Volume 3 no.6 June 1976 Nucleic Acids Research

Joining of ribooligonucleotides with T4 RNA ligase and identification of the oligonucleotide-adenylate intermediate.[1]

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Received 3 May 1976

ABSTRACT

T4 RNA ligase was found to join A-A-A-A-A-A and ^{32}pU-U-U-U in the presence of ATP as cofactor. In this reaction the pyrophosphate of *pU-U-U-U and pA was isolated by chromatography on a RPC-5 column, besides the joined product and the starting materials. This pyrophosphate was shown to be an intermediate in the joining reaction because of the fact that coupling with A-A-A-A-A-A to give the decanucleotide could be performed in the absence of ATP. The structure of the oligonucleotide-adenylate was determined by enzymatic digestion with base-nonspecific nuclease and venom phosphodiesterase. Further evidence for the proposed structure was obtained by isolation of the intermediate obtained by using *pU-U-U-U and [α-32p]ATP. This pyrophosphate gave *pA and *pU by treatment with venom phosphodiesterase. Several other joining reactions between various purine- and pyrimidine ribooligonucleotides to 5'-phosphorylated ribooligonucleotides are discussed.

INTRODUCTION

RNA ligase has been isolated from T4 infected E.coli[3] and detected in mammalian tissues[4,5] as an activity for joining a terminal 5'-phosphate with a 3'-hydroxyl group. Although the biological roles of this enzyme have yet to be clarified, it's properties are extremely useful. Recently DNA was also reported as being joined to DNA or RNA by this enzyme.[6] Originally, the ligase activity was found to promote the cyclization of ribopolynucleotides of chain length around 40 particularly well[3] and a minimum chain length for cyclization was reported as being eight.[7] Shorter oligonucleotides were joined using an excess of the 3'-hydroxyl component[8] and the joining of tRNA fragments obtained by the depurination of Y base was reported.[9] It was also demonstrated that chemically synthesized short tRNA fragments could be joined with RNA ligase to produce larger oligo-

nucleotides[10] which may be useful in studies on recognition of tRNA analogs by aminoacyl tRNA synthetases. In the present communication we report the joining of short ribooligonucleotides and the characterization of the oligonucleotide-adenylate intermediate.

MATERIALS AND METHODS

Ribooligonucleotides. U-U-U-U[11] and C-C-C-Cp[12] were synthesized chemically. $(A)_{3-7}$ were prepared either by dephosphorylation of chemically polymerized products[13] or by digestion of poly(A) as described by Silber et al.[3] [5'-^{32}p]r$(A)_{\sim 36}$ was prepared as described previously[3] and purified by gel filtration on Sephadex G-50 in 50 mM triethylammonium bicarbonate. Labeled short oligonucleotides were isolated by DEAE-cellulose (DE-81) chromatography in 0.35 M ammonium formate. In the case of U-U-U-U an H-meromyosin treatment was given after the kinase reaction.

Enzymes and ATP. Bacterial alkaline phosphatase (BAPF), venom phosphodiesterase and Micrococcal nuclease were purchased from Worthington Biochemical Corp. Polynucleotide kinase (Dr.M Takanami), H-meromyosin (Dr.Y. Tonomura) and RNase M[14] were generous gifts. [γ-^{32}p]ATP was prepared by the method of Glynn and Chappell.[15] [α-^{32}p]ATP was a gift from Drs. K. Miura and K. Shimotono.

Assays of RNA ligase. The intramolecular cyclization reaction[3] was used as the method of assay during purification of the enzyme. A part of the incubation mixture (30 µl out of 50 µl) was added to BAPF (0.26 unit in 0.2 M Tris-HCl, pH 8.0, 10 µl), treated at 65° for 30 min, spotted to a disc of Whatman 3MM (ϕ 2.4 cm) presoaked with sodium phosphate (0.1M,10µl),washed with 5% Cl_3CCOOH-0.01 M NaH_2PO_4 (100 ml) 3 times for 10min and the radioactivity of acid-insoluble material was counted after drying the paper disc with 1 : 1 ethanol-ether (50 ml) twice for 5 min. The rest of the mixture was used to estimate nuclease activities; the mixture was further incubated for 2.5-4 hr, heated at 100° for 2 min and 10 µl was applied to a strip of DEAE-cellulose paper (DE-81). DE-81 was irrigated with 0.35 M ammonium formate containing 7 M urea for 1 hr (1-10 cm). The radioactivity

which moved from the origin was measured by cutting the chromatogram and counting the strips.

