Supporting Information for

A copper-sulfenic acid complex from oxidation of a cavity mutant of *Pseudomonas aeruginosa* azurin

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Site-directed mutagenesis and protein purification. Plasmid containing the M121G mutation was constructed by site-directed mutagenesis using wild-type azurin (pET9a) as the template using the Quik-Change mutagenesis procedure with the forward primer 5'-CAC-TCC-GCA-CTG-GGG-AAA-GGT-ACC-3' and the corresponding complementary reverse primer. The mutation was confirmed via sequencing using a T7 promoter. The protein was expressed in BL-21* *E. Coli* (Novagen, Madison, WI) and purified according to published procedures.(1, 2) Electrospray ionization mass spectrometry confirmed the identity of the isolated variant (MW = 13871; MW_{cal} = 13871.67).

Spectroscopic measurements. UV-vis absorption spectra were obtained at room temperature on an HP Agilent 8453 diode array spectrometer. An extinction coefficient of 9800 $M^{-1}cm^{-1}$ at 280 nm was used to determine the concentration of purified protein.(3) All UV-vis spectra were recorded in 50 mM potassium phosphate pH 7.0 buffer unless otherwise noted. X-band EPR measurements were collected at 30 K on a Varian-122 spectrometer equipped with an Air Products Helitran cryostat and temperature controller, with a collection frequency of 9.05 GHz, power of 0.2 mW and field modulation of 4.0 G. EPR spectra were recorded with 20% glycerol in 50 mM temperature-independent-pH (TIP) pH 7.0 buffer (4).

Mass spectrometry measurements. The mass spectra of the proteins were acquired using a Waters Quattro II spectrometer operating in positive-ion mode. Samples (10 µL of 20 µM protein in ammonium acetate buffer) were injected into a flow of 50 µL/min of 50% CH_3CN/H_2O mobile phase and integrated over the first minute of detection. Unless otherwise noted, protein samples were first treated with a 1:10 volume of 1% v/v formic acid immediately prior to injection. Syringe pump mass spectra were obtained with directly injecting 0.2 mM protein in 10 mM ammonium acetate pH 6.0 into the spectrometer at a rate of 5 µL/min. The mass spectra were collected from 500-2000 m/z and were deconvoluted using the MassLinx software package with a 1 Da resolution and a 10,000-20,000 Da calculation window.

Preparation of Cu(II)-M121G azurin. Aliquots of metal-free (apo-) M121G azurin (1.5-2.0 mL) in 50 mM ammonia acetate buffer pH 6.35 were titrated with 2 eq. Cu(II) (from a stock solution of 50 mM $CuSO_4$) and stirred at room temperature for 30 minutes. Excess copper was removed using a PD-10 desalting column containing 8.3 ml of Sephadex™ G-25 medium. The buffer was exchanged to 50 mM

potassium phosphate buffer (pH 7.0) with Amicon® Ultra-4 centrifugal filter devices with a 10 kDa molecular weight cutoff.

Crystallography. Crystals of Cu(II)-M121G azurin suitable for X-ray diffraction grew over three days at 4 °C using the hanging drop method, which consisted of suspension of a 2 µL droplet containing 1 µL apo-M121G azurin (1.3 mM) in 100 mM NaOAc pH 5.6 buffer and 1 µL PEG buffer (25% PEG 4000 containing 100 mM $LiNO_3$, 10 mM $CuSO_4$ and 100 mM Tris pH 8.0) above 250 µL well buffer (25% PEG 4000 containing 100 mM $LiNO_3$ 10 mM $CuSO_4$ and 100 mM Tris pH 8.0). The structure was solved by molecular replacement using AutoMR docked in Phenix (5). The crystal structure of wild type azurin (PDB: 4AZU) was used as a searching model.

Preparation of Cu(I)-M121G azurin. The Cu(II)-M121G azurin (1.0 ml of 2.0 mM protein in 50 mM potassium phosphate buffer, pH 7.0) was degassed with argon on a Schlenk line and was then transferred into a glove bag. The Cu(II)-M121G azurin was then reduced by slow addition of solid sodium ascorbate containing 10%mol N, N, N', N' – tetramethyl-p-phenylenediamine (TMPD) as a mediator until the protein solution turned completely colorless. The resulting solution was purified via PD-10 desalting column to remove excess reductant.

Reaction of Cu(I)-M121G azurin with H_2O_2. To 2.0 ml 0.9 mM Cu(I)-M121G azurin in 50 mM potassium phosphate buffer (pH 7.0) was added 5 eq. H_2O_2 at room temperature under stirring. The color of the solution changed from colorless to green in an hour. The excess H_2O_2 was removed either by running through PD-10 desalting column or buffer exchanging with Amicon® Ultra-0.5 centrifugal filter devices with a 10 kDa molecular weight cutoff.

Resonance Raman Spectroscopy. Resonance Raman (rR) spectra were obtained in an ~135° backscattering configuration with incident powers ranging from ~20 – 50 mW using either a Coherent I90C-K Kr^+ CW ion laser (647.1 nm laser line) or a Coherent Innova Sabre 25/7 Ar^+ CW ion laser (457.9 nm laser line). Scattered light was dispersed through a triple monochromator (Spex 1877 CP, with 1200, 1800, and 2400 groove/mm gratings) and detected with an Andor Newton charge-coupled device (CCD) detector cooled to -80° C. Samples were contained in NMR tubes immersed in a liquid nitrogen finger dewar. Background spectra were obtained using either buffer or charcoal at 77 K in an NMR tube. Raman energies were calibrated using Na_2SO_4 and citric acid. Frequencies are accurate to within 2 cm^{-1}.

Capture and detection of sulfenic acid with dimedone. The green solution from the previous step (200 µL of 0.2 mM protein) was treated with dimedone (64 µL of 250 mM stock) immediately followed by addition of 200 µL denaturation solution containing 6 M guanidine hydrochloride, 10 mM EDTA and 100

mM Bis-Tris, pH 7.4. The mixture was stirred at room temperature for 30 minutes. After labeling, the resulting solution was exchanged into water by ultrafiltration using an Amicon® Ultra-0.5 10 K centrifugal filter device for further mass spectroscopic studies.

