84. Nucleotides

Part XXXVI[1])

Syntheses and Biological Characterization of Phosphorothioate Analogues of (3′–5′)Adenylate Trimer

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(25.IV.91)

The four protected diastereoisomers **7a/7b** and **8a/8b** of *P*-thioadenylyl-(3′–5′)-*P*-thioadenylyl-(3′–5′)-adenosine were synthesized, separated, and deblocked to the free oligonucleotides (*Scheme*). Biochemical characterization of these (3′–5′)phosphorothioate analogues of adenylate trimer indicate that these compounds, and the corresponding 5′-monophosphates, neither bind to nor activate RNase L, and are considered to be valuable control compounds in screening experiments.

1. Introduction. – Phosphorothioate analogues of nucleotides and oligonucleotides play an important role in elucidating the stereochemical course of many biochemical reactions [2] and have proven to be extremely valuable probes in studies of DNA conformation, DNA/protein interactions, and antiviral agents. *Zon* and coworkers have shown that substitution of one of the diastereotopic O-atoms with an S-atom in the 3′–5′-internucleotide linkages in DNA results in two phosphorothioate-containing diastereoisomers which possess different structural and electronic features as well as altered physicochemical properties [3].

We have reported on the synthesis, separation, and biological characterization of all four diastereoisomers of the phosphorothioate analogues of (2′–5′)adenylate trimer [4–7]. These analogues have been used to study the binding and activation process of the (2′–5′)oligoadenylate-dependent RNase (RNase L) leading to a better understanding of the (2′–5′)A pathway that can be used to assess the role of the (2′–5′)A synthetase/RNase L approach in virus infection, cell growth, and cell differentiation [8].

We report here the synthesis, separation, and biological characterization of all four possible isomers of phosphorothioate analogues of the (3′–5′)adenylate trimer in order to provide valuable standards for *in vivo* and in *vitro* studies of (2′–5′)oligonucleotide analogues. The syntheses were performed using the phosphoramidite method and the 2-cyanoethyl group for phosphate protection in contrast to our former syntheses of (2′–5′)trimers based upon the 2-(4-nitrophenyl)ethyl blocking group. The chemical synthesis of (*PR*)- and (*PS*)-*P*-thioadenylyl-(3′–5′)adenosine has already been reported by

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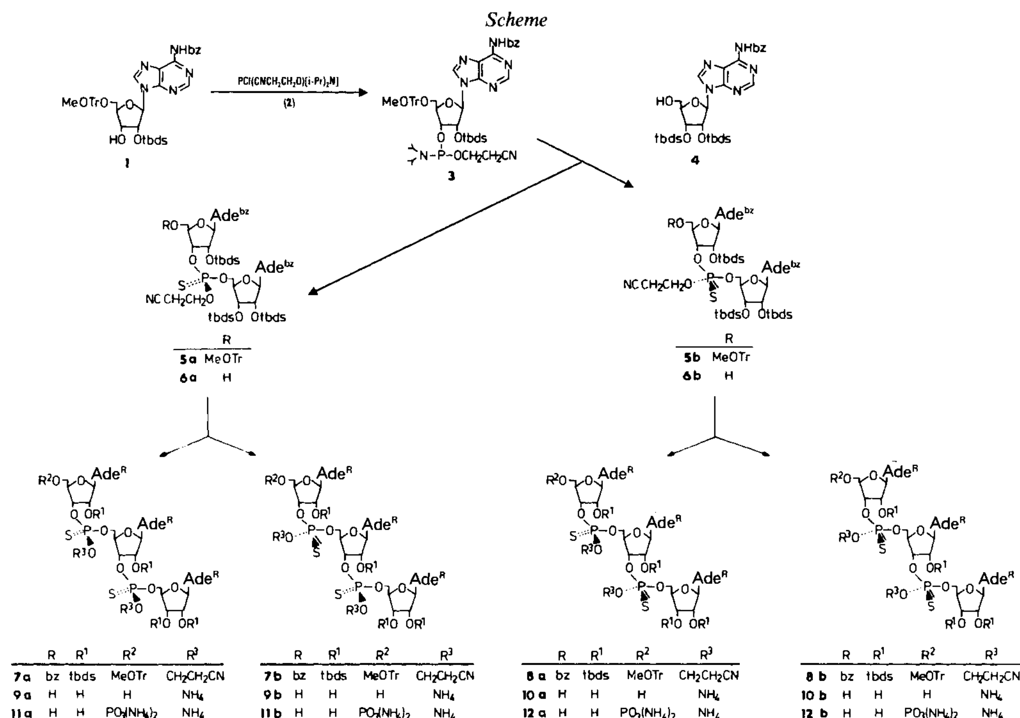
Marlier and *Benkovic* [9] applying the phosphite triester approach and assigning the configuration of the new chiral center by ^{31}P-NMR chemical shift comparisons.

