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Crystallization of L-Cysteine in Heavy Water Induces Intrinsic Fluorescence / Banerjee, D., Chibh, S., Tiwari, O.S., Mirón, G.D., Monti, M., Yakir, H.R., Pawar, S., Fixler, D., Shimon, L.J.W., Gazit, E., Hassanali, A..  
- In: ANGEWANDTE CHEMIE. INTERNATIONAL EDITION. - ISSN 1433-7851. - 64:29(2025).  
[10.1002/anie.202505331]

### *Availability:*

This version is available at: 20.500.11767/153176 since: 2026-09-16T14:07:46Z

### *Publisher:*

### *Published*

DOI:10.1002/anie.202505331

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A Journal of the Gesellschaft Deutscher Chemiker

# Angewandte Chemie

GDCh

International Edition

[www.angewandte.org](http://www.angewandte.org)

## Accepted Article

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This manuscript has been accepted after peer review and appears as an Accepted Article online prior to editing, proofing, and formal publication of the final Version of Record (VoR). The VoR will be published online in Early View as soon as possible and may be different to this Accepted Article as a result of editing. Readers should obtain the VoR from the journal website shown below when it is published to ensure accuracy of information. The authors are responsible for the content of this Accepted Article.

**To be cited as:** *Angew. Chem. Int. Ed.* **2025**, e202505331

**Link to VoR:** <https://doi.org/10.1002/anie.202505331>

# Crystallization of L-Cysteine in Heavy Water Induces Intrinsic Fluorescence

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Developing non-invasive techniques that can probe how solvents modulate the nucleation pathways of bioorganic molecules in solution remains an active and open area of research. Herein, we investigate the crystallization of the amino acid L-Cysteine and show that both the structure of the crystal and its intrinsic fluorescence can be drastically altered by solvent isotope effects. Crystals formed in heavy water exhibit markedly different intermolecular packing as well as strikingly different monomer conformations compared to those in light water. Remarkably, these differences in the supramolecular packing result in significantly elevated intrinsic fluorescence in the crystal that is formed in heavy water. Using a combination of experimental techniques and advanced electronic structure approaches, we elucidate the molecular interactions within the crystals that govern both the electronic origins and the intensity of their emission. These findings demonstrate how tuning the solvent by changing its isotope leads to the emergence of design principles for new intrinsic fluorophores that could serve as novel sensing probes for biomedical applications.

[\*] D. Banerjee  
Scuola Internazionale Superiore di Studi Avanzati (SISSA), via  
Bonomea 265, 34136 Trieste, Italy

D. Banerjee, G.D. Mirón, M. Monti  
International Centre for Theoretical Physics (ICTP), Strada  
Costiera 11, 34151 Trieste, Italy

S. Chibh, O.S. Tiwari, E. Gazit  
The Shmunis School of Biomedicine and Cancer Research, The  
George S. Wise Faculty of Life Sciences, Tel Aviv University,  
6997801 Tel Aviv, Israel

H.R. Yakir  
Institute of Chemistry, The Hebrew University of Jerusalem,  
Edmond J. Safra Campus, 9190401 Jerusalem, Israel

S. Pawar, D. Fixler  
Faculty of Engineering, The Institute of Nanotechnology and  
Advanced Materials, Bar-Ilan University, 5290002 Ramat-Gan,  
Israel

L.J.W. Shimon  
Department of Chemical Research Support, Weizmann Institute  
of Science, 7610001 Rehovot, Israel

A. Hassanali  
International Centre for Theoretical Physics (ICTP), Strada  
Costiera 11, 34151 Trieste, Italy  
E-mail: ahassana@ictp.it

## Introduction

The formation of ordered supramolecular assemblies by various metabolites including amino acids, has been extensively studied in recent years. These simple and biocompatible building blocks can assemble into ordered architectures with unique chemical, physical and optical properties<sup>[1–5]</sup>. One of the most intriguing observations within this context, is non-traditional intrinsic luminescence (NTIL)<sup>[6,7]</sup>, a phenomenon where non-aromatic or more generally, non-conjugated groups under physico-chemical confinement exhibit visible emission. Curiously, this phenomenon appears to contradict the conventional chemical paradigm that fluorescence in chemical and biological systems arises from aromatic or, more generally, conjugated groups<sup>[8]</sup>.

NTIL is typically associated with hetero-atomic sub-luminophores (HASLs), which are chemical moieties rich in lone-pair electrons or polar bonds, such as amines, amides, ethers, hydroxyls, esters, nitriles, imines, carbonyls (C=O) or sulfur/phosphorus groups. The key principle behind NTIL is that when these HASLs are forced into close proximity and their molecular motion is restricted due to chemical-crosslinking<sup>[9,10]</sup> and various forms of confinement<sup>[11,12]</sup> due to, for example, non-covalent interactions such as hydrogen-bonding. This unexpected non-aromatic fluorescence (NAF) has now been observed in a variety of biological and synthetic supramolecular assemblies constituting amino acids<sup>[13–16]</sup>, peptides<sup>[17,18]</sup>, peptide-based dendrimers<sup>[19,20]</sup>, oligosaccharides<sup>[21]</sup>, amyloid fibrils<sup>[22–27]</sup>, and other proteins<sup>[28–31]</sup>, all of which lack aromatic building blocks. In the last decade, there have been several proposals on the electronic and spectroscopic origins of this phenomenon. This includes the delocalization of electrons across the peptide backbone structure<sup>[17]</sup>, the existence of short hydrogen-bonds that permit proton transfer<sup>[15,23]</sup>, charge transfer excitations between polar amino acids<sup>[29]</sup>, and constrained carbonyl groups leading to fluorescence<sup>[32–35]</sup>. The manifestation of these various effects in different types of aggregates or assemblies<sup>[30,36–41]</sup> with different chemical properties, remains an open area of study from both experimental and theoretical perspectives.

