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REGULAR ARTICLE

Design synthesis and anti-proliferative activity of some new coumarin substituted hydrazide–hydrazone derivatives

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Abstract. A series of 21 coumarin hydrazide–hydrazone derivatives were designed, synthesized and evaluated potential cytotoxicity effects at 25 μg/mL for 48 h against liver cancer (HepG2) cell line *in vitro*. Then, seven out of 21 compounds with % cell viability lower than 60% were selected for evaluation of *in vitro* anti-proliferative activity against liver cancer (HepG2), breast cancer (SKBR-3) and human colon cancer (Caco-2) cell lines. Among the test compounds, **5g**, **6d** and **6f** showed potent activities against both Hep-G2 and SKBR-3 cell lines. More significantly, compound **6d**, having a 4-bromophenyl moiety, exhibited best cytotoxic activity against Hep-G2 cell line with IC_{50} value of 2.84 ± 0.48 μg/mL which is comparable to the standard doxorubicin (IC_{50} = 2.11 ± 0.13 μg/mL). In addition, compound **6f**, having 4-methoxyphenyl moiety, demonstrated the most potent activity (IC_{50} = 2.34 ± 0.68 μg/mL) against SKBR-3 cell line on comparison with other tested coumarin hydrazide–hydrazone derivatives. Unfortunately, all test compounds, as well as doxorubicin, showed no cytotoxicity toward drug-resistant cell line, Caco-2. Our preliminary results indicated that coumarin hydrazide–hydrazone derivatives could be exploited as leading structures for further anticancer-drug development.

Keywords. Anti-proliferative activity; Cancer; Coumarin; Hydrazide–hydrazone; Molecular hybridization.

1. Introduction

Cancer is one of the leading causes of morbidity and mortality in every world region. There were an estimated 18.1 million new cancer cases and 9.6 million cancer deaths in 2018 worldwide.[1] To decrease the mortality rate of cancer, the development of safe and selective anticancer drugs with minimal side effects is still highly desirable and can be achieved by the structural hybridization approach.

Drug combination therapy is most often used to treat patients because a synergistic or additive effect leads to a lower therapeutic dosage of each drug.[2] Thus, molecular hybridization of two or more bioactive pharmacophores into a single chemical backbone has been applied widely for the design and synthesis of lead compounds against serious diseases such as bacterial infections, HIV, cancer, malaria and tuberculosis.[3–5]

Coumarin and its derivatives are widely distributed throughout nature and have attracted much attention due to their numerous biological activities including anticoagulant,[6] anticancer,[7,8] antifungal,[9] anti-HIV,[10] antimicrobial,[11] anti-osteoporosis,[12] antioxidant[13] and anti-inflammatory[14] activities. Similarly, hydrazide–hydrazone derivatives exist as structural subunits in many pharmacologically active compounds and possess a wide spectrum of biological activities, such as antimicrobial,[15] anticancer,[16] anticonvulsant,[17] antifungal,[18] antiviral,[19] anti-tubercular,[20] anti-inflammatory,[21] and antiprotozoal[22] activities.

Recently, the hybrid compounds containing coumarin and aryl hydrazide–hydrazone pharmacophoric

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units have been synthesized and evaluated their cytotoxic activity. The combination of these biologically active moieties has resulted in several novel compounds with improved anticancer activity.[23,24]

Prompted by these findings and in continuation of our interest in the synthesis of coumarin derivatives as well as our recent interest in the discovery and development of novel anticancer agents,[25,26] we have combined 7-Hydroxy-4-methylcoumarin (**1**) with different aryl hydrazide–hydrazones and investigated their cytotoxic activity against human hepatic carcinoma (HepG2), breast carcinoma (SKBR-3), and colorectal adenocarcinoma (Caco-2) cell lines *in vitro*.

2. Experimental

2.1 *Materials and physical measurements*

All chemicals were purchased from commercial sources and used without further purification. The coumarin precursor **1** was prepared *via* Pechmann reaction.[25] Column chromatography was performed on silica gel (Kieselgel 60, 70–230 mesh, Merck) in common glass columns. Melting points (°C) were determined with the Gallenkamp melting point apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker spectrometer. Chemical shifts (δ) are reported in ppm and relative to TMS or the residual undeuterated solvent as the internal standard and coupling constants (J) in Hertz. Splitting patterns are designated as follows: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, sext = sextet, m = multiplet, dd = double doublet, br.s = broad singlet. ESI mass spectra were performed with a Thermo Finnigan LCQ Advantage Mass Spectrometer. High-Resolution Mass Spectrometry was measured with a MicroTOFLC, Bruker Daltonics. Infrared spectra were recorded on a Perkin Elmer FT-IR Spectrum GX.

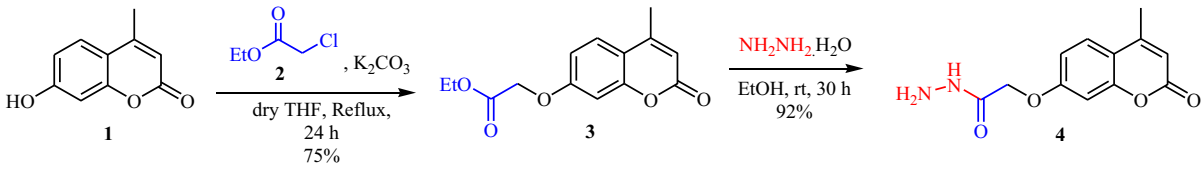
2.2 *Chemistry*

2.2.1 *Synthesis of ethyl [(4-methyl-2-oxo-2H-chromen-7-yl)oxy]acetate (**3**) (Scheme 1)*: To a solution of 7-Hydroxy-4-methylcoumarin (**1**, 10 mmol) in dry THF (25 mL), anhydrous potassium carbonate (20 mmol) and ethyl chloroacetate (20 mmol) were added under N_2 atmosphere. The mixture was stirred under reflux for 24 h, cooled and then the precipitated solid was filtered and washed with cold acetone. The filtrate was concentrated under reduced pressure and purified by crystallization from ethanol to obtain light yellow solid of **3**. Yield 75%, R_f 0.60 (50% EtOAc/hexanes), M.p. 116–117 °C (from ethanol) [Ref[27] 112–114 °C (from ethanol)]. IR (KBr): ν_{max} 3010, 2950, 1732, 1710, 1614, 1569, 1472, 1215, 1076 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.70 (d, J = 9.5 Hz, 1H), 7.04–6.95 (m, 2H), 6.24 (d, J = 0.9 Hz, 1H), 4.93 (s, 2H), 4.18 (q, J = 7.1 Hz, 2H), 2.40 (d, J = 0.7 Hz, 3H), 1.22 (t, J = 7.1 Hz, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.17 (C), 160.57 (C), 160.03 (C), 154.51 (C), 153.32 (C), 126.53 (CH), 113.66 (C), 112.30 (CH), 111.47 (CH), 101.55 (CH), 64.93 (CH_2), 60.79 (CH_2), 18.10 (CH_3), 14.01 (CH_3). MS (ESI$^+$), *m/z* (% rel. intensity) 263.6 (M + H$^+$, 100).

2.2.2 *Synthesis of 2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy]acetohydrazide (**4**) (Scheme 1)*: To a solution of Ethyl [(4-methyl-2-oxo-2H-chromen-7-yl)oxy]acetate (**3**, 1 mmol) in ethanol (6 mL), hydrazine hydrate (1 mmol) was added slowly. The mixture was stirred at room temperature for 30 h and the solid was filtered and washed with cold ethanol. The solid was recrystallized from chloroform/methanol and gave white solid of **4**. Yield 92%, R_f 0.60 (20% MeOH/EtOAc), M.p. 216–218 °C (from methanol/chloroform) [Ref[28] 212 °C (from methanol)]. IR (KBr): ν_{max} 3310, 3250, 3079, 2949, 1744, 1670, 1607, 1501, 1398, 1151, 1076 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 9.43 (s, 1H), 7.71 (d, J = 8.8 Hz, 1H), 7.06–6.88 (m, 2H), 6.23 (s, 1H), 4.62 (s, 2H), 4.37 (s, 2H), 2.40 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 166.03 (C), 160.76 (C), 160.06 (C), 154.49 (C), 153.38 (C), 126.47 (CH), 113.56 (C), 112.51 (CH), 111.40 (CH), 101.56 (CH), 66.50 (CH_2), 18.13 (CH_3). MS (ESI$^+$), *m/z* (% rel. intensity) 249.7 (M + H$^+$, 100).

