

An unique fluorescence biosensor for selective detection of tryptophan and histidine †

Pinkesh G Sutariya^a, Alok Pandya^a, Anand Lodha^b and Shobhana K Menon^{ab*}

^a Department of Chemistry, School of sciences
Gujarat University, Navrangpura, Ahmedabad 380009, ^b Department of Forensic Science, Gujarat University, Navrangpura,
Ahmedabad 380009
shobhanamenon07@gmail.com

Contents

1. Materials and method	
2. Absorption and Luminescence	
3. Binding Study	
4. Real sample preparation	
5. Synthesis procedure	
6. Emission spectra of TDPC ligand with amino acids.....	S1
7. Binding constant curve for L-Trp, L-His, DL-Ala and L-ser.....	S2-S5
8. Absorption spectra of TDPC ligand with L-Trp and L-His	S6-S7
9. Absorption spectra of TDPC ligand with L-Trp and L-His at various concentrations.S8-S9	
10. ESI-MASS spectra of TDPC ligand with L-His.....	S10
11. Job's plot obtained from the absorption titration of TDPC with L-Trp and L-His...S11-S12	
12. ¹H NMR spectrum of TDPC ligand with L-Trp.....	S13
13. pH study of TDPC ligand with L-Trp and L-His	S14-S15
14. Visual colour changes obtained upon addition of TDPC with various amino acids....	S16
15. Competitive emission spectra of TDPC ligand with L-Trp complex with other amino acids.	S17
16. Proposed binding mechanism through hydrogen bonding with TDPC ligand and (A) L-His (B) L-Trp	S18
17. Time-dependent study of L-Trp. and L-His. With TDPC.....	S19-S20
18. Real sample analysis table.....	Table S1- S2

1. Materials and methods

All the reagents and chemicals were used of analytical grade purchased from sigma aldrich. Silica gel (Merck, 0.040-0.063mm) was used for column chromatography. Melting points were taken on Opti-Melt (Automated melting point system). The FT-IR spectra were recorded as KBr pellet on Bruker TENSOR-27 in the range of 4000-400 cm^{-1} . Discover BenchMate system-240 V (CEM Corporation) microwave synthesizer was used for synthesis. ^1H NMR spectra was scanned on 400 MHz FT-NMR Bruker Avance-400 in the range of 0.5 ppm -15 ppm and ^{13}C NMR spectra was recorded on a Bruker DPX-300 spectrometer using internal standard tetramethylsilane (TMS) and deuterated DMSO as a solvent in the range of 0.5 ppm to 250 ppm. ESI Mass spectra were taken on a Shimadzu GCMS-QP 2000A. Emission spectrum was recorded on Horiba Jobin, Fluorolog, and Edinburgh F900. UV-Vis absorption spectra were acquired on a Jasco V-570 UV-Vis. Spectrometer. Working standard solutions were prepared daily in deionized water.

2. Absorption and luminescence

Absorption spectra of compound lower rim substituted calix[4]arene (TDPC) was recorded in acetonitrile and the data are given in experimental section. This compound show absorption band in the region between 290-390 nm, the band at 327 nm and 357 nm indicates π - π^* transition of pyrene system to calix[4]arene . This compound shows two strong luminescence band at 440 nm and 475 nm in acetonitrile with excitation at the absorption maxima (λ_{max}) of the pyrene moiety, which is at 370-380 nm. The fluorescence study has been carried out at $\lambda_{\text{ex}}= 380$ nm, $\lambda_{\text{em}}= 440$ nm.

3. Binding study

Stock solutions of the TDPC (1×10^{-8} M) and that of amino acids (DL-Alanine, DL-Aspartic acid, DL-DOPA, L-Histidine, L-Leucine, DL-Methonine, L-Proline, L-Tryptophan, DL-2-Amino butyric acid, L-Cystenine, L-Glutamic acid, L-Hydroxyproline, DL-Nor-Leucine, L-Ornithine, DL-Serine, L-Tyrosine, L-Arginine, L-Cystine, Glycine, L-Isoleucine, L-Lysine, DL-Phenyl alanine, DL-Threonine, DL-Valine) (1×10^{-6} M) were prepared in freshly purified acetonitrile. Then 2 mL stock solution of the complex and 2 mL stock solution of each amino acids were taken in a 5 mL volumetric flask, so that the effective concentration of the complex is 1×10^{-8} M and that of the amino acids are 1×10^{-6} M (100 fold). The spectra of the amino acids added solutions were compared with that of the original solution to ascertain the interactions of the amino acids with the ionophore. For emission titration study, the same stock solutions of the complexes were used and amino acids solutions of desired concentration (1.0 – 120.0 equivalents) were prepared by proper dilution of the stock solution. The molecular binding property of fluoroionophore TDPC was investigated with a large number of amino acids (DL-Alanine, DL-Aspartic acid, DL-DOPA, L-Histidine, L-Leucine, DL-Methonine, L-Proline, L-Tryptophan, DL-2-Amino butyric acid, L-Cystenine, L-Glutamic acid, L-Hydroxyproline, DL-Nor-Leucine, L-Ornithine, DL-Serine, L-Tyrosine, L-Arginine, L-Cystine, Glycine, L-Isoleucine, L-Lysine, DL-Phenyl alanine, DL-Threonine, DL-Valine) in acetonitrile. Then solution for L-Trp and L-His were prepared in the concentration range of (0-120 nM) and (0-120 nM) respectively with 1×10^{-8} M concentration of TDPC ligand. The biomolecule recognition process was monitored by luminescence, UV-Vis and ESI-Mass spectral changes. The binding constants were calculated by fluorescence titration data. Here, we have shown representative spectra showing the changes observed in emission intensities upon the addition of increasing

concentration of amino acids are shown in the **Figure 1 A and B**. According to this procedure, the fluorescence intensity (F) scales with the amino acids concentration ([M]) through $(F_0-F)/(F-F_\infty) = ([M]/K_{\text{diss}})^n$. The binding constant (K_s) is obtained by plotting $\text{Log}[(F_0-F)/(F-F_\infty)]$ vs. $\text{Log}[M]$, where F_0 and F_∞ are the relative fluorescence intensities without addition of guest amino acids and with maximum concentration of amino acids (when no further change in emission intensity takes place), respectively. The value of $\text{Log}[M]$ at $\text{Log}[(F_0-F)/(F-F_\infty)] = 0$ gives the value of $\text{log}(K_{\text{diss}})$, the reciprocal of which is the binding constant (K_s). We have also calculated binding constant for DL-Ala and L-Ser. for major interferent during this analysis.

4. Preparation of serum samples

Tack a blood sample collected in oxalated container, add 4 part of 5% trichloroacetic acid in it which forms the precipitation then add $\text{Ba}(\text{OH})_2$ to decompose the precipitation. Add HgSO_4 in 5% H_2SO_4 , centrifuge it, and filter it. Resultant solution used for the histidine estimation now. For determination of total tryptophan, blood was drawn into evacuated blood-collection tubes without anticoagulant and was allowed to clot spontaneously on standing for 10 to 15 mm. To each milliliter of serum, 2 ml of a cold solution of trichloroacetic acid (60 g/liter) was added. Precipitated protein was then removed by centrifuging the mixture and the supernatant fluid was filtered through a plastic Millipore filter. The pH of the aqueous (top) layer, which contained the compounds of interest, was adjusted to a pH of approximately 7 with solid tris(hydroxymethyl)-aminomethane solution¹. The results are shown in **Table S1 and S2**. This result confirms the use of calix[4]arene as fluoroionophore have high sensitivity and specificity towards L-Trp and L-His in blood serum.

