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Diastereomers of 5′-*O*-Adenosyl 3′-*O*-Uridyl Phosphorothioate: Chemical Synthesis and Enzymatic Properties†

Peter M. J. Burgers and Fritz Eckstein*

ABSTRACT: A procedure is described for the synthesis of the title compounds via phosphotriester intermediates. The 2-cyanoethyl group is used to protect the P–SH function during the course of the synthesis. Resolution of the phosphorus diastereomers is accomplished at the phosphotriester stage. Removal of the 2-cyanoethyl group without racemization, followed by removal of the other protective groups, affords the optically pure diastereomers of 5′-*O*-adenosyl 3′-*O*-uridyl phosphorothioate. Their designation as *Rp* and *Sp* follows from the stereospecificity in the hydrolysis catalyzed by RNase A. These diastereomers are useful for the investigation of the stereospecificity as well as of the stereochemical course of action of nucleases. Snake venom exonuclease hydrolyses only the *Rp* diastereomer, whereas both diastereomers are substrates for RNases A and T_2. The results with the latter indicate that RNase T_2 also operates by an in-line mechanism.

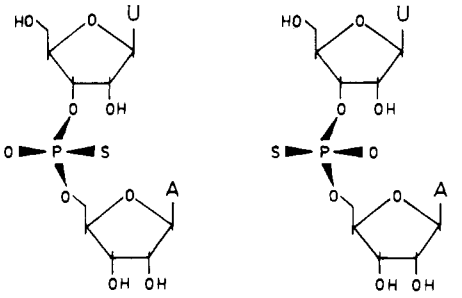
Diastereomeric phosphorothioate analogues of nucleotides are important tools for the elucidation of the stereochemistry of action of different classes of enzymes. For instance, the stereochemical course of action of RNase A has been established by its reaction with the diastereomers of cyclic uridine 2′,3′-*O*,*O*-phosphorothioate (Eckstein, 1975).

To obtain a more complete insight into enzyme–substrate interactions of exo- and endonucleases in general, one needs the two diastereomeric phosphorothioate analogues of an appropriate diribonucleoside monophosphate in their optically pure form. In addition, the study of these diastereomers by CD and NMR spectroscopy could give important information about the influence of the phosphodiester function on the conformation of dinucleoside monophosphates in general and on intramolecular base-stacking interactions in particular.

In this publication we describe the synthesis and separation of the diastereomers of 5′-*O*-adenosyl 3′-*O*-uridyl phosphorothioate [Up(S)A][1] and their enzymatic hydrolysis by the ribonucleases A and T_2 and by the exonucleolytic phosphodiesterase from snake venom. An alternative method of preparation of Up(S)A did not allow the separation of the diastereomers at any stage of the synthesis (Burgers & Eckstein, 1978a).

General Methods and Materials

Thin-layer chromatography was performed on Merck Kieselgel 60 F_{254} plates in system A ($CHCl_3$–MeOH, 92:8 v/v) and on Merck DC Alufolien Cellulose F_{254} plates in system B (1 M NH_4OAc–EtOH, 3:7 v/v). Merck Kieselgel 60H was used for column chromatography.



Up(S)A (R_p) Up(S)A (S_p)

A Packard-Becker 8200 chromatograph, equipped with a Packard 1170 UV detector operating at 254 nm and a Servogor RE 511 recorder, was employed for the high-performance liquid chromatography. The strong anion-exchanger Nucleosil 10 SB from Macherey & Nagel was stirred with three lots of 0.5 M NH_4OAc (pH 4.5), decanted to remove the fine particles and then packed into a stainless steel column (40 cm × 2 mm) according to the slurry method. Isocratic elution of the column was performed with a varying range of buffers, all containing 0.05 M KH_2PO_4 and 0.2–0.5 M KCl at pH 4.5. The flow rate was 1.0 mL/min at pressures of 160–220 atm.

