid over ~5 min to neutralize the $NaHCO_3$, and stir another 5 min.

14. Collect the product by vacuum filtration and wash it twice with 5 mL cold water and then twice with 5 mL cold 95% ethanol.

15. Without removing it from the funnel, dry **S.3** over P_2O_5 in a vacuum desiccator overnight.

    The yield is usually >95%.

    The synthesis is continued using either diethoxymethylacetate in dimethylformamide (DMF) to give ^{12}C at the 8 position (steps 16a to 26a) or [^{13}C]sodium ethyl xanthate to give ^{13}C at the 8 position (steps 16b to 22b).

Perform ring closure

*For [7-^{15}N]-2-thioxohypoxanthine **(S.4a):***

16a. Scrape out most of **S.3** from the funnel into a 100-mL round-bottom flask. Rinse the funnel with portions of 96% formic acid and add them to the flask to give a final volume of 25 mL.

Table 1.6.1 Molecular Weights, TLC and HPLC Mobilities, and UV λ_{max} of ^{15}N-Labeled Adenosine and Guanosine Intermediates[a]

Compound	mol. wt. (Da)	TLC R_f[b]	HPLC retention time (min)[c]	UV λ_{max} (nm)
S.2	173	0.2	1.2	360
S.3	159	0.0	0.8	301
S.4a	169	0.2	0.7	280
S.4b	202	0.0	1.4	299
S.5a/b	137/138	0.1	0.8	250
S.6a/b	155/156	0.3	2.4	265
S.7a/b	287/288	0.2	4.5	264
S.8a/b	269/270	0.1	2.6	259
S.9a/b	285/286	0.0	0.9	295
S.12a/b	316	0.1	3.3	280
S.13a/b	287	0.0	1.4	253

[a]Abbreviations: HPLC, high-performance liquid chromatography; mol. wt., molecular weight; TLC, thin-layer chromatography.

[b]R_f values determined with 10:90 (v/v) CH_3OH/CH_2Cl_2.

[c]Gradient of 2:98 to 40:60 (v/v) acetonitrile/0.1 M triethylammonium acetate, pH 6.8, over 5 min, on a Waters NovaPak C18 column.

17a. Add a stir bar, attach a condenser, and reflux the solution for 1 hr to make the formate salt.

18a. Concentrate to dryness using a rotary evaporator and scrape down the sides of the flask with a spatula, if necessary.

19a. Insert a rubber septum and displace the air with nitrogen.

20a. Use syringes to add the following through the septum:

20 mL anhydrous DMF
1.63 mL DEMA (10 mmol, 2 eq)
0.24 mL of 96% formic acid (6 mmol, 1.2 eq).

21a. Heat the mixture for 3 hr in an oil bath set at 130°C. Follow the reaction by HPLC.

The flask is lifted from the oil bath and allowed to cool briefly, and then a small syringe with a long, dry needle is used to get a sample for HPLC.

22a. Cool the flask, concentrate the solution to a solid using a rotary evaporator, and loosen it with a spatula if necessary.

23a. Add 15 mL acetonitrile to the flask, attach a condenser, and reflux it for 10 min using the 130°C oil bath.

24a. Cool the flask to room temperature, add 10 mL acetonitrile, and then chill it in an ice bath.

25a. Collect **S.4a** by vacuum filtration and wash it twice with 5 mL cold acetonitrile.

26a. Without removing it from the funnel, dry **S.4a** over P_2O_5 in the vacuum desiccator overnight. Proceed to step 27.

The yield is usually >95%.

*For [8-^{13}C-7-^{15}N]-2,8-dithioxohypoxanthine (**S.4b**):*

16b. Scrape out most of **S.3** from the funnel into a 100-mL round-bottom flask and add 0.80 g (5.5 mmol) [^{13}C]NaSCSOEt.

17b. Insert a condenser into the flask, attach a nitrogen line and vent needle, and displace the air for 5 min.

18b. Add 15 mL DMF and reflux the mixture under nitrogen for ~3 hr, using HPLC to monitor the reaction.

