Electronic Supporting Information

Elucidation of the intrinsic optical properties of hydrogen-bonded and protonated flavin chromophores by photodissociation action spectroscopy

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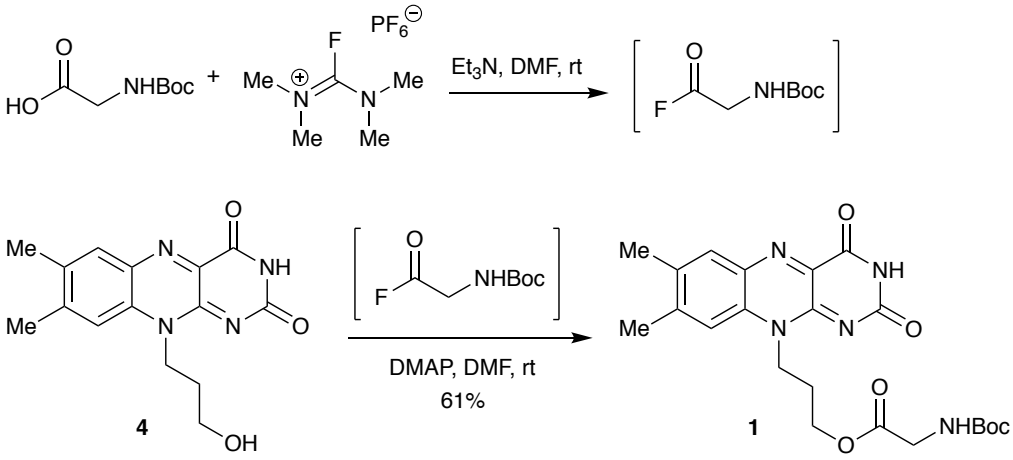
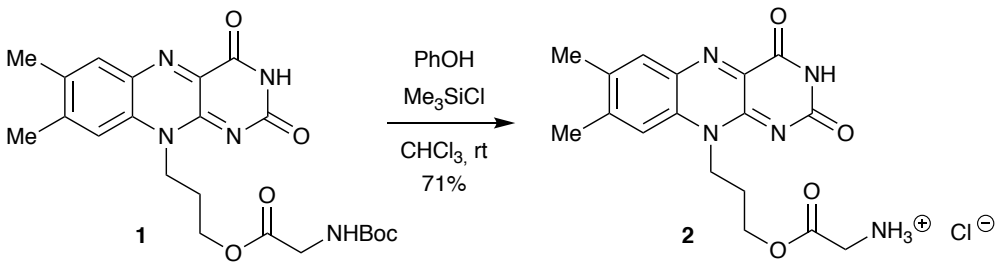
Synthesis protocols



*Synthesis of **1**:* To a solution of (*tert*-butoxycarbonyl)glycine (43.2 mg, 0.25 mmol) in anhydrous DMF (10 mL; dried over 4Å molecular sieves) under argon was added tetramethyl fluoroformadinium (TFFH) (81.8 mg, 0.31 mmol) at rt. Then NEt_3 (0.2 mL) was added, and after stirring for ca. 15 min. (during which *tert*-butyl (2-fluoro-2-oxoethyl)carbamate was formed), compound **4** (35.5 mg, 0.12 mmol) dissolved in dry DMF (ca. 3 mL) was added followed by *N*,*N*-dimethylaminopyrine (DMAP) (8.9 mg). The mixture was stirred overnight at rt and then concentrated *in vacuo* in the presence of Celite. Dry column vacuum chromatography (SiO_2, Gradient: heptane → $CHCl_3$ → 4 % v/v MeOH in $CHCl_3$) gave **1** as an orange solid (33.4 mg, 61%). M.p. 210 °C (decomp.). ^{1}H-NMR (300 MHz, $CDCl_3$) δ: 11.31 (s, 1H); 7.87 (s, 1H), 7.77 (s, 1H), 7.21 (t, J = 6.7 Hz, 1H), 4.64 (t, 2H, J = 6.5 Hz), 4.19 (t, J = 6.5 Hz, 2H), 3.71 (d, J = 6.7 Hz, 2H), 2.50 (s, 3H), 2.38 (s, 3H), 2.08 (m, J = 6.5 Hz, 2H), 1.36 ppm (s, 9H) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 170.1, 159.6, 155.4 (2 C), 149.9, 146.3, 136.9, 135.5, 133.5, 130.8, 130.4, 115.6, 78.0, 61.6, 41.7, 40.9, 27.9, 25.6, 20.3, 18.5; MS (FAB): m/z 457 (M^+); Calc. for $C_{22}H_{27}N_5O_6 \bullet 1/3\ H_2O$: C, 57.01; H, 6.02; N, 15.11; Found: C, 56.90; H, 5.85 %; N, 14.85.



*Synthesis of **2**:* To a solution of compound **1** (16.5 mg, 0.036 mmol) in $CHCl_3$ (1 mL) was added phenol (100 mg, 0.92 mmol) and trimethylsilylchloride (0.12 mL, 1.1 mmol), resulting in an orange precipitate. The mixture was stirred at another 3 h at rt. The product was filtered and triturated with $CHCl_3$, and filtered to provide the product **2** as an orange solid (9.1 mg, 71%). M.p. 212 ^{o}C (decomp.). ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 11.39 (s, 1H), 8.34 (br s, 3H), 7.94 (s, 1H), 7.86 (s, 1H), 4.73 (t, J = 6.5 Hz, 2H), 4.30 (t, J = 6.5 Hz, 2H), 3.95 (s, 2H), 2.53 (s, 3H), 2.41 (s, 3H), 2.13 (m, J = 6.5 Hz, 2H) ppm; ^{13}C-NMR (75 MHz, DMSO-d_6) δ: 168.4, 160.7, 156.9, 150.9, 147.5, 137.8, 136.7, 134.7, 131.8, 131.4, 116.7, 63.4, 41.6, 26.6, 21.3, 19.5 ppm, one signal overlapping; MS (ESP): *m/z* 358 ([M-Cl]$^{+}$). HR-MS (ESP): *m/z* 358.1510; calc. for $C_{17}H_{20}N_5O_4$: 358.1510.

