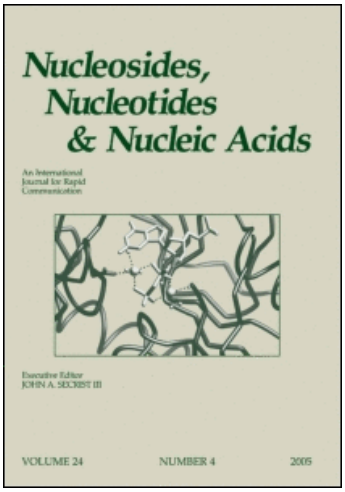

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SYNTHESIS AND RNASE L BINDING AND ACTIVATION OF A 2-5A-(5′)-DNA-(3′)-PNA CHIMERA, A NOVEL POTENTIAL ANTISENSE MOLECULE

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ABSTRACT: Fully automated solid-phase synthesis gave access to a hybrid in which 5′-phosphorylated-2′-5′-linked oligoadenylate (2-5A) is connected to the 5′-terminus of DNA which, in turn, is linked at the 3′-end to PNA [2-5A-(5′)-DNA-(3′)-PNA chimera]. This novel antisense molecule retains full RNase L activation potency while suffering only a slight reduction in binding affinity.

INTRODUCTION

Since their introduction in 1991, peptide nucleic acids (PNAs)[1] have received increasing attention for application in a antisense strategy. PNAs are stable towards enzymatic degradation[2] and recognize target RNA sequences with high affinity and specificity.[3] However, since the PNA•RNA duplex is not a substrate for RNase H, which cleaves the RNA in a DNA•RNA duplex, inhibition of gene expression by PNA is limited to steric blocking.[4]

In our laboratory, several strategies have been explored to overcome this drawback.[5] For example, we reported the synthesis of several classes of PNA-DNA chimeras.[6] In this type of hybrids, the PNA part is responsible for high affinity recognition of the target mRNA and provides protection against degradation by exonucleases,[7] which are the most

prominently active of the nucleases in cells and serum.[8,9] The presence of a DNA window in the conjugate leads to activation of RNase H upon complex formation with mRNA,[10] prevents self-aggregation and improves the solubility and cellular uptake of the molecule.[11] Alternatively, covalent attachment of 5′-phosphorylated-2′-5′-linked oligoadenylate [(pp)p5′A2′(p5′A2′)$_n$p5′A or 2-5A] to the amino terminus of PNA gave access to an antisense probe with the ability to induce rapid degradation of mRNA through activation of ubiquitous latent RNase L.[12]

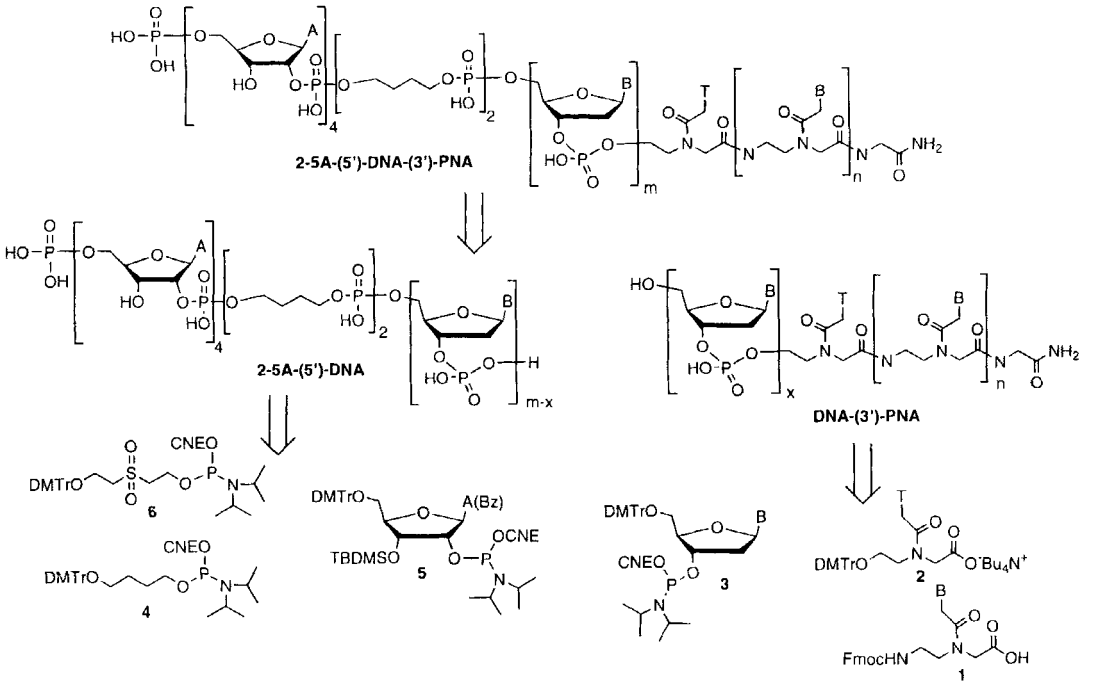
The merits of the two individual constructs mentioned above were an incentive for the design and synthesis of a hybrid [*i.e.* a 2-5A-(5′)-DNA-(3′)-PNA chimera] in which 2-5A is connected to the 5′-terminus of DNA which, in turn, is linked at the the 3′-end to PNA. Such hybrids would have the ability of degrading their target sequences *via* activation of both RNase L and RNase H. In addition, the PNA part provides protection against digestion by 3′-exonucleases and it may be expected that the presence of the 2-5A moiety protects the DNA from degradation by 5′-exonucleases.

