Supporting Information for:

Precise small molecule degradation of a non-coding RNA identifies cellular binding sites and modulates an oncogenic phenotype

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Table S1. Sequences of primers used in this study.

Primer	Sequence (5’ to 3’)
miR-96	TTTGGCACTAGCACATTTTTGCT
pri-miRNA-96-F	AGAGAGCCCGCACCAGT
pri-miRNA-96-R	CTTGAGGAGGAGCAGGCT
RNU6	ACACGCAAATTCGTGAAGCGTTC
Universal reverse	GAATCGAGCACCAGTTACGC
miR-10b	TACCCTGTAGAACCGAATTTGTG
miR-21	TAGCTTATCAGACTGATGTTGA
miR-27a	TTCACAGTGGCTAAGTTCCGC
miR-9	TCTTTGGTTATCTAGCTGTATGA
miR-194	TGTAACAGCAACTCCATGTGGA
miR-15a	TAGCAGCACATAATGGTTTGTG
miR-16	TAGCAGCACGTAAATATTGGCG
miR-139	TCTACAGTGCACGTGTCTCCAGT
miR-182	TTTGGCAATGGTAGAACTCACACT
miR-196a	TAGGTAGTTTCATGTTGTTGGG
miR-128a	TCACAGTGAACCGGTCTCTTT
miR-142	TGTAGTGTTTCCTACTTTATGGA
miR-223	TGTCAGTTTGTCAAATACCCC
miR-101	TACAGTACTGTGATAACTGAA
miR-132	TAACAGTCTACAGCCATGGTCG
miR-212	TAACAGTCTCCAGTCACGGCC
miR-135a	TATGGCTTTTTATTCCTATGTGA
miR-1271	CTTGGCACCTAGCAAGCACTCA

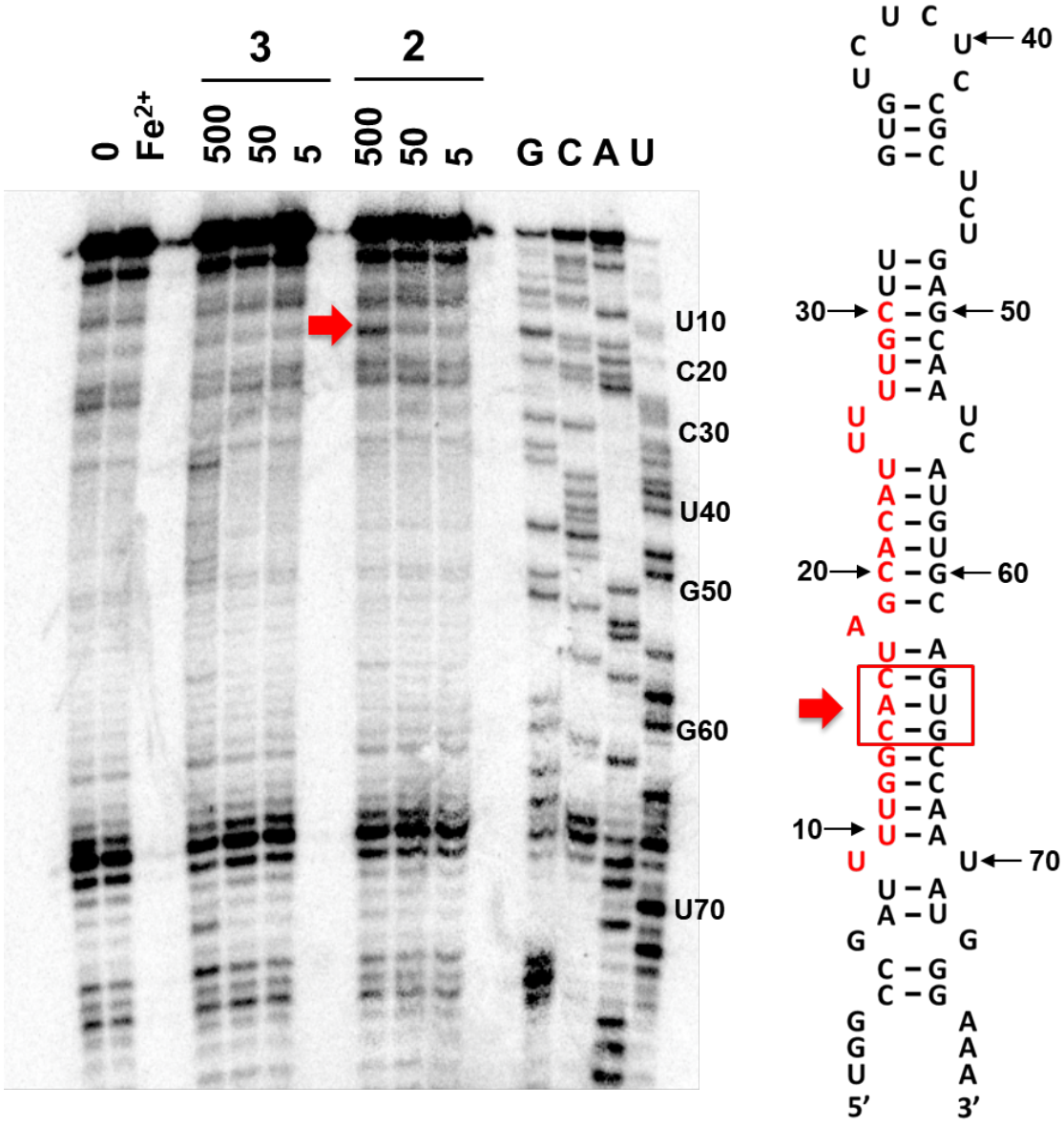


Figure S1. In vitro cleavage of pri-miR-96 by **2** and **3**, as assessed by reverse transcription/primer extension using a 5’-^{32}P-labeled primer. The red arrow indicates a cleavage band unique to **2** at an AU pair near the Drosha site, as designed. Bands that appear in the “0” (untreated) and “Fe^{2+}” (treated solely with Fe^{2+}) were not considered as they are due to “RT stops” and not to compound treatment.

