Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Mechanistical model for the firefly luciferin regeneration in biomimetical conditions: a model for the *in vivo* process?

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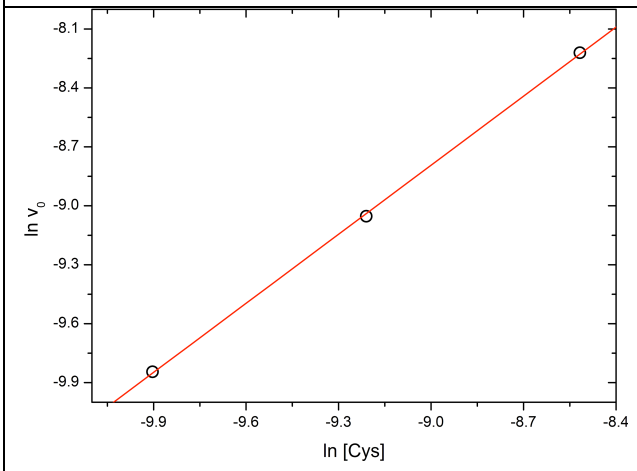
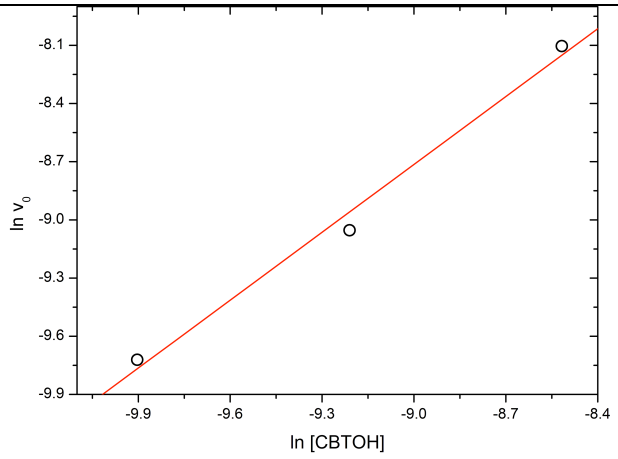
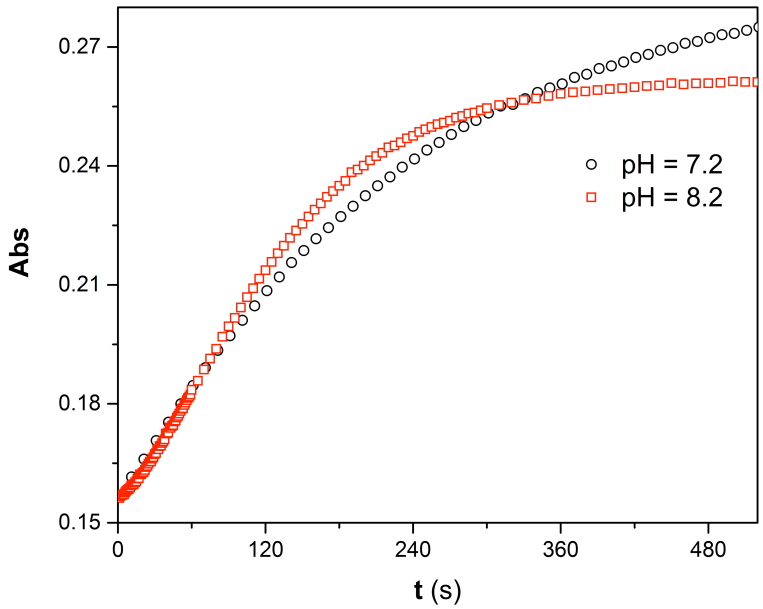
Figure S1: Plots of $\ln v_0$ versus ln[reactant] to determine the rate law for the reaction between CBTOH and cysteine at pH = 7.2. The initial rate (v_0) was determined through UV-absorption at 343 nm by fixing the concentration of one reactant and varying the other.

Varying cysteine order = 1.17 ± 0.02	Varying CBTOH order = 1.2 ± 0.1
	

The values obtained fit the following rate law for the reaction:

$v = k_{bim}$ [Cysteine][CBTOH]

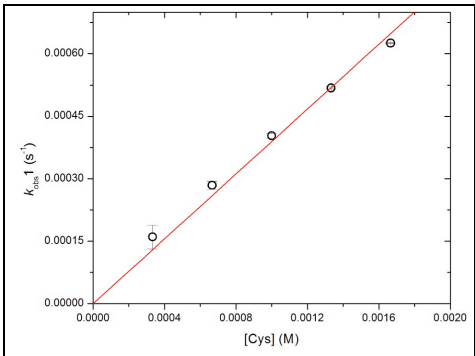
Figure S2: Time profile for the absorption of luciferin at 343 nm produced upon reaction of CBTOH and cysteine at pH = 7.2 and 8.2. The Equation 1 and Equation 2 were used to obtain the observed rate constant from the experimental data using mono- and biexponential curve fitting, respectively. The results are listed at **Tables S1** to **S12**.

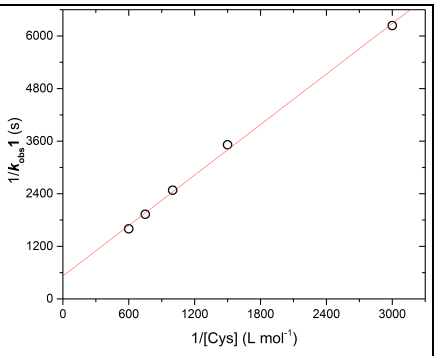


Equation 1: $y = y_0 + A*[1 - \exp(-k_{obs}1*t)]$

Equation 2: $y = y_0 + A_1*[1 - \exp(-k_{obs}1*t)] + A_2*[1 - \exp(-k_{obs}2*t)]$

Table S1: Observed rate constant ($k_{obs}1$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH = 6.0.

