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Synthesis of novel indolizine, diazepinoindolizine and Pyrimidoindolizine derivatives as potent and selective anticancer agents

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Abstract A series of new indolizine **9a–c, 10a–c,** diazepinoindolizine **7a–c, 8a–c**, and pyrimidoindolizine **11** derivatives were synthesized and structures of the newly synthesized compounds were confirmed by spectral and elemental analyses. Anti-tumor activity evaluation was carried out using sulphorhodamine-B assay method against lung adenocarcinoma (A549), breast (MCF7), hepatoma (Hep3B) cancer cell lines and normal fibroblast cells. Compounds **7a, 9c, 10a,c** and **11** showed to be the most active against the lung cancer cell line with IC_{50} in nanomole range (16–85 nmol/ml) and compound **11** was the best selective one (S. I. = 19). The most potent compounds against MCF7 are **8c, 9b,c, 10a,b**, and **11**. Their IC_{50} range is 4–46 nmol/ml and the best selectivity was assigned for compound **11** (S. I. = 133). As for the hepatoma cancer cell line, compounds **7a, 8a–c, 9a–c**, and **10a,b** were found to be the most potent with IC_{50} range 3–90 nmol/ml and compound **8c** was the most selective one (S. I. = 42), the rest of the new compounds showed IC_{50} value >100 nmol/ml. Compound **10a** showed a broad spectrum activity and selectivity against the tested cell lines with a much lesser effect on normal fibroblast cells ($IC_{50} > 200$ nmol/ml).

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Abbreviations

SRB Sulphorhodamine-B
S. I. Selectivity index
μM Micromole

Introduction

Indolizine is an important N-containing heterocyclic nucleus. It has several previous names in the chemical literature such as pyrindole, pyrrodine, pyrrocoline, and pyrrole[1,2-a]pyridine, and it is also considered as an indole isomer due to the ten delocalized Π-electrons. The indolizine system consists of two fused 5- and 6-membered rings with one nitrogen bridge at the ring junction. Its numbering system is shown in Fig. 1 [1]. The aromatic indolizine system does not appear to occur naturally; however, its perhydro derivatives, named indolizidines, represent a scaffold for several alkaloids [2]. The biological potential of indolizine derivatives was observed through their reported pharmacological activities, e.g., anti-inflammatory and anticonvulsant [3], antioxidant [4], antimicrobial [5], antitubercular [5] and antidiabetic [6].

The role of indolizine scaffolds in cancer treatment was also proven through the observed anticancer activity by several indolizine derivatives, e.g., indolizine derivative-bearing cyano group **I** showed good antiproliferative activity against the human hepatocellular liver carcinoma (Hep-G2) cell line at IC_{50} value equal 0.20 μg/ml [7]. 6,7-diphenyl-2,3,8,8a-tetrahydro-1*H*-indolizin-5-one **II**, an analogue of septicine, showed cytotoxic activity against eight cancer cell lines with GI_{50} values in the range of 0.4–9 μM in addition to its good pharmacokinetic properties [8]. In addition, piperidin-1-yl-containing compounds **IIIa,b** showed greater in vitro cytotoxic activity than perillyl alcohol. They showed inhibitory properties against α-1 Na/K-ATPase and Ras oncogene activity in cancerous cells [9]. Schiff bases showed different biological activities and proved to be a versatile pharmacophore useful in designing new bioactive agents [10]. Moreover, the *p*-nitro benzylidine derivative **IV** revealed significant anticancer activity against human ovarian cancer cell line A2780, Co rectal cancer cell line HCT116 and human liver cancer cell line HepG2 [11]. All these facts guided us to synthesize our target

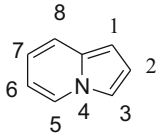


Fig. 1 Structure and numbering of indolizine nucleus

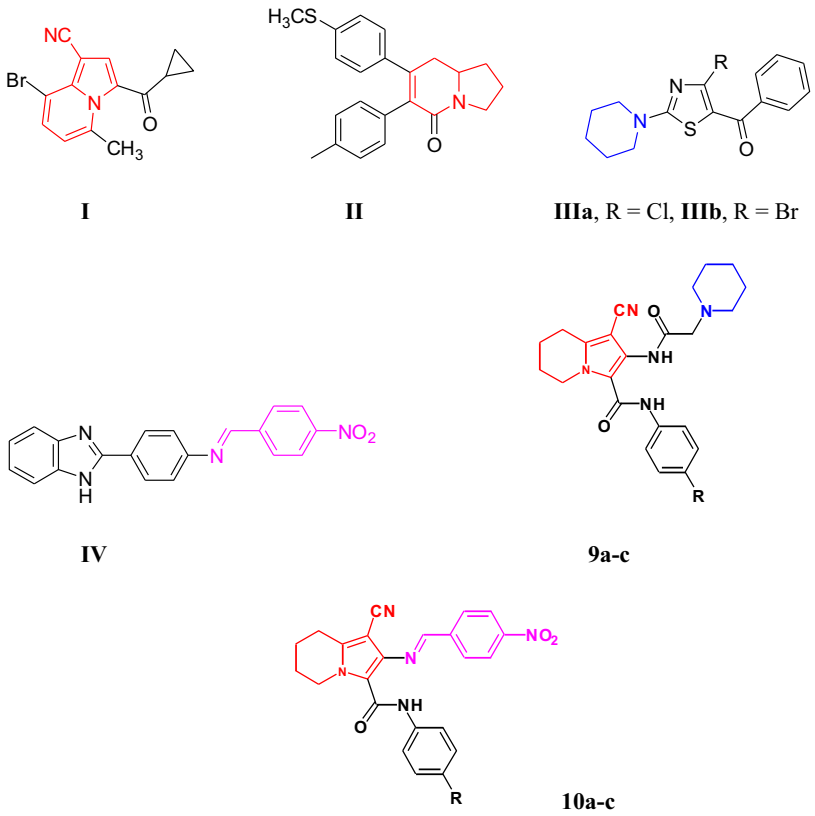
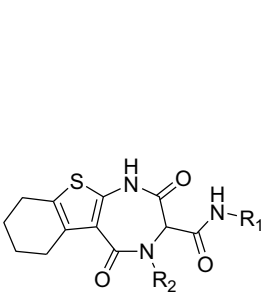


Fig. 2 Indolizine, piperidine, and *p*-nitro Schiff base derivatives (**I–IV**) as antitumor active agents and our target compounds (**9a–c** and **10a–c**)

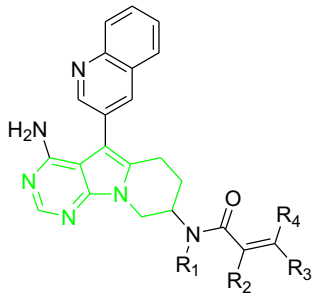
compounds that represent hybrid molecules of indolizine scaffold with piperidin-1-yl moiety **9a–c** or with the active pharmacophore *p*-nitro substituted Schiff base **10a–c** (Fig. 2).

