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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Synthesis, antimicrobial and cytotoxic evaluation of spirooxindole [pyrano-bis-2*H*-l-benzopyrans]

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Abstract Microwave-assisted tandem double condensation between isatins and 4-hydroxycoumarin under zinc triflate catalysis to afford spirooxindoles in excellent yield is reported. The synthesized compounds were screened for their in vitro antibacterial and antifungal activities. The compounds that showed promising antimicrobial activity (**3a**, **3j** and **3m**) were studied for their binding affinity towards AmpC-β-lactamase receptor, which revealed that compound **3a** is highly stabilized by strong hydrogen bond interactions with in the binding pocket. The synthesized spirooxindoles were also evaluated for their cytotoxic potential against COLO320 adenocarcinoma colorectal cancer cells. Biological assay and in silico studies indicated compound **3n** as the most active in terms of low IC_{50} value (50.7 µM) and least free energy of binding (−8.89 kcal/mol) respectively.

Keywords Spirooxindoles · Antibacterial · Antifungal · Cytotoxicity · Molecular docking

Introduction

Spirooxindoles have received paramount importance in medicinal chemistry, because of their prevalence as substructure in numerous bioactive agents and natural products (Williams and Cox, 2003; Tang et al., 2001; Tsuda et al., 2003; Cui et al., 1996; Wang et al., 2005). On the other hand, 4-hydroxycoumarin is a structural component present in various pharmaceuticals and clinical drugs like warfarin, phenprocoumon, acenocoumarol, tioclomarol and brodifacoum (Fig. 1; Bo et al., 2010). Based on their unique pharmacological properties, we speculated that combining the structural characteristics of both spirooxindole and

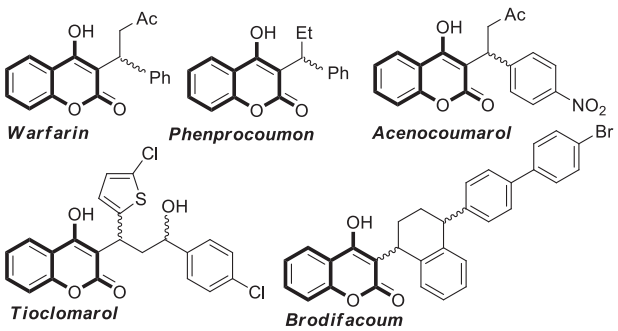

Fig. 1 Pharmaceuticals containing 4-hydroxycoumarin scaffold

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4-hydroxycoumarin moieties by a hybrid pharmacophore approach could substantially enhance the biological activity of the resulting compounds. Consequently, these structurally interesting heterocycles could be utilized in the synthesis of improved chemical entities and new drug discovery research. However, the synthesis of these types of compounds has been scarce and a great deal of interest exists in view of their structural complexity (Bo et al., 2010; Jain et al., 1996). To the best of our knowledge, only limited reports were documented for this type of transformations though with narrow substrate scope. For example, a substituent at the isatinyl nitrogen was not reported in the previously reported *p*-TSA and I_2 catalysed protocols (Almansour et al., 2012a–c). In view of the aforementioned facts, we intended a simple and straightforward synthesis of hybrid-spirooxindoles via tandem Knoevenagel/Michael reaction between substituted isatins and 4-hydroxycoumarin. Towards this end, we herein report the microwave-assisted synthesis of spirooxindole[pyrano-bis-2*H*-l-benzopyrans], their antimicrobial and cytotoxic properties. In addition, molecular docking of the compounds with appropriate receptors was performed and all these results were disclosed in this article.

Materials and methods

All commercially available solvents and reagents were used without further purification. Melting points were determined in capillary tubes and are uncorrected. Microwave reactions were performed in CEM-Discover microwave reactor. Infrared (IR) spectra were recorded on a Perkin-Elmer FTIR spectrophotometer as KBr pellets. ^{1}H and ^{13}C NMR spectra were obtained in $CDCl_3$, Acetone-d_6, dimethylsulphoxide (DMSO)-d_6 on a Bruker spectrometer at 400 and 100 MHz, respectively. Proton chemical shifts (δ) are relative to tetramethylsilane (TMS, $\delta = 0.00$) as internal standard and expressed in parts per million. Spin multiplicities are given as s (singlet), d (doublet), t (triplet) and m (multiplet). Coupling constants (*J*) are given in hertz. Mass spectra were recorded on a PE-SCIEX API 300 mass spectrometer. HRMS data were collected on a maxis 10138 mass spectrometer. Elemental analyses were recorded using a Thermo Finnigan FLASH EA 1112CHN analyzer. All the compounds gave C, H and N analysis within ±0.5 % of the theoretical values. Analytical thin-layer chromatography (TLC) was performed on precoated plastic sheets of silica gel G/UV-254 of 0.2 mm thickness (Macherey-Nagel, Duren, Germany) using analytical grade solvents and visualized with iodine spray (10 % (w/w) I_2 in silica gel) or UV light ($\lambda = 254$ and 365 nm). Synthetically good yields of N-substituted isatins **3b** (Shmidt et al., 2012), **3c** (Chen et al., 2010), **3d** (Diaz et al., 2008), **3e** (Bacchi et al., 2005), **3f** (Chowdhury et al., 2013), **3g** (Shi et al., 2012), **3h** (Sharma et al., 2014), **3i** (Shakoori et al., 2013), **3j** (Rassu et al., 2012), **3k** (Vintonyak et al., 2011), **3o** (Wee et al., 2012) and **3p** (Rahmati and Khalesi, 2012) were prepared by several closely related N-alkylation procedures. The bacterial strains (*Shigella flexneri* MTCC 1457, *Micrococcus luteus* MTCC 106, *Enterobacter aerogenes* MTCC 111, *Staphylococcus aureus* MTCC 96, *Staphylococcus epidermidis* MTCC 3615, *Klebsiella pneumoniae* MTCC 109, *Salmonella typhimurium* MTCC 1251, *Bacillus subtilis* MTCC 441, *Staphylococcus aureus* (MRSA-methicillin resistant)) were obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India-160 036. Fungal strains (*Malassesia pachydermatis* and *Candida albicans* MTCC 227) were obtained from the Department of Microbiology, Christian Medical College, Vellore, Tamil Nadu, India. Mueller Hinton agar (MHA) was purchased from Hi-media Pvt. Ltd, Mumbai, India. COLO320 adenocarcinoma colorectal cancer cell line was obtained from National Institute of Cell Sciences, Pune. Absorbance (λ_{max}) was red at 570 nm in an ELISA reader. Cytotoxicity of the compounds were statistically analysed by Duncan multiple range test at $P = 0.05$ with the help of SPSS 11.5 version software package. All computations for molecular docking studies were carried out on a Dell Desktop D510 personal computer (2.4 GHz Pentium 4 processor, Intel, Santa Clara, CA) running Red Hat Enterprise Linux Client release 5.5. The time required for each simulation run was on the order of Real = 1 h 13 min 54.67 s, CPU = 1 h 08 min 54.57 s, System = 11.75 s on the Pentium machine.

General procedure for the synthesis of compounds 3a-3p

To a pyrex reaction vessel containing a solution of isatin **1** (1.0 mmoL) and $Zn(OTf)_2$ (5 mol%) in DMSO (1 mL) was added 4-hydroxycoumarin **2** (2.0 mmoL). The resulting mixture was stirred at 100 °C for 30 min at a power level of 200 W. After completion of the reaction as evidenced by TLC, the mixture was cooled to room temperature and poured into ice-cold water (10 mL). The solid obtained was collected by filtration and washed with copious amount of cold water (100 mL) and dried under vacuum at 40 °C for 6 h to afford 400 mg of analytically pure product **3a**.

*1,2-Dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo [12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]-docosan]-1′(14′),3′(12′),4′ (9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3a**)*

Off-white powder (MeOH); this compound was prepared from isatin **1a** (147 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain an off-white powder (400 mg, 92 % yield). mp 178–180 °C (MeOH); FTIR (KBr) ν_{max}: 3411, 1732, 1707,

1662, 1608, 1347, 1101, 751 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ_H: 6.82–6.86 (m, 2H, Ar-H), 7.19 (d, $J = 7.6$ Hz,1H, Ar-H), 7.23 (d, $J = 7.7$ Hz, 1H, Ar-H), 7.49 (d, $J = 8.2$ Hz, 2H, Ar-H), 7.56 (t, $J = 7.6$ Hz, 2H, Ar-H), 7.79 (t, $J = 7.6$ Hz, 2H, Ar-H), 8.46 (t, $J = 7.7$ Hz, 2H, Ar-H), 10.85 (brs, 1H, D_2O exchangeable, -NH); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 46.5 (C, C-3), 10.33 (C, C-10 and C-10′), 108.9 (CH, C-5), 112.4 (C, C-17 and C-20), 116.5 (CH, C-13 and C-13′), 121.5 (CH, C-6), 123.6 (CH, C-16 and C-16′), 124.3 (CH, C-7), 125.1 (CH, C-15 and C-15′), 1291 (CH, C4), 131.9 (C, C-9), 133.9 (CH, C14 and C-14′), 144.1 (C, C-8), 151.8 (C, C-12 and C-12′), 154.4 (C, C-18 and C-18′), 157.9 (C=O, C-11 and C-11′), 176.4 (C=O, C-2); HRMS(ESI): $m/z_{(obs.)} = 458.0641$ $[M+Na]^+$; Anal. Calcd for $C_{26}H_{13}NO_6$: C, 71.72; H, 3.01; N, 3.22. Found C, 71.65; H, 2.98; N, 3.20.

