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Artificial ribonucleases: From combinatorial libraries to efficient catalysts of RNA cleavage

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Abstract

Combinatorial libraries of small organic compounds capable of cleaving RNA were synthesized. The compounds contain benzene ring substituted with two residues of bis quaternary salt of diazabicyclo[2.2.2]octane (DABCO) bearing hydrophobic fragments of different length and structure, attached to DABCO at the bridge position. These compounds, lacking traditional functionalities involved in transesterification reaction, exhibit pronounced RNA cleavage activity. To identify the most active artificial ribonucleases, sublibraries and truncated libraries, containing compounds lacking one of substituents were synthesized. Analysis of ribonuclease activity of truncated libraries resulted in identification of the most active compounds, which are characterized by the presence of at least one long oligomethylene substituent.

Keywords: Artificial ribonucleases; Combinatorial libraries; Lipophilicity; Phosphodiester bonds cleavage; RNA

1. Introduction

Catalysts capable of efficient and specific cleavage of RNA under physiological conditions can find important applications in molecular biology and biotechnology [1,2]. RNA can be cleaved by a number of simple compounds [3–20], which can be divided in two

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classes: (1) complexes of transition metal (Cu^{2+} [3,4], Zn^{2+} [5–7], Eu^{3+} [8,9], Pb^{2+} [10,11] and reviews [2,12,13]) and (2) organic molecules such as biogenic amines [14,15], some peptides [16–19], detergents [20,21] and specially designed synthetic compounds [22,23]. Cleavage of RNA by the second group of compounds usually yields 2′,3′-cyclophosphate and 5′-hydroxyl at the cleavage site [15,24]. Attempts have been made to develop small catalysts of RNA cleavage by mimicking active centers of natural ribonucleases [25,26]. Amino acids found in the catalytic centers of these enzymes were attached to molecules capable of binding to RNA, e.g. intercalating molecules [27–29], cationic structures [22,23,27,30–32], or oligonucleotides [33–39], were designed. Some of the conjugates displayed reasonable ribonuclease activity [27,30,34,37] and entirely cleaved RNA substrate under physiological conditions within several hours (up to 20 h) [31,34,37].

Recently we synthesized artificial ribonucleases—conjugates of 1,4-diazabicyclo[2.2.2]octane (DABCO) and imidazole [30,31]. Surprisingly, some truncated compounds synthesized in this study lacking imidazole residue were found to exhibit noticeable ribonuclease activity [31]. This finding suggested a possibility of development of artificial ribonuclease by conjugation of hydrophobic and cationic structures. In the present study we synthesized combinatorial libraries of compounds containing two cationic diazabicyclo[2.2.2]octane residues, connected by a rigid benzene ring as core structure, and substituted with hydrophobic fragments of different length and structure. Analysis of the libraries revealed several compounds capable of efficiently cleaving RNA.

2. Materials and methods

2.1. Miscellaneous chemicals, enzymes and RNAs

Chemicals for electrophoresis were purchased from Sigma (USA), [γ-^{32}P]ATP was from Biosan (Russia). Solutions for RNA handling were prepared using Milli-Q water, filtered through membranes with 0.2 μm pore size (Millipore, USA) and autoclaved. T4 polynucleotide kinase was from Fermentas (Lethuania), ribonuclease T1 was from Sigma (USA). Oligonucleotide ON21 r(UCGAAUUUCCACAGAAUUCGU) used as RNA substrate was synthesized by Dr. M. Repkova (this institute) by standard phosphoramidite chemistry and purified using RP-HPLC.

Melting points were determined on a Kofler melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on “Bruker AC-200” (200.13 MHz (^{1}H), 50.32 MHz (^{13}C)), “Bruker AM-400” (400.13 MHz (^{1}H), 100.61 MHz (^{13}C)) spectrometers. All coupling constants are given in Hertz, chemical shifts—in ppm. UV spectra were recorded on a “Specord M-40” spectrophotometer with H_2O as the solvent. Elemental analyses were carried out by the microanalytical section of the Novosibirsk Institute of Organic Chemistry. Chemical reagents were purchased from Aldrich (USA). Solvents and reagents were used as received.

The polycationic salts were extensively dried in vacuum but crystallization water remained.

Combinatorial libraries **3** (see Fig. 1) were prepared by reaction of α,α′-dichlorxylenes (**1**) with the corresponding monosubstituted 1,4-diazabicyclo[2.2.2]octane salts **2** (compounds **2** were obtained as described in [40] with high yields. Solution of α,α′-dichlorxylene **1** ($n/2$ mmol; n = amount of monosubstituted 1,4-diazabicyclo[2.2.2]octane salts **2**) in acetonitrile was added to equimolar mixture of 1-alkyl-4-aza-1-azoniabicyclo[2.2.2]octanes

