

2-Bromopalmitate targets retinoic acid receptor alpha and overcomes all-trans retinoic acid resistance of acute promyelocytic leukemia

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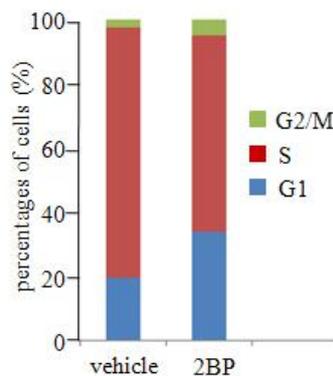
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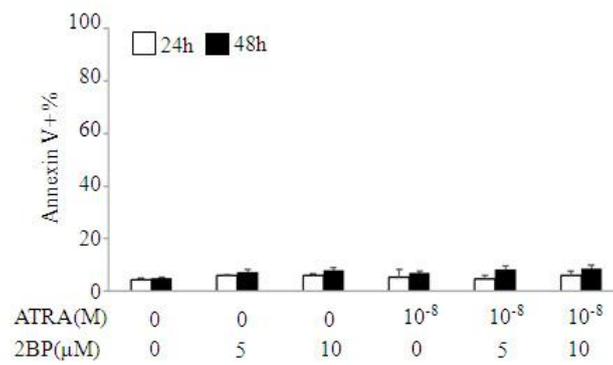
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Supplementary Figures

Supplementary Figure 1. 2BP induces cell cycle arrest of NB4 cells. NB4 cells were treated with 10 μ M 2BP for 48 hours, and percentages of cells at the indicated phases of cell cycle were detected by flow cytometry.



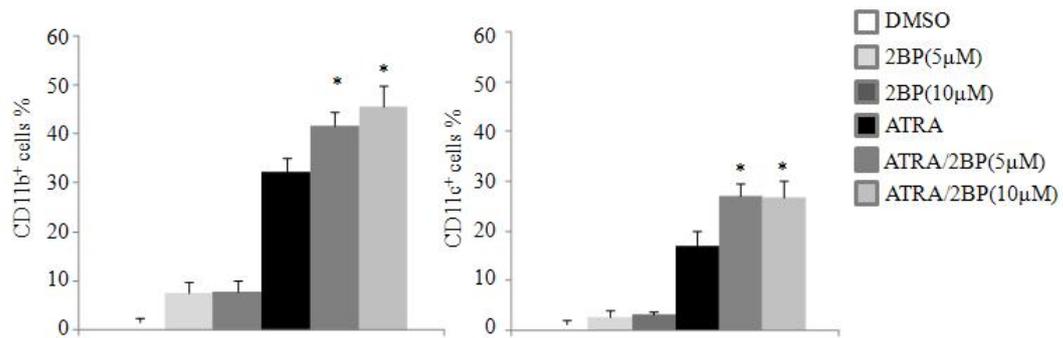
Supplementary Figure 2. Effects of co-treatment of 2BP and ATRA on cell apoptosis. NB4 cells were incubated with 5 or 10 μ M 2BP and/or 10⁻⁸M ATRA for the indicated hours and the percentage of Annexin V⁺ cells were evaluated by flow cytometry.



