Supporting information

Facile Functionalization of FK506 for Biological Studies by the Thiol-Ene ‘Click’ Reaction

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Table of content

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supplementary Figure 1</td>
<td>2</td>
</tr>
<tr>
<td>Supplementary Figure 2</td>
<td>3</td>
</tr>
<tr>
<td>Experiment section</td>
<td>4</td>
</tr>
<tr>
<td>Chemicals and instrument</td>
<td>4-7</td>
</tr>
<tr>
<td>Synthesis of compounds</td>
<td>7</td>
</tr>
<tr>
<td>Plasmid construction</td>
<td>7</td>
</tr>
<tr>
<td>Cell Culture and transfection</td>
<td>7-8</td>
</tr>
<tr>
<td>Luciferase assay</td>
<td></td>
</tr>
<tr>
<td>Compound Characterization</td>
<td></td>
</tr>
<tr>
<td>NMR of compound 17</td>
<td>9</td>
</tr>
<tr>
<td>NMR of compound 18</td>
<td>10</td>
</tr>
<tr>
<td>NMR and HRMS of compound 19</td>
<td>11-12</td>
</tr>
<tr>
<td>NMR and HRMS of compound 20</td>
<td>12-13</td>
</tr>
<tr>
<td>NMR and HRMS of compound 21</td>
<td>14-15</td>
</tr>
<tr>
<td>NMR and HRMS of compound 9</td>
<td>15-16</td>
</tr>
<tr>
<td>1H NMR and HRMS of compound 3</td>
<td>17</td>
</tr>
<tr>
<td>1H NMR and HRMS of compound 4</td>
<td>18</td>
</tr>
<tr>
<td>1H NMR and HRMS of compound 6</td>
<td>19</td>
</tr>
<tr>
<td>1H NMR and HRMS of compound 11</td>
<td>20</td>
</tr>
<tr>
<td>1H NMR and HRMS of compound 12</td>
<td>21</td>
</tr>
<tr>
<td>1H NMR and HRMS of compound 13</td>
<td>22</td>
</tr>
<tr>
<td>1H NMR and HRMS of compound 15</td>
<td>23</td>
</tr>
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</table>
**Fig. S1.** TEC reaction setup. Reaction was carried out using handheld UV lamp at room temperature.
Fig. S2. TEC reaction time profile. The conversion was based on FK506 consumption followed under HPLC.
**Experiment section**

**Chemicals and instruments:**

Bulk solvents were obtained from EMD. Cysteamine, 3-thiopropanoic acid, Cysteine, Dithiothreitol, 5-hexynoic acid, N-Ethyl-N’-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI), Et3N, 1-Hydroxybenzotriazole hydrate (HOBr), 3-Aminopropanol, di-tert-butyl dicarbonate (Boc2O), 4-(Dimethylamino)pyridine (DMAP), p-Toluensulfonyl chloride (TsCl), triethylsilane, NaN3 and 2,2-Dimethoxy-2-phenylacetophenone (DPAP) were obtained from Sigma-Aldrich and Alfa-Aesar and were used directly without further purification. Other chemicals are commercially available. Boc-cysteamine was synthesized from Cysteamine following the general procedure. 3-(tritylthio)propinoic acid was synthesized following the reported literature (Langmuir, 2008, 24, 13581). NMR spectra were recorded on a Bruker instrument (300 MHz). Mass and NMR spectra for new compounds were recorded at the Mass Spectrometry and NMR Facilities, Department of Chemistry and Chemical Biology, University of New Mexico.

**Synthesis of compounds:**

![Scheme S1](image_url)

Scheme S1. a) (Boc)2O, DIPEA, MeOH (99%); b) TsCl, DMAP, Et3N, DCM (96%); c) NaN3, DMF, rt, 13 h (89%); d) TFA/DCM, rt, 1 h (99%); e) HATU, DIPEA, DCM, rt, overnight (92%); f) TFA/DCM(v/v = 2: 8), Et3SiH, rt, 1h (94%).

