http://dx.doi.org/10.21577/0103-5053.20180153

J. Braz. Chem. Soc., Vol. 30, No. 1, 198-209, 2019
Printed in Brazil - ©2019 Sociedade Brasileira de Química

Short Report

A Facile Synthesis of Novel Isatinspirooxazine Derivatives and Potential *in vitro* Anti-Proliferative Activity

Iara S. Santos,[a,b] Fabiana S. Guerra,[c] Lucas F. Bernardino,[a] Patrícia D. Fernandes,[c] Lidilhone Hamerski[b] and Bárbara V. Silva*[,a]

[a]*Instituto de Química (IQ), Universidade Federal do Rio de Janeiro (UFRJ), Av. Athos da Silveira Ramos, 149, Cidade Universitária, Ilha do Fundão, 21949-900 Rio de Janeiro-RJ, Brazil*

[b]*Instituto de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373, Edifício do CCS, Bloco H, Cidade Universitária, 21941-902 Rio de Janeiro-RJ, Brazil*

[c]*Laboratório de Farmacologia da Dor e da Inflamação, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Avenida Carlos Chagas Filho, 373, Edifício do CCS, Bloco J, Cidade Universitária, 21941-902 Rio de Janeiro-RJ, Brazil*

Novel isatinspirooxazine derivatives were designed and synthesized as potential anti-proliferative agents. The new compounds were obtained from aldol condensation reactions between isatin and 3-(hydroxyimino)butan-2-one in the presence of an organic base in order to generate an aldol adduct, followed by cyclization in trifluoroacetic acid, providing the desired isatinspirooxazines in 30 to 80% yield. All the synthesized compounds, including the starting material and the synthetic intermediates, were tested for *in vitro* anti-proliferative activity against cell lines MCF-7 and MDA-MB231 (breast cancer) and A549 (lung cancer), highlighting the compound 4-methyl,5'-methyl-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] with an IC_{50} (half maximal inhibitory concentration) = 0.34 μM, more potent than the reference drug, doxorubicin (IC_{50} = 1.88 μM), in breast cancer line MDA-MB231.

Keywords: isatinspirooxazine, anti-proliferative activity, aldol adduct

Introduction

Cancer is the second largest cause of death globally, and despite technological and social development, it is estimated that in 2018, in the United States alone, 1,735,350 new cases of cancer and 609,640 cases of cancer deaths will be identified. Additionally, there is an alarming rise in the incidence of new types of cancer, highlighting the issue as a public health problem for health systems worldwide. Although the number of new anticancer therapies has increased in the past decade, the mechanisms for decreasing the incidence of cancer remains unclear. New anticancer therapies include new chemotherapeutic agents, but severe side effects persist. Thus, there is a constant need to develop alternative anticancer drugs with minimal side effects.[1]

*e-mail: barbara@iq.ufrj.br

Isatin (1*H*-indole-2,3-dione) is a small, versatile and widely applicable pharmacological molecule. These characteristics make isatin and its derivatives attractive to many research groups as resources for chemical and pharmacological studies. This molecule and its analogues display diverse types of biological activity, such as trypanocidal, anticonvulsant, antimicrobial, antiprotozoal and anti-inflammatory activities.[2-8]

Furthermore, isatin and its derivatives are effective against several cancer cell lines, capable of reducing the proliferation of cancer cells through the inhibition of several proteins, particularly protein kinases.[9-12]

The oxazines are heterocyclic derivatives of 2*H*- and 4*H*-pyran. They are formed by a six-membered ring that contains an oxygen and nitrogen heteroatom.[13] Oxazines and their derivatives have broad pharmacological activity, including antimalarial, antimicrobial, anti-HIV, antidepressant and anticancer activities.[14-21]

In continuation of our interest in the synthesis and biological activities of isatin derivatives, we describe herein the synthesis of a novel series of isatin derivatives containing the 4*H*-1,2-oxazin-4-one nucleus, and the *in vitro* evaluation of cytotoxic activity of all products, including the key intermediates, against three human cancer cell lines.

Results and Discussion

Chemistry

The anticancer role of isatin derivatives is well-established in the literature. The most significant is sunitinib malate (Sutent®), used in the treatment of advanced renal carcinoma, gastrointestinal stromal tumors, and pancreatic neuroendocrine tumors. This compound is a multikinase inhibitor targeting VEGFR-1, VEGFR-2, PDGFRb, and c-Kit.[22,23] Furthermore, the pharmaceutical company Boehringer reported a new isatin-based triple angiokinase inhibitor, BIBF1120 II, in phase III clinical trials for non-small cell lung cancer.[24,25] The fluorine atom at C-5 of sunitinib malate is essential to its activity, and several structure-activity relationship (SAR) studies revealed that substitution at position 5 was favored over positions 4, 6 or 7, providing greater anti-oncogenic activity.[10,11]

For these reasons, we decided to use as our starting material isatin and its derivatives containing chloro-, bromo-, iodo-, fluoro-, nitro-, and methyl-groups at the 5 and/or 7 positions of the aromatic ring (**1a**-**i**). These compounds were synthesized according to previous reports.[26-29]

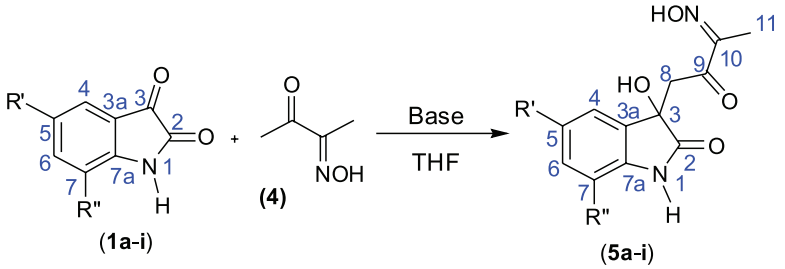
The continuous SAR studies on isatin have shown that C-3-substituted isatins have several biological activities. Examples include the hydrazone, imine and hydrazide moieties, which exhibited specific and potent receptor tyrosine kinases (RTKs) and cyclin-dependent kinases (CDKs) inhibition.[30,31]

Therefore, isatins (**1a**-**i**) were condensed with 3-(hydroxyimino)butan-2-one (**4**) in tetrahydrofuran, and in the presence of a catalytic amount of diethylamine or triethylamine, to directly yield the aldol adduct derivatives (Table 1).

In the reactions with diethylamine,[32,33] there was total consumption of the starting material over periods that varied from 7 to 21 days at room temperature. The different yields can be justified by the different solubility of the products in the liquid-liquid extraction solvent (ethyl acetate). Compounds **5a**, **5b** and **5i** were obtained in the lowest yields, while **5d** showed excellent yields.

Reactions with substrates (**1e**-**g**), containing two chlorine or bromine atoms at positions 5 and 7, respectively, and a nitro group at position 5 of the aromatic ring, failed when we used the diethylamine base. In this cases, 2-(2-aminophenyl)-*N*,*N*-diethyl-2-oxoacetamides were obtained in 50, 65 and 70% yield by employing

Table 1. Reaction conditions and yields for the synthesis of aldol adduct derivatives



1	5	R'	R''	Base	Temperature	time	Yield / %
1a	**5a**	H	H	Et_2NH	r.t.	7 d	47
1b	**5b**	Br	H	Et_2NH	r.t.	9 d	44
1c	**5c**	Cl	H	Et_2NH	r.t.	7 d	57
1d	**5d**	I	H	Et_2NH	r.t.	8 d	91
1e	**5e**	Cl	Cl	Et_3N	reflux	13 h	45
1f	**5f**	Br	Br	Et_3N	reflux	13 h	65
1g	**5g**	NO_2	H	Et_3N	reflux	12 h	70
1h	**5h**	F	H	Et_2NH	r.t.	7 d	61
1i	**5i**	Me	H	Et_2NH	r.t.	21 d	36

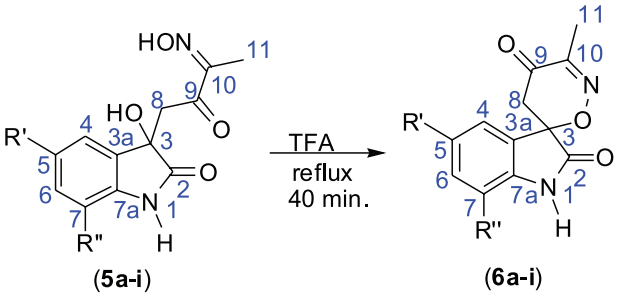
r.t.: room temperature.

substrates **1e**, **1f** and **1g**, respectively. The formation of the oxoacetamides involves the opening of the heterocycle ring, i.e., nucleophilic attack of the pair of electrons from diethylamine to the amide carbonyl (C-2). At first this was not expected, since the amide carbonyl (C-2) is less electrophilic than the ketone (C-3). However, the highly electron withdrawing effect of the two chlorine or bromine atoms and the nitro group probably increased the electrodeficiency of both carbonyls. The nucleophilic attack on C-3 is reversible, while attack on C-2 leads to a nucleophilic acylic substitution.

