

**Isolation and characterization of antibacterial compound from a mangrove-endophytic fungus,
Penicillium chrysogenum MTCC 5108**

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Abstract Microorganisms, especially endophytic fungi that reside in the tissue of living mangrove plants, seem to play a major role in meeting the general demand for new biologically active substances. During the course of screening for biologically active secondary metabolites from marine microorganisms, an antibiotic compound containing an indole and a diketopiperazine moiety was isolated from the culture medium of *Penicillium chrysogenum*, (MTCC 5108), an endophytic fungus on the mangrove plant *Porteresia coarctata* (Roxb.). The cell free culture medium of *P. chrysogenum* showed significant activity against *Vibrio cholerae*, (MCM B-322), a pathogen causing cholera in humans. Bioassay guided chemical characterization of the crude extract led to the isolation of a secondary metabolite possessing a molecular formula $C_{19}H_{21}O_2N_3$. Its antibacterial activity was comparable with standard antibiotic, streptomycin. This compound (1) was found to be (3,1'-didehydro-3[2''(3''',3'''-dimethyl-prop-2-enyl)-3''-indolylmethylene]-6-methyl piperazine-2,5-dione) on the basis of mass spectrometry (MS), infrared spectroscopy (FTIR) and one and two-dimensional nuclear magnetic resonance (NMR) analysis.

Keywords Marine fungi • *Penicillium chrysogenum* • secondary metabolite • *Vibrio cholerae*

Introduction

The search for antibiotics from fungi started with the discovery of penicillin, produced by *Penicillium notatum* [1]. Penicillin is a potent antibiotic against Gram-positive bacteria, which became a “wonder drug” and saved millions of lives. It is still a “front-line” antibiotic, although the development of penicillin-resistance in several pathogenic bacteria now limits its effectiveness. Due to the development of drug resistance in pathogenic microbes, combined with the increasing frequency of infectious diseases in immunocompromised individuals, a need to search for newer antibiotics has increased several folds. Screening of fungal metabolites led to the discovery of novel as well as rediscovery of high numbers of previously described metabolites. For this reason, attention is focused towards isolating fungi from less investigated habitats and ecological niches like the oceans and those inhabiting the body of marine organisms (flora and fauna). The astounding chemical variety of biologically active secondary metabolites from endophytic fungi [2] has led to further research on these organisms. The most well-known example being that of taxol, the multimillion dollar anticancer compound produced in yew plant *Taxus brevifolia* by the terrestrial fungus *Taxomyces andreanae* [3]. It is reported that between 1987 and 2000, approximately 140 new natural products were isolated from endophytic fungi [4], whereas a similar number was subsequently characterized in half of this time between 2000 and 2006 [5]. Therefore, a number of metabolites were isolated from fungi which found their way into medical applications as natural products.

Mangrove plants have been proved to be well established source for structurally diverse and biologically active secondary metabolites [6]. More than 200 species of endophytic fungi have been isolated and identified from mangrove plants and several reports on the isolation of antibiotic compounds from endophytic fungi have been reported. To mention a few griseofulvin, [7], chaetomugilin A and D [8], cytosporone B and C [9], 3-O-methylalaternin and altersolanol A [10], phomoenamides [11], phomodione [12], ambuic acid [13], isopestacin [14], munumbicin A, B, C and D [15], pestalachlorides A and B, [16], brefeldin A [17], coronamycin, [18], pumilacidin [19], cytochalasin D [20], polyketide citrinin [21,22], fumigaclavine C, fumitremorgin C, physcion [23] and several others.

These examples illustrate the enormous chemical diversity and biological potential of endophytic fungi. Hence, in the present study, we report on the isolation of endophytic fungi from a mangrove plant *Porteresia coarctata* (Roxb). Laboratory culturing of all isolates on a small scale followed by preliminary antibiotic screening of their crude extracts against clinical bacterial pathogens led to the selection of *Penicillium chrysogenum* (MTCC 5108) for further study. Its mass culture in the laboratory followed by bioassay guided chromatographic isolation and detailed chemical characterization leading to the identification of the active compound 1 is reported here.

