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Synthesis of ribonucleoside 3′,5′-cyclic phosphorothioates using a modified hydroxybenzotriazole phosphotriester approach

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Abstract. Phosphorothioylation of 3′,5′-dihydroxyl ribonucleosides with *O*-(2-chlorophenyl) *O,O*-bis[6-(trifluoromethyl)-1-benzotriazolyl] phosphorothioate, followed by addition of *N*-methylimidazole and removal of protecting groups, gives ribonucleosides 3′,5′-cyclic phosphorothioates. The latter compounds could also be prepared by phosphorothioylation of 2′,3′,5′-trihydroxyl ribonucleosides.

Introduction

Phosphorothioate-containing analogues of nucleosides play an important role in elucidating the stereochemical course of many biochemical reactions in which nucleotides take part[1].

The first synthesis of adenosine 3′,5′-cyclic phosphorothioate (cAMPS) was achieved by *Eckstein* et al.[2] via a base-promoted cyclisation of adenosine 5′-*O*-bis(*p*-nitrophenyl)-phosphorothioate. Subsequently, *Stec* et al.[3] succeeded in preparing the individual diastereoisomers of cAMPS by converting *N*6,*N*6,2′-*O*-tribenzoyladenosine 3′,5′-cyclic phosphate into a diastereoisomeric mixture of *N*6,*N*6,2′-*O*-tribenzoyladenosine 3′,5′-cyclic phosphoroanilidates. After separation of the diastereoisomers, conversion into cAMPS (Rp) and cAMPS (Sp) was accomplished by reaction with potassium and carbon disulfide followed by removal of all protecting groups. The study of *Stec* et al. also indicated that the cAMPS previously prepared by *Eckstein* et al. was the pure Sp isomer.

As part of a programme to synthesize DNA fragments containing a chiral phosphorothioate linkage[4], we focussed our attention on the development of a rapid and efficient method for the preparation of 3′,5′-cyclic phosphorothioates of ribonucleosides.

Results and discussion

Reese et al.[5] used the reagent *O*-(2,5-dichlorophenyl) *O,O*-bis(1-benzotriazolyl) phosphorothioate for the preparation of thymidine-3′,5′-thymidine phosphorothioate. Unfortunately, the reactivity of the latter reagent was rather low.

On the basis of earlier studies on the phosphorylation properties of the reagent 2-chlorophenyl phosphorodichloridate activated with 6-nitro-[4] or 6-(trifluoromethyl)-[6] substituted 1-hydroxybenzotriazoles[7], we investigated the properties of the bifunctional phosphorothioylating reagent *O*-(2-chlorophenyl)*O,O*-bis[6-(trifluoromethyl)-1-benzotriazolyl] phosphorothioate **3** (see Scheme 1).

As previously reported[6], trifluoromethyl-substitued benzotriazolyl-activated phosphorylating reagents are equally as reactive as their nitro-substituted congeners. Since the latter reagents gave strongly coloured reaction products, we used the easily accessible phosphorothioylating reagent **3** in a two-step synthesis of the ribonucleoside 3′,5′-cyclic phosphorothioates **5a-d**.

In the preparation of phosphorothioate-containing nucleotides using reagent **3**, or similarly activated reagents, activation of the second phosphorylation step by *N*-methylimidazole is required[4]. However, addition of *N*-methyl imidazole may enhance the formation of nucleobase-phosphorylated by-products[6]. We therefore protected the lactam functions in guanosine and uridine.