For intermolecular reactions, incubation was at 37° in 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 0.1 mM ATP and 0.5-2 µg BSA in a total volume of 10 µl. All concentrations of oligonucleotides are expressed as termini concentrations.

Nearest neighbour frequency analysis. The reaction mixture (2 µl) was incubated with RNase M (2 µg) plus added 1 M ammonium acetate (2 µl) at 37° for 4 hr and subjected to paper chromatography using a mixture of 0.1 M phosphate (pH 6.8)-ammonium sulfate - n-propanol (100 : 60 : 2, v/w/v). The chromatogram was cut into strips 1 cm wide and counted using a liquid scintillation counter.

Purification of RNA ligase. RNA ligase was isolated from E. coli A19 infected with T4 AmN82XE1140 at a multiplicity of infection of 5 by the procedure published earlier [3,4] except that Sephadex G-100 replaced Sephadex G-75. The enzyme was further purified by a hydroxyapatite column and a second Sephadex G-100 column. The preparation was concentrated through a collodion bag under reduced pressure and kept at 0° in 20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1 M KCl, 5% glycerol. One unit of the enzyme refers to the amount which cyclyzes 1 nmole of $[5'-^{32}p]rA_{\sim 36}$.

RESULTS

Characterization of the oligonucleotide-adenylate intermediate. ^{32}pU-U-U-U (I, in Chart 1) (210 pmoles) and A-A-A-A-A-A (10 nmoles) were treated with RNA ligase (2 units) using the standard conditions for intermolecular reaction. The reaction was stopped after 15 min by heating at 100° for 2 min.

Chart 1

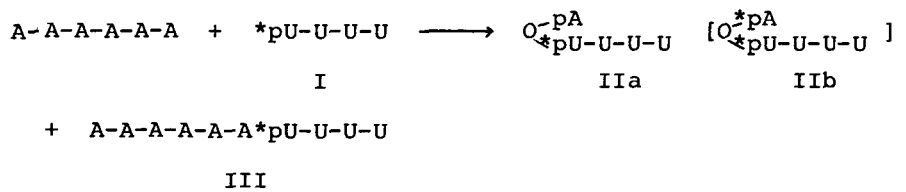


Fig.1

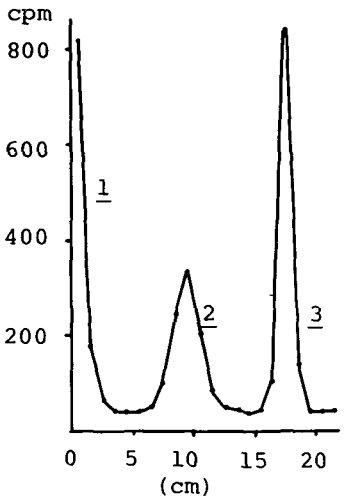


Fig.2

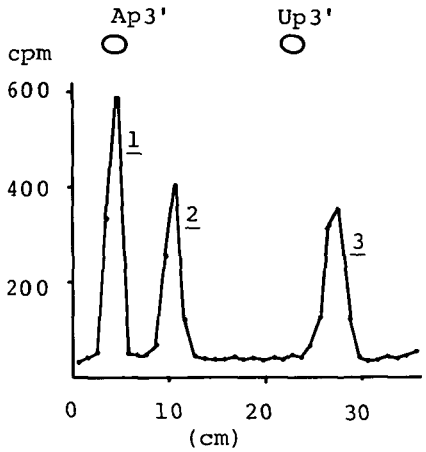


Figure 1. DEAE-cellulose paper chromatography of the BAPF treated products in the reaction of *pU-U-U-U with A-A-A-A-A-A. The paper was irrigated with 0.4 M ammonium formate, cut into strips 1 cm wide and counted.

Figure 2. Paper chromatography of the reaction between *pU-U-U-U and A-A-A-A-A-A after treatment with RNase M for nearest neighbour analysis. Ap and Up indicate markers detected by UV absorption.