Ultraviolet absorption spectra were measured on a Shimadzu UV-200 spectrophotometer. Kinetic measurements were carried out using a Zeiss PMQ II spectrophotometer equipped with an automatic cuvette changer.

The ^1H and ^{31}P nuclear magnetic resonance spectra were recorded with a Bruker-Physics HFX 60 spectrometer

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[1] Abbreviations used: Up(S)A, 5′-*O*-adenosyl 3′-*O*-uridyl phosphorothioate; *Rp* and *Sp*, diastereomers of Up(S)A; U>pS, cyclic uridine 2′,3′-*O*,*O*-phosphorothioate; LC, high-performance liquid chromatography.

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equipped with a Bruker-Data-System B-NC12 Fourier transform unit. Chemical shifts are given in ppm downfield from tetramethylsilane as internal standard for ^1H spectra and 85% aqueous phosphoric acid as external standard for ^{31}P spectra.

2,4,6-Triisopropylbenzenesulfonyl chloride was obtained from Aldrich-Europe (Belgium). 5′-*O*-*p*-Chlorophenoxyacetyl-2′-*O*-methoxytetrahydropyranyluridine was prepared by the method of van Boom et al. (1971). 2′,3′-Isopropylideneadenosine (Waldhof, Germany) was N-benzoylated according to the method of Griffin et al. (1967). Uridylyl-(3′–5′)adenosine was a product from Boehringer, Mannheim (Germany).

Snake venom phosphodiesterase (*Crotalus terr. terr.*; 1 mg/mL, 1.5 U/mg) and pancreatic ribonuclease A (50 U/mg) were purchased from Boehringer, Mannheim (Germany). Ribonuclease T_2 (1000 U/mg) was a product from Sigma, St. Louis, MO.

Chemical Syntheses

S-2-Cyanoethyl Phosphorothioate. A solution of trisodium phosphorothioate dodecahydrate (5 g) in water (50 mL) was cooled to 0 °C and acrylonitrile (5 mL) added with vigorous stirring. After 1 h at 0 °C, ethanol (50 mL) was added and the mixture was set aside at 0 °C for 10 min. The precipitate formed was then filtered off and more ethanol (250 mL) was added to the filtrate. After 1 h at 0 °C the crystals of disodium *S*-2-cyanoethyl phosphorothioate (1.65 g, 62%) were filtered off, washed with ethanol (50 mL) and ether (50 mL) and dried in vacuo over P_2O_5 at 25 °C. Anal. Calcd for $C_3H_4NNa_2O_3PS$ (211.09): C, 17.07; H, 1.91; P, 14.67; S, 15.19. Found: C, 17.10; H, 2.20; P, 14.57; S, 15.04. ^{31}P NMR (D_2O): 15.5 ppm. The product is unstable at room temperature and should be stored at −20 °C. About 2% decomposition is observed, by ^{31}P NMR, after 6 months at −20 °C.

3′-O-[5′-O-p-Chlorophenoxyacetyl-2′-O-methoxytetrahydropyranyluridyl] 5′-O-[N⁶-Benzoyl-2′,3′-O-isopropylideneadenosyl]phosphorothioate S-2-Cyanoethyl Ester (**3a**). Anhydrous pyridine (10 mL) was added to a dried mixture of 5′-*O*-*p*-chlorophenoxyacetyl-2′-*O*-methoxytetrahydropyranyluridine (**1**, 526 mg, 1 mmol), disodium *S*-2-cyanoethyl phosphorothioate (316 mg, 1.5 mmol), 2,4,6-triisopropylbenzenesulfonyl chloride (1.35 g, 4.5 mmol), and Dowex 50-WX8 (50–100 mesh, H^+ form, 3 g). The mixture was stirred in the dark at 20 °C. After 5 h TLC (system A) indicated that all starting nucleosidic material had reacted and N^6-benzoyl-2′,3′-*O*-isopropylideneadenosine (**2**, 945 mg, 2.3 mmol) was added. After another 12 h, when TLC (system A) showed that no nucleotidic material remained at the origin, the resin was filtered off and washed with pyridine (5 mL) and ethanol (2 × 20 mL). The filtrate was concentrated to an oil. A solution of the oil in chloroform (100 mL) was washed with 5% aqueous sodium bicarbonate (100 mL) followed by water (100 mL). The dried ($MgSO_4$) organic layer was concentrated to an oil which was triturated with petroleum ether (40–60 °C, 200 mL). The precipitate was redissolved in chloroform and applied to a column (12 × 4.5 cm) of Kieselgel H (100 g), equilibrated in $CHCl_3$–MeOH–AcOH (96:4:0.05, v/v). The column was eluted with the same solvent (500 mL). The appropriate fractions were collected, 10% v/v of toluene was added, and the mixture was concentrated to dryness. A solution of the residue in chloroform (10 mL) was added with stirring to petroleum ether (40–60 °C, 100 mL). The precipitate of **3a** was filtered off and dried in vacuo (P_2O_5): yield 710 mg (66%); R_f (system A) 0.39; UV (95% EtOH) λ_{max} 275, 262 (ε 25 600, 24 000), λ_{min} 265, 239 nm (ε 23 900, 17 900).

