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ORIGINAL PAPER

Synthesis and anti-rotavirus activity of some nitrogen heterocycles integrated with pyrazole scaffold

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Abstract
Herein, the building block synthon 2-cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acryloyl chloride **4** was synthesized and utilized for construction of a wide variety of nitrogen heterocycles encompassing a pyrazole scaffold via treatment with some nitrogen nucleophiles. The seven membered heterocycles **14**–**16** and **20** were constructed from the reaction of the acid chloride **4** with 1,4-binucleophilic reagents such as 2-aminophenol, 2-aminoaniline, 2-aminothiophenol, and thiosemicarbazide, respectively. The structures of all the synthesized heterocycles were elucidated from their elemental and spectral analysis. Antiviral activity screening of products obtained against rotavirus (RV) revealed that compounds **3**, **12**, **13**, **15**, and 1**6** exhibited high reduction effect on RV titer of ≥ 2 $\log_{10}$ $TCID_{50}$. Thus, these compounds can be possible candidates of anti-RV agents.

Keywords Rotavirus · Diarrhea · Pyrazole · Thiazolopyrimidine · Benzothiazepine

Introduction

Pyrazoles can be traced in a number of well-established drugs and different categories with diverse therapeutic activities and occupy a prime position in medicinal and pesticide chemistry [1–12]. For instance, the antiviral activity of ethyl 5-amino-1-(4*b*,5,10,10*a*-tetrahydronaphtho[2′,3′:4,5]thieno[2,3-d]pyrimidin-4-yl)-1*H*-pyrazole-4-carboxylate (**I**) (Fig. 1) revealed the highest anti-HAV activity at a concentration of 20 µg/10^5 cells, in comparison with the other tested compounds [5]. Pyrazole analogues have been found to be used as building blocks in organic synthesis for designing pharmaceuticals and agrochemicals and as bifunctional ligands for metal catalysis. The recent pharmaceutical applications of 2-propenoylamides [13, 14] and other heterocyclic skeletons have stimulated us to synthesize new derivatives of these classes of compounds hoping to obtain structures with possibly enhanced antiviral potency.

In spite of over 40 years of clinical and basic research on rotavirus (RV) following their discovery in 1973, RV infection continues to be a worldwide health concern in human [15]. RV, a member of the Reoviridae family, is a major cause of serious gastroenteritis and diarrhea in young kids [16] and is responsible for about one-third of all diarrheal cases requiring hospital admission [17]. RV gastroenteritis results in > 500,000 deaths per year in children < 5 years [18]. Although two approved oral, live, attenuated RV vaccines (Rotarix and RotaTeq) are available internationally, > 90% of rotavirus-mediated childhood mortalities are recorded within developing countries due to limited access to these vaccines, sanitation concerns, high cost, and lack of availability to routine healthcare services [19]. Thus, it is necessary to develop a new antiviral drug with high safety, low cost, and high efficacy against RV infection in which our study is aimed to.

Results and discussion

Chemistry

In continuation of our efforts toward constructing heterocyclic compounds and evaluating their pharmaceutical efficiency [20–29], the present work investigated the proclivity of high functionality compound:

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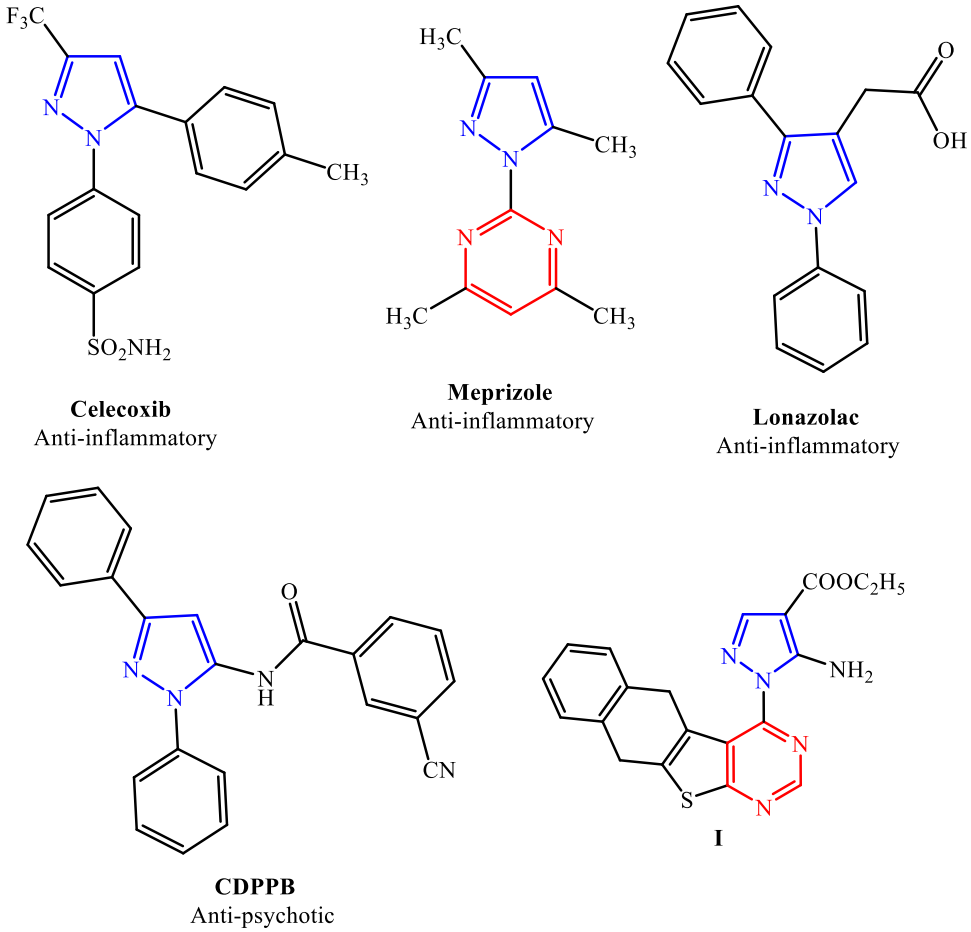
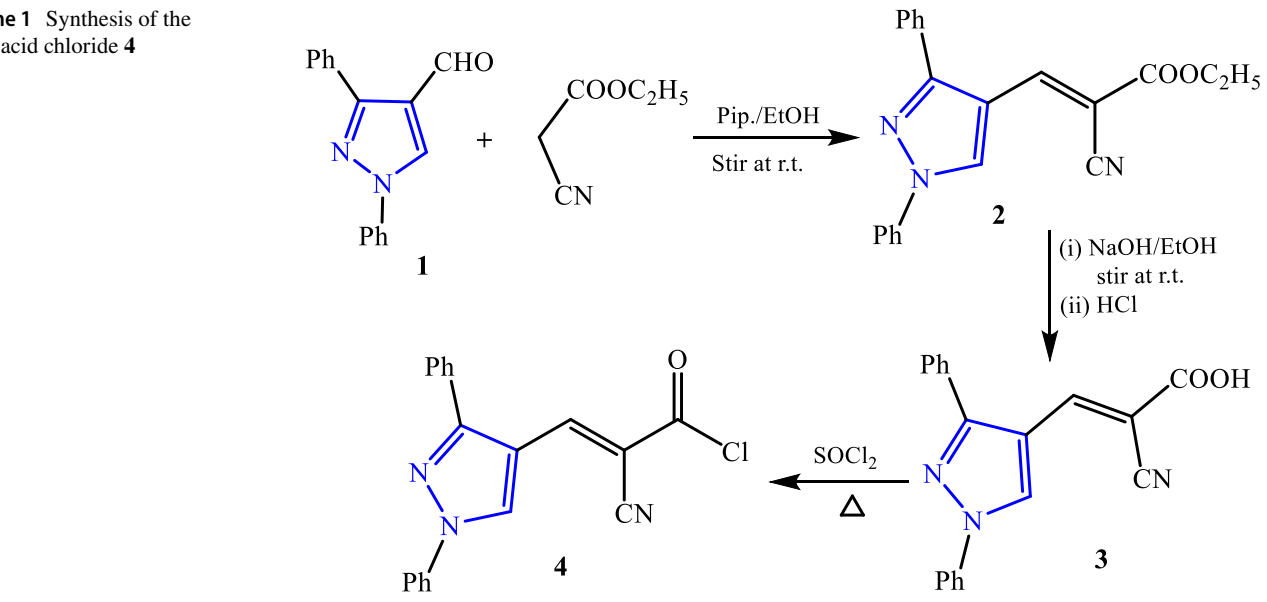


