Chemical synthesis of branched RNA

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ABSTRACT

A branched tetranucleotide consisting of adenosine linked 2' and 5' to guanosine and 3' to cytidine was synthesized from appropriately protected nucleoside phosphoramidites as synthons. The product was characterized enzymatically.

INTRODUCTION

Recent progress directed toward understanding the mechanism of RNA splicing in eukaryotes has demonstrated that excised introns exist as lariat structures, a single-stranded circular RNA with a tail (1-5). A modified nucleotide having 2'-5' as well as normal 3'-5' phosphodiester bonds was shown to exist at the branch point and to be an intrinsic part of splicing as first proposed by Wallace and Edmonds (6). The splicing mechanism, including the steps leading to the lariat structure and the cellular machinery responsible for this process, still remains to be illucidated (7,8). Clearly the branch nucleotide is a key intermediate. Its availability could perhaps be of considerable value as an aid to understanding the mechanism of mRNA splicing in eukaryotes. This manuscript reports the chemical synthesis of a branched tetranucleotide in milligram quantities. An outline of the synthesis is presented in Fig. 1.

MATERIALS AND METHODS**Reagents and Solvents**

Silica Gel H60 and Fractosil 500 were purchased from Merck. Nucleosides were obtained from Pharma Waldhof. Other reagents such as solvents, enzymes, and reagents used for protecting nucleosides were purchased from various commercial sources.

³¹P NMR spectra were recorded on a Bruker WM-250 spectrometer and the chemical shifts were reported relative to an external capillary standard at 85% phosphoric acid.

Thin layer chromatography (tlc) was performed on EM plastic backed sheets (silica gel 60 F₂₅₄, 0.2 mm, cat #5775) in acetone:hexanes:triethylamine (45:45:10) (solvent A) and dichloromethane:methanol (85:15) (solvent B). Preparative column chromatography was performed on Merck silica gel H60. Concentrations *in vacuo* were carried out at 40°C or lower using an aspirator or an oil vacuum line. Solids were dried at 25°C and 0.02 torr. All reactions were at room temperature.

Anhydrous solvents were obtained as follows: dichloromethane by distillation first from phosphorus pentoxide and then calcium hydride; acetonitrile by successive distillation from calcium hydride, phosphorus pentoxide, and then calcium hydride; pyridine and triethylamine by distillation first from toluene sulfonyl chloride and then calcium hydride; dichloroacetic acid by distillation and dimethylformamide by storage over molecular sieves followed by distillation from calcium hydride.

Bis-diisopropylamino- β -cyanoethoxyphosphine, tetrazole, and diisopropylammonium tetrazolide were prepared according to the previously published procedures of Barone et al. (9). Triethylammonium hydrofluoride was also prepared according to a published procedure (10). 5'-O-Dimethoxytrityl-2'-O-*t*-butyldimethylsilyl-N⁶-benzoyladenine, 2',3'-O-benzoyl-N⁴-benzoylcytidine, 2',3'-O-isobutyryl-N²-isobutyrylguanosine, and 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyl-N²-isobutyrylguanosine were prepared using published procedures (11-14).

Synthesis of 1

5'-O-Dimethoxytrityl-2'-O-*t*-butyldimethylsilyl-N⁶-benzoyladenine (1.18 g, 1.5 mmol) and diisopropylammonium tetrazolide (0.13 g, 0.75 mmol) were dried *in vacuo*. Acetonitrile (6 ml) and bis-diisopropylamino- β -cyanoethoxyphosphine (0.58 ml, 1.9 mmol) were added. After stirring for 12 hr, TLC analysis in solvent A indicated the reaction was complete. The reaction mixture was transferred to a separatory funnel using saturated aqueous sodium bicarbonate (50 ml) and the product was isolated by extraction with dichloromethane (2 x 50 ml). The organic layer was washed

with saturated aqueous sodium chloride, dried over anhydrous Na_2SO_4 (30 min) and concentrated to the product as a dry foam. The yield of 1 after 12 hr drying *in vacuo* was 98% (1.45 g). ^3lp NMR 150.784, 149.202.

