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Synthesis, structure, and sugar dynamics of a 2′-spiroisoxazolidine thymidine analog

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ABSTRACT

Bicyclic nucleoside analogs have shown promise in Antisense and RNA interference strategies. Ribofuranosyl ring conformation is a controlling factor in this regard. We have introduced a spiroisoxazolidine ring at the 2′-position of the sugar to gauge the effect it has on sugar dynamics. Proton relaxation measurements and coupling constant analysis indicate the sugar is locked in the North conformation with substantial sugar rigidity evident.

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1. Introduction

Nucleoside analogs originally gained their importance as medicinal agents to combat forms of cancer and several types of viral disease states.[1] More recently the ability to functionalize either the heterocycle or the sugar portion of a nucleoside has led to their use in Antisense[2] and RNA Interference[3] strategies. In these processes specifically functionalized nucleotides are incorporated into oligodeoxyribonucleotides (ODN's) targeted to specific RNA or DNA. In the case of RNA, endogenous enzymes then destroy the heteroduplexes formed between the ODN and the target RNA resulting in the control of the flow of genetic information. With m-RNA destroyed disease causing proteins cannot be synthesized in the cell.[4] A Synthetic RNAi has been shown to silence genes in vitro in cultured human cells.[5] The sugar portion of the nucleoside has become an important region for alteration since the conformation of the sugar ring appears to control whether the ODN binds to RNA or DNA.

As shown in Figure 1 the ribofuranosyl ring can exist in two low energy twist conformations designated as C3′-*endo* (N-type) and C3′-*exo* (S-type).[6] ODN's in which the sugar rings favor the N conformation bind to RNA, whereas the S sugar ODN's favor DNA as a target.[7] Several types of fused, spiro and bridged bicyclo nucleoside analogs known as LNA's (Locked Nucleic Acids) have been studied that can lock the sugar conformation in either the N or S conformation.[8] Some of these compounds incorporated into ODN's have shown strong RNA binding affinities.[9] Substitution at the 2′ position of the sugar can also strongly control the sugar conformational equilibrium. Electronegative groups, such as OH, F, and NH_2 shift the equilibrium toward the N conformation due to the *gauche* effect.[10] These types of substituents increase the affinity of the ODN toward RNA targets. Our interests centered on the generation of a spiro ring junction at the 2′ position of the ribofuranose ring of Thymidine. We seek to decrease the flexibility of the sugar and thus lock it in a specific conformation. At the same time we would like to introduce acidic or basic functionality to allow conformational control through pH regulation. We chose to incorporate an isoxazolidine ring via dipolar cycloaddition methodology.

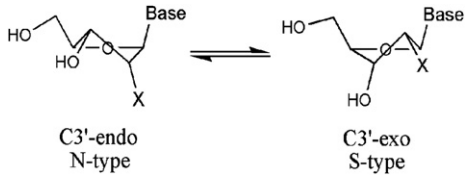

Figure 1. Twist Conformations of 2′-Substituted Nucleosides.

2. Results and discussion

2.1. Synthesis and structure elucidation

A few 2′-spiro oxetanes and oxolanes have been synthesized via an intramolecular S_N2 strategy.[11] Spiro ring formation via cycloaddition onto 2′ sugar dipoles has not been reported. Oximes can be used as

