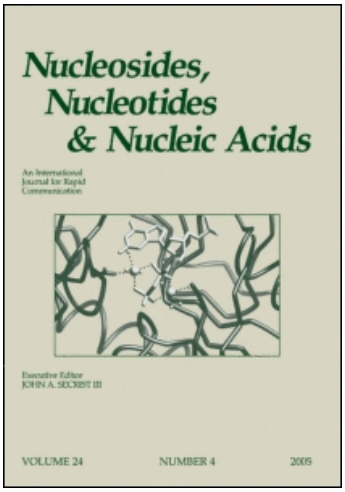

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The Evaluation OF 2', 3'-Dideoxy-β-D-*Erythro*-Hex-2'-Enopyranosyl Nucleosides as Potential Antisense Constructs: Synthesis, Biophysical Properties and Enzymatic Stability of 2'-Deoxyadenosine-(3'-6')-[1-(2', 3'-Dideoxy-β-D-*Erythro*-Hex-2'-Enopyranosyl)thymine] Phosphate

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THE EVALUATION OF 2',3'-DIDEOXY-β-D-*ERYTHRO*-HEX-2'-ENOPYRANOSYL NUCLEOSIDES AS POTENTIAL ANTISENSE CONSTRUCTS: SYNTHESIS, BIOPHYSICAL PROPERTIES AND ENZYMATIC STABILITY OF 2'-DEOXYADENOSINE-(3'-6')-[1-(2',3'-DIDEOXY-β-D-*ERYTHRO*-HEX-2'-ENOPYRANOSYL)THYMINE] PHOSPHATE

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ABSTRACT: 1-(2',3'-dideoxy-β-D-*erythro*-hex-2'-enopyranosyl)thymine was used as a nucleoside substitute in the synthesis of the dimer ApT*. CD studies on the dimer show that it adopts stacked, B-form mini-helices in solution. The title compound, relative to natural ApT, possesses an increased resistance to degradation by nucleases.

Introduction

In recent years, the use of antisense constructs as a potential therapeutic tool has received much attention. A major problem with this approach, however, is the rapid enzymatic degradation of natural oligodeoxynucleotides. Therefore, enzymatically stable oligonucleotide analogues that form stable duplexes with their natural complements must be developed. While extensive studies have been carried out on oligonucleotides modified in their bases and phosphate backbones, sugar modifications have only received a small amount of attention. In several recent studies by Augustyns *et al.*[1,2], the effects on base-pairing properties and enzymatic stability of the incorporation of fully saturated hexose nucleoside analogues into antisense oligonucleotides have been examined. While the modified hexose nucleosides studied conferred greater resistance against enzymatic degradation when incorporated at various positions in natural oligonucleotide strands, they suffered from a reduced duplex-forming capacity with the natural complement. In addition to possible steric problems, a second deterrent to duplex formation is the decrease in entropy (due to the loss of conformational freedom) when an oligonucleotide containing flexible hexose sugars binds to its natural complement. Therefore, assuming the presence of identical intermolecular forces in duplex formation, complexation between single strands

containing more rigid sugars is more energetically favorable. To minimize the effects of this entropic factor, we have, as a first step, synthesized the DNA dimer 2'-deoxyadenosine-(3'-6')-[1-(2',3'-dideoxy-β-D-*erythro*-hex-2'-enopyranosyl)thymine] phosphate (ApT*, compound (4)), which contains the conformationally rigid unsaturated sugar 2',3'-dideoxy-β-D-*erythro*-hex-2'-enopyranose, as an antisense construct with potentially increased hybridization capabilities. We have selected to study a 3'-end protected molecule, since (3'-5')-exonuclease activity is the major cause of the degradation of natural oligonucleotides in serum[3]. We present here the synthesis of ApT*, the results of CD studies on the compound, and enzyme degradation results.

