Chapter 5

Synthesis of quinoline bearing dispiropyrrolidines

Chapter-5

Multicomponent route to the synthesis of quinoline bearing dispiropyrrolidines as cytotoxic agents

5.1. Abstract

A series of novel functionalized quinoline based isatin, acenaphthalene and ninhydrin bearing dispiropyrrolidines derivatives have been synthesized through 1, 3-dipolar cycloaddition reaction of azomethine ylide which is generated from sarcosine with the dipolarophile. The asymmetric three-component 1, 3-dipolar cycloaddition products of a broad range of spirooxindole derivatives provide high yield with unusual regiochemistry and excellent stereoselectivities (up to 82%) under mild conditions. The regio and stereochemical outcomes of the products were established by single crystal X-ray structure and spectroscopic techniques. Cell based anticancer evaluation of selected compounds showed significant cell proliferation against cervical as well as breast cancer cell lines. The straightforward construction of spirooxindole skeletons with high stereo and regioselectivity suggests a new avenue to medicinal chemistry and diversity-oriented synthesis.

5.1.1. General Features

Multicomponent reactions (MCRs) have been designed to produce a range of biologically active compounds and have become an active area of research in organic, combinatorial, and medicinal chemistry.\textsuperscript{1} The MCR strategy offers significant advantages over conventional linear-type synthesis because of its flexible, convergent, and atom efficient nature.\textsuperscript{2} The development of new efficient methods to synthesize nitrogen heterocycles with structural diversity is one of the major objectives of modern synthetic organic chemists. As discussed in chapter 1, the 1, 3-dipolar cycloaddition of azomethine ylides is a convenient protocol for the construction of highly functionalized five-membered N-heterocycles.\textsuperscript{3} For example azomethine ylides, generated \textit{in situ} from isatin/acenaphthalene/ninhydrin with sarcosine which reacts with \( \alpha, \beta \)-unsaturated quinolinyl compounds afforded quinoline based dispiropyrrolidines.


Moreover the structural motif of spiro and dispiro pyrrolidine is commonly present in a number of natural products as exemplified by gelsemium and marcfortine alkaloids. It also comprises a core component of various biologically active compounds. Recently, it has been found serving as antagonists of MDM2 interactions and hence hold great potential to be selective and potent anticancer agents. During the past several years, significant advances have been made on the development of synthetic methods to access spirooxindole derivatives with a concomitant creation of an all-carbon quaternary stereogenic center in an enantioselective manner. For e.g. Overman et al., established an asymmetric intramolecular Heck reaction rendering the synthesis of spiro[pyrrolidin-3, 3′-oxindole] derivatives with high enantiomeric purity. Trost and coworkers developed an elegant asymmetric alkylation reaction of an oxindole enolate enabling a concise synthesis of horfeline. Recently, Barbas and Melchiorre independently reported organocatalytic conjugate addition reactions of 3-substituted oxindoles to electronically deficient olefins creating an all-carbon stereogenic center with high levels of stereo chemical control. Chen and co-workers presented a Mannich reaction of 3-substituted oxindoles to imines, yielding oxindole derivatives with high enantioselectivity. While each of the reported synthetic approaches have its own advantages, in the final product, the functionality incorporated by this reaction is projected from the compact spirocyclic core into different areas in space. The challenges associated with the synthesis of natural products containing the pyrrolidinyl-
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spirooxindole core have made them to try elegant synthetic investigations.\(^{13-17}\) The interesting biological activity associated with this core has also made it the focus on medicinal chemistry investigations. The most notable of these is a structure-based design approach to inhibit protein-protein interactions,\(^{18,19}\) in which Wang and co-workers designed pyrrolidinyl-spirooxindole that inhibited the proliferation of human prostate cancer cells.\(^{20,21}\) In continuation of our research on the synthesis of spiro heterocycles comprising quinoline core unit, herein we disclosed a catalytic free 1, 3-dipolar addition of azomethine ylide to dipolarophiles, leading to a structurally diverse synthesis of optically active spiro [pyrrolidin-3, 3'-oxindole] derivatives. The primary advantage of this strategy is that diverse sets of molecules can be made in a short span of time.

5.2. Multicomponent route to the synthesis of quinoline bearing dispiropyrrolidines as cytotoxic agents- The Present study

5.2.1. Results and discussion

5.2.2. Chemistry

The starting precursor 2-methoxy-3-formylquinoline 1 was prepared as per the literature method.\(^{22}\) The required dipolarophiles 4 and 5 were prepared by the reaction of 2-methoxy-3-formyl quinoline with various cyclic ketones (Scheme 5.1). The dipolarophile 4 was prepared by treating equimolar mixture of 2-methoxy-3-formylquinoline 1 either with indanone 2a or \(\alpha\)-tetralone 2b in aqueous ethanol with drop wise addition of NaOEt in ethanol at 0 °C.

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Scheme 5.1 Synthesis of dipolarophiles 4 & 5

Similarly the dipolarophile 5 was prepared by the same manner as mentioned for 4 by treating 2-methoxy-3-formylquinoline 1 with cyclopentanone 3a/cyclohexanone 3b which resulted in the formation of bicondensed product 5 in good yield (Table 5.1) as the starting cyclic ketone is bearing two active methylene sites. The structure and geometry of dipolarophiles were assigned by spectroscopic analysis.

Table 5.1 Synthesis of quinoline based dipolarophiles

<table>
<thead>
<tr>
<th>Product</th>
<th>2-methoxy-3-formyl quinolines/Aromatic aldehydes</th>
<th>Cyclic ketones</th>
<th>Product</th>
<th>Yield (%)</th>
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<td><img src="image2.png" alt="structure" /></td>
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<tr>
<td>4b</td>
<td><img src="image4.png" alt="structure" /></td>
<td><img src="image5.png" alt="structure" /></td>
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<tr>
<td>4c</td>
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<td><img src="image8.png" alt="structure" /></td>
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<table>
<thead>
<tr>
<th>5a</th>
<th><img src="image" alt="Chemical Structure" /></th>
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<tbody>
<tr>
<td>5b</td>
<td><img src="image" alt="Chemical Structure" /></td>
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</tr>
<tr>
<td>17a</td>
<td><img src="image" alt="Chemical Structure" /></td>
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</tr>
<tr>
<td>17c</td>
<td><img src="image" alt="Chemical Structure" /></td>
<td>82</td>
</tr>
</tbody>
</table>

*Synthesis of dipolarophile by treatment with 2-methoxy-3-formylquinoline 1 (1 mmol), cyclic ketones 2,3 (1 mmol) in ethanol at 0 °C

**Isolated yield**

The reaction of non-stabilized azomethine ylide generated *in situ* from decarboxylative condensation of isatin 6/ ninhydrin 8/ acenaphthalene 9 and sarcosine 7 with dipolarophiles 4 in refluxing methanol furnished quinoline based dispiropyrrlidines 10, 11, 12 and 13 in good yields (68–82%) with excellent selectivity (Table 5.2).
Scheme 5.2 Synthesis of quinoline based dispiropyrrrolidines 10, 11(a, b), 12, 13.

