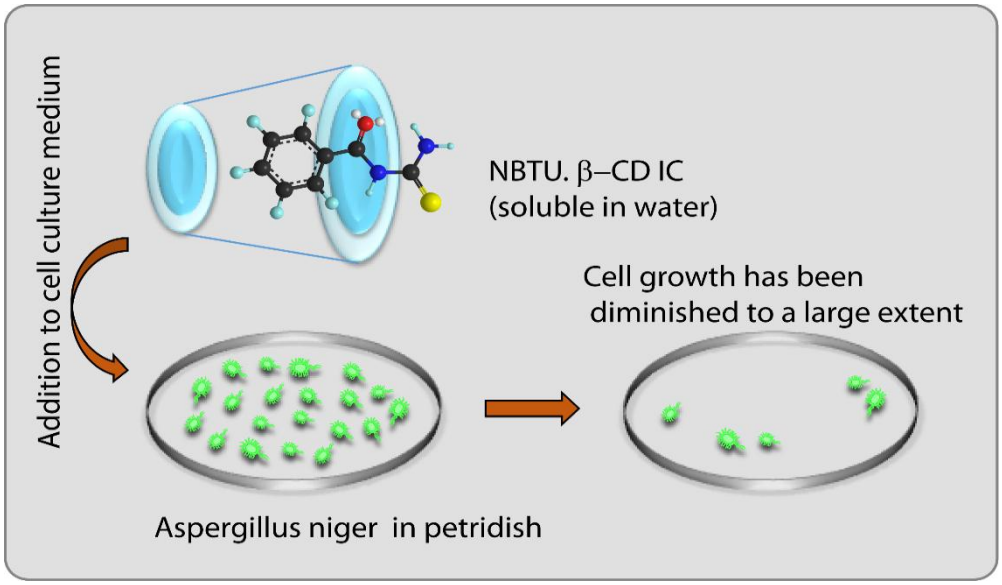
CHAPTER VIII

Exploring Host Guest Inclusion Complex of N-Benzoylthiourea inside β-Cyclodetrin Molecule for Enhanced Antimicrobial Application by Physicochemical Methodologies



ABSTRACT

The inclusion complexes of N-Benzoylthiourea with β-Cyclodextrin (β-CD) was prepared, the resultant complex was characterised by FTIR, UV-Visible Spectroscopy, proton nuclear magnetic resonance spectroscopy (1H NMR), fluorescence spectroscopy, 2D ROESY, High Resolution Mass Spectrometry and Scanning Electron Microscope (SEM) study . The stoichiometry was established using a Job plot method of continuous variation and it was found to be 1:1. The inclusion procedure was clarified by applying 2D ROESY study. The stability of the inclusion complex was confirmed by the means of the values of ΔH^0, ΔG^0, ΔS^0 and association constants derived from UV-Visible spectroscopy and Fluorescence Spectroscopy. The 1:1 stoichiometry was visually demonestrated by HRMS study. Our results showed that less polar part of the guest molecule that is aromatic ring was deeply inserted into the cavity of β-CD. By complexation with β-CD, both the water and thermal of NBTU were prominently improved and NBTU.β-CD complex showed antifungal activity against *Aspergillus niger* (fungal pathogen).

Keywords

N-Benzoylthiourea (NBTU), β-Cyclodextrin (β-CD), Inclusion complex(IC), HRMS study, Job Plot.

Chapter VIII

1. INTRODUCTION

Cyclodextrins (CDs) are torus-shaped cyclic oligosaccharide molecules that are made up of α-D-glucopyranose residues linked by glycosidic linkages and considered as a propitious synthetic molecular host. It has a doughnut shape, in which the cavity has a hydrophobic character compared with water, whereas the rims, bearing the OH groups, are hydrophilic. This structure makes CDs capable of forming inclusion compounds with a variety of guest molecules both insolution and in the solid state [297].The primary condition for a molecule to form an inclusion complex with CD is to fit in the cavity, either completely or partially. In addition, a suitable. energetic balance is always required, which depends on the nature of the guest, the inner cavity diameter of the CD and its substitution degree. The formation of these inclusion compounds has been widely used to improve the aqueous solubility of poorly soluble drugs, together with their bioavailability, dissolution rate, permeability and stability. Natural CDs can be modified by substitution of the hydroxyl groups by methyl or hydroxypropyl groups in order to improve properties such as the solubility or to avoid undesired effects. 2-Hydroxypropyl-b-cyclodextrin (HPβ-CD) is a hydroxyl alkyl β-CD derivative that has been studied mostthoroughly. It is highly hydrophilic and generally forms. The benzoyl thioureas derivatives are quite well known in the field and has been extensively studied. Benzoyl thiourea derivatives can be considered as useful chelating agents because of having suitable. C=O and C=S function groups and hence are capable of encapsulating into the coordinating moiety metal ions [298]. Therefore, new thiourea derivatives and their structures have gained intterest over years of several researchers because of their complexation capacity with various host molecules [299, 300] [301] [302].

Few of the benzoylthiourea derivatives are found to be biologically potent, such as pharmacological [303], antifungal [304] [305], antitumour [306], antibacterial [307-309] and herbicidal properties.

Fungi are progressively important reasons of acute embedded human infections, particularly recurrent mucosal, and nail infections [310]. Very few no of drugs are available for their treatment but most of them fungi static and come up with permanent resistance, which boosts the search for alternatives and drive us to develop some cost effective and less toxic water soluble drugs. Fungicides are acquiring great attention due to their cost effectiveness and eco-friendly nature. If a compound has antifungal potency, it will be a tremendously helpful because of the development of drug resistance in pathogenic fungi. The present work aims to assess the anti-aspergillus activity of NBTU.β-CD inclusion complex [311] [312] [313, 314] [314].We

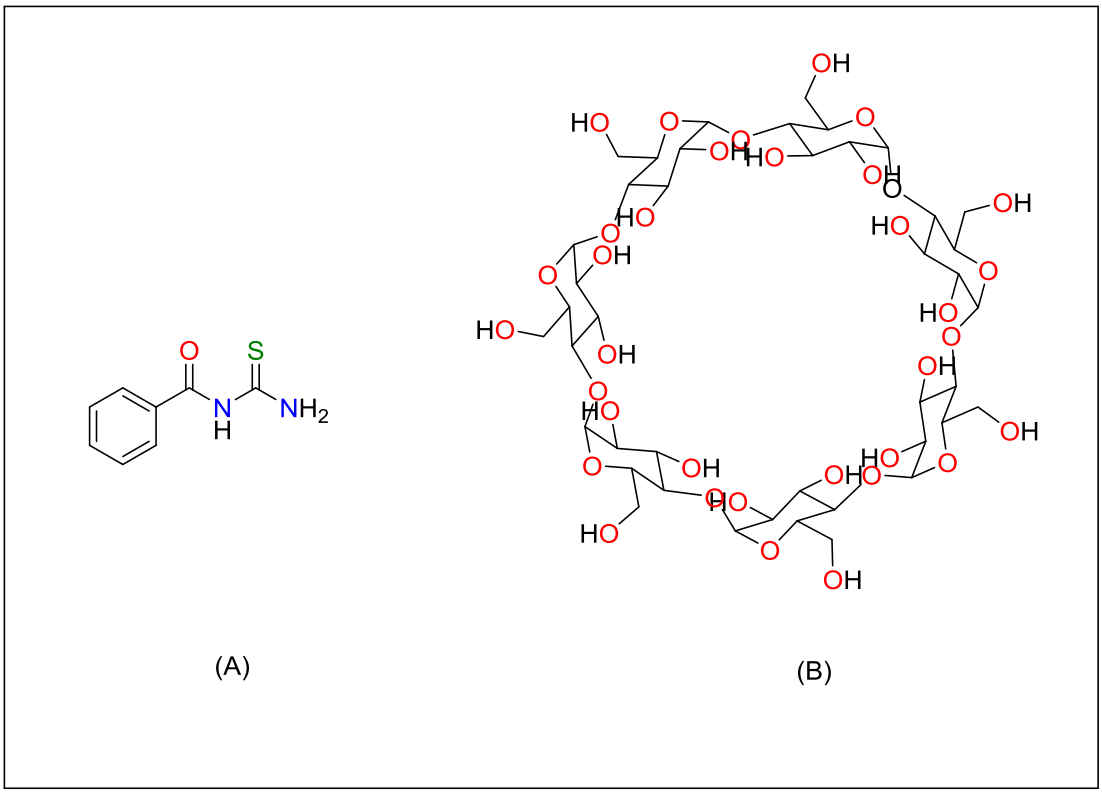
synthesised the inclusion complex of NBTU (guest molecule) with β-CD (host molecule) and characterised it with the help of FTIR, UV-Visible spectroscopy, 1H NMR, 2D-ROESY, Fluorescence spectroscopy, HRMS study and SEM study. The aim of this work was to investigate the impact of NBTU.β-CD IC on antimicrobial activity of some selected gram positive and gram negative bacteria and pathogens. Hence we *B. subtilis* (gram positive), *E. coli* (gram negative), *Aspergillus niger* (fungal pathogen) as our model organisms and checked the antimicrobial activity of the IC for further applications in medicinal field.

2. EXPERIMENTAL SECTION

2.1. Source and purity of samples

N-benzoyl thiourea was purchased from TCI Chemicals, India and β-Cyclodextrin (β-CD) was purchased from Sigma-Aldrich and used without further purification. The details of the chemicals used in this work are listed in **Table.S1**. Distilled water (specific conductivity < 1μS·cm-1) was used for the preparation of all solutions.

The Structures of the chemicals used is given in **Scheme1**.