The following four criteria showed the presence of the oligonucleotide-intermediate (II). Similar intermediates have been characterized in the case of T4[16] and E. coli[17] DNA ligases. i) An aliquot (2 µl) was treated with BAPF and subjected to chromatography on DE-81. As shown in Fig. 1, the counts at the origin (1, 34%) indicated joining and the second BAPF resistant spot (2, 30%) was assumed to be the oligonucleotide-adenylate (IIa) by virture of it's mobility. The unreacted starting material (I) was degraded to give ^{32}Pi (3, 36%) which ran at the solvent front. ii) Another aliquot (2 µl) was treated with RNase M for nearest neighbour analysis and the products were separated by paper chromatography. The results are shown in Fig. 2. The spot (1, 34%) represented the transfer of ^{32}p from ^{32}pU-U-U-U to Ap by coupling. The second spot (2, 30%) maywell result from the oligonucleotide-adenylate (peak 2, in Fig. 1) and contained P^1,5'-adenosine-P^2,5'-uridine 3'-phosphate ($O\overset{pA}{*pUp}$). The spot 3 had the same mobility as *pUp obtained by hydrolysis of

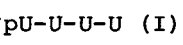
*pU-U-U-U with RNase M. iii) Homochromatography[18] of an aliquot (1.5 µl) using homo-mix III[19] showed two spots (Fig. 3). The faster travelling spot had the same mobility as the 5'-labeled tetranucleotide (I) and the slower moving spot was the joined product. In this system the tetranucleotide (I) and it's adenylated pyrophosphate (IIa) were not resolved. v) The rest of the reaction mixture was subjected to chromatography on RPC-5[20] to isolate the products.[8] The elution pattern is shown in Fig. 4. Each peak was identified by homochromatography: peak 1, *pU-U-U-U (I); peak 2,  (IIa); peak 3, A-A-A-A-A-A*pU-U-U-U (III). The isolated pyrophosphate (IIa) (14 pmoles) was further treated with A-A-A-A-A-A (6.7 nmoles) using RNA ligase (1.8 units) in the absence of ATP. The reaction mixture was applied to homochromatography as shown in Fig. 5. The main part of the radioactivity was found at the same position as joined product isolated by RPC-5. This indicates that IIa served as the intermediate in the joining reaction. Further evidence

Fig. 4

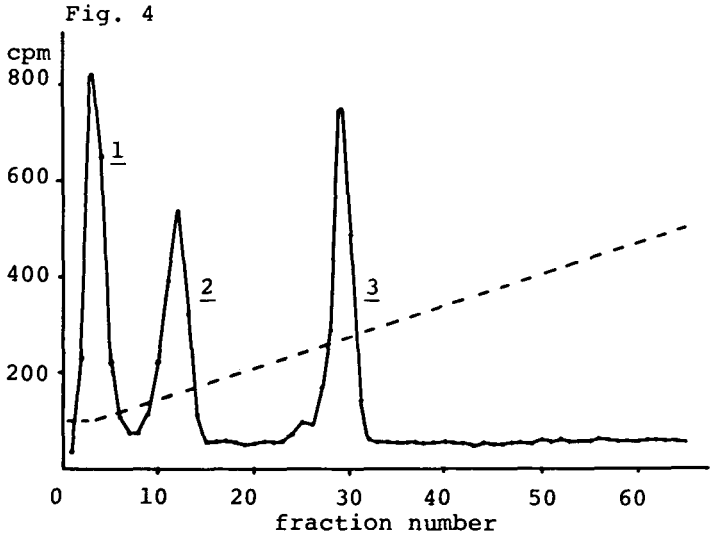


Figure 4. Chromatography of the products in the reaction of *pU-U-U-U with A-A-A-A-A-A on a column (0.7 x 10 cm) of RPC-5 using a gradient of KCl (0.2 - 1 M) in 10 mM Tris-HCl (pH 7.5, 100 ml). Fractions of 1.6 ml were collected every 2 min. Radioactivity was counted by Cerenkov's method.