1-Methyl-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione ***(3b)***

Off-white powder (MeOH); this compound was prepared from 1-methyl isatin **1b** (161 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain an off-white powder (427 mg, 95 %). mp 186–188 °C (MeOH); FTIR (KBr) ν_{max}: 2986, 1718, 1662, 1610, 1344, 1097, 754 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6) δ_H: 3.43 (s, 3H, -NCH_3), 6.94–6.96 (m, 2H, Ar-H), 6.99–7.01 (m 1H, Ar-H), 7.31 (t, $J = 7.6$ Hz, 1H, Ar-H), 7.35 (d, $J = 8.3$ Hz, 2H, Ar-H), 7.45 (t, $J = 7.4$ Hz, 2H, Ar-H), 7.65 (t, $J = 7.6$ Hz, 2H, Ar-H), 8.09 (t, $J = 7.7$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 27.1 (CH_3), 51.4 (C, C-3), 104.2 (C, C-10 and C-10′), 108.3 (CH, C-5), 112.9 (C, C-17 and C-20), 117.1 (CH, C-13 and C-13′), 122.5 (CH, C-6), 123.2 (CH, C-16 and C-16′), 124.8 (CH, C-7), 129.8 (CH, C-4), 130.0 (C, C-9), 133.4 (C, C-9′), 145.8 (C, C-8), 152.7 (C, C-12 and C-12′), 154.7 (C, C-18), 158.3 (C=O, C-11), 175.6 (C=O, C2); MS(ESI): $m/z = 450$ $[M+H]^+$; Anal. Calcd for $C_{26}H_{13}NO_6$: C, 72.16; H, 3.36; N, 3.12. Found C, 72.25; H, 3.28; N, 3.10.

1-Ethyl-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione ***(3c)***

Off-white powder (MeOH); this compound was prepared from 1-ethyl isatin **1c** (175 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain an off-white powder (440 mg, 95 % yield). mp 176–178 °C; FTIR (KBr)ν_{max}: 2983, 2931, 2868, 1737, 1714, 1663, 1609, 1345, 1100, 757 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ_H: 1.43 (t, $J = 7.2$ Hz, 3H, -CH_3), 3.94 (q, $J = 7.2$ Hz, 2H, -CH_2–), 6.91–6.93 (m, 2H, Ar-H), 6.95–7.01 (d, $J = 7.1$ Hz, 1H, Ar-H), 7.30–7.34 (m, 3H, Ar-H), 7.46 (t, $J = 7.6$ Hz, 2H, Ar-H), 7.66 (t, $J = 7.6$ Hz, 2H, Ar-H), 8.11 (d, $J = 7.9$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 11.9 (-CH_3), 35.8 (-CH_2-Me), 46.7 (C, C-3), 104.4 (C, C-10 and C-10′), 108.5 (CH, C-5), 113.1 (C, C-17 and C-20), 117.2 (CH, C-13 and C-13′), 122.4 (CH, C-6), 122.8 (CH, C-16 and C-16′), 123.6 (CH, C-7), 124.9 (CH, C-4), 129.9 (C, C-9), 131.4 (CH, C-14 and C-14′), 133.6 (C, C-9′), 145.2 (C, C-8), 152.8 (C, C-12 and C-12′), 154.8 (C, C-18), 158.4 (C=O, C-11 and C-11′), 175.1 (C=O, C2); MS(ESI): $m/z = 464$ $[M+H]^+$; Anal. Calcd for $C_{26}H_{13}NO_6$: C, 72.57; H, 3.70; N, 3.02. Found C, 72.67; H, 3.76; N, 3.12.

1-Propyl-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione ***(3d)***

Red powder (MeOH); this compound was prepared from 1-propyl isatin **1d** (189 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain a red powder (448 mg, 94 % yield). mp 179–181 °C (MeOH); FTIR (KBr)ν_{max}: 2950, 2927, 2853, 1738, 1717, 1666, 1609, 1344, 1102, 751 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ_H: 0.90 (t, $J = 6.6$ Hz, 3H, -CH_3), 1.61–1.65 (m, $J = 7.1$ Hz, 2H, -CH_2-CH_3), 4.17 (t, $J = 7.3$ Hz, 2H, -NCH_2-), 6.91–6.94 (m, 1H, Ar-H), 7.29–7.35 (m, 3H, Ar-H), 7.44–7.56 (m, 4H, Ar-H), 7.67 (t, $J = 7.5$ Hz, 2H, Ar-H), 8.12 (d, $J = 7.9$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 11.3 (-CH_3), 21.8 (-CH_2-Me), 42.4 (-N-CH_2-), 47.2 (C, C-3), 102.4 (C, C-10 and C-10′), 107.6 (CH, C-5), 112.7, (C, C-17 and C-20), 117.6 (CH, C-13 and C-13′), 122.6 (CH, C-6), 122.9(CH, C-16 and C-16′), 123.8 (CH, C-7), 124.5 (CH, C-4), 129.9 (C, C-9), 131.8 (C, C-9′), 133.7 (C, C-8), 145.9 (C, C-14 and C-14′), 152.4 (C, C-18), 154.3 (C, C-14 and C-14′), 158.1 (C=O, C-11), 175.3 (C=O, C2); MS(ESI): $m/z = 478$ $[M+H]^+$; Anal. Calcd for $C_{29}H_{19}NO_6$: C, 72.95; H, 4.01; N, 2.93. Found C, 72.90; H, 3.97; N, 2.99.

1-Butyl-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione ***(3e)***

Off-white powder (MeOH); this compound was prepared from 1-butyl isatin **1e** (203 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain an off-white powder (466 mg, 95 % yield). mp 185–187 °C (MeOH); FTIR (KBr) ν_{max}:

2950, 2923, 2852, 1738, 1712, 1664, 1611, 1341, 1102, 752 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ_H: 0.90 (t, $J = 6.8$ Hz, 3H, -CH_3), 1.37–1.39 (m, 2H, -CH_2-CH_3), 1.41–1.44 (m, 2H, -CH_2-CH_2CH_3), 3.99 (t, $J = 7.4$ Hz, 2H, -NCH_2-), 6.91–6.94 (m, 1H, Ar-H), 7.31–7.35 (m, 3H, Ar-H), 7.46 (t, $J = 7.5$ Hz, 2H, Ar-H), 7.53–7.57 (m, 2H, Ar-H), 7.66 (t, $J = 7.5$ Hz, 2H, Ar-H), 8.10 (d, $J = 7.8$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 13.9 (-CH_3), 21.8 (-CH_2Me), 28.0 (-CH_2Pr), 39.8 (-NCH_2-), 45.7 (C, C-3), 103.9 (C, C-10 and C-10′), 108.9 (CH, C-5), 113.1 (C, C-17 and C-20), 117.2 (CH, C-13 and C-13′), 122.1 (CH, C-6), 122.9 (CH, C-16 and C-16′), 123.8 (CH, C-7), 124.6 (CH, C-4), 129.8 (C, C-9), 131.3 (C, C-9′), 133.6 (C, C-8), 145.6 (C, C-14 and C-14′), 152.2 (C, C-12 and C-12′), 154.5 (C, C-18), 158.3 (C=O, C-11), 175.2 (C=O, C2); MS(ESI): $m/z = 492$ $[M+H]^+$; Anal. Calcd for $C_{30}H_{21}NO_6$: C, 73.31; H, 4.31; N, 2.85. Found C, 73.25; H, 4.28; N, 2.93.

*1-Pentyl-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3f**)*

Off-white powder; this compound was prepared from 1-pentyl isatin **1f** (217 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain an off-white powder (460 mg, 91 % yield). mp 190–192 °C (MeOH); FTIR (KBr) ν_{max}: 2959, 2905, 2856, 1741, 1710, 1664, 1604, 1349, 1100, 754 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ_H: 0.91 (t, $J = 6.5$ Hz, 3H, -CH_3), 1.37–1.43 (m, 4H, -CH_2-CH_2-CH_3), 1.47 (q, $J = 7.4$ Hz, 2H, -N-CH_2-), 4.08 (t, $J = 7.8$ Hz, 2H, Ar-H), 6.90–6.63 (m 2H, Ar-H), 6.99 (d, $J = 7.3$ Hz, 1H, Ar-H), 7.30–7.35 (m 3H, Ar-H), 7.47 (t, $J = 7.6$ Hz, 2H, Ar-H), 7.69 (t, $J = 7.8$ Hz, 2H, Ar-H), 8.11 (d, $J = 7.8$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 14.2 (-CH_3), 22.9 (-CH_2Me), 27.6 (-CH_2Et), 29.1, (-CH_2Pr), 41.5 (-NCH_2-), 45.7 (C, C-3), 104.0 (C, C-10 and C-10′), 108.9 (CH, C-5), 113.1 (C, C-17 and C-20), 117.1 (CH, C-13 and C-13′), 122.6 (CH, C-6), 122.9 (CH, C-16 and C-16′), 123.3, (CH, C-7), 124.9 (CH, C-4), 129.7 (C, C-9), 131.6 (C, C-9′), 133.7 (C, C-8), 145.1 (C, C-14 and C-14′), 152.6 (C, C-12 and C-12′), 154.7 (C, C-18), 158.5 (C=O, C-11), 175.0 (C=O, C2); MS(ESI): $m/z = 506$ $[M+H]^+$; Anal. Calcd for $C_{31}H_{23}NO_6$: C, 73.65; H, 4.49; N, 2.77. Found C, 73.59; H, 4.48; N, 2.83.

*1-Hexyl-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3g**)*

Off-white solid (MeOH); this compound was prepared from 1-hexyl isatin **1g** (231 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain an off-white powder (451 mg, 87 % yield). mp 193–195 °C (MeOH); FTIR (KBr) ν_{max}: 2952, 2925, 2856, 1739, 1716, 1665, 1609, 1345, 1103, 754; cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ_H: 0.91 (t, $J = 6.6$ Hz, 3H, -CH_3), 1.37–1.38 (m, 4H, -CH_2-CH_2-CH_3), 1.47–1.84 (m, 4H, -NCH_2-CH_2-CH_2-), 3.84 (t, $J = 7.8$ Hz, 2H, -NCH_2-), 6.91–6.96 (m, 2H, Ar-H), 6.98 (d, $J = 7.1$ Hz, 1H, Ar-H), 7.29–7.44 (m, 3H, Ar-H), 7.46 (t, $J = 7.5$ Hz, 2H, Ar-H), 7.66 (t, $J = 7.5$ Hz, 2H, Ar-H), 8.10 (d, $J = 7.8$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C 14.3 (-CH_3), 22.8 (-CH_2Me), 27.0 (-CH_2Et), 27.1 (-CH_2Pr), 31.8 (-CH_2Bu), 41.4 (-NCH_2-), 46.7 (C, C-3), 104.4 (C, C-10 and C-10′), 108.6 (CH, C-5), 113.0 (C, C-17 and C-20), 117.1 (CH, C-13 and C-13′), 122.4 (CH, C-6), 122.8, (CH, C-16 and C-16′), 123.5 (CH, C-7), 124.9 (CH, C-4), 129.8 (C, C-9), 131.4 (C, C-9′), 133.5 (C, C-8), 145.7 (C, C-14 and C-14′), 152.7 (C, C-12 and C-12′), 154.8 (C, C-18), 158.3 (C=O, C-11), 175.4 (C=O, C2); MS(ESI): $m/z = 520$ $[M+H]^+$, Anal. Calcd for $C_{32}H_{25}NO_6$: C, 73.98; H, 4.85; N, 2.70. Found C, 73.93; H, 4.80; N, 2.66.

