151. Nucleotides

Part XXX[1])

Chemical Synthesis of Adenylyl-(2′ → 5′)-adenylyl-(2′ → 5′)-8-azidoadenosine, and Activation and Photoaffinity Labelling of RNase L by [^{32}P]p5′A2′p5′A2′p5′N_3^8A

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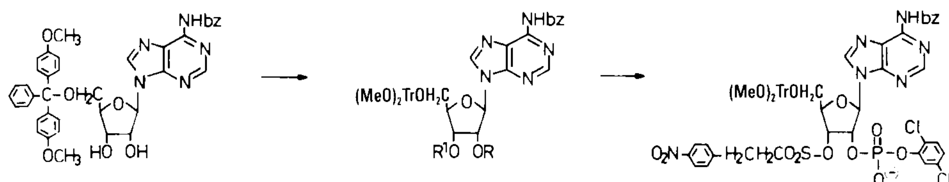
The chemical synthesis of adenylyl-(2′–5′)-adenylyl-(2′–5′)-8-azidoadenosine (**15**) was performed by the phosphotriester approach. Enzymatic phosphorylation of **15** by [γ-^{32}P]ATP led to the corresponding labelled 5′-monophosphate **16**. Photoinsertion of **16** took place on UV irradiation by covalent cross linking to a protein of M_r 80 K known to be RNase L. Radiobinding and core-cellulose assays as well as photoaffinity labelling experiments with **16** are described.

1. Introduction. – In earlier studies, *Suhadolnik et al.* [2] reported the successful enzymatic synthesis of 2′–5′ linked [α-^{32}P]ppp5′N_3^8A trimer from [α-^{32}P]N_3^8ATP and its use as a photoprobe to label covalently specific proteins of unfractionated extracts of interferon-treated L929 cells. Six proteins have been characterized in this manner, and the determination of their amino-acid sequences of the 2′–5′A binding site is currently under investigation. In order to elucidate which of the azido groups on C(8) of the three purine rings is covalently inserted into the 2′–5′A binding domain, we have now chemically synthesized one of the three possible 8-monoazido-substituted 2′–5′A trimers, core A2′p5′A2′p5′N_3^8A, which has been phosphorylated at the 5′-end with [^{32}P]phosphate from [γ-^{32}P]ATP. It was found that this photoprobe covalently links to a protein of M_r 80 K known to be RNase L. We are presently completing the rigorous chemical and enzymatic syntheses of pA2′p5′N_3^8A2′p5′A and pN_3^8A2′p5′A2′p5′A which will permit us to delineate even further the structural requirements of the 2′–5′A molecule in binding to RNase L, in activating this enzyme, and in finding out which amino-acid sequence is involved at the 2′–5′A binding site. The studies described here show great promise for the evaluation of the biological role of the 2′–5′A molecule and its substrate target RNase L in antiviral/antineoplastic chemotherapy as well as the role of this molecule in cell growth and cell differentiation.

2. Syntheses. – The chemical approach to synthesize 2′–5′ connected oligonucleotides was based on the phosphotriester methodology [3–5] which has been proven by experi-

[1]) Part XXIX, see [1].

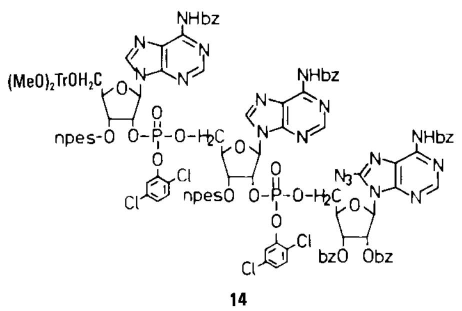
Scheme



	R	R[1]
2	npes	H
3	H	npes
4	npes	npes

	R	R[1]	R[2]	R[3]
6	H	H	H	H
7	bz	bz	bz	bz
8	bz	H	H	H
9	bz	H	H	MeOTr
10	bz	bz	bz	MeOTr
11	bz	bz	bz	H

	R
12	$(MeO)_2Tr$
13	H



14

	R	
15	H	
16	$[^{32}P]O_3H^{\ominus}$	$NH_4^{\oplus}$

bz = benzoyl, MeOTr = monomethoxytrityl, $(MeO)_2Tr$ = dimethoxytrityl, npes = 2-(4-nitrophenyl)ethylsulfonyl

