Vibration Ball Milling for the Synthesis of 5'-Thioadenosine 5'-Pyrophosphate (P'→5') Adenosine (dASppA)

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Application of Vibration Ball Milling to the Synthesis of 5′-Thioadenosine 5′-Pyrophosphate (P′→5′) Adenosine (dASppA)

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Abstract:	Using vibration ball milling, 5′-chloro-5′-deoxyadenosine (ClдA) reacts cleanly with 4-methoxybenzyl mercaptan (MobSH) under basic conditions, to the corresponding thioether (MobSдA) which is isolated following precipitation and trituration. Under acidic conditions, in a one-pot, two-step process, MobSдA is transformed into 5′-deoxy-5′-(5-nitropyridyl-2-disulfanyl)-adenosine (NPySSдA). Michaelis-Arbuzov (M-A) reaction of NPySSдA with tris(trimethylsilyl) phosphite proceeds to completion within 30 minutes by ^{31}P NMR and the persilylated M-A product thus formed can be stored in solution under anhydrous conditions at room temperature for several days (in contrast, the anionic phosphorothiolate monoester is labile to hydrolysis). Following evaporation, mechanochemical mixing of the crude M-A product with the nucleotide donor adenosine 5′-monophosphomorpholidate under acidic activation in the presence of additional water gives rapid hydrolytic desilylation and phosphate coupling so that essentially complete reaction is observed after 90 minutes and dASppA isolated following C-18 reversed phase HPLC and desalting (>99% pure - monitoring at 260 nm).

CURRENT PROTOCOLS
The Fine Art of Experimentation

Application of Vibration Ball Milling to the Synthesis of 5′-Thioadenosine 5′-Pyrophosphate (P′→5′) Adenosine (dASppA)

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Significance Statement Procedures for the preparation of the 5′,5′-pyrophosphorothiolate-linked dinucleoside dASppA over three steps are described. Two of these steps are considerably expedited by the mechanical mixing of reagents and substrates in a vibration ball mill using minimal added solvent. Most significantly, phosphate coupling in solution using phosphoromorpholidates requires extended pre-treatment of (poly)anionic substrates to render them soluble in anhydrous solvents and can take several days to reach completion. Such conditions are incompatible with unstable intermediates such as phosphorothiolate monoesters. By using vibration ball milling (VBM) in the presence of low volumes of water, hydrolytic unmasking of 5′-thioadenosine-5′-*S*-phosphate monoester and coupling with adenosine 5′-monophosphomorpholidate can be achieved within 90 minutes. The crude reaction mixture can be purified using reversed-phase HPLC.

ABSTRACT Using vibration ball milling, 5′-chloro-5′-deoxyadenosine (ClddA) reacts cleanly with 4-methoxybenzyl mercaptan (MobSH) under basic conditions, to the corresponding thioether (MobSdA) which is isolated following precipitation and trituration. Under acidic conditions, in a one-pot, two-step process, MobSdA is transformed into 5′-deoxy-5′-(5-nitropyridyl-2-disulfanyl)-adenosine (NPySSdA). Michaelis-Arbuzov (M-A) reaction of NPySSdA with tris(trimethylsilyl) phosphite proceeds to completion within 30 minutes by ^{31}P NMR and the persilylated M-A product thus formed can be stored in solution under anhydrous conditions at room temperature for several days (in contrast, the anionic phosphorothiolate monoester is labile to hydrolysis). Following evaporation, mechanochemical mixing of the crude M-A product with the nucleotide donor adenosine 5′-monophosphomorpholidate under acidic activation in the presence of additional water gives rapid hydrolytic desilylation and phosphate coupling so that essentially complete reaction is observed after 90 minutes and dASppA isolated following C-18 reversed phase HPLC and desalting (>99% pure - monitoring at 260 nm).

Keywords: ball mill; mechanochemistry; Michaelis-Arbuzov; phosphate coupling; thionucleosides

INTRODUCTION

A previous unit has described the value of nucleoside and nucleotide analogues containing thiol functions (UNIT 1.34) and outlines issues associated with performing oxidation-sensitive reactions of thiols under basic conditions (Reddy et al., 2015). The groups of Reese (Divakar and Reese, 1982; Divakar et al., 1990; Marriott et al., 1990) and Engels (Jahn-Hofmann and Engels, 2004) have described solution-phase chemistry for the preparation of thionucleosides in which the thiol function is masked by an acid-sensitive group (e.g., 4-methoxybenzyl, *t*-butyl, dimethoxytrityl or thiopixyl) and one-pot deprotection-activation as *S*-aryl disulfides of both nucleoside and peptide substrates under acid conditions has been described. (Divakar et al., 1990; Schroll et al., 2012) The application of such disulfides for the preparation of internucleoside phosphorothiolate linkages via Michaelis-Arbuzov chemistry is well established (Vyle et al., 1992; Gaynor and Cosstick, 2011; Li et al., 2011) and was adapted by our group to the preparation of a stable, silylated intermediate (Eguaogie et al., 2016; Eguaogie et al., 2017) from which the target pyrophosphorothiolate-linked dinucleoside analogue was accessed using mechanochemistry. In contrast to solution-phase mixing of reagents and substrates under standard conditions, grinding has only recently been explored in the context of organic chemistry using vibration (Wang, 2013) or planetary (Do and Friščić, 2017) ball milling or single- / twin-screw extrusion (Crawford, 2017). These references are examples of review / prospective articles which also provide some useful discussions on differences in both the scales and applications of the different grinding actions. For this unit, those reactions using ball-milling were performed using a vibration mill in which symmetric oscillation at 30 Hz of two counter-balanced vessels containing balls leads to high energy impact of the ball(s) with materials contained within the vessel. The rate accelerations observed may result from the rapid and intensive mixing of these materials and / or transfer of impact energy to reactive centers.