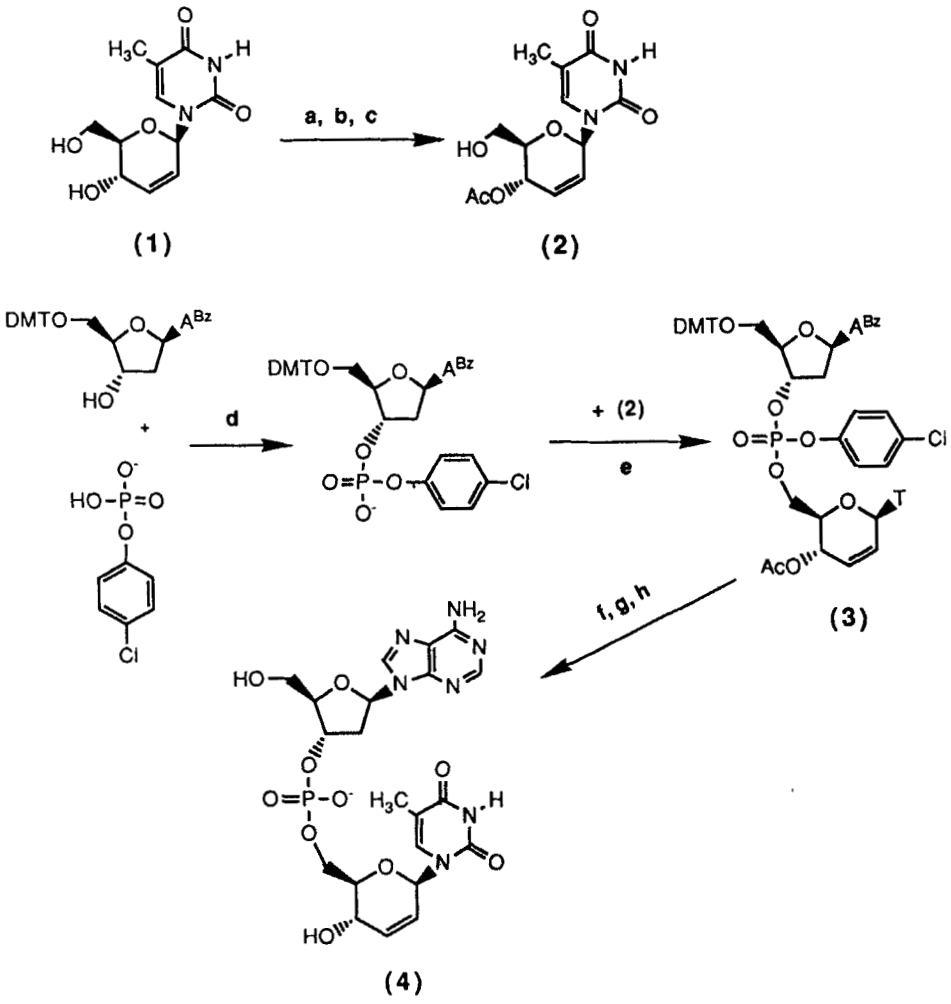
Results and Discussion

*Synthesis of ApT**

Using solution phase phosphotriester chemistry (see Fig. 1) the dimer ApT* (4) was obtained by coupling 5'-O-4,4'-dimethoxytrityl-6-N-benzoyl-2'-deoxyadenosine-3'-p-chlorophenyl phosphate with 1-(2',3'-dideoxy-4'-O-acetyl-β-D-*erythro*-hex-2'-enopyranosyl) thymine (2). After deprotection with zinc bromide, oximate reagent and base, and purification by RP-HPLC, the final yield of fully deprotected dimer (4) was 17%. The coupling step was extremely slow, (24 hrs. to reach completion). This was probably due to the conformational rigidity of the unsaturated hexopyranose sugar, which may have hindered the formation of the proper transition state complex. Coupling was initially attempted using H-phosphonate chemistry, but was unsuccessful. H-phosphonate couplings were always dominated by a side reaction, where the activating agents tried, (pivaloyl chloride, TPS-Cl and TPS-tetrazole) reacted preferentially with O6' of (2). The synthesis of (1) is described in a previous publication[4].