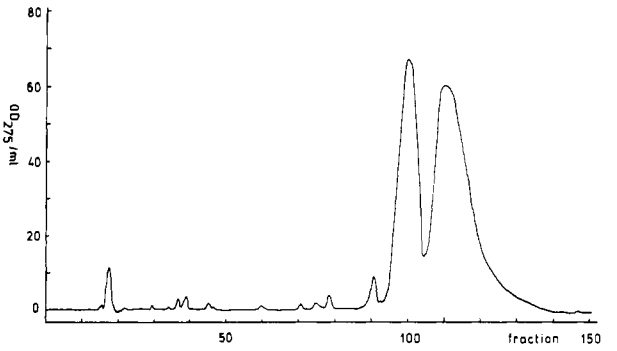


FIGURE 1: Separation of the diastereomers of **3b** by silica gel chromatography (for details see Chemical Syntheses).

Partial Deblocking of **3a** *to* **3b** *and Separation of Diastereomers.* Aqueous ammonia (5%, 20 mL) was added to a solution of the fully protected dinucleoside monophosphorothioate **3a** (650 mg, 0.61 mmol) in dioxane (20 mL). After 10 min at 20 °C the solution was flash-evaporated and the residue partitioned between chloroform (100 mL) and water (100 mL). The organic layer was dried over $MgSO_4$ and concentrated to dryness. A solution of this material in chloroform (1 mL) was applied to a column (28 × 2.5 cm) of Kieselgel H (100 g), equilibrated in $CHCl_3$–EtOH–AcOH (94:6:0.05 v/v). The column was eluted with the same solvent under a pressure of 1.2 atm. Fractions of 10 mL were collected. Aliquots of the fractions were diluted 50-fold with methanol and their absorption at 275 nm was measured. The UV tracing of the column elution is given in Figure 1. The appropriate fractions were collected and, after addition of 10% v/v of toluene, concentrated to dryness. The residue was redissolved in chloroform (5 mL) and added with stirring to petroleum ether (40–60 °C, 100 mL). The precipitated material was filtered off and dried in vacuo (P_2O_5). Fractions 95–103: high R_f isomer of **3b** (152 mg, 28%); R_f (system A) 0.27; UV (95% EtOH) λ_{max} 275, 262 (ε 26 300, 25 200), λ_{min} 265, 241 nm (ε 25 000, 18 300); ^{31}P NMR ($CDCl_3$) 26.2 ppm. Fractions 104–107: mixture of isomers (88 mg, 16%). Fractions 108–120: low R_f isomer of **3b** (219 mg, 40%); R_f (system A) 0.25; UV (95% EtOH) λ_{max} 275, 262 (ε 26 800, 25 600), λ_{min} 265, 241 nm (ε 25 300, 18 400); ^{31}P NMR ($CDCl_3$) 27.3 ppm.

