ANTIBACTERIAL ACTIVITY AND PHOTOLYTIC STABILITY OF SYNTHESIZED 5-CHLOROISATIN-3-HYDRAZONE

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Isatin is an important precursor for the synthesis of various pharmacologically active heterocyclic compounds. Its derivatives possess antimicrobial, antiviral, anti-inflammatory, anticonvulsive, anti-HIV, anticancer activities, etc. In this paper, 5-Chloroisatin-3-hydrazone was synthesized and identified by elemental microanalysis, as well as FTIR and UV/VIS methods. Its antibacterial activity was tested against *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 43895 and *Proteus vulgaris* ATCC 8427 in different concentrations. In case of Gram-negative bacteria *Proteus vulgaris*, 5-Chloroisatin-3-hydrazone showed to be the most efficient at all tested concentrations (inhibition zone is 25 mm) at the concentration of 500 $\mu\text{g}\cdot\text{cm}^{-3}$. The photolytic stability of synthesized 5-Chloroisatin-3-hydrazone was also investigated by using UV-B and UV-C cumulatively irradiation at different intervals of time. With the increase in the energy of the incoming photons, the rate of degradation 5-Chloroisatin-3-hydrazone also increases. This suggests that the stability of the compound depends on the radiation dose, i.e., on the energy of the incoming photons. The results show that photolysis kinetics obeys the equation of the pseudo-first order. The mechanism of photolysis can be suggested as hydrogen abstraction from DMF by the triplet state of the isatin carbonyl group, with the formation of ketyl and DMF radicals.

Keywords: 5-Chloroisatin-3-hydrazone, synthesis, spectroscopic analysis, antibacterial activity, photolytic stability

Introduction

Isatin and its derivatives are very important intermediates in the synthesis of different pharmaceutical active components [1]. As an endogenous compound, isatin can be found in plants of the genus *Isatis*, in *Calanthe discolor* LINDL and in *Couroupita guianensis* Aubl [2]. It also represents a consistent component of the secretion from the parotid gland of *Bufo* frogs [2] and is a constituent of different alkaloids, drugs, dyes, and pesticides [3]. Antimicrobial, antiviral, antiretroviral (anti-HIV), anticonvulsive, antitumor and anti-inflammatory activities of isatin and its derivatives are well described in the literature [4]. Hydrazones have shown to possess antibacterial, anticancer, tuberculostatic and fungicidal properties, making them important in medicine and pharmacy [5]. Previous studies have shown that isatin and some of its derivatives in combination with benzophenone are used as auxiliary UV absorbers in pharmaceutical and plastic industries [6]. Isatin derivatives possess a significant photolytic stability against UV-A irradiation, suggesting the implying kinetic to be pseudo-first order [7,8]. Although similar compounds have been investigated as UV-absorbers, the basic mechanisms related to their interactions with UV-light are still undefined [8].

With the aim of the possible application in cosmetic and pharmaceutical formulations, 5-Chloroisatin-3-hydrazone

was synthesized, identified and tested for antibacterial activity and photolytic stability. Antibacterial activity was tested against *E. coli*, *E. faecalis*, *S. aureus*, *P. aeruginosa*, and *P. vulgaris* by the disc diffusion method. Photolytic stability of 5-Chloroisatin-3-hydrazone toward UV-B and UV-C irradiation has been investigated, too.

Experimental

Chemicals

All chemicals were of reagent grade and used without further purification. 5-Chloroisatin was provided from Fluka Chemie AG (Switzerland), and the hydrazine solution was purchased from Sigma Aldrich (Munich, Germany).

Materials and methods

Elementary microanalysis of carbon, hydrogen and nitrogen was performed using a Carlo Erba 1106 microanalyzer (Devon, United Kingdom). The purity of the synthesized compound was gained by thin layer chromatography (TLC) on silica gel using benzene: chloroform = 55:45. The compound being visualized by iodine vapors. The UV/VIS spectra were recorded on a Varian Cary-100 UV/VIS

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spectrophotometer (United States) using 1×10^{-4} mol·dm⁻³ solutions in ethanol, *N,N*-dimethylformamide (DMF) and dimethylsulfoxide (DMSO).

