A trimethoprim conjugate of thiomaltose has enhanced antibacterial efficacy *in vivo*.

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SUPPLEMENTARY INFORMATION

ABBREVIATIONS

Ac……..……Acetyl

AcOH…….……Acetic acid

C…….…….…….Carbon

$CDCl_3$……..……....Deuterated chloroform

CD_3OD…….....Deuterated methanol

CFU…….Colony forming unit

CH_3CN……..Acetonitrile

CuI……..……...Copper iodide

DBU…….....1,8-Diazabicyclo[5.4.0]undec-7-ene

DCC…….……. N,N'-Dicyclohexylcarbodiimide

DCM/CH_2Cl_2……Dichloromethane/Methylene chloride

DIPEA……..*N,N*-Diisopropylethylamine

DI water…...…….....Deionized water

DMAP…….4-Dimethylaminopyridine

DMF……..……..*N, N*-Dimethylformamide

DMSO..…….....Dimethyl sulfoxide

DMSO-d_6..……...……Deuterated Dimethyl sulfoxide

D_2O……...…….Deuterium oxide

ESI.....Electrospray ionization

EtOAc…….....…….…….....Ethyl acetate

H……..…….…….....Proton

HCl……..…….….....Hydrogen chloride

H_2O.....Water

HPLC.....High-performance liquid chromatography

HRMS…….…….....High resolution mass spectrometry

Hz……..…….....……..…Hertz

LB……. Luria-Bertani

LiOH……..……Lithium hydroxide

M…….…….…..……...Molar

MALDI……...Matrix assisted laser desorption ionization spectroscopy

MeOH/CH_3OH..……..…….…Methanol

mL……..Milliliter

$NaHCO_3$……..…….Sodium bicarbonate

NaOH……...……...…….....Sodium hydroxide

$NaSO_4$…….....…….…….....Sodium sulfate

NMR……Nuclear magnetic resonance

N_2……...……...…….....Nitrogen

OD...…….Optical density

PBS...……...……..……Phosphate buffered PBS

Py…..……..Pyridine

s.e.m.……...…Standard error of the mean

TEA……...Triethyl amine

TLC…...……Thin layer chromatography

UV……...……Ultraviolet

µL…….……..Micro litter

EXPERIMENTAL PROCEDURES

^{1}H-NMR spectra were recorded in $CDCl_3$, CD_3OD, DMSO-d_6 or D_2O on a Bruker AVB-400 spectrometer at 298K. TMS ($\delta\ (ppm)_H = 0.00$) was used as the internal reference. ^{13}C-NMR spectra were recorded in either $CDCl_3$, CD_3OD, DMSO-d_6 or D_2O at a 100MHz on a Bruker AVB-400 spectrometer, using the central resonances of $CDCl_3$ ($\delta\ (ppm)_C = 77.0$) and methanol ($\delta\ (ppm)_C = 50.4$) as the internal references. Chemical shifts are reported in ppm and multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), and m (multiplet). Coupling constants, *J*, are reported in hertz (Hz). High-resolution mass spectra (HRMS) were obtained on a AB SCIEX TOF/TOF 5800 system and are reported as m/z (relative intensity). Accurate masses are reported for the molecular ion (M^+) or a suitable fragment ion. Chemicals and solvents were purchased from Sigma-Aldrich or VWR and used without further purification. Flash chromatography was carried out using silica gel (230-400 mesh). All reactions were performed under anhydrous conditions under N_2 or Argon and monitored by TLC on MD Millipore Silica Gel 60 F254 Glass Plates. Detection was accomplished by examination under UV light (254 nm) and by charring with 20 % sulfuric acid in methanol.

S1. Synthesis

S1.1. Synthesis of 4-(but-3-yn-1-yldisulfanyl)butanoic acid (1)

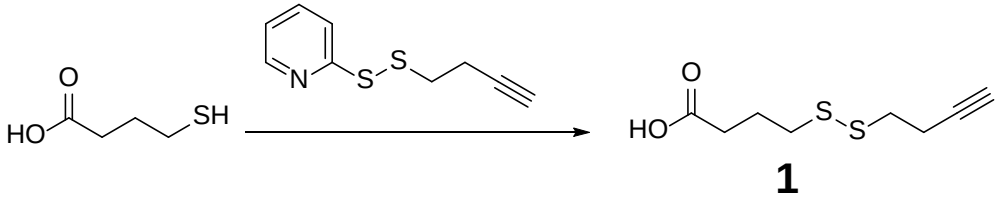


Figure S1. Synthesis of compound **1**.

