RESEARCH ARTICLE

Synthesis and Evaluating the Antimicrobial Activities of Various Adducts Prepared from Isatins and Proline

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Received: 31st January, 2022; Revised: 24th March, 2022; Accepted: 10th April, 2022; Available Online: 25th June, 2022

ABSTRACT

Isatin (indolines) play a well-known role as building units for so many compounds synthesized to obtain certain agents that target characteristic tissues and cells, exerting their pharmacological aspects and alleviating a lot of diseased processes. Accordingly, this research is about introducing some isatins to be nucleophilically attacked at C3 forming products of azomethine ylide functionality. These iminium compounds were made by allowing certain isatins to be reacted with the secondary amino acid, proline, at acetic acid and methanol medium and then collected after purification to be identified with total Leukocyte count (TLC) and melting point. The structural characterization was performed by fourier-transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (^{1}H-NMR), and community health nursing (CHN) analysis. The microbiological evaluation was proved with the disc diffusion method on cultured agars of *Staphylococcus aureus,* some Gram-negative *bacilli,* and the fungus *Candida albicans* using more than one concentration of the prepared molecules. It was found that the isatin adduct has no activity, whereas the others, having changed in substituents at position 5, are fluctuated in their action results.

Keywords: Adducts, Antimicrobial activity, Azomethine ylide, Isatin, L-proline.

International Journal of Drug Delivery Technology (2022); DOI: 10.25258/ijddt.12.2.38

How to cite this article: Abdulkadir MQ, Abdulwahab AH, Al-Mudhafar MMJ. Synthesis and Evaluating the Antimicrobial Activities of Various Adducts Prepared from Isatins and Proline. International Journal of Drug Delivery Technology. 2022;12(2):682-687.

Source of support: Nil.

Conflict of interest: None

INTRODUCTION

New molecules, having antimicrobial properties, are continuously prepared to be active against serious infestations with infectious microbes that resist the classical chemotherapeutic agents.[1,2] The endogenously isolated substance in many organisms is isatin (1H-indole-2, 3-dione) which shows widely ranged vital roles[3] and its ring makes moiety necessary in many medicinally effective molecules.[4] Isatin-derived compounds possess antibacterial[5] and antifungal[6] actions whereas isatin-based Schiff and Mannich bases have exerted anti-human immunodeficiency virus (HIV) activities too.[7-9] Isatin-based compounds have variable chemical properties so that they can be used as reactants to synthesize many biologically active products.[10-12] Isatin-established hydrazines were discovered to act against Walker carcinosarcoma 256.[13] Also, acetone and ketone-derivatives of isatin exhibited anticonvulsant properties.[14] Thiosemicarbazones were also prepared as derivatives of isatin to serve as research reagents in physiology.[15-17] In the same manner, a lot of newly made ingredients; that include isatin nucleus; were tested for their anti-tuberculosis,[18,19] antileprotic,[13] antifungal,[20,21] anti-viral,[22] antibacterial,[23,24] and as anticonvulsants.[25,26] Also, an isatin Schiff base 5'-(2,3-Dihydroxybenzylidene amino)spiro[1,3] dioxolane-2,3'-indoline]-2'-on was displayed the highest antioxidative activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) way of analysis.[27] Moreover, different dispiropyrrolidines derived from azomethine ylide of isatin and pyrrolidine-2, 5-dione derivatives showed high antibacterial activity against *Bacillus subtilis*, *S. aureus*, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Proteus vulgaris*.[28]

Since few years, Azomethine ylides have become a great target for synthesizing unnatural pyrrolidine and proline scaffolds, demonstrating the preferred structure of these polysubstituted pyrrolidine rings.[29]

Accordingly, adduct products or ylides of different isatins are going to be planned and prepared to be chemically represented as an iminium, using the secondary hetero-nitrogen-containing amino acid; L-proline, in which the quaternary nitrogen together with the neighboring anion creates a structure resembling *Zwitterion* that may help the new molecules to enter the bacterial cell of some Gram-negative *bacilli* via porins to exert their antibacterial action intracellularly.

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EXPERIMENTAL

Materials

Isatin was obtained from Hi-Media Laboratories, India, 5-fluoroisatin, 5-bromoisatin, and 5-methoxyisatin from Hangzhou Hyper Chemicals Limited, China. L-Proline was bought from BDH chemicals, England.

