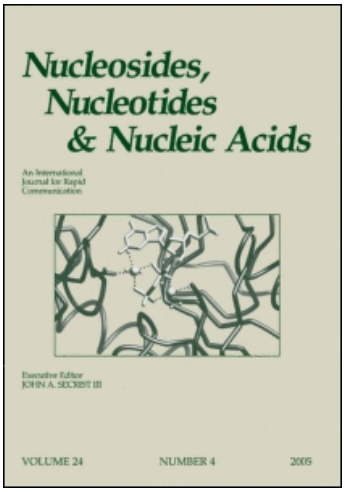


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# Synthesis and NMR Characterization of Diastereomeric $C_{PSMe}G$ Derivatives

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NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(5&6), 903–915 (2000)

# SYNTHESIS AND NMR CHARACTERIZATION OF DIASTEREOMERIC $C_{PSMe}G$ DERIVATIVES

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## ABSTRACT

Synthesis and stereochemical characterization of enantiomerically pure $C_{PSMe}G$ derivatives by NMR methods are reported. The effect of 5'-dimethoxytrityl on the conformational properties is described. It was found that in P-diastereomers the conformational differences about the C3'-O3' bond, as discernible from the $\Delta J$ values, are enhanced by the presence of this protecting group.

## INTRODUCTION

Introduction of various modifications at the phosphorus atom results in chiral phosphate derivatives. Considering their biological importance, several research groups have undertaken the preparation of synthetic modified oligonucleotides. The stereocontrolled synthesis of oligonucleoside phosphorothioates and the structural and biochemical consequences of diastereomerism have been extensively studied by Stec and coworkers[1,2]. The role of the configuration of the phosphorothioate group in the preference for particular conformers was described by Cosstick and Eckstein for structurally modified oligonucleotides[3,4]. These products are generally characterized by the use of nuclease P1 digestion, snake venom phosphodiesterase, HPLC, CD spectroscopy and, in a few cases, by X ray analysis. The use of NMR methods, however, is mostly restricted for $^{31}P$ NMR spectroscopy.

As an extension of our previous work on the NMR studies of phosphate-modified nucleotides we now wish to describe the synthesis and structural analysis of two pairs of diastereomerically pure deoxynucleoside-SMe-phosphorothioate dimers. Recently we have reported for some phosphate-modified mono- and dinucleotide derivatives that the vicinal $^{13}C$-$^{31}P$ coupling data can be applied for configurational assignment of diastereomers [5-7]. Since the $^{3}J_{C4',P}$ and $^{3}J_{C2',P}$ data reflect the conformational equilibrium about the C3' - O3' bond, the different preference of conformers in the diastereomers appeared as systematic differences in the $\Delta J$ (= $^{3}J_{C4',P}$ - $^{3}J_{C2',P}$) values. The configurations assigned this way were consistent with the results obtained from NOE and T-ROESY experiments.

The $\Delta J$ values are influenced also by factors other than the absolute configuration. Thus the nature of the pendant group on the phosphorus atom and the aromatic solvent effect may also affect the extent of base overlap, and consequently, the conformational properties of the molecules. Accordingly, in our recent studies [5-7] where the side chain of the compounds was benzyl moiety, stacking interactions between the bases and the phenyl rings could be assumed.