The FTIR spectra was recorded on a Michaelson Bomen MB-series spectrophotometer (Canada), using the KBr pastille (1.5 mg/150 mg) technique, in the range of 4000–400 cm⁻¹ wave number, and 2 cm⁻¹ resolution. The mixture is vacuumed, pressed under the pressure of 200 MPa, so as to form thin, permeable pastilles.

Cumulatively UV irradiations of the sample are carried in a cylindrical photochemical reactor "Rayonnet" with 7 symmetrically placed lamps with emission maxima $\lambda = 300$ nm for UV-B and $\lambda = 254$ nm for UV-C. The samples (DMF solution with $c = 5 \cdot 10^{-5}$ mol·dm⁻³) were irradiated in quartz cuvettes (1 x 1 x 4.5 cm) placed on rotating circular holder. The total energy flux 10.5 W·m⁻² for $\lambda = 300$ nm was calculated from the ratio of the energy of UV photons and 12.5 W·m⁻² for $\lambda = 254$ nm was calculated by using UV-meter Solarmeter SM 8.0 UVC, „Solartech" Inc.

Synthesis of 5-Chloroisatin-3-hydrazone

5-Chloroisatin-3-hydrazone was prepared by the reaction of the equimolar amount of 5-Chloroisatin (1.81 g) and hydrazine solution (0.41 cm³) in 95% ethanol, following the standard procedure [9,10]. The reaction mixture was refluxed for 45 min at 50 °C. pH was adjusted to 4.5 by adding H₂SO₄. The product, precipitated as dark-yellow solid, was collected by filtration, washed with ethanol and dried over CaCl₂.

Antibacterial activity

The compound was evaluated for its *in vitro* antibacterial activity against Gram-positive bacteria *S. aureus* and *E. faecalis* and Gram-negative bacteria *E. coli*, *P. aeruginosa* and *P. vulgaris*. The antimicrobial screening was performed by the agar diffusion method using a paper disc. The sterilized agar (autoclaved at 120 °C for 30 min) was inoculated (1 cm³/100 cm³ medium) with the suspension of microorganisms (matched to a McFarland barium sulphate standard) and poured into a Petri dish. The paper discs impregnated with 5-Chloroisatin-3-hydrazone (125 µg·cm⁻³, 250 µg·cm⁻³ and 500 µg·cm⁻³) in DMF were placed on the solidified medium. The zones of inhibition were measured after 72 h of incubation at 37 °C [9,10].

Photolytic activity

The synthesized compound dissolved in DMF (1×10^{-4} mol·dm⁻³) was irradiated by lamps with emission maxima $\lambda_{\max} = 300$ nm for UV-B and $\lambda_{\max} = 254$ nm for UV-C. 5-Chloroisatin-3-hydrazone was UV-B irradiated at intervals of 0, 15, 30, 45 and 60 min, and UV-C irradiated at intervals of 0, 5, 10 and 16 min. Changes in absorbance were monitored as a function of time.

Results and discussion

The synthesis of 5-Chloroisatin-3-hydrazone was carried out by the carbonyl-amine condensation reaction of

5-chloroisatin and hydrazine, in the presence of ethanol (Fig. 1). The formation of 5-Chloroisatin-3-hydrazone is carried out in an acidic medium (pH = 4.5) not only because of the greater reaction velocity, but also because of the water elimination, which occurs after the nucleophilic attack of hydrazone onto the isatin carbonyl group. The attack on the C=O group of isatins usually happens in the position C-3 (i.e. in the β -position), while C-2 (i.e. α -position) reacts with nucleophiles only under specific conditions, due to a negative inductive effect of the amino group [2].

Figure 1. Scheme of the synthesis reaction of 5-Chloroisatin-3-hydrazone by the carbonyl-amine condensation

5-Chloroisatin-3-hydrazone was obtained as a dark-yellow powder (yield, 90%), and it was successfully dissolved in ethanol, DMF and DMSO. The experimental and theoretical values from the elemental analysis are in a good accordance, which confirms the suggested molecular formula C₈H₆N₃OCl of 5-Chloroisatin-3-hydrazone: Experimental value: C 49.20, H 3.01, N 21.45; Theoretical value: C 49.11, H 3.07, N 21.48. The purity was confirmed by thin layer chromatography, and *R_f* value of 0.75 is in accordance with similar isatin derivatives [9].