*1-(Prop-2-en-yl)-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′- pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3h**)*

Off-white solid (MeOH); this compound was prepared from 1-allyl isatin **1h** (187 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain an off-white powder (422 mg, 89 % yield). mp 167–169 °C (MeOH); FTIR (KBr) ν_{max}: 3065, 1735, 1714, 1664, 1609, 1345,1104, 754 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ_H: δ 4.54 (d, $J = 4.9$ Hz, 1H, =CH_2-) 5.31 (d, $J = 10.4$ Hz, 1H, =CH_2-), 5.61 (d, $J = 17.2$ Hz, 1H, -CH=CH_2), 6.02–6.08 (m, 1H, Ar-H), 6.94 (d, $J = 7.4$ Hz, 2H, -NCH_2-), 7.00 (d, $J = 7.5$ Hz, 1H, Ar-H), 7.29 (t, $J = 7.7$ Hz, 1H, Ar-H), 7.35 (d, $J = 8.3$ Hz, 2H, Ar-H), 7.46 (d, $J = 7.6$ Hz, 2H, Ar-H), 7.65 (d, $J = 7.7$ Hz, 2H, Ar-H), 8.11 (d, $J = 7.9$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 43.7 (-NCH_2-), 46.5 (C, C-3), 104.2 (C, C-10 and C-10′), 109.3 (CH, C-5), 112.9 (C, C-17 and C-20), 117.1 (CH, C-13 and C-13′), 117.8 (allyl=CH_2), 122.4 (CH, C-6), 122.6 (CH, C-16 and C-16′), 123.2 (CH, C-7), 124.8 (CH, C-4), 129.6 (C, C-9), 130.9 (C, C-9′), 131.6 (allyl-CH=), 133.4 (C, C-8), 145.2 (C, C-14 and C-14′), 152.7 (C, C-12 and C-12′), 154.6 (C, C-18), 158.2 (C=O, C-11), 175.2 (C=O, C2); MS(ESI): $m/z = 476$ $[M+H]^+$, Anal. Calcd for $C_{29}H_{17}NO_6$: C, 73.26; H, 3.60; N, 2.95. Found C, 73.31; H, 3.55; N, 2.99.

1-(Prop-2-yn-yl)-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′- pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′

*(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3i**)*

Off-white solid; this compound was prepared from 1-propargyl isatin **1i** (185 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain an off-white powder (445 mg, 91 % yield). mp 177–179 °C (MeOH); FTIR (KBr) ν_{max}: 3240, 2123, 1734, 1722, 1665, 1609, 1345, 1104, 754 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ_H: 2.37 (s, 1H, acetylenic-CH), 4.72 (s, 2H, -NCH_2-), 6.97–7.03 (m, 2H, Ar-H), 7.17 (d, $J = 7.8$ Hz, 1H, Ar-H), 7.35–7.37 (m, 3H, Ar-H), 7.46 (t, $J = 7.5$ Hz, 2H, Ar-H), 7.66 (t, $J = 7.5$ Hz, 2H, Ar-H), 8.10 (d, $J = 7.8$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 29.6 (-NCH_2-), 46.0 (C, C-3), 74.6 (internal propargyl C), 77.5 (terminal propagyl C), 102.8 (C, C-10 and C-10′), 108.6 (CH, C-5), 112.4, (C, C-17 and C-20), 116.6 (CH, C-13 and C-13′), 122.5 (CH, C-6), 123.7 (CH, C-16 and C-16′), 124.3 (CH, C-7), 125.2 (CH, C-4), 129.2 (C, C-9), 130.9 (C, C-9′), 134.0 (C, C-8), 143.8 (C, C-14 and C-14′), 151.9 (C, C-12 and C-12′), 154.6 (C, C-18), 157.9 (C=O, C-11), 174.2 (C=O, C2); MS(ESI): $m/z = 474$ $[M+H]^+$, Anal. Calcd for $C_{29}H_{15}NO_6$: C, 73.57; H, 3.19; N, 2.96. Found C, 73.51; H, 3.23; N, 3.00.

*1-Benzyl-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3j**)*

Off-white solid (MeOH); this compound was prepared from 1-benzyl isatin **1j** (237 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain an off-white powder (493 mg, 94 % yield). mp 154–156 °C; FTIR (KBr) ν_{max}: 1723, 1664, 1611, 1345, 1100, 756 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ_H: 5.15 (s, 2H, -NCH_2-), 6.68 (d, $J = 7.9$ Hz, 1H, Ar-H), 6.90 (t, $J = 7.4$ Hz, 1H, Ar-H), 7.01 (d, $J = 7.2$ Hz, 1H, Ar-H), 7.15 (t, $J = 7.6$ Hz, 1H, Ar-H), 7.29–7.34 (m, 2H, Ar-H), 7.37–7.42 (m, 3H, Ar-H); 7.47 (t, $J = 7.5$ Hz, 2H, Ar-H), 7.63 (d, $J = 7.6$ Hz, 2H, Ar-H), 7.67 (t, $J = 7.8$ Hz, 2H, Ar-H), 8.12 (d, $J = 7.8$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 45.6 (C, C-3), 50.2 (-NCH_2Bn), 104.3 (C, C-10 and C-10′), 109.7 (CH, C-5), 112.9 (C, C-17 and C-20), 117.2 (CH, C-13 and C-13′), 122.8 (CH, C-6), 123.4 (CH, C-16 and C-16′), 123.9 (CH, C-7), 124.9 (CH, C-4), 127.4 (benzyl *para* C), 127.5 (benzyl *meta* C), 127.9 (benzyl *ortho* C), 128.8 (benzyl *ipso* C), 129.8 (C, C-9), 130.5, 131.0 (C, C-9′), 133.7 (C, C-8), 135.9, 145.4 (C, C-14 and C-14′), 152.7, (C, C-12 and C-12′), 154.8 (C, C-18), 158.5 (C=O, C-11), 176.0 (C=O, C2); MS(ESI): $m/z = 526$ $[M+H]^+$, Anal. Calcd for $C_{33}H_{19}NO_6$: C, 75.42; H, 3.64; N, 2.67%. Found C, 75.45; H, 3.59; N, 2.64 %.

*1-[(2-Fluorophenyl)methyl]-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′- pentacyclo [12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3k**)*

Pink solid (MeOH); this compound was prepared from 1-(2-fluorobenzyl)isatin **1k** (255 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain a pink solid (500 mg, 92 % yield). mp 168–170 °C; FTIR (KBr) ν_{max}: 1726, 1654, 1619, 1355, 1106, 750 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ_H: 5.18 (s, 2H, -NCH_2-), 6.88 (d, $J = 7.9$ Hz, 1H, Ar-H), 7.10–7.13 (m, 1H, Ar-H), 7.18 (d, $J = 7.2$ Hz, 1H, Ar-H), 7.20 (t, $J = 7.6$ Hz, 1H, Ar-H), 7.29–7.35 (m, 2H, Ar-H), 7.38–7.41 (m, 2H, Ar-H); 7.44 (t, $J = 7.5$ Hz, 2H, Ar-H), 7.68 (d, $J = 7.6$ Hz, 2H, Ar-H), 7.73 (t, $J = 7.8$ Hz, 2H, Ar-H), 8.15 (d, $J = 7.8$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 43.6 (-NCH_2-), 49.8 (C, C-3), 104.5 ((C, C-10 and C-10′), 109.3 (CH, C-5), 112.5(C, C-17 and C-20), 115.8 (CH, C-13 and C-13′), 116.1 (CH, C-6), 116.7 (CH, C-16 and C-16′), 117.2 (CH, C-7), 122.8 (CH, C-4), 123.4 ($J_{C\text{-}F} = 35.6$ Hz), 123.9 ($J_{C\text{-}F} = 205.1$ Hz), 124.1 (C, C-6, Bn-2F), 124.9 (C, C-5, Bn-F), 127.4 (C, C-4, Bn-F), 127.5, 127.9, 128.8, 129.8 (C, C-9), 130.5 (C, C-3 Bn-2F), 131.0 (*ipso*-Bn C), 131.1 (C, C-9′), 133.1 (C, C-9′), 135.96 (2-F-Bn-C), 145.40 (C, C-8), 152.3 (C, C-14 and C-14′), 154.4 (C, C-12 and C-12′), 158.6 (C, C-18), 161.0 (C=O, C-11), 175.8 (C=O, C2); MS (ESI): $m/z = 544$ $[M+H]^+$, Anal. Calcd for $C_{33}H_{18}FNO_6$: C, 72.93; H, 3.34; N, 2.58. Found C, 72.89; H, 3.40; N, 2.62.

*5-Chloro-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3l**)*

Off-white solid (MeOH); this compound was prepared from 5-chloroisatin **1l** (181 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain an off-white powder (416 mg, 89 % yield). mp 164–166 °C; FTIR (KBr) ν_{max}: 3395, 1730, 1706, 1663, 1610, 1345, 1108, 761 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ_H: 6.85 (d, $J = 8.3$ Hz, 1H, Ar-H), 7.24 (dd, $J = 8.3$ and 2.1 Hz, 1H, Ar-H), 7.46 (d, $J = 2.1$ Hz, 1H, Ar-H), 7.49 (d, $J = 8.3$ Hz, 2H, Ar-H), 7.55 (t, $J = 7.6$ Hz, 2H, Ar-H), 7.80 (t, $J = 7.6$ Hz, 2H, Ar-H), 8.45 (d, $J = 7.6$ Hz, 2H, Ar-H), 10.99 (brs, D_2O exchangeable, 1H, -NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 46.6 (C, C-3), 102.7 (C, C-10 and C-10′), 110.2 (C-Cl, C-5), 112.5 (C, C-17 and C-20), 116.6 (CH, C-13 and C-13′), 123.7 (CH, C-6), 124.7 (CH, C-16 and C-16′), 125.2 (CH, C-7), 125.5 (CH, C-4), 128.9 (C, C-9), 133.7 (C, C-9′), 134.0 (C, C-8), 143.1 (C, C-14 and C-14′), 151.9 (C, C-12 and C-12′), 154.8 (C, C-18),

158.0 (C=O, C-11), 176.3 (C=O, C2); MS(ESI): $m/z = 468$ $[M-H]^-$, Anal. Calcd for $C_{26}H_{12}ClNO_6$: C, 66.47; H, 2.57; N, 2.98. Found C, 66.43; H, 2.51; N, 3.03.

