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An approach to the stereoselective synthesis of Sp-dinucleoside phosphorothioates using phosphotriester chemistry

R.Cosstick and D.M.Williams

The Robert Robinson Laboratories, Department of Organic Chemistry, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK

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ABSTRACT

An approach to the stereoselective synthesis of Sp- dinucleoside phosphorothioates has been investigated which utilizes phosphotriester chemistry. The stereoselectivity of internucleotide bond formation between N^4-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-O-(S2-cyanoethyl)phosphorothioate (3) and 3'-O-acetylthymidine has been studied using either mesitylenesulphonyl-5-(pyridin-2-yl)tetrazole (MSPy) or 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) as the activating agent.

The removal of the cyanoethyl group from the protected dinucleoside phosphorothioate has been studied, and conditions are reported which provide rapid deprotection without concomittant desulphurisation.

INTRODUCTION

Oligonucleotides containing diastereomerically pure phosphorothioate linkages have been extensively used to study enzymatic cleavage of phosphodiester bonds[1-4], and conformational properties of DNA[5-7]. The phosphorothioate function can be introduced into oligonucleotides through routes based upon both the phosphotriester[8,9] and phosphoramidite [1,3,5,6,10] method of oligonucleotide synthesis. Unfortunately neither of these approaches is stereospecific and both yield mixtures of Rp- and Sp-epimers. Chirally pure phosphorothioate containing oligonucleotides have been obtained either through dimer couplings of pre-separated diastereomerically pure dinucleoside phosphorothioate derivatives [1,5,9], or by hplc separation of diastereomeric oligonucleotides which result from the sulphurisation of the epimeric phosphite triesters[3,6]. However in both strategies the chromatographic resolution of individual diastereomers is often difficult, and the result is oligonucleotides which are contaminated with a phosphorothioate group of the unwanted configuration.

Recently a stereospecific synthesis for the Rp-configuration of dinucleoside phosphorothioates has been reported[11]. In order to complement this procedure we have become interested in developing a stereo-

selective route to the Sp-diastereomers. Ohtsuka et al[12] have reported that the use of arenesulphonyl-5-(pyridin-2-yl)tetrazolides as activating agents leads to the stereoselective synthesis of dinucleoside phosphate aryl esters. On the basis of proton and phosphorus nmr and CD studies the authors have assigned the Sp-configuration to the dinucleoside triester[13]. It has been proposed that the protected nucleoside-3'-phosphate undergoes selective sulphonylation at the pro-S phosphate oxygen which leads, after subsequent displacement by the tetrazolide, to the new phosphodiester bond being formed with the same oxygen atom. The potential use of this type of activating agent for stereoselective synthesis of the Sp-diastereomer of dinucleoside phosphorothioates was apparent.

In this paper we wish to report the application of mesitylene-sulphonyl-5-(pyridin-2-yl)tetrazole to the synthesis of dinucleoside phosphorothioates. Our results show that the new phosphodiester bond is formed selectively with the pro-S oxygen atom, confirming the conclusions drawn by Ohtsuka et al. However, the degree of stereoselectivity is less than that obtained for the synthesis of dinucleoside phosphate aryl esters[12]. We have reinvestigated the use of the cyanoethyl group to protect the sulphur atom of the phosphorothioate linkage, and developed conditions under which this group can be efficiently removed without the accompanying desulphurisation which had been previously noted[14].

EXPERIMENTAL

Analytical tlc was carried out on Alugram Sil G/UV_{254} plates and eluted with 10% MeOH/$CHCl_3$ unless otherwise stated. Preparative silica tlc was performed on Merck 7747 kieselgel 60 PF_{254} Plates. Merck 9385 kieselgel 60 was used for flash chromatography. Approximately 0.1% pyridine was added to all eluants used for flash chromatography. Hplc was performed on a Varian 5000 liquid chromatograph equipped with a Varian UV50 detector operating at 256 nm. Separations were performed on a column (250 x 4.6 mm) packed with 5μ Spherisorb ODS silica. The following elution systems were used: system 1, a linear gradient of acetonitrile (0-25% in 20 min) in 50 mM KH_2PO_4, pH 6.0; system 2, a stepwise gradient of acetonitrile (0-25% in 20 min, 25-50% in an additional 10 min) in 50 mM KH_2PO_4, pH 6.0 A flow rate of 1 ml/min was used for both systems.

