Chapter 2

Dinucleotides Containing 3′-*S*-Phosphorothiolate Linkages

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Abstract

The 3′-*S*-phosphorothiolate (3′-SP) linkage has proven to be a very useful analogue of the phosphodiester group in nucleic acid derivatives; it is achiral and also shows good resistance to nucleases. Whilst oligonucleotides containing a 3′-SP linkage are best prepared using phosphoramidite chemistry, the corresponding dinucleotides are most efficiently synthesised using a Michaelis–Arbuzov reaction between a nucleoside 5′-phosphite and a nucleoside 3′-*S*-disulphide. The method described here is for a thymidine dinucleotide and is based on the use of a silyl phosphite, which is more reactive than simple alkyl phosphites and also simplifies the deprotection strategy. Full experimental details and spectroscopic data for the synthetic intermediates and the target dinucleotide are provided.

Key words: Phosphorothiolate, dinucleotide, oligonucleotide, Michaelis–Arbuzov reaction, silyl phosphate, chemical synthesis.

1. Introduction

Historically, dinucleotides (or more correctly dinucleoside phosphates) have been prepared as model compounds to evaluate the potential of different chemical approaches to the synthesis of oligonucleotides (1) and have not therefore attracted much attention in their own right. However, in the last decade dinucleotides have become recognised as a distinct group of anti-viral agents that can inhibit the activity of HIV integrase (2, 3) and show activity against hepatitis B (4, 5).

As a potential means to enhance biological activity of nucleotides, the phosphodiester bond is usually modified in order to increase resistance to nucleases and phosphodiesterases. The phosphorothioate modification, in which sulphur replaces

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a non-bridging oxygen atom, is an effective analogue for this purpose (4) and is convenient to prepare by chemical procedures (6, 7). This modification results in an asymmetric phosphorus atom and therefore produces mixtures of diastereoisomers, unless the stereochemistry is controlled (8). Replacement of one of the bridging oxygen atoms leads to an achiral phosphorothiolate linkage that can be prepared with either the 3′-oxygen (9) or the 5′-oxygen (9, 10) substituted by sulphur.

The 3′-*S*-phosphorothiolate (3′-SP) linkage (**Fig. 2.1**) initially attracted interest as a probe to obtain detailed mechanistic information about enzymes that process nucleic acids (9). However, subsequently it has been shown through NMR studies on both dinucleotides (11) and oligonucleotide duplexes (12) that a single 3′-*S*-phosphorothiolate modification shifts the conformation of its attached 2′-deoxyribose sugar almost completely to what is described as the north or (C-3′ *endo*) conformation, which is the sugar conformation found in RNA. Thus, oligodeoxynucleotides containing a phosphorothiolate linkage are conformationally good mimics of RNA and as a result they form thermodynamically more stable duplexes with complementary RNA than the corresponding unmodified oligodeoxynucleotides (13). This property suggests that oligonucleotides containing 3′-SP linkages may be of use as both antisense agents and in RNA interference, where enhanced binding to an RNA target is required. It is also interesting to note that dinucleotides containing sugars that are expected to exist predominantly in the north conformation also show good activity against hepatitis B (4).

