Microwave-assisted Synthesis and Antimicrobial Activity of Novel Spiro 1,3,4-thiadiazolines From Isatin Derivatives

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(Additional Supporting Information may be found in the online version of this article.)

Abstract. This work describes the synthesis of spiro 1,3,4-thiadiazolines from isatin-β-thiosemicarbazone acetylation, using microwave irradiation as a source of heating the reaction medium. *N*-substituted isatin derivatives were used as substrates to obtain thiosemicarbazones by adding thiosemicarbazide to the isatin ketone carbonyl. The final synthetic step was the reaction of thiosemicarbazones with acetic anhydride under microwave irradiation to get the spiro compounds. Reaction times ranged from 6 to 18 minutes resulting in yields of up to 90%. Biological assays have shown promising antibacterial and antifungal activity, especially spiro thiadiazolines derived from allylated isatins. All the proposed molecules proved to be potential drug candidates based on the results of the *in silico* investigation, with satisfactory drug-likeness and drug-score, respecting Lipinski's Rule. The use of the microwave reactor was efficient for the synthesis of thiosemicarbazones and spiro-compounds, resulting in a significant reduction in reaction times with conventional heating. Taking into account the threat of antimicrobial resistance, this work presents a series of bioactive molecules that are easily obtained via microwave reaction.

Keywords: isatin derivatives; spiro compounds; isatin-β-thiosemicarbazones; acetylation; biological activity.

Introduction

Isatin is a heterocyclic compound widely used as a building block in the synthesis and development of new drug candidates. Its synthetic versatility has allowed the synthesis of new compounds endowed with biological activity, such as spirocyclic derivatives.[1,2]

These polycyclic compounds have rings attached through an ordinary atom, usually, a sp^3 hybridized carbon. Medicinal chemists worldwide have shown enormous interest in spiro compounds, especially molecules with 1,3,4-thiodiazolinic rings since this characteristic is recurrent in many alkaloids of natural origin widely studied due to their pharmaceutical properties.[3,4]

Moreover, synthetic derivatives have obeying this trend, showing up as promising anticancer,[5,6] antifungals,[6] and especially antibacterial agents,[7–9] whose biological activities are possibly associated with the N=C–S portion of the 1,3,4-thiadiazolinic ring.[10]

The synthesis of thiadiazolinic compounds represents a challenge for organic chemists. Several synthetic strategies are used for its execution, among then oxidative condensation and cycloaddition reactions from molecules with multiple carbon-nitrogen bonds,[11] such as diazo compounds,[12] isothiocyanates,[13] and nitrilimines.[14] However, the main route of obtaining is based on the acetylation of thiosemicarbazones under conventional heating,[15] for example the incorporation of spiro 1,3,4-thiadiazolines into α-pinene[16] and piperidine derivatives[9] (Figure 1 a-b).

In general, these protocols are based on conventional heating, whose main limitation is the non-uniform temperature throughout the system, which compromises the yield and reaction time.[17] This difficulty has been gradually eliminated due to the variety of new methodologies reported in recent bibliographic reviews that show the application of microwave irradiation as an indispensable tool for synthesizing these compounds.[18–20]

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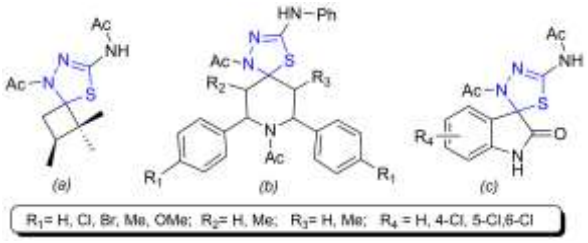


Figure 1. α-pinene (*a*), piperidine (*b*), and isatin derivatives (*c*) containing 1,3,4-thiadiazolinic moiety.

The literature reports few studies using microwave radiation to obtain spiro 1,3,4-thiadiazolines. However, the results published so far reinforce the advantages mentioned above. Mamun et al.,[21] for example, report the synthesis of spiro thiadiazolines by acetylation of isatin-β-thiosemicarbazones (Figure 1 c) with reaction times of 3 to 4 minutes under microwave irradiation. In turn, the reaction time can reach 4 hours in protocols based on conventional heating for similar molecules.[22]

Based on this context, this work aimed to evaluate the use of microwave irradiation in the synthesis of new spiro 1,3,4-thiadiazolines derived from isatin.

Paying attention to antimicrobial resistance as one of our most serious health threats, as well as the wide exploitation of spirooxindoles as antimicrobial agents,[23] we also propose an *in silico* study of the pharmacokinetics and toxicological properties of the proposed molecules and *in vitro* investigation of their antimicrobial and antifungal activity.

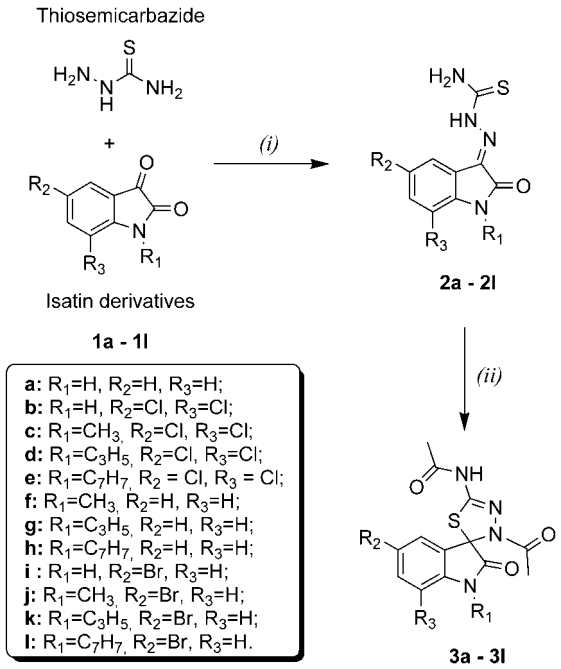
Results and Discussion

Synthesis of isatin-derived thiosemicarbazones (2a-2l)

Thiosemicarbazones (**2a-2l**) were obtained via condensation reactions involving thiosemicarbazide and ketone carbonyl present in isatin derivatives (**1a-1l**) using methanol solvent under microwave irradiation at 100 °C, as shown in Scheme 1.