Synthesis

The synthesis of different adducts is illustrated in Figure 1:

Synthesis of the isatin-proline adduct (I) [1, 3-dihydro-3-{(1-pyrrolidinyl)iminium}-2H-indol-2-one]

Isatin (1 gm, 6.8 mmol) was solubilized together with proline (0.94 g, 8.16 mmol) in 20 mL methanol and then three drops of glacial acetic acid were added allowing the mixture to stir for 1/2 hours. At room temperature followed by reflux for 1.5 hours. The flask was cooled, the mixture was filtered, and the residue was dried and collected for washing with hot water and 5% sodium bicarbonate. The washed solid was recrystallized using N, N- dimethylformamide (DMF)/water system, dried, and collected to provide identification methods.

Synthesis of 5-methoxyisatin-proline adduct (II) [1, 3-dihydro-3-{(1-pyrrolidinyl)iminium}-5-methoxy-2H-indol-2-one]

5-Methoxyisatin(1g,5.65 mmol) and proline (0.95g, 8.16 mmol) were stirred together at room temperature, in 20 mL methanol and glacial acetic acid (3 drops), for 1/2 hour. which is then followed by reflux for 1.5 hours. The system was then cooled and filtered to let the solvent evaporate, leaving the residue, which was then dried and collected for washing with cold water and 5% sodium bicarbonate. The washed powder was then recrystallized from DMF/water mixture, dried, and collected for later characterization procedures.

Synthesis of 5-fluoroisatin-proline adduct(III), [1, 3-dihydro-3-{(1-pyrrolidinyl)iminium}-5-fluoro-2H-indol-2-one]

5-Fluoroisatin (1 g, 6.06 mmol) and proline (0.95 g, 8.16 mmol) were mixed in presence of methanol (20 mL), and of the catalyst, glacial acetic acid (3 drops). This mixture was then refluxed for 3hr, cooled, and filtered. Methanol was then evaporated to leave the residual solid, which was then washed well with water and 5% sodium carbonate. Then it is recrystallized from a methanol/water mixture, dried, and collected for further analysis ways.

Synthesis of 5-bromoisatin- proline adduct(IV)[1, 3-dihydro-3-{(1-pyrrolidinyl)iminium}-5-bromo-2H-indol-2-one]

The amino acid, proline (0.95 g, 8.16 mmol) was reacted with 5-bromoisatin (1 g, 4.425 mmol) in the acidic medium; methanol (20 mL) containing anhydrous acetic acid (3 drops); and left for 3 hr-reflux distillation. The distillate was then cooled, filtered, and allowed to evaporate methanol solvent leaving the solid product which was then washed with water and 5% sodium bicarbonate. The compound was then recrystallized from a methanol/water system, and dried to be ready for identifying techniques.

Identification and Characterization

Melting Point

The melting point was measured for each of the ylides produced under atmospheric pressure, and the values are illustrated in Table 1.

Solubility

Solubility of the obtained adducts was studied under laboratory conditions using protic and aprotic solvents like water, methanol, ethanol, DMF, chloroform, and 5% sodium bicarbonate solution. The results were varied and are shown in Table 2.

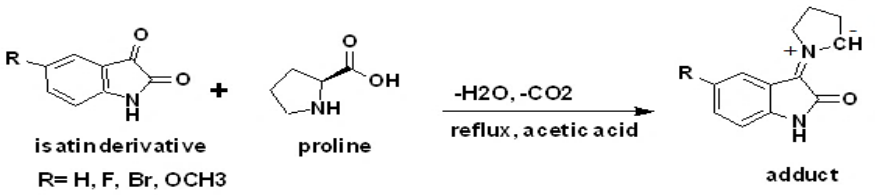


Figure 1: Synthesis of Azomethine ylides (adducts) of Isatins

Table 1: The melting points of the final compounds compared to the starting precursors isatins

Isatins	*Melting points °C*	*Adducts*	*Melting points °C*
Isatin	203(d)	I	296(d)
5-Methoxyisatin	195–198	II	163(d)
5-Fluoroisatin	224	III	380(d)
5-Bromoisatin	253	IV	303(d)