Specifically, this unit describes, sequentially: synthesis of 5′-*S*-(4-methoxybenzyl)-5′-thioadenosine (**2**) in a vibration ball mill (see Basic Protocol 1); preparation of 5′-deoxy-5′-(5-nitropyridyl-2-disulfanyl)-adenosine (**3**) in solution (see Basic Protocol 2); and synthesis of 5′-thioadenosine 5′-pyrophosphate (P′→5′) adenosine (**5**) over two steps using both solution-phase conditions and vibration ball milling (see Basic Protocol 3).

CAUTION: The vibration ball mill used in this protocol creates significant noise levels over prolonged times and according to local regulations requires housing away from a general work area.

BASIC PROTOCOL 1

SYNTHESIS OF 5′-*S*-(4-METHOXYBENZYL)-5′-THIOADENOSINE (2):

This protocol describes the rapid and clean transformation of 5′-chloro-5′-deoxyadenosine into the target without intramolecular cyclisation (a common side-product of the displacement reaction in solution). The ball mill vessel is charged with the nucleoside analogue substrate and reagents, sealed and vibrated on the mill. The reaction mixture containing pure product is extracted from the vessel, precipitated and the solids triturated.

Materials

Argon
5′-Chloro-5′-deoxyadenosine (**1**) - Carbosynth NC05644
1,1,3,3-Tetramethylguanidine (TMG)
4-Methoxybenzyl mercaptan (MobSH) (4-methoxy-α-toluenethiol)
Acetonitrile
(TLC eluting solvent: 8:2; v/v; $CHCl_3$:EtOH)
10% (w/v) KH_2PO_4 (aq) cooled on ice
2M HCl (aq) cooled on ice
Ice
18.2 MΩ Water (purified by reverse osmosis) cooled on ice
5:1 Diethyl ether (Et_2O) : *n*-hexane cooled on ice

Vacuum desiccator containing phosphorus pentoxide and (separately) KOH as desiccants.
2 x Zirconia-lined vessel (25 ml internal volume; Retsch, 01.462.0201)
1 or 2 x 15 mm zirconia ball (10.70 g; Retsch, 05.368.0094) (N.B. Do NOT include ball in second zirconia vessel if this is only used as a counterbalance).
Argon-filled balloons
Gilson (automatic) pipette and tips (1000 µL)
Retsch Mixer Mill MM 400 (Retsch, 20.745.0001)
(Silica gel TLC plate with fluorescent indicator)
(254-nm UV lamp)
Conical flask (250 mL)
Magnetic stirring bar
Magnetic stirrer
pH paper
Bath Sonicator
Sintered funnels (porosity 4)
Büchner flasks (250mL)
100 mL Conical flask with ground glass joint and stopper

Procedure

1. Dry the zirconia-lined vessels (comprised of a larger (male threaded), lower body, a teflon O-ring and an upper (female threaded) cap) and zirconia ball(s) in a drying oven at 65° C overnight. Transfer to a desiccator and evacuate. After the vessels have attained ambient temperature, release the vacuum using an argon-filled balloon, ensure that the O-ring is securely located in the upper cap and the ball is in the lower body of each vessel to be used to perform the reaction.

2. Under a gentle stream of argon, transfer one 286 mg (1.0 mmol) aliquot of 5′-chloro-5′-deoxyadenosine (**1**) to each vessel body containing a ball.

3. Using an automatic pipette, under a gentle stream of argon, transfer 420 µL (3.0 mmol) of 4-methoxybenzyl mercaptan to the vessel body and then (using a fresh tip) immediately add 630 µL (5.0 mmol) of 1,1,3,3-tetramethylguanidine.

   CAUTION: 4-methoxybenzyl mercaptan has an unpleasant odour. It should be handled strictly within a fume-cupboard wearing appropriate personal protective equipment. Cleaning with concentrated bleach is NOT recommended for the ball mill vessels.