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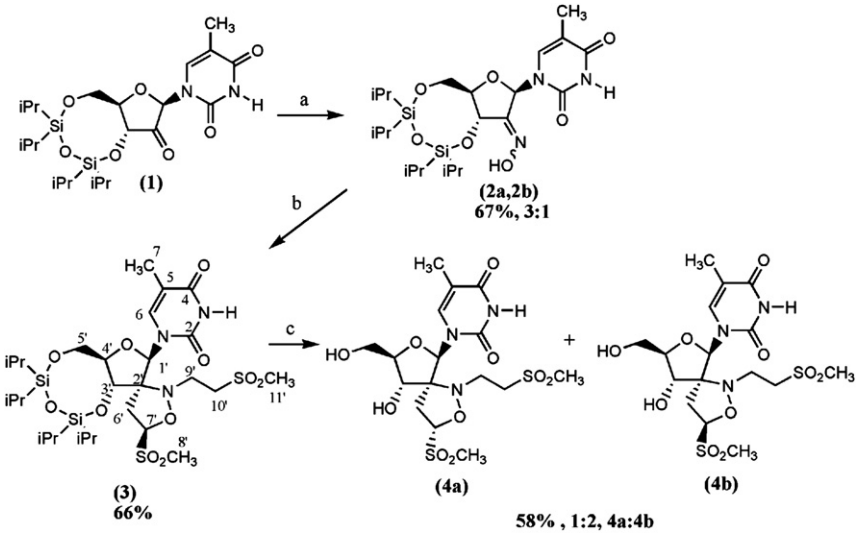
1,3-dipoles at elevated temperatures via a 1,2-prototropic rearrangement to the *H*-nitrone.[12] As shown in Scheme 1 our synthesis began with the disiloxane protected ketone (**1**) obtained by chromium trioxide oxidation of the corresponding alcohol.[13] Subsequent reaction with hydroxylamine hydrochloride gave the 2′-oxime (**2a**,**b**) as a separable mixture of geometric isomers and gave ^{1}H and ^{13}C NMR spectrum identical with a literature reference.[14] The cycloaddition of the oxime (**2b**) was conducted in refluxing toluene with 3 equiv of the electron deficient dipolarophile, methylvinylsulfone. Compound (**3**) was isolated in 66% yield with no other regio- or stereoisomers present. Stereoselectivity in this process is not unusual given the presence of chiral centers in the reactant. Addition of organometallics to 3′,5′-disiloxy protected 2′-keto nucleosides show good stereoselectivity with addition coming from the least hindered bottom face.[15] Similar results are obtained whether a mixture of oxime isomers is used or from either pure isomer. This indicates that both oxime isomers are present and in equilibrium under these conditions. This regioisomer would generally not be the preferred one since the electron rich oxygen of the nitrone would more likely attack the electron poor unsubstituted carbon of the methylvinylsulfone.[12c] Alkylation of the isoxazolidine nitrogen occurs through a conjugate addition of a second equivalent of methylvinylsulfone. The structure of (**3**) is supported through the use of several 2D NMR experiments including COSY, HETCOR, HMBC, and NOESY. Figure 2



a: H_2NOH-HCl in pyridine at 25 °C for 48 hrs. b: 3 eq. Methylvinylsulfone in refluxing toluene, 28 hrs. c. 2.5 eq. TBAF in THF 0 °C for 1 hr then H_2O.

Scheme 1. Synthetic procedure for synthesis of 2′-spiroisoxazolidine nucleosides.