2.2.3 *General procedure for synthesis of 2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy]-N'-(substituted methylene)acetohydrazide **5**(**a**–**n**) (Table 1)*: A mixture of 2-[(4-Methyl-2-oxo-2*H*-chromen-7-yl)oxy]acetohydrazide (**4**, 0.5 mmol) and the appropriate aromatic aldehyde (0.5 mmol) in 1:1 methanol/chloroform (15 mL) and acetic acid (0.05 mL) was stirred at reflux for 5 h. The mixture was cooled and then the precipitated solid was filtered and recrystallized from methanol to obtain a white solid of **5a**–**n**.

2.2.3a *2-[(4-Methyl-2-oxo-2H-chromen-7-yl)oxy]-N′-(benzylidene)acetohydrazide (**5a**)*: Yield 98%, R_f 0.83 (20% MeOH/EtOAc), M.p. 273–275 °C (from methanol) [Ref[29, 30] 276–278 °C and 268–269 °C (from methanol)]. IR (KBr): ν_{max} 3450, 2905, 1720, 1635, 1520, 1490, 1225, 870 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.67 (s, 1H, N*H*), 11.65 (s, 1H, N*H*)8.35 (s,1H, C*H*=N), 8.03 (s, 1H, C*H*=N), 7.75–7.62 (m, 6H), 7.46–7.44 (m, 6H), 7.08–6.98 (m, 4H), 6.24 (m, 2H), 5.31 (s, 2H, OCH_2) and 4.82 (s, 2H, OCH_2); resolved signals for 67:33 mixture of E_{syn}:E_{anti} conformers, 2.41 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6): δ 168.96, 164.18, 161.80, 161.15, 160.70, 160.61, 155.00, 154.96, 153.99,153.94, 148.64, 144.63, 134.42, 134.35, 130.78, 130.52, 129.33, 129.28, 127.65, 127.47, 127.08, 126.89, 114.20, 113.83, 112.90, 111.95, 111.65, 102.14, 101.98, 67.02 (CH_2), 65.69 (CH_2), 18.59 (CH_3). MS (ESI$^+$) *m/z* (% rel. intensity) 337.4 (M + H$^+$, 100).



Scheme 1. Synthesis of hydrazine **4**.

2.2.3b *2-[(4-methyl2–oxo-2H-chromen7–yl)oxy]-N′-(2-fluorobenzylidene)acetohydrazide* (***5b***):[31] Yield 97%, R_f 0.85 (70% EtOAc/hexane), M.p. 250–251 °C (from methanol). IR (ATR): ν_{max} 3298, 3086, 1689, 1677, 1549, 1154, 1085, 766 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.77 (s, 2H, N*H*), 8.59 (s, 1H, C*H*=N), 8.24 (s, 1H, C*H*=N), 8.04–7.86 (m, 2H), 7.77–7.65 (m, 2H), 7.55–7.44 (m, 2H), 7.36–7.24 (m, 4H), 7.11–6.96 (m, 4H), 6.24 (s, 1H), 6.22 (s, 1H), 5.31 (s, 2H, OCH_2) and 4.82 (s, 2H, OCH_2); resolved signals for 68:32 mixture of E_{syn}:E_{anti} conformers, 2.40 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.03, 165.20, 164.23, 162.80, 161.78, 161.10, 160.59, 160.52, 159.65, 159.49, 155.03, 154.99, 153.85, 153.81, 141.39, 141.34, 137.33, 137.27, 132.73, 132.61, 132.45, 132.34, 127.05, 126.85, 125.36, 125.32, 122.03, 121.98, 121.89, 116.56, 116.28, 114.23, 113.84, 112.87, 112.00, 111.70, 102.21, 102.02, 67.12 (CH_2), 65.69 (CH_2), 18.58 (CH_3). MS (ESI) m/z (% rel. intensity) 354.4 (M + H^+, 100).

2.2.3c *2-[(4-methyl2–oxo-2H-chromen7–yl)oxy]-N′-(3–fluorobenzylidene)acetohydrazide* (***5c***):[31] Yield 70%, R_f 0.60 (70% EtOAc/hexane), M.p. 254–257 °C (from methanol). IR (ATR): ν_{max} 3080, 2967, 1709, 1682, 1609, 1394, 1265, 1135, 778 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.75 (s, 2H, N*H*), 8.33 (s, 1H, C*H*=N), 8.02 (s, 1H, C*H*=N), 7.77–7.44 (m, 8H), 7.30–7.27 (m, 2H), 7.11–6.95 (m, 4H), 6.24 (s, 1H), 6.22 (s, 1H), 5.32 (s, 2H, OCH_2) and 4.82 (s, 2H, OCH_2); resolved signals for 69:31 mixture of E_{syn}:E_{anti} conformers, 2.41 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.13, 164.50, 164.47, 164.26, 161.83, 161.27, 161.16, 160.58, 160.49, 155.04, 155.00, 153.85, 153.80, 147.18, 147.15, 143.05, 143.02, 137.06, 136.96, 131.44, 131.35, 131.24, 127.05, 126.84, 124.08, 123.97, 117.58, 117.33, 117.04, 114.21, 113.82, 113.76, 113.48, 113.18, 112.88, 111.99, 111.69, 102.16, 102.06, 67.08 (CH_2), 65.75 (CH_2), 18.59 (CH_3). MS (ESI) m/z (% rel. intensity) 354.2 (M^+, 100).

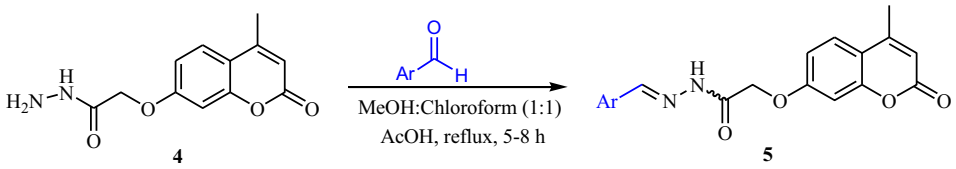
2.2.3d *2-[(4-methyl2–oxo-2H-chromen7–yl)oxy]-N′-(4–fluorobenzylidene)acetohydrazide* (***5d***): Yield 97%, R_f 0.61 (80% EtOAc/hexane), M.p. 264–282 °C (from methanol) [Ref[32] 238–240 °C (from acetic acid)]. IR (ATR): ν_{max} 3082, 2968, 1681, 1614, 1391, 1270, 1230, 1138, 1085, 834 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.65 (s, 2H, N*H*), 8.33 (s, 1H, C*H*=N), 8.02 (s, 1H, C*H*=N), 7.85–7.66 (m, 6H), 7.35–7.23 (m, 4H), 7.07–6.92 (m, 4H), 6.2 (s, 1H), 6.21 (s, 1H), 5.30 (s, 2H, OCH_2) and 4.81 (s, 2H, OCH_2); resolved signals for 65:35 mixture of E_{syn}:E_{anti} conformers, 2.41 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.91, 165.16, 164.09, 161.88, 161.81, 161.18, 160.59, 160.51, 155.03, 154.99, 153.85, 153.81, 147.44, 143.33, 131.07, 131.03, 129.88, 129.77, 129.72, 129.61, 127.04, 126.84, 116.52, 116.44, 116.23, 116.15, 114.19, 113.81, 112.87, 111.98, 111.68, 102.16, 102.01, 67.10 (CH_2), 65.70 (CH_2), 18.59 (CH_3). MS (ESI) m/z (% rel. intensity) 355.1 (M + H^+, 100).

