In Vitro Evolution of a Friedel-Crafts Deoxyribozyme

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Materials and Methods

Materials

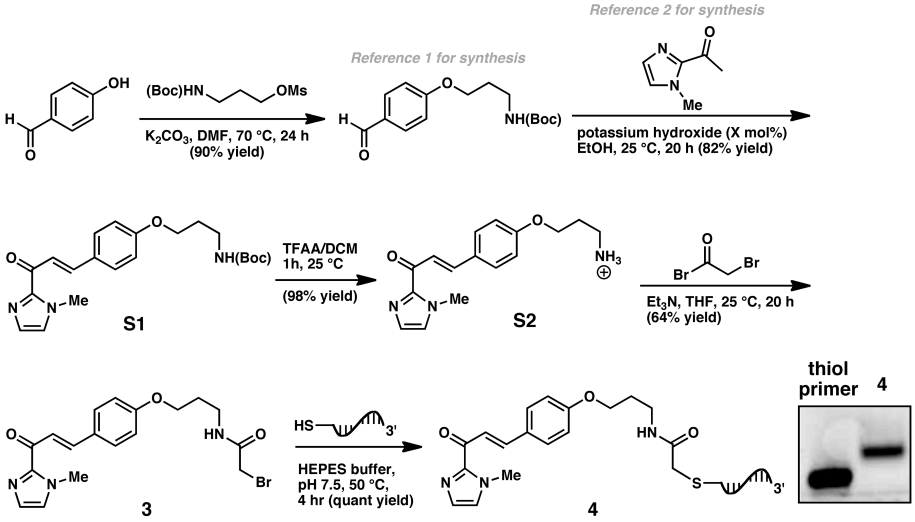
Oligonucleotides - IDT
RNas/DNase-free water - Sigma Aldrich
5-methoxy indole - Sigma Aldrich
Mini Protean TBE-Urea gels - BioRad
Criterion TBE-Urea gels - BioRad
Glycogen - Sigma Aldrich
SyBr Gold stain - Sigma Aldrich
MOPS - BioRad
Dithiothreitol - Sigma Aldrich
Sodium acetate - Sigma Aldrich
Absolute Ethanol - Sigma Aldrich

Equipment

Thermo Cycler - BioRad
Miniprotean PAGE Apparatus - BioRad
Criterion PAGE Apparatus - BioRad
GelDoc - BioRad

Experimental Details and Supporting Data

Synthesis of compound **3**.



Synthesis of S1. See the following references for the preparation of reactants.[1,2] To a solution of 2-acetyl-1-methylimidazole (0.62 g, 5.0 mmol) in EtOH (10.0 mL) was added the benzaldehyde derivative (1.4 g, 5.0 mmol) and a catalytic amount of KOH (100 mg). The solution was stirred for 20 h then transferred to a separatory funnel. The reaction mixture was concentrated, saturated NaCl (50.0 mL) was added and the mixture was extracted with EtOAc (3 x 30 mL). The combined organic extracts were dried over sodium sulfate, filtered, and concentrated in vacuo. The resulting residue was purified by silica gel column chromatography using ethyl acetate: hexanes (55: 45) to provide the chalcone (1.58 gm, 82%) as yellowish thick oil.
^{1}H NMR (300 MHz, CD_3Cl): δ 7.92 (d, *J* = 18.0 Hz, 1H), 7.75 (d, *J* = 18.0 Hz, 1H), 7.60 (d, *J* = 9.0 Hz, 2H), 7.17 (s, 1H), 7.04 (s, 1H), 6.86 (d, *J* = 9.0 Hz, 2H), 4.85 (bs, 1H), 4.05 (s, 3H), 4.02 (t, *J* = 6.0 Hz, 2H), 3.30 (q, *J* = 6.0 Hz, 2H), 1.96 (p, *J* = 6.0 Hz, 2H), 1.41 (s, 9H).