Table 5.2 Synthesis of quinoline based dispiropyrrrolidine

<table>
<thead>
<tr>
<th>Dipolarophiles</th>
<th>Dipoles</th>
<th>Product</th>
<th>Yield $^b$</th>
</tr>
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<td><img src="image4" alt="Image" /></td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
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</tbody>
</table>
Synthesis of quinoline based dispiropyrrrolidines by treatment with dipolarophiles 4 & 5, isatin 6/ ninhydrin 8/ acenaphthalene 9 with sarcosine 7

b Isolated yield

From stereoechemochemical point of view, the dipole was attacked from the top side of the dipolarophile resulted in the formation of the cycloadduct 12 & 13 as shown below.
Scheme 5.3 Transition state for the formation of cycloadduct 12 & 13

In Scheme 5.4, the reaction of non stabilized azomethine ylide generated in situ from isatin 6 and sarcosine 7 was treated with bicondensed dipolarophile 5 in refluxing methanol to furnish cycloadduct 14 in excellent yield. Contrary to the expected bicycloadduct we obtained only a mono cycloadduct as the major product and is shown in Table 5.2.

Scheme 5.4 Synthesis of quinoline based dispiro cycloadduct 14

Similar 1, 3-dipolar cycloaddition had also been carried out by treating acridine based dipolarophile 17 with isatin 6 and sarcosine 7, where the expected cycloadduct was not formed even though the reaction was carried out with various conditions in diverse solvents (Scheme 5.5). It may be assumed that the reason for not forming the product is owing to the steric hindrance due to the presence of phenyl group at the 4th position of the (E)-2-benzylidene-9-phenyl-3, 4-dihydroacridin-1(2H)-one 17.
5.2.3. $^1$H, $^{13}$C NMR chemical shifts as well as IR and Mass spectral assignments of quinoline based dispiropyrrlidines

The structures of dipolarophile were established by its spectral and elemental analysis (Fig. 5.1-5.5). The $^1$H NMR spectrum of 4b (Fig. 5.1) exhibiting two triplets at $\delta$ 2.98 and 3.12 and was assignable to methylene protons of cyclic ketone, a singlet at $\delta$ 4.06 showed proton assignment for OCH$_3$ function. A singlet which appeared at $\delta$ 8.34 was due to C4-H of dipolarophile. The protons which displayed from $\delta$ 7.39-8.34 were due to quinoline unit of 4b. The $^{13}$C NMR spectrum (Fig. 5.2) of 4b showed peaks at $\delta$ 27.61, 28.89, 53.77 ppm indicating the presence of methylene and methoxy carbons. The cyclic keto and ‘OCH$_3$’ attached carbons exhibited peaks at $\delta$ 187.41 and 160.48 respectively. The IR spectrum and elemental analysis (Fig. 5.3) further confirmed the formation of the product. This was factual for all the other derivatives of the series.

On the other hand, the $^1$H NMR spectrum of 5b (Fig. 5.4) exhibiting two triplets at $\delta$ 1.84 and 2.96 were assignable to methylene protons of cyclic ketone. A singlet at $\delta$ 4.13 showed proton assignment for OCH$_3$ group. A singlet peak exhibiting at $\delta$ 7.26 was attributed to olefin.
proton of dipolarophile. From the aforementioned proton assignment it was assumed that even though the central cyclic ketone unit bears two planar quinoline units on either side, it showed proton assignment for a mono condensed unit since it comprises planar conformation. The IR and elemental analysis (Fig. 5.5) further supported the formation of condensed product.

The structures of quinoline based dispiropyrrolidines are in accordance with their 1D and 2D NMR spectroscopic data. In particular, the regiochemistry proposed for the product 11b was decided on the basis of its $^1$H NMR (Fig. 5.6) spectrum exhibiting triplets at δ 5.05, 4.12 and 3.33 owing to C-4-H and C-5-H respectively. The $^{13}$C NMR spectrum (Fig. 5.7, 5.8) of 11b showed two peaks at δ 59.36 and 75.81 ppm reflecting the presence of two spiro carbons, the cyclic keto and indole-1,2-dione carbonyl carbons exhibited peaks at δ 196.66 and 177.14 ppm, respectively which further confirmed the formation of cycloadduct. This is also supported by its $^1$H-$^1$H COSY (Fig. 5.9, 5.10) correlation with C-5-H. Further the benzylic proton at C-4 shows HMBC (Fig. 5.11, 5.12) correlation with C-5 at δ 54.78 ppm, besides being correlated with spiro carbon C-3 at δ 59.36 ppm, carbonyl carbon C-1 at δ 196.0 and methoxy bearing quinoline carbon C-2 at δ 160.35 ppm respectively. The methylene protons of pyrrolidine ring showed two triplets at δ 4.12 and 3.33 related by a $^1$H-$^1$H COSY correlation. This is also supported by the HMBC correlation of the signal besides being correlated with C-2 carbon of quinoline ring at δ160.35 ppm and spiro carbon C-3 at δ 59.87 ppm. The C-5 methylene protons appears as triplets at δ 4.12 and 3.33 ppm which was revealed from its $^1$H-$^1$H COSY correlation with C-4 benzylic protons at δ 5.05 ppm. Among C-5 methylene protons, the proton which appears at δ 3.33 showed HMBC correlations with spiro carbons C-2 and C-3 at δ 59.87 and 76.12 ppm respectively, as it showed HMBC correlation with N-CH$_3$ proton at δ 2.00 ppm and NH proton of oxindole at δ 10.69 ppm respectively. The aromatic protons appeared in the region of 6.58-8.46 ppm. The structure of other quinoline based spiropyrrolidines was also assigned similar straightforward method as discussed for 11b. Further elemental analysis was consistent with the proposed structure. Identical results were furnished by other compounds with identical stereochemistry.
To enhance the scope of the above methodology, we treated the same dipolarophiles with azomethine ylide generated from, ninhydrin 8 and sarcosine 7. The reaction yielded novel dispiropyrrrolidine derivative 12 in good yield 68-74%. For instance the $^1$H NMR spectrum (Fig. 5.16) of 12 showed a triplet at $\delta$ 5.35 for benzylic proton, which confirmed the regiochemistry of the cycloadduct. The methylene protons of pyrrolidine ring showed two triplets at $\delta$ 4.25 and 3.59. These results again confirm the regiochemistry of compound 12. The $^{13}$C NMR spectrum (Fig. 5.17) showed resonances at $\delta$ 79.70 and 61.35 ppm reflecting the presence of two spiro carbons. Peaks at $\delta$ 207.20, 205.15 and 197.24 ppm for of indene-1, 2, 3-trione systems and cyclic keto carbonyl carbons respectively. The IR and elemental analysis was also found to be satisfactory. The structure of 12 was established by X-ray (Fig. 5.18) single crystal analysis, which also proved the regiochemistry of cycloadduct.