The activity of thienodiazepinediones **Va,b** to antagonize the p53-Mdm2 interaction was proven using FP screening assay [12]. This encouraged us to apply isosteric replacement strategy and synthesize diazepinindolizine system **7a–c**, **8a–c**, which have not been investigated yet as a potential pharmacophore (Fig. 3). In 2014, Sagara et al. [13] invented novel derivatives **VI** with a pyrimidoindolizine scaffold useful as anticancer agents due to their ability of inhibiting EGFR tyrosine kinases. Based on this finding and for further exploration of anticancer activity of this biologically important scaffold, it was of our interest to construct the pyrimidoindolizine system **11** (Fig. 3) aiming to obtain new compounds with potential activity as anticancer.

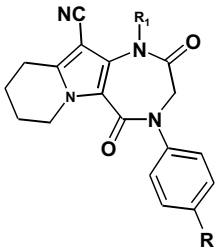


Va, R_1 = benzyl, R_2 = 4-chlorobenzyl

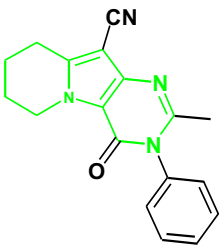
Vb , R_1 = 3,4-dichlorobenzyl, R_2 = 4-chlorobenzyl



VI



7a-c, 8a-c



11

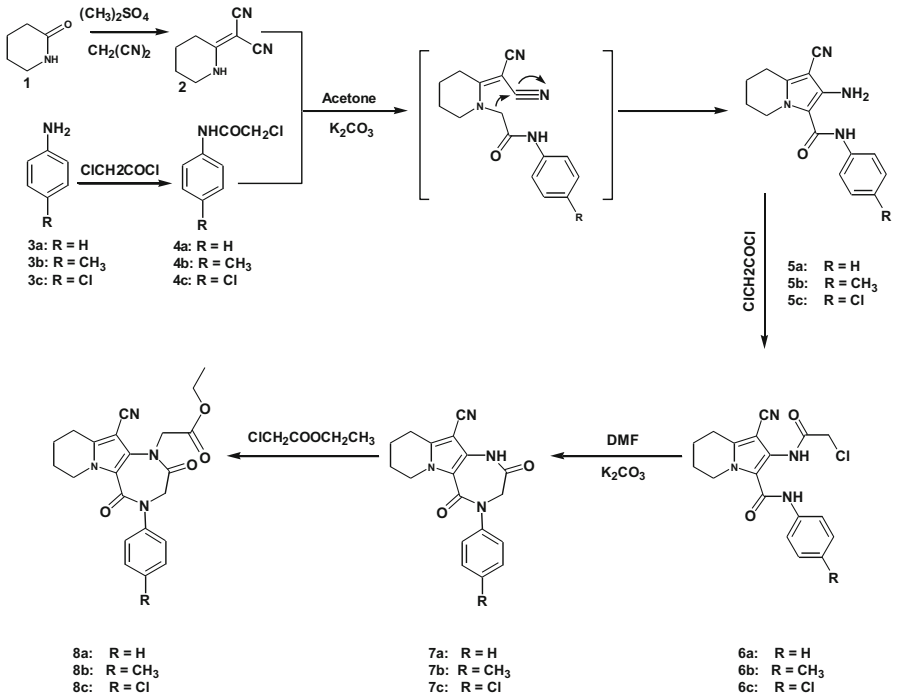
Fig. 3 Anticancer active agents (**Va,b** and **VI**) and our target compounds (**7a–c, 8a–c** and **11**)

Results and discussion

Chemistry

The present investigation involves the preparation of four classes of novel indolizine derivatives; (1) diazepinoindolizines **7a–c, 8a–c**, (2) 6-(2-(piperidin-1-yl) acetamido) derivatives **9a–c**, (3) *p*-nitro Schiff base compounds **10a–c**, (4) pyrimidoindolizine derivative **11**. The starting materials **2**, **4a–c**, **5a**, and **6a** were prepared according to the previously reported methods [14], equimolar amount of compounds **2** and **4a–c** was refluxed in dry acetone for 24 h in presence of dry K_2CO_3 to afford compounds **5a–c**, respectively. Chloroacylated indolizine derivatives **6a–c** were obtained through reaction of **5a–c** with chloroacetyl chloride (Scheme 1).

Stirring these compounds **6a–c** in DMF in the presence of dry potassium carbonate afforded diazepinoindolizines **7a–c**. The IR spectra of the cyclized products **7a–c** showed the presence of a stretching band at 3,280–3,217 cm^{-1} corresponding to the NH group, a sharp absorption band at 2,230–2,221 cm^{-1}



Scheme 1 Synthesis of diazepinoindolizine derivatives **7a–c** and **8a–c**

attributed to the cyano group. ^{1}H-NMR spectra of compounds **7a–c** revealed the presence of a singlet signal at 4.24–4.28 ppm corresponding to the $-CH_2$ group of the diazepine ring and a singlet exchangeable signal at 10.92–10.98 ppm assigned for the amidic proton. Additionally, ^{13}C-NMR spectrum of compounds **7a–c** was used as a tool in confirming their structures. Moreover, the mass spectrum of compound **7a** revealed the molecular ion at 320 *m/z*.

