SUPPORTING INFORMATION

Synthesis and Evaluation of a Fluorescent Ritterazine-Cephalostatin Hybrid

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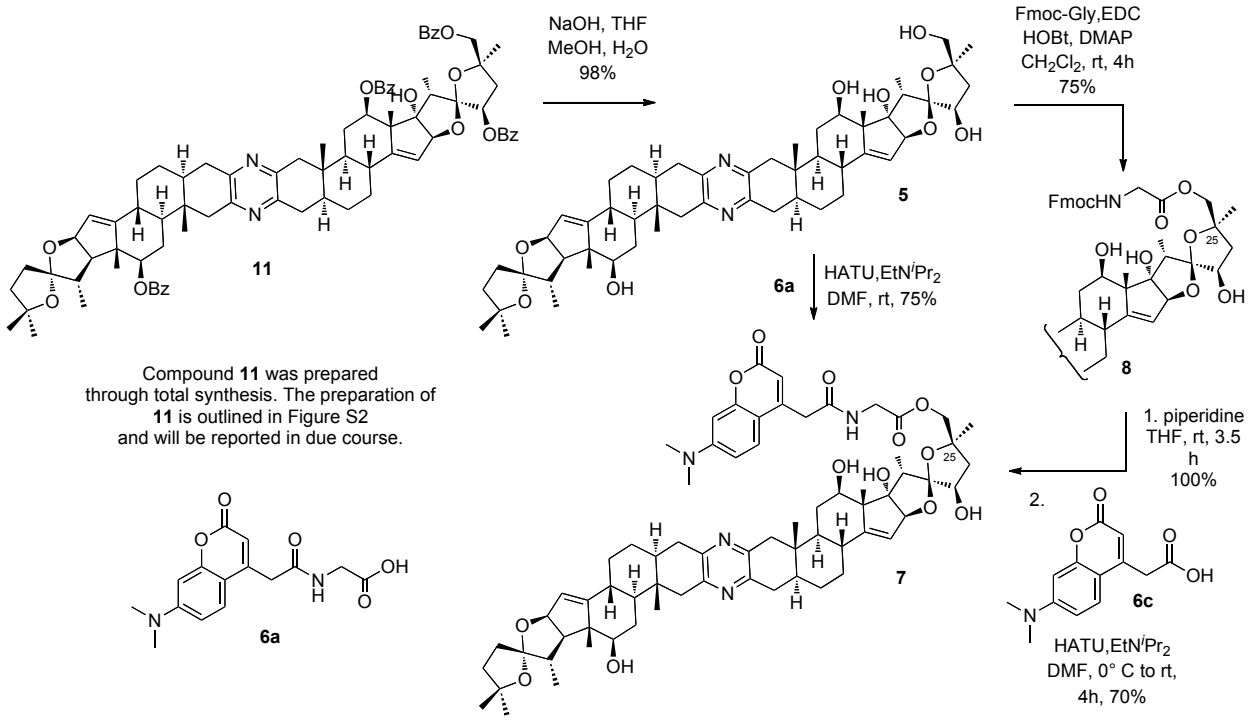
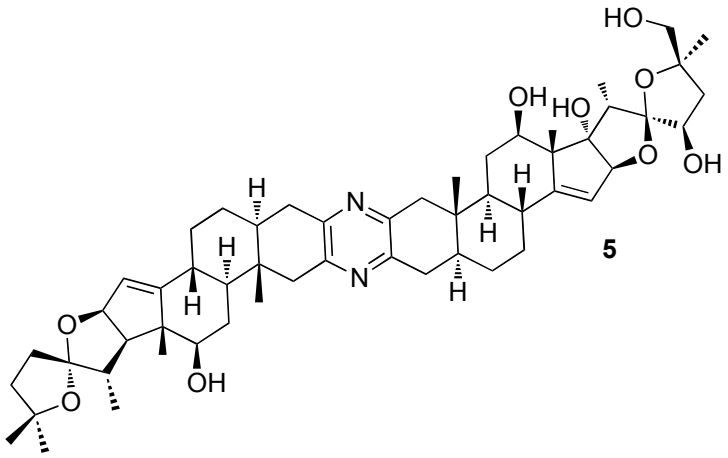
1. Synthetic Procedures

1.a. General Synthetic Procedures. All reagents used from commercial source as received. Tetrahydrofuran (THF) was distilled from benzophenone ketyl and dichloromethane (CH_2Cl_2) distilled from CaH_2. *N,N*-Dimethylformamide (DMF) was distilled from CaH_2. All other solvents used for workup and purifications were distilled. Progress of reactions was monitored by thin layer chromatography (TLC) using 0.25 mm silica gel 60 F-254 plates (EM Biosciences). The plates were visualized with a UV lamp (254 nm) and/or with visualizing solutions activated with heat. The two visualizing agents were used including *p*-anisaldehyde solution (1350 mL absolute ethanol, 50 mL concentrated H_2SO_4, 37 mL *p*-anisaldehyde) or I_2 on silica gel. ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker AMX-400 or Bruker AMX-500 at 400 or 500 MHz, respectively. NMR spectra were determined in $CDCl_3$ (7.26 ppm and 77.0 ppm) and CD_3OD (3.34 ppm and 49.86 ppm) solutions and are reported in parts per million (ppm). Peak multiplicities in ^{1}H-NMR spectra, when reported, are abbreviated as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and/or br (broad). Mass spectral analyses were run by the Purdue University campus wide mass spectrometry facility.

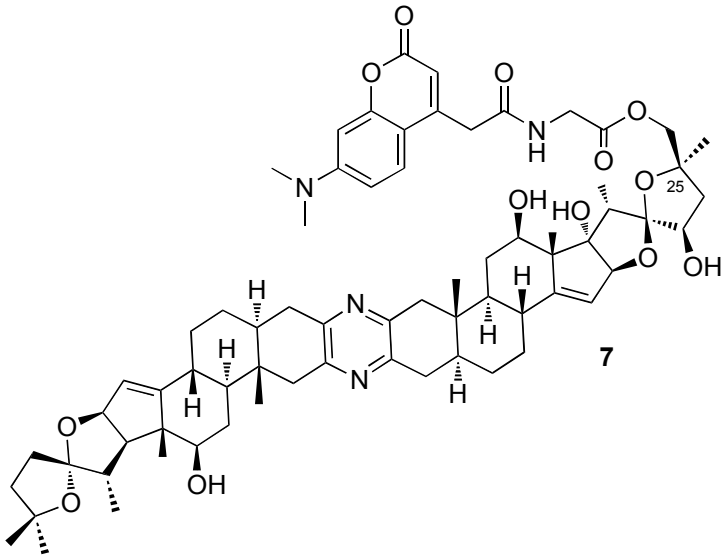
1.b. Synthesis of 25-*epi* Ritterostatin G_N1_N probe 7. A two-step procedure was used that began by the preparation of **5**. The following section provides conditions and methods for the preparation of **5** from its corresponding tetra-benzoyl protected derivative **11**, followed by labeling with IAF tag **6** to afford probe **7** (as shown in Scheme S1).



Scheme S1. Two routes were developed to prepare 25-*epi*-ritterostatin G_N1_N probe **7**. The first route arises through a direct labeling of 25-*epi*-ritterostatin G_N1_N (**5**) with IAF tag **6a**. The second route involves a stepwise addition via **8** followed by coupling with the IAF tag **6c**.