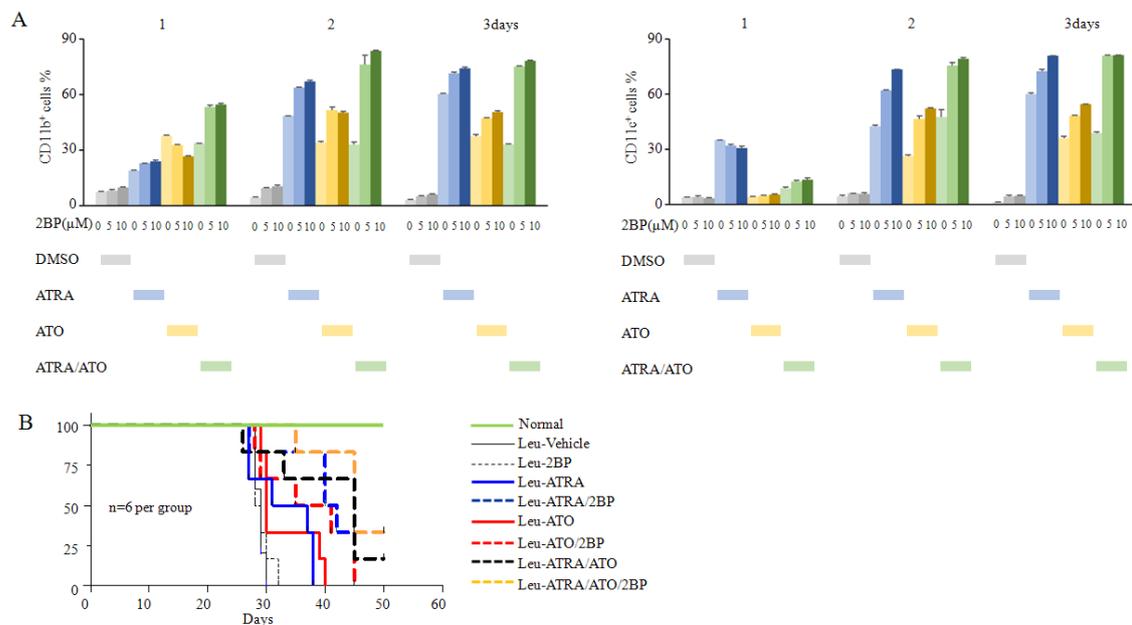
Supplementary Figure 3. 2BP enhances ATRA-induced cell differentiation in HL60

cells. HL60 cells were treated with 5 μ M or 10 μ M 2BP in combination with ATRA for 3 days. The CD11b-positive and CD11c-positive cells were counted by flow cytometry.

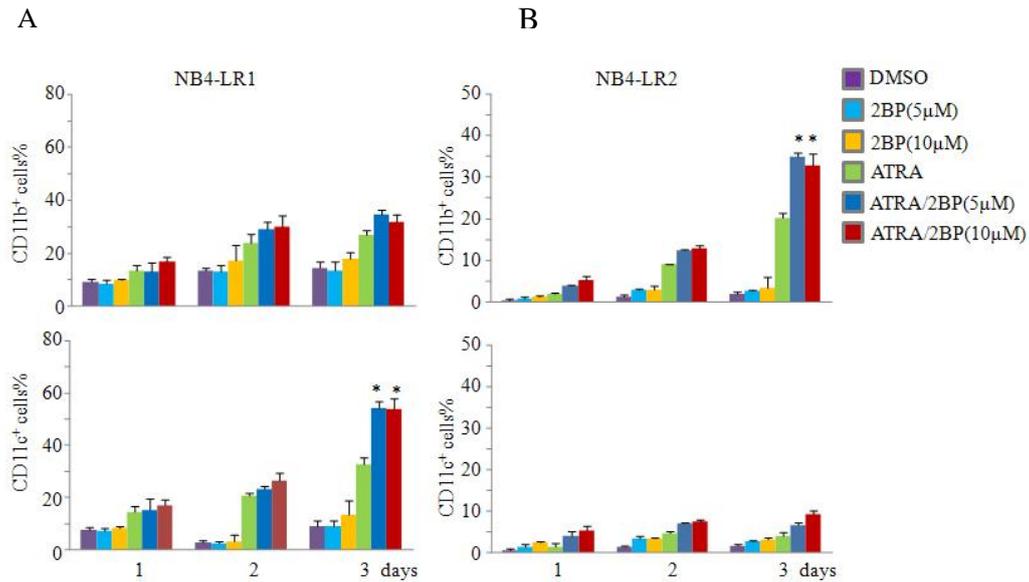
* $p < 0.01$ against ATRA-treated group.



