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Vibrational dynamics of inclusion complexes by Raman scattering: an experimental and numerical study

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Raman spectroscopy has been applied to the study of interactions involved in the formation of inclusion complexes between cyclodextrins and indomethacin. Raman spectra of free molecules and of their inclusion complexes have been recorded in the energy ranges 10-50 and 100-3500 cm^{-1}, and significant energy shifts, intensity variations and broadening have been observed due to complexation. The experimental data have been interpreted by comparison with the results of numerical simulation.

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1 Introduction

Inclusion complexes are supramolecular assembly systems at nanometric scale, formed by molecular encapsulation of a guest molecule into a suitable host one, where non-covalent interactions, like Van der Waals, hydrophobic and hydrogen bonds are involved. Generally, these complexes are formed in aqueous solution, but they preserve their structure in the solid state as well. The investigation of the interactions between host and guest molecules is of interest both from a general point of view (for example, as a prototype of enzyme-substrate interaction mechanism), and from a technological one. In particular, the inclusion complexes between drugs and cyclodextrins (CD) are a topic of current interest for pharmaceutical industry, because these systems improve the solubility, stability and bioavailability of the guest molecules. CD consist of six (αCD), seven (βCD) or eight (γCD) glucose units, linked by glycosidic bonds. In water, these cyclic molecules take the peculiar three-dimensional shape of a hollow truncated cone with a slightly hydrophilic outer surface, while the hydrophobic central cavity is able to encapsulate any guest molecule having an appropriate chemical affinity, and whose shape and size fit inside the CD [1]. CD are known also to be good models for enzyme action, combining the cage effects with the conformational control of the guest molecule [2].

Inclusion complexes have been studied in aqueous solution, usually by UV and visible absorption spectroscopy, fluorescence, and NMR spectroscopy, while X-ray and neutron diffraction are typically utilized in the solid state. These complexes have been little studied by means of Raman spectroscopy, although the usefulness of this technique in such systems is now acknowledged [3, 4]. In the cases where Raman scattering has been applied to investigate the effect of the inclusion process on the guest molecules [5–7], the studies on the interactions between CD and some non-steroidal anti-inflammatory agents [8–10] have evidenced significant changes in the vibrational spectrum of the complexed guest molecules with respect to the free ones.

In this work, Raman scattering has been applied to the study of the interactions involved in the formation of complexes between CD and indomethacin (IMC), an anti-inflammatory agent whose chemical structure is reported in figure 1.

IMC has been characterized by different spectroscopic techniques, and it is known that, depending on the sample history, in the solid state the molecule can show a variety of polymorphic forms, and that it can also be found in amorphous form [11]. On the other hand, in solution IMC can exist in two equilibrating forms produced by the hindered rotation around the partial N-CO double bond, generating the *syn* (=*E* stereochemistry) and *anti* (= *Z* stereochemistry) isomers, according to the relative positions of amide C=O and methyl group, as shown in figure 1. X-ray studies indicated that the *syn*-isomer is the most thermodynamically stable form in the solid [12]. Our Raman spectra of the free molecules and of their inclusion complexes, recorded in the spectral ranges 10-50 cm^{-1} and 100-3500 cm^{-1}, show that, after the complex formation, the Raman peaks undergo significant shifts, intensity variations and broadening. These results have been complemented by ^{1}HNMR and mass spectrometry measurements, and interpreted on the basis of vibrational analysis performed by both *ab initio* quantum chemical calculations and molecular dynamics. In particular, our aim was to identify the normal modes corresponding to the peaks which undergo the largest variations under complexation; by singling out the atoms which have the largest displacements in such normal modes, we obtain information on the geometry of the complex. Relevant information is also provided by molecular mechanics (MM) calculations, and a full conformational analysis on bond rotamers of free IMC was performed in order to get information about the structures of minimum energy of the molecule.

2 Experimental results

Samples of inclusion complexes of IMC and of its sodium salt, with natural βCD and with the synthetically modified hydroxypropyl-βCD (HPβCD), were prepared in aqueous solution, according to the procedure adapted from the literature [13]; the samples were characterized by Electrospray-Ionization Mass Spectrometry (ESI-MS).

The Raman spectra were generally recorded using dried samples; in some cases, when the amount of

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available complex was small, in order to improve the Raman signal the technique of Surface Enhanced Raman Scattering (SERS) was used. **Drops of aqueous solution of the sample were repeatedly deposited on a suitable metallic substrate (Ti-6Al-4V alloy) and dried by evaporation, thereby increasing the complex concentration.**

The ESI-MS technique provided information on the stoichiometry of the complex; in particular, in the case of the IMC-βCD complexes, we were able to detect the cluster signals for the pseudo-molecular ion $[M\text{-}H]^-$of the 1:1 complex at m/z 1490, whereas no signals arising from 1:2 complexation were detected. The same behaviour was observed for all examined complexes, supporting 1:1 stoichiometry in these cases as well.