Up(S)A, Rp and Sp Isomers. A solution of the high R_f diastereomer of **3b** (45 mg, 0.05 mmol), in dioxane (3.2 mL), triethylamine (4 mL), and water (0.8 mL), was incubated at 50 °C for 2 h. The solution was concentrated to dryness and redissolved in 15% aqueous ammonia and the sealed flask incubated for another 3 h at 50 °C. The solution was then again concentrated and the residue dissolved in dioxane (1 mL) and 0.1 N HCl (5 mL). The pH was brought to 1.0 with 1 N HCl. After 12 h at 20 °C the solution was neutralized with dilute ammonia and the product chromatographed over a column (14 × 1.3 cm) of DEAE-Sephadex A-25. Elution was performed with a linear gradient of 500 mL each of 0.02 M and 0.15 M triethylammonium bicarbonate. Fractions of 10 mL were collected. Fractions 17–27 (290 A_{260} units, 24%) contained UpA. Fractions 35–55 (760 A_{260} units, 63%) contained Up(S)A. Reaction with RNAse A showed this isomer to be the *Sp* diastereomer. Analytical details are given in Table I.

The low R_f diastereomer of **3b** was deprotected in exactly the same way to yield after chromatography over DEAE-Sephadex 160 A_{260} units (13%) of UpA and 820 A_{260} units

Table I: Analytical Data of UpA and Up(S)A

	R_f[a]	R_T (min)[b]	$\delta^{31}P$ (ppm)	k ($\times 10^{-4}$ s^{-1})[c]	h (%)[d]
UpA	0.38	6.2	−1	3.7 (±0.2)	4.2 (±0.3)
Up(S)A, *Rp*	0.46	14.1	56.1	2.8 (±0.2)	5.1 (±0.3)
Up(S)A, *Sp*	0.46	16.4	55.5	3.5 (±0.2)	3.7 (±0.3)

[a] On cellulose plates in system B. [b] LC retention times in 0.05 M KH_2PO_4, 0.25 M KCl, pH 4.5. [c] In 0.5 M KOH at 37 °C. [d] Hypochromicity determined by alkaline degradation.

(65%) of Up(S)A. The latter was ascertained to be the *Rp* isomer by its stereospecific hydrolysis by RNase A. Analytical details are given in Table I.

Enzymatic Procedures

Ribonuclease A. Michaelis–Menten constants at 25 °C were obtained according to Follmann et al. (1967). The enzymic digestion of 1 mM solutions of Up(S)A (*Rp* and *Sp*) was also followed by LC and the ratio U>pS:A after complete hydrolysis of the dinucleotide measured. For Up(S)A (*Rp*), U>pS (endo): A = 0.98:1.00. For Up(S)A (Sp), U>pS (exo): A = 0.95:1.00.

Ribonuclease T_2. The kinetic constants were measured at 25 °C according to the method of Imazawa et al. (1968). Quantitation by LC of the digestion products after complete hydrolysis of 1 mM solutions of the dinucleotides gave the following ratios. For Up(S)A (*Rp*), U>pS (endo): A = 1.00:1.00. For Up(S)A (Sp), U>pS (exo): A = 1.03:1.00.