^{31}P Nmr spectra were obtained on a Jeol FXQ60 spectrometer operating at 24.15 MHz. with ^{1}H broad-band decoupling. Peaks were referenced to an external standard of 85% H_3PO_4 and the downfield deuterium signal was used

to lock samples run in d5-pyridine. ^{1}H nmr spectra were obtained on a Perkin Elmer R34 spectrometer operating at 220 MHz. Chemical shifts are given in ppm downfield from an internal standard of tetramethylsilane.

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. 3-Nitrobenzylalcohol was used as the matrix.

Pyridine, toluene, CH_3CN and CH_2Cl_2 were dried by refluxing with and then distilling from calcium hydride. Dioxan was refluxed over sodium in the presence of benzophenone and then distilled. Et_3N and $^{t}BuNH_2$ were distilled from potassium hydroxide and then refluxed and distilled from calcium hydride. Petroleum-ether was distilled, washed with concentrated HCl, water and refluxed and distilled from $CaCl_2$.

An authentic sample of Rp-and Sp-dCp(S)dT was a kind gift from Professor F. Eckstein.

Synthesis of N^4-benzoyl-5'-O-4,4'-dimethoxytrityl-2'-deoxycytidine-3'-O-(S2-cyanoethyl)phosphorothioate (3)

Disodium (S2-cyanoethyl) phosphorothioate[14] (0.96 g, 4.55 mmol) was dissolved in water (20 ml) and stirred with Dowex-pyridinium form resin (10 g) for 30 min. The resin was removed by filtration and the filtrate concentrated to a gum. The resulting pyridinium salt was dried by co-evaporation with dry pyridine (2 x 10 ml). N^4-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine[15] (0.48 g, 0.758 mmol) was added and the mixture dried by coevaporation with dry pyridine (3 x 10 ml). The mixture was suspended in dry pyridine (1 ml), and N,N'-dicyclohexylcarbodiimide (2.35 g, 11.39 mmols) added and the flask sealed. After 30 hours at room temperature tlc indicated that no starting material remained. Water (2 ml) was added and the mixture left overnight. Precipitated dicyclohexylurea was filtered off, washed with 50% aqueous pyridine (50 ml) and the combined washings and filtrate extracted with petroleum-ether (3 x 20 ml). The aqueous pyridine layer was evaporated, dissolved in chloroform (70 ml) and extracted with a 0.3 M solution of triethylammonium bicarbonate (100 ml). The combined chloroform extracts were dried over sodium sulphate and evaporated. The crude product was purified by flash chromatography (15 g of silica) using a gradient of 0 - 10% MeOH in $CHCl_3$. Appropriate fractions containing the product were pooled, evaporated, coevaporated with toluene to remove pyridine, dissolved in a small amount of chloroform and precipitated into pentane. After drying the filtered material over P_2O_5 a cream-coloured powder (316 mg, 0.358 mmol, 47%) was obtained.

^{31}P Nmr δ (d5-pyridine) 12.252 ppm.

