^{1}H NMR and Molecular Modeling Study on the Inclusion Complex β-Cyclodextrin−Indomethacin

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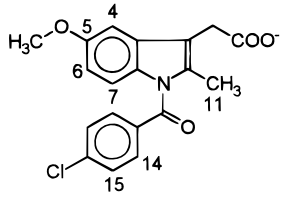
The solution structure of the inclusion complex between β-cyclodextrin (**2**) and the nonsteroidal anti-inflammatory agent indomethacin sodium salt (**1**) [1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindoleacetic acid sodium salt] is investigated in D_2O solution via ^{1}H NMR spectroscopy. The guest molecule **1** exists in solution as a mixture of *E* and *Z* isomers in fast exchange on the NMR time scale, as shown by NOE experiments. Low-temperature ^{1}H NMR spectra on indomethacin methyl ester (**3**) (soluble in CH_2Cl_2-d_2) showed that the *Z* isomer is the most thermodynamically stable. Geometrical features of the host−guest inclusion complex between **2** and **1** are inferred from intermolecular dipolar contacts obtained by 1D NOE difference spectra and 2D ROESY experiments. A more detailed picture of the solution structure of the complex is obtained by combining NMR structural information and molecular dynamics and energy calculations on the inclusion complexes. Computations took into account the existence of two diastereomeric forms of the guest (*E* and *Z*), the two possible sites of interaction of the guest molecule with the cavity (*i.e.* the *p*-chlorobenzoyl ring and the indole ring system), and the different topologies for the entry of the guest into the host's cavity. The main finding that can be obtained from both experimental and theoretical data is that the complexation selectively stabilizes the *E* isomer with respect to *Z*. The inclusion complex is characterized by the interaction of the *p*-chlorobenzoyl moiety of **1***E* with the lipophilic cavity of the host **2**, the entry being through the larger rim of the truncated cone of **2**.

Introduction

The inclusion complex chemistry with cyclodextrins (cyclohexa-, cyclohepta-, and cyclooctaamylose, respectively, αCD, βCD, and γCD) is concerned with small molecules with hydrophobic regions which, under the influences of noncovalent interactions, can be accommodated in the host cavity, leading to structures of higher complexity in the solid and solution state. These complexes can increase the stability and improve the solubility and bioavailability of molecules of pharmaceutical interest.[1,2] That stimulated a great deal of research toward the synthesis of new host−guest complexes and the structural characterization of the supramolecular adducts in terms of geometry and conformational preferences *via* a variety of physical methods, with solid state and high-resolution NMR playing primary roles.[3]

Among the several hundred cyclodextrin−drug inclusion complexes prepared and patented, the adduct of the nonsteroid anti-inflammatory indomethacin sodium salt (**1**) [1-(*p*-chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid sodium salt] with CDs[4,5] and their derivatives[9]



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was one of the most widely studied. Although a complete characterization of the complex of **1** with βCD (**2**) in terms of stoichiometry, association constant, and stability has been fully accomplished in aqueous solution by ^{1}H NMR, UV spectroscopy, and circular dichroism, a detailed description of the geometry of the complex in solution remains unreported, expecially as far as the mode of inclusion and the conformation of the drug within the host cavity are concerned. Indeed literature data[4,6−8] do not lead to unequivocal conclusions on this issue. We present here some results of a conformational study of the complex in solution using intermolecular nuclear Overhauser enhancement in the laboratory frame (NOE)[10] and in the rotating frame (ROE).[10] The geometry inferred from experimental data is further supported by energy calculations carried out on eight possible topologies for the inclusion complex.

Results and Discussion

A classical NMR method for the investigation of host−guest compounds relies upon the analysis of chemical

