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Dr.Y.H.Rao
Email: submitijca@gmail.com

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Research Article

Synthesis of C-Diprenylated-Trihydroxy-Xanthone Derivatives as Lung Anticancer Agents and *In vitro* Test Against Cancer Cells

Kejiya Rani Manchikalapudi[1], Rajeev Chalivendra[2]
[1]*Junior Lecturer in Chemistry, Bhashyam Junior College (Viswa karma Campus), Chandramouli Nagar, Guntur. Email. kejiyaranimanchikalapudi@gmail.com.*
[2]*Research Scholar, Department of Chemistry, Krishna University, Machilipatnam. Email. raajeev.nriha@gmail.com.*

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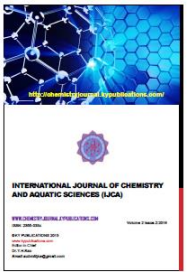

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ABSTRACT

This study emphasizes that incorporating the C-prenylation group mainly modifies the isolated xanthone framework at different locations. There are two methods for obtaining xanthone compounds: synthesis and isolation. This study aims to synthesize novel drug compounds with anti-lung cancer activity derived from C-prenylated polyhydroxy xanthone and test them in vitro on NCI-H661 cells. The target compound was synthesised from 2,5-dihydroxybenzoic acid and 1,3,5-trihydroxybenzene in two steps: C-prenylation of xanthone compounds and an acylation-dehydration reaction modified by the Grover, Shah, and Shah (GSS) method. The 1,3,7-trihydroxy xanthone and 1,3,7- trihydroxy- 2,4- diprenyl xanthone compounds were successfully synthesised, purified by column and preparative thin layer chromatography, and characterized by FT-IR, ^{1}H-NMR, and ^{13}C-NMR. In vitro tests on NCI-H661 cells revealed that 1,3,7-trihydroxy-2,4-diprenylxanthone was superior to 1,3,7-trihydroxyxanthone in preventing lung cancer.
Keywords: Synthesis of xanthone derivative. Characterization, *in vitro* studies, anti-lung cancer agent.

1. INTRODUCTION

Cancer presently ranks as the second foremost cause of mortality in developing nations, including Indonesia. Cancer predominantly manifests in the elderly, with individuals over 65 years exhibiting a tenfold increased risk compared to their younger counterparts. Cancer results from both endogenous and exogenous factors that induce genetic alterations [1]. According to the WHO (2022) and the Indian Ministry of Health [2], the prevalence of cancer among individuals of all ages in India was 1.4‰, with the highest prevalence observed in the northern Indian region at 4.1‰, followed by Central Java and Bali Provinces at 2.1‰ and 2.0‰, respectively. Lung cancer is a malignant neoplasm that is the leading cause of mortality globally. Annually, over 1.61 million cases of lung cancer are reported, resulting in approximately 1.38 million fatalities, constituting 18.2% of all cancer patients globally [3].

Researchers' interest in developing, constructing, and discovering more effective anticancer drugs is rapidly escalating in medicinal chemistry. Cancer cells can be treated using radiotherapy, chemotherapy, surgery, hormone therapy, and immunotherapy [4]. Chemotherapy and surgery are typically the predominant treatments for patients with lung cancer. Nonetheless, the application of surgical intervention in treatment is significantly constrained by factors such as tumour dimensions and insufficient medical personnel. Radiotherapy can induce mutagenicity in certain living cells within the tissue. Chemotherapy is extensively utilised in the medical field due to its cost-effectiveness, efficiency, ease of administration, and capability to address malignant tumours [5].

An initiative to address the issue of cancer is the discovery of a novel pharmaceutical agent. Drug development typically involves the isolation of active constituents from medicinal plants and the synthesis of analogue compounds derived from natural substances [6]. Medicinal plants containing active compounds effective against cancer cells, such as *Garcia* species. These plants are rich in phenolic compounds (secondary metabolites) derived from xanthones [7]. These compounds exhibit anticancer, anti-HIV, antioxidant, anti-inflammatory, and antibacterial properties. Despite possessing anticancer properties, the yield of the isolate is exceedingly low at 0.55%, rendering it ineffective as a treatment for lung cancer cells in both home and commercial settings. Consequently, the synthesis of xanthone derivative compounds represents a promising approach in lung cancer treatment due to its benefits in health, scientific efficacy, and cost-effectiveness relative to isolation methods [8].

