The First Stereocontrolled Solid-Phase Synthesis of Di-, Tri-, and Tetra[adenosine (2′,5′) phosphorothioate]s

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Oligoadenylates with a 2′,5′-linkage are generated in mammalian cells in response to viral infection.[1] During the past two decades several analogues of 2′,5′-polyadenylates were synthesized and used as effective tools in studies of biological function of oligoadenylate (2′,5′) phosphates.[2] Phosphorothioate analogues are of special interest due to their expected enhanced stability against cellular endo- and exonucleases.[3] The first chemical synthesis of triadenosine 2′,5′-phosphorothioate and separation of its diastereomers was published by Nelson et al.[4] while the enzymatic synthesis of a [*all-R*$_P$]-trimer and -tetramer with 2′,5′-internucleotide phosphorothioates was described by Lee and Suhadolnik.[5] These pioneering works were followed by extensive efforts of several research establishments.[6] All possible diastereomers of triadenosine 2′,5′-phosphorothioate have been obtained and studied as stereoselective activators (agonists and antagonists) of RNase L[7] and potential antivirals.[8] These properties induced the renaissance of chemistry and biology of 2′,5′-polyadenylate analogues in conjunction with the *antisense approach* to downregulation of the biosynthesis of preselected proteins.[9] Torrence et al. described a new approach to the selective regulation of mRNA expression[10] and also demonstrated that the 5′-O-phosphorothioylation of triadenosine 2′,5′-phosphorothioate dramatically enhanced resistance of obtained derivatives to degradation by phosphatases, although $_P$5′A2′$_P$5′A2′$_P$5′A showed an identical IC_{50} (5×10^{-10} M) as $_{PS}$5′A2′$_P$5′A2′$_P$5′A to activate 2′,5′-oligoadenosine-dependent RNase.[11] It was also found that activation of RNase L strongly depends on the configuration at phosphorus of internucleotide phosphorothioate linkages.[7,9b]

Besides [S_P]-stereoselective synthesis described by Battistini[6a,b] all the described methods for the chemical synthesis of oligoadenylate 2′,5′-phosphorothioate analogues were nonstereospecific, and the synthesis of any particular diastereomer was accomplished as the result of stepwise separations of intermediate products. In this report we present the first approach to the solid-phase stereocontrolled synthesis of oligo[adenosine (2′,5′) phosphorothioate]s. This approach is based upon the oxathiaphospholane method developed in this laboratory.[12]

The synthesis of monomer N^6-benzoyl-5′-*O*-DMT-3′-*O*-TBDMS-adenosine 2′-*O*-(2-thiono-1,3,2-oxathiaphospholane) (**1**) is depicted in Scheme 1.

N^6-Benzoyl-5′-*O*-DMT-3′-*O*-TBDMS-adenosine (**2**)[13] was phosphitylated by means of 2-(*N*,*N*-diisopropylamino)-1,3,2-oxathiaphospholane[12] in a dichloromethane solution in the presence of 1*H*-tetrazole. The resulting 1,3,2-oxathiaphospholane intermediate (**3**) was in situ sulfurized with elemental sulfur to give **1** in 84−90% yield as a mixture of two diastereomers in the ratio of 58:42 (^{31}P NMR assay). Because 3′-O → 2′-O migration of TBDMS-group was sometimes observed[14] during the chemical synthesis of ribonucleoside phosphoramidites, we examined if **1** is contaminated with its regioisomer **4**.

