Luminescence phenomena of carbon dots derived from citric acid and urea – molecular insight.

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Electronic Supplementary Information (ESI)

Experimental

Synthesis of gCDs and bCDs

Citric acid (Aldrich) (0.77 g) was mixed with urea (Avator, Poland)(2.6 g) and above reactants were dissolved in double distilled water (20 ml). For the synthesis of gCDs reagents were placed in a glass vessel and heated in a microwave oven (Prolabo Synthewave 402, 300 W) for 5 minutes at 140°C using 100% power of the reactor. For fabrication of bCDs reactants were placed in Teflon vessel and heated in the high-pressure microwave reactor (Ertec Magnum II, 600 W) for 4 h at 160-180°C, 8.5-9.0 bar using 90% power of the reactor. Afterward, bCD reaction mixture was transferred to a dialysis bag (Spectra/Por cut off ~1000 Da), while gCD reaction mixture was dissolved in double distilled water (20 ml) prior placing in a dialysis bag (Spectra/Por cut off ~1000 Da). Then the dialysis membranes with CDs were placed in separate containers filled with 1000 ml of double distilled water each and left for separation. Solution outside dialysis bags (containing low molecular fractions) was exchanged with distilled water every day until complete removal of small molecular fluorescent compounds from the dialysis bags (~7 days of separation turn out sufficient after visual testing under UV light). Low molecular fractions (collected every day for seven days of separation) (gCDout and bCDout) were mixed together and reduced to 1/10 of initial volume using rotary evaporator (Heidolph Hel-VAP, 42 mbar, 50°C), freeze-dried (Alpha 2-4 LO Plus, 0.37 mbar, 5 days) and weighted (gCDout – ~2.5 g and bCDout - ~2.6 g). For fractions which remained in dialysis bags after seven days of separation (gCDin and bCDin), these were also freeze-dried using the same conditions as for CDout and weighted (gCDin – ~0.27 g and bCDin – ~0.08 g).

Synthesis of bCD in anhydrous conditions

Citric acid (0.192 g) was mixed with urea (0.06 g) and heated at 160°C for 2 hours using classical heating method[1]. The mixture was then dissolved in distilled water (0,5 mg/ml) and analyzed using low and high-resolution LC-ESI-MS techniques.

Determination of molecular weight and elemental formula of fluorophores

The identification of fluorescent compounds in low molecular fractions (CDout) was achieved by HR-ESI-MS and ESI-MS/MS fragmentation patterns of molecular ions. High-resolution mass spectra were acquired using the MALDISynapt G2-S HDMS (Waters Corporation, Milford, MA, USA), coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadruple). For low-resolution LC-ESI-MS and MS/MS analyses LCMS-8030 (Shimadzu, Japan) mass spectrometric system coupled to a LC-20ADXR pump utilizing the LC gradient was used. The LC analyses were carried out on a 100 mm × 4.6 mm × 5.0 μm Kinetex C18 chromatographic

column from Phenomenex (Torrance, United States). The ion chromatograms and mass spectra (electrospray voltage 4.5 kV; capillary temperature 250 °C; sheath gas: N2) were recorded by LabSolutions software (Schimadzu, Japan). Argon was used as the collision gas for CID experiments.

Purification of green fluorescing molecule

Separation and isolation of green fluorescing fraction (HPPT) from gCDout solution (30 mg/ml) on an HPLC (preparative HPLC system with LC-20AP pumps, UV-Vis SPD-20AV detector and LabSolutions 5.51 operating software, Shimadzu Corp., Japan) equipped with preparative column C18 (250 mm × 50 mm i.d., 30 μm) (Interchim, France) with a 30 mm x 10 mm i.d. guard column of the same material under the following gradient system: (t[min], % A, % B, % C), (0, 100, 0, 0), (10, 100, 0, 0), (50, 0, 10, 90), (60, 0, 70, 30), (62, 0, 70, 30). The mobile phases were: A – demineralized water, B- pure acetone, C – 0.1% formic acid in water. The injection volume was 30 mL and the flow rate was 50 mL/min. Detection was performed at 404 and 254 nm with a PDA UV –Vis detector; column temp. 30 ºC. The eluates were pooled and freeze-dried to obtain a pure pigment of HTTP.

NMR analyses of green fluorescing molecule

NMR analyses were recorded on Avance III HD 400 MHz (Bruker) in dimethyl sulfoxide-d_6 (Aldrich).

Optical properties

Absorption spectra of aqueous solutions of gCDin (0.025 mg/ml), bCDin (0.025 mg/ml), gCDout (0.025 mg/ml), bCDout(0.025 mg/ml), HPPT (0.025 mg/ml), citrazinic acid (Aldrich) (0.025 mg/ml) and ethanol solution of coumarin 153 (Aldrich) (0.025 mg/ml) were acquired using BLACK-Comet-SR (StellarNet) spectrometer using quartz cuvettes with 10 mm optical path.

Fluorescence spectra of aqueous solutions of gCDin (0.0125 mg/ml), bCDin (0.0125 mg/ml), gCDout (0.0125 mg/ml), bCDout (0.0125 mg/ml), HPPT (0.0125 mg/ml), citrazinic acid (0.0125 mg/ml) and ethanol solution of coumarin 153 (0.0125 mg/ml) were acquired using Quanta MasterTM 40 (PTI) both emission and excitation slits were set to 2 nm. Excitation spectra of HPPT were collected using the same equipment with emission set to 543 nm.

Fluorescence quantum yield (QY) determinations were conducted using a comparative method using coumarin 153 (fluorescence max 530 nm, useful excitation range 370-450 nm, absolute QY=58% in ethanol[2]) as standard. All solutions were prepared in absorption range 0.05-0.1 (408 nm) in order to eliminate concentration quenching effects; aqueous solutions of gCDin (0.0125 mg/ml), gCDout (0.0125 mg/ml), HPPT (0.00125 mg/ml), and ethanol solution of coumarin 153 (0.00125 mg/ml). The samples were excited at 408 nm and the fluorescence spectra were acquired between 415-700 nm. The QY was calculated as:

$$\Phi = \Phi_{st} \frac{n^2(1-10^{-A_{st}})\int I_{em}dv}{n_{st}^2(1-10^{-A})\int I_{em}^{st}dv}$$

where:

Φ and Φ_{st} - quantum yield of a sample and the standard
A and A_{st} – absorption of a sample and the standard at 408 nm
$\int I_{em} dv$ and $\int I_{em}^{st} dv$ – integral of fluorescence intensity of a sample and the standard
n and n_{st} - refractive index of solvent used for a sample and the standard

Determination of chemical structure of fluorophores and verification of literature theses concerning molecular fluorescence of CDs derived from citric acid and urea

For the blue fluorescing CDs, it was stated that the origin of their high quantum yield fluorescence lay in the formation of citrazinic acid and its derivatives[3]. Thus we conducted LC-DAD-ESI-MS and HR-ESI-MS analyses of the solution outside the dialysis bag and we have found that this solution contains 2 main fluorescent fractions with retention times 4.8 and 6.2 minutes. The latter fraction is citrazinic acid since it has the same retention time, UV-VIS spectra, *m/z* value and fragmentation spectra as standard (Figures S1A, S1E-G, S2A-D and Table S1). Low-resolution LC-DAD-ESI-MS analyses and elemental formula from HR-ESI-MS for a fraction with retention time 4.8 minutes indicate that it comprises of citrazinic amide (Figures S1B-D and Table S1). Thus confirming the thesis proposed in the literature reports.

For the green fluorescing CDs, it was suggested that the source of efficient green fluorescence of these materials is a citrazinic amide or its salt[1]. However, our analyses showed that green fluorescent fraction from gCDout has properties distinct from citrazinic amide (Figure S3A-D and Table S1). Thus above results are in contradiction with literature thesis.

Table S1. HR-ESI-MS results for main fluorescent fractions detected in bCDout and gCDout solutions.

CDout fraction	Exact mass (*m/z* ESI+)	Formula (ESI+)	% fit
bCDout	155.0461	$C_6H_7N_2O_3$	99
	156.0306	$C_6H_6NO_4$	98
gCDout	181.0252	$C_7H_5N_2O_4$	97

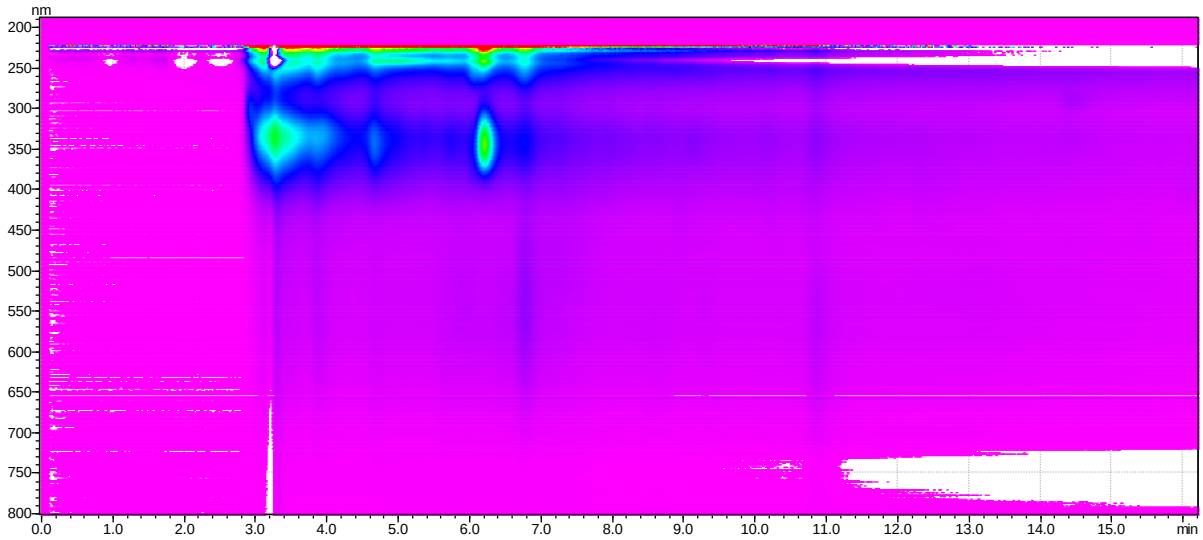


Figure S1A. LC-DAD chromatogram of bCDout (retention time of the blue fluoresce fraction is 6.2 min). Chromatographic separation conditions applied: The separation of the analytes was performed with a binary gradient elution. The injection volume was 20 µL, and the flow rate was 0.5 mL/min. The column (Kinetex C18) was thermostated at 40 ^{o}C. The mobile phases were - A - 2% formic acid in water, and B - pure methanol. The gradient profile was: (t[min], %B), (0, 0.1), (13, 10), (14, 80), (16, 80).