25-*epi*-ritterostatin G_N1_N (5). 12,12',23,26-tetra-O-benzoyl 25-*epi* ritterostatin G_N1_N[1] (**11**, 5 mg, 0.0114 mmol) was dissolved in 1.5 mL of THF/MeOH/H_2O (2:2:1). NaOH (5.0 mg, 10 eq.) was added and the mixture was stirred for 12 h at rt. The solvent was evaporated under vacuum, and the crude product was dissolved in EtOAc, extracted with EtOAc (4×5 mL) and CH_2Cl_2 (4x 5 mL) and dried over Na_2SO_4. The solvent was evaporated, and the crude product was purified by flash chromatography with 5% MeOH in CH_2Cl_2 to provide **5** as a colorless solid (10.1 mg, 98%). R_f = 0.25 (2 % MeOH:EtOAc). ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.39 (1H, s), 5.35 (1H, s), 4.93 (1H, d, *J* = 8.23 Hz), 4.81 (1H, s), 4.69 (1H, s), 4.18 (1H, m), 3.99 (1H, br), 3.86-3.82 (1H, m), 3.62 (1H, d, *J* = 12.0 Hz), 3.55 (1H, d, *J* = 12.0 Hz), 3.32 (1H, m), 3.24 (1H, dd, *J* = 16.0, 7.8 Hz), 2.90 (1H, s), 2.85 (1H, s), 2.82 (1H, d, *J* = 5.6 Hz), 2.78 (1H, d, *J* = 4.8 Hz), 2.63 (1H, s), 2.60 (1H, s), 2.59 (1H, d, *J* = 5.6 Hz), 2.55-2.49 (4H, m), 2.48-2.43 (2H, m), 2.41-2.31 (2H, m), 2.10-1.97 (3H, m), 1.95-1.90 (3H, m), 1.88-1.80 (3H, m), 1.75-1.68 (3H, m), 1.65-1.50 (5H, m), 1.28-1.23 (2H, t), 1.19 (3H, s), 1.18 (3H, s), 1.15 (4H, s), 1.09 (3H, d, J = 6.8 Hz), 1.06-1.01 (6H, s), 0.99 (2H, br), 0.97 (1H, s), 0.95 (1H, s), 0.87 (1H, s), 0.85 (3H, s), 0.83 (3H, s); ^{13}C NMR: (100 MHz, $CDCl_3$): δ 156.9, 153.1, 148.6, 148.5, 148.3, 147.9, 120.6, 119.8, 117.4, 116.0, 92.7, 91.7, 84.2, 82.6, 81.9, 78.6, 75.0, 10.8, 67.9, 55.7, 54.7, 52.7, 52.0, 45.4, 45.3, 41.2, 37.1, 36.0, 35.9, 34.5, 31.5, 30.0, 29.8, 29.6, 29.1, 28.9, 28.7, 28.4, 28.2, 28.0, 27.9, 27.8, 27.7, 25.2, 24.4, 22.5, 20.6, 14.0, 13.9, 13.3, 11.9, 11.7, 11.6, 8.5; MS (ESI) calculated for $C_{54}H_{76}N_2O_9$ 896.56, found 897.52 (M+H).

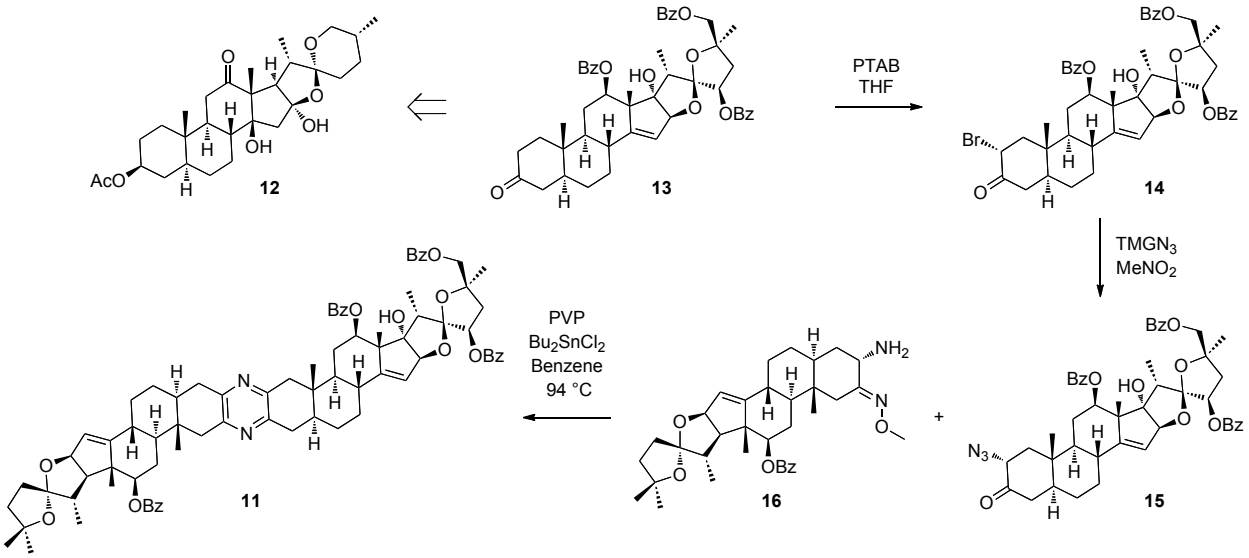


7

Probe 7. 25-*epi* Ritterostatin G_N1_N (**5**, 4.0 mg, 0.0045 mmol) and IAF tag **6a**[2] (1.4 mg, 0.0045 mmol) were dissolved in DMF (0.2 mL). The solution was cooled to 0 °C and O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU) (2.6 mg, 0.0067 mmol) was added followed by *N*,*N*-diisopropylethylamine (DIPEA) (2.5 μL, 0.0154 mmol). The reaction mixture was stirred at 25 °C in the dark (protection of reaction flask by aluminum foil) and monitored by TLC. After 4 h, the reaction was diluted with EtOAc (3 mL) and H_2O (1.5 mL). The mixture was extracted with EtOAc (3×3 mL) and combined organic layers was dried over Na_2SO_4, filtered, and concentrated in vacuum to give the fluorescent yellow solid crude product which was purified by chromatography on a silica gel column using a gradient of 1:1 EtOAc:hexanes to EtOAc and then to 1:10 MeOH:EtOAc to afford **7** (3.9 mg, 75 %). Multiple purifications were required to compound **7** with sufficient purity for cellular applications. R_f = 0.20 (10:1 MeOH:EtOAc). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.62 (1H, d, *J* = 7.2 Hz), 7.46 (1H, d, *J* = 7.2 Hz), 6.87 (1H, t, *J* = 6.5 Hz), 6.65 (1H, dd, *J* = 11.5, 6.4 Hz), 6.51 (1H, d, *J* = 2.5 Hz), 6.31 (1H, s), 6.11 (1H, s), 5.43 (2H, d, *J* = 21.5 Hz), 5.18 (1H, s), 4.95 (1H, d, *J* = 8.5 Hz), 4.91 (1H, d, *J* = 8.0 Hz), 4.72 (1H, s), 4.67 (1H, s), 4.47 (1H, d, *J* = 11.5 Hz), 4.45-4.41 (1H, m), 4.38 (1H, t), 4.25-4.19 (2H, m), 3.89 (1H, br), 3.85 (1H, d, *J* = 2.8 Hz), 3.83 (1H, d, *J* = 2.5 Hz), 3.77 (2H, d, *J* = 14.5 Hz), 3.71 (2H, s), 3.69 (1H, s), 3.60 (1H, d, *J* = 14.5 Hz), 3.25 (1H, dd, *J* = 14.8, 7.0 Hz), 3.06 (1H, s), 3.05 (6H, s), 2.91-2.86 (2H, m), 2.83-2.75 (3H, m), 2.66-2.60 (2H, m), 2.58-2.46 (2H, m), 2.44 (2H, q), 2.38-2.30 (1H, m), 2.15 (1H, t), 2.10-1.94 (4H, m), 1.93-1.83 (2H, m), 1.80-1.70 (2H, m), 1.68-1.57 (2H, m), 1.47-1.35 (6H, br), 1.28 (3H, s), 1.23