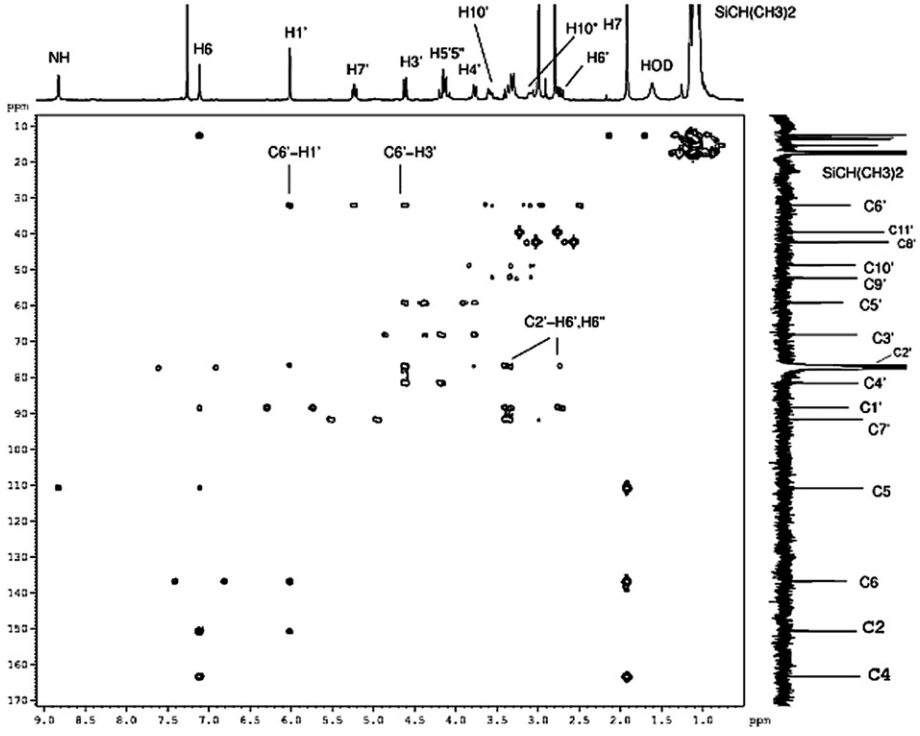


Figure 2. HMBC spectrum of (**3**).

shows the HMBC spectrum of (**3**), which allows us to determine the connectivities between the sugar atoms and the 6-position of the isoxazolidine ring.

The assigned regiochemistry of (**3**) is supported by strong 3-bond correlations between 2′C–9′H, 6′C–1′H, and 6′C–3′H. A 2-bond correlation between 2′C–6′H is also evident. In principle the dipolarophile could attack either face of the intermediate *H*-nitrone giving rise to four possible stereoisomers. The assigned stereochemistry is confirmed through the use of the 2D NOESY experiment shown in Figure 3. Positive NOE contacts for sugar-heterocycle relative orientation are observed between 6H–3′H, 1′H–4′H, and 3′H–5′H. NOE contacts are also evident between 1′H–7′H, 1′H–6′H, and 3′H–9′H, which set the position of the isoxazolidine ring relative to the sugar. Compound (**3**) was then deprotected with fluoride ion and an aqueous workup to give a mixture of stereoisomers (**4a**) and (**4b**) in 58% yield. We believe that under these conditions epimerization occurred at 7′C because of the presence of the carbanion stabilizing methylsulfonyl group.[16]