Organic crystals offer excellent model systems for exploring the microscopic origins of NAF since one can determine the key interactions governing this phenomenon in a more controlled setting. It is widely recognized that molecular packing plays a crucial role in organic solid-state emission<sup>[42]</sup>, where even minor changes in molecular arrangement can lead to substantial variations in photophysical properties<sup>[43–46]</sup>. For example, different crystal polymorphs of the same compound can display variations in emission wavelengths or engage in fundamentally different photophysical processes, including thermally activated delayed fluorescence (TADF), and persistent room temper-

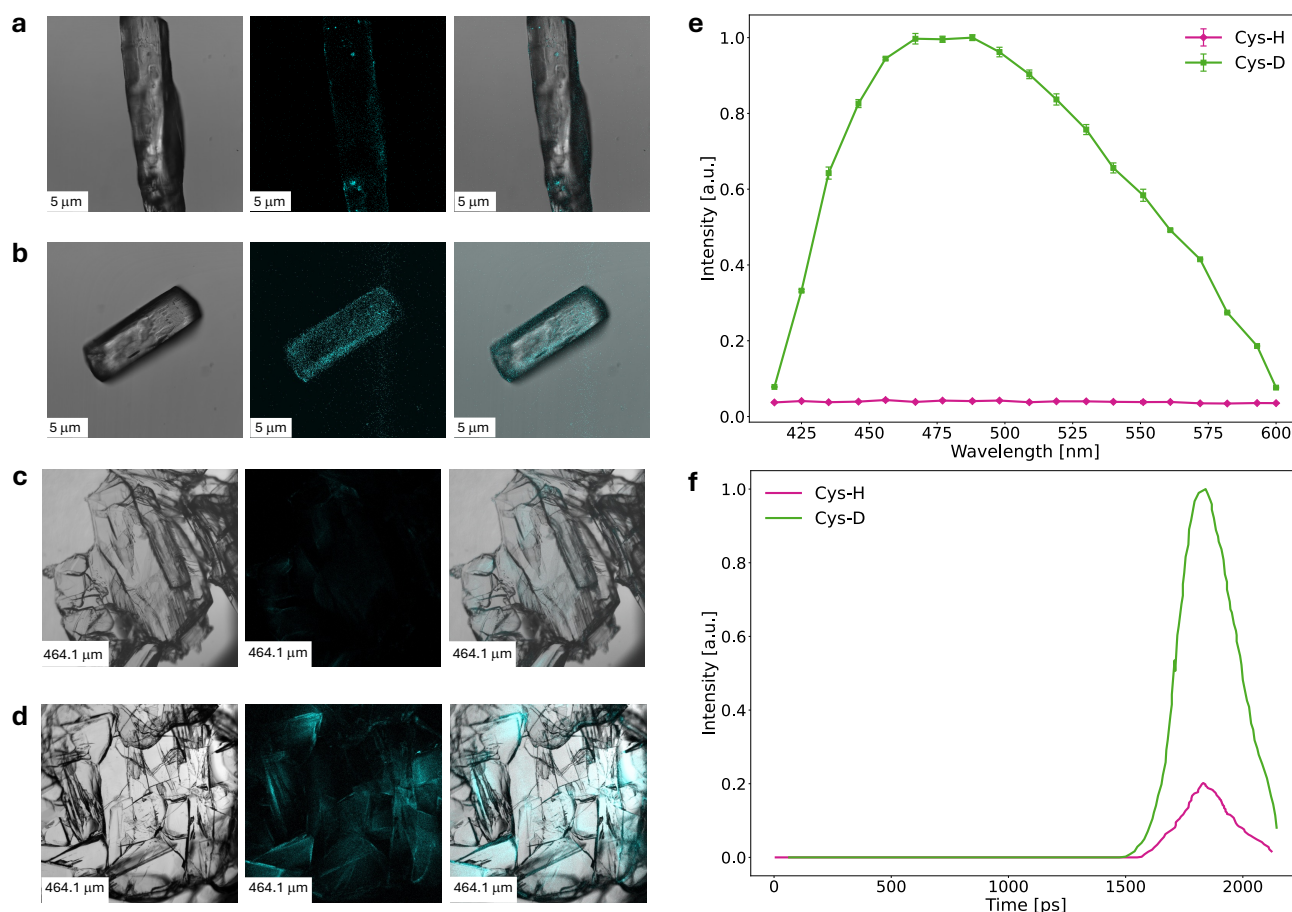


**Figure 1.** In panels **a** and **b**, the confocal microscopic images of single crystals of Cys-H and Cys-D that are excited at a wavelength of 405 nm are shown, respectively. In panels **c** and **d**, the confocal microscopic images of aggregate of crystals of Cys-H and Cys-D that are excited at a wavelength of 405 nm are shown, respectively. Each of these four panels consist of brightfield, fluorescence, and merged image of the crystals (from left to right). In panel **e**, the emission spectra of Cys-H (pink) and Cys-D (green) captured with a confocal microscope is shown. The standard error over 3 independent measurements are highlighted with vertical bars as well. In panel **f**, the FLIM spectra after excitation at 480 nm of crystal aggregates captured with a time-correlated single-photon counting (TCSPC) system for Cys-H (pink) and Cys-D (green) is shown.

ature phosphorescence (p-RTP)<sup>[47–52]</sup>. Interestingly, very recent work has shown that changing the chemistry and physical interactions associated with amino acids in crystals through the introduction of sugars, can significantly enhance luminescence<sup>[53]</sup>.

Our search for novel compounds exhibiting NAF, has led us to investigate how crystal polymorphism can affect optical properties in L-Cysteine (Cys). The thiol chemistry associated with Cys is essential for stabilizing tertiary and quaternary protein structures<sup>[54–56]</sup>. Furthermore, the thiol group also makes Cys extremely sensitive to redox chemistry – a factor that has been linked to the medical pathology of various diseases associated with protein aggregation<sup>[57–61]</sup>. Within the context of fluorescence spectroscopy, Cys has also been suggested to function as a quencher of fluorescence of aromatic and heterocyclic compounds<sup>[62–65]</sup>.

In this work, we present a joint experimental and theoretical study expanding our understanding of non-aromatic fluorescence focusing on Cysteine crystals grown in H<sub>2</sub>O (Cys-H) and D<sub>2</sub>O (Cys-D). Over the last decades, there has been a growing appreciation that biophysical processes<sup>[66]</sup> such as protein folding and aggregation can be subtly modulated using either light (H<sub>2</sub>O) or heavy water (D<sub>2</sub>O). Surprisingly, these two crystals have starkly different crystal structures as

well as optical properties. Interestingly, Cys-D exhibits enhanced fluorescence compared to Cys-H. This enhancement originates from subtle differences in the molecular packing and hydrogen-bonding networks of Cys crystals formed in light versus heavy water. By deploying excited-state non-adiabatic molecular dynamics simulations, we unravel the nature of the electronic states that trigger non-radiative decay pathways involving the amine (C–N) and thiol (S–H) groups differently for Cys structures crystallized in light and heavy water. Our findings form part of a broader effort elucidated in previous studies showing that single amino acids or polypeptide chains in isolation do not display these anomalous optical properties. However, crystals (including L-Cysteine) appear to display blue fluorescence emission, a feature which is strongly modulated by the nature of the interactions that stabilize the crystal or aggregate.<sup>[6,7,13,67]</sup> We also show the importance of the coupling of both singlet and triplet state emission for Cys-D indicating a much more complex origin in the underlying photoluminescence than is evident at first sight. These insights could guide the rational design of bioorganic fluorophores with tunable emission, opening new avenues for imaging, sensing, and photonics in biological systems.