   *Retsch recommends that, for the 25 mL jar described in this protocol, the volume of material should be in the range 4.0 – 10.0 mL. The procedure described here uses less than the recommended minimum but has been reliable in our hands over many repetitions (see also Basic Protocol 3). However, our experience has been that in the complete absence of solids, extended vibration of a zirconia-lined vessel **with a zirconia ball** can damage the lining.*

4. Close each vessel by screwing tight the upper cap and O-ring onto the lower body and move to the location of the ball mill. Secure the vessel in place by tightening the outer metal holder ensuring that both ends of the vessel are fully flush against each holder. Repeat with the second vessel so that the mass is sufficiently balanced on each side of the arm.

   Several visual sources both on the Retsch website and YouTube may prove useful in understanding ball mill use. In particular, we have found that inexperienced users may not secure the vessel in the holder properly or use a counterbalancing jar which includes a ball but no other materials (leading to cracking of the zirconia over several hours of milling).

5. Program the machine so that it will vibrate at 30 Hz for 30 minutes and start.

6. Allow the reaction vessel to cool to room temperature, remove from the ball mill and orientate so that the lower body is at the bottom. Unscrew the vessel.

   *The neat reaction mixture is an homogenous clear, viscous liquid. We have found that this procedure is reproducible but, if desired, a small sample of the reaction mixture can be dissolved in acetonitrile and TLC (8:2; v/v; $CHCl_3$:EtOH) of this solution used to show complete conversion of **1** (Rf = 0.22) into **2** (Rf = 0.33).*

7. Using a Gilson pipette, rinse both parts of the vessel thoroughly with acetonitrile (total volume 10 mL) and transfer the washings to a 250 mL conical flask and add a magnetic stirring bar.

8. Cool this diluted reaction mixture on ice at 0 - 4 °C and stir at a low speed to avoid spillage.

9. Gently poor in 100 mL of the ice-cold aqueous KH_2PO_4 and increase the rate of stirring.

During addition of the aqueous solution, a white amorphous precipitate forms.

10. Adjust the pH of this solution to 3.0-3.5 by adding ice-cold 2M aqueous HCl dropwise whilst still maintaining cooling and stirring.

11. Using a bath sonicator, sonicate the suspension for a minimum of 15 minutes in order to fully break up any solids which have not been properly dispersed by stirring.

12. Cool the suspension on ice with gentle stirring.

13. Filter the suspension through a sintered funnel (porosity 4).

14. In the funnel, triturate the solids thoroughly with ice-cold H_2O (25 mL), filter and repeat.

15. Dry the solids *in vacuo* over P_2O_5 / KOH for at least 3 hours at less than 8 mbar.

16. Transfer the dried solids into a stoppered 100-mL conical flask, add 30 mL of ice-cold 5:1 (v/v) Et_2O : *n*-hexane and sonicate until the solids are fully dispersed throughout the liquid (typically only one minute is sufficient).

17. Filter the suspension through a sintered funnel (porosity 4).

18. In the funnel, triturate the solids thoroughly with ice-cold 30 mL 5:1 (v/v) Et_2O : *n*-hexane.

19. Dry over P_2O_5 / KOH. Characterize the purified compound (**2**) by ^{1}H- and ^{13}C-NMR, and ES-MS.

*5'-S-(4-Methoxybenzyl)-5'-thioadenosine (**MobSdA**) – **2**. White solid. Yield: 0.323 g (80%) Rf (SiO_2) = 0.33 (8:2; v/v; $CHCl_3$/EtOH).*
^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.34 (1H, s, H8), 8.14 (1H, s, H2), 7.30 (2H, s, NH_2), 7.13 (2H, d, $^{3}J_{HH}$ = 8.5 Hz, ArH, m- to OMe), 6.81 (2H, d, $^{3}J_{HH}$ = 8.3 Hz, ArH, o to OMe), 5.89 (1H, d, $^{3}J_{HH}$ = 5.3 Hz, H1'), 5.50 (1H, d, $^{3}J_{HH}$ = 5.8 Hz, 2'OH), 5.30 (1H, d, $^{3}J_{HH}$ = 5.0 Hz, 3'OH), 4.70-4.78 (1H, m, H2'), 4.15-4.20 (1H, m, H3'), 3.98-4.05 (1H, m, H4'), 3.71 (3H, s, OCH_3), 3.67 (2H, s, $ArCH_2$), 2.75-2.85 and 2.55-2.65 (2H, 2 x m, H5', H5'');
^{13}C NMR (101 MHz, DMSO-d_6) δ = 158.09, 156.07, 152.63, 149.40, 139.93, 130.11, 129.94 (2C), 119.19, 113.67 (2C), 87.61, 83.76, 72.62, 72.52, 54.98, 34.98, 33.15;
ES + MS ($C_{18}H_{21}N_5O_4S$), HRMS (ESI, positive ion). Calculated m/z for $C_{18}H_{21}N_5O_4SNa$ [M+Na]: 426.1212; found 426.1215.