ence to work very reliably and successfully in preparative scale-up experiments [6–9]. N^6-Benzoyl-5′-*O*-(dimethoxytrityl)adenosine (**1**) [10] was chosen as starting material and was first treated with 2-(4-nitrophenyl)ethanesulfonyl chloride [11] [12] to introduce a new type of protecting group into the sugar moiety of the molecule. In $CHCl_3$ and in presence of Et_3N at −6° resulted a mixture of the two isomeric N^6-benzoyl-5′-*O*-(dimethoxytrityl)-2′-*O*- (**2**) and -3′-*O*-[(4-nitrophenyl)ethylsulfonyl]adenosine (**3**) as well as the 2′,3′-bis-*O*-(4-nitrophenyl)ethylsulfonyl derivative **4** in a molar ratio of 28:32:14. Separation of the three components was achieved by silica-gel column chromatography and the structural assignment by NMR-spectral means. Compound **3** was then phospho-

rylated to the crude 2,5-dichlorophenyl phosphodiester **5** by treatment with 2,5-dichlorophenyl di(triazolido)phosphate in pyridine and hydrolytic workup. The second component **10** was prepared from 8-azidoadenosine (**6**) [13] in a series of reactions. Benzoylation led to the N^6,N^6,2′-*O*,3′-*O*,5′-*O*-pentabenzoyl derivative **7** which gave, on alkaline hydrolysis, the 8-azido-N^6-benzoyladenosine (**8**). Monomethoxytritylation proceeded in the usual manner in pyridine to 8-azido-N^6-benzoyl-5′-*O*-(monomethoxytrityl)adenosine (**9**) which was then benzoylated to **10** with benzoyl cyanide/Bu_3N and subsequently partially deblocked by acid treatment to give 8-azido-N^6,2′-*O*,3′-*O*-tribenzoyladenosine (**11**). Condensation of the phosphodiester **5** and the 5′-OH component **11** was catalyzed by a mixture of 2,4,6-triisopropylbenzenesulfonyl chloride and 3-nitro-1,2,4-triazole to give the fully protected dinucleoside phosphotriester **12** in 74% yield. Detritylation of **12** worked best with 2% TsOH in CH_2Cl_2/MeOH yielding the new 5′-OH building block **13** which was needed in the final condensation step for the extension of the chain with the phosphodiester **5** to form the trimer **14** in 82% yield.

The complete deprotection of the various blocking groups in **14** was the result of a series of subsequent reactions which were performed in the order: acid treatment to split off the dimethoxytrityl group, 4-nitrobenzaldoximate cleavage to remove the 2,5-dichlorophenyl group, DBU (1,8-diazabicyclo[5.4.0]undec-7-ene) reaction for the β-elimination of the (4-nitrophenyl)ethylsulfonyl residue, and final ammonolysis to free the molecule from the benzoyl groups. The adenylyl-(2′–5′)-adenylyl-(2′–5′)-8-azidoadenosine (**15**) was purified on a *DEAE-Sephadex* column using a linear gradient of $Et_3NH_2CO_3$ buffer (pH 7.5) to get a 64% yield of the triethylammonium salt of **15**. Its conversion into the more stable ammonium salt was finally achieved by paper chromatography using the system i-PrOH/conc. NH_3/H_2O 6:1:3 and followed by lyophilization of the eluate. The radioactive labelling of **15** at the 5′-terminus was performed with [γ-^{32}P]ATP under the catalysis of T4 polynucleotide kinase to give **16**.

3. Physical Data. – The protected nucleosides and nucleotides were characterized by elemental analysis and UV and NMR spectra *(Table)*. Since the ^{1}H-NMR spectra are generally of complex nature due to many overlapping signals and the fact that the protected phosphotriesters always consist of diastereoisomeric mixtures derived from the chiral P centers, only some distinct signals are reported for characterization. The azido groups were detected by IR spectroscopy.

4. Biochemical Application. – The ability of [^{32}P]pA2′p5′A2′p5′N_3^8A (**16**) to compete with p_3A_4[^{32}P]pCp for binding to the RNase L in L929 cell extracts was compared to that of p_3A2′p5′A2′p5′A and pA2′p5′A2′p5′A in radiobinding assays. A dose-dependent displacement of the radioactive probe from RNase L was observed *(Fig. 1)*; [^{32}P]pA2′p5′A2′p5′N_3^8A (**16**) binds with an IC_{50} of $1 \cdot 10^{-8}$ M.

The activation of RNase L by [^{32}P]pA2′p5′A2′p5′N_3^8A (**16**) was then determined by the hydrolysis of poly(U)[^{32}P]pCp using partially purified enzyme and measured in the core-cellulose assay [14]. The new synthetic oligonucleotide **16** showed the same activation of RNase L with an IC_{50} of $5 \cdot 10^{-6}$ M as authentic pA2′p5′A2′p5′A as determined previously [15].

The photoaffinity labelling of RNase L by [^{32}P]pA2′p5′A2′p5′N_3^8A (**16**) was achieved by incubating L929 cell extracts first with the reagent followed then by UV irradiation.