The exchange of this for the trimethylamine, a non-nucleophilic base, and heating under reflux conditions afforded the desired products in moderate yields.

On the other hand, heterocycles such as the oxazines and their derivatives have been shown to have a wide range of pharmacological activity including anticancer.[20,21] Thus, in the following step, the new isatin derivatives were utilized for oxazine derivative addition on C-3. This strategy could potentially provide compounds that exhibit anticancer activity.

The reaction of aldol adducts in trifluoroacetic acid (TFA) under reflux for 40 min afforded an array of isatinspirooxazines in moderate to good yields (Scheme 1).



(**5a-i**) (**6a-i**)

Substituents	Yield
(a) R' = H; R'' = H	56%
(b) R' = Br; R'' = H	50%
(c) R' = Cl; R'' = H	58%
(d) R' = I; R'' = H	37%
(e) R' = Cl; R'' = Cl	60%
(f) R' = Br; R'' = Br	71%
(g) R' = NO_2; R'' = H	40%
(h) R' = F; R'' = H	51%
(i) R' = CH_3; R'' = H	80%

Scheme 1. Synthesis of isatinspirooxazines derivatives.

Biological studies-cytotoxic activity

All compounds, including starting materials, were evaluated for their cytotoxic activity against MCF-7 (human mammary epithelial adenocarcinoma, ATCC No. HTB-22), A-549 (human lung adenocarcinoma, ATCC No. CCL-185), MDA-MB231 (human mammary epithelial adenocarcinoma triple negative, ATCC No. HTB-26) by employing the 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. The results are summarized in Table 2. Half maximal inhibitory concentration (IC_{50}) values are expressed in μM and compared against doxorubicin as the positive control.

The results obtained for the halogenated, nitro and methyl isatin derivatives at C-5 and C-7 (Table 2) did not corroborate those reported in the literature, except for the mono-halogen compounds (Br, Cl and I) at C-5, which were cytotoxic to the three cancer cell lines tested. 5-Methyl-isatin (**1i**) demonstrated a very intriguing result as, besides cytotoxicity, selectivity of the compound for the MDA-MB231 cell line (IC_{50} = 8.84 μM) was observed. It was inactive against the MCF-7 cell line. This was of particular interest as both lineages are breast tumor-derived, but the MDA-MB231 tumor is more aggressive than the MCF-7 tumor. This compound was also cytotoxic for the A549 cell line (IC_{50} = 6.24 μM).

The addition of 3-(hydroxyimino)butan-2-one (**4**) at C-3 to yield intermediate aldol adducts (**5a-i**) also generated compounds that had no significant effect on the cytotoxicity of this compound on MCF-7, MDA-MB231, and A549 cells. However, the derivative obtained from 5-methyl-isatin (**5i**) showed a slight improvement in cytotoxic activity, and this compound displayed MCF-7 cytotoxicity (IC_{50} = 7.14 μM).

Additionally, aldol adducts (**5d**) (IC_{50} = 2.95, 2.79 and 3.66 μM in the MCF-7, MDA-MB231 and A549 lines, respectively) and (**5i**) (IC_{50} = 7.14, 6.31 and 5.43 μM in the MCF-7, MDA-MB231 and A549 lines, respectively) exhibited an increase in cytotoxic activity when compared to their respective starting materials, isatins (**1d**) and (**1i**). The substance (**5a**) also presented activity superior to (**1a**).

The last series of compounds tested were the oxazine derivatives. Again, the mono-halogenated compounds displayed similar cytotoxicity to their precursors. However, again the 5-methyl-isatin derivative (**6i**) showed an intriguing result: the spirooxazine group at C-3 increased cytotoxic activity specific to the MDA-MB231 cell line (IC_{50} = 0.34 μM) that was also more potent than doxorubicin (IC_{50} = 1.88 μM). This can also be observed for substance (**6b**). Though it did not exceed the activity of the reference standard, it was more active than (**1b**) and (**5d**), indicating the importance of the oxazine nucleus for these derivatives.

It is worth mentioning that tumors formed by the MDA-MB231 lineage are very aggressive, with rapid proliferation and formation of metastases. Additionally, the cells are triple negative, i.e., not responsive to hormonal

Table 2. Cytotoxic effect of compounds against MCF-7, MDA-MB, and A549 tumor human cell lines

	IC_{50}[a] / μM				
	MCF-7	MDA-MB231	A549	Human blood leukocytes	Erythrocytes[b]
1a	> 30.0	> 30.0	6.01	> 100.0	> 100.0
1b	7.29	7.67	5.23	> 100.0	> 100.0
1c	3.87	4.47	3.62	> 100.0	> 100.0
1d	5.97	20.44	5.20	> 100.0	> 100.0
1e	> 30.0	> 30.0	NT	NT	NT
1f	> 30.0	> 30.0	NT	NT	NT
1g	> 30.0	> 30.0	NT	NT	NT
1h	> 30.0	> 30.0	NT	NT	NT
1i	> 30.0	8.84	6.24	> 100.0	> 100.0
5a	6.69	> 30.0	5.31	> 100.0	> 100.0
5b	10.97	> 30.0	5.14	> 100.0	> 100.0
5c	6.63	6.6	5.64	> 100.0	> 100.0
5d	2.95	2.79	3.66	> 100.0	> 100.0
5e	> 30.0	> 30.0	NT	> 100.0	> 100.0
5f	> 30.0	> 30.0	NT	> 100.0	> 100.0
5g	> 30.0	> 30.0	NT	> 100.0	> 100.0
5h	> 30.0	> 30.0	NT	> 100.0	> 100.0
5i	7.14	6.31	5.43	> 100.0	> 100.0
6a	> 30.0	14.62	8.53	> 100.0	> 100.0
6b	5.58	2.66	5.91	> 100.0	> 100.0
6c	19.3	6.09	5.50	> 100.0	> 100.0
6d	> 30.0	25.81	6.86	> 100.0	> 100.0
6e	> 30.0	> 30.0	NT	> 100.0	> 100.0
6f	> 30.0	> 30.0	NT	> 100.0	> 100.0
6g	> 30.0	> 30.0	NT	> 100.0	> 100.0
6h	> 30.0	> 30.0	NT	> 100.0	> 100.0
6i	> 30.0	0.34	13.2	> 100.0	> 100.0
Doxorubicin[c]	4.49	1.83	0.55	NT	NT

[a]Data are presented as half maximal inhibitory concentration (IC_{50}) values (μM) obtained by nonlinear regression for all cell lines from three independent experiments; [b]concentration of compound that induced erythrocyte lysis; [c]doxorubicin was used as positive control. Only compounds with an IC_{50} value lower than 10 μM for at least one cell line were considered active; NT: not tested.

treatments, implying limitations in available therapeutic approach for patients with this type of tumor. This has a greater degree of clinical implication than for tumors formed by MCF-7 cells, making **6i** a promising prototype molecule for the treatment of this type of cancer.

In general, our biological results suggest that the insertion of hydrophobic substituents and electron-withdrawing groups such as chlorine, bromine, and iodine at the 5-position of the aromatic ring make isatins and derivatives more cytotoxic; this is in agreement with the literature.[10,11] However, the most active **6i** has a methyl group, an electron donor by inductive effect or via hyperconjugation. In a review, Barreiro *et al.*[34] described that the methyl group is able to block metabolic active sites, thereby increasing the biological stability of many compounds and generally contributing to increased lipophilicity, making the molecule less water soluble.

All substances tested showed a lytic effect at > 100 μM, indicating that a concentration greater than 100 μM would be necessary for cell death and that these derivatives may be selective agents against tumor cells.

Conclusions

In this work, isatinspirooxazines derivatives were designed, synthesized and evaluated against the breast cancer lineages MCF-7 and MDA-MB231, and some were evaluated using the lung cancer line A549. These types of cancer are responsible for high mortality in the modern world.

The anti-proliferative screening results, with the exception of the substances containing fluorine atoms, suggested that the attachment of halogens to the aromatic ring of the isatin nucleus results in more active derivatives

against the investigated cell lines. However, oxazine (**6i**), which has a methyl group attached to the aromatic ring, was the most active against the MDA-MB231 cell line compared to all other synthesized substances.

It should be noted that, as the new compounds investigated are structurally simple and easily accessible, they represent a very promising starting point for the design and synthesis of more potent agents for the treatment of cancer. Mechanism of action studies of these compounds are underway.