19b. Cool the mixture in an ice bath and add 50 mL cold acetonitrile to precipitate **S.4b**.

20b. Collect the solid **S.4b** by vacuum filtration and wash it twice with 5 mL cold acetonitrile. Save the filtrate and both washes.

21b. Concentrate the filtrate and washes, and purify this portion of **S.4b** by preparative reversed-phase chromatography.

22b. Dry the combined portions of **S.4b** over P_2O_5 in a vacuum desiccator overnight. Continue with step 27.

The yield is usually >95%.

Synthesize [7-^{15}N]hypoxanthine and [8-^{13}C-7-^{15}N]hypoxanthine (S.5a/b)

27. Scrape out most of **S.4a/b** from the funnel into a 100-mL round-bottom flask.
28. Rinse the funnel with portions of water and add them to the flask to give a final volume of 30 mL.
29. Add a stir bar and 2 mL of 96% formic acid.
30. Weigh 4.5 g of 50% aqueous RaNi slurry into a small glass vial or beaker.

    CAUTION*: RaNi is pyrophoric (will spontaneously burst into flames) if it is allowed to dry out. To weigh it, shake the bottle and immediately transfer some of the suspended RaNi to the vial. Continue adding the suspension (shaking the bottle each time) until 4.5 g of the 50:50 mixture of RaNi/water has been measured out.*
31. Using a dropper, transfer the RaNi suspension over 5 min to the reaction flask, and add 1.5 g dipotassium salt of EDTA.

    Traces of remaining RaNi in the vial and dropper should be destroyed with 6 M HCl.
32. Connect a condenser to the flask. Using the 130°C oil bath, reflux for ~2 hr while monitoring the reaction for completeness by HPLC.
33. Remove the flask from the oil bath and allow it to cool only briefly.
34. Remove the condenser from the flask and carefully filter the hot reaction mixture to remove the RaNi. Rinse the flask and then the funnel with three 10-mL portions of boiling water, adding these washes to the filtrate.

    To destroy the RaNi remaining in the funnel, the funnel should be transferred to a large beaker, and portions of 6 M HCl should be slowly added until no black particles can be observed.
35. Concentrate the filtrate and washes to dryness in a 100-mL round-bottom flask using the rotary evaporator.
36. Dry **S.5a/b** over P_2O_5 in the vacuum desiccator overnight.

    *The yield is usually >95%. **S.5a/b** can be purified by reversed-phase chromatography if desired.*

Synthesize [7-^{15}N]- and [8-^{13}C-7-^{15}N]-6-chloropurine (S.6a/b)

37. Weigh 0.69 g (5.0 mmol) **S.5a/b** into a very dry 100-mL round-bottom flask.
38. Add 20 mL (215 mmol, 43 eq) $POCl_3$ and 2 mL (16 mmol) *N*,*N*-dimethylaniline.

    Use great care with $POCl_3$; it is very reactive.
39. Attach a condenser and reflux 20 min under nitrogen.

    The resulting solution should be black and homogeneous. It is essential for this reaction to remain anhydrous.
40. Monitor the reaction for completeness by HPLC and continue refluxing the mixture for ≤30 min more.
41. Concentrate the mixture to a very small volume using a rotary evaporator, first with an aspirator and then with a vacuum pump protected with a dry-ice trap. Add 10 mL *N*,*N*-dimethylaniline and continue the evaporation.

    It is necessary to remove all traces of $POCl_3$, or localized heating from later neutralization with NH_3 may cause the reaction to reverse. Be extremely careful to dry the evaporator condenser and trap prior to use, to keep dry ice in the traps throughout this process, and then to pour the collected $POCl_3$ into a plastic container of ice to destroy it.

42. Cool the flask in an ice bath and very slowly add 30 mL of 5% NH_3 to dissolve the black gum.

    As a neutral compound, 6-chloropurine is insoluble in water, but under basic conditions it will ionize and therefore dissolve.

