Tetrahedron Vol. 49, No. 5, pp. 1115-1132, 1993
Printed in Great Britain

0040-4020/93 $6.00+.00

STEREOSELECTIVE SYNTHESIS OF CYCLIC DINUCLEOTIDE PHOSPHOROTHIOATES

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(*Received in UK* 19 *October* 1992)

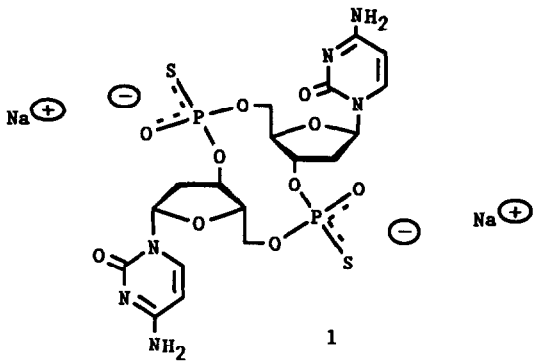
Key Words: cyclic oligonucleotides; phosphorothioates; hydrogen phosphonate; stereoselective cyclization; cytidine dinucleotide.

Abstract- Cyclic dicytidyl phosphorothioates have been synthesized by using the hydrogenphosphonate methodology followed by oxidative sulfurization with a complete stereoselectivity in the cyclization step.

INTRODUCTION

Our choice to synthesize cyclic oligodeoxynucleotide phosphorothioates came out from the occurrence of activity of linear oligodeoxycytidine phosphorothioates (14-28 mers) in inhibiting the reverse transcription process within the HIV life cycle[1] and a reported case of inhibition of DNA-dependent RNA polymerase by cyclic diribonucleotides (with normal phosphate moieties)[2]. Indeed these two unrelated findings suggested us to consider cyclic oligodeoxyribonucleotide phosphorothioates as potential anti-HIV agents. So our specific target was the synthesis of cyclo-(3′→5′)-bis-P-thiocytidylyl-(3′→5′) (structure **1**).

With this purpose in mind we had to face two new synthetic problems: the unprecedented cyclization through a modified phosphate group like the phosphorothioate moiety and the stereochemical aspect arising from the formation of a new stereogenic centre in the cyclization step.



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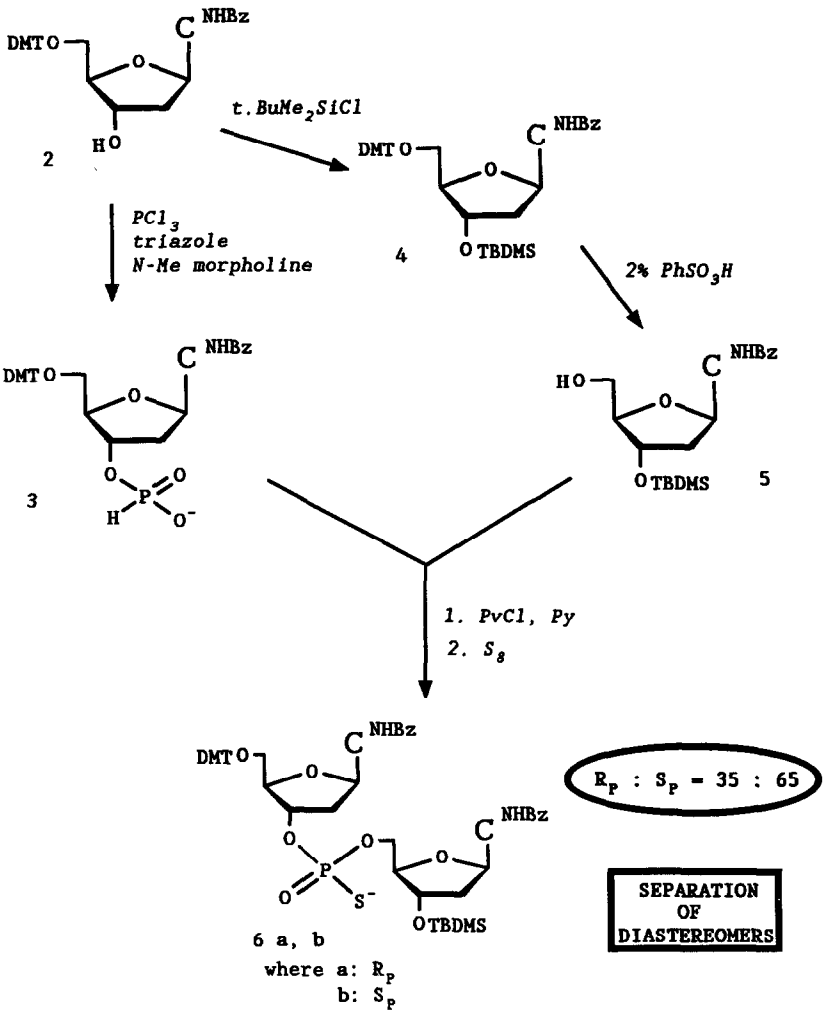
Besides the random homopolymerizations leading to some cyclic oligonucleotides in a mixture with linear analogues, previous synthetic approaches to cyclic oligonucleotides almost exclusively deal with unmodified phosphate groups, basically according to two strategies within methodologies well established for linear oligomerizations. Mayol et al. used to anchor a first term of the oligomer on a solid support through the amino group of the base providing elongation and cyclization by the "phosphotriester method"[3]. The cyclization through the normal phosphate moiety have been also performed in diluted solution always using the "phosphotriester method" in the classic form, namely through a diprotected phosphate group[4-6] or in the hydroxybenzotriazole modification by van Boom et al. after deprotection of a terminal allylphosphate moiety[7] and by Garbesi, Colonna et al. providing phosphorylation and cyclization in a one-pot reaction[8]. Actually cyclic dinucleotides containing one phosphorothioate group have been also synthesized but again an usual phosphate group was involved in the cyclization step[9]. In this paper we wish to report an efficient and stereoselective synthesis of cyclic dinucleotide phosphorothioates. Among the methods used for the formation of the modified internucleotide bond, we chose the "hydrogenphosphonate" method followed by thiooxidation because in a previous study of ours it was found to have a certain degree of stereoselectivity in the case of ribonucleotides[10].