UV/VIS spectral analysis of 5-Chloroisatin-3-hydrazone

The UV/VIS spectra of 5-Chloroisatin-3-hydrazone recorded in the solution of ethanol, DMF and DMSO ($c = 1 \times 10^{-4}$ mol·dm⁻³), and the numerical data are presented in Table 1. The spectra were recorded in different solvents to show the impact of their different polarity on bands position in this compound.

The electronic spectra of 5-Chloroisatin-3-hydrazone which are covered by with far more intense $\pi \rightarrow \pi^*$ band assignation do not possess a visible transition of $n \rightarrow \pi^*$ type, which is also characteristic of the synthesized compound [11, 12]. Ethanol, as a protic polar solvent ($\mu = 1.69$ D), allows the full range spectrum recording, starting from $\lambda_{\max} = 190$ nm. Aprotic polar solvents such as DMF and DMSO enable spectrum recording starting from $\lambda_{\max} = 270$ nm, because of their own absorption. The band that appears in the spectrum recorded in ethanol at $\lambda_{\max} = 357$ nm is bathochromically shifted for $\Delta\lambda = 11$ nm ($\lambda_{\max} = 368$ nm) and for $\Delta\lambda = 16$ nm ($\lambda_{\max} = 373$ nm) in the spectrum recorded in DMF and DMSO, respectively. Those shifts were expected, since the values of dipolar moments of these two solvents are $\mu = 3.82$ D for DMF and $\mu = 3.96$ D for DMSO [7].

Table 1. UV/VIS spectral data of 5-Chloroisatin-3-hydrazone

Solvent	$\lambda_{\max}(\text{nm})/\epsilon \cdot 10^3 (\text{mol}^{-1} \cdot \text{dm}^3 \cdot \text{cm}^{-1})$	Band assignment
Ethanol	357.00/1.3819	$\pi \rightarrow \pi^*$
	249.00/1.1114	$\pi \rightarrow \pi^*$
	229.00/1.3733	$\pi \rightarrow \pi^*$
	221.00/0.5664	$\pi \rightarrow \pi^*$
	215.00/0.4849	$\pi \rightarrow \pi^*$
DMF	368.00/1.4795	$\pi \rightarrow \pi^*$
	298.00/1.2286	$\pi \rightarrow \pi^*$
DMSO	373.00/0.8389	$\pi \rightarrow \pi^*$
	358.00/0.6179	$\pi \rightarrow \pi^*$

FTIR spectral analysis of 5-Chloroisatin-3-hydrazone

The main IR spectral bands of 5-Chloroisatin-3-hydrazone and numerical data are presented in Figure 2 and Table 2. The stretching vibration $\nu(\text{C}=\text{O})$ is bathochro-

mic shifted (1691 cm^{-1}) due to the presence of the intramolecular hydrogen bond (Fig. 3). In isatin-hydrazones, a hydrogen bond between secondary amino and keto group in the position 2 is a common one, forming a stable chelate structure. The spectrum of 5-Chloroisatin-3-hydrazone, besides the absorption vibration $\nu_{\text{S}}(\text{NH}_2)$ at 3177 cm^{-1} , exhibits a new band at 3396 cm^{-1} , as absorption vibration $\nu_{\text{AS}}(\text{NH}_2)$ of the hydrazone part of the molecule. The symmetric vibration is hardly noticeable, since it is overlapped with the absorption vibration of the secondary amino group. This confirms the presence of the already mentioned intramolecular hydrogen bond. FTIR spectrum of 5-Chloroisatin-3-hydrazone contains a band at 1599 cm^{-1} , as a newly created $\text{C}=\text{N}$ bond, which correlates with the literature, and can not be found in the spectra of 5-Chloroisatin [2, 13, 14].