Table. *Physical Data of Nucleosides and Nucleotides*

	UV Spectra (MeOH)						IR Spectra (KBr) [cm^{-1}]	^{1}H-NMR Spectra ($CDCl_3$, δ[ppm])			
	λ_{max} [nm]			lg ε				H–C(2)	H–C(8)	H–C(1′)	MeO
2	233	276		4.52	4.47			8.49 (*s*)	8.15 (*s*)	6.02 (*d*)	3.72 (*s*)
3	233	276		4.51	4.46			8.60 (*s*)	8.16 (*s*)	6.27 (*d*)	3.73 (*s*)
4	234	274		4.54	4.58			8.56 (*s*)	8.20 (*s*)	6.23 (*d*)	3.73 (*s*) 3.74 (*s*)
7	229	274	296	4.78	4.19	4.22	2120	8.40 (*s*)	–	6.30 (*m*)	–
8	227		297	4.36		4.19	2157	8.68 (*s*)[a])		5.76 (*d*)	
9	230		298	4.55		4.20	2150	8.26 (*s*)		5.91 (*d*)	3.72 (*s*)
10	229	280 (sh)	298	4.81	4.21 (sh)	4.32	2157	8.62 (*s*)		6.58 (*d*)	3.74 (*s*)
11	230		299	4.66		4.29	2157	8.77 (*s*)		6.36 (*d*)	
12	228	237 (sh)	279	4.84	4.75 (sh)	4.64	2157	8.62 (*s*) 8.59 (*s*)	8.16 (*s*)	6.09–6.42 (*m*)	3.70 (*s*)
13	228	239 (sh)	281	4.85	4.68 (sh)	4.64	2157	8.73 (*s*) 8.65 (*s*)	8.09 (*s*)	6.04–6.38 (*m*)	
14	228		279	5.01		4.82	2157	8.81 (*s*) 8.60 (*s*) 8.58 (*s*)	8.35 (*s*) 8.29 (*s*)	6.04–6.48 (*m*)	3.69 (*s*)
15		261[b])					2161	8.12 (*s*)[c]) 8.07 (*s*) 8.03 (*s*)	7.78 (*s*)	6.10 (*d*) 6.01 (*d*) 5.72 (*d*)	

[a]) In (D_6)DMSO.
[b]) In H_2O.
[c]) In D_2O.

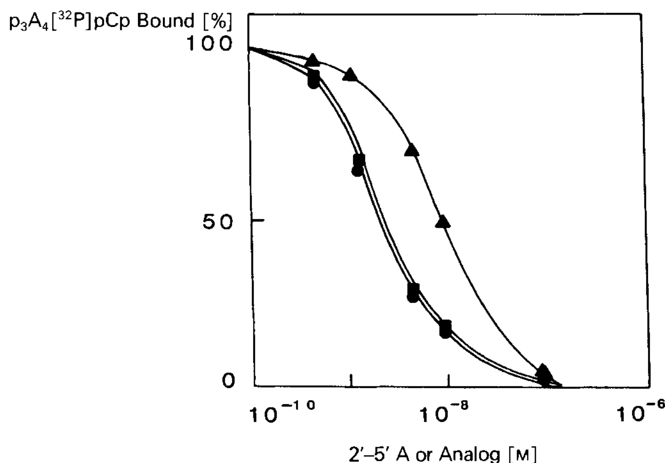


Fig. 1. *Ability of [^{32}P]pA2′p5′A2′p5′N_3^8A* (**16**) *to compete with p_3A_4[^{32}P]pCp for binding to RNase L in L 929 cell extracts. Ca.* 50% of the p_3A_4[^{32}P]pCp was bound in the absence of added oligonucleotide (total dpm = 20000). ● p_3A2′p5′A2′p5′A; ■ pA2′p5′A2′p5′A; ▲ [^{32}P] pA2′p5′A2′p5′$N_3$8A (**16**).

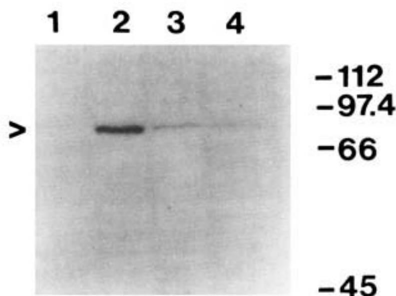


Fig. 2. *Photoincorporation of [^{32}P]pA2′p5′A2′p5′N_3^8A* (**16**) *into RNase L.* Incubation of L929 cell extracts was with the reagent followed by UV irradiation for 60 s. Lane 1 = no photolysis control; lane 2 = **16**; lane 3 = **16** in competition with p_3A2′p5′A2′p5′A; lane 4 = p_3A_4[^{32}P]pCp.