Scheme 1. Structures of compounds used: (A) N-Benzoylthiourea, (B) β-Cyclodextrin.

2.2 Apparatus and Procedure

Samples were weighed using Mettler Toledo AG-285 (uncertainty ±0.0003 g). Solutions of different strengths were prepared by mass dilution at 298.15 K.

FT-IR spectroscopic study was performed by Perkin Elmer FT-IR Spectrometer 8300 applying KBr Desk technique with scanning range 400-4000 cm^{-1}.

UV-Visible spectroscopy was performed using Agilent 8453 spectrophotometer.

2D ROESY and 1H NMR study, spectra of the pure solutions and the ICs were recorded at 400 MHz BRUKER ADVANCE at 298.15K in d6-DMSO. Signals were denoted as δ values in ppm using TMS as internal standard (d6-DMSO: δ 2.50 ppm). The differences in chemical shifts were recorded to determine the interaction between NBTU and β-CD molecules.

The Mass Spectroscopic analysis was performed in Agilent Accurate-Mass Q-TOFLC/MS6520.

Scanning Electron Microscope (SEM) study was performed in JEOL JSM-IT100 instrument.

Fluorescence spectra were recorded using QuantaMaster 40 spectrofluorometer.

2.3. Synthesis of the inclusion compound

N-Benzoylthiourea (0.2 mmol) was dissolved in 10 mL hot methanol and stirred for 2 hours. β-CD.12H_2O (0.4 mmol) was dissolved in water (20 mL) at 50°C and stirred for 30 minutes. The aqueous solution of β-CD was added dropwise to the hot methanolic solution of NBTU and stirred for 48 hours. The inclusion complex of N-Benzoylthiourea and β-CD was prepared by coevaporation method [269]. Then it was filtered and cooled to room temperature, put the mixture into the refrigerator at 277 K overnight. The white precipitate was filtered and washed with hot methanol. The solid was dried in a vacuum drying oven to obtain a white NBTU.β-CD inclusion complex.

2.4. Antimicrobial activity assay

In this experiment, *B. subtilis* (gram positive), *E. coli* (gram negative), *Aspergillus niger* (fungal pathogen) were considered as model organisms. Tests were done according to the Agar cup method [315]. In short, organisms were inoculated by spread plate technique in Muller-Hinton agar and the compounds (host-guest complexes) were applied in agar cup at 1 mg/ml concentration in separate plates and incubated at 37 °C for 24 hrs. Double distilled water was

used as the control. Antimicrobial activity was determined by means of the clear zone (zone of inhibition) surroundings agar cup. Each of the experiments was done in triplicate.

3. RESULT AND DISCUSSION

3.1. FTIR Analysis:

FTIR spectroscopy is used to approve the formation of inclusion complex by the means of the deviation in shift and intensity pattern of the absorption peak between inclusion complex and pure compounds [316]. The formation of inclusion complex is reflected in broadening, widening, disappearance or change in intensity patterns of the peaks recorded [317] [316]. The FTIR spectra of pure compounds (NBTU and β-CD) and the inclusion complex (NBTU.β-CD) were recorded and the spectra assigned are shown in **Table. S2. and Fig.1**.

The infrared spectra of the guest molecule (NBTU) showed some few characteristic peaks at 3308 cm-1 for secondary thioamide (N-H) group and 3232 cm^{-1} for primary amine group. A broad peak at 3154 cm-1 was associated with the stretching vibration of the aromatic C-H bond. The strong C=O stretching vibrational peak was observed at 1681 cm^{-1}, which is in agreement with the literature data [318] The aromatic benzene appeared at 1604 cm^{-1}. Usually, the bending vibration of N-H in NH2 group observed at 1533 cm^{-1}. The C-N asymmetric stretching mode was observed at 1419 cm-1, whereas symmetric stretching vibration of C-N occurred at 1030 cm^{-1}. The C=S group showed its asymmetric stretching vibration at 1237 cm^{-1}. However, the symmetric stretching mode of C=S appeared at 710 cm^{-1} [319].

In the β-CD spectrum, a strong absorption peak at 3392 cm^{-1} attributed to the stretching of the bonds of (O-H) groups. The stretching vibrations of the C-H bonds in the -CH and -CH_2 groups appeared at 2926 cm^{-1}. Deformation vibrations of the O-H bonds were observed at 1639 cm^{-1} in the C-O-H groups and in the water molecules. Whereas, deformation vibrations appeared at 1410 cm-1 and 1366 cm-1 due to the C-H bond in the -CH_2OH and -CHOH groups. The absorption peaks at 1156 cm^{-1} were due to (C–O), (C–C) and (C–O–C) [320].

After the formation of inclusion complex with NBTU, the absorption peaks at 3154 cm^{-1} and 1604 cm^{-1} almost disappeared in the NBTU.β-CD inclusion complex, indicating that the benzene group was included into the host cavities. A distinct shift in the peak from 1681 cm^{-1} due to C=O group in pure NBTU to 1627 cm^{-1} in inclusion complex was observed, which might arise due to the change in environment of C=O group on entering hydrophilic environment to hydrophobic cavity of β-CD. The complex got stabilised at the wider rim of host moiety. However, the

characteristic absorption peak for asymmetric stretching of C=S bond at 1237 cm^{-1} (found in pure NBTU) remained almost unaltered in the inclusion complex (appeared at around 1233 cm^{-1}). All these phenomena indicated that not whole guest molecule rather part of the guest molecule i.e. only benzoyl (Ph-CO) part was encapsulated inside the host molecules. The most possible structure depicted from FTIR spectroscopy is given in **Fig.1.**

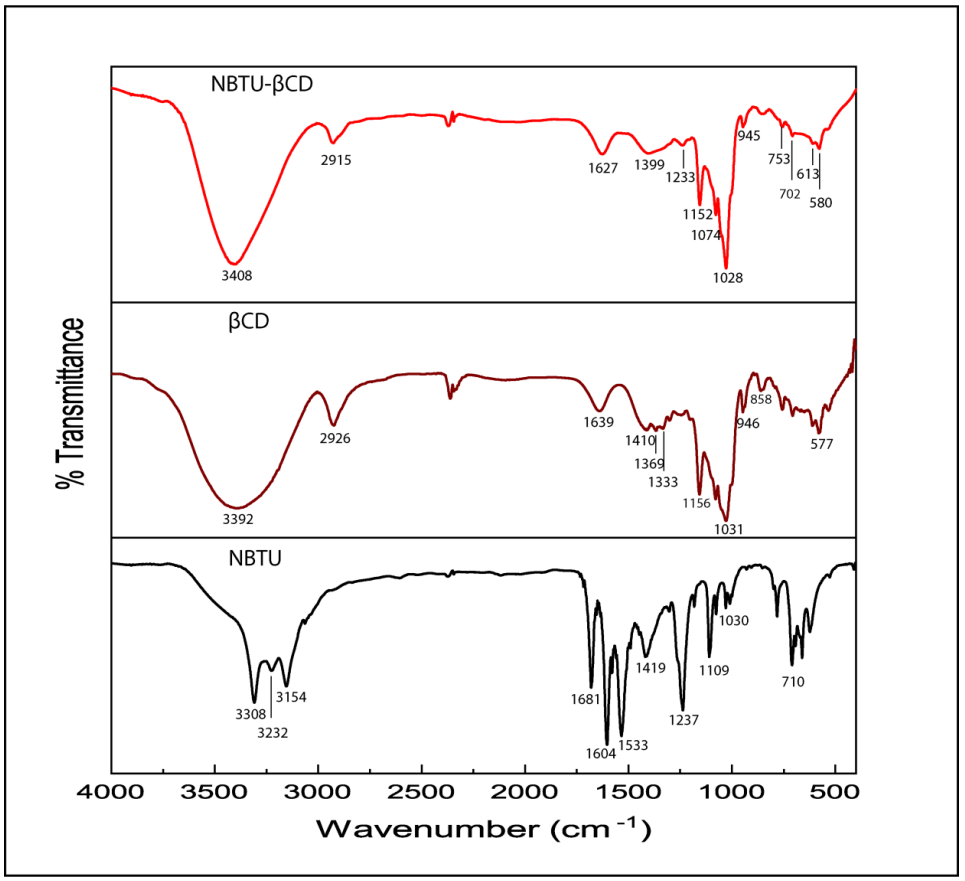


Fig.1. Stretching frequency of pure NBTU, pure β-CD and NBTU.β-CD inclusion complex formed.

3.2. UV-Vis Spectroscopy

3.2.1 Stoichiometric studies by Job's plot:

A continuous variation method (Job's plot) was performed with the help of Agilent-8453 UV-Vis spectrophotometer in order to confirm the stoichiometry of the inclusion complex [288] [190, 321]. The total concentration of guest and host in every cases were kept constant i.e, [NBTU]+[β-CD] = 100 μM and the mole fraction (R) i.e, {[NBTU]/([NBTU]+[β-CD])} for all sets were taken in the range between 0 to 1. After preparing the sample solutions, UV absorbance for each case were found at 241 nm and The plot of ΔA × R versus R gives final Job's plot diagram, where ΔA stands for difference in absorbance of pure NBTU and in presence of cyclodextrin molecules.

