Research Article

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Synthesis and in vitro cytotoxicity evaluation of isatin-pyrrole derivatives against HepG2 cell line

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Abstract: This paper reports the synthesis and in vitro cytotoxicity evaluation of isatin-pyrrole derivatives 5–8, obtained from the appropriate isatins with pyrrole, with good yields and purity. The product structures were confirmed through spectroscopy methods. Furthermore, the MTT assay on the human liver cancer HepG2 cell lines revealed moderate activity in all compounds, which was highest in sample 6 (IC50 0.47 µM). The anticancer activity was affiliated with the presence of a nitro group at C-5 and N-methyl of the isatin scaffold.

Keywords: isatin, isatin-pyrrole derivatives, anticancer, HepG2 cell line

1 Introduction

Cancer is a serious threat to human health and a leading cause of death globally [1]. According to the GLOBOCAN 2018 database, 18.1 million people of all ages have various types of cancer, leaving almost half of the total affected individuals dead [2]. Additionally, chemotherapy is one of the most common treatments and is known to confer several disadvantages, including toxicity to normal cells [3]. Hence, there is a need to develop drugs with lower cytotoxicity. Moreover, the most promising approach is molecular hybridization or pharmacophore hybrid [4]. This involves the combination of two distinct pharmacophore functions to produce synergistic, more powerful, selective, and safer drugs [5].

Great efforts have been made to promote this technique, based on the isatin skeleton to develop cancer drugs [6], and recent studies show good anticancer activity in isatin 1 and its derivatives [7]. For example, the isatin-podophyllotoxin and nitromidazole-isatin hybrid were reported to be active against human leukaemia and breast cancer cells, respectively [8,9]. These effects are altered by modifications at the C-3, amide group, and phenyl ring of the isatin hybrid, as better activity was detected with the presence of a nitro group at C-5 isatin and a methyl or benzyl group at N-isatin [10–12].

Pyrrole is another important active chromophore with heterocyclic aromatic characteristics. It contains a nitrogen atom and is part of the cofactors and natural products of vitamin B12 and porphyrinogens [13,14]. Furthermore, pyrrole possesses broad-spectrum bioactivities, including anticancer and antibacterial functions [15,16], while molecular hybrid derivatives, including oroidin and sophoridine, recently exhibited remarkable anticancer activity against MCF-7 and HepG2 cancer cell lines [15,17]. Moreover, the trimethoxybenzaldehyde-pyrrole hybrid demonstrated good effects against HeLa and MCF-7 [18].

These findings suggest the need to investigate the combination of pharmacophoric elements including isatin and pyrrole, in a single chemical framework, and to investigate their cytotoxicity. Additionally, the effect of the nitro and amino group at the C-5 region of isatin and the methyl group of N-isatin on the compound’s anticancer activity was also investigated. This study, therefore, reports on the synthesis of isatin-pyrrole derivatives
alongside with their anticancer activity against HepG2 cancer cell lines.

2 Materials and method

All chemicals were purchased from commercial suppliers and used without purification. Melting points were measured using a Fisher John apparatus and are uncorrected. The Fourier-Transform Infrared (FT-IR) spectrum was confirmed using FTIR spectrophotometer Shimadzu 8400S. The mass spectra were recorded using LC-MS Mariner Biospectrometer Hitachi L 6200 with ESI Waters LCT Premier XE or TOF-MS Waters LCT Premier XE mass spectrometer. The proton (^1H) and carbon (^13C) nuclear magnetic resonance (NMR) were measured in acetone-d6 solvent using FT-NMR JNM-ECA500 500 MHz and FT-NMR JNM-ECS400 400 MHz.