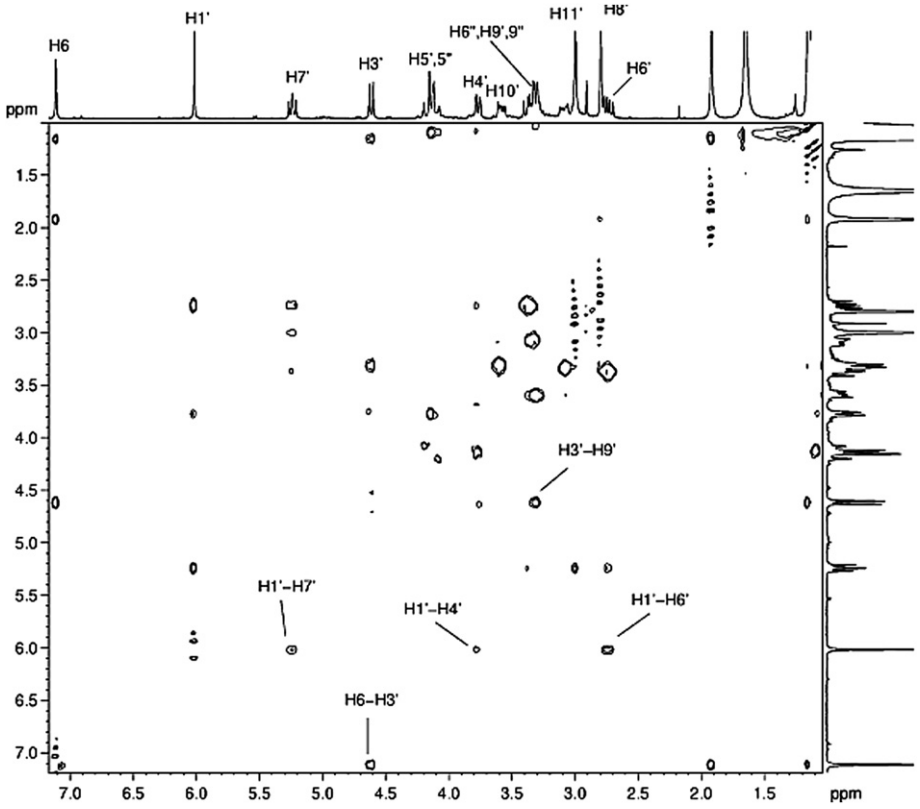


Figure 3. NOESY spectrum of (**3**).

As with (**3**) the structures of (**4a**) and (**4b**) were verified with several 2D NMR techniques. The relative stereochemistries of both were again determined by NOESY experiments. The NOESY spectrum of (**4b**), Figure 4, shows positive NOE contacts between H1′ and one of the diastereotopic H6′ protons. Significantly, a strong contact is also evident between H1′ and H7′ thus confirming the relative configuration at C7′. The NOESY spectrum of (**4a**) lacks this contact (data not shown).

The mechanism for the formation of (**3**) could follow one of two possible pathways as shown in Scheme 2. The oximes (**2a**) and (**2b**) could undergo conjugate addition at nitrogen to form an intermediate *N*-alkylnitrone (**II**). This nitrone could then perform the cycloaddition with a second mole of alkene to give product. This pathway is active in cases were the oxime is relatively simple in structure.[17] The other pathway involves initial 1,2-prototropic rearrangement to the *H*-nitrone (**III**) and cycloaddition to the isoxazolidine (**IV**). Subsequent conjugate addition to (**IV**) gives the observed product (**3**).

2.2. Sugar conformation and flexibility

Our approach to the study of sugar ring flexibility was led by reports that ^{1}H relaxation rates can be useful tools in this regard.[18] ^{1}H spin-lattice (T_1) relaxation measurements were performed on degassed solutions of (**4a**), (**4b**), and 5-Methyluridine using the Inversion-Recovery method as shown in Table 1.[19] Qualitative analysis of the results allows us to make several observations. As expected, increasing sample temperature leads to increased relaxation times due to higher molecular motion and less efficient relaxation. Relative to the heterocycle protons H6 and H7 of the standard, 5-Methyluridine, both (**4a**) and (**4b**) give somewhat shorter T_1′s indicating some resistance to rotation of the heterocycle about the N1–C1′ bond. The largest decreases in T_1 values relative to 5-methyluridine are seen for the sugar H1′, H3′, H4′, and H5′ protons indicating significant loss of flexibility. Several empirical formulas have been developed to relate nucleoside sugar conformation to ^{1}H–^{1}H vicinal coupling constants.[20] We chose the method of Remin which utilizes $J_{3'4'}$ and estimates the %S conformation according to the formula: $S=(J_N-J_{exp})/(J_N-J_S)$ where J_{exp} is the experimentally determined $J_{3'4'}$ coupling constant and $J_N=8.4$ Hz and $J_S=1.1$ Hz.[21] Under conditions of deuterium exchange to remove the H3′–3′OH coupling, compound (**4a**) gave a H3′–H4′ coupling of 8.3 Hz, which gives a %S value of 1.4%, (or 98.6% N). In concert with the relaxation data the sugar ring of (**4a**) does appear to be rigidly locked in the N conformation. The same analysis for