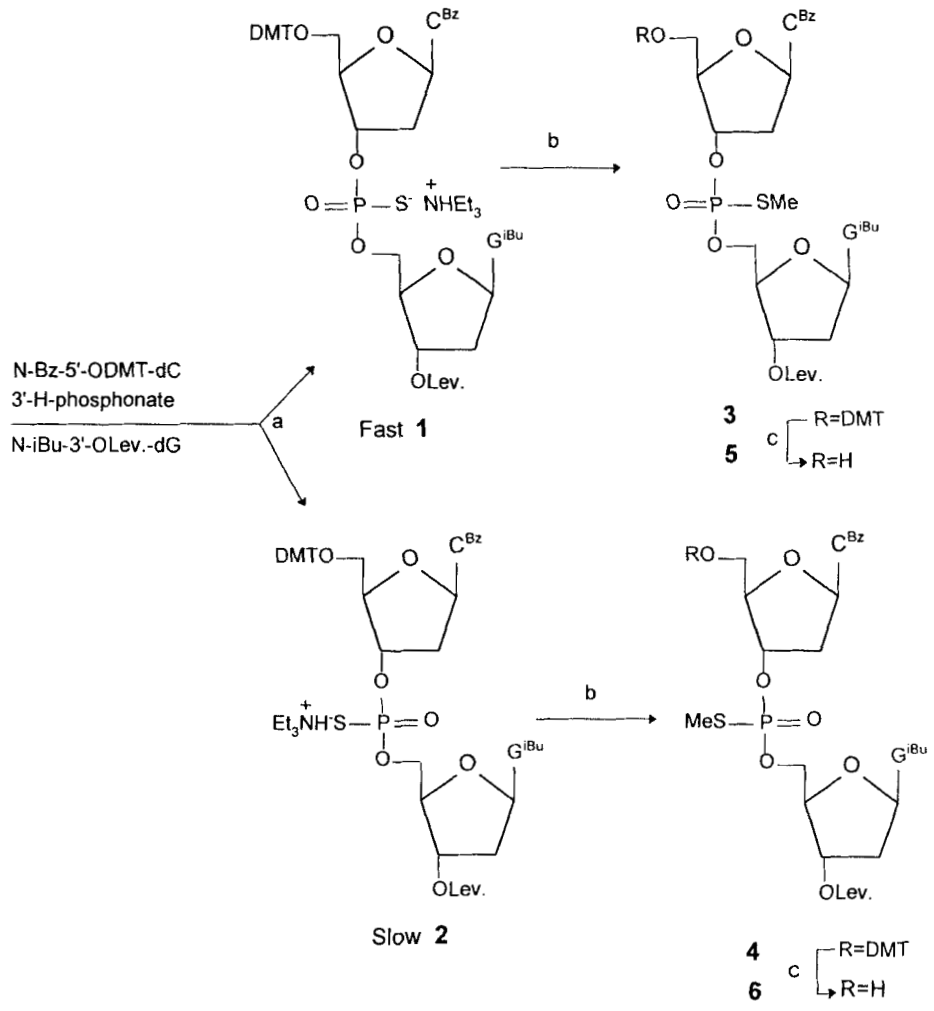


In this paper our results, applying the method based on the use of the $\Delta J$ values for configurational determination and conformational characterization of diastereomeric dinucleoside-S-methyl phosphoro-thioates (**3,4,5,6**), are presented. The presence of the S-methyl, instead of the benzyl group, resulted in altered spectral properties, which can be attributed to the fact that the overall geometry has been changed upon introduction of the S-methyl group on the phosphorus.

From our earlier work and other ongoing studies also appeared that decomposition (detritylation) in solution affects the spectral characteristics of both the neighbouring and the remote protons. This observation has prompted us to examine whether the substitution of the dimethoxytrityl group by hydrogen will have far-reaching influence on the three-dimensional spatial arrangement.

## RESULTS AND DISCUSSIONS

The diastereomerically pure Sp and Rp dinucleoside phosphorothioates (**1** and **2**) were synthesized using H-phosphonate chemistry (Scheme). Although separation of




a, i. pivaloyl chloride, pyridine, followed by sulfurization ii. $SiO_2$ chromatography

b, MeI, pyridine, $CH_3CN$

c, i. pTsOH/$CHCl_3$-MeOH ii. $NEt_3$ iii. $SiO_2$ chromatography

**Scheme**

P-diastereomers can be achieved for intermediate H-phosphonate-diesters as well [8], it can be effected more safely and efficiently after thiooxidation because of the larger $R_f$ difference between the P-isomers and resistance of thiophosphoric acid diesters to solvolysis in basic and protic medium.

**TABLE 1**

| Comp. | $\delta$ $^{31}$P[ppm] | | $^{3}JC_{4',P}$ | $^{3}JC_{2',P}$ | $\Delta J$ | $\delta$H1' | $\delta$H1" | $\delta$H6 | $\delta$H8 |
|---|---|---|---|---|---|---|---|---|---|
| | $CDCl_3$ | DMSO | [Hz] | [Hz] | [Hz] | [ppm] | [ppm] | [ppm] | [ppm] |
| **3** | 30.06 | 29.69 | 5.0 | 4.8 | 0.2 | 6.32 | 6.16 | 8.13 | 7.65 |
| **4** | 30.35 | 29.80 | 6.6 | 3.1 | 3.5 | 6.23 | 6.15 | 8.03 | 7.63 |
| **5** | 31.14 | 29.68 | 5.1 | 4.6 | 0.5 | 6.29 | 6.19 | 8.29 | 7.68 |
| **6** | 30.74 | 29.63 | 5.7 | 4.2 | 1.5 | 6.17 | 6.19 | 8.24 | 7.72 |



After the chemoselective S-methylation of **1** and **2** we obtained pure Sp and Rp 5'-ODMT-S-methyl-phosphorothioate dimers (**3** and **4**, respectively). In order to study the dependence of the NMR spectral parameters on 5'-protection, **3** and **4** were detritylated and after the necessary silica gel chromatographic purifications the corresponding 5'-free-S-methyl-dimers (**5** and **6**) were isolated in good overall yields.

The tendency of $^{31}$P chemical shifts of nucleotides measured in polar solvents is generally accepted for configurational determination of diastereomers. However, it is well documented that structurally modified nucleotides can exhibit anomalous chemical shifts [9]. Our compounds, where detritylation resulted in interchanged $^{31}$P chemical shifts, may present an example for these observations (Table 1).

The complete assignment of the proton spectra in deuterochloroform was achieved by homonuclear decoupling and by T-ROESY experiments. In order to avoid overlap of some of the pertinent protons these measurements were repeated in deuterobenzene-deuteromethanol solvent mixture (4:1) for **3** and **4**. T-ROESY experiments provided also the configurational assignment of the chiral phosphorus atom. In the spectrum of **3** strong NOEs of the S-methyl protons were measured to the H2$\alpha$' proton while the weak NOE connection with the H4' proton was near to the detection limit. On the contrary, in the T-ROESY spectrum of **4** the strongest crosspeaks resulted from the NOE connection of the S-methyl protons with H4' and H5' protons while the crosspeak with the H2'$\alpha$ was negligible. The NOE correlation between the S-methyl and

H3' protons was not distinctive since it appeared in the ROESY spectra of both isomers with about the same intensity. These results established the configurational designation as: **3**: Sp; **4**: Rp .The ROESY spectra of the detritylated compounds **5** and **6** did not show such differences in the NOE correlations of the S-Me protons. Nevertheless, since **5** and **6** are derived from **3** and **4**, respectively, their configuration directly follows from that of the parent compounds . The starting materials **1** and **2** do not contain proper groups to display diagnostic NOE effects, thus their configurational assignment is based on the well established structures of **3** and **4** considering also that S-methylation can not change the original P- configuration.