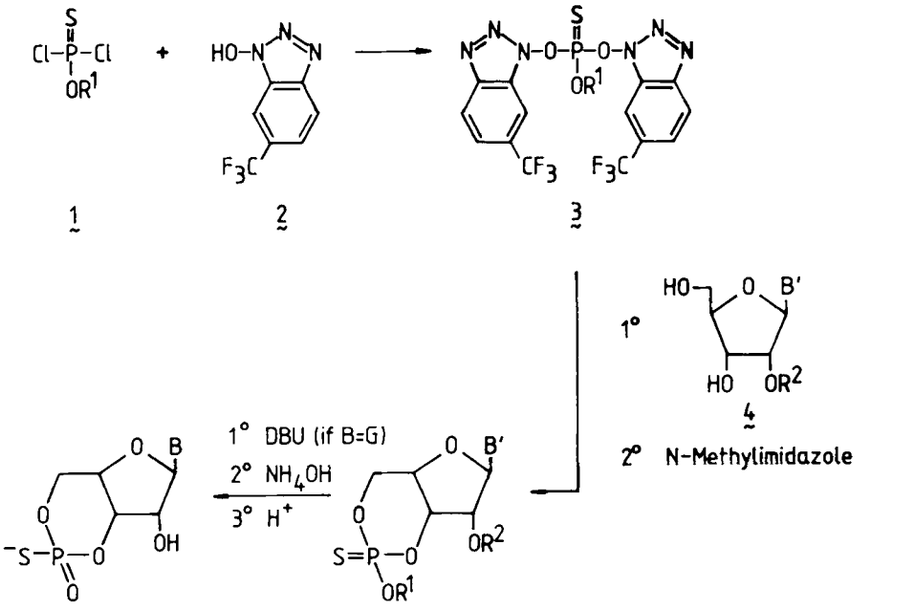
Thus, a properly protected 3′,5′-dihydroxyl ribonucleoside (*e.g.* **4** in Scheme 1) was phosphorothioylated with a slight excess of **3**, a stock solution of which was prepared by adding **2**[8] to **1**[9] in the presence of pyridine. TLC analysis, after 5 min, revealed complete conversion of **4** into material with zero mobility. *N*-Methylimidazole was added and, after 1 h, TLC analysis showed the presence of a product with high mobility. After work-up and short-column chromatography[10], compounds **5a-d** could be isolated in an average yield of 76%. In the case of **5b**, diastereoisomers could be separated by silica-gel column chromatography (for details see Table I).

Table I Relevant data of fully protected 3′,5′-cyclic phosphorothioates **5a-d**.

Compound[a]	Yield (%)	R_f values[b]	^{31}P NMR data[c]
5a (Rp + Sp)	74	0.68	55.94/54.75
5b (Rp)	38	0.56	63.89
5b (Sp)	45	0.70	59.91
5c (Rp + Sp)	67	0.75	60.08/57.39
5d (Rp + Sp)	80	0.72	59.59/56.94

[a] The assignment of the individual diastereoisomers is based on data from *Stec* et al.[3]. [b] Eluens: CH_2Cl_2/CH_3OH (92/8, v/v). [c] ^{31}P NMR in CH_2Cl_2. D_2O was used as external reference. Chemical shifts are in ppm relative to 85% H_3PO_4.

The fully protected 3′,5′-cyclic phosphorothioates **5a-d** were completely deblocked by the following procedure. In the



6 a: B = Adenin-9-yl
b: B = Cytosin-1-yl
c: B = Guanin-9-yl
d: B = Uridin-1-yl

5 a: B' = N6-Benzoyl-A
b: B' = N4-Anisoyl-C
c: B' = N2-Acetyl-O6-(4-nitrophenylethyl)-G
d: B' = O4-(4-nitrophenylsulfonylethyl)-U

R^1 = 2-Chlorophenyl R^2 = Tetrahydropyranyl (THP)

Scheme 1

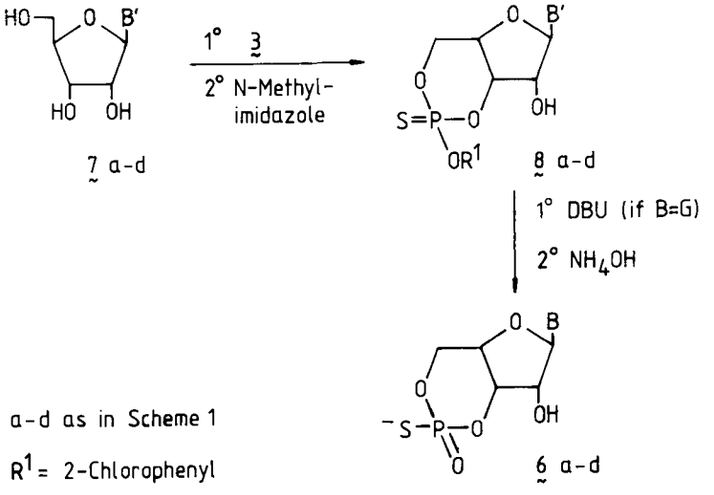
case of **5c** (B' = $G^{Ac,NPE}$), the 2-(4-nitrophenyl)ethyl group was first removed by treatment with DBU[11]. The 2-chlorophenyl, the *N*-acyl and the *O*4-[2-(4-nitrophenylsulfonyl)-ethyl][12] groups were then deblocked by ammonolysis at 50°C. Cleavage of the acid-labile tetrahydropyranyl groups was effected by acid hydrolysis[13]. After neutralization with aqueous ammonia, the reaction mixtures were purified by DEAE-Sephadex anion-exchange chromatography. The identity of the ribonucleoside 3',5'-cyclic phosphorothioates **6a-d** thus obtained was established by ^{31}P and ^1H NMR spectroscopy (see Table II).

