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Probing the Activation Site of Ribonuclease L with New N^6-Substituted 2′,5′-Adenylate Trimers

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Abstract—2-5A trimer [5′-monophosphoryladenylyl(2′-5′)adenylyl(2′-5′)adenosine] activates RNase L. While the 5′-terminal and 2′-terminal adenosine N^6-amino groups play a key role in binding to and activation of RNase L, the exocyclic amino function of the second adenylate (from the 5′-terminus) plays a relatively minor role in 2-5A's biological activity. To probe the available space proximal to the amino function of the central adenylate of 2-5A trimer during binding to RNase L, a variety of substituents were placed at that position. To accomplish this, the convertible building block 5′-*O*-dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-6-(2,4-dinitrophenyl)thioinosine 2′-(2-cyanoethyl*N*,*N*-diisopropylphosphoramidite) was prepared as a synthon to introduce 6-(2,4-dinitrophenyl)thioinosine into the middle position of the 2-5A trimer during automated synthesis. Post-synthetic treatment with aqueous amines transformed the (2,4-dinitrophenyl)thioinosine into N^6-substituted adenosines. Assays of these modified trimers for their ability to bind and activate RNase L showed that activation activity could be retained, albeit with some sacrifice compared to unmodified p5′A2′p5′A2′p5′A. Thus, the spatial domain about thisN^6-amino function could be available for modifications to enhance the biological potency of 2-5A analogues and to ligate 2-5A to targeting vehicles such as antisense molecules.

Introduction

The 2-5A-antisense [2-5A, general formula $p_n5'A2'(p5'A2')_mp5'A$] strategy offers a great potential approach to therapeutic agents capable of control of gene expression.[1–7] The chimeric antisense compound, which consists of a 5′-phosphorylated 2-5A moiety and a covalently attached 3′,5′-nucleic acid, peptide nucleic acid (PNA) or PNA-nucleic acid domain, effects the selective and specific cleavage of RNA both in cell-free systems and in intact cells.[1,8–16] The antisense domain binds to a complementary RNA sequence, while the 2-5A portion activates RNase L for cleavage of the targeted RNA substrate to which the 2-5A chimera is bound.

To enhance the biological activity of 2-5A-antisense oligonucleotides, several structural modifications have been examined. Thus, a 3′-3′-terminal phosphodiester bond in the antisense domain[6] reduced exonuclease degradation, as did modification with 3′-terminal phosphorothioate linkages[7,12] or the introduction of a single terminal hydroxyprolinol moiety.[14] Ligation of 2-5A to peptide nucleic acid (PNA) not only provided enhance resistance to degradation, but also increased affinity to target RNA.[13,16] Modification of the 2-5A 5′-terminal monophosphate group to a monothiophosphate reduced to likelihood of phosphatase inactivation of the 2-5A-antisense chimera construct.[8] While the foregoing modifications addressed affinity and enzymatic inactivation issues, the inherent reduction of 2-5A biological potency resulting from 2-5A-antisense construction has not been the subject of structural modification studies. Thus, previous reports have shown that there is an approximate 10- to 50-fold reduction in ability to activate human 2-5A-dependent RNase L when 2-5A is ligated to a oligonucleotide (or analogue) of usual antisense length (12–18-mers).[9,13,16] This is most probably a result of the 2-5A-dependent RNase L structural requirements for maximal activation by 2′,5′-oligoadenylates. The usually employed activators of RNase L are trimeric and tetrameric 2′,5′-oligoadenylates, but we have found recently that longer 2′,5′-oligoadenylates are significantly less active than their counterparts of shorter lengths. (S. Bayly and P. F. Torrence, unpub-

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lished observations). If the 2′-terminus of 2′,5′-oligoadenylates is indeed limiting for full expression of the activation of RNase L, might another mode of conjugation of the antisense oligonucleotide domain lead to more biologically potent 2-5A-antisense chimeras? In this study, we have explored the adenine base of 2-5A as a potential point for linkage to antisense molecules.

Various analogues of 2-5A have been synthesized to examine the crucial structural parameters of 2-5A for interaction with RNase L.[17,18] The adenine rings of 2-5A play a critical and differential role in the interaction of RNase L with 2-5A. Exchange of the first (from the 5′-terminus) adenine base with inosine to yield ppp5′I2′p5′A2′p5′A led to a large decrease both in activation ability (200-fold loss) and in RNase L binding affinity. The replacement of the 2′-terminal adenine base by hypoxanthine to form ppp5′A2′p5′A2′p5′I resulted in a 1000-fold drop in activation ability with little change in RNase L binding ability. Finally, the exchange of the middle nucleoside by inosine to give ppp5′A2′p5′I2′p5′A effected only a minor drop both in RNase L activation (20-fold loss) and binding ability (2–3-fold decrease).[19,20] A model for 2-5A interaction with RNase L was developed[17,18,20,21] that posited separate binding and activation steps associated with nuclease activation, with the various functional groups of 2-5A playing different roles in these separable steps. Thus, the N^6-amino function of the first nucleotide of 2-5A was essential for RNase L binding, without which no activation would be possible. The exocyclic amino function of the last adenosine (2′-terminal) was crucial for the activation of RNase L, but not for the separable binding step. In other words, without the 2′-terminal adenineN^6-amino group, a 2-5A congener might bind quite well to RNase L, but still not activate its nuclease function. In contrast, theN^6-amino function of the middle adenosine was much less critical for the biological activity of the 2-5A system, both for activation and for binding, so that this amino function might be a candidate for chemical modification.

Additional studies with uridylate-substituted 2-5A congeners confirmed these observations with inosinate-substituted oligonucleotides.[22] Moreover, Player et al.[21] showed that the 5′-terminal adenosine purine N-1 moiety was key for RNase L binding while the 2′-terminal adenosine exocyclic N^6-amino group was critical for RNase L activation.

To probe the spatial relationships around this 2-5A central adenylate N^6-amino group during the interaction of 2′,5′-oligoadenylates with RNase L, we have synthesized a variety of analogues bearing N^6-amino-substituents that vary in steric bulk and hydrophobic properties. Evaluation of the RNase L binding and activation abilities of these congeners has provided a clearer picture of ligand–receptor contacts.

