Regio- and stereocontrolled synthesis of 2′-5′ linked phosphorothioate oligoadenylates by uranyl ion catalyst in aqueous solution

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Adenosine 5′-thiophosphoimidazolide (Imp(S)A) was prepared from adenosine, thiophosphoryl trichloride and imidazole. It hydrolyzed four times more slowly than the corresponding adenosine 5′-phosphoimidazolide. Epimerization between the diastereomers of Imp(S)A took place more quickly than the hydrolysis of Imp(S)A in aqueous solution. The uranyl ion catalyzed polymerization of Imp(S)A in neutral aqueous solution yielded, preferentially, a series of 5′-thiophosphorylated 2′-5′ linked thiophosphate oligoadenylates with R_p configuration. The corresponding adenosine 5′-thiophosphobenzimidazolide (Bzimp(S)A) was prepared in a similar way, and R_p and S_p diastereomers of Bzimp(S)A were separated by HPLC. (S_p)-Bzimp(S)A was polymerized by the uranyl ion catalyst efficiently giving 2′-5′ linked (R_p)-thiophosphate oligoadenylates up to a hexamer with both high regio- and stereoselectivity. On the other hand, uranyl-ion catalyzed polymerization of (R_p)-Bzimp(S)A proceeded inefficiently, and 2′-5′ linked (S_p)-thiophosphate di- and triadenylates were formed with low stereoselectivity. The polymerization took place by an S_N2 reaction pathway with inversion of the thiophosphoryl group of Imp(S)A or Bzimp(S)A. The mechanistic role of the uranyl ion in this regio- and stereocontrolled polymerization is described briefly.

Introduction

2′-5′ Oligoadenylates (2-5A) are prepared by 2-5A synthetase from ATP in interferon-treated cells and are involved in the antiviral action of interferon. The 2-5A binds and activates 2-5A dependent RNase (RNase L) which degrades viral mRNA, resulting in an inhibition of the protein synthesis.[1–4] Some 2-5A analogues have been known to possess anti-HIV activity.[5–7] During the past two decades, various 2-5A analogues have been synthesized to enable the biological function of 2-5A and its structure–activity relationship to be studied, and to explore potential antiviral activity. Thiophosphate analogues of 2-5A are of special interest because of their enhanced stability against cellular nuclease enzymes and biological activity that is comparable with the original 2-5A.[8–12] The thiophosphate linkage has a chiral centre at the phosphorous atom which is useful for investigating the stereochemical course of an enzymatic process such as the binding to and activation of RNase L. Enzymatically, 2′-5′ thiophosphate oligoadenylates with R_p configuration are produced from (S_p)-ATPαS by 2-5A synthetase.[11] 2′-5′ Thiophosphate oligoadenylates with all possible thiophosphoryl configurations have been prepared by conventional and stereocontrolled phosphite triester approaches, and their biological activities have been studied.[13–15] Stec and co-workers have reported the stereocontrolled synthesis of 2′-5′ thiophosphate oligoadenylates by using the oxathiaphospholane method.[16] It was reported that the all (R_p)-thiophosphate 2-5 A analogue binds and activates RNase L, while the all (S_p) analogue is devoid of any activity.[10] Torrence *et al.* developed a new approach to the selective down-regulation of a specific mRNA by conjugating the 2-5A analogue with antisense DNA,[17,18] and have shown that 5′-*O*-monothiophosphorylated 2-5A dramatically enhances resistance against degradation by phosphatase and exhibits strong biological activity.[19] In a previous communication, we have shown that the 2′-5′ linked (R_p)-thiophosphate oligoadenylates were obtained preferentially from adenosine 5′-thiophosphoimidazolide by a uranyl ion catalyst in aqueous solution.[20] The polymerization reaction took place with high regio-and stereoselectivity, although no protecting group was used. We further examined the uranyl ion catalyzed polymerization reactions of adenosine 5′-thiophosphoimidazolide (Imp(S)A) and adenosine 5′-thiophosphobenzimidazolide (Bzimp(S)A) under various conditions to optimize the synthesis of biologically active 2′-5′ all (R_p)-thiophosphate di-, tri- and tetraadenylates with 5′-terminal monothiophosphate, and to investigate the mechanism of the regio- and stereoselectivity of the polymerization. We report herein the syntheses and reactivities of adenosine 5′-thiophosphoimidazolide (Imp(S)A) and adenosine 5′-thiophosphobenzimidazolide (Bzimp(S)A) and their polymerization by the uranyl ion catalyst under various conditions. The mechanism of the regio- and stereocontrolled reaction is also described.

Results and discussion

Synthesis of adenosine 5′-thiophosphoazolides

Adenosine was first reacted with thiophosphoryl trichloride in triethyl phosphate to yield adenosine 5′-dichlorothiophosphate, which was treated with imidazole giving adenosine 5′-diimidazolothiophosphate in 32% yield. Subsequent partial hydrolysis of the diimidazolothiophosphate with an equimolar amount of water in pyridine gave adenosine 5′-thiophosphoimidazolide (Imp(S)A) (Scheme 1). These procedures were carried out in a one-pot reaction without isolation of the intermediates. The Imp(S)A was further purified by anion exchange column chromatography to give purified Imp(S)A as a triethylammonium salt in 17% yield from the starting adenosine. HPLC analysis showed that the resulting Imp(S)A was a 52 : 48 diastereomeric mixture, which could be separated by

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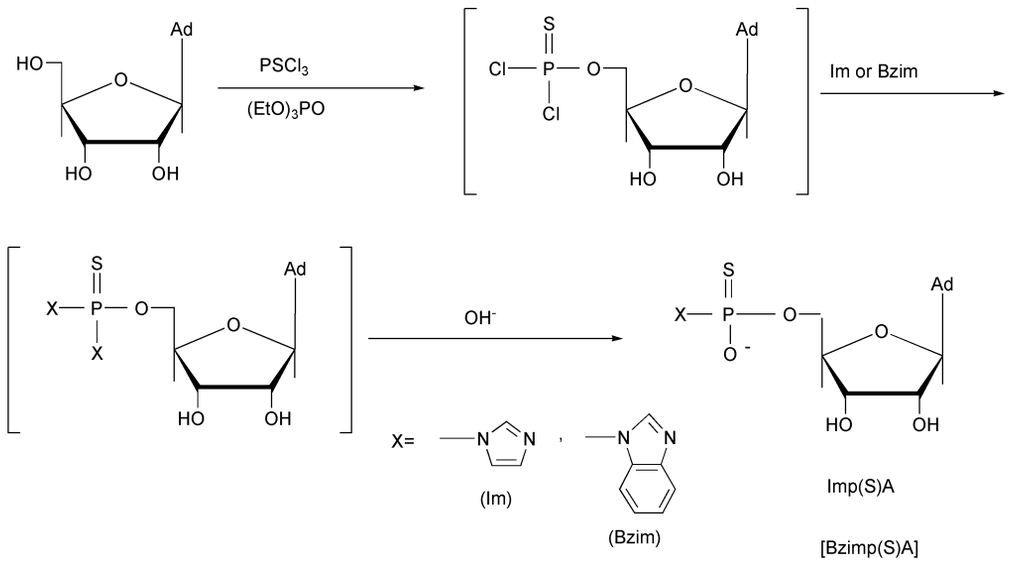


