

Article

Conformational Preferences of the Trypanocidal Drug Benznidazole by DFT-Guided Vibrational Spectroscopy

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Abstract

Chagas disease remains a major neglected parasitic illness in Latin America and other endemic regions, and benznidazole (BZN) is still the primary trypanosomacidal drug despite its incompletely understood mechanism of action. This work provides a detailed biophysical characterization of the conformational behavior and vibrational properties of benznidazole (BZN), a first-line trypanocidal drug still widely used for the treatment of Chagas disease. Using density functional theory combined with relaxed potential energy surface scans in vacuum and implicit water, two low-energy conformers (BZN1 and BZN2) were identified, separated by moderate rotational barriers and a small energy difference, indicating that both are intrinsically accessible at room temperature. For each conformer, infrared and Raman spectra were calculated and assigned via vibrational mode analysis, then compared with FT-IR and FT-Raman spectra recorded for pharmaceutical-grade polycrystalline BZN. The theoretical and experimental spectra show excellent agreement, with a Raman band in the 1350–1400 cm^{−1} region emerging as a sensitive conformational marker: the experimental maximum at 1359 cm^{−1} matches the most intense BZN1 mode, whereas the corresponding BZN2 band appears about 13 cm^{−1} higher in frequency. This clear spectroscopic fingerprint demonstrates that the solid drug is overwhelmingly composed of the BZN1 conformer, despite the theoretical accessibility of BZN2. Overall, the study links the conformational landscape of benznidazole to its vibrational signatures and highlights Raman spectroscopy, supported by quantum chemical calculations, as a powerful tool for conformational and potential polymorphic control of this clinically important nitroimidazole.

Keywords: benznidazole; conformational analysis; vibrational spectroscopy; Raman spectra; infrared spectra; Chagas disease



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1. Introduction

Chagas disease is a tropical parasitic illness that affects populations in regions of Latin America, Africa, and Asia [1] and remains a major public health problem more than a century after its discovery. The hemoflagellate protozoan parasite *Trypanosoma cruzi*, typically transmitted to humans by an insect vector (*Triatoma infestans*) [2], causes high morbidity, chronic cardiac and digestive complications, and significant socioeconomic impacts. The World Health Organization (WHO) estimates that more than 7 million people worldwide, predominantly in Latin America, are infected with *T. cruzi*, the etiological agent of Chagas disease [3]. In 2013, Lee et al. [4] used a Markov simulation model to estimate the global economic impact of Chagas disease, and global figures indicate an annual health care burden of 627.46 million, a lifetime cost of 188.80 billion, and millions of infected individuals and substantial lifetime healthcare costs worldwide. Despite this burden, therapeutic options are still limited: the nitroheterocycles nifurtimox (NFX, radanil, C₁₀H₁₃N₃O₅S, PubChem CID 6842999) and benznidazole (BZN) (BZN, PubChem CID 31593) [5–7] are the only drugs with proven efficacy against Chagas disease, and BZN is generally preferred due to its higher trypanocidal activity and lower toxicity [8].

In parallel with new drug design, formulation strategies, such as benznidazole-loaded lipid nanocapsules, have been developed to optimize drug release, permeability, and stability, aiming to improve therapeutic performance while mitigating toxicity [9]. Encapsulation of benznidazole into nanostructured lipid carriers, monitored by FT-IR, X-ray diffraction, and thermal analysis, has also demonstrated enhanced trypanocidal activity, emphasizing the central role of solid-state and vibrational characterization in the development of nanostructured dosage forms [10]. In addition, nanoencapsulation of benznidazole into calcium carbonate nanoparticles has been shown to increase its selectivity towards *T. cruzi*, further illustrating the potential of nanostructured delivery systems to improve the therapeutic index of this long-established drug [11]. Beyond their trypanocidal effects, benznidazole and other nitroheterocycles exhibit complex interactions with biomacromolecules, such as human serum albumin, which can modulate drug distribution and pharmacokinetics [12]. A recent review on nanocarriers for benznidazole and nifurtimox further underscores that, although numerous delivery systems have been proposed, systematic knowledge of benznidazole's solid-state properties and polymorphism remains comparatively scarce [13].

Benznidazole is a nitroimidazole derivative (N-benzyl-2-(2-nitroimidazol-1-yl) acetamide, C₁₂H₁₂N₄O₃) [14] whose structure can be viewed as three approximately planar fragments—an imidazole ring [A], a benzyl group [B], and an acetamide moiety [C]—see Figure 1. Recent efforts to design 2-nitroimidazole N-acylhydrazones analogous to benznidazole have yielded highly potent and selective anti-*Trypanosoma cruzi* agents, highlighting the nitroimidazole scaffold as a privileged pharmacophore in Chagas disease chemotherapy [15].

In the solid state, crystallographic data indicate that the imidazole and benzyl rings lie on opposite sides of the acetamide fragment, suggesting the existence of preferred conformations stabilized by intermolecular packing [16]. Experimental solid-state studies have already revealed several polymorphic forms of benznidazole, characterized by X-ray diffraction, vibrational spectroscopy, and thermal analysis, confirming that this API can crystallize in distinct packing arrangements with different physicochemical properties [17]. In addition, cocrystal polymorphs and solvates of benznidazole have been shown to markedly improve dissolution performance, underscoring how supramolecular arrangements and solid-state forms critically influence the drug's biopharmaceutical properties [18].

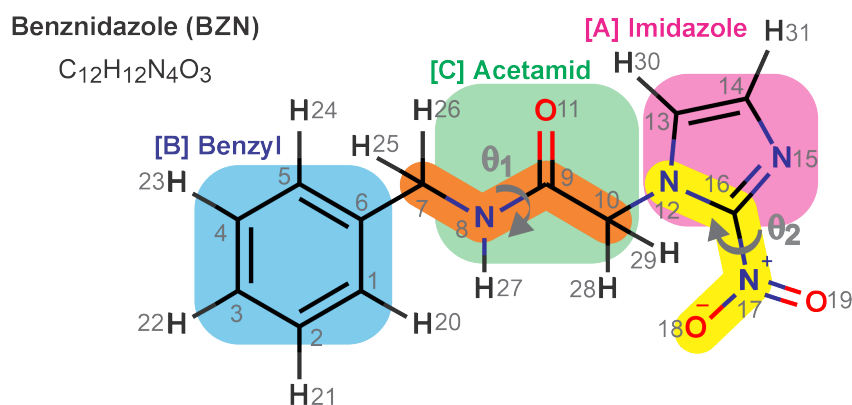


Figure 1. Planar molecular structure of benznidazole (BZN, C₁₂H₁₂N₄O₃, PubChem CID 31593) highlighting the three approximately planar fragments—[A] imidazole ring, [B] benzyl group, and [C] acetamide moiety—with full atom numbering and indication of the dihedral angles θ_1 (C7–N8–C9–C10) and θ_2 (N12–C16–N17–O18) that define the relative orientation of these groups.

However, a systematic theoretical characterization of the BZN conformational landscape, and of how different conformers manifest in infrared (IR) and Raman spectra—particularly in pharmaceutical solid samples—has not yet been fully established. Very recently, combined potential energy surface analysis, FT-IR/FT-Raman spectroscopy, and X-ray diffraction have identified two benznidazole conformers: one matching the room-temperature crystal structure and a second that becomes populated only upon heating, directly illustrating the relevance of conformational equilibria for the solid-state behavior of this drug [19].

A detailed understanding of BZN's conformational preferences and vibrational signatures is relevant for several reasons. First, conformational equilibria can influence physical properties such as solubility, stability, and polymorphism, which are critical for formulation and quality control of solid dosage forms. Second, IR and Raman spectroscopies are routinely used in analytical laboratories, and the ability to link specific spectral features to well-defined conformers provides a powerful tool for monitoring batch-to-batch consistency, detecting polymorphism, and supporting regulatory documentation. Finally, a reliable structural and spectroscopic description at the molecular level is an essential step toward future mechanistic studies that address the relationship between BZN structure and its biological activity in more detail. Previous quantum chemical studies on benznidazole have already combined DFT and TD-DFT calculations with experimental spectroscopy to assign its electronic transitions in solution and to rationalize the main absorption bands in water, thereby establishing a robust electronic-structure framework for this drug [20].

In this work, density functional theory (DFT) calculations combined with relaxed potential energy surface scans are employed to map the conformational space of benznidazole (BZN) in vacuum and in an implicit aqueous environment, identifying the most stable conformers and the barriers separating them. The corresponding IR and Raman spectra are computed, assigned using potential energy distribution analysis, and critically compared with experimental FT-IR and FT-Raman spectra recorded for pharmaceutical-grade BZN. Particular attention is given to spectral regions susceptible to conformational changes, allowing the predominant conformer in the solid drug to be identified and providing a consistent structural–spectroscopic framework for characterizing this clinically important nitroimidazole. Building on this framework, we further ask how the conformational information and vibrational signatures can be exploited in a systematic way for the analytical control of solid BZN.

In this context, our contribution is not to repeat previously reported electronic-structure analyses of benznidazole, but rather to establish a robust, experimentally accessible vi-

brational marker that can be used for routine conformational and polymorphic control of pharmaceutical-grade BZN solids. Specifically, we construct a two-dimensional relaxed potential energy surface (RPESS) as a function of the two key dihedral angles that govern the relative orientation of the imidazole, benzyl, and acetamide fragments, in both vacuum and PCM water, and we explicitly link this conformational landscape to a diagnostic Raman window ($1350\text{--}1400\text{ cm}^{-1}$). By showing that the experimental Raman band at 1359 cm^{-1} selectively identifies the BZN1 conformer and that the polycrystalline industrial material is overwhelmingly composed of this geometry, we provide a practical spectroscopic criterion for batch-to-batch quality control and for monitoring potential polymorphic or conformational deviations in benznidazole-based drug products.

2. Materials and Methods

2.1. Quantum Chemical Calculations and Conformational Analysis

Although benznidazole (BZN) has been in clinical use for more than five decades and remains the first-line trypanocidal drug for Chagas disease [21–24], a detailed theoretical structural and vibrational characterization is still needed to complement existing experimental data. In this work, the geometry of an isolated neutral BZN molecule at physiological pH was optimized using density functional theory (DFT). The hybrid exchange–correlation functional B3LYP [25,26] together with the 6-311+G(d,p) [27] basis set, as implemented in the Gaussian09 [28] package, was employed for all electronic-structure calculations [29]. Similar hybrid DFT levels have recently been applied to benznidazole monomer and dimer, in combination with FT-IR and FT-Raman measurements, yielding highly accurate vibrational spectra and further supporting the present computational protocol [30].