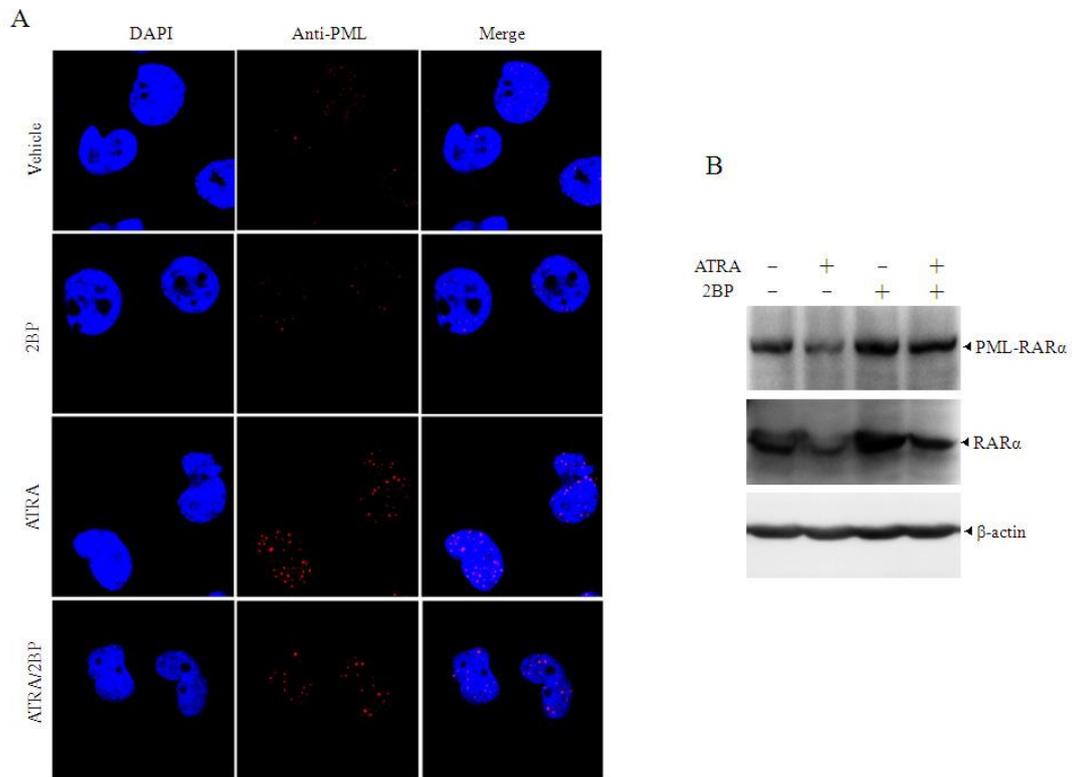
Supplementary Figure 4. 2BP enhances ATO-induced cell differentiation *in vitro* and *in vivo*. (A) NB4 cells were treated with 5 μ M or 10 μ M 2BP in combination with ATRA or ATO for indicated days. The CD11b-positive and CD11c-positive cells were counted by flow cytometry. (B) ATRA-sensitive leukemic (leu) mice were treated with vehicle, 2BP (5 mg per kg body weight, intraperitoneally), ATRA (10 mg per kg body weight, intraperitoneally), ATO (5 mg per kg body weight, intraperitoneally) or combination of the compounds daily for five continuous days a week. Normal FVB/N mice were taken as negative controls. The survival (%) and lifetime (days) of leukemic mice in each group were recorded and Kaplan–Meier survival analysis was shown.



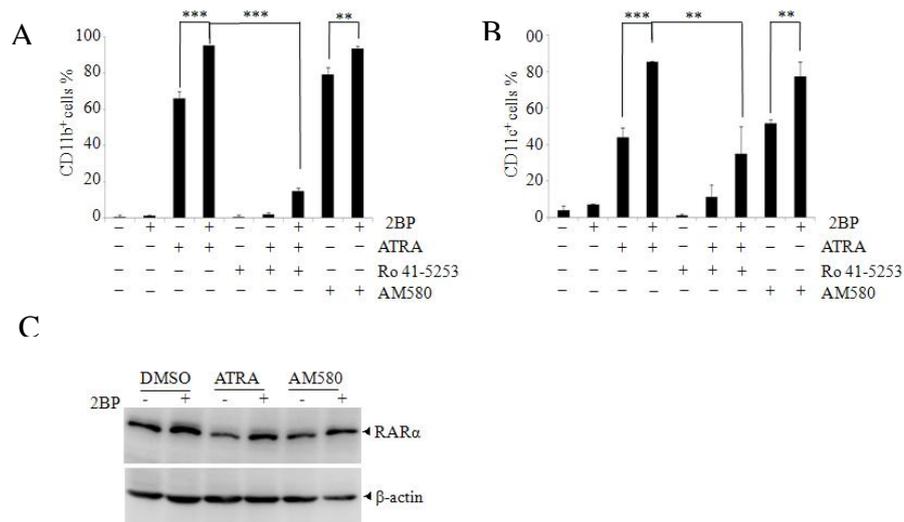
Supplementary Figure 5. 2BP plus ATRA induces differentiation of ATRA-resistant APL cells. NB4-LR1(A) and NB4-LR2(B) cells were treated with 5 μ M or 10 μ M 2BP in combination with ATRA for indicated days. The percentages of CD11b(upper panel) or CD11c(lower panel) expression were evaluated by flow cytometry. * $p < 0.01$ against ATRA-treated group.