RESULTS

Cyclic dinucleotide phosphorothioates were prepared starting from two properly protected monomers, the first with the reactive 3′-hydrogenphosphonate group and the second with the 5′-hydroxylic group free. A key point in the synthetic design was the appropriate choice of

SCHEME 1

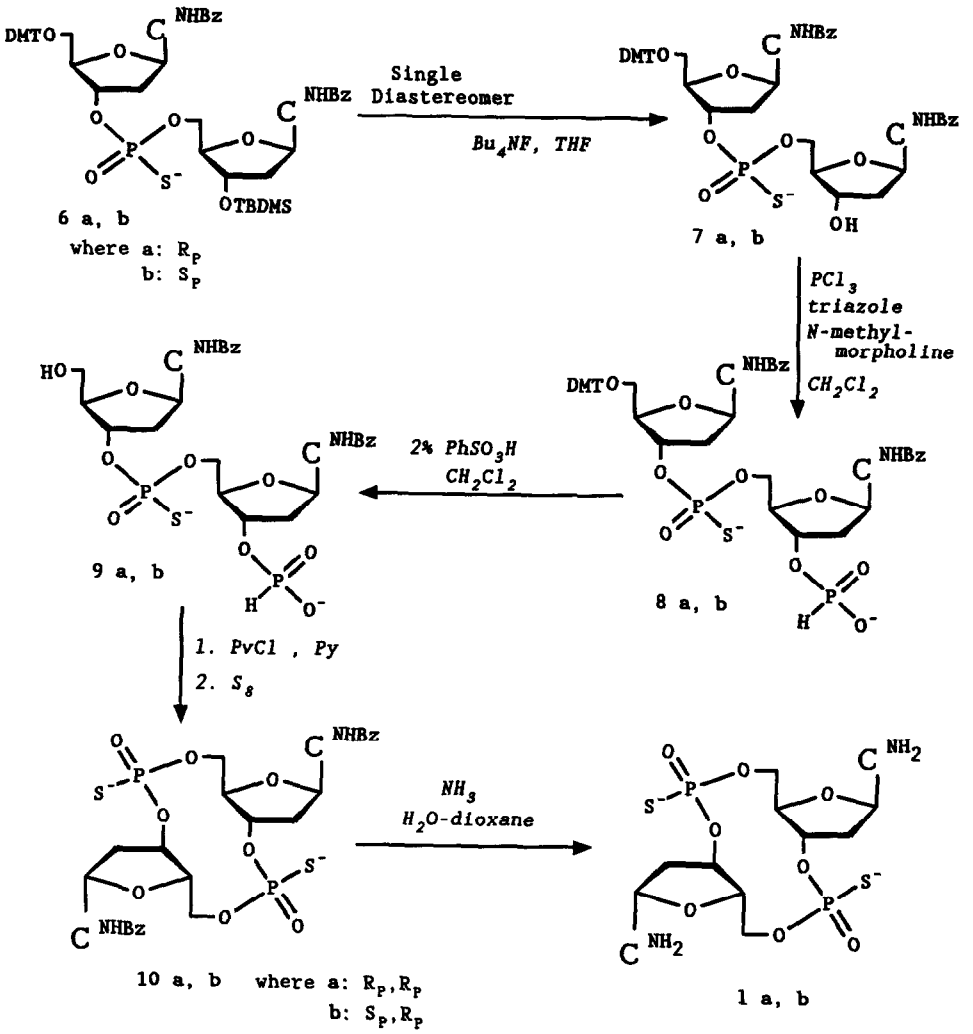


C(NH2)(I) = cytosine	DMT = dimethoxytrityl Bz = benzoyl TBDMS = t.butyldimethylsilyl

protecting groups. On the base of our previous experience the dimethoxytrityl and the t.butyldimethylsilyl group for the protection of the primary and secondary hydroxylic functions respectively and the benzoyl group for the protection of amino function of cytosine appeared to be suitable for our scope.

The first part of the synthesis is outlined in the scheme 1. The two protected nucleosides **3** and **5** were synthesized by literature procedures[11] starting from N^4-benzoyl-5'-O-(dimethoxytrityl)-2'-deoxycytidine (**2**). Coupling reaction between the 3'-hydrogenphosphonate **3** and the 5'-hydroxy monomer **5** was accomplished by using pivaloyl chloride as condensing agent in dry pyridine at room temperature, followed by oxidative sulfurization with elemental sulfur, to give the two diastereomers **6a** (R_P isomer) and **6b** (S_P isomer) in 70% yield and 35:65 ratio. Samples of the diastereomers were independently converted to completely deprotected dimers by usual procedures in order to assign the configuration at the phosphorus atom as it will be explained later in the stereochemical considerations. Separate diastereomers **6a** and **6b** were independently subjected to a sequence of reactions leading to the cyclic compounds as shown in the scheme 2. Indeed they were selectively deprotected by treatment with tetrabutylammonium fluoride in tetrahydrofuran to give the dimers **7a** and **7b** in 80% and 95% yield respectively. A second phosphorylation by using phosphorus trichloride, N-methylmorpholine and triazole gave the 3'-hydrogenphosphonate dimers **8a** and **8b** in 67% and 55% yield respectively. A critical step in our route was the deprotection of dimethoxytrityl group in 5'-position in the presence of the labile hydrogenphosphonate function. Several reaction conditions were tried as zinc bromide in methylene chloride at 0°C, trifluoroacetic and trichloroacetic acids in methylene chloride at 0°C, but only a short treatment of the 3'-hydrogenphosphonate dimers **8a** and **8b** with a 2% solution of benzenesulfonic acid in methylene chloride and methanol (70:30) at 0°C gave the dimers **9a** and **9b** in satisfactory yield (73% and 60% respectively). Another crucial point in our synthesis was the intramolecular condensation of the hydrogenphosphonate dimers **9a** and **9b** to give the cyclic dinucleotide phosphorothioates **10a** and **10b**. All the attempts to perform this reaction in diluted conditions, as previously described for the method of phosphate triester[7], failed and the better results were instead obtained by adding 3 equivalents of pivaloyl chloride as condensing agent to a solution of dimers **9a** and **9b** dissolved in dry pyridine and dimethylformamide in the usual conditions of concentration. The reaction was followed by HPLC and after disappearance of the starting compound, elemental sulfur was added. After complete