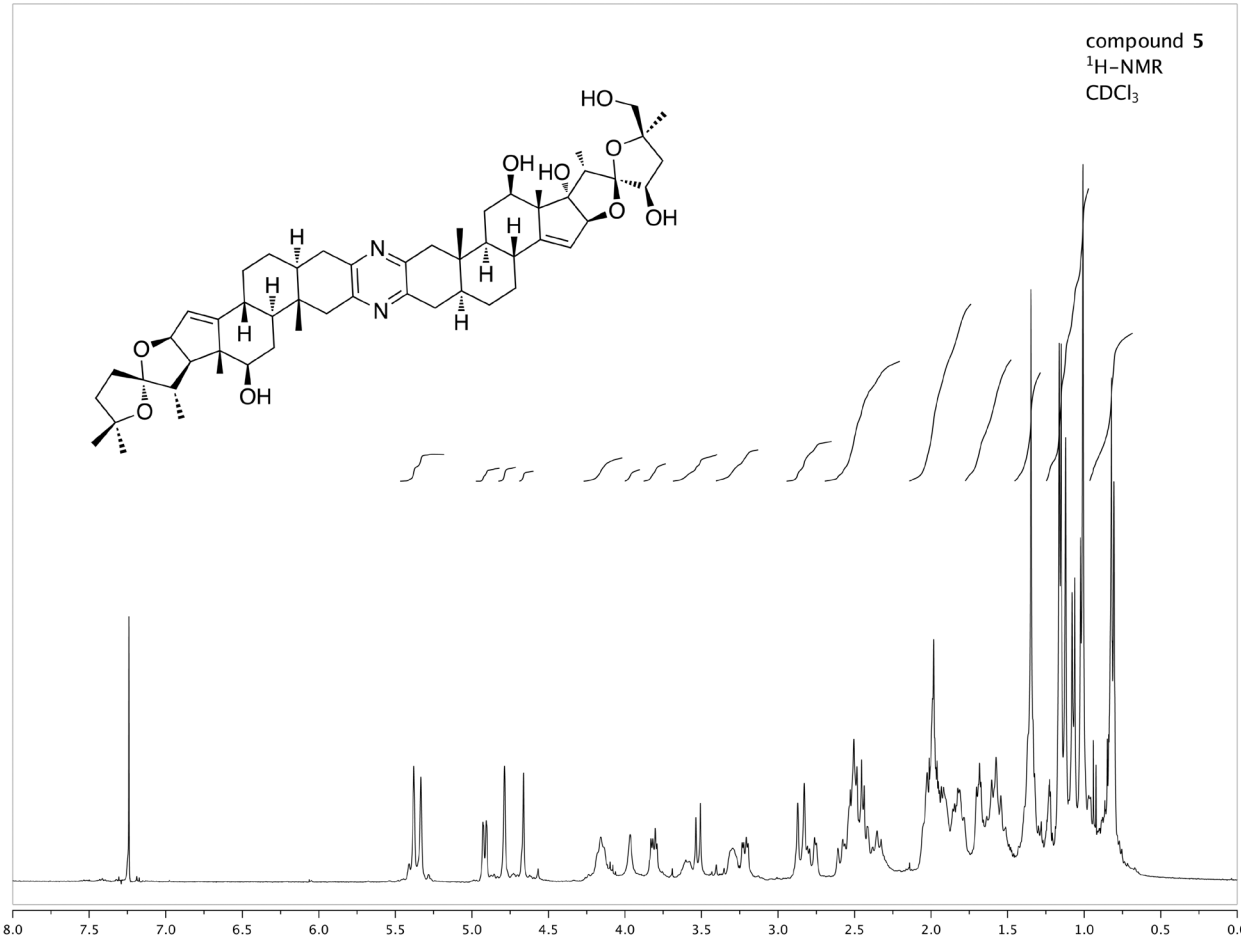
(3H, d, J = 7.5 Hz), 1.19 (1H, t), 1.16 (3H, s), 1.11 (3H, s), 1.08 (1H, d, J = 5.5 Hz), 1.06 (3H, d, J = 3.0 Hz), 0.99 (2H, d, J = 7.0 Hz), 0.88 (6H, s); ^{13}C NMR: (125 MHz, $CDCl_3$): δ 168.2, 167.5, 162.5, 156.0, 148.6, 148.4, 148.3, 126.2, 125.6, 120.0, 117.5, 116.7, 110.6, 109.6, 109.5, 109.3, 108.3, 98.1, 92.8, 91.1, 84.3, 82.1, 79.6, 79.0, 75.1, 69.9, 68.4, 55.8, 55.1, 53.2, 52.9, 52.2, 51.2, 45.6, 45.1, 43.4, 42.2, 41.6, 41.5, 41.3, 40.8, 40.2, 37.3, 37.1, 36.2, 36.0, 35.9, 35.3, 33.7, 33.5, 33.2, 30.0, 29.7, 29.2, 28.5, 28.3, 28.2, 28.1, 27.9, 24.6, 24.4, 22.7, 14.7, 14.1, 13.3, 11.9, 11.8, 11.7, 8.4; MS (ESI) calculated for $C_{69}H_{90}N_4O_{13}$ 1182.65, found 1183.10 (M+H) and 1205.27 (M+Na).

An alternate, stepwise approach as shown in Scheme S1 also provide access to compound **7**. This involved the preparation of intermediate **8**. The product of this route (shown in Figure S1) was identical to that made via the direct coupling method (described above).