**Fig. 5.15** Selected $^1$H and $^{13}$C NMR assignment and HMBC correlation of 11b
Further the methodology was extended by treating the same dipolarophiles with azomethine ylide generated from, acenathenequinone 9 and sarcosine 7. The reaction yielded novel dispiropyrrolidine derivative 13 in good yield 68-74%. For instance the $^1$H NMR spectrum (Fig. 5.19) of 13 showed a triplet at δ 5.18 for benzylic proton, which confirmed the regiochemistry of the cycloadduct. The methylene protons of pyrrolidine ring showed two triplets at δ 4.17 and 3.49. These results again confirm the regiochemistry of compound 13. The $^{13}$C NMR spectrum (Fig. 5.20) showed two peaks at δ 79.30 and 61.33 ppm indicating the presence of two spiro carbons. Peaks which appeared at δ 206.96 and 186.91 ppm are due to carbonyl carbon of acenapthenenone ring systems and keto carbonyl respectively. The IR (Fig. 5.21) and elemental analysis was also found to be satisfactory. The structure of 13 was established by X-ray (Fig. 5.22) single crystal analysis, which also proved the regiochemistry of cycloadduct.
The assignment of \(^1\)H (Fig. 5.23) and \(^{13}\)C NMR (Fig. 5.24, 5.25) of compound 14 has also been done by straightforward considerations as done for 13.

On the basis of its \(^1\)H NMR spectrum exhibiting a triplet at \(\delta 4.88\) ppm was assignable to benzylic protons which was supported by its \(^1\)H-\(^1\)H COSY (Fig. 5.26, 5.27) correlation with C5-H. Further it displayed HMBC correlation with C5-H at \(\delta 54.58\) ppm besides being correlated with spiro carbon C-3 at \(\delta 61.46\) ppm and keto carbonyl at C-1" at \(\delta 199.52\) ppm which determined proposed regiochemistry of the product. The C-5 methylene proton appeared at \(\delta 3.34\) and 4.16 and was supported by its \(^1\)H-\(^1\)H COSY correlation with benzylic proton C-4 at \(\delta 4.88\); The methylene proton at \(\delta 4.16\) showed HMBC (Fig. 5.28, 5.29) correlation with spiro carbon C-2 at \(\delta 76.51\) and C-3 at \(\delta 61.46\) ppm. The N-CH\(_3\) proton exhibited a singlet at \(\delta 2.0\) ppm and showed HMBC correlation with spiro carbons C-2 at \(\delta 76.51\) and C-3 at \(\delta 61.46\) ppm. The NH proton of oxindole ring showed a singlet at \(\delta 10.70\) ppm which showed HMBC correlation with oxindole carbonyl C-2 at \(\delta 176.43\). The aromatic protons were appeared in the region of \(\delta 8.38\)–6.79 ppm. The \(^{13}\)C NMR spectrum of 14 showed two peaks at \(\delta 76.51\) and 61.46 ppm reflecting the presence of two spiro carbons. Peaks which appeared at \(\delta 199.52\) and 176.43 ppm was due to carbonyl carbon of cyclic keto and oxindole ring respectively.

The dipolarophile (E)-2-benzylidene-9-phenyl-3,4-dihydroacridin-1(2H)-one 17 was achieved by treating 9-phenyl-3,4-dihydroacridin-1(2H)-one 15 with aromatic aldehydes 16 and was accordance with its \(^1\)H NMR and elemental analysis. The \(^1\)H NMR spectra (Fig. 5.32) of

![Fig. 5.31 Selected \(^1\)H and \(^{13}\)C NMR assignment and HMBC correlation of 14](image-url)
17a showed multiplets at $\delta$ 3.36 and 3.25 ppm suggesting the presence of methylene protons of acridine systems and a singlet which appeared at $\delta$ 7.72 was due to olefin proton. The aromatic regions appeared in the region of 7.29-8.11 and were in accordance with the proposed structure.

5.3. Intramolecular non covalent interaction

As discussed in chapter 4, the hydrogen bonding and $\pi$...$\pi$ stacking interactions often play an important role in controlling molecular conformation, molecular aggregation and the function of a vast number of chemical systems. They can cause critical influence on structure, geometry and stability of MOFs. To identify the non covalent interaction, coordinates of individual molecules were extracted from the relevant crystal structure of compound 13 (Fig. 5.33). The molecule showed a strong pi interaction with contact distance of 2.840 Å due to the intramolecular interaction between C17-H...$\pi$ with $\pi$. Further the strong $\pi$...$\pi$ stacking interaction was observed for ‘C14-H’ of quinoline ring and ‘O-1’ atom at $\alpha$-tetralone ring system with contact distance of 2.621 Å. The other non-covalent interactions observed are C35-H of quinoline ring with O-2 of acenaphthalene 1, 2-dione with contact distance of 2.616 Å. Similarly the other interactions observed within the systems are C8-H...O-1 with contact distance 2.577 Å. C12-H...O-2 with contact distances of 2.216 Å. It was assumed that the ‘O’ atom present in both indole as well as acenaphthalene system showed significant interaction with protons present in the molecules and this interaction could be a crucial for further binding studies with DNA, proteins and drug discovery.

![Fig. 5.33 Noncovalent interaction of compound 13](image-url)
5.4. Anticancer activity

5.4.1. Evaluation of cell viability against Cervical (HeLa) and breast (MCF-7) cancer cell lines

The decrease in cell viability was determined by MTT assay in terms of cell death. After treatment of the HeLa (human cervical cancer) cells for 48 h with the analogues in the concentration range of 10-100 (μg/mL), the inhibitory percentage against proliferation of cancer cells was determined. The cell viability (%) was obtained by continuous exposure for 48 h is depicted in Fig. 5.34. As shown in Fig. 5.34, the active analogues showed a distinctive potential pattern of selectivity as well as antitumor activity. Among quinoline bearing dispiropyrrolidines, analogues with disubstituted quinoline bearing spiropyrrolidine, analogue 14 displayed considerable potency towards the proliferation of cells with the IC₅₀ of the 40 μg/mL respectively. Similarly the other two analogues 10, 11b have also shown considerable potency toward cervical cancer cells with the IC₅₀ value of 50 and 64 μg/mL. The experiment results thus suggested that compounds 10, 11b and 14 inhibited the viability of cells in a dose dependent manner (Fig. 5.34, 5.34a).