2. METHODS

2.1 Research Tools and Materials

The employed instruments include laboratory glassware, 1H-NMR, ^{13}C-NMR, and FT-IR. The utilised materials comprise 2,5-dihydroxybenzoic acid, phloroglucinol (benzene-1,3,5-triol), potassium hydroxide (KOH), acetone, hydrochloric acid (HCl), dichloromethane, prenyl bromide, Eaton reagent, chloroform, n-hexane, ethyl acetate, distilled water, silica gel, silica gel plate thin layer chromatography 60 F254, and anhydrous sodium sulphate (Na_2SO_4).

2.2 Place and Time of Research

The research was executed in the laboratory of Acharya Nagarjuna University and the pharmacology laboratory, spanning from April 1, 2022, to June 30, 2023.

2.3 Methodology

2.3.1 Synthesis of 1,3,7-trihydroxy xanthone Compound

7.7 g of 2,5-dihydroxybenzoic acid was combined with 6.31 g of phloroglucinol in a 250 mL round-bottom flask. Then 100 mL of Eaton's reagent was added slowly to the mixture. The mixture was subjected to heating in a water bath at 80 ± 3 ^{o}C for 2 hours with continuous stirring. The mixture was subsequently cooled to ambient temperature using a cold-water bath. Additionally, the mixture was combined with small ice cubes and stirred for 15 minutes. The precipitate was subsequently filtered using a Buchner funnel and dried in an oven at 50 ^{o}C overnight. The resultant sample was subsequently purified via column chromatography and preparative thin-layer chromatography. The generated fractions were subsequently analysed utilising 1H-NMR, 13C-NMR, and FT-IR techniques.

2.3.2 Identification and Purification of Compound 1,3,7- trihydroxy xanthone

Using thin-layer chromatography, the compound 1,3,7-trihydroxyxanthone was identified. Two millilitres of n-hexane solution and one millilitre of ethyl acetate solution were combined, placed in a 100-millilitre beaker, and sealed tightly. Ethyl acetate was used to completely dissolve a few crystals of 1,3,7-trihydroxyxanthone, standard 2,5-dihydroxybenzoic acid, and phloroglucinol. Each of these was then spotted on a silica gel plate and eluted to the upper limit. UV light was used to identify the

spots that formed. Using column chromatography, the compound 1,3,7-trihydroxyxanthone was purified. To prepare the column, a layer of cotton, anhydrous sodium sulphate, silica gel slurry, target compound impregnated with sodium sulphate, and a large amount of n-hexane: ethyl acetate solvent (2:1) were created. The resulting fractions were subsequently gathered into vials, and thin layer chromatography was used to identify each vial. Additionally, the solvent was allowed to evaporate completely in each vial until only pale-yellow crystals were left.

2.3.3 Synthesis of 1,3,7-trihydroxy-2,4-diprenylxanthone Compound

A 250 mL round-bottom flask was filled with 50 mL of a 10% KOH solution. The flask was then filled with 0.5 g of 1,3,7-trihydroxyxanthone compound, which was swirled for 15 minutes. Additionally, a syringe was used to inject 7.16 g of prenyl bromide compound into 6 mL of acetone. At room temperature, the mixture was agitated for a full day. The mixture was periodically acidified using 100 millilitres of 10% HCl, and then dichloromethane was used for extraction. An evaporator was then used to gather and separate the organic layer from the solvent. Column chromatography and preparative thin-layer chromatography were used to purify the final product. 1H-NMR and 13C-NMR were then used to analyse the fractions that had formed.