BASIC PROTOCOL 2

SYNTHESIS OF 5'-DEOXY-5'-(5-NITROPYRIDYL-2-DISULFANYL)-ADENOSINE (3):

This protocol describes removal of the 4-methoxybenzyl function and concomitant disulfide formation without exposing the highly oxidation-prone thiol moiety. The unsymmetrical disulfide is a key intermediate in the construction of the P-S-C linkage using M-A chemistry. MobSdA is stirred with acid, a cation quench and a symmetric aryl disulfide. Pure product is isolated following silica gel column chromatography.

Materials

Argon
5'-*S*-(4-Methoxybenzyl)-5'-thioadenosine (**2**) from Basic Protocol 1
2,2'-dithiobis(5-nitropyridine)
Trifluoroacetic acid
Thioanisole
TLC eluting solvent: 9:1; v/v; CH_2Cl_2:MeOH
Methanol
Dichloromethane
Silica gel 60 Å for chromatography (40 -63 μm) dried at 130°C

Argon-filled balloons
25 mL oven-dried round-bottom flask
Magnetic stirring bar
Right-angled ground glass jointed balloon (tubing) adapter (2x)
Magnetic stirrer
50 mL ground glass jointed conical flask
Disposable syringes (1 mL and 20 mL) and needles
15 cm-long 21 gauge luer lock needle
Silica gel TLC plate with fluorescent indicator
254-nm UV lamp
Rotary evaporator
3 cm Diameter, glass-fritted column and test tubes for chromatography
500 mL round-bottom flask
Rotary evaporator
Vacuum desiccator containing phosphorus pentoxide and (separately) KOH as desiccants

Procedure

1. Add 0.81 g (2.0 mmol) of 5'-*S*-(4-methoxybenzyl)-5'-thioadenosine (**2**) and 1.24 g (4.0 mmol) of 2,2'-dithiobis(5-nitropyridine) to an argon-filled, oven-dried 25 mL round-bottom flask. Add a magnetic stirring bar and store under argon using a 90°-angled balloon adaptor equipped with an argon-filled balloon.

2. To a 50 mL conical flask under argon add 0.50 mL thioanisole and 19.5 mL trifluoroacetic acid. Gently swirl to effect mixing under argon using ground glass, 90°-angled balloon adaptor. Withdraw the solution into a syringe and immediately proceed to step 3.

3. Gently stir the round-bottom flask containing solids prepared in step 1 using a magnetic stirrer, remove the balloon adaptor and add the solution from step 2. Replace the balloon adaptor with argon-filled balloon.

4. Stir at ambient temperature for 2 hours.

   A dark orange color rapidly develops.

5. If desired, the progress of the reaction can be monitored by TLC *Rf = 0.24 TLC (9:1; v/v; DCM/MeOH); Rf value of starting material (**2**) = 0.25.*

6. Remove the stirring bar and concentrate the reaction mixture on a rotary evaporator to an oil and dilute with chloroform (supplied commercially with 1% v/v ethanol as stabilizer).

7. Purify the crude product by silica gel column column chromatography (30 g of silica gel packed in chloroform) eluting with 5%, 7.5% and 10% (v/v) MeOH in $CHCl_3$.

   *Compound **3** elutes with 7.5 - 10%* (v/v) MeOH *and its presence in solution can be detected visually by the pale yellow color of these fractions.*

8. Combine fractions containing pure **3** as determined by TLC (9:1 DCM:MeOH), remove the solvent on a rotary evaporator and dry in a vacuum desiccator.

9. Characterize the pure compound **3** by ^{1}H- and ^{13}C-NMR, and ES-MS.

   *5'-deoxy-5'-(5-nitropyridyl-2-disulfanyl)-adenosine: (**NPySSdA**) - **3**. Pale yellow solid. Yield: 0.66 g (75%); Rf (SiO_2) = 0.24 (9:1; v/v; DCM/MeOH).*
   ^{1}H NMR (400 MHz, DMSO-d_6) δ = 9.20 (1H, d, $^{4}J_{HH}$=2.7Hz, ArH), 8.51 (1H, dd, $^{3}J_{HH}$=2.7, 9.0Hz, ArH), 8.46 (1H, s, H_2), 8.21 (1H, s, H_8), 8.01 (1H, d, $^{3}J_{HH}$=8.8Hz, ArH), 7.87 (2H, brs, -NH_2), 5.89 (1H, d, $^{3}J_{HH}$=5.9Hz, H1'), 5.52 (2H, brs, 2x -OH) 4.79 (1H, ψt, $^{3}J_{HH}$=5.5Hz, H2'), 4.19 (1H, m, H3'), 4.09 (1H, m, H4'), 3.40-3.30 (2H, m, H5', H5''). ^{13}C NMR (101MHz, DMSO-d_6) δ = 167.31, 154.94, 151.27, 149.12, 144.66, 142.10, 140.61, 132.36, 119.52, 119.12, 87.70, 82.43, 72.55, 72.45, 41.49. HRMS (ESI, positive ion). Calculated m/z for $C_{15}H_{16}N_7O_5S_2$ [M+H]+ : 438.0654; found 438.0658.