Synthesis of S2. To a stirred solution of compound **S1** (0.385 g, 1.0 mmol, 1.0 equivalent) in DCM (3.0 mL) was added triethylsilane (50.0 μL) and trifluoroacetic acid (2.00 ml) and the resulting reaction mixture was stirred at rt for 1h, then concentrated under reduced pressure. The reaction mixture was dissolved in EtOAc and washed with Sat. Na_2HCO_3 solution. The combined organic extracts were dried over sodium sulfate, filtered, and concentrated in vacuo to provide **S2** (0.280 g, 98%) as a yellowish solid.
^{1}H NMR (300 MHz, DMSO-d_6): δ 7.87 (d, *J* = 16.0 Hz, 1H), 7.72 (m, 4H), 7.57 (s, 1H), 7.21 (s, 1H), 6.99 (d, *J* = 9.0 Hz, 2H), 4.84 (bs, 2H), 4.12 (t, *J* = 6.0 Hz, 2H), 3.99 (s, 3H), 2.95 (q, *J* = 6.0 Hz, 2H), 2.00 (p, *J* = 6.0 Hz, 2H).

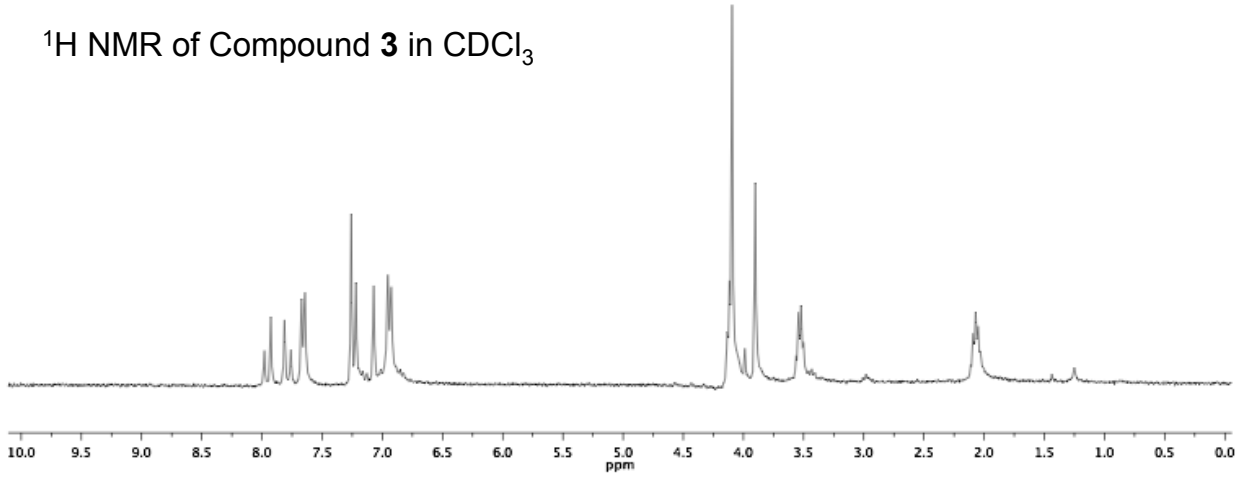
Synthesis of 3. To a stirred solution of compound **S2** (0.285 g, 1.0 mmol) in dry THF (10.0 mL) at 0 °C was added triethyamine (0.42 mL, 3 mmol) followed by the dropwise addition of bromoacetyl bromide (100.0 μL diluted with 5.0 mL of THF). The resulting reaction mixture was stirred at rt for 20 h. The reaction mixture was concentrated and dissolved in EtOAc and washed

[1] *Bioorganic & Medicinal Chemistry Letters* **2009**, *19*, 2763.
[2] *J. Am. Chem. Soc.* **2005**, *127*, 14675.

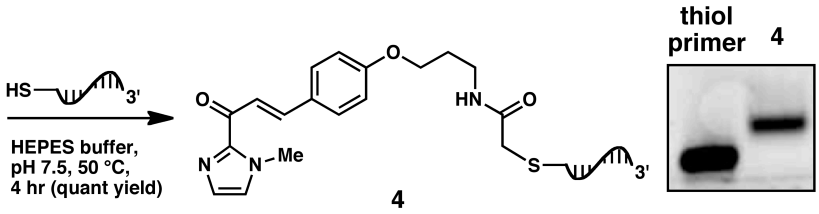
with brine. The combined organic extracts were dried over sodium sulfate, filtered, and concentrated in vacuo. The crude reaction mixture was by silica gel column chromatography using ethyl acetate: hexanes (70: 30) to provide the chalcone bromoacetamide **5** (0.260 g, 64%) as thick oil.