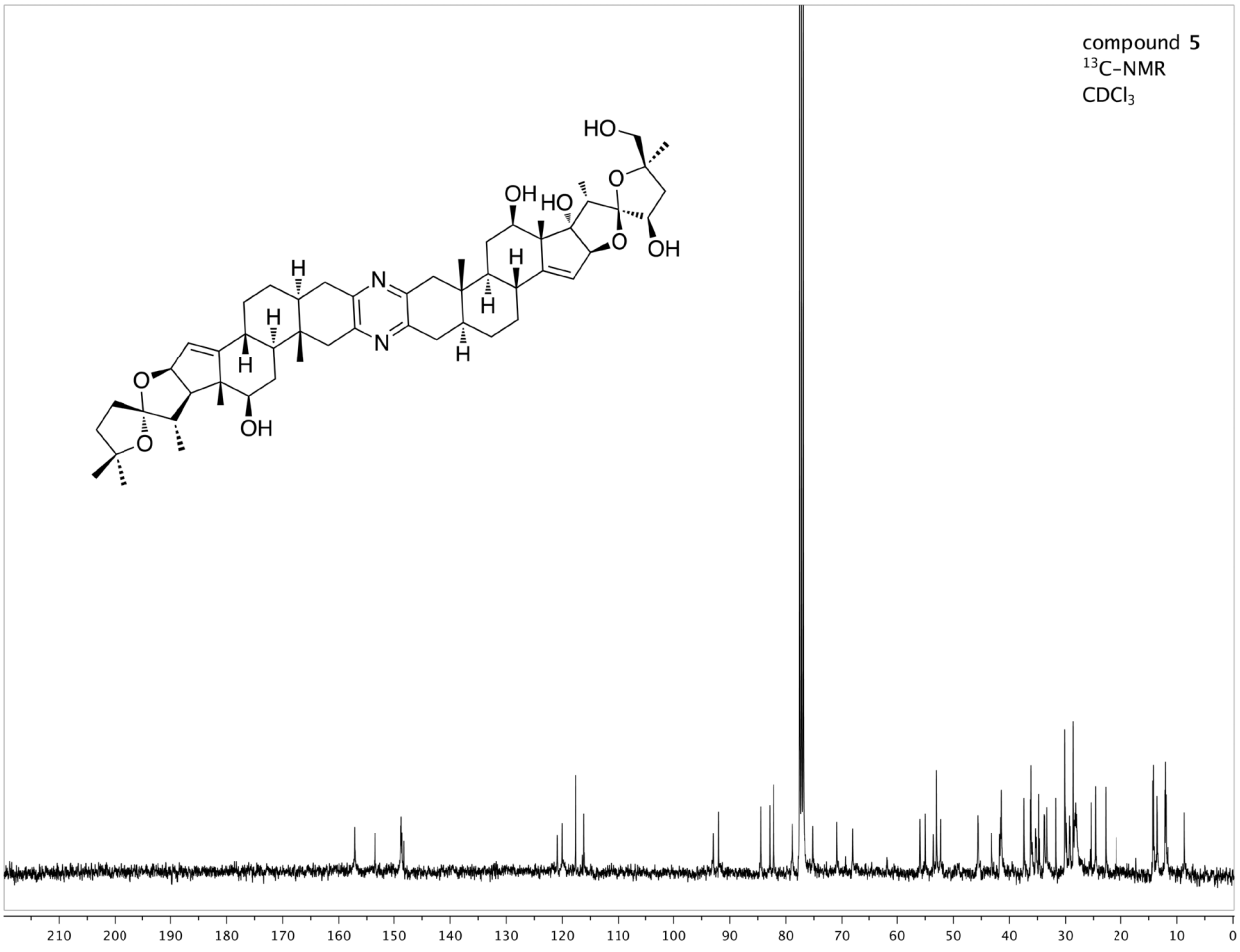


Scheme S2. General strategy for the synthesis of intermediate **11**. Intermediate **11** was prepared from 14,16-dihydroxyhecogeninacetate (**12**)[3] using methods developed in the Fuchs laboratory. This began by conversion of **12** to **13**. Ketone **13** was brominated using phenyltrimethylammonium tribromide (PTAB) and the resulting α-bromoketone **14** was displaced to give α-azidoketone **15**. Our unsymmetrical pyrazine coupling was used to prepare **11** from **15** and **16**, by using similar methods to that described in the synthesis of ritterazine M.[4] The synthesis of **11** and key intermediate **5** will be reported in due course.

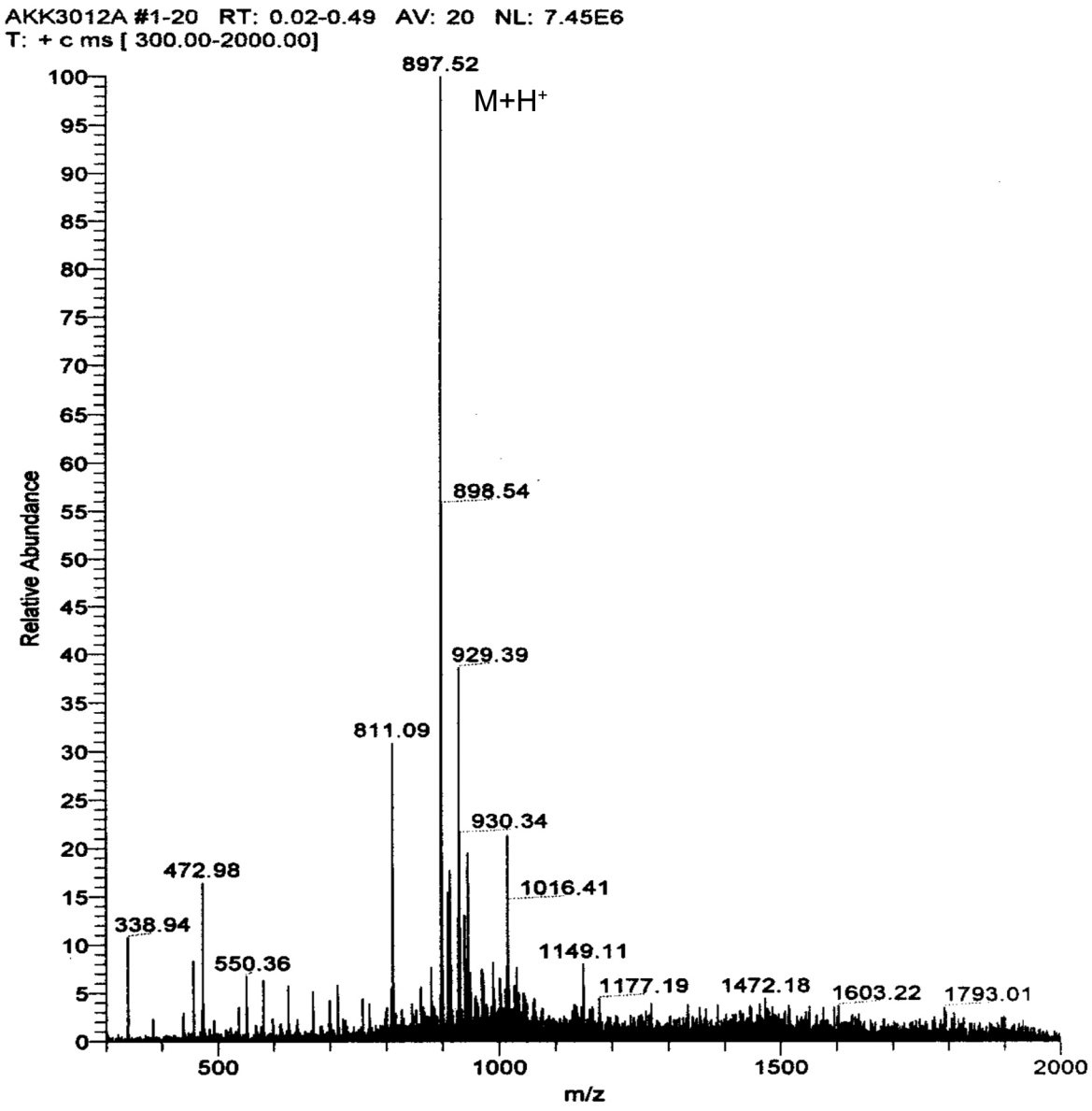
2. Spectral Data.