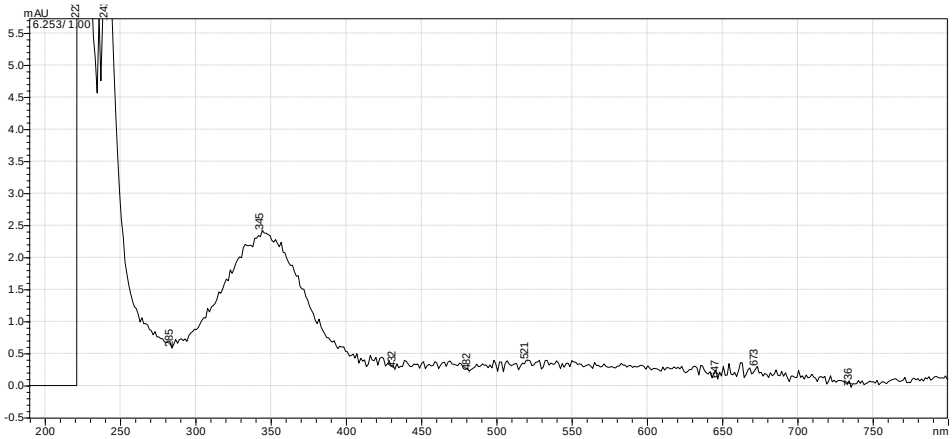


Figure S1B. UV-VIS spectra of bCDout acquired after chromatographic separation – the blue fluorescing fraction with retention time 6.2 min

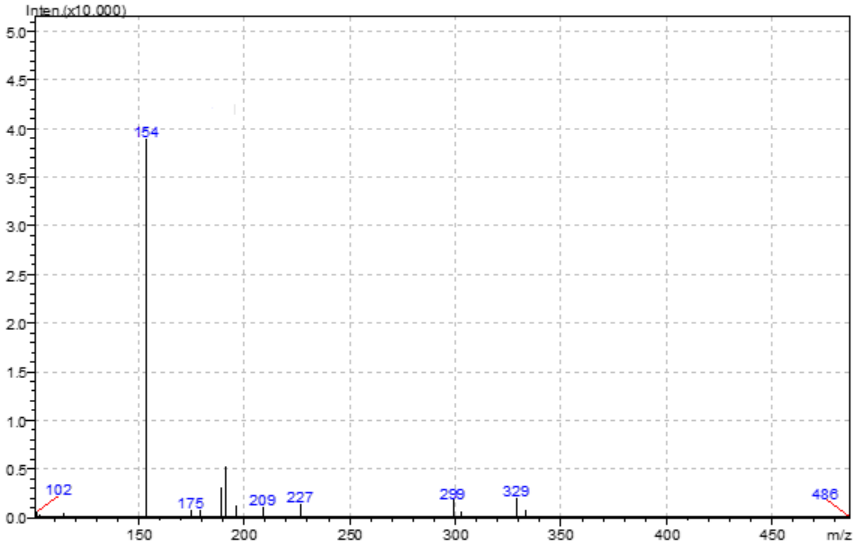


Figure S1C. Mass spectra of bCDout (ESI-) – the blue fluorescing fraction with retention time 6.2 min

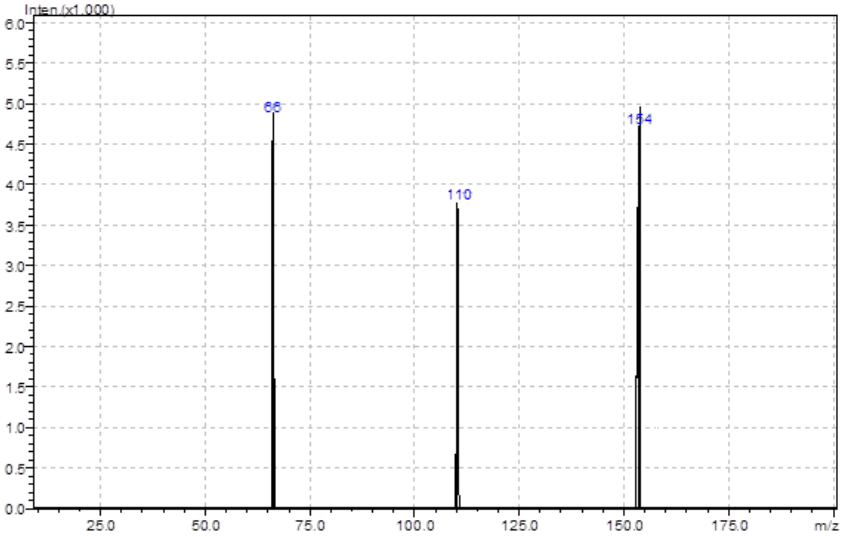


Figure S1D. Fragmentation spectra of bCDout (ESI-) – the blue fluorescing fraction with retention time 6.2 min

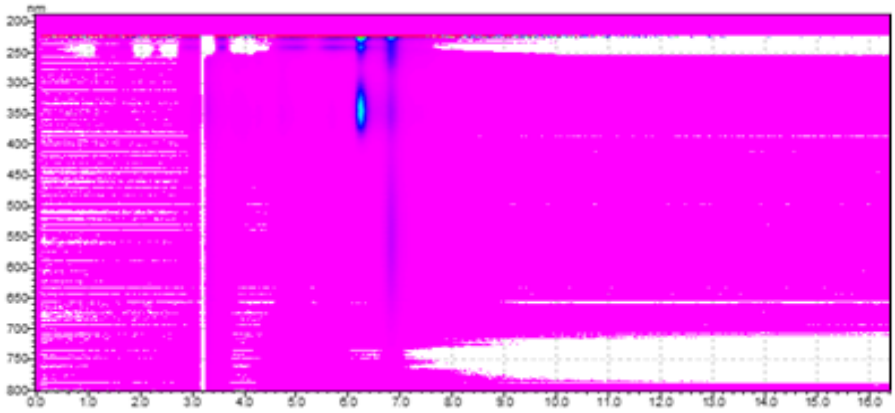


Figure S2A. LC-DAD chromatogram of citrazinic acid standard (retention time 6.2 min). Chromatographic separation conditions applied: The separation of the analytes was performed with a binary gradient elution. The injection volume was 20 µL, and the flow rate was 0.5 mL/min. The column (Kinetex C18) was thermostated at 40 ^{o}C. The mobile phases were - A - 2% formic acid in water, and B - pure methanol. The gradient profile was: (t[min], %B), (0, 0.1), (13, 10), (14, 80), (16, 80).

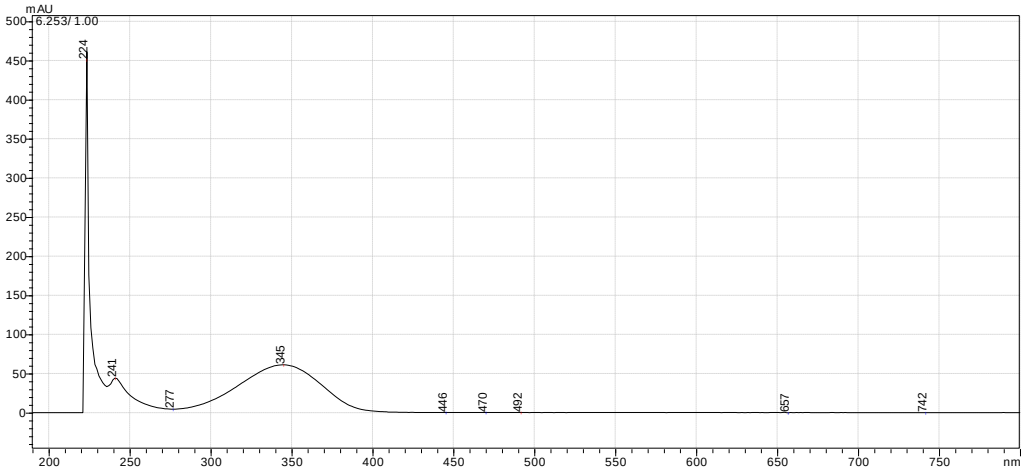


Figure S2B. UV-VIS spectra of citrazinic acid standard acquired after chromatographic separation (retention time 6.2 min)

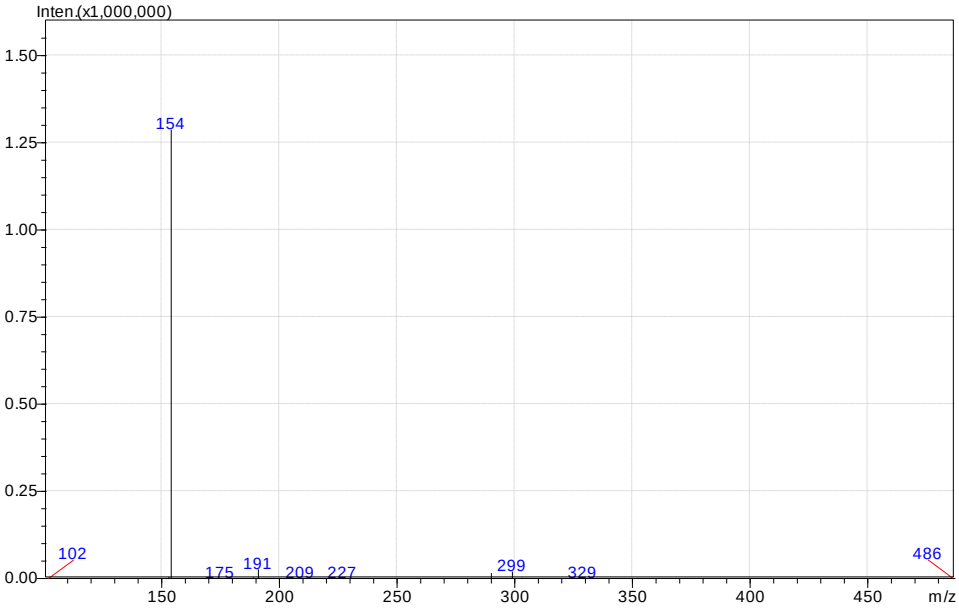


Figure S2C. Mass spectra of citrazinic acid standard (ESI-) (retention time 6.2 min)