In figure 2, left panel, we report the Raman spectra of free IMC (a) and its inclusion complexes formed with HPβCD (b) and βCD (c), as well as the spectrum of the physical mixture of 1:10 IMC and βCD (d), in the energy range of 1500-1750 cm^{-1}. **First of all, it is important to note that the spectra taken with SERS (b and c) do not differ qualitatively from the one taken with conventional Raman apparatus (f) on a slightly different system, although in principle changes might be expected.**

We focused on this energy range because there are no peaks of free CD, so that complexation-induced changes on IMC peaks can be readily observed. Moreover, this is also the range where C=O stretching energies are expected, so that by combining experiment and calculations, useful information on the geometry of the complex (i.e., the position of the involved CO group) may be obtained from the features of Raman spectra (and relative normal modes). By comparing the Raman spectra (a), (b) and (c) of figure 2, we can observe that, actually, the sharp peak of free IMC at 1698 cm^{-1} exhibits a marked broadening and a shift to approximately 1670 cm^{-1} when the guest is included in CD cavity. On the other hand, only minor changes occur in the spectral region 1550-1630 cm^{-1}.

Comparison of the Raman spectrum of free IMC with those of two other molecules whose chemical structure reproduces subunits of the whole IMC, but which lack both the C=O amide group and the 1698 cm^{-1} peak [14], together with the *ab initio* calculation of the Raman spectrum, indicate that the peak of free IMC at 1698 cm^{-1} corresponds to the amide C=O stretch, in agreement with Taylor et al. [15]. Note that, besides the amide group (O=C(1')-N(1) in figure 1), IMC also contains a carboxylic COOH group, whose C=O stretching corresponds to a non-Raman-active normal mode. The observed large shift to lower energies ($\approx$30 cm^{-1}) of the IMC amide C=O peak after the complexation, is rather unusual, since generally, smaller energy variation are observed [8–10]. In principle, such large shift might be due to the complexation-induced breakdown of hydrogen bonding patterns of IMC dimers (figure 2 (a)). However, the Raman spectrum of the 1:10 physical mixture of IMC and βCD respectively, which is not expected to contain dimers (figure 2 (d)), shows the same characteristic peaks as in figure 2 (a), indicating that the shift in question must be actually ascribed to the formation of the complex. This conclusion is confirmed by molecular dynamics calculations, which show an effective involvement of the amide C=O group in the formation of the complex. A similar behaviour is observed also in the Raman spectra of the free IMC sodium salt and of its βCD complex, reported in figure 2 (e) and (f): in fact, the peak assigned to the amide C=O of the guest at 1676 cm-1 exhibits the same characteristic broadening and low-energy shift under the effect of the inclusion in the βCD cavity.

Significant changes in the spectra of complexed IMC with respect to the free molecule, have also been observed between 10 and 1500 cm^{-1}, and between 2700 and 3200 cm^{-1}. In the energy range 10-100 cm^{-1}, the spectra of free βCD and IMC, and of their inclusion complex, look like in figure 3 (a,b,c), respectively. However, mostly due to the presence of βCD peaks in the these ranges, the spectra are much more difficult to interpret, and their explanation requires further analysis.

3 Numerical simulation

Molecular mechanics calculations were carried out by using MM3 force field as implemented in the computer programme PCMODEL 7.0 (Serena Software, Bloomington, Indiana) and a full conformational analysis on bond rotamers of free IMC was performed by the subroutine GMMX of the PCMODEL 7.0 programme suite. All the *ab initio* calculations were performed with the GAUSSIAN 03 programme suite [16] utiliz-

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ing unrestricted DFT [17], and the non-local B3LYP functional hybrid method was employed [18]. The standard 6-31G(d) basis set [19] was used for the geometry optimization and frequency analysis of free IMC base pairs. Molecular dynamics runs, energy minimisations, and diagonalisation of the dynamical matrix were performed by the AMBER package [20]. In some runs, the solvent effects were incorporated explicitly, by including TIP3 water molecules [21]. The starting structures of IMC and βCD were obtained from the Protein Data Bank [22]. The coordinates for hydrogen atoms were generated by means of the *WWW PRODRG* server [23]. Partial charges were computed by means of the module *antechamber* of the AMBER package, utilizing the *AM1-BCC* model [24].

In order to obtain information on the structures of minimum energy of IMC, a full conformational search was first carried out through molecular mechanics. All the minimised structures falling in a strain-energy window of 2 kcal/mol were minimised with the MM3 force field. In almost all the minimized conformers, the carbon atom of the CH_3O group was found in the plane of the indole ring, and the C=O group of the carboxylic group was almost eclipsed with respect to the one of the CH_2C_3 hydrogens. The main outcome was the great conformational control imposed by the hindered rotation around the CO-N amide bond. Four distinct minima were calculated for rotation around the torsion angle θ O=C(1')-N(1)-C(2). Among them, two showed *syn* relationship of the carbonyl group with respect to the CH_3-C(2) group (θ = + 49 ,- 49), and two had *anti* relationship between the same groups (θ = +113, -111). For the sake of clarity, we will label such stationary conformers as *syn1* (+49), *syn2* (-49), *anti1*(+113) and *anti2* (-111). The *syn* conformers resulted more stable than the *anti* ones by about 0.6 kcal/mol; if we consider that in such conformational equilibria the entropic contribution to Gibbs free-energy difference among *syn* and *anti* rotamers should be negligible, their relative Boltzmann population at 298 K is expected to lead to a *syn*/*anti* ratio of 7:3. A slightly lower energy (0.2 kcal/mol) for the *syn1* with respect to *syn2* conformers was found. A rough estimate of the activation barriers connecting these minima was obtained by dihedral angle option subroutine. This allowed us to identify four transition states: TS1 (+0) between *syn2* and *syn1* minima, TS2 (+90) between *syn1* and *anti1*, TS3 (180) between *anti1* and *anti2*, and finally TS4 (270) connecting *anti2* to *syn2*. The activation energy for the conformational interconversion processes of the *syn*/*syn* or *anti*/*anti* type, were evaluated to be very low (about 1 kcal/mol), whereas the *syn*/*anti* were found to be 4 kcal/mol.

