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Structural Characterization of Imazalil/β-Cyclodextrin Inclusion Complex

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An equimolar inclusion complex between imazalil, a selected fungicide, and β-cyclodextrin using an aqueous standard solution procedure has been obtained. The complex has been investigated in solution by ^{1}H and ^{13}C NMR techniques in combination with computational methods in order to establish a valuable analytical protocol through which to gain insight into the interactions of the inclusion complex in aqueous solution. Intramolecular NMR distance constraints have been detected and used for three-dimensional complex structure determination.

KEYWORDS: Imazalil; cyclodextrins; inclusion complex; NMR spectroscopy; computational methods

Microencapsulation of biologically active compounds into biopolymers using the molecular inclusion technique promises a variety of applications in drugs as well as in agrochemical industry because biologically active compounds can be slowly released from the biopolymer for a specified period of time (*1*−*3*). Besides important benefits in dispensing active agents, some disadvantages related to biopolymeric matrices such as cost, water solubility, use of organic solvent and problems concerning molecular encapsulation of drugs and agrochemicals are present (*4*−*6*).

β-Cyclodextrin (β-CD), a glucose cyclic oligomer consisting of seven glucose residues linked by α(1→4) glycosyl bonds (**Figure 1**), is a promising nontoxic, cheap, and water soluble biopolymeric matrix (*7*−*11*). Concerning the topology, β-CD can be schematically represented by a toroid with a lipophilic cavity and a hydrophilic external surface (*7*, *8*). Axial protons, namely, H_3 and H_5, point inside the toroid cavity, playing the role of detectors for inclusion complex formation; primary and secondary hydroxyl groups are placed on the smaller and on the larger rim, respectively.

Stable β-CD inclusion complexes can be obtained by using nonpolar molecules, which can form electrostatic interactions with the lipophilic cavity. This guest−host binding is mainly due to dipole−dipole and hydrophobic interations (*5*, *12*, *13*).

Imazalil (IMZ) is one of the few fungicides permitted in the postharvest treatment of fruits (**Figure 2**). Despite its intrinsic high antifungal activity (*14*−*17*), the fungicide effectiveness of IMZ is strongly affected by its very low water solubility (0.018 g/100 mL); thus, water soluble formulations are largely investigated.

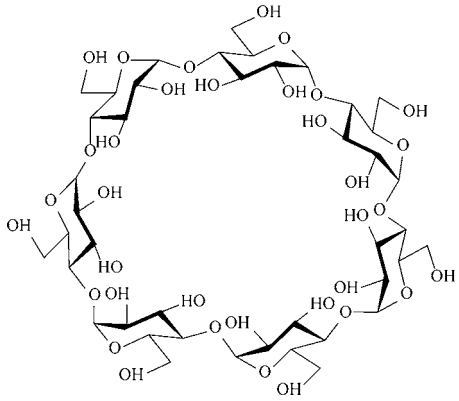

Figure 1. Structure of β-cyclodextrin (β-CD).

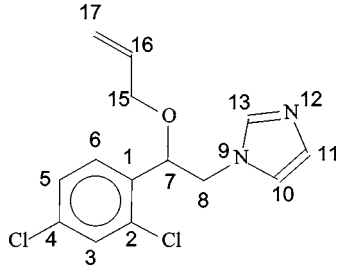

Figure 2. Structure of 1-[2-(2,4-dichlorophenyl)-2-(2-propenyloxy)ethyl]-1*H*-imidazole (IMZ).

Recently, we reported the preparation of an equimolar stable inclusion complex IMZ/β-CD in water under mild and direct method (*18*) and some studies concerning its antifungal activity in the postharvest treatment of citrus fruits.

The aim of the present work is to elucidate the structural details of the IMZ/β-CD inclusion complex in aqueous solvent

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by high-resolution multidimensional NMR techniques in combination with molecular dynamic calculations, a characterization not yet performed. We hope to establish an analytical protocol of the inclusion complex that can be applied to other β-CD inclusion complexes in order to gain insight into interactions in solution between host and guest.

MATERIALS AND METHODS

Preparation of Imazalil/β-Cyclodextrin Inclusion Complex (IMZ/β-CD). β-CD (Cavamax 7 Pharma) was obtained from Wacker Chemie Italia SpA and was used as reported. IMZ (97%) was a gift from Dr. Mario Schirra (Oristano, SS) and used without any purification.

