Summary

The phosphorylation of unprotected nucleoside, 9-β-erythrityladenine and adenosine, was attempted by the use of P1-diphenyl P2-morpholino pyrophosphorochloridate. Whereas in the former case 23% of 4'-monophosphate was obtained, the yield of 5'-AMP was very low. Isopropylideneadenosine was phosphorylated with Wilsmeier complex derived from morpholinophosphorodichloridate in 50% yield. The use of DMF in the reaction of isopropylidene-AMP-morpholidate was also investigated. 2',3'-O-Isopropylidene-ATP was synthesized and characterized.

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212. Morio Ikehara and Eiko Ohtsuka: Studies of Nucleosides and Nucleotides. XXI.*1 A New Synthesis of Thymidine 5'-Triphosphate and the Use of P1,P2-Di-(2-cyanoethyl)pyrophosphate in the Nucleoside Triphosphate Synthesis.

(Faculty of Pharmaceutical Sciences, School of Medicine, Hokkaido University**)

The synthesis of nucleoside triphosphate by the use of P1-diphenyl P2-morpholino pyrophosphorochloridate was reported in the series of papers from this laboratory.1-3) In these cases we used acetyl group for the protection of hydroxyl group of nucleosides and the acetyl group was removed after initial phosphorylation by the alkaline treatment prior to the reaction with pyrophosphate salt. However, in the case of 9-β-β-xylofuranosyladenine, the alkaline removal of 2'- and 3'-acyetyl group after phosphorylation caused the cyclization of 5'-phosphoramophoridolate to 3'-hydroxyl and gave 3',5'-cyclic phosphate exclusively.4) In order to circumvent this cyclization, the deprotection after the triphosphate formation would be necessary.

Conditions for alkaline treatment of nucleosides and phosphates appeared in the literature were listed in Table I, together with those obtained by us. The results of this investigation were applied to the synthesis of thymidine 5'-triphosphate.

3'-O-Acetylthymidine was phosphorylated with P1-diphenyl P2-morpholinopyrophosphorochloridate according to the procedure reported earlier.5)

The resulting 3'-O-acetylthymidine 5'-morphismolinophosphorochloridate was reacted with 5 equivalents of bis(tributylammonium)pyrophosphate in the presence of 1 equivalent of tributylamine. In this case the reaction could be expected to occur in the following 2 ways: i) reaction of phosphoramophidlate with pyrophosphate salt and ii) reaction of phosphoramorphidlate residue with pyrophosphate. Though it is hard to

*2 Kita 12-Jo, Nishi 5-chome, Sapporo (池原森男, 大塚栄子).
2) Same as *1.
4) Unpublished experiments by M. Ikehara and E. Ohtsuka.
differentiate these 2 mechanisms, it would be reasonably prospected that the phosphorochloridate is much more unstable than phosphoromorpholidate, especially in the alkaline media. The authors favored the mechanism involving the pathway ii), because labile chloridate residue must be hydrolyzed during the handlings. The extent of reaction estimated by paper electrophoresis was 50, 67, and 85% at the end of 16, 40, and 55 hours' reaction at 20°. After the usual work-up procedure, the acetyl group of thymidine was removed with N lithium hydroxide at pH 13. Ion-exchanger column chromatography showed the following results: TMP\(^{a3}\) 11.8%, TDP 19.3%, TTP 24.8% and T-tetra-P 11.6%. Total yield of TTP calculated from acetyltymidine was 12%. Pattern of ion-exchanger column chromatography was shown in Fig. 1. This sample of TTP was analyzed and compared with an authentic specimen by the paper chromatography and paper electrophoresis.

\[ \text{Chart 1.} \]

\(^{a3}\) Abbreviations used were: TMP, thymidine 5'-monophosphate; TDP, thymidine 5'-diphosphate; TTP, thymidine 5'-triphosphate; FAD, flavin adenine dinucleotide; DCC, N,N-dicyclohexylcarbodimide; DMF, N,N-dimethylformamide.