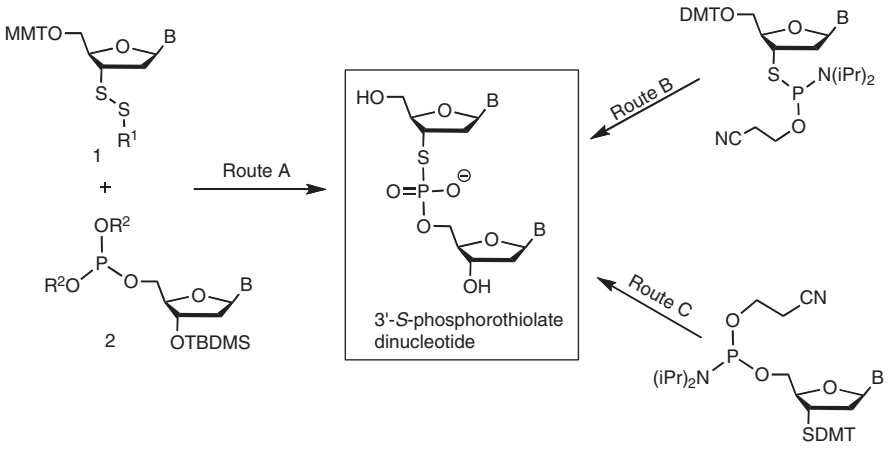

Fig. 2.1. Synthetic routes for the preparation of phosphorothiolate dinucleotides and oligonucleotides. Route A shows the Michaelis–Arbuzov reaction, which is the preferred method for the preparation of dinucleotides containing a phosphorothiolate linkage. Routes B and C show methods based on phosphoramidite chemistry, which have mainly been used for the automated synthesis of oligonucleotides with a 3′-SP modification. B = nucleobase, R^1 = aryl, R^2 = alkyl or trimethyl silyl, MMT = monomethoxytrityl, DMT = dimethoxytrityl, TBDMS = *tertiary*-butyldimethylsilyl.

The chemical synthesis of oligonucleotides containing 3′-SP linkages requires manipulation of the deoxyribose sugar and is therefore more challenging than the synthesis of the phosphorothioate analogues. A number of different chemical approaches have been used to prepare dinucleotides and oligonucleotides containing 3′-SP linkages. Our own research has shown that the most efficient route to prepare phosphorothiolate dinucleotides is through a Michaelis–Arbuzov reaction between a nucleoside 3′-*S*-disulphide (1, **Fig. 2.1**) and a nucleoside 5′-*O*-phosphite (2, **Fig. 2.1**) (Route A, **Fig. 2.1**) (14, 15). An attractive feature of this reaction is that a number of different alkyl or silyl substituents can be used in the phosphite component and this provides a means to control reactivity and vary the protecting group that remains on the phosphorothiolate linkage at the completion of the reaction. In this respect the silyl phosphites are particularly valuable in that they are more reactive than simple alkyl phosphites and are easily prepared from the corresponding *H*-phosphonates (14, 15). In addition, the silyl group on the phosphorothiolate linkage is removed when the reaction is quenched by the addition of water (Step 8 of **Section 3.3**). For these reasons the Michaelis–Arbuzov reaction based on the use of silyl phosphites is presented below as the method of choice for preparing dinucleotides containing 3′-SP linkages and the overall reaction route is shown in **Fig. 2.2**. The specific example chosen is a thymidine dinucleotide although 3′-thionucleosides have been prepared from both purine and pyrimidine deoxynucleosides (16, 17).

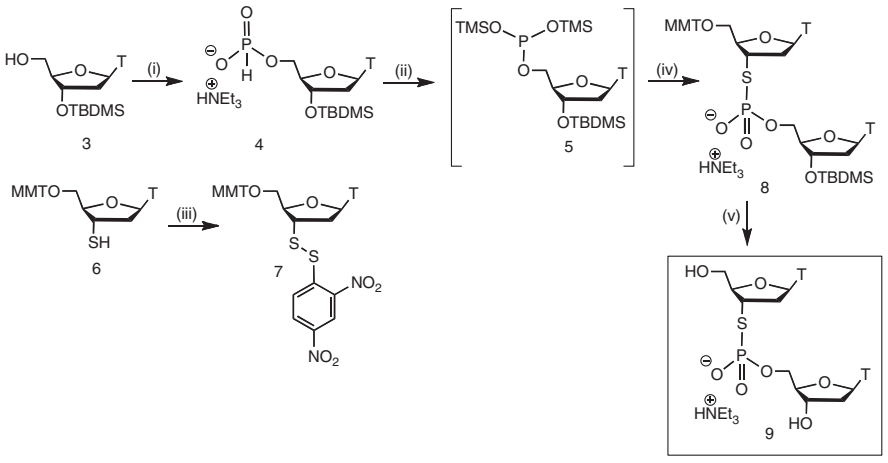

Fig. 2.2. Reagents: (i) phosphorus trichloride, *N*-methylmorpholine, 1,2,4-triazole in dichloromethane; (ii) bis(trimethylsilyl)trifluoroacetamide in dichloromethane; (iii) 2,4-dinitrophenylsulfenyl chloride, pyridine in THF; (iv) compound 7 in acetonitrile; (v) acetic acid:H_2O (4:1) followed by $NEt_3.3HF$. MMT = monomethoxytrityl, TBDMS = *tertiary*-butyldimethylsilyl.