BASIC PROTOCOL 3

SYNTHESIS OF 5'-THIOADENOSINE 5'-PYROPHOSPHATE (P'→5') ADENOSINE (5):

This protocol describes the Michaelis-Arbuzov reaction between persilylated **3** and $(TMSO)_3P$ under anhydrous conditions to give a relatively stable intermediate phosphorothiolate monoester (**4**). Ball-milling **4** in the presence of AMP-morpholidate, activators and water engenders hydrolytic desilylation and phosphate coupling. Pure dASppA (**5**) is isolated following RP-HPLC and desalting.

Materials

Chloroform
Phosphorus pentoxide
Activated basic aluminium oxide
Argon
Bis(trimethylsilyl)acetamide (BSA)
5'-deoxy-5'-(5-nitropyridyl-2-disulfanyl)-adenosine (**3**)
Tris(trimethylsilyl)phosphite
Adenosine 5'-monophosphomorpholidate 4-morpholine-*N*,*N*'-dicyclohexylcarboxamidine salt (AMP-morpholidate)
1*H*-Tetrazole
Magnesium chloride hexahydrate (>99%)
18.2 MΩ water
pH paper
100 mM triethylammonium acetate (TEAA) pH 6.5 in 100% H_2O
100 mM triethylammonium acetate (TEAA) pH 6.5 in 95:5 H_2O:MeCN
100 mM triethylammonium acetate (TEAA) pH 6.5 in 35:65 H_2O:MeCN
100 mM triethylammonium bicarbonate (TEAB) pH 7.8 in 100% H_2O
100 mM triethylammonium bicarbonate (TEAB) pH 8.3 in 35:65 H_2O:MeCN

2 x Zirconia-lined vessel (25 ml internal volume; Retsch, 01.462.0201)
1 or 2 x 15 mm zirconia ball (10.70 g; Retsch, 05.368.0094) (N.B. Do NOT include ball in second Zirconia vessel is only used as counterbalance).
Oven-dried ground glass jointed 50 mL conical flasks (x3)
Oven-dried round-bottom flasks (5 mL, 10 mL)
Oven-dried magnetic stirring bar
Oven-dried Hamilton gas-tight syringes (100 µL, 500 µL and 2.5 mL)
Oven-dried rubber septa (B14 and as needed for glassware)
Oven-dried 5 mm diameter NMR tubes
Oven-dried balloon adaptors
Argon-filled balloons
Vacuum desiccator containing phosphorus pentoxide and (separately) KOH as desiccants
10 mL Disposable syringes (include one with the top 10% of the barrel removed)
Retsch Mixer Mill MM 400 (Retsch, 20.745.0001)
Gilson (automatic) pipette and tips (1000 µL)

0.45 µm Spin-X® cellulose acetate membrane filter for 1.5 mL Eppendorf tubes
2 mL Eppendorf tubes
Microcentrifuge capable of 12000 rpm
50 µL syringe with blunt-ended needle for HPLC injection
5 mL disposable syringe with 22 ga Blunt-tipped, Kel-F hubbed 51 mm needle
HPLC system capable of pumping at 8 mL min^{-1} – the system used to develop this protocol was a ThermoFinnigan SpectraSYSTEM consisting of a P2000 binary gradient pump with UV1000 sample detector. Samples were injected manually via a Rheodyne injection valve into a 20 µL or 5 mL injection loop.
Sterile tubes (25mL)
100 mL round-bottom flask
Cold-finger rotary evaporator
Vacuum pump (capable of < 10 mm Hg)
Quartz UV cuvette 1 cm pathlength, 1.5 mL capacity
UV-Vis spectrometer

Procedure

1. Dry the zirconia-lined vessel, zirconia ball, glassware, septa, stirring bar, syringes and NMR tube(s) in a drying oven at 65° C overnight. Transfer to a desiccator, evacuate, allow to cool to room temperature and re-equilibrate to atmospheric pressure with argon (see Basic Protocol 1).

2. Add *ca*. 5 g of P_2O_5 to a dry 50 mL conical flask, 3/4 fill with chloroform and seal with a rubber septum. Gently swirl the suspension and store for 30 minutes.

3. Assemble a short column for the basic alumina by cutting off the top 10% of a 10 mL disposable syringe barrel, insert a piece of lintless tissue sufficient to plug the nozzle (gently press into place with a Pasteur pipette) and pack *ca*. 4 mL of basic alumina in the barrel. Attach a needle, insert a septum into the barrel and secure the septum in place with parafilm.

4. Filter 30 mL of the dried chloroform under gravity through the column into an argon-filled oven-dried 50 mL conical flask.

