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Isolation of Meliacins from Chisocheton Paniculatus Hiern, their Chemical Transformations to New Limonoids and Screening for Antifungal Activity

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ABSTRACT

Paniculatin (**1**) (6α-acetoxyazadirone) and Paniculatol (**2**) (dihydroxycompound of 6α-acetoxyazadirone) are two naturally occurring meliacins have been isolated from the fruits and wood of chisocheton paniculatus Hiern. Chemical transformations of paniculatin and paniculatol gave 2-aminothiazolo [4,5-d] [1,2,20,21,22,23-hexahydro] paniculatin (**4a**), 2-aminothiazolo [4,5-d] [6α,7α-dihydroxy-1,2,20,21,22,23-hexahydro] paniculatin (**4b**), Dialdehyde (**5**), 3-hydroxy paniculatol (**6**), Dialdehyde **7**, 2-methyloxazolo [4,5-d][1,2,20,21,22,23-hexahydro] paniculatin(**9a**), 2-methyloxazolo [4,5-d][6α,7α –dihydroxy-1,2,20,21,22,23-hexahydro] paniculatin(**9b**). Antifungal activity of the above compounds and their derivatives are reported here. The structures of the new compounds have been elucidated on the basis of elemental analysis and spectral data.

1. INTRODUCTION:

The plants belonging to meliaceae family, are abundantly growing in Assam. Specially, in this family, the neem tree (Azadirachta indica, A Juse, family-meliaceae) has attained world wide prominence in recent years.

It is an age old practice to use the various types of plants (leaves) especially of the Meliaceae family to destroy or to keep away the harmful insects from the crop fields in rural areas. No systematic studies have been undertaken to evaluate the active principles of these plants. The idea of this is also based on the fact that traditional knowledge still effectively used by the rural people. At present natural pesticide is becoming acceptable to all, because of the toxic effects of the commercial synthetic

pesticides. They cause tremendous damage to the environment as well as to human being due to its residual effect.

Limonoids [1,2] are the most distinctive secondary metabolites of the plant order Rutales. Recent works have established a wide range of biological activities for these compounds including insect antifeedant and growth regulating properties[14], a variety of medicinal effects in animals and humans and antifungal and antiviral activity. Over 300 limonoids have been isolated to date. In particular, they characterize members of the family Meliaceae where they are diverse and abundant. Recently limonoids[3] have attracted much attention because of their marked insect antifeedant and growth regulating activities of Azadirachtin and related highly oxidized c-seco limonoids from the tree seeds, Azadirachta indica A Juse and Melia azedarach Linn. Information on the biological activities of over seventy limonoids have been published over the decade. More recently limonoids have shown anticarcinogenic and antitumourogenic activities[1,4a-c].

During the investigation, a number of new limonoids (tetranortriterpenoids) have been isolated from the plant Chisocheton paniculatus (meliaceae family). There is very good scope for further investigation of limonoids occurring in the plants of Meliaceae family.

Chisocheton paniculatus [5] Hiern occurs as a tree in the forests of Assam, the fruit which is capsule, 1.5-3 inches across, globose with pyriform base. It has been reported that a number of species of this family exhibit physiological activity[6].

In our continuing studies [7,8] on the Meliaceae of the North-Eastern Region of India we have investigated the fruits and heart-wood of Chisocheton paniculatus, a member of the Meliaceae from this region. The plant material was collected from tropical evergreen forest of Nambor Reserve Forest of Golaghat district of Assam, India.

Limonoids have attracted much attention because of the marked insect antifeedant and growth regulating, antifungal activity of azadirachtin and related limonoids isolated from the neem tree and also of few limonoids isolated from the fruits of Chisocheton paniculatus Hiern[4,8-9.] We report here the isolation, identification of known paniculatin (**1**), and paniculatol (**2**), their chemical transformation to new limonoids and their antifungal activities.

Petroleum ether extract of powdered fruit yielded a complex mixture of several compounds. After careful Chromatographic separation one compound was isolated (TLC) and on crystallization (benzene: MeOH:: 9:1) gave pure crystalline white solid (plates) with a m.p. 191.9 ^{0} C. The compound gave a purple spot on t.l.c. plate when sprayed with vanillin / H_2SO_4 followed by heating at 100 ^{0}C. a positive Libermann-Burchard reaction and yellow coloration with tetranitromethane (TNM) indicated that the compound is an unsaturated triterpene. The pure compound was identified as Paniculatin (**1**) by comparison of their m.p., IR and ^{1}H-NMR data with the reported values[7], as well as by comparing with authentic samples.

