Synthesis of 2'(3')-O-DL-alanyl hexainosinic acid using T4 RNA ligase: suppression of the enzymic reverse transfer reaction by alkaline phosphatase

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ABSTRACT

2'(3')-Q-DL-Alanyl (Ip)₅I was synthesized by a new method. An alanine ortho ester of inosine 5'-phosphate was added to (Ip)₄I using the ATP-independent reaction of T4 RNA ligase, and the product was converted smoothly to the desired ester. The enzymic reverse transfer reaction was conveniently suppressed by the dephosphorylation of the adenosine 5'-phosphate coproduct, catalyzed *in situ* by alkaline phosphatase.

INTRODUCTION

Chemically synthesized 2'(3')-Q-aminoacyl oligoribonucleotides have found considerable use as analogs of the 3'-terminus of aminoacyl tRNA. In this capacity, they have served as probes of the donor and acceptor sites of peptidyltransferase,¹⁻³ and have helped to elucidate the interactions between tRNA and elongation factor T_u.⁴ The reactions of these compounds also may aid in determining the origin of the genetic code and of biological peptide synthesis. Our investigation of these latter problems required the racemic 2'(3')-Q-DL-alanyl derivative of hexainosinic acid.

Previous syntheses of aminoacyl oligonucleotides have been restricted to derivatives of dimers and trimers. Most commonly, an acylated nucleoside has been extended by the sequential addition of protected mononucleotide units,⁵⁻⁹ though the acylation of protected oligomers with activated amino acid derivatives has also found use.^{9,10} Both methods would be cumbersome for oligomers of longer chain lengths, the former due to the number of coupling steps required and the latter to the extensive protection that is needed if acylation at a single position is desired.^{9,10} Chladek and Zemlicka¹¹ selectively

glycylated the 2'(3')-position of unprotected dinucleoside monophosphates by the initial formation of a 2',3'-cyclic ortho ester. Subsequent acid-catalyzed hydrolysis to the carboxylic ester and deprotection of the glycyl amino group afforded the desired products in high yield. This promising approach was less successful for longer oligomers, though a tRNA was so derivatized under carefully controlled conditions.¹² Unfortunately, the triethyl ortho ester starting materials, though available for several common amino acids, have thus far been restricted to racemic mixtures, and this has perhaps limited the application of the technique.¹³

In a novel procedure, N-protected 2'(3')-Q-aminoacyl esters of adenosine 5'-phosphate were coupled to truncated tRNA's using T4 RNA ligase.¹⁴ This enzyme catalyzes the formation of a phosphodiester bond between the 5'-phosphate of a donor and the 3'-hydroxyl of a suitable acceptor with the concomitant hydrolysis of ATP.¹⁵ The minimum donor for this reaction is a nucleoside 3',5'-diphosphate.¹⁶⁻¹⁸ However, adenylylated compounds of the form A-5'pp5'-N, which are known to be intermediates in the enzymic reaction,¹⁹⁻²¹ may be used in an ATP-independent reaction for the addition of a number of standard or modified nucleoside 5'-phosphates to the 3'-end of an oligonucleotide.^{22,23} The synthetic usefulness of the enzyme has been limited by a recently recognized "reverse transfer reaction".¹⁵ In this process, the adenosine 5'-phosphate that is liberated in the ligation attacks the nascent product or the reactants to form new pyrophosphates and mixtures of oligomers. These intermediates will then undergo coupling to form several unwanted by-products.

In this paper we report that the reverse transfer reaction in ATP-independent ligations can be effectively suppressed by the in situ dephosphorylation of adenosine 5'-phosphate catalyzed by alkaline phosphatase. This has enabled us to prepare 2'(3')-Q-DL-alanyl(Ip)₅I via the ligase-catalyzed joining of an ortho ester derivative of inosine 5'-phosphate and pentainosinic acid. Hydrolysis of the ortho ester and deprotection of the amino group afforded the alanyl ester in high yield. We have found that the tert-butoxycarbonyl (Boc) function is a suitable amino protecting group for acid-catalyzed ortho ester exchange reactions, and is

more easily and efficiently removed from aminoacyl oligonucleotides than the benzyloxycarbonyl (Cbz) group.