2. Syntheses. – N^6-Benzoyl-2′-*O*-[(*tert*-butyl)dimethylsilyl]-5′-*O*-(monomethoxytrityl)adenosine (**1**) [10] was converted into the corresponding 3′-phosphoramidite **3** by treatment with chloro(2-cyanoethoxy)(diisopropylamino)phosphine (**2**) [11] in the presence of *Hünig*'s base in 86% yield, after purification and drying (see *Scheme*). This building block was used for further condensation with N^6-benzoyl-2′,3′-bis-*O*-[(*tert*-butyl)dimethylsilyl]adenosine (**4**) [10] under catalysis of 3-nitro-1,2,4-triazole [12] in MeCN, and followed by oxidation with sulfur, to give a (*PR*)- and (*PS*)-isomer mixture of the fully protected phosphorothioate dimers **5a** and **5b**, respectively. The two diastereoisomers could be separated by prep. TLC (silica gel), and after isolation, the pure **5a** and **5b** were separately detritylated with 2% TsOH in CH_2Cl_2/MeOH to give the corresponding 5′-hydroxy dimers **6a** and **6b** in 78 and 81% yield, respectively.

For the preparation of the four diastereoisomeric trimers, having (*PR*,*PR*)-, (*PS*,*PR*)-, (*PR*,*PS*)-, and (*PS*,*PS*)-configuration, phosphoramidite **3** was condensed with the (*PR*)- and (*PS*)-dimer **6a** and **6b**, respectively, in an analogous manner and oxidized finally with elemental sulfur. Starting from the (*PR*)-dimer **6a**, the two isomeric trimers with (*PR*,*PR*)- and (*PS*,*PR*)-configuration **7a** and **7b** could be isolated in pure form and yields of 22 and 31%, respectively, after a tedious, difficult separation by prep. TLC (silica gel). The same condensation between phosphoramidite **3** and the (*PS*)-dimer

Scheme



6b led to the (*PR,PS*)/(*PS,PS*)-mixture **8a/8b** which was separated by prep. TLC (26 and 36% yield, resp.).

The deprotection of the four isomers **7a**, **7b**, **8a**, and **8b** was then achieved in each case by the same sequence of reactions. At first, the 5′-*O*-(monomethoxy)trityl group was cleaved by 2% TsOH in CH_2Cl_2/MeOH 4:1 and after chromatographical purification, DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) treatment eliminated the cyanoethyl groups, methanolic ammonia removed the benzoyl groups, and Bu_4NF deblocked the silyl groups. Isolation and purification of the free oligonucleotides **9a**, **9b**, **10a**, and **10b** resulted from *DEAE-Sephadex* and paper chromatography. Phosphorylation of **9a**, **9b**, **10a**, and **10b** at the 5′-terminus was performed with ATP under the catalysis of T4 polynucleotide kinase to give **11a**, **11b**, **12a**, and **12b**, respectively.