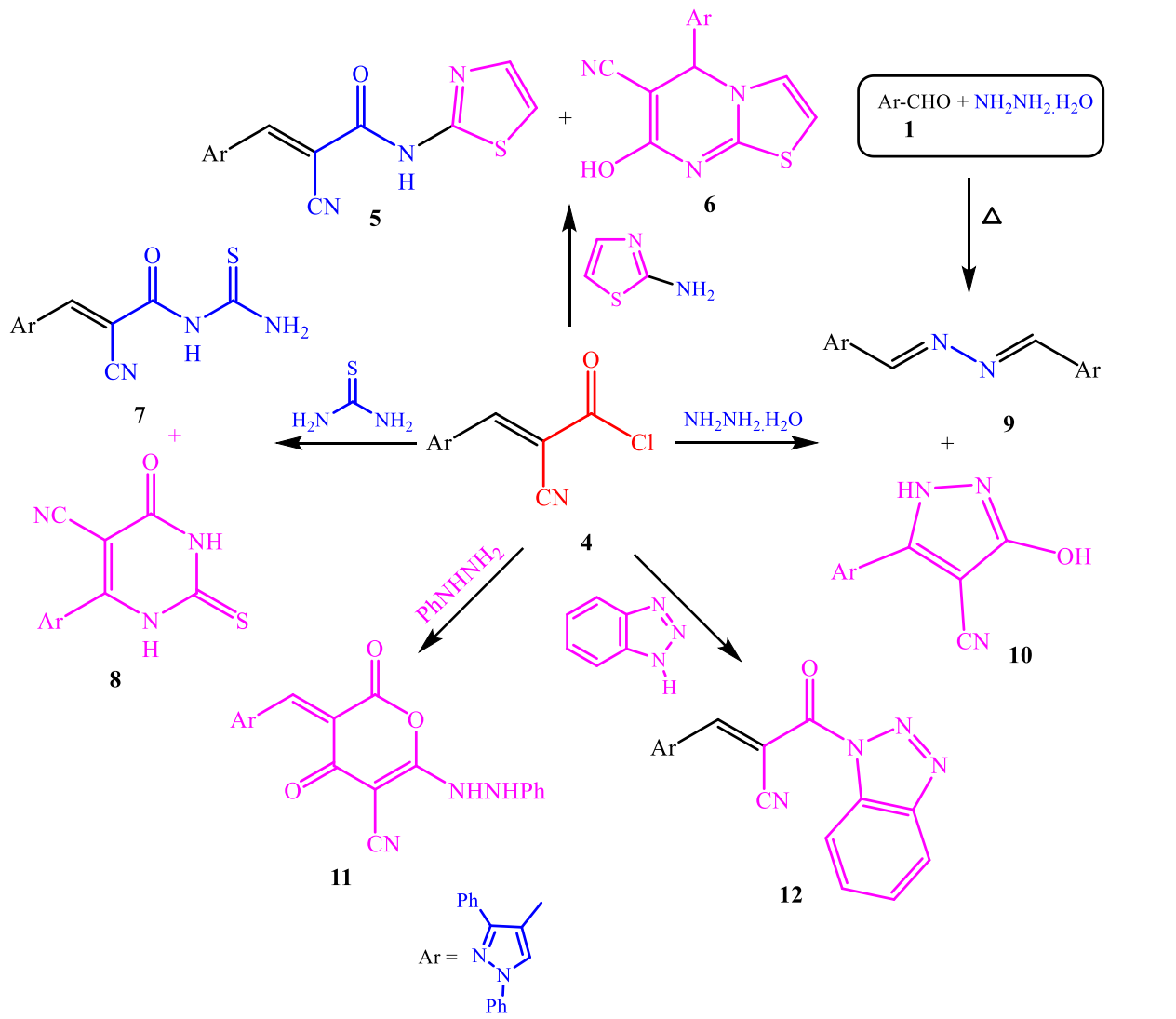
Fig. 1 Pyrazole derivatives with diverse therapeutic activities

2-cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acryloyl chloride (**4**) with some nitrogen nucleophiles. Condensation of pyrazole aldehyde **1** with ethyl cyanoacetate afforded the corresponding arylidene derivative **2** [30, 31] which was easily transformed into the corresponding acid **3** via hydrolysis using ethanolic sodium hydroxide solution followed by acidification with dilute hydrochloric acid (20%). Treating the acid **3** with thionyl chloride acquired the targeted acid chloride **4** (Scheme 1). The chemical structures of compounds **3** and **4** were elucidated from their analytical and spectral data. Thus, IR spectrum of the acid **3** revealed the absence of ester carbonyl group and showed the characteristic absorption bands for OH and C=O of the carboxylic moiety at ν 3453 and 1697 cm^{-1}, respectively. Also, the IR spectrum of acid chloride **4** displayed the absence of OH group and the appearance of a carbonyl group at ν 1754 cm^{-1}. The ^{1}H-NMR spectra were completely consistent with the assigned structures. Furthermore, the mass spectrum of **4** supported its structure as it exhibited the correct molecular ion peak at *m/z* 333.20 (52.03%) in addition to [$M^{+\cdot}$+2] peak at *m/z* 335.11 (15.44%) attributable for the presence of chlorine atom (cf. "Experimental" section).

The proclivity of the acid chloride **4** was studied toward some *N*-nucleophiles, such as 1,3-binucleophiles including 2-aminothiazole and thiourea as well as 1,2-binucleophiles including hydrazine and phenylhydrazine (Scheme 2). Thus, treatment of the acid chloride **4** with 2-aminothiazole in refluxing benzene and in the presence of triethylamine as a base afforded the open-chain compound, the amide derivative **5** as well as the fused heterocyclic compound, thiazolopyrimidine derivative **6** (Scheme 2). The IR spectrum of compound **5** retained the stretching absorption band of the nitrile function, which rules out its cyclization on it. Meanwhile, its ^{1}H-NMR spectrum retained the singlet signal of the olefinic proton at δ 8.09 ppm. Furthermore, its mass spectrum strongly confirmed the assigned structure by showing the correct molecular ion peak as the base peak at *m/z* 397 (100%). IR spectrum of compound **6** lacked to ν C=O and provided the characteristic absorption bands of cyano and hydroxyl groups. Also, its ^{1}H-NMR spectrum lacked to the olefinic proton singlet and displayed a new singlet for methine proton which supported the cyclization step. Presumably, the reaction pathway of compound **4** with 2-aminothiazole is illustrated in Scheme 3.

Scheme 1 Synthesis of the target acid chloride **4**



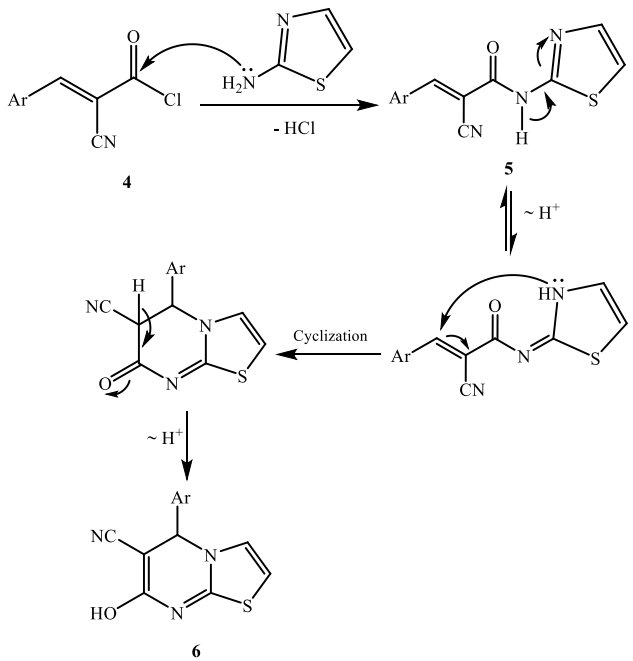


Scheme 2 Reaction of acid chloride **4** with some nitrogen nucleophiles

On the other hand, thiourea reacted with acid chloride **4** to furnish a mixture of thiourea derivative **7** as canary yellow crystals and tetrahydropyrimidinethione derivative **8** as deep yellow crystals which were separated by fractional recrystallization (cf. "Experimental" section). The spectral data were fit with the assigned structures. The ^{1}H-NMR spectrum of compound **7** provided exchangeable singlet signals for NH and NH_2 protons.

It was interesting that hydrazinolysis of the acid chloride **4** at 0 °C afforded a mixture of diheterylazine **9** as pale yellow crystals and 5-hydroxy-1′,3′-diphenyl-1′*H*,2*H*-[3,4′-bipyrazole]-4-carbonitrile (**10**) as yellow crystals (Scheme 2). The chemical structure of the azine **9** was evidenced from its spectral data and supported by direct comparison with an authentic sample prepared from refluxing ethanolic solution of the pyrazole aldehyde **1** with hydrazine [3]. The ^{1}H-NMR spectrum of pyrazole derivative **10** displayed the disappearance of the olefinic hydrogen, and the presence of two labile hydrogen atoms corresponds to both NH and OH protons. The hydrazinolysis of acid chloride **4** is displayed in Scheme 4.