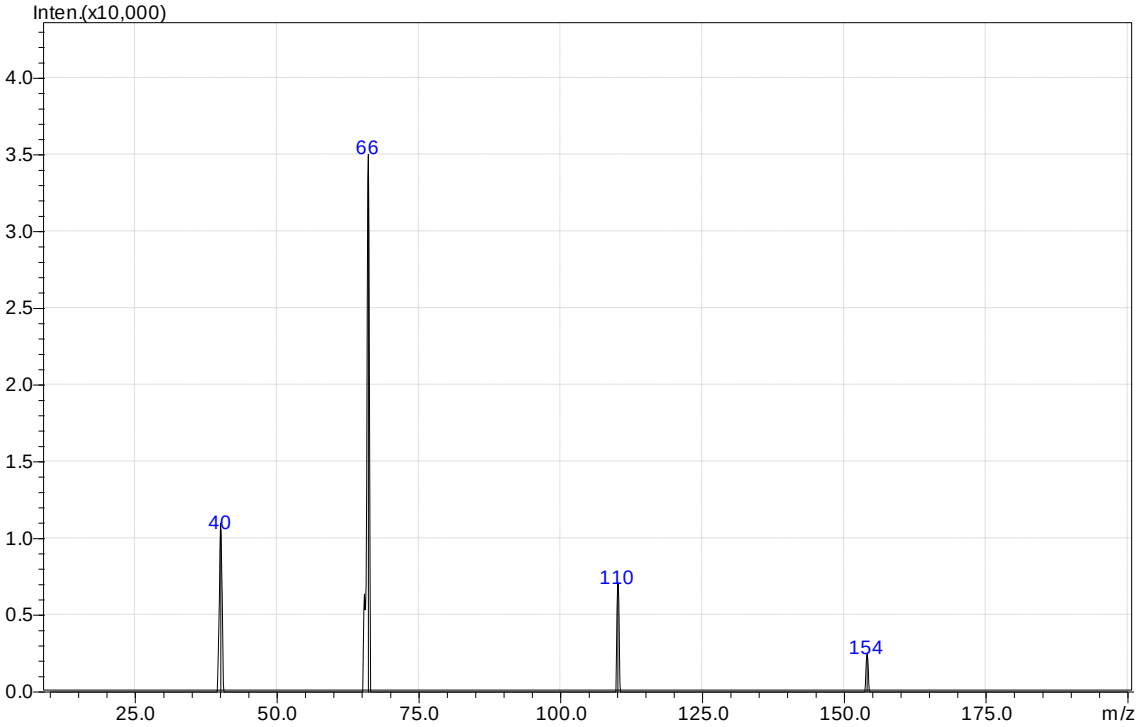


Figure S2D. Fragmentation spectra of citrazinic acid standard (ESI-) (retention time 6.2 min)

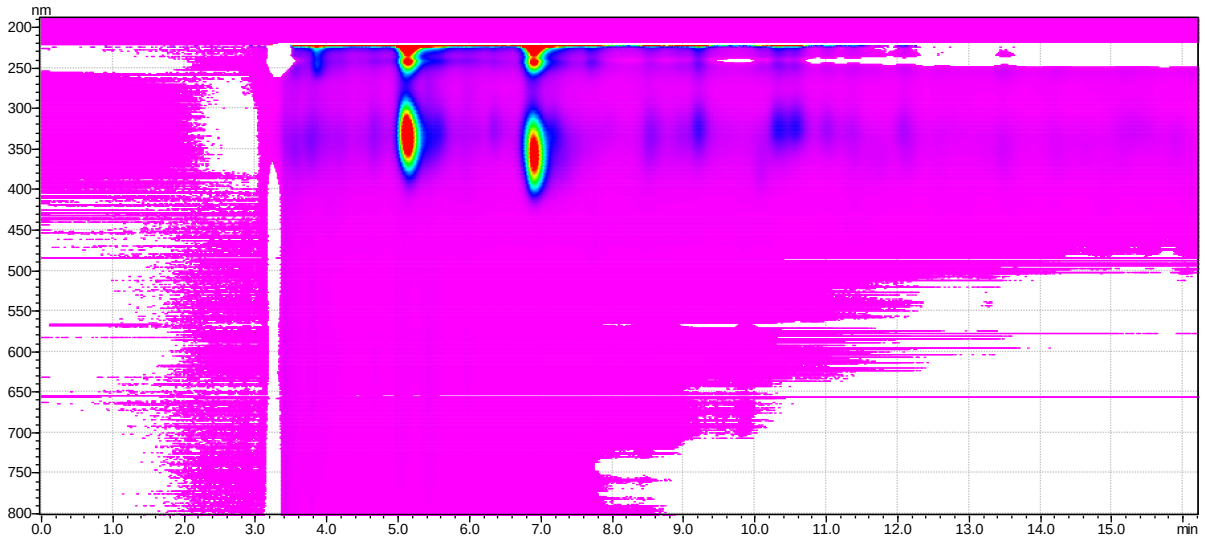


Figure S3A. LC-DAD chromatogram of bCD synthesized in water-free conditions (retention times of the blue fluoresce fractions are 5.0 for citrazinic amide and 6.8 min for citrazinic acid). Chromatographic separation conditions applied: The separation of the analytes was performed with a binary gradient elution. The injection volume was 20 µL, and the flow rate was 0.5 mL/min. The column (Kinetex C18) was thermostated at 40 ºC. The mobile phases were - A - 2% formic acid in water, and B - pure methanol. The gradient profile was: (t[min], %B), (0, 0.1), (13, 10), (14, 80), (16, 80).

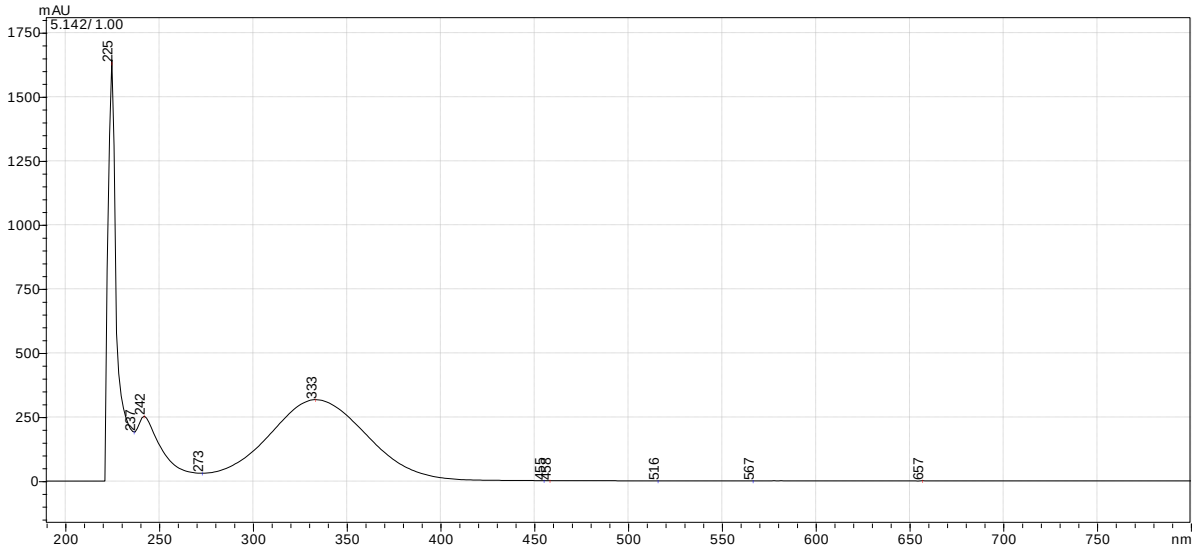


Figure S3B. UV-VIS spectra of bCD synthesized in water-free conditions acquired after chromatographic separation – the blue fluorescing fraction with retention time 5.0 min is citrazinic amide