The photoinsertion of the ^{32}P-labelled photoprobe was determined by SDS-polyacrylamide gel electrophoresis and subsequent autoradiography. The photolabelling of L929 cell extracts by **16** resulted in the specific labelling of a protein of M_r 80 K, the reported molecular weight of RNase L (*Fig. 2*, lane 2) [16]. Authentic p_3A2′p5′A2′p5′A at 1×10^{-6} M competes for the photoinsertion of **16** (lane 3). Under the same photolabelling conditions, p_3A_4[^{32}P]pCp showed little insertion (lane 4). The photolabelling of RNase L by **16** was UV-irradiation-dependent, and no reaction could be detected in absence of UV light (lane 1).

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Experimental Part

General. The 5′-*O*-triphosphoryl-2′-5′-adenylate trimer and tetramer, the 5′-*O*-phosphoryl-2′-5′-adenylate trimer and poly(U) were purchased from *Pharmacia* [^{32}P]pCp (3000 Ci/mmol) from *Amersham*, [γ-^{32}P]ATP (4500 Ci/mmol) from ICN, and media and sera for cell culture from *GIBCO*. The enzymes T4 RNA ligase and T4 polynucleotide kinase were from *Bethesda Research Laboratories*. L929 cells were maintained in monolayer culture in *Dulbecco*'s modified *Eagle* medium supplemented with 5% bovine serum. The L929 cells were collected by trypsinization, washed once with PBS (phosphate buffer saline), and an unfractionated cytosolic extract was prepared by lysing in glycerol buffer as described [17]. Radiobinding assays were performed with L929 cell extracts as the source of RNase L (50 µg protein per assay). The T4 RNA ligase was used to synthesize p_3A_4[^{32}P]pCp from p_3A_4 and [^{32}P]pCp [18] as well as for poly(U)[^{32}P]pCp from poly(U) and [^{32}P]pCp [19]. Core-cellulose assays for RNase L activation were performed as previously described [19]. Photoaffinity labelling experiments were done as follows: 10 µl of L929 cell extract (100 µg of protein per assay) were combined with 10 µl of buffer (140 mM NaCl and 35 mM *Tris*-HCl (2-amino-2-(hydroxymethyl)propane-1,3-diol hydrochloride), pH 7.5) and were incubated in the presence of $1 \cdot 10^{-6}$ M [^{32}P]pA2′p5′A2′p5′N_3^8A (**16**; 17 µCi/mmol) or $1 \cdot 10^{-8}$ M p_3A_4[^{32}P]pCp (10 µCi/mmol) in a 30-µl volume. In competition photolabelling experiments, p_3A_3 ($1 \cdot 10^{-6}$ M) was also added. After incubation in microcentrifuge tubes at 0° for 90 min, the samples were transferred to ice-cold porcelain spot plates and photolyzed for 60 s with a 254-nm *UVG-11* mineralight lamp from *Ultraviolet Products, Inc.*, at a distance of 2 cm (1.0 J/m^2). After photolabelling, 30 µl of a reductive, protein-solubilizing mixture (15.4 mg/ml DTT (dithiothreitol), 25% *w/v* sucrose, 4% SDS (dodecyl sodium sulfate), 125 mM *Tris*-HCl, pH 8 and 0.0025% pyronin Y) were added to the samples. The photolabelled proteins were analyzed by 8% SDS-polyacrylamide gel electrophoresis

[20]. The *Coomassie* blue stained dried gels were subject to autoradiography at −70° with *X-Omat* film from *Kodak*. A *Beckman LS-100C* liquid scintillation spectrometer was used for all radioactive measurements (counting efficiency, 99% for ^{32}P). TLC: precoated silica-gel thin-layer sheets *F 1500 LS 254* and cellulose thin-layer sheets *F 1440* from *Schleicher & Schüll*. Prep. TLC: silica gel *6P PF$_{254}$ (Merck)*. Prep. column chromatography: silica gel (*Merck 60*, 0.063–0.2 mesh). Paper chromatography (PC): sheets (58 × 60 cm) from *Schleicher & Schüll*. Ion-exchange chromatography: *DEAE Sephadex A-25 (Pharmacia)*. M.p.: *Büchi* apparatus, model Dr. *Tottoli;* no corrections. UV/VIS: *Uvikon 820, Kontron* and *Lambda 5* and *15, Perkin Elmer;* λ_{max} in nm (lg ε). ^{1}H-NMR: *Bruker WM 250;* in δ (ppm) relative to TMS. IR: *Matson Polaris TM.*