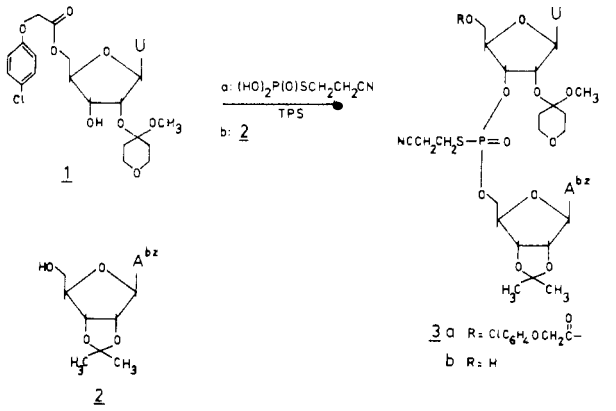
Snake Venom Phosphodiesterase. Hydrolysis of UpA and Up(S)A (*Rp*) was carried out at 37 °C in a system containing in a total volume of 50 μL, 200 mM Tris-HCl, pH 8.75, 2 mM $Mg(OAc)_2$, 0.1–0.6 mM dinucleotide, and 0.031 μg of enzyme for UpA and 0.5 μg of enzyme for Up(S)A (*Rp*). At appropriate times 10-μL aliquots were quenched with 2 μL of ice-cold 1 M acetic acid and analyzed by LC. Reaction rates were linear up to 20% hydrolysis. During the initial stage of the hydrolysis of Up(S)A (*Rp*), uridine and adenosine 5′-*O*-phosphorothioate were formed in the ratio 1.00:0.95. Desulfurization of adenosine 5′-*O*-phosphorothioate to adenosine 5′-phosphate became apparent after ca. 30% hydrolysis. The K_m and V_{max} values obtained from Lineweaver–Burk plots are given in Table II. Inhibition experiments with snake venom phosphodiesterase were carried out by an optical procedure, monitoring the rate of hydrolysis of thymidine 5′-*O*-*p*-nitrophenyl phosphate by the formation of *p*-nitrophenolate at 405 nm at a temperature of 25 °C (Figure 2).

Results

The possibility of thiophosphorylating nucleosides with *S*-2-cyanoethyl phosphorothioate has been mentioned in the literature but has not been pursued further because the starting material, *S*-2-cyanoethyl phosphorothioate, could not be obtained (Cook, 1970). We have achieved the synthesis of this material by the reaction of acrylonitrile with trisodium phosphorothioate. Although the reaction only went to 70% completion as indicated by ^{31}P NMR and phosphate was formed as a side product, the desired product could be obtained in pure form by stepwise crystallization. Phosphate and thiophosphate were quantitatively precipitated from 50% aqueous ethanol, while *S*-2-cyanoethyl phosphorothioate crystallized from 90% aqueous ethanol.

Coupling of the 3′-hydroxy nucleoside **1** (van Boom et al., 1971) with disodium *S*-2-cyanoethyl phosphorothioate activated by 2,4,6-triisopropylbenzenesulfonyl chloride (Lohrmann & Khorana, 1966), followed by condensation of the intermediate with the 5′-hydroxy nucleoside **2**, gave the fully protected dinucleoside monophosphorothioate **3a** as an approximately 1:1 mixture of diastereomers in good yield (Scheme I). Addition of excess dry Dowex cation exchanger (H^+ form) to the reaction mixture in pyridine solution effectively brought about the necessary exchange of sodium for pyridinium ion. After removal of the 5′-protecting group of **3a** by brief treatment with base, the diastereomers of **3b** were efficiently separated by chromatography on Kieselgel H (Figure 1).

Scheme I



Alkaline deblocking of **3b** was performed optimally with dioxane–triethylamine–water. Under these conditions removal of 71% of the 2-cyanoethyl group from the high R_f diastereomer of **3b** occurred via an elimination mechanism leaving the phosphorothioate moiety intact while the remaining 29% suffered direct nucleophilic attack at phosphorus resulting ultimately in the dinucleoside monophosphate UpA. For the low R_f diastereomer of **3b**, this ratio was 84:16.

After deblocking of the phosphorothioate function, the *N*-benzoyl protective group was removed by treatment with ammonia. Finally, treatment with 0.1 N HCl at pH 1.0 for 12 h removed the remaining acid-labile groups. Although it is known that under these strongly acidic conditions, which are necessary for the complete removal of the isopropylidene group, natural dinucleoside monophosphates isomerize partially to give a 2′-5′ internucleotide linkage (Smith et al., 1962), such an isomerization was not observed for the phosphorothioate analogues. When Up(S)A was allowed to stand in 0.1 N HCl at pH 1.0 for 48 h and was subsequently treated with RNase A, analysis by LC showed that no undigested material remained (results not described in experimental part). This indicates that phosphorothioate analogues of dinucleoside monophosphates are much more stable in acid than the parent compounds.