Materials and Methods

Isolation of marine fungus

The leaves of the mangrove plant *Porteresia coarctata* (Roxb) were collected from Chorao Island, along the Mandovi estuary of Goa, India. Collection was made using sterile polythene bags and transported to the laboratory. On reaching the laboratory, the leaves were rinsed with sterile seawater (SSW) to remove adherent particles and detritus material. The leaves were then immersed in methanol (70%) for 60-120s for surface sterilization and later rinsed once again in SSW. The leaves were next held with sterile tweezers and cut into smaller fragments using a sterile razor and placed on potato dextrose agar (PDA, HiMedia) medium in a Petri dish so that the freshly cut edges come in direct contact with the PDA surface. The plates were observed regularly for fungal growth and the fungal hyphae were picked aseptically and placed on fresh PDA plates. The individual strains were repeatedly subcultured on potato dextrose agar plates until pure fungal isolates were obtained (Fig. 1). The fungal strains were identified at Agarkar Research Institute, Pune, India. These cultures were grown on a small scale (100ml) in the laboratory in potato dextrose broth medium for 15 days. The cell free fermentation medium was extracted in chloroform and subjected to primary antibiotic screening which led to the selection of *P. chrysogenum* for further study (Table 1). This culture was deposited at the Institute of Microbial Technology (IMTEC), Chandigarh, India, bearing accession no. MTCC 5108.

Preparation of inoculum and mass culture

For small scale fermentation, fungal spores were collected from 8-day-old culture grown on Potato dextrose agar (PDA) slants with sterile seawater (5 ml). The spore suspension

containing about 5×10^4 spores ml^{-1} was used to inoculate 300ml of liquid potato dextrose broth (200 g/l potato starch, 20 g/l dextrose, pH 7.4) prepared in seawater:distilled water (1:1). Fermentation was carried out on a shaker for a week. This was used as inoculum for mass culturing three 5-litre flasks, each containing 2 litres of sterile, potato dextrose fermentation medium. The flasks were incubated at 28-30°C in a stationary condition for 15 days. At the end of the incubation period, the fermentation medium was filtered free of fungal mat.

Chemical isolation and characterization of active compound

All solvents used for extractions, isolation and purification were of HPLC grade. Following incubation, cell free culture medium was concentrated under vacuum to reduce the volume to approximately 800ml. This concentrated filtrate was transferred to separating flask and extracted repeatedly (4 times) with chloroform (300ml). The chloroform fractions were pooled and concentrated under vacuum and low temperature to yield crude chloroform extract (30 mg). Slurry of the crude extract was prepared in silica gel (100 mg) and after slow drying under stream of nitrogen, was loaded onto a glass column (60 x 1.5 cm) using silica gel (60-120 mesh size). The column was eluted initially with 100ml of petroleum ether (PE) followed by a gradient of ethyl acetate (EA) in petroleum ether (5%, 10%, 15% and 25% EA in PE 100 ml each). Thin layer chromatographic (TLC) analysis of the various fractions was carried out to check the purity of the fluorescent compound. Further purification of the yellow compound on a Sephadex- LH₂₀ column using chloroform:methanol (1:1) yielded pure yellow solid compound (9 mg) as shown by a single spot on TLC. The solvent system used for developing the spot on TLC was 3% methanol in chloroform and the spots were visualized with iodine vapours in an iodine chamber. The spot of the compound could also be viewed by using ceric sulphate as a spraying agent and later heating the TLC plate in an oven at 100°C for 2 min. The active yellow compound was soluble in chloroform, ethyl acetate and methanol.

Analytical methods

Thin Layer Chromatography (TLC)

TLC was performed on silica gel coated aluminium sheets (60 F₂₅₄, Merck). The compound was spotted using a fine capillary tube (2-5 μ l) and detected as a fluorescent spot in a UV-chamber at 366 nm. The plate was developed using 3% methanol in chloroform and visualized after keeping the plates in iodine chamber for 2 minutes.

Nuclear Magnetic Resonance Spectrometry

^1H , ^{13}C , COSY, HMBC and HMQC spectral data were generated in deuterated chloroform (CDCl_3) at 300 MHz, using a Bruker Avance AMX300 instrument. Tetramethyl silane (TMS) was used as an internal standard at $\delta 7.24$ for ^1H NMR and $\delta 77.0$ for ^{13}C NMR.