UV-Vis absorption spectra in solution

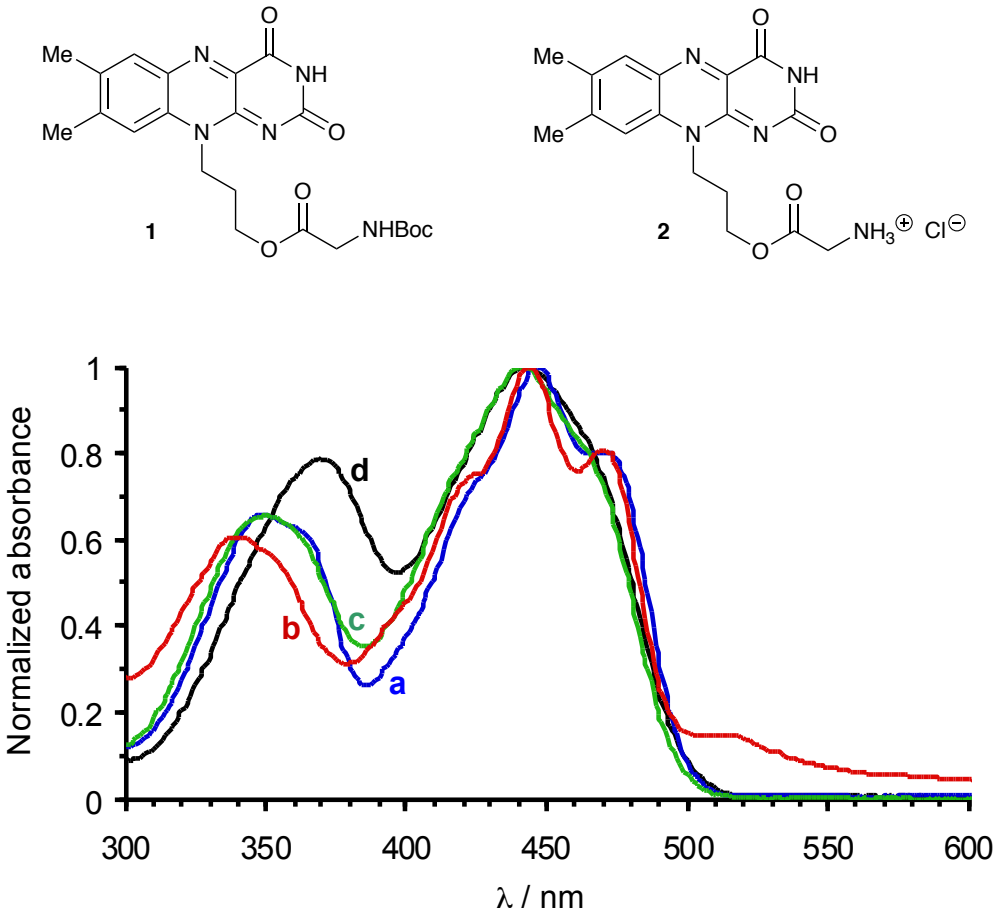
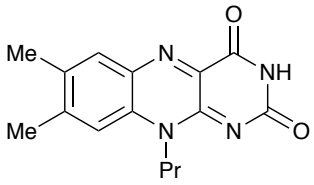


Figure S1. UV-Vis absorption spectra of a) **1** in $CHCl_3$ (blue curve), b) **1** in PhMe (red curve), c) **1** in MeOH (green curve), and d) **2** in H_2O (black curve).

Protonation studies



3

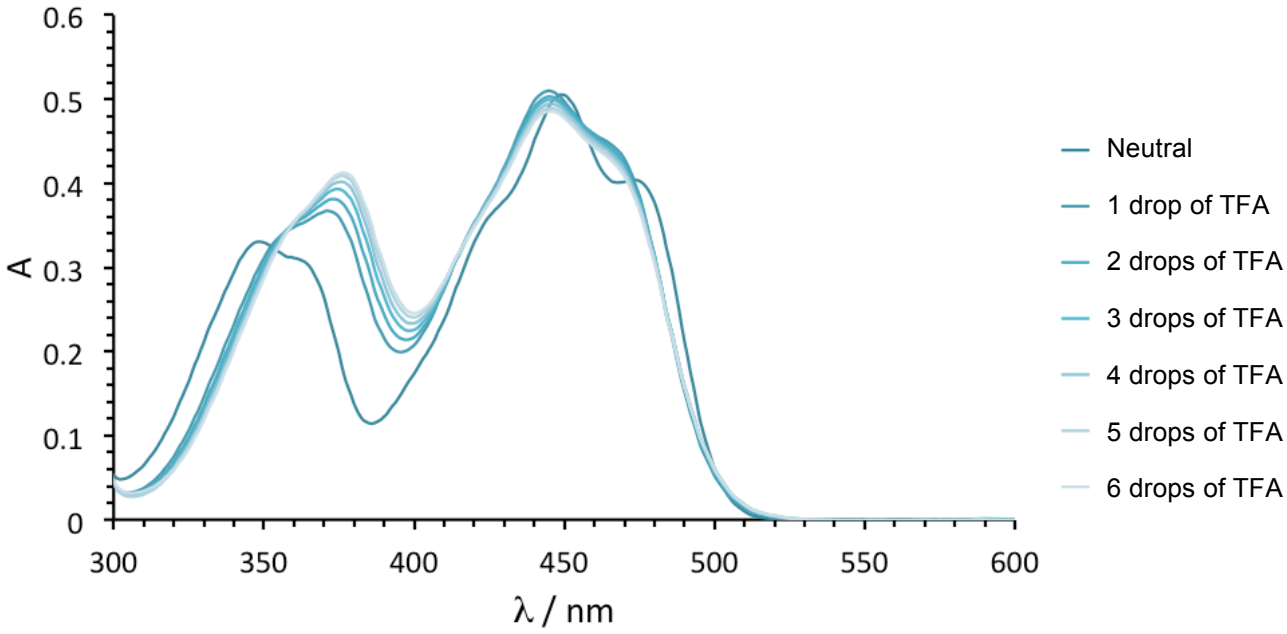


Figure S2. UV-Vis absorption spectra **3** in chloroform before and after addition of drops of trifluoroacetic acid (TFA).

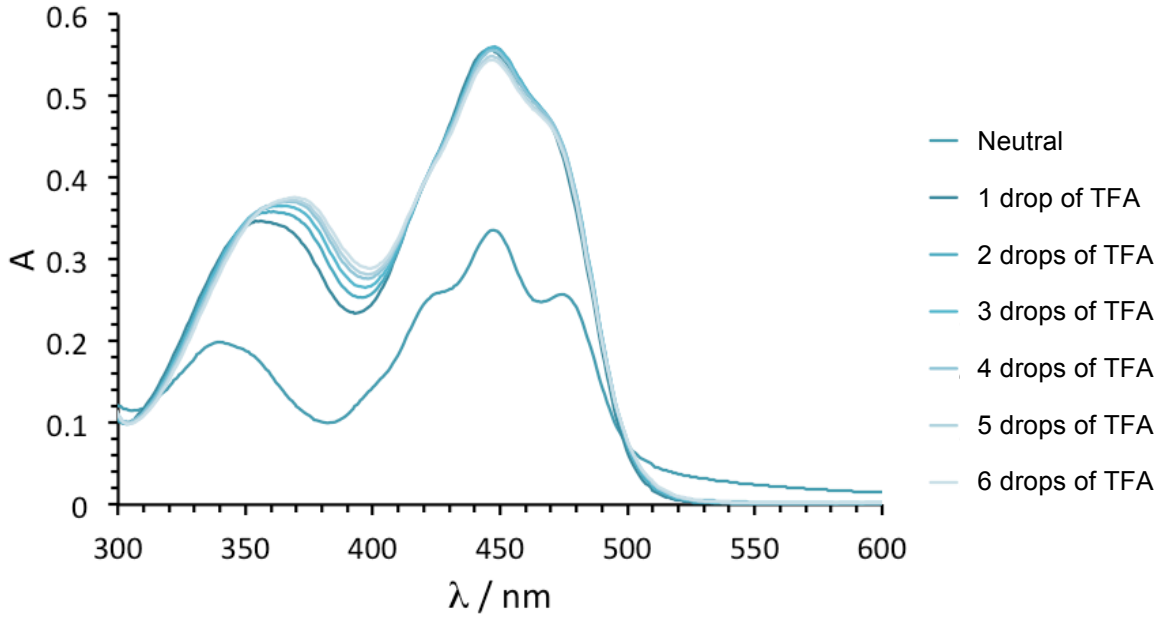


Figure S3. UV-Vis absorption spectra **3** in toluene before and after addition of drops of trifluoroacetic acid (TFA).

