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Spectral characterization of fluconazole

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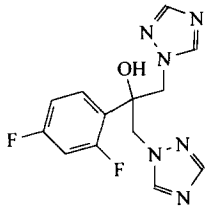
Abstract

Reference ^{1}H, ^{19}F and ^{13}C NMR, mass, IR and Raman spectra are provided to the open literature for the first time for the potent antifungal agent fluconazole, α-(2,4-difluorophenyl)-α-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-1,2,4-triazole-1-ethanol. The ^{1}H, ^{19}F and ^{13}C NMR spectra were analyzed in detail to attribute shifts, including ^{19}F chemical shifts and C–F and F–F coupling constants. The EI mass spectrum, although rich in fragment ions, lacked a molecular ion. FAB and MS/MS experiments were undertaken in support of the structure in order to validate the EI spectrum as a reference mass spectrum. IR and Raman spectra are compared to show the complementary nature of their features and discussed in terms of principal group vibrations. NMR and vibrational data together with assignments are summarized in tabulated form for convenience of use. All these data are consistent with the structure of fluconazole.

Keywords: Fluconazole; ^{1}H NMR; ^{19}F NMR; ^{13}C NMR; EI, FAB and MS/MS; FT-IR spectra; FT-Raman spectra

1. Introduction



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Fluconazole (**I**), α-(2,4)-difluorophenyl)-α-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-1,2,4-triazole-1-ethanol, an orally active bistriazole antifungal agent, has been known since 1982–83 when Pfizer acquired the innovator patents for it [1]. This drug is highly active against a variety of fungal pathogens that cause systemic mycoses [2]. It is well absorbed and has been found to be safe and effective in treating superficial [3] and systemic [4] infections with candida species and as a maintenance therapy for cryptococcal meningitis [5,6], particularly in patients with AIDS. Fluconazole has also been shown to be effective in preventing fungal infections in patients undergoing bone marrow transplantation[7].

Given the importance of fluconazole as a new antifungal agent, it was surprising to find virtually no molecular spectroscopic data on this substance in the chemical literature. It was on this account that this spectroscopic characterization of

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fluconazole (^1H, ^{13}C and ^{19}F NMR, mass and vibrational spectra) was undertaken. These data together with their structural attribution not only provide assurance of the authenticity of the assigned structure, but also constitute the basis for the pharmaceutical identification of fluconazole.

2. Experimental

2.1. Materials

Fluconazole (Lot 836-920-03) was obtained from Pfizer Central Research (Groton, CT, USA) as a finely divided white power.

2.2. Equipment and procedures

^1H, ^{19}F and ^{13}C NMR spectra were obtained from deuterodimethyl sulfoxide (DMSO-d_6) solutions of the fluconazole using Bruker AM 400 and AC 200 cryospectrometers. All spectra on the Bruker AM 400 were acquired at 300 K using a standard Bruker software micro-program (Version DISRVKØ1). Chemical shift assignments were made using heteronuclear correlation experiments employing the microprogram XH-CORR.AUR. Proton (400.13 MHz) and carbon (100.6 MHz) spectra were acquired with a 5 mm dual probe. Fluorine (376.5 MHz) spectra were acquired with a fluorine 5 mm probe, using appropriate bandpass and bandstop filters. Acquisitions of proton (200 MHz) and carbon (50 MHz) spectra on the Bruker AC 200 spectrometer were performed at ambient temperature.

A mass spectral study was initiated using a Finnigan Mat 4610 mass spectrometer operated in the EI mode and employing a gas chromatograph (DB-5, J&W Scientific, 15 m × 0.25 mm i.d., 0.25 μm film thickness) and the direct exposure probe (DEP); the study was continued using a VG AutoSpect Q high-resolution instrument equipped with a liquid secondary ion (Cs) source in the fast atom bombardment (FAB) mode using a glycerol matrix. The sample was dissolved in methanol (1 mg ml^{-1}), then injected into the GC (injection port temperature 250°C; oven program: 200°C (1 min), then increased at 20°C min^{-1} to 280°C (15 min)).

