

Synthesis, Characterisation and its Anti Bacterial Activity of Dalfampridine Genotoxic Impurity 1, 2-DI (PYRIDINE-4-YL) Hydrazine

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Abstract: *Objective:* Dalfampridine and its impurities are used as intermediates for synthesis of chemicals, pharmaceuticals and agrochemical industry, antineoplastic, anticoagulant, anti-inflammatory, antispasmodic and antiasthmatic drugs, fungicide and as additives for foodstuffs [1]. Moreover its derivatives mainly used to treat multiple sclerosis so [2], the drug availability should be within the limit as per ICH guidelines. In the present study was aimed to synthesize 1, 2-di (pyridine-4-yl) hydrazine and evaluate antibacterial activity by using agar cup method. *Methods:* 1, 2-di (pyridine-4-yl) hydrazine synthesized by number of steps which is N-Oxidation, Nitration, Azo coupling and Deoxygenation followed by Reduction. Synthesized molecule was characterized by LC-MS, HPLC, ¹H NMR and serially diluted test solution of three doses of 1, 2-di (pyridine-4-yl) hydrazine (50µg/ml, 75µg/ml and 100µg/ml) were added in the cups which is present in bacteria spreaded petridish. All the plates stood incubated for 24 hrs at 37^oC [3]. MIC (Minimum Inhibitory Concentration) was measured and compared with gentamycin as a positive control. *Results:* 1, 2-di (pyridine-4-yl) hydrazine molecule was synthesized and characterized as per methods. The synthesized 1, 2-di (pyridine-4-yl) hydrazine (100µg/ml) was found to be effective and it exhibited significant antibacterial activity against its direction of Escherichia-coli > Pseudomonas-aeruginosa > Bacillus-subtilis > Staphylococcus-aureus which activity is lesser than standard drug gentamycin. Whereas 100µg/ml dose showed higher anti bacterial activity with more zone of inhibition than 75µg/ml and 50µg/ml dose of synthesized molecule. *Conclusions:* The results proposed 100µg/ml of synthesized 1, 2-di (pyridine-4-yl) hydrazine showed significant antibacterial activity but less activity than standard drug gentamycin.

Keywords: Dalfampridine, Genotoxic impurities, Oxidation, Nitration, Azo coupling, Reduction, Deoxygenation, LC-MS, ¹H NMR, Melting point, Anti bacterial activity, Agar well cup method

1. Introduction

Several 4-aminopyridine derivatives are known to be important intermediates for the preparation of various herbicides, antibacterial, antiviral drugs and dyes [4]. For example, 4-aminopyridine, as fampridine, is used for the treatment of multiple sclerosis [5]. Its derivatives non-selectively block K⁺ channel, which is actively blocking a wide variety of potassium channels with different state dependences. Therefore, in search for clues for the structural determinants of K⁺ channels that are important for the state dependences of drug-channel interactions, 4-AP serves as a useful tool. Potassium channel blocker used to help multiple sclerosis patients walk.

The common adverse effects in patients administered dalfampridine include urinary tract infections, insomnia, dizziness, headache, nausea, weakness, back pain, ataxia and visual disturbances[6].

2. Materials and Methods

2.1 Materials

- All reactions were carried out in dry glassware under dry nitrogen atmospheric condition.
- Glass wares used for all the reactions which is borosilicate quality.
- Rotavapor-B.U.CHI is a device used to removal of solvents from samples by evaporation.
- The suitable mobile phases (solvent system for TLC) as applicable were developed using silica Gel-G ready

plates. Pre-coated aluminium silica plates were used with suitable mobile phase.

- Progress of Reaction and monitoring for each stage is performing by LC-MS.
- Mass of all synthesized compounds can be determined by using Thermo- DSQ-Trace and Advantage Max, Agilent 6130 LC/MS single quad mass spectrometer.
- Separation and purification of synthesized impurities carried out by using column chromatography and automated chromatography COMBI FLASH Rf device.
- The purity of all synthesized compounds identified by HPLC Agilent-1200 with PDA detector.
- The media used for biological evaluation was purchased from Hi Media and media were prepared according to manual.
- Biological evaluation was done under Laminar Air Flow.