^{1}H-NMR (400 MHz, $CDCl_3$) of compound 5



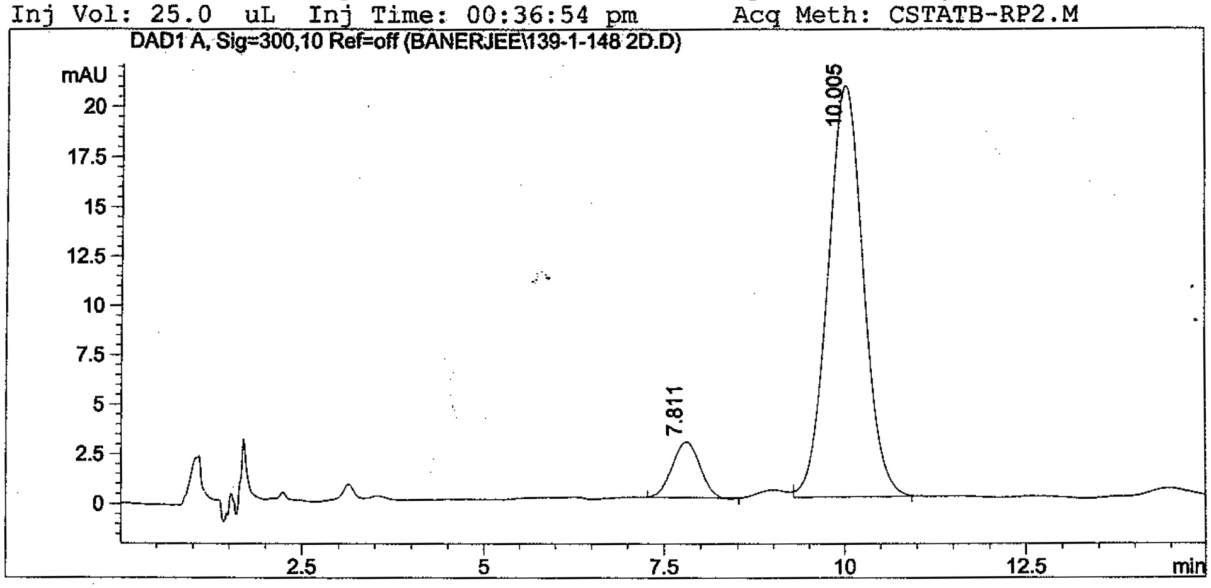
^{13}C-NMR (100 MHz, $CDCl_3$) of compound 5



ESI Mass spectral data of 25-*epi* Ritterostatin G_N1_N, 5

Sample Name: 139-1-148
Comments : 25-epi Ritterostatin GN1N
75/25 MeOH/H20 , 1.0mL/min
Econosphere C18 5u, 4.6x150mm [606020586.1], no guard

Vial No: 50 Inj Date: 07/23/2008 Operator: Douglas Lantrip
Inj Vol: 25.0 uL Inj Time: 00:36:54 pm Acq Meth: CSTATB-RP2.M