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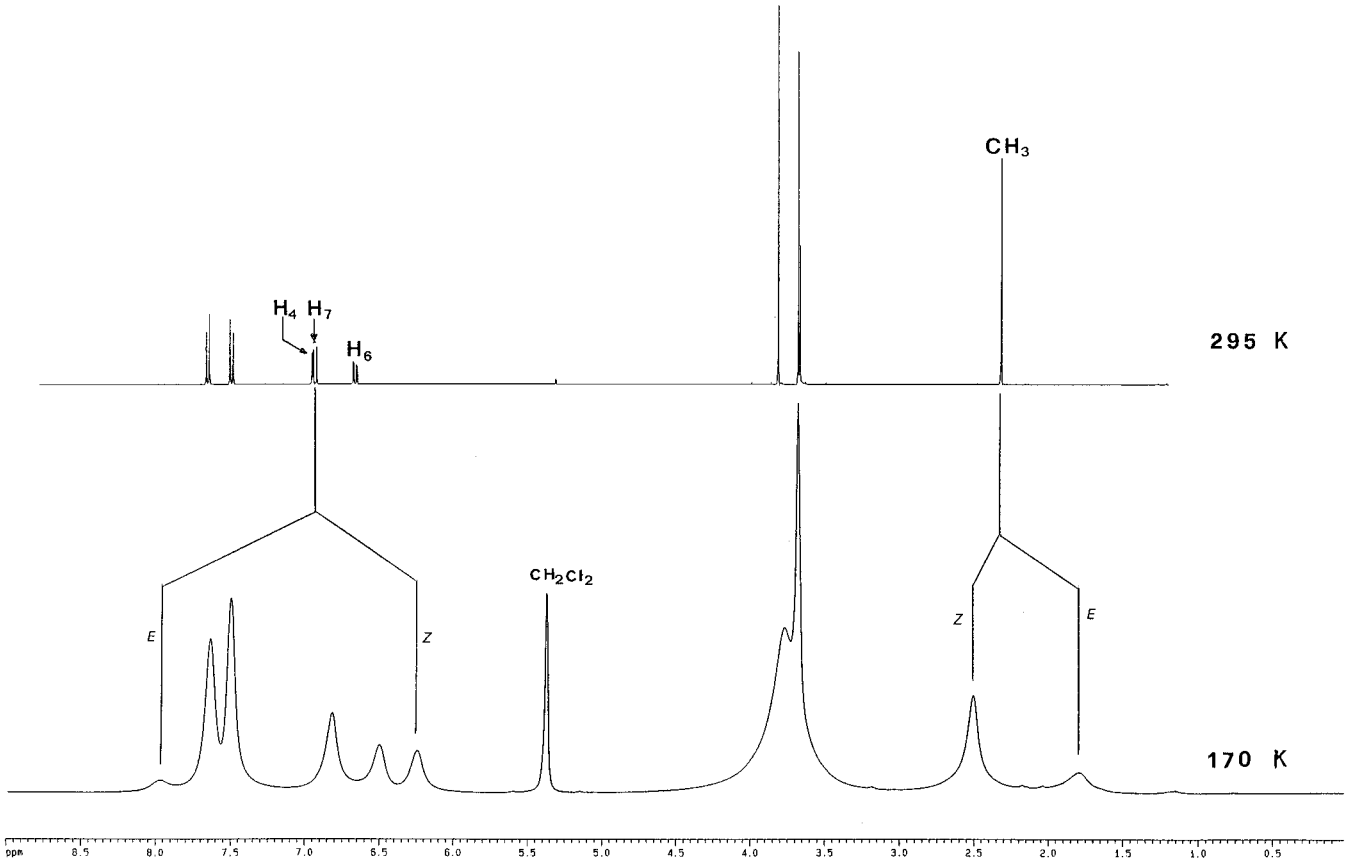


Figure 1. ^{1}H NMR spectrum of **3** in CD_2Cl_2. Upper trace: 295 K. Lower trace: 170 K.

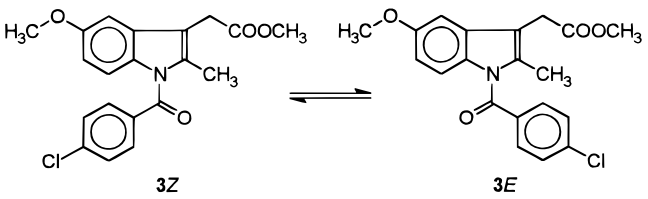
Table 1. Proton Chemical Shifts[a] of Indomethacin Sodium Salt (1)

proton	free **1**	1:1 complex of **1** with **2**
Me11	2.18	2.12
$CH_2$10	3.53	3.59
OMe	3.84	3.88
H6	6.67	6.85
H7	6.95	7.33
H4	7.04	7.08
H14,18	7.64	7.68
H15,17	7.55	7.58

[a] Chemical shifts in ppm from external DSS (sodium 2,3-dimethyl-2-silapentane-5-sulfonate); solvent D_2O; temperature 298 K.

shift variations of protons directly involved in the interaction: in the case of aromatic guests, H3′ and H5′ of the glucose units, namely the protons inside the cavity, undergo high-field shifts due to anisotropic shielding by the aromatic ring of the guest, whereas the protons of the guest that interacts with the nonpolar cavity of βCD experience low-field shifts.[11] A selection of chemical shift data for **1** in its free form and complexed with **2** is presented in Table 1. Chemical shift variations of all the aromatic protons of **1** in the presence of βCD are a clear evidence for the formation of the complex. However the chemical shift variation (up to 0.4 ppm) for H7 of **1**, already reported in literature,[7] is unexpectedly large in comparison to the shifts usually observed for aromatic protons upon inclusion and therefore can hardly be entirely attributed to the intermolecular effects of the host−guest interaction only. On the other hand **1** exhibits conformational flexibility in solution and can