1. N^6*-Benzoyl-5′-*O*-(dimethoxytrityl)-2′-*O*-[2-(4-nitrophenyl)ethylsulfonyl]adenosine* (**2**), N^6*-Benzoyl-5′-*O*-(dimethoxytrityl)-3′-*O*-[2-(4-nitrophenyl)ethylsulfonyl]adenosine* (**3**), *and* N^6*-Benzoyl-5′-*O*-(dimethoxytrityl)-2′,3′-*O*-bis[2-(4-nitrophenyl)ethylsulfonyl]adenosine* (**4**). A soln. of 1.0 g (1.48 mmol) of *N*^{6}-benzoyl-5′-*O*-(dimethoxytrityl)adenosine (**1**) [10] in dry $CHCl_3$ (6 ml) was cooled to −6°, and then 0.062 g (0.62 mmol) of Et_3N and 0.55 g (2.24 mmol) of 2-(4-nitrophenyl)ethanesulfonyl chloride [21] in $CHCl_3$ (2 ml) were added dropwise. After 45 min, the mixture was allowed to warm up to r.t., diluted with $CHCl_3$ (100 ml), washed with H_2O (2 × 25 ml), dried (Na_2SO_4), and evaporated. The residue was taken up in $CHCl_3$ (5 ml) and purified by chromatography on a silica-gel column (10 × 2 cm). Elution with $CHCl_3$ gave first, after evaporation, 0.234 g (14%) of **4** as an amorphous solid. Anal. calc. for $C_{54}H_{49}N_7O_{15}S_2$ (1100.2): C 58.95, H 4.49, N 8.91; found: C 59.10, H 4.74, N 8.75.

Elution with $CHCl_3$/MeOH 100:1 gave **2**/**3** (0.866 g, 65%), followed finally by **1** (0.1 g, 10%). Another silica-gel chromatography (column 10 × 3 cm) of **2**/**3** with toluene/AcOEt 4:1 gave, in the 1st fraction, 0.42 g (32%) of **3**, and then with toluene/AcOEt 3:1, 0.367 g (28%) of **2** as colourless amorphous foams. Anal. calc. for $C_{46}H_{42}N_6O_{11}S$ (886.9): C 62.29, H 4.77, N 9.47; found for **2**: C 62.59, H 4.96, N 9.10; found for **3**: C 62.88, H 4.83, N 8.91.

2. *8-Azido-*N^6,N^6*,2′-*O*,3′-*O*,5′-*O*-pentabenzoyladenosine* (**7**). In pyridine (30 ml) was treated 1 g (3.24 mmol) of 8-azidoadenosine [13] [22] with 3 ml (25.8 ml) of benzoyl chloride for 3 h at 50–60° with stirring. The mixture was poured onto ice, treated with $CHCl_3$ (100 ml), and the extract washed with 5% $NaHCO_3$ soln. and then with H_2O. The org. phase was dried (Na_2SO_4), evaporated, and co-evaporated with toluene to give a solid. The product was crystallized from EtOH to give 2.4 g (89%) of **7**. M.p. 146–147°. Anal. calc. for $C_{48}H_{32}N_8O_9$ (828.8): C 65.22, H 3.89, N 13.52; found: C 64.79, H 3.73, N 13.41.

3. *8-Azido-*N^6*-benzoyladenosine* (**8**). In dry pyridine (6 ml) and dry EtOH (6 ml) were suspended 1.66 g (2 mmol) of **7** and, after cooling in an ice bath, 2N NaOH (8 ml) and EtOH (8 ml) were added. After stirring for 30 min, a clear soln. was obtained, and TLC showed no starting material. Pyridinium *Dowex* (50 × 4) was added to the mixture till neutral pH. It was filtered off and the *Dowex* washed with EtOH (200 ml). The united filtrates were evaporated and coevaporated with toluene (2 × 10 ml). The residue was taken up in H_2O (200 ml), washed with CH_2Cl_2 (2 × 100 ml), and the aqueous phase then evaporated carefully to keep foaming to a minimum. The residue was co-evaporated with MeOH (3 × 10 ml) to give a pale yellow foam: 0.66 g (80%). Anal. calc. for $C_{17}H_{16}N_8O_5$ (412.4): C 49.51, H 3.91, N 27.17; found: C 49.40, H 3.85, N 27.05.

