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SUPPORTING INFORMATION

Synthesis and Evaluation of "*Ama-Flash*", a Photocaged Amatoxin Prodrug for Light-Activated RNA Pol II Inhibition and Cell Death

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Materials and Methods

General: All reactions were performed under argon atmosphere in flame-dried glassware and dried solvents at room temperature, unless otherwise stated. Controlled temperature reactions were performed using a mineral oil bath and a temperature controlled hot plate (IKA Ceramag Midi). Temperatures below room temperature were achieved in an ice/water bath (0°C), dry ice/ethylene glycol bath (-20°C), dry ice/ethanol/ethylene glycol bath (-20°C to -75°C) and dry ice/acetone bath (-78°C). Solvents were removed under reduced pressure using a Büchi rotary evaporator. Anhydrous solvents were prepared by distillation under nitrogen atmosphere or drying over 3Å or 4Å molecular sieves for at least 48 hours. Ethers were distilled from sodium in the presence of benzophenone as indicator. Triethylamine, methylene chloride and hexanes were distilled over calcium hydride. Methanol was distilled from magnesium. DMSO and DMF were dried over 4Å molecular sieves under argon atmosphere. All reagents and solvents were purchased from Sigma-Aldrich, Alfa Aesar, Acros Organics, Strem Chemicals, Matrix Scientifics, AK Scientific, Oakwood Chemicals or TCI America, unless otherwise stated. Authentic α-amanitin was purchased from Sigma-Aldrich.

Thin-layer chromatography (TLC) was performed using silica gel 60 F_{254} precoated aluminum plates (EM Science). Detection of TLC spots was performed using UV lamp at 254 nm, or by staining with *p*-anisaldehyde, potassium permanganate, ninhydrin or 2,4-dinitrophenylhydrazine, prepared according to literature procedures. Flash column chromatography purifications were performed using silica gel 60 (230-400 mesh, Silicycle, Quebec). Low-resolution mass spectra (LRMS ESI) in electrospray ionization (ESI) mode were obtained from a Bruker Esquire spectrometer. High-resolution mass spectra (HRMS ESI) in electrospray ionization (ESI) mode were obtained from a Waters/Micromass LCT spectrometer. Proton (^{1}H-NMR) and carbon (^{13}C-NMR) spectra were obtained using Bruker AV-300 (300 MHz) and AV-400inv (400 MHz) spectrometers. Circular Dichroism (CD) spectra were obtained using a Jasco J-815 spectrophotometer.

HPLC purification methods: All HPLC chromatograms were generated on an Agilent 1100 system equipped with an auto injector, a fraction collector, and a diode array detector. Analytical injections were performed on an Agilent Eclipse XDB C-18 (4.6 x 250 mm) column with a flow rate of 2 mL/min. The column was fitted with a column guard. In cases of closely-eluting peaks, integration was performed by standard data analysis software package whereby a line was drawn between both peaks and then integration was performed without peak correction. Chromatograms were obtained with a solvent gradient of 0.1% formic acid in water (Solvent B) and 0.1% formic acid in acetonitrile (Solvent A). The solvent gradients were:

Gradient **A**: 0-18 min 5%-50% A, 18-21 min 50%-100% A; 21-26 min 100%A, 26-31 min 100%-5% A.

Gradient **B**: 0-17 min 10%-35% A, 17-19 min 35%-100% A; 19-23 min 100% A, 23-24 min 100%-10% A, 24-29 min 10% A.

Gradient **C**: 0-25 min 20%-65% A, 25-27 min 65%-100% A; 27-32 min 100% A, 32-34 min 100%-20% A, 34-38 min 20% A.

Peptide quantification: Quantifications of different peptides were performed using a reported extinction coefficient of 10,000 $M^{-1}cm^{-1}$ at the wavelength with the maximum absorbance (290-305nm), except for α-amanitin that has an extinction coefficient of 13,500 $M^{-1}cm^{-1}$. All UV wavelength scans and measurements were performed using a Cary5000 Spectrophotometer and readings were acquired at values of approximately 0.1-0.5 AU. Peptides were purified by HPLC and eluates were lyophilized. The dry compound was re-suspended in a known amount of solvent (0.1% formic acid in H_2O:MeCN 1:1) and the concentration was measured using the UV absorbance at the λ_{max}, assuming the extinction coefficient of 10,000 $M^{-1}cm^{-1}$ for all tryptathionine-containing peptides, with the exception of α-amanitin that has an extinction coefficient of 13,500 $M^{-1}cm^{-1}$. Typically, 5.4-65.1 nmol (5-60 µg) is obtained and accurately quantified by UV-Vis spectroscopy using a 0.5 mL cuvette. For illustrative purposes, a quantity of 5.4 nmol of α-amanitin in a volume of 0.5 mL gives a concentration of 10.8 µM and an absorbance reading of 0.136 AU, a value that is fully within the ideal range for quantitative UV-Vis spectroscopy.

Sample preparation for cell toxicity assays: Following quantification, solutions with toxic peptides were re-lyophilized and re-suspended in a given volume of DMSO or H_2O to provide a 1 mM solution, which was then used in cell toxicity assays.

Cell culture: Cells were cultured in a-MEM or high-sucrose DMEM, purchased from Gibco. Fetal bovine serum (FBS), 0.25 % trypsin (with 1.3 mM EDTA), 0.85% Trypan blue, and the antibiotic mixture Pen/Strep (10K U/mL penicillin, 10K mg/mL streptomycin) were also purchased from Gibco. All cell culture plastic ware was obtained from Corning or Falcon. Cells were cultured at 37°C in a humidified chamber with 5% CO_2. When used in cell culture, DMSO was purified by filtration through a 0.2 mm filter. All experiments are carried out in a laminar flow culture cabinet, unless otherwise noted. Absorbance measurements of the 96-well plates were obtained using a Beckman-Coulter DTX 880 multimode detector, equipped with an excitation filter of 595 nm.

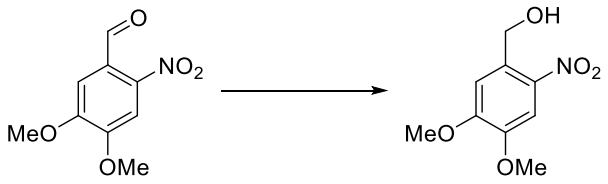
Immortalized CHO cells had been stored in liquid nitrogen. To revive cells, a 1-mL tube of the frozen cells in medium containing 10% DMSO was warmed in a 30°C water bath and diluted with 9 mL of fresh media. Media contained 10% FBS and 100 U/mL penicillin and 100 mg/mL streptomycin, unless otherwise indicated. The cells were incubated in a T-25 flask at 37°C at 5% CO_2. After 24 hours, the medium was aspirated and replaced with fresh medium. When cells reached a level of 90-100% confluence, they were sub-cultured. The medium was removed, and the cells were treated with 0.25% trypsin

containing 1.3 mM EDTA in the incubator. Once the cells were detached from the tissue culture flask, 3-5 mL media was added to quench the trypsin and transferred to a 10 mL centrifuge tube. The mixture was centrifuged for 5 min at 8000 rpm, and the supernatant was discarded. The cells were suspended in fresh medium, diluted as required, and transferred to a new culture flask.

To assay cell viability, a nearly confluent tissue culture flask was trypsinized, and the cells were counted following treatment with Trypan blue, using a hemocytometer. The cells were then diluted to the appropriate stock concentrations in fresh medium and transferred in 100 µL to a 96-well plate using a multi-channel pipette. The number of cells plated varied from experiment-to-experiment. These were incubated a 37°C and 5% CO_2 for a 24-hour period to allow for adherence. The medium was aspirated, and fresh medium was added, which contained the desired additives in DMSO. The cells were then re-incubated for 72 h. At the completion of the experiment, a 100 µL aliquot of 2.5 mg/mL MTT in PBS was added to each well. The plate was incubated three hours further to allow for the formation of the formazan product in viable cells. The media was carefully aspirated, and the purple product was solubilized in DMSO. The absorbance of each well was recorded at 590 nm. Data was processed in Microsoft Excel and GraphPad Prism. Experiments were performed in triplicates unless otherwise noted, and the error bars were calculated as the standard error of the mean.

Trypsinized cells were diluted to a concentration of 3.3×10^4 cells/mL for CHO cells. Each cell line was plated in a 96-well plate, with 100 µL of the stock solution per well (5000 cells per well) and incubated for 24 hours. Stocks of α-amanitin or analogs were prepared at various concentrations, containing a maximum of 1% DMSO, and added to various wells, according to the desired final concentration. The cells were incubated for 72 hours, at which point viability was assessed as described.

Synthetic Procedures and NMR Characterizations



2 **3**

(4,5-Dimethoxy-2-nitrophenyl)methanol, 6-nitroveratryl alcohol (3).

To a solution of 6-nitroveratraldehyde **2** (5 g, 23.7 mmol) in MeOH (100 mL) was slowly added $NaBH_4$ (0.45 g, 11.85 mmol). The mixture was stirred at room temperature for 1 hour. The solvent was then evaporated under reduced pressure and the residue was partitioned between EtOAc (50 mL) and H_2O (50 mL). The aqueous phase was extracted with EtOAc (3 x 20 mL), the combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Recrystallization from EtOAc/hexanes yielded the product as a yellow solid (3.53 g, 70%).

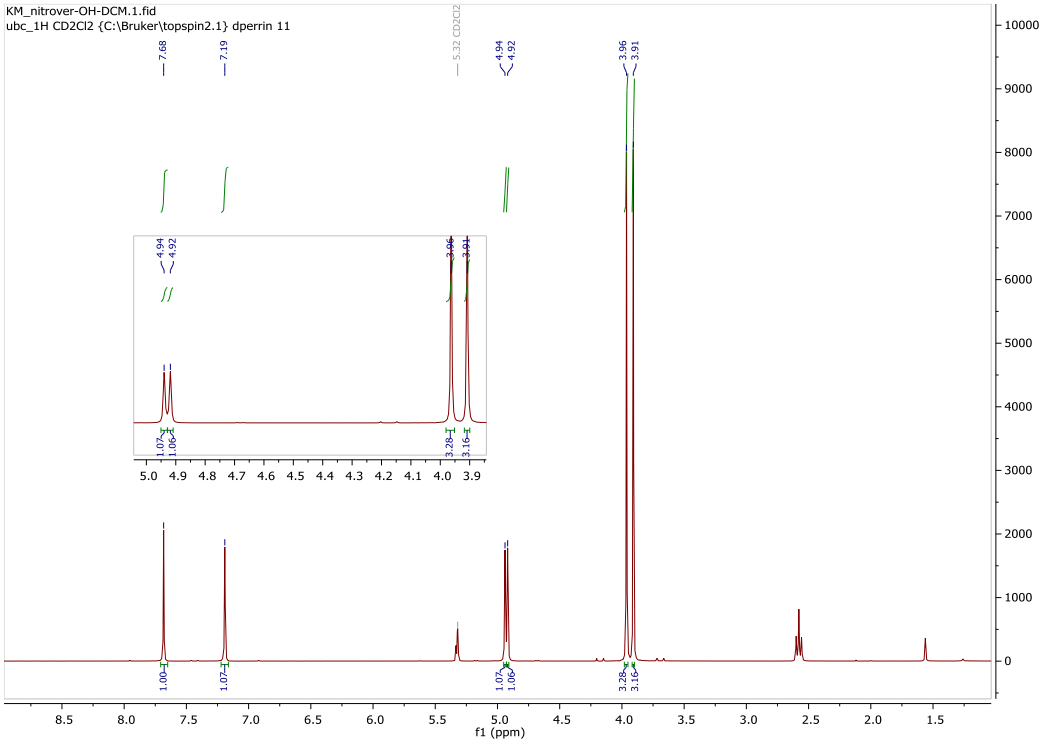
TLC (EtOAc:hex 80:20 v/v): R_f = 0.48

^{1}H NMR (300 MHz, Methylene Chloride-d_2) δ 7.69 (s, 1H), 7.19 (s, 1H), 4.94 (s, 1H), 4.92 (s, 1H), 3.97 (s, 3H), 3.91 (s, 3H).

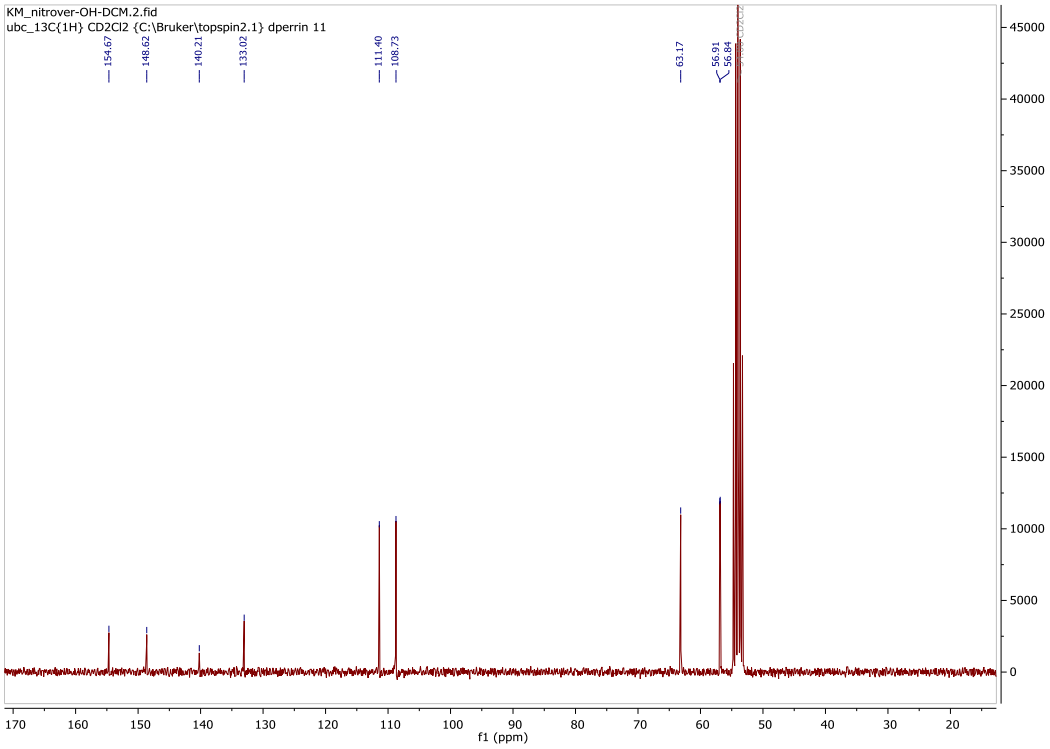
^{13}C NMR (75 MHz, Methylene Chloride-d_2) δ 154.7, 148.6, 140.2, 133.0, 111.4, 108.7, 63.2, 56.9, 56.8.

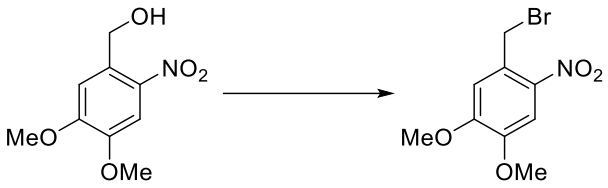
HRMS ESI (m/z) calculated for $C_9H_{11}NO_5Na$ $[M+Na]^+$ 236.0535; found 236.0525.

^{1}H-NMR of **3**, 300 MHz, CD_2Cl_2



^{13}C-NMR of **3**, 75 MHz, CD_2Cl_2





3 **4**

1-(Bromomethyl)-4,5-dimethoxy-2-nitrobenzene, 6-nitroveratryl bromide (4).

To a stirred solution of PPh_3 (2.46 g, 9.38 mmol) in dry DCM (95 mL) at room temperature was added Br_2 (0.48 mL, 9.38 mmol) dropwise over 5 minutes. The light orange solution was allowed to stir under Argon for another 10 minutes. Then, **3** (2 g, 9.38 mmol) was added at once. The reaction was allowed to proceed for 2h at RT, at which point the contents were transferred to a separatory funnel containing saturated aqueous $NaHSO_3$ (35 mL). The organic phase was separated and washed once with H_2O (35 mL). The combined aqueous layers were extracted with DCM (3 x 30 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude residue was purified by flash column chromatography using silica gel (EtOAc/hex 30:70) to afford the product as a light yellow solid (2.38 g, 92%).

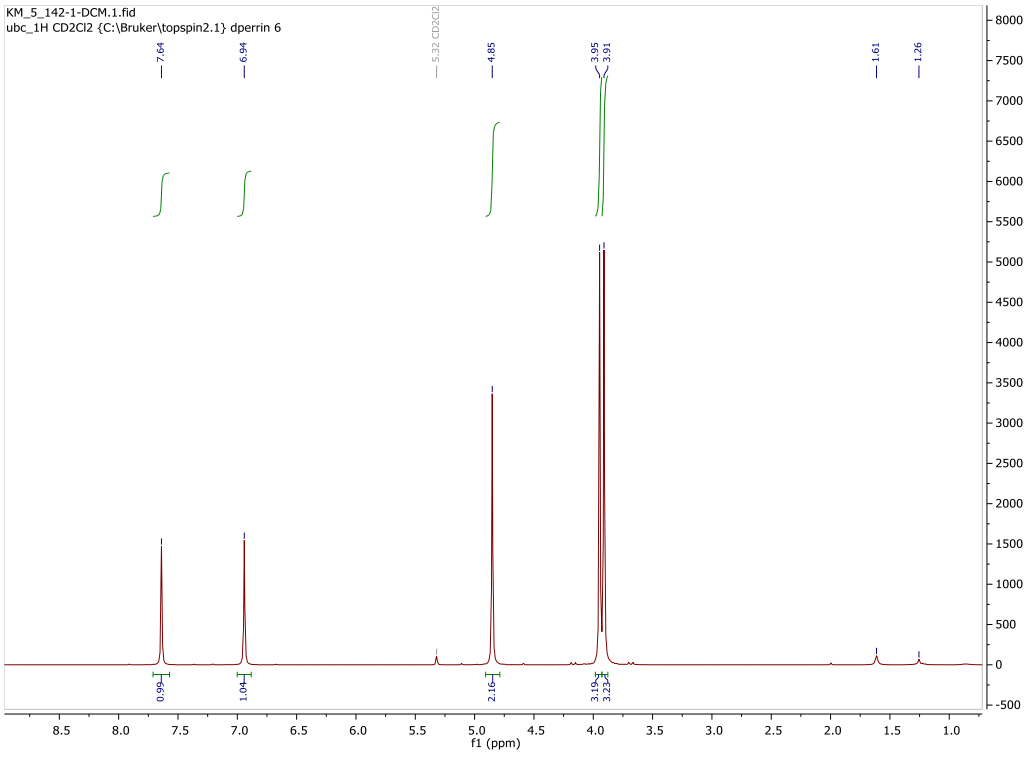
TLC (EtOAc:hex 50:50 v/v): R_f = 0.45

^{1}H NMR (300 MHz, Methylene Chloride-d_2) δ 7.64 (s, 1H), 6.94 (s, 1H), 4.85 (s, 2H), 3.95 (s, 3H), 3.91 (s, 3H).

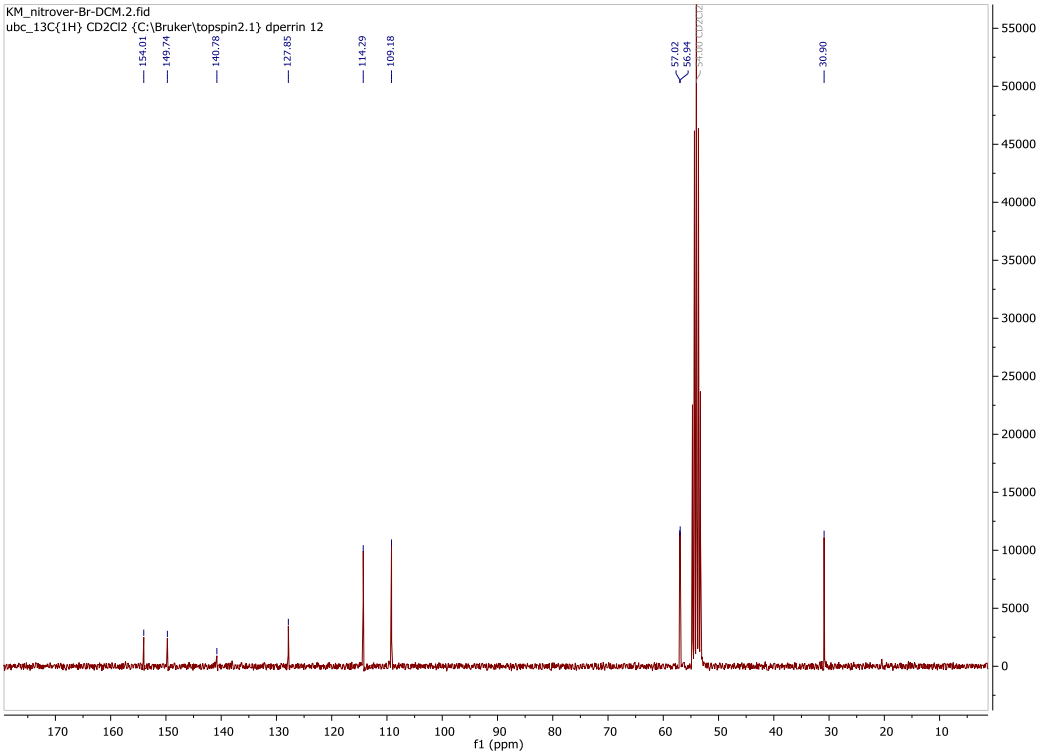
^{13}C NMR (75 MHz, Methylene Chloride-d_2) δ 154.0, 149.7, 140.8, 127.9, 114.3, 109.2, 57.0, 56.9, 30.9.

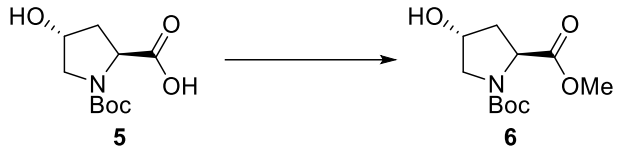
HRMS ESI (m/z) calculated for $C_9H_{10}NO_4NaBr$ $[M+Na]^+$ 297.9691; found 297.9692.

^{1}**H-NMR** of **4**, 300 MHz, CD_2Cl_2



^{13}**C-NMR** of **4**, 75 MHz, CD_2Cl_2





1-(*tert*-Butyl)-2-methyl-(2*S*,4*R*)-4-hydroxypyrrolidine-1,2-dicarboxylate, N^{α}-Boc-*trans*-Hyp-OMe (6).