Scheme 1. *E/Z* Equilibrium for Indomethacin Methyl Ester (3)



exist in two diastereomeric forms (Scheme 1) due to hindered rotation about the partial C−N double bond: the *E* isomer, with the *p*-chorobenzoyl ring *syn* to Me11, and the *Z* isomer, with the *p*-chlorobenzoyl ring in spatial proximity to H7. Single-crystal X-ray diffraction studies[12] and theoretical calculations[13] showed that the *Z* isomer is the most thermodynamically stable form of indomethacin. This latter form appeared to be the dominant isomer in solution as well (*vide ultra*). Steady-state NOE experiments carried out on the free molecule allowed us to prove the presence of both isomers in solution in fast exchange on the NMR time scale (data not reported here). In order to obtain detailed informations on the chemical shift of the pure *E* and *Z* conformers, low-temperature proton spectra were carried out on indomethacin methyl ester (**3**), a functional derivative of **1** showing good solubility in CH_2Cl_2-d_2, a suitable solvent for low-temperature experiments. An array of ^{1}H NMR spectra was collected from room temperature to 170 K. The spectrum at this temperature is reported in Figure 1 together with the control recorded at room temperature.

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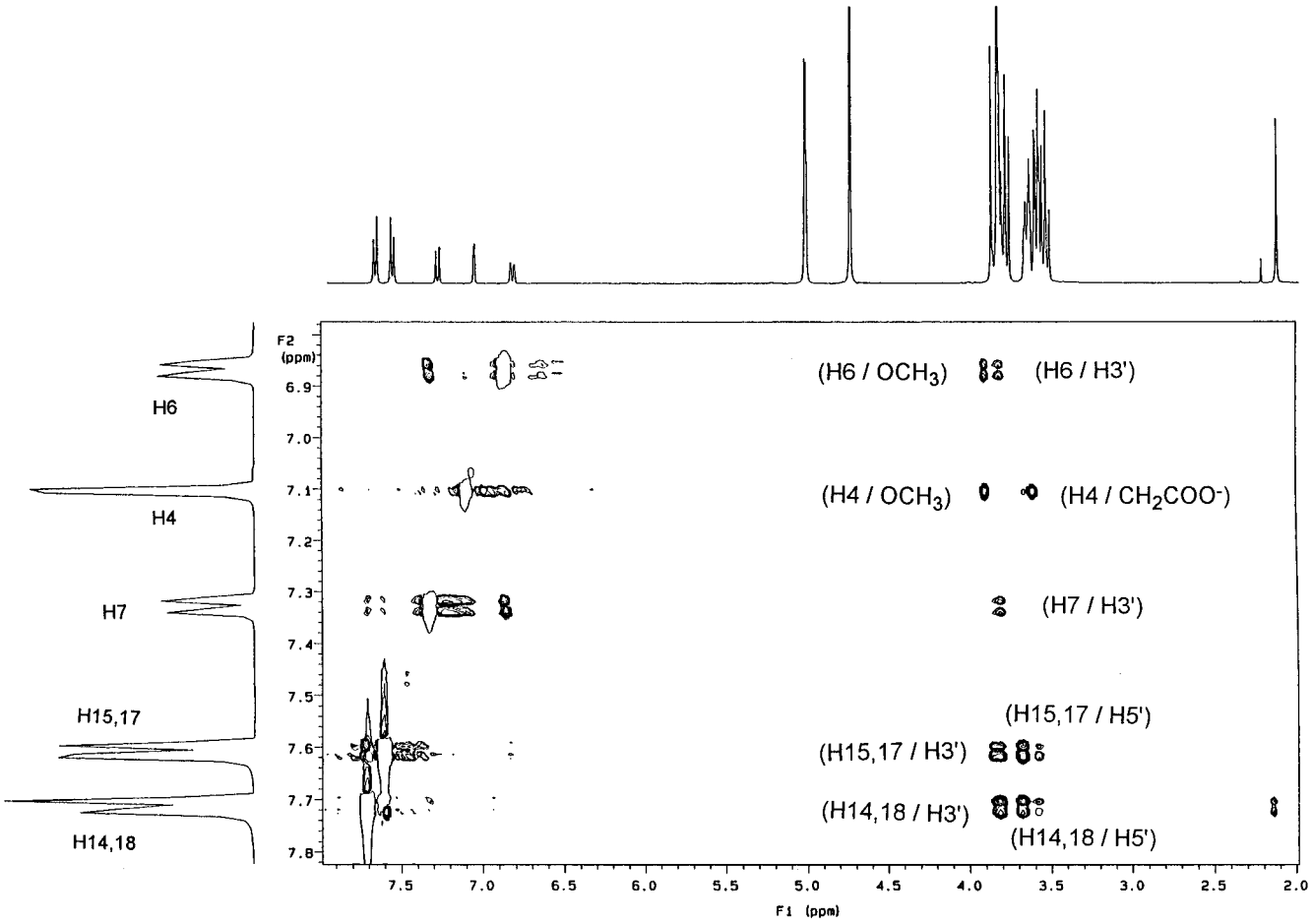