In order to obtain a more reliable picture of the thermodynamic and kinetic parameters, *ab initio* DFT calculations were carried out on the stationary points of the reaction coordinate. The results were not so different from those obtained by MM calculations, as shown in figure 4. In fact, *syn1* (used as reference for energy differences) was again the full minimum structure, but the differences of the total electronic energies of *syn2* (+0.2 kcal/mol), *anti1* (0.8 kcal/mol), and *anti2* (+0.7 kcal/mol), were low enough to allow them to play a role in the thermally averaged rotamer distribution. The geometries of TS1 and TS2 were evaluated by using the corresponding MM3 minimized structures as input files. As shown in figure 4, the electronic energies of TS1 (θ = +90) and TS3 (θ = -90), were found to be 6.3 and 6.4 kcal/mol higher than *syn1* (θ = +28) minimum, respectively, whereas those of TS2 (θ = 180) and TS4 ((θ = 0) were found at about 4.0 kcal/mol above that of *syn1*. In this sense, our calculations are in agreement: i) with single crystal X-ray diffraction studies whereby *syn* was found to be the most thermodinamically stable form [12], and ii) with NMR data reported by Fronza et al. [25], who observed that same conformer (here represented by *syn1*+*syn2*) was more abundant than the *anti* conformer (here *anti1*+*anti2*). At room temperature, the two rotamers were found to be in fast exchange on the NMR timescale, thus showing only averaged NMR signals. However, by lowering the temperature of a solution of the IMC methyl ester in deuterated dichloromethane, Fronza et al. [25] showed that at 170 K such *syn*/*anti* equilibrium became slow enough to allow the detection of signals ascribable to single rotamers. Since the experimental activation energy of IMC methyl ester in dichloromethane is higher (6.4 kcal/mol, estimated rate constant of about 10^6 s^{-1} at 298 K) than that obtained with our *ab initio* level of theory in the gas phase, other factors besides enthalpy changes must play a role. While the presence of a methyl group on the carboxylic moiety is expected to play a minor role, differential solvation of stationary states could give a contribution to the energy-barriers trough which such interconversion occurs. It is worth of mention that the dipole moments of the four minimua (1.10±0.10 D for *syn1*-*anti2*), as obtained by DFT calculations, are significantly lower than those obtained, at the same level of theory, for the two relevant transition states TS1 and TS3

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(2.85±0.07 D). This difference is large enough to change the activation barrier. In non polar solvents, such as dichloromethane, the less polar ground states are expected to be more stable than transition states with higher dipole moments, thus leading to a significant increase of activation energy. Moreover, a negative activation entropy due to more ordered TS1 and TS3 structures with respect to minima, might explain a free energy of activation high enough to justify the slow exchange rate observed in low temperature NMR spectra.

However, it is important to note that at finite temperature, it is the (Gibbs) *free* energy which is minimised by the system, and not the potential energy. Thus, entropic effects might be important. These are not accounted for by the above *ab initio* calculations, but may be included by means of molecular dynamics simulations. We have simulated at $300K$) i) a single IMC molecule in vacuum, ii) a single IMC molecule in a box of 1114 molecules of water, iii) a single inclusion complex in a box of 633 molecules of water. In the latter case, the complex was prepared by docking the Cl atom of IMC into the cavity of the βCD, and minimising the energy of the resulting configuration.

During these runs we recorded the dihedral angle θ defined above. With these data we built the histograms shown in figure 5, representing the probability distribution $P(\theta)$ of the dihedral angles in thermodynamic equilibrium, for the three cases studied. Consistently with the *ab inito* computation, in free IMC the angle θ moves back and forth between configurations close to the *anti2* and *syn2* minima, the time spent in the two regions being almost equivalent. In this situation we expect that entropy does not play any relevant role. The slight preference for the *anti2*-like rather than *syn2*-like configurations (as the *ab initio* calculation would suggest), is likely to be a failure of the force field utilized. On the other hand, for IMC embedded in a box of water, *anti2* is clearly more stable than *syn2*. Since the dipole moments of both kinds of structures are similar, we expect that the interaction energy with a polar solvent like water should be similar as well. This suggest that it could be entropic effects to stabilise the *anti2* conformation. Finally, for IMC complexed with βCD in a box of water, all values of the angle θ are almost equiprobable, thus suggesting that the interaction energy between βCD and IMC should be larger than all the energy-barriers between the four minima.

