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Synthesis and reactivity of intermediates formed in the T_4 RNA ligase reaction

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ABSTRACT: The intermediate adenylated donor derivatives A(5')pp(5')dTp and A(5')pp(5')GpGpGp have been prepared from suitable phosphorylating reagents activitated by 1-hydroxybenzotriazole. Phosphodiester bond formation between donor and acceptor oligonucleotides as catalyzed by T_4 RNA ligase is shown to be more efficient when the adenylated form of the donor molecule is used.

INTRODUCTION

T_4 RNA ligase catalyzes the synthesis of a 3'-5' phosphodiester between an "acceptor" oligonucleotide containing a free 3'-hydroxyl and a "donor" oligonucleotide containing a 5'-terminal phosphomonoester (for reviews see 1,2). A trinucleoside diphosphate (NpNpN) and a nucleoside-3',5'-bisphosphate (pNp) are the minimum required substrates for the acceptor and donor molecules respectively. The mechanism of T_4 RNA ligase has been shown to involve essentially the same mechanistic steps reported for DNA ligase (3,4). The process involves three distinct steps (5,6):

$$\text{E} + \text{ATP} \rightleftharpoons \text{E-AMP} + \text{PPi}$$

$$\text{E-AMP} + \text{pNp} \rightleftharpoons \text{E[A(5')pp(5')Np]}$$

$$\text{E[A(5')pp(5')Np]} + \text{XpYpZ} \rightleftharpoons \text{XpYpZpNp} + \text{E} + \text{AMP}$$

The efficiency of the overall joining reaction appears to be dependent upon the nature of both the donor and acceptor oligonucleotide (7.8). Secondary structure effects as well as variations in nucleotide sequence can have dramatic effects upon reaction yield (7-9). In general, single stranded fragments containing purines, particularly with adenosine in the 3' terminal position, function as good acceptors while those containing pyrimidines, particularly cytidine, are good donors and result in high yields of

joined product. The effectiveness of the enzyme in oligoribonucleotide synthesis is limited by the fact that it does not function equally well with a variety of acceptor and donor molecules (7,8).

Previous studies have indicated that yields of joined product appear to be related to the efficiency of the donor adenylation reaction, the second step in the mechanistic pathway (7,10). Using the preadenylated donor molecules, higher yields of joined product were obtained and the dependence of the reaction upon the nucleoside composition of the acceptor was significantly reduced (10,11).

We wish to report a procedure for the chemical synthesis of preadenylated donor molecules such as A(5')pp(5')dTp and A(5')pp(5')GpGpGp and their reactivity in the oligoribonucleotide joining reaction catalyzed by T_4 RNA ligase.

EXPERIMENTAL

Materials

High performance liquid chromatograpy was performed using a C_{18} reversed-phase silica (ODS-Hypersil), or an anion-exchange matrix (APS-Hypersil) products of Shandon Southern, Runcorn England. The columns were prepared by slurry packing as described elsewhere (12). Thin layer chromatography was performed using 5 x 10 cm glass plates of Kieselgel 60 (Merck, Darmstadt, W. Ger). Morpholino-O,O-bis(1-benzotriazolyl)phosphate, 2-chlorophenyl-O,O-bis(1-benzotriazolyl)phosphate and 2,4-dichlorophenyl-O,O-bis(1-benzotriazolyl)phosphate were prepared as described (13, 14). Snake venom phosphodiesterase, bacterial alkaline phosphatase and RNase T_1, were products of Boehringer (Mannheim, W. Ger.). T_4 RNA ligase in the unadenylated form was isolated according to published procedures (10). Triethylammoniumbicarbonate (TEAB) buffer was prepared at pH 7.5. ^{31}P-NMR spectra were obtained at 81.01 MHz and ^{1}H-NMR spectra were obtained at 200 MHz using a Bruker WP005SY instrument (Karlsruhe, W. Ger.).

Methods

5'-O-(9-phenylxanthen-9-yl)-thymidilyl-3'-[2,4-dichlorophenyl-2-(4-nitrophenyl)ethyl]phosphate 1a.