5. Experimental

Synthesis of compound A

A mixture of 1-amino pyrene (3.0 g, 0.013 mol), K_2CO_3 (1.90 g, 0.013 mol), KI (2.56 g, 0.013 mol), TEA (1.96 ml, 0.013 mol) and dichloromethane (60 ml) was stirred at 40°C for 24 hours. The progress of reaction was monitored by using tlc of hexane:ethylacetate (6:4) at regular interval of time. Then crude product was added to 0.1M HCl solution and stirred. at room temperature and then filtered out. Resulted product was crystallised with absolute methanol. Yield 3.4 g (75%). Elemental analysis, Anal.calc: $C_{22}H_{22}ClNO_2$: C, 71.83; H, 6.03; Cl, 9.64; N, 3.81; O, 9.45% Found: C, 71.38; H, 6.01; Cl, 9.52; N, 3.43, O, 9.38%. FT-IR (KBr)v: 3280 cm^{-1} (-NH), 3300 cm^{-1} (-CH), 1H NMR: δ_H (DMSO,400MHZ), 3.56 (4H, -OCH₂CH₂O-,s), 3.42 (4H, -OCH₂CH₂O-,s), 3.76 (2H, -OCH₂CH₂ NH-, t), 3.38(2H, -OCH₂CH₂ NH-, q), 4.12(1H, -OCH₂CH₂ NH-, s), 8.12 (2H, Ar-H, s), 8.02 (1H, Ar-H, s), 7.78 (4H, Ar-H, s), 7.23 (2H, Ar-H, s). m.p. 178°C. ESI MAAS (m/z) 368.1 (M+1).

Synthesis of compound 1 Microwave assisted synthesis of p-tert-butylcalix[4]arene

A mixture of p-tert-butyl phenol (4.0 g, 0.33 mM), sodium hydroxide (NaOH) (1 g) and formaldehyde(1.8 ml,0.18 mM) solution was taken in an open vessel and was irradiated with 50 W power in a microwave synthesizer Discover(CEM)by stirring for 3 min. After cooling for 10 min, resulted yellow solid mass. Next, 4 ml of toluene and 30 ml of diphenyl ether was added in this yellow solid, again irradiated with microwave power of 100 W for 5 min with stirring and obtained a dark brown solution. Further, this solution was added in to 75 ml of ethylacetoacetate and kept for 2 h. Finally, white precipitate was obtained which was filtered and washed with ethylacetoacetate and finally dried. Yield, 3.5 g (96%).Elemental analysis for $C_{44}H_{56}O_4$:Calcd.C, 81.44; H, 8.70; O, 9.80% Found: C, 80.11; H, 8.26; O, 9.90% 1HNMR : δ_H DMSO,400 MHZ):

1.18(36H, t-butyl, s), 3. 81 (8H, ArCH₂Ar, s), 7.12 (8H, s, Ar-H), 9.71(4H, Ar-OH, s). ESI-MASS (m/z) 648 (M+1).

Synthesis of 25, 27-bis (methane)-26, 28-dihydroxy-p-tert- butyl calix[4]arene,2

A mixture of p-tert-butylcalix[4]arene 1 (3.5 g, 0.80 mM), K₂CO₃(1.9 g, 14.0 mM) and 1-iodomethane (4 ml, 14.0 mM) in dry acetone (150 ml) was stirred at 56°C for 24 hrs . The actual reaction time was considered by taking tlc at regular interval of time by using mixture of (ethylacetate:hexane, 8:2)The solvent was then evaporated under vacuum and the residue taken up with CH₂Cl₂ .The organic phase was washed with 0.1 M HCl up to neutrality and dried over anhydrous Na₂SO₄ . After complete evaporation of the solvent, the resulting crude product was purified by column chromatography (silica gel, hexane: ethyl acetate 1); 2.9 g, yield (81%). Elemental analysis, Anal.calc: C₄₆H₆₀O₄ : C,81.61; H,8.93; O,9.45% Found: C,81.38; H,8.52; O,9.18%. ¹H NMR: δH (DMSO,400MHZ), 1.20 (18H, t-butyl, s) 0.96(18H, t-butyl, s), 4.28 (4H, -OCH₂, t), 3.64(H, -OCH₂, t), 3.83 (4H, ArCH₂Ar ,d), 3.97(4H, OCH₂, s), 4.30(4H, ArCH₂Ar, d), 6.42(4H, Ar-H, s), 6.85(4H, Ar-H, s), 9.19 (2H, OH, s), m.p. 223-228°C. ESI MAAS (m/z) 677.1 (M+1).

Synthesis of compound 3:

A mixture of compound 2 (1.5 g, 0.0007 mol), K₂CO₃ (3.1 g, 0.0014 mol) and compound A (0.548 g, 0.0014 mol) in dry acetonitrile (50 ml) was stirred at reflux temperature for 24 hrs. The actual reaction time was considered by taking tlc at regular interval of time by using mixture of (ethylacetate:hexane, 8:2)The solvent was then evaporated under vacuum and the residue taken up with CH₂Cl₂ .The organic phase was washed with 0.1 M HCl up to neutrality and dried over anhydrous Na₂SO₄ . After complete evaporation of the solvent, the resulting crude product was purified by column chromatography (silica gel, hexane: ethyl acetate 8:2), 2.7 g, yield (78%).

Elemental analysis, Anal.calc: C₉₀H₁₀₂N₂O₈: C, 80.68; H, 7.67; N, 2.09; O, 9.55% Found: C, 80.31; H, 7.52; O, 9.38%. FT-IR (KBr)v: 3180 cm⁻¹(-NH), 3230 cm⁻¹(-CH), ¹H NMR: δH (DMSO,400MHZ), 1.32 (18H, t-butyl, s) 0.92(18H, t-butyl, s), 4.28 (4H, -OCH₂, t), 3.91(16H, O-CH₂-CH₂-O, S), 3.73 (4H, O-CH₂-CH₂t), 3.42 (4H, O-CH₂-CH₂-NH-,q), 8.92 (2H, O-CH₂-CH₂-NH-,s), 3.64(6H, -OCH₃, s), 3.83 (4H, ArCH₂Ar ,d), 4.30(4H, ArCH₂Ar, d), 7.02(4H, Ar-H, s), 7.20 (4H, Ar-H, s), 7.62 (4H, Ar-H, s), 7.91 (8H, Ar-H, s), 8.14 (4H, Ar-H, s), 8.18 (2H, Ar-H, s) m.p.:215⁰C. ¹³C NMR, 151.2, 152.6, 144.1, 142.6, 134.2, 129.7, 126.1, 125.2, 124.7, 74.2, 70.6, 44.3, 34.8, 31.3. ESI MAAS (m/z), 1339 (M+1).