M/z (FAB$^-$) 782 (M-H)$^-$, 479(M-DMT)$^-$, 214(cytosine^{Bz}-H)$^-$

1-Mesitylenesulphonyl-5-(pyridin-2-yl)tetrazole (MSPy)

To an ice-cold solution of mesitylenesulphonyl chloride (10.0 g, 45.72 mmol) in dry dioxan (174 ml), 5-(pyridin-2-yl)tetrazole[12] (7.40 g, 50 mmol) and Et_3N (6.95 ml, 50 mmol) were added. The mixture was stirred in the dark for three hours at room temperature after which time it was filtered and evaporated. The residue was dissolved in CH_2Cl_2 (300 ml), washed with saturated aqueous $NaHCO_3$ (2 x 50 ml), H_2O (2 x 50 ml), dried over sodium sulphate and evaporated to dryness. The crude product was redissolved in CH_2Cl_2 (50 ml), filtered with suction through silica gel (100 g) packed in a sinter funnel and eluted with CH_2Cl_2. Fractions containing the MSPy were concentrated and precipitated into dry petroleum-ether. Isolated yield, 8.22 g, 25 mmol, 52%.

^1H Nmr; δ ($CDCl_3$); 8.78 (d, 1H), 8.20 (d, 1H), 7.86 (m,1H), 7.43 (m, 1H) all pyridine ring protons, 7.04 (s, 2H mesitylene ring protons), 2.78 (s, 6H, $2CH_3$). 2.32 (s, 3H, CH_3) ppm.

Synthesis of Rp-and Sp-3'-O-(N^4-benzoyl-5'-O-4,4'-dimethoxytrityl-2'-deoxycytidyl)- 5'-O-(3'-O-acetyl-2'-deoxythymidyl)-(S2-cyanoethyl)-phosphorothioate. DMTdC^{Bz}p(SCH_2CH_2CN)dT_{OAc}(Rp- and Sp-(4)).

A. Using 1-mesitylenesulphonyl-3-nitro-1,2,4-triazole (MSNT) as an activating agent

The triethylammonium salt of (3) (300 mg, 0.339 mmol) and 3'-O-acetylthymidine[16] (77 mg, 0.271 mmol) were dried by coevaporation with dry pyridine (3 x 10 ml), and finally dissolved in dry pyridine (2 ml). MSNT[17] (251 mg, 0.847 mmol) was added and the resulting solution stirred for 50 min. Water (1 ml) was then added and after a further 20 mins the mixture was poured into a saturated $NaHCO_3$ solution (20 ml). The product was extracted into $CHCl_3$ (6 x 25 ml), the combined extracts washed with water (20 ml), dried over $MgSO_4$, and finally evaporated to dryness. The crude product was purified by column chromatography (30 g silica gel). Elution was carried out with a 0-10% gradient of MeOH in $CHCl_3$ and the appropriate fractions were pooled and concentrated . The product was purified further by preparative tlc using 2% MeOH/$CHCl_3$ and 3 x 5% MeOH/$CHCl_3$ as eluant. The purified product was dissolved in a small volume of $CHCl_3$ and precipitated into petroleum-ether. A white powder (151 mg, 0.144 mmol, 53%) was obtained.

^{31}P Nmr, δ ($CDCl_3$) 27.0 ppm.

M/z (FAB^-), 1048$(M-H)^-$, 995$(M-CH_2CHCN-H)^-$, 782 ($DMTdC^{Bz}p(O, SCH_2CH_2CN))^-$.

B. Using MSPy as an activating agent

The triethylammonium salt of (3) (303 mg, 0.343 mmol) and 3'-O-acetylthymidine (77 mg, 0.271 mmol) were dried by coevaporation with dry pyridine (2 x 5ml) followed by dry toluene (2 x 5 ml). MSPy (226 mg, 0.687 mmol) was then added, followed by dry CH_2Cl_2: pyridine (2ml, 20:1, v/v). After three hours the reaction was quenched by the addition of water (1 ml) and left for a further 15 min. The solution was poured into saturated aqueous $NaHCO_3$ (20 ml) and extracted into $CHCl_3$ (6 x 25 ml). The combined extracts were washed with water (2 x 20ml), dried over Na_2SO_4 and evaporated. The crude product was purified by silica gel (36 g) chromatography using a gradient of 0 - 3% MeOH in $CHCl_3$. The appropriate fractions were evaporated, dissolved in a small amount of $CHCl_3$ and precipitated into petroleum-ether. The filtered material was dried in vacuo to afford a white powder (138 mg, 0.132 mmol, 46%).