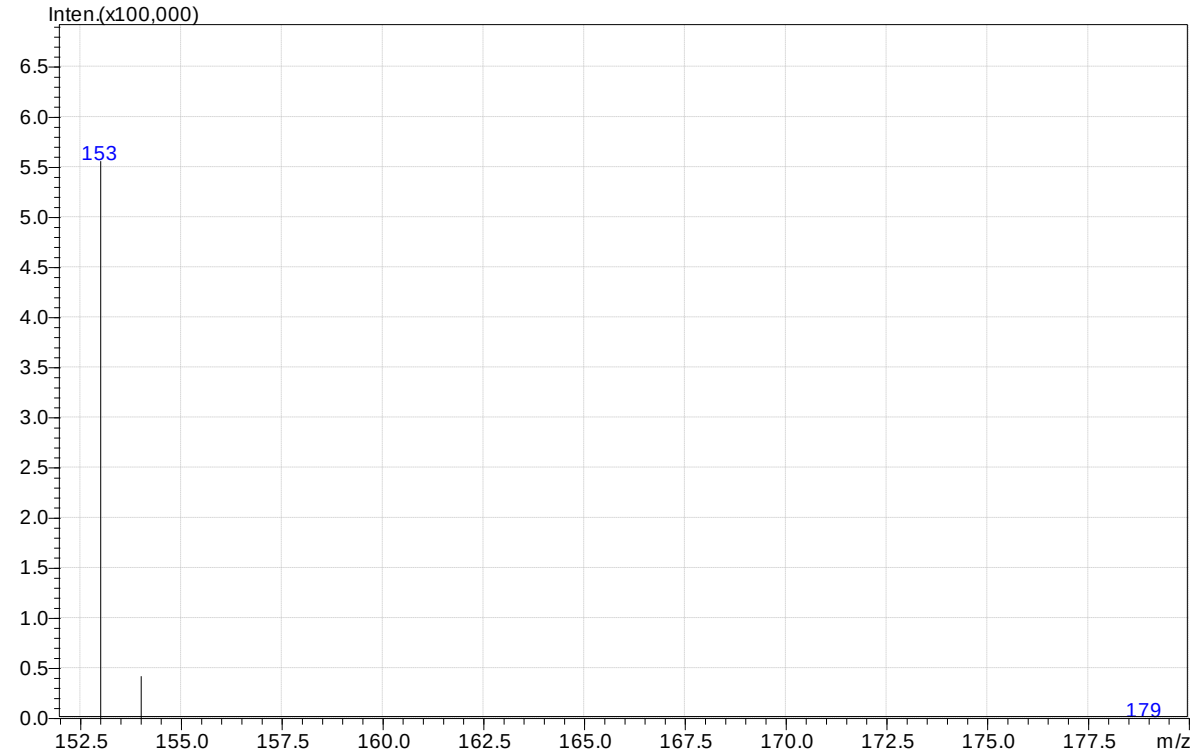


Figure S3C. Mass spectra of bCD synthesized in water-free conditions (ESI-) – the blue fluorescing fraction with retention time 4.8 min is citrazinic amide

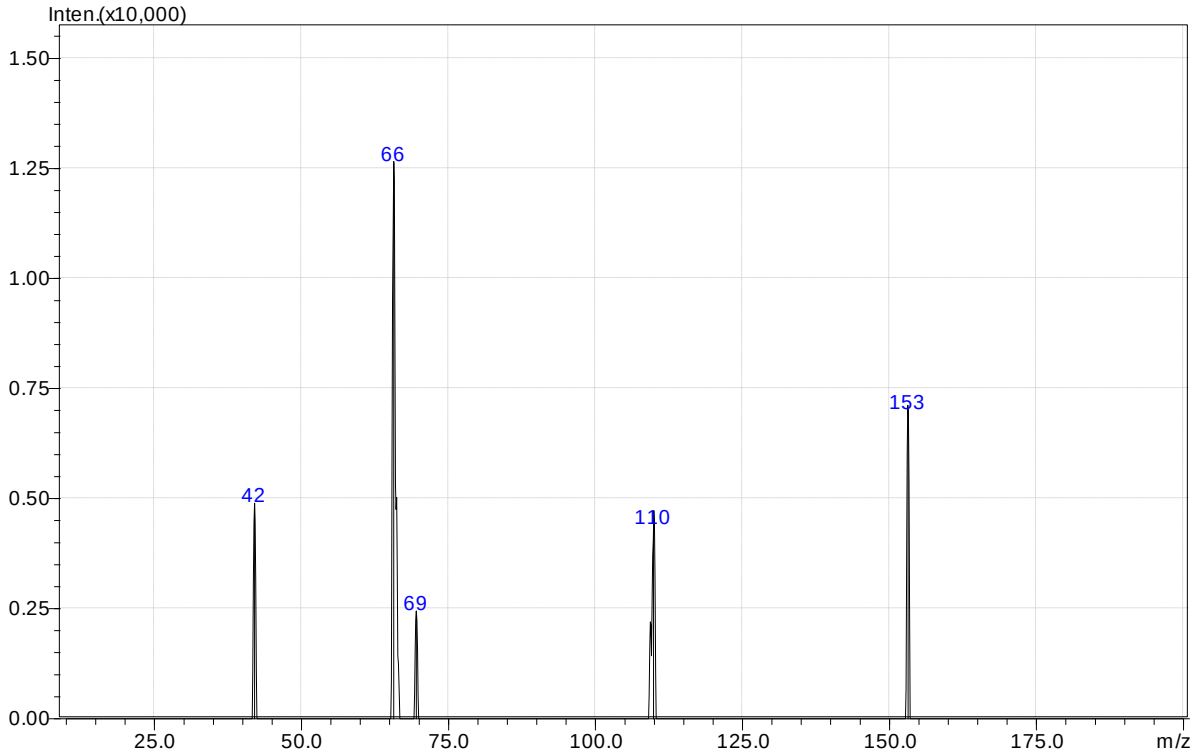


Figure S3D. Fragmentation spectra of bCD synthesized in water-free conditions (ESI-) – the blue fluorescing fraction with retention time 4.8 min

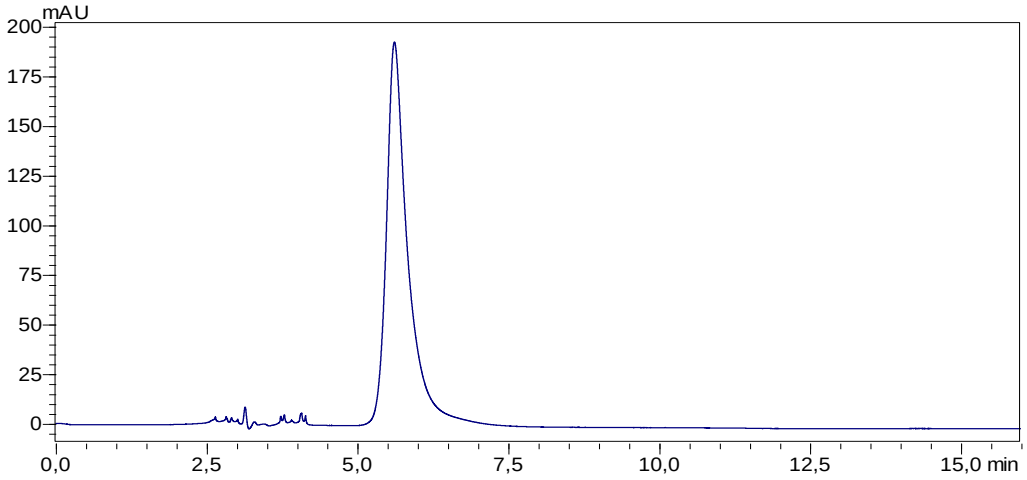
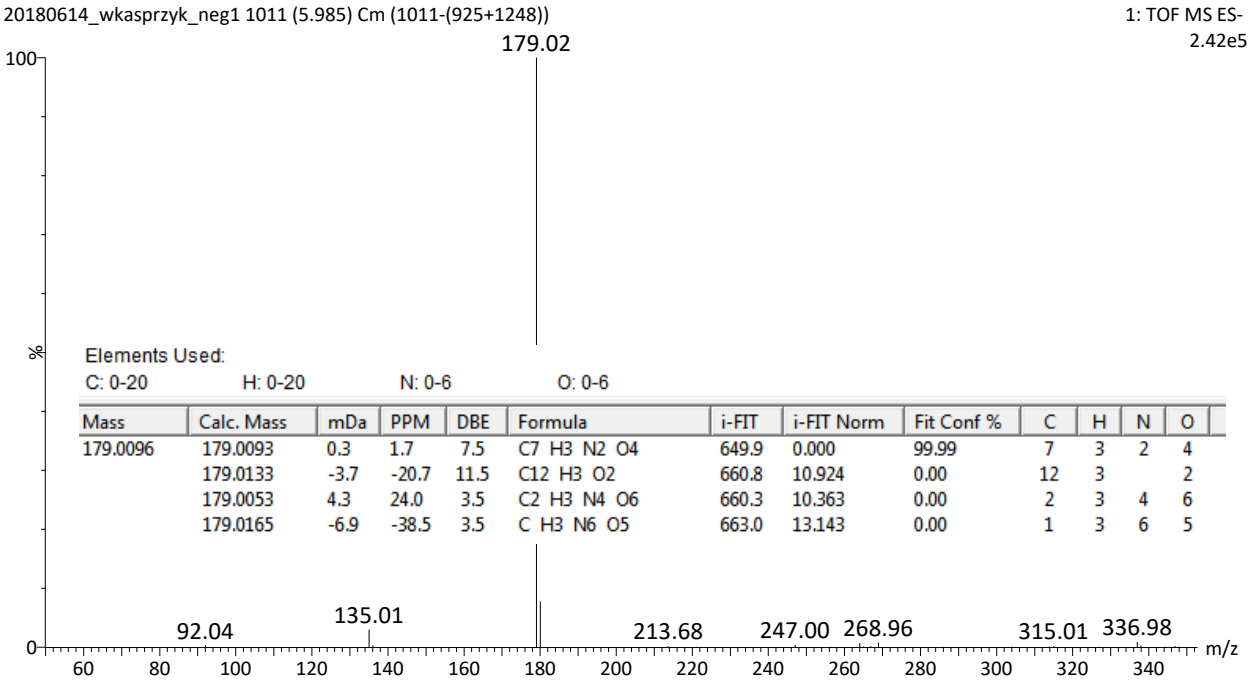


Figure S4A. UV-VIS (max plot 200-700 nm) chromatogram of HPPT after separation from gCDout showing >99% purity of obtained compound. Chromatographic separation conditions applied: The separation of HPPT was performed with a binary gradient elution. The injection volume was 20 µL, and the flow rate was 0.5 mL/min. The column (Acquity UPLC BEH C18 1.7 um) was thermostated at 40 ºC. The mobile phases were - A – 0.1% formic acid in water, and B - pure methanol. The gradient profile was: (t[min], %B), (0, 5), (5, 5), (15, 100), (17, 100), (17.1, 5), (20, 5).