To explore the conformational space of BZN, relaxed potential energy surface scans (RPESS) were performed in both the gas phase and in implicit water, the latter described by the polarizable continuum model (PCM). In these scans, the dihedral angles C7–N8–C9–C10 (θ_1) and N12–C16–N17–O18 (θ_2), indicated in Figure 1, systematically varied in 11 steps of 30° along each coordinate, generating a two-dimensional map of the relative energy ΔE . Stationary points on this surface were re-optimized at the same level of theory, allowing the identification of the two most stable room-temperature conformers (BZN1 and BZN2) and their corresponding interconversion barriers. This protocol thus provides a coherent picture of the low-energy conformational landscape and how it is modulated by the surrounding medium.

Relaxed potential energy surface scans were performed both in vacuum and in an implicit aqueous environment described by the PCM, in order to assess the influence of solvation on the relative stability of the conformers and their interconversion barriers. Using the same electronic-structure description for both the conformational and vibrational calculations ensures that the energetic landscape and the simulated spectra are internally consistent.

The B3LYP/6-311+G(d,p) level was chosen because it has already been successfully employed for benznidazole monomers and dimers, reproducing FT-IR and FT-Raman spectra with high accuracy, and because similar hybrid DFT protocols have performed reliably for related nitroimidazole and benzimidazole systems. In the present work, this level yields an excellent correlation ($r^2 > 0.9989$) between calculated and experimental wavenumbers in the $1000\text{--}1800\text{ cm}^{-1}$ region and correctly predicts the key Raman conformational marker at $1359\text{--}1360\text{ cm}^{-1}$, which is central to our solid-state assignment. Although London dispersion corrections (e.g., D3/D4 or dispersion-aware functionals) could further refine the relative energies and barrier heights, our main conclusions are anchored in the experimental Raman spectrum of the polycrystalline solid, which directly

shows that BZN1 dominates the pharmaceutical material, and are therefore not expected to be qualitatively altered by such refinements.

2.2. Vibrational Spectra: Calculation and Experiment

Harmonic vibrational frequencies, IR intensities and Raman scattering activities were computed at the B3LYP/6-311+G(d,p) level for each optimized conformer. No empirical frequency scaling factor was applied; instead, the systematic deviations of the harmonic frequencies were quantified a posteriori by linear regression against the experimental wavenumbers. In the 1000–1800 cm^{-1} region, this procedure yields a correlation coefficient $r^2 > 0.9989$ for both IR and Raman data, indicating that the chosen level provides a highly consistent description of the vibrational spectrum and that residual errors are essentially uniform across this window. This approach allows us to use the unscaled harmonic frequencies directly for mode assignment and for identifying conformationally sensitive spectral regions, while relying on the regression parameters as an explicit validation of the method.

On this basis, the internal structure of the normal modes was examined in greater detail. Normal-mode compositions were analyzed using the VEDA program [31,32], and the resulting PED information was complemented by visual inspection of the eigenvectors and by comparison with characteristic vibrational patterns of related benzimidazole and benzyl systems. Full Cartesian coordinates of the optimized BZN1 and BZN2 conformers in vacuum and PCM water, together with the complete sets of calculated harmonic frequencies and their dominant PED contributions, are provided as Appendix A, ensuring full reproducibility of the theoretical analysis.

Experimental FT-IR and FT-Raman spectra were recorded for pharmaceutical-grade polycrystalline benzimidazole (BZN, 99% purity) supplied by the Pernambuco State Pharmaceutical Laboratory (LAFEPE, Recife, Pernambuco, Brazil). FT-IR spectra were collected in the 40–4000 cm^{-1} range with 40 scans at a resolution of 2 cm^{-1} , and FT-Raman spectra were acquired with a Nd:YAG laser (excitation wavelength 1064 nm) in the 35–4000 cm^{-1} range with 1024 scans at the same resolution. Experimental bands were deconvoluted into Gaussian components using a second-derivative procedure to determine peak positions [33], and an experimental–theoretical wavenumber correlation was obtained by weighted linear regression with the SPECA program [34,35], taking literature tables of IR and Raman characteristic group frequencies as reference [31,36]. This combined computational and spectroscopic protocol serves as the basis for the conformational and vibrational analyses presented in Sections 3 and 4. Similar combined DFT and FT-IR/FT-Raman approaches have been successfully applied to related heteroaromatic systems such as benzimidazole and other nitroimidazole derivatives, yielding reliable assignments for ring, nitro and amide vibrations. The characteristic patterns identified in those studies, particularly for C=N, C=C and NO₂ stretching and bending modes, are consistent with the normal-mode compositions obtained here for BZN1 and BZN2, further supporting the present vibrational analysis.

The polycrystalline sample was also characterized by powder X-ray diffraction (PXRD), and the resulting pattern was compared with the diffraction data reported in Refs. [16–18]. Within the resolution of our measurements, the PXRD profile is consistent with the room-temperature polymorph (Form I) described by Soares-Sobrinho et al. and Honorato et al., with no additional reflections indicative of other polymorphs or cocrystals, ensuring that the vibrational signatures discussed in this work correspond to a well-defined crystalline form of BZN.

3. Results

3.1. Conformation Landscape of Benznidazole

The two-dimensional RPES as a function of the dihedral angles θ_1 (C7–N8–C9–C10) and θ_2 (N12–C16–N17–O18) reveals a rich but well-defined conformational landscape for benznidazole, as shown in Figure 2. The values of θ_1 , θ_2 and other relevant torsional angles for the optimized BZN1 and BZN2 geometries are listed in Tables A1 and A2 in the Appendix A. Two minima are clearly identified and denoted BZN1 and BZN2, which are represented in Figure 2a; they differ mainly in the relative orientation of the benzyl and imidazole rings with respect to the acetamide fragment, which lie on opposite sides in BZN1 and on the same side in BZN2. The energy difference between these minima is small, about $0.4 \text{ kcal mol}^{-1}$, and the rotational barriers connecting them are approximately $6.8 \text{ kcal mol}^{-1}$ —as shown in the top panel of Figure 2—indicating that both conformers are, in principle, thermally accessible at room temperature in vacuum and in the PCM water model.

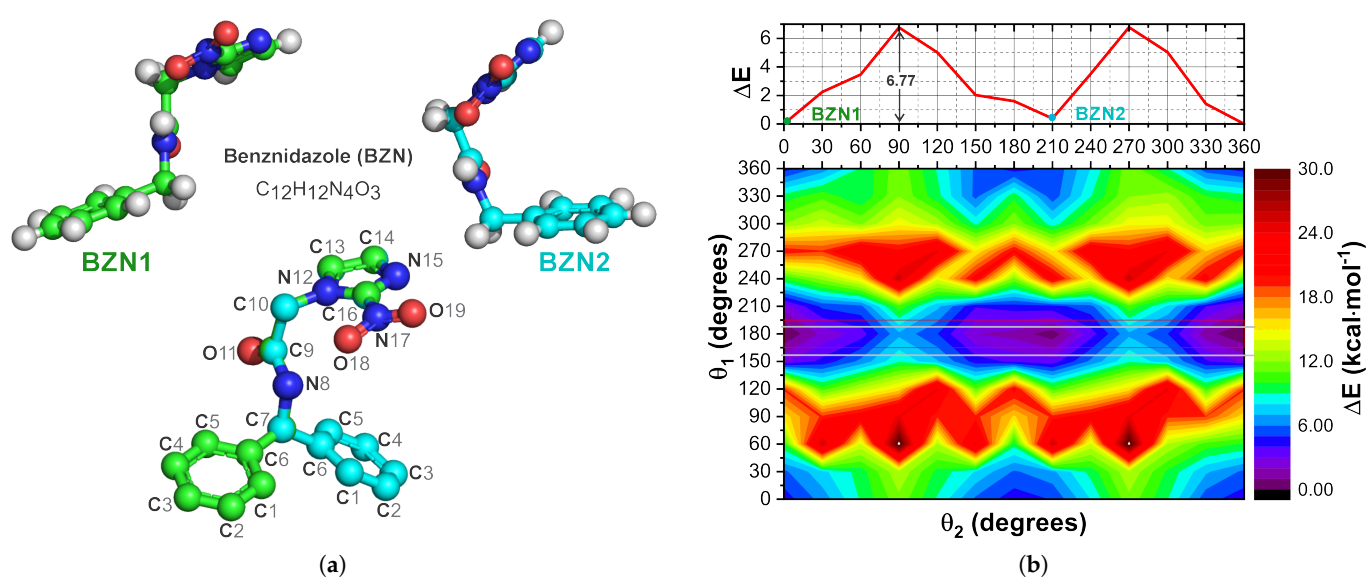


Figure 2. (a) Ball-and-stick representations of the two lowest-energy benznidazole conformers, BZN1 (left) and BZN2 (right), showing the atom numbering scheme and highlighting the dihedral angles θ_1 and θ_2 that define the relative orientation of the three fragments; the central superposed view aligns the imidazole and acetamide groups, making clear that the conformational difference between BZN1 and BZN2 arises predominantly from the orientation of the benzyl group. (b) Energy profile and relaxed potential energy surface scan (RPES) for benznidazole. The top panel shows the one-dimensional energy barrier connecting the two lowest-energy conformers, BZN1 and BZN2, along the C7–N8–C9–C10 dihedral angle. The bottom panels show the two-dimensional contour map of the RPES as a function of θ_1 = C4–N8–C9–C10 (abscissa) and θ_2 = N12–C16–N17–O18 (ordinate) dihedral angles (in degrees), with the color scale indicating the relative energy ΔE in kcal mol^{-1} .

The present two-dimensional RPES is intentionally focused on the dihedral angles that control the relative orientation of the imidazole, benzyl and acetamide fragments, and is therefore designed to capture the dominant large-amplitude motions connecting the major conformers. Other dihedral degrees of freedom, particularly those adjacent to θ_1 , can give rise to additional local minima within a few kcal mol^{-1} of BZN1/BZN2, as indicated by recent conformational searches, and may become especially relevant in solution or in biological environments, where explicit solvation and entropic effects play a role. A more comprehensive conformational analysis including such dihedrals, Gibbs free energies and explicit water molecules would be highly valuable for future studies

of BZN in aqueous media; however, this exceeds the scope of the present work, which is centered on the solid-state conformational preference and its Raman signature in the pharmaceutical material. Nevertheless, the solid-state Raman fingerprint discussed below shows that, under the crystallization conditions employed for the pharmaceutical-grade material, BZN1 is overwhelmingly selected, even if a richer conformational manifold exists in gas phase or solution.

3.2. Calculated Vibrational Spectra of BZN1 and BZN2

For each conformer, 87 normal modes were obtained at the B3LYP/6-311+G(d,p) level [37], allowing the construction of theoretical IR and Raman spectra in the 1000–1800 cm^{-1} interval (Figure 3) and detailed mode assignments from PED analysis. In both BZN1 and BZN2 the overall band pattern is similar, reflecting the common nitroimidazole–benzyl–acetamide scaffold, but subtle frequency shifts and intensity changes arise from the different relative orientations of the fragments. In particular, modes involving coupled motions of the acetamide and nitroimidazole groups, such as those around 1350–1400 cm^{-1} , are especially sensitive to the conformational change and therefore serve as potential spectroscopic markers.