MATERIALS AND METHODS

General methods.

UV measurements were made on a Cary 15 recording spectrophotometer (Varian Associates). A Radiometer PHM 26c pH meter equipped with an MI-710 Tiny Combination pH Probe (Microelectrodes, Inc.) was used to measure the pH of small volumes (ca. 10-100 μ l), and was calibrated at pH 7.0 and 10.0 with standard buffers (Fisher). Solvents were removed on a Buchi Rotovapor R rotary evaporator, and lyophilization was carried out on a Labconco Lab-Con 3.

High performance liquid chromatography (HPLC).

HPLC instrumentation (all Waters Associates) included two pumps (models 6000 and 6000A), a model 660 solvent programmer, a model U6K injector, and a model 440 absorbance detector operating at 254 nm. Solvents from the pumps were tangentially mixed in a specially designed chamber.²⁴ The detector output was monitored on a Hewlett-Packard Moseley 7101B Strip Chart Recorder. Chromatography was performed at ambient temperature using one of four sets of conditions (Table 1). Flow rates were 1.0 ml/min, and all programs were linear. Radial-PAK C18 octadecylsilyl bonded-phase columns were purchased from Waters, and compressed in a model RCM-100 radial compression module. RPC-5 was prepared

Table 1. HPLC conditions.^a

System	Gradient	Column
A	0.05 M ammonium acetate (pH 4.7) 4-68% acetonitrile in 90 min	Radial-PAK C18 5 mm i.d., 10 μ m
B	0.05 M ammonium acetate (pH 4.7) 4-68% acetonitrile in 90 min	Radial-PAK C18 8 mm i.d., 5 μ m
C	0.01 M sodium phosphate (pH 7.0) 3-25% acetonitrile in 50 min	Radial-PAK C18 8 mm i.d., 10 μ m
D	0.01 M Tris-HCl (pH 8.1) 5-50 mM KClO ₄ in 50 min	RPC-5 0.4 x 100 cm

^a All flow rates were 1.0 ml/min, and all gradients were linear.

using a modification of the method of Pearson *et al.*²⁵ and packed into a 0.4 x 100 cm stainless steel column. Solvents were passed through Millipore HA or FH filters (0.5 μ m) before use.

HPLC was used for both the characterization and isolation of reaction products. Compounds were identified by coelution with authentic markers when possible, and all retention times on the octadecylsilyl columns were consistent with expected relative hydrophobicities (Table 2). Purification of up to several milligrams of nucleotidic material was routinely effected using Radial-PAK cartridges. Chromatographic peaks were integrated off-line using a Summagraphics Bitpad interfaced with a Cromemco Z-80 computer.

Reagents.

Water was distilled and passed through a Milli-Q Reagent Grade Water system (Millipore) to give a specific resistivity of 15 megaohm-cm. DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals. N-Boc-DL-alanine was obtained from Chemical Dynamics Corp., N-Cbz-DL-alanine from Sigma, and propionitrile, 1,1'-carbonyldiimidazole, benzyl chloroformate, di-~~tert~~-butyl dicarbonate, and 2,4-dinitrofluorobenzene from Aldrich. Phosphoric acid (99%, crystalline) was purchased from Matheson, Coleman, and Bell. All chemicals were reagent grade and used without further purification unless otherwise indicated. N,N-Dimethylformamide (DMF) was dried over Davison Type 4A molecular sieves and distilled under reduced pressure. Tris was recrystallized from water-ethanol. Acetic acid for ammonium acetate buffers was distilled from chromium trioxide. Triethylammonium bicarbonate (1 M) was prepared by bubbling CO₂ through a mixture of freshly distilled triethylamine (139 ml) in 700 ml of water at 0 °C to give a pH of 7.6, warming to ambient temperature, and diluting to 1 liter.

Enzymes.

Phosphodiesterase I (*Crotalus atrox* venom, EC 3.1.4.1), spleen exonuclease (bovine spleen, EC 3.1.16.1), RNA ligase (T4 infected *E. coli*, EC 6.5.1.3, RNase-free grade), and ribonuclease T₁ (*A. oryzae*, EC 3.1.27.3) were purchased from P-L Biochemicals, and alkaline phosphatase (*E. coli*, EC 3.1.3.1, BAPF grade) was from Worthington Biochemicals. All were used without further

Table 2. HPLC retention times on octadecylsilyl columns.

