JSc-EUSL(2001) **Vol.2 No.1**, p 51-60

ISSN 1391-586X; ©2001 Published by Eastern University, Sri Lanka

A NOVEL SYNTHETIC METHOD FOR FUNGAL MELANIN BIOSYNTHESIS INTERMEDIATE, 1,3,6,8-TETRAHYDROXYNAPTHALENE.

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(Received 24 January 2001; Accepted 17 January 2002)

Abstract

A novel route was designed to synthesize 1,3,6,8-Tetrahydroxynaphthalene (T4HN), an intermediate of fungal melanin biosynthesis, using 3,5-dimethoxybenzoic acid as the starting material. The method is efficient and more convenient than the previously published methods and overall yield of the final product was ~26%.

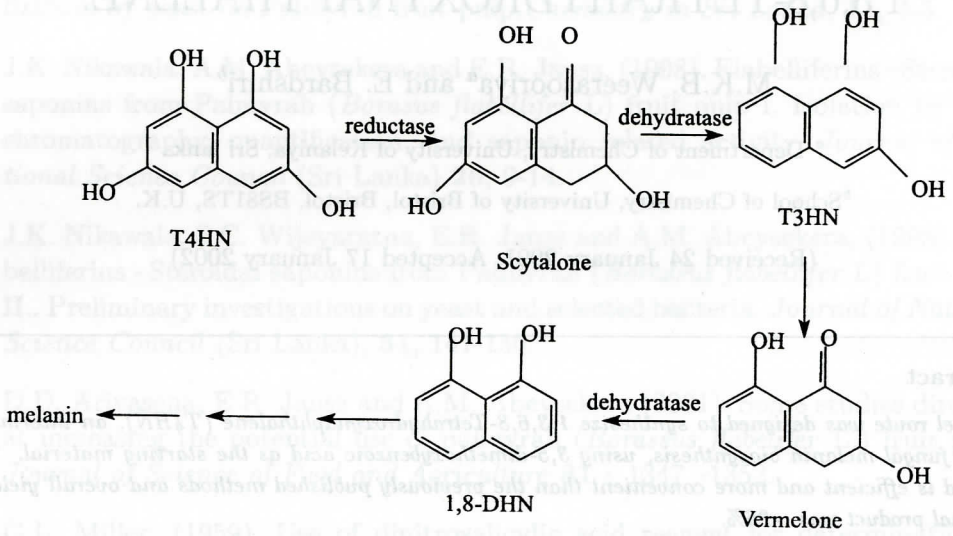
keywords :DHN melanin, 1,3,6,8-Tetrahydroxynaphthalene, synthesis

1 Introduction:

Melanin, derived from 1,8-dihydroxynaphthalene (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 largerbergii, Pyricularia oryzae, Colletotrichum lindemuthanum,* and *C.lindemutianum*[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 deffi-cient 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, phthalide, pyriquilon and carpropamide are the currently used melanin inhibitors and at non-fungitoxic concentration these inhibitors prevent rice blast disease caused by *P.oryzae* by preventing DHN melanin synthesis in cell walls[7][8][8][9][10].

The biosynthesis of DHN melanin is well documented and 1,3,6,8-Tetrahydroxynaphthalene. (T4HN), (+)-scytalone, 1,3,8-Trihydroxynaphthalene (T3HN), (-)-vermelone and 1,8-Dihydroxy naphthalene (1,8-DHN) have been found as the major intermediates of the biosynthetic pathway (Scheme 1).



Scheme 1. Pentaketide Pathway of 1,3-Dihydroxynaphthalene (DHN) melanin biosynthesis

The pentaketide intermediate, assembled from C_3 acetate units is cyclised to form symmetrical T4HN, presumably by a specific polyketide synthase. Reduction of T4HN to scytalone followed by dehydration of scytalone to T3HN is accomplished by reductase and dehydratase enzymes respectively. Subsequent reduction and dehydration steps give rise to vermelone and 1,8-Dihydroxy naphthalene (1,8-DHN) respectively[11][12][13][14][15]. 1,8-DHN is then polymerised to fungal melanin by air oxidation.