Electrospray Ionization Tandem Mass spectrometry (ESI-MS/MS)

Mass data was obtained using Electrospray Ionization Mass Spectrometer (ESI-MS/MS, QSTAR XL System) equipped with electrospray ionization source using a spray voltage of 5.5 kv. Typically about 10 μg of sample was dissolved in methanol containing 1% formic acid. About 10 scans were averaged to get a mass spectrum. IR spectral data was recorded on FTIR-8201 PC Shimadzu spectrometer.

Antibiotic assay

Antibacterial activity was tested against seven bacterial pathogens (listed in Table 1), obtained from Goa Medical College, Goa. Antibacterial activity was carried out using the standard paper disc diffusion assay method as described earlier [22]. Briefly, sterile Whatman No. 1 filter paper (GF/C) discs of 6 mm diameter were impregnated with the compound under study ($1\text{mg}\cdot\text{disc}^{-1}$ in the case of crude extract; $10\mu\text{g}\cdot\text{disc}^{-1}$ in case of pure compound) and placed on Nutrient agar (NA) plates which was surface inoculated and uniformly spread with the test organism (approximately 1.2×10^8 CFU/ml). Standard streptomycin discs ($10 \mu\text{g}\cdot\text{disc}^{-1}$) served as positive control and discs containing only solvent (chloroform), served as negative control. Same concentration of standard and pure compound was used during the assay for the purpose of comparison. Nutrient agar plates were incubated at 37°C for 24h. The zone of growth-inhibition around each disc was measured in millimeters. The assay was carried out in triplicate.

Results and Discussion

Fungi are ubiquitous eukaryotic, heterotrophes which have gained importance as rich sources of biologically active secondary metabolites. Mangrove-associated fungi are still poorly investigated and thus present a promising source of bioactive compounds. Among the several endophytic fungi isolated from the mangrove plant *Porteresia coarctata* (Roxb) (Fig.1), preliminary screening using clinical bacterial pathogens showed that all the strains were insensitive to the crude fungal extracts (Table 1), except for that of *P. chrysogenum* which

showed significant activity against *V. cholerae*. This resulted in the selection of *P. chrysogenum* for further chemical investigation.

Penicillium chrysogenum was mass cultured in the laboratory. At the end of the fermentation period, the culture was harvested and the cell free medium was subjected to a series of chromatographic isolation techniques combined with thin layer chromatographic application for purity testing to obtain the active compound **1**. The purified Compound **1** was a diketopiperazine (DKP) derivative and its antibacterial screening using clinical pathogens showed it to be significantly active selectively against human pathogen, *Vibrio cholerae* with an inhibition zone of 14-16mm (Fig. 2).

Natural penicillins obtained from culture-filtrate of *P. notatum* or *P. chrysogenum* are penicillin G and penicillin V. Both are active against Gram-positive bacteria. In the present study, we isolated an antibacterial compound which is active against Gram negative *V. cholerae* (inhibition zone of 14-16 mm). Its antibacterial activity was comparable with streptomycin, which also showed 14-16 mm inhibition zone. All the other clinical pathogens viz. *Escherichia coli*, *Salmonella typhi*, *Shigella flexineri*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Staphylococcus aureus* were insensitive to compound **1** (Table 1).

Structure elucidation of compound **1** was arrived at by considering all the spectroscopic data. Mass data (ESI-MS) of compound "1" exhibited ($M^+ + Na^+$) and ($2M^+ + Na^+$) signals at m/z 346 and 669 respectively, indicative of M^+ 323 corresponding to molecular formula of $C_{19}H_{21}O_2N_3$ (Fig. 3).

A close inspection of the ^{13}C NMR spectra of "1" (Table 2) disclosed signals for 19 carbons: These included one secondary methyl (C-7), two tertiary methyls (C-4", C-5"), one sp^3 quaternary carbon (C-3"), one sp^2 hybridized methylene (C-1"), one sp^3 hybridized methine (C-6), six sp^2 methines (C-1', C-4", C-5", C-6", C-7" and C-2") and seven sp^2 quaternary carbons including amide carbonyls (C-2, C-3, C-5, C-2", C-3", C-3"a and C-7"a). The presence of two secondary amide groups were inferred from signals at 165.5 and 159.6 ppm from its ^{13}C NMR spectra ($CDCl_3$), sharp and strong IR absorptions at 3350 cm^{-1} and 1676 cm^{-1} (Fig. 4), and also from the presence of two D_2O exchangeable protons at δ 6.4 and 7.4 (these signals appeared at δ 8.2 and 8.6 respectively in DMSO). The IR absorption (Fig. 4) at 1676 cm^{-1} was also indicative of α - β unsaturated carbonyl functionality. The presence of a third