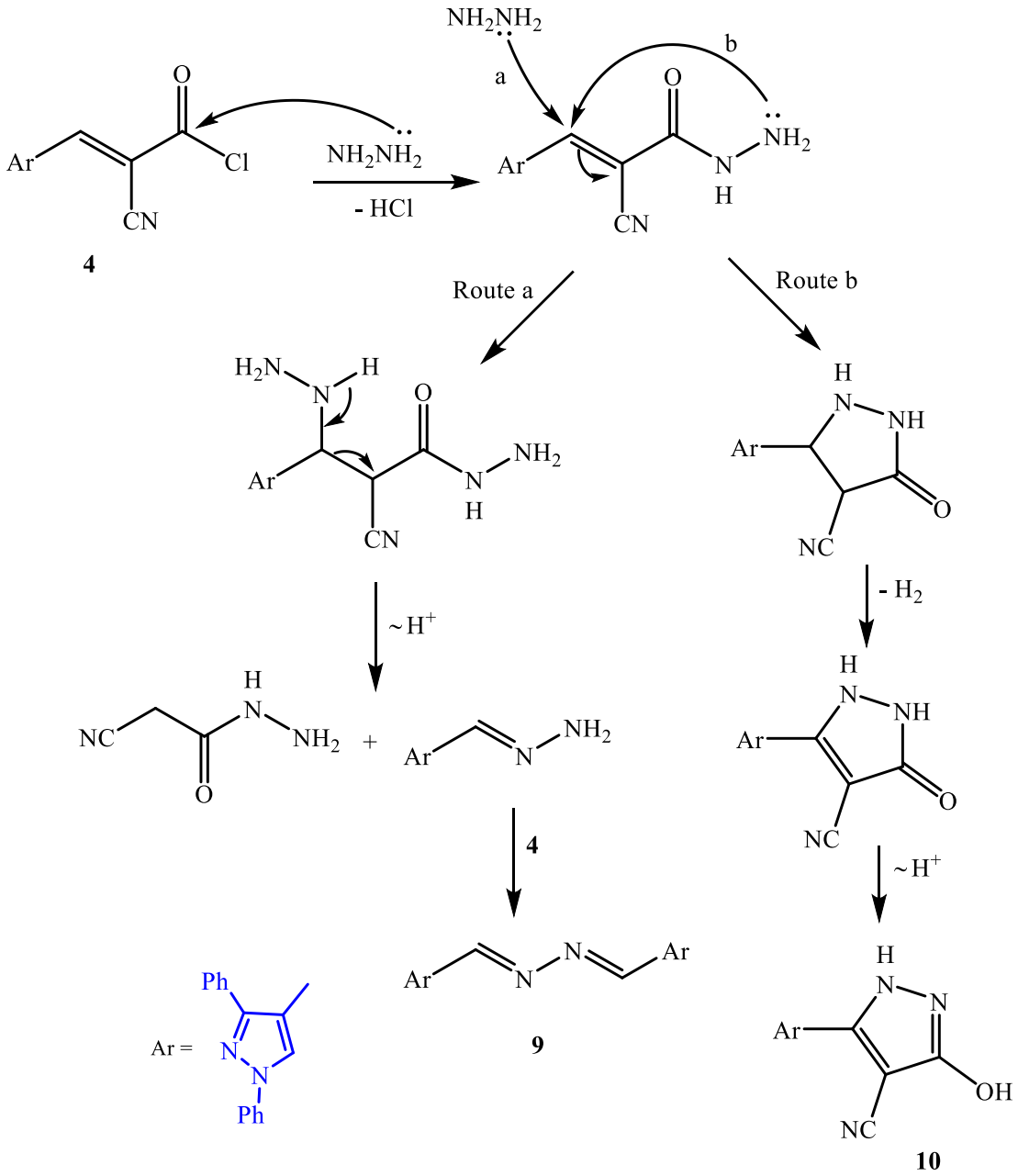
In turn, reaction of phenylhydrazine with acid chloride **4** led to the construction of pyranedione derivative **11** as brown crystals (cf. Scheme 2). Its IR spectrum displayed the absorption bands for lactone C=O at ν 1734 cm^{-1}, ketone C=O at ν 1687 cm^{-1}, CN at ν 2240 cm^{-1} and NH at ν 3139 cm^{-1} groups. Perhaps, formation of pyranedione **11** could be explained as depicted in Scheme 5. From synthetic and biological point of views, benzotriazole reacted with acid chloride **4** to obtain the corresponding acrylamide derivative bearing benzotriazole moiety **12** as yellow crystals (cf. Scheme 2). Its IR spectrum displayed the absorption bands for C≡N and C=O groups. The ^{1}H-NMR spectrum showed signals for the olefinic and aromatic protons. Furthermore, the mass spectrum exhibited the highest recorded peak at *m/z* 416 (10.56%) attributable for the molecular ion peak, as well as some of the abundant peaks (cf. "Experimental" section).



Scheme 3 Formation of thiazolopyrimidine **6**

Condensation of acid chloride **4** with some 1,4-binucleophilic reagents, namely 2-aminophenol, 2-aminoaniline, 2-aminothiophenol, 1,2-diaminoethane, 2-aminoethanol, and thiosemicarbazide, is displayed in Scheme 6. Thus, treating **4** with 2-aminophenol afforded a mixture of acrylamide derivative **13** and benzoxazepine derivative **14**. The IR spectrum of amide **13** exhibited phenolic OH absorption band which disappeared in that of compound **14**. The ^{1}H-NMR spectrum of amide **13** retained singlet signal for olefinic proton at δ 8.19 ppm and provided two exchangeable singlet signals for amide NH and phenolic OH protons at δ 10.13 and δ 7.37 ppm, respectively. Such a reaction, treating the acid chloride **4** with 2-aminoaniline or 2-aminothiophenol, acquired the benzodiazepine **15** and benzothiazepine **16**, respectively. In the ^{1}H-NMR spectra of compounds **14–16**, the absence of the olefinic singlet confirmed the cyclization step. The reaction could be visualized to occur via elimination of HCl molecule to afford the corresponding amide derivative which underwent cyclization followed by dehydrogenation to construct benzoxazepine, benzodiazepine and benzothiazepine derivatives **14–16** (Scheme 7).

In turn, treatment of acid chloride **4** with 1,2-diaminoethane afforded *N*,*N*′-(ethane-1,2-diyl)bis(2-cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acrylamide) (**17**) as yellow crystals. Its IR spectrum displayed the absorption bands for NH at ν 3319 cm^{-1}, C≡N at ν 2206 cm^{-1} and C=O at ν 1672 cm^{-1}. Also, the ^{1}H-NMR spectrum showed singlet signal for NH, olefinic and methylene protons as well as signals for aromatic protons (cf. "Experimental" section). In case of ethanolamine, the reaction prompted the acrylamide derivative **18** as pale yellow crystals. IR spectrum of compound **18** showed bands for OH, NH, C≡N and C=O groups. Meanwhile, the ^{1}H-NMR spectrum displayed two exchangeable singlet signals for NH and OH protons, two triplet signals for CH_2–CH_2 protons, as well as signals for olefinic and aromatic protons. Furthermore, the mass spectrum provided its molecular ion peak as the base peak at *m/z* 358.28 (100%). It was fortunate that thiosemicarbazide reaction with acid chloride **4** produced a mixture of the open-chain amide **19** in addition to the cyclic structure, triazepine derivative **20**. The IR spectra exhibited absorption bands for NH, C≡N, C=O and C=S groups. Full analysis of the ^{1}H-NMR and mass spectra