Scheme 1 Preparation of adenosine 5′-thiophosphoazolide.

preparative HPLC. The Bzimp(S)A was prepared in the same way as that for Imp(S)A from adenosine and benzimidazole in 7% yield after purification by anion exchange column chromatography. The diastereomeric mixture of Bzimp(S)A in a 53 : 47 ratio was separated by HPLC. The structures of Imp(S)A and Bzimp(S)A were confirmed by ESI-mass spectrometry and NMR spectroscopy. The configuration of the thiophosphoryl groups of the adenosine 5′-thiophosphoazolides was determined by a comparison of the HPLC retention times and the ^{31}P NMR spectra. (R_p) diastereomers showed shorter reverse-phase HPLC retention times and higher-field ^{31}P NMR chemical shifts compared to the corresponding (S_p) diastereomers.[21–24]

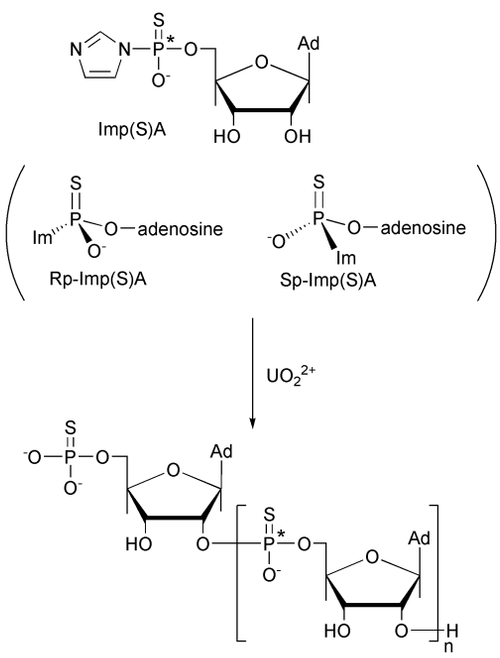
Hydrolytic stability and epimerization of the diastereomers of Imp(S)A

The hydrolytic stability of Imp(S)A was measured in 0.2 M *N*-ethylmorpholine buffer (pH 7.0) at 25 °C. The remaining Imp(S)A was analyzed by HPLC, and the hydrolysis reaction rate of Imp(S)A was estimated by assuming that the hydrolysis reaction obeyed pseudo first-order kinetics. The hydrolytic stability of ImpA was also measured under the same conditions for comparison. The pseudo first-order rate constant and half-life of Imp(S)A in neutral aqueous solution at 25 °C were 3.5×10^{-3} h^{-1} and 8.4 d, respectively. The rate constant and half-life of the corresponding adenosine 5′-phosphoimidazolide (ImpA) were 1.4×10^{-2} h^{-1} and 2.1 d, respectively. Thus, replacement of the phosphoryl group of ImpA with a thiophosphoryl group resulted in a four-fold increase in hydrolytic stability.

Epimerization of the (R_p) and (S_p) diastereomers of Imp(S)A was measured under the same conditions as used for the hydrolysis of Imp(S)A. Each diastereomer of Imp(S)A was dissolved in the buffer solution and kept at 25 °C immediately after separation by HPLC. The solution was analyzed by HPLC after various time periods and the epimerization rates were estimated from the ratio of (R_p) and (S_p) diastereomers, with the assumption that the epimerization obeyed first-order opposing reaction kinetics. The epimerization rates of $R_p \longrightarrow S_p$ and $S_p \longrightarrow R_p$ of Imp(S)A were estimated to be 6.7×10^{-3} and 6.1×10^{-3} h^{-1}. The corresponding half-lives of the epimerization were 4.3 and 4.7 d, respectively. The epimerization of diastereomeric Imp(S)A took place approximately twice as fast as than the hydrolysis of Imp(S)A. This result indicates that the stereochemical course of the reaction of the diastereomeric Imp(S)A is affected by the epimerization.

Polymerization of Imp(S)A by a uranyl ion catalyst

We conducted the polymerization of Imp(S)A in the presence of a uranyl nitrate catalyst in *N*-ethylmorpholine buffer (pH 7.0) (Scheme 2). Fig. 1 shows a typical HPLC profile of the



Scheme 2 Oligomerization of adenosine 5′-thiophosphoazolide by a uranyl ion catalyst.

reaction mixture in which a 1 : 25 molar ratio of the uranyl ion to Imp(S)A was used as the catalyst. A series of 2′-5′ linked (R_p)-thiophosphate oligoadenylates were formed preferentially. The polymerization was carried out on a large scale in order to prepare a series of thiophosphate 2′-5′ oligoadenylates for physicochemical and biochemical studies. The reaction mixture was separated by DEAE-TOYOPEARL anion exchange column chromatography, and the diastereomeric mixture of trimers was further purified by HPLC on an ODS silica gel column. 2′-5′ Linked thiophosphate (R_p)- and (S_p)-diadenylates, (R_pR_p), (R_pS_p) and (S_pR_p)-triadenylates and an ($R_pR_pR_p$)-tetraadenylate were isolated in 15.5, 4.6, 15.2, 3.0, 1.8 and 4.9% yields, respectively, from the starting Imp(S)A. The formation of 3′-5′ linked thiophosphate oligoadenylates was too small to isolate in substantial yields. The structures of the

Table 1 ^{31}P NMR, HPLC retention time, enzymatic stability and ESI-mass spectra of adenosine 5′-thiophosphoazolides and thiophosphate oligoadenylates

	^{31}P NMR (ppm)[a]				HPLC	SVPD digestion	
	P(1)	P(2)	P(3)	P(4)	RT/min	Half life/h	ESI-mass[f]
Imp(S)A							
(R_p)	47.31				35.7[b]		412.1 (412.1)
(S_p)	47.15				36.2[b]		412.1 (412.1)
Bzimp(S)A							
(R_p)	46.75				46.4[b]		462.1 (462.1)
(S_p)	46.16				47.8[b]		462.1 (462.1)
2′-5′ p(S)Ap(S)A							
(R_p)-2	44.17	57.66			13.4[c]	8.5	707.2 (707.1)
(S_p)-2	44.17	56.73			15.7[c]	⩾48.0[d]	707.2 (707.1)
2′-5′ p(S)Ap(S)Ap(S)A							
(R_pR_p)-3	44.65	58.35	58.08		20.2[c]	5.5	1051.9 (1052.1)
(R_pS_p)-3	44.65	58.34	57.35		21.7[c]	⩾24.0[d]	1052.0 (1052.1)
(S_pR_p)-3	44.66	56.68	58.20		22.3[c]	6.0[e]	1052.2 (1052.1)
2′-5′ p(S)Ap(S)Ap(S)Ap(S)A							
($R_pR_pR_p$)-4	44.60	58.75	58.04	58.10	28.4[c]	5.4	1397.3 (1397.1)

[a] Decoupled spectra. [b] HPLC retention time on an ODS silica gel column. [c] HPLC retention time on an RPC-5 column. [d] No digestion detected by the time indicated. [e] Cleavage to an (S_p)-dimer and p(S)A was observed. [f] ESI-mass spectrum obtained in the negative mode. Calculated mass number [M − H$^+$] is shown in parentheses. The corresponding multivalent ion peaks were also observed in the case of oligomers.