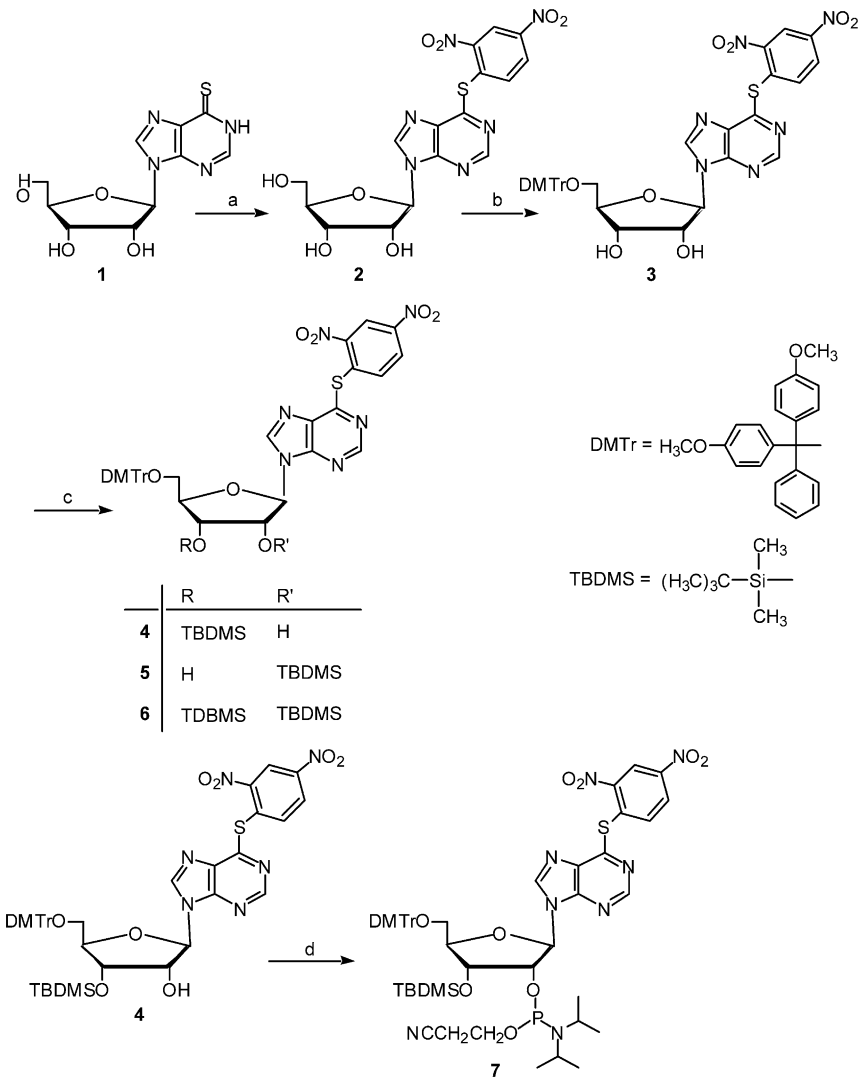
Results and Discussion

A simplified chemical approach to these various trimers recommends a post-synthetic conversion strategy, whereby a phosphoramidite of a versatile base is introduced into the oligomer sequence. After synthesis, the DNA is treated with appropriate nucleophiles, which displaces the leaving group of the modified base and converts the oligomer into a series of different substituted products (Scheme 1).[23–31] In 1992, Xu et al.[28] presented the incorporation of 6-(2,4-dinitrophenyl)thiodeoxyguanosine phosphoramidite as a convertible monomer into oligomers and their conversion into DNA containing deoxyguanosine modified at the 6-position with S-, N- and O-derivatives. This leaving group is reported to be stable towards the reagents of automated DNA synthesis and can be displaced under mild condition of room temperature. Subsequently, the same working group has extended this strategy for the preparation of correspondingN^6-substituted deoxyadenine analogues, using the phosphoramidite monomer of 6-(2,4-dinitrophenyl)thiodeoxyinosine.[29]

Herein, we have employed the (2,4-dinitrophenyl)thio leaving group for the generation of central adenylate-modified 2-5A trimers, which are subsequently referred to with the general formula p5′A2′p5′$A^{NRR'}$2′p5′A ($A^{NRR'}$, N^6-R,R′-substituted adenosine) (Fig. 1). This required the preparation of the versatile phosphoramidite **7**, its incorporation into RNA during automated synthesis giving p5′A2′p5′$A^{S\text{-}dnPh}$2′p5′A ($A^{S\text{-}dnPh}$, 6-(2,4-dinitrophenyl)thioinosine) (**8**) and the conversion of **8** into trimers p5′A2′p5′$A^{NRR'}$2′p5′A upon treatment with aqueous amine.

Monomer synthesis

The synthesis of phosphoramidite **7** (Scheme 1) began with the introduction of the 2,4-dinitrophenyl group into 6-mercaptopurine riboside (**1**) according to Xu et al.[28] The reaction of commercially available compound **1** with 2,4-dinitrofluorobenzene in presence of triethylamine resulted in 6-(2,4-dinitrophenyl)thioinosine (**2**) in 91% yield. Derivative **2** was then tritylated in the 5′-position with dimethoxytrityl chloride in the presence of DMAP/triethylamine to give 5′-*O*-dimethoxytrityl-6-(2,4-dinitrophenyl)thioinosine (**3**) in 84% yield. Subsequent silylation of compound **3** with *tert*-butyldimethylsilyl chloride and imidazole in DMF led to a mixture of all three possible products **4**–**6**, from which the 2′,3′-bis-*O*-substituted derivative **6** was isolated after silica gel column purification in 8% yield. For the separation of the mono-protected isomers **4** and **5**, a second column purification had to be performed. The desired 3′-*O*-*tert*-butyldimethylsilyl product **4** was obtained in a yield of 51% while the yield of the 2′-*O*-*tert*-butyldimethylsilyl side product was 27%. The assignments of structures of the respective 3′-*O*-TBDMS- and 2′-*O*-TBDMS-substituted isomers **4** and **5** were effected by ^{1}H NMR spectra irradiation experiments. By irradiation of the resonance frequency of the H–C(1′) proton, the signal of the neighbouring H–C(2′) proton was simplified. Subsequent irradiation of the resonance frequency of the H–C(2′) proton led to a simplification of the signals of the H–C(1′) and H–C(3′) protons. In this way, the assignments of the chemical shifts to the corresponding H–C(2′) or H–C(3′) protons



