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A novel antifungal pyrrole derivative from *Datura metel* leaves

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Phytochemical investigation of the leaves of *Datura metel* Linn. led to the isolation of a new pyrrole derivative **1** which was characterised as 2β-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1′-methylethyl pentanoate on the basis of spectral data analyses and chemical reactions. Compound **1** was endowed with antifungal activity and its MIC was found to be 87.5 μg/ml. Two proteins having molecular weights of 42 and 58 kD of *Aspergillus fumigatus* are potential targets for compound **1**.

1. Introduction

Datura metel Linn. (Solanaceae) has an important place in the traditional system of medicine as a narcotic, anodyne and antispasmodic drug similar to *Belladonna* and *Stramonium* (Kiritikar and Basu 1999; Nadkarni 2002; Wealth of India 2002). The chemical constituents steroids (Ali and Shuaib 1996), tropane alkaloids (Wealth of India 2002; Siddiqui et al. 1986, 1988), withanolides (Kazushi et al. 1989; Mahmood et al. 1988; Manickam et al. 1994; Rastogi and Mehrotra 1998; Shingu et al. 1990; Mohini et al. 1992) and 15, 18-dihydroxy-abietatriene (Ali and Shuaib 1996) have been reported from the plant. This communication describes the isolation and characterization of a novel antifungal pyrrole derivative from this plant.

2. Investigations, results and discussion

2.1. Isolation of metabolite 1

The chloroform fraction of *D. metel* leaves was fractionated to identify the active antifungal component. The examination of the TLC under UV light revealed the presence of 9–10 major compounds in the chloroform fraction of *D. metel* most of which reacted positively with Dragendorff's reagent. These findings suggested that the chloroform fraction of *D. metel* contained a number of alkaloids. The existence of large number of alkaloids in *D. metel* has already been reported (Siddiqui et al. 1988; Wealth of India 1992).

Further sub-fractionation of the chloroform fraction was carried out by column chromatography to identify and purify the active component. A gradient of chloroform: methanol from 100 : 0 to 0 : 100 v/v was applied to elute various components of the chloroform fraction to isolate the alkaloidal compounds from *D. metel*. In total 78 subfractions were collected and pooled based on their R_f values. The pooling and analysis of fractions for antifungal activity revealed that sub fraction-11 (SF-11) was active against *A. fumigatus*.

The TLC profile of SF-11 showed the presence of three compounds and their positive reactivity with Dragendorff's reagent suggested that these compounds were alkaloidal in nature. However, we did not know if the alkaloids recovered in SF-11 were the same or different from those reported earlier from *D. metel* (Siddiqui et al. 1988). An alkaloidal compound of SF-11 called APC-II and having R_f values of 0.22 and 0.42 in the solvent systems chloroform : methanol : formic acid and chloroform : methanol : diethylamine, respectively, was found to be active against *A. fumigatus*. The HPLC resolved APC-II into two peaks at retention times 1.91 and 2.71, respectively. The compound recovered in peak-I at a retention time of 1.91 presented compound **1**.

The results of the carbon disulphide test supported the presence of a secondary amino group in **1** (Furniss et al. 1989). The reaction of **1** in ethanol with ferric chloride and hydrochloric acid indicated the existence of an ester group in the compound. However, the tests performed for aldehyde groups were negative (Furniss et al. 1989).

2.2. Structural elucidation of 1

The prominent absorption maximum at 206 nm in the UV spectrum indicated the presence of an ester group. The characteristic absorption bands in IR were observed at 3434 (NH) and 1725 (ester) cm^{-1}. The molecular mass of **1** was established as 239.2 on the basis of its FAB mass spectrum consistent with the molecular formula $C_{14}H_{25}O_2N$. The mass spectrum of **1** displayed the base peak at m/z 58, which was generated due to the $C_{1'}-C_2$ and OC–O fission, suggesting secondary nature of the amino group. A prominent ion peak at m/z 102 arose due to the formation of a pentanoic acid moiety ($C_5H_{10}O_2^+$). Cleavage of the $C_{1''}-C_{2''}$ linkage resulted in the formation of an ion peak at m/z 57 ($C_4H_9^+$).