Petroleum ether extract of powdered heart-wood of Chisocheton paniculatus yielded a complex mixture of gummy material. After careful Chromatographic separation one compound was isolated (TLC) and on crystallization (benzene: MeOH:: 9:1) gave pure crystalline white solid (plates) with a m.p. 181.1 ^{0} C. The

compound gave a purple spot on t.l.c. plate when sprayed with vanillin / H_2SO_4 followed by heating at 95 ^{0}C. A positive Libermann-Burchard reaction and yellow coloration with tetranitromethane (TNM) indicated that the compound is an unsaturated triterpene. The pure compound was identified as Paniculatol (**2**) by comparison of their m.p., IR and ^{1}H-NMR data with the reported values[7], as well as by comparing with authentic samples. In the ^{1}H-NMR spectrum of **1**, the two protons geminal to the acetoxy groups appearing at 5.42 and 5.47 in its diacetate compound, shifted and appeared as multiplet centered at 4.30.

Mild alkaline hydrolysis of **1** with methanolic KOH gave the corresponding diol (**2**), $C_{26}H_{34}O_4$, m.p. 181.1^{0}C. In the ^{1}H NMR spectrum of **2** the 2 protons geminal to the acetoxy groups appearing at 5.42 and 5.47 in **1** shifted and appeared as multiplet centered at 4.30.

On hydrogenation of Paniculatin **1** in presence of palladium / calcium carbonate gave the hydrogenated product **3a** and characterized as 1,2,20,21,22,23-hexahydropaniculatin, $C_{30}H_{44}O_6$, m.p. 212.7^{0}C. In ^{1}H NMR spectrum, the appearance of new peaks at 2.06, 2.61, 2.80, 4.14, 4.18 suggest the saturated carbon and disappearance of unsaturated carbon protons peak at 6.39, 6.62, 6.80 and 7.53.

And on mild alkaline hydrolysis of **3a** with methanolic KOH gave the corresponding diol **3b**, $C_{26}H_{40}O_4$, m.p. 95.2 ^{0}C and characterized as 6α,7α-dihydroxy-1,2,20,21,22,23-hexahydro paniculatin.

On treatment of **3a** with thiourea in presence of chlorine gas gave the heterocyclic compound **4a** (2-amino thiazolo [4,5-d] [1,2,20,21,22,23-hexahydro] paniculatin), $C_{31}H_{44}O_5N_2S$, m.p. 88.3^{0}C. In the ^{1}H NMR spectrum singlet is seen at 6.43 for $–NH_2$ proton.

Again, On treatment of **3b** with thiourea in presence of chlorine gas gave the heterocyclic compound **4b** (2-amino thiazolo [4,5-d] [6α,7α-dihydroxy-1,2,20,21,22,23-hexahydro] paniculatin), $C_{31}H_{44}O_5N_2S$, m.p. 88.3^{0}C. In the ^{1}H NMR spectrum singlet is seen at 6.43 for $–NH_2$ proton.

Treatment of diol **2** with aqueous sodium periodate ($NaIO_4$) at room temperature gave the corresponding dialdehyde **5**, $C_{26}H_{32}O_4$, m.p. 138.5^{0}C indicating that the two hydroxyl groups are vicinal.

On reduction of **2** with sodium borohydride gave compound **6**, $C_{26}H_{36}O_4$, m.p. 142.7^{0}C. In the IR spectrum broad hydroxyl peak is seen instead of α,β-unsaturated ketone.

Treatment of diol **3b** with aqueous sodium periodate ($NaIO_4$) at room temperature gave the corresponding dialdehyde **7**, $C_{26}H_{38}O_4$, m.p. 145.4^{0}C.