43. Make sure the pH of the solution is >10 (add more NH_3 if necessary) and then pour it into a separatory funnel. Wash it first with 30 mL ethyl acetate and then twice with 30 mL ethyl ether. Check the layers by HPLC.

44. Combine all organic layers that contain traces of product in a separatory funnel, backwash with 5% NH_3, and add this aqueous layer to the main reaction mixture.

45. Concentrate this aqueous solution to dryness to remove all the NH_3.

    It is necessary to remove all traces of NH_3, or localized heating from later neutralization with HCl may cause the reaction to reverse. As the NH_3 evaporates, it is very likely to bump, so a large enough flask should be used.

46. Add 20 mL water and chill the flask in an ice bath. Slowly acidify the solution to pH 2 using 1 M HCl.

    The mixture will turn cloudy.

47. Set up a continuous extraction apparatus for solvents lighter than water. Pour the aqueous layer into the extractor and add ethyl ether until the level is just under the side arm.

48. Fill a 250-mL round-bottom flask with ethyl ether, add a stir bar, connect to the extractor, and place it in an oil bath. Attach a condenser to the top of the extractor, and start heating the oil bath to 45°C.

49. Continue the extraction for 3 to 4 days, using fresh ether each day. Check both the aqueous and ether layers each day by HPLC. Verify that the pH of the aqueous layer is still <2 and, if it is not, adjust it with 1 M HCl.

50. Concentrate the ether layers to a small volume, whereupon a significant amount of **S.6a/b** should crystallize out. Collect **S.6a/b** by vacuum filtration and check it for purity by HPLC. Save the filtrate.

51. Concentrate the filtrate to dryness and dissolve it in 10 mL water. Purify by reversed-phase preparative chromatography.

52. Concentrate the fractions containing pure product to dryness. Dry both portions of **S.6a/b** in a vacuum desiccator over P_2O_5 overnight.

    The yield is usually between 80% and 90%.

Synthesize [7-^{15}N]- and [8-^{13}C-7-^{15}N]-6-chloro-9-(β-D-erythropentofuranosyl) purine (S.7a/b)

53. Place 2.12 g (7.5 mmol, 1.5 eq) of 7-methylguanosine and 0.78 g (5 mmol) **S.6a/b** into a 100-mL round-bottom flask and add 20 mL of 0.02 M K_2HPO_4. Using pH paper, adjust the pH to 7.4 with 3 M NaOH, if necessary.

54. Add 250 units of purine nucleoside phosphorylase. Insert a septum and heat the mixture ~3 days in an oven at 30°C with gentle agitation. Monitor the reaction for completeness each day by HPLC.

55. Pour the mixture into 10 mL DMF and stir ~1 hr at room temperature.

Table 1.6.2 Nuclear Magnetic Resonance Chemical Shifts (ppm) for ^{15}N-Labeled Adenosine (**S.8**) and Guanosine (**S.13**)[a]

Compound	N1**H**	H8	H2	N**H**$_2$	1′H	2′H	3′H	4′H	5′H	N1	N7	**N**H$_2$	C2	C8
S.8a/b	—	8.35	8.14	7.40	5.87	4.60	4.14	3.96	3.6	—	242	83	152	140
S.13a/b	10.6	7.92	NA	6.44	5.68	4.38	4.07	3.85	3.6	148	244	75	154	135

[a]All NMR samples in DMSO-d_6. ^{15}N data are relative to $^{15}NH_3$ using external 1M [^{15}N]urea in DMSO at 77.0 ppm as a reference. Additional data (e.g., for intermediates) are available in Pagano et al. (1995), Zhao et al. (1997), and Shallop and Jones (2000).

Table 1.6.3 Nuclear Magnetic Resonance Coupling Constants (Hz) for ^{15}N-Labeled Adenosine (**S.8**) and Guanosine (**S.13**)

Compound	^{13}C8-^{15}N7	^{13}C2-^{15}N1	^{13}C2-$^{15}NH_2$
S.8a/b	<1	NA	NA
S.13a/b	<1	12	24

56. Filter the suspension to remove most of the solid 7-methylguanine. Suspend the solid in 5 mL fresh DMF, stir 15 min, and filter.