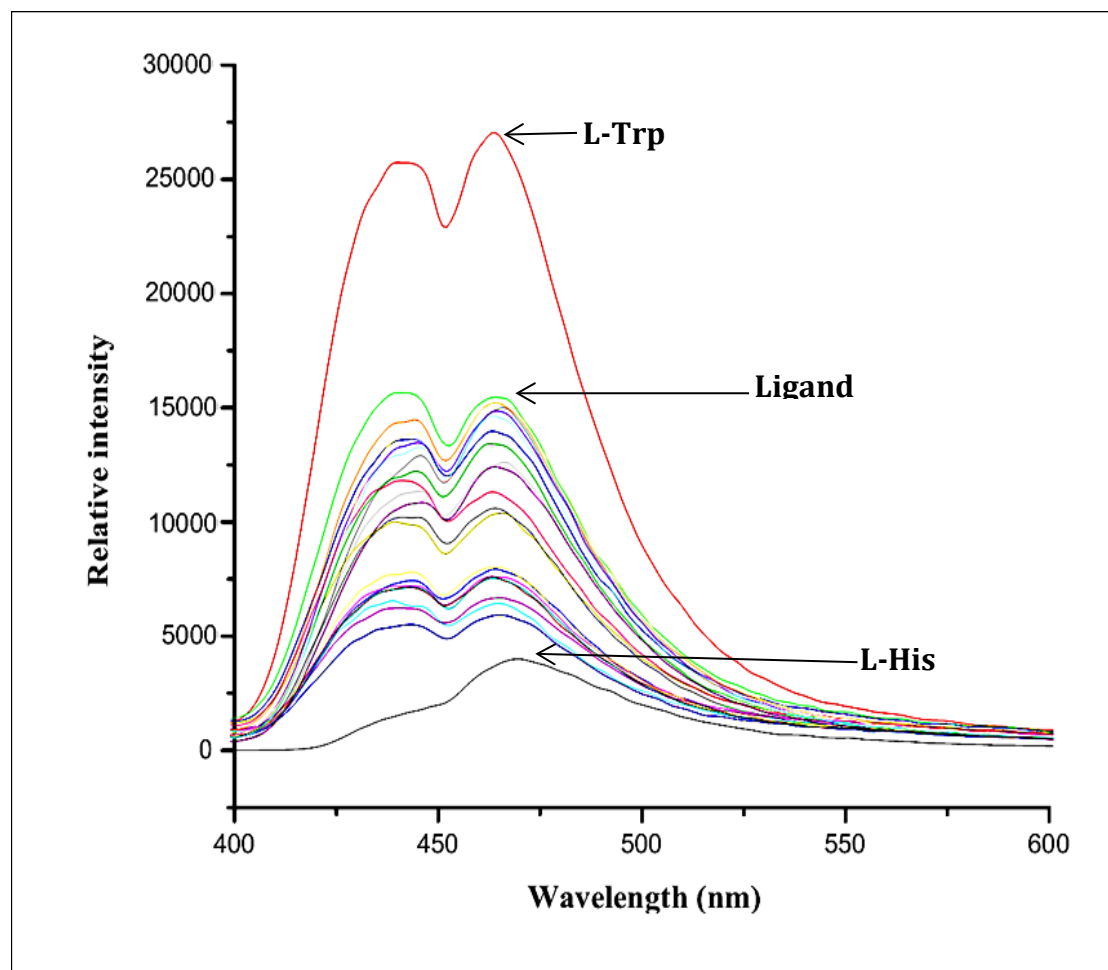


Fig. S1 Emission spectra of TDPC ligand with amino acids from top to down (a) DL-Alanine (b) DL-Aspartic acid (c) DL-DOPA (d) L-Leucine (e) DL-Methonine (f) L-Proline (g) DL-2-Amino butyric acid (h) L-Cystenine (i) L-Glutamic acid (j) L-Hydroxyproline (k) DL-Nor-Leucine (l) L-Ornithine (m) DL-Serine (n) L-Tyrosine (o) L-Arginine (p) L-Cystine (q) Glycine (r) L-Isoleucine (s) L-Lysine (t) DL-Phenyl alanine (u) DL-Threonine and (v) DL-Valine. (Ex= 380 nm, Em= 440 nm)

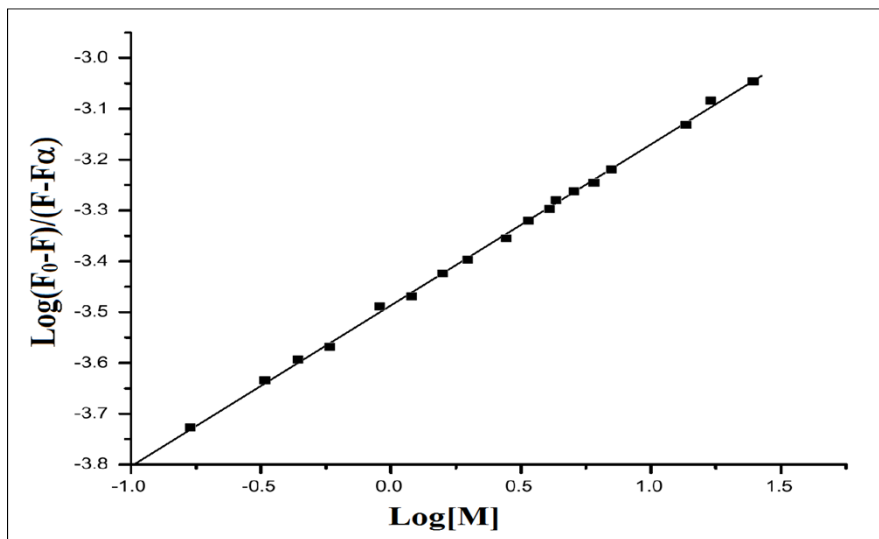


Fig. S2 Binding constant curve for L-Trp.

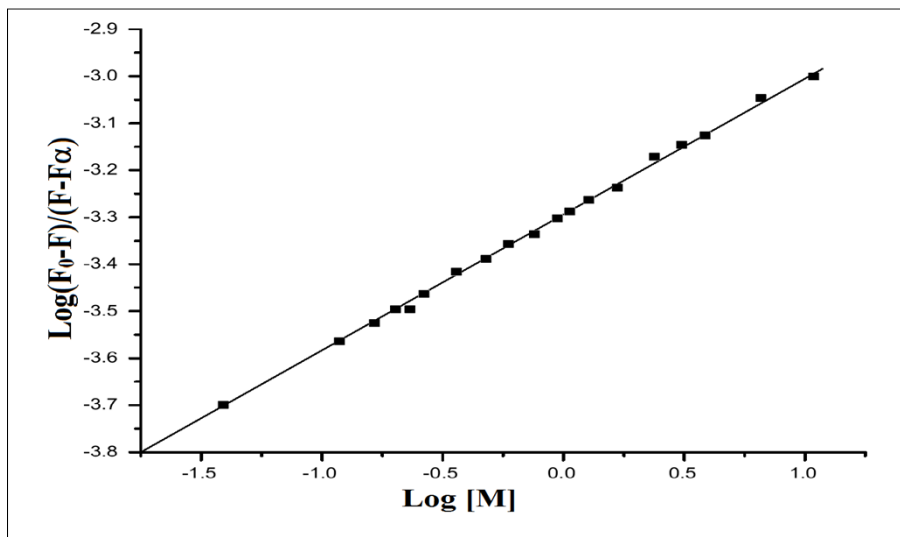


Fig. S3 Binding constant curve for L-His.

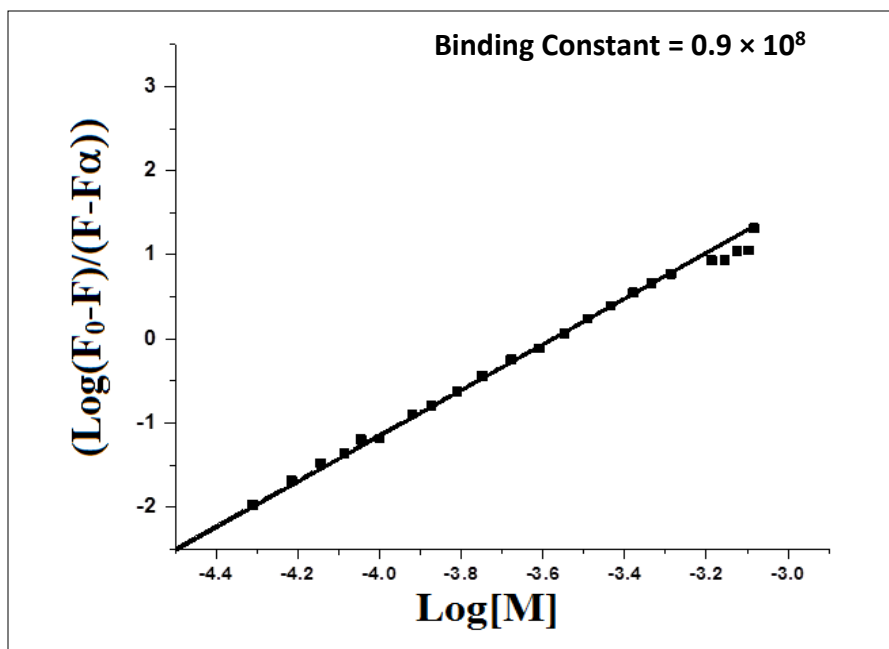


Fig. S4 Binding constant curve for DL-Ala.