^{1}H NMR (300 MHz, CD_3Cl): δ 7.95 (d, *J* = 16.0 Hz, 1H), 7.77 (d, *J* = 16.0 Hz, 1H), 7.67 (d, *J* = 9.0 Hz, 2H), 7.22 (s, 1H), 7.07 (s, 1H), 6.94 (d, *J* = 9.0 Hz, 2H), 4.12 (t, *J* = 6.0 Hz, 2H), 4.09 (s, 3H), 3.90 (s, 2H), 3.52 (q, *J* = 6.0 Hz, 2H), 2.07(p, *J* = 6.0 Hz, 2H).
Mass Spectrometry: HRMS-ESI (m/z): Calcd for $C_{18}H_{21}N_3O_3$ $[M+H]^+$, 408.0746. Found 408.0754.

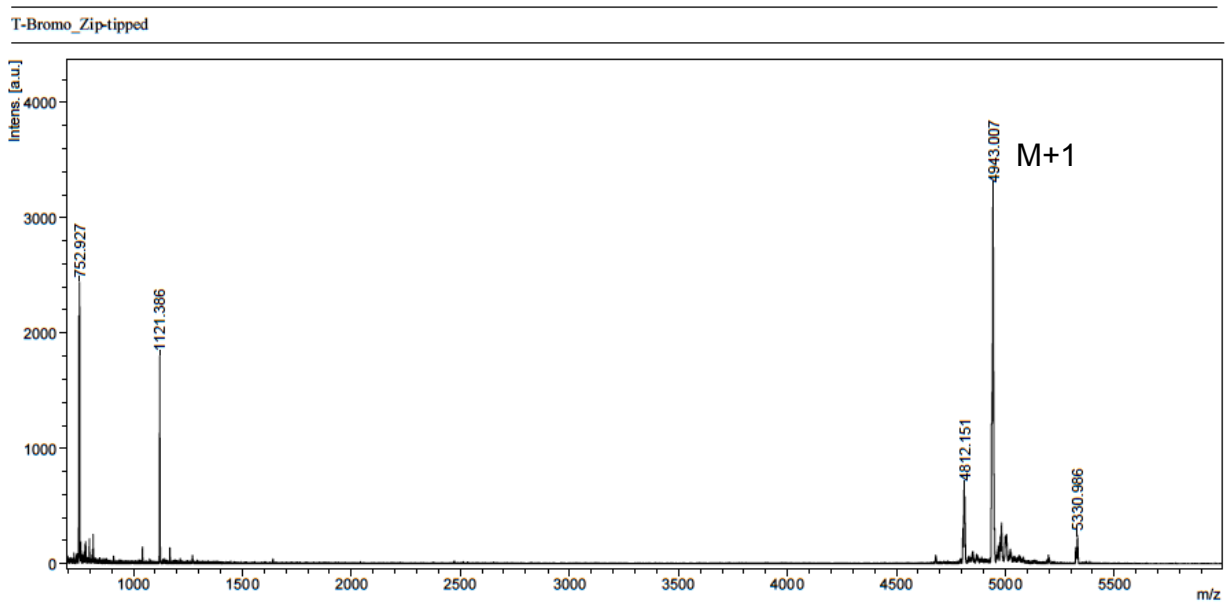
^{1}H NMR of Compound **3** in $CDCl_3$



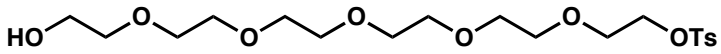
Synthesis of forward primer 4



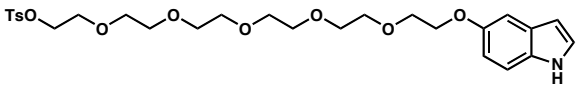
5 ′-thiol primer was reacted with 1.2 equivalents of **3** in HEPES buffer (pH = 7.5), 150mM NaCl, at 50 °C for 4 hours. The reaction was deemed quantitative by PAGE electrophoresis and staining with SYBR Gold. The 5'-acyl imidazole DNA primer was purified by PAGE electrophoresis or Zip-Tip (Sigma) and characterized by MALDI.