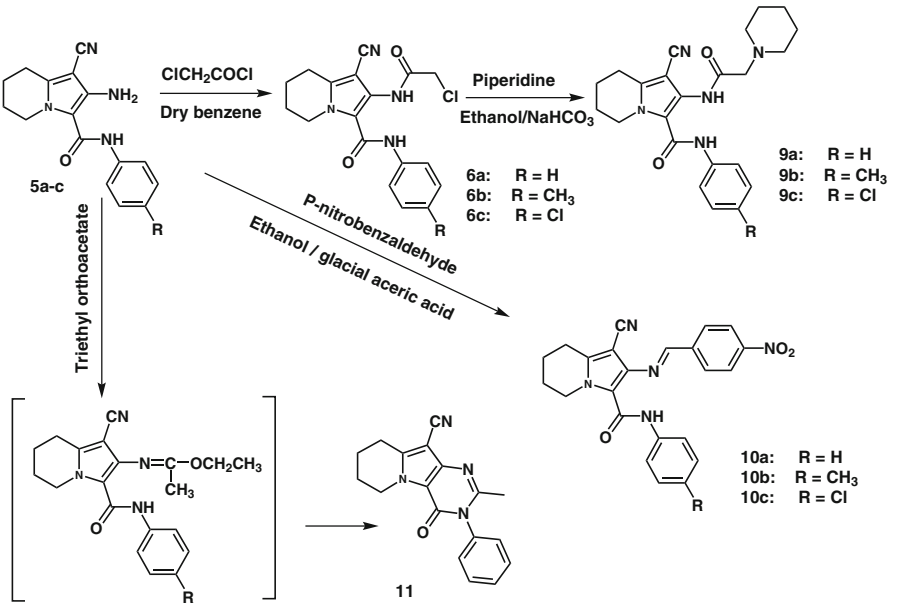
In the present work, diazepinoindolizines were treated with ethyl chloroacetate in dry acetone and in the presence of potassium carbonate as an acid-binding agent to afford the corresponding ethyl ester derivatives **8a–c**. The IR spectra revealed the disappearance of the NH group absorption band and the appearance of another absorption band at 1,751–1,742 cm^{-1}, indicating the additional carbonyl group of the ethyl esters. Also, ^{1}H-NMR spectra of compounds **8a–c** revealed the disappearance of the exchangeable NH signal found in the starting materials and the presence of a triplet signal at 1.26–1.27 ppm assigned for methyl group of the ethyl moiety, a quartet signal at 4.22–4.23 ppm assigned for methylene group of the ethyl moiety, and a singlet signal at 4.71–4.75 ppm assigned for methylene group of the acetate moiety. Additionally, ^{13}C-NMR spectrum of compounds **8a–c** demonstrated the presence of a signal at 14.08–14.09 ppm attributed to the methyl group of the ethyl moiety, 48.32 ppm attributed to the methylene group of the acetate group,

61.91–61.99 ppm attributed to the methylene group of the ethyl moiety, and 167.56–167.63 ppm attributed to the carbonyl carbon of the acetate group.

Reacting the chloroacylated indolizines **6a–c** with piperidine in absolute ethanol and in the presence of sodium bicarbonate afforded the piperidin-1-yl indolizines **9a–c**. The ^{1}H-NMR spectrum of compounds **9a–c** confirmed the substitution of the piperidine nucleus for the chlorine atom as it shows two signals at 1.47–1.67 ppm attributed to six protons belonging to the three methylene groups in the piperidine nucleus and a signal at 2.58–2.63 ppm attributed to four protons belonging to the two methylene groups adjacent to the nitrogen atom in the piperidine nucleus. Furthermore, ^{13}C spectrum of compounds **9a–c** revealed the presence of new signals at 23.43–23.47 ppm, 26.09–26.11 ppm, and 55.02–55.03 ppm attributed to methylene groups of piperidine moiety.

In the present work, the target compounds **10a–c** were prepared via refluxing the aminonitriles **5a–c** with an equimolar amount of aromatic aldehyde in absolute ethanol and a few drops of glacial acetic acid to catalyze the reaction. The structure of Schiff bases **10a–c** was confirmed using spectral and elemental analysis. The IR spectra revealed the disappearance of the primary amino group absorption band. Also, ^{1}H-NMR spectra of compounds **10a–c** show two signals at 8.09–8.41 ppm attributed to four protons belonging to additional aromatic protons and a singlet signal appeared at 9.23–9.28 ppm assigned for CH group. Furthermore, ^{13}C spectrum of the compounds revealed the presence of six new aromatic carbons and a signal at 157.06–157.30 ppm assigned for C=N.

Herein, compound **11** was prepared via refluxing the aminonitriles **5a** in excess triethyl orthoacetate where cyclization occurred between the imidate carbon and



Scheme 2 Synthesis of the target compounds **9a–c**, **10a–c** and **11**

nitrogen atom of the amino group generating the fused pyrimidine ring directly (Scheme 2). The structure of compound **11** was confirmed using spectral and elemental analysis. The IR spectrum revealed the disappearance of the primary amino group absorption band. ^{1}H-NMR spectrum of compound **11** revealed the disappearance of the exchangeable NH_2 and NH signals found in the starting materials and the presence of a singlet signal at 2.24 ppm assigned for methyl group of triethyl orthoacetate moiety. Furthermore, ^{13}C spectrum of the compound revealed the presence of new signal at 24.35 ppm attributed to methyl group and a signal at 154.39 ppm assigned for C=N.

Pharmacological screening

The four novel classes of indolizines were screened against lung adenocarcinoma (A549), breast (MCF7), hepatoma (Hep3B) cancer cell lines, and normal fibroblast cells (non-tumorous cell line) using sulphorhodamine-B assay method [15]. IC_{50} values in μmol/ml and selectivity index values that were calculated by dividing the IC_{50} value of the tested compound against normal fibroblasts over that against the cancer cell line [16] are represented in Table 1. Nine compounds (**7a, 8a–c, 9a–c**, and **10a,b**) out of 13 are highly potent against Hep3B cancer cell line with IC_{50} range (3–90 nmol/ml) and all these nine compounds are selective to Hep3B. Six compounds (**8c**, **9b,c, 10a,b**, and **11**) are highly potent against MCF7 cancer line with IC_{50} range equal 4–46 nmol/ml, and all are selective to MCF7 except for compounds **9b,c**. Five compounds of the tested compounds (**7a, 9c, 10a,c**, and **11**) are potent against the A549 cell line. Their IC_{50} range is 16–85 nmol/ml, all are selective to A549 except for **9c**. Compound **7a** is the best active compound against

Table 1 IC_{50} values in μmol/ml and selectivity index for the tested indolizine derivatives

Comp. no.	A549		MCF7		Hep3B		Normal fibroblast cells IC_{50}
	IC_{50}	S. I.	IC_{50}	S. I.	IC_{50}	S. I.	
7a	0.016	1.0625	0.726	0.023416	0.003	5.666667	0.017
7b	0.136	0.235294	0.192	0.166667	0.869	0.036824	0.032
7c	0.477	0.314465	0.199	0.753769	1.181	0.127011	0.15
8a	0.453	0.86755	0.182	2.159341	0.079	4.974684	0.393
8b	0.678	0.057522	0.129	0.302326	0.023	1.695652	0.039
8c	0.287	3.965157	0.018	63.22222	0.027	42.14815	1.138
9a	2.559	0.115279	0.139	2.122302	0.09	3.277778	0.295
9b	0.167	0.035928	0.046	0.130435	0.051	0.117647	0.006
9c	0.085	0.223529	0.038	0.5	0.087	0.218391	0.019
10a	0.038	5.605263	0.039	5.461538	0.017	12.52941	0.213
10b	0.394	0.345178	0.028	4.857143	0.01	13.6	0.136
10c	0.038	5.421053	0.177	1.163842	0.254	0.811024	0.206
11	0.028	18.96429	0.004	132.75	0.198	2.681818	0.531

Hep3B, IC_{50} = 3 nmol/ml and S. I. = 5.7 and also the best active one against A549 with IC_{50} value = 16 nmol/ml and S.I. = 1.1. The best active compound against MCF7 is compound **11**, IC_{50} = 4 nmol/ml and S. I. = 132.