The ^1H NMR spectrum of **1** displayed two one-proton carbinol multiplets at δ 4.15 ($w_{1/2} = 13.5$ Hz) and 3.75 ($w_{1/2} = 11.5$ Hz) assigned to H-2′ and H-2 α, respectively.

A two-proton double doublet at δ 3.17 (J = 7.44,7.44 Hz) was ascribed to methylene protons H_2-5a and H_2-5b attached to the amino group. Two one-proton signals at δ 2.31 (t, J = 6.48 Hz) and δ 1.95 (m) were associated with the H_2-2″a and H_2-2″b methylene protons, adjacent to the esteric carbonyl group. A six-proton broad singlet at δ 1.62 was attributed to C-6 and C-7 methyl protons, attached to C-3 and C-4 olefinic carbons. Two one-proton doublets at δ 1.42 (J = 6.96 Hz) and δ 1.35 (J = 7.28 Hz) were accounted to H_2-2′a and H_2-2′b methylene protons linked to the ester bearing carbon. A three-proton triplet at δ 0.87 (J = 5.96 Hz) was due to the Me-5″ primary methyl protons. The remaining C-3′ Me, CH_2-3″a, CH_2-3″b, CH_2-4″a and CH_2-4″b, methylene protons resonated as a seven-proton broad signal at δ 1.25. The absence of any signal beyond δ 4.15 suggested tetra-substituted olefinic linkage in the molecule. The existence of a methyl group linked to the secondary amino group was ruled out due to absence of any signal between δ 2.0 and δ 3.0 in the ^{1}H NMR spectrum. The ^{13}C NMR spectrum of **1** displayed important signals at δ 171.3 (COO-1″), 138.1 (C-3) and 139.3 (C-11).

D. sanguinea contains 3α-tigloyloxy-tropan-6β-ol having a molecular mass of 239.0 (Evan and Lampard 1972). But the possibility of any similarity between the 3α-tigloyloxy-tropane and 2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate described in the current study was ruled out on the basis of mass fragmentation pattern and NMR spectrum analyses (Evan and Major 1968). Further the absence of any signal beyond δ 4.15 in the NMR spectrum and the presence of a base peak at m/z 57.9 and 102 in the FAB spectrum did not support the possibility of a tropane skeleton in compound **1** (Evan and Major 1968).

Based on these evidences and analysis of COSY spectrum, which proved the proton to proton relationship between CH_2-5 and Me-7, CH-2 and CH_2-1′, CH_1-2′ and Me-3′ and Me-5″ and CH_2-4″ the structure of the molecule was formulated as 2β-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1′-methylethyl pentanoate. This is an unknown pyrrole derivative reported for the first time from a natural or synthetic source.

2.3. Antifungal activity of 1

Compound **1** was endowed with antifungal activity. The MIC of the purified compound **1** against pathogenic species of *Aspergillus* such as *A. fumigatus*, *A. flavus* and *A. niger* was found to be 87.5 μg/ml by microbroth dilution and percentage spore germination inhibition assays. The antimycotic potential of the compound was evident from the disc diffusion assay also as a concentration of 5.0 μg/disc inhibited the growth of *Aspergillus* fungi (Rajesh and Sharma 2002).

A number of alkaloids including hyoscine, hyoscyamine, meteloidine, scopolamine, tigloidine, tropine, withametelline and datumetine etc. have been reported from *Datura* species (Rastogi and Mehrotra 1998). Some of these alkaloids have found application in health care. However, none of the compounds isolated from *D. metel* has been evaluated for its antimycotic properties. The results of the current study show that a new alkaloid from *D. metel* exhibits antifungal activity.

2.4. Fungal protein target(s) for 1

The exact mechanism of action of **1** is not known since it is a novel molecule isolated first time from a plant source. However, we attempted to study its possible mechanism of action by identifying gene/protein targets for **1** in the pathogen. The secretory proteins of untreated and compound **1** treated cultures of *A. fumigatus* after 12 h were analyzed by 12% SDS-PAGE to determine the effect of the compound on gene products of the pathogen. It was observed that the expression of two proteins having molecular weights of 42 and 58 kD was completely inhibited or down regulated by compound **1**. The results indicated that these two proteins were the possible potential targets for this novel compound **1** and their depletion at the time of germination of *A. fumigatus* spores could be an important mechanism of action of **1** on pathogenic fungi.