Characterization of Inclusion Complexes in Solution by NMR. ^{1}H and ^{13}C NMR spectra of the IMZ/β-CD complex in D_2O and H_2O were acquired at 11.7 and 9.39 T on Bruker DMX and DRX spectrometers, respectively, at 300 K. Homo- and heteronuclear bidimensional spectra have been recorded with the use of a 5 mm *z*-gradient reverse probe on both instruments. ^{1}H and ^{13}C chemical shifts have been referred to an external reference (trimethylsilylpropionic acid, sodium salt). Solvent signal was suppressed by presaturation with low irradiation power. Mixing times of 1 s and 300 ms for NOESY (*19*) and ROESY (*20*) spectra have been used, respectively. ^{1}H assignment was performed by the use of bidimensional TOCSY (*21*) and DQF COSY (*22*) experiments. Complete ^{13}C assignment of the inclusion complex has been gained by the use of GE-HMBC and GE-HMQC (*23*) experiments.

Computational Methods. The starting structure of the IMZ/β-CD complex was assembled by using the Builder module of the InsightII program and energy minimized with the Discover program. The interproton distance constraints were derived from cross-peak volume integration of the NOESY spectrum and divided into ranges of distance classes. The starting energy-minimized molecule of the IMZ/β-CD complex was subjected to restrained molecular dynamics (RMD) followed by restrained energy minimization (REM) protocols consisting of 500 steps of steepest descents and 1000 steps of conjugate gradient procedures with distance-variable dielectric constant. The dynamic simulations were performed by heating from 300 to 1000 K in 1 ps, followed by 10 ps of equilibration at 700 K, cooling to 300 K in 5 ps, and a final 5 ps of equilibration at 300 K. A complete set of 50 structures have been generated. A maximum derivative of 0.01 kcal mol^{-1} was assumed for convergence criterion, and best structures have been selected on the basis of minimum violations of distances. All simulations were performed by using a CVFF force field (*24*) on a Silicon Graphics-Octane workstation. Molecular dynamic calculations and energy minimization programs were part of the InsightII software from MSI.

RESULTS AND DISCUSSION

NMR Properties of the IMZ/β-CD Inclusion Complex. Reasonable water solubility (>2 mM) of the IMZ/β-CD complex at 27 °C gave feasible ^{1}H and ^{13}C NMR experiments, performed in both H_2O and D_2O, thus providing further experimental evidence of the complex formation because free IMZ is practically water insoluble. The use of homo- (TOCSY and DQF COSY) and heteronuclear (GE-HMQC and GE-HMBC) bidimensional experiments gave the complete proton and carbon signal assignments of the IMZ/β-CD inclusion complex (**Table 1**).

The complexation is a dynamic process, in which the included molecule is in fast exchange (relative to the NMR time scale) between the free and bound states. Here significant high-field shifts for all β-CD proton resonances in the IMZ/β-CD complex with respect to the free β-CD have been measured (see **Table 2**), as a consequence of inclusion complex formation.

H_3 and H_5 protons located inside the CD cavity both experienced the highest upper field shift values observed upon complex formation (50 Hz shift). The other β-CD protons, H_1, H_2, and H_4, experienced lower upper field shifts, 5, 3, and 1 Hz, respectively, whereas H_6, located on the narrow end rim cavity, showed an upfield shift of 15 Hz, thus indicating the interaction of this portion of the molecule with the included guest. Concerning the included IMZ molecule, proton H_{13} showed particularly acidic properties, exchanging very rapidly with deuterons of D_2O, thus avoiding its detection in pure D_2O solution, whereas ^{1}H NMR spectra recorded in H_2O revealed the appearance of the mentioned signal, as a singlet, with two distinct chemical shifts at 7.64 and 7.67 ppm, respectively (**Figure 3**). This phenomenon did not seem to appear in the same ^{1}H NMR spectra performed recently with a supercritical carbon dioxide complex preparation (*25*).