Dimethylsulfate (1.85 mL, 19.5 mmol) was added to a mixture of K_2CO_3 (4 g, 39 mmol) and Boc-Hyp-OH **5** (3 g, 13 mmol) in dry acetone (20 mL) at room temperature. The mixture was vigorously stirred overnight at RT and concentrated under reduced pressure to remove acetone. The residue was diluted with EtOAc (40 mL), washed with saturated aqueous $NaHCO_3$ (2 x 20 mL) and brine (40 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure followed by drying *in vacuo* to yield the product as a white solid (2.86 g, 90%), which was used in the next step without further purification.

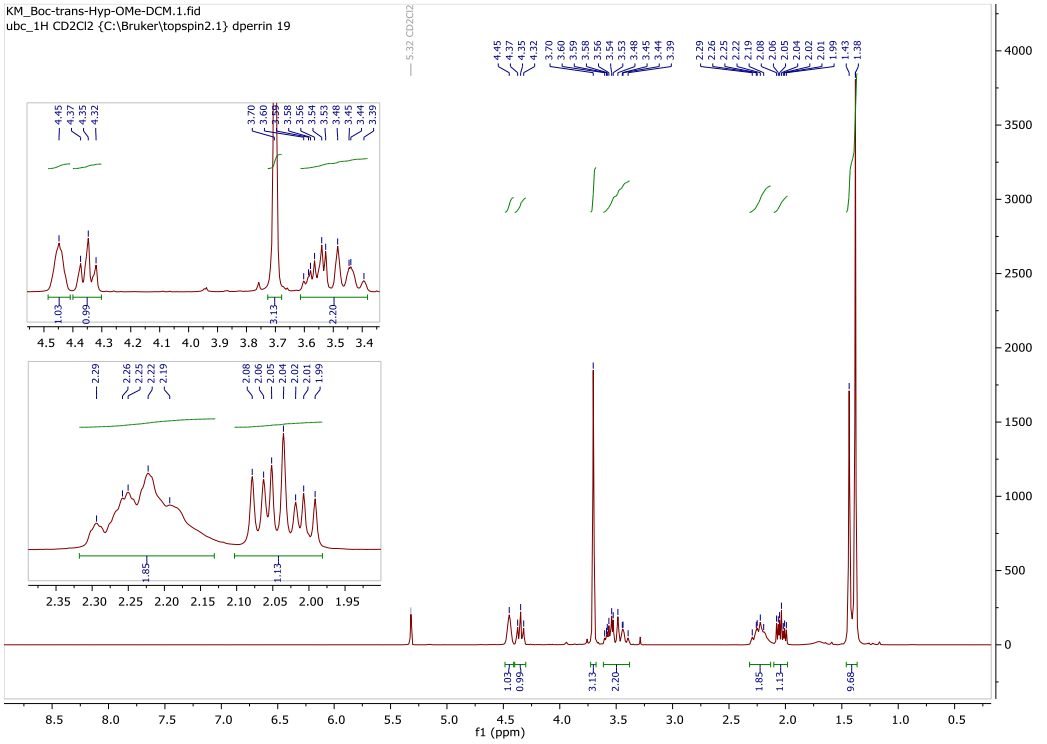
TLC (EtOAc:hex 60:40 v/v): R_f = 0.17

^{1}H NMR (300 MHz, Methylene Chloride-d_2) δ 4.50 – 4.41 (m, 1H), 4.40 – 4.30 (m, 1H), 3.70 (s, 3H), 3.61 – 3.37 (m, 2H), 2.33 – 2.11 (m, 1H), 2.04 (ddd, *J* = 13.2, 8.1, 4.7 Hz, 1H), 1.43 (s, 4H), 1.38 (s, 5H).

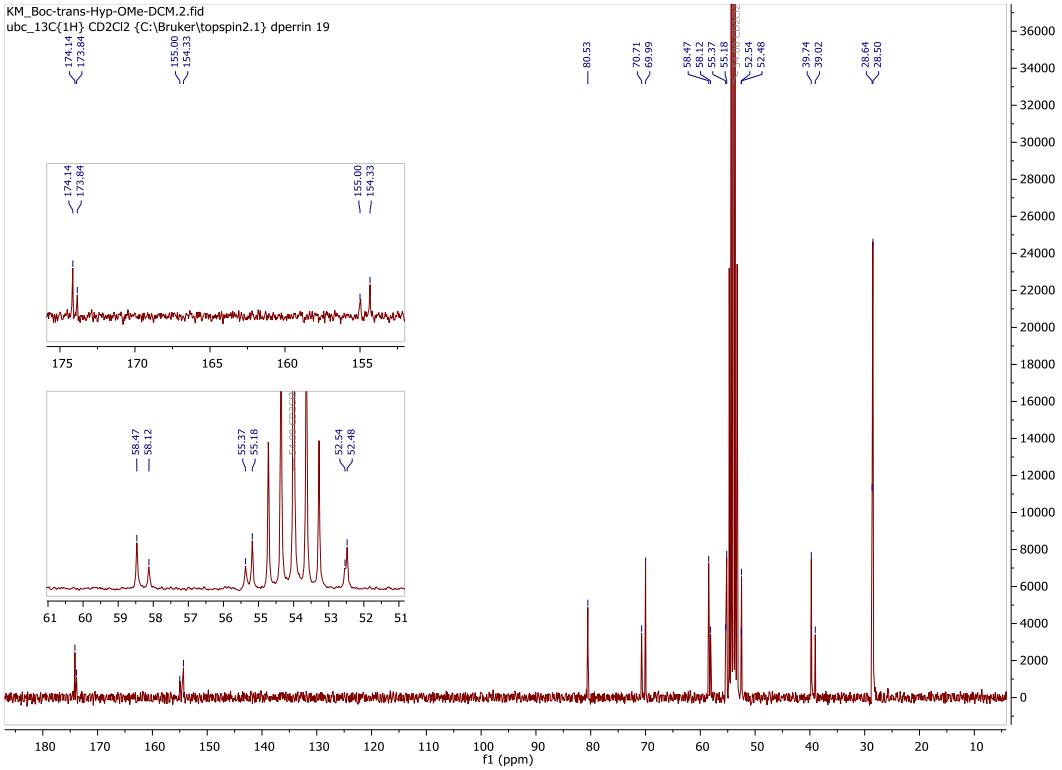
^{13}C NMR (75 MHz, Methylene Chloride-d_2) δ 174.1, 173.8, 155.0, 154.3, 80.5, 70.7, 70.0, 58.5, 58.1, 55.4, 55.2, 52.5, 52.5, 39.7, 39.0, 28.6, 28.5.

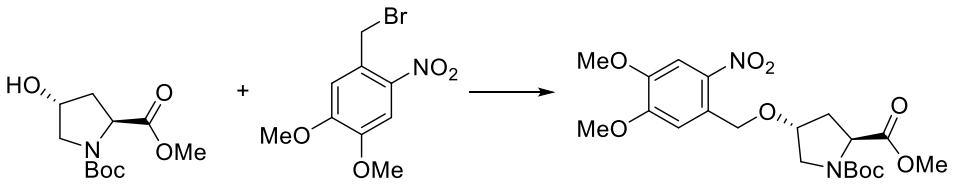
HRMS ESI (m/z) calculated for $C_{11}H_{19}NO_5Na$ $[M+Na]^+$ 268.1161; found 268.1158.

^{1}H-NMR of **6**, 300 MHz, CD_2Cl_2



^{13}C-NMR of **6**, 75 MHz, CD_2Cl_2





6 **4** **7**

1-(*tert*-Butyl)-2-methyl-(2*S*,4*R*)-4-((4,5-dimethoxy-2-nitrobenzyl)oxy)pyrrolidine-1,2-dicarboxylate, N^{α}-Boc-*trans*-(6-Nitroveratryl)-Hyp-OMe (7).

The Boc-protected hydroxy-proline methyl ester (**6**) (3.94 g, 16.08 mmol) was dissolved in DCM (38 mL). This solution was added to a solution containing nBu$_4$NHSO$_4$ (690 mg, 4.82 mmol) in DCM (38 mL) and 2.5M aqueous NaOH (38 mL) and was shielded from light. A solution of freshly prepared 6-nitroveratryl bromide (**4**) (1.84 g, 6.7 mmol) in DCM (38 mL) was added, and the reaction mixture was vigorously stirred at room temperature for 2.5 h. The layers were separated, and the aqueous phase was extracted with DCM (3 x 30 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude residue was purified by flash column chromatography using silica gel (EtOAc/hex 25:75 to 35:65, gradient) to afford the product as a light yellow solid (1.17 g, 40%).

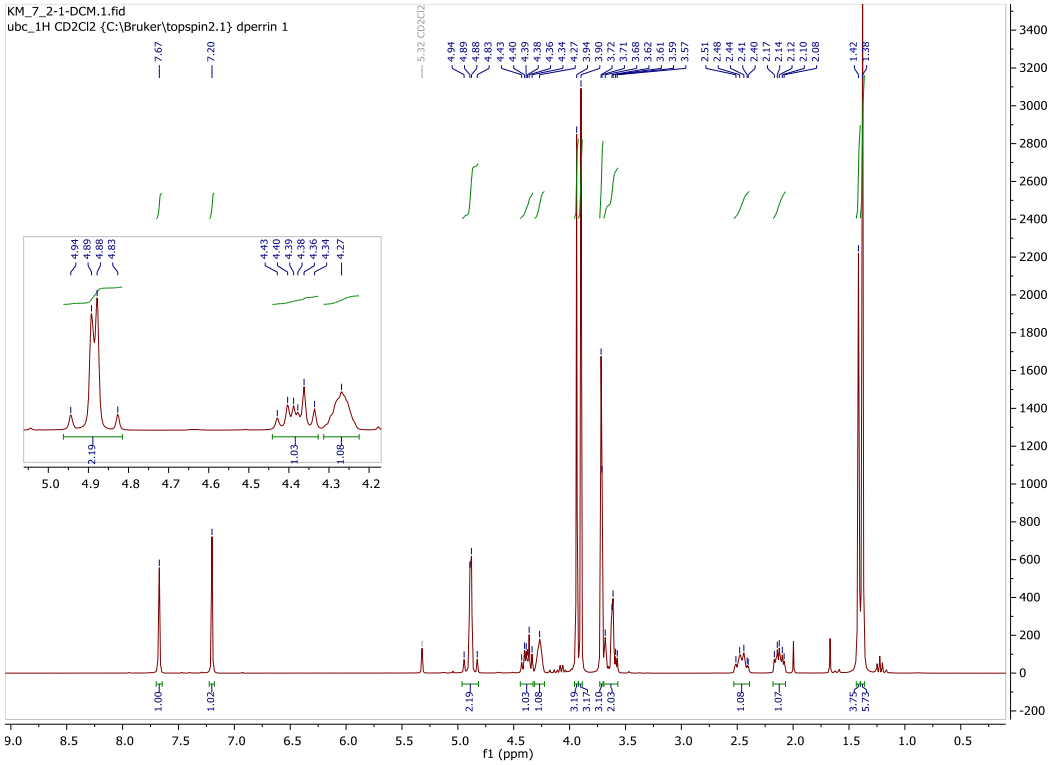
TLC (EtOAc:hex 60:40 v/v): R_f = 0.44

^{1}H NMR (300 MHz, Methylene Chloride-d_2) δ 7.65 (s, 1H), 7.19 (s, 1H), 4.87 (dd, J = 15.6, 4.3 Hz, 2H), 4.44 – 4.32 (m, 1H), 4.31 – 4.22 (m, 1H), 3.93 (s, 3H), 3.89 (s, 3H), 3.71 (d, J = 3.3 Hz, 3H), 3.69 – 3.56 (m, 2H), 2.54 – 2.38 (m, 1H), 2.20 – 2.05 (m, 1H), 1.41 (s, 3H), 1.37 (s, 6H).

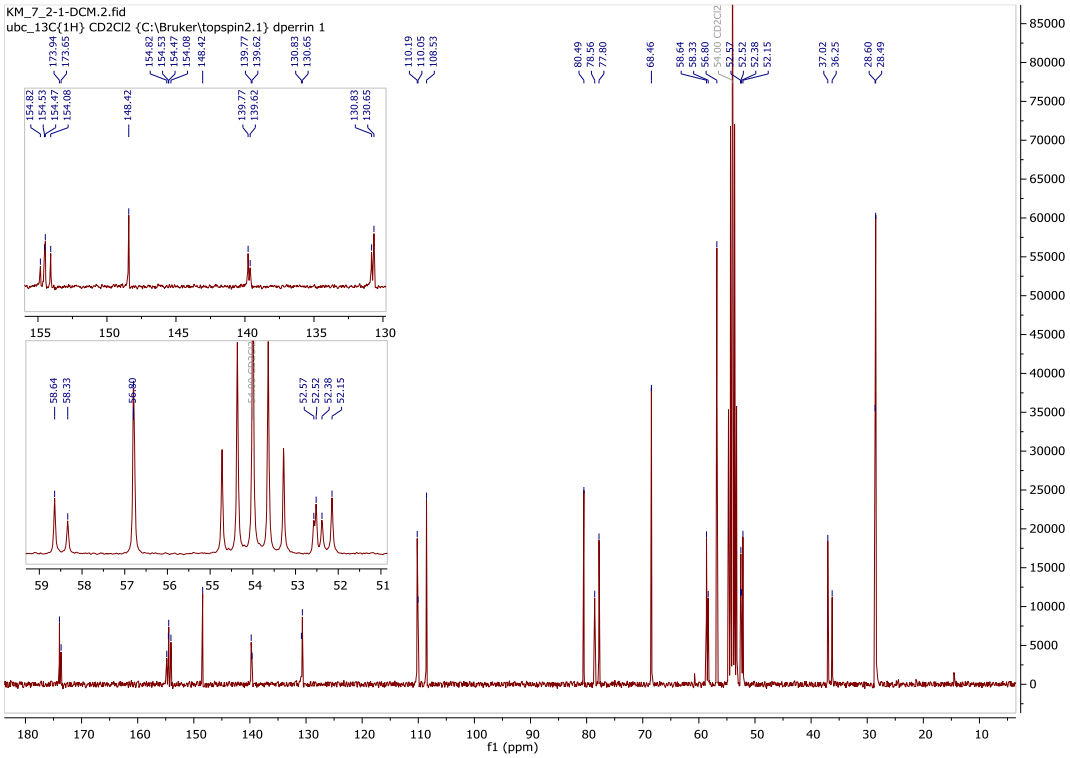
^{13}C NMR (75 MHz, Methylene Chloride-d_2) δ 173.9, 173.7, 154.8, 154.5, 154.5, 154.1, 148.4, 139.8, 139.6, 130.8, 130.7, 110.2, 110.1, 108.5, 80.5, 78.6, 77.8, 68.5, 58.6, 58.3, 56.8, 52.6, 52.5, 52.4, 52.1, 37.0, 36.3, 28.6, 28.5.

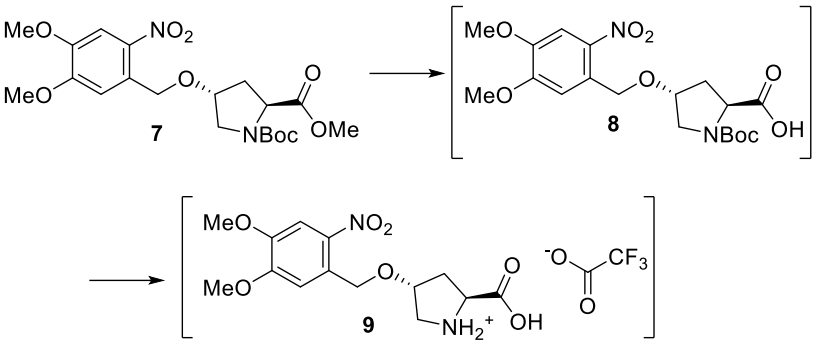
HRMS ESI (m/z) calculated for $C_{20}H_{28}N_2O_9Na$ $[M+Na]^+$ 463.1693; found 463.1701.

^{1}H-NMR of **7**, 300 MHz, CD_2Cl_2



^{13}C-NMR of **7**, 75 MHz, CD_2Cl_2

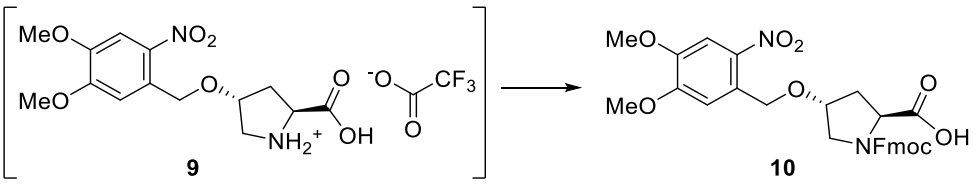




(2*S*,4*R*)-2-carboxy-4-((4,5-dimethoxy-2-nitrobenzyl)oxy)pyrrolidin-1-ium-2,2,2-trifluoro acetate (9).

The starting material (**7**) (424 mg, 0.96 mmol) was dissolved in 1,4-dioxane/H_2O (6 mL:3 mL) and stirred to mix. LiOH (230 mg, 9.6 mmol) was added and the reaction was stirred at room temperature for 1.5 h. H_2O (5 mL) was added to the reaction mixture and was extracted with DCM (3 x 7 mL). The aqueous phase was acidified to pH~1 with 1M aq. HCl and extracted with EtOAc (3 x 10 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford the crude free acid (**8**). The resulting oil was re-dissolved in DCM (10 mL), and TFA (3 mL) was added. The reaction was stirred at room temperature for 1 h. The solvent and TFA were evaporated and co-evaporated with diethyl ether and toluene under reduced pressure. The crude product was dried in vacuo to yield the TFA salt **9** as an off-white solid, which was used in the next step without further purification.

HRMS ESI (m/z) calculated for $C_{14}H_{19}N_2O_7$ $[M+H]^+$ 327.1192; found 327.1187.



(2*S*,4*R*)-1-(((9*H*-fluoren-9-yl)methoxy)carbonyl)-4-((4,5-dimethoxy-2-nitrobenzyl) oxy) pyrolidine-2-carboxylic acid, *N*$^{\alpha}$-Fmoc-Hyp(ONv)-OH (10).

9 (1.03 g crude, 2.34 mmol) was dissolved in 1,4-dioxane/H_2O (12 mL:5 mL) and $NaHCO_3$ (312 mg, 5.15 mmol) was added at once. If necessary, a saturated aqueous solution of Na_2CO_3 was added to adjust the pH to 8. The reaction was stirred at room temperature for 10 min, followed by the addition of Fmoc-OSu (907 mg, 2.69 mmol). After 3 h, H_2O (8 mL) was added to the reaction, which was then extracted with EtOAc (3 x 15 mL). The EtOAc was back-extracted with H_2O (2 x 10 mL), and the combined aqueous layers were acidified to pH~1 with 1 M aq. HCl. This was extracted with EtOAc (3 x 40 mL). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. The crude residue was purified by flash column chromatography using silica gel (DCM/MeOH/HOAc 98:2:0.1) to yield the product as a yellow foamy solid (1.15 g, 90% over 3 steps).

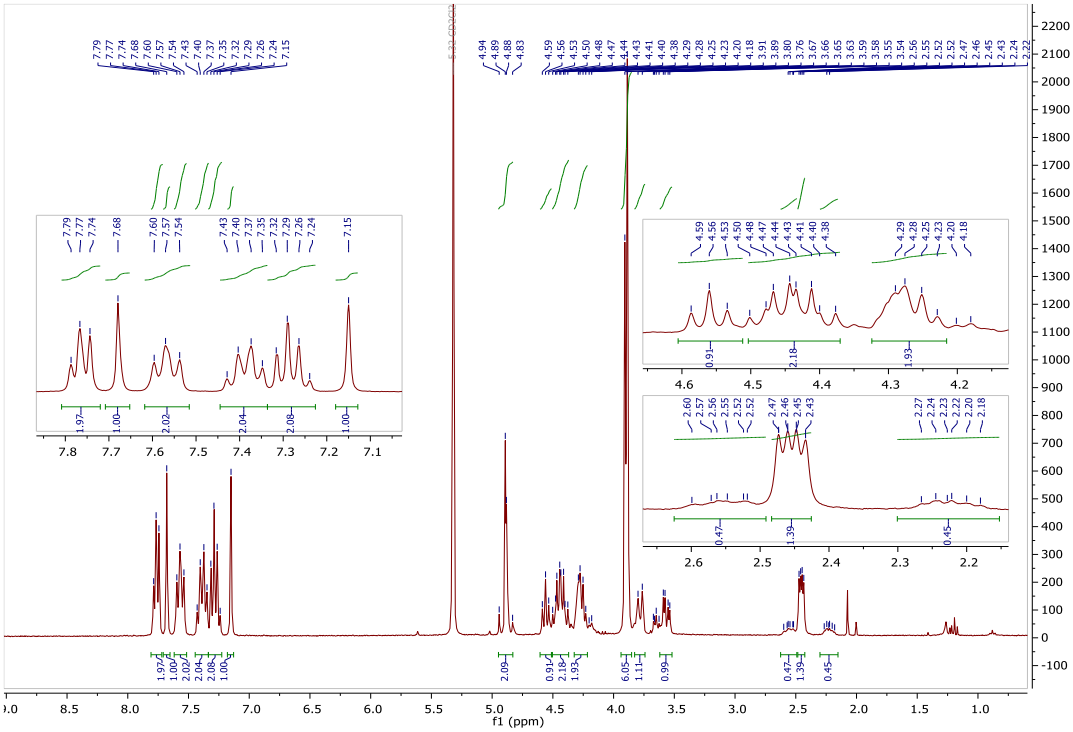
TLC (DCM:MeOH:HOAc 95:5:1 v/v/v): R_f = 0.33

^{1}H NMR (300 MHz, Methylene Chloride-d_2) δ 7.77 (t, *J* = 6.5 Hz, 2H), 7.68 (s, 1H), 7.57 (t, *J* = 9.4 Hz, 2H), 7.39 (q, *J* = 7.7 Hz, 2H), 7.28 (q, *J* = 7.5 Hz, 2H), 7.15 (s, 1H), 4.89 (d, *J* = 2.8 Hz, 2H), 4.56 (t, *J* = 7.9 Hz, 1H), 4.50 – 4.35 (m, 2H), 4.33 – 4.22 (m, 2H), 3.91 (s, 3H), 3.89 (s, 3H), 3.82 – 3.74 (m, 1H), 3.67 – 3.53 (m, 1H), 2.62 – 2.50 (m, 0.5H), 2.45 (dd, *J* = 7.7, 4.0 Hz, 1H), 2.29 – 2.16 (m, 0.5H).