![Fig. 5.34 Cell viability of compds 10, 11b, 14 determined against Cervical (HeLa) cancer cell lines by MTT assay](image-url)
Fig. 5.34a Cell cycle analysis of compounds 10, 11b, 14

Similarly the cell viability of compounds 4a, 4c, 5b and 13 were evaluated against breast cancer cell lines (MCF-7). After treatment of the MCF-7 (human breast cancer cells) for 48 h with the compounds in the concentration range of 10-100 (μg/mL), the inhibitory percentage against proliferation of cancer cells was determined. The cell viability (%) was obtained by continuous exposure for 48 h, which is depicted in Fig. 5.35. The experimental results thus reveal that the active analogue 13 showed a distinctive potential pattern of anticancer against MCF-7 cells with the IC$_{50}$ value of 32 μg/mL, the IC$_{50}$ values of compounds 4a, 4c and 5b is depicted as 60, 65, 40 respectively, which were also shown marked activities against the breast cancer cell line. Thus from the results the selected compounds inhibited the cell viability in a dose dependent manner.
5.4.1. DNA fragmentation analysis

The DNA fragmentation analysis was performed for the selected compounds such as 10, 11b, 14 and 4a, 4c, 5b 13. DNA was extracted from cultures of Hela (cervical cancer) and MCF-7 (breast cancer) cells which were treated with compound 10, 11b, 14 (HeLa) as well as 4a, 4c, 5b and 13 (MCF-7) cells at IC₅₀ concentrations for 6-24 h. The occurrence of necrosis (1%) was detected by gel electrophoresis, specific degradative necrotic degeneration was prominent in cells incubated with the treated compounds for 6 h and the fragmented DNA increased greatly in cells treated for 24 h, which is shown in Fig. 5.36.
5.4.2. Fluorescence microscopic method against HeLa and HCF-7

The Hela and MCF-7 cells were treated with aforementioned analogues for 24 h, and viability was observed by staining live and dead cells with DAPI, Rhodamine and FITC respectively. Fluorescence stained cervical and breast cancer cells after treatment with the control were shown in Fig. 5.37 & 5.38 respectively. From the results, it was observed that a remarkable morphological change was observed by the fluorescence microscopic analysis for the cells under treatment. In all the cases fluorescence was noticed in the cytoplasm, whereas in few cases fluorescence appeared to be located inside or around the nuclei.

![Fluorescence microscopic analysis](image)

**Fig. 5.37** Fluorescence microscopic analysis of cervical cancer cells after treatment with control (DAPI, FITC) and compounds 10, 11b and 14

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M. Sankaran, 2012

Fig. 5.38 Fluorescence microscopic analysis of breast cancer cells after treatment with control (DAPI, Rhodamine) and compounds 4a, 4c, 5b and 13

5.5. Conclusion

In summary, we have described a simple one-pot three component reaction involving isatin, sarcosine, and α, β-unsaturated carbonyl compounds for the synthesis of 2-methoxy quinoline dispiro pyrrolidines in methanol. Particularly, valuable features of this method include the good yields, broader substrate scope together with mild reaction conditions make these molecules useful and attractive process for the synthesis of these interesting compounds. Moreover, this method can be considered as an ideal tool for synthesis because (1) rapid assembly of heterocyclic molecules by a three-component process minimizes the generation of...
waste (2) both azomethine ylide and olefinic dipolarophiles are simultaneously generated in situ without the need to isolate intermediates. Therefore, this one-pot, autocatalytic transformation clearly represents an appealing methodology for the synthesis of dispiropyrrolidines. Furthermore, compounds 10,11b, 14 and 4a, 4c, 5b and 13 have shown promising antiproliferation and apoptotic activities against both cervical and breast cancer cell lines.

5.6. Experimental

5.6.1. General procedure for the synthesis of quinoline based α, β-unsaturated carbonyl systems (4, 5)

A 1:2 molar mixture of cyclic ketones (indanone, α-tetralone, cyclopentanone, cyclohexanone) was treated with various substituted 2-methoxy-3-formyl quinoline by the addition of NaOEt at 0 °C. Then the reaction mixture was stirred at room temperature for 5-7 h. After completion of the reaction as inferred by the TLC, the mixture was poured into crushed ice and neutralized with dil. HCl. The precipitate thus formed after adding into crushed ice was filtered off and the residue subjected to column chromatography using petroleum ether: ethyl acetate mixture (3:2) v/v as eluent and compound obtained as a pale yellow solid.

5.6.1.1. (E)-2-((2-methoxyquinolin-3-yl)methylene)-2, 3-dihydro-1H-inden-1-one (4a)

Pale brown solid (70%) mp. 125–130 °C; IR (KBr) νmax: 3056.62, 2949.59, 1692.23, 1601.59, 1467.56, 1441.53, 1399.10, 1263.15, 1011.48, 952.66, 734.74 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ 8.74 (s, 1H, C4-H), 8.07 (d, 1H, J=8.00 Hz, ArH), 7.80-7.85 (m, 3H, ArH), 7.72-7.77 (d, 3H, ArH), 7.51 (dt, 2H, J=7.00 Hz, ArH), 4.27 (s, 2H, CH₂), 4.11 (s, 3H, OCH₃); ¹³C NMR (100 MHz, DMSO-d₆) δ 138.05, 137.93, 137.00, 134.69, 130.56, 128.02, 127.14, 126.14, 124.86, 53.84, 32.32; Anal. Calcd. For C₂₀H₁₃NO₂ (301) C, 79.72; H, 5.02; N, 4.65; Found, C, 79.70; H, 4.97; N, 4.61%.

5.6.1.2. (E)-2-((2-methoxyquinolin-3-yl) methylene)-3, 4-dihydronaphthalen-1(2H)-one (4b)

Grey solid (78%) mp. 129–132 °C; IR (KBr) νmax 3062.41, 2943.80, 1663.30, 1604.48, 1438.64, 1394.28, 1265.07, 1018.23, 949.77, 735.71 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆) δ 8.34 (s, 1H, C4-H), 7.96-8.01 (m, 2H, ArH), 7.81 (d, 2H, J=7.92 Hz, ArH), 7.16 (t, 1H, J=8.16 Hz, ArH), 7.60 (t, 1H, J=7.44 Hz, ArH), 7.39-7.50 (m, 3H, ArH), 4.06 (s, 3H, OCH₃), 3.12 (t, 2H, J=5.88 Hz, CH₂-H), 2.98 (t, 2H, J=6.52 Hz, CH₂-H); ¹³C NMR (100 MHz, DMSO-d₆)
δ 187.41, 160.48, 146.38, 143.21, 138.00, 137.25, 133.36, 130.88, 130.05, 128.30, 127.66, 127.06, 124.60, 120.86, 53.77, 28.89, 27.61; Anal. Calcd. For C_{21}H_{17}NO_{2} (315) C, 79.98; H, 5.43; N, 4.44; Found, 70.02; H, 5.46; N, 4.40%.