Table 2: The solubility of the synthesized products in different solvents

Adducts	*Water*	*Methanol*	*Ethanol*	*DMF*	*Chloroform*
I	Insoluble	Insoluble	Very slightly soluble	Soluble	Very slightly soluble
II	Insoluble	Soluble	Slightly soluble	Soluble	Slightly soluble
III	Very slightly soluble	Soluble	Slightly soluble	Soluble	Soluble
IV	Very slightly soluble	Soluble	Slightly soluble	Soluble	Soluble

Table 3: Physical description, percent yield, empirical formula, and molecular weight of the synthesized products

Compound	*Physical description*	*Percent yield (%)*	*Empirical formula*	*Molecular weight (g/mole)*
I	Blue powder	84	$C_{13}H_{13}N_2O_3$	245
II	Brown powder	71	$C_{14}H_{15}N_2O_4$	275
III	Red-deep brown crystalline powder	58	$C_{13}H_{12}N_2O_3F$	263
IV	Light brown crystalline powder	53	$C_{13}H_{12}N_2O_3Br$	324

Table 4: The obtained wavelengths of maximum visible light absorbance values of resultant molecules

The Adduct	*Max (nm)λ*
I	580
II	245
III	240
IV	242

Table 5: The elements percent of each produced compound

Compound	*C% found (calculated)*	*H% found (calculated)*	*N% found (calculated)*
I	72.66(71.98)	6.44 (6.04)	13.07 (13.99)
II	68.53 (67.81)	6.45(6.13)	11.98(12.17)
III	67.124(66.05)	4.897(5.08)	11.728(12.84)
IV	52.87(51.63)	4.02 (3.97)	9.89(10.04)

Table 6: FTIR frequencies of characteristic groups of the final compounds

Compound	*>CH (υ in cm^{-1})*	*N-H (υ in cm^{-1})*	*C=O (υ in cm^{-1})*	*C=N (υ in cm^{-1})*
I	2866.2	3406.3	1716.6	1616.4
II	2858.5	3251.4	1708.9	1604.8
III	2873.9	3213.5	1708.9	1627.9
IV	2831.5	3197.9	1716.6	1616.3

Physical Description

The solids that represent the iminium compounds prepared are crystalline powders with different colors for each as mentioned in Table 3.

Thin-layer Chromatography

This was achieved by using a water/ethanol solvent mixture with two different ratios of 1/1 and 4/1.

Ultraviolet Spectroscopy

This measurement was done by preparing solutions of the target compounds in DMF at a concentration of 1 mg/25 mL to be ready for determining UV-visible spectra at the range of 200–750 nm to fix the length of the wave at which the highest value of excitation occurs. This is illustrated in Table 4.

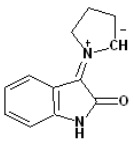
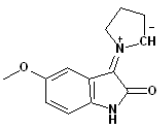
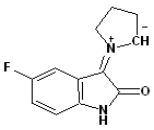
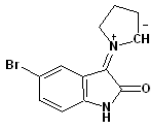
CHN Analysis

Elemental evaluation for the carbon, hydrogen, and nitrogen forming the new molecules, was analyzed, and the values were as accurate as those calculated theoretically as illustrated in Table 5.

Infrared Spectrophotometry

The synthesized isatins-proline conjugates were analyzed by the FTIR instrument giving charts that ensure that the reaction proceeded in the forward direction of forming the desired substances or not. The obtained data are illustrated in Table 6.

Table 7: ^{1}H-NMR spectral data (δ ppm) for synthesized compounds

Compound	*Structures*	*^{1}HNMR Spectral data(δ ppm)*
I		1.19(m,2H,-methylene); 1.66(m,4H,two methylene groups);7.14-7.5 (m,4H,Ar-H); 11.1(s,1H,N-H)
II		1.20(m,2H,-methylene); 1.66(m,4H,two methylene groups); 3.80(S,3H, OCH3); 6.9-7.48 (m,3H,Ar-H); 9.08(s,1H,N-H)
III		1.15(m,2H,-methylene); 1.68(m,4H,two methylene groups); 6.89-7.97 (m,3H,Ar-H); 8.58(s,1H,N-H)
IV		1.22(m,2H,-methylene); 1.78(m,4H,two methylene groups); 7.44-7.51 (m,3H,Ar-H); 9.1(s,1H,N-H)

^{1}H-NMR Spectroscopy

The structural composition was further approved by determining the protons of each moiety with the aid of the nuclear magnetic resonance measurement technique, and it was the expected data as appeared in Table 7.