Synthesis of 3

Compound 1 (0.73 g, 0.73 mmol), 2',3'-O-benzoyl-N4-benzoyl-cytidine (0.27 g, 0.49 mmol), and tetrazole (0.16 g, 2.4 mmol) were added to a reaction flask and dried *in vacuo* at 0.02 torr for 12 hr. Anhydrous acetonitrile (5 ml) was added and the solution stirred for ten minutes. Analysis by tlc using solvent A indicated the reaction was complete. A solution (1.5 ml) of 0.1 M iodine in THF:lutidine:water (2:2:1, v/v/v) was added, followed in 5 min by 1 M $\text{Na}_2\text{S}_2\text{O}_3$ (50 ml). The product was isolated by extraction into CH_2Cl_2 , washed with NaHCO_3 , dried with anhydrous Na_2SO_4 , conc. to 3 ml, and fractionated by column chromatography on Merck H60 silica gel using CH_2Cl_2 : CH_3OH (96:4, v/v). The isolated yield was 79% (0.65 g, 0.43 mM).

Synthesis of 4

2',3'-O-Isobutyryl-N2-isobutyrylguanosine (1.22 g, 2.5 mmol) and diisopropylammonium tetrazolide (0.21 g, 1.25 mmol) were placed in a reaction flask and dried *in vacuo* for 1.5 hr. Acetonitrile (12 ml) and bis-diisopropylamino- β -cyanoethoxyphosphine (0.82 ml, 2.75 mmol) were added and the reaction mixture stirred for 12 hr. Analysis by tlc in solvent A showed the reaction was complete. The reaction mixture was transferred to an extraction funnel using saturated aqueous sodium bicarbonate (50 ml) and the product was extracted into the organic phase using CH_2Cl_2 (2 x 50 ml). The organic layer was then washed with saturated aqueous sodium chloride (3 x 50 ml), dried over anhydrous Na_2SO_4 and concentrated to the product as a dry foam. After drying *in vacuo* (12 hr.), the yield was 88% (153 g). ^3lp NMR 149.572, 149.000.

Synthesis of 5

The first step was removal of the 2'-O-*t*-butyldimethylsilyl group from 3 using a published procedure (14). Compound 3 (0.65 g, 0.43 mmol) was dissolved in an anhydrous solution (20 ml) of triethylamine:pyridine (1:3, v/v) and stored 12 hr. The solution containing 3 was then concentrated *in vacuo* to an oil and

re-concentrated twice to an oil with added anhydrous pyridine. The resulting gum was dissolved in 1 M triethylammonium fluoride in anhydrous pyridine (8.6 ml, 20 eq.). After 48 hr, tlc analysis in solvent B indicated that removal of the *t*-butyldimethylsilyl group was complete. Aqueous sodium phosphate (100 ml, pH = 7.0) was added to the reaction mixture and the product was extracted into CH₂Cl₂ (3 x 50 ml). The organic extracts containing the product were combined, dried over anhydrous Na₂SO₄, concentrated to 5 ml and added dropwise to 300 ml *n*-hexane. The product as a white precipitate was dried *in vacuo* to yield 0.53 g (87%). ³¹P NMR -4.88 (singlet).

Compound 3 after removal of the 2'-O-*t*-butyldimethylsilyl group (0.49 g, 0.35 mmol), 4 (0.98 g, 1.40 mmol), and tetrazole (0.29 g, 4.2 mmol) were placed in a reaction flask and dried *in vacuo* for 12 hr. Acetonitrile was added and the solution stirred for 10 min. Analysis by tlc in solvent B indicated the appearance of a new compound. A solution (1.5 ml) of 0.1 M iodine in THF:lutidine:H₂O (2:1:1, v/v/v) was then added. After 5 min, a 1 M aqueous solution of Na₂S₂O₃ was added to quench the oxidation reaction. The product (5) was isolated by extraction into CH₂Cl₂, washed with NaHCO₃, dried over anhydrous Na₂SO₄, concentrated to 3 ml, loaded on to a silica gel H60 column, and eluted with dichloromethane:methanol (96:4, v/v) to yield 0.50 g (72%). ³¹P NMR -3.33, -5.08 (broad peaks).