2.2.3e *2-[(4-methyl2–oxo-2H-chromen7–yl)oxy]-N′-(3–chlorobenzylidene)acetohydrazide* (***5e***): Yield 91%, R_f 0.52 (70% EtOAc/hexane), M.p. 234–235 °C (from methanol) [Ref[33] 242–244 °C (from methanol)]. IR (ATR): ν_{max} 3094, 2973, 1709, 1682, 1615, 1390, 1273, 1135, 731 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.75 (s, 2H, N*H*), 8.31 (s, 1H, C*H*=N), 8.00 (s, 1H, C*H*=N), 7.85–7.62 (m, 6H), 7.55–7.43 (m, 4H), 7.10–6.48 (m, 4H), 6.23 (s, 1H), 6.21 (s, 1H), 5.33 (s, 2H, OCH_2) and 4.83 (s, 2H, OCH_2); resolved signals for 68:32 mixture of E_{syn}:E_{anti} conformers, 2.40 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.15, 164.35, 161.84, 161.22, 161.15, 160.61, 155.02, 153.87, 146.86, 142.88, 136.76, 136.65, 134.13, 131.22, 131.12, 130.34, 130.11, 128.16, 127.05, 126.89, 126.84, 126.60, 126.38, 126.30, 114.22, 113.84, 112.90, 112.00, 111.68, 102.15, 102.04, 67.07 (CH_2), 65.78 (CH_2), 18.61 (CH_3). MS (ESI), m/z (% rel. intensity) 370.6 (M^+, 100).

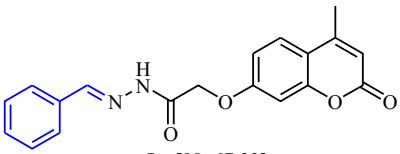
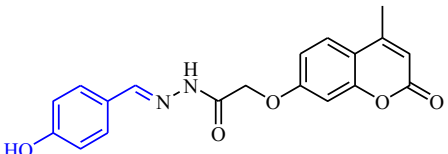
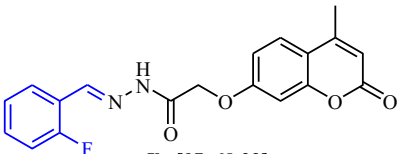
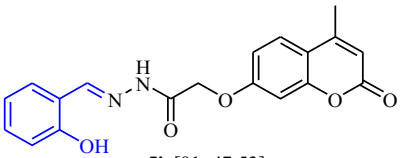
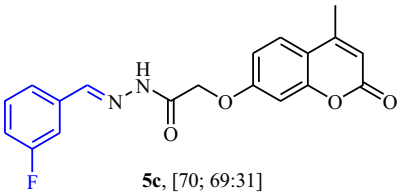
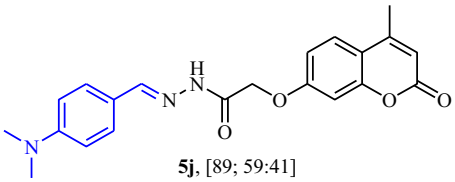
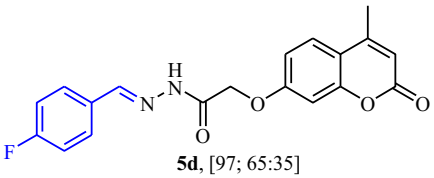
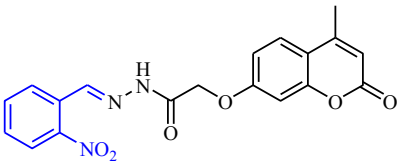
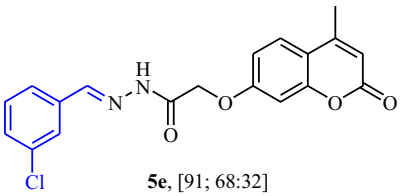
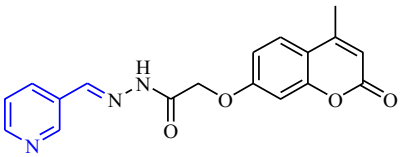
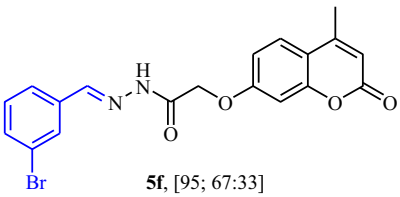
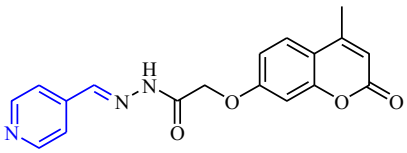
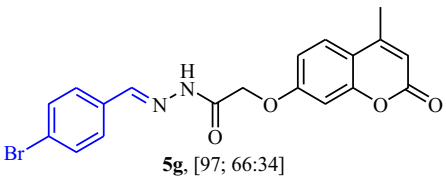
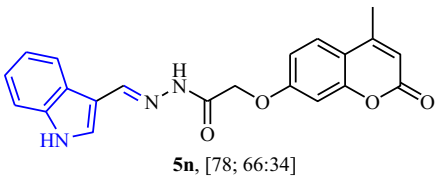
2.2.3f *2-[(4-methyl2–oxo-2H-chromen7–yl)oxy]-N′-(3–bromobenzylidene)acetohydrazide* (***5f***):[31] Yield 95%, R_f 0.50 (70% EtOAc/hexane), M.p. 236–238 °C (from methanol). IR (ATR): ν_{max} 3278, 3091, 1723, 1681, 1621, 1537, 1299, 1263, 1150, 1018, 842 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.76 (s, 2H, N*H*), 8.30 (s, 1H, C*H*=N), 7.99 (s, 1H, C*H*=N), 7.97–7.85 (m, 2H), 7.83–7.52 (m, 6H), 7.50–7.30 (m, 2H), 7.15–6.89 (m, 4H), 6.24 (s, 1H), 6.22 (s, 1H), 5.33 (s, 2H, OCH_2) and 4.83 (s, 2H, OCH_2); resolved signals for 67:33 mixture of E_{syn}:E_{anti} conformers, 2.41 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.13, 164.32, 161.84. 160.60, 155.03, 153.86, 146.77, 142.82, 138.81, 136.99, 136.88, 133.20, 132.99, 131.48, 131.37, 129.74, 129.50, 127.05, 126.83, 126.72, 122.65, 113.83, 112.89, 112.00, 111.068, 102.17, 102.06, 67.08 (CH_2), 65.79 (CH_2), 18.58 (CH_3). MS (ESI), m/z (% rel. intensity) 415.9 (M^+, 100).

2.2.3g *2-[(4-methyl2–oxo-2H-chromen7–yl)oxy]-N′-(4-bromobenzylidene)acetohydrazide* (***5g***): Yield 97%, R_f 0.51 (70% EtOAc/hexane), M.p. 266–268 °C (from methanol) [Ref[30] 273–275 °C (from methanol)]. IR (ATR): ν_{max} 3069,

Table 1. Condensation reaction of hydrazide **4** with aromatic aldehydes.[a]



4 **5**

Entry	Product [Yield,[b] (%); E_{syn}:E^{c}_{anti}]	Entry	Product [Yield,[b] (%); E_{syn}:E^{c}_{anti}]
1	 **5a**, [98; 67:33]	8	 **5h**, [82; 63:37]
2	 **5b**, [97; 68:32]	9	 **5i**, [91; 47:53]
3	 **5c**, [70; 69:31]	10	 **5j**, [89; 59:41]
4	 **5d**, [97; 65:35]	11	 **5k**, [83; 70:30]
5	 **5e**, [91; 68:32]	12	 **5l**, [80; 69:31]
6	 **5f**, [95; 67:33]	13	 **5m**, [90; 70:30]
7	 **5g**, [97; 66:34]	14	 **5n**, [78; 66:34]

[a]Reaction conditions: 0.5 mmol of **4**, 0.5 mmol of aldehyde in 15 mL of MeOH/$CHCl_3$ (1:1) and 0.05 mL of AcOH under reflux for 5–8 h.

[b]Isolated yield

[c]E_{syn}:E_{anti} was determined by the integration of the duplicate OCH_2 peaks in the ^{1}H NMR spectra.

