Synthesis of Oligoribonucleotides Containing N^6-Alkyladenosine and 2-Methylthio-N^6-Alkyladenosine

This unit describes synthesis of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-*tert*-butyldimethylsilyl-3′-*O*-[(2-cyanoethoxy)-(*N,N*-diisopropylamino)]phosphinyl-6-methylthiopurine riboside (**S.9**, Fig. 4.23.1; see Basic Protocol 1) and 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-*tert*-butyldimethylsilyl-3′-*O*-[(2-cyanoethoxy)-(*N,N*-diisopropylamino)]phosphinyl-2-methylthio-6-chloropurine riboside (**S.18**, Fig. 4.23.2; see Alternate Protocol). Both modified phosphoramidites and natural ribonucleoside phosphoramidites are used for the synthesis of precursor forms of oligoribonucleotides, which are then converted into oligoribonucleotides containing N^6-alkyladenosine and 2-methylthio-N^6-alkyladenosine by treatment with an appropriate primary amine (Kierzek and Kierzek, 2003a,b; see Basic Protocol 2).

SYNTHESIS OF THE 6-METHYLTHIOPURINE RIBOSIDE PHOSPHORAMIDITE

BASIC PROTOCOL 1

The synthesis of 5′-*O*-DMTr-2′-*O*-TBDMS-3′-*O*-(2-cyanoethyl-*N,N*-diisopropyl)-6-methylthiopurine riboside phosphoramidite starts with complete conversion of inosine (**S.1**; Fig. 4.23.1) into 2′,3′,5′-tri-*O*-acetylinosine (**S.2**) by reaction with acetic anhydride in pyridine. **S.2** is quantitatively transformed into 2′,3′,5′-tri-*O*-acetyl-6-thiopurine riboside (**S.3**) by treatment with Lawesson's reagent for 1.5 hr at 100°C (Cava and Levinson, 1985; Kierzek and Kierzek, 2003a). This product is transformed into 2′,3′,5′-tri-*O*-acetyl-6-methylthiopurine riboside (**S.4**) with methyl iodide in the presence of potassium carbonate in *N,N*-dimethylformamide (DMF) over 2 hr at 50°C in ~81% yield (Wetzel and Eckstein, 1975). Acetyl groups are removed with ammonium hydroxide in pyridine, and the resulting 6-methylthiopurine riboside (**S.5**) is treated with 4,4′-dimethoxytrityl chloride (DMTr-Cl). After purification by short-column chromatography, the 5′-*O*-DMTr-6-methylthiopurine riboside (**S.6**) is obtained in ~74% yield (Smith et al., 1962). This product is reacted with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) in pyridine in the presence of imidazole to give a mixture of 2′- (**S.7**), 3′- (**S.8**), and 2′,3′-silylated derivatives, which after purification and subsequent 3′-*O*-TBDMS derivative isomerization gives 5′-*O*-DMTr-2′-*O*-TBDMS-6-methylthiopurine riboside (**S.7**) in ~66% yield (Ogilvie at al., 1978). The phosphoramidite **S.9** is prepared by reaction of **S.7** with 2-cyanoethyl diisopropylchlorophosphoramidite in anhydrous acetonitrile in the presence of diisopropylethylamine (Beaucage and Caruthers, 1981). The product is obtained in ~85% yield after purification by short-column chromatography on silica gel.

Materials

Inosine (**S.1**)
Pyridine (reagent grade or better)
Acetic anhydride
Dichloromethane (anhydrous)
Methanol (anhydrous)
Saturated aqueous sodium bicarbonate solution
Sodium sulfate (anhydrous)
Toluene

Contributed by Elzbieta Kierzek and Ryszard Kierzek
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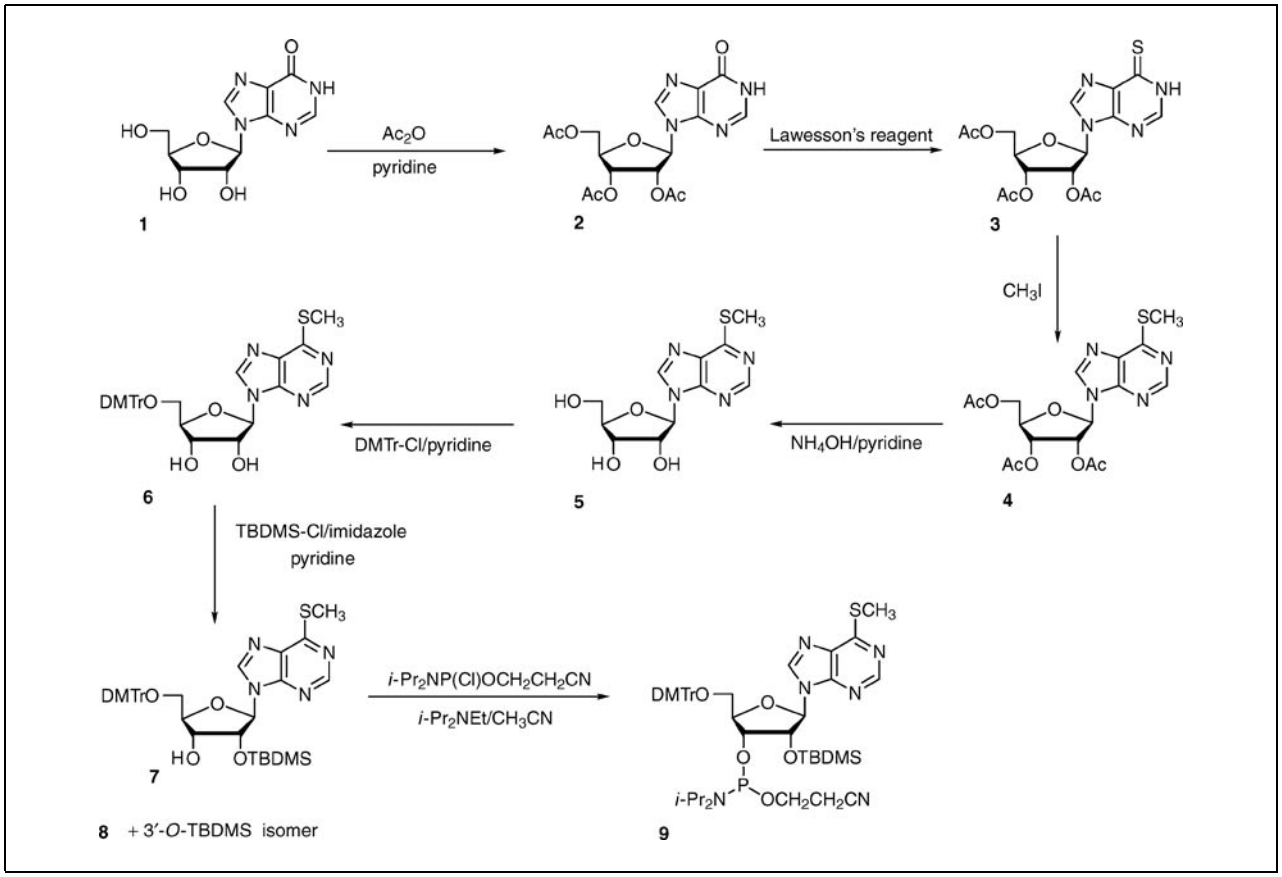

Figure 4.23.1 Acetylation, thiolation, methylation, acetyl deprotection, dimethoxytritylation, silylation, and phosphinylation of purine riboside derivative. Abbreviations: Ac, acetyl; DMTr, 4,4′-dimethoxytrityl; *i*-Pr, isopropyl; TBDMS, *tert*-butyldimethylsilyl.

1,4-Dioxane (anhydrous)
Lawesson's reagent (Aldrich)
N,*N*-Dimethylformamide (DMF; anhydrous)
Potassium carbonate (anhydrous), ground
Methyl iodide
Celite 545
Silica gel 60H (Merck)
Concentrated aqueous ammonium hydroxide (NH_4OH; 28% to 30%)
4,4′-Dimethoxytrityl chloride (DMTr-Cl)
Imidazole
tert-Butyldimethylsilyl chloride (TBDMS-Cl)
Ethyl acetate
Hexanes (anhydrous)
10% (w/v) aqueous sodium phosphate, monobasic (NaH_2PO_4)
Benzene (anhydrous)
Dry ice/ethanol bath
Acetonitrile (commercial with <20 ppm water *or* dried over 3Å molecular sieves)
Diisopropylethylamine (DIPEA; anhydrous)
2-Cyanoethyl diisopropylchlorophosphoramidite
Acetone
Triethylamine (TEA; anhydrous)

250- and 25-mL round-bottom flasks
Silica gel 60F thin-layer chromatography (TLC) plates (Merck)

250-mL and 1-L separatory funnels
Rotary evaporator
Vacuum pump and water aspirator
Reflux condenser
100° and 50°C oil baths (silicone oil)
60- and 150-mL sintered glass funnels
Rubber septa
Vacuum adaptor for 250-mL round-bottom flask and appropriate traps
Vacuum desiccator
1-mL and 10-mL glass syringes with needles
Silanized silica gel 60F TLC plates (Merck), optional

Additional reagents and equipment for TLC (*APPENDIX 3D*) and column chromatography (*UNIT 2.4 & APPENDIX 3E*)

Acetylate inosine

1. Suspend 8.01 g (30 mmol) inosine (**S.1**) in 75 mL anhydrous pyridine in a 250-mL round-bottom flask and begin stirring. Add 9.90 mL (105 mmol) acetic anhydride and leave 16 hr at room temperature.

2. Analyze the reaction mixture by TLC (*APPENDIX 3D*) on silica gel 60F TLC plates using 9:1 (v/v) dichloromethane/methanol (**S.2** $R_f = 0.54$).

   The TLC analysis is performed with a short-wave length UV lamp at 254 nm.

3. Pour the reaction mixture carefully into a 1-L separatory funnel containing 400 mL saturated aqueous sodium bicarbonate solution and extract three times with 200 mL dichloromethane.

4. Dry combined organic layers with ~10 g anhydrous sodium sulfate and evaporate on a rotary evaporator with a water aspirator. Coevaporate residue three times with ~50 mL toluene. Dry under vacuum pump.