The hexahydro product **3a** on treatment with hydroxyl amine hydrochloride in presence of pyridine gave the keto oxime **8a**, The oxime when reacted [10,11] with acetyl chloride in presence of acetic anhydride with pyridine as solvent gave the new heterocyclic compound **9a**, characterized as 2-methyl oxazolo [4,5-d] [1,2,20,21,22,23-hexahydro] paniculatin, $C_{32}H_{47}NO_6$ (gummy). In the ^{1}H NMR spectra a characteristic peak at δ2.60 was observed and assignable to $–N=C-CH_3$ in a five membered rings.

In a similar reaction sequence the compound **3b** on treatment with hydroxyl amine hydrochloride in presence of pyridine gave the keto oxime **8b**, which on treatment

with acetyl chloride in presence of acetic anhydride with pyridine as solvent gave the new heterocyclic compound **9b**, characterized as 2-methyl oxazolo [4,5-d] [6α,7α-dihydroxy-1,2,20,21,22,23-hexahydro] paniculatin, $C_{28}H_{42}NO_4$ (gummy). In the ^1H NMR spectra the characteristic peak for –N=C-CH_3 was observed in the same position as in the case of compound **9a**.

2. Results and discussion:

Antifungal activities of the above compounds have been shown against two different fungi Drechslera oryzae and Rhizoctonia solani using methanol as solvent by Inhibition Zone Technique. **Table-1** shows the results of antifungal tests for different compounds. Experiment was observed for 10 days.

From the **table-1** it is seen that the activity of the compound (**1**) is due to the presence of the α,β-unsaturated carbonyl group and the furan ring and also due to the presence of two acetoxy group in small extent. It is confirmed by the fact that the compound (**3a**) which is the reduced product of (**1**) is some what less active. The compound (**2**) is found to be more active than the compound (**1**). Similarly the compound (**6**) is more active than (**2**). From this point of view we can say that the better activity of compounds (**6**) and (**2**) is claimed to be due to the participation of hydroxyl group in the compounds. In comparision with (**2**) the compound (**3b**) is some what less active. Thus we can say that the activity of the compound (**2**) is not only due to the presence of the two hydroxyl groups at 6,7 positions but also the presence of the α,β-unsaturated carbonyl and the furan ring. Out of all the compounds in the scheme, the most active compounds are (**4a**) and (**4b**). The additional activity of these compounds is due to the presence of the sulphur atom, which enhances the activity of the compound against the fungal pathogens. Other compounds viz. (**5**), (**7**), (**8a**), (**8b**), (**9a**) and (**9b**) have moderate activity against the fungal pathogens.

3. Experimental section

3.1. General Experimental Procedures:

Melting points were determined in open capillaries on a Mettler air heated (Model FP 62) apparatus and are uncorrected. Optical rotations were taken on a Optical activity polarimeter (Model AA1000). UV spectra were recorded in MeOH on a Hitachi 320 and Jasco UV-Vis 7800 spectrophotometers; IR, on a Perkin-Elmer system 2000 FT IR spectrometer (ν_{max} in cm^{-1}), and NMR spectra on a Varian EM-360 L NMR spectrometer using TMS as internal reference (chemical shifts in δ ppm). Mass spectra were recorded on LC-MS, Bruker (Model esquire 3000) mass spectrometer. Thin Layer Chromatography (TLC) and Preparative TLC were performed on Silica gel-G (E. Merck) and Column Chromatography were on Silica gel (BDH) of 60-120 mesh size.

3.2. Plant Material:

The fruits and heart-wood of Chisocheton paniculatus Hiern were collected from Nambor Reserve Forest of Golaghat, Assam, India during May 2000. Identification

was made by Dr. S.C. Nath, Scientist, Regional Research Laboratory, Jorhat from the Herbarium species maintained in the Institute.

3.3. Extraction and Isolation:
Collected fruits of Chisocheton paniculatus were first shed dried and grind. 500 gm of powdered fruits were extracted with (60-80^{0}C) petroleum ether in a soxhlet apparatus. The extract on concentration under reduced pressure gave 15 gm of light yellow material. These materials were washed several times by petroleum ether followed by ethanol. After careful chromatographic separation one compound was isolated and on crystallization (benzene: MeOH:: 9:1) gave pure crystalline solid (plates) compound.

Paniculatin **1**, the reported limonoid was isolated from the oil obtained after trituration of the gummy material with pet. ether, as a pure compound (PTLC, TLC, Column Chromatography) and identified by comparing with an authentic sample (TLC. IR, m. m.p).

