Unconventional aliphatic fluorophores discovered as the luminescence origin in citric acid-urea carbon dots

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Materials and Methods

Materials: Citric acid (Sigma-Aldrich, ≥99.5%) and urea (Mallinckrodt, ≥99.6%) were purchased and used without further purification. Milli-Q water was from Direct-Q® 3, 5, 8 laboratory water purification system by MilliporeSigma. 5-oxopyrrolidine-3-carboxylic acid (Combi-Blocks, 97%), 5-oxopyrrolidine-3-carboxamide (Ambeed, 97%), ethyl 5-oxopyrrolidine-3-carboxylate (Miilipore Sigma, 95%), and N-ethyl 5-oxopyrrolidine-3-carboxylate (Chembridge Corporation, 95%) were purchased and used without further purification.

ESI-MS/MS method: A Sciex ExionLC UHPLC system coupled to a Sciex X500R quadrupole-time-of-flight mass spectrometer (UHPLC/QTOF-MS) was used for separation, detection, and structural characterization of 5-oxopyrrolidine-3-carboxylic acid separated from CD synthesis. A Phenomenex Kinetix (2.6 μm) F5 (100 Å), 2.1 mm x 30 mm column at 40 °C was used during the following 5 min gradient rapid elution with A: Water containing 0.1% formic acid and B: Methanol containing 0.1% formic

acid, at a flow rate of 0.5 mL/min: 5% B, 0 min to 1.0 min; 5% B to 50% B, 1.0 min to 2.0 min; 50% B to 95% B, 2.0 min to 3.0 min; 95% B, 3.0 min to 3.5 min; 95% B to 5% B, 3.5 min to 4.0 min; 5% B 4.0 min to 5.0 min. For electrospray ionization mass spectrometry in positive ionization mode (5 min), parameters were as follows: scan time, 0.127s; ion source gas1, 45; ion source gas2, 45; curtain gas, 30; CAD gas, 7; temperature 500 °C; spray voltage, 5500 V; declustering potential (DP), 50; DP spread, 0; scan range, *m/z* 50-500; accumulation time, 0.1 s; collision energy, 10; collision energy spread, 0. For electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ionization mode, parameters were as follows: scan time, 0.102 s; ion source gas1, 45; ion source gas2, 45; curtain gas, 30; CAD gas, 7; temperature 500 °C; spray voltage, 5500 V; declustering potential (DP), 50; DP spread, 0; scan range, *m/z* 50-1200; accumulation time, 0.1 s; collision energy, 35; collision energy spread, 15.

Sample preparation for single molecule imaging: 5-oxopyrrolidine-3-carboxylic acid solid powder (Catalog No. CA-5625, combi-blocks) was dissolved in distilled water at a concentration of 0.8 µg/ml. Solutions were sonicated in ice water using Misonix Sonicator 3000, operated at 10 W for 4×2.5 min. The solution was diluted 10 times, and a10 ml drop was spin-coated on a coverslip at the bottom of a cell culture dish.

Single molecule fluorescence microscopy: Imaging was performed using Olympus IX-71 inverted microscope with a 100× oil immersion objective, four solid-state lasers (405, 488, 542, 594) with similar power, and an Electron Multiplying CCD camera (Andor iXon Ultra 897). The camera's pixel size was 16 µm and the microscope magnification was 100X. Thus, the scale factor was 160 nm/pixel. 1,000 images of 512×512 pixels were collected within 100 s and stored as 16-bit FITS files. The analysis was performed using MATLAB routines available upon request.

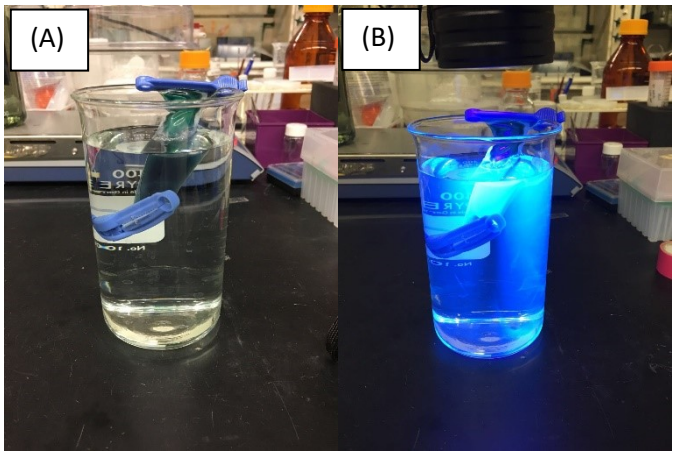


Figure S1. Carbon dots (CDs) undergoing dialysis under white light (A); and under 400 nm illumination (B).

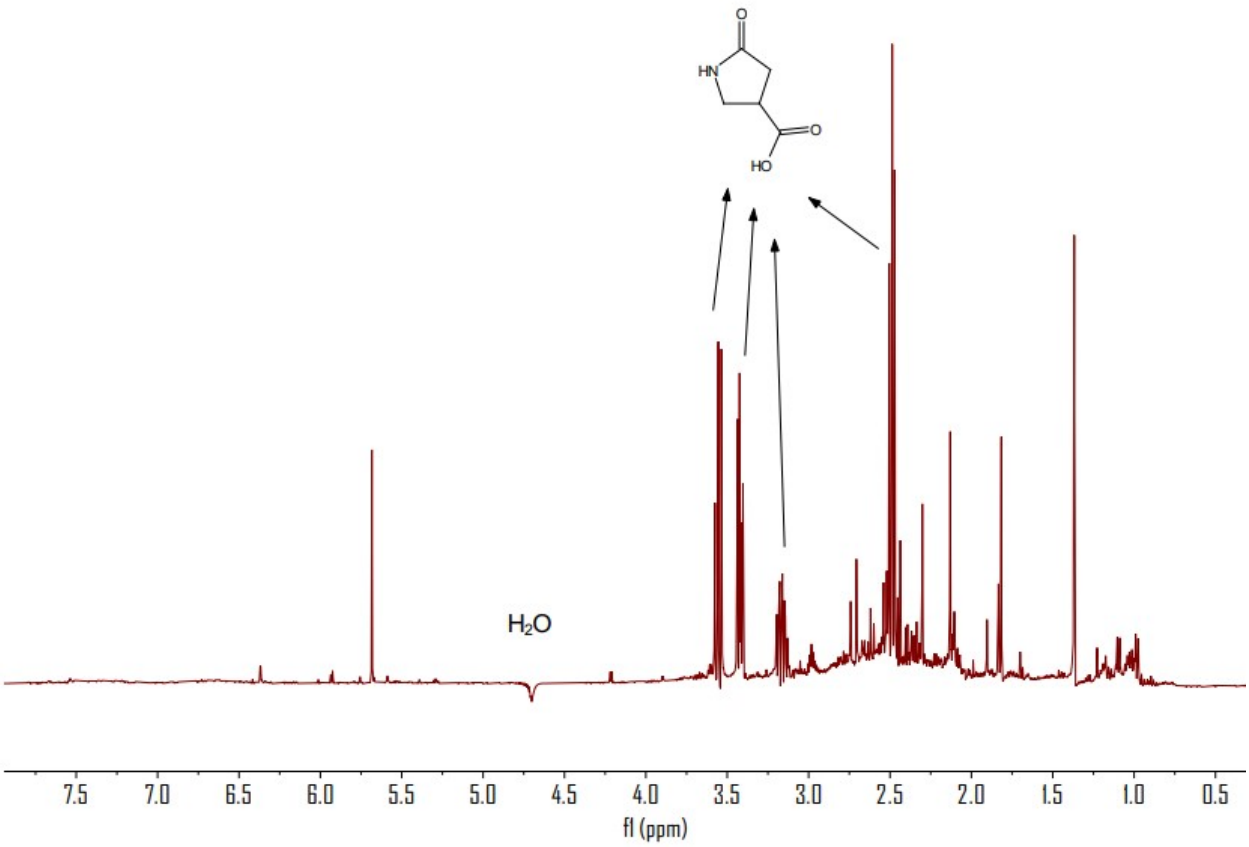
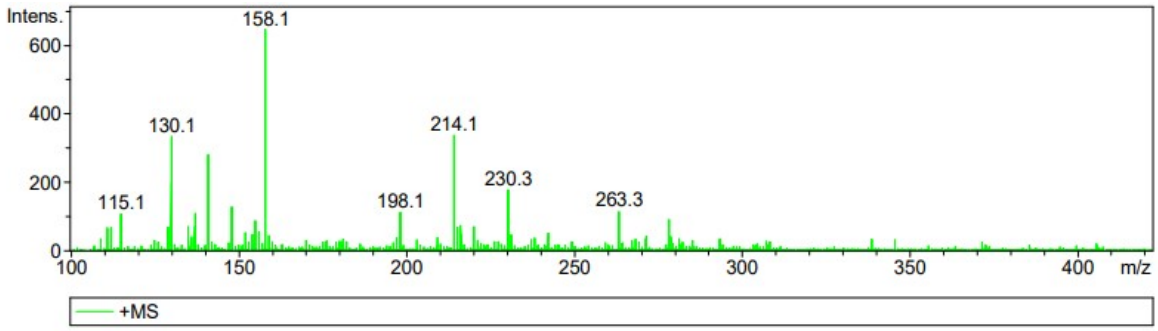