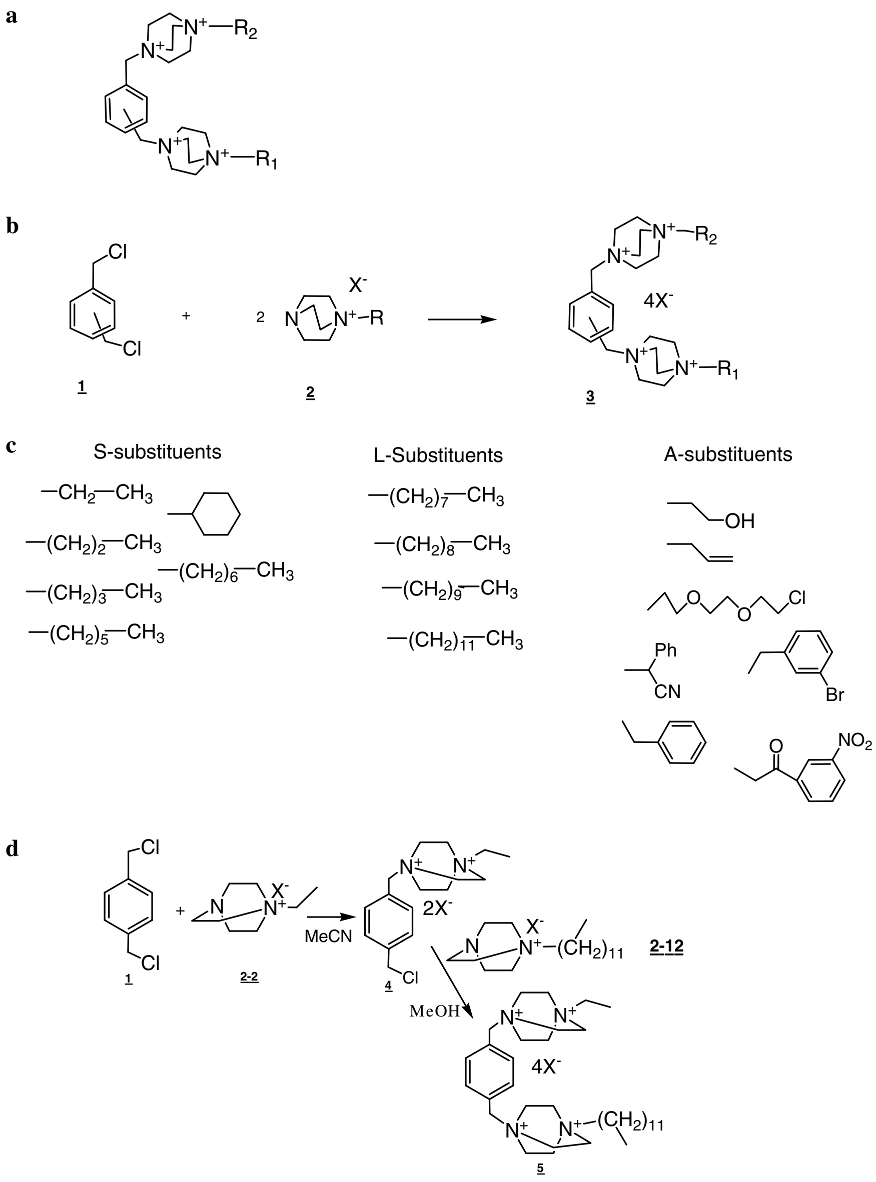

Fig. 1. Combinatorial libraries of artificial ribonucleases. (a) General structure of compounds of the combinatorial libraries. (b) Scheme of the synthesis. (c) List of substituents: S-, L- and A-short saturated, long saturated and mixture of aromatic and unsaturated substituents. (d) Scheme of Dp2/12 synthesis.

(**2**) in acetonitrile. After stirring at 60 °C for 48–72 h, the formed precipitate was filtered, washed with acetonitrile and dried under vacuum to give white hygroscopic microcrystals.

Sublibraries and truncated libraries were prepared using the same protocol, omitting particular substituent or a group of substituents in the course of the synthesis.

2.1.1. Method of synthesis of compounds Dp6 and Dp12 (see Fig. 1b)

Solution of α,α′-dichlor-*p*-xylene (**1**) (175 mg, 1 mmol) in acetonitrile (5 ml) was added to 1-alkyl-4-aza-1-azoniabicyclo[2.2.2]octane salt (**2**) (2.1 mmol) in acetonitrile (20 ml). After stirring at room temperature for 36 h the formed precipitate was filtered, washed with acetonitrile (3 × 15 ml) and dried under vacuum to give white hygroscopic microcrystals.

2.1.2. Method of synthesis of compound Dp2/12 (see Fig. 1d)

1-Ethyl-4-aza-1-azoniabicyclo[2.2.2]octane bromide (221 mg, 1 mmol) (**2–2**) in acetonitrile (10 ml) was added to solution of α,α′-dichlor-*p*-xylene (**1**) (875 mg, 5 mmol) in acetonitrile (15 ml). After stirring at 60 °C for 36 h the formed precipitate was filtered, washed with acetonitrile (3 × 15 ml) and dried under vacuum to give white hygroscopic microcrystals (**4**).

1-Dodecyl-4-aza-1-azoniabicyclo[2.2.2]octane chloride (**2–12**) (349 mg, 1.1 mmol) in methanol (10 ml) was added to solution of compound **4** (396 mg, 1 mmol) in methanol (10 ml). After stirring at 60 °C for 36 h the formed precipitate was filtered, washed with methanol (3 × 15 ml) and dried under vacuum to give white hygroscopic microcrystals.