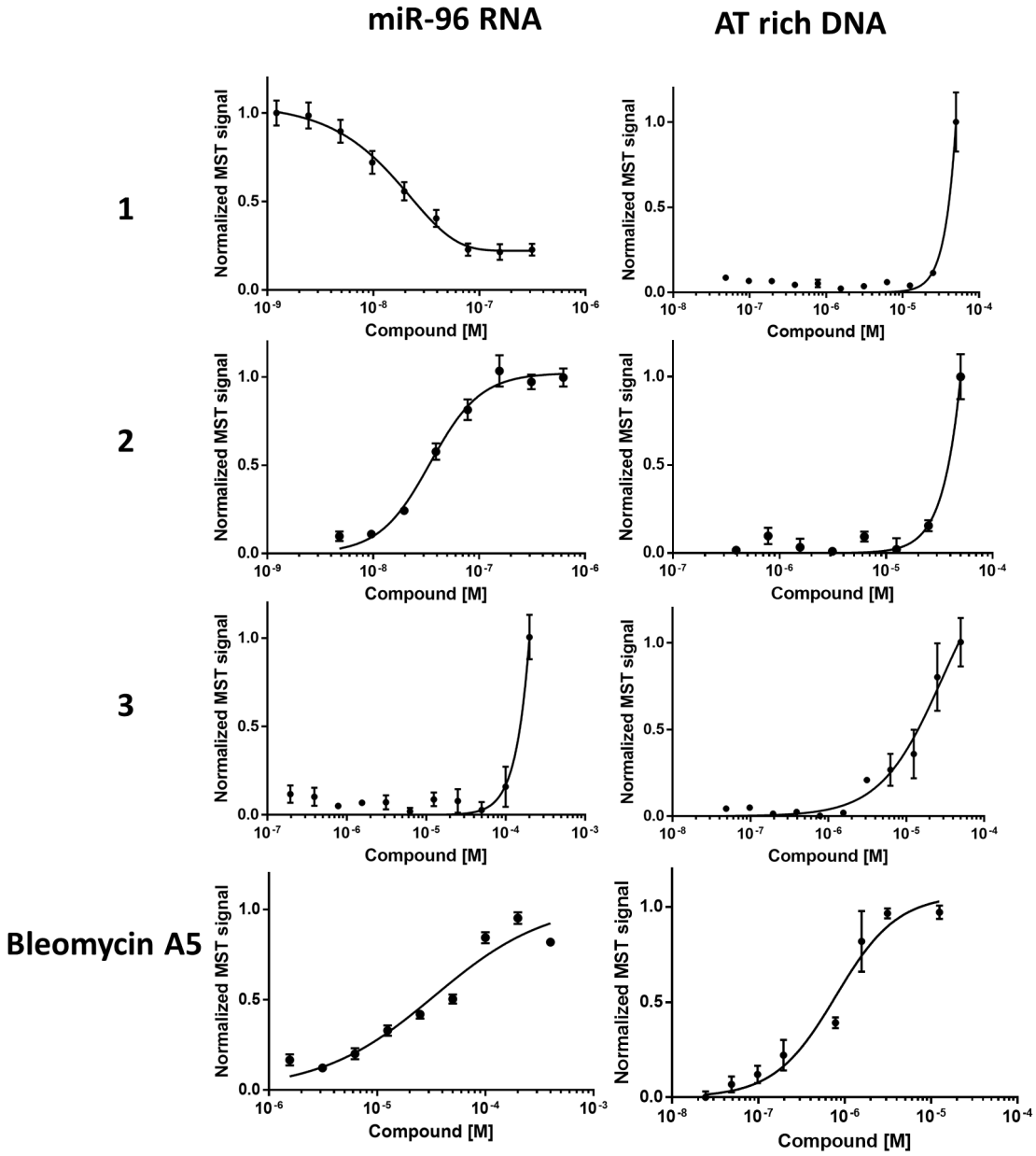


Figure S2. Binding affinities of **1**, **2**, **3** and bleomycin A5 for pri-miR-96 or an AT-rich DNA hairpin. The MST signal was normalized and the binding curve of compounds with RNA or DNA obtained. The MST signal for **1** with RNA decreased as a function concentration while MST signals for **2** (bleomycin conjugate), **3** (bleomycin conjugate), and bleomycin A5 decreased as a function of nucleic acid concentration, suggesting that the bleomycin moiety changes the thermophoresis of bound state[1].

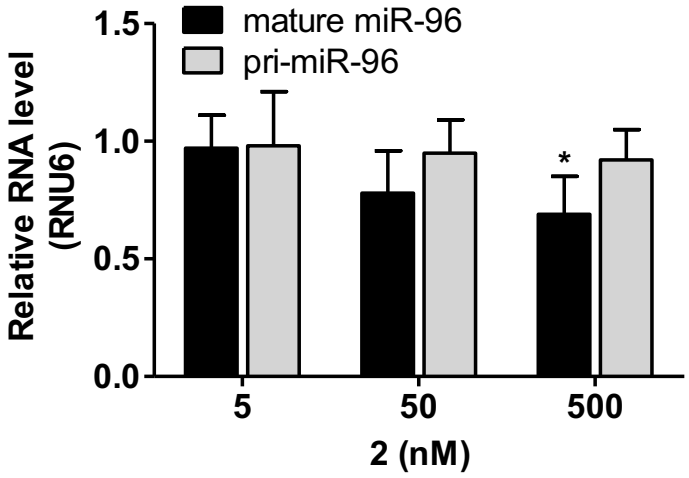


Figure S3. RT-qPCR analysis of mature and pri-miR-96 levels of MDA-MB-231 cell treated with **2** prepared in the absence of Fe^{2+}. Mature miR-96 levels were downregulated while pri-miR-96 levels were unaffected in the absence of Fe^{2+}. Data are expressed as mean ± s.e.m. ($n \geq 3$). *$p<0.05$, **$p<0.01$, as determined by a two-tailed Student *t* test by comparison to levels in untreated cells.

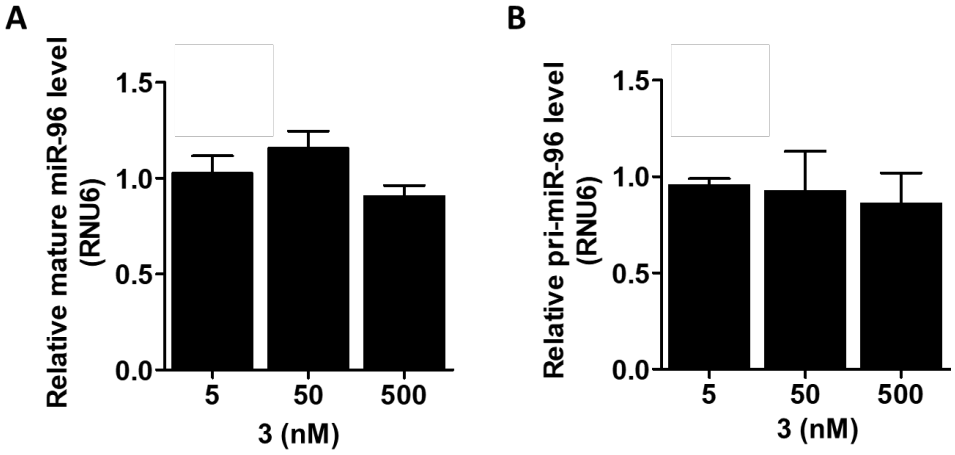
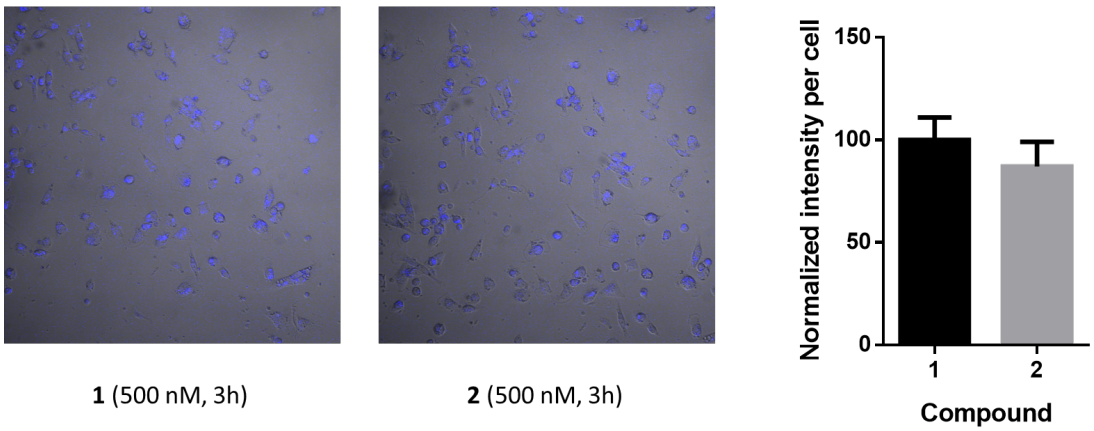


Figure S4. Effect of **3** on mature miR-96 (**A**) and pri-miR-96 (**B**) levels. Data are expressed as mean ± s.e.m. (n ≥ 3).



1 (500 nM, 3h) **2** (500 nM, 3h)

Figure S5. Cellular permeability of **1** and **2** as measured by confocal microscopy. MDA-MB-231 cells were treated with 500 nM **1** or **2** for 3 h and cellular uptake was visualized by compound fluorescence. Images were quantified by Image J. Data are expressed as mean ± s.e.m. ($n \geq 3$).