To a stirred solution of 4-mercaptobutanoic acid (0.36 g, 3.0 mmol) in DCM (10 mL) was added 2-(but-3-yn-1-yldisulfanyl)pyridine (0.65 g, 3.3 mmol) in DCM (5 mL) and TEA (0.5 mL), and the reaction mixture was stirred at room temperature overnight under

N_2. The reaction was concentrated *in vacuo* and purified by flash column chromatography on silica gel (hexane/EtOAc/AcOH, 7:3:0.05) to afford **1** (0.34 g, 56%). ^{1}H NMR (400 MHz, $CDCl_3$): *δ (ppm)* 2.82 (t, *J* = 7.2 Hz, 2H), 2.76 (t, *J* = 7.0 Hz, 2H), 2.60 (td, *J* = 7.0, 2.3 Hz, 2H), 2.51 (t, *J* = 7.2 Hz, 2H), 2.13 – 1.99 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): *δ (ppm)* 178.7, 82.1, 69.6, 37.7, 37.1, 32.2, 23.9, 19.0. HRMS m/z Found: 203.0207, calculated: 203.0200 for $C_8H_{11}O_2S_2$ [*M*-H]$^-$.

S1.2. Synthesis of Trimethoprim alkyne (2)

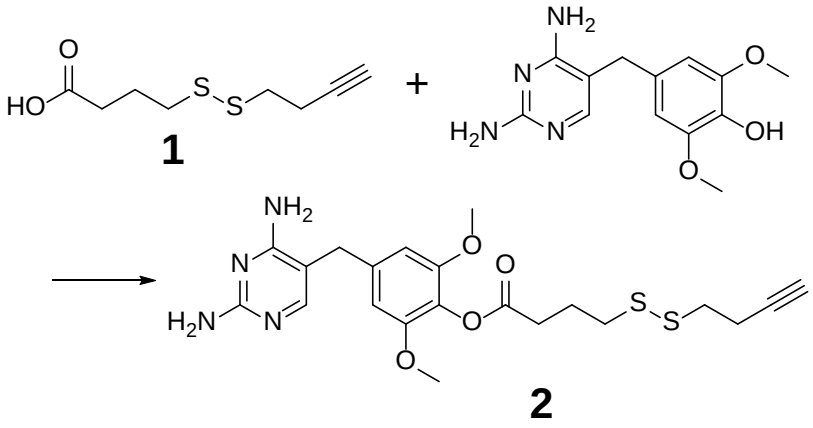
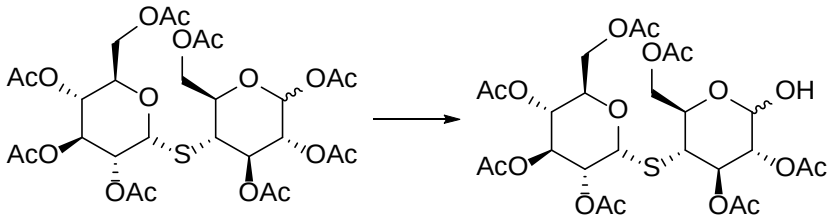


Figure S2. Synthesis of compound **2**.

To a stirred suspension of 4'-OH-trimethoprim[1] (1.1 g, 3.8 mmol) and the acid **1** (0.31 g, 1.5 mmol) in DMF (10 mL) was added DCC (0.34 g, 1.6 mmol), DMAP (0.30 g, 2.4 mmol) and TEA (0.3 mL). The reaction mixture was stirred at room temperature overnight under N_2 and then 200 mL of EtOAc was added. The mixture was extracted with water (50 mL x 2) and brine (50 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated to dryness *in vacuo*. The residue was purified by flash column chromatography on silica gel (5% MeOH in DCM) to afford **2** (193 mg, 28%). ^{1}H NMR (400 MHz, MeOD): *δ (ppm)* 7.55 (s, 1H), 6.57 (s, 2H), 3.76 (s, 6H), 3.69 (s, 2H), 2.85 (dm, 4H), 2.70 (t, J = 7.1 Hz, 2H), 2.59 (td, J = 7.1, 2.5 Hz, 2H), 2.32 (t, J = 2.6 Hz, 1H), 2.12 (m, 2H). ^{13}C NMR (100 MHz, MeOD): *δ (ppm)* 172.9, 153.7, 138.6, 108.7, 106.4, 83.2, 70.9, 61.7, 56.8, 51.9, 40.6, 38.4, 34.6, 33.4, 33.2, 32.1, 27.1, 26.7, 26.2, 25.8,

19.8. HRMS m/z Found: 463.1461, calculated: 463.1474 for $C_{21}H_{27}N_4O_4S_2$ $[M+H]^+$.

S1.3 Synthesis of α-D-glucopyranose, 4-S-(2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl) -4-thio-2,3,6-triacetate (4).



4

Figure S3. Synthesis of compound **4**.