Fourier transform infrared (FT-IR) spectra were recorded from 4000 to 400 cm^{-1} with a resolution of 2 cm^{-1} using a Nicolet 60SX spectrometer with a DTGS (deuterated triglycine sulfate) detector. Samples for IR studies were prepared as fused KBr discs (0.3% sample) with spectral-grade potassium bromide. Samples for FT-Raman spectroscopy were prepared in the form of discs, 3 mm in diameter, with a KBr backing. The discs were formed in a hand-held minipress. The FT-Raman spectra were recorded at the Thornton Research Centre, Shell Research, Chester, UK. Details of the experimental arrangement have been given in two earlier publications [8,9]. The excitation source was a cw Nd:YAG laser operating at 1064.1 nm (9397.6 cm^{-1}). The laser beam was focussed to a 1 mm diameter spot at the sample, which was placed at one focus on an ellipsoidal mirror. The scattered radiation was collected by this mirror and directed into the Jacquinot stop of a Perkin-Elmer Model 1760 near-IR spectrometer. A laser power of 300 mW (at the sample) was used and 200 scans at a nominal resolution of 4 cm^{-1} were collected. The nitrogen-cooled germanium detector

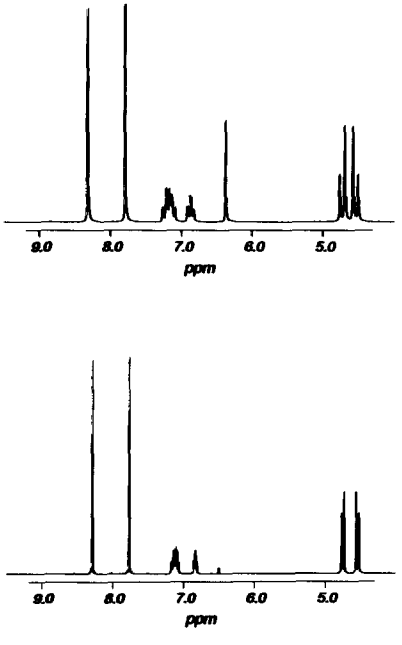
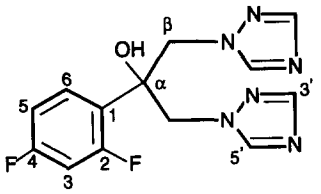


Fig. 1. ^1H NMR spectra of fluconazole in DMSO-d_6 recorded at 200 MHz (top) and after D_2O exchange at 400 MHz (bottom).

Table 1
^{1}H, ^{13}C and ^{19}F NMR assignments



Position No.	$\delta^{1}H$ [a]	$\delta^{13}C$ [b,c]	$\delta^{19}F$ [d]
1	–	123.49 ($^{2}J_{CF}$ = 12.8, $^{4}J_{CF}$ = 3.7 Hz)	–
2	–	162.25 ($^{1}J_{CF}$ = 249.7, $^{3}J_{CF}$ = 12.7 Hz)	−106.93 ($^{4}J_{FF}$ = 8.1 Hz)
3	7.17 [e] (m)	104.35 ($^{2}J_{CF}$ = 26.8 Hz)	–
4	–	159.46 ($^{1}J_{CF}$ = 247.4, $^{3}J_{CF}$ = 12.4 Hz)	−110.93
5	6.87 (m)	111.37 ($^{2}J_{CF}$ = 20.7, $^{4}J_{CF}$ = 3.0 Hz)	–
6	7.17 [e] (m)	130.08 ($^{3}J_{CF}$ = 5.9, 9.8 Hz)	–
α	–	74.04 ($^{3}J_{CF}$ = 5.1 Hz)	–
β	4.72,4.54 (AB)	55.30 ($^{4}J_{CF}$ = 5.1 Hz)	–
3′	7.78 (s)	151.09	–
5′	8.31 (s)	145.48	–

[a] ppm from DMSO-d_5 at δ 2.49 in DMSO-d_6.
[b] Magnitudes of the fluorine couplings are given in parentheses.
[c] ppm from DMSO-d_6 at δ 39.5.
[d] ppm from external $CFCl_3$ at δ 0.00.
[e] Value approximate owing to overlap of the two multiplets.

covered the spectral range 9400–6200 cm^{-1}, equivalent to a Raman shift range of 0–3200 cm^{-1}. The 0–200 cm^{-1} region, however, was obscured by the filters needed to remove the intense Rayleigh scattering and any unscattered laser radiation. The detector response was not linear and was very low in the CH stretching region of the Raman spectrum near 3000 cm^{-1}. The OH stretching region near 3450 cm^{-1} could not be observed. The Raman spectra shown in Figs. 6 and 7 have not been corrected for detector response and are presented here only for qualitative comparisons.