2.2. Characterization of compounds Dp12 and Dp2/12 and intermediate compound 4

2.2.1. 1,4-Bis-(4-dodecyl-1,4-diazoniabicyclo[2.2.2]octan-1-ylmethyl)benzene tetrachloride (Dp12)

^{1}H NMR (D_2O): $\delta = 0.86$ (t, 6H, $C\mathit{H}_3(CH_2)_{11}N^+$), 1.28 (m, 32H, $CH_3(C\mathit{H}_2)_8(CH_2)_3N^+$), 1.41 (4H, $CH_3(CH_2)_8C\mathit{H}_2(CH_2)_2N^+$), 1.83 (4H, $CH_3(CH_2)_8CH_2C\mathit{H}_2CH_2N^+$), 3.71 (m, 4H, $CH_3(CH_2)_{10}C\mathit{H}_2N^+$), 4.18 and 4.22 (m, 24H, $^+N(C\mathit{H}_2C\mathit{H}_2)_3N^+$), 5.09 (s, 4H, $PhC\mathit{H}_2N^+$), 7.96 (s, 4H, H_{arom}).

^{13}C NMR (D_2O): 14.55 (q, 2C, $\mathit{C}H_3(CH_2)_{11}N^+$); 22.61 (t, 2C, $CH_3\mathit{C}H_2(CH_2)_{10}N^+$); 23.29 (t, 4C, $CH_3CH_2(\mathit{C}H_2)_2(CH_2)_8N^+$); 26.62 (t, 2C, $CH_3(CH_2)_3\mathit{C}H_2(CH_2)_7N^+$); 29.68 (t, 2C, $CH_3(CH_2)_4CH_2(CH_2)_6N^+$); 30.01 (t, 4C, $CH_3(CH_2)_5(CH_2)_2(CH_2)_4N^+$); 30.08 (t, 4C, $CH_3(CH_2)_7(\mathit{C}H_2)_2(CH_2)_2N^+$); 32.62 (t, 2C, $CH_3(CH_2)_9\mathit{C}H_2CH_2N^+$); 51.80 (t, 6C, $^+N(CH_2\mathit{C}H_2)_3N^+(CH_2)_{11}CH_3$); 52.14 (t, 6C, $^+N(\mathit{C}H_2CH_2)_3N^+(CH_2)_{11}CH_3$); 66.08 (t, 2C, $CH_3(CH_2)_{10}\mathit{C}H_2N^+$); 68.98 (t, 2C, $Ph\mathit{C}H_2N^+$); 129.37 (s, 2C, $C_{1,4}$); 135.01 (d, 4C, $C_{2,3,5,6}$).

Yield 65%; M.p. > 225 °C (dec); UV (λ_{max} (ε)): 270 (760); 278 (700).

2.2.2. 1-(4-Ethyl-1,4-diazoniabicyclo[2.2.2]octan-1-ylmethyl)-4-(4-dodecyl-1,4-diazoniabicyclo[2.2.2]octan-1-ylmethyl)benzene triclroride bromide (Dp2/12)

^{1}H NMR (CD_3OD): $\delta = 0.93$ (br. t, 3H, $C\mathit{H}_3(CH_2)_{11}N^+$, $J = 6.8$), 1.32 (m, 16H, $CH_3(C\mathit{H}_2)_8(CH_2)_3N^+$), 1.42 (m, 4H, $CH_3C\mathit{H}_2(CH_2)_7C\mathit{H}_2(CH_2)_2N^+$), 1.51 (t, 3H, $C\mathit{H}_3CH_2N^+$, $J = 7.2$); 1.85 (2H, $CH_3(CH_2)_9C\mathit{H}_2CH_2N^+$), 3.59 (m, 2H, $CH_3(CH_2)_{10}C\mathit{H}_2N^+$), 3.68 (m, 2H, $CH_3C\mathit{H}_2N^+$); 4.12 (m, 24H, $^+N(C\mathit{H}_2C\mathit{H}_2)_3N^+$), 5.02 (m, 4H, $PhC\mathit{H}_2N^+$), 7.86 (s, 4H, H_{arom}).

^{13}C NMR (CD_3OD): 8.02 (q, 1C, $\mathit{C}H_3CH_2N^+$); 14.40 (q, 1C, $\mathit{C}H_3(CH_2)_{11}N^+$); 23.10 (t, 1C, $CH_3\mathit{C}H_2(CH_2)_{10}N^+$); 23.69 (t, 1C, $CH_3(CH_2)_2\mathit{C}H_2(CH_2)_8N^+$); 27.12 (t, 1C, $CH_3(CH_2)_3\mathit{C}H_2(CH_2)_7N^+$); 30.11 (t, 1C, $CH_3(CH_2)_4\mathit{C}H_2(CH_2)_6N^+$); 30.42 (t, 2C, $CH_3(CH_2)_5(CH_2)_2(CH_2)_4N^+$); 30.58 (t, 2C, $CH_3(CH_2)_7(CH_2)_2(CH_2)_2N^+$); 30.69 (t, 1C, $CH_3(CH_2)_9\mathit{C}H_2CH_2N^+$); 33.02 (t, 1C, $CH_3CH_2\mathit{C}H_2(CH_2)_9N^+$); 52.03 (t, 6C,

$^{+}N(\mathit{C}H_2CH_2)_3N^{+}CH_2$); 52.11 (t, 3C, $^{+}N(CH_2\mathit{C}H_2)_3N^{+}CH_2CH_3$); 52.47 (t, 3C, $^{+}N(\mathit{C}H_2CH_2)_3N^{+}(CH_2)_{11}CH_3$); 62.11 (t, 1C, $CH_3\mathit{C}H_2N^{+}$); 66.08 (t, 1C, $CH_3(CH_2)_{10}\mathit{C}H_2N^{+}$); 68.54 (t, 2C, $Ph\mathit{C}H_2N^{+}$); 130.32 (s, 2C, $C_{1,4}$); 135.47 (d, 4C, $C_{2,3,5,6}$).