57. Concentrate the combined filtrates to a small volume using a rotary evaporator and add 15 mL water (more if necessary to dissolve the solid).

58. Purify the crude **S.7a/b** by preparative reversed-phase HPLC and dry it over P_2O_5 in the vacuum desiccator overnight.

    The yield is usually 85% to 95%.

Synthesize [7,NH$_2$-^{15}N$_2$]- and [8-^{13}C-7,NH$_2$-^{15}N$_2$]adenosine (S.8a/b)

59. Place 1.44 g (5 mmol) **S.7a/b** into a clean Teflon liner of a bomb and add 0.54 g (10 mmol, 2 eq) of [^{15}N]NH_4Cl and 7 mL anhydrous DMSO.

60. Add 1.5 g (15 mmol, 3 eq) $KHCO_3$ and immediately seal the bomb.

61. Heat the bomb 3 days in an 80°C oven, swirling the mixture once or twice each day.

62. Cool the bomb to room temperature and then to −20°C for ≥30 min. Open it carefully and dilute the mixture with 10 mL water. Adjust the pH to 7 with glacial acetic acid.

    Care should be taken when opening the bomb because CO_2 will have been generated.

63. Check the reaction by HPLC.

64. Purify **S.8a/b** by preparative reversed-phase HPLC and dry it over P_2O_5 in the vacuum desiccator overnight.

    The yield is usually 80% to 90%. See Tables 1.6.2 and 1.6.3 for NMR chemical shifts and coupling constants.

SYNTHESIS OF 7-METHYLGUANOSINE

SUPPORT PROTOCOL

Although 7-methylguanosine can be purchased from Sigma, it is quite easy to make. Commercial 7-methylguanosine is very expensive and the purity may not be as high.

Materials

Guanosine
N,*N*-Dimethylacetamide
Nitrogen gas source
Dimethyl sulfate
Concentrated aqueous NH_3
Acetone, 4°C
95% (v/v) ethanol
Ethyl ether

250-mL round-bottom flask
Rubber septum
10-mL syringes

CAUTION: Dimethyl sulfate is very dangerous because it is a potent alkylating agent; wear gloves and use caution.

Synthesize 7-methylguanosine

1. Place 9.91 g (35 mmol) guanosine in a 250-mL round-bottom flask along with a stir bar.
2. Add 80 mL *N*,*N*-dimethylacetamide as a solvent, insert a rubber septum, and displace the air with nitrogen gas.
3. Carefully withdraw 7.0 mL (74 mmol, 2.1 eq) dimethyl sulfate with a 10-mL syringe and add it to the suspension.
4. Stir mixture ~6 hr and monitor the reaction for completeness by HPLC.
5. Slowly add 10 mL concentrated aqueous NH_3 and then check the pH using pH paper. Continue to add more NH_3 slowly, frequently checking the pH, until the mixture is pH 10.

   This step is essential and quenches the excess dimethyl sulfate.

6. Slowly add the mixture to 300 mL cold acetone in an ice bath to precipitate the product.
7. Collect the white precipitate by vacuum filtration.
8. Check the acetone filtrate by HPLC for product. If there is a significant amount, concentrate the filtrate to a small volume, chill it, and collect the additional product by vacuum filtration. Combine it with the rest.

Purify 7-methylguanosine

9. Suspend the solid crude product in 300 mL of 95% ethanol, stir 5 min, and collect it by filtration.
10. Suspend the solid product in 300 mL ethyl ether, stir 5 min, and collect it by filtration.
11. Dry the pure product over P_2O_5 in a vacuum desiccator overnight.

    The yield is usually 80% to 90%. It is very important to keep this compound dry. It should be transferred to a bottle with a tight lid, the air should be displaced with nitrogen, and it should be stored at −20°C. It can be kept for up to 3 months.