5.6.1.3. *(E)-2-((2-methoxy-8-methylquinolin-3-yl)methylene)-3,4-dihydronaphthalen-1(2H)-one (4c)*

Pale brown solid (68%) mp. 142-145 °C; IR (KBr) v_{max} 3051.80, 3003.59, 2940.91, 1775.15, 1667.16, 1610.27, 1442.49, 1344.14, 1246.75, 1136.83, 1014.37, 943.98, 754.03 cm\(^{-1}\); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) δ 7.25 (s, 1H, C4-H), 7.46 (d, 1H, J=7.20 Hz, ArH), 7.26 (d, 1H, J=9.60 Hz, ArH), 7.06 (t, 2H, J=7.50 Hz, ArH), 6.80-6.92 (m, 4H, ArH), 3.54 (s, 3H, OCH\(_3\)), 2.55 (t, 2H, J=6.00 Hz, CH\(_2\)-H) 2.43 (t, 2H, J=6.00 Hz, CH\(_2\)-H), 2.12 (s, 3H, CH\(_3\)); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) δ 188.52, 160.25, 147.70, 142.21, 138.11, 137.22, 134.55, 130.15, 130.00, 128.30, 127.66, 126.06, 124.21, 121.86, 53.77, 28.89, 27.61, 15.66; Anal. Calcd. For C\(_{22}\)H\(_{19}\)NO\(_2\) (329) C, 80.22; H, 5.81; N, 4.25; Found C, 80.25; H, 5.77; N, 4.30%.

5.6.1.4. *(2E,5E)-2,5-bis(2-methoxyquinolin-3-yl)methylene)cyclopentanone (5a)*

Pale yellow solid (72%) mp. 144–148 °C; IR (KBr) v_{max} 3054.69, 2940.91, 1951.61, 1682.59, 1588.09, 1442.49, 1398.14, 1347.03, 1218.79, 1007.62, 752.10 cm\(^{-1}\); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) δ 8.13 (s, 1H, C4-H), 7.97 (s, 1H, ArH), 7.79 (m, 2H, ArH), 7.64 (t, 1H, J=7.00 Hz, ArH), 7.39 (t, 1H, J=7.20 Hz, ArH), 4.10 (s, 3H, OCH\(_3\)), 3.14 (s, 2H, CH\(_2\)); \(^{13}\)C NMR (100 MHz, DMSO-\(d_6\)) δ 160.58, 146.49, 139.50, 138.01, 130.47, 128.02, 127.18, 124.48, 120.97, 53.83, 26.92; Anal. Calcd. For C\(_{27}\)H\(_{22}\)N\(_2\)O\(_3\) (422) C, 76.76; H, 5.25; N, 6.63; Found, C, 76.70; H, 5.21; N, 6.59%.

5.6.1.5. *(2E,6E)-2,6-bis(2-methoxyquinolin-3-yl)methylene)cyclohexanone (5b)*

Yellow solid (80%) mp. 148–152 °C; IR (KBr) v_{max} 3055.66, 2950.55, 1658.48, 1599.66, 1473.35, 1442.49, 1400.07, 1348.00, 1263.15, 1146.47, 1012.45, 745.35 cm\(^{-1}\); \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) δ 7.98 (d, 2H, J=10.68 Hz, ArH), 7.85 (d, 1H, J=8.32 Hz, ArH), 7.74 (d, 1H, J=7.92 Hz, ArH), 7.65 (q, 1H, ArH), 7.40 (t, 1H, J=7.56 Hz, ArH), 7.26 (s, 1H,Ar CH=CH), 4.13 (s, 3H, OCH\(_3\)), 2.96 (t, 2H, J=5.40 Hz, CH\(_2\)), 1.84 (t, 1H, J=5.80 Hz, CH-H); Anal. Calcd. For C\(_{28}\)H\(_{24}\)N\(_2\)O\(_3\) (436) C, 77.04; H, 5.54; N, 6.42; Found, C, 76.98; H, 5.57; N, 6.46%.
5.6.2. General procedure for the synthesis of acridone based \( \alpha, \beta \)-unsaturated carbonyl systems (17)

A 1:2 molar mixture of 9-phenyl-3,4-dihydroacridin-1(2H)-one 15 was treated with various substituted aromatic aldehydes 16 in the presence of NaOH were stirred at room temperature for 5-7 h. After completion of the reaction as inferred by the TLC, the mixture was poured into crushed ice and neutralized with dil HCl. The precipitate thus formed after adding into crushed ice was filtered off and the residue subjected to column chromatography using petroleum ether: ethyl acetate mixture (3:1) v/v as eluent and compound obtained as a pale yellow solid.

5.6.2.1. (E)-2-(4-chlorobenzylidene)-9-phenyl-3,4-dihydroacridin-1(2H)-one (17a)

Pale green solid (82%) mp. 177–181 °C; IR (KBr) \( \nu_{\text{max}} \) cm\(^{-1}\); \( ^{1}H \) NMR (500 MHz, DMSO-\( d_{6} \)) \( \delta \) 8.11 (d, 1H, J=8.50 Hz, ArH), 7.80 (t, 1H, J=7.00 Hz, ArH), 7.72 (bs, 1H, Cl'-H), 7.53-7.57 (m, 4H, ArH), 7.46 (t, 1H, J=8.00 Hz, ArH), 7.37-7.41 (d, 2H, J=10.68 Hz, ArH), 7.29 (d, 2H, J=7.50 Hz, ArH), 3.36 (q, 2H, CH\(_2\)), 3.25 (q, 2H, CH\(_2\)); Anal. Calcd. For C\(_{26}\)H\(_{18}\)ClNO \( (395) \) C; 78.88; H; 4.58; N; 3.54; Found, C; 78.85; H; 4.61; N; 3.50%.

5.6.2.2. (E)-2-benzylidene-9-phenyl-3,4-dihydroacridin-1(2H)-one (17b)

Pale green solid (84%) mp. 182–185 °C; IR (KBr) \( \nu_{\text{max}} \) cm\(^{-1}\); \( ^{1}H \) NMR (500 MHz, DMSO-\( d_{6} \)) \( \delta \) 8.12 (d, 1H, J=8.00 Hz, ArH), 7.80 (t, 1H, J=8.00 Hz, ArH), 7.53-7.57 (m, 4H, ArH), 7.42-7.47 (m, 4H, ArH), 7.37 (d, 1H, J=6.00 Hz, ArH), 7.28-7.30 (m, 2H, ArH), 3.35 (q, 2H, CH\(_2\)), 3.29 (q, 2H, CH\(_2\)); Anal. Calcd. For C\(_{26}\)H\(_{19}\)NO \( (361) \) C; 86.40; H; 5.30; N; 3.88; Found, C; 86.44; H; 5.32; N; 3.91%.

5.6.2.3. (E)-2-benzylidene-7-chloro-9-phenyl-3,4-dihydroacridin-1(2H)-one (17c)

Pale green solid (82%) mp. 150–155 °C; IR (KBr) \( \nu_{\text{max}} \) cm\(^{-1}\); \( ^{1}H \) NMR (500 MHz, DMSO-\( d_{6} \)) \( \delta \) 8.05 (d, 1H, J=9.00 Hz, ArH), 7.79 (s, 1H, olefin-H), 7.73 (dd, 1H, \( J_{1}=2.50 \), \( J_{2}=9.00 \) Hz, ArH), 7.56-7.58 (m, 3H, ArH), 7.51 (d, 1H, J=2.50 Hz, ArH), 7.44-7.46 (m, 4H, ArH), 7.26-2.28 (m, 3H, ArH), 3.33-3.35 (q, 2H, CH\(_2\)), 3.26-3.29 (q, 2H, CH\(_2\)); Anal. Calcd. For C\(_{26}\)H\(_{18}\)ClNO \( (395.88) \) C; 78.88; H; 4.58; N; 3.54; Found, C; 78.92; H; 4.55; N; 3.51%.
5.6.3. General procedure for the synthesis of quinoline based dispiropyrrolidines 10, 11, 12, 13.