Prepare of compound 17

3-Aminopropanol (7.0 g, 92 mmol) dissolved in methanol (100 mL) was successively treatd with di-tert-butyl dicarbonate (22.0 g, 101.2 mmol) and di-iso-propylethylamine (32 mL, 184 mmol) stir overnight at room temperature. After removal of the solvent under reduced pressure, the product was dissolved in DCM (100 mL). The organic layer was washed with 10 % citric acid (X 2). The aqueous layers were extracted with DCM. The combined organic layers were dried over sodium sulfate. Removal of the solvent under vacuum gave compound 17 as colorless viscous oil (17.5 g, yield: 99 %). 1H NMR (CDCl3, 300 MHz): 4.78 (bs, 1H), 3.67-3.63 (t, J = 11.4 Hz, 2H), 3.29-3.25 (t, J = 12.3 Hz, 2H), 1.69- 1.63 (m, 2H), 1.44 (s, 9H). 13C NMR (CDCl3, 300 MHz): 157.50, 79.64, 59.26, 36.97, 32.89, 28.36.

Prepare of compound 18

Compound 17 (3.50 g, 20 mmol), Et3N (2.02 g, 20 mmol) and DMAP (0.244g, 2 mmol) were stirred in DCM about 5 min, then add TsCl (4.00 g, 21 mmol) and stirred further 2 h at room temperature. Colorless viscous solid was
obtained after purification by silica gel column chromatography using hexane/ethyl acetate (v/v = 2: 1) as an eluting solvent (Rf = 0.72). Yield: 96 %. 1H NMR (CDCl3, 300 MHz): 7.79-7.77 (d, J = 8.4 Hz, 2H), 7.36-7.33 (d, J = 8.1 Hz, 2H), 4.61 (bs, 1H), 4.09-4.05 (t, J = 12.0 Hz, 2H), 3.15 (s, 2H), 2.44 (s, 3H), 1.87-1.79 (m, 2H), 1.41 (s, 9H). 13C NMR (CDCl3, 300 MHz): 155.87, 144.91, 129.92, 127.88, 127.47, 67.17, 49.66, 37.10, 35.40, 28.5, 28.35, 21.64.

Prepare of compound 19

Compound 18 (3.29 g, 10 mmol) and NaN3 (3.30 g, 30 mmol) were stirred in THF/H2O (v/v = 5: 1) for 2 h. The mixture was extracted with ethyl acetate; the organic layers were combined, washed three times with NaHCO3 solution, and subsequently dried with anhydrous Na2SO4. Colorless viscous solid was obtained after the solvent was removed under reduced pressure. Yield: 89%. 1H NMR (CDCl3, 300 MHz): 4.66 (bs, 1H), 3.37-3.33 (t, J = 13.2 Hz, 2H), 3.22-3.18 (t, J = 12.9 Hz, 2H), 1.80-1.71 (m, 2H), 1.43 (s, 9H). 13C NMR (CDCl3, 300 MHz): 155.94, 79.42, 49.11, 38.10, 29.29, 28.37. TOF-HRMS (m/z) found (calcd.) for C8H16N4OS (M): [M+Na]+, 223.1171 (223.1171).

Prepare of compound 20

Compound 19 (1g, 5 mmol) was stirred in DCM/TFA (v/v = 5: 1) for 1 h, white solid was obtained after the solvent was removed under reduced pressure. Yield: 99%. 1H NMR (CDCl3, 300 MHz): 8.30 (bs, 2H), 3.57 (s, 2H), 3.18 (s, 2H), 2.09 (s, 2H). 13C NMR (CDCl3, 300 MHz): 48.51, 37.81, 26.80. TOF-HRMS (m/z) found (calcd.) for C3H3N4 (M): [M+H]+, 101.0814 (101.0827).

Prepare of compound 21

3-(tritylthio)propanoic acid (697 mg, 2.0 mmol), HATU (836 mg, 2.2 mmol), DIPEA (516 mg, 4.0 mmol), and compound 20 (220 mg, 2.2 mmol) were stirred at room temperature overnight in DCM. White solid was obtained after purification by silica gel column chromatography using hexane/ethyl acetate (v/v = 1: 1) as an eluting solvent (Rf = 0.65). Yield: 96 %. 1H NMR (Acetone-d6, 300 MHz): 7.41-7.39 (m, 6H), 7.34-7.28 (m, 6H), 7.26-7.20 (m, 3H), 7.14 (bs, 1H), 3.39-3.35 (t, J = 13.5 Hz, 2H), 3.27-3.20 (q, J = 19.2 Hz, 2H), 2.44-2.39 (t, J = 14.7 Hz, 2H), 2.22-2.17 (t, J = 14.7 Hz, 2H), 1.77-1.68 (m, 2H). 13C NMR (Acetone-d6, 300 MHz): 171.11, 145.83, 130.34, 128.70, 127.47, 67.17, 49.66, 37.10, 35.40, 28.53. TOF-HRMS (m/z) found (calcd.) for C25H26N4O3S (M): [M+Na]+, 453.1717 (453.1725), [2M+Na]+, 883.3400 (883.3552).