Trypsin digest and HPLC MS/MS analysis. Trypsin digest and HPLC MS/MS analysis were carried by the Protein Science Facility in the Biotechnology Center at the University of Illinois at Urbana-Champaign. Samples were digested with trypsin for 15 minutes at 55 °C using a CEM Liberty microwave digester (CEM Corporation, Matthews, NC). The digested products were analyzed on a Thermo Scientific Velos Pro mass spectrometer with a Dionex RSLCnano Ultimate 3000 UPLC front end using an Acclaim PepMap RSLC (75 micron X 15 cm, C18 2 micron 100 Angstrom) column (Thermo Scientific) with a linear gradient of 1 to 60 % acetonitrile in 0.1 % formic acid over 120 minutes. The data were analyzed using the Mascot database search engine (Matrix Science, London) and searched against a custom database containing the mutant M121G Azurin sequence.

Calculations. All DFT calculations were carried out using the Gaussian 09 software (6). For all calculations the B3LYP (7) functional was used in the unrestricted format. For the small models, a TZVP (8) basis set on all atoms was used for all calculations. For the large models, a split 6-311G(d) (on Cu, ligating atoms, and O's on the oxidized thiolate, either bound or unbound)) and 6-31G(d) (9-11) (all other atoms) basis set was used. All molecular orbital compositions were determined using the QMForge program (12) (Mulliken population analyses), and all orbital surfaces were generated using the β-LUMO program (13). The M121G crystal structure was used for a starting point for all models considered. Geometry optimizations using small models involved C_α constraints, while large model constraints included C_α and protein backbone O and N atoms. All reported energies and single point calculations (e.g., EPR and time dependent DFT (TDDFT)) have been environment corrected using a PCM model ($\varepsilon = 4.0$) (14). TDDFT calculations were simulated using the SWizard program revision 4.6 (15, 16) using Gaussian band-shapes with half-widths of 2000 cm^{-1}. Wave function stability checks were carried out to ensure they represented energetic minima.

Supporting References:

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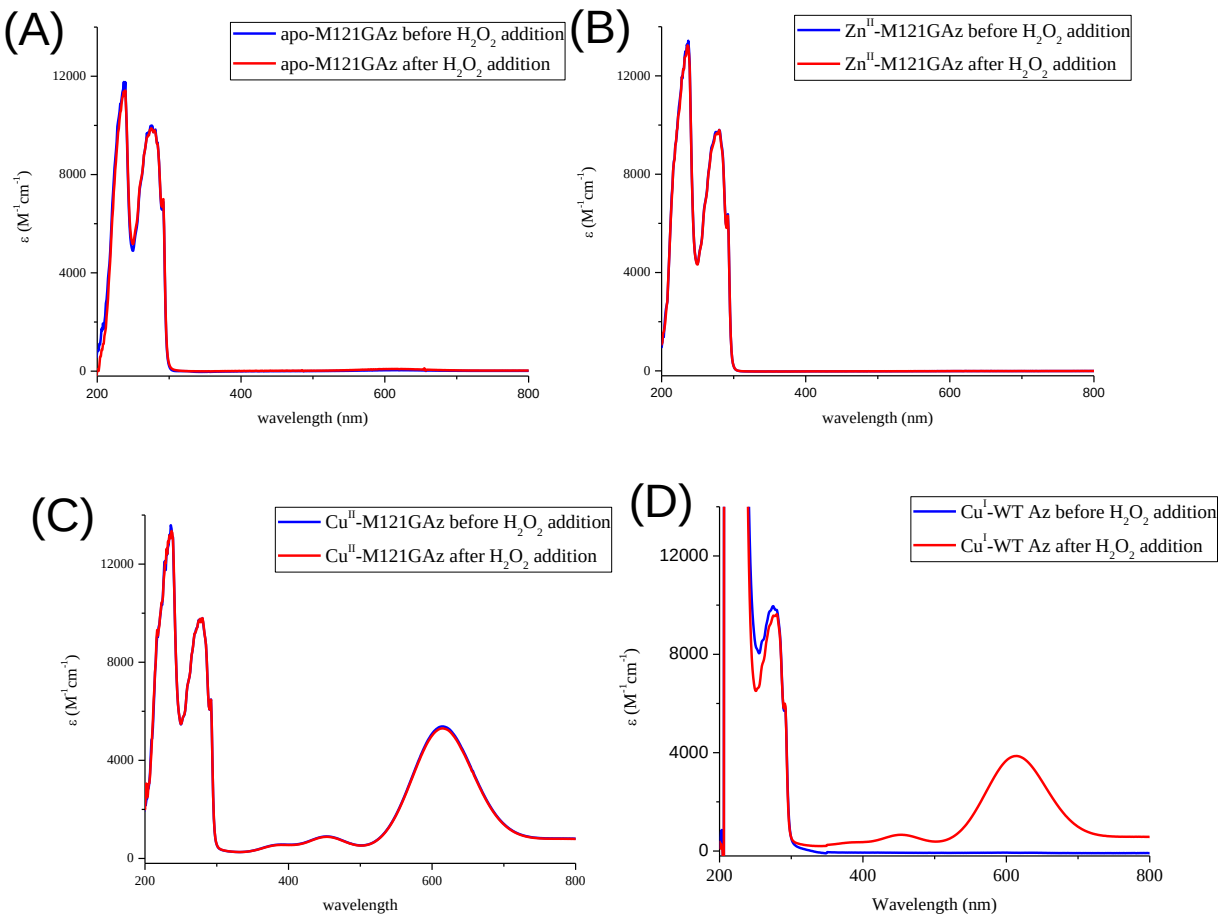

Fig. S1. UV-vis spectrum upon addition of 5 eq. H_2O_2 to (A) apo-M121G Az, (B) Zn(II)-M121G Az, (C) Cu(II)-M121G azurin and (D) Cu(I)-WT azurin in 50 mM KPi buffer pH 7.0. Spectra in blue were obtained before H_2O_2 addition, while spectra in red were obtained 30 minutes after H_2O_2 addition.

