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# Characterization, phase-solubility, and molecular modeling of inclusion complex of 5-nitroindazole derivative with cyclodextrins

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**Abstract**—The slightly water-soluble 5-nitroindazole derivative (5-NI) and its inclusion with either β-cyclodextrin (βCD) or Heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (DMβCD) were investigated. The stoichiometric ratios and stability constants describing the extent of formation of the complexes were determined by phase-solubility measurements obtaining type-$A_L$ diagrams in both cases. According to the continuous variation method (Job's plot) a 1:1 stoichiometry has been proposed for the complexes. Also electrochemical studies were carried out on both CDs complexes, where the observed change in the $E_{PC}$ value for DMβCD indicated a lower feasibility of the nitro group reduction. The detailed spatial configuration is proposed based on two-dimensional NMR methods. These results are further interpreted using molecular modeling studies. The latter results are in good agreement with the experimental data.


## 1. Introduction

Cyclodextrin are well-known cyclic oligomers composed of several D-glucose units bonded by α(1,4) linkages providing an hydrophobic internal cavity able to include various drug molecules, thus forming non-covalent inclusion complexes.[1] Cyclodextrin complexation has been widely used in the pharmaceutical field to improve properties of drugs, such as solubility, dissolution rate, chemical and physical stability, and, as consequence, bioavailability, as well as to reduce their irritancy and toxicity.[2,3] The fit of the entire or at least a part of the guest molecule in the cyclodextrin-host cavity determines the stability of the inclusion complex and the selectivity of the complexation process. Therefore, the stability constant value of drug–cyclodextrin complexes is a useful index of the binding strength of the complex and is of great importance for the understanding and evaluation of the inclusion complex formation.[4] It is important to accurately determine this parameter, in order to predict changes in the physico-chemical properties of the drug after inclusion in the cyclodextrin cavity and to select the most suitable cyclodextrin-host molecule for a given drug-guest, so that inclusion complexation may be successfully exploited at its best.[5]

Many of the compounds showing antiprotozoal activity bear a nitro group in their heterocyclic structure, such as nifurtimox and benznidazole for the treatment of Chagas' disease or metronidazole in trichomoniasis chemotherapy. The ability to produce radical species capable to induce a cascade of reduced materials, which are toxic toward the parasite, has been the proposed mechanism of action of these nitro compounds.[6,7] 5-Nitroindazole derivatives were identified as good in vitro antiparasites, and good trypanosome/mammal agent.[8–10] But these therapeutic agents possess low intrinsic solubility. One of the promising approaches is to encapsulate the drug in the hydrophobic cavity of cyclodextrin and with it to try to increase the bioavailability which may produce better biological activity.

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This study was undertaken in order to determine the binding constants of the inclusion complexes of 1-(2-dimethylamino)ethyl)-3-methoxy-5-nitro-1H-indazole (5-NI) Figure 1, with β-cyclodextrin (βCD) and Heptakis (2,6-di-*O*-methyl)-β-cyclodextrin (DMβCD). The structural information, such as the stoichiometry and the geometry of the complexes, and also thermodynamic parameters such us the stability constant, are necessary to clarify the complexation mechanism so as to determine the driving forces governing this interaction.

## 2. Results and discussion

### 2.1. Phase-solubility measurements

Both CDs enhanced the poor aqueous solubility of 5-NI, thus proving a certain degree of its inclusion complexation in aqueous solutions, Figure 2. The phase-solubility diagrams of 5-NI with βCD and DMβCD within the concentration range studied displayed a typical $A_L$ type diagram (i.e., linear increase of 5-NI solubility with increasing CD concentration), consistent with a 1:1 molecular complex formation for both CDs. The result observed showed a linear behavior which is unequivocal for both CDs studied. The binding constant $K_a$ of the complexes, 187 and 3688 $M^{-1}$ for βCD and DMβCD, respectively, was calculated from the slopes of the linear phase-solubility plots according to the methodology described in the experimental part. The binding constant for 5-NI determined for both CDs, followed the rank order DMβCD > βCD, reflecting an enhancement of binding and solubility with increasing substitution and hydrophilicity of the CDs.