   *2′,3′,5′-Tri-O-acetylinosine (**S.2**): 11.89 g (100%). TLC (dichloromethane/methanol 9:1 [v/v]): $R_f = 0.54$. UV (MeOH): $\lambda_{max} = 224, 256$ nm; $\lambda_{min} = 228$ nm. ^{1}H NMR ($CDCl_3$): δ 2.10 (s, 3H, CH_3), 2.15 (s, 3H, CH_3), 2.16 (s, 3H, CH_3), 4.41–4.47 (m, 3H, H4′, H5′, H5″), 5.61 (t, J = 5.8 Hz, 1H, H3′), 5.88 (t, J = 6.5 Hz, 1H, H2′), 6.19 (d, J = 5.1 Hz, 1H, H1′), 8.11 (s, 1H, H2), 8.31 (s, 1H, H8). ^{13}C NMR ($CDCl_3$): δ 20.3, 20.5, 20.7 (C(O)$\underline{CH_3}$), 63.0 (C5′), 70.5 (C3′), 73.3 (C2′), 80.4 (C4′), 86.6 (C1′), 125.1 (C5), 138.6 (C8), 145.8 (C4), 148.7 (C2), 158.7 (C6), 169.3, 169.5, 170.3 ($\underline{C}$(O)CH_3).*

Thionylate inosine derivative

5. Dissolve 11.83 g (30 mmol) **S.2** in 100 mL 1,4-dioxane in a 250-mL round-bottom flask and add 7.28 g (18 mmol) Lawesson's reagent. Attach a reflux condenser and reflux the reaction mixture in an oil bath for 1.5 hr at 100°C.

   CAUTION: *Reaction must be run in an efficiently working hood.*

6. Periodically analyze the reaction mixture by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol (**S.3** $R_f = 0.64$).

7. On the rotary evaporator with water aspirator, remove approximately half the volume of dioxane.

8. Add 10 mL methanol followed by 200 mL saturated aqueous sodium bicarbonate solution.

   Cooling the reaction mixture and evaporation of dioxane result in gel formation, but addition of methanol dissolves the reaction mixture, thus allowing easy workup.

9. Extract the reaction mixture three times with 150 mL dichloromethane.

10. Dry combined organic layers with ~20 g anhydrous sodium sulfate and rotary evaporate to a solid foam.

*2′,3′,5′-Tri-O-acetyl-6-thiopurine riboside (**S.3**): TLC (dichloromethane/methanol 9:1 [v/v]): $R_f = 0.64$. UV (MeOH): $\lambda_{max} = 233, 325$ nm; $\lambda_{min} = 268$ nm.*

Methylate 6-thioinosine derivative

11. Coevaporate the reaction mixture with 50 mL DMF in a 250-mL round-bottom flask and dissolve the residue in 50 mL DMF.

12. Add 24.4 g (33 mmol) ground anhydrous potassium carbonate and 2.07 mL (33 mmol) methyl iodide. Heat the reaction mixture in the oil bath for 2 hr at 50°C with intensive stirring.

Anhydrous potassium carbonate must be ground to a powder before addition to the reaction mixture.

13. Periodically analyze the reaction mixture by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol (**S.4** $R_f = 0.86$).

14. Cool the reaction mixture to room temperature and filter off potassium carbonate by vacuum filtration through a Celite 545 cake on a 60-mL sintered glass funnel. Transfer the filtrate to a 1-L separatory funnel and add 150 mL saturated aqueous sodium bicarbonate solution.

15. Extract the reaction mixture three times with 150 mL dichloromethane. Dry combined organic layers with ~20 g anhydrous sodium sulfate and rotary evaporate with water aspirator.

16. Purify the reaction mixture by short-column chromatography (*UNIT 2.4 & APPENDIX 3E*). Prepare the column (150-mL sintered glass funnel, ~100 g silica gel 60H) in dichloromethane, and form the gradient by increasing the amount of methanol by 0.5% (v/v) for every 100 mL dichloromethane, up to 2.5% methanol. Analyze composition of the fractions (~30 mL) on silica gel 60F TLC plates in 9:1 (v/v) dichloromethane/methanol.

17. Combine the fractions containing product (**S.4**) and rotary evaporate with water aspirator.

*2′,3′,5′-Tri-O-acetyl-6-methylthiopurine riboside (**S.4**): 10.27 g (81% relative to **S.2**). TLC (dichloromethane/methanol 9:1 [v/v]): $R_f = 0.86$. UV (MeOH): $\lambda_{max} = 221, 284$ nm; $\lambda_{min} = 241$ nm. ^{1}H NMR ($CDCl_3$): δ 2.09 (s, 3H, CH_3), 2.13 (s, 3H, CH_3), 2.16 (s, 3H, CH_3), 2.75 (s, 3H, SCH_3), 4.35–4.49 (m, 3H, H4′, H5′, H5″), 5.67 (t, J = 5.6 Hz, 1H, H3′), 5.96 (t, J = 5.4 Hz, 1H, H2′), 6.22 (d, J = 5.4 Hz, 1H, H1′), 8.15 (s, 1H, H2), 8.76 (s, 1H, H8).*

Remove acetyl groups and perform dimethoxytritylation

18. To 10.27 g (24.19 mmol) **S.4** in a 250-mL round-bottom flask, add 40 mL pyridine and 20 mL concentrated aqueous NH_4OH. Close the flask with a rubber septum and leave for 16 hr at room temperature.

19. Analyze reaction mixture by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol (**S.5** $R_f = 0.33$).

20. Rotary evaporate the reaction mixture and coevaporate residue three times with anhydrous pyridine (~50 mL each time).

21. Dissolve the oily residue in 125 mL anhydrous pyridine and add 8.99 g (26.61 mmol) DMTr-Cl. Stir the reaction mixture for 1 to 1.5 hr at room temperature.

22. Periodically analyze reaction mixture by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol (**S.6** $R_f = 0.40$). If reaction is not complete after 1.5 hr, add 5% to 10% (w/w) DMTr-Cl, based on the initial amount added, and stir 1 hr.

23. Carefully add the reaction mixture to 250 mL saturated aqueous sodium bicarbonate solution in a 1-L separatory funnel. Extract three times with 200 mL dichloromethane.

24. Dry combined organic layers with ~20 g anhydrous sodium sulfate and rotary evaporate with water aspirator. Coevaporate residue three times with 50 mL toluene.

25. Purify the reaction mixture by short-column chromatography. Prepare the column (150-mL sintered glass funnel, ~100 g silica gel 60H) in dichloromethane and make the gradient by increasing the amount of methanol by 0.5% (v/v) for every 100 mL dichloromethane, up to 2% methanol.

26. Analyze composition of fractions (~30 mL) by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol. Combine fractions containing product (**S.6**) and rotary evaporate with water aspirator to a solid foam.

*5′-O-(4,4′-Dimethoxytrityl)-6-methylthiopurine riboside (**S.6**): 10.73 g (74%). TLC (dichloromethane/methanol 9:1 [v/v]): $R_f = 0.40$. FAB-MS: m/z = 601.5. UV (MeOH): $\lambda_{max} = 215, 226, 280$ nm; $\lambda_{min} = 220, 256$ nm. ^{1}H NMR ($CDCl_3$): δ 2.64 (s, 3H, SCH_3), 3.31–3.36 (m, 1H, H5′), 3.43–3.48 (m, 1H, H5″), 3.72 (s, 6H, OCH_3), 4.37 (d, J = 3.8 Hz, 1H, H4′), 4.46–4.49 (m, 1H, H3′), 4.85 (t, J = 7.0 Hz, 1H, H2′), 6.06 (d, J = 6.0 Hz, 1H, H1′), 6.73 (d, J = 9.0 Hz, 4H, DMTr), 7.14–7.28 (m, 9H, DMTr), 8.20 (s, 1H, H2), 8.54 (s, 1H, H8). ^{13}C NMR ($CDCl_3$): δ 11.8 (SCH_3), 55.2 (OCH_3), 63.5 (C5′), 72.9 (C3′), 76.1 (C2′), 86.3 (C4′), 91.1 (C1′), 113.2 (DMTr), 126.9 (C5), 127.8, 128.0, 129.1, 129.9, 135.4, 135.5 (DMTr), 140.9 (C8), 144.2 (C4), 151.2 (C2), 158.6 (C6).*

Silylate 2′-OH

27. To a 250-mL round-bottom flask, add 10.56 g (17.59 mmol) **S.6** and coevaporate twice with 50 mL anhydrous pyridine using the water aspirator. Dissolve the residue in 80 mL anhydrous pyridine.

28. Add 3.14 g (45.73 mmol) imidazole and 3.45 g (22.87 mmol) TBDMS-Cl. Stir the reaction mixture 2 hr at room temperature.

29. Periodically analyze reaction mixture by TLC on silica gel 60F plates using 1:1 (v/v) ethyl acetate/hexanes. If reaction is not complete after 2 hr, add 5% to 10% (w/w) TBDMS-Cl and 10% to 20% (w/w) imidazole, based on the original amounts added, and stir 1 hr.

*One should be able to observe the disappearance of substrate (**S.6** $R_f = 0.07$) and appearance of the silylated derivatives. The reaction mixture contains the 2′-silylated isomer (**S.7** $R_f = 0.77$) and 3′-silylated isomer (**S.8** $R_f = 0.58$), as well as the 2′,3′-disilylated derivative ($R_f = 0.95$).*

30. Carefully add the reaction mixture to 250 mL saturated aqueous sodium bicarbonate solution in a 1-L separatory funnel and extract three times with 200 mL dichloromethane.

31. Extract combined organic layers twice with 200 mL of 10% aqueous NaH_2PO_4.

During extraction with aqueous monobasic sodium phosphate, the reaction mixture forms an emulsion with relative ease. Vigorous shaking of the mixture should be avoided.

32. Dry combined organic layers with ~10 g anhydrous sodium sulfate and rotary evaporate. Coevaporate residue three times with ~50 mL toluene.

33. Purify the reaction mixture by short-column chromatography. Prepare the column (150-mL sintered glass funnel, ~100 g silica gel 60H) in dichloromethane and make the gradient by increasing the amount of methanol by 0.25% (v/v) for every 200 mL dichloromethane, up to 0.5% methanol.

    This column chromatography is used to separate the mixture of 2′-silylated and 3′-silylated isomers (which run together) from the 2′,3′-disilylated derivative and other impurities.

34. Analyze the composition of the fractions (~30 mL) by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol. Collect only the fractions containing 2′- and 3′-silylated isomers ($R_f = 0.61$), and rotary evaporate to a solid foam. Dispose of fractions carrying 2′,3′-disilylated derivative ($R_f = 0.81$).