During the experiment, the temperature was kept constant at 298.15K and for the entire sample, experiment was performed in triplicate. The stoichiometry of an inclusion complex is generally determined by taking the maximum point of R value obtained from the graph i.e, if R is 0.33, it indicates the ratio between guest to host is 1:2 and 1:1 if R~0.50 whereas, 2:1 when R~0.66. In **Fig. 2**, it was observed that the highest R value was obtained at 0.50 which indicates the formation of 1:1 inclusion complex [322] **(Table.S3.)**

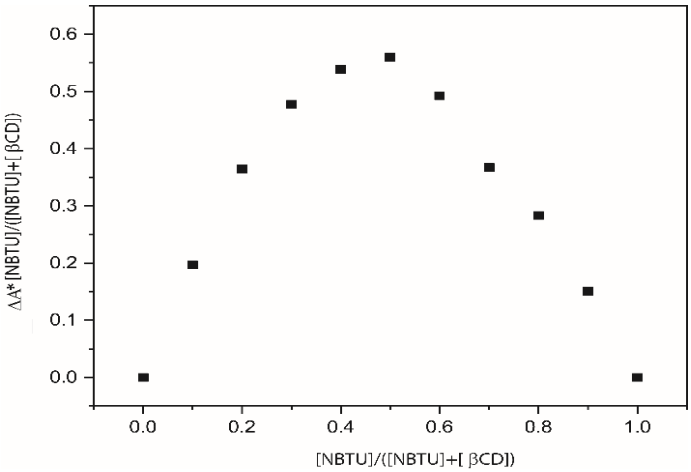


Fig.2. Job's Plot for (NBTU+β-CD) system

3.2.2. Association Constant and Thermodynamic Parameters

UV-Visible spectroscopy is quite useful for determining the stoichiometry of the inclusion complex formed. The association constant can also be determined by monitoring continuous change in the optical density (OD) along with or without a spectral shift depending upon the addition of host molecule to the guest solution [281] [282]. The stoichiometry obtained from the Job's method was further verified by Modified Benesi-Hildebrand method and association constant value was determined accordingly. Three different temperatures ranging from 293.15K to 313.15 K were chosen to record the UV-Vis spectra. At first, a typical double reciprocal plot of $1/(A\text{-}A_o)$ versus $1/(CD)$ was obtained for NBTU v/s β-CD with R^2 regression correlation factor 0.9908 .It indicated 1:1 stoichiometry was followed for the given inclusion complex (**Fig.S2**.).

Association constant is one of the key factors to determine the non-covalent binding behaviour of host-guest interactions. The binding constant of N-Benzoylthiourea (guest molecule) with Cyclodextrins (host molecule) has been calculated using the modified form of Benesi-Hildebrand equation [274].

$$\frac{1}{[A\text{-}A_0]} = 1Ka[NBTU]0\Delta\varepsilon.\frac{1}{[\beta\text{-}CD]_0} + \frac{1}{\Delta\varepsilon} \quad (1)$$

Where, $(A-A_0)$ = difference in absorbance of NBTU with and without adding β-CD.

Δε = Difference in molar extinction coefficient of NBTU.

K_a = Association constant

$[NBTU]_0$ and $[\beta\text{-CD}]_0$ = initial concentration of NBTU and β-CD respectively.

Successive addition of CD up to 50 μM was done and gradual increase in the spectral absorbance was observed with increasing concentration of cyclodextrin molecules (**Fig.3.**). These spectra **(Table. S4)** show two distinct peaks at two different positions. The peak at 241 nm appeared due to the π-π*transition of the aromatic system, whereas the transition at 279 nm took place due to n– π* transition of the C=O and C=S groups. From the intercept and slop of the double reciprocal plot, Ka value can be determined (Fig. The association constant (K_a) for 1:1 NBTU.β-CD was found to be 9.1303M^{-1}, 20514.05 M^{-1}, 22615.82 M^{-1} at 293.15K, 303.15K and 313.15K respectively (**Table.S4.**)(**Fig. S2, S3 and S4**). Basically, association constant is a measure of the extent of complexation o guest and host and is temperature dependent. In our case, k_a of NBTU.βCD has been found to be increasing with rise the in temperature. It simply indicates that the formation of inclusion complex is favoured at relatively higher temperature.

A good linear correlation can be found from the 1/ΔA versus 1/CDs plot (**Table.S4. & Fig.S2. , Fig.S3. Fig.S4.**) [126] [187] [286].

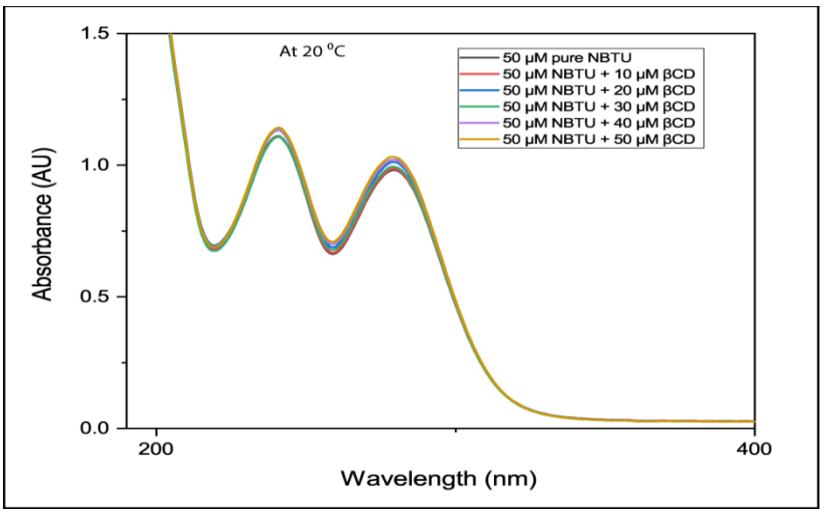


Fig.3. UV-vis spectra of 50 μM of NBTU and successive addition of βCD upto 50 μM at 20 ^{0}C.

3.2.3 Thermodynamic parameters evaluation:

Basic thermodynamic parameters like complexing enthalpy and entropy values can be calculated by using van Hoff equation;

$$lnK_a = \frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} \quad (2)$$

Where, ΔH^0 and ΔS^0 stands for change in enthalpy and entropy respectively. T is the absolute temperature and R is the Universal Gas Constant.

The calculated thermodynamic parameters like ΔG^0, ΔH^0, ΔS^0 have been shown in the **Table.1.** At all the three temperatures, ΔG^0 was found to be negative (-22.33kJ/mol, -24.70kJ/mol, and -25.88kJ/mol at 20^0C, 30^0C and 40^0C respectively) (**Table.1.**), which dictates spontaneity of inclusion phenomena between NBTU and β-CD. ΔH^0 value for NBTU.β-CD inclusion complex was found to be largely positive, which gave an indication that large amount of energy was required during the formation of non-covalent bond. However, positive entropy change (ΔS^0) suggested that during the process randomness in the system was increasing possibly due to the hydrophobic interactions which result in the breaking and removal of the water molecules present inside the cyclodextrin cavity [323].

Table.1. Association constants (Ka) obtained by the Benesi–Hildebrand method at 303.15K[a] at 313.15K[a], 323.15K[a], and corresponding Gibb's free energy (ΔG^0), enthalpy (ΔH^0) and entropy(ΔS^0) of (NBTU+β-CD) system

Temp(K)	K_a/M^{-1}	ΔH^0/KJ mol^{-1}	ΔS^0/KJ mol^{-1} K^{-1}	ΔG^0 KJ mol^{-1}
293.15	9230.89			-22.33
303.15	20514.05	46.999	0.23665	-24.70
313.15	22615.82			-25.88

3.3 Fluorescence Spectroscopy

The UV-Vis measurements showed that the association constant (K_a) for 1:1 (NBTU+β-CD) complex ranges between 9.2×10^3 M^{-1} to 22.6×10^3 M^{-1} (**Table.S4.**). Steady state fluorescence titration measurement was performed to verify the data obtained. During the experiments, initially 20 μM solution of NBTU was taken in cuvette and subsequently β-CD was added upto 100 μM. Modified Benesi-Hildebrand equation was applied to calculate Association constant (K_a) given below [162],

$$\frac{1}{F\text{-}F_o} = \frac{1}{(F_{max}\text{-}F_o)\times K_a \times [CD]^n} + \frac{1}{F_{max}\text{-}F_o}$$

Where, F and F_o represents the fluorescence intensity of NBTU on adding β-CD and pure NBTU (without the addition of CD molecule) respectively. F_{max} is the saturation fluorescence intensity. K_a is the association constant obtained by dividing intercept by slope. n is the binding stoichiometry between β-CD and NBTU.

Fig.4. showed the fluorescence spectral variation of NBTU with increasing concentration of β-CD. Pure NBTU showed its fluorescence emission maxima at 316 nm but with increasing concentration of β-CD, the fluorescence spectra got quenched [324]. But no change in the position of emission maxima was observed on further addition of CD molecules. The decrease in fluorescence intensity with increasing β-CD concentration indicates the formation of inclusion complex..

From the Benesi Hildebrand equation, K_a has been calculated for the NBTU.β-CD complex. When $1/(F\text{-}F_0)$ was plotted against 1/[CD] shown in **Fig.S5.**, a good linear relationship was found with regression coefficient (R^2) at 0.9825. The linearity of the plot clearly depicts that the stoichiometry of the inclusion complex must be 1:1. From the plot, the formation constant was found to be 19.9×10^3 M^{-1} at 313.15 K (**Table.S5.**). The obtained data is in good corelation with those obtained from UV-Visible measurements and Fluorescence confirmed the process of inclusion phenomena.