Studying the Antibacterial and Antifungal Activity

Determination of anti-infective effects for these prepared products was done with the aid of the microbiology center laboratory of *Ibn Al-Haitham* Science College using Gram-positive *S. aureus* and Gram-negative *Pseudomonas, Klebsiella,* and *Escherichia coli.* In addition, the antifungal effect was also tested against *Candida albicans.* The method used is the agar diffusion method using the culture-sensitivity test in which these compounds were dealt with to obtain discs of 200 µg/mL and 400 µg/mL of each employed powder dissolved in DMF. The results were viewed at Tables 8 and 9, respectively.

RESULTS AND DISCUSSION

Synthesis

The molecules synthesized are in the form of iminium adduct, and they were formed with the aid of reflux and acidic catalyst.

Table 8: The zone of inhibition (mm) using a concentration of 200ug/ml of each iminium adduct produced in DMF compared with a control

Adducts	*Staphylococcus aureus*	*Escherichia coli*	*Klebsiella pneumonia*	*Pseudomonas aeruginosa*	*Candida albicans*
I	---	---	---	---	---
II	12	---	---	12	---
III	---	---	---	---	---
IV	13	---	---	14	---
Control	---	---	---	---	---

Table 9: The inhibition zone values (mm) resulted in a concentration of 400µg/ml of the four new compounds in DMF in comparison to those of Amoxicillin and Cefepime as reference antibiotics and to control

Adducts	*Staphylococcus aureus*	*Escherichia coli*	*Klebsiella pneumonia*	*Pseudomonas aeruginosa*	*Candida albicans*
I	---	---	---	---	---
II	20	12	---	25	---
III	19	11	---	16	---
IV	18	---	---	14	---
Amoxicillin	50	---	17	40	---
Cefepime	55	30	44	24	---
Fluconazole	---	---	---	---	30
Control	---	---	---	---	---

(---) = No1activity, slightly1active (inhibition1zone between 5-10 mm), moderately active (inhibition1zone between 10-20 mm), highly1active (inhibition1zone more than 20 mm)[30]

This reaction is a nucleophilic substitution that begins with the nucleophilic attack as the same as Michael's addition, followed by dehydration and decarboxylation. The C3 of isatin is sensitive to a nucleophile attack higher than proline ring C2 at which the possibility of positive charge to concentrate is low because of the sure resonance (of cyclic amide part, lactam) with the neighboring isatins secondary hetero-nitrogen, which tends to donate its pair of electrons. So, the electron deficiency will be more likely obvious at C3, which is ready for accepting the nucleophilic species represented by the secondary hetero-nitrogen of proline which would easily replace the carbonyl oxygen at C3 only.

Identification and Characterization

Melting Point Determination

The melting point values are at centigrade. All adducts prepared had shown higher melting points than those of their corresponding isatins. This might be due to the appearance of two adjacent opposite charges along with the same bond. These charges are represented by the positively charged quaternary iminium nitrogen and the neighboring α-carbanion. The formation of such molecular crystals could make the compound highly ionic even in the solid state, and it may be the reason for unique strong intermolecular ionic forces rendering salt-like molecules by exerting elevated melting point values.

Solubility

Adducts **I** and **II** are water-insoluble to the extent that they are non-wettable by water and remain fluffy whereas compounds **III** and **IV** (5-haloadducts) had some water solubility to the extent of being very slightly soluble. This might be due to the same intra-molecular ionic forces previously mentioned. This interaction can be obvious, especially when we washed adduct **I** and **II** even with hot water, which is un-sufficient to break it and solvate the molecule. In contrast, adduct **III** and **IV** have formed, in addition to those forces, weak hydrogen bonding (between its halogen atom and water hydrogen) with water molecules and this force is enough to solvate a little number of molecules in water and also it may be the causative factor for halo-isatins and their prepared derivatives to exert highest melting point values among others.