4 Conclusions

In the present paper we have reported the results of a multi-technique study of the inclusion complexes formed by IMC with βCD. In particular Raman scattering experiments in the energy range 1600-1700 cm^{-1} and the comparison with the results of *ab initio* and molecular dynamics simulations, suggest that the geometry of the IMC-βCD complex is the one characterised by the 4-chlorobenzoyl unit inserted into the cavity of βCD through its larger rim. Moreover *ab initio* and molecular dynamics calculations allowed to study the structures of minima of energy, both for free and complexed IMC.

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FIGURE CAPTIONS

FIG. 1. Chemical structure of *syn-* (a) and *anti-* (b) indomethacin rotamers.

FIG. 2 Raman spectra of (a): free IMC ; (b): IMC-HPβCD complex; (c): IMC-βCD complex; (d): 1:10 IMC-βCD physical mixture; (e): free sodium salt of IMC; (f): sodium salt of IMC included in βCD.

FIG. 3 Raman spectra of (a): free βCD ; (b) free IMC; (c): IMC-βCD inclusion complex, in the energy range 10-100 cm^{-1}.

FIG. 4 Energy profile of the indomethacin *syn-anti* interconversion from *ab initio* calculations.

FIG. 5 Probability distribution of the dihedral angle θ (see text) for free IMC (solid line), IMC in water (dashed line), and IMC-βCD complex (dotted line).

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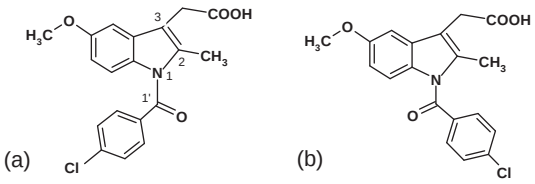

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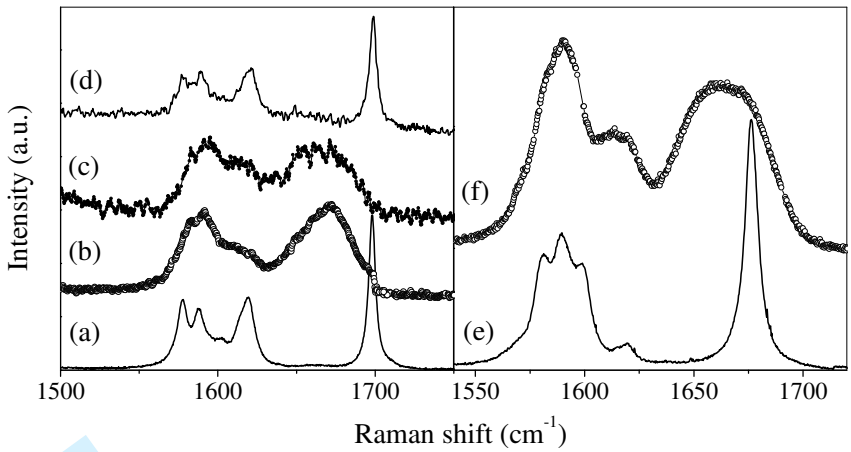

Figure 2. Raman spectra of (a): free IMC ; (b): IMC-HPβCD complex; (c): IMC-βCD complex; (d): 1:10 IMC-βCD physical mixture; (e): free sodium salt of IMC; (f): sodium salt of IMC included in βCD.

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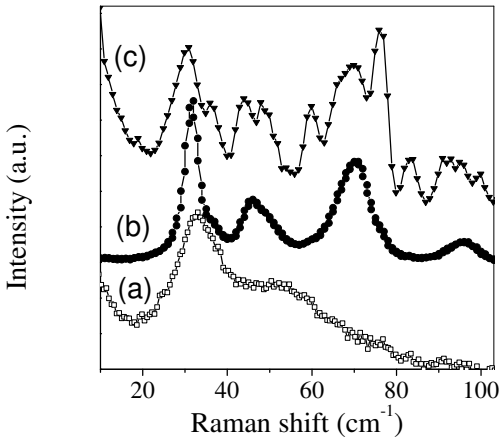

Figure 3. Raman spectra of (a): free βCD ; (b) free IMC; (c): IMC-βCD inclusion complex, in the energy range 10-100 cm^{-1}.

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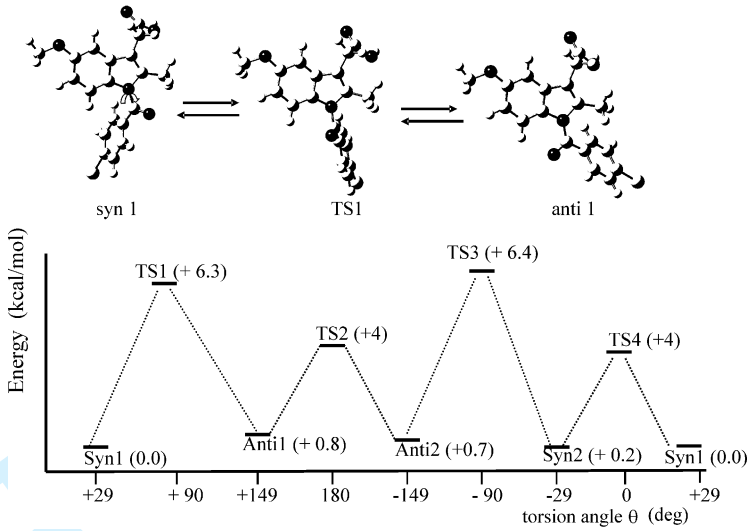

Figure 4. Energy profile of the indomethacin *syn-anti* interconversion from *ab initio* calculations.