35. To purify the mixture of 2′- and 3′-silylated isomers, prepare a column (150-mL sintered glass funnel, ~100 g silica gel 60H) in toluene and make the gradient by increasing the amount of ethyl acetate by 0.5% (v/v) for every 100 mL toluene, up to 4% ethyl acetate.

36. Analyze composition of the fractions (~30 mL) by TLC on silica gel 60F plates using 1:1 (v/v) ethyl acetate/hexanes. Collect separate fractions for pure 2′-isomer, mixed fractions containing mostly the 2′-isomer, and mixed fractions containing mostly the 3′-isomer.

37. Add ~100 mL methanol to the fraction containing mostly 3′-isomer and leave at room temperature for ~2 days.

    Isomerization of tert-butyldimethylsilyl groups usually takes ~2 days and gives similar amounts of 2′- and 3′-isomers.

38. Rotary evaporate methanol with water aspirator and combine the residue with the mixed fractions containing mostly 2′-isomer. Purify this mixture as described in steps 35 and 36.

    If the synthesis is run on a large scale, a second isomerization of the fraction containing mostly 3′-isomer should be carried out, and the purification should be repeated.

39. Combine the fractions containing pure product, rotary evaporate, and coevaporate three times with ~30 mL benzene in a 250-mL round-bottom flask. Dissolve in ~10 mL benzene per 1 g product.

40. To lyophilize the product, freeze benzene solution in a dry ice/ethanol bath, rotating the flask in such a way that the freezing liquid evenly covers most of the surface of the round-bottom flask. Connect flask with a vacuum adaptor to a vacuum line and apply vacuum for 10 to 16 hr. Be sure that vacuum traps are large enough to condense all benzene and that solid benzene will not clog the vacuum line.

    Lyophilized product is very stable and can be stored safely for many years at 4°C.

    *5′-O-(4,4′-Dimethoxytrityl)-2′-O-tert-butyldimethylsilyl-6-methylthiopurine riboside (**S.7**): 8.29 g (66%). TLC (ethyl acetate/hexanes 1:1 [v/v]): $R_f = 0.77$. ^{1}H NMR ($CDCl_3$): δ −0.20 (s, 3H, $SiCH_3$), −0.04 (s, 3H, $SiCH_3$), 0.76 (s, 9H, t-butyl), 2.67 (s, 3H, SCH_3), 3.30–3.36 (m, 1H, H5′), 3.43–3.48 (m, 1H, H5″), 3.73 (s, 6H, OCH_3), 4.37 (d, J = 3.5 Hz, 1H, H4′), 4.45–4.50 (m, 1H, H3′), 4.85 (t, J = 6.1 Hz, 1H, H2′), 6.06 (d, J = 5.4 Hz, 1H, H1′), 6.73 (d, J = 9.0 Hz, 4H, DMTr), 7.14–7.28 (m, 9H, DMTr), 8.20 (s, 1H, H2), 8.54 (s, 1H, H8). ^{13}C NMR ($CDCl_3$): δ −5.2, −5.0 ($SiCH_3$), 11.7 (SCH_3), 17.8 ($C(CH_3)$), 25.5 ($C(CH_3)_3$), 55.2 (OCH_3), 63.4 (C5′), 71.6 (C3′), 75.6 (C2′), 84.2 (C4′), 88.2 (C1′), 113.2 (DMTr), 126.9 (C5), 127.9, 128.1, 129.1, 130.0, 131.8, 135.6 (DMTr), 140.3 (C8), 144.5 (C4), 152.0 (C2), 158.5 (C6).*

Convert to phosphoramidite

41. Place 1.10 g (1.54 mmol) **S.7** in a 25-mL round-bottom flask closed with rubber septum equipped with a ventilation needle. Dry overnight under vacuum in a desiccator.

42. With 1- and 10-mL glass syringes and needles, add the following:

    7.5 mL anhydrous acetonitrile (<20 ppm water)
    0.40 mL (2.31 mmol) DIPEA
    0.44 g (1.85 mmol, 0.41 mL) 2-cyanoethyl diisopropylchlorophosphoramidite.

    Stir reaction mixture 1 hr at room temperature.

    Because of its high viscosity, the phosphine should be added through the rubber septum into the flask as it sits on a balance to accurately measure the amount added by weight. This is especially important for small-scale syntheses.

43. Analyze reaction mixture by TLC on silica gel 60F plates using 45:45:10 (v/v/v) acetone/hexanes/TEA (**S.9** $R_f = 0.73$) or on silanized silica gel 60F TLC plates using 7:3 (v/v) acetone/water ($R_f = 0.28$). If reaction is not complete after 1 hr, add 10% to 20% (w/w) DIPEA followed by 5% to 10% (w/w) 2-cyanoethyl diisopropylchlorophosphoramidite, based on the original amounts added, and stir 30 min.

    Silica gel TLC plates should be saturated with 45:45:10 (v/v/v) acetone/hexanes/TEA and dried before spotting the reaction mixture.

44. Add the reaction mixture to 50 mL saturated aqueous sodium bicarbonate in a 250-mL separatory funnel and extract three times with 50 mL dichloromethane containing 1% (v/v) TEA.

45. Dry combined organic layers with ~5 g anhydrous sodium sulfate and rotary evaporate with water aspirator to a foam.

46. Purify the reaction mixture by short-column chromatography. Prepare the column (60-mL sintered glass funnel, ~30 g silica gel 60H) in 90:10:1 (v/v/v) hexanes/ethyl acetate/TEA and make the gradient by increasing the amount of ethyl acetate by 5% (v/v) for every 100 mL mixture, up to 30% ethyl acetate. Keep amount of triethylamine constant (1%) during the entire purification.

47. Analyze composition of the fractions (~20 mL) by TLC on silica gel 60F plates in 45:45:10 (v/v/v) acetone/hexanes/TEA. Collect separate fractions containing the phosphoramidite (**S.9**) and evaporate.

48. Lyophilize the product as described in steps 39 and 40.

    Lyophilized product is very stable and can be stored safely for at least 2 years at −20°C.

    *5′-O-(4,4′-Dimethoxytrityl)-2′-O-tert-butyldimethylsilyl-3′-O-[(2-cyanoethoxy)-(N,N-diisopropylamino)]phosphinyl-6-methylthiopurine riboside (**S.9**): 1.20 g (85%). TLC (acetone/hexanes/triethylamine 45:45:10 [v/v/v]): $R_f = 0.73$; on silanized plates (acetone/water 7:3 [v/v]): $R_f = 0.28$. FAB-MS: m/z = 915.2. ^{1}H NMR ($CDCl_3$): δ −0.22 (d, 3H, $SiCH_3$), −0.04 (d, 3H, $SiCH_3$), 0.75 (s, 9H, t-butyl), 1.04–1.21 (m, 12H, 2i-Pr), 2.73 (s, 3H, SCH_3), 3.28–3.35 (m, 1H, H5′), 3.49–3.66 (m, 5H, H5″, CH_2CH_2), 3.78 (s, 6H, OCH_3), 3.85–4.00 (m, 2H, 2CH), 4.34–4.44 (m, 2H, H4′, H3′), 5.08 (t, J = 5.6 Hz, 1H, H2′), 6.01 (d, J = 6.0 Hz, 0.5H, H1′), 6.07 (d, J = 6.0 Hz, 0.5H, H1′), 6.79–6.83 (m, 4H, DMTr), 7.21–7.48 (m, 9H, DMTr), 8.17 (s, 0.5H, H2), 8.20 (s, 0.5H, H2), 8.60 (s, 0.5H, H8), 8.62 (s, 0.5H, H8). ^{13}C NMR ($CDCl_3$): δ −5.2, −4.7 (Si($\underline{C}H_3$)), 11.7 (SCH_3), 17.9 (C($\underline{C}H_3$)), 20.0, 20.4, 24.6, (NC($\underline{C}H_3$)$_2$), 25.6 ($\underline{C}(CH_3)_3$), 42.9, 43.5 (N$\underline{C}$(CH$_3$)$_2$), 55.2 (OCH_3), 57.6, 58.8 ($\underline{C}H_2CH_2O$-), 63.3 (C5′), 72.7 (C3′), 73.4, 74.5 ($CH_2\underline{C}H_2O$-), 75.2 (C2′), 83.9 (C4′), 88.1 (C1′), 113.2 (DMTr), 117.4 ($\underline{C}N$), 126.9 (C5), 127.9, 128.1, 129.1, 130.0, 131.9, 135.6 (DMTr), 140.6 (C8), 144.5 (C4), 151.9 (C2), 158.5 (C6). ^{31}P NMR ($CDCl_3$): δ 149.4, 150.8.*