Figure 2. Expansion of 2D ROESY spectrum of the complex. Cross peaks labeled (H*a*/H*b*) indicate dipolar contact between proton *a* and proton *b*.

At 170 K the spectrum displays a splitting of the signals of H7 (7.9 and 6.5 ppm) and of the Me11 group (2.5 and 1.6 ppm) indicating that the rotation about the amide bond is slow on the NMR time scale. As the carbonyl group is known to strongly deshield nearly coplanar hydrogen atoms,[14] the signal at 7.9 ppm can be stright-forwardly assigned to H-7 of *E* and the broad singlet at 6.3 ppm to H-7 of the *Z* isomer. A complementary pattern is found for the chemical shift of Me11: low-field shift for Me11 of *Z*, high-field shift for Me11 of *E*. Thus the downfield shift experienced by the aromatic protons, in particular H7, of the indole moiety, and the upfield shift showed by the methyl group of **1** after complexation with the host **2** is likely to be accounted for by a change in the conformer population of the guest molecule in such a way as to favor the *E* isomer, which is expected to exhibit the two aromatic ends in a more favorable conformation for the entry into the cyclodextrin cavity with respect to the *Z* isomer.

A more detailed picture of the solution geometry of the host−guest complex can be obtained by measuring intermolecular nuclear Overhauser enhancements (NOEs). Often these complexes fall in the region where $\omega\tau_c$ is close to 1 and consequently small NOEs are expected. Therefore we decided to perform the experiments either in the laboratory frame or in the rotating frame. The expansion of the 2D ROESY spectrum (Figure 2) shows the aromatic region of the spectrum along the F2 dimension and the whole spectrum along F1. Both protons belonging to the *p*-chlorobenzoyl ring give rise to intense cross peaks with H3′ and H5′ of β-cyclodextrin, demonstrating the full involvement of this end of the molecule in the complexation. The intramolecular cross peak between H14 and Me11 suggests a large contribution of the *E* isomer to the complex. Interestingly, also H6 and H7, belonging to the six-membered ring of the indole moiety, exhibit dipolar contacts with H3′ of cyclodextrin, while H4 experiences intramolecular dipolar contacts only. We also carried out some $^1H\{^1H\}$ transient NOE experiments[10] which can provide a semiquantitative picture of the degree of penetration of the *p*-chlorobenzoyl and indole ring into the host cavity. These experiments were run at a higher temperature (310 K) in order to increase τ_c and thus reach the positive NOE regime. The inner protons H3′ and H5′ of cyclodextrin were selectively inverted, and the intensities of the various aromatic protons in the NOE difference spectra were plotted as a function of the mixing time. The slope of the NOE buildup curve for null mixing time is proportional to the reciprocal of the sixth power of the internuclear distance of the protons dipolarly coupled; hence this approach can provide information on the spatial relationships between host and guest. However, the possibility of a quantitative evaluation of the internuclear distances is severely hampered by the intrinsic complexity of the system, characterized by one of the components, the guest, partly free and partly included in the other and also undergoing