2965, 1712, 1683, 1614, 1389, 1274, 1137, 1082, 830 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.71 (s, 2H, N*H*), 8.30 (s, 1H, C*H*=N), 8.00 (s, 1H, C*H*=N), 7.75–7.63 (m, 10H), 7.07–6.98 (m, 4H), 6.24 (s, 1H), 6.22 (s, 1H), 5.30 (s, 2H, OCH_2) and 4.82 (s, 2H, OCH_2); resolved signals for 66:34 mixture of E_{syn}:E_{anti} conformers, 2.40 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.03, 164.20, 161.81, 160.60, 155.03, 153.88, 147.34, 143.33, 133.79, 133.72, 132.23, 129.49, 129.35, 127.05, 126.85, 123.68, 113.82, 112.88, 111.97, 111.69, 102.16, 102.01, 67.08 (CH_2), 65.70 (CH_2), 18.59 (CH_3). MS (ESI), m/z (% rel. intensity) 415.9 (M^+, 100).

2.2.3h *2-[(4-methyl2–oxo-2H-chromen7–yl)oxy]-N′-(4-hydroxybenzylidene)acetohydrazide (**5h**)*: Yield 82%, R_f 0.4 (70% EtOAc/hexane), M.p. 281–284 °C (from methanol) [Ref[29] 280–282 °C (from methanol)]. IR (ATR): ν_{max} 3357, 3328, 3273, 1696, 1669, 1598, 1540, 1392, 1275, 1071 819 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.45 (s, 1H, N*H*), 11.42 (s, 1H, N*H*), 9.92 (s, 2H), 8.22 (s, 1H, C*H*=N), 7.92 (s, 1H, C*H*=N), 7.74–7.67 (m, 2H), 7.57–7.52 (m, 4H), 7.07–6.95 (m, 4H), 6.84 (s, 2H), 6.81 (s, 2H), 6.24 (s, 1H), 6.21 (s, 1H), 5.26 (s, 2H, OCH_2) and 4.78 (s, 2H, OCH_2); resolved signals for 63:37 mixture of E_{syn}:E_{anti} conformers, 2.40 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.50, 163.63, 161.86, 161.22, 160.59, 160.51, 160.01, 159.78, 155.03, 154.99, 153.84, 153.81, 148.85, 144.79, 129.40, 129.19, 127.03, 126.84, 125.42, 116.16, 116.11, 114.16, 113.78, 112.87, 112.82, 111.96, 111.67, 102.16, 102.02, 67.15 (CH_2), 65.68 (CH_2), 18.59 (CH_3). MS (ESI), m/z (% rel. intensity) 352.1 (M^+, 100).

2.2.3i *2-[(4-methyl2-oxo-2H-chromen7–yl)oxy]-N′-(2-hydroxybenzylidene)acetohydrazide (**5i**)*: Yield 91%, R_f 0.38 (70% EtOAc/hexane), M.p. 292–294 °C (from methanol) [Ref[33] 284–286 °C (from methanol)]. IR (ATR): ν_{max} 3281, 3101, 2916, 1722, 1679, 1614, 1535, 1392, 1299, 1151, 745 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.84 (brs, 1H, N*H*), 11.59 (brs, 1H, N*H*), 11.02 (brs, 1H), 10.08 (brs, 1H), 8.56 (s, 1H, C*H*=N), 8.32 (s, 1H, C*H*=N), 7.75–7.50 (m, 4H), 7.35–7.20 (m, 2H), 7.10–6.84 (m, 8H), 6.24 (s, 1H), 6.21 (s, 1H), 5.27 (s, 2H, OCH_2) and 4.84 (s, 2H, OCH_2); resolved signals for 47:53 mixture of E_{syn}:E_{anti} conformers, 2.41 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.56, 164.00, 161.82, 161.11, 160.60, 160.51, 157.81, 156.88, 155.02, 154.98, 153.86, 153.81, 148.73, 142.08, 132.04, 131.71, 129.65, 127.06, 126.85, 120.45, 119.85, 119.09, 116.59, 114.24, 113.81, 112.85, 112.01, 111.68, 102.24, 102.04, 67.02 (CH_2), 65.74 (CH_2), 18.59 (CH_3). MS (ESI), m/z (% rel. intensity) 352.7 (M^+, 100).

2.2.3j *2-[(4-methyl2–oxo-2H-chromen7–yl)oxy]-N′-(4-dimethylamino)benzylidene)aceto-hydrazide (**5j**)*: Yield 89%, R_f 0.58 (70% EtOAc/hexane), M.p. 236–249 °C (from methanol) [Ref[29] 260–262 °C (from methanol)]. IR (ATR): ν_{max} 3315, 1703, 1682, 1614, 1524, 1365, 1152, 1081, 801 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.37 (s, 1H, N*H*), 11.32 (s, 1H, N*H*), 8.17 (s, 1H, C*H*=N), 7.89 (s, 1H, C*H*=N), 7.75–7.68 (m, 2H), 7.54–7.50 (m, 4H), 7.07–6.94 (m, 4H), 6.76–6.72 (m, 4H), 6.24 (s, 1H), 6.21 (s, 1H), 5.25 (s, 2H, OCH_2) and 4.76 (s, 2H, OCH_2); resolved signals for 59:41 mixture of E_{syn}:E_{anti} conformers, 2.97 (s, 12H), 2.41 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.26, 163.36, 161.90, 161.24, 160.60, 155.03, 154.99, 153.86, 153.82, 152.09, 151.91, 149.37, 145.35, 128.98, 128.74, 127.03, 126.84, 121.74, 121.68, 114.15, 113.78, 112.88, 112.82, 111.94, 111.65, 102.15, 102.00, 67.19 (CH_2), 65.70 (CH_2), 40.22 ($2CH_3$), 18.59 (CH_3). MS (ESI), m/z (% rel. intensity) 380.3 (M + H^+, 100).

2.2.3k *2-[(4-methyl2–oxo-2H-chromen7–yl)oxy]-N′-(2-nitrobenzylidene)acetohydrazide (**5k**)*:[34] Yield 83%, R_f 0.65 (70% EtOAc/hexane), M.p. 218–222 °C (from methanol). IR (ATR): ν_{max} 3297, 3082, 1696, 1611, 1513, 1342, 1152, 1070, 742 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 12.00 (s, 1H, N*H*), 11.94 (s, 1H, N*H*), 8.74 (s, 1H, C*H*=N), 8.39 (s, 1H, C*H*=N), 8.17-8.05 (m, 4H), 7.83-7.65 (m, 6H), 7.08-7.00 (m, 4H), 6.24 (s, 1H), 6.22 (s, 1H), 5.29 (s, 2H, OCH_2) and 4.85 (s, 2H, OCH_2); resolved signals for 70:30 mixture of E_{syn}:E_{anti} conformers, 2.41 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.22, 164.53, 161.74, 161.15, 160.59, 160.52, 155.04, 154.99, 153.87, 153.82, 148.47, 143.93, 139.91, 134.26, 133.96, 131.36, 131.31, 131.07, 129.12, 129.00, 128.57, 128.49, 127.05, 126.86, 125.14, 124.93, 113.85, 112.89, 111.99, 111.72, 102.17, 101.98, 67.04 (CH_2), 65.66 (CH_2), 18.59 (CH_3). MS (ESI), m/z (% rel. intensity) 382.3 (M + H^+, 100).

2.2.3l *2-[(4-methyl2–oxo-2H-chromen7–yl)oxy]-N′-[(pyridin-3-yl)methylene]aceto-hydrazide (**5l**)*: Yield 80%, R_f 0.29 (70% EtOAc/hexane), M.p. 250-267 °C (from methanol). IR (ATR): ν_{max} 2969, 1707, 1683, 1612, 1274, 1137, 1081, 838 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.81 (s, 2H), 8.97-8.79 (m, 2H), 8.68-8.54 (m, 2H), 8.39 (s, 1H, C*H*=N), 8.06 (s, 1H, C*H*=N), 8.23–8.09 (m, 2H), 7.82–7.63 (m, 2H), 7.55–7.42 (m, 2H), 7.12–6.92 (m, 4H), 6.25 (s, 1H), 6.23 (s, 1H), 5.33 (s, 2H, OCH_2) and 4.84 (s, 2H, OCH_2); resolved signals for 69:31 mixture of E_{syn}:E_{anti} conformers, 2.41 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.15, 164.33, 161.80, 161.80, 161.15, 160.63, 160.54, 155.04, 154.99, 153.90, 153.87, 151.33, 151.04, 149.27, 149.04, 145.90, 141.70, 134.11, 134.06, 130.45, 130.37, 127.08, 126.87, 124.50, 124.37, 114.22, 113.84, 112.92, 111.99, 111.72, 102.19, 102.05, 67.05 (CH_2), 65.75 (CH_2), 18.66 (CH_3). MS (ESI), m/z (% rel. intensity) 338.3 (M + H^+, 100). HRMS (ESI-TOF): calcd for $C_{18}H_{16}N_3O_4$ [M + H]$^+$: 338.1141, found: 338.1138.