SYNTHESIS OF [2-^{13}C-1,7,NH_2-$^{15}N_3$]- AND [8-^{13}C-1,7,NH_2-$^{15}N_3$]GUANOSINE

As shown in Figure 1.6.2, in the adenosine to guanosine transformation (Zhao, 1997; Shallop and Jones, 2000), the adenosine amino group becomes the guanosine N1, while the guanosine amino and C2 come from potassium cyanide. Thus, to make [2-^{13}C-1,7,NH_2-$^{15}N_3$]guanosine (**S.13a**), [7,NH_2-$^{15}N_2$]adenosine (**S.8a**) is used with [^{13}C,^{15}N]KCN, and to synthesize [8-^{13}C-1,7,NH_2-$^{15}N_3$]guanosine (**S.13b**), [8-^{13}C-7,NH_2-$^{15}N_2$]adenosine (**S.8b**) is used with [^{15}N]KCN.

The first step is the oxidation of adenosine (**S.8a/b**) to the N1 oxide (**S.9a/b**), which is followed by a one-flask set of reactions without purification to give **S.12a/b**. Labeled cyanogen bromide is generated in situ from labeled potassium cyanide and bromine, and its reaction with **S.9a/b** forms **S.10a/b**. Treatment with triethylamine opens the oxazolidine ring, allowing the N1 oxide to be methylated by methyl iodide to give **S.11a/b**. Aqueous sodium hydroxide then opens the pyrimidine ring, which deformylates, rearranges, and closes again upon neutralization and heating to give **S.12a/b**. Enzymatic deamination then gives the final product, **S.13a/b**.