2.3.4 Culture Preparation

NCI-H661 cells (lung carcinoma cells) were cultured in DMEM medium supplemented with 10% foetal bovine serum (FBS), 100mU/mL penicillin, and 100mg/mL streptomycin, and incubated at 37°C in a 5% CO2 atmosphere. NCI-H661 cells were cultivated until they attained 70-80% confluence under optimal conditions. The old medium was subsequently evaporated from the petri dish and rinsed twice with phosphate-buffered saline (PBS). Two millilitres of trypsin-EDTA were applied to the cultured cell surface and incubated at 37°C in 5% CO2 for five minutes. Subsequently, 2 mL of 10% FBS medium was added to the cells, followed by centrifugation. The cells were subsequently transferred to a new tissue culture flask containing fresh medium and incubated at 37°C in 5% CO2.

2.3.5 *In Vitro* Test

NCI-H661 cells were harvested in 100 mL of medium (± 4000 cells) grown on sterile petri dishes and incubated overnight with CO2 gas. Cytotoxicity test was carried out using the MTT method (3-[4,5-dimethylthiazol-2-yl]- 2,5- diphenyltetrazolium bromide) for 24 hours. A series of solutions of the synthesized compound 1,3,7-trihydroxy-2,4- diprenylxanthone were made with concentrations of 12.5; 25; 50; 100; and 200mg/mL with medium solvents of 3 replications each. Three types of controls were also made, namely NCI-H661 cell control (100 mL NCI-H661 cells + 100 L medium), medium control (200 L medium), and sample control (100 mL 1,3,7-trihydroxy-2,4- diprenylxanthone + 100mL medium). A total of 100mL of 1,3,7-trihydroxy-2,4-diprenylxanthone solution of each concentration was put into the microplate wells containing 100mL of cancer cells. The microplates were then incubated for 24 hours in a CO2 incubator, then 10mL of MTT was added to each microplate well and re-incubated for 4 hours in a CO2 incubator. The MTT reaction was stopped by adding 10% sodium dodecyl sulphate (SDS), and then the microplates were re-incubated for 12 hours in the dark at room temperature. After incubation, the absorbance of each well was measured using a microplate reader spectrophotometer at a wavelength of 540 nm.

3. RESULTS AND DISCUSSION

3.1. Characterization of 1,3,7-trihydroxy xanthone Compound

The synthesis of 1,3,7-trihydroxyxanthone was conducted by reacting 2,5-dihydroxybenzoic acid with phloroglucinol using Eaton's reagent. The amalgamation was subjected to heating for 30 minutes at a temperature of 80 °C. The synthesis of 1,3,7-trihydroxyxanthone is an acylation-dehydration reaction marked by the liberation of small molecules, including water (H2O). This method

was devised and refined by Grover, Shah, and Shah (GSS) to achieve a xanthone framework that is non-stereospecific and racemic, thereby facilitating the production of a distinct and readily separable product.

The results yielded the formation of an orange solid. The orange solid was subsequently analysed using thin-layer chromatography. The thin layer chromatography data indicates that the resultant product is a mixture exhibiting four spots when eluted with n-hexane: ethyl acetate (2:1). The resultant product mixture was purified via column chromatography employing a solvent polarity gradient of n-hexane and ethyl acetate (2:1). Fraction 18-50 is hypothesised to be 1,3,7-trihydroxyxanthone, as indicated by the emergence of two spots on the TLC plate. Pale yellow crystals of 1,3,7-trihydroxyxanthone were isolated from the fraction and subsequently characterised using FT-IR, 1H-NMR, and 13C-NMR spectrophotometry. The FT-IR spectrum in Figure 4.1 exhibits a peak corresponding to a hydroxyl group (O-H), resulting in an absorption band at 3229 cm-1. Subsequently, absorption occurs in the aromatic group C = Csp2 within the range of 1617 - 1518 cm-1. The absorption at 1654 cm-1 signifies the presence of a carbonyl group (C = O). Furthermore, the absorptions at wave numbers approximately 1200 and 1050 cm-1 correspond to asymmetric and symmetric C-O-C stretching vibrations, respectively.