Thiomaltose acetate was prepared following the reported method.[2] To a stirred solution of thiomaltose acetate (0.35 g, 0.50 mmol) in DMF (10 mL) was added $N_2H_4 \cdot HOAc$ (46.0 mg, 0.50 mmol). The reaction mixture was kept at room temperature for 12 hours under nitrogen, and the mixture was concentrated *in vacuo*. The residue was dissolved in EtOAc (100 mL) and washed with water (30 mL x 2) and brine (30 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated to dryness *in vacuo*. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 1:1) to afford **4** (α/β mixture, 1:1, 0.26 g, 82%). ^{1}H NMR (400 MHz, $CDCl_3$): *δ (ppm)* 5.88 (m, 1H), 5.59 (t, *J* = 10.3 Hz, 0.5H), 5.39 (d, *J* = 3.4 Hz, 0.5H), 5.26 (m, 2H), 5.06 (m, 1H), 4.96 (m, 1H), 4.78 – 4.60 (m, 2H), 4.32 (m, 1H), 4.28 – 4.15 (m, 3H), 4.10 (m, 1H), 3.69 (m, 1H), 3.01 (m, 1H), 2.11-1.97 (m, 21 H). ^{13}CNMR (100 MHz, $CDCl_3$): *δ (ppm)* 170.8, 170.6, 170.0, 169.9, 169.8, 169.5, 95.0, 90.3, 82.4, 75.1, 74.7, 72.8, 72.3, 72.2, 70.3, 69.8, 69.7, 68.5, 68.1, 67.9, 63.8, 63.5, 61.5, 43.8, 20.9, 20.8, 20.7, 20.6, 20.5, 20.5, 20.4. MS (HR-ESI) m/z Found: 675.1555, calculated: $C_{26}H_{36}O_{17}SNa$ $[M+Na^+]$ 675.1571.

S1.4 Synthesis of α-D-glucopyranose, 3-azidopropyl 4-S-(2,3,4,6-tetra-O-acetyl-β-D -glucopyranosyl)-4-thio-2,3,6-triacetyl (6).

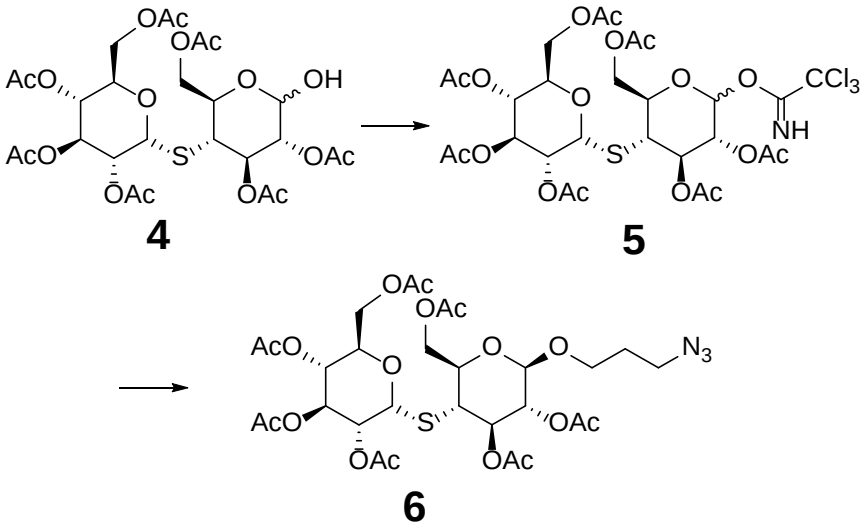


Figure S4. Synthesis of compound **6**.

To a stirred solution of **4** (0.20 g, 0.31 mmol) in dry DCM (5 mL) was added trichloroacetonitrile (0.6 mL, 6.0 mmol), and the solution was cooled to 0 ºC. DBU (23 µL, 0.15 mmol) was then added and the suspension was stirred at 0 ºC for 6 hours under nitrogen. The reaction mixture was concentrated *in vacuo* to afford crude compound. The residue was purified by flash column chromatography on silica gel (hexane/EtOAc, 2:1) to afford **5** (0.18 g, 77%) and used immediately after purification. To a stirred solution of trichloroimidate **5** (0.16 g, 0.20 mmol) and 3-azidopropanol (0.10 g, 1.0 mmol) in dry DCM (5 mL) was added 4Å M.S. The mixture was stirred under nitrogen at 0 ºC for 1 hour. TMSOTf (45 µL, 0.22 mmol) was then added and the mixture was stirred at 0 ºC for 2 hour. The reaction was quenched with Et_3N and concentrated *in vacuo*. The residue was dissolved in EtOAc (20 mL) and washed with water (5 mL x 2) and brine (10 mL). The organic phase was dried over Na_2SO_4, filtered and evaporated to dryness *in vacuo*. The residue was purified by flash column chromatography on silica gel (hex-