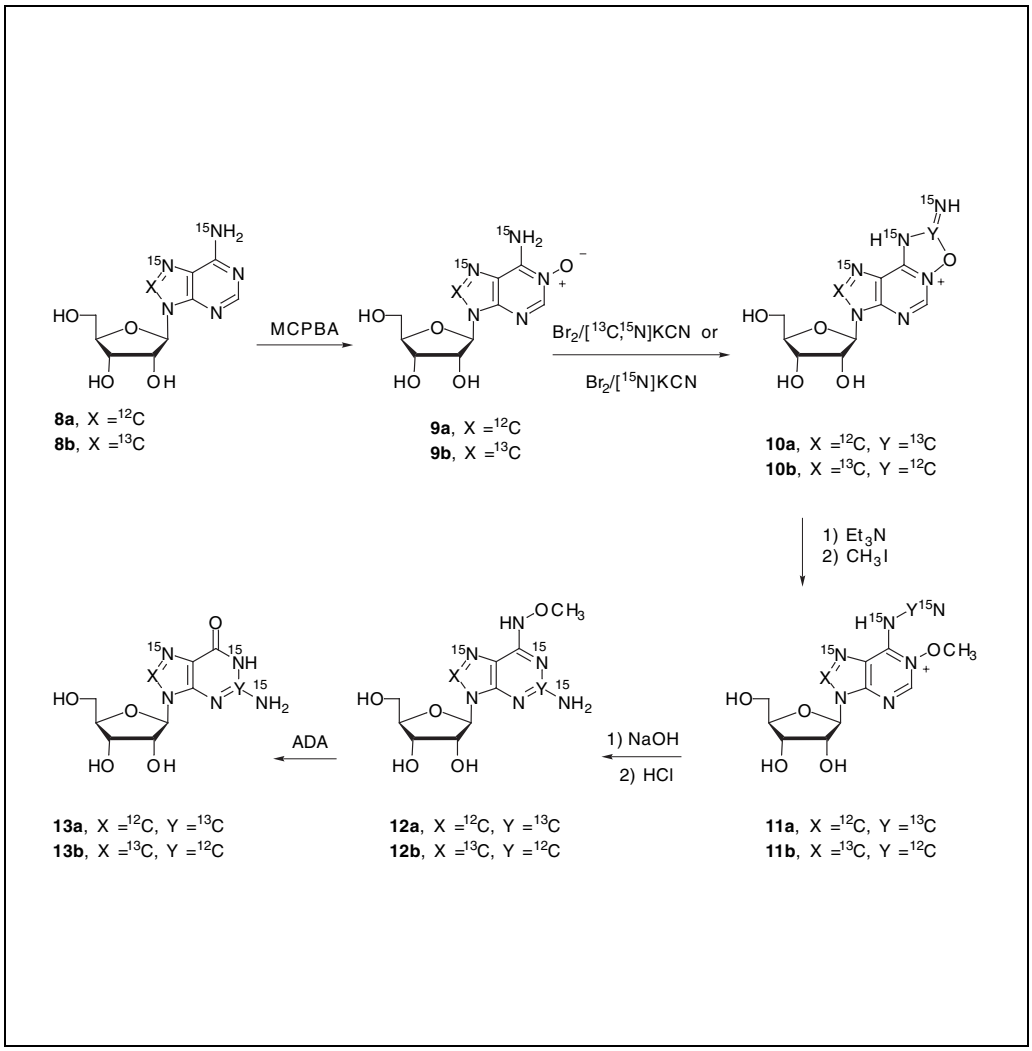

Figure 1.6.2 Steps for synthesis of [2-^{13}C-1,7,NH_2-$^{15}N_3$]- and [8-^{13}C-1,7,NH_2-$^{15}N_3$]guanosine using Basic Protocol 2. ADA, adenosine deaminase; Et_3N, triethylamine; MCPBA, 3-chloroperoxybenzoic acid.

Materials

[7,NH_2-$^{15}N_2$]Adenosine or [8-^{13}C-7,NH_2-$^{15}N_2$]adenosine (**S.8a** or **S.8b**; see Basic Protocol 1)
50% (v/v) methanol
3-Chloroperoxybenzoic acid (MCPBA), purified (see recipe)
Ethyl ether
Phosphorous pentoxide (P_2O_5)
[^{13}C,^{15}N]Cyanogen bromide or [^{15}N]cyanogen bromide, freshly prepared (see recipe)
0.1 M potassium phosphate (KH_2PO_4), pH 7.5
Dimethyl formamide (DMF), anhydrous
Acetonitrile
Triethylamine
Nitrogen gas source
Methyl iodide
0.1 M NaOH
1 M HCl
95% (v/v) ethanol
Adenosine deaminase (Sigma)

100-mL round-bottom flasks
Rotary evaporator
Vacuum desiccator
Oil bath (silicone oil), 60°C

Additional reagents and equipment for analytical and preparative reversed-phase high-performance liquid chromatography (HPLC; *UNIT 10.5*)

Synthesize [7,NH_2-$^{15}N_2$]- and [8-^{13}C-7,NH_2-$^{15}N_2$]adenosine-N1-oxide (S.9a/b)

1. Weigh 1.35 g (5.0 mmol) of [7,NH_2-$^{15}N_2$]adenosine (**S.8a**) or [8-^{13}C-7,NH_2-$^{15}N_2$]adenosine (**S.8b**) into a 100-mL round-bottom flask. Add a stir bar and 50 mL of 50% methanol.

2. Add 1.72 g (10 mmol, 2 eq) purified MCPBA, cover the flask with aluminum foil, stir 3 to 4 hr, and monitor the reaction for completeness by HPLC.

   Any reversed-phase column can be used.

3. Dilute the solution with 25 mL water and wash it with three 50-mL portions of ethyl ether.

4. Use a rotary evaporator to concentrate the aqueous solution to a small volume, purify **S.9a/b** by preparative reversed-phase HPLC, and dry it over P_2O_5 in a vacuum desiccator overnight in a 100-mL round-bottom flask.

   The yield is usually 90% to 95%. See Table 1.6.1 for various data on this and other intermediates and products.

Synthesize [2-^{13}C-1,7,NH_2-$^{15}N_3$]- and [8-^{13}C-1,7,NH_2-$^{15}N_3$]-2-amino-6-(methoxyamino)-9-(β-D-ribofuranosyl)purine (S.12a/b)

5. Dissolve 1.43 g (5.0 mmol) **S.9a/b** in 40 mL water.

6. Add 7.5 mmol freshly prepared [^{13}C,^{15}N]cyanogen bromide or [^{15}N]cyanogen bromide, stir for 2 hr, and monitor the reaction for completeness by HPLC.