It should be noted for the purpose of completeness that the phosphoramidite approach has been widely used for the synthesis of oligonucleotides containing up to five 3′-SP linkages (16) and this has been achieved using 3′-*S*-phosphorothioamidites as shown in **Fig. 2.1** (Route B). A detailed protocol for the automated solid-phase synthesis of oligonucleotides incorporating 3′-SP linkages has been published (18), which could certainly be used for the preparation of dinucleotides containing this modification. More recently the amidite method has also been used in a reverse approach, through reaction of a 3′-thionucleoside with a 5′-*O*-phosphoramidite (**Fig. 2.1**, Route C) (17). This route however proved to be relatively inefficient and was most suitable for preparing oligonucleotides that terminated with a 3′-thionucleoside.

2. Materials

2.1. Chemicals

1. 5′-*O*-Monomethoxytrityl-3′-deoxy-3′-thiothymidine (6, **Fig. 2.2**) [synthesised via previously reported protocol using monomethoxytrityl chloride in place of dimethoxytrityl chloride (18)]
2. 3′-*O*-(*tert*-Butyldimethylsilyl)-thymidine (Berry & Associates)
3. Acetic acid
4. Bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich)
5. C_{18} reversed-phase silica gel
6. Deuterated chloroform
7. Deuterated water
8. 2,4-Dinitrophenylsulfenyl chloride (Sigma-Aldrich)
9. Dowex 50W-X8 (Hydrogen form) ion exchange resin
10. Magnesium sulphate
11. *N*-methylmorpholine
12. Nitrogen (oxygen-free or white-spot)
13. Phosphorus trichloride
14. Sand (low in iron, 40–100 mesh)
15. Silica gel (particle size 40–63 μm)
16. Sodium bicarbonate aqueous solution (saturated)
17. Sodium sulphate
18. Thin-layer silica gel plated on aluminium backing
19. 1,2,4-Triazole

20. Triethylamine trihydrofluoride
21. Triethylamine

2.2. Reagent Setup

1. Triethylammonium bicarbonate (TEAB) solution (1 M): this is prepared by bubbling carbon dioxide gas (warming solid carbon dioxide) through a solution of triethylamine (139 mL) in distilled water (800 mL) until the required pH is attained (~8.5). The volume of the solution is then adjusted to 1 L using additional distilled water (*see* **Note 1**).
2. TEAB solution (0.1 M): dilute 1 M TEAB (100 mL) into 1 L of distilled water.
3. *p*-Anisaldehyde stain: prepared by mixing *p*-anisaldehyde (6 mL) with sulphuric acid (8 mL), acetic acid (2.4 mL) and ethanol (218 mL).
4. Acetic acid:water (4:1).

2.3. Solvents and Solvent Setup

1. Acetonitrile, anhydrous DNA grade (Link Technologies)
2. Dichloromethane
3. Dichloromethane, anhydrous (Sigma-Aldrich). Can also be distilled from calcium hydride (*see* **Note 2**)
4. Ethanol, absolute
5. Methanol
6. Petroleum ether (40–60)
7. Pyridine, anhydrous (Sigma-Aldrich). Can also be distilled from calcium hydride (*see* **Note 2**)
8. Tetrahydrofuran
9. Tetrahydrofuran, anhydrous (Sigma-Aldrich). Can also be distilled from sodium and benzophenone (*see* **Note 2**)
10. Toluene

2.4. Equipment

1. Disposable 21-gauge hypodermic syringe needles with Luer connection
2. Disposable plastic syringes with Luer connections
3. Reusable, non-sterile 19-gauge metal needles with Luer connection
4. Pasteur pipettes
5. Rubber septum, various sizes
6. Standard organic laboratory glassware

2.5. Equipment/ Procedural Setup

1. Silica gel (*see* **Note 3**) column chromatography: pack the column using the appropriate eluent. Dissolve the impure compound into a minimum volume of eluent and place the resulting crude oily mixture on top of the silica bed using

a Pasteur pipette (*see* **Note 4**). Allow the sample to enter the silica bed. Cover the top of the silica with layer of sand (2.5 cm thick) and run the column using the appropriate eluent system. Use gentle air or nitrogen pressure to aid elution and identify fractions containing pure product by eluting thin-layer silica gel plates on aluminium backing and staining with anisaldehyde (Step 3 of **Section 2.2**). Combine fractions containing pure product and remove all solvents using a rotary evaporator.