^{13}C NMR (75 MHz, Methylene Chloride-d_2) δ 174.9, 157.0, 155.0, 154.5, 148.5, 144.6, 144.3, 141.8, 139.8, 139.8, 130.4, 128.3, 128.2, 127.6, 127.6, 125.5, 125.4, 120.5, 110.3, 110.2, 108.6, 78.1, 77.6, 68.7, 68.6, 68.1, 58.9, 58.0, 52.7, 52.2, 47.7, 47.6, 37.2, 35.6.

HRMS ESI (m/z) calculated for $C_{29}H_{28}N_2O_9Na$ $[M+Na]^+$ 571.1693; found 571.1692.

^{1}H-NMR of **10**, 300 MHz, CD_2Cl_2



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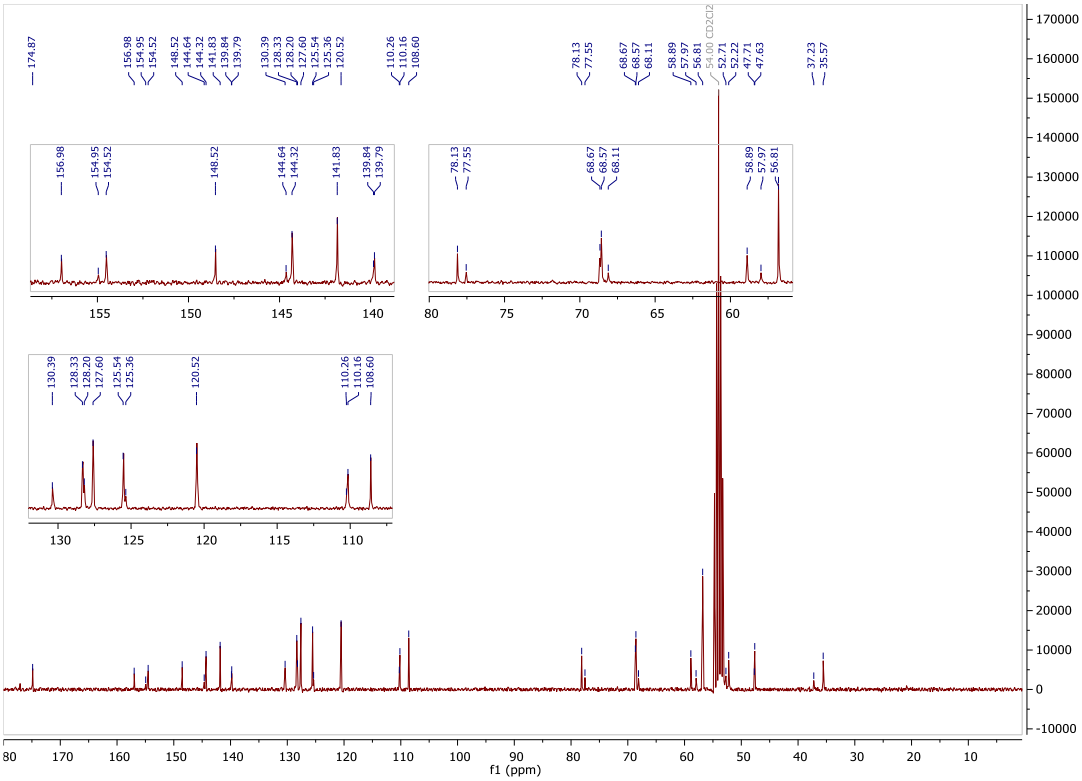
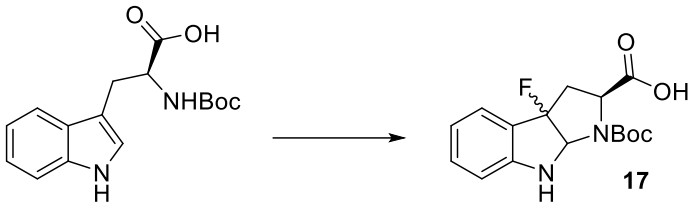
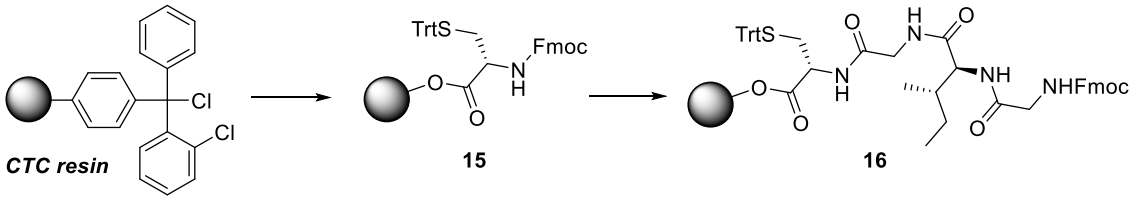




(2*S*)-1-(*tert*-butoxycarbonyl)-3a-fluoro-1,2,3,3a,8,8a-hexahydropyrrolo[2,3-*b*]indole -2-carboxylic acid, *N*$^{\alpha}$-Boc-Fpi-OH (17).

N-Fluorocollidinium triflate (FP-T300, 5 g, 17.3 mmol) was added to a solution of Boc-Trp-OH (2.63 g,8.65 mmol) in dry DCM (120 mL) in a dry round-bottom flask equipped with a condenser under Argon. The resulting solution was stirred at 40°C for 3 hours. Upon completion of the reaction (TLC), its contents were transferred to a separatory funnel and washed with 0.0001 M aq. HCl (2 x 100 mL) and brine (100 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to yield the crude product as a light-brown foam, which was used in the next step without further purification.

TLC (MeOH:DCM:HOAc 10:90:1 v/v/v): R_f = 0.42

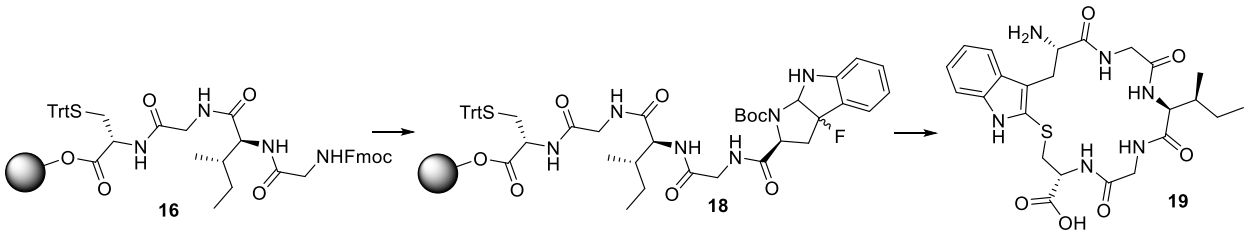


Synthesis of the linear tetrapeptide (16).

Fmoc-Cys(Trt)-OH was loaded on the chlorotrityl resin according to the following protocol. To a flame-dried flask was added chlorotrityl resin (1 g, 1.2 mmol/g, 200-400 mesh), which was then suspended in dry CH_2Cl_2 (9 mL). To this flask was added Fmoc-Cys(Trt)-OH (1.59 g, 2.7 mmol) and DIPEA (1.17 mL, 6.8 mmol). The reaction was stirred at room temperature and under Argon overnight and transferred to a Zeba spin column. The resin was washed three times with DMF and DCM (8 mL, each). Unreacted sites of the resin were capped by applying a solution of CH_2Cl_2:MeOH:DIPEA (8 mL of an 80:15:5 mixture, 20 min), and then washed with CH_2Cl_2 (3 x 8 mL) then DMF (3 x 8 mL) then CH_2Cl_2 (3 x 8 mL) again. The resin was dried on *in vacuo* over P_2O_5 to remove residual solvent. Resin loading was determined using manufacturer's protocols. Briefly, a weighed amount of resin was treated with a 2% solution of DBU in DMF for 30 minutes. The solution was diluted and the UV absorbance of the liberated dibenzofulvene was measured at 304 nm, with an absorption coefficient of ε_{304}=7624 $M^{-1}cm^{-1}$. The measured resin loadings generally ranged from 0.6-0.7 mmol/g. Then, unreacted sites of the resin were capped with a solution of CH_2Cl_2:MeOH:DIPEA (8 mL of a 80:15:5 mixture, 20 min), and then washed with CH_2Cl_2 (3 x 8 mL) then DMF (3 x 8 mL) then CH_2Cl_2 (3 x 8 mL) again.

The N-terminal Fmoc protecting group of **15** was removed by washing with 20% piperidine in DMF (8 mL for 5 min, 8 mL for 10 min). Following deprotection, the resin was washed with DMF (3 x 8 mL), followed by CH_2Cl_2 (3 x 8 mL) and again with DMF (3 x 8 mL). Five equivalents of the following N^{α}-Fmoc-amino acids (Gly, Ile, Gly) and five equivalents of coupling agents (HBTU/HOBt) in DMF were applied sequentially to the growing N-terminus. In general, the following protocol was followed for coupling: Resin was placed in a Zeba spin column (up to 400 mg in a 5 mL column or 1 g in a 10 mL column) and pre-swollen in DMF (8 mL) for 30 min (3 x 8 mL, 10 min each, draining DMF after each swelling). A pre-mixed solution of the next amino acid, coupling reagent(s) and DIPEA in DMF were added to the spin column. The resulting suspension was gently shaken on a vortexer for 1-2 h. The solvent was drained, and the resin was washed with CH_2Cl_2 (3 x 8 mL) then DMF (3 x 8 mL) then CH_2Cl_2 (3 x 8 mL) again. Often, a Kaiser test was performed to check for complete couplings (if Kaiser test positive: repeat coupling; if negative: proceed to the next step). Alternatively, a small amount of resin was removed from the batch and was deprotected with 25 % hexafluoroisopropanol (HFIP) in CH_2Cl_2, and the released peptide was analyzed by LRMS-ESI.

Finally, the unreacted amine sites were capped with EtOAc/collidine/Ac2O (2:2:1, 8 mL) for 20 minutes before washing the resin with CH_2Cl_2 (3 x 8 mL) then DMF (3 x 8 mL) then CH_2Cl_2 (3 x 8 mL) again.



Synthesis of the monocyclic di-deoxy-pentapeptide (the eastern ring) (19).

N$^{\alpha}$-Boc-Fpi-OH (**17**) (crude, 1.15 g, 6 eq) was coupled to the N-terminus of the tetrapeptide **16** (1 g of resin, 0.6 mmol/g loading) as previously described for amino acid couplings on CTC resin. After the coupling was completed (to yield the corresponding linear pentapeptide **18**), the resin was washed with DMF (3 x 8 mL), MeCN (3 x 8 mL), EtOAc/EtOH 1:1 (3 x 8 mL) and CH_2Cl_2 (3 x 8 mL). The resin-bound linear pentapeptide was transferred to a round-bottom flask and stirred in TFA/DCM 1:1 (18 mL) for 1 hour to induce the Savige-Fontana reaction and the global deprotection of acid-labile protecting groups. Triisopropyl silane (TIS, 0.36 mL) and H_2O (0.36 mL) were added to the reaction and stirring was continued for 1 hour. The resin was filtered over glass wool and washed with CH_2Cl_2 (10 mL). The combined filtrate was evaporated *in vacuo*, followed by co-evaporation with Et_2O (2 x 10 mL), and then dried under reduced pressure. The residue was then re-dissolved in minimum amount of 0.1% FA (formic acid) in H_2O/MeCN 1:1 and purified by C18 SEP-PAK. Fractions containing the desired product were detected by analytical HPLC and mass spectrometry. These fractions were combined, lyophilized, and re-suspended in a known volume of MeOH. Concentration and mass of the product were determined by its UV absorbance at 290 nm, with an extinction co-efficient of 10,000 $M^{-1}cm^{-1}$, that is presumably lower than that of the natural product (13,500 $M^{-1}cm^{-1}$).

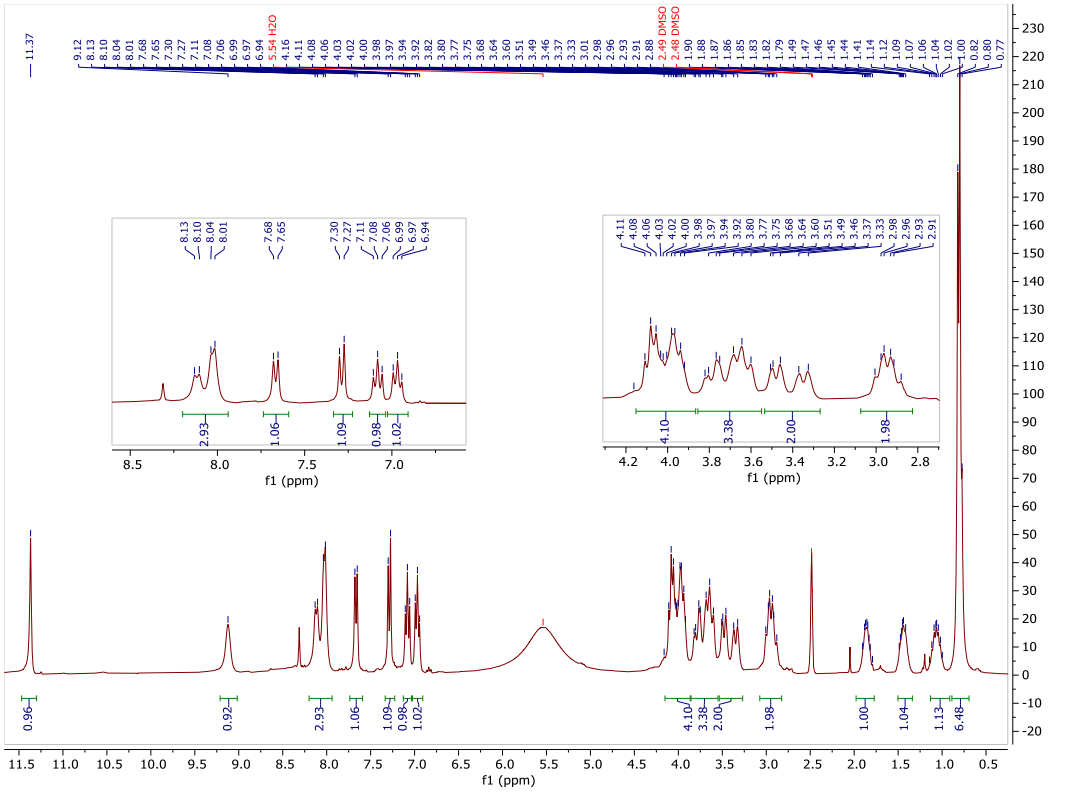
HPLC (gradient A): $\mathbf{t_R}$ = 11.1 min; $\boldsymbol{\lambda_{max}}$ 290 nm

^{1}H NMR (400 MHz, DMSO-d_6) δ 7.70 – 7.61 (m, 1H), 7.39 – 7.27 (m, 1H), 7.26 – 7.11 (m, 1H), 7.11 – 7.01 (m, 1H), 6.96 – 6.88 (m, 0.3H), 5.08 – 4.97 (m, 1H), 4.65 – 4.44 (m, 1H), 4.41 – 4.18 (m, 3H), 4.16 – 3.71 (m, 7H), 3.63 – 3.53 (m, 1H), 3.43 (dd, *J* = 13.7, 4.7 Hz, 1H), 3.19 (ddd, *J* = 35.0, 14.2, 7.6 Hz, 1H), 2.79 – 2.46 (m, 2H), 2.40 – 2.26 (m, 1H), 2.23 – 2.12 (m, 1H), 1.95 – 1.79 (m, 1H), 1.68 – 1.50 (m, 1H), 1.18 (dt, *J* = 14.4, 8.4 Hz, 1H), 1.00 – 0.86 (m, 6H).

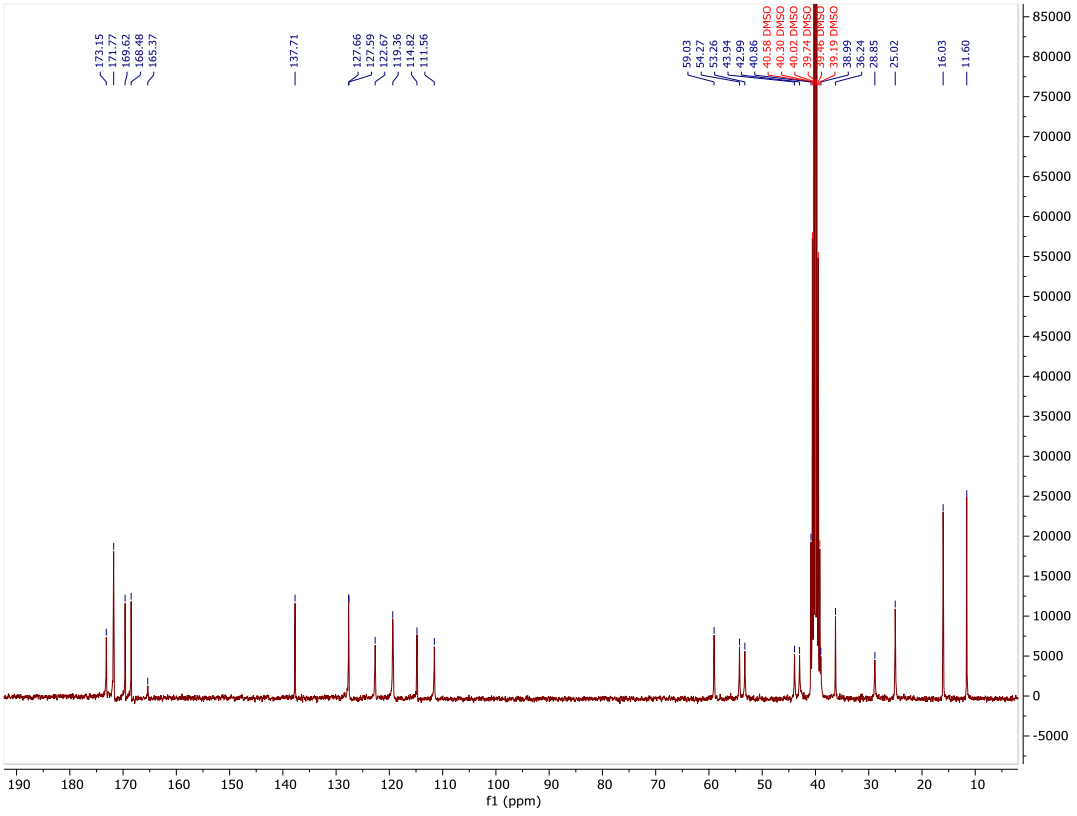
^{13}C NMR (101 MHz, DMSO-d_6) δ 11.60, 16.03, 25.02, 28.85, 36.24, 38.99, 40.86, 42.99, 43.94, 53.26, 54.27, 59.03, 111.56, 114.82, 119.36, 122.67, 127.59, 127.66, 137.71, 165.37, 168.48, 169.62, 171.77, 173.15.

HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{24}H_{32}N_6O_6S$, 532.2108; found 532.2104

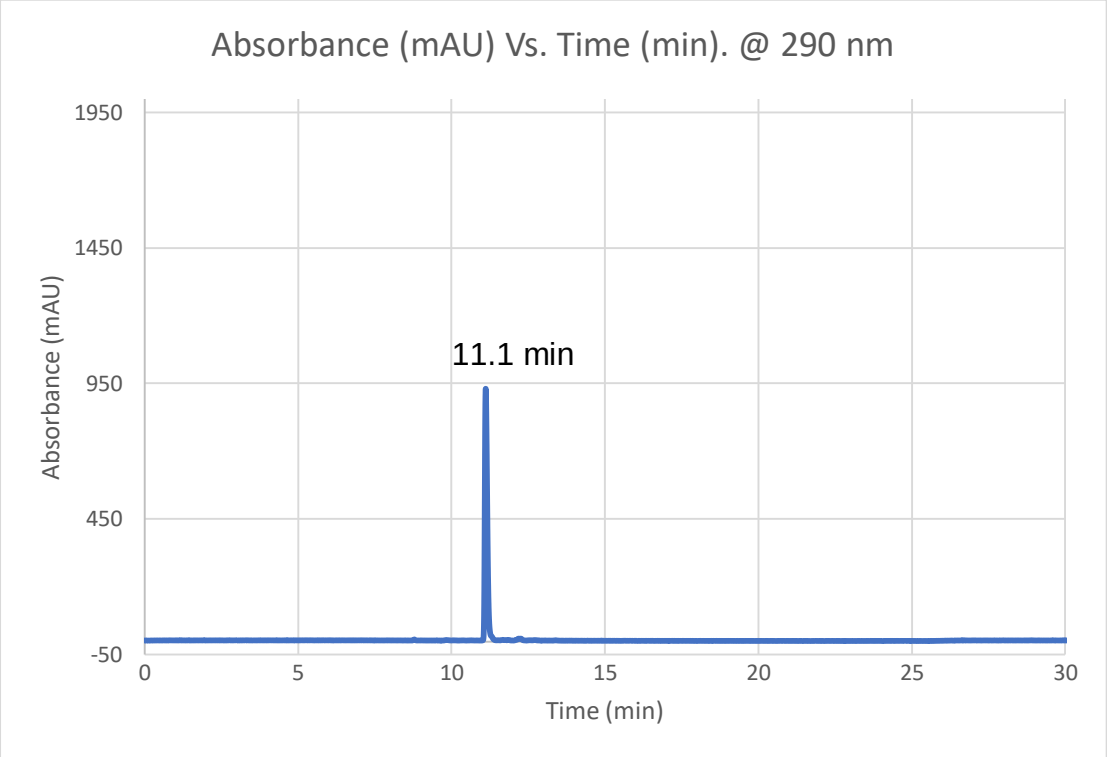
^{1}**H-NMR** of **19**, 400 MHz, $(CD_3)_2SO$



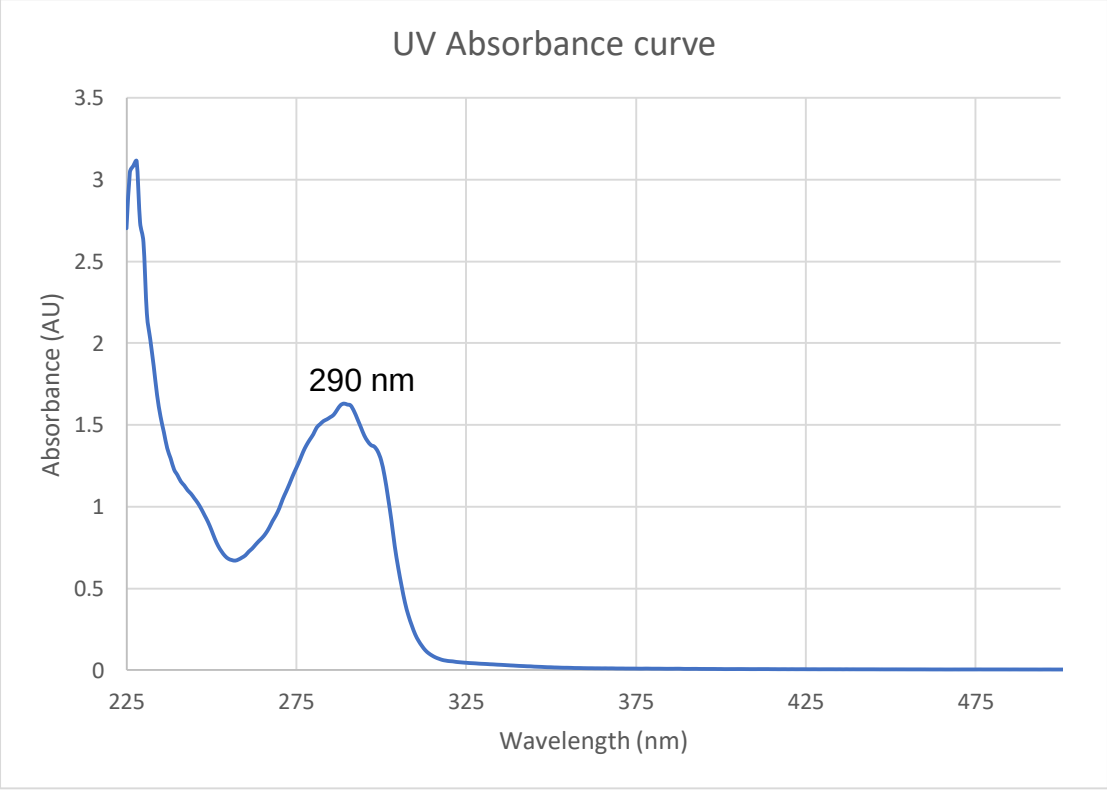
^{13}**C-NMR** of **19**, 101 MHz, $(CD_3)_2SO$

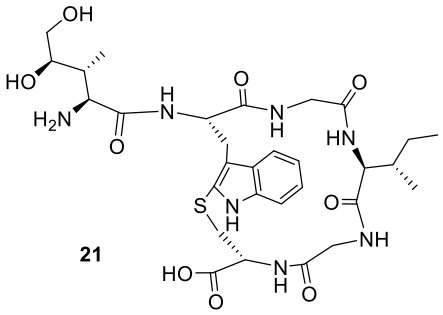


HPLC chromatogram for pure **19**, 290 nm



UV Absorbance curve for **19**





21

Synthesis of the monocyclic di-deoxy-hexapeptide, [5+1] (21).