In this report, we present the first fully automated solid-phase synthesis of a 2-5A-(5′)-DNA-(3′)-PNA chimera, namely p2-5A-bu$_2$-AAA.AAt*.ggg.gca.aat.a-GlyNH$_2$ (*i.e.* **14** in Scheme 2), complementary to a consensus sequence in the RNA of the respiratory syncytial virus (RSV), identified previously as a suitable target for regulation of gene expression by 2-5A-antisense conjugates.[13] In addition, it will be shown that this novel construct retains full capacity in binding and activation of RNase L.

RESULTS AND DISCUSSION

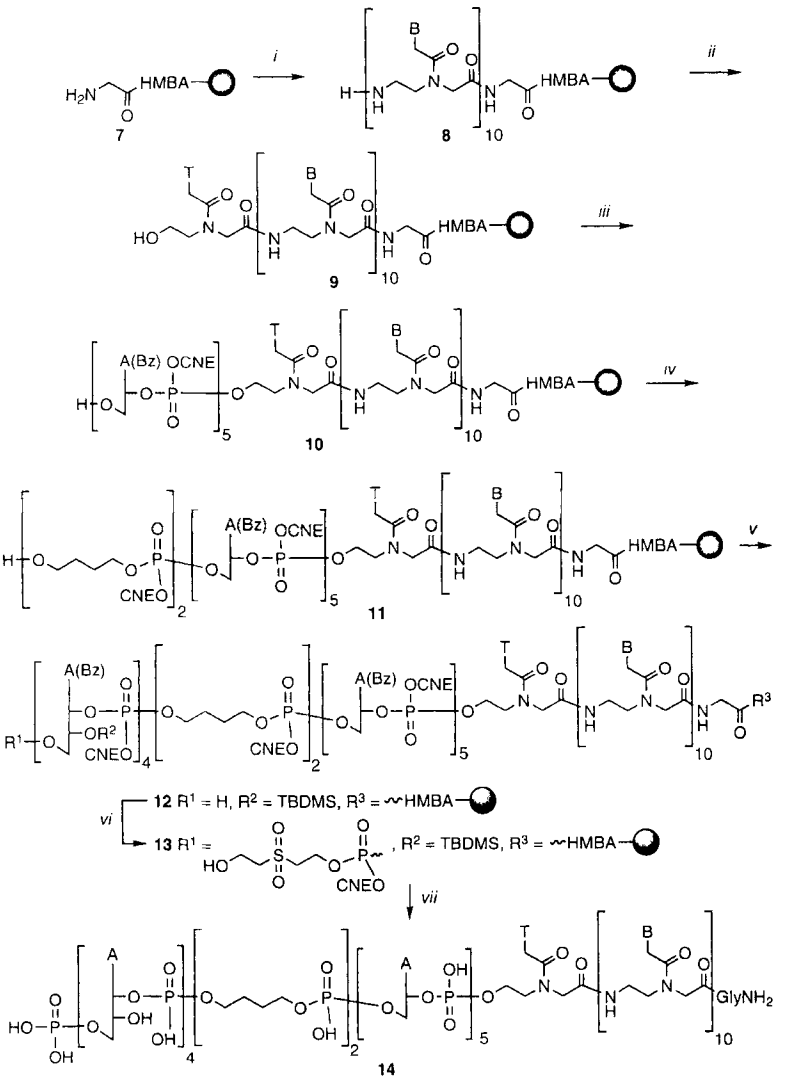
Retrosynthetic inspection of the target structure reveals that an on-line solid-phase synthesis approach is feasible by adapting the synthetic procedures devised for the construction of 2-5A-(5′)-DNA and DNA-(3′)-PNA chimeras (Scheme 1). Consequently, the DNA-PNA part will be accessible using building blocks **1**-**3** and the 2-5A-DNA domain from phosphoramidites **3**-**6**.