Figure S2. ^{1}H NMR spectrum of CDs dialysate in 90% H_2O+10% D_2O in water suppression mode.



#	m/z	I
1	115.1	107
2	130.1	333
3	135.1	71
4	137.1	109
5	141.0	280
6	148.1	128
7	155.1	88
8	156.1	54
9	158.1	645
10	198.1	113
11	214.1	336
12	230.3	177
13	263.3	114

Figure S3. ESI-MS spectrum of CDs dialysate in positive mode.

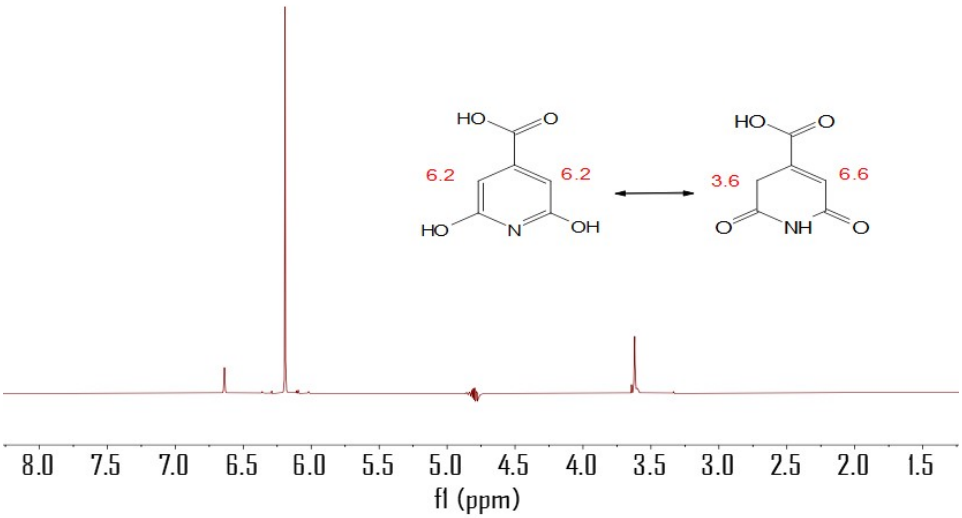


Figure S4. ^{1}H NMR spectrum of citrazinic acid in 90% H_2O+10% D_2O in water suppression mode

