Preparation of 2'-thio-2'-deoxycytidine 2':3'-phosphorothioate

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ABSTRACT

The synthesis of a novel ribonucleotide analog 2'-thio-2'-deoxycytidine 2':3'-O:S-phosphorothioate is described. In the first step, 2,2'-anhydro-1-ß-D-arabinosylcytosine was thiophosphorylated by the action of dithio-phosphate, a process which gave predominantly the 3'-O-phosphorothioate isomer. An intramolecular displacement reaction led to the formation of the title compound. Structure and reactivity of this thioanalogue differ substantially from 2':3'-CMP.

INTRODUCTION

The substitution of sulfur for the 2'-oxygen of ribonucleotides could lead to compounds of both theoretical and practical interest. The availability of 2'-thio analogs may bring forth some insight into the role of the hydroxyl group in all natural nucleotides containing the ribose moiety, such as cAMP, several coenzymes and DNA itself. Because of structural similarities but different chemical reactivities, the 2'-thio analogs may also turn out to be valuable antimetabolites. One ribonucleoside analog, 2'-thio-2'-deoxyuridine has already been described, but no attempts were made to prepare any of the phosphate esters. Among the possible nucleotide analogs, a major challenge appeared to be the construction of 2'-thio-2'-deoxyribonucleoside 2':3'-cyclic phosphorothioates. The synthesis of these analogs involves the familiar difficulty of introducing the cis 3'-O,2'-S substitution along with the labile 5-member O,S-phosphorothioate ring. The same ring system in ethylene O,S-cyclic phosphorothioate was reported to be 25 times more reactive than the natural phosphate ester in alkaline hydrolysis. In this paper we report the synthesis of 2'-thio-2'-deoxycytidine 2':3'-phosphorothioate (\(\Phi\), scheme 1) and a process which represents a useful addition to the art of nucleotide chemistry.

The crucial discovery which led to this work was the observation that \(\Phi\), 2'-anhydro-1-ß-D-arabinosylcytosine 3'-phosphate \(\Phi\) could undergo spontaneous rearrangement to form cytidine 2':3'-phosphate.
We rationalized that the same type of rearrangement is possible using the 3'-O-phosphorothioate.

\[ \text{RESULTS} \]

In several attempts to realize the above rationale, experimental difficulties arose due to the fact that anhydro-araC and thiophosphate esters are labile to alkali and acid, respectively, and the end-product \( \delta \) is labile to both agents. Therefore, a novel thiophosphorylation method had to be developed which would directly yield phosphorothioate esters in neutral medium. We found that inorganic dithiophosphate \( ^6 \) could serve the purpose of transferring the thiophosphoryl group under the cleavage of one P-S bond as the driving force. Anhydro-araC reacted smoothly with a 5-fold excess of dithiophosphate in anhydrous dimethylformamide at room temperature. It was found advisable to terminate the reaction before all the anhydro-araC reacted (8-12 hrs) because of the increase of polyphosphate byproducts.

The isomeric anhydro-araC 5'-O-phosphorothioate \( \zeta \) and 3'-O-phosphorothioate \( \xi \) were isolated by ion exchange chromatography in 5 and 43 percent yields, respectively. While compound \( \xi \) gave acceptable elemental analysis, compound \( \zeta \) has not been fully analysed. The presence of O-phosphorothioate in both \( \xi \) and \( \zeta \) was indicated by their relative stabilities to alkaline phosphatase and their increased acidity relative to the corresponding phosphate esters. Attempts of desulfurization by the methods of Eckstein \( ^7 \) resulted in the hydrolysis of the ester linkage. Mild oxidation of both \( \xi \) and \( \zeta \) led to the formation of zwitterionic disulfides. The assignment of structure to the major product as the 3'-isomer was aided by its similarity to anhydro-araC 3'-phosphate in chemical and spectral properties. \( ^3, ^4 \) The pmr spectrum of \( \xi \) (Table 1) shows the characteristic deshielding of the 2' and 3' protons.

At neutral pH, \( \zeta \) slowly rearranged to 2'-thio-2'-deoxycytidine 2':3'...

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0-2'-phosphorothioate (‡). The factors which determine the rate of rearrangement are the same as reported for the natural phosphate, i.e. pH, ionic strength and solvent. We obtained the best yield so far (69%) in 90% pyridine solution at 20° for 48 hr.

Comparison of the pmr spectra of ‡ with that of 2':3'-CMP revealed an upfield shift of the C2'-H resonance which is in accord with previous observations9 of increased shielding upon replacement of oxygen with sulfur in nucleoside phosphate esters. A further proof of 2'-thio substitution is the large coupling constant of the glycosidic proton in the pmr spectrum. This phenomenon was noted most recently by Imazawa et al.1 who interpreted it in terms of a general substituent effect which forces the C2' into an extreme endo position. As a result, there is an increase in the population of the anti conformer which, in turn, leads to the observed downfield shift of the C6'-H resonance. These findings are in sharp contrast to the behavior of the natural 2':3'-CMP10 for which the syn configuration was proven.