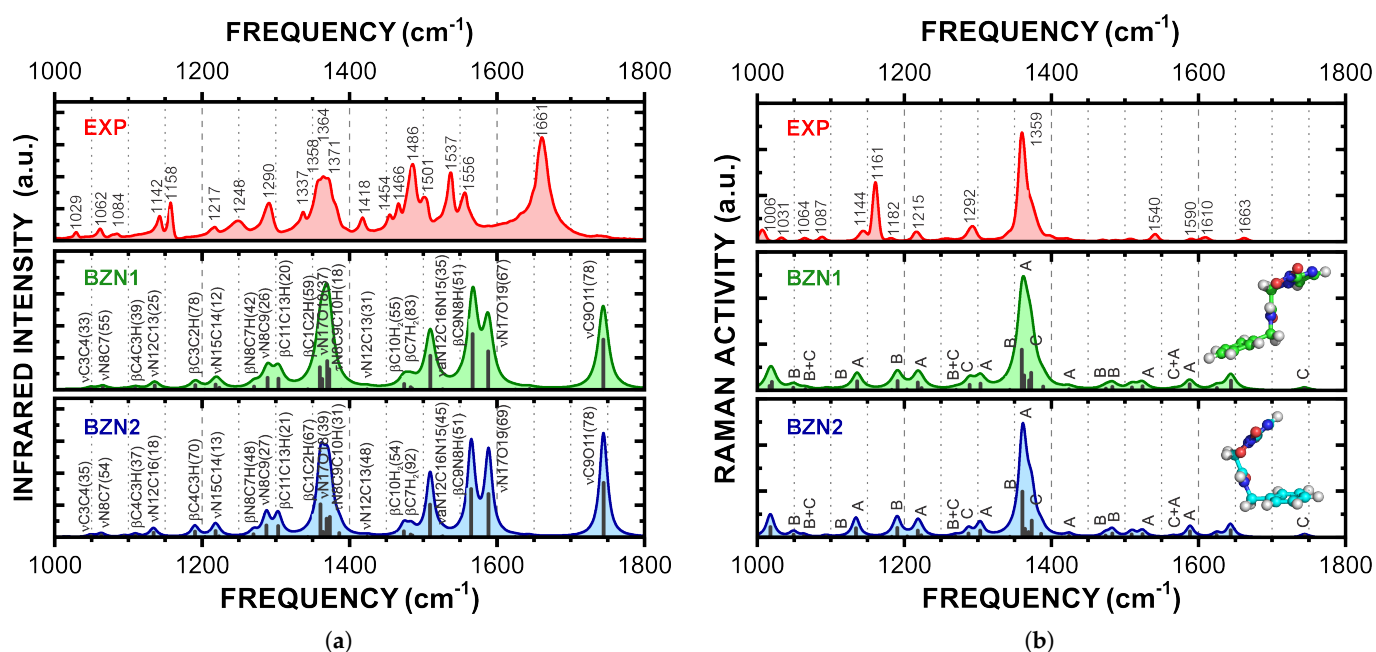


Figure 3. (a) Infrared spectra of benznidazole in the 1000–1800 cm^{-1} region. The top panel shows the experimental FT-IR spectrum (EXP) of pharmaceutical-grade BZN, while the middle and bottom panels display the DFT-calculated spectra for the BZN1 (green) and BZN2 (blue) conformers, respectively, with the main normal-mode assignments indicated above the corresponding bands; (b) Raman spectra of benznidazole in the 1000–1800 cm^{-1} region. The top panel shows the experimental Raman spectrum (EXP) of pharmaceutical-grade BZN, while the middle and bottom panels display the DFT-calculated Raman spectra for the BZN1 (green) and BZN2 (blue) conformers, respectively, with the main normal-mode contributions indicated by the labels A (imidazole), B (benzyl) and C (acetamide) and representative conformer geometries shown in the insets.

Consistently with the conformational analysis, these vibrational calculations were first carried out for isolated molecules. The IR and Raman spectra shown in Figure 3 were computed for isolated BZN1 and BZN2 molecules. PCM calculations at the same level of theory yield very similar vibrational patterns and relative shifts for the internal modes considered here and are therefore not shown; their main effect is to slightly modify the

conformational energetics along the RPES rather than to alter the qualitative spectral signatures discussed below.

3.3. Experimental IR and Raman Spectra of BZN

The experimental FT-IR and FT-Raman spectra of the experimental FT-IR and FT-Raman spectra of pharmaceutical-grade polycrystalline BZN show well-resolved bands across the 1000–1800 cm^{-1} region, with intensities and positions compatible with the calculated spectra. Weighted linear regression between experimental and theoretical wavenumbers yields correlation coefficients ($r^2 > 0.9989$) for both IR and Raman data, confirming the reliability of the chosen quantum chemical level and the mode assignments. Gaussian deconvolution of the experimental bands, aided by second-derivative analysis, provides precise peak positions and reveals that many features can be reproduced with a small number of components, facilitating comparison with the simulated spectra. For completeness, the full experimental FT-IR and FT-Raman spectra in the 0–3600 cm^{-1} range, together with the corresponding theoretical spectra, are provided in the Appendix A (Figures A1–A5). These expanded plots show that the overall agreement between calculated and experimental band positions and patterns extends beyond the 1000–1800 cm^{-1} window, although larger deviations are observed for high-frequency X–H stretching modes, where harmonic DFT is known to overestimate wavenumbers.

Within this generally very good agreement, some specific discrepancies between the experimental and calculated spectra deserve explicit comment. In particular, the intense experimental IR band at 1661 cm^{-1} is assigned to the amide I C=O stretching mode in the solid, whereas the corresponding isolated-molecule harmonic calculation places this band around 1750 cm^{-1} . This overestimation is typical for carbonyl stretches at the harmonic DFT level and is further amplified by the absence of explicit hydrogen bonding and crystal-packing effects, which lower the C=O frequency in the polycrystalline material. Likewise, the bands observed experimentally at 1158/1161 and 1248 cm^{-1} involve coupled C–N, C–O and ring modes that are especially sensitive to intermolecular interactions and anharmonicity; in a single-conformer, gas-phase harmonic model, these effects are only approximated, leading to shifts in wavenumbers and differences in band intensities. In addition, the solid-state spectra contain lattice modes, overtones and combination bands that are not captured within the harmonic approximation.

3.4. Spectroscopic Identification of the Predominant Conformer

Although BZN1 and BZN2 give rise to broadly similar spectra, the 1350–1400 cm^{-1} region exhibits clear conformer-dependent signatures. In the Raman spectra (Figures 3b and 4), the experimental band at 1359 cm^{-1} coincides with the most intense calculated mode of BZN1 (1360 cm^{-1}), whereas the corresponding dominant mode of BZN2 appears at 1374–1375 cm^{-1} , about 13 cm^{-1} higher in frequency. This shift is significantly larger than the experimental resolution and persists after band deconvolution, leading to a Raman profile in which the pattern and relative intensities of the components match BZN1 but not BZN2. In contrast, the IR spectra (Figures 3a and 4) in the same interval are less discriminatory, because both conformers reproduce the main experimental band within a few wavenumbers, making Raman the more sensitive probe for conformational analysis in this case.

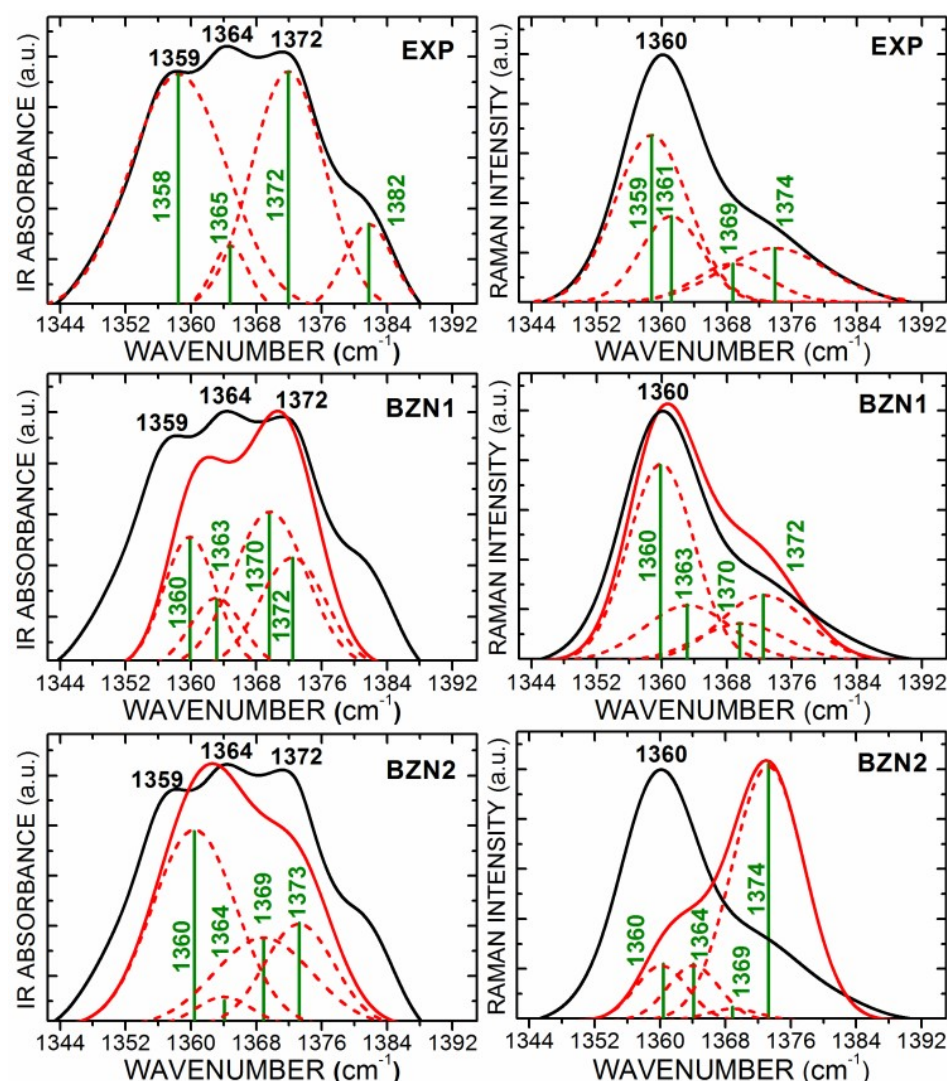


Figure 4. Gaussian fits applied to the experimental (top), theoretically calculated BZN1 (middle), and BZN2 (bottom) IR and Raman modes in the 1350–1400 cm^{-1} spectral range. In this region, the main BZN1 vibrational mode at 1360 cm^{-1} was assigned to $\nu_a\text{N12C16N17(21)} + \beta\text{C1C2H(13)} + \beta\text{C9C10H(13)} + \nu\text{N15C16(12)} + \tau\text{N8C9C10H(10)}$, and the main BZN2 vibrational mode at 1375 cm^{-1} was assigned to $\tau\text{N8C9C10H(31)} + \beta\text{C10H}_2(11)$.