Acid catalysis was not used to obtain the products, considering that some authors claim that acid is a dispensable condition in obtaining these molecules.[24–26] Reaction conditions and yields for getting thiosemicarbazones can be seen in Table 1.

It can be observed that the parameters used to obtain thiosemicarbazones were satisfactory, providing moderate yields (51-88%) with reaction times between 30 and 45 minutes, with no formation of co-products. These yields can be associated with the use of methanol as a solvent since its high polarity can result in solubilization and consequent loss of part of the product by filtration.



Scheme 1. *(i)* MeOH, 100 ºC (microwave reactor), 30-45 min; *(ii)* Ac_2O, 120 ºC (microwave reactor), 6-18 min.

Table 1. Reaction times and yields for obtaining thiosemicarbazones **2a-2l**.

Compound	R_1	R_2	R_3	Time (min)	Yield (%)
2a	H	H	H	45	67
2b	H	Cl	Cl	45	51
2c	CH_3	Cl	Cl	30	63
2d	C_3H_5	Cl	Cl	30	53
2e	C_7H_7	Cl	Cl	30	57
2f	CH_3	H	H	30	75
2g	C_3H_5	H	H	30	64
2h	C_7H_7	H	H	30	87
2i	H	Br	H	30	77
2j	CH_3	Br	H	45	88
2k	C_3H_5	Br	H	45	73
2l	C_7H_7	Br	H	45	81

^{1}H NMR spectra (see supporting information) indicate the formation of the desired products due to the appearance of the three characteristic peaks of thiosemicarbazone: *i)* a singlet around 12.0 ppm, corresponding to thioamides hydrogen; *ii)* two diastereotopic hydrogens singlets between near to 9.3 and 8.7 ppm, corresponding to the -NH_2 portion of thiosemicarbazone due to the restricted rotation of the bond C-NH_2 by resonance effects.[27,28] We also highlight the presence of peaks of thioamides carbons in the most unshielded region of the ^{13}C NMR spectrum (about 179.0 ppm) that attest to the formation of compounds **2a-2l**.

Synthesis of isatin-derived 1,3,4-thiadiazolines (3a-3l)

Few synthetic protocols are described in the literature for obtaining spiro 1,3,4-thiadiazolines derived from isatin. It is commonly a reaction that uses thiosemicarbazone derived from isatin and excess of acetic anhydride. Reaction times vary from 1 to 4 hours under conventional heating[22,29,30] and from 3 to 4 minutes using an adapted domestic microwave oven.[21]

Being aware of this information, we submitted the first three reactions for the preparation of spiro 1,3,4-thiadiazoline (**3a**) to microwave irradiation, varying the temperature and evaluating by TLC the time required for total consumption of thiosemicarbazone (Table 2). All synthetic protocols described in the literature use an excess of acetic anhydride in the cyclization reactions, leading us to use a fixed amount of this reagent in the reactions carried out in this study (3.5 mL).

Table 2. Investigation of the influence of temperature on the synthesis of **3a**.

Entry [a]	Temp. (°C)	Time (min)	Yield (%)[b]
1	80	18	85
2	100	12	87
3	120	6	82

[a] Reactions using 0.5 mmol of thiosemicarbazone (2a) and 3.5 mL of acetic anhydride; [b] isolated yield.

Based on the results obtained in the preparation of the spiro 1,3,4-thiadiazoline **3a**, we can observe that the increase in temperature in the microwave reactor promotes a gradual decrease in reaction time. It was also observed the total conversion of the starting material and the formation of the same products, regardless of the temperature used.

Considering that reference used does not detail synthetic aspects such as the possible formation of co-products and subsequent purification of the desired products, we proceeded to synthesize the other spiro compounds using microwave irradiation at 120 °C. These results are shown in Table 3.

The values in Table 3 reveal that two compounds (**3b** and **3i**) showed significantly lower yields than the other synthesized compounds due to the formation of co-products which made purification via chromatographic column difficult. These compounds come from isatin that was not subjected to substitution reactions in the NH portion of the indole nucleus (R_1=H), an essential factor for the low yields obtained, since this problem was not verified for the *N*-substituted derivatives. Compound **3a** was an exception, which yielded 82% even though it belonged to the class of compounds **3b** and **3i**.

Table 3. Reaction times and yields for obtaining 1,3,4-thiadiazolines **3a**-**3l**.

Compound	R_1	R_2	R_3	Time (min)	Yield (%)[a]
3a	H	H	H	6	82
3b	H	Cl	Cl	6	36
3c	CH3	Cl	Cl	6	88
3d	C_3H_5	Cl	Cl	6	81
3e	C_7H_7	Cl	Cl	6	65
3f	CH_3	H	H	6	62
3g	C_3H_5	H	H	6	70
3h	C_7H_7	H	H	6	90
3i	H	Br	H	6	25
3j	CH_3	Br	H	18	57
3k	C_3H_5	Br	H	18	61
3l	C_7H_7	Br	H	18	65

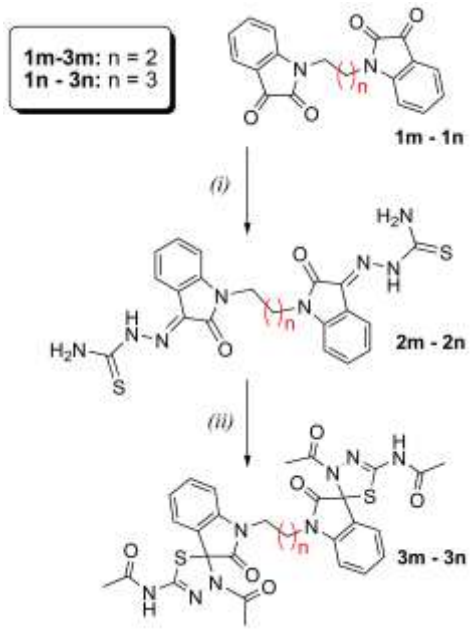
[a] Isolated yield.

