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CHARACTERIZATION OF SIMVASTATIN AND ITS CYCLODEXTRIN INCLUSION COMPLEXES BY ABSORPTION AND CIRCULAR DICHROISM SPECTROSCOPIES AND MOLECULAR MECHANICS CALCULATIONS

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The spectral properties of simvastatin (SIM) in organic and aqueous media are reported and discussed on the basis of its electronic features investigated via semiempirical PM3 and molecular mechanics MM+ calculations. The interaction of SIM with α-, β-, 2-hydroxypropyl-β- and γ-cyclodextrin (CyD) was studied by UV absorption and circular dichroism (CD) spectroscopies. The hyperchromism of the absorption bands in presence of CyD provides evidence for the complexation process. Complexation also produces significant modifications in the CD spectrum of SIM, correlated to the parallel and antiparallel orientations of the transition moment of the SIM molecule with respect to the CyD symmetry axis. Non-linear regression analysis was used to determine the stoichiometries and association constants of the complexes. A discussion on the difficulty to distinguish between the 1:2 stoichiometry and a mixture of 1:1 and 1:2 stoichiometries, in the experimental conditions imposed by the CyD solubility, was made. The experimental data on the inclusion complexes were correlated with the results of in vacuo and water-dependent molecular mechanics calculations.

INTRODUCTION

Simvastatin (SIM) is a hypolipidemic drug belonging to the statins class, used to control hypercholesterolemia and to prevent cardiovascular diseases. Its structure (Figure 1) consists of three main fragments: dimethylbutanoate (DMB), dimethyl-hexahydronaphtalene (HHN) and ethyl-hydroxy-tetrahydropyranone (THP), joined by flexible bonds characterized by the dihedrals shown in Figure 1 and hereafter labeled as $\varphi_1 - \varphi_4$. SIM is very lipophilic and is found in the form of an inactive lactone that is rapidly hydrolyzed after ingestion to produce the active agent, simvastatin acid.

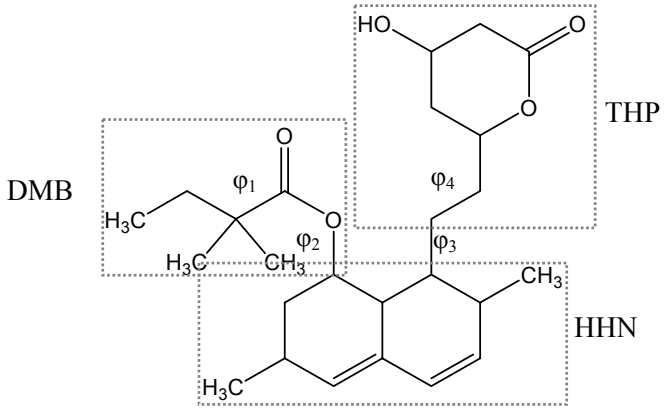

Fig. 1 – Molecular structure of simvastatin (DMB – dimethylbutanoate, HHN – dimethyl-hexahydronaphtalene and THP – ethyl-hydroxy-tetrahydropyranonyl moieties). $\varphi_1 - \varphi_4$ denote the main dihedrals along the molecular backbone.

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Due to their unique structural features, cyclodextrins (CyDs) have become an indispensable tool for the pharmaceutical industry, being commonly used to improve drug physicochemical properties, such as solubility, stability and bioavailability.[1] CyDs are cyclic oligosaccharides consisting of 6 (α), 7 (β) or 8 (γ) glucopiranose units linked α-(1,4) and forming a relatively hydrophobic cavity, with a hydrophilic outer surface, capable of incorporating various drug molecules.[2] A large number of CyDs modified by synthesis is also known.[3] CyDs are also optically active, thus offering a potential discrimination of enantiomeric compounds, as it is the case of most drug molecules.[4] They also act as carriers, delivering the necessary amount of drug to the target site.

The literature data on SIM and its CyD inclusion complexes are mostly related to their pharmacological use,[5] with relatively few data concerning their electronic and structural features.[6] Most up-to-date studies refer to innovative methods for their preparation, identification and characterization and involve techniques such as mass spectrometry,[6] high performance liquid chromatography/diode array detection,[7] derivative spectrometry, [8, 9] X-ray diffraction,[10] supercritical antisolvent technique[11] and solubility studies.[12]

The present study aims to determine the spectral properties of SIM in several media and to investigate, on both experimental and theoretical grounds, its electronic features and interaction with various CyDs.