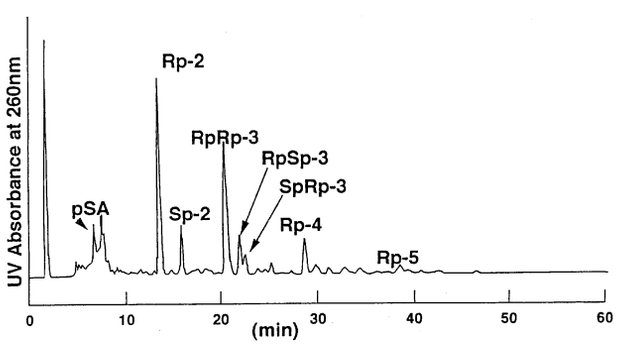


Fig. 1 HPLC profiles of the products from the uranyl-ion catalyzed polymerization of Imp(S)A. The reaction was run at 25 °C and pH 7.0 for 4 d. Imp(S)A (50 mM) and uranyl nitrate (2mM) were used for the reaction. (R_p)-2, 2′-5′ (R_p)-p(S)Ap(S)A; (S_p)-2, 2′-5′ (S_p)-p(S)Ap(S)A; (R_pR_p)-3, 2′-5′ (R_pR_p)-p(S)Ap(S)Ap(S)A; (R_pS_p)-3, 2′-5′ (R_pS_p)-p(S)Ap(S)Ap(S)A; (S_pR_p)-3, 2′-5′ (S_pR_p)-p(S)Ap(S)Ap(S)A; (R_p)-4 and (R_p)-5, 2′-5′ all (R_p) thiophosphate tetra- and pentaoligoadenylates, respectively.

2′-5′ linked thiophosphate oligoadenylates were confirmed by comparison of their HPLC retention times, ESI-mass spectra, ^{31}P NMR spectra and their stability against venom phosphodiesterase and RNase T2. These data are listed in Table 1. (R_p)-thiophosphate oligonucleotides showed shorter retention times compared to the corresponding (S_p)-thiophosphate oligonucleotides.[21] ^{31}P NMR of the resulting thiophosphate oligoadenylates demonstrated the presence of a 5′-terminal thiophosphate with the appropriate number of internucleotide thiophosphates .^{31}P NMR signals of the internucleotide thiophosphate with higher-field chemical shifts indicate an (R_p) configuration, while the corresponding signals with lower-field chemical shifts are due to the (S_p) configuration.[21–24] All the isolated oligomers were completely resistant to digestion by RNase T2, which suggests that they possess a 2′-5′ linkage. RNase T2 is reported to degrade 3′-5′ linked thiophosphate oligonucleotides with both (R_p) and (S_p) configuration, but not 2′-5′ linked oligonucleotides.[25] The (R_p)-thiophosphate oligonucleotides are reported to be hydrolyzed by venom phosphodiesterase from the 2′(3′)-terminal, but this is not the case for the (S_p)-thiophosphate oligonucleotides.[21] Thus all 2′-5′ linked (R_p)-thiophosphate oligoadenylates were cleaved to adenosine 5′-thiophosphate by venom phosphodiesterase. The 2′-5′ (S_pR_p)-thiophosphate triadenylate was hydrolysed to 2′-5′ (S_p)-thiophosphate diadenylate and adenosine 5′-thiophosphate, while no cleavage was observed in the case of the 2′-5′ (R_pS_p)-thiophosphate triadenylate under the conditions examined.

The uranyl ion-catalyzed polymerization of Imp(S)A was conducted under various conditions to investigate the effects of catalyst concentration and pH of the solution and to optimize the yield of biologically active 2′-5′ linked (R_p)-thiophosphate tri- and tetraadenylates. Table 2 shows the effect of the concentration of the uranyl ion on the thiophosphate oligoadenylate formation. Low catalyst concentration ($UO_2(NO_3)_2$ = 0.5 mM; [Imp(S)A/[UO_2^{2+}] = 100) gave preferentially the 2′-5′ linked (R_p)-thiophosphate diadenylate. On the other hand, a high catalyst concentration increased the yield of the 2′-5′ linked (S_p)-thiophosphate oligoadenylates, although the total yield of thiophosphate oligoadenylates was nearly the same as that obtained using the low catalyst concentrations examined in this study. A catalyst concentration of 1–2 mM ([Imp(S)A/[UO_2^{2+}] = 50–25) was adequate for the synthesis of the biologically active 2′-5′ linked all (R_p)-thiophosphate tri- and tetraadenylates. Table 3 shows the effect of the pH of the medium on polymerization. A low pH of the medium enhanced the hydrolysis of the thiophosphoimidazolide bond and suppressed the chain-elongation reaction. Thus, the 2′-5′ linked (R_p)-thiophosphate diadenylate and hydrolyzed product p(S)A were preferentially formed at pH 6.3. A difference in pH between 7.0 to 7.8 showed a slight effect on the polymerization when the reaction time was 5 d. A higher pH somewhat retarded both the polymerization and the hydrolysis rates of Imp(S)A, and longer reaction times were required for completion of the reaction at higher pH compared to lower pH. Fig. 2 shows the time course of the polymerization reaction of the diastereomeric mixture of (R_p)- and (S_p)-Imp(S)A in *N*-ethylmorpholine buffer (pH 7.0) using 2 mM catalyst at 25 °C. The polymerization was almost complete in 1 d, and the reaction produced the (R_p)-thiophosphate linkage in over 80% yield compared to the (S_p)-thiophosphate linkage, although both the (R_p)- and (S_p)-

Table 2 Effect of the catalyst uranyl ion concentration on the polymerization of Imp(S)A[a]

[Imp(S)A] / [UO_2^{2+}]	HPLC yield (%)[b]							
		2′-5′ Dimer		2′-5′Trimer			2′-5′Tetramer	
	pSA	(R_p)-2	(S_p)-2	(R_pR_p)-3	(R_pS_p)-3	(S_pR_p)-3	(R_p)-4	High oligomers
5	12.5	12.7	8.8	20.1	8.8	2.4	6.8	4.9
10	11.5	12.1	8.6	19.2	7.9	3.0	6.4	6.4
25	8.6	15.2	8.9	24.6	8.9	3.2	8.5	6.9
50	8.4	24.1	7.6	21.9	6.1	3.3	6.0	4.4
100	14.4	37.2	9.5	16.1	4.2	2.3	2.9	0.8

[a] The reaction was conducted in 0.2 M *N*-ethylmorpholine–HNO_3 buffer (pH 7.0) at 25 °C for 5 d. [b] a) Correction for hypochromicity of each oligoadenylate was not made for the calculation of the yield.

Table 3 Effect of the pH of the medium on the polymerization of Imp(S)A[a]

pH	HPLC yield (%)[b]							
		2′-5′ Dimer		2′-5′Trimer			2′-5′ Tetramer	
	pSA	(R_p)-2	(S_p)-2	(R_pR_p)-3	(R_pS_p)-3	(S_pR_p)-3	(R_p)-4	High oligomers
6.3[c]	18.8	38.5	7.6	14.9	3.0	2.6	2.9	2.1
7.0[d]	8.6	15.2	8.9	24.6	8.9	3.2	8.5	6.9
7.4[d]	6.6	12.8	7.9	24.6	8.2	3.2	9.1	7.1
7.8[d]	5.8	18.5	9.2	24.8	6.8	3.7	8.4	5.4

[a] The reaction was conducted using 2 mM uranyl ion catalyst ([UO_2^{2+}]/[Imp(S)A] = 25) at 25 °C for 5 d. [b] Correction for hypochromicity of each oligoadenylate was not done for calculation of the yield. [c] 0.2 M Imidazole–HNO_3 buffer was used. [d] 0.2 M *N*-ethylmorpholine buffer was used.

