Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 15, pp. 1843-1848, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00328-9

SYNTHESIS AND RIBONUCLEASE H RELATED BIOCHEMICAL PROPERTIES OF α-ANOMERIC 2'-DEOXYPYRIMIDINE HOMOOLIGOMER AND ITS PHOSPHOROTHIOATE ANALOG

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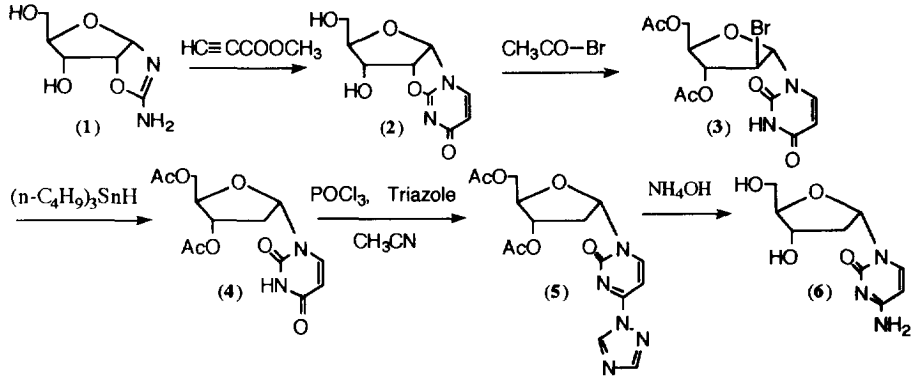
Key Words: α-anomeric 2'-deoxycytidine; DNA-RNA duplex; RNase H; inhibition of RNase H

Abstract: RNase H mediated degradation of RNA on the β-DNA•RNA duplex was inhibited by the presence of extra 2'-deoxypyrimidine homooligomer. The effect was dependent on the nature of the nucleobase, glycoside bond configuration, and the internucleotide linkage of the extra homooligomer used. Thus, α-anomeric 2'-deoxycytidylate phosphorothioate exhibited the most potent inhibitory effect (> 40 %) on RNase H.

Antisense technique utilizing single-stranded DNAs or RNAs has been attracting great interest as a potential new therapeutic method since the discovery of its possible application as an anti-HIV agent.[1] The phosphorothioate analog of β-anomeric DNA (β-S-DNA) is regarded to be a suitable antisense agent because of its enhanced stability towards the action of a variety of nucleases compared to natural DNA.[1] In the antisense technique, it was also suggested that the action of RNase H which degrades the RNA component of the DNA-RNA duplex is crucial to obtain a high degree of the antisense effect.[2] From this point of view, β-S-DNA is again advantageous over natural DNA since the duplex of β-S-DNA and RNA is reportedly a better substrate of RNase H than the duplex of natural DNA and RNA.[3]

Meanwhile, a 28-mer long homooligomer of oligodeoxycytidylate phosphorothioate (β-S-dC_{28}) was also found to possess potent anti-HIV activity.[1] Interestingly, the other pyrimidine homooligomer, oligothymidylate phosphorothioate (β-S-dT_{28}), showed rather limited anti-HIV activity.[1] Recently, the same phosphorothiate homooligomer but consisting of α-anomeric 2'-deoxycytidine residue (α-S-dC_{28}) was prepared. The oligomer exhibits potent anti-HIV activity *in vitro*. In contrast, the corresponding oligomer having phosphodiester linkage exclusively (α-dC_{28}) was almost inactive.[4]

These facts prompted us to investigate some basic physicochemical and biochemical properties of α- and β-anomeric deoxypyrimidine homooligomers (dC_{12}, and dT_{15}) and their phosphorothioate analogs. During the study, we found that the presence of an extra deoxypyrimidine homooligomer inhibits RNase H mediated degradation of RNA on the DNA–RNA duplex. The effect was dependent on the nature of the nucleobase, glycoside bond configuration, and the internucleotide linkage of the extra homooligomer used. We wish to report these findings along with facile a stereospecific preparation of α-anomeric 2'-deoxycytidine.



Scheme 1. The stereospecific synthesis of a-anomeric 2'-deoxycytidine.