N^6-Benzoyl-5′-*O*-DMT-2′-*O*-TBDMS-adenosine 3′-*O*-(2-thiono-l,3,2-oxathiaphospholane) (**4**) was synthesized as a mixture of two diastereomers in the ratio of 43:57 with 82% yield via the same procedure as described for preparation of **1**.[15] Careful inspection of the ^{31}P NMR spectrum of **1** showed that this compound was not contaminated with its regioisomer **4**. Without separation

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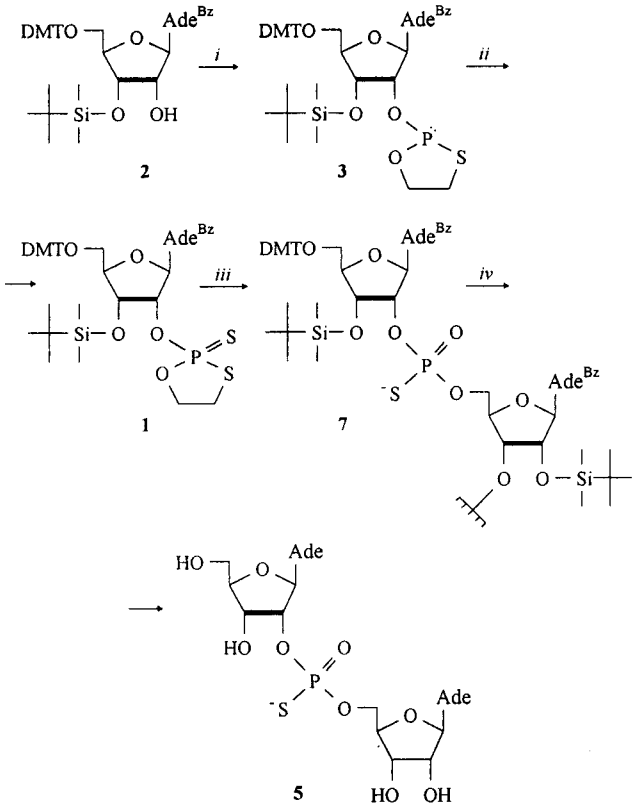
Scheme 1



i: 2-*N,N*-diisopropylamino-1,3,2-oxathiaphospholane, 1*H*-tetrazole; *ii:* elemental sulfur; *iii:* N^6-benzoyl-2'-*O*-TBDMS-adenosine on CPG, DBU; *iv:* 1. 3% DCA in CH_2Cl_2, 2. NH_4OH, 3. *n*-$Bu_4N^+F^-$.

into diastereomers, monomer **1** was used for the manual, solid-phase synthesis of diadenosine (2′,5′) phosphorothioate (**5**) (Scheme 1). Condensation was preceded by the synthesis on the controlled-pore glass of immobilized N^6-benzoyl-5′-*O*-DMT-2′ -*O*-TBDMS-adenosine (**6**) via DBU resistant succinyl-sarcosinyl linker.[16] Then by using different ratio of **1**:**6**:DBU and different coupling time, optimal conditions for a 1 μmol scale synthesis were established, similar to those described for the synthesis of diribonucleoside (3′,5′) phosphorothioates.[15] High molar excess of a mixture of diastereomers **1** (30-fold) and DBU (90-fold) was necessary to complete the reaction within 25 min with a satisfactory yield (ca. 95% according to HPLC assay; more than 98% from DMT^+ cation assay). The corresponding protocol is presented in Table 1.

The condensation step, producing protected dimer **7**, was followed by detritylation (3% DCA in dichlorornethane) and cleavage from the solid support/debenzoylation [32% aqueous ammonia diluted with ethanol (3:1, v/v, 1 mL) at room temperature for 16 h]. The removal of the *tert*-butyldimethylsilyl protecting group was accomplished by further treatment of the resulting product with 300 μL of 1 M tetra-*n*-butylammonium fluoride (TBAF) in THF for 12 h at room temperature. This time was sufficient for complete desilylation. No traces of the 2′-O → 3′-O migration of phosphorothioate diester could be detected by RP-HPLC. Extension of the desilylation time to 24 h gave only 1% of diadenosine 3′,5′-phosphorothioate, which was confirmed via coinjection with the genuine