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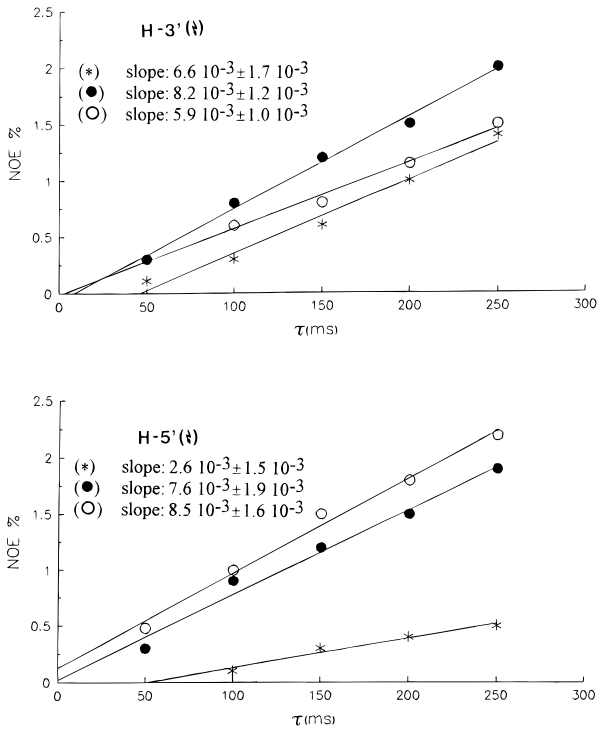

Figure 3. Top: NOE buildup curves for the guest's protons H7 (*), H14,18 (○), and H15,17 (●) after irradiation of H3′ of the host. Bottom: NOE buildup curves for the guest's protons H7 (*), H14,18 (○) and H15,17 (●) after irradiation of H5′ of the host.

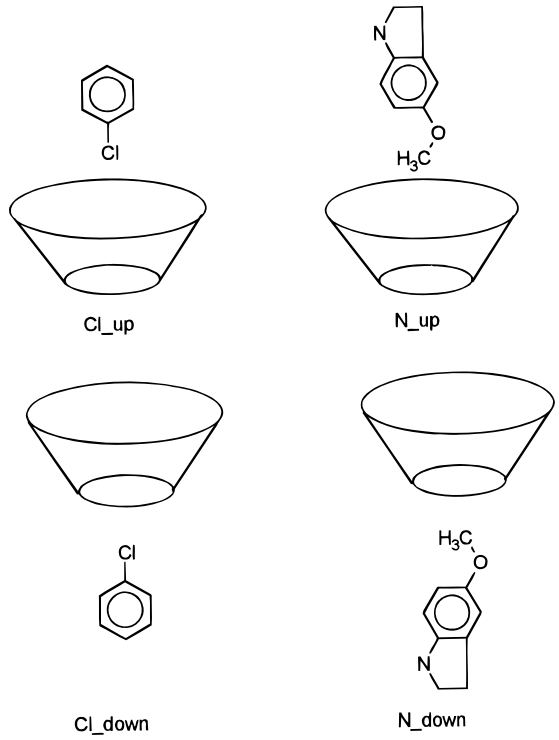

Figure 4. Sketch of the different topologies for the entry of the guest **1** into the cavity of the host **2**.

an oscillatory motion inside the cavity leading to periodic variation of the internuclear distances about the equilibrium position. Figure 3 displays, from top to bottom, the linear region of the NOE buildup curves for H14, H15, and H7 obtained perturbating H3′ and H5′, respectively. For H7, belonging to the six-membered ring of the indole moiety, the curve at the top shows a slope greater than that for the curve at the bottom, indicating that H7 lies close to the large rim of the torus-shaped host. As far as the protons belonging to *p*-chlorobenzoyl ring (H14 and H15) are concerned, the NOE buildup curves obtained by irradiating both H3′ and H5′ (Figure 3) show approximatively the same slope, indicating that the benzoyl ring is deeply inserted into the host's cavity.

On the basis of the experimental data, the solution geometry of the complex cannot be unambiguously assessed and several different hypotheses on the solution geometry of the complex can be formulated. We believe that at least the following two can be regarded as reasonable: (i) the presence of two diastereomeric 1:1 adducts, each one involving inclusion of a different end of the molecule, with the complex with the *p*-chlorobenzoyl ring inserted being predominant, or (ii) the presence in solution of a single complex, presumably indomethacin *E*, with the guest in a suitable conformation for the interaction with the lipophilic cavity through both the *p*-chlorobenzoyl ring and part of the indole moiety. To test the reliability of these hypotheses, we carried out calculations on all the possible complexes originating from indomethacin *E* and *Z* with *β*-cyclodextrin taking into account the different modes of inclusion. A conformational search was attempted by employing simulated annealing and constant temperature molecular dynamics with periodical temperature jumps (see section Computational Details). Indeed, **1** possesses two different lipophilic parts, namely the phenyl ring and the indole ring, able to interact with the nonpolar cavity of CD, giving rise to two different modes of inclusion. Moreover the entry of **1** may occour through either the larger or the smaller rim of the truncated cone of CD, giving rise to four different topologies, as shown in Figure 4. The nomenclature used to designate the different topologies will be referred to as Cl_up, Cl_down, N_up, and N_down. The overall number of binding topologies totals eight taking into account the equilibrium mixture of *E* and *Z* isomers. The results of global energy minima for the eight possible topologies are reported in Tables 2 and 3.