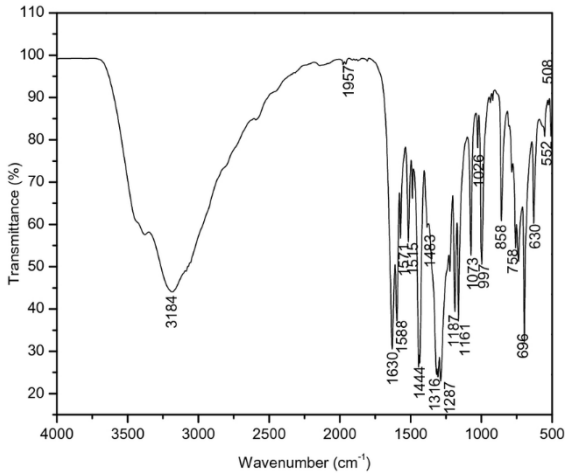

Figure 4.1 FT-IR Spectra of Compound 1,3,7-trihydroxy xanthone

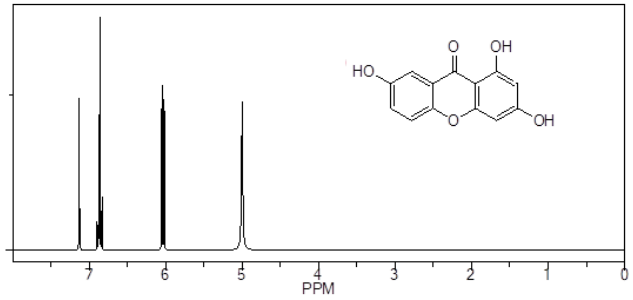

Figure 4.2 1H-NMR Spectra of 1,3,7-trihydroxyxanthone

Table 4.1 1H-NMR spectral analysis data for the compound 1,3,7-trihydroxy xanthone

H-Position	Chemical shift (ppm)	Multiplicity	Integration	Proton Types
2	6.140-6.143 (*J*=1.8)	Doublet		Aril
4	6.285-6.287 (*J*=1.2)	Doublet		Aril
5	7.585-7.600 (*J*=9)	Doublet		Aril
6	7.732-7.752 (J=3;9)	Doublet of doublet		Aril
8	7.908-7.912 (J=2.4)	Doublet	^{1}H	Aril
1-OH	12.829	Singlet		Hydroxyl
3-OH	12.480	Singlet		Hydroxyl
7-OH	12.585	Singlet		Hydroxyl

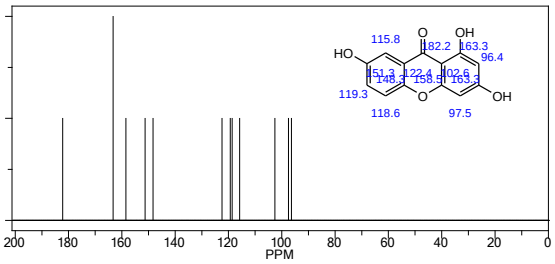

Figure 4.3 ^{13}C-NMR Spectra of Compound 1,3,7-trihydroxyxanthone

The ^{1}H-NMR spectrum of the synthesized product in Figure 4.2 shows that there are 5 protons in the chemical environment of the aromatic group and 3 protons from the hydroxyl group. The signals at δ 6.140-6.143 ppm and 6.285-6.287 ppm belong to the protons of the aromatic group H2 and at the meta position. The signal at δ 7.732-7.752 ppm indicates the presence of protons from the aromatic group H6 paired with protons at the ortho and meta positions. The signal at δ 7.585- 7.600 ppm comes from protons from the aromatic group H5 paired with protons from the ortho position, while the signal that appears at δ 7.908-7.912 ppm with doublet multiplicity pairs with each other at the meta position. The presence of a hydroxyl group is indicated by the signal at δ 12.585- 12.829 ppm. The ^{13}C-NMR spectrum in Figure 4.3 shows that in the chemical environment, there are 2 types of carbon types identified, namely aryl and carbonyl carbon types (C = O). The δ signal of 179.12 ppm shows the specifications of carbon in carbonyl which is a bridge between 2 benzene in the xanthone structure. The δ signal in the range of 94.60-166.61 ppm shows the type of carbon, namely aryl at various positions in the xanthone framework. 1H-NMR and ^{13}C-NMR instrument data show that the compound analysed has aryl, carbonyl and hydroxyl groups, then reinforced again with the FT-IR spectrum which shows that there is C-O-C (ether) absorption at 1200 and 1050 cm-1. Based on the analysis using the FT-IR, 1H-NMR and ^{13}C-NMR spectrophotometers, it can be concluded that the compound 1,3,7-trihydroxyxanthone was successfully synthesized. The synthesis reaction of 1,3,7-trihydroxy xanthone is shown in Figure 4.4.