^{31}P Nmr δ ($CDCl_3$) 27.0 ppm.

M/z (FAB^-) 1048 $(M-H)^-$

Deprotection

A. Removal of dimethoxytrityl group

Rp- and Sp-(4) (10-50 mg) Was treated with 80% aqueous acetic acid (5 ml) for one hour. The solution was then evaporated to dryness and the residue dissolved in water (20ml). The resulting solution was concentrated to about 5 ml and then extracted with ether (5 x 10 ml). The aqueous phase was evaporated to dryness, dissolved in dioxan and analysed. Deprotection was quantitative as judged by hplc, and gave a single peak at 31.9 min (System 2).

B. Removal of cyanoethyl group

A portion of the dioxan solution from the previous experiment was evaporated, dried by coevaporation with pyridine and subjected to one of the following set of deprotection conditions:

(A) Treatment with Et_3N/dioxan/water (5:4:1, v/v/v, 2 ml) for 3 hrs at room temperature.

(B) Treatment with dry CH_3CN/Et_3N (1:1, v/v, 2 ml) for 48 hrs at room temperature.

(C) Treatment with dry Et_3N/pyridine (1:2, v/v, 3 ml) for 5 days at room temperature.

(D) Treatment with dry $^tBuNH_2$/pyridine (1:10, v/v, 1.1 ml) for 3 hrs at room temperature.

The reactions were monitored by tlc and when complete each reaction mixture was evaporated. The individual residues were coevaporated with water and dissolved in dioxan/water (1:1, v/v, 2 ml). The resultant solutions were analysed by hplc using system 2. Retention times were as follows: $dC^{Bz}pdT_{OAc}$ 25.5 min and Rp-and Sp-$dC^{Bz}p(S)dT_{OAc}$ 27.3 and 27.7 min respectively.

C. Removal of the benzoyl and acetyl groups

Samples from the previous deprotection were evaporated to dryness and treated with concentrated aqueous ammonia solution (35%, w/w) at 40°C for 16 hrs in a screw-top flask. The solutions were then evaporated, dissolved in distilled water, and extracted with $CHCl_3$ (1 x 10ml) and finally analysed by hplc using system 1. Deprotection appeared to be quantitative as judged by hplc. Retention times were as follows: dCpdT (14.0 min); Rp-dCp(S)dT (15.5 min) and Sp-dCp(S)dT (15.9 min).

RESULTS AND DISCUSSION

Synthesis of the protected dinucleoside phosphorothioates

The application of the phosphotriester approach to the synthesis of dinucleoside phosphorothioates requires the protection of the sulphur atom during formation of the internucleotide linkage. There are few examples of S-protected dinucleoside phosphorothioates in the literature and to our knowledge only two protecting groups have been studied; the 2-cyanoethyl group [14, 17, 18] which is removed under mildly basic conditions and the methyl group[19] which is removed by treatment with thiophenoxide anion. Of the two groups the former is the more attractive since removal of the methyl group was accompanied by about 20% of internucleotide bond cleavage[19].

Burgers and Eckstein[14] had previously prepared 5'-O-adenosyl-3'-O-uridyl phosphorothioate (Up(S)A) using S2-cyanoethyl phosphorothioate (1) that had been activated with 2,4,6-triisopropylbenzenesulphonyl chloride. Our initial attempts to prepare $DMTdC^{Bz}p(SCH_2CH_2CN)$ (3) using a similar procedure were unsuccessful; the product was isolated in poor yield and FAB^- mass spectrometry indicated that it was contaminated with derivatives of the triisopropylbenzenesulphonyl chloride. Low yields had previously