Table 2. Calculated Energy (kcal mol^{-1}) of the Complexes of *β*-Cyclodextrin (2) and Indomethacin *Z* (1*Z*)

conformer	topology	$E_{tot.}$	$E_{interaction}$	E_{bond}	E_{vdW}	$E_{Coulomb}$
i	*β*CD+1*Z*	296.36		131.64	116.53	48.20
ii	Cl_up	218.76	−77.60	133.61	82.96	2.19
iii	N_up	215.14	−81.22	139.02	95.37	−19.25
iv	Cl_down	249.06	−47.30	145.43	79.53	24.10
v	N_down	239.45	−56.91	137.90	83.23	18.32

Table 3. Calculated Energy (kcal mol^{-1}) of the Complexes of *β*-Cyclodextrin (2) and Indomethacin *E* (1*E*)

conformer	topology	$E_{tot.}$	$E_{interaction}$	E_{bond}	E_{vdW}	$E_{Coulomb}$
vi	*β*CD+1*E*	297.13		131.53	117.13	48.47
vii	Cl_up	213.10	−84.03	140.37	85.51	−12.78
viii	N_up	215.90	−81.23	138.02	93.65	−15.77
ix	Cl_down	236.52	−60.61	139.26	81.50	15.76
x	N_down	236.04	−61.09	138.21	81.07	16.77

Conformers i and vi represent the reference system, namely the two molecules at infinite distance. The corresponding energy values are therefore the sum of the calculated energies of host and guest when apart. The bond, van der Waals, and Coulombic contributions to the total energy are reported for all the calculated geometries. The entry of the guest through the smaller rim of CD can be ruled out on the basis of the high values of the total energy (conformers iv, v, ix, and x). The most

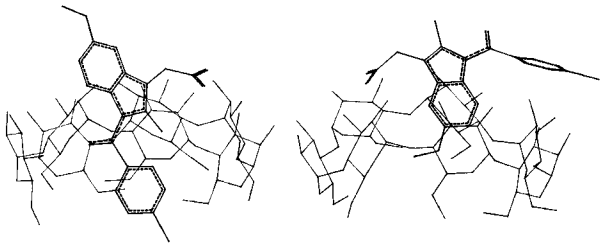

Figure 5. Left: energy-minimized structure of the complex **1***E*−**2**. Right: energy-minimized structure of the complex **1***Z*−**2**.

favourable interaction energies are obtained for the topologies Cl_up and N_up for both *Z* and *E* isomers. Hence, the *Z* isomer interacts with **2** mainly through the indole ring (conformer iii), while for the *E* isomer the *p*-chlorobenzoyl ring penetrates inside the torus of the host molecule **2** (conformer vii). The graphical output for the calculations of the geometries of complexes iii and vii is displayed in Figure 5. The remaining two low-energy conformations, ii and viii, are not expected to be appreciably populated at room temperature as they differ by 3.62 and 2.80 kcal mol^{-1} from the most stable conformations, iii and vii, respectively. The most important feature arising from the energy calculation is that the complexation of indomethacin **1** with β-cyclodextrin causes a reversal in the thermodynamical stability of the two isomers of the guest. CVFF calculations on the isolated guest gave a total energy for indomethacin *Z* 0.77 kcal lower than for indomethacin *E*, in good agreement with our solution state measurements at low temperature and with reported solid state[12] and gas phase investigations.[13] Conversely, Tables 2 and 3 show that the energy of the complex host−**1***E* (conformer vii) is 2.04 kcal more stable than the corresponding adduct of **1***Z* (conformer iii). van der Waals interactions appear to be the most important stabilizing factor of the complex of indomethacin *E* (E_{vdW} (vii) − E_{vdW} (iii) = −9.86 kcal); these favorable interactions are only partially compensated by the major ability of the carboxylate group of the *Z* isomer of indomethacine to establish H-bonds with the secondary OH groups located on the larger rim of the cyclodextrin torus, as demonstrated by the coulombic contribution to the total energy ($E_{Coulomb}$ (vii) − $E_{Coulomb}$ (iii) = +6.47 kcal). These data lead therefore to the conclusion that free **1** exists in solution mainly as the *Z* isomer, while **1** complexed with **2** is mainly in the *E* form. However, since the energy data are obtained by *in vacuo* calculations they can only be used in a semiquantitative way. Any attempt to derive a more quantitative picture (*e.g.* Boltzmann populations) should be avoided since solvatation effects, not taken into account in the calculations, are expected to play a role. Calculations also provide a rationale for the unexpectedly large deshielding experienced by H7 of **1** upon complexation: the low-field shift is due to two different effects that coherently sum up, the complexation and the stabilization of the *E* form of **1** in the complex.