3. Results and discussion

3.1. ^{1}H NMR spectral features

The 200 and 400 MHz ^{1}H NMR spectra of DMSO-d_6 solutions of fluconazole are shown in Fig. 1 for comparison of magnetic field strength effects and spectral resolution. The 400 MHz proton spectrum was recorded when it was found more expedient to use the higher field strength to obtain good ^{13}C NMR detail. By means of deuterium oxide (D_2O) exchange, the singlet resonance near δ 6.4 was identified as arising from the

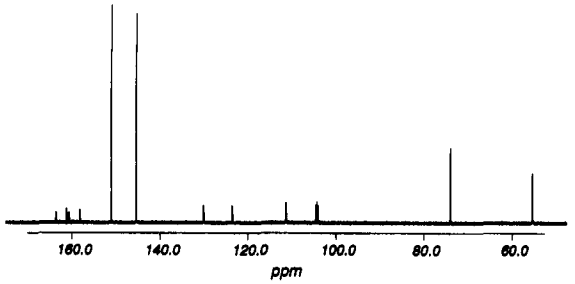


Fig. 2. ^{13}C NMR spectrum of fluconzaole in DMSO-d_6 recorded at 100 MHz.

hydroxyl proton of fluconazole. The remainder of the ^1H NMR spectra consisted of two singlets near δ 8.3 and 7.8, each integrating for two protons (consistent with the two protons of the two equivalent triazole rings of fluconazole), complex, highly coupled resonance patterns integrating with the ratio of 2:1 protons seen centred near δ 7.2 and 6.9 (consistent for the three aromatic protons of the *m*-difluorophenyl ring of fluconazole) and an AB pattern integrating for four protons centred near δ 4.65 (consistent for the two sets of β-methylene protons of fluconazole). The phenyl ring protons were assigned using carbon–hydrogen heteronuclear correlations and the triazole ring protons were assigned using NOE difference experiments. These features are consistent with the structure of fluconazole, and the ^1H NMR chemical shifts have been assigned as summarized in Table 1.

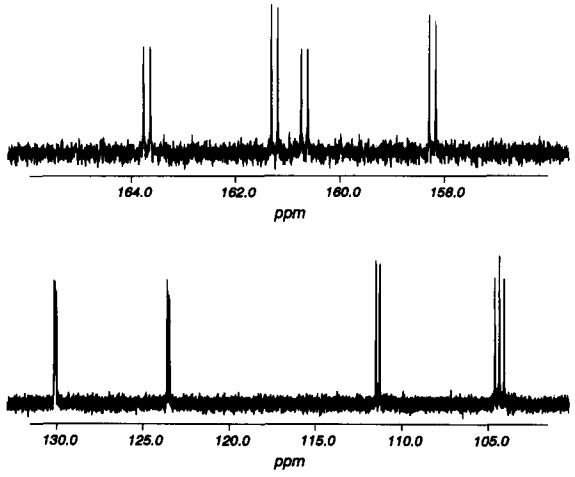


Fig. 3. ^{13}C NMR expansions of two aromatic regions of the carbon spectrum.