Recently, *Eckstein* et al.[14] described a procedure in which nucleoside 3',5'-cyclic phosphorothioates could be prepared starting from 2',3',5'-trihydroxyl nucleosides (*e.g.* **7** in Scheme 2) in an overall yield of 8–15%. In this respect we found that **3** could also be used to attain the same result.

Table II Relevant data of nucleoside 3',5'-cyclic phosphorothioates **6a-d**.

Compound[a]	^{31}P NMR[b]	^1H NMR (H_1·)[c]	^1H NMR (base protons)[c]
6a (Sp)	54.81	6.00 (d)	8.06 (H_2) /8.08 (H_8)
6a (Rp)	56.37	5.95 (d)	8.01 (H_2) /8.02 (H_8)
6b (Sp)	54.58	5.79 (d)	6.01 (H_5,d)/7.63 (H_6,d)
6b (Rp)	56.54	5.92 (d)	5.97 (H_5,d)/7.84 (H_6,d)
6c (Sp)	53.33	5.77 (d)	7.69 (H_8)
6c (Rp)	54.94	5.80 (d)	7.72 (H_8)
6d (Sp/Rp)	54.78/56.33	5.66 (2×)	5.72 (H_5) /7.52 (H_6)

[a] The assignment of the individual diastereoisomers is based on data from *Stec* et al.[3]. [b] ^{31}P NMR in D_2O. Chemical shifts are in ppm relative to 85% H_3PO_4. [c] ^1H NMR in D_2O. Tetramethylammonium chloride (TMA) was used as internal reference. Chemical shifts are in ppm relative to tetramethylsilane (TMS).



7 a-d

8 a-d

6 a-d

a-d as in Scheme 1

R^1 = 2-Chlorophenyl

Scheme 2

For example, the properly protected ribonucleoside **7** (Scheme 2) was allowed to react with a slight excess of **3**. After 5 min, TLC analysis indicated complete conversion of **7** into baseline material. *N*-Methylimidazole was added and, after 60 min, TLC analysis revealed the presence of two new products: a higher-running component (presumably 3',5'-cyclic), which was present in excess over the lower-running one (presumably 2',3'-cyclic). In order to prevent decomposition of the required product, work-up was carried out rapidly keeping the pH of the solution at 5.5. Complete deblocking of the crude 3',5'-cyclic phosphorothioates **8a,b,d** thus obtained was achieved by ammonolysis at 50°C. Crude compound **8c** was deblocked by treatment with DBU[11], followed by ammonolysis at 50°C. The crude products were purified by DEAE-Sephadex anion-exchange chromatography. Compounds **6a-d** were isolated in an overall yield of 14–25% and the ^{31}P NMR data, apart from some minor impurities in the cases of **6b** (B = C) and **6d** (B = U), were identical with those recorded for the same compounds obtained earlier.

The data presented in this paper clearly indicate that the bifunctional phosphorothioylating reagent **3** is very convenient for the introduction of a cyclic 3',5'-phosphorothioate linkage in ribonucleosides.