Mass	Calc. Mass	mDa	PPM	DBE	Formula	i-FIT	i-FIT Norm	Fit Conf %	C	H	N	O
179.0096	179.0093	0.3	1.7	7.5	C7 H3 N2 O4	649.9	0.000	99.99	7	3	2	4
	179.0133	-3.7	-20.7	11.5	C12 H3 O2	660.8	10.924	0.00	12	3		2
	179.0053	4.3	24.0	3.5	C2 H3 N4 O6	660.3	10.363	0.00	2	3	4	6
	179.0165	-6.9	-38.5	3.5	C H3 N6 O5	663.0	13.143	0.00	1	3	6	5

Figure S4B. High resolution mass spectra of HPPT after separation from gCDout showing molecular ion peak with m/z (ESI-) = 179.0093 and indicating molecular formula $C_7H_4N_2O_4$.

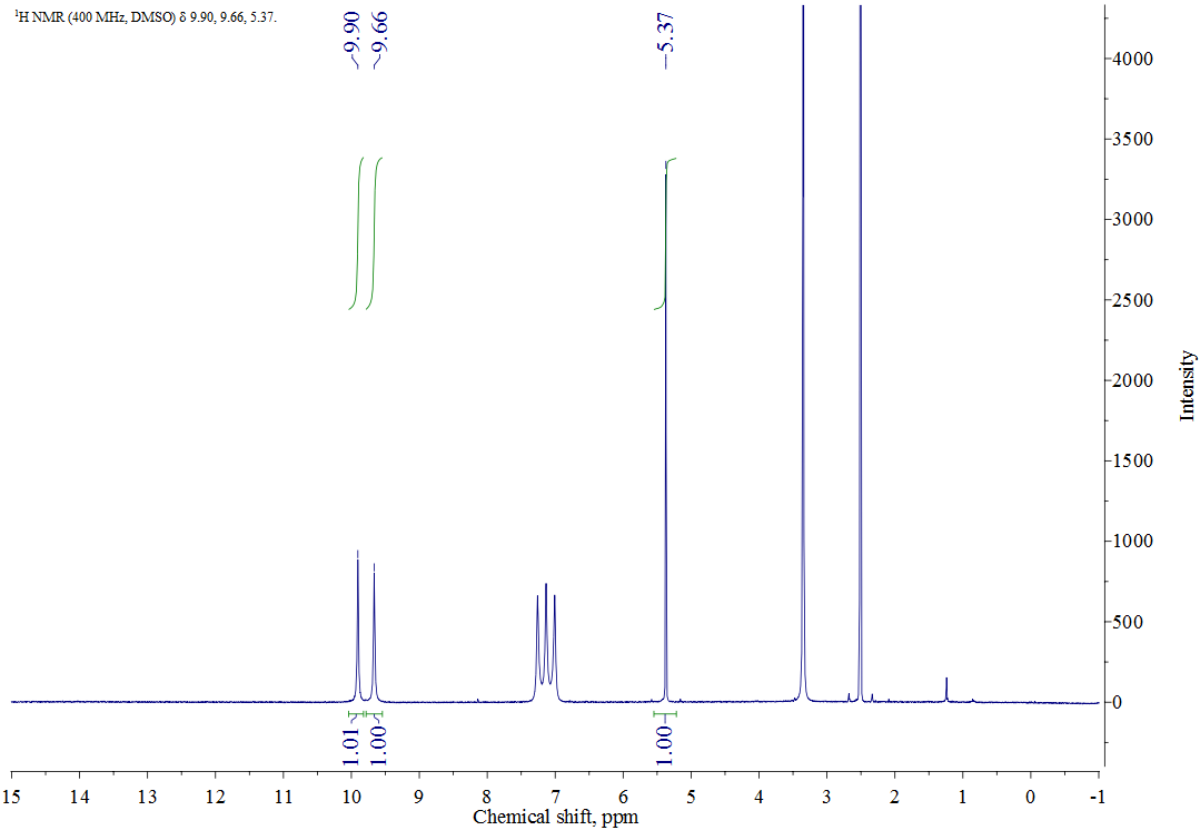


Figure S5. ^{1}H NMR spectra of HPPT (signals around 2.5 ppm comes from DMSO, ~3.3 ppm - H_2O, ~7.1 ppm – NH_4^+)

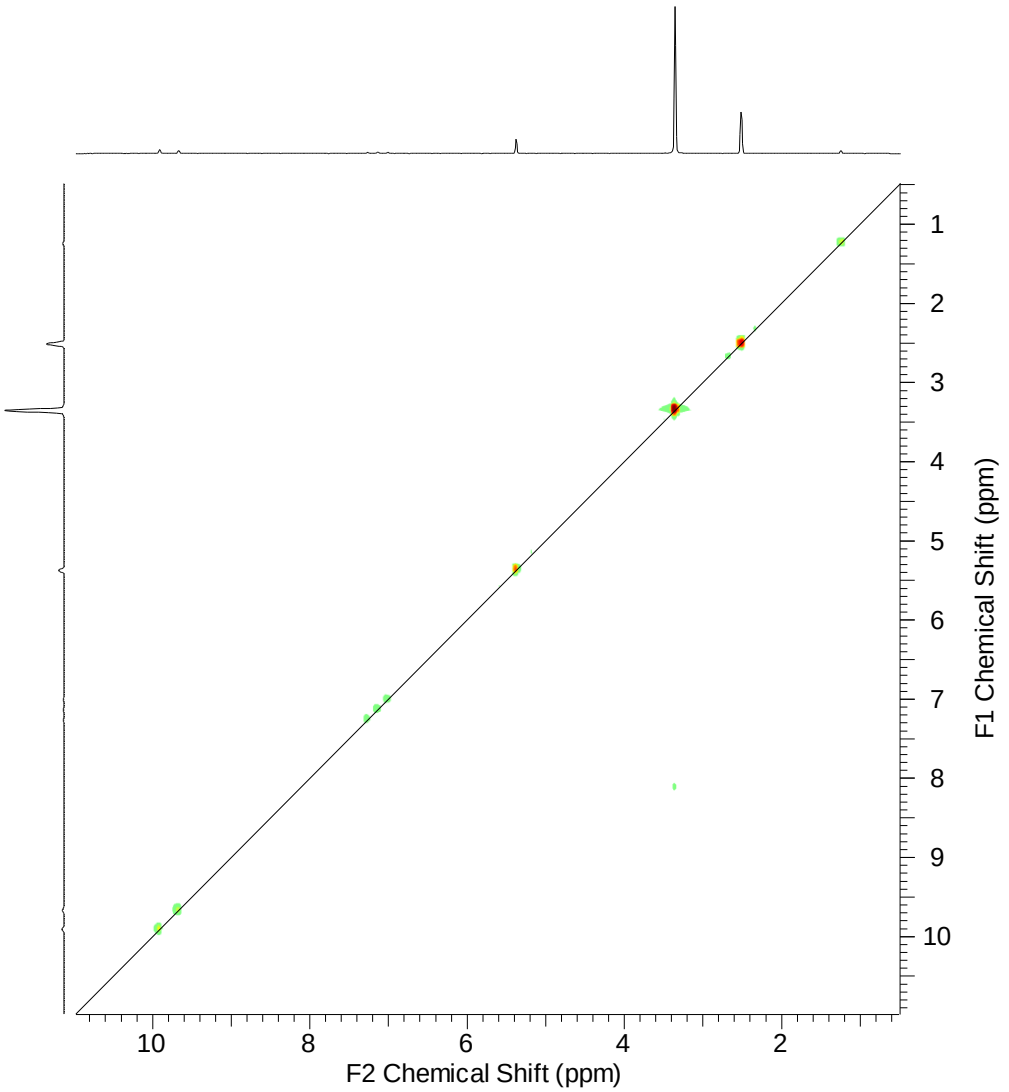


Figure S6. COSY ^{1}H-^{1}H NMR spectra of HPPT (signals around 2.5 ppm comes from DMSO, ~3.3 ppm - H_2O, ~7.1 ppm – NH_4^+)

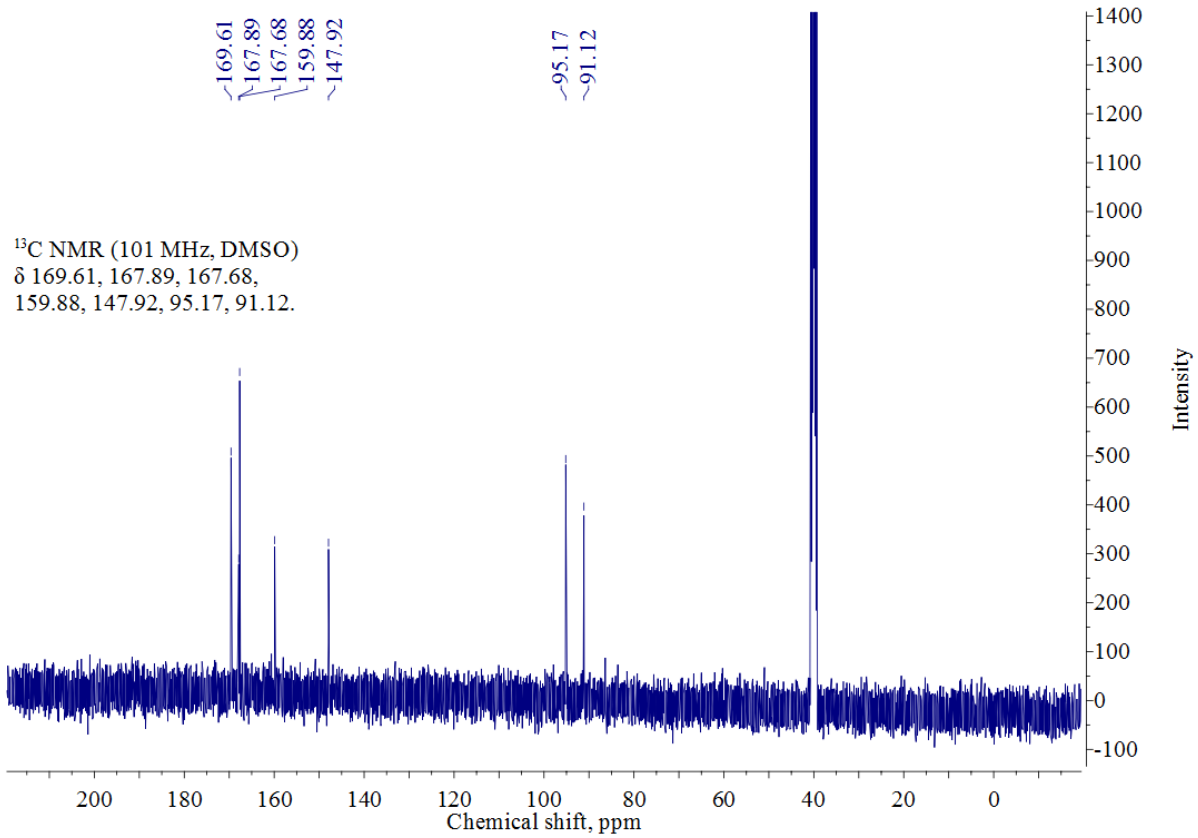


Figure S7. ^{13}C NMR spectra of HPPT (signals around 40 ppm comes from DMSO)