3.2. ^{13}C and ^{19}F NMR spectral features

When a ^{13}C NMR spectrum of fluconazole in DMSO-d_6 was recorded at 50 MHz, only four resonances (δ 150, 145, 74 and 55) were apparent after 120 scans. By examining the same solution at 100 MHz for an accumulation of 5000 scans, a highly characteristic ^{13}C NMR spectrum was obtained showing intricacies of the various ^{19}F–^{13}C couplings (Fig. 2). Two singlet resonances for the two non-^{19}F-coupled carbon atoms of the equivalent triazole rings are seen at δ 151 and 145. The two doublet resonances centred near δ 74.0 and 55.3 arise from the α-quaternary carbon and the two equivalent β-methylene carbon atoms of fluconazole coupled by the proximate *o*-fluoro atom. The two doublet of doublets pattern seen at δ 164–158 (Figs. 2 and 3) are due to the two aromatic carbon atoms substituted with fluorine. The pseudo-triplet (doublet of doublets) at δ 104.4 (Figs. 2 and 3) is due to the aromatic carbon located between the *m*-fluoro-substituted carbons. The remaining ^{13}C (doublet of doublets) patterns near δ 130, 123 and 111 are due to the remaining three aromatic carbons coupled to the two *m*-fluoro atoms. From these features, the ^{13}C NMR spectrum is consistent with the expected structure of fluconazole, and the chemical shifts and C–F coupling constants (absolute values) and ^{19}F chemical shifts and their coupling constant are presented in Table 1.

3.3. Mass spectral features

Because of its high polarity, fluconazole was first introduced into a Finnigan Mat 4610 mass spectrometer, operated in the EI mode, by means of the direct exposure probe (DEP). The mass spectrum obtained under these conditions (Fig. 4) did not show a molecular ion ($M^{\cdot+}$) at *m/z* 306, and the highest mass fragment was seen to be 224 Da. This ion arises by facile cleavage of either triazole group α to the hydroxy group. In our GC/MS analysis, however, the compound eluted as a sharp peak with a retention time of 2.5 min and gave an EI spectrum that was virtually identical with that of the DEP/EI spectrum shown in Fig. 4. It is worth noting that the corresponding spectrum in the Wiley Registry of Mass Spectral