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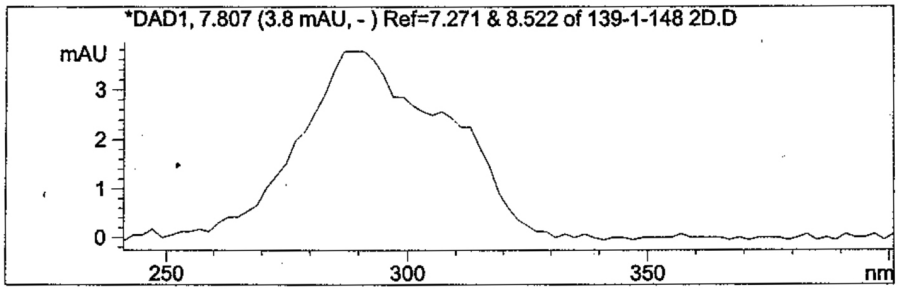
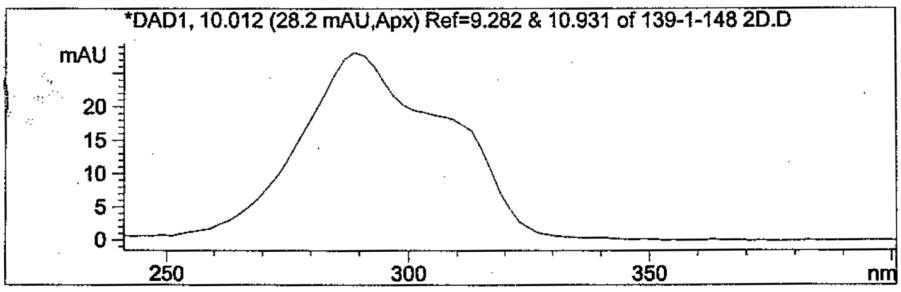
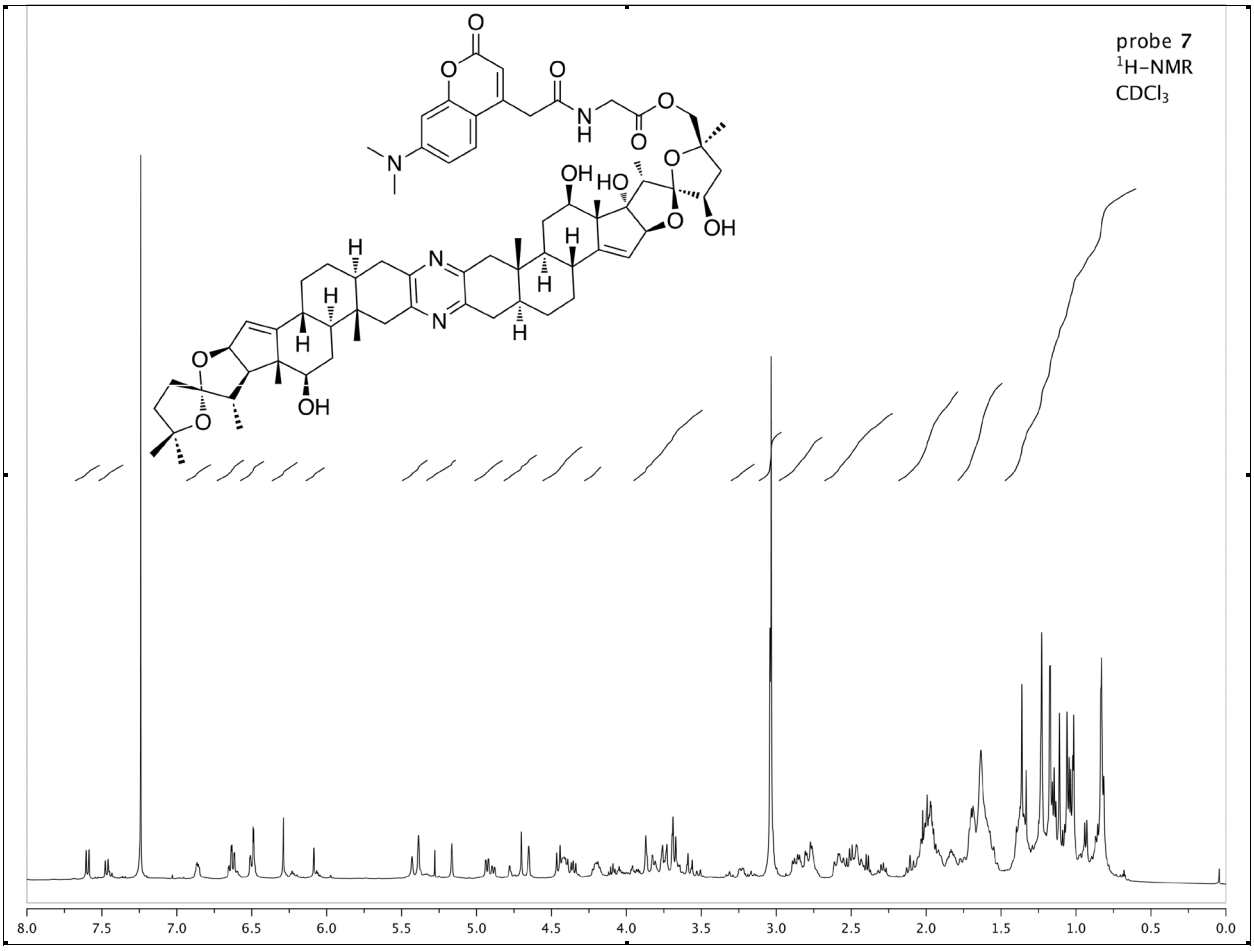
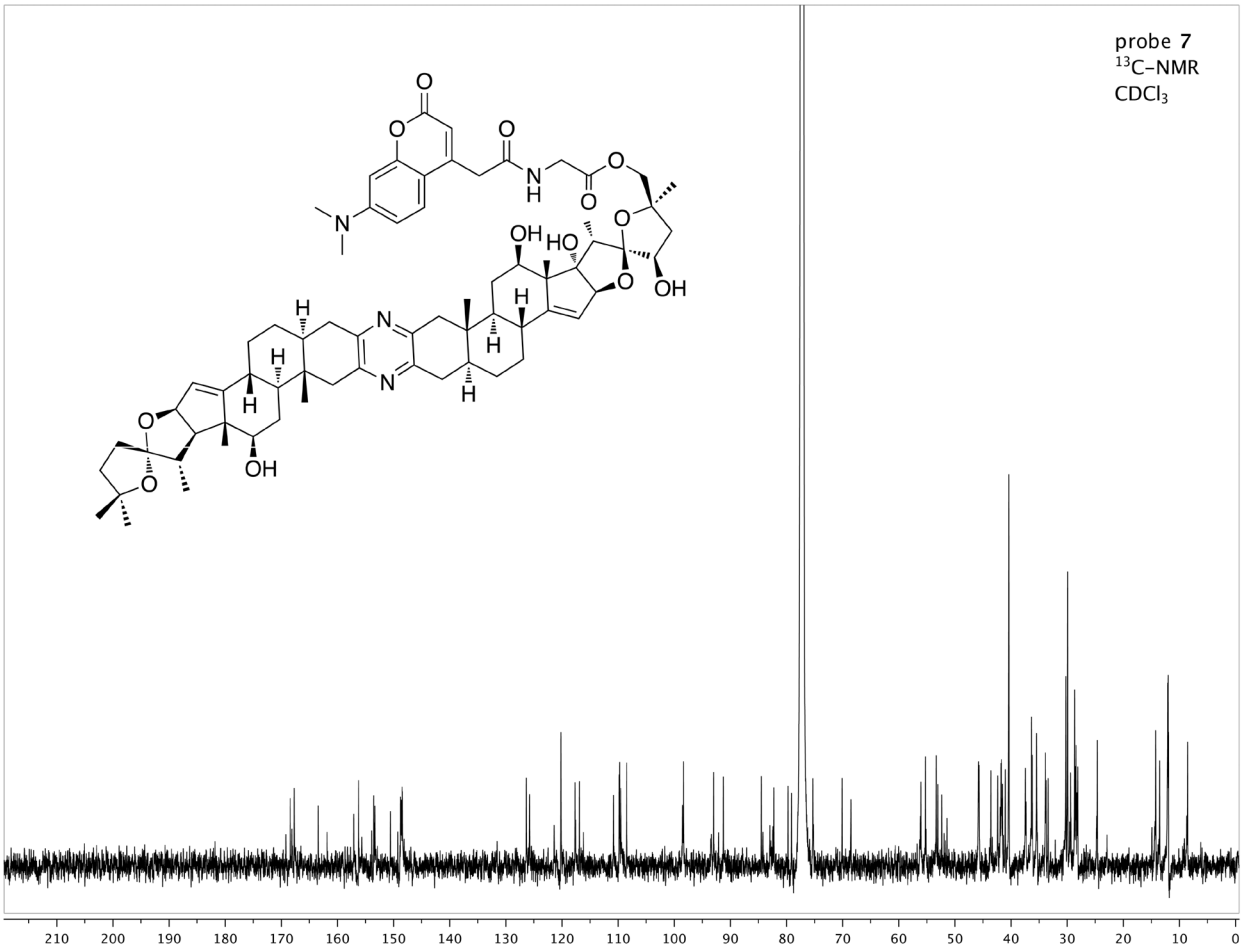
7.81	73	9.2	
10.01	715	90.8	