Synthesis of 6

5'-O-Dimethoxytrityl-2'-O-tetrahydropyranyl-N2-isobutyryl-guanosine (0.53 g, 0.75 mmol) and diisopropylammonium tetrazolide (0.064 g, 0.37 mmol) were placed in a reaction flask and dried *in vacuo* for 1.5 hr. Acetonitrile (3.5 ml) and bis-diisopropyl-amino- β -cyanoethoxyphosphine were added and the reaction solution stirred for 12 hr. Analysis by tlc in solvent A indicated that the reaction was complete. The reaction mixture was transferred to a separatory funnel using saturated aqueous sodium bicarbonate (50 ml) and the product was extracted into dichloromethane (2 x 50 ml). The organic layer containing the product was then washed with saturated sodium chloride (3 x 50 ml), dried over anhydrous Na₂SO₄, and concentrated to a dry foam. After drying *in vacuo* for 12 hr, the yield of 6 was 90% (0.62 g). ³¹P NMR 149.909, 149.270.

Synthesis of 7 In Protected Form

Compound 5 (0.50 g, 0.25 mmol) was dissolved in a solution (50 ml) of 2% dichloroacetic acid (w/v) in dichloromethane. After 2 min, a saturated aqueous solution of sodium bicarbonate was added to neutralize the acid. The organic layer was removed and the aqueous layer was extracted with dichloromethane (2 x 50 ml). The combined organic layers were dried over anhydrous Na_2SO_4 , concentrated to 3 ml, and added dropwise to n-hexane (200 ml). The product was collected by centrifugation and dried *in vacuo* to yield 0.34 g (79%).

Compound 5 after removal of the 5'-dimethoxytrityl group as described above (0.31 g, 0.18 mmol), compound 6 (0.58 g, 0.64 mmol), and tetrazole (0.17 g, 2.4 mmol) were placed in a reaction flask and dried *in vacuo* for 12 hr. Acetonitrile (4 ml) was added and the solution stirred for 10 min. Analysis by tlc in solvent B indicated formation of a new product. Sequential additions of a solution (1.5 ml) of 0.1 M iodine in THF:lutidine:water (2:2:1, v/v/v) and then, after 5 min, a solution (50 ml) of 1 M $\text{Na}_2\text{S}_2\text{O}_3$ completed the synthesis. The reaction mixture was transferred to an extraction funnel and the product isolated in the organic phase by extraction with dichloromethane (3 x 50 ml). The combined organic layers were washed with saturated aqueous sodium bicarbonate (100 ml), dried over anhydrous Na_2SO_4 , concentrated to 3 ml and loaded on to a silica gel H60 column. The product was eluted with dichloromethane:methanol (93:7, v/v). The isolated yield was 71% (0.33 g). $^3\text{1P}$ NMR -2.93 (broad peak).

Purification of 7 Free of Protecting Groups

Compound 7 in protected form (241 mg, 0.095 mmol) was dissolved in 2 ml pyridine and 2.4 ml cold concentrated aqueous ammonia added. The sealed flask was stored at 55°C for 48 hr. After concentration *in vacuo* to a dry residue followed by re-concentration with water, the product was dissolved in 0.01 M HCl and the pH adjusted to 2 with 1 M HCl. After 48 hr at room temperature, the reaction was neutralized (pH = 8) with aqueous ammonia and concentrated *in vacuo*. The product was dissolved in water (10 ml), charged on to a DEAE Sephadex A-25 column (1.4 x 4.5 cm) and eluted using a gradient (0.2 M to 0.7 M) of triethylammonium acetate (TEAC). The main peak containing 7 eluted at

Fig. 1. Reaction scheme for synthesis of a branched tetranucleotide. A^{Bz}, N6-benzoyladenine; C^{Bz}, N4-benzoylcytosine; G^{iB}, N2-isobutyrylguanine; DMTr, dimethoxytrityl; TBDMS, *t*-butyldimethylsilyl; Bz, benzoyl; iB, isobutyryl; thp, tetrahydropyranyl; DACEP, diisopropylamino- β -cyanoethoxyphosphinyl; TEAF, tetraethylammonium fluoride.

0.67 M TEAC. The product was collected, concentrated *in vacuo*, desalted on a Sephadex G-25 column (1.4 x 45 cm) by eluting with water, and lyophilized. The yield was 59% (78.2 mg).

