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Supplemental Information

Bright Green Biofluorescence in Sharks

Derives from Bromo-Kynurenine Metabolism

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Transparent Methods

General

^{1}H and 2D (gCOSY, gHSQCAD, and gHMBCAD) NMR spectra were obtained from an Agilent 600 MHz spectrometer with a cold probe (Agilent, Santa Clara, CA, USA) in methanol-d_4 (Cambridge Isotope Laboratories, Inc. Tewksbury, MA, USA). High-resolution (HR) ESI-QTOF-MS data were recorded using an Agilent iFunnel 6550 system with a Phenomenex Kinetex C_{18} (100 Å) 5 µm (250 × 4.6 mm) column. For general HR-ESI-QTOF-MS analysis, extracts were prepared by resuspension in 100 µl methanol (LC-MS grade, Fisher, USA). 2 µl of samples were injected and analyzed at 25 ºC and 0.7 ml/min with a water:acetonitrile gradient solvent system containing 0.1% formic acid: 0-30 min, 10-100% acetonitrile; hold for 5 min, 100% acetonitrile; 2 min, 100-10% acetonitrile; 5 min equilibration time 10% acetonitrile. Mass spectra were acquired in the range of 25-1700 *m/z* in positive ion mode. Collected data was analyzed using Agilent MassHunter Qualitative Analysis Software (Version B.06.00, Agilent Technologies), and all targeted mass ions were extracted and analyzed within a 10 ppm error range [extracted ion count (EIC) chromatograms]. Sep-Pak® Vac 35 cc (10 g) C_{18} cartridge (Waters Corporation, Milford, MA) was used for the flash column chromatography. Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) was performed on an Agilent Prepstar HPLC system. Two reversed-phase columns, an Agilent Polaris C18-A 5 µm (250 × 21.2 mm) column and a Phenomenex Luna C_{18}(2) (100 Å) 10 µm (250 × 10.0 mm) column, were used for the separation and purification of metabolites, respectively. Routine low-resolution HPLC-MS analysis was acquired by using an Agilent 1260 Infinity Quaternary LC system with an autosampler and a photo diode array (PDA) detector coupled to an Agilent 6120 single quadrupole Electrospray Ionization (ESI) mass spectrometer.

Shark skin collection and extraction

Specimens of *Cephaloscyllium ventriosum* were obtained from Marinus Scientific, LLC. This study was approved and carried out in strict accordance with the recommendations in the Guidelines for the Use of Fishes in Research of the American Fisheries Society and the John B. Pierce Laboratory's Institutional Animal Care and Use Committee (IACUC) # VP5-2019. The *Scyliorhinus retifer* skin used in this study was from Gruber et al (2016). A small fragment of the fluorescent skin of each shark species was dissected and used for extraction studies. As the fluorescent molecules are soluble in 0.1 M sodium hydroxide, samples of the shark were cut in 1 cm x 1 cm pieces and allowed to incubate in 0.1 M sodium hydroxide for 1 h before experiments were conducted. Fluorescence spectra of shark skin extracts were recorded on a Hitachi F-7000 Fluorimeter.

Larger-scale extraction and isolation of metabolites

Crude materials from skin of the swell shark were extracted in a 0.1 M sodium hydroxide solution, followed by subsequent extraction of the small molecule fraction with butanol. The small molecules were fractionated by reversed-phase analytical HPLC system, eluting 30 HPLC fractions using a Phenomenex Kinetex C_{18} (2) 5 µm column (250 × 4.6 mm) with a gradient of 0.01% trifluoroacetic acid in acetonitrile/water (Flow rate: 1 ml/min; 0-60 min, 10/90→100/0%, 1 min fraction window). HPLC fraction 12 was further subjected to reversed-phase HPLC [Column: Phenomenex Luna C_{18} (100Å) 10 µm (250 × 4.6 mm)] with a linear gradient in acetonitrile/water mobile phase elution (Flow rate: 1 ml/min; 0-60 min, 10/90→100/0%) to yield a 6-bromo-tryptophan (**5**) (t_R = 10.5 min). The butanol-soluble fraction was subjected to a Sep-Pak® Vac 35 cc (10 g) C_{18} cartridge with a step gradient elution (20%, 40%, 60%, 80%, and 100% methanol in water). The 80% methanol fraction was further fractionated using an Agilent Prepstar HPLC system [Agilent Polaris C_{18}-A 5 µm (250 × 21.2 mm) column] with a linear gradient elution (50-100% acetonitrile in water over 60 min, 8 ml/min, 1 min fraction collection window). The HPLC fraction 18 was purified on an Agilent Phenyl-Hexyl 5 µm (250 × 9.4 mm) column using a linear gradient separation (50-100% acetonitrile in water, 2 ml/min) to yield 6-bromo-DKP (**4**) (t_R = 10.1 min). The purified compounds were dried under reduced pressure on a Genevac HT-4X system over 12 h for NMR analysis. All chemical structures of materials were fully characterized by LC-MS co-injection with standards, 2D-NMR, and MS analysis.

Chemical synthesis of metabolites

6-Bromo-DL-tryptophan was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). To synthesize 8-bromo-DL-*N*-formyl-kynurenine, 6-bromo-DL-tryptophan (50 mg, 0.177 mmol, 1.0 eq) was added to a 10 ml round-bottom flask under air atmosphere. The chemical was suspended in 2 ml water and *meta*-chloroperoxybenzoic acid (0.32 g, 1.854 mmol, 10.5 eq) was subsequently added. The reaction was stirred at room temperature under air atmosphere for 20 h and completion of the reaction was monitored by LC-MS analysis. Upon completion, the reaction was transferred to a 20 ml scintillation vial using methanol and completely dried down using nitrogen gas. The reaction material was fractionated on a Sep-Pak® Vac 35 cc (10 g) C_{18} cartridge with a step gradient elution (20, 40, and 60% methanol in water). The 40% methanol fraction containing 8-bromo-DL-*N*-formyl-kynurenine (t_R = 22.4 min, 3 mg) was then purified using a reversed-phase HPLC with a Phenomenex Luna C_{18} (100Å) 10 µm column (250 × 4.6 mm) and a gradient of 20% to 80% aqueous methanol for 30 min with a flow rate of 2 ml/min. For the deformylation, 8-bromo-DL-*N*-formyl-kynurenine (2 mg, 0.006 mmol, 1.0 eq) was dissolved in a 1:1 mixture of trifluoroacetic acid:water (600 µl). The reaction was stirred for 4 h at room temperature and dried using nitrogen

gas. The 8-bromo-DL-kynurenine (t_R = 24.3 min, 2 mg) was then isolated on a Phenomenex Luna C_{18} (100Å) 10 µm column (250 × 4.6 mm) at a flow rate of 2 ml/min with a linear gradient from 10%-80% over 30 min. Syntheses of enantiopure 8-bromo-L-*N*-formyl-kynurenine (**2**) and 8-bromo-L-kynurenine (**1**) were conducted as above, but with using enantiopure 6-bromo-L-tryptophan (**5**) as starting material. For the deamination, 8-bromo-DL-kynurenine (2 mg) was dissolved in anhydrous dimethylformamide (1 ml) and the reaction was initiated at 70 °C and completed after 3 h. The reaction mixture was dried and was purified by reversed-phase HPLC [Phenomenex Luna C_{18} (100Å) 10 µm column (250 × 4.6 mm), 2 ml/min] with a linear gradient of 10% acetonitrile/0.1% formic acid-100% acetonitrile/0.1% formic acid in water over 60 min with a flow rate of 2 ml/min to yield 8-bromo-CKA (carboxyketoalkene) (**3**) (t_R = 36.1 min, 0.6 mg).

For the chemical synthesis of compound **8**, 7-bromoquinoline-2-carboxylic acid (**8a**) was purchased from Millipore Sigma (St. Louis, MO, USA). Compound **8a** (200 mg) was suspended in a solvent mixture (acetone/water = 10:1). Methyl iodide (300 µl) and potassium carbonate (600 mg) were added and refluxed for 2 h. The reaction mixture was dried and extracted with ethyl acetate to yield **8b** (~200 mg). Reduction of **8b** in glacial acetic acid (5 ml) was performed utilizing sodium cyanoborohydride (150 mg) at 25 °C for 3 h. The reaction mixture was neutralized with saturated sodium bicarbonate followed by the ethyl acetate extraction twice. The crude reaction materials were directly treated with acetic anhydride (8 ml) and heated at 90 °C for 3 h and was separated by reversed-phase HPLC [Phenomenex Luna C_{18} (100Å) 10 µm column (250 × 4.6 mm), 2 ml/min] with a linear gradient of 20% acetonitrile/0.1% formic acid-60% acetonitrile/0.1% formic acid in water for 30 min with a flow rate of 2 ml/min to yield **8d** (t_R = 16.2 min, 110 mg). The benzylic oxidation on **8d** (80 mg) was initiated with chromium hexacarbonyl (50 mg) and *tert*-butyl hydroperoxide (70 wt% in water, 600 µl) in acetonitrile and refluxed for 24 h. The product **8e** was eluted at t_R = 16.5 min under the aforementioned purification condition for **8d**. The deprotection of **8e** (15 mg) was achieved in water containing potassium carbonate (20 mg) and refluxed for 2 h, yielding **8** (13 mg).

Biotransformation of 6-bromo-tryptophan to 8-bromo-kynurenine in *E. coli*

E. coli Nissle 1917 (EcN) colonies were used to inoculate 5 ml of lysogeny broth (LB) (BD, Franklin Lakes, NJ, USA; 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) sodium chloride) for a total of three biological replicates and incubated at 37 °C with shaking at 250 rpm for 16 h. After overnight, 20 µl of each seed culture was used to inoculate 2 ml of M9 minimal medium (VWR Funding Inc., West Chester, PA, USA; 0.6% (w/v) Na_2HPO_4, 0.3% (w/v) KH_2PO_4, 0.05% (w/v) NaCl, 0.1% (w/v) NH_4Cl, 0.2% (w/v) glucose, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$)

supplemented with 10 mM 6-bromo-D-tryptophan, 10 mM 6-bromo-L-tryptophan, 1 mM 6-bromo-D-tryptophan, 1 mM 6-bromo-L-tryptophan, or nothing (negative control). The inoculated cultures were grown at 37 °C with shaking at 250 rpm. After 20 h cultivation, each culture was extracted with 3 ml butanol, vortexed, and centrifuged. The organic layers were dried and resuspended in 80 µl methanol for LC-MS analysis. These experiments were performed with biological triplicates. Addition of the amino acids required solubilization in 1N sodium hydroxide, followed by neutralization with 1N hydrochloric acid.

Fluorescence spectra

Fluorescence spectra from all compounds **1**-**8** were measured at a concentration of 5 mM in methanol on a Hitachi-F-7000 Spectrophotometer.