Circular Dichroism Studies

The CD spectra of ApT* at 10° C and 70° C, and the CD spectrum of natural ApT at 10° C (from ref. 5) are shown in Fig. 2. The virtually identical positioning of λ max., λ min. and the cross over points for ApT and ApT*, indicate that substitution of a 2'-deoxyribose sugar with 2',3'-dideoxy-β-D-*erythro*-hex-2'-enopyranose at the 3'-terminus of ApT has a minimal influence on the global structural parameters. The positive and negative Cotton bands of the ApT* spectra are highly symmetric, and as the temperature is increased to 70°C, the magnitudes of the CD maxima decrease by approximately 60%. Overall, these features (and the signs of the Cotton bands) are consistent with the formation of stacked, B-form minihelices in solution[6,7].



a) 1.1 eq. DMT-Cl, 0.1 eq. DMAP. in pyridine **b)** 2.0 eq. Acetic anhydride. **c)** 80:20 acetic acid:H_2O. **d)** 3.3 eq. TPS-Cl in pyridine. **e)** TPS-tetrazolide (1 eq. TPS-Cl + 3 eq. 1H-tetrazole) in pyridine. **f)** $ZnBr_2$ (1.0 M in 85:15 CH_2Cl_2:Isopropanol). **g)** 10 eq. o-Nitrobenzaldoxime + 8.8 eq. TMG in 50:50 dioxane:acetonitrile. **h)** conc. NH_4OH

Figure 1

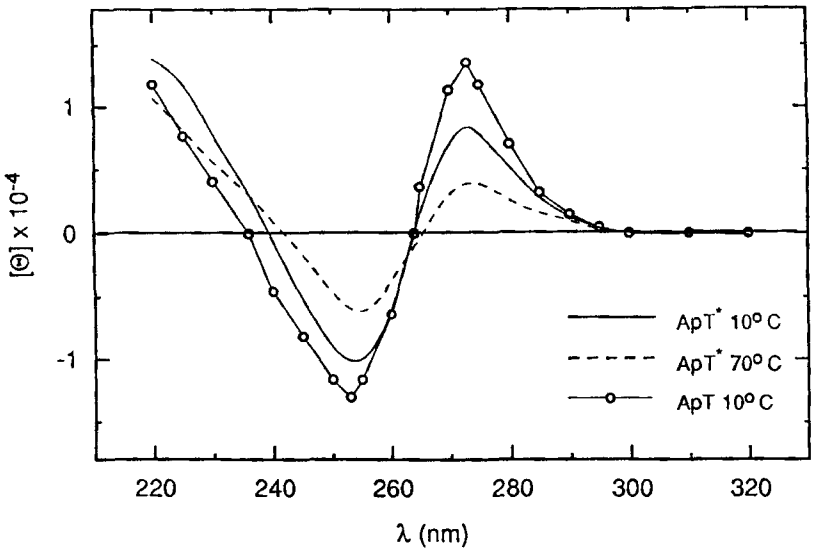


Figure 2. Circular Dichroism (CD) spectra of ApT and ApT* at 10° C, and ApT* at 70° C. The ApT spectrum is from (5). The vertical axis gives [Θ] in deg M^{-1} cm^{-1}, the molar ellipticity per residue.

Enzymatic Hydrolysis of ApT and ApT*

As a means to assess the enzymatic stability of ApT*, relative to its natural analogue ApT, the following nucleases were used: nuclease S1 (endonuclease), snake venom phosphodiesterase (SV PDE, (3'-5')-exonuclease) and bovine spleen phosphodiesterase (BS PDE, (5'-3')-exonuclease). RP-HPLC was used to monitor the degradation of the dimers, which follows first order kinetics. With each enzyme (Table 1), ApT* showed an increased resistance to degradation relative to ApT.

ApT* was degraded more slowly than ApT by factors of 4, 3 and 2 in nuclease S1, SV PDE and BS PDE, respectively. These results suggest that a 2',3'-dideoxy-β-D-*erythro*-hex-2'-enopyranose moiety at the 3'-end of an oligonucleotide can provide significant protection against (3'-5')-exonuclease degradation. However, compared to results obtained from TpT# dimers, where T# refers to a thymine nucleoside containing the fully saturated 2',3'-dideoxy-β-D-*erythro*-hexopyranose sugar[1], the unsaturated sugar moiety seems considerably less effective. Since the spatial distribution of important functional groups on nucleosides containing both the saturated and unsaturated hexopyranose moieties is almost identical (Fig. 3), structural differences are probably not the cause of the increased rates of degradation observed in ApT*. Also, the equal abundance of the 6'- and 3'-phosphate fragment ions in the negative ion FAB mass