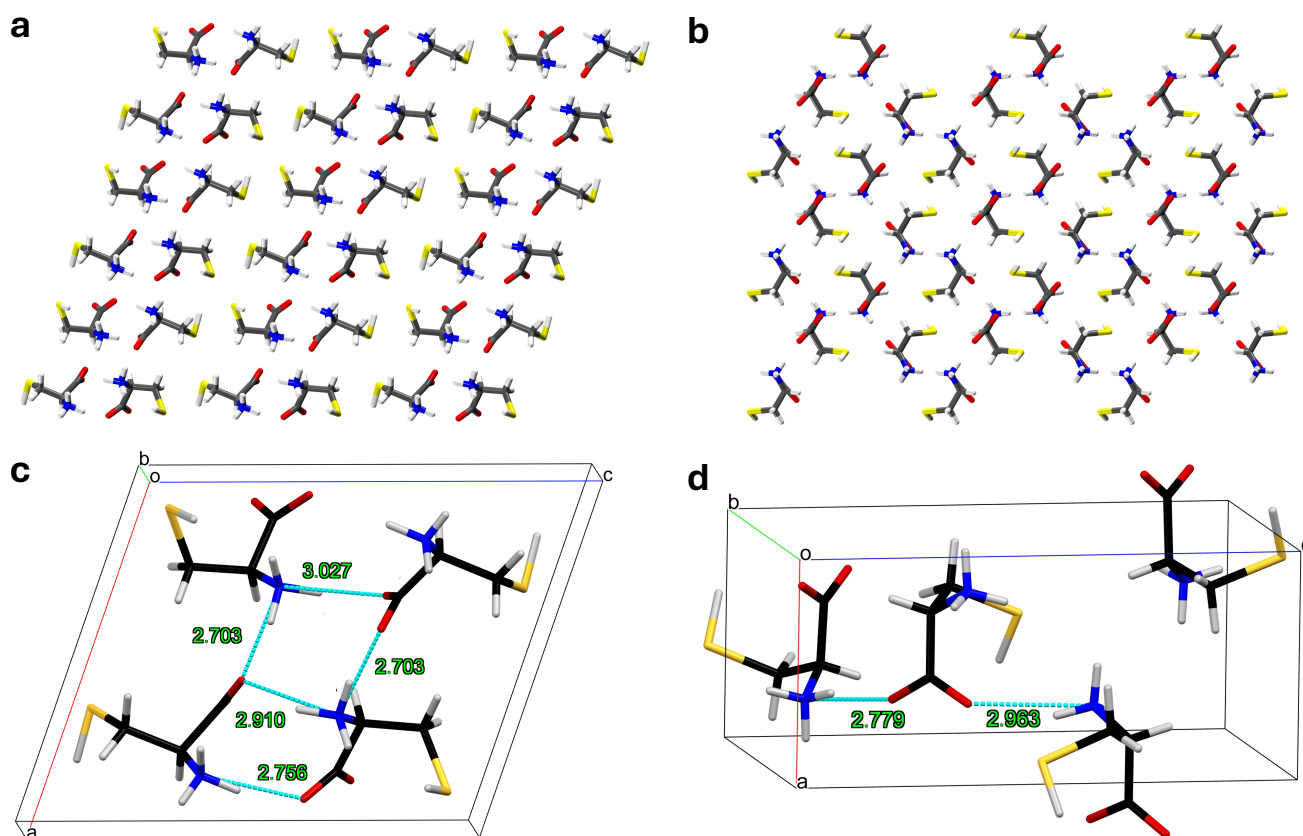


**Figure 2.** Panels **a** and **b** show an overview of the crystal packing in Cys-H<sup>[68]</sup> and Cys-D respectively. Panel **c** shows the monoclinic unit cell of Cys-H, while **d** shows the orthorhombic unit cell of Cys-D, each containing 4 monomers, with some typical hydrogen bonds and their lengths between O (red) and N (blue) atoms highlighted.

## Results and Discussion

### Experimental Measurements Reveal Enhanced Intrinsic Fluorescence of Cysteine in D<sub>2</sub>O vs H<sub>2</sub>O

We prepared crystals of L-Cys in D<sub>2</sub>O and H<sub>2</sub>O using the heating and cooling method<sup>[69]</sup>. Specifically, L-Cys was dissolved in H<sub>2</sub>O and D<sub>2</sub>O at 90 °C until the solution became clear and transparent. Subsequently, the amino acid solutions were cooled to room temperature to allow crystals to form.

Following crystallization, confocal microscopy was used to investigate the optical properties of the crystals. The crystals were illuminated by laser light, and their fluorescence was recorded. An excitation wavelength of 405 nm was used to capture images of fluorescence and brightfield. It was evident from the images taken of the crystals formed in H<sub>2</sub>O that the fluorescence was very low, as seen in Fig. 1a. Crystals formed in D<sub>2</sub>O however, showed significantly higher fluorescence than crystals formed in H<sub>2</sub>O (Fig. 1b). Confocal microscopy was also performed on multiple crystals together and the same trend was observed with crystals formed in D<sub>2</sub>O displaying higher fluorescence (Fig. 1c and Fig. 1d).

To obtain a more quantitative comparison of the fluorescence intensity arising from the crystals formed in H<sub>2</sub>O versus D<sub>2</sub>O, the emission spectra were extracted from the confocal microscope and the fluorescence signals were quantified. The crystals prepared in D<sub>2</sub>O produced more enhanced fluorescence signals that were approximately an order of

magnitude higher than those prepared in H<sub>2</sub>O (Fig. 1e). Note that these features were reproduced on three independent measurements on multiple crystals and the standard error of these measurements is shown in Fig. 1e.

Although the preceding results indicate that the optical properties of the crystals in H<sub>2</sub>O and D<sub>2</sub>O are different, the setup of the confocal microscope has a limited sensitivity for resolving the fluorescence intensity. We thus turned to using a time-correlated single-photon counting (TCSPC) system to conduct fluorescence lifetime imaging microscopy (FLIM). Cys crystals were uniformly distributed on a microscope slide prior to imaging. The FLIM system, equipped with a TCSPC card and two detectors, was used to capture images and record the fluorescence lifetimes of the crystals in both H<sub>2</sub>O and D<sub>2</sub>O under identical conditions. Using FLIM, we also find that the crystals in D<sub>2</sub>O display enhanced fluorescence compared to those in H<sub>2</sub>O (Fig. 1f). In addition, the absolute quantum yield was determined for both Cys-H and Cys-D at room temperature on multiple crystals, the former being 0(±0.05)% (that is, there was no measurable quantum yield within the error bars of the instrument), and the latter at 8(±0.02)% (see SI Figure S1 for details).