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Table 1. Protocol for the Manual, Solid-Phase Synthesis of Oligoadenosine (2′,5′) Phosphorothioate

no.	step	reagent/solvent	volume	time (min)
1	detritylation	3% DCA in dichloromethane	2 mL	2
2	washing	acetonitrile	10 mL	1
3	drying	argon and vacuum line		5
4	condensation	30 μmol of monomer in 95 μL of acetonitrile + 45 μL of 2 M DBU in acetonitrile	140 μL	25
5	washing	acetonitrile	10 mL	1
6	drying	argon and vacuum line		5

sample.[15] The structure of obtained dimer **5** was confirmed by FAB-MS. Since DBU-assisted 1,3,2-oxathiaphospholane ring opening condensation of **1** with the 5′-OH function of the corresponding nucleoside occurred with satisfactory yield under conditions required by solid phase synthesis, efforts were focused on the synthesis of diastereomerically pure monomers **1**. Their separation was achieved by silica gel column chromatography using a mixture of chloroform and *n*-hexane (10:1, v/v) as an eluting system. Pure diastereomers, fast-eluted **1** (*Fast*-**1**) and slow-eluted **1** (*Slow*-**1**), were isolated in 15−20% yield and in 25−30% yield, respectively. The relatively low efficiency of separation of diastereomerically pure **1** results from insufficient separability of **1** [Rf values of *Fast*-**1** and *Slow*-**1** on HP TLC plates developed twice in a mixture of chloroform/*n*-hexane 15:1 (v/v) were 0.20 and 0.16, respectively]. The condensation of each isomer, *Fast*-**1** and *Slow*-**1**, with **6** was further examined according to the conditions established and presented above. The reaction of diastereomerically pure *Fast*-**1** gave pure *Fast*-**5** in 95% yield (RP-HPLC assay, >98% yield, DMT^+ release assay) and *Slow*-**1** provided pure *Slow*-**5** in 94% yield (RP-HPLC assay, ca. 98% yield from DMT^+ release assay). It has been noticed that condensation of **1** (*Fast*-**1**:*Slow*-**1** = 47:53) with **6** (molar ratio of **1**:**6** = 30:1) gave the diastereomeric mixture of product **5** in the ratio *Fast*-**5**:*Slow*-**5** = 68:32, indicating slightly different kinetics for the condensation process for both diastereomers of **1**.

The absolute configuration at phosphorus in both diastereomers *Fast*-**5** and *Slow*-**5** was assigned enzymatically. According to the literature,[4] snake-venom phosphodiesterase (*sv*PDE) preferentially cleaves the [R_P]-A2′$_{PS}$5′A providing adenosine 5′-*O*-phosphorothioate (AMPS) and adenosine, while the [S_P]-diester is completely resistant toward *sv*PDE-assisted hydrolysis. It appeared that *Fast*-**5** was hydrolyzed when incubated with *sv*PDE, while *Slow*-**5** was completely resistant toward *sv*PDE hydrolysis. Therefore, *Fast*-**5** has the [R_P]-configuration while *Slow*-**5** has the [S_P]-configuration. For the purpose described below it is worth mentioning that the [R_P]-*Fast*-**5** was prepared from oxathiaphospholane *Fast*-**1**, while the [S_P]-*Slow*-**5** was obtained from *Slow*-**1**.

Applying the protocol of manual synthesis, the [*all*-R_P]- and [*all*-S_P]-diastereomers of trimer **8** and tetramer **9** were prepared from the individual pure diastereoisomers of *Fast*-**1** and *Slow*-**1**, respectively. After the final deprotection (conditions as described for dimer **5**), the fully deprotected oligonucleotides **8** and **9** were isolated by RP-HPLC (Figure 1).

The results are summarized in Table 2. The structures of **8** and **9** were confirmed by MALDI MS; for each