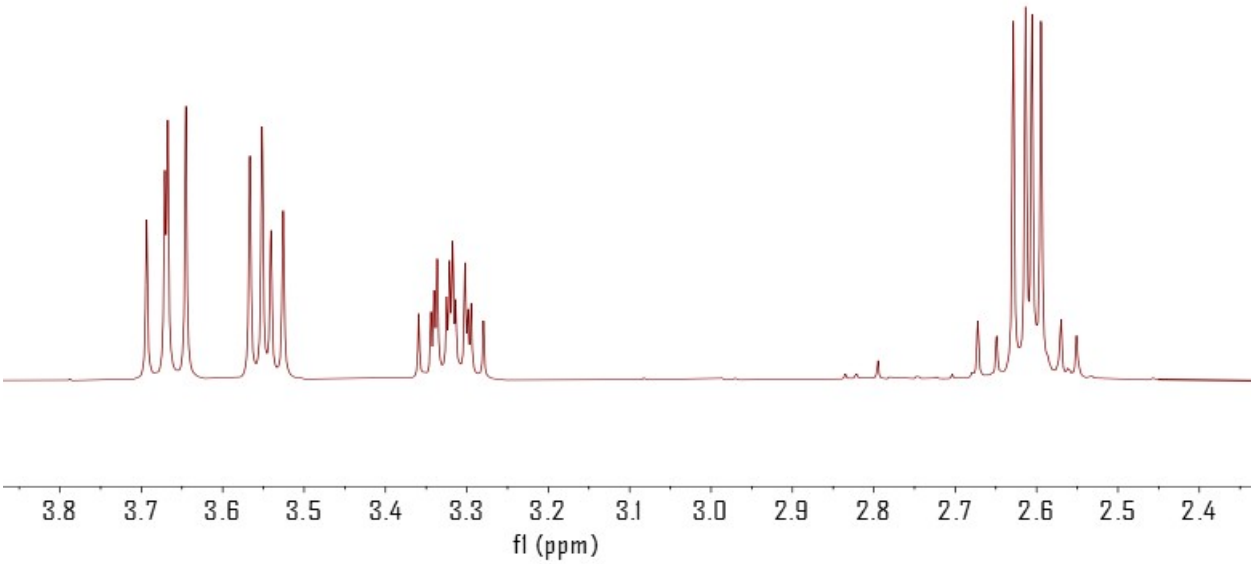


Figure S5. ^{1}H NMR spectrum of **1** in D_2O

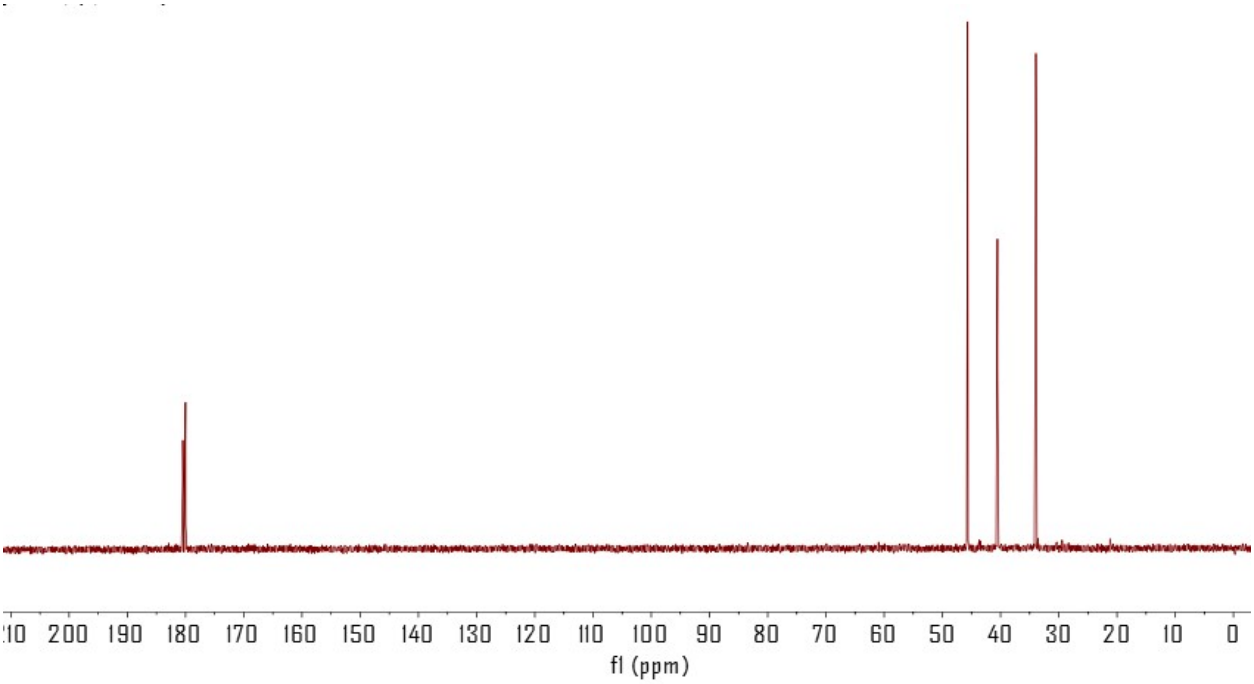


Figure S6. ^{13}C CPT spectra of **1** in D_2O

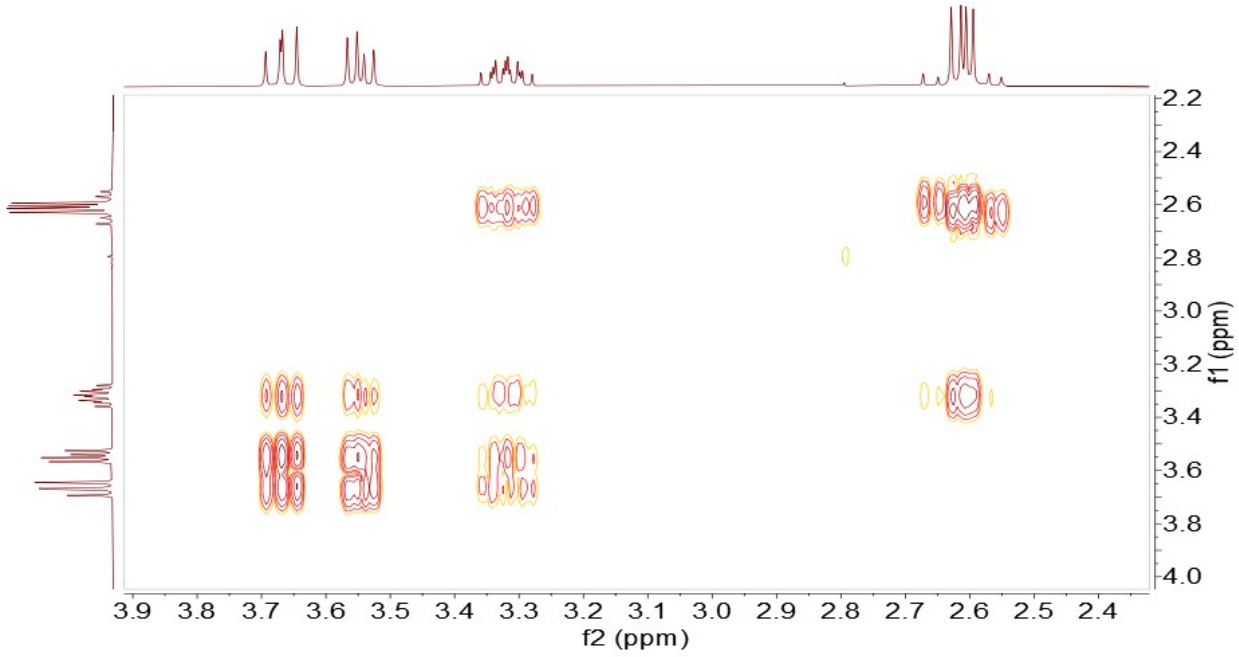


Figure S7. ^{1}H COSY spectra of **1** in D_2O

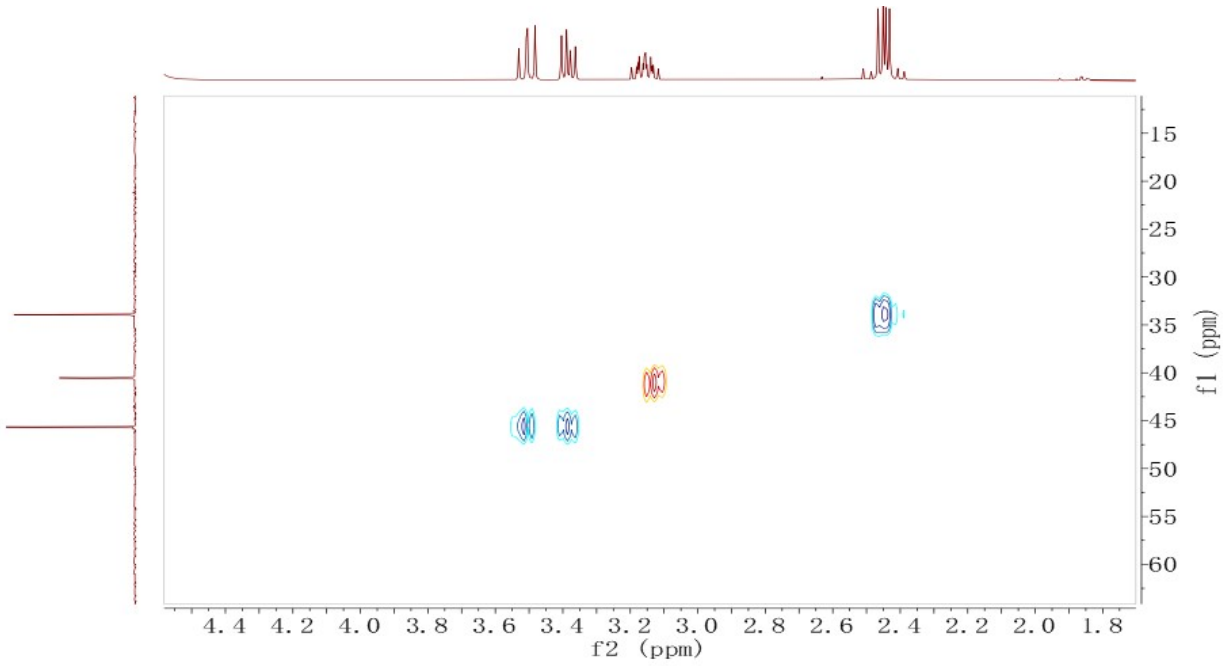


Figure S8. ^{1}H-^{13}C HSQC spectrum of **1** in D_2O