From the results obtained as above, it was clarified that the de-acetylation of protected nucleoside polyphosphate could be achieved without cleavage of pyrophosphate bond by the mild alkaline treatment. Therefore, we attempted the use of pyrophosphate, which is bearing the alkaline labile protecting group, for the second step of triphosphate synthesis described above. Moreover, by the use of protected pyrophosphate, unfavored disproportionation reaction\textsuperscript{11} of the resulting triphosphate was assumed to be avoidable. As reported by Todd, et al.,\textsuperscript{9} tri-substituted pyrophosphate seems to be unsuitable for the triphosphate synthesis, because of the easier decomposition of fully esterified triphosphate.

The successful results of 2-cyanoethyl protecting found by Tener,\textsuperscript{12} was applied to the synthesis of P\textsubscript{1},P\textsubscript{2}-di-(2-cyanoethyl)pyrophosphate. 2-Cyanoethyl group would be removed after triphosphate synthesis by the alkaline treatment in nearly the same condition as listed in the Table I. 2-Cyanoethylphosphate was then condensed by the use of DCC and the structure of the dicyanoethyl pyrophosphate was confirmed by paper chromatography and paper electrophoresis. The pyrophosphate was then derived to its bis-tributylammonium salt and was reacted with 2',3'-O-isopropylideneadenosine 5'-phosphoromorpholinocloridate, which was prepared from 2',3'-O-isopropylideneadenosine and morpholinophosphoric dichloridate\textsuperscript{13} in the presence of DMF. When this reaction was carried out in acetonitrile for 4 days at room temperature, 4 spots corresponding to nucleoside (R\textsubscript{AMP} 0.15), phosphoromorpholidate (0.60), monosubstituted diphasphate (0.91) and dissubstituted triphosphate (1.20) were detected by paper electrophoresis. The reaction mixture was then treated with 9N ammonia at 60° for 1.5 hours in order to remove the cyanoethyl group. By this deprotection procedure 26% of isopropylideneadenosine, 54% of morpholidate and 20% of diphasphate, accompanied with a trace amount of mono and triphosphate, were obtained. When the solvent of the above reaction was changed to pyridine, 18% nucleoside, 19% of morpholidate and 36% of diphasphate accompanied with 10% of unidentified substance having R\textsubscript{AMP} 0.81 and trace of triphosphate were obtained. It was deduced from the results obtained as above, that, in

contrary to our expectation, the alkaline treatment of resulting P<sup>3</sup>,P<sup>4</sup>-dicyanoethyl-isopropylideneadenosine 5'-triphosphate caused the total cleavage of pyrophosphate between P<sup>1</sup>, P<sup>2</sup>, and P<sup>3</sup> prior to the elimination of cyanoethyl group. This observation seems to be consistent with the fact that trisubstituted pyrophosphate linkage is rather reactive against nucleophilic displacement on the less substituted phosphorus atom. Therefore, the cleavage had occurred on the pyrophosphate bond between P<sup>1</sup>~P<sup>2</sup> and P<sup>2</sup>~P<sup>3</sup> and would form P<sup>2</sup>-cyanoethylidiphosphate (A) directly or via the intermediary expected metaphosphate (B).

Regarding the results obtained as above, the reaction of acetylprotected nucleoside phosphoromorpholidate with dicyanoethyl pyrophosphate was not investigated so far.

**Experimental**

**Paper Chromatography**—All chromatographies were conducted on Toyo Filter Paper No. 51 A. Solvent A, iso-PrOH-12% (NH₄)₂SO₄ = 3:2; solvent B, EtOH-M AcONH₄ = 7:3, pH 7.5.

**Paper Electrophoresis**—All electrophoreses were carried out on Toyo Filter Paper No. 51 A and in 0.05M triethylammonium bicarbonate, pH 7.5, 20 v./cm. for 1 hr.