   *To make **S.13a**, [^{13}C,^{15}N]cyanogen bromide is used, and to make **S.13b**, [^{15}N]cyanogen bromide is used.*

CAUTION: *The waste in the evaporator trap may contain excess cyanogen bromide. Dispose of this waste in an appropriately designated area, following the guidelines provided by the local safety officer.*

7. Concentrate the solution to a very small volume using a rotary evaporator, add 10 mL anhydrous DMF and 10 mL acetonitrile, and concentrate again. Repeat this drying process two more times.

   CAUTION: *Be very careful to dispose of the waste from the evaporator trap according to accepted regulations, because it may contain excess cyanogen bromide.*

8. Add 25 mL anhydrous DMF and 2.8 mL (20 mmol, 4 eq) triethylamine under nitrogen gas.

9. Stir 45 min and then slowly add 2.5 mL (40 mmol, 8 eq) methyl iodide.

   Do not wait >1 hr to add the methyl iodide.

10. Cover the flask with aluminum foil and stir 3 to 4 hr while monitoring the reaction for completeness by HPLC.

11. Concentrate the solution to a yellow oil and add 85 mL of 0.1 M NaOH.

    Do not wait >4 hr to concentrate the solution and add the NaOH.

    CAUTION: *The waste from the evaporator trap must be carefully disposed according to accepted regulations, because it may contain the excess methyl iodide.*

12. Stir 20 min and then adjust the pH to 7.4 with 1 M HCl.

13. Add 80 mL of 95% ethanol, attach a condenser, and heat the solution in an oil bath at 60°C for 4 hr while monitoring the reaction for completeness by HPLC.

14. Concentrate the solution to a small volume using a rotary evaporator, purify **S.12a/b** by reversed-phase HPLC, and dry it over P_2O_5 in the vacuum desiccator overnight in a 100-mL round-bottom flask.

    The yield is usually 80% to 90%.

Synthesize [2-^{13}C-1,7,NH$_2$-^{15}N$_3$]- and [8-^{13}C-1,7,NH$_2$-^{15}N$_3$]guanosine (S.13a/b)

15. Dissolve 1.58 g (5 mmol) **S.12a/b** in 80 mL of 0.1 M KH_2PO_4, pH 7.5.

16. Add 300 units of adenosine deaminase, stopper the flask, and heat 4 days at 37°C with gentle agitation.

    Most of the product should crystallize out during this time.

17. Cool the mixture in an ice bath and collect crude **S.13a/b** by filtration.

18. Recrystallize **S.13a/b** by recrystallization from water and dry it over P_2O_5 in a vacuum desiccator overnight.

    The yield is usually 75% to 85%. See Tables 1.6.2 and 1.6.3 for NMR chemical shifts and coupling constants.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

3-Chloroperoxybenzoic acid (MCPBA)

Dissolve 10 g MCPBA in 200 mL ether and wash with three 150-mL portions of 0.1 M aqueous potassium phosphate, pH 7.5. Concentrate to dryness and dry over P_2O_5 in a vacuum desiccator overnight. Store up to 3 months at −20°C in a bottle with a tight cap.

CAUTION: *MCPBA is potentially explosive and must be handled with care.*

Commercial MCPBA is contaminated with 40% to 50% 3-chlorobenzoic acid, which can be removed by extraction.

[^{13}C,^{15}N]Cyanogen bromide

Place 3 mL water into a small pear-shaped flask and add 1.2 g (0.38 mL, 7.5 mmol) bromine. Chill the flask in an ice bath, slowly add 0.50 g (7.5 mmol, 1 eq) [^{13}C,^{15}N]potassium cyanide dissolved in 20 mL water, and stir 30 min. Draw the solution into a syringe in order to add it to a reaction.