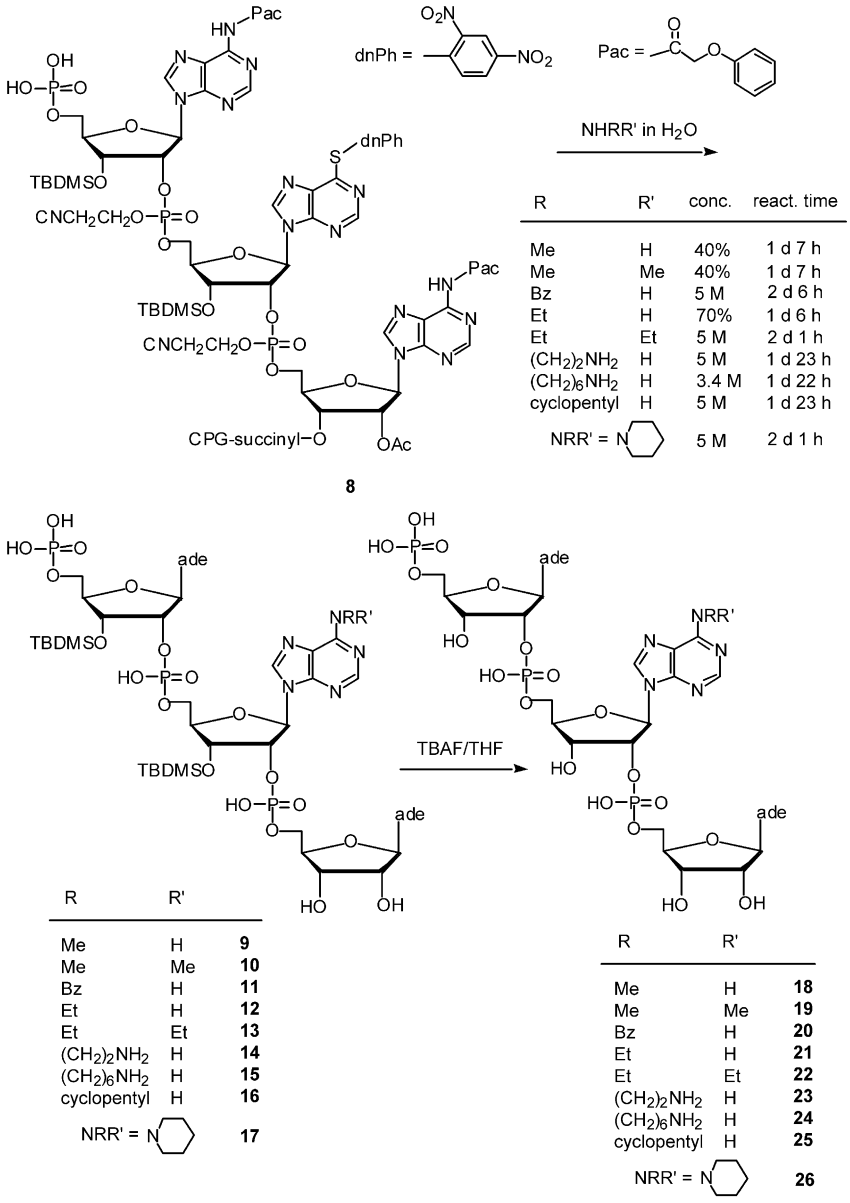
Scheme 1. Synthesis of 5′-*O*-dimethoxytrityl-6-(2,4-dinitrophenyl)-3′-*O*-(*tert*-butyldimethyl-silyl)thioinosine 2′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (**7**): (a) 2,4-dinitrofluorobenzene, Et_3N, CH_2Cl_2, 91%; (b) DMTrCl, DMAP, Et_3N, pyridine, 84%; (c) TBDMSCl, imidazole, DMF, 51% (**4**), 27% (**5**), 8% (**6**); (d) 2-cyanoethyl tetraisopropylphosphorodiamidite, tetrazole, CH_2Cl_2, 77%.

were unambiguous. By comparison of the chemical shifts for the H–C(2′) and H–C(3′) protons of the silylated isomers **4** and **5**, and of the unsilylated compound **3**, it was possible to determine the ^1H NMR spectra of the respective 3′-*O*-TBDMS- and 2′-*O*-TBDMS-substituted isomers **4** and **5**. Thereby silylation at a carbohydrate hydroxyl group led to a downfield movement of the chemical shift for the proton, which stands geminal to this silylated hydroxyl group. Irradiation of the resonance frequency of the H–C(2′) or H–C(3′) proton, respectively, resulted in a singlet for an eventually geminal standing OH-function instead of a doublet. Finally, phosphoramidite **7** was obtained in 77% yield by treatment of compound **4** with 2-cyanoethyl tetraisopropylphosphorodiamidite in presence of tetrazole in CH_2Cl_2.

Trimer synthesis and derivatization

The convertible trimer p5′A2′p5′A$^{S\text{-}dnPh}$2′p5′A (**8**) was synthesized on a DNA/RNA synthesizer using standard phosphoramidite chemistry as described earlier.[32–35] The convertible phosphoramidite **7**, which was prepared as a 0.1 M solution in dry CH_2Cl_2, was employed without modification to the coupling protocol. The stepwise condensation yield, as monitored by the released DMTr cation, generally ranged from 96% to 100%.

The structure of the resulting convertible p5′A2′p5′A$^{S\text{-}dnPh}$2′p5′A trimer **8** is illustrated in Scheme 2. In this form, oligomer **8** was protected at the exocyclic amino functions, the 3′-hydroxyl groups and at the internucleotide phosphate linkages, and was still attached to the solid support. The resin-bound intermediate **8** was treated with different aqueous base solutions at room temperature for 1–2 days. This base treatment effected simultaneously the displacement of the 6-(2,4-dinitrophenyl)thio leaving group, the cleavage of the oligomer from the solid support and, except for the TBDMS group, complete deblocking of the protection groups to give the functionalized trimers **9**–**17**. For displacement reactions involving volatile amines, the excess of base



Scheme 2. Derivatization and deprotection of convertible trimer **8**.

was removed simply by lyophilization. In all other cases, the reaction mixtures had to be neutralized with aqueous AcOH to avoid degradation of the RNA internucleotide linkages during evaporation. The resulting salts were removed with a C-18 SepPac cartridge, and the product-containing fractions combined and reduced to dryness. Subsequent cleavage of the 3′-*O*-TBDMS protecting group by treatment with TBAF/THF and semi-preparative purification of the products on a PRP-1HPLC column using standard methods,[34–37] led to the converted trimers **18**–**26**. In order to obtain completely pure 2-5A analogues for biological testing (oligomers **21** and **26** were especially problematical), an additional Dionex HPLC at pH 8 with a Tris–HCl/ammonium chloride gradient were performed. A final desalting step led to the desired trimers. The purity of oligonucleotides was analysed using both ion-pair exchange and reversed phase chromatography, while their identity was confirmed by mass spectrometry.

RNase L activation by 2-5A analogues

The ability of the N^6-substituted 2-5A analogues to activate RNase L for the degradation of poly(U) was determined in an in vitro assay, employing poly(U) as the substrate to be degraded. The concentrations EC_{50}, which cause cleavage of 50% of the poly(U) are listed in Table 1. To be able to compare the respective EC_{50} values, each was divided by the EC_{50} of the unsubstituted 2-5A trimer to give the term C_{rel} (Table 1). This term expresses the relative concentration of the other trimers needed to effect 50% cleavage. The higher the C_{rel}, the less effective the activation compared to the effectiveness of the standard 2-5A trimer, the C_{rel} of which is defined as 1.