2.1 Synthesis of N-methyl-5-nitroisatin (3)

The synthesis of 3 was performed by stirring 5-nitroisatin (2) (200 mg, 1.04 mmol) and sodium hydride (100 mg, 4.16 mmol) in anhydrous dimethyl sulfoxide (10 mL) at rt for 1 h. Dimethyl sulfate (0.40 mL, 4.16 mmol) was added, and the mixture was cooled with ice with stirring for 2 h before adding cold water. The resulting precipitate was filtered off, washed with water, and dried to yield N-methyl-5-nitroisatin (3) as a yellow solid (200 mg, 95%), mp 145–146°C (lit. 132–134°C [19]). IR (KBr) v cm⁻¹: 3,063 (C–H aromatic), 2,945 (C–H sp³), 1,743 (C=O), and 1,608 cm⁻¹ (C=C aromatic). ^1H-NMR (500 MHz, acetone-d6): δ, ppm 3.36 (s, 3H, CH₃), 7.41 (d, J = 9.1 Hz, 1H, ArH), 8.32 (d, J = 2.6 Hz, 1H, ArH), and 8.60 (dd, J = 9.1, 2.6 Hz, 1H, ArH).

2.2 Synthesis of 3-hydroxy-3-(1H-pyrrol-2-yl) indolin-2-one (5)

A solution of isatin (1) (0.15 g, 1.02 mmol) in methanol-water (1:1) (20 mL) was stirred at 50°C, and then potassium carbonate (7.0 mg, 0.051 mmol) and pyrrole (4) (71µL, 1.02 mmol) were added. After stirring for 30 h, cold water was then incorporated, and the product was extracted several times with dichloromethane. The extracts were combined, dried over magnesium sulfate, followed by evaporation under reduced pressure. Subsequently, the crude product was purified using column chromatography with chlorofom:ethyl acetate (3:1) eluant to yield 3-hydroxy-3-(1H-pyrrol-2-yl)indolin-2-one (5) as a black solid (100 mg, 45%), mp 151–152°C. IR (KBr) v cm⁻¹: 3,375 (N–H), 3,198 (O–H), 1,710 (C=O), and 1,622 cm⁻¹ (C=C aromatic). ^1H-NMR (500 MHz, acetone-d6): δ, ppm 5.37 (s, 1H, O–H), 5.67 (1H, d, ArH pyrrole), 5.90 (t, 1H, ArH pyrrole), 6.80 (d, 1H, ArH pyrrole), 6.89 (1H, d, ArH isatin), 7.02 (1H, t, ArH isatin), 7.23 (1H, t, ArH isatin), 7.27 (1H, d, ArH isatin), 9.26 (bs, 1H, N–H isatin), and 10.04 (bs, 1H, N–H pyrrole). ^13C-NMR (125 MHz, acetone-d6): δ, ppm 73.3, 107.1, 107.4, 110.0, 119.4, 122.0, 125.2, 129.4, 129.8, 131.8, 141.9, and 177.3. HRMS (ESI): m/z calcd for C₁₂H₅N₃O₄, [M – H]+ 213.2121; found: 213.1722.

2.3 Synthesis of 3-hydroxy-5-nitro-3-(1H-pyrrol-2-yl)indolin-2-one (6)

A solution of 5-nitroisatin (2) (72 mg, 0.37 mmol) in methanol:water (1:1) (10 mL) was stirred at 50°C, and then potassium carbonate (2.6 mg, 0.019 mmol) and pyrrole (4) (0.026 µL, 0.37 mmol) were added. After stirring for 5 h, cold water was added, and the product was extracted several times with dichloromethane. These combined extracts were dried over anhydrous magnesium sulfate and evaporated under reduced pressure to produce 3-hydroxy-5-nitro-3-(1H-pyrrol-2-yl)indolin-2-one (6) as a green solid (49 mg, 51%), mp 163–164°C. IR (KBr) v cm⁻¹: 3,375 (N–H), 3,279 (O–H), 1,720 (C=O), and 1,627 cm⁻¹ (C=C aromatic). ^1H-NMR (500 MHz, acetone-d6): δ, ppm 5.82–5.83 (m, 1H, ArH pyrrole), 5.84 (bs, 1H, OH), 5.97–5.99 (m, 1H, ArH pyrrole), 6.89–6.90 (m, 1H, ArH pyrrole), 7.18 (d, J = 9.1 Hz, 1H, ArH isatin), 8.28 (dd, J = 9.1, 2.6 Hz, 1H, ArH isatin), 8.36 (d, J = 2.6 Hz, 1H, ArH isatin), 9.95 (bs, 1H, NH pyrrole), and 10.30 (bs, 1H, NH isatin). ^13C-NMR (125 MHz, acetone-d6): δ, ppm 74.9, 108.6, 108.8, 111.3, 121.3, 121.9, 127.0, 129.0, 134.3, 144.9, 149.1, and 180.3. HRMS (ESI): m/z calcd for C₁₂H₁₀N₃O₄, [M + H]+ 260.0671; found: 260.0674.