*5-Bromo-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3m**)*

Brown solid (MeOH); mp 187–189 °C; this compound was prepared from 5-bromoisatin **1m** (225 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain a brown solid (450 mg, 88 % yield). FTIR (KBr) ν_{max}: 3395, 1730, 1706, 1663, 1610, 1345, 1108, 761 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 6.80 (d, $J = 7.4$ Hz, 1H, Ar-H), 7.37 (d, $J = 7.6$ Hz, 1H, Ar-H), 7.49 (d, $J = 7.5$ Hz, 2H, Ar-H), 7.57–7.63 (m, 3H, Ar-H), 7.82–7.86 (m, 2H, Ar-H), 8.45 (d, $J = 7.0$ Hz, 2H, Ar-H), 11.00 (brs, D_2O exchangeable, 1H, -NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 46.5 (C, C-3), 102.7 (C, C-10 and C-10′), 110.7 (C-Br, C-5), 112.5 (C, C-17 and C-20), 113.1 (CH, C-13 and C-13′), 116.5 (CH, C-6), 123.7 (CH, C-16 and C-16′), 125.1 (CH, C-7), 127.3 (CH, C-4), 131.7 (C, C-9), 133.9 (C, C-9′), 143.5 (C, C-8), 151.9 (C, C-14 and C-14′), 154.8 (C, C-12 and C-12′), 156.0 (C, C-18), 156.1 (C=O, C-11); 176.1 (C=O, C2); MS(ESI): $m/z = 512$ $[M-H]^-$, Anal. Calcd for $C_{26}H_{12}BrNO_6$: C, 60.72; H, 2.35; N, 2.72. Found C, 60.69; H, 2.40; N, 2.68.

*5-Nitro-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3n**)*

Yellow solid (MeOH); this compound was prepared from 5-nitroisatin **1n** (192 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain a yellow solid (411 mg, 86 % yield). mp 209–211 °C (MeOH); FTIR (KBr) ν_{max}: 3395, 1730, 1706, 1663, 1610, 1345, 1108, 761 cm^{-1}. ^{1}H NMR (400 MHz, Acetone-d_6) δ_H: δ 7.17 (d, $J = 8.7$ Hz, 1H, Ar-H), 7.45 (d, $J = 8.3$ Hz, 2H, Ar-H),7.58 (t, $J = 7.9$ Hz, 2H, Ar-H), 7.81 (t, $J = 8.6$ Hz, 2H, Ar-H), 8.23 (dd, $J = 8.6$ and 2.2 Hz, 1H, Ar-H), 8.31 (d, $J = 2.2$Hz, 1H, Ar-H), 8.46 (d, $J = 8.0$ Hz, 2H, Ar-H), 10.59 (brs, D_2O exchangeable, 1H, -NH); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 46.7 (C, C-3), 102.9 (C, C-10 and C-10′), 109.5 (CH, C-5), 112.6 (C, C-17 and C-20), 116.9 (CH, C-13 and C-13′), 119.6 (CH, C-6), 122.9 (CH, C-16 and C-16′), 125.1 (CH, C-7), 126.6 (CH, C-4), 132.3 (C, C-9), 133.9 (C, C-8), 142.7 (C, C-14 and C-14′), 150.6 (C-NO2, C-5), 152.6 (C, C-12 and C-12′), 155.2 (C, C-18), 158.4 (C=O, C-11), 177.3 (C=O, C2); MS(ESI): $m/z = 479$ $[M-H]^-$, Anal. Calcd for $C_{26}H_{12}N_2O_8$: C, 65.01; H, 2.52; N, 5.83. Found C, 65.06; H, 2.50; N, 5.78.

*5-Fluoro-1-methyl-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′- pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octane-2,11′,15′-trione (**3o**)*

Colourless solid (MeOH); this compound was prepared from 5-fluoro-1-methyl isatin **1o** (179 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 2.0 mmoL) according to the general procedure to obtain a colourless solid (416 mg, 89 % yield). mp 181–183 °C; FTIR (KBr) ν_{max}: 1734, 1716, 1669, 1613, 1355, 1103, 760 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ_H: 3.38 (s, 3H, -NCH_3) 6.88–6.92 (m, 1H, Ar-H), 7.19 (d, $J = 8.7$ Hz, 1H, Ar-H), 7.33 (d, $J = 8.3$ Hz, 2H, Ar-H),7.56 (t, $J = 7.7$ Hz, 2H, Ar-H), 7.76–7.80 (m, 1H, Ar-H), 7.83 (dd, $J = 8.8$ and 2.2 Hz, 1H, Ar-H), 8.34 (d, $J = 2.2$ Hz, 1H, Ar-H), 8.49 (d, $J = 8.0$ Hz, 2H, Ar-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ_C: 35.8 (-CH_3), 46.7 (C, C-3), 102.9 (C, C-10 and C-10′), 109.5 (CH, C-5), 112.6 (C, C-17 and C-20), 116.6 (CH, C-13 and C-13′), 116.9 (C, C-9), 119.6 (C, C-9′), 122.9 (CH, C-6), 123.9 (CH, C-16 and C-16′), 124.1, (CH, C-7), 126.6, 131.3, 131.4, 133.9, 142.7, 150.6 (C, C-8), 152.6 (C, C-14 and C-14′), 155.2 (C, C-12 and C-12′), 158.4 (C, C-18), 160.8 (C=O, C-11), 177.3 (C=O, C2); MS (ESI): $m/z = 468$ $[M+H]^+$, Anal. Calcd for $C_{27}H_{14}FNO_6$: C, 69.38; H, 3.02; N, 3.00. Found C, 69.43; H, 3.07; N, 2.97.

*1-(3-{2,11′15′-trioxo-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′- pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octaen-1-yl} propyl)-1,2-dihydro-2′,10′,16′-trioxaspiro[indole-3,13′-pentacyclo[12.8.0.0^{3,12}.0^{4,9}.0^{17,22}]docosan]-1′(14′),3′(12′),4′(9′),5′,7′,17′(22′),18′,20′-octaene-2,11′,15′-trione (**3p**)*

Pale yellow solid (MeOH); this compound was prepared from bis-isatin **1p** (334 mg, 1.0 mmoL) and 4-hydroxycoumarin **2** (324 mg, 4.0 mmoL) according to the general procedure to obtain a pale yellow solid (737 mg, 81 % yield). mp > 250 °C (MeOH); FTIR (KBr) ν_{max}: 2928, 2877, 1742, 1734, 1706, 1689, 1612, 1359, 1109, 7601 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ_H: 1.72–1.88 (m, 2H, -CH_2-), 3.82–4.02 (q, $J = 7.1$ Hz, 4H, -NCH_2-), 6.84–6.94 (m, 4H), 7.19 (d, $J = 7.9$ Hz, 2H, Ar-H), 7.26 (d, $J = 7.9$ Hz, 2H, Ar-H), 7.49–7.55 (m, 4H, Ar-H), 7.55–7.63 (m, 4H, Ar-H), 7.79–7.90 (m, 4H, Ar-H), 8.46–8.54 (m, 4H, Ar-H), MS(ESI): $m/z = 911$ $[M+H]^+$.

Experimental procedure for the evaluation of antimicrobial activity

All the synthesized compounds were tested for their antibacterial (against *S. epidermidis*, *S. typhimurium*, *K. pneumonia*, *B. subtilis*, *S. flexneri*, *M. luteus*, *E. aerogenes*, *S. aureus* and *S. aureus* MRSA) and antifungal activities (against *C. albicans*, *M. pachydermatis*) using disc diffusion method (Bauer et al., 1966). Petri plates were prepared with 20 mL of sterile MHA. The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min and a specific amount of synthesized compound at 1 mg/disc was added to each disc separately. The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. *Negative control was prepared using DMSO. Streptomycin was used as a positive control against bacterial strains and ketoconazole was used as a positive control for fungal strains. The plates were incubate*d for 24 h at 37 °C for bacteria and 48 h at 28 °C for fungi. The radii of inhibition zones were recorded in millimeters and the experiment was repeated twice. Bacterial inoculums were prepared by growing cells in Mueller Hinton broth for 24 h at 37 °C. The filamentous fungi were grown on sabouraud dextrose agar slants at 28 °C for 10 days and the spores were collected using sterile doubled distilled water and homogenized. Yeast was grown on sabouraud dextrose broth at 28 °C for 48 h.

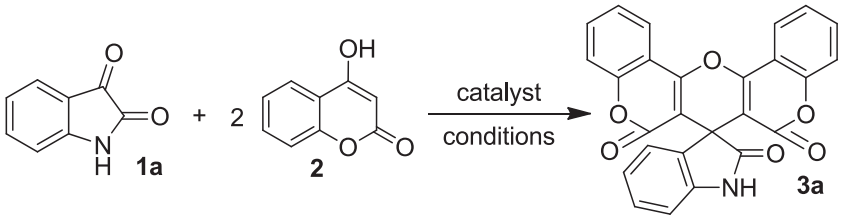
Experimental procedure for the evaluation of cytotoxicity

The MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyl-tetrazolium bromide] cell proliferation assay (Mossman, 1983) was used to evaluate the cytotoxic activity of the synthesized compounds against COLO320 adenocarcinoma colorectal cancer cell lines. COLO320 cancer cell line was maintained in complete tissue culture medium RPMI with 10 % fetal bovine serum and 2 mM L-glutamine, along with antibiotics (about 100 IU/mL of penicillin, 100 µg/mL of streptomycin) with the pH adjusted to 7.2. Cells (5×10^5) were seeded in 96 well plates containing medium with different concentrations such as 500 µg/mL, 250 µg/mL and 100 µg/mL. The cells were cultivated at 37 °C with 5 % CO_2 and 95 % air in 100 % relative humidity. After various durations of cultivation, the solution in the medium was removed. An aliquot of 100 µL of medium containing 1 mg/mL of MTT was loaded to the plate. Incubation at 37 °C for 4 h allowed reduction of MTT by mitochondrial dehydrogenase to an insoluble formazan product. Well contents were removed and the formazan product was solubilized by the addition of 100 µL of DMSO, which led to the formation of purple colour. The amount of formazan product is directly proportional to the number of living cells. Absorbance of the converted dye in each well was read on ELISA reader at 570 nm. From the absorbance, % of inhibition was calculated by using the formula, % of inhibition $= A_c - A_t / A_c \times 100$, where A_c is the mean absorbance of control and A_t is that of test. From the results, nonlinear regression graph was plotted between % cell growth inhibition and $\log_{10}$ concentration (µM). The half maximal inhibitory concentration (IC_{50} value) was determined averaged from three replicate experiments. Cytotoxicity were statistically analysed by Duncan multiple range test at $P = 0.05$ with the help of SPSS 11.5 version software package.