Table 2. Yields and Physicochemical Characteristics of Tri- and Tetraadenosine (2′,5′) Phosphorothioates

compound	RP-HPLC[a] t_R (min)	yield (%) HPLC[b]	yield (%) DMT+	^{31}P-NMR[c] δ (ppm)	molecular formula[d]	molecular weight calcd	molecular weight measured[e]
			trimer **8**				
[*all*-R_P] $A_{PS}A_{PS}A$	24.07	90.8	97	58.15, 57.92	$C_{30}H_{37}N_{15}O_{14}P_2S_2$	957.2	956.4
[*all*-S_P] $A_{PS}A_{PS}A$	25.28	84.3	87	57.19, 57.11	$C_{30}H_{37}N_{15}O_{14}P_2S_2$	957.2	956.5
			tetramer **9**[f]				
[*all*-R_P] $A_{PS}A_{PS}A_{PS}A$	25.77	85.6	82	58.09, 58.08, 57.84	$C_{40}H_{49}N_{20}O_{19}P_3S_3$	1302.3	1301.2
[*all*-S_P] $A_{PS}A_{PS}A_{PS}A$	27.42	77.5	74	57.14, 57.01, 56.94	$C_{40}H_{49}N_{20}O_{19}P_3S_3$	1302.3	1301.6

[a] Condition of analyses see the Experimental Section. [b] Calculated from intergrated HPLC chromatograms. [c] ^{31}P NMR spectra were run in D_2O solution. [d] Molecular formula described for a fully acid form. [e] Negative MALDI $(M - H)^-$ ions. [f] For preparation of **9**, capping step with 0.5 mL of Ac_2O:lutidine:THF (1:1:8, v/v/v) plus 0.5 mL of DMAP:THF (7:93, w/v) preceded each detritylation step.

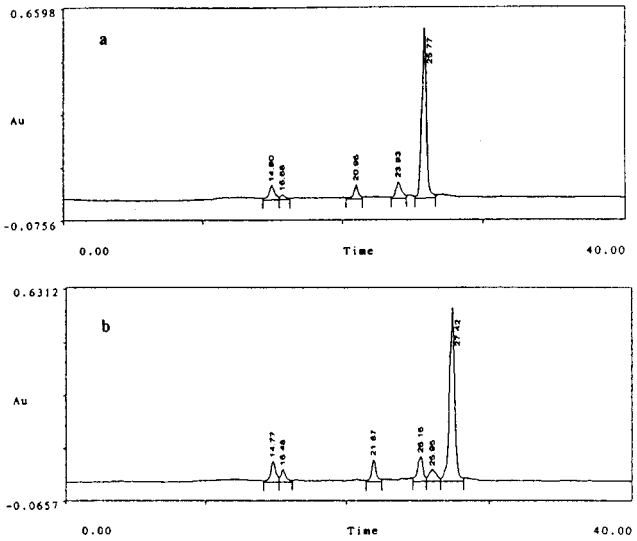

Figure 1. RP-HPLC chromatograms of crude (a) [*all*-R_P] and (b) [*all*-S_P]-A2′$_{PS}$5′A2′$_{PS}$5′A2′$_{PS}$5′A.

compound the expected (M − H) ion was observed. ^{31}P NMR spectra were consistent with those described in the literature.[4]

Independently, the diastereomeric purities of **8** and **9** were confirmed by *sv*PDE enzymatic digestion; only [*all*-R_P] **8** and **9** were hydrolyzed into adenosine and AMPS, while [*all*-S_P] isomers remained intact.

Within the frame of the project presented in this communication, efforts were made toward assignment of absolute configuration at phosphorus in both diastereomers of **1**. Because all attempts at crystallization of individual diastereomers of **1** have failed, the DMT-O-protective group was removed **1** with *p*-toluenesulfonic acid for each **1**.

Crystallization of *Slow*-**1A** (obtained from *Slow*-**1**) from dichloromethane/diethyl ether (1:1, v/v) provided crystals suitable for X-ray analysis. The ORTEP plot of *Slow*-**1A** is presented in Figure 2,[17] and the absolute configuration is described in terms of Cahn−Ingold−Prelog convention[18] is [S_P].