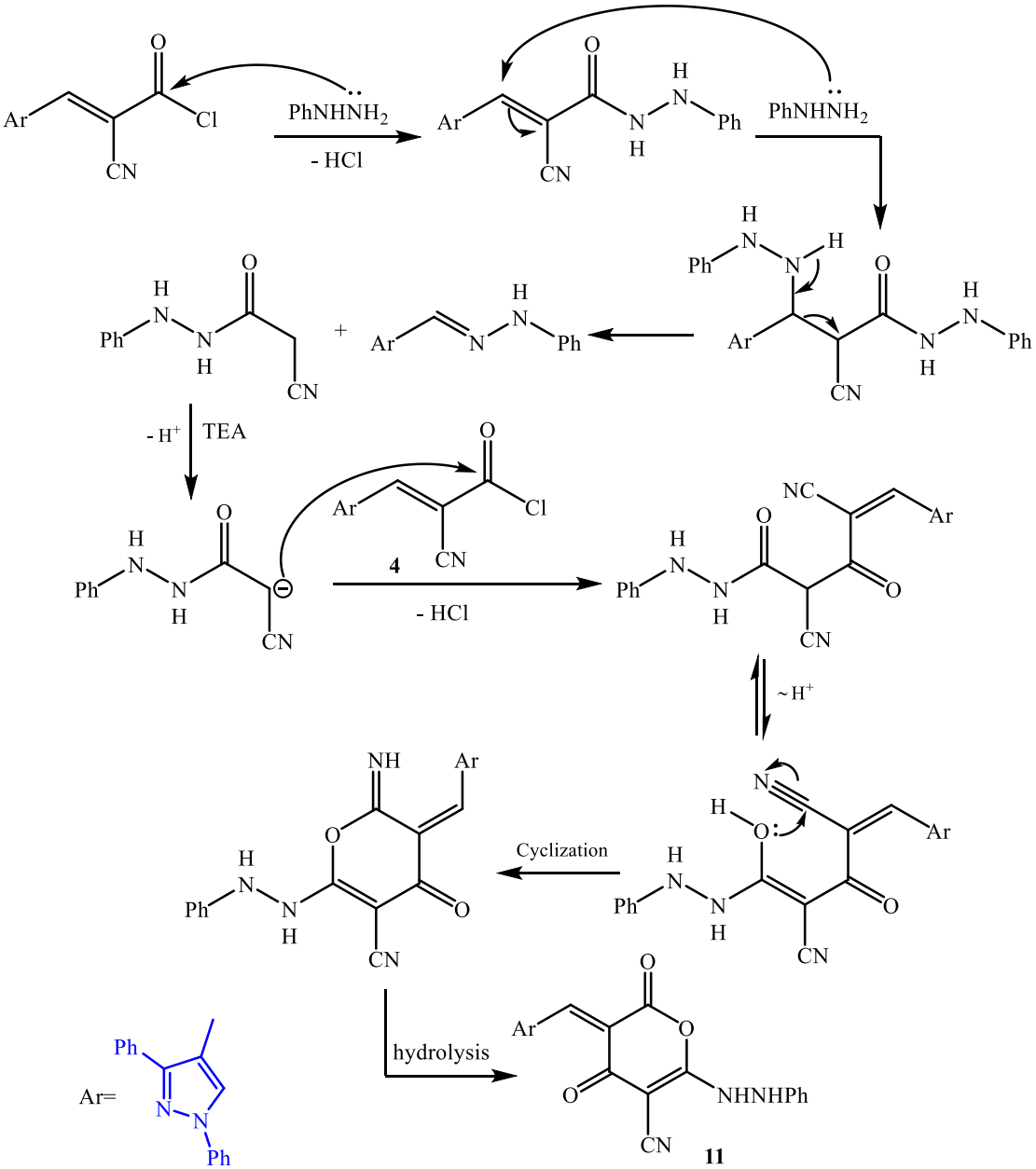


Scheme 4 Suggested pathways for hydrazinolysis of acid chloride **4**

strongly confirmed the proposed structures (cf. "Experimental" section).

Antiviral screening

In the present study, anti-RV activities were carried out for the synthetic ten compounds. First of all, cytotoxicity assay was made. Evaluation of cytotoxicity is clearly a significant step of the assessment of a potential antiviral drug since a useful drug should show either long-term toxicity or chronic against the host. Such a compound should be totally selective for specific viral processes with no or little impacts on cellular metabolism [32]. Several techniques have been developed for investigating the antiviral activities of compounds in cell culture. Evaluation of cytotoxicity in vitro is usually performed by cell viability assays, such as the dye uptake by non-viable cells after breakdown of the permeability of the cellular barrier (e.g., eosin Y, trypan blue, etc.) or function of mitochondria (e.g., XTT or MTT assay), but other methods, such as changes in cell culture morphology under microscopic investigation, have also been applied as

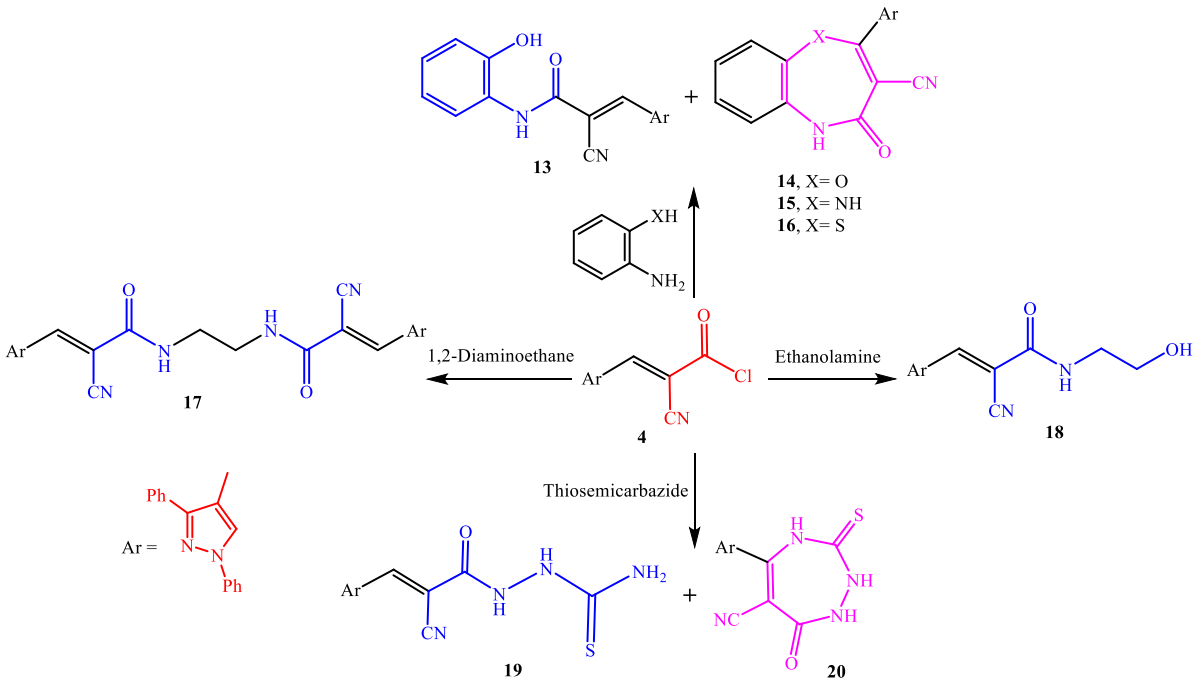


Scheme 5 Plausible pathway for the formation of pyranedione **11**

indicators of compound toxicity [33]. Our results demonstrated that the CC_{50} of the tested compounds ranged from 750 to 1500 µg/mL as summarized in Table 1 and Fig. 2.

Safe concentrations of the synthetic compounds were then evaluated against RV infection using $TCID_{50}$ measurement. The tested compounds showed reduction effect in the virus titer ranging from 0 to 3.25 $\log_{10}$ $TCID_{50}$. Two compounds (**8** and **11**) did not show antiviral activities against RV infection. Week antiviral activities with reduction in virus titers < 1 $\log_{10}$ $TCID_{50}$ were shown from **4**, **6**, and **10** compounds against RV infection. Strong antiviral activities with reduction in virus titers ≥ 2 $\log_{10}$ $TCID_{50}$ were observed from compounds **3**, **12**, **13**, **15**, and **16**. These five compounds are therefore regarded to be powerful anti-rotavirus drug candidates (Fig. 3).

Viral life cycle involves various steps: attachment, penetration, replication of viral proteins and genetic materials, assembly, and viral exit from infected cells. These steps can be used as targets of anti-rotavirus SA-11 agents [34]. Our results demonstrated that our tested compounds might be affected on the RV infections via reacting with viral capsid, preventing the virus attachment with the cell host. Further studies on the antiviral activities of these



Scheme 6 Behavior of acid chloride **4** toward some 1,4-binucleophiles

Scheme 7 A suggested mechanism for synthesis of compounds **13–16**

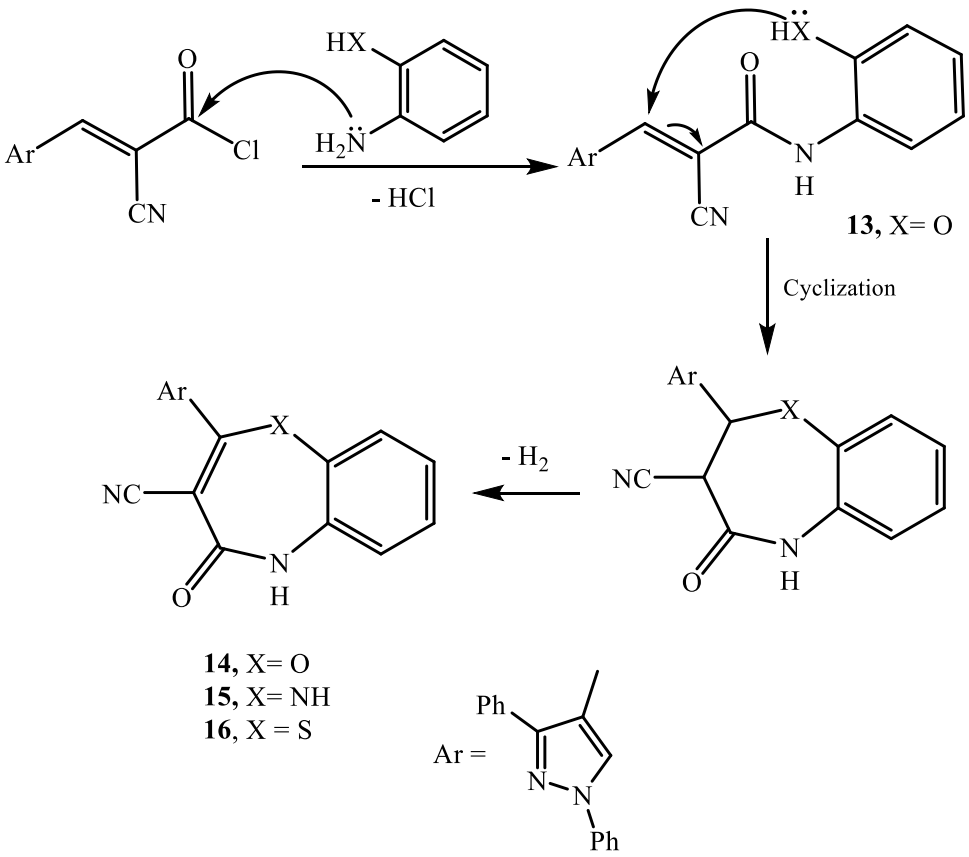


Table 1 Results of antiviral activity of compounds on RV SA11 by $TCID_{50}$/0.1 mL measurement