   This dried, filtered chloroform (after removing the ethanol stabilizer and acid residues) should be used on the same day it is prepared.

5. Withdraw 20 mL of the dried, filtered chloroform under argon and transfer to a new argon-filled, oven-dried 50 mL conical flask.

6. Add 5 mL of BSA and swirl to fully mix the 4:1; v/v; $CHCl_3$:BSA silylation stock solution.

7. Transfer 50 mg (0.114 mmol) of 5′-deoxy-5′-(5-nitropyridyl-2-disulfanyl)-adenosine (**3**) into an oven-dried 5 mL round-bottom flask containing a stirring bar. Add 1.5 mL of the silylation stock solution under argon and stir the suspension for 30 min.

   When transferring precise quantities of the silylation stock solutions, use Hamilton-type gas-tight syringes (the polymer gaskets in disposable syringes rapidly expand when in contact with this mixture). Dissolution of the silylated nucleoside gives a clear solution.

8. In an oven-dried 5 mL round-bottom flask under argon, mix 0.55 mL of silylation stock solution and 50 µL (0.125 mmol) of $(TMSO)_3P$. Withdraw 0.5 mL of this solution and add to the stirring solution prepared in step 7 above.

   Immediately, a yellow color is apparent which intensifies. If using ^{31}P NMR to evaluate the suitability of (especially older) batches of $(TMSO)_3P$ for the M-A reaction, this should be done in the silylation mix using an external lock (D_2O-filled capillary insert).

9. Continue stirring for 30 minutes.

   *If desired ^{31}P-NMR can be used analyse the reaction mixture by withdrawing 0.5 mL and adding to an oven-dried, argon-filled 5 mm NMR tube; with a D_2O insert. One predominant peak at ~ δ = 4.8 (compound **4**) is present which integrates for ca. 80% of the total phosphorus content.*

10. Release the vacuum on the vacuum desiccator containing the ball mill vessel using an argon balloon.

    Unlike Basic Protocol 1, the ball should not be in the lower body at this stage.

11. Transfer the whole M-A reaction mixture into the lower body of the ball mill vessel using a gas tight syringe. Rinse out the glassware (and NMR tube, if used) with 0.5 - 1.0 mL of the residual dried, deacidified chloroform from step 4 and add this to the main solution.

12. Place the lower body of the ball mill vessel containing crude M-A reaction mixture and washings into the vacuum desiccator and gradually increase the vacuum to *ca.* 50 mm Hg paying close attention to avoid bumping. Maintain these conditions for a minimum of 3 hours.

    This can also be left overnight; a yellow paste is formed.

13. At least 30 minutes prior to the next step, adenosine 5′-monophosphomorpholidate 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt (AMP-morpholidate) should be removed from the freezer and allowed to equilibrate to room temperature under an inert atmosphere.

14. Carefully release the vacuum of the desiccator using an argon-filled balloon, ensure that the Teflon O-ring is securely located in the upper cap and remove the lower body.

15. In quick succession, add 120 mg (0.171 mmol) AMP-morpholidate, 17 mg (0.24 mmol 1*H*-tetrazole, 35 mg (0.17 mmol) $MgCl_2 \cdot (H_2O)_6$, 25 µL H_2O (1.37 mmol) and a 15 mm zirconia ball.

16. Close the vessel by screwing tight the upper cap and O-ring onto the lower body. Check that the vessel is fully screwed tight and move to the location of the ball mill.

17. Load the ball mill with balanced vessels (see Basic Protocol 1), program the machine so that it will vibrate at 30 Hz for 90 minutes and start.

18. Allow the reaction vessel to cool to room temperature, remove from the ball mill and orientate so that the lower body is at the bottom. Unscrew the vessel.

    The neat reaction mixture is a pale yellow paste.

19. Using a Gilson (automatic) pipette, rinse both parts of the ball mill with a total volume of 2 mL 18.2 MΩ deionized water and transfer the washings into two separate 1.5 mL Eppendorf tubes.

    If no further washes are required (step 22), the concentrated yellow suspensions can be stored frozen at this stage. If storing, the pH of the solution should be checked at this stage and if not in the range 6-8, adjusted using HPLC eluent A (100% water).

20. Sonicate the Eppendorf tubes for five minutes, and filter each through a 0.45 µm cellulose acetate Spin-X® centrifuge filter at 12,000 rpm (typically for 3 minutes). Rinse the solids with 0.5 mL deionized H_2O and combine with the original 2 mL.