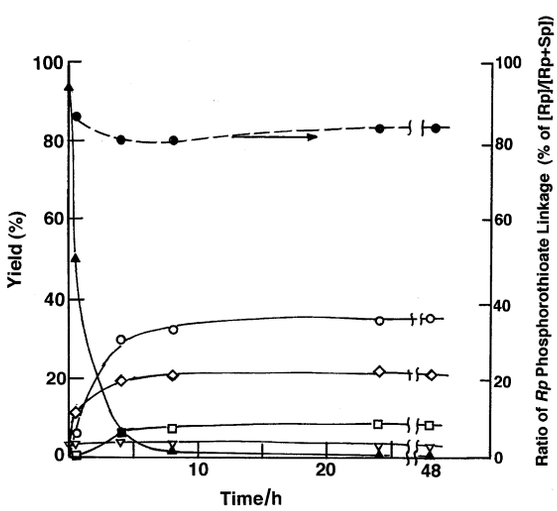


Fig. 2 Time course for the formation of thiophosphate oligoadenylates from Imp(S)A. Reaction conditions: Imp(S)A, 50 mM; uranyl nitrate, 2 mM; 25 °C; pH 7.0. Imp(S)A, -▲-; p(S)A, -▽-; 2′-5′ dimer (R_p + S_p), -◇-; 2′-5′ trimers (R_pR_p + R_pS_p + S_pR_p), -○-; 2′-5′ tetramer (all R_p), -□-. Ratio of R_p linkage in the resulting 2′-5′ thiophosphate oligoadenylates, -●-.

Imp(S)A were consumed in the same ratio. The (R_p)- and (S_p)-Imp(S)A epimerize more quickly than they polymerize using the uranyl ion catalyst.

We further prepared and conducted uranyl-ion catalyzed polymerization of Bzimp(S)A to investigate the mechanism of the regio- and stereoselectivity of the polymerization reaction, as the diastereomeric mixture of Bzimp(S)A could be separated by HPLC more easily than that of Imp(S)A and epimerization hardly takes place between the diastereomers of Bzimp(S)A. Thus, we could study the stereochemical course of the polymerization using (R_p)- and (S_p)-Bzimp(S)A. The reactivity of the Bzimp(S)A was lowered, because the condensed benzene ring lowered the basicity of imidazole, which results in the stabilization of the P–N bond of Bzimp(S)A. The presence of bulky benzimidazole also decreased the reactivity of the phosphobenzimidazolide bond. The time course of the polymerization of the diastereomeric mixture of Bzimp(S)A is shown in Fig. 3.

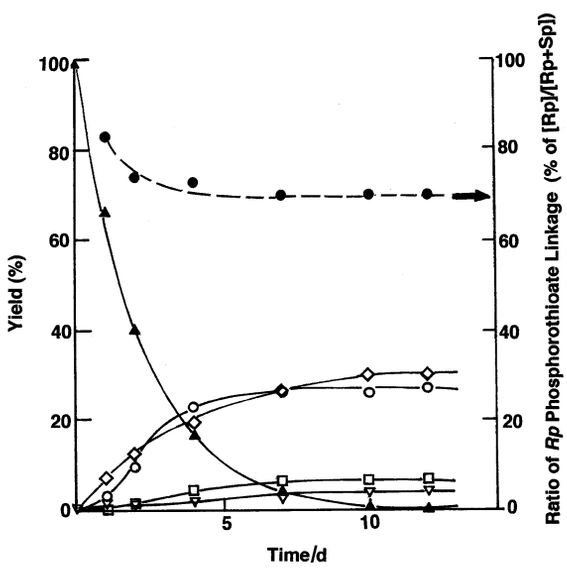


Fig. 3 Time course of the thiophosphate oligoadenylate formation from Bzmp(S)A. Reaction conditions: Imp(S)A, 50 mM; uranyl nitrate, 2mM; 25 °C; pH 7.0. Bzmp(S)A, -▲-; p(S)A, -▽-; 2′-5′ dimer (R_p + S_p), -◇-; 2′-5′ trimers (R_pR_p + R_pS_p + S_pR_p), -○-; 2′-5′ tetramer (all R_p), -□-. Ratio of R_p linkage in the resulting 2′-5′ thiophosphate oligoadenylates, -●-.

More than 10 d was required for the completion of the polymerization reaction, while polymerization of the corresponding Imp(S)A took place in 10 h under the same conditions. 2′-5′ Linked (R_p)-thiophosphate oligoadenylates were formed mainly in the reaction. The R_p to S_p ratio of the thiophosphate linkage of the resulting oligoadenylates was nearly 7 : 3, and (S_p)-Bzimp(S)A disappeared more quickly than (R_p)-Bzimp(S)A during the reaction.

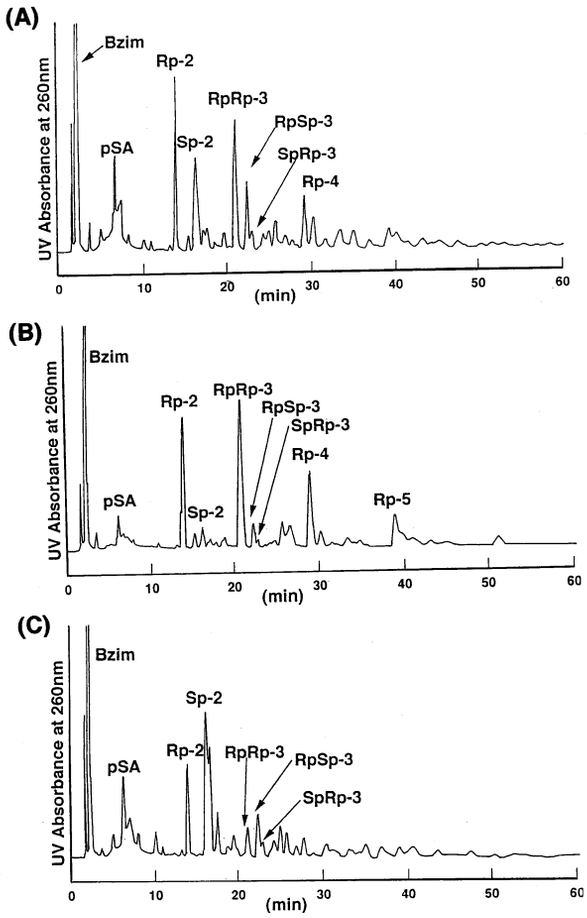
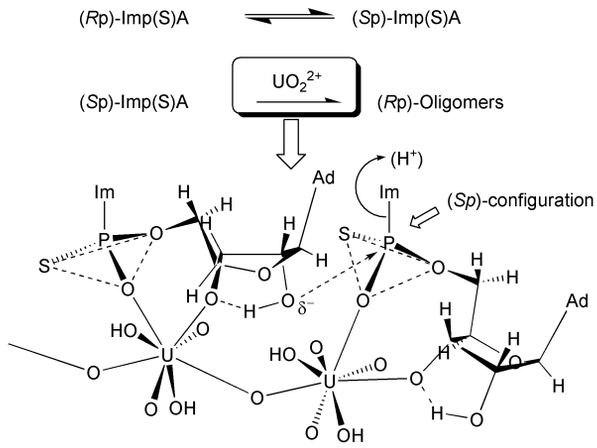