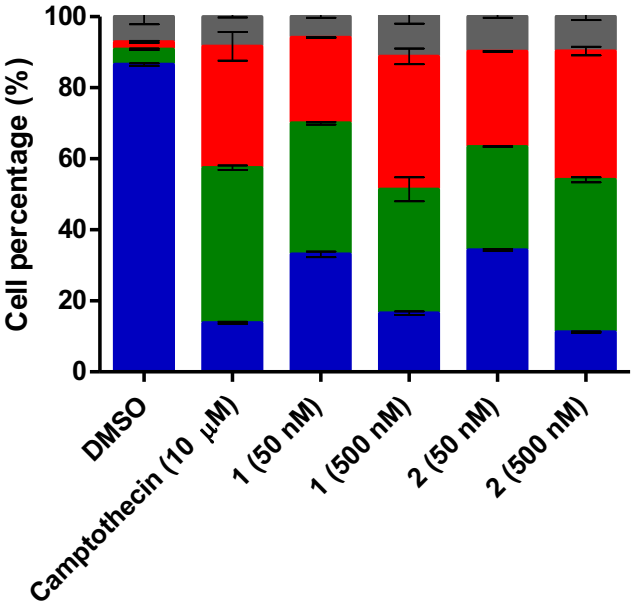


Figure S6. Studying reversal of phenotype by **1** and **2** in MDA-MB-231 cells. The percentage of cells undergoing apoptosis was determined by Annexin V/PI staining and flow cytometry. MDA-MB-231 cells were treated with **1** or **2** at the indicated concentrations for 48 h; 10 μM camptothecin was used as positive control. Data are expressed as mean ± s.e.m. ($n \geq 3$).

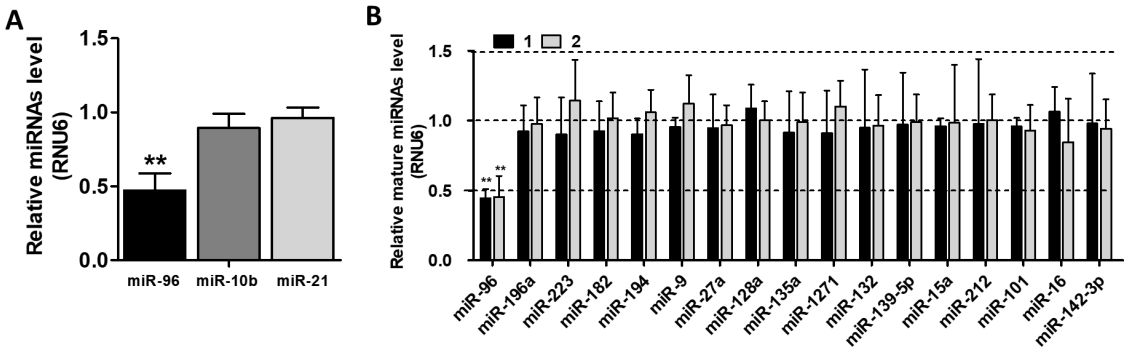


Figure S7. Studying the selectivity of **2. A,** Effect of **2** (500 nM) on mature levels of miR-96, miR-10b and miR-21. **B**, effects of **1** and **2** on levels of all miRNAs predicted to target the FOXO1 3' UTR by TargetScan.[2] Data are expressed as mean ± s.e.m. (n ≥ 3). *p<0.05, **p<0.01, as determined by a two-tailed Student *t* test by comparison to levels in untreated cells.

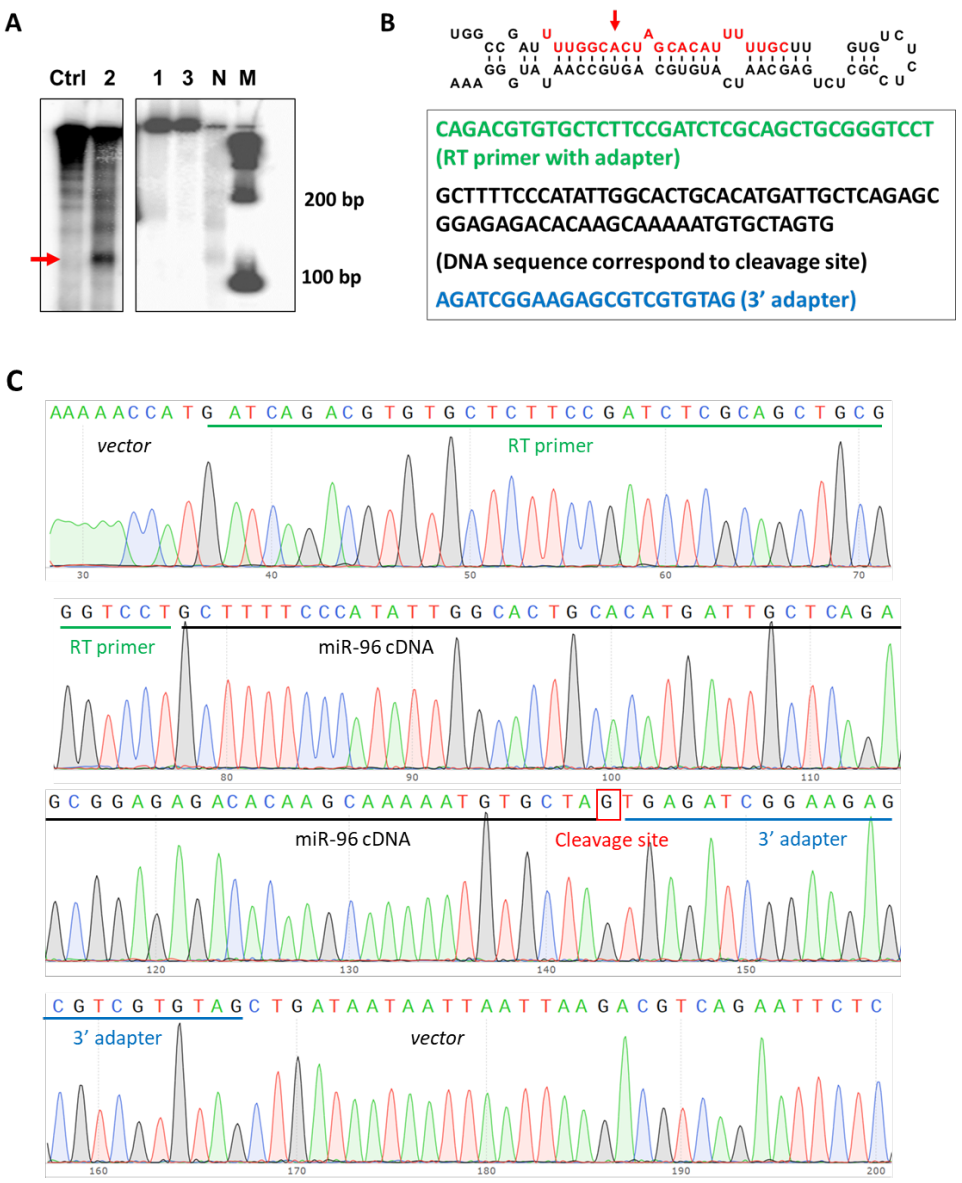


Figure S8. Ribo-SNAP-Map identifies **2**'s binding site within pri-miR-96 in cells. **A**, representative gel image of PCR products from cells treated with **1**, **2**, or **3**. **M** indicates a "marker", or 100 bp ladder with bands at 100 bp and 200 bp labeled. The lane **N** in the right gel was the sample of **2** with not enough radioactivity. **B**, the cleavage site should produce a PCR product that is 128 bp. A band corresponding to the predicted size was observed in the PCR products from cells treated with **2** for 6 h while no band at 128 bp was observed from cells treated with **1** or **3** for 6 h. **C**, Sanger sequencing data for the mapping of cleavage sites by **2**, containing vector, RT primer, miR-96 DNA, and 3' adapter sequences. The cleavage site was identified as indicated in the red box and lettering.