Supplementary Figure 6. The effects 2BP on PML-RAR α fusion protein. (A) After treated with 5 μ M 2BP in combination with ATRA for 2 days, NB4 cells were fixed and incubated with the antibody specifically against PML, followed by Texas red labeled second antibody and DAPI. Fluorescence signals were detected on a Bio-Rad MRC-1024 laser scanning confocal microscope. Scale bars are 7.5 μ m. **(B)** NB4 cells were treated with 5 μ M 2BP in combination with ATRA for 2 days and western blot analysis were performed using anti-PML-RAR α antibody.



Supplementary Figure 7. 2BP presents synergistic effect with RAR α agonist and RAR α antagonist. NB4 cells were treated with 5 μ M 2BP, ATRA (10⁻⁸ M), AM580 (10⁻⁶ M) or Ro 41-5253(10⁻⁶ M) alone or their combination for 3 days. CD11b(A) and CD11c(B) expression was measured by flow cytometry. All values represent means with bar as S.D. of three independent experiments, each of which with triplicate samples. The symbol ** represented $p < 0.005$ and *** represented $p < 0.001$ between the line-pointed group. (C) NB4 cells were treated with 5 μ M 2BP, ATRA (10⁻⁸ M), or AM580 (10⁻⁶ M) as indicated for 48 hours and RAR α protein was detected by western blot. β -actin was used as internal control. Each experiment was repeated for at least three times.



Supplementary Materials and Methods.

Cell cycle analysis

Cells were collected, rinsed, and fixed overnight with 75% cold ethanol at -20 °C. Then, cells were treated with 100µg/mL RNase A in Tris-HCl buffer (pH 7.4) and stained with 25µg/mL propidium iodide (PI, Sigma-Aldrich, St. Louis, MO). Cell-cycle distribution was determined by flow cytometry (Beckman Coulter, Miami, FL).

Plasmids

Human RAR α cDNA was amplified from NB4 cells by RT-PCR and then cloned into psumo3 vector provided by Dr Zhou to generate a His-SUMO3 tagged fusion protein. The primers for amplifying RAR α cDNA are listed below.

RAR α Forward: 5'-cgcgatccatggccagcaacagc-3'

RAR α Reverse: 5'-cccaagctttcacggggagtgg-3'

To get cysteine-mutated RAR α proteins (Cys105 to Ala105 and Cys174 to Ala174), plasmids with site-directed mutants were constructed using QuikChangeTM site-directed mutagenesis kit (Stratagene, Palo Alto, CA). The specific primers used for point mutation of human RAR α gene are listed below. After confirming a 100% identities match by DNA sequencing, the *E. coli* strain BL21 was transformed with constructs for next step of proteins purification.

RAR α (Cys174Ala) Forward: 5'-gtgccaagcccaggcctctgagagctacac-3'

RAR α (Cys174Ala) Reverse: 5'-gtgtagctctcagaggcctcgggcttgggcac-3'

RAR α (Cys105Ala) Reverse: 5'-cttgagccctcagcggcgctgaccca-3'

RAR α (Cys105Ala) Forward: 5'-tggggtcagcggcgctgagggtgcaag-3'

Purification of Recombinant Proteins

His-tagged WT and mutant RAR α were expressed in bacteria BL21 (DE3) by induction

with 0.5mM isopropylthiogalactopyranoside (IPTG) at 30 °C and purified from the cytosol of the expressed cells by affinity chromatography on Ni-NTA-agarose (Qiagen).

RNA interference and transfection

Pairs of complementary oligonucleotides against RAR α from Open Biosystems (Open Biosystems, Huntsville, AL) shRAR α were annealed and ligated into pSIREN Vector (Clontech Laboratories, Mountain View, CA). Scrambled shRNA (Open Biosystems, Huntsville, AL) was used as the negative control. These shRNA vectors were co-transfected with packaging plasmids VSV-G and Gag-Pol into 293T cells to produce retrovirus. Forty-eight hours after transfection, supernatants containing retrovirus were collected and were used to infect NB4 cells. After 48 h, puromycin (0.5 μ g/mL, Calbiochem, Darmstadt, Germany) was added to the medium for selection.