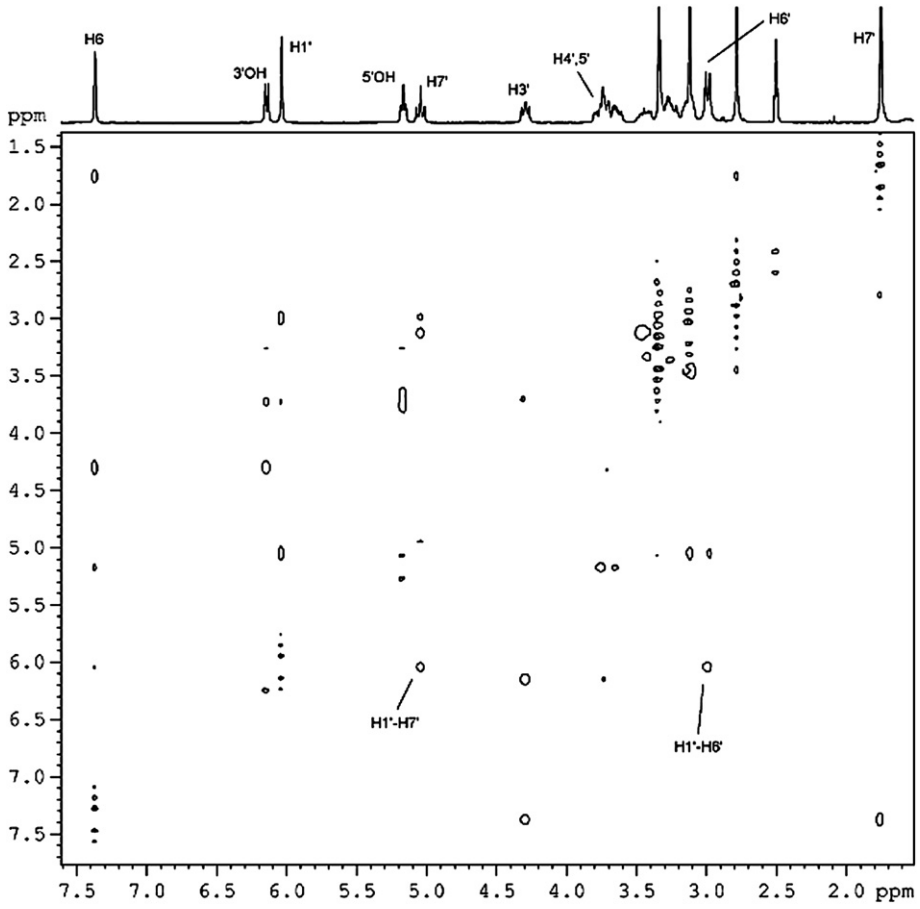
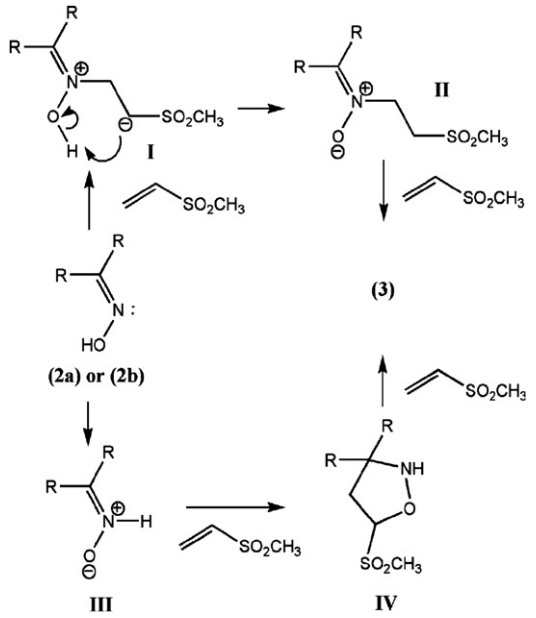


Figure 4. NOESY spectrum of (**4b**).

(**4b**) is less straightforward since its H3′-H4′ coupling constant of 8.8 Hz gives erroneous results when applied to this equation. J_N and J_S are the limiting values for $J_{3'4'}$ calculated from average phase angles and puckering amplitudes determined for 2′-deoxynucleosides in the solid state.[22] Since our compounds appear to be rigidly locked in one conformation it is probable that assumptions inherent in this equation are not applicable in this instance.