3.5. Solid-State Conformational Preference

Taken together, the RPES mapping and the combined theoretical–experimental vibrational analysis indicate that, although BZN1 and BZN2 are both low-energy conformers in the isolated molecule, only BZN1 is significantly populated in the pharmaceutical solid. The small intrinsic energy difference explains why BZN2 remains a plausible solution-phase conformer, whereas the strong agreement between the experimental Raman spectrum and the BZN1 calculation in the diagnostic 1350–1400 cm^{-1} window shows that crystallization conditions select BZN1 almost exclusively. These findings establish a direct link between benznidazole’s conformational landscape and its vibrational fingerprints, providing a consistent basis for using Raman and IR spectroscopy to monitor conformational and potential polymorphic forms in benznidazole-based drug products.

4. Discussion

The combined quantum chemical and spectroscopic results provide a consistent picture of benznidazole as a flexible, yet conformationally biased, molecule whose solid-state

behavior is dominated by a single geometry. The RPES analysis shows that BZN1 and BZN2 differ only modestly in energy and are separated by moderate rotational barriers, indicating that both conformers are thermally accessible for the isolated molecule in vacuum and in aqueous continuum. This intrinsic flexibility explains why nitroimidazole derivatives can adapt to different environments, but it also implies that the experimentally observed conformational distribution will depend sensitively on intermolecular interactions and crystal-packing effects.

The calculated IR and Raman spectra for BZN1 and BZN2 reproduce the main features of the experimental spectra with excellent accuracy, as evidenced by the high linear correlation between theoretical and experimental wavenumbers. This agreement validates the use of the chosen DFT level and the PED-based assignments as a reliable basis for interpreting the vibrational signatures of benznidazole. The same DFT framework has also been successfully employed to reproduce the optical absorption spectrum of benznidazole in aqueous solution, with TD-DFT calculations within a PCM solvation model yielding excellent agreement with the experimental UV–Vis bands [20]. Comparable levels of theory have been shown to reproduce the vibrational response of benznidazole aggregates, including hydrogen-bonded dimers, confirming the robustness of such DFT-based spectroscopic analyses for this nitroimidazole drug [30]. At the same time, the comparison reveals that only a few spectral regions—most notably 1350–1400 cm^{-1} in the Raman spectra—are truly diagnostic for distinguishing between the two conformers, while most other bands are only weakly sensitive to their structural differences. The excellent linear correlation between unscaled B3LYP/6-311+G(d,p) harmonic frequencies and experimental wavenumbers ($r^2 > 0.9989$) justifies the use of unscaled values for our conformational analysis and for the identification of the Raman conformational marker.

The Raman window around 1360 cm^{-1} emerges as a key conformational probe. In this region, the experimental band at 1359 cm^{-1} coincides with the most intense BZN1 mode but is clearly separated from the corresponding BZN2 maximum at 1374–1375 cm^{-1} , a shift far larger than the instrumental resolution. Deconvolution of the Raman band confirms that its internal structure mirrors the BZN1 pattern, and no additional components attributable to BZN2 are required to fit the data within experimental uncertainty. In contrast, the IR spectra in the same region can be fitted reasonably well by either conformer, highlighting that Raman spectroscopy is significantly more sensitive than IR to the subtle changes in coupling between the acetamide and nitroimidazole fragments that distinguish BZN1 from BZN2. Although the present analysis emphasizes the 1350–1400 cm^{-1} Raman region because it provides a particularly intense and well-resolved marker for distinguishing BZN1 and BZN2 in the solid, other vibrational modes are also sensitive to conformational changes. In particular, high-frequency X–H stretching bands in the 2700–3700 cm^{-1} region can be affected by local geometry and hydrogen bonding, but their quantitative description at the harmonic DFT level is more challenging due to anharmonicity and strong environmental effects. For practical solid-state conformational and polymorphic control of pharmaceutical-grade BZN, we therefore focus on the mid-frequency region, where the DFT model reproduces the experimental band patterns most reliably and where a clear conformer-selective Raman marker is observed.

These observations have direct implications for the drug's solid-state behavior. The small computed energy difference indicates that solution or gas-phase populations of BZN1 and BZN2 should be comparable, but the Raman evidence shows that the polycrystalline pharmaceutical material is overwhelmingly composed of BZN1. This predominance of a single conformer in the room-temperature solid is consistent with a recent vibrational and crystallographic study, which detected only the lowest-energy benznidazole conformer under ambient conditions, while the higher-energy conformer emerged upon thermal

activation [19]. Thus, the crystallization conditions used in the preparation of benznidazole tablets or bulk drug substance likely stabilize packing motifs compatible with the BZN1 geometry, effectively “freezing out” BZN2 even though it is a valid low-energy conformer in isolation. This illustrates how crystal engineering factors can override purely intramolecular preferences and underscores the importance of combining conformational analysis with experimental spectroscopy when characterizing active pharmaceutical ingredients.

From a broader perspective, the work demonstrates that a carefully calibrated combination of DFT calculations, RPES mapping, and IR/Raman measurements can deliver a detailed, molecular-level understanding of the conformational landscape of a clinically important drug. The identification of BZN1 as the unique detectable conformer in the solid state, together with the assignment of a specific Raman marker band, provides practical tools for routine quality control, detection of possible polymorphs, and comparison of different manufacturing batches of benznidazole. More generally, the strategy adopted here can be readily extended to other nitroimidazole derivatives, where subtle conformational and packing effects may also influence stability, bioavailability, and formulation performance. Such conformational and solid-state insights are particularly relevant in the context of nanostructured formulations, where benznidazole-loaded lipid carriers and inorganic nanoparticles have already demonstrated improved efficacy and selectivity profiles in experimental *T. cruzi* models [9–11].

Although the present analysis emphasizes the 1350–1400 cm^{-1} Raman region because it provides a particularly intense and well-resolved marker for distinguishing BZN1 and BZN2 in the solid, other vibrational modes are also sensitive to conformational changes. In particular, high-frequency X–H stretching bands in the 3700–2700 cm^{-1} region can be affected by local geometry and hydrogen bonding, but their quantitative description at the harmonic DFT level is more challenging due to anharmonicity and strong environmental effects. For practical solid-state conformational and polymorphic control of pharmaceutical-grade BZN, we therefore focus on the mid-frequency region where the DFT model reproduces the experimental band patterns most reliably and where a clear conformer-selective Raman marker is observed.

We note that the use of dispersion-corrected functionals (such as B3LYP-D3/D4, ω B97X-D or M06-2X) could refine the quantitative conformational energetics of BZN1 and BZN2, particularly for the more compact BZN2 geometry, and we point to such an investigation as a natural extension of the present study.

The excellent linear correlation between unscaled B3LYP/6-311+G(d,p) harmonic frequencies and experimental wavenumbers ($r^2 > 0.9989$) justifies the use of unscaled values for our conformational analysis and for the identification of the Raman conformational marker.

From a mechanistic viewpoint, the conformational and solid-state preferences of BZN are relevant to the free-radical based mechanism of nitroheterocyclic drugs, in which reductive activation and generation of reactive intermediates play a central role. Although our work does not explicitly model radical pathways, the identification of a well-defined low-energy conformer in the pharmaceutical solid provides a structural baseline for future studies that combines conformational information with kinetic data on radical formation. Moreover, formulation strategies such as lipid nanocapsules, nanostructured lipid carriers and inorganic nanoparticles have already demonstrated improved trypanocidal activity and selectivity for BZN [9–13], and the DFT-guided Raman marker established here offers a practical means of monitoring whether the drug maintains its polymorphic and conformational state when incorporated into such delivery systems.

5. Conclusions

The present study provides a comprehensive conformational and vibrational characterization of benznidazole, integrating DFT calculations, RPES mapping, and experimental IR/Raman spectroscopy of a pharmaceutical-grade solid sample. Two low-energy conformers (BZN1 and BZN2) were identified, separated by moderate rotational barriers and a small energy difference, confirming that both are intrinsically accessible at room temperature for the isolated molecule in vacuum and in an implicit aqueous environment.

Comparison between calculated and experimental spectra shows excellent overall agreement and reveals a Raman band in the 1350–1400 cm^{-1} region as a highly sensitive conformational marker. The experimental maximum at 1359 cm^{-1} coincides with the most intense mode of BZN1 and differs significantly from the corresponding feature of BZN2, which appears around 1374–1375 cm^{-1} . This spectral evidence, supported by band deconvolution, indicates that the polycrystalline pharmaceutical material is overwhelmingly composed of the BZN1 conformer, with no detectable contribution from BZN2 under the crystallization conditions investigated.

These findings establish a direct link between the conformational landscape of benznidazole (BZN) and its vibrational fingerprints, highlighting Raman spectroscopy—complemented by IR and quantum chemical analysis—as a powerful tool for conformational and potential polymorphic control of this drug. The structural and spectroscopic framework developed here can support the quality control of benznidazole formulations and provides a robust basis for future studies aimed at correlating conformational preferences with solid-state properties and, ultimately, with biological activity.

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Appendix A

Table A1. Optimized geometric parameters (bond length, in Å) for the two BZN conformers: BZN1 and BZN2. The atom labels are presented in Figure 1.