4. *8-Azido-*N^6*-benzoyl-5′-*O*-(monomethoxytrityl)adenosine* (**9**). Compound **8** (0.66 g, 1.2 mmol) was coevaporated with dry pyridine (3 × 6 ml) and then taken up in dry pyridine (12 ml), 0.62 g (2 mmol) of monomethoxytrityl chloride was added and the mixture stirred at r.t. for 20 h. After addition of MeOH (2 ml) and stirring for another 15 min, the volume was concentrated to ¼, the residue taken up in $CHCl_3$ (200 ml), washed with H_2O (2 × 100 ml), dried (Na_2SO_4), and then evaporated. After coevaporation with toluene (2 × 10 ml), the residue was dissolved in little $CHCl_3$ and applied onto a silica-gel column (15 × 2 cm). The pure product was eluted with $CHCl_3$/MeOH 25:1 to give, after evaporation, **8** as a colourless solid: 0.593 g (72%). Anal. calc. for $C_{37}H_{32}N_8O_6$ (684.7): C 64.90, H 4.71, N 16.36; found: C 65.44, H 5.00, N 16.90.

5. *8-Azido-*N^6*,2′-*O*,3′-*O*-tribenzoyl-5′-*O*-(monomethoxytrityl)adenosine* (**10**). In dry CH_3CN (10 ml) were dissolved 1.47 g (2.15 mmol) of **9**, and 2 drops of Bu_3N and 0.62 g (5.2 mmol) of benzoyl cyanide were added. After stirring at r.t. for 18 h, the soln. was evaporated, the residue dissolved in $CHCl_3$ (5 ml) and applied onto a silica-gel column (15 × 2.5 cm) for chromatography with $CHCl_3$. The main fraction gave, on evaporation, **9** as an amorphous solid: 1.53 g (80%). Anal. calc. for $C_{51}H_{40}N_8O_8 \cdot 1\ H_2O$ (910.9): C 67.24, H 4.61, N 12.55; found: C 67.09, H 4.45, N 12.17.

6. *8-Azido-*N^6*,2′-*O*,3′-*O*-tribenzoyladenosine* (**11**). In a 2% soln. of TsOH in CH_2Cl_2/CH_3OH 4:1 (20 ml) were treated 0.85 g (0.95 mmol) of **10** for 10 min till TLC showed no starting material. The mixture was diluted with $CHCl_3$ (50 ml), washed with H_2O (2 × 20 ml), dried (Na_2SO_4), and evaporated. Purification by silica-gel column chromatography (15 × 2.5 cm) with $CHCl_3$, evaporation, and drying under high vacuum at 40° gave an amorphous

solid: 0.452 g (82%) of **11**. Anal. calc. for $C_{31}H_{24}N_8O_7 \cdot 1\ H_2O$ (638.6): C 58.30, H 4.10, N 17.54; found: C 58.21, H 3.95, N 16.98.

7. N^6-*Benzoyl-5′-*O-*(dimethoxytrityl)-3′-*O-*[2-(4-nitrophenyl)ethylsulfonyl]adenylyl-{2′-[*O^P-*(2,5-dichlorophenyl)]→5′}-8-azido-*N^6,*2′-*O,*3′-*O-*tribenzoyladenosine* (**12**). To an ice-cold soln. of 0.255 g (0.9 mmol) of 2,5-dichlorophenylphosphorodichloridate and 0.122 g (1.8 mmol) of 1,2,4-triazole in dry pyridine (2.5 ml) was added slowly and dropwise a soln. of 0.532 g (0.6 mmol) of **3** in dry pyridine (2.5 ml). After 30 min stirring, the mixture was treated with 90% aq. pyridine (1.2 ml) and stirred for further 15 min. The mixture was diluted with $CHCl_3$ (100 ml), washed with H_2O (2 × 50 ml), dried (Na_2SO_4), evaporated, and finally co-evaporated with dry pyridine (5 × 10 ml) to give the phosphodiester **5**. The residue was taken up in dry pyridine (5 ml) and 0.324 g (0.52 mmol) of **11**, 0.369 g (1.2 mmol) of 2,4,6-triisopropylbenzenesulfonyl chloride, and 0.41 g (3.6 mmol) of 3-nitro-1,2,4-triazole were added and stirred for 18 h at r.t. The mixture was extracted with $CHCl_3$ (100 ml), washed with H_2O (2 × 50 ml), evaporated, and finally co-evaporated with toluene (3 × 10 ml). Purification by silica-gel column chromatography (15 × 2.5 cm) using $CHCl_3$ gave from the main fractions after drying under high vacuum, an amorphous solid: 0.89 g (74%) of **12**. Anal. calc. for $C_{83}H_{67}Cl_2N_{14}O_{20}PS \cdot 2\ H_2O$ (1750.5): C 56.95, H 4.08, N 11.43; found: C 57.04, H 4.19, N 11.20.