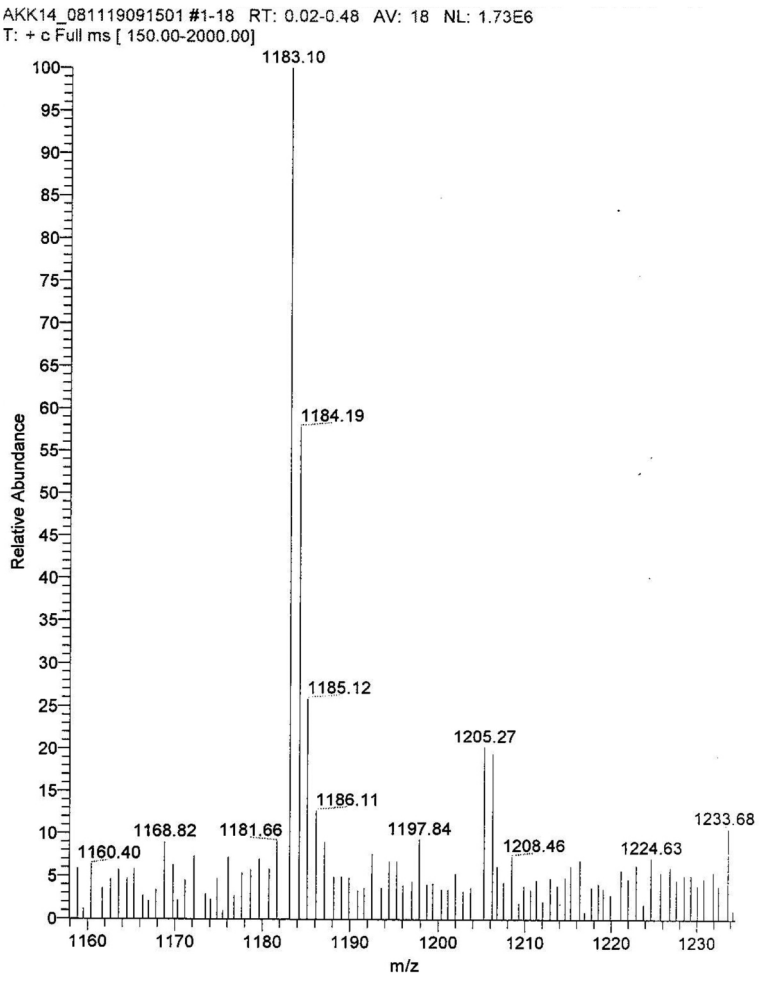
HPLC data of Compound 5



^{1}H NMR (500 MHz, $CDCl_3$) of compound 7



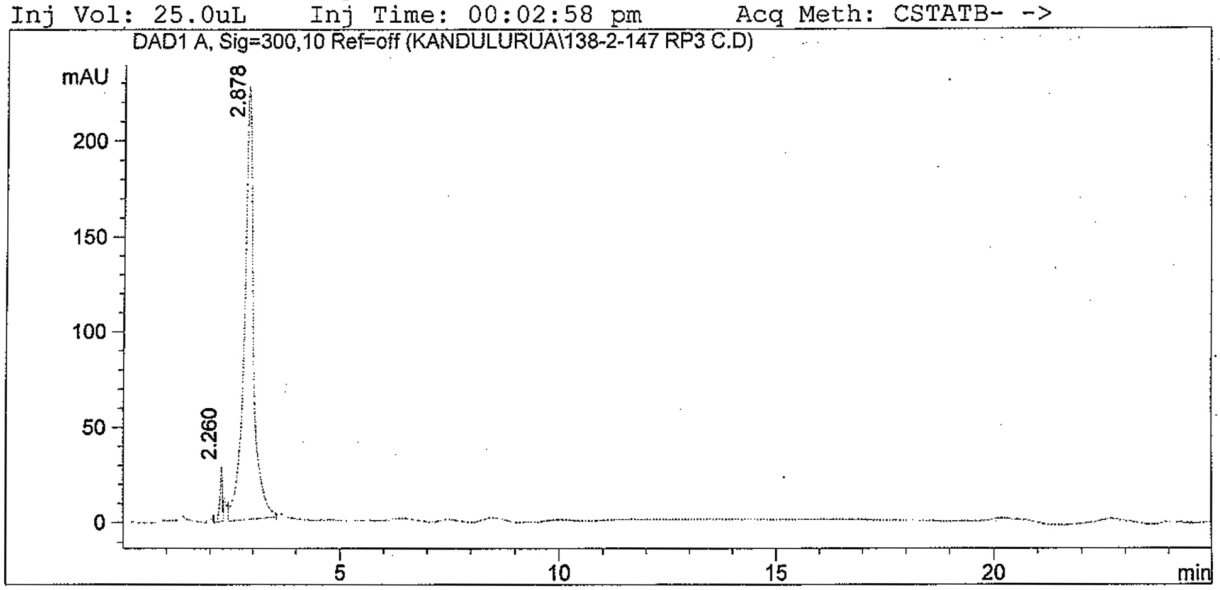
^{13}C NMR (125 MHz, $CDCl_3$) of compound 7

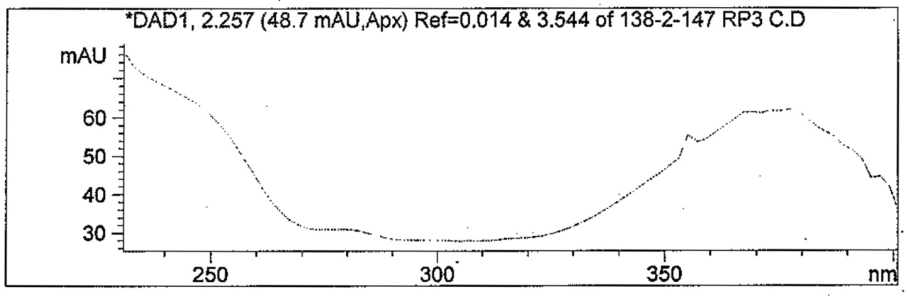
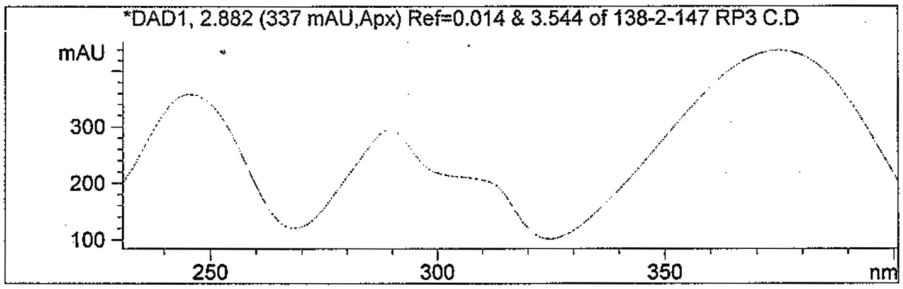


ESI Mass spectral data of compound 7

Sample Name: 138-2-147
Comments : 25-epi-Ritterostatin GN1N-glycine-coumarin
50/50->100/0 MeOH/H20 , 1.0mL/min
Econosphere C18 5u, 4.6x150mm [606020586.1]

Vial No: 74 Inj Date: 11/17/2008 Operator: Douglas Lantrip
Inj Vol: 25.0uL Inj Time: 00:02:58 pm Acq Meth: CSTATB- ->



RT(min)	Area	Area%	Spectra
2.26	116	3.3	
2.88	3459	96.7	

HPLC data of Compound 7

3. Cellular Imaging Studies

3.a. Cell lines. HCT–116 cells (ATCC CCL–247) were propagated in McCoy's SA media (GIBCO–BRL, Invitrogen), supplemented with 10% heat–inactivated fetal calf serum (FCS) and penicillin/streptomycin (GIBCO–BRL, Invitrogen). HeLa cells were cultured in DMEM (GIBCO–BRL, Invitrogen) containing 10% FCS. All cells were incubated at 37 °C in a 5% CO_2 atmosphere. For routine passage, cells were split 1:6 when they reached confluence, generally every 1–3 days.