RESULTS AND DISCUSSION

1. Spectral properties and theoretical calculations on the isolated simvastatin molecule

The main spectral features of the absorption spectra of SIM recorded in polar protic solvents (methanol and ethanol) and in a polar non protic solvent (DMSO) are listed in Table 1. As the inclusion process in CyDs is studied in a methanol:water 1:9 v:v mixture, the respective data are included too. The low solubility of SIM prevents determinations in a wider range of solvents. The spectrum consists of two bands characterized by a large difference in their intensity. The values of the molar absorptivity coefficient, $\varepsilon > 10^4$ M^{-1} cm^{-1} suggest that the first band, located at ~238 nm, is due to a π–π^* transition. The nature of the weak second band, located at ~280 nm, is more difficult to asses. It could be ascribed to an n–π^* transition but, as we have already mentioned, the low solubility of SIM does not allow the use of other solvents in order to obtain more information on this band. Therefore, all further experiments will be focused on the change in position/intensity of the electronic band at 238 nm.

Table 1

Spectral characteristics of SIM in the used solvents

Solvent	λ_a (nm)	ε ($M^{-1}cm^{-1}$)
Methanol	238	22452
Ethanol	238; 275	14512; 677
DMSO	275	1766
Methanol:water (1:9, v:v)	238; 278	18903; 1338

In order to obtain more information on the electronic structure of SIM, molecular orbital (MO) calculations were performed. As the presence of the single bonds ensures an enhanced flexibility of the molecule, a conformational search was firstly performed at both molecular mechanics (MM+) and semiempirical (PM3) levels; the dihedrals chosen for this purpose are the angles φ in Figure 1. The conformations found in an energy range of 5 kcal mol^{-1} are quite similar in what concerns these angles (φ values will be given in Table 4 in connection with the change of the molecular conformation upon inclusion in CyD).

The most important observation is that for all these conformations the energy and shape of the frontier MOs implied in the electronic transitions are the same. Both HOMO and LUMO are localized on the trans-butadiene skeleton of HHN and represent the bonding and antibonding orbitals between the two double bonds. We expect therefore that the electronic spectrum will be dominated by the π electron system of the HHN moiety and that its experimental change in the presence of CyD will reflect the perturbation of this part of the molecule by the inclusion process. Calculations predict the $S_0 \rightarrow S_1$ transition at

276 nm (oscillator strength 0.93), in a qualitative agreement with the experimental 238 nm value, considering the approximations in the computational method. The $S_0 \rightarrow S_1$ transition moment is located in the plane of the HHN moiety, parallel to the double bonds, as for the trans-butadiene molecule.

2. The inclusion complexes of simvastatin with cyclodextrins

2.1. Stoichiometries and association constants via absorption measurements

The changes in the spectral properties of the guest upon inclusion into the CyD cavity are due, on one hand, to the hydrophobic character of the cavity and, on the other hand, to the restrictions imposed by the host upon the guest's movement. In the case of SIM, an increase in the absorbance was observed upon inclusion, allowing for a better observation of the band at 280 nm, without any modifications in the position of the bands. A similar behavior was reported by El-Kemary *et al*,[13] in the case of a pyrimidine derivative. The authors considered the absorption increase as evidence for the occurrence of complexation. As an example, the evolution of the SIM absorption spectrum in presence of 2-hydroxypropyl-*β*-CyD (2-HP-*β*-CyD) is given in Figure 2.

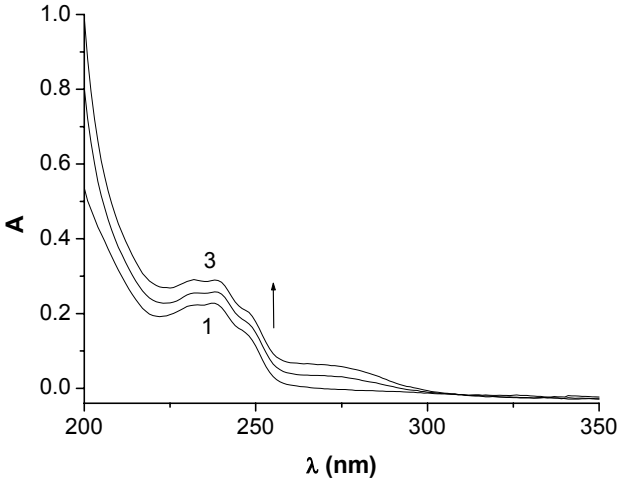

Fig. 2 – Absorption spectra of SIM in absence and presence of various 2-HP-*β*-CyD concentrations: (1) 0 M, (2) $9.46 \cdot 10^{-3}$ M and (3) $1.65 \cdot 10^{-2}$ M.