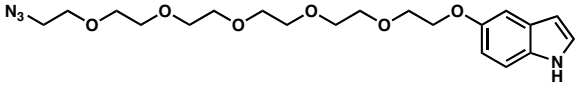
Synthesis of 5



Hexaethylene glycol (1.0 g, 3.54 mmol) and triethylamine (0.493 mL, 3.54 mmol) were dissolved in acetronitrile (15 mL). Toluene –*p*-sulfonyl chloride (0.675 g, 3.54 mmol) was dissolved in acetonitrile (10 mL) and added dropwise over 20 min and kept stirring at room temperature for 20h. The white solid was filtered and washed with acetronitrile.The solution was concentrated under vacuum and diluted with water and extracted with dichloromethane (50 mL). The organic layer was dried over Na_2SO_4 and then concentrated in vacuum. The crude product was purified by flash column chmatography using acetone: chloroform (30: 70) as an eluant to provide a thick oil (0.7 g, 45%).^{1}H NMR (300 MHz, $CDCl_3$) δ 7.72 (d, *J* = 9 Hz, 2H), 7.27 (d, *J* = 9 Hz, 2H), 4.08 (t, *J* = 5.5 Hz, 2H), 3.62-3.50 (m, 22H), 2.90 (s, 1H), 2.37 (s, 3H).

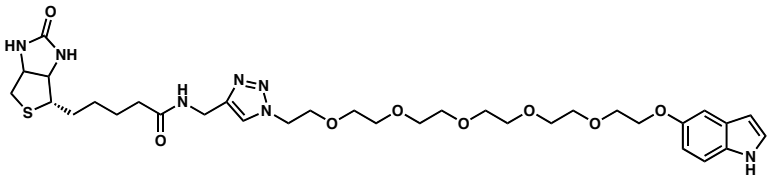


In a 25 mL round bottom flask PPh_3 (0. 290 g, 1.1 mmol) and DIAD (0.220 g, 1.1 mmol) were taken in dry THF (5.0 mL) under argon atmosphere and cooled to 0°C. To this solution 5-hydroxy indole (0.133 g, 1.0 mmol) and mono tosylated hexa PEG (0.436 g, 1.0 mmol) in THF (5.0 mL) was added slowly and stirred at room temperature for 24 h. The reaction mixture was concentrated under vacuum and purified by column chromatography using ethylacetate: hexanes (60: 40) then with methanol /dichlorometahne (1: 99) to provide sticky oil (0.363 g, 66%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.75 (d, J = 8.0 Hz, 2H), 7.29 (d, *J* = 8.0 Hz, 2H), 7.24 (m, 1H), 7.14-7.05 (m, 2H), 6.80 (dd, *J* = 9.0, 3.0 Hz, 1H), 6.38 (m, 1H), 4.92 (bs, 1H), 4.10 (m, 4H), 3.83 (t, *J* = 5.5 Hz, 2H), 3.68-3.52 (m, 18H), 2.38 (s, 3H).