A mixture of quinoline based α, β unsaturated carbonyl systems 4, 5 (1 mmol), indole-2, 3-dione 6 / indene-1, 2, 3-trione 8/ acenaphthalene 9 (1 mmol) and sarcosine (2 mmol) in methanol (2:1) 30 ml was refluxed over a water bath for 2-3 h. After completion of the reaction as monitored by TLC the excess solvent was removed under vacuum and the residue subjected to column chromatography using petroleum ether: ethyl acetate mixture (4:1 v/v) as eluent, this compound was obtained as a yellow solid. Most of the products were sedimented from the reaction medium after attaining room temperature, they were filtered off and recrystallized from MeOH and DMF mixture to obtain pure products.

5.6.3.1. 4-(2-methoxyquinolin-3-yl)-1-methyl-pyrrolidine-spiro[2,3']oxindole-spiro[3,2'']-
dihydro-indene-1''-one (10)

Pale grey solid (72%) mp. 158–160 °C; [α]D = -0.33 (c 0.5 μL, DMSO); IR (KBr) umax
3393.14, 3153.04, 3069.16, 1941.97, 1719.23, 1616.06, 1468.53, 1328.71, 1262.18, 1016.30,
785.85, 749.20 cm⁻¹; 1H NMR (500 MHz, DMSO-d6) δ 10.47 (s, 1H, NH), 8.44 (s, 1H, C-4-H),
7.97 (d, 1H, J=7.50 Hz, ArH), 7.70 (d, 1H, J=8.00 Hz, ArH), 7.64 (t, 1H, J=7.00 Hz, ArH),
7.58 (d, 1H, J=8.00 Hz, ArH), 7.41-7.47 (m, 2H, ArH), 7.29 (t, 1H, J=10.50 Hz, ArH), 7.15-7.19
(m, 2H, ArH), 7.04 (t, 1H, J=7.50 Hz, ArH), 6.83 (t, 1H, J=7.50 Hz, ArH), 6.63 (d, 1H, J=7.50
Hz, ArH), 5.32 (t, 1H, J=9.20 Hz, C-4'-H), 4.32 (t, 1H, J=9.80 Hz, C-3'-H), 3.37 (t, 1H, J=9.50
Hz, C-3'-H), 3.13 (s, 3H, CH3), 2.72 (d, 1H, J=19.00 Hz, CH), 2.41 (d, 1H, J=17.50 Hz, ArH),
2.12 (s, 3H, N-CH3); 13C NMR (125 MHz, DMSO-d6) δ 206.73, 178.07, 160.93, 152.10, 145.01,
143.68, 137.14, 135.69, 135.02, 129.82, 128.33, 127.83, 127.37, 126.75, 126.10, 125.52, 124.77,
123.67, 122.09, 109.90, 78.00, 63.78, 55.04, 52.57, 43.27, 35.22; Anal. Calcd. For C30H25N3O3
(475) C, 75.77; H, 5.30; N, 8.84; Found, C, 75.72; H, 5.33; N, 8.79%.

5.6.3.2. 4-(2-methoxyquinolin-3-yl)-1-methyl-pyrrolidine-spiro[2,3']oxindole-spiro[3,2''']
tetrahydro-naphthalen-1'''-one (11a)

Pale brown crystal (75%) mp. 160–164 °C; [α]D = -0.17 (c 0.5 μL, DMSO); IR (KBr)
umax 3062.41, 2937.06, 1665.23, 1605.45, 1439.60, 1395.25, 1266.04, 1018.23, 951.69, 754.03,
736.67, 701.96 cm⁻¹; 1H NMR (400 MHz, DMSO-d6) δ 10.65 (s, 1H, NH), 7.94-8.02 (m, 2H,
ArH), 8.81 (d, 1H, J=8.28 Hz, ArH), 7.73 (d, 1H, J=8.36 Hz, ArH), 7.63 (t, 1H, J=8.16 Hz,
ArH), 7.40-7.50 (m, 2H, ArH), 7.20-7.28 (m, 2H, ArH), 6.95 (t, 1H, J=7.80 Hz, ArH), 6.87
(d, 1H, J=6.96 Hz, ArH), 6.72 (d, 1H, J=7.40 Hz, ArH), 6.65 (d, 1H, J=7.64 Hz, ArH), 6.55 (t, 1H, J=7.60 Hz, ArH), 5.01 (t, 1H, J=9.08 Hz, C-4'-H), 4.10 (t, 1H, J=9.32 Hz, C-3'-H), 3.68 (s, 3H, OCH₃), 3.34 (t, 1H, J=6.00 Hz, C-3'-H), 2.98-3.20 (m, 2H,CH₂-H), 2.07 (m, 1H,CH₂-H), 1.97 (s, 3H, N-CH₃), 1.20 (m, 1H, CH₂-H); ¹³C NMR (100 MHz, DMSO-d₆) δ 186.44, 176.67, 160.79, 144.63, 143.60, 142.61, 138.62, 137.34, 133.72, 132.06, 130.48, 129.22, 128.31, 127.73, 126.41, 125.68, 124.61, 123.82, 121.24, 119.88, 109.44, 75.66, 59.40, 54.81, 53.24, 33.99, 29.17, 27.90, 26.90, 24.89; Anal. Calcd. For C₃₃H₂₇N₃O₃ (489) C, 76.05; H, 5.56; N, 8.58; Found, C, 74.98; H, 5.51; N, 8.60%.

5.6.3.3. 4-(2-methoxy-8-methylquinolin-3-yl)-1-methyl-pyrrolidine-spiro[2,3ʹ]oxindole-spiro[3,2ʺ]tetrahydronaphthalen-1ʹ ones (11b)

Pale brown crystal (77%) mp. 165–170 °C; [α]D = -0.60 (c 0.5 μL, DMSO); IR (KBr); ¹H NMR (300 MHz, DMSO-d₆) δ 10.69 (s, 1H, NH), 8.46 (s, 1H, ArH), 8.05 (d, 1H, J=9.00 Hz, ArH), 7.79 (d, 1H, J=8.10 Hz, ArH), 7.52 (d, 1H, J=7.00 Hz, ArH), 7.36 (t, 1H, J=7.80 Hz, ArH), 7.25-7.30 (m, 2H, ArH), 6.97 (t, 1H, J=7.80 Hz, ArH), 6.89 (d, 1H, J=7.00 Hz, ArH), 6.75 (d, 1H, J=7.50 Hz, ArH), 6.69 (d, 1H, J=7.80 Hz, ArH), 6.58 (t, 1H, J=7.50 Hz, ArH), 5.05 (t, 1H, J=9.05 Hz, C-4ʹ-H), 4.12 (t, 1H, J=9.13 Hz, C-3ʹ-H), 3.39 (t, 1H, J=6.00 Hz, C-3ʹ-H), 3.73 (s, 3H, OCH₃), 2.80 (m, 2H, CH₂-H), 2.62 (s, 3H, CH₃), 2.30 (m, 2H, CH₂-H), 2.0 (s, 3H, N-CH₃); ¹³C NMR (125 MHz, DMSO-d₆) δ 196.66, 177.14, 160.35, 143.81, 143.08, 137.95, 134.32, 133.09, 132.54, 129.74, 128.31, 127.30, 126.16, 125.98, 124.31, 123.76, 121.69, 109.89, 76.12, 59.87, 55.30, 53.40, 34.45, 29.66, 25.35, 17.69; Anal. Calcd. For C₃₂H₂₉N₃O₃ (503) C, 76.32; H, 5.80; N, 8.34; Found, C, 76.33; H, 5.86; N, 8.30%.