2.4 Synthesis of 3-hydroxy-N-methyl-5-nitro-3-(1H-pyrrol-2-yl)indolin-2-one (7)

A solution of N-methyl-5-nitroisatin (3) (110 mg, 0.53 mmol) in methanol:water (1:1) (20 mL) was stirred at 50°C, and then potassium carbonate (5.37 mg, 0.039 mmol) and pyrrole (4) (26 µL, 0.37 mmol) were added. After stirring for 2 h, cold water was added, and the product was extracted
several times with dichloromethane. These extracts were dried over magnesium sulfate and evaporated under reduced pressure to generate 3-hydroxy-5-nitro-3-(1H-pyrrro-2-yl)indolin-2-one (7) as a green solid (88 mg, 63%), mp 139–140°C (lit. 102–103°C [20]). IR (KBr) ν cm⁻¹: 3,543 (N–H), 3,325 (O–H), 1,710 (C=O), and 1,614 cm⁻¹ (C=C aromatic). ¹H-NMR (500 MHz, acetone-d₆): δ, ppm 5.83–5.82 (m, 1H, ArH pyrrole), 5.89 (bs, 1H, OH), 5.96–5.98 (m, 1H, ArH pyrrole), 6.83–6.81 (m, 1H, ArH pyrrole), 7.25 (d, J = 7.8 Hz, 1H, ArH isatin), 8.34–8.37 (m, 2H, ArH isatin), and 10.32 (bs, 1H, NH pyrrole). ¹³C-NMR (125 MHz, acetone-d₆): δ, ppm 29.9, 73.8, 108.1, 109.6, 121.0, 121.1, 127.3, 129.3, 133.3, 144.3, 150.3, and 176.9. HRMS (ESI): m/z calc'd for C₁₃H₁₂N₃O₄, [M + H]+ 274.2521, found: 274.2660.

2.5 Synthesis of 5-amino-3-hydroxy-3-(1H-pyrro-2-yl)indolin-2-one (8)

5-Amino-3-hydroxy-3-(1H-pyrro-2-yl)indolin-2-one (8) was produced by reducing 3-hydroxy-5-nitro-3-(1H-pyrrro-2-yl)indolin-2-one (6). This reduction involved heating a mixture of 6 (75 mg, 0.29 mmol) and Pd/C (7 mg) in ethanol (10 mL) at reflux for 1 h. Then, hydrazine hydrate (40 equiv) was added dropwise, followed by heating at reflux for an additional 1 h and subsequently filtered after cooling. The filtrate was evaporated under reduced pressure, followed by crude product purification using column chromatography with chloroform:ethyl acetate (1:3). Then 8 was generated as a brown solid (31 mg, 47%), mp 170–171°C. IR (KBr) ν cm⁻¹: 3,365 (NH₂), 3,225 (O–H), 1,703 (C=O), and 1,624 cm⁻¹ (C=C aromatic). ¹H-NMR (500 MHz, DMSO-d₆): δ, ppm 4.80 (2H, bs, NH₂), 5.59 (1H, s, OH), 5.91 (1H, d, ArH pyrrole), 5.90–5.91 (m, 1H, ArH pyrrole), 5.99–6.00 (m, 1H, ArH pyrrole), 6.68–6.79 (m, 1H, ArH pyrrole), 7.23 (d, J = 9.0 Hz, 1H), 8.26 (dd, J = 9.0, J = 2.6 Hz, 1H, ArH isatin), 8.36 (d, J = 2.6 Hz, 1H, ArH isatin), 9.88 (bs, 1H, NH isatin), and 10.76 (bs, 1H, NH pyrrole). ¹³C-NMR (125 MHz, DMSO-d₆): δ, ppm 74.3, 106.8, 107.0, 110.4, 112.7, 114.3, 119.3, 131.1, 131.7, 133.5, 144.3, and 177.7. HRMS (ESI): m/z calc'd for C₁₂H₁₀N₂O₂, [M + Na]+ 252.2224, found: 252.1999.