**Thymidine 5'-Triphosphate**—Into a solution of 3'-O-acetylthymidine<sup>16</sup> (283 mg., 1 m mole) dissolved in 3 ml. of anhyd. dioxane, a dioxane (2 ml.) solution of P<sup>1</sup>-diphenyl P<sup>2</sup>-morpholinopyrophosphorochloridate (freshly prepared from morpholinophosphorodichloridate (416 mg., 2 m mole), diphenyl phosphate (500 mg., 2 m mole) and 2,6-lutidine (0.642 ml., 6 m mole) was added. The whole solution was kept in standing at room temperature for 48 hr. under exclusion of moisture. At the end of reaction aliquot was examined by paper electrophoresis. A single spot having R<sub>amp</sub> 0.65 (TMP-morpholidate) was

15) Whether monometaphosphate is the true reacting species or not is awaiting for further investigations.
observed. Precipitated white solid was removed by filtration and the filtrate and washings (dioxane) were combined. After addition of 0.238 ml. (1 mole) of tributylamine, dioxane was evaporated in vacuo. A solution of 5 ml of bis-tributylammonium pyrophosphate dissolved in 10 ml. of pyridine was added to the residue. The whole mixture was incubated for 55 hr. in a thermostat. Aliquots examined by paper electrophoresis at 16, 40 and 55 hr. showed that the extents of the reaction proceeded were 50, 67, and 85%, respectively. After the addition of 2 ml. of H₂O, pyridine was evaporated under reduced pressure. The residue was taken up in 5 ml. of H₂O and extracted thoroughly with Et₂O at pH 4 to 5. The residual aqueous solution was adjusted to pH 8 with N LiOH and extracted again with Et₂O. Unreacted nucleoside was removed almost completely by these Et₂O extractions. H₂O layer was adjusted to pH 13 with N LiOH and kept for 1 hr. at room temperature. pH of the solution was readjusted to 5.0 and the solution was adsorbed on activated charcoal (ca. 5 g.). The yield calculated from TODH at this stage was 60%. Charcoal was extracted with EtOH containing 2% NH₃ and the extract were evaporated up to 100 ml. in a rotary evaporator (82% recovery). Solution was adjusted to pH 8.5 and applied to a column (2 × 8 cm.) of Dowex I-X8 (Cl⁻ form, 100~200 mesh) ion-exchanger. Results of chromatography was shown in the following Table II.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Eluting buffer</th>
<th>Substance</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.003 N HCl + 0.015 M LiCl</td>
<td>TMP</td>
<td>11.8</td>
</tr>
<tr>
<td>II</td>
<td>0.003 N HCl + 0.1 M LiCl</td>
<td>TDP</td>
<td>19.3</td>
</tr>
<tr>
<td>III</td>
<td>0.003 N HCl + 0.2 M LiCl</td>
<td>TTP</td>
<td>24.8</td>
</tr>
<tr>
<td>IV</td>
<td>0.003 N HCl + 0.4 M LiCl</td>
<td>T-tetra P</td>
<td>11.6</td>
</tr>
</tbody>
</table>

TTP fractions were collected, neutralized with N LiOH and evaporated in a rotary evaporator at 20~30°C to a small bulk. After the addition of 2 volumes of MeOH and 20 volumes of Me₂CO, the whole solution was stored overnight in a refrigerator. Precipitated TTP-Li₂(51.2 mg.) was collected by centrifugation and washed with Me₂CO-EtOH mixture, EtOH and finally with anhyd. EtOH. Purity estimated photometrically on the weight basis after drying in a P₂O₅ desiccator at 3 mm. Hg was 72.8% (total yield from acetylthymidine 12.0%). Anal. Calcd. for C₆H₃O₃N₂P₂Li₄: Total P, 17.77; labile P, 11.83. Found: Total P, 12.86; labile P, 8.58. Base-labile P~total P~1.00:1.99:2.98 (theory, 1:2:3).

Paper chromatography: Rₐₐₕ 1.0 (solvent A). Paper electrophoresis: Rₐₐₕ 1.5. In both tests the sample was revealed as a single spot detected by metaperiodate, UV irradiation. Chromatographical comparison with an authentic sample of TTP showed excellent coincidence.