Fig. 4 HPLC profiles of the products from the uranyl-ion catalyzed polymerization of Bzmp(S)A. The reaction was run at 25 °C and pH 7.0 for 10 d. 50 mM of Bzmp(S)A and 2 mM of uranyl nitrate were used for the reaction. (A) Reaction of a diastereomeric mixture of Bzimp(S)A[R_p + S_p]; (B) reaction of (S_p)-Bzimp(S)A; (C) reaction of (R_p)-Bzimp(S)A. Peak assignment is the same as that in the Fig. 1.

Fig. 4 (A), (B) and (C) illustrate the HPLC profiles of the uranyl-ion catalyzed polymerization products from the diastereomeric mixture (S_p + R_p)-, (S_p)- and (R_p)-Bzimp(S)A, respectively, under the same conditions. (S_p)-BzimpA underwent chain-elongation reaction effectively forming oligoadenylates up to hexamer with mainly (R_p) thiophosphate 2′-5′ linkages. Digestion of the reaction mixture with venom phosphodiesterase resulted in the disappearance of these peaks in the HPLC, which indicates that the thiophosphate oligoadenylates were composed of the R_p configuration. The R_p to S_p ratio of the thiophosphate oligoadenylates configuration was 94 : 6. On the other hand, (R_p)-Bzimp(S)A disappeared slowly compared to (S_p)-BzimpA, and di- and triadenylates with mainly (S_p) thiophosphate configuration (4 : 6 of R_p to S_p ratio) were obtained in the reaction. The fact that (S_p)-Bzimp(S)A gave (R_p) thiophosphate oligoadenylates and (R_p)-Bzimp(S)A gave (S_p) thiophosphate oligoadenylates indicates that the uranyl-ion-catalyzed polymerization takes place by a S_N2-type mechanism with inversion of the thiophosphate configuration.

The mechanism of the selective formation of the 2′-5′ linked thiophosphate oligoadenylates with (R_p) configuration is not clear; however, we postulate a possible hypothesis for this regio- and stereocontrolled oligomerization from the available results and the structure of the uranyl-ion–nucleotide complex proposed by Kainosho[26] and Feldman[27] and their co-workers. They reported that the uranyl ion forms a cluster-type complex with adenosine 5′-monophosphate of which the phosphate group and the 3′-hydroxy group coordinate to the UO_2^{2+} ion. The uranyl-ion catalyzed polymerization may take place *via* a similar complex formed from the UO_2^{2+} ion and Imp(S)A as



Scheme 3 Proposed mechanism of the regio- and stereoselectivity of the uranyl-ion catalyzed polymerization.

shown in Scheme 3. The nucleophilicity of the 2′-hydroxy group of Imp(S)A could be enhanced in the coordination state, and the 2′-hydroxy group could attack the thiophosphate group of the adjacently coordinated Imp(S)A forming a 2′-5′ internucleotide linkage with ejection of the imidazole group. ImpA was reported to yield 2′-5′ linked oligoadenylates selectively by the uranyl ion catalyst in a similar way.[28] The thiophosphate group of Imp(S)A could coordinate to the UO_2^{2+} ion *via* the oxygen, but not *via* the sulfur, because oxygen is a stronger electron donor than sulfur toward the uranyl ion. A hard base oxygen anion has a higher affinity for the hard acid uranyl ion than the soft base sulfur anion.[29,30] The orientation of Imp(S)A imposed by the coordination of the thiophosphate oxygen to the UO_2^{2+} ion could control the stereoselectivity of the polymerization as shown in Scheme 3. Both (R_p)- and (S_p)-Imp(S)A form complexes with the UO_2^{2+} ion. The leaving imidazole group may be oriented in the apical position with the incoming 2′-OH group in the complex of (S_p)-Imp(S)A, which favours (R_p)-thiophosphate internucleotide bond formation by an S_N2 mechanism. Both the leaving and incoming groups could not be oriented in the apical position in the case of the (R_p)-Imp(S)A complex. Thus, the stereocontrolled thiophosphate oligoadenylate formation was suppressed by (R_p)-Imp(S)A.

That the diastereomeric mixture of Imp(S)A yielded mainly (R_p)-thiophosphate oligoadenylates can be explained as follows; (S_p)-Imp(S)A polymerizes effectively into a 2′-5′ linked (R_p)-thiophosphate oligoadenylate *via* the UO_2^{2+} ion catalyst, while the corresponding (R_p)-Imp(S)A cannot polymerize efficiently and rather epimerizes into the (S_p)-Imp(S)A which undergoes polymerization as shown in Scheme 3. The epimerization takes place easily in the case of Imp(S)A, but not easily in the case of Bzimp(S)A. The mechanism of the epimerization of Imp(S)A and Bzimp(S)A is unclear. However, we can postulate that a thiometaphosphate is produced by scission of the P–N bond of Imp(S)A as a possible intermediate for the epimerization. Free metaphosphate or thiometaphosphate is proposed as an intermediate for the racemization of the phosphorus in the solvolysis of *p*-nitrophenyl phosphate[31,32] or thiophosphate,[33] respectively. The benzene ring of benzimidazole lowers the basicity of the imidazole nitrogen, and thus stabilizes the P–N bond of the Bzimp(S)A resulting in the suppression of thiometaphosphate formation, which is an intermediate in the epimerization of Bzimp(S)A. Thus, the slow epimerization of Bzimp(S)A suppresses the 2′-5′ linked (R_p)-thiophosphate oligoadenylate formation from its diastereomeric mixture compared with that from the diastereomeric mixture of Imp(S)A.

In conclusion, 5′-thiophosphorylated 2′-5′ linked thiophosphate oligoadenylates with an R_p configuration can be obtained preferentially from Imp(S)A or Bzimp(S)A by the uranyl ion catalyst in aqueous solution without any protecting groups. The 2′-5′ linked (R_p)-thiophosphate oligoadenylate has strong

biological activity[8–12] as 2-5A and the 5′-terminal thiophosphate group provide strong metabolic stability for 2-5A.[19] Thus this reaction provides a simple method for the regio- and stereocontrolled synthesis of biologically active 2-5A analogues.

Experimental

Materials and Method

Adenosine was purchased from Seikagaku Kogyo. Thiophosphoryl trichloride, benzimidazole and imidazole were obtained from Wako Pure Chemicals. Venom phosphodiesterase and alkaline phosphatase were from Worthington Biochemicals and RNase T2 was obtained from Sigma. DEAE-TOYOPEARL 650 M was obtained from Toso Co., and DEAE-Sephadex A-25 from Pharmacia. Other reagents were obtained commercially. Triethyl phosphate and *N*-ethylmorpholine were distilled before use. UV measurements were carried out on a Hitachi 3200 instrument or a Shimadzu UV-1200 spectrophotometer. ^{31}P NMR and ^{1}H NMR spectra were taken on a Bruker AMX 500 or JEOL A500 spectrometer using 85% orthophosphoric acid and HDO, respectively, as standards. ESI-mass spectra were recorded with a Perkin-Elmer Sciex API-100 instrument in negative mode.