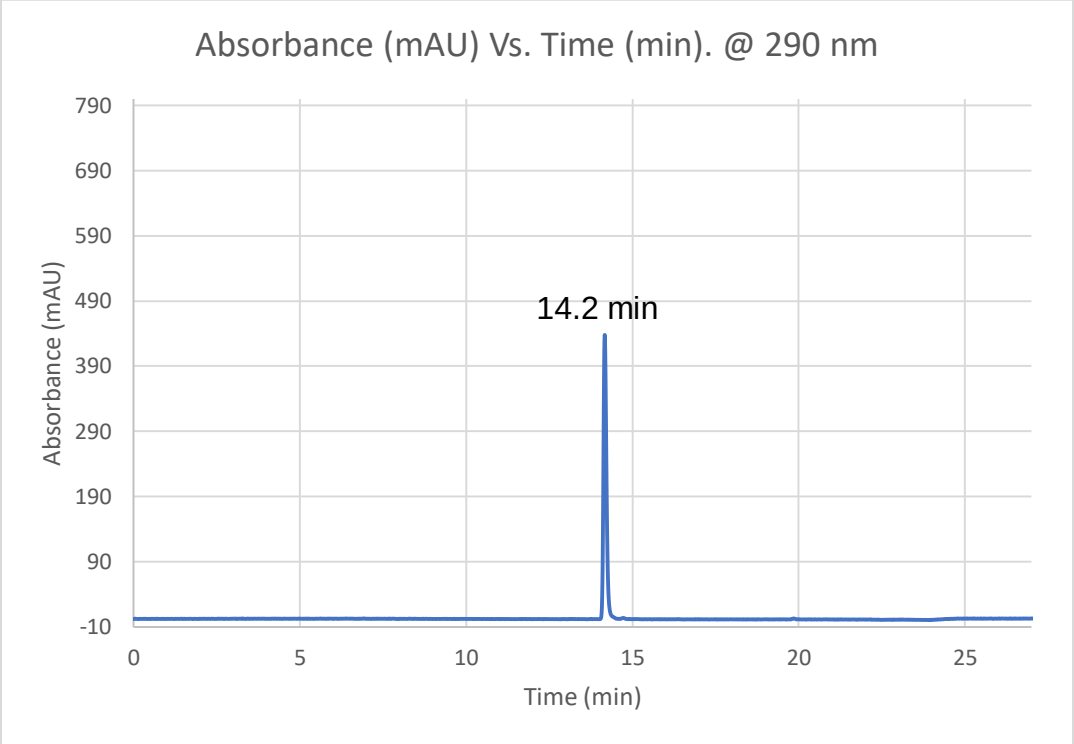
Monocyclic di-deoxy-pentapeptide **19** (30 mg, 56.4 µmol, 1 eq) and (2*S*,3*R*,4*R*)-O^{γ},O^{δ}-bis-TBS-N^{α}-Fmoc-dihydroxyisoleucine-OSu (**20**) (60 mg, 84.5 µmol, 1.5 eq) were dissolved in 400 µL DMF. To this solution was added DIPEA (13 µL, final pH 9). The resulting mixture was let stand at room temperature for 24 hours. After completion of the coupling (HPLC, MS), 28 µL of Et_2NH was added to the reaction mixture, and the reaction was stirred for another 2 hours. To the reaction mixture was added 400 µL of 1M TBAF in THF and 10 µL of HOAc, and the resulting solution was let stand at room temperature for 2 hours. The reaction was further acidified to pH~3 with 1 M aq. HCl, and the solvent was removed *in vacuo*. The crude octapeptide was resuspended in 0.1% formic acid in H_2O/MeCN (1:4) and purified using C18 SEP-PAK (isolated yield: 12 mg, 32% over 3 steps).

Note: Fully protected, NHS-ester of DHIle (**20**) was prepared according to *JACS*, **2018**, *140* (21), 6513-6517.

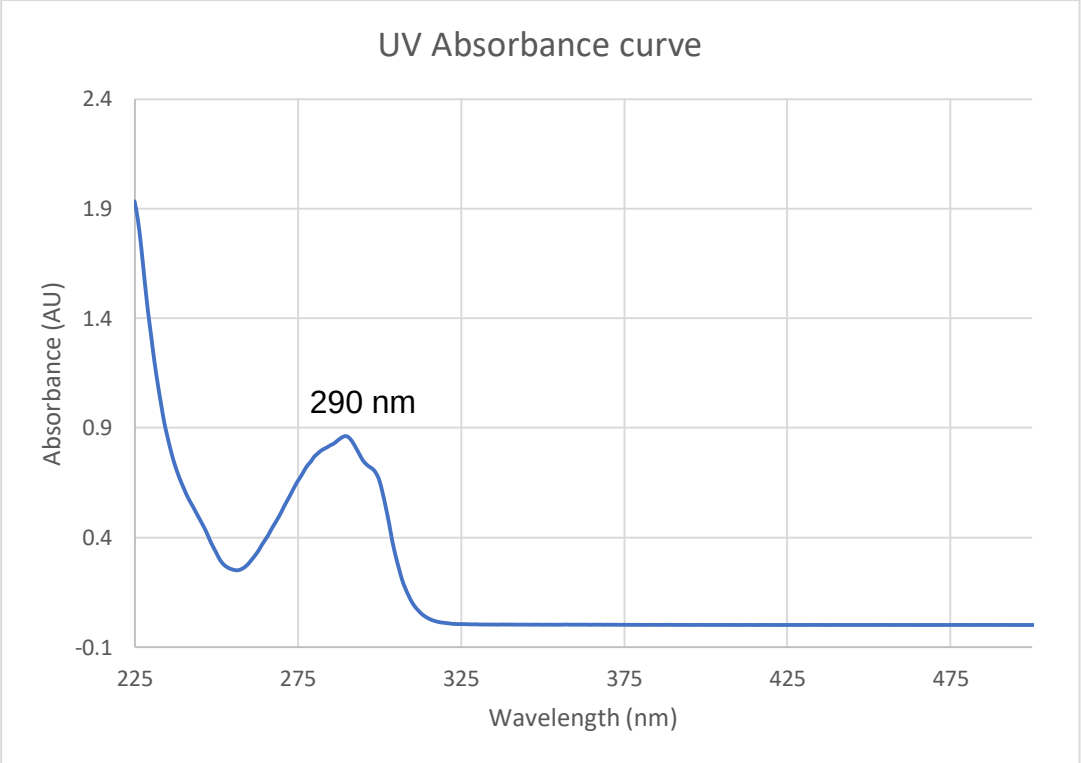
HPLC (gradient B): $\mathbf{t_R}$ = 14.2 min; $\boldsymbol{\lambda_{max}}$ 290 nm

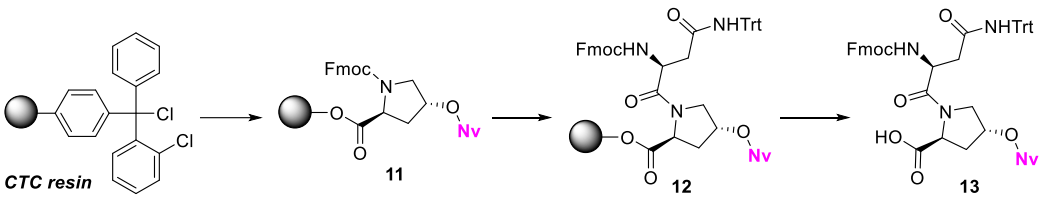
HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{30}H_{44}N_7O_9S$, 678.2916; found 678.2913

HPLC chromatogram for pure **21**, 290 nm



UV Absorbance curve for **21**





Synthesis of N^{α}-Fmoc-Asn(Trt)-Hyp(ONv)-OH dipeptide (13).

Fmoc-Hyp(ONv)-OH (**10**) was loaded on the 2-chlorotrityl chloride (CTC) resin according to the following protocol. To a flame-dried flask was added CTC resin (450 mg, 1.2 mmol/g, 200-400 mesh), which was then suspended in dry CH_2Cl_2 (5 mL). To this flask was added Fmoc-Hyp(ONv)-OH (**10**) (450 mg, 0.82 mmol) and DIPEA (0.36 mL, 2.07 mmol). The reaction was stirred at room temperature and under Argon overnight and transferred to a Zeba spin column. The resin was washed three times with DMF and DCM (5 mL, each). Unreacted sites of the resin were capped by applying a solution of CH_2Cl_2:MeOH:DIPEA (5 mL of an 80:15:5 mixture, 20 min), and then washed with CH_2Cl_2 (3 x 5 mL) then DMF (3 x 5 mL) then CH_2Cl_2 (3 x 5 mL) again. The resin was dried on *in vacuo* over P_2O_5 to remove residual solvent. Resin loading was determined using manufacturer's protocols. Briefly, a weighed amount of resin was treated with a 2% solution of DBU in DMF for 30 minutes. The solution was diluted and the UV absorbance of the liberated dibenzofulvene was measured at 304 nm, with an absorption coefficient of ε_{304}=7624 $M^{-1}cm^{-1}$. The measured resin loadings generally ranged from 0.6-0.7 mmol/g (in this case, the loading was measured to be **0.65 mmol/g**). Then, unreacted sites of the resin were capped with a solution of CH_2Cl_2:MeOH:DIPEA (5 mL of a 80:15:5 mixture, 20 min), and then washed with CH_2Cl_2 (3 x 5 mL) then DMF (3 x 5 mL) then CH_2Cl_2 (3 x 5 mL) again.

The N-terminal Fmoc protecting group of **11** was removed by washing with 20% piperidine in DMF (5 mL for 5 min, 5 mL for 10 min). Following deprotection, the resin was washed with DMF (3 x 5 mL), followed by CH_2Cl_2 (3 x 5 mL) and again with DMF (3 x 5 mL). Then, a coupling cocktail consisting of N^{α}-Fmoc-Asn(Trt)-OH (866 mg, 1.46 mmol, 5 eq), PyBOP (758 mg, 1.46 mmol, 5 eq) and DIPEA (0.5 mL, 2.92 mmol, 10 eq) in DMF (6 mL) was applied to the Fmoc-deprotected **11**. The resulting suspension in a Zeba spin column was gently shaken on a vortexer for 1-2 h. The solvent was drained, and the resin was washed with CH_2Cl_2 (3 x 8 mL) then DMF (3 x 8 mL) then CH_2Cl_2 (3 x 8 mL) again. As the Kaiser test was positive, the coupling cycle was repeated once more. Following the second coupling reaction, the unreacted amine sites were capped with Ac_2O/collidine/EtOAC (8 mL, 1:2:2 v/v/v). Then, the resin-bound dipeptide (**12**) was cleaved from the resin using the following procedure: the air-dried resin was transferred to a 25-mL RBF. To this was added 8 mL of HFIP/DCM (1:4) and the resulting suspension was gently stirred at RT for 5 minutes. Then, the resin was filtered off and the filtrate was concentrated under reduced pressure to yield the crude product (**13**) as an off-white solid

(487 mg). Based on the TLC analysis, the crude product was sufficiently pure to be used in the next step without further purification.

Note: during this synthesis, all flasks/vials were protected from light to avoid the photodecomposition of the photolabile starting material/products.

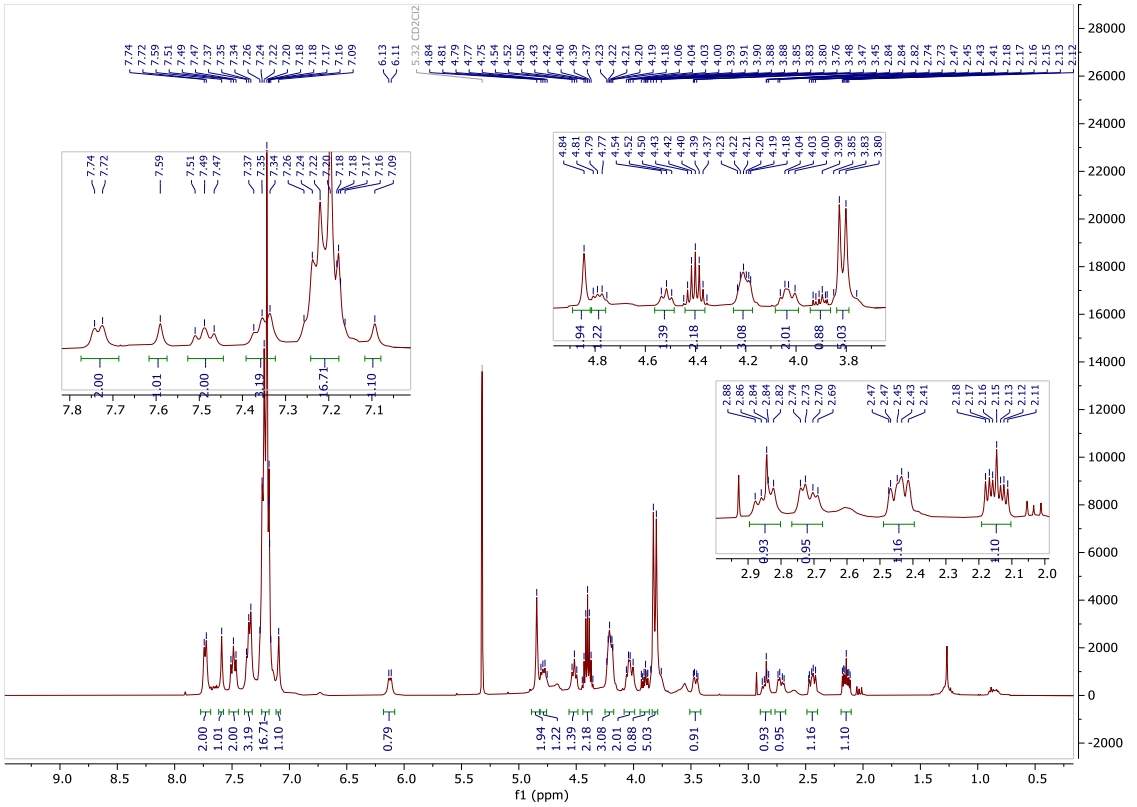
TLC (MeOH:DCM 10:90 v/v): R_f = 0.37

^{1}H NMR (400 MHz, Methylene Chloride-d_2) δ 7.73 (d, *J* = 7.4 Hz, 2H), 7.59 (s, 1H), 7.49 (t, *J* = 8.8 Hz, 2H), 7.40 – 7.31 (m, 3H), 7.25 – 7.17 (m, 16H), 7.09 (s, 1H), 6.12 (d, *J* = 8.5 Hz, 1H), 4.84 (s, 2H), 4.78 (q, *J* = 7.1 Hz, 1H), 4.52 (t, *J* = 8.3 Hz, 1H), 4.40 (hept, *J* = 6.2 Hz, 2H), 4.26 – 4.16 (m, 3H), 4.10 – 3.96 (m, 2H), 3.95 – 3.83 (m, 1H), 3.81 (d, *J* = 10.5 Hz, 5H), 3.46 (dd, *J* = 11.7, 4.0 Hz, 1H), 2.90 – 2.80 (m, 1H), 2.71 (dd, *J* = 15.2, 6.1 Hz, 1H), 2.44 (dd, *J* = 13.5, 8.1 Hz, 1H), 2.15 (ddd, *J* = 13.4, 8.7, 4.6 Hz, 1H).

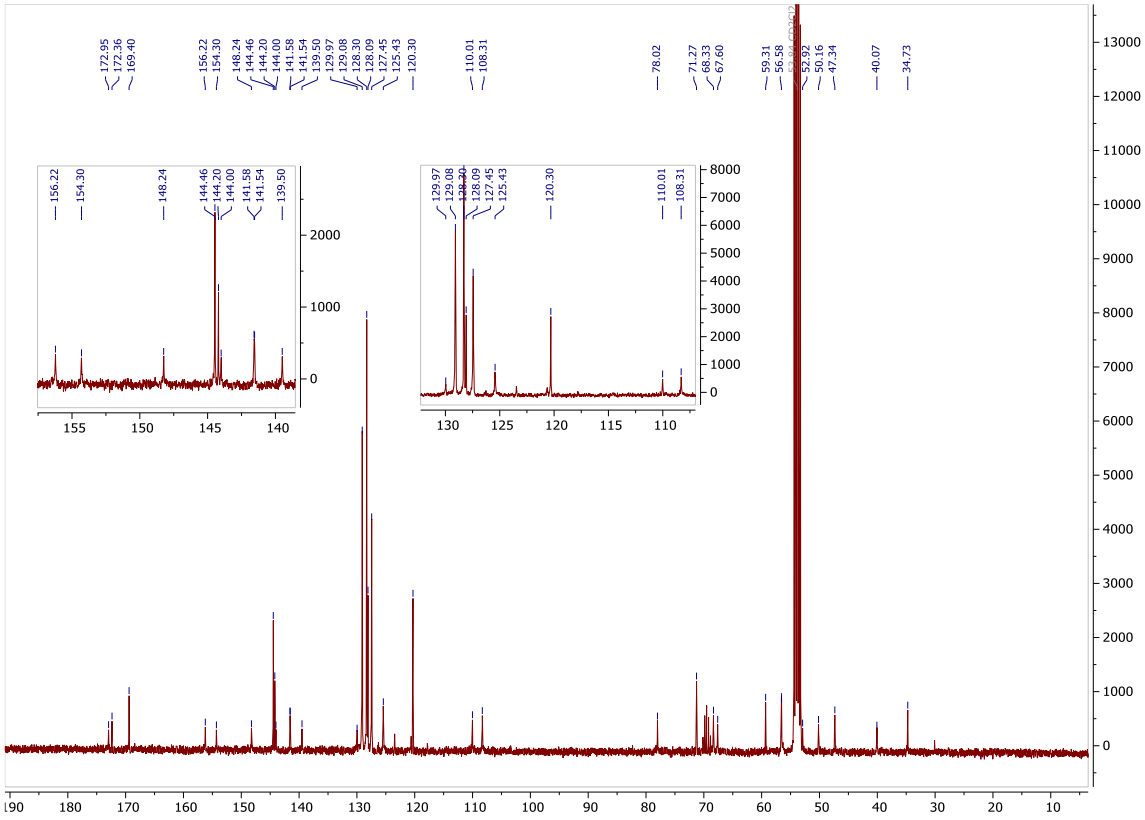
^{13}C NMR (101 MHz, Methylene Chloride-d_2) δ 172.95, 172.36, 169.40, 156.22, 154.30, 148.24, 144.46, 144.20, 144.00, 141.58, 141.54, 139.50, 129.97, 129.08, 128.30, 128.09, 127.45, 125.43, 120.30, 110.01, 108.31, 78.02, 71.27, 68.33, 67.60, 59.31, 56.58, 52.92, 50.16, 47.34, 40.07, 34.73.

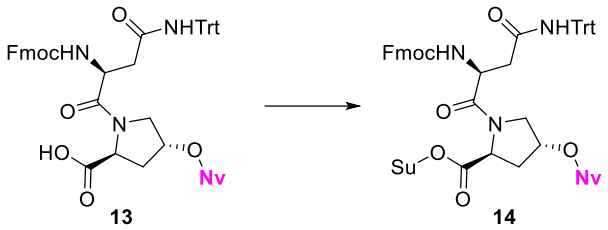
HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{52}H_{49}N_4O_{11}$, 905.3392; found 905.3390

^{1}**H-NMR** of **13**, 400 MHz, CD_2Cl_2



^{13}**C-NMR** of **13**, 101 MHz, CD_2Cl_2

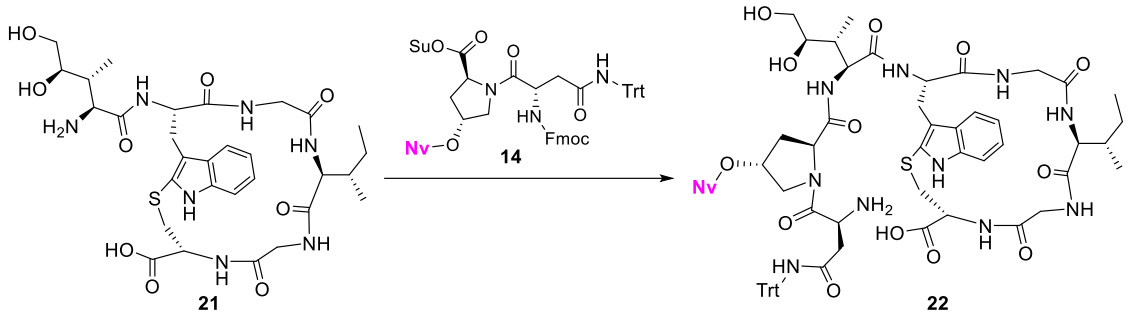




Synthesis of the NHS-ester of *N*$^{\alpha}$-Fmoc-Asn(Trt)-Hyp(ONv)-OH dipeptide (14).