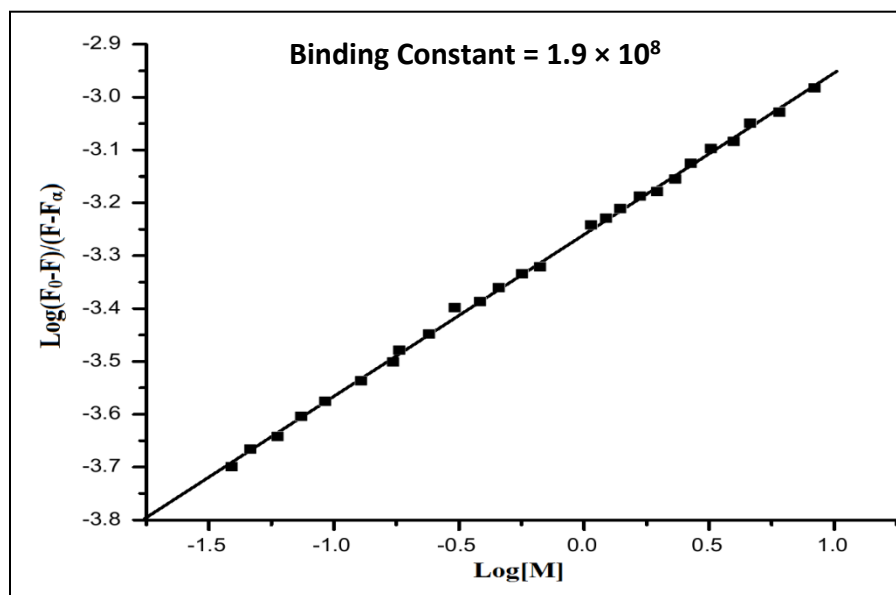


Fig. S5 Binding constant curve for L-Ser.

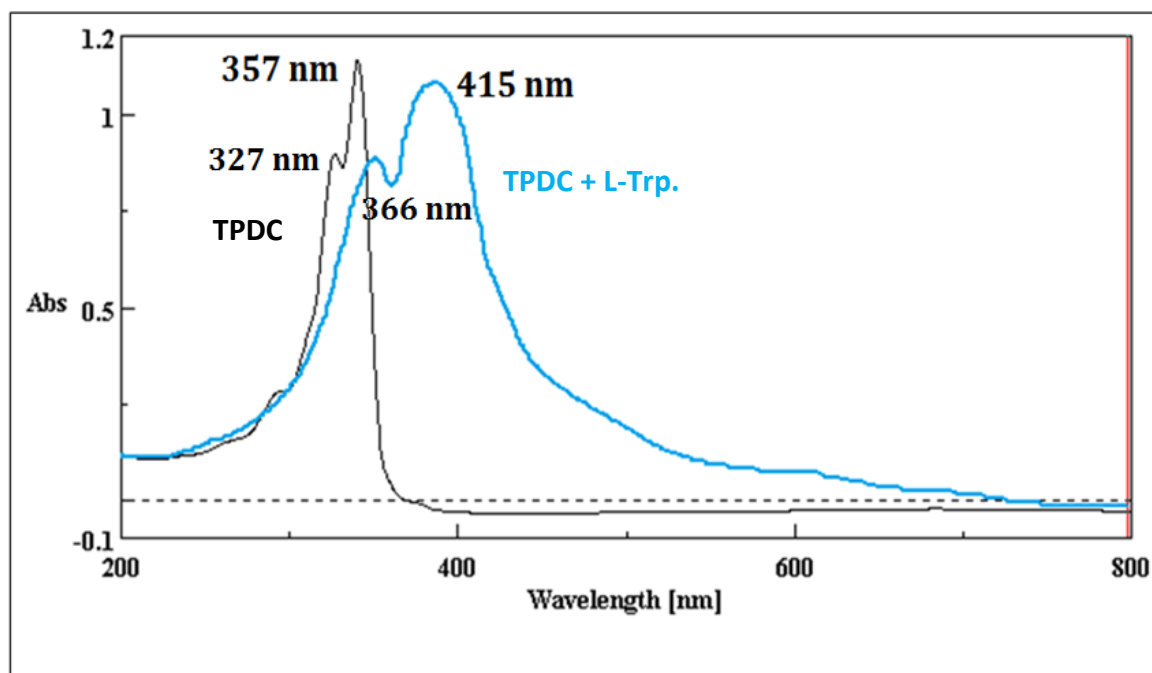


Fig. S6 Absorption spectra of TDPC ligand with L-Trp

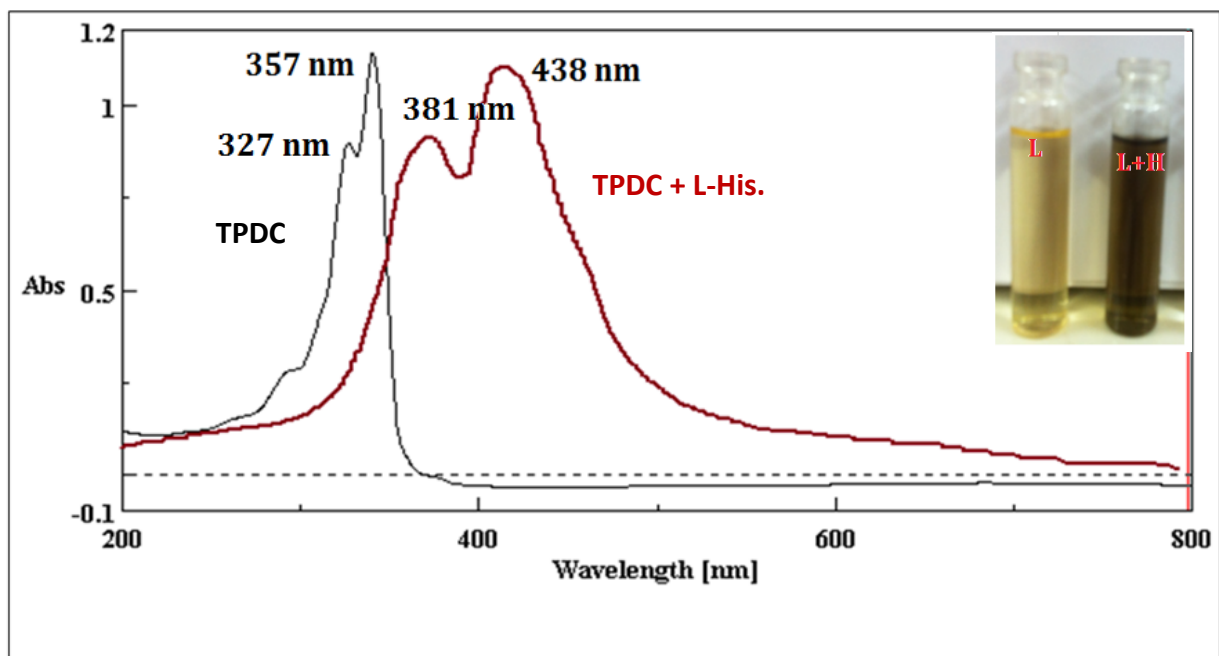


Fig. S7 Absorption spectra of TDPC ligand with L-His.

