Sequence and structural analysis of clones 10 and 29



Primary sequences and predicted consensus secondary structures of clones 10 and 29. The nucleotides in the constant regions are shown in lower cases. The identical nucleotides are shown in red. The nucleotides forming consistent base-pairings in the consensus secondary structures are shown in fuchsia. (A) Primary sequence alignment of clones 10 and 29. (B) The predicted consensus secondary structures of clones 10 and 29.

Mass analysis of the ribozyme product and of the authentic compound



MALDI-TOF mass analyses of the product. (A) The structure of biotin-LC-Ac-TEG-SH and mass spectrum of the chloroform extract of the ribozyme reaction. (B) Mass spectrum of the authentic, chemically synthesized compound.

## Gel mobility shift assay of clone 10 and 29 including some selected clones



Gel mobility shift assay of clone 10 and 29 including several selected clones. The amount of S-A RNA: **10**, 8.8%; **18**, 2.9%; **19**, 4.9%; **20**, 0.9%; **25**, 1.3%; **26** and **27**, 0%; **29**, 13%.

## Materials and methods.

A 222-mer DNA library with 142 randomized nucleotides was constructed as previously reported<sup>[1]</sup> except a different ratio of monomer solution (A:C:G:T=3:3:2:2.4) was used for the random positions during automated solid-phase oligonucleotide synthesis. The modified base HS-TEG-GMP was synthesized as previously reported<sup>[2]</sup>. Restriction enzymes were purchased from New England Biolabs, *Taq* polymerase from Invitrogen, DNAase-free RNAase inhibitor from Ambion, strepavidin from Rockland, sulfo-NHS-LC-biotin from Molecular Biosciences, and coenzyme-A and inorganic pyrophosphatase

from Sigma. All other chemicals and solvents were from Aldrich Chemical Co. <sup>1</sup>H NMR spectra were recorded on Bruker ARX-500 spectrometer. All spectra used the residual solvent peak as reference signals. Mass spectra were recorded on a Voyager-DE SYR (Applied Biosystems) MALDI spectrometer or on a MDS Sciex Qstar Pulsar ESI mass spectrometer. Preparative HPLC was performed on an HP 1100 series instrument using an Alltech Econosil C18 column.

Preparation of malonyl modifier (malonyl-TEG-thiosulfate). A mixture of 3,6-dioxa-1,8-octanedithiol (3.85 g, 21.1 mmol) and malnonyl monochloride (2.46 g, 20.1 mmol) was dried under vacuum over KOH pellets in a vacuum desiccator for 1 h. The solid residue was dissolved in ethyl acetate, washed with water and saturated NaHCO<sub>3</sub> solution. The aqueous layer was cooled to 0 °C and acidified with 1 N HCl solution to pH 1. The precipitate was filtered, washed with excess water and dried. The resulting solid (4.11 g, 15.3 mmol) was treated with sodium tetrathionate dihydrate (4.69 g, 15.3 mmol) in water for 4 h and lyophilized. The residue was dissolved in water and purified by reverse phase HPLC with a gradient of water and acetonitrile to give malonyl-TEG-thiosulfate. <sup>1</sup>H NMR  $(500 \text{ MHz}, D_2\text{O}) \delta 2.98 \text{ (t, } J = 5.3 \text{ Hz}, 2 \text{ H}), 3.12 \text{ (t, } J = 5.3 \text{ Hz}, 2 \text{ H}), 3.39 \text{ (s, } 2 \text{ H}), 3.51$ (s, 6 H), 3.69 (t, J = 5.3 Hz, 2 H);  $^{13}$ C NMR (500 MHz, D<sub>2</sub>O)  $\delta$  28.62, 34.09, 68.77, 68.88, 69.31, 69.38, 173.66, 197.72; MS(ESI) calculated for C<sub>9</sub>H<sub>15</sub>O<sub>8</sub>S<sub>3</sub> [M-H]<sup>-</sup>: 346; found: 346. Preparation of the malonyl-modified RNA pool (Malonyl-PEG-RNA). The 5'-thiol modified RNA (HS-TEG-RNA) was generated in a transcription reaction (20 mL) containing 40 mM Tris-HCl (pH = 7.9), 12 mM MgCl<sub>2</sub>, 2 mM spermidine, 10 mM dithiothreitol with 2 mM each ATP, UTP, GTP, CTP, 16 mM HS-TEG-GMP, 800 µg PCR-amplified DNA template, 4000 U RNase inhibitor, 80 U inorganic pyrophosphatase. and 20,000 U T7 RNA polymerase. When labeled RNA was desired, the reaction also contained 100  $\mu$ Ci  $\alpha$ -[<sup>32</sup>P]ATP. After incubation at 37 °C for 7 h., the reaction was quenched by addition of 400 U RNAse-free DNAse I and 10 mM CaCl<sub>2</sub> followed by incubation at 37 °C for 2 h., ethanol precipitation and gel purification. The malonyl-PEG-RNA was prepared by an overnight incubation of HS-TEG-RNA with a 1000-fold excess of malonyl-TEG-thiosulfate in 6 mL of reaction buffer containing 20 mM HEPES, 150 mM NaCl and 1 mM EDTA at room temperature.