Overall, the modified 2-5A trimers display a trend of decreased activity as the substituents at the amino function increased in steric bulk. Thus, the N^6-mono-

Table 1. Relative activities C_{rel} of the N^6-functionalized trimers to unsubstituted 2-5A compound in cleavage activity and binding assay

Compd	R	R′	EC_{50} (nM)[a]	C_{rel}[b]	IC_{50} (nM)[c]	C_{rel}[d]
Series 1						
18	Me	H	0.44±0.05 (4)	1.5	26 (2)	2.6
19	Me	Me	2.2±0.4 (4)	7.6	53–58 (2)	5.5
20	Bz	H	1.2±0.4 (3)	4.3	16–22 (2)	1.9
21	Et	H	0.65±0.20 (4)	2.2	27–33 (2)	3.0
p5′A2′p5′A2′p5′A			0.29±0.01 (4)	1.0	9.2–11 (2)	1.0
Series 2						
22	Et	Et	3.1±0.4 (4)	17		
23	$(CH_2)_2NH_2$	H	1.5±0.4 (4)	8.5		
24	$(CH_2)_6NH_2$	H	2.4±0.3 (4)	13		
25	Cyclopentyl	H	0.34±0.03 (4)	1.9		
26	NRR′ = 		1.8±0.6 (4)	10.2		
p5′A2′p5′A2′p5′A			0.18±0.04 (5)	1.0		

These values are given as the mean ± standard deviation, with the number of experiments (*n*) given in parentheses.
[a]EC_{50} is defined as the concentration of trimer required to cleave 50% of poly(U).
[b]C_{rel} is the EC_{50} value divided by the EC_{50} of the 2-5A standard, with 2-5A arbitrarily assigned a C_{rel} of 1.
[c]IC_{50} is defined as the concentration of trimer required to displace 50% of bound radioactive probe to RNase L.
[d]C_{rel} is the IC_{50} value divided by the IC_{50} of the 2-5A standard, with 2-5A arbitrarily assigned a C_{rel} of 1.

methylated trimer **18** possessed a C_{rel} of 1.5 while the dimethylated congener **19** displayed a decrease in activation ability with a C_{rel} of 7.6, a 5-fold activity drop. Similarly, the monoethylated trimer **21** showed a C_{rel} value of 2.2, only slightly less effective than unmodified 2-5A. In contrast, the diethylated analogue **22** was about 8-fold less active than **21** with a C_{rel} of 17. The trend continued with the monobenzyl-substituted compound **20** that showed a 4.3 reduction in activation ability compared to parent 2-5A trimer. However, a single N^6-cyclopentyl group (**25**) had less effect than the single benzyl substitution with a diminution in activity ($C_{rel}=1.9$) that was similar to that of the methyl or ethyl substitutions. This result may imply that the lone cyclopentyl moiety (in compound **25**) can be accommodated in the RNase L 2-5A activation site, but that the increased bulk of the benzyl group could not. That some critical steric threshold is involved is suggested by the significant decreases in activation upon dimethyl or diethyl substitution. In addition, the analogue **26** ($C_{rel}=10.2$) also demonstrated a major activity loss; however, its activity was significantly greater than the diethyl derivative **22**. It might be speculated that the restriction or 'tying back' of the methylene groups in the piperidine ring structure reduces the entropy cost associated with the accommodation in the RNase L activation site; however, its chair-like conformation would require greater space accommodation than the cyclopentyl substituent ($C_{rel}=1.9$).

The N^6-aminoethyl (**23**, $C_{rel}=8.5$) and N^6-aminohexyl (**24**, $C_{rel}=13$) congeners also suggest a role for steric bulk in the determination of activator potency. However, both analogues are significantly less effective activators than other monosubstituted derivatives This results suggest that a hydrophilic group could be less compatible with the 2-5A activator site than a more hydrophobic substituent.

Binding assays

The ability of certain of the modified 2-5A trimers to bind to RNase L was examined in a radiobinding assay for the set of compounds **18**–**21**. The assay ascertained the ability of the trimer to displace the radiolabelled probe p5′A2′(p5′A2′)$_2$p5′A3′[^{32}P]p5′C3′p from RNase L. The IC_{50} values, listed in Table 1, give the concentration required to inhibit radioactive probe binding by 50%. Here again, the data were normalized to the 2-5A standard, whose relative IC_{50} is defined as 1. According to the C_{rel} values, allN^6-substituted trimers **18**–**21** showed a decrease in binding ability compared to the unmodified 2-5A. There was a fair correlation between loss of RNase L binding and loss of ability to activate RNase L for the oligonucleotides **18**, **19**, and **21**. However, analogue **20**, the benzyl congener, displayed a contrary behavior with an activation ability that was less than would have been expected, based on the behavior of **18**, **19**, and **21**. Binding assay were not conducted on compounds **22**–**26**.

Conclusions

The foregoing experiments show a tendency to decreased RNase L activation ability as the N^6-substituents of the central adenylate of 2-5A increase in steric bulk. In addition, the data suggest a preference for hydrophobic substituents at that site. Thus, 2-5A trimers, which are appropriately substituted in the N^6-position of the central adenylate, are still capable of productive binding and activation 2-5A-dependent RNase L. Consequently, further modifications at this position could be possible, such as attachment of antisense deoxyoligonucleotides. 2-5A-Antisense chimeras with such a mode of linkage between the 2-5A moiety and the antisense domain have not yet been examined for their biological properties. To date, 2-5A-antisense chimeras have been constructed only through ligation of 2-5A either at the 5′-terminus or the 3′-terminus of the antisense chain. Since attachment of antisense to the central adenylate likely would alter the spatial orientation of RNase L relative the antisense-hybridized RNA target, this structural alteration may bring about different sites of RNA cleavage and could provide access to RNA sequences not available to 2-5A-antisense based on 5′- or 3′-terminus ligation. In any case, the results of this study open a path to a novel form of 2-5A-antisense; however, the present study suggests that while RNase L-active 2-5A-antisense may be generated by linking an antisense chain to the N^6-amino of the central adenylate of 2-5A, such chimeric molecules, like their 2-5A 2′-terminus-conjugatde counterparts,[9,13,16] would be significantly muted in their RNase L activation potency.

Experimental

Monomer synthesis

Chemical reagents were obtained from Aldrich (Milwaukee, WI, USA). All reactions were performed under an inert atmosphere of argon in anhydrous solvents (SureSeal packing, Aldrich) and used without further purification or treatment. Analytical thin layer chromatography was run on precoated plates of Whatman silica gel, 60 Å on polyester with fluorescence indicator (Whatman, Clifton, NJ, USA). The compounds were localized at 254 nm using a UV lamp and in case of the tritylated products by staining with TFA gas. Flash column chromatography was run on Merck silica gel 60 (0.040–0.063 mm). The melting point was measured on a Laboratory Devices, Mel-Temp II apparatus and is uncorrected. UV spectra were determined on a Hewlett Packard 8452 spectrophotometer. ^{1}H NMR spectra were recorded on a Varian Gemini 300 MHz, the ^{31}P NMR spectra was obtained on a Varian Mercury 300 MHz spectrometer. The chemical shifts are reported in parts per million (ppm) relative to internal tetramethylsilane in $CDCl_3$ or DMSO-d_6 (^{1}H NMR) or relative to H_3PO_4 (^{31}P NMR). The coupling constants (J) are reported in Hertz (Hz). Chemical ionisation (CI) mass spectrum using NH_3 was recorded on a Finnigan MAT 4600 apparatus. Fast atom bombardment (FAB) mass spectra were performed with nitrobenzylalcohol (NBA) as a matrix on an Extrel EL 400 spectrometer. Elemental analysis were carried out at Atlantic Microlab, Inc. (Norcross, GA, USA).