Yield 592 mg (83%). M.p. > 200 °C (dec). UV (λ_{max} (ε)): 219 (12482); 268 (845); 275 (788).

2.2.3. 1,4-Bis-(4-hexyl-1,4-diazoniabicyclo[2.2.2]octan-1- ylmethyl) benzene dichloride dibromide (Dp6)

^{1}H NMR (D_2O): $\delta = 0.94$ (t, 6H, $C\mathit{H}_3(CH_2)_5N^{+}$, $J = 6.7$), 1.41 (m, 12H, $CH_3(C\mathit{H}_2)_3(CH_2)_2N^{+}$), 1.89 (m, 4H, $CH_3(CH_2)_3C\mathit{H}_2CH_2N^{+}$), 3.64 (m, 4H, $CH_3(CH_2)_3CH_2C\mathit{H}_2N^{+}$), 4.14 (m, 24H, $^{+}N(C\mathit{H}_2C\mathit{H}_2)_3N^{+}(CH_2)_5CH_3$), 5.00 (s, 4H, $PhC\mathit{H}_2N^{+}$), 7.87 (s, 4H, H_{arom}).

^{13}C NMR (D_2O): 13.99 (q, 2C, $\mathit{C}H_3(CH_2)_5N^{+}$); 22.19 (t, 2C, $CH_3\mathit{C}H_2(CH_2)_4N^{+}$); 22.45 (t, 2C, $CH_3CH_2\mathit{C}H_2(CH_2)_3N^{+}$); 25.70 (t, 2C, $CH_3(CH_2)_2\mathit{C}H_2(CH_2)_2N^{+}$); 31.09 (t, 2C, $CH_3(CH_2)_3\mathit{C}H_2CH_2N^{+}$); 51.85 and 52.02 (t, 6C, $^{+}N(CH_2\mathit{C}H_2)_3N^{+}(CH_2)_5CH_3$); 66.35 (t, 2C, $CH_3(CH_2)_4\mathit{C}H_2N^{+}$); 68.71 (t, 2C, $Ph\mathit{C}H_2N^{+}$); 129.14 (s, 2C, $C_{1,4}$); 135.04 (d, 4C, $C_{2,3,5,6}$).

Anal. Calcd. for $C_{32}H_{58}Br_2Cl_2N_4 \cdot 6H_2O$: C 45.88, H 8.42, N 7.02; found: C 45.30, H 7.89, N 7.02. Yield 548 mg (75%). M.p. 235–240 °C. UV (λ_{max} (ε)): 268 (1040), 275 (980).

*2.2.4. 1-(4-Ethyl-1,4-diazoniabicyclo[2.2.2]octan-1-ylmethyl)-1-clroromethylbenzene clroride bromide (Intermediate compound **4**)*

^{1}H NMR (CD_3OD): $\delta = 1.46$ (t, 6H, $C\mathit{H}_3CH_2N^{+}$, $J = 7.2$); 3.75 (q, 2H, $CH_3C\mathit{H}_2N^{+}$, $J = 7.2$); 4.10 (m, 12H, $^{+}N(C\mathit{H}_2C\mathit{H}_2)_3N^{+}CH_2CH_3$); 4.75 (s, 2H, $PhC\mathit{H}_2N^{+}$); 4.94 (s, 2H, $PhC\mathit{H}_2Cl$); 7.68 (s, 4H, H_{arom}).

^{13}C NMR (CD_3OD): 8.14 (q, 1C, $\mathit{C}H_3CH_2N^{+}$); 45.81 (t, 1C, $Ph\mathit{C}H_2Cl$); 52.04 (t, 3C, $^{+}N(\mathit{C}H_2CH_2)_3N^{+}$); 52.15 (t, 3C, $^{+}N(CH_2\mathit{C}H_2)_3N^{+}CH_2CH_3$); 62.00 (t, 1C, $CH_3\mathit{C}H_2N^{+}$); 68.89 (t, 1C, $Ph\mathit{C}H_2N^{+}$); 127.00 (s, 1C, C_1); 131.15 (d, 2C, $C_{3,5}$); 134.75 (d, 2C, $C_{2,6}$); 143.20 (s, 1C, C_4). Yield 226 mg (57%). M.p. > 190 °C (dec). UV (λ_{max} (ε)): 224 (10898).

Analytical data for obtained compounds Dp6, Dp12 and Dp2/12 are listed in Table 1.