The assembly of the 2-5A-(5′)-DNA-(3′)-PNA chimera was performed on a fully automated DNA synthesizer on a 1 μmol scale starting from a glycine unit (**7**, Scheme 2), anchored *via* a base-labile 4-hydroxymethylbenzoic acid (HMBA) linker to highly cross-linked polystyrene beads. As outlined above, the PNA part of the molecule was



SCHEME 1

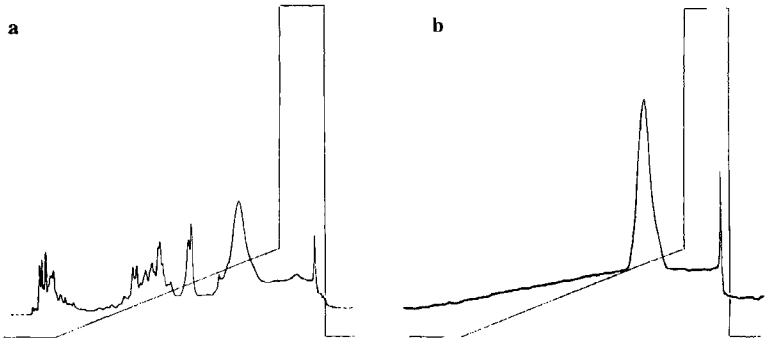
constructed with Fmoc/acyl protected PNA monomers[14] **1** using HATU as the condensing agent to give PNA decamer **8**. The hydroxyethylglycine linker was appended to the resin-bound decamer by coupling of building block **2**[15] followed by detritylation. Sequential elongation of **9** with DNA phosphoramidite **3** {B=A(Bz)} using 5-(2-nitrophenyl)tetrazole as the activator led to the immobilized DNA-(3')-PNA chimera **10**. At this stage, two butanediol spacers were linked to the 5'-end of oligomer **10** using known 4-(4,4'-dimethoxytrityloxy)butyl-2-cyanoethyl-*N,N*-di-*iso*-propyl phosphoramidite (**4**)[16] to give resin-bound **11**. Next, **11** was extended with the 2-5A-moiety using 2'-phosporamidite **5** in four consecutive coupling cycles to provide immobilized 2-5A-(5')-DNA-(3')-PNA **12**. A masked 5'-terminal phosphate function was introduced by coupling of 2-[2-(4,4'-dimethoxytrityloxy)ethanesulfonyl]ethyl-2-cyanoethyl-*N,N*-di-*iso*-propyl phosphoramidite (**6**) to give the protected target structure **13**. Ammonolysis (NH_3/MeOH) of **13** resulted in release from the resin and concomitant deprotection of the exocyclic amines and phosphates. The silyl protective groups were removed with triethylammine



Reagents and conditions: *i)* Elongation with **1**. *ii)* Elongation with **2**. *iii)* Elongation with **3**. *iv)* Elongation with **4**. *v)* Elongation with **5**. *vi)* Elongation with **6**. *vii)* 1. NH_3/MeOH, 16h, 50 °C. 2. Et_3N/HF/NMP, 1.5h, 65 °C. For the steps involved in the different elongation cycles, see Experimental Section.

SCHEME 2

trihydrofluoride to give, after desalting (G25) and reversed-phase high performance liquid chromatography purification (RP-HPLC), the 2-5A-(5′)-DNA-(3′)-PNA chimera **14**. The homogeneity of the chimera was confirmed by analytical HPLC (Fig. 1) as well as polyacrylamide gel electrophoresis (PAGE).