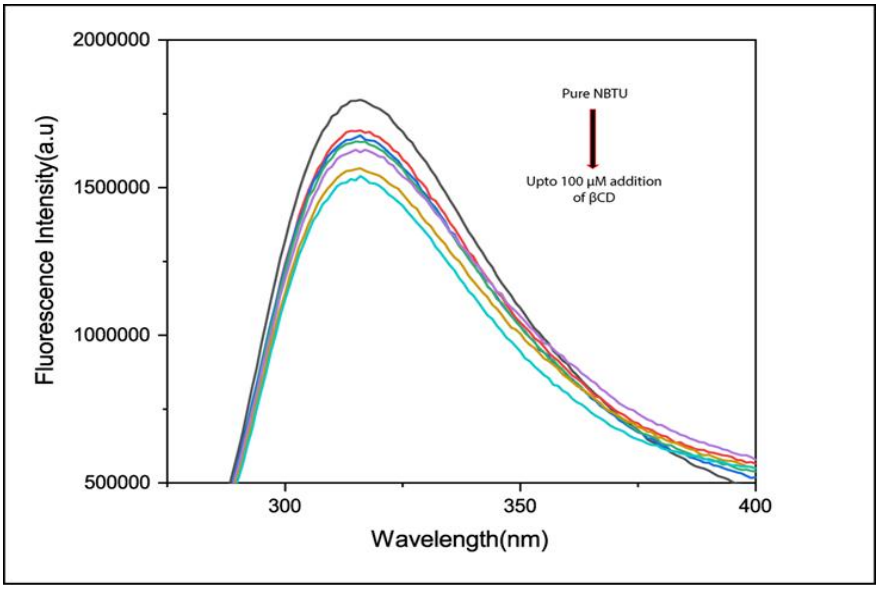
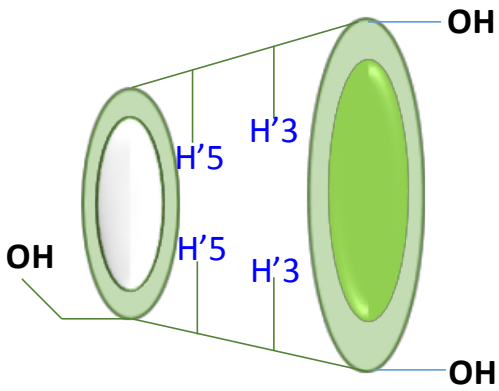


Fig.4. Fluorescence spectral variation of 20 µM NBTU with increasing concentration of β-CD upto 100µM.

3.4 1H NMR Spectroscopy

The diffrence in chemical shift values between the pure compound and newly formed inclusion complex in 1H NMR gives an indication about the fact that inclusion phenomena between NBTU & CD molecule has taken place.

The chemical shift values of the aromatic protons inside the cavity of β-CD molecule,after the inclusion procedure are controlled by the presence of aromatic ring in NBTU compound [325]. From **Scheme 2**, , it is already known that H3 and H5 protons are located inside the hollow cavity, whereas H3 is situated closer to the wider rim rather than the H5 and H1, H2, H4 protons are spotted at the exterior part of the β-CD molecule [294]. The free rotation of the primary hydroxyl on the narrower rim directs to the steric effect and hence the most plausible binding mode is the insertion of the less polar part of the guest molecule inside the β-CD cavity through the wider rim. This phenomena of insertion reflects in upfield shifts for the signals for H3 than H5 [326][327][325, 328][329].When the complex was formed, the H3 proton located above the ring current of the phenyl ring made it to be shielded and upfield shift, same logic stands for the H5 proton but due to its location, the chemical shift experienced by it is smaller than the H3 one. [33] (**Fig.,S6.**, **Fig.S7.**, **Fig.S8.**). The change in chemical shifts definitely carries the evidence of incorporating NBTU molecule inside cavity of β-CD molecule.



Scheme2. Location of H3 and H5 protons of Cyclodextrin molecule.

3.6. 2D-ROESY

2D ROSEY NMR was preformed to have a better understanding of the conformation of NBTU.β-CD inclusion complex (**Fig.5**). In this experiment, through space interactions between protons takes place, when they are present in the vicinity of 3-5 Å [330] [331] either in the same

molecule or different molecules. In NBTU.β-CD inclusion complex, the correlation of protons only occurs between the aromatic protons of NBTU and β-CD. The NOE cross-peaks between aromatic protons of guest moleule with H'3 and H'5 of CD are clearly shown in Fig. 5.

2D ROESY spectrum (**Fig.3.**) of the inclusion complex showed intermolecular cross-peaks between aromatic protons of NBTU (H1, H2, H3, H4, H5) and β-CD cavity protons (H'3 & H'5). These appearances of crosspeaks confirm the position of the aromatic ring inside the cavity of β-CD. Although it is not clearly depictable whether the guest molecule enters into the cavity through the narrower rim or the wider rim because of poor resolution, still the shape and size of β-CD makes NBTU to approach from the wider side. In such cases, the cross-peak intensity of H'3 proton of β-CD should be higher than that of the H'5 one. In this work, the same trend is followed, which is a definite indication of the fact that H'3 of NBTU is located near the wider rim (Fig. 10).

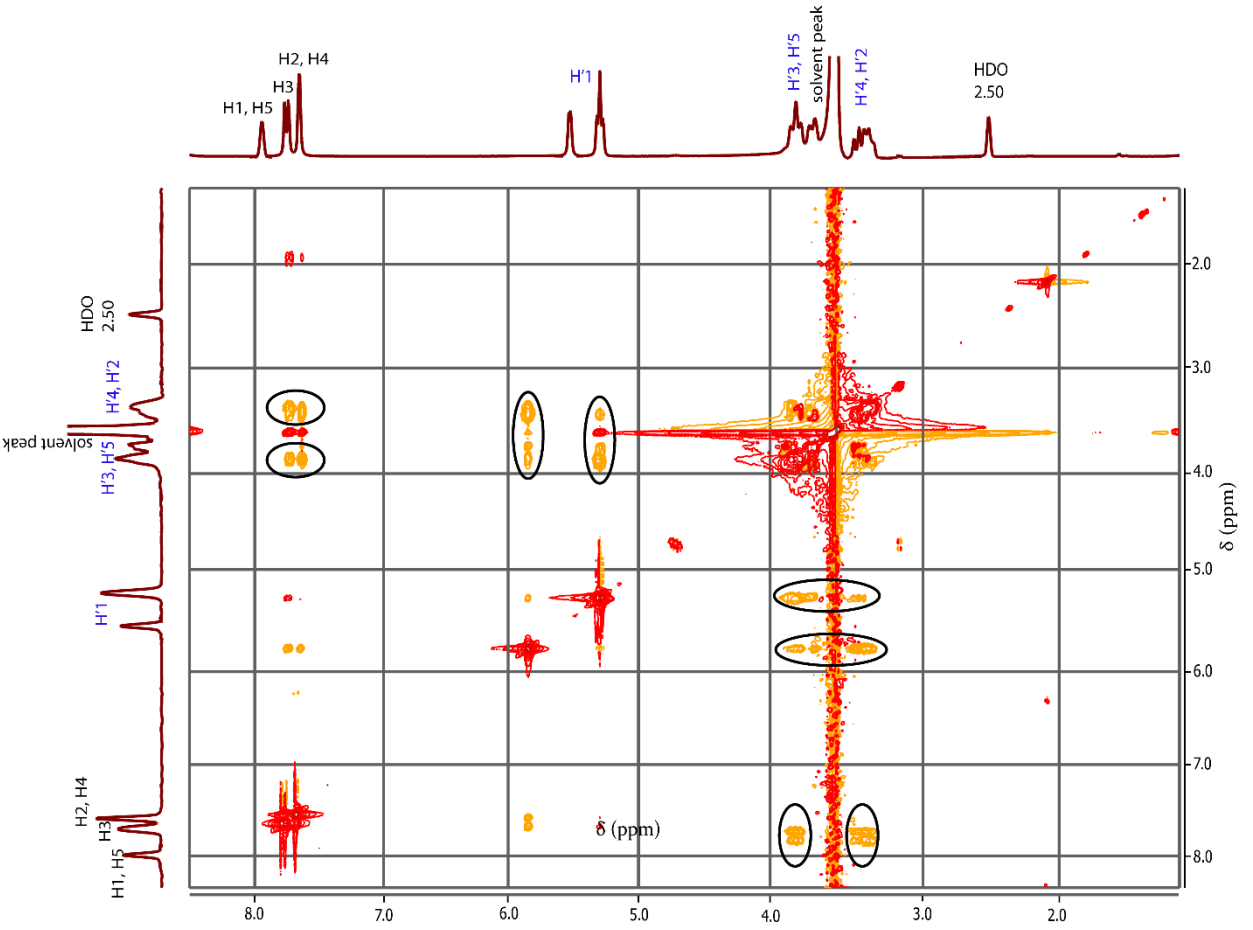


Fig.5. 2D-ROESY spectra of NBTU.β-CD inclusion complex in d6-DMSO solvent.

3.5. HRMS Study

Electrospray ionization (ESI) mass spectrometry (MS) experiment was performed on an Agilent 6530 Accurate-Mass Q-TOF LC/MS system (Agilent Technologies).The sample was prepared in methanol and the detection was performed in the positive ion mode. The peaks were given in m/z (% of basis peak).

The peak at 1315.4115 in $[M+H]^+$ mode confirms the formation of NBTU.β-CD ICs respectively and for $[M+Na]^+$ mode we got m/z values at 1337.3934. The m/z values have been given in **Table.2.** and the spectrum has been given in Fig.S9, which is almost equal to the sum of host and guest molecule. It indicates the formation of the IC of 1:1 stoichiometric ratio [187].