Since the *Slow*-**1** conversion to *Slow*-**1A** does not change of spatial arrangement of ligands surrounding the P atom, the absolute configuration of diastereomer *Slow*-**1** is also [S_P]. Because [S_P]-**1** constitutes the precursor of [S_P]-**5**, the conclusion can be drawn that the DBU-assisted 1,3,2-oxathiaphospholane ring opening

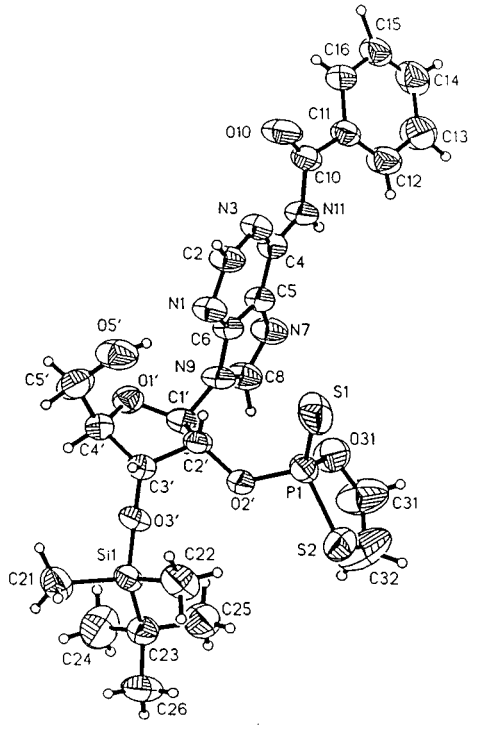

Figure 2. ORTEP plot of [S_P]-N^6-benzoyl-3′-*O*TBDMS-adenosine 2′-*O*-(2-thiono-1,3,2-oxathiaphospholane).

condensation process occurs with retention of configuration at the phosphorus atom. Such a conclusion is in agreement with an "adjacent" type mechanism for that process, as it was proposed in our earlier publications[12a,19] and well supported by results of ab initio studies.[20]

Experimental Section

Dichloromethane was dried over calcium hydride and distilled before use. Chloroform was distilled from potassium carbonate. Long chain alkylamine controlled-pore glass (LCA CPG) 500 A (Mesh size 80−120) and snake venom phosphodiesterase (*sv*PDE) were obtained from Sigma. Column chromatography and thin-layer chromatography (TLC) were performed on 230−400 mesh silica gel and silica gel F 254 plates (Merck), respectively.

Reversed-phase high performance liquid chromatography (RP-HPLC) was performed using an ECONOSPHERE C-18 5 μm, 4.6 × 250 mm column, with the following gradients. Buffer A: 0.1 M TEAB, buffer B: 40% CH_3CN in 0.1 M TEAB, pH 7.0. System A: 0−35 min, 0−60% B; system B: 0−20 min, 0−60% B. N^6-Benzoyl-5′-*O*-DMT-3′-*O*-TBDMS-adenosine and N^6-ben-

(17) Detailed description of crystal structure of *Slow*-**1A** will be published elsewhere.

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zoyl-5′-*O*-DMT-2′-*O*-TBDMS-adenosine were synthesized according to the literature.[13] N^6-Benzoyl-5′ -*O*-DMT-2′-*O*-TBDMS-adenosine was succinylated by the standard procedure[14] and attached to sarcosinylated LCA CPG as described by Brown et al.[16]

***N*⁶-Benzoyl-5′-*O*-DMT-3′-*O*-TBDMS-adenosine 2′-*O*-(2-Thiono-l,3,2-oxathiaphospholane) (1).** To a stirred solution of N^6-benzoyl-5′-*O*-DMT-3′-*O*-TBDMS-adenosine (**2**) (3.17 g, 4.03 mmol) and l*H*-tetrazole (389 mg, 5.5 mmol) in dry dichloromethane (25 mL) at room temperature was added 2-(*N*,*N*-diisopropylamino)-1,3,2-oxathiaphospholane[12a] (892 μL, 4.31 mmol) dropwise by syringe over 20 min under argon atmosphere. Reaction was maintained for 5 h, and then elemental sulfur (360 mg, 11.25 mmol) was added. Stirring was continued for 12 h. An excess of sulfur was filtered off, and solvent was evaporated to dryness. A 30 mL volume of acetonitrile was added to the residue, and the precipitated sulfur was filtered off again. After removing of the solvent, the residue was dissolved in 100 mL of chloroform and washed with 5% $NaHCO_3$ (2 × 30 mL), saturated brine (30 mL), and water (2 × 20 mL). The chloroform layer was dried over anh $MgSO_4$. The $MgSO_4$ was filtered off, and the solvent was evaporated to dryness. The crude product was dissolved in ca. 5 mL of chloroform, and this solution was applied to silica gel column (230−400 mesh, 80 g). The product was eluted with a gradient of 0−1% methanol in chloroform. Appropriate fractions were combined and evaporated under reduced pressure to give 3.2 g (85% yield) of title compound as a mixture of two diastereomers in the form of an amorphous powder. ^{31}P NMR (81 MHz, CD_3CN) 108.00 ppm, 106.77 ppm; MS: FAB +ve, *m*/*z* 926 $(M + l)^+$, FAB -ve, *m*/*z* 924 $(M - l)^-$.