In the proton spectrum of all four SMe-compounds the sugar proton chemical shifts and coupling values of the pG residues reflect the presence of the C3''-O-levulinyl substituent, while the proton couplings of the Cp sugar moieties differ from those of the other derivatives studied by us. From the sum of the H2'α couplings, with the help of an empirical expression [10], the percentage of the S-type (C2'-endo) conformer can be determined. These values in compounds **3** and **4** for the Cp sugar ring are lower (76 - 79 %) than those calculated for dinucleoside phophorothioates and phosphoramidates (84 - 93%) having a phenyl group in the side chain[5-7], which indicates that the conformational equilibrium is somewhat biased towards the N-puckered form. The presence of the S-Me substituent instead of the benzyl may be responsible for this change in the Cp sugar ring pucker. Detritylation caused a further slight S→N shift in the Cp sugar ring conformation of compounds **5** and **6**, as calculated from the sum of the H2'α coupling values.

Conformational preferences about the various bonds in nucleotides are known to be strongly interdependent, thus the change in sugar ring pucker is expected to be accompanied by torsional variation about the C3' - O3' bond [11]. The vicinal $^{13}C$ - $^{31}P$ couplings, as discussed by Lankhorst and coworkers, reflect the conformational differences along the C3'- O3' bond, while neither $^{2}J_{C3',P}$ nor $^{3}J_{H3',P}$ were found to show much variation[12]. They have concluded that when $\varepsilon^{t}$ is the preferred conformer about the C3' - O3' bond, the $^{3}J_{C4',P}$ coupling is much larger than $^{3}J_{C2',P}$. In our approach this means that smaller $\Delta J$ values refer to a shift towards the $\varepsilon^{-}$ conformational state.

Similarly to our earlier findings the ΔJ values for compounds **3** and **4** display characteristically different conformational properties in the diastereomers. ( Table 1). It should be noted, however, that the ΔJ values reflect sterical arrangement, while the designation of the absolute configuration changes according to the different priorities of the substituents at the phosphorus. For the present compounds in agreement with other, structurally related, SMe derivatives[13] the Cahn-Ingold-Prelog rules define the priority of the four groups about the phosphorus as: MeS > nucleoside 3'-O > nucleoside-5"-O> O. The ΔJ values of **3** and **4** confirm the stereochemistry identified by the T-ROESY experiments, and reveal that the different configuration at the phosphorus is accompanied by conformational differences. The larger ΔJ in **4** (+3.5 Hz) than in **3** (+0.2 Hz) reflect that in **4** the $\varepsilon^t$ conformer is more preferred than in **3.**



Detritylation of **4** resulted in a noteworthy change of the $^{13}C$-$^{31}P$ couplings, consequently in the ΔJ values, while these values hardly varied on going from **3** to **5**. As a result, the difference in the ΔJ value is definitely less for the diastereomeric detritylated derivatives (ΔJ =+0.5 Hz for **5** and +1.5 Hz for **6** ) than between **3** and **4** (see Table 1). The smaller $^3J_{C4',P}$ and larger $^3J_{C2',P}$ values in **6** reveal a shift towards the $\varepsilon^-$ conformer while the conformational change is small and opposite for **5**.

The chemical shifts of the anomeric protons (H1' in Cp and H1'' in pG) and base protons (H6 and H8) are known to be sensitive to changes in base stacking of nucleobases[14]. By analogy, the 0.16 and 0.21 ppm downfield shifts of the H6 resonances in **5** and **6**, in comparison with their values in **3** and **4**, respectively, denotes a major change when the interaction between the dimethoxytrityl and cytidyl moieties has ceased. Although the impact on the H8, H1' and H1'' chemical shifts is of lesser extent, the change in the conformation of the glycosidic bond in the Cp residue upon detritylation readily followed from the T-ROESY spectra as well. In compounds **3** and **4** the NOE correlation from H6 to H2'β and H1' was about of the same intensity suggesting the presence of both *syn* and *anti* conformation as an equilibrium mixture[15]. On the contrary, the stronger cross peaks from H6 to H1' in **5** and **6** indicated the predominance of the *syn* conformer in both compounds.

Detritylation affected also the nonequivalence of the C5''-2H methylene protons. The chemical shift difference of these protons varied in opposite way in the Sp and Rp isomers ( $\Delta\delta AB$ = 0.52 ppm and 0.30 ppm in **3** and **4**, respectively, while 0.75 ppm and 0.05 ppm in **5** and **6** , respectively). In addition, the $J_{H4'',C5''-2H}$ and $J_{C5''-2H,P}$ couplings also showed noteworthy differences upon detritylation. These latter changes, similarly to the vicinal carbon-phosphorus couplings, were again larger for **6** than for **5**.