5.6.3.4. 4-(2-methoxy-8-methylquinolin-3-yl)-1-methyl-pyrrolidine-spiro[2,2ʹ]indolinone-spiro[3,2ʺ]tetrahydronaphthalen-1ʹ one (12)

Pale brown crystal (68%) mp.162–168 °C; [α]D = -0.114 (c 0.5 μL, DMSO); IR (KBr); ¹H NMR (500 MHz, DMSO-d₆) δ 8.54 (s, 1H, C-4-H), 8.19 (d, 1H, J=7.80 Hz, ArH), 7.98 (t, 1H, J=8.10 Hz, ArH), 7.86 (d, 1H, J=6.60 Hz, ArH), 7.76 (d, 1H, J=7.50 Hz, ArH), 7.66 (t, 1H, J=8.10 Hz, ArH), 7.58 (d, 1H, J=8.10 Hz, ArH), 7.45 (d, 1H, J=7.00 Hz, ArH), 7.25-7.34 (m, 3H, ArH), 7.16 (t, 1H, J=7.20 Hz, ArH), 7.06 (t, 1H, J=7.50 Hz, ArH), 5.35 (t, 1H, J=7.50 Hz, C-4ʹ-H), 4.25 (t, 1H, J=7.20 Hz, C-3ʹ-H), 3.59 (t, 1H, J=7.20 Hz, C-3ʹ-H), 3.15-2.90 (m, 1H, CH₂-H), 2.66 (s, 3H, CH₃), 2.05 (s, 3H, N-CH₃), 1.80-2.00 (m, 1H, CH₂-H); ¹³C NMR (125
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Synthesis of quinoline bearing dispiropyrrrolidines

MHz, DMSO-$d_6$) δ 207.20, 205.15, 197.24, 160.45, 144.11, 142.86, 142.32, 137.62, 136.04, 134.73, 132.91, 131.83, 130.15, 129.08, 128.32, 127.74, 126.21, 125.47, 124.87, 123.54, 120.18, 79.70, 61.35, 56.62, 53.13, 40.77, 34.66, 29.37, 26.24, 17.50; Anal. Calcd. For C33H28N2O4 (516) C, 76.73; H, 5.46; N, 5.42; Found, C, 76.70; H, 5.41; N, 5.39%; CCDC number for 12: CCDC 886308. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK. Fax: + 44(0)-1223-336033 or e-mail: deposit@ccdc.cam.ac.uk.

5.6.3.5. 4-(2-methoxyquinolin-3-yl)-1-methyl-pyrrolidine-spiro[2,2']acenaphthalene-spiro[3,2'']tetrahydronaphthalen-1''-one (13)

Pale brown crystal (74%) mp. 157–160 °C; [α]D = -0.29 (c 0.5 μL, DMSO); IR(KBr); IR (KBr) $v_{max}$ 3392.17, 3348.78, 3055.66, 2943.80, 2861.84, 1704.76, 1681.62, 1599.66, 1446.35, 1402.00, 1259.29, 1012.45, 754.03 cm$^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$) δ 8.60 (s, 1H, C-4-H), 8.17 (d, 1H, $J$=8.00 Hz, ArH), 8.03 (d, 1H, $J$=7.00 Hz, ArH), 7.74-7.79 (m, 2H, ArH), 7.52 (d, 1H, $J$=7.00 Hz, ArH), 7.33-7.39 (m, 2H, ArH), 7.14-7.21 (m, 3H, ArH), 6.58 (d,1H, $J$=7.50 Hz, ArH), 5.18 (t, 1H, $J$=8.50 Hz, C-4'-H), 4.17 (t, 1H, $J$=9.50 Hz, C-3'-H), 3.73 (s, 3H, OCH$_3$), 3.49 (t, 1H, $J$=8.50 Hz, C-3'-H), 3.00-3.23 (m, 2H, CH$_2$-H) 2.60 (s, 3H, CH$_3$), 1.90 (s, 3H, N-CH$_3$), 1.65 (m, 1H, CH$_2$-H), 1.30 (m, 1H, CH$_2$-H); $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 206.96, 186.91, 160.34, 159.43, 144.76, 143.86, 139.37, 137.72, 134.59, 134.17, 133.19, 130.98, 129.97, 129.12, 128.75, 128.07, 127.54, 126.56, 124.70, 120.98, 119.87, 79.30, 61.33, 56.24, 53.44, 39.47, 34.44, 29.55, 27.37, 25.84, 17.71; Anal. Calcd. For C$_{36}$H$_{30}$N$_2$O$_3$ (538) C, 80.27; H, 5.61; N, 5.20; Found, C, 80.30; H, 5.55; N, 5.15%

5.6.3.6. 4-(2-methoxyquinolin-3-yl)-1-methyl-pyrrolidine-spiro[2,3']oxindole-spiro[3,2'']6''-methylidine-(2-methoxyquinolin-3-yl)cyclohexan-1''-one (14)

Pale yellow needles (82%) mp. 174–178 °C; [α]D = -0.145 (c 0.5 μL, DMSO); IR (KBr) IR(KBr) $v_{max}$ 3307.32, 2951.52, 2863.77, 1714.41, 1668.12, 1594.84, 1467.56, 1440.56, 1397.17, 1263.15, 1015.34, 914.03, 755.95 cm$^{-1}$; $^1$H NMR (500 MHz, DMSO-$d_6$) δ 10.69 (s, 1H, NH), 8.38 (s, 1H, QC4-H), 8.00 (t, 1H, $J$=8.16 Hz, olefin-H), 7.96 (d, 1H, $J$=8.00 Hz, ArH), 7.87 (d, 1H, $J$=8.00 Hz, ArH), 7.81 (d, 1H, $J$=9.50 Hz, ArH), 7.72-7.79 (m, 2H, ArH), 7.66 (t, 2H, $J$=7.50 Hz, ArH), 7.58 (s, 1H, ArH), 7.46 (t, 1H, $J$=7.50 Hz, ArH), 7.41 (t, 1H, $J$=7.50 Hz, ArH), 7.21 (t, 1H, $J$=8.00 Hz, ArH), 7.06 (d, 1H, $J$=7.50 Hz, ArH), 7.00 (t, 1H, $J$=7.50 Hz, ArH), 6.79