Experimental

General procedures

All the compounds obtained in this work were characterized by spectroscopic and spectrometric methods. The nuclear magnetic resonance spectra of hydrogen (^{1}H NMR) and carbon (^{13}C NMR) were collected on a Bruker apparatus, XRD-400 model, operating at 200, 300 or 500 MHz for ^{1}H nuclei and 50, 75 or 125 MHz for ^{13}C. The solvent used was deuterated dimethyl sulfoxide (DMSO-d_6, δ 2.50, q, J 1.9 Hz and ^{1}H chemical shift of HOD (chemical shift of H_2O) δ 3.3, s) or deuterated chloroform ($CDCl_3$, δ 7.26, s, and ^{1}H chemical shift of HOD δ 1.5, s). Chemical shifts (δ) were expressed in parts *per* million (ppm) values and coupling constants (J) in hertz (Hz). The relative areas of the hydrogen signals were obtained by electronic integration, and the multiplicity of absorption bands was indicated according to the convention: s (singlet), brs (broad singlet), d (doublet), doublet of doublets (dd), t (triplet), q (quartet), quint (quintet), sext (sextet) and m (multiplet). The quaternary carbons of compounds, described by the letter q, were differentiated from CH, CH_2 and CH_3 with the aid of distortionless enhancement by polarization transfer (DEPT)-135.

The exact masses of compounds were obtained using a quadrupole-time-of-flight mass spectrometer (MS, Micromass, UK) in positive electrospray ionization (ESI) mode, using formic acid for the ionization of the substances and concentrated phosphoric acid for equipment calibration.

Melting points of the products were determined on a Mel-Temp apparatus using glass capillaries. Values were not corrected.

Synthesis of isatins

5-Bromo-1*H*-indoline-2,3-dione (**1b**)

In an Erlenmeyer flask, 40 mmol isatin (5.9 g) was solubilized in a mixture of ethanol (120 mL) and distilled water (40 mL) with subsequent addition of 40 mmol bromine (6.4 g). The reaction mixture was kept at room temperature under magnetic stirring. The progress of the reaction was monitored by thin layer chromatography. After the total consumption of the starting material, the reaction mixture was poured into ice and the precipitate formed was vacuum-filtered.[35] Orange solid; yield 70%; ^{1}H NMR (400 MHz, DMSO-d_6) δ 6.88 (d, J 8.0 Hz, 1H, H-7), 7.65 (s, 1H, H-4), 7.73 (d, J 8.0 Hz, 1H, H-6); ^{13}C NMR (100 MHz, DMSO-d_6) δ 114.76 (CH), 120.03 (C), 127.36 (CH), 140.53 (CH), 150.08 (C), 159.45 (C), 183.68 (C); ^{13}C NMR DEPT-135 (100 MHz, DMSO-d_6) δ 114.76 (CH), 127.36 (CH), 140.52 (CH); IR (KBr) ν_{max} / cm^{-1} 3488, 3209, 3075, 1751, 1708, 1614, 1469, 1448, 1292, 1272, 844.

5-Chloro-1*H*-indoline-2,3-dione (**1c**)

A solution of 9 mmol (2.09 g) of trichloroisocyanuric acid (TCCA) in 12 mL of concentrated sulfuric acid (225.3 mmol) was prepared in an Erlenmeyer flask. Subsequently, 20 mmol (2.94 g) of isatin (**1a**) was added to the solution at 0 °C. The mixture was kept under magnetic stirring for 15 min. The mixture was poured onto crushed ice. The obtained precipitate was filtered under vacuum into a Büchner funnel and washed with ice water.[26] Orange solid; yield 70%; ^{1}H NMR (500 MHz, $CDCl_3$ + DMSO-d_6) δ 6.92 (d, J 10.0 Hz, 1H, H-7), 7.54 (d, J 10.0 Hz, 1H, H-4), 7.61 (dd, J 10.0 and 5.0 Hz, 1H, H-6), 11.15 (s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 114.33 (CH), 119.59 (C), 124.60 (CH), 127.29 (C), 137.74 (CH), 149.69 (C), 159.62 (C), 183.82 (C); ^{13}C NMR DEPT-135 (125 MHz, DMSO-d_6) δ 114.32 (CH), 124.59 (CH), 137.74 (CH); IR (KBr) ν_{max} / cm^{-1} 3479, 3093, 3066, 1760, 1702, 1616, 1469, 1452, 1284, 846.

5-Iodo-1*H*-indoline-2,3-dione (**1d**)

In a 500 mL flask containing 80 mL of methanol and an equal amount of $KICl_2$ solution (freshly prepared), 27 mmol (4 g) of isatin (**1a**) was added. This reaction was stirred under magnetic stirring for five days, after which time another 20 mL of methanol and 20 mL of $KICl_2$ solution was added, and the suspension remained under stirring for another three days. The reaction medium was filtered and washed with water to exhaustion. The product was recrystallized from ethanol and the final yield was 86%.[29] Red solid; yield 86%; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6) δ 6.73 (d, J 12.0 Hz, 1H, H-7), 7.70 (s, 1H, H-4), 7.80 (d, J 12.0 Hz, 1H, H-6), 11.09 (s, 1H, NH); ^{13}C NMR (100 MHz, $CDCl_3$ + DMSO-d_6) δ 85.40 (C), 115.06 (CH), 119.96 (C), 133.00 (CH), 146.25 (CH), 150.52 (C), 158.86 (C), 183.47 (C); ^{13}C NMR DEPT-135 (100 MHz, $CDCl_3$ + DMSO-d_6) δ 115.06 (CH), 133.0 (CH), 146.25 (CH); IR (KBr) ν_{max} / cm^{-1} 3467, 3239, 3091, 1747, 1731, 1604, 1459, 1438, 885.

5,7-Dichloro-1*H*-indoline-2,3-dione (**1e**)

A solution of 20 mmol (4.64 g) of trichloroisocyanuric acid (TCCA), used as the chlorinating agent, in 12 mL of sulfuric acid was prepared in an Erlenmeyer flask. Subsequently, 20 mmol (2.94 g) of isatin (**1a**) was added to the solution at 0 °C. The mixture was kept under magnetic stirring for 30 min. The mixture was poured onto crushed ice. The obtained precipitate was filtered under vacuum into a Büchner funnel and washed with ice water.[26] Orange solid; yield 80%; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6) δ 7.39 (s, 1H, H-6), 7.54 (s, 1H, H-4), 11.55 (s, 1H, NH); ^{13}C NMR (100 MHz, $CDCl_3$ + DMSO-d_6) δ 118.27 (C), 120.07 (C), 123.24 (CH), 128.33 (C), 136.83 (CH), 147.05 (C), 159.32 (C), 182.91 (C); ^{13}C NMR DEPT-135 (100 MHz, $CDCl_3$ + DMSO-d_6) δ 123.24 (CH), 136.83 (CH); IR (KBr) ν_{max} / cm^{-1} 3209, 3088, 1757, 1699, 1614, 1454, 1400, 1290, 876.

5,7-Dibromo-1*H*-indoline-2,3-dione (**1f**)

In an Erlenmeyer flask, the isatin (**1**) 20 mmol (3.0 g) was solubilized in a mixture of acetic acid (70 mL) followed by addition of 47 mmol of bromine (7.5 g). The reaction mixture was heated at 70-80 °C for 1 h. The progress of the reaction was monitored by thin layer chromatography. After the total consumption of the starting material, the reaction medium was poured onto ice and the precipitate formed was vacuum filtered. Subsequently the formed product was treated with 50 mL of 10 M HCl and redissolved.[35] Orange solid; yield 70%; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6) δ 7.53 (s, 1H, H-6), 7.77 (s, 1H, H-4), 11.38 (s, 1H, NH); ^{13}C NMR (100 MHz, $CDCl_3$ + DMSO-d_6) δ 106.55 (C), 115.60 (C), 120.51 (C), 126.52 (CH), 142.23 (CH), 149.07 (C), 159.19 (C), 183.02 (C); ^{13}C NMR DEPT-135 (100 MHz, $CDCl_3$ + DMSO-d_6) δ 126.52 (CH), 142.23 (CH); IR (KBr) ν_{max} / cm^{-1} 3459, 3180, 3079, 1743, 1608, 1448, 1288, 873.