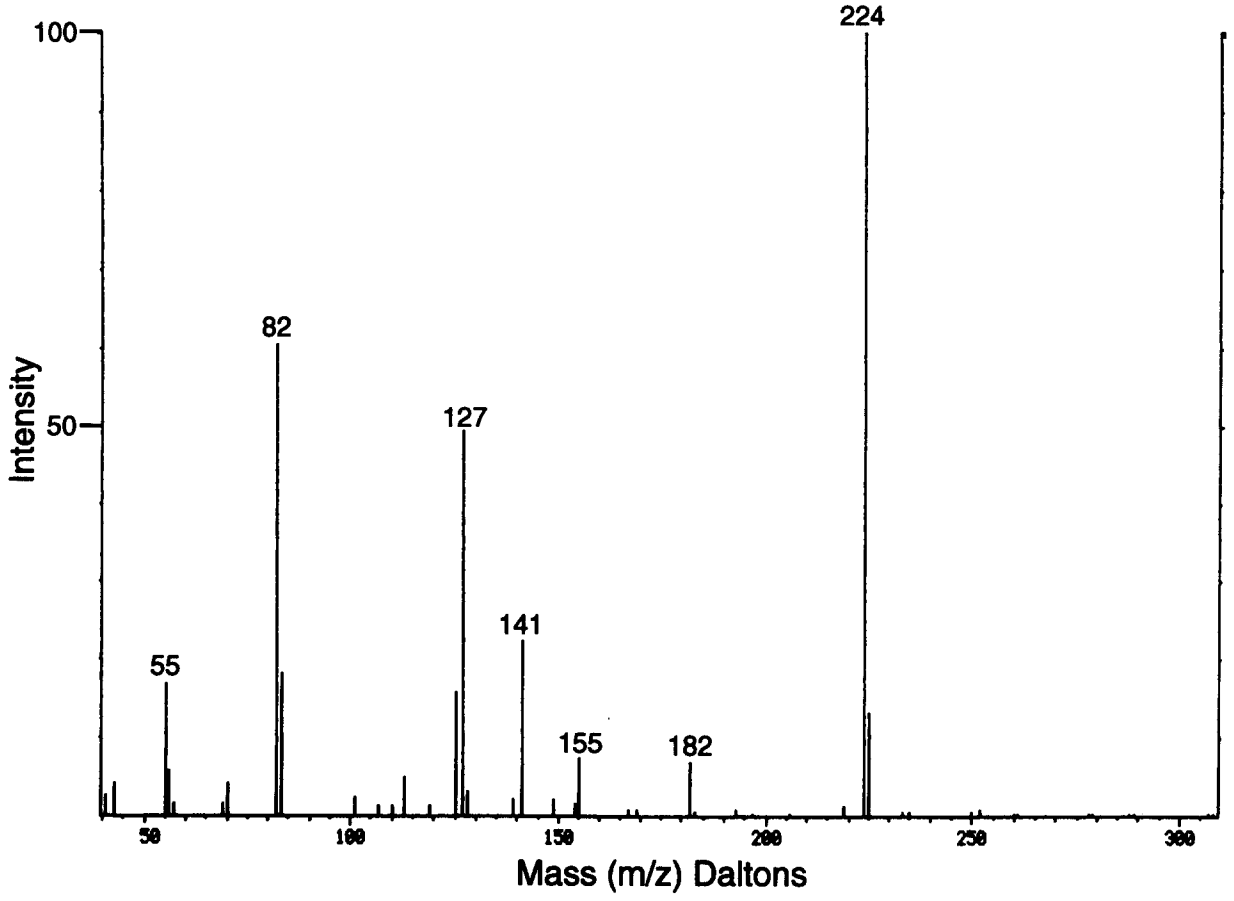


Fig. 4. DEP mass spectrum of fluconazole in the EI mode.

Data [10] contained additional peaks at m/z 142 (63%) and 170 (20%) which were not present in either our GC/MS or DEP/MS spectra.

Brammer et al. [11] reported obtaining a pseudo-molecular ion (M + 1 = 307 Da) for fluconazole by LC thermospray MS analysis of fluconazole metabolites. Interestingly, however, no $M^{\cdot+}$ was produced for the TMS derivative of fluconazole in recent GC/MS work reported by Wu et al. [12]. Because no good reference mass spectrum of underivatized fluconazole was available, the substance was examined on the AutoSpect Q high-resolution instrument in the FAB mode using a glycerol matrix. Under these conditions, an M + 1 peak at 307 Da was found, consistent with the expected MW, in addition to peaks at m/z 185 and 277 from the glycerol matrix.

In order to obtain additional structural information from this material, the FAB experiment was repeated as an MS/MS experiment in which the M + 1 ion was selected, then allowed to collide with argon gas (10^{-6} mbar) to obtain a daughter spectrum dominated by cleavage β to the tertiary alcohol and dehydration, viz. a peak at 238 Da (from loss of 69 Da for one triazole ring) which loses both water (−18 Da) and the other triazole ring to give peaks at 220 and 169 Da, respectively (Fig. 5). This indicates that the alcohol is being protonated prior to ionization. Since the mass spectral characteristics generated by this MS/MS study are consistent with the expected structure for fluconazole, one can now also be assured that the DEP/EI mass spectrum of fluconazole constitutes a valid reference spectrum for this substance.