According to the continuous variation method, if a physical parameter directly related to the concentration of the complex can be measured for a set of samples with continuously varying molar fraction of its components. The maximum concentration of the complex will be present in the sample where the molar ratio *R* corresponds to the complexation stoichiometry. In Figure 3, the maximum absorbance variation for 5-NI in both CDs was observed for $R = 0.5$, which might indicate that the main stoichiometry is 1:1, in agreement with the stoichiometry suggested from the phase-solubility study.

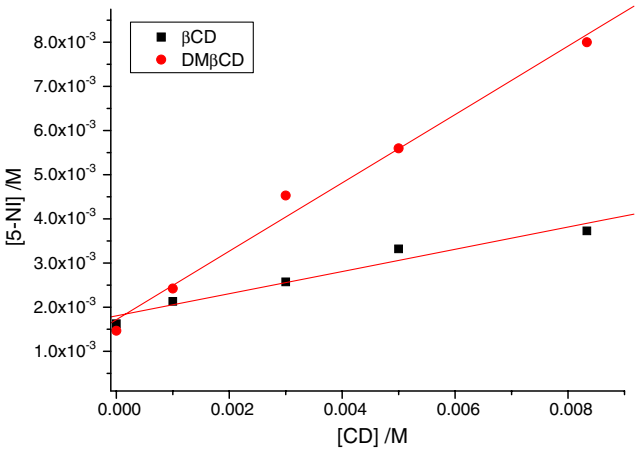


**Figure 2.** Phase-solubility diagrams of 5-NI-βCD and 5-NI-DMβCD system in water at 30 °C.

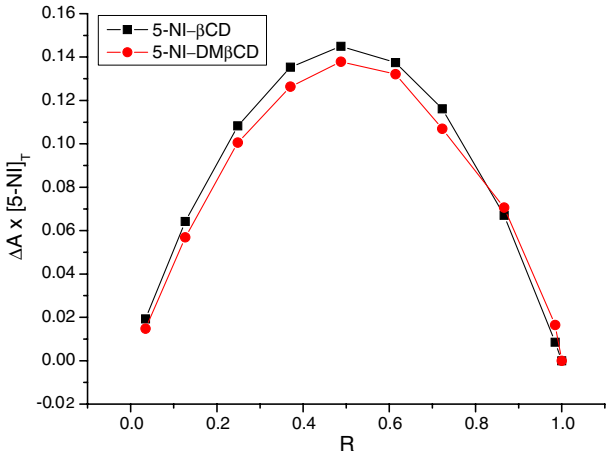


**Figure 3.** Continuous variation plot for the 5-NI-βCD and 5-NI-DMβCD system from absorbance measurements.

### 2.2. DPP studies

The electrochemical behavior in protic media for the 5-nitroindazole derivatives has been previously studied,[11] indicating that 5-NI is reduced on the mercury electrode due to the four-electron and four-proton irreversible reduction of the nitroaromatic group to yield the

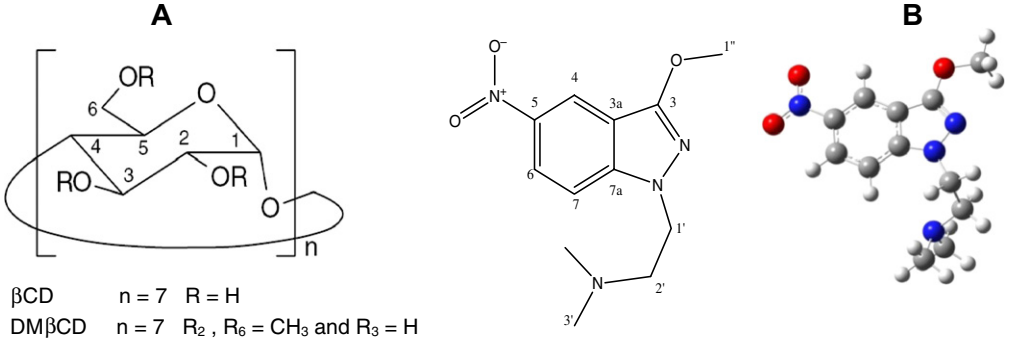


**Figure 1.** Molecular structures of β-cyclodextrin and Heptakis-2,6-di-*O*-methyl-β-cyclodextrin (A). Plane and spatial representations of the 5-NI molecular structure (B).

