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Original article

New pyrazoles incorporating pyrazolylpyrazole moiety: Synthesis, anti-HCV and antitumor activity

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ABSTRACT

Three series of novel pyrazole derivatives **2b–d**, **4a–d** and **6a–d** were synthesized *via* two step procedure that utilizes hydrazonoyl chlorides **1a–d** and enaminones **3a–d** and **5a–d**, respectively as starting materials. The structures of all the newly synthesized products have been established on the basis of analytical and spectral data. Moreover, some of the products **2–6** were tested against HCV and Subacute Sclerosing Panencephalitis (SSPE). In addition, compounds **2–6** were also tested for the inhibition of peroxynitrite-induced tyrosine nitration and antioxidant activity. The tested compounds are highly effective at very low concentration as anti-HCV, SSPE antioxidant and anticancer in the following ascending order **2d**, **4c**, **6b**, **3b**, **6c**, **4d**, **2b**, **2c**, **2a**, **6a**, **5b**, **5a**, **3a**, **4b** and **5c**. It is worth to mention that all tested compounds are more potent than the reference standards used for comparing activity. All the measurements revealed that the mechanism of action of the anti cancer activities of all the tested compounds is topoisomerase I inhibitor.

1. Introduction

A survey of literature revealed that pyrazoles belong among the most representative five-membered heterocyclic systems [1–3]. Numerous synthetic pyrazole derivatives found use in various pharmaceutical, agrochemicals, photographic and other applications. Such examples of important pyrazole derivatives are natural products (*S*)-pyrazolylalanine [4], pyrazomycin [5] and synthetic compounds sildenafil, ionazolac [6–8], difenamizole [9–11], mepirizole [12–14], phenidone [15–17] and bicyclic pyrazolidinone LY 186826 [18–26]. On the other hand, synthesis of heterocyclic compounds represents the major topic of our research interest [27–37]. So, based on these findings, we prepare in this context, a series of novel pyrazole derivatives containing one and two pyrazole rings as substituents, using enaminones and hydrazonoyl chloride as starting materials. Our objective from synthesis of these pyrazole derivatives is to study the effect of introducing pyrazole moiety in position-4 on the biological activity of target compounds.

2. Chemistry

The starting materials 1-(1-aryl-4-benzoyl-1*H*-pyrazol-3-yl) ethanones **2a–d** were prepared in one step by reaction of 1-phenyl-3-(*N*,*N*-dimethylamino)-propenone with *N*-aryl 2-oxo-propane-hydrazonoyl chlorides **1a–d** in dioxane and in the presence of triethylamine under reflux as previously described [38]. Reactions of compounds **2a–d** with dimethylformamide–dimethylacetal (DMF–DMA) in dry toluene under reflux for 10 h, afforded products that were identified as 1-(1-aryl-4-benzoyl-1*H*-pyrazol-3-yl)-3-dimethylamino-propenones **3a–d** (Scheme 1). The structures of the latter products were established from their microanalyses, mass, IR and ^{1}H NMR spectra (see Experimental). When each of **3a–d** was reacted with *N*-phenyl 3-oxo-propane-hydrazonoyl chloride **1a** in dioxane in the presence of triethylamine under reflux, a single product was obtained in each case as evidenced by TLC analysis of the crude product. The microanalyses and mass spectral data of the isolated products are in consistent with the assigned structure **4** (cf. Scheme 1). Besides, the IR spectra of products **4** revealed in each case three absorption bands for the carbonyl groups near ύ 1699–1685, 1681–1673 and 1653–1642 cm^{-1} (see Experimental). ^{1}H NMR spectra exhibited one singlet signal near δ 2.69–2.63 ppm due to the acetyl protons, in addition to the signals at δ 9.18–9.05 and 8.94–8.86 ppm assigned for the proton at position-5 for each

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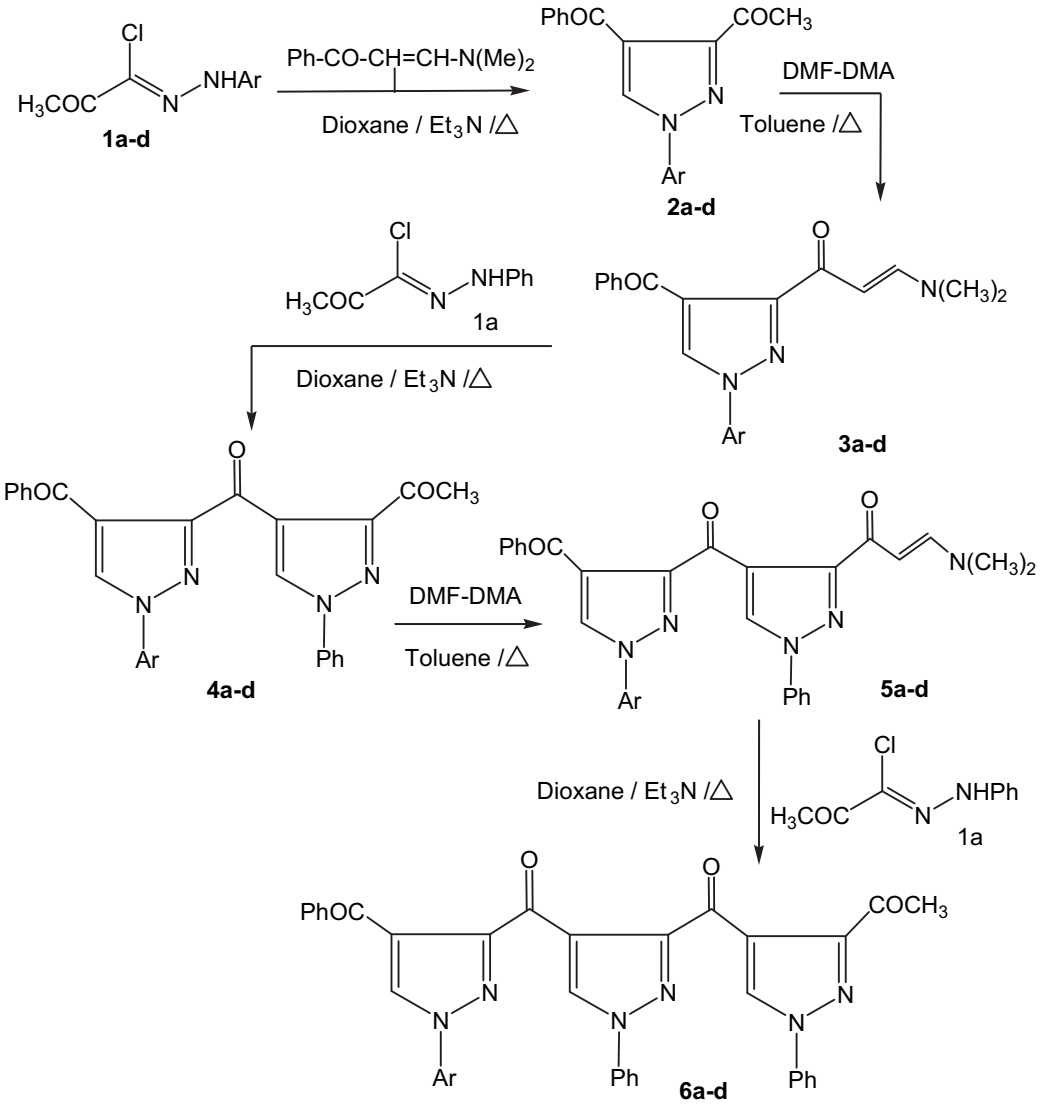