2.3. End labeling of ON21

[5′-^{32}P] labeling of ON21 was performed using [γ-^{32}P]ATP and T4 polynucleotide kinase as described in [41]. After labeling, ON21 was purified by electrophoresis in 8% denaturing polyacrylamide gel. The labeled RNA was eluted from gel by three portions (300 μl each) of 0.5 M ammonium acetate, containing 0.5 mM EDTA and 0.1% SDS and ethanol precipitated. RNA pellet was centrifuged, rinsed twice with 80% ethanol, dissolved in water and stored at −20 °C.

Table 1
Analytical data for obtained compounds

Compound	Found				Calculated		
	C	H	N		C	H	N
Dp6	45.30	7.89	7.02	$C_{32}H_{58}Br_2Cl_2N_4 \cdot 6H_2O$	45.88	8.42	7.02
Dp2/12	47.60	9.23	7.12	$C_{34}H_{62}Br_2Cl_2N_4 \cdot 6H_2O$	48.17	8.56	6.61
Dp12	61.30	9.87	6.43	$C_{44}H_{82}Cl_4N_4 \cdot 3H_2O$	61.24	10.28	6.49

2.4. Cleavage experiments

5′-End labeled ON21 was diluted with unlabeled ON21 to a final concentration of 5×10^{-6} M. Standard reaction mixture (10 μl) contained 50 mM Tris–HCl buffer, pH 7.0, 0.2 M KCl, 0.5 mM EDTA, from 1 μM to 5 mM of one of the tested compounds or libraries and 0.5 μM of [5′-^{32}P]ON21 (about 50,000 Cherenkov's cpm). Reaction mixtures were incubated at 37 °C for different times (from 1 to 20 h) and quenched by RNA precipitation with 100 μl of 2% lithium perchlorate solution in acetone. The RNA pellet was collected by centrifugation and dissolved in loading buffer (7 M urea containing leading dyes). The cleavage products were analyzed in 15% PAGE containing 8 M urea. The cleavage sites were assigned by comparison of the electrophoretic mobilities of cleavage products with those of ON21 cleavage products with RNase T1 and in 2 M imidazole buffer, pH 7.0. Quantitative data were obtained using Phosphoimager System (Molecular Imager, Bio-Rad). Cleavage extent corresponds to the ratio of radioactivity found in the cleavage products to the radioactivity applied on the gel lane.

3. Results

3.1. Design of combinatorial libraries

Compounds of the combinatorial libraries were built of two positively charged 1,4-diazabicyclo[2.2.2]octane residues (here and after DABCO), substituted at the bridge nitrogen atom by hydrophobic fragments of different length and complexity, and connected by a rigid linker—benzene. General structure of the compounds, substituents, used in the study, and scheme of combinatorial library synthesis are shown in Fig. 1. The synthetic scheme was designed to yield compounds differing with respect to location of the DABCO residues in the benzene ring (*ortho*, *meta* and *para*) and in length and structure of substituents.

Monoquaternary salts of 1,4-diazabicyclo[2.2.2]octane bearing different alkyl-substituents were obtained with high yields (compounds **2** in Fig. 1b) according to described protocol [42]. Interaction of compounds **2** with α,α′-dichlorxylene (**1**) in acetonitrile results in formation of products **3**. These products are formed with high yields regardless the structure of the alkyl substituents. For different substituents, yields of the products **3** varied from 40% to 80%.

In these compounds positively charged DABCO residues provide affinity to RNA backbone. Geometry of the compound is different due to different locations of DABCO residues in the benzene ring (*ortho*, *meta* and *para*). Hydrophobic substituents of the compounds were either saturated oligomethylene fragments or unsaturated fragments (Fig. 1c). The following sublibraries were prepared: S-sublibrary contains the compounds with short oligomethylene units and cyclohexyl fragment; L-sublibrary contains the compounds with longer fragments; and A-sublibrary, which contains the compounds with different aromatic and unsaturated substituents. In the synthesis of sublibraries SL, SA and LA, reaction mixtures contained two groups of substituents: short and long, short and aromatic or long and aromatic substituents, respectively.

All substituents in reaction mixture were taken in equimolar quantities. In case the substituents are incorporated into the libraries at a similar rate, which at its best is a rough approximation, in products will be the equimolar quantities of all libraries. Degeneracy of combinatorial library was assumed to be $\boldsymbol{n} + (\boldsymbol{n} - 1) + \ldots + 1 = (\boldsymbol{n}^2 + \boldsymbol{n})/2$, where $\boldsymbol{n}$ is the number of substituents in the reaction mixture. For example, for S-library with 6 substituents, the number of constituent compounds is 21. Taking into account the degeneracy of combinatorial library, concentration of each individual compound at concentration $\boldsymbol{M}$ of combinatorial library, containing $\boldsymbol{n}$ substituents is $2\boldsymbol{M}/(\boldsymbol{n}^2 + \boldsymbol{n})$.

3.2. Ribonuclease activity of combinatorial libraries

Ribonuclease activity of combinatorial libraries and individual compounds was assayed at 37 °C and neutral pH (50 mM Tris–HCl buffer, pH 7.2 containing 0.2 M KCl, 0.5 mM EDTA). The substrate was [5′-^{32}P]-labeled oligonucleotide ON21. Concentration of oligonucleotide was 5 μM. To identify cleavage sites imidazole ladder and G-ladder produced by partial RNA cleavage with 2 M imidazole buffer (pH 7.0) and with RNase T1, respectively, were run in parallel. Combinatorial libraries under the study cleaved ON21 (see Fig. 2) at C_{10}–A_{11} and C_{12}–A_{13} phosphodiester bonds located in the loop present in the secondary structure of this oligoribonucleotide.