Table.2. The calculated values and obtained m/z values of the solid inclusion complex

Ions	Exact Mass (calculated) $[M+H]^+$	m/z values Obtained	Exact Mass (calculated) $[M+Na]^+$	m/z values Obtained
[β-CD+ NBTU]	1315.4133	1315.4115	1337.3952	1337.3934

3.6. SEM STUDY

Surface morphology of NBTU, β-Cyclodextrin and NBTU.β-CD were examined by Scanning Electron Microscope (JEOL JSM-IT100). Samples were diffused onto double-adhesive carbon coated tape on aluminum stubs. These sample stubs were coated with a thin layer of gold (25 Å). Samples were throughly examined by SEM and photographed under various magnifications.

From SEM analysis, NBTU was seen as irregular shaped crystals that formed aggregates and pure β-CD was observed as three-dimensional parallelogram shaped particles [202] . NBTU.β-CD was found to contain neither irregular shaped crystals nor three-dimensional parallelogram shaped particles, but showed irregular shaped clumps or aggregates of NBTU.β-CD complexes. Although SEM micrographs do not confirm the formation of IC, but it indicated some morphological changes definitely took place.

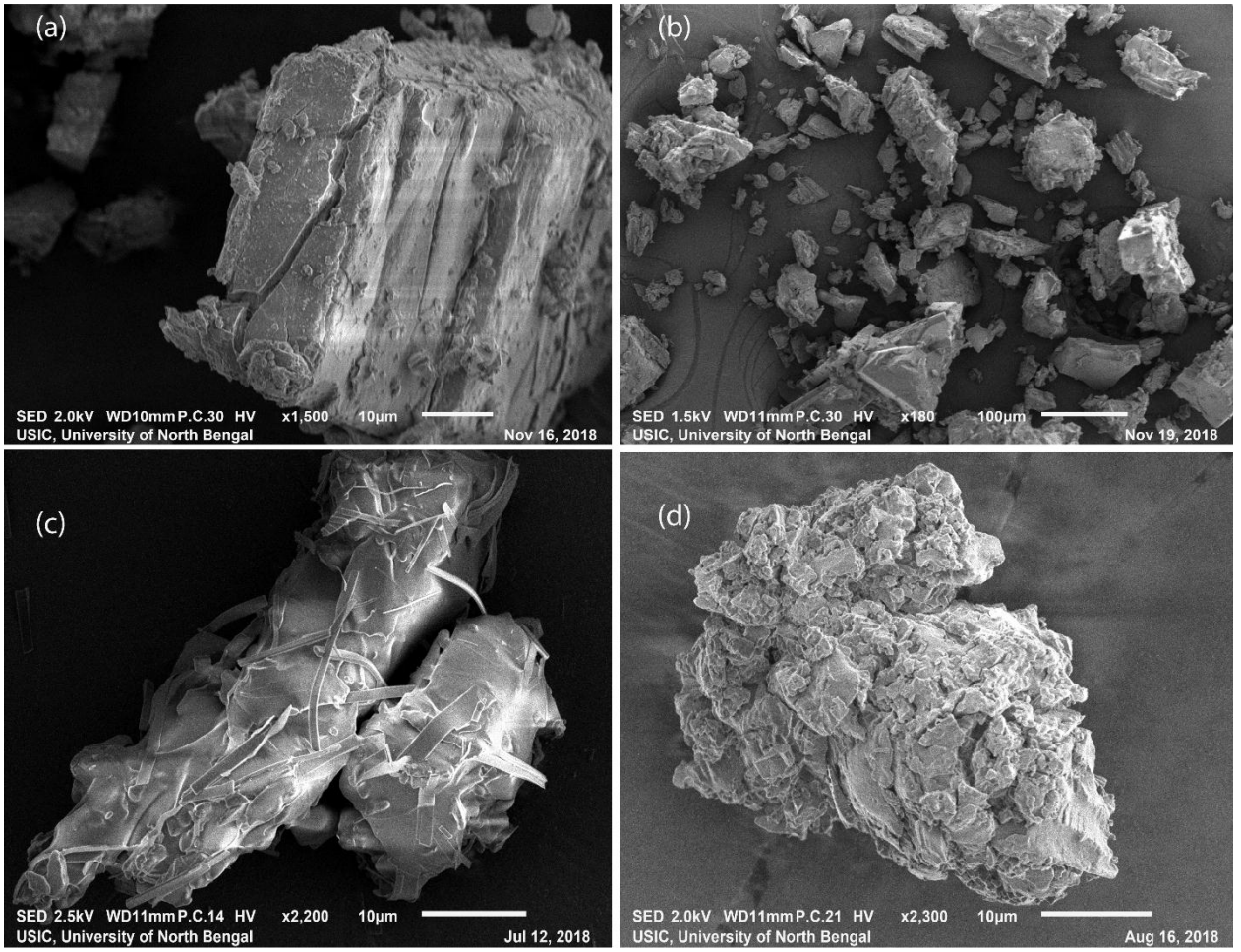


Fig.6 Scanning Electron Micrograph of pure compounds, physical mixture of compounds and inclusion compound formed (a) Pure β-CD, (b) Equimolar (1:1) Physical mixture of NBTU and β-CD, (c) Pure NBTU and (d) NBTU.β-CD inclusion complex.

3.7. Applications

3.7.1. Cytotoxic activity of IC

In this experiment, B. subtilis (gram positive), E. coli (gram negative), Aspergillus niger (fungal pathogen) were considered as model organisms. Tests were done according to the Agar cup method [315]. In short, organisms were inoculated by spread plate technique in Muller-Hinton agar and the compounds (host-guest complexes) were applied in agar cup at 1 mg/ml concentration in separate plates and incubated at 37 °C for 24 hrs. Double distilled water was used as the control. Antimicrobial activity was determined by means of the clear zone (zone of inhibition) surroundings agar cup (**Fig.7.)**. Each of the experiments was done in triplicate.

Fig.7. simultaneously shows that on applying IC at a concentration of 1000µg/mL the (marked by **S2**) and at 600µg/mL(marked by **S1**), there occurs huge difference in the fungal growth in the petridish. When the IC concentration is high (**S2**), fungal growth has been decreased to a considerable extent but when IC was applied at lower concentration, the growth can not be diminished to such huge extent. The MIC value of the IC applied was found to be 1mg/mL.

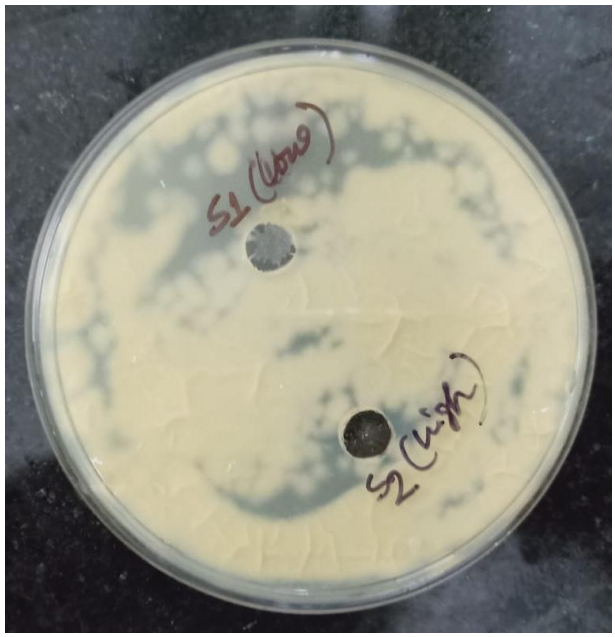


Fig.7. No Zone of inhibition found for *Aspergillus niger* (Fungal pathogen) by Agar Cup method.

4. CONCLUSION

The present work describes the the synthesis of inclusion complex between NBTU and cyclodextrin molecules. The Job's plot method and Steady state fluorescence study both confirms the 1:1 stoichiometry for the inclusion complex formed. From the spectral pattern obtained in UV-visible spectroscopic study and Steady state measurement, it has been observed that NBTU molecule successfully enters in the hollow cavity of the β-CD molecule, which is in good agreement with HRMS study. The morphological analysis also supports that the inclusion has taken place successfully. Although great diagnostic and therapeutic advances in antifungal

Research has been observed so far but aspergillosis still exists as a question mark for morbidity and mortality. We took strategy to fight against this problem by developing a cost effective and environment friendly cyclodextrin based inclusion complex, which will also exhibit less toxicity. We applied the IC in model organisms such as *B. subtilis* (gram positive), *E. coli* (gram negative), *Aspergillus niger* (fungal pathogen) and it has been found that no zone of inhibition is found for

Aspergillus niger, which indicates on addition of the inclusion complex in cell culture medium pathogen growth of *Aspergillus niger* is diminished to a considerable extent.