2.2.3m *2-[(4-methyl-2-oxo-2H-chromen7–yl)oxy]-N′-[(pyridin-4-yl)methylene]aceto-hydrazide (**5m**)*: Yield 90%, R_f 0.14 (60% EtOAc/hexane), M.p. 236–240 °C (from methanol). IR (ATR): ν_{max} 3056, 2957, 1728, 1686, 1612, 1398, 1270, 1158, 1085, 975 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.92 (s, 2H, N*H*), 8.65 (s, 4H), 8.33 (s, 1H, C*H*=N), 8.01 (s, 1H, C*H*=N), 7.84–7.55 (m, 6H), 7.13–6.91 (m, 4H), 6.24 (s, 1H), 6.22 (s, 1H), 5.34 (s, 2H, OCH_2) and

4.86 (s, 2H, OCH_2); resolved signals for 70:30 mixture of E_{syn}:E_{anti} conformers, 2.40 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.37, 164.59, 161.78, 161.13, 160.61, 155.06, 153.89, 150.73, 150.67, 146.15, 142.02, 141.55, 127.07, 126.87, 121.52, 121.42, 113.86, 112.91, 112.00, 111.71, 102.18, 102.04, 67.04 (CH_2), 65.85 (CH_2), 18.60 (CH_3). MS (ESI), m/z (% rel. intensity) 338.3 (M + H$^+$, 100). HRMS (ESI-TOF): calcd for $C_{18}H_{16}N_3O_4$ [M + H]$^+$: 338.1141, found: 338.1142.

2.2.3n *2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy]-N′-[(1H-indol-3-yl)methylene]aceto-hydrazide (**5n**)*: Yield 78%, R_f 0.17 (70% EtOAc/hexane), M.p. 286–288 °C (from methanol). IR (ATR): ν_{max} 3325, 3068, 2969, 1676, 1607, 1392, 1267, 1139, 1079, 733 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.58 (s, 2H, N*H*), 11.36 (s, 1H), 11.31 (s, 1H), 8.49 (s, 1H, C*H*=N), 8.23 (s, 1H, C*H*=N), 8.21–8.10 (m, 2H), 7.85–7.65 (m, 4H), 7.46–7.42 (d, J = 12.0 Hz, 2H), 7.23–6.95 (m, 8H), 6.23 (s, 1H), 6.21 (s, 1H), 5.34 (2 s, 2H, OCH_2) and 4.75 (2 s, 2H, OCH_2); resolved signals for 66:34 mixture of E_{syn}:E_{anti} conformers, 2.41 (s, 6H). ^{13}C NMR (75 MHz, DMSO-d_6): δ 168.01, 163.14, 161.99, 161.34, 160.61, 160.56, 155.02, 153.87, 153.84, 145.76, 142.02, 137.55, 137.48, 131.05, 127.03, 126.88, 124.75, 123.10, 122.34, 122.19, 121.06, 120.90, 114.13, 113.79, 112.91, 112.71, 112.34, 111.93, 111.85, 111.65, 102.20, 102.05, 67.32 (CH_2), 65.87 (CH_2), 18.61 (CH_3). MS (ESI), m/z (% rel. intensity) 376.9 (M + H$^+$, 100). HRMS (ESI-TOF): calcd for $C_{21}H_{18}N_3O_4$ [M + H]$^+$: 376.1297, found: 376.1287.

2.2.4 *General procedure for synthesis of 2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy]-N′-(1-substituted ethylidene)acetohydrazide **6(a–g)** (Table 2)*: A mixture of 2-[(4-Methyl-2-oxo-2*H*-chromen-7-yl)oxy]-acetohydrazide (**4**, 0.5 mmol) and the acetophenone derivatives (0.5 mmol) in acetic acid (10 mL) was stirred at room temperature for 18 h. The precipitated solid was filtered and recrystallized from methanol to obtain white solid of **6a**–**g**.

2.2.4a *2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy)]-N′-(1-phenylethylidene)acetohydrazide (**6a**)*:[35] Yield 85%, R_f 0.80 (20% MeOH/EtOAc), M.p. 211–213 °C (from methanol). IR (KBr): ν_{max} 3400, 2995, 1775, 1604, 1520, 1450, 1215, 895 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6): δ 10.94 (s, 1H, N*H*), 10.67 (s, 1H, N*H*), 7.82 (m, 4H), 7.70 (d, J = 8.8 Hz, 2H), 7.46–7.42 (m, 6H), 7.02–6.96 (m, 4H), 6.23 (s, 2H), 5.35 (s, 2H, OCH_2) and 4.92 (s, 2H, OCH_2); resolved signals for 77:23 mixture of E_{syn}:E_{anti} conformers, 2.41 (s, 6H, 2CH_3), 2.32 (s, 3H, CH_3), 2.29 (s, 3H, CH_3). ^{13}C NMR (100 MHz, DMSO-d_6): δ 169.77, 161.90, 160.61, 155.03, 153.89, 149.05, 138.43, 129.67, 128.83, 126.85, 126.73, 113.77, 112.83, 111.65, 102.02, 66.11, 18.61, 14.06. MS (ESI$^+$), *m/z* (% rel. intensity) 352.0 (M + H$^+$, 12).

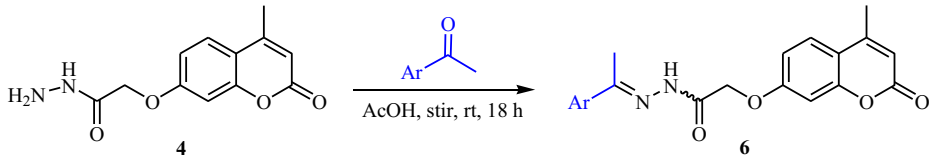
2.2.4b *2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy)]-N′-[1-(2-fluorophenyl)ethylidene]aceto-hydrazide (**6b**)*: Yield 78%, R_f 0.14 (60% EtOAc/hexane), M.p. 231–232 °C (from methanol). IR (ATR): ν_{max} 3198, 3052, 2979, 1713, 1671, 1611, 1552, 1395, 1284, 1195, 1158, 766 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.01 (s, 1H, N*H*), 10.74 (s, 1H, N*H*), 7.74–7.68 (m, 4H), 7.48–7.43 (m, 2H), 7.30–7.24 (m, 4H), 7.00–6.93 (m, 4H), 6.22 (s, 2H), 5.27 (s, 2H, OCH_2) and 4.92 (s, 2H, OCH_2); resolved signals for 75:25 mixture of E_{syn}:E_{anti} conformers, 2.41 (s, 6H, 2CH_3), 2.31 (s, 3H, CH_3), 2.27 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.83, 161.84, 160.58, 155.02, 153.86, 146.92, 131.51, 131.41, 130.44, 130.27, 127.38, 126.84, 125.01, 124.96, 116.77, 116.47, 113.78, 112.81, 111.66, 101.96, 66.72 (CH_2), 66.01 (CH_2), 18.59 (CH_3), 17.34 (CH_3). MS (ESI$^+$), *m/z* (% rel. intensity) 369.6 (M + H$^+$, 100). HRMS (ESI-TOF): Calcd for $C_{20}H_{17}FN_2O_4$ [M + H]$^+$: 369.1251, found: 369.1259.