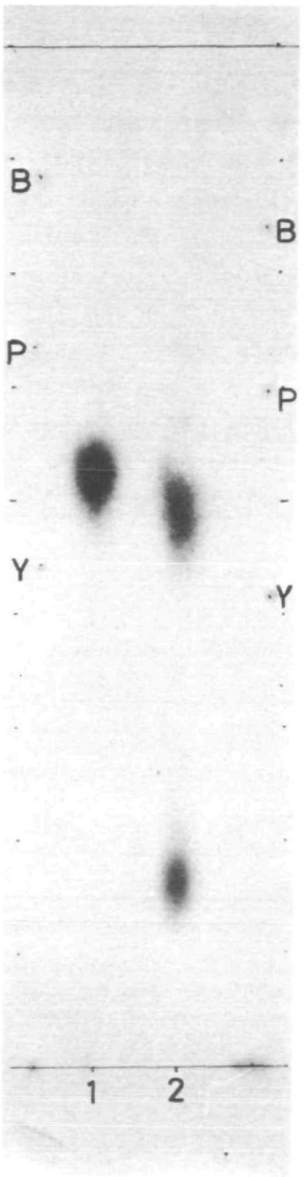


Figure 3. Homochromatography of the products in the reaction of *pU-U-U-U with A-A-A-A-A-A. B, P and Y represent dye markers: blue, xylene cyanol; pink, acid fuchsin; yellow, methyl orange.[19] 1 shows the marker of *pU-U-U-U and 2 shows the reaction mixture.

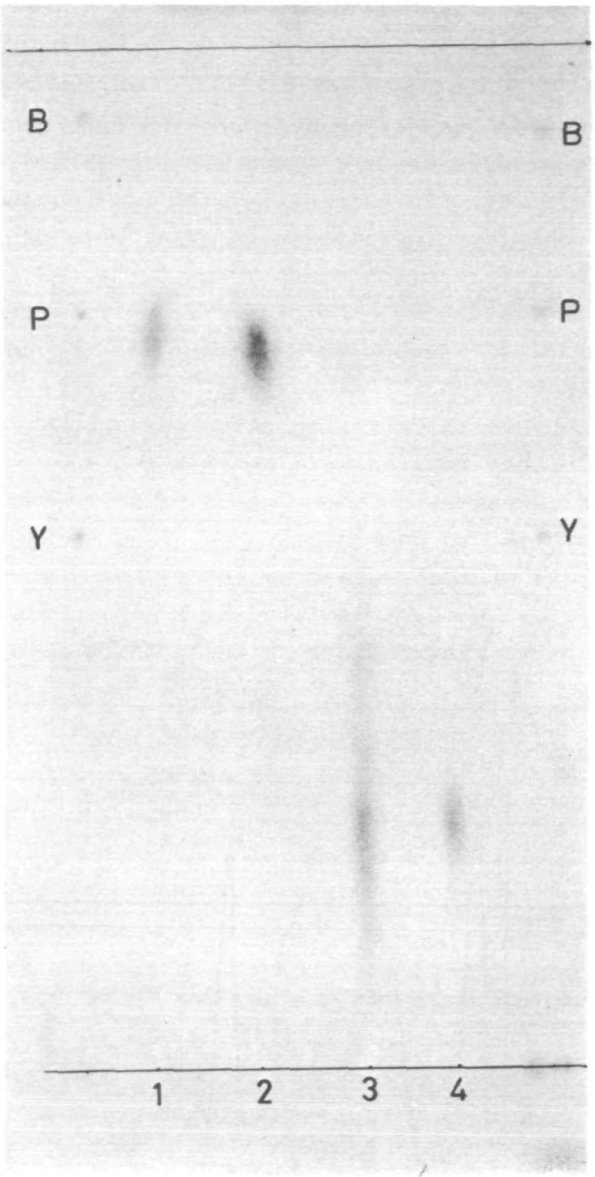


Figure 5. Homochromatography of the products in the reaction of the adenylated pyrophosphate (IIa) with A-A-A-A-A-A in the absence of ATP. The dye markers are the same as the ones used in Fig. 3. 1, *pU-U-U-U; 2, A(5'p)O*pU-U-U-U; 3, the reaction mixture; 4, A-A-A-A-A-A *pU-U-U-U from peak 3 in Fig. 4.

for the structure of IIa as postulated was obtained by venom phosphodiesterase hydrolysis. The product was identified as *pU by paper chromatography as shown in Fig. 6. An independent joining reaction as above but using [α-^{32}p]ATP was performed to obtain further information on the structure of the pyrophosphate intermediate. The structure in this case would be IIb. After isolation of the products by RPC-5 chromatography IIb was digested with venom phosphodiesterase and analyzed by paper chromatography as shown in Fig. 7. *pA and *pU were obtained in a ratio of ca. 1 to 1. Accumulation of this type of compound was observed in several experiments discussed in subsequent sections.