**Synthesis of Biotin-LC-CoA** . The mixture of coenzyme A (168 mg, 0.18 mmol), Sulfo-NHS-Biotin-LC (Pierce, 100 mg, 0.18 mmol) and NaHCO<sub>3</sub> (170 mg, 2.02 mmol) in water (3 mL) was stirred for 5 h and lyophilized. The residue was purified by a reverse-phase HPLC with a gradient of aqueous acetonitrile. <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O)  $\delta$  0.70 (s, 3 H), 0.85 (s, 3 H), 1.22-1.67 (m, 12 H), 2.18 (m, 3 H), 2.38 (m, 2 H), 2.57 (m, 2 H), 2.72 (d, 1 H), 2.95 (m, 3 H), 3.12 (m, 2 H), 3.23-3.27 (m, 4 H), 3.40 (m, 2 H), 3.51 (m, 1 H), 3.79 (m, 1 H), 4.00 (s, 1 H), 4.21 (m, 2 H), 4.37 (m, 1 H), 4.56 (m, 2 H), 6.14 (d, *J* = 6.9 Hz, 1 H), 8. 23 (s, 1 H), 8.53 (s, 1 H); <sup>31</sup>P NMR (500 MHz, D<sub>2</sub>O)  $\delta$  2.88, -10.00 (d, *J*<sub>P-P</sub> = 18.6)

and -10.56 (d,  $J_{P-P} = 18.6$ ); HRMS(ESI) calculated for  $C_{37}H_{60}N_{10}O_{19}P_3S_2$  [M-H]<sup>-</sup>: 1105.2691; found: 1105.2684.

*In vitro* selection of ketosynthase ribozymes. To select ketosynthase ribozymes, 10  $\mu$ M malonyl-PEG-RNA was incubated with 10 mM Biotin-LC-CoA in 360  $\mu$ L of reaction buffer (50 mM HEPES, pH 7.4, 300 mM KCl, 50 mM MgCl<sub>2</sub>) at room temperature for 20 h. The reaction mixture was extracted with equal volumes of chloroform:phenol (1:1) and chloroform:isoamyl alcohol (24:1), and precipitated with 3.0 volumes of ethanol. The pellet was redissolved in 1 mL binding buffer (1.0 M NaCl, 20 mM HEPES, pH 7.4, 5 mM EDTA) and transferred to a tube containing 1 mL of 50 % streptavidin-agarose gel slurry (prewashed twice with binding buffer). The slurry mixture was mixed gently for 4-5 min, and then washed with 10 mL binding buffer, 10 mL water, 10 mL 7.0 M urea, 10 mL water. Bound RNA was treated with 1 mL 100 mM DTT in binding buffer for 1 h and eluted from the column. The eluted RNA was precipitated with 3 volumes of ethanol and 100  $\mu$ g glycogen. To isolate sufficient quantities of product for mass analysis, the reaction was scaled up 8 fold.

The RNA thus selected was converted to cDNA by reverse transcription. A 20  $\mu$ L annealing mixture (50 mM Tris-HCl, pH 8.3, 10 mM DTT, 75 mM KCl, 3 mM MgCl<sub>2</sub>, 2 mM each dNTP, 5 pmol 3'-primer) was incubated at 42 °C for 2 min. Then, 1  $\mu$ L (200 U) of SuperScript III Reverse Transcriptase was added and the mixture incubated at 42 °C for 50 min followed by inactivation by heating at 70 °C for 15 min. The cDNA was amplified by PCR in a 500  $\mu$ L reaction containing 20  $\mu$ L of the reverse transcription reaction mixture, 1.5 mM MgCl<sub>2</sub>, 20 mM Tris-HCl, pH 8.3, 10 mM KCl, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 0.1% Triton X-100, 0.2 mM each dNTP, 1.0  $\mu$ M primers, and 5 U Taq DNA polymerase. The PCR product was purified and used in the next round of selection as described above.