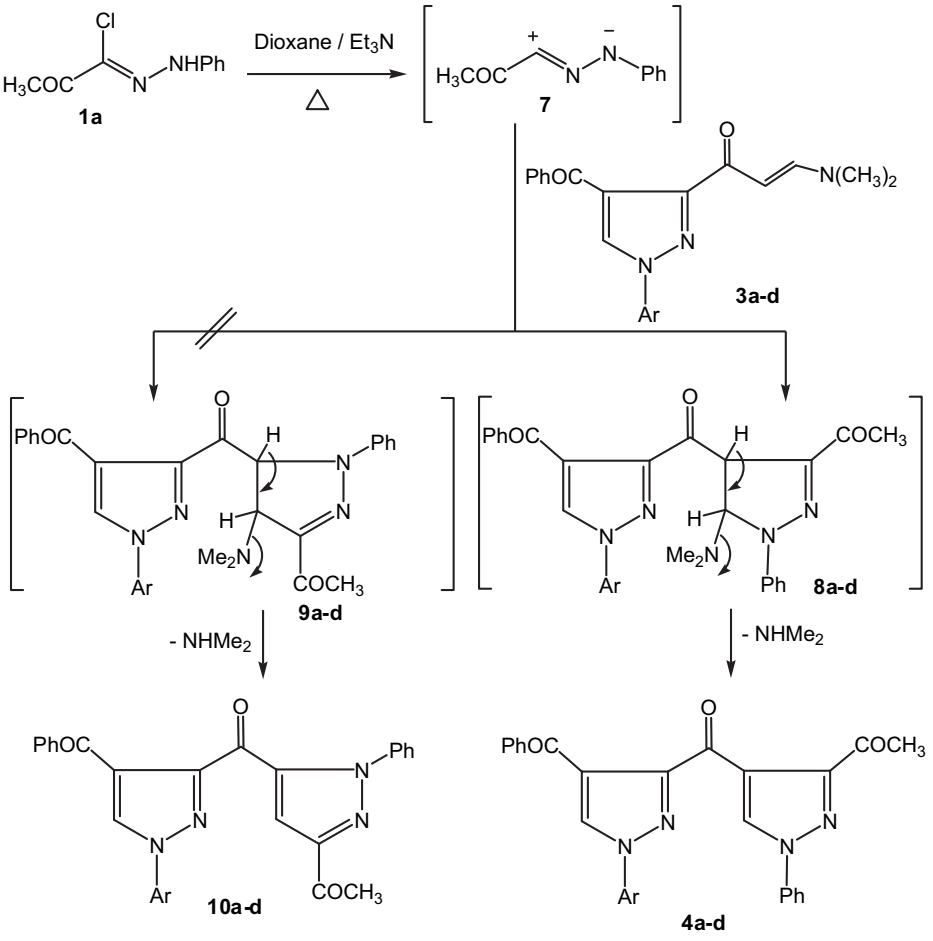
Scheme 1. Synthesis of pyrazoles **2–6**.

pyrazole ring. In a similar manner pyrazole derivatives **6a–d** were prepared following the same sequence of reactions as for products **4** (cf. Scheme 1). Thus, reaction of each of **4a–d** with dimethylformamide–dimethylacetal in dry toluene under reflux for 10 h afforded the enaminones **5a–d**. The structures of the latter products were established based on microanalyses and spectral data (see Experimental). For example, ^{1}H NMR spectra of products **5a–d** showed in each case two characteristic singlet signals near δ 5.87–5.81 and 7.78–7.68 ppm assignable to the olefinic protons (CH═CH) of the enaminone residue, in addition to the signals of the two protons at position-5 of pyrazole rings. Reaction of compounds **5a–d** with *N*-phenyl 2-oxo-propane-hydrazonoyl chloride **1a** in dioxane under reflux for 8 h in the presence of triethylamine afforded pyrazole derivatives **6a–d** in high yields. The structures of the latter products were evidenced by their microanalyses and spectroscopic (mass, IR, ^{1}H NMR) data (see Experimental).

It is interesting to suggest a mechanism for the reaction of enaminones with hydrazonoyl chlorides to know the regioselectivity of such reaction. To achieve this, we have taken reaction of enaminones **3a–d** with *N*-phenyl 2-oxo-propane-hydrazonoyl chloride **1a** as a typical example for the prepared compounds. As depicted in Scheme 2, it is suggested that the reaction of **3** with **1** starts with a regioselective 1,3-dipolar cycloaddition of the *N*-phenyl-*C*-acetylnitrilimine intermediate **7**, generated *in situ* by base-catalyzed dehydrohalogenation of the hydrazonoyl chloride **1a**, to the carbon–carbon double bond of the enaminones **3a–d** to afford the non-isolable cycloadducts **8a–d**. Elimination of dimethylamine from the latter intermediates **8** yielded the corresponding pyrazole derivatives **4a–d** as end products and the other isomers **10a–d** were discarded. This is because the ^{1}H NMR spectra of such products revealed, in each case, two singlet signals for the pyrazole ring proton in the region δ 9.18–9.05 and 8.94–8.86 ppm assignable to pyrazole-5H. Literature reports [39,40] indicated that the ^{1}H NMR spectra of 5-unsubstituted pyrazoles exhibit the characteristic singlet signals of 5-CH at δ 8.12–9.0 ppm, while, the 4-unsubstituted pyrazoles showed the 4-CH signal at δ 5.81–5.89 [41,42].

3. Pharmacology

Some pyrazole derivatives have anticancer activities [43]. So, we devoted our study to investigate the anticancer, anti-HCV and



Ar : **a**, C_6H_5; **b**, 4-$CH_3C_6H_4$; **c**, 4-ClC_6H_4; **d**, 4-$NO_2C_6H_4$

Scheme 2. The mechanism of reaction of hydrazonoyl halide with enaminone.

antioxidant activities of some representative examples of the newly synthesized compounds **2**–**6**.

3.1. Results and discussion

3.1.1. Hepatitis C Virus (HCV) NS3-4A protease inhibitory activities in both HCV replicon cells and in hamster brains

In order to monitor the potential of fifteen new synthesized compounds to inhibit HCV we used two methods for determination of minimum inhibitory concentration (MIC) of these compounds. Firstly, their antiviral activities in HCV Replicon Cells were measured by their Hepatitis C Virus (HCV) NS3-4A protease inhibitory activities. Secondly, their antiviral chemotherapy for Subacute Sclerosing Panencephalitis (SSPE) was determined in Hamster Brains. Ribaverin was used in both methods as positive control. Table 1 showed the MIC values for the tested compounds and ribaverin against HCV and SSPE. The results showed that all the tested compounds are highly effective at very low concentration compared to ribaverin in the following ascending order **2d**, **4c**, **6b**, **3b**, **6c**, **4d**, **2b**, **2c**, **2a**, **6a**, **5b**, **5a**, **3a**, **4b** and **5c**. It is worth to mention that all tested compounds are more potent than Ribaverin.