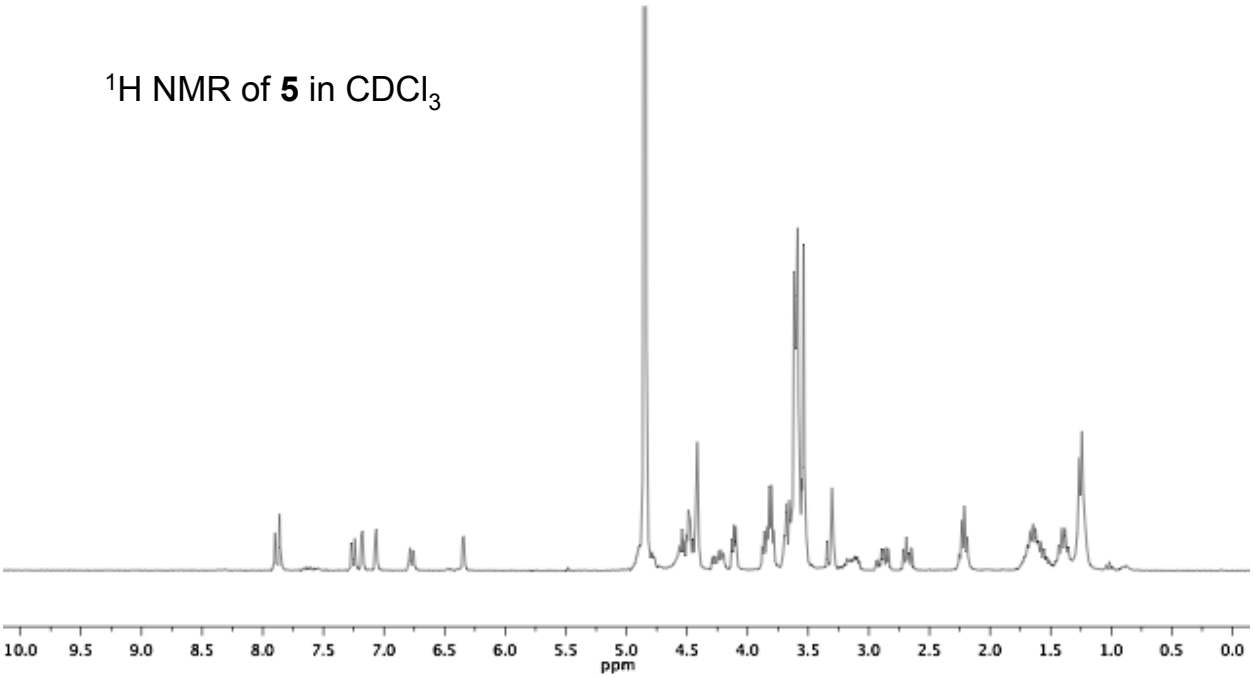
The tosylated hexapeg-indole (0.551 g, 1.0 mmol) was taken in dry DMF (5.0 mL). NaN_3 (0.325 g, 5.0 mmol) and tetrabutylamonium iodide (0.037g, 10 mol%) was added to the solution and stirred for 20 h at 80^{0}C under argon atmosphere. The reaction mixture was diluted with brine

solution (30.0 mL) and extracted with ethyl acetate. The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to provide pure product (0.380 g, 90%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.25 (d, *J* = 8.5 Hz, 1H), 7.15 (m, 1H), 7.08 (d, *J* = 3.0 Hz, 1H), 6.84 (dd, *J* = 9.0, 3.0 Hz, 1H), 6.42 (m, 1H), 4.14 (t, *J* = 5.5 Hz, 2H), 3.84 (t, *J* = 5.5 Hz, 2H), 3.68-3.58 (m, 18H), 3.33 (t, *J* = 5.5 Hz, 2H).



The azide (0.295 g, 0.70 mmol) and biotin alkyne (0.196 g, 0.70 mmol) were taken in acetone (3 mL). To this solution $CuSO_4.5\ H_2O$ (0.018 g, 10 mol%) in water (1.5 ml) and Sodium ascorbate (0.028 g, 20 mol% in 1.5 mL water) was added respectively and stirred at room temperature for 24 h. The reaction mixture was concentrated, diluted with water (30.0 mL) and extracted with dichloromethane (30.0 mL), The organic layer was dried over Na_2SO_4 and purified by flash colum chromatography using methanol /dichloromethane (7: 93) as an eluant to provide pure product (0.344 g, 70%). ^{1}H NMR (300 MHz, CD_3OD) δ 7.90 (s, 1H), 7.25 (d, *J* = 8.5 Hz, 1H), 7.18 (d, *J* = 2.5 Hz, 1H), 7.08 (d, *J* = 2.5 Hz, 1H), 6.76 (dd, *J* = 9.0, 2.5 Hz, 1H), 6.34 (d, *J* = 2.5 Hz, 1H) 4.55 (t, *J* = 4.9 Hz, 2H), 4.43 (s, 2H), 4.29-4.24 (m, 1H), 4.24-4.22 (m, 1H), 3.86 (t, *J* = 4.9 Hz, 4H), 3.69-3.58 (m, 18H), 3.21-3.15 (m, 1H), 2.88 (dd, *J* = 12.8, 4.9 Hz, 1H), 2.68 (d, *J* = 12.8 Hz, 1H), 2.21 (t, *J* = 7.5 Hz, 2H), 1.76-1.54 (m, 4H), 1.46-1.39 (m, 2H).Mass Spectrometry: HRMS-ESI (m/z): Calcd for $C_{33}H_{50}N_7O_8S$ $[M+H]^+$, 704.3442. Found 704.3439.

