Vidyodaya J. of Sci., (2000) Vol. 9, pp 111-127

Synthesis and attempted ^{2}H labeling of 2,4-positions of 1, 3, 8-Trihydroxynapthalene, an intermediate of fungal melanin biosynthesis

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Received on :
Accepted on : 01-01-01

Abstract

1, 3, 8 - Trihydroxynaphthalene (T3HN), an intermediate of fungal melanin biosynthesis was successfully synthesized using 3,5-dimethoxybanzoic acid as the starting material. 2-and 4- positions of T3HN was attempted to label with deuterium as it is expected to be important to study the stereochemistry of the reduction of 1,3- dihydroxy ring of T3HN. The study showed that similar to H-2 and H-4 protons of 1, 3, 6, 8 - tetrahydroxynapthalene (another intermediate of fungal melanin biosynthesis) those of T3HN also rapidly exchange with the protons in the medium and T3HN does exist as an equilibrium mixture or tautomeric forms in pH 7 buffered aqueous solution. Hence stereochemical study of reduction of T3HN using 2-and 4-deuterated T3HN was impossible.

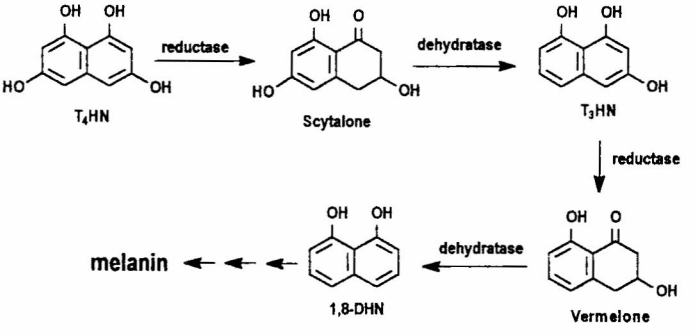
Key words : ***Melanin biosynthesis, 1, 3, 8 - Trihydroxynaphthalene, Reduction, Stereochemistry, Deuterium label.***

1. Introduction

Melanin, derived from 1,8 - dihdroxynaphthalene (DHN) and related pentaketide metabolites have been isolated from the cell walls of number of imperfect and ascomycetes fungi[1,2]. These include human pathogens such as *Wanigella dermatitidis*, and plant pathogens such as *Verticillim dahlae, Phialaphora largerberigii, Pyricularia oryzae, Colletotrichum lindemuthianum,* and *Colletiotrichum, lagenarium*[3,4,5,6]. Melanized cells of some of these fungi are directly involved in pathogenesis. For example, studies with inhibitors of melanin synthesis and melanin defficient strains have shown that melanin formed in the fungal appressoria provide the necessary strength and rigidity to the pathogen for the penetration into host

plant tissue. Tricyclazole, fthalide, pyriquilon and carpropamide are the currently used, melanin inhibitors. At non-fungitoxic concentration these inhibitors prevent rice blast disease caused by *P. oryzae* by preventing DHN melanin synthesis in cell walls.[7,8,9,10].

The biosynthetic pathway to DHN melanin (scheme. 1) is well established, but important question remain is about the stereochemistry of the reduction and elimination process, involved. As described in fig. 1 reduction (s) of 1,3,6,8, tetrahydroxynaphthalene (T4HN) to scytalone and 1,3,8-trihydroxynaphthalens (T3HN) to vermelone are the reductive steps involved in the pathway, and the same reductase is able to accomplish both reductions [11,12]. During the reduction(s) of T3HN to vermelone and T4HN to scytalone hydrogen at the 3-position of vermelone and scytalone is provided by NADPH. The absolute configuration of this center has been established as R. Two additional hydrogens at the 2-and 4-positions of vermelone and scytalone are presumably derived from the medium. The stereochemistry of the addition of these protons are not known.



Scheme 1. Pentaketide pathway of 1,8-Dihydroxynaphthalene (DHN) melanin biosynthesis

Labeling studies employing NMR detection of hydrogen isotopes would appear to be utility in this because of the insight, they would offer into the biological and chemical mechanisms. The most widely used isotope is deuterium. It is a stable isotope, readily available at high level of enrichment The chemical shift values in ^2H NMR are almost similar to those equivalent protons. Accordingy 2,4-deuterated T3HN or T4HN is expected to be important to study the stereochemistry of reduction of 1,3-dihydroxy rings. However, all the ring protons of T4HN had been reported to undergo rapid exchange with the protons in the medium whereas those of T3HN are not [13,14]. Bases on these observations, we decided to synthesize 2,4- deuterated T3HN for the above stereochemica/study. Present paper describes a succesful route to synthesize substrate T3HN (scheme 2) and methodology to incorporate the deuterium label into 2- and 4- positions of T3HN and the fate of the incorporated label due to the existence of keto-enol tautomers of T3HN.