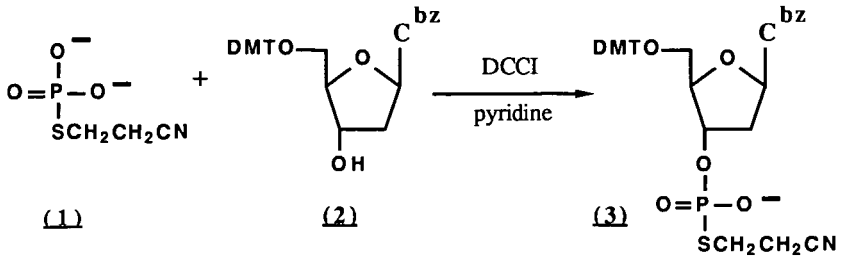
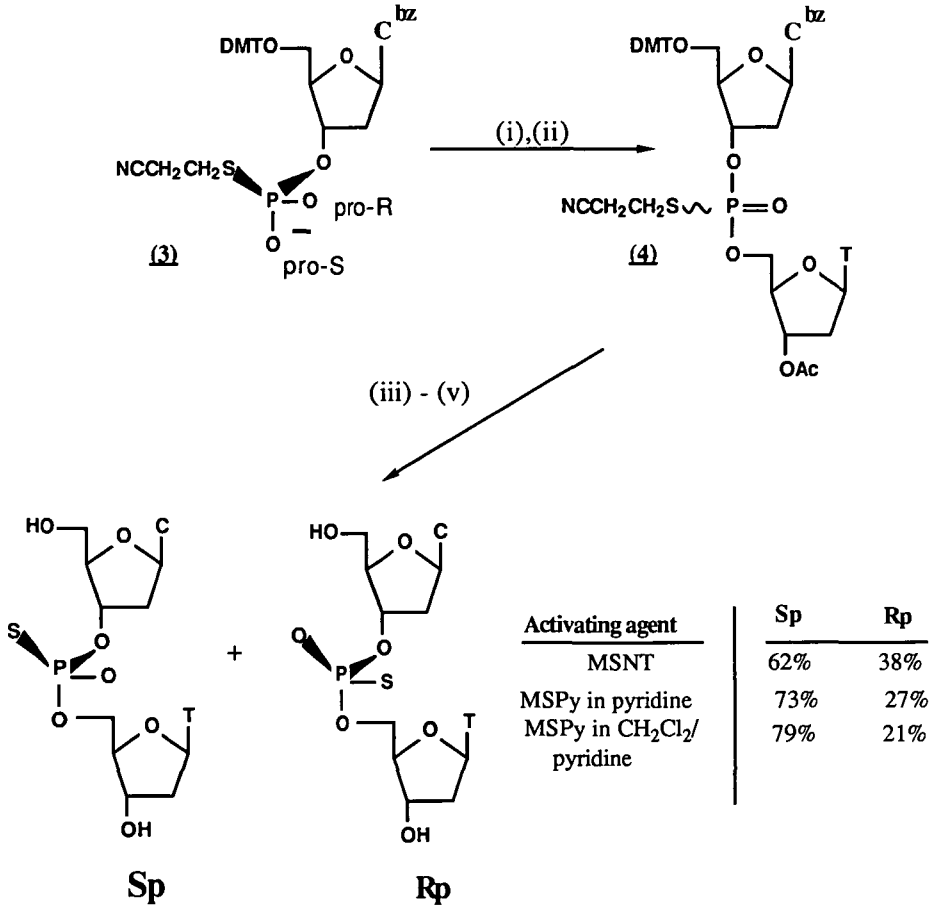


Figure 1. Synthesis of $DMTdC^{Bz}p(SCH_2CH_2CN)$

been reported for a reaction very similar to this [20]. A much more satisfactory preparation of (3) was achieved by reaction of $DMTdC^{Bz}$ (2) with the pyridinium salt of (1) which had been activated with dicyclohexylcarbodiimide (DCCI) (Figure 1). This procedure is similar to that previously reported by Schaller et al., for the synthesis of the corresponding O2-cyanoethyl phosphates.[21] (3) Was obtained in a yield of 47% and appeared to be pure by both ^{31}P nmr and tlc. Confirmation of the identity of the product was obtained by FAB^- mass spectrometry which gave the expected $(M-H)^-$ ion signal. In a subsequent experiment (not reported) it was demonstrated that this procedure could also be applied to the synthesis of N2-isobutyryl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyguanosine-3'-O-(S2-cyanoethyl) phosphorothioate; a yield of about 50% was achieved.