To 5'-O-(9-phenylxanthene-9-yl)thymidine (15) (2.0g; 4.0 mmol), which had been coevaporated from dry pyridine (x3), was added 0.8 ml dry pyridine and 22 ml (4.4 mmol) of a 0.2 M solution of 2,4-dichloro-O,O-bis(1-benzotriazolyl)phosphate. After

stirring for 30 min, tlc analysis (CH_2Cl_2/CH_3OH, 9/1) indicated complete disappearance of starting material. p-Nitrophenylethanol (0.87 g, 5.2 mmol) was coevaporated from dry pyridine (x3) dissolved in 2 ml of dry dioxane and added with 1.4 ml of N-methylimidazole to the reaction mixture. After 2h stirring the reaction was stopped with 20 ml 1M TEAB buffer pH 7.5. The mixture was partitioned between 50 ml dichloromethane and 50 ml 1M TEAB buffer. The organic phase was dried ($MgSO_4$) and the solvent removed. The residue was chromatographed on silica gel using flash chromatography. The product eluted in dichloromethane containing 1-1.5% methanol. Yield: 2.9g (2.6 mmol) 65%.
R_f (CH_2Cl_2/CH_3OH) 9/1): 0.72
^{31}P-NMR (CD_3CN): δ = -7.38 ppm.
^{1}H-NMR (d_6-DMSO + Trace D_2O): δ = 1.49 ppm (s, 3H, C5-CH_3); 2.1-2.6 (m, H2'); 2.52 (m, DMSO); 2.75-3.25 (m, 4H, -CH_2CH_2-); 3.63 (s, HOD); 4.1 (m, 1H, H4'); 4.44 (m, 2H, H5'); 5.13 (m, 1H, H3'); 6.16 (t, 1H, H1', J = 7 Hz); 6.9-7.6 (m, 17H, Ar-H); 7.7 (s, 1H, H6); 8.1 (m, 2H, 4-nitrophenyl-H, ortho).

Thymidilyl-3'-[2,4-dichlorophenyl-2-(4-nitrophenyl)ethyl]-phosphate 2a.

To 1.0g (1.2 mmol) of 1a was added 40 ml of 2% p-toluenesulfonic acid in dichloromethane/methanol (7/3) which had been precooled to 0°C (16). After stirring 15 min at 0°C the reaction was stopped with 10 ml of 10% sodium bicarbonate (w/v). The reaction mixture was partitioned between 50 ml dichloromethane and 50 ml water. The organic phase was dried ($MgSO_4$) and evaporated to dryness. The product was purified by flash chromatography, eluting with 1.5% methanol in dichloromethane.
Yield: 0.57g (0.96 mmol) 83%. R_f (CH_2Cl_2/CH_3OH, 9/1): 0.45.
^{31}P-NMR (CD_3CN): δ = -6.85; -6.92 ppm
^{1}H-NMR (d_6-DMSO + Trace D_2O): δ = 1.77 ppm (s, 3H, C5-CH_3); 2.1-2.9 (m, H2' and Ar-$\underline{CH_2}CH_2$-O); 2.51 (m, DMSO); 3.13 (m, 2H, Ar-$CH_2\underline{CH_2}$-O); 3.5 (s, HOD); 4.02 (m, 1H, H4'); 4.53 (m, 2H, H5'); 5.10 (m, 1H, H3'); 6.18 (t, 1H, H1', J = 7 Hz); 7.2-7.8 (m, 5H, Ar-H and H6); 8.15 (m, 2H, 4-nitrophenyl-H, ortho).

Thymidilyl-5'-O-[(1H-benzotriazol-1-yloxy)-4-morpholinylphosphinyl]-3'-[2,4-dichlorophenyl-2-(4-nitrophenyl)ethyl]phosphate 3a.

To 0.42g (0.71 mmol) of 2a was added 7 ml of a 0.2M solution of morpholino-O,O-bis(1-benzotriazolyl)phosphate (17) in dry tetra-

hydrofuran, 0.25 ml of N-methyl-imidazole and 0.2 ml of pyridine. After stirring for 18h at ambient temperature the reaction was stopped with 1 ml 1M TEAB buffer pH 7.5 and partitioned between 20 ml dichloromethane and 20 ml 1M TEAB buffer pH 7.5. The organic phase was dried ($MgSO_4$) and the solvent removed by evaporation. The crude product (0.47g) was generally pure enough to be used directly in the following synthesis. Small quantities could be further purified by flash chromatography on silica gel.

R_f (CH_2Cl_2/CH_3OH, 9/1): 0.49.