### Supramolecular Packing of Cysteine Crystals are Different in D<sub>2</sub>O vs H<sub>2</sub>O

Cysteine has two well-known crystal polymorphs when crystallized in H<sub>2</sub>O at both ambient and low temperatures. One of them is a monoclinic crystal<sup>[68,70]</sup>, while the other is or-

thorhombic<sup>[71–73]</sup>. The striking differences between the optical properties of Cys-H and Cys-D indicate that there must be some important structural differences between the two crystals. Therefore, to understand the structural features contributing to the differences in the optical properties between the two crystals in H<sub>2</sub>O and D<sub>2</sub>O, we first investigated their crystal packing. We resolved the latter structure by performing single-crystal X-ray diffraction (SC-XRD) (see Methods for more details), while the former was taken from Ref. 68. Crystal data collection and refinement parameters are shown in SI Table S1. To the best of our knowledge, this is the first time the structure of Cys has been crystallized and characterized using D<sub>2</sub>O as a solvent. Neither Cys-H nor Cys-D contains any crystallographic light/heavy water molecules. One interesting feature to note is that the Cys-D structure bears a striking similarity to the orthorhombic structures that have been observed in Refs. 71–74, indicating an underlying crystal polymorphism. We have independently verified that single-crystal X-ray diffraction analysis of Cysteine in H<sub>2</sub>O yields a monoclinic structure consistent with previous reports in the literature (see SI Table S2 for more details).

The two different structures thus formed by crystallizing cysteine in H<sub>2</sub>O (Cys-H) and D<sub>2</sub>O (Cys-D) are shown in Fig. 2a (monoclinic) and Fig. 2b (orthorhombic). Both crystals consist of 4 monomers in the unit cell with subtle differences in the packing arrangement, specific interactions involving the hydrogen bonds (HBs), and the conformations of the cysteine molecules. Specifically, the N-C termini HBs typically range between 2.70–3.02 Å in Cys-H, (Fig. 2c) while in Cys-D (Fig. 2d) they range between 2.76–2.96 Å. Differences along other dihedral angles involving the O–C–C<sub>α</sub>–C<sub>β</sub> are also observed (see SI Figure S2) which ultimately play an important role in affecting the fate of cysteine on the excited state.

While the XRD experiments provide insight into the different crystal structures of Cys-H and Cys-D, they do not reveal the positions of hydrogen atoms, as these cannot be resolved. However, given the subtle differences between Cys-H and Cys-D, it is important to establish whether these crystallographic differences arise from a hydrogen isotope effect or an environmental solvent effect. Therefore, electrospray ionization mass spectrometry (ESI-MS) in positive ion mode was performed on both crystals and the results are shown in SI Figure S3. The mass spectrum of Cys-H (SI Figure S3a) shows an intense *m/z* peak at 122.1, which is consistent with the fragment of the protonated molecule ([CysH+H]<sup>+</sup>)<sup>[75]</sup>. The same *m/z* peak is observed at 122.4 for Cys-D (SI Figure S3b), thus confirming the presence of hydrogen atoms in both crystals. Other characteristic peaks of cysteine, i.e. at *m/z* 152 and 241, can be also observed in the spectra, as well as the ion peak corresponding to sodiated cystine ([CysScy+Na]<sup>+</sup>) at *m/z* 263. The detected ion peaks provide further validation that the differences in the physical properties in the two structures do not originate from isotope differences - all non-carbon, non-nitrogen, and non-sulfur atoms are hydrogen in both crystals.

## Molecular Mechanisms and Electronic Origins Behind Intrinsic Fluorescence of Cysteine in Heavy Water

The preceding experimental results show a striking effect of the solvent (H<sub>2</sub>O vs D<sub>2</sub>O) on the resulting cysteine crys-

tal structures. To understand the differences in the optical properties of Cys-H and Cys-D, we turned to conducting mixed quantum/classical molecular (QM/MM) electronic structure calculations that probe the absorption, emission, and non-radiative decay mechanisms. Time-Dependent Density Functional Theory (TDDFT) with the CAM-B3LYP functional was used for all our electronic structure calculations (see Methods for more details). We carved out two monomers from each unit cell forming a dimer which was treated at QM level (see SI Figure S4). Note that the criterion for choosing these QM dimers was based on making sure that the highest number of HBs were formed between the N and C termini. This was then embedded in a periodic environment consisting of MM cysteine molecules. Given the high computational cost of these simulations, we strike a careful balance between efficiency and accuracy, guided by previous approaches introduced by some of us<sup>[35,76]</sup>. In the Methods section, we present validation tests using more accurate electronic structure methods and a larger QM region to justify our approach.

We first determined the absorption spectra for both systems using TDDFT, based on a sample of 40 independent configurations from the ground state equilibrium simulations (see Methods section). Figure 3a shows the absorption spectra for Cys-H and Cys-D. In both cases, the lowest excitation energies are found to be around 210 nm (5.9 eV) (see SI Figure S5a for a zoomed-in version). To gain further insight into the nature of the low-lying electronic transitions, we examined the molecular orbitals involved in the S<sub>0</sub> → S<sub>1</sub> excitations. Figure 3b illustrates the molecular orbitals associated with two typical excitations observed in both systems: i) n → π\* transition involving the C-terminus carboxylate groups and ii) n → σ\* transition localized on the S–H (thiol) group.

Although both of these transitions can characterize the S<sub>0</sub> → S<sub>1</sub> excitation, there are differences in their occurrence in both systems. In Cys-H, the initial excitation is more or less equally probable to be of either type, while for Cys-D, it is much more likely to be of type i) than type ii) (see SI Table S3 for details). The differences in crystal packing between Cys-H and Cys-D seem to significantly influence the nature of electronic transitions in the Franck-Condon region. Consequently, the variations observed in fluorescence are expected to become more pronounced as the system evolves in its electronically excited states.