The key nucleoside α-anomeric 2'-deoxycytidine (**6**)[5] was synthesized by a facile stereospecific method starting from the 2,2'-anhydrouridine (**2**) which was obtained from riboaminooxazoline (**1**) and methyl propiolate.[6] The anhydro nucleoside was treated with acetyl bromide to give α-anomeric 3', 5'-di-*O*-acetyl-2'-bromouridine (**3**). After removal of the bromine atom on the foregoing compound with *n*-tributyltin hydride, the obtained α-anomeric 2'-deoxyuridine (**4**) was converted to the corresponding triazolyl nucleoside derivative (**5**) followed by treatment with ammonium hydroxide in dioxane at room temperature[7] to afford α-anomeric 2'-deoxycytidine. The total yield of **6** from **2** was quite satisfactory (> 50 %).[8] This method gives better result than the method recently reported by us in which riboaminooxazoline (**1**) was also utilized.[9] α-Anomeric thymidine was prepared by the method previously reported.[10] The nucleosides were converted to the corresponding protected 3'-phosphoramidites and assembled to the desired homooligomers by the standard procedure[11] on a DNA synthesizer (ABI-381A) The oligomers were purified by reverse phase HPLC, detritylation, ethanol precipitation and Sephadex G-25 gel filtration as usual. It should be noted that the detritylation of α-anomeric oligomers required slightly more severe conditions (10 % AcOH in 20 min.) than the corresponding β-anomeric oligomers (10 % AcOH in 10 min.).

The thermal stability of the duplexes consisting of 2'-deoxypyrimidine homooligomer, (dT_{15} or dC_{12}), and poly RNA (poly rA or poly rI)[12] was examined by the temperature dependent change of UV absorption at 260nm. The values of melting temperature (*Tm*) for each sample are listed in Table 1. As we have reported previously, the duplex consisting of α-dT_{15} (or α-S-dT_{15}) and polyriboadenylate (poly rA) possesses higher *Tm* values compared to the corresponding β-dT_{15} (or β-S-dT_{15})•poly rA duplex.[13] However, as shown in Table 1,

oligocytidylate with α-configuration formed a less stable duplex with its complementary poly rI compared to its β-counterpart. The results are in agreement with a recent report although the experimental condition is slightly different.[14]

Table 1. The Thermal Stability of 2'-Deoxypyrimidine Homooligomer • RNA Duplex

Duplex	β-dC_{12} Poly rI	β-S-dC_{12} Poly rI	α-dC_{12} Poly rI	α-S-dC_{12} Poly rI	β-dT_{15} Poly rA	β-S-dT_{15} Poly rA	α-dT_{15} Poly rA	α-S-dT_{15} Poly rA
Tm (°C)	27	20	21	19	40	27	60	43

Condition: Duplex concentration, 5.8 μM; Buffer, 20 mM Tris-HCl (pH. 7.5) containing 120 mM KCl and 10 mM $MgCl_2$. The samples are pre-melted at 90 °C for 30 min and allowed to thermally equilibrate. *Tm* values were determined from the first differentials of the absorbance vs. temperature plot using an Igor graphing and data analysis program (Wave Matrix, Inc.) and a Macintosh IIci computer (Apple Computer, Inc).

The duplex consisting of β-S-DNA•RNA is reportedly a better substrate compared to the β-DNA•RNA duplex towards RNase H mediated degradation of the RNA component.[3] On the other hand, the duplex consisting of α-DNA•RNA is known to be resistant towards the action of the enzyme.[15] We observed that this is true even when α-S-DNA, either α-S-dC_{12} or α-S-dT_{15}, is used as the DNA component in the DNA•RNA duplex. Thus, the change in UV absorption at 260 nm, indicating the degradation of the RNA component,[3] was quite small (< 5%) in the mixture of α-S-dC_{12}•poly rI duplex and *E. coli* RNase H compared to that in the mixture of the corresponding β-DNA•RNA duplex and *E. coli* RNase H (Fig. 1). The same result was obtained for the α-S-dT_{15}•poly rA duplex and the enzyme mixture (data not shown).