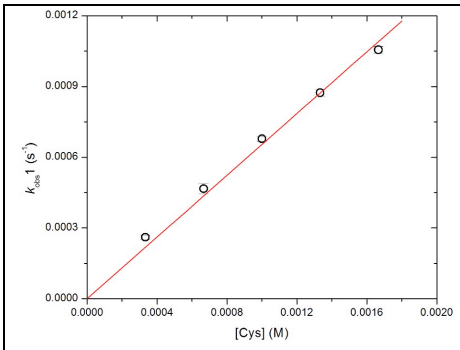


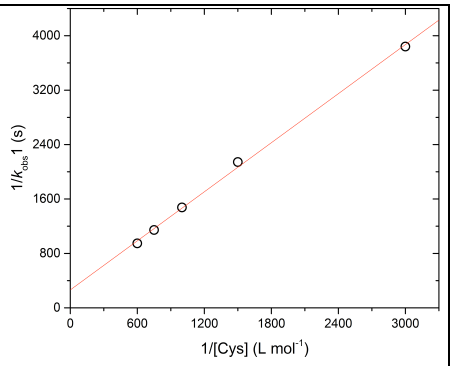


[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})
0.00033	0.00016 ± 0.00003
0.00067	0.00028 ± 0.00001
0.00100	0.000403 ± 0.000005
0.00133	0.000518 ± 0.000003
0.00167	0.000625 ± 0.000001
k_{bim} (L mol^{-1} s^{-1})	0.39 ± 0.01
k_{sat} (s^{-1})	0.0019 ± 0.0003

Conditions: in 0.10 mol L^{-1} phosphate at pH 6.0, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 343 nm.

Table S2: Observed rate constant ($k_{obs}1$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 6.20.

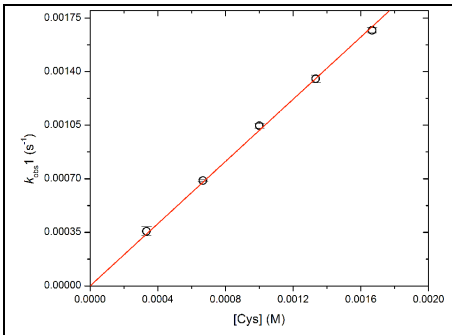
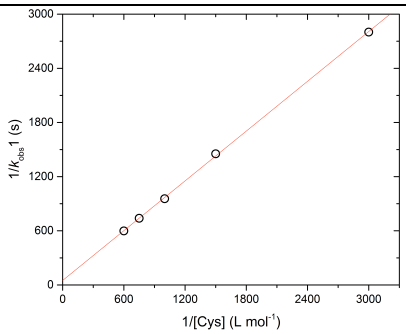




[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})
0.00033	0.00026 ± 0.00001
0.00067	0.00047 ± 0.00002
0.00100	0.00068 ± 0.00001
0.00133	0.00087 ± 0.00001
0.00167	0.00106 ± 0.00001
k_{bim} (L mol^{-1} s^{-1})	0.65 ± 0.01
k_{sat} (s^{-1})	0.0038 ± 0.0006

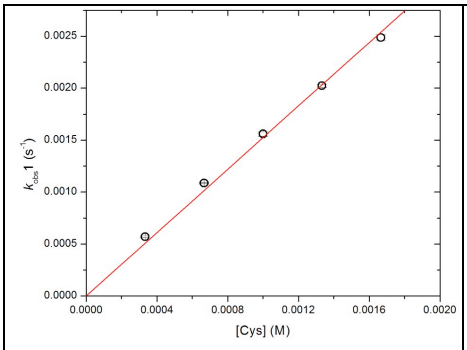
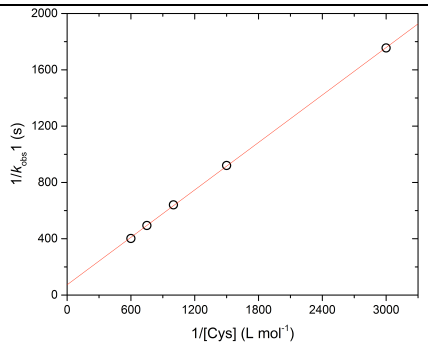
Conditions: in 0.10 mol L^{-1} phosphate at pH 6.2, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 343 nm.

Table S3: Observed rate constant ($k_{obs}1$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 6.40.

	
[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})
0.00033	0.00036± 0.00003
0.00067	0.000688 ± 0.000004
0.00100	0.00105 ± 0.00001
0.00133	0.00135 ± 0.00002
0.00167	0.00167 ± 0.00002
k_{bim} (L mol^{-1} s^{-1})	1.017 ± 0.009
k_{sat} (s^{-1})	0.020 ± 0.005

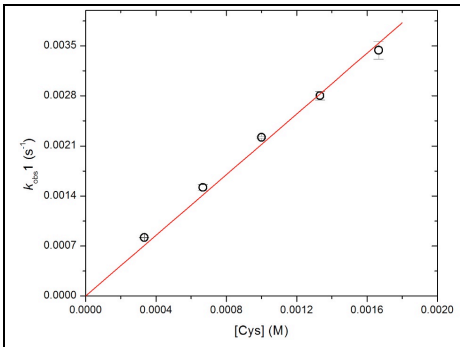
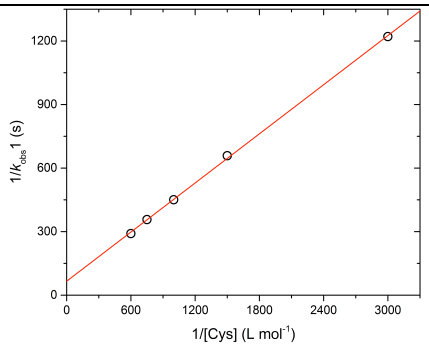
Conditions: in 0.10 mol L^{-1} phosphate at pH 6.4, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 343 nm.