HPLC

HPLC was performed with a Hitachi 638 or a JASCO PU880/UV870 apparatus on an RPC-5[34] or an ODS silica gel (Wakosil 5C18) column (40 × 250 mm). HPLC was carried out on an ODS silica gel column using a linear gradient elution of methanol (2.4–51.2%) buffered with 10 mM triethylammonium acetate (pH 7.3) for 60 min with a flow rate of 1.0 ml min^{-1}. HPLC on an RPC-5 column was conducted with a linear gradient of $NaClO_4$ solution (0–70 mM) buffered with 2 mM Tris–acetate buffer (pH 7.5) and 0.1 mM EDTA in 60 min with a flow rate of 1.0 ml min^{-1}.

Preparation of adenosine 5′-thiophosphoimidazolide [Imp(S)A]

To a suspension of dried adenosine (1.60 g, 6.0 mmol) in 30 ml dry triethyl phosphate, thiophosphoryl trichloride (720 µl, 7.2 mmol) was added with stirring in an ice bath. The mixture was stirred for 2 h in an ice bath, then overnight at room temperature. A homogeneous solution of adenosine 5′-dichlorothiophosphate was formed. A solution of imidazole (2.94 g, 43 mmol) in 15 ml dry triethyl phosphate was added to the above solution with stirring in an ice bath, and the mixture was stirred for 20 min giving adenosine 5′-diimidazolothiophosphate. The resulting precipitate of imidazole hydrochloride was removed by filtration with a stream of dry nitrogen. The filtrate was treated with an aqueous pyridine solution (pyridine : water = 30 ml : 139 µl) for 1 h at room temperature. The mixture was poured into a 305 ml solution of acetone–ether–saturated sodium perchlorate in acetone (150 ml : 150 ml : 5 ml) with stirring. The resulting white precipitate was collected on a glass filter with a slow stream of dry nitrogen, and washed with acetone, then with ether. After drying under vacuum for 1 h, Imp(S)A was obtained as a crude sodium salt (1965 mg, 29 900 A_{260}, 32%). The crude Imp(S)A was purified by anion exchange column chromatography on a DEAE-TOYOPEARL 650 M (HCO_3^- form: 30 mm × 450 mm) column. The elution was conducted at 4 °C by a linear gradient [0.3 M (1.6 L)–1.0 M (1.6 L)] of triethylammonium bicarbonate buffer (pH 7.5) at a flow rate of 1.2 ml min^{-1}. The appropriate fractions containing Imp(S)A, which were eluted at approximately 0.45 M of triethylammonium bicarbonate buffer, were collected and lyophilized to give purified Imp(S)A as a triethylammonium salt (15,600 A_{260}, 17% from adenosine). HPLC on an ODS silica gel column showed that Imp(S)A is a 52 : 48 diastereomeric mixture (R_p and S_p). A portion of the diastereomeric mixture of Imp(S)A was separated by HPLC on an ODS silica gel column (10 × 250 mm) with a linear gradient elution of methanol (18.4–30.4%) buffered with 10 mM triethylammonium acetate (pH 7.3) in 30 min with a flow rate of 3.0 ml min^{-1}.

(R_p)-Imp(S)A. Selected ^{1}H NMR (D_2O) δ 8.34 (1H, s, 2-H or 8-H), 8.30 (1H, s, 2-H or 8-H), 7.92 (1H, s, imidazole 2-H), 7.14 (1H, s, imidazole 4-H or 5-H), 6.91 (1H, s, imidazole 4-H or 5-H), 6.15 (1H, d, J = 5.0 Hz, 1′-H).

(S_p)-Imp(S)A. Selected ^{1}H NMR (D_2O) δ 8.26 (1H, s, 2-H or 8-H), 8.23 (1H, s, 2-H or 8-H), 7.92 (1H, s, imidazole 2-H), 7.20 (1H, s, imidazole 4-H or 5-H), 6.93 (1H, s, imidazole 4-H or 5-H), 6.07 (1H, d, J = 4.8 Hz, 1′-H).

Preparation of adenosine 5′-thiophosphobenzimidazolide (Bzimp(S)A)

To a suspension of dried adenosine (267 mg, 1.0 mmol) in 5 ml dry triethyl phosphate, thiophosphoryl trichloride (120 µl, 1.2 mmol) was added with stirring in an ice bath. The mixture was stirred for 2 h in an ice bath then overnight at room temperature. A homogeneous solution was formed with the formation of adenosine 5′-thiophosphoryl dichloride. A solution of benzimidazole (780 mg, 6.6 mmol) in 5 ml dry triethyl phosphate was added to the above solution with stirring in an ice bath, and the mixture was stirred for 30 min giving adenosine 5′-dibenzimidazolothiophosphate. The resulting precipitate of benzimidazole hydrochloride was removed by filtration with a stream of dry nitrogen. The filtrate was treated with 10 ml pyridine containing 23 µl water for 1 h at room temperature. The mixture was poured into a 140.5 ml solution of acetone–ether–saturated sodium perchlorate in acetone (70 ml : 70 ml : 0.5 ml) with stirring. The resulting white precipitate was collected on a glass filter with a slow stream of dry nitrogen, and washed with acetone and then with ether. After drying under vacuum for 1 h, Bzimp(S)A was obtained as a crude sodium salt (259 mg, 34%). The crude Bzimp(S)A was purified by anion exchange column chromatography on a DEAE-Sephadex A-25 (HCO_3^- form: 30 × 500 mm) column. The elution was conducted at 4 °C using a linear gradient [0 M (2.0 L)–1.0 M (2.0 L)] of triethylammonium bicarbonate buffer (pH 7.5) with a flow rate of 1.5 ml min^{-1}. The appropriate fractions containing Bzimp(S)A, which were eluted at approximately 0.65 M of triethylammonium bicarbonate buffer, were collected and lyophilized to give purified Bzimp(S)A as a triethylammonium salt (1050 A_{260}, 7% from adenosine). HPLC on an ODS silica gel column showed that Bzimp(S)A is an R_p and S_p diastereomeric mixture in a 53 : 47 ratio. A portion of the diastereomeric Bzimp(S)A (420 A_{260}) was further separated by HPLC on an ODS silica gel column (10 × 250 mm) giving (R_p)-Bzimp(S)A (54 A_{260}, 92% diastereomeric excess) and (S_p)-Bzimp(S)A (63 A_{260}, 80% diastereomeric excess).

(R_p)-Bzimp(S)A. Selected ^{1}H NMR (D_2O) δ 8.45 (1H, s, benzimidazole 2-H), 8.07 (1H, s, 2-H or 8-H), 8.03 (1H, s, 2-H or 8-H), 7.55 (1H, d, J = 7.5 Hz, benzimidazole 5-H or 8-H), 7.53 (1H, d, J = 7.5 Hz, benzimidazole 5-H or 8-H), 7.13 (1H, t, J = 7.5 Hz, benzimidazole 6-H or 7-H), 6.97 (1H, t, J = 7.5 Hz, benzimidazole 6-H or 7-H), 5.91 (1H, d, J = 5.0 Hz, 1′-H), 4.48 (1H, m, 3′-H), 4.35 (1H, m, 4′-H), 4.16 (1H, m, 5′-H), 4.08 (1H, m. 5″-H).