Conclusions

According to the results obtained during this work, we can conclude that: (1) indolizine represents a hopeful scaffold for designing new potent and selective anticancer agents; (2) diazepinoindolizines showed to be highly potent and selective anticancer agents; compounds **8a–c** are active and selective to Hep3B. In addition, compound **8c** showed good activity and selectivity to MCF7 (IC_{50} = 18 nmol/ml, S. I. = 63). Moreover, compound **7a** was the most active against A549 and Hep3B; (3) pyrimidoindolizine **11** is the most potent and selective compound to MCF7. Its IC_{50} and S.I. are 4 nmol/ml and 132, respectively, in addition to its activity and selectivity to A549 with IC_{50} = 28 nmol/ml and S.I. = 19; (4) both hybrid molecules (indolizine with piperidine) **9c** and (indolizine with *p*-nitro Schiff base) **10a** exhibited broad spectrum activity against all three cancer cell lines. However, hybridization with *p*-nitro Schiff base leads to obtaining more selective compound **10a** than **9c**.

Experimental

Chemistry

Melting points were uncorrected and were carried out by open capillary tube method using IA 9100MK-Digital Melting Point Apparatus. Microanalyses were carried out at the microanalytical Center, Faculty of Science, Cairo University. Infrared spectra were made on a BRUKER Vector 22 (Japan) infrared spectrophotometer and were expressed in wavenumber (cm^{-1}) using a potassium bromide disc at the Microanalytical Center, Faculty of Science, Cairo University. The ^{1}H-NMR spectra were recorded in $CDCl_3$ and DMSO-d_6 on a Varian Mercury spectrometer (400 MHz) (NMR Lab. Faculty of Pharmacy, Beni-Suef University). Chemical shifts were reported on the δ scale and were related to that of the solvent and *J* values are given in Hz. ^{13}C NMR spectra were obtained on a Bruker APX400 at 100 MHz at the Faculty of Pharmacy, Beni-Suef University, Beni-Suef, Egypt. Mass spectra were recorded on Finnigan MAT, SSQ 7000, mass spectrometer at 70 eV (EI) at the Microanalytical Center, Faculty of Science, Cairo University and Waters Micromass Q-Tof Micro mass spectrometer (ESI) and Waters Acquity Ultra Performance LC with ZQ detector in ESI mode. IUPAC chemical nomenclature were assigned using CS Chemdraw ultra version 5.0. thin-layer chromatography, using Macherey–Nagel AlugramSil G/UV254 silica gel plates and benzene-ethanol (9.5:0.5) as the eluting system.

General procedure for the preparation of **7a**–**7c**

A mixture of compounds **6a–c** (3.58 mmol) and anhydrous potassium carbonate (0.49 g, 3.58 mmol) in dry DMF (10 ml) was stirred at room temperature for 48 h. The reaction mixture was poured onto ice-cooled water; the obtained precipitate was filtered, washed with water, and recrystallized from ethanol/acetone.

2,5-Dioxo-4-phenyl-2,3,4,5,7,8,9,10-octahydro-1*H*-[1, 4] diazepino[5,6-b] indolizine-11-carbonitrile **7a**

Compound **7a** was prepared from **6a** and anhydrous potassium carbonate. Yellowish crystals, 0.53 g; 47 % yield, m.p. 268–270 °C. IR (KBr, cm^{-1}): $\nu_{max.} = 3{,}217$ (NH), 3,049 (CH aromatic), 3,008, 2,952 (CH_2), 2,214 (CN), 1,688, 1,644 (COs), 1,553, 1,540 (C=C, NH), 1,490, 1,443, 1,326 (C–N, C–O). ^{1}H-NMR (DMSO-d_6, 400 MHz): δ 1.81 (m, 2H, CH_2-9) 1.91 (m, 2H, CH_2-8), 2.86 (t, 2H, $J = 6$ Hz, CH_2-10), 4.19 (t, 2H, $J = 6$ Hz, CH_2-7), 4.28 (s, 2H, CH_2-3), 7.29–7.45 (m, 5H, aromatic protons) and 10.93 (s, H, NH, which disappeared on deuteration). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 18.8 (CH_2), 22.4 (CH_2), 22.5 (CH_2), 45.9 (CH_2), 54.8 (CH_2), 82.7 (C), 114.3 (CN), 115.7 (C), 126.1 (2CH), 126.9 (CH), 129.4 (2CH), 130.3 (C), 142.7 (C), 143.3 (C), 159.8 (CO), 168.4 (CO). Anal. Calcd. for $C_{18}H_{16}N_4O_2$ (320.35): C, 67.49; H, 5.03; N, 17.49. Found C, 67.62; H, 5.30; N 17.19.

2,5-Dioxo-4-(p-tolyl)-2,3,4,5,7,8,9,10-octahydro-1*H*-[1, 4] diazepino [5,6-b] indolizine-11-carbonitrile **7b**

Compound **7b** was prepared from **6b** and anhydrous potassium carbonate. Yellow crystals, 0.69 g; 58 % yield, m.p. 267–269 °C. IR (KBr, cm^{-1}): $\nu_{max.} = 3{,}280$ (NH), 3,077 (C–H aromatic), 2,961, 2,924 (CH_3, CH_2), 2,215 (CN), 1,689, 1,640 (COs), 1,591, 1,554, 1,534 (C=C,NH), 1,444, 1,367, 1,305 (C–N, C–O).^{1}H-NMR (DMSO-d_6, 400 MHz): δ 1.81 (m, 2H, CH_2-9) 1.90 (m, 2H, CH_2-8) 2.31 (s, 3H, $\underline{CH_3}$Ph), 2.85 (t, 2H, $J = 6$ Hz, CH_2-10), 4.18 (t, 2H, $J = 6$ Hz, CH_2-7), 4.24 (s, 2H, CH_2-3), 7.22 and 7.24 (2 × d, 2 × 2H, $J = 8.4$ Hz, para-substituted phenyl ring) and 10.92 (s, H, NH, which disappeared on deuteration).^{13}C NMR (DMSO-d_6, 100 MHz): δ 23.5 (CH_2), 25.7 (CH_3), 27.2 (CH_2), 27.3 (CH_2), 50.6 (CH_2), 59.7 (CH_2), 87.4 (C), 119.1 (CN), 120.5 (C), 130.7 (2CH), 134.5 (2CH), 135.0 (C), 141.0 (C), 145.0 (C), 147.9 (C), 164.5 (CO), 173.1 (CO). Anal. Calcd. for $C_{19}H_{18}N_4O_2$ (334.37): C, 68.25; H, 5.43; N, 16.76. Found C, 68.55; H, 5.73; N 16.73.