Paniculatin 1: Crystalline solid (MeOH); m.p. 191.9^{0}C (lit[7],m.p. 186-188^{0}C) $[\alpha]^{25}_D$ 118^0 (c=0.5) (lit7 $[\alpha]^{25}_D$ 119^0(c=0.5); UV λ_{max}220 nm (ε 10,000); IR ν_{max} 1750, 1675, 1505 and 875 cm^{-1}; EIMS (70 eV) m/z [M$^+$] 494, calcd. For $C_{30}H_{38}O_6$.

3.4. Extraction and Isolation of Paniculatol:
Collected heartwood of Chisocheton paniculatus were first shed dried and grind. 500 gm of powdered heartwood material were extracted with (60-80^{0}C) petroleum ether in a soxhlet apparatus. The extract on concentration under reduced pressure gave 12.5 gm of light yellow material. These materials were washed several times by petroleum ether followed by ethanol. After careful chromatographic separation one compound was isolated and crystallization (benzene: MeOH:: 9:1) gave pure crystalline white solid (plates) compound.

Paniculatol (**2**): Crystalline solid (MeOH); m.p. 181.1^{0}C (lit[3], m.p. 180-82^{0}C); $[\alpha]^{25}_D$ +21.5 (C=0.67, $CHCl_3$) (lit[3] $[\alpha]^{25}_D$ +22.5 (0.67, $CHCl_3$); UV λ_{max}220 nm (ε 11,7000); IR ν_{max} 3400, 1675, 1500 and 875 cm^{-1}; EIMS (70 eV) m/z [M$^+$] 410, calcd. For $C_{26}H_{34}O_4$.

Preparation of compound 2:
Paniculatin (**1**) (1.01 mmol, 0.5gm) was left at room temperature for 12 hr. in methanolic KOH (5%, 25 ml) then it was refluxed for 2 hr. in a water bath. On usual work up gave a corresponding diol (**5**) which was crystallized from methanol, m.p. 181.1^{0}C.

Preparation of compound 3a:
6α-acetoxy azadirone (**1**) (paniculatin) (2.02 mmol,1gm,) in methanol (40 ml) was stirred with palladium/calcium carbonate (0.5 gm) under an atmosphere of hydrogen for 6 hr. in a PAR hydrogenation apparatus. The mixture was filtered, Removal of solvent under reduced pressure gave 1,2,20,21,22,23-hexahydro paniculatin (**3**), which was crystallized from methanol, m.p. 212.7^{0}C. The spectral data are given in the **Table-1**.

Preparation of compound 3b:
1,2,20,21,22,23-hexahydro paniculatin (**3a**), (1 mmol, 0.5gm) was left at room temperature for 12 hr. in methanolic KOH (5%, 25 ml) then it was refluxed for 2 hr. in a water bath. On usual work up gave a corresponding diol 6α,7α-dihydroxy-1,2,20,21,22,23-hexahydropaniculatin (**3b**), which was crystallized from methanol, m.p. 95.2 ^{0}C.

Preparation of compound 4a:
A mixture of (**3a**) (2 mol, 1gm) and thiourea (10 mmol, 0.15 gm) was taken in a two necked 250 ml r.b. flask fitted with a reflux condenser provided with a calcium chloride guard tube. Chlorine gas was passed through the solution. The reaction mixture was warmed slightly in an water bath. A sudden exothermic reaction occurs and all the thiourea passes into solution. The resulting solution was refluxed for 30 minutes and then cooled in an ice bath and then extracted with a suitable solvent. Recrystallized from 2N HCl solution. The spectral data are given in the **Table-1**.

Preparation of compound 4b:
A mixture of (**3b**) (0.5mmol, 0.25gm) and thiourea (10 mmol, 0.15 gm) was taken in a two necked 250 ml r.b. flask fitted with a reflux condenser provided with a calcium chloride guard tube. Chlorine gas was passed through the solution. The reaction mixture was warmed slightly in an water bath. A sudden exothermic reaction occurs and all the thiourea passes into solution. The resulting solution was refluxed for 30 minutes and then cooled in an ice bath and then extracted with a suitable solvent. Recrystallized from 2N HCl solution. The spectral data are given in the **Table-1**.