ane/EtOAc, 1:1) to afford **6** (93 mg, 63%). ^{1}H NMR (400 MHz, $CDCl_3$): *δ (ppm)* 5.87 (d, *J* = 5.9 Hz, 1H), 5.23 (dd, *J* = 19.0, 9.4 Hz, 2H), 5.04 (t, *J* = 9.8 Hz, 1H), 4.94 (dd, *J* = 10.4, 5.9 Hz, 1H), 4.77 (t, *J* = 9.8 Hz, 1H), 4.64 (dd, *J* = 11.8, 2.1 Hz, 1H), 4.44 (d, *J* = 8.0 Hz, 1H), 4.31 (dd, *J* = 12.5, 4.1 Hz, 1H), 4.25 - 4.14 (m, 2H), 4.09 (dd, *J* = 12.4, 1.7 Hz, 1H), 3.91 - 3.82 (m, 1H), 3.68 – 3.52 (m, 2H), 3.39 – 3.27 (m, 2H), 2.98 (t, *J* = 10.9 Hz, 1H), 2.11 - 2.01 (m, 21H), 1.86 - 1.75 (m, 2H). ^{13}CNMR (100 MHz, $CDCl_3$): *δ (ppm)* 170.5, 170.4, 170.1, 170.0, 169.8, 169.5, 169.4, 100.3, 82.3, 75.7, 72.7, 72.6, 70.3, 69.7, 68.6, 67.9, 66.3, 63.6, 61.5, 47.9, 43.7, 28.9, 20.8, 20.7, 20.6, 20.6, 20.5, 20.4. HRMS (HR-ESI) m/z Found: 758.2035, calculated: 758.2054 for $C_{29}H_{41}N_3O_{17}SNa$ $[M+Na]^+$.

S1.5. Synthesis of Azidothiomaltose (7).

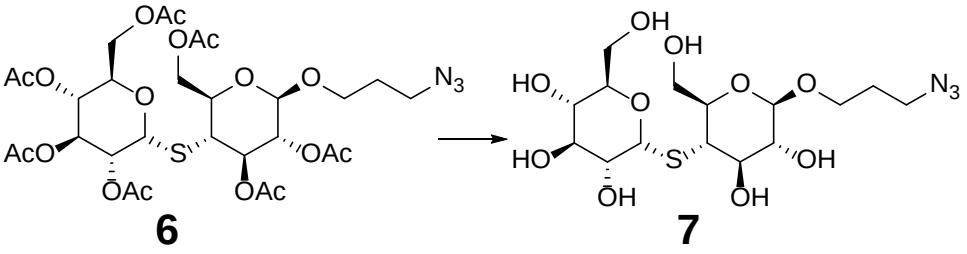


Figure S5. Synthesis of compound **7**.

To a stirred solution of **6** (74 mg, 0.10 mmol) in anhydrous CH_3OH (5 mL) was added $NaOCH_3$ (5.4 mg, 1.0 mmol) under an atmosphere of N_2 at room temperature, and the stirring was continued at room temperature for 12 hours. The reaction mixture was neutralized by adding acidic resins (Dowex 50W, hydrogen form), filtrated, concentrated *in vacuo* and used in the following step without further purification. ^{1}H-NMR (400 MHz, MeOD): *δ (ppm)* 5.68 (d, 1H, *J* = 5.6 Hz), 4.28 (d, 1H, *J* = 8.0 Hz), 4.00-3.89 (m, 5H), 3.73-3.59 (m, 4H), 3.50-3.44 (m, 4H), 3.28-3.18 (m, 2H), 2.91-2.88 (m, 1H), 1.90-1.87 (m, 2H). ^{13}C NMR (100 MHz, D_2O): *δ (ppm)* 102.8, 86.6, 77.1, 75.6, 74.6, 74.2, 73.7, 71.9, 66.1, 61.8, 61.3, 28.8. MS (MALDI) m/z Found: 464.1, calculated: $C_{15}H_{27}N_3O_{10}SNa$ $[M+Na^+]$ 464.13.

S1.6. Synthesis of Thiomaltose-trimethoprim (3)