hydroxylamine derivative according to the following overall reaction:

$$\text{R-NO}_2 + 4e^- + 4\text{H}^+ \rightarrow \text{R-NHOH} + \text{H}_2\text{O}$$

In order to evaluate the changes in the polarograms due to the addition of βCD/DMβCD into a 5-NI solution, several different CDs/5-NI mixtures in aqueous media at pH 7 were used. The solutions were equilibrated in a Julabo thermostatic shaking water bath for 24 h at 30 °C. As shown in Figure 4a, the presence of different concentrations of βCD in the medium do not shift the cathodic peak potential ($E_{PC}$, −0.56 V) indicating that the formation of the inclusion complexes do not affect the charge transference process. The anodic peak potential ($I_{pc}$) decreases when the βCD concentration increases. The decrease of the peak current, reveals a diminution of the diffusion current which has been previously ascribed as a reduction of the apparent diffusion coefficient in inclusion complexes with regards to the unbound 5-NI.[12]

DMβCD shows a different behavior (Fig. 4b) under the same experimental conditions. We observed a change in the $E_{PC}$, which reveals that 5-NI is reduced less favorably when the nitroindazole is included in the hydrophobic cavity. Probably this enhanced difficulty is due to the nitro group which is located inside the cavity of CD hindering the interaction with the electrode.

### 2.3. $^1$H NMR studies

$^1$H NMR data for 5-NI dissolved in deuterated water ($D_2O$) shows well resolved signals in two distinct spectral regions: two triplets corresponding to methylene protons, H-1′ and H-2′, and two singlets corresponding to methoxy group and both methyl bounded to nitrogen in the high frequency region, and three signals corresponding to the aromatic protons, see Table 1.

The formation of inclusion complex can be proved from the changes of chemical shifts of 5-NI or CDs in $^1$H NMR spectra. Table 2 lists the detailed variation of the chemical shifts of 5-NI and internal protons of both cyclodextrins before and after forming inclusion complex.

The H-3 and H-5 protons of the glucose units are facing the interior of the CD cavity, whereas H-6 protons are located at the rim with the primary alcohols. H-2 and

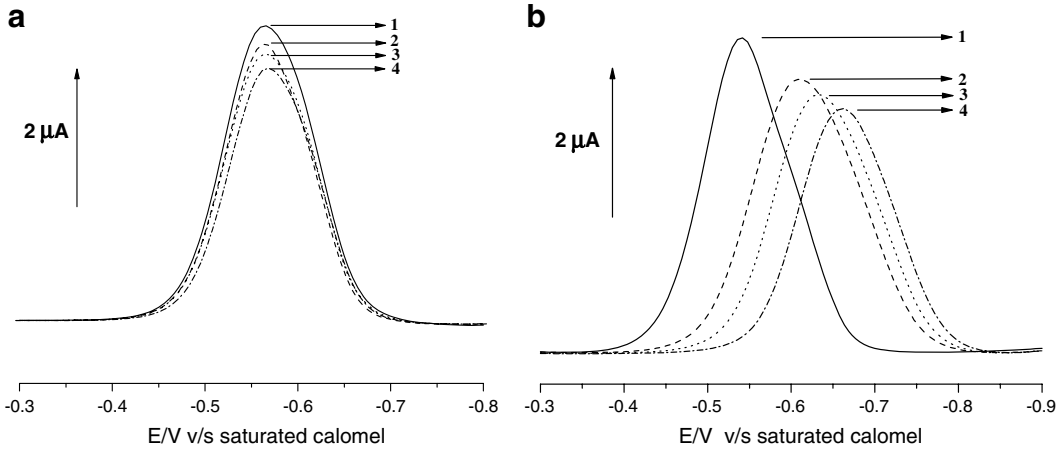


**Figure 4.** DPP of 1 mM of 5-NI in 0.1 M of 70/30 Britton–Robinson/EtOH (pH 7.1). (a) In absence (1) and presence of (2) 3, (3) 5 and (4) 7 mM β-CD, (b) in absence (1) and presence of (2) 3, (3) 5 and (4) 7 mM DMβCD. Conditions: scan rate 10 mVs$^{-1}$, pulse amplitude 50 mV, pulse width 50 ms.