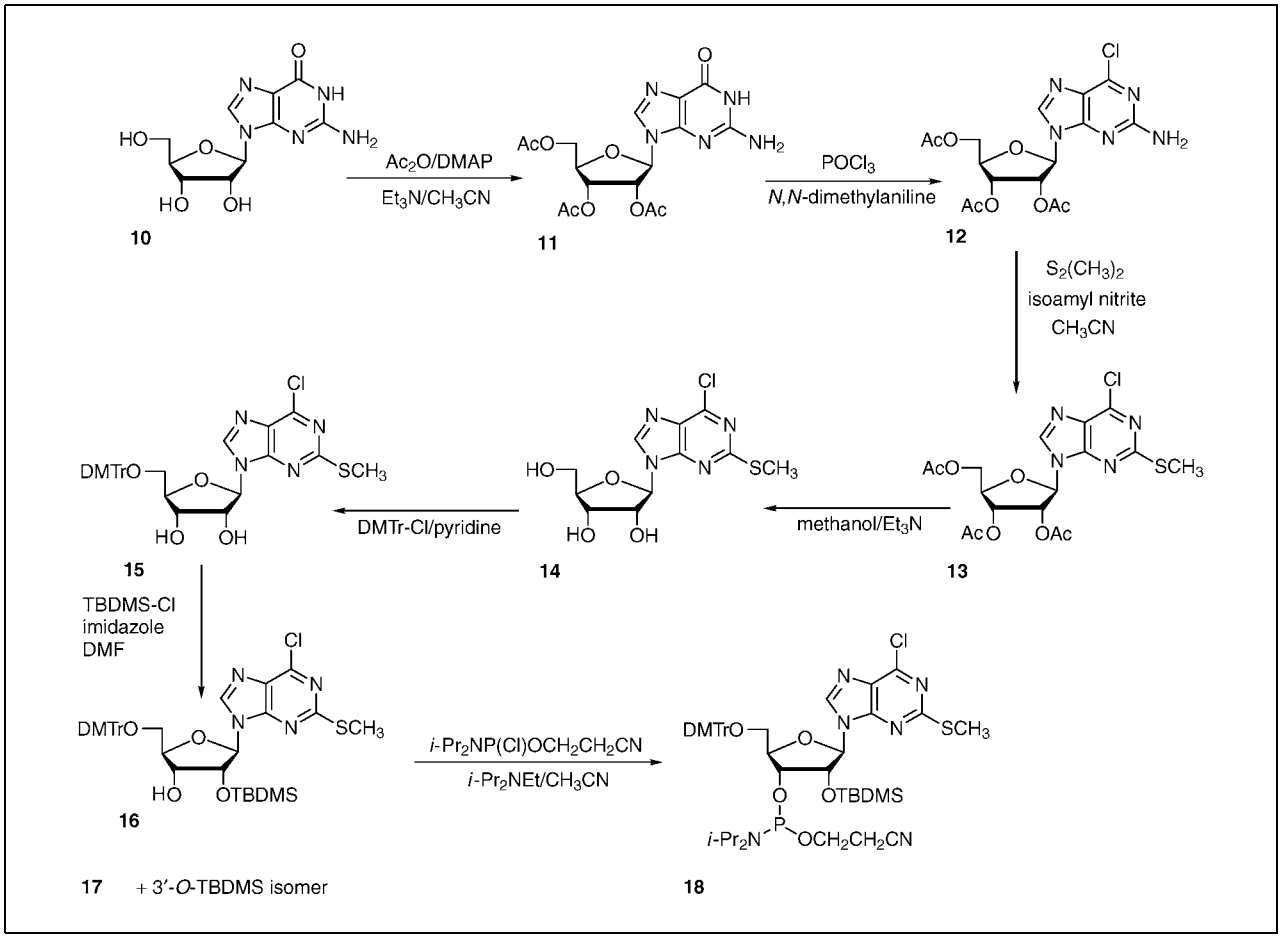

Figure 4.23.2 Acetylation, chlorination, introduction of methylthio group, acetyl deprotection, dimethoxytritylation, silylation, and phosphinylation of purine riboside derivative. Abbreviations: Ac, acetyl; DMAP, 4-dimethylaminopyridine; DMF, *N,N*-dimethylformamide; DMTr, 4,4′-dimethoxytrityl; Et, ethyl; *i*-Pr, isopropyl; TBDMS, *tert*-butyldimethylsilyl.

ALTERNATE PROTOCOL

SYNTHESIS OF THE 2-METHYLTHIO-6-CHLOROPURINE RIBOSIDE PHOSPHORAMIDITE

Guanosine (**S.10**; Fig. 4.23.2) is *O*-peracetylated with acetic anhydride in acetonitrile (Matsuda, 1986) and the resulting 2′,3′,5′-tri-*O*-acetylguanosine (**S.11**) is converted to 2′,3′,5′-tri-*O*-acetyl-2-amino-6-chloropurine riboside (**S.12**) by phosphorus oxychloride treatment (Robins and Uznañski, 1981). The 2-amino group of **S.12** is transformed into 2-methylthio with dimethyl disulfide and isoamyl nitrite in acetonitrile using a 45-min treatment at 45°C (Nair and Fasbender, 1993). After column purification, 2′,3′,5′-tri-*O*-acetyl-2-methylthio-6-chloropurine riboside (**S.13**) is obtained in ~53% yield. Acetyl groups are removed with triethylamine (TEA) in methanol. The product, 2-methylthio-6-chloropurine riboside (**S.14**) is treated with 4,4′-dimethoxytrityl chloride (DMTr-Cl) in pyridine for 2 hr at room temperature. After column chromatography, 5′-*O*-DMTr-2-methylthio-6-chloropurine riboside (**S.15**) is obtained in ~75% yield (Smith et al., 1962). The reaction of **S.15** with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) gives a mixture of 2′- and 3′-isomers (**S.16** and **S.17**; Ogilvie et al., 1978). The 2′-*O*-TBDMS isomer (**S.16**) is separated (~57% yield) and treated with 2-cyanoethyl diisopropylchlorophosphoramidite in anhydrous acetonitrile in the presence of diisopropylethylamine. After purification by silica gel column chromatography, the phosphoramidite **S.18** is obtained in ~84% yield (Beaucage and Caruthers, 1981).

Additional Materials *(also see Basic Protocol 1)*

Guanosine (**S.10**; anhydrous)
4-Dimethylaminopyridine (DMAP)
Phosphorus oxychloride ($POCl_3$), freshly distilled
N,*N*-Dimethylaniline
Dimethyl disulfide
Isoamyl nitrite

500-mL and 1-L round-bottom flasks
140° and 60°C oil baths (silicone oil)
250-μL and 5-mL glass syringes with needles

Acetylate guanosine

1. Add 8.49 g (30 mmol) anhydrous guanosine (**S.10**) and 0.64 g (2.25 mmol) DMAP to 375 mL anhydrous acetonitrile in a 1-L round-bottom flask and begin stirring.

   It is important to use anhydrous guanosine and not the hydrated form.

2. Add 16.7 mL (118 mmol) TEA and 10.2 mL (108 mmol) acetic anhydride and stir 30 min at room temperature.

   After a few minutes, solid guanosine dissolves.

3. Analyze reaction mixture by TLC (*APPENDIX 3D*) on a silica gel 60F TLC plate using 9:1 (v/v) dichloromethane/methanol (**S.11** $R_f = 0.31$).

   The TLC analysis is performed with a short-wavelength UV lamp at 254 nm.

4. Concentrate to ~50 mL on a rotary evaporator equipped with a water aspirator.

5. Add the reaction mixture carefully to 400 mL saturated aqueous sodium bicarbonate solution in a 1-L separatory funnel and extract three times with 150 mL dichloromethane.

6. Dry combined organic layers with ~10 g anhydrous sodium sulfate and rotary evaporate.

   *2′,3′,5′-Tri-O-acetylguanosine (**S.11**): TLC (dichloromethane/methanol 9:1 [v/v]): $R_f = 0.31$.*

Introduce 6-chloro group

7. To dried residue of **S.11** (30 mmol) in a 500-mL round-bottom flask, add 82.5 mL (900 mmol) freshly distilled $POCl_3$ and 5.24 mL (36 mmol) *N*,*N*-dimethylaniline. Close flask with a reflux condenser and place in a 140°C oil bath. Continue heating until 4 min from the moment it starts to reflux.

   CAUTION: *Phosphorous oxychloride can be dangerous and should be handled carefully. It is recommended to run the reaction first on a small scale.*

8. Cool flask in a wet ice bath (~10 min).

9. Remove excess phosphorous oxychloride on the rotary evaporator.

   It is important to clean the rotary evaporator very well with dichloromethane before and after evaporation of phosphorus oxychloride.

10. Add 200 mL dichloromethane to the residue and transfer to a 1-L separatory funnel.

11. Wash four to six times with 500 mL water to remove most of the hydrochloric acid (until pH is ~4). For the final wash, use 500 mL saturated aqueous sodium bicarbonate.

12. Dry the combined organic layers with ~30 g anhydrous sodium sulfate and rotary evaporate with water aspirator.

13. Purify the reaction mixture by short-column chromatography (*UNIT 2.4 & APPENDIX 3E*). Prepare the column (150-mL sintered glass funnel; ~100 g silica gel 60H) in dichloromethane and wash with dichloromethane only.

14. Analyze composition of the fractions (~20 mL) by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol (**S.12** $R_f = 0.71$).

15. Pool fractions containing product and rotary evaporate.

*2′,3′,5′-Tri-O-acetyl-2-amino-6-chloropurine riboside (**S.12**): 9.49 g (74% relative to **S.10**). TLC (dichloromethane/methanol 9:1 [v/v]): $R_f = 0.71$. UV (MeOH): $\lambda_{max} = 224, 247, 310$ nm; $\lambda_{min} = 234, 268$ nm. ^{1}H NMR ($CDCl_3$): δ 2.05 (s, 3H, CH_3), 2.07 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 4.31–4.49 (m, 3H, H4′, H5′, H5″), 5.71 (t, J = 5.6 Hz, 1H, H3′), 5.93 (t, J = 5.6 Hz, 1H, H2′), 5.98 (d, J = 4.8 Hz, 1H, H1′), 7.86 (s, 1H, H8). ^{13}C NMR ($CDCl_3$): δ 20.3, 20.4, 20.6 (C(O)$\underline{C}H_3$), 62.6 (C5′), 70.1 (C3′), 72.9 (C2′), 80.0 (C4′), 87.0 (C1′), 120.3 (C5), 142.2 (C8), 151.1 (C4), 151.9 (C6), 167.3 (C2), 169.2, 169.4, 170.2 ($\underline{C}$(O)CH_3).*

Introduce 2-methylthio group

16. In a 500-mL round-bottom flask, dissolve 7.23 g (16.9 mmol) **S.12** in 140 mL anhydrous acetonitrile.

17. Add 15.2 mL (169 mmol) dimethyl disulfide and 4.53 mL (33.8 mmol) isoamyl nitrite. Heat the reaction mixture in a 60°C oil bath under refluxing condenser for 45 min.

CAUTION: *The reaction must be run in a well-ventilated fume hood.*

18. Periodically analyze the reaction mixture by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol (**S.13** $R_f = 0.64$) or on silanized silica gel 60F TLC plates in 7:3 (v/v) acetone/water ($R_f = 0.70$).

19. Concentrate to ~30 mL on the rotary evaporator with water aspirator. Add the reaction mixture to 300 mL saturated aqueous sodium bicarbonate in a 1-L separatory funnel and extract three times with 100 mL dichloromethane.

20. Dry combined organic layers with ~30 g anhydrous sodium sulfate and rotary evaporate.

21. Purify the reaction mixture by short-column chromatography. Prepare the column (150-mL sintered glass funnel; ~100 g silica gel 60H) in dichloromethane and make the gradient by increasing the amount of methanol by 0.25% (v/v) for every 100 mL mixture, up to 2% methanol.

22. Analyze the composition of the fractions (~30 mL) by TLC on silica gel plates using 9:1 (v/v) dichloromethane/methanol.