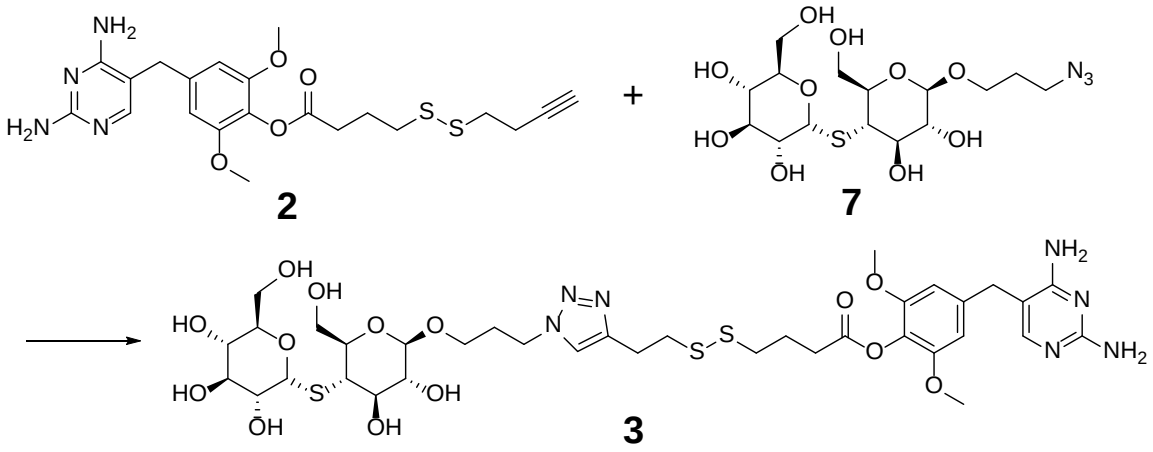


Figure S6. Synthesis of compound **3**.

To a stirred solution of **7** (10 mg, 0.023 mmol) and trimethoprim alkyne **2** (10.6 mg, 0.023 mmol) in DMSO (2 mL) was added CuI (0.2 mg, 1 µmol) and DIPEA (1.2 mg, 0.093 mmol). The mixture was stirred at room temperature for 12 hours under nitrogen and then diluted with 20 mL of water, filtered and purified by HPLC to afford **3** (14.7 mg, 71%, >90% pure on HPLC). ^{1}H-NMR (400 MHz, D_2O): *δ (ppm)* 7.69 (s, 1H), 7.32 (s, 1H), 6.60 (s, 2H), 5.63 (d, J = 5.5 Hz, 1H), 4.37 (m, 2H), 4.26 (d, J = 7.9 Hz, 1H), 3.94 – 3.91 (m, 2H), 3.86 - 3.37 (m, 20H), 3.25 (t, J = 8.5 Hz, 1H), 3.08 - 2.86 (m, 4H), 2.81 - 2.59 (m, 5H), 2.12 - 1.92 (m, 4H). ^{13}C NMR (100 MHz, D_2O): *δ (ppm)* 172.99, 164.40, 163.04, 162.68, 154.32, 151.74, 139.73, 135.86, 126.57, 123.55, 117.82, 109.00, 105.77, 102.13, 86.22, 76.22, 75.16, 74.10, 73.50, 73.03, 71.05, 69.46, 66.10, 61.56, 60.47, 56.13, 47.06, 37.13, 36.84, 32.72, 31.90, 29.69, 24.61, 24.10. HRMS (HR-ESI) m/z Found: 904.2862, calculated: 904.2891 for $C_{36}H_{54}N_7O_{14}S_3$ $[M+Na]^+$.

S1.7. Synthesis of Thiomaltose-perylene.

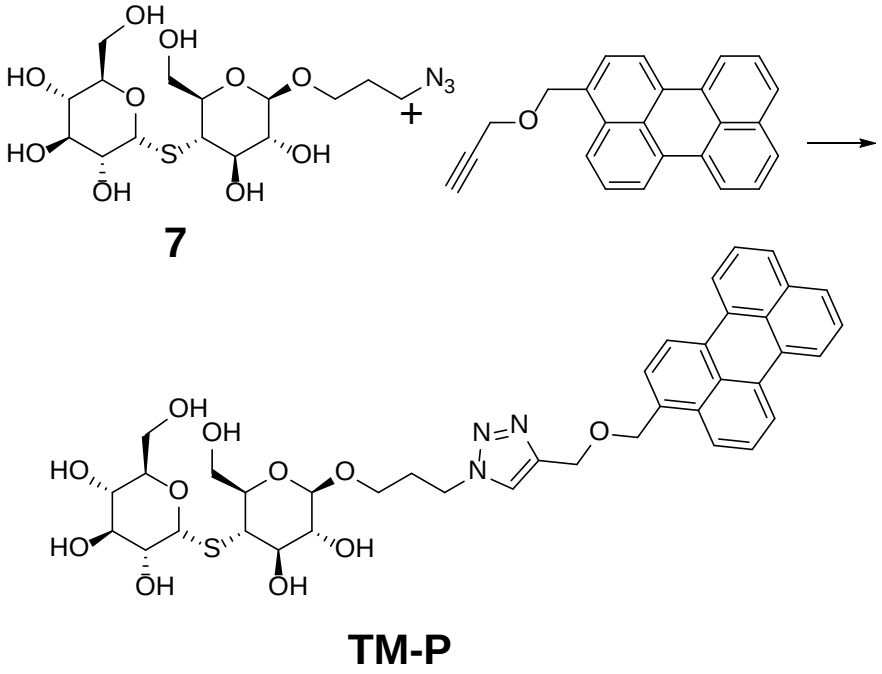


Figure S7. Synthesis of compound **TM-P**.