4-(4-chlorophenyl)-2,5-dioxo-2,3,4,5,7,8,9,10-octahydro-1*H*-[1, 4] diazepino [5,6-b] indolizine-11-carbonitrile **7c**

Compound **7c** was prepared from **6c** and anhydrous potassium carbonate. Yellow crystals, 0.57 g; 45 % yield, m.p. 293–294 °C. IR (KBr, cm^{-1}): $\nu_{max.} = 3{,}274$ (NH), 3,114 (C–H aromatic), 2,963, 2,901 (CH_2), 2,220 (CN), 1,693, 1,639 (COs), 1,561, 1,536 (C=C,NH), 1,490, 1,441, 1,370 (C–N, C–O). ^{1}H-NMR (DMSO-d_6,

400 MHz): δ 1.80 (m, 2H, CH_2-9) 1.89 (m, 2H, CH_2-8), 2.85 (t, 2H, $J = 6$ Hz, CH_2-10), 4.18 (t, 2H, $J = 6$ Hz, CH_2-7), 4.28 (s, 2H, CH_2-3), 7.47–7.49 (m, 4H, aromatic protons) and 10.98 (s, H, NH, which disappeared on deuteration).^{13}C NMR (DMSO-d_6, 100 MHz): δ 23.5 (CH_2), 27.2 (CH_2), 27.3 (CH_2), 50.6 (CH_2), 59.3 (CH_2), 87.5 (C), 119.0 (CN), 120.3 (C), 132.1 (2CH), 134.0 (2CH), 136.0 (C), 144.0 (C), 146.2 (C), 148.2 (C), 169.2 (CO), 173.0 (CO). Anal. Calcd. for $C_{18}H_{15}ClN_4O_2$ (354.79): C, 60.94; H, 4.26; N, 15.79. Found C, 60.64; H, 4.44; N, 15.49.

General procedure for the preparation of **8a**–**8c**

A mixture of compounds **7a**–**c** (3.12 mmol), ethyl chloroacetate (0.4 g, 3.12 mmol), and anhydrous potassium carbonate (0.45 g, 3.26 mmol) in dry acetone (50 ml) was stirred under reflux for 6 h. The mixture was filtered, concentrated, and left to cool, whereby white crystals were formed, collected, dried, and recrystallized from ethanol.

Ethyl 2-(11-cyano-2,5-dioxo-4-phenyl-2,3,4,5,7,8,9,10-octahydro-1*H*- [1, 4] diazepino[5,6-b]indolizin-1-yl)acetate **8a**

Compound **8a** was prepared from **7a** and ethyl chloroacetate. White crystals, 0.69 g; 55 % yield, m.p. 263–265 °C. IR (KBr, cm^{-1}): $\nu_{max.} = 3{,}060$ (C–H aromatic), 2,977, 2,937 (CH_2), 2,222 (CN), 1,742, 1,693, 1,644 (COs), 1,595, 1,549 (C=C), 1,468, 1,373, 1,307 (C–N, C–O).^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.26 (t, 3H, $J = 7.2$ Hz, $\underline{CH_3}CH_2$), 1.93 (m, 2H, CH_2-9), 2.07 (m, 2H, CH_2-8), 2.94 (t, 2H, $J = 6.4$ Hz, CH_2-10), 4.22 (q, 2H, $J = 7.2$ Hz, $\underline{CH_2}CH_3$), 4.54 (t, 2H, $J = 6.4$ Hz, CH_2-7), 4.62 (s, 2H, CH_2-3), 4.75 (s, 2H, $N\underline{CH_2}CO$) and 7.28-7.43 (m, 5H, aromatic protons).^{13}C NMR ($CDCl_3$, 100 MHz): δ 14.0 (CH_3), 18.8 (CH_2), 22.5 (CH_2), 22.7 (CH_2), 45.9 (CH_2), 48.3 (CH_2), 54.3 (CH_2), 61.9 (CH_2), 83.6 (C), 114.0 (CN), 117.0 (C), 125.9 (2CH), 127.3 (CH), 129.3 (2CH), 131.7 (C), 141.6 (C), 143.0 (C), 159.4 (CO), 166.8 (CO), 167.6 (COO). Anal. Calcd. for $C_{22}H_{22}N_4O_4$ (406.43): C, 65.01; H, 5.46; N, 13.78. Found C, 65.30; H, 5.37; N, 13.48.

Ethyl 2-(11-cyano-2,5-dioxo-4-(*p*-tolyl)-2,3,4,5,7,8,9,10-octahydro-1*H*-[1, 4] diazepino[5,6-b]indolizin-1-yl)acetate **8b**

Compound **8b** was prepared from **7b** and ethyl chloroacetate. White crystals, 0.81 g; 62 % yield, m.p. 190–192 °C. IR (KBr, cm^{-1}): $\nu_{max.} = 3{,}074$ (C–H aromatic), 2,966, 2,938 (CH_3, CH_2), 2,218 (CN), 1,742, 1,691, 1,644 (COs), 1,591, 1,549, 1,511 (C=C), 1,470, 1,373, 1,306 (C–N, C–O).^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.27 (t, 3H, $J = 7.2$ Hz, $\underline{CH_3}CH_2$), 1.92 (m, 2H, CH_2-9), 2.07 (m, 2H, CH_2-8), 2.36 (s, 3H, $\underline{CH_3}$Ph), 2.93 (t, 2H, $J = 6.4$ Hz, CH_2-10), 4.23 (q, 2H, $J = 7.2$ Hz, $\underline{CH_2}CH_3$), 4.54 (t, 2H, $J = 6.4$ Hz CH_2-7), 4.62 (s, 2H, CH_2-3), 4.71 (s, 2H, $N\underline{CH_2}CO$) and 7.14-7.29 (m, 4H, aromatic protons).^{13}C NMR ($CDCl_3$, 100 MHz): δ14.0 (CH_3), 18.8 (CH_2), 21.0 (CH_3), 22.5 (CH_2), 22.6 (CH_2), 45.8 (CH_2), 48.3 (CH_2), 54.4 (CH_2), 61.9 (CH_2), 83.5 (C), 114.1 (CN), 117.1 (C), 125.7 (2CH), 129.9 (2CH), 131.7 (C), 137.2 (C), 139.0 (C), 142.9 (C), 159.5 (CO), 166.9 (CO), 167.6

(COO). Anal. Calcd. for $C_{23}H_{24}N_4O_4$ (420.46): C, 65.70; H, 5.75; N, 13.33. Found C, 66.00; H, 6.02; N, 13.03.