Interestingly, H_{10} and H_{11} did not show a well-resolved vicinal coupling constant, thus appearing as singlets either in D_2O or in H_2O solvent; furthermore, H_{11} presented a unique chemical shift value, whereas the H_{10} singlet showed two distinct chemical shifts. The same holds true for the H_{15} and H_{17} protons: each of them appeared with the appropriate multiplicity. The same effect is experienced in a more sensitive way by the corre-

Table 1. IMZ/β-CD Inclusion Complex ^{13}C and ^{1}H Chemical Shifts Measured in D_2O and in H_2O, Respectively

	^{13}C, ppm	^{1}H, ppm
C_1H	104.16	5.06
C_2H	74.13	3.65
C_3H	75.38	3.87
C_4H	83.33	3.85
C_5H	74.32	3.77
C_6H	62.33	3.86
N–C_{13}H═N	140.31/140.25	7.67/7.64
Cl–C_2(Ar)	136.37	
Cl–C_4(Ar)	136.33	
C_1 (Ar)	136.25	
–C_3H═(Ar)	130.99	7.60
–C_5H═(Ar)	129.60	7.43
–C_6H═(Ar)	130.85	7.39
N–C_{11}H═	129.30	7.03
N–C_{10}H═	123.03	7.17/7.14
O–CH	77.75	5.17
O–$C_{15}H_2$	72.44/72.31	5.24/5.17
C_{16}H═CH_2	135.38/135.47	5.78
$C_{17}H_2$═CH	120.98/121.06	4.04/3.87
N–CH_2	52.98	4.35

Table 2. Chemical Shift Values for β-CD in the Free and in the Complex State Measured in H_2O

proton	β-CD	β-CD/IMZ	$-\Delta\delta$[a]
H_1	5.075	5.065	0.010
H_2	3.652	3.653	0.006
H_3	3.973	3.873	0.100
H_4	3.857	3.855	0.002
H_5	3.878	3.769	0.109
H_6	3.891	3.861	0.030

[a] $\Delta\delta$ represents the chemical shift differences between the two states.

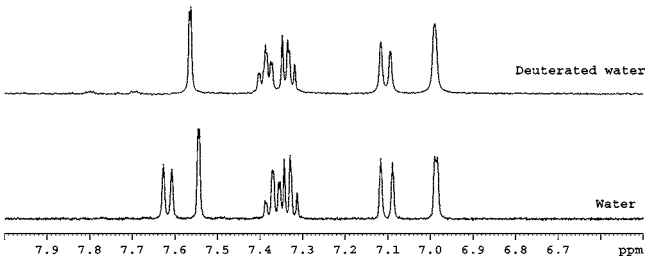

Figure 3. ^{1}H NMR spectra of IMZ/β-CD complex recorded in pure (bottom) and deuterated water (top): 1D aromatic region expansion is represented.

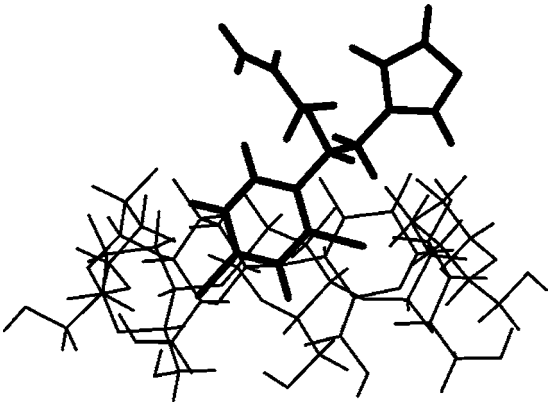

Figure 4. IMZ/β-CD complex structure stick representation of one molecule obtained after RMD and REM.

sponding attached carbons C_{13}, C_{15}, and C_{17} (see **Table 1**) and by C_{16}, thus suggesting the presence of diastereomeric complexes arising from the IMZ racemic form. Therefore, a chiral host−guest interaction (*26*, *27*), due to the stereogenic C_7 center, has to be considered. The ratio of the duplicated protons did not change when the NMR experiment was carried out at different times. The IMZ/β-CD complex stability can be followed in both water and D_2O by NMR during time evolution. The 1D ^{1}H spectrum recorded 18 h after sample preparation showed significant shifts for all IMZ proton resonances, in combination with different integral ratios between IMZ and β-CD protons, thus indicating that modifications of the complex are in progress, most likely due to the slow release process.