Table S4: Observed rate constant ($k_{obs}1$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 6.60.

	
[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})
0.00033	0.000570 ± 0.000007
0.00067	0.00109 ± 0.00001
0.00100	0.00156 ± 0.00002
0.00133	0.00202 ± 0.00001
0.00167	0.00249 ± 0.00003
k_{bim} (L mol^{-1} s^{-1})	1.52 ± 0.02
k_{sat} (s^{-1})	0.014 ± 0.001

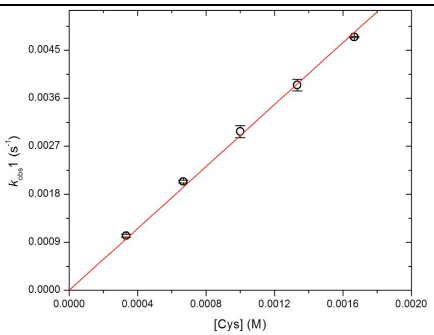
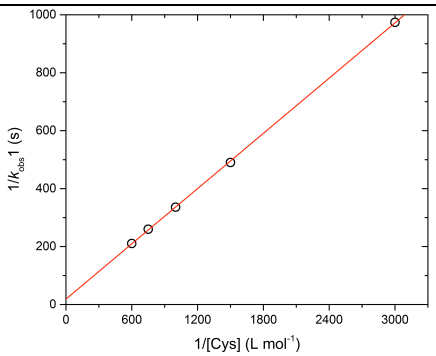
Conditions: in 0.10 mol L^{-1} phosphate at pH 6.6, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 343 nm.

Table S5: Observed rate constant ($k_{obs}1$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 6.80.

	
[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})
0.00033	0.000819 ± 0.000005
0.00067	0.00152 ± 0.00004
0.00100	0.00222 ± 0.00001
0.00133	0.00280 ± 0.00006
0.00167	0.0034 ± 0.0001
k_{bim} (L mol^{-1} s^{-1})	2.12 ± 0.04
k_{sat} (s^{-1})	0.015 ± 0.002

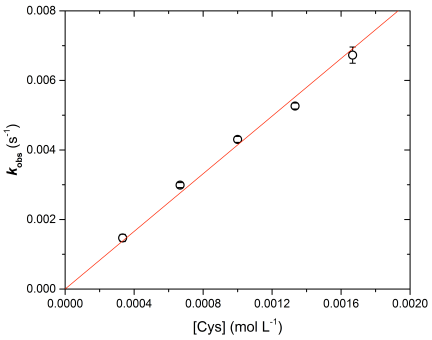
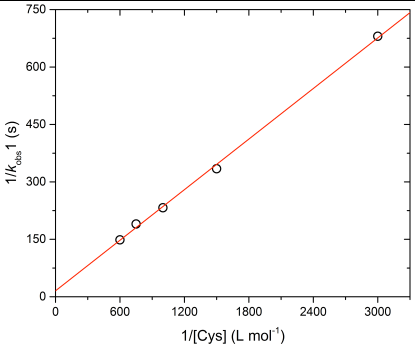
Conditions: in 0.10 mol L^{-1} phosphate at pH 6.8, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 343 nm.

Table S6: Observed rate constant (k_{obs}), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 7.00.

	
[Cysteine] (mol L^{-1})	k_{obs} (s^{-1})
0.00033	0.00103 ± 0.00002
0.00067	0.00204 ± 0.00003
0.00100	0.0030 ± 0.0001
0.00133	0.0038 ± 0.0001
0.00167	0.00475 ± 0.00001
k_{bim} (L mol^{-1} s^{-1})	2.90 ± 0.03
k_{sat} (s^{-1})	0.054 ± 0.009

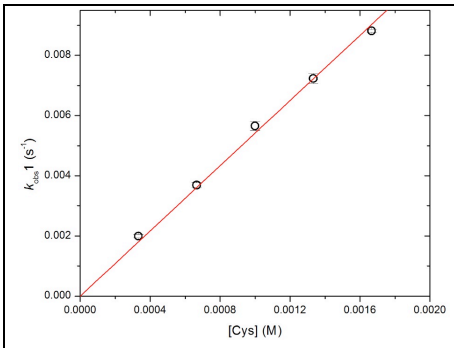
Conditions: in 0.10 mol L^{-1} phosphate at pH 7.0, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 343 nm.

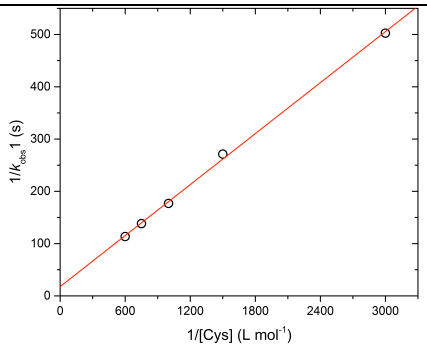
Table S7: Observed rate constant ($k_{obs}1$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 7.20.

	
[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})
0.00033	0.0015 ± 0.0001
0.00067	0.00299 ± 0.00007
0.00100	0.00430 ± 0.00008
0.00133	0.00526 ± 0.0008
0.00167	0.0067 ± 0.0002
k_{bim} (L mol^{-1} s^{-1})	4.09 ± 0.08
k_{sat} (s^{-1})	0.06 ± 0.03

Conditions: in 0.10 mol L^{-1} phosphate at pH 7.2, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 343 nm.

Table S8: Observed rate constant ($k_{obs}1$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 7.40.