Sample ID	CC_{50} (µg/mL)	Virus titers without compound[a]	Virus titers with compound[b]	Reduction value of virus titers[c]
3	850	$10^{6.25}$	10^{3}	$10^{3.25}$
4	900	10^{6}	$10^{5.25}$	$10^{0.75}$
6	1400	$10^{6.25}$	$10^{5.75}$	$10^{0.5}$
8	1500	10^{6}	10^{6}	0
10	850	$10^{6.25}$	10^{6}	$10^{0.25}$
11	1200	$10^{6.25}$	$10^{6.25}$	0
12	750	$10^{6.25}$	10^{6}	$10^{2.25}$
13	800	$10^{6.25}$	10^{4}	$10^{2.25}$
15	900	$10^{6.25}$	$10^{4.25}$	10^{2}
16	1200	10^{6}	$10^{3.25}$	$10^{2.75}$

[a]Titer of positive control

[b]Titer of rotavirus when incubated with the compound prior to infection

[c]Reduction of virus titer was calculated as "virus titer without extract–virus titer with extract"

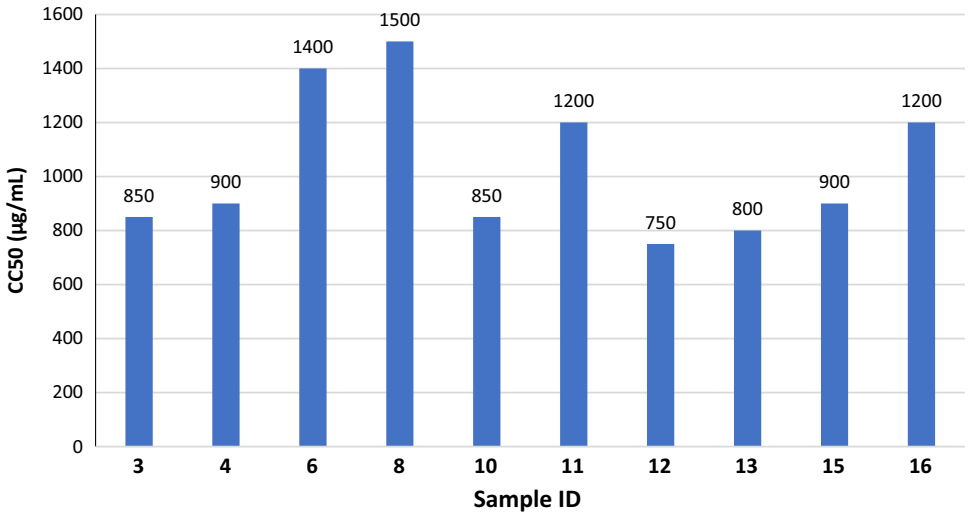


Fig. 2 CC_{50} of the tested compounds

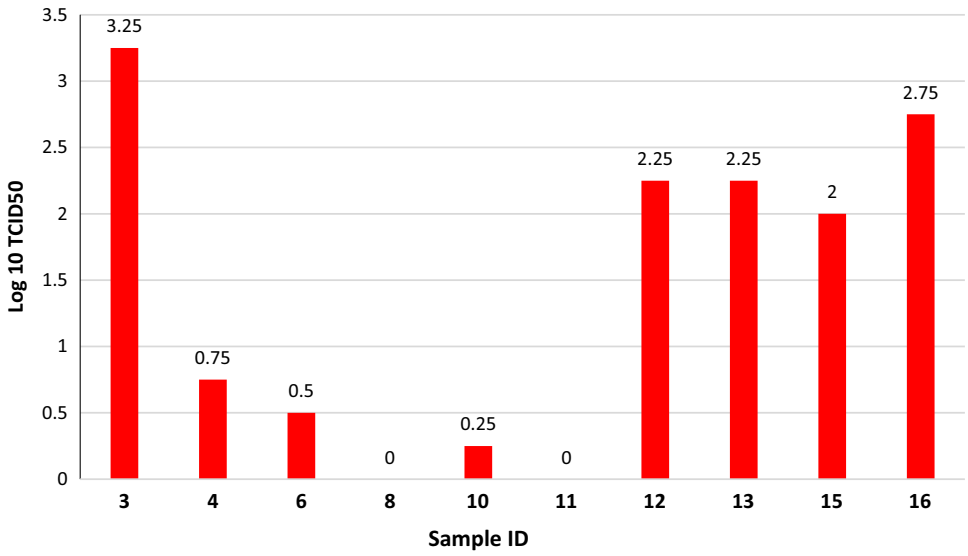


Fig. 3 Reduction effect in the virus titer

compounds in cell culture to investigate whether these compounds can have a effect on the virus via other replication steps are required.

Conclusion

Synthesis of some nitrogen heterocycles such as thiazolopyrimidine, pyrimidinethione, hydroxypyrazole, pyranedione, benzotriazole, benzoxazepine, benzodiazepine, benzothiazepine, and triazepinethione derivatives encompassing pyrazole scaffold starting with treatment of acryloyl chloride derivative **4** with some nitrogen nucleophiles has been reported. The antiviral screening of the synthesized products showed promising anti-RV activities of five compounds (**3**, **12**, **13**, **15**, and **16**) which can be considered as novel anti-rotavirus drugs.

Experimental

Chemistry

Chemicals and solvents are of commercial grade and obtained from Sigma-Aldrich, Merck, Fluka, and El-Nasr pharmaceutical chemicals companies and were purified and dried by standard techniques. Melting points were measured on a Gallenkamp electric melting point apparatus and were uncorrected. The infrared spectra were recorded using potassium bromide disks on a FTIR Thermo Electron Nicolet iS10 (USA) infrared spectrometer and expressed in wave number (ν, cm^{-1}) at the Chemistry Department, Faculty of Science, Ain Shams University. The ^{1}H-NMR spectra were run at 400 MHz on a Bruker NMR spectrometer using tetramethylsilane (TMS) as an internal standard in deuterated dimethyl sulfoxide (DMSO-d_6) at Faculty of Pharmacy, Cairo University, Giza, Egypt. Chemical shifts (δ) are quoted in ppm. The abbreviations used are as follows: s, singlet; d, doublet; t, triplet; and m, multiplet. All coupling constant (J) values are given in hertz. The mass spectra were recorded on a Shimadzu GC–MS-QP-1000 EX mass spectrometer operating at 70 eV at the regional center for Mycology and Biotechnology (RCMB) of Al-Azhar University, Nasr City, Cairo, Egypt. Elemental analyses were carried out at the microanalytical unit, Faculty of Science, Ain Shams University. The reactions were monitored by thin-layer chromatography using Merck Kieselgel 60 F_{254} obtained from Fluka. The antiviral activity was performed at Microanalytical Center of Ain Shams University, Egypt. The starting pyrazole aldehyde **1** and arylidene derivatives, namely ethyl 2-cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acrylate (**2**), were previously prepared [30, 31].

2-Cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acrylic acid (3)

To a solution of ethyl 2-cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acrylate (**2**) [30, 31] (3 mmol, 1 g) in ethanol (15 mL), sodium hydroxide (3 mmol, 0.11 g) in ethanol (5 mL) was added. The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was poured onto crushed ice and acidified with cold dilute HCl. The separated solid was filtered off, washed with water, dried, and recrystallized from ethanol to give the acid **3** as pale yellow crystals, mp. 298–300 °C, yield 93%. IR (KBr, ν, cm^{-1}): 3453 (OH), 2226 (CN), 1697 (C=O). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 13.87 (*br*.s, 1H, OH, exchangeable), 9.19 (s, 1H, CH pyrazole), 8.10 (s, 1H, CH=), 7.95–7.93 (d, 2H, *N*-phenyl, $J = 7.92$ Hz), 7.66–7.56 (m, 7H, Ar–H), 7.49–7.45 (t, 1H, C4–H, *N*-phenyl, $J = 7.36$ Hz). EIMS *m*/*z* (%): 315.38 ($M^{+\cdot}$, 40.82), 263.58 (100.00), 170.05 (80.13), 104.19 (68.96), 41.70 (85.17). Anal. Calcd. for $C_{19}H_{13}N_3O_2$ (315.33): C, 72.37; H, 4.16; N, 13.33. Found: C, 72.11; H, 4.02; N, 13.36%.