The association constants, *K*, characterizing the inclusion process give the magnitude of the drug–CyD interaction that is responsible for the biodisponibility and therapeutic effects of the complex. *K* can be evaluated considering two models: Benesi-Hildebrand (BH) or non-linear regression analysis of the absorbance, *A*, as a function of the CyD concentration, [*CyD*]. The non-linear equations derived from the analysis of the equilibria present in solution, corresponding to the formation of 1:1, 1:2 or of mixtures of 1:1 and 1:2 complexes are given below:[14]

$$G + nCyD \xleftrightarrow{K_{1n}} G\text{–}CyD_n \text{, with } A = \frac{A_0 + K_{1n}A_{1n}[CyD]^n}{1 + K_{1n}[CyD]^n}, \quad (1)$$

or

$$G + CyD \xleftrightarrow{K_{11}} G\text{–}CyD$$

$$G\text{–}CyD + CyD \xleftrightarrow{K'_{12}} G\text{–}CyD_2, \text{ with } A = \frac{A_0 + K_{11}A_{11}[CyD] + K_{11}K'_{12}A_{12}[CyD]^2}{1 + K_{11}[CyD] + K_{11}K'_{12}[CyD]^2}. \quad (2)$$

A_0 and A_{1n} (A_{11} or A_{12}) are the absorbances of the guest in absence of CyD and of the 1:n (1:1 or 1:2) complex (at maximum CyD concentration, when all guest is considered to be in the complexed form). K_{11} and K_{12} are the association constants of the 1:1 and 1:2 complexes, respectively. Attention should be paid to the fact that when a mixture of complexes is present K'_{12} is the product between

the association constants of the 1:1 and 1:2 complexes in the mixture, $K'_{12} = K_{11}K_{12}$.

The analysis of the experimental data was firstly done using the BH model.[15] The equations characterizing the formation of solely 1:1 or 1:2 complexes are based on the dependency $1/(A-A_0)$ vs. $1/[CyD]^n$. When $1/(A-A_0)$ is plotted against 1/[CyD], we obtained a non-linear curve (not shown), which excludes the formation of complexes with 1:1 stoichiometry.[15] The same result was obtained for all CyDs. This is in accordance with the absence of isosbestic points in the absorption spectra and suggests high order associations present in solution.[16]

If $1/(A-A_0)$ is plotted against $1/[CyD]^2$, the dependence seems linear (R = 0.99) but the standard errors (intercept 0.854±1.845, slope 0.003±4.676) and standard deviation (6.36) are very large. Thus, this model offers inconclusive data concerning the formation of complexes with 1:2 stoichiometry. It is known that the linearization employed in the BH model may introduce significant errors in the evaluated binding parameters.[4, 17]

More accurate results are usually obtained using the non-linear regression model, depicted in Eqs. (1) and (2). In the case of SIM, the non-linear model also rules out the formation of 1:1 complexes, but the non-linear fits using Eq. (1) for a 1:2 stoichiometry gave good results for all CyDs except α-CyD, with small errors (<15%) and very good correlation coefficients (r^2 >0.99). The respective fits are given in Figure 3 and the association constants are listed in Table 2.