NMR spectra

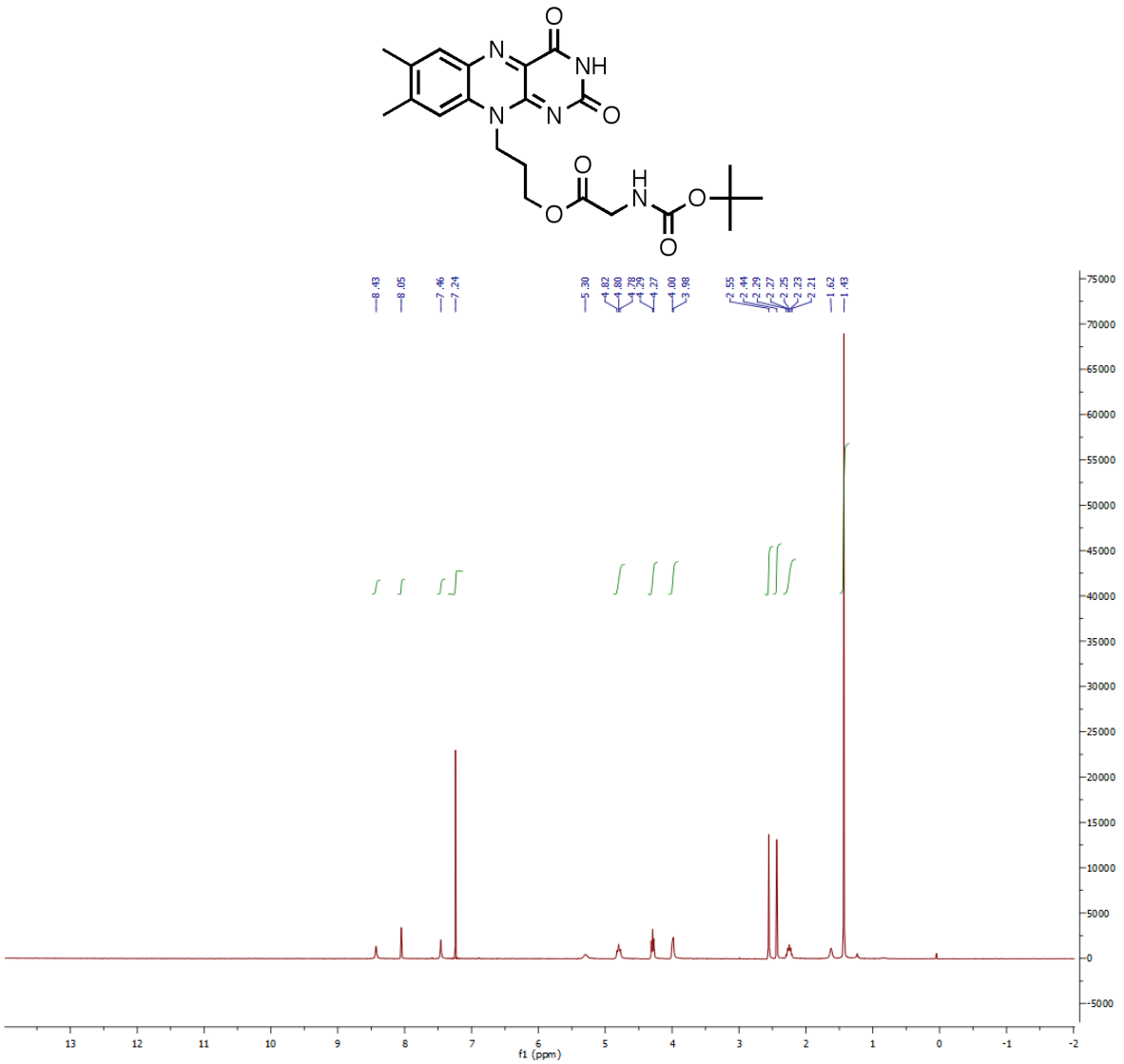


Figure S4. ^{1}H NMR spectrum (300 MHz) of **1** in $CDCl_3$.