Table 1

	$t_{1/2}$ (min.)		
	nuclease S1	SV PDE	BS PDE
ApT	10.6	2.1	1.6
ApT*	42.1	6.2	3.1

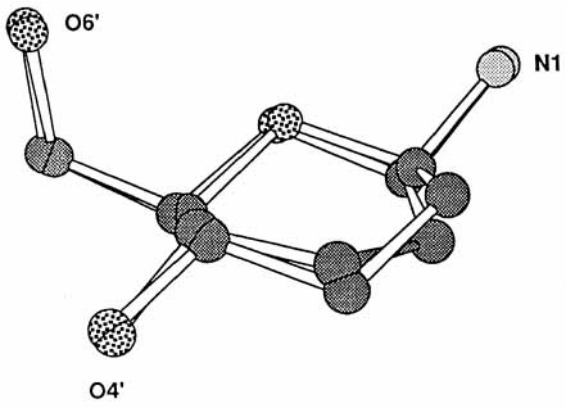


Figure 3 N1, O4' and O6' of 1-(2',3'-dideoxy-β-D-*erythro*-hex-2'-enopyranosyl) thymine (T*, compound (1), double bond is darkened) superimposed on the same atoms from 1-(2',3'-dideoxy-β-D-*erythro*-hexopyranosyl) thymine (T#). Except for N1, the remaining base atoms from each nucleoside are not shown. Coordinates for both sugars were obtained from crystal structures. (Coordinates for T* are from ref. 4, while those for T# are from ref. 8). The average r.m.s. difference in atomic coordinates between models is 0.09 Å.

spectrum (see experimental) indicate that the stability of the 3'-6'-phosphodiester linkage is not compromised by unsaturation of the hexopyranose sugar, ruling it out as a contributing factor. Thus, the more rapid degradation of the title compound is probably the result of sequence and/or entropic effects. Experimental evidence suggests that sequence is important, since under similar experimental conditions, natural ApT is digested almost five times more rapidly than TpT (from ref. 1). Additionally, entropic factors (see Introduction) dictate that the rigidity of the T* relative to T# may favor the binding of oligonucleotides containing the rigid sugar to nucleases.

Summary

Our results demonstrate that substitution of a 2'-deoxyribose sugar at the 3'-end of the dinucleotide ApT with 2',3'-dideoxy-β-D-*erythro*-hex-2'-enopyranose provides a

substantial increase in (3'-5')-exonuclease resistance. ApT* maintains a global structural isomorphism with ApT, suggesting that the hybridization properties of oligonucleotides containing the unsaturated hexose sugar at the 3'-end should not be compromised. Because of favorable entropic factors, the conformational rigidity of the sugar used in this study may, actually, increase the hybridization capabilities of oligonucleotides containing it in positions other than at the 5'- or 3'-ends, relative to oligonucleotides containing more flexible saturated hexopyranose sugars. Currently, we are attempting to synthesize longer hybrid oligonucleotide sequences incorporating 2',3'-dideoxy-β-D-*erythro*-hex-2'-enopyranose nucleotides at the ends and the interior of the strands, to assess their structural and hybridization properties, as well as their enzymatic stabilities.

Experimental Section

^{1}H NMR spectra were obtained at 300.1 MHz (Bruker AM300). Signals are quoted as δppm downfield from internal tetramethylsilane unless otherwise stated.

Negative ion FAB mass spectra (glycerol matrix, 8-keV xenon atoms) were obtained on a VG 7070E-HF spectrometer.

CD spectra were obtained on a Jasco J-500A spectropolarimeter using a 1.0 mm cell equipped for temperature control with a water bath. ApT* was studied at 4.41 x 10^{-4} M (calculated from UV absorbance measurements) in 0.01 M sodium phosphate buffer (pH 6.8), and 0.1 M NaCl to maintain a constant ionic strength. The CD spectrum of ApT[5], was obtained in the same buffer (with 0.001 M EDTA) at approximately the same concentration.

Final purification of ApT* and analysis of enzyme degradation products were carried by reverse phase HPLC on an analytical C-18 (phenomenex) column using a Varian 9010 HPLC solvent delivery system equipped with a Varian 9050 UV/vis detector. The flow rate was 1 ml/min. For purification of ApT*, the solvent system used was: A (0.1 M NH_4OAc) and B (MeOH). A linear gradient was used, where A was varied from 100% to 40% over 40 minutes. For the enzyme degradation studies, isocratic elution was performed using a ratio of C (5% MeOH in 0.1 M triethyl-ammonium acetate (pH 7.0)) and D (50% MeOH in 0.1 M triethyl-ammonium acetate (pH 7.0)) of 2:1. The hydrolysis of the dimers was followed by integration of the dimer signal. The decrease in the dimer concentration with time was fitted to a simple exponential function.