SCHEME 2


DMTO
NHBz
OTBDMS
6 a, b
where a: R_P
b: S_P
Single
Diastereomer
Bu_4NF, THF
OH
7 a, b
PCl_3
triazole
N-methyl-morpholine
CH_2Cl_2
8 a, b
2% $PhSO_3H$
CH_2Cl_2
HO
9 a, b
1. PvCl , Py
2. S_8
NH_3
H_2O-dioxane
NH_2
10 a, b where a: R_P,R_P
b: S_P,R_P
1 a, b

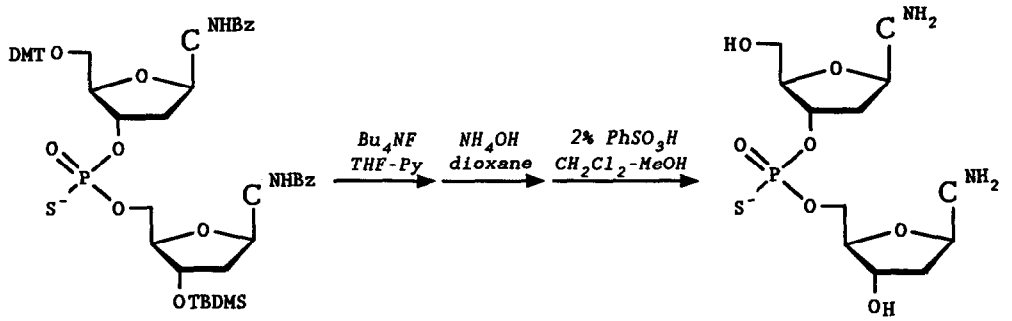
oxidation of the H-phosphonate to phosphorothioate the reaction mixture was quenched by adding triethylamine and after usual work-up was purified by reverse phase column chromatography on RP8 silica gel. Both **9a** and **9b** gave only one of the two expected cyclic dimers, so the reaction showed a complete stereoselectivity. Cyclic dimers **10a** (R_P,R_P isomer) and **10b** (S_P,R_P isomer) were isolated as triethylammonium salts in 55% and 49% yield respectively. The absolute configuration at the new phosphorus stereocentre was determined by using NMR spectroscopy as explained in the stereochemical considerations. Finally, the deprotection of benzoylcytosine moiety was performed by ammonia aqueous solution in dioxane at 50°C to obtain the cyclic dimers **1a** (R_P,R_P isomer) and **1b** (S_P,R_P isomer) in 85% and 72% yield respectively.

STEREOCHEMICAL CONSIDERATIONS

As it can be seen in formula **1**, the target molecule has two stereogenic phosphorus atoms and hence three possible diastereomers could be obtained: R_P,R_P , R_P,S_P and S_P,S_P , providing not to change the stereogenic carbon atoms of the glycosidic parts already present in the starting materials.

In order to ascertain the phosphorus configuration in the first internucleotidic phosphorothioate moiety introduced along the synthetic route, samples of the two diastereomers **6a** and **6b** have been independently and completely deprotected to obtain the dinucleotides **11a** and **11b** as shown in the scheme 3. The two diastereomers have two different signals

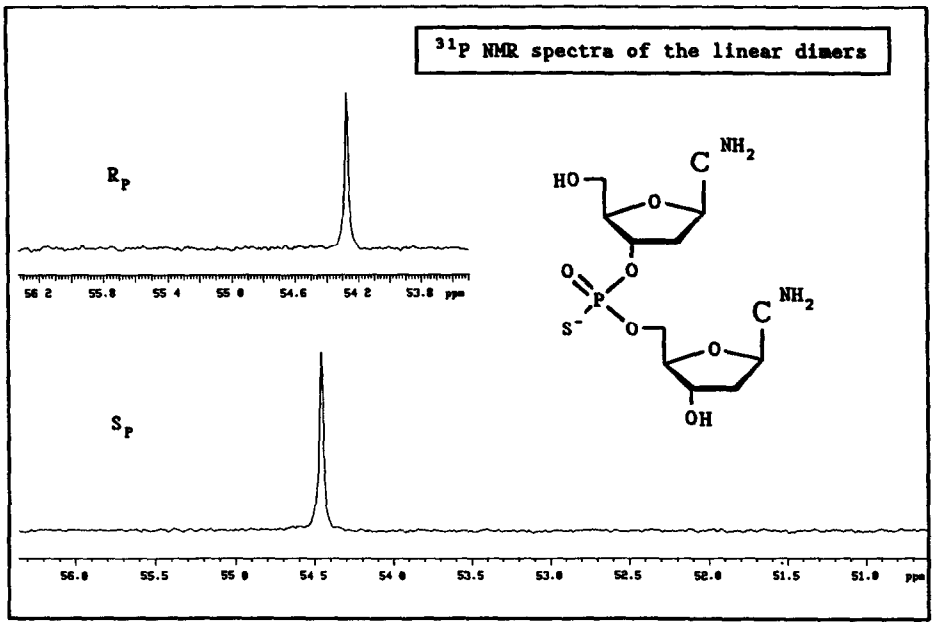
SCHEME 3

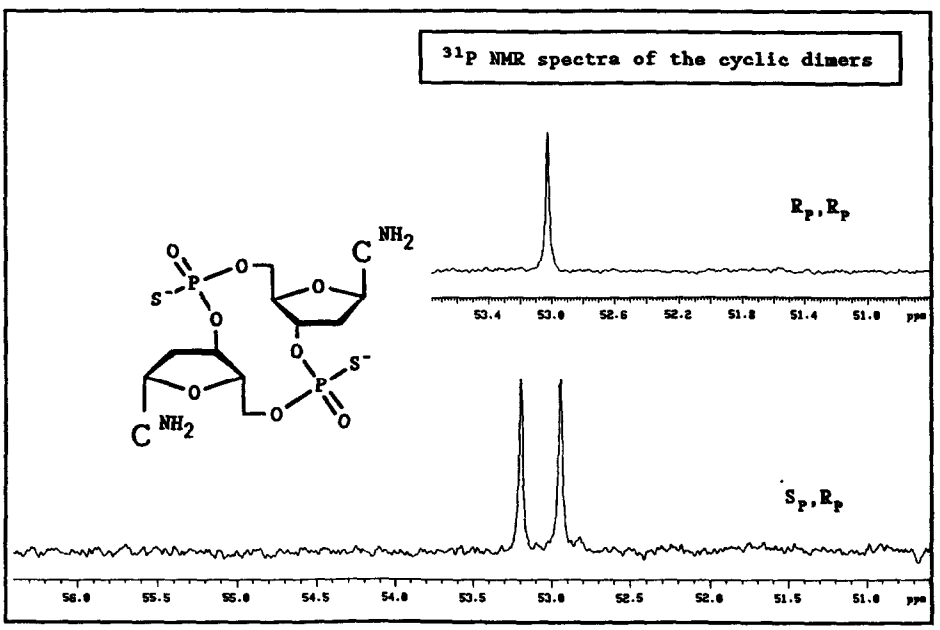


6 a or 6 b

11 a or 11 b

FIGURE


31P NMR spectra of the linear dimers
Rp
Sp
HO
NH2
OH
56.0 55.5 55.0 54.5 54.0 53.5 53.0 52.5 52.0 51.5 51.0 ppm