Compound	Retention time (min)		
	System A	System B	System C
Ino	8.2		
Ado	14.8	21.2	
pI	2.0		
pA	3.5	12.0	
Ip	2.5		
I>p	5.1		
IpI	14.7		
(Ip) ₄ I		18.4	
(Ip) ₅ I		18.4	
2'(3')-Cbz-Ala-I	37.3, 37.7, 40.9		
2'(3')-Boc-Ala-I	31.0, 31.2, 35.0, 35.5		
2'(3')-Ala-I	16.3, 16.8		
5'-Boc-Ala-I	31.0		
2'(3')-Cbz-Ala-pI	31.8, 34.7		
2'(3')-Boc-Ala-pI	25.0, 26.8, 27.6		
Boc-Ala isomers of IpI:			
terminal 2'	27.1		
3'	28.6, 29.0		
internal 2'	24.4, 25.5		
5'	25.5		
2a	51.3, 52.3		
2b	44.1, 44.8		
3a	40.0, 40.9		40.4, 42.7, 44.3, 46.0
	41.7		35.4, 36.0, 37.7, 39.0
3b	34.3, 35.4		55.3, 56.8, 57.0
4a			51.8, 52.3, 53.1, 53.4
4b			40.2, 41.4, 42.4, 43.2
5a		35.6, 36.3	35.4, 35.9, 36.8, 37.2
		37.3	
5b	28.6, 29.7	33.6	
6a		29.2, 30.0	
		30.3	
6b		27.4, 27.8	
7a		33.4, 34.2	
		34.7	
7b		30.5, 30.9	
8a		25.2, 26.2	
8b		23.5, 24.0, 25.2	
9		19.6	
DNP-Ala	23.5		

purification, and reported activities were assumed to be correct.

Enzymic digestions were carried out using the following conditions: a) (0.1 ml total volume) 0.1 M Tris-HCl (pH 7.5 or 8.9), 0.1 M NaCl, 15 mM MgCl₂, phosphate ester (ca. 0.1 mM), and phosphodiesterase I (500 units/ml) incubated for 15 min at ambient temperature;²⁶ b) (0.1 ml) 0.1 M ammonium acetate (pH 4.7), phosphodiester bond (ca. 1.0 mM), and spleen exonuclease (1.7 units/ml) incubated for 1 hr at 37 °C;²⁷ c) (20 µl) 40 mM Tris-HCl (pH 7.5), 2 mM EDTA, phosphodiester bond (ca. 0.1 mM), and ribonuclease T₁ (500 units/ml) incubated for 15 min at 37 °C.²⁸

Nucleosides and nucleotides.

Inosine, the sodium salts of inosine 5'-phosphate and inosine 2'(3')-phosphate, and the potassium salts of poly(C) and poly(I) were purchased from P-L Biochemicals. Poly(C) was dialyzed against 0.01 M Tris-HCl (pH 7.0) containing 1 mM EDTA, and then against 0.01 M triethylammonium bicarbonate (pH 8.1). Oligoinosinates were prepared from poly(I) by limited alkaline hydrolysis, dephosphorylation, and DEAE-Sephadex chromatography,²⁹ and compared to authentic samples from Miles or P-L Biochemicals (HPLC System D). Inosine 2',3'-cyclic phosphate was prepared from inosine 2'(3')-phosphate.³⁰

Acylation with N-protected alanyl imidazolides.

Mixtures of the Cbz- or Boc-DL-alanyl esters of inosine and inosine 5'-phosphate were prepared by a modification of the method of Gottikh *et al.*³¹ To form the imidazolides, 1,1'-carbonyldiimidazole (7.2 mg, 44 µmol) was dissolved in 50 µl of dry DMF, Cbz-DL-alanine (8.9 mg, 40 µmol) or Boc-DL-alanine (7.6 mg, 40 µmol) was added, and the solution stirred for 30 min at ambient temperature. To this was added inosine (0.45 mg, 1.7 µmol), or a solution of inosine 5'-phosphate (3.0 mg, 7.0 µmol) in 0.1 ml of H₂O. The reaction was stirred for 3-12 hr at ambient temperature and products were separated by HPLC (System A). Monoacylated derivatives were characterized by relative rates of alkaline hydrolysis and by sensitivity to periodate oxidation. 2'(3')-Boc-DL-alanylinosine was deprotected by treatment with 95% trifluoroacetic acid at room temperature for 5 min.