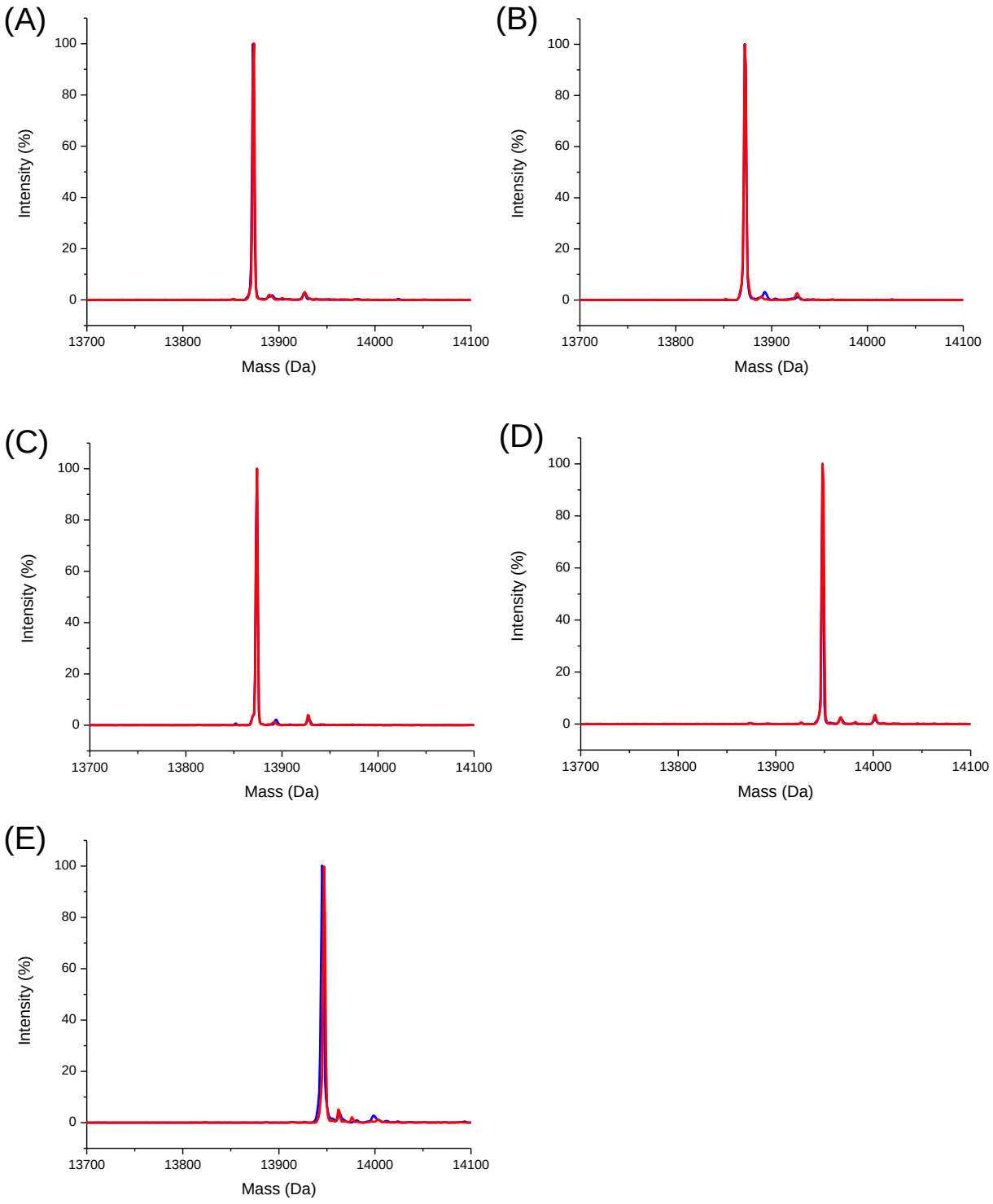

Fig. S2. Electrospray mass spectrum upon addition of 5 eq. H_2O_2 to (A) Cu^{II}-M121G; (B) apo-M121G; (C) Zn^{II}-M121G; (D) Cu^{II}-Wild-Type; and (E) Cu^{I}-Wild-Type. Spectra in blue were obtained before oxidant addition, while spectra in red were obtained 30 minutes after H_2O_2 addition. (For M121G azurin, cal. 13871.67 and obs. 13871 Da; for wild type azurin, cal. 13945.81 and obs. 13945 Da)