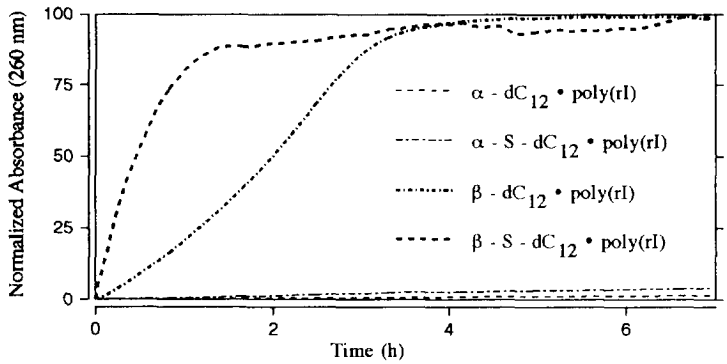


Fig. 1. RNase H mediated degradation of RNA. RNase H (8 units) was added to the DNA•RNA hybrids (5.8 μM as a duplex concentration) in a 20 mM Tris-HCl buffer solution (pH 7.5) containing 120 mM potassium chloride, 10 mM magnesium chloride, 5 % sucrose, and 0.2 mM DTT at 16 °C.[3] The whole mixture was incubated at that temperature and the change of UV absorption at 260 nm was monitored.

We further examined some non-specific inhibitory effect of α-S-DNAs, as well as other oligomers, on the action of RNase H. 2'-Deoxypyrimidine homooligomers were added to a mixture containing the β-dC_{12}•poly rI

duplex, the natural substrate of RNase H, and *E. coli* RNase H and the change in UV absorption in the mixture was monitored at 260 nm as above. Without the additional DNA strand, the absorption increased steadily and

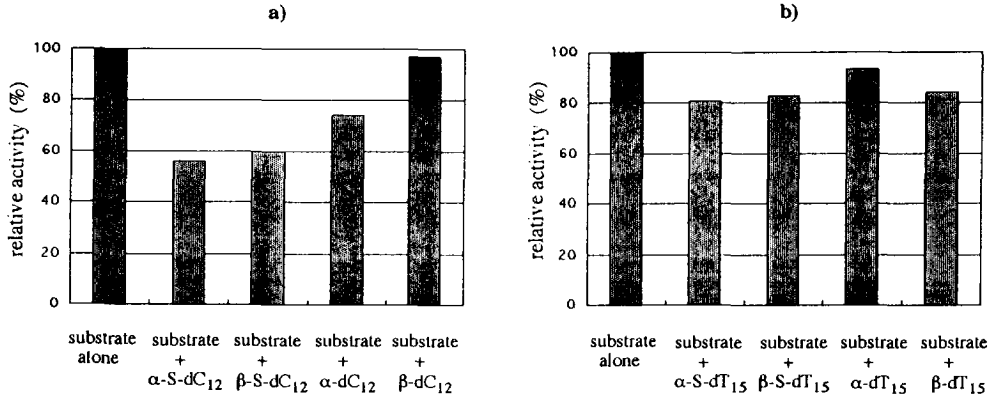


Fig. 2. Inhibition of RNase H activity by 2'-deoxypyrimidine homooligomer. OligoDNA (5.8 μM) was added immediately after the addition of RNase H (8 units) to the solution of the β-dC_{12}•poly rI hybrid (5.8 μM as a duplex concentration) in the same buffer as above at 16 °C. The whole mixture was incubated at that temperature for 4h. The relative activities were obtained from the total change of UV absorption at 260 nm relative to that of the reference reaction in which the extra DNA was absent. Each experiment was repeated three times and the results were averaged. a). Inhibition of RNase H activity by oligodeoxycytidylate. b). Inhibition of RNase H activity by oligodeoxythymidylate.

reached the plateau at about 3.5 h. The phenomenon reflects the fact that the poly rI strand in the duplex was degraded by the action of RNase H within the indicated period. Meanwhile, RNase H induced degradation of RNA was suppressed by the addition of an extra DNA strand. The extent of the suppression, however, is not the same in all cases and there is some correlation between the effect and the nature of the added DNA. Figure 2 summarizes the extent of the inhibitory effect on the RNase H mediated RNA degradation brought about by the addition of the extra DNA. S-DNAs always exhibit higher effect compared to the corresponding DNAs having normal phosphodiester linkages. It is also seen that α-DNAs exhibit higher inhibitory effect compared to the corresponding β-DNAs. Furthermore, homocytidylates are more effective compared to the corresponding homothymidylates despite the fact that the homocytidylates used are shorter in length than the homothymidylates. Thus, we observed both the sugar configuration effect and the base composition effect in addition to the effect of internucleotide linkages. As the result, α-S-dC_{12} possesses the most potent inhibitory effect on the RNase H mediated RNA degradation among the DNA samples tested.[16]