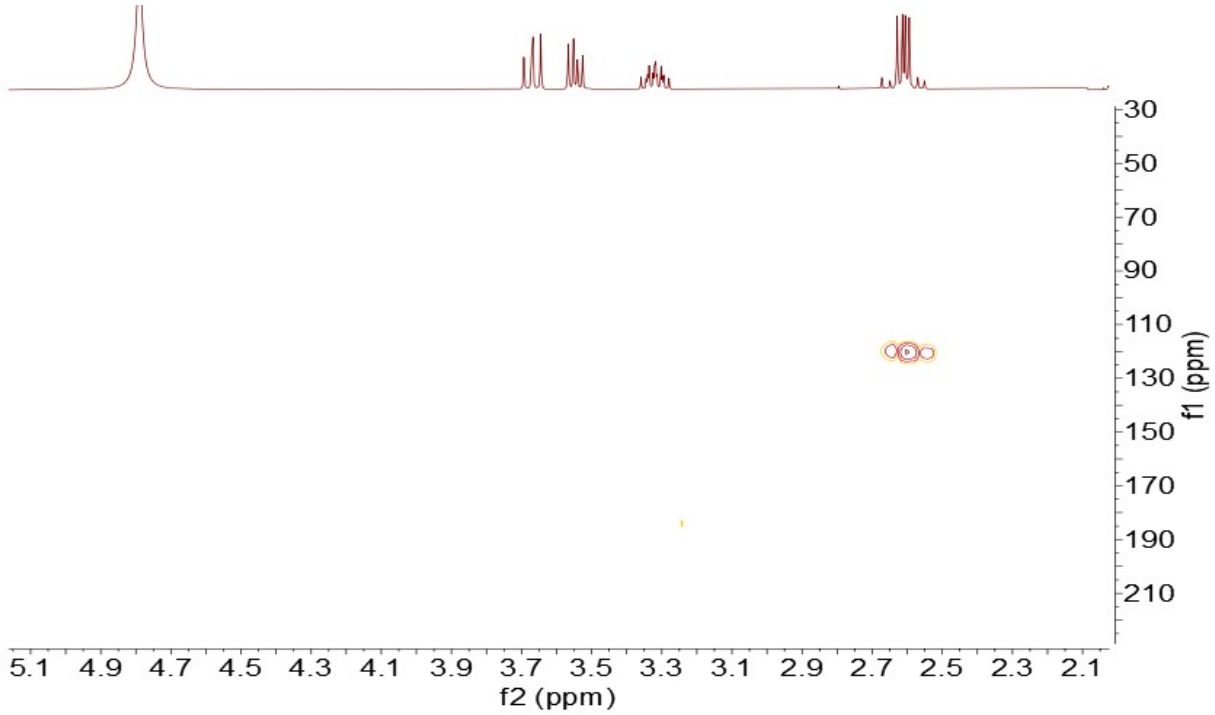


Figure S9. ^{1}H-^{15}N HMBC spectrum of **1** in D2O.

Note: only two protons at 2.6 ppm showed coupling to the lactam nitrogen. This is probably due to the ring strain leading to other protons having J coupling constants that are outside of the detection range.

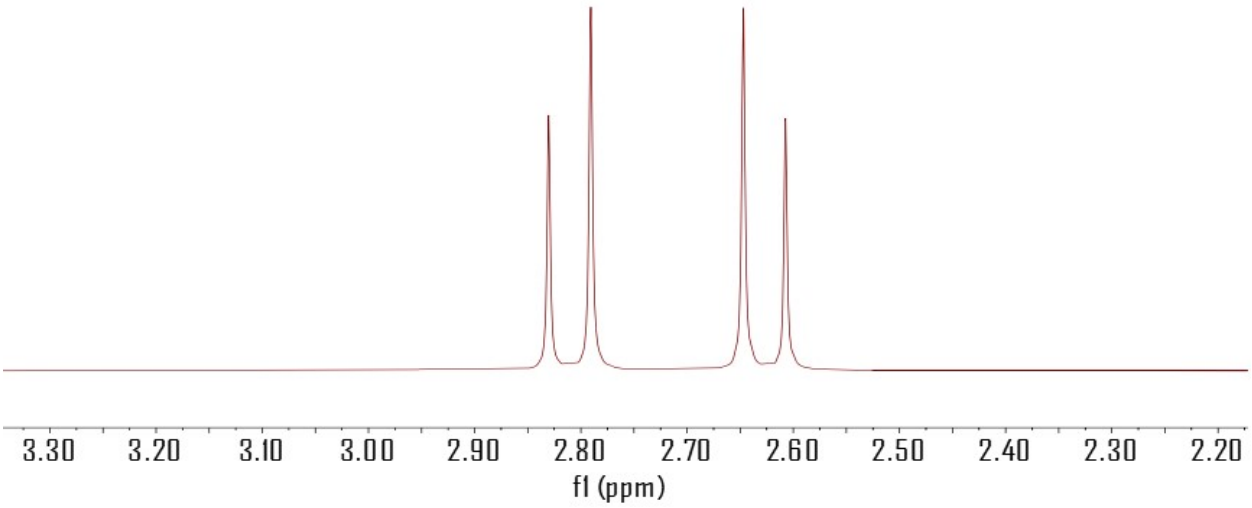


Figure S10. ^{1}H NMR spectrum of citric acid.