Experimental

General methods and materiels

Pyridine and dioxane were dried by refluxing with CaH_2 for 16 h and were then distilled. Pyridine was redistilled from *p*-toluenesulfonyl chloride (60 g per litre) and KOH (25 g per litre) and stored under nitrogen. Dioxane was redistilled from $LiAlH_4$ (5 g per litre) and stored under nitrogen. *N*-Methylimidazole was distilled under reduced pressure and stored under nitrogen. 1-Hydroxy-6-(trifluoromethyl)benzotriazole was prepared according to the procedure described by *König* and *Geiger*[8] and dried *in vacuo* (P_2O_5) for 70 h at 50°C. Schleicher and Schüll DC Fertigfolien F1500 LS254 were used for TLC in CH_2Cl_2/CH_3OH (92/8, v/v). Short-column chromatography was performed on Kieselgel 60 (230–400 mesh ASTM) suspended in CH_2Cl_2. DEAE-Sephadex A25 was purchased from Pharmacia (Uppsala, Sweden). ^{31}P NMR spectra were measured at 80.7 MHz (proton-noise decoupled) using a JEOL JNM-FX 200 spectrometer. Chemical shifts are given in ppm (δ) relative to 85% H_3PO_4 as external standard. ^{1}H NMR spectra were measured at 300 MHz, using a Bruker WM-300 spectrometer equipped with an ASPECT-2000 computer, operating in the Fourier Transform mode. TMA was used as internal reference; δ values are given in ppm relative to TMS ($\delta_{TMA} - \delta_{TMS} = 3.19$ ppm). Triethylammonium bicarbonate (TEAB) buffer was prepared by passing a stream of CO_2 gas through a cooled (ice-water bath) solution of triethylamine in deionized water (1 M) until a neutral solution was obtained. Partially protected nucleosides were prepared as described previously[12,15]. Cation-exchange resin (sodium form): a solution of sodium hydroxide (2 M; 100 ml) was passed over a column of cation-exchange resin (Dowex 50W × 8, 100–200 mesh; Fluka, 44514, H^+-form, 1 × 10 cm) followed by eluting the column with sterile water until pH 7.0.

Synthesis of O-(2-chlorophenyl) O,O-bis[6-(trifluoromethyl)-1-benzotriazolyl] phosphorothioate (**3**)

A solution of *O*-(2-chlorophenyl) phosphorodichloridothioate[9] (**1**; 1.92 g; 7.3 mmol) in anhydrous dioxane (7.0 ml) was added dropwise to a stirred solution of dry 1-hydroxy-6-(trifluoromethyl)-benzotriazole (**2**; 3.01 g; 14.8 mmol) and anhydrous pyridine (1.2 ml; 15.0 mmol) in anhydrous dioxane (30 ml) at room temperature. The solution was stirred for 1 h at 20°C and the pyridine-HCl salt was filtered off. The 0.2-M stock solution of **3** (^{31}P NMR: δ_P 66.2 ppm) thus obtained could be stored for several weeks at −20°C.

Synthesis of O-(2-chlorophenyl) 3',5'-O-[2'-O-(tetrahydropyranyl)-N6-benzoyladenosine] phosphorothioate (**5a**)

A solution of phosphorylating agent **3** (0.2 M; 5.5 ml; 1.1 mmol) in dioxane was added to 2'-*O*-(tetrahydropyranyl)-*N*6-benzoyladenosine (**4**; $B' = A^{Bz}$; 456 mg; 1.0 mmol) which had been dried by repeated coevaporation with anhydrous pyridine (3 × 20 ml). The reaction mixture was stirred for 5 min at 20°C. TLC analysis indicated that **4** had been converted into baseline material. Anhydrous *N*-methylimidazole (0.4 ml; 5.0 mmol) was added and the reaction mixture was stirred for 1 h at 20°C. TLC analysis showed the reaction to be complete and a few drops of TEAB buffer (1 M) were added. The reaction mixture was diluted with CH_2Cl_2 (75 ml) and the solution was washed twice with TEAB buffer (1 M; 50 ml, 0.1 M; 3 × 20 ml). The organic layer was dried with $MgSO_4$ (5 g) and concentrated to a small volume. The crude reaction product was applied to a column of silica gel (10 g) suspended in CH_2Cl_2. The column was eluted with CH_2Cl_2, applying a 0 → 4% gradient of CH_3OH. The fractions containing pure **5a** were collected and concentrated to a yellow glass (477 mg). See Table I for other relevant data.

Synthesis of O-(2-chlorophenyl) 3',5'-[2'-O-(tetrahydropyranyl)-N4-anisoylcytidine] phosphorothioate (**5b**)

3',5'-Cyclic phosphorothioate **5b** was prepared from **4** ($B' = C^{An}$) as described above. Rp and Sp isomers were separated on a silica gel column. Yield Rp isomer 249 mg, Sp isomer 289 mg. See Table I for other relevant data.