2-Cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acryloyl chloride (4)

A mixture of **3** (5 mmol) and thionyl chloride (5 mL) was heated on a water bath at 65 °C for 4 h. The excess thionyl chloride was distilled under reduced pressure. The obtained yellowish green solid was collected and used without further purification, mp. 164–166 °C, yield 84%. IR (KBr, ν, cm^{-1}): 2228 (C≡N), 1754 (C=O). ^{1}H-NMR (400 MHz, DMSO-d_6): 9.19 (s, 1H, CH pyrazole), 8.12 (s, 1H, CH=), 7.94–7.92 (d, 2H, *N*-phenyl, $J = 7.92$ Hz), 7.66–7.50 (m, 7H, phenyl), 7.48–7.45 (t, 1H, C4–H, *N*-phenyl, $J = 7.32$ Hz). EIMS *m*/*z* (%): 333.20 ($M^{+\cdot}$, 52.03), 298.23 (100.00), 270.00 (52.78), 241.06 (10.31), 77.04 (86.33). Anal. Calcd. for $C_{19}H_{12}ClN_3O$ (333.78): C, 68.37; H, 3.62; N, 12.59. Found: C, 68.04; H, 3.40; N, 12.61%.

Reaction with 2-aminothiazole

To a solution of **4** (2 mmol, 0.7 g) in dry benzene (20 mL) containing two drops of triethylamine, 2-aminothiazole (2 mmol, 0.21 g) was added. The reaction mixture was heated under reflux for 2 h and then cooled to room temperature. The precipitated solid was collected and found to be a mixture of two compounds **5** (recrystallized from dioxane) and **6** (recrystallized from ethanol).

2-Cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)-*N*-(thiazol-2-yl)acrylamide (5)

Pale yellow crystals, mp. 288–290 °C, yield 30%. IR (KBr, ν, cm^{-1}): 3150 (NH), 2215 (C≡N), 1672 (C=O amide). ^{1}H-NMR (400 MHz, DMSO-d_6): (*E*- and *Z*-isomers, 1:1) δ 13.51, 13.48 (br.s, 1H, NH, exchangeable), 9.21, 9.19 (s, 1H, CH pyrazole), 8.28, 8.09 (s, 1H, CH=), 7.95–7.93 (d, 2H, *N*-phenyl, J = 7.92 Hz), 7.72–7.70 (d, 1H, C4–H thiazole, J = 6.38 Hz), 7.6–7.51 (m, 7H, phenyl), 7.49–7.45 (t, 1H, C4–H, *N*-phenyl, J = 7.36 Hz), 7.21–7.19 (d, 1H, C5–H thiazole, J = 6.35 Hz). EIMS *m*/*z* (%): 397.24 ($M^{+\cdot}$, 100.00), 298.22 (19.92), 77.15 (18.19), 51.15 (4.14). Anal. Calcd. for $C_{22}H_{15}N_5OS$ (397.46): C, 66.48; H, 3.80; N, 17.62. Found: C, 66.23; H, 3.68; N, 17.60%.

5-(1,3-Diphenyl-1*H*-pyrazol-4-yl)-7-hydroxy-5*H*-thiazolo[3,2-*a*]pyrimidine-6-carbonitrile (6)

Pale yellow crystals, mp. 292–294 °C, yield 70%. IR (KBr, ν, cm^{-1}): 3333 (OH), 2216 (C≡N), 1614 (C=N). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 13.33 (*br*.s, 1H, OH, exchangeable), 9.21 (s, 1H, CH pyrazole), 7.95–7.93 (d, 2H, *N*-phenyl, J = 7.80 Hz), 7.72–7.70 (d, 1H, C4–H thiazole, J = 6.68 Hz), 7.67–7.51 (m, 7H, phenyl), 7.58–7.56 (d, 1H, C5–H thiazole, J = 6.70 Hz), 7.49–7.46 (t, 1H, C4–H, *N*-phenyl, J = 7.40 Hz), 7.20 (s, 1H, C4–H pyrimidine). EIMS *m*/*z* (%): 397.29 ($M^{+\cdot}$, 16.48), 298.21 (17.43), 77.13 (100.00), 45.09 (54.21). Anal. Calcd. for $C_{22}H_{15}N_5OS$ (397.46): C, 66.48; H, 3.80; N, 17.62. Found: C, 66.19; H, 3.62; N, 17.58%.

Reaction with thiourea

A mixture of **4** (2 mmol, 0.7 g) in dry benzene (20 mL) containing few drops of triethylamine and thiourea (2 mmol, 0.16 g) was heated under reflux for 2 h. The solvent was evaporated under vacuum. The residue was found to be a mixture of compounds **7** (recrystallized from ethanol) and **8** (recrystallized from benzene).

N-Carbamothioyl-2-cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acrylamide (7)

Canary yellow crystals, mp. 254–256 °C, yield 29%. IR (KBr, ν, cm^{-1}): 3321, 3290 (NH, NH_2), 2218 (C≡N), 1709 (C=O), 1639 (C=N), 1222 (C=S). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 11.66 (br.s, 1H, NH, exchangeable), 9.25 (s, 1H, CH pyrazole), 8.13 (s, 1H, CH=), 7.98–7.96 (d, 2H, *N*-phenyl, J = 7.72 Hz), 7.72–7.54 (m, 7H, phenyl), 7.51–7.49 (t, 1H, C4–H, *N*-phenyl, J = 7.60 Hz), 7.47 (br.s, 2H, NH_2, exchangeable). Anal. Calcd. for $C_{20}H_{15}N_5OS$ (373.43): C, 64.33; H, 4.05; N, 18.75. Found: C, 64.19; H, 3.92; N, 18.78%.

6-(1,3-Diphenyl-1*H*-pyrazol-4-yl)-4-oxo-2-thioxo-1,2,3,4-tetrahydropyrimidine-5-carbonitrile (8)

Deep yellow crystals, yield 71%, mp. 190–192 °C [Lit. [35, 36], mp. 190–192 °C].

Reaction with hydrazine hydrate

To a solution of **4** (2 mmol, 0.7 g) in dry toluene (20 mL) in the presence of two drops of triethylamine, hydrazine hydrate (2 mmol, 0.15 mL, 80%) was added dropwise at 0 °C, and the reaction mixture was stirred for 1 h. The separated solid was collected by filtration and found to be a mixture of two compounds **9** and **10** which was fractionally recrystallized from ethanol to afford the azine **9**. The insoluble part was recrystallized from ethanol/dioxane mixture (1:1) to give compound **10**.

(1*E*,2*E*)-1,2-Bis((1,3-diphenyl-1*H*-pyrazol-4-yl)methylene)hydrazine (9)

Pale yellow crystals, mp. 236–238 °C [Lit. [3], mp. 240–242 °C].

5-Hydroxy-1′,3′-diphenyl-1′*H*,2*H*-[3,4′-bipyrazole]-4-carbonitrile (10)

Yellow crystals, mp. 306 °C (decomp.), yield 49%. IR (KBr, ν, cm^{-1}): 3441 (OH), 3185 (NH), 2212 (C≡N), 1618 (C=N). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.67 (br.s, 2H, NH + OH, exchangeable), 9.23 (s, 1H, CH pyrazole), 7.97–7.95 (d, 2H, *N*-phenyl, J = 7.84 Hz), 7.77–7.48 (m, 7H, phenyl), 7.42–7.38 (t, 1H, C4–H, *N*-phenyl, J = 7.24 Hz). EIMS *m*/*z* (%): 327.35 ($M^{+\cdot}$, 47.19), 317.70 (80.49), 251.12 (100), 183.21 (45.53), 138.35 (90.52), 55.39 (54.74). Anal. Calcd. for $C_{19}H_{13}N_5O$ (327.35): C, 69.71; H, 4.00; N, 21.39. Found: C, 69.52; H, 3.83; N, 21.38%.