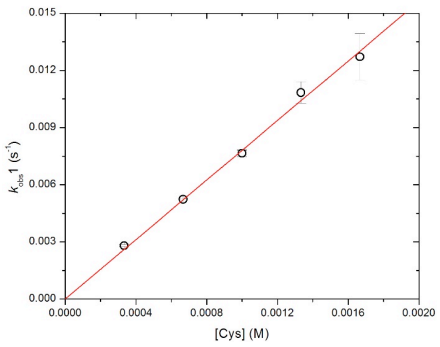
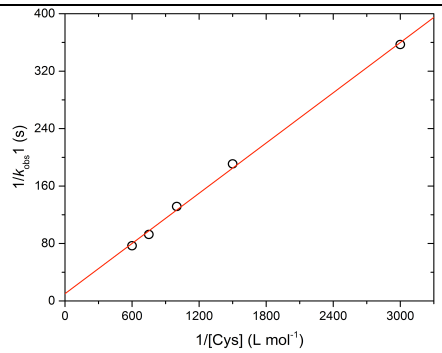




[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})
0.00033	0.00199 ± 0.00006
0.00067	0.00369 ± 0.00008
0.00100	0.0056 ± 0.0001
0.00133	0.0072 ± 0.0001
0.00167	0.00881 ± 0.00005
k_{bim} (L mol^{-1} s^{-1})	5.42 ± 0.08
k_{sat} (s^{-1})	0.06 ± 0.02

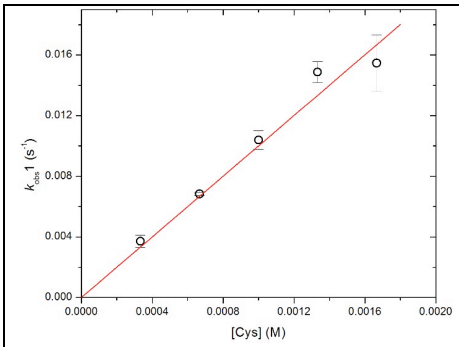
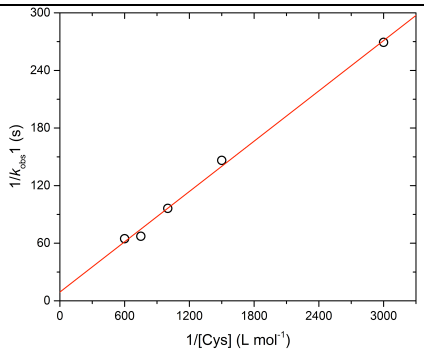
Conditions: in 0.10 mol L^{-1} phosphate at pH 7.4, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 343 nm.

Table S9: Observed rate constants ($k_{obs}1$ and $k_{obs}2$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 7.60.

		n/a
[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})	$k_{obs}2$ (s^{-1})
0.00033	0.00280 ± 0.00005	0.03 ± 0.01
0.00067	0.00524 ± 0.00003	0.0232 ± 0.0002
0.00100	0.0076 ± 0.0002	0.024 ± 0.003
0.00133	0.0108 ± 0.0006	0.028 ± 0.008
0.00167	0.013 ± 0.001	0.029 ± 0.009
k_{bim} (L mol^{-1} s^{-1})	7.8 ± 0.1	0.027 ± 0.02
k_{sat} (s^{-1})	0.10 ± 0.05	–

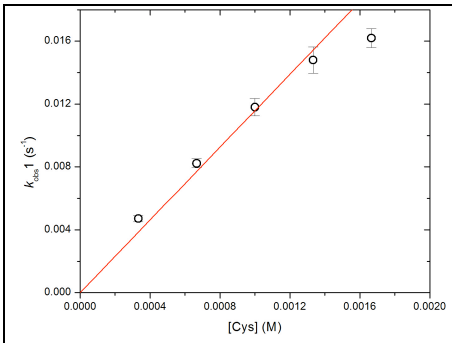
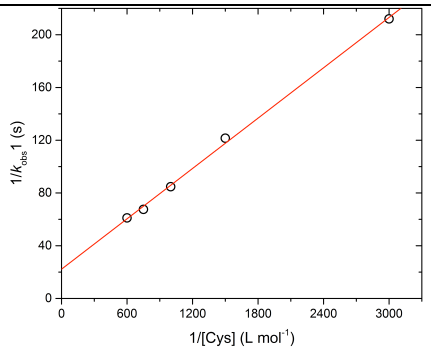
Conditions: in 0.10 mol L^{-1} phosphate at pH 7.6, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 344 nm.

Table S10: Observed rate constants ($k_{obs}1$ and $k_{obs}2$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 7.80.

		n/a
[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})	$k_{obs}2$ (s^{-1})
0.00033	0.0037 ± 0.0004	0.021 ± 0.006
0.00067	0.0068 ± 0.0001	0.023 ± 0.001
0.00100	0.0149 ± 0.0007	0.020 ± 0.003
0.00133	0.015 ± 0.002	0.018 ± 0.001
0.00167	10.0 ± 0.9	0.024 ± 0.006
k_{bim} (L mol^{-1} s^{-1})	10.0 ± 0.9	0.021 ± 0.009
k_{sat} (s^{-1})	0.11 ± 0.06	

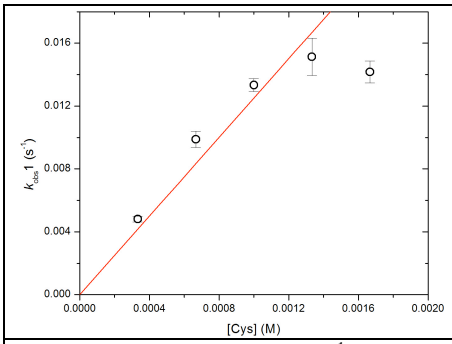
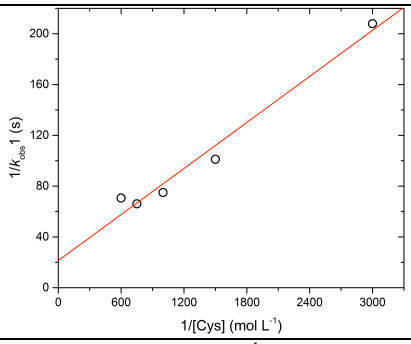
Conditions: in 0.10 mol L^{-1} phosphate at pH 7.8, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 339 nm.