3.1.2. DPPH radical scavenging activity

Radical scavenging activities of new synthesized compounds were measured using model colorimetric test: DPPH radical scavenging test. The results are summarized in Table 2. The stable radical DPPH has been used widely for the determination of primary antioxidant activity, that is, the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts and food materials. The assay is based on the reduction of DPPH radicals in methanol which causes an absorbance drop at 515 nm. Antioxidant activity of the tested compounds using DPPH method

Table 1
MIC of Ribaverin and the tested compounds against HCV and SSPE.

Tested compound	MIC μg/ml	
	HCV	Subacute Sclerosing Panencephalitis (SSPE)
Ribaverin	16.15	77.89
2a	0.162	3.615
2b	0.159	3.424
2c	0.161	3.516
2d	0.144	3.089
3a	0.32	4.00
3b	0.150	3.178
4b	0.322	4.120
4c	0.147	3.111
4d	0.155	3.216
5a	0.291	3.992
5b	0.181	3.821
5c	0.323	4.150
6a	0.171	3.714
6b	0.149	3.123
6c	0.151	3.198

Table 2
Decrease of DPPH absorbance (%) by the tested compounds.

Compound number	Decrease of DPPH absorbance (%) [mean ± SD ($n = 3$)]
Ascorbic acid (standard)	79.042 ± 2.928
2a	95.16 ± 0.031
2b	95.80 ± 0.045
2c	95.55 ± 0.023
2d	98.19 ± 0.024
3a	94.55 ± 0.044
3b	96.60 ± 0.066
4b	94.00 ± 0.089
4c	97.70 ± 0.043
4d	95.98 ± 0.066
5a	94.65 ± 0.033
5b	94.90 ± 0.056
5c	93.00 ± 0.054
6a	95.00 ± 0.033
6b	96.90 ± 0.056
6c	96.11 ± 0.055

exhibited good scavenging activity against the DPPH radical in the following descending order **2d**, **4c**, **6b**, **3b**, **6c**, **4d**, **2b**, **2c**, **2a**, **6a**, **5b**, **5a**, **3a**, **4b** and **5c**. It is worth to mention that all tested compounds are more potent than ascorbic acid.

3.1.3. Inhibition of peroxynitrite-induced tyrosine nitration

The reactive nitrogen species peroxynitrite ($ONOO^-$) has been implicated in numerous human disease pathologies and its role is usually inferred from the measurement of 3-nitrotyrosine (3-NT). Peroxynitrite and species derived from it can oxidise nitrate lipids [44], proteins [45], DNA [46,47] and carbohydrates [48] leading to tissue damage in a number of pathological conditions in humans and in experimental animals. Due to the cytotoxicity of ($ONOO^-$) and its apparent formation at sites of tissue injury [49,50], there has been considerable interest in the ability of natural and synthetic antioxidants to diminish ($ONOO^-$) damage. In the present work, the ability of the synthetic compounds to prevent ($ONOO^-$)-mediated damage was examined using the model system: ($ONOO^-$)-mediated tyrosine nitration. When the amino acid tyrosine is exposed to ($ONOO^-$) at pH 7.4, 3-NT is formed [51]. The relative inhibitory activity of these compounds is summarized in Table 3 as IC_{50} values. Under our experimental conditions the relative potencies of the tested compounds are in ascending order **2d**, **4c**, **6b**, **3b**, **6c**, **4d**, **2b**, **2c**, **2a**, **6a**, **5b**, **5a**, **3a**, **4b** and **5c**. It is worth to mention that all tested compounds are more potent than Trolox (Table 3).

Table 3
IC_{50} values of the compounds tested for the inhibition of ($ONOO^-$)-mediated 3-nitrotyrosine formation.

Compound number	IC_{50} (μM) [mean ± SD ($n = 3$)]
Trolox	58.430 ± 5.9
2a	39.502 ± 0.0376
2b	37.777 ± 0.0399
2c	38.123 ± 0.0388
2d	31.542 ± 0.0212
3a	43.987 ± 0.0334
3b	34.201 ± 0.0245
4b	44.123 ± 0.0356
4c	32.189 ± 0.0212
4d	36.550 ± 0.0289
5a	43.981 ± 0.0333
5b	42.167 ± 0.0343
5c	44.550 ± 0.0466
6a	42.111 ± 0.0354
6b	33.983 ± 0.0233
6c	35.150 ± 0.0267

Table 4
Antitumor activities of the tested compounds.

Compound No	Average GI50	Relative potency to etoposide
2a	0.0178	168.5393258
2b	0.0159	188.6792453
2c	0.0168	178.5714286
2d	0.0115	260.8695652
3a	0.022	136.3636364
3b	0.01152	260.4166667
4b	0.078	38.46153846
4c	0.0115	260.8695652
4d	0.0154	194.8051948
5a	0.021	142.8571429
5b	0.02	150
5c	0.079	37.97468354
6a	0.0199	150.7537688
6b	0.01151	260.6429192
6c	0.0153	196.0784314

3.1.4. In vivo efficacy study

The *in vivo* activity of the tested compounds and Etoposide against murine colon cancer Colon 38 was tested (Table 4). Treatment of Colon 38 implanted into mice with ER-37328 induced a clear dose-dependent inhibition of tumor growth. Moreover, marked tumor regression was observed at 3 mg/kg. No body weight was observed at 3 mg/kg. All the tested compounds showed potent antitumor activities with the order of activity in descending order **2d**, **4c**, **6b**, **3b**, **6c**, **4d**, **2b**, **2c**, **2a**, **6a**, **5b**, **5a**, **3a**, **4b** and **5c**. The cytotoxicity results of the tested compounds compared to the activity of etoposide where the tested compound active at low concentration and also at the same level of toxicity of etoposides where the author had mentioned no death or no weight variation so the toxicity nearly more or less equal to that of etoposide.

In a progress to determine the mechanism of action of the antitumor activities, the correlation coefficients with topoisomerase I inhibitor, and cisplatin, calculated according to the NCI COMPARE analysis procedure using the tested compounds as the benchmark, were measured (Table 5). These calculations culminated on the newly synthesized compounds act as topoisomerase I inhibitors. The correlation coefficients are indicative measure to the possibility of the mechanism of the antitumor activity, where as the value is high to certain drug, this means that the compound is similar in mechanism of action to this drug and vice-versa. Upon this rational the values of correlation coefficients of the tested

Table 5
The correlation coefficients of the newly synthesized compounds with topoisomerase I inhibitors.