***In situ* and microscope fluorescence imaging**

To excite a fluorescence response of the sharks, a Aquatica Rouge underwater housing was fitted with custom designed blue excitation lighting. The LED light (Royal Blue) was collimated to ensure its perpendicular incidence on the scientific grade 450/70 nm interference filter surface (Semrock, Inc., Lake Forest, IL), minimizing the transmission of out-of-band energy. The ultra-bright LEDs, collimating lenses, filters, and exit diffusers were contained in custom-made water- and pressure-proof housings and powered by NiMH Battery Packs (Ikelite Underwater Systems, Indianapolis, IN). To image and record biofluorescence, a scientific-grade 514 nm long-pass emission filter (Semrock, Inc.) was placed in front of the sensor of the camera. Cross-sections of both sharks were taken on an AxioZoom v16 stereo fluorescent microscope using a PlanNeoFluar Z 2.3×/0.57 objective and 38 HE GFP filter set.

Marfey's analysis for absolute configuration determination

Dried 6-bromo-DKP (diketopiperazine) (**4**) (~0.1 mg) was acid-hydrolyzed in 1 N hydrochloric acid (250 µl) at 110 °C for 1 h with stirring. After cooling down, the hydrolysate was dried under the purge of nitrogen gas. The standards D- and L-6-bromo-tryptophan were purchased from LabNetwork, Inc. (South Portland, ME, USA). The standards D- and L-8-bromo-kynurenines and 8-bromo-*N*-formyl-kynurenines were prepared as described in the chemical synthesis section. The dried hydrolysates, naturally-purified compounds, and commercial standards, were treated with 25 µl of a solution of N_{α}-(2,4-dinitro-5-fluorophenyl)-L-alaninamide (FDAA) (10 mg/ml in acetone) followed by the addition of 100 µl of 1 N sodium bicarbonate to yield L-FDAA derivatized compounds. The reactions were heated at 80 °C for 3 min and neutralized with 25 µl of 2 N hydrochloric acid. The Marfey's derivatives were then diluted to 20-100 µl methanol for single quadrouple LC-MS and/or HR-ESI-QTOF-MS analysis. 2-10 µl

samples were analyzed using a Phenomenex Kinetex C_{18} (100Å) 5 µm (250 × 4.6 mm) column with a flow rate (0.7 ml/min) and a solvent system of water and acetonitrile containing 0.1% formic acid (v/v). The retention times of L-FDAA derivatized compounds were determined as follows: a linear gradient elution with 50-100% acetonitrile, 6.2 min for L-FDAA derivatized 6-bromo- L-tryptophan, 6.8 min for L-FDAA derivatized 6-bromo-D-tryptophan, 6.5 min for L-FDAA derivatized 8-bromo- L-kynurenine, and 7.1 min for L-FDAA derivatized 8-bromo-D-kynurenine.

Antibacterial activity of metabolites

Antibacterial activities of metabolites were evaluated using the minimum inhibitory concentration (MIC) assay in 96-well plates. Stock solutions of metabolites were prepared in sterile dimethyl sulfoxide with a concentration of 10 mM. Dimethyl sulfoxide was used as a negative control, and chloramphenicol was prepared as positive control for the antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Vibrio parahaemolyticus*. The stock solutions of compounds (10 mM) were serially diluted and adjusted to ten different final concentrations (100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39, and 0.19 µM) in the same concentration of fresh dimethyl sulfoxide. MRSA was grown in tryptic soy broth (TSB) and *V. parahaemolyticus* was grown in Marine Broth. Media (50 µl) containing compound at the varying concentrations was added to each well. Overnight cultures of the bacteria were diluted to OD_{600} = 0.05, 50 µl of the cell culture broth was then dispensed into individual wells, and the plates were sealed and incubated at 37 °C overnight. Plates were then read for OD_{600} using a PerkinElmer Envision 2100 multimode plate reader (PerkinElmer, Waltham, MA, USA). All samples were tested in triplicate.

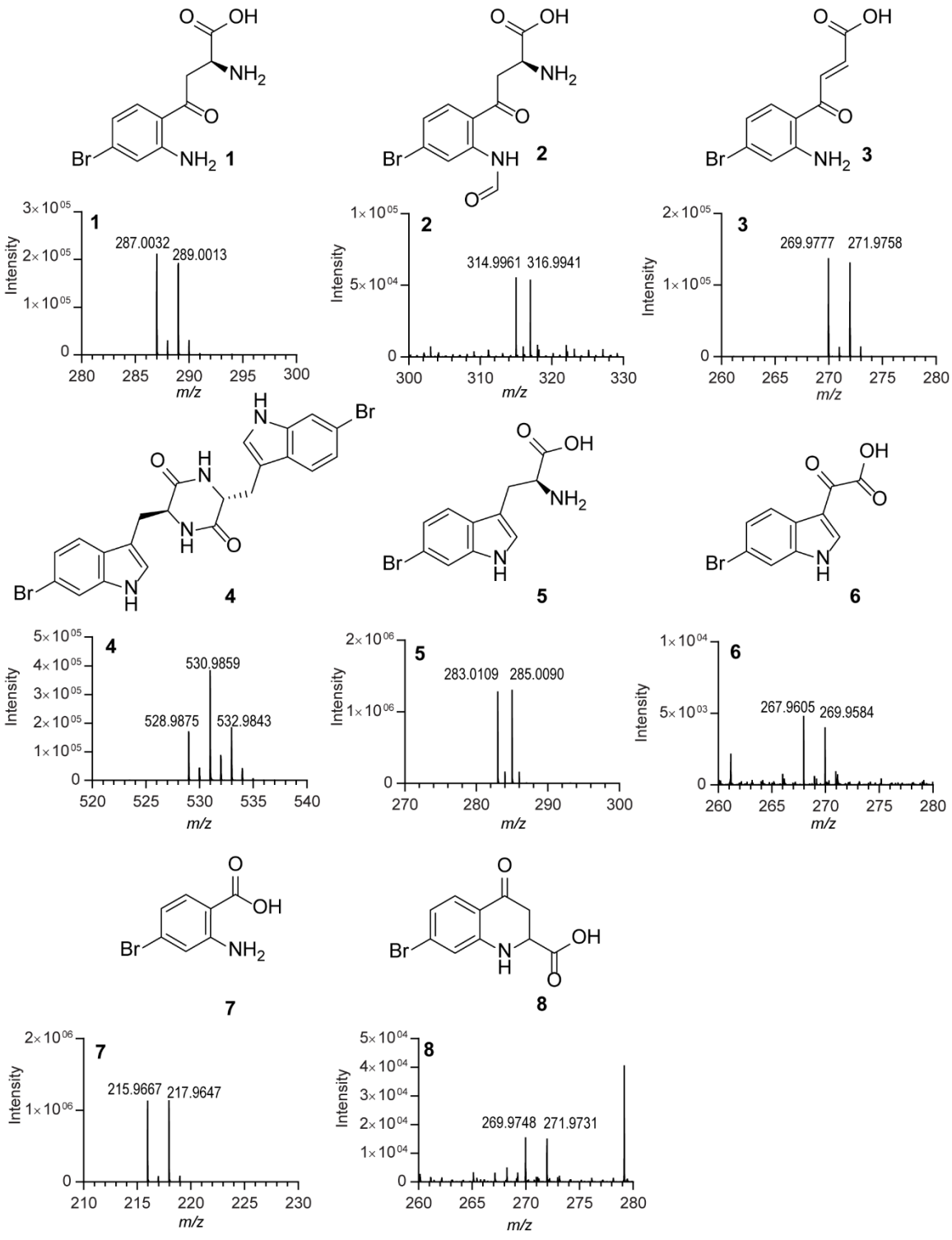

Figure S1. HR-ESI-QTOF-MS spectra of metabolites **1**-**8**. Related to Figure 2.

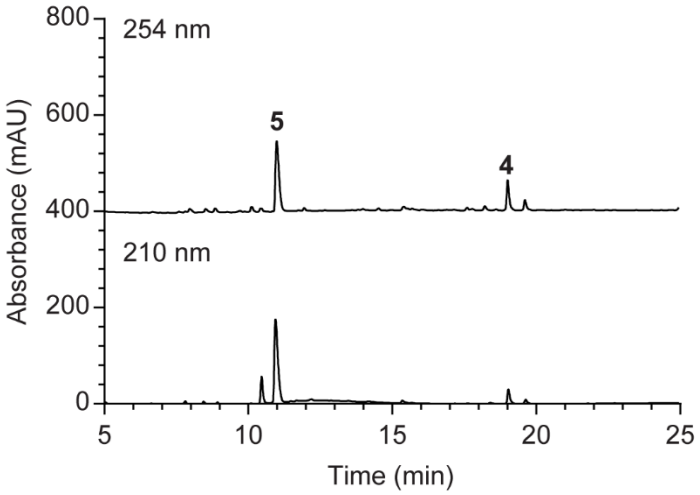

Figure S2. HPLC chromatogram of crude extract from the skin of *C. ventriosum*. The 10 mg of crude samples were dissolved in 100 µl methanol and 10 µl of samples were analyzed for HPLC-MS analysis. This figure shows the two major metabolites, **4** and **5** (210 and 254 nm spectra are shown). Related to Figure 2.

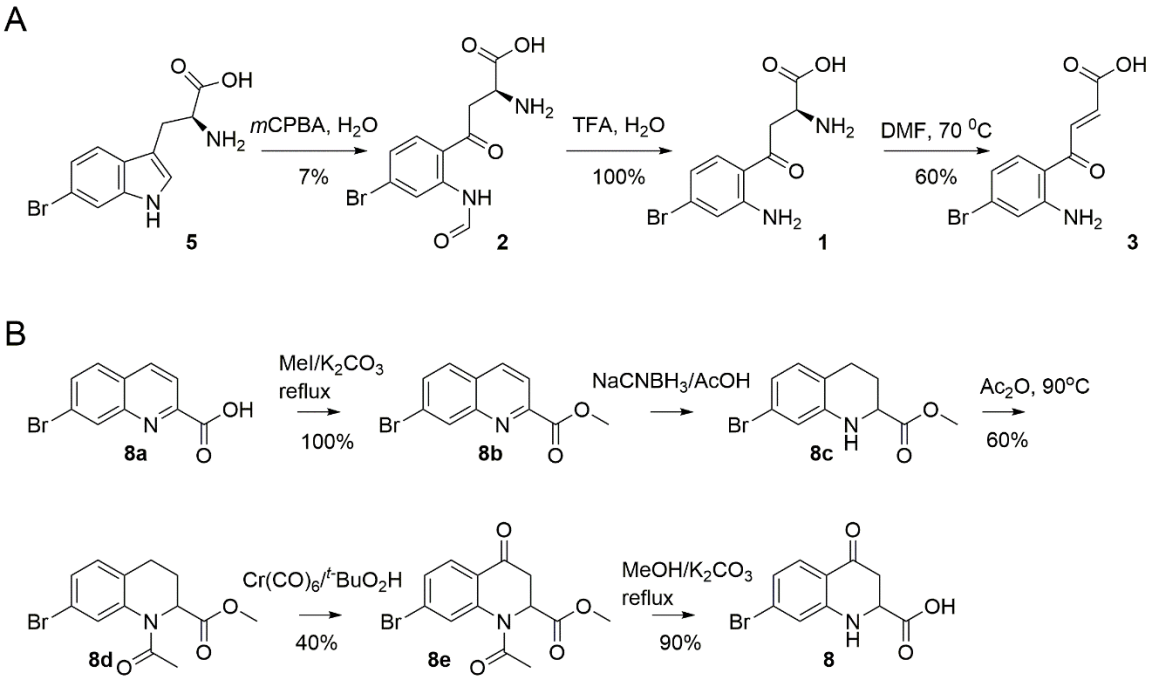

Figure S3. Synthetic scheme of metabolites **1**-**3** (A) and **8** (B). Related to Figure 2.