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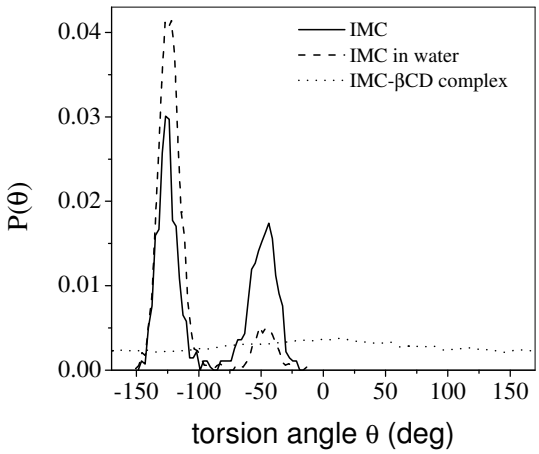

Figure 5. Probability distribution of the dihedral angle θ (see text) for free IMC (solid line), IMC in water (dashed line), and IMC-βCD complex (dotted line).

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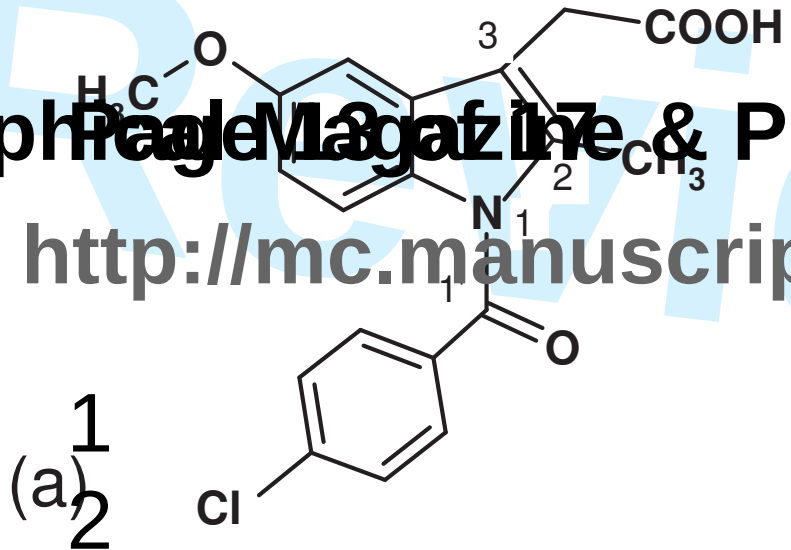

(a)

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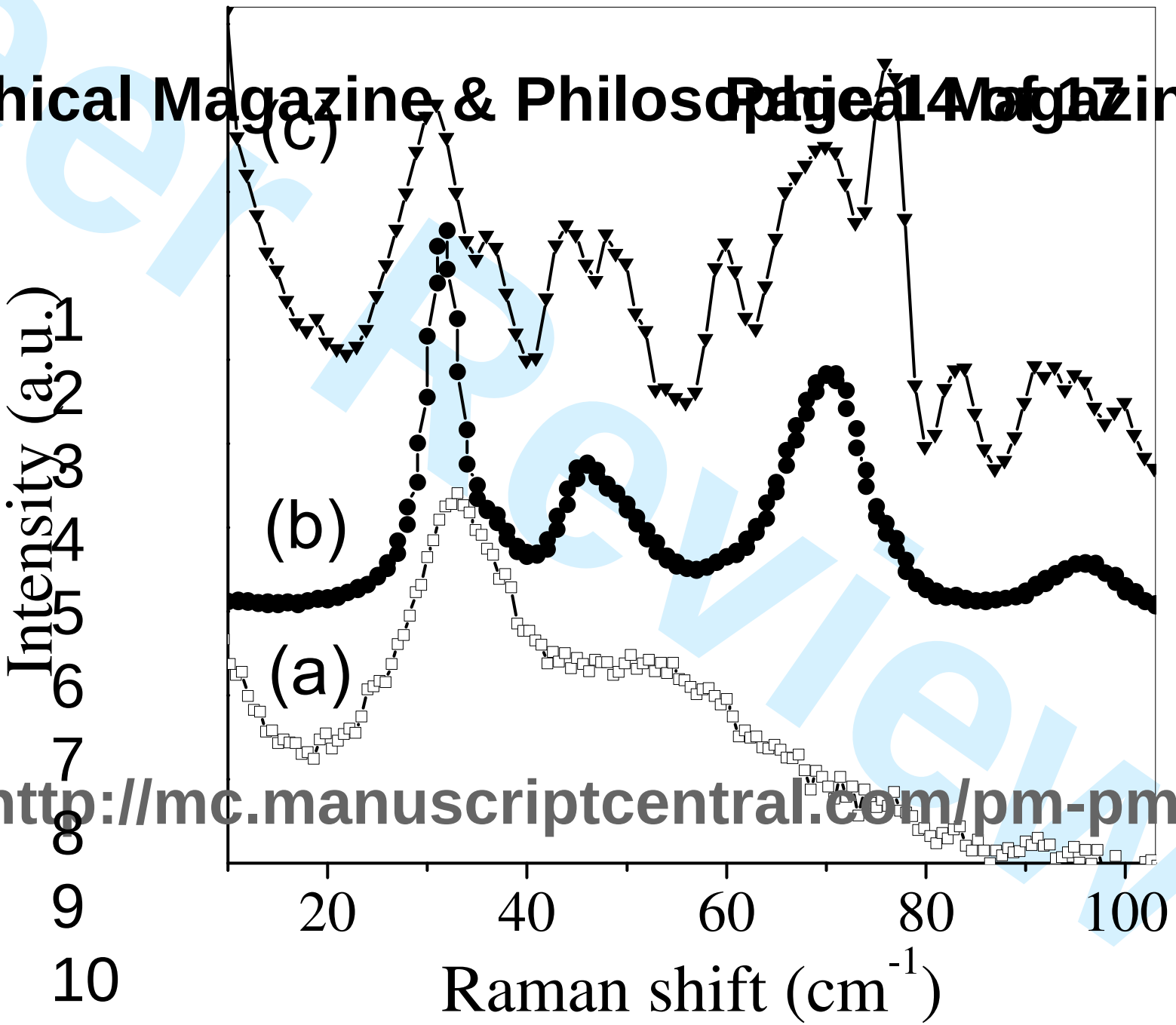
2