#	Bond	Bond Length (Å)				
		BZN1	BZN2	BZNpcm	cBZN	BZN _{XRD}
1	C1–C2	1.395	1.395	1.392	1.396	1.391
2	C1–C6	1.397	1.397	1.397	1.399	1.388
3	C2–C3	1.393	1.393	1.393	1.396	1.383
4	C3–C4	1.396	1.395	1.397	1.399	1.384
5	C4–C5	1.392	1.393	1.393	1.396	1.391
6	C5–C6	1.400	1.400	1.401	1.403	1.396
7	C6–C7	1.516	1.516	1.516	1.507	1.505
8	C7–N8	1.465	1.467	1.463	1.475	1.468
9	N8–C9	1.352	1.352	1.349	1.348	1.329
10	C9–C10	1.543	1.543	1.539	1.535	1.528
11	C9–O11	1.222	1.222	1.226	1.239	1.228
12	C10–N12	1.471	1.471	1.460	1.464	1.463
13	N12–C13	1.369	1.369	1.364	1.372	1.358
14	N12–C16	1.378	1.378	1.373	1.385	1.362
15	C13–C14	1.381	1.381	1.383	1.389	1.370
16	C14–N15	1.358	1.358	1.357	1.368	1.362
17	N15–C16	1.312	1.312	1.317	1.335	1.314
18	C16–N17	1.442	1.442	1.432	1.436	1.435
19	N17–O18	1.243	1.214	1.235	1.247	1.226
20	N17–O19	1.214	1.243	1.227	1.248	1.232
21	N8–H27	1.012	1.013	1.011	1.032	0.836
22	C1–H20	1.086	1.086	1.085	1.091	0.950
23	C2–H21	1.084	1.084	1.084	1.089	0.950
24	C3–H22	1.084	1.084	1.084	1.088	0.950
25	C4–H23	1.084	1.084	1.084	1.091	0.950
26	C5–H24	1.085	1.085	1.085	1.090	0.950
27	C7–H25	1.091	1.092	1.091	1.098	0.990
28	C7–H26	1.093	1.090	1.093	1.098	0.990
29	C10–H28	1.086	1.086	1.088	1.096	0.990
30	C10–H29	1.088	1.088	1.091	1.091	0.990
31	C13–H30	1.078	1.078	1.078	1.085	0.950
32	C14–H31	1.079	1.079	1.078	1.084	0.950

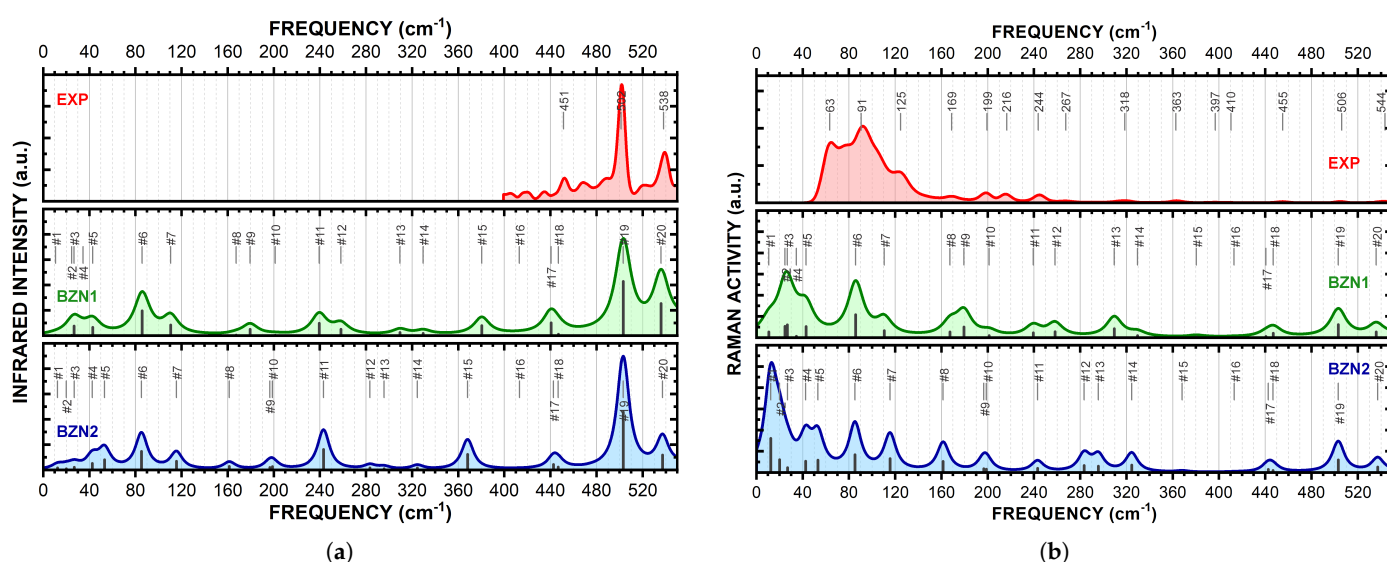


Figure A1. IR (a) and Raman (b) spectra of pharmaceutical-grade benznidazole in the 0–550 cm^{-1} region (EXP, top panels, red), compared with B3LYP/6-311+G(d,p) spectra for the BZN1 (middle panels, green) and BZN2 (bottom panels, blue) conformers, highlighting CH and NH stretching modes; vertical sticks indicate the calculated harmonic frequencies and normal-mode labels (#1–#20), and shaded regions mark the most intense conformer-dependent bands.

Table A2. Optimized geometric parameters (angle in degree) for the two BZN conformers: BZN1 and BZN2. The atom labels are presented in Figure 1.

#	Bond Angle	Angle (Degree, °)				
		BZN1	BZN2	BZNpcm	cBZN	BZN _{XRD}
1	C2–C1–C6	120.68	120.68	120.69	119.0	120.75
2	C2–C1–H20	119.66	119.63	119.73	120.14	119.60
3	C6–C1–H20	119.66	119.69	119.59	119.19	119.60
4	C1–C2–C3	119.99	119.99	120.06	119.88	119.74
5	C1–C2–H21	119.85	119.86	119.78	120.4	120.10
6	C3–C2–H21	120.16	120.15	120.16	119.71	120.10
7	C2–C3–C4	119.72	119.74	119.64	120.0	120.29
8	C2–C3–H22	120.14	120.13	120.22	119.63	119.90
9	C4–C3–H22	120.13	120.14	120.15	120.37	119.90
10	C3–C4–C5	120.20	120.22	120.17	119.89	119.86
11	C3–C4–H23	120.01	120.01	120.04	120.43	120.10
12	C5–C4–H23	119.79	119.78	119.79	119.70	120.10
13	C4–C5–C6	120.45	120.42	120.58	120.52	120.41
14	C4–C5–H24	120.10	120.19	119.71	119.43	119.80
15	C6–C5–H24	119.44	119.39	119.71	120.04	119.80
16	C1–C6–C5	118.95	118.96	118.86	119.02	118.93
17	C1–C6–C7	120.75	120.66	120.32	119.90	120.41
18	C5–C6–C7	120.30	120.38	120.82	121.07	120.65
19	C6–C7–N8	113.11	113.24	112.81	111.25	110.86
20	C6–C7–H25	110.60	110.19	110.76	110.95	109.50
21	C6–C7–H26	110.08	110.65	109.82	110.13	109.50
22	N8–C7–H25	106.63	107.74	106.40	107.81	109.50
23	N8–C7–H26	108.14	106.71	109.33	109.77	109.50
24	H25–C7–H26	108.09	108.12	107.53	106.81	108.10
25	C7–N8–C9	123.03	122.64	123.77	121.38	121.09
26	C7–N8–H27	118.99	118.68	117.43	119.57	119.90
27	C9–N8–H27	117.98	117.51	118.76	118.43	118.20
28	N8–C9–C10	114.91	114.89	113.56	114.23	114.48
29	N8–C9–O11	125.10	125.13	124.81	124.47	124.46
30	C10–C9–O11	119.99	119.98	121.60	121.16	120.91
31	C9–C10–N12	111.43	111.16	111.74	111.80	110.81
32	C9–C10–H28	112.34	112.52	111.03	110.74	109.50
33	C9–C10–H29	106.94	106.99	108.80	108.80	109.50
34	N12–C10–H28	108.90	108.90	109.72	110.73	109.50
35	N12–C10–H29	107.27	107.27	106.98	106.25	109.50
36	H28–C10–H29	109.81	109.84	108.43	107.75	108.10
37	C10–N12–C13	123.32	123.42	124.30	123.74	124.17
38	C10–N12–C16	131.80	131.70	130.53	130.75	130.67
39	C13–N12–C16	104.88	104.88	105.11	105.41	104.99
40	N12–C13–C14	106.68	106.67	106.76	106.58	106.76
41	N12–C13–H30	121.05	121.05	121.54	121.17	126.60
42	C14–C13–H30	132.27	132.28	131.70	132.39	126.60
43	C13–C14–N15	110.29	110.30	110.13	110.73	110.69
44	C13–C14–H31	128.04	128.04	127.84	127.44	124.70
45	N15–C14–H31	121.67	121.66	122.03	121.83	124.70
46	C14–N15–C16	105.07	105.07	104.96	104.54	103.74
47	N12–C16–N15	113.07	113.08	113.03	112.78	113.81
48	N12–C16–N17	124.30	124.27	123.96	124.24	123.42
49	N15–C16–N17	122.62	122.66	123.01	122.94	122.74
50	C16–N17–O18	117.37	118.15	117.77	117.49	118.30
51	C16–N17–O19	118.11	117.35	118.09	118.09	117.62
52	O18–N17–O19	124.51	124.51	124.13	124.42	124.07

Table A3. Assignment of low frequency ($\leq 550\text{ cm}^{-1}$) normal mode of BZN1 and BZN2.

Normal Mode	BZN1			BZN2		
	Freq. (cm^{-1})	Vibrational Assignment (>10%)	Frag.	Freq. (cm^{-1})	Vibrational Assignment (>10%)	Frag.
#1	10.75	$\tau\text{C6C7N8C9}(76)$	B+C	12.38	$\tau\text{C1C6C7N8}(78)$	B+C
#2	24.65	$\tau\text{C1C6C7N8}(67)+$ $\tau\text{N12C16N17O18}(10)$	B+C A	19.92	$\tau\text{C1C6C7N8}(51)$	B+C
#3	26.67	$\tau\text{N8C9C10N12}(68)$	A+C	26.90	$\tau\text{C1C6C7N8}(55)$	B+C
#4	34.55	$\tau\text{C7N8C9C10}(61)$	B+C	42.53	$\tau\text{C6C7N8C9}(72)$	B+C
#5	42.81	$\tau\text{C1C6C7N8}(69)$	B+C	53.07	$\tau\text{N8C9C10N12}(71)$	A+C
#6	85.81	$\tau\text{C9C10N12C13}(49)+$ $\beta\text{C9C10N12}(21)$	A+C A+C	85.13	$\tau\text{C9C10N12C13}(36)+$ $\beta\text{C9C10N12}(15)+$ $\tau\text{C1C6C7N8}(11)$	A+C A+C B+A
#7	110.43	$\tau\text{N12C16N17O18}(57)$	C	115.43	$\tau\text{N12C16N17O18}(61)$	C
#8	167.33	$\tau\text{C7N8C9C10}(65)$	B+C	161.39	$\tau\text{C2C1C6C7}(68)$	B
#9	179.37	$\tau\text{C2C1C6C7}(44)+$ $\beta\text{N8C9C10}(16)$	B C	196.46	$\phi\text{N12N15C16N17}(53)+$ $\tau\text{C9C10N12C13}(10)$	A A+C
#10	201.23	$\beta\text{N8C9C10}(50)+$ $\tau\text{C2C1C6C7}(17)$	C B	198.80	$\beta\text{N8C9C10}(20)+$ $\beta\text{C7N8C9}(20)+$ $\beta\text{N15C16N17}(16)+$ $\beta\text{C10N12C16}(13)$	C B+C A A+C
#11	239.32	$\beta\text{C7N8C9}(59)$	B+C	243.03	$\beta\text{C7N8C9}(25)+$ $\beta\text{N15C16N17}(15)+$ $\beta\text{C1C6C7}(21)$	B+C A B
#12	258.14	$\beta\text{N8C9C10}(17)+$ $\tau\text{C9C10N12C13}(10)+$ $\tau\text{C7N8C9C10}(10)$	C A+C B+C	283.37	$\beta\text{C9C10N12}(20)+$ $\nu\text{C9C10}(13)$	A+C C
#13	309.39	$\beta\text{C10N12C16}(72)$	A+C	295.61	$\beta\text{C16N17O19}(22)+$ $\beta\text{N15C16N17}(17)+$ $\beta\text{C6C7N8}(11)$	A A B+C
#14	329.46	$\beta\text{C1C6C7}(24)+$ $\tau\text{C1C6C7H25}(12)+$ $\beta\text{C2C1C6}(11)$	B B B	324.44	$\beta\text{C1C6C7}(19)+$ $\beta\text{C10N12C16}(13)+$ $\beta\text{C6C7N8}(11)$	B A+C B+C
#15	380.39	$\beta\text{C1C6C7}(23)$	B	368.02	$\beta\text{C1C6C7}(26)+$ $\beta\text{C10N12C16}(15)+$ $\tau\text{C1C6C7H25}(11)+$ $\beta\text{C7N8C9}(11)$	B A+C B B+C
#16	412.96	$\tau\text{C1C2C3C4}(77)+$ $\tau\text{C4C3C2H21}(18)$	B B	413.22	$\tau\text{C1C2C3C4}(70)+$ $\tau\text{C4C2C2H21}(22)$	B B
#17	440.50	$\beta\text{N8C9C10}(23)+$ $\nu\text{C16N17}(22)+$ $\beta\text{N17O18O19}(10)$	C A A	442.53	$\nu\text{C16N17}(41)+$ $\beta\text{N17O18O19}(17)$	A A
#18	446.90	$\nu\text{C16N17}(25)+$ $\beta\text{C13N12C16}(15)+$ $\tau\text{C1C2C3C4}(11)$	A A B	446.67	$\beta\text{N8C9C10}(26)+$ $\tau\text{C1C2C3C4}(15)+$ $\nu\text{C16N17}(10)$	C B A
#19	503.02	$\tau\text{C1C2C3C4}(17)+$ $\beta\text{C2C3C4}(10)+$ $\beta\text{C2C1C6}(10)$	B B B	502.94	$\tau\text{C1C2C3C4}(21)$	B
#20	535.88	$\beta\text{C16N17O18}(34)+$ $\phi\text{O11C10N8C9}(11)+$ $\nu\text{N12C10}(10)$	A C A+C	537.07	$\beta\text{C16N17O18}(25)+$ $\phi\text{O11C10N8C9}(11)$	A C