5-Nitro-1*H*-indol-2,3-dione (**1g**)

In an Erlenmeyer flask, a solution containing 20 mmol (3.00 g) of isatin (**1a**) was completely solubilized in 40 mL of concentrated sulfuric acid (H_2SO_4). This solution was then cooled to 0 °C in an ice bath and a solution of 19 mmol (1.92 g) of potassium nitrate (KNO_3) in 4 mL of concentrated sulfuric acid (H_2SO_4) was added to the reaction medium, which in turn was kept under stirring at 0 °C for 30 min. After this time, the reaction was poured over crushed ice and the yellow precipitate formed was vacuum-filtered on a Büchner funnel and washed with ice water.[36] Yellow solid; yield 70%; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6) δ 7.07 (d, *J* 8.0 Hz, 1H, H-7), 8.23 (s, 1H, H-4), 8.39 (d, *J* 8.0 Hz, 1H, H-6), 11.66 (s, 1H, NH); ^{13}C NMR (100 MHz, $CDCl_3$ + DMSO-d_6) δ 113.10 (CH), 117.85 (C), 120.31 (CH), 133.45 (CH), 143.30 (C), 155.71 (C), 159.83 (C), 182.92 (C); ^{13}C NMR DEPT-135 (100 MHz, $CDCl_3$ + DMSO-d_6) δ 113.10 (CH), 120.30 (CH), 133.45 (CH); IR (KBr) ν_{max} / cm^{-1} 3332, 3093, 1767, 1751, 1620, 1533, 1471, 1335, 748.

General procedure for the preparation of fluoro-1*H*-indoline-2,3-dione and methyl-1*H*-indoline-2,3-dione (**1h-i**)

In an Erlenmeyer flask containing 9 mL of sulfuric acid, 18 mmol of the respective isonitrosoacetanilide formed from the anilines was slowly added and kept under magnetic stirring at room temperature. The end of the reaction was identified by elevated temperature and gradual color change. The solution was poured onto crushed ice, and the precipitate formed was vacuum-filtered on Büchner funnel and washed with ice water.[28]

5-Fluoro-1*H*-indoline-2,3-dione (**1h**)

Red solid; yield 85%; ^{1}H NMR (400 MHz, $CDCl_3$ + DMSO-d_6) δ 6.89 (dd, *J* 8.0 and 4.0 Hz, 1H, H-7), 7.27-7.29 (m, 1H, H-6), 7.33-7.38 (m, 1H, H-4), 11.02 (s, 1H, NH); ^{13}C NMR (100 MHz, $CDCl_3$ + DMSO-d_6) δ 111.66 (d, *J* 24.0 Hz, CH), 113.98 (d, *J* 8.0 Hz, CH), 118.7 (d, *J* 7.0 Hz, C), 124.99 (d, *J* 24.0 Hz, CH), 147.45 (C), 158.58 (d, *J* 236.0 Hz, C), 159.80 (C), 184.36 (C); ^{13}C NMR DEPT-135 (100 MHz, $CDCl_3$ + DMSO-d_6) δ 111.66 (d, *J* 23.0 Hz, CH), 113.88 (d, *J* 8.0 Hz, CH), 124.4 (d, *J* 24.0 Hz, CH); IR (KBr) ν_{max} / cm^{-1} 3446, 3228, 3099, 1761, 1738, 1620, 1489, 1288, 1194, 891.

5-Methyl-1*H*-indoline-2,3-dione (**1i**)

Red solid; yield 84%; ^{1}H NMR (500 MHz, DMSO-d_6) δ 2.21 (s, 3H, CH_3), 6.76 (d, *J* 10.0 Hz, 1H, H-7), 7.27 (s, 1H, H-4), 7.35 (d, *J* 10.0 Hz, 1H, H-6), 10.90 (s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 20.62 (CH_3), 112.56 (CH), 118.30 (C), 125.32 (CH), 132.54 (C), 139.32 (CH), 149.08 (C), 159.99 (C), 185.12 (C); ^{13}C NMR DEPT-135 (125 MHz, DMSO-d_6) δ 20.60 (CH_3), 112.52 (CH), 125.29 (CH), 139.28 (CH); IR (KBr) ν_{max} / cm^{-1} 3442, 3284, 3029, 1743, 1716, 1490, 1303, 1128, 829.

General procedure for the preparation of (2'-oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5a-d**, **5h-i**)

To a round bottom flask 1 mmol of the isatin (**1a-i**), 2 mmol of 3-(hydroxyimino)butan-2-one (**4**), 20 mL of tetrahydrofuran (THF) and 1 mL of diethylamine were added. The reaction was maintained at room temperature and was monitored by thin layer chromatography until

the total consumption of the starting material. Then, liquid-liquid extraction was performed with ethyl acetate (3 × 20 mL), the organic phase was dried over anhydrous sodium sulfate, filtered and concentrated in vacuum to give a brown solid, which was purified by silica gel column chromatography.

(2'-Oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5a**)

Light brown solid; yield 47%; mp 160-164 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.62 (s, 3H, H-11), 3.26 (d, *J* 24.0 Hz, 1H, H-8a), 3.82 (d, *J* 24.0 Hz, 1H, H-8b), 5.99 (s, 1H, OH), 6.78 (d, *J* 8.0 Hz, 1H, H-7), 6.87 (t, *J* 8.0 Hz, 1H, H-6), 7.13-7.18 (m, 2H, H-4 and H-5), 10.21 (s, 1H, NH), 12.39 (s, 1H, NOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 7.72 (CH_3), 44.08 (CH_2), 72.86 (C), 109.41 (CH), 121.17 (CH), 123.60 (CH), 128.96 (CH), 131.50 (C), 142.73 (C), 154.75 (C), 178.22 (C), 195.13 (C); ^{13}C NMR DEPT-135 (100 MHz, DMSO-d_6) δ 8.16 (CH_3), 44.51 (CH_2), 109.85 (CH), 121.62 (CH), 124.04 (CH), 129.42 (CH); IR (KBr) ν_{max} / cm^{-1} 3376, 3245, 3062, 2913, 1704, 1679, 1618, 1471, 1193, 933; high-resolution (HR)MS (ESI) calcd. for $C_{12}H_{13}N_2O_4^+$ [M + H]$^+$: 249.08754, found: 249.08685, error (ppm): 2.73; calcd. for $C_{24}H_{26}N_4NaO_8^+$ [2M + Na]$^+$: 519.14919, found: 519.14833, error (ppm): 1.65.

5'-Bromo-(2'-oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5b**)

Brown solid; yield 44%; mp 184-186 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.67 (s, 3H, H-11), 3.36 (d, *J* 16.0 Hz, 4H, H-8a), 3.82 (d, *J* 16.0 Hz, 1H, H-8b), 6.16 (s, 1H, OH), 6.77 (d, *J* 8.0 Hz, 1H, H-7), 7.35 (d, *J* 8.0 Hz, 1H, H-6), 7.41 (s, 1H, H-4), 10.39 (s, 1H, NH), 12.46 (s, 1H, NOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 8.20 (CH_3), 44.58 (CH_2), 73.35 (CH), 111.87 (CH), 113.35 (C), 127.11 (CH), 132.01 (CH), 134.65 (C), 142.57 (C), 155.10 (C), 178.25 (C), 195.67 (C); ^{13}C NMR DEPT-135 (100 MHz, DMSO-d_6) δ 8.20 (CH_3), 44.58 (CH_2), 111.86 (CH), 127.11 (CH), 132.00 (CH); IR (KBr) ν_{max} / cm^{-1} 3485, 3217, 1713, 1697, 1618, 1182, 991; HRMS (ESI) calcd. for $C_{12}H_{11}BrN_2NaO_4^+$ [M + Na]$^+$: 350.97794, found: 350.97717, error (ppm): 2.19; calcd. for $C_{24}H_{22}Br_2N_4NaO_8^+$ [2M + Na]$^+$: 676.96712, found: 678.96611, error (ppm): 2.95.

5'-Chloro-(2'-oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5c**)

Brown solid; yield 57%; mp 155-159 ºC; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.65 (s, 3H, H-11), 3.34 (d, *J* 20.0 Hz, 1H, H-8a), 3.60 (d, *J* 20.0 Hz, 4H, H-8b)*, 6.16 (s, 1H, OH), 6.80 (d, *J* 10.0 Hz, 1H, H-7), 7.21 (dd, *J* 10.0 and 5.0 Hz, 1H, H-6), 7.29 (d, *J* 5.0 Hz, 1H, H-4), 10.38 (s, 1H, NH), 12.44 (s, 1H, NOH), *signal overlap occurred of solvent; ^{13}C NMR (125 MHz, DMSO-d_6) δ 8.19 (CH_3), 44.57 (CH_2), 73.37 (C), 111.30 (CH), 124.41 (CH), 125.67 (C), 129.15 (CH), 134.24 (C), 142.14 (C), 155.11 (C), 178.39 (C), 195.66 (C); ^{13}C NMR DEPT-135 (125 MHz, DMSO-d_6) δ 8.20 (CH_3), 44.56 (CH_2), 111.30 (CH), 124.41 (CH), 129.16 (CH); IR (KBr) ν_{max} / cm^{-1} 3504, 3207, 1699, 1621, 1483, 1473, 1184, 993; HRMS (ESI) calcd. for $C_{12}H_{11}ClN_2NaO_4^+$ [M + Na]$^+$: 305.03050, found: 305.02975, error (ppm): 2.45; calcd. for $C_{24}H_{22}Cl_2N_4NaO_8^+$ [2M + Na]$^+$: 587.07021, found: 587.07021, error (ppm): 3.40.