(S_p)-Bzimp(S)A. Selected ^{1}H NMR (D_2O) δ 8.30 (1H, s, benzimidazole 2-H), 7.97 (1H, s, 2-H or 8-H), 7.94 (1H, s, 2-H or 8-H), 7.51 (1H, d, J = 7.2 Hz, benzimidazole 5-H or 8-H), 7.46 (1H, d, J = 7.2 Hz, benzimidazole 5-H or 8-H), 7.08 (1H, t, J = 7.2 Hz, benzimidazole 6-H or 7-H), 6.93 (1H, t, J = 7.2 Hz, benzimidazole 6-H or 7-H), 5.87 (1H, d, J = 3.8 Hz, 1′-H), 4.52 (1H, m, 3′-H), 4.34 (1H, m, 4′-H), 4.24 (1H, m, 5′-H), 4.15(1H, m, 5″-H).

Hydrolytic stability of Imp(S)A

Diastereomeric Imp(S)A (3.0 A_{260}) was dissolved in 200 μl of 0.2 M *N*-ethylmorpholine buffer (pH 7.0) at a 1 mM concentration and kept at 25 °C. Each aliquot of the solution was taken at appropriate intervals and analyzed by HPLC. The pseudo-first order rate constant of the hydrolysis and half-life of Imp(S)A was estimated from the remaining proportion of Imp(S)A. The hydrolytic stability of ImpA was also measured under the same conditions as that for Imp(S)A for comparison.

Epimerization of (R_p)- and (S_p)-Imp(S)A

The (R_p)- and (S_p)-Imp(S)A were lyophilized immediately after separation by HPLC, and dissolved in 200 μl of 0.2 M *N*-ethylmorpholine buffer (pH 7.0) at a 1 mM concentration and kept at 25 °C. Each aliquot of the solution was taken at appropriate intervals and analyzed by HPLC. The rate constant for epimerization and the half-life of epimerization were estimated from the proportion of each Imp(S)A diastereomer that was analyzed by HPLC.

Polymerization of Imp(S)A or Bzimp(S)A

Reactions were carried out in an Eppendorf tube. The reaction mixture was prepared in an ice bath by addition of compounds in the following order: buffer solution, Imp(S)A solution and uranyl nitrate solution. Samples of the mixture were agitated vigorously and kept at 25 °C for various periods of time. A typical reaction mixture (50 μl) contained Imp(S)A (50 mM) and a catalytic amount of uranyl nitrate (0.5–50 mM) in *N*-ethylmorpholine–HNO_3 buffer (0.2 M, pH 7.0, 7.4 or 7.8) or imidazole–HNO_3 buffer (0.2 M, pH 6.0 and 7.0). The reactions were stopped by adding 10 μl of 0.25 M EDTA solution, and the solutions were analyzed by HPLC on an RPC-5 column.

Polymerizations of (R_p)-, (S_p)- and the diastereomeric mixture (R_p- + S_p-) of Bzimp(S)A were carried out in a 100 μl reaction mixture containing 50 mM Bzimp(S)A, 2 mM uranyl nitrate and 0.2 M *N*-ethylmorpholine buffer (pH 7.0) at 25 °C for 12 d. Aliquots of solution were taken and analyzed by HPLC at various periods of time.

Large-scale syntheses of thiophosphate oligoadenylates by polymerization of Imp(S)A were carried out in a reaction mixture (12.8 ml) containing 50 mM Imp(S)A (9750 A_{260}), 2 mM uranyl nitrate in 0.2 M *N*-ethylmorpholine–HNO_3 buffer (pH 7.0) at 25 °C for 10 d with stirring. The reaction mixture was passed through a Chelex 100 column (Na^+ form, 16 × 150 mm) to remove the uranyl ion, and was subjected to DEAE-Sephadex A-25 anion column chromatography (HCO_3^- form, 30 × 500 mm). The elution was carried out with a linear gradient [0.4 M (2 l)–1.2 M (2 l)] of triethylammonium bicarbonate buffer (pH 7.5) containing 25% methanol with a flow rate of 1.35 ml min^{-1}. The isolated yields of thiophosphate oligoadenylates were as follows: (R_p)-p(S)A2′p(S)5′A (1330 A_{260}, 15.5%), (S_p)-p(S)A2′p(S)5′A (390 A_{260}, 4.6%), (R_pR_p)-p(S)A2′p(S)5′A2′p(S)5′A (1300 A_{260}, 15.2%), (R_pS_p- + S_pR_p)-p(S)A2′p(S)5′A2′p(S)5′A (420 A_{260}, 4.9%), ($R_pR_pR_p$)-p(S)A2′p(S)5′A2′p(S)5′A2′p(S)5′A (421 A_{260}, 4.9%) and other diastereomeric mixtures of thiophosphate tetraadenylates and higher oligoadenylates (620 A_{260}, 7.2%). A portion of diastereomeric mixture of 2′-5′ thiophosphate triadenylates (R_pS_p and S_pR_p) was further purified by HPLC to give (R_pS_p)-p(S)A2′-p(S)5′A2′p(S)5′A (16.8 A_{260}) and (S_pR_p)-p(S)A2′p(S)5′A2′-p(S)5′A (10.0 A_{260}).

Characterizaion of 2′-5′ linked thiophosphate oligoadenylates

Identification of the products was carried out by comparing the HPLC retention times, ^{31}P and ^1H NMR spectra, ESI-mass spectra and enzyme digestions with snake venom phosphodiesterase, RNase T2 and alkaline phosphatase.

Snake venom phosphodiesterase degrades both the 2′-5′ and 3′-5′ linked thiophosphate internucleotide linkages from the 2′(3′) end with R_p configuration but not with (S_p) configuration.[21] Digestion with venom phosphodiesterase was carried out at 37 °C for 2 d in a mixture (100 μl) containing the substrate (0.6 A_{260}), 0.05 units of the enzyme, 0.002 M $MgCl_2$ in 0.02 M Tris–acetate (pH 8.8) buffer. An aliquot of the solution was taken at appropriate intervals, analyzed by HPLC, and the half-life of each oligomer in respect to reaction with the enzyme was estimated.

RNase T2 degrades the 3′-5′ linked thiophosphate oligoadenylates with both R_p and S_p configuration but not the 2′-5′ linkage.[25] The reaction with RNase T2 digestion was carried out at 37 °C for 1 and 2 d in a mixture (100 μl) containing the substrate (0.6 A_{260}), 0.05 unit of the enzyme, 2 mM EDTA and 0.05 M sodium acetate (pH 4.5). The sample was analyzed by HPLC.