3-((1,3-Diphenyl-1*H*-pyrazol-4-yl)methylene)-2,4-dioxo-6-(2-phenylhydrazinyl)-3,4-dihydro-2*H*-pyran-5-carbonitrile (11)

To a solution of **4** (2 mmol, 0.7 g) in dry dioxane (20 mL) containing few drops of triethylamine, phenylhydrazine (2 mmol, 0.21 g) was added, and the reaction mixture was stirred at room temperature for 1 h. The reaction mixture was poured onto ice/water and acidified with dilute HCl. The precipitated solid was filtered off and recrystallized from benzene to give **11** as brown crystals, mp. 260–262 °C,

yield 72%. IR (KBr, ν, cm^{-1}): 3139 (NH), 2240 (C≡N), 1734 (C=O lactone), 1687 (C=O ketone). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 13.64 (br.s, 1H, NHPh, exchangeable), 9.19 (s, 1H, CH pyrazole), 8.09 (s, 1H, CH=), 7.95–7.93 (d, 2H, *N*-phenyl, J = 7.84 Hz), 7.66–7.55 (m, 12H, phenyl), 7.49–7.47 (t, 1H, C4–H, *N*-phenyl, J = 7.40 Hz), 6.38 (br.s, 1H, NH, exchangeable). EIMS *m/z* (%): 447.40 ([$M^+ - CN^-$], 24.65), 378.30 (75.56), 277.48 (87.46), 174.59 (64.96), 117.98 (100.00). Anal. Calcd. for $C_{28}H_{19}N_5O_3$ (473.49): C, 71.03; H, 4.04; N, 14.79. Found: C, 70.96; H, 3.91; N, 14.81%.

2-(1*H*-Benzo[*d*] [1,2,3] triazole-1-carbonyl)-3-(1,3-diphenyl-1*H*-pyrazol-4-yl) acrylonitrile (12)

A solution of **4** (2 mmol, 0.7 g) and benzotriazole (2 mmol, 0.25 g) in dry benzene (20 mL) was heated under reflux for 1 h in the presence of two drops of triethylamine. The separated solid after cooling was collected and recrystallized from ethanol to give **12** as yellow crystals, mp. 234–236 °C, yield 71%. IR (KBr, ν, cm^{-1}): 2220 (C≡N), 1686 (C=O). ^{1}H-NMR (400 MHz, DMSO-d_6): (*E*- and *Z*-isomers, 53:47%) δ: 9.41, 9.19 (s, 1H, CH pyrazole), 8.64, 8.09 (s, 1H, CH=), 8.33–8.30, 8.25–8.23 (d, 1H, C4–H benzotriazole, J = 8.06 Hz), 8.01–7.99, 7.95–7.93 (d, 1H, C7–H benzotriazole, J = 7.90 Hz), 7.85–7.82 (dd, 1H, C5–H benzotriazole, J = 8.04 and 7.36 Hz), 7.77–7.75 (d, 2H, *N*-phenyl, J = 6.04 Hz), 7.68–7.46 (m, 8H, phenyl), 7.51–7.48 (dd, 1H, C6–H benzotriazole, J = 7.88 and 7.37 Hz). EIMS *m/z* (%): 416.79 ($M^{+\cdot}$, 10.56), 291.58 (44.76), 163.35 (34.74), 119.48 (37.66), 54.70 (100.00). Anal. Calcd. for $C_{25}H_{16}N_6O$ (416.44): C, 72.10; H, 3.87; N, 20.18. Found: C, 71.97; H, 3.71; N, 20.15%.

Reaction with 2-aminophenol

A solution of **4** (2 mmol, 0.7 g) and 2-aminophenol (2 mmol, 0.22 g) in dry dioxane or benzene (20 mL) containing two drops of triethylamine was refluxed for 1 h. The precipitated solid was collected and found to be a mixture of two compounds **13** (recrystallized from benzene) and **14** (recrystallized from ethanol).

2-Cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)-*N*-(2-hydroxyphenyl)acrylamide (13)

Pale yellow crystals, mp. 270–272 °C, yield 55%. IR (KBr, ν, cm^{-1}): 3365 (OH), 3242 (NH), 2211 (C≡N), 1660 (C=O). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.13 (br.s, 1H, NH, exchangeable), 9.21 (s, 1H, CH pyrazole), 8.19 (s, 1H, CH=), 7.98–7.96 (d, 2H, *N*-phenyl, J = 8.00 Hz), 7.87–7.85 (d, 1H, C6–H hydroxyphenyl, J = 7.80 Hz), 7.71–7.69 (d, 2H, phenyl, J = 6.8 Hz), 7.64–7.56 (m, 5H, phenyl), 7.50–7.46 (t, 1H, *N*-phenyl, J = 7.40 Hz), 7.37 (br.s, 1H, OH, exchangeable), 7.05–7.01 (dd, 1H, C4–H hydroxyphenyl, J = 7.96 and 7.36 Hz), 6.95–6.93 (d, 1H, C7–H hydroxyphenyl, J = 7.92 Hz), 6.85–6.81 (dd, 1H, C5–H hydroxyphenyl, J = 8.00 and 7.44 Hz). EIMS *m/z* (%): 406.18 ($M^{+\cdot}$, 100.00), 343.20 (3.28), 298.14 (74.94), 80.14 (69.57). Anal. Calcd. for $C_{25}H_{18}N_4O_2$ (406.45): C, 73.88; H, 4.46; N, 13.78. Found: C, 73.61; H, 4.27; N, 13.81%.

2-(1,3-Diphenyl-1*H*-pyrazol-4-yl)-4-oxo-4,5-dihydro benzo[b] [1,4] oxazepine-3-carbonitrile (14)

Pale yellow crystals, mp. 290–292 °C, yield 45%. IR (KBr, ν, cm^{-1}): 3360 (NH), 2212 (C≡N), 1656 (C=O). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.12 (br.s, 1H, NH, exchangeable), 9.20 (s, 1H, CH pyrazole), 7.97–7.95 (d, 2H, *N*-phenyl, J = 8.08 Hz), 7.88–7.86 (d, 1H, C9–H oxazepine, J = 7.92 Hz), 7.71–7.69 (d, 2H, phenyl, J = 7.36 Hz), 7.64–7.54 (m, 5H, phenyl), 7.50–7.46 (t, 1H, *N*-phenyl, J = 7.60 Hz), 7.05–7.01 (dd, 1H, C6–H oxazepine, J = 7.76 and 7.60 Hz), 6.95–6.93 (d, 1H, C8–H oxazepine, J = 8.00 Hz), 6.85–6.81 (dd, 1H, C7–H oxazepine, J = 7.76 and 7.56 Hz). EIMS *m/z* (%): 404.03 ($M^{+\cdot}$, 4.98), 298.24 (76.34), 108.18 (8.98), 80.13 (67.15), 77.12 (100.00). Anal. Calcd. for $C_{25}H_{16}N_4O_2$ (404.43): C, 74.25; H, 3.99; N, 13.85. Found: C, 74.01; H, 3.73; N, 13.80%.

4-(1,3-Diphenyl-1*H*-pyrazol-4-yl)-2-oxo-2,5-dihydro-1*H*-benzo[*b*] [1,4] diazepine-3-carbonitrile (15)

A solution of **4** (2 mmol, 0.7 g) and 2-aminoaniline (2 mmol, 0.22 g) in dry dioxane or benzene (20 mL) containing two drops of triethylamine was refluxed for 1 h. The precipitated solid was collected and recrystallized from dioxane to afford **15** as yellow crystals, mp. > 360 °C, yield 83%. IR (KBr, ν, cm^{-1}): 3351 (NH), 2209 (C≡N), 1686 (C=O). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.91 (br.s, 1H, NHCO, exchangeable), 9.21 (s, 1H, CH pyrazole), 8.22 (br.s, 1H, NH, exchangeable), 7.96–7.94 (d, 2H, *N*-phenyl, J = 8.00 Hz), 7.69–7.67 (d, 2H, phenyl, J = 7.28 Hz), 7.64–7.57 (m, 6H, phenyl), 7.54–7.31 (m, 4H, Ar–H, diazepine). EIMS, *m/z* (%): 403.53 ($M^{+\cdot}$, 21.63), 291.42 (64.22), 178.51 (62.62), 126.73 (100.00), 73.70 (44.85). Anal. Calcd. for $C_{25}H_{17}N_5O$ (403.45): C, 74.43; H, 4.25; N, 17.36. Found: C, 74.19; H, 4.08; N, 17.39%.

2-(1,3-Diphenyl-1*H*-pyrazol-4-yl)-4-oxo-4,5-dihydro benzo[*b*] [1,4] thiazepine-3-carbonitrile (16)

A solution of **4** (2 mmol, 0.7 g) and 2-aminothiophenol (2 mmol, 0.26 g) in dry benzene (20 mL) containing two

drops of triethylamine was heated under reflux for 1 h. The precipitated solid upon cooling was collected and recrystallized from benzene to furnish benzothiazepine **16** as beige crystals, mp. 176–178 °C, yield 68%. IR (KBr, ν, cm^{-1}): 3324 (NH), 2202 (C≡N), 1689 (C=O). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.69 (br.s, 1H, NH, exchangeable), 8.57 (s, 1H, CH pyrazole), 7.95–7.93 (d, 2H, *N*-phenyl, J = 7.68 Hz), 7.88–7.86 (d, 2H, phenyl, J = 7.84 Hz), 7.76–7.54 (m, 5H, phenyl), 7.51–7.48 (t, 1H, *N*-phenyl, J = 8.32 Hz), 7.37–7.26 (m, 4H, Ar–H, thiazepine). EIMS, *m*/*z* (%): 420.09 ($M^{+\cdot}$, 54.07), 383.79 (100.00), 327.44 (79.95), 271.94 (64.86), 138.89 (58.72), 76.00 (91.76). Anal. Calcd. for $C_{25}H_{16}N_4OS$ (420.49): C, 71.41; H, 3.84; N, 13.32. Found: C, 71.26; H, 3.75; N, 13.21%.