Table S11: Observed rate constants ($k_{obs}1$ and $k_{obs}2$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 8.00.

		n/a
[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})	$k_{obs}2$ (s^{-1})
0.00033	0.0047 ± 0.0002	0.014 ± 0.001
0.00067	0.0082 ± 0.0003	0.018 ± 0.002
0.00100	0.0118 ± 0.0006	0.018 ± 0.001
0.00133	0.0148 ± 0.0008	0.018 ± 0.001
0.00167	0.0162 ± 0.0006	0.0208 ± 0.0009
k_{bim} (L mol^{-1} s^{-1})	10.0 ± 0.9	0.018 ± 0.003
k_{sat} (s^{-1})	0.045 ± 0.005	

Conditions: in 0.10 mol L^{-1} phosphate at pH 8.0, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 338 nm.

Table S12: Observed rate constants ($k_{obs}1$ and $k_{obs}2$), bimolecular rate constant (k_{bim}) and saturation constant (k_{sat}) obtained for the reaction between CBTOH and cysteine at pH 8.20.

		n/a
[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})	$k_{obs}2$ (s^{-1})
0.00033	0.0048 ± 0.0002	0.020 ± 0.003
0.00067	0.0099 ± 0.0005	0.0134 ± 0.0007
0.00100	0.0133 ± 0.0004	0.0151 ± 0.0005
0.00133	0.015 ± 0.001	0.017 ± 0.002
0.00167	0.0142 ± 0.0007	0.025 ± 0.003
k_{bim} (L mol^{-1} s^{-1})	12.5 ± 0.8	0.018 ± 0.005
k_{sat} (s^{-1})	0.05 ± 0.02	

Conditions: in 0.10 mol L^{-1} phosphate at pH 8.2, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 337 nm.

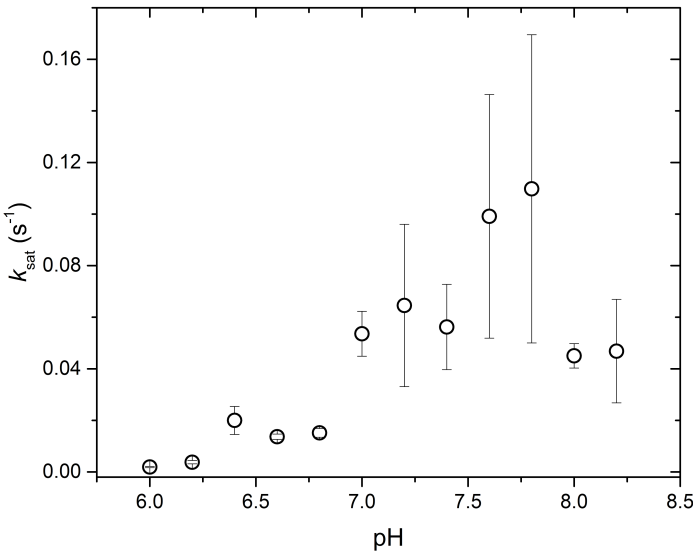


Figure S3: Plot of k_{sat} versus pH for the reaction between CBTOH and cysteine. Conditions: see **Tables S1** to **S12**.

Data fitting

The plots of $k_{obs}1$ and k_{bim} *versus* pH were fitted with a simple logistic function (**Equation S1**) at the Origin 7.0 software (Microcal, USA), which generates a sigmoidal curve. The parameters obtained are: a (maximum value for the y axis), k (steepness of the curve) and x_0 (sigmoid mid-point).