Molecular docking studies

The level of antimicrobial activity was studied computationally by Molegro Virtual Docker (MVD) by automated docking of the most active compounds (**3a**, **3j** and **3l**) to the binding site of AmpC-*β*-lactamase. Experimentally, X-ray crystal structures of AmpC-*β*-lactamase from *Escherichia coli* were retrieved from Protein Data Bank (PDB) database-1KE4 (Powers and Shoichet, 2002). Computationally, MVD was used to predict potential binding site on AmpC-*β*-lactamase. The MolDock scoring function used by MVD is derived from the PLP scoring functions (Yang and Chen, 2004). To investigate the potential binding mode of inhibitors, all compounds were subjected to molecular docking using the AutoDock 1.5.4 docking program. The X-ray crystal structure of Checkpoint kinase 1 (CHK1) in complex with pyrazolo[1,5-*a*]pyrimidine was downloaded from the protein data bank (PDB ID: 3OT3) and was used for the docking study. Ligand 2D structures were drawn using ChemDraw Ultra 7.0. Chem3D Ultra 7.0 was used to convert 2D structure into 3D and the energy minimized using semi-empirical AM1 method. Minimize energy to minimum RMS gradient of 0.100 was set in each iteration. All structures were saved as .pdb file format for input to AutoDockTools (ADT) version 1.5.4 (http://mgltools.scripps.edu/). All the ligand structures were then saved in PDBQT file format, for input into AutoDock version 1.5.4 (http://vina.scripps.edu/). All the ligand structures were then saved in PDBQT file format, for input into AutoDock version 1.5.4. For the molecular docking study, protein structure was obtained from the PDB; the CHK1 structure PDB ID was 3OT3. The co-crystallized ligand pyrazolo [1,5-*a*]pyrimidine in the CHK1 structure was removed. For the protein structure, all hydrogen atoms were added, lower occupancy residue structures were deleted, and any incomplete side chains were replaced using the ADT version 1.5.4. Further ADT was used to remove crystal water, added Gagteiger charges to each atom, and merged the non-polar hydrogen atoms to the protein structure. The distance between donor and acceptor atoms that form a hydrogen bond was defined as 1.9 Å with a tolerance of 0.5 Å, and the

Scheme 1 Proposed synthesis of target **3a**



acceptor–hydrogen–donor angle was not less than 120°. The structures were then saved in PDBQT file format, for input into AutoDock version 1.5.4. A grid box with dimension of $40 \times 40 \times 40$ Å^3 and was centred on 15.837, −2.391, 11.647 was created around the binding site of pyrazolo[1,5-*a*]pyrimidine on CHK1 protein using autodock tools. The centre of the box was set at pyrazolo[1,5-*a*] pyrimidine and grid energy calculations were carried out. For the Autodock docking calculation, default parameters were used and 50 docked conformations were generated for each compound. In order to verify reproducibility of the docking calculations, the bound ligand (pyrazolo[1,5-*a*] pyrimidine) was extracted from the complexes and submitted for one-ligand run calculation. This reproduced top scoring conformations of 10 falling within root-mean-square deviation (rmsd) values of 0.832 to 1.124 Å from bound X-ray conformation for CHK1, suggesting this method is valid enough to be used for docking studies of other compounds. The outputs were exported to VMD and Pymol for visual inspection of the binding modes and interactions of the compounds with amino acid residues in the active sites. Docking of different ligands to protein was performed using Autodock, the same protocol used in as that of validation study. All docking were taken into 2.5 million energy evaluations were performed for each of the test molecules. Docked ligand conformations were analysed in terms of energy, hydrogen bonding, and hydrophobic interaction between ligand and receptor protein CHK1. Detailed analyses of the ligand-receptor interactions were carried out, and final coordinates of the ligand and receptor were saved as pdb files. For display of the receptor with the ligand binding site, VMD software was used.

Results and discussion

Chemistry

Based on our previous experience with transition metal catalysis (Praveen and Perumal, 2011a; 2016a, b; Praveen et al., 2009a–d, 2010a–c, 2011a, 2012a, b, 2013; Praveen and Ananth, 2016; Jeyaveeran et al., 2016a, b; Parthasarathy et al., 2013), we initially screened a few metal catalysts for the model reaction between 1.0 mmoL of isatin and 2.0 mmoL of 4-hydroxycoumarin at 80 °C (Scheme 1, Table 1).

Table 1 Screening of Lewis acids[a]

Entry	Catalyst	mol %	Solvent	Temp (°C)	Time (h)	Yield (%)[b]
1	$InCl_3$	10	CH_3CN	80	12	25
2	$InCl_3$	10	DMSO	80	12	36
3	$In(OTf)_3$	10	DMSO	80	12	32
4	$NiBr_2$	10	CH_3CN	80	12	15
5	$NiBr_2$	10	DMSO	80	12	18
6	AgOTf	10	DMSO	80	12	28
7	$Cu(OTf)_2$	10	DMSO	80	12	39
8	$Zn(OTf)_2$	10	DMSO	80	12	57
9	$Zn(OTf)_2$	5	DMSO	80	12	59
10	$Zn(OTf)_2$	5	DMSO	25	48	10
11	$Zn(OTf)_2$	5	DMSO	150	12	79
12	**$Zn(OTf)_2$**	**5**	**DMSO**	**100**	**0.5**	**92**[c]

The bold values are used to highlight the condition which gives maximum yield of product

[a] All reactions were performed using 1.0 mmoL of isatin and 2.0 mmoL of 4-hydroxycoumarin

[b] Isolated yield after filtration

[c] The reaction was performed under microwave irradiation with a power level of 200 W

In this effort, we found that the use of indium(III) salts afforded poor yields of product **3a** in both DMSO and acetonitrile (entries 1–3). Similarly, poor results were observed, when $NiBr_2$ in acetonitrile and DMSO was employed (entries 4 and 5). Among the coinage metal catalysts, $Cu(OTf)_2$ gave slightly better yield over AgOTf (entries 7 and 6 respectively) and $Zn(OTf)_2$ gave a much improved yield of 57 % (entry 8). Reducing the catalytic amount of $Zn(OTf)_2$ from 10 to 5 mol% resulted in no appreciable change in yield (entry 9) and thus evaluated for subsequent screening. A drastically reduced yield was observed, when the reaction was performed at room temperature (entry 10). Conversely, by increasing the temperature to 150 °C, the yield was significantly improved to 79 % (entry 11). In order to reduce the reaction time, we investigated the use of microwave irradiation instead of conventional heating. To this end, microwave heating of the model reaction at 100 °C at a power level of 200 W for 30 min afforded an excellent yield of the product **3a** (entry 12). Consequently, this reaction condition was applied to a range of substituted isatins with 4-hydroxycoumarin (Scheme 2,

Scheme 2 $Zn(OTf)_2$ catalysed microwave synthesis of spirooxindoles **3a**-**3o**

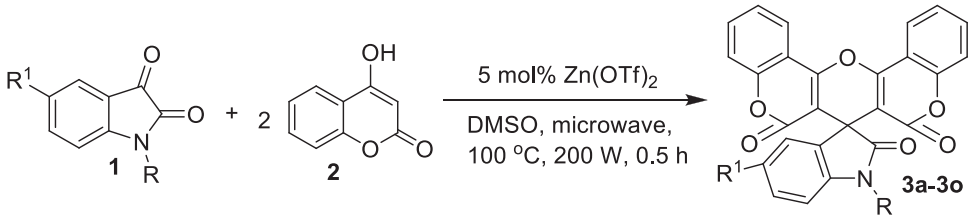


Table 2 Synthesis of targeted spirooxindoles **3a**-**3o**

Entry	R	R^1	Product[a]	Yield (%)[b]
1	H	H	**3a**	92
2	Methyl	H	**3b**	95
3	Ethyl	H	**3c**	95
4	Propyl	H	**3d**	94
5	Butyl	H	**3e**	95
6	Pentyl	H	**3f**	91
7	Hexyl	H	**3g**	87
8	Allyl	H	**3h**	89
9	Propargyl	H	**3i**	91
10	Benzyl	H	**3j**	94
11	2-Flurorobenzyl	H	**3k**	92
12	H	Cl	**3l**	89
13	H	Br	**3m**	88
14	H	NO_2	**3n**	86
15	Methyl	F	**3o**	89

[a] All products were characterized by IR, NMR and mass

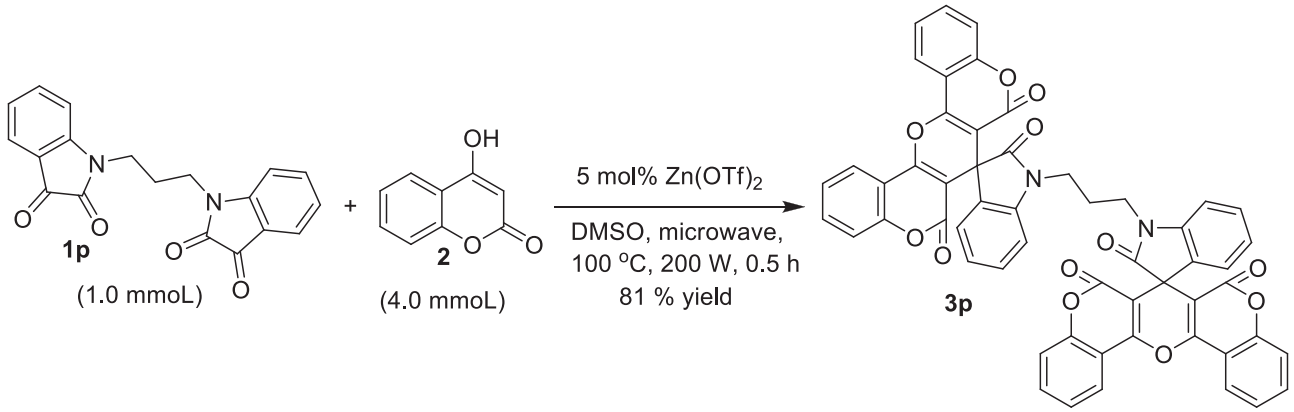
[b] Isolated yield after filtration

Table 2). The reaction appears to be general and works well regardless of the electronic nature of the peripheral substituents (R and R^1) on the isatin moiety and offered excellent yield of products **3a**-**3o** (Table 2). The utility of this chemistry was further manifested by its applicability to afford product with propargyl tether **3i** without observing any allene-isomerization (Nandi and Kundu, 2001; Fortes and Garrote, 1997; Noguchi et al., 1996; Abbiati et al., 2005). In order to extend the scope of this methodology, we subjected 1.0 mmoL of bis-isatin **1p** with 4.0 mmoL of 4-hydroxycoumarin under our optimized reaction condition. The reaction worked well and afforded the corresponding bis-spiro skeleton **3p** in 81 % yield (Scheme 3).