The starting material (**13**) (20 mg, 22.1 µmol, 1 eq), EDC.HCl (6.8 mg, 33.2 µmol, 1.5 eq) and HOSu (6.4 mg, 55.2 µmol, 2.5 eq) were dissolved in dry DMF (200 µL) in an Eppendorf tube. The resulting mixture was protected from light and shaken on a vortexer for 3 hours at RT. The reaction mixture was diluted with EtOAc (400 µL) and washed with 5% aq. citric acid (800 µL), 5% aq. $NaHCO_3$ (800 µL) and brine. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to yield the crude NHS-ester **14** as a white foamy solid. The crude product (~20 mg) was used in the next step without further purification.

TLC (MeOH:DCM 6:94 v/v): R_f = 0.65



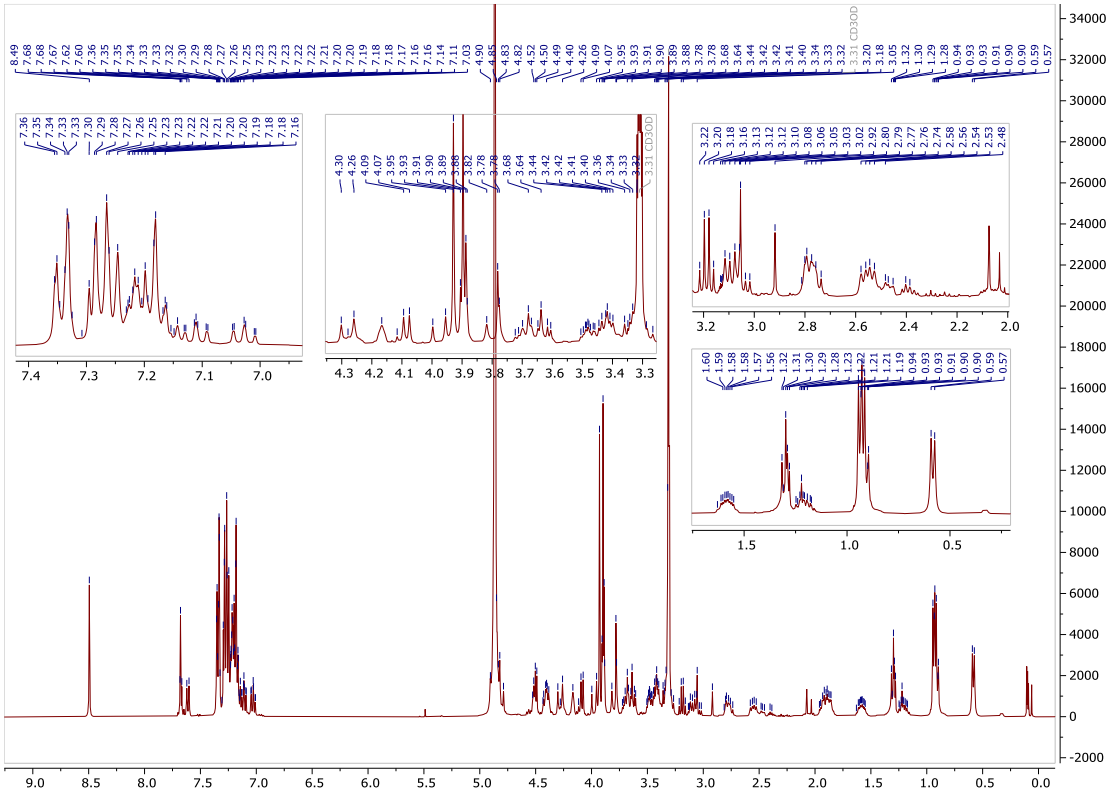
Synthesis of the monocyclic octapeptide (22).

Monocyclic hexapeptide **21** (4 mg, 6 µmol, 1 eq), **14** (crude, 20 mg, 20 µmol, 3.4 eq) and DIPEA (6 µL) were dissolved in DMF (400 µL) in an Eppendorf tube. The reaction was protected from light and let stand at RT for 18 hours. Upon completion of the coupling (MS), Et_2NH (6.4 µL) was added to the reaction mixture. After 2 hours, 460 µL of 0.1% formic acid in H_2O/ACN (330 µL:130 µL) was added to the reaction. The resulting solution was directly purified by HPLC. The purified fractions were lyophilized to yield the pure product (**22**) (6.95 mg, 5.18 µmol, 86%).

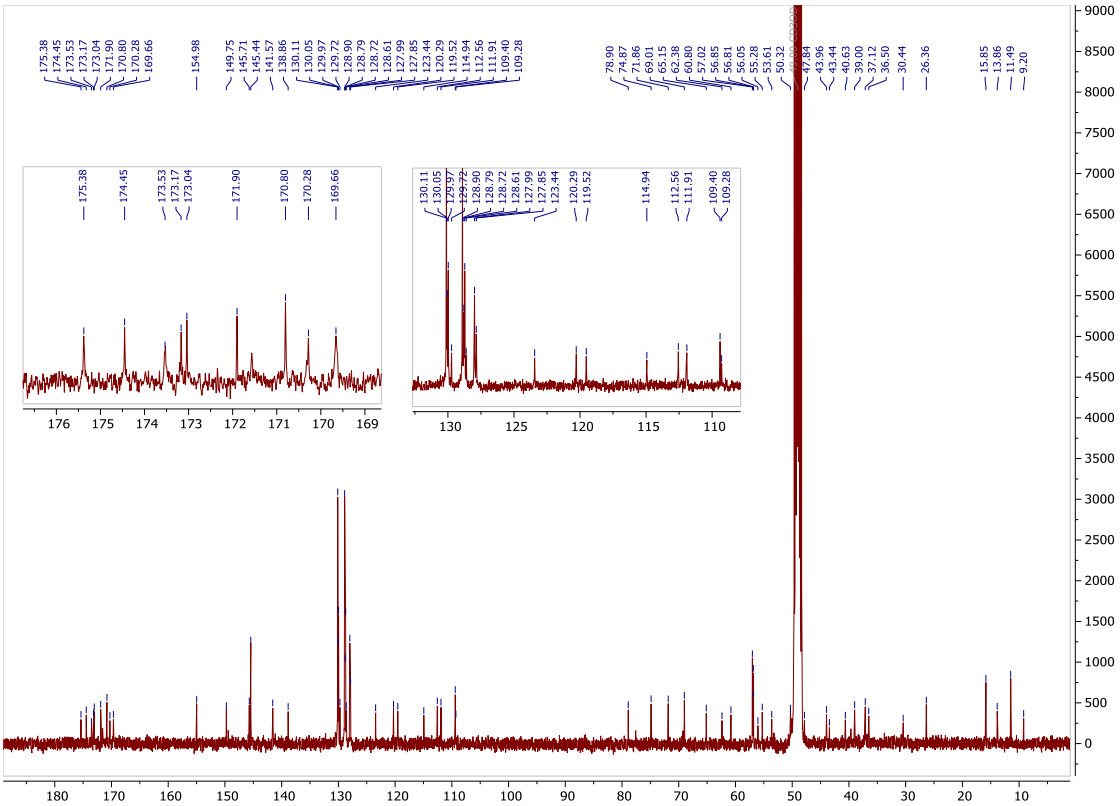
HPLC (gradient C): $\mathbf{t_R}$ = 20.1 min; $\boldsymbol{\lambda_{max}}$ 290 nm

HRMS-ESI (*m/z*): $[M+H]^+$ calcd. for $C_{67}H_{80}N_{11}O_{17}S$, 1342.5449; found 1342.5438

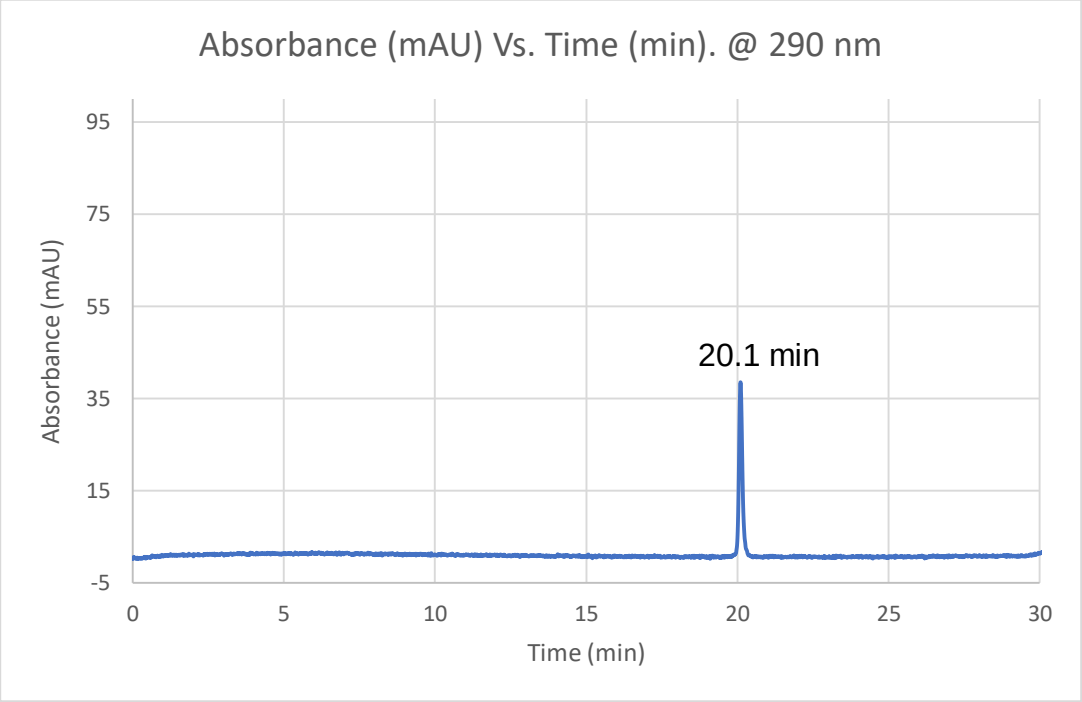
^{1}H-NMR of **22**, 400 MHz, CD_3OD



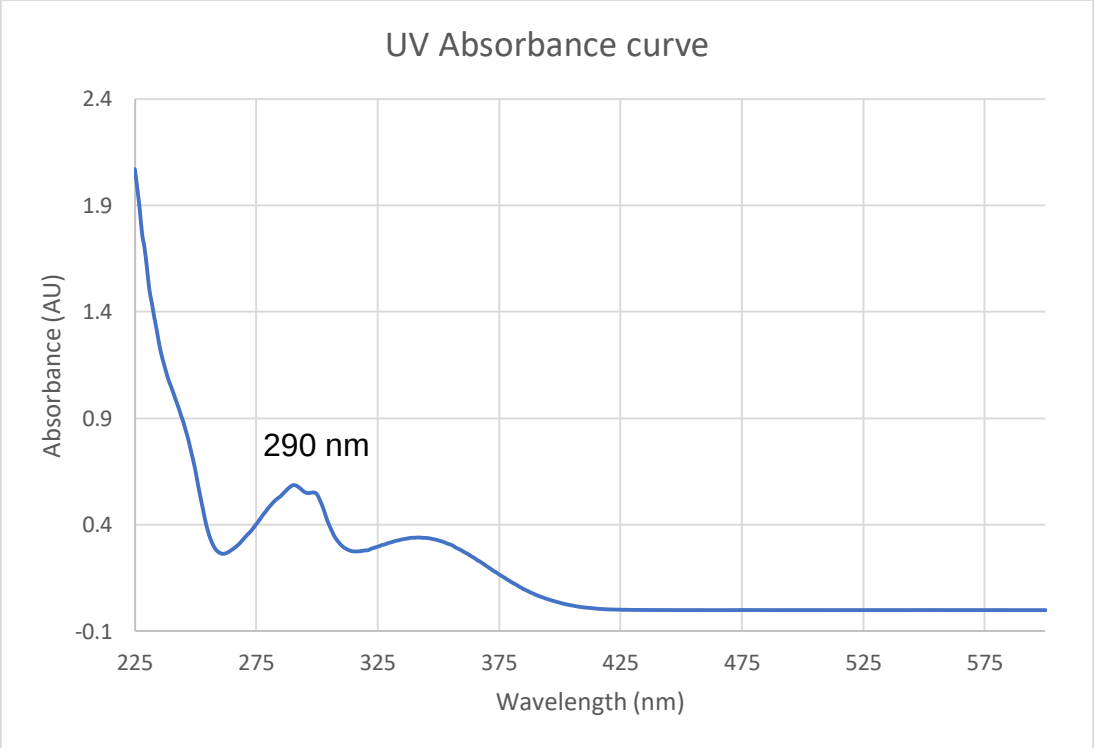
^{13}C-NMR of **22**, 101 MHz, CD_3OD

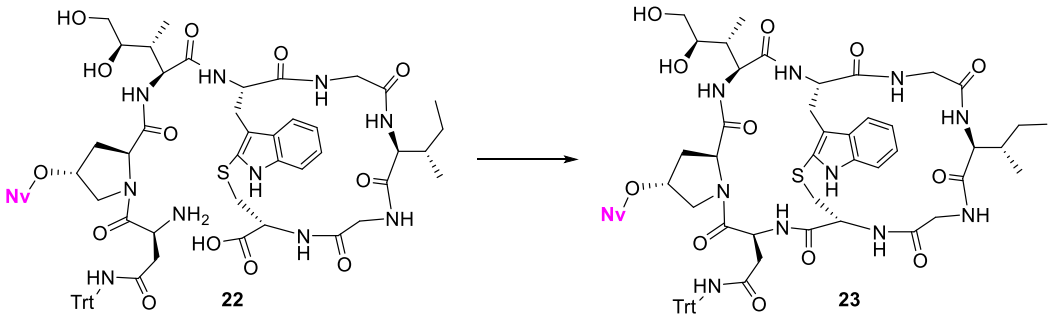


HPLC chromatogram for pure **22**, 290 nm



UV absorbance curve for **22**





Macrolactamization for the synthesis of Trt-protected bicyclic octapeptide (23).

Monocyclic octapeptide **22** (6.95 mg, 5.18 µmol, 1 eq), PyBOP (23.1 mg, 44.4 µmol, 8.5 eq) and DIPEA (6.21 µL) were dissolved in *N*,*N*-dimethyl acetamide (DMA) (1.5 mL) in a 15-mL falcon tube. The resulting mixture was protected from light and shaken on a vortexer at RT for 16 hours. The reaction mixture was diluted with 1.5 mL of 0.1% formic acid in H_2O/ACN (1.1 mL:400 µL) and directly purified by HPLC (1.76 mg, 1.33 µmol, 26%). It is noteworthy that the actual yield (based on HPLC) was noticeably larger than the isolated yield, possibly due to unwanted retention of the greasy product on the HPLC column.

Note: PyBOP and DIPEA were used as stock solutions as follow:

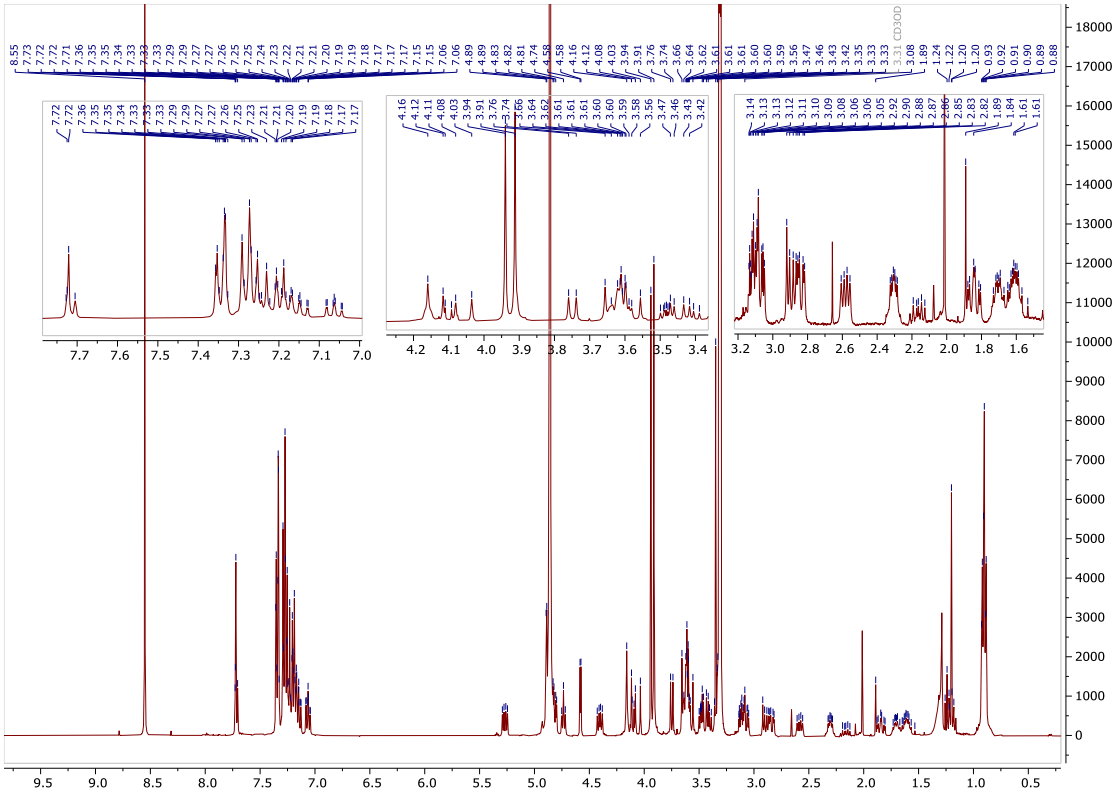
PyBOP stock solution: 34.4 mg of PyBOP in 1 mL DMA. Added 665.5 µL to the reaction.

DIPEA stock solution: 9.2 µL of DIPEA in 190.8 µL DMA. Added 100 µL to the reaction.

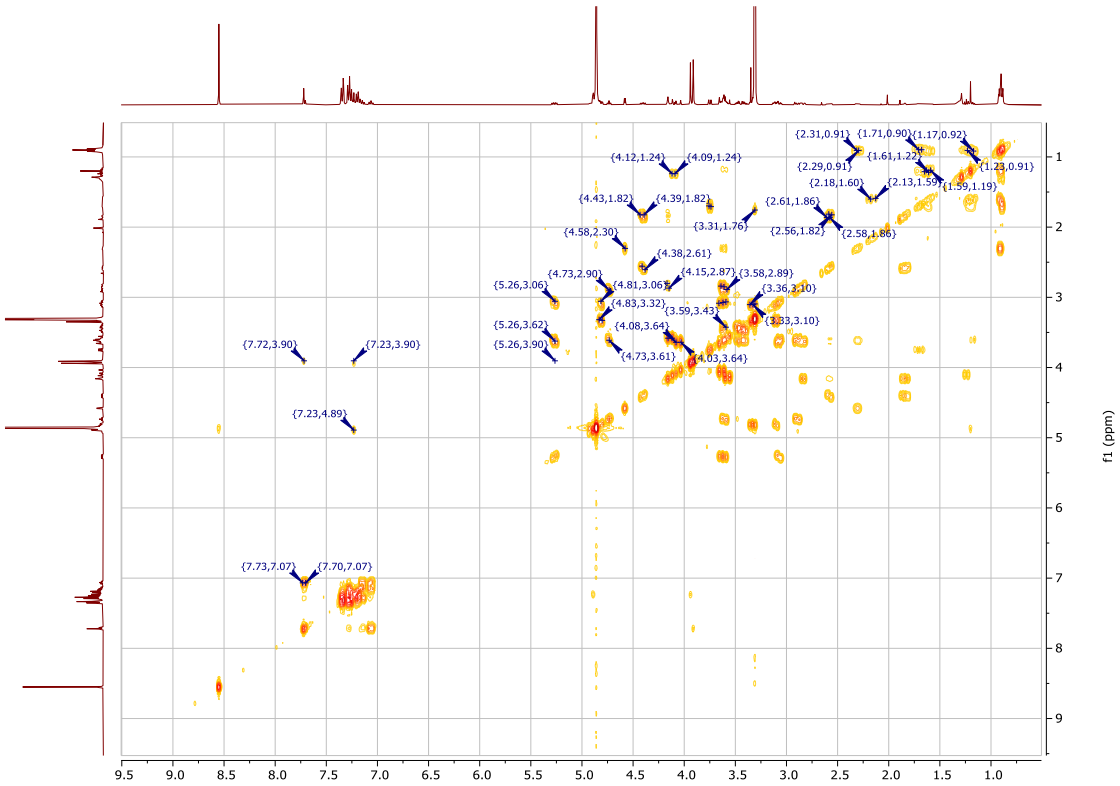
HPLC (gradient C): $\mathbf{t_R}$ = 29.2 min; $\boldsymbol{\lambda_{max}}$ 291 nm

HRMS-ESI (*m/z*): $[M+Na]^+$ calcd. for $C_{67}H_{77}N_{11}NaO_{16}S$, 1346.5168; found 1346.5154