Figure 2. FTIR spectrum of 5-Chloroisatin-3-hydrazone**Table 2.** FTIR spectral data of 5-Chloroisatin-3-hydrazone

5-Chloroisatin-3-hydrazone	Band assignment (cm^{-1})
3177	$\nu(\text{CONH})$
3396, 3240	$\nu_{\text{AS}}(\text{NH}) + \nu_{\text{S}}(\text{NH})$
1691	$\nu(\text{C}=\text{O})$
1666	$\nu(\text{C}=\text{C})$
1599	$\nu(\text{C}=\text{N})$
1552	$\delta(\text{NH}) + \nu(\text{C}-\text{N})$
1476	$\delta(\text{NH})$
1243	$\nu(\text{C}-\text{N}) + \delta(\text{NH})$
1201	$\nu(\text{C}-\text{O})$
814	$\nu(\text{C}-\text{Cl})$

Figure 3. Structural presentation of intramolecular hydrogen bond formed in 5-Chloroisatin-3-hydrazone

Antibacterial activity

In vitro antibacterial activity of 5-Chloroisatin-3-hydrazone was tested against Gram-positive bacteria (*S. aureus* and *E. faecalis*) and Gram-negative bacteria (*E. coli*, *P. aeruginosa*, and *P. vulgaris*). Experimental results of the antibacterial activities of 5-Chloroisatin-3-hydrazone are given in Table 3. The Table also contains the values of antibacterial activity of isatin-3-hydrazone, synthesized under the same condition and tested against the same microorganisms [15].

Table 3. Zone of inhibition (mm) of 5-Chloroisatin-3-hydrazone and Isatin-3-hydrazone

Microorganism	5-Chloroisatin-3-hydrazone			Isatin-3-hydrazone [15]		
	$\mu\text{g}\cdot\text{cm}^{-3}$			$\mu\text{g}\cdot\text{cm}^{-3}$		
	125	250	500	125	250	500
<i>E. coli</i>	17	19	22	12	17	19
<i>E. faecalis</i>	13	19	21	13	19	21
<i>S. aureus</i>	14	20	22	14	20	22
<i>P. aeruginosa</i>	16	20	23	16	20	23
<i>P. vulgaris</i>	22	22	25	18	21	25

5-Chloroisatin-3-hydrazone showed to be the most efficient at all tested concentrations against bacteria *P. vulgaris* and the inhibition zone is the largest (25 mm) at the concentration of $500 \mu\text{g}\cdot\text{cm}^{-3}$. 5-Chloroisatin-3-hydrazone exhibits the lowest activity against *E. faecalis* at the tested concentrations of 125 and $500 \mu\text{g}\cdot\text{cm}^{-3}$ (inhibition zones 13 and 21 mm, respectively), while at the concentration of $250 \mu\text{g}\cdot\text{cm}^{-3}$ the weakest activity was reported against both *E. faecalis* and *E. coli* (inhibition zones are 19 mm). Comparing to isatin-3-hydrazone [15], 5-Chloroisatin-3-hydrazone possesses the almost similar antibacterial activities against *E. faecalis*, *S. aureus* and *P. aeruginosa* (under the same concentrations). At all concentrations, 5-Chloroisatin-3-hydrazone is more efficient than isatin-3-hydrazone [15] against *E. coli*, as well as against *P. vulgaris*. Comparing the activities of two compounds, better results achieved with 5-Chloroisatin-3-hydrazone can be explained by the presence of chloro substituent at position 5, which is the only difference in the structure of two isatin derivatives.

Photolytic activity

UV-VIS absorption spectra of 5-Chloroisatin-3-hydrazone after UV-B and UV-C irradiation, as well as, the kinetic $\ln A$ are shown in Figures 4b and 5b. Although UV-VIS spectra were recorded in three different solvents, photolytic stability was only investigated in DMF in order to continued our previously work [7,8]. 5-Chloroisatin-3-hydrazone was subjected to UV-A radiation, too, at intervals of 15 min within 60 min. The results showed no degradation of the compound after 60 min. of irradiation, and therefore were not shown in the paper. Absorption maxima decrease in both cases (Figures 4a and 5a), and a slight bathochromic shifts have been observed at the end of any particular UV-B and UV-C irradiation. The values of $\ln A$ plotted against irradiation time as a linear function