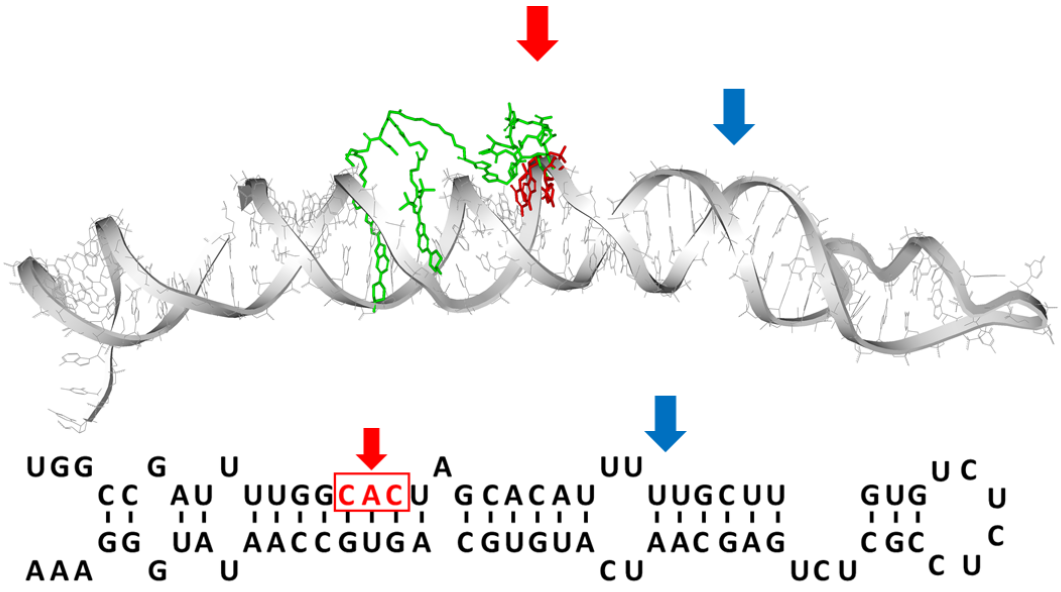


Figure S9. Molecular modeling of **2** bound to pri-miR-96 (**2**, green; pri-miR-96, grey; CAC region shown to be cleaved by **2**, red). The molecular modeling of the linker length suggested that the bleomycin moiety in **2** prefers to bind the CAC region near the binding site and not bleomycin's canonical AU rich sequence (indicated with a blue arrow) distal to **2**'s binding site, as expected.

Experimental Procedures

RNA preparation: The single-stranded DNA template (5'-GGGTGGCCGATTTTGGCACTAGCACATTTTTGCTTGTGTCTCTCCGCTCTGAGCAATCATGTGCAGTGCCAATATG GGAAA-3') for PCR amplification was purchased from Integrated DNA Technologies, Inc. (IDT) and used without further purification. PCR amplification was performed in 1× PCR Buffer (10 mM Tris, pH 9.0, 50 mM KCl, and 0.1% (v/v) Triton X-100), 2 μM T7 promoter forward primer (5'-GGCCGGATCCTAATACGACTCACTATAGGGTGGCCGATTTTGGC-3'), 2 μM reverse primer (5'-TTTCCCATATTGGCA-3'), 4.25 mM $MgCl_2$, 330 μM dNTPs, and 1 μL of Taq DNA polymerase in a 300 μL reaction. The cycling conditions used for PCR were 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. The resulting double stranded DNA template was transcribed by T7 RNA polymerase in 1× Transcription Buffer (40 mM Tris HCl, pH 8.1, 1 mM spermidine, 0.001% (v/v) Triton X-100 and 10 mM DTT) containing 2.25 mM of each rNTP and 5 mM $MgCl_2$ at 37 °C overnight. The RNA product was purified on a denaturing 15% polyacrylamide gel, and concentrations were determined by UV absorbance at 260 nm.

In vitro RNA cleavage and primer extension: The pri-miR-96 RNA (1 μM) was folded as previously described[3] by heating at 65 °C for 5 min and slowly cooling to room temperature. Different concentrations of **2** or **3** (5, 50, 500 nM) were pre-activated by the addition of 1 eq Fe^{2+} and added to the folded RNA in a total volume of 20 μL. Next, 1 eq Fe^{2+} was added 30 min and then 60 min later, and the reaction mixtures were incubated at 37 °C overnight.[4] After ethanol precipitation and quantification by Nanodrop, reverse transcription was performed by using SuperScript™ III Reverse Transcriptase (ThermoFisher Scientific) per the manufacturer's protocol using 5' ^{32}P-labeled primer (~10,000 counts). The A, T, G and C sequencing ladders were generated by using a ratio of ddNTP/dNTP of 3:1. The RNA was digested by the addition of RNase A and RNase H and incubated at 37 °C for 30 min. Then, an equal volume of Loading Buffer (95% formaldehyde, 50 mM EDTA, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol) was added to each reaction. The final mixture was resolved on a denaturing 15% polyacrylamide gel.

Microscale thermophoresis[1]: MST measurements were performed on a Monolith NT.115 system (NanoTemper Technologies) with Cy5-labeled AT-rich DNA hairpin (CGCGAATTCGCGTTTTCGCGAATTCGCG; IDT) or Cy5-labeled pri-miR-96 RNA (GGAUCCGAUUUUGGGAAACCAAUAUGGGAUCC; Dharmacon, Inc). Samples were prepared with constant RNA or DNA concentration (5 nM) and varying concentrations of compound in the presence of 0.05% (v/v) Tween-20. The following parameter were used: 5 – 20 % LED, 20 – 80% MST power, Laser-On time = 30 s, Laser-Off time = 5 s. Fluorescence was detected using excitation wavelengths of 605–645 nm and emission wavelengths of 680–685 nm. The resulting data were analyzed by thermophoresis analysis and fitted by quadratic binding equation in MST analysis software (NanoTemper Technologies). The dissociation constant was then determined using a single-site model to fit the curve.

DNA cleavage *in vitro*: Different concentrations of **2** or **3** (5, 50, 500, 5000 and 10000 nM) were pre-activated by the addition of 1 eq Fe^{2+} and then 2 μL of a plasmid encoding GFP (150 ng/μL) was added in

a final volume of 20 µL. An additional equivalent of Fe^{2+} was added 30 min and then 60 min later, and the reaction mixture was incubated at 37 °C overnight. The reaction mixture was loaded on 0.8% agarose gel with 6×Gel Loading Dye, Purple (NEB) and stained with ethidium bromide for 10 min.

Visualization of DNA damage: MDA-MB-231 cells were grown in a glass bottom 96-well plate and treated with compound for 24 h. Cells were washed with 1× DPBS three times and then fixed with 100 µL of 4% paraformaldehyde for 10 min at 37 °C. Cells were washed with 1× DPBS three times and then with 0.1% Triton X-100 in 1× DPBS three times for 5 min at 37 °C. Cells were then incubated with a 1:500 dilution of anti-γH2AX (Abcam)[5] at 37 °C for 1 h, washed three times with 0.1% Triton X-100 in 1× DPBS for 5 min at 37 °C, and incubated with a 1:200 dilution of goat anti-mouse IgG-DyLight 488 conjugate (Thermo Scientific) at 37 °C for 1 h. After washing the cells with 0.1% Triton in 1× DPBS and twice with 1× DPBS for 5 min at 37 °C, nuclei were stained with DAPI (100 µL of 1 µg/mL), and cells were imaged.

RT-qPCR of miRNAs: Cells (~70% confluency) were treated with various concentrations of compound for 24 h. Total RNA was extracted using a Quick-RNA Miniprep Kit (Zymo Research) per the manufacturer's protocol. Approximately 200 ng of total RNA, as determined by Nanodrop, was used for reverse transcription using a miScript II RT Kit (Qiagen) per the manufacturer's protocol. RT-qPCR was performed on a 7900HT Fast Real Time PCR System (Applied Biosystem) using Power SYBR Green Master Mix (Applied Biosystems). All primers were purchased from IDT and listed in Table S1. The primers for unbiased miRNA profiling were purchased from Eurofins Genomics in a 384-well plate. The expression levels of miRNAs were normalized to U6 small nuclear RNA.[6]

Western blotting: Cells in 6-well plates (~70% confluency) were treated with 500 nM of **2** for 48 h. Total protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce Biotechnology) following the manufacturer's protocol and quantified using a Micro BCA Protein Assay Kit (Pierce Biotechnology). Approximately 20 µg total protein was separated on a 10% SDS-polyacrylamide gel, and then transferred to a PVDF membrane. The membrane was washed with 1× Tris-buffered saline (TBS) and then blocked in 5% milk in 1× TBST (1× TBS containing 0.1% Tween-20) for 1 h at room temperature. After incubation in 1:1000 FOXO1 primary antibody (Cell Signaling Technology) in 1× TBST containing 5% milk overnight at 4 °C, the membrane was washed with 1× TBST and incubated with 1:2000 anti-rabbit IgG horseradish-peroxidase secondary antibody conjugate (Cell Signaling Technology) in 1×TBS for 1 h at room temperature. The membrane was washed with 1× TBST and protein expression was quantified using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) per the manufacturer's protocol. To quantify β-actin expression, the membrane was stripped using 1× Stripping Buffer (200 mM glycine, pH 2.2 and 0.1% SDS) followed by washing in 1× TBST. The membrane was blocked and probed for β-actin similarly using 1:5000 β-actin primary antibody (Cell Signaling Technology) in 1× TBST containing 5% milk at room temperature for 1 h. The membrane was washed with 1× TBST and incubated with 1:10,000 antirabbit IgG horseradish-peroxidase secondary antibody conjugate (Cell Signaling Technology) in 1× TBS for 1 h at room temperature. β-actin protein expression was quantified using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology) per the manufacturer's

protocol. The fold change of FOXO1 expression was calculated by normalizing FOXO1 band intensity to β-actin band intensity.