exchangeable proton at δ 11.15 in DMSO spectrum and at δ 8.27 in CDCl_3 spectrum along with the pattern of ^1H NMR signals in DMSO (7.47, 7.21, 7.14, 7.06 and 6.96) was suggestive of a conjugated indole nucleus, as present in dipodazine, [(Z)-1',3-didehydro-3-(3"-indolylmethylene)-piperazine-2,5-dione], a metabolite from *Penicillium dipodomis*, (Fig. 6) [24]. The only exception observed was that olefinic methine proton signal at δ 7.93 of the indole nucleus in dipodazine was absent in Compound **1** (Fig. 5), indicating that C-2" position was also substituted in the latter.

The ^{13}C NMR spectrum of dipodazine and compound **1** are virtually identical with the following changes. The C-2" carbon at 143 ppm in Compound **1** is a singlet and has undergone \sim 17.0 ppm downfield shift appropriate for tertiary alkyl group substitution [25]. Four new signals (27.2, 39, 113 and 144.2 ppm) have appeared in the spectrum of compound **1**. The intensity of the signal at δ 27.2 is suggestive of two similar carbons. These new carbon signals are attributed to α , α -dimethyl (reversed isopentenyl) substituent which must be attached to the C-2" of the indole moiety. The cross peaks originating from the vinylic proton $^2\text{J}_{\text{C-3}''', \text{H-2}''}$ and $^3\text{J}_{\text{C-2}'', \text{H-2}''}$ and $^2\text{J}_{\text{C-1}'', \text{H-2}''}$ in HMBC spectrum (Fig. 7) confirmed the position and the nature of the isopentenyl substituent (this substituent may also be taken as 1,1 dimethyl-2-propenyl unit).

Considering the formula, the conjugated moiety, isopentenyl substituent and the presence of two secondary amide groups, it was suggestive of tryp-alanine derived cyclic dipeptide. The cross peaks, in the HMBC spectrum, $^3\text{J}_{\text{C-3}''\text{a}, \text{H-1}'}$; $^3\text{J}_{\text{C-2}'', \text{H-1}'}$ and $^3\text{J}_{\text{C-2}', \text{H-1}'}$ connected C-1' to the indole and diketopiperazine moieties. HMBC connectivities are also observed with the C-7 secondary methyl and the C-6 methine with the C-5 and C-2 carbonyls of diketopiperazine moiety respectively (Fig. 7).

All the above data indicated that compound **1** is dipodazine, extended by a reversed isopentenyl or 1,1 dimethyl 2-propenyl moiety attached at position 2" of the pyrazole ring of indole moiety, as shown in Fig. 6, dipodazine is tryp-glycine derived cyclic dipeptide whereas compound **1** is tryp-alanine derived cyclic dipeptide. The 2,5-DKP (Diketopiperazine), are known to be frequently generated as unwanted by-products or degradation products in the syntheses of oligopeptides [26]. Some piperazine derivatives are reported to exhibit activities towards the central nervous systems, such as anti-anxiety activity and anti-convulsive activity,

as described in U.S. Patent No. 3,362,956 [27]. Piperazine derivatives are also known to possess calmodulin inhibitory activity [28]. Some of the compounds with calmodulin inhibitory activity have been revealed to be antihypertensive and vasodilatory in action [29]. Hence, marine fungi continue to yield compounds showing newer activities not reported earlier.

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Caption to figures

Fig. 1. Pure fungal strains isolated from *Porteresia coarctata* (Roab).

Fig. 2. Growth inhibition zone against *Vibrio cholerae*

Fig. 3. Mass spectrum of Compound 1.

Fig. 4. FTIR of Compound 1.