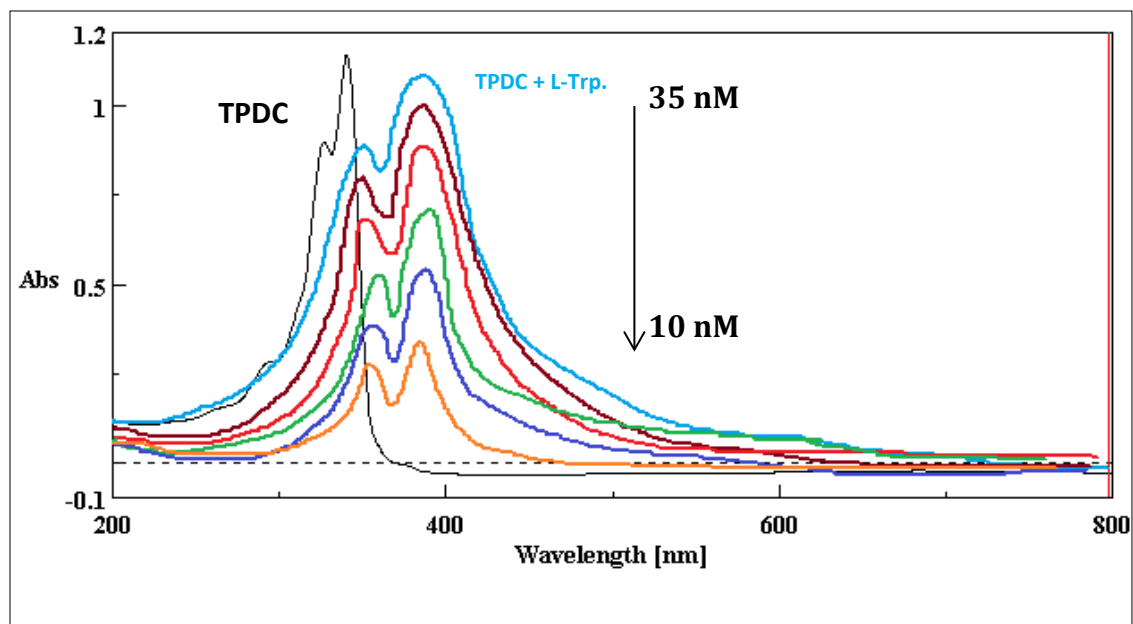


Fig. S8 Absorption spectra of TDPC ligand with L-Trp at various concentrations (35 nM, 30 nM, 25 nM, 15 nM and 10 nM).

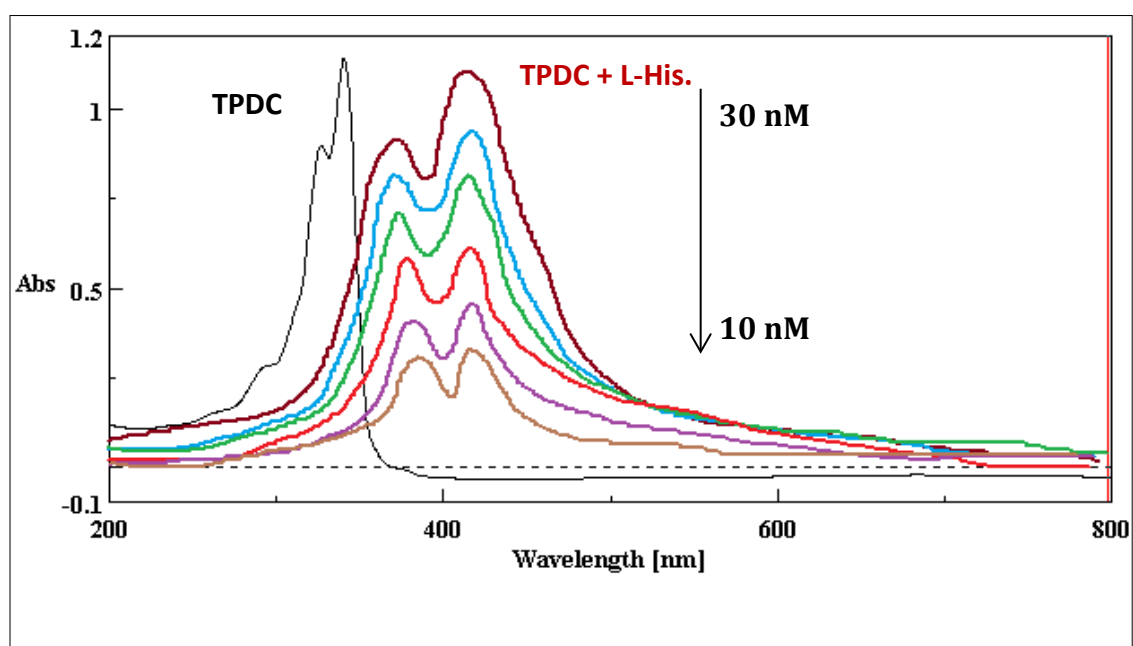


Fig. S9 Absorption spectra of TDPC ligand with L-His at various concentrations (30 nM, 28 nM, 24 nM, 20 nM, 15 nM and 10 nM).

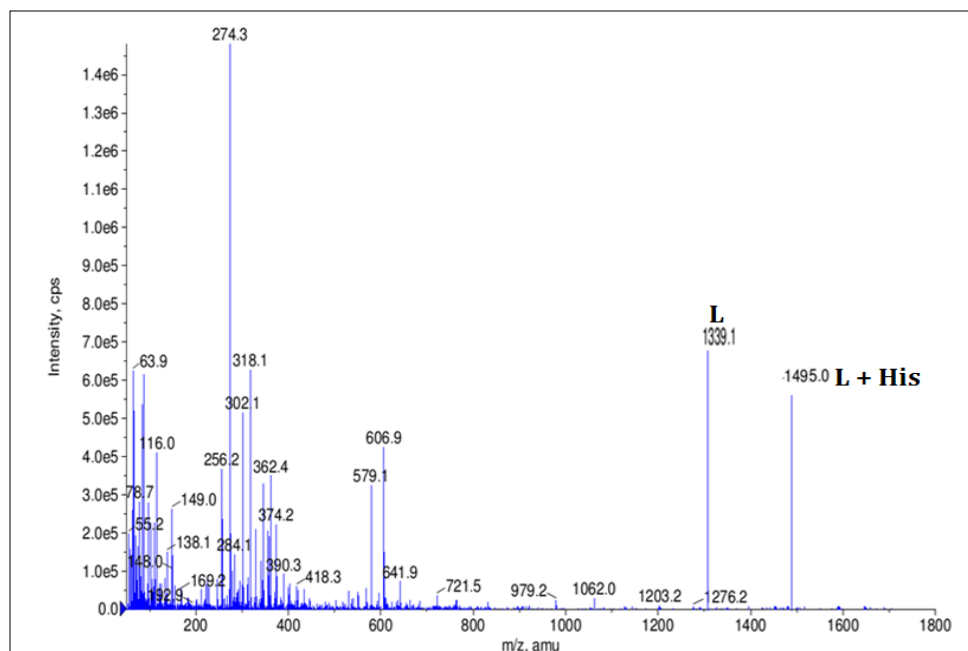


Fig. S10 ESI mass spectrum showing the isotopic peak pattern of molecular ion peak for 1:1 complex formed between TDPC and L-His.

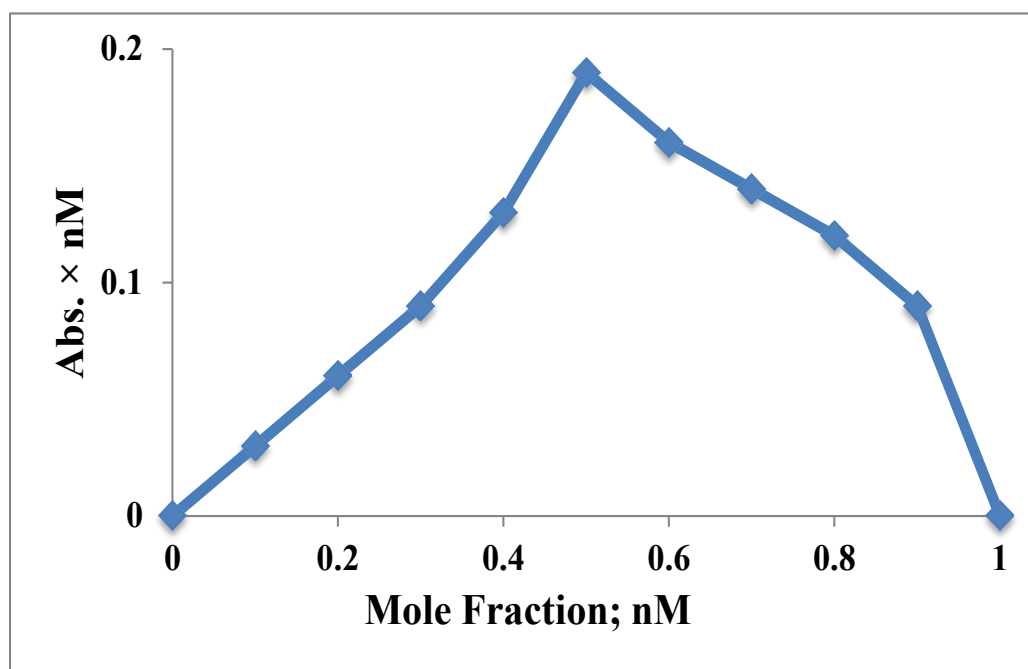


Fig. S11 Job's plot obtained from the absorption titration of TDPC with L-Trp.