Western Blots

Protein extracts were equally loaded on 8-10% SDS-polyacrylamide gel, electrophoresed, and transferred to nitrocellulose membrane (Amersham Bioscience). The blots were stained with 0.2% Ponceau S red to ensure equal protein loading. After blocking with 5%(w/v) nonfat milk in PBS, the membranes were incubated with indicated antibodies, followed by horseradish peroxidase (HRP)-linked secondary antibodies (Cell Signaling). Detection was performed by chemiluminescence phototope-HRP kit (Cell Signaling).

Confocal Microscopy

Cells were harvested on slides and fixed. After permeabilization with 0.1% (v/v) Triton X-100 in PBS and blocking with 2% (w/v) BSA in PBS, cells were incubated overnight with the anti-PML antibody followed by secondary antibodies conjugated either with Alexa Fluor 555 dye (Invitrogen) for 1 h. Cellular DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Fluorescence signals were

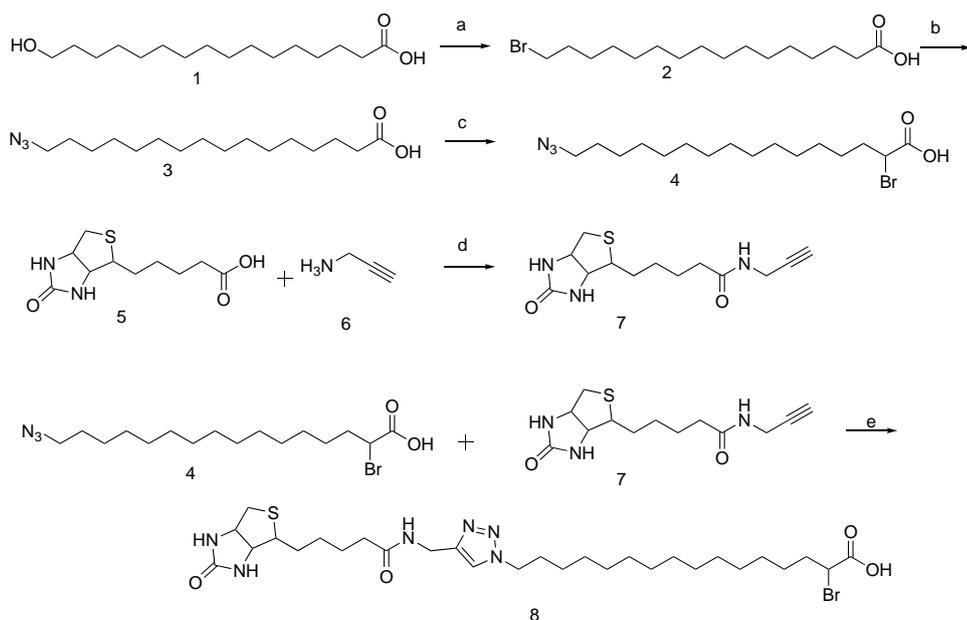
detected on a Nikon A1R confocal laser microscope.

Surface Plasmon Resonance (SPR) assay

The SPR experiments were performed on Biacore T200 (GE Healthcare). Full-length recombinant RAR α was immobilized on a CM5 chip (GE Healthcare) aiming at 500 response units using amine coupling chemistry. Compound binding with RAR α was analyzed in a single-cycle kinetic analysis at escalated concentration of 2BP at a flow rate of 30 μ l/min. Biacore T200 Evaluation Software 1.0 (GE Healthcare) was used in data analysis.

Synthesis of biotin-tagged 2BP and its intermediates

The synthesis of biotin-16-azido-2-bromopalmitate (2BPN₃) conjugate **8** was started from the synthesis of 16-azido-2-bromopalmitate (2BPN₃) **4** and N-(Prop-2-ynyl)biotinamide **7**. 16-Azido-2-bromopalmitate (2BPN₃) **4** was synthesized from 16-hydroxyhexadecanoic acid **1** by a known procedure with variations in order to obtain good yields of intermediates [1,2]. Treatment of compound **1** with hydrobromic acid in AcOH under reflux for 2 days gave compound **2** in good yield, followed by replacement of bromide by treatment with sodium azide in N,N-dimethylformamide produced compound **3** in high yield. Then the key intermediate 16-azido-2-bromopalmitate (2BPN₃) **4** was prepared by a Hell-Volhard-Zelinsky α -bromination of the corresponding acid **3** with molecular bromine and thionyl chloride. Amidation of 2-propynylamine with biotin gave compound **7** in high yield. Finally, reaction of 2BPN₃ **4** with compound **7** in the presence of CuSO₄ 5H₂O and sodium ascorbate produced conjugate **8** in moderate yield.