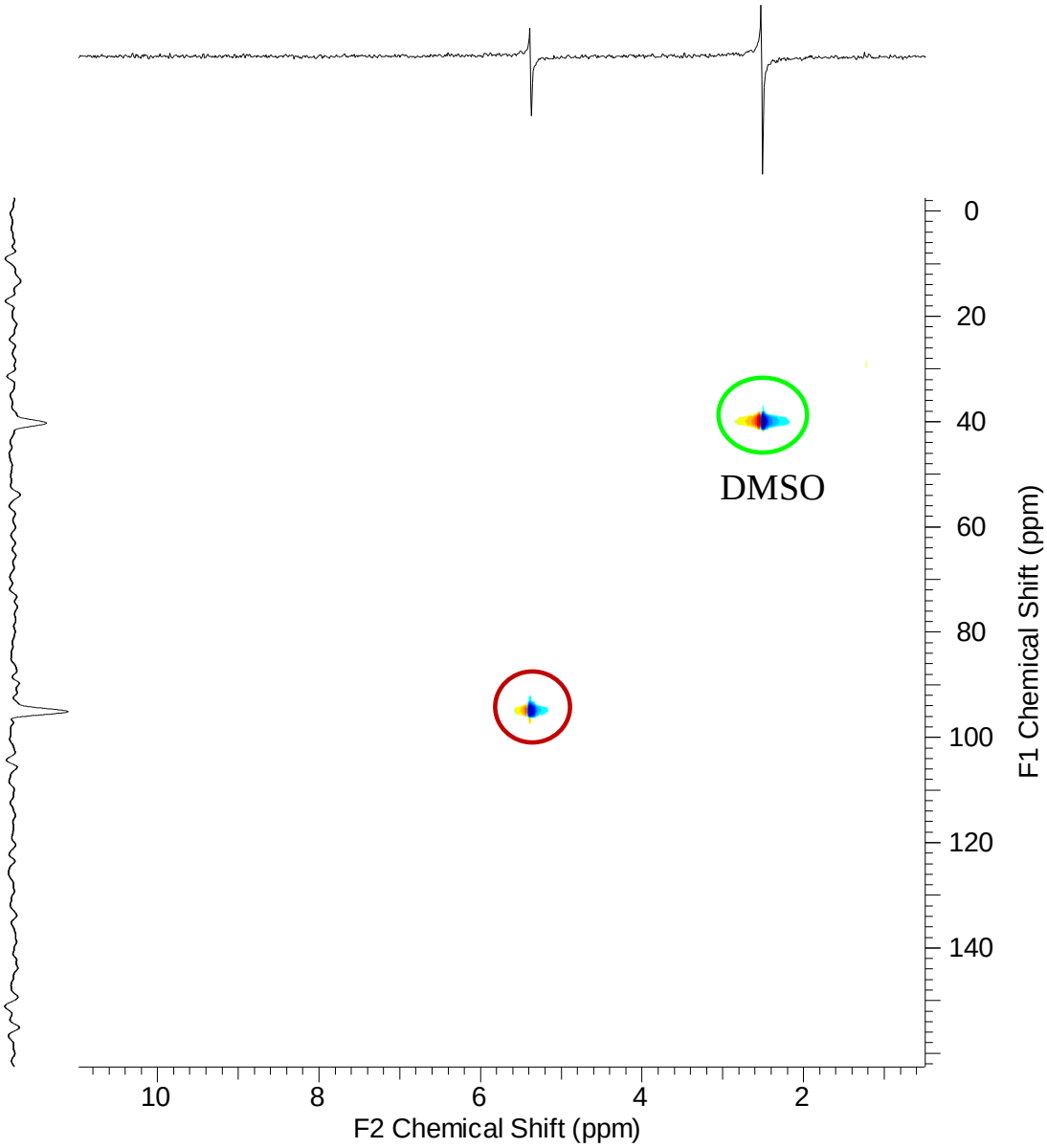


Figure S8. HSQC ^{13}C-^{1}H NMR spectra of HPPT

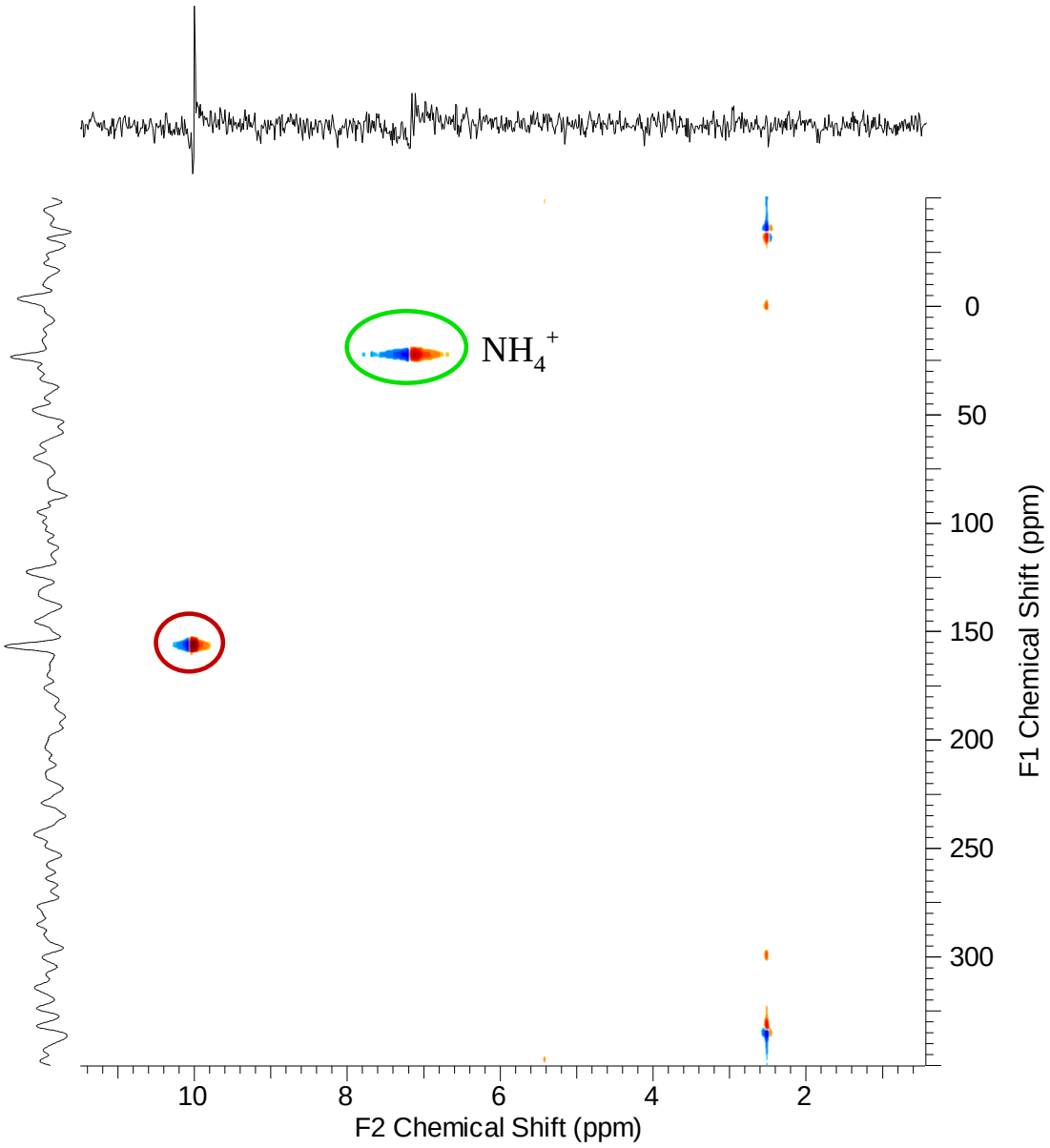


Figure S9. HSQC ^{15}N-^{1}H NMR spectra of HPPT

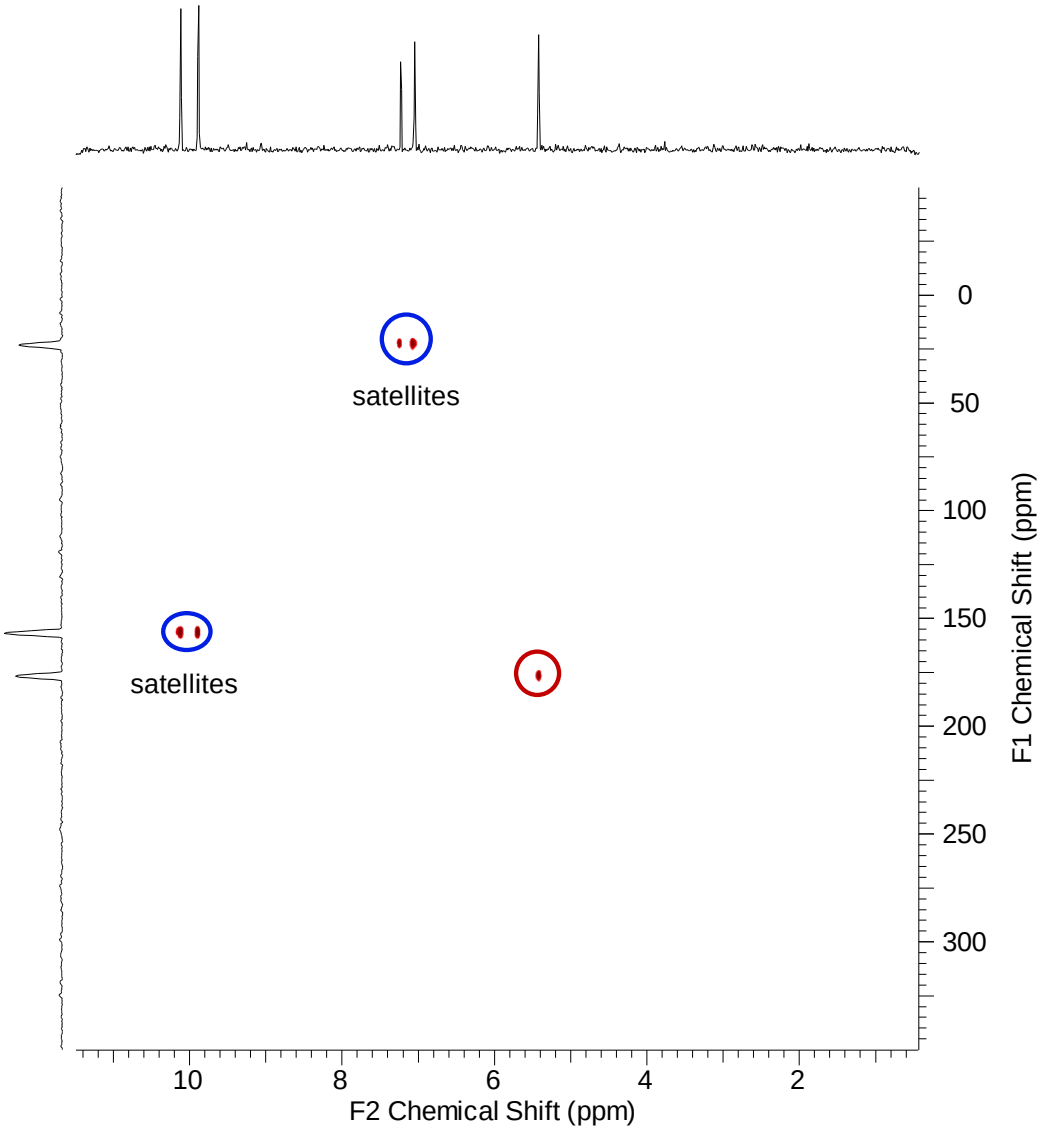


Figure S10. HMBC ^{15}N-^{1}H NMR spectra of HPPT

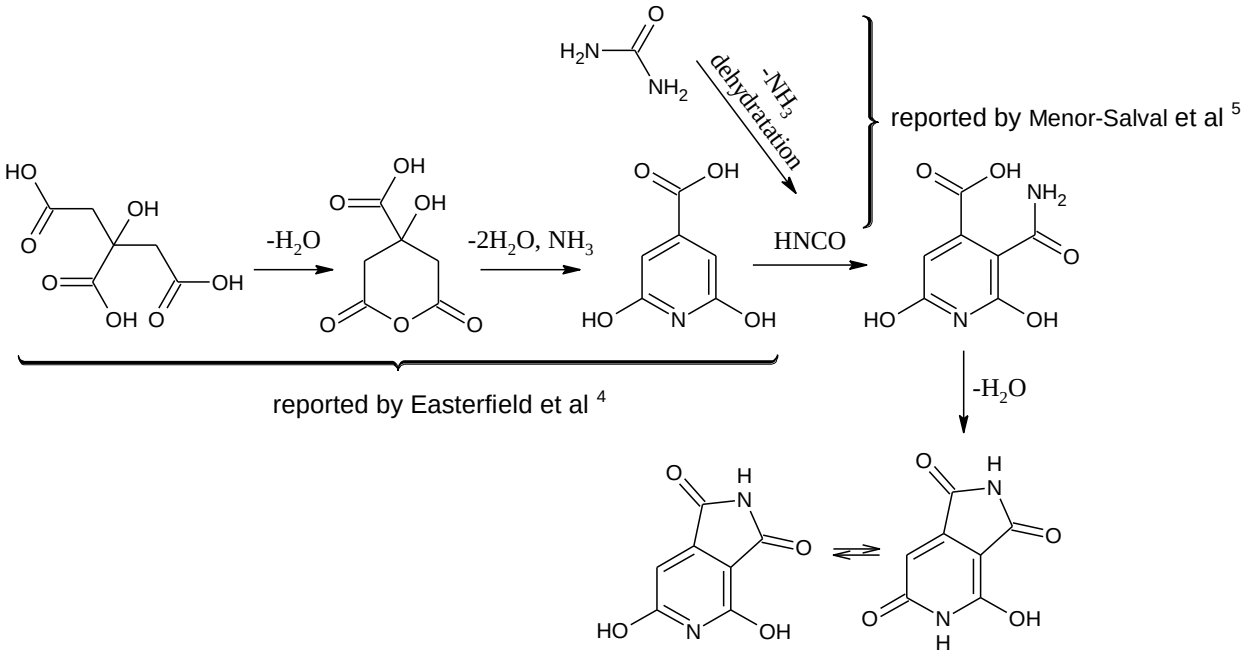