Analysis of dipolar correlation experiments (NOESY and ROESY) revealed strong intermolecular interactions among protons H_3/H_5 of β-CD and H_3 of IMZ and to a lesser extent between protons H_3 of β-CD and H_6 of IMZ, respectively. It is worth noting that these NOEs were not distinguishable for each single sugar moiety due to the β-CD structural symmetry. These three intermolecular NOEs in combination with another nine intramolecular NOEs have been weighed on the basis of their intensity, converted into qualitative distance constraints, and used in RMD and REM protocols (*28*). The use of molecular dynamics (*29*) takes into account the known CD flexibility and gives much more reliable results. In the starting complex structure for the restrained molecular dynamic protocol, guest and host were initially located far away from each other, facing the larger rim of CD, and the complex formation was driven by applying the NMR constraints. In particular, the introduction of the guest molecule in the CD cavity took place from the wider secondary hydroxyl groups side, as classically described. To account for bimodal complexation, we have also computed the starting complex structure with IMZ facing the narrow rim of CD. The final complex structures presented the same properties from the energetical point of view of the previous complex structures, but on the basis of distance violation with respect to the NMR constraints imposed, only the complex structure obtained from large-rim introduction can be accepted. In particular, the complex derived from larger rim inclusion displayed a violation of ~0.4 Å for distance restraint between H_5 of CD and H_3 of IMZ for only 2 of 50 computed structures, whereas the complex derived from narrow-rim inclusion displayed violations of up to 2 Å for distance restraint between H_3/H_5 of CD and H_6 of IMZ for all structures computed. This result strongly supports the existence in solution of the mostly populated complex derived from large-rim inclusion.

The resulting structure of the IMZ/β-CD complex is represented in **Figure 4** (only one is represented for clarity) and consists of the aromatic phenyl moiety inserted into the toroid template of β-CD, whereas the allylic chain and the imidazole ring are distributed close to the larger rim. In particular, the axis connecting protons H_6 and H_3 of IMZ lies on the C7 symmetry axes of the β-CD, fully consistent with the experimental NMR data. The IMZ is quite deeply inserted into the hydrophobic macrocycle, as confirmed by the affected chemical shift values of H_6 β-CD protons located at the narrow end of the toroid. Furthermore, comparison of the complexes obtained with the use of the two different starting structures showed a preferential imidazole group orientation stabilized by H bonds among external hydroxyl groups of β-CD and one/both nitrogen atoms of the guest molecule (N_9/N_{12} of imidazole ring) only for the complex with the large-rim inclusion mechanism. The other possible insertion mechanism explored (via the narrow rim) did not result in any complex structures stabilized by H bonds, most likely due to a less deep insertion mode.

Conclusions. The complete analysis of the IMZ/β-CD complex in solution was investigated by ^{1}H and ^{13}C NMR techniques in D_2O and H_2O without further treatment (*30*). Two diastereomeric IMZ/β-CD complexes have been observed as well as the presence of a privileged conformer. The solution structure of the complex was determined by NMR techniques in combination with computational methods that allowed important information concerning the insertion of the IMZ molecule into the β-CD toroid to be obtained. The aromatic ring is deeply inserted in the cavity, whereas the allyl group and the imidazole ring were excluded from the cavity, but the latter was in close contact with the larger rim. In particular, in all of the complex structures the imidazole group orientation was stabilized by H bonds among hydroxyl groups of β-CD and even both nitrogen atoms of the guest (N_9/N_{12} of imidazole ring). Interestingly, the rapid exchange of the acid H_{13} proton of the imidazole ring with bulk deuterons occurred as observed by comparison of proton NMR spectra recorded in D_2O and H_2O, respectively. The possible presence of water molecules, which came from the IMZ/β-CD complex preparation, improves the acidity of H_{13}, and therefore the proton−deuteron exchange takes place with a different time scale in the complex obtained under supercritical carbon dioxide conditions (*25*).

Despite the wide use of β-CD in therapy, food, cosmetics, and toiletry (*7*, *9*, *31*), there has been little investigation of its use in agro-food formulations (*11*, *32*−*34*). These latter applications have not been largely developed because of the huge amount of inclusion complexes required and the lack of their structural characterizations in aqueous solutions. The inclusion modality of the IMZ into the β-CD from the aromatic side allows other β-CD complexes with synthetic imidazoles useful in agriculture (*35*) to be investigated. Studies are in progress in our laboratories.

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