Compound ‡ was found more susceptible to hydrolysis than natural 2':3'-CMP. The difference was relatively small at pH 1.3 but became very substantial at pH 12.5. Pancreatic ribonuclease had no effect on this analog. The hydrolysis product(s) has not yet been fully analysed, but preliminary observations indicate that the stable end product of acidic hydrolysis is the natural (3') phosphate ester, while the 2'-S-phosphorothioate is formed at high pH.

DISCUSSION

Several aspects of the above work contain the element of surprise and deserve emphasis. It is the first time in the area of nucleosides that the 3' hydroxyl group exhibited a superior reactivity. The gauche-gauche configuration11 of the -CH2OH alone seems insufficient to explain the observed difference. The reduction of nucleophilicity due to 5' oxygen-base interaction is a more likely cause. In contrast, similar thio phosphorylation of 2'-deoxythymidine produced the 5'-and 3'-0-phosphorothioates in 2:1 ratio at a much reduced rate12. This points to the uniqueness of anhydro-araC in phosphorylations which may involve strong coulombic interactions. Considering the high nucleophilicity of thiophosphate esters, the relatively slow rearrangement of ‡ is surprising. The stereochemistry of ‡ must greatly differ from that of 2':3'-CMP because the 2' endo configuration is apparent on the NMR time scale. It follows that the conformation of the 5-member ring containing the phosphorus and also the torsion angle of the base had to be affected. However, the causes of resistance to pancreatic ribonuclease A
may not necessarily be related to the different stereochemistry since alkaline hydrolysis results in P-O cleavage. The lower energy of the P-S bond may be offset by the reluctance of sulfur to occupy the apical positions of the pentacovalent intermediate in cyclic phosphorothioates.

Our understanding of the mechanism of thiophosphorylation is incomplete and there may be more than one reactive species considering the formation of polyphosphates. Further research will center around the polymerization of 4 and the use of 2 and 3 as potential antileukemic agents. The reaction reported here may also be a model of prebiotic nucleotide chemistry.

**EXPERIMENTAL**

**Analytical methods.** Thin layer chromatography was performed on Eastman Chromagram sheets with fluorescent indicator in the solvent mixture 1-butanol/95% ethanol/1.0 M \( \text{NH}_4\text{OOCCH}_3 \) (30:15:5). High voltage electrophoresis was carried out on a Savant flat plate apparatus in 0.05 M \( \text{NH}_4\text{OOCCH}_3 \) at pH 5.2 and pH 7.0. Elemental analyses were conducted by M-H-W Laboratories. The pmr spectra were recorded by a Varian HA-100 spectrometer at ambient temperature in 0.2 M solution of the nucleotides in \( \text{D}_2\text{O} \), pH 7.0 with TMS as external standard.

2,2'-Anhydro-araC tosylate was a gift of Dr. W. J. Wechter, The Upjohn Company. Alkaline phosphatase (BAPF) and ribonuclease A were purchased from Worthington and Sigma respectively. Analytical grade solvents were dried over molecular sieve Linde type 4A.

**Thiophosphorylation of anhydro-araC**

An aqueous solution of \( (\text{NH}_4)_2\text{PS}_2\text{O}_6 \) (21 mmoles) was converted to the triethylammonium salt by repeated evaporation from pyridine and triethylamine. The anhydrous pyridine solution of dithiophosphate was mixed with a solution of 2,2'-anhydro-araC tosylate (1.828 g, 4 mmoles) in 30 ml dimethylformamide. The resulting solution was concentrated to 20 ml and stirred at 25° for 8 hr. Water (200 ml) was added, and the nucleotide mixture was absorbed on Dowex 1 resin (formate, 100 ml). Approximately, 1.5 mmoles of the nucleoside were recovered from the water wash. Elution with 0.02 M acetic acid produced first the minor product, anhydro-araC 5'-O-phosphorothioate (2, 1950 O.D. units) followed by the 3'-O-phosphorothioate (3, 18070 O.D. units, 1.8 mmoles). Subsequent elution with 0.1 M formic acid yielded a trace amount of 2'-thio-2'-deoxycytidine 2':3'-phosphorothioate (4) while a final rinsing with 1 M NaCl released a considerable amount of polyphosphates (4500 units) which were not further analysed. Compound 2 gave correct elemental analysis for C, H, N, P, but
low values were obtained for sulfur due to partial loss on drying. The freeze-dried powder of the zwitterionic form of \( \frac{\lambda}{2} \) gave the following analysis:

for the formula \( C_9H_{12}N_0PS + 1.5 H_2O \) (mw 348.26), calc. C 31.03, H 4.19, N 12.06, P 8.89, S 9.20; found C 31.09, H 4.13, N 12.26, P 9.01, S 9.13. 