Supporting Information

Exploring Host Guest Inclusion Complex of N-Benzoylthiourea inside Beta Cyclodetrin Molecule for Antimicrobial Application by Physicochemical Methodologies

Table. S1. Details of the chemicals used

Name of chemicals	Source	CAS no	Purification method	Mass purity
N-Benzoylthiourea	TCI Chemicals(India) Pvt. Limited	614-23-3	Used as purchased	>98.0%
β-Cyclodextrin	Sigma-Aldrich, Germany	7585-39-9	Used as purchased	*w*≥97%
Distilled Water	Sigma-Aldrich, Germany	7732-18-5	Used as purchased	*w*≥99%

Table.S2. Stretching frequency of funcional groups present in pure NBTU**,** pure β-CD and NBTU.β-CD inclusion complex

Pure NBTU	**β-CD**	**NBTU+β-CD**
3308: ν (N-H) secondary	**3392**: ν (O-H) of primary & secondary	**3408**: ν (O-H) of beta-cyclodextrin
3232: ν (N-H) primary	**2926**: ν (C-H) of CH and CH_2 groups	**2915**: ν (C-H) stretching vibrations
3154: ν (Aromatic =C-H)	**1639**: δ(O-H, H bonded) of O-H bonds in the C-O-H groups and in the water molecules	**1627**: ν (C=O)
1681: ν (C=O)	**1410** & **1366**: δ (C-H, vibrations of CH_2OH and CHOH groups)	**1399**: ν(C-N)
1604: ν (C=C) aromatic ring	**1156**: ν(C–O), ν(C–C), δ(C–O–C)	**1233**: ν(C=S)

1533: bending (N-H)	**1031**: vibration of C-O-H of alcohol.	**1152** & **1074**: ν(C–O), ν(C–C), δ(C–O–C)
1419: ν(C-N) asymmetric	**858**: δ(C-C-H), ν(C-O), ν(C-C) from anomeric vibration	**945**: skeletal vibration involving -1,4 linkage
1237: ν(C=S) asymmetric		**860**: δ(C-C-H), ν(C-O), ν(C-C) from anomeric vibration
1030: ν(C-N) symmetric		**702**: ν(C=S) symmetric
710: ν(C=S) symmetric		

Table.S3. Data obtained from Job Plot of (NBTU+β-CD) system in UV-Visible Spectroscopy at 298.15 K^a

NBTU(ml)	β-CD (ml)	NBTU(μM)	β-CD (μM)	[NBTU]/([NBTU]+[βCD])	Absorbance (A)	ΔA	ΔA*[NBTU]/([NBTU]+[βCD])
4	0	100	0	1	2.271625519	0.000000000	0.000000000
3.6	0.4	90	10	0.9	2.104566574	0.167058945	0.150353050
3.2	0.8	80	20	0.8	1.917884979	0.353740540	0.282992432
2.8	1.2	70	30	0.7	1.747245789	0.524379730	0.367065811
2.4	1.6	60	40	0.6	1.450400257	0.821225262	0.492735157
2	2	50	50	0.5	1.152246475	1.119379044	0.559689522
1.6	2.4	40	60	0.4	0.925057888	1.346567631	0.538627052
1.2	2.8	30	70	0.3	0.680891037	1.590734482	0.477220345
0.8	3.2	20	80	0.2	0.450273514	1.821352005	0.364270401
0.4	3.6	10	90	0.1	0.304224968	1.967400551	0.196740055
0	4	0	100	0	0.000000000	2.271625519	0.000000000

Table.S4. Data obtained from Benesi-Hildebrand double reciprocal plot of (NBTU+β-CD) system in UV-Visible Spectroscopy at 298.15 K^a

Temp/k	[NBTU]/μM	[βCD]/μM	$A_\circ$	A	1/[βCD](M^{-1})	1/ΔA	Intercept	Slope	Ka(M^{-1})
	50	10	1.110046	1.117588	100000	132.5925			
	50	20	1.110046	1.123524	50000	74.19744			
293.15	50	30	1.110046	1.127348	33333	57.79660	11.3540	0.001230	9230.89
	50	40	1.110046	1.134323	25000	41.19251			
	50	50	1.110046	1.142199	20000	31.10163			
	50	10	1.085968	1.100063	100000	70.95031			
	50	20	1.085968	1.105392	50000	51.48407			
303.15	50	30	1.085968	1.113244	33333	36.66286	12.6623	0.000617	20514.05
	50	40	1.085968	1.124133	25000	26.20210			
	50	50	1.085968	1.138734	20000	18.95191			
	50	10	1.114446	1.123108	100000	115.4470			
	50	20	1.114446	1.125944	50000	86.97098			
313.15	50	30	1.114446	1.132634	33333	54.98113	22.2992	0.000986	22615.82
	50	40	1.114446	1.137354	25000	43.65254			
	50	50	1.114446	1.142443	20000	35.71785			

Table.S5. Association constant data for NBTU & β-CD obtained using Fluorescence method.

[NBTU] /μM	[β-CD] /μM	F_o	F	$\Delta F = F_o - F$	1/[β-CD] /M^{-1}	1/ΔF	Intercept	Slope	K_a /M^{-1}
20	20	1797377	1693359	104017	50000	9.61379E-06			
20	40	1797377	1650384	146992	25000	6.80306E-06			
20	60	1797377	1605927	191450	16666	5.2233E-06	2.79E-06	1.40E-10	19928.57
20	80	1797377	1577763	219614	12500	4.55344E-06			
20	90	1797377	1565255	232121	11111	4.30808E-06			
20	100	1797377	1538779	258597	10000	3.86702E-06			

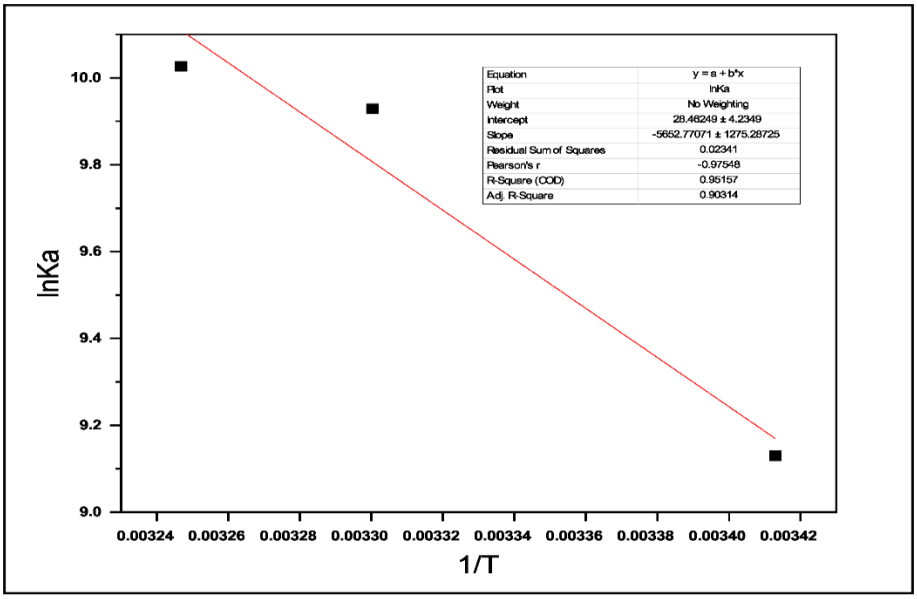


Fig.S1. Plot of ln Ka v/s 1/T for (NBTU+β-CD) system

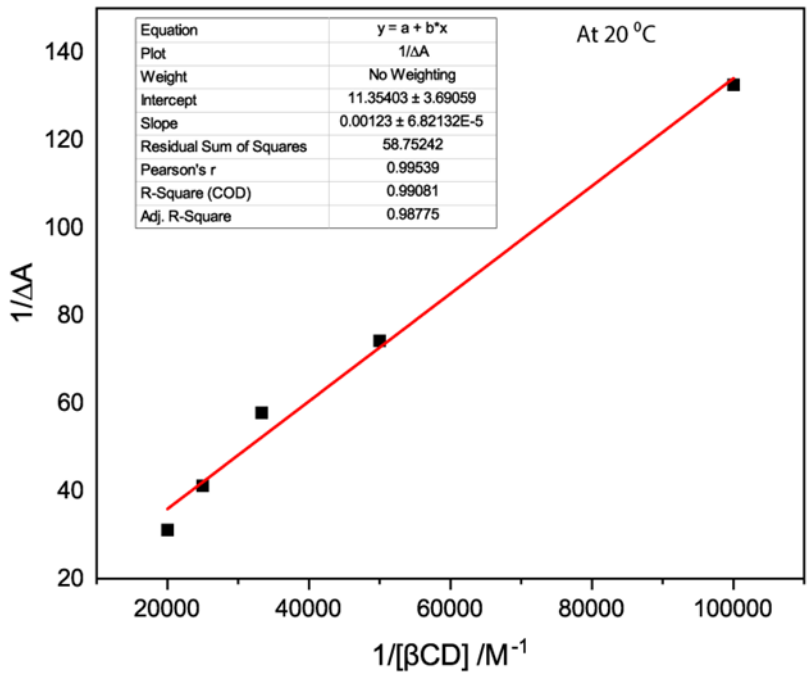


Fig.S2. The Benesi-Hildebrand double reciprocal plot of NBTU.CD inclusion complexes at 20^{0} C

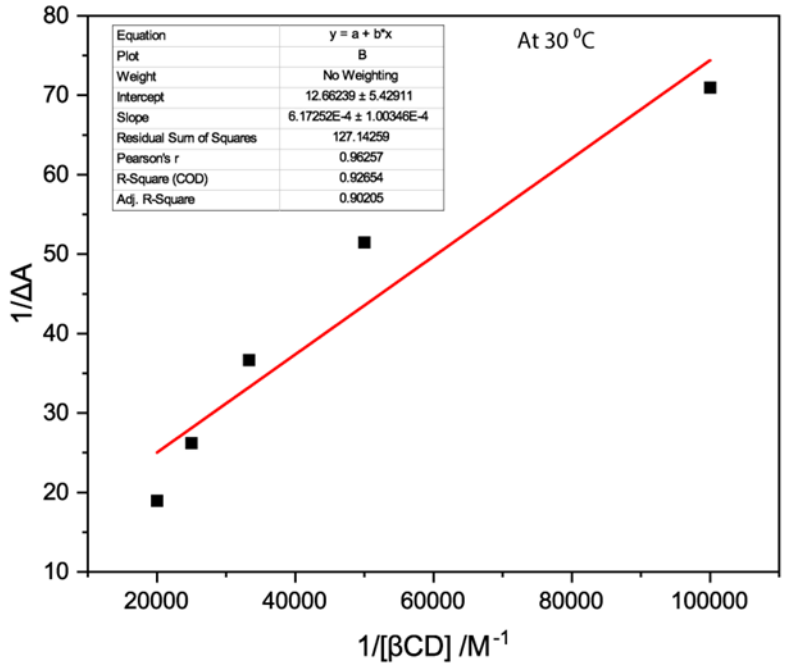


Fig.S3. Benesi-Hildebrand double reciprocal plot of NBTU.CD inclusion complexes at 30^{0} C