**Table 1.** $^1$H and $^{13}$C NMR assignment for 0.5 mM 5-NI in $D_2O$ at 300 K

| $^{13}$C RMN | | $^1$H RMN | | | HMBC C–H long range |
|---|---|---|---|---|---|
| Carbon | δ/ppm | Proton | *J*/Hz | δ/ppm | |
| $C_3$ | 158.3 | — | — | — | $H_4$, $H_{1''}$ |
| $C_{3a}$ | 111.9 | — | — | — | $H_7$ |
| $C_4$ | 118.6 | $H_4$ | d; $^4J$:1.90 | 8.62 | $H_6$, $H_7$ |
| $C_5$ | 140.8 | — | — | — | $H_4$, $H_6$, $H_7$ |
| $C_6$ | 122.5 | $H_6$ | dd; $^4J$:1.90; $^3J$:9.47 | 8.20 | $H_4$ |
| $C_7$ | 108.6 | $H_7$ | d; $^3J$:9.47 | 7.45 | — |
| $C_{7a}$ | 143.0 | — | — | — | $H_4$, $H_6$, $H_7$ |
| $C_{1'}$ | 58.2 | $H_{1'}$ | t; 6.50 | 4.35 | $H_{2'}$ |
| $C_{2'}$ | 47.4 | $H_{2'}$ | t; 6.50 | 2.86 | $H_{1'}$, $H_{3'}$ |
| $C_{3'}$ | 45.7 | $H_{3'}$ | s | 2.21 | $H_{2'}$ |
| $C_{1''}$ | 56.5 | $H_{1''}$ | s | 4.00 | — |

**Table 2.** Complexation shifts ($\Delta\delta$ ppm) for 1:1 complex 0.5 mM of 5-NI-βCD and 5-NI-DMβCD at 300 K

| Compound | Proton | $\Delta\delta$(ppm) = ($\delta_{free} - \delta_{complex}$) 5-NI-βCD | $\Delta\delta$(ppm) = ($\delta_{free} - \delta_{complex}$) 5-NI-DMβCD |
|---|---|---|---|
| 5-NI | H-4 | 0.13 | 0.35 |
| | H-6 | 0.08 | 0.21 |
| | H-7 | 0.06 | 0.14 |
| | H-1′ | 0.08 | 0.16 |
| | H-2′ | 0.23 | 0.28 |
| | H-3′ | 0.23 | 0.20 |
| | H-1″ | −0.03 | −0.02 |
| CD | H-3 | 0.11 | 0.22 |
| | H-5 | 0.08 | 0.08 |
| | H-6 | 0.02 | −0.01 |
| Complex | $K_a$ | 187 $M^{-1}$ | 3688 $M^{-1}$ |

H-4 are at the opposite entrance of the cavity. High frequency shifts of the interior proton signals of CDs are indicative that aromatic guest molecules are located close to the protons for which a shift is observed. This displacement is due to the anisotropic magnetic effect induced by the presence of the aromatic group of the guest molecule. The analysis of the variations undergone by the CDs protons as a consequence of the presence of 5-NI strongly suggests complexation involving inclusion into the cavity of the host, as the external protons H-1, H-2 and H-4 were slightly affected, whereas the H-3 and H-5 protons were significantly shifted.

Table 2 reports the chemical shift values of 5-NI and CDs protons in the native and complexed forms. The induced shift $\Delta\delta$ is defined as the difference in chemical shift in the absence and presence of the other reactants. In the present case, the induced shifts were calculated by the following equation: $\Delta\delta = \delta(\text{free}) - \delta(\text{complex})$. In this convention, positive and negative signs show high and low frequency shifts, respectively. In the presence of 5-NI, H-3 and H-5 protons of the CDs are shifted to higher frequency, these protons are inside the CD cavity, their shifts suggest that the indazole ring is included inside the CD.[13] Since the signal due to H-6, which is located at the narrow end of the cyclodextrin, is not significantly shielded by the guest molecule, it is likely that the 5-NI molecule enters from the wider end of the cyclodextrin where the secondary hydroxyls are located. The high frequency shift observed for H-4, H-6, and H-7 of 5-NI are more pronounced for DMβCD than for βCD, indicating a stronger interaction for the complex 5-NI-DMβCD, which is confirmed by the higher value of the association constant obtained.