Interproton distances between H3′ and H5′ of the seven glucose units of β-cyclodextrin and H7, H6, and H4 of **1** measured on the minimized geometries of vii and iii are consistent with the ROEs and transient NOE data (we assumed 4.5 Å as cutoff distance for detectable Overhauser enhancements). The complex with the lowest energy, **1***E*−βCD (vii), showed nonbonded distances of H7 of indomethacin with the seven H3′ and H5′ of the host in the range 8.71 to 2.19 Å and 8.54 to 4.11 Å, respectively. The NOE buildup curves shown in Figure 3 provide experimental evidence that the distance H7−H3′ is greater than H7−H5′, as correctly reproduced by the geometry optimization. A more interesting picture comes out if we compare the distance H4−H3′ for both complexes, namely vii and iii: in the former it ranges from 8.76 to 5.35 Å, therefore outside a suitable interval of distances for the buildup of NOE, and in the latter spans the range 6.40−2.60 Å. The presence of a significant amount of the complex indomethacin *Z*−β-cyclodextrin in solution would be consequently proved by an intense dipolar correlation between H4 and H3′ in the ROESY spectrum. Experimentally no intermolecular ROE between H4 and H3′ was observed.

Conclusions

Free indomethacin **1** exists in solution as a mixture of *E* and *Z* isomers, the latter being the prevalent form, as demonstrated by low-temperature NMR spectra carried out on the methyl ester **3**. Complexation of **1** with βCD (**2**) leads to a selective stabilization of the *E* isomer with respect to *Z*; hence the major complex in solution is **1***E*−βCD. This conformational change was experimentally proven by chemical shift varations on the guest molecule upon complexation, intermolecular NOEs and ROEs, and energy calculations. The solution conformation of the inclusion complex is characterized by the *p*-chlorobenzoyl ring of **1***E* deeply inserted into the lipophylic cavity of the host, the entry occurring through the larger rim of the βCD truncated cone.

Experimental Section

^{1}H NMR spectra were recorded at 200.13, 300.13, and 400.13 MHz. The ROESY experiment on the complex was performed using a mixing time of 500 ms.

The variable temperature spectra were carried out on indomethacin methyl ester **3** in the temperature range 298−170 K in CH_2Cl_2-d_2 (Merck Co. Italy).

1-(*p*-Chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic Acid Sodium Salt (1). Indomethacin was obtained from Sigma Co. (Italy) and converted into sodium salt **1** by NaOH titration. The water solution was then freeze-dried, dissolved in D_2O, and freeze-dried two times. The NMR characterization is reported in Table 1.

Complex of the Indomethacin Sodium Salt with β-Cyclodextrin. A 5.3 mM solution of **1** in water was added to a 5.3 mM solution of **2**. The reaction mixture was allowed to stand overnight and then freeze-dried. The solid was in turn redissolved in D_2O and freeze-dried two times. A selection of the ^{1}H NMR data for the complex are reported in Table 1.

1-(*p*-Chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid Methyl Ester (3). 1-(*p*-Chlorobenzoyl)-5-methoxy-2-methylindole-3-acetic acid (206 mg) dissolved in 5 mL of 2,2-dimethoxypropane was added of 0.5 mL of 37% HCl. The solution was stirred at room temperature until the reaction was complete (ca. 2 h), as monitored by TLC. Successively the solvent was evaporated at reduced pressure and the crude material suspended in water and extracted several times with ethyl acetate. After flash cromatography (eluant hexane−EtOAc = 75:25) 192 mg of a pale yellow solid was obtained, with an overall yield of 90%: mp 92−93 °C; ^{1}H NMR (CH_2Cl_2-d_2) δ 7.65 (m, 2H), 7.49 (m, 2H), 6.94 (d, *J* = 2.6 Hz, 1H), 6.93 (d, *J* = 9.1 Hz, 1H), 6.66 (dd, *J* = 2.6 and 9.1 Hz, 1H), 3.81 (s, 3H), 3.68 (s, 3H), 3.67 (s, 2H), 2.32 (s, 3H).

Computational Details

Molecular modeling and dynamics simulations were carried out on a Silicon Graphics Personal IRIS 4D-35

workstation. The force field used for energy minimizations and molecular dynamics (MD) calculations was CVFF[15] as implemented in the BIOSYM software[16] packages Insight II (release 3.1) and Discover (release 2.9). No attempt to include solvent effects was done in any calculation. The dielectric constant was set to 1.0.