5'-Iodo-(2'-oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5d**)

Brown solid; yield 91%; mp 162-164 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.66 (s, 3H, H-11), 3.33 (d, *J* 20.0 Hz, 1H, H-8a), 3.80 (d, *J* 20.0 Hz, 2H, H-8b)*, 6.12 (s, 1H, OH), 6.66 (d, *J* 8.0 Hz, 1H, H-7), 7.51 (dd, *J* 8.0 and 4.0 Hz, 1H, H-6), 7.54 (d, *J* 4.0 Hz, 1H, H-4), 10.36 (s, 1H, NH), *signal overlap occurred of solvent; ^{13}C NMR (100 MHz, DMSO-d_6) δ 8.21 (CH_3), 44.58 (CH_2), 73.19 (C), 84.37 (C), 112.43 (CH), 132.57 (CH), 134.93 (C), 137.85 (CH), 143.05 (C), 155.11 (C), 178.03 (C), 195.67 (C); ^{13}C NMR DEPT-135 (100 MHz, DMSO-d_6) δ 8.21 (CH_3), 44.58 (CH_2), 112.43 (CH), 132.56 (CH), 137.85 (CH); IR (KBr) ν_{max} / cm^{-1} 3250, 2887, 1714, 1697, 1616, 1443, 1180, 827; HRMS (ESI) calcd. for $C_{12}H_{11}IN_2NaO_4^+$ [M + Na]$^+$: 396.96630, found: 396.96525, error (ppm): 2.64; calcd. for $C_{24}H_{22}I_2N_4NaO_8^+$ [2M + Na]$^+$: 770.94284, found: 770.94151, error (ppm): 1.72.

5'-Fluoro-(2'-oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5h**)

Brown solid; yield 61%; mp 180-182 ºC; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.62 (s, 3H, H-11), 3.25 (d, *J* 15.0 Hz, 1H, H-8a), 3.76 (d, *J* 15.0 Hz, 1H, H-8b), 6.12 (s, 1H, OH), 6.72-6.75 (m, 1H, H-7), 6.94-6.98 (m, 1H, H-6), 7.08 (d, *J* 10.0 Hz, 1H, H-4), 10.24 (s, 1H, OH), 12.41 (s, 1H, NOH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 8.26 (CH_3), 44.62 (CH_2), 73.65 (CH), 110.60 (d, *J* 7.5 Hz, CH), 112.12 (d, *J* 25.0 Hz, CH), 115.52 (d, *J* 23.8 Hz, CH), 133.93 (d, *J* 7.5 Hz, C), 139.41 (d, *J* 1.25 Hz, C), 155.20 (C), 158.33 (d, *J* 235.0 Hz, C), 178.73 (C), 195.65 (C); ^{13}C NMR DEPT-135 (125 MHz, DMSO-d_6) δ 8.22 (CH_3), 44.58 (CH_2), 110.56 (d, *J* 7.5 Hz, CH), 112.08 (d, *J* 25.0 Hz, CH), 115.48 (d, *J* 23.8 Hz, CH); IR (KBr) ν_{max} / cm^{-1} 3325, 3224, 1716, 1682, 1493, 1188, 1032, 822; HRMS (ESI) calcd. for $C_{12}H_{11}FN_2NaO_4^+$ [M + Na]$^+$: 289.06006, found: 289.05928, error (ppm): 2.69; calcd. for $C_{24}H_{22}F_2N_4NaO_8^+$ [2M + Na]$^+$: 555.13035, found: 555.12926, error (ppm): 1.96.

5'-Methyl-(2'-oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5i**)

Brown solid; yield 36%; mp 156-158 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.64 (s, 3H, H-12), 2.20 (s, 3H, H-11), 3.26 (d, *J* 16.0 Hz, 1H, H-8a), 3.77 (d, *J* 16.0 Hz, 1H, H-8a), 5.96 (s, 1H, OH), 6.67 (d, *J* 8.0 Hz, 1H, H-7), 6.96 (d, *J* 8.0 Hz, 1H, H-6), 7.02 (s,1H, H-4), 10.12 (s, 1H, NH), 12.40 (s, 1H, NOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 8.18 (CH_3), 21.10 (CH_3), 44.65 (CH_2), 73.41 (CH), 109.58 (CH), 124.76 (CH), 129.53 (CH), 130.34 (CH), 132.05 (C), 140.71 (C), 155.18 (C), 178.67 (C), 195.56 (C); ^{13}C NMR DEPT-135 (100 MHz, DMSO-d_6) δ 8.18 (CH_3), 21.11 (CH_3), 44.61 (CH_2), 109.57 (CH), 124.76 (CH), 129.53 (CH); IR (KBr) ν_{max} / cm^{-1} 3346, 3230, 2926, 1709, 1678, 1497, 1041, 1020, 731; HRMS (ESI) $C_{13}H_{14}N_2NaO_4^+$ [M + Na]$^+$: 285.08513, found: 285.08434, error (ppm): 2.77; calcd. for $C_{26}H_{28}N_4NaO_8^+$ [2M + Na]$^+$: 547.18049, found: 547.17939, error (ppm): 2.0.

General procedure for the preparation of (2'-oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5e-g**)

To a round bottom flask under magnetic stirring coupled to a condenser 2 mmol of 3-(hydroxyimino)butan-2-one (**4**), 20 mL of dry tetrahydrofuran and 1 mL of distilled triethylamine were added. After the mixture was kept under stirring for 15 min, 1 mmol of the desired isatin was added. The reaction remained under reflux and was monitored by thin layer chromatography. After consuming all the starting material, liquid-liquid extraction was performed with ethyl acetate (3 × 20 mL), the organic phase was dried over anhydrous sodium sulfate, filtered into a flask and concentrated in vacuum to give a brown solid that was purified by silica gel column chromatography using 1:1 hexane:ethyl acetate as the eluent.

5',7'-Dichloro-(2'-oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5e**)

Brown solid; yield 45%; mp 212-215 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.66 (s, 3H, H-11), 3.43 (d, *J* 16.0 Hz, 1H, H-8a), 3.84 (d, *J* 16.0 Hz, 1H, H-8b), 6.31 (s, 1H, OH), 7.33 (d, *J* 8.0 Hz, 1H, H-6), 7.41 (d, *J* 8.0 Hz, 1H, H-4), 10.85 (s, 1H, NH), 12.47 (s, 1H, NOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 8.19 (CH_3), 44.6 (CH_2), 73.89 (C), 114.68 (C), 123.26 (CH), 126.30 (C), 128.69 (CH), 135.64 (C), 140.16 (C), 155.02 (C), 178.31 (C), 195.74 (C); ^{13}C NMR DEPT-135 (100 MHz, DMSO-d_6) δ 8.19 (CH_3), 44.76 (CH_2), 123.25 (CH), 128.68 (CH); IR (KBr) ν_{max} / cm^{-1} 3294, 3082, 1720, 1689, 1618, 1460, 1389, 1115, 1034, 708; HMRS (ESI) calcd. for $C_{12}H_{10}Cl_2N_2NaO_4^+$ [M + Na]$^+$: 338.99146, found: 338.99074, error (ppm): 2.12; calcd. for $C_{24}H_{20}Cl_4N_4NaO_8^+$ [2M + Na]$^+$: 654.99074, found: 656.98925, error (ppm): 3.03.

5',7'-Dibromo-(2'-oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5f**)

Light brown solid; yield 65%; mp 181-184 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.68 (s, 3H, H-11), 3.43 (d, *J* 16.0 Hz, 1H, H-8a), 3.84 (d, *J* 16.0 Hz, 1H, H-8b), 6.33 (s, 1H, OH), 7.48 (s, 1H, H-6), 7.63 (s, 1H, H-4), 10.75 (s, 1H, NH), 12.49 (s, 1H, NOH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 8.90 (CH_3), 44.76 (C), 73.89 (C), 114.68 (C), 123.26 (CH), 126.30 (C), 128.69 (CH), 135.64 (C), 140.16 (C), 155.02 (C), 178.31 (C), 195.74 (C); ^{13}C NMR DEPT-135 (100 MHz, DMSO-d_6) δ 8.19 (CH_3), 44.76 (CH_2), 123.25 (CH), 128.68 (CH); IR (KBr) ν_{max} / cm^{-1} 3485, 3199, 1713, 1697, 1618, 1375, 1182, 650; HRMS (ESI) calcd. for $C_{12}H_{10}Br_2N_2NaO_4^+$ [M + Na]$^+$: 428.88646, found: 428.88804, error (ppm): 3.68; calcd. for $C_{24}H_{20}Br_4N_4NaO_8^+$ [2M + Na]$^+$: 834.78815, found: 834.78816, error (ppm): 1.20.