Scheme 2. Possible pathways of formation of (**3**).

3. Conclusions

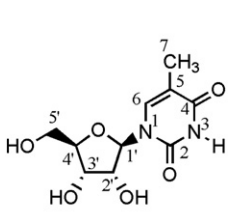
A 2′-spiroisoxazolidine nucleoside analog was synthesized with complete stereo- and regioselectivity. Cycloadditions onto 2′ sugar dipoles provide an efficient strategy to introduce spirocyclic rings in a stereoselective manner. Relaxation measurements and coupling constant analysis indicate substantial loss of flexibility of the ribofuranosyl ring of the nucleoside. These compounds also possess weakly acidic (C7′–H) and weakly basic (isoxazolidine-N) groups that could be used to introduce charge as a function of pH, which may allow further conformational control.

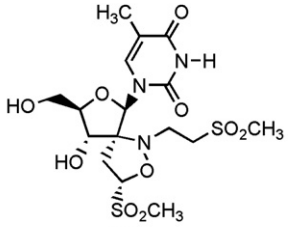
4. Experimental

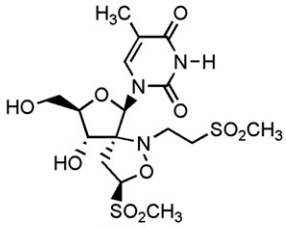
4.1. General procedures

All reagents were purchased commercially from Sigma–Aldrich, Fisher Scientific or Alfa Aesar and used without further purification. NMR spectra were obtained using a Bruker AVANCE 300, with ^{1}H recorded at 300.13 MHz and ^{13}C at 75.5 MHz, in DMSO-d_6 or $CDCl_3$. NOESY spectra were acquired on nitrogen degassed samples at room temperature using a mixing time of 450 ms. Inversion-Recovery T_1 relaxation times were determined on nitrogen degassed samples at the indicated probe temperature. Individual experiments used relaxation delays ranging from 0.1 s to 10 s. A semi logarithmic plot of $[M_{\text{infinite}}-M(t)]/M_{\text{infinite}}$ against (t) gave

Table 1
Proton relaxation times of sugar protons of (**4a**), (**4b**), and 5-methyluridine


5-Methyluridine


(4a)


(4b)

Proton / Compound	H_6	H_7	$H_{1'}$	$H_{2'}$	$H_{3'}$	$H_{4'}$	$H_{5'}$
5-MeUrd 295 °K	0.735[a]	0.616	2.13	0.679	0.845	0.987	0.432
5-MeUrd 330 °K	1.499	1.145	3.524	1.434	1.727	1.982	0.639
4b 295 °K	0.707	0.595	0.637	NA	0.300	0.333	0.241
4b 330 °K	1.078	0.696	0.844	NA	0.552	0.493	0.362
4a 295 °K	0.525	0.581	0.544	NA	0.389	Ovr.[b]	Ovr.
4a 330 °K	0.768	0.730	0.817	NA	0.589	0.383	0.383

[a] Experiments performed on 2.5 mM N_2 degassed samples in 95% DMSO-d_6/5% D_2O. Relaxation time given in seconds.
[b] T_1 could not be determined due to overlapped peaks.

calculated T_1 values with standard deviations of 5–10 ms. High resolution TOF mass spectra were recorded at the Ohio State CCIC (Columbus, OH). Analytic TLC was performed using Whatman Partisil K6F silica gel plates with UV indicator. Silica gel (230–400 mesh, 60 Å) was used for flash chromatography. IR spectra were obtained using a Perkin–Elmer FT-IR GX spectrometer.