Ethyl 2-(4-(4- chlorophenyl)-11-cyano-2,5-dioxo-2,3,4,5,7,8,9,10-octahydro-1*H*- [1, 4] diazepino[5,6-b]indolizin-1-yl)acetate **8c**

Compound **8c** was prepared from **7c** and ethyl chloroacetate. White crystals, 0.72 g; 53 % yield, m.p. 215–217 °C. IR (KBr, cm^{-1}): $\nu_{max.}$ = 3,093 (C–H aromatic), 2,976 (CH_2), 2,222 (CN), 1,751, 1,685, 1,648 (COs), 1,595, 1,594 (C=C), 1,472, 1,375, 1,306 (C–N, C–O). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.27 (t, 3H, J = 7 Hz, $\underline{CH_3}CH_2$), 1.94 (m, 2H, CH_2-9), 2.10 (m, 2H, CH_2-8), 2.95 (t, 2H, J = 6.4 Hz, CH_2-10), 4.23 (q, 2H, J = 7 Hz, $\underline{CH_2}CH_3$), 4.54 (t, 2H, J = 6.4, Hz CH_2-7), 4.62 (s, 2H, CH_2-3), 4.73 (s, 2H, $N\underline{CH_2}CO$), 7.38 and 7.43 (2xd, 2x2H, J = 8.8 Hz, *para*-substituted phenyl ring). ^{13}C NMR ($CDCl_3$, 100 MHz): δ14.0 (CH_3), 18.7 (CH_2), 22.5 (CH_2), 22.7 (CH_2), 45.9 (CH_2), 48.3 (CH_2), 54.2 (CH_2), 61.9 (CH_2), 83.8 (C), 113.9 (CN), 116.9 (C), 127.2 (2CH), 129.4 (2CH), 131.9 (C), 132.9 (C), 140.0 (C), 143.2 (C), 159.3 (CO), 166.7 (CO), 167.5 (COO). Anal. Calcd. for $C_{22}H_{21}ClN_4O_4$ (440.88): C, 59.93; H, 4.80; N, 12.71. Found C, 60.14; H, 4.76; N, 12.41.

General procedure for the preparation of **9a**–**9c**

A mixture of compounds **6a**–**c** (2.8 mmol), sodium bicarbonate (0.5 g, 5.9 mmol) and piperidine (0.5 g, 5.6 mmol) in absolute ethanol (10 ml) was refluxed for 8 h. The reaction mixture was filtered while hot, concentrated, cooled, and the separated crystals were collected, dried, and recrystallized from ethanol.

1-Cyano-*N*-phenyl-2-(2-(piperidin-1-yl)acetamido)-5,6,7,8-tetrahydroindolizine-3-carboxamide **9a**

Compound **9a** was prepared from **6a** and piperidine. White crystals, 0.74 g; 66 % yield, m.p. 230-232 °C. IR (KBr, cm^{-1}): $\nu_{max.}$ = 3,306, 3,240 (NHs), 3,054 (C–H aromatic), 2,929, 2,856 (CH_2), 2,220 (CN), 1,654 (COs), 1,570, 1,505 (C=C,NH), 1,444, 1,323 (C–N, C–O). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.48 (m, 2H, CH_2-4″), 1.67 (m, 4H, CH_2-3″–5″), 1.89 (m, 2H, CH_2-7), 1.98 (m, 2H, CH_2-6), 2.63 (t, 4H, J = 5.2 Hz, CH_2-2″–6″), 2.91 (t, 2H, J = 6.2 Hz, CH_2-8), 3.22 (s, 2H, $CO\underline{CH_2}$), 4.29 (t, 2H, J = 5.8 Hz, CH_2-5), 7.12 (t, 1H, J = 7.4 Hz, CH-4′), 7.34 (t, 2H, J = 7.6 Hz, CH-3′–5′), 7.63 (d, 2H, J = 8 Hz, CH-2′, CH-6′), 9.42 (s, H, $\underline{NH}COCH_2$, which disappeared on deuteration) and 9.83 (s, H, $CO\underline{NH}$ phenyl, which disappeared on deuteration). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.0 (CH_2), 22.7 (CH_2), 22.8 (CH_2), 23.4 (CH_2), 26.1 ($2CH_2$), 45.8 (CH_2), 55.0 ($2CH_2$), 62.0 (CH_2), 88.3 (C), 113.8 (CN), 119.3 (2CH), 121.6 (C), 123.5 (C), 124.1 (CH), 129.0 (2CH), 138.3 (C), 140.4 (C), 157.6 (CO), 173.5 (CO). Anal. Calcd. for $C_{23}H_{27}N_5O_2$ (405.49): C, 68.13; H, 6.71; N, 17.27. Found C, 68.40; H, 6.89; N, 17.24.

1-Cyano-2-(2-(piperidin-1-yl)acetamido)-*N*-(*p*-tolyl)-5,6,7,8-tetrahydroindolizine-3-carboxamide **9b**

Compound **9b** was prepared from **6b** and piperidine. Yellowish crystals, 0.8 g; 69 % yield, m.p. 226–228 °C. IR (KBr, cm^{-1}): $\nu_{max.}$ = 3,262, 3,188 (NHs), 3,065 (C–H aromatic), 2,929, 2,852 (CH_3, CH_2), 2,219 (CN), 1,659 (COs), 1,601, 1,545, 1,516 (C=C,NH), 1,450, 1,392, 1,324 (C–N, C–O). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.47 (m, 2H, CH_2-4″), 1.65 (m, 4H, CH_2-3″–5″), 1.89 (m, 2H, CH_2-7), 1.98 (m, 2H, CH_2-6), 2.33 (s, 3H, $\underline{CH_3}$Ph), 2.58 (t, 4H, J = 5.2 Hz, CH_2-2″–6″), 2.91 (t, 2H, J = 6 Hz, CH_2-8), 3.15 (s, 2H, CO$\underline{CH_2}$), 4.27 (t, 2H, J = 5.6 Hz, CH_2-5), 7.13 and 7.5 (2 × d, 2 × 2H, J = 8 Hz, *para*-substituted phenyl ring), 9.34 (s, H, $\underline{NH}COCH_2$, which disappeared on deuteration) and 9.81 (s, H, CO$\underline{NH}$ phenyl, which disappeared on deuteration).^{13}C NMR ($CDCl_3$, 100 MHz): δ 19.0 (CH_2), 20.9 (CH_3), 22.7 (CH_2), 22.7 (CH_2), 23.4 (CH_2), 26.1 ($2CH_2$), 45.7 (CH_2), 55.0 ($2CH_2$), 62.0 (CH_2), 88.2 (C), 113.9 (CN), 119.2 (2CH), 121.5 (C), 123.6 (C), 129.5 (2CH), 133.8 (C), 135.8 (C), 140.3 (C), 157.4 (CO), 173.4 (CO). Anal. Calcd. for $C_{24}H_{29}N_5O_2$ (419.52): C, 68.71; H, 6.97; N, 16.69. Found C, 68.45; H, 7.18; N, 16.39.

