

Synthesis of Coumarin Derivatives and Assessment of their Anti Microbial Activity

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Abstract: A series of iodo and nitro coumarin derivatives have been synthesized and the structures of newly synthesized compounds were confirmed on the basis of IR and CO-IR data. The synthesized compounds were tested for their *in vitro* antimicrobial activity in a disc diffusion technique and streak plate isolation method against four strains of bacteria and three fungal strains. The tested compounds have shown different activity in terms of growth inhibition of microorganism.

Keywords: Iodo coumarin derivatives, Nitro coumarin derivatives, Disc diffusion technique, Streak plate isolation method, antibacterial activity, and antifungal activity

1. Introduction

Coumarin stands in special place in the realm of synthetic organic chemistry. Coumarin, the parent molecule of coumarin derivatives, is the simplest class of naturally occurring phenolic substances made of fused benzene and pyrone ring.

The coumarins are varied in structure, due to the various type of substitution in their basic structures, which can influence their biological activity. A careful structure – system-activity relationship study of coumarins with special respect to carcinogenicity, mutagenicity and cancer preventing activities should be conducted. Vast majority of coumarins completely innocuous may be beneficial in a variety of human disorders, in spite of some ongoing controversy. Coumarins are documented widely in literature and their preparation has received much attention due to variety of biological activities they possess. A direct efficient and operationally convenient approach to the synthesis of iodo and nitro coumarin derivatives is presented here. Due to the importance of iodo-substituted organic compounds as synthos or valuable precursors in organic synthesis, in addition to their use as radioactively-labeled markers in medical diagnosis, the introduction of iodine into organic molecules has received significant attention among the scientific community. Since molecular iodine itself is poorly reactive, substantial efforts have been invested in the development of efficient, selective and mild methods for direct introduction of iodine into organic compounds. The nitro group in organic synthesis focuses on reactions that proceed under mild conditions, important functional groups that can be synthesized by conversion of nitro group and the stereo selectivity of reaction of nitro compounds. The issues are of great importance in pharmaceutical, agrochemical and fine chemical industries.

2. Materials and Methods

Solvents and reagents used for the synthesis were of reagent grade and were purified by standard methods. Melting points were determined by using a (Joshibha) Model melting point approach and are uncorrected IR spectra were recorded on

Brucker Model. The reaction progress and purity of all prepared compounds was followed by TLC in the system benzene/ethyl acetate and benzene/pet ether visualizing spots with UV lamp and iodine vapour. Laminar airflow Cabinet (Kemi), autoclave (Osworld, "Autoclave Steam Sterilizer" JRIC-39) and incubator (Genuine) were used for microbial activity.

3. Preparation of Coumarins (1A)

1. Preparation of 4-hydroxycoumarin (1A)

A mixture of phenol (4.5g), malonic acid (5.2 g), fused zinc chloride (18.5 g) and phosphorus oxychloride (15 ml) was irradiated in a microwave oven for 11/2 hours. The progress of reaction was monitored by TLC. The product was cooled and decomposed with ice water and allowed to stand. The resulting 4-hydroxy coumarin was dissolved in 10% sodium carbonate and acidified. At about neutral point some oily by product separated out and was removed. Acidification of the remaining solution gave 4-hydroxy coumarin. On recrystallisation from dilute alcohol pure 4-hydroxy coumarin was obtained.

2. Preparation of 7-hydroxy-4-methyl coumarin (2A)

A Solution of resorcinol (11 g) in ethyl acetoacetate (13g) is added to cold concentrated sulphuric acid (100 ml) with stirring. The reaction mixture was kept for 20 hours at room temperature and poured with vigorous stirring onto crushed ice. The separated product was filtered, washed with water and crystallized from dilute alcohol.

3. Preparation of 4, 7-dihydroxycoumarin (3A)

A mixture of dry resorcinol (5g), malonic acid (5.2g), fused zinc chloride (18.5 g) and phosphorus oxychloride (15 ml) was irradiated in a microwave oven for 11/2 hours. After the reaction was complete as monitored by TLC, the crude product obtained from the reaction was recrystallised from hot water, as pale yellow crystals.