(b)



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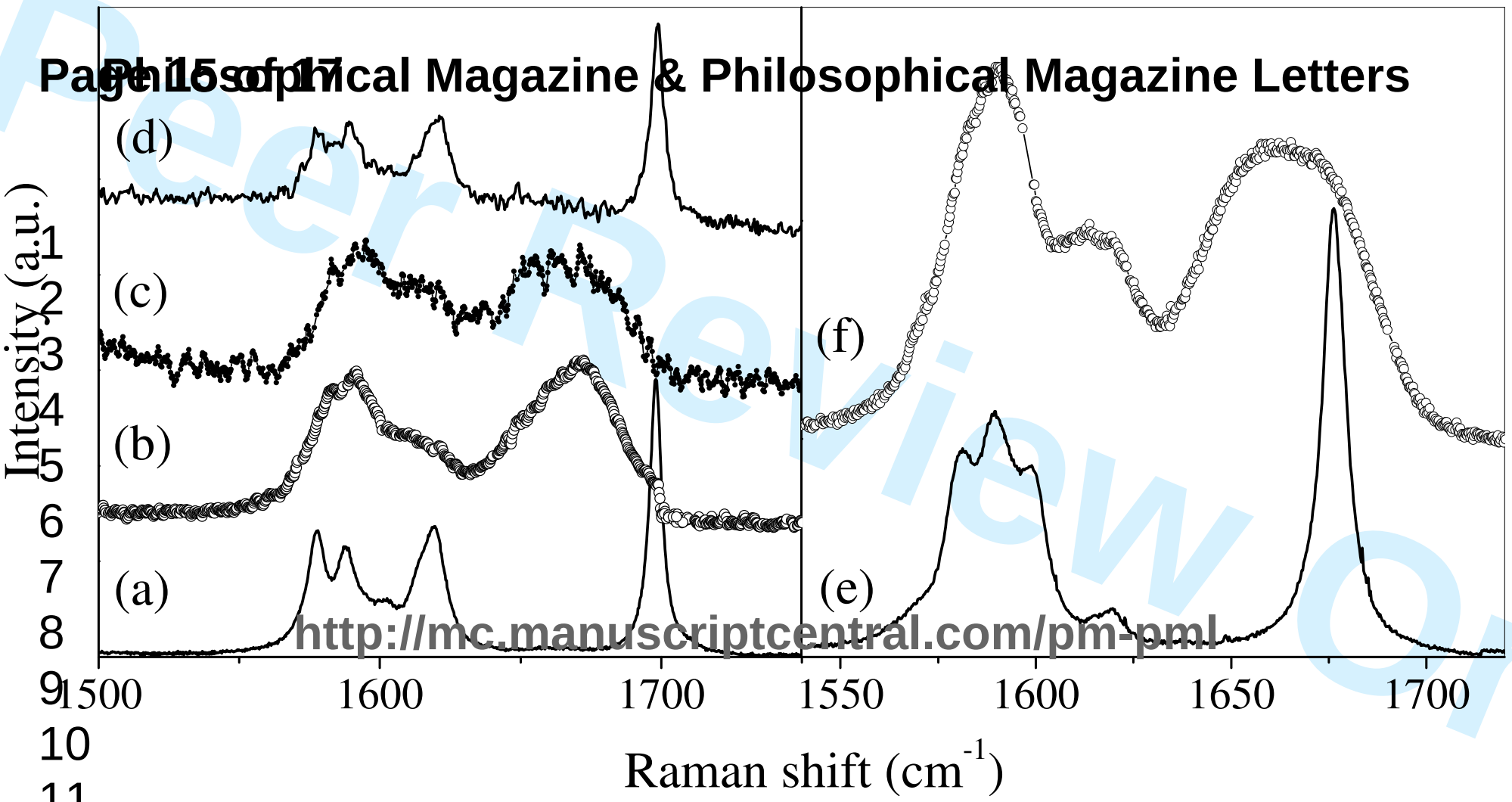