Scheme 1. a) aq. HBr (40%), AcOH, 2 days, 83.3%; b) NaN₃, DMF, 6 h, 91.0%; c) i: SOCl₂, reflux 1 h, ii: bromine, reflux, 3 h, iii: H₂O, 12 h, 52.1%; d) HATU, DIPEA, 0 °C to r.t., 8 h, 82.9%; e) CuSO₄ · 5H₂O, sodium ascorbate, t-BuOH-THF-H₂O (1:1:1), 12 h, 75.5 %.

To a stirring solution of 16-hydroxyhexadecanoic acid **1** (2.0 g, 7.34 mmol) in AcOH (20 mL) was added aqueous HBr (40%, 10 mL) at room temperature, and the resulting reaction mixture was refluxed at 85 °C for 1 day, then cooled to room and aqueous HBr (40%, 10 mL) was added to the reaction mixture, and the reaction mixture was further refluxed at 85 °C for 1 day. The solid was filtered off and washed with ice-cold water (10 mL × 2), and dried to yield 16-bromohexadecanoic acid **2** (2.05 g, 6.11 mmol, 83.3%) as a white solid. ¹H NMR (500 MHz, Chloroform-*d*) δ 3.41 (t, *J* = 6.9 Hz, 2H), 2.35 (t, *J* = 7.5 Hz, 2H), 1.85 (p, *J* = 7.0 Hz, 2H), 1.63 (p, *J* = 7.5 Hz, 2H), 1.42 (t, *J* = 7.4 Hz, 2H), 1.27 (d, *J* = 10.8 Hz, 20H); ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.52 (t, *J* = 6.7 Hz, 2H), 2.18 (t, *J* = 7.4 Hz, 2H), 1.79 (p, *J* = 6.8 Hz, 2H), 1.48 (t, *J* = 7.3 Hz, 2H), 1.37 (t, *J* = 7.4 Hz, 2H), 1.25 (d, *J* = 4.5 Hz, 20H); HRMS-ESI (*m/z*) [M - H]⁻ calcd for C₁₆H₃₀BrO₂, 333.1429; found, 333.1435.

To a stirring solution of compound **2** (750 mg, 2.24 mmol) in DMF (5 mL) was added NaN₃ (291 mg, 4.48 mmol) at room temperature, and the resulting reaction mixture was refluxed for 6 hours. Upon cooling of the mixture to room temperature and removing of the solvent the mixture was diluted with CH₂Cl₂, washed with brine and water, dried over anhydrous NaSO₄, filtered and concentrated under reduced pressure. Then the residue was purified by flash chromatography to give compound **3** (607 mg, 2.04 mmol, 91.0%) as white solid. ¹H NMR (500 MHz, Chloroform-*d*) δ 3.25 (t, *J* = 7.0 Hz, 2H), 2.35 (t, *J* = 7.5 Hz, 2H), 1.69 – 1.50 (m, 4H), 1.43 – 1.10 (m, 22H); HRMS-ESI (*m/z*) [M - H]⁻ calcd for C₁₆H₃₀N₃O₂, 296.2338; found, 296.2335.

Compound (**3**) (1.0 g, 3.36 mmol) was added in thionyl chloride (10 mL), and refluxed for 1 hours. Then, bromine (0.25 mL) was added dropwise over 2 h and the resulting reaction mixture was further refluxed for 3 h. Then, cooled to room temperature, concentrated and dissolved in H₂O (20 mL) and stirred at room temperature for 12 h. Then the mixture was diluted with CH₂Cl₂, washed with 0.1 N HCl, brine and water, dried over anhydrous NaSO₄, filtered and concentrated under reduced pressure and purified by flash chromatography to give compound **4** (659mg, 1.75 mmol, 52.1%) as white solid. ¹H NMR (500 MHz, DMSO-*d*₆) δ 3.52 (t, *J* = 6.7 Hz, 2H), 2.18 (t, *J* = 7.4 Hz, 2H), 1.79 (p, *J* = 6.8 Hz, 2H), 1.55 – 1.42 (m, 2H), 1.36 (q, *J* = 7.1 Hz, 2H), 1.25 (d, *J* = 4.6 Hz, 20H); ¹H NMR (500 MHz, Chloroform-*d*) δ 3.25 (t, *J* = 7.0 Hz, 2H), 2.35 (t, *J* = 7.5 Hz, 2H), 1.69 – 1.50 (m, 4H), 1.43 – 1.10 (m, 22H); HRMS-ESI (*m/z*) [M - H]⁻ calcd for C₁₆H₂₉BrN₃O₂, 374.1443; found, 374.1449.