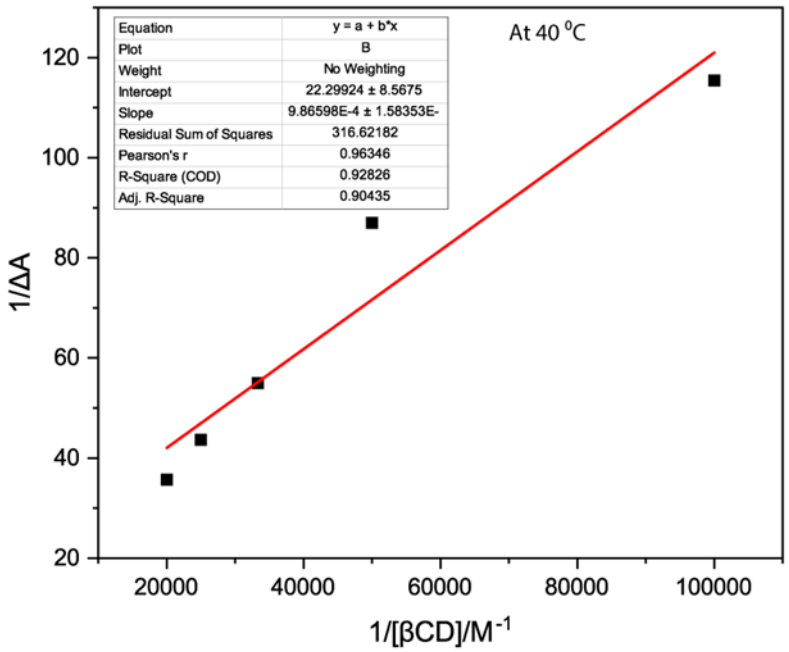


Fig.S4. Benesi-Hildebrand double reciprocal plot of AA.CD inclusion complexes at 40^{0} C

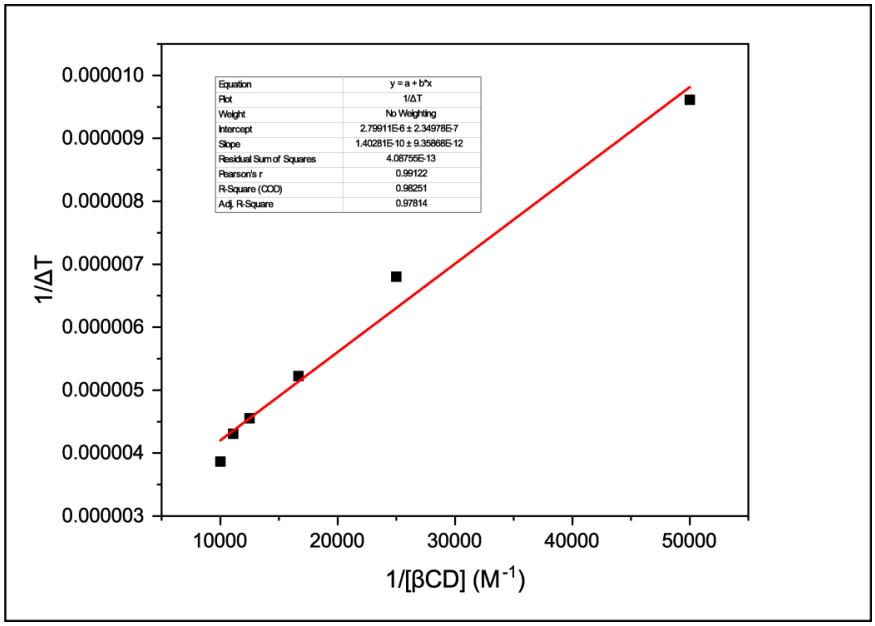


Fig.S5. Benesi-Hildebrand double reciprocal plot of $1/(F_0-F)$ against 1/[β-CD] between NBTU and β-CD from Fluorescence Spectroscopy

Table.S6. ^{1}H-NMR spectra of pure NBTU, pure β-CD, (NBTU+β-CD) inclusion complexes

NBTU (400 MHz, d6-DMSO) **δ /ppm**	**β-Cyclodextrin (400 MHz, d6-DMSO) δ /ppm**
7.93(d, 1H, *J*=4 Hz) 7.63 (t, 1H, *J*= 8 Hz), 7.51(t, 1H, *J*=4 Hz), 11.26 (s, 1H, -NH), 9.58-9.86 (-NH_2, s, 2H)	3.55 (7H, d, *J* = 4Hz), 3.63 (21H, m, *J* =4Hz), 3.30(7H, t, *J* = 4 Hz), 5.71-5.78 (6H, dd, *J* =8 Hz)
[NBTU+β-CD] δ /ppm	
3.57 (7H, d, J = 4Hz), 3.53 (21H, m, J=4Hz), 3.28(7H, t, J = 4 Hz), 5.70-5.77 (6H, dd, J =8 Hz),7.86-7.88(d, 1H, *J*=4 Hz) 7.58-7.59 (d, 1H, *J*= 4 Hz), 7.44(t, 1H, *J*=8 Hz)	

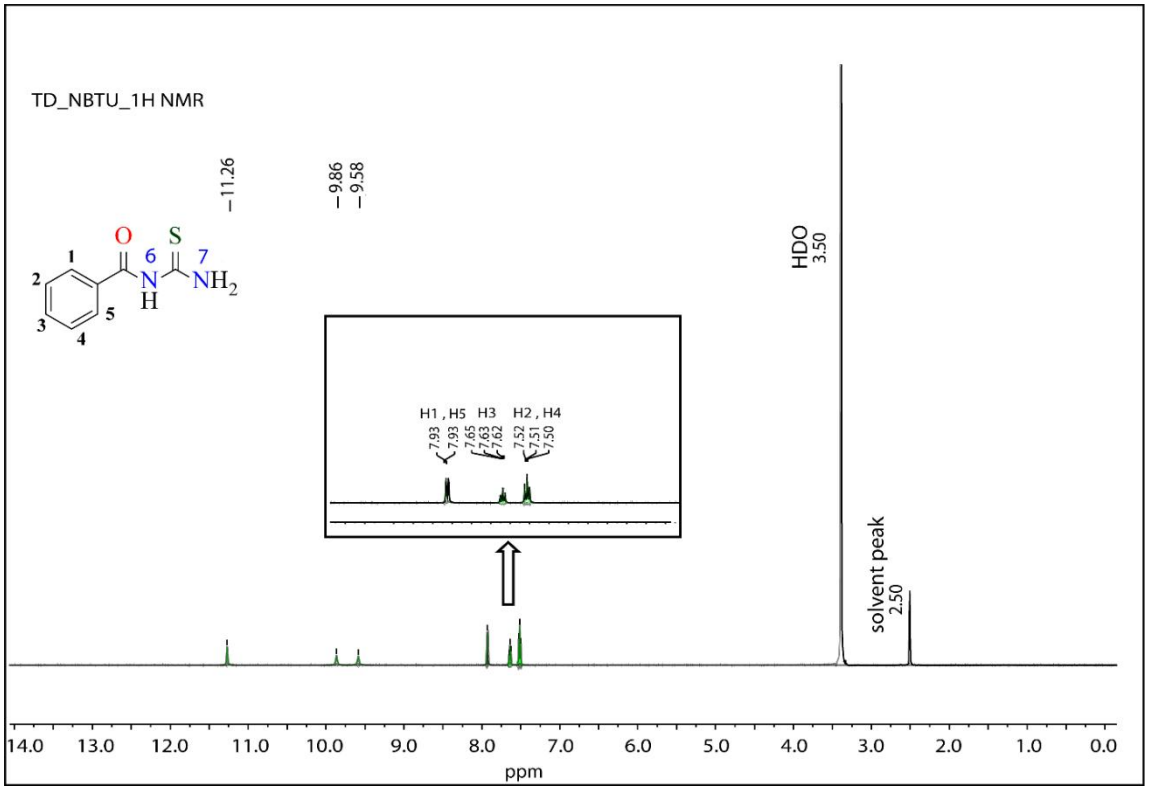


Fig.S6. 1H NMR spectra of pure NBTU in d6-DMSO.