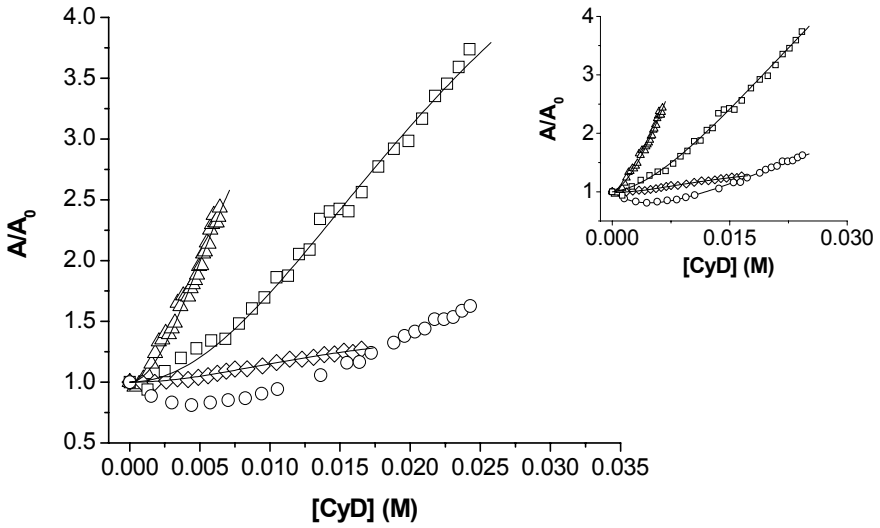

Fig. 3 – Dependence of the normalized absorption on the α-CyD (○), β-CyD (Δ), 2-HP-β-CyD (◊) and γ-CyD (□) concentrations. The solid curves represent the best fits according to Eq. (1) for 1:2 stoichiometry. Inset: best fits according to Eq. (2) for 1:1+1:2 stoichiometries. The resulted parameters are listed in Tables 2 and 3.

Table 2

Estimated 1:2 association constants (K_{12}) and ratios of absorbance of the complex *vs.* absorbance in the absence of CyD (A_{12}/A_0) for the studied SIM–CyD complexes

CyD	K_{12} (M^{-1})	A_{12}/A_0	r^2	F-stat
β	14085.2 ± 1937.4	4.85 ± 0.35	0.991	3503
2-HP-β	4385.5 ± 430.1	1.49 ± 0.04	0.994	3450
γ	1523.2 ± 135.6	6.56 ± 0.31	0.994	4370

Although the fit of the experimental data to Eq. (1) for n = 2 can be considered as satisfactory, a more careful examination of the first segment of the curve, at low CyD concentration, reveals some deviations of the experimental points. Therefore, the data were also fitted considering Eq. (2), *i.e.* assuming the presence of both 1:1 and 1:2 complexes (inset of Figure 3). This treatment leads to higher correlation coefficients and a better adjustment of the function on the experimental points (lower and more randomly distributed residuals), but also to large standard errors, especially in the evaluation of K'_{12} and A_{12} (Table 3).

Table 3

Estimated association constants (K_{11}, K'_{12}) and normalized absorbances (A_{11}/A_0, A_{12}/A_0) for a mixture of 1:1+1:2 SIM–CyD complexes

CyD	K_{11} (M^{-1})	K'_{12} (M^{-1})	A_{11}/A_0	A_{12}/A_0	r^2	F-stat
α	98.0	11.8	0.00027	8.1	0.997	2194
β	16.6	232.7	4.4	10.0	0.992	1194
2-HP-β	68.3	101.0	0.9	1.6	0.995	1291
γ	39.6	12.2	1.4	21.1	0.996	1941

In order to check the two possibilities, *i.e.* the presence of only the 1:2 complex or of both complexes, two theoretical curves were generated with the aid of the TableCurve program. Using the *K* and *A* parameters obtained above for the 1:2 complex (Table 2) and for the mixture of complexes (Table 3) we extended the curves depicting Eqs. (1) and (2) at higher values of CyD concentration. As shown in Figure 4 for the case of 2-HP-β-CyD and γ-CyD complexation, the experimental points are well fitted by both equations. It can be seen that up to a CyD concentration of ~10^{-2} M the curves are identical and only at much higher CyD concentrations the differences can be observed. Unfortunately, the CyDs solubility prevents the use of such concentrations, and therefore it is difficult to ascertain which are the species present in the system. α- and γ-CyD allow the use of somewhat higher but still insufficient concentrations.

By mass spectrometry, Wen *et al.*[6] showed the formation of stable SIM–α-CyD and SIM–β-CyD complexes of 1:1 stoichiometry. In the case of β-CyD, they also found evidence for the formation of 2:1 complexes. 2:1 complexes were not observed in our case, probably due to the very different experimental conditions.