Obtained **1** (2.5 g) was dissolved in ca. 3 mL of a chloroform−*n*-hexane (15:1, v/v) mixture and applied onto a column (30 × 6 cm) containing 150 g of silica gel (Silica gel 60H). The column was eluted with a mixture of chloroform−*n*-hexane (15:1, v/v), and fractions were collected [TLC control of the eluate on HP TLC plates, developed in chloroform−*n*-hexane (15:1, v/v) twice]. Appropriate fractions were combined and concentrated to dryness to give the pure, separated diastereomers **1** as follows: (1) fast-eluted diastereomer (*Fast*-**1**): 335 mg, ^{1}H NMR (200 MHz, C_6D_6) 0. 19 (s, 3H), 0.33 (s, 3H), 1.02 (s, 9H), 2.15 (m, 2H), 3.28 (m, 2H), 3.30 (s, 6H), 3.65 (m, 2H), 4.40 (m, 4H), 5.00 (dd, J = 4.7, 2.8, 1H), 6.20 (m, 1H), 6.50 (d, J = 6.7, 1H), 6.70 (m, 20H); ^{31}P NMR (81 MHz, C_6D_6) 104.88 ppm; R_f 0.20 (chloroform−*n*-hexane, 15:1, v/v, double development); MS: FAB +ve, *m*/*z* 926 $(M + l)^+$, FAB -ve, *m*/*z* 924 $(M - 1)^-$. (2) Slow-eluted diastereomer (*Slow*-**1**): 472 mg, ^{1}H NMR (200 MHz, C_6D_6) 0.15 (s, 3H), 0.29 (s, 3H), 1.00 (s, 9H), 2.30 (m, 2H), 3.25 (m, 2H), 3.29 (s, 6H), 3.55 (m, 2H), 4.40 (m, 4H), 5.05 (t, J = 4.1, 1H), 6.25 (m, 1H), 6.47 (d, J = 5.6, 1H), 6.70 (m, 20H); ^{31}P NMR (81 MHz, C_6D_6) 106.49 ppm, R_f 0.16 (chloroform−*n*-hexane, 15:1, v/v, double development); MS: FAB +ve, *m*/*z* 926 $(M + l)^+$, FAB -ve, *m*/*z* 924 $(M - l)^-$.