2.2 Methods

Scheme to synthesis of 1, 2-di (pyridine-4-yl) hydrazine:

Step 1: Pyridine Oxidation:

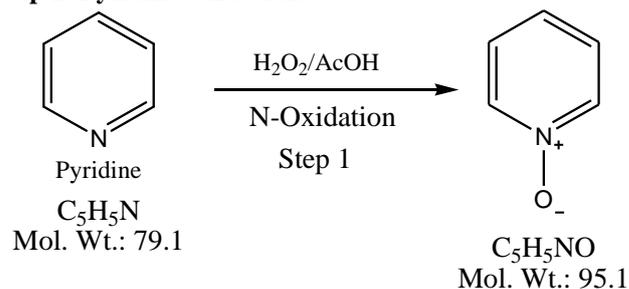


Figure 4: Step 1 Oxidation reaction of 1, 2-di (pyridine-4-yl) hydrazine

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Pyridine is oxidized into pyridine N oxide which on treatment with H₂O₂/AcOH. 10 grams of Pyridine was taken in the single neck RB flask, to this 30 ml of acetic acid was added and stirred for ten minutes. 50 ml of hydrogen peroxide was added and closed the flask with stopper. The reaction mixture was stirred over night.

Workup:

After completion of the reaction, excess acetic acid removed and peroxide by concentrating in the rotavapor and dried completely. Three times added a toluene and removed the solvent in rotavapor to expel the traces of water. Product Yield is 10 gm.

Step 2: Nitration of Pyridine N- Oxide:

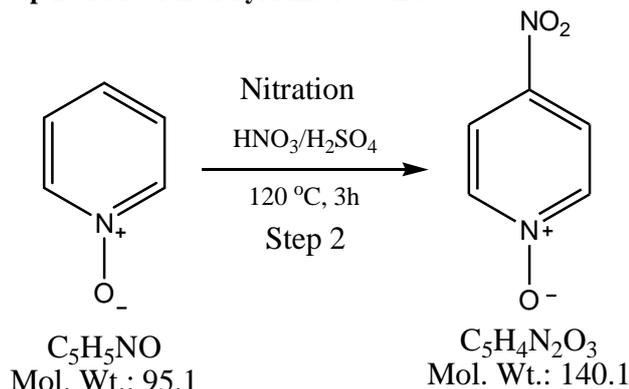


Figure 5: Step 2 Nitration reaction of 1, 2-di (pyridine-4-yl) hydrazine

10ml of fuming HNO₃ are mixed with 10ml of conc. H₂SO₄ are added under stirring and cooling in an ice bath. The nitrating mixture is brought to a temperature of 20°C. 10gms of pyridine-N-oxide are filled in the reaction flask and heated to 60°C. The nitrating mixture is transferred and added drop wise within 30 minutes. Thereby the internal temperature drops to about 40°C. Afterwards the reaction mixture is refluxed for 3 hours to 125-130°C internal temperature.

Work up:

After cooling down to room temperature the reaction mixture is poured in 150g finely crunched ice. Then about 170 mL of a saturated sodium bicarbonate solution are added carefully in portions until a pH- is neutral. A yellow crystalline solid precipitates formed, which contains product and sodium sulfate. Crude yield is 5.5 gm.

Step 3: Azo Coupling of 4-Nitro Pyridine N- Oxide [7]

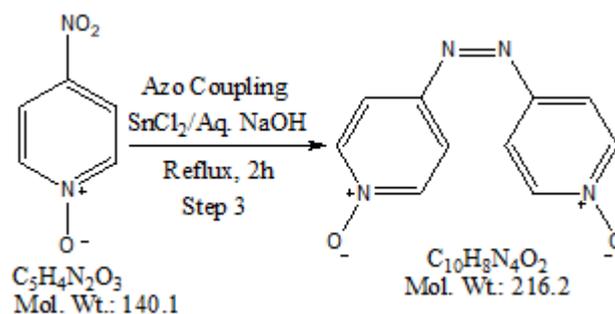


Figure 6: Step 3 Azo Coupling reaction of 1, 2-di (pyridine-4-yl) hydrazine

Three-neck round-bottomed flask, fitted with a stirrer and a reflux condenser, is placed on a steam cone. In the flask are placed 3.5 gm of 4-nitro pyridine -N- oxide is dissolved in 200 ml of 10% Aq NaOH. To the mixture is added 10 gm of SnCl₂, the stirrer is started, and the mixture is refluxed for 1 hr at 100°C. The initial addition of the reactants resulted in a cloudy yellow solution with a black deposit on the bottom of the flask (metallic tin). At the end of two hours, the solution was a vivid red with dark red oil on the surface.