It is well documented that perturbation of the geometry due to altered stacking of nucleobases causes a series of conformational adjustments. Accordingly, altered stacking interaction perturbs the conformation about the glycosidic bond, which induces an S↔N shift in the sugar ring conformation. This shift in the conformer equilibrium then causes a change in the torsion about the C3'-O3' bond[11]. By analogy, all the aforementioned changes can be explained by assuming that the presence of the dimethoxytrityl ring system exerts an influence on the stacking mode of the bases. In the detritylated molecules the absence of this effect results in an altered overall conformation. The course of these conformational changes, as it follows from the $\Delta J$ and $\Delta\delta AB$ values, is different for the diastereomers and reflect a shift towards the $\varepsilon^-$ conformation for the Rp isomer, while the conformational equilibrium is somewhat shifted towards the $\varepsilon^t$ domain for the Sp isomer.

In summary, it can be concluded that, since the effect of the various factors on the proton NMR parameters can not be clearly separated from the conformational effects, the determination of $\Delta J$ is an expedient method for configurational assignment of the diastereomeric phosphate-modified dinucleotides and to inform about the accompanied conformational differences along the C3'-O3' bond. Our studies confirmed that the dimethoxytrityl group essentially contributes to enhance the conformational differences along the C3'-O3' bond in the diastereomers. In the detritylated molecules the loss of the interaction between the dimethoxytrityl and base moieties led to more conformational flexibility about the C3'-O3' bond, as reflected by the closer but still different values of $\Delta J$ in compounds **5** and **6**. The stereochemical conclusions were in agreement with the results of the T-ROESY experiments.

## EXPERIMENTAL

**General.** $^{1}H$ , $^{13}C$ and $^{31}P$ spectra were recorded in $CDCl_3$ and $C_6D_6$+$CD_3OD$ solutions at room temperature using a Varian Unity Inova 400 spectrometer. Chemical shifts (δ) are reported relative to internal TMS ($^{1}H$ and $^{13}C$ spectra) and to external $H_3PO_4$ ($^{31}P$ spectra). Information about the $^{1}H$-$^{31}P$ coupling constants were obtained from the comparison of the proton spectra with and without phosphorus decoupling. Coupling constant values of the pertinent sugar protons were ultimately confirmed by spectral simulation using the LAOCOON program incorporated in the Varian software package. The $^{13}C$-$^{31}P$ coupling constants were determined from proton-decoupled $^{13}C$ spectra. The assigned protons and protonated carbons were correlated by HSQC experiment. T-ROESY experiments were applied for the determination of the spatial connectivities of pertinent protons , using 400 msec mixing time.



**Materials.** N-Bz-5'-ODMT-dC and N-i.Bu-5'-O-DMT-dG were purchased from Pharma-Waldhof GmbH (Dusseldorf, Germany). Pyridine was distilled from $CaH_2$ and stored over molecular sieves (4A), pivaloyl chloride was freshly distilled before use.

**Chromatography**. For column chromatography Kieselgel 60/0.04-0.63 mm/ (Merck), was used. Progress of reactions were followed by TLC using Kieselgel 60 $HF_{254}$ plates. Solvent systems used were the following: A) $CHCl_3$-MeOH 3%,
B) $CHCl_3$-MeOH 10% - TEA 5%, C) $CHCl_3$, D) $CHCl_3$-MeOH 15%.

*Sp and Rp $N^4$-Benzoyl-5'-O-dimethoxytrityl-2'-deoxycytidilyl(3'→5')P-thio-$N^2$-isobutyryl-3'-O-levulinyl-2'-deoxyguanosine triethylammonium salts* (**1** and **2**).

N-BZ-5'-O-DMT-dC-3'-H-phosphonate DBU salt[15] /1.40g, 1.65 mmole/ and N-iBu-3'-levulinyl-dG[16] /0.65g, 1.50 mmole/ were dissolved in dry pyridine /25 mL/and evaporated. The residue was redissolved in the same volume of dry pyridine and pivaloyl chloride /0.52 mL, 4.5 mmole/ was added. The mixture was stirred at ambient temperature for 15 min. then diluted with $CH_2Cl_2$ /120 mL/ and washed with saturated $NaHCO_3$ solution /2x40 mL/. The organic phase was dried with $Na_2SO_4$, filtered and evaporated. The residual diastereomeric mixture of H-phosphonate diesters obtained was dissolved in pyridine /30 mL/ and $CS_2$ /10 mL/, then sulfur powder /0.32 g, 10 mmole was added in one portion. The homogeneous mixture was stirred at room temperature overnight then evaporated to dryness. To remove pyridine traces it was repeated with