(R_p)-p(S)A2′p(S)5′A (R_p-dimer). Selected ^1H NMR (D_2O) δ 8.54 (1H, s, 2-H or 8-H), 8.25(1H, s, 2-H or 8-H), 8.24 (1H, s, 2-H or 8-H), 8.04 (1H, s, 2-H or 8-H), 6.26 (1H, d, J = 4.9 Hz, 1′-H A(1)), 5.93 (1H, d, J = 3.4 Hz, 1′-H A(2)), 5.58 (1H, dd, J = 4.9 Hz, J = 4.9 Hz, 2′-H A(1)), 4.38 (1H, dd, J = 3.4 Hz, J = 4.1 Hz, 2′-H A(2)); ESI-MS *m*/*z* 707.2, 353.1, 235.2. Calcd for $[M - H]^{1-}$ 707.1, $[M - 2H]^{2-}$ 353.0, $[M - 3H]^{3-}$ 235.1; RNase T2 digestion: no digestion detected.

(S_p)-p(S)A2′p(S)5′A (S_p-dimer). Selected ^1H NMR (D_2O) δ 8.61 (1H, s, 2-H or 8-H), 8.27 (1H, s, 2-H or 8-H), 8.21 (1H, s, 2-H or 8-H), 8.09 (1H, s, 2-H or 8-H), 6.28 (1H, d, J = 5.5 Hz, 1′-H A(1)), 5.96 (1H, d, J = 4.0 Hz, 1′-H A(2)), 5.43 (1H, dd, J = 5.5 Hz, J = 4.9 Hz, 2′-H A(1)), 4.43 (1H, dd, J = 4.0 Hz, J = 4.7 Hz, 2′-H A(2)); ESI-MS *m*/*z* 707.2, 353.1, 235.0. Calcd for $[M - H]^{1-}$ 707.1, $[M - 2H]^{2-}$ 353.0, $[M - 3H]^{3-}$ 235.2; RNase T2 digestion: no digestion detected after 48 h.

(R_pR_p)-p(S)A2′p(S)5′A2′p(S)5′A (R_pR_p-trimer). Selected ^1H NMR (D_2O) δ 8.35 (1H, s, 2-H or 8-H), 8.31 (1H, s, 2-H or 8-H), 8.20 (1H, s, 2-H or 8-H), 8.07 (1H, s, 2-H or 8-H), 7.92 (1H, s, 2-H or 8-H), 7.89 (1H, s, 2-H or 8-H), 6.08 (1H, d, J = 3.1 Hz, 1′-H A(1)), 5.99 (1H, d, J = 5.3 Hz, 1′-H A(2)), 5.82 (1H, d, J = 3.8 Hz, 1′-H A(3)), 5.33 (1H, dd, J = 3.1 Hz, J = 5.9 Hz, 2′-H A(1)), 5.27 (1H, dd, J = 5.3 Hz, J = 5.6 Hz, 2′-H A(2)), 4.27 (1H, dd, J = 3.8 Hz, J = 4.3 Hz, 2′-H A(3)); ESI-MS *m*/*z* 1051.9, 525.5, 350.0. Calcd for $[M - H]^{1-}$ 1052.1, $[M - 2H]^{2-}$ 525.4, $[M - 3H]^{3-}$ 350.0; RNase T2 digestion: no digestion detected after 48 h.

(R_pS_p)-p(S)A2′p(S)5′A2′p(S)5′A (R_pS_p-trimer). Selected ^1H NMR (D_2O) δ 8.35 (1H, s, 2-H or 8-H), 8.33 (1H, s, 2-H or 8-H), 8.16 (1H, s, 2-H or 8-H), 8.10 (1H, s, 2-H or 8-H), 8.01 (1H, s, 2-H or 8-H), 7.90 (1H, s, 2-H or 8-H), 6.09 (1H, d, J = 3.0 Hz, 1′-H A(1)), 5.97 (1H, d, J = 5.1 Hz, 1′-H A(2)), 5.86 (1H, d, J = 4.6 Hz, 1′-H A(3)), 5.38 (1H, dd, J = 3.0 Hz, J = 6.1 Hz, 2′-H A(1)), 5.04 (1H, dd, J = 5.1 Hz, J = 4.2 Hz, 2′-H A(2)), 4.34 (1H, dd, J = 4.6 Hz, J = 4.6 Hz, 2′-H A(3)); ESI-MS *m*/*z* 1052.0, 525.6, 350.0. Calcd for $[M - H]^{1-}$ 1052.1, $[M - 2H]^{2-}$ 525.4, $[M - 3H]^{3-}$ 350.0; RNase T2 digestion: no digestion detected after 48 h.

(S_pR_p)-p(S)A2′p(S)5′A2′p(S)5′A (S_pR_p-trimer). Selected ^1H NMR (D_2O) δ 3.36 (1H, s, 2-H or 8-H), 8.24 (1H, s, 2-H or 8-H), 8.17 (1H, s, 2-H or 8-H), 8.05 (1H, s, 2-H or 8-H), 7.99 (1H, s, 2-H or 8-H), 7.91 (1H, s, 2-H or 8-H), 6.16 (1H, d, J = 2.2 Hz, 1′-H A(1)), 6.02 (1H, d, J = 5.0 Hz, 1′-H A(2)), 5.83 (1H, d, J = 3.7 Hz, 1′-H A(3)), 5.13 (1H, dd, J = 2.2 Hz, J = 5.8 Hz, 2′-H A(1)), 5.28 (1H, dd, J = 5.0 Hz, J = 4.0 Hz, 2′-H A(2)), 4.24 (1H, dd, J = 3.7 Hz, J = 4.0 Hz, 2′-H A(3)); ESI-MS *m*/*z* 1051.9, 525.5, 350.0. Calcd for $[M - H]^{1-}$ 1052.1, $[M - H]^{2-}$ 525.4, $[M - 3H]^{3-}$ 350.0; RNase T2 digestion: no digestion detected after 48 h.

($R_pR_pR_p$)-p(S)A2′p(S)5′A2′p(S)5′A2′p(S)A ($R_pR_pR_p$-tetramer). Selected ^{1}H NMR (D_2O) δ 8.29 (1H, s, 2-H or 8-H), 8.24 (1H, s, 2-H or 8-H), 8.18 (1H, s, 2-H or 8-H), 8.09 (1H, s, 2-H or 8-H), 8.05 (1H, s, 2-H or 8-H), 7.91 (1H, s, 2-H or 8-H), 7.09 (1H, s, 2-H or 8-H), 7.85 (1H, s, 2-H or 8-H), 6.08 (1H, d, J = 3.5 Hz, 1′-H A(1)), 5.95 (1H, d, J = 5.2 Hz, 1′-H A(2)), 5.86 (1H, d, J = 3.3 Hz, 1′-H A(3)), 5.81 (1H, d, J = 4.2 Hz, 1′-H A(4)), 5.36 (1H, dd, J = 3.5 Hz, J = 5.8 Hz, 2′-H A(1)), 5.28 (1H, dd, J = 5.2 Hz, J = 4.5 Hz, 2′-H A(2)), 4.96 (1H, dd, J = 3.3 Hz, J = 5.5 Hz, 2′-H A(3)), 4.25 (1H, dd, J = 4.2 Hz, J = 4.0 Hz, 2′-H A(4)); ESI-MS *m/z* 698.2, 465.1, 348.6. Calcd for $[M - 2H]^{2-}$ 698.1, $[M - 3H]^{3-}$ 465.0, $[M - 4H]^{4-}$ 348.5; RNase T2 digestion: no digestion detected after 48 h.

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