The isomeric Boc-DL-alanyl esters of IpI were prepared by

treating the dimer (2.0 A₂₄₈ units) in 25 μ l of H₂O with the imidazolide of Boc-DL-alanine (10 μ mol) in 10 μ l of DMF. Four monoacylated products were isolated by HPLC (System A). The derivatives were characterized by digestion with phosphodiesterase I (pH 7.5) and by hydrolysis rates of the deprotected esters.³²

Mixtures of the Cbz- or Boc-DL-alanyl esters of (Ip)₅I were prepared by treating the oligomer (1.2 A₂₄₈ units) in 6 μ l of H₂O with the imidazolide (2.4 μ mol) in 3 μ l of DMF. In one experiment, poly(C) (0.67 A₂₆₇ units) was included. The products were analyzed by HPLC (System B).

Ortho ester exchange reactions.

Ethyl DL-orthoalanate was synthesized from propionitrile by published methods.^{13,33} The amino group was Cbz-protected by reaction with benzyl chloroformate¹³ or Boc-protected with di-~~tert~~-butyl dicarbonate³⁴ to give the triethyl orthoalanates 1a and 1b respectively.

In a modification of the method of Hwang *et al.*,¹² the triethylammonium salt of (Ip)₅I (23 A₂₄₈ units) and ortho ester 1a (59 μ mol) were dried by the successive addition and evaporation of DMF, treated with phosphoric acid (0.19 mmol) in 5 ml of DMF, and stirred for ca. 10 hr at reduced pressure (0.15-0.2 mm) while the volume was maintained at 5 ml with added DMF. The mixture was evaporated to ca. 0.5 ml and chromatographed (HPLC System B). Yields of the desired ortho ester product (6a) were variable but never exceeded 25%, and a number of by-products were observed. The triethyl ortho ester 1a failed to react with (Ip)₅I when methanesulfonic or trifluoroacetic acids were used as catalysts.

The 2',3'-cyclic alanine ortho esters of inosine (2a and 2b) were prepared from 1a and 1b, respectively, by a modification of the procedure used to prepare the Cbz-glycine ortho ester.³⁵ The products were stable to alkali (1 M KOH, 2 hr) and were hydrolyzed by formic acid (0 °C, 15 min) to the corresponding 2'(3')-esters.

For the preparation of the ortho ester derivatives of inosine 5'-phosphate (3a and 3b), 1a or 1b (0.5 mmol), disodium inosine 5'-phosphate (3.83 mg, 8.9 μ mol), and 98% trifluoroacetic

acid (26 μ l) were combined in 0.3 ml of dry DMF, stirred for 90 min at ambient temperature, and neutralized with 65 μ l of triethylamine. Volatile components were removed in vacuo and the residue treated with 1 ml of 0.01 N sodium hydroxide and extracted with four 1-ml portions of diethyl ether. Excess ether was evaporated from the aqueous phase and 1.0 ml of 0.01 M triethylammonium bicarbonate (pH 8.1) was added. A yellow impurity was removed by chromatography on a 14.7 x 1.1 cm DEAE-Sephadex A-25 column (bicarbonate form) with a gradient of 0.01-1.0 M triethylammonium bicarbonate. The absorbance of the eluate was monitored at 254 nm using a Zeiss PMQII spectrophotometer equipped with a flow-through cell, and the product was collected and lyophilized. Yields of ca. 60% from inosine 5'-phosphate were estimated from the absorbance. Significant losses occurred during the chromatography. The products were stable to base (1M KOH, 2hr), and treatment with 80% formic acid (0 °C, 15 min) gave the expected N-protected 2'(3')-esters of inosine 5'-phosphate.

Synthesis of ortho ester derivatives of A-5'pp5'-I (5a and 5b).