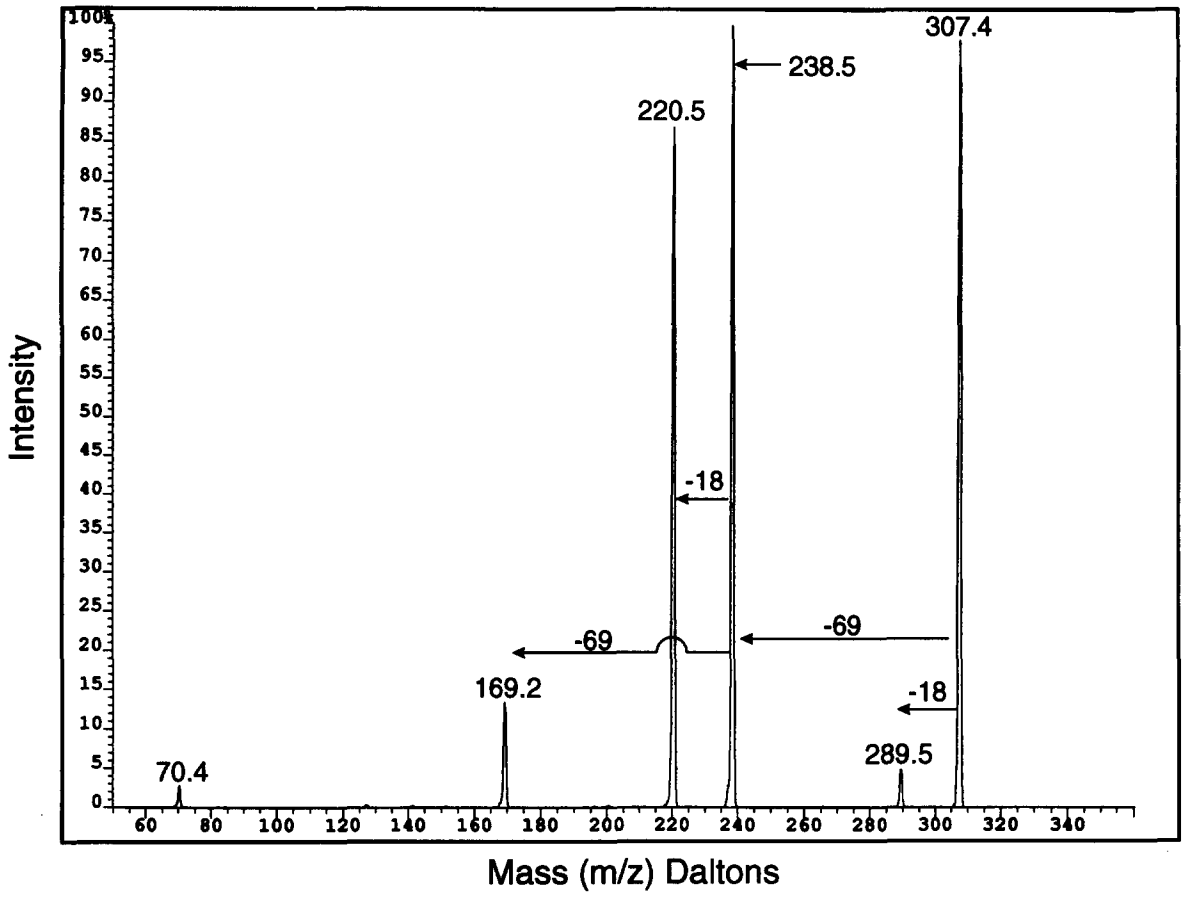


Fig. 5. Tandem mass spectrum of the fluconazole pseudo-molecular ion (M + 1 = 307 Da).

3.4. IR and Raman spectral features

IR and Raman spectra of fluconazole are shown and compared in Figs. 6 and 7. Observed wavenumbers are listed in Table 2, with suggested assignments to vibrations of the various functional groups present in the molecule. The observed features can be attributed to an *N*-substituted 1,2,4-triazole ring, a 1,2,4-trisubstituted benzene ring and a tertiary alcohol group.

3.5. Vibrations of the 1,2,4-triazole ring

From studies of the IR spectrum and a normal coordinate analysis, Bougeard et al. [13] have identified the 18 normal modes of vibration of free (gas-phase) 1,2,4-triazole. These comprise two CH stretching modes (ν_{CH}) near 3140 cm^{-1}, four ring stretching modes ($\nu_{triazole\ ring}$) between 1510 and 1280 cm^{-1}, a ring breathing mode at 1155 cm^{-1}, two in-plane CH bending modes (β_{CH}) at 1260 and 1040 cm^{-1}, two in-plane ring bending modes near 970 cm^{-1}, two out-of-plane CH bending modes at 883 and 842 cm^{-1} and two out-of-plane ring deformation modes near 670 cm^{-1}. The frequencies of the CH stretching vibrations are lowered by about 20 cm^{-1} in the solid crystalline state, whereas the frequencies of the ring stretching and CH bending modes are raised by between 20 and 50 cm^{-1}. In free 1,2,4-triazole, there are also three vibrations involving the N–H group. These vibrations are affected by hydrogen bonding in the solid state. The N–H bond becomes an N–C bond in fluconazole, so that N–H · · · N bonding is no longer possible.