Workup:

The reaction mass was concentrated by removing sodium hydroxide. The reaction mass quenched in 20 ml of water and extracted with 100 ml of DCM and concentrated. Product Yield is 2gm.

Step 4: Deoxygenation [8]:

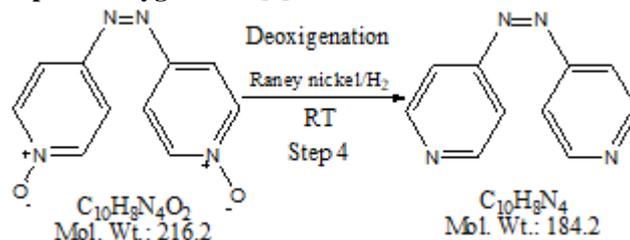


Figure 7: Step 4 Deoxygenation reaction of 1, 2-di (pyridine-4-yl) hydrazine

The catalytic reduction over Raney nickel at atmospheric temperature and pressure was suitable for this reaction. The catalytic reduction of 2gm of step 3 product in 100 ml of MeOH, with 1 mole equivalent of Raney nickel added. The reaction is carried under hydrogen pressure. The reaction is stopped after 3-4 hrs reduction. The reaction is monitored by TLC and LC-MS.

Workup:

The reaction mass is filtered off by using high vacuum remove Raney nickel. The filtrate is concentrated to remove methanol by using rotary evaporator. Product Yield is 1.5 gm.

Step 5: Reduction:

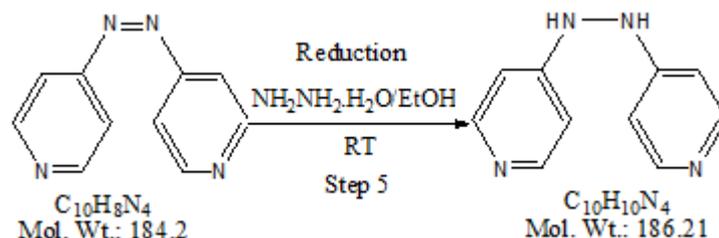


Figure 8: Step 5 Reduction reaction of 1, 2-di (pyridine-4-yl) hydrazine

1.5gms of step 4 product was dissolved in 30 ml of ethanol and 0.3ml of hydrazine hydrate was added. The reaction was carried out to color change from deep orange to brown color. The reaction mass was monitored by TLC and LC-MS.

Workup:

The reaction mass was concentrated by removing ethanol and unreacted hydrazine hydrate. The reaction mass quenched in 20 ml of water and extracted with 100 ml of DCM and concentrated. Product yield is 1gm.

Evaluation of Anti Bacterial Activity

Agar Diffusion Method

Following are the techniques which were used for agar diffusion method

- 1) Agarcup
- 2) Agarditch
- 3) Paperdisc

For our study, we used the first method, to assess the minimum inhibitory concentration (MIC).

Non-automated invitro bacterial tests. This method gives accurate, precise results to identify the antimicrobial agent which is required to inhibit the growth of precise microorganisms.

Identification of MIC (Minimal Inhibitory Concentration) by using agar cup method

- 1) Synthesized molecule are tested for antibacterial activity.
- 2) Necessary controls were used. Like,
 - Drug (Standard) control
 - Organism control
 - Gentamycin (Known antibacterial agent) as reference was used in the present study.
 - Muller Hinton Agar (MHA) (Hi Media) was used as nutrient medium for growth of microorganisms.
 - The cultures used for testing were obtained from NCL which were equivalent to ATCC cultures.
 - All microorganisms were inoculated in Tryptic Soya Bean Broth.
- 3) The dissolved drugs were serially diluted according to NCCLS guidelines.