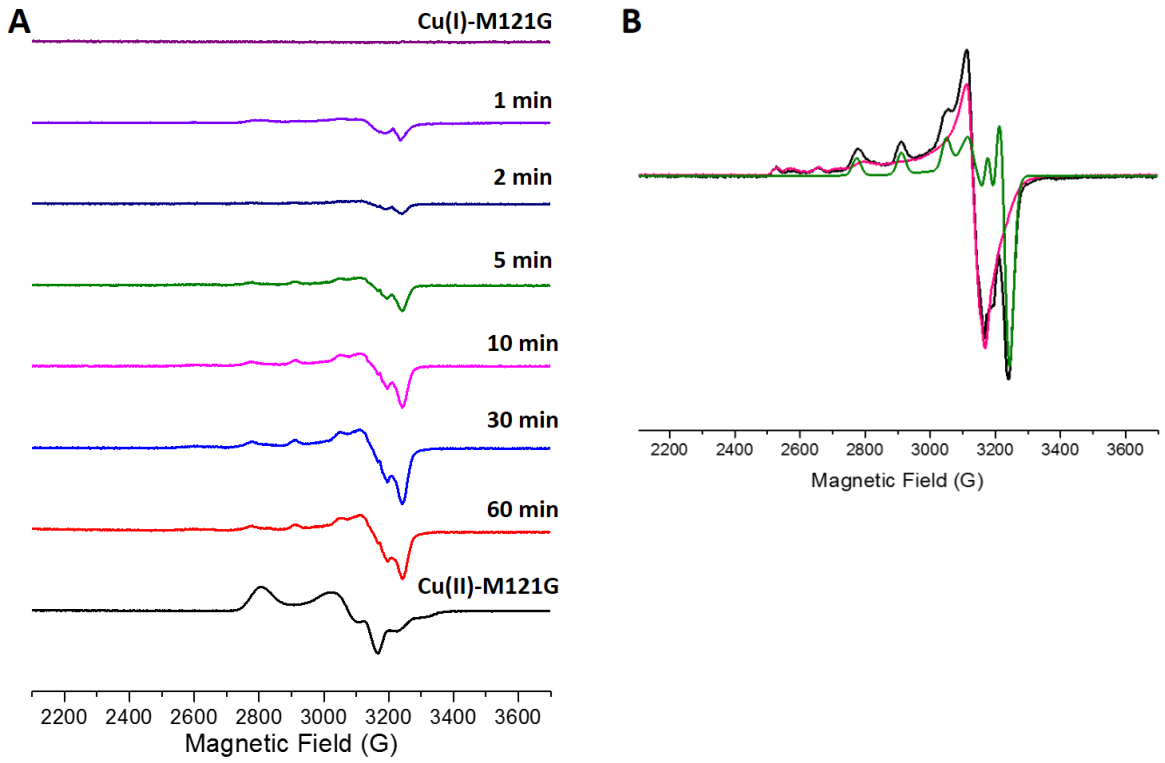

Fig. S3. EPR study and simulation of the air sensitive species. (A) Time-resolved X-band EPR spectra of a solution of 0.6 mM Cu(I)-M121G after addition of 5 eq. H_2O_2 in 50 mM TIP7 buffer (pH 7.0) along with authentic Cu(II)-M121G (bottom). Reactions were performed anaerobically in an EPR tube and quenched via freezing in liquid N_2. Spectra were collected at 30 K and were an average of 10 scans. Microwave frequency = 9.05 GHz; field modulation = 4.0 G; microwave power = 0.2 mW. (B) X-Band EPR spectra of Cu(I)-M121G azurin after incubating with 5 eq. H_2O_2 for 30 min in 50 mM TIP7 buffer pH 7.0 (black), the resulted solution exposed to air (pink) and simulation of the air-sensitive species (green).

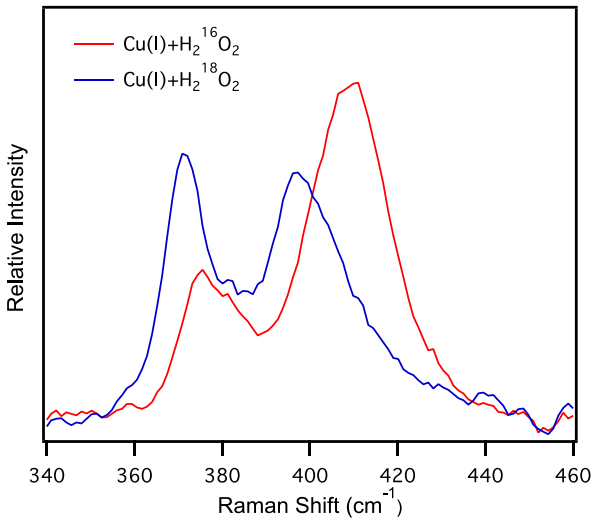

Fig. S4. Resonance Raman data (λ_{ex} = 457.9 nm) for Cu(I)M121G reacted with $H_2{}^{16}O_2$ and $H_2{}^{18}O_2$ at pH 7.

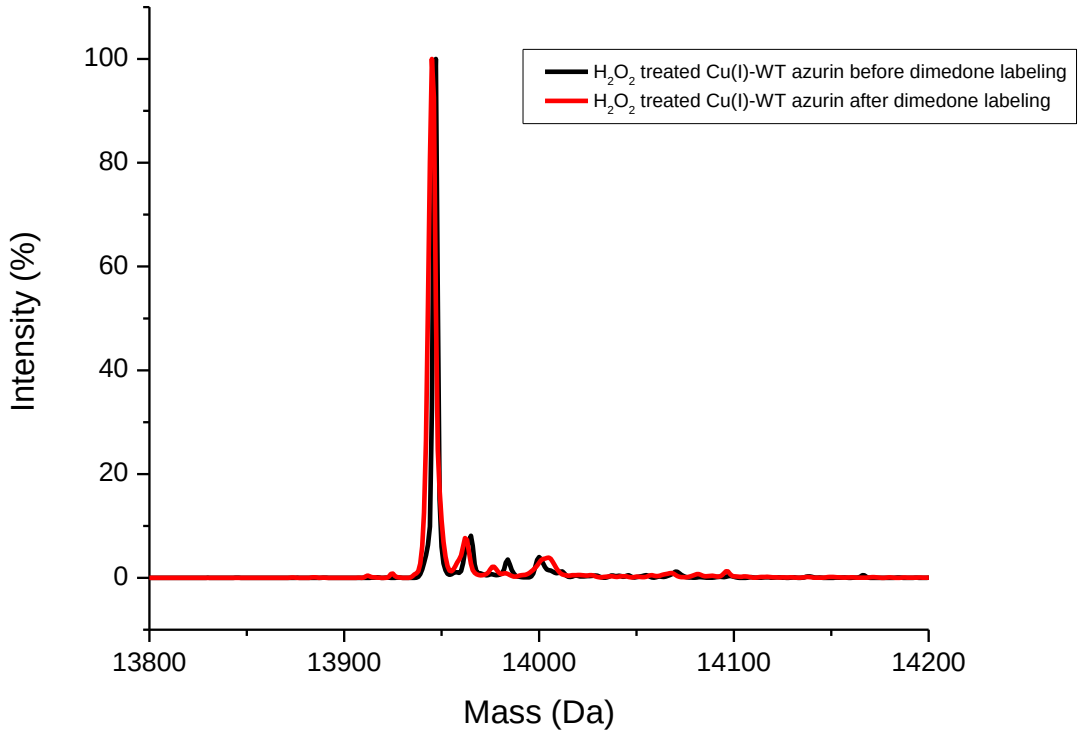

Fig. S5. Control experiment: ESI-MS of Cu(I)-Wild Type after H_2O_2 treatment and dimedone labeling. (Cal. WT azurin: 13945.81, obs. 13946 Da)