toluene /2x20 mL/. The solid residue was taken up in $CHCl_3$ /6 mL/ and applied to a column /150 g/ packed in $CHCl_3$ - $Et_3N$ (1%). The column was eluted with linear gradient of systems A and B. The appropriate pure fractions were combined and evaporated to give 0.75 g , 0.60 mmole (40%) of faster moving Sp (**1**) and 0.67 g, 0.54 mmole (36%) of slower moving Rp isomer (**2**) of protected dinucleoside phosphorothioate TEA salts. NMR data: **1** : $^1$H NMR ($CDCl_3$, 25°C): δ 2.30 (1H, $J_{gem}$=-14.1, $J_{1',2'\beta}$=6.7, $J_{2'\beta,3'}$=5.9 Hz; H2'β), 2.36 (1H, $J_{gem}$=-14.3, $J_{1'',2''\alpha}$=5.9, $J_{2''\alpha,3''}$=1.0 Hz, H2''α), 2.99 (1H, $J_{gem}$=-14.1, $J_{1',2'\alpha}$=6.0, $J_{2'\alpha,3'}$=3.2 Hz, H2'α), 3.0 - 3.46 (2H, $J_{4',5'}$=3.1+3.3 Hz, H5'), 3.47 (1H, $J_{gem}$=-14.3, $J_{1'',2''\beta}$=9.3, $J_{2''\beta,3''}$=5.1 Hz; H2''β), 3.85 (1H, $J_{gem}$=-10.5, $J_{4'',5''A}$=4.6, $J_{5''A,P}$=5.9 Hz; H5''A), 4.34 (1H, $J_{3'',4''}$=1.0, $J_{4'',5''A}$=4.6, $J_{4'',5''B}$=7.8 Hz; H4''), 4.36 (1H, $J_{3',4'}$=3.0, $J_{4',5'}$=3.1+3.3 Hz; H4') 4.81 (1H, $J_{gem}$=-10.5, $J_{4'',5''B}$=7.8, $J_{5''A,P}$= 10.4 Hz; H5''B), 5.13 (1H, $J_{2'\alpha,3'}$=3.2, $J_{2'\beta,3'}$=5.9, $J_{3',4'}$=3.0, $J_{3',P}$=8.4 Hz; H3'), 5.64 (1H, $J_{2''\alpha,3''}$=1.0, $J_{2''\beta,3''}$=5.1, $J_{3'',4''}$=1.0 Hz, H3''), 6.15 (1H, $J_{1'',2''\alpha}$=5.9, $J_{1'',2''\beta}$=9.3 Hz; H1''), 6.27 (1H, $J_{1',2'\alpha}$=6.0, $J_{1',2'\beta}$=6.7 Hz; H1'), 7.28 (1H, $J_{5,6}$=7.5Hz; HC5), 7.79 (1H, HG8), 8.17 (1H, $J_{5,6}$=7.5 Hz; HC6). $^{31}$P NMR ($CDCl_3$, 25 °C),: δ 57.56 ppm, (DMSO-$d_6$, 25°C): δ 55.48 ppm.

**2** : $^1$H NMR (CDCl3, 25 °C): δ 2.31 (1H, $J_{gem}$=-14.3, $J_{1',2'\beta}$=6.6, $J_{2'\beta,3'}$=6.0Hz; H2'β), 2.33 (1H, $J_{gem}$=-14.0,$J_{1'',2\alpha''}$=5.8, $J_{2''a,3''}$=1.1Hz; H2''α), 2.98 (1H, overlapped), 3.28 (1H, $J_{gem}$=-14.0, $J_{1'',2''\beta}$=9.2, $J_{2''\beta,3''}$=5.5 Hz; H2''β), 3.4 - 3.5 (2H, $J_{4',5'}$=3.0+3.3 Hz; H'5), 4.13 (1H, $J_{gem}$=-10.8, $J_{4'',5''A}$=4.7, $J_{5''A,P}$=6.4 Hz; H5''A), 4.26 (1H, $J_{3'',4''}$=1.2, $J_{4'',5''A}$=4.7, $J_{4'',5''B}$=5.4Hz; H4''), 4.34(1H, $J_{gem}$=-10.8, $J_{4'',5''B}$=5.4, $J_{5''B,P}$=7.7 Hz; H5''B), 4.46 (1H, $J_{3',4'}$=3.0, $J_{4',5'}$=3.0+3.3 Hz; H4'), 5.30 (1H, $J_{3',4'}$=3.0, $J_{2'\alpha,3'}$=2.8, $J_{2'\beta,3'}$=6.0, $J_{3',P}$=8.8 Hz; H3'), 5.48 (1H, $J_{2''\alpha,3''}$=1.1, $J_{2''\beta,3''}$=5.5, $J_{3'',4''}$=1.2 Hz; H3''), 6.15 (1H, $J_{1'',2''\alpha}$=5.8, $J_{1'',2''\beta}$=9.2 Hz; H1''), 6.28 (1H, $J_{1',2'\alpha}$=6.1, $J_{1',2'\beta}$=6.6 Hz; H1'), 7.30 (1H, $J_{5,6}$=7.5 Hz; HC5), 7.87 (1H; HG8), 8.18 (1H, $J_{5,6}$=7.5Hz; HC6). $^{31}$P NMR ($CDCl_3$, 25°C): δ 57.04 ppm, (DMSO-d6, 25°C): δ 55.51 ppm.

*(Sp)$N^4$-Benzoyl-5'-O-dimethoxytrityl-2'-deoxycytidilyl(3'→5')P-methylthio-$N^2$-iso-butyryl -3'-O-levulinyl-2'-deoxyguanosine* (**3**).