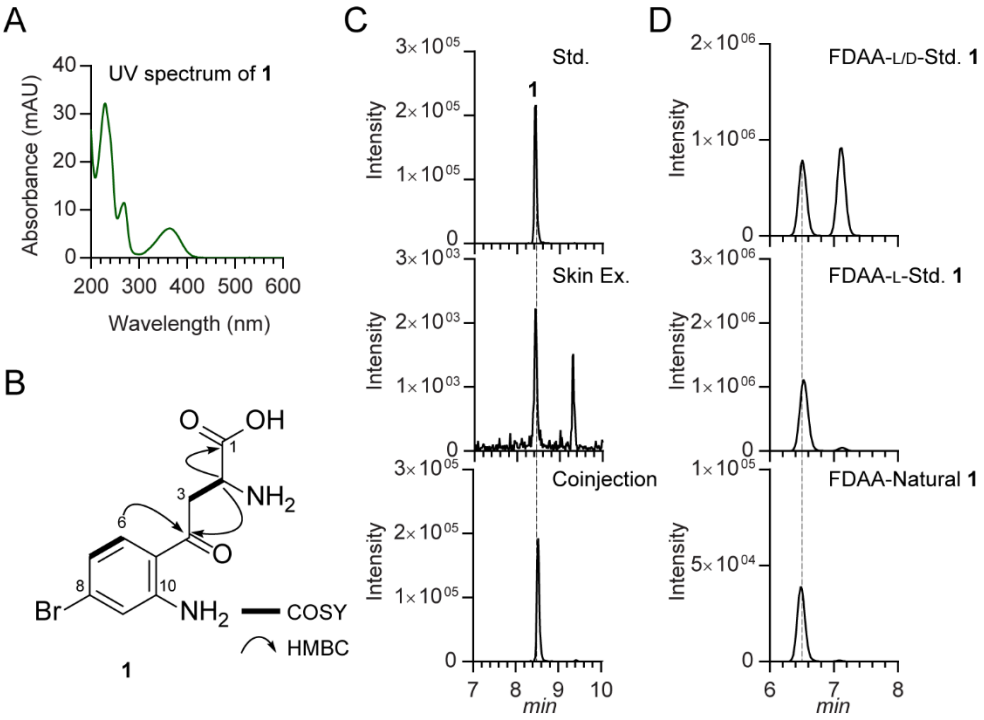

Figure S4. Structural characterization of **1**. (A) UV-visible spectrum of purified **1**. (B) Key 2D NMR (COSY and HMBC) correlations of **1**. (C) Co-injection studies using skin extract and standard **1**. (D) Marfey's analysis. Related to Figure 2.

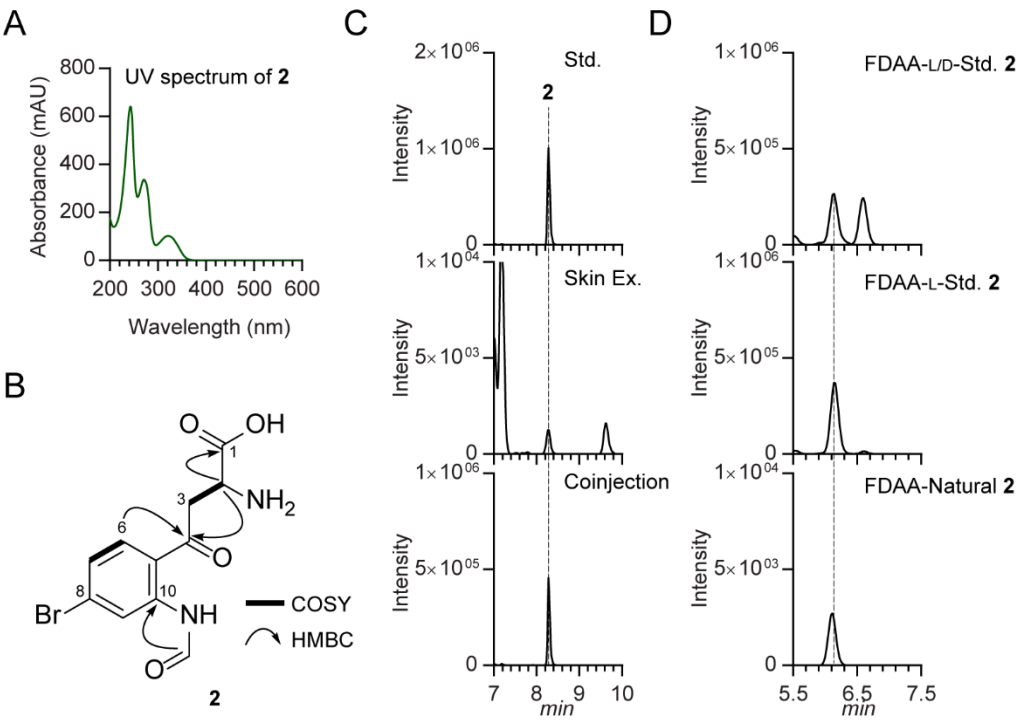

Figure S5. Structural characterization of **2**. (A) UV-visible spectrum of purified **2**. (B) Key 2D NMR (COSY and HMBC) correlations of **2**. (C) Co-injection with skin extract and standard **2**. (D) Marfey's analysis. Related to Figure 2.

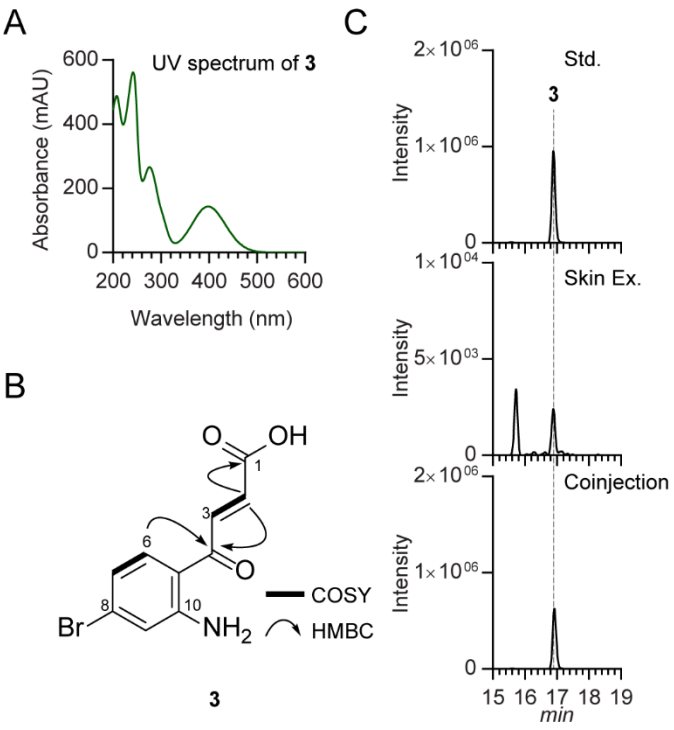

Figure S6. Structural characterization of **3**. (A) UV-visible spectrum of purified **3**. (B) Key 2D NMR (COSY and HMBC) correlations of **3**. (C) Co-injection with skin extract and standard **3**. Related to Figure 2.

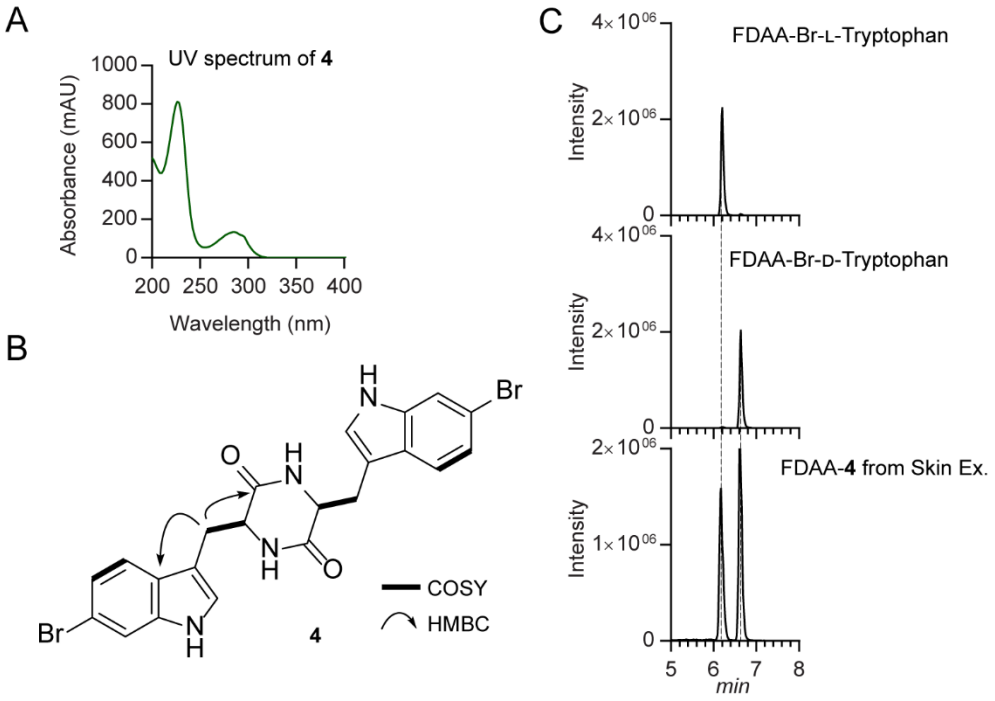

Figure S7. Structural characterization of **4**. (A) UV-visible spectrum of purified **4**. (B) Key 2D NMR (COSY and HMBC) correlations of **4**. (C) Marfey's analysis. Related to Figure 2.

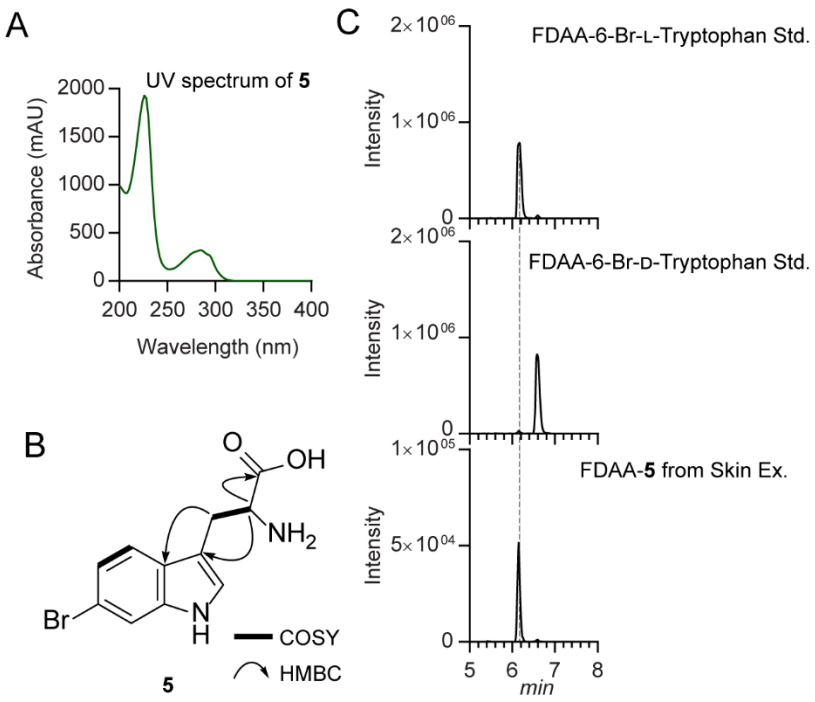

Figure S8. Structural characterization of **5**. (A) UV-visible spectrum of purified **5**. (B) Key 2D NMR (COSY and HMBC) correlations of **5**. (C) Marfey's analysis. Related to Figure 2.