Fig. 5. Compound 1

Fig. 6. Dipodazine

Fig. 7. HMBC Correlation of Compound 1

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Fig. 1 Pure fungal strains on PDA agar plates isolated from *Porteresia coarctata* (Roxb).
a) *Aspergillus terreus* b) *Mucor* sp. c) *Aspergillus flavus* d) *Aspergillus fumigatus*
Fusarium sp. f) *Penicillium chrysogenum*.

e)

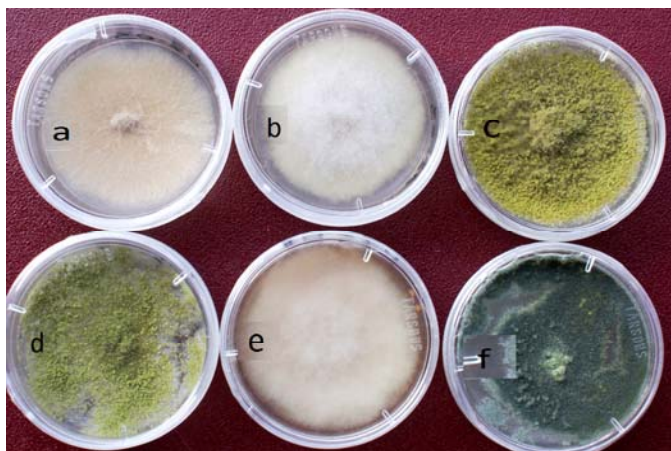


Fig. 2 Growth inhibition zone against *Vibrio cholerae*



Fig. 3 Mass spectrum of compound 1