The results presented here raise several interesting questions regarding the contribution of RNase H toward the antisense effect using the DNA phosphorothioate analog as the antisense agent because, as is indicated here, it would be possible that the action of RNase H may be interrupted by the presence of excess amount of DNA phosphorothioate. Another intriguing question is that whether there is any relationship between the observed RNase H inhibitory effect and the potent anti-HIV effect of α- and β-S-dCn. It has been reported that RNase H activity of HIV-RT is essential for viral replication.[17] It is also known that reverse transcriptase of HIV (HIV-RT) possesses an RNase H domain in it and this domain is well conserved, particularly around its active center

bearing four acidic amino acids, between HIV-RT and *E. coli* RNase H.[18] Thus, further investigation to elucidate the relationship of the inhibition of RNase H type of action of HIV-RT by the phosphorothioate analog of 2'-deoxypyrimidine homooligomer is currently in progress.

Acknowledgment. This work was supported in part by a Grant-in-Aid from Gilead Sciences.

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8. Physical properties of the intermediates (**3**), (**4**), and (**5**) are as follows; $O^{3'}$,$O^{5'}$-Diacetyl-α-2'-bromo-2'-deoxyuridine (**3**), mp. (recrystallized from ethanol) 174.5 - 175.5 °C. Calcd. for $C_{13}H_{15}N_2O_7Br$: C, 40.00; H, 3.88; N, 7.18. Found: C, 40.01; H, 3.86; N, 7.21. ^{1}H-NMR ($CDCl_3$) δ: 8.37 (1H, s, br, NH), 7.32 (1H, d, J = 8.2 Hz, H-6), 6.14 (1H, d, J = 3.4 Hz, H-1'), 5.78 (1H, dd, J = 8.2, 2.2 Hz, H-5), 5.46 - 5.39 (1H, m, H-2'), 4.62 - 4.42 (4H, m, H-3', 4', 5'), 2.15 (3H, s, CH_3 of Acetyl), 2.08 (3H, s, CH_3 of Acetyl). $O^{3'}$,$O^{5'}$-Diacetyl-α-2'-deoxyuridine (**4**), mp. (recrystallized from CH_2Cl_2 - benzene) 135.5 - 136.0 °C. Calcd. for $C_{13}H_{16}N_2O_7$: C, 49.98; H, 5.17; N, 8.97. Found: C, 50.14, H, 5.18, N, 9.15. ^{1}H-NMR ($CDCl_3$) δ: 8.78 (1H,s, br, NH), 7.48 (1H, d, J = 8.3 Hz, H-6), 6.22 (1H, dd, J = 7.0, 1.7 Hz, H-1'), 5.76 (1H, dd, J = 8.3, 1.6 Hz, H-5), 5.28 - 5.21 (1H, m, H-3'), 4.63 - 4.55 (1H, m, H-4'), 4.28 - 4.13 (2H, m, H-5'), 2.84 - 2.73 (1H,m, H-2'), 2.38 - 2.27 (1-H, m, H-2'), 2.13 (3H, s, CH_3 of Acetyl), 2.04 (3H, s, CH_3 of Acetyl). $O^{3'}$,$O^{5'}$-Diacetyl-4-triazolyl-α-2'-deoxyuridine (**5**), mp. (recrystallized from ethanol) 206.0 - 207.0 °C. Calcd. for $C_{15}H_{18}N_5O_6$: C, 49.57; H, 4.72; N, 19.28. Found: C, 49.77, H, 4.86, N, 19.52. ^{1}H-NMR ($CDCl_3$) δ: 9.30 (1H, s, H-5 of triazole), 8.15 (1H, s, H- 3 of triazole), 8.14 (1H, d, J = 7.2 Hz, H-6), 7.10 (1H, d, J = 7.2 Hz, H-5), 6.27 (1H, dd, J = 6.9, 1.2 Hz, H-1'), 5.30 - 5.26 (1H, m, H-3'), 4.80 - 4.67 (1H, m, H-4'), 4.34 - 4.17 (2H, m, H-5'), 2.98 - 2.82 (1H, m, H-2'), 2.58 - 2.47 (1-H, m, H-2'), 2.14 (3H, s, CH_3 of Acetyl), 1.95 (3H, s, CH_3 of Acetyl).

The mp. of α-2'-deoxycytidine thus obtained was 196-197 °C (lit [5] 193 - 194 °C).

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(*Received in Japan* 27 *May* 1996; *accepted* 8 *July* 1996)