Synthesis of O-(2-chlorophenyl) 3',5'-[2'-O-(tetrahydropyranyl)-N2-acetyl-O6-[2-(4-nitrophenyl)ethyl]guanosine] phosphorothioate (**5c**)

3',5'-Cyclic phosphorothioate **5c** was prepared from **4** ($B' = G^{Ac,NPE}$) as described above. Yield 500 mg. See Table I for other relevant data.

Synthesis of O-(2-chlorophenyl) 3',5'-[2'-O-(tetrahydropyranyl)-O4-[2-(4-nitrophenylsulfonyl)ethyl]uridine] phosphorothioate (**5d**)

3',5'-Cyclic phosphorothioate **5d** was prepared from **4** ($B' = U^{NPSE}$) as described above. Yield 521 mg. See Table I for other relevant data.

Removal of the 2-(4-nitrophenyl)ethyl group from **5c**

3',5'-Cyclic phosphorothioate **5c** (200 mg; 0.27 mmol) was coevaporated with dry pyridine (3 × 20 ml). A solution of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU; 152 mg; 1.0 mmol) in dry pyridine (2 ml) was added and the mixture was stirred with the exclusion of moisture for 2 h at room temperature. The reaction mixture was brought to pH 6 by the addition of an aqueous solution of acetic acid (0.5 M). The solution of **5c** ($B' = G^{Ac}$) thus obtained was evaporated to a yellow oil.

Deprotection of 3',5'-cyclic phosphorothioates **5a-d**

Compounds **5a**, **5b** and **5d** (150 mg) were dissolved in dioxane (3 ml) and concentrated ammonia (14.8 M; 50 ml) was added. The same procedure was used for compound **5c** after removal of the 2-(4-nitrophenyl)ethyl group (see above). The reaction vessels were sealed and maintained at 50°C for 24 h. Each solution was evaporated to near dryness and acidified with hydrochloric acid (0.01 M; 50 ml). The pH was adjusted to 2.00 by the addition of hydrochloric accd (0.1 M). Each mixture was left for 16 h at 20°C, followed by neutralization with diluted ammonia (5 M) to pH 8.0 and concentrated to a small volume (2 ml).

Synthesis of ribonucleoside 3',5'-cyclic phosphorothioates **6a-d** *via intermediates* **8a-d**

A solution of phosphorylating agent **3** (0.2 M; 2.75 ml; 0.55 mmol) in dioxane was added to base-protected ribonucleoside **7** (0.5 mmol) which had been dried by repeated coevaporation with anhydrous pyridine (3 × 10 ml). The reaction mixture was stirred for 5 min at 20°C after which time TLC analysis indicated complete conversion of **7** into material with zero mobility. Dry *N*-methylimidazole (0.2 ml; 2.5 mmol) was added and stirring was continued for a further 60 min. TLC analysis showed complete conversion into a high-running component (90%) and a low-

running component (10%). A few drops of ammonium hydrogen phosphate buffer [$(NH_4)_2HPO_4$ in water; 1 M; acidified with H_3PO_4 to pH 5.5] were added and the reaction mixture was diluted with CH_2Cl_2 (40 ml). The resulting solution was washed with ammonium hydrogen phosphate buffer (1 M; 50 ml) and the organic layer was concentrated to a small volume. After removal of the 2-(4-nitrophenyl)ethyl group from **8c** (see above), all four mixtures were dissolved in dioxane (3 ml) and concentrated ammonia (14.8 M; 50 ml) was added. After 24 h at 50°C, each solution was evaporated to a small volume (2 ml).

Purification of deblocked 3',5'-cyclic phosphorothioates **6a-d**

After centrifugation, each solution containing crude **6a-d** as obtained above was applied to a column of DEAE-Sephadex A25 (2 × 30 cm) suspended in TEAB buffer (0.05 M). The column was eluted with a linear gradient of 0.05 → 1.0 M TEAB buffer for 24 h at a flow-rate of 40 ml/h. The appropriate fractions were pooled, concentrated to small volume and coevaporated with sterile water (5 × 50 ml) to remove triethylammonium bicarbonate. All four 3',5'-cyclic phosphorothioates were brought into the sodium form by passing them over a column (1 × 10 cm) of Dowex 50W cation-exchange resin (100–200 mesh, sodium form). The resulting aqueous solutions were lyophilized, dissolved in D_2O and relyophilized from D_2O (3 × 2 ml). ^1H and ^{31}P NMR data are shown in Table II.

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