The concentrations of combinatorial libraries, used in the cleavage assays were 10 μM, 50 μM, 200 μM, 1 mM and 5 mM. For some libraries concentrations higher than 5 mM could not be reached because of solubility limitation. Ten micromolar concentration was the lowest, where ribonuclease activity could be detected for the most active libraries.

Ribonuclease activity of full combinatorial libraries SLA depends on the location of substituted DABCO residues in the benzene ring (Fig. 3). SLA library with *para* location of DABCO residues displays somewhat higher activity then *ortho*-, *meta*- and full *omp*-SLA libraries. Therefore, in our study we focused on the libraries containing DABCO residues located in *para*-position of the benzene ring.

Experiments with *para*-sublibraries, composed of the compounds with one or two groups of substituents demonstrate that the structure and length of hydrophobic substituents strongly affect ribonuclease activity of the compounds (Fig. 4a). Among S, L and A sublibraries, only L-sublibrary displayed high cleavage activity. Activities of S and A sub-

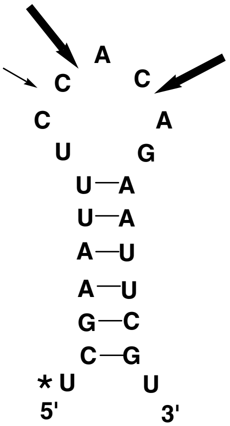

Fig. 2. 21-mer oligoribonucleotide used as an RNA substrate. Arrows show the sites of cleavages induced by the compounds. Arrow size corresponds to the cleavage intensity. Asterisk shows [^{32}P]-label location.

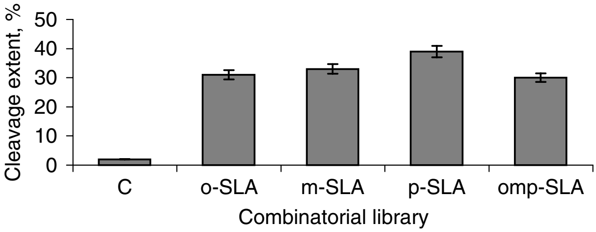

Fig. 3. Ribonuclease activity of combinatorial libraries is affected by location of substituents in the benzene ring: *ortho (o), meta (m) or para (p).* Combinatorial libraries x-SLA (where x- is *o*-, *m*- or *p*-) contain mixture of short (S), long (L) and aromatic (A) substituents. *omp*-Library contains mixture of *o*, *m* and *p* -isomers. C corresponds to cleavage extent of incubation control. Cleavage extent (%), percent of the oligoribonucleotide cleaved under standard assay conditions. The standard assay conditions: ON21 (5 μM) was incubated in the presence of one of the combinatorial libraries at 1 mM concentration for 18 h at 37 °C.

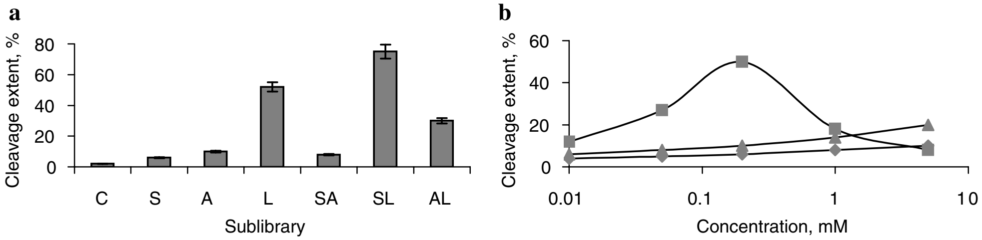

Fig. 4. Cleavage of ON21 by the sublibraries. (a) Effect of substituent type (S, L or A) on ribonuclease activity of the sublibraries. C corresponds to cleavage extent of incubation control. (b) Concentration effect. Rhombuses correspond to S-sublibrary, squares to L-library and triangles to A-library. Assay conditions: see legend to Fig. 3.

libraries were 6–10 times lower. Experiments with sublibraries, formed by compounds with two groups of substituents (SL, SA and LA sublibraries) clearly demonstrated importance of L-fragments for the activity of the compounds (Fig. 4a).

Investigation of concentration dependences of the oligonucleotide cleavage by different sublibraries (Fig. 4b) has demonstrated bell-shaped concentration dependence curve with the maximum at 0.2 mM for the L-library. Bell-shaped concentration dependences were also observed for AL, SL and other sublibraries, with fragments L (data not shown). Previously, bell-shaped concentration dependence of ribonuclease activity was observed for DABCO–histidine conjugates, containing tetradecamethylene substituent at one of bridge nitrogens of DABCO residue [26,27]. The decrease in the oligonucleotide cleavage rate at high concentrations of combinatorial libraries may be explained by limited solubility of compounds in water or by micelle formation.