31P NMR spectra of the cyclic dimers
Rp,Rp
Sp,Rp
NH2
53.4 53.0 52.6 52.2 51.8 51.4 51.0 ppm
56.0 55.5 55.0 54.5 54.0 53.5 53.0 52.5 52.0 51.5 51.0 ppm

in the ^{31}P NMR spectra, as shown in figure. They were subjected to the enzymatic action of the snake venom phosphodiesterase, being known that R_P configuration is more labile while S_P configuration is largely resistant. Indeed the disappearance of the starting sample was followed by HPLC along the time and the sample **11a** was degraded much faster than the sample **11b**. Accordingly the configuration R_P has been assigned to **11a** and the configuration S_P to **11b**. So the process to a dideoxyribonucleotide phosphorothioate involving the hydrogenphosphonate method followed by thiooxidation showed a modest stereoselectivity in favor of the S_P configuration, that is S_P : R_P 65 : 35 .

Anyway much more striking and unexpected was the result of the stereochemical elucidation of the phosphorothioate moiety formed in the cyclization step, namely the second stereogenic phosphorus introduced along the synthetic pathway. The assignment of configuration at this phosphorus atom has been deduced from the NMR data and from the fact that both the open dinucleotide R_P (**9a**) and its diastereomer S_P (**9b**) after cyclization to the structure **10** gave only one of the two possible diastereomers and the product of the reaction was different in the two cases. Indeed **10a** and hence **1a** could be the R_P,R_P-diastereomer or the R_P,S_P-diastereomer, on the other hand **10b** and hence **1b** could be the S_P,R_P-diastereomer (being identical to the R_P,S_P-diastereomer) or the S_P,S_P-diastereomer. The molecule having the two phosphorus atoms with the same configuration occur to have a twofold axis of symmetry so accordingly their ^{1}H and ^{31}P NMR spectra are expected to show a single set of signals due to the two magnetically equivalent nucleotidic parts of the molecule. This equivalence doesn't occur when the phosphorus atoms have different configuration causing a splitting in two set of signals. Indeed the ^{31}P NMR spectrum of **1a** showed only one signal that is the case of the R_P,R_P-diastereomer, on the contrary the ^{31}P NMR spectrum of **1b** shows two signals of equal intensity indicating unequivocally that **1b** is the S_P,R_P-diastereomer, as shown in figure. ^{1}H NMR spectra also are consistent with these assignments.

In conclusion either starting from the R_P uncyclized dinucleotide **9a** or the S_P analogue **9b** the cyclization step followed by thiooxidation is characterized by a complete R_P stereoselectivity. The phosphorothioate formation by the present methodology actually involves two steps: the internucleosidic coupling to an H-phosphonate derivative and a subsequent thiooxidation. It has been recently proved by F. Seela et al.[12] that the thiooxidation of separated H-phosphonate diastereomers is a "stereospecific" process. This finding leads to the conclusion that in both the cases of asymmetric induction occurring in our process, the

stereoselectivity actually takes place at the level of the first new stereogenic centre formation, namely at the level of the H-phosphonate diester formation. The obliged direction of attack by the nucleophile caused by the reduced conformational freedom in the forming cycle can be thought to be responsible for the complete stereoselectivity occurred in the cyclization step.

EXPERIMENTAL

^{1}H NMR spectra were recorded using VARIAN VXR 200 and VXR 400 spectrometers. ^{31}P NMR were recorded on a VARIAN VXR 200 spectrometer. Coupling constants are measured in Hertz. NMR data are given indicating with A and B respectively the first and the second deoxyribose ring from the 5′ end to the 3′ end of the dinucleotide molecule. Mass spectra were recorded using a VARIAN Mat-311A spectrometer and in the reported data M is the molecular weight of the acid form. Column chromatography was performed on Carlo Erba silica gel 60 (230-400 mesh). Reverse phase column chromatography was performed on Merck LiChroprep RP8 (230-400 mesh). HPLC was performed using Beckman System Gold with Partisphere Whatman column. Analytical thin layer chromatography was performed using pre-coated glass-backed plates (Merck Kieselgel 60 F_{254}) and visualized by ultraviolet light and acidic ammonium molybdate (IV) solution. Snake venom phosphodiesterase (SVPD from Crotalus durissus) for enzymatic hydrolysis was purchased from Boehringer.

R_P and S_P N^4-Benzoyl-5′-O-(dimethoxytrityl)-2′-deoxy-P-thiocytidylyl-(3′→5′)-N^4-benzoyl-3′-O-(t.butyldimethylsilyl)-2′-deoxycytidine, triethylammonium salt (6a and 6b)

N^4-Benzoyl-5′-O-(dimethoxytrityl)-2′-deoxycytidine-3′-(hydrogen phosphonate), triethylammonium salt (3) (8.8 g, 11.0 mmol) and N^4-benzoyl-3′-O-(t.butyldimethylsilyl)-2′-deoxycytidine (5) (4.9 g, 11.0 mmol) were dried by coevaporation with dry pyridine and dissolved in the same solvent (120 ml). Distilled pivaloyl chloride (3.39 ml, 27.5 mmol) was added dropwise and the reaction mixture was stirred under nitrogen atmosphere for 30 minutes at room temperature. Elemental sulfur (3.52 g, 110 mmol) was added and, after 3 hours, the reaction was quenched by addition of triethylamine (10 ml). The reaction mixture was concentrated under vacuum, the residue dissolved in methylene chloride, washed with water, dried (Na_2SO_4) and evaporated under vacuum. Purification and separation of the diastereomers 6 was accomplished by two silica gel column chromatographies eluting with a stepwise gradient from ethyl acetate to ethyl acetate/methanol 95:5. The two products were obtained

with a total yield of 70%.