In an IR and Raman study of a series of halogen-substituted *N*-methyl-1,2,4-triazoles [14], most of the normal modes of the triazole ring were observed at frequencies close to those observed for the parent molecule [13]. Two sharp lines at 3125 and 3097 cm^{-1} in the Raman spectra of fluconazole and a sharp IR band at 3118 cm^{-1} are assigned to the CH stretching vibrations of the triazole rings. Strong features in either the IR or Raman spectra at 1507, 1420, 1368 and 1318 cm^{-1} are attributed to triazole ring stretching modes and a very strong IR band at 1144 cm^{-1} is assigned to the ring breathing mode. Very strong IR bands at 966 and 851 cm^{-1} are attributed to in-plane and out-of-plane CH bending modes, respectively.

3.6. Vibrations of the 1,2,4-trisubstituted benzene ring

Aromatic CH stretching modes give rise to the Raman lines at 3072 and 3022 cm^{-1} and sharp IR absorptions at 3072 and 3010 cm^{-1}. The strong IR band at 1620 cm^{-1} and the corresponding Raman line at 1618 cm^{-1} are assigned to an aromatic ring stretching mode ($\nu_{C=C}$). The strong IR band at 1105 cm^{-1} is assigned to a C–F stretching mode (ν_{CF}) and the very strong Raman line at 1018 cm^{-1} is attributed to an in-plane CH bending mode (β_{CH}). The very strong Raman line at 737 cm^{-1} is characteristic of a 1,2,4-trisubstituted benzene derivative [15] and is attributed to an out-of-plane CH bending mode (ν_{CH}). The very strong IR band at 680 cm^{-1} and the strong band at 524 cm^{-1} are assigned to benzene ring deformations. The IR band of medium intensity at 569 cm^{-1} is assigned to a CF in-plane bending mode. Other CF bending modes and benzene ring deformations give riise to weak Raman lines below 500 cm^{-1}. The three weak IR absorptions at 1898, 1844 and 1766 cm^{-1} are characteristic of a 1,2,4-trisubstituted benzene ring [16].

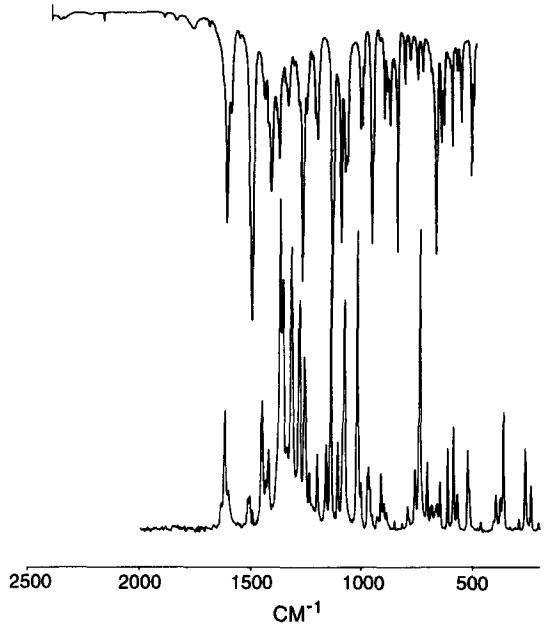


Fig. 6. FT-Raman (bottom) and FT-IR (top) spectra of fluconazole over the range 2500–400 cm^{-1}.