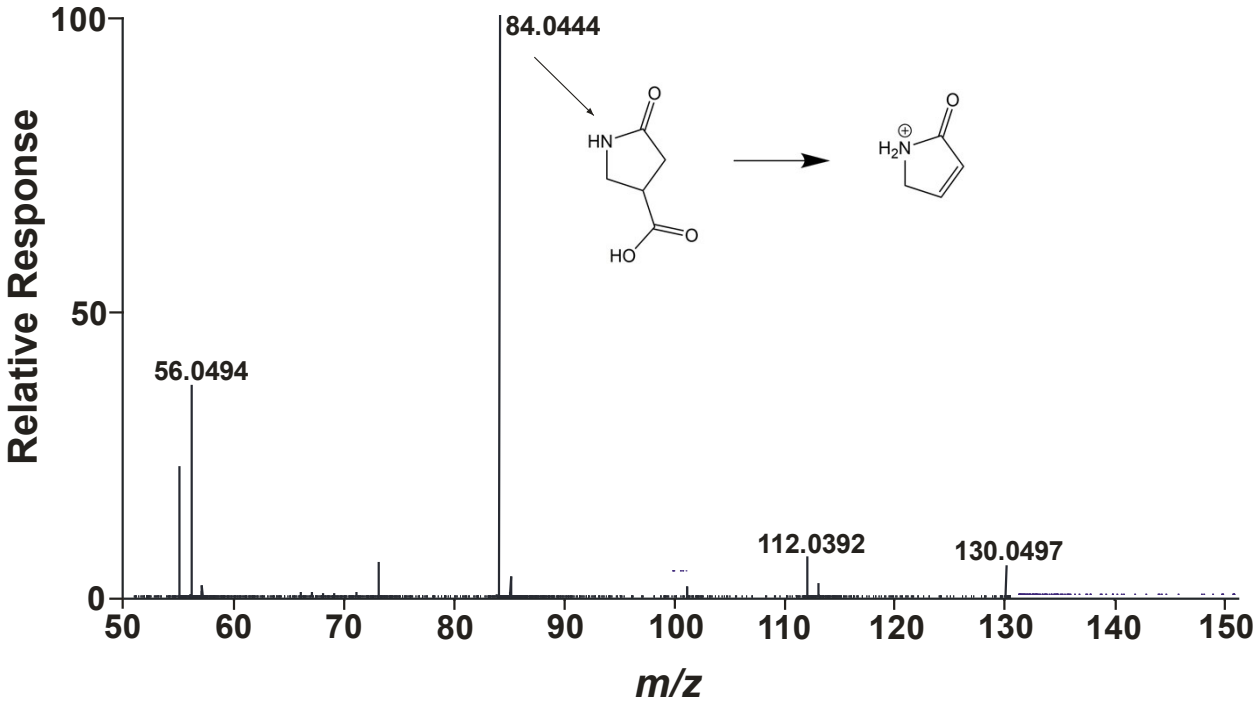


Figure S11. ESI-MS/MS spectrum of **1** separated from CD synthesis. The inserted scheme illustrates the proposed fragmentation of **1.**

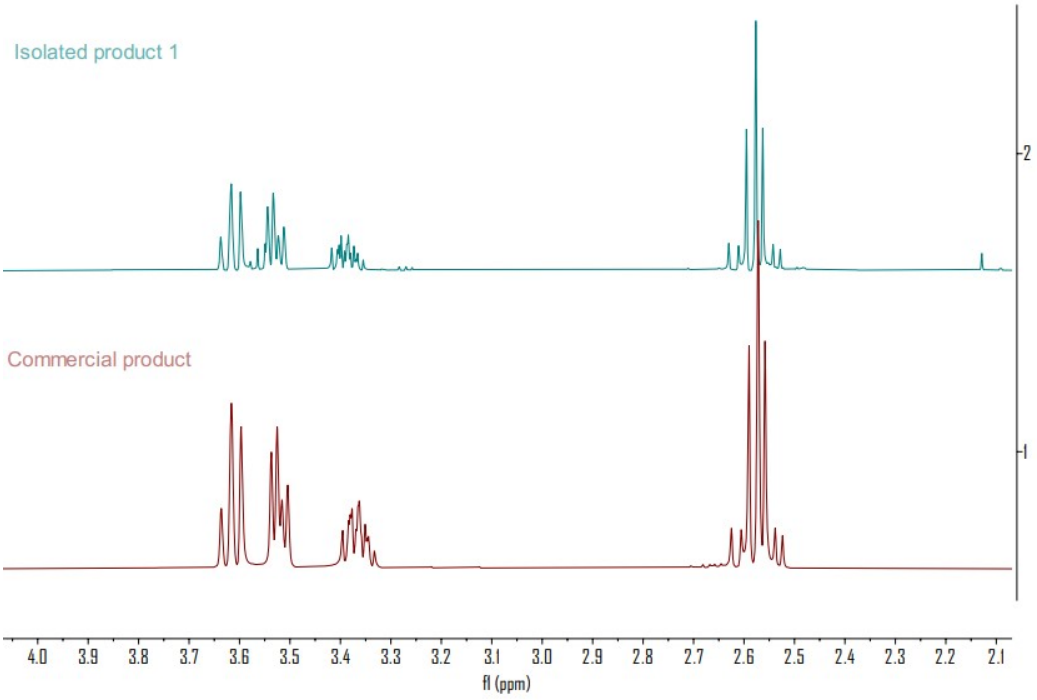


Figure S12. Stacked ^{1}H NMR spectra of commercial product 5-oxopyrrolidine-3-carboxylic acid and the isolated product **1**.

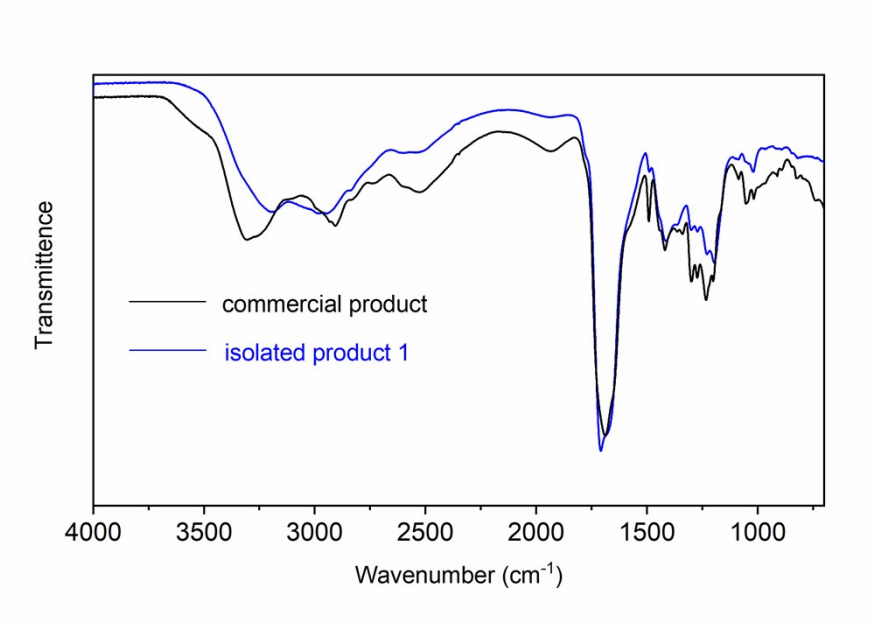
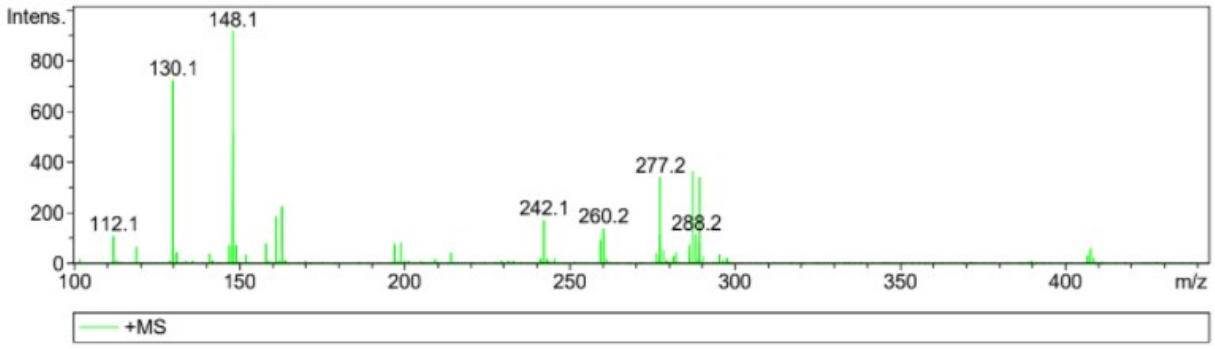


Figure S13. Stacked FT-IR spectra of commercial product 5-oxopyrrolidine-3-carboxylic acid and the isolated product **1**.