Experimental

^{1}H and ^{13}C NMR spectra were recorded on a JEOL GX 270 spectrometer. In the case of ^{2}H NMR, spectra were recorded on a JEOL GX 400 instrument. Samples which contained water or deuterium oxide were referenced to sodium 2,2-dimetthy 1-2 silapentane-5-sulphonate (DSS) whereas all the others were referenced to tetramethy1 silane. Preparative thin-layer chromatography was carried out on 20x20 cm glass plates coated with silica gel (0.5 or 0.75 cm thickness, Merck, Art. 7747, Kiesei gel PF_{254}). The bands were visualized by the use of ultra-violet (254 nm). Flash colum chromatography was performed using silica gel (Fluka, 60378 Kiesel gel 60 220 - 440 mesh). All the solvents were dried and distilled prior to use according to the standard procedures. Nitrogen was dried by passage through a silica-gel-calcium-chloride column.

Synthesis of 1,3,8-trihydroxynaphthalene

Metyl 3,5-dimethoxybenzoate

3,5-Dimethoxybenzoic acid (**1**, 10g) was dissolved in HCi saturated methanol (100m) and the reaction mixture stirred for 48 hours. Methanol was distilled off and the remaining aqueous solution was extracted with ethyl acetate (3x50ml). The organic layer was washed thoroughly with water, then with sodium bicarbonate solution followed by water and brine. The organic layer was dried over $MgSO_4$ and the solvent was evaporated to yield crude methy1 3,5-dimethoxybenzoate (**2**), 9.8g (98%). Recrystallisation from diethyl ether yieded white needle shaped crystals m.p. 40-43°C (lit. m.p. 42-44°C)[15]; $\delta^{1}H$ 3.81 (6H, s, Ar-OC**H**$_3$), 3.90 (3H, s, CO-OC**H**$_3$), 6.63 (IH, t, *J* 2.4 Hz, 4-**H**), 7.17 (2H, d, *J* 2.4 Hz, 2-**H**); $\delta^{13}C$ 52. 13 (CO_2-**C**H$_3$), 55.41 (2-O**C**H$_3$), 105.62 (**C**-4), 106.98 (**C**-6 and **C**-2), 131.88 (**C**-1), 160.53 (**C**-5 and **C**-3), 166.74 (CO_2-CH$_3$); m/e 196 (100%, M^+), 165 (59.4% , M^+-OCH^3), 138 (29.4%), 122 (231.2%), 107 (10%), 63 (10%)

3,5 - Dimethoxybenzylalcohol

Methyl 3,5-dimethoxybenzoate (**2**, 9.92g) in dry THF (30ml) was added dropwise to a stirring suspension of lithium aluminium hydide (1.35g, 0.66eq), in dry THF (50ml). The flask was fitted to a reflux condenser and the system was flushed with nitrogen over the whole period. Once the addition was complete, the reaction mixture was stirred overnight and then heated to reflux for 2 hours. After cooling, aqueous methanol (10% 330 ml) was added cautiously to the reaction mixture, followed by water (30ml) to destroy the excess lithium aluminium hydride complex. The mixture was

filterd off and the filtrate was evaporated to remove THF. The resultant aqueous solution was extracted with ethyl acetate (3x50ml), washed with birne, and dried over $MgSO_4$. Evaporation of the solvent yielded 3,5-dimethoxybenzoyl alcohol (**3**) 7.5g (89%) as a white solid. The Product was further purified by recrystallisation from diethyl ether: petroleum ether 2:1 (v/v). m.p. 47-51°C (Lit. m.p. 48-45°C) [15];δ ^1H 3.25 (1H, s, **OH**) 33.75 (6H, s, OCH_3) 4.5 (2H, s, CH_2-OH) 6.35 (1H, s, Ar-**H**), 6.5 (2H, s, Ar-**H**); δ ^{13}C 55.25 (2-OCH_3), 64.73 (CH_2-OH), 99.43 (**C**-4), 101.57 (**C**-6 and **C**-2), 143.74 (**C**-1), 160.9 (**C**-5 and **C**-3); m/e 168 (100%M^+), 139 (32%), 109 (11%)

3,5- Dimethoxybenzyl bromide

Phosphorous tribromide (8.3g, 15eq) was added dropwise to a stirring solution of 3,5 dimethoxybenzl alcohol (3,7.5g) in dry diethyl ether (50ml). The reaction mixture was left to stirr for a period of 12 hrs in the dark. The solution was poured into ice water (50ml) and the ether layer separated. As the bromide is not very soluble in ether, the flask was extracted with ethyl acetate (3x50ml), washed with water, dried over $MgSO_4$ and concentrated to yield pure 3,5-dimethoxybenzoyl bromide (**4**) 9.7g (93%). Recrystallisation form diethyl ether gave white needle shaped crystals having m.p. 46-50°C (lit.m.p. 45-48°C)[15], δ ^1H 3.79 (6H, s, OCH_3) 4.42 (2H, s, CH_2Br), 6.39(1H, s, Ar-**H**), 6.53(2H, s, Ar-H); δ^{13}C 33.6 (CH_2Br), 55.36 (2-OCH_3), 100.54 (C-4) 106.91 (**C**-6 and C-2), 139.7 (**C**-1), 160.84 (**C**-5 and **C**-3); m/e 232 (19.4%, M^{+2}), 230 (19.6%M^+), 151(100%, M^+-Br)