Equation S1	$y = \frac{a}{1+e^{-k(x-xc)}}$

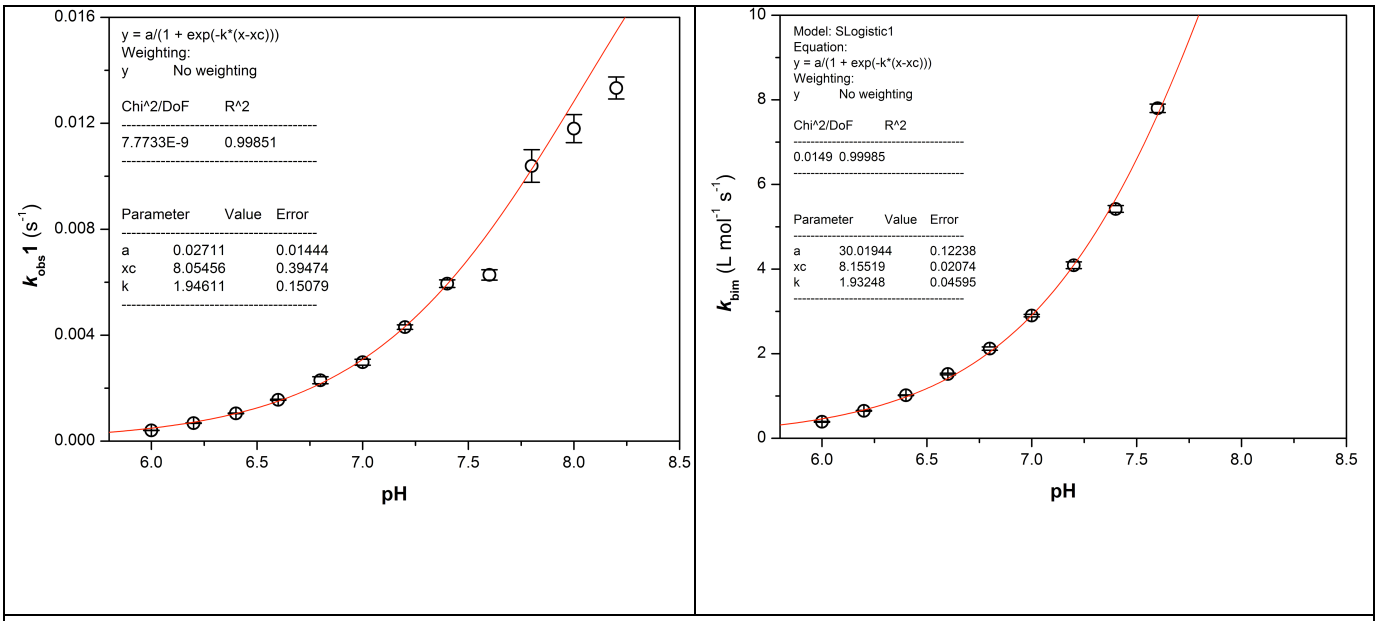
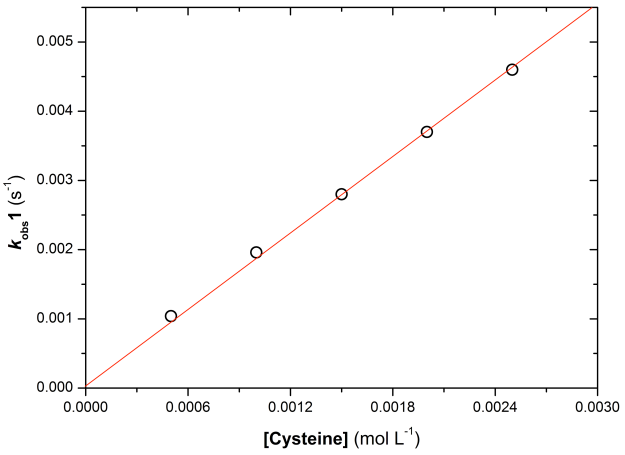
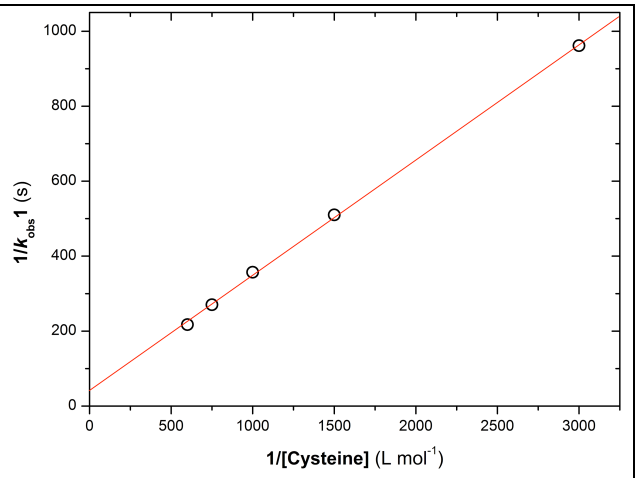


Figure S4: Sigmoidal fitting using the Simple Logistics (Equation S1) for the plots of $k_{obs}1$ and k_{bim} *versus* pH for the reaction between CBTOH and cysteine. Conditions: see **Tables S1** to **S12**.

Reaction kinetics in buffered D_2O

Table S13: Observed rate constant ($k_{obs}1$) for the reaction of CBTOH and cysteine at pD = 7.20 in D_2O. The values for the bimolecular rate constant (k_{bim}) and saturation rate constant (k_{sat}) have been obtained from this data.





[Cysteine] (mol L^{-1})	$k_{obs}1$ (s^{-1})
0.0033	0.00104
0.0067	0.00196
0.0010	0.00280
0.0013	0.00370
0.0017	0.00460
k_{bim} (L mol^{-1} s^{-1})	1.86 ± 0.02
k_{sat} (s^{-1})	0.024 ± 0.004

Conditions: in 0.10 mol L^{-1} phosphate at pD 7.2, [CBTOH] = 2.0×10^{-5} mol L^{-1}, T = 25.0 °C, 342 nm.

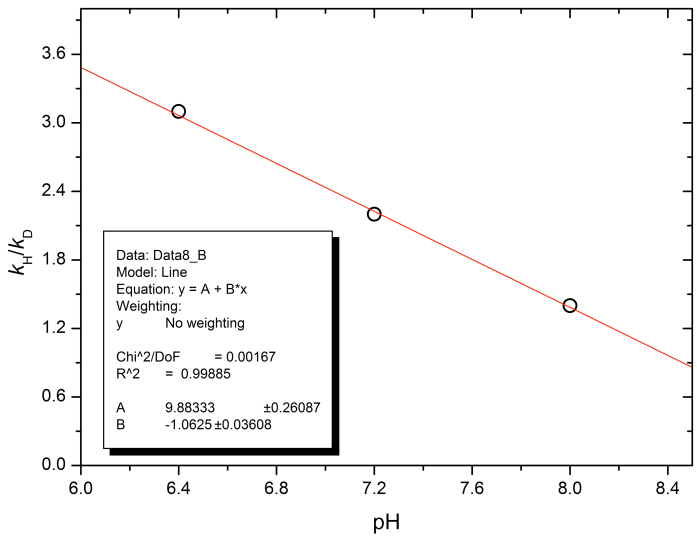
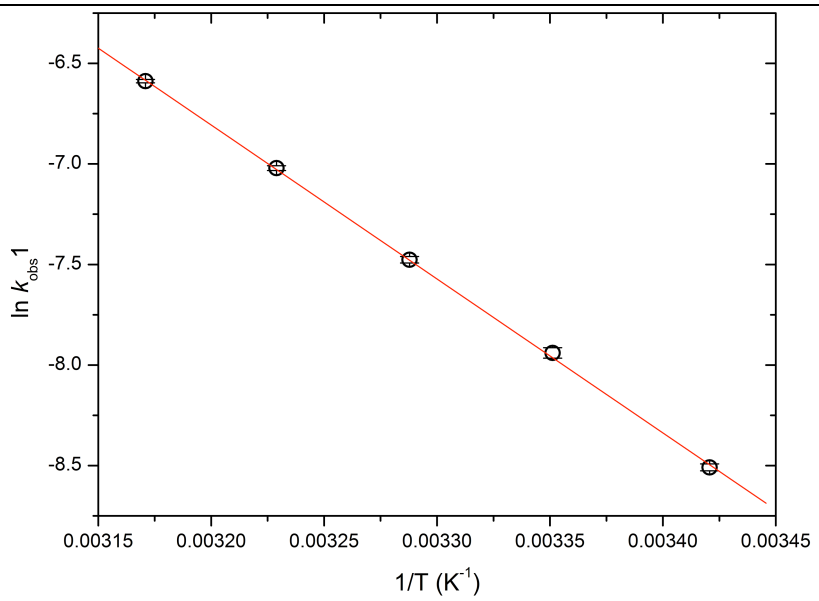