The high Rf compound was the R_P isomer (**6a**) (3.87 g, 28% yield). TLC: Rf 0.34, ethyl acetate/methanol 85:15.

^{1}H NMR (400 MHz, DMSO-d_6): δ= 0.07 (s, 6H, two $SiCH_3$); 0.66 (s, 9H, $SiC(CH_3)_3$); 2.1-2.6 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.2-3.4 (m, 2H, $CH_2$5'A); 3.72 (s, 6H, two OCH_3); 3.7-4.1(m, 3H, H4'B + $CH_2$5'B); 4.32 (m, 1H, H4'A); 4.50 (m, 1H, H3'B); 5.06 (m, 1H, H3'A); 6.15, 6.18 (two dd, J= 6.3 Hz, 2H, H1'A + H1'B); 6.87 (d, J= 8.5 Hz, 4H, aromatic hydrogens ortho to OCH_3); 7.0-7.6 (m, 17H, aromatic hydrogens + H5A + H5B); 8.00 (d, J= 7.3 Hz, 4H, aromatic hydrogens ortho to CONH); 8.12, 8.52 (two d, J= 7.3 Hz, 2H, H6A + H6B); 11.20 (bs, 2H, two NHCO); + triethylammonium signals.

^{31}P NMR (81 MHz, DMSO-d_6): δ= 54.22 (H_3PO_4 as external reference).

FAB-MS (positive ions): m/z 1201 ($[M+2Na-H]^+$); 1179 ($[M+Na]^+$).

The low Rf compound was the S_P isomer (**6b**) (5.81 g, 42% yield). TLC: Rf 0.25, ethyl acetate/methanol 85:15.

^{1}H NMR (400 MHz, DMSO-d_6): δ= 0.02 (s, 6H, two $SiCH_3$); 0.82 (s, 9H, $SiC(CH_3)_3$); 2.0-2.7 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.2-3.5 (m, 2H, $CH_2$5'A); 3.67 (s, 6H, two OCH_3); 3.90 (m, 2H, $CH_2$5'B); 3.96 (m, 1H, H4'B); 4.28 (m, 1H, H4'A); 4.44 (m, 1H, H3'B); 5.04 (m, 1H, H3'A); 6.12, 6.16 (two dd, J= 6.4 Hz, 2H, H1'A + H1'B); 6.83 (d, J= 8.5 Hz, 4H, aromatic hydrogens ortho to OCH_3); 7.0-7.6 (m, 17H, aromatic hydrogens + H5A + H5B); 7.94 (d, J= 7.3 Hz, 4H, aromatic hydrogens ortho to CONH); 8.12, 8.45 (two d, J= 7.3 Hz, 2H, H6A + H6B); 11.20 (bs, 2H, two NHCO); + triethylammonium signals.

^{31}P NMR (81 MHz, DMSO-d_6): δ= 54.70 (H_3PO_4 as external reference).

FAB-MS (positive ions): m/z 1201 ($[M+2Na-H]^+$); 1179 ($[M+Na]^+$).

N^4-Benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-2'-deoxycytidine, tetrabutylammonium salt (7a)

A 0.2M tetrabutylammonium fluoride solution in tetrahydrofuran/pyridine 4:1 (40 ml, 8 mmol) was added to N^4-benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-O-(t.butyldimethylsilyl)-2'-deoxycytidine, triethylammonium salt (**6a**) (4.05 g, 3.2 mmol) and the resulting solution was stirred overnight at room temperature. The reaction mixture was evaporated under reduced pressure, the residue was dissolved in methylene chloride and the organic solution washed with water. The purification was performed by silica gel column chromatography eluting with ethyl acetate/methanol 8:2 to give the title compound (**7a**) as a white solid (3.28 g, 80% yield).

^{1}H NMR (200 MHz, DMSO-d_6): δ= 2.0-2.3 (m, 2H, $CH_2$2'B); 2.3-2.7 (m, 2H, $CH_2$2'A); 3.0-3.5 (m, 2H, $CH_2$5'A); 3.8-4.0 (m, 3H, H4'B + $CH_2$5'B);

4.30 (m, 2H, H3'B + H4'A); 5.02 (m, 1H, H3'A); 6.14, 6.16 (two dd, J= 6.3 Hz, 2H, H1'A + H1'B); 6.86 (d, J= 8.8 Hz, 4H, aromatic hydrogens ortho to OCH_3); 7.0-7.7 (m, 17H, aromatic hydrogens + H5A + H5B); 7.97 (d, J= 7.0 Hz, 4H, aromatic hydrogens ortho to CONH); 8.09, 8.50 (two d, J= 7.5 Hz, 2H, H6A + H6B); + tetrabutylammonium signals.

FAB-MS (negative ions): m/z 1041 ($[M-H]^-$).

FAB-MS (positive ions): m/z 1043 ($[M+H]^+$).

N^4-Benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-2'-deoxycytidine, tetrabutylammonium salt (7b)

N^4-Benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-O-(t.butyldimethylsilyl)-2'-deoxycytidine, triethyl ammonium salt (**6b**) (4.15 g, 3.3 mmol) was treated as described in the above preparation to give the title compound (**7b**) (4.02 g, 95% yield).

^1H NMR (200 MHz, DMSO-d_6): δ= 1.9-2.3 (m, 2H, $CH_2$2'B); 2.3-2.6 (m, 2H, $CH_2$2'A); 3.0-3.4 (m, 2H, $CH_2$5'A); 3.69, 3.70 (two s, 6H, two OCH_3); 3.90 (m, 2H, $CH_2$5'B); 3.96 (m, 1H, H4'B); 4.28 (m, 2H, H3'B + H4'A); 5.02 (m, 1H, H3'A); 6.13, 6.17 (two m, 2H, H1'A + H1'B); 6.85 (d, J= 8.8 Hz, 4H, aromatic hydrogens ortho to OCH_3); 7.0-7.7 (m, 17H, aromatic hydrogens + H5A + H5B); 7.98 (d, J= 7.1 Hz, 4H, aromatic hydrogens ortho to CONH); 8.10, 8.47 (two d, J= 7.3 Hz, 2H, H6A + H6B); 11.17, 11.23 (two bs, 2H, two NHCO); + tetrabutylammonium signals.

FAB-MS (positive ions): m/z 1043 ($[M+H]^+$).