Activation of (3) with MSNT[22] and subsequent reaction with 3'-O-acetylthymidine in pyridine under standard conditions afforded the fully protected dinucleoside phosphorothioate as a mixture of two diastereomers in an overall yield of 53% (Figure 2). No separation of the diastereomers was obtained on tlc using 10% $MeOH/CHCl_3$ and the possibility of using other eluants to achieve resolution was not explored. Our previous experience[5] with dinucleoside phosphorothioates indicates that the best resolution is normally achieved once the 3'-protecting group is removed. The ^{31}P nmr chemical shift (27.0 ppm) was consistent with that expected for a single P-S bond in a triester;[14] signals corresponding to the two diastereomers were not resolved at 24.15 MHz. Confirmation of the identity of the product was obtained by FAB^- mass spectrometry which gave the expected $(M-H)^-$ ion signal.

Condensation reactions with MSPy were generally performed in CH_2Cl_2 containing about 5% pyridine. Under these conditions the combined isolated



Activating agent	Sp	Rp
MSNT	62%	38%
MSPy in pyridine	73%	27%
MSPy in CH_2Cl_2/ pyridine	79%	21%

Figure 2. Synthesis of dCp(S)dT and diastereomeric composition.
(i) Activating agent; (ii) 3'-O-acetylthymidine; (iii) 80% acetic acid; (iv) ^{t}BuNH$_2$/pyridine(1:10, v/v) and (v) conc. aq. NH$_3$.

yield of Rp-(4) and Sp-(4) was about 46%. Reactions carried out with MSPy activation in neat pyridine gave significantly lower yields (30 - 35%).

Deprotection studies and analysis of diastereomeric ratios

Quantitative removal of the dimethoxytrityl group was obtained by treatment with 80% acetic acid for one hour at room temperature. The resultant partially protected dinucleoside phosphorothioate triester was dried prior to decyanoethylation.

The removal of the cyanoethyl group from dinucleoside phosphorothioate triesters occurs by a β-elimination mechanism and, has previously been

Table 1 Decyanoethylation of dinucleoside phosphorothioate triesters.

Method	Conditions[a]	Time	% β-elimination[b]	% Desulphurisation[b]
A	Et_3N/dioxan/ H_2O (5:4:1,v/v/v)	3 hrs	77[c]	20[c]
B	Et_3N/CH_3CN (1:1, v/v)	2 days	>99	<1
C	Et_3N/pyridine (1:2, v/v)	5 days	94	6
D	$^tBuNH_2$/pyridine (1:10, v/v)	3 hrs	>99%	nd

nd = none detected.

a. All reactions carried out at room temperature.

b. Calculated from hplc analysis of the reactions using gradient system 2. Retention times for the products are as follows; $dC^{Bz}pdT_{OAc}$ (desulphurised product) 25.5 min, Rp- and Sp-$dC^{Bz}p(S)dT_{OAc}$ 27.3 and 27.7 min respectively and Rp-and Sp-$dC^{Bz}p(SCH_2CH_2CN)dT_{OAc}$ (starting material) 31.9 and 32.3 min respectively.

c. Trace of starting material remained.