Analysis of the 40 simulated trajectories for each system revealed distinct non-radiative decay pathways for Cys-H and Cys-D. In Cys-H, 90% of trajectories decayed non-radiatively to the ground electronic state, while in Cys-D, this pathway was observed in only 33% of trajectories. This difference corroborates the experimentally observed enhancement of emission intensity upon the change of packing from the crystal structure of Cys-H to that of Cys-D. Furthermore, Cys-D displays sizable intersystem crossing (ISC), with an additional 33% of trajectories populating the triplet states. In contrast, ISC was less prevalent in Cys-H, accounting for only 7.5% of the simulated trajectories. At the end of our 2 ps NAMD simulations, only 5% of trajectories remained in S<sub>1</sub> for Cys-H, while 33% did so for Cys-D. For the latter, we can use the trajectories that remain on S<sub>1</sub> to estimate the fluorescence emission spectrum which is shown by the blue curve in Figure 3a. This emission spectrum is blue-shifted compared to the experimental spectra by about 80 nm (0.5 eV) which is within the theoretical error bars of

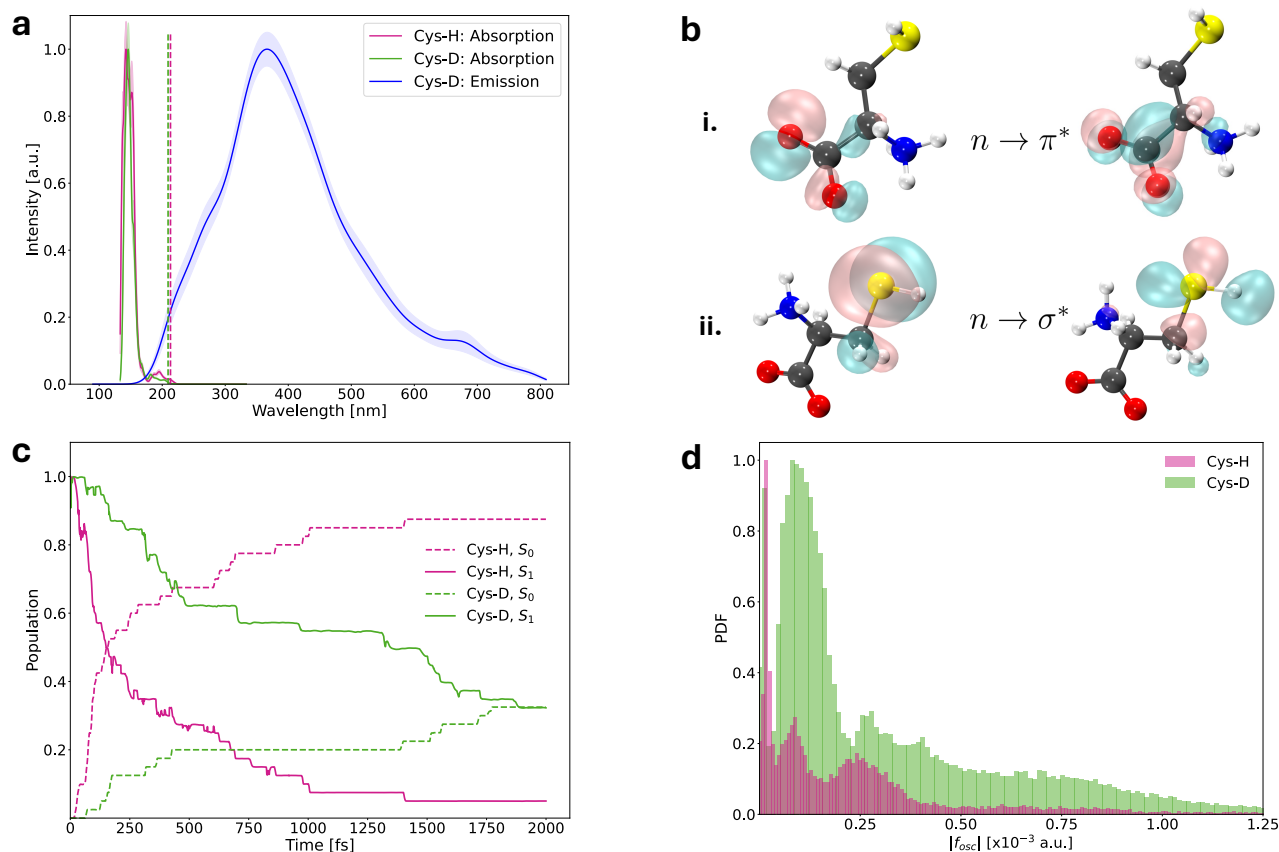


**Figure 3.** In panel **a**, the theoretically computed absorption spectra for Cys-H (pink) and Cys-D (green) and fluorescence emission spectrum (blue) for Cys-D is shown. The shaded blue region denotes the standard error in theoretical estimation since it is an average over 40 independent trajectories. The dashed vertical lines (green - 209 nm, pink - 215 nm) represents the wavelength corresponding to the  $S_0 \rightarrow S_1$  transition in our electronic structure calculations for Cys-D and Cys-H, respectively. Panel **b** shows the 2 types of  $S_0 \rightarrow S_1$  transitions that occur commonly. In **(b.i)**, we see an  $n \rightarrow \pi^*$  transition that occurs on the carbonyl (CO) group, whereas in **(b.ii)** we see an  $n \rightarrow \sigma^*$  transition that is present in the SH moiety. Panel **c** shows the evolution of the average population of the singlet electronic states, where  $S_1$  and  $S_0$  are denoted by solid and dashed lines respectively. Results corresponding to Cys-D and Cys-H are shown in green and pink, respectively. In panel **d**, the oscillator strengths obtained from the TSH simulations, corresponding to  $S_1 \rightarrow S_0$  emission are shown. Results corresponding to Cys-D and Cys-H are shown in green and pink, respectively.

the electronic structure methods we are employing. Later in the paper, we present benchmarks to determine theoretical error bars for our choice of electronic structure method and discuss their implications for comparisons with experimental results.

We begin first by examining the behavior of the excited state dynamics involving the singlet states. Figure 3c shows the average electronic populations of the  $S_0$  and  $S_1$  states for both Cys-H and Cys-D. Here we clearly observe that in Cys-H, the  $S_1$  population reduces significantly within the first 250 fs, and thereafter continues decaying further, while for Cys-D, the population decay occurs over a significantly longer time-scale. The corresponding curves for the  $S_0$  state are also shown mirroring the behavior of the  $S_1$ . These theoretical predictions are thus fully consistent with the experimental observation of photoluminescence only in Cys-D.

The lower probability of non-radiative decay in Cys-D, on its own, is not sufficient to lead to non-aromatic fluorescence. Specifically, we also need the excited state on which the system exists to be *bright*. This is characterized in terms of the oscillator strength between  $S_1$  and  $S_0$ . In Figure 3d we see the probability distribution function of the oscillator strengths for the  $S_1 \rightarrow S_0$  transition, and we can see clearly that Cys-D has a higher probability of having a larger oscillator strength than Cys-H, thereby contributing to a *bright*

photoluminescence which is, again, in good agreement with the experimental results.