To a stirred solution of **7** (10 mg, 0.023 mmol) and alkyne functionalized perylene dye[3] (13 mg, 0.041 mmol) in DMF (2 mL) was added CuI (0.2 mg, 1.0 µmol) and DIPEA (1.2 mg, 0.093 mmol). The mixture was stirred at room temperature for 12 hours under nitrogen and then diluted with 20 mL of water, filtered and purified by HPLC to afford **TM-P** (13 mg, 75%, >90% pure on HPLC). ^{1}H-NMR (400 MHz, DMSO-d_6): *δ (ppm)* 7.85-7.76 (m, 3H), 7.72 (d, *J* = 8.0 Hz, 1H), 7.51 (d, *J* = 8.0 Hz, 1H), 7.39 (s, 1H), 7.21 (d, *J* = 8.0 Hz, 2H), 7.13-7.03 (m, 4H), 5.96 (d, *J* = 6.0 Hz, 1H), 4.96-4.91 (m, 2H), 4.71 (m, 2H), 4.55 (s, 2H), 4.45 (*d*, *J* = 8.4 Hz, 1H), 3.78-3.71 (m, 5H), 3.69-3.67 (m, 2H), 3.43-3.31 (m, 5H), 3.22-3.20 (m, 3H), 2.91-2.89 (m, 1H), 1.91 (m, 2H). ^{13}C NMR (100

MHz, DMSO-d_6): *δ (ppm)* 143.3, 134.8, 133.1, 132.8, 131.9, 131.7, 131.0, 128.9 128.5, 127.9, 127.9, 126.7, 126.5, 126.1, 123.7, 123.6, 120.1, 120.04, 120.01, 119.5, 103.6, 97.8, 81.5, 79.1, 77.3, 76.9, 75.7, 73.8, 73.1, 72.8, 70.6, 68.1, 64.3, 62.0, 48.7, 47.9, 28.5. HRMS (HR-ESI) m/z Found: 784.2543, calculated: 784.2516 for $C_{39}H_{43}N_3O_{11}SNa$ $[M+Na]^+$.

S2. Serum Stability

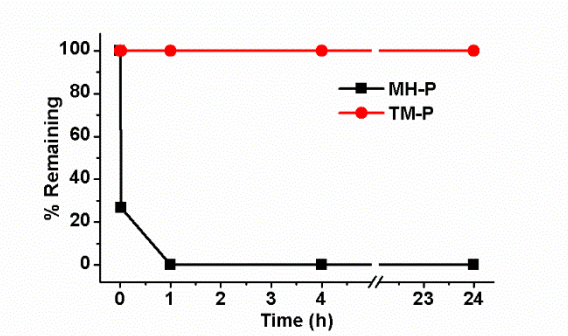


Figure S8. Serum stability of MH-P.

To 146 μL of FBS was added 4 μL of 2.5 mM TM-P or MH-P. The solution was incubated at 37 °C and analyzed with HPLC at different time points. The column used for the HPLC analysis was an XBridge BEH C8 column (5μm, 4.6x150mm), and the elution was performed with 0.1% TFA in H_2O (A) and 0.1% TFA in acetonitrile (B). The gradient used was: 0-4 min, 5% B; 4-23 min, 5% to 100% B; 23-25 min, 100% B. The chromatography was monitored at 435 nm for the absorption of perylene. All peaks were integrated for the area and the % remaining TM-P or MH-P was calculated as: TM-P (or MH-P) peak area/the sum of all peak area.

S3. Metabolic Stability

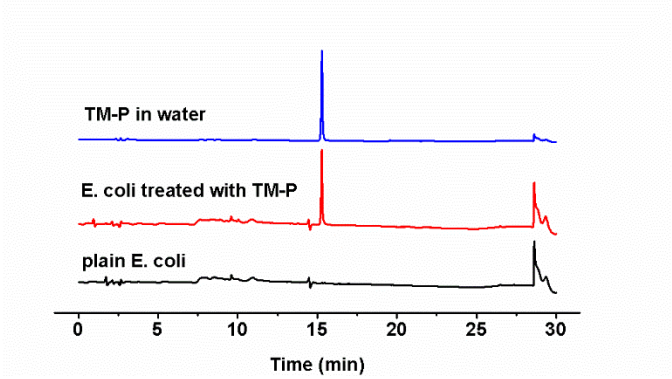


Figure S9. HPLC chromatography of TM-P after bacterial uptake.

To a 1 mL suspension of *E. coli* in LB medium (OD600 = 0.6) was added 10 μL of 2 mM TM-P. The mixture was incubated at 37 °C for 1 h and then centrifuged at 10000 rpm for 5 min. The supernatant was discarded and the cell pellet was washed with 1 mL of PBS for 3 times, via centrifugation. The cell pellet was then lysed with 200 μL of bacteria lysis buffer and the supernatant was analyzed with HPLC using the same condition used in section S2. The intracellular concentration of TM-P (about 2.8 mM) was calculated based on the integrated area of TM-P comparing to TM-P standard and the approximate intracellular volume of 134 nanoliters per *E. coli*.