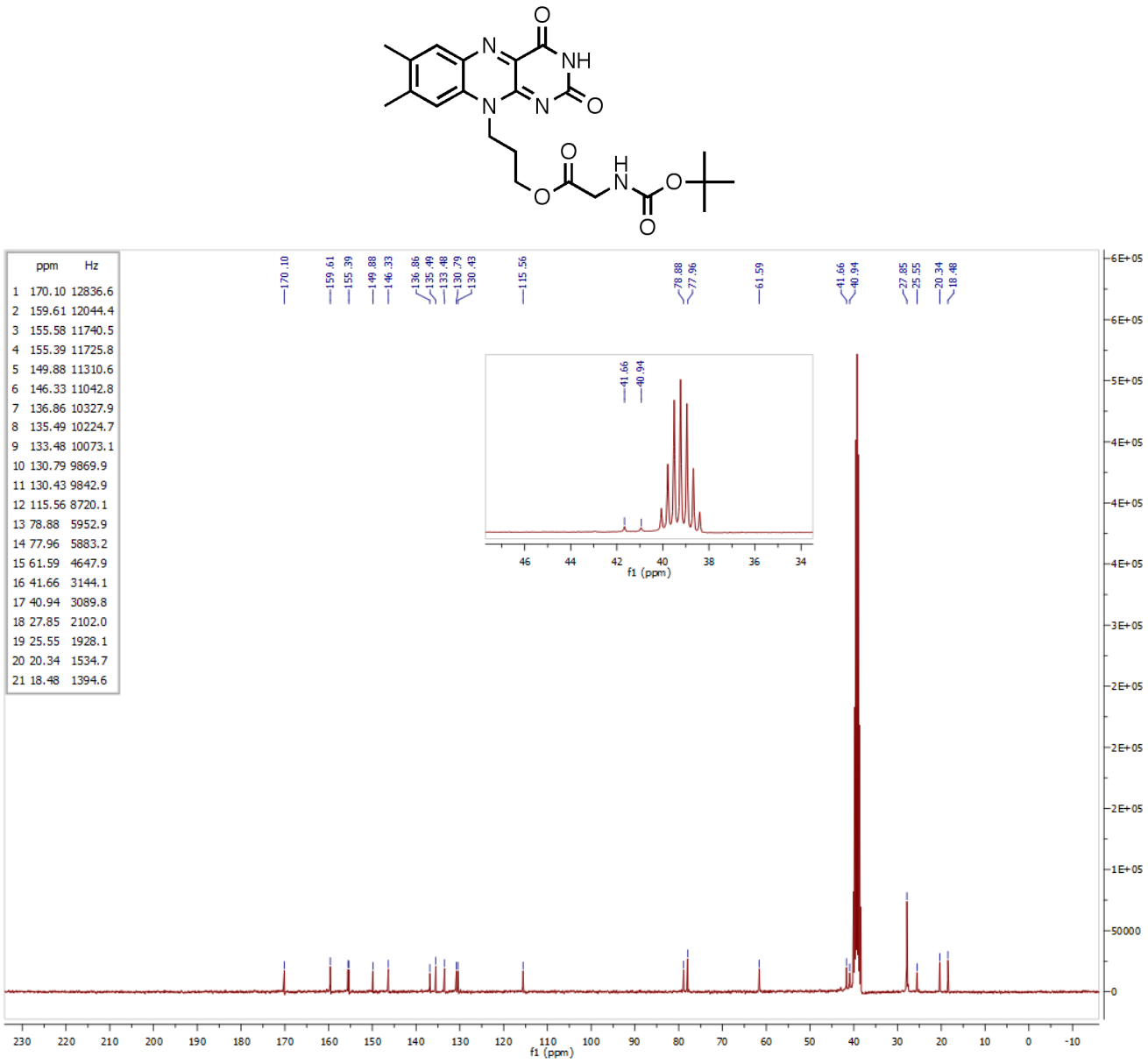


Figure S5. ^{13}C NMR spectrum (75 MHz) of **1** in DMSO-d_6.

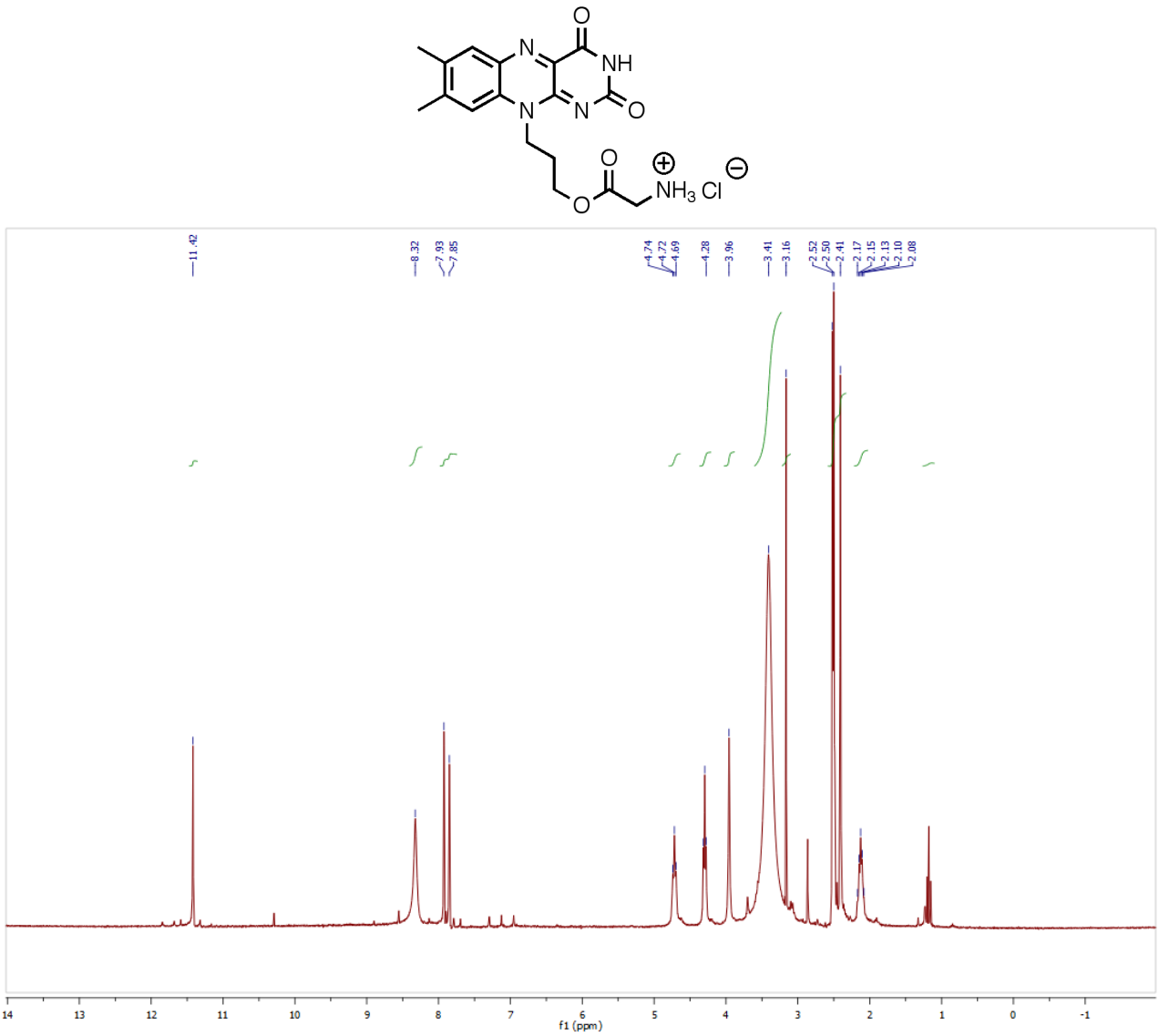


Figure S6. ^{1}H NMR spectrum (300 MHz) of **2** in DMSO-d_6.