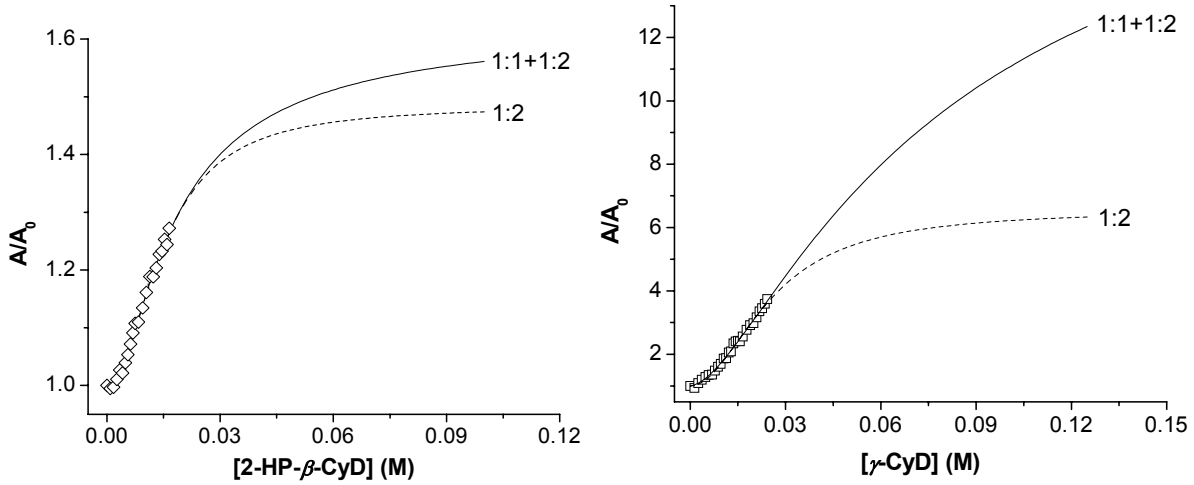

Fig. 4 – Plot of the 1:2 and 1:1+1:2 curves generated in a higher CyD concentration range than our experimental data, figured as dots, using the parameters in Tables 2 and 3, respectively.

2.2. Circular dichroism study of simvastatin–cyclodextrin inclusion complexes

The CD spectrum of SIM in absence of CyD (Figure 5) shows a positive signal at ~238 nm, corresponding to the $\pi\rightarrow\pi^*$ $S_0\rightarrow S_1$ absorption band, as stated by the theoretical calculations. In presence of CyD, the ellipticity of this signal is modified, confirming the inclusion of SIM in the asymmetric locus of the CyD cavity: an increase in ellipticity is observed for the complex with β-CyD, while complexation with α- and 2-HP-β-CyD diminishes the SIM signal. For a better observation of the CyD effect, difference CD spectra obtained by substracting the SIM signal from the spectra of the complexes were also given as inset in Figure 5. We observe a positive CD signal in the case of β-CyD and negative signals for α- and 2-HP-β-CyD.

The sign of the CD exhibited by a molecule incorporated in the CyD cavity is determined by the angle between the vector of the electronic transition moment of the molecule and the 6- or

7-fold symmetry axis of *α*- and *β*-CyD ring, respectively. If the transition moment is parallel to the symmetry axis, the sign of the circular dichroism is positive (axial inclusion). If the transition moment is perpendicular to this axis, the sign is negative (equatorial inclusion).[18] The positive CD signal for *β*-CyD shows inclusion of the SIM molecule with the $S_0 \rightarrow S_1$ transition moment parallel to the symmetry axis of *β*-CyD. This is supported by the molecular mechanics calculations on 1:1 and 1:2 inclusion complexes (Section 2.3). In the case of *α*- and 2-HP-*β*-CyD, the negative signals suggest similar geometries for the two complexes, with SIM included in such a position that allows the transition moment correlated with the HHN fragment to be directed equatorially in respect with the cavity axis.

A second CD signal can also be observed to appear around 195 nm, but this is a less reliable spectral region from experimental point of view and therefore it was not considered.