3. Physical Data. – The protected nucleosides and nucleotides were characterized by C,H,N analyses, UV spectra, ^{1}H- and ^{31}P-NMR spectra, and their chromatographic behavior (see *Table*). The ^{1}H-NMR spectra are in general of complex nature due to many overlapping signals; therefore, only some distinct signals like those of the base moieties and anomeric protons are listed in the *Table*. Characterization of the fully deprotected trimers were based upon HPLC retention times and ^{31}P-NMR spectra, which indicate that the phosphorothioate P-atom resonates at lower field when (*PR*)-configurated and at higher field when (*PS*)-configurated. The (*PR,PR*)-trimer **9a** show two close signals at *ca.* 57.2 ppm, whereas the (*PS,PS*)-trimer **10b** offers only one collapsed signal at 56.464 ppm. The mixed (*PS,PR*)- (**9b**) and (*PR,PS*)-configurations (**10a**) are expectedly revealed by distinct chemical-shift differences of the characteristic P-signals.

The internucleotide configurations were also checked by enzymatic digestions using snake-venom phosphodiesterase (SVPDE) and nuclease P1. HPLC was used to separate the (3′–5′)phosphorothioate diesters in the enzyme digestion products. SVPDE preferentially cleaved (*PR*)-(3′–5′)phosphorothioate linkages from the 3′-terminus: after 8 h digestion, the (*PR,PR*)- and (*PS,PR*)-trimer cores but not the (*PS,PS*)- nor the (*PR,PS*)-trimer cores, were partially digested. Nuclease P1, on the other hand, preferentially cleaved (*PS*)-(3′–5′)phosphorothioate linkages, also from the 3′-terminus [14]: after 4 h digestion, the (*PR,PS*)- and (*PS,PS*)- but not the (*PS,PR*)- and (*PR,PR*)-trimer cores were digested. Although the (3′–5′)phosphorothioates cannot be completely digested even after 24 h, (3′–5′)A trimer was completely cleaved by these two enzymes in 1 h.

4. Biochemical Application. – The ability of the 3′–5′phosphorothioates **9a**, **9b**, **10a**, and **10b** and the corresponding 5′-monophosphates **11a**, **11b**, **12a**, and **12b** to compete with (2′–5′)p_3ApApApA [^{32}P]pCp for binding to RNase L was compared to that of (2′–5′)p_3ApApA in radiobinding assays [15], using L929 cell extracts as the source of RNase L [7]. Similarly to previous reports [17] on the specificity of RNase L for the (2′–5′)-internucleotide linkage, these 3′–5′ isomers and the corresponding 5′-monophosphates do not bind to RNase L (*Fig. 1*). More importantly, simultaneous incubation of (2′–5′)p^3ApApA and compound **11a** does not interfere with the binding affinity of authentic (2′–5′)p^3ApApA (*Fig. 1*).

Similar results have been obtained using the core-cellulose assay [16] for quantitative RNase L activation (data not shown). These data indicate that these (3′–5′)A *P*-thioanalogues are completely without activity and have no effect on RNase L activation by authentic (2′–5′)A trimer.

Table. *Physical Data of Phosphorothioate Analogues of Adenylate Dimers and Trimers*