^{1}H NMR of **5** in $CDCl_3$



DNA library construction. A template library with the sequence CCTCGAGCGAACAGC-N_{40}-TCGACTAGGACTACC-3′ was purchased from IDT. Library DNA was prepared by polymerase chain reaction (PCR), using forward primer **4** ((acyl imidazole-GGAGCTCGCTTGTCG-3′)) and reverse primer 5′-$(AAC)_4$-Glenn spacer 18-CCATCAGGATCAGCT-3′. A 50 µL sample containing 50 pmol of library template, 100 pmol forward primer and reverse primer, 1x of NEB ThermoPol buffer (20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100), 200 µM of dNTP, and 5 units of Taq polymerase was subjected to 25 PCR cycles (conditions: 94 °C for 2 min, then cycles of 94 °C for 30 s, 47 °C for 30 s, and 72 °C for 30 s, with the final cycle continued for 5 min at 72 °C).

Strand separation was performed on a 15% denaturing Criterion PAGE gel and the corresponding gel fragment was excised. Gel extraction was done using a crush & soak method. The crushed fragment was resuspended in 350 µl water and kept at 65 °C for 4 hours. The supernatant was retrieved by centrifugation and was freeze dried. 20µl water was added to this dried gel-extracted library and salt was removed by ethanol precipitation.

In Vitro Selection

1. 150 pmol DNA library was dissolved in 20 mM MOPS buffer (pH 6.5). DNA was denatured by heating to 94°C for 4 minutes and cooling on ice for 10 minutes. To the denatured sample was added 400 nmol biotinylated indole and 150 µM $Cu(NO_3)_2$, to a total volume of 100 µl. The sample was incubated at room temperature. On completion of the reaction, alcohol precipitation was performed to isolate salt-free DNA.

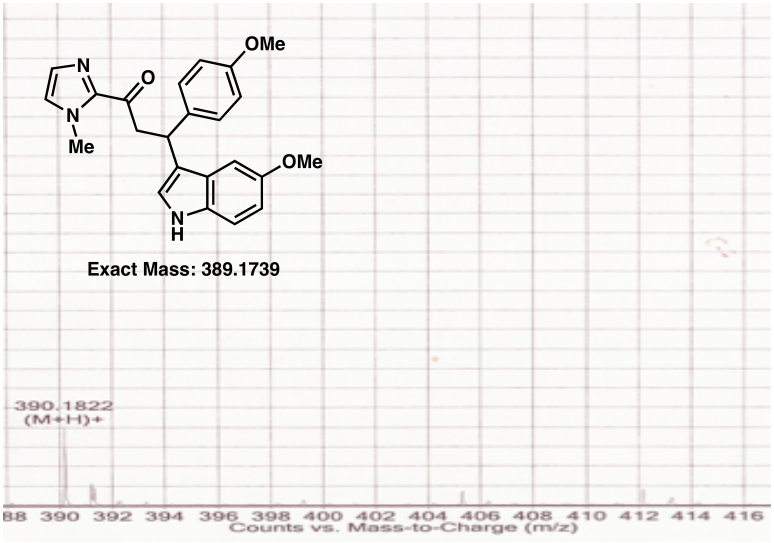
2. Alcohol precipitated DNA library was run on a 15% TBE-urea criterion gel and the higher molecular weight DNA was excised. DNA was isolated as described above (“crush and soak method”).

3. In each selection round, enriched DNA was used as a template for amplifying the next round library. 25 cycles of PCR were performed in 50 µl with 25 pmol of forward primer, 100 pmol of reverse primer, 0.2 mM of each dNTP, 1× NEB ThermoPol buffer, and 2.5 units of Taq polymerase. The amplification program was: 94°C for 2 min, 25 cycles of 94°C for 30 s, 50°C for 45 s, and 72°C for 45 s, with the final cycle continued for 5 min at 72 °C.

Cloning after 7th round of selection. After 7 rounds of selection, the enriched DNA was used as a template for 10 cycles of PCR amplification of the active deoxyribozyme molecules. 1 µl of 10-cycle PCR product was used further as template for 25 cycles of PCR. This PCR product was digested using *EcoRI* and *HindIII* and run on 1.5% agarose gel. The 100 bp restriction digested product was excised from the gel and cloned into doubly digested pUC 19 vector, then transformed in TOP10 chemically competent *E. coli*. The individual colonies were screened for the presence of insert using colony PCR and positive clones were selected.