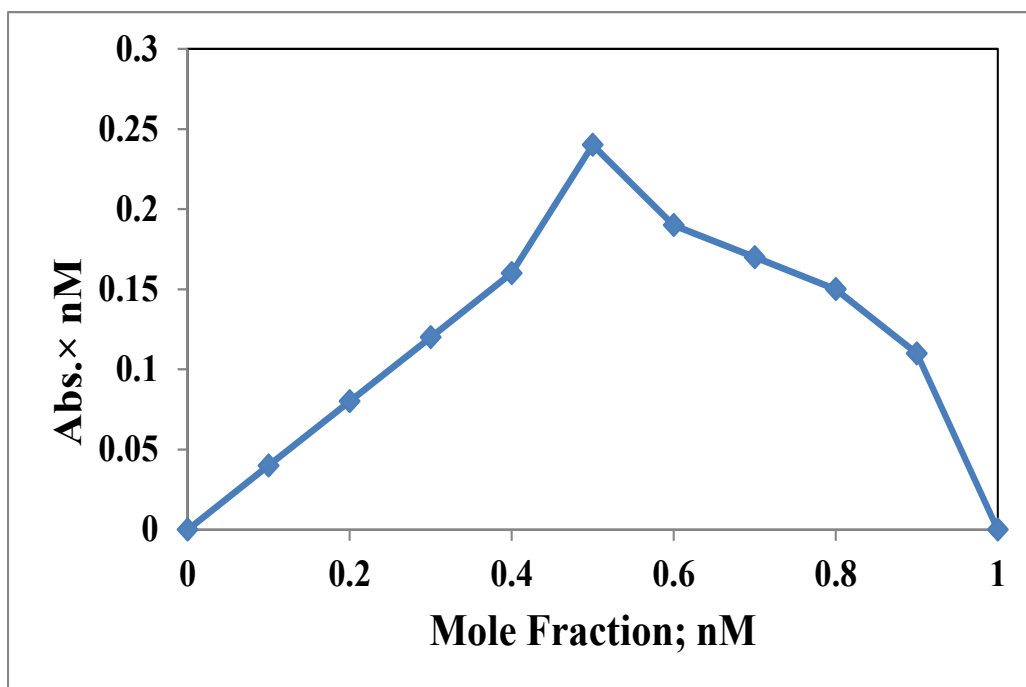


Fig. S12 Job's plot obtained from the absorption titration of TDPC with L-His.

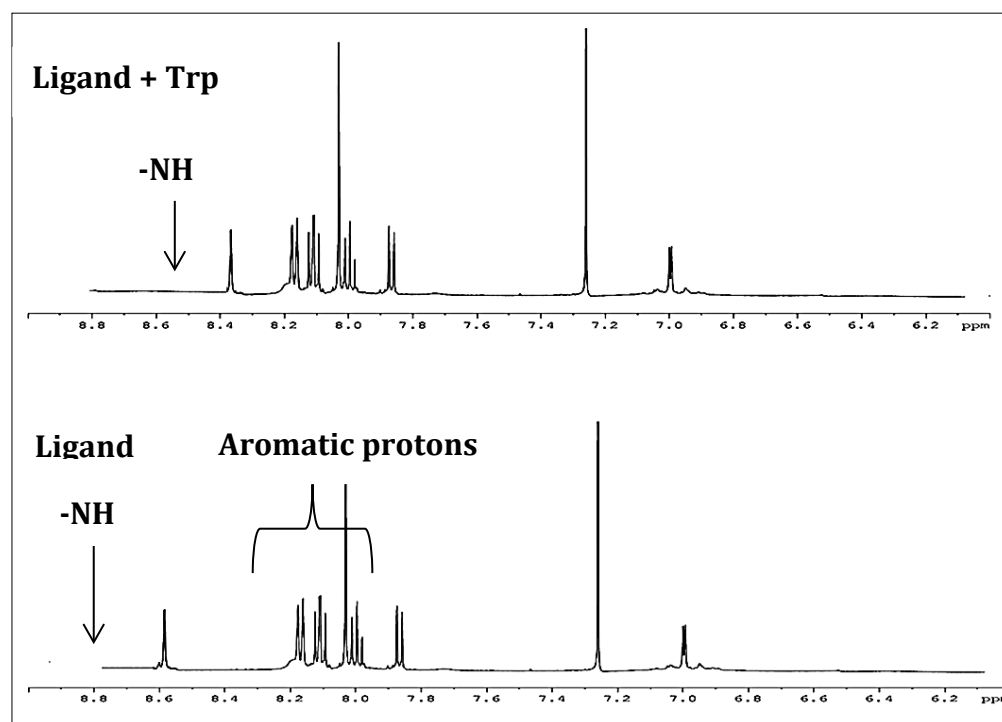


Fig. S13 ¹H NMR spectrum of TAQC ligand with L-Trp recorded in CDCl₃

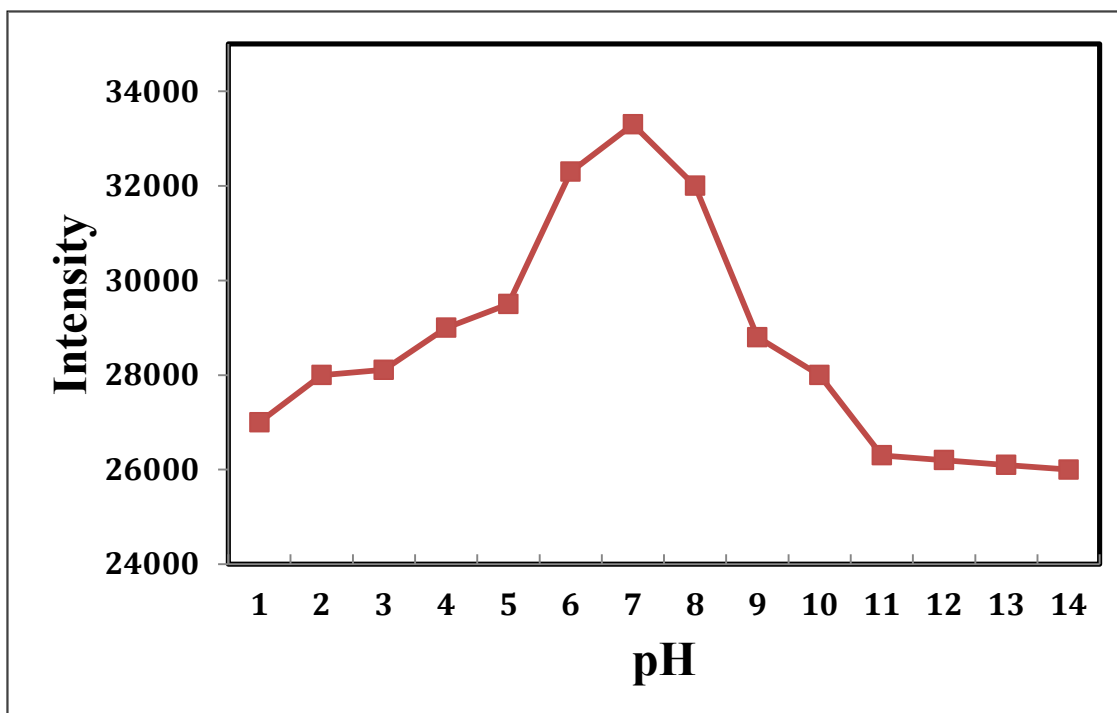


Fig. S14 Shows the effect of fluorescence intensities of TDPC with L-Trp complex by varying pH.