The pure Sp dinucleoside phosphorothioate (**1**) /150 mg, 0.12 mmole/ was dissolved in dry pyridine /3mL/ and evaporated to remove MeOH traces. The residues was dissolved in dry $CH_3CN$ /1.5 mL/ and dry pyridine /0.5 mL/ then MeI /15 μL, 0.24 mmole/ was

added. The mixture was stirred at ambient temperature overnight then diluted with $CH_2Cl_2$ /30 mL/ and washed with aq. saturated $NaHCO_3$ solution /2x10mL/ and water /10 mL/. The organic phase was dried with $Na_2SO_4$, filtered and evaporated finally with toluene /2x10 mL/ to remove pyridine traces. The yellow amorphous residue (0.16g) was dissolved in ether /3 mL/ and precipitated by dropwise addition to cold petroleum ether /12 mL/. After filtration and drying on air we isolated 130 mg of white semisolid product (**3**), $R_f$ (D): 0.66. NMR data: **3** : $^1$H NMR ($CDCl_3$, 25°C): δ 2.21 (3H, $J_{Me,P}$=15.7 Hz; SMe), 2.38 (1H, $J_{gem}$ = -14.4, $J_{1'',2''\alpha}$=6.2, $J_{2''\alpha,3''}$=1.4Hz; H2"α), 2.45(1H, $J_{gem}$=-14.3, $J_{1',2'\beta}$=6.7, $J_{2'\beta,3'}$=5.9Hz, H2'β), 3.00 (1H, $J_{gem}$=-14.3, $J_{1',2'\alpha}$=6.0, $J_{2'\alpha,3'}$=2.6Hz; H2'α), 3.44(1H, $J_{gem}$=-14.4,$J_{1'',2''\beta}$=8.5, $J_{2''\beta,3''}$=6.3Hz; H2"β), 3.48(2H, $J_{4',5'}$=2.9+2.9 Hz; H5'), 4.27 (1H, $J_{gem}$=-10.5, $J_{4'',5''A}$=4.7, $J_{5''A,P}$=5.5 Hz; H5"A), 4.35 (1H, $J_{3'',4''}$ =1.6, $J_{4'',5''A}$ =4.7, $J_{4'',5''B}$ =6.1, $J_{4'',P}$=1.2Hz; H4"), 4.52 (1H,$J_{3',4'}$=2.9, $J_{4',5'}$=2.9+2.9Hz; H4') 4.79 (1H,$J_{gem}$= -10.5,$J_{4'',5B}$=6.1, $J_{5''B,P}$=7.9 Hz; H5"B), 5.26 (1H,$J_{2'\alpha,3'}$=2.6, $J_{2'\beta,3'}$=5.9, $J_{3',4'}$=2.9, $J_{3',P}$=7.8 Hz; H3'), 5.51 (1H,$J_{2''\alpha,3''}$ =1.4, $J_{2''\beta,3''}$=6.3, $J_{3'',4''}$ =1.6Hz; H3"), 6.16 (1H,$J_{1'',2''\alpha}$=6.2, $J_{1'',2''\beta}$=8.5 Hz; H1"), 6.32(1H, $J_{1',2'\alpha}$=6.0, $J_{1',2'\beta}$=6.7Hz; H1'), 7.3(1H, $J_{5,6}$=7.5Hz; HC5), 7.65 (1H; HG8), 8.13 (1H, $J_{5,6}$=7.5Hz; HC6). $^{31}$P NMR ( $CDCl_3$,25°C); δ 30.06ppm, (DMSO-d6, 25°C): δ29.69ppm. $^{13}$C NMR ($CDCl_3$,25°C): δ 11.85ppm($J_{C,P}$=4.5Hz,SMe) 35.11(C2''),40.42($J_{C,P}$=4.8Hz,C2'),62.51(C5'),66.43($J_{C,P}$=6.1Hz,C5")75.13 (C3"), 78.25 ($J_{C,P}$=5.9Hz,C3'),2.65($J_{C,P}$=9.4Hz,C4"),85.33($J_{C,P}$=5.0Hz,C4'),86.69(C1"),87.26(C1').

*(Rp)$N^4$-Benzoyl-5'-O-dimethoxytrityl-2'-deoxycytidilyl(3'→5')P-methylthio-$N^2$-iso-butyryl-3'-O-levulinyl-2'-deoxyguanosine* (**4**).

Starting from 150 mg /0.12 mmole/ pure **2**, similar S-methylation followed by the same work-up resulted in 135 mg of chromatographically pure **4** as off-white solid, Rf(D):0.64. NMR data:. **4**: $^1$H NMR ($CDCl_3$, 25°C) δ 2.28(3H, $J_{Me,P}$=15.8Hz; SMe), 2.35(1H,$J_{gem}$=--14.4,$J_{1',2'\beta}$=6.5,$J_{2'\beta,3'}$=5.8Hz;H2'β),2.42(1H,$J_{gem}$=-14.3,$J_{1'',2''\alpha}$=5.8,$J_{2''\alpha,3''}$=1.4Hz ;H2"α), 2.93(1H, $J_{gem}$=-14.4, $J_{1',2'\alpha}$=6.0, $J_{2'\alpha,3'}$=2.9Hz; H2'α), 3.16(1H, $J_{gem}$=-14.3, $J_{1'',2''\beta}$=8.9, $J_{2'',\beta,3''}$ =6.0Hz; H2"β), 3.36 (2H, $J_{4',5'}$=3.0+3.0 Hz; H'5), 4.32(1H, $J_{3',4'}$= 2.9, $J_{4',5'}$= 3.0 + 3.0Hz;H4'), 4.35(1H, $J_{3'',4''}$= 1.6, $J_{4'',5''B}$=5.0, $J_{4'',5''A}$ = 5.8, $J_{4'',P}$= 1.1Hz; H4''), 4.39(1H,$J_{gem}$=-10.7, $J_{4'',5''A}$=5.8, $J_{5''A,P}$=5.7 Hz; H5''A), 4.69 (1H, $J_{gem}$=-10.7, $J_{4'',5''B}$=5.0, $J_{5''B,P}$= 6.7Hz, H5"B), 5.16(1H, $J_{2'\alpha,3'}$=2.9, $J_{2'\beta,3'}$=5.8, $J_{3',4'}$=2.9, $J_{3',P}$=7.9Hz;H3') 5.44(1H, $J_{2''\alpha,3''}$ =1.4, $J_{2''\beta,3''}$ =6.0, $J_{3'',4''}$ =1.6 Hz; H3"), 6.15(1H, $J_{1'',2''\alpha}$=5.8, $J_{1'',2''\beta}$=8.9Hz, H1"),