4. Preparation of 4-methyl-7, 8-dihydroxycoumarin (4A)

A mixture of phenolic substrate (trihydroxybenzaldehyde) (20ml) and ketoester (21 ml) was heated at 70°C in the presence of concentrated sulphuric acid. After completion of

the reaction, the reaction mixture was cooled at room temperature and poured onto crushed ice (50 g). The solid product obtained was filtered off, washed with ice-cold water and recrystallised from hot ethanol, to obtain the pure product.

5. Preparation of 4, 7-dimethylcoumarin (5A)

A mixture of p-cresol (21ml) and ethyl acetoacetate (26 ml) in sulphuric acid (100 ml) was heated on a water bath for 5 hours. The reaction mixture was then cooled and poured into ice water. The product was filtered, washed with water, dried and then crystallized from dilute alcohol to give white crystals.

6. Preparation of 7-hydroxycoumarin (6A)

An intimate mixture of resorcinol (3g), malonic acid (2.4 g) and 60ml of concentrated sulphuric acid were irradiated in a microwave oven for 2 ½ hours until the effervescence ceased. It was cooled and treated with excess of crushed ice. The mixture became clear in 1½ hours and the heating was stopped. The crude product thus obtained was recrystallised from ethanol as dark red powder.

4. Nitration of coumarins

1) Nitration of 4-hydroxycoumarin (1B)

4-hydroxycoumarin (100mg) was mixed with acetic acid (3ml) and concentrated nitric acid (2 ml). This mixture was heated on a water bath for 15 to 25 minutes. The reaction mixture was cooled and then poured into ice water. The product was filtered, washed with water and then dried and obtained as yellow solid. The melting point was noted in table-1. The product was positive to Lassaigne's test. CO-IR of reactant and product was recorded.

2) Nitration of 4-methyl-7-hydroxycoumarin (2B)

4-methyl-7-hydroxycoumarin (100mg) was mixed with acetic acid (3 ml) and concentrated nitric acid (2 ml). This mixture was heated on a water bath for 15 to 25 minutes. The reaction mixture was cooled and then poured into ice water. The product was filtered, washed with water and then dried and obtained as yellow solid. The melting point was noted in table-1. The product was positive to Lassaigne's test. CO-IR of reactant and product was recorded.

3) Nitration of 4, 7- dihydroxycoumarin (3B)

4, 7- dihydroxycoumarin (100mg) was mixed with acetic acid (3 ml) and concentrated nitric acid (2 ml). This mixture was heated on a water bath for 15 to 20 minutes. The reaction mixture was cooled and then poured into ice water. The product was filtered, washed with water and then dried and obtained as yellow solid. The melting point was noted in table-1. The product was positive to Lassaigne's test. CO-IR of reactant and product was recorded.

4) Nitration of 4-methyl-7, 8-dihydroxycoumarin (4B)

4-methyl-7, 8-dihydroxycoumarin (100mg) was mixed with acetic acid (3 ml) and concentrated nitric acid (2 ml). This mixture was heated on a water bath for 15 to 25 minutes. The reaction mixture was cooled and then poured into ice water. The product was filtered, washed with water and then dried and obtained as yellow solid. The melting point was noted in table-1. The product was

positive to Lassaigne's test. CO-IR of reactant and product was recorded.

5) Nitration of 4, 7-dimethylcoumarin (5B)

4, 7-dimethylcoumarin (100mg) was mixed with acetic acid (3 ml) and concentrated nitric acid (2 ml). This mixture was heated on a water bath for 15 to 25 minutes. The reaction mixture was cooled and then poured into ice water. The product was filtered, washed with water and then dried and obtained as yellow solid. The melting point was noted in table-1. The product was positive to Lassaigne's test. CO-IR of reactant and product was recorded.

6) Nitration of 7-hydroxycoumarin (6B)

7-hydroxycoumarin (100mg) was mixed with acetic acid (3 ml) and concentrated nitric acid (2 ml). This mixture was heated on a water bath for 15 to 25 minutes. The reaction mixture was cooled and then poured into ice water. The product was filtered, washed with water and then dried and obtained as yellow solid. The melting point was noted in table-1. The product was positive to Lassaigne's test. CO-IR of reactant and product was recorded.