Compound number	The correlation coefficients with (topoisomerase I inhibitor), and cisplatin, calculated according to the NCI COMPARE analysis procedure using the tested compounds as the benchmark			
	Etoposide	Doxorubicin	SN-38	Cisplatin
2a	17.14	16.77	15.98	14.66
2b	20.11	19.12	18.76	17.24
2c	18.16	18.11	17.67	16.18
2d	31.12	30.14	30.000	29.12
3a	5.11	4.88	4.67	3.66
3b	27.18	27.01	26.15	25.24
4b	4.78	4.11	3.90	3.16
4c	30.11	29.11	28.16	27.28
4d	21.15	21.00	20.15	20.00
5a	13.67	12.13	11.88	10.12
5b	15	14.88	13.99	12.13
5c	4.12	4.00	3.89	3.14
6a	16.18	15.88	14.78	13.88
6b	28.56	28.16	27.14	26.15
6c	22.13	22.00	21.17	20.16

Table 6
The relative potencies of the newly synthesized compounds to Etoposide.

Compound number	Relative potency to etoposide
2a	168.50
2b	188.60
2c	178.50
2d	260.80
3a	136.30
3b	260.40
4b	38.40
4c	260.80
4d	194.80
5a	142.80
5b	150
5c	37.90
6a	150.70
6b	260.60
6c	196.00

agents is high near the side of etoposide and low in other side of antitumor agents so we can got to the final conclusion is the confirmation of the mechanism of action of the tested agent is similar to that of etoposide as topoisomerase II inhibitor.

3.1.5. Inhibition of topoisomerase II activity

The effects of the tested compounds and etoposide on topoisomerase II activity were analyzed by means of relaxation assay. The tested compounds and etoposide inhibited topoisomerase II activity and the relative potency of each compound were determined and confirmed in the following descending order **2d**, **4c**, **6b**, **3b**, **6c**, **4d**, **2b**, **2c**, **2a**, **6a**, **5b**, **5a**, **3a**, **4b** and **5c** (Table 6).

All the previous mentioned measurements revealed that all the tested compounds are more active than Etoposide and act similarly to its mechanism of action as topoisomerase I inhibitor.

4. Conclusion

A series of pyrazole derivatives incorporating one and two pyrazole rings as substituent were prepared using hydrazonoyl chlorides and enaminones as starting materials. It is worth noting that the presence of different substituents on the aromatic rings in position-1 of parent pyrazole is responsible for the remarkable difference in the inhibition activity against HCV and Topoisomerase II.

The unsubstituted phenyl group has the highest activity than substituted ones, and the 4-nitrogroup greatly reduces the activities than the 4-methyl one.

Moreover, the newly synthesized compounds were found to have radical scavenging activity than ascorbic acid. Based on the results of biological activity, we can conclude that the new synthetic compounds are more potent than the reference standards and are promising candidates to further advance drug discovery efforts.

5. Experimental protocol

5.1. Chemistry

Melting points are uncorrected. IR spectra were recorded (KBr) on Pye Unicam SP-1000 Spectrophotometer. ^{1}H NMR spectra were obtained on Varian Gemini 300 MHz spectrometer using TMS as internal reference and DMSO-d_6 as solvent. Chemical shifts are expressed as δ (ppm). Mass spectra were recorded on a GCMS-QP 1000 EX mass spectrometer operating at 70 eV. The elemental analyses were performed at the Microanalytical Center at Cairo University, Giza, Egypt. Hydrazonoyl chloride **1a–d** was prepared as following the literature method [52].

*5.2. Synthesis of 1-(1-aryl-4-benzoyl-1H-pyrazol-3-yl)-ethanone **2a–d***

To a mixture of equimolar amounts of enaminone and the appropriate hydrazonoyl chlorides **1a–d** (50 mmol of each) in dioxane (100 mL) was added triethylamine (7 mL, 50 mmol). The reaction mixture was refluxed for 8 h. The solvent was evaporated and the residue was washed by petroleum ether and treated with methanol. The solid that formed was filtered and crystallized from ethanol to give compounds **2a–d**.

*5.2.1. 1-(4-Benzoyl-1-phenyl-1H-pyrazol-3-yl)-ethanone (**2a**)*

Yellow crystals, (11.6 g, 80%), mp 133–135 °C (Lit. mp 132 °C) [21]; IR (KBr) υ = 1687, 1650 (2 CO), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 2.57 (s, 3H, $COCH_3$), 7.23–7.91 (m, 10H, Ar–H), 9.01 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 290 (M^+, 25), 213 (20), 105 (50), 77 (100). *Anal.* Calcd. for $C_{18}H_{14}N_2O_2$ (290.32): C, 74.47; H, 4.86; N, 9.65. Found: C, 74.29; H, 4.68; N, 9.58%.

*5.2.2. 1-[4-benzoyl-1-(4-methylphenyl)-1H-pyrazol-3-yl]-ethanone (**2b**)*

Yellow crystals, (11.9 g, 80%), mp 150–152 °C; IR (KBr) υ = 1685, 1649 (2 CO), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 2.24 (s, 3H, Ar–CH_3), 2.53 (s, 3H, $COCH_3$), 7.29–8.05 (m, 9H, Ar–H), 9.02 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 304 (M^+, 20), 227 (25), 105 (40), 77 (100). *Anal.* Calcd. for $C_{19}H_{16}N_2O_2$ (304.35): C, 74.98; H, 5.30; N, 9.20. Found: C, 74.82; H, 5.22; N, 9.34%.

*5.2.3. 1-[4-benzoyl-1-(4-chlorophenyl)-1H-pyrazol-3-yl]-ethanone (**2c**)*

Yellow crystals, (13.3 g, 82%), mp 178–180 °C; IR (KBr) υ = 1688, 1644 (2 CO), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 2.57 (s, 3H, $COCH_3$), 7.49–8.02 (m, 5H, Ar–H), 8.04 (d, 2H, *J* = 8 Hz, Ar-H), 8.05 (d, 2H, *J* = 8 *Hz*, Ar–H), 9.03 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 326 (M^+ + 2, 6), 325 (M^+ + 1, 12), 324 (M^+, 20), 281 (40), 111 (20), 105 (50), 77 (100). *Anal.* Calcd. for $C_{18}H_{13}ClN_2O_2$ (324.77): C, 66.57; H, 4.03; N, 8.63. Found: C, 66.39; H, 4.12; N, 8.54%.

*5.2.4. 1-[4-benzoyl-1-(4-nitrophenyl)-1H-pyrazol-3-yl]-ethanone (**2d**)*

Brown crystals, (12.6 g, 75%), mp 217–219 °C; IR (KBr) υ = 1693, 1639 (2 CO), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 2.59 (s, 3H, $COCH_3$), 7.49–8.02 (m, 5H, Ar–H), 8.14 (d, 2H, *J* = 8 *Hz*, Ar–H), 8.21 (d, 2H, *J* = 8 *Hz*, Ar–H), 9.08 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 337 (M^+ + 2, 15), 336 (M^+ + 1, 30), 335 (M^+, 40), 258 (50), 105 (60), 77 (100). *Anal.* Calcd. for $C_{18}H_{13}N_3O_4$ (335.32): C, 64.48; H, 3.91; N, 12.53. Found: C, 64.39; H, 4.11; N, 12.44%.