Detail analysis of above biochemical conversions requires the isolation and synthesis of intermediates such as T4HN, T3HN, scytalone and vermelone that can be used as substrates for the corresponding enzymatic conversion step. Scytalone has been isolated from number of fungi such as *Scytallidium sp*[16], *Phialphora largerbergii*[17] and *Verticillium dahliae*[18] whereas vermelone has been isolated from *Verticillium dahliae brm-1*[19]. However, no reports were found for the isolation of T4HN and T3HN from fungi probably due to their high instability. In an alternative way, Bell

et al. has shown that T3HN can be synthesized by dehydration of scytalone[18]. Previously, a method has also been designed for the synthesis of T4HN by Viviani and co-workers[20]. In their work, Diethyl 1,3-acetonedicarboxylate has been used as the starting material and the synthetic route has proceeded via Ethyl 2,4-di (ethoxycarbonyl) 3,5-dihydroxxyphenylacetate, 3,5-Dihydroxyphenyl acetic acid, Methyl 3,5-dimethoxyphenylacetate, Methyl 2-acetyl 3,5-dimethoxyphenylacetate and 6,8-Dihydroxy 1,3-dimethoxynaphthalene to 1,3,6,8-Tetrahydroxynaphthalene. In this synthetic route, acylation of 3,5-dihydroxyphenylacetic acid has been more difficult than expected. Further, the conversion of Methyl 3,5-dimethoxyphenylacetate to Methyl 2-acetyl 3,5-dimethoxyphenylacetate also has led to mixtures of starting material and mono- and di-acetylated esters. As the consequences of all these problems, the yield of the final product has been very poor. As an alternative to above route here, an efficient, convenient and reproducible novel method for the synthesis of T4HN with an overall yield of ~26% is described in this paper.

2 Experimental:

^1H and ^{13}C NMR spectra were recorded on a JEOL GX 270 spectrometer. In the case of ^2H NMR, spectra were recorded on a JEOL GX 400 instrument. Samples which contained water or deuterium oxide were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) whereas all the others were referenced to tetramethyl silane. Preparative thin-layer chromatography was carried out on 20×20 *cm* glass plates coated with silica gel (0.5 or 0.75 *cm* thickness, Merck, Art. 7747, Kiesel gel PF_{254}). The bands were visualized by the use of ultra-violet (254 *nm*). Flash column 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.

2.1 Synthesis of 1,3,6,8-tetrahydroxynaphthalene

2.1.1 *Methyl 3,5-dimethoxybenzoate*

3,5-Dimethoxybenzoic acid (**1**, 10 *g*) was dissolved in HCl saturated methanol (100 *ml*) and the reaction mixture stirred for 48 hours. Methanol was distilled off and the remaining aqueous solution was extracted with ethyl acetate (3×50 *ml*). The organic layer was washed thoroughly with water, then with sodium bicarbonate solution followed by water and brine. Drying the organic layer over $MgSO_4$ and evaporation of the solvent yielded crude methyl 3,5-dimethoxybenzoate (**2**),9.8 *g* (98%). Recrystallisation from diethyl ether yielded white needle shaped crystals m.p. 40-43°C (lit. m.p. 42-44°C) [21]. δ^1H 3.81 (6H, s, Ar-OC**H**$_3$), 3.90 (3H, s, CO-OC**H**$_3$), 6.63 (1H, t, *J* 2.4Hz, 4-**H**), 7.17 (2H, d, *J* 2.4Hz, 2-**H**); $\delta^{13}C$ 52.13

(CO_2-CH_3), 55.41 (2-OCH_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%)

2.1.2 *3,5-Dimethoxybenzylalcohol*

Methyl 3,5-dimethoxybenzoate (**2**, 9.92 *g*) in dry THF (30 *ml*) was added dropwise to a stirring suspension of lithium aluminium hydride (1.35 *g*, 0.66eq), in dry THF (50 *ml*). 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 a reflux for 2 *hours*. After cooling, aqueous methanol (10%, 30 *ml*) was added cautiously to the reaction mixture, followed by water (30 *ml*) to destroy the excess lithium aluminium hydride complex. The mixture was filtered off and the filtrate was evaporated to remove THF. The resultant aqueous solution was extracted with ethyl acetate (3 × 50 *ml*), washed with brine, and dried over $MgSO_4$. Evaporation of the solvent yielded 3,5-dimethoxybenzoyl alcohol (**3**) 7.5 *g* (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) [21] δ^1H 3.25 (1H, s, **OH**), 3.75 (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%)