Caspase assay: Cells in 96-well plates (~60% confluency) were treated with various concentrations of compound for 48 h. In the case of miR-96 overexpression, cells were transfected with a plasmid encoding pri-miR-96 with Lipofectamine 2000 (Thermo Fisher Scientific) per the manufacturer's protocol, split into 96-well plates, and treated with compounds for 48 h. MCF 10A cells were cultured in Dulbecco's modified eagle medium/F12 (DMEM/F12) supplemented with 20 ng/μL epidermal growth factor (EGF), 0.5 μg/mL hydrocortisone, cholera toxin 0.1 μg/mL, bovine insulin 10 μg/mL, 1% penicillin/streptomycin and 10% FBS (complete growth medium).Caspase-3/7 activities were measured by using Caspase-Glo® 3/7 Assay Systems (Promega) per the manufacturer's protocol. Luminescence of compound-treated wells was normalized to untreated cells, and the fold change of caspase activities was calculated.

Annexin V/PI Assay: Cells in 6-well plates (~60% confluency) were incubated with **2** or **3** for 48 h. As a positive control, cells were treated with 10 μM camptothecin for 24 h. The cells were detached from the surface by using accutase and washed twice with ice-cold 1× DPBS and then three times with 1× Annexin Binding Buffer (50 mM Hepes (pH 7.4), 700 mM NaCl and 12.5 mM $CaCl_2$). The cells were suspended in 100 μL 1× Annexin Binding Buffer containing 5 μL Annexin V-APC (eBioscience). The cells were incubated for 10 min at room temperature followed by washing with 1× Annexin Binding Buffer. The cells were then stained with 1 μg/mL propidium iodide in 300 μL of 1× Annexin Binding Buffer for 15 min at room temperature. Flow cytometry was performed using a BD LSRII instrument (BD Biosciences). For data analysis, the appropriate quadrant was assigned, and the early and late apoptosis percentages, as shown by Annexin V and PI staining, were calculated.

Molecular modeling: Modeling of the binding of **2** to pri-miR-96 was generated as previously reported[7]. Briefly, we first modeled the binding of **1** to pri-miR-96. The target RNA structure was modeled in RNAComposer (http://rnacomposer.cs.put.poznan.pl/) using the secondary structure predicted with ViennaRNA (http://rna.tbi.univie.ac.at/). The RNA-binding small molecule was energy minimized using MacroModel (Schrodinger, LLC, NY) and placed proximal to the RNA. Energy minimization of the RNA-small molecule complex was performed until the gradient of energy was less than 0.01 kcal/mol/Å. The bleomycin A5 moiety was then manually added and allowed flexibility during an additional energy minimization to model the binding of **2** to pri-miR-96. Energy minimization was performed until the gradient of energy was less than 0.01 kcal/mol/Å. The conformation with lowest energy is shown in Figure S8.

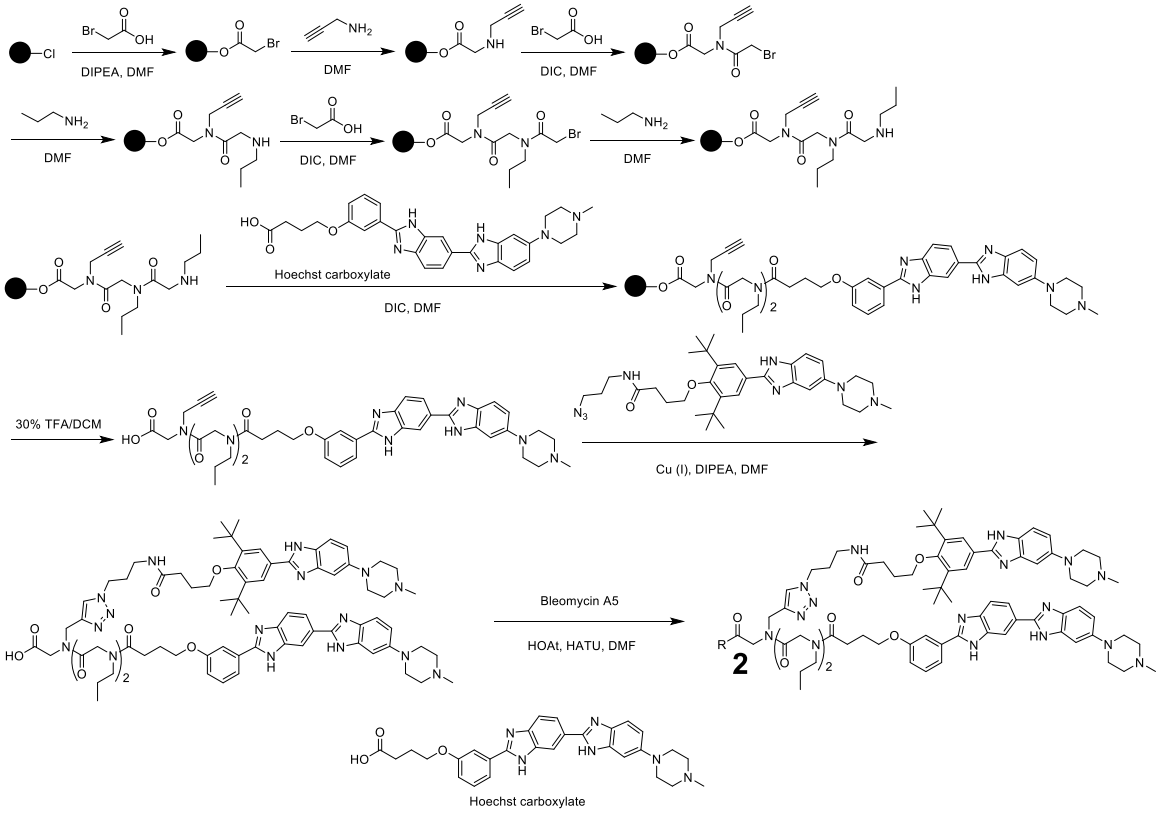
Ribo-SNAP-Map[8, 9]**:** Cells were grown in 100 mm dishes to ~70% confluency and treated with **1** (control), **2**, or **3** (control) for 6 h. Total RNA was then extracted by treatment with TRIzol (ThermoFisher Scientific) and quantified by Nanodrop. Approximately 10 μg of total RNA was used for reverse transcription with a pri-miR-96 specific primer (5'-CAGACGTGTGCTCTTCCGATCTCGCAGCTGCGGGTCCT-3'; 2 pmol) using Superscript III (SSIII; Life Technologies). 10 μg RNA with 2 pmol of gene-specific primer