Further information about the inclusion mode of 5-NI in the CDs included in this study can be derived from the evidence of spatial proximities between protons of CDs and 5-NI. Two-dimensional rotating-frame noe spectroscopy experiments have often been successfully applied to prove through-space intermolecular interactions in CD complexes. Indeed, in the ROESY experiments (Rotational nuclear Overhauser effect spectroscopy), dipolar interactions between protons at a distance less than 3–4 Å are detected as cross-peaks in a bi-dimensional map, indicating the portion of the guest situated in the torus cavity. In this study, ROESY spectra were collected to gain additional insights. The effects were only qualitatively used.

Figure 5 shows a partial contour plot of 2D-ROESY spectra of the inclusion complex of 5-NI and βCD. (To attribute unambiguously the protons H-3, H-5, and H-6 of the cyclodextrin region, a correlation heteronuclear $^{1}H$–$^{13}C$ spectrum HSQC (heteronuclear single quantum correlation ) of 5-NI-βCD system was performed in the same conditions as those used for the ROESY spectrum.) There are intermolecular cross-peaks between H-4 of 5-NI with H-3 and H-5 of βCD, and also we observed an interaction between hydrogen H-7 of the substrate with H-3 or H-6 of the cyclodextrin, and a less intense cross-peak between H-6 of 5-NI and H-3/H-6 of CD. However, in agreement to the chemical shift observed for the H-6 of CD, practically it is not perturbed by the substrate, indicating that H-3 of the CD interact with 5-NI. We also observe dipolar intramolecular interaction between H-1′, H-2′ and H-3′ with the aromatic proton H-7 of 5-NI, (data not shown), indicating that the aliphatic chain is folded over the aromatic moiety as the 5-NI free in solution. Both, the aromatic and the alkyl moieties are included into the cyclodextrin ring and are protected from the aqueous due to the upfield chemical shift observed for these protons. The analysis of the dipolar interactions generated by the internal protons of the cyclodextrin, suggest that the nitroindazole ring is inserted in the cyclodextrin cavity with the nitro group oriented toward the secondary hydroxyl group of the CD.

Figure 6 depicts the ROESY spectrum of the 5-NI-DMβCD complex. This complex shows correlations between H-4, H-6, and H-7 (less intense) of nitroindazole derivative with H-3 or H-6 of the DMβCD. However,

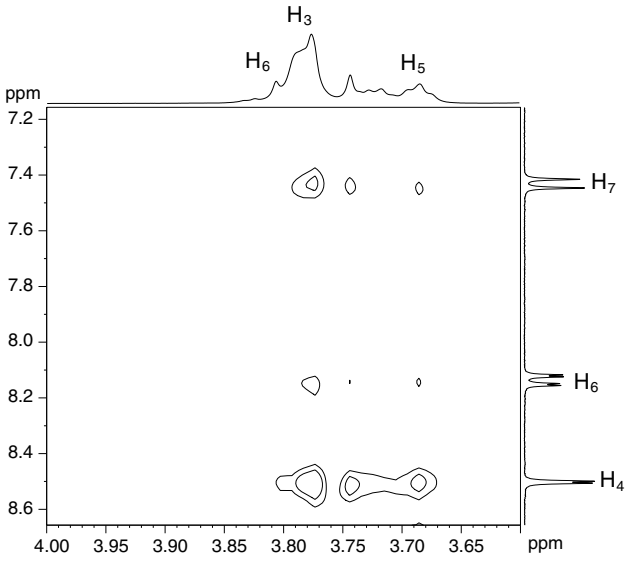


**Figure 5.** Partial contour plot of two-dimensional ROESY spectrum of a solution containing the 1:1 5-NI-βCD inclusion complex (0.5 mM in $D_2O$).