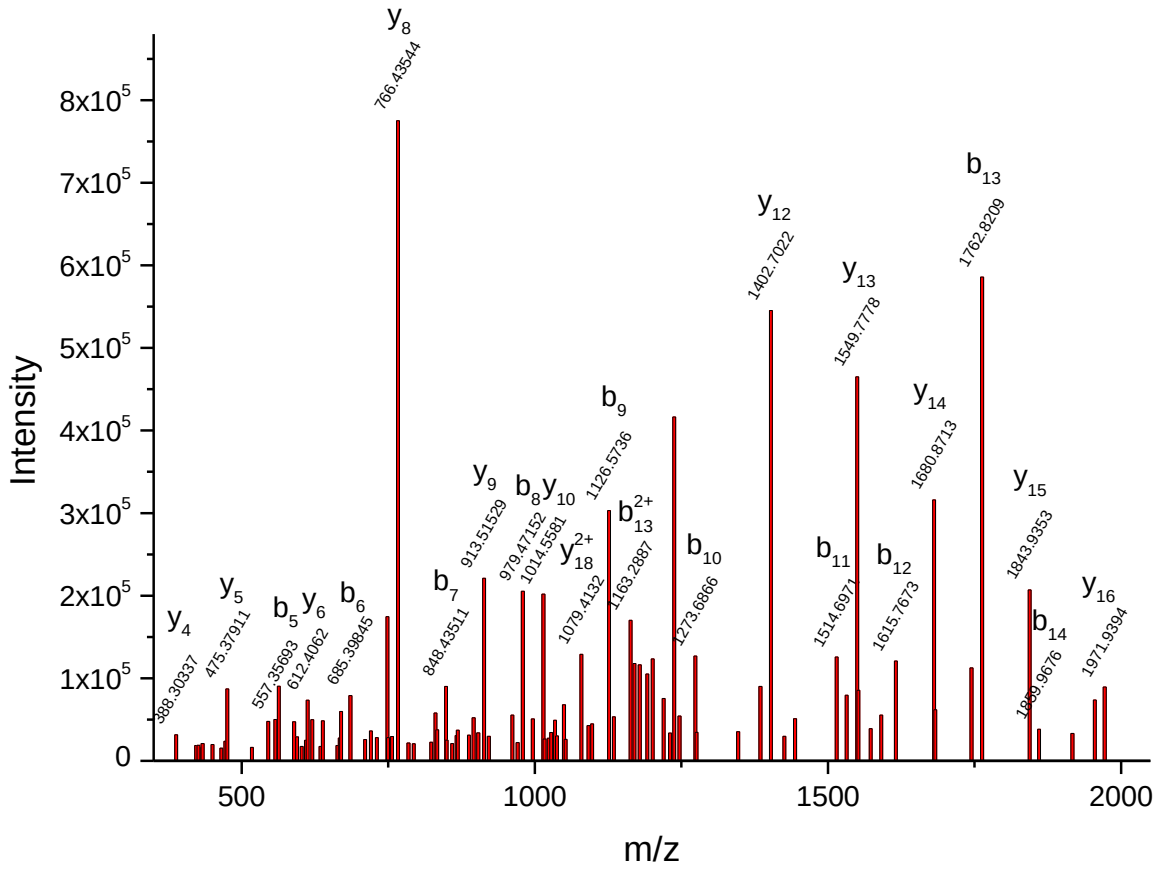

Fig. S6. MS/MS data of NH_2-LKEGEQYMFF(C-dimedone)TFPGHSALGK-COOH formed from trypsin digestion of dimedone treated reaction solution. The precursor mass was m/z (2+) = 1264.5855 (cal. m/z (2+) = 1264.0998).