N^4-Benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-(hydrogenphosphonate)-2'-deoxycytidine, triethyl ammonium salt (8a)

A stirred solution of fresh distilled phosphorus trichloride (1.1 ml, 12.5 mmol) and N-methylmorpholine (13.8 ml, 125 mmol) in dry methylene chloride (125 ml) was added, at room temperature, to 1,2,4-triazole (2.93 g, 42.5 mmol), dried under vacuum in the presence of phosphorus pentoxide. After 30 minutes the reaction mixture was cooled to 0°C and a solution of N^4-benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-2'-deoxycytidine, tetrabutylammonium salt (**7a**) (3.2 g, 2.5 mmol) (dried by coevaporation with pyridine) in methylene chloride (40 ml) was added dropwise over 20 minutes. The reaction mixture was stirred for 10 minutes then poured into 1.0M aqueous triethylammonium hydrogencarbonate (100 ml), shaken and separated. The aqueous phase was extracted with methylene chloride, then the combined organic phase was dried (Na_2SO_4) and evaporated to a foam. Purification by silica gel column chromatography eluting with methylene chloride/

methanol/triethylamine 70:30:2 gave the title compound (**8a**) (2.2 g, 67% yield) as a white foam.

^{1}H NMR (400 MHz, DMSO-d_6): δ= 2.2-2.6 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.2-3.4 (m, 2H, $CH_2$5'A); 3.70 (s, 6H, two OCH_3); 3.9-4.4 (m, 4H, $CH_2$5'B + H4'A + H4'B); 4.90, 5.08 (two m, 2H, H3'A + H3'B); 6.65 (d, J= 605 Hz, 1H, P-H); 6.18 (m, 2H, H1'A + H1'B); 6.87 (d, J= 8.5 Hz, 4H, aromatic hydrogens ortho to OCH_3); 7.0-7.6 (m, 17H, aromatic hydrogens + H5A + H5B); 7.98 (m, 4H, aromatic hydrogens ortho to CONH); 8.11, 8.60 (two m, 2H, H6A + H6B); 11.18, 11.24 (two bs, 2H, two NHCO); + triethylammonium signals.

FAB-MS (negative ions): m/z 1127 ($[M+Na-2H]^-$) ; 1105 ($[M-H]^-$).

N^4-Benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-(hydrogenphosphonate)-2'-deoxycytidine, triethyl ammonium salt (8b)

N^4-Benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-2'-deoxycytidine, tetrabutylammonium salt (**7b**) (3.85 g, 3 mmol) was treated as described in the above preparation to give the title compound (**8b**) (2.16 g, 55% yield).

^{1}H NMR (400 MHz, DMSO-d_6): δ= 2.1-2.7 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.2-3.4 (m, 2H, $CH_2$5'A); 3.71 (s, 6H, two OCH_3); 3.9-4.2 (m, 3H, $CH_2$5'B + H4'B); 4.38 (, 1H, H4'A); 4.80, 5.10 (two m, 2H, H3'A + H3'B); 6.18 (m, 2H, H1'A + H1'B); 6.65 (d, J= 605 Hz, 1H, P-H); 6.85 (d, J= 8.5 Hz, 4H, aromatic hydrogens ortho to OCH_3); 7.0-7.6 (m, 17H, aromatic hydrogens + H5A + H5B); 8.00, 8.01 (two d, J= 7.3 Hz, 4H, aromatic hydrogens ortho to CONH); 8.07, 8.38 (two d, J= 7.3 Hz, 2H, H6A + H6B); 11.09 (bs, 2H, two NHCO); + triethylammonium signals.

FAB-MS (positive ions): m/z 1129 ($[M+Na]^+$); 1107 ($[M+H]^+$).

N^4-Benzoyl-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-(hydrogenphosphonate)-2'-deoxycytidine, triethylammonium salt (9a)

Benzenesulfonic acid (1.53 g, 9.67 mmol) was added to an ice-cooled solution of N^4-benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-(hydrogenphosphonate)-2'-deoxycytidine, triethylammonium salt (**8a**) (2.0 g, 1.53 mmol) in methylene chloride/methanol 7:3 (76 ml). The reaction mixture was stirred for 10 minutes, then was poured into 1.0M aqueous triethylammonium hydrogencarbonate (100 ml). The organic phase was extracted several times with water then the aqueous extracts were collected and concentrated under reduced pressure. The crude was purified by reverse phase column chromatography on RP8 using a linear gradient from 0% to 20% of acetonitrile in water to give

after freeze-drying the title compound (**9a**) as a white lyophile (1.12 g, 73% yield).

^{1}H NMR (400 MHz, DMSO-d_6): δ= 2.0-2.6 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.68 (m, 2H, $CH_2$5'A); 4.2-4.4 (m, 4H, H4'A + H4'B + $CH_2$5'B); 4.88 (, 2H, H3'A + H3'B); 6.17 (m, 2H, H1'A + H1'B); 6.62 (d, J= 605 Hz, 1H, P-H); 7.2-7.6 (m, 8H, aromatic hydrogens + H5A + H5B); 7.98 (d, J= 7.4 Hz, 4H, aromatic hydrogens ortho to CONH); 8.40, 8.61 (two d, J= 8.0 Hz, 2H, H6A + H6B); 11.20 (bs, 2H, two NHCO); + triethylammonium signals.

FAB-MS (negative ions): m/z 825 ([M+Na-2H]$^-$).

FAB-MS (positive ions): m/z 849 ([M+2Na-H]$^+$); 827 ([M+Na]$^+$).

N^4-Benzoyl-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-(hydrogenphosphonate)-2'-deoxycytidine, triethylammonium salt (9b)

N^4-Benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→ 5')-N^4-benzoyl-3'-(hydrogenphosphonate)-2'-deoxycytidine, triethyl ammonium salt (**8b**) (2.19 g, 1.67 mmol) was treated as described in the above preparation to give the title compound (**9b**) (1.01 g, 60% yield).

^{1}1H NMR (400 MHz, DMSO-d_6): δ= 2.1-2.7 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.66 (m, 2H, $CH_2$5'A); 4.0-4.3 (m, 4H, H4'A + H4'B + $CH_2$5'B); 4.94 (, 1H, H3'B); 5.01 (, 1H, H3'A); 6.16, 6.20 (two m, 2H, H1'A + H1'B); 6.65 (d, J= 600 Hz, 1H, P-H); 7.2-7.7 (m, 8H, aromatic hydrogens + H5A + H5B); 7.98, 8.01 (two d, J= 7.0 Hz, 4H, aromatic hydrogens ortho to CONH); 8.33, 8.39 (two d, J= 7.3 Hz, 2H, H6A + H6B); 11.15 (bs, 2H, two NHCO); + triethylammonium signals.