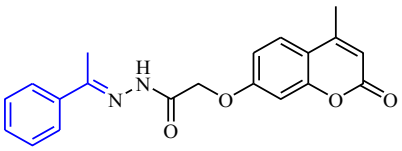
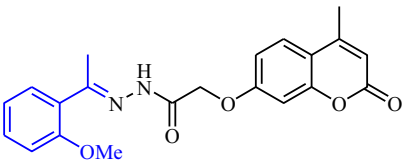
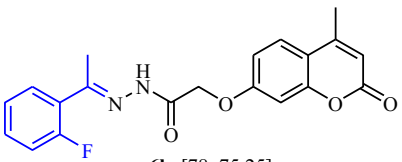
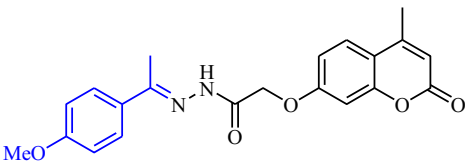
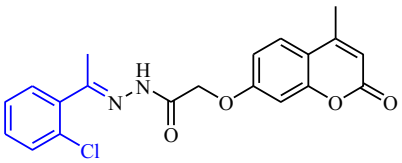
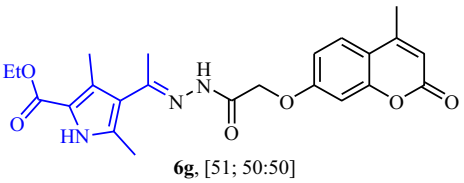
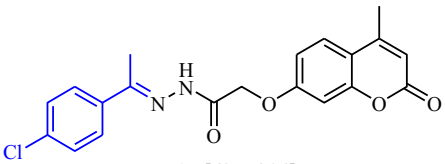
2.2.4c *2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy)]-N′-[1-(2-chlorophenyl)ethylidene]-aceto-hydrazide (**6c**)*: Yield 76%, R_f 0.73 (70% EtOAc/hexane), M.p. 233–235 °C (from methanol). IR (ATR): ν_{max} 3176, 3075, 1675, 1612, 1512, 1392, 1252, 1077, 819 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.00 (s, 1H, N*H*), 10.72 (s, 1H, N*H*), 7.74–7.40 (m, 10H), 7.01–6.91 (m, 4H), 6.22 (s, 2H), 5.21 (s, 2H, OCH_2) and 4.92 (s, 2H, OCH_2); resolved signals for 73:27 mixture of E_{syn}:E_{anti} conformers, 2.40 (s, 6H, 2CH_3), 2.29 (s, 3H, CH_3), 2.27 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.78, 161.80, 160.57, 155.01, 153.84, 149.75, 139.11, 131.59, 131.03, 130.69, 130.31, 127.76, 126.84, 113.78, 112.78, 111.87, 111.67, 101.94, 66.63 (CH_2), 65.97 (CH_2), 18.58 (CH_3), 18.34 (CH_3). MS (ESI), *m/z* (% rel. intensity) 385.7 (M + H$^+$, 100). HRMS (ESI-TOF): Calcd for $C_{20}H_{18}ClN_2O_4$ [M + H]$^+$: 385.0955, found: 385.0938.

2.2.4d *2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy)]-N′-[1-(4-chlorophenyl)ethylidene]-aceto-hydrazide (**6d**)*: Yield 69%, R_f 0.48 (70% EtOAc/hexane), M.p. 220–222 °C (from methanol) [Ref[36] 209 °C]. IR (ATR): ν_{max} 3240, 1692, 1612, 1389, 1257,1160, 1085, 828 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.97 (s, 1H, N*H*), 10.71 (s, 1H, N*H*), 7.88–7.67 (m, 6H), 7.48 (d, J = 9.0 Hz, 4H), 7.02–6.95 (m, 4H), 6.22 (s, 2H), 5.34 (s, 2H, OCH_2), 4.92 (s, 2H, OCH_2); resolved signals for 76:24 mixture of E_{syn}:E_{anti} conformers, 2.40 (s, 6H, 2CH_3), 2.31 (s, 3H, CH_3), 2.27 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.80, 161.87, 160.57, 155.03, 153.84, 150.06, 147.87, 137.25, 134.36, 128.82, 128.52, 126.82, 113.77, 112.84, 111.66, 102.00, 66.11 (CH_2), 18.59 (CH_3), 13.93 (CH_3). MS (ESI), *m/z* (% rel. intensity) 385.4 (M + H$^+$, 100).

2.2.4e *2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy)]-N′-[1-(2-methoxyphenyl)ethylidene]-acetohydrazide (**6e**)*: Yield 71%, R_f 0.54 (70% EtOAc/hexane), M.p. 222–224 °C (from methanol). IR (ATR): ν_{max} 3328, 3169, 1715, 1608, 1389, 1274, 1158, 1076, 764 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.82 (s, 1H, N*H*), 10.56 (s, 1H, N*H*), 7.70–7.66 (m, 2H), 7.48–6.70 (m, 12H), 6.24 (s, 1H), 6.21 (s, 1H), 5.22 (s, 2H, OCH_2) and 4.89 (s, 2H, OCH_2); resolved signals for 79:21 mixture of Esyn:Eanti conformers, 3.83 (s, 3H,

Table 2. Condensation reaction of hydrazide **4** with acetophenone derivatives.



Entry	Product [Yield,[b] (%); E_{syn}:E^{c}_{anti}]	Entry	Product [Yield,[b] (%); E_{syn}:E^{c}_{anti}]
1	 **6a**, [85; 77:23]	5	 **6e**, [71; 79:21]
2	 **6b**, [78; 75:25]	6	 **6f**, [61; 74:26]
3	 **6c**, [76; 73:27]	7	 **6g**, [51; 50:50]
4	 **6d**, [69; 76:24]		

[a]Reaction conditions: 0.5 mmol of **4**, 0.5 mmol of acetophenone derivative in 10 mL of AcOH at room temperature for 18 h.

[b]Isolated yield.

[c]E_{syn}:E_{anti} was determined by the integration of the duplicate OCH_2 peaks in the ^{1}H NMR spectra.

OCH_3), 3.77 (s, 3H, OCH_3), 2.40 (s, 6H, $2CH_3$), 2.23 (s, 3H, CH_3), 2.20 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.61, 161.88, 160.59, 157.64, 155.01, 153.85, 151.13, 131.57, 130.73, 129.91, 129.30, 128.39, 127.16, 126.81, 121.57, 120.81, 113.73, 112.79, 112.56, 112.09, 111.63, 101.97, 101.90, 66.00 (CH_2), 56.04 (CH_3), 56.00 (CH_3), 18.58 (CH_3), 18.03 (CH_3). MS (ESI), *m/z* (% rel. intensity) 380.4 (M^+, 100). HRMS (ESI-TOF): Calcd for $C_{21}H_{21}N_2O_5$ $[M + H]^+$: 381.1459, found: 381.1441.

2.2.4f *2-[(4-methyl-2-oxo-2H-chromen-7-yl)oxy)]-N′-[1-(4-methoxyphenyl)ethylidene]-acetohydrazide* (***6f***): Yield 61%, R_f 0.52 (70% EtOAc/hexane), M.p. 231–235 °C (from methanol) [Ref[36] 199 °C]. IR (ATR): ν_{max} 3178, 3077, 1713, 1675, 1612, 1514, 1392, 1252, 1136, 1078, 819 cm^{-1}. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.81 (s, 1H, N*H*), 10.57 (s, 1H, N*H*), 7.80–7.68 (m, 6H), 7.05–6.94 (m, 8H), 6.24 (s, 1H), 6.22 (s, 1H), 5.32 (s, 2H, OCH_2), 4.89 (s, 2H, OCH_2); resolved signals for 74:26 mixture of E_{syn}:E_{anti} conformers, 3.80 (s, 6H, OCH_3), 2.41 (s, 6H, $2CH_3$), 2.28 (s, 3H, CH_3), 2.25 (s, 3H, CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.53, 166.60, 161.91, 160.66, 160.59, 155.03, 153.84, 148.91, 130.87, 128.37, 128.19, 126.96, 126.82, 114.17, 113.75, 113.03, 112.83, 111.87, 111.64, 102.15, 102.00, 66.74 (CH_2), 66.11 (CH_2), 55.70 (CH_3), 18.59 (CH_3), 13.57 (CH_3), 13.93 (CH_3). MS (ESI), *m/z* (% rel. intensity) 381.7 ($M + H^+$, 100).