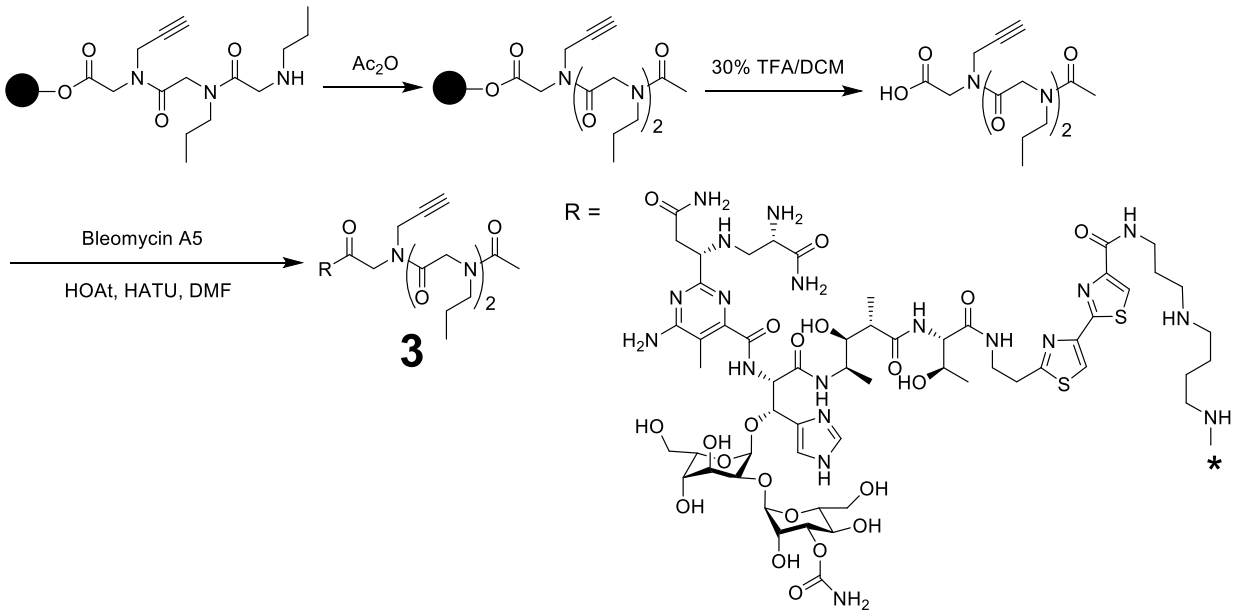
and 1 µL 10 mM dNTP Mix in total 13 µL was kept at 65°C for 5 min and in ice for 5 min. Then 4 µL 5X First-Strand Buffer, 1 µL 0.1 M DTT, 1 µL RNaseOUT and 1 µL SuperScript™ III RT were added and incubated at 50°C for 1 h and then 85°C for 10 min. After digesting the RNA with RNase A and RNase H, the cDNA was purified by using RNAClean XP beads (Beckman Coulter; 1.8 volumes of beads and 3 volumes of isopropanol).

The purified cDNA was ligated with a 3' adapter (/5Phos/AGATCGGAAGAGCGTCGTGTAG/3Bio/) by T4 RNA ligase 1 (New England BioLabs; NEB) following the manufacturer's recommended protocol (2 µL 10× T4 RNA ligase buffer, 1 µL of 1 mM ATP, 10 µL 50% PEG 8000, 5 µL cDNA, 1 µL of 20 µM ssDNA adaptor, and 1 µL of T4 RNA ligase). Then, the cDNA ligated to the adaptor was purified with RNAClean XP beads as described above. PCR amplification was performed with the ligated cDNA by using Phusion polymerase (NEB) with cycles of 98 °C for 20 s, 64 °C for 20 s and 72 °C for 90 s and the following primers: forward - (5'-CAGACGTGTGCTCTTCCGATC-3'); reverse - (5'-CTACACGACGCTCTTCCGATCT-3'). The PCR products were then 5'- end labeled with ^{32}P as described above and separated on a denaturing 15% polyacrylamide gel. A 100 bp ladder (NEB) was used as a marker. For Sanger sequencing, the PCR products were separated on a denaturing 15% polyacrylamide gel, and the target band was excised from gel and ethanol precipitated. The purified DNA was ligated into a vector by using NEB's PCR Cloning Kit per the manufacturer's protocol. Antibiotic-resistant colonies were selected and subjected to Sanger sequencing by Genewiz.

General protocol for compound synthesis:

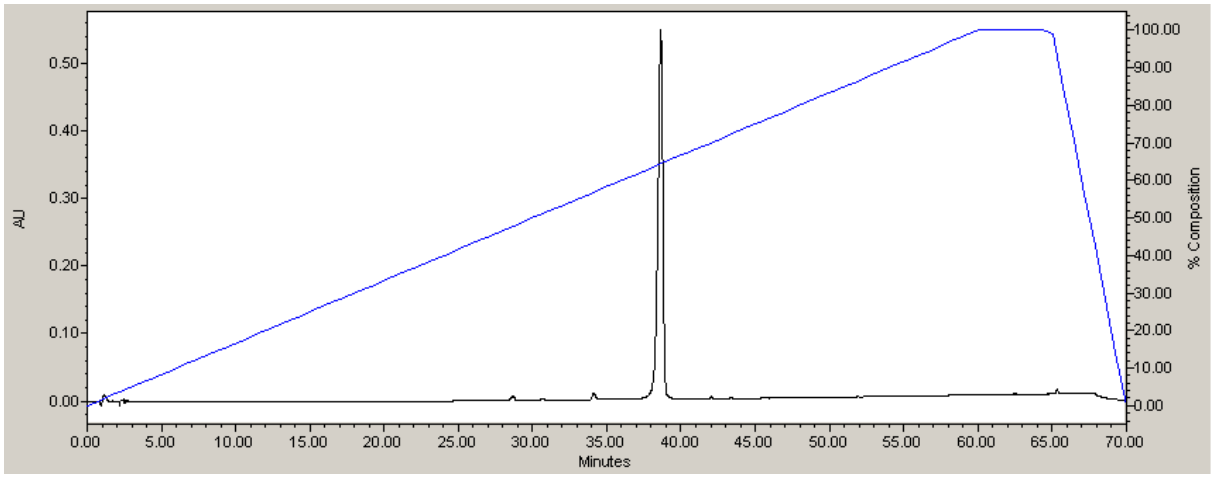
Scheme 1. Synthetic route for **2** and **3:**




Ac2O
30% TFA/DCM
Bleomycin A5
HOAt, HATU, DMF
3
R =

Synthesis of compound 1: Compound **1** was synthesized as previously described.[6] Briefly, Rink amide resin (500 mg, 345 µmol) with a substitution level of 0.69 mmol/g was shaken in *N*,*N*-dimethyl formamide (DMF) for 5 min and then deprotected with 20% piperidine in DMF (10 mL, 2x 30 min) at room temperature. The resin was washed with 10 mL DMF three times. Next, 5 eq of bromoacetic acid in 10 mL DMF solution and 5 eq of DIC (*N,N*'-diisopropylcarbodiimide) were added, and the resin was shaken at room temperature for 30 min. After washing with 10 mL DMF three times, 5 eq of propargylamine in 10 mL DMF was added, and the reaction mixture was shaken at room temperature for 2 h. The bromoacetic acid coupling step was repeated and 5 eq propylamine in 10 mL DMF was added, and the reaction mixture was shaken at room temperature for 2 h. The bromoacetic acid coupling step and propylamine step were repeated. The peptoid in the resin was then treated with a solution of 2 eq Hoechst carboxylate (synthesized as previously described)[10], 2 eq DIC and 2 eq DIPEA (*N,N*'-diisopropylethylamine) in 10 mL DMF. The reaction mixture was shaken at room temperature for 2 h and washed with 10 mL DMF three and then 10 mL dichloromethane (DCM) three times. The product was cleavage from resin by adding 10 mL 30% trifluoroacetic acid (TFA) in DCM and shaken 30 min at room temperature. The eluate was concentrated under vacuum, and the product was precipitated with excess amounts of ether.

The resulting yellow solid was directly treated with a solution of 1.1 eq azide[3], 0.2 eq Cu(I) catalyst and 2 eq DIEA in 2 mL DMF. The reaction mixture was kept at 65 °C overnight, and the reaction process was monitored by MALDI-TOF MS. After the starting material was no longer detectable, the product was precipitated with excess amounts of ether and dissolved in 50% methanol in water with 0.1%TFA. HPLC purification was performed with a linear gradient from 0% to 100% B (methanol or acetonitrile + 0.1%TFA) in A (water + 0.1%TFA) over 60 min and a flow rate of 5 mL/min. The pure fractions were collected, and the solvent was concentrated under vacuum. Excess amounts of ether were added, and the resulting solid was obtained as product. Purity was evaluated on a reverse phase Waters Symmetry C18 5 µm 4.6 × 150 mm column at room temperature with a flow rate of 1 mL/min and a linear gradient of 0–100% B in A. Absorbance was monitored at 254 nm and 345 nm. Mass spectra were recorded on a 4800 plus MALDI-TOF/TOF analyzer. MALDI: $[M+H]^+$ calculated: 1391.8179; $[M+H]^+$ observed: 1391.9153.