2.6 Cell culture conditions

The HepG2 cell line was obtained from the Agency for Assessment and Application of Technology, Indonesia. The cells were routinely maintained and grown at 37°C, 5% CO₂ in a 95% humidified atmosphere. Additionally, the growth medium was prepared from Roswell Park Memorial Institute (RPMI) 1640 (Gibco) using phenol red, 2 mM glutamine, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 1 mM sodium pyruvate, and 10% foetal bovine serum (FBS), which was previously inactivated at 56°C for 30 min. Cell passaging was performed using 4 mL of trypsin-EDTA at room temperature for 3 minutes. A total of 10 mL of media with 10% FBS was then used to reduce the action of trypsin on cells, and the resulting cells were plated after centrifugation.

2.7 Preparation of cytotoxicity test solutions

The stock solutions of 5–8 and the doxorubicin control compound were individually combined with dimethyl sulfoxide (DMSO) and diluted serially in RPMI to yield the varying concentrations (12.5, 25, 50, 100, 200, and 400 µg/mL). A final concentration of 0.1% DMSO was obtained in the medium, and this was also used in the corresponding control. Additionally, no serum or antibiotics were introduced to the test and control mediums. All solutions were freshly prepared and protected from light.

2.8 Cytotoxicity test

The cytotoxicity test was performed using the MTT method [21]. The HepG2 cells were maintained as monolayer cultures in RPMI 1640 medium and supplemented with antibiotics, including 100 IU/mL penicillin and 100 µg/mL streptomycin, and 10% FBS in a humidified incubator containing 5% CO₂ at 37°C. The subcultures were obtained by trypsin treatment of confluent cultures, and the resulting suspension (100 µL) (5 × 10⁵ cells) was transferred to 96 well plates. These were then incubated in a CO₂ incubator for 24 h. The cell culture medium in each well was discarded and replaced with 100 µL of test solutions at various concentrations or the positive control (DMSO) before incubation for 24 h. Phosphate-buffered saline solution and 100 µL of MTT (0.5 mg/mL) were added to the wells, and the cells were incubated for an additional 4 h until blue coloured formazan crystals were observed. Subsequently, a 10% solution of sodium dodecyl sulfate in 0.1 N HCl was added, and the cells were incubated for the next 4 h at room temperature. The
absorbance was measured using an ELISA plate reader at 570 nm, and the percentage of cell viability was then calculated. The IC\textsubscript{50} value was determined by plotting the percentage of cell viability against sample concentration, and the assay was performed in triplicate.

**Ethical approval:** The conducted research is not related to either human or animal use.

3 Results and discussion

\(N\)-Methyl-5-nitroisatin (3) was synthesized using techniques from previous works [22–24]. This synthesis involved a reaction between 5-nitroisatin (1) and sodium hydride and dimethyl sulfoxide, followed by a reaction with dimethyl sulfate to generate the yellow solid \(N\)-methyl-5-nitroisatin (2) (Scheme 1). Subsequently, the structure of 3 was confirmed with (1) FT-IR, where the spectrum showed peaks at 3,063, 2,945, 1,743, and 1,608 cm\(^{-1}\) designating C–H aromatic, C–H sp\(^2\), C=O, and C=C aromatic groups, respectively. (2) In \(^1\)H NMR, the spectrum showed a singlet at 3.55 ppm which indicated methyl group protons and two doublets at 7.41 and 8.32 ppm and another doublet at 8.60 ppm for aromatic protons. A previous report [25] showed the presence of singlet signal at 3.38 ppm for methyl group protons, based on \(^1\)H NMR data (in CDCl\(_3\)). However, the chemical shift reported in this research at 3.55 ppm due to measurement was carried out in different solvents (in acetone-\(d_6\)); and the absence of NH proton signal in the NMR data suggests the successful synthesis of compound 3.