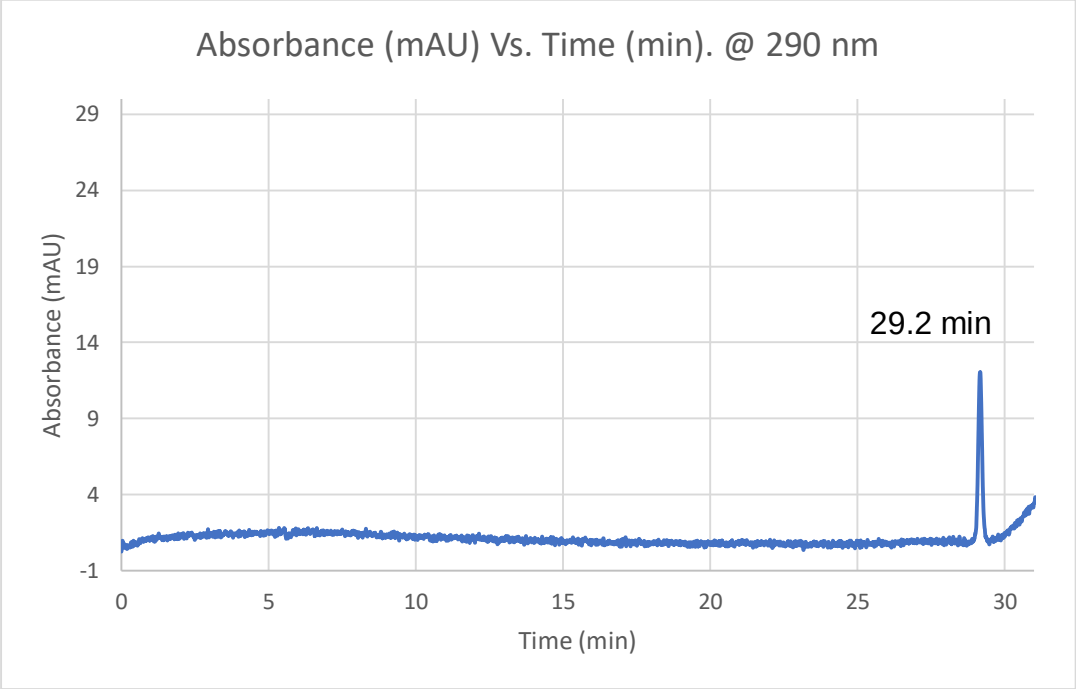
^{1}H-NMR of **23**, 400 MHz, CD_3OD



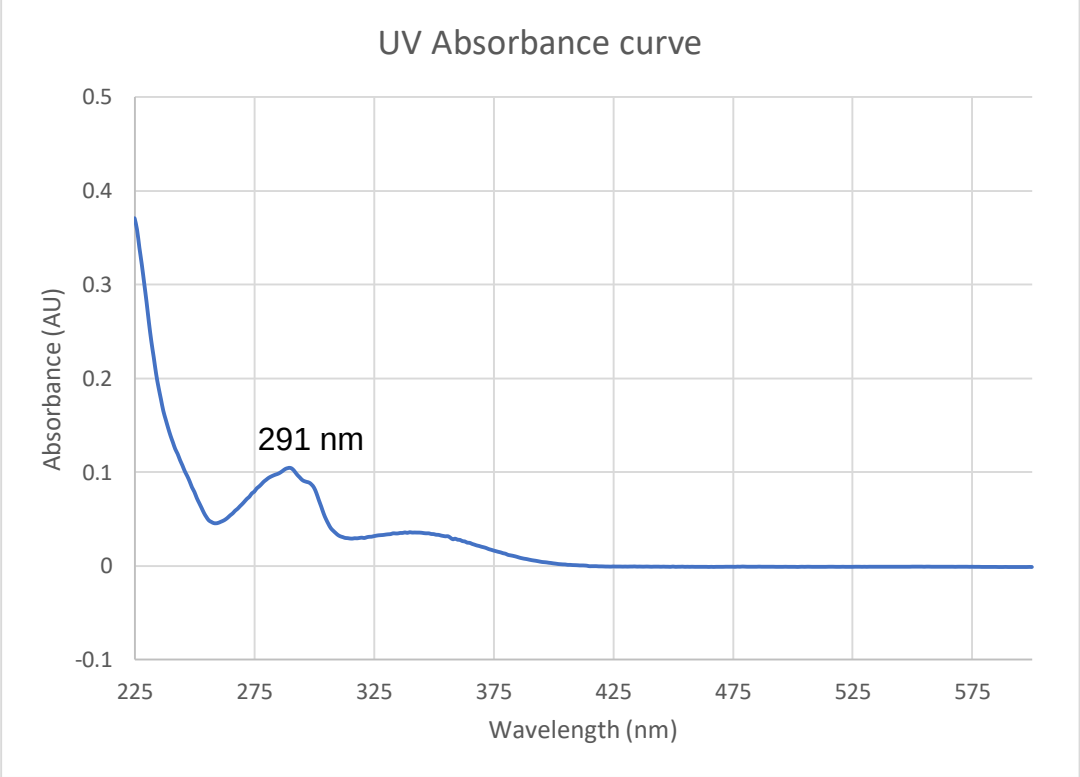
COSY45 of **23**, 400 MHz, CD_3OD

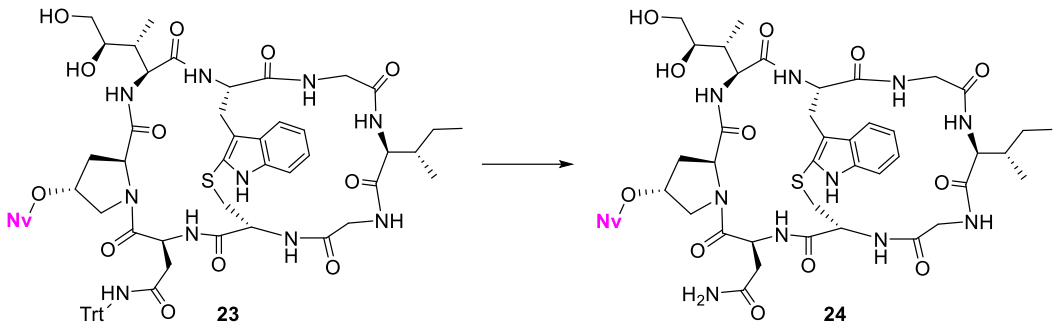


HPLC chromatogram for pure **23**, 290 nm



UV absorbance curve for **23**





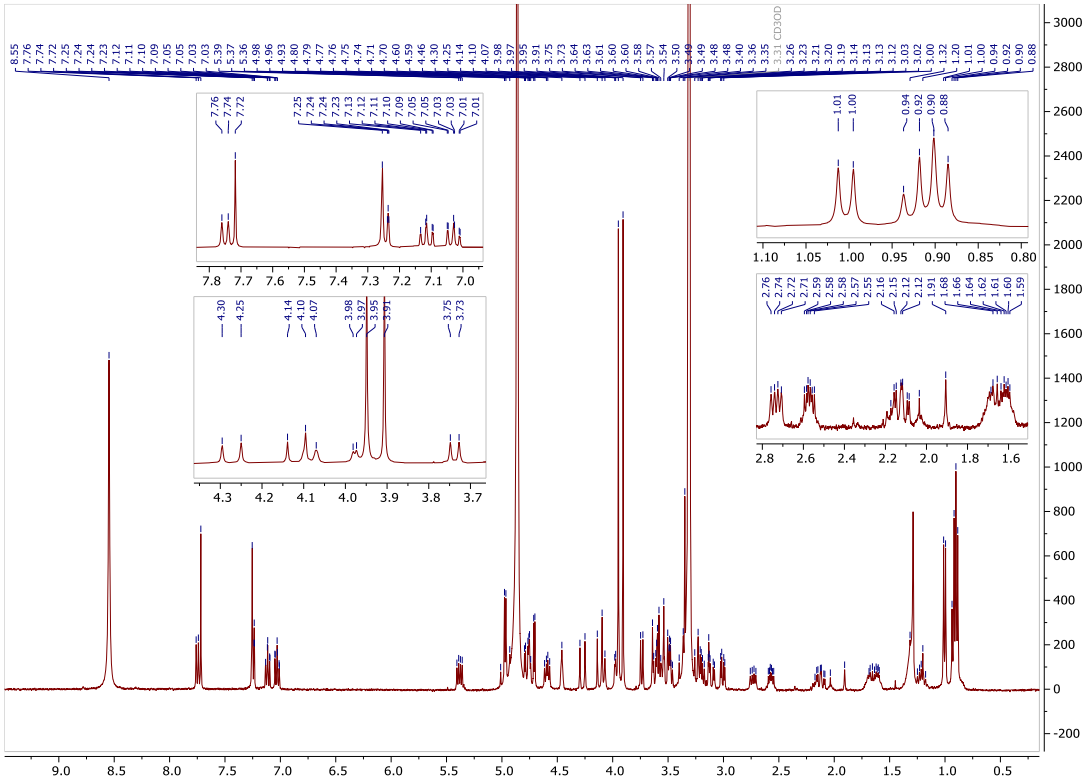
Trt deprotection for the synthesis of bicyclic octapeptide, photolabile di-deoxy-α-amanitin (Ama-Flash, 24).

To the Trt-protected bicyclic octapeptide **23** (1.33 µmol) in an Eppendorf tube was added TFA/DCM (800 µL:800 µL). The resulting solution was protected from light and let stand at RT for 1 hour. Then, TIPS/H_2O (32 µL:32 µL) were added and the reaction continued for another hour. The volatiles were removed under reduced pressure and the residue was dissolved in 1.2 mL of 0.1% formic acid in H_2O/ACN (2:1). The resulting solution was directly purified by HPLC to yield the pure photolabile di-deoxy-α-amanitin (761 nmol, 57%).

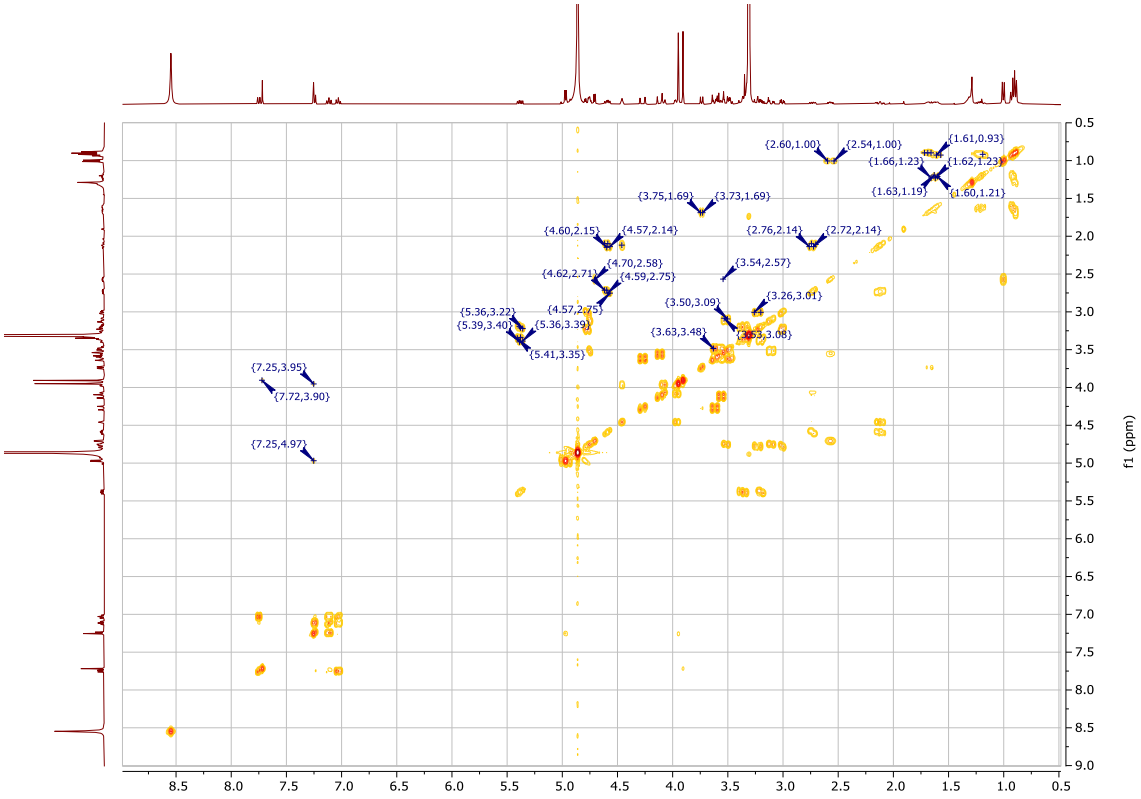
HPLC (gradient C): $\mathbf{t_R}$ = 17.1 min; $\boldsymbol{\lambda_{max}}$ 291 nm

HRMS-ESI (*m/z*): $[M+Na]^+$ calcd. for $C_{48}H_{63}N_{11}NaO_{16}S$, 1104.4073; found 1104.4062

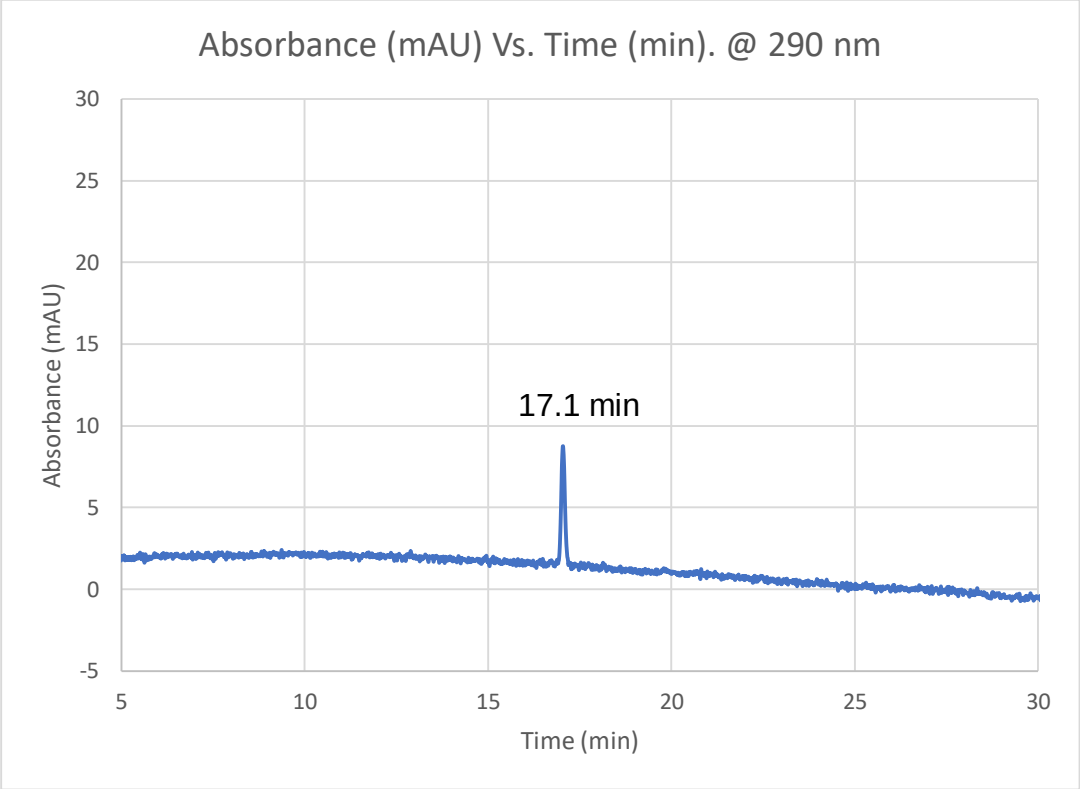
^{1}**H-NMR** of **24**, 400 MHz, CD_3OD



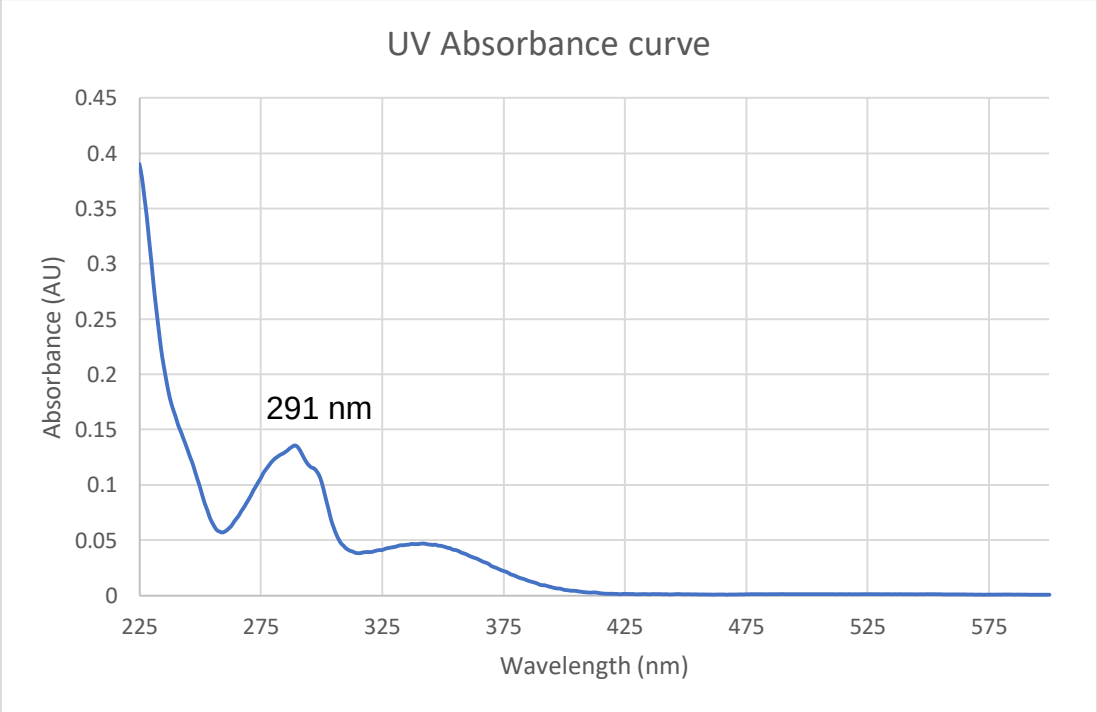
COSY45 of **24**, 400 MHz, CD_3OD



HPLC chromatogram for pure **24**, 290 nm



UV absorbance curve for **24**



Kinetic studies: photo-deprotection of Ama-Flash (24) in solution

To investigate the rate of deprotection for the nitroveratryl-protected dideoxy-α-amanitin (**24**), 30 nmol of **24** was dissolved in 1 mL of PBS (with 1% DMSO), at a final concentration of 30 µM. The resulting solution was placed in a UV cuvette and was subjected to irradiation with light (λ=366 nm). At various timepoints (see below), aliquots (100 µL) were taken out and analyzed by HPLC to determine the percentage of deprotection. The set-up for this experiment is shown below.

UV irradiation: hand-held UV lamp, 115 V, 60 Hz, 0.16 Amps

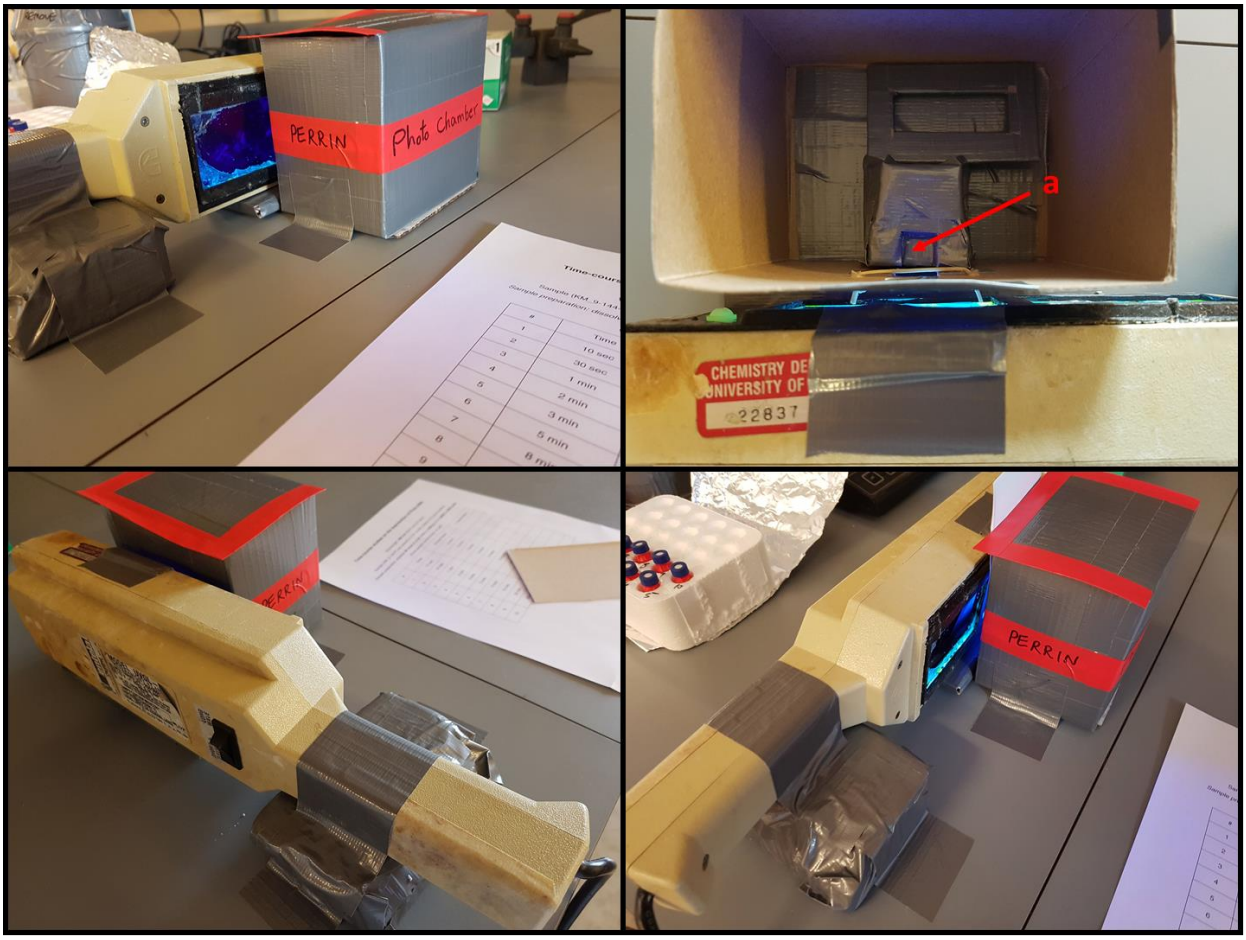


Figure S1. Set-up for UV irradiation of Ama-Flash (**24**) to determine the deprotection half-life. The cuvette containing the solution described above was positioned in holder (**a**) and was subjected to UV light (366 nm) from the side, while the surroundings were covered form other sources of light.

#	Time (min)	Ama-Flash (**24**) area under the peak	Dideoxy-α-AMA area under the peak	% deprotected
1	0	472	0	0
2	0.5	458	negligible	3
3	1	433	44	8
4	2	368	82	22
5	3	321	120	32
6	4	279	149	41
7	5	246	169	48
8	8	168	221	64
9	15	62	282	87
10	45	0	345	100

Table S1. Percentage of photo-deprotection vs. irradiation time for Ama-Flash.