	UV Spectra (MeOH)		^{1}H-NMR Spectra ($CDCl_3$, δ [ppm])			^{31}P-NMR Spectra	TLC	HPLC[a])
	λ_{max} [nm]	logε	H–C(1')	H–C(8) +	H–C(2)	($CDCl_3$)	R_f	t_R [min]
3	230, 279	4.43, 4.33	6.08 (*d*), 6.03 (*d*)	8.70 (*s*), 8.67 (*s*)	8.22 (*s*), 8.19 (*s*)	151.97, 149.84		
5a	230, 279	4.59, 4.63	6.05 (*d*), 5.96 (*d*)	8.80 (*s*), 8.65 (*s*)	8.27 (*s*), 8.26 (*s*)	69.49	0.37[b])	
5b	230, 279	4.60, 4.62	6.01 (*d*), 5.99 (*d*)	8.77 (*s*), 8.57 (*s*)	8.22 (*s*), 8.19 (*s*)	68.49	0.15[b])	
6a	229, 279	4.43, 4.59	5.99 (*d*), 5.84 (*d*)	8.85 (*s*), 8.79 (*s*)	8.23 (*s*), 8.21 (*s*)		0.11[b])	
6b	229, 279	4.44, 4.60	6.01 (*d*), 5.82 (*d*)	8.81 (*s*), 8.77 (*s*)	8.26 (*s*), 8.21 (*s*)		0.15[b])	
7a (*PR,PR*)	228, 279	4.79, 4.75	6.12 (*d*), 6.05 (*d*), 5.96 (*d*)	8.79 (*s*), 8.78 (*s*), 8.62 (*s*)	8.29 (*s*), 8.27 (*s*) 8.25 (*s*)		0.33[c])	
7b (*PS,PR*)	228, 279	4.68, 4.73	6.06 (*d*), 6.01 (*d*) 5.95 (*d*)	8.78 (*s*), 8.76 (*s*) 8.55 (*s*)	8.29 (*s*), 8.27 (*s*) 8.25 (*s*)		0.19[c])	
8a (*PR,PS*)	227, 279	4.67, 4.78	6.08 (*d*), 6.05 (*d*) 6.02 (*d*)	8.78 (*s*), 8.76 (*s*) 8.66 (*s*)	8.33 (*s*), 8.30 (*s*) 8.25 (*s*)		0.15[c])	
8b (*PS,PS*)	227, 279	4.71, 4.78	6.03 (*d*), 6.00 (*d*)	8.79 (*s*), 8.75 (*s*)	8.25 (*s*, 3 H)		0.10[c])	
9a (*PR,PR*)	257[d])		5.99 (*d*)	8.55 (*s*)		57.236, 57.205[e])	0.27[f])	22.75
9b (*PS,PR*)	257[d])					57.05, 56.40[e])	0.20[f])	29.85
10a (*PR,PS*)	257[d])					57.29, 56.27[e])	0.20[f])	29.30
10b (*PS,PS*)	257[d])					56.464[e])	0.20[f])	34.20

[a]) *A*, 50 mM $(NH_4)H_2PO_4$ (pH 7); *B*, MeOH/H_2O 1:1. Linear gradient: t = 0–1 min, 20% *B* in *A*; 1–31 min→50% *B* in *A*; 31–50 min→50% *B*.

[b]) SiO_2, CH_2Cl_2/AcOEt/hexane 1:1:1.

[c]) SiO_2, CH_2Cl_2/AcOEt/hexane 1:1:0.5.

[d]) In H_2O.

[e]) Instead of $CDCl_3$, D_2O buffer pH 8.5 0.1M *Tris*·HCl, 1 mM EDTA/10 mM NaCl 1:1, reference H_3PO_4.

[f]) Cellulose, i-PrPH/conc. NH_3/H_2O 65:10:25.