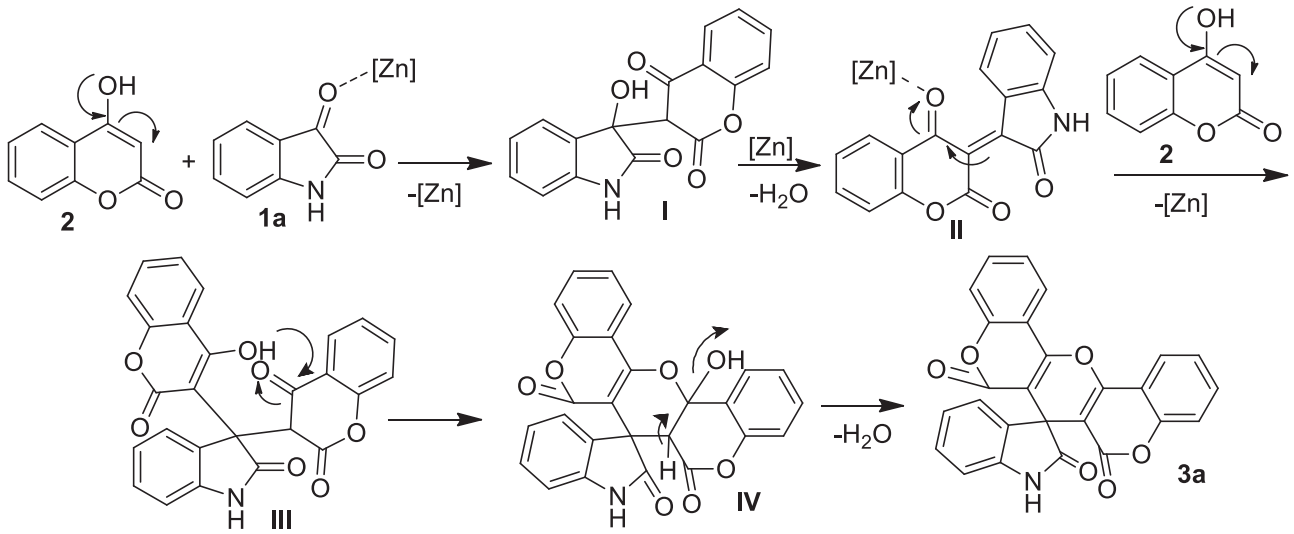
The structure of all products was confirmed by spectral data (FTIR, ^{1}H NMR, ^{13}C NMR and MS) and elemental analyses. Representatively, the IR spectrum of compound **3a** showed a broad peak at 3411 cm^{-1} and a sharp peak at 1708 cm^{-1} corresponds to the –NH and carbonyl functionalities of the oxindole core respectively. Sharp stretching bands at 1732 and 1662 cm^{-1} suggested the presence of carbonyl group characteristic of the δ-lactone ring. The ^{1}H NMR spectrum of **3a** recorded in DMSO-d_6 showed 13 protons. Out of which, 12 protons resonated in the aryl region from δ_H 6.84–8.48 ppm and a broad singlet at δ_H 10.85 ppm corresponds to -NH proton (D_2O exchangeable) of the oxindole moiety. A less intense peak at δ_C 46.5 ppm indicates the presence of a quaternary carbon in the aliphatic region (spiro carbon). Two peaks at δ_C 157.9 and 176.4 ppm corresponds to the carbonyl carbon of the coumarin and oxindole, respectively. This observation was supported by DEPT-135 and 2D chemical shift correlation experiments (see supplementary Data). Finally, HRMS data showed a peak at $m/z_{(obs\cdot)} = 458.0641$ corresponds to $[M+Na]^+$, where M is the molecular mass of **3a** is in well agreement with the expected $m/z_{(expd.)} = 458.0635$. All these experiments and data unambiguously confirmed the structure of compound **3a**. Based on the observed results, a tentative mechanism was proposed (Scheme 4) for the formation of representative compound **3a** (Boroujeni and Ghasemi, 2013). According to which, 4-hydroxycoumarin **2** undergoes nucleophilic addition at the C3 carbon of the zinc-activated isatin to form intermediate **I**. Dehydration of intermediate **I** results in the formation of intermediate **II**. The oxophilic zinc again enters the catalytic circle by interacting with the carbonyl oxygen of **II** and drives the addition of another molecule of 4-hydroxycoumarin **2** leading to **III**. The later upon intramolecular cyclization results in intermediate **IV** and subsequent dehydration affords the product **3a**.

Antimicrobial activity

In the present study, all compounds were tested for their antibacterial activity against nine bacterial strains (*S. epidermidis*, *S. typhimurium*, *K. pneumonia*, *B. subtilis*, *S. flexneri*, *M. luteus*, *E. aerogenes*, *S. aureus* and *S. aureus* MRSA) and antifungal activity against two fungal strains (*C. albicans*, *M. pachydermatis*) using disc diffusion method (Bauer et al., 1966). Analysis of the screening results revealed that most of the compounds exhibited comparable antimicrobial activities against the reference drugs (Table 3). The most active derivatives of the target compounds against all bacterial and fungal strains were **3a**, **3j** and **3m** bearing in their structures NH, NBn and C_5-Br moieties, respectively. Compounds **3d**, **3e**, **3f** and **3h** possessing propyl, butyl, pentyl and allyl chains respectively emerged as the least active as evidenced by their insignificant zones of inhibition. Other compounds **3b**, **3c**, **3g**, **3i**,



Scheme 3 Synthesis of bis-spiro framework **3p**



Scheme 4 Plausible mechanism for the formation of compound **3a**

3k and **3l** exhibited moderate activities against the tested strains. Compound possessing the bis-spiro skeleton **3p** exhibited very poor activity against all the strains.

Minimum inhibitory concentration

The activity of the test compounds which gave positive results (**3a**-**3c**, **3g** and **3i-3m**) was assessed in terms of minimum inhibitory concentration (MIC) by standard reference methods (Duraipandiyan and Ignacimuthi, 2009; Raj et al., 2013). MIC is defined as the maximum dilution of the test compound that inhibits the growth of the microorganism. The selected compounds were dissolved in DMSO to prepare a series of descending concentration (1000 µM, 500 µM, 250 µM, 125 µM, 62.5 µM, 31.25 µM, 15.62 µM and 7.81 µM). These solutions were then diluted to give serial two-fold dilutions and were added to each medium in 96 well plates. An inoculum of 100 µL from each well was inoculated. The references, ketoconazole for fungi and streptomycin for bacteria, were included in the assays as positive controls. For fungi, the plates were incubated for 48-72 h at 28 °C and for bacteria the plates were incubated for 24 h at 37 °C. The MIC for fungi was defined as the lowest concentration showing no visible fungal growth after the incubation time. Five microlitres of tested broth was placed on the sterile MHA plates for bacteria and incubated at respective temperature. The MIC for bacteria was determined as the lowest concentration of the compound inhibiting the visual growth of the test cultures on the agar plate. The MIC values of active compounds against bacteria and fungi are given in Table 4, which revealed that compound **3a** devoid of any substitution showed activity against *E. aerogenes* (62.5 µM) and *S. aureus* MRSA (62.5 µM). Compound **3j** possessing N-benzyl substituent showed activity against *S. epidermidis* (62.5 µM), *S. aureus* (62.5 µM) and *C. albicans* (62.5 µM).

Table 3 Zone of inhibition (mm) of compounds **3a**-**3o** (1 mg/disc)

Organism	3a	3b	3c	3d	3e	3f	3g	3h	3i	3j	3k	3l	3m	3n	3o	control
Bacteria																
S. epidermidis	**21**	8	8	2	4	3	11	3	8	**22**	9	10	**21**	7	10	25
S. typhimurium	**16**	9	2	1	2	5	8	1	10	**15**	8	8	**14**	8	9	18
K. pneumoniae	**18**	10	8	3	3	2	9	5	8	**17**	11	10	**16**	2	10	20
B. subtilis	**22**	11	8	2	3	6	10	2	8	**23**	9	9	**21**	1	9	25
S. flexneri	**25**	8	8	1	1	1	8	2	9	**26**	9	8	**25**	3	6	30
M. luteus	**21**	9	8	1	2	1	10	2	8	**22**	8	9	**20**	1	2	26
E. aerogenes	**18**	10	9	3	2	5	11	3	8	**20**	9	10	**19**	1	11	22
S. aureus	**11**	12	9	2	2	4	11	4	10	**13**	13	9	**14**	2	4	14
S.aureus MRSA	**26**	12	9	4	3	2	10	6	8	**25**	10	9	**28**	2	**7**	30
Fungi																
C. albicans	**24**	10	8	2	2	2	3	4	2	**23**	8	3	**26**	2	8	28
M. pachydermatis	**12**	2	2	2	3	3	2	3	2	**12**	8	8	**13**	3	4	26

The bold values are used to highlight those compounds which gives maximum zone of inhibition values

Control: Streptomycin (standard antibacterial agent) and Ketoconazole (standard antifungal agent)

Table 4 MIC of selected compounds

Organism	3a	3b	3c	3g	3i	3j	3k	3l	3m	Control
Bacteria										
S. epidermidis	500	500	500	250	500	**62.5**	500	500	**62.5**	25
S. typhimurium	500	500	-	500	500	125	500	500	500	30
K. pneumoniae	500	500	500	500	500	250	250	500	250	6.25
B. subtilis	250	250	500	500	500	125	500	500	250	25
S. flexneri	250	500	500	500	500	125	500	500	250	6.25
M. luteus	250	500	500	250	500	125	500	500	125	6.25
E. aerogenes	**62.5**	250	500	250	500	125	500	250	250	25
S. aureus	500	250	500	250	250	**62.5**	125	500	**62.5**	6.25
S. aureus MRSA	**62.5**	250	500	250	500	250	250	500	125	6.25
Fungi										
C. albicans	500	250	500	250	250	**62.5**	500	500	500	25

The bold values are used to highlight the minimum MIC values

Control: Streptomycin (standard antibacterial agent) and Ketoconazole (standard antifungal agent)

Compound **3m** possessing bromo substituent at the C5-carbon of the oxindole core exhibited activity against *S. epidermidis* (62.5 µM) and *S. aureus* (62.5 µM).