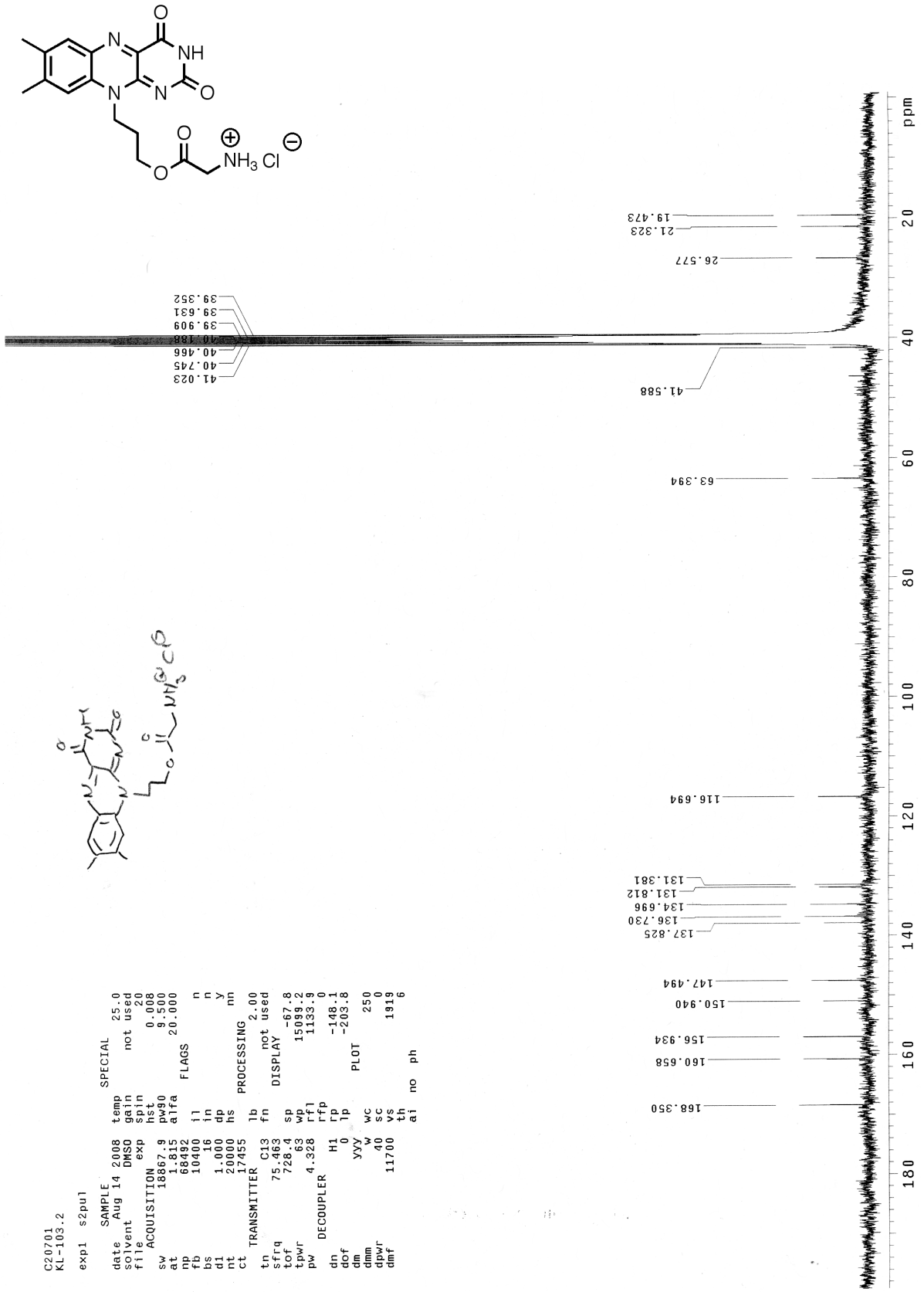


Figure S7. ^{1}H NMR spectrum (75 MHz) of **2** in DMSO-d_6.

Gas-phase action spectroscopy studies

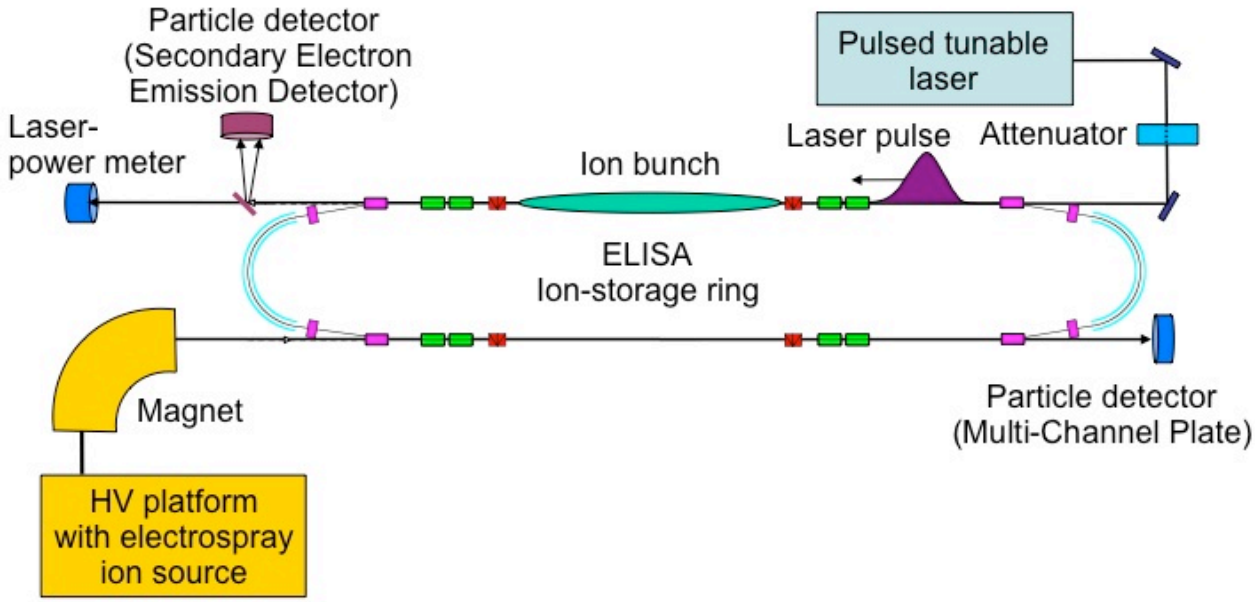


Figure S8. Schematic drawing of the ELISA storage ring in Aarhus, where the action-absorption spectra were obtained.