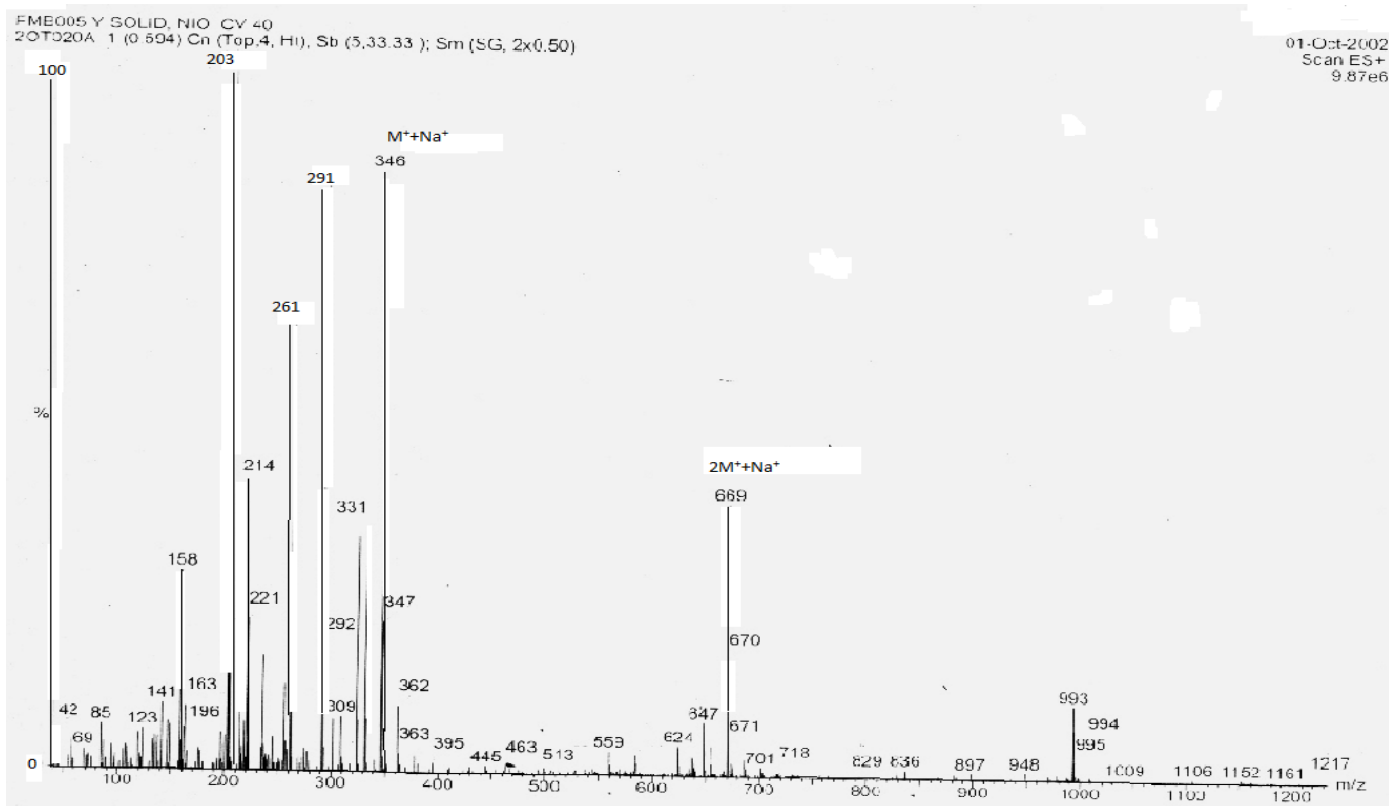


Fig. 4 FTIR of compound 1

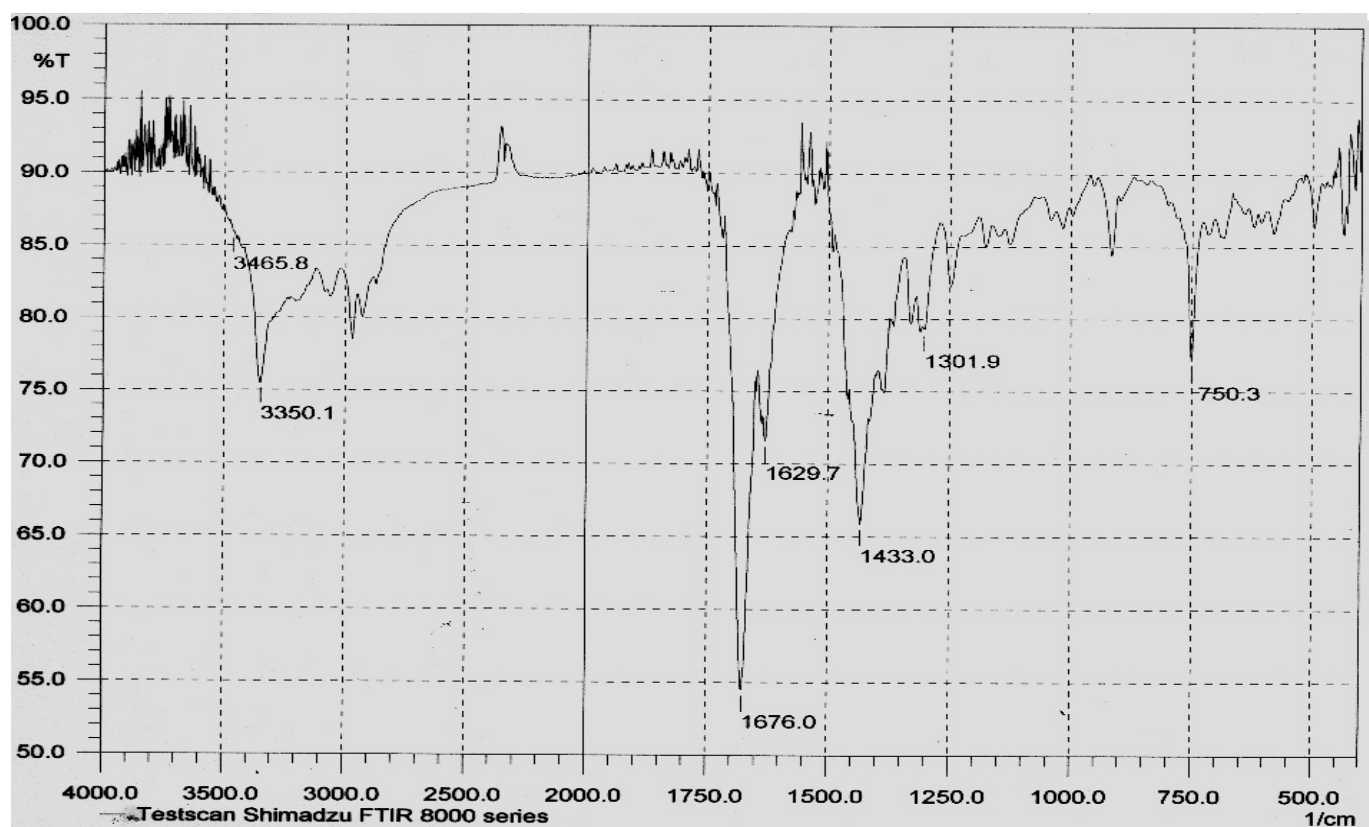


Fig. 5. Compound 1

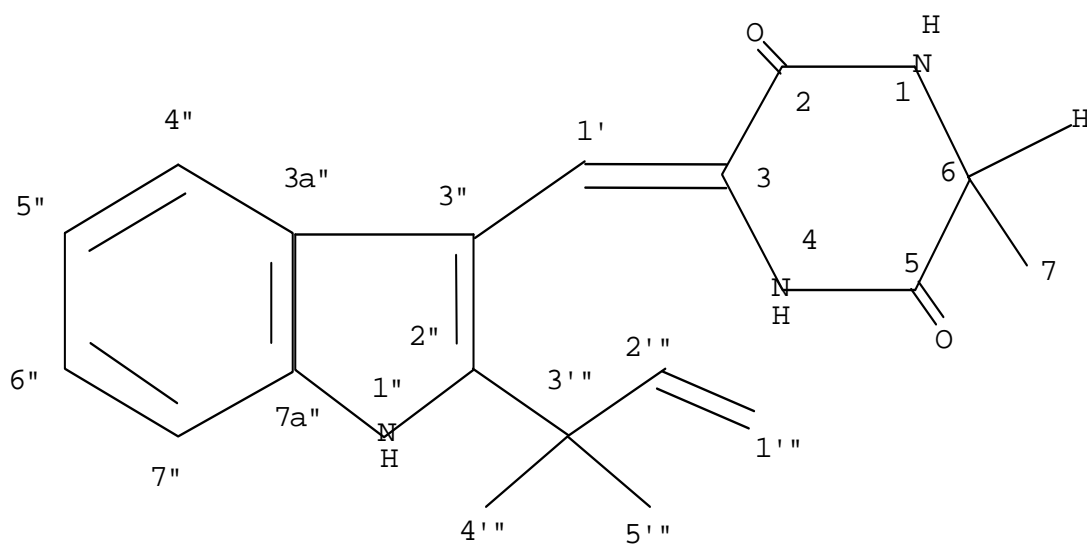


Fig. 6. Dipodazine

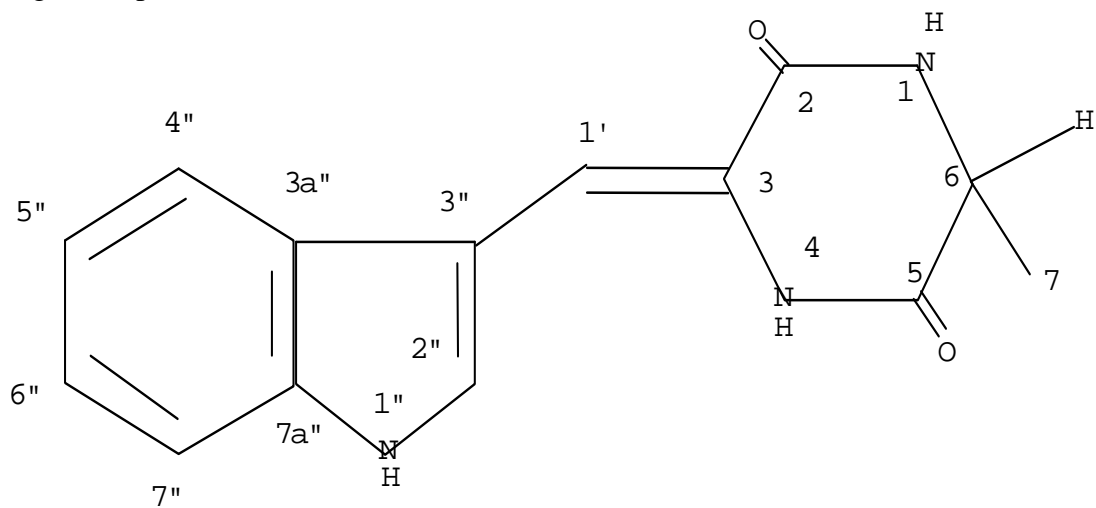


Fig. 7. HMBC correlation of compound 1.