4.2. Procedures for the synthesis of (3), (4a), and (4b)

*4.2.1. 1-[2-N-(2-Methylsulfonylethyl)-2-C-(1-methylsulf-onyl)oxo-ethano-3,5-di-O-(1,1,3,3-tetraisopropyldisiloxane-1,3-diyl)β-D-2′-dideoxyribofuranosyl]thymine (**3**).* The less polar oxime isomer (**2a**, 0.210 g (4.1×10^{-4} mol)) was reacted with 3 equiv, 0.108 mL (0.00123 mol), of methylvinylsulfone in 4 mL toluene under an N_2 environment and allowed to reflux for 18 h. The product was isolated using flash chromatography on silica gel with 5% Methanol–Chloroform as eluent. The reaction yielded 0.155 g of Compound (**3**) with a percent yield of 52%. The second, more polar, oxime isomer (**2b**) was also reacted. Compound (**2b**) (0.6886 g, 0.00135 mol) was reacted with 3 equiv, 0.3534 mL (0.00404 mol), of methylvinylsulfone in 15 mL toluene under the same conditions and yielded 0.648 g of (**3**) with a percent yield of 67%. ^{1}H NMR ($CDCl_3$); δ 8.75 (s, 1H, N_3H), 7.11 (s, 1H, H_6), 6.01 (s, 1H, H_1'), 5.23 (dd, 1H, J=9.4, 7.6, H_7'), 4.61 (d, 1H, J=9.0, H_3'), 4.14 (m, 2H, $H_5'+H_5''$), 3.77 (dd, 1H, H_4'), 3.59 (m, 1H, $H_{10'}$), 3.37 (dd, 1H, J=13.7, 7.6, H_6'), 3.31 (m, 2H, $H_9'+H_9''$), 3.08 (m, 1H, $H_{10''}$), 3.00 (s, 3H, $H_{11'}$), 2.79 (s, 3H, H_8'), 2.75 (dd, 1H, J=13.7, 7.8, H_6''), 1.92 (s, 3H, H_7), 1.10 (m, 28H, $SiCH(CH_3)_2$). ^{13}C NMR ($CDCl_3$); δ 163.21 (CO, C_4), 150.58 (CO, C_2), 136.75 (CH, C_6), 110.60 (C quat., C_5), 91.57 (CH, C_7'), 88.23 (CH, C_1'), 81.33 (CH, C_4'), 77.22 (C quat., C_2'), 68.02 (CH, C_3'), 59.15 (CH_2, C_5'), 52.14 (CH_2, C_9'), 48.70 (CH_2, $C_{10'}$), 42.14 (CH_3, C_8'), 39.47 (CH_3, $C_{11'}$), 31.89 (CH_2, C_6'), 17.59, 17.54, 17.40, 17.31, 17.20, 17.09, 17.83 (CH_3, Si–$CH(CH_3)_2$), 15.08, 13.57, 13.02 (CH, Si–$CH(CH_3)_2$), 12.44 (CH_3, C_7). UV (MeOH); λ_{max} 266 nm. IR (KBr): 3218, 2947, 2869, 1694, 1465, 1299, 1131, 1062, 885, 756 cm^{-1}. HRMS: $C_{28}H_{51}N_3S_2Si_2O_{11}$ calcd M+Na 748.2401 found 748.2427.

*4.2.1.1. Deprotection of (**3**).* Compound (**3**) (0.155 g, 2.14×10^{-4} mol) was reacted with 0.642 mL (0.00268 mol) 1 M TBAF in 10 mL THF at ice bath temperature. The reaction was allowed to warm to 25 °C and stirred overnight under an N_2 atmosphere. After 24 h, 0.2 mL of H_20 was added. Thin layer chromatography indicated the presence of two isomers. The isomers were isolated using flash chromatography on silica gel with 12% Methanol–Chloroform. The isolated yield was 58% with 0.040 g of the less polar isomer (**4a**) and 0.019 g of the more polar isomer (**4b**).