2. C_{18} reversed-phase silica gel column chromatography: performed in an analogous way to regular column chromatography above.

3. Methods

Michaelis-Arbuzov chemistry is the best approach for synthesising dinucleotides containing a 3′-*S*-phosphorothiolate linkage. Various disulphides and phosphite triesters have been investigated and the procedure below, as outlined in **Fig. 2.2**, is considered to be the most efficient. The key step in the synthesis is the reaction between a nucleoside silyl phosphite (5, **Fig. 2.2**), which is prepared in situ by silylation of the *H*-phosphonate (4, **Fig. 2.2**), and the nucleoside disulphide (7, **Fig. 2.2**). The resultant 3′,5′-protected dimer (8, **Fig. 2.2**) is then deprotected to give the product dinucleotide (9, **Fig. 2.2**).

3.1. Preparation of 5′-O-Monomethoxytrityl-3′-deoxy-3′-(2,4-dinitrophenyldisulfanyl)-thymidine

See 7, **Fig. 2.2**.

1. Weigh 2,4-dinitrophenylsulfenyl chloride (0.89 g, 3.8 mmol) into a 100-mL round bottomed flask (flask 1), add a Teflon-coated magnetic stir bar and seal the top with a rubber septum.
2. Insert a nitrogen (*see* **Note 5**) inlet and vent (consisting of disposable syringe needles) through the rubber septum and allow the flask to be filled with nitrogen (at least 20 min, s*ee* **Note 6**).
3. Add anhydrous THF (40 mL) to flask 1, making sure the stock anhydrous THF solution is kept anhydrous by way of a nitrogen inlet.
4. Place flask 1 into an ice bath ensuring the ice bath is maintained at 0–2°C. Leave solution to cool (15 min).
5. Whilst flask 1 is cooling, weigh 5′-*O*-monomethoxytrityl-3′-deoxy-3′-thiothymidine (6, **Fig. 2.2**) (1.0 g, 1.9 mmol) into a separate 50-mL round bottomed flask (flask 2) and add anhydrous pyridine (20 mL). Co-evaporate the pyridine on

a rotary evaporator (to help remove any traces of water from the nucleoside) leaving a thick oil.

6. Seal flask 2 with a rubber septum and insert a nitrogen inlet and vent (consisting of disposable syringe needles) through the rubber septum and allow the flask to be filled with nitrogen (at least 20 min, *see* **Note 7**).
7. Add anhydrous THF (20 mL) to flask 2 and manually agitate to aid dissolution. Transfer the contents of flask 2 into flask 1 using a long metal needle (dropwise) and leave the combined solution to stir at 0–2°C for 30 min.
8. Remove the flask from the ice bath and allow the flask to warm to room temperature. Allow the solution to stir for an additional 1 h.
9. Transfer the contents of the flask to a 250-mL separating funnel containing saturated aqueous $NaHCO_3$ (75 mL) and dichloromethane (75 mL). Shake the flask and discard the aqueous layer (*see* **Note 8**).
10. Wash the dichloromethane layer with two more portions of saturated aqueous $NaHCO_3$ (2 × 75 mL). Dry the combined organic layer by adding magnesium sulphate (generally 4–5 spatulas) until the powdered magnesium sulphate settles slowly ('snowstorm' effect) when the flask is agitated. Filter off the magnesium sulphate under gravity through a fluted filter paper in a glass funnel. Concentrate the filtrate to dryness using a rotary evaporator.
11. Purify the impure compound by column chromatography following the procedure given in **Section 2.5**. Use dichloromethane containing 1% methanol to pack the column, dissolve impure compound using dichloromethane and elute product by slowly increasing the proportion of methanol from 1 to 3%. 5′-*O*-Monomethoxytrityl-3′-deoxy-3′-(2,4-dinitrophenyldisulfanyl)-thymidine will appear as a yellow amorphous solid.

