Pergamon

Tetrahedron Letters, Vol. 38, No. 6, pp. 1021-1024, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)00084-1

5′-Deoxy-5′-thioribonucleoside-5′-triphosphates

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Abstract: 5′-Deoxy-5′-thiouridine- and 5′-deoxy-5′-thioadenosine-5′-triphosphate **6** and **7** were chemically synthesised by two methods. Their incorporation into RNA to produce a 5′-S-bridging phosphorothioate internucleotide linkages, using T7 RNA polymerase, was investigated.

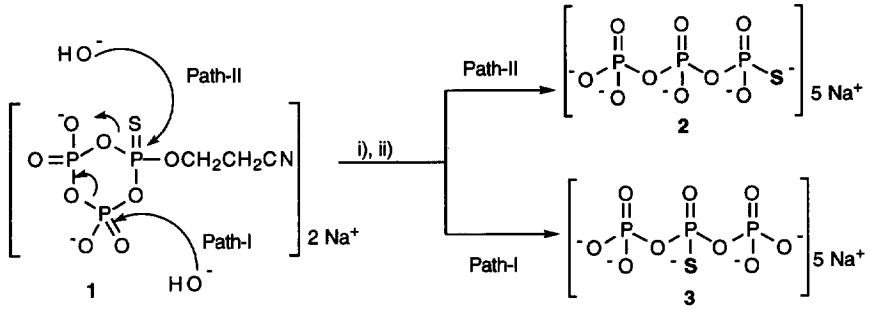
INTRODUCTION

Phosphorothioate nucleosides and oligonucleotides have found diverse applications in biochemistry and molecular biology.[1,2] The emergence of antisense oligodeoxynucleotides, containing phosphorothioate linkages for nuclease protection, as modulators of gene expression has stimulated the interest in achiral phosphorothioate linkages such as the 3′-O-P-S-5′ linkage. Similar to the 3′-S-P-O-5′ linkage for the characterisation of enzyme- or ribozyme-catalysed reactions, [3,4] the incorporation of the 3′-O-P-S-5′ linkage into the substrate has been used for the characterisation of the mechanism catalysed by the hammerhead ribozyme.[5,6] In these two examples the linkage was incorporated chemically using the phosphoramidite stepwise procedure as it was done in an other publication.[7] Alternative methods have been reported by other authors.[8,9] Given these wide applications it seemed warranted to explore the possibility to incorporate the 3′-O-P-S-5′ linkage enzymatically into DNA or RNA. We report here the synthesis of 5′-deoxy-5′-thiouridine- and 5′-deoxy-5′-thioadenosine-5′-triphosphate by two different synthetic routes to investigate their substrate properties for T7 RNA polymerase.

RESULTS AND DISCUSSION

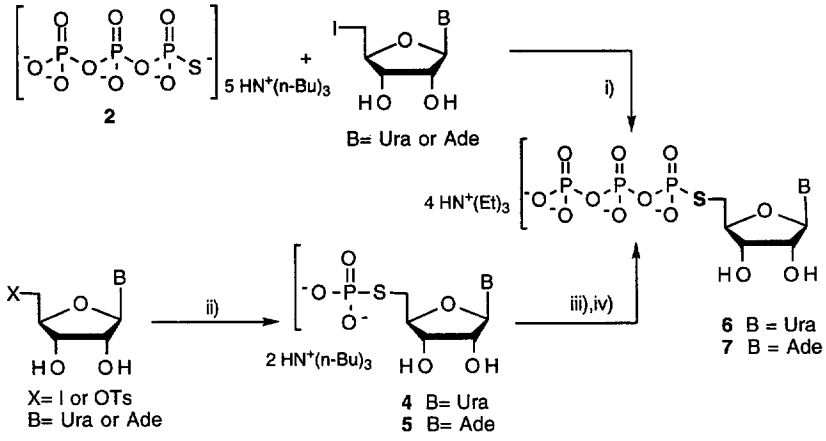
The synthesis of 5′-deoxy-5′-thioribonucleoside-5′-triphosphates and their 2′-deoxy derivatives has been reported previoulsy and were found not to be substrates for *E.coli* DNA dependent RNA polymerase and DNA polymerase I.[10] However, these compounds were only characterised by paper chromatography and phosphate analysis. Here we report two alternative methods for the synthesis of two 5′-deoxy-5′-thioribonucleoside-5′-triphosphates. P^1-O-(cyanoethyl)-P^1-cyclic thiotriphosphate **1**, prepared by a modified procedure,[11] underwent hydrolytic opening exclusively to the P^1-O-(cyanoethyl)-P^1-thiotriphosphate.[12] Removal of cyanoethyl group with 1N NaOH resulted in the formation of **2** (Scheme-1).[13] For characterisation the P^1-(S)-thiotriphosphate **2** was desulphurised with a 1% solution of I_2 in pyridine / water (98:2 v/v) to inorganic triphosphate, with the

characteristic ^{31}P NMR spectrum. The triphosphate obtained from **2** was compared with the triphosphate obtained by the opening of tri-sodium trimetaphosphate with 1N NaOH and was found to be identical in all respects.[14]



Scheme 1 *Reagents and conditions*: i) Water ; ii) aq NaOH, RT, 40 min.