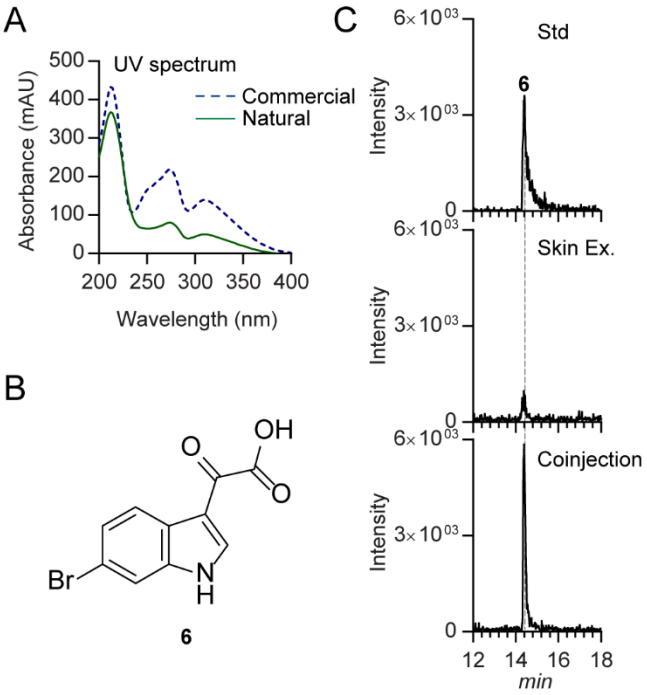

Figure S9. Structural characterization of **6**. (A) UV-visible spectrum of **6**. (B) Chemical structure of **6**. (C) Co-injection with skin extract and standard **6**. Related to Figure 2.

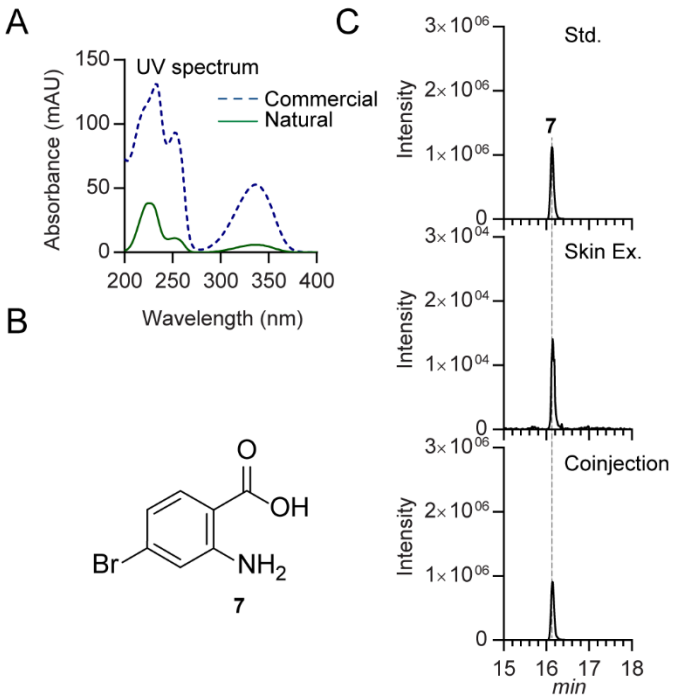

Figure S10. Structural characterization of **7**. (A) UV-visible spectrum of **7**. (B) Chemical structure of **7**. (C) Co-injection with skin extract and standard **7**. Related to Figure 2.

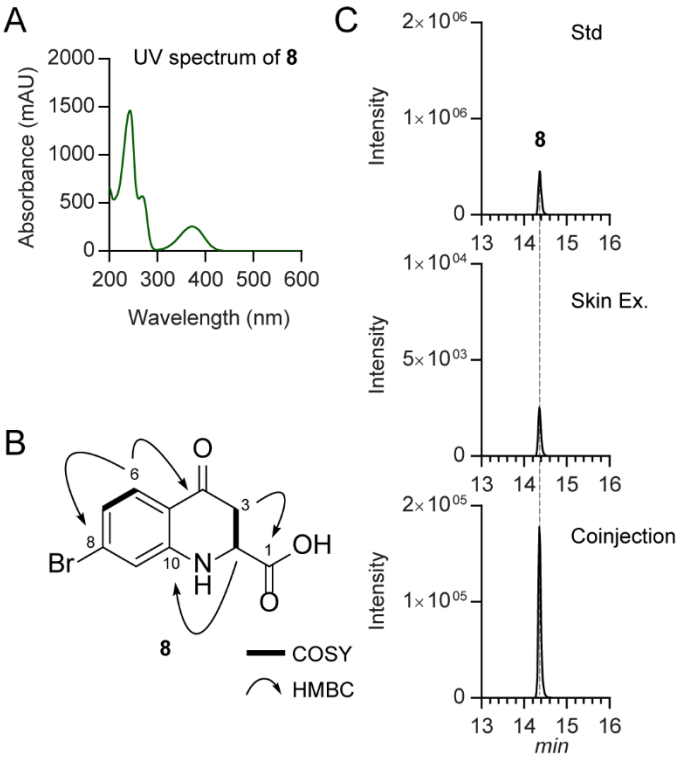

Figure S11. Structural characterization of **8**. (A) UV-visible spectrum of **8**. (B) Key 2D NMR (COSY and HMBC) correlations of **8**. (C) Co-injection with skin extract and standard **8**. Related to Figure 2.

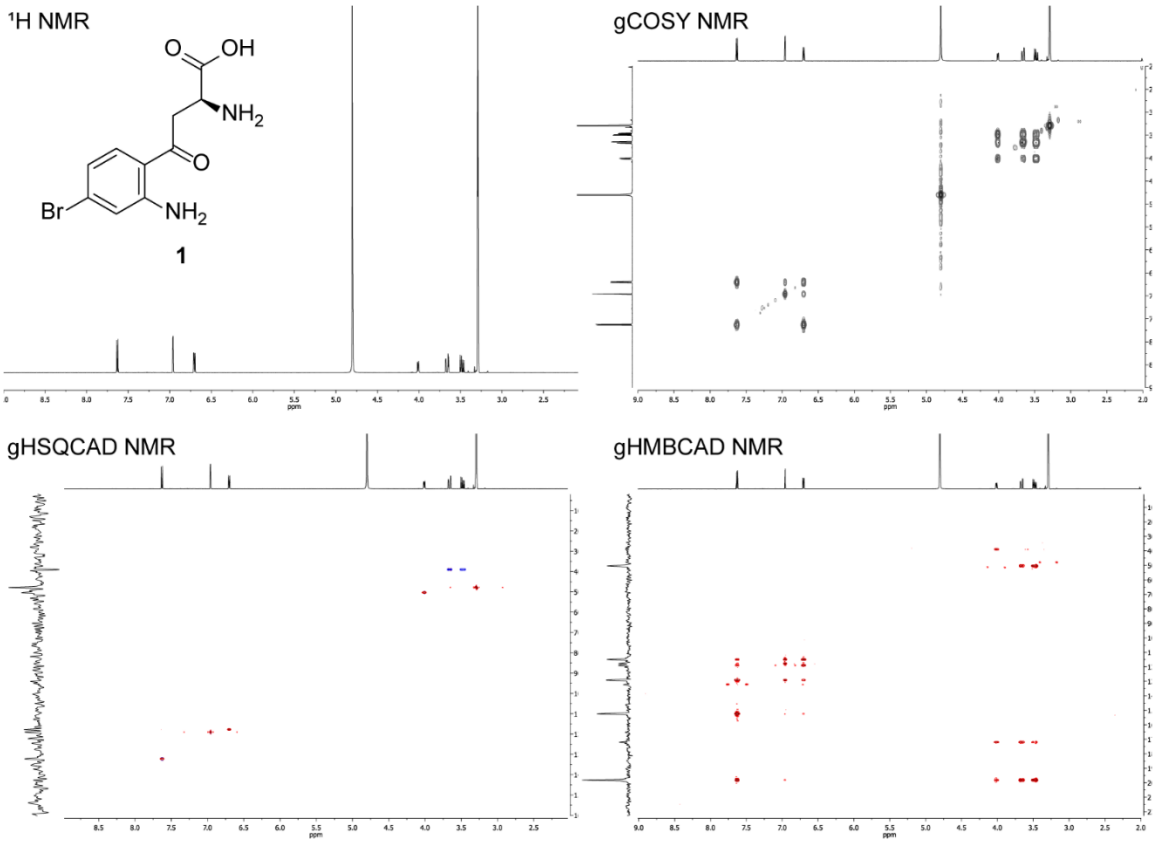

Figure S12. NMR spectra of **1**. Related to Figure 2.