Greek letters ν , β , ϕ and τ denote stretching, bending, out-of-plane, torsion, respectively. Capital letters A, B and C represent the imidazole group, the benzyl group and the acetamide fragment, respectively. Capital letters separated by “+” indicate contributions from both fragments.

Table A4. Assignment of middle frequency (>550 and ≤ 1000 cm^{-1}) normal mode of BZN1 and BZN2.

Normal Mode	BZN1			BZN2		
	Freq. (cm^{-1})	Vibrational Assignment ($>10\%$)	Frag.	Freq. (cm^{-1})	Vibrational Assignment ($>10\%$)	Frag.
#21	583.40	$\beta\text{C16N17O18(24)+}$ $\tau\text{C10C9N8H27(22)+}$ $\nu\text{C9C10(10)}$	A C C	577.60	$\beta\text{C16N17O18(17)+}$ $\tau\text{C10C9N8H27(13)+}$ $\beta\text{N8C9O11(11)+}$ $\nu\text{C9C10(10)}$	A C C C
#22	607.42	$\beta\text{C2C3C4(22)+}$ $\beta\text{C6C7N8(14)+}$ $\tau\text{C13N12C16N15(10)}$	B B+C A	618.34	$\beta\text{C6C7N8(17)+}$ $\beta\text{C3C4C5(11)+}$ $\tau\text{C13N12C16N15(11)}$	B+C B A
#23	626.40	$\tau\text{C13N12C16N15(32)+}$ $\tau\text{C10C9N8H27(21)+}$ $\tau\text{C14N15C16N12(10)}$	A C A	627.25	$\tau\text{C13N12C16N15(53)+}$ $\tau\text{C14N15C16N12(10)}$	A A
#24	635.21	$\beta\text{C1C2C3(75)+}$ $\beta\text{C2C1C6(11)}$	B B	635.19	$\beta\text{C1C2C3(76)+}$ $\beta\text{C2C1C6(11)}$	B B
#25	646.68	$\tau\text{C10C9N8H27(41)+}$ $\tau\text{C13N12C16N15(16)}$	C A	659.51	$\tau\text{C14N15C16N12(62)+}$ $\tau\text{C13N12C16N15(12)}$	A A
#26	659.83	$\tau\text{C14N15C16N12(66)+}$ $\tau\text{C13N12C16N15(16)}$	A A	660.95	$\tau\text{C10C9N8H27(58)}$	C
#27	692.98	$\beta\text{N8C9O11(27)+}$ $\nu\text{N12C10(23)}$	C A+C	690.80	$\nu\text{N12C10(23)+}$ $\beta\text{N8C9O11(20)}$	A+C C
#28	713.45	$\tau\text{C5C4C3H22(55)+}$ $\tau\text{C2C3C4C5(32)}$	B B	712.60	$\tau\text{C5C4C3H22(56)+}$ $\tau\text{C3C2C1C6(31)}$	B B
#29	747.85	$\phi\text{O19C16O18N17(81)}$	A	747.33	$\phi\text{O19C16O18N17(81)}$	A
#30	767.35	$\tau\text{C5C4C3H22(31)+}$ $\tau\text{C2C3C4C5(24)+}$ $\beta\text{C2C3C4(12)}$	B B B	767.93	$\tau\text{C5C4C3H22(30)+}$ $\tau\text{C3C2C1C6(20)+}$ $\beta\text{C3C4C5(13)}$	B B B
#31	789.61	$\tau\text{N15C14C13H30(82)}$	A	788.82	$\tau\text{N15C14C13H30(81)}$	A
#32	825.31	$\phi\text{O11C10N8C9(20)}$	C	818.56	$\phi\text{O11C10N8C9(19)+}$ $\beta\text{C9C10N12(10)}$	C A+C
#33	828.77	$\beta\text{C2C3C4(22)+}$ $\phi\text{O11C10N8C9(12)}$	B C	832.63	$\beta\text{C3C4C5(20)+}$ $\phi\text{O11C10N8C9(13)+}$ $\nu\text{C6C7(13)}$	B C B+C
#34	856.66	$\beta\text{N17O18O19(51)+}$ $\nu\text{N17O18(12)}$	A A	855.64	$\beta\text{N17O18O19(46)+}$ $\nu\text{N17O18(10)}$	A A
#35	861.14	$\tau\text{C3C2C1H20(95)}$	B	860.99	$\tau\text{C3C2C1H20(92)}$	B
#36	863.77	$\nu\text{C9C10(15)+}$ $\nu\text{N8C9(14)+}$ $\tau\text{C1C6C7H25(13)+}$ $\phi\text{O11C10N8C9(10)}$	C C B C	867.85	$\nu\text{C9C10(13)+}$ $\nu\text{N8C9(13)+}$ $\phi\text{O11C10N8C9(11)+}$ $\tau\text{C1C6C7H25(10)}$	C C C B
#37	891.69	$\tau\text{N15C14C13H30(79)}$	A	891.45	$\tau\text{N15C14C13H30(78)}$	A
#38	922.78	$\beta\text{C14N15C16(57)+}$ $\nu\text{N12C16(19)}$	A A	922.61	$\beta\text{C14N15C16(54)+}$ $\nu\text{N12C16(12)}$	A A
#39	934.83	$\tau\text{C3C2C1H20(73)}$	B	936.12	$\tau\text{C3C2C1H20(72)}$	B
#40	954.69	$\beta\text{C9C10H28(22)+}$ $\tau\text{N8C9C10H28(11)+}$ $\nu\text{N8C7(10)+}$ $\beta\text{C14N15C16(10)}$	C C B+C A	954.50	$\beta\text{C9C10H28(21)+}$ $\tau\text{N8C9C10H28(13)+}$ $\nu\text{N8C7(10)}$	C C B+C
#41	990.31	$\tau\text{C4C3C2H21(76)+}$ $\tau\text{C1C2C3C4(12)}$	B B	990.43	$\tau\text{C4C3C2H21(70)+}$ $\tau\text{C1C2C3C4(17)}$	B B

Greek letters ν , β , ϕ and τ , denote stretching, bending, out-of-plane, torsion, respectively. Capital letters A, B and C represent the imidazole group, the benzyl group and the acetamide fragment, respectively. Capital letters separated by “+” indicate contributions from both fragments.

Table A5. Assignment of middle frequency (>1000 and ≤ 1360 cm^{-1}) normal mode of BZN1 and BZN2.