^{31}P-NMR ($CDCl_3$): δ = 9.836, 9.815, 9.261, 9.214 ppm (5'-Phosphate)
δ = -7.028, -7.213, -7.361, -7.398 ppm (3'-Phosphate)

Synthesis of A(5')pp(5')dTp

Formation of the pyrophosphate linkage and 3'-terminal phosphomonoester generally proceeded from 3a without isolation of any of the partially protected intermediates. The procedure is given in steps A through D and was monitored by ^{31}P-NMR.

A. Thymidilyl-3'-[2,4-dichlorophenyl-2-(4-nitrophenyl)ethyl phosphoryl]-5'-[4-morpholinyl]phosphonate 4a.

To 0.47g of 3a was added 3 ml acetonitrile, 3 ml triethylamine and 0.75 ml water and the mixture was stirred at ambient temperature (17). The mixture was then evaporated to dryness.

R_f ($CH_2Cl_2/CH_3OH/Et_3N$ 9/1/0.1): 0.34.

^{31}P-NMR ($CDCl_3$): δ = 6.637, 6.615 ppm (5'-phosphate);
δ = -7.615, -7.633 ppm (3'-phosphate).

B. P^1-5'-Adenosyl-P^2-5'-(thymidilyl-3'-[2,4-dichlorophenyl-2-(4-nitrophenyl)ethyl]phosphoryl)diphosphate 5a.

To 0.48g of the crude hydrolysis product (4a), which was dried by repeated evaporation from toluene and dissolved in 35 ml dimethylformamide, was added a solution of the trioctylammonium salt of 5'-AMP (35 ml dimethylformamide containing 1.07 mmol AMP) and the mixture stirred 20h at 50°C. The reaction mixture was then evaporated to dryness.

^{31}P-NMR (CD_3CN): δ = -7.558, -7.657 ppm (3'-phosphate). δ = -10.373, -10.384, -10.602, -10.619-11.226, -11.278, -11.497, -11.512 ppm (J = 19 Hz) (P-O-P).

C. P^1-5'-Adenosyl-P^2-5'-(thymidilyl-3'-[2-(4-nitrophenyl)ethyl]-phosphoryl)diphosphate 6a.

The residue containing 5a was treated with 20 ml of a 50% aqueous dioxane solution containing 1.25g (7.5 mmol) 4-nitrobenzaldoxime

and 0.75g (6.7 mmol) 1,1,2,2,-tetramethylguanidine and stirred overnight at ambient temperature. The reaction was stopped with the addition of an excess of Dowex (pyridinium form). The ion-exchange resin was filtered and washed with 50% aqueous pyridine and the filtrate evaporated to dryness. The residue was dissolved in 50 ml water and extracted with an equivalent amount of diethylether (3x). The aqueous solution was evaporated to dryness.
^{31}P-NMR (D_2O + 1 mM EDTA): δ = -0.104 ppm (3'-phosphate).
δ = -10.335, -10.584, -10.723, -10.971 ppm(J = 20 Hz)(P-O-P).

D. P^1-5'-Adenosyl-P^2-5'-(thymidilyl-3'-phosphoryl)diphosphate [A(5')pp(5')dTp] 7a.

The residue containing 6a was evaporated from dry pyridine (x3), treated with 1.5 ml 1,8-diazabicyclo[5.4.0]undec-7-ene in 4.5 ml pyridine and stirred at ambient temperature for 24h (18). The reaction was stopped with an excess of Dowex (pyridinium form). The ion-exchange resin was filtered and washed with water. The filtrate was reduced in volume and added to a column (7 x 25 cm) of Sephadex A-25 which had been equilibrated with 0.02 M TEAB buffer pH 7.5. The column was developed with an 8 liter gradient 0.02M-0.8M TEAB pH 7.5. The product, eluting from 0.47-0.49M TEAB, was collected and evaporated to dryness repeatedly from water.
Yield: 0.14g (27% yield from 2a).
^{31}P-NMR (D_2O, 1 mM EDTA): δ = 1.594 ppm (3'-Phosphate)
δ = -10.342, -10.593, -10.638, -10.89 ppm (J = 20 Hz)(P-O-P).
^{1}H-NMR (D_2O): δ - 1.72 ppm (s, 3H, $-CH_3$; Thymidine); 2.1-2.3 (m, 1H, H2'; Thymidine); 2.35-2.60 (m, 1H, H2"; Thymidine); 4.0-5.0 (m, H2', H3', H4', H5') 4.82 (s, HOD); 6.07 (d, 1H, H1', J = 6.0 Hz; Adenosine); 6.24 (t 1H, H1', J =6.3 Hz, Thymidine); 7.51 (s, 1H, H6; Thymidine); 8.20 (s, 1H, H8; Adenosine); 8.45 (s, 1H, H2; Adenosine).
UV (H_2O): λ_{max} = 261 nm, λ_{min} = 232 nm.
Analysis by HPLC using an ion-exchange column (APS-Hypersil) indicated a homogeneous product. Treatment with bacterial alkaline phosphatase yielded a product which co-eluted with an authentic sample of A(5')pp(5')dT. Treatment with snake venom phosphodiesterase yielded one equivalent each of 5'-AMP and pTp.