After complete deblocking, the diastereomers of Up(S)A were purified by DEAE-Sephadex chromatography. From ^{31}P NMR spectroscopy and LC, it followed that their chemical and optical purity was greater than 98%.

The absolute configuration of the diastereomers of Up(S)A was established by studying the hydrolysis of these compounds catalyzed by RNase A. It is known that transesterification of a dinucleoside monophosphorothioate in the *Rp* configuration by RNase A yields the endo configuration of the cyclic nucleoside 2′,3′-*O*,*O*-phosphorothioate (Usher et al., 1972; Saenger et al., 1974). Analogously, the diastereomer of Up(S)A resulting from deprotection of the high R_f diastereomer of **3b** was converted upon treatment with RNase A into the exo isomer of cyclic uridine 2′,3′-*O*,*O*-phosphorothioate (U>pS) and adenosine. This demonstrates that this dia-

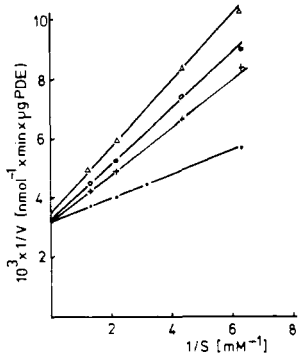


FIGURE 2: Lineweaver–Burk plot of hydrolysis of thymidine 5′-*p*-nitrophenyl phosphate by snake venom phosphodiesterase with dinucleotides as inhibitors. All solutions contained 200 mM Tris-HCl, pH 8.75, 2 mM $Mg(OAc)_2$, and 0.050 µg of PDE/mL: (●) no inhibitor; (○) 0.192 mM UpA; (+) 0.187 mM Up(S)A, *R*p; (△) 0.375 mM Up(S)A, *S*p.

stereomer of Up(S)A has the *S*p configuration at phosphorus. Similarly, RNase treatment of the other diastereomer of Up(S)A gave the endo isomer of U>pS and adenosine, showing that the low R_f diastereomer of **3b** had the *R* configuration. Identification of the diastereomers of U>pS was routinely carried out by LC (Burgers & Eckstein, 1978b).

The diastereomers of Up(S)A were almost as labile to alkali as UpA (Table II). LC showed that the endo isomer of U>pS was a transient product of the saponification of Up(S) A (*R*p), while the exo isomer was formed from Up(S)A (*S*p).

Both diastereomers of Up(S)A were equally well hydrolyzed by RNase T_2, while RNase A showed a definite preference for Up(S)A *R*p. LC established that Up(S)A (*R*p) was hydrolyzed by RNase T_2 to give U>pS (endo) and adenosine and Up(S)A (*S*p) to U>pS (exo) and adenosine. Only the *R*p diastereomer of Up(S)A was a substrate for snake venom phosphodiesterase. For RNase A and RNase T_2 nearly equal K_M values were found for UpA and the two diastereomers of Up(S)A. In the case of snake venom phosphodiesterase the diastereomers of Up(S)A as well as UpA were competitive inhibitors of the hydrolysis of thymidine 5′-*O*-*p*-nitrophenyl phosphate (Figure 2). Their K_i values, obtained with two different concentrations of inhibitor (0.2 and 0.4 mM), were comparable (Table II).

Discussion

The elucidation of the stereochemical course of action of RNase A with the use of cyclic uridine 2′,3′-*O*,*O*-phosphorothioate (Eckstein, 1975) and of *E. coli* DNA dependent RNA polymerase with the help of adenosine 5′-*O*-(1-thiotriphosphate) (Eckstein et al., 1976; Burgers & Eckstein, 1978b) has shown that nucleoside phosphorothioates are powerful tools for the investigation of enzyme mechanisms. To extend this type of study to the general class of nucleases, it is important to have the diastereomers of a simple phosphorothioate substrate analogue available. We have chosen as such a compound the phosphorothioate analogue of UpA mainly because it would facilitate the stereochemical assignment of the diastereomers on the basis of earlier work with RNase A and cyclic uridine 2′,3′-*O*,*O*-phosphorothioate.