Joining of ^{32}pU-U-U-U with oligoadenylates of different sizes. As shown in Table I, ^{32}pU-U-U-U was joined with oligoadenylates of chain lengths 3 to 5. The reaction was analyzed by homochromatography, phosphatase resistance and/or nearest neighbour analysis with RNase M. The amounts of the joined products could be estimated by transfer of ^{32}p to A*p. The pyrophosphorylated intermediate would yield $O\begin{matrix}pA\\ *pUp\end{matrix}$. The yields of intermolecular reactions increased with increasing chain length, when the same amount of the ligase was used. However,

Fig. 6

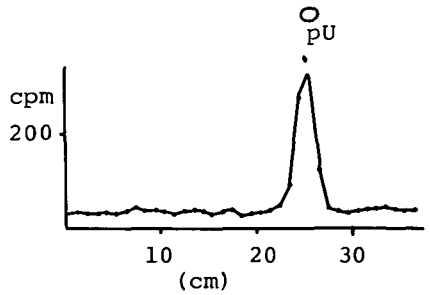


Fig. 7

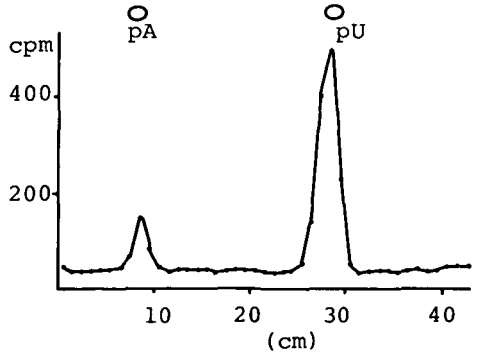


Figure 6. Paper chromatography of the venom phosphodiesterase digested products of IIa (2 in Fig. 4). The solvent system was the same as that used for the nearest neighbour analysis.

Figure 7. Paper chromatography of the venom phosphodiesterase digested mixture of the oligonucleotide-adenylate (IIb) obtained by using [α-32p]ATP. Chromatographic conditions were the same as in Fig. 6.

Table I

Acceptor nmole	Donor pmole	En unit	Time min	Joining %	RNase M digestion pA / pUp	*pUp	A*p	U*p
A-A-A 15	*pU-U-U-U 93	0.4	45	10	15	75	10	0
A-A-A-A 15	*pU-U-U-U 93	0.4	45	33	42	25	33	0
A-A-A-A-A 15	*pU-U-U-U 93	0.4	45	53	35	12	53	0
A-A-A-A-A 15	*pU-U-U-U 53	1.2	15	~100[a]	—	—	—	—

a, observed in homochromatography.

an excess of the enzyme completed the reaction after 15 min as shown in the last line in Table I. Neither cyclization nor self-polymerization of *pU-U-U-U was observed.

Joining of ^{32}pA-A-A-A-A with pyrimidine oligonucleotides. *pA-A-A-A-A (1 nmole) was allowed to react with U-U-U-U as shown in the first line of Table II. Phosphatase treatment showed that 57% of the terminal phosphate became resistant and RNase M digestion of the reaction mixture also showed approximately the same degree of transfer of ^{32}p to the mononucleotides. To identify the joined product the rest of the reaction mixture was subjected to RPC-5 chromatography. The isolated products (peaks 1-3 in Fig. 8) were characterized by homochromatography and nearest neighbour analysis. Peak 1 and 2 contained *pA-A-A-A and *pA-A-A-A-A, respectively. Peak 3 consisted mainly of the cyclic decaadenylate contaminated with the linear decanucleotide in the latter part of the peak. Self-condensation of the pentanucleotide and subsequent cyclization of the decanucleotide seemed to have occurred, since RNase M digestion of the compounds in peak 3 gave A*p and a trace of *pAp as estimated by paper chromatography. This is in contrast to the other experiments shown in Table I, where the transfer of ^{32}p took place from ^{32}pU-U-U-U to A^{32}p solely. The joining of ^{32}pA-A-A-