Table 4.2 ^{13}C- NMR spectral analysis data for the compound 1,3,7-trihydroxy xanthone

H-Position	Chemical shift (ppm)	Types of carbon
1	165.51	Carbon type
2	97.71	Ariel
3	162.01	Ariel
4	93.5	Ariel
4a	156.53	Ariel
5	117.25	Ariel
6	119.91	Ariel
7	152.94	Ariel
8	107.27	Ariel
9	178.02	Ariel
9a	101.16	carbonyl (C=O)
10a	144.14	Ariel

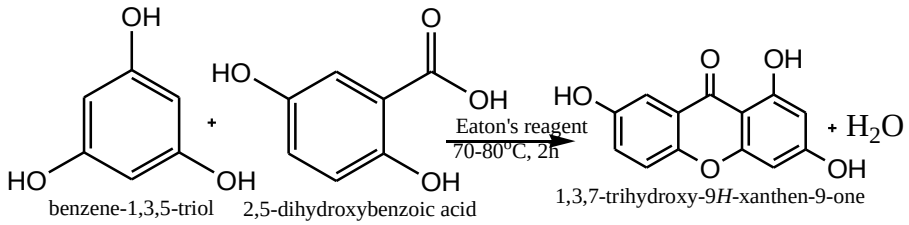

Figure 4.4 Compound Synthesis Reaction

3.2. Characterization of Compound 1,3,7- trihydroxy-2-prenylxanthone

The synthesis of C-prenylated polyhydroxy xanthone derivatives was conducted by reacting 1,3,7-trihydroxyxanthone with prenyl bromide (1-bromo-3-methylbut-2-ene) in a 10% potassium hydroxide solution. The product obtained was extracted using chloroform slightly acidified with 10% HCl. From the extraction, 2 layers were obtained, where the chloroform layer (brown) was taken and concentrated with an evaporator to remove the remaining chloroform solvent. The target compound extract was then dried and weighed. Purification was carried out by column chromatography and preparative thin-layer chromatography. The purified compound 1,3,7- trihydroxy-2-prenylxanthone was then characterized by 1H-NMR and ^{13}C-NMR spectrometers.