Synthesis of the trimer 1b

To 0.98g (1.31 mmol) of a guanosine derivative containing N-benzoyl, 2'-0-tetrahydropyranyl and 5'-0-dimethyoxytrityl protect-

ing groups, which had been coevaporated from dry pyridine (x3), was added 0.4 ml pyridine and 7.2 ml (1.44 mmol) of 2,4-dichlorophenyl-O,O-bis(1-benzotriazolyl) phosphate. After 30 min stirring at ambient temperature 0.33g (2 mmol) 4-nitrophenylethanol and 0.4 ml of N-methylimidazole were added as described for 1a. The isolated phosphotriester derivative was elongated twice in the 3' to 5' direction using 2-chlorophenyl-O,O-bis(1-benzotriazolyl) phosphate and suitably protected quanosine derivative similar to the procedure described previously (19). The trimer 1b was isolated by flash chromatography and evaporated to dryness to yield a total of 0.77g.

Synthesis of the trimer 2b.

The 4,4'-dimethoxytrityl group was hydrolyzed by the procedure described for 2a (16). After flash chromatograpy on silica gel 0.51g (78%) of 2b was obtained. R_f = 0.43.

Synthesis of A(5')pp(5')GpGpGp 7b.

The adenylated trimer 7b was prepared by the series of reactions A through E analagous to those used to prepare 7a.

A. Synthesis of 3b.

To 68mg of 2b was added 0.83 ml (0.17 mmol) of a 0.2M solution of morpholino-O,O-bis(1-benzotriazolyl)phosphate, 0.1 ml of pyridine and 0.1 ml N-methylimidazole (20). The reaction was monitored by tlc (CH_2Cl_2/CH_3OH, 9/1). After a normal workup 3b was obtained as a white foam.

B. Synthesis of 4b.

Hydrolysis of the product obtained for 3b to yield 4b was done as described for 3a to yield 4a.

C. Synthesis of 5b.

The trioctylammonium salt of 5'-AMP (0.1 mmol in 3 ml of dimethylformamide) was added to a solution of 4b in 3 ml of dimethylformamide essentially as described in the synthesis of 5a.

D. Synthesis of 6b.

The residue containing 5b was treated with 2.5 ml of a dimethylformamide solution containing 0.188g 4-nitrobenzaldoxime and 0.11g 1,1,3,3-tetramethylguanidine. After stirring overnight at ambient temperature the reaction was stopped with an excess of Dowex (pyridinium form). The ion-exchange resin was filtered and

washed with dimethylformamide, dimethylsulfoxide, 50% aqueous dimethylsulfoxide and water. The filtrate was evaporated and worked up as described for 6a.

E. Synthesis of 7b.

The residue containing 6b was dissolved in 20 ml concentrated ammonia and heated at 50° for 36h. After removal of the ammonia by evaporation the residue was dissolved in 1 ml of dimethylsulfoxide. 1,8-diazobicyclo[5.4.0]undec-7-ene) (0.6 g., 4 mmol) and 1 ml pyridine were added and the reaction mixture stirred at ambient temperature for 24h (18). The reaction was stopped with an excess of Dowex (pyridinium form). The ion-exchange resin was washed with dimethylsulfoxide, 50% aqueous pyridine and water. The filtrate was evaporated to dryness, dissolved in 20 ml 0.01 N HCl and adjusted to pH 2. After 18h at ambient temperature the solution was neutralized and evaporated.