(Figures 4b and 5b), suggesting the photolytic kinetics to be probably of pseudo-first order. Degradation kinetics highly depends of UV-irradiation energy input. With the increase in the energy of the incoming photons the rate of degradation 5-Chloroisatin-3-hydrazone increases, also. This suggests that the stability of compound depends on the radiation dose, i.e., on the energy of the incoming photons. Also, the results for reaction rate constants (Figures 4b and 5b), showed that photolysis of 5-Chloroisatin-3-hydrazone under the UV-C irradiation ($k = -0.05579 \text{ min}^{-1}$) is faster, than photolysis under the UV-B irradiation ($k = -0.01071 \text{ min}^{-1}$). This suggests that photolytic reduction of 5-Chloroisatin-3-hydrazone is slower under the UV-B irradiation and therefore more stable. The results are in a good agreement with previous investigation of the similar isatin derivatives [7,8]. Based on the previous research [7,8], mechanism can be suggested as a hydrogen abstraction from DMF by triple state of isatin carbonyl group (induced by irradiation energy absorption) as a very reactive chromophore (Figure 6). Radicals, such as ketyl and DMF, suggested in this mechanism can only be speculated, since the suitable techniques, such as flash one, were not available.

Figure 4. Changes in the absorption spectra of 5-Chloroisatin-3-hydrazone with the increase of UV-B light irradiation in DMF (a) and the kinetics of the UV-B induced degradation of the 5-Chloroisatin-3-hydrazone at $\lambda = 368 \text{ nm}$ (b)

Figure 5. Changes in the absorption spectra of 5-Chloroisatin-3-hydrazone with the increase of UV-B light irradiation in DMF (a) and the kinetics of the UV-B induced degradation of the 5-Chloroisatin-3-hydrazone at $\lambda = 368$ nm (b)

Figure 6. The proposed mechanism of 5-Chloroisatin-3-hydrazone photoreduction in DMF

Conclusion

The synthesized 5-Chloroisatin-3-hydrazone possesses various antibacterial activities against all tested microorganisms at the concentrations of 125, 250 and $500 \mu\text{g}\cdot\text{cm}^{-3}$. The best results are achieved against Gram-negative bacteria *P. vulgaris* at the concentration of $500 \mu\text{g}\cdot\text{cm}^{-3}$ (inhibition zone 25 mm). 5-Chloroisatin-3-hydrazone showed a significant photostability against UV-B and UV-C irradiation. The photolytic kinetics is probably of pseudo-first order depending of the irradiation energy input, UV-B vs UV-C. The mechanism can be suggested as a hydrogen abstraction from DMF by triplet state of the isatin carbonyl group, with the formation of ketyl and DMF radicals.

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Izvod

ANTIBAKTERIJSKA AKTIVNOST I FOTOLITIČKA STABILNOST SINTETISANOG 5-HLOROIZATIN-3-HIDRAZONA

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Izatin je važan prekursor u sintezi farmaceutski aktivnih heterocikličnih jedinjenja. Njegovi derivati poseduju različitu biološku aktivnost, kao što su antimikrobna, anti-viralna, antiinflamatorna, antikonvulzivna, itd. U ovom radu je izvršena sinteza 5-hloro-izatin-3-hidrazona i identifikacija pomoću elementarne mikroanalize, FTIR i UV/VIS metode. Antibakterijska aktivnost je testirana disk-difuzionom metodom u odnosu na bakterije *E. coli*, *E. faecalis*, *S. aureus*, *P. aeruginosa* i *P. vulgaris*. U odnosu na Gram-negativnu bakteriju *P. vulgaris*, 5-hloroizatin-3-hidrazona pokazuje najbolju aktivnost pri testiranim koncentracijama (zona inhibicije je 25 mm pri koncentraciji od 500 $\mu\text{g}\cdot\text{cm}^{-3}$). Fotolitička stabilnost sintetisanog jedinjenja. 5-hloroizatin-3-hidrazona je praćena u odnosu na UV-B i UV-C zračenje u različitim vremenskim intervalima. Porastom energije upadnih fotona raste brzina degradacije 5-hloroizatin-3-hidrazona pa se može pretpostaviti da stabilnost jedinjenja zavisi i od doze zračenja, odnosno od energije upadnih fotona. Rezultati pokazuju da se kinetika fotolize pokorava jednačini pseudo-prvog reda, te da predloženi mehanizam podrazumeva apstrakciju vodonika iz DMF-a kao rastvarača pomoću tripletne C=O grupe.

Ključne reči: 5-hloroizatin-3-hidrazon, sinteza, spektroskopska analiza, antibakterijska aktivnost, fotolitička stabilnost.