Reaction of acid chloride 4 with 1,2-diaminoethane or ethanolamine

To a stirred solution of acid chloride **4** (2 mmol, 0.7 g) in dry dioxane (10 mL) containing two drops of triethylamine, 1,2-diaminoethane (2 mmol, 0.14 mL) or 2-aminoethanol (2 mmol, 0.13 mL) was added, and stirring was continued for 10 min. The precipitated solid was collected and recrystallized from the suitable solvents to afford compounds **17** and **18**, respectively.

2-Cyano-*N*-(2-(2-cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acrylamido)ethyl)-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acrylamide (17)

Yellow crystals, mp. 292–294 °C (dioxane), yield 83%. IR (KBr, ν, cm^{-1}): 3319 (NH), 2206 (C≡N), 1672 (C=O). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.15 (s, 2H, CH pyrazole), 8.53 (br.s, 2H, 2NHCO, exchangeable), 8.06 (s, 2H, CH=), 7.95–7.93 (d, 4H, *N*-phenyl, J = 7.96 Hz), 7.65–7.52 (m, 14H, phenyl), 7.49–7.45 (t, 2H, *N*-phenyl, J = 7.12 Hz), 2.89 (s, 4H, $2CH_2$). EIMS, *m*/*z* (%): 403.01 (28.94), 258.49 (39.25), 217.38 (75.42), 149.55 (92.51), 55.63 (100.00). Anal. Calcd. for $C_{40}H_{30}N_8O_2$ (654.73): C, 73.38; H, 4.62; N, 17.11. Found: C, 73.29; H, 4.54; N, 17.09%.

2-Cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)-*N*-(2-hydroxyethyl)acrylamide (18)

Pale yellow crystals, mp. 204–206 °C (benzene), yield 65%. IR (KBr, ν, cm^{-1}): 3467 (OH), 3352 (NH), 2215 (C≡N), 1669 (C=O). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.15 (s, 1H, CH pyrazole), 8.32 (br.s, 1H, NH, exchangeable), 8.07 (s, 1H, CH=), 7.95–7.93 (d, 2H, *N*-phenyl, J = 8.2 Hz), 7.67–7.55 (m, 7H, Ar–H), 7.49–7.45 (t, 1H, *N*-phenyl, J = 7.16 Hz), 4.75 (br.s, 1H, OH, exchangeable), 3.51–3.47 (t, 2H, CH_2O, J = 5.76 Hz), 3.31–3.26 (t, 2H, CH_2N, J = 5.76 Hz). EIMS, *m*/*z* (%): 358.28 ($M^{+\cdot}$, 100.00), 298.19 (56.63), 77.08 (65.88), 51.11 (10.60). Anal. Calcd. for $C_{21}H_{18}N_4O_2$ (358.40): C, 70.38; H, 5.06; N, 15.63. Found: C, 70.21; H, 4.89; N, 15.60%.

Reaction with thiosemicarbazide

A mixture of **4** (2 mmol, 0.7 g) in dry benzene (20 mL) containing few drops of triethylamine and thiosemicarbazide (2 mmol, 0.19 g) was heated under reflux for 2 h. The solvent was evaporated under vacuum. The residue was found to be a mixture of compounds **19** (recrystallized from benzene) and **20** (recrystallized from ethanol/dioxane mixture, 1:1).

2-(2-Cyano-3-(1,3-diphenyl-1*H*-pyrazol-4-yl)acryloyl)hydrazine-1-carbothioamide (19)

Yellow crystal, mp. 220–222 °C, yield 44%. IR (KBr, ν, cm^{-1}): 3333, 3285, 3195 (NH, NH_2), 2214 (C≡N), 1686 (C=O), 1229 (C=S). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 10.49 (br.s, 1H, NHCO, exchangeable), 9.93 (br.s, 2H, NH_2, exchangeable), 9.19 (br.s, 1H, NH, exchangeable), 9.18 (s, 1H, CH pyrazole), 8.09 (s, 1H, CH=), 7.95–7.93 (d, 2H, *N*-phenyl, J = 7.88 Hz), 7.67–7.37 (m, 8H, Ar–H). EIMS, *m*/*z* (%): 388.04 ($M^{+\cdot}$, 13.20), 321.67 (100.00), 277.69 (77.43), 218.51 (54.06), 107.90 (39.32). Anal. Calcd. for $C_{20}H_{16}N_6OS$ (388.45): C, 61.84; H, 4.15; N, 21.64. Found: C, 61.72; H, 3.94; N, 21.67%.

5-(1,3-Diphenyl-1*H*-pyrazol-4-yl)-7-oxo-3-thioxo-2,3,4,7-tetrahydro-1*H*-1,2,4-triazepine-6-carbonitrile (20)

Pale yellow crystals, mp. > 300 °C, yield 56%. IR (KBr, ν, cm^{-1}): 3216 (NH), 2222 (C≡N), 1684 (C=O), 1228 (C=S). ^{1}H-NMR (400 MHz, DMSO-d_6): δ 9.24 (br.s, 1H, NHCO, exchangeable), 9.21 (s, 1H, CH pyrazole), 8.28 (br.s, 1H, NHCS, exchangeable), 7.96–7.94 (d, 2H, *N*-phenyl, J = 7.56 Hz), 7.72 (s, 1H, HN<u>NH</u>CS, exchangeable), 7.70–7.56 (m, 7H, Ar–H), 7.50–7.46 (t, 1H, *N*-phenyl, J = 7.28 Hz). EIMS, *m*/*z* (%): 386.06 ($M^{+\cdot}$, 30.82), 322.07 (17.77), 197.73 (52.5), 90.25 (59.30), 42.23 (100.00). Anal. Calcd. for $C_{20}H_{14}N_6OS$ (386.43): C, 62.16; H, 3.65; N, 21.75. Found: C, 62.01; H, 3.49; N, 21.70%.

Antiviral bioassays

Virus and cells

A MA 10^4 cell line was obtained from VACSERA, Cairo, Egypt, and used for antiviral assays. Cells were propagated in DMEM (Gibco, BR), supplemented with fetal bovine

serum and antibiotic. Rotavirus RV SA-11 was kindly provided by Dr. Mohamed N. Shaheen, NRC, Giza, Egypt.

Cytotoxicity assay

Different concentrations of the tested compounds were prepared in DMEM containing 2% antibiotic and 2% FBS. The cytotoxic effect of the extracts on MA 10^4 cell lines was determined by using 3-(4,*Z*-5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [37]. This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to a purple formazan derivative by mitochondrial succinate dehydrogenase in viable cells. Briefly, MA 10^4 cells were grown in 96-well microtiter plates at concentration of 5×10^3 cells/well. After 24 h at 37 °C in a 5% CO_2-humidified atmosphere, the culture medium was removed and the different concentrations of the tested extract were added to wells and incubated with cell lines for an additional 48 h at 37 °C. The culture medium containing the tested extracts was removed, and 100 µL of MTT solution (5 mg/mL) was added each well. The plates were further incubated for 4 h at 37 °C to allow MTT formazan formation. After the removal of MTT, 50 µL dimethyl sulfoxide (DMSO) was added to each well. The plates were further incubated for 30 min at 37 °C for solubilization of formazan crystals. The absorbance was read on a multi-well ELISA reader at a test wavelength of 540 nm. The percentage of cytotoxicity was calculated as $[A-B/A]\times100$, where A and B are the mean of three optical densities of untreated and treated cells, respectively. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that can reduce the cell viability by 50%.

Antiviral activities of compounds by measurement of $TCID_{50}$

For $TCID_{50}$ determination, the non-toxic concentration from each compound was selected to be evaluated against RV infection. Tenfold dilution of activated RV SA-11 was prepared in cell culture medium, and then, 100 µL of viral dilutions 10^{-4} to 10^{-9} was incubated with 100 µL of each culture medium containing the compound for 1 h at 37 °C in a CO_2 incubator. Virus dilutions either with or without compound were added into four parallel cell culture wells. All plates were incubated at 37 °C in a CO_2 incubator for 72 h, then the cytopathic effect was observed under inverted microscope, and virus titration was calculated and expressed as 50% tissue culture infection dose ($TCID_{50}$) by using Spearman–Kärber method [38]. The reduction in virus titer was calculated as differences between the values of treated and untreated virus.

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Compliance with ethical standards

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

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