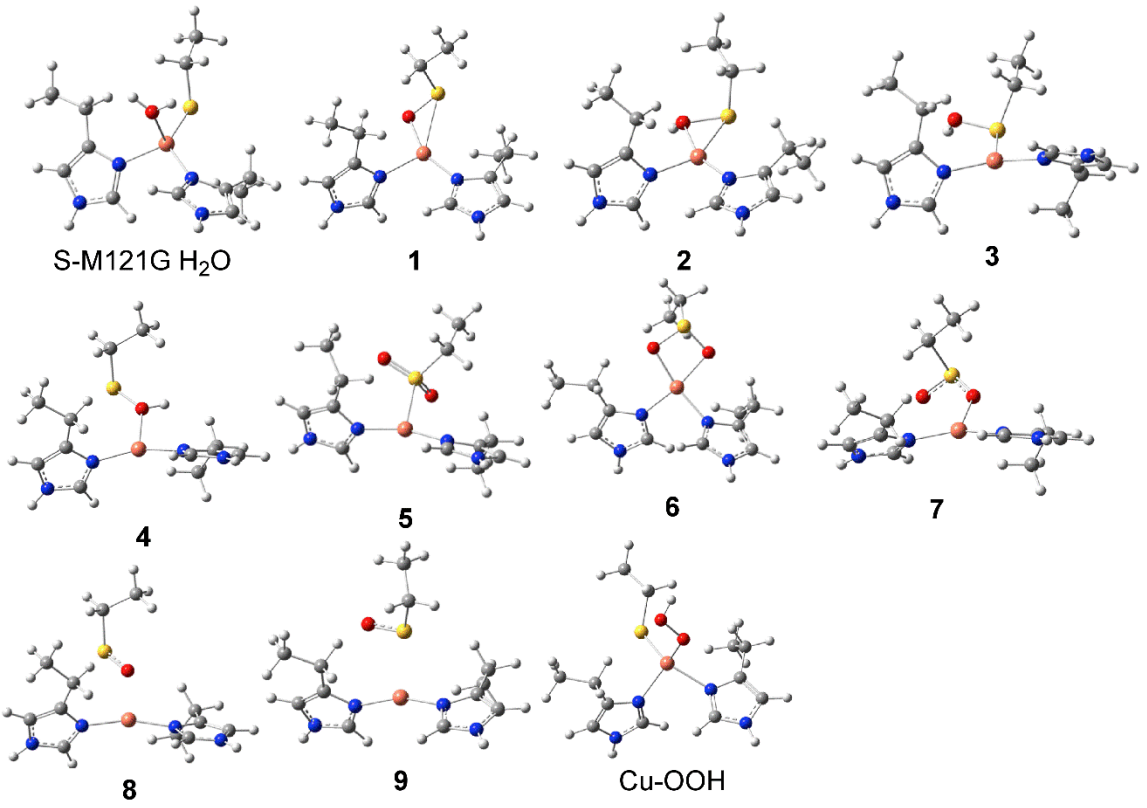

Fig. S7. Small DFT structures for M121G-H_2O and models for the air-sensitive species. Atom coloring: Cu, pink; S, yellow; O, red; N, blue; C, grey; H, white.

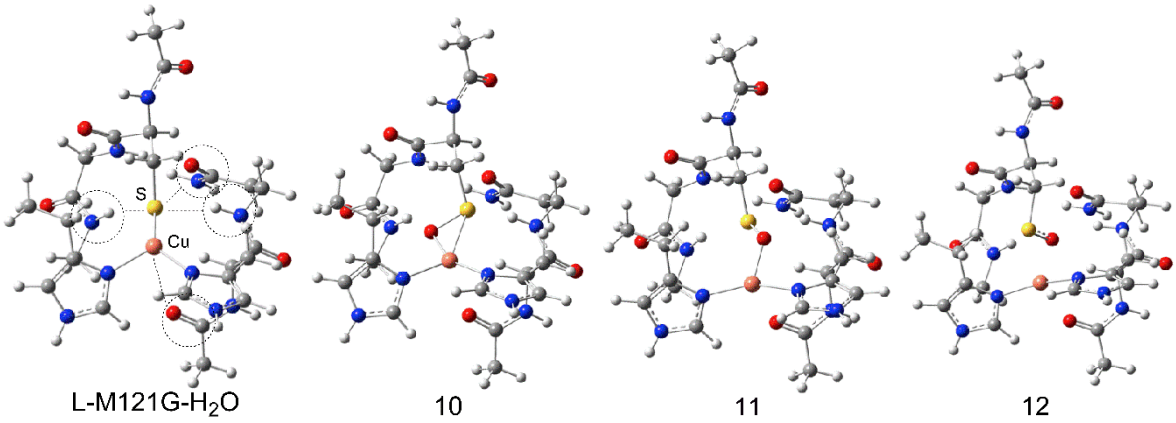

Fig. S8. Large DFT structures for M121G-H_2O (L-M121G-H_2O) and the air-sensitive species: pseudo-side-on (**10**), O-bound (**11**), and S-bound (**12**). Specific second sphere interactions are labeled in the L-M121G-H_2O model. Atom coloring: Cu, pink; S, yellow; O, red; N, blue; C, grey; H, white.

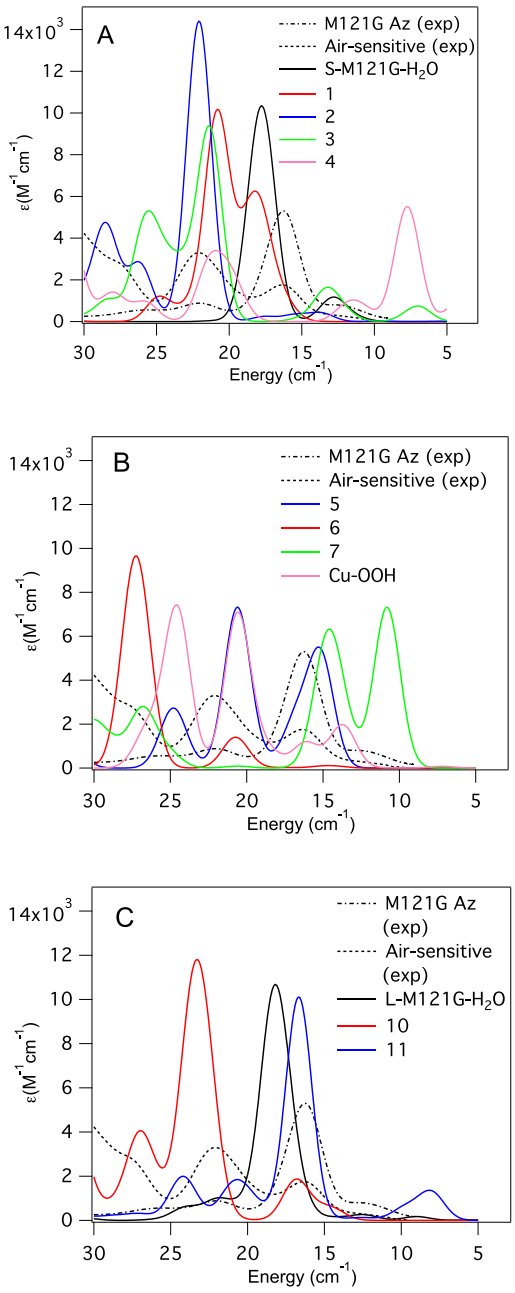

Fig. S9. Time dependent DFT calculated absorption spectra. TDDFT calculationed absorption spectra for potential air-sensitive species (A) 1 – 4 and Cu(II)-M121G azruin (S-M121G-H_2O), (B) 5 – 7 and Cu-OOH species (small models) and (C) 10 -11 and Cu(II)-M121G azurin (L-M121G-H_2O).