Preparation of compound 5:
The dihydroxy compound **2** (0.2 mmol, 0.085 gm) was taken in 30 ml ethanol and treated with aqueous sodium periodate ($NaIO_4$) (57mmoll, 12 ml) for 3 hr at room temperature. During this period the reaction was completed (TLC). This was then diluted with water. It was then extracted with ether (3 x 30ml) and dried. Removal of the solvent gave crude product as gum (70 mg). The crude product on preparative thin layer chromatography on silica gel G plate (20 x 20 cm, 0.5 mm) using 5 % ethyl acetate in benzene, yielded pure dialdehyde as crystalline solid (50 mg). m.p. 138.5^{0}C.

Preparation of compound 6:
Dihydroxy compound of **1** (**2**) (250 mmol, 102.5 gm,) was dissolved in 50 ml dried methanol. Solution was cooled in ice bath. Now excess amount of Sodium Borohydride was added gradually under stirring condition until effervescences was ceased.

After completion of the reaction (TLC), solvent was evaporated completely and worked up by usual procedure. The product (white crystal) was recrystallized in alcohol (m.p. 157.3^{0}C), Yield 98%.

Preparation of compound 7:
The dihydroxy compound (**3b**) (0.24 mmol, 0.1 gm) taken in 30 ml ethanol was

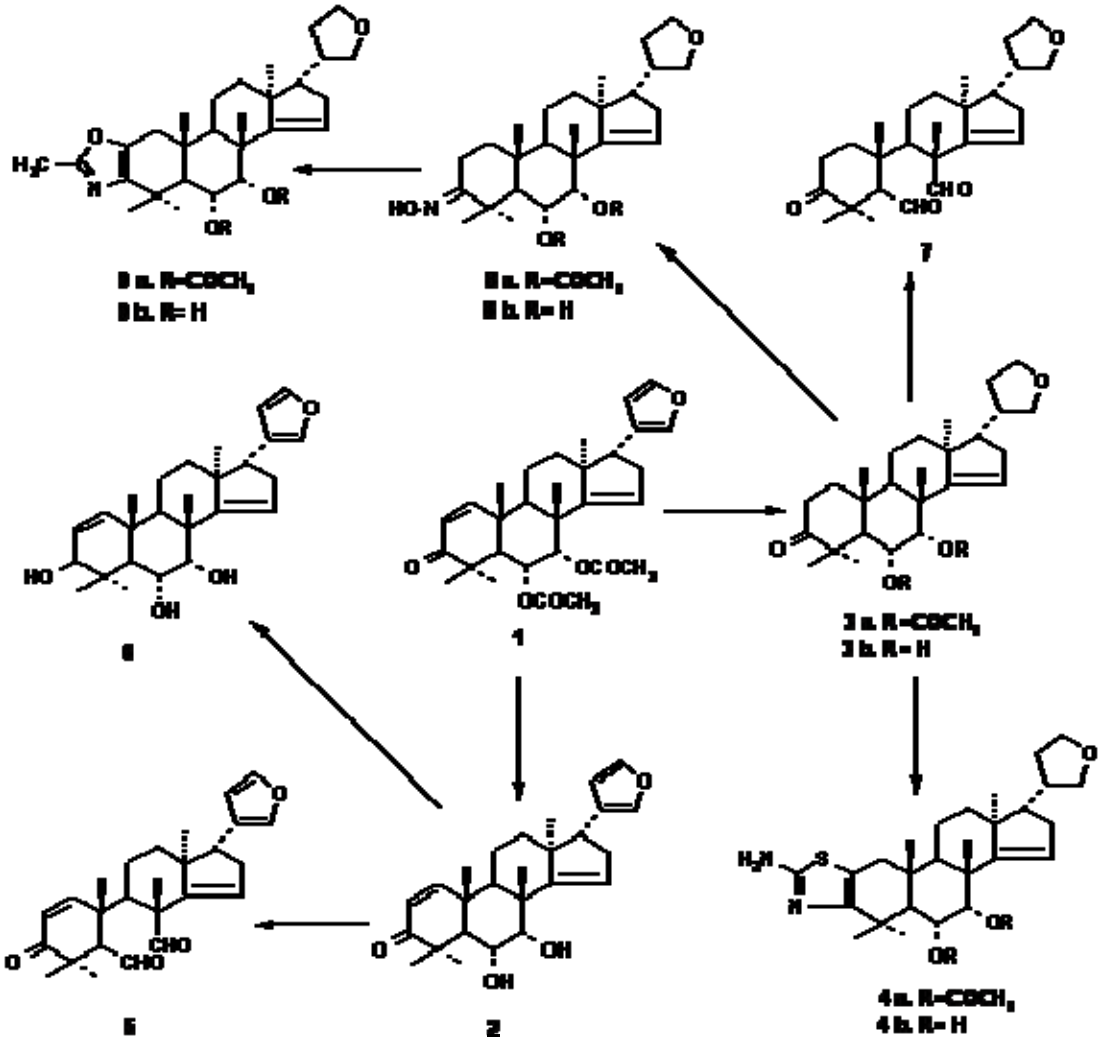
treated with aqueous sodium periodate ($NaIO_4$) (57 mmol, 12 ml) for 3 hr at room temperature. During this period the reaction was completed. This was then diluted with water. It was then extracted with ether (3 x 30 ml) and dried. Removal of the solvent gave crude product as gum (82 mg). The crude product on preparative thin layer chromatography on silica gel G plate (20 x 20 cm, 0.5 mm) using 5 % ethyl acetate in benzene, yielded pure dialdehyde as crystalline solid (60 mg),m.p. 145.4^{0}C.