Prepare of compound 9

To a solution of compound 21 (215 mg, 0.5 mmol) in trifluoroacetic acid (TFA, 1mL) and CH2Cl2 (2 mL) was added triethylsilane (174 mg, 1.5 mmol). The resulting mixture was stirred for a half hour at room temperature. After evaporating the reaction solvent, CH2Cl2 (20 mL) was added to the resulting residues. The organic layer was extracted with H2O (10 mL). The aqueous layer was then evaporated to give the transparent oil product. Yield: 94 %. 1H NMR (CDCl3, 300 MHz): 5.93 (s, 1H), 3.41-3.36 (m, 4H), 2.84-2.80 (q, J = 12.9 Hz, 2H), 2.52-2.49 (t, J = 7.8 Hz, 2H), 1.84-1.79 (m, 2H), 1.62-1.59 (t, J = 9.9 Hz, 1H). 13C NMR (CDCl3, 300 MHz): 171.58, 49.45, 40.46, 37.48, 28.79, 20.54. TOF-HRMS (m/z) found (calcd.) for C6H12N4OS (M): [M+Na]+, 211.0628 (211.0630), [2M+Na-2H]+, 397.1207 (397.1207).

Synthesis of compound 3

FK506 (201.0 mg, 0.25 mmol), Boc-cysteamine (46.0 mg, 0.26 mmol), DPAP (3.2 mg, 12.5 mmol) and 0.4 mL dichloromethane were put in a vials, and stirred 15 min under UV light. White solid (246 mg, 98% yield) was
obtained after purification by silica gel column chromatography using ethyl acetate as an eluting solvent (Rf = 0.5). 1H NMR (300 MHz, CDCl3): 5.33-5.21 (d, J= 36.8 Hz, 1H), 5.12-5.08 (m, 2H), 4.88-4.40 (m, 1H), 3.94-3.57 (m, 3H), 3.41-3.29 (m, 9H), 3.05-2.95 (m, 2H), 2.78-2.50 (m, 6H), 2.38-1.26 (m, 45H), 1.07-0.82 (m, 13H). TOF-HRMS (m/z) found (calcd.) for C51H48N2O14S (M): [M+Na]+, 1003.5583 (1003.5541).

Synthesis of compound 4
Method A: compound 3 dissolved in DCM/TFA and stir 1 h at room temperature.
Method B: FK506 (201.0 mg, 0.25 mmol), cysteamine (19.3 mg, 0.26 mmol), DPAP (3.2 mg, 12.5 nmol) and 0.4 mL methanol were put in a vials, and stirred 15 min under UV light. White solid (211 mg, 96% yield) was obtained after purification by silica gel column chromatography using ethyl DCM/methanol (v/v= 5: 1) as an eluting solvent (Rf = 0.56). 1H NMR (300 MHz, Acetone-d6): 6.93-6.80 (m, 1H), 6.45-6.29 (m, 1H), 5.30-4.98 (m, 3H), 4.66-4.34 (m, 1 H), 4.13-4.02 (m, 2H), 3.80-3.32 (m, 11H), 3.02-3.00 (m, 2H), 2.68-2.50 (m, 4H), 2.49-1.58 (m, 36 H), 1.21-0.88 (m, 13H). TOF-HRMS (m/z) found (calcd.) for C46H76N2O12S (M): [M+H]+, 881.5215 (881.5197).

Synthesis of compound 6
5-hexynoic acid (28.0 mg, 0.25 mmol), EDCI (52.7 mg, 0.275 mmol), HOBr (37.1 mg, 0.275 mmol), and Et3N (50.5 mg, 0.5 mmol), stir about 1 h in DCM, then add compound 4 (242 mg, 0.275 mmol) and stir overnight. White solid (190 mg, 78% yield) was obtained after purification by silica gel column chromatography using ethyl acetate as an eluting solvent (Rf = 0.42). 1H NMR (300 MHz, CDCl3): 6.80-6.69 (m, 1H), 6.34-6.10 (m, 1H), 5.97 (s, 1H), 5.26-4.85 (m, 2H), 4.75-4.72 (m, 1H), 4.49-4.16 (m, 1H), 3.92-3.72 (m, 1H), 3.59-3.26 (m, 11H), 3.06-2.92 (m, 2H), 2.63-2.61 (m, 2H), 2.49-2.23 (m, 6H), 2.12-1.23 (m, 39H), 1.80-0.83 (m, 13H). TOF-HRMS (m/z) found (calcd.) for C52H82N2O13S (M): [M+H]+, 979.5360 (976.2860).