2.1.3 *3,5-Dimethoxybenzyl bromide*

Phosphorous tribromide (8.3 *g*, 15eq) was added dropwise to a stirring solution of 3,5-dimethoxybenzyl alcohol (**3**, 7.5 *g*) in dry diethyl ether (50 *ml*). The reaction mixture was left to stir for a period of 12 *hours* in the dark. The solution was poured into ice water (50 *ml*) and the ether layer was separated. As the bromide is not very soluble in ether, the flask was extracted with ethyl acetate (3 × 50 *ml*), washed with water, dried over $MgSO_4$ and concentrated to yield pure 3,5-dimethoxybenzoyl bromide (**4**) 9.7 *g* (93%). Recrystallisation from diethyl ether gave white needle shaped crystals having m.p. 46-50°C (lit. m.p. 45-48 °C) [21],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)

2.1.4 *3.5-Dimethoxybenzyl cyanide*

Aqueous potassium cyanide solution (4 *g* in 6 *ml* of water) was added to a methanolic solution of 3,5-dimethoxy benzyl bromide (**4**, 9.7 *g*) in methanol (50 *ml*) and the reaction mixture was heated to reflux for 4-5hours. The cooled reaction mixture was

evaporated on a warm water bath and the aqueous layer was extracted with ethyl acetate (3×70 *ml*). 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**) 7.2 g (97%) m.p. 48-52°C (Lit. m.p. 50-51°C)[21] δ^1H 3.68 (2H, s, C**H**$_2$CN), 3.79 (6H, s, OC**H**$_3$), 6.4 (1H, s, Ar-**H**), 6.46 (2H, s, Ar-**H**); δ^{13}C 23.56 (**C**H$_2$CN), 55.26 (2-O**C**H$_3$), 99.7 (**C**-4), 105.88 (**C**-6 and **C**-2) 117.66 (**C**N), 131.86 (**C**-1), 161.15 (**C**-5 and **C**-3); m/e 177 (100%, M^+), 146 (10%, M^+ -OCH_3), 121 (11.9%), 108 (22.9%), 77 (10.5%)

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

Ethyl bromoacetate (9 *ml*, 2eq) was slowly added dropwise over a period of an hour (most addition was during the last 30 *minute*) to a refluxing and vigorously stirring mixture of 3,5-dimethoxybenzyl cyanide (**5**, 7.2 *g*, 1eq) and activated zinc (7.9 *g*, 3eq) in THF (40 *ml*). 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 stirred for 30 *minute*. Unreacted zinc was removed by filtration and all the volatile materials were evaporated. The aqueous solution was extracted with ethyl acetate (3×100 *ml*) and the organic layer was washed with water, sodium bicarbonate, water 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 polymers formed during the reaction. Evaporation of ether yielded ethyl 4-(3',5' -dimethoxyphenyl)-butan-onoate (**6**) as a green yellow oil 8.9 g (82%) which was almost pure. δ^1H 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 (**C**O$_2$CH$_2$CH$_3$), 200.46 (CO); m/e 266 (80%, M^+), 178 (100%, M^+ -CH_3CO_2Et), 151 (27%)

2.1.6 *4-(3 ',5 ' -Dimethoxyphenyl)-butan-3-onoic acid*

Ethyl 4-(3 ',5 ' -dimethoxyphenyl)-butan-3-onoate (**6** , 4.5 *g*) in methanol (5 *ml*) was slowly added to a cooled (0°C) stirring solution of NaOH (40 *ml* of 4N). The basic reaction mixture stirred for 2 days at room temperature. Then the mixture was cooled (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′ -dimethoxyphenyl)-butan-3-onoic acid (**7**) as a thick syrup 2.61 *g* (65%).. δ^1H 3.61 (2H, s, Ar-CH_2-CO), 3.79 (2H, s, CO-CH_2-CO), 3.85 (6H, s, OCH_3), 6.4-6.3 (3H, m, Ar-H); δ^{13}C 41.30 (Ar-CH_2CO), 55.34 (CO-CH_2CO), 55.59 (2-OCH_3), 99.40 (C-4), 107.34 (C-6 and C-2), 135.34 (C-1), 160.68 (C-3 and C-5), 177.47 (CO_2H), 200.46 (CH_2CO); m/e 194 (99%, M^+ -CO_2), 151 (100%, M^+ -$COCH_2CO_2H$), 43 (99%)

