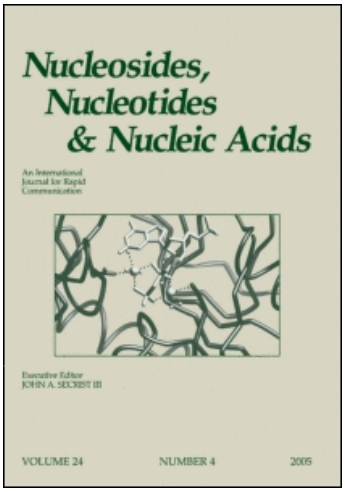

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Reinvestigation of 4-Thiothymidine-5'-triphosphate Synthesis

H. Bazin[a]; S. Sauvaigo[b]
[a] CIS biointernational/DIVT/Research and New Technologies, Bagnols/Cèze Cedex, France [b] CEA Grenoble/DRFMC/SCIB/Lésions des Acides Nucléiques, Grenoble Cedex 9, France

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REINVESTIGATION OF 4-THIOTHYMIDINE-5'-TRIPHOSPHATE SYNTHESIS

Bazin, H.[1]* and Sauvaigo, S.[2]
[1]CIS biointernational/ DIVT/ Research and New Technologies, BP 175, F-30203 Bagnols/Cèze Cedex, France. E-mail: hbazin@compuserve.com
[2]CEA Grenoble/DRFMC/SCIB/Lésions des Acides Nucléiques, 17 rue des Martyrs, F-38054 Grenoble Cedex 9, France.

ABSTRACT : The 4-Thiothymidine-5'-triphosphate **1** (S^4TTP) was known to be a substrate for polymerase, however a commercial sample of this compound failed to be incorporated into DNA. Mass spectrometry combined to alkaline phosphatase digestion and ^{31}P-NMR showed that this sample was in fact 5'-chloro-5'-deoxy-4-thiothymidine-3'-triphosphate **2**. The desired S^4TTP was synthesized by two alternate routes, was fully characterized and was shown to be incorporated in a DNA polymerase assay.

Sample analysis: The commercial dNTP (USB #77153, Amersham) gave the following MS-(ES^-), after exchange with TEA : $(M-H)^-$ 515.1 (100%) and 517.2 (40%). These data were inconsistent with a mass of 498 expected for S^4TTP (Calc. for free acid $C_{10}H_{17}N_2O_{13}P_3S$). The 18 units shift and the isotopic motive suggested the replacement of a hydroxyl by chlorine. The ^{31}P-NMR showed the presence of a triphosphate and the UV (λmax = 335nm in H_2O) was consistent[1] with S^4T structure. The dNTP (2.5 µl of the 10mM commercial solution) in 50µl buffer (0.1M Tris pH 9, 0.1M NaCl, 15 mM $MgCl_2$) was treated with 5µl Snake Venom Phosphodiesterase (Pharmacia, 5U/µl) no change was observed in HPLC[2] in contrast with dTTP which was hydrolysed to dTMP. The dNTP (10µl of 10mM) in 85µl buffer (as above) was treated with 5µl of alkaline phosphatase (125U), within 15mn HPLC[3] showed complete hydrolysis to a new product (Rt= 20mn)[3]. This product was distinct from authentic 4-thiothymidine[1] (Rt= 13.1 mn)[3] and the ES-MS data [$(M-H)^-$ 275.1 (50%), 277.2 (15%)] was consistent with chlorine substituted 4-thio-thymidine. The proton coupled ^{31}P-NMR of the dNTP sample displayed a quartet at -11.2

ppm (Pα) with J_{PH}= 8.2Hz and J_{PP}=19Hz. Upon selective decoupling of $H_{3'}$ (5.13ppm), the quartet collapsed to a doublet (J=19Hz) and upon $H_{5'}$, $_{5''}$ decoupling (4.1ppm), the signal remained unaffected. These data indicated a triphosphate chain at 3'position, correlated with a resistance to SVP and in favour of structure **2** for commercial dNTP.

4-STTP Synthesis: To understand the origin of compound **2**, and considering the reported S^4TTP synthesis[4], one striking feature was the use of pyridine/$(EtO)_3PO$ mixture for phosphorylation (presumably to prevent glycosyl cleavage). Since the S^4TTP used in ref.5 was prepared in $(EtO)_3PO$ alone, we tested the action of pyridine. S^4T [1] (50µmol) in pyridine (24µl)/$(EtO)_3PO$ (150µl) mixture (0°C) was reacted with $POCl_3$ (50µl) for 5h. The main product was eluted on DEAE-sepharose with TEAB 0.18M (Rt=19.5mn)[2], the ^1H coupled ^{31}P-NMR (doublet, J_{PH}=7.3Hz) and MS-(ES^-) [$(M-H)^-$ 355.1(100%), 357.1 (35%)] were compatible with 5'-chloro-5'-deoxy-S^4T-3'-phosphate structure. Similarly S^4T was phosphorylated in $(EtO)_3PO$ alone, the main product eluted on DEAE-sepharose with TEAB 0.17M (Rt=7.7mn)[2]. The ^{31}P-NMR and MS-(ES^-) [$(M-H)^-$ 337.1(100%)] were compatible with 4-thiothymidine-5'-monophosphate **3** structure. The S^4TMP **3** was converted to S^4TTP **1** by the carbonyl-diimidazole procedure[4], the product (8.7µmol) was eluted on DEAE-sepharose with TEAB 0.38M (Rt=16.3mn)[2], MS-(ES^-): $(M-H)^-$ 497.1. The S^4T was also phosphorylated by an alternate procedure[6] and yielded S^4TTP as above.

CONCLUSIONS: Pyridine induced the formation of 5'-chlorinated nucleotide in the $POCl_3$ phosphorylation, and reported S^4TTP data[4] were questionable. Authentic S^4TTP was synthesized and was incorporated in a polymerase assay (Pol I Klenow fragment).

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3. Same column. Eluant A: TEAB 50mM pH7; B : ACN. 0 to 60%B in 30mn. 1ml/mn.

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