The tri-n-butylammonium salt of **2** was treated with 1 eq of the appropriate 5′-iodo-5′-deoxynucleoside[15] in anhydrous DMF to yield 5′-deoxy-5′-thioribonucleoside-5′-triphosphate **6** or **7** (Scheme 2). ^{31}P NMR spectroscopy of the reaction product, after purification over DEAE indicated in addition to the signal of 5′-thio-5′-deoxynucleoside-5′-triphosphate some inorganic triphosphate impurities. This could be due to incomplete thiolation of **2** at the initial stage. The product was further purified by HPLC. The yield of **6** and **7** prepared by this method was nearly 20%.[16]



OTs = p-toluene sulphonyl; Ura = uracil; Ade = adenine

Scheme 2 *Reagents and conditions*: i) DMF, RT, 24 hr; ii) thiophosphoric acid, tri-n-butylammonium salt, DMF, RT, 24 hr; iii) N,N′-carbonyldiimidazole; iv) tri-n-butylammonium pyrophosphate; DMF, RT, 24 hr.

Alternatively, the synthesis of **6** and **7** was started by the reaction of the tri-n-butylammonium salt of thiophosphoric acid with the appropriate 5′-tosyl-5′-deoxyadenosine (Aldrich) or 5′-iodo-5′-deoxyadenosine

or 5′-iodo-5′-deoxyuridine, prepared by a slightly modified published procedure,[17] to yield the 5′-deoxy-5′-thioribonucleoside-5′-monophosphates **4** and **5**.[18] These were activated with N,N′-carbonyldiimidazole and treated with pyrophosphate to give **6** and **7**, respectively.[19] DEAE-Sephadex purification was followed by purification by preparative HPLC (see footnote 16 for programme gradient and buffer system). The yield of **6** and **7** prepared by this method was 25 and 38%, respectively.[20] They were identical in all respects to the material obtained by the previous method.

Nucleoside triphosphate **6** was dephosphorylated by alkaline phosphatase to 5′-thio-5′-deoxyuridine. The latter is slowly oxidised to the disulphide which can be reduced again by addition of DDT.

Nucleoside triphosphates **6** and **7** were used together with three unmodified nucleoside 5′-triphosphates in transcription reactions to determine whether they could serve as substrates for T7 RNA polymerase. Two plasmid-derived DNA templates, encoding *E.coli* tRNA Asp[21] or a hammerhead ribozyme substrate, were used as templates. Even under a variety of conditions, such as changing time (15 min - 2h), temperature (0, 5, 15, 25, 37°C), addition of detergents, inorganic pyrophosphatase and increasing the concentration of the thio-triphosphates to 3 mM, full length transcripts were not observed.[22] This is consistent with the observation that **6** and **7** were not substrates for DNA dependent RNA polymerase from *E.coli* but weak competitive inhibitors (Stütz and Scheit).[10b] These authors also showed that the corresponding 2′-deoxy derivatives are not substrates for *E.coli* DNA polymerase I.[10a]

Acknowledgements: We are thankful to P. A. Heaton, S. Verma, N. K.Vaish, S. Alefelder for the critical reading of the manuscript, S. Vörtler for discussion, U. Kutzke and A. Zeuch for technical assistance, and B. Seegar for NMR spectroscopy. This work was supported by the Deutsche Forschungsgemeinschaft.

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12. The nucleophilic attack by water occurs exclusively at the phosphate and not at the phosphorothioate since the phosphorothioate as a monoanion has pKa 1.7 and is thus a better leaving group than either of the phosphates with pKa 6.6 as dianion. The product was purified by chromatography over a DEAE-A-25 Sephadex column which was eluted with a linear gradient of triethylammonium bicarbonate (TEAB) (0.1-0.8 M, total volume 1 l) to yield the triethylammonium salt of P^1-O-(cyanoethyl)-P^1-thiotriphosphate (87%). ^{31}P NMR (D_2O) 43.5 (dt, $J_{1,2}$ = 28.2 Hz, $J_{P,H}$ = 8.3, P^1), -10.4 (d, $J_{3,2}$ = 20

Hz, P^3), -23.7 (dd, $J_{2,3}$ = 20 Hz, P^2). No branched phosphorothioate corresponding to **3** was seen in the ^{31}P NMR spectrum.