5'-Nitro-(2'-oxo-3'-hydroxy-3'-indolyl)-3-oxo-4-hydroxy-imino-butane (**5g**)

Light brown solid; yield 70%; mp 209-211 ºC; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.61 (s, 3H, H-11), 3.41 (d, *J* 20.0 Hz, 1H, H-8a), 3.94 (d, *J* 20.0 Hz, 1H, H-8b), 6.36 (s, 1H, OH), 6.98 (d, *J* 10.0 Hz, 1H, H-7), 8.12 (d, 1H, *J* 10.0 Hz, H-6), 8.12 (dd, *J* 10 and 5.0 Hz, 1H, H-4), 10.98 (s, 1H, NH), 12.47 (s, 1H, NOH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 8.27 (CH_3), 44.74 (CH_2), 72.94 (CH), 110.15 (CH), 120.03 (CH), 126.99 (CH), 133.30 (C), 142.47 (C), 150.00 (C), 155.10 (C), 179.14 (C), 195.89 (C); ^{13}C NMR DEPT-135 (125 MHz, DMSO-d_6) δ 8.23 (CH_3), 44.70 (CH_2), 110.10 (CH), 119.99 (CH), 126.95 (CH); IR (KBr) ν_{max} / cm^{-1} 3458, 3250, 1724, 1689, 1628, 1325, 1016, 648; HRMS (ESI) calcd. for $C_{12}H_{11}N_3NaO_6^+$ [M + Na]$^+$: 316.05456, found: 316.05371, error (ppm): 2.69; calcd. for $C_{24}H_{22}N_6NaO_{12}^+$ [2M + Na]$^+$: 609.11935, found: 609.118227, error (ppm): 1.77.

General procedure for the preparation of 4-methyl-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] (**6a-i**)

In a round bottom flask coupled to a condenser, 1 mmol of the aldol adduct (**6a-i**) and 5 mL of trifluoroacetic acid (TFA) were added. The reaction medium was maintained under reflux under magnetic stirring for 40 min. The consumption of the starting material was checked by thin layer chromatography. Isolation was done with the addition of saturated sodium carbonate solution (Na_2CO_3) to pH 9.0

and liquid-liquid extraction with ethyl acetate, washing the organic phase with brine, drying the organic phase with anhydrous sodium sulfate, filtration, and evaporation of the solvent, resulting in a dark brown solid that was purified by silica gel column chromatography using hexane: ethyl acetate in concentrations of 10 to 100% as eluent.

4-Methyl-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] (**6a**)

Light brown solid; yield 46%; mp 196-199 ºC; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.95 (s, 3H, H-11), 3.20 (d, *J* 18.2 Hz, 1H, H-8a), 3.15 (d, *J* 18.2 Hz,1H, H-8b), 6.93 (d, *J* 10.0 Hz, 1H, H-7), 7.04 (t, *J* 7.5 Hz, 1H, H-6), 7.35 (t, *J* 7.5 Hz, 1H, H-5), 7.49 (d, *J* 10.0 Hz, 1H, H-4), 11.03 (s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 8.10 (CH_3), 42.24 (CH_2), 79.54 (C), 110.99 (CH), 123.13 (CH), 125.67 (CH), 125.77 (C), 131.71 (CH), 142.92 (C), 144.38 (C), 173.02 (C), 196.07 (C); IR ν_{max} / cm^{-1} 3410, 3157, 3097, 1728, 1716, 1560, 1475, 1101, 746; HRMS (ESI) calcd. for $C_{12}H_{10}N_2NaO_3^+$ [M + Na]$^+$: 253.0583, found: 253.0585, error (ppm): 0.40; calcd. for $C_{24}H_{20}N_4NaO_6^+$ [2M + Na]$^+$: 483.1275, found: 483.1279, error (ppm): 0.80; GC-MS (gas chromatography-mass spectrometry) (70 eV) *m/z* 230 [M]$^+$.

4-Methyl-5'-bromo-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] (**6b**)

Brown solid; yield 50%; mp 198-200 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.96 (s, 3H, H-11), 3.12 (d, *J* 16.0 Hz, 1H, H-8a), 3.22 (d, *J* 16.0 Hz, 1H, H-8b), 6.91 (d, *J* 8.0 Hz, 1H, H-7), 7.56 (d, *J* 8.0 Hz, 1H, H-6), 7.87 (s, 1H, H-4), 11.17 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 8.05 (CH_3), 42.03 (CH_2), 79.22 (C), 112.82 (CH), 114.64 (C), 128.01 (C), 128.78 (CH), 134.25 (CH), 143.09 (C), 143.69 (C), 172.66 (C), 195.70 (C); ^{13}C NMR DEPT-135 (100 MHz, DMSO-d_6) δ 8.05(CH_3), 42.03 (CH_2), 112.82 (CH), 128.78 (CH), 134.25 (CH); IR (KBr) ν_{max} / cm^{-1} 3207, 2981, 2846, 1747, 1616, 1562, 1390, 1105, 814; HRMS (ESI) calcd. for $C_{12}H_{10}BrN_2NaO_3^+$ [M + H]$^+$: 308.98748, found: 308.98698, error (ppm): 1.62; calcd. for $C_{24}H_{18}Br_2N_4NaO_6^+$ [M + Na]$^+$: 638.94907, found: 640.94646, error (ppm): 3.11; GC-MS (70 eV) *m/z* 308 [M]$^+$, 310 [M + 2]$^+$.

4-Methyl-5'-chloro-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] (**6c**)

Light brown solid; yield 58%; mp 214-217 ºC; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.92 (s, 3H, H-11), 3.09 (d, *J* 20.0 Hz, 1H, H-8a), 3.18 (d, *J* 20.0 Hz, 1H, H-8b), 6.92 (d, *J* 10.0 Hz, 1H, H-7), 7.39 (dd, *J* 10.0 and 5.0 Hz, 1H, H-6), 7.71 (d, *J* 10,0 Hz, 1H, H-4), 11.13 (s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 8.30 (CH_3), 42.08 (CH_2), 79.35 (C), 112.41 (CH), 126.19 (CH), 127.12 (C), 127.72 (C), 131.49 (CH), 143.16 (C), 143.34 (C), 172.84 (C), 195.83 (C); ^{13}C NMR DEPT-135 (125 MHz, DMSO-d_6) δ 8.09 (CH_3), 42.05 (CH_2), 112.37 (CH), 126.15 (CH), 131.45 (CH); IR (KBr) ν_{max} / cm^{-1} 3209, 1738, 1713, 1562, 1477, 1358, 1174, 823; HRMS (ESI) calcd. for $C_{12}H_9ClN_2NaO_3^+$ [M + Na]$^+$: 287.0194, found: 287.0202, error (ppm): 2.70; GC-MS (70 eV) *m/z* 264 [M]$^+$, 266 [M + 2]$^+$.

4-Methyl-5'-iodo-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] (**6d**)

Dark brown solid; yield 30%; mp 215-217 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.94 (s, 3H, H-11), 3.10 (d, *J* 20.0 Hz, 1H, H-8a), 3.20 (d, *J* 20.0 Hz, 1H, H-8b), 6.79 (d, *J* 8.0 Hz, 1H, H-7), 7.70 (d, *J* 8.0 Hz, 1H, H-6), 7.96 (s, 1H, H-4), 11.12 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 8.05 (CH_3), 42.06 (CH_2), 79.03 (C), 85.78 (C), 113.22 (CH), 128.21 (C), 134.12 (CH), 140.00 (CH), 143.06 (C), 144.12 (C), 172.49 (C), 195.72 (C); IR (KBr) ν_{max} / cm^{-1} 3294, 2922, 2850, 1751, 1707, 1614, 1562, 1346, 823; HRMS (ESI) calcd. for $C_{12}H_9IN_2NaO_3^+$ [M + Na]$^+$: 378.9550, found: 378.9549, error (ppm): 0.26; calcd. for $C_{24}H_{18}I_2N_4NaO_6^+$ [2M + Na]$^+$: 734.9208, found: 734.9212, error (ppm): 0.54; GC-MS (70 eV) *m/z* 356 [M]$^+$.