2.2.4g *ethyl 4-[1-(2-(4-methyl-2-oxo-2H-chromen-7-yloxy)acetoylimino)ethyl]-3,5-dimethyl-1H-pyrrole-2-carboxylate (**6g**)*: Yield 51%, R_f 0.45 (70% EtOAc/hexane), M.p. 163–169 °C (from methanol). IR (ATR): ν_{max} 3292, 3223, 3057, 1706, 1649, 1613, 1392, 1276, 1207, 1158, 1080, 830 cm^{-1}. ^1H NMR (300 MHz, DMSO-d_6): δ 11.73 (s, 1H, N*H*), 9.59 (s, 1H, N*H*), 10.64 (s, 1H, N*H*), 10.30 (s, 1H, N*H*), 7.74–7.68 (m, 2H), 7.06–6.82 (m, 4H), 6.24 (s, 1H), 6.22 (s, 1H), 5.16 (s, 2H, OCH_2) and 4.78 (s, 2H, OCH_2); resolved signals for 50:50 mixture of E_{syn}:E_{anti} conformers, 4.23 (q, J = 7.2 Hz, 4H, CH_2CH$_3$), 2.41 (s, 6H, CH_3), 2.35 (s, 6H, CH_3), 2.31 (s, 6H, CH_3), 2.17 (s, 3H, CH_3), 2.14 (s, 3H, CH_3), 1.28 (t, J = 7.2 Hz, 6H, CH$_2$CH_3). ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.20, 166.61, 161.85, 161.23, 161.07, 160.56, 160.50 155.00, 154.97, 153.84, 148.12, 133.20, 126.96, 125.87, 126.47, 122.25, 117.21, 114.18, 113.76, 113.04, 112.67, 111.97, 111.66, 102.15, 101.90, 66.73 (CH_2), 66.12 (CH_2), 59.59 (CH_2), 18.59 (CH_3), 18.27 (CH_3), 14.93 (CH_3), 13.32 (CH_3), 12.25 (CH_3). MS (ESI), *m/z* (% rel. intensity) 440.2 ($M + H^+$, 100). HRMS (ESI-TOF): Calcd for $C_{23}H_{26}N_3O_6$ $[M + H]^+$: 440.1822, found: 440.1826.

2.3 *Biology*

2.3.1 *Cell culture*: The hepatocellular carcinoma (HepG2), breast carcinoma (SKBR-3) cell lines were obtained from ATCC (MD, USA). Colorectal adenocarcinoma (Caco-2) was provided from Assoc. Prof. Dr. Kobtham Sathirakul, Mahidol University. The HepG2 and Caco-2 cells were cultured in EMEM medium whereas SKBR-3 was cultured in DMEM medium. All media (Gibco, Langley, VA, USA) were supplemented at 10% with fetal bovine serum (Gibco) and streptomycin plus penicillin (100 µg/mL and 100 U/mL, respectively; Sigma Co., Madrid, Spain). Cell cultures were maintained under standard conditions: incubation at 37 °C, 95% relative humidity with 5% CO_2 atmosphere.

2.3.2 *Cell viability assay*: Firstly, all the candidates, coumarin-aryl hydrazide–hydrazone hybrids, **5–6** were screened for their anti-proliferative activity against HepG2 at a concentration of 25 µM for 48 h. Then the potent coumarin-aryl hydrazide–hydrazone hybrids, **5–6** were further determined the IC_{50} values for HepG2, SKBR-3 and Caco-2 cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described.[37] In brief, cells were seeded into 96-well tissue culture plates in appropriated basal medium for each cell line containing 10% FBS to a final volume of 100 µL. The cells were subjected to different treatments after 24 h of seeding and incubated for 48 h with test compounds, doxorubicin as a positive control, or vehicle (DMSO). Then, MTT solutions were added and cells were incubated for 3 h. Formazan crystals formed were dissolved in 200 µL of DMSO and the optical density was determined at 570 nm using a microplate reader (Varioskan™ Flash Multimode Reader; Thermo Scientific™). Results were calculated by subtracting blank readings.

2.3.3 *Data analysis*: The concentration-response analysis was performed using Calcusyn™ version 1.1 (Biosoft Software, UK) to calculate the IC_{50} values.

3. Results and Discussion

3.1 *Chemistry*

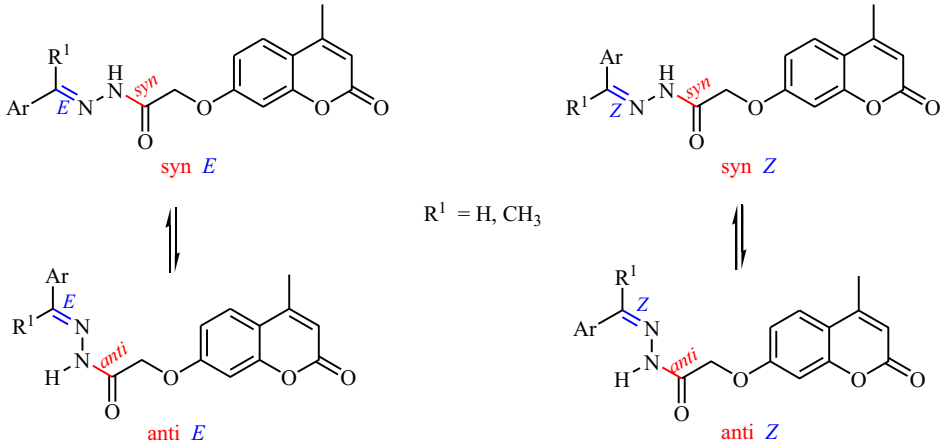
The hydrazine **4**, a precursor to the target coumarin hydrazide–hydrazone derivatives **5** and **6**, was synthesized as described in Scheme 1. The starting coumarin **1**, prepared *via* Pechmann reaction,[25] was treated with ethyl chloroacetate (**2**) in the presence of potassium carbonate under reflux in dry THF for 24 h to afford compound **3** in 75% yield. Then, hydrazinolysis of ethyl ester **3** with hydrazine hydrate in ethanol at room temperature for 30 h gave hydrazide **4** in excellent yield.

The condensation reaction of hydrazide **4** with aromatic aldehydes in the presence of a catalytic amount of glacial acetic acid in methanol and chloroform (1:1) under reflux for 5–8 h led to hydrazide–hydrazones **5(a–n)** in 70–98% yield (Table 1).

Similarly, the condensation reaction of compound **4** with acetophenone derivatives in acetic acid at room temperature for 18 h yielded the desired product **6(a–g)** in 51–85% yield (Table 2).

For the hydrazide–hydrazone compounds **5** and **6**, the duplication pattern of some ^1H and ^{13}C NMR signals revealed the presence of a mixture of syn/anti conformers around the amide bond, not *E*/*Z* stereoisomers of imine double bond. The only *E* geometric isomer, the most stable isomer, has been proved to be found in DMSO-d_6 solution by several experiments such as NOE and 2D-NOESY experiments, X-ray diffraction crystallography as well as conformation analysis as described in the literature[38–40] (Scheme 2).

The ^1H NMR spectra of compound **5** displayed two sets of singlet signals at 4.75–4.86/5.25–5.34 and 7.89–8.62 / 8.03–8.90 ppm corresponding to the OCH_2 and N=CH group, respectively. Similarly, the ^1H NMR spectra of compound **6** displayed only the separated singlet signals at 4.78–4.92/5.16–5.35 ppm corresponding to the OCH_2 protons. The downfield line of OCH_2 protons and upfield line of N=CH proton were assigned to syn conformer whereas the upfield line of OCH_2 protons and downfield line of N=CH proton were assigned to anti-conformer of the amide



Scheme 2. Stereochemistry of coumarin hydrazide–hydrazone derivatives.