Cis Reaction. 150 pmol (1.5 μM) acyl imidazole-lined M14 DNA in 20mM MOPS buffer (pH 6.5) was denatured by heating at 94°C for 4 minutes and cooling on ice for 10 minutes. To the refolded sample was added 400 nmol (400 μM) biotinylated indole **5** and 150 μM $Cu(NO_3)_2$, to a total volume of 100 μl. On completion of the reaction, M14 DNA was isolated by ethanol precipitation and run on a 15% TBE-urea gel.

Trans Reaction. 2 mM acyl imidazole **1**, 10mM indole **5**, 0.6 mM $Cu(No_3)_2$, and 1 mM M14 DNA were reacted in 1 mL of MOPS buffer (pH = 6.5). The reaction was carried out in a glass vial and kept at 37ºC Incubator for 24 hours. Organics were extracted with EtOAc, solvent was removed under vacuum, and product was isolated by flash chromatography on a silica column.[3] Yield was calculated by measuring the dry weight of obtained product, which is represented as a single spot on thin layer chromatography (visualized by UV radiation). Spectra agree with previous reports[4], and are supported by the mass spectrometry data below.



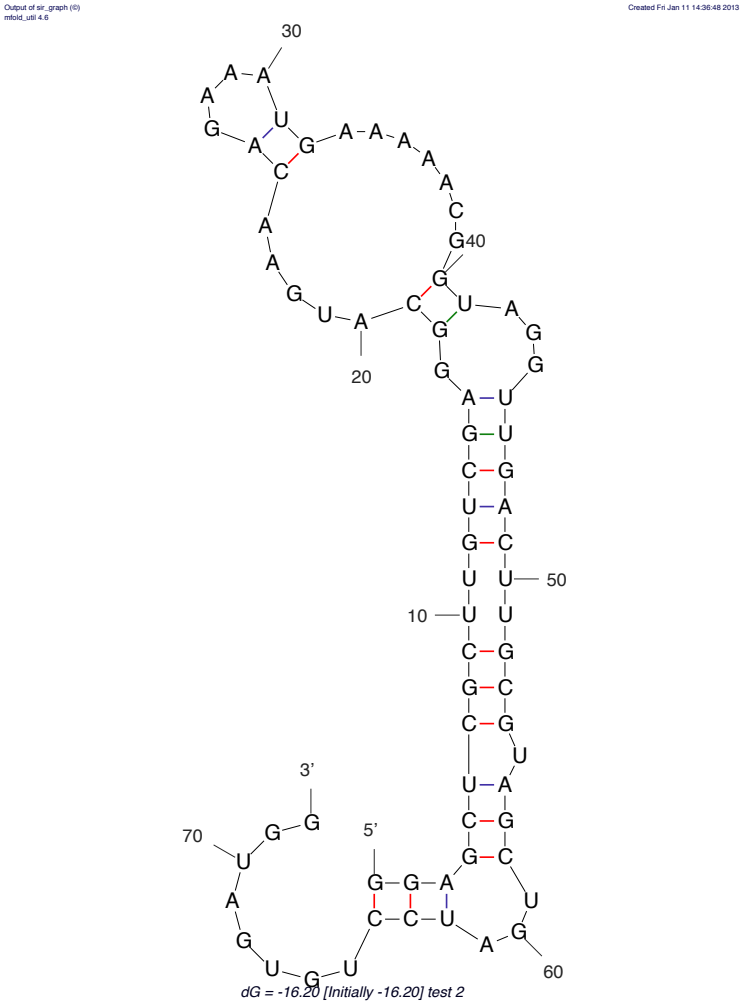
[3] *J. Org. Chem.* **1978**, *43*, 2923.
[4] *Angew Chem Int Ed Engl*. **2009**, *48*, 3346.

M14 sequence.

Insert sequence of M14:

5′-AGGCATGAACAGAAATGAAAAACGGTAGGTTGACTTGCGT-3′

mFold[5] structure prediction.



[5] *Nucleic Acids Res*. **2003**, *31*, 3406.