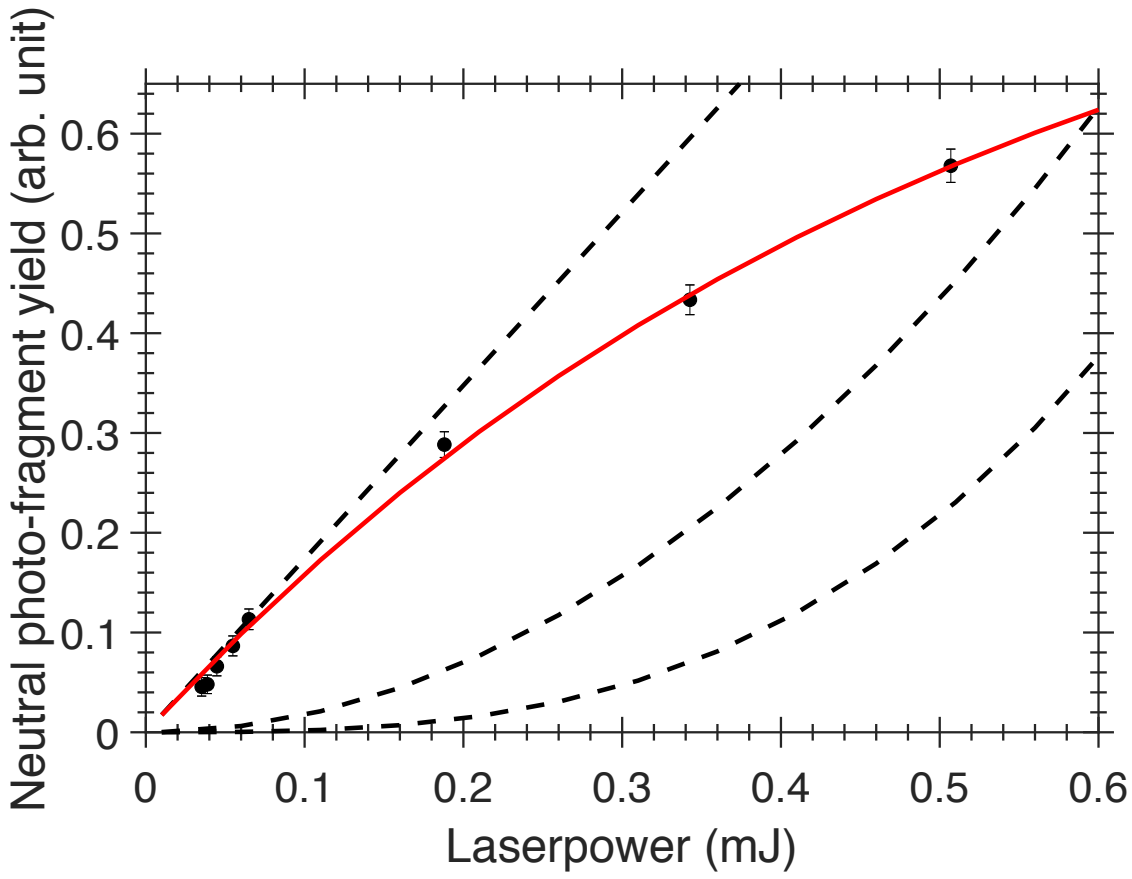


Figure S9. Yield of neutral photo-fragments of **3•H⁺** as a function of laser power (power dependence) at 260 nm. At low power (before saturation) the yield is proportional to P^1, which is consistent with a one-photon process. Dashed lines indicate 1, 2 and 3 photon dependencies respectively.

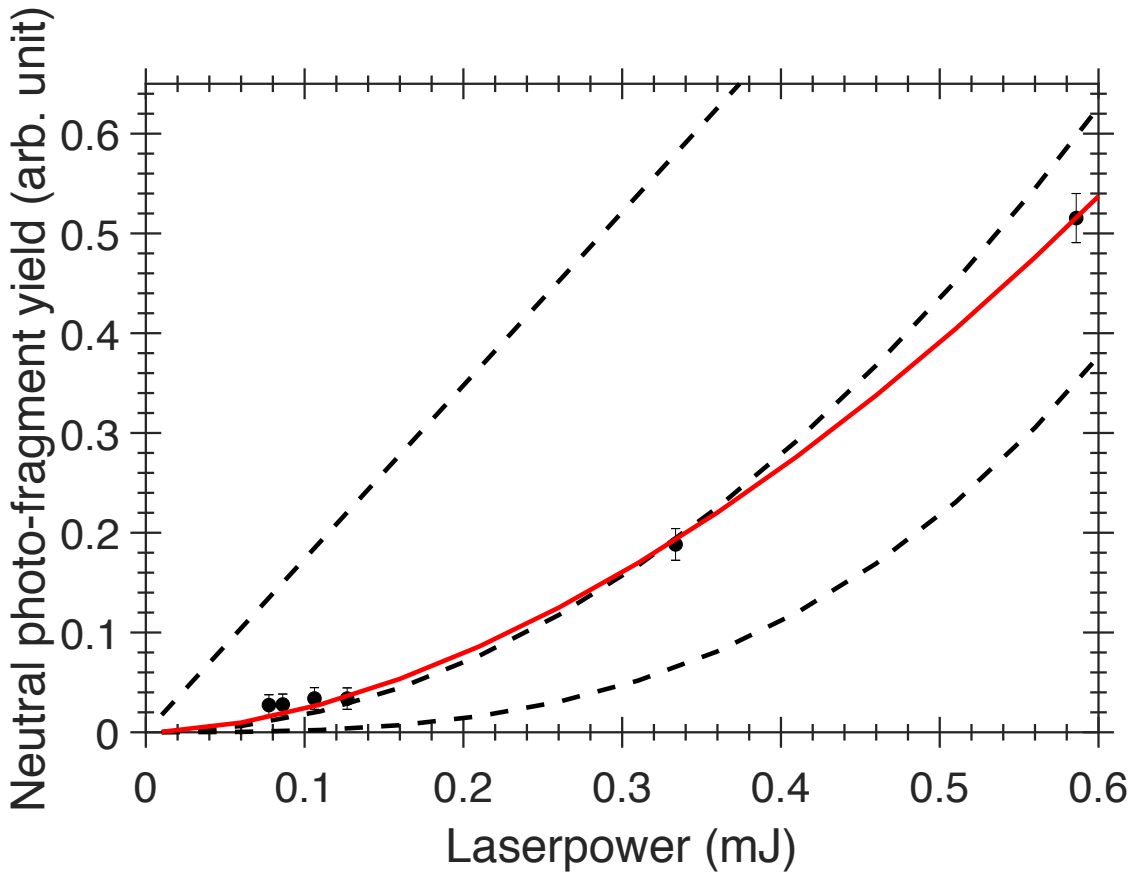


Figure S10. Yield of neutral photo-fragments of **3•H⁺** as a function of laser power (power dependence) at 410 nm. Measurement is consistent with a two-photon process. The dashed lines represent 1, 2 and 3 photon dependencies respectively.

Calculational data

Table S1. Relative Gibbs free energies in eV (calculated as electronic energy with thermal correction at 298.15 K) of **3•H**$^+$ with the proton located at each of the various heteroatoms obtained computationally using different methods and the 6-311+G(d,p) basis set. Boltzmann factors for the distribution between protonated species are included in brackets.