23. Collect fractions containing product (**S.13**) and rotary evaporate with water aspirator.

*2′,3′,5′-Tri-O-acetyl-2-methylthio-6-chloropurine riboside (**S.13**): 4.13 g (53%). TLC (dichloromethane/methanol 9:1[v/v]): $R_f = 0.64$; on silanized TLC plates (acetone/water 7:3 [v/v]): $R_f = 0.70$. UV (MeOH): $\lambda_{max} = 233, 263, 306$ nm; $\lambda_{min} = 248, 281$ nm. ^{1}H NMR ($CDCl_3$): δ 2.10 (s, 3H, CH_3), 2.11 (s, 3H, CH_3), 2.15 (s, 3H, CH_3), 2.65 (s, 3H, SCH_3), 4.29–4.48 (m, 3H, H4′, H5′, H5″), 5.65 (t, J = 5.8 Hz, 1H, H3′), 5.99 (t, J = 5.0 Hz, 1H, H2′), 6.12 (d, J = 4.5 Hz, 1H, H1′), 8.10 (s, 1H, H8). ^{13}C NMR ($CDCl_3$): δ 14.8 (SCH_3), 20.3, 20.4, 20.6 (C(O)$\underline{C}H_3$), 62.7 (C5′), 70.1 (C3′), 72.9 (C2′), 80.0 (C4′), 87.0 (C1′), 120.3 (C5), 142.1 (C8), 151.2 (C4), 151.9 (C6), 167.3 (C2), 169.2, 169.4, 170.2 ($\underline{C}$(O)CH_3).*

Remove acetyl groups and perform dimethoxytritylation

24. To 7.96 g (17.34 mmol) **S.13** in 250-mL round-bottom flask, add 140 mL of 9:1 (v/v) methanol/TEA and leave for ~48 hr at room temperature.

25. Periodically analyze reaction mixture by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol (**S.14** $R_f = 0.17$).

26. When the deprotection of acetyl groups is completed, evaporate reaction mixture on the rotary evaporator and coevaporate the residue three times with ~30 mL anhydrous pyridine.

27. Dissolve the residue in 75 mL anhydrous pyridine and add 6.45 g (19.07 mmol) DMTr-Cl. Stir reaction mixture for 1 to 1.5 hr at room temperature.

28. Periodically analyze reaction mixture by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol (**S.15** $R_f = 0.59$). If reaction is not complete after 1.5 hr, add 5% to 10% (w/w) DMTr-Cl, based on initial amount added, and stir 1 hr.

29. Add the reaction mixture to 350 mL saturated aqueous sodium bicarbonate solution in a 1-L separatory funnel. Extract three times with 250 mL dichloromethane.

30. Dry combined organic layers with ~30 g anhydrous sodium sulfate and rotary evaporate with water aspirator. Coevaporate the residue three times with ~50 mL toluene.

31. Purify the reaction mixture by short-column chromatography. Prepare the column (150-mL sintered glass funnel; ~100 g silica gel 60H) in dichloromethane and make the gradient by increasing the amount of methanol by 0.25% (v/v) for every 100 mL dichloromethane, up to 3% methanol.

32. Analyze composition of the fractions (~30 mL) by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol. Combine the fractions containing product (**S.15**) and rotary evaporate to a solid foam.

*5′-O-(4,4′-Dimethoxytrityl)-2-methylthio-6-chloropurine riboside (**S.15**): 8.26 g (75%). TLC (dichloromethane/methanol 9:1 [v/v]): $R_f = 0.59$. FAB-MS: m/z = 635.3. ^{1}H NMR ($CDCl_3$): δ 2.49 (s, 3H, SCH_3), 3.37–3.40 (m, 2H, H5′, H5″), 3.74 (s, 6H, OCH_3), 4.34 (q, J = 3.2 Hz, 1H, H4′), 4.46–4.49 (m, 1H, H3′), 4.85 (t, J = 5.9 Hz, 1H, H2′), 6.01 (d, J = 5.4 Hz, 1H, H1′), 6.74 (d, J = 9.0 Hz, 4H, DMTr), 7.17–7.24 (m, 9H, DMTr), 8.14 (s, 1H, H8). ^{13}C NMR ($CDCl_3$): δ 14.8 (SCH_3), 55.2 (OCH_3), 63.5 (C5′), 72.4 (C3′), 75.6 (C2′), 81.4 (C4′), 86.8 (C1′), 113.2 (DMTr), 127.1 (C5), 127.8, 129.1, 129.9, 135.3, 139.5 (DMTr), 142.4 (C8), 144.1 (C4), 147.3 (C6), 158.6 (C2).*

Silylate 2′-OH

33. To a 250-mL round-bottom flask, add 5.44 g (8.57 mmol) **S.15** and coevaporate with 30 mL anhydrous DMF.

34. Add 70 mL anhydrous DMF followed by 1.50 g (21.44 mmol) imidazole and 1.54 g (10.28 mmol) TBDMS-Cl. Stir the reaction mixture 5 hr at room temperature.

35. Periodically analyze reaction mixture by TLC on silica gel 60F plates using 4:6 (v/v) ethyl acetate/hexanes. If reaction is not complete after 5 hr, add 5% to 10% (w/w) TBDMS-Cl and 10% to 20% (w/w) imidazole, based on the original amounts added, and stir 2 hr.

*Silylated derivatives should appear in the reaction mixture. After reaction is complete, the mixture is composed of the 2′-silylated isomer (**S.16** $R_f = 0.70$), the 3′-silylated isomer (**S.17** $R_f = 0.45$), and the 2′,3′-disilylated derivative ($R_f = 0.86$).*

36. Add the reaction mixture to 250 mL saturated aqueous sodium bicarbonate solution in a 1-L separatory funnel and extract three times with 100 mL dichloromethane.

37. Extract combined organic layers twice with 100 mL of 10% aqueous NaH_2PO_4.

During extraction with aqueous monobasic sodium phosphate, the reaction mixture forms an emulsion with relative ease. Vigorous shaking of the mixture should be avoided.

38. Dry combined organic layers with ~20 g anhydrous sodium sulfate and rotary evaporate with water aspirator. Coevaporate residue three times with 30 mL toluene.

39. Purify the reaction mixture by short-column chromatography. Prepare the column (60-mL sintered glass funnel; ~30 g silica gel 60H) in dichloromethane and elute products with dichloromethane.

This column chromatography separates 2′-silylated and 3′-silylated isomers (which run together) from the 2′,3′-disilylated derivative and other impurities.

40. Analyze composition of the fractions (~20 mL) by TLC on silica gel 60F plates using 9:1 (v/v) dichloromethane/methanol. Collect only the fractions containing 2′- and 3′-silylated isomers ($R_f = 0.59$), and rotary evaporate to a solid foam. Dispose of fractions carrying 2′,3′-disilylated derivative ($R_f = 0.82$).

41. Purify the mixture of 2′- and 3′-silylated isomers again. Prepare the chromatography column (60-mL sintered glass funnel; ~30 g silica gel 60H) in toluene and make the gradient by increasing the amount of ethyl acetate by 0.5% (v/v) for every 100 mL toluene, up to 5% ethyl acetate.

42. Analyze composition of the fractions (~30 mL) by TLC on silica gel plates using 4:6 (v/v) ethyl acetate/hexanes. Collect separate fractions for pure 2′-isomer, mixed fractions containing mostly 2′-isomer, and mixed fractions containing mostly 3′-isomer.

43. Add ~70 mL methanol to the fraction containing mostly 3′-isomer and leave at room temperature for ~2 days.

Isomerization usually takes ~2 days and gives equimolar amounts of 2′- and 3′-isomers.

44. Evaporate methanol and combine the residue with the mixed fractions containing mostly 2′-isomer. Purify this mixture as described in steps 41 and 42.

If the synthesis is run on a large scale, a second isomerization of the fraction containing mostly 3′-isomer should be carried out, and the purification should be repeated.

45. Combine the fractions containing product, rotary evaporate, and coevaporate three times with ~30 mL benzene in a 250-mL round-bottom flask. Dissolve in ~10 mL benzene per 1 g product.

46. To lyophilize the product, freeze benzene solution in a dry ice/ethanol bath, rotating the flask in such a way that the freezing liquid evenly covers most of the surface of the round-bottom flask. Connect flask with a vacuum adaptor to a vacuum line and apply vacuum for 10 to 16 hr. Be sure that vacuum traps are large enough to condense all benzene and that solid benzene will not clog the vacuum line.

Lyophilized product is very stable and can be stored safely for many years at 4°C.

*5′-O-(4,4′-Dimethoxytrityl)-2′-O-tert-butyldimethylsilyl-2-methylthio-6-chloropurine riboside (**S.16**): 3.66 g (57%). TLC (ethyl acetate/hexanes 4:6 [v/v]): R_f = 0.70. ^{1}H NMR ($CDCl_3$): δ −0.15 (s, 3H, $SiCH_3$), 0.12 (s, 3H, $SiCH_3$), 0.85 (s, 9H, t-butyl), 2.55 (s, 3H, SCH_3), 3.41–3.52 (m, 2H, H5′, H5″), 3.79 (s, 6H, OCH_3), 4.27 (q, J = 3.5 Hz, 1H, H4′), 4.37 (q, J = 4.2 Hz, 1H, H3′), 4.85 (t, J = 7.2 Hz, 1H, H2′), 6.08 (d, J = 5.4 Hz, 1H, H1′), 6.80 (d, J = 9.0 Hz, 4H, DMTr), 7.22–7.43 (m, 9H, DMTr), 8.20 (s, 1H, H8). ^{13}C NMR ($CDCl_3$): δ −5.1, −4.9 (Si($\underline{C}H_3$)), 14.7 (SCH_3), 17.9 (C($\underline{C}H_3$)), 25.5 ($\underline{C}(CH_3)_3$), 55.2 (OCH_3), 63.4 (C5′), 71.6 (C3′), 75.3 (C2′), 84.3 (C4′), 88.1 (C1′), 113.3 (DMTr), 127.1 (C5), 127.97, 128.02, 130.0, 135.3, 135.4 (DMTr), 142.1 (C8), 150.9 (C4), 158.6 (C6), 166.9 (C2).*

Convert to phosphoramidite

47. Place 0.51 g (0.68 mmol) **S.16** in a 25-mL round-bottom flask closed with rubber septum equipped with a ventilation needle. Dry overnight under vacuum in a desiccator.

48. With 250-μL and 5-mL glass syringes and needles, add the following:

    4.0 mL anhydrous acetonitrile (<20 ppm water)
    0.18 mL (1.02 mmol) DIPEA
    0.19 g (0.82 mmol, 0.18 mL) 2-cyanoethyl diisopropylchlorophosphoramidite.