3.2. Preparation of 3′-O-(tert-Butyldimethylsilyl)-thymindin-5′-yl Phosphonate Triethylammmonium Salt

See 4, **Fig. 2.2**.

1. To the side neck of a two-necked flask (flask 1), fit a glass stopper with appropriately sized Keck clip. Insert a Teflon-coated magnetic stir bar to the flask and fit the main neck with a pressure-equalising dropping funnel.
2. Attach a nitrogen bubbler to the top of the pressure-equalising dropping funnel and allow the flask to be filled with nitrogen (at least 20 min, *see* **Note 6**).
3. Remove the stopper from the side neck of flask 1 and add anhydrous dichloromethane (14 mL), making sure the stock anhydrous dichloromethane solution is kept anhydrous by way of a nitrogen inlet. Return the stopper to flask 1.

4. Remove the stopper from the side neck of flask 1 and add phosphorus trichloride (1.2 mL, 14 mmol, *see* **Note 9**) and *N*-methylmorpholine (15.7 mL, 140 mmol). Return the stopper to flask 1 and leave the solution to stir for 5 min to ensure dissolution.
5. To the stirring solution in flask 1, use a spatula to slowly add 1,2,4-triazole (3.2 g, 47 mmol) to flask 1 via the side neck. Leave mixture stirring for 30 min.
6. Whilst flask 1 is stirring, weigh 3′-*O*-(*tert*-butyldimethylsilyl)-thymidine (3, **Fig. 2.2**) (1 g, 2.8 mmol) into a separate 50-mL round bottomed flask (flask 2) and add anhydrous acetonitrile (20 mL). Co-evaporate the acetonitrile on a rotary evaporator to help remove any traces of water from the nucleoside.
7. Seal flask 2 with a rubber septum and insert a nitrogen inlet and vent (consisting of disposable syringe needles) through the rubber septum and allow the flask to be filled with nitrogen (at least 20 min).
8. After flask 1 has been stirred for 30 min, place flask 1 into an ice bath ensuring the ice bath is maintained at 0–2°C. Leave solution to cool (15 min).
9. Whilst flask 1 is cooling, add anhydrous dichloromethane (38 mL) to flask 2 and manually agitate to aid dissolution. Using a long metal needle, transfer the contents of flask 2 to the top of the pressure-equalising dropping funnel on flask 1, ensuring the tap on the funnel is closed.
10. Slowly add the contents of the dropping funnel into the stirring solution in flask 1 over a 20-min period. After addition, allow the mixture to stir for an additional 10 min.
11. To a 500-mL separating funnel, add triethylammonium bicarbonate (TEAB, 1 M, pH 8.5; 110 mL). Transfer the contents of the reaction flask to the separating funnel. Shake the flask and collect the organic layer into a conical flask or another suitable receptacle.
12. To the separating funnel, add a further portion of dichloromethane (200 mL) and wash the aqueous layer. Collect the organic layer and combine with the previous organic layer.
13. Dry the combined organic layer by adding sodium sulphate (generally 4–5 spatulas) until the powdered sodium sulphate settles slowly ('snowstorm' effect) when the conical flask is agitated. Filter off the sodium sulphate under gravity through a fluted filter paper in a glass funnel. Concentrate the filtrate to dryness using a rotary evaporator.

14. Purify the impure compound by column chromatography following the procedure given in **Section 2.5**. Use dichloromethane containing 2% triethylamine and 3% methanol to pack the column, dissolve impure compound using dichloromethane and elute product by slowly increasing the proportion of methanol from 3 to 5% (maintaining 2% triethylamine). 3′-*O*-(*tert*-Butyldimethylsilyl)-thymindin-5′-yl phosphonate triethylammmonium salt will appear as a white amorphous solid (*see* **Note 10**).

3.3. Preparation of 5′-O-Monomethoxytrityl-3′-thiothymidylyl-(3′→5′)-[3′-O-(tert-butyldimethylsilyl)-thymidine] Triethylammonium Salt

See 8, **Fig. 2.2**.