Normal Mode	BZN1			BZN2		
	Freq. (cm^{-1})	Vibrational Assignment ($>10\%$)	Frag.	Freq. (cm^{-1})	Vibrational Assignment ($>10\%$)	Frag.
#42	1007.79	$\tau\text{C6C5C4H23(69)+}$ $\tau\text{C1C2C3C4(10)+}$ $\tau\text{C2C3C4C5(10)}$	B B B	1008.10	$\tau\text{C6C5C4H23(67)+}$ $\tau\text{C1C2C3C4(10)}$	B B
#43	1016.64	$\beta\text{C3C4C5(42)+}$ $\nu\text{C3C4(22)}$	B B	1015.45	$\tau\text{C1C6C7H25(16)+}$ $\beta\text{C3C4C5(14)}$	B B
#44	1019.72	$\tau\text{C1C6C7H25(16)}$	B	1017.95	$\nu\text{C3C4(22)+}$ $\beta\text{C3C4C5(21)+}$ $\nu\text{C4C5(13)}$	B B B
#45	1049.17	$\nu\text{C3C4(33)+}$ $\beta\text{C3C4C5(32)+}$ $\beta\text{C4C5H24(21)}$	B B B	1049.37	$\nu\text{C3C4(35)+}$ $\beta\text{C3C4C5(32)+}$ $\beta\text{C5C4H23(16)}$	B B B
#46	1065.38	$\nu\text{N8C7(55)}$	B+C	1063.21	$\nu\text{N8C7(54)}$	B+C
#47	1094.25	$\beta\text{C14C13H30(48)+}$ $\beta\text{C14N15C16(22)}$	A A	1093.68	$\beta\text{C14C13H30(49)+}$ $\beta\text{C13N12C16(22)}$	A A
#48	1109.26	$\beta\text{C4C3H22(39)+}$ $\nu\text{C1C2(32)}$	B B	1109.72	$\beta\text{C4C3H22(37)+}$ $\nu\text{C2C3(33)}$	B B
#49	1135.92	$\nu\text{N12C13(25)+}$ $\beta\text{C9C10H28(17)+}$ $\beta\text{C14C13H30(10)}$	A C A	1134.26	$\nu\text{N12C16(18)+}$ $\beta\text{C9C10H28(18)+}$ $\beta\text{C14C13H30(11)+}$ $\nu\text{N12C13(10)}$	A C A A
#50	1182.75	$\beta\text{C3C2H21(78)+}$ $\nu\text{C1C6(10)}$	B B	1183.01	$\beta\text{C4C3H22(70)+}$ $\nu\text{C1C6(10)}$	B B
#51	1190.66	$\nu\text{N15C14(36)+}$ $\beta\text{C14C13H30(20)+}$ $\beta\text{C13N12C16(12)}$	A A A	1190.27	$\nu\text{N15C14(33)+}$ $\beta\text{C14C13H30(18)+}$ $\beta\text{N12C16N15(12)}$	A A A
#52	1203.64	$\nu\text{C4C5(15)+}$ $\beta\text{C5C4H23(73)}$	B B	1203.94	$\beta\text{C5C4H23(73)+}$ $\nu\text{C4C5(17)}$	B B
#53	1218.28	$\nu\text{N15C14(12)+}$ $\beta\text{C14C13H30(11)+}$ $\beta\text{C13N12C16(11)}$	A A A	1218.13	$\nu\text{N15C14(13)+}$ $\beta\text{C14C13H30(12)+}$ $\beta\text{N12C16N15(11)}$	A A A
#54	1223.65	$\nu\text{C6C7(49)+}$ $\beta\text{C4C5H24(13)}$	B B	1222.92	$\nu\text{C6C7(33)+}$ $\beta\text{C5C4H23(12)}$	B B
#55	1270.44	$\beta\text{N8C7H25(42)+}$ $\nu\text{C1C6(13)}$	B+C B	1269.69	$\beta\text{N8C7H25(48)+}$ $\nu\text{C1C6(12)+}$ $\tau\text{C1C6C7H25(10)}$	B+C B B
#56	1288.84	$\nu\text{N8C9(26)+}$ $\beta\text{C9N8H27(16)}$	C C	1287.26	$\nu\text{N8C9(27)+}$ $\beta\text{C9N8H27(17)}$	C C
#57	1303.70	$\beta\text{C14C13H30(20)+}$ $\nu\text{N12C10(14)+}$ $\nu\text{N17O18(13)+}$ $\nu\text{N12C16(10)}$	A A+C A A	1303.11	$\beta\text{C14C13H30(21)+}$ $\nu\text{N12C10(14)}$	A A+C

Greek letters ν , β and τ , denote stretching, bending, torsion, respectively. Capital letters A, B and C represent the imidazole group, the benzyl group and the acetamide fragment, respectively. Capital letters separated by "+" indicate contributions from both fragments.

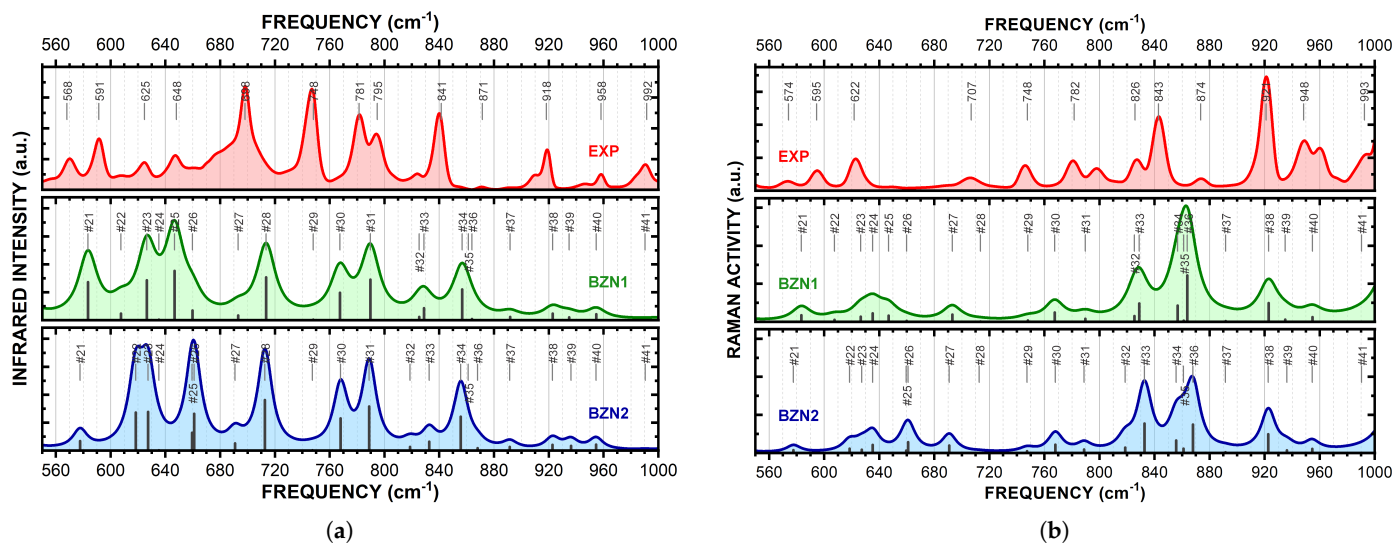


Figure A2. Infrared (a) and Raman (b) spectra of pharmaceutical-grade benznidazole in the 550–1000 cm^{-1} region (EXP, top panels, red), compared with B3LYP/6-311+G(d,p) spectra for the BZN1 (middle panels, green) and BZN2 (bottom panels, blue) conformers; vertical sticks indicate the calculated harmonic frequencies and normal-mode labels (#21 to #41), and shaded regions mark the conformer-dependent bands.

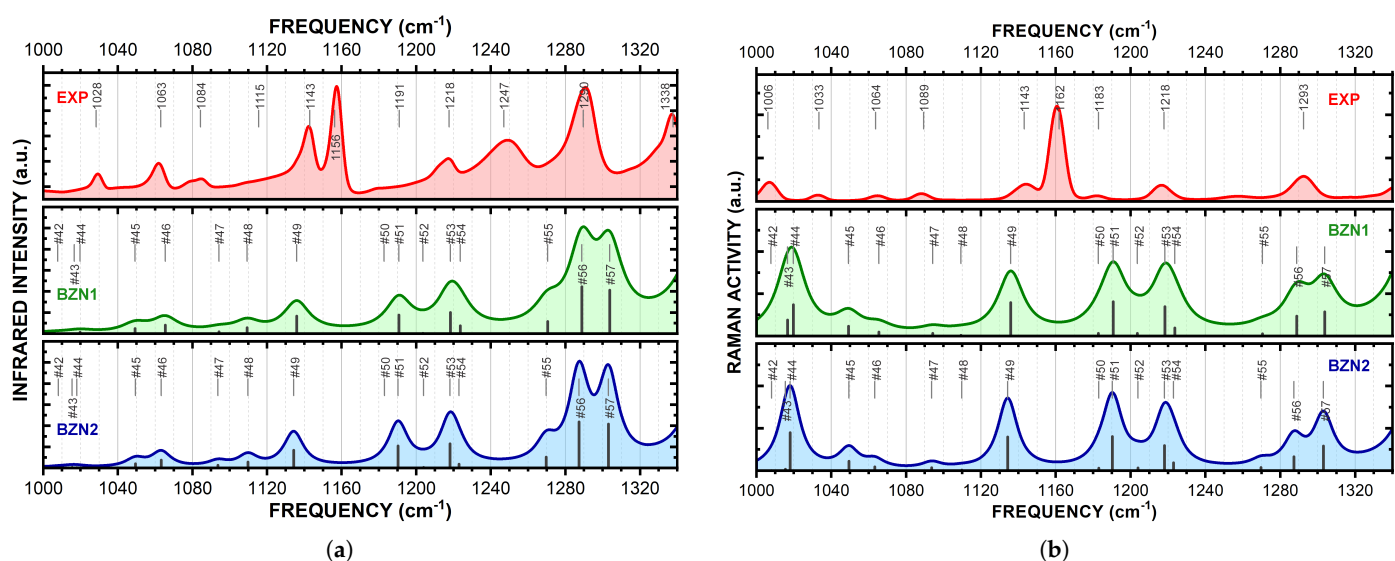


Figure A3. Infrared (a) and Raman (b) spectra of pharmaceutical-grade benznidazole in the 1000–1340 cm^{-1} region (EXP, top panels, red), compared with B3LYP/6-311+G(d,p) spectra for the BZN1 (middle panels, green) and BZN2 (bottom panels, blue) conformers; vertical sticks indicate the calculated harmonic frequencies and normal-mode labels (#42 to #57), and shaded regions mark the conformer-dependent bands.

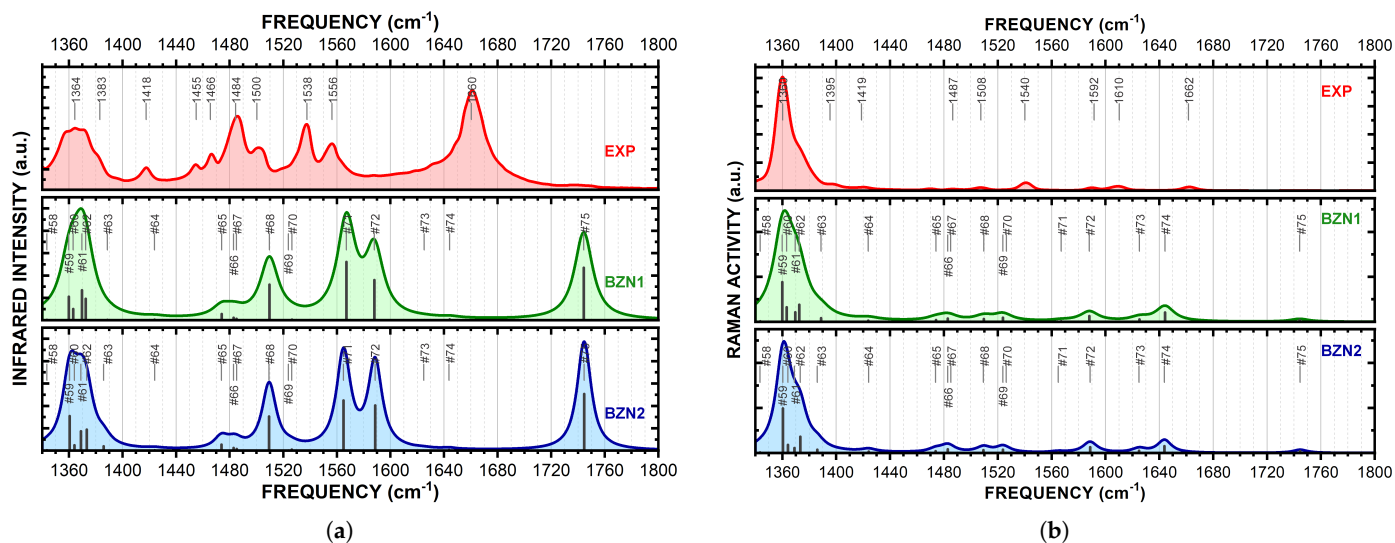


Figure A4. Infrared (a) and Raman (b) spectra of pharmaceutical-grade benznidazole in the 1340–1800 cm^{-1} region (EXP, top panels, red), compared with B3LYP/6-311+G(d,p) spectra for the BZN1 (middle panels, green) and BZN2 (bottom panels, blue) conformers; vertical sticks indicate the calculated harmonic frequencies and normal-mode labels (#58 to #75), and shaded regions mark the conformer-dependent bands.