Method	*N1*	*O2*	*N3*	*O4*	*N5*	*N10*
ωB97XD	0.0002	0	0.306	0.748	0.550	1.979
	(0.991)	(1.0)	(10^{-6})	(10^{-13})	(10^{-10})	(10^{-34})
CAM-B3LYP	0.025	0	0.277	0.423	0.562	2.014
	(0.382)	(1.0)	(10^{-5})	(10^{-8})	(10^{-10})	(10^{-35})
HSEH1PBE	0.049	0	0.344	0.362	0.478	2.092
	(0.147)	(1.0)	(10^{-6})	(10^{-7})	(10^{-9})	(10^{-36})
LSDA	0.042	0	0.340	0.210	0.243	2.278
	(0.193)	(1.0)	(10^{-6})	(10^{-4})	(10^{-5})	(10^{-39})

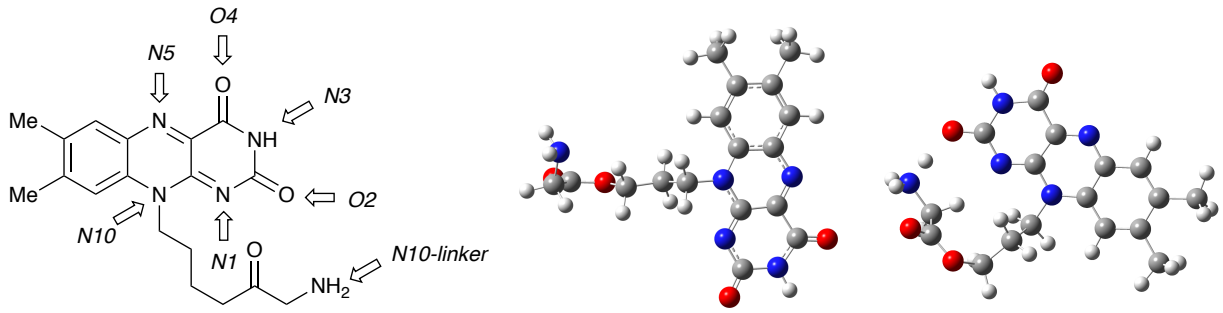


Figure S11. Left: Numbering of protonation sites of the neutral, deprotonated compound **2**. Middle: Optimized structure of the neutral, deprotonated compound **2**. Right: Optimized structure of the *N10-linker*-protonated compound revealing a hydrogen bond between NH_3^+ and *O2*.

Table S2. Relative Gibbs free energies in eV (calculated as electronic energy with thermal correction at 298.15 K) of **2** with the proton located at each of the various heteroatoms obtained computationally using different methods and the 6-311+G(d,p) basis set. Boltzmann factors for the distribution between protonated species are included in brackets.

Method	*N1*	*O2*	*N3*	*O4*	*N5*	*N10*	*N10-linker*
ωB97XD	0.706	0.678	0.977	1.405	1.217	2.693	0
	(10^{-12})	(10^{-12})	(10^{-17})	(10^{-24})	(10^{-21})	(10^{-46})	(1)
CAM-B3LYP	0.640	0.593	0.875	1.306	1.139	2.648	0
	(10^{-11})	(10^{-11})	(10^{-15})	(10^{-23})	(10^{-20})	(10^{-45})	(1)
HSEH1PBE	0.697	0.624	1.021	1.284	1.090	2.747	0
	(10^{-12})	(10^{-11})	(10^{-18})	(10^{-22})	(10^{-19})	(10^{-47})	(1)

Coordinates – Optimized structures were obtained with functional ωB97X-D and basis set 6-311+G(d,p).

Deprotonated compound 2 (with neutral amino group)

Energy: -774274.7631122 Hartrees

C	1.17303	3.42883	0.19883
C	0.54041	2.21909	0.42305
C	1.22987	1.00623	0.28956
C	2.58319	1.03775	-0.09189
C	3.21358	2.27326	-0.31097
C	2.54098	3.46517	-0.17234
C	2.72652	-1.23807	-0.08843
C	1.32587	-1.39048	0.29167
C	1.38901	-3.70462	0.25298
C	3.51259	-2.49901	-0.28937
H	-0.50806	2.22739	0.68904
H	4.25844	2.24531	-0.59946
H	3.21872	-4.51752	-0.22141
N	3.31287	-0.10713	-0.26403
N	0.63702	-0.22648	0.51232
N	0.70338	-2.52203	0.43754
N	2.75208	-3.62885	-0.09944
O	4.67993	-2.53099	-0.58553
O	0.87941	-4.79075	0.37893
C	-0.76428	-0.33047	0.92676
C	-1.70873	-0.29282	-0.27081
H	-0.97075	0.47418	1.63404
H	-0.85908	-1.28010	1.45138
C	-3.14094	-0.50511	0.17719
H	-1.63463	0.66691	-0.78989
H	-1.42397	-1.07685	-0.97631
H	-3.26547	-1.52129	0.56724
H	-3.43041	0.21549	0.94712
O	-3.96727	-0.32681	-0.97676
C	-5.30739	-0.30108	-0.88697
O	-5.95917	-0.16618	-1.88456
N	-5.75006	0.84917	1.21785
C	-5.95411	-0.40430	0.49076
H	-6.25275	1.60265	0.76318
H	-6.11134	0.77323	2.16050
C	3.23515	4.77735	-0.41560
C	0.40037	4.71012	0.34394
H	3.20993	5.41225	0.47514
H	2.75604	5.33705	-1.22454
H	4.27945	4.62064	-0.68776
H	0.40190	5.27561	-0.59250
H	-0.63637	4.52202	0.62522
H	0.84959	5.35350	1.10634

H	-7.00316	-0.65948	0.31319
H	-5.49902	-1.21695	1.06305

Compound 2 with proton at *N10-linker* (but no counter anion) – with the NH_3^+ group H-bonded to *O2*

Energy: -774531.9415302 Hartrees

C	4.00630	1.58140	0.09979
C	2.64235	1.53976	0.32905
C	1.93901	0.33216	0.25705
C	2.64014	-0.84707	-0.07655
C	4.03034	-0.78879	-0.29777
C	4.72428	0.39210	-0.21290
C	0.73119	-2.09154	-0.03565
C	-0.08014	-0.94583	0.29351
C	-2.05063	-2.14289	0.22380
C	0.03517	-3.40468	-0.18886
H	2.13242	2.46872	0.54609
H	4.52618	-1.72071	-0.54393
H	-1.88645	-4.14219	-0.15579
N	2.01321	-2.04331	-0.19887
N	0.57281	0.23039	0.48730
N	-1.39728	-0.96719	0.41968
N	-1.34550	-3.29547	-0.03154
O	0.57259	-4.44714	-0.42651
O	-3.28653	-2.21692	0.26569
C	-0.22221	1.38538	0.92099
C	-0.92384	2.05966	-0.25888
H	0.42904	2.06556	1.46550
H	-0.95385	1.00023	1.63139
C	-2.03910	3.01225	0.18951
H	-0.19975	2.58237	-0.88711
H	-1.36601	1.27706	-0.87876
H	-2.33982	2.83113	1.22629
H	-1.74370	4.05640	0.11680
O	-3.17868	2.91249	-0.68136
C	-4.01820	1.89251	-0.60177
O	-4.82101	1.67036	-1.46559
N	-4.87346	-0.14916	0.39754
C	-3.98094	1.00828	0.64781
H	-4.26524	-1.03819	0.31983
H	-5.36489	0.02959	-0.49102
H	-5.56305	-0.28315	1.13274
C	6.20663	0.43598	-0.45362
C	4.73273	2.89211	0.17689