General Procedure for the Solid-Phase Synthesis of Di-(5), Tri-(8) and Tetra[adenosine(2′,5′)-phosphorothioate]s (9). N^6-Benzoyl-5′-*O*-DMT-2′-*O*-TBDMS-adenosine (**6**) (1 μmol, loading 41 μmol/g) was detritylated with 3% DCA in dichloromethane (2 mL), washed thoroughly with 10 mL of dry acetonitrile, and then dried under vacuum. For the coupling step a solution of *Fast*-**1** (30 μmol) or *Slow*-**1** (35 μmol) in dry acetonitrile and a 2 M DBU (45 μL, 90 μmol) solution in dry acetonitrile (total volume 140 μL) were prepared and instantly introduced onto the column. After 25 min the column was washed with dry acetonitrile (10 mL) and then treated with 3% DCA in dichloromethane (2 mL) for 2 min. The column was washed with acetonitrile (10 mL) again. For the synthesis of trimer **8** and tetramer **9** coupling steps as described above were repeated once or twice, respectively. For tetramer **9** the capping step [with 0.5 mL of Ac_2O, lutidine, THF (1:1:8, v/v/v) and 0.5 mL of DMAP, THF (7:93, w/v); time 2 min] preceded each following detritylation. After drying with argon, the support was transferred into a glass vial and treated with a mixture of 32% concentrated ammonia and ethanol (3:1, v/v, 1 mL). The vial was tightly closed and shaken at room temperature for 12 h to cleave the product from the solid support and remove the N^6-benzoyl protective groups. The ammonia solution was filtered, and the CPG support was washed with a new portion of ammonia solution (2 × 0.5 mL). The combined filtrates were evaporated to dryness. The residue was treated with a 1 M solution of tetra-*n*-butylammonium fluoride in THF overnight at room temperature. The resulting mixture was then evaporated for 4 h in Speed Vac. The crude deprotected oligonucleotides (**5**, **8**, and **9**) were dissolved in water (ca. 1 mL) and purified by RP-HPLC (system A).

***sv*PDE−Assisted Hydrolysis of Oligo[adenosine (2′,5′) phosphorothioate]s.** A sample of **5**, **8**, or **9**, respectively, (0.2 OD unit) was dissolved in 100 μL of 100 mM Tris-HCl buffer (pH 8.5) containing 15 mM $MgCl_2$. Then 2 μg of *sv*PDE was added, and the resulting mixture was incubated at 37 °C for 2 h. The enzyme was heat-denaturated (2 min, 95−99 °C) and removed by centrifugation (10000 rpm, 10 min). The obtained mixture was analyzed on RP-HPLC (system B). It appeared that fast-eluted diastereomers underwent hydrolysis to adenosine and AMPS, while slow-migrating isomers remained intact.

***N*⁶-Benzoyl-3′-*O*-TBDMS-adenosine 2′-*O*-(2-thiono-1,3,2-oxathiaphospholane). (*Slow*-1A).** To a stirred solution of slow-eluted isomer, N^6-benzoyl-5′-*O*-DMT-3′-*O*-TBDMS-adenosine 2′-*O*-(2-thiono-1,3,2-oxathiaphospholane) (*Slow*-**1**) (105 mg, 0.11 mmol), in dry dichloromethane (2 mL) was added 10 mg of *p*-toluenesulfonic acid. The removal of the 5′-*O*-dimethyoxytrityl protecting group was followed by TLC. Then, the reaction was quenched by adding pyridine (0.3 mL), and the resulting solution was concentrated to dryness under vacuum. The residue was loaded on silica gel column (230−400 mesh, 7 g) and eluted with a gradient of methanol (0−2%) in chloroform to give the title compound (61 mg, 86% yield). This compound was dissolved in dichloromethane/diethyl ether (1:1, v/v) and allowed to stand for crystallization. Obtained crystals [Rf 0.42, chloroform/ethanol (19:1, v/v); mp 187−188 °C; ^{1}H NMR (500 MHz, $CDCl_3$): 0. 17 (s, 3H), 0.20 (s, 3H), 0.96 (s, 9H), 3.40 (m, 2H), 3.90 (m, 2H), 4.27 (m, 1H), 4.39 (m, 2H), 4.63 (m, 1H), 5.75 (m, 1H), 6.08 (d, J = 7.5, 1H), 6.16 (m, 1H), 7.80 (m, 5H), 8.08 (s, 1H), 8.81 (s, 1H), 9.10 (br s, 1H); ^{31}P NMR (202 MHz, $CDCl_3$) 105.86 ppm; MS: FAB, +ve, *m*/*z* 624 $(M + H)^+$, 646 $(M + Na)^+$, FAB -ve, *m*/*z* 644 $(M + Na - 2H)^-$] were subjected for X-ray analysis, which showed that the absolute configuration at phosphorus is [S_P] (Figure 2).

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