5. Preparation of Iodocoumarins

1. Preparation of iodo- 4-hydroxycoumarin (1C)

In a 100ml beaker, provided with mechanical stirrer, a mixture of 100mg of 4-hydroxycoumarin, 60mg of sodium hydrogen carbonate and 15ml of water were taken. The mixture was cooled at 15°C by the addition of crushed ice. It was stirred and 120 mg of powdered resublimed iodine was introduced in portions of 5-6 mg at interval of 2-3 minutes, so that all the iodine was added during 30 minutes. This was followed by continuous stirring for 3 hours. After 3 hours, the TLC showed that the reactant disappeared and a brown coloured spot appeared. After the reaction was complete, the mixture was extracted with diethyl ether and then ether layer was evaporated. Then treated with ethanol, the product was obtained as a semisolid. The product was very hygroscopic in nature, so the yield and melting point could not be recorded. CO-IR of reactant and product was recorded.

2. Preparation of iodo-7-hydroxy-4 methyl coumarin (2C)

In a 100ml beaker, provided with mechanical stirrer, a mixture of 100mg of 7-hydroxy-4 methyl coumarin, 60mg of sodium hydrogen carbonate and 15ml of water were taken. The mixture was cooled at 15°C by the addition of crushed ice. It was stirred and 120 mg of powdered resublimed iodine was introduced in portions of 5-6 mg at interval of 2-3 minutes, so that all the iodine was added during 30 minutes. This was followed by continuous stirring for 48 hours. After 48 hours, the TLC showed that the reactant disappeared and a brown coloured spot appeared. After the reaction was complete, the mixture was extracted with diethyl ether and then ether layer was evaporated. Then treated with ethanol, the product was obtained as a semisolid. The product was very hygroscopic in nature, so the yield and melting point could not be recorded. CO-IR of reactant and product was recorded.

3. Preparation of iodo-4, 7 –dihydroxycoumarin (3C)

In a 100ml beaker, provided with mechanical stirrer, a mixture of 100mg of 4, 7 –dihydroxycoumarin, 60mg of sodium hydrogen carbonate and 15ml of water were taken. The mixture was cooled at 15°C by the addition of crushed ice. It was stirred and 120 mg of powdered resublimed iodine was introduced in portions of 5-6 mg at interval of 2-3 minutes, so that all the iodine was added during 30 minutes. This was followed by continuous stirring for 5 hours. After 5 hours, the TLC showed that the reactant disappeared and a brown coloured spot appeared. After the reaction was complete, the mixture was extracted with diethyl ether and then ether layer was evaporated. Then treated with ethanol, the product was obtained as a semisolid. The product was very hygroscopic in nature, so the yield and melting point could not be recorded. CO-IR of reactant and product was recorded.

4. Preparation of iodo-4-methyl-7, 8-dihydroxycoumarin (4C)

In a 100ml beaker, provided with mechanical stirrer, a mixture of 100mg of 4-methyl-7, 8-dihydroxycoumarin, 60mg of sodium hydrogen carbonate and 15ml of water were taken. The mixture was cooled at 15°C by the addition of crushed ice. It was stirred and 120 mg of powdered resublimed iodine was introduced in portions of 5-6 mg at interval of 2-3 minutes, so that all the iodine was added during 30 minutes. This was followed by continuous stirring for 42 hours. After 42 hours, the TLC showed that the reactant disappeared and a brown coloured spot appeared. After the reaction was complete, the mixture was extracted with diethyl ether and then ether layer was evaporated. Then treated with ethanol, the product was obtained as a semisolid. The product was very hygroscopic in nature, so the yield and melting point could not be recorded. CO-IR of reactant and product was recorded.

5. Preparation of iodo-4, 7-dimethylcoumarin (5C)

In a 100ml beaker, provided with mechanical stirrer, a mixture of 100mg of 4, 7-dimethylcoumarin, 60mg of sodium hydrogen carbonate and 15ml of water were taken. The mixture was cooled at 15°C by the addition of crushed ice. It was stirred and 120 mg of powdered resublimed iodine was introduced in portions of 5-6 mg at interval of 2-3 minutes, so that all the iodine was added during 30 minutes. This was followed by continuous stirring for 42 hours. After 42 hours, the TLC showed that the reactant disappeared and a brown coloured spot appeared. After the reaction was complete, the mixture was extracted with diethyl ether and then ether layer was evaporated. Then treated with ethanol, the product was obtained as a semisolid. The product was very hygroscopic in nature, so the yield and melting point could not be recorded. CO-IR of reactant and product was recorded.