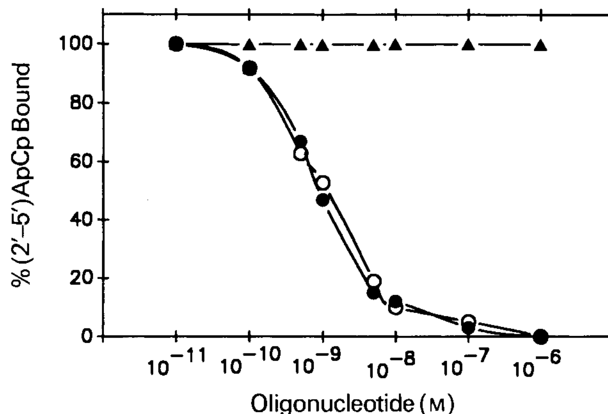


Fig. 1. *The (3'–5')phosphorothioate trimer analogues* **11a**, **11b**, **12a**, *and* **12b** *do not compete with (2'–5')p³ApApApA-[³²P]pCp for binding to RNase L in L929 cell extracts.* 65% of the (2'–5')p³ApApApA[³²P]pCp was bound in the absence of added oligonucleotide (total dpm = 18000). (●, p_3A2'p5'A2'p5'A; ○, p_3A2'p5'A2'p5'A + 1 µM pA3'sp5'A3'sp5'A **11a**; ▲, pA3'sp5'A3'sp5'A **11a**; sp = phosphorothioate.

Finally, these *P*-thioadenylyl-(3'–5')*P*-thioadenylyl-(3'–5')-adenosine 5'-monophosphates **11a**, **11b**, **12a**, and **12b** were analyzed in the rRNA cleavage assay, a specific assay for (2'–5')A and functional (2'–5')A analogues [6] [7] (*Fig. 2*). It is observed that RNase L specifically degrades rRNA following activation by (2'–5')A or functional (2'–5')A analogues, but not with the reported (3'–5')oligo(*P*-thioadenylates) [17]. Consistent with the results are the facts that these analogues do neither activate RNase L (*Fig. 1*) nor do they affect activation of RNase L by authentic (2'–5')A oligomers (*Fig. 1*).

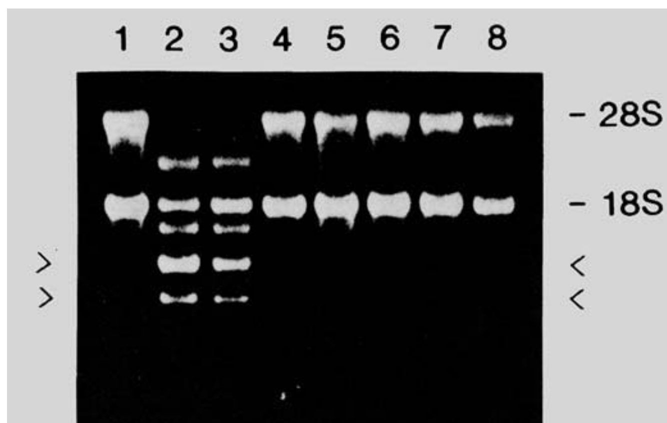


Fig. 2. *Ribosomal RNA cleavage assay with (3'–5')phosphorothioate trimer analogues.* L929 cell extracts were incubated in the absence (lane 1) or the presence of (2'–5')p_3ApApA (1 · 10^{-8} M; lane 2), (2'–5')p_3ApApA (1 · 10^{-8} M) **11a** (1 · 10^{-6} M; lane 3), (3'–5')pApApA (5 · 10^{-6} M; lane 4), **11a** (5 · 10^{-6} M; lane 5), **11b** (5 · 10^{-6} M; lane 6), **12a** (5 · 10^{-6} M; lane 7), or **12b** (5 · 10^{-6} M; lane 8). The positions of 28S and 18S rRNA are shown; the arrows indicate the positions of the well characterized specific cleavage products (SCP) of RNase L.

Experimental Part

General. See [1]. Moreover: Snake-venom phosphodiesterase (SVPDE) from *Sigma Chemicals* and nuclease P1 from *Pharmacia*. Prep. TLC: silica-gel *60 PF_{254}* (*Merck*). HPLC: *Merck-Hitachi D-2000;* column *RP-18*, 125–4 cm, 5 μm (*Merck*); flow rate 1 ml/min; *A*, 50 mM $(NH_4)H_2PO_4$ (pH 7); *B*, MeOH/H_2O 1:1; linear gradient: 0.1 min, 20% *B*; 1–31 min→50%; 31–50 min, 50% *B*. UV/VIS: *Perkin-Elmer, Lambda 5*; λ_{max} in nm (log ε). ^{31}P-NMR: *Jeol* 400 MHz, δ in ppm rel. to H_3PO_4.