^{1}H NMR spectroscopic data confirm the successful formation of the desired thiadiazolines, evidenced by the disappearance of the two singlets mentioned above, characteristic of the hydrogens of the -NH_2 portion of the precursor thiosemicarbazones. The appearance of two singlets around 2.0 ppm is also noted, which are related to α-amidic hydrogens resulting from the acetylation of thiosemicarbazone,[28] consistent with the literature data for similar compounds previously described.[21,22]

Regarding the ^{13}C NMR spectra obtained for the synthesized 1,3,4-thiadiazolines, there is an increase in the number of carbon signals compared to thiosemicarbazones. Two of these signals are attributed to the methyl carbons introduced by acetylation and are located close to 22.0 ppm. Based on the spectroscopic associations available in the literature for similar compounds, we highlight the signals' appearance in the most unshielded region of the APT spectra (~170.0 ppm), referring to the carbonyl carbons resulting from acetylation. The presence of a discrete signal around 74.0 ppm, attributed by Somogyi[22] to the carbon spiro, confirms the formation of the desired compounds.

Based on the twin drug proposal, widely explored in the literature, we also performed the synthesis of two dimeric thiadiazolines derived from isatin.

The dimeric compounds (**1m** and **1n**) were submitted to addition reactions with thiosemicarbazide to generate homodimeric thiosemicarbazones (**2m** and **2n**) and their subsequent conversion to homodimeric 1,3,4-thiadiazolines (**3m** and **3n**), according to the synthesis protocols adopted in this research. Scheme 2 shows the synthetic route for obtaining these homodimers.



Scheme 2. *(i)* MeOH, 100 ºC (microwave reactor), 30 min; *(ii)* Ac_2O, 120 ºC (microwave reactor), 30 min.

The synthesis of dimeric compounds demanded a longer reaction time concerning monomeric thiadiazolines. The total consumption of the starting thiosemicarbazones (**2m** and **2n**) occurred after 30 min under heating in a microwave reactor, resulting in yields of 52 and 27% for **3m** and **3n**, respectively. These compounds were fully characterized, presenting behavior similar to monomers at ^{1}H and ^{13}C NMR spectra.

The main objective of homodimer synthesis is to obtain more selective and potent drugs than the monomeric units that compose it, exhibiting a different selectivity profile and pharmacokinetic properties.[31]

In silico study

The *in silico* study has become an excellent strategy to assist medicinal chemists in discovering new possible drugs. This study is based on a theoretical investigation of the pharmacokinetic and pharmacodynamic properties and toxicological effects (ADMET - Absorption, Distribution, Metabolization, Excretion, and Toxicity) of compounds with potential biological activity, making it possible to reduce the number of compounds prepared with undesirable physicochemical properties.[32]

One of the most important theoretical approaches to pharmacokinetics *in silico* study is the Lipinski's Rule of Five.[33] This rule is based on the physical-chemical criteria that the compounds must obey to be absorbed and distributed by the human organism. These parameters also indicate that the drugs can present metabolic stability, remaining in the body for the time necessary to act appropriately instead of being only excreted. To better understand this behavior in spiro 1,3,4-thiadiazolines, the prediction of the compounds' physical-chemical properties was made.

Values of physicochemical properties, molecular weight, number of acceptors (HBA), and hydrogen donors (HBD), Log P and molar refractivity were calculated using the *SwissADME* software.[34]

The results given in Table 4 show that the monomeric spiro 1,3,4-thiadiazolines evaluated do not violate any parameter of Lipinski's Rule. All compounds have a molecular mass below 500 g mol^{-1}, indicating good solubility and cross cell membranes without difficulty. On the other hand, the studied homodimers (**3m** and **3n**) violated this rule, presenting respectively molar mass values of 648.71 and 662.74 g mol^{-1}.

The increase in the number of possibilities for hydrogen bonds, represented as a function of HBA and HBD, contributes to increasing the compound's polarity. A high number of hydrogen bonds hinders the drug's penetration through the lipid bilayer of cell membranes. These bonds need to be broken as the molecule passes through the hydrophilic to hydrophobic medium.[33]

Lipophilicity of a compound is expressed as Log P. Compounds with cLog P > 5 are poorly soluble in aqueous solutions; therefore, they cannot access the membrane surface.[35] In an optimal situation for effective gastrointestinal absorption after oral administration of the drug, the Log P value must be between 0 and 3.[36] All synthesized molecules obey this parameter, except for the dimeric molecule **3n**. This compound showed cLog P = 3.48 so it did not violate the limit established by Lipinski's Rule (cLog P > 5).

Molar refractivity is a constitutive-additive physical-chemical property related to the adjustment of the molecule to its receptor site. Positive values indicate the presence of dispersive forces in the active center that allow the ligand-bioreceptor interaction. In contrast, negative values represent the ligand's ability to distort the bioreceptor conformation, causing a stereochemical impediment that makes molecular interaction difficult.[37] All compounds analyzed, except homodimers **3m** and **3n**, showed values within the reference standard established (40 ≤ molar refractivity ≤ 130),[38] indicating an excellent ligand-bioreceptor interaction.

Studies for a possible prediction of the toxicological properties (mutagenic, tumorigenic, irritant, and reproductive effects) of the proposed compounds were also carried out using the *Osiris Property Explorer*.[39] Among all the synthesized 1,3,4-thiadiazolines, only compounds **3b-3e** presented a single risk group: irritation with a medium level of risk (Table 5).