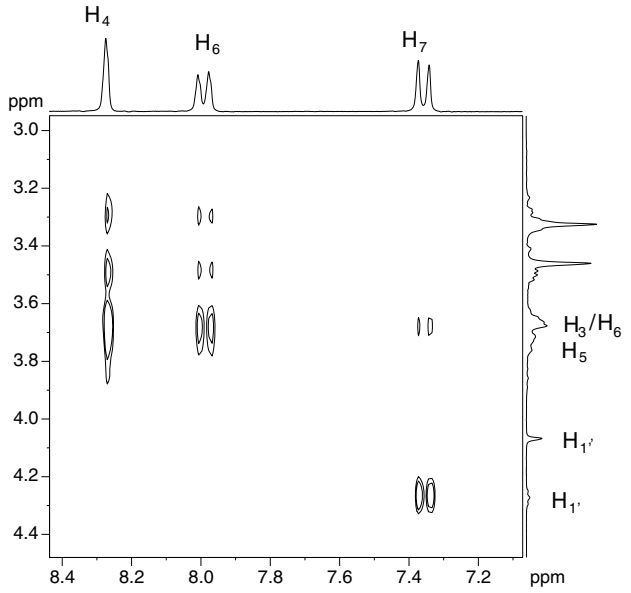


**Figure 6.** Partial contour plot of two-dimensional ROESY spectrum of a solution containing the 1:1 5-NI-DMβCD inclusion complex (0.5 mM in $D_2O$).

in agreement to the chemical shift observed for the H-6 of CD, practically not perturbed by the substrate, indicating that H-3 of the CD interacts with 5-NI, see Table 2. The analysis of the dipolar interactions generated by the internal protons of the cyclodextrin, suggest that the nitroindazole ring is inserted with the nitro group oriented toward the secondary hydroxyl group of the CD. Seemingly, the aliphatic chain is also folded to the aromatic region but with minor a degree as in the 5-NI-βCD complex, because the only intramolecular interaction observed is between H-7 and H-1′ of the aliphatic chain. Nonetheless, it must be included in the hydrophobic cavity due to the upfield shift observed for these protons.

### 2.4. Molecular modeling studies

In order to rationalize the NMR experimental results described above, we carried out molecular modeling studies of the complexes. These studies revealed that a preferred final orientation for all the complexes studied occurs in spite of the different initial configurations arbitrarily imposed. The minimum energy complexes obtained for the CDs under study are shown in Figure 7. It is worth to note that although no fixed distances were imposed during the docking calculations, the results are in very good agreement with that obtained by the 2D ROESY spectra. Noticeable differences between the βCD and its derivatized forms can be observed, being the orientation of the ligand once inside of the host molecule the most relevant. In the case of the 5-NI-βCD complex, the conformation obtained by molecular modeling was in agreement with the ROESY results. The complex has the indazole-ring inserted in the cyclodextrin cavity, in such a way that the nitro and methoxy groups are placed toward the secondary rim. The aliphatic chain is folded over the indazole ring closer to the primary rim. However, the theoretical results obtained from the 5-NI-DMβCD complex indicate a different form of inclusion compared to the βCD complex. The nitroindazole ring is inserted in the DMβCD cavity with the nitro group oriented to the secondary rim and the methoxy group is exposed to the outside by the primary rim. As we can see in Figure 7a and c, the nitro group is more protected in the DMβCD than in the