#	m/z	I
1	112.1	108
2	130.1	720
3	148.1	916
4	242.1	170
5	260.2	136
6	277.2	340
7	288.2	112

Figure S14. Positive mode ESI-MS of the raw product from itaconic acid and urea reaction.

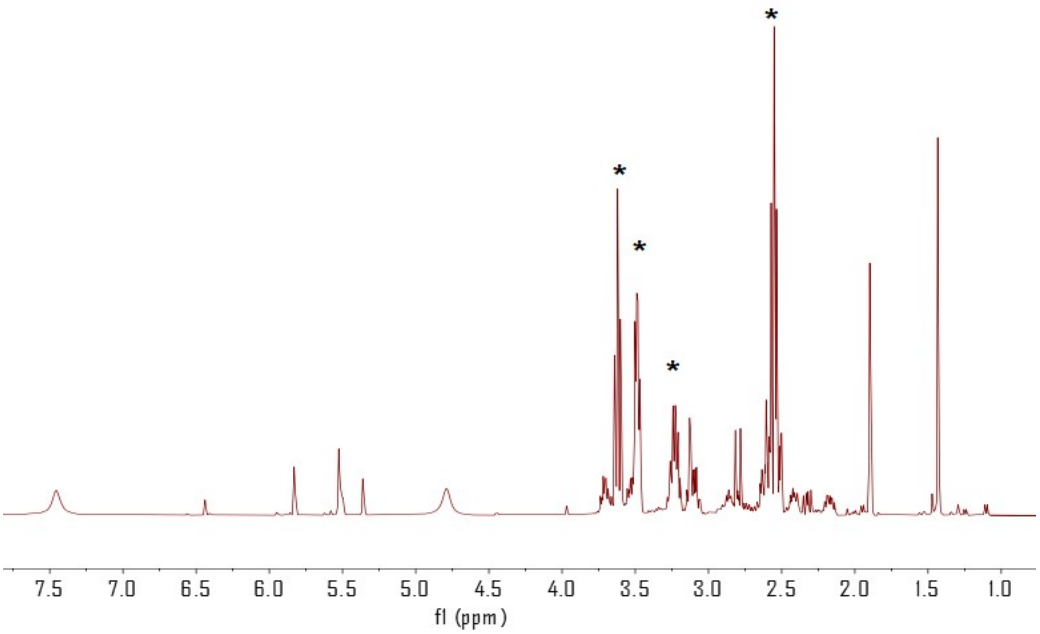


Figure S15. ^{1}H NMR of the raw product from itaconic acid and urea reaction, with * indicating the formation of 5-oxopyrrolidine-3-carboxylic acid.

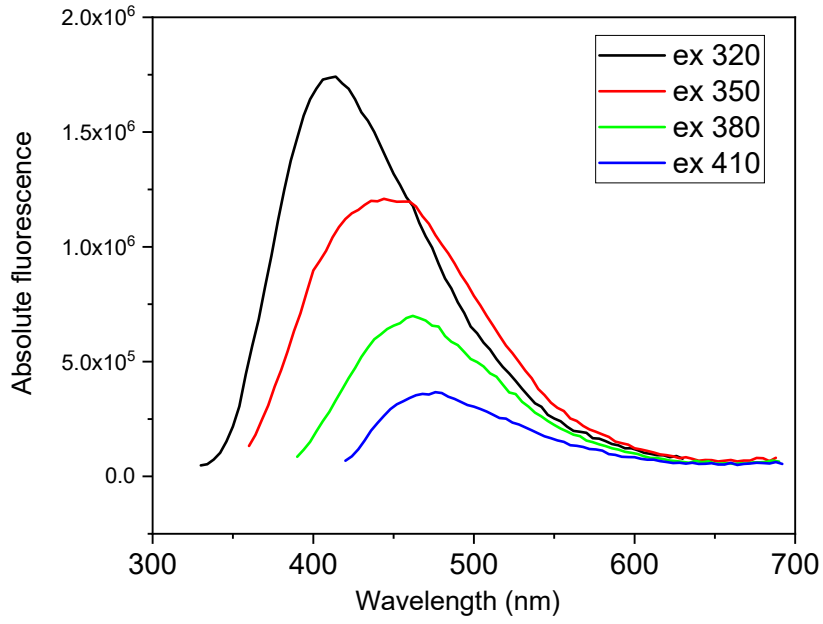


Figure S16. The fluorescence spectra of the raw product of the hydrothermal reaction between itaconic acid and urea with varied excitation wavelength.

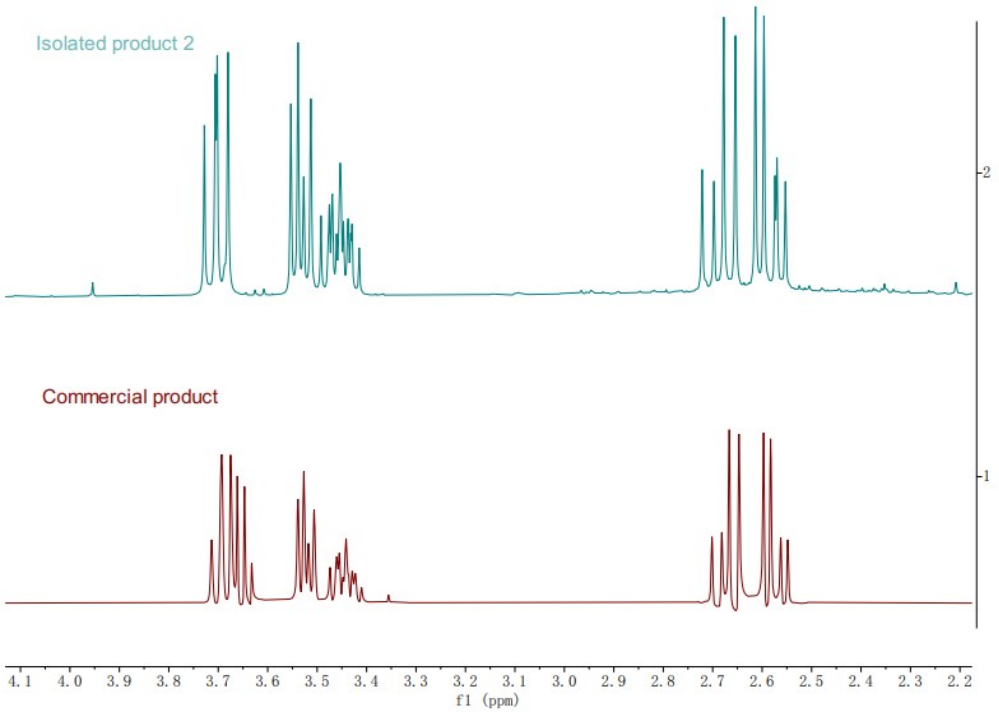


Figure S17. Stacked ^{1}H NMR spectra of commercial product 5-oxopyrrolidine-3-carboxamide and the isolated product **2**.