    Stir reaction mixture 1 hr at room temperature.

    Because of its high viscosity, the phosphine should be added through the rubber septum into the flask as it sits on a balance to accurately measure the amount added by weight. This is especially important for small-scale syntheses.

49. Analyze reaction mixture by TLC on silica gel 60F plates using 45:45:10 (v/v/v) acetone/hexanes/TEA (**S.18** $R_f = 0.72$) or on silanized 60F plates using 8:2 (v/v) acetone/water ($R_f = 0.32$). If reaction is not complete after 1 hr, add 10% to 20% (w/w) DIPEA followed by 5% to 10% (w/w) 2-cyanoethyl diisopropylchlorophosphoramidite, based on original amounts added, and stir for 0.5 hr.

    Silica gel TLC plates should be saturated with 45:45:10 (v/v/v) acetone/hexanes/TEA and dried before spotting the reaction mixture. For silanized plates for this derivative, 8:2 (v/v) acetone/water is preferred.

50. Add the reaction mixture to 50 mL saturated aqueous sodium bicarbonate solution in a 250-mL separatory funnel and extract three times with 50 mL dichloromethane containing 1% (v/v) TEA.

51. Dry combined organic layers with ~5 g anhydrous sodium sulfate and rotary evaporate.

52. Purify the reaction mixture by short-column chromatography. Prepare the column (60-mL sintered glass funnel; ~30 g silica gel 60H) in 90:10:1 (v/v/v) hexanes/ethyl acetate/TEA and make the gradient by increasing the amount of ethyl acetate by 5% (v/v) for every 100 mL mixture, up to 30% ethyl acetate. Keep the amount of TEA constant (1%) during the entire purification.

53. Analyze composition of the fractions by TLC on silica gel 60F plates in 45:45:10 (v/v/v) acetone/hexanes/TEA. Collect fractions containing phosphoramidite (**S.18**) and rotary evaporate.

54. Lyophilize the product as described in steps 45 and 46.

    Lyophilized product is very stable and can be stored safely for at least 2 years at −20°C.

    *5′-O-(4,4′-Dimethoxytrityl)-2′-O-tert-butyldimethylsilyl-3′-O-[(2-cyanoethoxy)-(N,N-diisopropylamino)]phosphinyl-2-methylthio-6-chloropurine riboside (**S.18**): 0.54 g (84%). TLC (acetone/hexanes/triethylamine 45:45:10 [v/v/v]): $R_f = 0.72$; on silanized TLC plates (acetone/water = 8:2 [v/v]): $R_f = 0.32$. FAB-MS: m/z = 949.6. ^{1}H NMR ($CDCl_3$): δ −0.20 (s, 3H, $SiCH_3$), −0.04 (s, 3H, $SiCH_3$), 0.78 (s, 9H, t-butyl), 1.16–1.22 (m, 12H, 2i-Pr), 2.55 (s, 3H, SCH_3), 3.33–3.40 (m, 1H, H5′), 3.45–3.50 (m, 1H, H5″), 3.58–3.70 (m, 4H, -CH_2CH_2-), 3.78 (s, 3H, OCH_3), 3.79 (s, 3H, OCH_3), 3.84–4.02 (m, 2H, 2CH), 4.33–4.41 (m, 2H, H4′, H3′), 4.87–4.91 (m, 1H, H2′), 6.08 (d, J = 6.0 Hz, 0.5H, H1′), 6.11 (d, J = 6.0 Hz, 0.5H, H1′), 6.80–6.84 (m, 4H, DMTr), 7.22–7.45 (m, 9H, DMTr), 8.22 (s, 1H, H8). ^{13}C NMR ($CDCl_3$): δ −5.1, −4.7 (Si($\underline{C}H_3$)), 14.6 (SCH_3), 17.9 (C($\underline{C}H_3$)), 20.37, 20.45, 24.6 (NC($\underline{C}H_3$)$_2$), 25.5 ($\underline{C}$(CH$_3$)$_3$), 42.9, 43.5 (N$\underline{C}$(CH$_3$)$_2$), 55.2 (OCH_3), 57.5, 58.7 ($\underline{C}H_2CH_2O$-), 63.4 (C5′), 72.8 (C3′), 73.6, 75.3 ($CH_2\underline{C}H_2O$-), 76.1 (C2′), 83.9 (C4′), 87.7 (C1′), 113.3 (DMTr), 117.4 ($\underline{C}N$), 127.1 (C5), 128.0, 128.1, 130.0, 135.2, 135.5 (DMTr), 142.2 (C8), 150.8 (C4), 158.7 (C6), 166.7 (C2). ^{31}P NMR ($CDCl_3$): δ 149.7, 150.7.*

SYNTHESIS OF OLIGORIBONUCLEOTIDES CONTAINING N^6-ALKYLADENOSINE OR 2-METHYLTHIO-N^6-ALKYLADENOSINE

The procedure starts with chemical synthesis of a precursor oligoribonucleotide carrying the 6-methylthiopurine riboside (**S.19**; Fig. 4.23.3) or 2-methylthio-6-chloropurine riboside (**S.23**; Fig. 4.23.4), which is then postsynthetically converted into N^6-alkyladenosine (**S.22**) or 2-methylthio-N^6-alkyladenosine (**S.24**), respectively. The synthesis was performed on an Applied Biosystems 392 synthesizer but could be done on any commercially available synthesizer (see *APPENDIX 3C* for additional details on automated synthesis). Commercially available 5′-*O*-DMTr-2′-*O*-TBDMS-3′-*O*-[(2-cyanoethoxy)-(*N,N*-diisopropylamino)]phosphinyl derivatives of uridine and *N*-protected cytidine, adenosine, and guanosine are used in addition to the modified precursor phosphoramidite **S.9** or **S.18**. In both cases, the coupling yield of modified phosphoramidite based on the trityl assay (*APPENDIX 3C*) is comparable to that of standard commercial 2′-*O*-silylated phosphoramidites.

When the synthesis is completed (with DMTr-OFF), the solid support carrying the precursor oligoribonucleotide **S.19** is oxidized with a 20 mM solution of magnesium monoperoxyphtalate in 9:1 (v/v) dioxane/water for 2.5 hr at room temperature to give a mixture of sulfoxide (**S.20**) and sulfone (**S.21**) derivatives of the precursor oligoribonucleotide. Under these conditions, oxidation of the thiomethyl group does

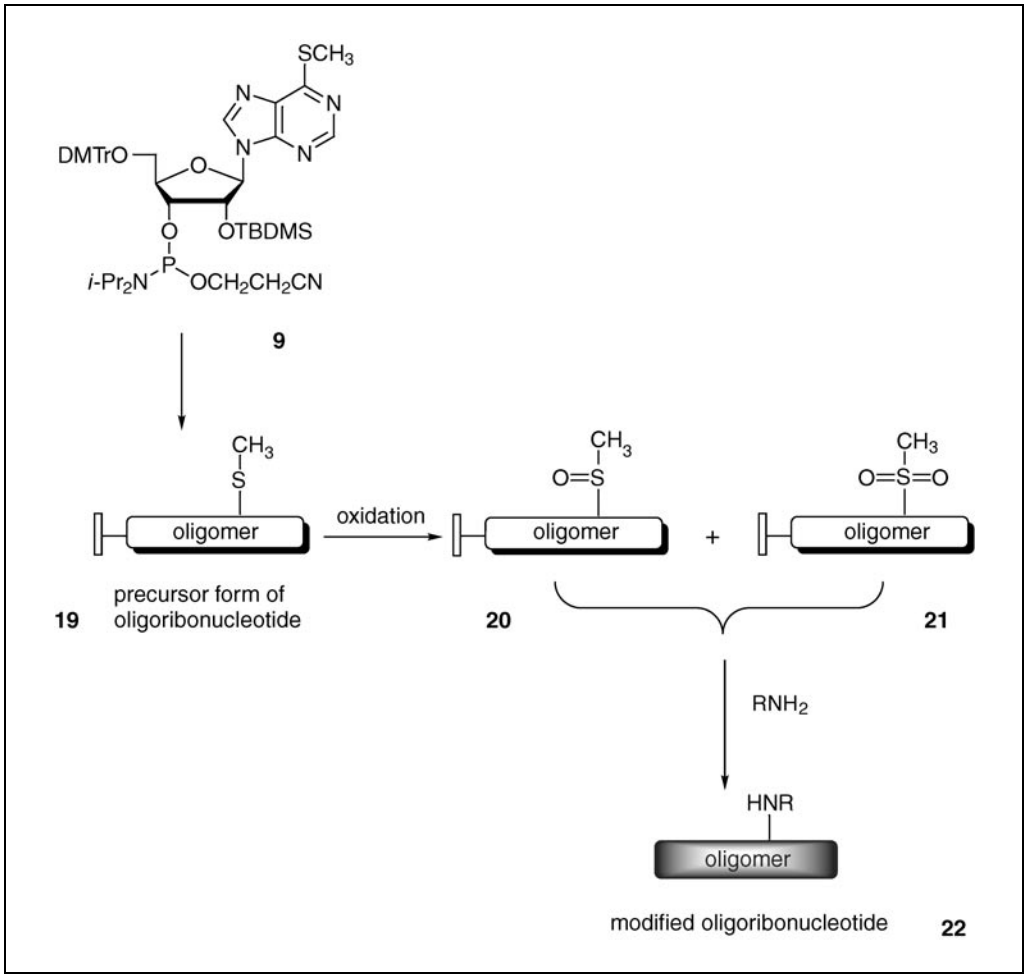

Figure 4.23.3 Oxidation and aminolysis of precursor oligoribonucleotide carrying 6-methylthiopurine riboside to give the modified oligoribonucleotide containing N^6-alkyladenosine. Abbreviations: DMTr, 4,4′-dimethoxytrityl; *i*-Pr, isopropyl; R, alkyl chain of primary amine; TBDMS, *tert*-butyldimethylsilyl.