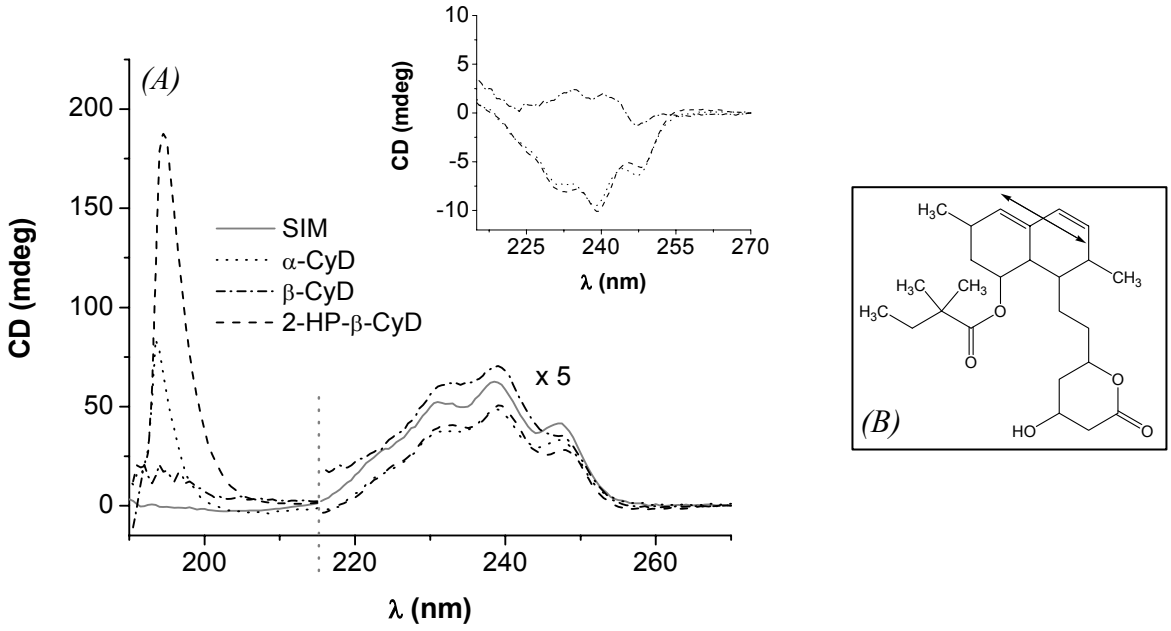

Fig. 5 – (A) CD spectra of SIM ($1.2 \cdot 10^{-5}$ M in methanol:water 1:9, v:v) alone and in presence of *α*-CyD ($4.8 \cdot 10^{-2}$ M), *β*-CyD ($1.3 \cdot 10^{-2}$ M) and 2-HP-*β*-CyD ($2.0 \cdot 10^{-2}$ M). Inset: Difference CD spectra obtained by substracting the SIM spectrum from the spectra of the complexes. (B) Orientation of the $S_0 \rightarrow S_1$ transition moment of SIM.

2.3. Molecular mechanics calculations

Molecular modeling was used in order to gain information on the binding mechanism at molecular level. The strategy employed is extensively described in the Experimental part. Since the analysis of the experimental data attested the presence of 1:2 complexes or, at least, of a mixture of 1:1 and 1:2 complexes, we started our investigation with the 1:1 SIM–*β*-CyD complex. Three possible structures were generated by introducing the guest with the dimethylbutanoate (DMB), hexahydronaphtalene (HHN) and tetrahydropyranonyl (THP) moiety, respectively, inside the *β*-CyD cavity, from the narrow rim as well as from the wide cavity rim. After full optimization it was observed that the most stable structure of the complex corresponds to the THP fragment included in the cavity through the narrow rim, slightly surpassing the cavity, the complex being stabilized by the formation of a hydrogen bond between the carbonyl group of DMB and a secondary hydroxyl group of *β*-CyD. The structure of the complex is depicted in Figure 6 (A) and the binding energies obtained after are given in Table 4. The same predilection for the inclusion of THP, but from the wide CyD ring, was observed by Wen *et al.*,[6] by means of a semiempirical PM3 study. The guest–host interaction is mainly due to van der Waals forces, as shown by the relative contribution of the van der Waals and electrostatic energy terms also listed in Table 4. As expected, the presence of surrounding water molecules determines a

diminution of the electrostatic term. Table 4 also includes the guest perturbation energies that are most likely due to a modification of the molecular conformation upon complexation. The examination of the dihedral angles along the molecular backbone reveals different values in the case of complexed SIM as compared to the lowest energy conformer (Table 5).