accomplished using Et_3N/dioxane/water (5:4:1, v/v/v)[14]. Under these conditions deprotection was accompanied by approximately 20% desulphurisation. It appeared that desulphurisation resulted from hydrolysis of the triester since the phosphorothioate diesters are unaffected by this treatment and suggested that this problem could be eliminated if anhydrous reaction conditions were employed. A variety of amines have previously been used in non-aqueous conditions to effect removal of a cyanoethyl group from dinucleoside phosphate triesters. Those conditions which have commonly been used include tertiary amines in acetonitrile or pyridine[23-27] or primary amines in pyridine.[28-29]

In the present study the decyanoethylation of $dC^{Bz}p(SCH_2CH_2CN)dT_{OAc}$ was carried out using either Et_3N or $^tBuNH_2$ under the conditions outlined in Table 1. From this table it can be seen that the best results were obtained with $^tBuNH_2$/pyridine (1:10, v/v) (Method D), after 3 hours the reaction was > 99% complete and more importantly there was no evidence of desulphurisation. This procedure was adopted in all subsequent preparations of dCp(S)dT. The comparative rates of deprotection obtained with $^tBuNH_2$ and Et_3N are in agreement with the results of Hsiung,[29] where deprotection was found to be quickest with primary amines. However, the much slower rates of cleavage observed in this study indicate that the

S-cyanoethyl group is significantly more stable to β-elimination than the analogous O-cyanoethyl group.

Removal of benzoyl and acetyl groups was accomplished using standard conditions. Byproducts from the deprotection were removed from the reaction mixture by extraction with chloroform, and the resulting Rp- and Sp-dCp(S)dT mixture was greater than 98% pure as judged by hplc. The absolute configuration of diastereomers was assigned on the basis of the following established criteria: the Sp-diastereomer is hydrolysed by nuclease P1 whilst the Rp-diastereomer remains undigested[30] and the Sp-diasteromer always has a greater retention time on reverse phase silica than the Rp-diastereomer.[31] In additon, the products of our syntheses were compared to authentic samples of Rp-and Sp-dCp(S)dT.

The ratio of the diasteromers present in each of the preparations of dCp(S)dT was obtained directly from hplc analysis of the samples using gradient system 1 and are tabulated in figure 2. Activation with MSPy afforded a diastereomeric excess of 58% of the Sp-epimer in $CHCl_2$/pyridine which was reduced to 46% in pyridine alone. However, the degree of stereoselectivity obtained in this study is significantly less than that reported for the dinucleoside aryl phosphate triesters (approximately 90%). The results are in accordance with those of Ohtsuka et al., since they demonstrate that MSPy activation results in the new phosphodiester bond being formed selectively with the pro-S oxygen atom of the original nucleoside-3'- phosphate diester. This confirmatory evidence is particularly significant since the configuration of dinucleoside phosophorothioates can be assigned with greater certainty than those of the dinucleoside aryl phosphate triesters; (configuration assignment of dinucleoside phosphorothioates is based upon the substrate specificities of phophodiesterases[30] and the X-ray structural analysis of the substrates[7]). The stereoselectivity of MSPy activation has been attributed to the coordination the pyridine nitrogen atom in MSPy to the pro-R oxygen atom of the nucleoside phosphate diester. However, our results demonstrate that MSNT also exerts a small degree of diastereoselectivity indicating that additional factors, such as steric effects, are likely to be involved.

It is interesting to note that synthesis of dinucleoside phosphorothioates by the hydroxybenzotriazole[8,9] and phosphoramidite[5] approaches favours the Rp-configuration. The present phosphotriester synthesis appears to be the method of choice if dinucleoside phosphorothioates enriched with the Sp-configuration are required.

To conclude we have demonstrated that the synthesis of dinucleoside S2-cyanoethyl phosphorothioate triesters using MSPy is stereoselective for the Sp-diastereomer (58% diastereomeric excess). In addition the cyanoethyl group can be removed from the triesters without accompanying desulphurisation by treatment with $^{t}BuNH_2$/pyridine (1:10). Bulkier S-protecting groups are currently being studied with the aim of increasing the stereoselectivity of the reaction.

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