21. Analyse a 0.5 mL aliquot of these first concentrated washings by ^{31}P NMR (using an external D_2O lock).

    *^{31}P NMR analysis provides a valuable check on the efficiency of the coupling reaction enabling the relative quantities of peaks in the HPLC profile to be estimated. Typical spectra can be found in the ESI for Eguaogie et al., 2016. Resonances are observed at: δ ca. 17 (dASMP this slowly degrades to dASH and inorganic phosphate); δ ca. 8 and –12 (doublets) arising from the product **5**; δ ca. 7 (AMP-morpholidate); δ ca. 0 (AMP and inorganic phosphate); δ ca. -2 and -11 (two doublets arising from the R_P- and S_P-diastereoisomers of Ap(M)pA, δ ca. -11.5 (Ap_2A - also with δ ca. -22 Ap_3A). If broad peaks are evident, add 5 mM EDTA·Na_2 (pH 7.0) to a final concentration of 2 mM.*

22. Repeat steps 19 and 20 to remove residual materials from the jar.

    The same Spin-X® components used in step 20 can be used for this operation.

23. Combine all solutions and analyze this crude reaction mixture by C18-reversed phase-HPLC; inject 5 µL. (All water used in the following operations was 18.2 MΩ deionized quality).

    Column: 150 x 4.60 mm Phenomenex Clarity 5µm Oligo-RP
    Flow rate: 1 mL min^{-1}
    Eluent A: 100 mM TEAA (pH 6.5) in H_2O
    Eluent B: 100 mM TEAA (pH 6.5) in 35:65 H_2O:MeCN
    Gradient: 0-5 min, 0% Eluent B; 5-25 min, 0-20% Eluent B; 25-30 min, 20-100% Eluent B; 30-32 min, 100% Eluent B; 32-40 min, 100-0% Eluent B; 40-50 min, 0% Eluent B
    UV detection at 260 nm.

24. Purify the combined solutions in two runs; collecting the eluent containing pure **5**.

    Column: 250 x 21.2 mm Phenomenex Clarity 5µm Oligo-RP
    Flow rate: 8 mL min^{-1}
    Eluent A: 100 mM TEAA (pH 6.5) in 95:5 H_2O:MeCN
    Eluent B: 100 mM TEAA (pH 6.5) in 35:65 H_2O:MeCN
    Gradient: 0-10 min, 0% Eluent B; 10-60 min; 0-14% Eluent B; 60-70 min, 14% Eluent B; 70-90 min, 14-100% Eluent B; 90-100 min, 100% Eluent B; 100-120 min, 100-0% Eluent B; 120-140 min, 0% Eluent B
    UV detection at 280 nm.

25. Concentrate the pure fractions to ca. 15 mL on a rotary evaporator and desalt in a single run with multiple loading / injection cycles.

    Column: 250 x 21.2 mm Phenomenex Clarity 5µm Oligo-RP
    Flow rate: 8 mL min^{-1}
    Eluent A: 100 mM TEAB (pH 7.8) in H_2O
    Eluent B: 100 mM TEAA (pH 8.3) in 35:65 H_2O:MeCN
    Gradient: 0-10 min, 0% Eluent B; 10-60 min, 0-30% Eluent B; 60-80 min, 30-100% Eluent B; 80-90 min, 100% Eluent B; 90-110 min, 100-0% Eluent B; 110-120 min, 0% Eluent B
    UV detection at 280 nm.

26. Evaporate the product-containing fractions to dryness and coevaporate with water until no more TEAB (white salt) residues are present.

27. Extract the residues from the flask in 1 mL 18.2 MΩ deionized water. Remove 2 µL and dilute to 1 mL. Measure the absorbance at 260 nm and calculate the concentration of dinucleotide in the stock solution according to the following equation:

    $c\ (mM) = A^{260\ nm} \times 19.9$

28. Characterize the pure compound **5** by ^{31}P-NMR and ES-MS.

*5'-thioadenosine 5'-pyrophosphate (P'→5') adenosine (**dASppA**) – **5**. 531 OD^{260nm} units 25 % from (**3**). ^{31}P NMR (162 MHz, D_2O) δ = 7.32 (d, $^2J_{PP}$=29.9Hz, Pβ), 11.88 (d, $^2J_{PP}$=28.9Hz, Pα). HRMS (ESI, negative ion). Calculated m/z for $C_{20}H_{25}N_{10}O_{12}P_2S$ $[M+H]^-$: 691.0849, found 691.0871*