*HPLC pattern of crude (a) and purified (b) 2-5A-(5')-DNA-(3')-PNA chimera **14**.*

FIG. 1

Having the chimera at our disposal, the biological relevance of this new molecular architecture was explored. To this end, two different methodologies were employed.[17,18] In the first assay,[17] competition of 2-5A-antisense constructs (**14-16**) with p5'A2'p5'A2'p5'A2'p5'A3'[^{32}P]p5'C3'p provided a measure of the ability of the modified 2-5A-antisense to bind to human RNase L. In this assay, the parent 2-5A trimer **15** had an IC_{50} value of 7.3 nM (entry 1 in Table 1). Covalent attachment of a DNA antisense sequence to 2-5A (as in **16**) led to a small (4-fold) decline in binding affinity (entry 2). A slightly larger decrease in binding was observed when the isosequential 2-5A-DNA-PNA chimera was used. It turned out that compound **14** was three times less effective, compared to the native 2-5A-DNA sequence **16**, in replacing the radiolabeled 2-5A-probe.

In the second assay,[18] the relative ability of the analogs in inducing cleavage of poly(U)p[^{32}P]Cp *via* activation of RNAse L was explored. It was established that the unsubstituted 2-5A **15** was a very effective inducer (EC_{50} value of 0.30 nM) of RNase L mediated cleavage of the RNA substrate. In this model, the activation potency of the parent 2-5A-DNA hybrid **16** is two orders of magnitude lower. Interestingly, the 2-5A-DNA-PNA chimera **14** was not significantly different in activation ability, in spite of its somewhat less effective binding.

TABLE 1. Binding and activation of RNase L by 2-5A derivatives **14-16**.

Entry	Compound	Sequence[a]	IC_{50} (nM)[b]	EC_{50} (nM)[c]
1	**15**	p(5'A2')$_3$	7.3	0.30 ± 0.02
2	**16**	p2-5A-bu$_2$-AAA.AAT.GGG.GCA.AAT.A	23 ± 2	31 ± 3
3	**14**	p2-5A-bu$_2$-AAA.AAt*.ggg.gca.aat.a	75 ± 13	21 ± 8

[a] "bu" indicates a butyl spacer, t* stands for a *N*-2-hydroxyethyl-*N*-(thymin-1-ylacetyl)glycine linker. Capital letters stand for DNA, lower case letters for PNA.
[b] Radiobinding assays were performed in human RNase L-containing CEM cell lysate (n=4). IC_{50} is the concentration of compound required to displace 50% of ^{32}P-labeled 2-5A probe.
[c] RNase L activation assays were performed *in vitro* with a poly(U) substrate (n=5). EC_{50} is the concentration of compound required to induce RNase L cleavage of 50% of the substrate.

CONCLUSION

The results presented in this paper reveal that 2-5A-DNA-PNAs retain their capacity to bind and activate RNase L and are comparable to previously described 2-5A-DNA[19] and 2-5A-PNA chimeras in their behavior towards RNase L. These constructs are attractive candidates for further evaluation as antisense regulators of gene expression because of their high stability and, in comparison to 2-5A-PNAs, potentially increased cellular uptake. In addition, it is of interest to note that the presence of a small DNA window is sufficient, as demonstrated for 2'-*O*-methylated 2-5A-antisense oligonucleotides[20] and PNA-(5')-DNA-(3')-PNA chimeras,[21] for activation of RNase H. Antisense probes with a dual mode of action are of interest since RNase L possesses a different substrate specificity and subcellular distribution than RNase H.[20] The convenient, fully automated synthesis described in this paper opens the way of performing comparitive studies on the relative activity of 2-5A-DNA, 3'-stabilized 2-5A-DNA,[22-24] 2-5A-PNA and 2-5A-DNA-PNA antisense constructs. A full report on the biological properties of 2-5A-DNA-PNA chimeras (*i.e.* stability, specificity and RNase H activity) will be published in due course.

EXPERIMENTAL SECTION

Synthesis of the 2-5A-(5')-DNA-(3')-PNA chimera (14): All solvents (Biosolve, DNA