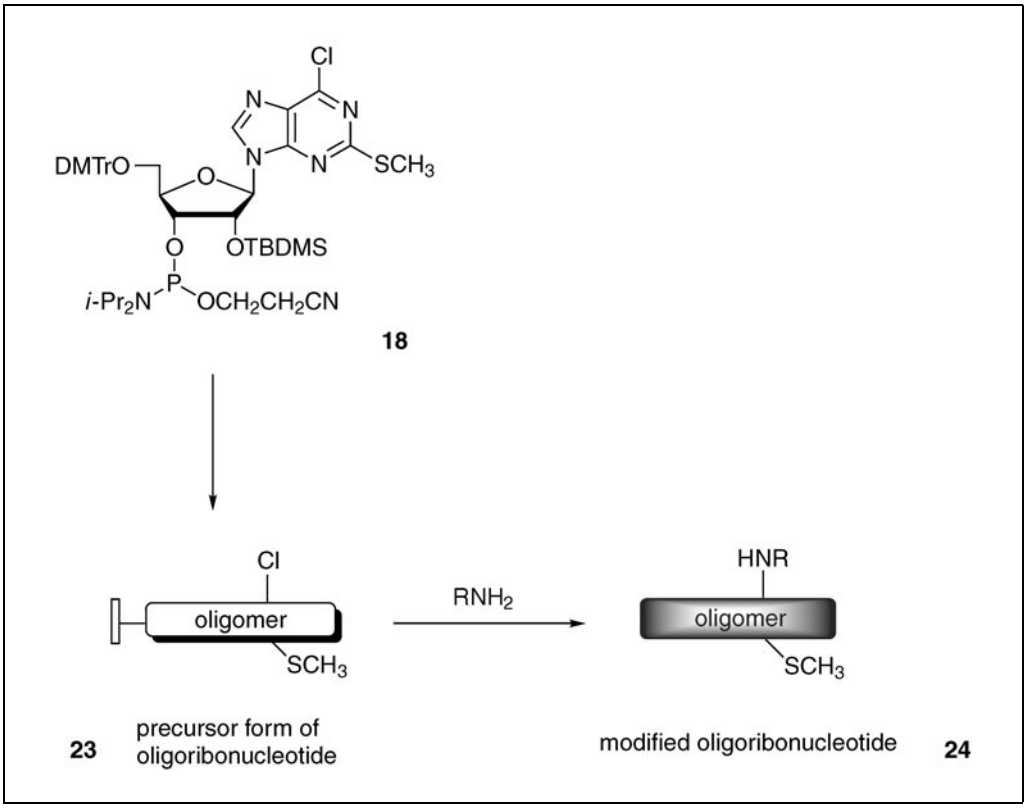

Figure 4.23.4 Aminolysis of precursor oligoribonucleotide carrying 2-methylthio-6-chloropurine riboside to give the modified oligoribonucleotide containing 2-methylthio-N^6-alkyladenosine. Abbreviations: DMTr, 4,4′-dimethoxytrityl; *i*-Pr, isopropyl; R, alkyl chain of primary amine; TBDMS, *tert*-butyldimethylsilyl.

not cause any side reactions (e.g., modification of native nucleobases). The support is washed with 9:1 (v/v) dioxane/water followed by acetonitrile and is then dried. The oxidation procedure is not required for the solid support carrying the precursor oligoribonucleotide **S.23**.

Next, the solid support (with **S.20/S.21** or **S.23**) is treated with the appropriate primary amine in acetonitrile. After amine treatment, the samples are evaporated, and a mixture of 3:1 (v/v) aqueous ammonia/ethanol is used (8 hr at 55°C) to deprotect and cleave the modified oligomer from the support. The support is filtered off and the solution dried down. The residue is coevaporated with anhydrous pyridine and treated with 1 M triethylammonium fluoride in pyridine for 48 hr at 55°C. The reaction mixture is evaporated and desalted on a Sep-Pak cartridge, and modified oligonucleotides are purified by TLC (*APPENDIX 3D*), high-performance liquid chromatography (HPLC; *UNIT 10.5*), or polyacrylamide gel electrophoresis (PAGE; *UNIT 10.4*).

Materials

Commercial phosphoramidites: 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-*tert*-butyldimethylsilyl-3′-*O*-[(2-cyanoethoxy)-(*N,N*-diisopropylamino)]phosphinyl uridine and *N*-protected cytidine, adenosine, and guanosine

Modified phosphoramidite: 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-*tert*-butyldimethylsilyl-3′-*O*-[(2-cyanoethoxy)-(*N,N*-diisopropyl)]phosphinyl-6-methylthiopurine riboside (**S.9**; see Basic Protocol 1) or -2-thiomethyl-6-chloropurine riboside (**S.18**; see Alternate Protocol)

Acetonitrile, anhydrous

Magnesium monoperoxyphthalate hexahydrate (for oligomers containing **S.9**)

Dioxane (for oligomers containing **S.9**)
Primary amine: e.g., isopentenylamine hydrochloride and triethylamine (TEA; for i^6A or ms^2i^6A) *or* methylamine (for m^6A or ms^2m^6A)
Pyridine (reagent grade or better)
2 M methylamine in tetrahydrofuran (for m^6A or ms^2m^6A)
Concentrated aqueous ammonia (28% to 32%)
Ethanol
1 M triethylammonium fluoride in pyridine (or other desilylating agent)
10 mM ammonium acetate

Automated oligonucleotide synthesizer (Applied Biosystems 392)
1.5- and 5-mL screw-top tubes
15-mL sintered glass funnels
Water aspirator
55°C water bath
Speedvac evaporator (Savant)
Spin filters
Sep-Pak cartridges (Waters)
10-mL disposable syringes

Additional reagents and equipment for automated oligoribonucleotide synthesis (*APPENDIX 3C*)

Synthesize desired oligoribonucleotide

1. Synthesize precursor oligoribonucleotide (**S.19** or **S.23**) on a 1-μmol scale in DMTr-OFF mode on an automated oligonucleotide synthesizer using the protocol provided by the manufacturer (also see *APPENDIX 3C*). Use phosphoramidites (commercial and modified) at 0.1 M in anhydrous acetonitrile. To achieve a 0.1 M solution of modified phosphoramidite, dissolve 0.25 g **S.9** in 2.73 mL acetonitrile or 0.25 g **S.18** in 2.64 mL acetonitrile.

Oxidize thiomethyl group (for S.19 only)

2. Transfer the oligoribonucleotide-support from the synthesis column to a 1.5-mL screw-top microcentrifuge tube.
3. Dissolve 98.8 mg (0.2 mmol) magnesium monoperoxyphthalate hexahydrate in 10 mL of 9:1 (v/v) dioxane/water. Add 1 mL of this solution to the solid support and leave for 2.5 hr at room temperature with occasional shaking.
4. Filter off the oxidizing solution using 15-mL sintered glass funnel.
5. Wash the solid support three times with 5 mL of 9:1 (v/v) dioxane/water and three times with 5 mL anhydrous acetonitrile. Dry support with a water aspirator.

Treat with primary amine

6. Transfer oligoribonucleotide-support (mixture of **S.20** and **S.21** from step 5 *or* **S.23** from step 1) to a 5-mL screw-top tube.

    a. For general procedure, add 0.45 mL anhydrous acetonitrile and 0.05 mL of the appropriate primary amine. Seal tube tightly and leave for 12 hr at room temperature with shaking.
    b. For N^6-isopentenyladenosine or 2-methylthio-N^6-isopentenyladenosine (**S.22** or **S.24**; R = isopentenyl), use 25 mg (0.21 mmol) isopentenylamine hydrochloride in 0.45 mL anhydrous pyridine and 0.05 mL (0.42 mmol) TEA, and incubate 12 hr at 55°C with shaking for **S.22** or 2 to 5 hr at 55°C for **S.24**.

c. For N^6-methyladenosine or 2-methylthio-N^6-methyladenosine (**S.22** or **S.24**; R = methyl) use 0.5 mL of 2 M methylamine in tetrahydrofuran and incubate 12 hr at 55°C with shaking for **S.22** or 2 to 5 hr at 55°C for **S.24**.

*To convert precursor oligoribonucleotides into oligomers containing N^6-neopentylyladenosine, N^6-(1-methylpropyl)adenosine, N^6-(1-methylbutyl)adenosine, N^6-isopentyladenosine, N^6-propargyladenosine, and 2-methylthio-N^6-isopentyladenosine, the following primary amines are used: neopentylamine (2,2-dimethylpropylamine), 1-methylpropylamine (sec-butylamine), 1-methylbutylamine, isoamylamine (3-methylbutylamine), propargylamine, and isoamylamine (3-methylbutylamine), respectively. When synthesizing these or any other modified adenosines, conversion yields will be affected by the choice and concentration of the primary amine used, as well as the temperature and time of the substitution reaction. When **S.19** is used, the oxidative activation of the SCH_3 is also critically important (Kierzek and Kierzek, 2003a).*

7. Cool the reaction mixture at −20°C for 5 min, carefully open the sealed screw-top tube, and evaporate to dryness in a Speedvac evaporator.

Deprotect and cleave oligomer from support

8. Add 0.8 mL of 3:1 (v/v) concentrated aqueous ammonia/ethanol and leave tightly sealed for 8 hr at 55°C.

9. Cool at −20°C for 5 min and filter off the solid support using a spin filter. Wash the support twice with 1 mL water.

10. Transfer combined filtrates to a 5-mL screw-top tube and evaporate to dryness in the Speedvac evaporator.

Desilylate oligomer

11. Coevaporate the residue with 0.5 mL anhydrous pyridine in the Speedvac evaporator.

12. Add 0.5 mL of 1 M triethylammonium fluoride in pyridine, close tightly, and leave for 48 hr at 55°C.

Removal of tert-butyldimethylsilyl groups can also be performed with tetra-n-butylammonium fluoride or triethylamine trihydrofluoride according to standard procedures.

13. Evaporate pyridine in the Speedvac evaporator.

Desalt product

14. Connect a Sep-Pak cartridge to the end of a 10-mL disposable syringe. Use the syringe to pass 10 mL acetonitrile followed by 10 mL of 10 mM ammonium acetate through the cartridge.

The reaction mixture can also be desalted with a Sephadex G-10 or G-25 column (Amersham Biosciences).

15. Dissolve the oligonucleotide residue in 10 mL of 10 mM ammonium acetate and load on the cartridge. Wash column with 10 mL of 10 mM ammonium acetate.

16. Elute crude mixture of modified oligoribonucleotide with 5 mL of 3:7 (v/v) acetonitrile/water and evaporate with the Speedvac evaporator.

17. Purify the modified oligoribonucleotide using any standard method, such as TLC (*APPENDIX 3D*), HPLC (*UNIT 10.5*), or PAGE (*UNIT 10.4*).