2.1.7 *1,3-Dihydroxy-6,8-dimethoxynaphthalene*

4-(3 ′,5 ′ -dimethoxyphenyl)-butan-3-onoic acid (**7**, 2.6 *g*) was stirred using a mechanical stirrer with polyphosphoric acid (19 *g*) for 1 *hour* at 45-50°C. The reaction mixture was cooled to room temperature and crushed ice (10 times of its weight) was added to the flask and continued stirring until all the ice melted. Once the hydrolysis was complete, the brown mixture was extracted into diethyl ether. Afterwards 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 performed by flash column (diameter 48 *mm*) eluting with 30% ethyl acetate in petroleum ether (v/v). R_f. value of the product was 0.38. Recrystallisation of the product from diethyl ether yielded 1,3-dihydroxy-6,8-dimethoxynaphthalene (**8**) as a needle shaped orange brown crystals. (1.7 *g*, 72%) m.p. 124-127°C (lit.m.p. 126-127°C)[21] δ^1H 3.8 (3H, s, 6-OCH_3), 3.92 (3H, s, 8-OCH_3), 6.27 (1H, d, *J* 2.13 Hz, H-7), 6.38 (1H, d, *J* 2.35 Hz, H-2), 6.47 (1H, d, *J* 2.13 Hz, H-5), 6.55 (1H, d, *J* 2.35 Hz, H-4); δ^{13}C 55.27 (6-OCH_3), 56.01 (8-OCH_3), 95.29 (C-7), 98.41 (C-2), 99.83 (C-5), 101.24 (C-4), 105.02 (C-8a), 138.57 (C-4a), 156.05 (C-1), 156.32 (C-3), 157.38 (C-8), 158.24 (C-6); m/e 220 (100%, M^+), 177 (46%, M^+ -CH_3CO)