6.23(1H,$J_{1',2'\alpha}$=6.0, $J_{1',2\beta'}$=6.5Hz,H1'),7.26(1H,$J_{5,6}$=7.5Hz;HC5),7.63(1H,HG8), 8.03(1H, $J_{5,6}$=7.5Hz, HC6). $^{31}$P NMR ($CDCl_3$, 25°C): δ 30.35 ppm, (DMSO-d6) , δ29.80 ppm. $^{13}$C NMR (CDCl3,25°C): δ 12.38 ppm($J_{C,P}$=4.6Hz, $SCH_3$), 35.72(C2''), 40.40($J_{C,P}$ =3.1Hz,C2'),62.30(C5'),66.59($J_{C,P}$=6.5Hz,C5''),74.93(C3''),78.20($J_{C,P}$=6.2Hz,C3'), 82.71 ($J_{C,P}$=9.4Hz,C4''), 85.00($J_{C,P}$=6.6Hz,C4'), 86.45(C1''),87.06(C1').

*(Sp)$N^4$-Benzoyl-2'-deoxycytidilyl(3'→5')P-methylthio-$N^2$-isobutyryl-3'-O-levulinyl-2'-deoxyguanosine* (**5**).

The pure **3** /120 mg/ was dissolved in $CHCl_3$ /4mL/ and MeOH /1 mL/, cooled in icebath then 1 M p.TsOH/MeOH solution /0.5 mL/ was added. Orange colour and TLC indicated complete detritylation within 5 min. The acidic mixture was diluted with $CHCl_3$ /30 mL/ extracted with sat. aq. $NaHCO_3$ solution /2x10 mL/ dried with $Na_2SO_4$, filtered and evaporated to give pale yellow amorphous residue /0.15 g/. It was purified on a silica gel column /15 g/ using gradient elution with systems C and D. Fractions containing the pure Sp detritylated S-methyl-dinucleoside-phosphorothioate (**5**) were combined and evaporated to give 73 mg, 84.8 μmole (71% overall yield from **1** ) of white solid foam, $R_f$ (D): 0.55. NMR data **5**: $^1$H NMR ($CDCl_3$, 30 °C): δ 2.27 (3H, $J_{Me,P}$=16.0 Hz, SMe), 2.45 (1H, $J_{gem}$= - 14.5,$J_{1'',2''\alpha}$=6.3, $J_{2'',\alpha,3''}$=1.3 Hz ; H2''α), 2.57 (1H,$J_{gem}$=-14.4, $J_{1',2'\beta}$=6.5, $J_{2'\beta,3'}$=5.8 Hz; H2'β), 2.85 (1H, $J_{gem}$=-14.4, $J_{1',2'\alpha}$=6.4, $J_{2'\alpha,3'}$=2.8 Hz; H2'α), 3.58 (1H, $J_{gem}$=-14.5, $J_{1'',2''\beta}$=8.3, $J_{2''\beta,3''}$=6.3 Hz; H2''β), 3.90 (1H, $J_{gem}$=-12.0, J4',5'A=2.6Hz; H5'A), 3.97 (1H, $J_{gem}$=-12.0, $J_{4',5'B}$=2.7 Hz, H5'B), 4.15 (1H, $J_{gem}$= - 10.7, $J_{4'',5''A}$=4.6, $J_{5''A,P}$=4.5 Hz, H5''A), 4.38 (1H, $J_{4',5'A}$=2.6, $J_{4',5'B}$=2.7, $J_{3',4'}$=2.9 Hz; H4'), 4.40 (1H, $J_{4'',5''A}$= 4.6, $J_{4'',5''B}$=6.7, $J_{3'',4''}$= 1.4, $J_{4'',P}$=1.2 Hz; H''4), 5.03 (1H, $J_{gem}$= -10.7, $J_{4'',5''B}$=6.7, $J_{5''B,P}$=8.0 Hz; H''5B), 5.33 (1H, $J_{2'\alpha,3'}$=2.8, $J_{2'\beta,3'}$=5.8, $J_{3',4'}$=2.9, $J_{3',P}$=8.3 Hz, H3'), 5.65 (1H, $J_{2''\alpha,3''}$=1.3, $J_{2''\beta,3''}$=6.3, $J_{3'',4''}$=1.4 Hz, H3''), 6.19 (1H, $J_{1'',2''\alpha}$=6.3, $J_{1'',2''\beta}$=8.3 Hz, H1''), 6.29 (1H, $J_{1',2'\alpha}$=6.4, $J_{1',2'\beta}$=6.5 Hz, H1'), 7.52 (1H, $J_{5,6}$=7.6 Hz, HC5), 7.68 (1H, HG8), 8.29 (1H, $J_{5,6}$=7.6 Hz, HC6). $^{31}$P NMR ($CDCl_3$, 25°C): δ 31.14 ppm, (DMSO-d6, 25°C) : δ 29.68 ppm. $^{13}$C NMR ($CDCl_3$, 30 °C): δ 12.12 ppm ($J_{C,P}$=4.7 Hz;$SCH_3$), 35.15(C2''), 39.76 ($J_{C,P}$=4.6 Hz, C2'), 61.52 (C5'), 65.82 ($J_{C,P}$=5.8 Hz, C5''), 75.30 (C3''), 78.15 ($J_{C,P}$=6.5 Hz; C3'), 82.76 ($J_{C,P}$=9.5 Hz; C4''), 86.53 ($J_{C,P}$=5.1 Hz; C4'), 86.64 (C1''), 88.22 (C1'), 96.88 (C5), 122.76 (G5), 139.04 (G8), 145.53 (C6), 147.71+147.75 (G4+G6), 155.0 (C2), 155.72 (G2), 166.50 (C4).