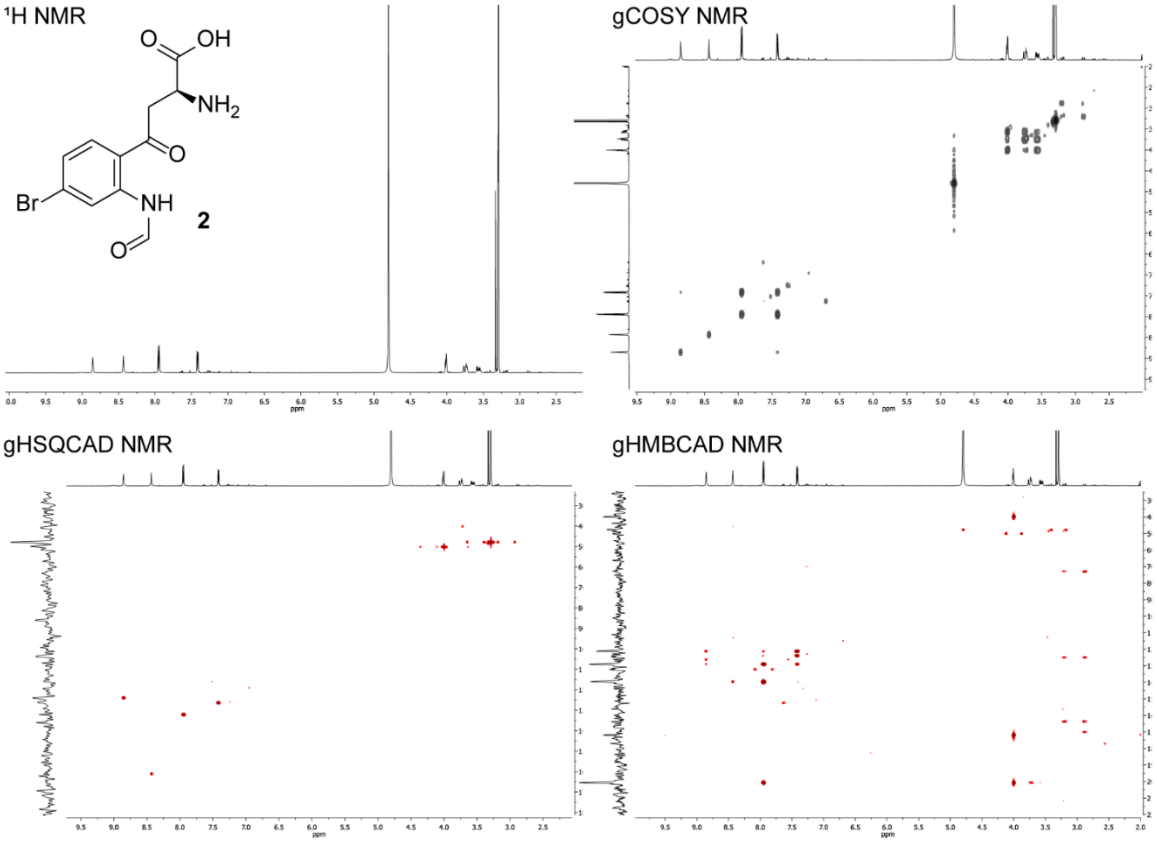

Figure S13. NMR spectra of **2**. Related to Figure 2.

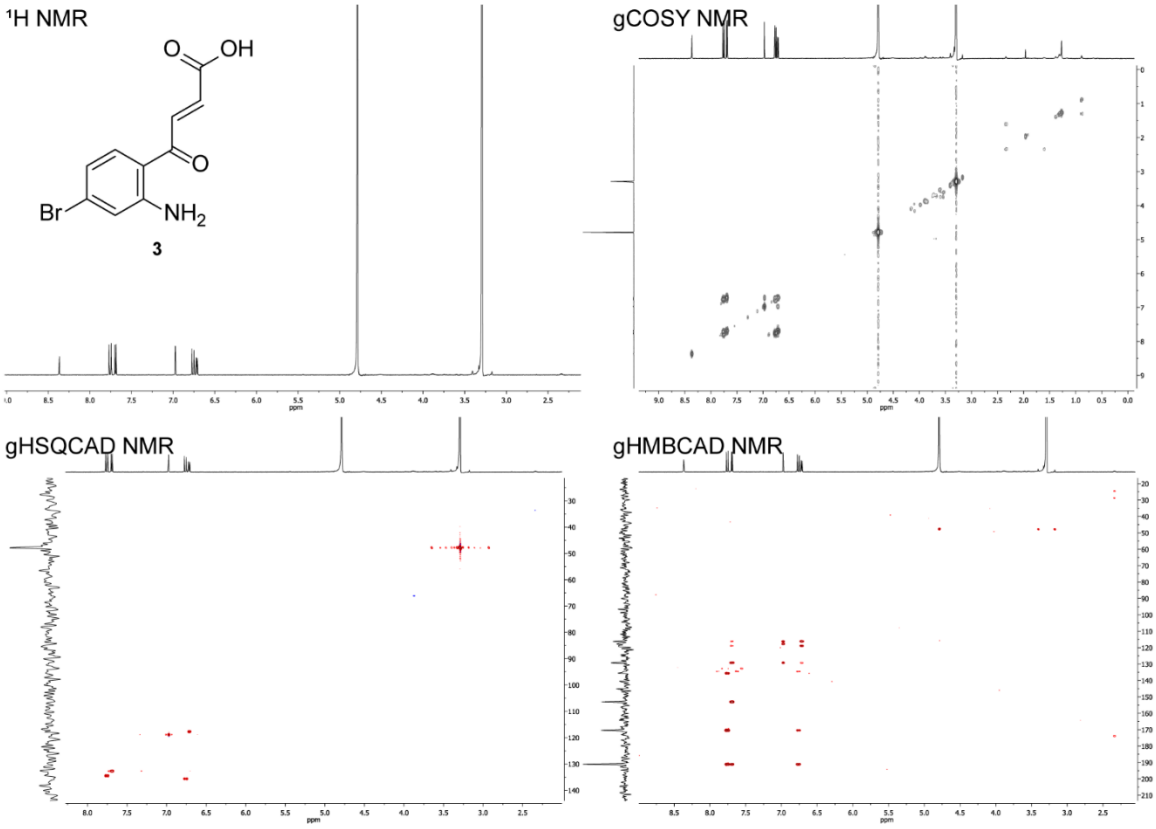

Figure S14. NMR spectra of **3**. Related to Figure 2.

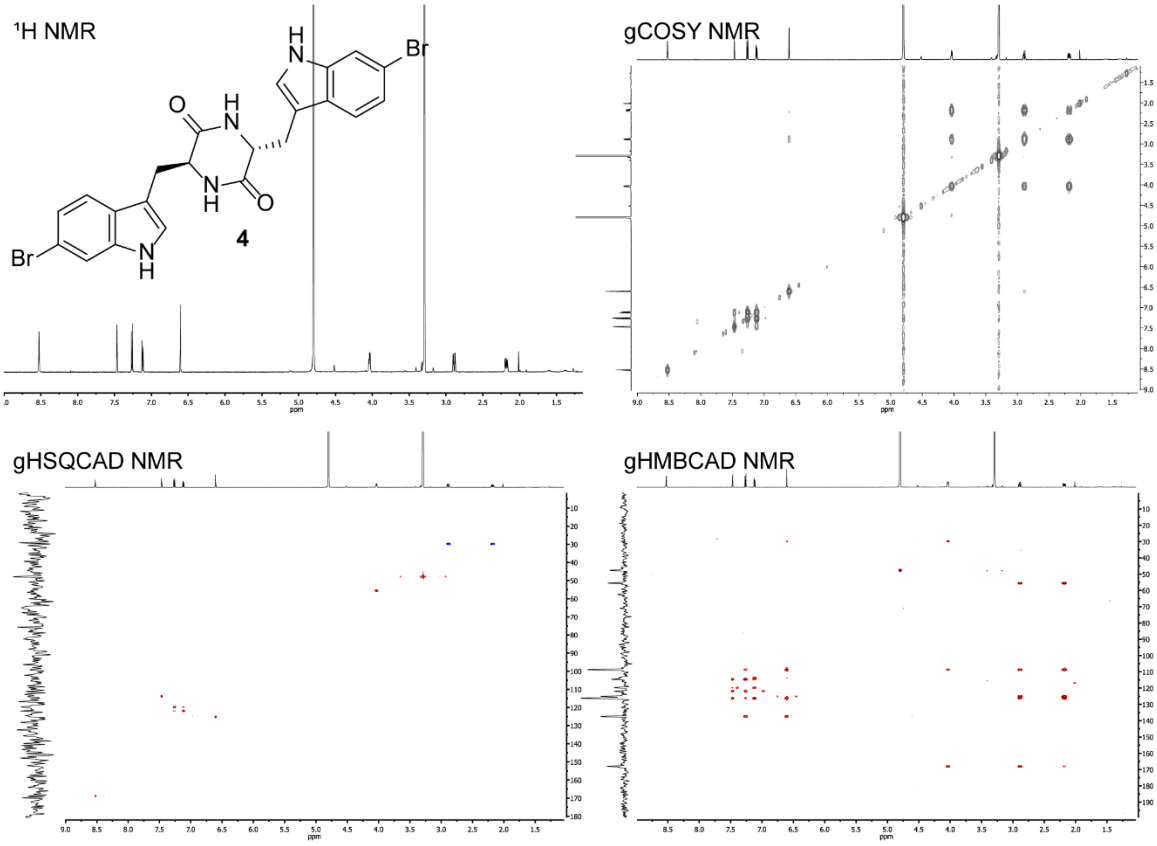

Figure S15. NMR spectra of **4**. Related to Figure 2.

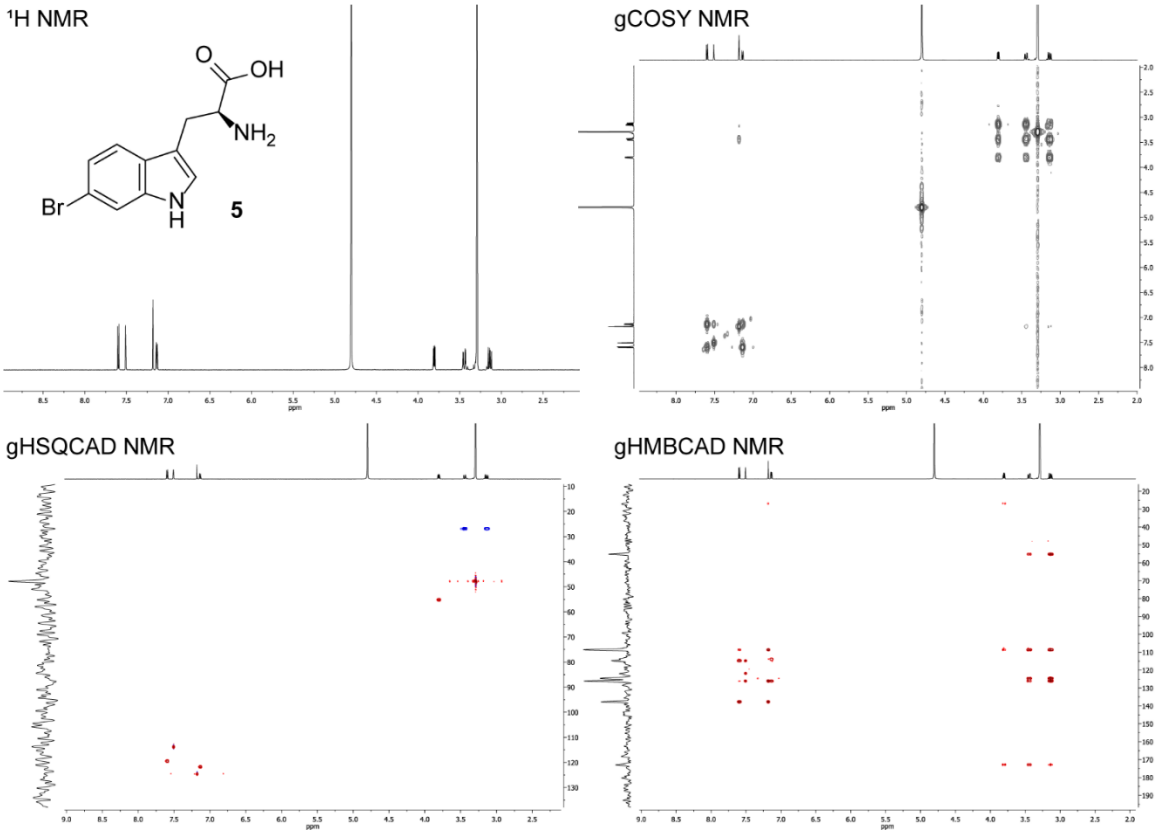

Figure S16. NMR spectra of **5**. Related to Figure 2.