3.3. Activity of truncated libraries

To identify the substituents, which are essential for the activity of the compounds, truncated library approach was used. ***n*** truncated libraries were synthesized by excluding one of the ***n*** substituents from the reaction mixture. For example, L-8, L-9, L-10, L-12 truncated libraries, are L-sublibraries lacking octa-, nona-, deca or dodecamethylene

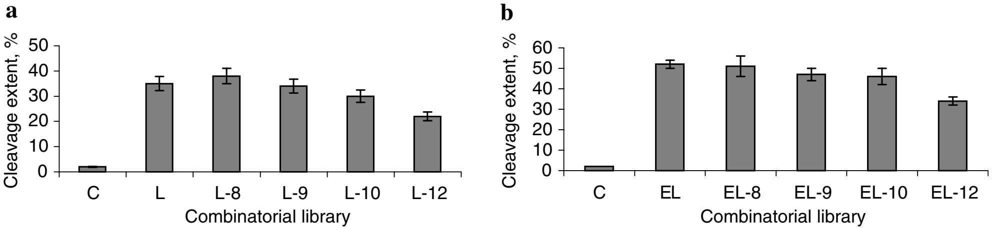

Fig. 5. Ribonuclease activity of truncated L- (a) and EL- (b) libraries. C corresponds to cleavage extent of incubation control. Assay conditions: ON21 (5 μM) was incubated under standard conditions in the presence of one of the combinatorial libraries (0.1 mM) for 8 h at 37 °C.

substituents, respectively (Fig. 5a). The data show that L-8 library is the most active among the four truncated libraries and L-12 library exhibits the lowest cleavage activity. From the data shown in Fig. 5a it can be concluded, that the longer are oligomethylene fragments in truncated libraries, the higher cleavage activity is observed.

For LS sublibrary $n = 10$, and it was necessary to analyze 10 truncated LS sublibraries. Taking into account the data on the role of L and S substituents in the catalysts activity, limited truncated EL library was synthesized, where one of the two DABCO residues was substituted with ethyl (E) fragment and the other DABCO residue was substituted with one of the L-fragments ($n = 4$ for this library). As in the case of L-truncated libraries, EL-8 truncated library displayed the highest activity and EL-12 displayed the lowest activity (Fig. 5b).

From results of the described studies it can be concluded that long hydrophobic substituents are essential for high cleavage activity of the compounds.

3.4. Activity of individual compounds Dp12 and Dp2/12

According to the obtained data two individual compounds Dp12 and Dp2/12 (Fig. 6) were synthesized and investigated. The first compound, Dp12 contains two dodecamethylene (12) fragments that substitute DABCO (D) residues being in the *para*-position (p) of benzene ring. The second compound, Dp2/12 contains DABCO residues substituted with one ethylene (2) and one dodecamethylene (12) fragments.

Both Dp12 and Dp2/12 compounds display high ribonuclease activity and cleave ON21 entirely in 36 h under the standard reaction conditions (see Fig. 7a for analysis of ON21 cleavage products). Analysis of sequence-specificity of Dp12 and Dp2/12 showed that both compounds cleave RNA at two adjacent C–A bonds in the loop. Dp12 preferentially cleaves at the site C_{12}–A_{13}, while Dp2/12 cleaves preferentially at the site C_{10}–A_{11}. When the compounds are presented in the reaction mixture at optimal concentrations, the activ-

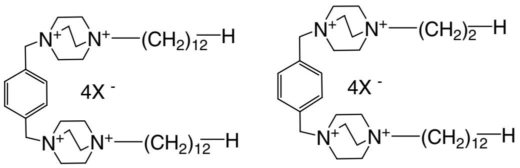

Fig. 6. The most active artificial ribonucleases Dp12 and Dp2/12.