The drug-likeness parameter assesses the presence of functional groups and physical properties similar to most known drugs. Negative values represent minimal similarities, and positive values represent a more remarkable similarity between the molecules

studied and the drugs in use.[40] On the other hand, the drug-score combines values of some parameters into a single numerical value that is useful for judging the potential of the compound to become a possible drug.[39] All spiro compounds provided positive values that indicate substantial similarities with pharmacophoric subunits and properties present in already known drugs.

We emphasize here that *in silico* studies are essential, but not decisive for searching for new drug candidates. These results guide the development of new structures, but the biological activity and toxicity of the compounds must be considered.

Table 4. Physicochemical properties are predicted via *SwissADME*.

Compound	Lipinski's parameter					Molar Refractivity
	MW	HBA	HBD	cLog P	nViol	
3a	304.32	4	2	1.42	0	88.15
3b	373.21	4	2	1.95	0	98.17
3c	387.24	4	1	2.37	0	103.07
3d	413.28	4	1	2.54	0	112.21
3e	463.34	4	1	2.92	0	127.56
3f	318.35	4	1	1.90	0	93.05
3g	344.38	4	1	2.22	0	102.19
3h	394.45	4	1	2.49	0	117.54
3i	383.22	4	2	1.80	0	95.85
3j	397.25	4	1	2.28	0	100.75
3k	423.28	4	1	2.75	0	109.89
3l	473.34	4	1	2.92	0	125.24
3m	648.71	8	2	2.87	1	188.80
3n	662.74	8	2	3.48	1	193.61

MW: molecular weight; HBA: hydrogen bond acceptor; HBD: hydrogen bond donor; MW: cLog P: octanol/water partition coefficient; nViol: number of violations;

Table 5. Physicochemical properties predicted via *Osiris Property Explorer*.

Compound	Mutagenic effects	Irritating effects	Tumorigenic effects	Reproductive effects	Drug-likeness	Drug-score
3a	NR	NR	NR	NR	7.04	0.81
3b	NR	MR	NR	NR	6.74	0.51
3c	NR	MR	NR	NR	7.27	0.50
3d	NR	MR	NR	NR	5.02	0.43
3e	NR	MR	NR	NR	7.96	0.34
3f	NR	NR	NR	NR	7.57	0.83
3g	NR	NR	NR	NR	5.31	0.76
3h	NR	NR	NR	NR	8.24	0.63
3i	NR	NR	NR	NR	4.91	0.71
3j	NR	NR	NR	NR	5.44	0.69
3k	NR	NR	NR	NR	3.19	0.60
3l	NR	NR	NR	NR	6.13	0.47
3m	NR	NR	NR	NR	8.30	0.30
3n	NR	NR	NR	NR	7.15	0.28

NR: no risk; MR: medium risk.

In vitro biological evaluation

Strains of bacteria (*Staphylococcus aureus*, ATCC-13150; *Escherichia coli*, ATCC-18739; *Pseudomonas*

aeruginosa, ATCC-9027) and fungi (*Candida albicans*, ATCC-90028; *Candida tropicalis*, ATCC-13803; *Cryptococcus neoformans*, FCF-119) were used to evaluate the biological activity of the synthesized spiro compounds.

Biological activity of the compounds was based on the values of minimum inhibitory concentration (MIC) obtained *in vitro*, and their activity was considered according to the following criteria: intense activity (MIC < 600 μg mL^{-1}); moderate exercise (600 ≤ MIC ≤ 1500 μg mL^{-1}); inadequate training or inactive product (MIC > 1500 μg mL^{-1}).[41–43] Negative control was carried out in parallel with the antimicrobials gentamicin (64 μg mL^{-1}) inhibiting bacteria and amphotericin B (32 μg mL^{-1}) inhibiting fungi. Table 6 shows the results of the antimicrobial activity of the tested compounds.

As observed in Table 6, only five compounds were active against at least one of the studied microorganisms. Among the tested molecules, it was found that the *N*-allylated compounds **3d** and **3g** had the lowest MIC values (137.5 μg mL^{-1}) for all microorganisms tested. The only exception concerns the performance of **3g** against *P. aureuginosa*, whose MIC was 550.0 μg mL^{-1}.

Compound **3l** showed activity on all trial strains, whose MIC values varied between 125.0 and 500.0 μg mL^{-1}. An opposite behavior was observed for the compound *N*-methylated **3f**, which showed only antifungal activity with MIC ranging between 137.5 and 550.0 μg mL^{-1}. Compound **3h** was active only against *P. aureuginosa* with MIC of 275 μg mL^{-1}.

Despite recent reports regarding the use of dimerization as a viable alternative to improve the biological activity of monomeric precursors,[44–47] the evaluated dimers (**3m** and **3n**) did not show physical activity against the tested microorganisms. These results reinforce the need for studies involving aryl-substituted isatin derivatives, because of the promising results of some thiadiazolines derived from chlorinated and brominated isatins.

Table 6. Minimum inhibitory concentration (μg mL^{-1}) of the 1,3,4-thiadiazolines against bacterial and fungal strains.

Compound	Bacteria			Fungi		
	S. aureus **ATCC-13150**	***E. coli*** **ATCC–18739**	***P. aeruginosa*** **ATCC-9027**	***C. albicans*** **ATCC-90028**	***C. tropicalis*** **ATCC-13803**	***C. neoformans*** **FCF-119**
3b	+	+	+	+	+	+
3c	+	+	+	+	+	+
3d	137.5	137.5	137.5	137.5	137.5	137.5
3e	+	+	+	+	+	+
3f	+	+	+	550.0	137.5	137.5
3g	137.5	137.5	550.0	137.5	137.5	137.5
3h	+	+	275.0	+	+	+
3j	+	+	+	+	+	+
3k	+	+	+	+	+	+
3l	250.0	125.0	500.0	+	+	+
3m	+	+	+	+	+	+
3n	+	+	+	+	+	+
Culture media	-	-	-	-	-	-
Microorganism	+	+	+	+	+	+
Amphotericin B	NT	NT	NT	-	-	-
Gentamicine	-	-	-	NT	NT	NT

MIC: minimum inhibitory concentration; ATCC: American Type Culture Collection; (+): the presence of microbial growth; (-): no microbial growth; NT: Not tested.