synthesis grade) were used as received. All reactions were performed on a Pharmacia Gene Assembler using highly cross-linked polystyrene (loading: 26-28 μmol/g) as a solid support on a 1 μmol scale. The support was functionalized with a glycine moiety *via* a 4-hydroxymethylbenzoic acid linker. Assembly of the PNA part was established using solutions of 0.3 M of monomers **1** and **2** in dimethylformamide (containing 25% dimethylsulfoxide in case of the pyrimidine building blocks **1**), 0.3 M DIPEA in acetonitrile/dimethylformamide (1/1, v/v) and 0.3 M HATU in acetonitrile/dimethylformamide (1/1, v/v). Prior to coupling, the monomers were pre-activated for 1 min by mixing equal amounts of the PNA monomer (15 equiv per μmol support), HATU and DIPEA solutions. The protocol for one PNA chain extension cycle consisted of (1) wash: acetonitrile/dimethylformamide (1/1, v/v), 2.5 mL; (2) coupling: PNA + HATU + DIPEA in acetonitrile/dimethylformamide (1/1, v/v), 15 min; (3) wash: acetonitrile/dimethylformamide (1/1, v/v), 2.5 mL (4) capping: Ac_2O/lutidine/*N*-methylimidazole/tetrahydrofuran (1/1/1/7, v/v/v/v), 2.0 mL; (5) wash: acetonitrile/dimethylformamide (1/1, v/v), 2.5 mL; (6) Fmoc deprotection: 20% piperidine in acetonitrile/dimethylformamide (1/1, v/v), 3 min; (7) wash: acetonitrile/dimethylformamide (1/1, v/v), 2.5 mL. Chain elongation was then continued with 15 equiv of commercially available phosphoramidites **3-6** for the DNA and 2-5A part using 5-(*o*-nitrophenyl)tetrazole (8 equiv) as the activator. Standard DNA capping, washing, oxidation and detritylation cycles were used. Coupling yields were gauged spectrophotometrically (254 nm) by the absorption of the released trityl cation after each deprotection step. After the last elongation step, the oligomers were cleaved from the support with concomitant deprotection of the phosphate groups and exocyclic amino groups by treatment with methanolic ammonia (1.5 mL) at 50 °C for 16h. The samples were filtered and the silyl protective groups were removed by treatment with a $Et_3N.3HF$/NMP (1/1, v/v) solution at 65 °C for 1.5h.[25] Desalting was effected using a G-25 column with a 0.15 M solution of ammonium bicarbonate as the eluting agent. RP-HPLC purification and analysis were carried out on a Jasco HPLC system equipped with a Platinum EPS C-18 5U column (10.0x250 mm and 4.6x150 mm, respectively). Gradient elution was performed at 40 °C by building up a gradient starting with buffer A (50 mM triethylammonium acetate in water) and applying buffer B (50 mM triethylammonium acetate in acetonitrile/water, 3/1, v/v) with a flow rate of 1.0 mL/min

or 5.0 mL/min for analysis and purification, respectively. The purity of the products was confirmed by further analysis on a denaturing 20% polyacrylamide electrophoresis gel while the identity was confirmed with ESI-MS. For **16**: m/z = 1688.7 $[M+4H]^{4+}$, 1351.1 $[M+5H]^{5+}$; calculated for $C_{207}H_{263}N_{92}O_{125}P_{23}$: 6749.1. For **14**: m/z = 1598.5 $[M+4H]^{4+}$, 1278.9 $[M+5H]^{5+}$; calculated for $C_{220}H_{277}N_{115}O_{93}P_{12}$: 6388.7.

RNase L activation and cleavage of a polyuridylic acid (poly(U)) substrate: Pure recombinant human RNase L was prepared by a modification of a previously described procedure.[17] 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.[17,18] 2 μL of a 10x 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 10x solution of 2-5A-DNA (final concentrations 10^{-5} to 10^{-9} M) and recombinant RNase L enzyme (final concentration of 100 nM) were added, and lastly 2μL 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 5mg/ml carrier (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 x 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 cytoplasmic extracts of SF21 insect cells expressing low levels of human RNase L were used in these assays. The 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.[17,18] To assay a given 2-5A-DNA chimera, serial dilutions were prepared in water. Each binding assay consisted of 5 μL of 2-5A-DNA, 10 μL of cell lysate, and 10 μL of a master mix to give a final concentration of 20 mM Tris HCl (pH 7.5), 85 mM KCl, 5 mM $Mg(OAc)_2$, 0.2 mM ATP, 2% (v/v) glycerol and 0.1 nM 2-

5A-[^{32}P]pCp probe. The order of addition for each assay was 2-5A-DNA, then master mix, then lysate. Assay mixtures were incubated at 4 °C for 2 h, after which they were applied to nitrocellulose filters that were subsequently washed (3x) with water. The filters were placed in scintillant and counted in a liquid scintillation counter.[17,18]

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