The isatin-pyrrole derivatives 5–8 were prepared through a reaction between the appropriate isatins 1–3 and pyrrole (4), using method from previous work for indoles [26]. This process was initiated by dissolving the isatins in methanol:water, followed by the introduction of potassium carbonate as a catalyst. Then pyrrole (4) was added to obtain the final derivative products 5–8 (Scheme 2). The yields were of acceptable purity and were further subjected to analysis using FT-IR, \(^1\)H-NMR, \(^{13}\)C-NMR, and mass spectrometry. Additionally, the FT-IR spectra showed peaks at the 3,375–3,426 cm\(^{-1}\) region, indicating an N–H group, at 3,198–3,325 cm\(^{-1}\) for an O–H group, at 1,703–1,720 cm\(^{-1}\) for a carbonyl group, and at 1,624–1,627 cm\(^{-1}\) for a C–C aromatic. The \(^1\)H NMR spectra showed greater deshielding in the NH pyrrole than the NH isatin, and the inverse was the case with the aromatic protons. This was due to the relatively lesser aromatic characteristics of pyrrole. The isatin-pyrrole derivative 7 showed a singlet resonance in its \(^1\)H NMR at 3.27 ppm (in acetone-\(d_6\)) for the methyl group protons, which is similar with Li et al. data [20] at 3.25 ppm (in CDCl\(_3\)). Moreover, \(^{13}\)C NMR spectra exhibited peaks corresponding to quartener carbons (C-3) at 73.3–74.9 ppm, carbonyls at 176.9–180.3 ppm, and the quaternary aromatic carbons were less deshielded than the tertiary form. The treatment of isatin derivative (6) with hydrazine and palladium on charcoal in ethanol led to the production of compound (8), following nitro group reduction method of previous work [27]. This exhibited an FT-IR spectrum with an NH\(_2\) peak at 3,543 and 3,423 cm\(^{-1}\) for unsymmetrical and symmetrical N–H, respectively.

The cytotoxicity test of isatin derivatives (5–8) against the liver cancer cell line HepG2 was performed using a colorimetric method. This method was based on the ability of mitochondrial dehydrogenase enzyme to convert 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan, indicated by a colour change from yellow to blue. Furthermore, the result was analysed using an ELISA reader, and the IC\textsubscript{50} values of 5–8 against HepG2 cells are shown in Table 1. The results showed the ability for substitutions at C-5 and the presence of N-methyl on the isatin scaffold to influence bioactivity. Meanwhile, isatin-pyrrole 6 bearing a nitro group at C-5 of the isatin scaffold was identified as the most active compound, due to the IC\textsubscript{50} of 0.47 \(\mu\)M, although the N-methyl group tends to reduce the effect.

**Scheme 1:** Synthesis of 3. Reagents/conditions: (a) (i) NaH (4 eq), DMSO, rt 1 h; (ii) DMS (4 eq), cold 2 h, 95%.

**Scheme 2:** Synthesis of 5–8. Reagents/conditions: (a) 4 (1 eq), MeOH:H\(_2\)O (1:1), K\(_2\)CO\(_3\), 50°C 2–3 h, 5 (45%), 6 (51%), 7 (63%); (b) (i) Pd/C, EtOH, reflux 1 h; (ii) NH\(_2\)NH\(_2\):H\(_2\)O (40 eq), EtOH, reflux 1 h, 8 (47%).
Table 1: Anticancer activity of synthesized compounds

| Compounds                  | IC50 (µM) |
|----------------------------|-----------|
| (5)                        | 10.33     |
| (6)                        | 0.47      |
| (7)                        | 1.33      |
| (8)                        | 4.64      |
| Doxorubicin                | 0.00035   |

4 Conclusions

A total of four isatin-pyrrole derivatives (5–8) were successfully synthesized in good yield and purity, and the structure was confirmed using FTIR, NMR, and MS. These products were tested for anticancer activity using the liver cancer cell line HepG2, and their IC50 values were calculated. The cytotoxicity assay of all compounds showed moderate action, although (6) exhibited the highest effect, with an IC50 of 0.47 µM.

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Data availability statement: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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