Table II

Acceptor nmole	Donor pmole	En unit	Time min	BAP resist. %	RNase M digestion *pAp	A*p	U*p	C*p
U-U-U-U 10	*pA-A-A-A-A 1000	2.0	15	57	43	55	2	-
U-U-U-U 15	*pA-A-A-A-A 68	0.4	45	27	63	24	3	-
U-U-U-U 15	*pA-A-A-A-A 72	0.17	120	9	91	9	0	-
C-C-C-C 15	*pA-A-A-A-A 72	0.17	120	30	70	24	-	6

Fig. 8

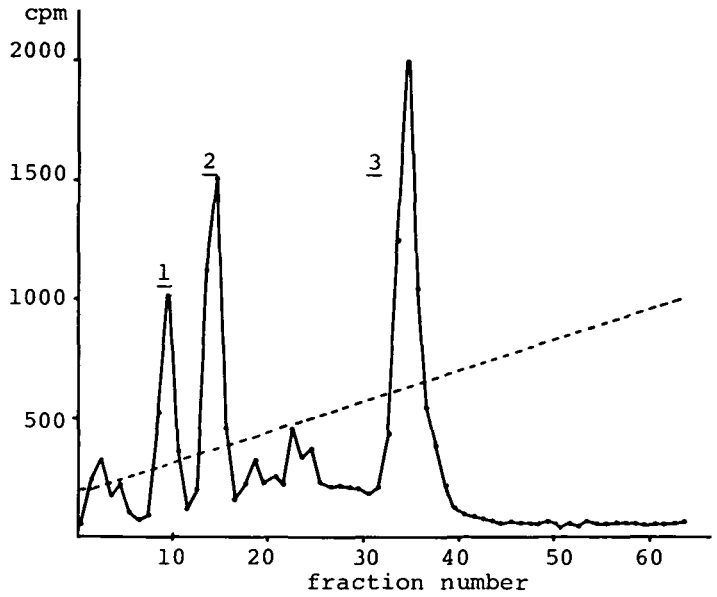


Figure 8. Chromatography of the products in the joining of *pA-A-A-A-A with U-U-U-U on a column (0.7 x 10 cm) of RPC-5. The conditions for elution were the same as described in Fig.4.

A-A-A with U-U-U-U occurred in only 2% yield as evidenced by transfer of ^{32}p to $U^{32}p$ and similar reactions were observed even using larger excesses of pyrimidine oligonucleotides as acceptor molecules. These results are summarized in Table II.

DISCUSSION

A DNA-adenylate intermediate has been characterized in the T4[16] and E.coli[17] DNA ligase reactions. Since the T4 RNA ligase requires ATP as a cofactor, a similar intermediate might be expected to be formed. The present experiments showed the presence of the ribooligonucleotide-adenylate intermediate and further conversion of this intermediate to joined products has been demonstrated. The presence of a RNA ligase-[^{32}p]AMP complex has been demonstrated by Cranston et al.[4] and this complex was shown to be dissociated by the addition of 5'-p-poly(A) of an average chain length of 29 nucleotides to give acid-soluble radioactivity. They also found that the substrate-mediated dissociation of the complex depended on Mg^{2+}, and characterized

the acid-soluble product as ATP. Presumably, AMP was released from a pyrophosphate such as we have identified, when the reaction was terminated by addition of acid. The present experiments do not enable us to predict under what conditions the pyrophosphate intermediate tends to accumulate. Further studies on this problem are in progress.

In the reaction of *pU-U-U-U with oligoadenylate, transfer of the radioactive phosphate to A*p occurred whereas *pA-A-A-A-A seemed to react with itself even in the presence of 10 - 200 fold excess of U-U-U-U. This may be explained in terms of a difference in affinity of the enzyme for oligonucleotides with different base sequences. It will be of interest to determine whether this base preference of the ligase, if any, is based only on the 3'-terminal nucleotide or on the whole molecule. The self-condensed pentaadenylate was almost completely cyclized to the cyclic decanucleotide, since the nearest neighbour analysis of the joined product gave A*p as practically the sole products. The linear decanucleotide should yield A*p and *pAp in 1 to 1 ratio. This finding is consistent with previous studies which showed that oligoadenylate of this size cyclized rather easily.[7] Identification of compounds in peaks in Fig. 8 showed no significant amount of the oligonucleotide-adenylate intermediate but some 3'-exonucleolytic degradation of the pentanucleotide to the tetranucleotide was detected. Further purification of this ligase is under investigation.

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