1. Weigh 3′-*O*-(*tert*-butyldimethylsilyl)-thymindin-5′-yl phosphonate triethylammmonium salt (4, **Fig. 2.2**) (0.521 g, 1 mmol) into a 25-mL round bottomed flask (flask 1) and add anhydrous acetonitrile (10 mL). Co-evaporate the acetonitrile on a rotary evaporator to help remove any traces of water from the nucleoside.
2. Add a Teflon-coated magnetic stir bar to flask 1 and seal the top with a rubber septum. Insert a nitrogen inlet and vent (consisting of disposable syringe needles) through the rubber septum and allow the flask to be filled with nitrogen (at least 20 min).
3. Add anhydrous dichloromethane (2 mL), making sure the stock anhydrous dichloromethane solution is kept anhydrous by way of a nitrogen inlet. Stir the solution until dissolution is achieved.
4. Add bis(trimethylsilyl)trifluoroacetamide (0.4 mL, 1.47 mmol) to flask 1 and leave to stir for 15 min. This forms 3′-*O*-(*tert*-butyldimethylsilyl)-thymidin-5′-yl-bis(trimethylsilyl) phosphite (5, **Fig. 2.2**) in situ in almost quantitative yield.
5. Weigh 5′-*O*-monomethoxytrityl-3′-deoxy-3′-(2,4-dinitrophenyldisulfanyl)-thymidine (7, **Fig. 2.2**) (0.698 g, 0.96 mmol) into a 10-mL round bottomed flask (flask 2) and seal the top with a rubber septum.
6. Insert a nitrogen inlet and vent (consisting of disposable syringe needles) through the rubber septum and allow the flask to be filled with nitrogen (at least 10 min)
7. Add anhydrous dichloromethane (1 mL) and manually agitate until dissolution occurs; then transfer the contents of flask 2 to flask 1 dropwise, using a long metal needle. A deep red solution will occur; leave the reaction to stir for 30 min.
8. Quench the reaction with distilled water (0.1 mL) and transfer the contents of the flask to a 50-mL separating funnel containing saturated aqueous $NaHCO_3$ (20 mL) and dichloromethane (20 mL). Shake the separating funnel,

discard the aqueous layer and transfer the organic layer to a conical flask.

9. Dry the organic layer by adding magnesium sulphate (generally 4–5 spatulas) until the powdered magnesium sulphate settles slowly ('snowstorm' effect) when the conical flask is agitated. Filter off the magnesium sulphate under gravity through a fluted filter paper in a glass funnel. Concentrate the filtrate to dryness using a rotary evaporator.
10. Purify the impure fully protected dinucleotide by column chromatography following the procedure given in **Section 2.5**. Use dichloromethane containing 2% triethylamine and 2% methanol to pack the column, dissolve impure fully protected dinucleotide using dichloromethane and elute product by slowly increasing the proportion of methanol from 2 to 5% (maintaining 2% triethylamine). 5′-*O*-Monomethoxytrityl-3′-thiothymidylyl-(3′→5′)-[3′-*O*-(tert-butyldimethylsilyl)thymidine] triethylammonium salt (8, **Fig. 2.2**) will appear as a white amorphous solid.

3.4. Preparation of 3′-Thiothymidylyl-(3′→5′) thymidine Triethylammonium Salt

See 9, **Fig. 2.2**.

1. Weigh out the protected dinucleotide (compound 8, **Fig. 2.2**) (0.25 mmol) into a 50 mL-round bottomed flask and add acetic acid:H_2O (4:1) (25 mL). Add a Teflon-coated magnetic stir bar and leave the solution to stir overnight and evaporate the acetic acid using a rotary evaporator. Wash the residue with ethanol (25 mL) and evaporate.
2. Using petroleum ether (40–60, 25 mL), triturate the residue and decant off the petroleum ether. Add toluene (25 mL) and co-evaporate using a rotary evaporator.
3. Dissolve the residue in THF (4 mL) and add $NEt_3.3HF$ (8.5 mL, 50 mmol). Add a Teflon-coated magnetic stir bar and leave to stir overnight.
4. Add water (1 mL) to quench the reaction and then add triethylamine until the reaction mixture becomes neutral by spotting on pH paper. Evaporate the solution to dryness using a rotary evaporator.
5. Purify the fully deprotected dinucleotide by C_{18} reverse-phase column chromatography following the procedure given in **Section 2.5**. Use methanol to pack the column and wash the column with methanol containing an increasing amount of distilled water (20%, 40%, etc.) until the column is packed in pure water. Dissolve the impure fully deprotected dinucleotide and elute the product by slowly increasing the proportion of methanol from 0 to 5%.