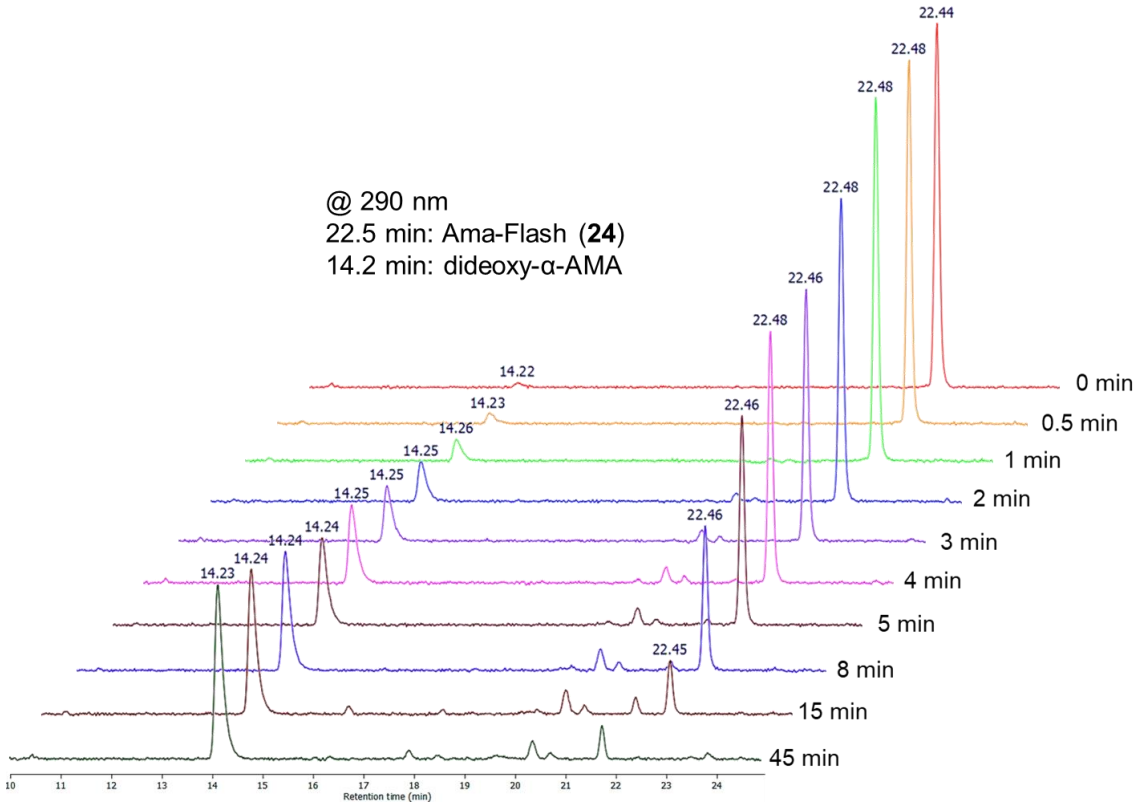


Figure S2. HPLC chromatograms for the photo-deprotection of Ama-Flash. HPLC gradient: A

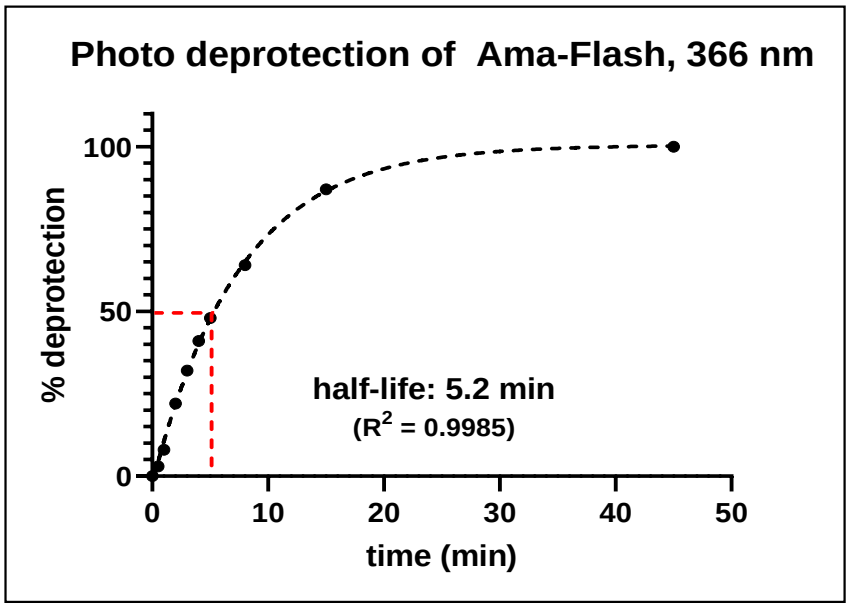


Figure S3. Half-life curve for the uncaging of Ama-Flash (**24**).

General procedures

To assay cell viability using MTT assay, CHO cells were trypsinized and seeded on to 96-well plates*. The cells, suspended in 50 µL of MEM_{α} media, were allowed to form a monolayer over a period of 24 hours. The compounds analyzed were dissolved in 50 µL of 2% DMSO-containing MEM_{α} media and added to each well to achieve the desired concentration. The final volume/well in each assay was 100 µL unless otherwise stated and contained a final concentration of 1% DMSO in MEM_{α} media. Media, 1% DMSO, and blank controls were run in parallel and all readings were normalized against the controls. The cells were incubated at 37 °C for a specified duration of time in a humidified, 5% CO_2 atmosphere. Following a specified time period, 50 µL of MTT reagent solution (2.5mg/mL PBS buffer) were added to each well and the cells were incubated for another 3 hours at 37 °C in a humidified, 5% CO_2 atmosphere. The media was then aspirated and the well contents were dissolved in 100 µL of filtered DMSO. The 96-well plate was shaken for 5 minutes at 50 rpm and the absorbance reading was recorded at 590 nm using a 96-well plate reader. IC_{50} (drug concentration required to inhibit cell viability by 50%) values were calculated using GraphPad Prism 7 software with non-linear 3-parameter regression fit to the following equation: Y=Bottom + (Top-Bottom)/(1+10^((X-LogIC50))) with Hill slope of -1.0. Experiments were performed in triplicate, and the error bars were calculated as the standard error of the mean.

*cell count varied depending on the time duration of the assay, see each assay for exact cell count used.

Cell viability studies against CHO cells

Cell viability assay of caged Ama-Flash (24) against CHO cells (MTT)

To assess the toxicity of the caged amanitin (Ama-Flash, **24**) against CHO cells (5,000 cells/well), these cells were treated with varying concentrations of Ama-Flash, ranging from 0.1563 µM to 20 µM, and their viability was measured using an MTT colorimetric assay. As a control, the same assay was performed on α-amanitin (78 nM to 20 µM).

α-Amanitin

OD reading at 570nm				Mean	Net OD	% of alive cells
Blank	0.0351	0.033	0.0332	0.0338	0.0000	
medium ctrl	1.3481	1.2556	1.2801	1.2946	1.2608	100
0.078µM	1.0622	1.0222	1.0104	1.0316	0.9978	79
0.1563µM	0.9868	0.9942	0.8721	0.9510	0.9172	73
0.3125µM	0.6105	0.6146	0.6498	0.6250	0.5912	47
0.625µM	0.3768	0.3609	0.3703	0.3693	0.3355	27
1.25µM	0.1872	0.1853	0.1947	0.1891	0.1553	12
2.5µM	0.0895	0.0885	0.1064	0.0948	0.0610	5
5µM	0.0959	0.1036	0.1062	0.1019	0.0681	5
10µM	0.0833	0.0708	0.081	0.0784	0.0446	4
20µM	0.1018	0.0644	0.0881	0.0848	0.0510	4

Ama-Flash (24)

OD reading at 492nm				Mean	Net OD	% of alive cells
Blank	0.0348	0.0341	0.0337	0.0342	0.0000	
medium ctrl	1.2365	1.3047	1.3625	1.3012	1.2670	92
DMSO ctrl	1.1457	1.166	1.3014	1.2044	1.1702	100
0.1563µM	1.1444	1.1886	1.2618	1.1983	1.1641	99
0.3125µM	1.0636	1.2672	1.1723	1.1677	1.1335	97
0.625µM	0.9319	1.4122	1.2364	1.1935	1.1593	99
1.25µM	0.9979	1.0993	1.0958	1.0643	1.0301	88
2.5µM	1.076	1.0995	1.1003	1.0919	1.0577	90
5µM	0.9902	1.0334	1.2788	1.1008	1.0666	91
10µM	1.2595	1.0332	1.162	1.1516	1.1174	95
20µM	1.1134	1.1883	1.1548	1.1522	1.1180	96

Table S2. Raw data for the MTT assay of Ama-Flash and α-amanitin against CHO cells.

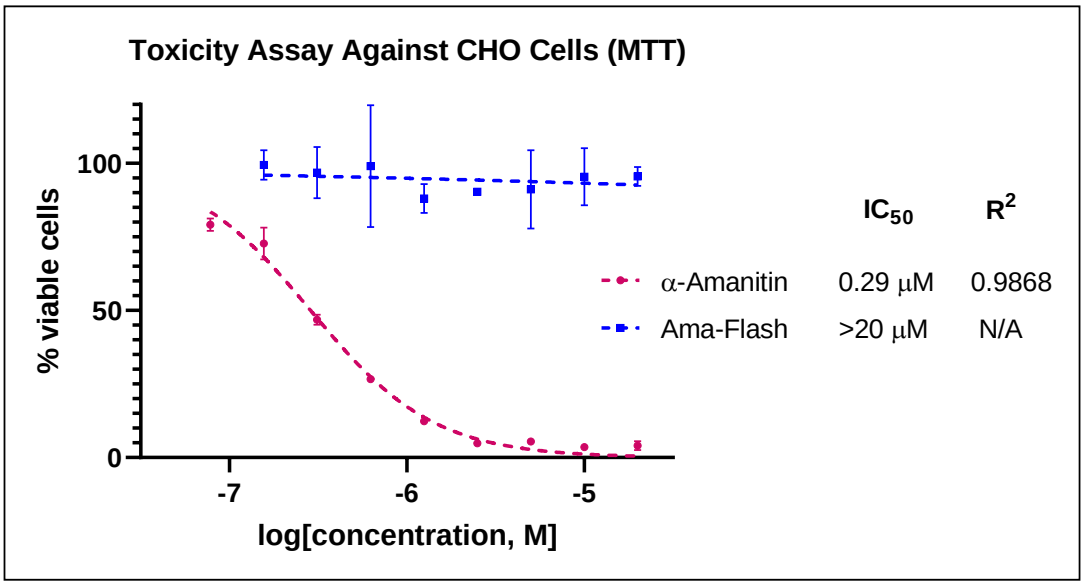


Figure S4. IC_{50} curves for CHO cell-based assays of authentic α-amanitin and Ama-Flash, **24** (MTT colorimetry).

Studying the effect of UV light (λ=366 nm) on the viability of CHO cells

To investigate the effect of UV light (λ=366 nm) on the viability of CHO cells (20,000 cell/well), these cells were irradiated with this light for varying durations, ranging from 15 minutes to 60 minutes, with 15-minute intervals. Then, their viability was measured using an MTT colorimetric assay at 590 nm. The cells for each irradiation time were placed in a separate 96-well plate.

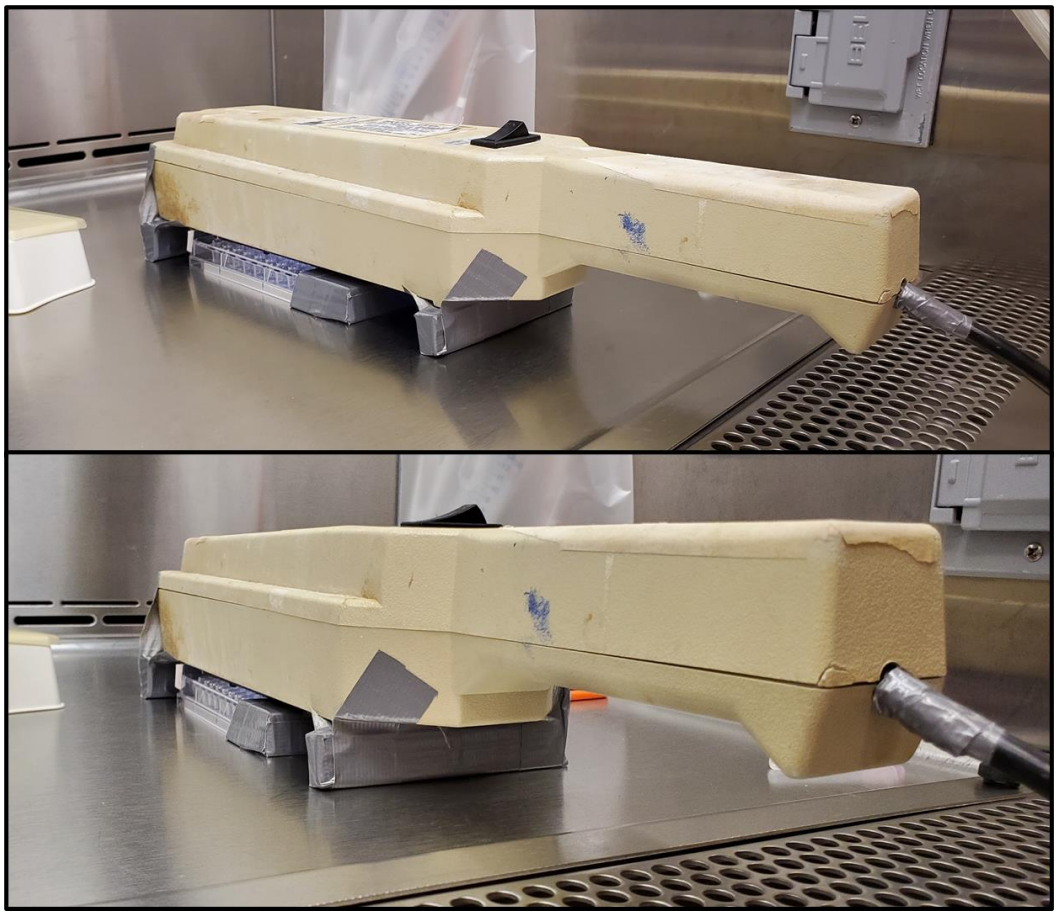


Figure S5. The set-up for irradiation of CHO cells placed in a 96-well plate. The distance between the UV source and the surface of the media in each well was kept consistent, and measured ca. 1 cm. UV irradiation: hand-held UV lamp, 115 V, 60 Hz, 0.16 Amps, λ=366 nm.

0 min

OD reading at 570nm							Mean	Net OD	% of alive cells
Blank	0.0365	0.0438	0.0396	0.0366	0.0372	0.0343	0.0380	0.0000	
medium ctrl	1.346	1.2609	1.4178	1.372	1.4348	1.3233	1.3591	1.3211	100
DMSO ctrl	1.4066	1.4453	1.3745	1.4255	1.3864	1.3609	1.3999	1.3619	100

15 min

OD reading at 570nm							Mean	Net OD	
Blank	0.0364	0.0338	0.0394	0.0367	0.0362	0.0345	0.0362	0.0000	
medium ctrl	1.336	1.2309	1.3178	1.3072	1.4048	1.2339	1.3051	1.2689	96
DMSO ctrl	1.4016	1.4053	1.3845	1.4055	1.3414	1.3709	1.3849	1.3487	99

30 min

OD reading at 570nm							Mean	Net OD	
Blank	0.0367	0.0354	0.0355	0.0354	0.0347	0.0362	0.0357	0.0000	
medium ctrl	1.2839	1.2084	1.1348	1.1223	1.1352	1.2691	1.1923	1.1566	88
DMSO ctrl	1.1357	1.0374	1.0188	0.9784	1.025	1.1038	1.0499	1.0142	74

45 min

OD reading at 570nm							Mean	Net OD	
Blank	0.0393	0.0365	0.0349	0.0349	0.0342	0.0344	0.0357	0.0000	
medium ctrl	1.096	0.913	0.8958	0.8737	0.8491	0.9908	0.9364	0.9007	68
DMSO ctrl	0.9216	0.8718	0.8649	0.8126	0.8083	0.9227	0.8670	0.8313	61

60 min

OD reading at 570nm							Mean	Net OD	
Blank	0.0358	0.0363	0.0366	0.0382	0.0382	0.0365	0.0369	0.0000	
medium ctrl	0.8747	0.8514	0.8565	0.8374	0.8262	0.8264	0.8454	0.8085	61
DMSO ctrl	0.8578	0.8358	0.8136	0.7881	0.8223	0.8115	0.8215	0.7846	58

Table S3. Raw data for the MTT assay of CHO cells, irradiated for various durations with λ=366 nm.

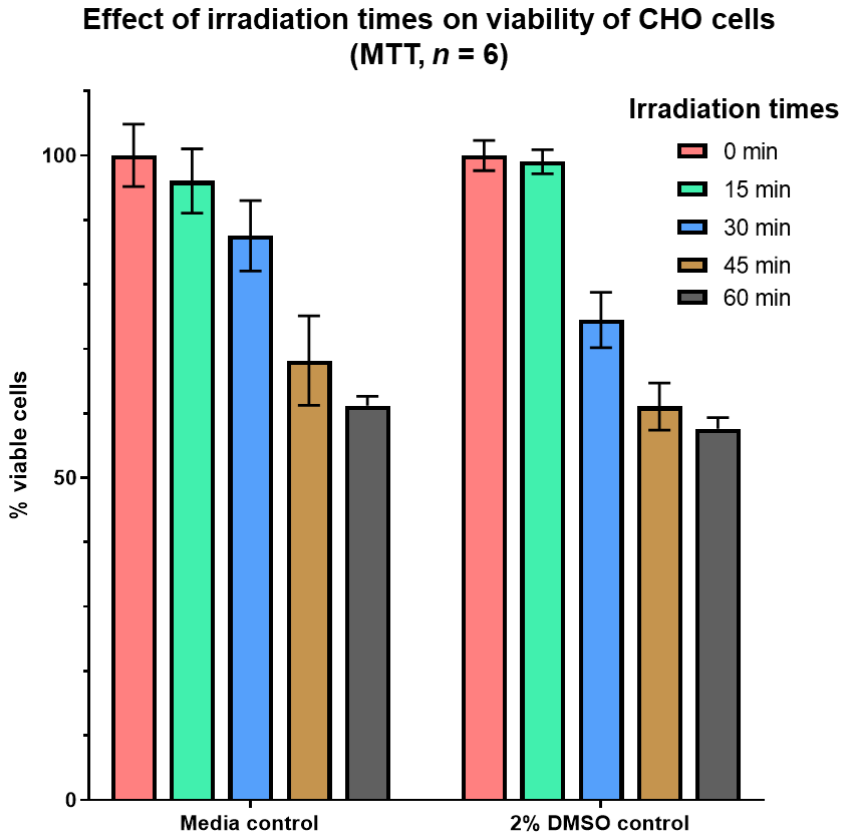


Figure S6. Cell viability of CHO cells following irradiation with l=366 nm for different durations.

Uncaging of Ama-Flash and its effect on the viability of CHO cells

A) Cell killing time-course studies of Ama-Flash (post-irradiation incubation time)

To measure the optimum time required for the uncaged Ama-Flash to effectively kill CHO cells, these cells (4,000 cells/well for 24 and 48 h post-irradiation duration; 3,000 cells/well for 72 h post-irradiation duration) were treated with Ama-Flash (11 µM) and incubated for 48 hours (protected from light) to ensure maximum internalization. At this point, the media was washed out and fresh media was added to each cell-containing well. Then, the cells were irradiated with λ=366 nm (according to the set-up shown in **Figure S5**) for 25 minutes. Following the irradiation, the cells were incubated for various durations: 24 h, 48 h and 72 h, and their viability was measured using an MTT colorimetric assay. For each time point, cells were placed in a separate 96-well plate.

	Ama-Flash (11 µM)	DMSO media ctrl	Blank (DMSO)	Media ctrl	Media (no-UV)
	1.2086	1.5665	0.0343	1.5487	1.827
	1.1439	1.4664	0.0334	1.6162	1.7955
	1.0961	1.5956	0.0339	1.6389	1.7286
			0.0359		1.8548
Average	1.149533333	1.542833333	0.034375	1.601266667	1.801475
Average-Blank	1.115158333	1.508458333		1.566891667	1.7671
% Alive	73.92702262	100		103.8737121	117.1460929

Table S4. Raw data for cell-killing time-course studies on uncaged Ama-Flash. Post-irradiation incubation time: **24 hours**.

	Ama-Flash (11 µM)	DMSO media ctrl	Blank (DMSO)	Media ctrl	Media (no-UV)
	0.1431	1.7492	0.033	1.7408	2.0636
	0.1636	2.0031	0.0339	1.9237	2.0003
	0.1599	2.3086	0.0325	2.158	2.0141
			0.0332		2.0902
Average	0.155533333	2.0203	0.03315	1.940833333	2.04205
Average-Blank	0.122383333	1.98715		1.907683333	2.0089
% Alive	6.158736549	100		96.00097292	101.0945324

Table S5. Raw data for cell-killing time-course studies on uncaged Ama-Flash. Post-irradiation incubation time: **48 hours**.

	Ama-Flash (11 µM)	DMSO media ctrl	Blank (DMSO)	Media ctrl	Media (no-UV)
	0.0636	1.5794	0.033	1.55	1.623
	0.0654	1.67	0.0335	1.6098	1.5529
	0.0614	1.6872	0.0332	1.6716	1.5483
			0.0336		1.5709
Average	0.063466667	1.645533333	0.033325	1.610466667	1.573775
Average - blank	0.030166667	1.612233333	2.5E-05	1.577166667	1.540475
% Alive	1.871149154	100		97.8269859	95.55111028

Table S6. Raw data for cell-killing time-course studies on uncaged Ama-Flash. Post-irradiation incubation time: **72 hours**.