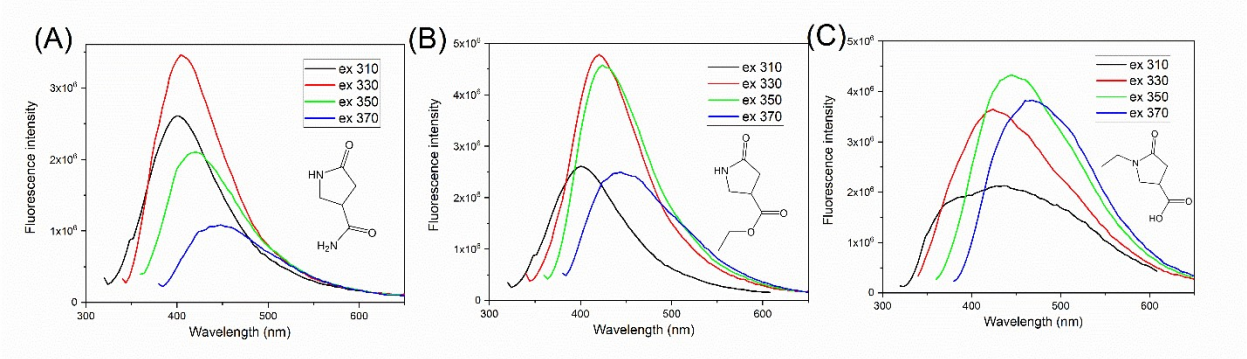


Figure S18. Fluorescence spectra of 5-oxopyrrolidine-3-carboxyamide (A); ethyl-5-oxopyrrolidine-3-carboxylate (B); and N-ethyl-5-oxopyrrolidine-3-carboxylic acid (C) with varied excitation wavelength.

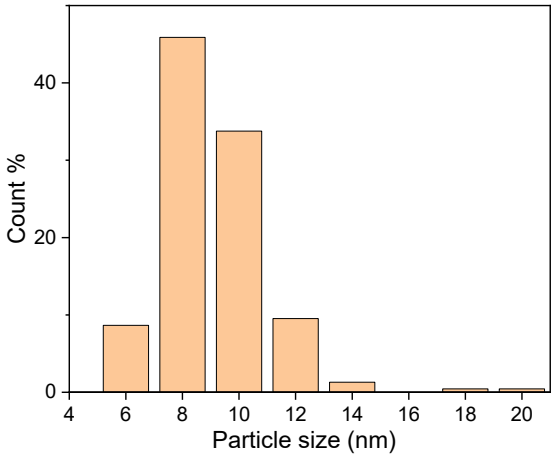


Figure S19. TEM particle size distribution histogram of CDs (n=231) using ImageJ analysis tools.

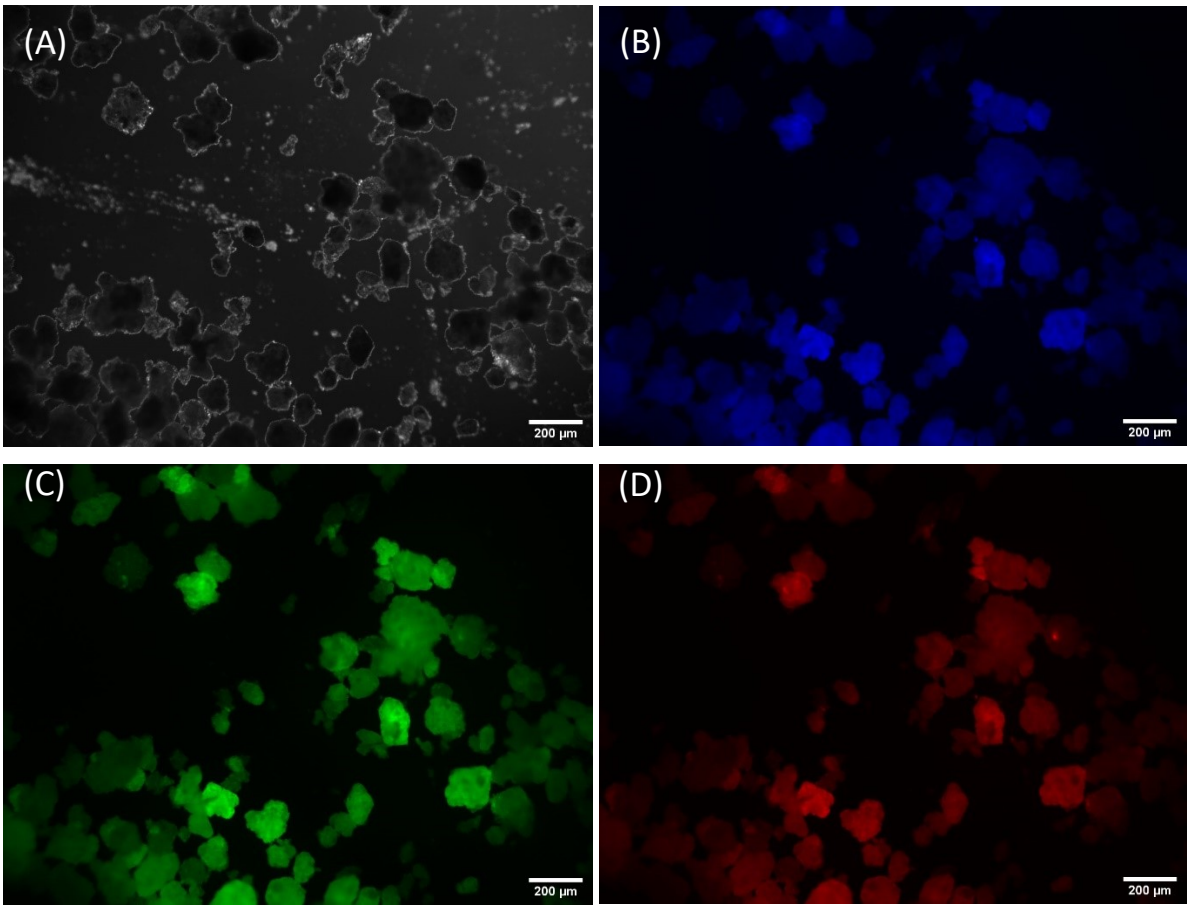


Figure S20. Macroscopic fluorescent images of solid **1** particles in bright field with differential interference contrast (A); DAPI channel, excitation 360-380 nm; emission 415 nm with longpass filter (B);

GFP channel, excitation 450-490 nm; emission 500-550 nm(C); and RFP channel, excitation 510-555 nm, emission 575-595 nm (D).