Figure S5: Plot of k_H/k_D *versus* pH for the reaction between CBTOH and cysteine. Conditions: see **Tables S7** and **S13**. The value for k_H/k_D reaches unity (no KIE) at pD = 8.3.

Activation parameters

The activation parameters were determined from the Arrhenius plots in different pH conditions.

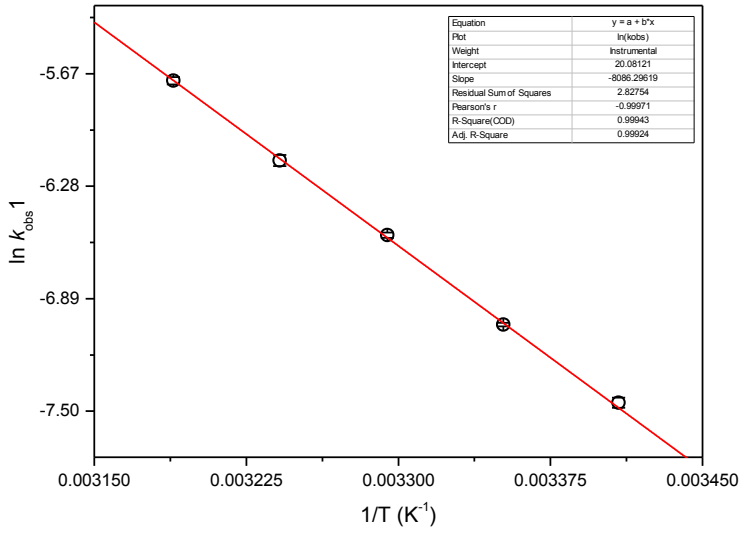
Table S14: Arrhenius plot determined from the $k_{obs}1$ at pH = 6.0



T (°C)	$k_{obs}1$ (s^{-1})
19.2	0.000202 ± 0.000003
25.2	0.00036 ± 0.00001
31.0	0.00057 ± 0.00001
36.5	0.00089 ± 0.00001
42.2	0.00138 ± 0.00001

Conditions: in 0.10 mol L^{-1} phosphate at pH 6.0, [CBTOH] = 2.0 × 10^{-5} mol L^{-1}, [Cysteine] = 1.0 × 10^{-3}, 342 nm.

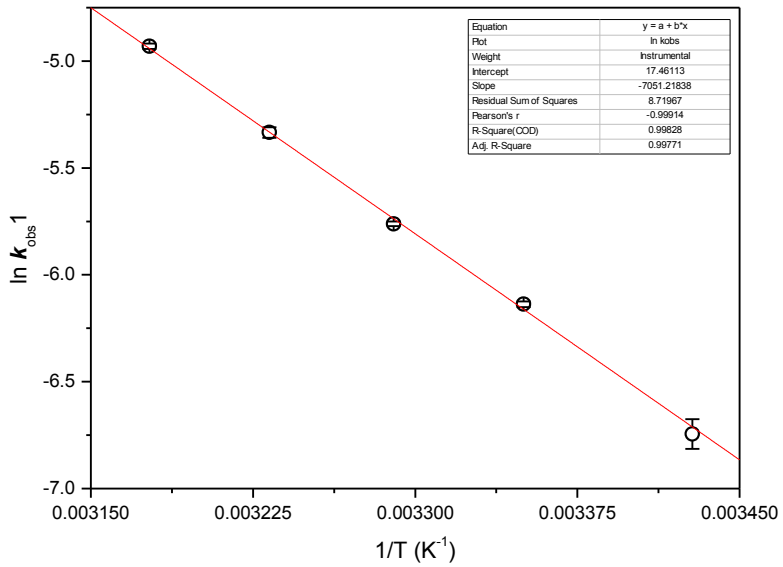
Table S15: Arrhenius plot determined from the $k_{obs}1$ at pH = 6.4



T (°C)	$k_{obs}1$ (s^{-1})
20.2	0.00058 ± 0.00002
25.2	0.000885 ± 0.000008
30.4	0.00144 ± 0.00002
35.4	0.00215 ± 0.00006
40.4	0.00332 ± 0.00007

Conditions: in 0.10 mol L^{-1} phosphate at pH 6.4, [CBTOH] = 2.0 × 10^{-5} mol L^{-1}, [Cysteine] = 1.0 × 10^{-3}, 342 nm.