It has been known for some time that the phosphorus diastereomers of dinucleoside monophosphate esters (triesters) with all 2′- and 3′-hydroxy functions protected show a large difference in chromatographic mobility on silica gel (Werstiuk & Neilson, 1972; van Boom et al., 1976). In combination with a method of removal of the protective group from the third acidic phosphorothioate function without racemization at phosphorus, this property should enable one to prepare pure diastereomers of dinucleoside monophosphorothioates. Protection of the phosphorothioate by way of a *S*-2-cyanoethyl function seemed to be the method of choice because removal of this protective group proceeds via a β elimination which does not involve attack at the phosphorus atom.

Table II: Enzymatic Degradation of UpA and Up(S)A

enzyme	UpA	Up(S)A, *R*p	Up(S)A, *S*p
RNase A			
K_M (mM)	1.1 (±0.1)	1.7 (±0.1)	1.6 (±0.1)
V_{max} (s^{-1})	770 (±30)	320 (±20)	11 (±1)
RNase T_2			
K_M (mM)	0.078 (±0.01)	0.13 (±0.02)	0.13 (±0.02)
V_{max} (s^{-1})	450 (±20)	220 (±10)	200 (±10)
snake venom phosphodiesterase			
K_M (mM)	0.16 (±0.02)	0.30 (±0.02)	
K_i (mM)	0.13 (±0.02)	0.17 (±0.03)	0.19 (±0.03)
V_{max}[a]	18 500 (±900)	170 (±10)	0.1[b]

[a] In nmol (mg of protein^{-1}) min^{-1}. [b] Rate of hydrolysis of a 1 mM substrate solution.

Synthesis of the fully protected dinucleoside monophosphorothioate **3a**, using *S*-2-cyanoethyl phosphorothioate as bifunctional phosphate donor and triisopropylbenzenesulfonyl chloride as the activating agent, followed by selective removal of the 5′-protecting group afforded **3b** as a mixture of phosphorus diastereomers. Although these diastereomers were only partially separated by TLC, they were almost completely separated by chromatography over a long column of Kieselgel H (Figure 1).

Alkaline deblocking of **3b** yielded a mixture of Up(S)A and UpA. The formation of the latter product is probably due to direct nucleophilic attack of the base on phosphorus involving 2-cyanoethylmercaptan as the leaving group. Before a survey of the enzymatic properties of the diastereomers of Up(S)A was undertaken, this contamination of UpA was thoroughly removed by DEAE-Sephadex chromatography.

The kinetic constants for the hydrolysis of the diastereomers by the different endo- and exonucleases are collected in Table II. A comparison of the V_{max} values of UpA with its phosphorothioate analogues immediately reveals the high reactivity of the *R*p and *S*p isomers with RNase T_2 and of the *R*p isomer with RNase A. This high reactivity is also reflected in the alkaline degradation of these compounds (Table I). From earlier studies it is known that alkali as well as RNase A hydrolyze the phosphodiester bond of a diribonucleoside monophosphate via an in-line mechanism (Usher et al., 1972). For instance, Up(S)A (*R*p) is transesterified to U>pS (endo) and adenosine. The present study with RNase T_2 demonstrates that this enzyme follows the same stereochemical course.

The virtual identity of the V_{max} as well as K_m values for the diastereomers of Up(S)A with RNase T_2 indicates that the substitution of oxygen by sulfur in the substrate does not seem to interfere with the optimal spatial arrangement of the reacting system. If it did one would expect different V_{max} values for the two diastereomers as is actually observed with RNase A.