Conclusion

The microwave radiation synthesis of was successfully applied, resulting in spiro compounds with yields between 25 and 90%. The use of the microwave reactor proved to be efficient for the synthesis of thiosemicarbazones and compound spiro, resulting in a significant reduction in reaction times with conventional heating. *In silico* studies have shown that the spiro compounds synthesized respected Lipinski's parameters, indicating a good oral availability. In addition, the results obtained for toxicity, drug-likeness and drug-score reinforce the potential of these thiadiazolines for drug candidates. The **3d**, **3f**, **3g**, **3h** and **3l** compounds showed intense antimicrobial activity, especially **3d** and **3g**. These *N*-allylated derivatives showed activity at a concentration of 137.5 μg mL^{-1} in practically all tests with microorganisms. Finally, the synthesized dimers (**3m** and **3n**) did not show activity against the microorganisms evaluated. This behavior reinforces the importance of further studies extending the series of dimeric compounds with substituent groups in the aromatic ring.

Experimental Section

General

All commercially available reagents were purchased from Aldrich and used without further purification. Reactions were monitored by thin-layer chromatography (TLC) using Silica gel 60 UV254 Macherey-Nagel pre-coated silica gel plates. Flash column chromatography was performed on a silica gel (300–400 mesh) using an ethyl acetate-hexane mixture as eluent. Organic phases were dried using anhydrous Na_2SO_4 prior to evaporation on a rotary evaporator. NMR spectra were recorded in DMSO-*d*6 with a Varian Mercury Spectra AC 20 or Bruker Advance II (400 MHz to ^{1}H NMR and 100 MHz to ^{13}C NMR). Chemical shift values are expressed as parts per million (ppm) downfield from TMS and J values are in hertz. Splitting patterns are indicated as singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), double doublet (dd), doublet of doublets of doublets(ddd) and broad peak (br).

Synthesis of the isatin derivatives (1b-1l)

*5,7-Dichloroisatin (**1b**)*
Isatin (**1a**) (10.0 mmol, 1 equiv.) was added to a solution of trichloroisocyanuric acid (10.0 mmol, 1 equiv.) in 6 mL of H_2SO_4 at 0 ºC. This mixture was stirring under ambient conditions for 30 min and accompanied by TLC. After this time, crushed ice was added to the reaction flask and the precipitate was filtered under reduced pressure and washed with ice water resulting in a solid yellow product **(1b)** with 89% yield.

*N-methyl isatin derivatives (**1c**, **1f** and **1j**)*
5.0 mmol of isatin (**1a**) or its derivatives 5,7-dichloroisatin (**1b**) and 5-bromoisatin (**1i**) were solubilized in 5 mL of DMF previously dried, followed by the addition of K_2CO_3 (1,2 equiv.) under magnetic stirring. Then methyl iodide (1.2 equiv.) was added and the reaction was carried out at room temperature under an inert argon atmosphere and monitored by TLC. After 2 hours of reaction liquid-liquid extraction was performed using ethyl acetate and distilled water. The organic phase was treated with anhydrous Na_2SO_4. After filtration and removal of the solvent under reduced pressure, solid red products were obtained with yields of 81% (**1c**), 93% (**1f**) and 87% (**1j**).

*N-allyl isatin derivatives (**1d**, **1g** and **1k**)*
5.0 mmol of **1a**, **1b** or **1i** were solubilized in 5 mL of DMF previously dried, followed by the addition of K_2CO_3 (1,2 equiv.) under magnetic stirring. Then allyl bromide (1.2 equiv.) was added and the reaction was carried out at room temperature under an inert argon atmosphere and monitored by TLC. After 2 hours of reaction liquid-liquid extraction was performed using ethyl acetate and distilled water. The organic phase was treated with anhydrous Na_2SO_4. After filtration and removal of the solvent under reduced pressure, solid red products were obtained with yields of 62% (**1d**), 79% (**1g**) e 71% (**1k**).

*N- benzyl isatin derivatives (**1e**, **1h** and **1l**)*
5.0 mmol of **1a**, **1b** or **1i** were solubilized in 5 mL of DMF previously dried, followed by the addition of K_2CO_3 (1,2 equiv.) under magnetic stirring. Then benzyl bromide (1.0 equiv.) was added and the reaction was carried out at room temperature under an inert argon atmosphere and monitored by CCDA. After 2 hours of reaction liquid-liquid extraction was performed using ethyl acetate and distilled water. The organic phase was treated with anhydrous Na_2SO_4. After filtration and removal of the solvent under reduced pressure, solid red products were obtained with yields of 78% (**1e**), 87% (**1h**) e 82% (**1l**).

General procedure for synthesis of homodimeric derivatives of isatin (1m and 1n)

2 mmol of **1a** was solubilized in 2 mL of DMF previously dried, followed by the addition of K_2CO_3 (1,2 equiv.) under magnetic stirring. Then 1 mmol of the corresponding dibromoalkane (1,3-dibromopropane for **1m** or 1,4-dibromobutane for **1n**) was added and the reaction was carried out at room temperature under an inert argon atmosphere and monitored by TLC. After 24 hours, ice water was added to the reaction flask and the precipitate was filtered under reduced pressure and washed with ethanol resulting in a solid orange product (**1m** and **1n**).