Having established the differences in the energetic and electronic properties on the excited state of Cys-H and Cys-D we next dissect the vibrational modes that lead to non-radiative decay in both systems. In recent works<sup>[15,23,33,35]</sup>, research by some of us has shown that specific vibrational modes such as the carbonyl stretch, proton transfer, and deplanarization play a critical role in modulating the excited-state lifetimes of glutamine, peptide chains, and amyloids. In particular, by constraining/locking these modes the barriers to accessing conical intersections (CIs) can be raised. As a result, this pathway is either delayed or completely inhibited, reducing the rate of the non-radiative decay and thereby increasing the likelihood of photon emission. The excitations involving the  $n-\pi^*$  and  $n-\sigma^*$  shown earlier in Figure 3b, prompted us to examine vibrational coordinates associated with these regions of the molecules. Specifically, in the case of the  $n-\pi^*$  transition, a natural expectation would be the lengthening of the C=O bond while for the  $n-\sigma^*$ , there would be an increase in the S-H bond length. Table S4 summarizes the statistics associated with the four main vibrational modes we identified that play a role in the photochemistry, details of which are now discussed.

A visual inspection of some of the excited-state trajec-

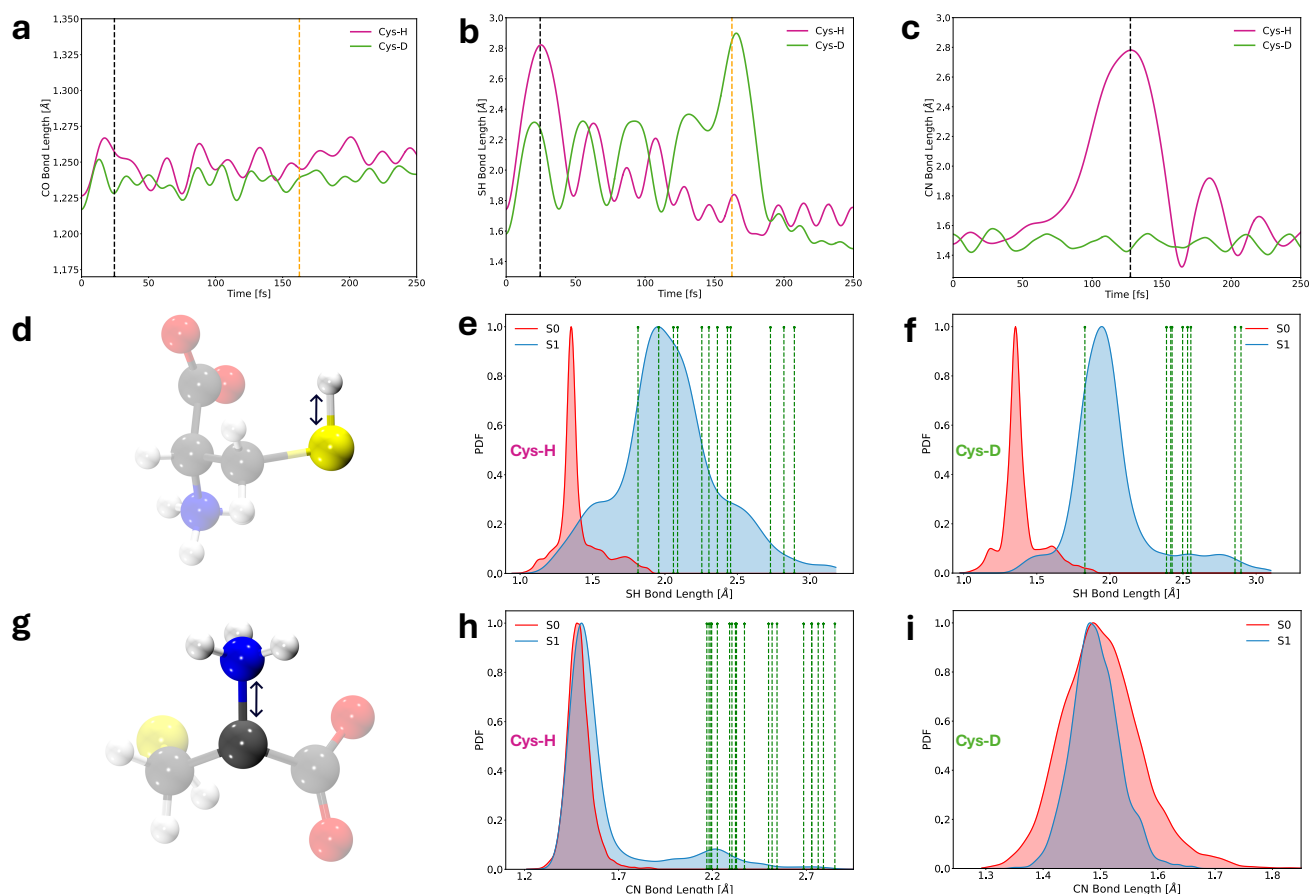


**Figure 4.** Panels a,b,c show the time-series evolution of CO, SH, CN bond lengths across model trajectories. The bond lengths for Cys-D and Cys-H are shown in green and pink respectively, while the time of encountering the conical intersection is shown with orange and black dashed vertical lines, respectively. The 2 major non-radiative decay modes are the stretching of the S–H and C–N bonds, depicted in panels d and g. The Probability Distribution Function (PDF) of the S–H bond for Cys-H and Cys-D are respectively shown in panels e and f, where the PDF corresponding to  $S_0$  is shown in red and the one corresponding to  $S_1$  is shown in blue. The dashed green lines depict the bond lengths at which the conical intersection is reached, and non-radiative decay occurs from  $S_1 \rightarrow S_0$ . Similarly, in panels h and i, the PDF of the C–N bond for Cys-H and Cys-D are shown.

ries indicated that most of the activity involves two vibrational modes, the S–H and C–N bond lengths. Although the initial excitation originates in an  $n-\pi^*$  transition localized on the carbonyl group, the C=O bond length in all our trajectories displays only moderate fluctuations ranging between 1.1–1.4 Å and it therefore does not appear to be a key player in the deactivation mechanism. On the other hand, for the excitations involving  $n-\sigma^*$  near the sulfide group, the S–H bond undergoes large geometrical changes which ultimately lead to non-radiative decays in both Cys-H and Cys-D.

Investigating a sample trajectory for both Cys-H and Cys-D that undergoes non-radiative decay, we show the time-series of the C=O bond (Fig. 4a) and observe no significant distortions that coincide with the system encountering the CI and decaying non-radiatively to the ground state. Instead, in the time series of the S–H bond (Fig. 4b) we observe a large extension of this bond at the point the system decays to  $S_0$ . For Cys-H, 12 out of the 35 decays to the ground state occur through this mechanism, whereas for Cys-D, 9 out of 13 non-radiative decays to the ground state follow this pathway.