Table 4

Energetic parameters of the most stable 1:1 and 1:2 SIM–*β*-CyD complexes. SIM enters the *β*-CyD cavity with the tetrahydropyranonyl (THP) fragment, through the primary rim; a second *β*-CyD molecule approaches the 1:1 complex from the dimethylbutanoate (DMB) side, with the secondary rim. Energies given in kcal mol^{-1}

Fragment	$E_{binding}$	% van der Waals	% electrostatic	$E_{perturbation\ guest}$
	1:1			
THP, vacuo	–39.92	82.36	17.64	15.40
THP, water	–35.15	88.42	11.58	9.87
	1:2			
DMB, vacuo	–78.60	87.42	12.58	9.22
DMB, water	–68.91	90.55	9.45	17.49

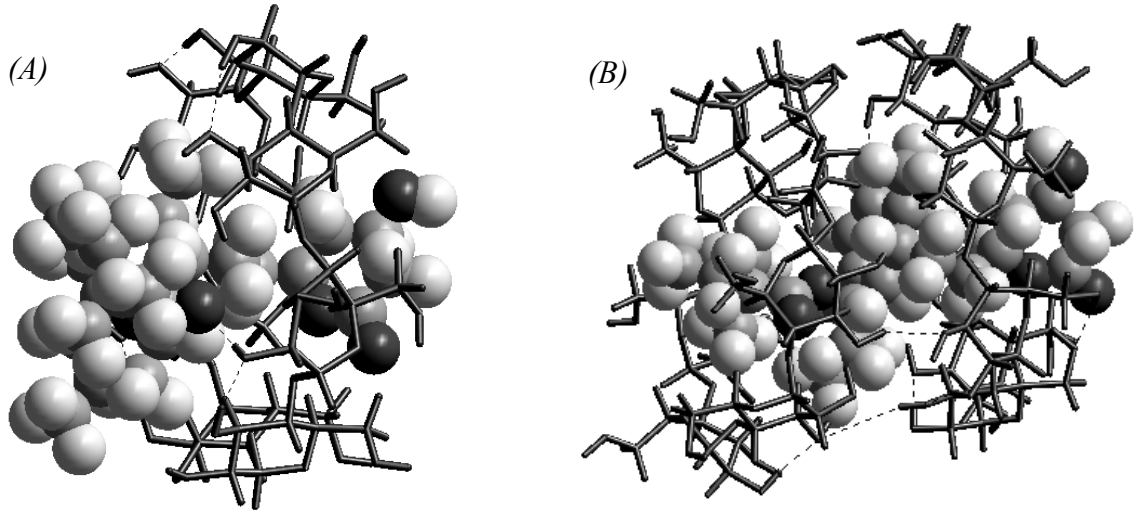

Fig. 6 – Proposed structures of the (A) 1:1 and (B) 1:2 SIM–*β*-CyD inclusion complexes. Dotted lines represent hydrogen bonds.

Table 5

Dihedral angles characterizing the conformation of free and *β*-CyD complexed SIM

Complex type	φ_1	φ_2	φ_3	φ_4
free SIM	–68.94	79.01	–59.71	–172.66
1:1	–70.97	115.48	–56.70	117.01
1:2	–146.17	76.26	–74.39	76.51

The 1:2 SIM–*β*-CyD complex was obtained by approaching a second *β*-CyD molecule to the DMB side of the 1:1 complex, with the narrow as well as with the wide rim (Table 4). The most stable 1:2 geometry corresponds to the *β*-CyD molecule approaching with its large cavity rim and is depicted in Figure 6 (B). The localization of the HHN moiety outside the *β*-CyD cavity in the case of the 1:1 complex and between the two *β*-CyD cavities in the case of the 1:2 stoichiometry explains why the position of the absorption band is not modified upon inclusion; the electronic features of the molecular fragment responsible for absorption remain unaltered by complexation.

The same procedure was followed in the study of the SIM–*γ*-CyD complexes. The energetic contributions for the 1:1 and 1:2 SIM–*γ*-CyD complexes are listed in Table 6. The most stable

1:1 complex corresponds to the HHN fragment inserted in the cavity through the wide rim. A deeper penetration of the SIM molecule is observed in the larger γ-CyD cavity. The most stable 1:2 complex is formed by approaching a second γ-CyD molecule towards the THP moiety of the 1:1 complex, with the narrow rim. The lower binding energy of the 1:2 SIM–β-CyD compared to the 1:2 SIM–γ-CyD complex is in accordance with the higher association constants found experimentally (Table 2) and suggests a more pronounced interaction between SIM and β-CyD.