General procedure for synthesis of isatin thiosemicarbazones (2a-2n)

1 mmol of isatin (**1a**) or its derivatives (**1b-1n**) were added to 4 mL of methanol in a glass tube specific for a microwave reactor (10 mL). Then thiosemicarbazide was add to the system under magnetic stirring, obeying 1 equiv. for monomeric (**1b-1l**) and 2 equiv. for dimeric derivatives (**1m-1n**). The reaction was carried out at 100 °C under microwave irradiation with closed vessel conditions and monitored by TLC every 15 minutes complete reagent consumption. At the end of the reaction, the mixture was cooled to room temperature in order to form a precipitate which was isolated by vacuum filtration, using ethanol for washing of the obtained solid.

Compound 2a: Yellow solid (67% yield), mp 220-225 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.48 (s, 1H), 11.19 (s, 1H), 9.03 (s, 1H), 8.67 (s, 1H), 7.64 (d, 1H), 7.33 (t, 1H), 7,06 (t, 1H), 6.91 (d, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.17, 163.10, 142.81, 132.52, 131.73, 122.84, 121.43, 120.43, 111.50.

Compound 2b: Orange solid (51% yield), mp 240-245 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.24 (s, 1H), 11.73 (s, 1H), 9.15 (s, 1H), 8.84 (s, 1H), 7.70 (d, J = 2.0 Hz, 1H), 7.51 (d, J = 2.0 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.24, 162.91,

138.99, 130.67, 129.88, 127.38, 123.77, 119.81, 116.28.

Compound 2c: Orange solid (63% yield), mp 230-235 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.17 (s, 1H), 9.21 (s, 1H), 8.93 (s, 1H), 7.84 (d, J = 2.1 Hz, 1H), 7.57 (d,J = 2.1 Hz, 1H), 3.49 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.24, 161.27, 138.34, 131.56, 129.34, 127.85, 124.36, 119.85, 116.62, 29.12.

Compound 2d: Orange solid (53% yield), mp 188-193 ºC.^{1}H NMR (400 MHz, DMSO-d_6) δ 12.12 (s, 1H), 9.22 (s, 1H), 8.95 (s, 1H), 7.87 (s, 1H), 7.54 (s, 1H), 6.01-5.92 (m, 1H), 5.14 (dd, J = 10.5. 1H), 5.07 (dd, J = 17.3, 1H), 4.63-4.61 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.23, 161.12, 137.41, 133.37, 131.66, 129.03, 128.07, 124.59, 120.00, 116.50, 116.38, 43.11.

Compound 2e: Brown solid (57% yield), mp 223-228 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.14 (s, 1H), 9.28 (s, 1H), 9.01 (s, 1H), 7.91 (d, J = 2.1 Hz, 1H), 7.48 (d, J = 2.1 Hz, 1H), 7.34-7.23 (m, 5H), 5.25 (s, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.23, 161.53, 137.34, 137.31, 131.57, 129.05, 128.91, 128.24, 127.66, 126.60, 124.79, 120.03, 116.37, 44.39;

Compound 2f: Yellow solid (75% yield), mp 220-225 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.38 (s, 1H), 9.07 (s, 1H), 8.70 (s, 1H), 7.66 (d, J = 6.8 Hz, 1H), 7.42 (td, J = 7.7 Hz, 1.3 Hz, 1H), 7.15-7.09 (m, 2H), 3.36 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.16, 161.21, 144.03, 131.64, 131.60, 123.33, 121.03, 119.70, 110.23, 26.14.

Compound 2g: Yellow solid (64% yield), mp 148-153 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.32 (s, 1H), 9.02 (s, 1H), 8.60 (s, 1H), 7.75 (d, 1H), 7.48 (t, 1H), 7.18 (t, 1H), 7.02 (d, 1H), 5.57 (m, 1H), 5.50 (t. 2H), 4.45 (d, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.72, 160.46, 142.64, 131.42, 131.04, 130.97, 122.89, 120.73, 119.36, 117.39, 110.27, 41.32.

Compound 2h: Yellow solid (87% yield), mp 250-255 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.41 (s, 1H), 9.11 (s, 1H), 8.76 (s, 1H), 7.71 (dd, J = 6.8 Hz, 1H), 7.39-7.30 (m, 5H), 7.26 (tt, 1H), 7.12 (td, J = 7.6 Hz, 1H), 7.02 (d, J = 7.9 Hz, 1H), 4.97 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.16, 161.29, 143.01, 136.20, 131.51, 131.42, 129.18, 128.08, 127.91, 123.50, 121.28, 119.97, 110.81, 42.98.

Compound 2i: Yellow solid (77% yield), mp 245-250 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.28 (s, 1H), 11.28 (s, 1H), 9.10 (s, 1H), 8.81 (s, 1H), 7.86 (d, J = 2.0 Hz, 1H), 7.48 (dd, J = 8.3, 2.1 Hz, 1H), 6.86 (d, J = 8.3 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.22, 162.70, 141.80, 133.64, 131.10, 123.92, 122.77, 114.63, 113.40.

Compound 2j: Orange solid (88% yield), mp 250-255 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.18 (s, 1H), 9.13 (s, 1H), 8.82 (s, 1H), 7.86 (d, J = 2.0 Hz, 1H), 7.55 (dd, J = 8.4, 2.1 Hz, 1H), 7.06 (d, J = 8.4 Hz, 1H), 3.34 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.21, 160.81, 143.02, 133.46, 130.17, 123.55, 121.92, 115.24, 112.18, 26.28.

Compound 2k: Yellow solid (73% yield), mp 205-210 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.18 (s, 1H), 9.15 (s, 1H), 8.86 (s, 1H), 7.92 (d, J = 2.0 Hz, 1H), 7.55 (dd, J = 8.4, 2.1 Hz, 1H), 7.01 (d, J = 8.4 Hz, 1H), 5.89-5.80 (m, 1H), 5.19 (ddd, J = 11.3, 9.2, 1.3 Hz, 2H), 4.36 (d,2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.22, 160.56, 142.12, 133.43, 131.70, 130.03, 123.75, 122.15, 117.93, 115.32, 112.73, 41.86.