3.5-Dimethoxybenzyl cyanide

Aqueous potassium cyanide solution (4g in 6 ml of water) was added to a methanolic solution of 3,5-dimethoxy benzyl bromide (4, 9.7g) in methanol (50ml) and the reaction mixture was heated to reflux for 4-5 hours. The cooled reaction mixture was evaporated on a warm water bath and the aqueous layer was extracted with ethyl acetate (3x70ml). The organic layer was washed with water repeatedly to remove all the unreacted potassium cyanide and dried over $MgSO_4$. The solvents were evaporated to give the pale yellow crude product. Recrystallisation from petroleum ether (30-40°C) gave pure pale yellow crystals of 3,5-dimethoxybenzyl cyanide (5) 7.2g (97%) m.p. 48-52°C) (Lit. m.p. 50-51°C) δ ^1H 3.68 (2H, S, CH_2CN) 3.79 (6H,s, OCH_3), 6.40(1H, S,Ar-H), 6.46 (2H, S, Ar-**H**);[13]δC 23.56 (CH_2CN), 55.26 (2-OCH_3), 99.7(C-4),105.88(**C**-6 and **C**-2)117.66 (CN), 131.86(C-1), 161.15(C-5 and C-3); m/e 177 (100%M^+), 146(10%M+OCH_3), 121 (11.9%), 77 (10.5%)

Ethyl 4-(3',5'- dimethoxyphenyl)- butan-3-onoate

Ethyl bromoacetate (9ml, 2eq) was slowly added dropwise over a period of an hour (most addition was during the last 30 min) to a refluxing and vigorously stirring mixture of 3.5-dimethoxybenzyl cyanide (**5**, 7.2g, leq) and activated zinc (7.9g, 3eq) in THF (40ml). After the addition was complete the reaction mixture was refluxed for another hour, then cooled. The reaction mixture was acidified to pH 3 using 3N HCl and sttirred for 30 min. Unreacted zinc was removed by filtrattion and all the volatile materials were evaporated. The aqueous solution was extracted with ethyl acetate (3x 100ml) and the organic layer was washed with water, sodium bicarbonate, followed by brine. Drying the organic layer over $MgSO_4$ and evaporation of the solvent yielded a crude product. It was re-dissolved in a minimum volume of diethyl ether and filtered through celite to remove pollymers formed during the reaction. Evaporation of ether yielded ethyl 4-(3'5' - dimethoxyphenyl) -butan-onoate (**6**) as a green yellow oil 8.9g (82%) which was almost pure. δ ^{1}H 1.27 (3H, t, *J* 7.14Hz, CH_2- C**H**$_3$), 3.45 (2H, s. Ar-C**H**$_2$-CO), 3.74 (2H, s, CO-C**H**$_2$-CO), 3.77 (6H,s, OC**H**$_3$) 4.16 (2H, q, *J* 7.14Hz) CH_3C**H**$_2$) 6.37 (3H, m, Ar-**H**); δ ^{13}C 14.03 (CH_2**C**H$_3$), 48.0 (Ar-**C**H$_2$CO), 50.19(CO-**C**H$_2$-$CO_2CH_2CH_3$), 55.21 (2-O**C**H$_3$), 61.31 (**C**H$_2$CH$_3$), 98.86 (**C** 4), 107.56 (**C**-6 and **C**-2), 135.37 (**C**-1), 160.83 (**C**-3 and **C**-5), 167.15 ($CO_2CH_2CH_3$), 200.46 (CO); m/e 266 (80%M$^+$), 178 (100%M$^+$-CH_3CO_2Et) 151 (27%)

4-(3',5,5' - Dimethoxyphenyl) 3-hydroxybutanoic acid

Ethyl 4(3',5' - dimethoxyphenyl)-butan-3-onoate (**6**, 4.5g) was dissolved in methanol (15ml) and cooled to 0°C. Sodium borohydride (2.3g) was added in small portions over a period of 5 min to the stirred methanolic solution. Once the addition was complete, stirring continued for another 1.5hrs at room temperature. The crude mixture was slowly added to a cooled (0°C) stirring solution of 4N NaOH (40ml). The basic reaction mixture stirred for 2 days at room temperature. Then the mixture was cooled (O^{0}C) and acidified cautiously using 3N HCl. All the methanol was evaporated and the aqueous solution was extracted with four portions of diethyl ether. The organic layer was washed with water, brine dried over $MgSO_4$, followed by evaporation of the solvent yielded 4-(3'-5'-dimethoxyphenl)3-hydroxybutanoic acid 2.6g (**7**, 64%) as a thick syrup. δ ^{1}H 2.54 (2H, AB of ABX, J_{AB} 13.47, J_{AX} 6.20, J_{BX} 7.26 Hz, Ar-C**H**$_2$-CHOH), 2.76 (2H, A'B' of A'B'X' J_{AB} 16.46, J_{AX} 8.33, J_{BX} 3.88 HZ, CHOH-C**H**$_2$- CO_2H), 3.74 (6H,s, 2-OC**H**$_3$), 4.26 (1H, m, H$_x$ of AB & A'B'), 6.36 (3H. m, Ar-**H**); δ ^{13}C 40.24 (Ar-