*After purification, from a 1-μmol-scale synthesis, ~10 to 30 OD_{260} units of modified oligoribonucleotide containing N^6-alkyladenosine (**S.22**) or 2-methylthio-N^6-alkyladenosine (**S.24**) are obtained (Table 4.23.1).*

Table 4.23.1 Retention Times, Synthesis Yields, and Spectrometric Mass Analysis of Oligoribonucleotides Obtained by Postsynthetic Modification

	N^6-Alkyladenosine ($\mathbf{A} = R^6A$)			2-Methylthio-N^6-alkyladenosine ($\mathbf{A} = ms^2R^6A$)		
R^6	Retention time (min)[a]	Yield (%)[b]	Mass[c]	Retention time (min)[a]	Yield (%)[b]	Mass[c]
	UACAUGUA			UACAUGUA		
H	24.0	64	2494.99	26.9	49	2540.91
Methyl	24.2	40	2508.93	30.0	35	2554.79
Neopentyl	33.8	47	2564.93			
1-Methylpropyl	30.4	42	2550.90			
1-Methylbutyl	34.3	61	2564.95			
Isopentyl	34.6	52	2564.78	44.7	24	2608.97
Isopentenyl	33.0	41	2563.75			
Propargyl	25.8	40	2532.64			
	UACAUGUA			UACAUGUA		
H	24.0	64	2494.99	25.3	48	2541.02
Methyl	24.5	47	2508.92	27.9	64	2555.04
Neopentyl	32.6	51	2564.87			
1-Methylpropyl	29.1	72	2550.88			
1-Methylbutyl	33.2	71	2565.01			
Isopentyl	33.2	45	2564.94	44.7	41	2608.93
Isopentenyl	31.4	56	2562.92			
Propargyl	25.2	64	2532.98			
	UACAUGUA			UACAUGUA		
H	24.0	64	2494.99	27.2	27	2541.01
Methyl	25.2	51	2508.61	31.0	54	2555.00
Neopentyl	35.1	58	2564.90			
1-Methylpropyl	31.6	77	2551.05			
1-Methylbutyl	35.9	51	2564.84			
Isopentyl	36.3	51	2564.78	48.8	65	2608.57
Isopentenyl	34.3	25	2563.30			
Propargyl	26.7	49	2532.81			
	ACAUGUA			ACAUGUA		
H	26.0	74	2188.35	26.3	35	2234.65
Methyl	27.1	49	2202.32	31.1	53	2249.03
Neopentyl	38.5	69	2258.49			
1-Methylpropyl	34.6	75	2244.46			
1-Methylbutyl	39.2	91	2258.52			
Isopentyl	39.7	55	2258.47	53.6	20	2302.92
Isopentenyl	38.0	65	2256.11			
Propargyl	26.0	42	2226.46			

continued

Table 4.23.1 Retention Times, Synthesis Yields, and Spectrometric Mass Analysis of Oligoribonucleotides Obtained by Postsynthetic Modification, *continued*

	N^6-Alkyladenosine ($\mathbf{A} = R^6A$)			2-Methylthio-N^6-alkyladenosine ($\mathbf{A} = ms^2R^6A$)		
R^6	Retention time (min)[a]	Yield (%)[b]	Mass[c]	Retention time (min)[a]	Yield (%)[b]	Mass[c]
				CCGGUCmUCCA**A**AACCGG		
H				29.9	16	5445.18
Methyl				32.9	24	5459.53

[a]HPLC: Supelco RP C18; eluent A = 100 mM triethylammonium acetate; eluent B = 1:1 (v/v) acetonitrile/A; gradient = 1% B/min.

[b]Overall yield refers to precursor oligomer synthesis, postsynthetic modification, and deprotection and is based on C18 HPLC analysis.

[c]Molecular weights measured by atmospheric pressure ionization–electrospray liquid chromatography/mass spectroscopy (API-ES LC-MS) in the negative ionization mode.

COMMENTARY

Background Information

There are 96 different natural modifications of nucleotides occurring in all types of RNA (Limbach et al., 1994; Bjork, 1995). Transfer RNAs are particularly rich in modified nucleotides, with 80 different modified nucleotides known. The modifications are mostly present in single-stranded regions of tRNA such as hairpin and multibranch loops. Positions 34 and 37 of the anticodon arm are particularly highly modified. The functions of modified nucleotides are not clearly defined, but it is known that they affect structure and biological activities of RNA, influence the secondary and tertiary interactions of tRNA, change hydrogen bond and stacking interactions as well as puckering of ribose residues, and influence binding of divalent cations (Persson, 1993; Grosjean et al., 1998).

Among 19 modifications of adenosine, 15 have either a substitution on the N^6-exocyclic amine group or a 2-methylthio group with a substitution on the N^6-exocyclic amine group at the same time. Examples include N^6-threonylcarbamoyladenosine (t^6A), N^6-isopentenyladenosine (i^6A), N^6-methyladenosine (m^6A), 2-methylthio-N^6-isopentenyladenosine (ms^2i^6A), and 2-methylthio-N^6-methyladenosine (ms^2m^6A). Because these residues are present at position 37 of the tRNA anticodon loop, studies of their biological function and of their influence on RNA structure are most important. The syntheses of oligoribonucleotides containing the last four modifications are performed by the methods described in this unit.

Studies of modified RNA activities are significantly limited by access to modified oligoribonucleotides. In the literature, syntheses of RNA carrying simple modifications are well described. Introduction of nucleosides bearing complex modifications is still challenging, however. In 1978, Wiewiórowski and co-workers described the first chemical synthesis of an anticodon loop heptamer containing t^6A (Adamiak et al., 1978). Recently, several chemical syntheses of anticodon loops and arms containing t^6A were published (Sochacka, 1999; Boudou et al., 2000; Stuart et al., 2000; Sundaram et al., 2000).

The most important improvement in the synthesis of oligoribonucleotides containing N^6-alkyladenosines and 2-methylthio-N^6-alkyladenosines is the application of a postsynthetic approach to introducing the modification, which is the approach described in this unit. This allows very simple simultaneous synthesis of several modified oligoribonucleotides from only one precursor oligoribonucleotide. The first crucial element of this approach is selection and development of the efficient synthesis of the modified phosphoramidites **S.9** and **S.18**. The second crucial element is setting conditions for postsynthetic modification of the precursor form of the oligoribonucleotides to obtain a very high yield of modification and at the same time keep elements of the oligoribonucleotides intact during the entire modification process.

The longest modified oligoribonucleotides that have been obtained by the described

postsynthetic modification of precursor oligonucleotides are octadecamers, which correspond to the length of the anticodon arm of tRNA. Because of the high yield of transformation of the precursor oligonucleotides, however, the final length of the modified oligoribonucleotides should be limited only by the general reliability of chemical RNA synthesis and purification methods. This high yield also should allow the introduction of several N^6-alkyladenosines and/or 2-methylthio-N^6-alkyladenosines into an oligoribonucleotide, if the N^6-alkyl substituent is the same.

Critical Parameters

The synthesis of modified phosphoramidites and transformation of precursor oligoribonucleotides into oligonucleotides containing N^6-alkyladenosine and 2-methylthio-N^6-alkyladenosine do not present significant difficulties. Some prior experience in the chemistry of nucleosides and oligonucleotides is nonetheless very useful.

It is important to be very careful during synthesis of 2′,3′,5′-tri-*O*-acetyl-2-amino-6-chloropurine riboside (**S.12**) because the reaction, particularly when performed on a large scale, uses several hundred milliliters of phosphorus oxychloride that has to be refluxed at 140°C for a short period of time only. It is best to perform this reaction first on a small scale for practice. In the next reaction, during synthesis of 2′,3′,5′-tri-*O*-acetyl-2-methylthio-6-chloropurine riboside (**S.13**), 10% to 20% 2′,3′,5′,N^2-tetraacetyl-6-chloropurine riboside is also formed, and this side product must be separated by short-column chromatography.

It is also very important to carefully separate the 2′- and 3′-*O*-TBDMS isomers during synthesis of 5′-*O*-DMTr-2′-*O*-TBDMS ribosides **S.7** and **S.16**. A first prepurification column allows for a relatively easy separation of both isomers during the second silica gel column purification.

The oxidation of the 6-methylthio group with magnesium monoperoxyphthalate in Basic Protocol 2 must be complete, because only the oxidation products (6-methylsulfone and 6-methylsulfoxide derivatives) can be converted into N^6-alkylated adenosine derivatives by treatment with the appropriate amine. Experiments carried out by the authors with protected 6-methylthiopurine riboside in this oxidation solution demonstrate that the oxidation of the 6-methylthio group was completed within 1 hr at room temperature. A longer incubation time is used for the precursor oligoribonucleotides (2.5 hr at room temperature) because of the heterogeneous conditions of oxidation and because no side products of oxidation have been observed.

During modification of the precursor form of the oligoribonucleotide, some problems may arise with transformation into N^6-isopentenyladenosine and 2-methylthio-N^6-isopentenyladenosine oligonucleotides (**S.22** and **S.24**, respectively; R = isopentenyl). This is due to the low solubility of isopentenylamine hydrochloride in acetonitrile or even in pyridine. This makes the deprotection process inconvenient. This salt is removed using a Sep-Pak cartridge before purification, however.

Anticipated Results

Synthesis of precursor phosphoramidites is not trivial but can be achieved by someone with basic experience in organic chemistry. The yield of particular steps of the synthesis ranges between 50% and 100%. Some reactions have high yield and no side products, which allows subsequent reaction without any purification of the intermediate. Precursor oligoribonucleotides are synthesized on an Applied Biosystems 392 synthesizer using a standard procedure of RNA synthesis. On the basis of HPLC analysis of the crude reaction mixture, the transformation of precursor oligoribonucleotide into oligoribonucleotide containing N^6-alkyladenosine or 2-methylthio-N^6-alkyladenosine proceeds with an 80% to 90% yield. The efficiency of transformation is dependent on structure (mostly a steric hindrance effect of the alkyl chain) and nucleophilicity of the primary amine. The modified oligoribonucleotides are purified from the entire crude reaction mixture by TLC or HPLC with a 20% to 60% overall yield (Table 4.23.1).

Time Considerations

Synthesis of modified phosphoramidites (**S.9** and **S.18**) takes ~2 to 3 weeks for each precursor. The transformation of precursor oligoribonucleotides (**S.19** and **S.23**) and purification of modified oligoribonucleotides (**S.22** and **S.24**) requires ~1 week.

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