The product was isolated on a 9.4 x 250 mm column of ODS-Hypersil at 60°C using 50 mM triethylammonium acetate pH 7.0 and a gradient of 0 to 11% acetonitrile in 60 min. The product A(5')pp(5')GpGpGp eluted with a retention time of 27 min. 96 A_{260} units were obtained.

^{31}P-NMR (D_2O, 1mM EDTA): δ = 2.696 ppm (3'-phosphate).

δ = -0.930, -1.061 ppm (phosphodiesters)

δ = -11.175, -11.414, -11.450, -11.690 ppm (J = 19 Hz)(P-O-P).

The following enzymatic reactions confirmed the identity of the product:

(a) Treatment with snake venom phosphodiesterase produced AMP and a product which coeluted with pGpGpGp.

(b) Treatment with RNase T_1 produced two equivalents of 3'-GMP and a product which coeluted with an authentic sample of A(5')pp(5')Gp.

T_4 RNA Ligase Reactions

Reaction mixtures of 50 µl at 37°C contained 50 mM Hepes pH 8.4, 20 mM $MgCl_2$, 3.3 mM dithiothreitol, 10 µg/ml bovin serum albumin and 64 µg/ml T_4 RNA ligase. The acceptor oligoribonucleotide concentration was typically 0.5 mM and that of the adenylated donor 0.6 mM. Reactions which did not use the adenylated donor contained 1.0 mM donor and 3.3 mM ATP (21).

Analysis of the reaction was by HPLC using a 4.6 x 250 mm column of ODS-Hypersil. The column was developed in 50 mM potassium dihydrogen phosphate pH 5.5 and a gradient of 0-5% methanol in 60 min.

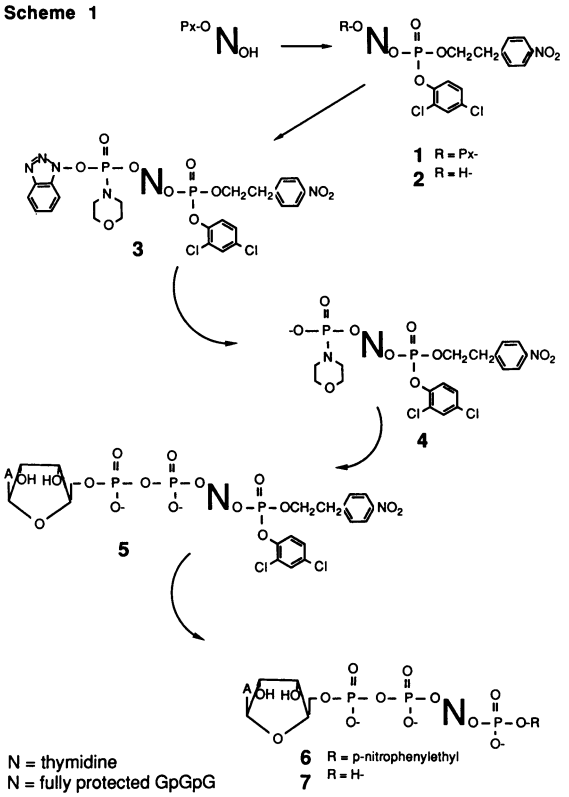
RESULTS

Chemical Synthesis of Adenylated Donors

It has been reported that T_4 RNA ligase will use a variety of substituted pyrophosphates of the form A(5')pp(5')N as donor substrates (11, 22). The substitutent N need not be a nucleoside for the reaction to proceed. However, in cases where N is a nucleoside or oligonucleotide derivative, synthesis of a homogenous product requires that 3'-hydroxyl group of the adenylated donor carry a blocking group in order that the initial joined product does not itself function as an acceptor molecule for further elongation. A number of blocking groups have been proposed (23, 24) but a 3'-phosphomonoester appears to be one of the more useful possibilities. Thus, the simplest derivative desired was the adenylated thymidine derivative A(5')pp(5')dTp. We wished, however, to approach the synthesis in such a manner that dT could be replaced by an oligonucleotide of choice. Therefore, the simplest initial target molecule would be thymidine containing protected 3' and 5' phosphate derivatives. In this case a suitable 5' phosphate could then be converted to the desired adenylated derivative and the 3' phosphate could produce the 3' phosphomonester blocking group. With this approach a similar strategy could then be used for the synthesis of an adenylated oligonucleotide, for example: A(5')pp(5')GpGpGp.