**Table 1**  
Pmr Data of Nucleotides

<table>
<thead>
<tr>
<th>Chemical shifts ( \delta ) ppm (coupling constants, Hz)</th>
<th>H-6</th>
<th>H-5</th>
<th>H-1'</th>
<th>H-2'</th>
<th>H-3'</th>
<th>H-4'</th>
<th>H-5'</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda )</td>
<td>8.62d (7.5)</td>
<td>7.14d</td>
<td>7.14d (6)</td>
<td>6.12s</td>
<td>5.30s</td>
<td>5.04s</td>
<td>4.32m</td>
</tr>
<tr>
<td>( \frac{\lambda}{2} )</td>
<td>8.66d (7.5)</td>
<td>7.16d</td>
<td>7.20d (6)</td>
<td>6.37d</td>
<td>5.63d</td>
<td>4.9</td>
<td>4.20m</td>
</tr>
<tr>
<td>( \frac{\lambda}{4} )</td>
<td>8.24d (7.5)</td>
<td>6.61d</td>
<td>6.53d (8)</td>
<td>4.88m</td>
<td>5.30</td>
<td>4.86m</td>
<td>4.33d</td>
</tr>
</tbody>
</table>

**Table 2**  
UV Maxima and Mobilities of Nucleotides

<table>
<thead>
<tr>
<th>UV max (pH 6)</th>
<th>( \epsilon )</th>
<th>Electrophoresis(^a) (pH 5.2)</th>
<th>TLC(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda )</td>
<td>262.5</td>
<td>10,400</td>
<td>0.61</td>
</tr>
<tr>
<td>( \frac{\lambda}{2} )</td>
<td>262.5</td>
<td>10,400</td>
<td>0.74</td>
</tr>
<tr>
<td>( \frac{\lambda}{4} )</td>
<td>270</td>
<td>9,200</td>
<td>0.89</td>
</tr>
<tr>
<td>AnhydroaraC 3'-P</td>
<td>262.5</td>
<td>10,300</td>
<td>0.35</td>
</tr>
</tbody>
</table>

\(^a\) relative to 2':3'-CMF

For alkaline phosphatase hydrolysis, one unit enzyme was added to 0.3 ml of 8 mM nucleotide solution in 0.1 M Tris-chloride, pH 8 at 37°. The products were observed by TLC and ion-exchange chromatography. The control anhydro-araC 3'-P was completely degraded within 1 hr. Compound \( \lambda \) hydrolysed 75% in 1 hr and showed no further change after 3 hr. Compound \( \frac{\lambda}{2} \) was essentially unaffected. Mild oxidation of \( \frac{\lambda}{2} \) and \( \frac{\lambda}{4} \) (50 mM) in 0.1 M Tris-Cl pH 7 with one equivalent of iodine at 20° for 30 min. was evaluated by electrophoresis at pH 5.2 and 7.0. The major products were zwitterionic compounds, probably desulfurized anhydro-araC phosphates.

2'-Thio-2'-deoxycytidine 2':3'-phosphorothioate (\( \frac{\lambda}{4} \)). Compound \( \frac{\lambda}{4} \) (400 mg., 1.0 mmole) was kept in 10 ml of pyridine-H\( _2\)O 9:1 at 20° for 48 hr. Upon dilution with water, the products were separated on a DEAE cellulose column.
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(HCO$_3^-$ form) using a linear gradient of triethylammonium bicarbonate. Following
the minor peak of unchanged $\frac{1}{2}$, the cyclic phosphorothioate $\frac{6}{7}$ appeared as the
major product (6460 O.D.-70 units, 69%). An equivalent amount of NaHCO$_3$ was
added and $\frac{6}{7}$ was freeze-dried several times for analyses. For C$_9$H$_{11}$N$_3$OPS$\cdot$Na$^+$
H$_2$O, calc. C 29.92, H 3.63, N 11.63, P 8.57, S 8.88; found C 30.06, H 3.71,

For alkaline hydrolysis, samples of 10 O.D. units of $\frac{6}{7}$ and 2':3'-CMP were
dissolved in 0.1 N KOH (0.4 ml) for 1 min at 20°, then neutralized with 0.1 N
acetic acid. The extent of hydrolysis was determined using a small DEAE
cellulose (1 ml) column. Only 6.5% of the analog remained unchanged while
2':3'-CMP was 71% intact. When acid hydrolyses were carried out in 0.1 N HCl
at 20° for 5 min, the amounts of unchanged materials were 53% for $\frac{6}{7}$ and 63%
for 2':3'-CMP.

Pancreatic RNase A (10 µg) and $\frac{6}{7}$ (2 mg) were incubated in 0.1 ml of
Tris-chloride, pH 8, at 20° for 3 hr. Analysis by TLC revealed no change.
The control 2':3'-CMP was completely hydrolysed.

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