6-(2,4-Dinitrophenyl)thioinosine (2). To a suspension of 6-mercaptopurine riboside (**1**; 2.00 g, 7.04 mmol) in CH_3CN (135 mL) was added Et_3N (6 mL, 43 mmol) and 2,4-dinitrofluorobenzene (1.06 mL, 8.38 mmol). After stirring 1 h at room temperature, the then clear solution was concentrated under reduced pressure to a yellow oil, which was co-evaporated with CH_2Cl_2 (3 × 30 mL). The hereby precipitated solid was filtered by suction, washed with CH_2Cl_2 and diethylether, and dried in vacuo to give 2.40 g of **2**. The filtrate was concentrated to dryness and the resulting yellow oil was purified by flash chromatography [silica gel, 30 g, 2 × 16 cm, CH_2Cl_2 (100 mL), CH_2Cl_2/MeOH 100:1 (101 mL), 100:2 (102 mL), 100:3 (103 mL), 100:4 (208 mL), 100:5 (210 mL), 100:6 (212 mL)]: additional 480 mg of **2**. Total yield 2.88 g (91%). Compound **2** was employed in next reaction step without further purification, for elemental analysis 52 mg of **2** were recrystallized in CH_2Cl_2/MeOH 19:5 (6 mL). Slight yellow solid. Mp 182–185 °C (dec.). R_f 0.35 (toluene/AcOEt/MeOH 5:4:1). UV (MeOH) λ_{max} 336, 276 nm. ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm) 8.93 (d, J = 1.8 Hz, 1H, H–C(3) of 2,4-dinitrophenyl); 8.89 (s, 1H, H–C(2)); 8.77 (s, 1H, H–C(8)); 8.47 (dd, J = 8.7, J = 2.9 Hz, 1H, H–C(5) of 2,4-dinitrophenyl); 8.10 (d, J = 9 Hz, 1H, H–C(6) of 2,4-dinitrophenyl); 6.05 (d, J = 5.1 Hz, 1H, H–C(1′)); 5.57 (d, J = 6 Hz, 1H, OH–C(2′)); 5.27 (d, J = 6 Hz, 1H, OH–C(3′)); 5.10 (t, J = 5.4 Hz, 1H, OH–C(5′)); 4.65–4.59 (m, 1H, H–C(2′)); 4.22–4.18 (m, 1H, H–C(3′)); 4.01–3.98 (m, 1H, H–C(4′)); 3.74–3.67 (m, 1H, H–C(5′)); 3.62–3.34 (m, 1H, H–C(5′)). CI-MS calcd for $(C_{16}H_{14}N_6O_8S–H)^+$: 451.06; found: 451. Anal. calcd for $C_{16}H_{14}N_6O_8S$ (450.39): C 42.67, H 3.13, N 18.66; found: C 42.62, H 3.09, N 18.45.

5′-*O*-Dimethoxytrityl-6-(2,4-dinitrophenyl)thioinosine (3). Compound **2** (1.65 g, 3.66 mmol) was co-evaporated with pyridine (3 × 10 mL) and taken up in pyridine (30 mL). After addition of dimethoxytrityl chloride (1.51 g, 4.46 mmol), DMAP (23 mg, 0.19 mmol) and Et_3N (715 μL, 5.15 mmol), the solution was stirred for 6 h. An additional amount of dimethoxytrityl chloride (370 mg, 1.09 mmol) was added and after another 16.5 h of stirring, the reaction mixture was diluted with CH_2Cl_2 (80 mL) and washed with saturated $NaHCO_3$ (100 mL). The aqueous layer was separated, extracted with CH_2Cl_2 (3 × 100 mL) and the combined organic layer dried (Na_2SO_4), evaporated and co-evaporated with toluene (4 × 10 mL). The residue was purified by flash chromatography (silica gel, 50 g, 3.5 × 16 cm, CH_2Cl_2 (200×mL), CH_2Cl_2/MeOH 100:1 (202 mL), 100:2 (204 mL), 100:3 (206 mL), 100:4 (208 mL)) to give 2.31 g (84%) of product **3**. Orange foam. R_f 0.55 (toluene/AcOEt/MeOH 5:4:1). UV (MeOH) λ_{max} 342, 274, 230 (sh) nm. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 9.01 (d, J = 1.8 Hz, 1H, H–C(3) of 2,4-dinitrophenyl); 8.71 (s, 1H, H–C(2)); 8.33 (s, 1H, H–C(8)); 8.26 (dd, J = 8.7, J = 3 Hz, 1H, H–C(5) of 2,4-dinitrophenyl); 7.88 (d, J = 8.7 Hz, 1H, H–C(6) of 2,4-dinitrophenyl); 7.27–7.14 (m, 9H, DMTr); 6.75–6.72 (m, 4H, *o* to MeO); 6.04 (d, J = 5.7 Hz, 1H, H–C(1′)); 5.06 (d, J = 3 Hz, 1H, OH–C(2′)); 4.97–4.92 (m, 1H, H-C(2′)); 4.50–4.48 (m, 1H, H–C(3′)); 4.45 (m, 1H, H–C(4′)); 3.77 (s, 6H, 2 MeO); 3.45 (dd, J = 10.8, J = 3.9 Hz, 1H, H-C(5′)); 3.33 (dd, J = 10.8, J = 3 Hz, 1H, H–C(5′)); 2.92 (broad s, 1H, OH-C(3′)). FAB-MS calcd for $(C_{37}H_{32}N_6O_{10}S–H)^+$: 753.20; found: 753. Anal. calcd for $C_{37}H_{32}N_6O_{10}S·1/2\ H_2O$ (761.77): C 58.34, H 4.37, N 11.03; found: C 58.14, H 4.38, N 10.77.