S4. Uptake of TM-IR780

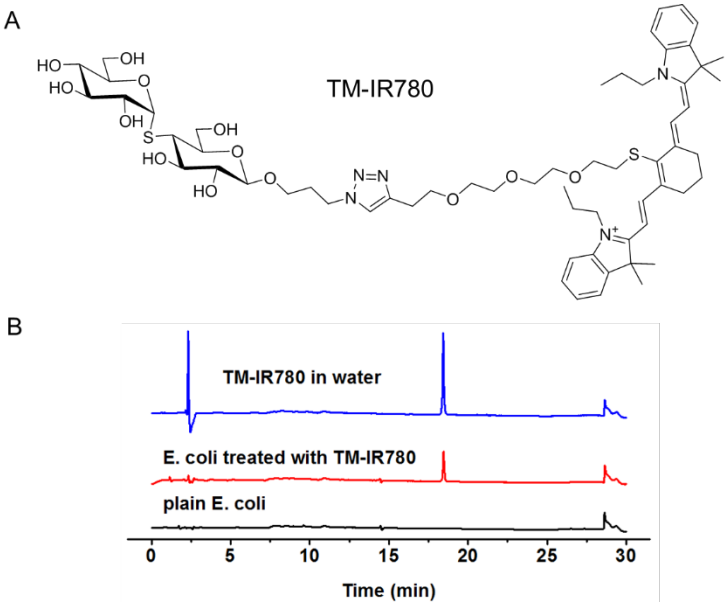


Figure S10. A. Structure of TM-IR780. B. HPLC chromatography of TM-IR780 after

bacterial uptake.

To a 1 mL suspension of *E. coli* in LB medium (OD600 = 0.6) was added 2 μL of 5 mM TM-IR780. The mixture was incubated at 37 °C for 1 h and then centrifuged at 10000 rpm for 5 min. The supernatant was discarded and the cell pellet was washed with 1 mL of PBS for 3 times, via centrifugation. The cell pellet was then lysed with 200 μL of bacteria lysis buffer and the supernatant was analyzed with HPLC using the same condition used in section S2, except the chromatography was monitored at 650 nm for the absorption of IR780. The intracellular concentration of TM-IR780 (about 3.2 mM) was calculated based on the integrated area of TM-IR780 comparing to TM-IR780 standard and the approximate intracellular volume of 134 nanoliters per *E. coli*.

S5. Fluorescent image of TM-P treated *E. coli*

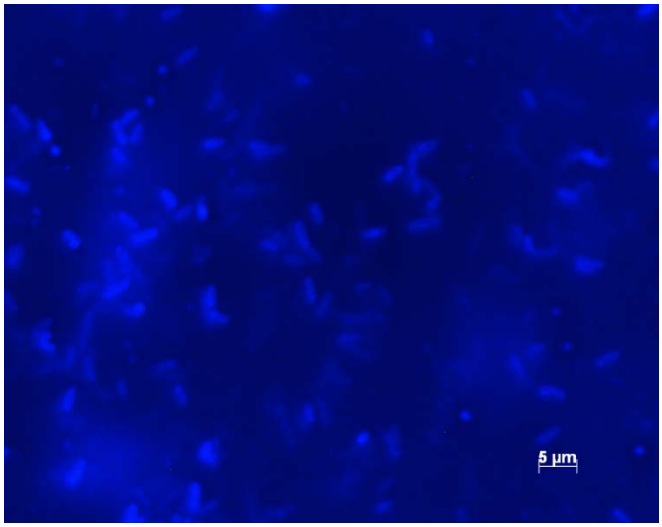


Figure S11. Fluorescent image of *E. coli* treated with TM-P.

E. coli (ATCC 33456) was incubated with 20 μM of TM-P in LB broth at 37 °C for 1 h and then washed 3 times with PBS via centrifugation. Bacteria was then resuspended in PBS and observed under fluorescent microscope (Axioskop 2 with AxiCcam, Zeiss).