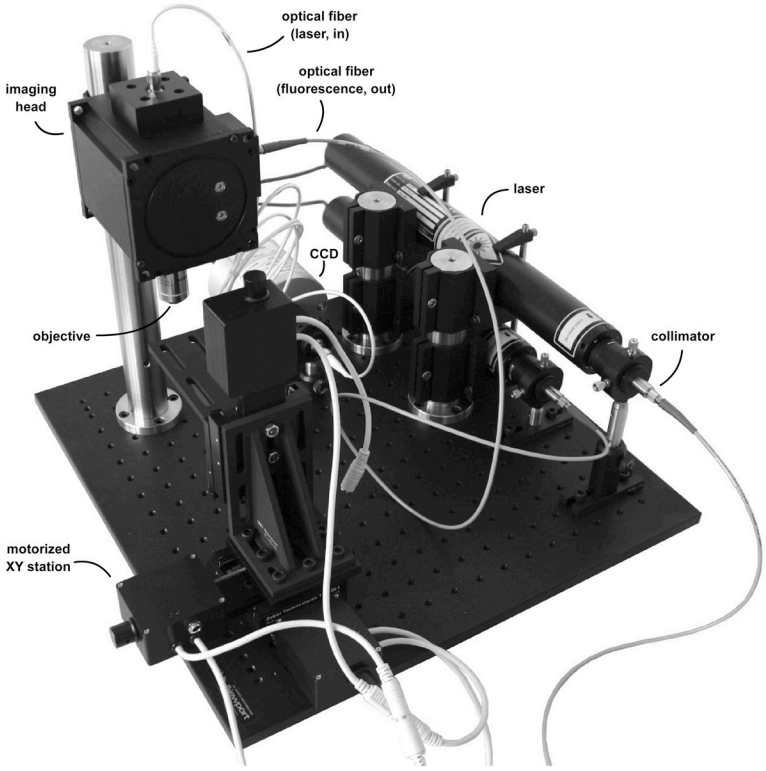


Figure S1. Time course imaging setup including laser or LED (not shown) light sources, imaging head, objective (Plan–Apochromat® 40×/1.25 na and 63×/1.40 na objective), motorized XYZ station (comprised of a Zaber KT-LS-28M and two Zaber T-LS28M), and CCD (CCD-1300QLN, Vosskuehler) camera. Lasers (Thorlabs) or LED (Ocean Optics) light was collimated (Fiber Collimation Package, Thorlabs) and directed to the head via fiber optic cables (Thorlabs) and the fluorescent light was collected using collimation and delivery to the CCD camera fiber optic cabling. The imaging head contained a dichroic mirror (Semrock BrightLine multiband set for DAPI, FITC, and Texas Red on a Zeiss Axio-Vert Filter Cube) capable of multicolor imaging. The bulk of the stands, boards, holders were either purchased from Thorlabs or machined on a CNC machine, polished and anodized in San Diego. For further information as to how to construct this instrument please email Dr. James J. La Clair (i@xenobe.org).

3.b. Time course imaging studies. Cell uptake and localization studies were conducted by treating cells at a density of 10^6 cells/cm^2 in a 35 mm glass–bottom dish (MatTek Corporation). Each compound was added as a 10× stock in media such that the net DMSO content remained under 0.5%. Time course imaging was conducted by incubating the cells at 37 °C under a 5% CO_2 atmosphere with a given concentration of probe and imaging over the course of 24 h. Images were collected on a self-built fiber optic laser-scanning microscope (Fig. S1). Cells were incubated using polycarbonate enclosure (not shown) that provide a sterile atmosphere at 37 °C. The CCD camera and XY motorized workstation were programmed to collect images at specific regions of each plate at select times over each incubation period. In each experiment, greater than 90% of the fluorescence from each probe absorbed within the cells within the first hour and therefore washing was not required. Images were collected at 10 or 15 min intervals with a 500 μs exposure to ensure minimal phototoxicity to the cells or photobleaching of the probes. Co-staining was conducted using established blue-fluorescent ER stain, dragmacidin D (**9**) and a nuclear stain, IAF-labeled nogalamycin **10** as described in the manuscript. Negative controls were conducted using IAF amide **6b** (a control for the dye). Each experiment was repeated 3-6 times and data from these studies are presented in Figure 2 in the manuscript. Select time points from these studies were then subjected to confocal microscopy (see Section 3.c).

3.c. Confocal microscopy. Confocal fluorescent images were collected on a Leica DMI6000 inverted confocal microscope with a Yokogawa spinning disk confocal head, ORCA–ER High Resolution B&W Cooled CCD camera (6.45 μm/pixel at 1×), Plan–Apochromat® 40×/1.25 na and 63×/1.40 na objective (Zeiss). After imaging the cells were fixed by treatment with 500 μL of 2% formaldehyde with 0.2% glutaraldehyde in Dulbecco's Phosphate-Buffered Saline (D-PBS) (2.6 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 8.1 mM Na_2HPO_4) pH 7.2 for 30 min followed by washing three times with D-PBS pH 7.2. Each experiment was repeated 3-5 times and select images are provided in Figure 3 in the manuscript.

3.d. Control Probes. Structures of the control probes are provided in Figure S2.

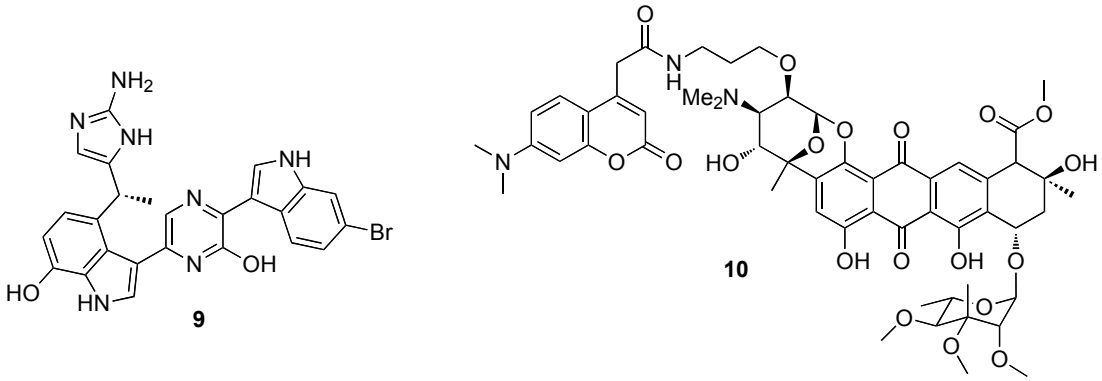


Figure S2. Structures of the blue fluorescent organelle stains dragmacidin D (**9**)[5] and IAF-labeled nogalamycin (**10**)[2]. The use of these probes as blue fluorescent cell stains in tumor cell lines has been described.[6] Samples of **9** were kindly provided by Stoltz group at Caltech (http://stoltz.caltech.edu/).

References:

1) Synthesis of 25-*epi* Ritterostatin G_N1_N (**5**) will be reported in due course.

2) Alexander, M. D.; Burkart, M. D.;Leonard, M. S.; Portonovo, P.; Liang, B.; Ding, X.; Joulli!, M. M.; Gulledge, B. M.; Aggen, J. B.; Chamberlin, A. R.; Sandler, J.; Fenical, W.; Cui, J.; Gharpure, S. J.; Polosukhin, A.;Zhang, H. R.; Evans, P. A.; Richardson, A. D.; Harper, M. K.; Ireland, C. M.; Vong, B. G.; Brady, T. P.;Theodorakis, E. A.; La Clair, J. J. *Chembiochem.,* **2006**, 7, 409.

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5) Garg, N. K.; Sarpong, R.; Stoltz, B. M. *J. Am. Chem. Soc*. **2002**, *124*, 13179-13184.

6) Forsyth, C. J.; Ying, L.; Chen, J.; La Clair, J. J. *J. Am. Chem. Soc*. **2006**, *128*, 3858-3859.