FAB-MS (negative ions): m/z 847 ([M+2Na-3H]$^-$); 825 ([M+Na-2H]$^-$); 803 ([M-H]$^-$).

FAB-MS (positive ions): m/z 849 ([M+2Na-H]$^+$); 827([+Na]$^+$).

Cyclo-N^4-benzoyl-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5'), triethylammonium salt (10a)

N^4-Benzoyl-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-(hydrogenphosphonate)-2'-deoxycytidine, triethylammonium salt (**9a**) (0.84 g, 0.834 mmol) was dried by coevaporation with pyridine, dissolved in pyridine (23 ml) and dimethylformamide (2 ml). Pivaloyl chloride (0.3 ml, 2.5 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 30 minutes under nitrogen atmosphere. Elemental sulfur (0.27 g, 8.34 mmol) was added, and after three hours the reaction was quenched by addition of triethylamine (5 ml). The reaction mixture was concentrated under reduced pressure, the crude was washed with water and the sulfur excess filtered off. The aqueous solution was purified by reverse phase column chromatography on RP8 using water/acetonitrile 9:1.

The fractions containing the product were combined and lyophilized to obtain the title compound (**10a**) as a white solid (0.47 g, 55% yield).

^{1}H NMR (200 MHz, DMSO-d_6): δ= 2.2-2.5 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.7-4.1 (m, 6H, H4'A + H4'B + $CH_2$5'A + $CH_2$5'B); 4.69 (m, 2H, H3'A + H3'B); 6.03 (m, 2H, H1'A + H1'B); 7.32 (d, J= 7.5 Hz, 2H, H5A + H5B); 7.46 (m, 4H, aromatic hydrogens meta to CONH); 7.55 (m, 2H, aromatic hydrogens para to CONH); 7.94 (d, J= 7.6 Hz, 4H, aromatic hydrogens ortho to CONH); 8.39 (d, J= 7.5 Hz, 2H, H6A + H6B); 11.17 (bs, 2H, two NHCO); + triethylammonium signals.

FAB-MS (negative ions): m/z 839 ($[M+Na-2H]^-$); 817 ($[M-H]^-$).

FAB-MS (positive ions): m/z 841 ($[M+Na]^+$); 819 ($[M+H]^+$).

Cyclo-N^4-benzoyl-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5'), triethylammonium salt (10b)

N^4-Benzoyl-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-(hydrogenphosphonate)-2'-deoxycytidine, triethylammonium salt (**9b**) (1.01 g, 1.0 mmol) was treated as described in the above preparation to give the title compound (**10b**) (0.5 g, 49% yield).

^{1}H NMR (400 MHz, DMSO-d_6): δ= 2.3-2.6 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.7-4.2 (m, 6H, H4'A + H4'B + $CH_2$5'A + $CH_2$5'B); 4.77 (m, 2H, H3'A + H3'B); 6.05 (m, 2H, H1'A + H1'B); 7.29, 7.37 (two bs, 2H, H5A + H5B); 7.47 (m, 4H, aromatic hydrogens meta to CONH); 7.58 (m, 2H, aromatic hydrogens para to CONH); 7.98 (d, J= 7.6 Hz, 4H, aromatic hydrogens ortho to CONH); 8.40, 8.66 (two d, J= 7.6 Hz, 2H, H6A + H6B); 10.96, 11.04 (two bs, 2H, two NHCO); + triethylammonium signals.

FAB-MS (negative ions): m/z 839 ($[M+Na-2H]^-$); 817 ($[M-H]^-$).

FAB-MS (positive ions): m/z 841 ($[M+Na]^+$); 819 ($[M+H]^+$).

Cyclo-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5'), sodium salt (1a)

Cyclo-N^4-benzoyl-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5'), triethylammonium salt (**10a**) (0.33 g, 0.32 mmol) was dissolved in 17% aqueous ammonia (87 ml) and stirred in a sealed vessel for 3 hours at 50°C. The reaction mixture was cooled to room temperature and concentrated under vacuum. The residue was dissolved in water and the insoluble benzoylamide was filtered off. The filtrate was purified by reverse phase column chromatography on RP8 eluting with water. The fractions containing the product were lyophilized and the white solid so obtained was dissolved in water and passed through a Dowex 50W-X8 sodium strong cation exchanger column. The resulting aqueous solution was freeze-dried to give the title compound as sodium salt (**1a**)

(180 mg, 85% yield).

^{1}H NMR (400 MHz, DMSO-d_6): δ= 2.0-2.4 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.6-4.0 (m, 6H, $CH_2$5'A + $CH_2$5'B + H4'A + H4'B); 4.68 (m, 2H, H3'A + H3'B); 5.66 (d, J= 7.5 Hz, 2H, H5A + H5B); 5.99 (m, 2H, H1'A + H1'B); 7.02, 7.16 (two bs, 4H, two NH_2); 7.82 (d, J= 7.5 Hz, 2H, H6A + H6B).

^{31}P NMR (81 MHz, DMSO-d_6): δ= 53.03 (H_3PO_4 as external reference).

FAB-MS (negative ions): m/z 653 ($[M+2Na-3H]^-$); 631 ($[M+Na-2H]^-$); 609 ($[M-H]^-$).

FAB-MS (positive ions): m/z 655 ($[M+2Na-H]^+$); 633 ($[M+Na]^+$).

Cyclo-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→5')-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5'), sodium salt (1b)

Cyclo-N^4-benzoyl-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5'), triethylammonium salt (**10b**) (0.5 g, 0.49 mmol) was treated as described in the above preparation to give the title compound (**1b**) (0.23g, 72% yield).

^{1}H NMR (400 MHz, DMSO-d_6): δ= 2.0-2.4 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.6-4.0 (m, 6H, $CH_2$5'A + $CH_2$5'B + H4'A + H4'B); 4.68 (m, 2H, H3'A + H3'B); 5.64, 5.73 (two d, J= 7.5 Hz, 2H, H5A + H5B); 5.99 (two m, 2H, H1'A + H1'B); 6.92, 7.00, 7.11, 7.15 (four bs, 4H, two NH_2); 7.78, 8.10 (two d, J= 7.5 Hz, 2H, H6A + H6B).