CH_2HOH), 43.03 ($CHOH-CH_2-CO_2H$), 55.25 (2-OCH_3) 68.42 (CH_2CHOH), 98.62 (Ar-**C**-4), 107.38 (Ar-**C-4** and **C-6**), 139.57 (Ar-**C**-1), 160.8 (Ar **C**-5 and **C**-3), 177.16 (CO_2H); m/e 240 (39.6%M^+), 152 (100%,M^+CH_2-OH-CH_2- CO_2H); found M, 240.99; $C_{12}H_{16}O_5$ requires M, 240.1)

1-Hydroxy-6,8-dimethoxynaphthalene

4-(3',5'-Dimethoxypheny1) 3-hydroxybutanoic acid (7, 2.6g) was stirred using a mechanical stirrer with polyphosphoric acid (18g) for 1 hr at 45-50°C. The reaction mixture was cooled to room temperature, then crushed ice (10 times of its weight) was added to the flask and stirring continued until all the ice-melted. The brown mixture was extracted into diethyl ether. The ether solution was washed with water, brine and dried over $MgSO_4$. Evaporation of the solvent gave a light brown gum which was practically pure. Further purification was achieved by flash chromatography (column diameter 48mm) eluting with 10% ethyl ether: petroleum ether v/v (R_f 0.30). Recrystallisation of the product with diethy1 ether: petroleum ether 30-40°C (3:7, v/v) gave bright yellow crystalline 1-hydroxy-6,8-dimethoxynaphthalene 1.4g (8,71%) m.p. 84-89°C (Lit. m.p. 86°C);δ^1H 3.8 (3H, s, 6-OCH_3), 4.0 (3H, s, 8-OCH_3) 6.45 (1H, d, *J*2.2Hz, Ar-**H**), 6.72 (2**H**, m, Ar-**H**), 7.19-7.29 (2H, AB quatret, *J* 7.87, Ar**H**), 9.12 (1H, s, **OH**); $\delta^{13}C$ 55.06 (6-OCH_3), 55.82 (8-OCH_3), 97.35 (**C**-7) 99.16 (**C**-2), 108.24 (**C**-5), 110.59 (**C**-8a), 117.67 (**C**-4), 128.29 (**C**-3), 137.26 (**C**-4a), 154.46 (**C**-1), 156.97 (**C**-8), 157.42 (**C**-6); m/e 204 (100%M^+), 161 (48.7%, M^+-CH_3C=0)

1,3,8-Trihydroxynaphthalene

1,hydroxy-6,8-dimethoxynaphthalene (8.14g) was dissolved under nitrogen in a mixture of glacial acetic acid and hydrobromic acid (11.0ml) and then refluxed under nitrogen for 3 hours. After cooling the reaction mixture, the solvent was evaporated and the residue was extracted with water, brine and dried over $MgSO_4$. The product was purified twice by preparative t. l.c. eluting with chloroform: acetone:formic acid (89:10:1). Pure 1, 3,8-trihydoxynaphthalene (9, 650mg, 69%, R_f 0.45) was obtained as pale green brown solid.

δ ^1H in $(CD_3)_2CO$,6.44 (1H, d,*J* 2.4**H**-2), 6.55 (1H, dd, *J*7.2, 1.3, **H**-7), 6,65 (1H, d *J* 2.4, **H**-4), 7.05 (1H, dd, *J*8.4, 1.3, **H**-5), 7.12 (1H, dd, *J*8.4, 7.2, **H**-6); $\delta^{13}C$ in $(CD_3)_2CO$, 101.7 (C-2), 102.3 (C-7), 106.67 (C-4), 110.73 (C-8a), 118.80 (C-5), 127.85 (**C**-6), 139.12 (**C**-4a), 154.94 (**C**-8), 156.04 (**C**-3), 157.06 (**C**-1);m/e 176 (99.1%M^+) 134 (100%, M^+-CH=C=O), 106 (20.6%), 77 (16.9%)

Incorporation of deuterium label into 2 -and 4 - positions of T3HN

T3HN (150mg) was dissolved in a mixture of deuterated trifluoroacetic acid (5ml) and deuterium oxide (2.00ml) and the resultant solution was stirred under nitrogen. After two hours all the acids were evaporated under high vacuum. The residue was dissolved in ethyl acetate and analysed by t.l.c. (Chloroform: acetone, 9:1, v/v, R_f 0.45). The pure product 120mg (80%) was analysed by ^1H and ^2H NMR spectroscopy and mass spectrometry.

Deuterium exchange of T3HN in the presence of deuterated trifluroacetic acid and deuterium oxide

T3HN (15mg) was dissolved in a mixture of deuterated trifluroacetic acid (0.2ml) and deuterium oxide (0.2ml). Then, the resultant solution was kept in a NMR tube under nitrogen and analysed by NMR spectroscopy at time zero and every 30 minutes upto 2 hours.

Incubation of T3HN with potassium phosphate buffer in deuterium oxide

T3HN (30mg), dissolved in acetone (0.6ml) was added to the potassium phosphate buffer (100mM, pH 7, 5ml) prepared in deuterium oxide and incubated overnight under nitrogen. Following the incubation period the assay mixture was acidified to pH 5 using 2M phosphoric acid, saturated with brine and the products were extracted into ethyl acetate (2x6ml). The isolation and purification of the product was performed by preparative t.l.c. eluting with chloroform:acetone (9:1, v/v, Rf. 045). The pure product 2.3mg (11.5%) was analysed by ^1H ^2H NMR and mass spectrometry.