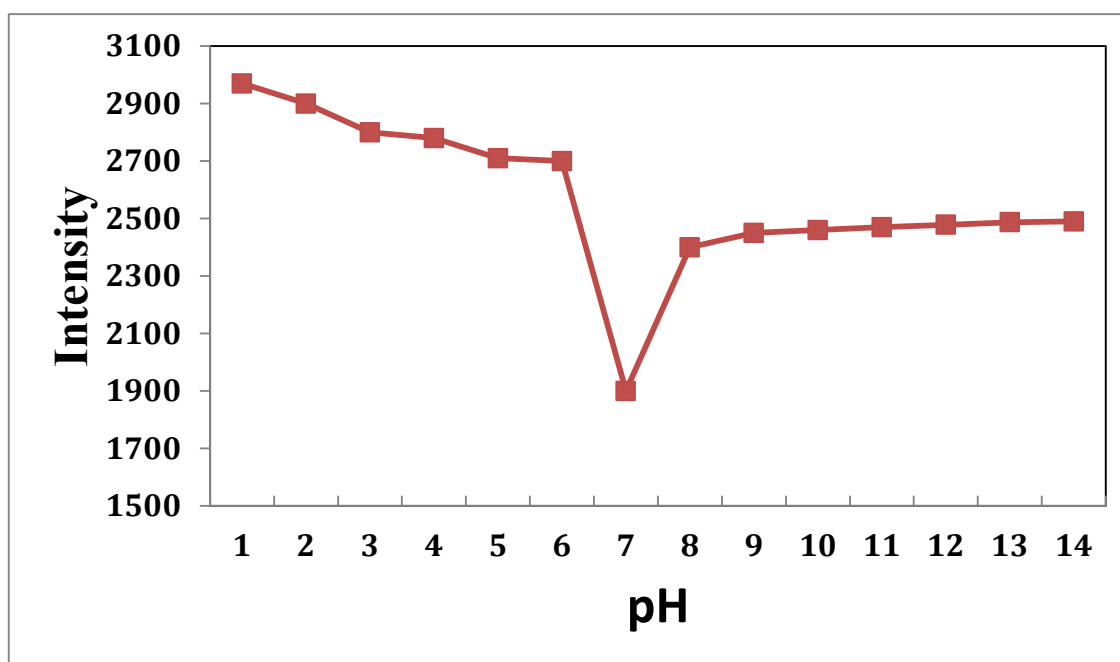


Fig. S15 Shows the effect of fluorescence intensities of TDPC with L-His complex by varying pH.

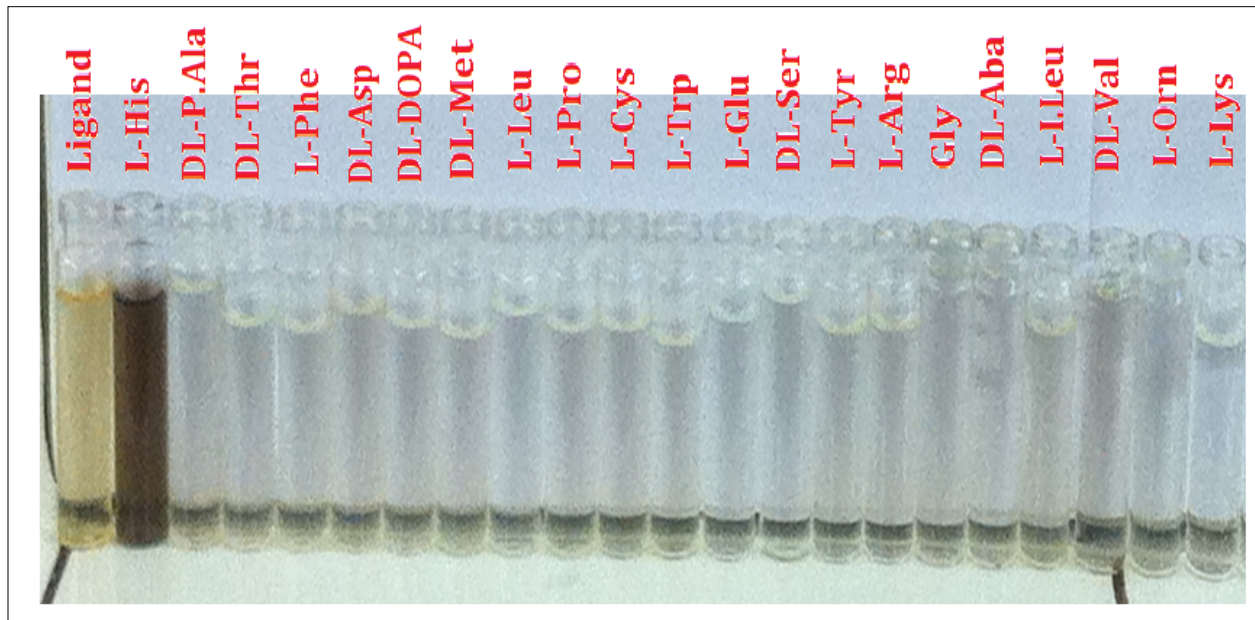


Fig. S16 Visual colour changes obtained upon addition of TDPC with various amino acids

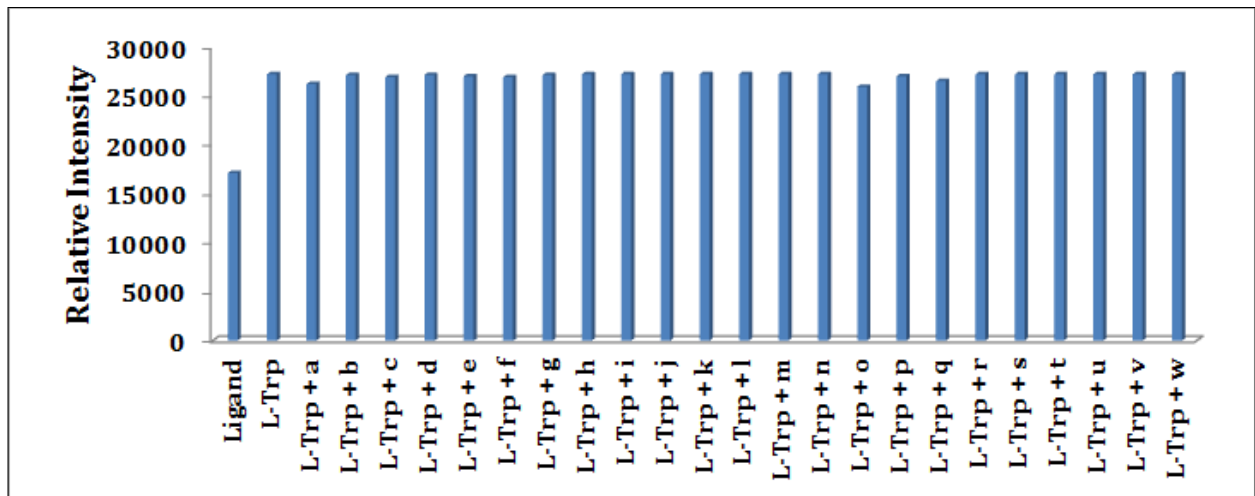


Fig. S17 Competitive emission spectra of TDPC ligand with L-Trp complex with other amino acids.