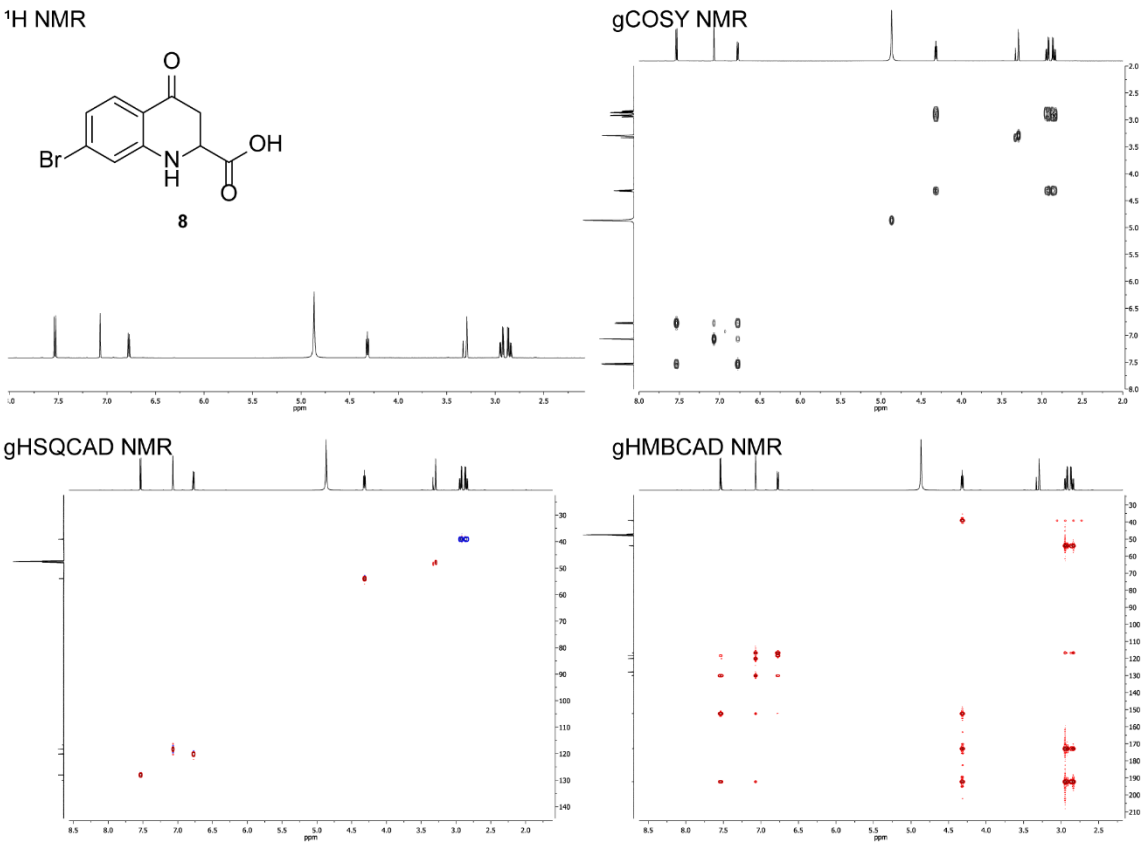

Figure S17. NMR spectra of **8**. Related to Figure 2.

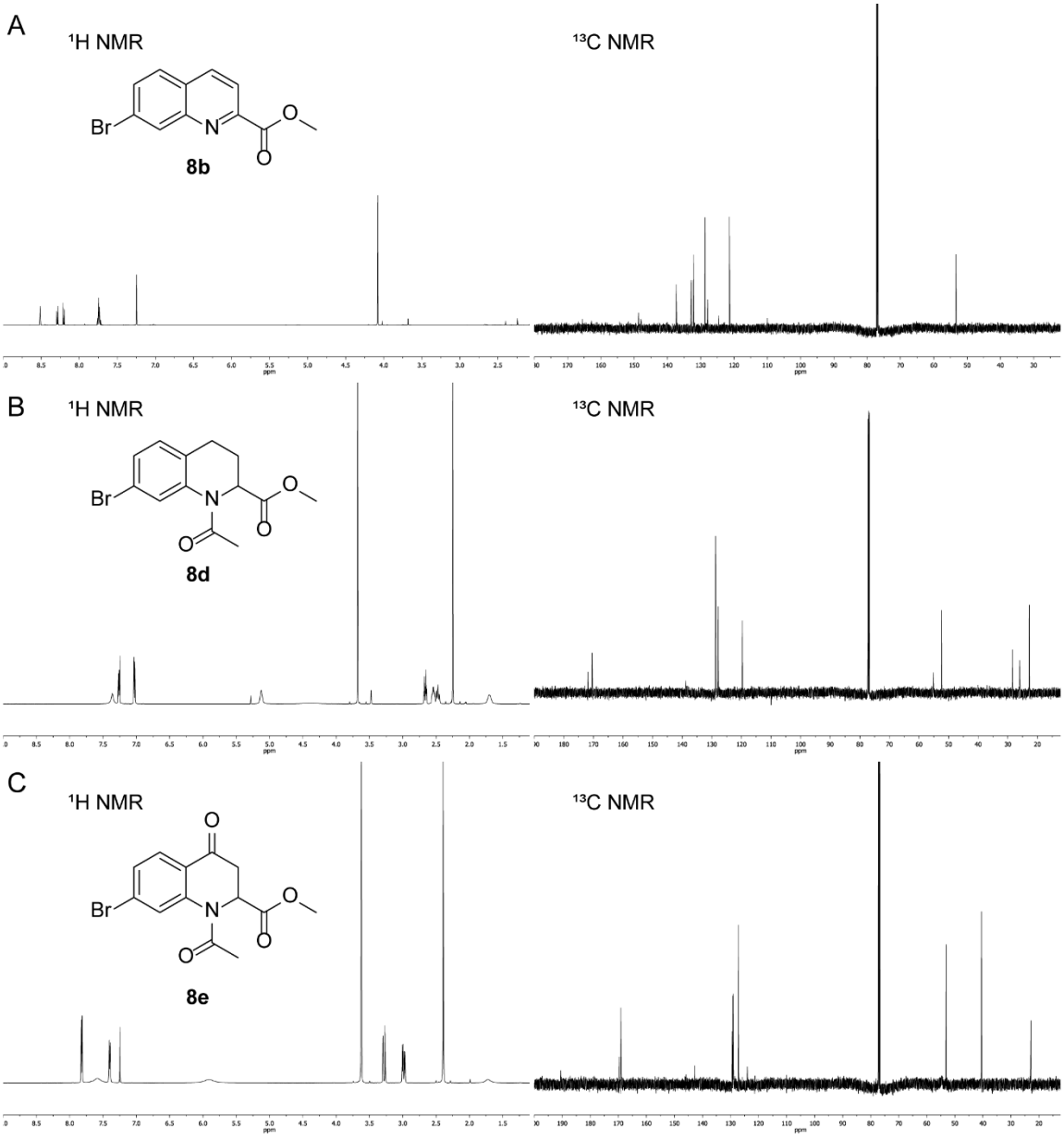

Figure S18. NMR spectra of synthetic intermediates **8b** (A), **8d** (B), and **8e** (C). Related to Figure 2.

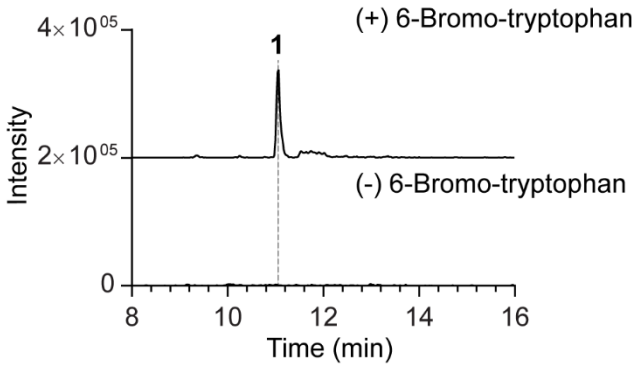

Figure S19. HPLC-MS analysis for the production of 8-bromo-kynurenine (**1**) from *E. coli* culture in the presence and absence of 6-bromo-tryptophan. Related to Figure 4.

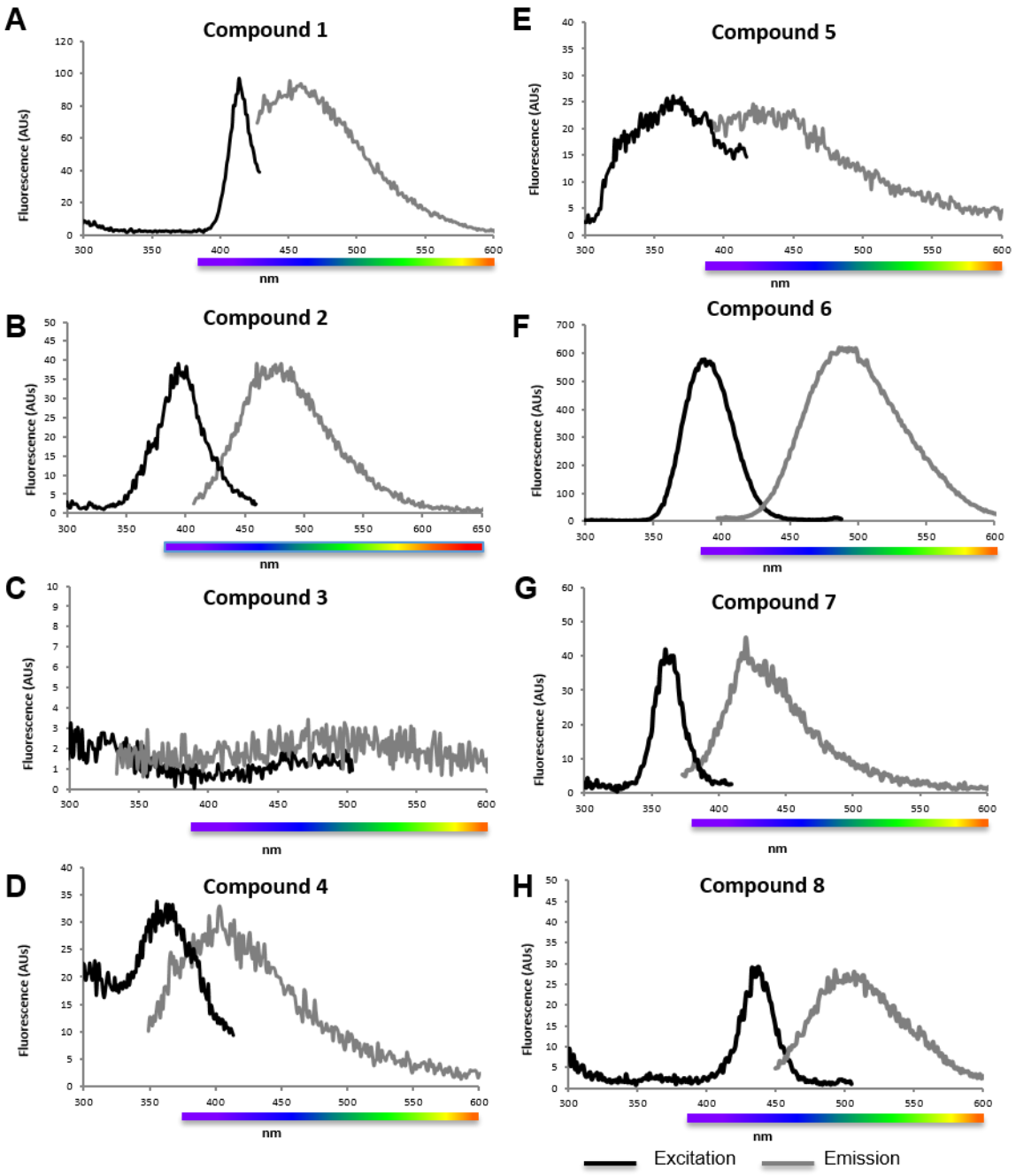

Figure S20. Excitation and emission spectra of compounds **1**-**8** in Phosphate Buffered Saline (PBS). Related to Figure 5.