Microbial strain:

Name of Microorganism Used	Type	Strain Used
A. Staphylococcus-aureus.	(Gram Positive)	ATCC 25923
B. Bacillus-subtilis	(Gram Positive)	ATCC 21332
C. Pseudomonas-aeruginosa.	(Gram Negative)	ATCC 27853
D. Escherichia-coli.	(Gram Negative)	ATCC 25922

- 4) Sterile molten MHA was poured aseptically under laminar air flow unit into sterile Petri plates containing the test microorganism and was allowed to solidify. After solidification of the media cups/wells were bored using 'T' borer.
- 5) The serially diluted antibacterial test solution(synthesized compounds) concentrations are

50µg/ml, 75µg/ml and 100 µg/ml were added in the cups and allowed to diffuse in the agar by placing the Petri plates under refrigeration for 10 minutes.

- 6) At 37⁰C all the plates stood incubated for 24 hours.

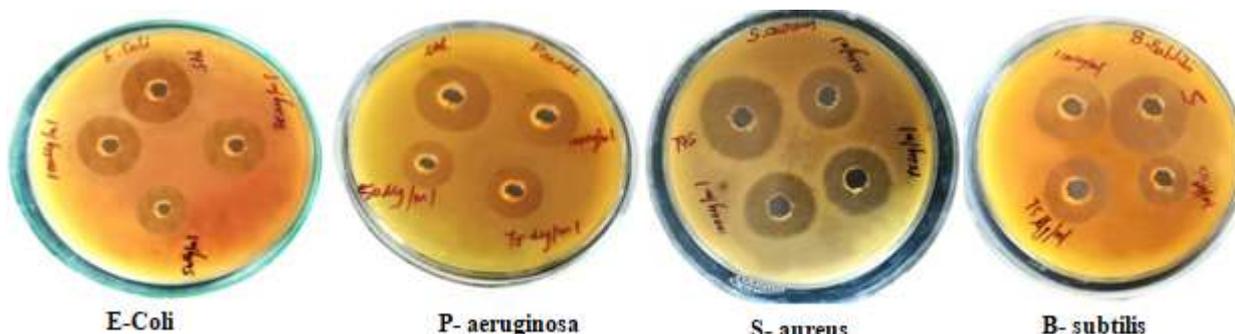


Figure: Photographs showing zone of inhibition of 1, 2-di (pyridine-4-yl) hydrazine