From the preceding analysis, the majority of the non-radiative decays for Cys-H remain unexplained and this led to us looking for alternative decay modes. In Fig. 4c we

show a different trajectory where the C–N stretches to a large value (2.8 Å) in concert with a decay of the system to the ground state. In fact, this turns out to be the dominant mode of non-radiative decay to the ground state for Cys-H, with 19 out of the 35 decays following this mechanism. Remarkably, this mode of decay appears unique to Cys-H and does not occur in Cys-D. In fact, the subtle changes in the underlying electronic structure are ultimately rooted in the differences of the O–C–C $\alpha$ –C $\beta$  dihedral angles between Cys-H and Cys-D. Specifically, looking at the probability distribution of this dihedral while the system is in  $S_1$  (see SI Figure S7b), we see that a planar geometry ( $0^\circ$ ,  $180^\circ$ ,  $360^\circ$ ) is far more probable in Cys-H and effectively not sampled in Cys-D. This means that the extended O–C–C $\alpha$ –C $\beta$  planarization is likely to lead to an enhancement of resonance within the molecule for the crystal formed in heavy water.

To conclude the discussion of decay mechanisms (which are summarized in SI Table S4), the remaining 4 out of 35 decays for Cys-H were through the stretching of the C–S bond, often involving a dissociation of the same. For Cys-D, there was 1 out of the 13 decays involving a C–S stretch. The remaining 3 decays for Cys-D were through a stretch of the C–COO $^-$  bond, but these decays, which followed ISC, will be elaborated on in Section “Cysteine in Heavy Water Exhibits Synergistic Singlet and Triplet Emission”.

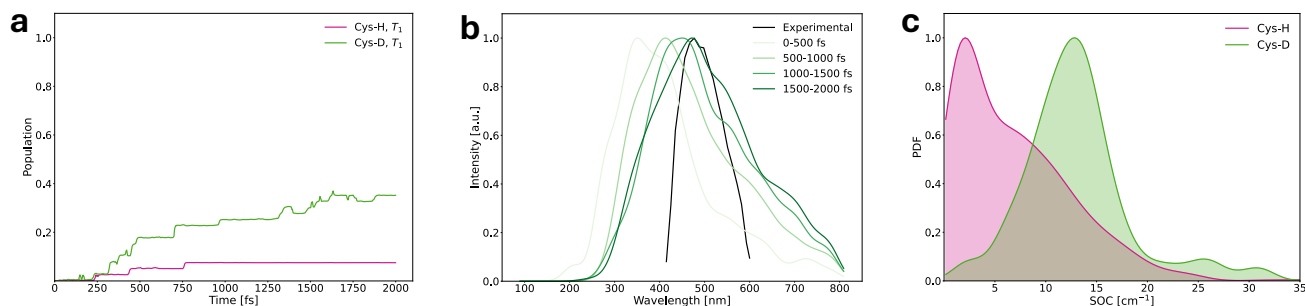


**Figure 5.** Panel **a** shows the cumulative triplet state population of Cys-H (pink) and Cys-D (green). In panel **b** we see the time-dependent emission spectra from the triplet state for Cys-D, in blocks of 500 fs, with the shades of green getting darker for successive blocks of time, while the experimental emission spectra is shown in black. Panel **c** shows a PDF of the SOC computed from 1000 random frames for both Cys-H (pink) and Cys-D (green).

To better quantify the differences in the ground and excited state distributions along some of these coordinates and how they affect the access to the CI, we show probability distributions of the S–H and C–N modes in the ground and excited state for Cys-H and Cys-D in Figure 4. Looking at the S–H mode (visualized in Fig. 4d), we clearly see that in both Cys-H (Fig. 4e) and Cys-D (Fig. 4f) the probability distribution of the S–H bond when the system is in  $S_1$  is peaked around 2.0 Å, while in  $S_0$  the equilibrium bond lengths are usually between 1.2–1.5 Å. The bond lengths at which the system encounters the CI are marked through green vertical lines, and they range between 2–3 Å. The position of the CI appears to be closer to the peak of the distribution of the  $S_1$  state in Cys-H compared to Cys-D, consistent with the idea of a less constrained mode in the former as observed in an earlier study for the carbonyl stretch<sup>[35]</sup>.

Looking at the probability distribution of the C–N mode (visualized in Fig. 4g) in both Cys-H (Fig. 4h) and Cys-D (Fig. 4i), we clearly see that the bond can stretch to larger values in  $S_1$  only in Cys-H, while in Cys-D it remains confined between 1.4–1.6 Å. This leads to the system decaying through the conical intersection along the C–N mode only in Cys-H, and the enhanced optical activity of Cys-D can be primarily attributed to the absence of this mode of decay. Furthermore, the large overlap in the distribution of the C–N mode in Cys-D creates a situation that makes accessing the CIs along this mode more difficult. We will elaborate more on the C–N mode and its uniqueness in Cys-H in the SI section “Inhibiting C–N Distortions in Cysteine Luminescence”.

## Cysteine in Heavy Water Exhibits Synergistic Singlet and Triplet Emission

As alluded to earlier, in Cys-D, we observe that the photochemistry is also heavily influenced by intersystem crossing. Figure 5a shows the time-evolution of the population of the triplet state for both Cys-H and Cys-D where one clearly sees that the significant increase in the triplet population appears to be a unique feature of Cys-D, where about 35% trajectories remain in a triplet state at the end of our 2 ps dynamics, while 7.5% of trajectories for Cys-H do the same. In fact, 17 out of 40 trajectories undergo ISC for Cys-D, and 3 of these decay to the ground state from the triplet states. While no clear vibrational mode could be associated to the  $S_1 \rightarrow T_1$  ISC transitions, the  $T_1 \rightarrow S_0$  transitions can be associated with a clear stretch of the C–COO<sup>−</sup> bond. This

also appears exclusively in Cys-D, and the few trajectories that do undergo ISC for Cys-H do not decay to the ground state via a further ISC.