^{31}P NMR (81 MHz, DMSO-d_6): δ= 52.94, 53.19, (H_3PO_4 as external reference).

FAB-MS (negative ions): m/z 653 ($[M+2Na-3H]^-$); 631 ($[M+Na-2H]^-$); 609 ($[M-H]^-$).

FAB-MS (positive ions): m/z 655 ($[M+2Na-H]^+$); 633 ($[M+Na]^+$).

2'-Deoxy-(R_P)-P-thiocytidylyl-(3'→5')-2'-deoxycytidine, sodium salt (11a)

0.2M Tetrabutylammonium fluoride solution in tetrahydrofuran/pyridine 4:1 (3.12 ml) was added to N^4-benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(R_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-O-(t.butyldimethylsilyl)-2'-deoxycytidine, triethylammonium salt (**6a**) (310 mg, 0.25 mmol) and the resulting solution was stirred overnight at room temperature and evaporated under vacuum. The residue, dissolved in dioxane (29 ml), was treated with 30% aqueous ammonia (39 ml) and stirred in a sealed vessel for 3 hours at 50°C. The reaction mixture was cooled to room temperature and concentrated under vacuum. Benzenesulfonic acid (0.25 g) was added to a ice-cooled solution of the above obtained crude in methylene chloride/methanol 7:3 (12.5 ml). The reaction mixture was stirred for 10 minutes then was poured into 1.0M aqueous triethylammonium hydrogencarbonate (20

ml). The aqueous layer was washed with diethyl ether and concentrated under reduced pressure. The crude was purified by reverse phase column chromatography on RP8 eluting with water. The fractions containing the product were lyophilized and the white solid so obtained was dissolved in water and passed through a Dowex 50W-X8 sodium strong cation exchanger column. The resulting aqueous solution was freeze-dried to give the title compound as sodium salt (**11a**) (80 mg, 58% overall yield).

^{1}H NMR (200 MHz, DMSO-d_6): δ= 1.8-2.3 (m, 4H, $CH_2$2'A + $CH_2$2'B); 3.57 (m, 2H, $CH_2$5'A); 3.8-3.9 (m, 3H, $CH_2$5'B + H4'B); 4.01 (m, 1H, H4'A); 4.25 (m, 1H, H3'B); 4.76 (m, 1H, H3'A); 5.71 (d, J= 7.5 Hz, 2H, H5A + H5B); 6.17 (m, 2H, H1'A + H1'B); 7.0-7.2 (bs, 4H, two NH_2); 7.79, 7.92 (two d, J= 7.5 Hz, 2H, H6A + H6B).

^{31}P NMR (81 MHz, DMSO-d_6): δ= 54.27 (H_3PO_4 as external reference).

FAB-MS (negative ions): m/z 553 ($[M+Na-2H]^-$); 531 ($[M-H]^-$).

FAB-MS (positive ions): m/z 555 ($[M+Na]^+$); 533 ($[M+H]^+$).

2'-Deoxy-(S_P)-P-thiocytidylyl-(3'→5')-2'-deoxycytidine, sodium salt (11b)

N^4-Benzoyl-5'-O-(dimethoxytrityl)-2'-deoxy-(S_P)-P-thiocytidylyl-(3'→5')-N^4-benzoyl-3'-O-(t.butyldimethylsilyl)-2'-deoxycytidine, triethyl ammonium salt (**6b**) (400 mg, 0.32 mmol) was treated as described in the above preparation to give the title compound (**11b**) (100 mg, 55% overall yield).

^{1}H NMR (400 MHz, DMSO-d_6): δ= 1.8-2.0 (m, 2H, C<u>H</u>(H)2'A + C<u>H</u>(H)2'B); 2.01 (m, 1H, C<u>H</u>(H)2'B); 2.25 (m, 1H, C<u>H</u>(H)2'A); 3.53 (m, 2H, $CH_2$5'A); 3.7-3.9 (m, 3H, $CH_2$5'B + H4'B); 3.95 (m, 1H, H4'A); 4.21 (m, 1H, H3'B); 4.73 (m, 1H, H3'A); 5.69, 5.70 (two d, J= 7.2 Hz, 2H, H5A + H5B); 6.11 (m, 1H, H1'A); 6.17 (m, 1H, H1'B); 6.9-7.1 (bs, 4H, two NH2); 7.75, 7.89 (two d, J= 7.2 Hz, 2H, H6A + H6B).

^{31}P NMR (81 MHz, DMSO-d_6): δ= 54.45 (H_3PO_4 as external reference).

FAB-MS (negative ions): m/z 553 ($[M+Na-2H]^-$); 531 ($[M-H]^-$).

FAB-MS (positive ions): m/z 555 ($[M+Na]^+$); 533 ($[M+H]^+$).

Enzymatic hydrolysis of the deprotected dinucleotide thiophosphates

The relative susceptivity of the two substrates **11a** and **11b** to hydrolysis by snake venom phosphodiesterase was determined by incubating a solution (3 mM) of each substrate in Tris buffer (25 mM) containing $MgCl_2$ (5 mM) at pH 8 with the enzyme (1 mg in 0.5 ml) (from Crotalus durissus) purchased from Boehringer and following the substrate disappearance by HPLC. In particular for each substrate a solution was prepared consisting of 700 μl of buffer, 200 μl of substrate solution

plus 50 µl of enzyme solution. For each substrate a control was prepared with the same components and volumes except for the enzyme that was replaced by an equal volume of buffer. The four solutions (the two substrates and the two controls) were incubated at 37°C taking aliquots from each solution at 0 time, 1 hour, 17 hours. Such aliquots were analyzed by HPLC (Whatman Partisphere C18, eluting with a linear gradient from 10% to 30% of methanol in 0.1M ammonium acetate) at 220 nm. Only **11a** showed a marked reduction in the area of the HPLC peak relatively to the control: 50% after 1 hour and 72% after 17 hours. This indicates the R_P configuration for **11a**. In the other case the dinucleotide **11b** remained almost unchanged both after 1 hour and after 17 hours indicating the S_P configuration for the substrate.

ACKNOWLEDGEMENTS

The Authors deeply thank Dr. Emanuele Arlandini for mass spectrometry studies.

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