13. After the removal of the cyanoethyl group, the doublet of the P^1 signal shifts to δP = 34.7 (J = 30.4 Hz) and ^1H coupled spectrum shows no splitting of the P^1 signal. The product was purified by chromatography over DEAE-A-25 Sephadex column which was eluted with a linear gradient of TEAB (0.1 - 0.8 M, total volume 1 l) to yield 85% of **2**. ^{31}P NMR (D_2O) 34.7 (d, $J_{1,2}$ = 30.4 Hz, P^1), -6.6 (d, $J_{3,2}$ = 21 Hz, P^3), -21.6 (dd, $J_{1,2}$ = 30.4 Hz, $J_{2,3}$ = 21 Hz, P^2). Even after purification the product was contaminated with a small quantity of pyrophosphate δP = -10.
14. Inorganic triphosphate ^{31}P NMR (D_20) -8.4 (d, J= 20 Hz, P^1 and P^3), -21 (t, J = 20 Hz, P^2),
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16. A solution of 5′-iodo-5′-deoxynucleoside (260 μmol) and P^1-thiotriphosphate *tetra* (tri-n-butylammonium) salt **2** (150 mg, 1 eq, 260 μmol) in N,N′-dimethylformamide (1 mL) was vigorously stirred at room temperature for 24 h. The solvent was then evaporated and the residue dissolved in water (2 mL) and purified by chromatography over DEAE-A-25-sephadex chromatography using a linear gradient of TEAB (0 - 0.4 M, total volume 2 l). Fractions containing product were evaporated to dryness and further purified by reverse phase HPLC using as eluant triethylammonium bicarbonate (100 mM) with a linear increase of acetonitrile from 0 % to 30% in 25 min. **6** [Yield 19%; ^{31}P NMR (D_2O): 8.8 (dt, $J_{\alpha,\beta}$ = 26.7 Hz, $J_{P,H}$ = 13.5 Hz, P_α); -7.0 (d, $J_{\gamma,\beta}$ = 20.1 Hz, P_γ); -22.1 (dd, $J_{\alpha,\beta}$ = 26.7 Hz, $J_{\gamma\beta}$ = 20.1 Hz, P_β)]. **7**, [Yield 21%; ^{31}P NMR (D_2O) 8.8 (dt, $J_{\alpha,\beta}$ = 26.7 Hz, $J_{P,H}$ = 13.5 Hz, $P_{\alpha,}$); -7.0 (d, $J_{\gamma,\beta}$ = 20.1 Hz, P_γ), -22.1 (dd, $J_{\alpha,\beta}$ = 26.7 Hz, $J_{\gamma\beta}$ = 20.1 Hz, P_β)].
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18. To a vigorously stirred solution of the 5′-iodo-5′-deoxynucleosides or 5′-tosyl-5′-deoxynucleoside (1 mmol) in N,N′-dimethylformamide (1 mL), was added the tri-n-butylammonium salt of thiophosphoric acid (1 g, 1.5 eq, 1.5 mmol) dissolved in N,N′-dimethylformamide (1 mL). The reaction mixture was stirred for 24 h and the solvent evaporated in vacuo. The crude reaction mixture was dissolved in water (2 mL) and purified by chromatography over a DEAE-A-25 sephadex column eluted with a linear gradient of TEAB (0 - 0.4 M, total volume 2 l). The appropriate fractions were collected and evaporated to dryness to yield the product. **4** [Yield 33%; ^{31}P NMR (D_2O) 16.9 (s)] and **5** [yield 30 %; ^{31}P NMR (D_2O) 16.9 (s)]. 5′-Deoxy-5′-thioribonucleotide-5′-monophosphate **5** is a substrate for alkaline phosphatase and is degraded to 5′-thio-5′-deoxyadenosine.
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20. To a vigorously stirred solution of **4** or **5** (26 mmol) in N,N′-dimethylformamide (0.5 mL), tri-n-butylamine (18.74 μl, 78 mmol) was added under an argon atmosphere. To this solution N,N′-carbonyldiimidazole (21 mg, 130 mmol) in N,N′-dimethylformamide (0.5 mL) was added and stirring was continued at RT for 4hr. Methanol (8.4 μl) was added followed by addition of tri-n-butyl ammonium pyrophosphate (119 mg, 130 mmol) in N,N′-dimethylformamide (200 μl) after 0.5 h. The reaction mixture was stirred at room temperatute for 20 hr. The reaction mixture was filtered to remove the imidazolium pyrophosphate, followed by addition of an equal volume of methanol, evaporation to dryness in vacuo. The residue was redissolved in water (2 mL) and purified by chromatography over DEAE-A-25-sephadex using a linear gradient of TEAB (0 - 0.4 M, total volume 2 l) followed by preperative HPLC (buffer system and gradent as in foot note 16) to yield the products.
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22. Full length transcripts were observed even in the presence of only three nucleoside 5′-triphosphates, presumably by misincorporation. This was not observed when either **6** or **7** were also present in the transcription mixture. It thus appears that these compounds act as inhibitors of misincorporation.

(*Received in Germany* 18 *December* 1996; *accepted* 14 *January* 1997)