4-Methyl-5',7'-dichloro-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] (**6e**)

Light brown solid; yield 36%; mp 220-223 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.97 (s, 3H, H-11), 3.16 (d, *J* 16.0 Hz, 1H, H-8a), 3.24 (d, *J* 16.0 Hz, 1H, H-8b), 7.65 (d, *J* 8.0 Hz, 1H, H-6), 7.79 (d, *J* 8.0 Hz, 1H, H-4), 11.68 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 8.08 (CH_3), 42.13 (CH_2), 79.73 (C), 115.66 (C), 125.03 (CH), 127.59 (C), 128.88 (C), 130.89 (CH), 141.45 (C), 143.13 (C), 172.80 (C), 195.51 (C); ^{13}C NMR DEPT-135 (100 MHz, DMSO-d_6) δ 8.07 (CH_3), 42.13 (CH_2), 125.03 (CH), 130.89 (CH); IR (KBr) ν_{max} / cm^{-1} 3167, 3066, 2922, 2850, 1753, 1720, 1570, 1470, 1163, 889; HRMS (ESI) calcd. for $C_{12}H_8Cl_2N_2NaO_3^+$ [M + Na]$^+$: 320.98097, found: 320.98034, error (ppm): 1.96; calcd. for $C_{24}H_{16}Cl_4N_4NaO_6^+$ [2M + Na]$^+$: 618.97217, found: 620.96882, error (ppm): 3.22; GC-MS (70 eV) *m/z* 298 [M]$^+$, 300 [M + 2]$^+$, 302 [M + 4]$^+$.

4-Methyl-5',7'-dibromo-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] (**6f**)

Yellow solid; yield 71%; mp 225-227 ºC; ^{1}H NMR (400 MHz, acetone-d_6 + TMS) δ 2.00 (s, 3H, H-11), 3.23 (d, *J* 20.0 Hz, 1H, H-8a), 3.30 (d, *J* 20.0 Hz, 1H, H-8b), 7.80 (d, *J* 8.0 Hz, 1H, H-6), 7.84 (d, *J* 8.0 Hz, 1H, H-4); ^{13}C NMR (100 MHz, CD_3OD-d_4 + DMSO-d_6) δ 7.70 (CH_3), 79.76 (C), 103.62 (C), 115.07 (C), 127.83 (CH), 129.00 (C),

136.02 (CH), 143.15 (C), 143.37 (C), 172.46 (C), 195.34 (C); ^{13}C NMR DEPT-135 (100 MHz, CD_3OD-d_4 + DMSO-d_6) δ 7.71 (CH_3), 127.82 (CH), 136.02 (CH); IR (KBr) ν_{max} / cm^{-1} 3413, 3174, 2921, 1712, 1613, 1575, 1458, 1307, 1156, 864; HRMS (ESI) calcd. for $C_{12}H_8Br_2N_2NaO_3^+$ [M + Na]$^+$: 410.87789, found: 410.87736, error (ppm): 1.29; calcd. for $C_{24}H_{16}Br_4N_4NaO_6^+$ [2M + Na]$^+$: 798.76601, found: 798.76636, error (ppm): 0.44; GC-MS (70 eV) *m/z* 386 [M]$^+$, 388 [M + 2]$^+$, 391 [M + 4]$^+$.

4-Methyl-5'-nitro-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] (**6g**)

Dark brown solid; yield 32%; mp 197-200 ºC; ^{1}H NMR (400 MHz, DMSO-d_6) δ 1.98 (s, 3H, H-11), 3.16 (d, *J* 20.0 Hz, 1H, H-8a), 3.31 (s, *J* 20.0 Hz, 4H, H-8b), 7.15 (d, *J* 8.0 Hz, 1H, H-7), 8.32 (dd, *J* 8.0 and 4.0 Hz, 1H, H-6), 8.68 (d, *J* 4.0 Hz, 1H, H-4), 11.74 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ 8.10 (CH_3), 41.97 (CH_2), 78.90 (C), 111.17 (CH), 122.28 (CH), 126.76 (C), 128.56 (CH), 143.34 (C), 150.75 (C), 173.56 (C), 195.58 (C); ^{13}C NMR DEPT-135 (100 MHz, DMSO-d_6) δ 8.10 (CH_3), 41.97 (CH_2), 111.17 (CH), 122.28 (CH), 128.55 (CH); IR (KBr) ν_{max} / cm^{-1} 3385, 3123, 1739, 1630, 1339, 833; HRMS (ESI) calcd. for $C_{24}H_{18}N_6NaO_{10}^+$ [2M + Na]$^+$: 573.09821, found: 573.09804, error (ppm): 0.29.

4-Methyl-5'-fluoro-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] (**6h**)

Dark brown solid; yield 51%; mp 198-203 ºC; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.92 (s, 3H, H-11), 3.10 (d, *J* 20.0 Hz, 1H, H-8a), 3.16 (d, *J* 20.0 Hz, 1H, H-8b), 6.90-6.92 (m, 1H, H-7), 7.17 (td, *J* 10.0 and 5.0 Hz, 1H, H-6), 7.52 (dd, *J* 10.0 and 5.0 Hz, 1H, H-4), 11.02 (s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 8.03 (CH_3), 42.06 (CH_2), 79.55 (C), 111.84 (d, *J* 7.5 Hz, CH), 113.75 (d, *J* 25.0 Hz, CH), 118.00 (d, *J* 23.75 Hz, CH), 127.25 (d, *J* 8.75 Hz, C), 140.57 (d, *J* 2.5 Hz, C), 142.99 (C), 158.70 (d, *J* 236.25 Hz, C), 172.94 (C), 195.79 (C); ^{13}C NMR DEPT-135 (125 MHz, DMSO-d_6) δ 8.04 (CH_3), 42.06 (CH_2), 111.84 (d, *J* 7.5 Hz, CH), 113.75 (d, *J* 25.0 Hz, CH), 118.00 (d, *J* 23.75 Hz, CH); IR (KBr) ν_{max} / cm^{-1} 3429, 3210, 1720, 1630, 1488, 820; HRMS (ESI) calcd. for $C_{12}H_9FN_2NaO_3^+$ [M + Na]$^+$: 271.04949, found: 271.04884, error (ppm): 2.40; calcd. for $C_{24}H_{18}F_2N_4NaO_6^+$ [2M + Na]$^+$: 519.10921, found: 519.10853, error (ppm): 1.30; GC-MS (70 eV) *m/z* 248 [M]$^+$.

4-Methyl-5'-methyl-spiro[(5-aza-4-eno-3-one-cyclohexane)-1,3'-(1*H*-indol-one)] (**6i**)

Light brown solid; yield 80%; mp 215-217 ºC; ^{1}H NMR (500 MHz, DMSO-d_6) δ 1.91 (s, 3H, H-11), 2.21 (s, 3H, H-12), 3.07 (d, *J* 20.0 Hz, 1H, H-8a), 3.12 (d, *J* 20.0 Hz, 1H, H-8b), 6.79 (d, *J* 10.0 Hz, 1H, H-7), 7.13 (d, *J* 10.0 Hz, 1H, H-6), 7.30 (s, 1H, H-4), 10.89 (s, 1H, NH); ^{13}C NMR (125 MHz, DMSO-d_6) δ 8.00 (CH_3), 21.01 (CH_3), 42.18 (CH_2), 79.54 (CH), 110.63 (CH), 125.77 (C), 126.02 (CH), 131.77 (CH), 132.16 (C), 141.77 (C), 172.90 (C), 195.94 (C); ^{13}C NMR DEPT-135 (125 MHz, DMSO-d_6) δ 8.00 (CH_3), 21.01 (CH_3), 42.18 (CH_2), 110.63 (CH), 126.02 (CH), 131.77 (CH); IR (KBr) ν_{max} / cm^{-1} 3411, 3224, 3027, 2921, 1724, 1627, 1560, 1353, 815; HRMS (ESI) calcd. for $C_{13}H_{12}N_2NaO_3^+$ [M + Na]$^+$: 267.0740, found: 267.0744, error (ppm): 1.50; calcd. for $C_{26}H_{24}N_4NaO_6^+$ [2M + Na]$^+$: 511.1588, found: 511.1593, error (ppm): 0.90; GC-MS (70 eV) *m/z* 244 [M]$^+$.

MTT assay-cytotoxic assay

Compounds (0.15-30.0 μM) were tested for cytotoxic activity against MCF-7 (human mammary epithelial adenocarcinoma, ATCC No. HTB-22), A-549 (human lung adenocarcinoma, ATCC No. CCL-185), MDA-MB231 (human mammary epitelial adenocarcinoma triple negative, ATCC No. HTB-26), freshly prepared human blood leukocytes and erythrocytes. All cell lines were maintained in Dulbecco's Modifed Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2.0 mM glutamine, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin at 37 °C with 5% CO_2. Each compound was dissolved with DMSO and diluted with cell culture medium to obtain a concentration of 100.0 μM. They were incubated with the cells for 48 h. The negative control received the same amount of DMSO (0.005% in the highest concentration). Doxorubicin was used as a positive control. The cell viability was determined by reduction of the yellow dye 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a blue formazan product after 3 h incubation as described by Denizot and Lang.[37]

Erythrocytes hemolysis

The test was performed as described by Campos *et al.*[38] in 96-well plates using a 2% human erythrocyte suspension in 0.85% NaCl containing 10 mM $CaCl_2$. The compounds diluted as mentioned above were tested at concentration of 100 μM. After incubation at room temperature for 30 min and centrifugation, the supernatant was removed and the liberated hemoglobin was measured spectrophotometrically at 540 nm. DMSO was used as a negative control and Triton X-100 (1%) was used as positive control.