Nuclease S1 1 OD^{260nm} of dimer in 270 μL of H_2O was mixed with 30 μl of the following buffer: 0.33 M sodium acetate (pH 4.8), 0.5 M sodium chloride, 0.01 M zinc chloride. Nuclease S1 (4.6 μL, 1068 U) (Sigma) was then added. The mixture was incubated at 37° C. 20 μl aliquots were removed at set times and mixed with 180 μL of the

following blocking buffer: 10 mM sodium phosphate (pH 6.8). This mixture was heat inactivated at 95° C for 5 min. and cooled. 10 μL aliquots were analyzed by HPLC.

Snake Venom Phosphodiesterase 1 OD^{260nm} of dimer in 200 μL of H_2O was mixed with 100 μL of the following buffer: 0.3 M Tris.HCl (pH 8.8), 0.3 M sodium chloride, 0.040 mM magnesium chloride. 3 μL (0.1 U) of a solution of snake venom phosphodiesterase (Sigma) in 5 mM Tris.HCl (pH 7.5), 50% glycerol (v/v) was then added. The mixture was incubated at 37° C. Inactivation and analysis was carried out as described for nuclease S1.

Bovine Spleen Phosphodiesterase 1 OD^{260nm} of dimer in 200 μL of H_2O was mixed with 100 μl of the following buffer: 300 mM sodium citrate (pH 6.0). 3 μL (0.1 U) of a solution of bovine spleen phosphodiesterase (Sigma) in 50 mM sodium citrate (pH 6.0), 50% glycerol (v/v) was then added. The mixture was incubated at 37° C. Inactivation and analysis was carried out as described for nuclease S1.

Synthetic Methods

1-(2',3'-dideoxy-4'-O-acetyl-β-D-*erythro*-hex-2'-enopyranosyl)thymine (2)

254 mg (1.0 mmol) of 1-(2',3'-dideoxy-β-D-*erythro*-hex-2'-enopyranosyl) thymine (**1**), was dried by co-evaporation 3 times in dry pyridine and then dissolved in 5 mL of 50/50 (v/v) anhydrous methylene chloride/pyridine. 373 mg (1.1 mmol) of dimethoxytrityl chloride and 12 mg (0.1 mmol) of 4-(dimethylamino)pyridine were then added to the solution, which was sealed and stirred for 10 hrs. At this point the reaction was complete (as determined by TLC, ether), and 0.19 mL (2.0 mmol) of acetic anhydride were added to the mixture, which was sealed again and stirred for 10 hrs. The reaction was then quenched with 1.0 mL of water, which was added dropwise, while the reaction mixture was immersed in an ice-bath. The reaction mixture was then washed twice with water, and concentrated to an yellowish solid on a rotary evaporator. The solid was dissolved in 3 mL of 80/20 (v/v) acetic acid/H_2O and stirred for 6 hrs. The reaction mixture was then concentrated on a rotary evaporator four times with ethyl acetate to remove all traces of acetic acid. Finally the product was concentrated to a syrup in ethyl acetate, and purified on silica gel (Aldrich, 60 grade 230-400 mesh) using ether as an eluent by dry column flash chromatography, yielding 170 mg (0.57 mmol, 57% yield) of (**2**) as a white powder.

^1H NMR ($CDCl_3$) δ_H 8.10 (1 H, br s, NH), 6.99 (1 H, s, 6-H), 6.53 (1 H, s, 1'-H), 6.20 (1 H, m, $J_{2',3'}$ 12.3 Hz, 2'-H), 5.76 (1 H, m, $J_{2',3'}$ 12.3 Hz, 3'-H), 5.48 (1 H, m, 4'-H), 3.82 (2 H, m, 6'-H), 3.70 (1 H, m, 5'-H), 2.13 (3 H, s, OAc), 1.93 (3 H, s, Me), 1.56 (1 H, br s, OH).