*4.2.2. 1-[2-N-(2-Methylsulfonylethyl)-2-C-(1-(R)-methylsulfonyl)oxo-ethano β-D-2′-dideoxyribofuranosyl]thymine (**4a**).* ^{1}H NMR (DMSO-d_6, 295 °K); δ 11.26 (s, 1H, N_3H), 7.37 (s, 1H, H_6), 6.15 (d, 1H, J=6.3, $C_3'OH$), 6.04 (s, 1H, H_1'), 5.17 (t, 1H, J=5.1, $C_5'OH$), 5.04 (t, 1H, J=8.4, H_7'), 4.28 (dd, 1H, J=6.3, 8.1, H_3'), 3.76–3.64 (m, 3H, H_4', H_5', H_5''), 3.45 (m, 2H, $H_{10'}$, $H_{10''}$), 3.26 (m, 2H, H_9', H_9''), 3.12 (s, 3H, H_8'), 2.99 (d, 2H, J=8.3, H_6'), 2.78 (s, 3H, $H_{11'}$), 1.75 (s, 3H, H_7). ^{13}C NMR (DMSO-d_6, 295 °K); δ 163.49 (CO, C_4), 150.23 (CO, C_2), 137.84 (CH, C_6), 108.26 (C, C_5), 91.40 (CH, C_7'), 87.08 (CH, C_1'), 81.41 (CH, C_4'), 76.46 (C, C_2'), 66.53 (CH, C_3'), 58.61 (CH_2, C_5'), 51.37 (CH_2, C_9'), 48.38 (CH_2, $C_{10'}$), 41.45 (CH_3, C_8'), 38.51 (CH_3, $C_{11'}$), 30.97 (CH_2, C_6'), 12.03 (CH_3, C_7). IR (KBr): 3368, 3026, 2928, 1686, 1476, 1412, 1282, 1127, 1050, 759 cm^{-1}. HRMS: $C_{16}H_{25}N_3S_2O_{10}$ calcd M+Na 506.0879 found 506.0909.

*4.2.3. 1-[2-N-(2-Methylsulfonylethyl)-2-C-(1-(S)-methylsulfonyl)oxo-ethano β-D-2′-dideoxyribofuranosyl]thymine (**4b**).* ^{1}H NMR (DMSO-d_6, 330 °K); δ 10.93 (s, 1H, N_3H), 7.57 (s, 1H, H_6), 5.97 (s, 1H, H_1'), 5.84 (d, 1H, J=6.0, $C_3'OH$), 5.25 (dd, 1H, J=9.4, 5.7, H_7'), 4.92 (s, 1H, $C_5'OH$), 4.53 (dd, 1H, J=8.3, 6.2, H_3'), 3.76–3.68 (m, 3H, H_4', H_5', H_5''), 3.35(dd, 1H, J=13.9, 9.5, H_6'), 3.31–3.21 (m, 4H, H_9', $H_{10'}$), 3.03 (s, 3H, H_8'), 2.88 (s, 3H, $H_{11'}$), 2.77 (dd, 1H, J=13.9, 5.7, H_6''), 1.75 (s, 3H, H_7). ^{13}C NMR (DMSO-d_6, 365 °K); 163.40 (CO, C_4), 150.49 (CO, C_2), 137.83 (CH, C_6), 108.51 (C, C_5), 90.12 (CH, C_7'), 90.02 (CH, C_1'), 82.94 (CH, C_4'), 77.26 (C, C_2'), 67.16 (CH, C_3'), 59.70 (CH_2, C_5'), 52.76 (CH_2, C_9'), 46.50 (CH_2, $C_{10'}$), 41.75 (CH_3, C_8'), 37.37 (CH_3, $C_{11'}$), 34.28(CH_2, C_6'), 11.86(CH_3, C_7). IR (KBr): 3422, 3018, 2929, 1686, 1475, 1412, 1286, 1128, 1054, 755 cm^{-1}. HRMS: $C_{16}H_{25}N_3S_2O_{10}$ calcd M+Na 506.0879 found 506.0840.

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