Our simulations indicate that the ISC in cysteine crystals occurs on a sub-picosecond timescale. Several previous theoretical and experimental studies in a wide-variety of systems have shown<sup>[77–82]</sup> that intersystem crossing can occur on similar timescales. This suggests that the observed luminescence observed on the nanosecond timescale in the experiments may arise from a mix of both singlet and triplet emission. In order to provide a theoretical prediction of the possible emission spectra arising from the lowest lying triplet state, the  $T_1 \rightarrow S_0$  energy gap, the corresponding transition-dipole moment, and oscillator strength need to be determined. We do this on a subset of configurations, selecting 100 random frames for each 50 nm block in the emission energies between 200 nm to 800 nm. This gives us mean oscillator strengths for each range of energies which we then use to compute the time-dependent emission spectra in blocks of 500 fs (Fig. 5b). Here we observe that the peak of the emission energies appears to converge, perhaps fortuitously, to the peak of the experimental emission spectra. On the other hand, if we look at the time-dependent emission spectra from  $S_1$ , it converges to approximately 400 nm which is an 80 nm (0.5 eV) blue-shift compared to the experimental results (see SI Figure S5b). It is important to note here that the oscillator strengths for triplet emission ( $\sim 10^{-6}$ ) are about three orders of magnitude smaller than those corresponding to the singlet emission ( $\sim 10^{-3}$ ). Consequently we would expect any emission arising from the triplet states to be much weaker than the singlet emission.

Since the vast majority of the ISC occurs from  $S_1 \rightarrow T_1$ , we look at the likely values of the spin-orbit coupling (SOC) for the two systems in Figure 5c. Having computed the PDF of the SOC over 1000 randomly selected frames while the system is in  $S_1$ , we see a clear difference in the two systems, with Cys-D (green) much more likely to have higher values of the SOC than Cys-H (pink), which is consistent with the behavior we observe in our simulations. The significant ISC observed in Cys-D can be further quantified, by visualizing for all trajectories, the cumulative probability of ISC while the system is in  $S_1$ . In SI Figure S6a, we clearly see that trajectories of Cys-D have a much higher cumulative probability of ISC than Cys-H. As we have already noted above, Cys-D is more likely to have a higher SOC than Cys-H, and this contributes to the increased ISC probability for the former. Part of the reason might also be the fact that Cys-H undergoes non-radiative decay to  $S_0$  so quickly that the sys-

tem has no opportunity to experience ISC. Another way of understanding the preferential ISC experienced by Cys-D is by looking at the distribution of energy gaps between  $S_1$  and the closest triplet state<sup>[83]</sup> (which is usually  $T_1$ ) in SI Figure S6b. In Cys-H, ISC from the singlet excited state is less probable due to the fact that there is a larger gap to the energetically closest triplet state. The distribution has a larger peak near 0 for Cys-D than for Cys-H, which coincides with more frequent occurrences of nearly-degenerate  $S_1$  and  $T_1$  states during Cys-D trajectories.

## Conclusion

In this joint experimental and theoretical work, we have taken an important step to understanding new governing principles underlying the phenomenon of intrinsic fluorescence in bioorganic crystals. Cysteine molecules crystallized in light and heavy water display rather striking differences in their crystallographic packing and optical properties. Using excited-state simulations, we rationalize the electronic and vibrational origins of the enhanced fluorescence observed in the crystal that is formed in heavy water.

Recent studies have highlighted that the careful incorporation of heavy atoms, such as sulfur, can improve the efficiency of thermally activated delayed fluorescence (TADF) photocatalysts<sup>[84]</sup>. Our findings indicate that Cys-D, with its relatively high SOC, could open new possibilities for the design of such materials. In addition, prior research<sup>[67]</sup> has shown that crystallised non-aromatic amino acids (L-Lysine, L-Serine, L-Isoleucine) can also display persistent room temperature phosphorescence (p-RTP) in addition to fluorescence. These previous findings reinforce the theoretical predictions made in the current work that synergistic singlet and triplet emission may be observed in L-Cysteine and that this is sensitive to differences in the molecular packing between the two crystals formed in light and heavy water.

The ability to probe in a time-resolved manner nucleation mechanisms of amino acids or proteins in solution remains an open challenge. The intrinsic fluorescence of amino-acids and how they potentially change as a function of clustering or aggregation offers a non-invasive manner to study both thermodynamics and kinetic pathways of crystallization without the introduction of external fluorescent probes.

In addition, solvent isotope effects in biophysical processes such as altering the secondary structure and aggregation of proteins have been observed in a wide class of biomolecular systems. Here we have shown that the simple effect of moving from light to heavy water alters the crystallization pathways and ultimately also the optical properties of the two crystals. It should be underscored that the solvent isotope effects we show in this work on the emission arise exclusively from the manner in which  $H_2O$  and  $D_2O$  alter the stability of the thermodynamically stable crystal packing of Cysteine molecules in solution. Since both crystals are devoid of any crystallographic water molecules ( $H_2O$  or  $D_2O$ ) and the mass-spectroscopy shows that both crystals consist of only protons (H not D), the effect on fluorescence emission is strongly modulated by the differences in the nature of the electronic states and non-radiative decay pathways as observed in our simulations, which originate from the distinct features in crystal packing. Our results set the stage for exploring the complexity of self-assembly combining both solvent-isotope effects along with changes in intrinsic fluorescence.

In summary, this study establishes a fundamental principle of non-aromatic fluorescence which is a clear testament to how relatively subtle structural packing changes can lead to massively different optical properties for crystal polymorphs of amino acids. Based on this molecular picture, we may be able to rationally design new generations of non-aromatic amino-acid based optical devices of unique photonic and electronic properties as well as intrinsic biocompatibility.

## Acknowledgements

DB, GDM, MM, and AH thank the European Commission for funding on the ERC Grant HyBOP 101043272. DB, GDM, MM, and AH also acknowledge CINECA supercomputing (project NAFAA-HP10B4ZBB2) and MareNostrum5 (project EHPC-EXT-2023E01-029) for computational resources.

## Conflict of Interest

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article

**Keywords:** Fluorescence • Ab initio calculations • Molecular Dynamics • Crystal growth • Amino acids

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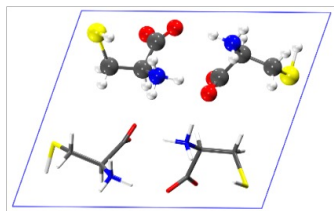
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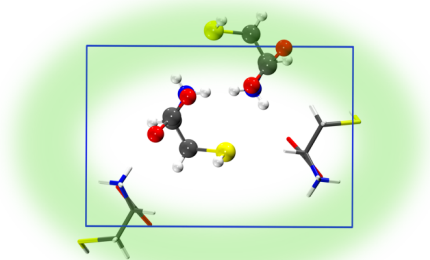
## Entry for the Table of Contents

Cysteine in H<sub>2</sub>O



Non-Fluorescent!

Cysteine in D<sub>2</sub>O



Fluorescent!

Crystallization of L-Cysteine in D<sub>2</sub>O generates a remarkably different structure compared to H<sub>2</sub>O. The crystals formed in D<sub>2</sub>O display enhanced intrinsic fluorescence and using state-of-the-art excited state simulations, we determine the molecular origins of this luminescence and propose design principles for new intrinsic fluorophores.