Figure S11A. Possible mechanism of HPPT formation (suggestion 1)

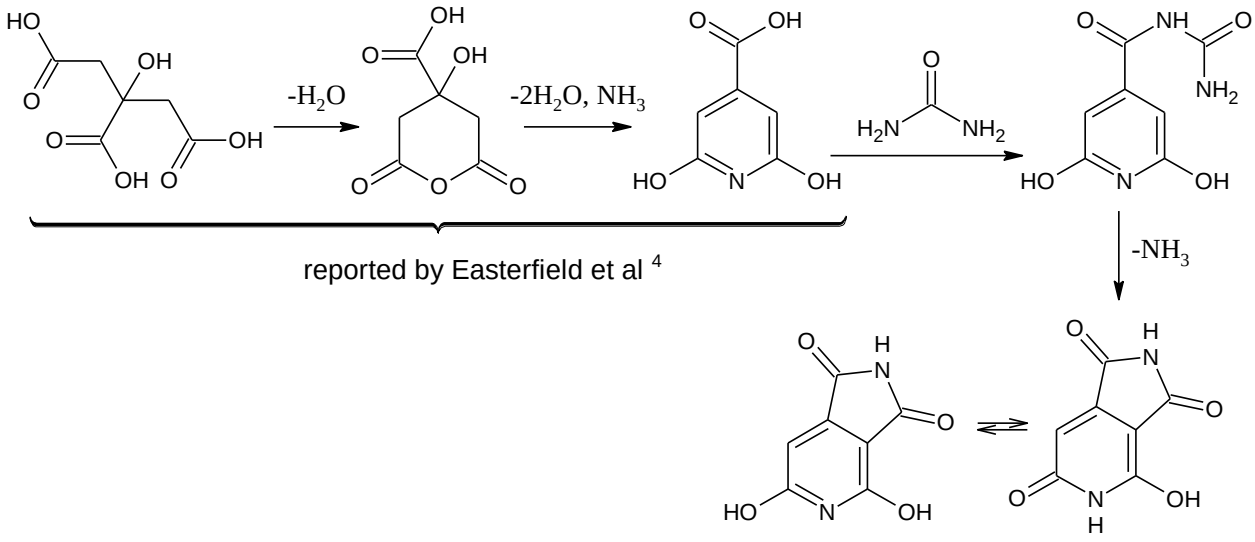


Figure S11B. Possible mechanism of HPPT formation (suggestion 2)

Optical properties characterization

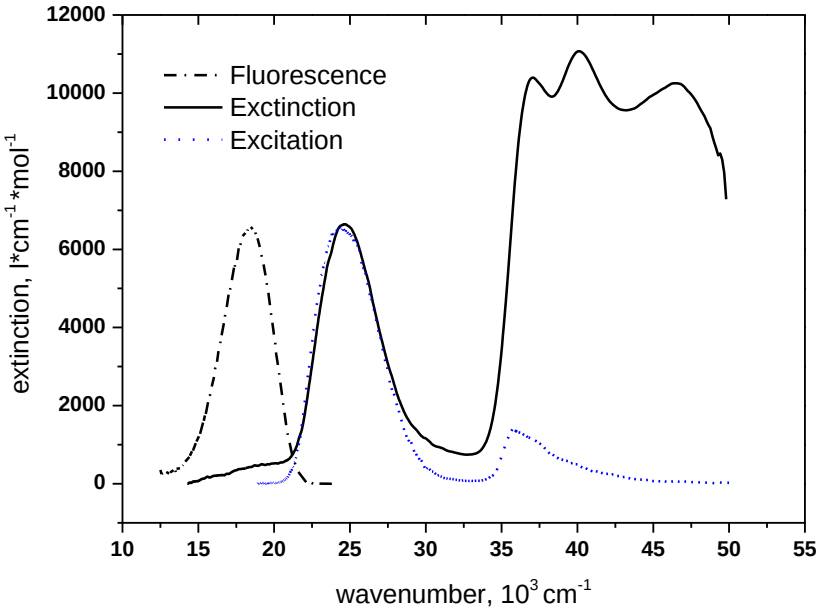


Figure S12. The absorption (extinction); normalized excitation and fluorescence spectra of HTTP.