B) Time-course studies on CHO cell killing by α-amanitin

To confirm the time required for α-amanitin to kill CHO cells, these cells (4,000 cells/well for 24 and 48 h duration; 3,000 cells/well for 72 h duration) were treated with α-amanitin (14.3 μM) and incubated for varying times: 24 h, 48 h and 72 h. Then, their viability was measured using an MTT colorimetric assay. The results are as follow:

24h OD reading at 590nm					**Mean**	**Net OD**	**% of alive cells**		
Blank	0.0336	0.0338	0.0337		0.0337	0.0000			
Medium ctrl	1.6557	1.7423	1.5964		1.6648	1.6311	102		
DMSO ctrl	1.7374	1.6725	1.6606		1.6902	1.6565	100		
14.3uM AMA	0.8703	0.7933	0.7921		0.8186	0.7849	47		
48h OD reading at 590nm					**Mean**	**Net OD**	**% of alive cells**		
Blank	0.0327	0.033	0.0321		0.0326	0.0000			
Medium ctrl	1.5633	1.5513	1.5971		1.5706	1.5380	104		
DMSO ctrl	1.5969	1.6737	1.616		1.6289	1.5963	100		
14.3uM AMA	0.082	0.0852	0.0912		0.0861	0.0535	3		
72h OD reading at 590nm					**Mean**	**Net OD**	**% of alive cells**		
Blank	0.0341	0.0339	0.0333		0.0338	0.0000			
Medium ctrl	1.4718	1.4909	1.4744		1.4790	1.4452	98		
DMSO ctrl	1.4544	1.4694	1.4312		1.4517	1.4179	100		
14.3uM AMA	0.0457	0.0666	0.063		0.0584	0.0246	2		
					Reading-blank			%alive	
α-AMA 24h	0.8703	0.7933	0.7921	0.8366	0.7596	0.7584	50.50407	45.85572	45.78328
α-AMA 48h	0.082	0.0852	0.0912	0.0494	0.0526	0.0586	3.114165	3.315892	3.694131
α-AMA 72h	0.0457	0.0666	0.063	0.0119	0.0328	0.0292	0.839269	2.31328	2.059384

Table S7. Raw data for the MTT assay of α-amanitin against CHO cells, various incubation times.

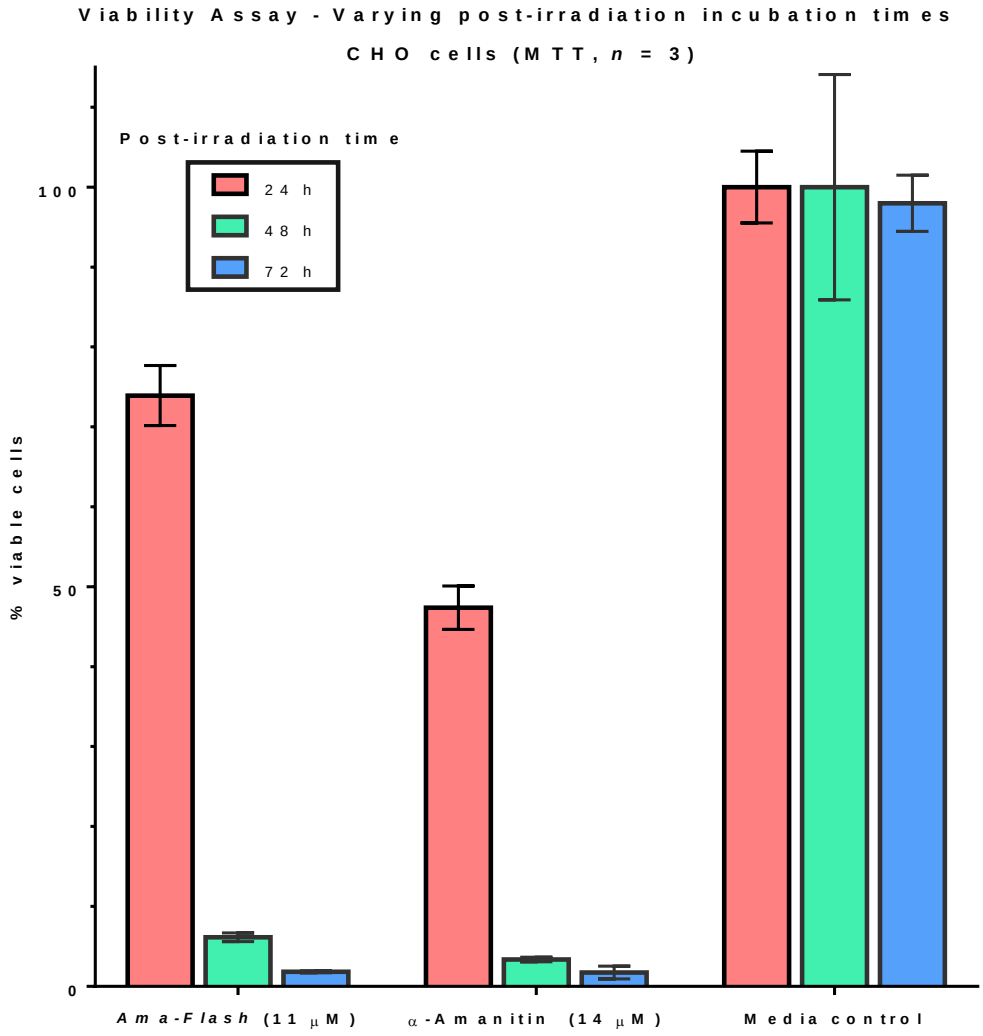


Figure S7. Assessing the required incubation time post-irradiation to observe death for CHO cells (cell killing time course), raw data is shown in **Tables S4**-**S7**.

C) Dose-response studies of uncaged Ama-Flash against CHO cells

To investigate the presence of a dose-response relationship between uncaging Ama-Flash and the death rate of CHO cells, CHO cells (4,000 cells/well for 24 and 48 h duration; 3,000 cells/well for 72 h duration) were treated with varying concentrations of Ama-Flash (ranging from 0.5 nM to 14.3 µM). Following an initial incubation of 48 hours, the media was washed out and fresh media was added to each cell-containing well. Then, cells were irradiated with λ=366 nm for 25 minutes and incubated for a second time and their viability was measured using an MTT assay. Two post-irradiation incubation times were explored in this experiment: 48 hours and 72 hours. In both experiments, α-amanitin was used as a standard and in similar concentration to those of Ama-Flash. The results are as follow:

Ama-Flash (M)	OD reading				Reading - Blank			Normalized Reading (%alive)		
5.88E-10	2.0239	1.7993	1.7925		1.9892	1.7646	1.7578	107.1158	95.02142	94.65525
5.88E-09	1.8276	1.7798	1.8354		1.7929	1.7451	1.8007	96.54534	93.97137	96.96536
5.88E-08	1.8046	1.7066	1.7744		1.7699	1.6719	1.7397	95.30682	90.02964	93.68059
1.76E-07	1.9732	1.8719	1.8206		1.9385	1.8372	1.7859	104.3857	98.93083	96.1684
5.29E-07	2.0728	1.8765	1.9229		2.0381	1.8418	1.8882	109.749	99.17854	101.6771
1.59E-06	0.9034	0.895	0.8493		0.8687	0.8603	0.8146	46.77837	46.32604	43.86515
4.76E-06	0.3299	0.2639	0.2534		0.2952	0.2292	0.2187	15.89614	12.34212	11.77671
1.43E-05	0.2874	0.2777	0.2624		0.2527	0.243	0.2277	13.60757	13.08523	12.26135
									Average	
DMSO ctrl	1.8611	1.843	1.9294	1.9286	1.82763	1.80953	1.89593	1.89513	1.857055	100%
Media ctrl	1.974	1.8345	1.6801	1.795	1.94053	1.80103	1.64663	1.76153	1.78743	96.25078
no-UV ctrl	1.9791	1.9704	1.8086	1.9782	1.94563	1.93693	1.77513	1.94473	1.900605	102.3451
Blank	**0.0339**	**0.0353**	**0.0367**	**0.0352**						
	0.0324									
Blank Avg	0.0347									

Table S8. Raw data for the MTT assay of Ama-Flash (0.5 nM to 14.3 µM) against CHO cells. Post-irradiation incubation time: **48 hours**.

α-amanitin (M)	OD reading				Reading - Blank			Normalized Reading (%alive)		
5.88E-10	1.9874	1.9664	1.9372		1.95272	1.93172	1.90252	103.5777	102.4638	100.915
5.88E-09	1.978	1.9656	1.9781		1.94332	1.93092	1.94342	103.0791	102.4214	103.0844
5.88E-08	1.8569	1.9198	1.9062		1.82222	1.88512	1.87152	96.65565	99.99204	99.27066
1.76E-07	1.9495	1.9206	1.96		1.91482	1.88592	1.92532	101.5674	100.0345	102.1244
5.29E-07	1.0255	1.0834	1.0909		0.99082	1.04872	1.05622	52.55587	55.62705	56.02487
1.59E-06	0.044	0.0462	0.0514		0.00932	0.01152	0.01672	0.494359	0.611053	0.886876
4.76E-06	0.052	0.0519	0.0528		0.01732	0.01722	0.01812	0.918701	0.913397	0.961136
1.43E-05	0.0454	0.0469	0.0476		0.01072	0.01222	0.01292	0.568619	0.648183	0.685313
									Average	
DMSO control	1.7976	1.9781	1.8991	2.005	1.76292	1.94342	1.86442	1.97032	1.88527	100%
Media control	1.9535	1.9214	1.7942	1.8846	1.91882	1.88672	1.75952	1.84992	1.853745	98.32783
No UV-control	2.0346	1.828	1.9489	2.0032	1.99992	1.79332	1.91422	1.96852	1.918995	101.7889
Blank	0.0333	0.0355	0.0364	0.0352						
	0.033									
Blank Average	0.03468									

Table S9. Raw data for the MTT assay of α-amanitin (0.5 nM to 14.3 µM) against CHO cells. Post-irradiation incubation time: **48 hours**.

Ama-Flash (M)	OD reading				Reading - Blank			Normalized Reading (%alive)		
5.88E-10		2.1292	1.7755	1.8972	2.09541	1.74171	1.86341	106.5832	88.59223	94.78251
5.88E-09		1.9798	1.8378	1.8651	1.94601	1.80401	1.83131	98.98397	91.76113	93.14974
5.88E-08		1.974	1.8608	1.8683	1.94021	1.82701	1.83451	98.68895	92.93102	93.31251
1.76E-07		1.9234	1.8987	1.9112	1.88961	1.86491	1.87741	96.11518	94.85881	95.49462
5.29E-07		2.0456	1.9574	2.1417	2.01181	1.92361	2.10791	102.3309	97.84459	107.219
1.59E-06		0.7393	0.7358	0.7949	0.70551	0.70201	0.76111	35.88583	35.7078	38.71393
4.76E-06		0.0696	0.0652	0.0655	0.03581	0.03141	0.03171	1.821479	1.597672	1.612932
1.43E-05		0.064	0.0569	0.0593	0.03021	0.02311	0.02551	1.536634	1.175492	1.297568
									Average	
DMSO control	1.9081	1.9824	2.0314	2.0772	1.87431	1.94861	1.99761	2.04341	1.965985	100%
Media control	2.1098	1.9658	1.8996	1.9375	2.07601	1.93201	1.86581	1.90371	1.944385	98.90131
No UV-control	1.9841	1.9	1.8917	2.0792	1.95031	1.86621	1.85791	2.04541	1.92996	98.16759
Blank		0.0329	0.0349	0.0353	0.0348					
		0.0323	0.0323	0.0338	0.0336					
		0.0336	0.0344							
Blank Average	0.03379									

Table S10. Raw data for the MTT assay of Ama-Flash (0.5 nM to 14.3 µM) against CHO cells. Post-irradiation incubation time: **72 hours**.

α-Amanitin (M)	OD reading				Reading - Blank			Normalized Reading (%alive)		
5.88E-10		2.3108	1.9275	1.9078	2.27696	1.89366	1.87396	104.3216	86.76027	85.85769
5.88E-09		2.0339	1.9593	1.9955	2.00006	1.92546	1.96166	91.63511	88.21722	89.87577
5.88E-08		1.9313	1.923	2.0044	1.89746	1.88916	1.97056	86.93437	86.5541	90.28353
1.76E-07		2.1227	2.0124	2.1135	2.08886	1.97856	2.07966	95.70359	90.65006	95.28208
5.29E-07		0.8615	0.8204	0.8541	0.82766	0.78656	0.82026	37.92022	36.03718	37.58118
1.59E-06		0.0447	0.0491	0.0429	0.01086	0.01526	0.00906	0.497564	0.699155	0.415095
4.76E-06		0.048	0.0449	0.0467	0.01416	0.01106	0.01286	0.648757	0.506727	0.589196
1.43E-05		0.0443	0.0458	0.0455	0.01046	0.01196	0.01166	0.479237	0.547962	0.534217
									Average	
DMSO control	2.1672	2.2286	2.1557	2.3144	2.13336	2.19476	2.12186	2.28056	2.182635	100%
Media control	2.1444	1.9766	2.0687	2.0771	2.11056	1.94276	2.03486	2.04326	2.03286	93.13788
No UV-control	2.0792	1.9149	1.9483	2.1043	2.04536	1.88106	1.91446	2.07046	1.977835	90.61685
Blank		0.0336	0.0338	0.0354	0.0337					
		0.0329	0.0325	0.0347	0.0339					
		0.0334	0.0345							
Blank Average	0.03384									

Table S11. Raw data for the MTT assay of α-amanitin (0.5 nM to 14.3 µM) against CHO cells. Post-irradiation incubation time: **72 hours**.

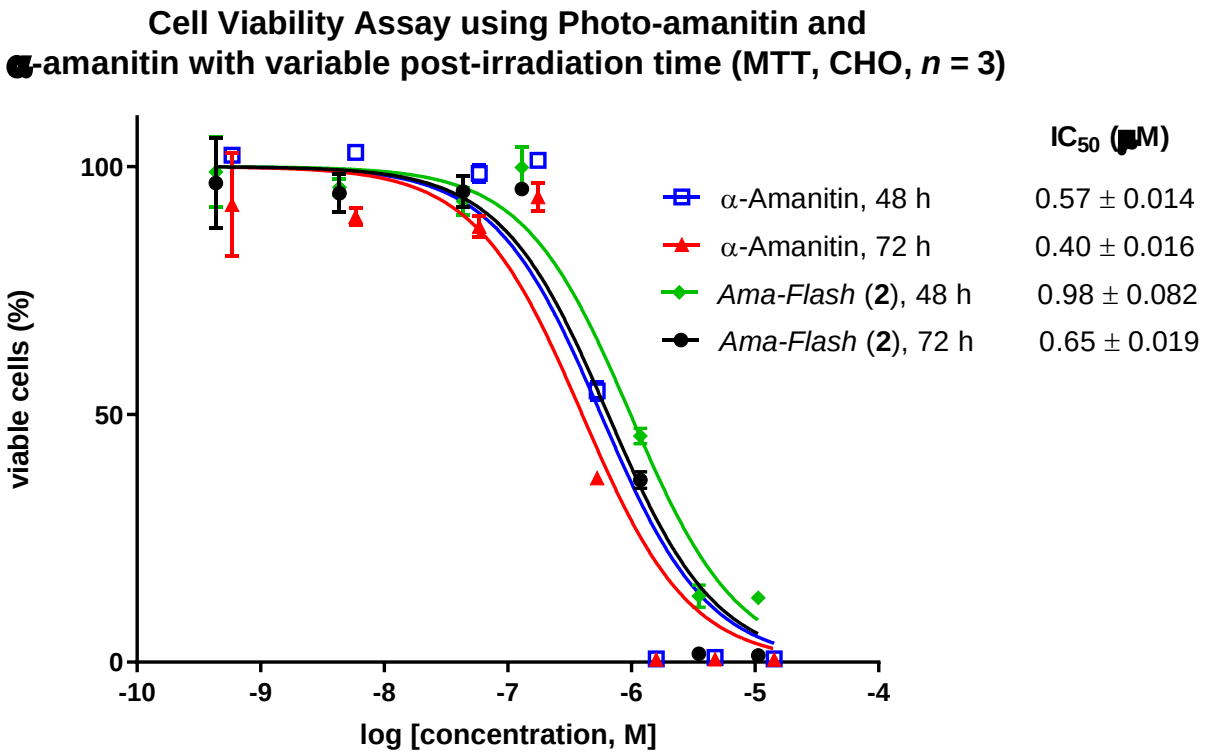


Figure S8. Dose-response curves of Ama-Flash against CHO cells for post-irradiation times of 48 h and 72 h.

In vitro transcription runoff assay of Ama-Flash (24)

Chemicals and Reagents

Primers were purchased from IDT *via* standard phosphoramidite chemistry. dNTP and rNTP were purchased from Thermofisher. *GoTaq* polymerase and HeLaScribe® Nuclear Extract in vitro Transcription System was purchased from Promega. *Taq* polymerase was purchased from NEB. [^{32}P] α-dGTP (3000Ci/mmol 10mCi/ml EasyTide) and [^{32}P] α-GTP (3000Ci/mmol 10mCi/ml EasyTide) were purchased from Perkin Elmer. QIAquick PCR purification kit was purchased from Qiagen. AcGFP1-N1 was a gift from Michael Davidson (Addgene plasmid # 54705; http://n2t.net/addgene:54705; RRID:Addgene_54705).

DNA Primer sequences

P1 AcGFP1.FOR.V4 CAGTCGACGGTACCGC;

P2 AcGFP1.REV.V4 GCCCTCGAACTTCACCTC;

P3 AcGFP1.FOR.V2 CGCGGGCCCGGGATCCAC;

P4 AcGFP.REV.V2 ACCTCGGCGCGCGACTTGT

Synthesis of the DNA template for transcription runoff

The DNA template for transcription runoff was synthesized by PCR with template pAcGFP-N1, and primers P1 and P2. To a final volume of 20 µL, 1 x GoTaq Buffer, 250 µM of each dNTP, 500 nM of each primers P1 and P2, 10 pg/µL pAcGFP-N1 and 0.05 U/µL GoTaq were employed to thermocycle for 30 cycles (30 s at 95 °C, 30 s at 50 °C and 60 s at 72 °C, Bio-Rad). A 1 µL aliquot of the amplified solution was resolved with 6 x DNA loading dye in a 1% agarose gel containing 1% ethidium bromide and then visualized using GelDoc XR imager (Bio-Rad). Purification was completed via QIAquick PCR purification kit (Qiagen).

Synthesis of the radioactive chromatographic standard

The radioactive chromatographic standard was synthesized by PCR with template pAcGFP-N1, and primers P3 and P4. To a final volume of 50 µL, 1 x Thermopol Buffer, 200 µM of each dNTP, 500 nM of each primers P3 and P4, 10 pg/µL pAcGFP-N1 and 0.02 U/µL Vent polymerase, 1 µL [^{32}P] α-dGTP were employed to thermocycle for 30 cycles (30 s at 95 °C, 30 s at 62 °C and 30 s at 72 °C, Bio-Rad). A 1 µL aliquot of the amplified solution was resolved with 6 x DNA loading dye in 8% denaturing PAGE and then visualized by autoradiography via the Typhoon 9200 imager (Molecular namics-Amersham-GE). Purification was completed via QIAquick PCR purification kit (Qiagen).

Transcription runoff assay and statistical analysis

The transcription runoff assay was modified from HeLaScribe® Nuclear Extract in vitro Transcription System (Promega). A 1.35X master mixture was formulated with 1.35X HeLa Nuclear Extract Transcription Buffer, 4.05 mM $MgCl_2$, 540 μM rATP, 540 μM rCTP, 540 μM rUTP and 21.6 μM rGTP, 5.4 ng/μL DNA template and 3 μL [^{32}P] α-GTP. To a final volume of 8 μL, 1 X master mixture, 0 - 3 μM aqueous solutions containing α-amanitin or amanitin analogs (B-H), 0.32 U/μL HeLaScribe® Nuclear Extract were combined to transcribe for 60 min. The reaction was quenched by 56 μL HeLa Extract Stop Solution, followed by phenol-chloroform extraction and EtOH precipitation. The pellet was resolved by 8% denaturing PAGE and then visualized by autoradiography via the Typhoon 9200 imager (Molecular dynamics-Amersham-GE). Dosimetry was calculated by ImageJ, [1] and the 3 parameter logistic fit was completed by Origin 2019 (OriginLab). For inhibitor I that demonstrates transcription activity E with half inhibitory concentration IC_{50},

$$E = \frac{IC_{50}}{IC_{50} + [I]} \quad (1)$$

This assumes symmetry around IC_{50} (asymmetry factor $S = 1$) and no cooperativity (Hill's slope $H = -1$). All data were presented in mean ± standard deviation ($n = 3$).

*Summarized transcription activities of α-amanitin and Ama-Flash (**24**)*

Concentration (nM)	Transcription activity (*E* %)	
	α-amanitin	Ama-Flash
1	67	-
100	7	-
300	-	115 ± 8
IC_{50} (nM)	ca. 8	> 1000

Table S12. Transcription activities of HeLa nuclear extract in α-amanitin and Ama-Flash (**24**). For α-amanitin: *n=1*; for Ama-Flash: *n=3*.

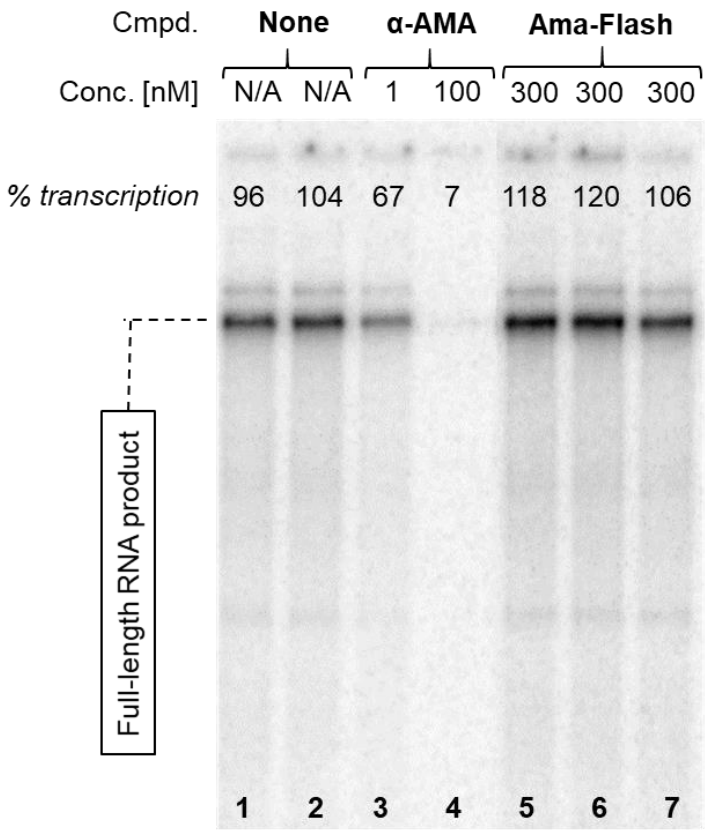


Figure S10. Characteristic inhibition of transcription by α-amanitin (*n=1*) and Ama-Flash (*n=3*). under varied concentrations ($n = 3$). [^{32}P]-autoradiogram of transcription runoff assay resolved by 8% denaturing PAGE.

References

(1) Schneider, C. A.; Rasband, W. S.; Eliceiri, K. W. NIH Image to ImageJ: 25 Years of Image Analysis. *Nat. Methods* **2012**, *9* (7), 671–675.