Table S1: Summary of EPR spectral parameters and percentages for select complexes

Species	g_x, g_y, g_z	A_x, A_y, A_z (Cu) ($\times 10^{-4}$ cm^{-1})	Percentage
Air-sensitive species	2.033, 2.041, 2.169	26.3, 17.8, 139.3	17.5 %
Cu(II)-M121G azurin (A)	2.024, 2.102, 2.294	42.3, 17.6, 18.1	10.9 %
Cu(II)-M121G azurin (B)	2.025, 2.042, 2.303	77.0, -, 11.5	8.1 %
Type 2 Copper Site	2.038, 2.067, 2.276	-, 19.7, 179.9	63.5 %

Table S2. Resonance Raman vibrational frequencies and isotope shifts obtained using λ_{ex} = 457.9 nm for Cu(I) + $H_2{}^{16}O_2$ and $H_2{}^{18}O_2$.

	$H_2{}^{16}O_2$ (cm^{-1})	$H_2{}^{18}O_2$ (cm^{-1})	Δ(16-18) (cm^{-1})
a	374	371	3
b	380	380	0
c	407	395	12
SO	837	807	30

Table S3. Main sequence ions detected within the error of the calculated value (highlighted in bold red). b* means b type ion after deamination and b^0 means b type ion after dehydration. y* means y type ion after deamination and y^0 means y type ion after dehydration.

No.	b	b^{2+}	b^*	b^{*2+}	b^0	b^{02+}	Seq.	y	y^{2+}	y*	y^{*2+}	y^0	y^{02+}	No.
1	114.0913	57.5493					L							21
2	242.1863	121.597	225.1598	113.0835			K	2415.1148	1208.061	2398.0883	1199.5478	2397.1042	1199.056	20
3	371.2289	186.118	354.2023	177.6048	353.2183	177.1128	E	2287.0198	1144.014	2269.9933	1135.5003	2269.0093	1135.008	19
4	428.2504	214.629	411.2238	206.1155	410.2398	205.6235	G	2157.9772	**1079.492**	2140.9507	1070.979	2139.9667	1070.487	18
5	**557.293**	279.15	540.2664	270.6368	539.2824	270.1448	E	2100.9558	1050.982	2083.9292	1042.4683	2082.9452	1041.976	17
6	**685.3515**	343.179	668.325	334.6661	667.341	334.1741	Q	**1971.9132**	986.4602	**1954.8866**	977.947	1953.9026	977.455	16
7	**848.4149**	424.711	831.3883	416.1978	**830.4043**	415.7058	Y	**1843.8546**	922.4309	1826.8281	913.9177	1825.844	**913.4257**	15
8	**979.4553**	490.231	962.4288	481.718	961.4448	481.226	M	**1680.7913**	840.8993	1663.7647	832.386	1662.7807	831.894	14
9	**1126.5238**	563.766	1109.497	555.2522	1108.513	554.7602	F	**1549.7508**	775.379	1532.7243	766.8658	1531.7402	**766.3738**	13
10	**1273.5922**	637.3	1256.566	628.7865	1255.582	628.2944	F	**1402.6824**	701.8448	1385.6558	693.3316	**1384.6718**	692.8395	12
11	**1514.6694**	757.838	1497.643	749.3251	1496.659	748.8331	C-dimedone	1255.614	628.3106	1238.5874	619.7973	**1237.6034**	619.3053	11
12	**1615.7171**	808.362	1598.691	799.8489	1597.707	799.3569	T	**1014.5367**	507.772	997.5102	499.2587	996.5261	498.7667	10
13	**1762.7855**	881.896	1745.759	873.3831	**1744.775**	872.8911	F	**913.489**	457.2482	896.4625	448.7349	895.4785	448.2429	9
14	**1859.8383**	930.423	1842.812	921.9095	1841.828	921.4175	P	**766.4206**	383.7139	749.3941	375.2007	748.41	374.7087	8
15	1916.8598	958.934	1899.833	950.4202	1898.849	949.9282	G	669.3679	335.1876	652.3413	326.6743	651.3573	326.1823	7
16	2053.9187	1027.46	2036.892	1018.9497	2035.908	1018.458	H	**612.3464**	306.6768	595.3198	298.1636	594.3358	297.6715	6
17	2140.9507	1070.98	2123.924	1062.4657	2122.94	1061.974	S	**475.2875**	238.1474	458.2609	229.6341	457.2769	229.1421	5
18	2211.9878	1106.5	2194.961	1097.9843	2193.977	1097.492	A	**388.2554**	194.6314	371.2289	186.1181			4
19	2325.0719	1163.04	2308.045	1154.5263	2307.061	1154.034	L	317.2183	159.1128	300.1918	150.5995			3
20	2382.0933	1191.55	2365.067	1183.037	2364.083	1182.545	G	204.1343	102.5708	187.1077	94.0575			2
21							K	147.1128	74.06	130.0863	65.5468			1

Table S4. Comparison between experimental bond distances and DFT structures for small M121G models and possible structures for the air-sensitive species.