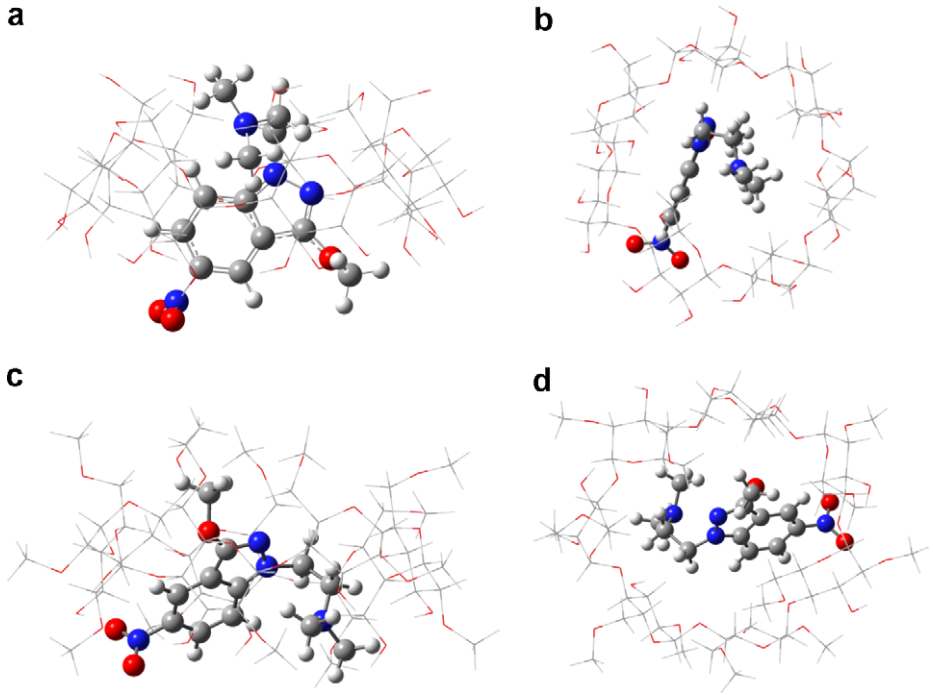


**Figure 7.** Relative host–guest geometry corresponding to the minimum of the energy of the formation of the 5-NI-βCD complex, (a) top primary rim view and (b) side view and 5-NI-DMβCD complex (c) top primary rim view and (d) side view.

βCD, which would agree with our electrochemical results. Beside, in both cases the methoxy group is exposed to the aqueous medium, which is in agreement with the low $\Delta\delta$ obtained for these protons, indicating practically no interaction with the cyclodextrin.

## 3. Conclusion

The aqueous solubility of 5-NI has been improved in neutral aqueous solutions through complexation with two cyclodextrins, βCD and DMβCD. These results indicated interaction between 5-NI and cyclodextrin in water forming 1:1 inclusion complex. The dissociated constant of the inclusion complex is measured as $187\ M^{-1}$ and $3688\ M^{-1}$ for βCD and DMβCD, respectively.

The effect of DMβCD on the polarographic behavior of nitroindazole can be summarized in a negative shift in the cathodic peak potential and a peak current decrease. However, in the complex 5-NI-βCD only small change in the $I_{PC}$ can be observed. From these changes we can assume that the nitroaromatic group in the DMβCD is hindered, whearas it is different for the βCD complex where the nitro group is exposed to the medium.

By means of experimental and theoretical methods, the present work unambiguously determined the geometrical inclusion parameters of 5-NI on both CDs. Besides the ROESY experiments showed that the inclusion of 5-NI in βCD has a different trend when it is compared with DMβCD. These results were corroborated by molecular modeling calculations.

## 4. Experimental

### 4.1. Apparatus

Spectrophotometric measurements were carried out with a $UV_2$ UNICAM spectrophotometer, using a 1 cm quartz cell.

All NMR experiments have been recorded on a Bruker AVANCE DRX300 spectrometer equipped with a pulse gradient unit. The spectra have been acquired in an inverse probe-head at 298 K in 5 mm tubes. All chemical shifts were relative to the DOH signal at 4.70 ppm. The NMR measurements have been done with standard BRUKER pulse sequences.

### 4.2. Reagents

The 5-NI derivative (1-(2-dimethylamino)ethyl)-3-methoxy-5-nitro-1H-indazole) was synthesized according to methods described earlier.[10,11]

β-Cyclodextrin (βCD), heptakis (2,6-di-*O*-methyl) β-cyclodextrin (DMβCD), and deuterated water ($D_2O$) were purchased from Sigma–Aldrich, Inc., St. Louis, MO. Other reagents were all analytical grade and double distilled water was used throughout.

### 4.3. Method

**4.3.1. Phase-solubility measurements.** Phase-solubility measurements were carried out according to the method of Higuchi and Connors.[14] Excess amount of 5-NI (5 mg) was added to 5 mL of deionized water containing increasing amounts of βCD and DM-βCD (ranging from 0 to 0.010 M). The resulting mixture was equilibrated in a Julabo thermostatic shaking water bath for 24 h at 30 °C after which the equilibrium was reached. The suspensions were filtered through 0.45 μm cellulose acetate membrane filter to remove undissolved solid. An aliquot from each vial was adequately diluted and spectrophotometrically analyzed at 362 nm.

The apparent stability constant ($K_a$) of the complexes was calculated from the phase-solubility diagrams according to the following equation:

$$K_a = \frac{\text{slope}}{S_o(1 - \text{slope})} \quad (1)$$

where $S_o$ is the solubility of 5-NI at 30 °C in the absence of cyclodextrin and slope means the corresponding slope of the phase-solubility diagrams, that is, the slope of the drug molar concentration versus CDs molar concentration graph.