Compounds (0.15-30.0 μM) were tested for cytotoxic activity against MCF-7 (human mammary epithelial

adenocarcinoma, ATCC No. HTB-22), A-549 (human lung adenocarcinoma, ATCC No. CCL-185), MDA-MB435 (human melanoma, ATCC No. HTB-129), freshly prepared human blood leukocytes and erythrocytes. All cell lines were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2.0 mM glutamine, 100 U mL^{-1} penicillin and 100 mg mL^{-1} streptomycin at 37 °C with 5% CO_2. Each compound was dissolved with DMSO and diluted with cell culture medium to obtain a concentration of 100.0 μM. They were incubated with the cells for 48 h. The negative control received the same amount of DMSO (0.005% in the highest concentration). Doxorubicin was used as a positive control. The cell viability was determined by reduction of the yellow dye MTT to a blue formazan product after 3 h incubation as described by Denizot and Lang.[37]

Supplementary Information

Supplementary information (spectroscopic data, ^{1}H NMR and ^{13}C NMR spectra and mass spectra (EI)) is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

The authors thank the Brazilian agencies National Counsel for Technological and Scientific Development (CNPq), State of Rio de Janeiro Research Foundation (FAPERJ) and Coordination for the Improvement of Higher Education Personnel (CAPES).

References

1. Siegel, R. L.; Miller, K. D.; Jemal, A.; *CA-Cancer J. Clin*. **2018**, *68*, 7.
2. Silva, B. V.; *J. Braz. Chem. Soc*. **2013**, *24*, 707; da Silva, J. F. M.; Garden, S. J.; Pinto, A. C.; *J. Braz. Chem. Soc.* **2001**, *12*, 273.
3. Manna, K.; Aggarwal, Y.; *Bioorg. Med. Chem. Lett.* **2009**, *19*, 2688.
4. Sriram, D.; Yogeeswari, P.; Gopal, G.; *Eur. J. Med. Chem.* **2005**, *40*, 1373.
5. Montes, G. C.; Silva, B. N. M.; Rezende, B.; Sudo, R. T.; Ferreira, V. F.; Silva, F. C.; Pinto, A. C.; Silva, B. V.; Sudo, G. Z.; *Molecules* **2017**, *22*, 800.
6. Panneerselvam, P.; Reddy, R. S.; Murali, K.; Kumar, N. R.; *Pharma Chem.* **2010**, *2*, 28.
7. Giorno, T.; Sardella, B.; Silva, B. V.; Pinto, A. C.; Fernandes, P. D.; *Life Sci.* **2016**, *151*, 189.
8. Nisha; Mehra, V.; Hopper, M.; Patel, N.; Hall, D.; Wrischnik, L. A.; Land, K. M.; Kumar, V.; *MedChemComm* **2013**, *4*, 1018.
9. Silva, B. V.; Horta, B. A. C.; Alencastro, R. B.; Pinto, A. C.; *Quim. Nova* **2009**, *32*, 453.
10. Vine, K. L.; Matesic, L.; Locke, J. M.; Ranson, M.; Skropeta, D.; *Anti-Cancer Agents Med. Chem.* **2009**, *9*, 397.
11. Vine, K. L.; Locke, J. M.; Ranson, M.; Pyne, S. G.; Bremner, J. B.; *Bioorg. Med. Chem.* **2007**, *15*, 931.
12. Saurav, P.; Ashalata, R.; Suman, J. D.; Subhankar, P.; Srivastava, G. N.; Trivedi, V.; Manna, D.; *MedChemComm* **2017**, *8*, 1640.
13. Weissberger, A.; *The Chemistry of Heterocyclic Compounds*, 17th ed.; Wiley: New York, 1962.
14. Gamenara, D.; Heinzen, H.; Moyna, P.; *Tetrahedron Lett.* **2007**, *48*, 2505.
15. Blaser, A.; Sutherland, B. D.; Palmer, H. S.; Kmentova, I.; Franzblau, S. G.; Wan, B.; Wang, Y.; Ma, Z.; Thompson, A. M.; Denny, W. A.; *J. Med. Chem*. **2012**, *55*, 312.
16. Palmer, B. D.; Thompson, A. M.; Sutherland, H. S.; Blaser, A. I. K.; Franzblau, S. G.; Wang, B.; Wan, Y.; Ma, Z.; Denny, W. A.; *J. Med. Chem.* **2010**, *53*, 282.
17. Mathew, B. P.; Kumar, A.; Sharma, S.; Shukla, P. K.; Nath, M.; *Eur. J. Med. Chem.* **2010**, *45*, 1502.
18. Cocuzza, A. J.; Chidester, D. R.; Cordova, B. C.; Jeffrey, S.; Parsons, R. L.; Bacheler, L. T.; Erickson-Viitanen, S.; Trainor, G. L.; Ko, S. S.; *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1177.
19. Zhou, D.; Harrison, B. L.; Shah, U.; Andree, T. H.; Hornby, G. A.; Scerni, R.; Schechter, L. E.; Smith, D. L.; Sullivan, K. M.; Mewshaw, R. E.; *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1338.
20. Morrison, R.; Al-Rawi, J. M. A.; Jennings, I. G.; Thompson, P. E.; Angove, M. J.; *Eur. J. Med. Chem*. **2016**, *110*, 326.
21. Ohno, M.; Ueki, J.; Sakagami, H.; Wakabayashi, H.; *In Vivo* **2013**, *27*, 507.
22. Delbaldo, C.; Faivre, S.; Dreyer, C.; Raymond, E.; *Ther. Adv. Med. Oncol.* **2011**, *4*, 18.
23. Sun, S.; Schiller, J. H.; *Crit. Rev. Oncol. Hematol.* **2007**, *62*, 93.
24. Roth, G. J.; Heckel, A.; Colbatzky, F.; Handschuh, S.; Kley, J.; Lehmann-Lintz, T.; Lotz, R.; Tontsch-Grunt, U.; Walter, R.; Hilberg, F.; *J. Med. Chem.* **2009**, *52*, 4466.
25. Singh, A.; Bains, T.; Hahn, H. J.; Liu, N.; Tam, C.; Cheng, L. W.; Kim, J.; Debnath, A.; Land, K. M.; Kumar, V.; *MedChemComm* **2017**, *8*, 1982.
26. Ribeiro, N. M.; Silva, B. V.; Violante, F. A.; Rezende, C. M.; Pinto, A. C.; *Org. Prep. Proced. Int.* **2005**, *37*, 265.
27. Sandmeyer, T.; *Helv. Chim. Acta* **1919**, *2*, 234.
28. Marvel, C. S.; Hiers, G. S.; *Org. Synth. Coll*. **1941**, *1*, 327.
29. Garden, S. J.; Torres, J. C.; Melo, S. C. S.; Lima, A. S.; Pinto, A. C.; Lima, E. L. S.; *Tetrahedron Lett*. **2001**, *42*, 2089.
30. Aboul-Fadl, T.; Radwan, A.; Attia, M.; Al-Dhfyan, A.; Abdel-Aziz, H.; *Chem. Cent. J*. **2012**, *6*, 49.
31. Fischer, P.; Lane, D.; *Curr. Med. Chem.* **2000**, *7*, 1213.
32. Garden, S. J.; Torres, J. C.; Ferreira, A. A.; Silva, R. B.; Pinto, A. C.; *Tetrahedron Lett.* **1997**, *38*, 1501.

33. Silva, R. B.; Torres, J. C.; Garden, S. J.; Violante, F. A.; Rezende, M. J. C.; Silva, B. V.; Pinto, A. C.; *Quim. Nova* **2008**, *31*, 924.
34. Barreiro, E. J.; Kümmerle, A. E.; Fraga, C. A. M.; *Chem. Rev.* **2011**, *111*, 5215.
35. Lindwall, H. G.; Bandes, J.; Weinberg, I.; *J. Prakt. Chem.* **1931**, *53*, 317.
36. Calvery, H. O.; Noller, C. R.; Adams, R.; *J. Am. Chem. Soc.* **1925**, *47*, 3058.
37. Denizot, F.; Lang, R.; *J. Immunol. Methods* **1986**, *89*, 271.
38. Campos, V. R.; Cunha, A. C.; Silva, W. A.; Ferreira, V. F.; Sousa, C. S.; Fernandes, P. D.; Moreira, V. N.; Rocha, D. R.; Dias, F. R. F.; Montenegro, R. C.; Souza, M. C. B. V.; Boechat, F. C. S.; Franco, C. F. J.; Resende, J. A. L. C.; *RSC Adv*. **2015**, *5*, 96222.

Submitted: June 15, 2018
Published online: August 15, 2018