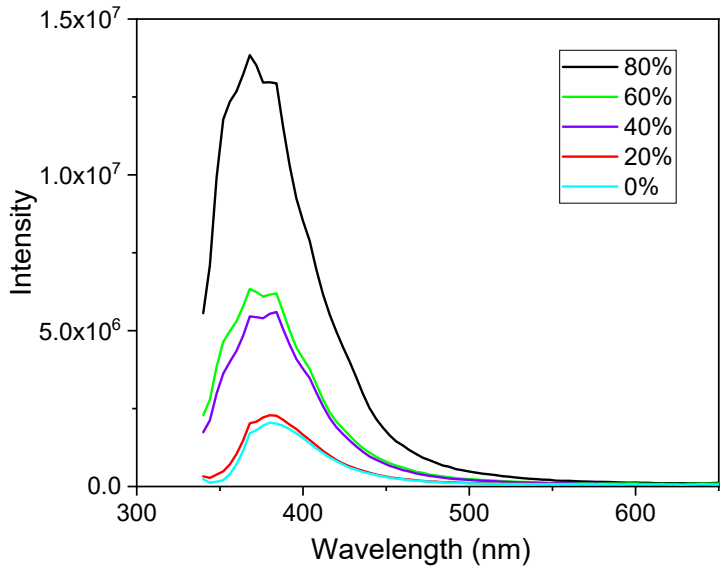


Figure S21. Fluorescence spectra of **2** in mixed solvents of DMSO and THF, with the percentage indicating the THF%.

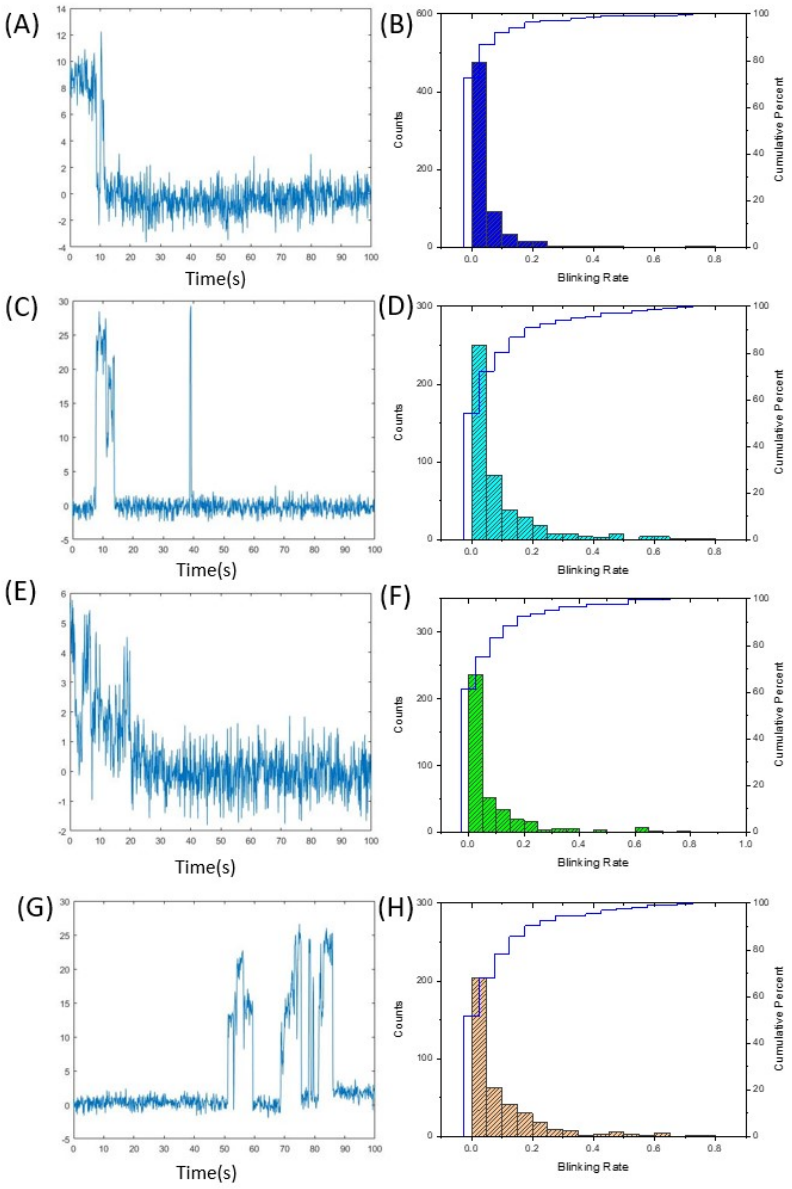


Figure S22. Characterization of on-time fraction for 5-oxopyrrolidine-3-carboxylic acid. Representative fluorescence traces from a single molecule on a glass coverslip in a culture dish excited by 405-nm, 488-nm, 542-nm, or 594-nm wavelengths are shown in (A), (C), (E), and (G), respectively. The histograms and cumulative percentages of the single molecule on-time fractions were calculated under excitation of 405-nm from 653 molecules (B), under 488-nm from 461 molecules (D), under 542-nm from 384 molecules (F), and under 594-nm from 394 molecules (H).

Figure S23. Photos of samples at the start of the room temperature stability test (A) and at the end (B). Bottles labeled 1-3 are replicate CDs; 4-6 are replicate 5-oxopyrrolidine-3-carboxylic acid; 7-9 are replicate citrazinic acid.

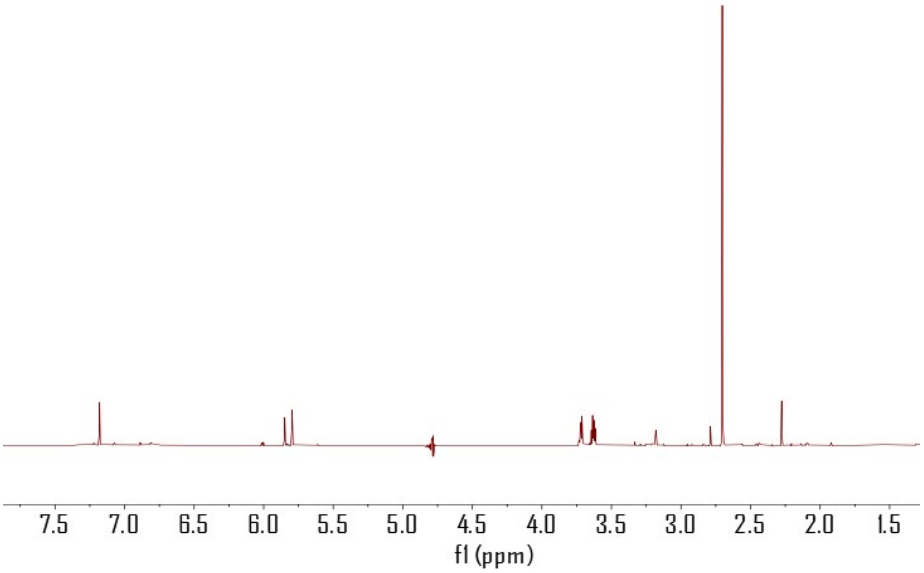


Figure S24. ^{1}H NMR spectra of citrazinic acid after dispersing in water for 7 days.

Table S1. Other fluorophores generated in CD synthesis.

Fluorophore number	Emission color	m/z in ESI-MS	^{1}H NMR chemical shift
3	other blue fluorophores	156, 179, 217	Singlet 8.3 ppm
4	green	236	
5	yellow	161, 163	1.8 ppm; 2.3 ppm, 2.7 ppm, 5.9 ppm
6	red	220	2.0 ppm
7 dark substances	little blue/cyan or no emissions	141, 135, 158, 214	4.3 ppm, 3.7 ppm, 1.2 ppm