**4.3.2. Stoichiometry determination by the continuous variation method (Job's plot).** The stoichiometry of inclusion was determined by the method developed by Job.[15] Equimolar $3.0 \times 10^{-4}$ M solutions of 5-NI and CD were mixed to a standard volume varying the molar ratio but keeping the total concentration of the species constant.

After stirring for 24 h, the absorbance at 362 nm was measured for all solutions and $\Delta A = A - A_o$, the difference in absorbance in the presence and in the absence of CDs, was plotted against $R$; $R$ = [5-NI]/{[5-NI] + [CD]}.

**4.3.3. Differential pulse polarography (DPP).** In protic media, the solutions were prepared starting from a stock solution 0.1 M of sample, 5-NI, in DMSO. The final solution was prepared through the corresponding dilution to obtain a sample final concentration, on the voltammetric cell, of 1.0 mM. DPP experiments were carried out using a Metrohm 693VA instrument with a 694VA Stand convertor and a 693VA Processor, keeping the concentration 1.0 mM of 5-NI in 70/30 Britton–Robinson/ethanol (ca. 1.0 mM, pH = 7.1) constant while varying the concentrations of CDs (0–10 mM), under a nitrogen atmosphere at room temperature, with KCl (ca. 0.1 M), using a three electrode cell. A hanging mercury drop electrode was used as the working electrode, a platinum wire as the auxiliary electrode, and saturated calomel as the reference electrode.

**4.3.4. Preparation of 5-NI-CDs complex for NMR study.** Inclusion complexes were obtained by mixing appropriate amounts of 5-NI and CDs in $D_2O$ with a molar ratio

of 1:1. The resulting mixture was equilibrated in a Julabo thermostatic shaking water bath for 24 h at 30 °C after which the equilibrium was reached.

Attributions of analyte proton resonances were realized by standard NMR experiments: COSY (correlation spectroscopy), HSQC (Heteronuclear Single Quantum Coherence), HMBC (Heteronuclear Multiple Bond Correlation). ROESY (Rotational nuclear Overhauser Effect Spectroscopy) experiments were performed as a general strategy to have complete identification of the geometry of the complex in solution. The measuring conditions for the two-dimensional ROESY spectra were: spectral width 3000 Hz; data size 16 K/8 K; relaxation delay 2 s and 32 scans with a mixing time of 400 ms. Phase sensitive spectra were acquired using TPPI scheme.

**4.3.5. Molecular modeling.** In silico build-up of βCD and DMβCD was carried out using the *Builder* module of the *InsightII* program[16] by adding to βCD 14 methyl in position 2 and 6 (DMβCD). The obtained models were subjected to optimization using a protocol of 300 steps of conjugate gradients to avoid steric hindrance and clashes that can appear in the building process. The 5-NI was build using *Gaussview* and then it was optimized using a semiempirical method such as AM1 as implemented in *Gaussian98* package of programs.[17]

Autodock3.0.5[18] with Lamarkian Genetic Algorithm (LGA) was used to generate the starting complexes. The parameters used for the global search was an initial population of 50 individuals, with a maximal number of energy evaluations of 1500,000 and a maximal number of generations of 50,000 as an end criterion. An elitism value of 1 was used, and a probability of mutation and crossing-over of 0.02 and 0.08 was used, respectively. From the best solutions obtained according to these parameters, some of them defined by the user as the best probabilities in our case 0.06 were further refined by a local search method such as pseudo Solis and Wets 'PSW'.

*Autodock* defines the conformational space implementing grids all over the space of the possible solutions. With the aim of testing the ability of *Autodock* to converge into solutions that are inside the βCD, a grid of 80 Å by the side and 0.3 Å spacing between each point was setup in such a way that it covered both the external surface and the internal cavity of the βCD.

The following procedure was employed on the CD docking simulations: 250 runs were done for each CD. At the end of each run, the solutions were separated into clusters according to their lowest RMSD and the best score value based on a free empiric energy function. Cluster solutions whose average score was not over 1 kcal $mol^{-1}$ with respect to the best energy obtained in the respective run were selected. Then, the solution that represents most of the complexes obtained in the run was compared with the NMR experimental data, assuring that this solution is able to represent it accurately.

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