	Bond Distances				Mulliken Spin Densities (PCM ε = 4.0)	
	Cu-S (Å)	Cu-O (Å)	Cu-N (Å)[a]	S-O (Å)	Cu	S/O
Cu(II)-M121G azurin (exp.)	2.2	2.9[b]	2.0	--	--	--
S-M121G-H_2O	2.18	2.52[b]	2.04	--	0.37	0.54/--
1	2.63	1.92	2.02	1.61	0.31	0.37/0.25
2	2.48	2.09	1.97	1.73	0.52	0.19/0.07
3	2.41	--	1.93	1.66	0.47	0.32/0.04
4	--	2.07	1.93	1.72	0.57	0.20/0.05
5	2.53	--	1.94	1.49[c]	0.22	0.21/0.38[d]
6	--	2.00[e]	2.01	1.59[c]	0.61	0.02/0.20[d]
7	--	2.04	1.96	1.53[c]	0.31	0.16/0.38[d]
8	--	2.32	1.94	1.55	0.03	0.55/0.40
9	2.77	--	1.94	1.54	0.07	0.44/0.42
Cu-OOH	2.28	1.90	2.15	1.44[f]	0.45	0.24/0.23
L-M121G-H_2O	2.17	2.48	1.99	--	0.59	0.25/--
10	--	1.90	1.98	1.60	0.50	0.16/0.28
11	2.79	--	1.92	1.54	0.15	0.46/0.34

[a] Averages of Cu-N(Im) distances.
[b] Axial Cu-OH_2 distance.
[c] Average distance for SO_2.
[d] Sum over both O atoms.
[e] Average of Cu-O distances.
[f] O-O distance.

Table S5. DFT vibrational frequencies for small M121G models and potential structures of the air-sensitive species.

Model	ν(Cu-S) (cm^{-1}) exp	ν(Cu-S) (cm^{-1}) calc		
M121G H_2O	400	408/413		
	ν($H_2{}^{16}O_2$)(cm^{-1})	ν($H_2{}^{18}O_2$)(cm^{-1})	Δ(cm^{-1})	assignment
1	418/442	412/431	6/11	Cu-O
	832	803	29	S-O
2	307/315/379	304/314/377	3/1/2	Cu-S(OH)
	713	689	24	S-O
3	303/391	302/390	1/1	Cu-S(OH)
	767/791	748/784	19/7	S-O
4	291	284	7	Cu-OH
	392	389	3	S-C_β-C_α + C_β-S-OH
	677	654	23	S-O
5	311	308	3	Cu-S
	415/418	401/417	14/1	O-S-O
	493	487	6	Cu-S
	1013/1174	977/1140	36/34	S-O (as)/S-O (s)
6	345	343	2	Cu-O(s) + Cu-N
	365	353	12	Cu-O(as)
	504/579	492/561	12/18	Cu-O(s) + O-S-O + S-C-C
	829/884	803/850	26/34	S-O(as)/S-O(s)
7	248	241	7	Cu-O

	341	331	10	SO_2-C_β
	376	370	6	C_β-S-O
	447	433	14	O-S-O
Cu-OOH	375	374	1	Cu-S
	459	437	12	Cu-OOH
	891	840	49	O-OH

Table S6. DFT vibrational frequencies for large M121G models and potential structures of the air-sensitive species.

Model	ν(Cu-S) (cm^{-1}) exp.	ν(Cu-S) (cm^{-1}) calc.		
L-M121G H_2O	400	384/403/406		
	$\nu(H_2{}^{16}O_2)(cm^{-1})$	$\nu(H_2{}^{18}O_2)(cm^{-1})$	$\Delta(cm^{-1})$	assignment
10 (SO)	448/457	432/445	16/12	Cu-O
	824	796	28	S-O
11 (O)	387/463/477	381/461/467	6/2/10	Cu-O
	820	801/772	19/48	S-O

Table S7. DFT structures for large M121G models and potential structures of the air-sensitive species upon removal of specific second sphere interactions.

	10	Cu-Dipole[a]	H-bonds	S-Dipole	Cu-Dipole/H-bonds	**11**	Cu-Dipole	H-bonds	S-Dipole	***Cu-Dipole/H-bonds[b]***
	Bond Distances									
Cu-O	1.93	1.92	1.91	1.92	1.90	1.91	1.96	1.94	1.90	***2.19***
Cu-S	2.42	2.40	2.43	2.45	2.43	--	--	--	--	--
Cu-N[c]	1.99	1.97	1.99	1.99	1.98	1.98	1.94	1.96	1.97	***1.90***
S-O	1.62	1.62	1.61	1.62	1.61	1.60	1.57	1.59	1.60	***1.55***
	Spin Densities									
Cu	0.48	0.43	0.47	0.51	0.39	0.44	0.26	0.33	0.49	***0.06***
O	0.20	0.21	0.23	0.19	0.23	0.30	0.36	0.33	0.29	***0.41***
S	0.21	0.25	0.25	0.18	0.30	0.19	0.33	0.31	0.16	***0.51***

[a] Indicator of which second sphere interaction has been removed.
[b] Only this combination results in reduction of Cu as discussed in the manuscript text.
[c] Average of two Cu-N(His) distance.

Table S8. Diffraction and refinement data for Cu(II)-M121G azurin crystal structure.

	Cu(II)-M121G Az
Data collection	
Beamline	21-ID-F
Wavelength (Å)	0.97872
Space Group	C 2 2 21
Cell dimension *a*, *b*, *c* (Å)	55.75 145.8 97.09
Resolution (Å)	50-1.54 (1.57-1.54)
R-merge	0.069 (0.376)
I/σI	33.88 (7.22)
No. of unique reflections	53091 (2616)
Completeness (%)	90.5 (91.1)
Redundancy	6.8 (6.5)
Refinement	
Resolution (Å)	29.15-1.54 (1.58-1.54)
Reflections in free set	2648 (5%)
Rwork/Rfree	0.1704(0.229)/0.1936(0.262)
No. reflections	50042
No. atoms	3291
Protein	2916
Cu	6
Water	340

Metal Ion	Cu
B-factor	13.00
Protein	12.10
Cu	13.38
Water	20.80
ESU(ML)	0.044
Rms. deviations	
Bond lengths (Å)	0.023
Bond angles (o)	2.123
Ramachandran favored (%)	98
Ramachandran outliers (%)	0