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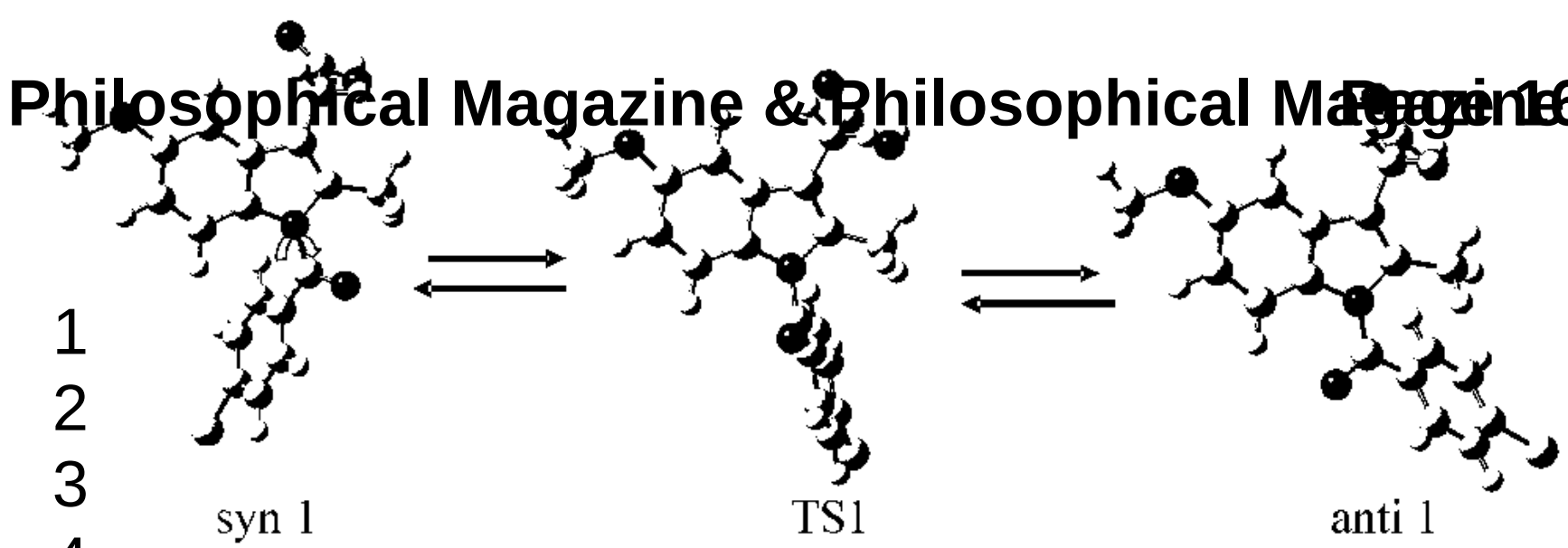