Cytotoxicity

In continuation of our ongoing project on bio-active heterocycles (Praveen et al., 2010c, 2011a–c, 2012a, b, 2013; Parthasarathy et al., 2013), an attempt has been made to evaluate the cytotoxicity of all compounds (**3a**-**3o**) against colorectal adenocarcinoma cell lines (COLO320) by MTT assay (Mossman, 1983). The cytotoxic results were compared with reference drug cyclophosphamide which showed 90 % inhibition at a concentration 150 µM. The tested compounds exhibited maximum cytotoxicity against COLO320 cells at a concentration of 200 to 50 µM. All concentrations used in the experiment could decrease the cell viability significantly ($P<0.05$) in a concentration-dependent manner. Cytotoxicity of each sample was expressed as IC_{50} value. The IC_{50} value is the concentration of test sample that causes 50 % inhibition of cell growth averaged from three replicate experiments (Table 5). Attempts to understand the SAR of the spirooxindoles started with the substituent at the C5 position, which revealed that replacement of C5-Cl (**3l**, $IC_{50} > 100$ µM) by a C5-Br (**3m**, $IC_{50} = 52.7$ µM) did increase the potency to a great extent. Similar trend was observed, when C5-Br (**3m**, $IC_{50} = 52.7$ µM) was replaced by C5-NO_2 (**3n**, $IC_{50} = 50.7$ µM) and resulted in slight escalation of activity. These observations could be attributed to the increase in size and electronegativity ($Cl < Br < NO_2$) of the respective substituent. It is apparent fro.m the data presented in Table 5 that with every increase in the number of $–CH_2$ spacer, the inhibitory potency also increased. This drift was observed in

Table 5 IC_{50} and FEB values of compounds **3a-o**

Entry	Compounds	IC_{50} (μM)	FEB (kcal/moL)[a]
1	**3a**	57.2	−8.24
2	**3b**	54.5	−8.44
3	**3c**	53.9	−8.34
4	**3d**	51.1	−8.63
5	**3e**	51.8	−8.87
6	**3f**	>100	−7.58
7	**3g**	>200	−6.71
8	**3h**	54.2	−8.55
9	**3i**	>100	−7.74
10	**3j**	53.1	−8.31
11	**3k**	51.8	−8.44
12	**3l**	>100	−7.73
13	**3m**	52.7	−8.77
14	**3n**	50.7	−8.89
15	**3o**	55.3	−8.33
16	Standard[b,c]	–	−9.53

[a] FEB Free Energy of Binding

[b] Cyclophosphamide was used as the standard for cytotoxic studies, which showed 90 % inhibition (156 µM).

[c] Pyrazolo[1,5-*a*]pyrimidine was used as the standard for docking

analogues containing NH (**3a**, $IC_{50} = 57.2$ µM), NMe (**3b**, $IC_{50} = 54.5$ µM), NEt (**3c**, $IC_{50} = 53.9$ µM) and NPr (**3d**, $IC_{50} = 51.1$ µM), which showed the activity increased in the order NPr > NEt > NMe > NH. Conversely, a completely opposite trend was observed when the carbon chain goes beyond the three methylene spacer **3d**. For example, a slightly reduced potency was observed for the NBu analogue (**3e**, $IC_{50} = 51.8$ µM) when compared to the NPr analogue (**3d**, $IC_{50} = 51.1$ µM). Higher congeners such as the N-pentyl (**3f**, $IC_{50} > 100$ µM), and N-hexyl (**3g**, $IC_{50} > 200$ µM) exhibited a drastically decreased cytotoxicity. Among the unsaturated systems, compound with a propargyl group (**3i**, $IC_{50} > 100$ µM) demonstrated significant cytotoxicity against its allyl counterpart (**3h**, $IC_{50} > 100$ µM). Assessment of the aryl groups revealed that compound with 2-fluorobenzyl substituent (**3k**, $IC_{50} = 51.8$ µM) exhibited an elevated cytotoxicity than those containing the benzyl (**3j**, $IC_{50} = 53.1$ µM). This enhanced activity is mainly attributed to the differences in size and electronegativity offered by the fluorine atom. However, when the fluorine atom was located on the C5-position (**3o**, $IC_{50} = 55.3$ µM), it renders the molecule less active than its unsubstituted counterpart (**3b**, $IC_{50} = 54.5$ µM). Analysis of the combined results as depicted in Table 5 indicates that compound with a -NO_2 group (**3n**, $IC_{50} = 50.7$ µM) positioned at the C5 of the oxindole core emerged as the most active. Compound with N-propyl substituent (**3d**, $IC_{50} = 51.1$ µM) was found to be almost active as **3n** and appeared as the second most active compound. Compounds possessing butyl (**3e**, $IC_{50} = 51.8$ µM) and 2-fluorobenzyl groups (**3k**, $IC_{50} = 51.8$ µM) respectively showed same but significant inhibitory potencies. Compounds **3f**, **3g**, **3i** and **3l** emerged as the least active among the tested series as evident from their poor cytotoxocity (IC_{50} values of >100 µM).

Molecular docking

The precise prediction of ligand-protein complexes is of fundamental importance in modern structure-based drug design (Taylor et al., 2002). The ligand matching and the corresponding docking score could confirm the binding efficiency of each molecule at the binding site. To this end, selected ligands were docked into AmpC-β-lactamase and CHK receptors to evaluate the antimicrobial and cytotoxic activities, respectively. Docking of ligands to the receptor was assessed using Moldock scoring function for antimicrobial and AUTODOCK for cytotoxicity and all the results were discussed in the following sections.

Binding towards AmpC-β-lactamase

The level of antimicrobial activity was studied computationally by MVD by automated docking of the most active compounds **3a**, **3j** and **3m** to the binding site of AmpC-β-lactamase. The MolDock scoring function used by MVD is derived from the PLP scoring functions. The MolDock further improves these scoring functions with new hydrogen bonding term and new charge schemes. The ligand matching and the corresponding scoring could confirm the binding efficiency of each molecule at the binding site which are evaluated and ranked according to Moldock scoring function (Vanderpool et al., 2009). Experimentally, X-ray crystal structures of AmpC-β-lactamase from *E. coli* were retrieved from PDB database-1KE4 (Powers and Shoichet, 2002). Computationally, MVD was used to predict the potential binding site on AmpC-β-lactamase. Based on the cavities detected, the ligands **3a**, **3j** and **3m** were docked with the active site of amino acids (Figs. 2, 3 and 4). The active site consisted of amino acid residue that bound to the corresponding amino acids such as ASN346, THR316, ALA318 and SER64. However, ASN346 was found to be conserved in the active site. The binding energy of ligand **3a** with protein active site was found to be −117.819 kcal mol^{-1}. The best poses for molecule **3j** shows two interactions with lowest energy of −113.067 kcal mol^{-1} which binds to active site amino acids ASN346 and ARG148 respectively. The best poses for molecule **3m** shows three interactions with lowest energy of −107.056 kcal mol^{-1} which binds to active site amino acids ASN346, GLU272 and ARG148 respectively. The docking study revealed that compound **3a** exhibited the minimum binding energy which indicates its

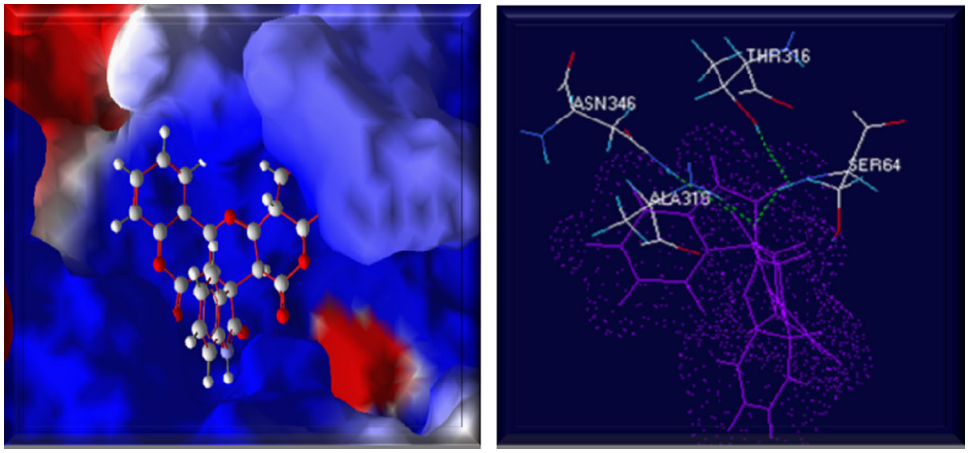


Fig. 2 Putative binding pose of compound **3a** in AmpC-*β*-lactamase (*left* panel). Docking of compound **3a** in AmpC-*β*-lactamase, where hydrogen bonding interactions are shown in dotted lines (*right* panel)

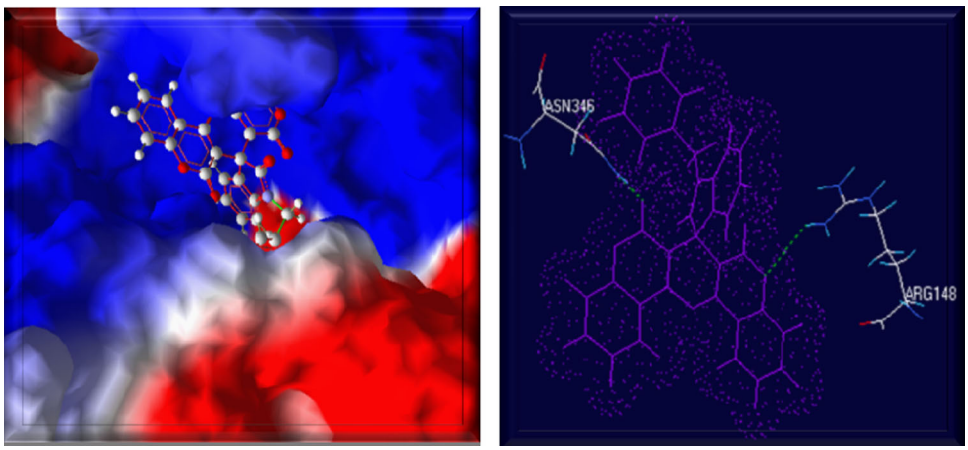


Fig. 3 Putative binding pose of compound **3j** in AmpC-*β*-lactamase (*left* panel). Docking of compound **3j** in AmpC-*β*-lactamase, where hydrogen bonding interactions are shown in dotted lines (*right* panel)

strong affinity with the protein and has been stabilized by strong hydrogen bond interaction in the binding pocket.