1. *N^6-Benzoyl-2′-O-[(*tert*-butyl)dimethylsilyl]-5′-O-(monomethoxytrityl)adenosine 3′-(2-Cyanoethyl N,N-Diisopropylphosphoramidite)* (**3**). To a soln. of 0.758 g (1 mmol) of **1** [10] and 0.52 g (4 mmol) of Et(i-Pr)$_2$N in CH_2Cl_2 (5 ml), 0.472 g (2.29 mmol) of chloro(2-cyanoethoxy)(diisopropylamino)phosphane (**2**) was added under N_2. After stirring at r.t. for 2 h, the mixture was diluted with CH_2Cl_2 (100 ml) and washed with sat. $NaHCO_3$/NaCl soln. (2 × 50 ml), dried (Na_2SO_4), and evaporated. The crude product was purified (Na_2SO_4), and evaporated. The crude product was purified by CC (silica gel, 20 × 3 cm; packed and eluted with AcOEt/Et_3N 119:1): Evaporation of the product fractions and coevaporation with CH_2Cl_2 gave 0.907 g (86%) of **3**. R_f (silica gel, toluene/AcOEt 1:1): 0.63 and 0.71 (diastereoisomers). ^{31}P-NMR ($CDCl_3$): 151.97, 149.84. Anal. calc. for $C_{52}H_{64}N_7O_7PSi \cdot 2\ H_2O$ (994.2): C 62.82, H 6.89, N 9.86; found: C 63.16, H 6.86, N 10.00.

2. *(*PR*)- and (*PS*)-N^6-Benzoyl-2′-O-[(*tert*-butyl)dimethylsilyl]-5′-O-(monomethoxytrityl)-P-thioadenylyl-{3′-[O^P-(2-cyanoethyl)]→5′}-N^6-benzoyl-2′,3′-bis-O-[(*tert*-butyl)dimethylsilyl]adenosine* (**5a** and **5b**, resp.). A mixture of 0.527 g (0.5 mmol) of **3** and 0.228 g (0.38 mmol) of N^6-benzoyl-2′,3′-bis-*O*-[(*tert*-butyl)dimethylsilyl]-adenosine (**4**) [10] was dried at r.t. under high vacuum for 20 h and then dissolved in MeCN (3.6 ml). After addition of 0.19 g (1.66 mmol) of 3-nitro-1,2,4-triazole, the soln. was stirred under N_2 at r.t. for 3 h, and the intermediate was stirred with 0.15 g (4.64 mmol) of sulfur in pyridine (1.5 ml) for 20 h. The mixture was diluted with $CHCl_3$ (100 ml), washed with sat. NaCl soln. (2 × 50 ml), dried, evaporated, and coevaporated with toluene (2 × 10 ml). The residue was dissolved in little $CHCl_3$, and the two isomers were separated by prep. TLC (silica gel, 40 × 20 × 0.2 cm plate, 2 × Et_2O): more unpolar **5a** (0.141 g, 25%) and more polar **5b** (0.175 g, 31%), after drying at 40°/high vacuum.

5a: TLC (CH_2Cl_2/AcOEt/hexane 1:1:1): R_f 0.38. ^{31}P-NMR ($CDCl_3$): 69.49. Anal. calc. for $C_{75}H_{94}N_{11}O_{12}PSSi_3 \cdot 2\ H_2O$ (1524.9): C 59.04, H 6.47, N 10.09; found: C 58.47, H 6.46, N 10.19.

5b: TLC (CH_2Cl_2/AcOEt/hexane 1:1:1): R_f 0.15. ^{31}P-NMR ($CDCl_3$): 68.49. Anal. calc. for $C_{75}H_{94}N_{11}O_{12}PSSi_3$ (1488.9): C 60.47, H 6.36, N 10.34; found: C 59.91, H 6.36, N 10.12.