Proton exchange of T3HN at pH 7

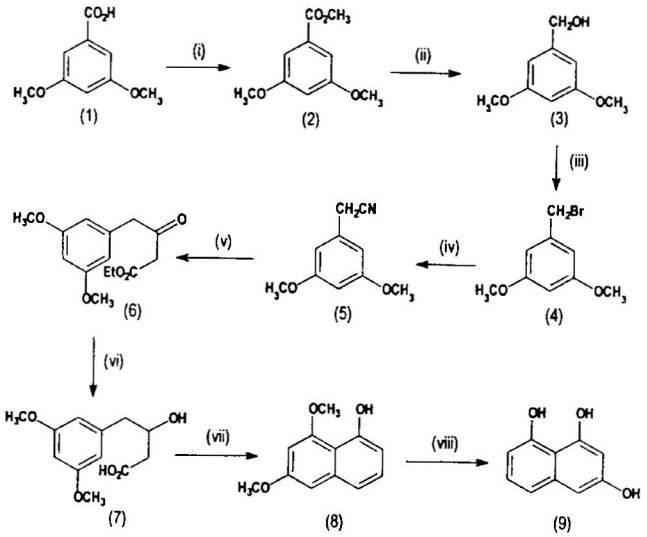
T3HN (9.0 mg) in 0.2ml of deuterated acetone and potassium phosphate buffer (100mM, pH 7, 0.2ml) was placed in a NMR tube under nitrogen and the sample was analysed ^1H, ^{13}C COSY NMR spectroscopy.

Another sample was prepared similarly with potassium phosphate buffer (100mM, pH 7) in deuterium oxide (0.2ml) and deuterated acetone (0.2ml) and monitored by ^1H NMR spectroscopy at specific time intervals (at time zero, every 30 minutes upto 5 hours, then at 12 hours, 24 hours and 48 hours)

Results and Discussion

Synthesis of 1,3,8 - trihydroxynaphthalene

1,3,8 - Ttrihydroxynaphthalene was synthesized according to the pathway shown in scheme 2. The synthesis was started with readily



Scheme 2. *Reagents and conditions*; i. MeOH/H+; ii.$LiALH_4$, dry THF, reflux 1hr, under N_2; iii. BBr_3; iv. KCN, reflux; v. Zn, $BrCH_2CO_2ET$, reflux, 1hr; vi. $NaBH_4$, NaOH/HCl; vii. Poly phosphorous acid, vigorous stirring, 45-50°C, under N_2; viii. HBr, glacial HAC, reflux, 3hrs

available 3,5-dimethoxybenzoic acid (**1**) and it was easily converted to the corresponding methyl ester by stirring with HCl saturated methanol for 48 hours. The ester was readily soluble in tetrahydrofuran and it was converted into alcohol in good yield. Bromination of the alcohol with phosphorous tribromide yielded the benzoyl bromide (**4**). The side chain was elongated by one carbon atom by treating the benzoyl bromide with potassium cyanide. Subsequent Reformatsky type reaction was performed by reacting the cyanide with ethyl bromoacetate in the presence of activated zinc to form the β keto ester (**6**). Ethyl bromoacetate tends to polymerise during the reaction. Therefore this reagent was added dropwise very slowly over a period of one hour. The yield of the reaction was 80%.

The Keto group of the ester was reduced to hydroxyl by $NaBH_4$/ MeOH and then ester was hydrolysed using 4N NaOH followed by acidifi-

cation to pH 5 with 3N HCl. The acid (**7**) was cyclised to I-hydroxy-6,8-dimethodynaphthalene using polyphosphoric acid. The produced naphthalene derivative (**8**) is very air sensitive, so the reaction was performed under an atmosphere of nitrogen. The purified 1-hydroxy-6,8 - dimethox ynaphthalene was demethylated by refluxing with glacial acetic acid and hydrobromic acid under nitrogen. Decomposition of the product, 1,3, 8 trihydroxynaphthalene (**9**) due to air was minimised by storing it under nitrogen at - 20°C.

Incorporation of Deuterium label into 2- and 4 positions of T3HN

T3HN was treated with deuterated trifluoroacetic acid and deuterium oxide with the aim of inserting deuterium label into 2- and 4- positions of T3HN ring. The experiment was carried out under nitrogen, avoiding any moisture contact. The isolated product was analysed by ^{1}H NMR and ^{2}H NMR and mass spectrometry. In the ^{1}H NMR spectrum of the deuterated T3HN three signals appeared as shown in fig.1b. In the ^{2}H NMR spectrum of the product two signals appeared at 6.59 and 7.10 ppm which corresponded to H-5 and H-7 respectively. The signals were broad and very intense. In addition to these, another small signal was seen at 6.69 ppm (Fig 1c). These results indicated a high level of incorporation of deuterium at the