Table 3. Percentage HepG2 cell viability after incubation with test compounds at 25 μM for 48 h using MTT assay.[a]

Compound	% cell viability at 48 h	Compound	% cell viability at 48 h
CTL	100	5l	66.99 ± 5.19
5a	59.83 ± 6.66	5m	104.21 ± 23.23
5b	75.48 ± 0.13	5n	44.06 ± 9.39
5c	66.17 ± 15.92	6a	58.40 ± 8.75
5d	76.45 ± 6.24	6b	66.51 ± 7.84
5e	33.22 ± 6.40	6c	55.88 ± 3.33
5f	59.35 ± 2.20	6d	19.57 ± 3.42
5g	50.75 ± 0.54	6e	80.41 ± 9.75
5h	51.06 ± 6.25	6f	48.82 ± 2.19
5i	79.88 ± 5.28	6g	92.16 ± 7.77
5j	62.42 ± 12.96	DOX	24.56 ± 8.33
5k	57.25 ± 12.14		

[a]The values are the mean ± SD of experiments performed in triplicate. The SD values were calculated using the expression: $\mathbf{SD} = \sqrt{\frac{\Sigma(\chi_1-\chi_2)^2}{2n}}$.

structure. The major conformer of almost all of the compound **5** and **6** was syn-*E* conformer, except for the compound **5i**, which was anti-*E* conformer (Table 1, entry 9). The ratio of the major (syn) and the minor (anti) conformers was determined by the integration of the separated singlet signals OCH_2 protons (Tables 1 and 2).

3.2 *Biology*

The synthesized compounds **5** and **6** were screened *in vitro* against human hepatocellular carcinoma (HepG2) cell line to investigate potential cytotoxicity effects at 25 μg/mL for 48 h (Table 3).

Compounds **5e**, **5n**, **6d** and **6f** exhibited less than 50% cell viability, which was considered cytotoxic, while all the other compounds exhibited more than 50% cell viability and supposed to have IC_{50} values ≥ 20 μM and show moderate to weakly cytotoxicity or even no cytotoxicity. Interestingly, the most active compound **6d** exhibited potent activity with 19.57 ± 3.42% cell viability, which lower than that of doxorubicin (24.56 ± 8.33% cell viability), the positive control drug. Compounds **5a**, **5f**, **5g**, **5h**, **5k**, **6a** and **6c** showed % cell viability ranging between 50 and 60%, which exhibited significant cytotoxicity. On further analyzing, it was observed that the position of substituent on phenyl ring and hetero-aromatic have a great impact on the cytotoxicity. The presence of a *para* hetero-substituent on the phenyl ring of compounds **5g**, **5h**, **6d** and **6f** appeared to be important for cell proliferation inhibitory activity comparing to the

Table 4. *In vitro* anticancer activity of the selected coumarin substituted hydrazide–hydrazone derivatives against HepG2, Caco-2 and SKBR-3 cancer cell lines using MTT assay[a]

Compound	IC_{50} (μg/mL)		
	HepG2	SKBR-3	Caco-2
5e	24.35 ± 3.18	15.88 ± 5.00	> 500
5g	4.67 ± 0.78	5.50 ± 1.55	> 500
5h	42.04 ± 1.70	61.27 ± 8.57	> 500
5n	31.31 ± 3.77	8.81 ± 1.19	> 500
6a	46.72 ± 6.57	50.97 ± 2.94	> 500
6d	2.84 ± 0.48	3.04 ± 0.22	> 500
6f	6.05 ± 0.37	2.34 ± 0.68	> 500
DOX	2.11 ± 0.13	0.19 ± 0.01	> 500

[a]The criteria used to categorize the cytotoxicity of coumarin substituted hydrazide–hydrazone derivatives against HepG2, SKBR-3, and Caco-2 based on U.S. National Cancer Institute (NCI) and Geran protocol[41,42] was as follows: $IC_{50} \leq 20$ μg/mL = highly cytotoxic, IC_{50} ranged between 21 and 200 μg/ml = moderately cytotoxic, IC_{50} ranged between 201 and 500 μg/mL = weakly cytotoxic and $IC_{50} > 501$ μg/mL = no cytotoxicity.

compounds **5f**, **5i**, **6c** and **6e**, respectively which have the hetero-substituent at *meta* or *ortho* position on the phenyl ring. However, compounds **5b**-**5d** and **6b**, containing fluoro group at any position on the phenyl ring, displayed lower activity than the parent compounds **5a** and **6a**, respectively. The replacement of phenyl ring in **5a** and **6a** by pyridine ring in **5l** and **5m** and substituted pyrrole ring in **6g** resulted in decreased or absent cytotoxicity. Except for compound **5n**, in which indole ring replaced the phenyl ring showed enhancement of activity. The substitution of methine hydrogen of the imines of **5a** and **5b** with a methyl substituent in **6a** and **6b** led to a slight increase in cytotoxicity. In addition, the influence of electronic properties at a *para* position on the benzene ring of electron-withdrawing substituent over electron-donating substituent on the cytotoxic activity could be observed in compounds **6d** and **6f**, containing imine methyl group, more than the compound **5g** and **5h**, having imine-hydrogen moiety.

Therefore, all the active compounds **5e**, **5n**, **6d** and **6f** along with **5g**, **5h** and **6a** were selected for evaluation of *in vitro* anti-proliferative activity against human hepatocellular carcinoma (HepG2), breast adenocarcinoma (SKBR-3), and colorectal adenocarcinoma (Caco-2) cell lines using MTT assay. The bioassay data are expressed as IC_{50}, as shown in Table 4.

From the results showed that all test compounds including the standard drug, doxorubicin, showed the $IC_{50} > 500$ μg/mL meaning that Caco-2 cell line resisted to these test compounds probably due to P-glycoprotein efflux pump activity within the cell line.

Compound **6d** with *N*′-(1-(4-chlorophenyl)ethylidene)acetohydrazide moiety (chloro group at *para* position on phenyl ring) was found consistent with the previous study to be the most active against liver HepG2 cell line with IC_{50} value of 2.84 ± 0.48 μg/mL, which was comparable to that obtained with the standard reference (doxorubicin) used (IC_{50} value of 2.11 ± 0.13 μg/mL). Surprisingly, compounds **5g** and **6f** containing *N*′-(4-bromobenzylidene)acetohydrazide and *N*′-(1-(4-methoxyphenyl)ethylidene)acetohydrazide moieties, respectively, displayed high cytotoxicity, while compounds **5e** and **5n** showed much lower activity against HepG2 cell line. Moreover, compounds **5h** and **6a** showed moderate cytotoxicity against both HepG2 and SKBR-3 cell lines. Remarkably, compound **6f** exhibited the most potent antiproliferative activity with IC_{50} 2.34 ± 0.68 μg/mL against breast adenocarcinoma SKBR-3 cell line, followed by compounds **6d**, **5g**, **5n**, and **5e** with potent cytotoxicity.

These findings suggested that the suitable *para*-substituents on the phenyl ring of coumarin hydrazide–hydrazone derivatives is responsible for its potent cytotoxicity, which deserve further modification and optimization to obtain the most potent compound.

4. Conclusions

In summary, the purpose of this study was to synthesize coumarin hydrazide–hydrazones derivatives and to screen their effects on cell viability in the HepG2 cell line. Furthermore, selected compounds were evaluated for *in vitro* antiproliferative activity against HepG2, SKBR-3, and Caco-2 cell lines. Regrettably, all synthesized compounds and also doxorubicin were inactive against Caco-2 cell line. Compound **6d** displayed the highest anti-proliferative activity (IC_{50} = 2.84 ± 0.48 μg/mL) against Hep-G2 cell line with IC_{50} value similar to the standard doxorubicin (IC_{50} = 2.11 ± 0.13 μg/mL), while compound **6f** emerged as the most potent compound exhibiting IC_{50} value of 2.34 ± 0.68 μg/mL against SKBR-3 cell line. These two compounds, **6d** and **6f**, might be promising for further investigation to develop new anti-cancer drugs. Further study of these compounds on the mechanism of action is in progress.

Supplementary Information (SI)

^{1}H and ^{13}C NMR spectra of all compounds, as well as HRMS spectra of compounds **5l**, **5m**, **6b**, **6c**, **6e** and **6g**, are given in the supplementary information at www.ias.ac.in/chemsci.

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