Synthesis of compound 11
FK506 (201.0 mg, 0.25 mmol), 3-thiopropanoic acid (28.0 mg, 0.26 mmol), DPAP (3.2 mg, 12.5 nmol) and 0.4 mL dichloromethane were put in a vials, and stirred 15 min under UV light. White solid (222 mg, 98% yield) was obtained after purification by silica gel column chromatography using ethyl acetate/ acetone (v/v= 1: 1) as an eluting solvent (Rf = 0.51). 1H NMR (300 MHz, CDCl3): 5.32-5.20 (d, J= 36.4 Hz, 1H), 5.10-5.00 (m, 2H), 4.75-4.30 (m, 1H), 3.94-3.54 (m, 3H), 3.40-3.29 (m, 11H), 3.05-2.95 (m, 2H), 2.75-2.51 (m, 6H), 2.38-1.25 (m, 36H), 1.06-0.82 (m, 13H). TOF-HRMS (m/z) found (calcd.) for C47H75N10O14S (M): [M-H]-, 908.4821 (908.4830).

Synthesis of compound 12
FK506 (201.0 mg, 0.25 mmol), compound 9 (48.9 mg, 0.26 mmol), DPAP (3.2 mg, 12.5 nmol) and 0.4 mL DCM were put in a vials, and stirred 15 min under UV light. Pale yellow solid (235 mg, 95% yield) was obtained after purification by silica gel column chromatography using ethyl acetate/ acetone (v/v= 4: 1) as an eluting solvent (Rf = 0.56). 1H NMR (300 MHz, CDCl3): 6.50 (s, 1H), 5.32-4.95 (m, 3H), 4.74-4.22 (m, 2H), 3.91-3.53 (m, 2H), 3.38-3.27 (m, 17H), 2.98-2.95 (m, 4H), 2.80-2.64 (m, 4H), 2.58-2.39 (m, 4H), 2.15-1.34 (m, 34H), 0.98-0.80 (m, 13H). TOF-HRMS (m/z) found (calcd.) for C50H81N3O15S (M): [M+Na]+, 1014.5485 (1014.5449).

Synthesis of compound 13
FK506 (201.0 mg, 0.25 mmol), Cysteine (31.5 mg, 0.26 mmol), DPAP (3.2 mg, 12.5 nmol) and 0.5 mL methanol/water (1: 1) were put in a vials, and stirred 15 min under UV light. White solid (220 mg, 95% yield) was
obtained after purification by silica gel column chromatography using acetone/methanol (v/v = 1:1) as an eluting solvent (Rf = 0.46). 1H NMR (300 MHz, CDCl3): 5.27-4.95 (m, 3H), 4.63-4.33 (m, 1H), 4.08-3.97 (m, 2H), 3.70-3.41 (m, 3H), 3.38-2.75 (m, 13H), 2.09-1.20 (m, 34H), 0.96-0.71 (m, 13H). TOF-HRMS (m/z) found (calcd.) for C47H76N2O14S (M): [M+H]+, 925.5076 (925.5096).

Synthesis of compound 15
FK506 (201.0 mg, 0.25 mmol), dithiothreitol (20.2 mg, 0.13 mmol), DPAP (3.2 mg, 12.5 mmol) and 0.4 mL dichloromethane were put in a vials, and stirred 15 min under UV light. White solid (220 mg, 99% yield) was obtained after purification by silica gel column chromatography using ethyl acetate/acetone (v/v=1:1) as an eluting solvent (Rf = 0.56). 1H NMR (300 MHz, CDCl3): 5.33-5.20 (d, J= 36.5 Hz, 2H), 5.10-5.06 (m, 4H), 4.74-4.28 (m, 4H), 3.92-3.57 (m, 8H), 3.41-3.30 (m, 22H), 3.06-2.97 (m, 4H), 2.74-2.54 (m, 14H), 2.30-1.25 (m, 64H), 1.08-0.83 (m, 26H). TOF-HRMS (m/z) found (calcd.) for C92H148N2O26S2 (M): [M+Na]+, 1783.9602 (1783.9659).