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(t, 1H, J=7.50 Hz, ArH), 4.87 (t, 1H, J=9.00 Hz, C-4'-H), 4.15 (t, 1H, J=9.50 Hz, C-5'-H), 4.10 (s, 3H, OCH₃), 3.95 (s, 3H, OCH₃), 3.34 (t, 1H, J=8.00 Hz, C-3'-H), 2.45 (m, 1H, CH₂-H), 2.03 (s, 3H, N-CH₃), 2.26-2.29 (m, 1H, CH₂-H), 2.03 (m, 1H, CH₂-H), 1.30-1.45 (m, 1H, CH₂-H), 1.04-1.34 (m, 2H, CH₂-H) ¹³C NMR (100 MHz, DMSO-d₆) δ 199.32, 176.43, 160.77, 159.75, 145.28, 144.59, 142.85, 138.35, 136.58, 130.27, 129.13, 128.19, 127.67, 126.29, 125.95, 124.98, 124.24, 124.16, 121.83, 119.94, 109.37, 76.57, 61.46, 54.58, 53.19, 41.75, 34.17, 29.55, 27.65, 18.26; Anal. Calcd. For C₃₈H₃₄N₄O₄ (610) C, 74.73; H, 5.61; N, 9.17; Found, C, 74.76; H, 5.55; N, 9.21%.

5.6.4. Biological Assays

5.6.4.1. MTT assay for cell viability

Cancer cells were seeded at a density of 10⁴ cells/well in 96-well plates for 24h, followed by the pre-treatment with different concentrations of the 10, 11b and 14 as well as 4a, 4c, 5b and 13 (10-100 µg/mL) for 1 h before the addition of the untreated ones. The viability of cells was measured after 24 h of exposure to the tested compounds by Elisa assay, based on the ability of mitochondria in viable cells to reduce MTT. A 0.5 mg/mL concentration of MTT solution was added to each well and, after 3 h of incubation at 37 °C, the medium was discarded and the formazan blue formed in the cells was dissolved in dimethyl sulfoxide (DMSO). Optical density at 570 nm was determined with Elisa reader (BMG Labtech). The optical density of the formazan formed in untreated cells was taken as 100% viability.

5.6.4.2. Fluorescence microscopy

Cellular viability produced by synthetic compounds was examined using fluorescence microscopy after staining the Hela and MCF-7 cells with the Live/Dead system (Molecular Probes, USA). After incubation with the samples in 96-well plates, cells were stained by adding DAPI (4’,6-diamino-2-phenylindole), Rhodamine 123 (red), and FITC (Fluorescence isothiocyanate-Green) to reach a final concentration in the well plates of DAPI(4µM), Rhodamine 123 (2µM) and FITC (2µM). DAPI undergoes fragment DNA conversion to fluorescent DAPI (blue340 nm, wavelength), which is retained well within live cells. Rhodamine 123 enters into cells and destroyed mitochondria membranes, and undergoes a fluorescence enhancement (red, 635 nm wavelength) upon binding to DNA fragments. FITC enters into cells
and damaged cytoplasm. A fluorescence enhancement (Green, 475 nm wavelength) cells were incubated with the dyes for 20 min in 100 μL PBS, and viewed under the fluorescence microscope (Nikon instrument Inc., USA) using an excitation wavelength of 488 nm.

5.6.4.3. Cell cycle analysis

The decrease in cell viability was determined by MTT assay (Mossman et al., 1983) in terms of cell death, the cell cycle analysis was studied by flowcytometry [Ganguly et al., 2010]. In brief, cancer cells were seeded in 90 mm tissue culture plate and treated with PHMBA. At various time points, cells were recovered, washed twice in PBS, fixed in 70% ethanol, and stored at 4°C until analyzed. Cells were washed twice in PBS, incubated for 1 h at room temperature with 250 mg/ml RNAse A for 20 min at 4 °C with 20 mg/mL PI. The cell cycle distribution and percentage of apoptotic cells were determined using a FACS Calibur flow cytometer (Becton Dickinson, USA). Ten thousand events were analyzed for each sample. Appropriate gating was used to select the single-cell population. The same gate was used on all samples, ensuring that the measurements were made on a standardized cell population. Within the experimental time periods used, non-apoptotic cells did not exhibit significant alterations in the cell cycle distribution in comparison to untreated cells.

5.6.4.4. DNA fragmentation assay

The cells with compounds were analyzed in a buffer containing 10 mM Tris-HCl, at pH 7.4 followed by the addition of 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 1 h at room temperature. After centrifugation at 12,000 rpm for 30 min, the collected supernatent was incubated with proteinase K (Invitrogen, Carlsbad, CA) for 3 h at 50 °C. The fragmented DNA in supernatent was extracted using neutral phenol:chloroform:isoamyl alcohol solution together with the addition of 300 ml isopropanol, 100 ml 5 M NaCl and RNase. After incubation for overnight at 4 °C, DNA was separated through 1% agarose gel and stained with ethidium bromide. Then DNA fragmentation pattern was visualized by ultraviolet light source.
Fig. 5.1 $^1$H NMR Spectrum of 4b

Fig. 5.2 $^{13}$C NMR Spectrum of 4b
Fig. 5.3 IR Spectrum of 4b

Fig. 5.4 ¹H NMR Spectrum of 5b
Fig. 5.5 IR Spectrum of 5b

Fig. 5.6 $^1$H NMR Spectrum of 11b
Fig. 5.7 $^{13}$C NMR Spectrum of 11b

Fig. 5.8 DEPT 135 Spectrum of 11b
Fig. 5.9 H-H COSY Spectrum of 11b

Fig. 5.10 H-H COSY (aromatic region) Spectrum of 11b
Fig. 5.11 HMBC Spectrum of 11b

Fig. 5.12 HMBC Spectrum of 11b
Fig. 5.16 $^1$H NMR Spectrum of 12

Fig. 5.17 $^{13}$C NMR Spectrum of 12
Fig. 5.19 $^1$H NMR Spectrum of 13

Fig. 5.20 $^{13}$C NMR Spectrum of 13
Fig. 5.21 IR Spectrum of 13

Fig. 5.23 ¹H NMR Spectrum of 14
Fig. 5.24 $^{13}$C NMR Spectrum of 14

Fig. 5.25 $^{13}$C DEPT Spectrum of 14
Fig. 5.26 $^1$H-$^1$H COSY (aliphatic) Spectrum of 14

Fig. 5.27 H-H COSY (aromatic region) Spectrum of 14
Fig. 5.28 HMBC (aliphatic region) Spectrum of 14

Fig. 5.29 HMBC Spectrum (aromatic region) of 14
Fig. 5.30 HMQC Spectrum of 14

Fig. 5.32 $^1$HNMR Spectrum of 17a