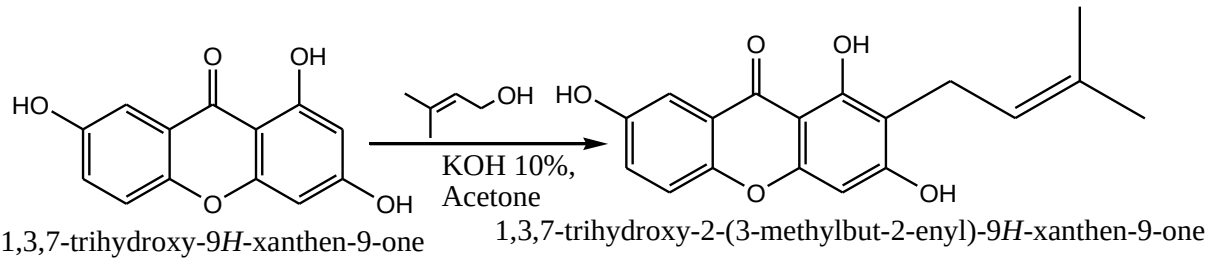

Figure 4.5 Synthesis Reaction of 1,3,7-trihydroxy-2-prenylxantone

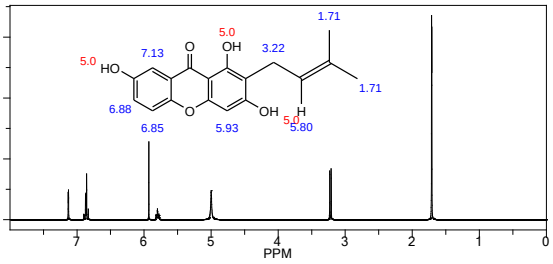

Figure 4.6 Spectral ^{1}H-NMR Compound 1,3,7-trihydroxy-2-prenylxantone

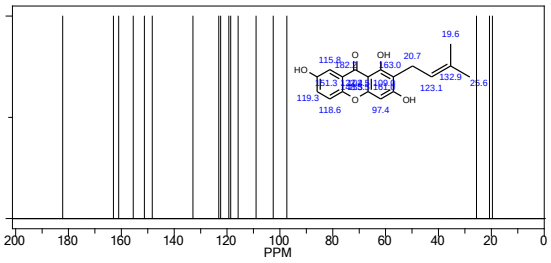

Figure 4.7 Spectra ^{13}C-NMR Compound 1,3,7-trihydroxy-2-prenylxantone

Table 4.3 Spectra analysis data of 1H-NMR and ^{13}C-NMR Compounds 1,3,7-Trihydroxy-2-prenylxantone

Position	"Chemical shifts in ^{1}H-NMR (ppm)"	Chemical shifts in ^{13}C-NMR (ppm)
1-OH	13.17 (s)	-
3-OH	9.98 (s)	-
s7-OH	11.05 (s)	-
1	-	165.41
2	6.51 (s)	97.61
3	-	161.91
4	-	93.4
4a	-	156.43
5	7.5-7.55 (d. *J*=9)	117.15
6	7.3-7.4 (dd. J = 8.7; 3)	119.81
7	-	152.84
8	7.5-7.6(d. J =3)	107.17
8a	-	177.92
9	-	101.06
9a	-	144.04
10a	-	165.41
1′	3.3-3.24(d. J=7.2)	97.61
2′	5.1-5.18 (m. J=7.2)	161.91
3′	-	93.4
4′	1.74 (s)	156.43
5′	1.63 (s)	117.15

The ^{1}H-NMR spectrum depicted in Figure 4.6 reveals a chemical environment characterised by hydroxyl hydrogen at δ 9.979-13.168 ppm at positions 1, 3, and 7 of the xanthone framework, aryl hydrogen at δ 6.443-7.479 ppm, and alkyl hydrogen (from prenyl) at δ 1.627-5.197 ppm. The ^{13}C-NMR spectrum (Figure 4.7) corroborates this, indicating a chemical environment with aryl carbon at δ 93.48-180.21 ppm and alkyl (from prenyl) at δ 18.16-122 ppm. Consequently, the analysis of 1H-NMR and ^{13}C-NMR spectrometers indicates that the compound 1,3,7-trihydroxy-2-prenylxanthone was successfully synthesised.

3.1 *In Vitro* Test Results on NCI-H661 Cells

The synthesis of xanthone derivative compounds via acylation-dehydration reactions employing the Grover, Shah, Shah (GSS) modification method is efficient and rapid, yielding specific products due to the absence of steric and racemic hindrances in the resultant xanthone framework. Furthermore, C-prenylation is performed to enhance the biological activity of xanthone compounds, creating an active site that serves as an effective anticancer agent. An increase in the number of C-prenyl substitutions on the xanthone framework correlates with enhanced biological activity of the compound. The synthesis of this C-prenylated polyhydroxy xanthone derivative compound is anticipated to represent a significant advancement in the development of effective lung cancer therapeutics. In vitro testing on NCI-H661 cells demonstrated that the compound 1,3,7-trihydroxy-2,4-diprenylxanthone exhibited superior lung cancer activity compared to 1,3,7-trihydroxyxanthone.

4.0 Conclusion

The compounds 1,3,7-trihydroxyxanthone and 1,3,7-trihydroxy-2,4-diprenylxanthone have been synthesised with yields of 52% and 22%, respectively. Evaluation of these compounds for cytotoxicity against NCIH661 cells indicated that 1,3,7-trihydroxy-2,4-diprenylxanthone exhibits superior anti-lung cancer efficacy compared to 1,3,7-trihydroxyxanthone. An increase in the number of C-prenyl substitutions on the xanthone framework correlates with enhanced biological activity of the compound. The synthesis of this C-prenylated polyhydroxy xanthone derivative is anticipated to represent a significant advancement in the development of effective anti-lung cancer therapeutics to address lung cancer cases.

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