RESULTS AND DISCUSSION

The synthesis strategy for 7, a typical branched tetranucleotide having a sequence consistent with current knowledge of these structures is outlined in Fig. 1. The nucleotide at the branch position is adenosine linked 2' to guanosine and 3' to cytidine. Although guanosine was arbitrarily chosen as the purine nucleoside 5' to the adenosine branch, most data now available (15) suggests that adenosine is the more common purine found at this site. Because of the possibility for steric crowding and consequently low yields during the synthesis of 2'-5' and 3'-5' internucleotide bonds, the less sterically con-

strained but highly reactive nucleoside phosphoramidites (16,17) were chosen as synthons. Thus the first step involved condensation of 1 with 2 using tetrazole in acetonitrile as an activating agent and oxidation of the phosphite to phosphate to form 3. Preparation of 1 began with 5'-O-dimethoxytrityl-2'-O-*t*-butyldimethylsilyl-N6-benzoyladenosine which was condensed with bis-diisopropylamino- β -cyanoethoxyphosphine using diisopropylammonium tetrazolide as catalyst.

The next major step was condensation of 4 at the 2' hydroxyl of adenosine. Model experiments, as expected from previous results (14), demonstrated that when 3 was treated directly with tetraethylammonium fluoride (TEAF) to remove the 2'-protecting group, internucleotide bond cleavage occurred (20%). Since cleavage resulted from formation of a cyclic phosphate triester, migration of cytidine 3' to 2' would also be expected if 3 were treated directly with TEAF. The synthesis of 5 therefore involved first the removal of the β -cyanoethyl group with triethylamine in pyridine in order to form the phosphate diester and then treatment with TEAF to remove the *t*-butyldimethylsilyl protecting group. Condensation with 4 in the presence of tetrazole followed by oxidation to the phosphate completed the synthesis of 5. Compound 4 can be prepared using standard conditions from 2',3'-di-O-isobutyryl-N2-isobutyrylguanosine and bis-diisopropylamino- β -cyanoethoxyphosphine. The possibility exists that the internucleotide phosphate diester, generated from 3 after removal of the β -cyanoethyl group, forms a mixed anhydride with 4 during the synthesis of 5. Other than consume one equivalent of 4 however, this reaction would not be deleterious to the synthesis since it is hydrolyzed during the aqueous work-up and may perhaps even enhance the yield. Based upon the expected reactivity of such mixed anhydrides (18), the free 2' hydroxyl would react preferentially with the less acidic tervalent phosphorus and lead to formation of 5.

Conversion of 5 to 7 was completed as a four step procedure. The dimethoxytrityl group was removed from 5 with dichloroacetic acid and the product was condensed with 6 (prepared from 5'-O-dimethoxytrityl-2'-O-tetrahydropyranyl-N2-isobutyrylguanosine and bis-diisopropylamino- β -cyanoethoxyphosphine) using tetrazole in acetonitrile to activate the reaction.

Fig. 2. Scheme for identification of the nucleotide at the branchpoint. Numbers in parentheses are mole fractions of products calculated from molar UV absorption coefficients at 260 nm of fractions pooled.

Fig. 3. Analysis of a branched tetranucleotide. An aliquot of 7 was labeled using [γ - ^{32} P]ATP and T4-polynucleotide kinase and analyzed by polyacrylamide gel electrophoresis with tris-borate buffer (pH 8.2) containing 7 M urea. Based on the calculated extinction coefficient, the phosphorylation was quantitative. Lane 1, the product of the kination; Lane 2, [γ - ^{32} P]ATP. The migration of bromphenol blue (BPB) and xylenecyanol (XC) are indicated.

After oxidation and silica gel column chromatography, compound 7 in protected form was isolated in 71% overall yield from 5. Following removal of protecting groups using concentrated aqueous ammonia and 0.01 M HCl, compound 7 was purified on a DEAE Sephadex column to yield 78.2 mg of the branched tetranucleotide. Based on a calculated extinction coefficient, compound 7 was then quantitatively converted to 8 (see Fig. 2), the 5'- ^{32}P labeled and branched tetranucleotide. Analysis of 8 by polyacrylamide gel electrophoresis indicated that the product as isolated by DEAE Sephadex was homogeneous (Fig. 3).