3′-Thiothymidylyl-(3′→5′)-thymidine triethylammonium salt (9, **Fig. 2.2**) will appear as a white amorphous solid.

6. If the sodium salt is required, dissolve the sample into water (5 mL) and pass the solution through a small column of Dowex 50W-X8 (Na^+ form) ion-exchange resin (prepared from the appropriate hydrogen form resin). Evaporate the solution using a rotary evaporator and 3′-thiothymidylyl-(3′→5′) thymidine sodium salt will appear as a glass or a white amorphous solid.

3.5. Yields and Spectral Information

Fast-atom bombardment (FAB) mass spectra were recorded on a VG Analytical 7070E mass spectrometer operating with a PDP 11/250 data system and an Ion Tech FAB ion gun working at 8 kV. High-resolution FAB mass spectra were obtained on a VG ZAB/E spectrometer at the SERC Mass Spectrometry Service Centre (Swansea, UK) and reported masses are accurate to 5 ppm. 3-Nitrobenzyl alcohol was used as a matrix unless stated otherwise. ^1H and ^{13}C NMR spectra were measured on either a Bruker AMX400 or a Bruker AC200 spectrometer, chemical shifts are given in ppm downfield from tetramethylsilane as an internal standard and *J* values are given in Hz. Peaks displaying obvious diastereoisomeric splitting are denoted with an asterisk. ^{31}P NMR spectra are referenced to 85% phosphoric acid.

1. **Compound 7**, 5′-*O*-monomethoxytrityl-3′-deoxy-3′-(2,4-dinitrophenyldisulfanyl)-thymidine): **Yield**, 72%. **Elemental analysis**, Found (C, 59.6; H, 4.5; N, 7.6%); $C_{36}H_{32}N_4O_9S_2$ requires C, 59.3; H, 4.42; N, 7.69%). **δ_H(200 MHz; $CDCl_3$)**, 9.24(1H, s, NH), 9.09(1H, s, ArH), 8.34(2H, s, ArH), 7.61 (1H, s, H6), 7.20–7.48(12H, m, ArH), 6.81(2H, d, *J* 8.8, Ph-OMe), 6.21(1H, t, *J* = 5.2, H1′), 4.09(1H, m, H4′), 3.80(3H, s, OMe), 3.75(1H, m, H3′), 3.65 (1H, m, H5″), 3.39(1H, m, H5′), 2.53(2H, m, H2′) and 1.47(3H, s, 5-Me); **m/z**, (FAB^+) 729 ($M + H^+$).
2. **Compound 4**, 3′-*O*-(*tert*-butyldimethylsilyl)-thymindin-5′-yl phosphonate triethylammmonium salt: **Yield**, 89%. **δ_P(81 MHz; $CDCl_3$)**, 6.3. **δ_H(400 MHz; $CDCl_3$)**, 9.18(1H, br s, NH), 7.74(1H, s, H6), 6.84(1H, d, J_{PH} = 617, HP), 6.34(1H, t, *J* = 7.2, H1′), 4.49(1H, m, H4′), 4.08(1H, m, H3′), 4.02(2H, m, H5′), 3.10(6H, q, *J* = 7.2, $N(C\underline{H}_2CH_3)_3$), 2.18(2H, m, H2′), 1.94(3H, s, 5-Me), 1.32(9 H, t, *J* = 7.2, $N(CH_2C\underline{H}_3)_3$), 0.88(9H, s, *t*Bu) and 0.08(6H, s, $SiMe_2$). **m/z**, (FAB^-) 419 $(M - Et,HN)^-$.
3. **Compound 5**, phosphite triester intermediate: **δ_P(81 MHz; $CDCl_3$)**, 116.0.
4. **Compound 8**, fully protected dinucleotide: **Yield**, 68%. **δ_P(81 MHz; $CDCl_3$)**, 14.1. **δ_H(200 MHz; $CDCl_3$)**,

7.65(1H, s, H6), 7.61(1H, s, H6), 7.19–7.37(12H, m, ArH), 6.78(2H, d, J = 8.1, ArH), 6.26(1H, t, H1′), 6.16(1H, t, H1′), 4.38(1H, m), 4.12(1H, m), 3.96(2H, m), 3.86(2H, m), 3.73(3H, s, OMe), 3.43(2H, m), 3.0(6H, q, $N(C\underline{H}_2CH_3)_3$), 2.67(2H, m, H2′), 2.10(2H, m, H2′), 1.84(3H, s, 5-Me), 1.36(3H, s, 5-Me), 1.27(9H, t, $N(CH_2C\underline{H}_3)_3$), 0.83 (9H, s, *t*Bu) and 0.01(6H, s, $SiMe_2$); **m/z**, (FAB^-) 947 (M^-).