COMMENTARY

Background Information Phosphate coupling for the preparation of nucleotides and their analogues was first reported as early as 1947 but required silver activation (Baddiley and Todd, 1947), high temperatures (Clark et al., 1957), strongly acidic conditions (Moffatt and Khorana, 1958) or water-sensitive condensing reagents (Khorana and Todd, 1953). Subsequently, Khorana introduced nucleoside phosphoromorpholidate monoesters as a compromise between their relative easy accessibility and P-N bond activation (Moffatt and Khorana, 1961) but could require several days for completion. Recent strategies with more expeditious coupling kinetics have been reviewed (Wagner et al., 2009; Jessen et al., 2014; Roy et al., 2016). However, the requirement for solvents with minimal water content may still make demands upon the integrity of labile intermediates during their processing. Such intermediates include phosphorothiolate monoesters; we observed nearly complete hydrolysis of dTSMP within 28 hours at room temperature under neutral conditions although during these investigations, we found that the integrity of the P-S bond was maintained if the monoester was silylated (Eguaogie et al., 2016). Rapid phosphate coupling using commercially-available substrates and reagents could be performed in a ball mill and was found to be expedited by addition of small volumes of water (Ravalico et al., 2011). The group of van Aalten have described the synthesis of UDP-*S*-GlcNAc (Dorfmueller et al., 2011) via phosphate coupling of the GlcNAc-α-*S*-phosphorothiolate monoester with UMP-*N*-methyl phosphoroimidazolide with an overall yield of 9% from the thiol (compared with the current protocol which gives 19% from the Mob-protected thionucleoside).

Critical Parameters and Troubleshooting

Safety precautions including the use of gloves, eye protection and fume hood ventilation for operations not involving the ball mill. Basic Protocol 2. Steps 2 - 4. Handling TFA with rubber septa will lead to leaching of the color into the reaction mixture. Step 5. In order to resolve the closely-running spots, the solvent front should be allowed to move at least 7 cm from the origin using a development tank in which a consistent vapour phase composition is maintained throughout the run. We have also found that using a reaction mixture / MobSdA co-spot is important in helping show the difference. Removing TFA can be effected by pre-eluting with diethyl ether. Basic Protocol 3. Step 12. Bumping can occur if the vacuum is applied too quickly; this is somewhat ameliorated by using the lower body of the ball milling vessel, but care should still be exercised.

Anticipated Results

Variability in the recovery of **2** was found to depend on the use of fully chilled washing solutions. In Basic Protocol 3, the quality of reagents (especially the phosphite but also BSA after prolonged storage) may require checking as described in the text. In addition, the use of the HPLC-grade solvents and high grade buffer reagents is critical.

Time Considerations

Two days should be allowed for the synthesis of **2**. Three days should be allocated for the subsequent reaction of **2** and isolation of **3**. Five days are required to proceed through to **5** and achieve the desalted material.

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INTERNET RESOURCES

Retsch website (MM400): http://www.retsch.com/products/milling/ball-mills/mixer-mill-mm-400/. Youtube: https://www.youtube.com/watch?v=ZaepF_-cXoc and https://www.youtube.com/watch?v=k6mPWPuR8PY.

FIGURE LEGENDS

Figure 1. Synthesis of 5′-*S*-(4-methoxybenzyl)-5′-thioadenosine (**2**); 5′-deoxy-5′-(5-nitropyridyl-2-disulfanyl)-adenosine (**3**) and 5′-thioadenosine 5′-pyrophosphate (P′→5′) adenosine (**5**). Key. DTNP 2,2′-dithiobis(5-nitropyridine); BSA *N*,*O*-bis(trimethylsilyl)acetamide; AMP-morpholidate Adenosine 5′-monophosphomorpholidate 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt

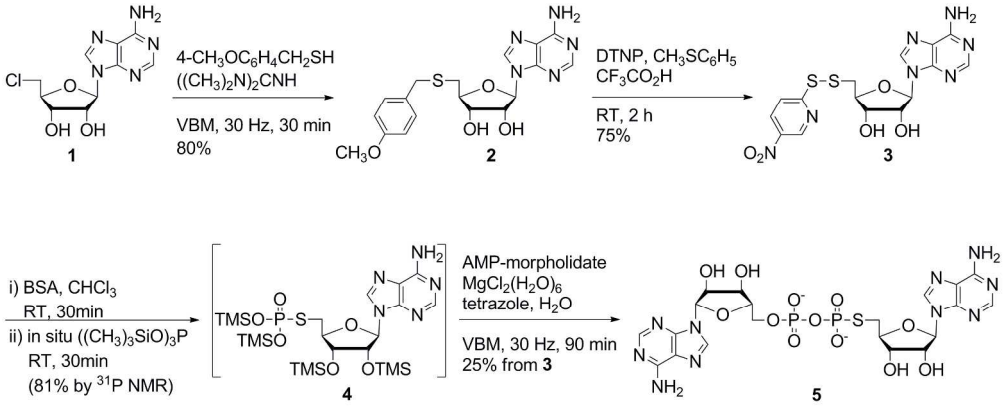

Figure 1. Synthesis of 5′-*S*-(4-methoxybenzyl)-5′-thioadenosine (**2**); 5′-deoxy-5′-(5-nitropyridyl-2-disulfanyl)-adenosine (**3**) and 5′-thioadenosine 5′-pyrophosphate (P′→5′) adenosine (**5**).Key. DTNP 2,2′-dithiobis(5-nitropyridine); BSA *N*,*O*-bis(trimethylsilyl)acetamide; AMP-morpholidate Adenosine 5′-monophosphomorpholidate 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt.

242x98mm (300 x 300 DPI)