CAUTION: *Use great care in handling bromine, because it is highly toxic, is fairly volatile, and can cause severe burns. Place a balance in a hood, draw up the required volume of bromine into a preweighed syringe, weigh the syringe, and adjust accordingly. Potassium cyanide and cyanogen bromide are also highly toxic and must be handled with great care.*

[^{15}N]Cyanogen bromide

Prepare as for [^{13}C,^{15}N]cyanogen bromide (see recipe), but use [^{15}N]potassium cyanide instead of [^{13}C,^{15}N]potassium cyanide.

[^{13}C]Sodium ethyl xanthate ([^{13}C]NaSCSOEt)

Dissolve 0.40 g (10 mmol) NaOH in 40 mL absolute ethanol. Add 0.77 g (10 mmol, 1 eq) [^{13}C]carbon disulfide ([^{13}C]CS_2; Isotec or Cambridge Isotope Laboratories). Cover the flask with aluminum foil and stir the solution overnight at room temperature. Concentrate to dryness and dry over P_2O_5 in a vacuum desiccator overnight. Store up to 6 months at −20°C.

The yield is usually quantitative.

COMMENTARY

Background Information

^{15}N NMR studies of specifically ^{15}N-labeled DNA and RNA fragments have provided significant information about local interactions at nitrogen atoms, such as hydrogen bonding, stacking, and protonation (Wang et al., 1991; Zhang et al., 1997, 1998). The use of one or more multilabeled nucleosides can provide more information than single-labeled nucleosides, but only as long as all signals can be distinguished. The use of ^{13}C tags adjacent to different nitrogens in a pair of nucleosides was designed to allow such differentiation (Zhao, 1997; Abad et al., 1998; Shallop and Jones, 2000). In addition, the ^{13}C chemical shifts can provide valuable information.

The route to labeled adenosine described here (Pagano et al., 1995) starts with a direct nitrosation, which is more convenient than the azo coupling described earlier (Gaffney et al., 1990). This route has been designed so as to remove both thiol groups (in the case of **S.4b**) in the same step. The enzymatic coupling is done with 6-chloropurine, because the subsequent displacement with [^{15}N]NH_3 can be done using much milder conditions. Also, the lipophilicity of the 6-chloro group helps in the purification of the nucleoside. The purine nucleoside phosphorylase uses 7-methylguanosine as a sugar donor and both generates the ribose-α-1-phosphate and couples it to the 6-chloropurine. The reaction is driven to completion by precipitation from solution of the very insoluble 7-methylguanine. The final amination has been optimized so that it only requires two equivalents of [^{15}N]NH_4Cl with $KHCO_3$.

Although not described here, [7,NH_2-$^{15}N_2$]adenosine can be converted to [1,7,NH_2-$^{15}N_3$]adenosine (Pagano et al., 1998).

The adenosine to guanosine transformation described here (Zhao, 1997; Shallop and Jones, 2000) is based on a previous method (Goswami and Jones, 1991) that was in turn derived from earlier work (Ueda et al., 1978).

Critical Parameters

In the chlorination that converts **S.5a/b** to **S.6a/b**, it is essential to remove all excess $POCl_3$, or heat generated during subsequent neutralization with ammonia will cause the reaction to reverse to some extent. During the one-flask set of reactions for the adenosine to guanosine transformation, the intermediates are not particularly stable, and the reactions should not be left for longer than the stated times. After formation of cyanogen bromide, there should be no excess bromine present (as indicated by an orange color). The order of addition during the formation of cyanogen bromide is also important. In the final enzymatic deammination to make **S.13a/b**, **S.12a/b** must be purified, or the enzymatic reaction will not work satisfactorily.

Anticipated Results

The early steps in these protocols generally give high yields, even for inexperienced workers. The later steps are more challenging, and, with experience, the stated yields can be obtained. Because most of these compounds are fairly polar, the use of TLC to monitor reactions is not very convenient. Reversed-phase HPLC is somewhat more informative, while UV λ_{max} data from an HPLC system with a diode array detector are particularly helpful.

Time Considerations

The total time for Basic Protocol 1 is 2 to 3 weeks and that for Basic Protocol 2 is 1 to 2 weeks. The Support Protocol requires 1 to 2 days.

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