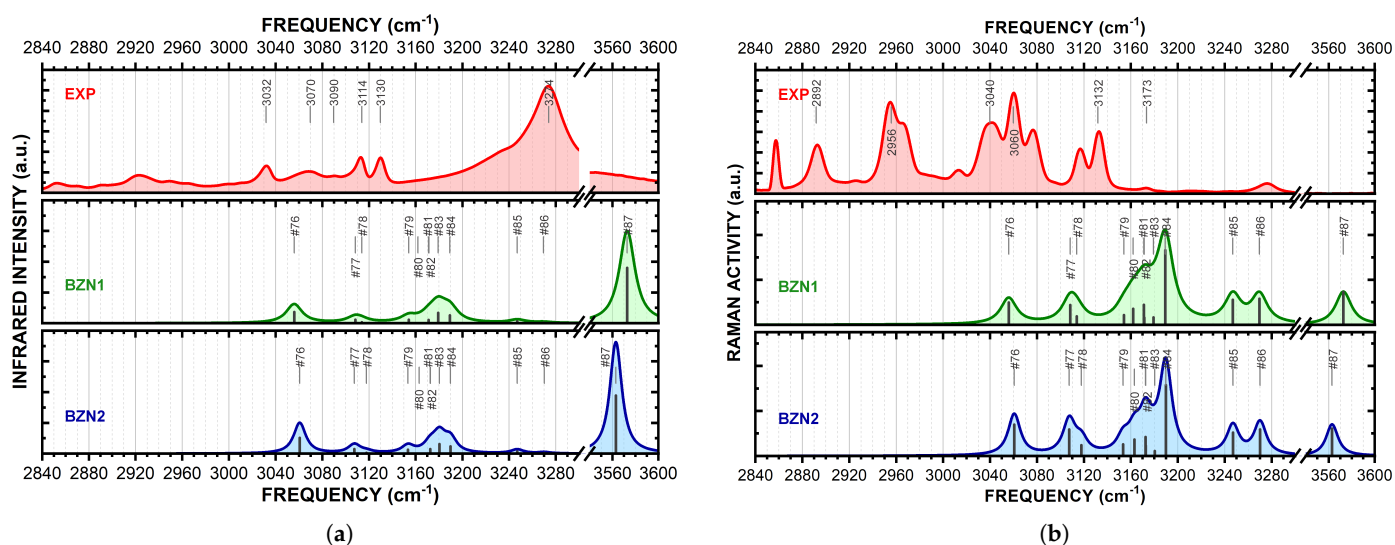


Figure A5. Infrared (a) and Raman (b) spectra of pharmaceutical-grade benznidazole in the 2840–3600 cm^{-1} region (EXP, top panels, red), compared with B3LYP/6-311+G(d,p) spectra for the BZN1 (middle panels, green) and BZN2 (bottom panels, blue) conformers; vertical sticks indicate the calculated harmonic frequencies and normal-mode labels (#76 to #87), and shaded regions mark the conformer-dependent bands.

Table A6. Assignment of middle frequency (>1360 and ≤ 1800 cm^{-1}) normal mode of BZN1 and BZN2.

Normal Mode	BZN1			BZN2		
	Freq. (cm^{-1})	Vibrational Assignment ($>10\%$)	Frag.	Freq. (cm^{-1})	Vibrational Assignment ($>10\%$)	Frag.
#58	1343.52	$\nu\text{C1C6(63)+}$ $\beta\text{N8C7H25(13)}$	B B+C	1343.44	$\nu\text{C1C6(70)+}$ $\beta\text{C3C4H22(12)}$	B B
#59	1359.87	$\nu\text{aN12C16N17(21)+}$ $\beta\text{C1C2H20(13)+}$ $\beta\text{C9C10CH28(13)+}$ $\nu\text{N15C16(12)+}$ $\tau\text{N8C9C10H28(10)}$	A B C A C	1360.41	$\nu\text{aN12C16N17(27)+}$ $\tau\text{N8C9C10H28(12)+}$ $\beta\text{C9C10H28(11)+}$ $\beta\text{C13N12C16(10)}$	A C C A
#60	1363.14	$\beta\text{C1C2H20(59)+}$ $\beta\text{N8C7H25(11)}$	B B+C	1364.09	$\beta\text{C1C2H20(67)}$	B
#61	1369.58	$\nu\text{N17O18(37)+}$ $\nu\text{N15C14(10)}$	A A	1368.87	$\nu\text{N17O18(39)+}$ $\nu\text{N15C14(10)+}$ $\beta\text{C10H28H29(10)}$	A A C
#62	1372.46	$\tau\text{N8C9C10H29(18)+}$ $\beta\text{C10H28H29(13)}$	C C	1373.26	$\tau\text{N8C9C10H29(31)+}$ $\beta\text{C10H28H29(11)}$	C C
#63	1388.67	$\tau\text{C1C6C7H26(63)}$	B	1385.86	$\tau\text{C1C6C7H26(73)}$	B
#64	1423.83	$\nu\text{N12C13(31)+}$ $\nu\text{N15C16(15)+}$ $\nu\text{N15C14(12)}$	A A A	1424.06	$\nu\text{N12C13(48)+}$ $\nu\text{N15C14(12)}$	A A
#65	1474.07	$\beta\text{C10H28H29(55)+}$ $\tau\text{N8C9C10H29(10)}$	C C	1473.78	$\beta\text{C10H28H29(54)+}$ $\tau\text{N8C9C10H29(13)}$	C C
#66	1482.87	$\beta\text{C7H25H26(83)}$	B	1482.83	$\beta\text{C7H25H26(92)}$	B
#67	1485.05	$\beta\text{C4C3H22(45)+}$ $\nu\text{C1C2(19)}$	B B	1485.13	$\beta\text{C4C3H22(42)+}$ $\nu\text{C2C3(25)}$	B B
#68	1509.54	$\nu\text{aN12C16N15(35)+}$ $\beta\text{C14C13H30(14)+}$ $\nu\text{N17O19(11)}$	A A A	1509.36	$\nu\text{aN12C16N15(45)+}$ $\beta\text{C14C13H30(13)+}$ $\tau\text{N8C9C10H29(13)}$	A A C
#69	1523.75	$\beta\text{C14N15C16(50)+}$ $\beta\text{C14C13H30(20)}$	A A	1523.81	$\beta\text{C13N12C16(25)+}$ $\beta\text{C14C13H30(19)+}$ $\beta\text{N12C16N15(15)+}$ $\nu\text{N12C16(11)}$	A A A A
#70	1526.38	$\beta\text{C4C5H24(59)+}$ $\beta\text{C3C4C5(11)}$	B B	1526.19	$\beta\text{C5C4H23(60)+}$ $\beta\text{C3C4C5(15)+}$ $\nu\text{C3C4(13)}$	B B B
#71	1567.08	$\beta\text{C9N8H27(51)+}$ $\nu\text{N8C9(18)+}$ $\nu\text{N17O19(11)}$	C C A	1564.94	$\beta\text{C9N8H27(51)+}$ $\nu\text{N8C9(17)}$	C A
#72	1587.98	$\nu\text{N17O19(67)}$	A	1588.50	$\nu\text{N17O19(69)}$	A
#73	1625.24	$\nu\text{C2C3(64)}$	B	1624.84	$\nu\text{C2C3(66)}$	B
#74	1644.18	$\nu\text{C4C5(47)+}$ $\beta\text{C5C4H23(18)+}$ $\nu\text{C3C4(12)+}$ $\beta\text{C2C1C6(10)}$	B B B B	1643.79	$\nu\text{C4C5(44)+}$ $\beta\text{C5C4H23(19)+}$ $\nu\text{C2C3(13)+}$ $\nu\text{C3C4(10)}$	B B B B
#75	1744.20	$\nu\text{C9O11(78)}$	C	1744.55	$\nu\text{C9O11(78)}$	C

Greek letters ν , β and τ , and the letter “a”, denote stretching, bending, torsion, and asymmetric, respectively. Capital letters A, B and C represent the imidazole group, the benzyl group and the acetamide fragment, respectively. Capital letters separated by “+” indicate contributions from both fragments.

Table A7. Assignment of high frequency (>3000 and ≤ 3600 cm^{-1}) normal mode of BZN1 and BZN2.

Normal Mode	BZN1			BZN2		
	Freq. (cm^{-1})	Vibrational Assignment ($>10\%$)	Frag.	Freq. (cm^{-1})	Vibrational Assignment ($>10\%$)	Frag.
#76	3055.83	$\nu\text{aC7H25H26}(100)$	B	3060.56	$\nu\text{aC7H25H26}(99)$	B
#77	3108.25	$\nu\text{sC10H28H29}(94)$	C	3107.41	$\nu\text{sC10H28H29}(94)$	C
#78	3113.87	$\nu\text{aC7H25H26}(100)$	B	3117.81	$\nu\text{aC7H25H26}(100)$	B
#79	3154.05	$\nu\text{C1H20}(77)$	B	3153.32	$\nu\text{C1H20}(79)+\nu\text{C5H24}(13)$	B B
#80	3161.96	$\nu\text{C2H21}(83)$	B	3162.93	$\nu\text{C4H23}(82)$	B
#81	3171.00	$\nu\text{C2H21}(88)$	B	3172.39	$\nu\text{C5H24}(73)$	B
#82	3171.37	$\nu\text{aC10H28H29}(100)$	C	3172.60	$\nu\text{aC10H28H29}(100)$	C
#83	3179.26	$\nu\text{C2H21}(89)$	B	3180.33	$\nu\text{C3H22}(87)$	B
#84	3189.33	$\nu\text{C2H21}(91)$	B	3189.73	$\nu\text{C2H21}(90)$	B
#85	3246.89	$\nu\text{C13H30}(17)+\nu\text{C14H31}(82)$	A A	3246.91	$\nu\text{C14H31}(83)+\nu\text{C13H30}(16)$	A A
#86	3269.49	$\nu\text{C13H30}(82)+\nu\text{C14H31}(17)$	A A	3270.08	$\nu\text{C13H30}(83)+\nu\text{C14H31}(16)$	A A
#87	3572.67	$\nu\text{N8H27}(100)$	C	3562.82	$\nu\text{N8H27}(100)$	C

Greek letter ν , and the letters “s” and “a”, denote stretching, symmetric and asymmetric, respectively. Capital letters A, B and C represent the imidazole group, the benzyl group and the acetamide fragment, respectively.

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