6. Preparation of iodo-7-hydroxycoumarin (6C)

In a 100ml beaker, provided with mechanical stirrer, a mixture of 100mg of 7-hydroxycoumarin, 60mg of sodium hydrogen carbonate and 15ml of water were taken. The mixture was cooled at 15°C by the addition of crushed ice. It was stirred and 120 mg of powdered resublimed iodine was introduced in portions of 5-6 mg at interval of 2-3 minutes,

so that all the iodine was added during 30 minutes. This was followed by continuous stirring for 7 hours. After 7 hours, the TLC showed that the reactant disappeared and a brown coloured spot appeared. After the reaction was complete, the mixture was extracted with diethyl ether and then ether layer was evaporated. Then treated with ethanol, the product was obtained as a semisolid. The product was very hygroscopic in nature, so the yield and melting point could not be recorded. CO-IR of reactant and product was recorded.

Antimicrobial activity of compounds

The synthesized coumarin and their iodo and nitro derivatives were tested for their antifungal and antibacterial activity and activity was compared.

Preparation of culture media for antibacterial antifungal studies

Preparation of Nutrient agar medium

Three grams of beef extract, 50g of peptone of NaCl and 15g of agar were taken in a beaker and then distilled water (1000ml) was added. The mixture was boiled and mixed thoroughly with a glass rod. After complete dissolution of agar the medium it was dispensed into several conical flask of 250ml volume. The conical flasks were closed with cotton plug and rapped with aluminum foil. It was there auto claved for 15 minutes at 121°C and 15 psi. After autoclaving, the medicine was used for culturing different micro organism.

Preparation of Sabouard Dextrose Agar. (SDA medium)

65g of SDA were suspended in 100ml distilled water. It was heated to boiling to dissolve the medium completely and the sterilized by autoclaving at 15 lbs, pressure (121°C) for 15min.

Antimicrobial testing

Disc method for determination of zone of inhibition of antibacterial

Paper discs of 4mm in diameter and glass Petri plates of 90mm in diameter were used throughout the experiment. Paper discs were sterilized in an autoclave and dried at 100°C in oven. Then the disc was soaked with test chemicals at the rate of 50g per disc for antibacterial analysis. One drop of bacterial suspension was taken in sterile Petri dish and then approximately 20ml of sterilized and melted NA (~45°C) was poured into the plate, and then mixed thoroughly. The paper discs after soaking with test chemicals were placed at the center of the inoculated pour plate. A control plate was also maintained in each case with alcohol. Firstly, the plates were kept for 4hrs at low temperature (4°C) the test chemicals diffused from disc to the surrounding medium by this time. The plates were then incubated at (35± 2) °C for growth of test organisms and were observed at 24 hours intervals for two days. The activity was expressed in terms of inhibition zone diameter in mm. Each experiment was repeated three times. The standard antibiotic, streptomycin and gentamycin was used as a positive control and compared with test chemicals under identical condition. The antimicrobial activities of the compounds were recorded.

Streak plate isolation method for determination of zone of inhibition of antifungal activity

The required amount of SDA medium was taken in a conical flask separately and was sterilized in an autoclave (at 120°C and 15psi) for 15min. A tube of SDA was liquefied and poured into a petridish. The plate was rotated gently for uniform distribution of the medium. The inoculating loop was held at a 60°C angle in the hottest part of the Bunsen burner flame. The entire tube was heated to redness. The loop was allowed to cool for 15 to 20 seconds before it touched the culture. A small amount of the culture was removed from the tube with the sterilized inoculating loop and the microorganisms were streaked in the plate. The stock solutions were prepared by dissolving the compounds in ethanol. Inoculation process was done under aseptical condition and the spores were inoculated in the medium and incubated for 5 days. A clear zone or ring was present on SDA plate. The diameters of the zone are measured.

The compounds synthesized will be designated as 1A, 1B, 1C, 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, 4C, 5A, 5B, 5C, 6A, 6B, 6C. for the purpose of easy reference.

6. Result

For the study 16 derivatives of coumarins have been prepared and characterized by their IR spectra. Their antimicrobial activities have been assessed.