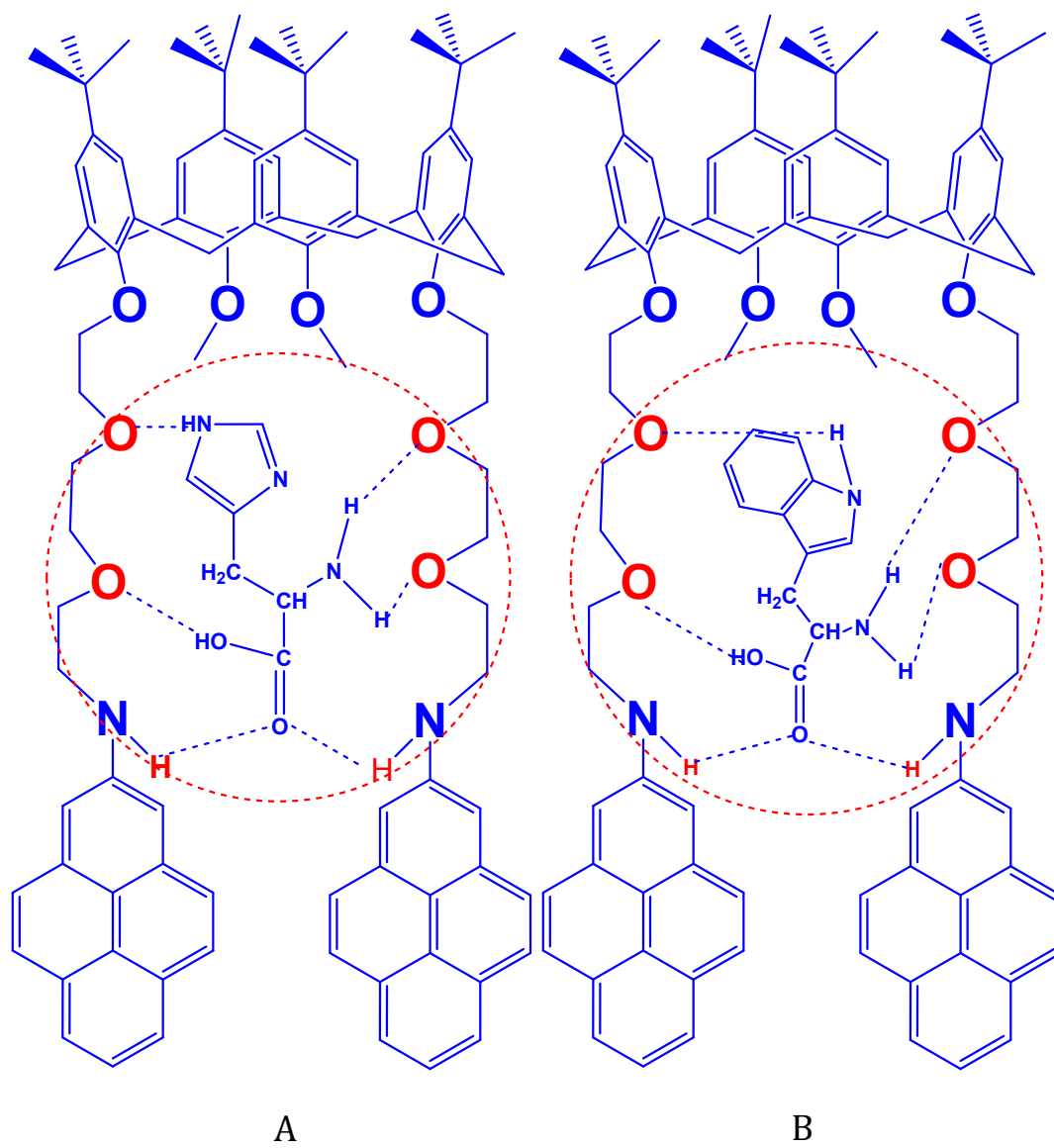


Fig. S18 Proposed binding mechanism through hydrogen bonding with TDPC ligand and (A) L-His (B) L-Trp .

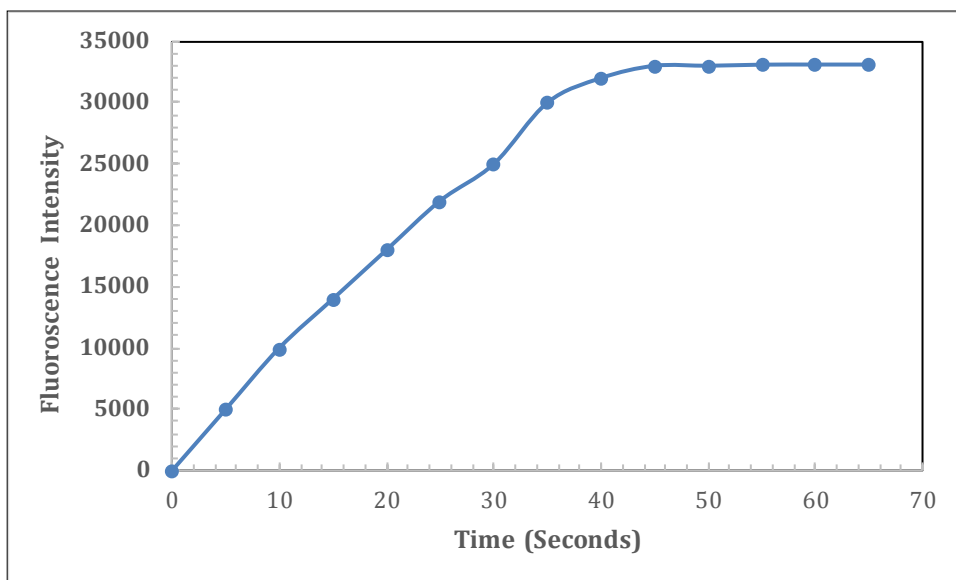


Fig. S19 The time-dependent fluorescence enhancement of TDPC Ligand (1×10^{-6} M) with L-Trp. (1×10^{-6} M).

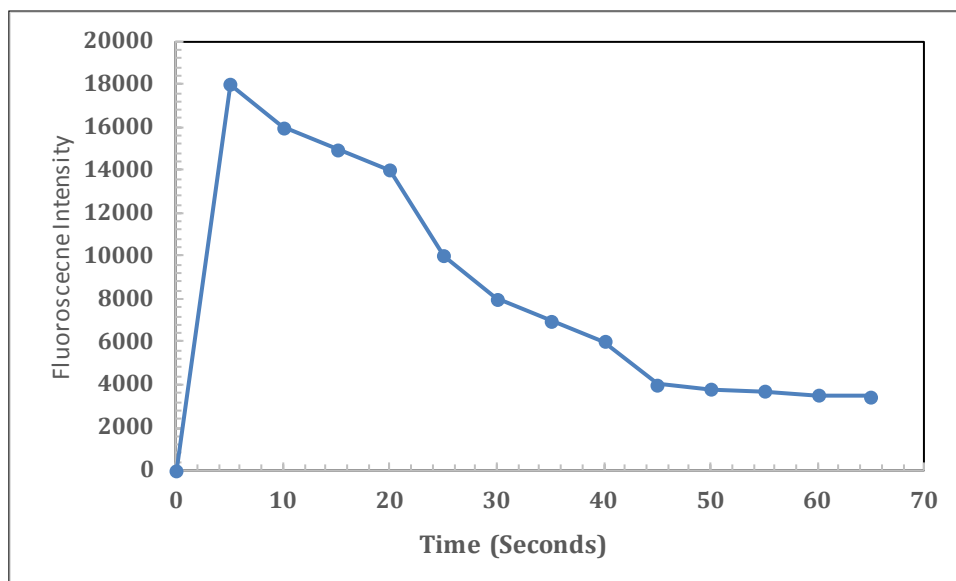


Fig. S20 The time-dependent fluorescence quenching of TDPC Ligand (1×10^{-6} M) with L-His. (1×10^{-6} M).

Sample	Added tryptophan (nM)	Found tryptophan (nM)	Recovery (%) \pm S.D (n=5)
Blood serum 1	0	8.5	-----
Blood serum 2	10	19.2	103.78 \pm 1.6
Blood serum 3	20	29.7	104.21 \pm 1.8
Blood serum 4	30	40.1	104.44 \pm 2.3
Blood serum 5	100	109.7	101.19 \pm 2.4

Table S1: Results of the determination of L-Trp in blood serum.

Sample	Added histidine (nM)	Found histidine (nM)	Recovery (%) \pm S.D (n=5)
Blood serum 1	0	10.5	-----
Blood serum 2	20	31.4	102.95 \pm 1.8
Blood serum 3	30	42.6	105.18 \pm 1.5
Blood serum 4	50	61.7	101.98 \pm 1.4
Blood serum 5	100	111.9	101.26 \pm 1.9

Table S2: Results of the determination of L-His in blood serum.

Reference

1. A. M. Krstulovic, P. R. Brown, D. M. Rosie, P. B. Champlin *Clinical chemistry*, **23**, 1977, 11.