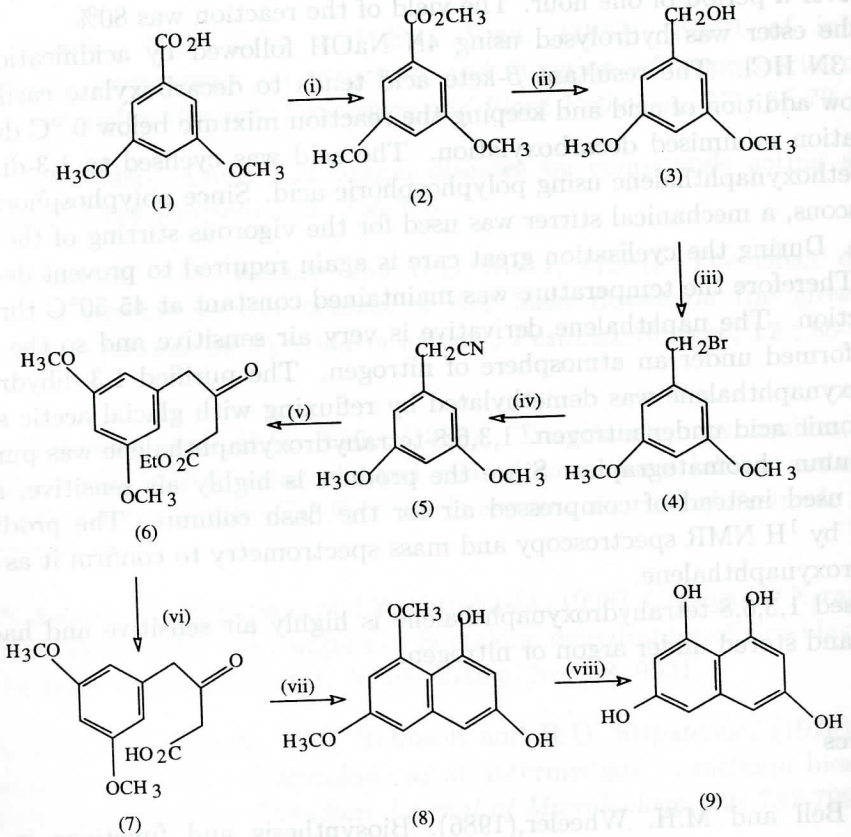
2.1.8 *1,3,6,8-Tetrahydroxynaphthalene*

1,3-dihydroxy-6,8-dimethoxynaphthalene (**8** , 1.7 *g*) was dissolved under nitrogen in a mixture of glacial acetic acid (37.5 *ml*) and hydrobromic acid (11.5 *ml*). The reaction mixture was refluxed under nitrogen for 3 hours. After cooling the reaction mixture, the solvent was evaporated and the residue was extracted into ethyl acetate. The organic layer was washed with water, brine and dried over $MgSO_4$ gave a brown solid shown to be practically pure by TLC. (acetone : hexane 60:40 v/v). Further purification was performed by flash column chromatography (diameter 35 *mm*) eluting with acetone:hexane- 60:40 (v/v). Nitrogen gas was used for the flash column instead of compressed air. R_f value of the product was 0.58. The pure compound 1,3,6,8-Tetrahydroxynaphthalene (**9**) which was a green-brown solid (1.43 *g*, 85%) was stored under nitrogen at -20°C. δ^1H in $(CD_3)_2CO$ 6.23 (2H, d, *J* 2.2 Hz, H-2 and H-7), 6.45 (2H, d, *J* 2.2 Hz, H-4 and H-5), 8.37 (broad, OH), 9.96 ((broad, OH); δ^{13}C in $(CD_3)_2CO$, 98.93 (C-7 and C-2), 100.93 (C-4 and C-5), 107.0

(C-8a), 140.18 (C-4a), 156.41 (C-3 and C-6), 157.45 (C-1 and C-8); m/e 192 (67.7%, M^+), 150 (100%, M^+ -CH_2CO), 43 (64.1%), 35 (61.5%)

3 Results and Discussion;

1,3,6,8-Tetrahydroxynaphthalene was synthesized according to the pathway shown in scheme 2.



Scheme 2. Reagent and conditions; i. $MeOH/H^+$; ii. $LiAlH_4$, dry THF, redflux 1 *hour*, under N_2; iii. PBr_3; iv. KCN, reflux; v. Zn, $BrCH_2CO_2Et$, reflux 1 *hour*; vi. NaOH/HCl; vii. PPA, Vigorous stirring, 1 *hour*, 45-50 ^0C, under N_2; viii. HBr, glacial HAC, reflux, 3hours.

The synthesis started with readily available 3,5-dimethoxybenzoic acid. The initial attempts to reduce the free acid to the benzoyl alcohol using lithium aluminium hydride resulted in a very poor yield. The poor solubility of the acid in tetrahydrofuran and the tendency of the acid to form a complex with lithium aluminium hydride were likely the reasons for the poor yield. To overcome this problem, the acid was con-

verted 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 in good yield into alcohol. Bromination of the alcohol with phosphorous tribromide yielded the benzoyl bromide. The side chain was elongated by one carbon atom by treating the benzoyl bromide with potassium cyanide. Subsequent Refromatsky type reaction was performed by reacting the cyanide with ethyl bromoacetate in the presence of activated zinc to form the β-keto ester. 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%.

Then the ester was hydrolysed using 4N NaOH followed by acidification to pH 5 with 3N HCl. The resultant β-keto acid tends to decarboxylate easily. However, slow addition of acid and keeping the reaction mixture below 0 °C during the acidification minimised decarboxylation. The acid was cyclised to 1,3-dihydroxy-6,8-dimethoxynaphthalene using polyphosphoric acid. Since polyphosphoric acid is very viscous, a mechanical stirrer was used for the vigorous stirring of the reaction mixture. During the cyclisation great care is again required to prevent decarboxylation. Therefore the temperature was maintained constant at 45-50°C throughout the reaction. The naphthalene derivative is very air sensitive and so the reaction was performed under an atmosphere of nitrogen. The purified 1,3-dihydroxy-6,8-dimethoxynaphthalene was demethylated by refluxing with glacial acetic acid and hydrobromic acid under nitrogen. 1,3,6,8-tetrahydroxynaphthalene was purified by flash column chromatography. Since the product is highly air sensitive, nitrogen gas was used instead of compressed air for the flash column. The product was analysed by ^{1}H NMR spectroscopy and mass spectrometry to confirm it as 1,3,6,8-tetrahydroxynaphthalene.

Synthesised 1,3,6,8-tetrahydroxynaphthalene is highly air sensitive and had to be handled and stored under argon or nitrogen.

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