*5.3. Synthesis of 1-(1-aryl-4-benzoyl -1H-pyrazol-3-yl)-3-dimethylamino-propenones (**3a–d**)*

A mixture of 1-(4-benzoyl-1-aryl-1*H*-pyrazol-3-yl)-ethanones **2a–d** (30 mmol of each) and dimethylformamide–dimethylacetal (DMF–DMA) (7 mL) in dry toluene (50 mL) was refluxed for 10 h. The solvent was evaporated under reduced pressure and methanol was added to the reaction mixture. The solid product was collected by filtration and crystallized from ethanol to give products **3a–d**.

*5.3.1. 1-(4-Benzoyl-1-phenyl-1H-pyrazol-3-yl)-3-dimethylamino-propenone (**3a**)*

Dark orange crystals, (8.8 g, 85%), mp 215–217 °C; IR (KBr) υ = 1683, 1640 (2 CO), cm^{-1}; ^{1}H NMR (DMSO-d_6) δ = 2.84 (s, 3H, CH_3),

2.96 (s, 3H, CH_3), 5.84 (d, 1H, J = 13 *Hz*, CH═), 7.23–7.99 (m, 10H, Ar–H), 7.65 (d, 1H, J = 13 *Hz*, CH═), 8.89 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 345 (M^+, 15), 240 (16), 105 (40), 98 (60), 77 (80), 70 (100). *Anal*. Calcd. for $C_{21}H_{19}N_3O_2$ (345.40): C, 73.03; H, 5.54; N, 12.17. Found: C, 73.11; H, 5.68; N, 12.28%.

*5.3.2. 1-[4-benzoyl-1-(4-methylphenyl)-1H-pyrazol-3-yl]-3-dimethylamino-propenone (**3b**)*

Orange crystals, (8.6 g, 80%), mp 187–189 °C; IR (KBr) υ = 1677, 1641 (2 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.36 (s, 3H, Ar–CH_3), 2.87 (s, 3H, CH_3), 3.08 (s, 3H, CH_3), 5.75 (d, 1H, J = 13 *Hz*, CH═), 7.34–7.50 (m, 5H, Ar–H), 7.65 (d, 1H, J = 13 *Hz*, CH═), 7.78 (d, 2H, J = 8 *Hz*, Ar–H), 7.85 (d, 2H, J = 8 *Hz*, Ar-H), 8.81 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 359 (M^+, 40), 254 (40), 105 (30), 98 (60), 77 (50), 70 (100). *Anal*. Calcd. for $C_{22}H_{21}N_3O_2$ (359.43): C, 73.52; H, 5.89; N, 11.69. Found: C, 73.38; H, 5.78; N, 11.48%.

*5.3.3. 1-[4-benzoyl-1-(4-chlorophenyl)-1H-pyrazol-3-yl]-3-dimethylamino-propenone (**3c**)*

Orange crystals, (9.12 g, 80%), mp 230–232 °C; IR (KBr) υ = 1680, 1639 (2 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.88 (s, 3H, CH_3), 3.09 (s, 3H, CH_3), 5.89 (d, 1H, J = 13 *Hz*, CH═), 7.46–7.59 (m, 5H, Ar–H), 7.65 (d, 1H, J = 13 *Hz*, CH═), 7.99 (d, 2H, J = 8 *Hz*, Ar–H), 8.03 (d, 2H, J = 8 *Hz*, Ar–H), 8.90 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 381 (M^+ + 2, 18), 380 (M^+ + 1, 25), 379 (M^+, 40), 274 (40), 105 (30), 98 (60), 77 (50), 70 (100). *Anal*. Calcd. for $C_{21}H_{18}ClN_3O_2$ (379.85): C, 66.40; H, 4.78; N, 11.06. Found: C, 66.31; H, 4.68; N, 11.18%.

*5.3.4. 1-[4-benzoyl-1-(4-nitrophenyl)-1H-pyrazol-3-yl]-3-dimethylamino-propenone (**3d**)*

Yellow crystals, (9.12 g, 78%), mp 195–197 °C; IR (KBr) υ = 1677, 1641 (2 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.89 (s, 3H, CH_3), 3.11 (s, 3H, CH_3), 5.89 (d, 1H, J = 13 *Hz*, CH═), 7.42–7.64 (m, 5H, Ar–H), 7.78 (d, 1H, J = 13 *Hz*, CH═), 8.23 (d, 2H, J = 8 *Hz*, Ar–H), 8.43 (d, 2H, J = 8 *Hz*, Ar–H), 9.09 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 392 (M^+ + 2, 18), 391 (M^+ + 1, 25), 390 (M^+, 40), 285 (40), 105 (30), 98 (60), 77 (50), 70 (100). *Anal*. Calcd. for $C_{21}H_{18}N_4O_4$ (390.40): C, 64.61; H, 4.65; N, 14.35. Found: C, 64.51; H, 4.48; N, 14.19%.

*5.4. Synthesis of 1-[1-aryl-4-(4-benzoyl-1H-pyrazole-3-carbonyl)-1-phenyl-1H-pyrazol-3-yl]-ethanone **4a–d***

To a mixture of 1-(1-aryl-4-benzoyl-1*H*-pyrazol-3-yl)-3-dimethylamino-propenones (**3a–d**) (20 mmol of each) and *N*-phenyl-2-oxo-propanhydrazonoyl chloride (**1a**) (3.93 g, 20 mmol) in dioxane (50 mL), triethylamine (2.8 mL, 20 mmol) was added. The reaction mixture was refluxed for 8 h. The solvent was evaporated and the residue was washed by petroleum ether and treated with methanol. The solid that formed was filtered and crystallized from ethanol to give compounds **4a–d**.

*5.4.1. 1-[4-(4-benzoyl-1-phenyl-1H-pyrazole-3-carbonyl)-1-phenyl-1H-pyrazol-3-yl]-ethanone (**4a**)*

Pale yellow crystals, (7.5 g, 82%), mp 127–129 °C; IR (KBr) υ = 1685, 1678, 1642 (3 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.65 (s, 3H, $COCH_3$), 7.38–7.98 (m, 15H, Ar–H), 8.86 (s, 1H, pyrazole-H-5), 9.14 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 460 (M^+, 25), 417 (16), 355 (40), 105 (40), 77 (100). *Anal*. Calcd. for $C_{28}H_{20}N_4O_3$ (460.50): C, 73.03; H, 4.38; N, 12.17. Found: C, 73.10; H, 4.28; N, 12.28%.