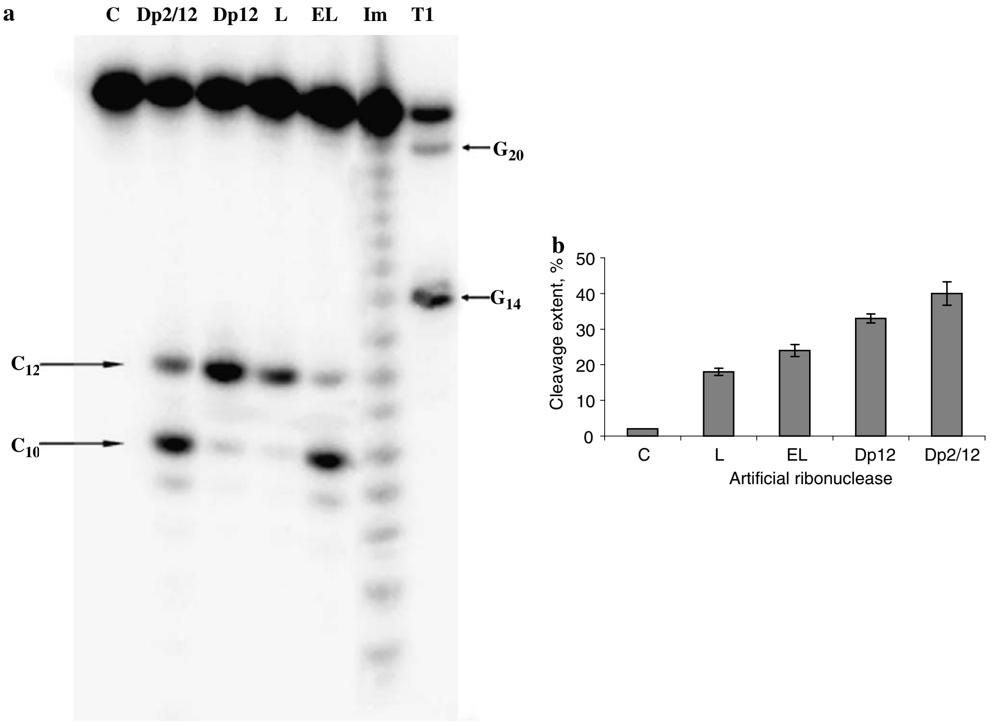

Fig. 7. Assay of ribonuclease activity of the compounds Dp12 and Dp2/12. (a) Specificity of RNA cleavage by the compounds. Autoradiograph of 15% denaturing PAAG after resolution of cleavage products. Lanes: C, incubation control; Im and T1, partial RNA cleavage in 2 M imidazole buffer, pH 7.0 and by RNase T1 under denaturing conditions. Lanes Dp2/12, Dp12, L and EL, incubation of ON21 in the presence of corresponding compounds or libraries. Experimental conditions: 5′-labled ON21 was incubated with one of the compounds or libraries at optimal concentrations (Dp2/12, 0.3 mM; Dp12, 10 μM; L, 0.1 mM; EL, 0.1 mM) for 4 h at 37 °C. (b) Comparison of RNA-cleavage activity of the compounds and combinatorial libraries under optimal conditions (L and EL sublibraries, 0.1 mM; Dp12, 10 μM; Dp2/12, 0.3 mM). C corresponds to cleavage extent of incubation control.

ity of Dp2/12 is somewhat higher then activity of Dp12 (Fig. 7b), and both the compounds are more active then combinatorial libraries L and EL.

4. Discussion

Combinatorial approach to design of compounds with specific activities provides a possibility to find the active structures that could not be predicted from the current knowledge of structure-function relations. In our study we synthesized and investigated libraries of compounds, containing cationic and lipophilic structures. The idea to investigate compounds of this type came from results of our recent study, which indicated a possibility, that some structures, built of cationic and lipophilic fragments can catalyze cleavage of RNA [27].

Synthesis of combinatorial libraries according to the scheme, shown at Fig. 1a, yielded a number of products with different substituents in equimolar quantities.

The libraries demonstrated ribonuclease activity, the most active compounds turned out to be the ones with *p*-location of DABCO residues in the benzene ring. The group of the most active compounds, containing L-substituents, displayed a bell-shaped concentration dependence of their activity. Similar bell-shaped concentration-activity curves in experiments with conjugates ABLkCm, containing tetradecamethylene fragment and one DABCO residue [26,27].

Experiments with sublibraries revealed the active group of compounds, containing long hydrophobic residues. Finally it was found, that substitution of DABCO residues by at least one long oligomethylene fragment provide for high ribonuclease activity of the compounds. The identified active structures Dp12 and Dp2/12 were synthesized as individual compounds. Ribonuclease activity of these compounds was found to be much higher, then the activity of initial combinatorial libraries. Total cleavage activity of compound Dp2/12 is somewhat higher than that of Dp12 (Fig. 7b).

The synthesized compounds cleave RNA at YA sequences within single stranded region, which is typical for the known artificial ribonucleases [38,43]. Compounds Dp12 and Dp2/12 display high ribonuclease activity: $\tau_{1/2}$ of RNA cleavage by these compounds is 5–7 h at 37 °C (primary data not shown). The cleavage activity of these compounds is comparable or higher then the activity of known artificial ribonucleases, containing traditional catalytic groups involved in transesterification reaction [22,31,44]. For example, conjugates of substituted diazabicyclo[2.2.2]octane and imidazole (ABLkCm) [31], which are among the most active artificial ribonucleases, display $\tau_{1/2}$ 4–7 h depending on RNA concentration and buffer type (entire RNA cleavage was achieved within 18–20 h, similarly to Dp12). Another artificial ribonucleases nLm [44], built of lysine and histamine residues, efficiently cleave RNA substrate at 37 °C, ($\tau_{1/2}$ 3–4 h), however this cleavage activity is observed at concentration of the compounds 1–5 mM that is 10–100 times higher, then the optimal concentration for ABLkCm and Dp12. The cleavage activity of recently designed artificial ribonuclease tris{2-[(benzimidazole-2-yl)amino]ethyl}amine [22] is similar to that of ABLkCm. This compound cleaved both single stranded and hairpin RNA substrates with efficiency 93% and 88%, respectively. This cleavage extent is achieved after 20 h of incubatuin at 1 mM concentration of artificial ribonucleases [22]. Other small artificial ribonucleases (antisense oligonucleotide based artificial ribonucleases are excluded from consideration) display lower cleavage activity [29,36,45,46, reviewed in 25].

The obtained data show, that some conjugates of cationic and hydrophobic structures can efficiently cleave RNA. The available knowledge does not allow suggesting exact mechanism of catalysis of RNA cleavage by these compounds. One can suggest that the compounds form micelles that bind RNA at their positive charged surface, where RNA chain can assume conformations favorable to transesterification to occur and where phosphodiester bonds are exposed to various microenvironments built of cationic structures and water molecules.

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