5′-*O*-Dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-6-(2,4-dinitrophenyl)thioinosine (4), 5′-*O*-dimethoxytrityl-2′-*O*-(*tert*-butyldimethylsilyl)-6-(2,4-dinitrophenyl)thioinosine (5) and 5′-*O*-dimethoxytrityl-2′,3′-bis-*O*-(*tert*-butyldimethylsilyl)-6-(2,4-dinitrophenyl)thioinosine (6). A solution of compound **3** (2.29 g, 3.04 mmol), imidazole (505 mg, 7.42 mmol) and *tert*-butyldimethylsilyl chloride (590 mg, 3.91 mmol) in DMF (5 mL) was stirred at room temperature overnight (17 h). After dilution with CH_2Cl_2 (80 mL), the solution was washed with saturated NaCl (100 mL). The aqueous layer was separated, extracted with CH_2Cl_2 (3 × 100 mL) and the combined organic layer dried (Na_2SO_4), evaporated and co-evaporated with toluene (4 × 10 mL). Separation of product mixture was achieved by flash chromatography [silica gel, 60 g, 3.5 × 20 cm, toluene (200 mL), toluene/AcOEt 18:1 (190 mL), 17:1 (180 mL), 16:1 (170 mL), 14:1 (150 mL), 12:1 (130 mL), 10:1 (110 mL), 9:1 (100 mL), 8:1 (90 mL), 6:1 (70 mL)]. First 244 mg (8%) of the 2′,3′-bis-*O*-substituted derivative **6** was eluted, then compound **5** and finally the 3′-*O*-substituted product **4**. A mixture of isomers **4** and **5** was separated by a second flash chromatography [silica gel, 15 g, 1.4 × 19 cm, toluene (100 mL), toluene/AcOEt 18:1 (95 mL),

17:1 (90 mL), 16:1 (85 mL), 14:1 (75 mL), 12:1 (65 mL)] to give a total yield of 1.34 g (51%) of **4** and 716 mg (27%) of **5**.

4. Yellow foam. R_f 0.19 (toluene/AcOEt 9:1). UV (MeOH) λ_{max} 340, 276, 228 (sh) nm. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 8.99 (d, J = 3 Hz, 1H, H–C(3) of 2,4-dinitrophenyl); 8.75 (s, 1H, H–C(2)); 8.33 (s, 1H, H–C(8)); 8.15 (dd, J = 8.7, J = 3 Hz, 1H, H–C(5) of 2,4-dinitrophenyl); 7.72 (d, J = 8.7 Hz, 1H, H–C(6) of 2,4-dinitrophenyl); 7.37–7.18 (m, 9H, DMTr); 6.79–6.77 (m, 4H, *o* to MeO); 6.08 (d, J = 4.8 Hz, 1H, H–C(1′)); 4.86 (t, J = 5 Hz, 1H, H–C(2′)); 4.69 (t, J = 4.4 Hz, 1H, H–C(3′)); 4.19–4.17 (m, 1H, H–C(4′)); 3.78 (s, 6H, 2 MeO); 3.53 (dd, J = 10.8, J = 3 Hz, 1H, H–C(5′)); 3.25 (dd, J = 11.1, J = 3.5 Hz, 1H, H–C(5′)); 3.10 (d, J = 6 Hz, 1H, OH–C(2′)); 0.91 (s, 9H, CMe_3); 0.12 (s, 3H, SiMe); 0.04 (s, 3H, SiMe). FAB-MS calcd for $(C_{43}H_{46}N_6O_{10}SSi–H)^+$: 867.28; found: 867. Anal. calcd for $C_{43}H_{46}N_6O_{10}SSi$ (867.02): C 59.57, H 5.35, N 9.69; found: C 59.66, H 5.47, N 9.58.

5. Yellow foam. R_f 0.28 (toluene/AcOEt 9:1). UV (MeOH) λ_{max} 342, 276, 226 (sh) nm. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 9.01 (d, J = 1.8 Hz, 1H, H–C(3) of 2,4-dinitrophenyl); 8.66 (s, 1H, H–C(2)); 8.33 (s, 1H, H–C(8)); 8.35–8.32 (m, 1H, H–C(5) of 2,4-dinitrophenyl); 7.91 (d, J = 8.7 Hz, 1H, H–C(6) of 2,4-dinitrophenyl); 7.44–7.18 (m, 9H, DMTr); 6.83–6.80 (m, 4H, *o* to MeO); 6.12 (d, J = 5.1 Hz, 1H, H–C(1′)); 4.99 (t, J = 4.8 Hz, 1H, H–C(2′)); 4.42–4.38 (m, 1H, H–C(3′)); 4.30–4.28 (m, 1H, H–C(4′)); 3.79 (s, 6H, 2 MeO); 3.55 (dd, J = 10.8, J = 3 Hz, 1H, H–C(5′)); 3.41 (dd, J = 10.8, J = 3.9 Hz, 1H, H–C(5′)); 2.67 (d, J = 3.9 Hz, 1H, OH–C(3′)); 0.84 (s, 9H, CMe_3); 0.01 (s, 3H, SiMe);−0.13 (s, 3H, SiMe). FAB-MS calcd for $(C_{43}H_{46}N_6O_{10}SSi–H)^+$: 867.28; found: 867. Anal. calcd for $C_{43}H_{46}N_6O_{10}SSi$ (867.02): C 59.57, H 5.35, N 9.69; found: C 59.40, H 5.43, N 9.64.

6. Yellow foam. R_f 0.66 (toluene/AcOEt 9:1). UV (MeOH) λ_{max} 344, 276, 226 (sh) nm. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 9.01 (d, J = 3 Hz, 1H, H–C(3) of 2,4-dinitrophenyl); 8.68 (s, 1H, H–C(2)); 8.35 (s, 1H, H–C(8)); 8.24 (dd, J = 8.7, J = 3 Hz, 1H, H–C(5) of 2,4-dinitrophenyl); 7.80 (d, J = 7.8 Hz, 1H, H–C(6) of 2,4-dinitrophenyl); 7.45–7.19 (m, 9H, DMTr); 6.83–6.80 (m, 4H, *o* to MeO); 6.07 (d, J = 4.8 Hz, 1H, H–C(1′)); 4.87 (t, J = 5 Hz, 1H, H–C(2′)); 4.33–4.31 (m, 1H, H-C(3′)); 4.26–4.25 (m, 1H, H–C(4′)); 3.79 (s, 6H, 2 MeO); 3.57 (dd, J = 10.8, J = 3.9 Hz, 1H, H-C(5′)); 3.32 (dd, J = 10.8, J = 3.9 Hz, 1H, H–C(5′)); 0.87 (s, 9H, CMe_3); 0.78 (s, 9H, CMe_3); 0.07 (s, 3H, SiMe); 0.00 (s, 3H, SiMe);−0.03 (s, 3H, SiMe);−0.28 (s, 3H, SiMe). FAB-MS calcd for $(C_{49}H_{60}N_6O_{10}SSi_2–H)^+$: 981.37; found: 981. Anal. calcd for $C_{49}H_{60}N_6O_{10}SSi_2$ (981.29): C 59.98, H 6.16, N 8.56; found: C 60.19, H 6.20, N 8.40.