Different mechanisms of action have been demonstrated for various antifungal drugs. Amphotericin B and other polyene drugs bind to a sterol present in the fungal cell wall (Kinsky 1970). The azoles may block dimethylation of lanosterol to inhibit *de novo* synthesis of ergosterol (Sud and Feingold 1981). 5-Flucytosine is reported to change the genetic code of the pathogen leading to inhibition of growth (Wain and Polak 1979). We, at present do not have enough data to clearly describe the complete mechanism of action of compound **1.** However, the preliminary results suggest that the compound **1** may exhibit fungicidal activity by inhibiting the expression of some important proteins at the time of germination of fungal spores.

3. Experimental

3.1. Plant material

Fresh leaves of *D. metel* were collected from the wild growth of the plant in Delhi and identified by Dr. M. P. Sharma, Reader, Department of Botany, Faculty of Science, Jamia Hamdard, New Delhi. The leaves were dried first in air and then in an electrical oven at 55 °C for 72 h.

3.2. Extraction

The dried and pulverized leaves (2 kg) were extracted exhaustively with $CHCl_3$ in a Soxhlet apparatus. The solvent was evaporated under reduced pressure to obtain a dark green mass (39.5 g).

3.3. Fractionation by column chromatography

The chloroform fraction of *D. metel* was subfractionated by modified column chromatography using silica gel column. Silica gel (60–120 μ) was packed in a glass column (5 × 35 cm) and equilibrated with chloroform. The slurry of the chloroform fraction was loaded on to the top of the column and components eluted with 250 ml of chloroform at a flow rate of 1.0 ml/min followed by the elution with different ratios of chloroform : methanol ranging from 100 : 0 to 0 : 100. In total 78 sub-fractions of 10 ml each were collected and analyzed by TLC using the solvent systems chloroform : methanol : diethylamine (8.5 : 1.5 : 0.1) and chloroform : methanol : formic acid (8.0 : 2.0 : 0.1) to pool the sub-fractions of similar R_f values. The pooled sub-fractions, apparently purified compounds (APC), were dried *in vacuo* and analyzed for antifungal activity against pathogenic fungi.

3.4. Thin layer chromatography (TLC)

The chloroform fraction of *D. metel* was chromatographed on TLC plates using the solvent system chloroform : methanol : formic acid (8.0 : 2.0 : 0.1). The chromatograms visualized under UV light at 254 nm revealed 9-10 major bands of compounds. The plates were also sprayed with various group specific reagents. It was observed that lanes sprayed with Dragendorff's reagent developed 10 major bands of alkaloidal compounds.

The pooling resulted in 15 sub-fractions (SF-1 to SF-15). The results of antifungal activity assays of the sub-fractions showed that SF-11 had activity against *A. fumigatus*. The TLC of SF-11 indicated the presence of Dragendorff's reagent reactive three major alkaloidal bands. The R_f values of three compounds in the solvent system chloroform : methanol : formic acid (8.0 : 2.0 : 0.1) were found to be 0.14, 0.22 and 0.37. The fractionation of SF-11 by the solvent system chloroform : methanol : diethylamine (8.5 : 1.5 : 0.1) on TLC also showed three alkaloidal compounds having R_f values 0.24, 0.42 and 0.49. Preparative TLC of SF-11 was performed and three apparently purified compounds (APC-I, APC-II and APC-III) were

obtained. Of these three compounds, APC-II having a R_f value of 0.42 was found to have antifungal activity against *A. fumigatus*.

3.5. High performance liquid chromatography (HPLC)

The APC having antifungal activity was subjected to HPLC for further purification of the active molecule. The mobile phase consisted of acetonitrile and water in a ratio of 70 : 30. The sample of APC was dissolved in the mobile phase and loaded to the column through the injector using a loading syringe. The components of APC were eluted with acetonitrile : water (70 : 30) at a flow rate of 1.0 ml/min to collect various HPLC peaks. The HPLC chromatogram was obtained by printing the data using CR-7A chromatopac. The compounds recovered in HPLC pooled peaks were analyzed for antifungal activity by a microbroth dilution assay to obtain the pure active compound. The antifungal activity of pure compound **1** was confirmed by disc diffusion and spore germination inhibition assays and results were expressed as MIC (Rajesh and Sharma 2002).