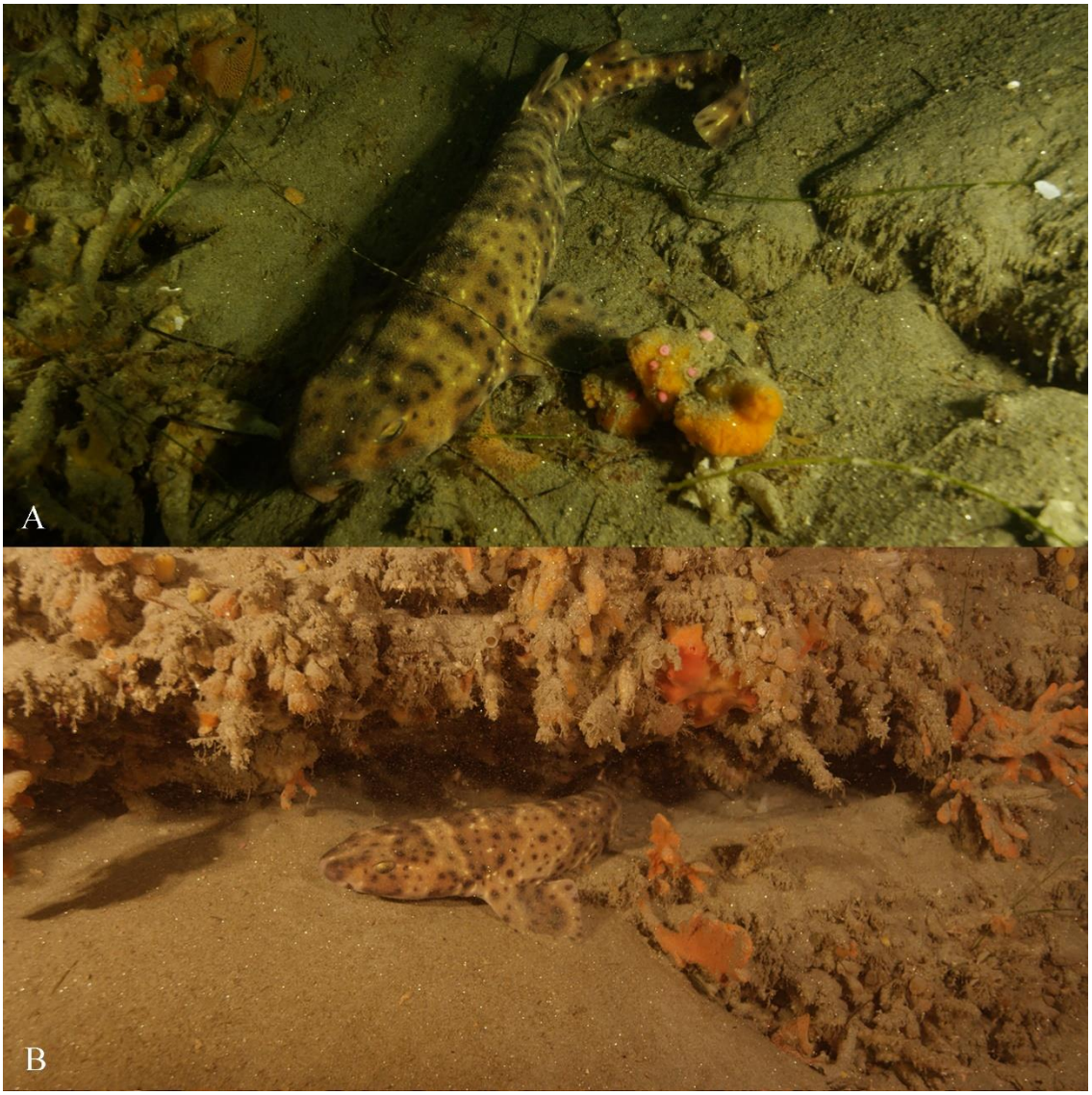

Figure S21. *Cephaloscyllium ventriosum* imagined in their natural habitat in the eastern Pacific, demonstrating their sedentary bottom-dwelling nature. Related to Figure 6.

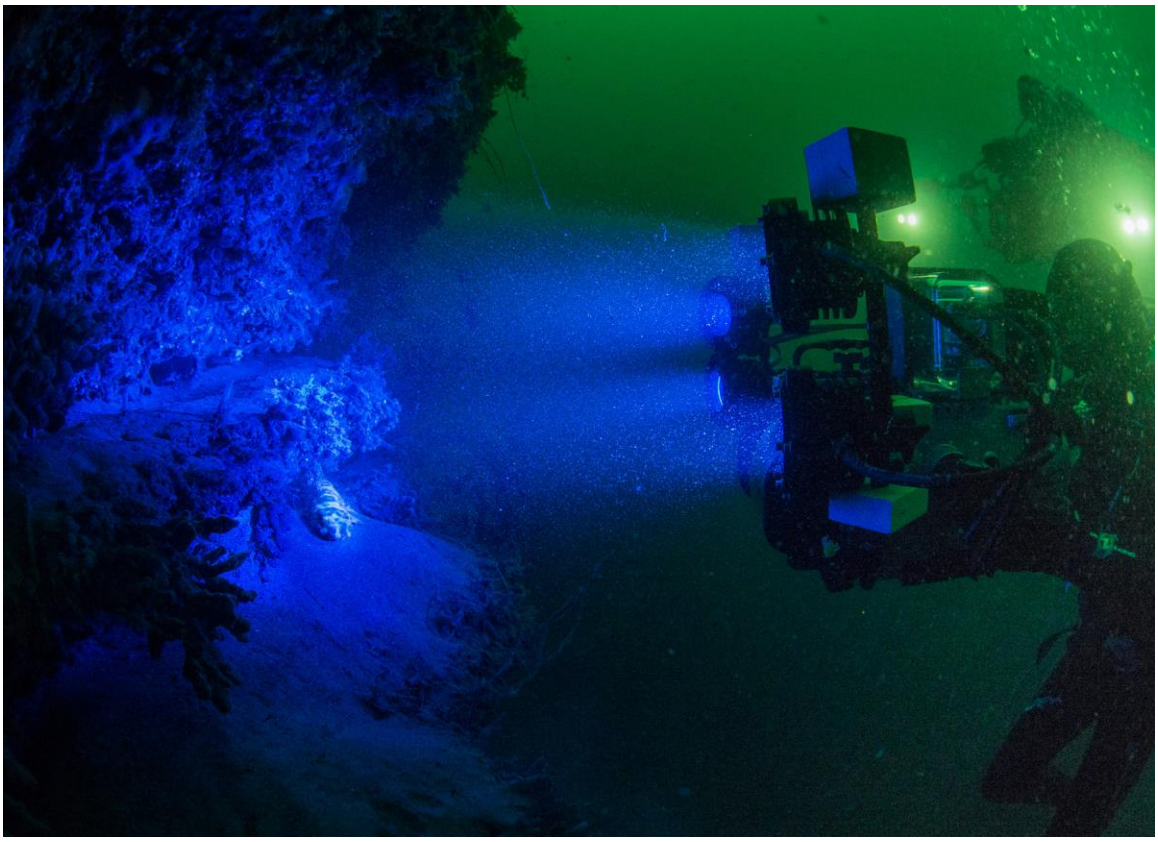

Figure S22. Fluorescent imaging camera system used for *Cephaloscyllium ventriosum* in its natural habitat in the eastern Pacific (30 m, Scripps Canyon, CA). Related to Figure 6.

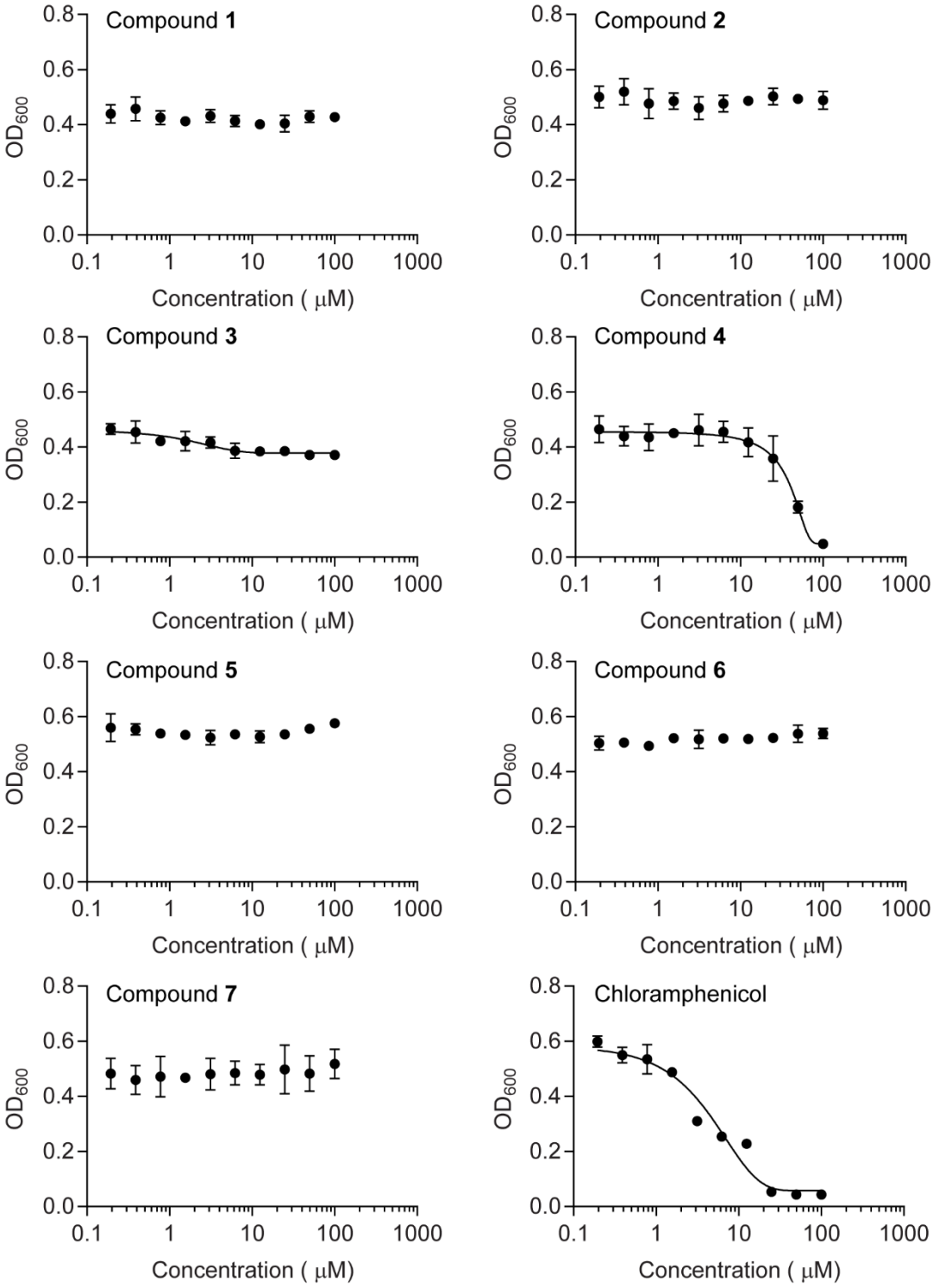

Figure S23. Growth inhibitory assay of metabolites against methicillin-resistant *S. aureus* (MRSA). Related to Figure 6.