*5.4.2. 1-{4-[4-benzoyl-1-(4-methylphenyl)-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-ethanone (**4b**)*

Pale yellow crystals, (7.5 g, 80%), mp 145–147 °C; IR (KBr) υ = 1692, 1673, 1648 (3 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.35 (s, 3H, Ar–CH_3), 2.63 (s, 3H, $COCH_3$), 7.46–7.99 (m, 14H, Ar–H), 8.89 (s, 1H, pyrazole-H-5), 9.05 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 474 (M^+, 17), 431 (20), 369 (15), 105 (100), 77 (80). *Anal*. Calcd. for $C_{29}H_{22}N_4O_3$ (474.52): C, 73.40; H, 4.67; N, 11.81. Found: C, 73.52; H, 4.58; N, 11.78%.

*5.4.3. 1-{4-[4-benzoyl-1-(4-chlorophenyl)-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-ethanone (**4c**)*

Brown crystals, (7.9 g, 80%), mp 93–95 °C; IR (KBr) υ = 1698, 1679, 1643 (3 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.69 (s, 3H, $COCH_3$), 7.46–7.79 (m, 10H, Ar–H), 7.95 (d, 2H, J = 8 *Hz*, Ar–H), 8.01 (d, 2H, J = 8 *Hz*, Ar–H), 8.90 (s, 1H, pyrazole-H-5), 9.15 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 496 (M^+ + 2, 12), 495 (M^+ + 1, 20), 494 (M^+, 30), 452 (16), 390 (40), 105 (50), 77 (100). *Anal*. Calcd. for $C_{28}H_{19}ClN_4O_3$ (494.94): C, 67.95; H, 3.87; N, 11.32. Found: C, 67.82; H, 3.78; N, 11.18%.

*5.4.4. 1-{4-[4-benzoyl-1-(4-nitrophenyl)-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-ethanone (**4d**)*

Yellow crystals, (7.8 g, 78%), mp 94–96 °C; IR (KBr) υ = 1699, 1681, 1653 (3 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.69 (s, 3H, $COCH_3$), 7.48–7.81 (m, 10H, Ar–H), 7.98 (d, 2H, J = 8 *Hz*, Ar–H), 8.05 (d, 2H, J = 8 *Hz*, Ar–H), 8.94 (s, 1H, pyrazole-H-5), 9.18 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 505 (M^+, 5), 462 (16), 300 (40), 105 (80), 77 (100). *Anal*. Calcd. for $C_{28}H_{19}N_5O_5$ (505.49): C, 66.53; H, 3.79; N, 13.85. Found: C, 66.32; H, 3.71; N, 13.82%.

*5.5. Synthesis of 1-[1-aryl-4-(4-benzoyl-1H-pyrazole-3-carbonyl)-1-phenyl-1H-pyrazol-3-yl]-3-dimethylamino-propenones **5a–d***

A mixture of 1-[1-aryl-4-(4-benzoyl-1*H*-pyrazole-3-carbonyl)-1-phenyl-1*H*-pyrazol-3-yl]-ethanones **4a–d**. (10 mmol of each) and dimethylformamide–dimethylacetal (DMF–DMA) (5 mL) in dry toluene (30 mL) was refluxed for 10 h. The solvent was evaporated under reduced pressure and the residue was triturated with methanol. The solid product formed was collected by filtration and crystallized from ethanol to give products **5a–d**.

*5.5.1. 1-[4-(4-benzoyl-1-phenyl-1H-pyrazole-3-carbonyl)-1-phenyl-1H-pyrazol-3-yl]-3-dimethylamino-propenone (**5a**)*

Brown crystals, (4.12 g, 80%), mp 210–212 °C; IR (KBr) υ = 1683, 1674, 1640 (3 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.86 (s, 3H, CH_3), 2.98 (s, 3H, CH_3), 5.81 (d, 1H, J = 13 *Hz*, CH═), 7.36–7.92 (m, 15H, Ar–H), 7.68 (d, 1H, J = 13 *Hz*, CH═), 8.85 (s, 1H, pyrazole-H-5), 9.09 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 515 (M^+, 25), 417 (16), 309 (10), 105 (40), 77 (100), 70 (35). *Anal*. Calcd. for $C_{31}H_{25}N_5O_3$ (515.58): C, 72.22; H, 4.89; N, 13.58. Found: C, 72.10; H, 4.78; N, 13.38%.

*5.5.2. 1-{4-[4-benzoyl-1-(4-methylphenyl)-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-3-dimethylamino-propenone (**5b**)*

Brown crystals, (4.2 g, 80%), mp 80–82 °C; IR (KBr) υ = 1684, 1678, 1647 (3 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.36 (s, 3H, Ar–CH_3), 2.84 (s, 3H, CH_3), 3.05 (s, 3H, CH_3), 5.81 (d, 1H, J = 13 *Hz*, CH═), 7.31–8.06 (m, 14H, Ar–H), 7.78 (d, 1H, J = 13 *Hz*, CH═), 8.96 (s, 1H, pyrazole-H-5), 9.01 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 529 (M^+, 35), 431 (16), 240 (40), 105 (100), 98 (80), 77 (70). *Anal*. Calcd. for $C_{32}H_{27}N_5O_3$ (529.60): C, 72.57; H, 5.14; N, 13.22. Found: C, 72.48; H, 5.22; N, 13.38%.

*5.5.3. 1-{4-[4-benzoyl-1-(4-chlorophenyl)-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-3-dimethylamino-propenone (**5c**)*

Yellow crystals, (4.4 g, 80%), mp 118–120 °C; IR (KBr) υ = 1689, 1677, 1643 (3 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.98 (s, 3H, CH_3), 3.17 (s, 3H, CH_3), 5.85 (d, 1H, J = 13 *Hz*, CH═), 7.51–7.99 (m, 10H, Ar–H), 7.68 (d, 1H, J = 13 *Hz*, CH═), 7.98 (d, 2H, J = 8 *Hz*, Ar–H), 8.05 (d,

2H, $J = 8$ *Hz*, Ar–H), 8.86 (s, 1H, pyrazole-H-5), 9.09 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 552 ($M^+ + 2$, 16), 551 ($M^+ + 1$, 20), 550 (M^+, 30), 445 (16), 309 (10), 105 (80), 98 (100), 77 (40). *Anal*. Calcd. for $C_{31}H_{24}ClN_5O_3$ (550.02): C, 67.70; H, 4.40; N, 12.73. Found: C, 67.58; H, 4.28; N, 12.68%.

5.5.4. 1-{4-[4-benzoyl-1-(4-nitrophenyl)-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-3-dimethylamino-propenone (**5d**)

Dark orange crystals, (4.2 g, 75%), mp 102–104 °C; IR (KBr) υ = 1691, 1672, 1644 (3 CO), cm^{-1}; ^1H NMR (DMSO-d_6) $\delta = 2.98$ (s, 3H, CH_3), 3.18 (s, 3H, CH_3), 5.87 (d, 1H, $J = 13$ *Hz*, CH═), 7.54–7.99 (m, 10H, Ar–H), 7.69 (d, 1H, $J = 13$ *Hz*, CH═), 8.08 (d, 2H, $J = 8$ *Hz*, Ar–H), 8.25 (d, 2H, $J = 8$ *Hz*, Ar–H), 8.89 (s, 1H, pyrazole-H-5), 9.17 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 560 (M^+, 30), 455 (26), 311 (10), 240 (20), 105 (100), 98 (40), 77 (50). *Anal*. Calcd. for $C_{31}H_{24}N_6O_5$ (560.57): C, 66.42; H, 4.32; N, 14.99. Found: C, 66.48; H, 4.18; N, 14.89%.