N-(4-chlorophenyl)-1-cyano-2-(2-(piperidin-1-yl)acetamido)-5,6,7,8-tetrahydro indolizine-3-carboxamide **9c**

Compound **9c** was prepared from **6c** and piperidine. Yellowish crystals, 0.78 g; 64 % yield, m.p. 253–255 °C. IR (KBr, cm^{-1}): $\nu_{max.}$ = 3,235, 3,181 (NHs), 3,055 (C–H aromatic), 2,933, 2,854 (CH_2), 2,222 (CN), 1,662 (COs), 1,601, 1,547 (C=C,NH), 1,486, 1,454, 1,391 (C–N, C–O). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.47 (m, 2H, CH_2-4″), 1.65 (m, 4H, CH_2-3″–5″), 1.90 (m, 2H, CH_2-7), 1.99 (m, 2H, CH_2-6), 2.59 (t, 4H, J = 5.2 Hz, CH_2-2″–6″), 2.92 (t, 2H, J = 6 Hz, CH_2-8), 3.15 (s, 2H, CO$\underline{CH_2}$), 4.26 (t, 2H, J = 5.4 Hz, CH_2-5), 7.3 and 7.6 (2 × d, 2 × 2H, J = 8.4 Hz, *para*-substituted phenyl ring), 9.39 (s, H, $\underline{NH}COCH_2$, which disappeared on deuteration) and 10.03 (s, H, CO$\underline{NH}$ phenyl, which disappeared on deuteration).^{13}C NMR ($CDCl_3$, 100 MHz): δ 18.9 (CH_2), 22.7 (CH_2), 22.7 (CH_2), 23.4 (CH_2), 26.0 ($2CH_2$), 45.8 (CH_2), 55.0 ($2CH_2$), 62.0 (CH_2), 88.3 (C), 113.7 (CN), 120.5 (2CH), 121.8 (C), 123.2 (C), 129.0 (2CH), 129.0 (C), 136.9 (C), 140.6 (C), 157.5 (CO), 173.6 (CO). Anal. Calcd. for $C_{23}H_{26}ClN_5O_2$ (439.94): C, 62.79; H, 5.96; N, 15.92. Found C, 62.85; H, 6.09; N, 15.62.

General procedure for the preparation of **10a–10c**

A mixture of compounds **5a–c** (3.56 mmol) and *p*-nitrobenzaldehyde (3.56 mmol) in absolute ethanol (20 ml) in the presence of glacial acetic acid (0.5 ml) was refluxed for 4 h. The reaction mixture was then concentrated, set aside to cool, where orange crystals were formed, collected, and recrystallized from ethanol.

1-Cyano-2-((4-nitrobenzylidene)amino)-*N*-phenyl-5,6,7,8-tetrahydroindolizine-3-carboxamide**10a**

Compound **10a** was prepared from **5a** and *p*-nitrobenzaldehyde. Orange crystals, 1.03 g; 70 % yield, m.p. 272–274 °C. IR (KBr, cm^{-1}): $\nu_{max.}$ = 3,278 (NH), 3,073 (C–H aromatic), 2,937 (CH_2), 2,208 (CN), 1,659 (CO), 1,545, 1,478 (C=C,NH), 1,434, 1,339 (C–N, C–O).^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.94 (m, 2H, CH_2-7), 2.06 (m, 2H, CH_2-6), 2.99 (t, 2H, J = 6.2 Hz, CH_2-8), 4.59 (t, 2H, J = 6 Hz, CH_2-5), 7.16 (t, 1H, J = 7.8 Hz, CH-4′), 7.39 (t, 2H, J = 7.6 Hz, CH-3′, -5′), 7.60 (d, 2H, J = 8 Hz, CH-2′, -6′), 8.1 and 8.41 (2 × d, 2 × 2H, J = 8.4 Hz, CH-2″, -3″, -4″, -5″, -6″), 9.25 (s, H, N=CH) and 10.62 (s, 1H, NH, which disappeared on deuteration).^{13}C NMR ($CDCl_3$, 100 MHz): δ 18.6 (CH_2), 22.9 (CH_2), 23.0 (CH_2), 47.2 (CH_2), 81.6 (C), 115.7 (CN), 119.7 (2CH), 120.6 (C), 124.2 (CH), 124.4 (2CH), 129.2 (2CH), 129.2 (2CH), 135.7 (C), 138.1 (C), 140.7 (C), 143.6 (C), 149.7 (C), 157.2 (CH), 158.6 (CO). Anal. Calcd. for $C_{23}H_{19}N_5O_3$ (413.43): C, 66.82; H, 4.63; N, 16.94. Found C, 67.12; H, 4.70; N, 16.91.

1-Cyano-2-((4-nitrobenzylidene)amino)-*N*-(*p*-tolyl)-5,6,7,8-tetrahydroindolizine-3-carboxamide**10b**

Compound **10b** was prepared from **5b** and *p*-nitrobenzaldehyde. Orange crystals, 1.11 g; 73 % yield, m.p. 280–282 °C. IR (KBr, cm^{-1}): $\nu_{max.}$ = 3,279 (NH), 3,069 (C–H aromatic), 2,925, 2,857 (CH_3, CH_2), 2,213 (CN), 1,659 (CO), 1,546, 1,518 (C=C,NH), 1,410, 1,338 (C–N, C–O).^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.93 (m, 2H, CH_2-7), 2.05 (m, 2H, CH_2-6), 2.36 (s, 3H, CH_3Ph), 2.98 (t, 2H, J = 5.8 Hz, CH_2-8), 4.59 (t, 2H, J = 5.4 Hz, CH_2-5), 7.20, 7.48 (2 × d, 2 × 2H, J = 8 Hz, CH-2′, -3′, -4′, -5′, -6′), 8.09 (d, 2H, J = 8 Hz, CH-2″, -6″), 8.37 (d, 2H, J = 8.4 Hz, CH-3″, -5″), 9.23 (s, H, N=CH) and 10.55 (s, 1H, NH, which disappeared on deuteration).^{13}C NMR ($CDCl_3$, 100 MHz): δ 18.6 (CH_2), 20.9 (CH_3), 22.9 (CH_2), 23.0 (CH_2), 47.2 (CH_2), 81.5 (C), 115.8 (CN), 119.7 (2CH), 120.8 (C), 124.3 (2CH), 129.2 (2CH), 129.7 (2CH), 133.9 (C), 135.4 (C), 135.5 (C), 140.7 (C), 143.5 (C), 149.6 (C), 157.0 (CH), 158.5 (CO). Anal. Calcd. for $C_{24}H_{21}N_5O_3$ (427.46): C, 67.44; H, 4.95; N, 16.38. Found C, 67.40; H, 4.85; N, 16.35.