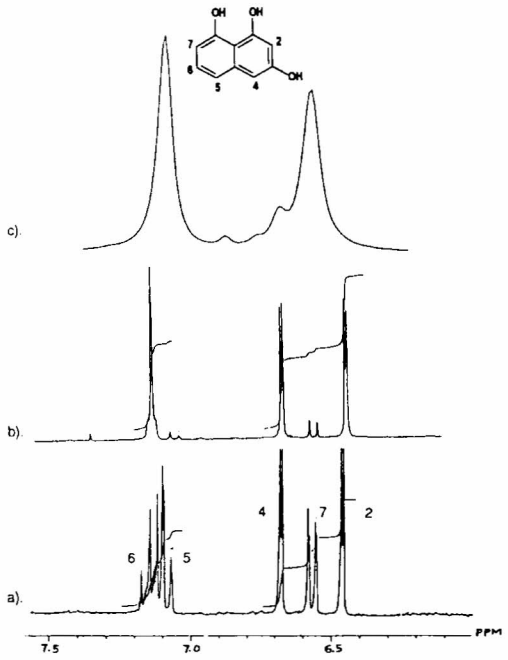


Fig 1

5-and the 7-positions and a small amount of incorporation at the 4-position. The base peak in mass spectrum was corresponded to M+1 (Fig 2b). Relative abundance and percentage of the each molecular ion was as follows (table 1).

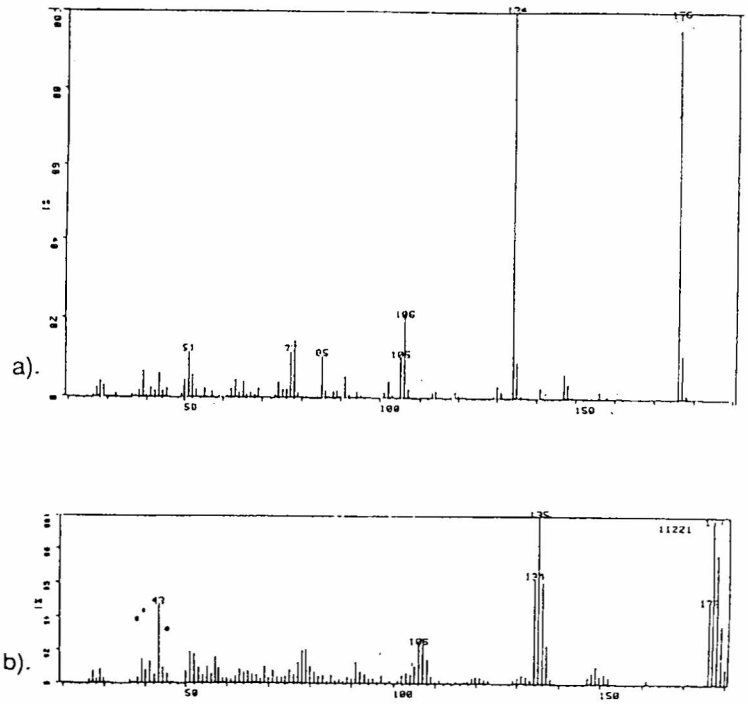


Fig2. Mass spectra of T3HN a). unlabelled b). Deuterium labeled

Table 1 . Relative percentage of each isotopic species in the molecular region of deuterated T3HN

Isotopic species	Relative abundance	Percentage (%)
M	49.3	19.06
M+1	95.9	37.46
M+2	77.5	29.9
M+3	36.2	13.9

Above data collectively suggests that the deuterium label has been incorporated into the 5- and 7- position of the ring rather than the expected 2- and 4- positions. The disappearance of the deuterium labeling at 2- and 4- positions of the isolated product could only be explained if extensive back exchange had occurred during the work-up of the reaction. Therefore, the proton exchange was monitored by NMR spectroscopy.

Monitoring the proton exchange process of T3HN in a NMR tube

T3HN was dissolved in a mixture of deuterated trifluroacetic acid and excess deuterium oxide and then the solution was kept in a NMR tube under nitrogen. The exchange was monitored by ^{1}H NMR spectroscopy at time zero and every 30 minutes upto two hours. Except, one signal all the others disappeared at time zero. The remaining signal was corresponded to H-6 and it did not disappear even after 2 hours (Fig. 3). This observation clarified that the deuterated trifluroacetic acid with excess deuterium oxide is capable of exchanging all the protons of T3HN except H-6. H-2 and H-4 are highly labile and possibly they back exchange during the work-up of the reaction.

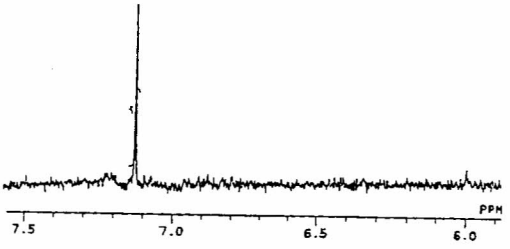


Fig 3 ^{1}H NMR spectrum of T3HN in the mixture of deuterated trifluoroacetic acid and deuterium oxide.

Investigating the stability of H-2 and H-4 of T3HN in aqueous buffered solution (pH 7).