Preparation of oxime 8a:
1,2,20,21,22,23-Hexahydro paniculatin (**3a**) (1.6 mmol, 0.8 gm), hydroxylamine hydrochloride (0.5 gm, 7.1 mmol), 5 ml of ethanol and 0.5 ml of pyridine were refluxed on a boiling water bath for 15-60 minutes. The ethanol was removed by distillation. 5 ml of water was added to the cooled residue, and cooled in an ice bath and stirred until the oxime crystallizes. Filtered off the solid, washed it with a little water and dried. Recrystallized from ethanol, m.p. 116.8 ^{0}C.

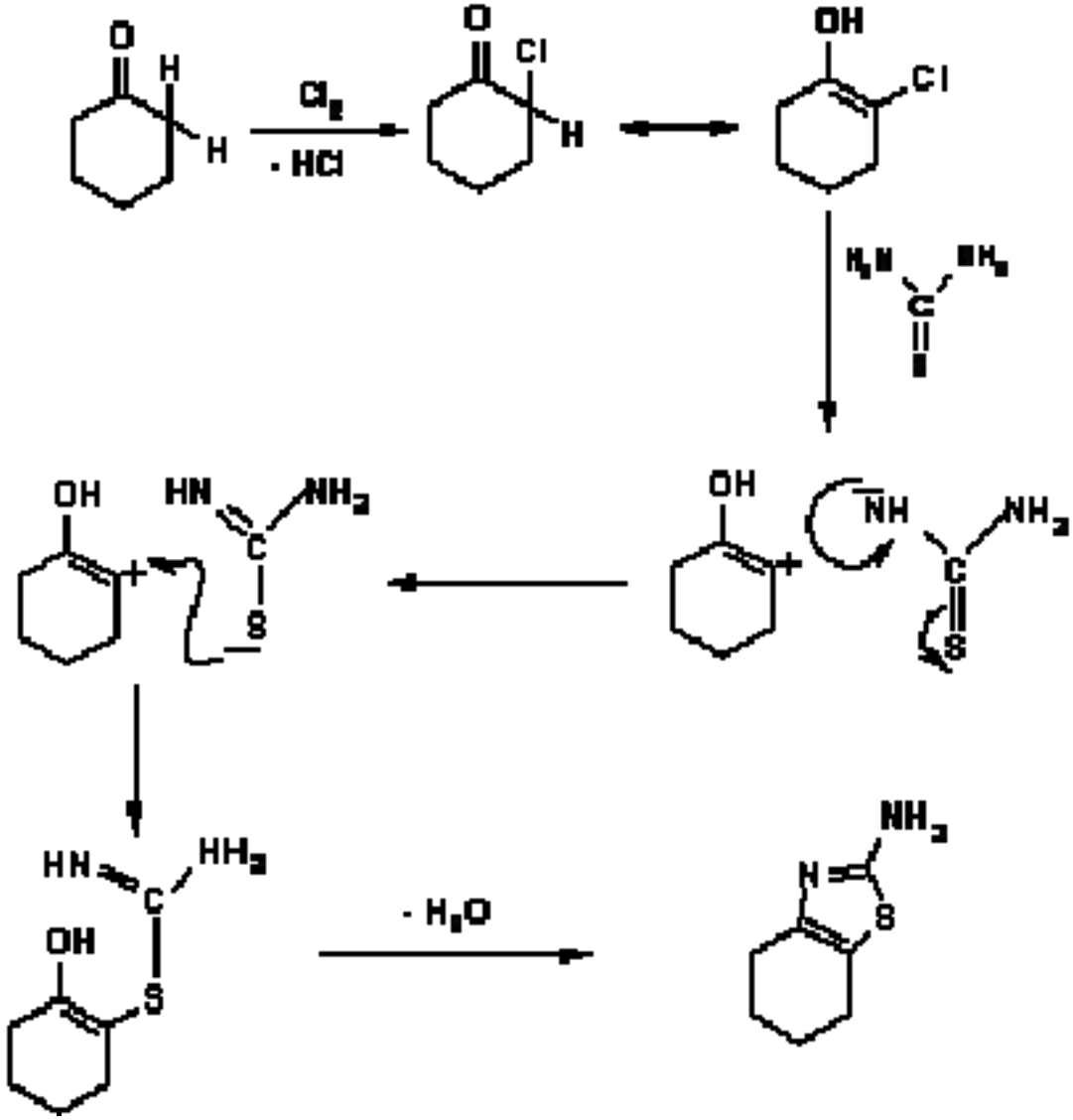
Preparation of compound 8b:
The compound (**3b**) (0.48 mmol, 0.24 gm), 0.2 gm (2.9 mmol) of hydroxylamine hydrochloride, 5ml of ethanol and 0.2 ml of pyridine were refluxed on a boiling water bath for 15-60 minutes. The ethanol was removed by distillation. 5 ml of water was added to the cooled residue, and cooled in an ice bath and stirred until the oxime crystallizes. Filtered off the solid, washed it with a little water and dried. Recrystallized from ethanol, m.p. 101.3 ^{0}C.