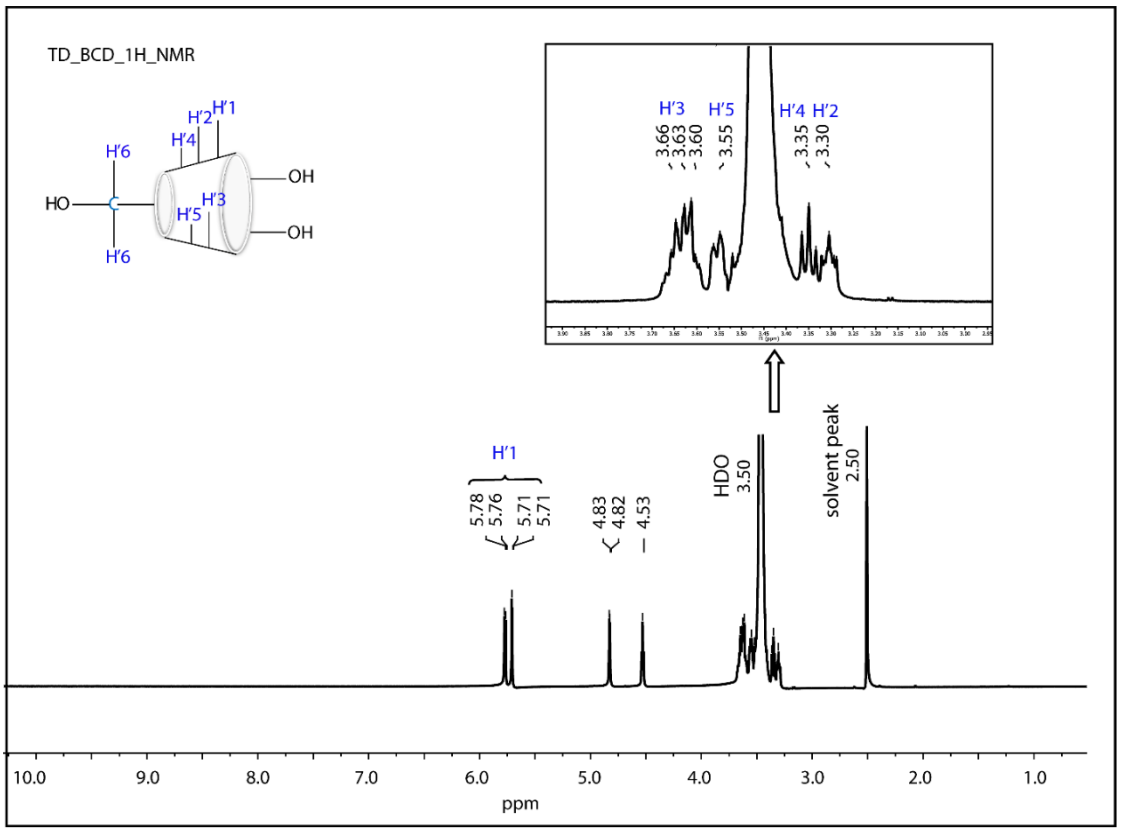


Fig.S7. 1H NMR spectra of pure β-CD in d6-DMSO.

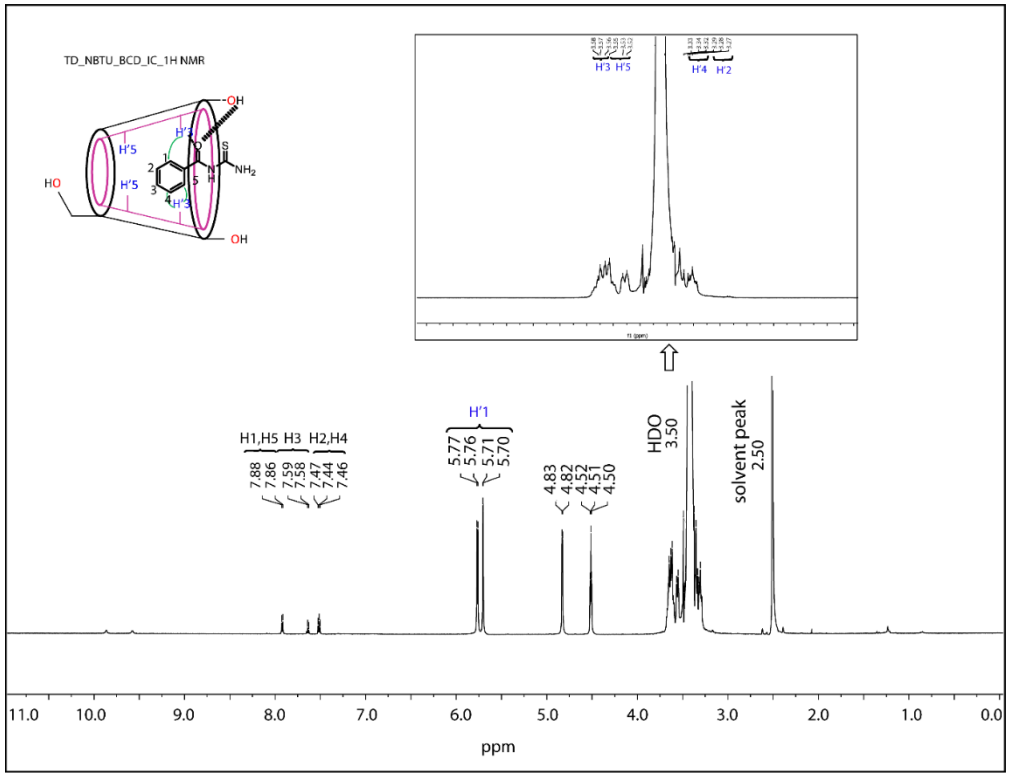


Fig.S8. 1H NMR spectra of pure IC in d6-DMSO.

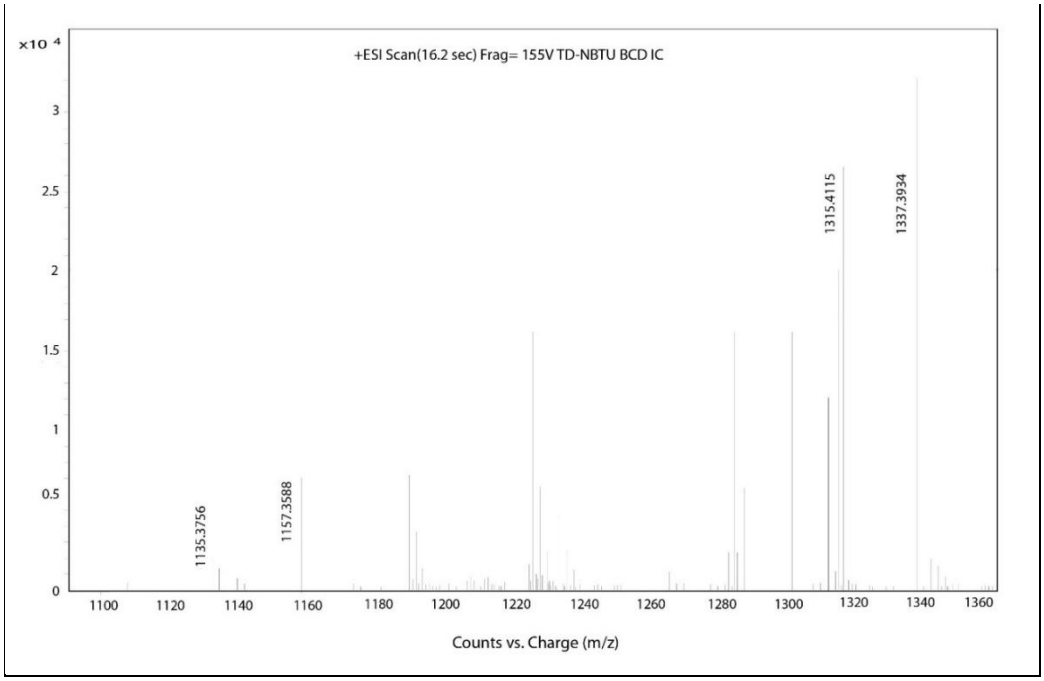


Fig.S9. HRMS Spectra of (NBTU+β-CD) inclusion complex.

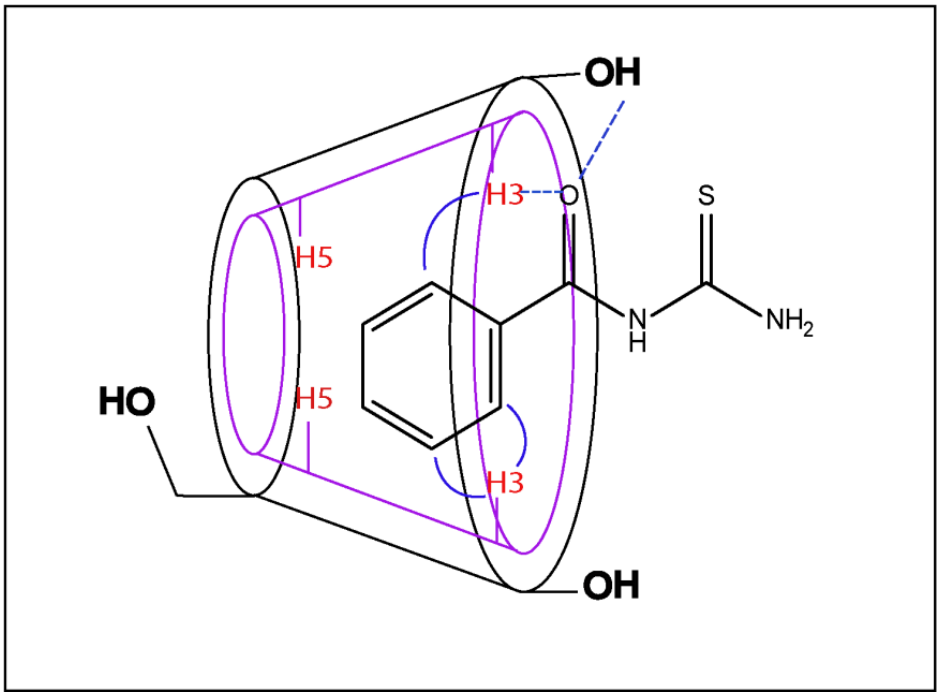


Fig.S10. The most plausible structure depicted from 2D-ROESY data