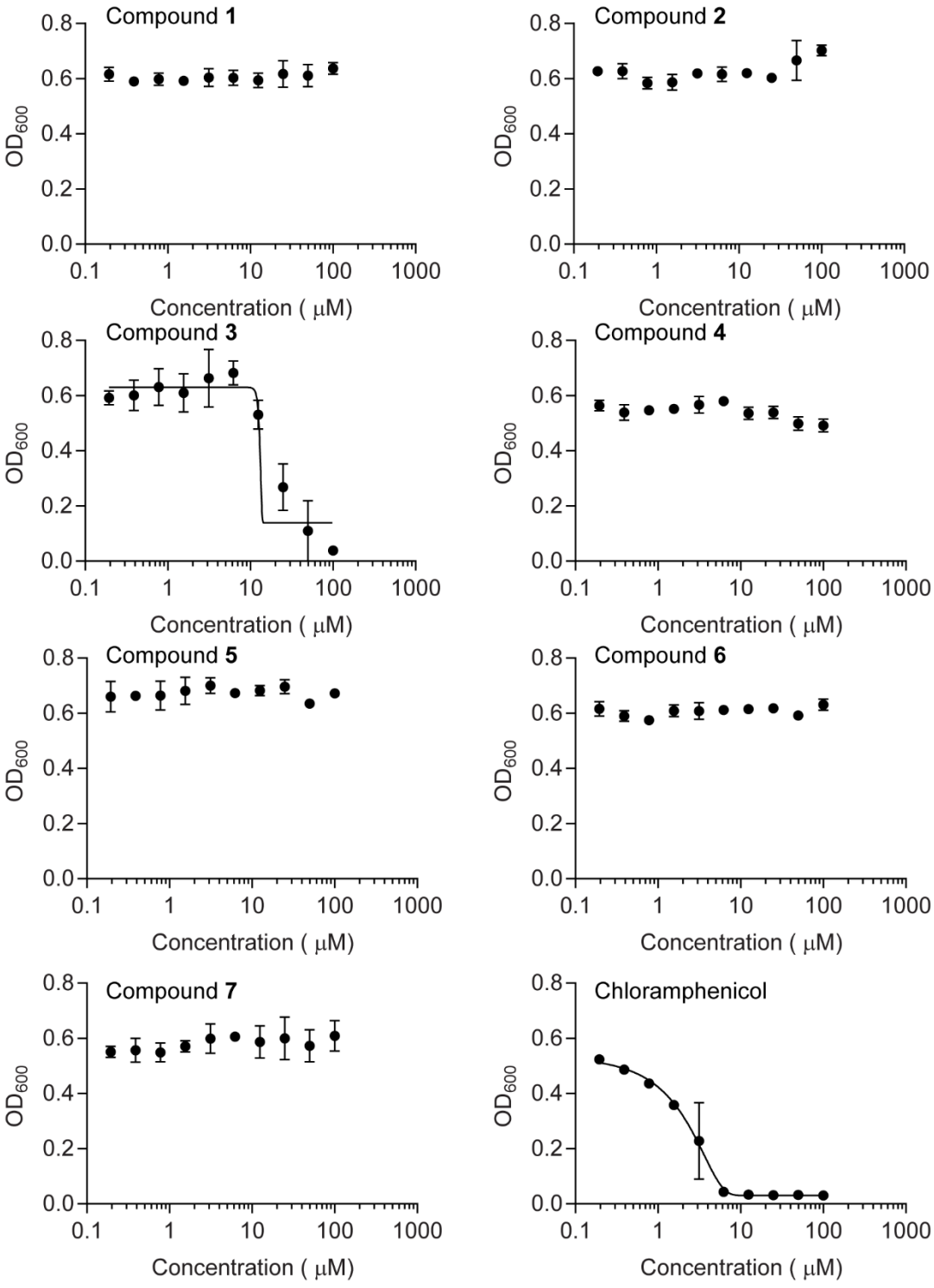

Figure S24. Growth inhibitory assay of metabolites against *Vibrio parahaemolyticus*. Related to Figure 6.

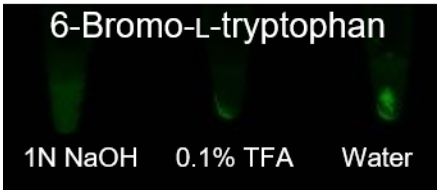

Figure S25. Solubility of 6-bromo-L-tryptophan in varying solvents. Figure shows fluorescence image of 6-bromo-L-tryptophan from typhoon instrument with a scanning progress of Alexa Fluor 488. Related to Figure 4.

Table S1. HR-ESI-QTOF-MS data of metabolites **1**-**8**. Related to Figure 2.

No	Obsd.			Calcd.			Chemical formula
	$[M]^+$	$[M+2]^+$	$[M+4]^+$	$[M]^+$	$[M+2]^+$	$[M+4]^+$	
1	287.0032	289.0013		287.0031	289.0011		$C_{10}H_{11}BrN_2O_3$
2	314.9991	316.9971		314.9980	316.9960		$C_{11}H_{11}BrN_2O_4$
3	269.9777	271.9758		269.9766	271.9745		$C_{10}H_9BrNO_3$
4	528.9875	530.9859	532.9843	528.9875	530.9854	532.9834	$C_{22}H_{18}Br_2N_4O_2$
5	283.0109	285.0090		283.0082	285.0062		$C_{11}H_{11}BrN_2O_2$
6	267.9605	269.9584		267.9609	269.9589		$C_{10}H_6BrNO_3$
7	215.9667	217.9647		215.9660	217.9640		$C_7H_6BrNO_2$
8	269.9748	271.9731		269.9766	271.9745		$C_{10}H_9BrNO_3$

Table S2. NMR assignments of compounds **1**-**3** in MeOD. Related to Figure 2.

	Compound **1**			Compound **2**			Compound **3**		
No	δ_H mult (*J*, Hz)	δ_C		δ_H mult (*J*, Hz)	δ_C		δ_H mult (*J*, Hz)		
1		172.0	C		172.0	C		170.6	C
2	4.01 dd (9.0, 2.9)	50.4	CH	4.01 dd (8.5, 3.3)	50.2	CH	6.76 d (15.4)	135.7	C H
3	3.66 dd (18.5, 2.9) 3.48 dd (18.5, 9.0)	39.0	CH_2	3.75 dd (18.6, 3.3) 3.57 dd (18.6, 8.5)	39.9	CH_2	7.76 d (15.4)	134.5	C H
4		198.1	C		200.7	C		191.1	C
5		115.0	C		121.2	C		116.2	C
6	7.63 d (8.7)	132.2	CH	7.95 d (8.5)	132.2	CH	7.69 d (8.7)	132.8	C H
7	6.70 dd (8.7, 2.0)	117.8	CH	7.42 dd (8.5, 2.0)	126.4	CH	6.72 dd (8.7, 1.9)	118.0	C H
8		129.2	C		129.2	C		129.2	C
9	6.96 d (2.0)	119.0	CH	8.85 brs	124.0	CH	6.97 d (1.9)	119.0	C H
10		152.5	C		139.8	C		153.1	C
N-formyl				8.43 brs	161.1	CH			

Table S3. NMR assignments of compounds **4** and **5** in MeOD. Related to Figure 2.

	Compound **4**				Compound **5**		
Position	δ_H mult (*J*, Hz)	δ_C		Position	δ_H mult (*J*, Hz)	δ_C	
1, 1'			NH	1			NH
2, 2'	6.60 s	125.1	CH	2	7.18 s	124.6	CH
3, 3'		108.7	C	3		108.7	C
3a, 3a'		126.2	C	3a		126.1	C
4, 4'	7.26 d (8.5)	119.8	CH	4	7.60 d (8.4)	119.5	CH
5, 5'	7.12 dd (8.5, 1.7)	121.9	CH	5	7.14 dd (8.5, 1.7)	121.8	CH
6, 6'		114.6	C	6		114.7	C
7, 7'	7.47 d (1.7)	113.8	CH	7	7.51 d (1.6)	113.9	CH
7a, 7a'		137.4	C	7a		137.8	C
8, 8'	2.89 dd (14.5, 3.8)	29.8	CH_2	8	3.44 dd (15.3, 4.2)	27.0	CH_2
	2.18 dd (14.5, 7.1)				3.14 dd (15.3, 9.0)		
9, 9'	4.04 dd (7.0, 3.9)	55.6	CH	9	3.81 dd (9.0, 4.2)	55.3	CH
10, 10'		168.1	C	10		172.8	C

Table S4. NMR assignments of compound **8** in MeOD. Related to Figure 2.

	Compound **8**		
No	δ_H mult (*J*, Hz)	δ_C	
1		173.0	C
2	4.32 m	54.0	CH
3	2.93 dd (16.5, 6.0)	39.1	CH_2
	2.85 dd (16.5, 7.5)		
4		192.3	C
5		116.8	C
6	7.54 d (8.5)	128.0	CH
7	6.78 dd (8.5, 1.5)	120.2	CH
8		130.1	C
9	7.07 d (1.5)	118.4	CH
10		152.4	C

Video S1. Footage of *Cephaloscyllium ventriosum*. Related to Figure 1.

Reference

Gruber, D.F., Loew, E.R., Deheyn, D.D., Akkaynak, D., Gaffney, J.P., Smith, W.L., Davis, M.P., Stern, J.H., Pieribone, V.A., and Sparks, J.S. (2016). Biofluorescence in catsharks (Scyliorhinidae): Fundamental description and relevance for elasmobranch visual ecology. Sci. Rep. 6, 24751.