5′-*O*-Dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-6-(2,4-dinitrophenyl)thioinosine 2′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) (7). A mixture of compound **4** (1.30 g, 1.50 mmol), tetrazole (58 mg, 0.83 mmol) and 2-cyanoethyl tetraisopropylphosphorodiamidite (0.7 mL, 2.20 mmol) was stirred in CH_2Cl_2 (10 mL) under argon at room temperature overnight (17 h). After dilution with CH_2Cl_2 (80 mL), the solution was washed with saturated $NaHCO_3$ (100 mL). The aqueous phase was separated, extracted with CH_2Cl_2 (3 × 100 mL) and the combined organic layer dried (Na_2SO_4), evaporated and the crude product purified by flash chromatography (silica gel, 50 g, 3 × 13 cm, petroleum ether/acetone 9:1 (200 mL), 8:1 (180 mL), 7:1 (160 mL), 6:1 (140 mL), 5:1 (120 mL), 9:2 (220 mL), 4:1 (200 mL), 7:2 (180 mL)): 1.23 g (77%) of **7**. Yellow foam. R_f 0.24 (toluene/AcOEt 9:1), 0.43 (petroleum ether/acetone 3:1). UV (MeOH) λ_{max} 344, 276, 230 (sh) nm. ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm) 8.98–8.97 [m, 1H, H–C(3) of 2,4-dinitrophenyl); 8.78, 8.74 (2s, 1H, H-C(2)]; 8.39, 8.37 (2s, 1H, H–C(8)); 8.04, 7.99 (2dd, J = 9, J = 8.7, J = 3, J = 2.9 Hz, 1H, H–C(5) of 2,4-dinitrophenyl); 7.63, 7.56 (2d, J = 9, J = 9 Hz, 1H, H–C(6) of 2,4-dinitrophenyl); 7.38–7.18 (m, 9H, DMTr); 6.80–6.76 (m, 4H, *o* to MeO); 6.32, 6.23 (2d, J = 3.9, J = 3.9 Hz, 1H, H–C(1′)); 5.13–5.09, 5.01–4.95 (2m, 1H, H–C(2′)); 4.73, 4.67 (2t, J = 4.8, J = 4.5 Hz, 1H, H–C(3′)); 4.25–4.15 (m, 1H, H–C(4′)); 3.78, 3.78 (2s, 6H, 2 MeO); 3.68–3.44 (m, $CNCH_2CH_2$, 2 $NCHR_2$, H–C(5′)); 3.23 (dd, J = 11.1, J = 3.5 Hz, 1H, H–C(5′)); 2.52, 2.41 (2t, J = 6.5, J = 6.5 Hz, 2H, $CNCH_2CH_2$); 1.33–1.10 (m, 12H, 2 CMe_2); 0.86, 0.85 (2s, 9H, CMe_3); 0.16, 0.06 (2s, 3H, SiMe); 0.13, 0.06 (2s, 3H, SiMe). ^{31}P NMR ($CDCl_3$, 121.4 MHz) δ (ppm) 151.14, 150.39. FAB-MS calcd for $(C_{52}H_{63}N_8O_{11}PSSi–H)^+$: 1067.39; found: 1067. Anal. calcd for $C_{52}H_{63}N_8O_{11}PSSi$ (1067.24): C 58.52, H 5.95, N 10.50; found: C 58.56, H 6.28, N 10.42.

Synthesis and derivatization of p5′A2′p5′A$^{S\text{-}dnPh}$2′p5′A

The synthesis of the 2-5A trimer followed the strategy described earlier.[32–35] All DNA synthesis reagents, the long chain alkylamino-controlled pore glass solid support (2′-*O*-acetyl-5′-*O*-dimethoxytrityl-N^6-phenoxyacetyladenosine-3′-lcaa-CPG, 500 Å), and the phosphorylation reagent for the 5′-terminus of the trimer (2-[2-*O*-(4,4′-dimethoxytrityl)ethylsulfonyl]ethyl-2′-(cyanoethyl *N*,*N*-diisopropylphosphoramidite)) were purchased from Glen Research (Sterling, VA, USA). The building block for the 2′,5′-adenylate domain (5′-*O*-dimethoxytrityl-3′-*O*-(*tert*-butyldimethylsilyl)-N^6-phenoxyacetyladenosine 2′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite)) was obtained from Pharmacia Biotech (Piscataway, NJ, USA). All phosphoramidites were used as 0.1 M solutions in CH_3CN, except for the functionalized phosphoramidite **7**, which was for solubility reasons employed as a 0.1 M solution in CH_2Cl_2 and connected at the optional port.

The trimer p5′A2′p5′A$^{S\text{-}dnPh}$2′p5′A (**8**) was synthesized on a 1.0 μM scale on a DNA/RNA synthesizer (ABI 392) under 4,5-dicyanoimidazole activation using standard reaction cycles: 1.0 μm RNA for chain elongation (600 s coupling time) and 1 μM Phosphorylation for 5′-terminal phosphorylation (60 s condensation time).

After the synthesis in the trityl-off modus, the oligonucleotide was washed with CH_3CN and dried under a stream of argon. The solid support with the attached trimer was treated with 2 mL of aqueous base solution in a screwed capped glass vial for 1–2 days at room temperature (base concentrations and reaction times are given in detail in Scheme 2). In the cases of methylamine, dimethylamine and ethylamine, the displacement mixture was separated from the resin by filtration over cotton wool and the excess of base was removed by lyophilization. All other amines were neutralized (pH 7) with 50% aqueous acetic acid under ice-bath cooling (about 1 equiv AcOH to base) before separation from resin and evaporation on a Speed-vac concentrator. The solutions were desalted using C-18 SepPak cartridges (12 g per 0.5 μmol scale). The loaded cartridge was washed with H_2O (40 mL), 5% MeOH/H_2O (20 mL), 10% MeOH/H_2O (20 mL) and 50% MeOH/H_2O (70–80 mL). 10 mL fractions were pooled and the oligomer was usually found in the fractions of 50% MeOH/H_2O, seldom of 10% MeOH/H_2O. For TBDMS cleavage, 1 M TBAF/THF (1 mL per 0.5 μmol scale) was added to the concentrated residues **9** to **17** for about 20 h at room temperature, then the reaction was stopped by addition of 1 mL H_2O under ice-bath cooling and the completely unprotected trimers **18–26** were lyophilized. The crude product was purified on a semi-prep Hamilton PRP-1 column (7 × 300 mm) (Hamilton, Reno, NV, USA): Solvent A: 10 mM tetrabutylammonium phosphate (TBAP), pH 7.5 in H_2O; solvent B: 10 mM TBAP, pH 7.5 in CH_3CN/H_2O 8:2; gradient: convex from 5% B to 95% B in 50 min; flow rate: 5 mL/min. Purified products were desalted with C-18 SepPak cartridges. The loaded cartridge was washed with H_2O (20 mL), 5% MeOH/H_2O (10 mL), 10% MeOH/H_2O (10 mL) and 50% MeOH/H_2O (20 mL). Solvents were collected in 5-mL fractions and the oligomer, usually found in fractions 9 and 10 (50% MeOH/H_2O) was concentrated on a Speed-vac concentrator. For additional purification, a Dionex NucleoPac PA-100 (9 × 250) column was used (Dionex, Sunnyvale, CA, USA): Solvent A: 25 mM Tris–HCl, pH 8 in H_2O; solvent B: 25 mM Tris and 1 M ammonium chloride, pH 8 in H_2O; gradient: isocratic 0% B for 2 min, linear from 0% B to 50% B in 38 min, linear from 50% B to 100% B in 10 min, isocratic 100% B for 5 min, linear from 100% B to 0% B in 3 min and isocratic 0% B for 5 min; flow rate: 1 mL/min. Trimers were finally desalted on a semi-prep Hamilton PRP-1 column (7 × 300 mm): Solvent A: 0.1 M triethylammonium acetate (TEAAc), pH 7 in H_2O; solvent B: MeOH; gradient: isocratic 0% B for 10 min, convex from 0% B to 100% B in 30 min; flow rate: 5 mL/min. The solutions of the resulting 2-5A trimers were quantitated as OD A_{260} by UV spectrophotometry using approximately the extinction coefficient for unmodified p5′A2′p5′A2′p5′A as reported earlier.[19]