5. **Compound 9**, 3′-*S*-phosphorothiolate dinucleotide: **δ_P(81 MHz; D_2O)**, 17.82. **δ_H(400 MHz; D_2O)**, 7.82(1H, s, H6), 7.78(1H, s, H6), 6.30(1H, t, J=4, H1′a), 6.00(1H, m, H1′b), 4.58(1H, m, H3′a), 4.25(1H, m, H5′a), 4.13(2H, m, H4′a+H5″a), 4.03(2H, m, H4′b+H5′b), 3.95(1H, m, H5″b), 3.55(1H, m, H3′b), 2.65(2H, m, H2′b+H2″b), 2.39(2H, m, H2′a+H2″a), 1.85(6H, m, dd, J = 12, 16, 5-Me). **δ_H(50.4 MHz; D_2O),**168.66(C4), 168.35(C4), 153.97(C2), 153.67(C2), 140.22(C6), 139.77(C6), 113.82(C5), 113.17(C5), 88.60 (C1′), 88.50(C1′), 87.94(C3′b), 87.26(C5′a), 87.17(C4′), 72.11(C4′), 66.74(C5′b), 62.05(C3′a), 61.34(C2′), 49.25 (C2′), 14.18(2C, 5-Me). **m/z**, (FAB^-) 561 (M^-). **HRMS**, Found 561.10426; $C_{20}H_{26}N_4O_{11}PS$ requires 561.10564 (M^-).

4. Notes

1. TEAB solution should be stored in the fridge and generally it can be stored for about 3 months. However, since the buffer is volatile, the pH can increase as carbon dioxide is lost. Test the pH every month and if required, bubble carbon dioxide through the solution to lower the pH (*see* Step 1 of **Section 2.2**).
2. If distillation is the preferred method of obtaining anhydrous solvents, check the COSHH assessments as both calcium hydride and sodium react violently with water. The flasks containing the drying agents and solvents should be quenched appropriately within 6 months of use, as peroxides can build up, which can be exceptionally dangerous.
3. Avoid pouring silica gel dry as silica particles are easily inhaled and prolonged exposure can cause breathing difficulties.
4. If for some reason, the sample does not dissolve in the eluent suggested, then pre-loading the impure material onto silica may be required. To achieve this, dissolve the impure

material into a suitable solvent and form a slurry with a small quantity of silica. Evaporate off the solvent and ensure the silica is dry. Pour the silica bearing the impure sample onto the top of the packed column and run it as usual.

5. Argon could be used instead of nitrogen.
6. A nitrogen inlet is usually in the form of a nitrogen balloon. However, a nitrogen bubbler can also be used, which negates the need for a rubber septum and syringe needles. In the case of a two-necked flask, a bubbler is the preferred route as you can remove the stopper from the side arm to aid nitrogen flow through the flask.
7. It is crucial that the conditions are kept strictly anhydrous whilst making the unsymmetrical disulphide. If 5′-*O*-monomethoxytrityl-3′-deoxy-3′-thiothymidine is maintained in solution under an air atmosphere for a prolonged period of time, the symmetrical disulphide might be formed. One way to avoid this is to place the nitrogen inlet directly into the solution and bubble nitrogen through the solution for approximately 30 min.
8. Take care when doing this extraction, as a by-product to the reaction is HCl; so during the extraction with $NaHCO_3$, carbon dioxide can be produced.
9. Phosphorus trichloride is highly toxic.
10. For some preparations, it might be necessary to remove small amounts of contaminating 1,2,4-triazole. This can be achieved by re-dissolving the sample in dichloromethane and washing with TEAB (0.1 M).

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