The hydrolysis of the diastereomers of Up(S)A by snake venom phosphodiesterase has already been the subject of a recent study (Burgers & Eckstein, 1978b). The kinetic experiments for that publication were performed on a mixture of the diastereomers in the presence of dithiothreitol. Although addition of this reagent stimulates the enzymatic hydrolysis of the phosphorothioates, it was omitted in this study since it slowly inactivates the enzyme and makes it difficult to obtain

linear kinetics. Both studies show that the *Rp* isomer is by far the preferred substrate for the snake venom exonuclease. It cannot be excluded that the small activity observed with the *Sp* isomer is due to a contamination by the *Rp* isomer. The fact that the *Sp* isomer is such a poor substrate is not due to a lack of binding. Both isomers are competitive inhibitors for the hydrolysis of thymidine 5'-*O*-*p*-nitrophenyl phosphate and have similar K_i values. Analogous results were found for the hydrolysis of the diastereomers of adenosine 5'-*O*-(1-thiotriphosphate) by snake venom phosphodiesterase (Burgers & Eckstein, 1978b). The formation of an abortive complex of the *Sp* diastereomer with the enzyme suggests that the functional group or groups at the active site involved in the catalysis of hydrolysis cannot function when one particular oxygen is replaced by sulfur. It is conceivable that protonation of a phosphate oxygen which might be necessary for hydrolysis becomes impossible or difficult when this oxygen is replaced by sulfur in the *Sp* diastereomer of the phosphorothioate.

Our data on the hydrolysis of Up(S)A by snake venom phosphodiesterase establish the stereoselectivity of this enzyme. To arrive at the stereochemistry of enzymatic hydrolysis, experiments with $H_2{}^{18}O$ will have to be performed. For such studies these compounds will also be suitable.

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Role of Histone H1 in the Conformation of Oligonucleosomes as a Function of Ionic Strength†

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ABSTRACT: The role of histone H1 with respect to the conformation of oligonucleosomes and the dependence on ionic strength was investigated by studying two functions: (1) compaction of single oligonucleosomal chains; (2) packaging of several nucleosomal chains into composite particles. The first function was examined by sedimenting homogeneous mono- to hexanucleosomes in isokinetic sucrose gradients containing various NaCl concentrations. Homogeneous oligonucleosomes were prepared from mildly digested rat liver nuclei using a NaCl concentration of 170 mM being present during nuclear lysis and sedimentation in isokinetic sucrose gradients. The high resolution obtained at this salt concentration results from the dissociation of composite particles into single nucleosomal chains. The second function was studied by analyzing the salt-dependent chain length heterogeneity of trimers and tetramers obtained by centrifugation in isokinetic sucrose gradients. The results show that, depending on the NaCl concentration, oligonucleosomes can exist in four discrete conformations. First, at very low ionic strength, oligonucleosomal chains exhibit an extended conformation and packaging of different chains into composite particles is negligible. Second, at about 5–20 mM NaCl, oligonucleosomes undergo a cooperative transition to a compact conformation. Above the transition, H1 mediates packaging of different nucleosomal chains into composite particles. Third, at 150 mM NaCl, single oligonucleosomes occur in a conformation slightly more compact than at 50 mM NaCl. At this salt concentration, however, packaging of several nucleosomal chains into composite particles is not observed. Fourth, above 200 mM NaCl, oligonucleosomes exhibit an extended conformation.

Nucleosomes are now recognized as the basic structural entities of chromatin. They are built by four of the five major histone classes forming a histone core around which the DNA helix is wrapped. Histone H1 appears not to be a structural component of the nucleosomal protein core, but to be located in the internucleosomal space (Varshavsky et al., 1976; Noll & Kornberg, 1977). Furthermore, histone H1 seems to specify the length of the internucleosomal DNA segment (Morris, 1976; Lipps & Morris, 1977; Spadafora & Geraci, 1975; Spadafora et al., 1976). In a number of tissues the nucleosomal repeat length was related to the size and the basicity of histone H1 (and histone H5). Studies on the structure of H1 under physiological salt conditions have revealed that a central region of H1 assumes a globular structure while the ends form unstructured tails (Hartman et al., 1977). These

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