The HPLC profile of APC-II showed two peaks, peak-I and peak-II, having retention times 1.91 and 2.71, respectively. The pure compound **1** recovered in peak-I at retention time 1.86–1.91 in various runs showed purity from 97–99.2% and was endowed with antifungal activity. The compound was further analyzed by TLC for purity and its chemical nature using two solvent systems (i) chloroform : methanol : diethylamine (8.5 : 1.5 : 0.1) and (ii) chloroform : methanol : formic acid (8.0 : 2.0 : 0.1). It gave only a single band in both the solvent systems. The R_f values of **1** with two solvent systems were found to be 0.22 and 0.42, respectively. The compound reacted positively with Dragendorff's reagent indicating, the nature of the compound to be alkaloidal. The spectral characteristics of **1** were: UV λ_{max} (MeOH): 206, 300 nm (log ε 5.7, 1.3); IR υ_{max} 3434, 3024, 1725, 1524, 1426,1238, 938, 760 cm^{-1}; + ve FABMS m/z: 239.2 $[M]^+$ (C_{14} H_{25} O_2 N) (16.3), 212 (13.2), 174 (16.2), 122 (34.2), 102 (96.4), 58 (100) and 57 (19.2). ^{1}H NMR ($CDCl_3$): δ 4.15 (1 H, m, $w_{1/2}$ = 13.5 Hz, H-2′), 3.75 (1 H, m, $w_{1/2}$ = 11.5 Hz, H-2), 3.17 (2 H, dd, J = 7.44, 7.44 Hz, H_2-5a, H_2-5b), 2.31 (1 H, t, J = 6.48 Hz, H_2-2″a), 1.95 (1 H, m, H_2-2″b), 1.62 (6 H, brs, Me-6, Me-7), 1.42 (1 H, d, J = 6.96 Hz, H_2-2′a), 1.35 (1 H, d, J = 7.28 Hz, H_2-2′b), 1.25 (7 H, brs, C-3′Me, H_2-3″a, H_2-3″b, H_2-4″a, H_2-4″b), 0.87 (3 H, t, J = 5.96 Hz, Me-5″).

3.6. Identification of targets for compound 1 in pathogene

Experiments were carried out to identify the gene product(s) of *A. fumigatus* targeted by the active molecule obtained form *D. metel*. The pathogenic *A. fumigatus* was cultured in absence and presence of sublethal doses of the compound **1**. The secretory proteins of *A. fumigatus* were isolated and separated on SDS-PAGE for comparison of their profile to identify those affected by treatment with compound **1**.

3.7. A. fumigatus culture

A. fumigatus was cultured in asparagine broth, a synthetic medium, prepared by dissolving asparagine (7.0 g), ammonium chloride (7.0 g), KH_2PO_4 (1.31 g), ferric chloride (0.30 g), dextrose (10.00 g), sodium citrate (0.90 g) and glycerol (25.0 ml) in 1.0 l of double distilled water. The medium was dispensed into 250 ml flasks and sterilized at 10 psi for 15 min. The flasks were divided in to two sets, the test set and the control set, each consisting of two flasks. An amount equivalent to half MIC (43.75 μg/ml) of the compound **1** was added to the test set of the flasks. In the control set, only solvent was added. The flasks of both sets were inoculated with spores of *A. fumigatus* and incubated at 37 °C in a BOD incubator for 12 h.

3.8. Study of differentially expressed proteins in A. fumigatus

The culture filtrate constituents of untreated and **1** treated *A. fumigatus* containing 12 h secretory proteins were collected and dialyzed against distilled water. The bicinchoninic acid assay (Singh et al. 1992; Smith et al. 1985) was used to estimate the concentration of proteins in the samples. The secretory proteins obtained from the different cultures were resolved by using 12.5% SDS-PAGE (Laemmli 1970). Appropriate molecular wt markers were used during electrophoresis.

The protein bands were visualized by staining the gels with silver stain (Merril 1986). The profile of protein bands of the secretory proteins obtained from untreated and **1** treated *A. fumigatus* were compared to identify the gene products targeted by the compound.

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