5'-O-(9-phenylxanthene-9-yl)thymidine could be phosphorylated with 2,4-dichlorophenyl-O,O-bis(1-benzotriazolyl)phosphate (14) and the initially formed monobenzotriazolyl adduct converted to the triester 1a (Scheme 1) by the addition of 4-nitrophenylethanol and a small amount of N-methylimidazole. The triester 1a could ultimately be converted to a 3'-phosphomonoester first by reaction with the oximate anion to remove the 2,4-dichlorophenyl group (25) and in a second step, reaction with a strong base to remove the 4-nitrophenyl ethyl group by ß-elimination (18, 26, 27). After removal of the 5' protecting group, the triester 2a was reacted with morpholino-O,O-bis(1-benzotriazaoly)phosphate (4, 17, 20) to form the bisphosphotriester 3a.

Scheme 1



Isolation of 3a by silica gel chromatography could be achieved but yields were very low, presumably as a result of hydrolysis of the benzotriazolyl group on the column. However, 3a was generally pure enough to be used in subsequent steps without chromatographic purification. The thymidine derivative 3a (Scheme 1) containing 3' and 5' phosphotriesters with four different phosphate protecting groups is a key intermediate in the synthesis of A(5')pp(5')Tp. Hydrolysis of the 5'-benzotriazolyl group provides a 5'-phosphomorpholidate which can be converted to the adenylated pyrophosphate. The 3'-phosphotriester is stable under the conditions used to form the pyrophosphate and can subsequently be converted to the 3'-phosphomonester as described above. The ^{31}P-NMR of 3a indicates the presence of the four diastereoisomers expected (Fig. 1). The transformation of 3a to A(5')pp(5')dTp could be efficiently monitored by ^{31}P-NMR and thus

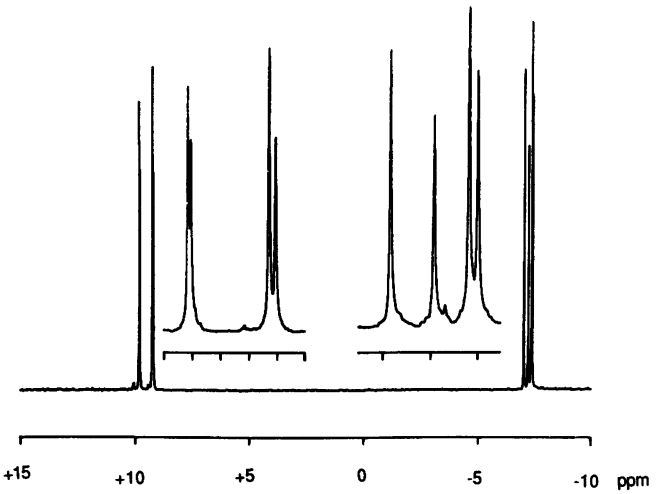


Figure 1. ^{31}P-NMR spectrum of the thymidine derivative 3a.

the various intermediates were not isolated. During the hydrolysis of 3a to 4a the eight peaks observed in the ^{31}P-NMR spectrum (Fig. 1) collapse to four peaks expected for the two diasterioisomers of 4a (see EXPERIMENTAL). Reaction with AMP to form 5a is accompanied by the presence of four peaks (two doublets) in the ^{31}P-NMR spectrum characteristic of the pyrophosphate, with a coupling constant of 19 Hz. The entire spectrum contains 10 peaks from the two diastereoisomers present. Deprotection of the 3'-phosphotriester of 5a (Scheme 1) proceeded in two steps. Removal of the 2,4-dichlorophenyl group with an oxime (25) produced 6a as a single diastereoisomer. The ^{31}P-NMR indicated a singlet for the 3'-phosphodiester and a quartet for the pyrophosphate (see EXPERIMENTAL). The final step involved removal of the 4-nitrophenylethyl group (18, 25, 26). After purification of 7a by Sephadex A-25 chromatography the expected ^{31}P-NMR spectrum was observed (Fig. 2a).

The identity of 7a could be confirmed by treatment with snake venom phosphodiesterase which produced one equivalent of AMP and one equivalent of pdTp. Treatment with bacterial alkaline phosphatase produced A(5')pp(5')dT and confirmed the presence of the phosphomonoester.