It has been found that 4-hydroxy coumarin, 7-hydroxy coumarin, 4, 7-dihydroxy coumarin got iodinated in 3-7 hours under cold condition. 7-hydroxy-4 methyl coumarin, 4, 7-dimethyl coumarin, 4-methyl-7, 8-dihydroxycoumarin did not form iodo derivatives even after 8 hours stirring in cold condition. This may be due to the bulky groups present in the reactant molecule. An Iodo compound was very hygroscopic in nature. So, the yield and the melting point could not be detected.

The IR spectrum of all the iodo coumarins suggested the presence of coumarin ring. Pending confirmation of the

exact position of the iodo substituent it can be probably suggested that the iodo substituent may be present in the 6 or 8 position of the ring of coumarin moiety.

All hydroxyl coumarins got nitrated in 25 minutes' under hot condition. The melting points are reported in Table-1. All nitro compounds were positive to Lassaigne's test. They were characterized by their IR spectra. Co-IR spectra also suggested positive reaction.

Anti bacterial screening was done for the various iodo and nitro coumarins against *Escherichia-coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*. It was found from the result presented in table-2, that all the compounds exhibited good activity against all the species. For *Escherichia-coli*, compounds 3C, 5C and 6C showed better activity than the control drug streptomycin. For *Staphylococcus aureus* compound 6A showed better activity than the control drug. Good activity was shown against *Klebsiella pneumoniae* by 2C, 6C. For *Pseudomonas aeruginosa* 5C showed better activity than the control drug streptomycin.

Antifungal screening was done using iodo and nitro coumarins against *Candida albicans*, *Aspergillus flavus*, *Mucor hiemalis*. In the case of *Candida albicans* compound 4C and 6C showed similar activity as that of the flucanazole. For *Mucor hiemalis* compound 1A showed good activity. For *Aspergillus flavus* 1C showed good activity compared to their other derivatives.

Table 1: Melting Points Of Nitrocoumarins

Compounds	Melting point(°C)	Formula (Molecular weight)
1B	230	C ₉ H ₅ NO ₅ (207)
2B	224-226	C ₁₀ H ₇ NO ₅ (221) C ₁₀ H ₆ N ₂ O ₇ (266)
3B	180(it decomposes)	C ₉ H ₅ NO ₆ (223)
4B	264-265	C ₁₀ H ₇ NO ₆ (237)
5B	118-120	C ₁₁ H ₈ N ₂ O ₆ (264)
6B	190(it decomposes)	C ₉ H ₅ NO ₅ (207)

Table 2: Zone of inhibition for antibacterial and antifungal activity studies of compounds

BACTERIA	Compounds						Compounds						Compounds						Control
	Zone of inhibition(mm)						Zone of inhibition(mm)						Zone of inhibition(mm)						
	1A	2A	3A	4A	5A	6A	1B	2B	3B	4B	5B	6B	1C	2C	3C	4C	5C	6C	Streptomycin (mm)
<i>Escherichia-coli</i>	7	5	7	6	6	7	6	5	6	5	5	5	8	7	9	8	9	9	7
<i>Staphylococcus aureus</i>	5	5	6	6	8	9	5	6	7	6	7	7	7	7	7	7	8	8	8
<i>Klebsiella pneumoniae</i>	6	8	5	6	6	6	6	5	4	3	5	4	8	9	6	8	6	9	11
<i>Pseudomonas aeruginosa</i>	4	4	7	7	8	6	5	5	5	6	6	7	6	6	8	8	10	8	8
FUNGUS																			Flucanazole (mm)
<i>Candida albicans</i>	4	4	6	5	5	6	4	5	5	4	5	5	5	5	4	7	6	7	7
<i>Mucor hiemalis</i>	7	4	5	6	5	4	5	4	4	4	5	4	4	4	5	5	5	5	8
<i>Aspergillus flavus</i>	6	4	6	5	5	5	5	5	6	6	6	6	7	6	5	6	5	6	12

7. Conclusion

An assessment of the antibacterial activities of the coumarin derivatives revealed that the iodo coumarins exhibited maximum activity against all the bacterial and fungal species taken up for the study.

This observation could be useful in carrying out further studies on the iodo derivatives particularly in clinical trials against various infections.

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