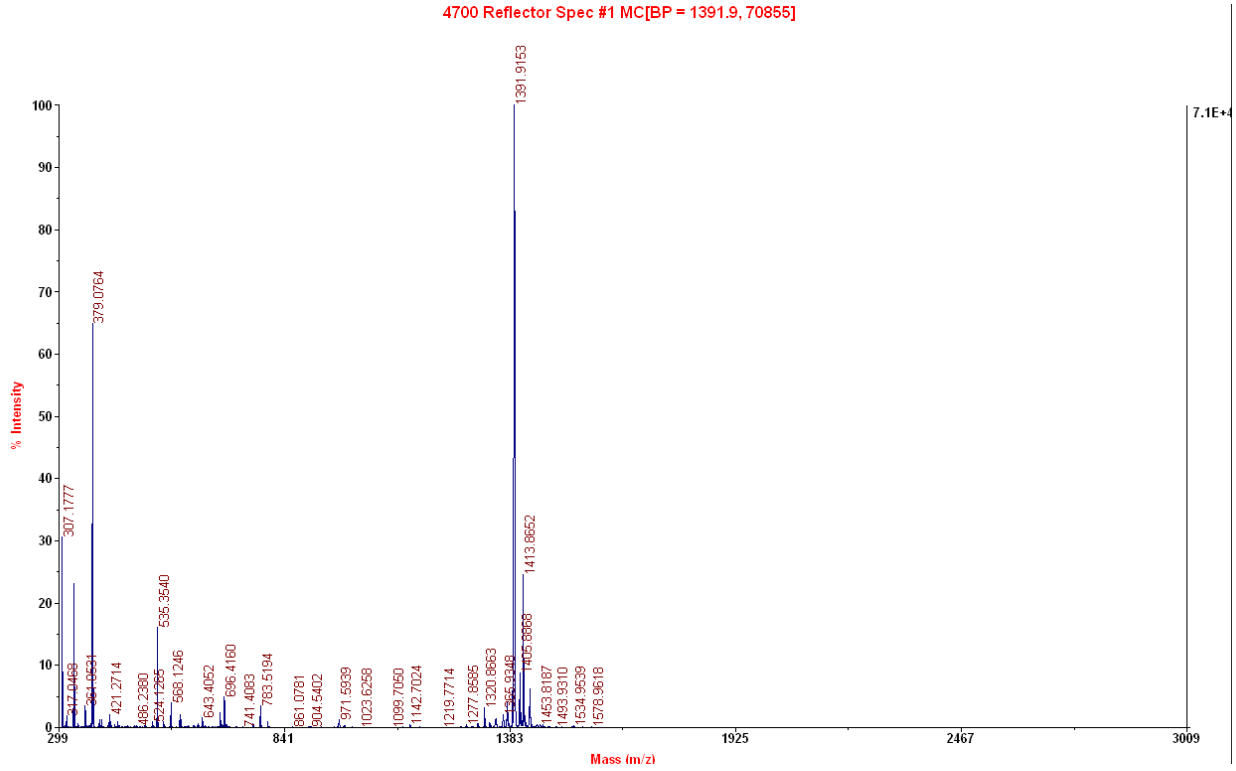


Figure S6. Characterization of compound **1** by HPLC (a linear gradient from 0% to 100% B in A over 60 min and a flow rate of 1mL/min) and mass spectrum obtained by MALDI-TOF MS.

Synthesis of compound 2: Chlorotrityl resin (500 mg, 580 μmol) was activated in 1 M HCl in 10 mL DCM for 10 min at room temperature, followed by washing with DCM three times and DMF three times. Next, 5 eq of bromoacetic acid and 5 eq DIPEA in 10 mL DMF were added, and the resin shaken at room temperature for 30 min. After washing with 10 mL DMF three times, 5 eq propargylamine in 10 mL DMF were added, and the reaction mixture was shaken at room temperature for 2 h. The resin was washed with 10 mL DMF three times and 5 eq of bromoacetic acid in 10 mL DMF solution and 5 eq DIC were added. The resin was shaken at room temperature for 30 min followed by addition of 5 eq propylamine in 10 mL DMF. The reaction mixture was shaken at room temperature for 2 h. The bromoacetic acid coupling step and propylamine step were repeated. The peptoid on the resin was then treated with a solution of 2 eq Hoechst carboxylate, 2 eq DIC and 2 eq DIPEA in 10 mL DMF solution. The reaction mixture was shaken at room temperature for 2 h and washed with 10 mL DMF three times and then 10 mL DCM three times. The product was cleaved from resin by adding 10 mL of 30% TFA in DCM and shaking the resin for 30 min at room temperature. The elutate was concentrated under vacuum, and the product was precipitated with an excess amount of ether.

The resulting solid was directly treated with a solution of 1.1 eq azide, 0.2 eq Cu(I) catalyst and 2 eq DIEA in 2 mL DMF. The reaction mixture was kept 65 °C overnight, and the reaction process was monitored by MALDI MS. After the starting material was no longer detectable, the product was precipitated with an excess amount of ether.

The Bleomycin A5 coupling reaction was performed by adding 2 eq HOAt (1-Hydroxy-7-azabenzotriazole), 2 eq HATU (hexafluorophosphate azabenzotriazole tetramethyl uronium), 5 eq DIEA, and 2 eq Copper-bleomycin A5. The reaction mixture was shaken at room temperature overnight and the product was precipitated with an excess amount of ether. The solid was dissolved in 50% acetonitrile in water with 0.1%TFA and subjected to HPLC purification. After injection of the solution, the column was washed with 50 mM EDTA (pH 6.7) for 30 min to remove the copper ion and then washed with water for another 30 min. Then the target product was separated with a linear gradient from 0% to 100% B (acetonitrile +0.1%TFA) in A (water +0.1%TFA) over 60 min and a flow rate of 5 mL/min. The pure product was obtained and characterized as described above. MALDI: $[M+H]^+$ calculated: 2814.3835; $[M+H]^+$ observed: 2814.4392.