*(Rp)$N^4$-Benzoyl-2'-deoxycytidilyl(3'→5')P-methylthio-$N^2$-isobutyryl-3'-O-levulinyl-2'-deoxyguanosine* (**6**).

Starting from 120 mg of pure **4**, detritylation and chromatographic purification were effected on similar way as described for **3**. Finally, we isolated 75.5 mg, 87.8 μmole (73% overall yield from **2**) of white solid main product (**6**), $R_f$(D):0.53. NMR data:

**6**: $^1$H NMR ($CDCl_3$, 30 °C): δ 2.28 (3H, $J_{Me,P}$=16.0 Hz, SMe), 2.40 ( 1H, $J_{gem}$=-14.4, $J_{1',2'\beta}$=6.3, $J_{2'\beta,3'}$=6.7 Hz; H2'β), 2.42 (1H, $J_{gem}$=-14.0, $J_{1'',2''\alpha}$=5.6, $J_{2''\alpha,3''}$=1.1 Hz;H2''α), 2.71 (1H,$J_{gem}$=-14.4, $J_{1',2'\alpha}$=6.1, $J_{2'\alpha,3'}$=2.9 Hz, H2'α), 3.14 (1H, $J_{gem}$=-14.0, $J_{1'',2''\beta}$=9.3, $J_{2''\beta,3''}$=6.0 Hz;H2''β), 3.71 (1H, $J_{gem}$=-12.1, $J_{4',5'A}$=2.2 Hz; H5'A), 3.84 (1H,$J_{gem}$=-12.1, $J_{4',5'B}$=2.5 Hz, H5'B), 4.26 (1H, $J_{3',4'}$=2.9, $J_{4',5'A}$=2.2, $J_{4',5'B}$=2.5 Hz;H4'), 4.35 (1H, $J_{3'',4''}$=1.2, $J_{4'',5''A}$=3.9, $J_{4'',5''B}$ = 5.9, $J_{4'',P}$= 1.1Hz; H4''), 4.48 (1H, $J_{gem}$= -10.7, $J_{4'',5''A}$= 3.9, $J_{5''A,P}$=5.9 Hz; H5''A), 4.53 (1H, $J_{gem}$=-10.7, $J_{4'',5''B}$=5.9, $J_{5''B,P}$=5.6 Hz;H5''B), 5.24 (1H, $J_{2'\alpha,3'}$=3.0, $J_{2'\beta,3'}$=5.7, $J_{3',4'}$=2.9, $J_{3',P}$=8.4 Hz; H3'), 5.38 (1H, $J_{2''\alpha,3''}$=1.1, $J_{2''\beta,3''}$=6.0, $J_{3'',4''}$=1.2Hz; H3'') 6.17 (1H, $J_{1',2'\alpha}$=6.1, $J_{1',2'\beta}$=6.3 Hz; H1'), 6.19(1H, $J_{1'',2''\alpha}$=5.6, $J_{1'',2''\beta}$=9.3 Hz; H1''), 7.42 (1H, $J_{5,6}$=7.5 Hz; HC5), 7.72 (1H, HG8), 8.24 (1H, $J_{5,6}$, HC6). $^{31}$P NMR ($CDCl_3$, 25°C): δ 30.74 ppm, (DMSO-d6, 25°C) : δ 29.63 ppm. $^{13}$C NMR ($CDCl_3$, 30°C): δ 12.23 ppm ($J_{C,P}$=4.5 Hz, SMe), 35.90 (C2''), 39.83 ($J_{C,P}$=4.2 Hz; C2'), 61.20 (C5'), 66.87 ($J_{C,P}$=6.6 Hz; C5''), 74.80 (C3''), 78.24 ($J_{C,P}$=7.3 Hz, C3'), 83.00 ($J_{C,P}$=9.3 Hz; C4''), 86.37 (C1''), 86.39 ($J_{C,P}$=5.7 Hz; C4'), 88.03 (C1'), 96.95 (C5), 122.54 (G5)' 138.75 (G8), 145.47 (C6), 147.95+148.07,(G4+G6), 155.0 (C2), 155.83 (G2), 166.66 (C4).



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