Following the method of Hecht et al.¹⁴ for the synthesis of A-5'pp5'-A, inosinate 3a or 3b (21 A₂₅₄ units) was treated with 1,1'-carbonyldiimidazole (1.22 mg, 7.5 μ mol) in 50 μ l of DMF and stirred for 2 hr. HPLC (System C) showed ca. 90% conversion to a product consistent with the imidazolid 4a or 4b. The reaction mixture was stirred with methanol (2 μ l) for 30 min and then treated with a solution of adenylic acid (2.8 μ mol) and tributylamine (2.8 μ mol) in 50 μ l of DMF. Stirring was continued for three days at ambient temperature, and HPLC then showed ca. 90% conversion to a new product which, using System A, was isolated and lyophilized. UV (5b, pH 4.7) $\lambda_{\max}$ = 250 nm; A(260/250) = 0.82; A(280/250) = 0.19.

The products 5a and 5b were stable to base (1 M KOH, 2 hr), and were degraded by phosphodiesterase I (pH 8.9) to give ortho ester 3a or 3b and adenosine in a molar ratio of 1:1.

Ligation of oligonucleotides and pyrophosphates 5.

Reactions were carried out in 0.1 ml total volume using the following conditions: (Ip)₄I (3-5 A₂₄₈ units), pyrophosphate 5a or 5b (4-5 A₂₅₀ units), 50 mM Tris-HCl (pH 8.1), 20 mM MgCl₂, 5 mM dithiothreitol, 50 μ g/ml bovine serum albumin, alkaline

phosphatase (3 units/ml, absent in controls), and RNA ligase (240-300 units/ml). The pH was measured before the addition of enzyme and adjusted if necessary. In certain reactions, a five-fold increase in scale was used. After 3-10 hr incubation at 37 °C, the reaction mixture was loaded onto a SEP-PAK C₁₈ cartridge (Waters Associates) to remove protein. The nucleotidic material was eluted with 0.05 M ammonium acetate (pH 4.7), 75% acetonitrile, and then lyophilized. For the reactions that contained alkaline phosphatase, HPLC (System B) showed 70-95% conversion to the desired ortho ester derivative of (Ip)₅I (6a or 6b) which was collected and lyophilized. In the large-scale preparations, the product was purified by chromatography on a 0.9 x 22 cm DEAE-Sephadex A-25 column (bicarbonate form), using a gradient of 0.1-1.0 M triethylammonium bicarbonate (pH 8.1). The resulting hexamer was free of the pentamer derivative. The Cbz- and Boc-protected alanine ortho esters of tetrainosinic acid (7a and 7b, respectively) were formed in a similar manner.

Spleen exonuclease degradation of the ligase reaction products (6a and 6b) gave inosine 3'-phosphate and the expected inosine ortho ester (2a or 2b) in a molar ratio of 5:1. In addition, hexamer 6a was chromatographically identical to the product obtained by direct ortho ester exchange.

Hydrolysis and deprotection of hexainosinates 6a and 6b.

Treatment of 6a or 6b with 80% formic acid (0 °C, 15 min) resulted in quantitative conversion to the 2'(3')-ester (8a or 8b) that was chromatographically identical to the corresponding products obtained by the acylation of (Ip)₅I with the imidazolides of Cbz- or Boc-DL-alanine (HPLC System B). The Cbz-protected derivative 8a could not be effectively deprotected by hydrogenolysis over Pd-BaSO₄. However, the Boc-containing 8b was quantitatively deprotected by brief treatment with 95% trifluoroacetic acid (room temperature, 5 min) to give ester 9.

The alanyl ester 9 was converted to (Ip)₅I and alanine by mild alkali, and the amino acid was analyzed by HPLC (System A) after conversion to the N-dinitrophenyl derivative.³⁶ Ribonuclease T₁ completely degraded the trifluoroacetic acid deprotected material to monomers (inosine 2',3'-cyclic phosphate, inosine 3'-phosphate, 2'(3')-Q-DL-alanylinosine, and inosine), the nucleo-

tide:nucleoside ratio being 5:1. The aminoacyl ester was partially hydrolyzed during this digestion.