Plasmid construction
All DNA fragments were amplified by PCR (Polymerase chain reaction) from other intermediate constructs. PCR was carried out with Phusion DNA Polymerase (New England Biolabs), PfuUltra II Fusion HotStart DNA Polymerase (Agilent Technologies) under S1000 thermal cycler with Dual 48/48 Fast Reaction Module (Bio-Rad).

1) For SV-ires-GalDBD-3FKBP12, a DNA construct SV-VP16-Frb-ires-GalDBD-FKBP12x3 (Sci. Signal., 2011, 4, rs2) was firstly digested by EcoRI and BamHI. The sticky ends of the vector were blunted by DNA Polymerase I (Klenow) Fragment (New England Biolabs) under the present of dNTPs. Finally, the blunt ends were ligated by T4 DNA Ligase (New England Biolabs).

2) For SV-VP16-FKBPI2x2, the DNA construct was first made as SV-VP16-FKBPI2. SV-VP16-Frb-ires-GalDBD-FKBPI2x3 was firstly digested by Ascl and NotI. The PCR product of FKBPI2 was inserted into the vector by recombination using the In-Fusion HD Enzyme Premix (ClonTech). The second copy of FKBPI2 was inserted via the Ascl site using T4 DNA ligase.

Cell Culture and transfection
CHO cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals), 1×glutamate (100× from Gibco) and 1×penicillin/streptomycin (Pen/Strep, 100X from Gibco). Cells were plated with the starting concentration of 50,000 cells per well in a 24-well plate (Greiner Bio-one) the day before transfection. An amount of 0.2µg of each DNA construct was mixed with Opti-MEM (Gibco) and PEI. After incubation at room temperature for 15min, the mixture was added to the cells and cultured for 24h. Then FK506, FK1012-DT and FK1012-ZE dissolved in DMSO were added into the cell culture with the final concentration of 200nM, 100nM and 100nM respectively. Each type of the experiments, including the one with transfected DNA but without drugs, was carried out as triplets. After the incubation of 10 hours, the cells were harvested and washed by PBS buffer (Gibco) for 3 times.

Luciferase assay
Cells in 24-well plates were frozen under the temperature of -80°C at first and then lysed with 100 µL of 1×Passive Lysis Buffer (Promega Corporation) at room temperature for 10min on a shaker. Cell lysates were then collected and centrifuged in tubes and 10µL of supernatant was added separately into a 96 well plate for Luciferase assay. 90µL
Luciferase substrate solution (5mg of D-luciferin and 7mg of coenzyme A in 33mL of Luciferase reading buffer, which includes 20mM tricine, 1.07mM (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67mM MgSO₄, 0.1mM EDTA, 33.3mM dithiothreitol and 0.53mM ATP in water) was added into each well with cell lysates. The signal was read with a 3s delay and 1s integration with Clomax Multi Detection System (Promega). Obtained data were analyzed by KaleidaGraph. The shown results are from 3 experimental repeats.
$^{1}$H NMR of compound 17

$^{13}$C NMR of compound 17
1H NMR of compound 18

13C NMR of compound 18
\(^1\)H NMR of compound 19

\(^{13}\)C NMR of compound 19
HRMS of compound 19

\(^1\)H NMR of compound 20
\[ ^{13}C \text{ NMR of compound 20} \]

\[ \text{HRMS of compound 20} \]
$^1$H NMR of compound 21

$^{13}$C NMR of compound 21
HRMS of compound 21

$^1$H NMR of compound 9
$^1$H NMR of compound 3

HRMS of compound 3
$^1$H NMR of compound 4

HRMS of compound 4
$^1$H NMR of compound 11

HRMS of compound 11
$^1$H NMR of compound 12

HRMS of compound 12
$^1$H NMR of compound 13
Elemental Composition Report

Single Mass Analysis
Tolerance = 20.0 PPM / DBE: min = -1.5, max = 50.0
Isotope cluster parameters: Separation = 1.0  Abundance = 1.0%

Monoisotopic Mass, Odd and Even Electron Ions
3 formula(e) evaluated with 3 results within limits (up to 50 closest results for each mass)

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HRMS of compound 15