Analysis of trimers

The ion-exchange method employed a Dionex NucleoPac PA-100 (4 × 250 mm) column: Solvent A: 25 mM Tris–HCl, pH 8 in H_2O; solvent B: 25 mM Tris and 1 M ammonium chloride, pH 8 in H_2O; gradient: isocratic 0% B for 2 min, linear from 0% B to 50% B in 18 min, linear from 50% B to 100% B in 10 min, isocratic B for 5 min, linear from 100% B to 0% B in 3 min and isocratic 0% B for 5 min; flow rate: 1 mL/min. For RP-18 analysis a Beckman Ultrasphere ODS (4.6 × 250 mm) column was used (Beckman, Fullerton, CA, USA): Solvent A: 50 mM ammonium phosphate, pH 7; solvent B: MeOH/H_2O 1:1; gradient: linear from 0% B to 50% B in 50 min, linear from 50% B to 0% B in 10 min, isocratic 0% B for 5 min; flow rate: 1 mL/min. The molecular weights were detected by electrospray mass spectrometry on a HP 1100 MSD and correspond to the calculated molecular masses for trimers **18** to **26**. The analytical data are listed in Table 2.

RNase L activation and cleavage of polyuridylic acid [Poly(U)] substrate

Pure recombinant human RNase L was prepared by a modification of a previously described procedure.[38] Poly(U) was obtained commercially as a mixture of high molecular weight uridine polymers. Using T4 RNA ligase, the poly(U) was 3′-labeled with 5′-[^{32}P]pCp and then HPLC purified. This procedure and the assay have been described previously.[38,39] 2 μL of a 10 × cleavage buffer (100 mM HEPES, pH 7.5, 1.0 M KCl, 50 mM $Mg(OAc)_2$, 10 mM ATP, and 143 mM 2-mercaptoethanol) and 12–16 μL of RNase-free water were used in each cleavage reaction. To this, 2 μL of a 10 × solution of 2-5A analogue (final concentrations 10^{-5}–10^{-10} M) and recombinant RNase L enzyme (final concentration 100 nM) were added, and lastly 2 μL of poly(U)-[^{32}P]pCp substrate (final concentration 10 μM in UMP equivalents) to make a final volume of 20 μL. After a 15-min incubation at 30 °C, 4 volumes of 5 mg/mL car-

Table 2. Physical data of trimers

Compd	Dionex pH 8	RP-18 pH 7	Empirical formula	ESPRAY-MS	
	R_t (min)	R_t (min)		Calcd	Found
18	9.3	16.7	$C_{31}H_{40}N_{15}O_{19}P_3$	1019.18	1019.50
19	9.2	19.9	$C_{32}H_{42}N_{15}O_{19}P_3$	1033.20	1033.36
20	9.9	42.5	$C_{37}H_{44}N_{15}O_{19}P_3$	1095.21	1095.4
21	9.3	21.4	$C_{32}H_{42}N_{15}O_{19}P_3$	1033.20	1033.6
22	9.3	34.0	$C_{34}H_{46}N_{15}O_{19}P_3$	1061.23	1061.49
23	7.5	15.6	$C_{33}H_{45}N_{15}O_{19}P_3$	1048.22	1048.43
24	7.4	26.2	$C_{37}H_{53}N_{15}O_{19}P_3$	1104.28	1104.49
25	9.4	37.1	$C_{35}H_{46}N_{15}O_{19}P_3$	1073.23	1073.47
26	9.3	36.8	$C_{35}H_{46}N_{15}O_{19}P_3$	1073.23	1073.47

rier (yeast) RNA was added, and then 10 M ammonium acetate to a final concentration of 2–2.5 M. After mixing with 2 volumes of cold ethanol, the reaction mixtures were left on ice for 30 min, and the precipitated RNA pelleted with a brief spin at 4 °C (12,000 *g* for 2 min). The presence of cleaved fragments of poly(U)-[^{32}P]pCp was assessed by counting aliquots of the supernatant in scintillation fluid.

Radiobinding assays

Crude lysate from insect cells (SF21), expressing a low concentration of recombinant human RNase L, was prepared according to previously described methodology.[40] The radiobinding assay probe p5′A2′(p5′A2′)$_2$p5′A3′[^{32}P]p5′C3′p, was synthesized by the T4 RNA ligase-catalyzed addition of [^{32}P]5′pCp to the 3′ end of 2-5A, using a published procedure with subsequent HPLC purification.[38,39] To assay a given 2-5A analogue, serial dilutions were prepared in water. Each binding assay consisted of 5 μL of 2-5A analogue, 10 μL of SF21 cell lysate, and 10 μL of a freshly prepared master mix that consisted of concentrated Tris buffer, ATP stock (pH 7.0), and sufficient 2-5A-[^{32}P]pCp probe to give final reaction concentrations of 10 mM Tris–HCl (pH 7.6), 85 mM KCl, 2 mM $Mg(OAc)_2$, 0.2 mM ATP, 5% (v/v) glycerol and ~0.1 nM probe. The order of addition for each assay was 2-5A or analogue, then master mix, then lysate. Assay mixtures were incubated at 4 °C for 2 h, after which they were applied to nitrocellulose filters pre-rinsed in 1 × Tris buffer (described above). Samples were left to stand for ~3 min, then washed (3 ×) with water. The filters were placed in scintillant and counted in a liquid scintillation counter.[38,39]

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