RESULTS AND DISCUSSION

A number of possible synthetic routes to 2'(3')-Q-DL-alanyl (Ip)₅I were investigated. A report of 50-70% specific acylation at the 3'-terminus of a tRNA with aminoacyl imidazolidines,^{37,38} though questioned,³⁹ led us to study the reactions of the latter compounds with oligonucleotides. The dimer IpI reacted at the internal 2'-, terminal 2'-, and 3'-hydroxyl groups in the proportion 1:1:2. There was very little reaction at the 5'-position.³² The relatively high extent of acylation at the internal 2'-positions implies reduced yields of 2'(3')-acylated products from longer oligomers, and indeed, the hexamer reacted predominantly at the internal sites. Weber and Lacey⁴⁰ observed that the internal hydroxyl groups of poly(A) were unreactive toward the imidazolidine of phenylalanine when the polymer was bound in a helix with poly(U). However, when we treated the (Ip)₅I:poly(C) helix with the activated N-protected alanyl imidazolidine, acylation at all sites was suppressed.

Complete specificity for the 2'(3')-positions can be achieved by the initial formation of a 2',3'-cyclic ortho ester.¹¹ As in the synthesis of the Cbz-glycine ortho ester of a tRNA,¹² the reaction of ethyl Cbz-DL-orthoalanate (1a) with (Ip)₅I required anhydrous conditions, reduced pressure (to remove product ethanol), and phosphoric acid catalysis. Nevertheless, yields were poor (less than 25%) due to by-products that may be analogous to the unidentified compounds observed in the reaction of dinucleotides with triethyl ortho esters.¹¹

The synthetic scheme shown in Figure 1 was used to prepare specifically 2'(3')-aminoacylated products in high yield. The N-protected alanine ortho esters of inosine 5'-phosphate (3) were coupled with adenosine 5'-phosphate to give the pyrophosphates 5 which served as the substrates for the ligase-catalyzed addition of the inosinates to (Ip)₄I.

It was considered advantageous to carry the aminoacyl group through the synthetic procedures masked as the ortho ester rather than as the N-protected 2'(3')-carboxylic ester. We found that

Figure 1. The synthesis of 2'(3')-Q-DL-alanyl(Ip)₅I. 1a-8a, R=Cbz; 1b-8b, R=Boc. CDI=1,1'-carbonyldiimidazole, BAP=bacterial alkaline phosphatase, P_i=inorganic orthophosphate.

the latter was unstable to the conditions of pyrophosphate formation. Therefore, use of the 2'(3')-ester would require the aminoacylation step to be carried out after formation of pyrophosphate, and this would be efficient only for the symmetrical A-5'pp5'-A. The ortho ester, however, is stable to the coupling reaction and as a result, any aminoacyl-bearing nucleoside may be incorporated into the pyrophosphate and subsequently ligated to the oligonucleotide. Furthermore, the ortho ester is stable in the pH range of the ligase reaction (7.5-8.3), so the enzymic

reaction time can be extended for sluggish acceptors without fear of ester hydrolysis.

We were unable to remove the Cbz-group efficiently from esters of oligonucleotides by hydrogenolysis over Pd-BaSO₄, and the deprotection of the Cbz-glycyl ester of tRNA was not reported.¹² The Boc-protected triethyl ortho ester 1b reacted with inosine 5'-phosphate without loss of the protecting group in the presence of trifluoroacetic acid, and was therefore used in preference to the Cbz-blocked compound. The product of this exchange (3b) was stable to alkali, while formic acid treatment afforded the 2'(3')-O-Boc-DL-alanyl ester.

Adenosine 5'-phosphate and ortho esters 3 were coupled via the imidazolides 4 to form the pyrophosphates 5. Our studies on the reaction of nucleoside 5'-phosphates with 1,1'-carbonyldiimidazole suggest that the formation of the imidazolidine, at least from unsubstituted 5'-nucleotides, is not straightforward and may involve a number of intermediates.⁴¹ The pyrophosphates 5 were stable to alkali, and phosphodiesterase I treatment yielded equimolar amounts of adenosine and inosinate 3a or 3b. The ortho ester derivatives were only slowly dephosphorylated by the 5'-phosphatase that contaminated the phosphodiesterase preparation.