2'-deoxyadenosine-(3'-6')-[1-(2',3'-dideoxy-β-D-*erythro*-hex-2'-enopyranosyl)thymine] phosphate (4)

430 mg (.65 mmol) of 5'-O-4,4'-dimethoxytrityl-6-N-benzoyl-2'-deoxyadenosine (Cruachem) and 150 mg (.70 mmol) of p-chlorophenyl phosphate were dried by co-evaporation (4 times) in dry pyridine. The resulting foam was dissolved in 5 mL dry pyridine and 610 mg (2.0 mmol) of 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) was added, and the mixture was sealed and stirred for 4 hrs. At this point, the phosphorylation was complete (determined by TLC, ether, followed by 12:1 CH_2Cl_2: MeOH), and 150 mg (0.5 mmol) of (2) (dried by co-evaporation with pyridine), an additional 370 mg (0.4 mmol) of TPSCl, and 250 mg (1.5 mmol) of 1H-tetrazole (dried by co-evaporation with dry acetonitrile) were added, and the mixture was sealed and stirred for 24 hrs (monitored by TLC, as above). 3 mL of H_2O were then added dropwise to the reaction mixture, which was placed in an ice-bath. The product was then extracted into methylene chloride, evaporated to a thick syrup, and purified on silica gel (same grade as used above), using dry column flash chromatography. A hexane/ethyl acetate solvent mixture was used as the eluent, changing the hexane content linearly from 100% to 0% through the course of the purification. Fractions were pooled, and solvent was removed, yielding 305 mg of semi-pure 5'-O-4,4'-dimethoxytrityl-6-N-benzoyl-2'-deoxyadenylyl-(3'-6')-[1-(2',3'-dideoxy-4'-acetyl-β-D-*erythro*-hex-2'-enopyranosyl)thymine]p-chlorophenyl phosphate (3) (~ 50% yield) as an orange solid.

Detritylation of (3) was carried out by adding 300 mg (0.26 mmol) to a 5 mL solution of 1.0 M zinc bromide in 85/15 (v/v) methylene chloride/isopropanol and stirring for 20 min. Solvent was then removed and the detritylated product dissolved in methylene chloride and washed with a minimum amount of H_2O. The solvent was then removed and the solid washed twice with ether to remove all traces of trityl alcohol. The phosphate group was then deprotected by adding 200 mg (0.2 mmol) of detritylated dimer to a solution of 330 mg (2.0 mmol) o-nitrobenzaldoxime and 0.22 mL (1.76 mmol) 1,1,3,3,N,N, N',N'-tetramethyl guanidinium in 3.0 ml 50/50 (v/v) dry dioxane/dry acetonitrile and stirred for 48 hrs. Solvent was removed, and the product was washed 5 times with ether, and dissolved in 10 mL of conc. NH_4OH. The ammonia solution was sealed in a pyrex tube, and heated at 60° C for 8 hrs. Finally, the ammonia was removed and the solid residue dissolved in H_2O. Purification of the crude product is described above. The purified product was de-salted on a C18 Sep-Pak (Sigma, using standard procedure), yielding 43 mg (17 %) of (4) as a free-acid.

UV (in CD buffer) λ_{max} 263, $\varepsilon = 20400\ l\ mol^{-1}\ cm^{-1}$

MS (negative ion FAB) *m/z* 566 (100, [M - H]$^-$), 333 (26, [6'-dT*MP - H]$^-$), 330 (24, [3'-dAMP - H]$^-$), 134 (107, [Adenine - H]$^-$), 125 (71, [thymine - H]$^-$)

^{1}H NMR (D_2O, using D_2O-signal as standard (δ=4.74)) δ_H 8.29 (1 H, s, 8-H(A)), 8.26 (1 H, s, 2-H(A)), 7.32 (1 H, s, 6-H(T)), 6.44 (1 H, t, 1'-H(A)), 6.36 (1 H, s, 1'-H(T)), 6.23 (1 H, d, $J_{2',3'}$ 12.0 Hz, 2'-H(T)), 5.69 (1 H, d, $J_{2',3'}$ 12.0 Hz, 3'-H(T)), 4.95 (1 H, m, 3'-H(A)), 4.54 (1 H, m, 4'-H(T)), 4.38 (1 H, m, 4'-H(A)), 4.21 (2 H, m, 6'-H(T)), 3.86 (1 H, m, 5'-H(T)), 3.79 (2 H, m, 5'-H(A)), 2.84-2.67 (2 H, m, 2'-H(A)), 1.64 (3 H, s, Me(T)).

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