Table 6

Energetic parameters of the most stable 1:1 and 1:2 SIM–γ-CyD complexes. The hexahydronaphtalene (HHN) fragment of SIM resides in the first γ-CyD cavity; a second γ-CyD molecule approaches the 1:1 complex from the tetrahydropyranonyl (THP) side, with the primary rim. Energies given in kcal mol^{-1}

Fragment	$E_{binding}$	% van der Waals	% electrostatic	$E_{perturbation\ guest}$
1:1				
HHN, vacuo	–38.92	94.45	5.55	2.87
HHN, water	–35.64	97.87	2.13	6.88
1:2				
THP, vacuo	–68.72	88.30	11.60	4.74
THP, water	–64.93	87.86	12.14	6.93

EXPERIMENTAL

1. Spectroscopic measurements

SIM was obtained from Helcor (Baia Mare, Romania). The absorption and circular dichroism spectra were recorded on a Jasco V-560 UV-VIS spectrophotometer and Jasco J-815 CD spectrometer, respectively, at room temperature. The solvents and CyDs from Fluka and Aldrich were used as received. The optimum drug concentrations were in the range 10^{-5} – 10^{-4} M. The inclusion complexes were prepared by adding to a drug stock solution (10^{-5} M in methanol:water 1:9, v:v) aliquots of CyD solution (10^{-2} M, prepared by adding CyD powder to the stock solution above).

2. Computational details

Since the SIM molecule has a very flexible structure, due to the presence of several molecular joints, a Conformational Search was firstly performed with the HyperChem 7.5 program in order to determine the most stable conformation. Four torsion angles were modified (angles φ in Figure 1). Semiempirical (PM3) and molecular mechanics (MM+) calculations on the ground state were then performed. The optimizations were done up to a RMS gradient of 0.05 kcal mol^{-1}Å^{-1}.

The investigation of the geometries of 1:1 and 1:2 complexes of SIM with β- and γ-CyD was done by molecular mechanics (MM+ force field, calculations in vacuo and in water) and consisted of two main steps. At first, the starting geometries and atomic charges of the free guest and host were obtained by PM3 semiempirical calculations (optimization method Eigenvector Following). The β-CyD geometry was taken from neutron diffraction studies[19] while the geometry of γ-CyD was taken from Ref [20]. Further, several starting structures for the inclusion complex were generated, considering all the possible ways the drug molecule can be docked into the CyD cavity. A search for the most stable structure was done by optimization under restrictions followed by full optimization. The energies characterizing the inclusion process were finally calculated: the binding energy between guest and host and the perturbation energy of the guest molecule: [21]

$$E_{binding} = E_{complex} - (E_{guest} + E_{CyD})_{\text{frozen in complex}} \quad (3)$$

$$E_{perturbation} = E_{guest\ frozen\ in\ complex} - E_{guest\ totally\ optimized} \quad (4)$$

In the case of the MM+ force field used, the binding energy represents the sum of the van der Waals and electrostatic energies, allowing for the estimation of their relative contributions.[22]

The 1:2 guest–host complex was obtained by approaching a second CyD molecule to the most stable 1:1 complex, according to the equilibrium established in solution.

The most stable 1:1 and 1:2 complexes were also optimized in a box of water molecules, having an edge of 30Å and comprising 892 molecules, thus offering a density close to the experimental one. Full optimizations were performed up to a gradient of 0.05 kcal Å^{-1} mol^{-1} for the in vacuo computations and 0.5 kcal Å^{-1} mol^{-1} for the water-dependent calculations.

CONCLUSIONS

This study explored the interaction of SIM with various CyDs, by means of absorption and CD spectroscopies as well as theoretical semiempirical and molecular mechanics calculations. The CyD effect on the spectral features of SIM was qualitatively and quantitatively investigated. The changes of the absorption and CD spectra suggested the inclusion of the SIM molecule inside the CyD cavity, the presumed structure of the inclusion complexes being confirmed by the theoretical results. The experimental data were analyzed by both linear and non-linear models, allowing for the calculation of 1:2 association constants. The experimental CyD concentration domain allowed by the CyD solubility does not permit a clear distinction between the 1:2 stoichiometry and a mixture of 1:1 and 1:2 stoichiometries.

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