When the ligation of pyrophosphate 5a or 5b and (Ip)₄I was attempted using published methods,²² the desired hexamer was accompanied by a mixture of longer and shorter oligomeric ortho esters. This problem was particularly severe when the reaction mixture was incubated for long periods with relatively small amounts of enzyme. The by-products apparently resulted from the reverse transfer reaction of RNA ligase. This was supported by the lack of side products from the ligation of 5 to (Ip)₂I. As the enzyme acceptor site requires a trinucleoside diphosphate,^{16,42,43} reversal in this case would lead only to the original reactants.

The reverse transfer reaction was eliminated by the degradation of the interfering adenosine 5'-phosphate with alkaline phosphatase that was added to the ligation reaction mixture. The chromatograms presented in Figure 2 show the products obtained from the ligase reaction when this enzyme was either absent or present at a concentration of 3 units/ml. The corresponding

Figure 2. Chromatograms of ligase reaction mixtures in the absence (A) and presence (B) of alkaline phosphatase. Products are: a) adenosine 5'-phosphate, b) 6b, c) 7b, d) 5b, and e) adenosine. The early shoulder on peak b) in chromatogram A may be due to ortho ester derivatives of oligomers longer than the hexamer.

yields are shown in Table 3. In both cases, the $(Ip)_4I$ acceptor was entirely converted to ortho ester products. In the chromatogram of the phosphatase-free reaction (A), ortho ester derivatives of the pentamer and tetramer are evident. As the latter is not degraded by the reverse transfer reaction, it accumulated to become the principal by-product. Longer oligomeric ortho esters are also evident. The hexamer, though still the major product, was obtained in relatively poor yield (57%). The chromatogram of the reaction mixture that contained alkaline phosphatase (B) shows the complete conversion of adenosine 5'-phosphate to adenosine, and a 91% yield of the desired hexamer. The pentamer arose from contaminating $(Ip)_3I$ in the acceptor preparation, and no tetramer was evident, though this product would have been expected had the reverse transfer reaction been operative.

Table 3. Product yields from the ligase reaction in the presence and absence of bacterial alkaline phosphatase (BAP).

Yield of oligomeric orthoester (%) ^a				
	I ₇₊	I ₆	I ₅	I ₄
-BAP	2	57	19	27
+BAP	-	91	9	-

^a Yields were calculated from chromatographic peak areas using appropriate weighting for different chain lengths.

It was found economical to reduce the amount of RNA ligase in the reaction mixtures while extending the reaction time. This could be done because the ortho ester was stable to the slightly alkaline conditions used and because the reverse transfer reaction was suppressed. The commercial enzyme preparation remained active for more than 8 hr at 37 °C, and yields of up to 2.5 nmol per unit of enzyme were obtained.

The hexameric ortho ester **6b** was converted without deprotection to the 2'(3')-Q-Boc-DL-alanyl ester **8b** by formic acid treatment. The product was chromatographically identical to the 2'(3')-isomers obtained upon acylation of (Ip)₅I with the imidazolid of Boc-DL-alanine, and was converted to (Ip)₅I in alkali. The ester **8b** was deprotected quantitatively by brief treatment with trifluoroacetic acid, the product (**9**) being hydrolyzed in mild alkali to (Ip)₅I and alanine. The 3'-5' phosphodiester bonds were shown to be 97% intact by the digestion of ester **9** to monomer with ribonuclease T₁.

This synthetic method provides an efficient and specific route to 2'(3')-Q-aminoacyl oligo- and polynucleotides of any composition. Unlike the similar approach of Hecht *et al.*,¹⁴ the aminoacyl group is not constrained to reside on an adenosine residue. Furthermore, the stability of the ortho ester would allow sluggish acceptors (e.g. DNA) to be used in the ligation.

The use of alkaline phosphatase to suppress the reverse transfer reaction in ATP-independent ligations is convenient, and applicable except when a phosphate monoester must be kept intact in the donor or acceptor molecules. By eliminating this reverse reaction, high yields may be obtained from small amounts of RNA

ligase, making the enzyme a valuable and economical synthetic tool.

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