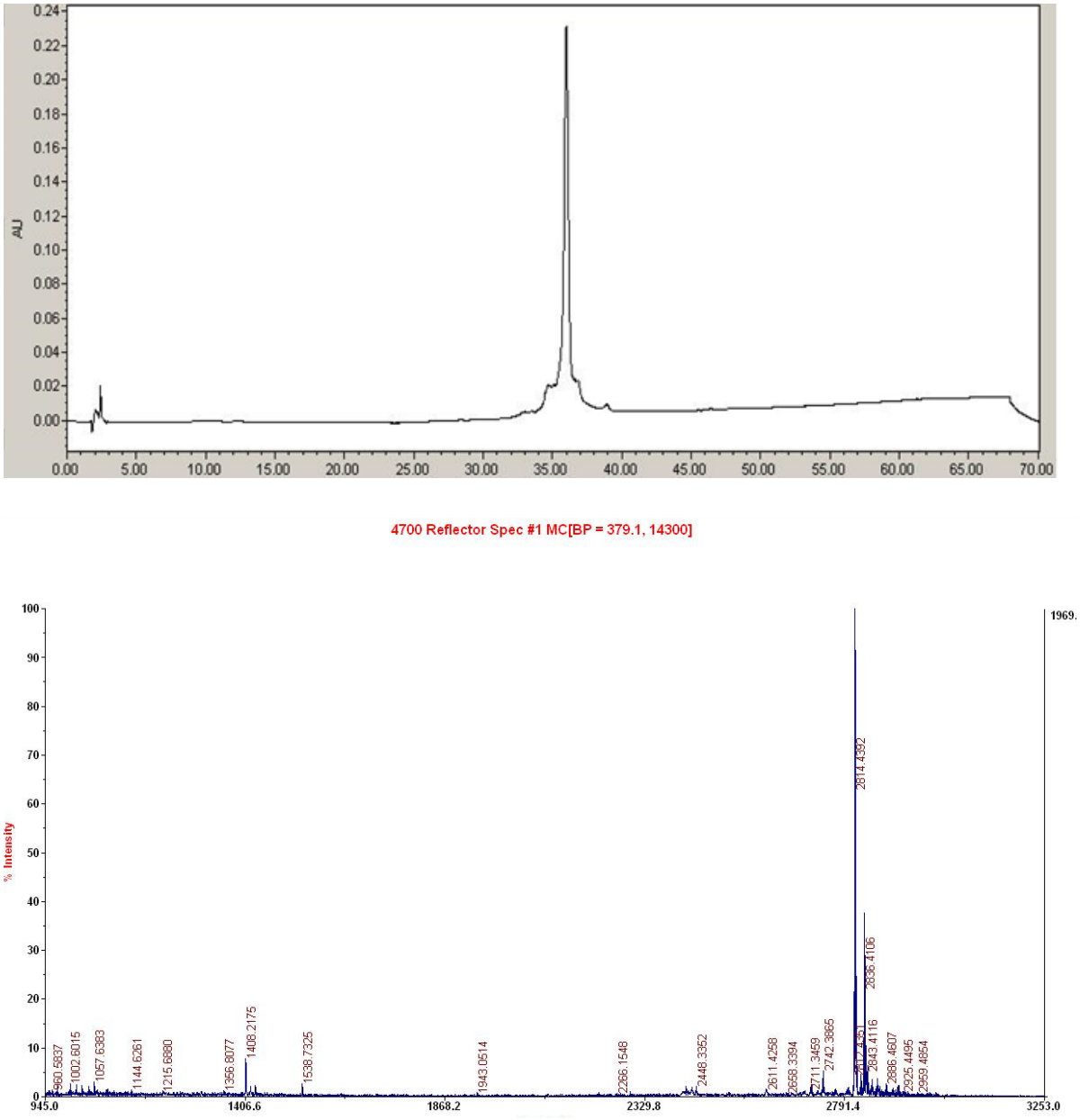


Figure S7. Characterization of compound **2** by HPLC (a linear gradient from 0% to 100% B in A over 60 min and a flow rate of 1mL/min) and MALDI mass spectrum.

Synthesis of compound 3: Chlorotrityl resin (500 mg,580 μmol) was activated in 1 M HCl in 10 mL DCM for 10 min at room temperature, followed by washing with DCM three times and DMF three times. Then, 5 eq bromoacetic acid and 5 eq DIPEA in 10 mL of DM were added, and the resin was shaken at room temperature for 30 min. After washing with 10 mL DMF three times, 5 eq propargylamine in 10 mL DMF was added, and the reaction mixture was shaken at room temperature for 2 h. The resin was washed with 10 mL DMF three times followed by addition of 5 eq of bromoacetic acid in 10 mL DMF solution and 5 eq DIC. The resin was shaken at room temperature for 30 min and then 5 eq propylamine in 10 mL DMF were added. After shaking the mixture at room temperature for 2 h, the bromoacetic acid coupling step and propylamine step were repeated. The peptoid on the resin was then treated with a solution of 2 eq acetic anhydride (Ac_2O) and 2 eq DIPEA in 10 mL DMF. The reaction mixture was shaken at room temperature for 30 min and washed with 10 mL DMF three times and then 10 mL DCM three times. The product was cleaved from the resin by adding 10 mL 30% TFA in DCM and shaking at room temperature for 30 min. The eluate was concentrated under vacuum, and the product was precipitated with excess amount of ether.

The Bleomycin A5 coupling reaction was performed by adding 2 eq HOAt, 2 eq HATU, 5 eq DIEA and 2 eq Copper-Bleomycin A5. The reaction mixture was shaken at room temperature overnight, and the product was precipitated with an excess amount of ether. The solid was dissolved in 50% acetonitrile in water with 0.1%TFA and subjected to HPLC purification. After injection of the solution, the column was washed with 50 mM EDTA (pH 6.7) for 30 min to remove the copper ion and then washed with water for another 30 min. Then the target product was separated with a linear gradient from 0% to 100% B (acetonitrile +0.1%TFA) in A (water +0.1%TFA) over 60 min and a flow rate of 5 mL/min. The pure product was obtained and characterized as described above. MALDI: $[M+H]^+$ calculated: 1775.7767; $[M+H]^+$ observed: 1775.8733.

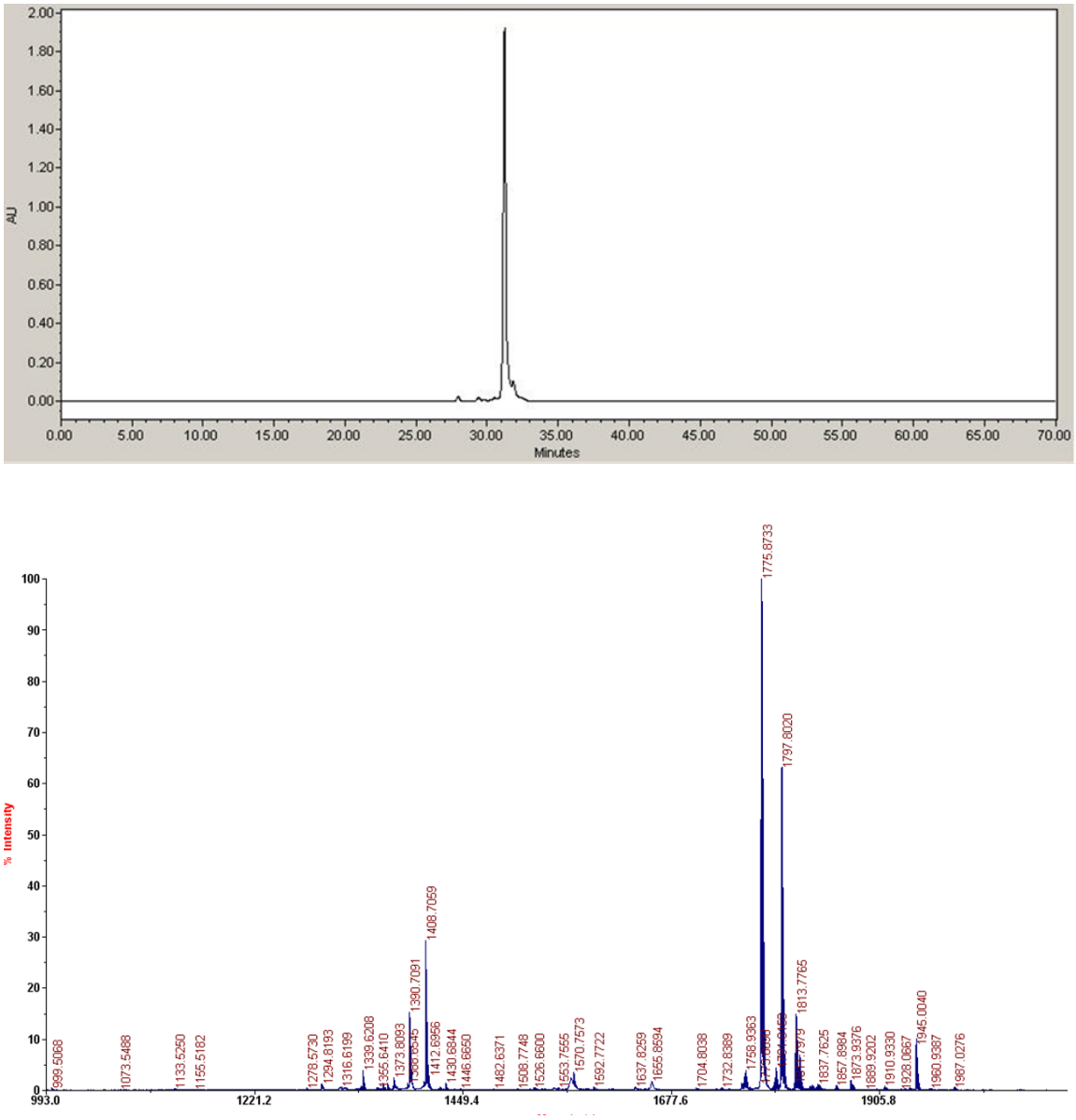


Figure S8. Characterization of compound **3** by HPLC (a linear gradient from 0% to 100% B in A over 60 min and a flow rate of 1mL/min) and MALDI mass spectrum.

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