The branched tetranucleotide was characterized by enzymatic degradation procedures. Compound 7, monoesterified at the 5' hydroxyl group with ^{32}P -phosphate to give 8 was first oxidized with sodium metaperiodate. The oxidized cis-glycol groups at 3' ends were then labeled by reduction with ^3H -sodium borohydride (19). The double-labeled product as analyzed by high voltage paper electrophoresis is shown in Panel A, Fig. 4. GMP at the 5' end was confirmed by the release of ^{32}P that comigrated with a pGp marker on treatment either with RNase T1 (panel D, Fig. 4) or with RNase T2 (panel E, Fig. 4). In each digest a slower migrating compound containing ^3H , but devoid of ^{32}P appeared as a single spot between the CMP and AMP markers. This we predict is the branch core structure (compound 11).

In order to characterize the 3' and 2' linked mononucleotides, doubly labeled compound 8 (2',3'-tritium and 5'- ^{32}P) was digested with snake venom phosphodiesterase (SVPdase). As expected essentially stoichiometric amounts of ^3H -GMP and ^3H -CMP were released by this enzyme (panel B, Fig. 4) indicating that both have the 2',3' cis-glycol structure expected for 3' or 2' terminal nucleotides of 8. Release of essentially all radioactivity into ^{32}P -GMP, ^3H -GMP, and ^3H -CMP confirms as well that

Fig. 4. Secondary analyses of end-labeled branched tetranucleotide. Compound 7 labeled with ^{32}P at the 5' end as described in Fig. 3 and at 3' ends by periodate oxidation followed by reduction with $^3\text{H-NaBH}_4$ was freed of reactants on a DEAE cellulose column using a sodium chloride gradient (6). After desalting, 10 to 20 pmol aliquots in 20 μl of 50 mM Tris, pH 8, and 10 mM MgCl_2 were treated with the following enzymes for 30 min at 37°C . A. None; B. 0.18 units (Worthington units) of a highly purified SVPDase (20); C. 0.18 units SVPDase followed by 15 units RNase T1 for an additional 30 min at 37°C ; D. 15 units RNase T1; E. 15 units RNase T2; F. 3 μg nuclease P1. Sources of these enzymes and a detailed description of the high voltage paper electrophoresis system used for the analysis are described in reference 6. In this separation system, 5'-GMP, 5'-CMP, and their corresponding 2',3'-cis-glycol derivatives have the same electrophoretic mobilities.

all internucleotide linkages are either 3'-5' or 2'-5'. Unfortunately in the solvent system routinely used for the high voltage electrophoretic analysis, 5'-GMP and the 5'-phosphorylated branched tetranucleotide comigrate (see panel A, Fig. 4).

Therefore in order to demonstrate unambiguously that the tritium comigrating with 5'-GMP (panel B, Fig. 4) was indeed tritium labeled 5'-GMP rather than undigested 8 (i.e. 8 containing 3'-3', 3'-2', or 2'-2' linkages), it was necessary to additionally treat the SVPDase digest with RNase T1. The results, as shown in panel C, Fig. 4, are essentially identical to those shown in panel B and confirm that the counts migrating with 5'-GMP are indeed ^3H -GMP and ^{32}P -GMP. If undigested 8 had been present, then RNase T1 would have released significant amounts of ^{32}P as pGp (panel D, Fig. 4).

Failure of RNase T2 to release ^3H -guanosine that migrates slightly behind the origin or ^3H -cytidine (well behind the origin) from 8 shows that the GMP and CMP at the 3' termini of 8 are not in the usual 3'-5' phosphodiester linkage (panel E, Fig. 4). On the other hand, the ability of SVPDase to release them as 5'-nucleotides from 8 would be expected were they attached to a branch point in RNA (6).

Finally 8 shows a resistance to nuclease P1 characteristic of branched nucleotides in RNA (6), since instead of releasing ^3H -mononucleotides, a single spot of ^3H with a mobility less than 8, but greater than branch core (panel F, Fig. 4) was released along with ^{32}P -GMP. The ^3H -labeled nuclease P1 resistant structure is presumed to be 9, the structure with a net charge of -4 obtained from nuclease P1 digests of nuclear RNA (6).