S6. Antibacterial activity of TMP-OH

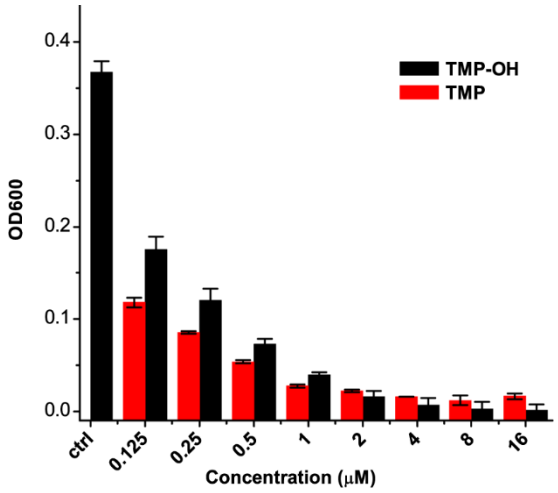
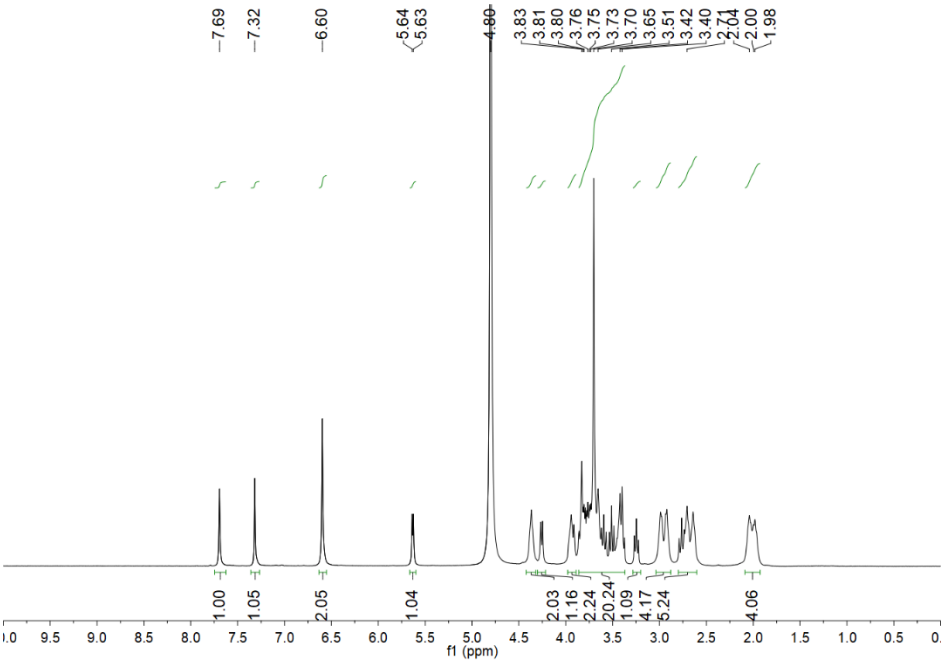


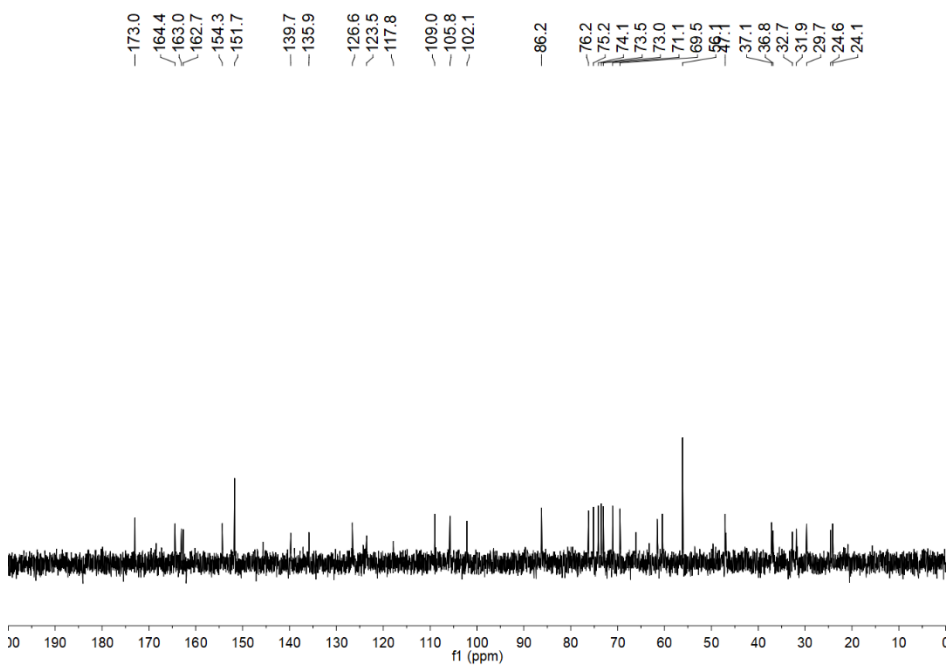
Figure S12. Antibacterial activity of TMP-OH versus trimethoprim

To a 96-well plate containing 200 μL of LB medium was added 1 μL of *E. coli* (OD600 = 0.5) and 2 μL of stock drug solution (TMP or TMP-OH) in DMSO. The plate was incubated at 37 °C, shaken at 190 rpm for 24 h, and the OD600 was measured using a Tecan Infinite 200 plate reader.

^{1}H-NMR spectra of **TM-TMP**



^{13}C-NMR spectra of **TM-TMP**



References

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