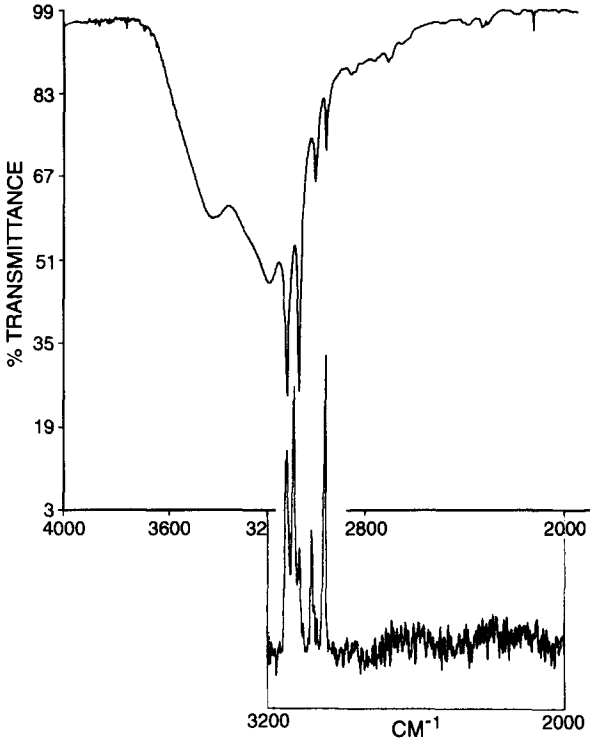


Fig. 7. FT-Raman (bottom) and FT-IR (top) spectra of fluconazole over the ranges 3200–2000 and 4000–2000 cm^{-1}, respectively.

Table 2
Wavenumbers (cm^{-1}) [a] of observed bands in the infrared and Raman spectra of fluconazole

IR	Raman	Assignment [b]
3190 m, br	–	H-bonded OH str.
3118 ms	3125	CH str. triazole ring
–	3097 m	CH str. triazole ring
3072 ms	3072 w	CH str. arom. ring
–	3022 w	CH str. arom. ring
3010 w	–	CH str. arom. ring
2966 w	2968 ms	CH_2 str.
1898 vw	–	Overtones and combs.
1844 vw	–	1,2,4-trisubst.
1766 vw	–	arom. ring
1620 s	1618 m	C=C str. arom. ring
1516 sh	1514 sh	C=C str. arom. ring
1507 vs	1506 vw	Triazole ring str.
1449 vw	1451 m	CH_2 def.
1420 m	1420 w	Triazole ring str.
1384 m	–	C=C str. arom. ring
–	1368 vs	Triazole ring. str.
1344 w	1355 s	C=C str. arom. ring
1317 vw	1318 vs	Triazole ring str.
1279 vs	1279 s	OH def.
1261 w	1257 ms	β-CH arom, ring
1210 m	1198 w	β-CH triazole ring
1144 vs	1137 s	Triazole ring breathing
1105 s	1106 w	CF str.
1085 ms	1077 s	C–OH str.
1018 m	1018 vs	β-CH arom. ring
966 s	966 w	β-CH triazole ring
909 m	909 w	Triazole ring def.
885 m	885 vw	γ-CH arom. ring
851 s	849 vw	γ-CH triazole ring
818 w	–	γ-CH arom. ring
794 vw	791 vw	γ-CH triazole ring
761 w	758 w	CH_2 rock
738 w	737 vs	γ-CH arom. ring
680 vs	685 vw	Arom. ring def.
646 m	646 w	Triazole ring def.
609 s	612 w	Arom. ring def.
–	586 m	Ring def.
569 m	569 vw	β-CF arom. ring
524 s	523 w	Ring def.
465 vw	465 vw	Skeletal
–	364 m	and
–	266 w	ring
–	240 vw	deformations

[a] Relative intensities are denoted by s = strong, m = medium, w = weak, v = very, sh = shoulder, br = broad.
[b] β = In-plane deformation, γ = out-of-plane deformation.

3.7. Vibrations of the tertiary alcohol and methylene groups

A broad IR band centred at 3190 cm^{-1} is attributed to the hydrogen bonded OH stretching mode of the tertiary alcohol group. The bending mode of this group is observed as strong features in both the IR and Raman spectra at 1279 cm^{-1}. A strong Raman line at 1077 cm^{-1} is assigned to the C–OH stretching mode. This corresponds to the lower frequency component of an IR doublet at 1085/1079 cm^{-1}. CH stretching of the CH_2 groups gives rise to bands at 2966 cm^{-1} in the IR and 2968 cm^{-1} in the Raman spectra. Bands at 1451 cm^{-1} (Raman) and 1449 cm^{-1} (IR) are assigned to CH_2 deformation modes.

Acknowledgements

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