Compound 2l: Yellow solid (81% yield), mp 235-240 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.22 (s, 1H), 9.18 (s, 1H), 8.89 (s, 1H), 7.94 (d, J = 2.1 Hz, 1H), 7.50 (dd, J = 8.4, 2.1 Hz, 1H), 7.37-7.24 (m, 5H), 6.95 (d, 1H), 4.96 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 179.23, 160.90, 142.00, 135.91, 133.43, 130.00, 129.18, 128.12, 127.85, 123.81, 122.28, 115.47, 112.73, 43.05.

Compound 2m: Orange solid (80% yield), mp 190-195 ºC.^{1}H NMR (400 MHz, DMSO-d_6) δ 12.33 (s, 2H), 9.09 (s, 2H), 8.70 (s, 2H) 7.65 (d, 2H), 7.36 (t, 2H), 7.16 (m, 2 H), 7.10 (m, 2H), 3.84 (t, 4H), 2.04 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.45, 160.58, 142.41, 137.78, 130.89, 122.67, 120. 57, 119.27, 109.84, 36.96, 24.72.

Compound 2n: Orange solid (78% yield), mp 240-245 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.37 (s, 2H). 9.05 (s, 2H). 8.67 (s, 2H). 7.64 (d, 2H). 7.39 (t, 2H). 7.37 (m, 2H). 7.09 (m, 2H). 3.76 (m, 4H). 1.68 (m, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 178.71, 160.89, 142.71, 131.09, 131.05, 122.86, 120.80, 119.39, 110.04, 38.79, 24.20.

General procedure for synthesis of the spiro thiadiazolines (3a-3n)

0.5 mmol of isatin thiosemicarbazones (**2a-2n**) were added under magnetic stirring to 3.5 mL of acetic anhydride in a glass tube specific for a microwave reactor (10 mL). The reaction was carried out at 120 °C under microwave irradiation with closed vessel conditions and monitored by TLC every 6 minutes until complete reagent consumption. At the end of the reaction the mixture was cooled to room temperature and diluted in ethyl acetate and treated with saturated solution of $NaHCO_3$ to neutralize the remaining acetic acid. washed with in order to form a precipitate which was isolated by vacuum filtration, using ethanol for washing of the obtained solid. After removal of the solvent under reduced pressure, the products (**3a-3n**) were purified by flash chromatography using silica gel and ethyl acetate/hexane as eluent, resulting in yellow solids.

Compound 3a: Yellow solid (82% yield), mp > 320 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.40 (d, J = 7.2 Hz, 1H), 7.34 (t, J = 7.5 Hz, 1H), 7.11 (t, J = 7.6 Hz, 1H), 6.85 (d, J = 8.1 Hz, 1H), 2.19 (s, 3H), 2.12 (s, 3H).

Compound 3b: Yellow solid (36% yield), the crystals became black without melting. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.49 (d, J = 2.0 Hz, 1H), 7.45 (d, J = 2.0 Hz, 1H), 2.16 (s, 3H), 2.09 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.60, 170.51, 167.57, 143.43, 138.57, 132.03, 129.75, 127.32, 123.34, 115.42, 75.11, 22.65, 22.40. HRMS (FTMS + pESI) m/z, calcd. for $C_{13}H_{10}Cl_2N_4O_3S$: 371.9851; found: 370.9783 (M-1).

Compound 3c: Yellow solid (88% yield), mp 277-282 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.00 (s, 1H), 7.51 (q, J = 2.1 Hz, 2H), 3.44 (s, 3H), 2.15 (s, 3H), 2.09 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.68, 170.52, 167.55, 143.65, 138.17, 132.93, 131.49, 127.88, 123.51, 115.74, 74.02, 30.31, 22.65, 22.37. HRMS (FTMS + pESI) m/z, calcd. for $C_{14}H_{12}Cl_2N_4O_3S$: 386.0007; found: 384.9900 (M-1).

Compound 3d: Yellow solid (81% yield), mp 218-223 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.09 (s, 1H), 7.56 (d, J = 2.1 Hz, 1H), 7.51 (d, J = 2.1 Hz, 1H), 5.97-5.88 (m, 1H), 5.25 (dd, J = 17.4. 1H), 5.12 (dd, J = 10.7. 1H), 4.58-4.57 (m, 1H), 2.18 (s, 2H), 2.09 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.66, 170.65, 167.64, 143.54, 137.26, 133.34, 133.05, 131.66, 128.11, 123.61, 116.06, 115.65, 73.99, 44.19, 22.67, 22.40. HRMS (FTMS + pESI) m/z, calcd. for $C_{16}H_{14}Cl_2N_4O_3S$: 412.0164; found: 411.0100 (M-1).

Compound 3e: Yellow solid (65% yield), mp 248-253 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.07 (s, 1H), 7.59 (d, J = 7.8 Hz, 1H), 7.44 (d, J = 2.1 Hz, 1H), 7.34-7.22 (m, 5H), 5.21 (q, 2H), 2.21 (s, 3H), 2.10 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.11, 170.62, 167.76, 143.51, 137.75, 137.28, 133.09, 131.61, 128.87, 128.28, 127.35, 126.28, 123.59, 115.65, 74.14, 45.63, 22.65, 22.38; HRMS (FTMS + pESI) m/z, calcd. for $C_{20}H_{16}Cl_2N_4O_3S$: 462.0320; found: 461.0238 (M-1).

Compound 3f: Yellow solid (62% yield), mp 245-250 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.03 (s, 1H), 7.37-7.33 (m, 2H), 7.09-7.02 (m, 2H), 3.14 (s, 3H), 2.13 (s, 3H), 2.08 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.30, 170.48, 167.33, 143.37, 143.07, 130.67, 128.37, 124.01, 123.61, 109.47, 74.62, 27.10, 22.76, 22.58.