Optimum pH for the reduction of T3HN to vermelone by enzyme reductase was found to be around pH 7. Therefore, we thought to monitor the stability of H-2 and H-4 protons in the pH 7 aqueous buffered solution. Accordingly, T3HN was dissolved in 0.2ml of acetone and incubated overnight in potassium phosphate buffer (pH 7, 100mM) prepared in deuterium oxide. The product was isolated, purified and analysed by ^{1}H NMR and ^{2}H NMR and mass spectrometry In the ^{1}H NMR spectrum of the product, the doublet of doublets at 6.55 ppm due to H-5 has been reduced with respect to the signal at 7.14 ppm. (Fig. 4a). The ^{2}H NMR spectrum of

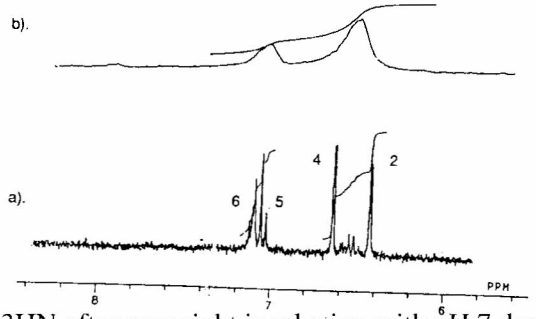


Fig 4 T3HN after overnight incubation with pH 7 deuterated potassium phosphate buffer a) ^{1}H HNMR spectrum in d_6-acetone b) ^{2}H NMR spectrum of in acetone

the product showed two broad signals at 6.58 and 7.10 ppm which corresponded to H-7 and H-5 respectively. The relative intensity of the latter signal was less than that of the former (Fig 4b). This observation clearly indicates the complete exchange of the H-7 and partial exchange of H-5. The mass spectrum of the same sample (Fig. 5) also provided the evidence for the incorporation. The relative abundance and percentage of the molecular ions are as follows (table 2).

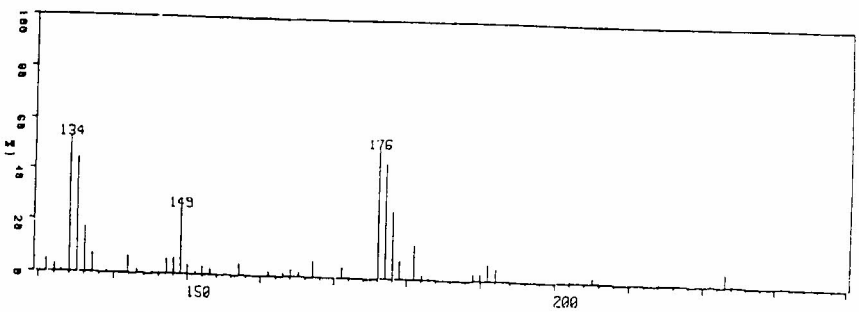


Fig 5. Mass spectrum T3HN after incubation with pH 7 deuterated potassium phosphate butter

Table 2. Relative percentage of each isotopic species in the molecular region of deuterated T3HN after incubation with pH 7 potassium phosphate buffer in deuterium oxide

Isotopic species	Relative abundance	Percentage (%)
M	50.9	39.9
M+1	44.1	34.1
M+2	26.0	20.0
M+3	7.4	5.7

Thus, it is reasonable to suggest that under these conditions complete exchange at H-7, a partial exchange at H-5 and no exchange at H-2 and H-4 of T3HN is taking place. The apparent lack of exchange at H-2 and H-4 is still a question. This might be due to the reverse exchange process, occurring during the work-up of the reaction.

To clarify this, the proton exchange of T3HN, under the above conditions was monitored by NMR. T3HN was dissolved in potassium phosphate buffer (pH 7) with the addition of an equal volume of acetone, to the aid of dissolution of T3HN and the resultant mixture was monitored by NMR.

^{1}H NMR spectrum of T3HN in aqueous buffered solution showed two set of signals which correspond to keto and phenol forms of T3HN in a ratio of 1:2 respectively (Table 3). The details are given in fig. 6.

Table 3 ^{1}H NMR spectum of T3HN in pH 7 potassium phoshate buffer and d_6 acetone (1:1, v/v)

δ [a](m,J[b])	Position	δ[b] (ppm)	Position
6.25 (s)	2	5.26	2'
6.4 (d, 7.15)	7	6.54	7'
6.51 (s)	4	3.56	4'
6.98 (d, 8.42)	5	6.65	5'
7.03 (dd, 7.15, 8.42)	6	7.14	6'