8. N^6-*Benzoyl-3′-*O-*[2-(4-nitrophenyl)ethylsulfonyl]adenylyl-{2′-[*O^P-*(2,5-dichlorophenyl)]→5′}-8-azido-*N^6,*2′-*O,*3′-*O-*tribenzoyladenosine* (**13**). In a soln. of 2% TsOH in CH_2Cl_2/MeOH 4:1 were stirred 0.61 g (0.357 mmol) of **12** for 30 min. The mixture was diluted with $CHCl_3$ (50 ml), washed with H_2O (2 × 20 ml), dried (Na_2SO_4), and evaporated. Purification was achieved by silica-gel column chromatography (10 × 2.5 cm) eluting **13** with $CHCl_3$/MeOH 50:1 and evaporation: 0.413 g (82%) of **13**. Anal. calc. for $C_{62}H_{49}Cl_2N_{14}O_{18}S$ (1412.1): C 52.73, H 3.49, N 13.33; found: C 52.30, H 3.57, N 13.33.

9. N^6-*Benzoyl-5′-*O-*(dimethoxytrityl)-3′-*O-*[2-(4-nitrophenyl)ethylsulfonyl]adenylyl-{2′-[*O^P-*(2,5-dichlorophenyl)]→5′}-*N^6-*benzoyl-3′-*O-*[2-(4-nitrophenyl)ethylsulfonyl]adenylyl-{2′-[*O^P-*(2,5-dichlorophenyl)]→5′}-8-azido-*N^6,*2′-*O,*3′-*O-*tribenzoyladenosine* (**14**). The phosphodiester **5** was prepared according to the procedure described in *Exper.* 7. The residue was mixed with 0.52 g (0.39 mmol) of **13**, coevaporated with dry pyridine (3 × 5 ml), and finally taken up in dry pyridine (4 ml). After addition of 0.37 g (12 mmol) of 2,4,6-triisopropylbenzenesulfonyl chloride and 0.46 g (3.6 mmol) of 3-nitro-1,2,4-triazole, the mixture was stirred at r.t. for 18 h, then diluted with $CHCl_3$ (100 ml), washed with H_2O (2 × 50 ml), evaporated, and co-evaporated with toluene (5 × 10 ml). The resulting residue was dissolved in $CHCl_3$ (7 ml), applied onto a silica-gel column (25 × 2.5 cm), and the trimer eluted with $CHCl_3$/MeOH 100:1. The main fraction was evaporated and dried under high vacuum to give 0.80 g (82%) of **14**. Anal. calc. for $C_{114}H_{92}Cl_4N_{20}O_{31}P_2S_2$ (2506.0): C 54.63, H 3.70, N 11.17; found: C 54.14, H 3.78, N 10.80.

10. *Adenylyl-(2′ → 5′)-adenylyl-(2′ → 5′)-8-azidoadenosine* (**15**). For deprotection, 0.53 g (0.217 mmol) of **14** was first treated with 2% TsOH in CH_2Cl_2/MeOH 4:1 at r.t. for 30 min. The mixture was diluted with $CHCl_3$ (50 ml), washed with H_2O (2 × 25 ml), dried, and evaporated. The product was purified by silica-gel column chromatography (10 × 2 cm) eluting with $CHCl_3$/MeOH 50:1 to give 0.305 g (66%) of the detritylated trimer. The next deblocking steps were performed subsequently using 0.095 g (0.038 mmol) of the preceding material for reaction with 0.063 g (0.38 mmol) of 4-nitrobenzaldehyde oxime in dioxane/H_2O/Et_3N 1:1:1 (2.1 ml). After stirring at r.t. for 3 h, the solvent was evaporated, co-evaporated with toluene (3 × 10 ml), applied onto a silica-gel column (10 × 2 cm), and the phosphodiester eluted with $CHCl_3$/MeOH/Et_3N 7:1:1. The main fraction was evaporated, then co-evaporated with pyridine (2 × 10 ml), and the residue treated with 0.5M DBU in pyridine (5 ml). After 20 h stirring at r.t., the soln. was neutralized with 1M AcOH in pyridine (2.5 ml) and again evaporated. The residue was then stirred with dioxane/conc. NH_3 soln. 1:3 (20 ml) for 48 h, the solvents were removed, the residue was taken up in H_2O (50 ml) and then washed with $CHCl_3$ (2 × 25 ml). The aq. phase was applied onto a *DEAE Sephadex A-25* column (60 × 1 cm) and the product eluted with 0.16M $Et_3NH_2CO_3$ buffer (pH 7.5). The combined product fractions were collected, evaporated, and finally co-evaporated with H_2O several time to give 880 *O.D.* units of **15**. Thereof, 500 *O.D.* units were further purified by paper chromatography using i-PrOH/conc. NH_3/H_2O 6:1:3. The product band was cut out, eluted with H_2O, and lyophilized to give 250 *O.D.* units (50% recovery) of the ammonium salt of **15**.