Compelling evidence for a branch point in 7 comes from the isolation of the branched nucleotide as an adenosine triphosphate by the path outlined in Fig. 2. Compound 8 was treated with nuclease P1 to remove quantitatively the terminal 5' GMP residue. The product of this reaction, compound 9 was then treated without purification, with sodium metaperiodate and the oxidized 3'-nucleosides were removed from 9 by β -elimination (21). The entire reaction mixture was then applied to a DEAE cellulose column and various products eluted from the column with a salt gradient as displayed in Fig. 5. Oxidized guanosine and cytidine released by β -elimination are not retained on the column. UV absorbing material in fractions 31-37 at low salt concentrations could not be identified as it had no UV absorption maxima in the range of 240 to 290 nm. Material eluting with a charge close to

Fig. 5. Recovery of the branch nucleotide from the tetranucleotide. About 350 μg of 7 labeled with ^{32}P at the 5' end to give 8 (Fig. 2) was purified by chromatography on DEAE cellulose as described except urea was omitted (6). Material released with a net charge of -5 (essentially all the sample, see for example the analysis in Fig. 3) was pooled and desalted before treatment with 200 μg of nuclease P1 in 200 μl of 50 mM Na-acetate, pH 4.5, for 60 min at 37°C to remove the 5' terminal GMP. The reaction product was oxidized with sodium metaperiodate and then β -eliminated. After dilution to 8 ml with H_2O , the mixture was applied to a DEAE cellulose column and eluted with a NaCl gradient (6). Peak fractions were pooled, desalted and taken up in distilled, deionized H_2O for UV spectral analysis.

-4 at fraction 50 was assumed to be 9 that had escaped either oxidation and/or β -elimination. However, UV spectral analysis failed to reveal a clear absorption maximum suggesting contamination with non-nucleotide material. The small amount of material eluting with a charge of -5 was not analyzed further.

The material eluting with a charge of -6 had a UV spectrum indistinguishable from ATP. It migrated as a single component with the same mobility as an authentic 2'3'5' adenosine triphosphate marker during high voltage electrophoresis and was entirely converted to adenosine by *E. coli* alkaline phosphatase (data not shown). The quantitative recovery of products is shown in the numbers in parentheses in Fig. 2. More than 90% of the starting material was recovered as the 2'3'5' adenosine triphosphate while the eliminated 3' nucleosides were recovered in similar yields. It is thus clear that adenosine is the branch point for the attachment of equimolar quantities of GMP and CMP. Our enzymatic

analysis cannot define which nucleotide is attached to the 2' or the 3' carbon of adenosine, but, as discussed previously, it is reasonable to assume that 7 is correct since cytidine esterified to the 3' phosphate of AMP as a phosphate diester served as the starting material for synthesis of the branch structure. Two dimensional $^1\text{H-NMR}$ studies which are now complete do indeed confirm that this is the case (22).

Since completion of this research, two manuscripts have appeared (23,24) that outline the synthesis of various branch structures (i.e. triribonucleoside diphosphates similar to compound 11). However, one route cannot be used for synthesizing a sequence defined branched structure (23). The other pathway as developed by Sekine and Hata as well as the method outlined in this manuscript yield sequence defined branch structures. We have observed however that branched core structures such as 11 which lack a 5'-nucleotide cannot be phosphorylated at the 5'-hydroxyl with T4-polynucleotide kinase. Thus, in order to radio label core structures such as 11 for biochemical experiments, a chemical method will be necessary.

Branched tetranucleotides may prove to be useful as substrates for isolating the processing machinery (25), as an antigen for obtaining antibodies useful for cytological and biochemical studies, and as a model system for obtaining biophysical information on the tertiary structure of potential branch sites. Moreover the protected, branched tetramer as shown in Fig. 1 (compound 7) can be extended chemically in the 5'-direction to yield oligoribonucleotides that contain conserved sequences 5' to the branch site which have now been implicated in lariat formation (15). Such extended structures may prove useful for understanding the RNA splicing process.

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