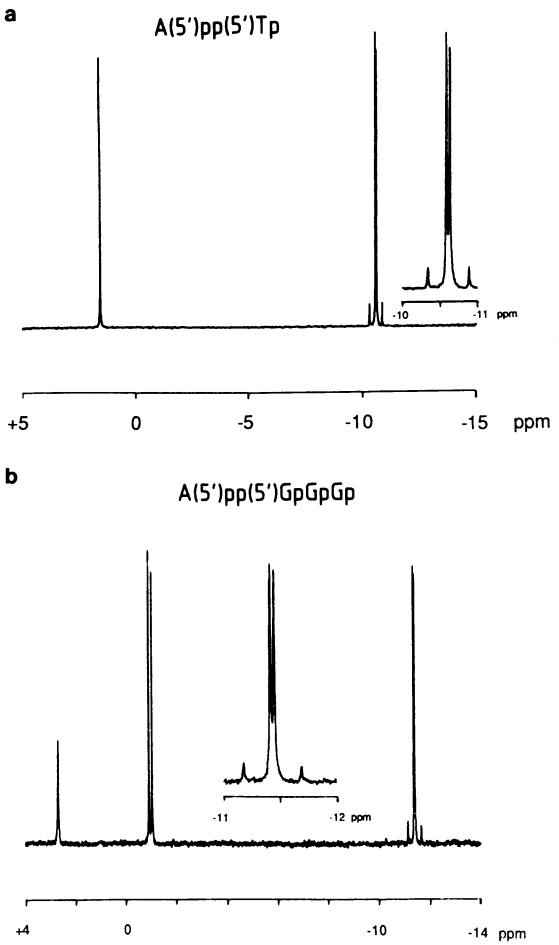


Figure 2. ^{31}P-NMR spectra of A(5')pp(5')dTp (above and A(5')pp(5')GpGpGp (below).

By an analogous series of reactions the trimer 3b containing the required 3' and 5'-phosphotriester derivatives could be converted to A(5')pp(5')GpGpGp (7b) (Scheme 1). In this case the final series of deprotection steps proceeded by an alternate route. After formation of the pyrophosphate, the 2,4-dichlorophenyl group and the internucleotidic 2-chlorophenyl groups were removed using an oxime. The nucleobase amides were then hydrolyzed with concentrated ammonia. The 4-nitrophenylethyl group was removed with diazabicy-

clo[5.4.0]undec-7-ene and finally treatment at pH 2 removed the 2'-O-tetrahydopyranyl protecting groups. The product was isolated by HPLC (see EXPERIMENTAL) and the expected ^{31}P-NMR spectrum was obtained (Fig. 2b).

The product 7b could be converted to one equivalent of AMP and pGpGpGp with snake venom phosphodiesterase. Bacterial alkaline phosphatase confirmed the presence of the 3'-phosphomonoester. Treatment with RNase T$_1$ produced two equivalents of 3'-GMP and one equivalent of A(5')pp(5')Gp.

Formation of the pyrophosphate linkage in the synthesis of A(5')pp(5')dTp and A(5')pp(5')GpGpGp could also be achieved by an alternate route. In this case the derivatives 3a and 3b were partially deprotected such that the 3'-terminal phosphate contained only the 4-nitrophenylethyl protecting group (with 3b the tetrahydropyranyl groups remained to protect the 2'-hydroxyls). The phosphotriesters were converted to phosphodiesters using an oxime in the presence of a strong base; the nucleobase amides (3b) were hydrolyzed with concentrated ammonia; and the morpholino group was removed by treatment with aqueous buffer pH 4.0. These partially protected derivatives were then reacted with adenosine-5'-phosphomorpholidate by essentially the same procedure described for the preparation of 5a and 5b. Subsequently, the terminal 4-nitrophenylethyl (and tetrahydropyranyl) group could be removed to yield 7a and 7b. The yields by this method were roughly the same as those described by the detailed procedures in the EXPERIMENTAL.