5.6. Synthesis of 1-{4-[1-aryl-4-(4-benzoyl-1H-pyrazole-3-carbonyl)-1-phenyl-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-ethanones **6a–d**

To a mixture of 1-[4-(1-aryl-4-benzoyl-1*H*-pyrazole-3-carbonyl)-1-phenyl-1*H*-pyrazol-3-yl]-3-dimethylamino-propenones **5a–d** (5 mmol of each) and *N*-phenyl-2-oxo-propane-hydrazonoyl chloride (**1a**) (0.98 g, 5 mmol) in dioxane (15 mL) was added triethylamine (0.7 mL, 5 mmol). The reaction mixture was refluxed for 8 h. The solvent was evaporated and the residue was washed by petroleum ether and treated with methanol. The solid that formed was filtered and crystallized from ethanol to give products **6a–d**.

5.6.1. 1-{4-[4-(4-benzoyl-1-phenyl-1H-pyrazole-3-carbonyl)-1-phenyl-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-ethanone (**6a**)

Yellowish brown crystals, (2.5 g, 80%), mp 94–96 °C; IR (KBr) υ = 1692, 1682, 1678, 1643 (4 CO), cm^{-1}; ^1H NMR (DMSO-d_6) δ = 2.61 (s, 3H, $COCH_3$), 7.26–7.97 (m, 20H, Ar–H), 8.86 (s, 1H, pyrazole-H-5), 9.02 (s, 1H, pyrazole-H-5), 9.14 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 630 (M^+, 15), 525 (20), 275 (40), 240 (20), 105 (100), 77 (70). *Anal*. Calcd. for $C_{38}H_{26}N_6O_4$ (630.67): C, 72.37; H, 4.16; N, 13.33. Found: C, 72.18; H, 4.23; N, 13.25%.

5.6.2. 1-{4-[4-[4-benzoyl-1-(4-methylphenyl)-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-ethanone (**6b**)

Brown crystals, (2.5 g, 80%), mp 86–88 °C; IR (KBr) υ = 1690, 1685, 1679, 1648 (4 CO), cm^{-1}; ^1H NMR (DMSO-d_6) $\delta = 2.34$ (s, 3H, Ar–CH_3), 2.65 (s, 3H, $COCH_3$), 7.16–7.95 (m, 19H, Ar–H), 8.89 (s, 1H, pyrazole-H-5), 9.01 (s, 1H, pyrazole-H-5), 9.12 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 644 (M^+, 15), 539 (20), 474 (40), 240 (20), 105 (100), 77 (70). *Anal*. Calcd. for $C_{39}H_{28}N_6O_4$ (644.70): C, 72.66; H, 4.38; N, 13.04. Found: C, 72.51; H, 4.42; N, 13.09%.

5.6.3. 1-{4-[4-[4-benzoyl-1-(4-chlorophenyl)-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-ethanone (**6c**)

Brown crystals, (2.6 g, 80%), mp 86–88 °C; IR (KBr) υ = 1694, 1687, 1679, 1648 (4 CO), cm^{-1}; ^1H NMR (DMSO-d_6) $\delta = 2.68$ (s, 3H, $COCH_3$), 7.06–7.95 (m, 19H, Ar–H), 8.89 (s, 1H, pyrazole-H-5), 9.03 (s, 1H, pyrazole-H-5), 9.15 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 667 ($M^+ + 2$, 6), 666 ($M^+ + 1$, 12), 665 (M^+, 15), 560 (20), 466 (20), 240 (40), 105 (80), 77 (100). *Anal*. Calcd. for $C_{38}H_{25}ClN_6O_4$ (665.11): C, 68.62; H, 3.79; N, 12.64. Found: C, 68.51; H, 3.62; N, 12.49%.

5.6.4. 1-{4-[4-[4-benzoyl-1-(4-nitrophenyl)-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazole-3-carbonyl]-1-phenyl-1H-pyrazol-3-yl}-ethanone (**6d**)

Brown crystals, (2.5 g, 75%), mp 90–92 °C; IR (KBr) υ = 1692, 1687, 1682, 1648 (4 CO), cm^{-1}; ^1H NMR (DMSO-d_6) $\delta = 2.69$ (s, 3H, $COCH_3$), 7.06–7.95 (m, 19H, Ar–H), 8.89 (s, 1H, pyrazole-H-5), 9.06 (s, 1H, pyrazole-H-5), 9.19 (s, 1H, pyrazole-H-5) ppm; MS, *m*/*z* (%) 675 (M^+, 12), 570 (20), 240 (30), 105 (80), 77 (100). *Anal*. Calcd. for $C_{38}H_{25}N_7O_6$ (675.67): C, 67.55; H, 3.73; N, 14.51. Found: C, 67.41; H, 3.68; N, 14.39%.

5.7. Determination of minimum inhibitory concentration (MIC), of ribavirin and different tested compounds in HCV replicon cells was performed as follow

Briefly, 1×10^4 replicon cells per well were plated in 96-well plates. On the following day, replicon cells were incubated at 37 °C for the indicated period of time with antiviral agents serially diluted in DMEM plus 2% FBS and 0.5% dimethyl sulfoxide (DMSO). Total cellular RNA was extracted using an RNeasy-96 kit (QIAGEN, Valencia, CA), and the copy number of HCV RNA was determined using a quantitative RTPCR (QRT-PCR) assay. Each datum point represents the average of five replicates in cell culture. The cytotoxicity of tested compounds was measured under the same experimental settings using a tetrazolium (MTS)-based cell viability assay (Promega, Madison, WI). For the cytotoxicity assay with human hepatocyte cell lines, 1×10^4 parental Huh-7 cells per well or 4×10^4 HepG2 cells per well were used.

5.8. MIC of the tested compounds in hamster brains for antiviral chemotherapy for Subacute Sclerosing Panencephalitis (SSPE)

Under ether anesthesia, 50 ml of ribavirin or tested compound solutions at dosages of 5, 10, and 20 mg/kg/day was injected for 10 days intracranially to a depth of 2 mm by using a 27-gauge needle and was placed within the subarachnoid space. At 1, 2, 3, 5, 7, 10, 12, 15, and 20 days after the initial injection, four hamsters from each group were sacrificed. The brains were aseptically removed, washed twice with phosphate-buffered saline (PBS), homogenized, and suspended in PBS. The suspension was centrifuged at 1600 3 g for 10 min. The supernatant was collected, ethanol was added to remove proteins, and the mixture was heated at 90 °C to evaporate the ethanol. The protein-free samples were used to evaluate the MIC in brain tissue by HPLC and bioassay.