Compound 3g: Yellow solid (70% yield), mp 220-225 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.03 (s, 1H), 7.39 (dd, J = 7.5, 0.8 Hz, 1H), 7.32 (td, J = 7.8, 1.2 Hz, 1H), 7.07 (td. J = 7.6, 0.8 Hz, 1H), 6.93 (d, J = 7.8 Hz, 1H), 5.87-5.78 (m, 1H), 5.30 (dd, J = 17.3, 1H), 5.14 (dd, J = 10.5, 1H), 4.40-4.22 (m, 2H), 2.15 (s, 3H), 2.09 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.13, 170.50, 167.39, 143.27, 142.02, 131.73, 130.53, 128.32, 124.12, 123.64, 117.18, 110.10, 74.68, 42.55, 22.76, 22.55.

Compound 3h: Yellow solid (90% yield), mp 225-230 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.06 (s, 1H), 7.40 (tt, 3H), 7.34-7.30 (m, 2H), 7.27-7.21 (m, 2H), 7.05 (td, J = 7.6, 0.9 Hz, 1H), 6.78 (d, J = 7.8 Hz, 1H), 5.02 (d, J = 16.2 Hz, 1H), 4.83 (d, J = 16.2 Hz, 1H), 2.19 (s, 3H), 2.09 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.49, 170.55, 167.52, 143.31, 141.86, 136.21, 130.53, 129.03, 128.40, 127.82, 127.46, 124.17, 123.79, 110.14, 74.81, 43.85, 22.77, 22.56.

Compound 3i: Yellow solid (25% yield), the crystals became black without melting. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.01 (s, 1H), 10.86 (s, 1H), 7.52 (d, J = 2.0 Hz, 1H), 7.43 (dd, J = 8.3, 2.1 Hz, 1H), 6.80 (d, J = 8.3 Hz, 1H), 2.15 (s, 1H), 2.08 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 173.45, 170.50, 167.50, 143.23, 140.88, 133.24, 131.24, 127.13, 114.42, 112.61, 74.66, 22.73, 22.55.

Compound 3j: Yellow solid (57% yield), mp 280-285 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.03 (s, 1H), 7.58 (d, J = 2.0 Hz, 1H), 7.54 (dd, J = 8.3, 2.0 Hz, 1H), 7.02 (d, J = 8.3 Hz, 1H), 3.13 (s, 3H), 2.14 (s, 3H), 2.08 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.98, 170.53, 167.48, 143.47, 142.47, 133.27, 130.55, 126.84, 115.11, 111.54, 74.23, 27.23, 22.73, 22.53. HRMS (FTMS + pESI) m/z, calcd. for $C_{14}H_{13}BrN_4O_3S$: 395.9892; found: 396.2807 (M+1).

Compound 3k: Yellow solid (61% yield), mp 225-230 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.61 (d, J = 2.0 Hz, 1H), 7.51 (dd, J = 8.4, 2.1 Hz, 1H), 6.91 (d, J = 8.4 Hz, 1H), 5.85-5.76 (m. 1H), 5.28 (dd, J = 17.3, 1.4 Hz, 1H), 5.14 (dd, J = 10.5, 1.4 Hz, 1H), 4.47-4.21 (m, 1H), 2.16 (s, 1H), 2.09 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.82, 170.58, 167.56, 143.35, 141.39, 133.17, 131.45, 130.53, 126.95, 117.26, 115.21, 112.16, 74.27, 42.65, 22.73, 22.50. HRMS (FTMS + pESI) m/z, calcd. for $C_{16}H_{15}BrN_4O_3S$: 422.0048; found: 420.9978 (M-1).

Compound 3l: Yellow solid (65% yield), mp 270-275 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.09 (s, 1H), 7.64 (d, J = 2.0 Hz, 1H), 7.44 (dd, J = 8.4, 2.1 Hz, 1H), 7.41-7.21 (m, 5H), 6.74 (d, J = 8.4 Hz, 1H), 5.04 (d, J = 16.3 Hz, 1H), 4.80 (d, J = 16.3 Hz, 1H), 2.21 (s, 3H), 2.10 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.18, 170.61, 167.67, 143.38, 141.23, 135.85, 133.18, 130.61, 129.05, 127.88, 127.42, 127.02, 115.40, 112.15, 74.40, 43.94, 22.73, 22.51. HRMS (FTMS + pESI) m/z, calcd. for $C_{20}H_{17}BrN_4O_3S$: 472.0205; found: 471.0100 (M-1).

Compound 3m: Yellow solid (52% yield), mp 220-225 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.03 (s, 2H), 7.39 (d, 2H), 7.31(ddd, J = 7.7, 1.2 Hz, 2H), 7.16-7,06 (m, 4H), 3.78 (m, 4H), 2.16 (s, 6H) 2.09 (s, 6H), 1.93 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 172.24, 170.51, 167.49, 142.29, 130.69, 128.33, 124.35, 123.63, 109.60, 75.03, 38.39, 38.35, 22.38. HRMS (FTMS + pESI) m/z, calcd. for $C_{29}H_{28}N_8O_6S_2$: 648.1573; found: 647.1487 (M-1).

Compound 3n: Yellow solid (27% yield), mp 245-250 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.02 (s, 2H), 7.39-7.31 (m, 4H), 7.11-7.15 (m, 4H), 3.72 (br, 4H), 2.16 (s, 6H), 2.10 (s, 6H), 1.68 (br, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.96, 170.08,

166.94, 142.86, 141.81, 130.26, 128.03, 123.74, 123.10, 109.42, 74.26, 39.52, 23.84, 22.36, 22.17. HRMS (FTMS + pESI) m/z, calcd. for $C_{30}H_{30}N_8O_6S_2$: 662.1730; found: 661.1658 (M-1).

Supporting Information

The data that supports the findings of this study are available in the supplementary material of this article.

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Conflict of Interest

The authors declare no conflict of interest, financial or otherwise.

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ARTICLE

Microwave-assisted Synthesis and Antimicrobial Activity of Novel Spiro 1,3,4-thiadiazolines From Isatin Derivatives

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