Reactions with T$_4$ RNA Ligase

Phosphodiester bond formation as catalyzed by T$_4$ RNA ligase was analyzed for the donor molecules pdTp and pGpGpGp in the presence of ATP and compared with the reaction involving the adenylated donors A(5')pp(5')dTp and A(5')pp(5')GpGpGp (Fig. 3). Under the conditions described (see EXPERIMENTAL) only small quantities of UpUpCpdTp were produced with donor pTp and acceptor UpUpC in the presence of ATP (Fig. 3). On the other hand quantitative conversion of UpUpC to UpUpCpdTp occurred within 30 min when the adenylation donor A(5')pp(5')Tp was used. With pGpGpGp as the donor molecule we were unable to detect any joined product. Using A(5')pp(5')pGpGpGp we were able to obtain a 60% yield

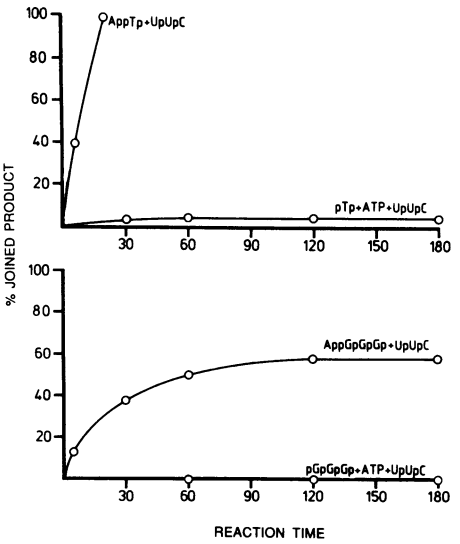


Figure 3. Reaction of the acceptor UpUpC with pdTp, pGpGpGp or corresponding adenylated donors.

of UpUpCpGpGpGp (Fig. 3). In both of the examples tested more efficient product formation was observed with the preadenylated form of the donor molecule.

DISCUSSION

Both derivatives 3a and 3b are key intermediates in the synthesis of the adenylated donors. They each contain a 5'-terminal and a 3'-terminal phosphotriester. The former can be easily hydrolyzed to a phosphomorpholidate which can be used to form the pyrophosphate linkage of the adenylated donor. the 3'-terminal phosphotriester can be converted to the diester with an oxime and finally to the phosphomonoester by a ß-elimination of the 4-nitrophenylethyl substituent.

In the conversion of 3a and 3b to A(5')pp(5')dTp and A(5')pp(5')GpGpGp respectively, each of the transformations involve reactions at the phosphorus atoms. In this respect, the reaction pathway can be easily monitored by ^{31}P NMR. The diastereoisomers of 3a and 3b or similar derivatives can result in numerous signals in the ^{31}P-NMR spectrum. However, the subsequent chemical steps; hydrolysis, formation of the pyrophosphate, etc.; tend to reduce the number of diasterisomeric products present and thus simplify the NMR analysis. Formation of the P-O-P

linkage can be easily confirmed by the appearance of the two doublets with a coupling constant of 19-20 Hz. With this approach it has been most efficient to avoid isolation of the various intermediates, other than to confirm the ^{31}P spectral information, in the conversion of 3a to 7a or 3b to 7b. Isolation of the final product was accomplished by soft gel ion-exchange chromatography and/or reversed phase HPLC. Except for various organic compounds released during deprotection reactions, product isolation involves resolution of the adenylated donor from excess AMP and unreacted donor molecule.

Both of the adenylated donors prepared, 7a and 7b were substrates for T_4 RNA ligase Both products functioned more efficiently in the joining reaction than either pdTp or pGpGpGp provided that the unadenylated form of the enzyme was used (10). A(5')pp(5')dTp resulted in quantitative conversion of UpUpC to UpUpCpdTp within 30 min. This is in agreement with previous results involving A(5')pp(5')Cp and A(5')pp(5')Gp (10). Although the reaction of A(5')pp(5')GpGpGp with UpUpC was more efficient than the corresponding reaction with pGpGpGp in the presence of ATP, we were unable to obtain quantitative formation of UpUpCpGpGpGp. This was likely to have resulted from aggregation of the quanosine oligonucleotide. It was necessary during the isolation of 7b as well as during analysis of the joining reaction between UpUpC and 7b to heat the HPLC column at 50°C. The higher temperature presumably resulted in denaturation of the oligonucleotide aggregates. This self association appears in part responsible for the relatively low isolated yield of 7b as well as the reduced efficiency in the joining reaction catalyzed by T_4 RNA ligase. The A(5')pp(5')GpGpGp derivative was prepared since it appeared to represent a "worst case" both for the chemical synthesis as well as subsequent enzyme reactions. We anticipate that other adenylated oligonucleotides will function as well or better than 7b in phosphodiester bond formation as catalyzed by T_4 RNA ligase.

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