5.9. Radical scavenging assays

5.9.1. DPPH radical scavenging assay

The antioxidant activity of the tested compounds and the standard were assessed on the basis of the radical scavenging effect of the stable DPPH• free radical [30]. Weighed quantities of the tested compounds were dissolved in distilled DMSO and used. Solution of ascorbic acid used as standard for this study was prepared in distilled H_2O. All these solutions were serially diluted with respective solvents to get lower dilutions. 10 μl of each compound or standard (from 0.0 μM/ml to 100 μM/ml) was added to 90 μl of DPPH• in methanol solution (100 μM) in a 96-well microtitre plate. After incubation in the dark at 37 °C for 30 min, the decrease in absorbance of each solution was measured at 515 nm using ELISA micro plate reader (Bio Rad Laboratories Inc., California, U.S.A., Model 550). Absorbance of blank sample containing the same amount of DMSO and DPPH• solution was prepared and measured as well. The experiment was carried out in triplicate. The scavenging potential was compared with a solvent control (0% radical scavenging) and ascorbic acid. Radical scavenging activity

was calculated by the following formula: % Reduction of absorbance $= [(A_B - A_A)/A_B] \times 100$, where: A_B: absorbance of blank sample; A_A: absorbance of tested extract solution ($t = 30$ min).

5.10. Reaction with reactive nitrogen species

5.10.1. Synthesis of peroxynitrite

Peroxynitrite ($ONOO^-$) was synthesized as previously described [42]. Briefly, an acidic solution (0.6 M HCl) of H_2O_2 (0.7 M) was mixed with KNO_2 (0.6 M) on ice for 1 s and the reaction quenched with ice-cold NaOH (1.2 M). Residual H_2O_2 was removed by mixing with granular MnO_2 prewashed with NaOH (1.2 M). The stock solution was filtered and then frozen overnight (−20 °C) and the top layer of the solution collected for the experiment. Concentrations of stock ($ONOO^-$) were redetermined before each experiment at 302 nm using a molar absorption coefficient of 1670 cm^{-1} M^{-1}. Concentrations of 200–250 mM were usually obtained. Once thawed, ($ONOO^-$) solutions were kept in ice for no longer than 30 min before use.

5.11. Reaction of tested compounds with peroxynitrite

The ability of tested compounds to inhibit peroxynitrite-induced tyrosine nitration was investigated *via* reaction of equimolar concentrations (100 μM) of tyrosine and peroxynitrite in the presence of increasing concentrations of each tested compound (0–300 μM) in 100 mM phosphate buffer, pH 7.4 at 37 °C for 15 min. Snap-freezing reaction mixtures prior to HPLC analysis successfully terminated reactions. The formation of 3-nitrotyrosine (3-NT) was monitored by HPLC analyzed with photodiode array detection (see below). 3-NT formed was characterised and quantified by use of an authentic standard (elution time and unique spectral characteristics).

5.12. HPLC analysis

Reaction mixtures were analyzed using reverse-phase HPLC. Analysis was performed on an Agilent 1100 system with a Zorbax ODS C18 column (150 × 4.6 mm i.d., 4 μm) and guard column (15 × 4.6 mm i.d., 4 μm). Mobile phase A consisted of methanol/water/5 N HCl (5/94.9/0.1 v/v/v) and mobile phase B of acetonitrile/water/5 N HCl (50/49.9/0.1 v/v/v). The following gradient system was used (min/% acetonitrile): 0/0, 5/0, 40/50, 60/100, 65/100, and 65.1/0 with a flow rate of 0.7 ml/min. The eluent was monitored by photodiode array detection at 280 nm for 3-NT measurements with spectra of products obtained over the 220–600 nm range.

5.13. Anticancer activities

5.13.1. Novel topoisomerase II inhibitors

The tested compounds inhibited topoisomerase II activity at 10 times lower concentration than etoposide in relaxation assay and Cells. Murine colon cancer Colon 38, murine leukemia P388 and human lung cancer LX-1 were obtained from the Cancer Chemotherapy Center, Japan Foundation for Cancer Research (Tokyo, Japan). Human lung cancer PC-1 and human gastric cancer MKN-1, MKN-7, MKN-28, and MKN-74 were obtained from Immuno Biology Laboratory (Gunma, Japan). Human lung cancer A549 and human colon cancer WiDr were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). Human gastric cancer HGC-27 and GT3TKB were obtained from Riken Cell Bank (Ibaraki, Japan). The other cell lines were purchased from American Type Culture Collection (Rockville, MD).

5.13.2. Growth-inhibitory effect

Exponentially growing solid tumor cells in 0.1 ml of medium were seeded in 96-well plates on day 0. On day 1, 0.1 ml aliquots of medium containing graded concentrations of test drugs were added to the cell plates. After incubation at 37 °C for 72 h, the cell number was estimated by sulforhodamine B (SRB) assay, as described by Skehan et al. Separately, the cell number on day 1 was also measured. GI50 values are the drug concentrations causing a 50% reduction in the net protein increase in control cells. The antitumor spectrum of ER-37328 was compared with those of other drugs by means of the NCI COMPARE analysis procedure. Exponentially growing P388 cells were seeded in 96-well plates on day 0. On day 1,0.1 ml aliquots of medium containing graded concentrations of test drugs were added to the wells. After incubation at 37 °C for 72 h, the cell number was determined by MTT assay. IC_{50} values were calculated as the drug concentrations that reduced the number of cells to 50% of the control number.

5.13.3. In vivo efficacy study

Female C57BL/6 mice (6 weeks of age; Charles River, Atsugi, Japan) were housed in barrier facilities on a 12 h light/dark cycle, with food and water ad libitum. About 30 mg of Colon 38 tumor tissue was inoculated s.c. into the flank of animals. Mice were randomized and separated into control and treatment ($n = 5$ each) groups when the tumor volume reached approximately 300 mm^3 on day 1. The tested compound was dissolved in 5% glucose. Etoposide was diluted in saline and doxorubicin was dissolved in saline. The tested compound was administrated on day 1 (single dose) by i.v. injection into the tail vein, and etoposide and doxorubicin were given at the maximum tolerated dose on the reported administration schedule. Control animals received 5% glucose. Tumor volume and body weight were measured on the days indicated in the figures. Tumor volume was calculated according to the following equation: tumor volume (mm^3) = (length−$width^2$)/2.

5.13.4. Relaxation assay

Topoisomerase II was purified from P388 cells. One unit of the enzyme was defined as the activity to relax completely 0.125 g of supercoiled pBR-322 DNA at 30 °C for 1 h. For the assay, 0.125 μg of supercoiled pBR-322 DNA (Takara Shuzo Co., Ltd., Tokyo, Japan) was relaxed with 1 unit of topoisomerase II in 20 μl of the assay buffer [50 mM Tris–HCl (pH 8.0), 100 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.5 μM EDTA, 1 mM ATP, and 30 μg/ml BSA] in the presence or absence of an inhibitor at 30 °C for 1 h. Samples were subjected to electrophoresis in 0.7% agarose gels with TBE buffer [89 mM Tris, 89 mM boric acid, and 2 mM EDTA (pH 8.0)]. The DNA was stained with ethidium bromide and photographed under UV light.

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