Binding towards CHK1

CHK1 is a protein kinase from the CAMKL kinase family, is a key element in the DNA damage response pathway and plays a crucial role in the S-G2-phase checkpoint (Labroli et al., 2011). Each phase of the cell cycle is regulated by several checkpoint mechanisms, such as DNA damage or spindle assembly checkpoints. First-line therapies for cancer include radiation and chemotherapy and are designed to induce cell death by damaging the DNA material of cancerous cells. Because cancer cells utilize the checkpoints to facilitate repair of their DNA, the inhibition of CHK1 represents an attractive strategy for sensitizing cancer cells to cytotoxic chemotherapeutics. To this end, all compounds were docked into the relative binding site of CHK1 crystal structures and the optimal conformations of these compounds were determined. To investigate the potential binding mode of inhibitors, all the compounds were subjected to molecular docking using the AutoDock 1.5.4 program. As a reference to other compounds, pyrazolo[1,5-*a*]pyrimidine was used as a model drug to show the binding modes of CHK1 inhibitors. From the docking scores, the free energy of binding (FEB) of all compounds were calculated (Table 5). The overlaid pose of bound and docked pyrazolo[1,5-*a*]pyrimidine ligand in the active site of CHK1 is shown in Figure 5. The reader will be reminded that the binding interactions of all the docked compounds (**3a-3o**) were shown in supplementary Fig. 6–21 (see supplementary Material). The docking results revealed that compound **3n** as the most active with a calculated binding energy of −8.89 kcal/mol. The least binding energy was exhibited by compound **3g** with a binding energy of –6.71 kcal/mol. To elucidate the interaction mechanism, the most potent inhibitors among the data set, were selected for more detailed analysis. The ligand-enzyme interaction analysis shows that Leu137, Glu91, Cys87, Asp148, Ser88, Gly90 and Tyr86 are the important residues present in the active site. As shown in supplementary Fig. 6, pyrazolo[1,5-*a*]pyrimidine binds to the kinase through key H-bond interactions: (i) Between the -NH of cyclohexyl ring and the -COO of Glu91 (–O···HN, 1.90 Å, 160.9°); (ii) Between the backbone carbonyl oxygen of Cys87 and the amino of

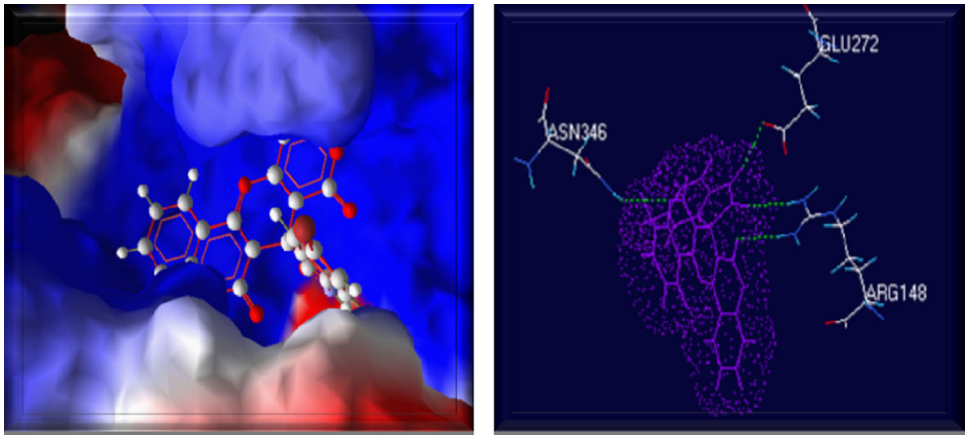


Fig. 4 Putative binding pose of compound **3m** in AmpC-β-lactamase (*left* panel). Docking of compound **3m** in AmpC-β-lactamase, where hydrogen bonding interactions are shown in dotted lines (*right* panel)

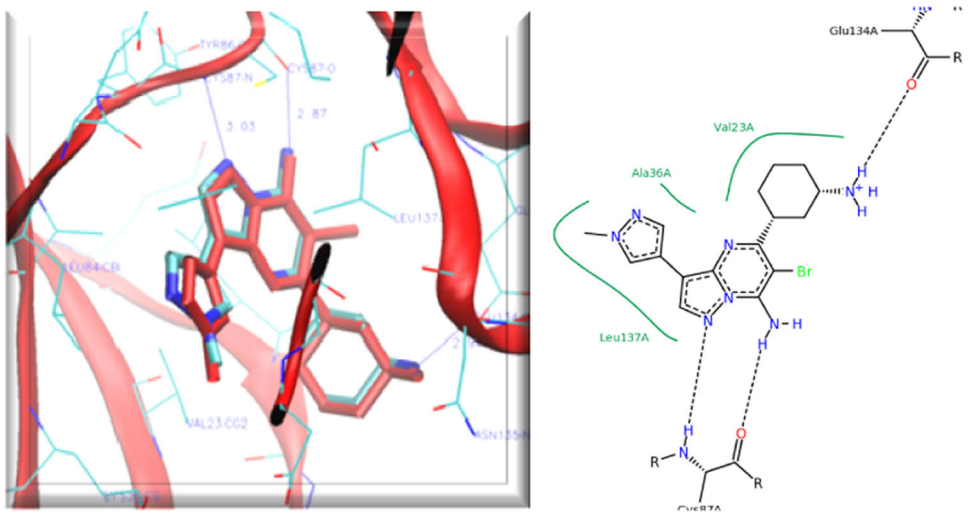


Fig. 5 Overlaid pose of bound and docked co-crystal

pyrimidine ring (–O···HN, 2.46 Å, 129.8°; -O···HN, 3.47 Å, 150.2°); (iii) Between the nitrogen atom of pyrazole ring and the -NH group of Cys87 residue (–N···HN, 2.37 Å, 157.6°); (iv) The interaction between the nitrogen atom of another pyrazole ring and the side chain of Asp148 is bridged by a structural molecule W502; (v) Pyrazolo[1,5-*a*] pyrimidine ring all form arene-cation interaction with Leu137 that further enhance the binding activity. The docking results were well consistent with the in vitro CHK1 inhibitory biological activity (IC_{50}). The binding mode of compounds **3a**-**3o** is shown in supplementary Figs. 6–21. The specific cleft in which the compounds bind in the active site contains polar residues (Glu85, Tyr86, Cys87, Ser88, Gly89, Gly90, Glu91, Ser147 and Asp148) and non-polar residues (Ala36, Leu84 and Val23). The compounds **3a**-**3e**, **3h** and **3m-3o** are well placed inside the active site and demonstrate the following interactions. (i) The oxygen atom of spirooxindole (supplementary Figs. 6–21) forms H-bond with –NH group of Cys87 (-O…HN, 2.15 Å). (ii) The –NH group of indole ring forms H-bond with the backbone –CO of Glu85 (-O…HN, 1.95 Å). It is remarkable to note that the large spiro-moiety has sufficient room to get introduced into the hydrophobic pocket composed by Ala36, Leu84 and Val23. The docking score was improved, when the R substituents were H, methyl, ethyl, propyl and butyl (**3a**-**3e**). Interestingly, the CHK1 inhibitory activity also improved in the same way. The similar binding mode and docking score as well as CHK1 inhibitory activity was also observed in compounds **3h** and **3m**-**3o**. The pentyl (**3f**) and hexyl (**3g**) groups were not able to accommodate into the active site; hence the docking score and CHK1 inhibitory activity also reduced. Ligands with bulky substituents like benzyl (**3j**) and 2-fluorobenzyl (**3k**) showed different binding mode on the same active site. The oxygen atom of indole ring (supplementary Figs. 15 and 16) forms H-bond with –NH group of Glu84 (-O…HN, 2.76 Å). The –NH group of indole ring forms H-bond with the backbone –CO of Glu85 (-O...HN, 2.15 Å). Based on the above observations, we conclude that the network of H-bonds formed between CHK1 and the compounds are crucial for molecular recognition.

Conclusion

In conclusion, we have disclosed $Zn(OTf)_2$ catalysed efficient synthesis of spirooxindole[pyrano-bis-2*H*-l-benzopyrans] via tandem double condensation reaction between 4-hydroxycoumarin and isatin. The beneficial advantages of this methodology are simple reaction conditions, no chromatographic techniques and excellent chemical yields. Antimicrobial evaluation of all compounds against a wide panel of antimicrobial strains revealed that three compounds (**3a**, **3j** and **3m**) showed comparable inhibitory ability against the reference drugs. Molecular docking studies of these compounds with AmpC-β-lactamase receptor revealed that compound **3a** which exhibited minimum FEB (−117.819 kcal/moL) indicates its strong affinity towards the targeted protein and has been stabilized by strong hydrogen bond interaction in the binding pocket. All compounds were also screened for their in vitro cytotoxicity against COLO320 cancer cells and computationally studied for their binding affinity towards CHKl receptor. Our overall results from biological assay and molecular in silico studies demonstrated that **3n** is the most active compound in terms of its low IC_{50} value (50.7 μM) and least FEB (−8.89 kcal/mol). We are currently engaged in understanding the effect of compounds on the host cell and their mechanism of action and will be reported in the due course.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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