Gel mobility shift assay. An *in vitro* selection reaction was performed as described above using  $\alpha$ -[<sup>32</sup>P]ATP-labelled malonyl-PEG-RNA. After isolation, 2 µg of the RNA was incubated with 15 µg streptavidin in a buffer containing 20 mM HEPES (pH 7.4), 1 M NaCl and 5 mM EDTA at room temperature for 20 min. The reaction mixture was analyzed by denaturing PAGE and the radioactive bands detected by phosphorimaging (Fugifilm, BAS-5000).

Identification of Biotin-LC-Ac-TEG-SH by MALDI-TOF-MS.  $8\mu$ M malonyl-PEG-RNA was prepared as described above using cDNA of clone 29 and incubated with 8 mM Biotin-LC-CoA in 1.5 mL reaction buffer at room temperature for 20 h. The mixture was extracted with chloroform (1.5mL × 2). The organic layer was concentrated and analyzed by MALDI-TOF-MS to give a peak at 564 ([M+H<sup>+</sup>]), 586 ([M + Na<sup>+</sup>]) and 602 ([M + K<sup>+</sup>]). The aqueous layer was precipitated with 3.0 volumes of ethanol. The pellet was treated with DTT (800  $\mu$ M) in 1.5 mL water at room temperature for 1 h. followed by extraction with chloroform and purified by DEAE-Sephadex column. The aqueous filtrate and chloroform extract were analyzed by MALDI-TOF-MS, in which, however, no trace of product was detected.

**Synthesis of an authentic sample of Biotin-LC-Ac-TEG-SH.** Solid magnesium ethoxide (67 mg, 0.58 mmol) was added to a solution of 3-(2-(2-(2-mercaptoethoxy)ethoxy)ethylthio)-3-oxopropanoic acid (malonyl-PEG)(313 mg, 1.17 mmol) in THF followed by stirring for 1 h at room temperature. Carbonyldiimidazole (95 mg, 0.58 mmol) was added to a suspension of Biotin-LC (179 mg, 0.50 mmol) in DMF at

80 °C in an oil bath. When the mixture started to dissolve, the oil bath was removed and the mixture was stirred for 1 h. The prepared Mg(O<sub>2</sub>CCH<sub>2</sub>COS(CH<sub>2</sub>CH<sub>2</sub>O)<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>SH) was added to this solution and stirred overnight at room temperature. The solvent was removed under reduced pressure, the residue dissolved in chloroform, washed with 0.5 N HCl, and washed with saturated NaHCO<sub>3</sub>. The organic layer was dried over MgSO<sub>4</sub> followed by removal of solvent. The product was purified on a HPLC using C18 reverse-phase column. The eluent gradient was 30% acetonitrile (42 min). <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  1.35-1.71 (m, alkyl protons), 2.21 (t, *J* = 5.3 Hz, 2 H), 2.57 (t, *J* = 5.3 Hz, 2 H), 2.70 – 2.80 (m, 1 H), 2.90-3.00 (m, 1 H), 3.08-3.23 (m, 7 H), 3.59-3.63 (m, 10 H), 4.33 (br, 1 H), 4.53 (br, 1 H), 6.12 (br, 1 H), 6.21 (br, 1 H). <sup>13</sup>C NMR (500 MHz, CDCl<sub>3</sub>)  $\delta$  25.15, 25.63, 26.18, 28.04, 28.11, 28.51, 28.81, 29.21, 30.57, 35.96, 39.20, 40.54, 43.79, 55.52, 60.16, 61.79, 69.77, 69.83, 70.21 (2 C), 163.68, 173.11, 195.60, 199.2. MS(ESI) calculated for C<sub>24</sub>H<sub>41</sub>N<sub>3</sub>O<sub>6</sub>S<sub>3</sub> : 563.2; found: 564.3 [M + H<sup>+</sup>].

<sup>1.</sup> B. Zhang, T. R. Cech, Nature 1997, 390, 96.

<sup>2.</sup> L. Zhang, L. Sun, Z. Cui, R. L. Gottlieb, B. Zhang, B. Bioconjugate Chem. 2001 12, 939.