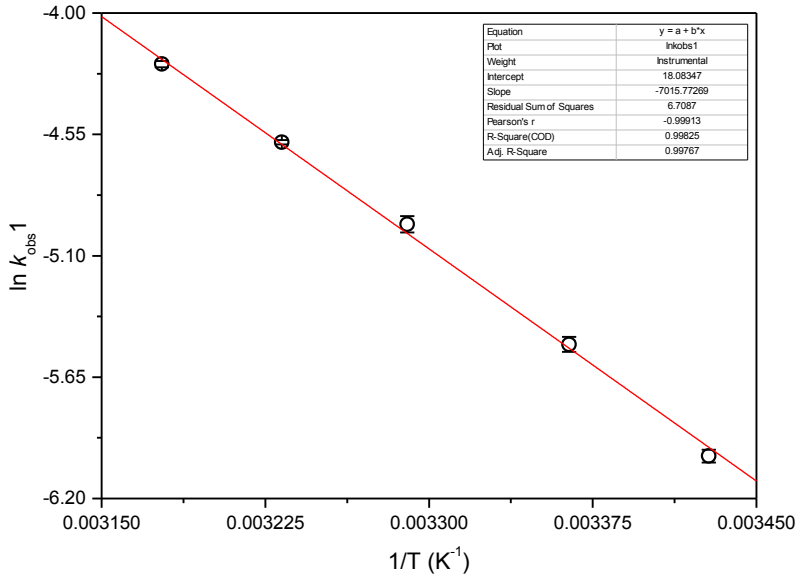
Table S16: Arrhenius plot determined from the $k_{obs}1$ at pH = 6.8



T (°C)	$k_{obs}1$ (s^{-1})
18.6	0.00118 ± 0.00008
25.4	0.00216 ± 0.00003
30.8	0.00315 ± 0.00004
36.2	0.0048 ± 0.0001
41.6	0.00722 ± 0.00009

Conditions: in 0.10 mol L^{-1} phosphate at pH 6.8, [CBTOH] = 2.0 × 10^{-5} mol L^{-1}, [Cysteine] = 1.0 × 10^{-3}, 342 nm.

Table S17: Arrhenius plot determined from the $k_{obs}1$ at pH = 7.2

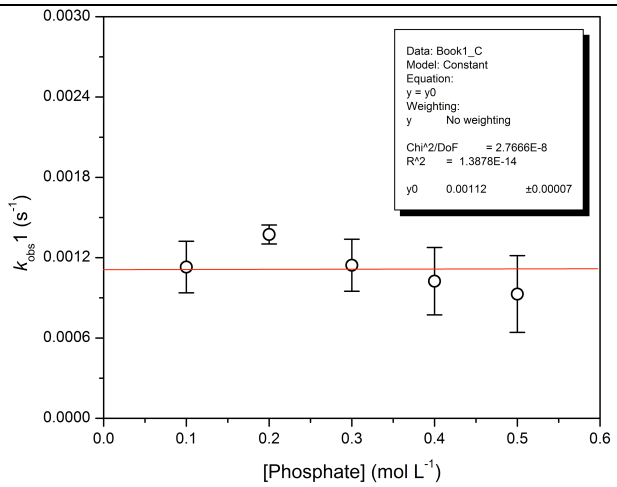


T (°C)	$k_{obs}1$ (s^{-1})
18.6	0.00246 ± 0.00007
24.1	0.0041 ± 0.0001
30.8	0.0070 ± 0.0003
36.2	0.0102 ± 0.0001
41.6	0.0145 ± 0.0002

Conditions: in 0.10 mol L^{-1} phosphate at pH 7.2, [CBTOH] = 2.0 × 10^{-5} mol L^{-1}, [Cysteine] = 1.0 × 10^{-3}, 342 nm.

Buffer concentration

Table S18: Rate constants determined varying the phosphate concentration at pH = 7.2.



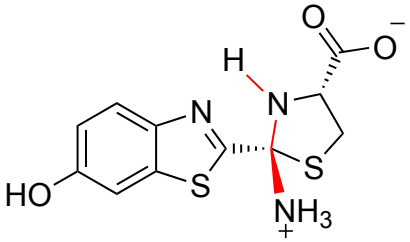
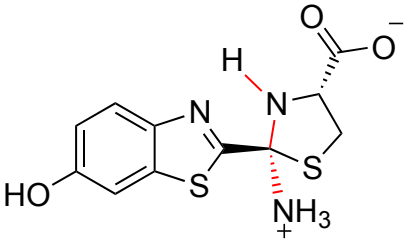
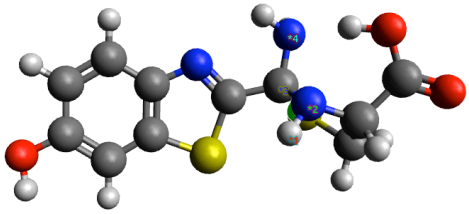
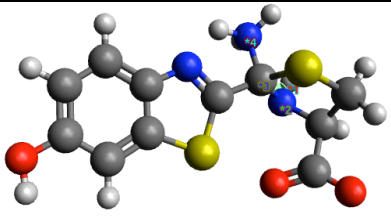
[Phosphate] (mol L^{-1})	$k_{obs}1$ (s^{-1})
0.1	0.0011 ± 0.0002
0.2	0.0014 ± 0.0001
0.3	0.0011 ± 0.0002
0.4	0.0010 ± 0.0002
0.5	0.0009 ± 0.0003
$k_{obs}1 = 0.0011 \pm 0.0001$ s^{-1}	

Conditions: in 0.10 mol L^{-1} phosphate at pH 6.4, [CBTOH] = 2.0×10^{-5} mol L^{-1}, [Cysteine] = 1.0×10^{-3} T = 25.0 °C, 342 nm.

Computational studies

The analysis of the structure of the intermediates, that is, the quantum calculation were made using the ORCA software (version 3.0.1). For the optimization of the geometry, necessary to determine the dihedral angle, DFT (Density Functional Theory) was used, because it is regarded as having the highest accuracy/computation cost ratio. B3LYP was used as functional and 6-31G(2d,2p) as basis set. The most energetically favourable conformation of the intermediates, as long as the dihedral angle H23-N11-C10-N16, were determined and are reported at **Table S19**.

Table S19: calculated parameters for the intermediates (*R*)-**2**. The bonds making up the dihedral angle along the H-N-C-N bonds plane are marked in red.

	(2*R*,4*R*)-**2**	(2*R*,4*S*)-**2**
Planar structure		
Optimized spatial structure		
Dihedral angle	146.4°	17.0°