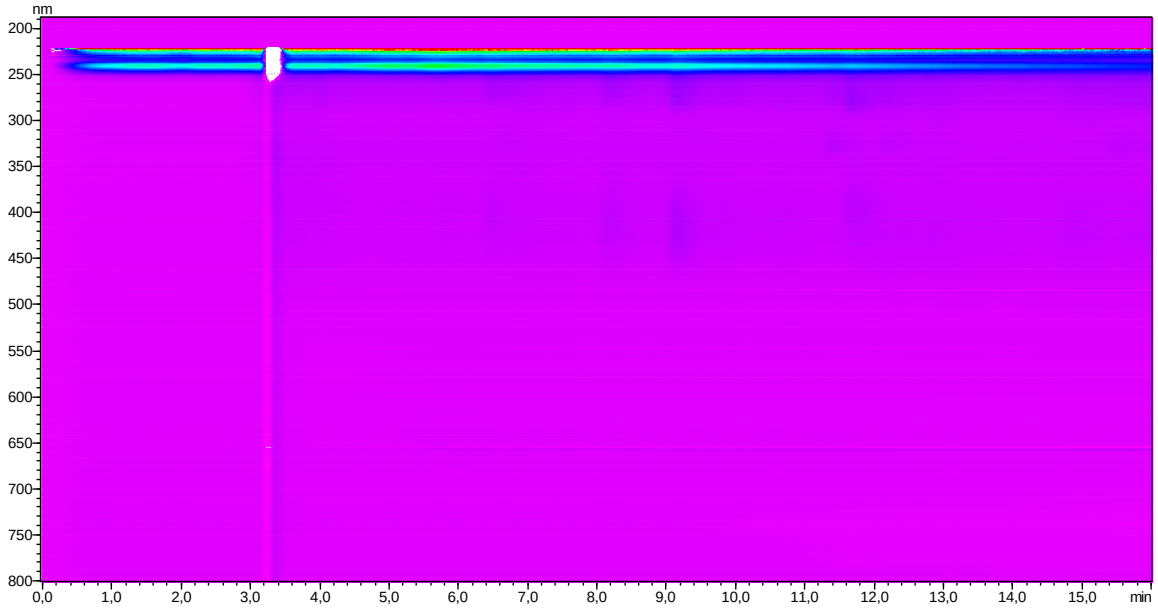


Figure S13A. LC-DAD chromatogram of gCDin showing that this fraction is free from unbounded HPPT fluorophore which should appear at rt ~6.2 minutes. Chromatographic separation conditions applied: The separation of the analytes was performed with a binary gradient elution. The injection volume was 20 µL, and the flow rate was 0.5 mL/min. The column (Kinetex C18) was thermostated at 40 °C. The mobile phases were - A - 2% formic acid in water, and B - pure methanol. The gradient profile was: (t[min], %B), (0, 0.1), (13, 10), (14, 80), (16, 80).

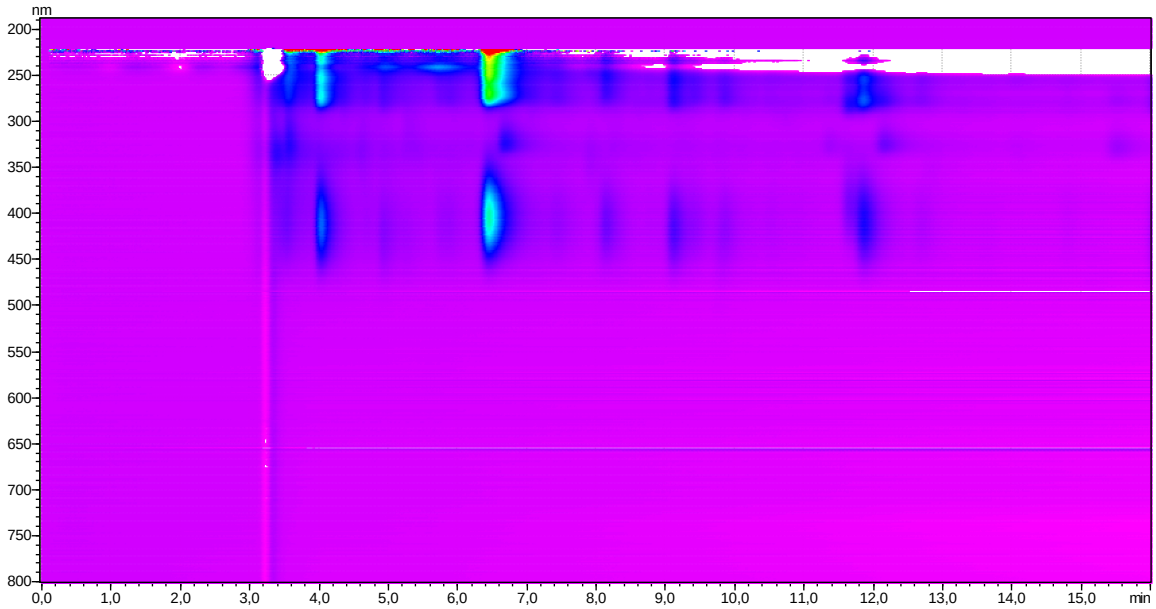


Figure S13B. LC-DAD chromatogram of gCDout. HPPT fraction appear at rt ~6.2. Chromatographic separation conditions applied: The separation of the analytes was performed with a binary gradient elution. The injection volume was 20 µL, and the flow rate was 0.5 mL/min. The column (Kinetex C18) was thermostated at 40 ºC. The mobile phases were - A - 2% formic acid in water, and B - pure methanol. The gradient profile was: (t[min], %B), (0, 0.1), (13, 10), (14, 80), (16, 80).

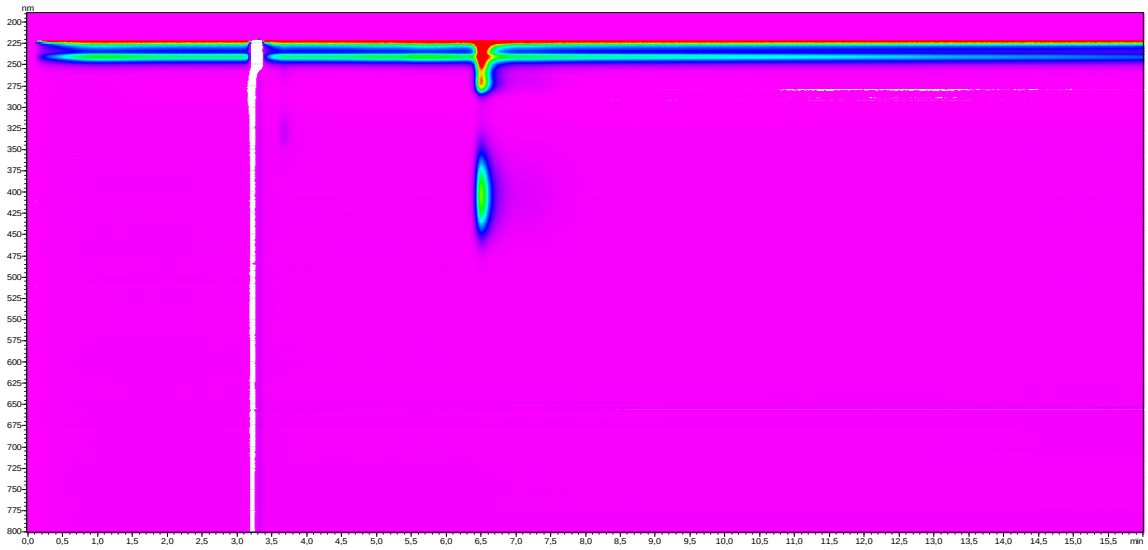


Figure S13C. LC-DAD chromatogram of HPPT. Chromatographic separation conditions applied: The separation of the analytes was performed with a binary gradient elution. The injection volume was 20 µL, and the flow rate was 0.5 mL/min. The column (Kinetex C18) was thermostated at 40 ºC. The mobile phases were - A - 2% formic acid in water, and B - pure methanol. The gradient profile was: (t[min], %B), (0, 0.1), (13, 10), (14, 80), (16, 80).:

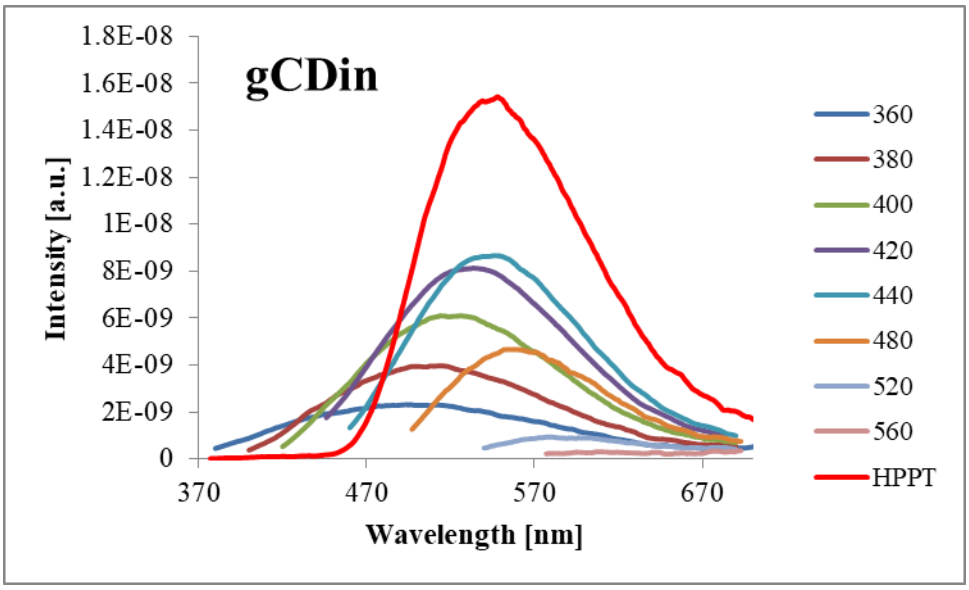


Figure S14. Fluorescence emission spectra of aqueous solutions of gCDin acquired using various excitation wavelengths.

Table S2. Calculated quantum yields of green fluorescing fractions.

Sample	QY [%]
HPPT	14.6
gCDin	6.4
gCDout	14.3

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