Calculations on the Guest Molecules. The geometries of indomethacin *E* and *Z* were built from scratch using library molecular fragments. The input geometry was first minimized using the steepest-descent minimizer to a maximum energy gradient of 0.05 kcal Å^{-1}; partial atomic charges were then calculated using the output of the first minimization as the input for a semiempirical SCF-MO calculation using the AM1 hamiltonian[17] (MOPAC[18] 6.0 distributed with Insight II). A final molecular mechanics energy minimization was then performed using Discover (quasi-Newton−Raphson minimizer to a maximum energy gradient less then 0.001 kcal Å^{-1}). The total energy difference between isomers *E* and *Z* was ($E_E - E_Z$) = 0.78 kcal mol^{-1}.

Calculations on the Host Molecule. The starting geometry of β-cyclodextrin was generated by connecting together with α-(1→4) glycosidic linkage seven D-glucopyranose monomers taken from the Insight II fragment library. This structure was in turn minimized with the CVFF force field. The geometry obtained at this stage did not show radial symmetry. It is known that the lower energy conformations of β-cyclodextrin do not show a perfect C_7 symmetry.[19] However, symmetrical structures can be regarded as a time-average of molecular conformations and are good models for simulating the solution behavior on the NMR time scale. Highly symmetrical computer models of cyclodextrins reported in the literature were obtained by constrained energy minimization[19,20] (*e.g.* fixing the glycosidic angles $C(1)_n-O(4)_{n+1}-C(4)_{n+1}$ and the distances $O(2)_n-O(3)_{n+1}$ at the average values taken from X-ray diffraction data[21]) or by force field minimization[22] starting from experimental atomic coordinates. We obtained a family of conformations showing a 7-fold symmetry through a short molecular dynamics simulation using the following protocol: 10 ps equilibration, 50 ps dynamics with minimization every ps (5000 iterations, steepest-descent, energy gradient ≤ 0.5 kcal Å^{-1}), and archiving of the structure. Four out of the fifty archived geometries showed the desired C_7 symmetry and were in turn allowed to fully relax (10000 iterations, quasi-Newton−Raphson minimizer, energy gradient ≤ 0.01 kcal Å^{-1}), obtaining structures very close in total energy (8 cal maximum energy difference). A quite large variety of geometrical descriptors can be used to characterize the conformation of cyclodextrins;[23] among them we considered the average value of the seven dihedral angles defined by four adjacent acetal oxygens (*e.g.* $O_A-O_B-O_C-O_D$, $O_B-O_C-O_D-O_E$, etc.), supposed to be zero for a perfectly planar arrangement. Our minimized structure showed an average positive dihedral = 2.4° and an average negative dihedral of −3.1°; we also took into account the deviation from C_7 symmetry by considering the angles between three consecutive acetal oxygens (e. g. $O_A-O_B-O_C$, $O_B-O_C-O_D$, etc.). The ideal value for a perfect heptagon is 128°, the average obtained in this work was 128.6°.

Calculations on the Complex. All the complexes were generated docking the guest to the host in the suitable orientation. In all cases the origin of a cartesian reference frame was placed on to the center of mass of CD and the *z* axis aligned with the C_7 symmetry axis of CD. A second reference frame was placed on the indomethacin molecules with the *z* axis passing through the Cl−C bond (to generate the adducts Cl_up and Cl_down) or with the O(5)−C(5) bond (to generate the adducts N_up and N_down). The *z* axis of the two frames were in turn aligned, and the energy of the ensemble CD−indomethacin was minimized with respect to translation along the mutual *z* axis and with respect to rotations around the same axis. At the end of this procedure we obtained eight starting geometries for MD simulations. The conformational search *via* molecular dynamics was performed using two different protocols: simulated annealing[24] and constant temperature dynamics with periodic temperature jumps.[25] In the first approach simulated annealing was done from 300 to 50 K in 50 K steps, 12 ps of simulation for each temperature step and minimization of the structure at the end of the cycle. The minimized structure was archived and used for the next cycle of simulated annealing. Five complete cycles were carried out. The second protocol consisted of equilibration at 300 K for 10 ps then 8 ps MD at the same temperature with minimization every ps, temperature jumps at 600 K for 4 ps to provide enough energy to pass conformational barriers, repetition of this cycle four times (overall length of MD: 48 ps, 32 minimized structures archived). Both methods were used for each calculation. The energy difference between the global minimum obtained by the first and the second protocol ranged between 40 and 300 cal. The lowest energy values were generally obtained by simulated annealing.

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