3. Results

Characterisation results

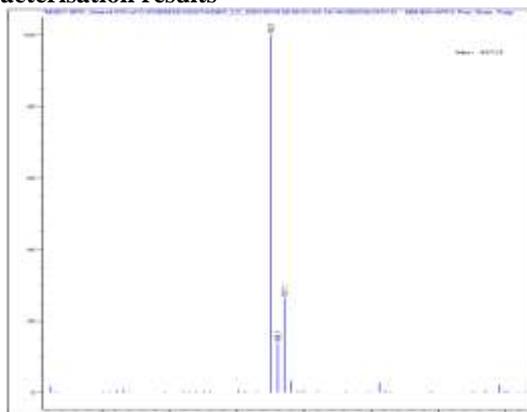


Figure: LC-MS Spectrum For 1, 2-di (pyridine-4-yl) hydrazine

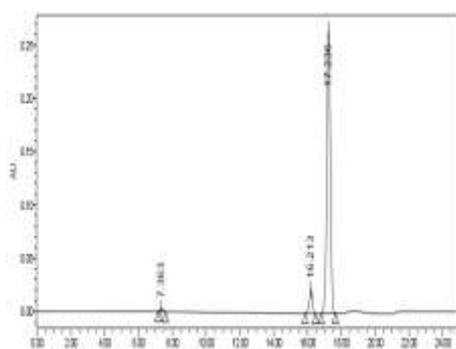


Figure: HPLC Spectrum For 1, 2-di (pyridine-4-yl) hydrazine

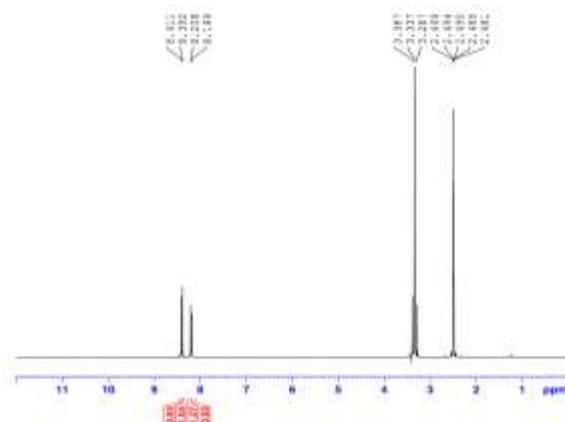


Figure: ¹H NMR Spectrum For 1, 2-di (pyridine-4-yl) hydrazine

Antibacterial Activity Results of 1, 2-di (pyridine-4-yl) hydrazine:

Table: Zone of Inhibition of 1, 2-di (pyridine-4-yl) hydrazine.

S. No.	Concentration (µg/ml)	Bacterial Culture Zone of Inhibition in mm			
		<i>E.coli</i>	<i>P.aeruginosa</i>	<i>S. aureus</i>	<i>B.subtilis</i>
1	Std (Gentamicin)	26	25	25	26
2	50	15	14	15	13
3	75	17	18	17	17
4	100	22	21	20	20

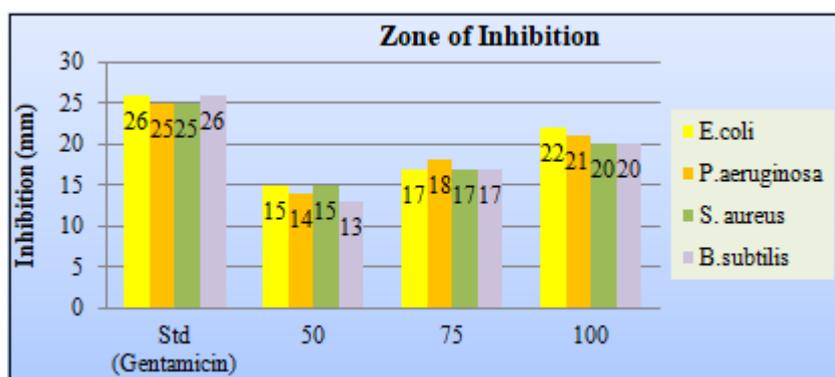


Figure: Zone of Inhibition of 1, 2-di (pyridine-4-yl) hydrazine

4. Discussion

During synthesis of 1, 2-di (pyridine-4-yl) hydrazine faced some difficulties. Such as;

N-oxidation: Pyridine N- oxidation was tried with m-CPBA but the result was not achieved since oxidation of nitrogen is very poor. Alternatively hydrogen peroxide was used in presence of acetic acid gives us oxidized product.

Azo formation: Chemistry involved in formation of diazo group by using nitro compounds is very new and coupling reagents were tried with titanium chloride in presence stannous chloride. And reaction was achieved by using stannous chloride in presence of aqueous base.

N- deoxygenatio6n: Selective N- deoxidation in presence of diazo group was very challenging since possibility of aza bond breakage observed by using palladium carbon metallic reagent in presence of hydrogen. Alternatively selection of

metallic reagents was done with repetitive trials with available literature source.

To achieve selective deoxygenation 1 equivalent of Raney nickel was used in presence of hydrogen 5 mbar pressure so controlled removal of two unit of oxygens in pyridine was achieved successfully.

5. Conclusions

The targeted molecule 1, 2-di (pyridine-4-yl) hydrazine was synthesized with good yield and high purity. To achieve high purity attention was drawn forwards automated combi flash and it was performed by developing different analytical methods for separation of synthesized genotoxic impurity. Characterized by LC-MS, HPLC, ¹H-NMR. 1, 2-di (pyridine-4-yl) hydrazine assessed for its in-vitro anti-bacterial activity compared to both gram-positive and gram-negative bacteria using the standard drug Gentamycin. We are concluded as molecule exhibits significant antibacterial activity against its direction of *Escherichia-coli* > *Pseudomonas-aeruginosa* > *Bacillus-subtilis* > *Staphylococcus-aureus*. 100 µg/ml of synthesized molecule showed more activity compared to other concentrations like 50µg/ml and 75µg/ml.

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