[a]ppm in d_6-acetone [b]Hz

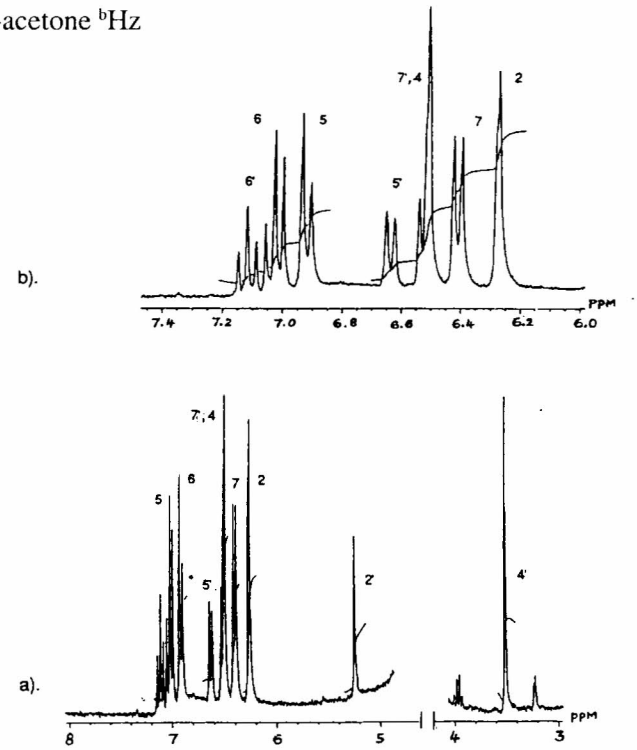


Fig 6 a)^{1}H NMR spectrum of T3HN in a mixture of pH 7 aqueous potassium phosphate buffer and d_6-acetono (1:1, v/v) and b). Expansion of the aromatic region of the same spectrum

^{13}C NMR spectrum of same sample showed twenty carbon signals, again these can be divided into two sets according to their intensities (table 4). The assignment of the protonated carbons in both tautomers were confimed by a ^{1}H-^{13} COSY experment of the same sample. The major species present could easily be ascribed to the excpected phenolic form of T3HN by comparison of its signal with those observed for T3HN in d_6-acetone. The second set contained only three signals assignable to aromatic hydrogens in addition to an olefinic (enolic) singlet (5.26 ppm) and an aliphatic methylene singlet (3.56 ppm). These signals provide clear evidence for the presence of a single keto-tautomer. Of the two possible tautomers (b) and (c) in fig. 7, the former is favoured due to the possibilty of hydrogen bonding to the 8-hydroxyl group. The diketo-tautomer (d) can be ruled out. The ^{13} C NMR spectra are consistent with the presence of the keto-tautomer, the enolic and ketonic carbons appearing at 101.0, 189.0 and 193.2 ppm respectively, and the methylene carbon at 39.4. ppm. The assignment of the protonated carbons in both tautomers was confirmed by a ^{1}H-^{13} C COSY experiment.

Table 4 ^{13}C NMR spectrum of T3HN at pH 7 in potassium phosphate buffer and d6 acetone (:1)

δa(ppm)	Position	δb(ppm)	Position
160.4	1	183.2	1'
101.2	2	101.0	2'
155.3	3	189.0	3'
102.6	4	39.4	4'
138.3	4a	104.2	4a'
116.6	5	117.9	5'
127.8	6	130.5	6'
104.7	7	106.0	7'
155.9	8	157.0	8'
110.7	8a	113.0	8a'

[a] Phenolic form [b] Keto form

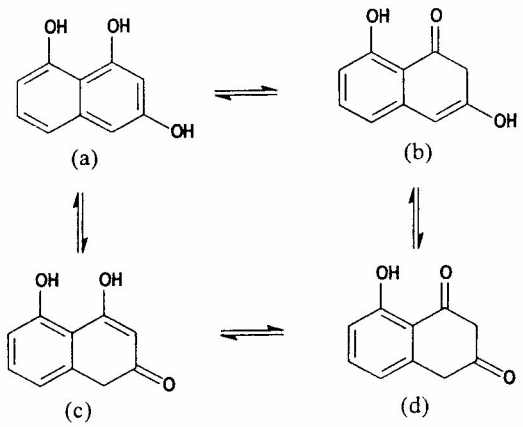


Fig. 7 Possible tautomers of T3HN

The rates of exchange of the ring protons of T3HN were examined by carrying out time course experiments in deuterated buffer. In this experiment T3HN (9.0mg) in d_6- acetone (0.2ml) and potassium phosphate buffer (100mM, pH 7, 0.2ml) prepared in deuterium oxide was placed in a NMR tube under nitrogen and the sample was analysed by NMR spectroscopy at specific time intervals, i.e. at time zero, every 30 min upto 5 hours, and then at 8, 12, 24 and 48 hours. Findings showed the half-life for exchange of 7-hydrogen and that of 5-hydrogen are about 3.5hrs and 48 hrs respectively. H-6 remain unexchanged.

In contrast to, *Vivani et al.* and *Sankawa et al.* our experiment clearly shows that similar to T4HN, T3HN also exist as an equilibrium mixture of tautomeric forms. H-2 and H-4 protons of T3HN are also very labile. All these findings collectively suggest that though the deuterium label can be inserted into 2- and 4- positions of T3HN, they rapidly back exchange possibly via-enol tautomerism.

Hence, investigation of the stereochemistry of reduction of T3HN using deuterium labeled T3HN was failed.

Conclusion

The study shows similar to T4HN, T3HN also does exist as a mixture of keto and enol forms in buffered aqueous solution. Hence, it is more likely similar to deoxygenations in other polyketide intermediates such as emodin (emodin-chrysapanol transformation) and versicolorin A[16,17] (in the biosynthesis of afalatoxin B1) reduction of 1,3-dihydroxy rings of T3HN and T4HN would also linked to the ket-enol tautomerism. However, further investigation are required to establish this farely conclusively.

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