Preparation of compound 9a:
Oxime (**8a**) (1.17 mmol, 0.6 gm) was dissolved in pyridine (1.17 mmol,0.1 gm) and acetic anhydride (0.5 ml). To this solution, a mixture of acetyl chloride (4 mmol,0.31 gm) and acetic anhydride (0.5 ml) was added slowly, with stirring at 0^{0}C. The mixture was then heated on a boiling water bath for 10 hr. cooled, poured into crushed ice (20 gm) and extracted with dichloromethane (6x30 ml). The organic extract was washed with water (100 ml) and saturated NaCl solution (60 ml), dried with anhydrous sodium sulphate and concentrated by distillation. The m.p. could not be obtained, since the compound was gummy.

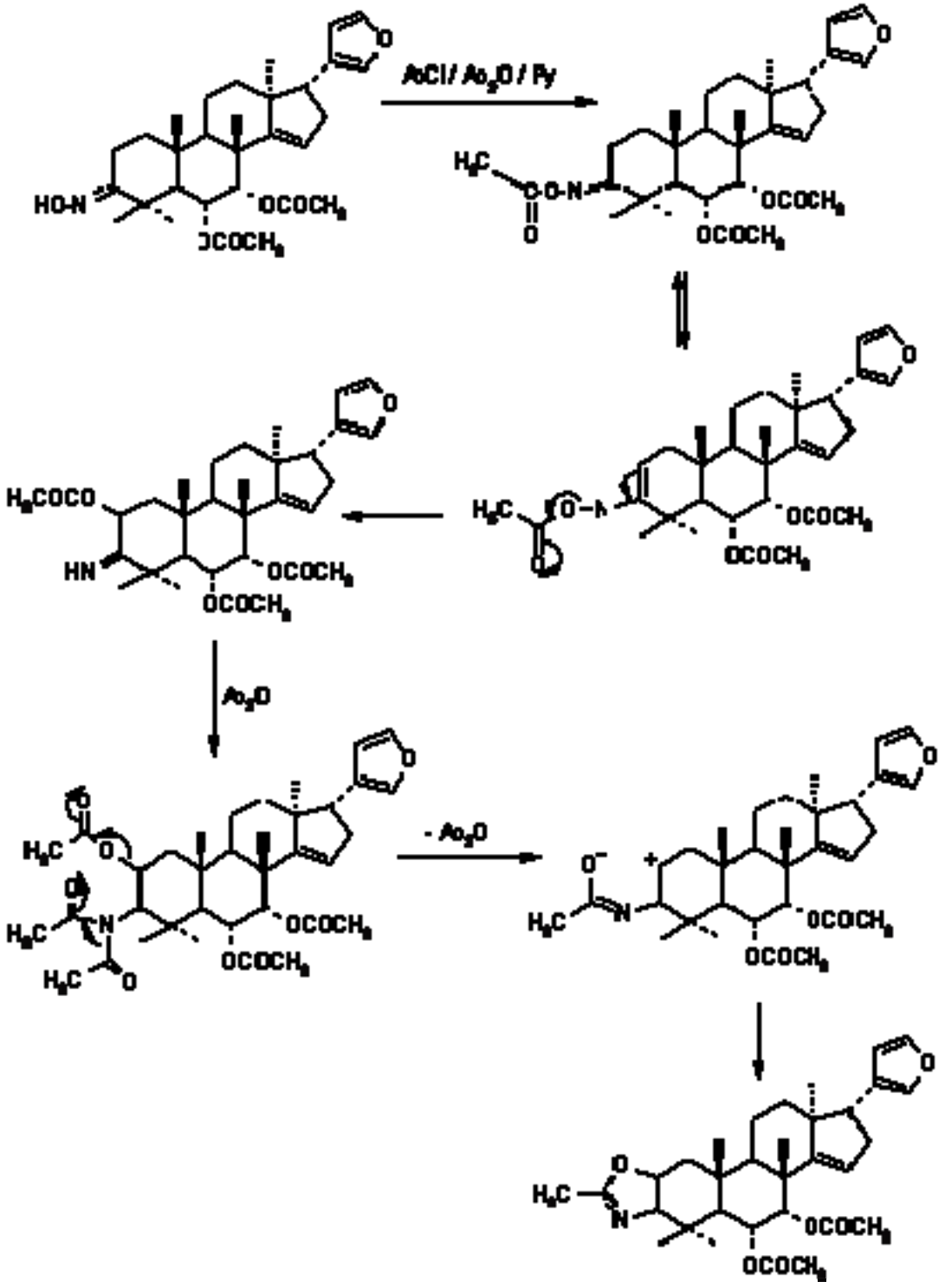
Preparation of compound 9b:
Oxime (**8b**) (0.23 mmol, 0.1 gm) was dissolved in pyridine (0.23mmol, 0.02 gm) and acetic anhydride (0.5 ml). To this solution, a mixture of acetyl chloride (5 mmol, 0.4 gm) and acetic anhydride (1 ml) was added slowly, with stirring at 0^{0}C. The mixture was then heated on a boiling water bath for 10 hr. cooled, poured into crushed ice (20 gm) and extracted with dichloromethane (6x30 ml). The organic extract was washed with water (100 ml) and saturated NaCl solution (60 ml), dried with anhydrous sodium sulphate and concentrated by distillation. The m.p. could not be obtained, since the compound was gummy.



Reaction Scheme



Proposed mechanism[12] for the formation of compound 4:



Proposed mechanism[12, 13] for the formation of compound 9:

Table-1 Antifungal Activities (Inhibition Zone Technique) (Inhibition in %)
F_1= Rhizoctonia solani, F_2= Drechslera oryzae, Host of the fungi = Rice

Sl. No.	No of Days	Sample No.	Concentration (100%)	
			F_1	**F_2**
i	10	1	73.75	75.90
ii	10	2	84.31	85.00
iii	10	3a	60.21	62.54
iv	10	3b	66.10	68.21
v	10	4a	72.31	76.35
vi	10	4b	94.23	96.23
vii	10	5	65.70	68.88
viii	10	6	94.21	94.82
ix	10	7	54.21	55.13
x	10	8a	65.28	66.42
xi	10	8b	67.43	69.32
xii	10	9a	67.42	69.43
xiii	10	9b	73.24	74.64

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