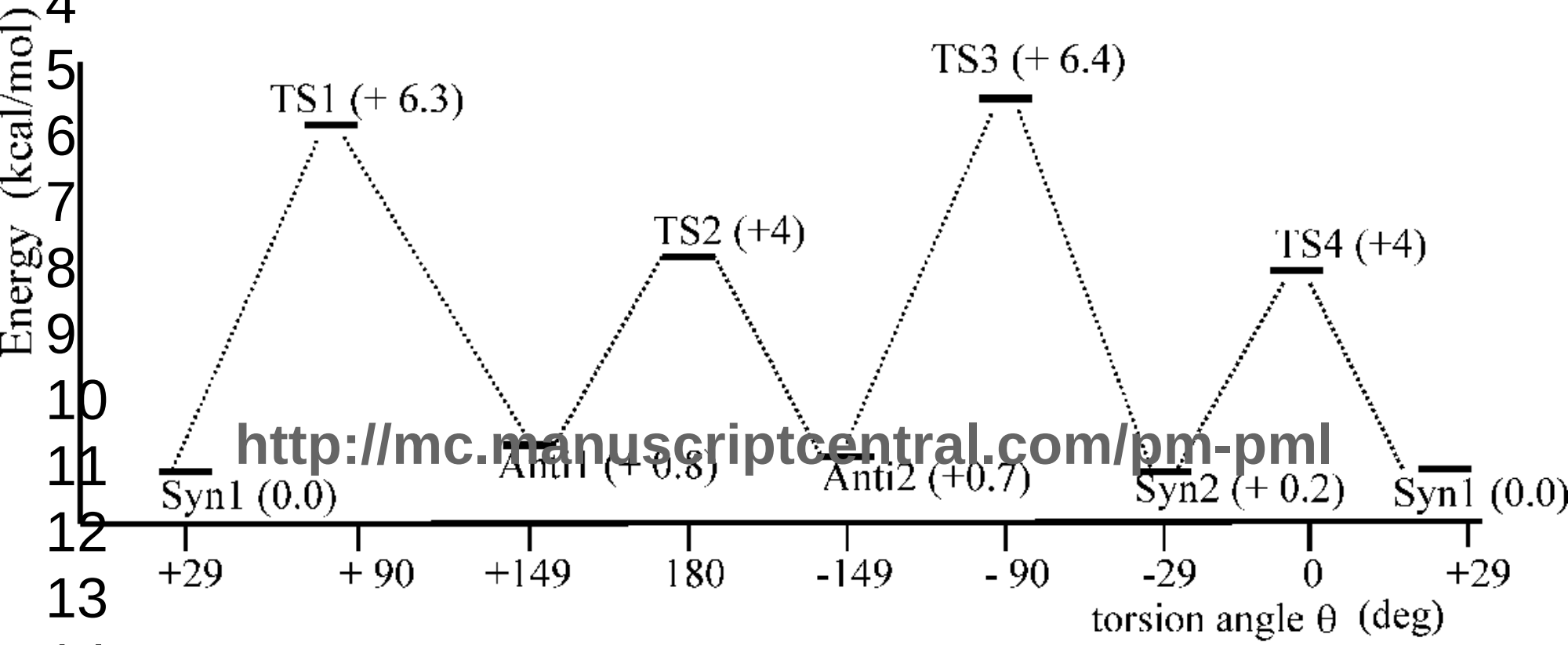

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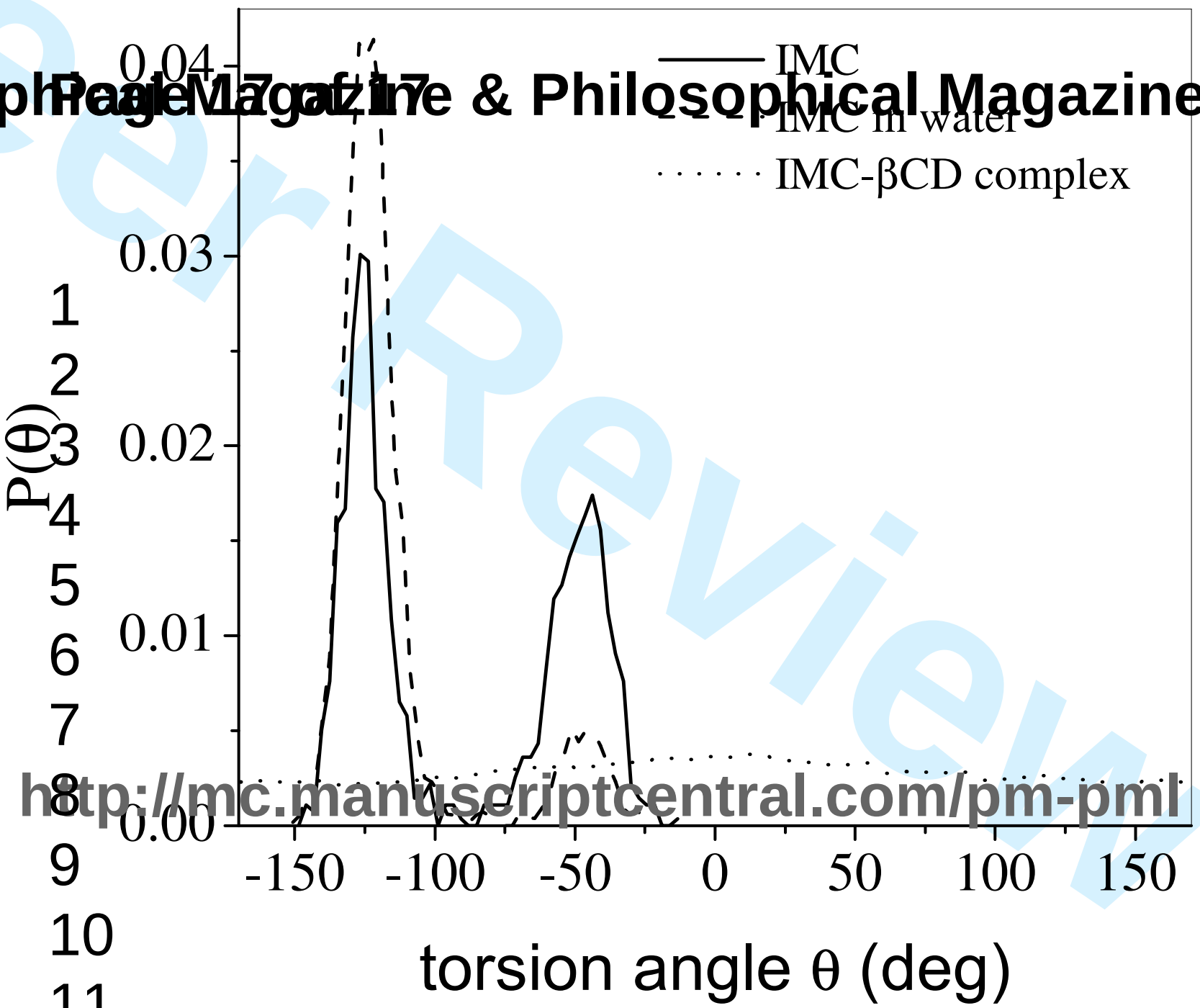

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