To a stirring solution of compound (**5**) (2.0 g, 8.19 mmol) in DMF (50 mL) was added HATU (4.7g, 12.4 mmol) and DIPEA (4.1 mL, 24.8 mmol) at 0 °C for 10 min, then 2-propynylamine (0.57 mL, 9.84 mmol) was added, and the resulting reaction mixture was further stirred at room temperature for 8 h. Then the mixture was

concentrated and purified by flash chromatography to give compound **7** (1.96 g, 6.79 mmol, 82.9 %) as white solid. ^1H NMR (500 MHz, Methanol- d_4) δ 4.49 (dd, $J = 7.9, 4.9$ Hz, 1H), 4.30 (dd, $J = 7.9, 4.4$ Hz, 1H), 3.94 (d, $J = 2.6$ Hz, 2H), 3.20 (ddd, $J = 9.0, 5.9, 4.5$ Hz, 1H), 2.92 (dd, $J = 12.7, 5.0$ Hz, 1H), 2.70 (d, $J = 12.8$ Hz, 1H), 2.33 – 2.11 (m, 2H), 1.85 – 1.53 (m, 4H), 1.44 (p, $J = 7.8$ Hz, 2H); HRMS-ESI (m/z) [$\text{M} + \text{H}$] $^+$ calcd for $\text{C}_{13}\text{H}_{20}\text{N}_3\text{O}_2\text{S}$, 282.1276; found, 282.1288.

To a stirring solution of compound (**4**) (120 mg, 0.32 mmol) and compound (**7**) (108 mg, 0.38 mmol) in t-BuOH-THF- H_2O (1:1:1, 6 mL) was added 1 N aq. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (6.4 μL , 0.0064 mmol) and 1 N aq. sodium ascorbate (64 μL , 0.64 mmol). Then the resulting reaction mixture was further stirred at room temperature for 12 h. The solvent was removed under reduced pressure, the residue was dispersed in EtOAc (10 mL) and the solution was saturated NH_4Cl and H_2O , dried over MgSO_4 , concentrated and purified by chromatography on silica gel to give compound **8** (158 mg, 0.24 mmol, 75.5 %) as white solid. ^1H NMR (600 MHz, DMSO- d_6) δ 8.27 (t, $J = 5.6$ Hz, 1H), 7.87 (s, 1H), 6.42 (s, 1H), 6.36 (s, 1H), 4.32 – 4.22 (m, 2H), 4.12 (ddd, $J = 7.6, 4.6, 1.6$ Hz, 1H), 3.08 (ddd, $J = 8.5, 6.1, 4.3$ Hz, 1H), 2.81 (dd, $J = 12.4, 5.1$ Hz, 1H), 2.57 (d, $J = 12.4$ Hz, 1H), 2.09 (t, $J = 7.5$ Hz, 2H), 2.03 – 1.87 (m, 2H), 1.76 (p, $J = 7.3$ Hz, 2H), 1.64 – 1.58 (m, 1H), 1.56 – 1.41 (m, 4H), 1.35 – 1.20 (m, 26H); ^{13}C NMR (151 MHz, DMSO) δ 172.41, 171.21, 163.16, 130.11, 123.09, 61.48, 59.66, 55.89, 49.69, 35.45, 34.60, 30.25, 29.51, 29.45, 29.36, 29.26, 28.88, 28.68, 28.49, 26.32, 25.66, 22.58; HRMS-ESI (m/z) [$\text{M} + \text{H}$] $^+$ calcd for $\text{C}_{29}\text{H}_{50}\text{BrN}_6\text{O}_4\text{S}$, 657.2798; found, 657.2792.