N-(4-chlorophenyl)-1-cyano-2-((4-nitrobenzylidene)amino)-5,6,7,8-tetrahydro indolizine-3-carboxamide **10c**

Compound **10c** was prepared from **5c** and *p*-nitrobenzaldehyde. Yellow crystals, 1.11 g; 70 % yield, m.p. 255–257 °C. IR (KBr, cm^{-1}): $\nu_{max.}$ = 3,274 (NH), 3,064 (C–H aromatic), 2,952, 2,873 (CH_2), 2,209 (CN), 1,666 (CO), 1,539, 1,482 (C=C,NH), 1,404, 1,340 (C–N, C–O). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.96 (m, 2H, CH_2-7), 2.07 (m, 2H, CH_2-6), 3.01 (t, 2H, J = 5.8 Hz, CH_2-8), 4.58 (t, 2H, J = 5.4 Hz, CH_2-5), 7.35 (d, 2H, J = 8 Hz, CH-3′, -5′), 7.57 (d, 2H, J = 8 Hz, CH-2′, -6′), 8.10 (d, 2H, J = 8.4 Hz, CH-2″, -6″), 8.41 (d, 2H, J = 8.4 Hz, CH-3″, -5″), 9.28 (s, H, N=CH) and 10.67 (s, 1H, NH, which disappeared on deuteration).^{13}C NMR ($CDCl_3$, 100 MHz): δ 18.7 (CH_2), 22.9 (CH_2), 23.0 (CH_2),

47.3 (CH_2), 81.5 (C), 115.9 (CN), 120.5 (2CH), 120.8 (C), 125.1 (2CH), 130.4 (2CH), 131.2 (2CH), 134.3 (C), 135.8 (C), 135.5 (C), 140.7 (C), 143.7 (C), 149.7 (C), 157.3 (CH), 158.8 (CO). Anal. Calcd. for $C_{23}H_{18}ClN_5O_3$ (447.87): C, 61.68; H, 4.05; N, 15.64. Found C, 61.98; H, 4.10; N, 15.34.

2-Methyl-4-oxo-3-phenyl-3,4,6,7,8,9-hexahydropyrimido[4,5-b]indolizine-10-carbonitrile **11**

A mixture of compound **5a** (3.56 mmol) and excess triethylorthoacetate was refluxed for 12 h. The mixture was then evaporated under reduced pressure and the residue was crystallized from ethanol. 0.62 g; 58 % yield, m.p. 244–246 °C. IR (KBr, cm^{-1}): $\nu_{max.} = 3{,}062$ (C–H aromatic), 2,954 (CH_3, CH_2), 2,215 (CN), 1,688 (CO), 1,555 (C=C), 1,422, 1,314 (C–N, C–O).^{1}H-NMR ($CDCl_3$, 400 MHz): δ 1.96 (m, 2H, CH_2-8), 2.07 (m, 2H, CH_2-7), 2.24 (s, 3H, $\underline{CH_3}$C=N), 3.08 (t, 2H, $J = 6.2$ Hz, CH_2-9), 4.45 (t, 2H, $J = 6$ Hz, CH_2-6), 7.24 (d, 2H, $J = 7.6$ Hz, CH-2′, -6′) and 7.56 (t, 3H, $J = 7$ Hz, CH-3′, -4′, -5′).^{13}C NMR ($CDCl_3$, 100 MHz): δ 18.9 (CH_2), 22.4 (CH_2), 23.0 (CH_2), 24.3 (CH_3), 45.6 (CH_2), 85.3 (C), 113.9 (CN), 115.1 (C), 128.0 (2CH), 129.4 (CH), 130.0 (2CH), 137.4 (C), 144.8 (C), 146.5 (C), 154.3 (C), 154.68 (CO). Anal. Calcd. for $C_{18}H_{16}N_4O$ (304.35): C, 71.04; H, 5.30; N, 18.41. Found C, 71.17; H, 5.39; N, 18.41.

Biological activity

Cytotoxic activity of the newly synthesized compounds was evaluated using sulphorhodamine-B (SRB) assay method and all the target compounds were screened against three cancer cell lines in addition to the non-tumorous cell line. Hep3B, MCF7, and A549 cancer cell lines were obtained from the American Type Culture Collection (ATCC, Minnesota, USA) through the Tissue Culture Unit, The Egyptian Organization for Biological Products and Vaccines (Vacsera, Egypt). Reagents and chemicals were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA). Anticancer activity evaluation was performed at the Center for Genetic Engineering, Al-Azhar University, Cairo, Egypt.

Cells were seeded for 24 h in 96-well microtiter plates at a concentration of 1,000–2,000 cells/well, 100 μl/well, then cells were incubated for 48 h with various concentrations (0, 6.25, 12.5, 25, 50, 100 μg/ml) of the tested compounds, three wells were used for each concentration, after incubation for 48 h the cells were fixed with 10 % trichloroacetic acid 150 μl/well for 1 h at 4 °C, washed by distilled water for three times. Wells were stained for 10-30 min at room temperature with 0.4 % SRB, dissolved in 1 % acetic acid 70 μl/well, washed with acetic acid 1 % to remove unbound dye until a colorless drainage was obtained. The plates were subjected to air drying for 24 h not exposed to UV. The dye was solubilized with 150 μl/well of 10 mM Trise-EDTA (pH 7.4) for 5 min on a shaker at 1,600 rpm. The optical density of each well was measured spectrophotometrically at 545 nm with an ELISA microplate reader. The percent of surviving cells was calculated and plotted against different concentrations of the tested compounds to obtain the survival curve. The IC_{50} values were calculated using sigmoidal concentration–

response curve fitting models (SigmaPlot software). Selectivity index was calculated by dividing the IC_{50} value of the tested compound against the normal fibroblast cells over that against the cancer cell line (Table 1).

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