H	6.73749	0.82017	0.42181
H	6.45132	1.09120	-1.29429
H	6.59592	-0.55741	-0.67553
H	5.22563	3.11906	-0.77255
H	4.05885	3.71447	0.41770
H	5.51511	2.85630	0.94021
H	-2.98189	0.60979	0.83703
H	-4.32531	1.57784	1.51235

Compound 3

C	-3.43000	0.20260	-0.15030
C	-2.23340	0.87340	-0.26950
C	-0.99370	0.19030	-0.19300
C	-1.00370	-1.24300	0.02860
C	-2.22830	-1.90590	0.14340
C	-3.43850	-1.23110	0.05800
C	1.27210	-1.35050	0.03130
C	1.39070	0.07890	-0.19390
C	3.74010	0.02680	-0.16630
C	2.54990	-2.14220	0.15070
H	-2.25240	1.94410	-0.41380
H	-2.19520	-2.97730	0.30390
N	0.13970	-1.97240	0.13230
N	0.20590	0.81480	-0.31830
N	2.51470	0.72240	-0.29250
N	3.68520	-1.35280	0.03890
O	2.57810	-3.33080	0.32680
O	4.78560	0.61960	-0.23780
C	0.31460	2.28730	-0.54230
C	0.28720	3.07940	0.77160
H	-0.49230	2.57400	-1.21450
H	1.25560	2.44780	-1.06070
C	0.40280	4.58160	0.50290
H	-0.63410	2.86280	1.32250
H	1.11870	2.74820	1.39980
H	0.39120	5.13530	1.44340
H	1.33570	4.82680	-0.01050
H	-0.42540	4.95270	-0.10750
C	-4.72950	-1.97450	0.18220
C	-4.72480	0.95470	-0.23480
H	-5.32520	-1.59800	1.02070
H	-5.34810	-1.84710	-0.71270
H	-4.56680	-3.04060	0.33320
H	-4.56330	2.02110	-0.38890

H	-5.34370	0.58450	-1.05780
H	-5.31520	0.83040	0.67820
H	4.57940	-1.82710	0.1170037

Compound 3 protonated at *O2* - 6•*O2*-H^+

Energy: -596798.9744427 Hartrees

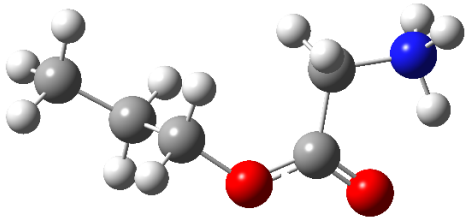
C	3.42672	0.19270	0.15320
C	2.22517	0.86177	0.27249
C	1.00919	0.16873	0.19662
C	1.03120	-1.23539	-0.01900
C	2.27351	-1.90204	-0.13292
C	3.45885	-1.22454	-0.04933
C	-1.23969	-1.33205	-0.03047
C	-1.35477	0.07218	0.18854
C	-3.61469	-0.00347	0.15230
C	-2.48951	-2.13349	-0.16224
H	2.24403	1.93346	0.41122
H	2.24012	-2.97355	-0.29171
N	-0.09783	-1.95452	-0.12453
N	-0.22001	0.78687	0.31846
N	-2.53595	0.71941	0.27374
N	-3.64626	-1.33512	-0.05242
O	-2.55735	-3.31024	-0.34118
O	-4.80057	0.54897	0.22403
C	-0.30588	2.24821	0.54126
C	-0.22296	3.02405	-0.77036
H	0.49238	2.51972	1.23101
H	-1.25291	2.43667	1.04006
C	-0.25677	4.52778	-0.51610
H	0.69195	2.75162	-1.30529
H	-1.06343	2.72765	-1.40474
H	-0.21187	5.07598	-1.45798
H	-1.17473	4.82379	-0.00166
H	0.59062	4.84819	0.09628
C	4.77065	-1.94382	-0.17145
C	4.71239	0.95854	0.23419
H	5.37800	-1.80851	0.72767
H	5.35168	-1.56186	-1.01532
H	4.61862	-3.01237	-0.32034
H	4.54010	2.02385	0.38500
H	5.29662	0.82972	-0.68129
H	5.32933	0.58955	1.05839
H	-4.54189	-1.80337	-0.13259
H	-4.70529	1.49813	0.37135

Compound 3 protonated at *N1* - 6•*N1*-H$^+$

Energy: -596798.5088771 Hartrees

C	3.44077	0.21749	0.10796
C	2.23419	0.88415	0.22890
C	1.02676	0.18145	0.18728
C	1.05052	-1.22167	0.00031
C	2.29074	-1.88561	-0.11192
C	3.47662	-1.20054	-0.05811
C	-1.22691	-1.34064	-0.01030
C	-1.33129	0.06882	0.17351
C	-3.78248	-0.05695	0.09355
C	-2.47718	-2.16499	-0.11961
H	2.25305	1.95992	0.33492
H	2.26016	-2.96048	-0.24711
N	-0.08201	-1.94796	-0.08093
N	-0.21661	0.79565	0.31661
N	-2.55525	0.64008	0.21337
N	-3.65299	-1.41757	-0.04249
O	-2.48135	-3.35003	-0.25782
O	-4.81587	0.54094	0.11664
C	-0.30287	2.25049	0.57341
C	-0.35868	3.06900	-0.71475
H	0.55761	2.52086	1.18192
H	-1.16948	2.42867	1.21334
C	-0.43644	4.56209	-0.41016
H	0.52909	2.84738	-1.31428
H	-1.22099	2.76079	-1.31548
H	-0.47121	5.13686	-1.33604
H	-1.33161	4.80822	0.16718
H	0.43484	4.89804	0.15807
C	4.78906	-1.91893	-0.17879
C	4.72226	0.99350	0.14758
H	5.40494	-1.76274	0.71107
H	5.36008	-1.55330	-1.03652
H	4.63901	-2.99083	-0.30318
H	4.54670	2.06096	0.27853
H	5.28983	0.84833	-0.77590
H	5.35715	0.64622	0.96753
H	-4.52165	-1.93381	-0.12066
H	-2.68208	1.64028	0.26687

Coordinates for protonated propyl glycinate



C	-0.222209	1.385378	0.920989
C	-0.923843	2.059664	-0.258876
H	0.399942	2.098195	1.420709
H	-0.954883	1.010218	1.604615
C	-2.039102	3.012251	0.189509
H	-0.199753	2.582367	-0.887111
H	-1.366014	1.277063	-0.878761
H	-2.339822	2.831134	1.226293
H	-1.743696	4.056405	0.116800
O	-3.178678	2.912486	-0.681357
C	-4.018203	1.892511	-0.601766
O	-4.821011	1.670362	-1.465586
N	-4.873459	-0.149165	0.397541
C	-3.980935	1.008281	0.647809
H	-5.360419	-0.014131	-0.465382
H	-5.533970	-0.228329	1.144173
H	-2.981889	0.609791	0.837031
H	-4.325310	1.577839	1.512349
H	0.379194	0.575974	0.563107
H	-4.327877	-0.985577	0.345060