Physical Description

Physical description is summarized in the below table:

Thin-layer chromatography and R*f values determination*

The effect of intra-molecular ionic interaction and the lipophilic property of the hydrophobic part of the molecule could be also seen on the R*f* values especially adduct I and II in that no movement if water: ethanol (4:1) system is used whereas a little movement (0.27 and 0.43) is recorded when (1:1) ratio respectively is applied. On the other hand, compounds III and IV have a higher tendency to move with the solvent system (4:1), achieving R*f* values of 0.18 and 0.29 respectively but, in the system (1:1), both reported the values of 0.52 and 0.49 respectively. These halogenated derivatives have such values due to the previously mentioned possible intermolecular hydrogen bonds that permit them to slight solvation and movement with water and ethanol during the TLC procedure.

UV-Visible Spectroscopy

Conversion of isatins to its iminium derivatives had been showing variable absorption bands depending on the obtained products excitability that will determine the energy needed to be absorbed in that if the derived molecule has sufficient stability and fewer electrons available to excite, this means needing higher energy to be absorbed and shorter wavelength

(hypsochromic shift from the original band position of the parent isatins). On the other hand, if the conjugate has enough electrons to absorb energy easily, this means less energy required and a longer length of wave (bathochromic shift). This stability may be confirmed with the possible resonance over the C=N=C axis. The driving force for this delocalization is the carbanion with the fixed quaternary nitrogen positive charge of iminium moiety, and the tendency of such negative charge translocation to occur might be affected with the existence of 5-substituent varied as electron-donating or -withdrawing and the values are illustrated with the Table 4.

CHN Analysis

CHN Analysis is summarized in the Table 5.

Infrared Measurement

The FTIR spectra for compounds **I-IV** was given in table 6**,** the characteristic IR bands for these adducts displayed at 1604.8-1627.9 cm^{-1} were due to stretching vibration of the new imine group (C=N).

^{1}HNMR Analysis

The protons of different structural functionalities were run using a nuclear magnetic resonance instrument, and the spectra were valuable results and with an acceptable precision range as shown in Tables 6 to 9.

Antimicrobial Survey

This study revealed a slight activity of compound **II** and **IV** at the lower used concentration against both *Staphylococcus aureus* and *Pseudomonas aeruginosa*, whereas they showed a moderate to good activity at the higher concentration, Tables 8 and 9. Additionally, *Escherichia coli* are faintly susceptible to the concentrated disc of compounds **II** and **III**. Isatin adduct (compound **I**) has no action against all the types of bacteria used at both concentrations. Also, all prepared molecules do not exert any zone of inhibition, at these concentrations, against *Klebsiella Spp.* and the fungus, *Candida albicans*. The possible rationalization about such findings is that methoxy and halogens substituents at the C5 of isatin ring will make these final *iminium Zwitterions* active against the used *Gram-negative Pseudomonas* and *Escherichia* via their specific protein, porin. The compound (**II**), substituted at position 5 with an electron-donating group (5-methoxyisatin adduct), has little more activity than those with electron-withdrawing halogens (5-fluoroisatin adduct (**III**) and 5-bromoisatin adduct (**IV**)). Also, the size of the 5-substituent may play a role in the activity of adduct **II** and **IV** in that the bromine atom and methoxy group have a larger size than the fluorine atom and the results gotten are the reflections for this illustration. On the other hand, compounds **I**, **II**, and **III** might have an ideal physicochemical property (partition coefficient) to penetrate the lipid membrane of the gram-positive *staphylococcal* cell. As a final consideration, these adducts can do all these probabilities as bactericidal agents according to the previous mechanisms of action. Finally, the explanation about the resistance of *Klebsiella* to all these prepared adducts is the possible ability of these microorganisms to change the nature of the porin proteins or download the number of the pores which permit this bacterial species to survive against the antibacterial properties of the user agents.

CONCLUSION

These final compounds were successfully prepared according to the mentioned chemical considerations and methods, analyzed qualitatively and identified by the explained techniques, and at last, tested and scanned for their antimicrobial effects. It was noticed that the un-substituted agent (adduct **I** or isatin derivative) was devoid of any activities with the used microorganisms, whereas those whose 5-substituents are either atoms (like electron-withdrawing halogens and exemplified with adducts **III** and **IV**) or a group (like electron-releasing methoxy represented with adduct **II**), were showing appreciable, variable and characteristic effects (ranging from moderate to high) against *S. aureus* and *P. aeruginosa* under the previously mentioned conditions and concentrations.

ACKNOWLEDGMENT

The continuous support of the University of Baghdad is greatly acknowledged.

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