P₃, P₅-Di-(2-cyanoethyl) Pyrophosphate—DCC (6.2 g., 30 m mole) was added into a solution of 2-cyanoethylphosphate (50 m mole) dissolved in 50 ml. of anhyd. pyridine. After 24 hr. at room temperature, spots having Rₐₐₕ 1.22 (starting material) and 1.39 (dicynoethyl pyrophosphate) were revealed by paper electrophoresis. Paper chromatographical test (in solvent B) showed 2 spots having Rf 0.32 (starting material) and 0.53 (dicynoethyl pyrophosphate). Additional 24 hrs. reaction with 3 g. of DCC could not complete the reaction. DCC (6 g.) was then added to the mixture and reaction was continued for more 2 days. Precipitated dicyclohexylurea was removed by filtration and the filtrate was evaporated to a syrup and extracted with Et₂O in order to remove unreacted DCC. H₂O layer was evaporated under reduced pressure and the residue was taken up in 100 ml. of pyridine. Though the solution showed the slight contamination with 2-cyanoethyl phosphate and inorganic phosphate, it contained mainly P₃,P₅-di-(2-cyanoethyl) pyrophosphate, which was confirmed by the behaviors in paper electrophoresis and paper chromatography.

Reaction of 2',3'-O-Isopropylidenedenosine 5'-Phosphoromorpholidate with P₃,P₅-Di-(2-cyanoethyl) Pyrophosphate—Into a cooled (0°C) solution of isopropylidenedenosine (93 mg., 0.3 m mole), triethylamine (61 mg., 0.6 m mole) and DMF (44 mg., 0.6 m mole) dissolved in 0.2 ml. of acetonitrile, morpholino-phosphorodichloridate (22 mg., 0.6 m mole) dissolved in 2 ml. of dioxane was added dropwise under stirring. The whole solution was kept in standing overnight at room temperature under exclusion of moisture.

i) Into a half of this solution (containing 0.15 m mole of isopropylidenedenosine) bis tributylammonium) P₃,P₅-di-(2-cyanoethyl) pyrophosphate (0.5 m mole) (prepared by the addition of 1 m mole of tributylamine into 2 ml. of pyridine solution obtained in the preceding section) was added. Pyridine was evaporated under reduced pressure and the residue was taken up in 2 ml. of acetonitrile. After 4 days at room temperature, an aliquot was examined by paper electrophoresis. The results were

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19) Purchased from Sigma Chemical Co., St. Louis, Mo., U. S. A.
summarized in Table II. Acetonitrile was evaporated in vacuo and the residue was dissolved in 9N NH₄OH. The solution was heated at 60° for 1.5 hr. An aliquot was applied to the paper electrophoresis, the spots were cut out and was eluted by H₂O. The yield of products was calculated from the UV absorption of the eluates (see Table III, line 3).

<table>
<thead>
<tr>
<th>Ns</th>
<th>Morpholidate</th>
<th>Unidentified substance</th>
<th>Monosubst.</th>
<th>Disubst.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.15</td>
<td>0.60</td>
<td>0.91</td>
<td>1.20</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td>0.60 (trace)</td>
<td>0.91</td>
<td>1.20</td>
</tr>
<tr>
<td>3</td>
<td>0.15 (26%)</td>
<td>0.60 (54%)</td>
<td>1.00 (trace)</td>
<td>1.25 (20%)</td>
</tr>
<tr>
<td>4</td>
<td>0.15 (18%)</td>
<td>0.60 (19%)</td>
<td>1.00 (17%)</td>
<td>1.25 (36%)</td>
</tr>
</tbody>
</table>

ii) The reaction was carried out in the same manner as described in i) with another half of the solution. In this case pyridine, which was used in the first step of the reaction, was not replaced by acetonitrile in the reaction with pyrophosphate. Results of the reaction was shown in Table III, line 2. Pyridine was evaporated in vacuo and the residue was heated in 9N NH₄OH for 1.5 hr. at 60°. Paper electrophoretical data was appeared in Table III, line 4.

Authors gratefully indebted to Prof. Lord Todd for his valuable suggestions on the reactivity of phosphoromorpholinochloridate.

**Summary**

A new synthesis of thymidine 5’-triphasphate was achieved by the phosphorylation with P¹-diphenyl P²-morpholino pyrophosphorochloridate of 3’-O-acetylthymidine, followed by the reaction with pyrophosphate salt and alkaline removal of acetyl group. The reaction of 2’,3’-O-isopropylideneadenosine 5’-phosphoromorpholinochloridate with P¹,P²-di-(2-cyanoethyl)pyrophosphate was also investigated. After the alkaline removal of protecting groups only isopropylidene-ADP was obtained.

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