3. *(*PR*)- and (*PS*)-N^6-Benzoyl-2′-O-[(*tert*-butyl)dimethylsilyl]-P-thioadenylyl-{3′-[O^P-(2-cyanoethyl)]→5′}-N^6-benzoyl-2′,3′-bis-O-[(*tert*-butyl)dimethylsilyl]adenosine* (**6a** and **6b**, resp.). At r.t. **5a** (or **5b**) (0.106 g, 0.71 mmol) was stirred with 2% TsOH in CH_2Cl/MeOH 4:1 (2 ml) for 40 min. The mixture was then diluted with $CHCl_3$ (25 ml), washed with H_2O (2 × 20 ml), dried, and evaporated. The crude product was purified by CC (silica gel, 5 × 2 cm, $CHCl_3$/MeOH 25:1). The dimer fraction was evaporated and the foam dried at 40°/high vacuum for 24 h: **6a** (0.075 g, 87%) or **6b** (0.068 g, 79%), resp.

6a TLC: (CH_2Cl_2/AcOEt/hexane 1:1:1): R_f 0.11. Anal. calc. for $C_{55}H_{78}N_{10}O_{11}PSSi_3$ (1217.3): C 54.26, H 6.46, N 12.65; found: C 53.96, H 6.64, N 12.30.

6b: TLC (CH_2Cl_2/AcOEt/hexane 1:1:1): R_f 0.15. Anal. calc. for $C_{55}H_{78}N_{10}O_{11}PSSi_3$ (1217.3): C 54.26, H 6.46, N 12.65; found: C 53.81, H 6.46, N 12.41.

4. *(*PR*)- and (*PS*)-N^6-Benzoyl-2′-[O-(*tert*-butyl)dimethylsilyl]-5′-O-(monomethoxytrityl)-P-thioadenylyl-{3′-[O^P-(2-cyanoethyl)]→5′}-(*PR*)-N^6-benzoyl-2′-O-[(*tert*-butyl)dimethylsilyl]-P-thioadenylyl-{3′-[O^P-(2-cyanoethyl)]→5}-N^6-benzoyl-2′,3′-bis-O-[(*tert*-butyl)dimethylsilyl]adenosine* (**7a** and **7b**, resp.) *and Their (*PR,PS*)- and (*PS,PS*)-Isomers* **8a** *and* **8b**, *Resp.* A soln. of **3** (0.109 g, 0.104 mmol) and 0.059 g (0.053 mmol) of **6a** in MeCN (0.8 ml) was stirred in presence of 0.04 g (0.35 mmol) of 3-nitro-1,2,4-triazole under N_2 at r.t. for 2 h. The intermediate was oxidized with 0.031 g (0.96 mmol) of sulfur in pyridine (0.31 ml). After 20 h stirring, the mixture was diluted with $CHCl_3$ (50 ml), washed with sat. NaCl soln. (2 × 25 ml), the org. phase dried, evaporated, and coevaporated with toluene (2 × 10 ml), and the crude product purified first by CC (silica gel, 10 × 2 cm, $CHCl_3$/MeOH 100:4) and, after evaporation, separated by prep. TLC (silica gel 20 × 20 × 0.2-cm 3 plates, CH_2Cl_2/AcOEt/hexane 90:90:60 and then 90:90:45). The product bands were cut out and eluted with $CHCl_3$/MeOH 8:2: more unpolar **7a** (0.024 g, 22%) and more polar **7b** (0.34 g, 31%).

7a ((*PR,PR*)): TLC (CH_2Cl_2/AcOEt/hexane 1:1:0.5): R_f 0.33. Anal. calc. for $C_{101}H_{127}N_{17}O_{18}P_2S_2Si_4$ (2105.6): C 57.61, H 6.07, N 11.30; found: C 56.89, H 6.19, N 10.92.