11. *5′-*O-*[^{32}P]Phosphoryladenylyl-(2′ → 5′)-adenylyl-(2′ → 5′)-8-azidoadenosine* (**16**). Compound **15** was phosphorylated at the 5′-terminus by a known, slightly modified procedure [23]. The mixture contained [γ-^{32}P]ATP (0.5 mCi, 4500 Ci/mmol), 0.15 mM of **15**, T4 polynucleotide kinase (500 units/ml) in 0.05M *Tris*-HCl (pH 7.6), 10 mM $MgCl_2$, 0.1 mM EDTA, and 0.5 mM ATP (total volume 100 µl). Incubations were kept at 37° for 40 h. The

progress of the reaction was monitored by reverse-phase HPLC using the following solvent system: solvent *A* = 50 mM ammonium phosphate (pH 7.0); solvent *B* = MeOH/H_2O 1:1; linear gradient (beginning at *t* = 1 min 10% *B*; *t* = 31 min 70% *B*). Fractions (500 µl) were collected and the radioactivity was determined. The peak fractions containing [^{32}P]pA2′p5′A2′p5′N_3^8A (**16**) as indicated by radioactive content and UV measurement, were pooled, dialyzed against glass-distilled H_2O at 4°, and lyophilized. The concentration of **16** was determined on the basis of the extinction coefficient at 260 nm (ε_M = 36000).

REFERENCES

[1] A.H. Beiter, W. Pfleiderer, *Synthesis* **1989,** 497.
[2] R.J. Suhadolnik, K. Kariko, R.W. Sobol, S.W. Li, N.L. Reichenbach, B.E. Haley, *Biochemistry* **1988,** *27,* 8840.
[3] C.B. Reese, *Phosphorus Sulfur* **1976,** *1,* 245.
[4] J.H. van Boom, *Heterocycles* **1977,** *7,* 1197.
[5] C.B. Reese, *Tetrahedron* **1978,** *34,* 3143.
[6] R. Charubala, E. Uhlmann, W. Pfleiderer, *Liebigs Ann. Chem.* **1981,** 2392.
[7] M. Ichiba, R. Charubala, R.S. Varma, W. Pfleiderer, *Helv. Chim. Acta* **1986,** *69,* 1768.
[8] E.I. Kvasyuk, T.I. Kulak, N.B. Khripach, I.A. Mikhailopulo, E. Uhlmann, R. Charubala, W. Pfleiderer, *Synthesis* **1987,** 535.
[9] R. Charubala, E. Uhlmann, F. Himmelsbach, W. Pfleiderer, *Helv. Chim. Acta* **1987,** *70,* 2028.
[10] R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, H.G. Khorana, *J. Am. Chem. Soc.* **1966,** *88,* 819.
[11] W. Pfleiderer, H. Schirmeister, T. Reiner, M. Pfister, R. Charubala, in 'Biophosphates and their Analogues – Synthesis, Structure, Metabolism and Activity', Eds. K.S. Bruzik and W.J. Stec, Elsevier Science Publ. B.V., Amsterdam, 1987, p. 133.
[12] M. Pfister, W. Pfleiderer, *Nucleos. Nucleot.* **1987,** *6,* 505.
[13] R.E. Holmes, R.K. Robins, *J. Am. Chem. Soc.* **1965,** *87,* 1772.
[14] R.H. Silvermann, *Anal. Biochem.* **1985,** *144,* 450.
[15] K. Kariko, S.W. Li, R.W. Sobol, R.J. Suhadolnik, R. Charubala, W. Pfleiderer, *Biochemistry* **1987,** *26,* 7136.
[16] G. Floyd-Smith, O. Yoshie, P. Lengyel, *J. Biol. Chem.* **1982,** *257,* 8584.
[17] K. Kariko, J. Ludwig, *Biochem. Biophys. Res. Commun.* **1985,** *128,* 695.
[18] M. Knight, D.H. Wreschner, R.H. Silverman, I. Kerr, *Meth. Enzymol.* **1981,** *79,* 216.
[19] K. Kariko, R.W. Sobol, L. Suhadolnik Jr., S.W. Li, N.L. Reichenbach, R.J. Suhadolnik, R. Charubala, W. Pfleiderer, *Biochemistry* **1987,** *26,* 7127.
[20] U.K. Laemmli, *Nature (London)* **1970,** *227,* 680.
[21] G. Ziegler, I.M. Sprague, *J. Org. Chem.* **1951,** *16,* 621.
[22] M. Ikehara, S. Yamada, *Chem. Pharm. Bull.* **1971,** *19,* 104.
[23] S.I. Oshevski, *FEBS Lett.* **1982,** *143,* 119.