7b ((*PS,PR*)): TLC (CH_2Cl_2/AcOEt/hexane 1:1:0.5): R_f 0.19. Anal. calc. for $C_{101}H_{127}N_{17}O_{18}P_2S_2Si_4$ (2105.6): C 57.61, H 6.07, N 11.30; found: C 56.76, H 6.18, N 10.76.

Exactly in the same manner, condensation of **3** and **6b** gave the more unpolar **8a** (0.029 g, 27%) and the more polar and **8b** (0.04 g, 37%).

8a ((*PR,PS*)): TLC (CH_2Cl_2/AcOEt/hexane 1:1:0.5): R_f 0.15. Anal. calc. for $C_{101}H_{127}N_{17}O_{18}P_2S_2Si_4$ (2105.6): C 57.61, H 6.07, N 11.30; found: C 57.15, H 6.13, N 10.72.

8b ((*PS,PS*)): TLC (CH_2Cl_2/AcOEt/hexane 1:1:0.5): R_f 0.1. Anal. calc. for $C_{101}H_{127}N_{17}O_{18}P_2S_2Si_4 \cdot 1\ H_2O$ (2123.6): C 57.12, H 6.12, N 11.21; found: C 57.00, H 6.16, N 10.90.

5. *(*PR*)- and (*PS*)- *P*-Thioadenylyl-3′→5′)-(*PR*)-*P*-thioadenylyl-(3′→5′)-*P*-thioadenosine* (diammonium salts; **9a** and **9b**, resp.) *and Their (*PR, PS*)- and (*SP, SP*)-Isomers* **10a** *and* **10b**, *Resp.* A soln. of 0.02 mmol (0.042 g) of **9a**, **9b**, **10a**, or **10b** in 0.6 ml of 2% TsOH in CH_2Cl_2/MeOH 4:1 was stirred at r.t. for 40 min and then diluted with $CHCl_3$ (50 ml). The org. phase was washed with H_2O (2 × 20 ml), dried, and evaporated. The residue was dissolved in little $CHCl_3$ and purified by prep. TLC (silica gel, 20 × 20 × 0.2-cm plates, $CHCl_3$/MeOH 196:4). The pure product band was cut out and eluted with $CHCl_3$/MeOH 8:2 to give, after evaporation, the corresponding 5′-hydroxy trimer in 85–90% yields. The 5′-hydroxy trimer (0.0275 g, 15 µM) was stirred with 0.5M DBU in dry pyridine (5 ml). After 24 h, the mixture was neutralized by addition of 1M AcOH in pyridine (2.5 ml) and evaporated. The residue was stirred with NH_3/MeOH (10 ml) for 48 h, and after evaporation, the residue was stirred with 1M Bu_4NF in THF (4 ml) for 48 h. The solvent was evaporated and the residue taken up in H_2O (10 ml) and applied onto a *DEAE Sephadex A-25* column (60 × 1 cm, linear gradient (0.001–1.0M) of $(Et_3NH)HCO_3$ buffer (pH 7.5)). The product fractions were evaporated, and final coevaporations were done with H_2O. Further purification by paper chromatography (i-PrOH/conc. NH_3 soln./H_2O 6:1:3) gave, after lyophilisation, colourless powders in 70–80% yield.

6. *(*PR*) and (*PS*)-5′-*O*-Phosphoryl-*P*-thioadenylyl-(3′→5′)-(*PR*)-*P*-thioadenylyl-(3′→5′)-*P*-thioadenosine* (tetraamonium salts; **11a** and **11b**, resp.) *and Their (*PR,PS*)- and (*SP, SP*)-Isomers* **12a** *and* **12b**. The appropriate purified deprotected trimer was phosphorylated at the 5′-terminus as previously described [18].

7. *Enzymatic Hydrolysis with Snake-Venom Phosphodiesterase* (SVPDE) *and Nuclease P1.* As previously described [6].

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